Université de Montréal

Semisynthetic Aminoglycoside Antibiotics – Toward Biomimetic Synthesis, Evasion of Bacterial Resistance and Reduced Toxicity

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Mémoire présentée à la Faculté des Études Supérieures
en vue de l'obtention du grade de
Maîtrise ès Sciences (M.Sc.) en chimie

July, 2010

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Université de Montréal
Faculté des Études Supérieures

Cette thèse intitulée:

Semisynthetic Aminoglycoside Antibiotics – Toward Biomimetic Synthesis, Evasion of Bacterial Resistance and Reduced Toxicity

présentée par
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est présentée à un jury composé des personnes suivantes:

Président du Jury	William Lubell
Directeur de Recherche	Stephen Hanessian
Membre du Jury	Joelle Pelletier

To my family.

Summary

Aminoglycosides are valuable and effective broad-spectrum bactericidal antibiotics against Gram-positive and Gram-negative pathogens, with several members of natural and semisynthetic origin occupying prominent roles in clinical practice since 1950. Nobel-prize winning crystallographic studies on the ribosome have revealed how their diverse polyaminated sugar framework is tailored to target a RNA helix within the decoding centre of the bacterial 30S subunit. By interfering with the affinity and kinetics of the tRNA selection and proof-reading steps, they induce error-prone protein synthesis, and translocation inhibition and lead to a lethal cycle of antibiotic uptake and membrane stress. In retaliation, bacterial pathogens have evolved and disseminated a number of enzymatic and efflux resistance mechanisms. These include *N*-acetyl-transferases, *O*-phosphotransferases and *O*-nucleotidyltransferases, which target the core hydroxyl and amino groups of aminoglycosides promiscuously; methyltransferases, which target the ribosomal binding-site; and energy-dependent drug efflux pumps for aminoglycoside-selective elimination, in Gram-negative pathogens.

The most problematic infectious pathogens which are currently resilient to most unrelated antibiotic classes and in the verge of *pan*-resistance have been defined '*ESKAPE'* bacteria, a mnemonic for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteriaceae*. The world-wide spread of aminoglycoside resistance to current clinical standards, such as tobramycin, amikacin and gentamicin, ranges from 20 to 60% of clinical isolates. Hence, the contemporary 4,6-disubstituted-2-deoxystreptamine aminoglycosides are currently inadequate as broad-spectrum anti-infective therapies.

The 4,5-disubstituted class of aminoglycosides are a challenging framework for medicinal chemistry, which includes butirosin, neomycin and paromomycin. Exploring the potential of these alternatives, colleagues in the Hanessian group and collaborators of Achaogen Inc. have demonstrated that paromomycin and neomycin analogs modified by deoxygenation of positions 3' and 4', as well as N1-substituted analogs possesing the α-hydroxy-γ-aminobutyryl amide (HABA) chain of butirosin, could produce promising antibiotics. Chapter 4 of this dissertation features the conception and development of an expedient semi-synthetic strategy to access novel aminoglycosides of the 4,5-disubstituted class, inspired from biosynthetic modifications of the sisomicin subfamily, that surmount

the wide-spread bacterial resistance mechanisms. This synthetic methodology relies on a novel Tsuji palladium-catalyzed hydrogenolysis developed on model monosaccharides, which was applied to generate a library of aminoglycosides comprising ring A hybrids of the neomycin and sisomicin families. The structure-activity relationships of this new class were assessed against a panel of 26 bacterial strains expressing modifying enzymes and efflux systems to provide an overview of *ESKAPE* pathogens. Two novel hybrid aminoglycoside analogs exhibited excellent antibacterial coverage, and may be promising candidates for preclinical development.

Aminoglycoside therapy is also invariably associated with a probability of nephrotoxic complications. Aminoglycoside toxicity has been largely correlated with the number of amino groups, and more loosely with the extent of deoxygenation. A long standing hypothesis in the field states that because the foremost interactions are effected by ammonium group salts, the tuning of pK_a parameters could provide a higher target dissociation rate, more effective clearance and overall less nephrotoxic analogs. Chapter 5 in this dissertation features the conception and asymmetric synthesis of isosteric β-substituted N1-HABA chains, modified by *mono*- and *bis*-fluorination. These chains covering a range of γ-N-pKa values from 10 to 7.5 were applied to advanced tetra-deoxygenated neomycin antibiotics. In spite of the important reduction in γ-N-pKa, broad-spectrum antimicrobial activity was not significantly disrupted for isosteric fluorinated analogs. Furthermore, structure-toxicity relationships, assessed by Achaogen's proprietary luciferase-coupled apoptosis assay, revealed that the novel β,β-difluoro-N1-HABA chain is less harmful in a Human Kidney 2 cell-line model and promising for the development as new generation neomycin antibiotics with improved therapeutic properties.

The final chapter in this dissertation features the proposal and validation of the concise biomimetic synthesis and self-assembly of *aminoglycoside 66-40C*, a remarkable C_2-symmetric 16-membered macrocyclic *bis*-imine dimer. The proposed structure was spectroscopically characterized as an anti-parallel *s-trans-bis*-azadiene macrocyclic system. Calculations indicate the anomeric effect of the α-glycosidic bond between rings A and B is important for pre-organization of the monomeric sisomicin 6'-aldehyde and favors the observed macrocycle product. Self-assembly in aqueous solutions was studied through the dimerization of three diverse analogs and cross-over experiments, which demonstrated the generality and stability of the macrocyclic motif of *aminoglycoside 66-40C*.

Keywords

aminoglycoside, antibiotic, neomycin, sisomicin, deoxygenation, Tsuji hydrogenolysis, HABA, fluorination, biomimetic, self-assembly.

Résumé

Les antibiotiques aminoglycosidiques sont des agents bactéricides de grande valeur et d'efficacité à large spectre contre les pathogènes Gram-positifs et Gram-négatifs, dont plusieurs membres naturels et semisynthétiques sont importants dans l'histoire clinique depuis 1950. Des travaux crystallographiques sur le ribosome, récompensés par le prix Nobel, ont démontré comment leurs diverses structures polyaminées sont adaptées pour cibler une hélice d'ARN dans le centre de codage de la sous-unité 30S du ribosome bactérien. Leur interférence avec l'affinité et la cinétique des étapes de sélection et vérification des tARN induit la synthèse de protéines à basse fidélité, et l'inhibition de la translocation, établissant un cercle vicieux d'accumulation d'antibiotique et de stress sur la membrane. En réponse à ces pressions, les pathogènes bactériens ont évolué et disséminé une panoplie de mécanismes de résistance enzymatiques et d'expulsion : tels que les *N*-acétyltransférases, les *O*-phosphotransférases et les *O*-nucleotidyltransférases qui ciblent les groupements hydroxyle et amino sur le coeur des aminoglycosides; des méthyl-transférases, qui ciblent le site de liaison ribosomale; et des pompes d'expulsion actives pour l'élimination sélective des aminoglycosides, qui sont utilisés par les souches Gram-négatives.

Les pathogènes les plus problématiques, qui présentent aujourd'hui une forte résilience envers la majorité des classes d'antibiotiques sur le bord de la *pan*-résistance ont été nommés des bactéries *ESKAPE*, une mnémonique pour *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* et *Enterobacteriaceae*. La distribution globale des souches avec des mécanismes de résistance envers les standards cliniques aminoglycosides, tels que la tobramycine, l'amikacine et la gentamicine, est comprise entre 20 et 60% des isolées cliniques. Ainsi, les aminoglycosides du type 4,6-disubstitués-2-deoxystreptamine sont inadéquats comme thérapies anti-infectieuses à large spectre.

Cependant, la famille des aminoglycosides 4,5-disubstitués, incluant la butirosine, la neomycine et la paromomycine, dont la structure plus complexe, pourrait constituter une alternative. Des collègues dans le groupe Hanessian et collaborateurs d'Achaogen Inc. ont démontré que certains analogues de la paraomomycine et neomycine, modifiés par désoxygénation sur les positions 3' et 4', et par substitution avec la chaîne N1-α-hydroxy-γ-

aminobutyramide (HABA) provenant de la butirosine, pourrait produire des antibiotiques très prometteurs. Le Chapitre 4 de cette dissertation présente la conception et le développement d'une stratégie semi-synthétique pour produire des nouveaux aminoglycosides améliorés du type 4,5-disubstitués, inspiré par des modifications biosynthétiques de la sisomicine, qui frustrent les mécanismes de résistance bactérienne distribuées globalement. Cette voie de synthèse dépend d'une réaction d'hydrogénolyse de type Tsuji catalysée par palladium, d'abord développée sur des modèles monosaccharides puis subséquemment appliquée pour générer un ensemble d'aminoglycosides hybrides entre la neomycine et la sisomicine. Les études structure-activité des divers analogues de cette nouvelle classe a été évalué sur une gamme de 26 souches bactériennes exprimant des mécanismes de résistance enzymatique et d'expulsion qui englobe l'ensemble des pathogènes *ESKAPE*. Deux des antibiotiques hybrides ont une couverture antibacterienne excellente, et cette étude a mis en évidence des candidats prometteurs pour le développement préclinique.

La thérapie avec les antibiotiques aminoglycosidiques est toujours associée à une probabilité de complications néphrotoxiques. Le potentiel de toxicité de chaque aminoglycoside peut être largement corrélé avec le nombre de groupements amino et de désoxygénations. Une hypothèse de longue date dans le domaine indique que les interactions principales sont effectuées par des sels des groupements ammonium, donc l'ajustement des paramètres de pK_a pourrait provoquer une dissociation plus rapide avec leurs cibles, une clairance plus efficace et globalement des analogues moins néphrotoxiques. Le Chapitre 5 de cette dissertation présente la conception et la synthèse asymétrique de chaînes N1-HABA β-substitutées par *mono-* et *bis*-fluoration. Des chaînes qui possèdent des γ-N-pKa dans l'intervale entre 10 et 7.5 ont été appliquées sur une neomycine tétra-désoxygénée pour produire des antibiotiques avancés. Malgré la réduction considérable du γ-N-pKa, le large spectre bactéricide n'a pas été significativement affecté pour les analogues fluorés isosteriques. De plus, des études structure-toxicité évaluées avec une analyse d'apoptose propriétaire d'Achaogen ont démontré que la nouvelle chaîne β,β-difluoro-N1-HABA est moins nocive sur un modèle de cellules de rein humain HK2 et elle est prometteuse pour le développement d'antibiotiques du type neomycine avec des propriétés thérapeutiques améliorées.

Le chapitre final de cette dissertation présente la proposition et validation d'une synthèse biomimétique par assemblage spontané du *aminoglycoside 66-40C*, un dimère C_2-symétrique *bis*-imine macrocyclique à 16 membres. La structure proposée du macrocycle a été affinée par spectroscopie nucléaire à un système *trans,trans*-*bis*-azadiène anti-parallèle. Des calculs indiquent que l'effet anomérique de la liaison α-glycosidique entre les anneaux A et B fournit la pré-organisation pour le monomère 6'-aldéhydo sisomicine et favorise le produit macrocyclique observé. L'assemblage spontané dans l'eau a été étudié par la dimérisation de trois divers analogues et par des expériences d'entre-croisement qui ont démontré la généralité et la stabilité du motif macrocyclique de l'*aminoglycoside 66-40C*.

Mots clés

aminoglycoside, antibiotique, neomycine, sisomicine, désoxygénation, hydrogénolyse de Tsuji, HABA, fluorination, biomimétique, assemblage spontané.

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Abbreviations

AAC	aminoglycoside *N*-acetyltransferase
Ac-CoA	acetyl coenzyme A
ADPNP	adenylyl-β-imidodiphosphate
Amk	amikacin
ANT	aminoglycoside *O*-nucleotidyltransferase
APH	aminoglycoside *O*-phosphotransferase
armA	ribosome methyltransferase 30S N1-G1405, gene armA
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
AUC	area under the curve
Btr	butirosin
CAM	ceric ammonium molybdate
Cbe	carboxyethyl
Cbz	carboxybenzyl
CHARMM-GUI	Chemistry at HARvard Macromolecular Mechanics - Graphical User Interface
CID	Center for Infectious Diseases
CLND	Chemiluminescent Nitrogen Detection
COSY	correlation spectroscopy
Cpx	Cpx A/R two-component signal transduction pathway of E. coli which regulates gene transcription
DAST	diethylaminosulfur trifluoride
dba	dibenzylideneacetone
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCL	dynamic combinatorial library
DCM	dichloromethane
D-HABA	*D*-(*R*)-4-amino-2-hydroxybutanoyl (or acid)
DHP	3,4-dihydro-[2H]-pyran
DIBAL	diisobutylaluminium hydride
DIPEA	*N,N*-diisopropylethylamine (or Hünig's base)
DIPHOS	1,2-bis(diphenylphosphino) ethane
DIPT	diisopropyl tartrate
DMAP	*N,N*-dimethyl-4-aminopyridine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPPA	diphenylphosphoryl azide
EC50	effective concentration for half-maximum activity
ECDC	European Centre for Disease Prevention and Control
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDP-I and II	energy dependent phase I and II
EF-Tu	elongation factor thermo unstable
EIP	energy independent phase
EMEA	European Medicines Agency
ESI	electrospray ionization
ESKAPE	mnemonic for bacterial pathogens: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacteriaceae*
FDA	Food and Drug Administration
FRET	Förster resonance energy transfer
FT-IR	Fourier transform infra-red
FtsH	membrane-bound ATP-dependent zinc metalloprotease
GDP	guanosine diphosphate
Gent	gentamicin complex
GTP	guanosine triphosphate
HABA	(*S*)-4-amino-2-hydroxybutanoyl (or acid)
HAPA	(*S*)-3-amino-2-hydroxypropanoyl (or acid)
HFBA	heptafluorobutyric acid
HMBC	heteronuclear multiple bond coherence
HMQC	heteronuclear multiple quantum coherence
H-Par	N1-HABA paromomycin
HPLC	high pressure liquid chromatography
HRMS	high resolution mass spectrometry
HSQC	heteronuclear single quantum coherence
IDSA	Infectious Diseases Society of America
iOWH	Institute of One World Health
KHMDS	potassium bis(trimethylsilyl)amide
L	ligand
LCMS	liquid chromatography mass spectrometry
LC-MSD	liquid chromatography - mass spectrometry detection
LG	leaving group
L-HABA	*L*-(*S*)-4-amino-2-hydroxybutanoyl (or acid)
LRMS	low resolution mass spectrometry
M-H	metal hydride
MIC	minimum inhibitory concentration

MIC-90	minimum inhibitory concentration for 90% of strains
mit-rRNA	mitochondrial ribosomal RNA
MOM	methoxymethyl
mRNA	mitochondrial ribonucleic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
Ms	methanesulfonyl
NADH	nicotinamide adenine dinucleotide
NeoB	neomycin B
NHS	*N*-hydroxysuccinimide
NIAID	National Institute of Allergy and Infectious Diseases (US)
nmpA	ribosome methyltransferase 30S N7-A1408, gene nmpA
nOe	nuclear Overhauser effect
Nu	nucleophile
ORTEP	Oak Ridge thermal Ellipsoid Program
Par	paromomycin
PCR	polymerase chain reaction
PDB	Protein Data Bank
PDF	Portable Document Format
py	pyridine
Ribo	ribostamycin
RMT	ribosome methyltransferase
rmtA-D	ribosome methyltransferase 30S N1-G1405, genes rmtA-D
RNA	ribonucleic acid
RND	resistance-nodulation-division gene family
RRF	ribosome release factor
rRNA	ribosomal ribonucleic acid
RT	room temperature
SAR	structure-activity relationship
Sec	Sec complexes, a major route of protein translocation across cell membranes
SENTRY	Antimicrobial Surveillance Program - JMI Laboratories
TBAF	tetra-n-butylammonium fluoride
TBAI	tetrabutylammonium iodide
TBS	*tert*-butyldimethylsilyl
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxy radical
TES	triethylsilyl
Tf	trifluoromethanesulfyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TOF	time of flight
tRNA	transfer ribonucleic acid
UV	ultra-violet
WHO	World Health Organization

Acknowledgements

I acknowledge Achaogen Inc. for financial support and *Fonds de la Recherche en Santé du Québec* (FRSQ) for a two-year fellowship.

This dissertation has taken a number of particularly interdisciplinary directions and it is certain that the bulk of this work would not have been achieved without the support of numerous talented colleagues.

Nuclear Magnetic Resonance support and 700 MHz acquisitions were performed by Dr. Phan viet Minh Tan, Dr. Sylvie Bilodeau and Dr. Cédric Malveau. I acknowledge the excellent heteronuclear correlation experiments and outstanding facilities.

Mass Spectrometry support and High-Resolution acquisitions were performed by Dr. Alexandra Furtos, Marie-Christine Tang and Karine Venne. I thank them for the outstanding level of services in the MS laboratory and Alexandra for performing the challenging LCMS analysis in Chapter 6.

Crystallographic X-ray acquisition and analysis were performed by the talented Ph.D. student Benoît Deschênes-Simard.

I acknowledge the helpful organization of the project and intellectual exchange with Achaogen scientists Adam A. Goldblum, Dr. Paola Dozzo, Dr. James Aggen, Dr. Martin Linsell and chemistry vice president Dr. Heinz Moser. I thank all the members of the *Aminoglycoside Project* of Achaogen for the enriching internship in San Francisco and a warm welcome to the lab by Timothy Kane, Darin Hildebrandt and Micah Gliedt.

Antibacterial testing, tissue culture and caspase assays were performed in Achaogen Inc. I acknowledge the high standard of science in their respective departments to Dr. Heinz Moser, Dr. George Miller and Dr. Eliana Armstrong.

I acknowledge the unconditional personal support throughout this work and also the careful review this dissertation to my loving wife Nicole Darricarrère.

I am very thankful to the contemporary colleagues of the *Aminoglycoside Team* for training and enriching exchange of ideas and protocols: Alexandre Guiguère, Dr. Justyna Grzyb and Dr. Janek Szychowski. Janek was an outstanding mentor during my early training with aminoglycosides.

Many thanks to Professor Jeffrey W. Keillor for useful discussions and encouragement.

I deeply thank all the contemporary Hanessian group members for their friendship and intellectual challenges throughout these years: Alexandre Giguère, Dr. Amy Xueling Mi, Dr. Andreas Larsson, Annmaria Baraldi, Dr. Benjamin Schroeder, Benoît Deschênes-Simard, Dr. Bradley Merner, Carol Major, Elaine Fournelle, Dr. Elias J. Stoffman, Étienne Chenard, Dr. Janek Szychowski, Jeremie Tessier, John Zhang Jianbin, Dr. Justyna Grzyb, Leo Bin Chen, Dr. Luciana Auzzas, Ludivine Riber, Nancy Xiao Tian Wang, Dr. Nicolas Boyer, Michele Ammouche, Dr. Ramkrishna Reddy Vakiti, Riccardo Matera, Rupal Oza, Dr. Sébastien Guesne, Stefano Rizzo, Stéphane Dorich, Dr. Thilo Focken, Dr. Thomas Jennequin, Dr. Uday Kumar Soma, Dr. Vincent Babonneau and Vu Linh Ly. I underline the particularly helpful contributions of Dr. Janek Szychowski, Dr. Nicolas Boyer, Benoît Deschênes-Simard, and Dr. Bradley Merner for frequent and useful discussions.

Finally and inclusively, I thank Professor Stephen Hanessian for his faith, outstanding mentorship and guidance.

Thank you all most sincerely.

Chapter 1

Introduction to Aminoglycoside Antibiotics: Structure, Nomenclature and Biosynthesis

1.1 - Discovery of Aminoglycosides

Aminoglycoside antibiotics are among the most ancient weapons in the ubiquitous microbial warfare through natural products.[1] Our knowledge of them dates back to the mid 20th century, post Second World War, shortly following the introduction of penicillin, the first β-lactam antibiotic, isolated by Alexander Fleming.[2] Streptomycin was found in 1943 by Albert Schatz, a graduate student in the laboratory of Selman A. Waksman at Rutgers University, New Jersey.[3] Waksman studied actinomycetes and the "antagonistic relationships of microorganisms"[4] and systematically developed isolation and screening methods for antibiotics, among which streptomycin and neomycin were identified.[3,5] Streptomycin would claim its place in history as the first effective treatment against *Mycobacterium tuberculosis*, fuelling the ongoing revolution in medicinal science and clinical practice. For his monumental contributions Waksman was awarded the Nobel prize in Physiology and Medicine in 1952.[6] The following two decades, 1950-1970, would witness the isolation of a vast series of natural aminoglycoside antibiotics from the soil microbial flora (Table 1.1). And in the next decade, 1970-1980, the efforts would turn to the preparation of the first generation of improved semisynthetic analogs (Table 1.2).

Table 1.1. Prominent examples of natural aminoglycosides from soil organisms.

Aminoglycoside	Microorganism	Year	Isolation
Streptomycin	*Streptomyces griseus*	1943	Waksman *et al*[3]
Neomycin B	*Streptomyces fradiae*	1949	Waksman *et al*[5]
Kanamycin A, B and C	*Streptomyces kanamyceticus*	1957	Umezawa *et al*[7]
Paromomycin	*Streptomyces rimosus*	1959	Parke, Davis & Co[8]
Gentamicin C complex	*Micromonospora purpurea*	1963	Schering-Plough[9]
Tobramycin	*Streptomyces tenebrarius*	1967	Eli-Lilly[10]
Ribostamycin	*Streptomyces ribosidificus*	1969	Meiji Seika[11]
Butirosin	*Bacillus circulans*	1970	Parke, Davis & Co[12]
Sisomicin	*Micromonospora inyoesis*	1970	Schering-Plough[13]
Lividomycin A and B	*Streptomyces lividus*	1970	Kowa Ltd.[14]

Table 1.2. Clinically-relevant semisynthetic aminoglycosides.

Aminoglycoside	Parent	Modification	Year	Developers[15]
Dibekacin	Kanamycin B	3',4'-dideoxy	1971	Meiji Seika[16]
Amikacin	Kanamycin A	N1-HABA	1972	Bristol-Myers[17]
Arbekacin	Dibekacin	N1-HABA	1973	Meiji Seika[18]
Netilmicin	Sisomicin	N1-Ethyl	1976	Schering-Plough[19]
Isepamicin	Gentamicin B	N1-HAPA	1978	Schering-Plough[20]

1.2 - General Aminoglycoside Nomenclature and Representation

Aminoglycosides are vastly heterogeneous in sugar structure, connectivity, substitution and stereochemistry. Hence, the field has developed a specific jargon, nomenclature and numbering which has its roots in the formal carbohydrate nomenclature.[21] Throughout the text in this work, the rings of aminoglycosides are referenced with letters, prioritizing the common core of glucosamine (ring A) and 2-deoxystreptamine (ring B) (Figure 1.1), while other authors in the field favour Roman numbers in the same sequence.

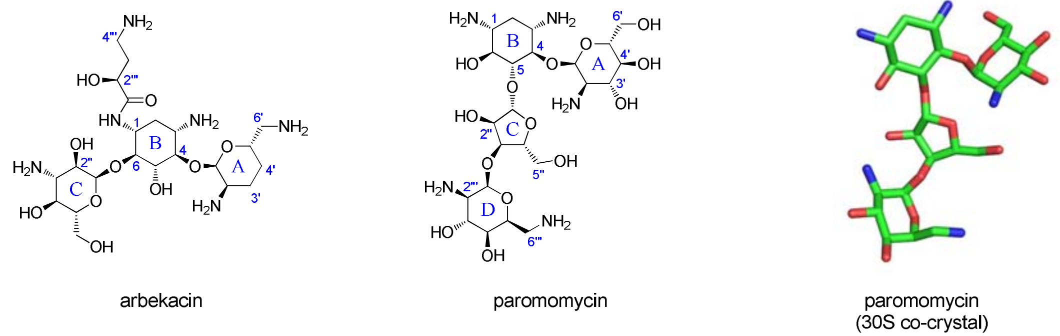

Figure 1.1. Basic numbering and representation of two classes of aminoglycoside antibiotics.

Aminoglycoside nomenclature is based on the most similar parent antibiotic as in carbohydrate rules, indicating modifications by a numbering and priming convention. The carbon numbering follows that of carbohydrates, but priming priority does not follow the aforementioned informal labelling of the rings (Figure 1.1). The 2-deoxystreptamine (ring B) is assigned first priority, thus numbered without priming, and next priority is always assigned to the ring attached at the 4 position (ring A). Priming priority then follows the sequence of any other rings attached to the 2-deoxystreptamine

at either position 5 or 6 and any others attached thereafter (e.g. Lividomycin A, Figure 1.4). In this work, this convention is also extended to label appendages on N1, assigned the lowest priority. Hence, we would name the semisynthetic aminoglycoside arbekacin as 1-*N*-((*S*)-4'''-amino-2'''-hydroxybutanoyl)-3',4'-dideoxy-kanamycin B (Figure 1.1).

The representation of aminoglycoside structures in two dimensions follows no set of rules. In the early days they were usually drawn as Haworth projections and in patent literature they are most often depicted flat without stereochemistry. In modern literature, either chair representations or Natta projections dominate, but the portrayed conformers and view-points can be very diverse. Throughout this work, the representation of aminoglycosides is inspired from the conformations observed in their target site within co-crystals of the 30S ribosomal subunit (Figure 1.1).[22,23] Taking into account the aesthetic value of straight angles and space economy, a slightly-tilted top-view from the A-site is optimal to clearly depict the biologically relevant conformations for several classes of aminoglycosides and their appendages (Figure 1.1).

1.3 - Aminoglycoside Classification and Structure

Several schemes of classification would be possible for such a diverse group of natural products. An old classification permeates in the antibiotic names: the suffix '-*mycin*' indicates isolation from the genus *Streptomyces*, '-*micin*' from *Micromonospora*, '-*osin*' from *Bacillus* (Table 1.1).[1] Aminoglycosides are most commonly classed by structural similarity, despite the fact that such classification schemes tend to exclude any information on biosynthetic kinship or mode of action. The work in this dissertation pertains to three subfamilies of aminoglycosides that are related structurally, biosynthetically, as well as by their mode of action and binding site: the 4,5-di-(aminoglycosyl)-2-deoxystreptamine or neomycin subfamily (Figure 1.4) and the 4,6-di-(aminoglycosyl)-2-deoxystreptamine subfamilies including the kanamycins and gentamicins (Figures 1.3 and 1.5). However, a simpler way to visualize the relationships of the pertinent natural and semisynthetic antibiotics is a biosynthetic genealogy tree (Figure 1.2).[1]

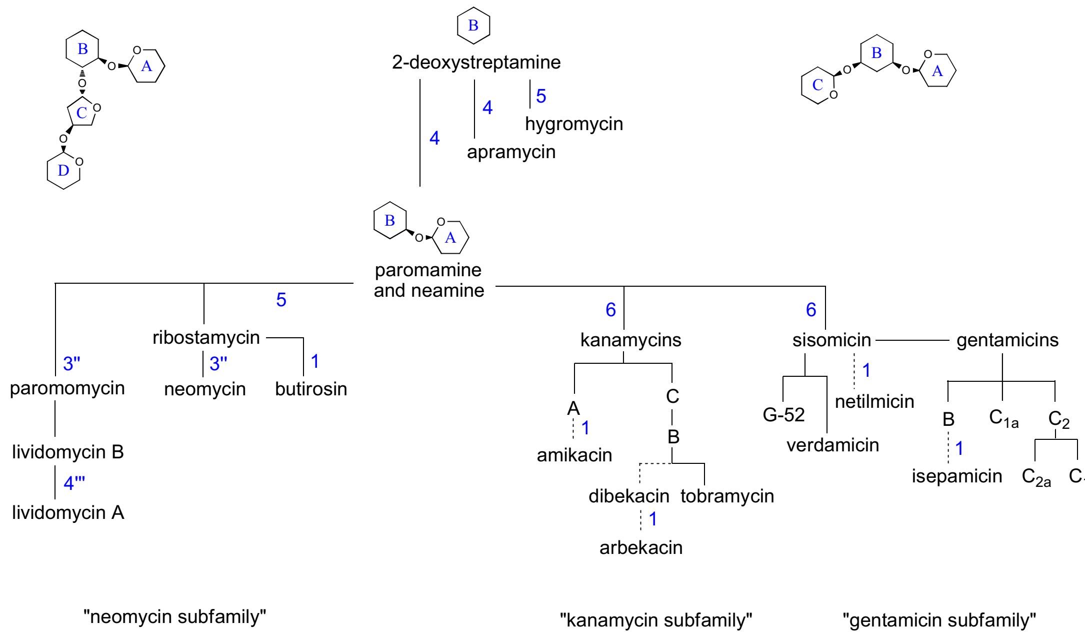

Figure 1.2. Structural and biosynthetic genealogy tree of the 2-deoxystreptamine derived aminoglycosides. The major glycosidic substitutions of aminoglycoside frameworks are indicated in blue. Semisynthetic antibiotics are indicated with doted lines. Adapted from reference 1.

Name	R^1	R^2	R^3	R^4	R^5
Kanamycin A	NH_2	OH	OH	OH	H
Kanamycin B	NH_2	OH	OH	NH_2	H
Kanamycin C	OH	OH	OH	NH_2	H
Tobramycin	NH_2	OH	H	NH_2	H
Amikacin	NH_2	OH	OH	OH	H_2N, O, OH
Dibekacin	NH_2	H	H	NH_2	H
Arbekacin	NH_2	H	H	NH_2	H_2N, O, OH

Figure 1.3. Structures of aminoglycosides in the "kanamycin family".[1]

Name	R^1	R^2	R^3	R^4
Ribostamycin	NH_2	OH	H	H
Butirosin	NH_2	OH	H_2N, O, OH	H
Paromomycin	OH	OH	H	H_2N, 2''', O, D, NH_2, HO, 6''', OH
Neomycin B	NH_2	OH	H	H_2N, 2''', O, D, NH_2, HO, 6''', OH
Lividomycin A	OH	H	H	H_2N, 2''', O, D, NH_2, HO, 6''', O, HO, HO, E, O, 6'''', HO, OH
Lividomycin B	OH	H	H	H_2N, 2''', O, D, NH_2, HO, 6''', OH

Figure 1.4. Structures of aminoglycosides in the "neomycin family".[1]

Representative members of other aminoglycoside families which are outside the scope of this work are shown in Figure 1.6. Nevertheless, the subfamilies of apramycin, hygromycin B, fortimicins and istamicins are somewhat related in biosynthetic, genetic and structural terms.[1]

Name	R^1	R^2	R^3	R^4	R^5	R^6
Gentamicin C_1	NHMe	Me	H	H	NH_2	H
Gentamicin C_{1a}	NH_2	H	H	H	NH_2	H
Gentamicin C_2	NH_2	Me	H	H	NH_2	H
Gentamicin C_{2a}	Me	NH_2	H	H	NH_2	H
Gentamicin B	NH_2	H	OH	OH	OH	H
Sisomicin	NH_2	H	*	H	NH_2	H
Antibiotic G-52	NHMe	H	*	H	NH_2	H
Verdamycin	NH_2	Me	*	H	NH_2	H
Netilmicin	NH_2	H	*	H	NH_2	Et
Isepamicin	NH_2	H	OH	OH	OH	H_2N, O, OH
ACHN-490	$NH(CH_2)_2OH$	H	OH	OH	OH	H_2N, O, OH

* Unsaturated between positions C4' and C5'.

Figure 1.5. Structures of aminoglycosides in the "gentamicin family".[1]

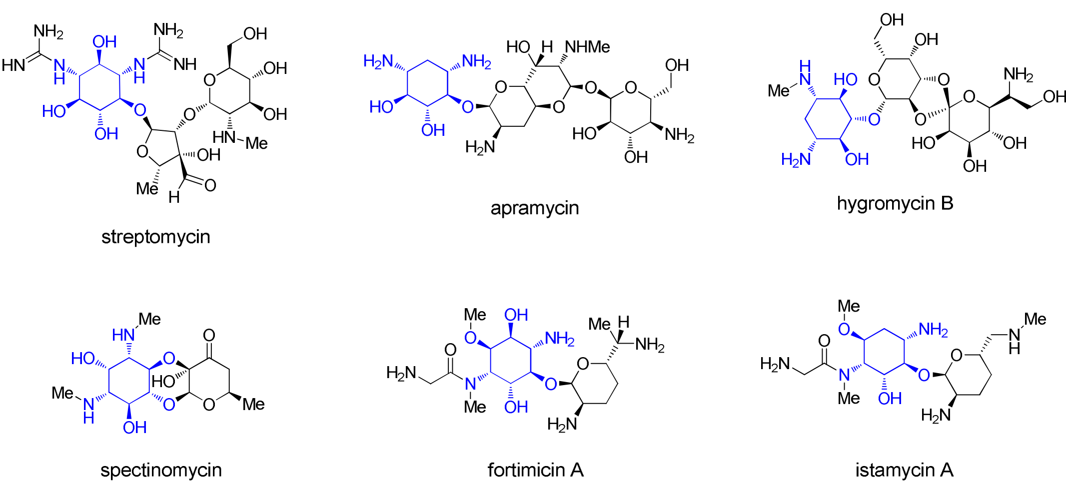

Figure 1.6. Representative aminoglycoside antibiotics not classified in the neomycin, kanamycin or gentamicin subfamilies. Their characteristic carbocyclic rings are highlighted in blue.[1]

1.4 - Aminoglycoside Biosynthesis

Despite the complex diversity of aminoglycoside structure, composition and stereochemistry, most of the carbon content can be directly traced back to glucose, or otherwise related metabolites from the main carbohydrate pathways (e.g. pentose phosphate shunt), such as fructose, xylose or mannose.[24,25,26] A representative example is lividomycin A, composed of five different carbohydrates (Figure 1.4).[1]

Most structural features of the final antibiotic framework are introduced in the precursors of the glycosidation steps. The elaboration of aminosugar precursors can include few simple steps, such as oxidation/amine transfer on C2 of glucose to furnish glucosamine (Figure 1.7) or a more complex series of steps, including a Ferrier type 2 rearrangement, which produces the carbocyclic ring of 2-deoxystreptamine (Figure 1.7).[1,24-26] These two essential precursors, glucosamine and 2-deoxystreptamine, are coupled to produce paromamine (Figure 1.7), which can be considered the structural and biosynthetic cornerstone of the aminoglycoside subfamilies studied herein (Figure 1.2).[24-26] Other classes, such as streptomycin (Figure 1.6), are assembled with a deceivingly similar ring B named streptamine (or streptidine when including the two guanidine groups), which is a derivative of *myo*-inositol obtained by a different aldol-type rearrangement of glucose.[24-26]

Figure 1.7. Biosynthetic pathways of glucosamine and 2-deoxystreptamine, the precursors of paromamine. DH = dehydrogenase, AT = aminotransferase.[24-26]

Crucial biosynthetic elaboration of the primordial aminoglycoside frameworks also occurs post-glycosidation, including oxidation/amino-transfer, epimerization, N- and C-methylation, N1-acylation, dehydration and deoxygenation (Figure 1.8).[1,24-26] Notably, when less elaborate precursors have been isolated as minor fermentation by-products or from biosynthetic mutants, they are invariably weaker antibiotics.

Figure 1.8. Representative examples of biosynthetic modifications (colored and labeled positions) occurring post-glycosidation of the aminoglycoside frameworks.

1.5 - Biosynthetic Deoxygenations of Ring A

The biosynthetic pathways of most aminoglycoside families have been well characterized.[24-26] Initially this characterization relied on feeding labeled precursors to monitor biotransformation, and later on classic genetic analysis of the biosynthetic operons and gene clusters through mutation and complementation studies.[1,24-26] An important driving force for this field has been the possibility of bioengineering improved antibiotics.[27] Paradoxically, the deoxygenation steps are very poorly understood despite being one of the most clinically-relevant biosynthetic transformations (Figure 1.3 – 1.5). This type of biotransformation is given specific attention herein due to the relevance for this work, both in terms of the naturally-available SAR and strategic inspiration for semisynthetic methodologies (Chapter 4).

The enzymatic activities for deoxygenation of aminoglycosides appear to have evolved twice independently.[1,24-26] One instance corresponds to the single

deoxygenation at 3' by the producers of lividomycins A and B, and the tobramycin/apramycin complex. The other instance corresponds to the double deoxygenations at 3' and 4' in the sisomicin and gentamicin subfamily, which share both genetic and structural homology with the family of the fortimicins and istamicins (Figure 1.2).

The better understood case is the single 3'-deoxygenation on the glucosamine derived rings. In tobramycin, it is thought to occur as a last biosynthetic step on kanamycin B by an enzyme belonging to the apramycin gene cluster.[1] These structurally dissimilar antibiotics are co-produced, and both are 3'-deoxygenated (Figure 1.9 A). The putative deoxygenating genes have counterparts only in the lividomycins cluster, which undergo an essentially identical modification (Figure 1.9 A).[1,24-26]

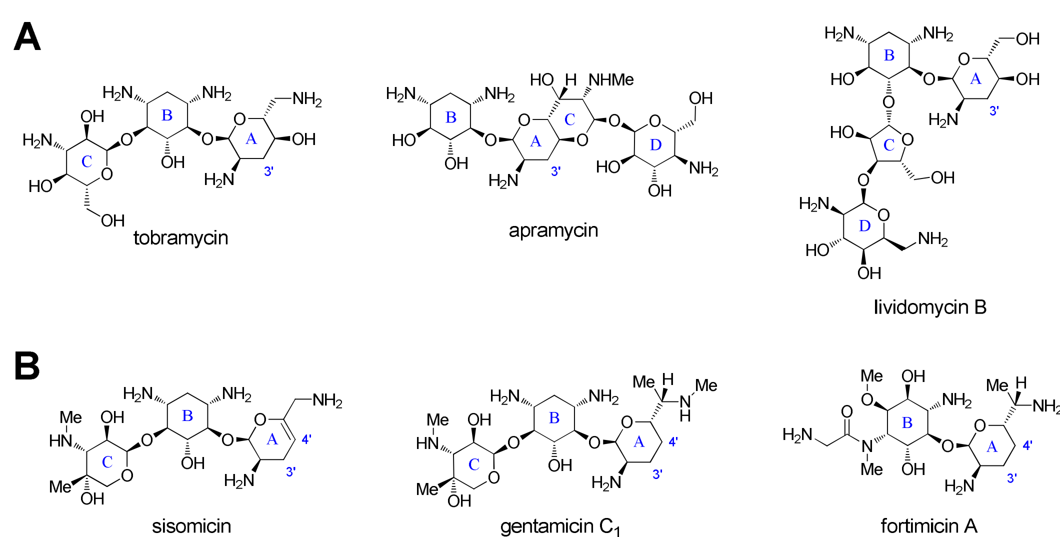

Figure 1.9. Aminoglycosides undergoing biosynthetic deoxygenations on ring A at either (A) position 3' or (B) both 3' and 4'.

On the other hand, both the genetics and enzymology of the double-deoxygenation at 3' and 4' positions remain mysterious. From preliminary biotransformation studies, a biosynthetic relationship was established between the unique 4',5'-olefin of the sisomicins (Figure 1.5) and the fully-reduced gentamicins.[28,29] Extensive mutation screens with these antibiotic producers failed to provide accumulation of 3'-deoxygenated or 4',5'-dehydrated aminoglycoside intermediates, and these pathways are usually depicted with unknowns for these last few steps.[1,24-26]

This situation is now becoming clarified due to the unexpected involvement of a 3'-phosphotransferase (related to the resistance genes discussed in Chapter 3).[30-33] Therefore, the biosynthetic pathways of 3',4'-dideoxygenation appear to require dehydration between 5'/4'-OH and activation by phosphorylation of 3'-OH, transforming the known precursors (e.g. JI-20A) into the primordial sisomicin ring A motif (Figure 1.10). These proposals may remain hypothetical until feeding studies with labeled precursors are conducted; however, consideration of the plausible pathways and mechanisms served as valuable inspiration for the development of synthetic methodology towards improved aminoglycoside antibiotics in the context of this work (Chapter 4).

Figure 1.10. A plausible 3',4'-dideoxygenation pathway accounting for 4',5'-dehydration and 3'-phosphorylation from precursor JI-20A to sisomicin by unassigned genes from references 30-31.

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Chapter 2

Mode of Action of Aminoglycoside Antibiotics: Ribosomal Target, Bactericidal Effects and Uptake

2.1 - Aminoglycoside Interactions with the Ribosome

The ribosome is a large (2.5 megaDalton) dynamic ensemble of catalytically active rRNA components (namely 5S, 16S, and 23S) embedded with over 50 proteins of structural and regulatory functions.[1] It is organized in two subunits, the 30S and 50S, which work in concert as a template-directed macromolecular catalyst for processive peptide synthesis (Figures 2.1 and 2.2). As a central pillar of cellular metabolism, the ribosome is the target of numerous unrelated classes of antibiotics, including aminoglycosides,[2] collectively accounting for over half of all known antibiotics.[3] The heavily positively-charged molecules target RNA pockets within the ribosome, where each antibiotic works by altering delicate balances between conformational states of the ribosome in fundamental processes of peptide synthesis. Most such antibiotics are known to inhibit protein synthesis by specifically blocking the translocation mechanism (e.g. tetracyclines, macrolides, oxazolidinones, chloramphenicols, reviewed in detail in reference 4). However, an effect unique to aminoglycosides is induction of error-prone protein synthesis by interfering with tRNA selection and proof-reading (Figure 2.1). [2,4]

The families of aminoglycosides that interfere with translation accuracy are the disubstituted 2-deoxystreptamine class, central to this work, and the relatively unrelated streptomycins, apramycin and hygromycin B.[2] Miscoding is, however, particularly subtle compared to translation inhibition for the former two antibiotics.[5] The crystal structures of the 30S ribosome subunit (Figure 2.2) as well as oligonucleotide models co-crystallized with several aminoglycosides have revealed that the members of the disubstituted 2-deoxystreptamine class, as well as apramycin and hygromycin B, explore overlapping binding sites in the eubacterial ribosome (Figure 2.3).[6,7,8] Streptomycin binds relatively nearby to the region of tRNA reception and codon-anticodon recognition known as the A-site.[4,7,8]

2.2 - Aminoglycoside Effects on Translation Accuracy

It has been long known that the difference in binding energy of recognition between codon and anticodon (i.e. Watson-Crick base pairing) belonging to cognate *versus* non-cognate tRNAs is too small – by orders of magnitude – to account for the high accuracy of translation (10^{-3} – 10^{-4} error frequency).[9] The proof-reading

mechanism has been subject of intense research for almost five decades, and aminoglycoside antibiotics have been instrumental for the development of this field. Molecular rationalization of both the mechanisms of proof-reading and its interference (10 – 100 fold increased error frequencies) was possible with the advent of high-resolution crystal structures of the ribosome subunits from 2000 to 2002 (Figure 2.2).[6-9] The importance of this work is highlighted by the recent Nobel prize awarded to key crystallographers in the field.[10] More recently, the detailed investigation of ribosomal functions has advanced towards single-molecule level FRET measurements.[11,12,13]

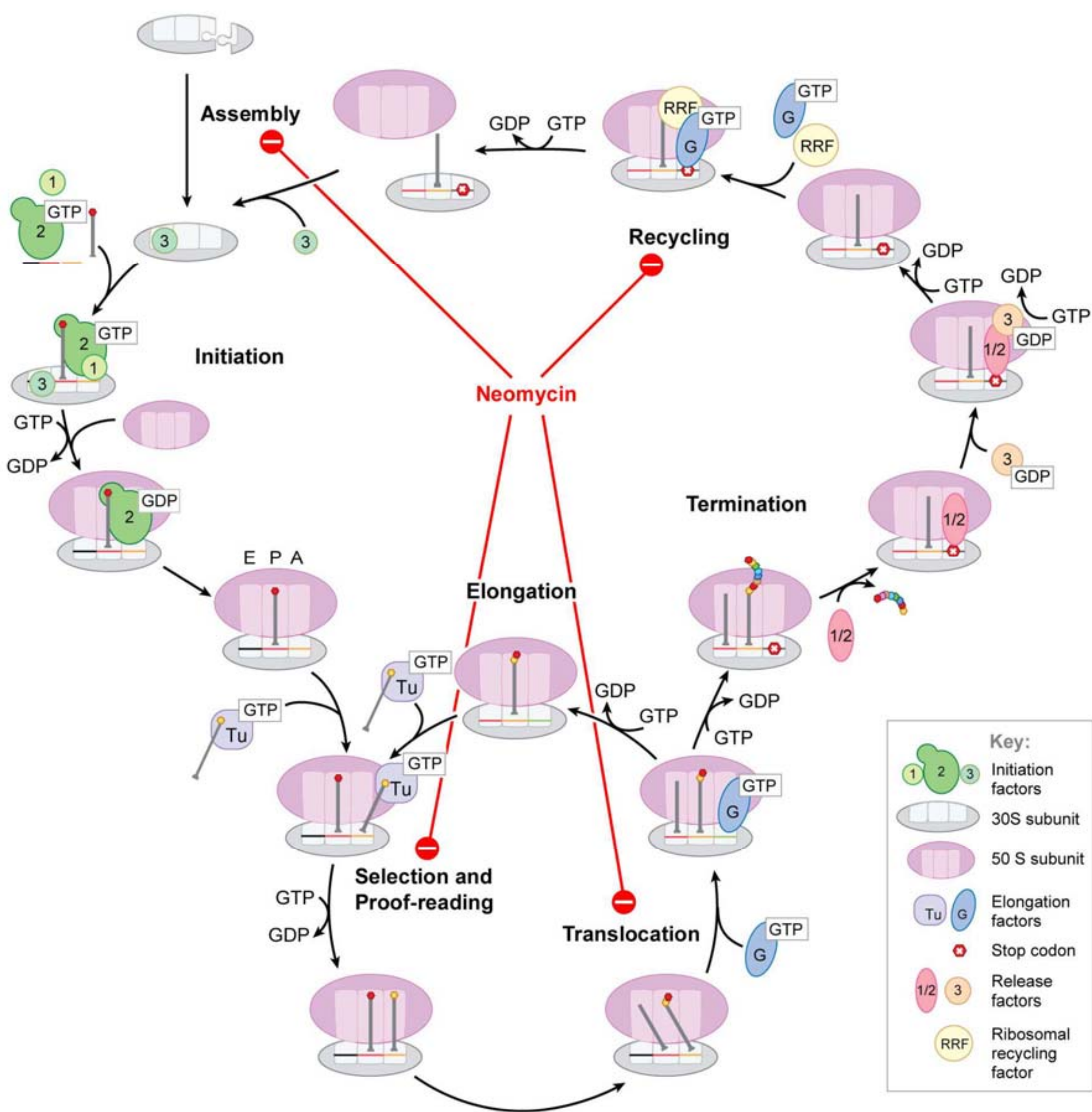

Figure 2.1. Representation of the translation cycle. The interference of neomycin on a number of ribosomal processes is highlighted to exemplify the effects of aminoglycoside antibiotics. The elongation cycle is shown in higher detail in Figure 2.4. Image adapted from reference 11.

It is thought that the ribosome achieves high translation accuracy employing repetition of discrimination steps separated by irreversible processes (Figure 2.4).[9,14] This is known as “kinetic proofreading”, endowing *n* steps with *s* selectivity a geometric enhancement of up to a s^n discrimination factor.[9,14] The ribosome is loaded with acyl-tRNAs invariably associated with Elongation Factor Tu (EF-Tu, Figure 2.4), which is in fact a tightly associated GTPase stalled with its GTP substrate. After an initial and reversible tRNA selection (k_2/k_{-2}), the GTPase activity of EF-Tu is activated by induced structural rearrangements of the ribosome (k_3) and serves as the energetic barrier separating a second tRNA exclusion event ($k_{6'}$) or its accommodation for peptide transfer (k_6). Hence, overall accuracy toward cognate *vs.* non-cognate and near-cognate tRNAs is achieved both by their different stabilities on the ribosome (differences in k_{-2}, $k_{-3'}$ and $k_{6'}$) and differentially induced forward rates of EF-Tu GTPase and tRNA accommodation (k_3 and k_6).[9,14]

Aminoglycosides of the disubstituted 2-deoxystreptamine family are thought to disrupt the finely-tuned kinetics through their interactions within helix H44 of the 30S subunit. The rRNA binding site of aminoglycosides has been well characterized by NMR,[15] high-definition ribosome structures,[6-9] and H44 oligonucleotide models,[16,17] providing a wealth of structures including co-crystallized aminoglycoside substrate variations (Figure 2.3). One of the observed effects of aminoglycoside binding within H44 is the extra-helical displacement of A1492 and A1493 by intra-helical interactions of the antibiotic (Figure 2.5 A – C).[6-9] These essential evolutionarily-conserved bases were known to be involved in decoding well before their characterization in crystal structures. Together with a highly conserved neighboring base G530, they are thought to interact with the sugar backbone of the codon-anticodon partners, recognizing and monitoring the geometry of a cognate pair independent of its sequence (Figure 2.5 D and E).[6-9] These decoding interactions are transmitted through the A-site tRNA to induce EF-Tu GTPase activity. Presumably, the favorable interactions of cognate tRNAs with mRNA and the A-site offset the barrier for stabilizing A1492 and A1493 in their extra-helical conformations. The 2-deoxystreptamine aminoglycosides interfere with H44 helix conformation through contacts of the core rings A and B, occupying the intra-helical space for these conserved adenines (Figure 2.6). These local and global structural changes (Figure 2.1)[6-7, 18] lead to enhanced affinity for non-cognate tRNAs and unbiased rates of EF-Tu activation and tRNA accommodation (Figure 2.4).[9,14]

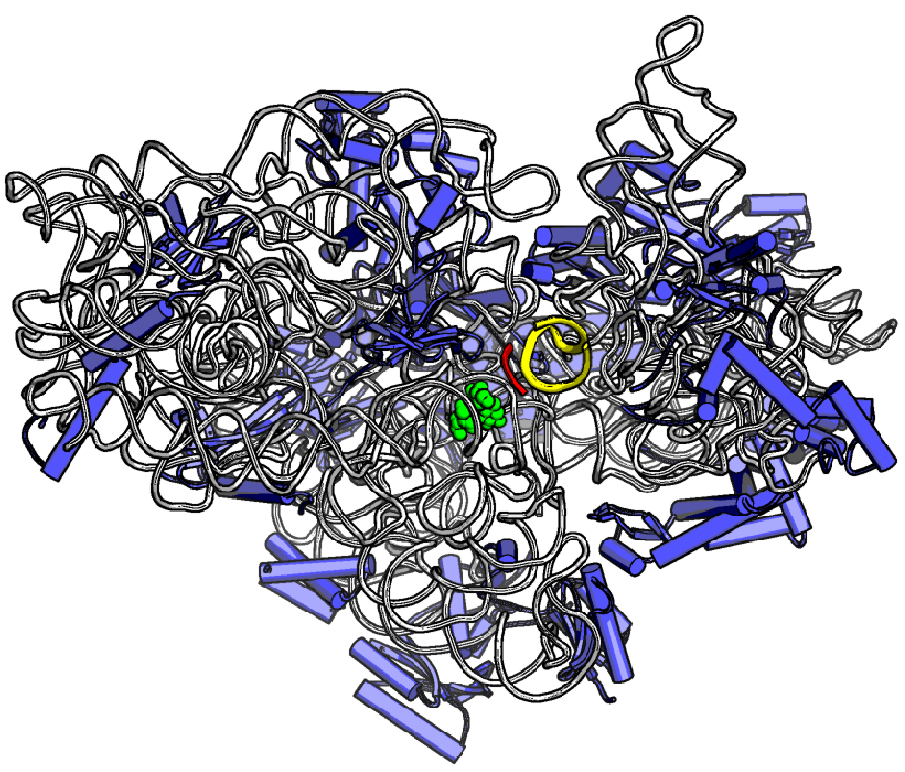

Figure 2.2. Representation of the ribosome 30S subunit (16S rRNA shown gray and associated proteins blue). A-site tRNA and mRNA fragments are shown red and yellow. Paromomycin is shown as green spheres. Adapted from reference 18, PDB entry 1IBL. Rendered in PyMol.[19]

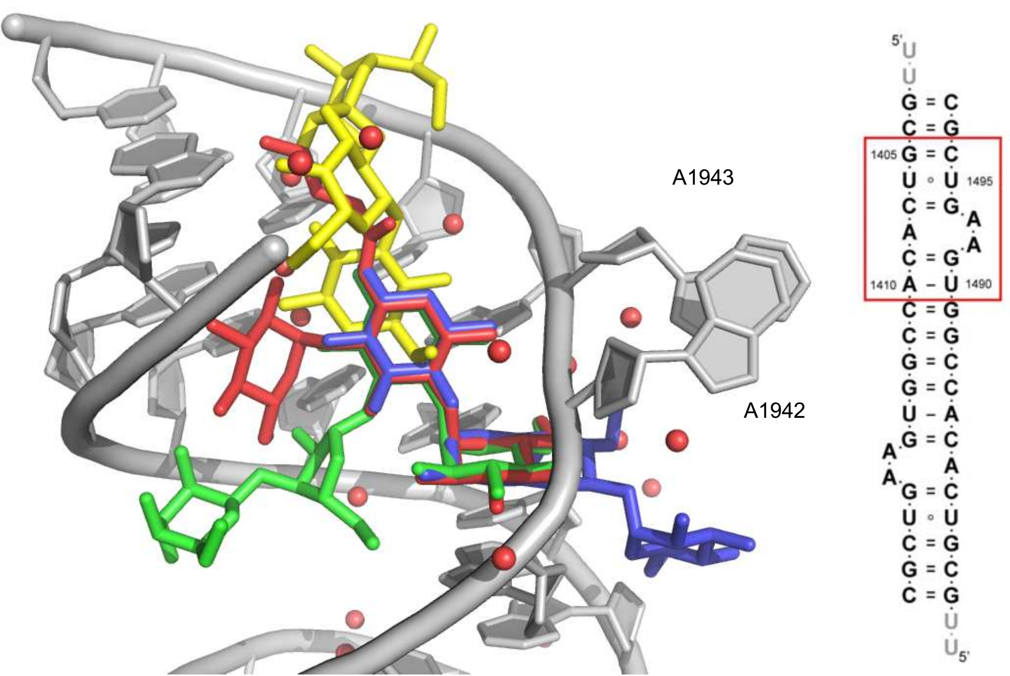

Figure 2.3. Representation of the H44 A-site model showing the overlapping binding sites of neomycin (green), amikacin (red) apramycin (blue) and hygromycin B (yellow). Respective structures compiled from references 17, 20, 21 and 8. PDB entries 2ET4, 2G5Q, 1YRJ and 1HNZ. Right: crystallized palindromic oligonucleotide with the A-site model highlighted in a red box. Rendered in PyMol.[19]

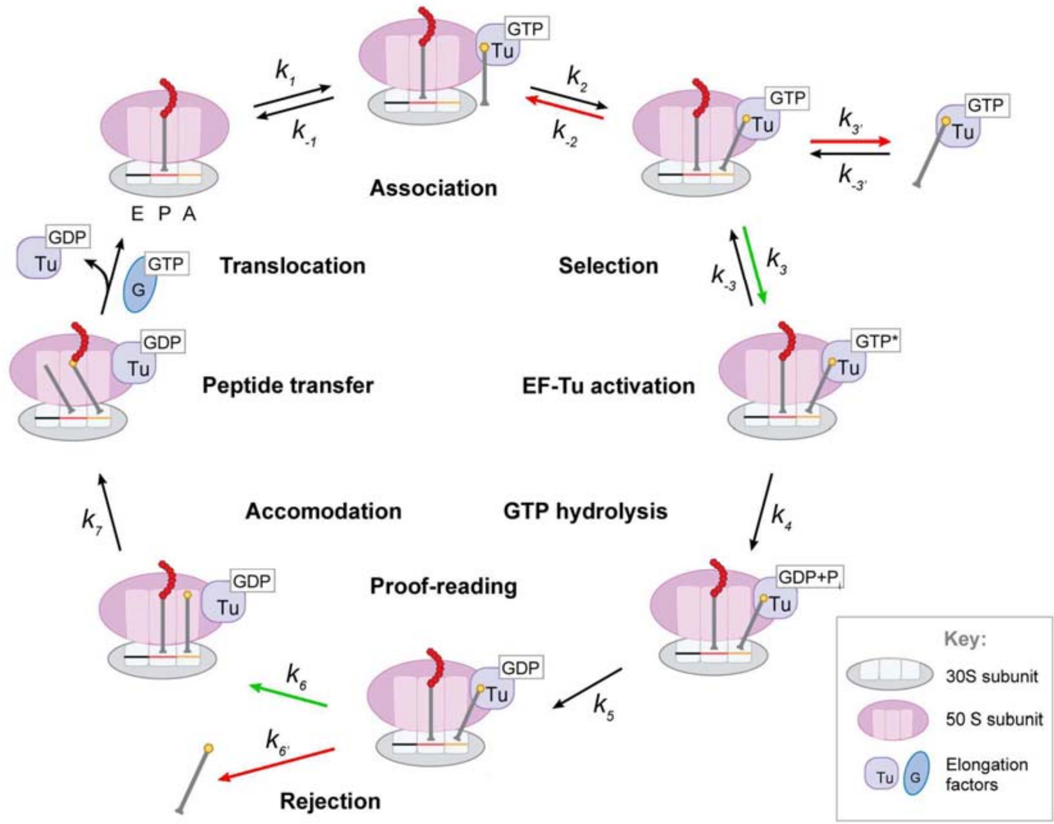

Figure 2.4. Peptide elongation cycle emphasizing reaction rates affected by aminoglycoside antibiotics. Red arrows indicate rates reduced by paromomycin through increased A-site tRNA affinity. Green arrows indicate processes altered by paromomycin through loss of rate discrimination between cognate and non-cognate tRNAs. Adapted from references 14 and 11.

The interactions of aminoglycosides within the A-site lead to slower tRNA selection and they also produce further inhibition of translocation over the mRNA template by blocking ribosome structural rearrangements after peptide transfer (Figure 2.1).[22] Moreover, the disubstituted 2-deoxystreptamine family interferes with *de novo* 30S subunit assembly[23] and post-translation ribosome recycling,[24,25] that is the separation of 30S and 50S subunits once the peptide product is released (Figure 2.1). Although inhibition of translocation may be explained through interactions with the aforementioned A-site helix H44 in the 30S subunit, the inhibition of ribosome recycling appears to be related to a second binding site in the 50S subunit H69 helix,[25] which was observed in 70S co-crystal preparations and also consistent with single-molecule FRET experiments.[13]

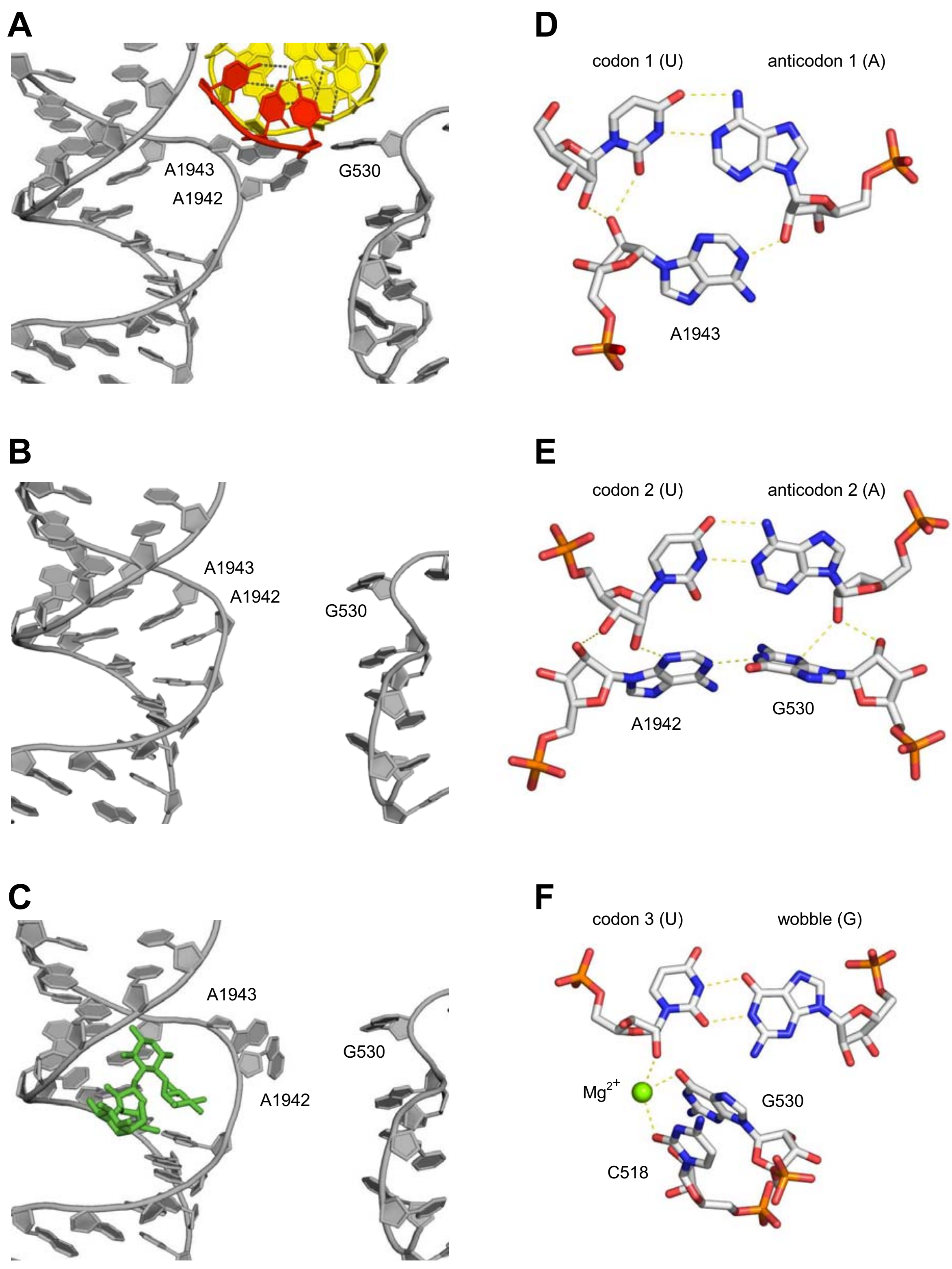

Figure 2.5. Conformations of the H44 helix and G530 loop in the 30S subunit in different crystals, (A) co-crystallized with fragment of cognate mRNA (red) and tRNA (yellow), (B) empty A-site and (C) with paromomycin (green). Panels D and E show detailed interaction contacts of panel A, between A1942, A1943, G530 and the sugar backbone of the (D) first, (E) second and (F) wobble codon-anticodon base pairs. Adapted from reference 18. Respective PDB entries are 1IBM, 1J5E and 1IBK. Rendered in PyMol.[19]

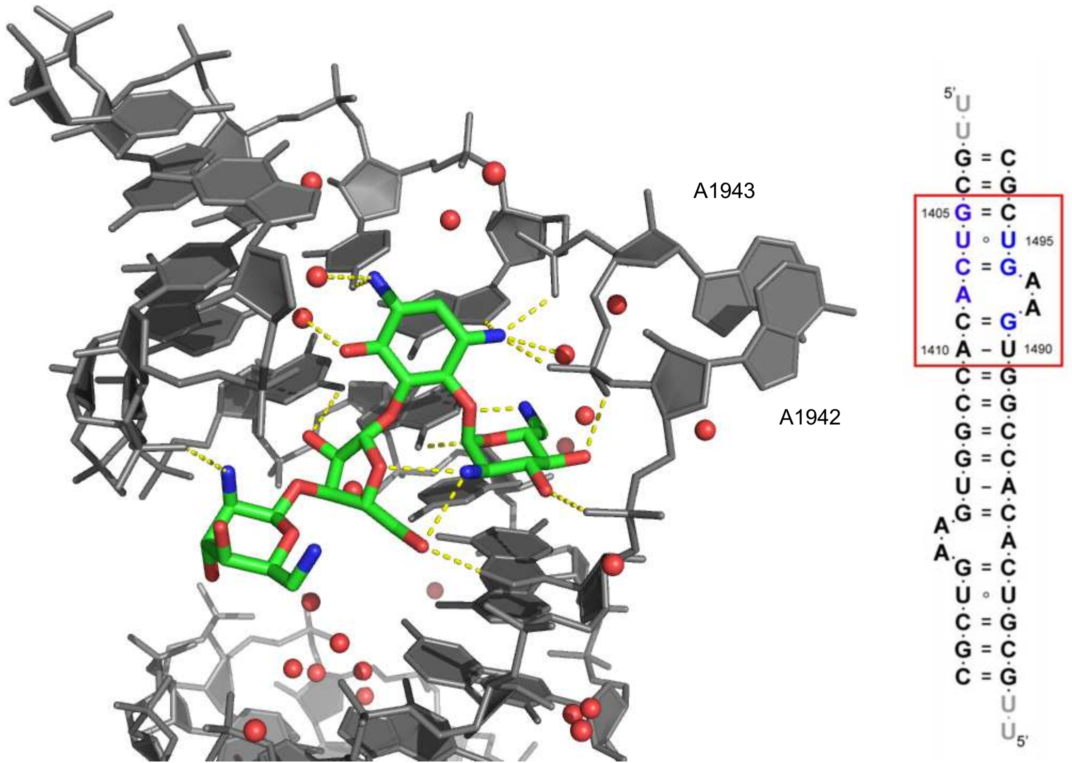

Figure 2.6. Contacts of neomycin co-crystallized within an oligonucleotide A-site H44 helix model. The H-bond and salt bridge contacts are numerous for the core rings A and B, effectively displacing adenines 1492 and 1493 to their extra-helical conformations. Right: crystallized palindromic oligonucleotide with the A-site model highlighted by the red box, and the nucleotides and phosphates (dots) making contacts with neomycin shown in blue. Adapted from reference 17, PDB entry 2ET4. Rendered in PyMol.[19]

2.3 - Bactericidal Action of Antibiotics

Despite the level of sophistication at which aminoglycoside interactions with the ribosome are understood, the mode of action of these antibiotics is incompletely understood. It still remains unknown exactly how translation inaccuracy and inhibition are connected to a *bactericidal* rather than a *bacteriostatic* effect. It is thought that many ribosome-targeting antibiotics produce their lethal effect by compromising the integrity of the inner membrane and triggering vicious cycles of membrane stress response pathways,[26,27] through inaccurate translation leading to misfolded proteins and blockage of the membrane translocation complex (Figure 2.7).[28,29] The study of the bactericidal effect of antibiotics is in resurgence.[28-30]

It is likely that the 2-deoxystreptamine aminoglycosides produce their bactericidal effect through a combination of error-prone translation and translocation inhibition.[28] Furthermore, they may prevent recovery of the affected bacterium due to their effects on ribosome recycling and 30S subunit assembly (Figure 2.1). Both misfolded transmembrane proteins and jammed translocation complexes can have toxic effects, which independently activate membrane stress responses *via* FtsH-dependent SecY degradation[28] and the Cpx two-component envelope stress response (Figure 2.7).[26] These effects lie upstream of other lethal carry-away physiological events such as the generation of reactive oxygen species, membrane depolarization and expression of periplasmic proteases.[26, 30]

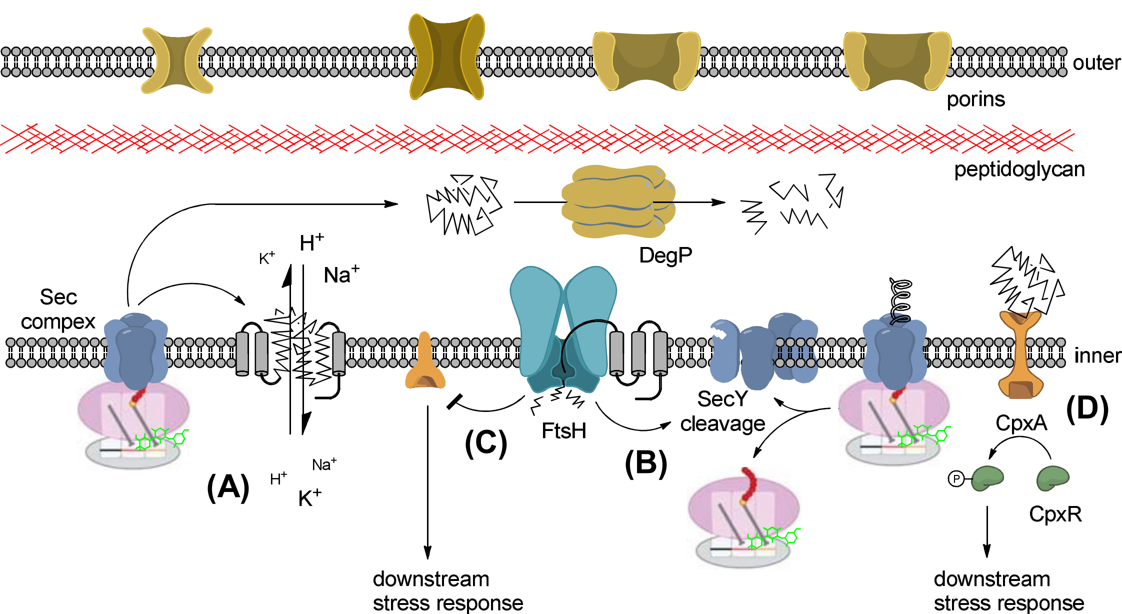

Figure 2.7. Compilation of possible mechanisms for aminoglycoside bactericidal effects. (A) Compromise of membrane permeability due to misfolded proteins. (B) Toxicity due to degradation of essential component SecY in jammed translocation complexes by FtsH holoenzyme,[28] a membrane-embedded ATP-dependent processive membrane protein metallo-endopeptidase.[31,32] (C) FtsH degradation of misfolded proteins and jammed translocation complexes, leads to increased stability of short-lived signaling proteins, which trigger a stress response. (D) Hydrophobic patches of misfolded proteins in the membrane and periplasmic space which activate the Cpx two-component system, and other signaling cascades for envelope stress.[26,30,33] Adapted from references 26 and 28.

2.4 - Aminoglycoside Uptake

Another long-standing question in the field of aminoglycosides is their mechanism of entry into bacterial cells. Unfortunately, progress in this field stopped in the 80's to 90's due to several experimental difficulties. Nevertheless, many measurements and membrane reconstruction experiments have revealed that aminoglycosides behave unlike any other drug class.[34,35,36]

The uptake mechanism appears to be *evolutionarily-conserved and essential* for survival, because resistance across Gram-negative or positive bacteria could not be developed by mutation of a transport mechanism. Considering the structural differences of aminoglycosides, the uptake mechanism operates by way of a *promiscuous and hydrophilic* cavity. Aminoglycoside uptake is *active*, including three stages: an initial energy independent phase (EIP) followed by energy dependent phase I and phase II (EDP-I and EDP-II, Figure 2.8).[35]

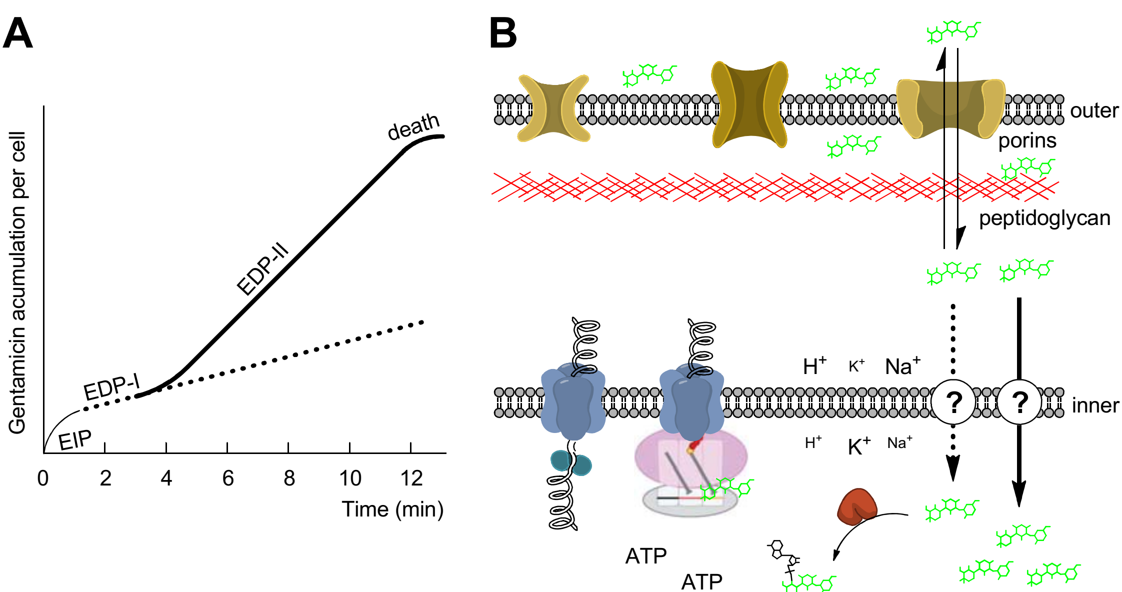

Figure 2.8. (A) Graphical representation of EIP, EDP-I and EDP-II for gentamicin, adapted from reference 36. (B) Representation of aminoglycoside intake requirements, including aminoglycoside-sensitive ribosomes, active protein synthesis and export, ATP and a chemiosmotic potential. Arrows indicate: solid line EIP, dashed EDP-I and bold EDP-II. Interference by an adenyltransferase resistance enzyme during EDP-I is also shown.[36]

The first stage EIP encompasses spontaneous charge interactions with the exterior components of bacterial envelopes and assisted diffusion into the periplasmic space of Gram-negative organisms.[35] During EDP-I and II, the energetic requirements for cytosolic uptake include *ATP* and a *threshold proton-motive force*.[35,36] In fact, bacteria grown under anaerobic conditions or chemiosmotic uncouplers become resistant to streptomycin.[36] Strangely, EDP I and II uptake also requires *aminoglycoside-sensitive ribosomes* and *active protein synthesis aimed for membrane translocation*.[35] For example, phage-infected bacteria uptake aminoglycosides only during lytic phase, when extracellular lysozyme is expressed, but not during cytosolic capsid protein synthesis.[36]

Currently, no targets or molecular mechanistic models for aminoglycoside uptake are available.[2,35,36] The characteristics of EDP-I and II discussed above are consistent with a vicious cycle starting with slow and intrinsic uptake (EDP-I) dependent on post- and co-translational translocation and the membrane-protein quality control machinery which, upon reaching a threshold concentration establishes an unfavorable carry-away response of uptake during EDP-II. Potential candidates for uptake mechanisms with these characteristics could include FtsH or the Sec translocation complex (Figure 2.7).[29, 31]

Bacterial expression of intracellular aminoglycoside modifying enzymes confers resistance by precluding the threshold accumulation of active antibiotic. Since aminoglycosides are modified as they enter the cell, the cycle of EDP-II uptake fails to become established and only a slow and harmless EDP-I uptake continues (Figure 2.8).[35,36] The vastly diverse and wide-spread array of resistance enzymes that have evolved to inactivate aminoglycoside antibiotics are discussed next in Chapter 3.

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Chapter 3

Issues Associated with Aminoglycoside Antibiotics: Bacterial Resistance Mechanisms and Toxicity

3.1 - Bacterial Resistance Overview

Much of modern medicine relies on the effectiveness of antibacterial agents. Procedures and therapies which are inherently invasive are performed under the guarantee that if an infection were to become established on the wound, it should be effectively treatable without outweighing the risk of the procedure.

Antibiotics are, in fact, an exhaustible resource.

The inevitable evolution and dissemination of resistance mechanisms can only be hoped to be managed and counteracted, striking a balance with the development and discovery of novel antibiotics.[1] Inevitably, one of the following mechanisms will become adapted and exploited by pathogens: impermeability,[2] efflux,[2] drug degradation or modification,[3] target mutation or enzymatic modification.[4,5] Prolonged clinical discontinuation is not a generally applicable strategy to reverse resistance mechanisms, because these are not an intrinsic burden to the fitness of the pathogen.[6,7]

Unfortunately, research and discovery of anti-infective agents has languished for two decades. This is the result of a complex scientific and socio-economic congruence, namely a disconnection between medical needs and pharmaceutical market reality.[8,9] Development of anti-infective agents is an inherently less attractive investment than other drug classes: therapy requires relatively short-courses and is self-limiting, efficacy studies are required for each type of infection, clinical trials in hospital settings are difficult to implement, vast competition and overlap with other antibiotics leads to a difficult market entry, and appropriate public health policies limit first-line use of new broad-spectrum agents, thus further postponing investment returns.[8,9] Considering the high development risks and costs of FDA approval,[10] there is an overall incentive to aim research efforts towards analgesics, anti-inflammatories, chronic diseases, cancer, mental illnesses and metabolic/endocrine disorders.[8,9] During the 90's many large pharmaceutical companies either directly abandoned or spun-off their anti-infective research platforms.[9] This was aggravated by disappointments in high-throughput screening ventures, crystal structure- and fragment-based design and the exhaustion of natural product classes. It could be said that anti-infective research underwent a slow reform, nowadays becoming more prevalent in smaller biotech companies with a

different business model,[11] but in general the field remains inadequately funded.[12,13] Most drastically, FDA antibiotic approvals declined steadily from 1990 to 2000 – to a current average of 1 drug approval per year.[8]

The most problematic pathogens which are currently *resilient* to many antibiotic classes and in the verge of *pan*-resistance have been defined *ESKAPE* bacteria,[12] a mnemonic for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteria*.

An overview of the current clinical situation can best be gained from the recent outstanding reports of the Infectious Diseases Society of America (IDSA)[13,14,15] and the European counterparts (ECDC and EMEA).[16] The secular importance of these documents cannot be overemphasized, and should be understood by all scientists associated with health research. These movements are no longer just for "raising awareness", they provide policy and government funding recommendations of critical priority and demand action and specific policy changes at the level of NIAID, FDA, US congress and EU commission.[14,17,18] This has led to the *10x'20 initiative* to revitalize research and development aiming to ensure approval of 10 new antibiotics by 2020 through increased funding, accelerated FDA review and intellectual propriety exemptions.[19] The periodical reports of these agencies review in great detail the current hospital costs and dangers of antibiotic resistance, and the status of the anti-infective drug pipelines:

- *Bad Bugs, No Drugs: No ESKAPE! An Update from the Infectious Diseases Society of America*. Clin. Infect. Dis. **2009**; *48* (1), 1-12 (reference 13)
- *IDSA Report: Bad Bugs, No Drugs: As Antibiotic Discovery Stagnates, a Public Health Crisis Brews.* PDF Informative Brochure. July 2004 (reference 14)
- See also references 15 and 16.

These documents are freely available at http://www.idsociety.org/10x20.htm.[20]

3.2 - Aminoglycoside Modifying Enzymes

The structures of aminoglycosides, in particular their abundant hydroxylation and numerous positive charges, sets this family apart both from drug-like small molecules and most classes of bioactive natural products.[21] Hence, not surprisingly, resistance mechanisms also diverge from those encountered conventionally in other antibiotic classes.[3] Even though, many modes of resistance have been described in the context of aminoglycosides, discussed further below, there is a strong prevalence of resistance by enzymatic modification.

Aminoglycoside modifying enzymes have been thoroughly studied and reviewed in recent literature from several valuable viewpoints: their evolution and genetics,[22,23] detailed mechanistic enzymology,[24,26] and their numerous X-ray crystal structures.[25,26,26] In this chapter, the focus is given specifically to their clinical distribution, their substrate preferences toward natural or semi-synthetic aminoglycosides and their impact on medicinal chemistry.[23,27,28]

The aminoglycoside modifying enzymes can be fit into three classifications: *N*-acetyltransferases, *O*-phosphotransferases and *O*-nucleotidyltransferases (Figure 3.1). These are respectively abbreviated AAC, APH and ANT, followed by the targeted aminoglycoside position(s) in brackets and an isoform identification, for example: AAC(6')-Ia. Each family is composed of numerous related isoforms, differentiated phylogenetically and by their specific target preferences (Table 3.1).[23] Each enzyme type is well represented in clinical collections worldwide by one or more of these isoforms, alone or in combinations, invariably encoded within mobile elements such as plasmids or transposons (Figure 3.2).[27] Due to the stochastic distribution of the resistance enzymes and the localized selective pressures, enzyme isoforms are not uniformly expressed by pathogenic clinical isolates in each geographical region (Figure 3.2 and 3.3).[27,28] This makes the distribution of aminoglycoside resistance a complex, diverse and dynamic global problem.[27-29]

Several enzymes which target streptomycin, spectinomycin and hygromycin B share the standard nomenclature. Although clinically-relevant these are not pertinent to the 2-deoxystreptamine families and will not be discussed herein (see reference 23).

Resistance enzymes have invariably evolved highly promiscuous active sites to target several aminoglycosides (Figure 3.4). Nevertheless, three characteristics dominate with respect to inactivation: firstly, the presence of a proper acceptor (amine *vs.* hydroxyl *vs.* deoxygenation); secondly, steric interference of an N1-group (HABA, HAPA or ethyl); and finally, the aminosugar substitution pattern (Figure 3.1).

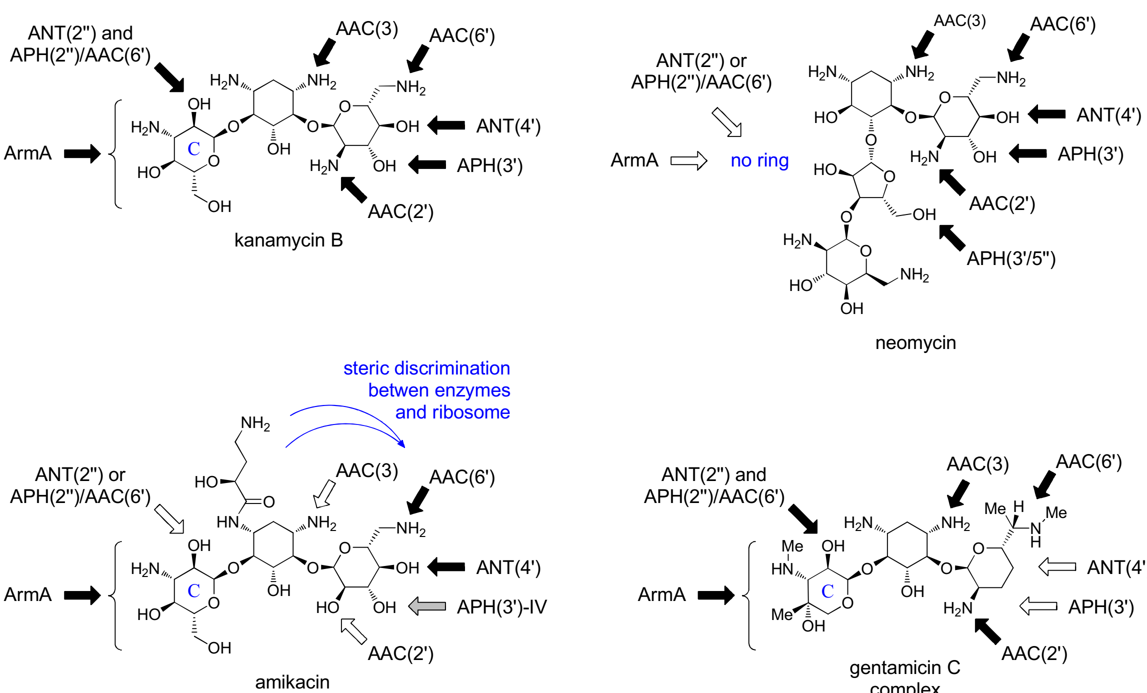


Figure 3.1. Resistance enzyme susceptibility of the disubstituted 2-deoxystreptamine families. Arrows indicate enzyme susceptibility, dark = severe, grey = one isoform, and white = none.[27]

Table 3.1. Distribution of aminoglycoside resistance enzymes in "ESKAPE bacteria"

Bacterium	Prevalent resistance mechanisms[a]	Ref.
P. aeruginosa	Efflux, AAC(6')-II, ANT(2")-I, APH(3')-II (chromosomal), AAC(3)-I	32,23,31,27
S. aureus	AAC(6')-Ie/APH(2")-Ia, ANT(4')-Ia, APH(3')-IIIa	32,23
***Acinetobacter* spp.**	Efflux, APH(3')-VI, AAC(6')-I, AAC(3)-I, ANT(2")-I, AAC(3)-II, ArmA	33,33,27
Enterobacteriaceae	AAC(6')-Ie, AAC(3)-I, ANT(2"), ArmA	34,23,27
Enterococci	AAC(6')-Ie/APH(2")-Ia, APH(3')-IIIa	32,35
K. pneumoniae	AAC(6')-Ib, AAC(3)-I, ANT(2")	33,36

a) ordered by percentage prevalence in the most recent reference, all included have been found in at least 10% of isolates displaying aminoglycoside resistance. See Figure 3.3 for a graphical representation.

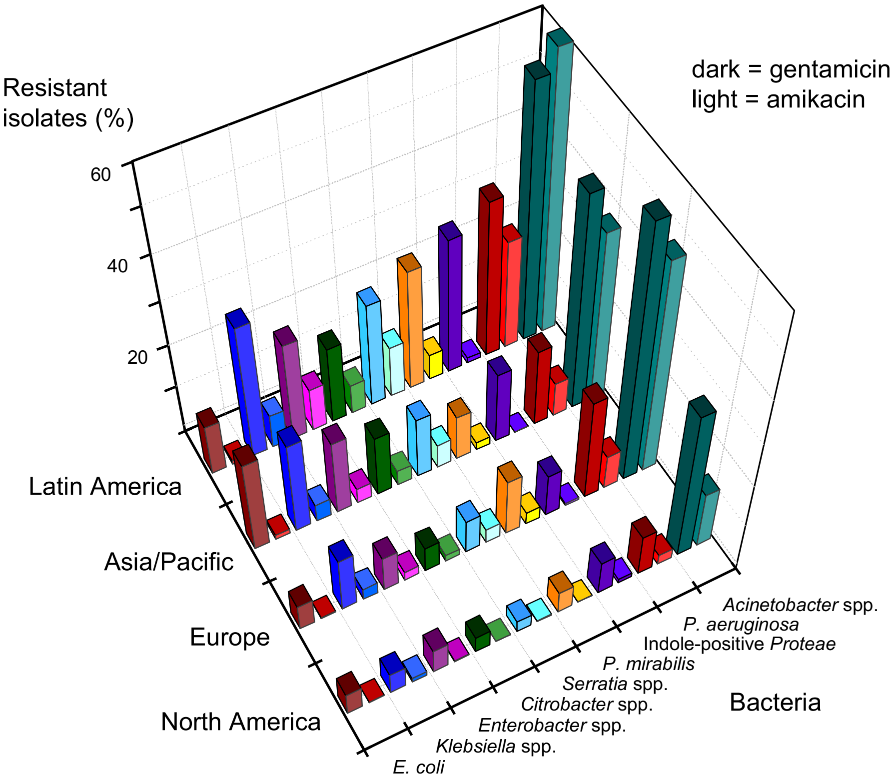


Figure 3.2. Prevalence of resistance to gentamicin (left darker bars) and amikacin (right lighter bars) in isolates from main pathogenic bacteria from the SENTRY Program collected between 1998-2007. This plot was constructed with raw data from reference 28.

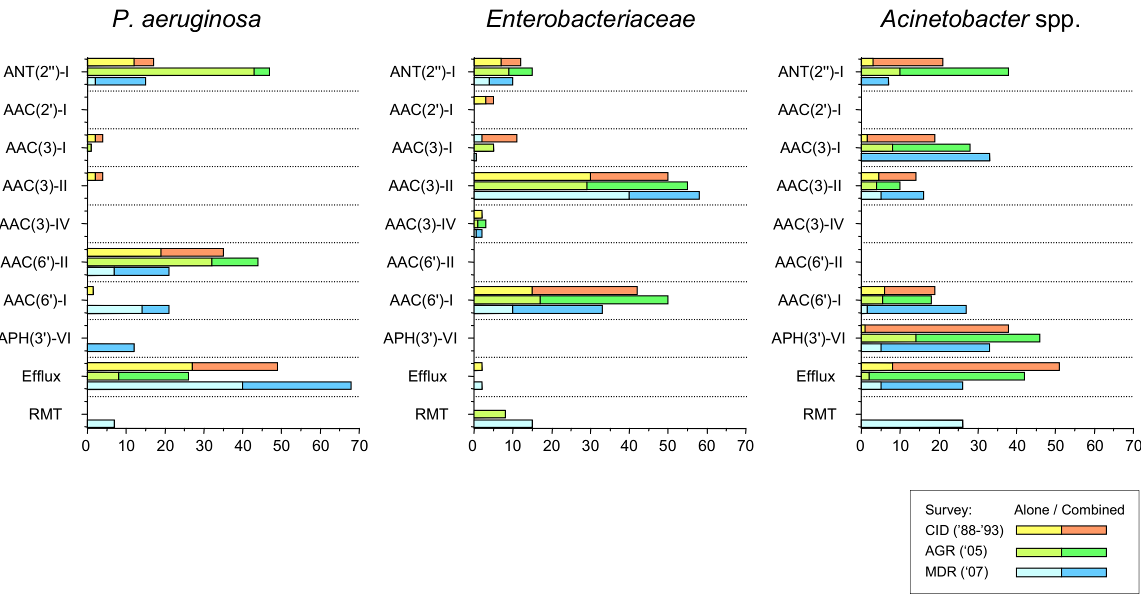


Figure 3.3. Distribution of aminoglycoside resistance mechanisms in isolates from the CID and SENTRY program collections from three time periods. Bars indicate the overall prevalence of each resistance mechanism, and the darker section indicates the sub-proportion expressed in combination with other mechanisms. Plot adapted from reference 27.

3.3 - Aminoglycoside Acetyltransferases

3.3.1 - AAC(6') are amongst the most frequently isolated enzymes. AAC(6')-I isoforms are common in *Acinetobacter*, other Gram-negative pathogens and *S. aureus*, in particular the fused bifunctional enzyme gene AAC(6')-Ie/APH(2"). A second isoform group, AAC(6')-II, is only common in *P. aeruginosa* (Figure 3.3).[27-29] An AAC(6')-I isoform has been characterized by X-ray crystal analysis bound to unproductive 6'-hydroxy aminoglycoside substrates, such as kanamycin C and paromomycin, which reveals the promiscuity of the active site and the extended negative electrostatic surface (Figure 3.4 A).[37,38]

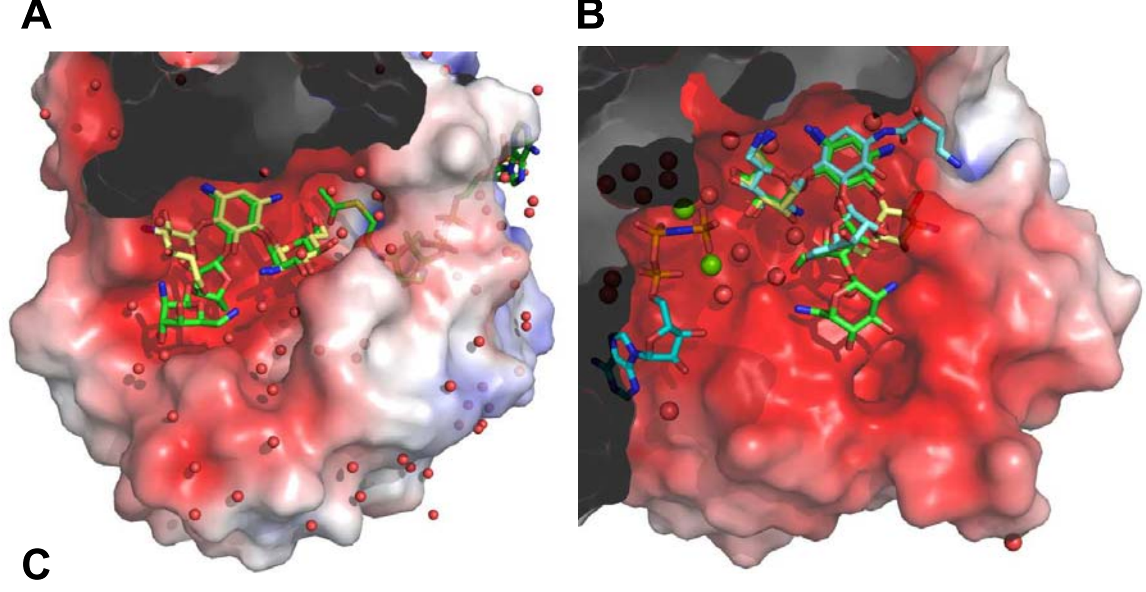
3.3.2 - AAC(3) are well represented in several Gram-negative pathogens, such as *P. aeruginosa* and *Acinetobacter* (Figure 3.3). In general, they provide high-level resistance against 4,6-disubstituted aminoglycosides without N1-groups, and to a lesser extent to the 4,5-disubstituted family.[27-39]

3.3.4 - AAC(1) is a rare enzyme with lesser clinical significance. There is little pressure for acquisition and selection of the gene due to the presence of N1-groups in several aminoglycosides.[27-29]

3.3.5. - AAC(2') is also a rarely found enzyme. Even though most aminoglycosides have a 2'-amino group, the pressure for acquisition and selection of this gene could have remained low due to the clinical importance of amikacin, bearing a 2'-hydroxyl group.[27-39]

3.4 - Aminoglycoside Phosphotransferases

3.4.1 - APH(2') kinase activity is associated only with the bifunctional enzyme AAC(6')-Ie/APH(2'). The protein C-terminus shares homology with other aminoglycoside phosphotransferases. This fused gene product is an important resistance determinant for the 4,6-disubstituted family, even those protected by N1-groups. As aforementioned this enzyme is prevalent in Gram-negatives and *S. aureus*, restricting the use of several aminoglycosides against several important pathogens (Figure 3.2).[27-39]



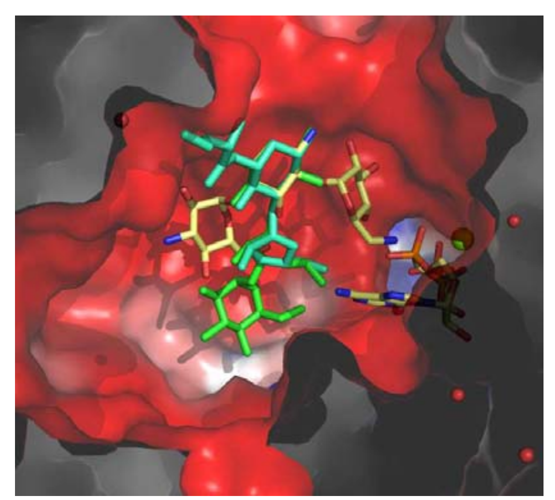


Figure 3.4. Active site pockets of three modifying enzymes, co-crystallized with both nucleotide cofactors and aminoglycoside substrate, displayed as semi- transparent van der Waals surfaces colored with the electrostatic potential. Color coding of the aminoglycosides is uniform: kanamycins are shown yellow, butirosin in cyan and neomycin or paromomycin in green. Clipping planes were used to expose the active sites. Water molecules and Mg^{2+} ions are shown as spheres. Panels: (A) AAC(6')-Ia co-crystallized with paromomycin and Ac-CoA, overlaying kanamycin C in the same protein.[37] PDB entries 2VQY and 1V0C.[37] (B) APH(3'/5")-IIIa co-crystallized with butirosin and ADPNP, overlaying neomycin and kanamycin B in the same protein.[40,41] PDB entries 3H8P,[40] 2B0Q and 1L8T.[41] (C) ANT(4')-I co-crystallized with kanamycin A and ADPNP,[42] overlaying frameworks of butirosin and neomycin. PDB entry 1KNY.[42] Electrostatic potentials were calculated with CHARMM-GUI (red = negative, blue = positive).[43] Substrate overlays were based on common ring AB atoms. Rendered in PyMol.[44]

3.4.2 - APH(3') enzymes are the most diverse subclass in terms of their resistance scope and phylogenetic relationships. There are at least seven isoforms, of which the most clinically relevant will be treated separately. In general they target both the 4,6- and 4,5-disubstituted families with 3'-hydroxyls, and certain isoforms are able to recognize aminoglycosides with N1-groups and several others phosphorylate both the 3' or the 5" primary hydroxyl group of 4,5-disubstituted aminoglycosides.[27-29]

- APH(3')-I is widespread in Gram-negatives, but its importance may have diminished since it is unable to target aminoglycosides with N1-groups.[27-29]

- APH(3')-II isoforms are chromosomally encoded in *P. aeruginosa*, providing intrinsic resistance to most aminoglycosides bearing 3'-hydroxyl groups, including the N1-substituted butirosin but not amikacin or isepamicin.[27-29]

- APH(3')-III is prevalent in *S. aureus* and imparts resistance to most aminoglycosides with 3' or 5'' hydroxyl groups, including butirosin, but only slightly affecting amikacin. The co-crystal structures of this isoform with several aminoglycosides and a non-hydrolysable ATP analog reveal how this enzyme can recognize such a vast collection of substrates: from the kanamycins to the N1-group of butirosin and the 4'''-glycosidated lividomycin A (Figure 3.4 B).[27-29]

- APH(3')-VI is primarily associated with *Acinetobacter* spp. and very significantly provides resistance against amikacin and isepamicin (see Figure 3.3).[27-39]

3.5 - Aminoglycoside Nucleotidyltransferases

This enzyme-type comprises the least diverse class, but responsible for resistance to important drugs in clinical use.

3.5.1 - ANT(4') isoform I is prevalent in *S. aureus*, and restricts the use of amikacin or isepamicin against Methicillin-resistant strains (MRSA). Another isoform is a minor resistance determinant in *P. aeruginosa* and other Gram-negatives pathogens. In general, this enzyme targets all 4'-hydroxyl groups of the 4,6- and 4,5-disubstituted families. Only one co-crystal structure has been reported, with kanamycin A, yet like the aforementioned resistance enzymes this homodimeric active site has the clear potential to host a diversity of substrates (Figure 3.4 C).

3.5.2 - ANT(2'') are widespread among Gram-negative pathogens, including *P. aeruginosa* and *Acinetobacter* spp (Figure 3.3). In general, they target ring C of both gentamicins and kanamycins, providing high-level resistance against 4,6-disubstituted aminoglycosides, except those bearing a N1-group (amikacin, arbekacin or isepamicin).

3.6 - Efflux Pumps and Permeability

Efflux is often classified as a minor resistance mechanism in the older literature compared to enzymatic modification of aminoglycosides.[26] This is true in regard to the number of species and overall fraction of isolates that express efflux systems.[27] However, the clinical impact of efflux pumps is considered equal or greater to that of the modifying enzymes. In fact, this mechanism of resistance provides broader target recognition than any enzyme, and hence it is a type of *pan*-resistance. Efflux is a major resistance determinant for aminoglycosides in some Gram-negative strains, such as the problematic nosocomial pathogens *P. aeruginosa* and environmental opportunists such as *Acinenetobacter* (Figure 3.3).[26,27] Impermeability, albeit only providing intermediate resistance, has also been associated with changes in outer membrane composition and electron-transport-chain modulation in *P. aeruginosa*.[26,45]

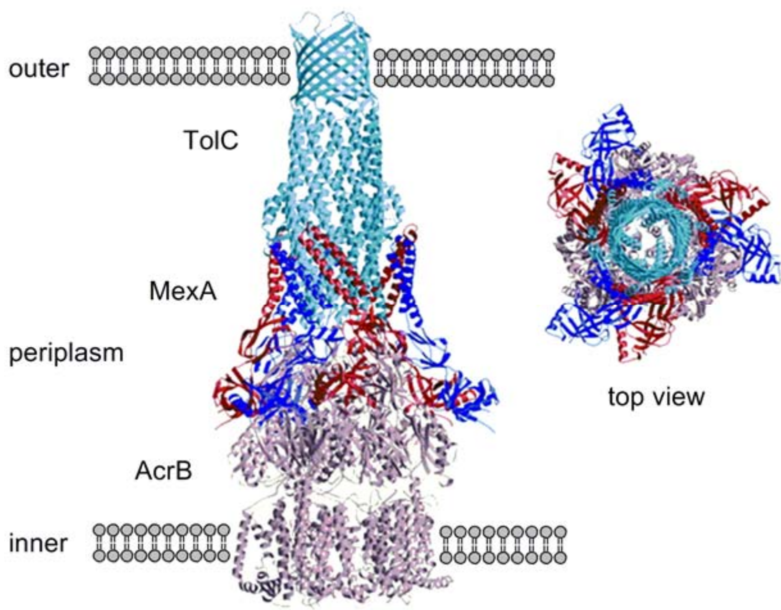


Figure 3.5. Reconstruction model of a RND class efflux system, the AcrA–AcrB–TolC drug efflux pump of *E. coli*. The periplasmic linker AcrA was modeled by the close homolog MexA of *P. aeruginosa* in a proposed 3-fold dimer association. Adapted from reference 44.

Fortunately, aminoglycoside pumps are distinct from those responsible for resistance towards other more lipophylic antibiotic classes.[46] Therefore, these genes have not undergone co-selection in geographical regions where aminoglycoside use has been regulated.[47] Aminoglycoside efflux complexes have evolved from one of the five major resistance determinant gene families described for other antibiotic drugs (reviewed in detail in reference 2), known as the *resistance-nodulation-division* (RND) gene family, named *MexAB* and *MexXY* in *P. aeruginosa* and *AdeABC* genes in *Acinetobacter*, which are homologous to the *AcrAB-TolC* efflux system of *E. coli*.[2] These efflux complexes consist of three major components, an inner membrane drug-proton antiporter, a periplasmic linker and also an outer membrane channel, which is usually associated with more than one efflux system. A representative model of the RND class complex has been built from several crystal structures (Figure 3.5).[47]

3.7 - Ribosomal Mutation and Enzymatic Modification

Target mutation of rRNA genes by base-substitution at the A-site binding pocket is considered rare, yet the single substitution A1408G can provide *pan*-resistance towards all aminoglycosides which bind the H44 helix.[26] This phenomenon has been assigned to the statistical improbability of changing the multiple copies of rRNA genes and the required compensatory mutations to maintain viable translation speed and accuracy.[48,26] It is worth noting the important exceptions in the genus *Mycobacterium*, including *M. tuberculosis*, which contain a single copy of rRNA genes.[49,50]

On the other hand, enzymatic modification of rRNA is compatible with high gene-copy numbers, and plasmid-encoded rRNA methyltransferases (RMT) have been characterized in clinical isolates. These target N7 of G1405, affecting the binding region of ring C of the 4,6-disubstituted families (Figure 3.6). These genes are known as *armA* and *rmtA-D*, the latter of which are thought to have derived by horizontal gene transfer from actinomycete aminoglycoside producers. Two crystal structures are available, revealing small globular enzymes which target only the isolated fully-assembled 30S subunit. Furthermore, a very rare RMT gene *nmpA* was detected in some isolates in Japan.[49] It was demonstrated to impart resistance to all aminoglycosides including neomycin and apramycin, differing in target preference for N1-methylation of A1408 (see Figure 3.6).[49,51]

In the light that all current clinically-useful intra-venous aminoglycoside antibiotics belong to the 4,6-disubstituted family, RMTs may be classified as *pan*-resistance mechanisms. Plasmids coding *armA* and *rmtA-D* have been isolated in virtually all pathogenic classes,[52] implying that they are not as genus-specific as efflux and impermeability. Although RMTs remain, to date, a somewhat latent source of resistance (Figure 3.3) and are relatively less spread than modifying enzymes or efflux pumps, there is growing concern for this mode of resistance expressed in literature and the pharmaceutical industry.[51,53,52]

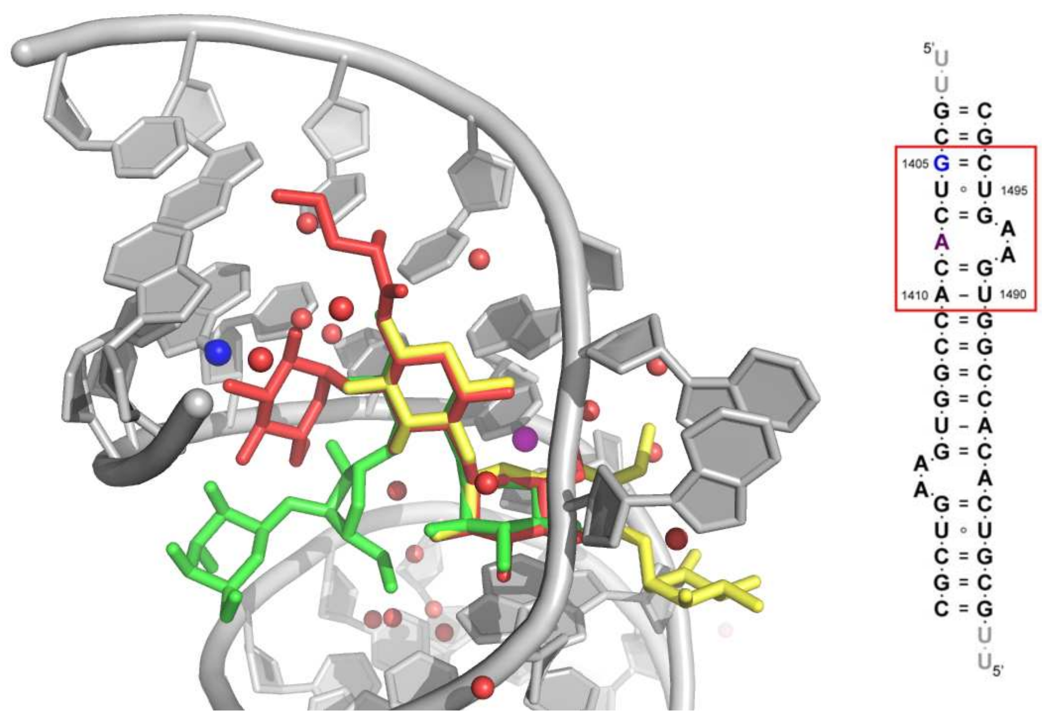


Figure 3.6. Representation of the H44 A-site model showing the overlapping binding sites of neomycin (green), amikacin (red) apramycin (yellow). The positions targeted for methylation by *armA* and *rmtA-D* (N7-G1405) are highlighted with a blue sphere and the target of *nmpA* (N1-A1408) is highlighted with a magenta sphere. Respective structures compiled from references 53, 54 and 55. PDB entries 2ET4, 2G5Q and 1YRJ. Right: crystallized palindromic oligonucleotide with the A-site model highlighted in a red box and the bases targeted for methylations respectively colored. Rendered in PyMol.[44]

3.8 - Antimicrobial Testing – the Primary Panel

In this dissertation, minimum inhibitory concentrations (MIC) were determined in collaboration with the Microbiology department of Achaogen Inc. The serial dilution MIC paradigm has been adapted to an automated 96-well plate system where growth is determined by optical density, allowing for rapid parallel screening of large strain collections.[56] Aminoglycoside purity and concentration have been established prior to antibiotic testing by HPLC with a Chemiluminescent Nitrogen Detector (CLND), requiring a purity minimum of 85%.

The isolate collection which was challenged throughout this work is a "primary panel" assembled by scientists of Achaogen Inc. led by renowned microbiologist and aminoglycoside expert George H. Miller, Ph. D.[27,28,32] The primary panel is composed of representative clinical isolates whose resistance profile has been well-characterized and their enzyme or efflux genotype has been confirmed by PCR.[27] Susceptible strains of ATCC origin serve as quality controls and indicators of the baseline bactericidal activity of each antibiotic. Resistant strains are representative of isolates which are difficult to treat, and would conform to the cap of MIC-90 curves for current aminoglycoside antibiotics (Figure 3.3).[57] Among several species, the primary panel provides an excellent overview of *ESKAPE* bacteria – *Enterococcus faecium, Staphylococcus aureus, Klebsiella* species*, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* species (Table 3.1).

3.9 - MIC Table Color-Code

MIC tables throughout this work include color-coding as a visual aid and to provide an intuitive categorization of relative therapeutic indexes.[36,56] Green highlighting implies good to excellent antibacterial activity in a range where toxic side-effects would be minimized; yellow implies borderline/intermediate inhibition *versus* the expected toxicity breakpoint; and red implies that an unacceptably high antibiotic concentration is required for inhibition so that effective dosing would likely not be achieved. The red/yellow/green margins vary from 4 to 16 μg/mL depending on the relative estimated therapeutic index or breakpoints of the antibiotic subfamily.[36,56]

Table 3.2. Minimum inhibitory concentration antibacterial testing against a range of susceptible and resistant isolates for antibiotics amikacin (Amk), gentamicin (Gent), neomycin B (NeoB), paromomycin (Par), N1-HABA paromomycin (H-Par), ribostamycin (Ribo) and butirosin (Btr). See Table 3.1 and Figures 3.1 to 3.3 for relevance of the resistance mechanisms.

			MIC (µg/mL)						
Bacterium	**Code**	**Description/Phenotype**	**Amk**	**Gent**	**NeoB**	**Par**	**H-Par**	**Btr**	**Ribo**
Wild Type Strains									
S. aureus	ASAU001	ATCC 29213 (QC)	2	0.5	0.5	2	2	8	16
E. coli	AECO001	ATCC 25922 (QC)	2	0.5	2	4	2	2	8
K. pnuemoniae	AKPN002	ATCC 10031	0.5	0.25	0.5	2	1	1	2
P. aeruginosa	APAE001	ATCC 27853 (QC)	2	0.5	32	>64	8	>32	>64
P. aeruginosa	APAE002	Wild type pump	2	2	8	>64	4	16	>64
A. baumannii	AABA1065	Susceptible	2	2	1	4	2	1	8
Efflux Strains									
P. aeruginosa	APAE010	MexXY upregulated	4	2	16	>64	8	32	>64
A . baumannii	AABA001	ATCC 19606	16	16	4	16	16	8	32
P. aeruginosa	APAE1024	Efflux system	16	4	32	>64	32	>32	>64
P. aeruginosa	APAE1094	Efflux system	16	8	64	>64	64	>32	>64
Modifying Enzymes and Methytransferase									
Providencia stuartii	APST001	AAC(2')-I	4	64	32	>64	>64	>32	>64
E. coli	AECO056	APH(3')-Ib	0.5	0.25	64	>64	2	1	>64
S. aureus	ASAU1029	APH(3')-III	8	0.5	>64	>64	32	>32	>64
A. baumannii	AABA1082	AAC(3)-I + APH(3')-VI + ANT(2')-I	>64	>64	>64	>64	64	>32	>64
Acinetobacter spp.	AASP004	APH(3')-VI	>64	0.5	16	>64	32	>32	>64
S. aureus	ASAU003	ANT(4')-I	64	0.5	>64	>64	>64	>32	>64
P. aeruginosa	APAE037	ANT(4')-II	32	2	8	>64	4	>32	>64
S. marcescens	ASMA004	AAC(6')-I	32	4	8	4	8	>32	>64
P. aeruginosa	APAE1010	AAC(6')-I	>64	64	>64	>64	16	>32	>64
P. aeruginosa	APAE1132	AAC(6')-II	4	32	8	>64	8	>32	>64
E. coli	AECO037	ANT(2")-I	4	64	2	4	8	4	8
E. cloacae	AECL004	ANT(2")-I + AAC(6') + APH(3')-I	64	32	>64	>64	8	32	>64
S. aureus	ASAU008	AAC(6')/APH(2")	64	>64	>64	>64	32	>32	>64
P. aeruginosa	APAE040	AAC(3)-I	4	32	8	>64	8	16	>64
E. coli	AECO1060	AAC(3)-IV	2	16	2	8	4	2	>64
E. coli	AECO045	ArmA	>64	>64	1	4	4	2	8

3.10 - Aminoglycoside Toxicity

The first clinical trials of Waskman's aminoglycosides, streptomycin and neomycin, immediately indicated nephrotoxicity and ototoxicity issues at higher doses, and these side-effects have been invariably associated with all clinically-approved aminoglycosides (data reviewed in reference 58). Rigorous study and characterization of aminoglycoside toxicity has lagged compared to their isolation and clinical use, rendering difficult prediction, prevention and research. Despite major leaps, this field remains controversial and speculative due to experimental difficulties. A detailed review regarding renal uptake is available in reference 59, mechanistic hypotheses were reviewed in reference 60 and major kidney biochemical pathways are reviewed in reference 61. Ototoxicity was recently reviewed in reference 62 and a chapter in book 26. This section will only highlight some of the better established details and their impact to medicinal chemistry.

3.11 - Aminoglycoside Nephrotoxicity

Nephrotoxicity is caused by uptake of a small but significant fraction (~5 – 10%) of total aminoglycoside dosage by the epithelium of the first sections of renal proximal tubules. Internalization occurs by megalin-dependent endocytosis at the luminal brushed surface contacting the glomerular filtrate of circulating blood.[60,59] These epithelial cells accumulate this massive amount of aminoglycosides (up to 2 mg/g tissue) in enlarged lysosomes and within layers of polar lipid deposits of lamellar structure known as myeloid bodies, an effect known for several cationic drugs. Thereafter, a number of morphological, metabolic, and functional alterations ensue.[60] The eventual outcome is apoptosis and necrosis of these cells which trigger renal shut-down through known inflammatory pathways.[60] Among these intermediary metabolic changes, the main aminoglycoside target(s) and the mechanistic origins of cellular toxicity have remained, however, difficult to identify.

Currently it is thought that low levels of aminoglycosides exit the myeloid bodies, which are considered protective structures, due to instability and oxidative injury or by retrograde transport through vesicular secretory pathways with final release to the cytoplasm from the endoplasmic reticulum.[63] Likewise, aminoglycosides are toxic

at very low levels if introduced into the cytoplasm by electroporation,[64] where they interfere with apoptotic and necrotic pathways at the level of the mitochondrial membrane, as well as inhibit proteasome degradation of pro-apoptotic signals. These mechanisms account for the apparent disconnections between nephrotoxicty and affinity or accumulation of each aminoglycoside in kidney cells.

3.12 - Aminoglycoside Ototoxicity

In general, aminoglycoside ototoxicity is different to nephrotoxicity, and it can be further divided into vestibulotoxicity (balance) and cochleotoxicity (hearing).[26] Expression of these symptoms is different for each antibiotic and appears to obey no clear structure-toxicity relationships or pharmacokinetic parameters.[65] Invariably, aminoglycosides target *hair cells* responsible for both auditory or balance sensations. Their inability to regenerate explains the irreversibility of the ototoxic effects. Other cell types, notably auditory neurons, can also be affected.[62] Unlike kidney dysfunction, ototoxicity is difficult to monitor or predict, with symptoms developing even weeks after cessation of antibiotic treatment.[26] Aminoglycoside ototoxicity is thought to arise from their particularly slow clearance from the ear tissues and fluids. Their half-lives are in the order of one month, despite the relatively low uptake levels, within 10% of blood concentrations, which is another important difference from nephrotoxicity.[26]

Mechanistically, ototoxicity has a stronger link to mitochondria, because carriers of a mit-rRNA mutation suffer enhanced sensitivity.[66] Weaker antibacterial action of analogs coincides with low ototoxicity.[66] Current mechanistic theories involve connections of aminoglycosides with the generation of reactive oxygen species, both by non-enzymatic and enzyme-mediated mechanisms.[26,62] One potential mechanism involves the aminoglycoside as a bridge for the interaction of redox-active metals and unsaturated arachidonic acids of phosphatidylinositides. The intermediacy of enzymatic pathways is supported by aminoglycoside-activation of Rho-GTPase Rac1, which activates NADPH oxidase, a generator of superoxide, and acts as a regulator of cytoskeleton architecture in these ciliated cells. The persistent redox imbalance generated by these sources, as well as the mitochondrial insults, are thought to eventually lead to activation of apoptotic and/or necrotic pathways in these rather fragile cells.[26,62]

3.13 - Aminoglycoside Dosing in the Clinic

As discussed above, controversy over few working hypotheses in this field, aggravated by unreliable *in vitro* and animal model systems all have hampered the design and screening of less-toxic aminoglycosides.[26] Deception occurred with the case of netilmicin (Figure 1.5), which was comparatively harmless in rodent models, but was later demonstrated to be as toxic as most aminoglycosides in clinical trials.[67]

A panoply of nephro-protectants have been identified from animal studies, including metal salts, bicarbonate, polycationic competitors and antagonists of megalin-uptake, polyanionic molecules which prevent phospholipid association during vesicular transport, antioxidants, anti-inflamatory and anti-apoptotic drugs.[26,60,62,68,70,69] However, co-administration of such agents with aminoglycosides in the setting of clinical studies has not been yet realized.

Two remarkable observations have influenced the understanding and clinical handling of aminoglycoside-induced nephrotoxicity.[60,70] Firstly, polycationic competition studies revealed that uptake by proximal tubule epithelial cells displayed saturable transport kinetics, with parameters relevant to clinical doses. Subsequently, animal model and human data have revealed that the standard fractionated daily dosing (3x or 2x *vs* once) results in higher concentration of drug in tubular epithelial cells, and led to higher kidney complications in randomized clinical trials.[71] Concurrently, it was recognized that the antibacterial effect of aminoglycosides was best correlated to AUC and not overall exposure time. These observations have prompted a review of the previous dosing paradigms which aimed at constant maintenance of blood concentrations above antibacterial breakpoints.[26,60,70] Currently, large once-daily doses in short courses (5–7 days) are recommended.[70,71] These paradigms have been demonstrated to improve clinical outcome with lower percentage of nephrotoxic side-effects, and are used in trials on the new antibiotic ACHN-490.[72]

Old aminoglycosides have declined in clinical use throughout North America and Europe in favor of novel generation antibiotics. However, they remain an important and affordable resource in underdeveloped regions of the world (hence the prevalence

of resistance observed in Figure 3.2).[5,26] Aminoglycosides are valuable for infectious emergencies in hospital settings, when facing undetermined pathogens or resistance profiles, due to their broad-spectrum antibacterial activity and their reliable lack of immune responses.[5,26] They are also useful for periodic inhalation therapy of cystic fibrosis patients throughout the world.[73] Their co-administration with β-lactams to profit from the synergistic antibacterial effects[74] was advocated for decades, but it is currently a source of debate.[75] Likewise, interactions with other drugs and nephrotoxins are becoming increasingly understood and avoided.[70] Aminoglycoside posology is being largely reformed.

Overall, it would appear that the future of aminoglycosides augurs well for the eventual elimination of nephrotoxicity by co-administration of protective agents. Curtailing unpredictable ototoxicity might also be possible through post-therapy follow-up with antioxidants. A paradox exists, however, between clinical need and the reality of pharmaceutical investments, because currently approved aminoglycoside antibiotics are off-patent and ineffective as first-line broad-spectrum treatments due to widespread bacterial resistance. Moreover, common antioxidants and nephroprotectants are neither new molecular entities nor novel in mechanistic terms. Hence, clinical trials may need to wait until novel generations of proprietary aminoglycosides require co-administration of nephro- and oto-protectants.

3.14 - References

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Chapter 4

Semisynthetic Hybrids of Aminoglycoside Antibiotics – Development and Application of Tsuji Palladium-Catalyzed Deoxygenation

4.1 - Design of New Aminoglycosides

As previously discussed, the detailed understanding of the mechanisms of antibacterial activity of natural and semisynthetic aminoglycosides is key for identifying the pitfalls of antibiotics in current clinical practice and streamlining the production of novel generations of analogs. Aminoglycoside medicinal chemistry could be considered to be in its 2nd generation, focused on the 4,6-disubstituted families up to the mid-80's.[1,2,3] By comparison, cephalosporins are currently in their 5th generation.[4] A particularly interesting text in reference 3 reviews the clinical candidates up to 1985 and effectively conveys the fierce competition among 2nd generation aminoglycosides at the time.[3] In 2010, the first aminoglycoside which could be considered 3rd generation entered clinical trials, ACHN-490 from Achaogen Inc (Figure 4.1).[5,6,7,8,9] Two structural variations were applied to the parent antibiotic sisomicin which is given using a modern dosing regime.[10] This antibiotic is a definitive step forward; however, it belongs to the well-exhausted 4,6-disubstituted family and due to holding particular kinship with arbekacin it does not overcome all of the resistance issues.[9]

The (L)-α-hydroxy-γ-aminobutyric amide (L-HABA) chain, which is found on the natural antibiotic butirosin, provides an enhancement which broadens the antibacterial spectrum compared to its congener ribostamycin (Table 3.2).[1] This side-chain has a snug fit within the distorted H44 helix, behind the bulged-out bases A1492 and A1493 (Chapter 2, Figure 2.1). Presumably, most aminoglycoside modifying enzyme genes have yet to evolve active sites able to recognize this unique modification in butirosin. Semisynthetic application of N1-groups to the kanamycins and gentamicins has provided current benchmark drugs such as amikacin, arbekacin and isepamicin (Figure 4.1).[1,2,3]

Although the 5,6-disubstituted class was discovered first (Table 1.1),[11] effective semisynthetic manipulations of the more complex paromomycin and neomycin antibiotics remained elusive during the 70's and 80's bloom of aminoglycoside medicinal chemistry due to the sizeable number of amino and hydroxyl groups on the parent scaffolds.[3] Within these difficulties has lied dormant the opportunity to explore the uncharted territory of these powerful antibiotics. Notably, analogs of the simpler butirosin did not reach clinical trials, despite numerous semisynthetic efforts.[3]

4.2 - Strategies for Countering Resistance – Evasion versus Inhibition

In the light that antibiotic resistance in the context of aminoglycosides is a complex, dynamic and diverse problem, the two possible remediation strategies might appear, at first glance, equally ambitious. One possibility is to develop aminoglycosides capable of evading all enzyme types and their multiple isoforms, as well as escape from efflux mechanisms and rRNA methyltransferases. The second strategy is to inhibit all the enzyme types and isoforms, reviving the use of the otherwise excellent aminoglycoside arsenal through inhibitor cocktails, as is currently applied with certain β-lactams and β-lactamase inhibitors, such as clavulanic acid.[12,13]

However, the genetic and structural diversity of the resistance enzyme isoforms would render difficult the development of broad-spectrum inhibitors, necessitating several compounds for each of the enzyme types. This problem is further amplified by the different cellular physiology of the many pathogens that express the enzymes (e.g. Gram-negative *vs.* positive). Therefore, design and synthesis of inhibitors of specific enzymes, have thus far remained in academic niches, providing probes for studying enzyme mechanisms,[14,15,16] or proof-of-concept for novel approaches of drug-design.[17,18,19]

In this work, we explore the more pragmatic evasion approach, which is, arguably, the only currently accessible, and likewise is most consistent with the current drug-approval policies. Nevertheless, the long-term continuation of aminoglycoside clinical use and development is likely to require a combination of these two major strategies, relying on inhibitors for particularly difficult and prevalent enzymes, such as AAC(6') isoforms I and II (Figure 3.3). Presumably, such prospective inhibitors would come from high-throughput screening efforts and may consequently explore the cofactor binding pocket and transition state electrostatics. On the other hand, inhibitor strategies strictly resembling aminoglycoside substrates or bisubstrates, will require broad spectrum of uptake and evasion of enzyme combinations and efflux pumps (e.g. tobramycin competitive inhibition of APH enzymes).[20]

4.3 - Deoxygenation for the Evasion of Aminoglycoside Modifying Enzymes

As discussed in the context of resistance mechanisms (Chapter 3), the 3' and 4' positions of the common ring A of aminoglycosides are highly susceptible to modification by phosphotransferases and adenyltransferases (Figure 3.1). Notably, several isoforms of these enzymes are not effectively evaded by introduction of an N1-group (Table 3.2, Chapter 3).

The highly promiscuous active sites of APH(3')s have adapted pockets for recognition of both 4,5- and 4,6-disubstituted deoxystreptamines, accommodating also N1-HABA groups (Figure 3.4 B).[21] Specific isoforms are also able to phosphorylate the distant ribose primary 5''-OH of several aminoglycosides, potentially by recognizing the substrate in different orientations or shifted within the active site.[22] These enzymes are often chromosomally encoded in *P. aeruginosa* strains, which coupled with their constitutively impermeable membranes and efflux capabilities leads to the overall incompetence of 3'-hydroxyl aminoglycosides against these important pathogens. Frequently, *S. aureus* strains of MRSA classification are carriers of ANT(4')s as their source of resistance to amikacin.[1] Therefore, the 4'-deoxygenated Amikacin derivative BB-K311 was studied in clinical trials, and the powerful semisynthetic 3',4'-dideoxygenated antibiotic arbekacin is in current application (Figure 4.1).[1,3] Such modifications were inspired directly from natural structure-activity relationships, since dideoxygenation is a biosynthetic modification in the gentamicin and sisomicin family (Chapter 2). Consequently, the complex of the 6'-multi-substituted 3',4'-dideoxygenated gentamicin C's remains clinically useful (Table 3.1, Chapter 3).[1]

Semisynthetic deoxygenation was applied early-on to the kanamycins (Figure 4.2), enforcing simple selective protection of ring C and dideoxygenation by classic methods, discussed further below. This provided dibekacin, which later became the substrate for N1-substitution towards arbekacin. Likewise, similar semisynthetic modifications have been carried out in the ribostamycin/butirosin subfamily (Figure 4.1).[23] Another significant approach to access novel deoxygenated aminoglycosides has been *mutational biosynthesis* or *mutasynthesis*,[24,25] a technique which was pioneered with these antibiotics, based on feeding mutant strains deficient in 2-deoxystreptamine biosynthesis with advanced synthetic precursors for their integration into novel

antibiotics, dubbed *mutamicins*. Relevant antibiotics include the 2-hydroxy gentamicin complex, Win 42322-2, which was originally advanced up to preclinical studies and 3',4'-dideoxygenated butirosins which were produced by feeding 3',4'-dideoxy neamine precursors (Figure 4.1). Nevertheless, the total screening of analogs available by these methods remained minimal.[24,25] These pioneering techniques should be considered predecessors of "synthetic biology", and were severely limited by either the ineptitude of the natural biosynthetic enzymes to process diverse synthetic precursors or deficiencies in the producer's self-immunity to the resulting antibiotic (e.g. APH(3')).[24,25] These problems could now be addressed with modern bioengineering technology, and although these applications are currently underdeveloped, they are regarded as potential sources of valuable advanced intermediates.[26]

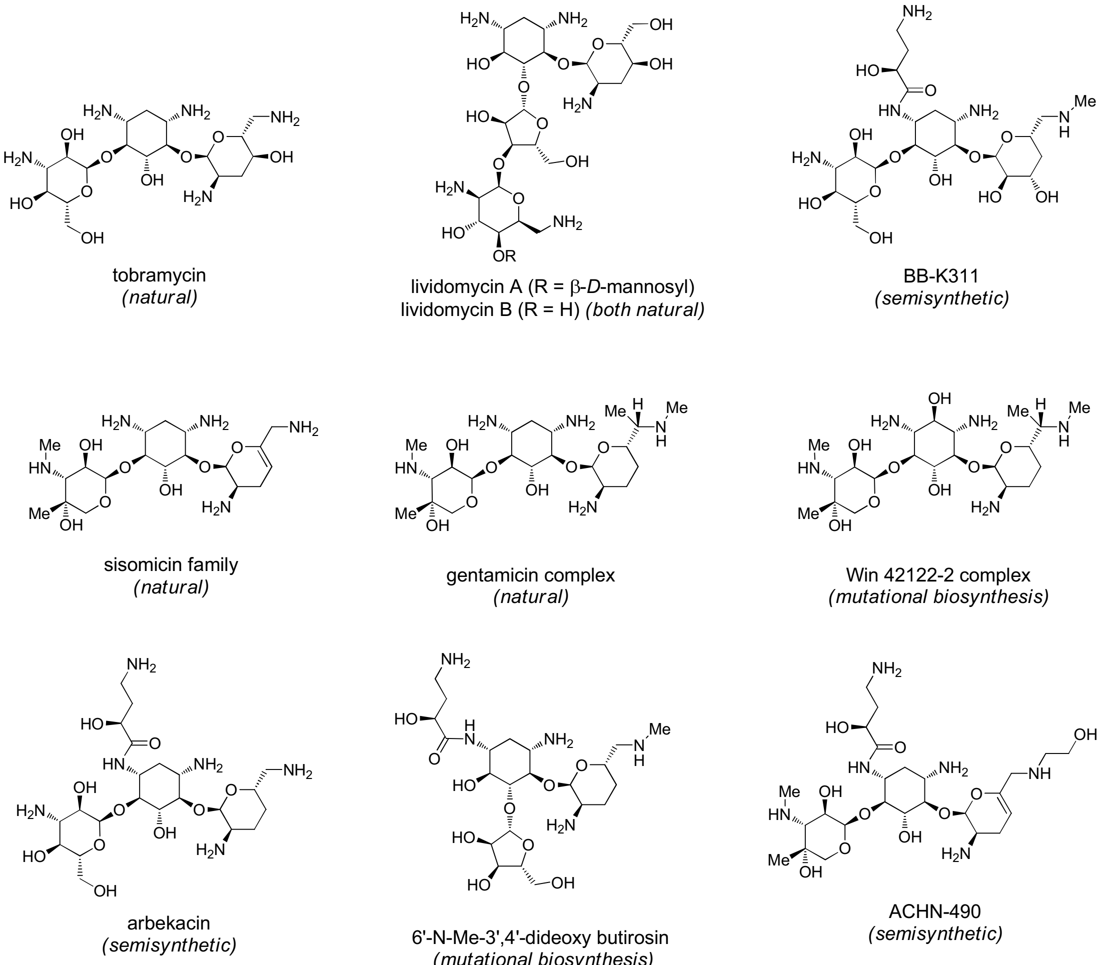


Figure 4.1. Examples of deoxygenated aminoglycosides from natural, semisynthetic and mutasynthetic sources.

4.4 - Synthetic Deoxygenation Methodologies

Throughout any synthetic endeavour, it is almost unavoidable to utilize molecular handles which are not required in the end-product, and a large proportion of these functionalities are C–O single or double bonds. Fortunately, deoxygenation methodologies are abundant and many options are mechanistically dissimilar and differentially selective or tolerant to a range of free or protected functionalities.

In general, deoxygenation at a single site is often achieved by hydroxyl activation for direct hydride displacement, β-elimination, Barton-McCombie radical reduction, or its nucleophilic displacement followed by dehalogenation or desulfuration by metal-catalyzed hydrogenation.[27] Alternatively, carbonyls are reduced to the methylene state *via* Clemmensen reduction, Wolf-Kishner reaction or milder variations.[27] Dideoxygenation methodologies for vicinal diols are dependent on their configuration. *Trans* diols require displacements to vicinal halides, in variations of the classic deoxygenation reactions by Tipson-Cohen and Kuhn-Winterstein.[28] *Cis* diols can also be deoxygenated through rearrangement of activated cyclic intermediates: thionocarbonates are deoxygenated with phosphine treatment in the Corey-Winter reaction,[27,28] various substituted dioxolanes undergo Eastwood deoxygenation,[28,29] and diol complexes with various metals produce olefins, for example by the McMurray method.[30] Most of these aforementioned deoxygenation methodologies have been applied to suitably protected carbohydrates.[31]

In the context of aminoglycosides, deoxygenation has been achieved by Barton-McCombie reduction of selected alcohols,[32,33] or alternatively dideoxygenation of the 3',4'-positions has involved variations of the Tipson-Cohen reduction (Figure 4.2).[34,35,36,37,38] The application of the diol-selective Samuelsson-Garegg modification[39] to aminoglycosides is of current interest within our research group.[40,37,38]

Regardless of the seemingly minimal chemical modifications required, the problem of aminoglycoside synthesis resides in accessing a suitably protected substrate for each reaction (Figure 4.2). The steps required to manipulate large aminoglycosides into the correct protection states can quickly grow to be quite numerous.[1,41] Often, these routes rely on protecting-group selectivity which can lead to unpredictable results and

difficult optimizations.[42] In this context a versatile and high-yielding semisynthetic route for 3',4'-deoxygenation in the paromomycin and neomycin subfamily is lacking (Figure 4.2 D).

A

from kanamycin A
5 steps, 75% yield

NaI, Zn dust
DMF, 95 °C
(86%)

B

from neomycin,
5 steps, 30% yield

1) NaI, $Na_2S_2O_3$, acetone, RT
2) H_2O, AcOH, dioxane
(80%)

C

from paromomycin
5 steps, 46% yield

1) NaOMe, MeOH
2) BzCl, py
(60%)

1) NaI, acetone, AcONa, AcOH
2) MsCl, py
(33%)

D

from paromomycin,
11 steps, 6% yield

1) NaOMe, MeOH
2) Ac_2O, py
(77%)

1) NaI, acetone, AcONa, AcOH
2) MsCl, py
(76%)

Figure 4.2. Examples of aminoglycoside 3',4'-dideoxygenation with variations of the Tipson-Cohen reaction. Data from references 34, 35, 43 and 38, respectively.

4.5 - Tsuji Palladium-Catalyzed Deoxygenation

Revision of the plausible biosynthetic deoxygenations in the sisomicin/gentamicin family (Chapter 1, Section 1.5) led to the hypothesis that the 3'-hydroxyl could be activated as a leaving group for reductive removal facilitated by stabilization from the neighboring electron-rich olefin, in a system well set for Ferrier type I reactions. The olefin would, in turn, arise from dehydration of 4'-OH through a 6'-aldehyde-equivalent under pyridoxal phosphate catalysis (Figure 4.3).[44]

Figure 4.3. Biosynthetic hypothesis for sisomicin accounting the involvement of APH(3') kinases.

The abiotic synthetic counterpart to this biosynthetic hypothesis would involve a Ferrier type I rearrangement intercepted by a hydride source.[27] The allyloxocarbenium species may, however, be difficult to reduce with regio- and stereochemical control without decomposition of such a labile ring A system (Figure 4.4, top).[45] Alternatively, invoking the participation of a metal complex, the reduction of the allylic leaving group may be attained with higher control and milder reaction conditions, such as through a (π-allyl)palladium-catalyzed hydrogenolysis reaction (Figure 4.4, bottom). Notably, this biosynthesis-inspired semisynthetic route is attractive, because it would circumvent numerous protection and deprotection steps required for isolation of 3'-OH from the remaining hydroxyl groups in the respective aminoglycoside.[46]

Figure 4.4. Comparison of biosynthesis-inspired routes for 3'-deoxygenation.

The variants of (π-allyl)palladium substitutions in which hydrides plays the role of nucleophile were extensively studied by Tsuji (Figure 4.5).[47] Mechanistically, they begin with coordination and oxidative insertion of Pd(0) with inversion of configuration to form a Pd(II) π-allyl intermediate as in the Tsuji-Trost allylation reaction.[27,47] The regiochemical outcome of the reduction of the intermediate depends on the choice of reducing agent (Figure 4.5). Metal hydride sources (boron, silicon, aluminum, etc) produce palladium hydride intermediates which undergo reductive elimination through the least-substituted σ-complex, leaving the most-substituted olefin as the major product (Figure 4.5, top).[47] The reaction is better known for the exception to the rule, in which formic acid or formate salts act as the reducing agents to provide the least substituted olefin product (Figure 4.5, bottom).

Current mechanistic understanding suggests that palladium hydride species are likely not involved.[47] Instead, hydride delivery from a formyl ligand is thought to be a concerted inner-sphere process, usually drawn as pericyclic reaction of 8-electrons 7-centers (as shown on Figure 4.5),[47] or as 6-electrons 6-centers by other proponents.[48]

Figure 4.5. Mechanistic details of variants of Tsuji palladium-catalyzed hydrogenolyses.

Steric interactions of the palladium complex with substrate in σ-coordination dictate the regiochemical outcome towards the least or most substituted olefin product with high control (Figure 4.5).[47] Furthermore, the stereochemistry of the product is dictated from initial inversion of configuration upon generation of the (π-allyl)palladium complex, followed by hydride delivery from this same face as the metal.[47,49] Common leaving groups (LG) include activated hydroxyl groups (esters, formates, carbonates and sulfonates), as well as halides, sulfones, alkoxides, nitro groups, and strained cycles such as epoxides, aziridines and cyclopropanes.[27,47]

Application of Tsuji allylic hydrogenolysis to carbohydrates has limited literature precedent (Figure 4.6 A), likely because glycals are often unreactive toward π-allyl complex formation compared to olefins elsewhere on carbohydrates,[50-55] a similar effect as that initially reported for linear enol ethers by Trost (Figure 4.6 panels A *vs.* B).[54] Literature examples of reactions involving (π-allyl)palladium complexes on carbohydrates and glycal substrates are shown in Figure 4.6, a rather short list, but exhaustive to the best of our knowledge. Furthermore, Tsuji allylic hydrogenolysis has been successfully applied to a variety of sterically-demanding carbocyclic substrates in a plethora of total syntheses of natural products.[47,56]

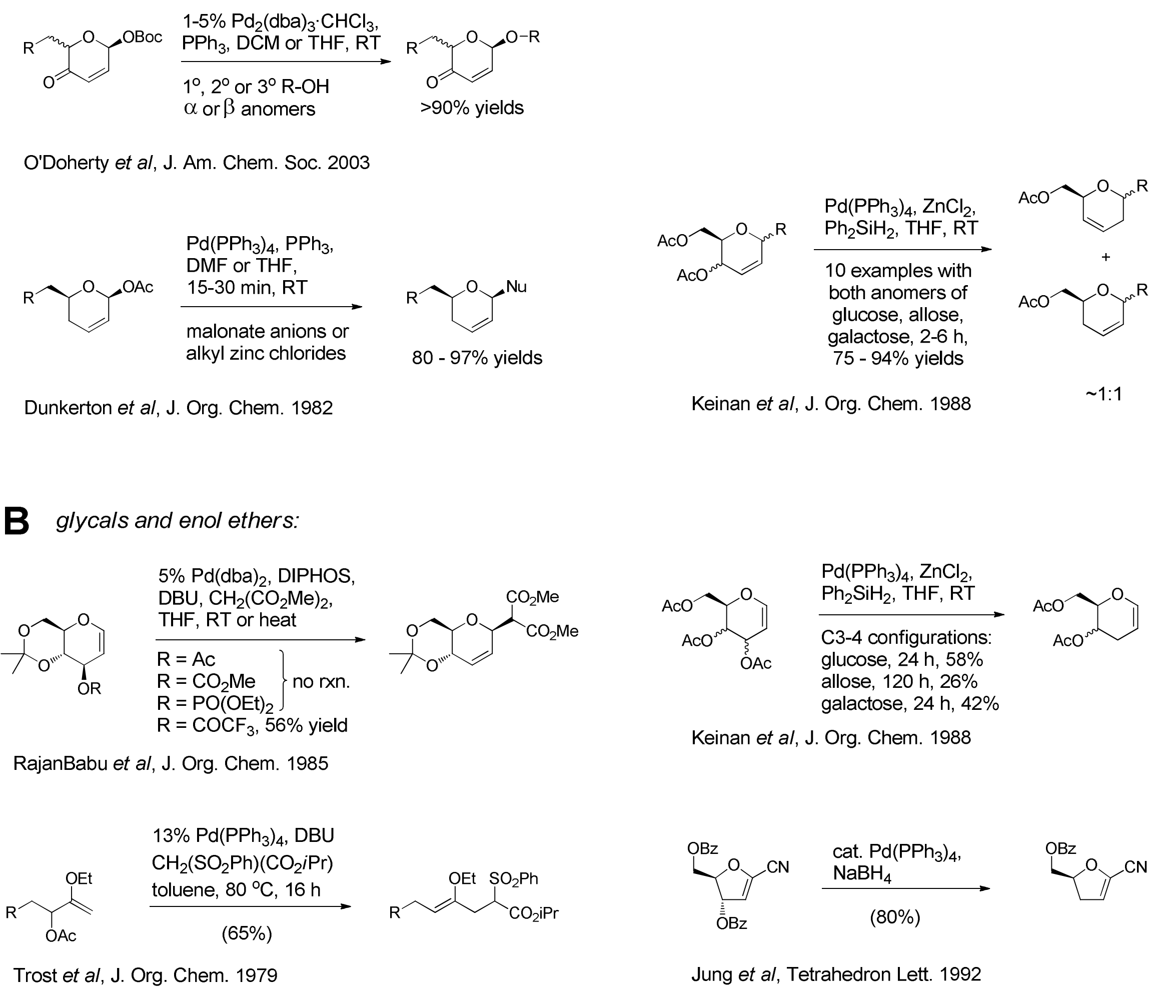


Figure 4.6. Examples of reactions involving (π-allyl)palladium complexes on (A) unsaturated carbohydrates and (B) glycals or enol ethers. Data from references 50, 51, 52, 53, 52, 54 and 55, respectively.

4.6 - Deoxygenation of Glucosamine Models of Paromomycin

A cautious approach required the construction of a compatible model system for the α-D-glucosamyl ring A of paromomycin, because the poorly defined and complex NMR spectra of aminoglycosides may render difficult the assignment and structural interpretation of regio- and stereochemical outcomes of the reaction.

Scheme 4.1 shows the synthesis of glucosamine models for testing the feasibility of the Tsuji deoxygenation methodology. Glucosamine hydrochloride was treated under classic Fischer glycosidation conditions, followed by Cbz-protection allowing column chromatographic purification of the α-anomer. Subsequent manipulations of the alcohols, leaving the 3-position free were effected *via* a 4,6-benzylidene (**4.1**), in expectation of using procedure later for paramomycin.[57] Subsequent installation of sufficiently acid-stable 3-leaving/protecting groups permitted benzylidene removal. Primary alcohol **4.2** was temporarily masked with a silyl group and the secondary alcohol was mesylated to give **4.3**, in preparation for oxidation-elimination. The protecting group manipulations were later expedited in a one-pot procedure. Oxidation and *in situ* β-elimination was effected with standard Parikh-Doering conditions leading to the α,β-unsaturated aldehyde **4.4** in 72% yield. The aldehyde was subsequently reduced to **4.5** and protected with a silyl group to **4.6**. Similar sequences were used with other acid-stable leaving/protecting groups for Tsuji deoxygenation (Table 4.1).

Scheme 4.1. Synthesis of 3-*O*-methylcarbonate substituted glucosamine model substrates for Tsuji deoxygenation reactions.

At this stage, several substrate variations were put to trial for Tsuji deoxygenation conditions towards 3,4-dideoxydehydro products (Table 4.1). Initial attempts with $Pd(PPh_3)_4$ from commercial sources or made *in situ* were unsuccessful, as were lower ligand stoichiometries (Table 4.1, entries 1 and 2). On the other hand, the complex of $Pd_2(dba)_3$ and PBu_3 produced the first successful reduction with

concomitant olefin translocation (Table 4.1, entry 3).[56] The C5-stereochemistry of the crystalline product was ascertained by X-ray crystallographic analysis (Figure 4.7). Similar yields of deoxygenated product with silyl protection (Table 4.1, entry 5) ruled out an interaction by primary alcohol coordination of the palladium complex.[56] In regard to leaving group preference, a significant improvement in yield occurred with the methylcarbonate-activated substrate was tested (Table 4.1, entry 6), however, its direct synthetic precursor was found unstable, and apparently underwent Ferrier type I rearrangement during workup or column chromatography to a compound assigned as **4.7** by NMR (Scheme 4.1, box). This defect was not observed for the analogs with 3-*O*-acetyl and benzoyl leaving groups.

Table 4.1. Representative results of Tsuji deoxygenation of glucosamine models

4.8 R1 = TBS
4.9 R1 = H

Entry	Compound	R_1	R_2	Catalyst and Ligand[a]	Time (h)	Yield (%)[b]
1	**4.10**	Bz	H	40% $Pd(PPh_3)_4$	48	no rxn
2	**4.10**	Bz	H	20% Pd_2dba_3 - 40% PPh_3	48	no rxn
3	**4.10**	Bz	H	20% Pd_2dba_3 - 40% PBu_3	20	61 (**4.9**)
4	**4.11**	Ac	H	20% Pd_2dba_3 - 40% PBu_3	20	27 (**4.9**)
5	**4.12**	Bz	TBS	20% Pd_2dba_3 - 40% PBu_3	36	57 (**4.8**)
6	**4.6**	CO_2Me	TBS	20% Pd_2dba_3 - 40% PBu_3	16	85 (**4.8**)
7	**4.5**	CO_2Me	H	20% Pd_2dba_3 - 40% PBu_3	16	decomp.

a) Conditions: THF, 60 ºC, 11 equiv. Et_3N, 10 equiv. HCO_2H, under Ar atmosphere. Scales of 50 - 75 mg for each substrate. b) Isolated yields after full conversion.

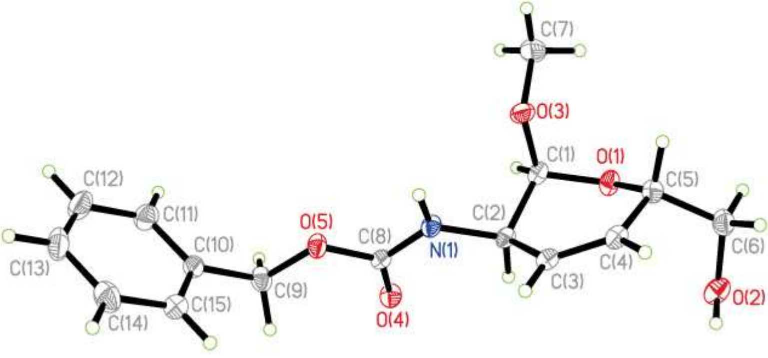


Figure 4.7. ORTEP drawing of **4.9** with thermal ellipsoids drawn at 30% probability level.

Tsuji deoxygenation of the highest yielding substrates was further investigated. Unfortunately, the high catalyst loading and long reaction times could not be improved by varying solvent (THF, benzene, CH_3CN, DCM, dioxane, DMSO and DMF), increasing reaction temperature, careful deoxygenation procedures or by changes in ligand stoichiometry. Notably, reductions of catalyst and ligand from the prohibitive 40 mol% palladium loading led to traces of conversion. Alternative reducing agents than triethylammonium formate did not promote conversion, including $NaCNBH_3$, $NaBH_4$ and Ph_2SiH_2.[47,53-55]

In view of the difficulties other substrates were surveyed and attention turned to aldehyde **4.4** (Scheme 4.2). Although this substrate lacked literature precedent in such reactions, this initially disregarded possibility provided deoxygenated product **4.10** several times faster than previous attempts in superior yield.

H, O, MeO, CbzHN, OCO_2Me — **4.4**

20% mol Pd_2dba_3, 40% mol Bu_3P — Et_3N, HCO_2H, THF, 60°C, 3 h — (92%)

H, O, MeO, CbzHN — **4.13**

Scheme 4.2. Tsuji palladium-catalyzed deoxygenation of aldehyde **4.4**.

The enhancements effected by conjugation of the electron withdrawing aldehyde function could be rationalized by taking into account that palladium(0) has higher affinity for electron-deficient olefins, as mentioned by Trost in the context of π-allyl complex formation with enol ether substrates.[54] Mechanistically, the regiochemistry of the olefin product is that expected using metal-hydride sources, but it may be explained by the mechanism involving an *endo*-face (π-allyl)palladium complex delivering hydride on a C5-*D*-configuration, followed by olefin conjugation (Figure 4.8). Employing DCO_2D as reducing agent the resulting deuteration at C3 was lost during workup and chromatography, indicative of enolization of aldehyde **4.13**. No deuteration was detected elsewhere (e.g. C4).

Figure 4.8. Mechanistic considerations of Tsuji deoxygenation for the generation of product **4.10**.

These encouraging results in the Tsuji deoxygenation model studies justified the challenge of the full aminoglycoside system, with the goal of obtaining paromomycin and neomycin products containing a 4',5'-unsaturation analogous to **4.13**.

4.7 - Tsuji Palladium-Catalyzed Deoxygenation of Paromomycin

The synthetic effort began with well-known and reliable manipulations of paromomycin (Scheme 4.3). Global *N*-Cbz protection was chosen, because these carbamates have provided better versatility and reliability in medicinal chemistry applications of aminoglycosides than other amine protecting groups. The Boc protecting group has been successfully applied; however, of aminoglycosides is contingent on their acid tolerance. Furthermore, *per-N*-Cbz aminoglycosides are generally soluble in organic solvents and purified by column chromatography. Two reliable options are available for reductive Cbz deprotection: hydrogenation catalyzed by $Pd(OH)_2/C$ (Pearlman's catalyst),[58] and Birch reduction with sodium in liquid ammonia.[27]

As aforementioned, the 4',6'-benzylidene (**4.14**) is the derivative of choice for site-selective protection of the ring A diol of paromomycin. Intermediate **4.14** is a valuable semisynthetic starting material and it has been industrially produced following the original preparations of three decades ago, in scales of hundred kilograms in quantitative yields and over 95% purity.[26,33, 59]

Scheme 4.3 structures: paromomycin; 1) CbzCl, $NaHCO_3$, MeOH/H_2O; 2) PhCHO, HCO_2H (91%) → 4.14; 1) DMAP, py, $O_2N-C_6H_4-OC(O)OMe$, THF reflux, 48 h; 2) 80% AcOH, 60 °C (91%) → 4.15; TBSOTf, py; then MsCl; then MeOH; then HF·py (96%) → 4.16; SO_3·py, DMSO, Et_3N, DCM (92%) → 4.17

Scheme 4.3. Semisynthetic manipulations of paromomycin toward α,β-unsaturated aldehyde intermediate **4.17** for Tsuji palladium-catalyzed 3'-deoxygenation.

The global protection of the six remaining alcohols of intermediate **4.14** with methylcarbonate groups could not be achieved following literature conditions involving methyl chloroformate. In spite of the addition of hundreds of equivalents, only complex mixtures of randomly protected intermediates were obtained. This result was rationalized by reacting methylchloroformate and triethylamine in DCM, an exotherm was observed with gas bubbling and rapid precipitation of triethylmethylamonium chloride.[60] Employing *para*-nitrophenyl methylcarbonate, which can be produced in bulk and cleanly precipitated as long needles from hot hexanes,[61] under transesterification conditions, intermediate **4.14** was protected in excellent yield. The six methylcarbonate protecting groups withstood the benzylidene deprotection with AcOH at 60 ºC to afford **4.15** in excellent yield. Subsequently, the remaining protecting group manipulations for ring A were expedited to a one-pot protocol, profiting from the highly selective silylation of 6'-OH and the higher reactivity of mesyl chloride at 4'-OH over the excess TBSOTf. Both electrophiles were then quenched with stoichiometric amounts of methanol and the mixture was directly exposed to excess pyridinium hydrogen fluoride overnight to produce mesylate **4.16**.[62] The Parikh-Doering oxidation and elimination procedure gave finally the desired α,β-unsaturated allylic carbonate **4.17**, with an overall 72% yield from commercial paromomycin sulfate.

The Tsuji deoxygenation conditions developed in model studies functioned well on the more sterically-demanding aminoglycoside scaffold **4.17**, yielding 82% of 3'-deoxygenation product **4.18** (Table 4.2, entry 1). Subsequent efforts to lower the catalyst loading led to proportional decrease in yield and geometric increase of reaction time (Table 4.2, entries 1-3). This trend suggested that the stoichiometry of metal and ligand was inappropriate for the formation of an active complex, likely a minor species despite the high metal loadings. A metal-ligand ratio to 1:2 was sufficient to surmount these issues at viable catalyst loadings (entry 4). Further reduction in catalyst loading was not pursued and may continue similar trend (entry 5). The competent 10 mol% palladium system was equally reliable at scales ranging from 10 mg to 1 g, leading to smooth 3'-deoxygenation in exceptional yields (Table 4.2, entry 4).

Table 4.2. Optimization of Tsuji palladium-catalyzed 3'-deoxygenation of paromomycin.

Entry	Pd_2dba_3 (mol%)	Bu_3P (mol%)	Time (h)	Yield (%)
1	20	40	3	82
2	10	20	9	73
3	5	10	24	46
4	**5**	**20**	**3**	**97**
5	2.5	5	5	78

Conditions: THF, 60 ºC, 11 equiv. Et_3N, 10 equiv. HCO_2H, under Ar atmosphere, scales of 100 mg of substrate **4.17**. Entry 4 was also performed in 1.0 g scale with identical yield.

In the optimized Tsuji deoxygenation reaction, a deep burgundy red THF solution of Pd_2dba_3, substrate **4.17** and triethylamonium formate was degassed with argon. The mixture underwent a striking color change to brown upon addition of Bu_3P, indicative of π-allyl complex formation. On the other hand, the color of unoptimized reactions was relatively opaque and black. Deoxygenation proceeded to completion equally well at 60 ºC for 3 h or at room temperature overnight, without requiring particularly strict degassing or anhydrous measures. Once starting material was consumed, the mixture was passed through a pad of silica to remove black palladium particles. The filtrate was partitioned between EtOAc and brine to remove the formate salts. The crude product was then purified from the ligands by precipitation in hexanes or column chromatography.

The Parikh-Doering and Tsuji palladium-catalyzed 3'-deoxygenation sequence competes well with to other methods for producing 3',4'-dideoxygenated aminoglycosides (Figure 4.2).[37,38] This scalable "spot-to-spot" reaction sequence provides an overall yield of 70% to furnish an advanced intermediate for 6'-functionalization toward novel sisomicin-hybrid scaffolds.

4.8 - Synthesis of Sisomicin-Hybrid Aminoglycosides

The production of gram quantities of 4',5'-dehydro-3',4'-dideoxy aldehyde **4.18** permitted the exploration of a novel subfamily of hybrid aminoglycosides containing the characteristic ring A of sisomicin. The specific objectives of the first generation of analogs included assessment of their antibiotic spectrum against resistant strains expressing modifying enzymes (primary panel, Chapter 3) and discernment of the structure-activity relationships for substitutions at the 6'-position, the overall contribution of ring D and spectrum enhancement by the N1-HABA group.

Mild reduction of the aldehyde functionality of **4.18** in the presence of the labile methylcarbonate groups could be effected in acidic conditions with sodium cyanoborohydride (Scheme 4.4), to produce the isolated 6'-alcohol **4.19**. Subsequent displacement with the simplified Mitsunobu conditions reported by Merck chemists[63] secured the allylic 6'-azide function on **4.20**, which had been previously proven to safely

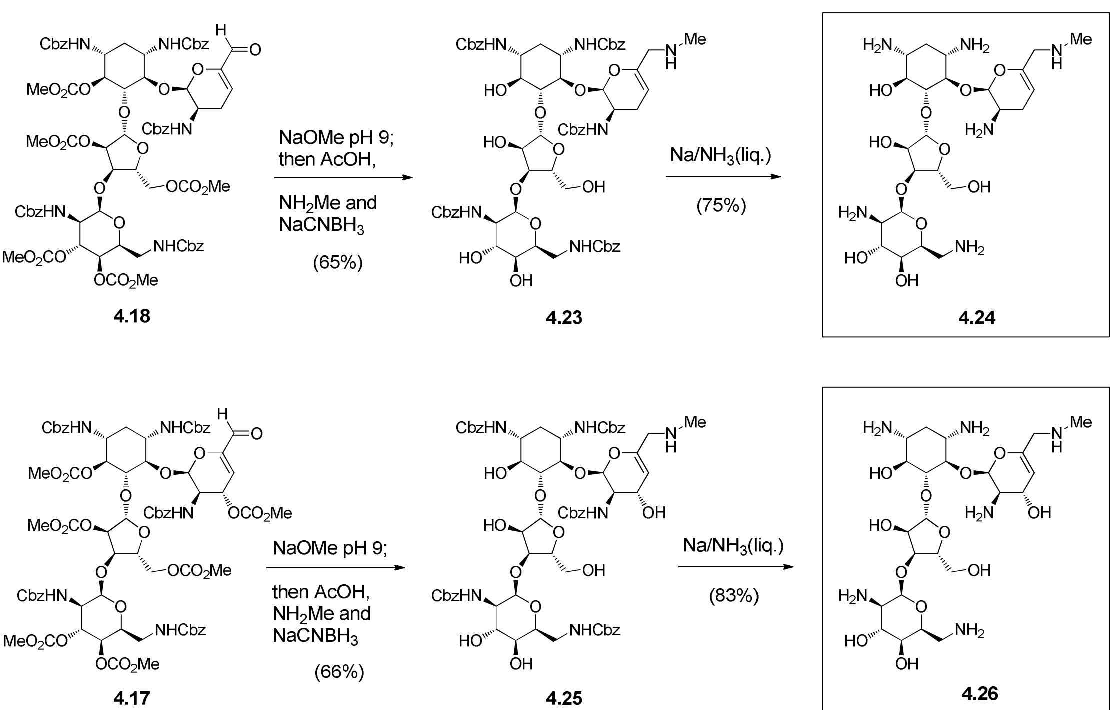
defy [3,3]-sigmatropic rearrangement on sisomicin (see Chapter 6).[64] Thereafter, global deprotection of the methylcarbonate groups was effected with catalytic sodium methoxide in methanol. Birch reduction of the 6'-azide and removal of the Cbz groups with sodium in liquid ammonia occurred in 5 to 10 minutes at -78 ºC, leaving intact the characteristic ring A olefin of the desired sisomicin-paromomycin **4.21** and sisomicin-neomycin **4.22** hybrids (Scheme 4.4).

Scheme 4.4. Synthesis of sisomicin-paromomycin **4.21** and sisomicin-neomycin **4.22** hybrids.

During a typical Birch reduction, complete deprotection within minutes was confirmed by mass spectrometry (direct injection, ESI positive mode). The most convenient quenching procedure was by adding excess acetic acid, leading to instant disappearance of the blue color from solvated electrons. The ammonia was then removed by bubbling argon, and the crude was purified by column chromatography using a modification of the solvent system for aminoglycoside TLC: mixtures of methanol, chloroform and conc. ammonium hydroxide in proportions varying from 2:3:0.5 to 2:3:2. Aminoglycosides were thus eluted as free bases, usually with a trace of

silica (<5% weight) which was easily removed by filtration after lyophilization and reconstitution in water. Purities were assessed in excess of 95% by NMR spectroscopy and HPLC using Chemiluminescent Nitrogen Detection (see Experimental Section for NMR spectra and HPLC reports, pages 140-163).

Methyl amine was introduced by reductive amination on aldehyde **4.18** (Scheme 4.5). One-pot deprotection of the methylcarbonate groups gave intermediate **4.23**, which was converted to **4.24**, a neomycin-hybrid of *antibiotic G-52* (natural analog of the sisomicin subfamily, see Chapter 1).[1] Although bulkier groups could be installed in this fashion (*N*-Et, *N*-*i*Pr or *N*-*t*Bu), previous analogs of sisomicin suggested loss of antibiotic activity would outweigh protection against AAC(6') isoforms, in particular for *P. aeruginosa* strains.[64] In this first generation, the aldehyde was not functionalized with Grignard reagents due to the potential lack of stereospecificity and associated complications.[64] In the form of a "negative control" for antibacterial testing, aldehyde **4.17** was likewise transformed into the analogous 3'-hydroxy hybrid **4.26** (Scheme 4.5).



Scheme 4.5. Synthesis of sisomicin-neomycin hybrids by reductive amination.

The removal of ring D to obtain the ribostamycin trisaccharide framework was performed taking advantage of the vicinal diols at 3''',4''' of paromomycin and neomycin hybrids **4.19** and **4.20** (Scheme 4.6).[65] After carbonate removal, oxidative cleavage with periodate followed by β-elimination with DBU, furnished intermediates **4.27** and **4.29**, reductive deprotection of which expanded the first generation library towards ribostamycin-sisomicin hybrid analogs **4.28** and **4.30**, respectively. The latter is a compound previously produced by Paulsen[66] and later by Le Goffic[67] through riboslylation at position 5-OH in sisamine (the core degradation product of sisomicin, see Chapter 6). The ^{13}C spectrum shifts of **4.30** matched identically the values previously reported by these authors (Table 4.3).[67]

Scheme 4.6. Oxidative diol cleavage toward sisomicin-ribostamycin hybrids **4.28** and **4.30**.

Table 4.3. Comparison of ^{13}C-NMR shifts of sisomicin-ribostamycin hybrid **4.30**.

	Reported 4.30 (20 MHz)[66]	Compound 4.30 (176 MHz)
Assignment	**δ_C (ppm)**	**δ_C (ppm)**
1	50.9	50.7
2	29.2	28.9
3	49.2	49.2
4	79.0	78.7
5	84.5	84.7
6	72.9	72.8
1'	97.5	97.5
2'	46.7	46.7
3'	24.1	23.7
4'	101.3	101.5
5'	144.3	144.2
6'	41.4	41.4
1"	111.2	111.4
2"	75.7	75.8
3"	70.6	70.5
4"	83.4	83.4
5"	63.0	62.8

Attention turned to the isolation of N1 for coupling of the HABA group. Selective N1 base-catalyzed deprotection *via* N1,O6 cyclic carbamates is a general and well-established procedure in aminoglycoside literature. It has been applied for N1-functionalization of the ribostamycin family,[66] as well as protected neamine, paromamine or sisamine.[67] In general, it is a feasible procedure using intermediates with a free 6-OH and neighboring Cbe or Cbz groups, if alternative cyclic carbamates are not possible elsewhere on the molecule. Once a cyclic carbamate is formed, it can be hydrolyzed faster than the linear Cbe or Cbz carbamates, liberating CO_2 to provide the free amine. Therefore, exposure of intermediate **4.29** to KHMDS produced cyclic carbamate **4.31**, which was hydrolyzed with saturated $Ba(OH)_2$, and coupled under standard conditions to (L)-*N*-Cbz-γ-amino-β-hydroxybutiric acid (Cbz-HABA) en route to the sisomicin-butirosin hybrid **4.33** (Scheme 4.7). Paulsen reported the synthesis up to the 3,6'-*N*-Cbe protected analog of **4.31**.[66]

Scheme 4.7. Selective N1-deprotection and appendage of L-HABA to sisomicin-butirosin hybrid **4.33**.

For the purpose of N1 modification of the more challenging neomycin scaffold, 6'-azido intermediate **4.20** was selected (Scheme 4.8). Isolation of N1 *via* cyclic carbamates in the neomycin/promomycin scaffold has been developed by colleagues in our laboratory.[40] Provided 3',4'-dideoxygenation prevents formation of alternative ring A cyclic carbamates, exposure to anhydrous strong base typically provides two cyclic carbamates in moderate yield: the N1,O6-oxazolidinone in ring B and N6''',O4'''-oxazinone in ring D. Notably, in these systems the ring B carbamate can be selectively hydrolyzed with mild aqueous LiOH, liberating N1 for coupling.[40] Structural assignments for these products were achieved by Mass Spectrometry ion decomposition studies and also validated with MIC data.[40] The remaining ring D cyclic carbamate can be cleaved with strong aqueous base, but the intermediates become difficult to purify due to their high polarity. However, equilibration in sodium benzyl oxide in benzyl alcohol drives the cyclic carbamate back to a Cbz group, almost to completion.[40] Likewise, careful exposure of **4.20** to KHMDS furnished *bis*-cyclic carbamate **4.34**, which was selectively hydrolyzed and acylated to N1-HABA intermediate **4.35** under standard coupling conditions. The ring D carbamate was then equilibrated to **4.36** for purification prior to global deprotection in Birch reduction conditions. The resulting aminoglycoside **4.37** evokes a sisomicin-neomycin-butirosin hybrid, which was thus given the name 'Frankenmycin' in our laboratory.

Scheme 4.8 (chemical scheme, not detected as an image): 4.20 → 4.34 (KHMDS, DMF 0 °C; 58%) → 4.35 (1) aq. LiOH, DMF; 2) EDC, NHS, HABA, DIPEA; 39%) → 4.36 (NaOBn, BnOH; 85%) → 4.37 (Na/NH_3(liq.); 63%).

Scheme 4.8. Selective N1-deprotection *via* bis-cyclic carbamate intermediates for appendage of L-HABA to sisomicin-butirosin-neomycin hybrid **4.37**.

4.9 - Analysis of Antibacterial Activity

The first generation of sisomicin-neomycin hybrid aminoglycosides were challenged with the Achaogen's primary panel (Table 4.4, Figure 4.9) representing a range of enzymatic and efflux resistance mechanisms and important pathogenic bacteria (see Chapter 3, Section 3.8). The accompanying Figure 4.9 includes all relevant structures for structure-activity comparisons. For color-coding in accordance with the approximate subclass therapeutic index see Chapter 3, Section 3.9.

Table 4.4. Antibacterial assesment of sisomicin-hybrids and controls. Minimum inhibitory concentration (μg/mL).

		MIC (μg/mL)														
Bacterium	**Description/Phenotype**	Amk	Gent	NeoB	Par	H-Par	Btr	Ribo	**4.28**	**4.30**	**4.33**	**4.21**	**4.26**	**4.24**	**4.22**	**4.37**
Wild Type Strains																
S. aureus	ATCC 29213 (QC)	2	0.5	0.5	2	2	8	16	>64	16	8	8	2	1	1	0.5
E. coli	ATCC 25922 (QC)	2	0.5	2	4	2	2	8	>64	64	8	>64	4	8	2	0.5
K. pnuemoniae	ATCC 10031	0.5	0.25	0.5	2	1	1	2	>64	16	4	>64	0.5	2	0.5	0.5
P. aeruginosa	ATCC 27853 (QC)	2	0.5	32	>64	8	>32	>64	>64	16	4	>64	>64	2	0.5	0.25
P. aeruginosa	Wild type pump	2	2	8	>64	4	16	>64	>64	64	8	>64	64	2	1	0.5
A. baumannii	Susceptible	2	2	1	4	2	1	8	>64	>64	4	>64	2	4	1	0.5
Efflux Strains																
P. aeruginosa	MexXY upregulated	4	2	16	>64	8	32	>64	>64	64	16	>64	>64	4	2	0.5
A . baumannii	ATCC 19606	16	16	4	16	16	8	32	>64	>64	32	>64	32	64	32	8
P. aeruginosa	Efflux system	16	4	32	>64	32	>32	>64	>64	>64	32	>64	>64	16	4	2
P. aeruginosa	Efflux system	16	8	64	>64	64	>32	>64	>64	>64	64	>64	>64	32	16	4
Modifying Enzymes and Methytransferase																
Providencia stuartii	AAC(2')-I	4	64	32	>64	>64	>32	>64	>64	>64	>64	>64	>64	>64	64	8
E. coli	APH(3')-Ib	0.5	0.25	64	>64	2	1	>64	>64	64	4	>64	>64	>64	>64	0.25
S. aureus	APH(3')-III	8	0.5	>64	>64	32	>32	>64	>64	>64	16	>64	>64	16	16	1
A. baumannii	AAC(3)-I + APH(3')-VI + ANT(2')-I	>64	>64	>64	>64	64	>32	>64	>64	>64	8	>64	>64	>64	>64	1
Acinetobacter spp.	APH(3')-VI	>64	0.5	16	>64	32	>32	>64	>64	64	4	64	8	4	1	1
S. aureus	ANT(4')-I	64	0.5	>64	>64	>64	>32	>64	>64	16	8	16	1	2	1	1
P. aeruginosa	ANT(4')-II	32	2	8	>64	4	>32	>64	>64	64	8	>64	16	4	1	0.5
S. marcescens	AAC(6')-I	32	4	8	4	8	>32	>64	>64	>64	>64	>64	4	8	32	32
P. aeruginosa	AAC(6')-I	>64	64	>64	>64	16	>32	>64	>64	>64	>64	>64	>64	32	>64	>64
P. aeruginosa	AAC(6')-II	4	32	8	>64	8	>32	>64	>64	>64	16	>64	>64	>64	>64	0.5
E. coli	ANT(2")-I	4	64	2	4	8	4	8	>64	>64	8	>64	8	16	2	4
E. cloacae	ANT(2")-I + AAC(6') + APH(3')-I	64	32	>64	>64	8	32	>64	>64	>64	>64	>64	>64	>64	>64	8
S. aureus	APH(2")/AAC(6')	64	>64	>64	>64	32	>32	>64	>64	>64	16	>64	>64	>64	64	2
P. aeruginosa	AAC(3)-I	4	32	8	>64	8	16	>64	>64	>64	16	>64	32	>64	2	1
E. coli	AAC(3)-IV	2	16	2	8	4	2	>64	>64	>64	8	>64	8	8	8	1
E. coli	ArmA	>64	>64	1	4	4	2	8	>64	64	8	>64	8	16	2	1

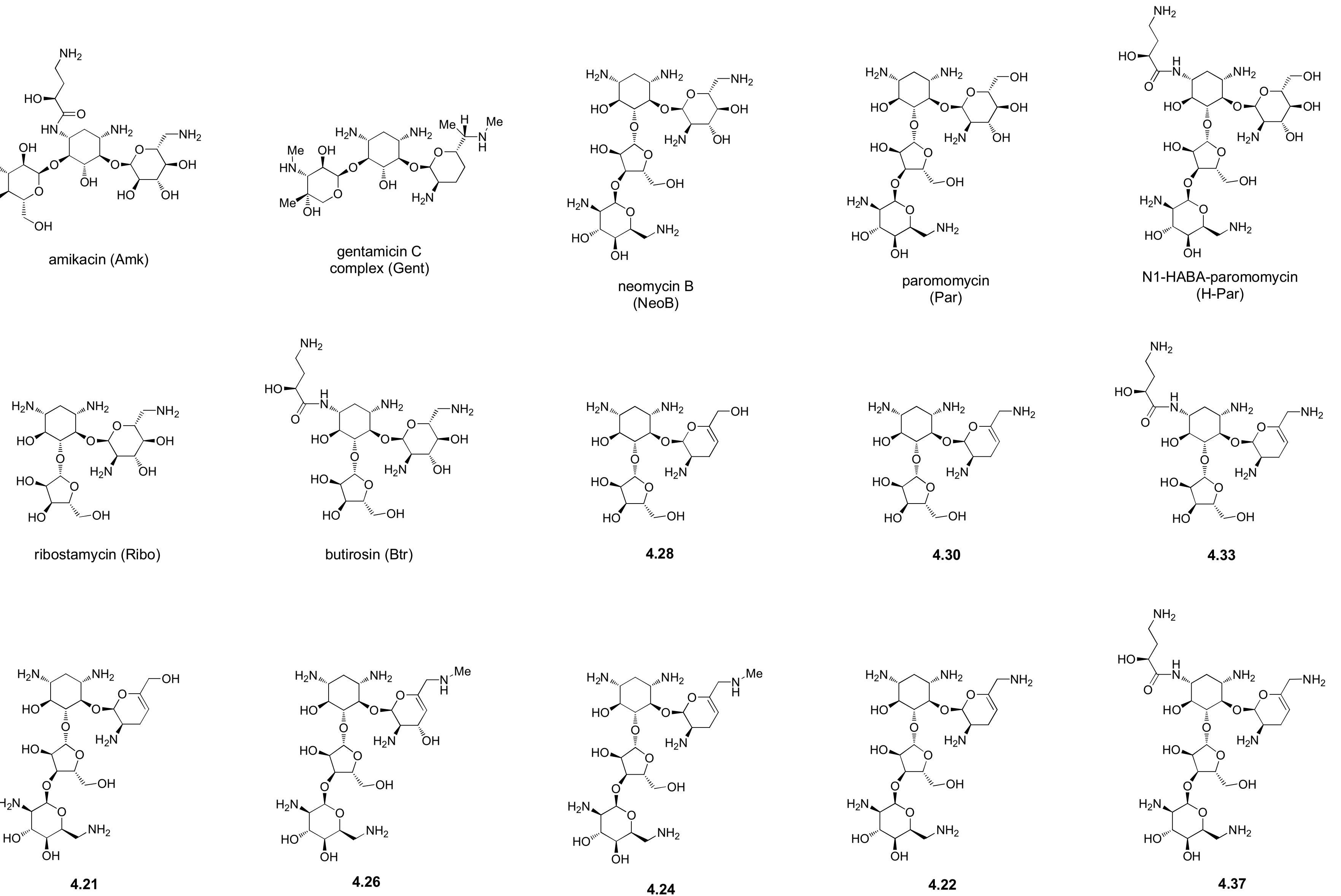


Figure 4.9. Structures of the novel sisomicin hybrid analogs presented in this chapter, compared with natural and semisynthetic control antibiotics.

Aminoglycosides of the paromomycin type, featuring a 6'-OH, **4.21** and **4.28** were less active compared to similar 6'-amino congeners **4.22** and **4.30**, respectively (Table 4.4). This for **4.28**, was consistent with other naturally derived 6'-OH ribostamycin analogs;[68] however, 3',4'-dideoxy paromomycins such as those prepared by Tipson-Cohen deoxygenation (Figure 4.2 C) have been reported to inhibit selected strains of *E. coli*, *S. aureus* and *S. epidermis*.[36] In our hands, most dideoxygenated paromomycins, such as **4.21** and analogs with N1-HABA groups, tend to be relatively poor antibiotics compared to their neomycin congeners (e.g. **4.22**), demonstrating the importance of testing and reporting an extended panel. This defect appears to be related to unforeseeable poor bacterial uptake of 3',4'-dideoxy paromomycins, which paradoxically have been show to be superior than paromomycin at inhibition of *in vitro* translation (Chapter 3).[36]

Unlike the 6'-amino aminoglycosides, paromomycin is active against a range of protozoa and cestode parasites. Notably, it has recently emerged as an important line of treatment against Leishmaniasis,[69] a disease caused by intracellular parasites of macrophages transmitted by sandflies in tropical and subtropical underdeveloped regions of the world,[69] which ranks high on the list of neglected diseases by the World Health Organization (WHO) and its partners.[70] Paromomycin analogs **4.21** and **4.28** were therefore provided to partners of the Institute of One World Health (iOWH) for antileishmanial assessment.[71] Furthermore, the 6'-hydroxyl aminoglycosides, which evade several enzymes could have potential as competitive inhibitors of AAC(6') isoforms,[72] much like tobramycin can inhibit APH(3') enzymes.[20]

The antibacterial activity of the sisomicin-ribostamycin hybrid **4.30** was lower across the spectrum of susceptible strains compared to its parent ribostamycin, reiterating that deoxygenation is an overall inactivating modification. However, both activity and spectrum were greatly enhanced by the N1-HABA group in **4.33** or the presence of ring D in **4.22**. The sisomicin-butirosin hybrid **4.33** displays clear enhancements *versus* the parent butirosin, and also neomycin B, paromomycin or HABA-paromomycin against a range of APH(3') and ANT(4') isoforms, with the exception of the APH(3'/5")-III which is known to effect phosphorylation of 5"-OH. This analog remained, however, defective against the effluxing strains of *P. aeruginosa* and *A. baumannii*, and its N1-HABA group appeared to provide poor protection against

most AAC(6') isoforms. In spite of its good antibiotic activity, **4.33** provides limited improvement over amikacin, a result consistent with similar butirosin analogs which were not pursued to clinical trials in the past.[3]

The sisomicin-neomycin hybrid series behave similarly in activity and spectrum to neomycin. With the exception of **4.26**, the "negative control" 3'-OH analog**,** the 6'-amino and 6'-methylamino hybrids **4.22** and **4.24** evade all ANT(4')s and most APH(3') isoforms, including those constitutive to *P. aeruginosa*. Here again, specific isoforms APH(3')-III and Ib appear able to phosphorylate 5''-OH. The effectiveness of **4.22** and **4.24** against effluxing strains of *P. aeruginosa* and *A. baumannii* is marginally better than the parent neomycin B, but superior to paromomycin or HABA-paromomycin. Likewise, as expected ribosomal modification by the ArmA methyltransferase does not significantly affect the neomycin family of aminoglycosides (see Chapter 3, Figure 3.6).

Finally, the combination of features of 3',4'-deoxygenation and N1-HABA in the sisomicin-butirosin-neomycin hybrid **4.37**, manifest strong synergism. In fact, the promiscuous APH(3'/5''') isoforms are completely evaded, much like all ANT(4')s. Four out of five AAC(6') isoforms are unable to target **4.37**, including the problematic bifunctional enzyme APH(2')/AAC(6'). Only the AAC(6')-I isoform in *P. aeruginosa* and *S. marcescens* strains provided resilience to **4.37**; however, it remains a relatively rare resistance determinant in this pathogen (Chapter 3, Figure 3.3). Protection against AAC(6')-I is conveyed by 6'*N*-methylation (e.g. **4.24** *vs.* **4.22**), implying this might be a desirable modification for **4.37** analogs. Other enzymes of the types AAC(3) and AAC(2'), which recognize the neomycin scaffold less efficiently than the kanamycin and gentamicin families, are further deterred by the N1-HABA group. Most importantly, **4.37** is potent against *P. aeruginosa* and *A. baumannii* strains with upregulated wild-type pumps or expressing the multi-aminoglycoside resistance efflux systems. Overall, 'Frankenmycin' **4.37** exceeds the antibacterial activity spectrum of current clinical standards amikacin and gentamicin. In fact, based on its extended *in vitro* inhibition spectrum it ranks as one of the most powerful aminoglycoside antibiotics to date.[38,37]

4.10 - Conclusions

The design and development of an efficient, high yielding, biosynthesis-inspired semisynthetic modification of the neomycin and paromomycin framework has led to 3',4'-dideoxy-4',5'-dehydro-aminoglycosides of the novel sisomicin-hybrid class. The structure-activity relationship of 8 structurally diverse analogs was assessed against 5 natural and 2 semisynthetic aminoglycosides, including two clinical standards, amikacin and gentamicin. Structure-activity relationships against a range of pathogenic strains expressing modifying enzymes and efflux systems have demonstrated the importance of *3',4'-dideoxygenation, 6'-amination, N1-substitution and the presence of ring D* for maximum antibacterial activity and enhanced spectrum. The powerful hybrid analog 'Frankenmycin' **4.37**, which includes all these features is proof that the current mechanisms of bacterial aminoglycoside resistance all may be overcome by carefully-designed antibiotic analogs of the neomycin family (Figure 4.10, Table 4.5). Additional modifications for improving analogs may include 6'-substitution (e.g., *N*-methyl or *N*-hydroxyethyl groups) and N1-side chain variations, which may be accessible from the presented semisynthetic strategy.

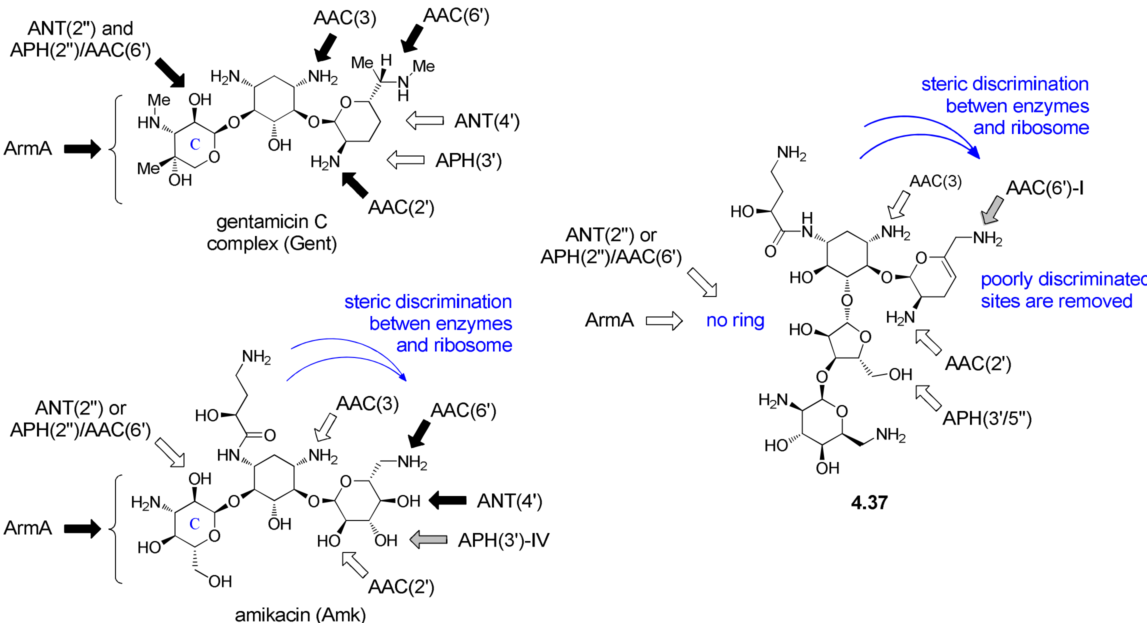


Figure 4.10. Comparative assessment of enzymatic resistance mechanisms targeting the gentamicin complex (Gent), amikacin (Amk) and 'Frankenmycin' **4.37**, see Table 4.4. Arrows indicate enzyme susceptibility, dark = severe, grey = one isoform, and white = none.

Examination of the prevalent resistance mechanisms in *ESKAPE* bacteria (Figure 3.3 and Table 3.1) indicates that such 3rd generation neomycin analogs may be effective and worthy of development, particularly for indications in urgent need of broad spectrum antibiotics. Considering that the neomycin families tend to rank among the more nephrotoxic aminoglycosides, one possible strategy would be to produce nebulizer formulations for nosocomial pneumonia and cystic fibrosis related infections,[73] followed by studies for indications requiring higher-risk intravenous administrations. Ideally, oto- and nephro-protectants would be simultaneously developed to provide the best therapeutic index for application as first-line treatments.

The bulk of this work has been included in U.S. provisional patent application No. 61/250,114, filed Oct. 9, 2009.[74]

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resistant (MDR) Gram-negative bacteria and methicillin-resistant *Staphylococcus aureus* (MRSA). Achaogen's most advanced compound, ACHN-490, a "neoglycoside" or next-generation aminoglycoside, is expected to be useful in multiple clinical indications, including complicated urinary tract infection (cUTI), hospital-acquired pneumonia (HAP) and blood stream infections (BSI). The compound completed a successful Phase 1 study in 2009 and is entering Phase 2 in 2010. Achaogen has several other innovative small molecule antibacterial programs rapidly nearing clinical development. Achaogen has numerous scientific programs addressing the global emergence of bacterial resistance. To date, Achaogen has secured more than $100 million in non-dilutive financing to support the development of these programs." Achaogen Fact Sheet Jan2010, http://www.achaogen.com/uploads/pdf/Achaogen%20Fact%20Sheet%20Jan2010.pdf (accessed May 16, 2010).

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Chapter 4 - Experimental Section

General Procedures

All reactions were carried out under an inert atmosphere of argon with dry solvents, using anhydrous conditions unless otherwise stated. Dry dichloromethane (DCM) and tetrahydrofuran (THF) were obtained from a solvent delivery system with activated alumina columns. Methanol (MeOH) was distilled from CaH_2 under argon. Reagents were purchased at the highest commercial quality and used without further purification. Flash column chromatography was performed with silica gel from SilicaFlash P60, particle size 40-63 µm, 230-400 mesh and distilled hexanes, ethyl acetate (EtOAc) or DCM. Free amines were purified with DCM applying gradients of 'ammoniacal MeOH' (referring to a 1:9 solution which was freshly prepared with 28% ammonia liquor before use). Deprotected free-base aminoglycosides were purified with homogeneous solvent systems consisting of $CHCl_3$/MeOH/$NH_4OH_{(aq)}$ in ratios ranging from 2:3:0.5 to 2:3:2. Yields refer to chromatographically and spectroscopically homogeneous material. Low temperature experiments conducted for longer than 3 h were conducted with a Cryocool apparatus with an acetone bath. Reactions were monitored by direct-injection low resolution mass spectrometry (LRMS) and thin layer chromatography (TLC) carried out on 0.25 mm E. Merck silica precoated plates (60F-254), visualized under UV light developed with acidified ammonium molybdate/cerium sulfate and heat. NMR spectra were recorded on Bruker ARX-400, AV-400 or AV-700 instruments and are calibrated using residual undeuterated solvent as an internal reference. The following abbreviations were used to explain multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Low resolution mass spectra (LRMS) were recorded on a Thermo Finnigan Surveyor MSQ and high resolution mass spectra (HRMS) were recorded on an Agilent Technologies LC-MSD TOF mass spectrometer by electrospray ionization in positive mode. Either protonated molecular ions $[M+H]^+$ or sodium adducts $[M+Na]^+$ were used for empirical formula confirmation. IR spectra were recorded on a Perkin-Elmer Spectrum One FT-IR using NaCl tablets. Optical rotations were recorded in a 1 dm cell at ambient temperature, on a Perkin-Elmer 343 polarimeter. Analytical HPLC was performed in Achaogen Inc. using mobile phases with 0.1% HFBA, column Sunfire C18, 3x50mm, 2.5 µm, flow of 0.5 mL/min at 40 ºC and Chemiluminescent Nitrogen Detection (CLND), water/MeOH gradient A: 25 to 95% in 20 min or gradient B: 30 to 75% in 30 min.

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4.1

Methyl 4,6-benzylidene-2-benzyloxycarbonylamino-2-deoxy-α-*D*-glucopyranoside (4.1).

Glucosamine hydrochloride (20 g, 92.7 mmol) and $NaHCO_3$ (20 g, 238 mmol) were dissolved in water (100 mL) and treated with benzyl chloroformate (16 mL, 112 mmol). After stirring overnight, the resulting white suspension was filtered, washing with copious amounts of water and $CHCl_3$. The white crude paste was dried under high-vacuum overnight and used without further purification. Acetyl chloride (45 mL, 570 mmol) was added dropwise slowly to ice-cold MeOH (250 mL) to generate a 3% w/w HCl solution. Crude *N*-Cbz-glucosamine was added to the HCl solution, which was stirred at RT for 24 h, when LRMS indicated complete glycosidation and TLC (12% $MeOH/CHCl_3$) displayed two products: major α (R_f = 0.3) and minor β (R_f = 0.25). The solution was neutralized with lead carbonate (121 g, 450 mmol) and the resulting white suspension was filtered. The filtrate was evaporated to dryness and absorbed onto silica gel for short column chromatography purification (4 → 6% MeOH in DCM) which eluted 12.5 g (41%, 38 mmol) of methyl 2-benzyloxycarbonylamino-2-deoxy-α-*D*-glucopyranoside (**4.S1**).

R_f = 0.3 in 12% $MeOH/CHCl_3$.
$[\alpha]^{22}_D$ 92.7° (*c* 0.8, MeOH), lit $[\alpha]^{22}_D$ 95.2° (*c* 1.0, MeOH).[1]
HRMS (ESI) calcd. for $C_{15}H_{21}NO_7$, M + H^+ = 328.1391, found 328.1387 (-1.12 ppm).
^{1}H NMR (CD_3OD, 300 MHz) δ 7.40-7.25 (m, 5H), 5.09 (s, 2H), 4.68 (d, *J* = 2.68 Hz, 1H), 3.83 (dd, *J* = 11.82, 2.05 Hz, 1H), 3.69 (dd, *J* = 11.85, 5.55 Hz, 1H), 3.64-3.60 (m, 1H), 3.60-3.46 (m, 2H), 3.37 (s, 3H), 3.39-3.30 (m, 1H).
^{13}C NMR (CD_3OD, 75 MHz) δ 159.0, 138.4, 129.6 - 129.0 (5C), 100.3, 73.8, 73.3, 72.4, 67.7, 62.9, 57.3, 55.7.

A solution of methyl α-*D*-glucosaminide **4.S1** (4 g, 12 mmol) in benzaldehyde (100 mL) was treated with formic acid (14 mL), and stirred for 24 h at RT. The solution was poured over a stirred sat. $NaHCO_3$ solution and the mixture was extracted with EtOAc. The volatiles were evaporated under high vacuum at 60 °C to a residue, which was dissolved in

EtOAc and washed with $NaHCO_3$ to remove benzoic acid traces, dried over $MgSO_4$, filtered and evaporated to a residue under vacuum. Purification by column chromatography (2% MeOH/DCM) yielded 4.4 g of the title compound **4.1** (87%, 10.5 mmol), as a white amorphous solid.

R_f = 0.27 in 1:2 EtOAc/hexanes.
HRMS (ESI) calcd. for $C_{22}H_{25}NO_7$, M + H^+ = 416.1704, found 416.1714 (2.48 ppm).
^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.54-7.45 (m, 2H), 7.42-7.29 (m, 8H), 5.54 (s, 1H), 5.23-5.07 (m, 3H), 4.73 (d, J = 3.22 Hz, 1H), 4.35-4.20 (m, 1H), 4.01-3.82 (m, 2H), 3.82-3.70 (m, 2H), 3.56 (t, J = 8.73, 8.73 Hz, 1H), 3.38 (s, 3H), 2.79 (s, 1H).
^{13}C NMR ($CDCl_3$, 75 MHz) δ 157.0, 137.3, 136.3, 129.4 - 126.5 (10C), 102.2, 99.4, 82.1, 70.7, 69.1, 67.5, 62.6, 55.9, 55.6.

OH
O
MeO
OH
CbzHN
OCO_2Me
4.2

Methyl 2-benzyloxycarbonylamino-2-deoxy-3-*O*-methoxycarbonyl-α-*D*-glucopyranoside (4.2).

Procedure A) A solution of alcohol **4.1** (922 mg, 2.22 mmol) in DCM (50 mL), was cooled in an ice bath, was treated pyridine (2.9 mL, 33.3 mmol) and methyl chloroformate (860 µL, 11.1 mmol), and stirred allowing to waram to RT over 1 h. Excess reagent was quenched with MeOH (1 mL), the solution was diluted with DCM, washed with 2 N HCl and sat. $NaHCO_3$, dried over Na_2SO_4, filtered and evaporated to a residue under vacuum. Purification by column chromatography (30% EtOAc/hexanes), yielded 1.0 g (95%, 2.11 mmol) of methyl 4,6-benzylidene-2-benzyloxycarbonylamino-2-deoxy-3-*O*-methoxycarbonyl-α-*D*-glucopyranoside (**4.S2**), as a single diastereomer of undetermined stereochemistry..

Procedure B) A solution of alcohol **4.1** (2.2 g, 5.3 mmol) in THF (50 mL) was treated with pyridine (570 µL, 7 mmol), DMAP (32 mg, 260 µmol) and *p*-nitrophenyl methyl carbonate (1.1 g, 5.7 mmol), and heated to reflux overnight. Excess reagent was quenched with 1 mL MeOH for 15 minutes. The solution was diluted with EtOAc, washed

with 2 N HCl, twice with 1 M NaOH and sat. NaCl. The organic fraction was dried over $MgSO_4$, filtered and evaporated under vacuum to a residue that was purified by column chromatography yielding 2.5 g (99%, 5.28 mmol) of methyl 4,6-benzylidene-2-benzyloxycarbonylamino-2-deoxy-3-*O*-methoxycarbonyl-α-*D*-glucopyranoside (**4.S2**), as a single diastereomer of undetermined stereochemistry.

R_f = 0.44 in 1:2 EtOAc/hexanes.
HRMS (ESI) calcd. for $C_{24}H_{27}NO_9$, M + H^+ = 474.1759, found 474.1747 (-2.75 ppm).
^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.46-7.38 (m, 2H), 7.37-7.25 (m, 8H), 5.49 (s, 1H), 5.19-5.00 (m, 4H), 4.73 (d, *J* = 3.51 Hz, 1H), 4.27 (dd, *J* = 9.79, 4.24 Hz, 1H), 4.07 (dt, *J* = 10.14, 10.13, 3.54 Hz, 1H), 3.90-3.80 (m, 1H), 3.80-3.68 (m, 2H), 3.66 (s, 3H), 3.37 (s, 3H).
^{13}C NMR ($CDCl_3$, 75 MHz) δ 156.10, 155.92, 137.15, 136.46, 129.30 - 126.45 (10C), 101.84, 99.45, 79.24, 74.61, 69.05, 67.19, 62.92, 55.64, 55.29, 54.64.

Benzylidene **4.S2** (1 g, 2.11 mmol) was dissolved in a minimum volume of DCM, treated with 80% AcOH (50 mL) and stirred heating at 60 °C overnight. The mixture was evaporated to a minimum volume under high-vacuum at 50 °C, diluted with DCM and washed with sat. $NaHCO_3$, dried over Na_2SO_4, filtered and evaporated to a residue under vacuum. Purification by column chromatography (2.5% MeOH/DCM) yielded 785 mg of the title compound (96%, 2.0 mmol), as a white amorphous solid.

R_f = 0.25 in 3:1 EtOAc/hexanes.
HRMS (ESI) calcd. for $C_{17}H_{23}NO_9$, M + H^+ = 386.1446, found 386.1436 (-2.56 ppm).
^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.39-7.32 (m, 5H), 5.24 (d, *J* = 9.84 Hz, 1H), 5.09 (q, *J* = 12.28, 12.22, 12.22 Hz, 2H), 4.89 (dd, *J* = 10.41, 9.38 Hz, 1H), 4.75 (d, *J* = 3.42 Hz, 1H), 3.99 (dt, *J* = 10.42, 10.35, 3.50 Hz, 1H), 3.91-3.83 (m, 2H), 3.83-3.62 (m, 1H), 3.70 (s, 4H), 3.54 (d, *J* = 4.73 Hz, 1H), 3.38 (s, 3H), 2.72 (t, *J* = 5.11, 5.11 Hz, 1H).
^{13}C NMR ($CDCl_3$, 75 MHz) δ 156.6, 156.2, 136.4, 128.7 - 128.3 (5C), 98.8, 78.5, 71.5, 68.9, 67.2, 61.9, 55.5, 55.4, 53.9.

CbzHN OCO$_2$Me MeO OH OTBS O

4.S3

Methyl 2-benzyloxycarbonylamino-2-deoxy-3-*O*-methoxycarbonyl-6-*tert*-butyldimethylsilyl-α-*D*-glucopyranoside (4.S3).

Diol **4.2** (760 mg, 1.97 mmol) was dried by evaporation three times from toluene, dissolved in DCM (12 mL), cooled to 0 °C and treated with Et_3N (360 μL, 2.56 mmol), DMAP (12 mg, 0.1 mmol), TBSCl (357 mg, 2.37 mmol) and stirred in an ice bath, which was allowed to warm to RT overnight. The reaction was quenched with sat. NH_4Cl, diluted with DCM and washed with 2 N HCl and sat. $NaHCO_3$, dried over Na_2SO_4, filtered and evaporated to a residue under vacuum. Purification by column chromatography (25% EtOAc/hexanes), yielded 913 mg of the title compound (93%, 1.82 mmol), as a white amorphous solid.

R_f = 0.4 in 1:2 EtOAc/hexanes.

HRMS (ESI) calcd. for $C_{23}H_{37}NO_9Si$, M + H^+ = 500.2310, found 500.2292 (-3.65 ppm).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.39-7.31 (m, 5H), 5.20-5.02 (m, 3H), 4.91 (dd, *J* = 10.64, 9.08 Hz, 1H), 4.72 (d, *J* = 3.49 Hz, 1H), 3.96 (ddd, *J* = 20.55, 10.40, 4.05 Hz, 2H), 3.87-3.74 (m, 2H), 3.72 (s, 3H), 3.66 (td, *J* = 9.60, 4.87, 4.87 Hz, 1H), 3.38 (s, 3H), 3.12 (d, *J* = 2.91 Hz, 1H), 0.91 (s, 9H), 0.10 (s, 6H).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 156.6, 156.1, 136.5, 128.7 - 128.3 (5C), 98.7, 78.2, 71.2, 70.6, 67.1, 64.3, 55.4, 55.3, 53.7, 26.0 (3C), 18.5, -5.3 (2C).

CbzHN OCO$_2$Me MeO OMs OTBS O

4.S4

Methyl 2-benzyloxycarbonylamino-2-deoxy-4-*O*-mesyl-3-*O*-methoxycarbonyl-6-*tert*-butyldimethylsilyl-α-*D*-glucopyranoside (4.S4).

Alcohol **4.S3** (887 mg, 1.78 mmol), was dried by evaporation three times from toluene, was dissolved in DCM (10 mL), treated with Et_3N (300 μL, 2.13 mmol) and mesyl

chloride (150 μL, 1.95 mmol), and stirred for 3 h at RT. The reaction was quenched with sat. NH_4Cl, diluted with DCM, washed with 2 N HCl and sat. $NaHCO_3$, dried over Na_2SO_4, filtered and evaporated to a residue under vacuum. Purification by column chromatography (25% EtOAc/hexanes) yielded 1.03 g of the title compound (99%, 1.77 mmol), as a white amorphous solid.

R_f = 0.4 in 1:2 EtOAc/hexanes (co-spots with **4.S3**).
HRMS (ESI) calcd. for $C_{24}H_{39}NO_{11}SSi$, , M + Na^+ = 600.1905, found 600.1890 (- 2.63 ppm).
^1H NMR ($CDCl_3$, 300 MHz) δ 7.39-7.32 (m, 5H), 5.20-5.02 (m, 4H), 4.78-4.66 (m, 2H), 4.06 (dt, J = 10.33, 10.21, 3.44 Hz, 1H), 3.97-3.88 (m, 1H), 3.85-3.68 (m, 3H), 3.71 (s, 1H), 3.39 (s, 3H), 3.03 (s, 3H), 0.91 (s, 9H), 0.09 (d, J = 1.72 Hz, 6H).
^{13}C NMR ($CDCl_3$, 75 MHz) δ 156.0, 155.6, 136.4, 128.7 - 128.3 (5C), 98.2, 75.7, 75.6, 70.4, 67.2, 61.8, 55.6, 55.5, 54.3, 38.7, 26.0 (3C), 18.5, -5.1 (2C).

4.3

Methyl 2-benzyloxycarbonylamino-2-deoxy-4-*O*-mesyl-3-*O*-methoxycarbonyl-α-*D*-glucopyranoside (4.3).

Procedure A) Silyl ether **4.S4** (1.02 g, 1.77 mmol) was dissolved in THF (10 mL), treated with acetic acid (220 μL, 2.64 mmol) and TBAF (1.0 M in THF, 2.64 mL, 2.64 mmol), and stirred overnight at RT. The mixture was evaporated under vacuum with gentle heating to a residue, which was purified by column chromatography (0.5 → 1% MeOH/DCM) yielding 740 mg of the title compound (91%, 1.6 mmol), as a white amorphous solid.

Procedure B) The protecting group manipulations above, leading to compound **4.3** were performed as a one-pot procedure. Compound **4.2** (1.18 g, 3.07 mmol) was dried by evaporating three times from toluene, dissolved in pyridine (10 mL), cooled to 0 °C and treated dropwise with TBSOTf (780 μL, 3.4 mmol). After stirring at 0 ºC for 2 h, TLC and LRMS indicated that only 6-OTBS intermediate **4.S3** was present. Mesyl chloride (1.4 mL,

18 mmol) was added to the mixture, which was stirred and allowed to warm to RT. After 3 h, LRMS analysis indicated that only 4-OMs,6-OTBS intermediate **4.S4** was present. The remaining mesyl chloride was quenched with excess MeOH (800 μL), at which point some pyridinium chloride precipitated. After 30 min, the crude mixture was cooled to 0 °C and treated with 3 mL of 70% HF in pyridine, and stirred at 0 °C overnight, when TLC and LRMS indicated complete silyl deprotection. The reaction was neutralized by pouring it over a stirred sat. $NaHCO_3$ solution. This mixture was extracted with EtOAc, and the organic fraction was evaporated under high-vacuum at 60 °C to a residue, which was diluted with EtOAc, washed with 2 N HCl and sat. $NaHCO_3$, dried over Na_2SO_4, filtered and evaporated to a residue under vacuum. Purificaiton by column chromatography (0.5 → 1% MeOH/DCM), yielded 1.42 g of the title compound (99%, 3.06 mmol), as a white amorphous solid.

R_f = 0.1 in 1:1 EtOAc/hexanes.
HRMS (ESI) calcd. for $C_{18}H_{25}NO_{11}S$, M + H^+ = 464.1221, found 464.1209 (-2.56 ppm).
^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.40-7.30 (m, 5H), 5.16 (d, J = 12.16 Hz, 2H), 5.08 (dd, J = 19.70, 10.99 Hz, 2H), 4.76 (dd, J = 17.20, 6.73 Hz, 2H), 4.11 (dt, J = 10.32, 10.28, 3.42 Hz, 1H), 3.97-3.70 (m, 4H), 3.70 (s, 2H), 3.40 (s, 3H), 3.07 (s, 3H).
^{13}C NMR ($CDCl_3$, 75 MHz) δ 155.9, 155.5, 136.3, 128.7 - 128.3 (5C), 98.5, 75.4, 75.3, 69.9, 67.2, 60.5, 55.8, 55.7, 54.3, 38.6.

H
O
MeO
CbzHN
OCO_2Me
4.4

Methyl 6'-aldehydo-2-benzyloxycarbonylamino-2-deoxy-3-*O*-methoxycarbonyl-α-*D*-glucopyranoside (4.4).

Alcohol **4.3** (1.42 g, 3.06 mmol), was dried by evaporation three times from toluene, dissolved in DCM (30 mL) and Et_3N (20 mL), and cooled to 0 °C. In a separate flask, a DMSO (12 mL) solution containing SO_3·pyridine complex (4.4 g, 27.6 mmol) was prepared. The DMSO solution was added dropwise to the alcohol mixture, which was

stirred at 0 ºC for 15 min and at RT for 3 h, when a single strongly UV-active spot was observed on TLC (1:1 EtOAc/Hex, R_f = 0.6). The reaction was quenched with water and evaporated under vacuum to a liquid residue, which was diluted with DCM and washed successively with 2 N HCl and sat. $NaHCO_3$, dried over Na_2SO_4, filtered and evaporated to a residue. Purification by column chromatography (0.5 → 1% MeOH/DCM) yielded 805 mg of the title compound (72%, 2.2 mmol), as a light yellow amorphous solid.

R_f = 0.6 in 1:1 EtOAc/hexanes, UV-active.
$[\alpha]^{22}_D$ 188.2° (*c* 0.5, $CHCl_3$).
HRMS (ESI) calcd. for $C_{17}H_{19}NO_8$, M + Na^+ = 388.1003, found 388.1005 (0.65 ppm).
^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.25 (s, 1H), 7.42-7.29 (m, 5H), 5.93 (d, *J* = 2.64 Hz, 1H), 5.43 (dd, *J* = 9.15, 2.61 Hz, 1H), 5.22-5.03 (m, 4H), 4.28 (dt, *J* = 9.07, 9.06, 2.22 Hz, 1H), 3.80 (s, 3H), 3.51 (s, 3H).
^{13}C NMR ($CDCl_3$, 75 MHz) δ 185.7, 155.9, 155.6, 149.4, 136.2, 128.8, 128.5, 128.4, 117.2, 99.9, 71.0, 67.5, 57.3, 55.6, 51.3.

OH
O
MeO
CbzHN
OCO_2Me
4.5

Methyl 2-benzyloxycarbonylamino-4,5-dehydro-2,4-dideoxy-3-*O*-methoxycarbonyl-α-*D*-glucopyranoside (4.5).

Aldehyde **4.4** (20 mg, 54.7 μmol) was dissolved in THF (3 mL), cooled to 0 °C, then treated with $NaBH_4$ (2 mg, 55 μmol) followed by 10 drops of EtOH. After stirring 30 min, the reaction was swiftly passed through a silica pad, washing with EtOAc, and the filtrate was washed with sat. NaCl, dried over $MgSO_4$, filtered and evaporated to a residue under vacuum. Purification by column chromatography on silica gel, which was previously neutralized with 3% Et_3N/hexanes and thoroughly washed with the elution solvent (60% EtOAc/hexanes), yielded 15.2 mg of the acid-sensitive title compound (75%, 39 μmol), as a white amorphous solid.

R_f = 0.2 in 1:1 EtOAc/hexanes.

^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.42-7.31 (m, 5H), 5.21-5.15 (m, 2H), 5.15-5.11 (m, 2H), 5.11-5.06 (m, 1H), 4.99 (d, J = 2.08 Hz, 1H), 4.20 (dt, J = 8.09, 8.03, 2.19 Hz, 1H), 4.07 (s, 2H), 3.79 (s, 3H), 3.52 (s, 3H), 1.86 (s, 1H).

^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.1, 155.5, 153.4, 136.3, 128.8 - 128.4 (5C), 99.2, 95.9, 71.5, 67.3, 62.2, 57.0, 55.2, 51.4.

4.7

((2*S*,3*R*)-3-carboxybenzylamino-2,6-dimethoxy-dihydropyran-6-yl)methanol (4.7).

By-product **4.7** was the only material isolated from chromatography (1% MeOH/DCM) of the preceding reaction product on silica gel which was not neutralized with Et_3N. From aldehyde **4.4** (88 mg, 0.24 mmol), 59 mg of the title compound (66%, 0.18 mmol) were isolated, as one diastereomer of undetermined stereochemistry.

R_f = 0.4 in 2:1 EtOAc/hexanes.

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.42-7.28 (m, 5H), 6.01 (dd, J = 10.39, 2.52 Hz, 1H), 5.75 (dd, J = 10.42, 2.34 Hz, 1H), 5.17 (d, J = 9.24 Hz, 1H), 5.14-5.09 (m, 2H), 4.95 (d, J = 3.83 Hz, 1H), 4.48 (ddd, J = 9.02, 5.98, 2.76 Hz, 1H), 3.62-3.56 (m, 2H), 3.49 (s, 3H), 3.46 (s, 1H), 3.30 (s, 3H).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 156.9 136.2, 130.8, 128.5 - 128.2 (5C), 126.3, 99.1, 98.2, 67.2, 67.1, 56.4, 49.9, 46.7.

OTBS
MeO
CbzHN
OCO_2Me
4.6

Methyl 2-benzyloxycarbonylamino-4,5-dehydro-2,4-dideoxy-3-*O*-methoxycarbonyl-6-*O*-*tert*-butyldimethylsilyl-α-*D*-glucopyranoside (4.6).

Procedure A) Alcohol **4.5** (50 mg, 0.136 mmol), was dried by evaporating three times from toluene, dissolved in dry DCM (3 mL) and cooled to -78 °C. Pyridine (37 µL, 0.50 mmol) and TBSOTf (94 µL, 0.41 mmol) were added to the alcohol solution, which was allowed to warm from -78 °C to 0 °C over 20 minutes. The reaction was quenched with sat. NH_4Cl, diluted with DCM, washed with sat. NaCl, dried over Na_2SO_4, filtered and evaporated to a residue under vacuum. Purification by column chromatography on silica gel, which was previously neutralized with 3% Et_3N/hexanes and thoroughly washed with elution solvent (20% EtOAc/hexanes), yielded 32 mg of the acid-sensitive title compound (49%, 66.6 µmol), as a white amorphous solid.

Procedure B) Aldehyde **4.4** (100 mg, 0.274 mmol), was dried by evaporating three times from toluene, dissolved in dry THF, cooled to -78 °C, treated with dropwise L-selectride (1.0 M in THF, 300 µL, 0.3 mmol) and monitored by TLC and LRMS. Pyridine (130 µL, 1.50 mmol) and TBSOTf (315 µL, 1.34 mmol) were added to the solution, and the reaction was allowed to warm from -78 °C to 0 °C over 20 min. The reaction was quenched with sat. NH_4Cl, diluted with DCM, washed with sat. NaCl, dried over Na_2SO_4, filtered and evaporated to a residue under vacuum. Purification by column chromatography using silica, which was previously neutralized with 3% Et_3N/hexanes and thoroughly washed with the elution solvent (20% EtOAc/hex), yielded 65 mg of the acid-sensitive title compound (49%, 0.135 mmol), as a white amorphous solid.

R_f = 0.4 in 1:4 EtOAc/hexanes.

HRMS (ESI) calcd. for $C_{23}H_{35}NO_8Si$, M + Na^+ = 504.2024, found 504.2034 (1.94 ppm).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.42-7.31 (m, 5H), 5.22-5.17 (m, 1H), 5.13 (d, J = 2.69 Hz, 2H), 5.11-5.02 (m, 2H), 4.95 (d, J = 1.33 Hz, 1H), 4.18 (dt, J = 7.89, 7.69, 1.79 Hz, 1H), 4.07 (s, 2H), 3.79 (s, 3H), 3.51 (s, 3H), 0.93 (s, 9H), 0.10 (s, 6H).

^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.1, 155.5, 154.0, 136.4, 128.7 - 128.3 (5C), 99.0, 94.7, 71.7, 67.3, 62.0, 56.8, 55.1, 51.6, 26.0 (3C), 18.5, -5.2 (2C).

4.8

Methyl 2-benzyloxycarbonylamino-3,4-dehydro-2,3,4-trideoxy-6-*O-tert*-butyldimethylsilyl-*α-D*-glucopyranoside (4.8).

In a typical Tsuji hydrogenolysis (Table 4.1), substrate **4.6** (62 mg, 129 µmol) was dissolved in dry THF (2 mL), treated with Et_3N (180 µL, 1.29 mmol) and formic acid (49.5 µL, 1.29 mmol), and degassed under a flow of argon three times by successive cooling at -78 ºC and sonication at RT. The reaction was started by successively adding tris(dibenzylideneacetone) dipalladium (23.8 mg, 26.0 µmol, 40% mol/mol Pd) and tributylphosphine (5.2 µL, 52 µmmol), which upon stirring at RT underwent a slow wine-red to black color change. After heating at 60 °C for 24 h, complete conversion was observed by LRMS and TLC (1:4 EtOAc/hexanes, UV-active R_f = 0.6). The reaction solution was filtered through a short silica pad washing with EtOAc. The filtrate was washed with sat. NaCl, dried over $MgSO_4$, filtered and evaporated to a residue. Purification by column chromatography (10 → 20% EtOAc/hexanes), yielded 44.5 mg of the title compound (85%, 109 µmol), as a white amorphous solid.

R_f = 0.6 in 1:4 EtOAc/hexanes, UV-active.
HRMS (ESI) calcd. for $C_{21}H_{33}NO_5Si$, M + H^+ = 408.2201, found 408.2195 (-1.46 ppm).
^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.41-7.29 (m, 5H), 5.84 (d, *J* = 10.50 Hz, 1H), 5.62 (d, *J* = 10.37 Hz, 1H), 5.16-5.10 (m, 1H), 5.12 (s, 2H), 4.84 (d, *J* = 4.12 Hz, 1H), 4.51-4.43 (m, 1H), 4.14-4.08 (m, 1H), 3.72 (dd, *J* = 10.20, 5.85 Hz, 1H), 3.60 (dd, *J* = 10.21, 6.14 Hz, 1H), 3.45 (s, 3H), 0.89 (s, 9H), 0.07 (s, 6H).
^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.2, 136.6, 128.8 - 128.1 (5C), 128.0, 125.4, 97.2, 69.0, 67.1, 65.7, 56.0, 47.6, 26.1 (3C), 18.5, -5.1 (2C).

4.9

Methyl 2-benzyloxycarbonylamino-3,4-dehydro-2,3,4-trideoxy-*α*-*D*-glucopyranoside (4.9).

Silyl ether **4.8** (14 mg, 28.7 μmol) was dissolved in THF (1 mL) and treated with TBAF (1.0 M in THF, 60 μL, 60 μmol), and stirred overnight. The mixture was evaporated under vacuum with gentle heating to a residue, which was purified by column chromatography (50% EtOAc/hexanes), yielding 8 mg (78%, 27 μmol) of the title compound. The white solid crystallized upon standing overnight in a mixture of hexanes containing a few drops of EtOAc, and provided diffracting crystals suitable for X-ray acquisition.

R_f = 0.3 in 1:1 EtOAc/hexanes.
$[\alpha]^{22}_{D}$ -6.6° (*c* 0.40, $CHCl_3$).
HRMS (ESI) calcd. for $C_{15}H_{19}NO_5$, M + Na^+ = 316.1169, found 316.1155 (4.22 ppm).
^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.43-7.31 (m, 5H), 5.75 (d, *J* = 10.65 Hz, 1H), 5.70 (d, *J* = 10.96 Hz, 1H), 5.18 (s, 1H), 5.14 (s, 2H), 4.90 (d, *J* = 4.13 Hz, 1H), 4.57-4.48 (m, 1H), 4.27-4.20 (m, 1H), 3.76 (dd, *J* = 11.50, 3.32 Hz, 1H), 3.64 (dd, *J* = 11.44, 6.26 Hz, 1H), 3.49 (s, 3H), 1.93 (s, 1H).
^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.1, 136.5, 128.8 - 128.4 (5C), 126.9, 126.8, 97.3, 68.9, 67.2, 65.1, 56.1, 47.5.

4.13

Methyl 6'-aldehydo-2-benzyloxycarbonylamino-4,5-dehydro-2,3,4-trideoxy-*α*-*D*-glucopyranoside (4.13).

Aldehyde **4.4** (100 mg, 0.274 mmol) was dissolved in dry THF (3 mL), then treated with Et_3N (420 μL, 3.0 mmol) and formic acid (100 μL, 2.7 mmol), degassed under a flow of argon three times by successive cooling to -78 ºC and sonication at RT, treated with

tris(dibenzylideneacetone) dipalladium (12.5 mg, 13.6 μmol) and tributylphosphine (13.6 μL, 54.4 μmmol), and stirred at RT, when the reaction mixture changed from a wine-red to yellow-brown color. After heating at 60 °C for 3 h, LRMS and TLC (1:1 EtOAc/hexanes, UV-active R_f = 0.45) indicated complete conversion. The reaction solution was filtered through a short silica pad washing with EtOAc. The filtrate was washed with sat. NaCl, dried over $MgSO_4$, filtered and evaporated to a residue. Purification by column chromatography (20 → 30% EtOAc/hexanes), yield 80 mg of the title compound (quantitative, 0.274 mmol), as an off-white amorphous solid.

R_f = 0.45 in 1:1 EtOAc/hexanes, UV-active.
$[\alpha]^{22}_D$ 43.4° (*c* 0.5, $CHCl_3$).
HRMS (ESI) calcd. for $C_{15}H_{17}NO_5$, M + Na^+ = 314.0999, found 314.0992 (2.22 ppm).
^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.19 (s, 1H), 7.41-7.30 (m, 5H), 5.94 (dd, *J* = 5.05, 2.14 Hz, 1H), 5.11 (s, 2H), 5.04 (d, *J* = 1.54 Hz, 1H), 5.00 (d, *J* = 9.38 Hz, 1H), 4.06 (ddd, *J* = 11.37, 10.10, 2.17 Hz, 1H), 3.47 (s, 3H), 2.57 (td, *J* = 18.46, 5.94, 5.94 Hz, 1H), 2.29 (ddd, *J* = 18.57, 11.40, 2.27 Hz, 1H).
^{13}C NMR ($CDCl_3$, 100 MHz) δ 186.1, 155.7, 148.8, 136.2, 128.8 - 128.5 (5C), 121.8, 97.8, 67.3, 56.6, 46.5, 24.9.

Methyl 2-benzyloxycarbonylamino-4,5-dehydro-2,3,4-trideoxy-*α*-*D*-glucopyranoside (4.S5).

Aldehyde **4.13** (71 mg, 0.244 mmol) was dissolved in THF (3 mL), treated with $NaBH_4$ (12 mg, 0.25 mmol) and 10 drops of MeOH, stirred 30 min, filtered through a silica pad washing with EtOAc, and the filtrate was evaporated to a residue under vacuum. Purification by column chromatography (50% EtOAc/hexanes) yielded 63 mg (89%) of title compound, as a white amorphous solid.

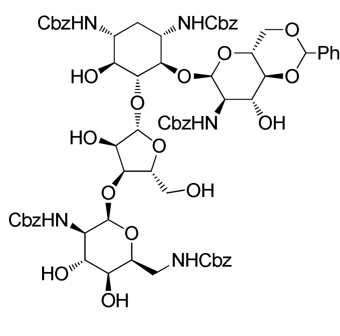
R_f = 0.25 in 1:1 EtOAc/hexanes.

$[\alpha]^{22}_D$ 85.0° (*c* 0.86, $CHCl_3$).

HRMS (ESI) calcd. for $C_{15}H_{19}NO_5$, M + H^+ = 294.1336, found 294.1329 (-2.47 ppm).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.47-7.31 (m, 5H), 5.15 (d, *J* = 9.22 Hz, 2H), 4.99 (d, *J* = 9.22 Hz, 1H), 4.92 (d, *J* = 2.23 Hz, 1H), 4.88 (d, *J* = 5.70 Hz, 1H), 4.09-3.97 (m, 3H), 3.49 (s, 3H), 2.27 (td, *J* = 16.16, 5.77, 5.77 Hz, 1H), 2.14-2.00 (m, 1H), 1.81 (s, 1H).

^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.9, 148.8, 136.4, 128.8 - 128.4 (5C), 97.9, 97.0, 67.2, 63.0, 56.2, 47.0, 23.6.



4′,6′-Benzylidene-*per*-*N*-Cbz-paromomycin (4.14).

Paromomycin sulfate (10 g) was dissolved in 20 mL of water and added through a dropper over 30 min to a rapidly stirring suspension of benzyl chloroformate (16 mL, 112 mmol) and Na_2CO_3 (24 g, 226 mmol) in 250 mL MeOH. After stirring overnight, the solution was diluted with 500 mL of DCM and the salts were filtered. The filtrate was evaporated to dryness, absorbed onto silica gel and purified by on a short column (4 → 6% MeOH, DCM) to yield 7.1 g of *per*-*N*-Cbz paromomycin (**4.S6**, 5.52 mmol).[2]

R_f = 0.3 in 20:5:3 $CHCl_3$/EtOAc/MeOH.

HRMS (ESI) calcd. for $C_{63}H_{75}N_5O_{24}$, M + H^+ = 1286.4875, found 1286.4853 (-1.66 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.45-7.22 (m, 25H), 5.30-5.23 (m, 1H), 5.23-5.14 (m, 2H), 5.14-4.98 (m, 9H), 4.83 (s, 1H), 4.64 (s, 1H), 4.08-3.89 (m, 4H), 3.88-3.82 (m, 2H), 3.82-3.74 (m, 2H), 3.74-3.69 (m, 1H), 3.69-3.58 (m, 3H), 3.56-3.51 (m, 2H), 3.50-3.35 (m, 5H), 3.35-3.31 (m, 2H), 2.02-1.93 (m, 1H), 1.42 (dd, *J* = 23.74, 12.07 Hz, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 157.4 - 156.8 (5C), 136.5 - 136.2 (5C), 127.9 - 126.9 (25C), 108.8, 98.7, 98.4, 85.5, 81.9, 79.4, 77.7, 76.3, 74.1, 73.7, 73.1, 72.7, 70.9, 69.8, 67.3, 66.2 - 65.7 (5C), 61.3, 61.0, 56.0, 52.3, 50.8, 50.5, 40.8, 33.6.

Intermediate **4.S6** (7.1 g, 5.52 mmol) was dried by evaporation three times from toluene, dissolved in freshly distilled benzaldehyde (70 mL), cooled to 0 °C, treated with formic acid (10 mL) and stirred with cooling for 24 h, when the *mono*-benzyldene product dominated, as judged by TLC (20:5:3, $CHCl_3$, EtOAc, MeOH, $R_f = 0.7$) and LRMS ($M+H^+ = 1374.5$). The reaction mixture was poured over stirring sat. $NaHCO_3$, which was extracted with EtOAc and dried over Na_2SO_4. The volatiles were evaporated under vacuum and the resulting solution of benzaldehyde was added dropwise to stirring hexanes. The resulting white gummy precipitate was collected and further purified by column chromatography (2 → 4% MeOH, DCM) to yield 5.3 g of the title compound **4.14** (70%, 38.6 mmol), as a single benzylidene anomer of undetermined configuration.

$R_f = 0.5$ in 20:5:3 $CHCl_3$/EtOAc/MeOH.
HRMS (ESI) calcd. for $C_{70}H_{79}N_5O_{24}$, $M + H^+ = 1374.5188$, found 1374.5187 (-0.04 ppm).
^1H NMR (CD_3OD, 400 MHz) δ 7.54-7.05 (m, 30H), 5.48 (s, 1H), 5.27 (s, 1H), 5.16-5.08 (m, 2H), 5.08-4.90 (m, 8H), 4.79 (s, 1H), 4.58 (s, 1H), 4.22 (dd, J = 8.67, 4.05 Hz, 1H), 4.04-3.78 (m, 7H), 3.79-3.63 (m, 2H), 3.63-3.50 (m, 3H), 3.50-3.37 (m, 5H), 3.37-3.28 (m, 3H), 3.28-3.24 (m, 1H), 1.91 (d, J = 10.96 Hz, 1H), 1.36 (dd, J = 23.30, 11.72 Hz, 1H).
^{13}C NMR (CD_3OD, 100 MHz) δ 157.4 - 156.6 (5C), 137.5 - 136.2 (5C), 128.1 - 126.9 (27C), 125.8 (4C), 108.9, 101.1, 98.9, 98.7, 85.8, 82.0, 81.3, 79.6, 76.6, 74.1, 73.8, 72.7, 69.8, 68.2, 68.1, 67.4, 66.3 - 65.8 (5C), 63.2, 61.7, 56.5, 52.3, 50.9, 50.2, 40.8, 33.6.

4.15

6,3′,2′′,5′′,3′′′, 4′′′-Hexa-*O*-methylcarbonate-*per*-*N*-Cbz-paromomycin (4.15).

A solution of 4′,6′-benzyldene intermediate **4.14** (2.43 g, 1.76 mmol) in dry THF (30 mL) was treated with pyridine (1.6 mL, 19.5 mmol), DMAP (54 mg, 0.44 mmol) and *p*-nitrophenyl methyl carbonate (3.49 g, 17.7 mmol), heated to reflux for 48 h, and quenched with MeOH (approx. 5 mL) for 15 minutes. The crude mixture was evaporated under vacuum to a reduced volume, which was diluted with EtOAc and washed successively with 2 N HCl, three times with 1 N NaOH and finally sat. NaCl. The organic fraction was evaporated under vacuum to a residue, which was purified by column chromatography (10 → 30% EtOAc/DCM) to yield 2.74 g of 6,3′,2′′,5′′,3′′′,4′′′-hexa-*O*-methylcarbonate-4′,6′-benzylidene-*per*-*N*-Cbz-paromomycin **4.S7** (91%, 1.60), as a white amorphous solid.

R_f = 0.4 in 40% EtOAc/$CHCl_3$.

HRMS (ESI) calcd. for $C_{82}H_{91}N_5O_{36}$, M + H^+ = 1722.5517, found 1722.5460 (-3.27 ppm)

^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.45-7.13 (m, 30H), 6.27-6.03 (m, 1H), 5.81 (s, 1H), 5.49-5.33 (m, 2H), 5.22-5.08 (m, 4H), 5.09-4.98 (m, 7H), 4.98-4.85 (m, 4H), 4.67 (s, 1H), 4.63-4.46 (m, 2H), 4.28 (dd, *J* = 11.15, 8.49 Hz, 3H), 4.11 (dd, *J* = 11.15, 8.49 Hz, 2H), 3.95-3.79 (m, 2H), 3.81 (s, 3H), 3.78-3.60 (m, 4H), 3.73 (s, 3H), 3.67 (s, 3H), 3.63 (s, 3H), 3.59 (s, 3H), 3.55 (s, 3H), 3.52-3.44 (m, 2H), 3.22-2.99 (m, 1H), 2.46-2.17 (m, 1H), 1.65-1.40 (m, 1H).

^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.8 - 154.0 (11C), 137.2 - 136.2 (6C), 129.2 - 126.5 (30C), 106.7, 101.6, 101.1, 98.4, 81.9, 79.7, 79.4, 78.8, 76.3, 74.6, 74.0, 72.8, 72.4, 70.4, 68.7, 67.1 - 66.9 (6C), 65.1, 63.7, 55.7 - 55.1 (6C), 54.5, 51.1, 50.1, 49.4, 41.6, 33.7.

The above intermediate **4.S7** (2.74 g, 1.60) was dissolved in a minimum volume of DCM, which was then treated with 80% AcOH (50 mL) and heated to 60 °C, forming a suspension. After stirring overnight, the acetic acid was evaporated under high-vacuum at 60 °C, the residue was partitioned between sat. $NaHCO_3$ and EtOAc, and dried over Na_2SO_4. The organic layer was filtered and evaporated to a residue that was purified by column chromatography (1 → 2% MeOH/DCM) to yield 2.38 g (92%) of the title compound, as a white amorphous solid. When intermediate **4.S7** was used without purification by chromatography the yield of **4.15** for the two-steps was 91%.

R_f = 0.7 in 20:5:3 $CHCl_3$/EtOAc/MeOH.

HRMS (ESI) calcd. for $C_{75}H_{87}N_5O_{36}$, M + H^+ = 1634.5204, found 1634.5126 (-4.77 ppm).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.56-7.17 (m, 25H), 5.93-5.73 (m, 2H), 5.26 (d, J = 9.12 Hz, 1H), 5.23-4.99 (m, 11H), 4.99-4.90 (m, 2H), 4.79 (dd, J = 10.20, 9.65 Hz, 1H), 4.71 (s, 1H), 4.68-4.53 (m, 2H), 4.41 (s, 1H), 4.27 (d, J = 10.56 Hz, 1H), 4.17-4.07 (m, 1H), 4.02-3.87 (m, 4H), 3.85 (s, 3H), 3.83-3.73 (m, 2H), 3.77 (s, 3H), 3.72 (s, 3H), 3.68 (s, 3H), 3.64 (s, 3H), 3.60 (s, 3H), 3.57-3.48 (m, 2H), 3.48-3.35 (m, 2H), 3.32-3.22 (m, 1H), 3.23-3.11 (m, 1H), 2.33-2.13 (m, 1H), 1.84-1.44 (m, 1H).

^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.5 - 153.7 (11C), 136.3 - 136.0 (5C), 128.4 - 127.8 (25C), 106.4, 99.7, 98.1, 81.6, 81.0, 79.0, 78.5, 78.1, 77.1, 76.2, 74.4, 73.0, 72.5, 72.1, 70.0, 69.8, 66.7 - 66.6 (5C), 65.1, 62.1, 55.4, 55.4, 55.1, 55.0, 54.8, 53.6, 51.1, 49.8, 49.1, 41.2, 33.1.

4.16

4′-*O*-Mesyl-6,3′,2″,5″,3‴,4‴-hexa-*O*-methylcarbonate-*per*-*N*-Cbz-paromomycin (4.16).

Compound **4.15** (2.38 g, 1.45 mmol), was dried by evaporation three times from toluene, dissolved in pyridine (10 mL), cooled to 0 °C and treated dropwise with TBSOTf (435 μL, 1.9 mmol). After 2 h, LRMS indicated only 6'-OTBS intermediate (**4.S8**) was present: HRMS (ESI) calcd. for $C_{81}H_{101}N_5O_{36}Si$, M + H^+ = 1748.6068, found 1748.5987 (-4.65 ppm). Mesyl chloride (340 μL, 4.37 mmol) was next added to the mixture, which was stirred allowing to warm to RT. After 3 h, LRMS indicated that only 4'-OMs,6'-OTBS intermediate (**4.S9**) was present: HRMS (ESI) calcd. for $C_{82}H_{103}N_5O_{38}SSi$, M + H^+ = 1826.5844 found 1826.5764 (-4.35 ppm). The remaining mesyl chloride was quenched with excess MeOH (300 μL), when some pyridinium chloride precipitated. After 30 min, the crude mixture was cooled to 0 °C, treated with 3 mL of 70% HF in pyridine and stirred with cooling overnight, when LRMS and TLC (R_f = 0.8, 20:5:3 $CHCl_3$/EtOAc/MeOH) indicated the presence of a single compound. The reaction was poured over a stirring sat. $NaHCO_3$ solution, partitioned with EtOAc, and the organic layer was reduced under high-vacuum at 60 °C to a residue, that was diluted with EtOAc and washed successively with 2 N HCl and sat. $NaHCO_3$, dried over Na_2SO_4 and filtered. The organic fraction was evaporated to a residue, which was purified by column chromatography (1 → 2% MeOH/DCM), to yield 2.41 g of the title compound (97%, 1.41 mmol), as a white amorphous solid.

R_f = 0.8, 20:5:3 $CHCl_3$/ EtOAc/MeOH

HRMS (ESI) calcd. for $C_{76}H_{89}N_5O_{38}S$, M + H^+ = 1712.4979 found 1712.4921 (-3.37 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.44-7.18 (m, 25H), 5.73 (d, *J* = 2.27 Hz, 1H), 5.24 (d, *J* = 3.18 Hz, 1H), 5.21 (s, 1H), 5.14-5.06 (m, 5H), 5.06-4.98 (m, 4H), 4.97 (d, *J* = 2.25 Hz,

1H), 4.93 (dd, J = 3.02, 2.18 Hz, 1H), 4.80 (d, J = 4.05 Hz, 1H), 4.75 (s, 1H), 4.72-4.64 (m, 2H), 4.63-4.56 (m, 1H), 4.34-4.21 (m, 2H), 4.15 (dd, J = 11.88, 2.83 Hz, 1H), 4.08-3.94 (m, 5H), 3.88-3.82 (m, 2H), 3.81 (s, 3H), 3.78-3.72 (m, 1H), 3.75 (s, 3H), 3.73 (s, 3H), 3.71 (s, 3H), 3.69 (s, 3H), 3.68-3.60 (m, 6H), 3.37 (dd, J = 13.88, 6.50 Hz, 1H), 3.26 (dd, J = 14.02, 6.59 Hz, 1H), 2.92 (s, 3H), 1.95 (ddd, J = 7.76, 4.14, 3.12 Hz, 1H), 1.69 (dd, J = 24.94, 12.50 Hz, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 158.9 - 155.5 (11C), 138.4 - 138.2 (5C), 129.7 - 129.0 (25C), 109.6, 99.2, 97.9, 84.7, 81.1, 79.9, 79.6, 78.8, 77.0, 76.7, 76.1, 73.7, 73.5, 71.5, 71.3, 68.3 - 67.6 (5C), 66.6, 61.6, 56.4 - 55.8 (6C), 55.0, 51.3, 51.1, 50.8, 41.7, 38.8, 34.4.

CbzHN NHCbz H O O MeO2CO O CbzHN OCO2Me O MeO2CO O O OCO2Me CbzHN O MeO2CO NHCbz OCO2Me

4.17

6'-Aldehydo-4',5'-dehydro-4'-deoxy-6,3',2'',5'',3''',4'''-hexa-*O*-methylcarbonate-*per*-*N*-Cbz-paromomycin (4.17).

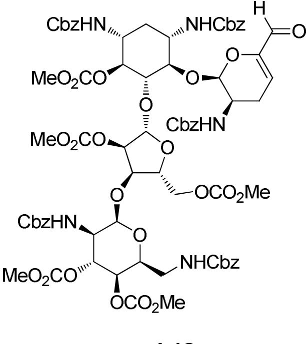
Alcohol **4.16** (2.41 g, 1.41 mmol), was dried by evaporation three times from toluene, dissolved in dry DCM (40 mL) and Et_3N (10 mL), and cooled to 0 °C. In a separate flask, a DMSO (6 mL) solution containing SO_3·pyridine complex (2.0 g, 12.5 mmol) was prepared. The DMSO solution was added dropwise to the alcohol mixture, which was allowed to warm to RT overnight. After 12 h, only a UV-active spot was observed on TLC (R_f = 0.45, 65% EtOAc/Hex). The reaction was quenched with water, diluted with EtOAc and washed successively with 2 N HCl and sat. $NaHCO_3$, dried over Na_2SO_4 and filtered. The organic layer was evaporated to a residue that was purified by column chromatography (10 → 30% EtOAc/DCM) to yield 2.08 g of the title compound (92%, 1.29 mmol), as an off-white amorphous solid.

R_f = 0.4-0.5 streak, 65% EtOAc/Hex, UV-active.

HRMS (ESI) calcd. for $C_{75}H_{83}N_5O_{35}$, M + Na^+ = 1636.4761, found 1636.4703 (-3.56 ppm).

^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.02 (s, 1H), 7.49-7.15 (m, 25H), 5.90 (d, J = 1.35 Hz, 1H), 5.73 (d, J = 1.34 Hz, 1H), 5.25-5.20 (m, 1H), 5.11-4.98 (m, 7H), 4.98-4.85 (m, 5H), 4.77 (s, 1H), 4.75 (d, J = 4.24 Hz, 1H), 4.62 (s, 1H), 4.45 (dd, J = 10.34, 9.83 Hz, 1H), 4.39 (dd, J = 6.87, 4.90 Hz, 1H), 4.26 (d, J = 10.45 Hz, 1H), 4.21-4.06 (m, 2H), 4.02 (dd, J = 7.00, 6.34 Hz, 1H), 3.95 (dd, J = 9.57, 7.99 Hz, 1H), 3.81-3.76 (m, 1H), 3.78 (s, 3H), 3.72 (s, 3H), 3.70-3.65 (m, 1H), 3.69 (s, 3H), 3.66 (s, 3H), 3.64 (s, 3H), 3.62 (s, 3H), 3.60-3.52 (m, 3H), 3.30-3.19 (m, 1H), 3.15-3.06 (m, 1H), 1.81-1.57 (m, 2H).

^{13}C NMR (DMSO-d_6, 100 MHz) δ 186.6, 156.2 - 153.5 (11C), 148.4, 137.3 - 136.9 (5C), 128.4 - 127.6 (25C), 116.9, 107.4, 96.9, 82.4, 79.7, 78.1, 78.1, 77.3, 73.9, 71.6, 71.2, 70.9, 69.3, 65.8 - 65.3 (5C), 65.0, 55.5 - 55.1 (6C), 54.6, 50.5, 49.4, 49.3, 49.2, 49.1, 32.8.



6'-Aldehydo-4',5'-dehydro-3',4'-dideoxy-6,3',2'',5'',3''',4'''-hexa-*O*-methylcarbonate-*per*-*N*-Cbz-paromomycin (4.18).

Under optimized conditions (Table 4.2, entry 4), 1.0 g of aldehyde **4.17** (0.62 mmol) was dissolved in dry THF (6 mL), treated with Et_3N (950 µL, 6.81 mmol), formic acid (240 µL, 6.19 mmol) and degassed under a flow of argon three times by successive cooling to -78 ºC and sonication at RT, treated with tris(dibenzylideneacetone) dipalladium (28.4 mg, 0.031 mmol, 10% mol/mol Pd) and tributylphosphine (31 µL, 0.124 mmol), and stirred at RT, when the reaction mixture changed from a wine-red to yellow-brown color. After heating at 60 °C for 3 h, LRMS and TLC (R_f = 0.35, 65% EtOAc/Hex, UV-active) indicated complete conversion. The reaction solution was filtered through a short silica pad washing with EtOAc. The filtrate was washed with sat. NaCl, dried over $MgSO_4$, filtered

and the volatiles were evaporated to give a residue which was purified by column chromatography (20 → 40% EtOAc/DCM), to yield 928 mg of the title compound (97%, 0.602 mmol), as an off-white amorphous solid.

R_f = 0.3-0.4 streak, 65% EtOAc/Hex, UV-active.
HRMS (ESI) calcd. for $C_{73}H_{81}N_5O_{32}$, M + H^+ = 1540.4937, found 1540.4930 (-0.46 ppm).
^{1}H NMR (DMSO-d_6, 700 MHz) δ 8.94 (s, 1H), 7.38-7.26 (m, 25H), 5.85 (dd, J = 4.06, 3.58 Hz, 1H), 5.62-5.58 (m, 1H), 5.23 (s, 1H), 5.10-4.97 (m, 7H), 4.97-4.92 (m, 2H), 4.92-4.87 (m, 2H), 4.77-4.71 (m, 2H), 4.61 (s, 1H), 4.46 (dd, J = 10.30, 9.55 Hz, 1H), 4.36 (dd, J = 6.55, 5.47 Hz, 1H), 4.28 (d, J = 10.69 Hz, 1H), 4.15 (dd, J = 11.29, 4.04 Hz, 1H), 4.11 (dd, J = 9.39, 8.56 Hz, 1H), 4.00 (dd, J = 7.02, 5.99 Hz, 1H), 3.86-3.81 (m, 1H), 3.78 (s, 3H), 3.75-3.71 (m, 1H), 3.68 (s, 3H), 3.67 (s, 3H), 3.64 (s, 3H), 3.61 (s, 3H), 3.62-3.48 (m, 4H), 3.28-3.20 (m, 1H), 3.16-3.09 (m, 1H), 2.32-2.22 (m, 2H), 1.69 (dd, J = 25.23, 12.62 Hz, 1H), 1.66-1.60 (m, 1H).
^{13}C NMR (DMSO-d_6, 175 MHz) δ 186.2, 156.2 - 153.5 (10C), 148.2, 137.3 - 136.9 (5C), 128.4 - 127.6 (25C), 121.5, 107.5, 96.9, 94.9, 82.5, 79.7, 79.2, 78.0, 77.7, 77.3, 73.9, 71.6, 71.1, 69.3, 65.7 - 65.4 (5C), 65.3, 65.2, 55.5 - 54.7 (5C), 49.3, 49.0, 46.7, 33.1, 23.5.

CbzHN NHCbz OH MeO2CO O O CbzHN MeO2CO O OCO2Me CbzHN O MeO2CO NHCbz OCO2Me

4.19

4',5'-Dehydro-3',4'-dideoxy-6,3',2'',5'',3''',4'''-hexa-*O*-methylcarbonate-*per*-*N*-Cbz-paromomycin (4.19).

Aldehyde **4.18** (928 mg, 0.602 mmol) was dissolved in THF (10 mL), treated with $NaCNBH_3$ (1.0 M in THF, 1.2 mL, 1.2 mmol) and 20 drops of AcOH, stirred overnight, and the reaction was filtered through a silica pad washing with EtOAc. The filtrate was washed with sat. $NaHCO_3$, dried over Na_2SO_4 and filtered. The organic layer was

evaporated to a residue, which was purified by column chromatography (1 → 2% MeOH/DCM), to yield 867 mg of the title compound (93%, 0.561), as a white amorphous solid.

R_f = 0.3-0.4 streak, 40% EtOAc/$CHCl_3$.
HRMS (ESI) calcd. for $C_{73}H_{83}N_5O_{32}$, M + Na^+ = 1564.4913, found 1564.4937 (1.53 ppm).
^{1}H NMR (CD_3OD, 400 MHz) δ 7.43-7.14 (m, 25H), 5.55-5.46 (m, 1H), 5.27-5.20 (m, 1H), 5.13-4.92 (m, 11H), 4.90 (dd, *J* = 2.94, 2.42 Hz, 1H), 4.83-4.79 (m, 1H), 4.73 (s, 1H), 4.68 (s, 1H), 4.57 (dd, *J* = 9.72, 9.22 Hz, 1H), 4.53-4.46 (m, 1H), 4.34-4.20 (m, 2H), 4.09 (dd, *J* = 11.89, 4.04 Hz, 1H), 4.06-4.00 (m, 1H), 3.99-3.94 (m, 1H), 3.86-3.80 (m, 2H), 3.78 (s, 3H), 3.77-3.74 (m, 2H), 3.74-3.64 (m, 4H),, 3.72 (s, 3H), 3.69 (s, 3H), 3.66 (s, 3H), 3.64-3.56 (m, 2H), 3.35 (dd, *J* = 13.93, 6.22 Hz, 1H), 3.24 (dd, *J* = 14.19, 6.95 Hz, 1H), 2.08-1.86 (m, 3H), 1.52 (dd, *J* = 25.05, 12.57 Hz, 1H).
^{13}C NMR (CD_3OD, 100 MHz) δ 158.9 - 155.5 (10C), 149.7, 138.5 - 138.4 (5C), 129.7 - 128.9 (25C), 109.4, 99.2, 98.9, 97.0, 84.3, 81.0, 79.9, 78.5, 78.2, 76.5, 73.8, 73.5, 71.4, 67.9 - 67.6 (5C), 67.1, 63.1, 56.4 - 55.8 (5C), 51.2, 51.1, 50.8, 48.6, 41.8, 35.2, 24.0.

General procedure for methylcarbonate deprotection

Sodium methoxide in MeOH was prepared by addition of a piece of sodium (~20 mg) to anhydrous MeOH (20 mL). The resulting alkaline solution was diluted with anhydrous MeOH to approx. pH 8 to 9 (Accutint pH paper roll). The methylcarbonate-protected aminoglycoside (50 to 500 μmol) was dissolved with the dilute NaOMe solution (5 to 15 mL) and stirred 3 to 5 h, monitoring by TLC and LRMS. The solution was neutralized with a few drops of AcOH, and then evaporated under vacuum to a minimum volume, which was diluted with DCM passed through a short silica pad washing with 10% MeOH/DCM. The volatiles were evaporated to a residue, which was purified by column chromatography (3 to 6% MeOH/DCM).

General procedure for Birch reduction

Approx. 5 to 7 mL of ammonia was condensed into a two-neck flask equipped with a cold finger condenser at -78 °C. A solution of protected aminoglycoside (25 to 50 μmol) in anhydrous THF (1 mL) was added to the ammonia solution, followed by a drop of *t*BuOH. Approx. 20 to 30 mg (~1 mmol) of sodium metal were added to the mixture, which was stirred vigorously at -78 °C until the reaction turned deep blue. After 5 min, LRMS analysis indicated complete removal of the protecting groups, and the reaction was quenched with excess AcOH (100 μL). The ammonia was slowly evaporated by bubbling argon at RT, to give a white residue of salts, which was dissolved in $CHCl_3$/MeOH/NH_4OH (2:3:0.5) and purified by column chromatography using the same solvent system, increasing the proportion of ammonia as required. The fractions containing aminoglycoside were identified by TLC, collected and evaporated under vacuum to furnish a wet residue, which was dissolved in a minimum volume of water and freeze-dried. The dry residue obtained was redissolved in a minimum of water, at which point insoluble traces of silica were generally observed, and were removed by filtration of the solution through a 0.45 μm syringe filter. Finally, freeze-drying the filtrate yielded the aminoglycoside analog as the free-base fluffy powder. For characterization purposes, the aminoglycoside was redissolved in a minimum volume of water, treated with AcOH (50 μL) and freeze-dried to provide the aminoglycoside acetate salts, which were obtained as yellow solids.

4',5'-Dehydro-3',4'-dideoxy-paromomycin (4.21).

Alcohol **4.19** (100 mg, 65 μmol) was submitted to the general procedure for methylcarbonate deprotection. The column chromatography solvents were 3 → 5% MeOH/DCM. The procedure yielded 65 mg (80%, 52 μmol) of 4',5'-dehydro-3'-deoxy-*per*-*N*-Cbz-paromomycin (**4.S10**), as a white amorphous solid.

R_f = 0.5, 20:5:3 $CHCl_3$/EtOAc/MeOH

HRMS (ESI) calcd. for $C_{63}H_{73}N_5O_{22}$, M + Na^+ = 1274.4639, found 1274.4615 (-1.91 ppm).

^{1}H NMR (CD_3OD, 700 MHz) δ 7.42-7.01 (m, 25H), 5.56 (s, 1H), 5.13-4.89 (m, 11H), 4.64-4.52 (m, 1H), 4.47 (s, 1H), 4.24-4.04 (m, 2H), 3.97-3.90 (m, 1H), 3.90-3.82 (m, 2H), 3.82-3.68 (m, 3H), 3.66-3.57 (m, 2H), 3.56-3.48 (m, 2H), 3.44 (s, 1H), 3.42-3.35 (m, 2H), 3.35-3.25 (m, 3H), 3.25-3.22 (m, 1H), 2.00 (dd, J = 14.69, 12.15 Hz, 1H), 1.96-1.86 (m, 2H), 1.27 (dd, J = 23.98, 11.50 Hz, 1H).

^{13}C NMR (CD_3OD, 175 MHz) δ 157.8 - 157.1 (5C), 148.1, 136.9 - 136.7 (5C), 128.2 - 127.1 (25C), 109.1, 98.8, 97.4, 95.0, 85.7, 82.2, 77.5, 75.6, 74.4, 74.1, 73.2, 70.2, 67.8, 66.5 - 66.1 (6C), 61.9, 61.6, 52.7, 51.5, 49.9, 41.2, 34.3, 22.1.

Alcohol **4.S10** (65 mg, 52 µmol) was submitted to the general Birch reduction procedure. The column chromatography solvents were $CHCl_3$/MeOH/NH_4OH, 2:3:0.5 followed by 2:3:1. The procedure yielded 33.9 mg (80%, 38 µmol) of the acetate salt of the title compound **4.21**, as a yellow amorphous solid.

R_f = 0.25, 2:3:2 $CHCl_3$/MeOH/NH_4OH. $[\alpha]^{22}_D$ 33.8º (c 1.10, H_2O).

HRMS (ESI) calcd. for $C_{23}H_{43}N_5O_{12}$, M + H^+ = 582.2981, found 582.2982 (0.19 ppm).

^{1}H NMR (D_2O, 700 MHz) δ 5.53 (d, J = 1.14 Hz, 1H), 5.21 (d, J = 1.14 Hz, 1H), 5.17 (d, J = 1.84 Hz, 1H), 4.98 (t, J = 3.48, 3.48 Hz, 1H), 4.43 (dd, J = 5.92, 5.34 Hz, 1H), 4.30 (dd, J = 4.66, 2.06 Hz, 1H), 4.23 (dd, J = 6.28, 3.59 Hz, 1H), 4.15 (t, J = 2.97, 2.97 Hz, 1H), 4.10 (dt, J = 5.88, 5.82, 3.60 Hz, 1H), 4.00 (t, J = 9.76, 9.76 Hz, 1H), 3.98-3.93 (m, 2H), 3.85 (dt, J = 5.07, 5.02, 1.51 Hz, 1H), 3.79 (dd, J = 12.17, 3.19 Hz, 1H), 3.74 (dd, J = 2.64, 1.21 Hz, 1H), 3.71 (t, J = 9.18, 9.18 Hz, 1H), 3.64 (dd, J = 12.11, 5.56 Hz, 1H), 3.59 (t, J = 9.84, 9.84 Hz, 1H), 3.52-3.49 (m, 1H), 3.42 (ddd, J = 12.70, 10.71, 4.12 Hz, 1H), 3.35 (dd, J = 13.62, 6.73 Hz, 1H), 3.30-3.27 (m, 1H), 3.28-3.23 (m, 1H), 2.55 (ddd, J = 18.11, 5.35, 3.24 Hz, 1H), 2.41 (td, J = 12.20, 3.97, 3.97 Hz, 1H), 2.25 (td, J = 8.28, 3.89, 3.89 Hz, 1H), 1.84 (s, 16H AcOD), 1.77 (dd, J = 12.62, 12.60 Hz, 1H).

^{13}C NMR (D_2O, 175 MHz) δ 180.9 (AcOD), 149.0, 110.4, 97.1, 96.3, 95.3, 83.7, 81.2, 77.9, 75.8, 73.1, 71.7, 70.1, 67.6, 67.2, 61.1, 60.7, 50.7, 49.6, 48.2, 45.9, 40.3, 27.8, 22.9 (AcOD), 22.8.

LC/CLND gradient A, R_t = 10.8 min, 98% purity.

4.20

6'-Azido-4',5'-dehydro-3',4'-dideoxy-*per*-*N*-Cbz-neomycin (4.20).

Alcohol **4.19** (866 mg, 0.561 mmol) was dissolved in THF (5 mL), cooled to 0 °C, was treated with diphenylphosphoryl azide (160 µL, 0.73 mmol) and DBU (110 µL, 0.73 mmol), stirred for 1 hour at RT and heated to 50 °C. After 5 h, the reaction was quenched with water, the volume was reduced under vacuum, diluted with EtOAc and washed successively with 2 N HCl and sat. $NaHCO_3$, dried over Na_2SO_4, filtered and evaporated to a residue. Purification by column chromatography (10 → 20% EtOAc/DCM), yielded 807 mg (92%, 0.515 mmol) of 6'-azido-4',5'-dehydro-3',4'-dideoxy-6,3',2'',5'',3''',4'''-hexa-O-methylcarbonate-per-*N*-Cbz-neomycin (**4.S11**), as an off-white amorphous solid.

R_f = 0.5, 40% EtOAc/$CHCl_3$.

HRMS (ESI) calcd. for $C_{73}H_{82}N_8O_{31}$, M + H^+ = 1567.5159, found 1567.5113 (-2.93 ppm).

^{1}H NMR (CD_3OD, 700 MHz) δ 7.38-7.11 (m, 25H), 5.51 (s, 1H), 5.21 (s, 1H), 5.08-4.96 (m, 7H), 4.96-4.90 (m, 3H), 4.86 (dd, *J* = 3.16, 2.47 Hz, 1H), 4.80-4.75 (m, 1H), 4.73-4.67 (m, 1H), 4.66-4.62 (m, 1H), 4.58-4.48 (m, 2H), 4.24 (dd, *J* = 7.54, 4.89 Hz, 1H), 4.21 (d, *J* = 10.84 Hz, 1H), 4.07 (dd, *J* = 11.75, 3.45 Hz, 1H), 3.98 (dd, *J* = 7.05, 5.68 Hz, 1H), 3.95-3.90 (m, 1H), 3.82 (dd, *J* = 8.87, 7.56 Hz, 1H), 3.75 (s, 3H), 3.74-3.70 (m, 2H), 3.69 (s, 3H), 3.68-3.67 (m, 1H), 3.66 (s, 3H), 3.62 (s, 3H), 3.62 (s, 3H), 3.60-3.53 (m, 3H), 3.45 (d, *J* = 13.93 Hz, 1H), 3.31 (dd, *J* = 13.87, 6.23 Hz, 1H), 3.20 (dd, *J* = 13.98, 6.83 Hz, 1H), 2.08-2.00 (m, 1H), 1.99-1.92 (m, 1H), 1.91-1.85 (m, 1H), 1.54 (dd, *J* = 23.40, 12.01 Hz, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 157.4 - 153.9 (10C), 144.6, 136.9 - 136.8 (5C), 128.2 - 127.4 (25C), 107.9, 99.4, 97.6, 95.9, 83.0, 79.6, 78.3, 77.8, 77.1, 74.7, 72.2, 72.0, 69.8, 66.3, 66.3, 66.2, 66.2, 66.0, 65.2, 54.9, 54.7 (2C), 54.6, 54.2, 52.4, 49.6, 49.6, 49.3, 46.9, 40.2, 33.2, 22.3.

FTIR (NaCl): 3367, 2102, 1754, 1725, 1519, 1442, 1257 cm^{-1}.

Azide **4.S11** (755 mg, 0.482 mmol) was submitted to the general procedure for methylcarbonate deprotection. The column chromatography solvents were 2 → 3% MeOH/DCM. The procedure yielded 488 mg of title compound **4.20** (79%, 0.382 mmol), as a white amorphous solid.

R_f = 0.65, 20:5:3 $CHCl_3$/EtOAc/MeOH
HRMS (ESI) calcd. for $C_{63}H_{72}N_8O_{21}$, M + H^+ = 1277.4885, found 1277.4864 (-1.59 ppm).
^{1}H NMR (CD_3OD, 400 MHz) δ 7.49-7.19 (m, 25H), 5.59 (s, 1H), 5.29 (s, 1H), 5.18-5.04 (m, 7H), 5.04-4.98 (m, 3H), 4.94 (dd, *J* = 3.11, 2.37 Hz, 1H), 4.89-4.82 (m, 1H), 4.81-4.75 (m, 1H), 4.72 (s, 1H), 4.66-4.56 (m, 2H), 4.32 (dd, *J* = 7.54, 4.89 Hz, 1H), 4.29 (d, *J* = 10.83 Hz, 1H), 4.15 (dd, *J* = 11.75, 3.45 Hz, 1H), 4.07 (dd, *J* = 6.91, 5.57 Hz, 1H), 4.01 (s, 1H), 3.90 (dd, *J* = 8.99, 7.69 Hz, 1H), 3.84 (s, 3H), 3.83-3.78 (m, 3H), 3.77 (s, 3H), 3.76-3.75 (m, 1H), 3.74 (s, 3H), 3.70 (s, 3H), 3.72-3.61 (m, 5H), 3.53 (d, *J* = 13.93 Hz, 1H), 3.39 (dd, *J* = 13.87, 6.23 Hz, 1H), 3.29 (dd, *J* = 13.99, 6.83 Hz, 1H), 2.16-2.08 (m, 1H), 2.08-2.00 (m, 1H), 2.00-1.93 (m, 1H), 1.63 (dd, *J* = 23.46, 12.14 Hz, 1H).
^{13}C NMR (CD_3OD, 100 MHz) δ 157.4 - 156.4 (5C), 144.2, 136.5 - 136.3 (5C), 127.8 - 126.7 (25C), 108.9, 98.9, 98.5, 95.2, 85.4, 81.9, 77.2, 76.6, 74.0, 73.8, 72.8, 69.8, 67.4, 66.1, 65.8 - 65.8 (5C), 61.5, 52.4, 52.0, 51.1, 49.4, 40.8, 33.5, 21.6.
FTIR (NaCl): 3368, 2101, 1699, 1524, 1245, 1040 cm^{-1}.

4',5'-Dehydro-3',4'-dideoxy-neomycin (4.22).

Azide compound **4.20** (29.5 mg, 23 μmol) was submitted to the general Birch reduction procedure. The column chromatography solvents were $CHCl_3$/MeOH/NH_4OH, 2:3:1 followed by 2:3:2. The procedure yielded 14.1 mg of the acetate salt of the title compound **4.22** (65%, 15 μmol), as a yellow amorphous solid.

R_f = 0.2, 2:3:2 $CHCl_3$/MeOH/NH_4OH.

$[\alpha]^{22}_D$ 32.6º (*c* 0.33, H_2O).

HRMS (ESI) calcd. for $C_{23}H_{44}N_6O_{11}$, M + H^+ = 603.2960, found 603.2957 (-0.47 ppm).

^{1}H NMR (D_2O, 400 MHz) δ 5.73 (d, *J* = 1.38 Hz, 1H), 5.33 (d, *J* = 1.54 Hz, 1H), 5.30 (d, *J* = 2.80 Hz, 1H), 5.25 (t, *J* = 3.52, 3.52 Hz, 1H), 4.52 (t, *J* = 5.44, 5.44 Hz, 1H), 4.39 (dd, *J* = 4.98, 2.85 Hz, 1H), 4.35 (ddd, *J* = 6.54, 3.99, 1.35 Hz, 1H), 4.27 (t, *J* = 3.10, 3.10 Hz, 1H), 4.23 (dt, *J* = 5.58, 5.52, 3.44 Hz, 1H), 4.08 (t, *J* = 9.70, 9.70 Hz, 1H), 4.00 (dt, *J* = 5.03, 4.99, 1.32 Hz, 1H), 3.90 (dd, *J* = 12.15, 3.47 Hz, 1H), 3.88-3.86 (m, 1H), 3.83 (t, *J* = 9.14, 9.14 Hz, 1H), 3.75 (dd, *J* = 12.04, 5.43 Hz, 1H), 3.74-3.67 (m, 3H), 3.63 (ddd, *J* = 2.91, 1.49, 0.93 Hz, 1H), 3.52-3.38 (m, 3H), 3.41-3.32 (m, 1H), 2.71 (ddd, *J* = 18.12, 5.26, 3.21 Hz, 1H), 2.49 (td, *J* = 12.50, 4.10, 4.10 Hz, 1H), 2.42 (td, *J* = 18.58, 4.19, 4.19 Hz, 1H), 1.97 (s, 18H AcOD), 1.85 (d, *J* = 1.38 Hz, 1H).

^{13}C NMR (D_2O, 100 MHz) δ 180.4 (AcOD), 143.0, 109.9, 100.1, 96.3, 95.3, 83.5, 81.2, 78.0, 75.9, 73.0, 71.7, 69.8, 67.3, 67.0, 61.0, 50.5, 49.5, 47.9, 45.5, 40.1, 40.1, 28.1, 22.7 (AcOD), 22.5.

LC/CLND gradient A, R_t = 11.6 min, 94% purity.

4.23

1,3,2',2''', 6'''-*N*-Cbz-6'-*N*-methyl-4',5'-dehydro-3',4'-dideoxy-neomycin (4.23).

Aldehyde **4.18** (94 mg, 61 μmol) was submitted to the general procedure for methylcarbonate deprotection. TLC and LRMS was used to indicate complete deprotection of the methylcarbonates to intermediate **4.S12**: HRMS (ESI) calcd. for $C_{63}H_{71}N_5O_{22}$, M + Na^+ = 1272.4483, found 1272.4472 (-1 ppm). The solution was acidified with 100 μL of AcOH, cooled to 0 °C, then treated with methylamine (91 μL, 2 M in THF, 0.18 mmol) and $NaCNBH_3$ (120 μL, 1 M in THF, 0.12 mmol), stirred and allowed to warm to RT overnight. The reaction was diluted with DCM, washed with sat. $NaHCO_3$, dried over Na_2SO_4 and filtered. The organic fraction was evaporated to a residue, which was purified by column chromatography (5 → 8% ammoniacal MeOH in DCM) yielding 44.5 mg (58%, 35 μmol) of the title compound **4.23**, as an off-white amorphous solid.

R_f = 0.3, 15% of ammoniacal MeOH/$CHCl_3$

HRMS (ESI) calcd. for $C_{64}H_{76}N_6O_{21}$, M + H^+ = 1265.5136, found 1265.5137 (0.05 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.44-7.12 (m, 25H), 5.52 (s, 1H), 5.17-4.94 (m, 11H), 4.90 (s, 1H), 4.50 (d, *J* = 2.97 Hz, 1H), 4.25-4.10 (m, 2H), 4.02-3.94 (m, 1H), 3.94-3.87 (m, 2H), 3.84 (s, 1H), 3.82-3.76 (m, 1H), 3.68-3.61 (m, 1H), 3.61-3.51 (m, 3H), 3.50-3.46 (m, 1H), 3.46-3.30 (m, 5H), 3.00 (d, *J* = 13.44 Hz, 1H), 2.81 (d, *J* = 13.56 Hz, 1H), 2.18 (s, 3H), 2.14-2.06 (m, 1H), 2.05-1.91 (m, 2H), 1.37 (dd, *J* = 24.33, 12.21 Hz, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 159.3 - 158.5 (5C), 147.9, 138.3 - 138.2 (5C), 129.7 - 128.7 (25C), 110.3, 100.4, 99.1, 97.2, 87.1, 83.7, 79.0, 78.6, 75.7, 75.7, 74.8, 71.7, 69.3, 68.0, 67.7 - 67.7 (5C), 63.3, 53.8, 52.9, 51.6, 47.0, 42.7, 35.5, 35.2, 23.6.

6'-*N*-Methyl-4',5'-dehydro-3',4'-dideoxy-neomycin (4.24).

Compound **4.23** (44.5 mg, 35 μmol) was submitted to the general Birch reduction procedure. The column chromatography solvents were $CHCl_3$/MeOH/NH_4OH, 2:3:1 followed by 2:3:2. The procedure yielded 25.1 mg of the acetate salt of title compound **4.24** (75%, 26 μmol), as a yellow amorphous solid.

R_f = 0.2, 2:3:2 $CHCl_3$/MeOH/NH_4OH.

$[\alpha]^{22}_D$ 29.0º (*c* 0.61, H_2O).

HRMS (ESI) calcd. for $C_{24}H_{46}N_6O_{11}$, M + H^+ = 595.3297, found 595.3286 (-1.91 ppm).

^{1}H NMR (D_2O, 700 MHz) δ 5.63 (d, *J* = 1.14 Hz, 1H), 5.22 (d, *J* = 1.42 Hz, 1H), 5.21 (t, *J* = 3.76, 3.76 Hz, 1H), 5.19 (d, *J* = 2.50 Hz, 1H), 4.42 (t, *J* = 5.48, 5.48 Hz, 1H), 4.29 (dd, *J* = 4.68, 2.82 Hz, 1H), 4.24 (dd, *J* = 5.92, 3.97 Hz, 1H), 4.16 (t, *J* = 3.04, 3.04 Hz, 1H), 4.11 (dt, *J* = 5.75, 5.63, 3.54 Hz, 1H), 3.96 (t, *J* = 9.73, 9.73 Hz, 1H), 3.88 (dt, *J* = 5.46, 5.19, 1.51 Hz, 1H), 3.80 (dd, *J* = 12.13, 3.14 Hz, 1H), 3.75 (d, *J* = 2.98 Hz, 1H), 3.65-3.60 (m, 1H), 3.65 (dd, *J* = 12.14, 5.56 Hz, 1H), 3.52-3.47 (m, 2H), 3.43-3.41 (m, 1H), 3.29-3.18 (m, 3H), 3.24 (dt, *J* = 11.86, 11.53, 4.19 Hz, 1H), 2.64 (s, 3H), 2.61 (ddd, *J* = 8.78, 5.76, 3.52 Hz, 1H), 2.36 (ddd, *J* = 13.35, 5.02, 3.90 Hz, 1H), 2.32 (ddd, *J* = 18.38, 4.71, 3.13 Hz, 1H), 1.85 (s, 18H AcOD), 1.73 (dd, *J* = 25.04, 12.83 Hz, 1H).

^{13}C NMR (D_2O, 175 MHz) δ 181.2 (AcOD), 141.7 110.3, 102.5, 96.5, 95.5, 83.9, 81.4, 78.4, 76.0, 73.2, 72.0, 70.1, 67.6, 67.2, 61.1, 50.8, 49.8, 49.3, 48.2, 45.6, 40.3, 31.9, 28.5, 23.1 (AcOD), 22.9.

LC/CLND gradient A, R_t = 11.7 min, 94% purity.

4.25

1,3,2',2''', 6'''-*N*-Cbz-6'-*N*-methyl-4',5'-dehydro-4'-deoxy-neomycin (4.25).

Aldehyde **4.17** (85 mg, 53 μmol) was submitted to the general procedure for methylcarbonate deprotection. LRMS was used to indicate complete deprotection of the methylcarbonates to intermediate **4.S13**: HRMS (ESI) calcd. for $C_{63}H_{71}N_5O_{23}$, M + Na^+ = 1288.4432, found 1288.4462 (2.33 ppm). The solution was treated with 100 μL of AcOH, methylamine (80 μL, 2 M in THF, 0.16 mmol) and $NaCNBH_3$ (100 μL, 1 M in THF, 0.10 mmol) as previously described for the preparation of **4.23**. After the procedure and purification by column chromatography (5 → 9% ammoniacal MeOH in DCM), 44.6 mg of title compound **4.25** (66%, 35 μmol) was obtained, as an off-white amorphous solid.

R_f = 0.3, 15% of ammoniacal MeOH/$CHCl_3$

HRMS (ESI) calcd. for $C_{64}H_{76}N_6O_{22}$, M + H^+ = 1281.5085, found 1281.5059 (-2.06 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.42-7.10 (m, 25H), 5.39 (s, 1H), 5.16-4.91 (m, 11H), 4.73 (s, 1H), 4.21 (d, *J* = 6.36 Hz, 1H), 4.15-4.00 (m, 3H), 3.96-3.84 (m, 3H), 3.83-3.76 (m, 2H), 3.71 (d, *J* = 11.54 Hz, 1H), 3.63-3.47 (m, 3H), 3.47-3.36 (m, 3H), 3.36-3.28 (m, 3H), 3.07-2.94 (m, 1H), 2.83 (d, *J* = 13.61 Hz, 1H), 2.21 (s, 3H), 2.03-1.89 (m, 1H), 1.35 (dd, *J* = 23.03, 11.42 Hz, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 157.4 - 156.7 (5C), 147.0, 136.5 - 136.2 (5C), 127.8 - 126.9 (5C), 108.9, 102.6, 98.5, 97.9, 84.8, 81.8, 79.4, 76.7, 74.0, 73.7, 72.8, 69.8, 67.4, 66.2, 65.9 - 65.7 (5C), 63.2, 61.6, 54.0, 52.3, 51.2, 51.0, 49.9, 40.8, 32.9.

6'-*N*-Methyl-4',5'-dehydro-4'-deoxy-neomycin (4.26).

Compound **4.25** (39.1 mg, 30 μmol) was submitted to the general Birch reduction procedure. The column chromatography solvents were $CHCl_3$/MeOH/NH_4OH, 2:3:1 followed by 2:3:2. The procedure yielded 24.4 mg of the acetate salt of the title compound **4.26** (83%, 25 μmol), as yellow amorphous solid.

R_f = 0.2, 2:3:2 $CHCl_3$/MeOH/NH_4OH.

$[\alpha]^{22}_D$ 55.4° (*c* 0.66, H_2O).

HRMS (ESI) calcd. for $C_{24}H_{46}N_6O_{12}$, M + H^+ = 611.3246, found 611.3244 (-0.46 ppm).

^{1}H NMR (D_2O, 700 MHz) δ 5.63 (d, *J* = 1.30 Hz, 1H), 5.42 (d, *J* = 3.99 Hz, 1H), 5.27-5.23 (m, 2H), 4.46 (t, *J* = 5.62, 5.62 Hz, 1H), 4.37 (dd, *J* = 3.85, 2.96 Hz, 1H), 4.28 (dd, *J* = 5.08, 2.93 Hz, 1H), 4.27 (dd, *J* = 6.48, 3.91 Hz, 1H), 4.18 (t, *J* = 3.04, 3.04 Hz, 1H), 4.18-4.14 (m, 1H), 4.11 (td, *J* = 5.30, 4.16, 4.16 Hz, 1H), 3.86-3.79 (m, 3H), 3.79-3.76 (m, 2H), 3.73 (dd, *J* = 12.39, 4.99 Hz, 1H), 3.71-3.66 (m, 2H), 3.54 (s, 1H), 3.50 (dd, *J* = 13.20, 8.35 Hz, 1H), 3.38 (dd, *J* = 13.59, 6.81 Hz, 1H), 3.35-3.29 (m, 2H), 2.70 (s, 3H), 2.46 (d, *J* = 11.84 Hz, 1H), 1.90 (s, 7H AcOD), 1.85 (dd, *J* = 25.62, 13.60 Hz, 1H).

^{13}C NMR (D_2O, 175 MHz) δ 180.3 (AcOD), 144.5, 109.4, 104.6, 96.2, 95.5, 82.6, 81.4, 78.5, 75.8, 73.2, 71.8, 70.1, 67.6, 67.2, 61.9, 60.7, 51.4, 50.7, 49.7, 49.1, 48.1, 40.4, 32.2, 27.9, 22.5 (AcOD).

LC/CLND gradient A, R_t = 11.7 min, 98% purity.

General procedure for periodate cleavage

The aminoglycoside diol (75 to 85 μmol) was dissolved in MeOH (5 mL), then treated with 10 equiv. $NaIO_4$ and stirred vigorously overnight, when LRMS indicated complete consumption of starting material. The major ion observed corresponded to the sodium adduct of the MeOH *bis*-hemiacetal of the 3''',4'''-*bis*-aldehyde intermediates. The suspension was filtered through a 0.45 μm syringe filter, and the filtrate was reduced under vacuum, diluted with DCM and washed with sat. NaCl. The combined organic layers were evaporated to a residue, which was dissolved in MeOH (3 mL), treated with 1 equiv. DBU and stirred for 4 h, when LRMS indicated complete elimination of the ring D system, leaving the 3'' alcohol free. The reaction was neutralized with AcOH, the volume was reduced under vacuum to a residue, which was diluted with DCM and passed through a silica pad washing with 10% MeOH/DCM. The filtrate was evaporated to a residue, which was purified by column chromatography (1 to 5% MeOH/DCM).

CbzHN NHCbz OH O HO O O CbzHN HO O HO OH

4.27

6'-Deamino-4',5'-dehydro-3',4'-dideoxy-6'-hydroxy-*per*-*N*-Cbz-ribostamycin (4.27).

4',5'-Dehydro-3',4'-dideoxy-6,3',2'',5'',3''',4'''-hexa-*O*-methylcarbonate-*per*-*N*-Cbz-paromomycin **4.S10** (106 mg, 85 μmol) was submitted to the general periodate cleavage procedure. The column chromatography solvents were 3 → 5% MeOH/DCM. The procedure yielded 28 mg of the title compound **4.27** (40%, 34 μmol), as a white amorphous solid.

R_f = 0.6, 20:5:3 $CHCl_3$/EtOAc/MeOH

HRMS (ESI) calcd. for $C_{41}H_{49}N_3O_{15}$, M + H^+ = 824.3236, found 824.3216 (-2.5 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.39-7.13 (m, 15H), 5.58 (s, 1H), 5.13 (s, 1H), 5.07-4.90 (m, 6H), 4.46 (d, *J* = 3.13 Hz, 1H), 4.06 (d, *J* = 3.83 Hz, 1H), 3.96 (dd, *J* = 5.84, 4.85 Hz,

1H), 3.86-3.68 (m, 4H), 3.68-3.57 (m, 2H), 3.56-3.45 (m, 3H), 3.45-3.33 (m, 1H), 3.34-3.24 (m, 1H), 2.06-1.81 (m, 3H), 1.29 (dd, J = 25.12, 12.63 Hz, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 156.9, 156.9, 156.8, 147.7, 136.5, 136.4, 136.3, 127.7 - 127.1 (15C), 108.9, 97.1, 94.5, 85.2, 83.0, 77.7, 75.1, 74.8, 74.1, 69.9, 66.1, 65.7, 65.7, 62.3, 61.2, 51.2, 49.5, 33.8, 21.6.

H2N NH2 OH O HO O O H2N HO O · 3 HOAc HO OH

4.28

6'-Deamino-4',5'-dehydro-3',4'-dideoxy-6'-hydroxy-ribostamycin (4.28).

Compound **4.27** (27.4 mg, 33 μmol) was submitted to the general Birch reduction procedure. The column chromatography solvent was $CHCl_3$/MeOH/NH_4OH, 2:3:0.5. The procedure yielded 15.8 mg of the acetate salt of the title compound **4.28** (79%, 26 μmol), as a yellow amorphous solid.

R_f = 0.2, 2:3:1 $CHCl_3$/MeOH/NH_4OH.

$[\alpha]^{22}_D$ 16.0º (c 0.79, H_2O).

HRMS (ESI) calcd. for $C_{17}H_{31}N_3O_9$, M + H$^+$ = 422.2133, found 422.2134 (0.24 ppm).

^{1}H NMR (D_2O, 700 MHz) δ 5.59 (d, J = 1.38 Hz, 1H), 5.17 (d, J = 1.36 Hz, 1H), 5.01 (t, J = 3.67, 3.67 Hz, 1H), 4.13 (dd, J = 4.76, 1.58 Hz, 1H), 4.09 (dd, J = 6.70, 4.81 Hz, 1H), 4.02 (dd, J = 10.41, 9.25 Hz, 1H), 3.98 (s, 1H), 3.97 (s, 1H), 3.94 (dt, J = 6.65, 6.51, 2.61 Hz, 1H), 3.86 (dt, J = 5.77, 5.73, 1.37 Hz, 1H), 3.81 (dd, J = 12.17, 2.89 Hz, 1H), 3.73 (t, J = 9.20, 9.20 Hz, 1H), 3.63-3.57 (m, 2H), 3.44 (ddd, J = 12.80, 10.64, 3.97 Hz, 1H), 3.28 (ddd, J = 12.37, 10.95, 4.05 Hz, 1H), 2.57 (ddd, J = 17.88, 5.47, 3.90 Hz, 1H), 2.42 (ddd, J = 12.63, 4.46, 3.90 Hz, 1H), 2.27 (ddd, J = 18.34, 4.79, 4.36 Hz, 1H), 1.87 (s, 13H AcOD), 1.79 (q, J = 12.64, 12.64, 12.60 Hz, 1H).

^{13}C NMR (D_2O, 175 MHz) δ 180.8 (AcOD), 148.8, 110.4, 97.3, 96.1, 83.8, 82.3, 77.7, 74.9, 71.8, 69.6, 62.0, 60.7, 49.7, 48.3, 46.0, 27.8, 22.9 (AcOD), 22.4.

LC/CLND gradient B, R_t = 9.3 min, 97% purity.

4.29

6'-Azido-4',5'-dehydro-3',4'-dideoxy-*per*-*N*-Cbz-ribostamycin (4.29).

Compound **4.20** (100 mg, 78 μmol) was submitted to the general periodate cleavage procedure. The column chromatography solvents were 1 → 3% MeOH/DCM. The procedure yielded 54 mg of the title compound **4.29** (64 μmol, 81%), as a white amorphous solid.

R_f = 0.7, 20:5:3 $CHCl_3$/EtOAc/MeOH

HRMS (ESI) calcd. for $C_{41}H_{48}N_6O_{14}$, M + Na^+ = 871.3121, found 871.3125 (0.53 ppm).

^{1}H NMR (CD_3OD, 700 MHz) δ 7.37-7.15 (m, 15H), 5.61 (s, 1H), 5.13 (d, *J* = 1.19 Hz, 1H), 5.07-4.89 (m, 6H), 4.51 (d, *J* = 4.18 Hz, 1H), 4.09 (d, *J* = 3.70 Hz, 1H), 3.97 (dd, *J* = 6.16, 4.98 Hz, 1H), 3.85-3.81 (m, 1H), 3.75 (ddd, *J* = 11.36, 6.27, 2.28 Hz, 1H), 3.60 (dd, *J* = 11.92, 2.76 Hz, 1H), 3.59-3.56 (m, 1H), 3.56-3.51 (m, 3H), 3.49 (dd, *J* = 11.93, 5.57 Hz, 1H), 3.45 (d, *J* = 13.83 Hz, 1H), 3.44-3.38 (m, 1H), 3.32-3.27 (m, 1H), 2.10-2.02 (m, 1H), 1.99-1.87 (m, 2H), 1.34 (dd, *J* = 21.88, 10.32 Hz, 1H).

^{13}C NMR (CD_3OD, 175 MHz) δ 157.3, 157.2, 156.9, 144.6, 136.9, 136.8, 136.7, 128.2 - 127.5 (m, 15C), 109.5, 99.3, 95.4, 85.8, 83.5, 76.8, 75.3, 74.5, 70.3, 66.5, 66.2, 66.1, 62.8, 52.3, 51.6, 49.9, 47.1, 33.9, 21.9.

FTIR (NaCl): 3337, 2101, 1698, 1531, 1253, 1028 cm^{-1}.

4.30

4',5'-Dehydro-3',4'-dideoxy-ribostamycin (4.30).

Compound **4.29** (19.1 mg, 22.5 μmol) was submitted to the general Birch reduction procedure. The column chromatography solvent was $CHCl_3$/MeOH/NH_4OH, 2:3:0.5. The procedure yielded 11.0 mg of the acetate salt of the title compound **4.30** (74%, 16.7 μmol), as a yellow amorphous solid.

R_f = 0.4, 2:3:2 $CHCl_3$/MeOH/NH_4OH.
$[\alpha]^{22}_D$ 16.7º (*c* 0.55, H_2O).
HRMS (ESI) calcd. for $C_{17}H_{32}N_4O_8$, M + H^+ = 421.2293, found 421.2290 (-0.58 ppm).
^{1}H NMR (D_2O, 700 MHz) δ 5.67 (d, *J* = 1.11 Hz, 1H), 5.17-5.14 (m, 2H), 4.12 (dd, *J* = 4.73, 1.80 Hz, 1H), 4.08 (dd, *J* = 6.66, 4.84 Hz, 1H), 4.03 (dd, *J* = 9.69, 9.24 Hz, 1H), 3.94 (dt, *J* = 6.48, 6.46, 2.88 Hz, 1H), 3.91 (dt, *J* = 5.15, 5.09, 1.40 Hz, 1H), 3.80 (dd, *J* = 12.23, 2.87 Hz, 1H), 3.74 (dd, *J* = 9.45, 8.86 Hz, 1H), 3.65-3.60 (m, 3H), 3.58 (dd, *J* = 12.19, 6.26 Hz, 1H), 3.44 (dt, *J* = 10.70, 10.55, 3.50 Hz, 1H), 3.28 (dt, *J* = 11.37, 11.23, 3.16 Hz, 1H), 2.62 (ddd, *J* = 17.96, 4.51, 3.81 Hz, 1H), 2.42 (d, *J* = 10.96 Hz, 1H), 2.32 (td, *J* = 18.41, 3.79, 3.79 Hz, 1H), 1.87 (s, 15H AcOD), 1.81 (dd, *J* = 24.21, 12.06 Hz, 1H).
^{13}C NMR (D_2O, 175 MHz) δ 181.5 (AcOD), 144.2, 111.4, 101.5, 97.5, 84.7, 83.4, 78.7, 75.8, 72.8, 70.5, 62.8, 50.7, 49.2, 46.7, 41.4, 28.9, 23.7 (AcOD), 23.6.
LC/CLND gradient B, R_t = 12.8 min, 90% purity.

4.32

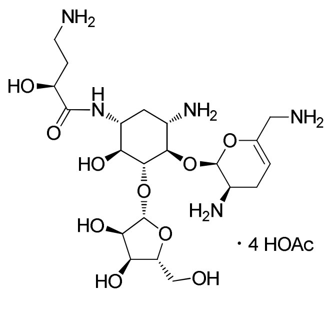
6'-Azido-4',5'-dehydro-3',4'-dideoxy-*per-N*-Cbz-butirosin (4.32).

Compound **16** (53 mg, 63 μmol) was dried by evaporation three times from toluene, dissolved in anhydrous DMF (2 mL), cooled to 0 °C, was treated dropwise with KHMDS (380 μL, 0.190 mmol, 0.5 M in toluene) and stirred with cooling for 5 h, when LRMS indicated complete consumption of the starting material and the major ions corresponded to the 1,6-oxazilidinone **4.31**: HRMS (ESI) calcd. for $C_{34}H_{39}N_6O_{13}$, M + H^+ = 741.2726, found 741.2712 (-1.85 ppm). The reaction was quenched with AcOH (20 μL) and the volume of DMF was reduced under high-vacuum at 50 °C to a residue, which was triturated with 10% Et_2O/Hex. The collected crude intermediate **4.31** was treated with 6 mL of 2:1 THF:sat. $Ba(OH)_2$, at 60 °C overnight, when starting material was not detected on TLC (20:5:3, $CHCl_3$, EtOAc, MeOH, Rf ~ 0) and LRMS indicated complete deprotection of the 1-amino group to intermediate **4.S14**: HRMS (ESI) calcd. for $C_{33}H_{42}N_6O_{12}$, M + H^+ = 715.2933, found 715.2938 (0.4 ppm). The suspension of salts were filtered out through a 0.45 μm syringe filter washing with THF, the filtrate was evaporated under high-vacuum at 60 °C, giving a residue that was diluted in THF and re-filtered. The filtrate was evaporated to a residue and used for amide coupling without further purification.

(*S*)-4-(Benzyloxycarbonylamino)-2-hydroxybutanoic acid (32 mg, 0.126 mmol) was dissolved in THF (1 mL), treated with *N*-hydroxysuccinamide (14.5 mg, 0.126 mmol), DIPEA (70 μL, 0.4 mmol) and EDC (27 mg, 0.141 mmol), and stirred for 2 h. A solution of free-amine intermediate **4.S14** dissolved in THF (1 mL) was treated with the active-ester mixture, which was stirred overnight, when LRMS indicated complete consumption of the free-amine intermediate **4.S14**, and the major ions corresponded to the N1-amide product. The reaction was quenched with sat. NH_4Cl, diluted with DCM, washed successively with 2 N HCl and sat. $NaHCO_3$, dried over Na_2SO_4 and filtered. The organic fraction was evaporated to a residue, which was purified by column chromatography (5 → 8%

ammoniacal MeOH in DCM), to yield 22.4 mg of the title compound **4.32** (38% for 3 steps, 23.6 μmol), as an off-white amorphous solid.

R_f = 0.4, 20:5:3 $CHCl_3$/EtOAc/MeOH
HRMS (ESI) calcd. for $C_{45}H_{56}N_7O_{16}$, M + Na^+ = 972.3598, found 972.3591 (-0.71 ppm).
^{1}H NMR (CD_3OD, 400 MHz) δ 7.39-7.18 (m, 15H), 5.62 (d, *J* = 1.42 Hz, 1H), 5.13 (s, 1H), 5.10-4.90 (m, 6H), 4.55 (d, *J* = 3.62 Hz, 1H), 4.10 (d, *J* = 3.69 Hz, 1H), 4.00 (ddd, *J* = 8.89, 7.12, 4.42 Hz, 2H), 3.84 (dt, *J* = 6.34, 5.97, 3.25 Hz, 1H), 3.77 (ddd, *J* = 10.97, 6.68, 2.29 Hz, 1H), 3.74-3.65 (m, 1H), 3.65-3.54 (m, 4H), 3.54-3.45 (m, 3H), 3.41 (dd, *J* = 9.92, 9.18 Hz, 1H), 3.25-3.14 (m, 2H), 2.10 (dd, *J* = 15.34, 11.95 Hz, 1H), 2.01 (dd, *J* = 6.32, 5.08 Hz, 1H), 1.97-1.84 (m, 3H), 1.76 (dt, *J* = 13.23, 13.20, 6.28 Hz, 1H), 1.42 (dd, *J* = 24.37, 12.03 Hz, 1H).
^{13}C NMR (CD_3OD, 100 MHz) δ 175.1, 157.2, 156.9, 156.4, 144.2, 136.6, 136.4, 136.4, 127.7 - 127.0 (15C), 109.0, 98.9, 95.0, 85.4, 83.1, 76.4, 74.8, 73.5, 69.9, 69.0, 66.1, 65.7, 65.6, 62.3, 52.0, 49.5, 49.2, 46.2, 36.3, 33.8, 32.9, 21.5.
FTIR (NaCl): 3366, 2102, 1693, 1433, 1041 cm^{-1}.



4',5'-Dehydro-3',4'-dideoxy-butirosin (4.33).

Compound **4.32** (22.4 mg, 23 μmol) was submitted to the general Birch reduction procedure. The column chromatography solvents were $CHCl_3$/MeOH/NH_4OH, 2:3:1 followed by 2:3:2. The procedure yielded 9.2 mg of the acetate salt of the title compound **4.33** (52%, 12.1 μmol), as a yellow amorphous solid.

R_f = 0.2, 2:3:2 $CHCl_3$/MeOH/NH_4OH.
$[\alpha]^{22}_D$ 12.6º (*c* 0.46, H_2O).
HRMS (ESI) calcd. for $C_{21}H_{40}N_5O_{10}$, M + H^+ = 522.2770, found 522.2766 (-0.67 ppm).
^{1}H NMR (D_2O, 400 MHz) δ 5.74 (d, *J* = 1.20 Hz, 1H), 5.21 (d, *J* = 1.26 Hz, 1H), 5.19 (dd, *J* = 4.00, 3.26 Hz, 1H), 4.29 (dd, *J* = 8.15, 3.97 Hz, 1H), 4.16 (dd, *J* = 4.64, 1.00 Hz, 1H), 4.12 (dd, *J* = 6.74, 4.82 Hz, 1H), 3.98 (dt, *J* = 6.64, 6.53, 2.68 Hz, 1H), 3.93-3.86 (m, 2H), 3.86-3.80 (m, 2H), 3.75 (dd, *J* = 9.50, 8.85 Hz, 1H), 3.66-3.60 (m, 3H), 3.56 (dd, *J* = 10.23, 9.25 Hz, 1H), 3.31 (dt, *J* = 10.89, 10.74, 3.60 Hz, 1H), 3.18-3.05 (m, 2H), 2.58 (ddd, *J* = 18.04, 5.34, 4.21 Hz, 1H), 2.40-2.29 (m, 1H), 2.18-2.09 (m, 2H), 1.97 (dt, *J* = 14.58, 14.49, 7.74 Hz, 1H), 1.88 (s, 7H AcOD), 1.62 (q, *J* = 12.50, 12.50, 12.46 Hz, 1H).
^{13}C NMR (D_2O, 100 MHz) δ 180.0 (AcOD), 175.2, 142.7, 110.0, 100.2, 96.0, 84.2, 81.8, 77.6, 74.6, 72.7, 69.1, 69.1, 61.4, 48.3, 48.2, 45.5, 40.1, 36.1, 30.5, 29.2, 22.2 (AcOD), 22.1.
LC/CLND gradient A, R_t = 9.7 min, 97.7% purity.

6'-Azido-1,6-4''',6'''-*bis*-carbamate-3,2',2'''-*N*-Cbz-4',5'-dehydro-3',4'-dideoxy-neomycin (4.34).

Compound **4.20** (100 mg, 78 μmol), was dried by evaporation three times from toluene, dissolved in anhydrous DMF (2 mL), cooled to 0 °C, and treated dropwise with KHMDS (470 μL, 0.235 mmol, 0.5 M in toluene) and stirred overnight at 0 °C, when LRMS indicated complete consumption of the starting material and the major ions corresponded to the *bis*-carbamate **4.34**. The reaction was quenched with AcOH (30 μL), evaporated under high-vacuum at 50 °C to a residue, which was diluted with THF and was filtered through a 0.45 μm syringe filter. The volatiles were evaporated to a residue, which

was purified by column chromatography (5 → 7 → 8% ammoniacal MeOH in DCM), to yield 48.9 mg of the title compound **4.34** (59%, 46 μmol), as an off-white amorphous solid.

R_f = 0.3, 20:5:3 $CHCl_3$/EtOAc/MeOH

HRMS (ESI) calcd. for $C_{49}H_{56}N_8O_{19}$, M + H^+ = 1061.3734, found 1061.3729 (-0.56 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.47-7.08 (m, 15H), 5.59 (s, 1H), 5.21-4.90 (m, 8H), 4.57 (s, 1H), 4.33-4.18 (m, 3H), 4.13 (dd, J = 7.80, 5.54 Hz, 1H), 4.07 (s, 1H), 4.02-3.94 (m, 1H), 3.92 (s, 1H), 3.86-3.74 (m, 2H), 3.74-3.58 (m, 4H), 3.56-3.35 (m, 5H), 3.27 (s, 1H), 2.21-1.89 (m, 3H), 1.51 (dd, J = 22.72, 11.37 Hz, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 160.6, 157.1, 156.8, 156.3, 153.2, 144.3, 136.4 - 136.2 (3C), 127.8 - 127.1 (15C), 107.7, 98.9, 97.2, 95.6, 83.0, 81.7, 78.3, 77.9, 76.2, 74.4, 72.8, 67.6, 66.3, 66.1, 65.9, 63.0, 62.0, 53.5, 51.9, 51.8, 50.9, 46.7, 43.1, 31.4, 21.5.

FTIR (NaCl): 3381, 2102, 1698, 1429, 1348, 1038 cm^{-1}.

4.35

6'-Azido-*N*-1-((*S*)-4-(benzyloxycarbonylamino)-2-hydroxybutanoyl)-3''',6'''-carbamate-3, 2',2'''-*N*-Cbz-4',5'-dehydro-3',4'-dideoxy-neomycin (4.35).

Bis-carbamate **4.34** (44 mg, 42 μmol) was dissolved in DMF (2 mL), treated with 0.5 M LiOH (530 μL, 0.266 mmol) and stirred overnight, when LRMS indicated deprotection of the 1-amino group (compound **4.S15**), and starting material was not detected on TLC (20:5:3, $CHCl_3$, EtOAc, MeOH, Rf ~ 0). The reaction was diluted with THF (10 mL) and filtered through a 0.45 μm syringe filter, and the filtrate was evaporated under high-vacuum at 60 °C, to give a residue which was redissolved in THF and refiltered. The filtrate was evaporated under vacuum to a residue, which was used without further purification.

(*S*)-4-(Benzyloxycarbonylamino)-2-hydroxybutanoic acid (22.3 mg, 88 μmol) was dissolved in THF (1 mL), treated with *N*-hydroxysuccinamide (10.1 mg, 88 μmol), DIPEA (46 μL, 0.155 mmol) and EDC (18.6 mg, 97 μmol), and stirred for 2 h. Free-amine intermediate **4.S15** was dissolved in dry THF (1 mL), and treated with the active-ester solution, which was stirred overnight, when LRMS indicated complete consumption of the free-amine intermediate, and the major ions corresponded to the N1-amide product **4.35**. The reaction was quenched with sat. NH_4Cl, evaporated under vacuum, diluted with DCM, washed successively with 2 N HCl and sat. $NaHCO_3$, dried over Na_2SO_4 and filtered. The organic layer was evaporated to a residue, which was purified by column chromatography (9 → 10% ammoniacal MeOH in DCM), to yield 21.1 mg of the title compound **4.35** (40% for 2 steps, 16.6 μmol), as an off-white amorphous solid.

R_f = 0.2, 20:5:3 $CHCl_3$/EtOAc/MeOH

HRMS (ESI) calcd. for $C_{60}H_{71}N_9O_{22}$, M + H^+ = 1270.4785 (-0.14 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.44-7.09 (m, 20H), 5.58 (s, 1H), 5.16-4.90 (m, 10H), 4.61-4.52 (m, 2H), 4.27-4.15 (m, 3H), 4.11 (s, 1H), 4.07-4.03 (m, 1H), 4.01 (dd, *J* = 7.43, 3.89 Hz, 1H), 3.95-3.86 (m, 2H), 3.76 (dd, *J* = 9.94, 6.41 Hz, 1H), 3.71-3.53 (m, 5H), 3.53-3.38 (m, 4H), 3.31 (d, *J* = 13.21 Hz, 1H), 3.24-3.12 (m, 2H), 2.18-2.05 (m, 1H), 2.05-1.96 (m, 1H), 1.96-1.83 (m, 2H), 1.75 (dt, *J* = 13.39, 13.35, 6.47 Hz, 1H), 1.41 (dd, *J* = 24.28, 12.06 Hz, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 175.12, 157.17 - 156.37 (4C), 153.17, 144.23, 136.59 - 136.31 (4C), 127.80 - 126.94 (20C), 108.51, 98.94, 97.79, 95.10, 85.24, 81.60, 77.70, 76.84, 76.37, 74.33, 73.56, 69.04, 67.50, 66.08, 66.04, 65.75, 65.66, 62.85, 61.53, 51.95, 51.77, 49.46, 49.15, 46.82, 43.09, 36.27, 33.79, 32.89, 21.50.

FTIR (NaCl): 3369, 2102, 1698, 1429, 1040 cm^{-1}.

4.36

6'-Azido-*N*-1-((*S*)-4''''-(benzyloxycarbonylamino)-2''''-hydroxybutanoyl)-3,2',2''',6'''-*N*-Cbz-4',5'-dehydro-3',4'-dideoxy-neomycin (4.36).

Compound **4.35** (21 mg, 16.6 μmol) was dried by evaporation three times from toluene, dissolved in anhydrous benzyl alcohol (2 mL), treated with sodium benzyloxide (100 μL, 1 M in benzyl alcohol, 0.10 mmol), and stirred for 16 h, when LRMS monitoring indicated that an equilibrium was reached, favouring the 6'''-*N*-Cbz product **4.36**. The reaction was quenched with AcOH (10 μL, 0.17 mmol), diluted with DCM (10 mL) and the solution was loaded onto silica gel for purification by column chromatography (2 → 4 → 8% MeOH/DCM), to yield 19.5 mg of the title compound **4.36** (85%, 14 μmol), as an off-white amorphous solid.

R_f = 0.4, 20:5:3 $CHCl_3$/EtOAc/MeOH
HRMS (ESI) calcd. for $C_{67}H_{79}N_9O_{23}$, M + Na^+ = 1400.5181, found 1400.5180 (-0.1 ppm).
^{1}H NMR (CD_3OD, 700 MHz) δ 7.37-7.11 (m, 25H), 5.55 (s, 1H), 5.11-4.89 (m, 11H), 4.57 (s, 1H), 4.55-4.51 (m, 1H), 4.15-4.10 (m, 2H), 3.98 (dd, *J* = 7.72, 3.88 Hz, 1H), 3.91 (dd, *J* = 7.95, 4.51 Hz, 1H), 3.87-3.82 (m, 2H), 3.77 (s, 1H), 3.74 (ddd, *J* = 11.36, 6.36, 2.13 Hz, 1H), 3.67-3.55 (m, 3H), 3.55-3.48 (m, 3H), 3.45 (d, *J* = 13.68 Hz, 1H), 3.43-3.34 (m, 3H), 3.34-3.26 (m, 2H), 3.22-3.18 (m, 1H), 3.14 (td, *J* = 13.28, 6.26, 6.26 Hz, 1H), 2.08 (dd, *J* = 15.57, 12.20 Hz, 1H), 2.02-1.94 (m, 1H), 1.92-1.87 (m, 1H), 1.85 (ddd, *J* = 14.01, 7.13, 4.28 Hz, 1H), 1.72 (dt, *J* = 13.66, 13.38, 6.88 Hz, 1H), 1.39-1.29 (m, 1H).
^{13}C NMR (CD_3OD, 100 MHz) δ 175.5, 157.8 - 156.7 (5C), 144.6, 137.0 - 136.7 (5C), 128.1 - 127.1 (25C), 109.2, 99.3, 98.8, 95.6, 85.7, 82.3, 77.5, 77.0, 74.1, 73.9, 73.2, 70.2, 69.4, 67.8, 66.5, 66.2, 66.2, 66.1, 66.0, 61.9, 52.7, 52.3, 49.8, 49.5, 48.1, 41.2, 36.6, 34.2, 33.3, 21.9.
FTIR (NaCl): 3380, 2102, 1693, 1431, 1527, 1031 cm^{-1}.

4.37

***N*-1-((*S*)-4-Amino-2-hydroxybutanoyl)-4',5'-dehydro-3',4'-dideoxy-neomycin (4.37).**

Compound **4.36** (31 mg, 22 μmol) was submitted to the general Birch reduction procedure. The column chromatography solvents were $CHCl_3$/MeOH/NH_4OH, 2:3:1 followed by 2:3:2. The procedure yielded 14.2 mg of the acetate salt of the title compound **4.37** (63%, 13.6 μmol), as a yellow amorphous solid.

R_f = 0.1, 2:3:2 $CHCl_3$/MeOH/NH_4OH.

$[\alpha]^{22}_D$ 27.5º (*c* 0.71, H_2O).

HRMS (ESI) calcd. for $C_{27}H_{51}N_7O_{13}$, $M + H^+$ = 682.3618, found 682.3620 (-0.29 ppm).

^{1}H NMR (D_2O, 700 MHz) δ 5.66 (d, *J* = 1.20 Hz, 1H), 5.25-5.24 (m, 1H), 5.21 (d, *J* = 2.45 Hz, 1H), 5.16 (t, *J* = 3.76, 3.76 Hz, 1H), 4.47 (dd, *J* = 5.92, 5.26 Hz, 1H), 4.32 (dd, *J* = 4.90, 2.53 Hz, 1H), 4.27 (dd, *J* = 8.16, 3.89 Hz, 2H), 4.19-4.17 (m, 1H), 4.12 (dt, *J* = 5.69, 5.65, 3.42 Hz, 1H), 4.06 (dd, *J* = 10.13, 9.41 Hz, 1H), 3.93 (dt, *J* = 4.96, 4.85, 1.33 Hz, 1H), 3.89 (ddd, *J* = 12.31, 10.60, 4.29 Hz, 1H), 3.82 (dd, *J* = 12.05, 3.29 Hz, 1H), 3.78-3.77 (m, 1H), 3.75 (t, *J* = 9.26, 9.26 Hz, 1H), 3.68 (dd, *J* = 12.14, 5.40 Hz, 1H), 3.65-3.58 (m, 3H), 3.55-3.52 (m, 1H), 3.46 (ddd, *J* = 12.69, 10.58, 4.27 Hz, 1H), 3.38 (dd, *J* = 13.64, 6.67 Hz, 1H), 3.32 (dd, *J* = 13.61, 3.91 Hz, 1H), 3.14-3.05 (m, 2H), 2.64 (ddd, *J* = 18.35, 4.68, 3.63 Hz, 1H), 2.34 (ddd, *J* = 18.52, 4.29, 3.58 Hz, 1H), 2.21 (td, *J* = 12.79, 4.31, 4.31 Hz, 1H), 2.12 (dddd, *J* = 14.22, 8.11, 6.62, 3.87 Hz, 1H), 1.93 (s, 18H AcOD), 1.98-1.94 (m, 1H), 1.75 (dd, *J* = 12.64, 12.63 Hz, 1H).

^{13}C NMR (D2O, 175 MHz) δ 179.3 (AcOD), 175.5, 143.3, 110.2, 100.4, 96.7, 95.4, 84.4, 81.2, 78.1, 75.9, 73.2, 72.9, 70.0, 69.4, 67.6, 67.2, 61.1, 50.7, 48.7, 48.5, 45.7, 40.5, 40.4, 36.5, 30.8, 29.5, 23.0 (AcOD), 22.0.

LC/CLND gradient A, R_t = 11.3 min, 96% purity.

Experimental references

1. Jeffs, P. W.; Chan, G.; Sitrin, R.; Holder, N.; DeBrosse, C., The structure of the glycolipid components of the aridicin antibiotic complex. *J. Org. Chem.* **1985,** *50* (10), 1726-31.
2. Hanessian, S.; Ogawa, T.; Takamoto, T., Aminoglycoside antibiotics: synthesis of pseudotrisaccharides derived from neamine and paromamine. *Can. J. Chem.* **1978,** *56* (11), 1500-8.

"Compound 4.21 (JPM4-189)" 1 1 D: Hanessian

"Compound 4.21 (JPM4-189)" 2 1 D: Hanessian

180.919
148.966
110.350
97.078
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81.178
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75.811
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67.163
61.114
60.704
50.719
49.642
48.224
45.892
40.280
27.778
22.905
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H_2N NH_2 OH O HO O O H_2N HO O OH · 5 HOAc O H_2N O NH_2 HO OH

4.21

[rel] 8 6 4 2 0

200 150 100 50 0 [ppm]

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Acq. Operator : rd Seq. Line : 15
Acq. Instrument : Chemstation 8 Location : Vial 10
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Different Inj Volume from Sequence ! Actual Inj Volume : 2 µl
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(modified after loading)
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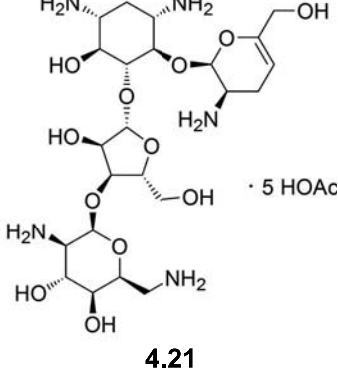
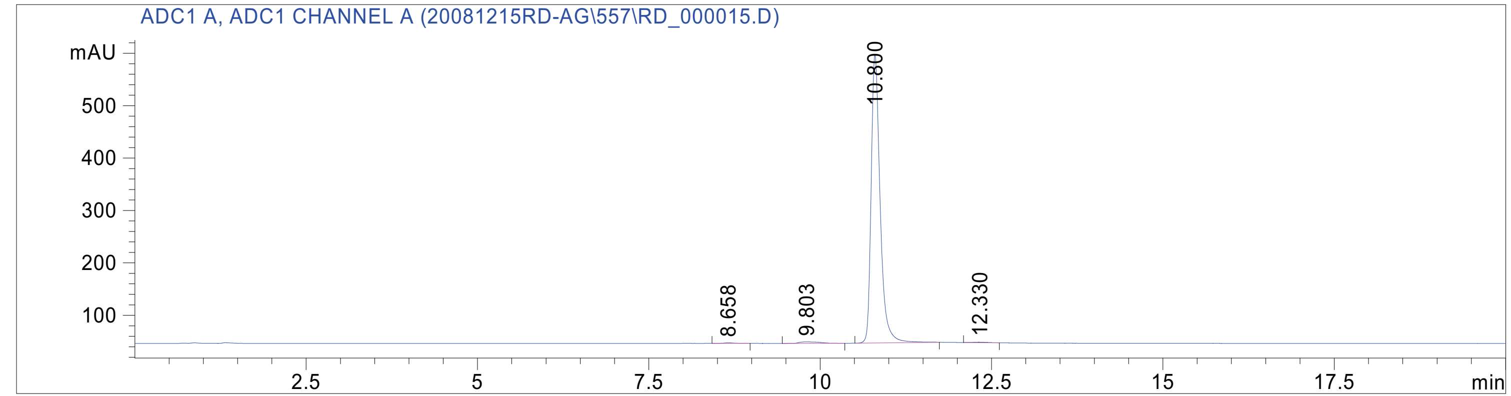
Sample Info : 20x dilution





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Area Percent Report

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Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: ADC1 A, ADC1 CHANNEL A

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	8.658	MM	0.1969	13.33785	1.12922	0.2611
2	9.803	BB	0.3603	61.65374	2.80684	1.2070
3	10.800	MM	0.1505	5015.08398	555.31445	98.1797
4	12.330	MM	0.2800	17.99119	1.07085	0.3522
Totals :				5108.06677	560.32136	

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*** End of Report ***

"Compound 4.22 (JPM4-191)" 1 1 D: Hanessian

5.306 5.299 5.260 5.251 5.242 4.534 4.521 4.507 4.397 4.390 4.385 4.378 4.363 4.352 4.340 4.281 4.274 4.266 4.243 4.235 4.230 4.221 4.216 4.207 4.107 4.083 4.059 4.010 3.999 3.988 3.921 3.912 3.890 3.882 3.873 3.869 3.865 3.855 3.832 3.809 3.775 3.761 3.744 3.731 3.713 3.685 3.638 3.635 3.632 3.628 3.625 3.506 3.497 3.481 3.463 3.447 3.430 3.420 3.396 3.386 3.369 3.364 3.343 3.333 2.743 2.733 2.722 2.698 2.689 2.677 2.515 2.505 2.495 2.484 2.474 2.464 2.454 2.444 2.434 2.408 2.397 2.387 1.969 1.901 1.870 1.838 1.808

H_2N, NH_2, NH_2, HO, O, O, O, H_2N, HO, O, OH, · 6 HOAc, O, H_2N, O, HO, NH_2, OH

4.22

0.910 1.014 0.989 0.984 1.012 2.047 1.082 1.034 1.001 1.016 3.142 3.949 1.082 4.087 1.000 1.987 18.067 1.135

7 6 5 4 3 2 1 [ppm]

[rel] 0.6 0.5 0.4 0.3 0.2 0.1 -0.0

"Compound 4.22 (JPM4-191)" 3 1 D: Hanessian

180.403
143.009
109.915
100.093
96.257
95.286
83.544
81.197
78.039
75.855
73.007
71.664
69.795
67.286
66.958
60.940
50.500
49.543
47.926
45.461
40.086
40.064
28.080
22.664
22.521

· 6 HOAc

4.22

[rel]

25 20 15 10 5 0

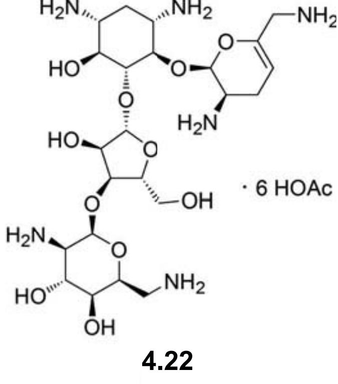
200 150 100 50 0 [ppm]

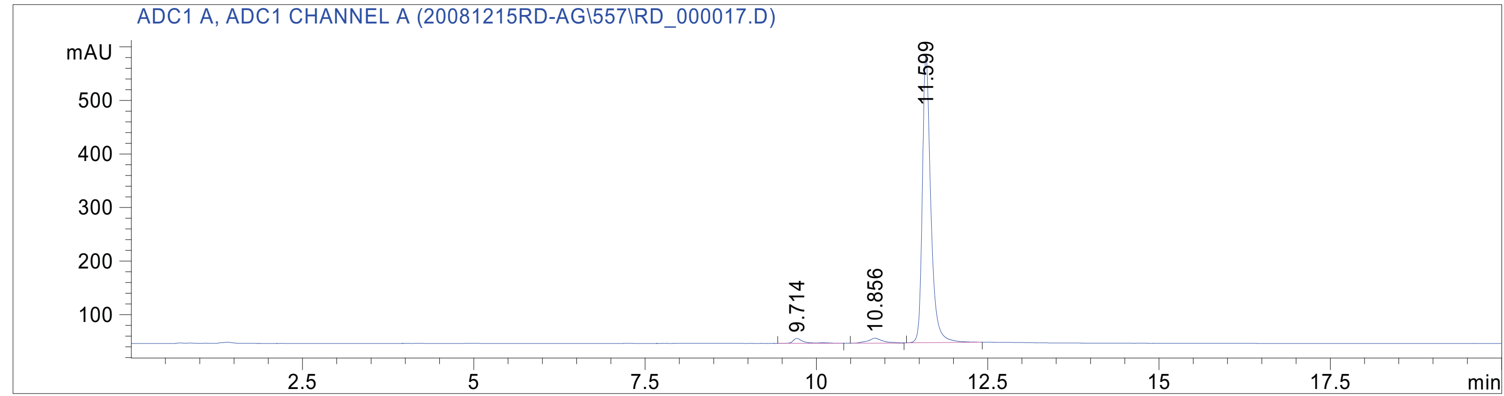
Data File C:\CHEM32\1\DATA\20081215RD-AG\557\RD_000017.D
Sample Name: JPM4-191

===

Acq. Operator : rd   Seq. Line : 17
Acq. Instrument : Chemstation 8   Location : Vial 11
Injection Date : 12/15/2008 10:55:45 PM   Inj : 1
                                                  Inj Volume : 20 µl
Different Inj Volume from Sequence !   Actual Inj Volume : 2 µl
Acq. Method : C:\Chem32\1\DATA\20081215RD-AG\557\05-25-95B_20M.M
Last changed : 10/24/2008 3:35:56 PM by rd
Analysis Method : C:\CHEM32\1\METHODS\INT_30MIN.M
Last changed : 12/16/2008 8:35:51 AM by rd
(modified after loading)
Method Info : Integration method.

Sample Info : 20x dilution





===

Area Percent Report

===

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: ADC1 A, ADC1 CHANNEL A

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	9.714	MM	0.1799	101.51895	9.40456	2.0827
2	10.856	BV	0.2273	150.06935	9.34979	3.0788
3	11.599	MM	0.1430	4622.69629	538.71045	94.8385

Totals : 4874.28459 557.46480

===

*** End of Report ***

"Compound 4.24 (JPM4-169)" 1 1 D: Hanessian

5.633, 5.224, 5.223, 5.218, 5.213, 5.208, 5.193, 5.190, 4.429, 4.422, 4.414, 4.296, 4.292, 4.289, 4.285, 4.249, 4.242, 4.234, 4.165, 4.160, 4.156, 4.125, 4.117, 4.112, 4.104, 3.971, 3.958, 3.944, 3.891, 3.884, 3.877, 3.809, 3.805, 3.792, 3.787, 3.755, 3.732, 3.719, 3.705, 3.703, 3.662, 3.654, 3.644, 3.636, 3.598, 3.587, 3.578, 3.574, 3.513, 3.372, 3.362, 3.352, 3.343, 3.324, 3.321, 3.318, 3.308, 3.302, 3.288, 3.283, 3.264, 3.258, 3.246, 3.243, 3.231, 3.225, 2.640, 2.629, 2.621, 2.615, 2.601, 2.595, 2.371, 2.366, 2.359, 2.353, 2.346, 2.339, 2.311, 1.846, 1.753, 1.735, 1.718, 1.700

H2N, NH2, N–Me, H, HO, O, O, H2N, HO, O, O, OH, · 6 HOAc, O, H2N, O, NH2, HO, OH

4.24

0.913, 0.949, 0.994, 0.976, 1.004, 1.041, 1.014, 1.034, 0.999, 2.009, 1.083, 2.041, 1.019, 1.998, 1.011, 1.082, 4.005, 2.000, 17.881, 1.090

[rel] 0.8 0.6 0.4 0.2 -0.0

7 6 5 4 3 2 1 [ppm]

"Compound 4.24 (JPM4-169)" 2 1 D: Hanessian

181.217
141.707
110.266
102.516
96.475
95.507
83.921
81.373
78.431
75.936
73.208
71.984
70.062
67.591
67.216
61.085
50.746
49.829
49.323
48.162
45.628
40.306
31.870
28.511
23.097
22.896

H_2N NH_2 N Me H O HO O O H_2N HO O O OH · 6 HOAc H_2N O HO NH_2 OH

4.24

[rel]
15
10
5
0

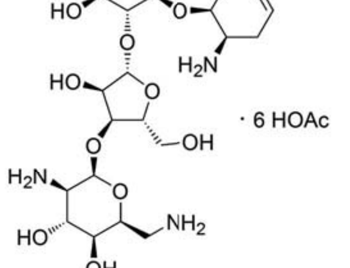
200 150 100 50 0 [ppm]

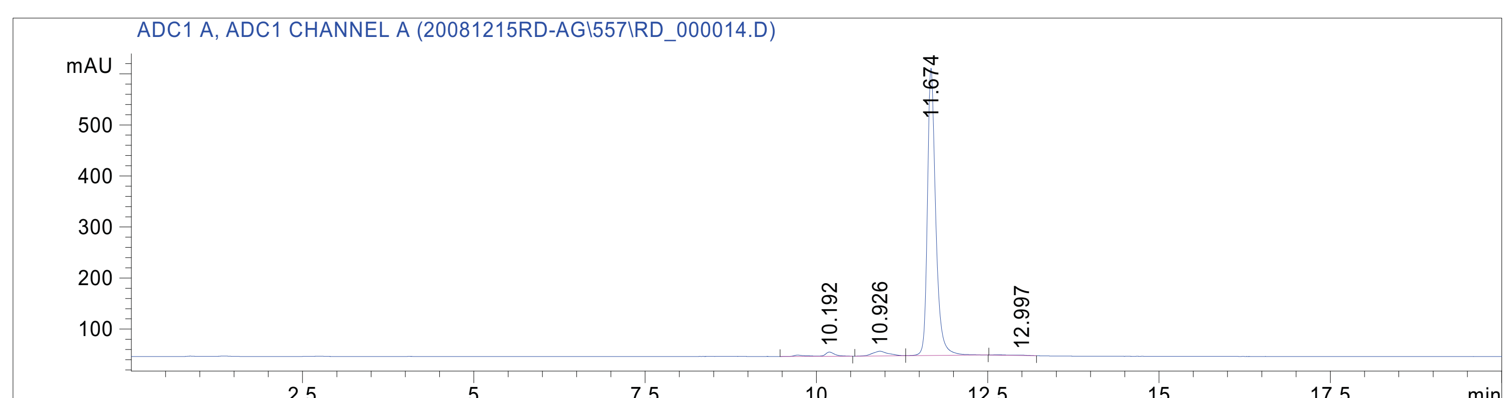
Data File C:\CHEM32\1\DATA\20081215RD-AG\557\RD_000014.D
Sample Name: JPM4-169

===

Acq. Operator : rd Seq. Line : 14
Acq. Instrument : Chemstation 8 Location : Vial 9
Injection Date : 12/15/2008 9:52:48 PM Inj : 1
Inj Volume : 20 µl
Different Inj Volume from Sequence ! Actual Inj Volume : 2 µl
Acq. Method : C:\Chem32\1\DATA\20081215RD-AG\557\05-25-95B_20M.M
Last changed : 10/24/2008 3:35:56 PM by rd
Analysis Method : C:\CHEM32\1\METHODS\INT_30MIN.M
Last changed : 12/16/2008 8:35:51 AM by rd
(modified after loading)
Method Info : Integration method.

Sample Info : 20x dilution





===

Area Percent Report

===

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: ADC1 A, ADC1 CHANNEL A

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	10.192	BB	0.1923	111.17039	8.09142	2.2071
2	10.926	BV	0.2445	150.17834	9.29495	2.9815
3	11.674	VB	0.1313	4749.79053	558.00244	94.2971
4	12.997	MM	0.4246	25.90739	1.01685	0.5143

Totals : 5037.04665 576.40566

===

*** End of Report ***

"Compound 4.26 (JPM4-193)" 1 1 D: Hanessian

5.635 5.421 5.415 5.254 5.252 4.472 4.464 4.456 4.375 4.370 4.366 4.290 4.286 4.283 4.278 4.268 4.260 4.187 4.183 4.178 4.162 4.148 4.124 4.116 4.110 4.103 3.845 3.842 3.836 3.824 3.819 3.810 3.797 3.782 3.774 3.740 3.733 3.722 3.715 3.694 3.682 3.673 3.543 3.513 3.501 3.487 3.397 3.387 3.378 3.368 3.336 3.330 3.324 3.310 3.305 2.701 2.464 2.453 2.447 1.898 1.886 1.863 1.846 1.828

H_2N, NH_2, N, Me, H, HO, O, O, O, H_2N, OH, HO, O, · 6 HOAc, O, OH, H_2N, O, NH_2, HO, OH

4.26

0.967 0.965 1.930 0.961 0.964 1.943 1.918 1.007 2.924 1.864 3.020 0.971 0.996 1.046 1.916 2.945 1.000 7.370 0.873

7 6 5 4 3 2 1 [ppm]

[rel] 0.6 0.4 0.2 -0.0

"Compound 4.26 (JPM4-193)" 3 1 D: Hanessian

180.264
144.516
109.432
104.608
96.181
95.477
82.579
81.396
78.462
75.746
73.182
71.806
70.083
67.588
67.146
61.860
60.709
51.414
50.732
49.733
49.081
48.112
40.362
32.225
27.882
22.525

H_2N NH_2 N Me H HO O O O H_2N OH HO O O OH · 6 HOAc O H_2N O NH_2 HO OH

4.26

[rel] 15 10 5 0

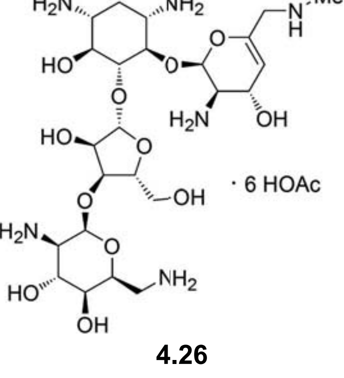
200 150 100 50 0 [ppm]

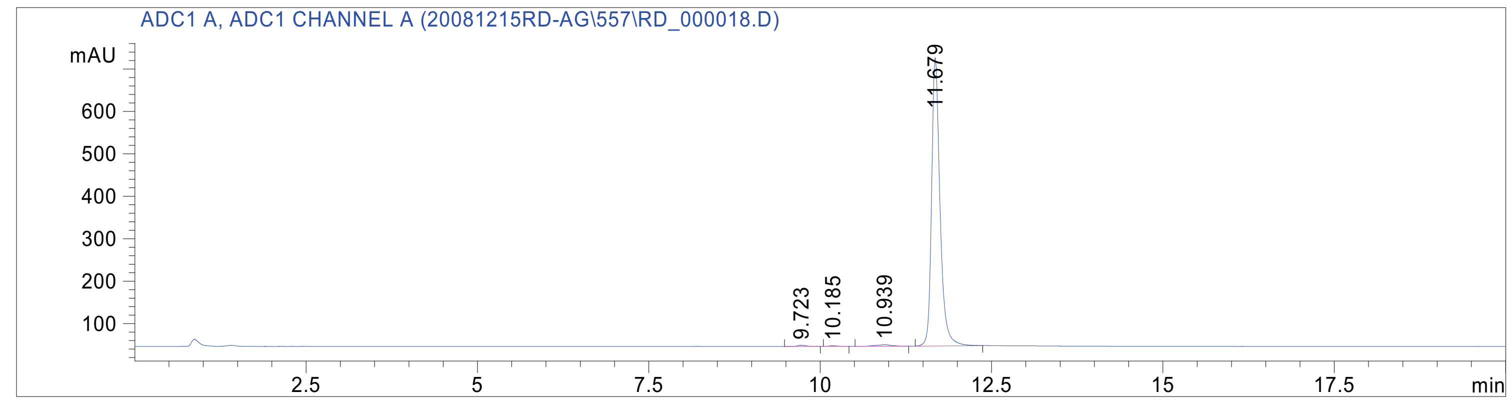
Data File C:\CHEM32\1\DATA\20081215RD-AG\557\RD_000018.D
Sample Name: JPM4-193

===

```
Acq. Operator   : rd                              Seq. Line :  18
Acq. Instrument : Chemstation 8                    Location : Vial 12
Injection Date  : 12/15/2008 11:16:43 PM                Inj :   1
                                                 Inj Volume : 20 µl
Different Inj Volume from Sequence !      Actual Inj Volume : 2 µl
Acq. Method     : C:\Chem32\1\DATA\20081215RD-AG\557\05-25-95B_20M.M
Last changed    : 10/24/2008 3:35:56 PM by rd
Analysis Method : C:\CHEM32\1\METHODS\INT_30MIN.M
Last changed    : 12/16/2008 8:35:51 AM by rd
                  (modified after loading)
Method Info     : Integration method.

Sample Info     : 20x dilution
```





===

Area Percent Report

===

```
Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs
```

Signal 1: ADC1 A, ADC1 CHANNEL A

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	9.723	BB	0.1310	23.25134	2.74062	0.3931
2	10.185	MM	0.1403	12.68547	1.50671	0.2145
3	10.939	BB	0.2216	61.72415	3.79780	1.0435
4	11.679	MM	0.1413	5817.34863	686.23798	98.3489

Totals : 5915.00959 694.28311

===

*** End of Report ***

"Compound 4.28 (JPM5-17)" 1 1 D: Hanessian

5.589 5.587 5.166 5.165 5.016 5.011 5.006 4.131 4.129 4.125 4.122 4.100 4.093 4.090 4.083 4.030 4.016 4.002 3.979 3.972 3.955 3.952 3.946 3.942 3.936 3.932 3.866 3.864 3.857 3.856 3.850 3.847 3.821 3.816 3.803 3.799 3.744 3.730 3.717 3.615 3.602 3.594 3.586 3.576 3.456 3.450 3.438 3.436 3.428 3.423 3.417 3.296 3.290 3.278 3.275 3.263 3.257 2.586 2.579 2.573 2.560 2.554 2.546 2.439 2.433 2.427 2.421 2.415 2.409 2.289 2.283 2.276 2.263 2.257 2.250 1.871 1.815 1.796 1.786 1.779 1.761

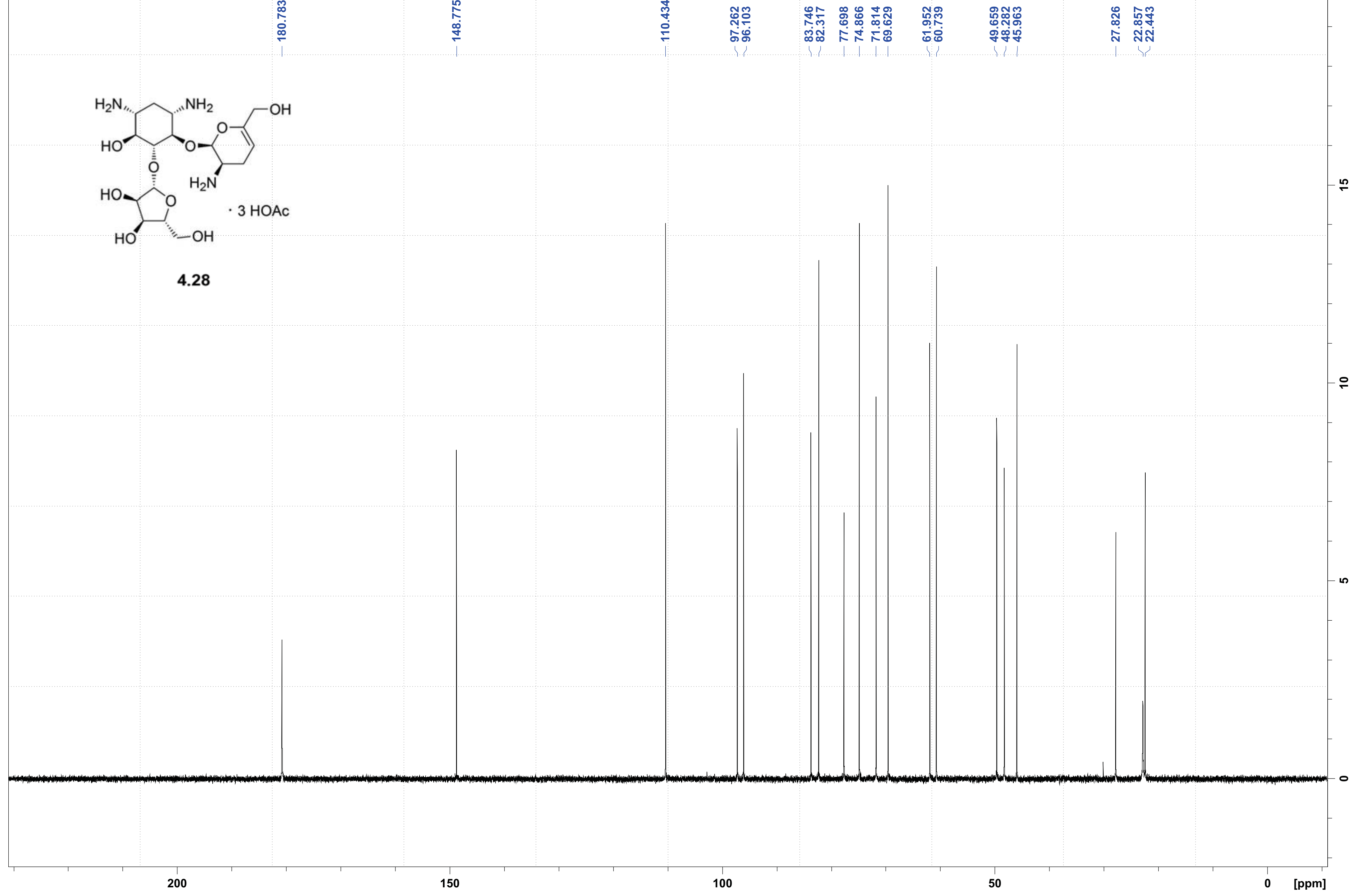
H_2N NH_2 OH O HO O O H_2N HO O · 3 HOAc HO OH

4.28

1.038 1.034 1.145 1.156 1.380 1.131 1.125 1.063 2.323 0.940 0.962 0.908 0.964 0.891 13.523 1.000

7 6 5 4 3 2 1 [ppm]

[rel] 1.0 0.8 0.6 0.4 0.2 -0.0

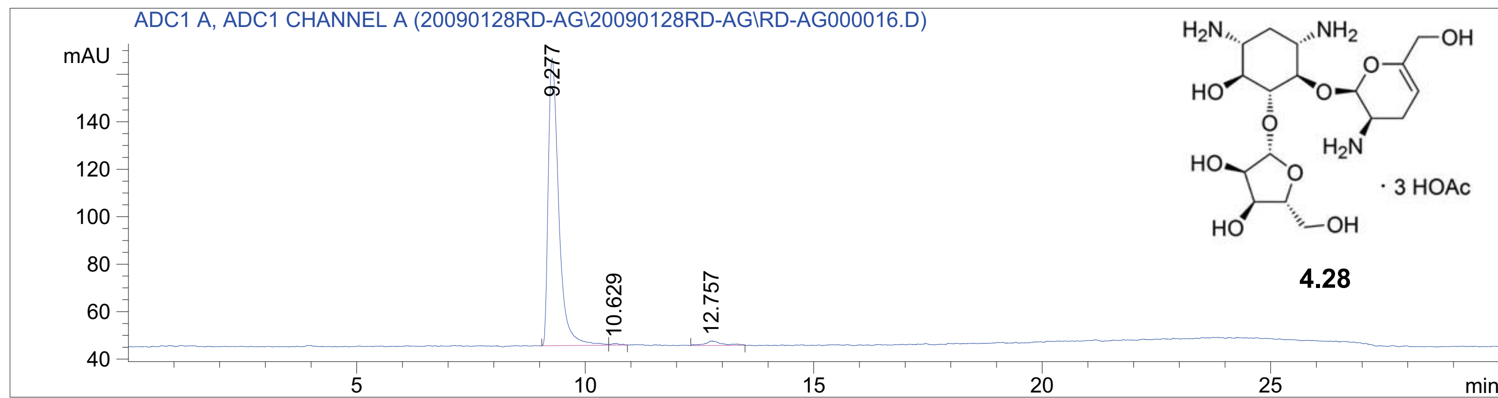

"Compound 4.28 (JPM5-17)" 3 1 D: Hanessian
180.783
148.775
110.434
97.262
96.103
83.746
82.317
77.698
74.866
71.814
69.629
61.952
60.739
49.659
48.282
45.963
27.826
22.857
22.443
H2N
NH2
OH
O
HO
O
O
H2N
HO
O
· 3 HOAc
HO
OH
4.28
[rel]
15
10
5
0
200
150
100
50
0
[ppm]

Data File C:\CHEM32\1\DATA\20090128RD-AG\20090128RD-AG\RD-AG000016.D
Sample Name: JPM-5-17

===

```
Acq. Operator   : rd                                Seq. Line :  16
Acq. Instrument : Chemstation 4                      Location : Vial 71
Injection Date  : 1/28/2009 11:48:42 PM                   Inj :   1
                                                   Inj Volume : 2 µl
Acq. Method     : C:\Chem32\1\DATA\20090128RD-AG\20090128RD-AG\05-30-75B_210_30M.M
Last changed    : 1/23/2009 12:23:25 PM by rc
Analysis Method : C:\CHEM32\1\METHODS\INT_30MIN.M
Last changed    : 1/28/2009 8:43:05 AM by rc
                  (modified after loading)
Method Info     : Integration method.

Sample Info     : 20x dilution
```



===

Area Percent Report

===

```
Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs
```

Signal 1: ADC1 A, ADC1 CHANNEL A

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	9.277	MF	0.2662	1937.68665	121.29505	96.9886
2	10.629	FM	0.2323	9.60944	6.89434e-1	0.4810
3	12.757	MM	0.4517	50.55310	1.86522	2.5304

Totals : 1997.84919 123.84971

===

*** End of Report ***

"Compound 4.30 (JPM5-19)" 1 1 D: Hanessian

5.666 5.162 5.161 5.155 5.150 4.121 4.119 4.115 4.112 4.089 4.082 4.079 4.072 4.040 4.027 4.013 3.948 3.944 3.938 3.934 3.929 3.925 3.912 3.905 3.899 3.810 3.806 3.793 3.789 3.752 3.739 3.726 3.614 3.598 3.589 3.580 3.571 3.439 3.426 3.295 3.279 3.268 2.639 2.633 2.626 2.612 2.607 2.601 2.430 2.416 2.337 2.331 2.326 2.310 2.305 2.299 1.880 1.832 1.815 1.797 1.781

H2N, NH2, NH2, HO, O, O, H2N, HO, O, HO, OH · 4 HOAc

4.30

0.850 1.931 1.011 1.021 0.932 1.068 0.940 1.045 1.029 0.971 1.000 0.817 0.876 0.902 15.109 1.055

7 6 5 4 3 2 1 [ppm]

[rel] 0.5 0.4 0.3 0.2 0.1 -0.0

"Compound 4.30 (JPM5-19)" 2 1 D: Hanessian

181.463, 144.178, 111.407, 101.528, 97.485, 84.722, 83.389, 78.741, 75.830, 72.793, 70.531, 62.828, 50.692, 49.155, 46.706, 41.399, 28.845, 23.724, 23.612

H_2N NH_2 NH_2 HO O O H_2N HO O HO OH · 4 HOAc

4.30

[rel] 15 10 5 0

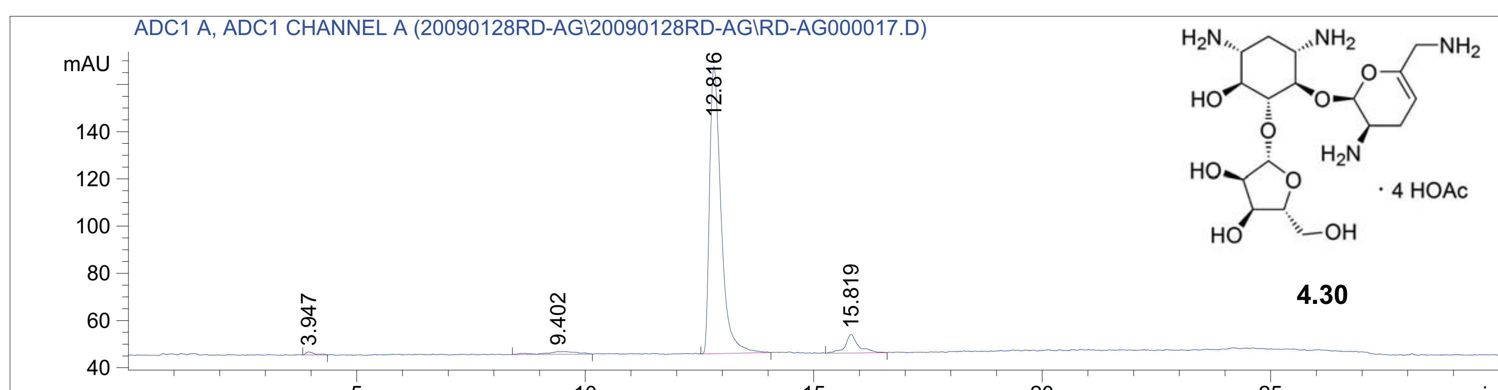
200 150 100 50 0 [ppm]

Data File C:\CHEM32\1\DATA\20090128RD-AG\20090128RD-AG\RD-AG000017.D
Sample Name: JPM-5-19

===

Acq. Operator : rd Seq. Line : 17
Acq. Instrument : Chemstation 4 Location : Vial 72
Injection Date : 1/29/2009 12:19:31 AM Inj : 1
Inj Volume : 2 µl
Acq. Method : C:\Chem32\1\DATA\20090128RD-AG\20090128RD-AG\05-30-75B_210_30M.M
Last changed : 1/23/2009 12:23:25 PM by rc
Analysis Method : C:\CHEM32\1\METHODS\INT_30MIN.M
Last changed : 1/28/2009 8:43:05 AM by rc
(modified after loading)
Method Info : Integration method.

Sample Info : 20x dilution



===

Area Percent Report

===

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: ADC1 A, ADC1 CHANNEL A

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.947	MM	0.2289	17.42037	1.26856	0.7351
2	9.402	MM	0.8175	59.04083	1.20362	2.4913
3	12.816	BB	0.2729	2134.35278	121.52830	90.0615
4	15.819	BB	0.2858	159.06853	7.80302	6.7121

Totals : 2369.88251 131.80350

===

*** End of Report ***

"Compound 4.33 (JPM5-29)" 1 1 D: Hanessian

5.739, 5.212, 5.197, 5.188, 5.179, 4.306, 4.296, 4.286, 4.276, 4.168, 4.157, 4.135, 4.118, 4.106, 3.997, 3.990, 3.981, 3.974, 3.964, 3.957, 3.915, 3.891, 3.880, 3.872, 3.849, 3.832, 3.817, 3.775, 3.752, 3.729, 3.652, 3.629, 3.606, 3.582, 3.558, 3.533, 3.338, 3.330, 3.312, 3.306, 3.301, 3.282, 3.274, 3.175, 3.157, 3.143, 3.132, 3.125, 3.116, 3.107, 3.097, 3.083, 3.065, 2.620, 2.606, 2.594, 2.574, 2.562, 2.550, 2.377, 2.371, 2.358, 2.333, 2.321, 2.312, 2.184, 2.165, 2.156, 2.147, 2.136, 2.126, 2.117, 2.100, 2.041, 2.016, 1.996, 1.979, 1.960, 1.944, 1.924, 1.884, 1.672, 1.641, 1.609, 1.578

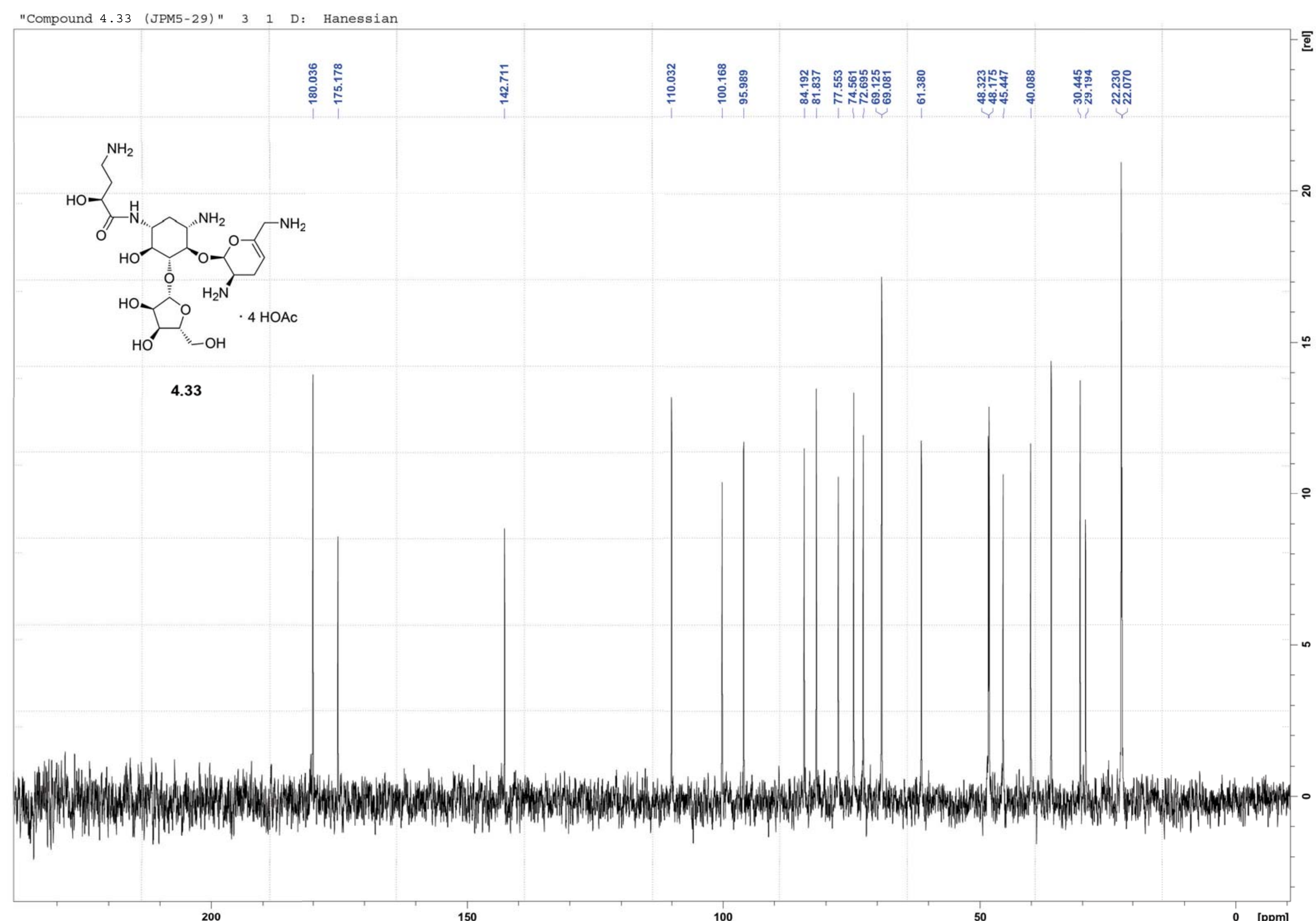
NH2, HO, H, N, NH2, O, NH2, O, HO, O, O, H2N, HO, O, · 4 HOAc, HO, OH

4.33

Integrals: 0.890, 1.778, 1.046, 0.934, 1.008, 1.152, 3.710, 1.069, 2.572, 1.094, 1.024, 1.983, 1.004, 1.030, 1.945, 1.211, 7.238, 1.049

[rel]: 0.15, 0.10, 0.05, 0.00

7, 6, 5, 4, 3, 2, 1 [ppm]

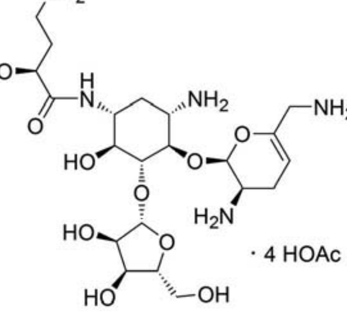


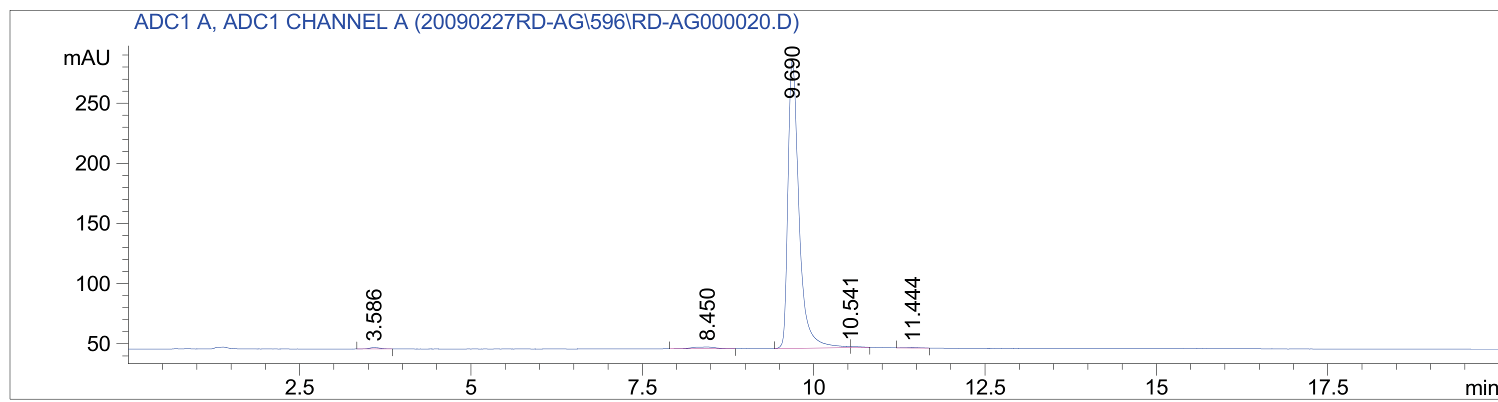
"Compound 4.33 (JPM5-29)" 3 1 D: Hanessian
180.036
175.178
142.711
110.032
100.168
95.989
84.192
81.837
77.553
74.561
72.695
69.125
69.081
61.380
48.323
48.175
45.447
40.088
30.445
29.194
22.230
22.070
[rel]
20
15
10
5
0
200
150
100
50
0
[ppm]
NH2
HO
H
N
O
NH2
NH2
O
HO
O
O
H2N
HO
O
· 4 HOAc
HO
OH
4.33

Data File C:\CHEM32\1\DATA\20090227RD-AG\596\RD-AG000020.D
Sample Name: JPM-5-29

===

Acq. Operator	: rd	Seq. Line :	20
Acq. Instrument	: Chemstation 8	Location :	Vial 26
Injection Date	: 2/27/2009 7:42:50 PM	Inj :	1
		Inj Volume :	2 µl
Acq. Method	: C:\Chem32\1\DATA\20090227RD-AG\596\05-25-95B_20M.M		
Last changed	: 2/23/2009 10:45:33 AM by rd		
Analysis Method	: C:\CHEM32\1\METHODS\INT_30MIN.M		
Last changed	: 2/27/2009 4:35:05 PM by rd (modified after loading)		
Method Info	: Integration method.		
Sample Info	: 20x dilution		





===
Area Percent Report
===

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: ADC1 A, ADC1 CHANNEL A

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.586	MM	0.1975	12.38992	1.04543	0.4474
2	8.450	MM	0.3210	33.89148	1.40172	1.2237
3	9.690	MF	0.1877	2705.54370	240.25909	97.6908
4	10.541	FM	0.1758	9.77732	9.27024e-1	0.3530
5	11.444	MM	0.2507	7.89570	5.24878e-1	0.2851

Totals : 2769.49811 244.15814

===
*** End of Report ***

"Compound 4.37 (JPM5-59)" 1 1 D: Hanessian

"Compound 4.37 (JPM5-59)" 2 1 D: Hanessian

179.315
175.536
143.324
110.225
100.429
96.740
95.363
84.435
81.206
78.132
75.878
73.161
72.880
70.041
69.435
67.581
67.191
61.143
50.743
48.662
48.525
45.740
40.450
40.346
36.483
30.787
29.477
23.018
21.950

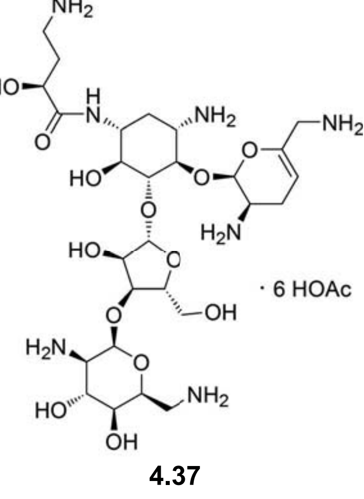
NH_2, HO, H, N, O, NH_2, NH_2, HO, O, O, H_2N, O, HO, O, · 6 HOAc, O, OH, H_2N, O, NH_2, HO, OH

4.37

[rel]
15
10
5
0

200 150 100 50 0 [ppm]

Data File C:\CHEM32\1\DATA\20090306RD-AG\603\RD-AG000007.D
Sample Name: JPM-5-59



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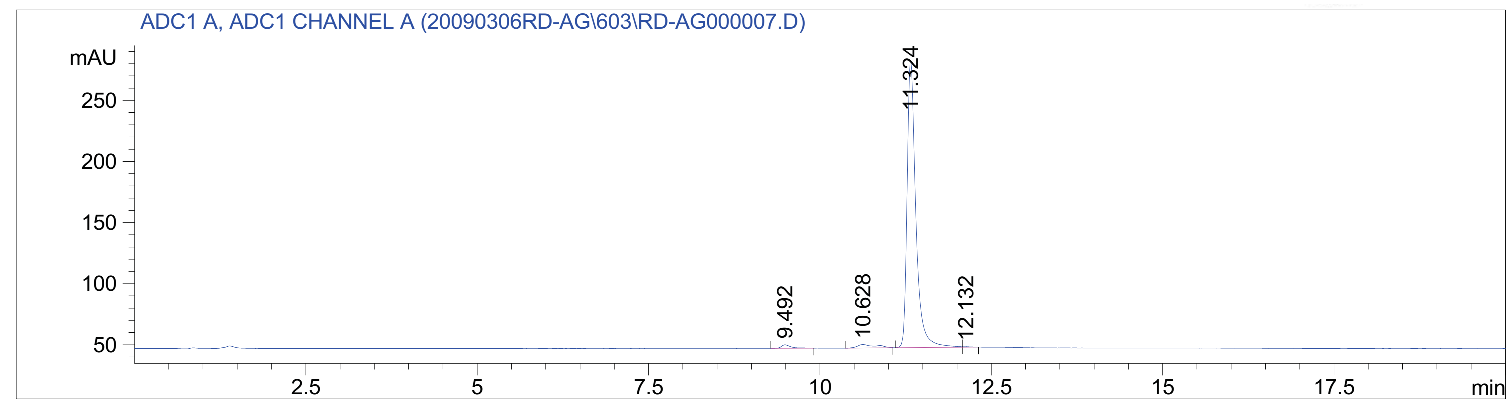

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Area Percent Report

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Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs
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Signal 1: ADC1 A, ADC1 CHANNEL A

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
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2	10.628	MM	0.3154	55.44510	2.92964	2.5553
3	11.324	MF	0.1457	2082.21240	238.20245	95.9642
4	12.132	FM	0.1330	3.64138	4.56144e-1	0.1678

Totals : 2169.78150 244.46314

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*** End of Report ***

Chapter 5

Toward Neomycin Analogs with Reduced Nephrotoxic Potential – Asymmetric Synthesis of β-Substituted HABA Groups

5.1 - Amines and Toxicity

In the context of the potential side effects of aminoglycosides (Chapter 3), the review of clinical and animal studies indicates that no structure-activity relationship is discernible for ototoxicity.[1,2,3] Compounds with any degree of kinship, at different doses, may unexpectedly insult different sections of the inner ear.[1,3,4] Therefore, this facet of toxicity remains empiric, and the first histological assessments on animal models are usually performed only late in preclinical testing.[5,6]

On the other hand, neprotoxicity has been largely correlated with the number of amino groups, and loosely with the extent of deoxygenation.[1,3,7] A large difference in acceptable dosage is apparent between the tetraamino antibiotics (kanamycin A, amikacin, ribostamycin, butirosin, etc) and the pentaamino congeners (kanamycin B, arbekacin, gentamicin, paromomycin, etc).[3] The differences in dosage for the pentaamino to hexaamino and deoxygenated antibiotics usually appear less pronounced.[3] Inconveniently, trends in nephrotoxicity tend to follow the requirements for good antibiotic activity. Therefore, the actual therapeutic index, as determined by the range of effective dosage with minimum side effects, and also the evasion of modifying enzymes in clinically-relevant bacteria has dictated the circumstantial usefulness and preclinical advancement of each antibiotic.[1,3,7,8,9]

5.2 - The pK_a Modulation Hypothesis and Literature Precedence

An unmet long-term goal in the aminoglycoside field is to systematically improve the therapeutic indices of known antibiotics. A hypothesis, almost as ancient as the field, suggests that the tuning of their pK_a parameters of the ammonium group salts could provide a higher target dissociation rate, more effective clearance and overall less nephrotoxic analogs.[1,3,7,10]

Extensive semisynthetic efforts have been carried out to modify ammonium group acidity (Figure 5.1).[1,2] and can be generally classified into three approaches. Firstly, antibiotic candidates with the least number of amines, such as those with amines replaced by alcohols, have been sought from wild-type and mutant antibiotic producers, and their development prioritized including those present as minor components in

fermentation mixtures. Examples include the important semisynthetic analogs amikacin (made from kanamycin A, not B nor C)[11] and later isepamicin (originating from the minor fermentation component gentamicin B).[3,12] Secondly, substitution of the electronegative groups of the sugars has been pursued by deoxofluorination at numerous positions of members of the kanamycin[13,14,15] and gentamicin/sisomicin[16] subfamilies (Figure 5.1). Likewise, *mutasynthetic* efforts have sought to include extra hydroxyls and fluorine (Figure 5.1).[5,17] Finally, electronegative atoms have been placed on appendages, such as N1 of propikacin,[3] 4'''-fluoro amikacin and arbekacin,[18] the shortened N1-group of isepamicin[12] as well as the N6' chain of ACHN-490 (Figure 5.1).[19] Desirable modifications involve positions concomitantly lowering toxicity and protecting against modifying enzymes, such as C3', N1 or N6' substitution.

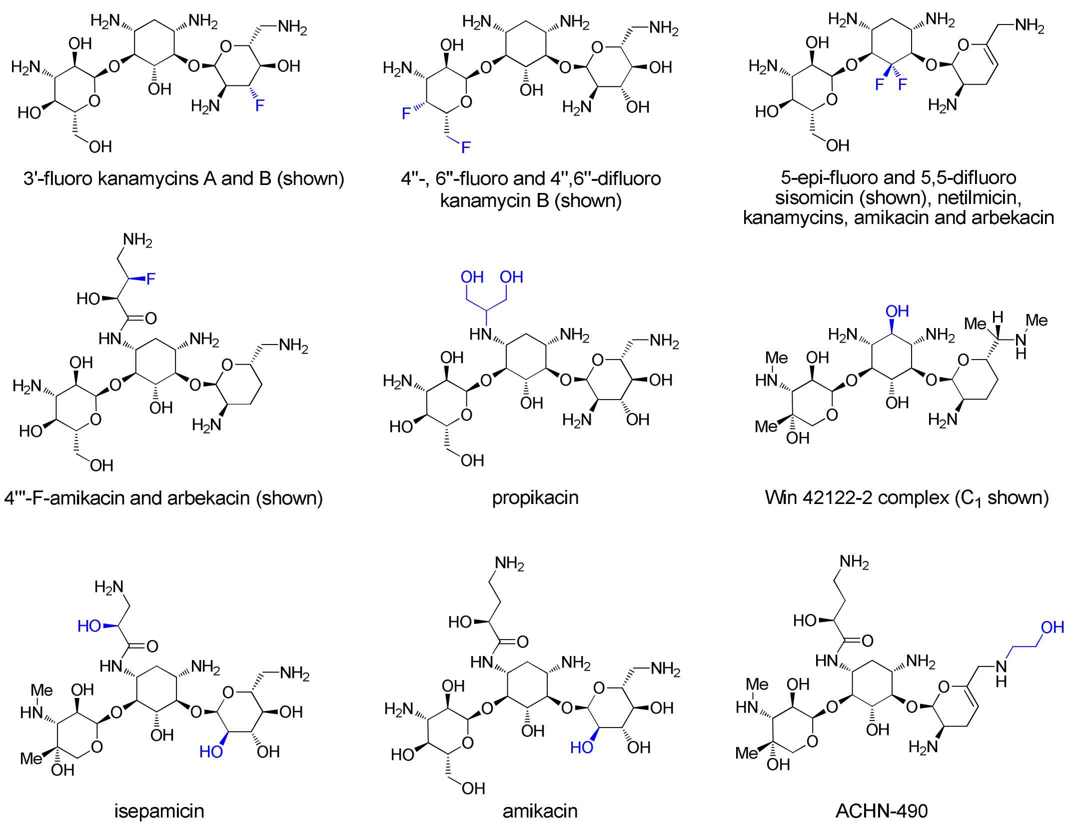

Figure 5.1. Examples of aminoglycoside modifications which modulate the pK_a of neighboring amines. Structures from references 13, 14, 15/16, 3, 18, 4/17, 12, 11 and 19, respectively.

The inosamycins are a mixture of natural aminoglycosides of the neomycin family (Figure 5.2 A),[20] that have received little attention due to their late discovery, when APH(3') and ANT(4') enzymes were already widespread. They feature a unique 2-deoxystreptamine structure lacking an amine at N1. Notably, the 6'-amino analog inosamycin A (Figure 5.2 A) has displayed good antibiotic properties, comparable to neomycin B, in particular against three *P. aeruginosae* and one *S. marcescens* strains displaying resistance to paromomycin.[20a]

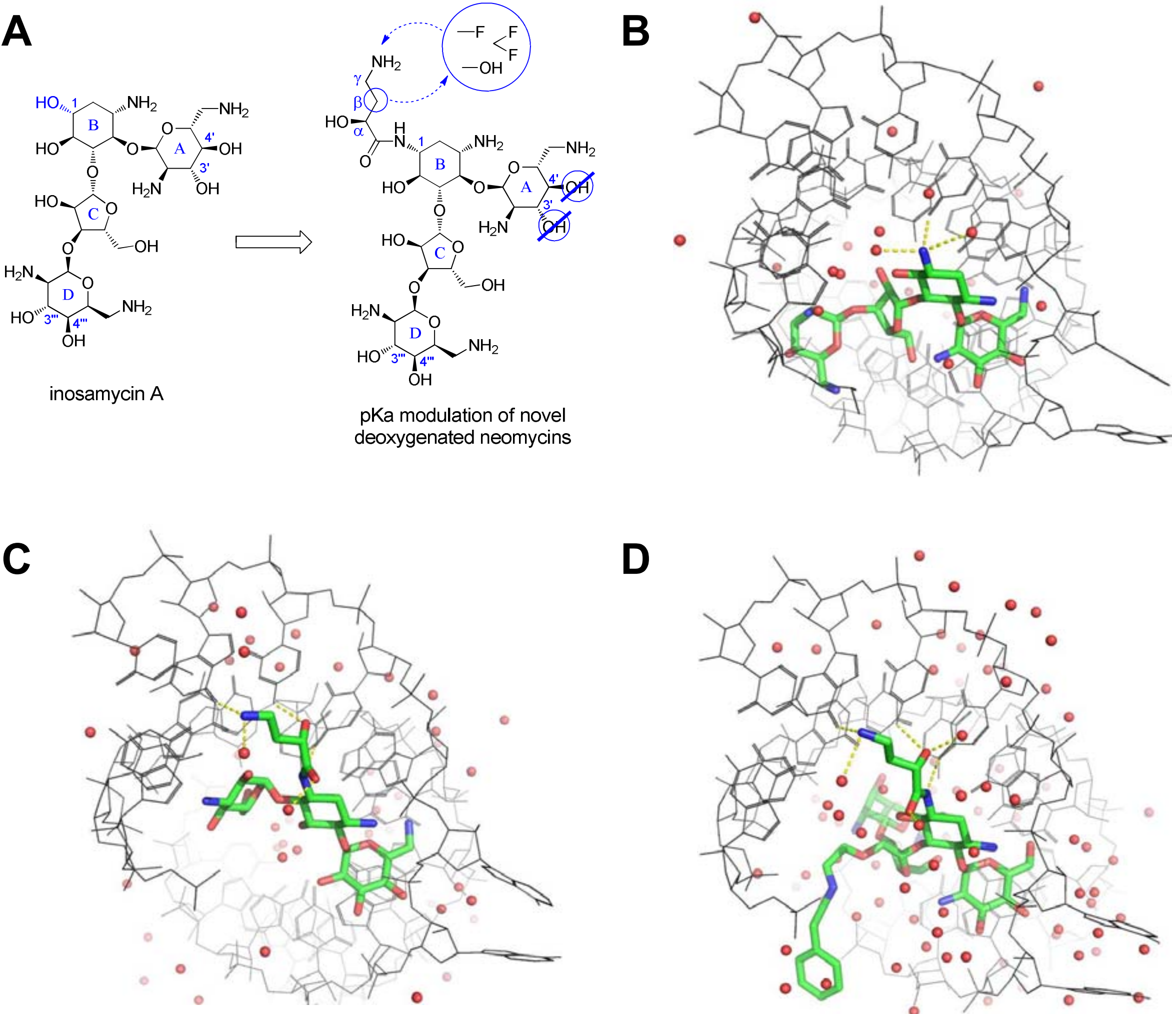

Figure 5.2. (A) Structure of inosamycin A and proposal of γ-amine pK_a modulation of the N1-HABA groups of next-generation 3',4'-deoxygenated neomycins. Panels B – D: top view of A-site model co-crystal structures with (B) neomycin B,[21] (C) amikacin[22] and (D) analog PM-II-162[23] focusing on the interactions of the N1 region (yellow dashed lines). PDB entries 2ET4, 2G5Q and 2PWT. Waters are shown as red spheres and RNA in gray. Key H-bonding contacts of the N1-region are shown as yellow dashed lines. Rendered in PyMol.[24]

The intriguing properties of the less regarded members of the neomycin family prompted inspection of the available co-crystal X-ray structures of aminoglycosides in oligonucleotide A-site helix models, which corroborated that neither the N1 nor γ-HABA amines are involved in salt bridges with nearby phosphate backbones (Figure 5.2).[21,22,23] Instead, interactions in this region are dominated by hydrogen bonding with a triad of bases,[25] N1 to O4-U1495, β-OH to O4-C1496 and γ-N to O6-G1497, as well as several nearby water molecules (Figure 5.2 B to D).[21,22,23] Taking into consideration these facts together with the high activity of antibiotic inosamycin A, we hypothesized that the N1-HABA section could be an optimal region for reduction of positive charge potential of advanced antibiotics of the neomycin family.

5.3 - Asymmetric Synthesis of β-Substituted HABA Fragments

This study required therefore a range of enantiopure, isosteric and minimally-modified β-substituted-HABA groups, for producing a range of γ-amine pK_a values. No general methods for the synthesis of such substrates were available, in particular the desirable β,β-difluoro HABA analog was not previously reported, even in racemic form. The literature precedent of a β-fluoro-HABA synthesis, which was applied to the aforementioned 4'''-fluoro amikacin and arbekacin analogs (Figure 5.1), was judged inadequate,[18] because of numerous protecting group manipulations and degradation steps from glucofuranose-1,2-isopropylidene, as well as lack of effective points for synthetic divergence (Figure 5.3 A). Notably, this synthesis did not allow β,β-difluorination,[26] a modification desired by the authors,[18,27] for reaching a γ-amine pK_a within the order of magnitude of physiological pH.[26] Hence, no success was attained in attenuating the toxic effects of these analogs (Figure 5.4 A to C). Several other studies have explored the steric and structural requirements of the N1-groups in butirosin,[28] kanamycin A[29] and neamine.[30] These efforts have consistently resulted in analogs displaying invariably weaker overall antibiotic activity when challenged against a sizeable library of isolates,[28-30] suggesting that the structure of the L-HABA N1-substituent may have undergone extensive refinement throughout the eons of natural selection challenges, accounting for its relatively intricate structure and biosynthetic origin.[31]

A Takahashi *et al*, *Carbohydr. Res.* **1993**, *249* (1), 57-76

primary ammonium pKa approx. 8.8 (applied to amikacin and arbekacin)

B Takahashi *et al*, *Carbohydr. Res.* **1998,** *306* (3), 349-360

primary ammonium pKa approx. 8.8 (applied to amikacin)

C Hoshi *et al*, *J. Antibiot. (Tokyo)* **1990**, *43* (7), 858-72

primary ammonium pKa range 8.8 - 10.7 (applied to amikacin)

n = 0, 1 and 2, *S* and *R*

Figure 5.3. Previous synthetic efforts to modulate the pK_a of N1 side chains of differing structures. Adapted from references 18, 27 and 32, respectively.

Review of current fluorination strategies,[33] including deoxofluorination and electropositive fluorine sources,[34] revealed that this small multi-substituted chain was a deceivingly difficult target. If unsuitably conceived, the synthetic strategy would lead to intermediates crowded by potential interfering nucleophilic groups and an adjacent acidic position (Figure 5.4). Hence, a new synthetic route for β-substituted L-HABA chains was conceived, involving a terminal olefin as a latent carboxylic acid function, which then traced back through uncharted azide and fluoride nucleophilic functionalizations of a known epoxide generated from desymmetrization of divinyl-carbinol by Sharpless epoxidation (Figure 5.4).[35,36] Evidently, azides, olefins and benzyl groups are fluorination-compatible but not completely devoid of issues when faced with an adjacent leaving group of the efficacy of dialkylaminosulfinyl difluoride. Nevertheless, the synthetic route was sufficiently expedient to be promptly tested (Scheme 5.1).[37]

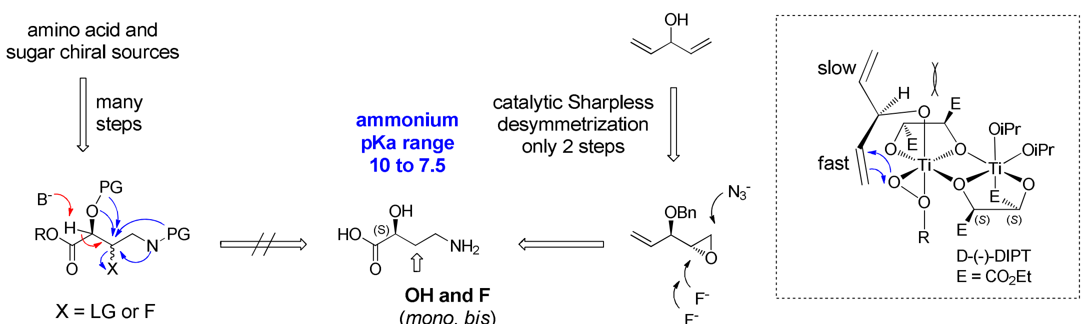

Figure 5.4. Issues associated with fluorine substitution of β-leaving groups in unsuitably protected L-HABA fragments, compared to the proposed divergent route from Sharpless desymmetrization of divinylcarbinol. The model of asymmetric epoxidation catalysis was adapted from references 38 and 39.

Following literature conditions for modified Sharpless desymmetrization of divinyl-carbinol followed by protection, benzylated epoxide **5.1** was obtained in good yield and an enantiopurity of >99% judged by chiral GC (Scheme 5.1).[35,36,40] The intermediate alcohol was best not evaporated to purity, because it produced azeotropes with solvents of low boiling point. Subsequently, treatment of **5.1** with a solution of ammonium azide in MeOH at room temperature over 4 days afforded smoothly **5.2** as a single diastereomer. Azide **5.2** underwent slow decomposition to a bright red and complex mixture, when the reaction was heated or when it was stored with the traces of acid, such as residual silica from chromatography. This was rationalized by the potential for cycloaddition by the proximal azide and olefin pair to form a triazoline,[41] which are known to lose N_2 to from aziridines and convert to imines.[42] Azido alcohol intermediate **5.2** could be stored for long-term in the freezer and was not explosive.

Direct deoxofluorination of the resulting alcohol **5.2** was found to provide only low yields of **5.3** with either DAST or Deoxofluor. However, the crude ^{1}H-NMR spectrum and chromatographic profiles (I_2, $KMnO_4$ and CAM development) of the reactions were usually clean, suggesting material loss due to volatility. Variations of the reaction protocol using various solvents and conditions produced often mixtures of elimination or chlorination[43] products in low yield rather than fluorination. Eventually, a low temperature procedure was established, in either dry DCM or benzene/pyridine, which reliably furnished **5.3** in 17% yield and good purity (Scheme 5.1). Thereafter, standard ozonolysis followed by Pinnick oxidation[39] were carried out uneventfully, affording the *syn* γ-azido-β-fluoro-HABA acid (*R*,*R*)-**5.4**, suitably protected for amide

coupling to the aminoglycoside and global deprotection (Scheme 5.1). The absolute stereochemistry of (*R*,*R*)-**5.4** and (*S*,*S*)-**5.4** was conveniently verified by X-ray crystallography (Figure 5.5). The overall yield was comparable with other sequences previously reported (Figure 5.3A).[18]

divinylcarbinol → 1) (-)-DIPT, Ti(*i*OPr)$_4$, 80% PhCMe$_2$OOH, MS 4Å, DCM, -35 °C; 2) NaH, BnBr, TBAI, THF, 0 °C to RT (47%) → (*R*,*S*)-**5.1** → NaN$_3$, NH$_4$Cl, MeOH/H$_2$O (66%) → (*R*,*S*)-**5.2**

(*R*,*S*)-**5.2** → DAST, PhH/py, -78 °C to RT (17%) → (*R*,*R*)-**5.3** → 1) O$_3$, DCM; Me$_2$S; 2) Pinnick oxidation (87%) → (*R*,*R*)-**5.4**

(*R*,*S*)-**5.2** → 1) (COCl)$_2$, DMSO, DCM -78 °C; then *N*-Me-morpholine; 2) DAST, DCM, -30 °C to RT (23%) → (*R*)-**5.5** → 1) O$_3$, DCM; Me$_2$S; 2) Pinnick oxidation (67%) → (*R*)-**5.6**

(*R*,*S*)-**5.2** → NaH, BnBr, TBAI, THF (68%) → (*R*,*S*)-**5.7** → 1) O$_3$, DCM; Me$_2$S; 2) Pinnick oxidation (75%) → (*S*,*S*)-**5.8**

products of an identical sequence with ligand (+)-DIPT: (*S*,*S*)-**5.4**, (*S*)-**5.6**, (*R*,*R*)-**5.8**

Scheme 5.1. Asymmetric synthesis of β-substituted HABA chains of (2*R*)- and (2*S*)-C$_\alpha$-stereochemistry.

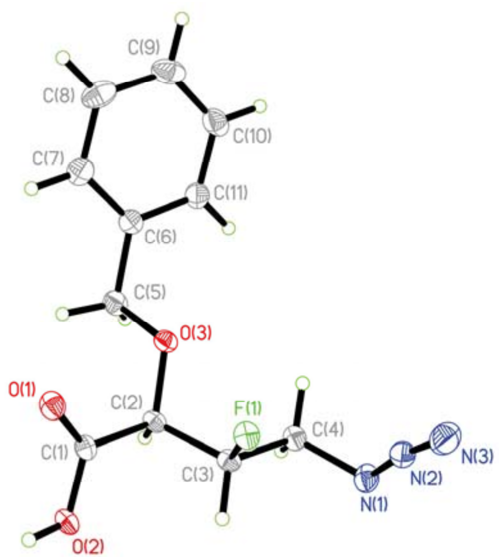

Figure 5.5. ORTEP drawing of (*R*,*R*)-**5.4** with thermal ellipsoids drawn at 30% probability.

Oxidation of **5.2** for bisfluorination was the next obstacle. A range of conditions reportedly for the production of homo-allylic ketones or epimerizable substrates failed, including mild TEMPO, Cornforth and Dess-Martin oxidations.[44,45] The typical variety of Swern conditions invariably led to isomerization of the terminal olefin into conjugation.[39] This problem was eventually overcome using a modification of the Swern oxidation, involving low-temperature deprotonation of the intermediate alkylsulfonium ylide with N-methylmorpholine,[46] followed by careful quench and workup with neutral phosphate buffer. The resulting sensitive ketone was then directly exposed to DAST in DCM, affording bisfluoride product **5.5** in 24% yield. Preparative TLC was required for complete removal of a minor close-eluting impurity (~7% by H-NMR). Thereafter, ozonolysis and Pinnick oxidation provided successfully the coveted β,β-difluoro-HABA intermediate (*R*)-**5.6** (Scheme 5.1).

Lastly, the more easily accessible *anti* α,β-hydroxy-HABA acid **5.8**, which was once previously used in its racemic form,[29] was produced as the weakest γ-amine pK_a modulator. This required protection of **5.2** to avoid neighbouring group interference during the ozonolysis step, otherwise unexceptionally furnishing (*S*,*S*)-**5.8** after Pinnick oxidation of the aldehyde intermediate (Scheme 5.1).

Both enantiomeric series of acids **5.4**, **5.6** and **5.8** were produced varying the Sharpless asymmetric epoxidation ligand (see Figure 5.3). As shown in Scheme 5.1, one series of β-substituted HABA analogs shares a matching relationship with the natural C_α-stereochemistry of L-HABA, whereas the enantiomeric series does not (see Experimental Section).

The 20-25% yields in fluorine substitutions are similar to those reported for unoptimized deoxofluorinations.[33] These reactions were not optimized herein, because efforts were focused on the goals of the goals of the medicinal chemistry applications. Although individual routes are conceivable, the divergent synthesis of acids (*R*,*R*)-**5.4**, (*R*)-**5.6** and (*S*,*S*)-**5.8** from a common intermediate has been successfully reproduced in an industrial setting with identical yields and purities on multi-gram scales (see Experimental Section for complete NMR spectral data, pages 212-227).[47]

5.4 - Synthesis of β-substituted HABA Neomycin Antibiotics

Enantiomeric β-substituted HABAs were added to the advanced intermediate 3',4',3''',4'''-tetradeoxygenated neomycin **5.9**, first described by colleagues in our group.[48,49] This intermediate has been produced in approx. 25% overall yield from neomycin, using a modification of the Tipson-Cohen reaction and alkaline N1-deprotection *via* the N1,O6-oxazolidinone (as discussed in Chapter 4).[49] This tetradeoxy neomycin scaffold was previously scaled-up for antibacterial testing of a variety of N1-groups and for rat nephrotoxicology studies of N1-HABA compound **5.10** (Scheme 5.2).[49] Therefore, **5.9** was ideal for exploration of the structure-activity relationships of the novel β-substituted HABA chains.

The sensitive β-substituted substrates were coupled with **5.9** under mild neutral conditions as previously reported.[18] Global hydrogenation of the aminoglycoside and HABA protecting groups produced compounds **5.11** to **5.13** of C_{α}-stereochemistry matching L-HABA and the unmatched diastereomers **5.14** to **5.16**, respectively (Scheme 5.2). Assessment by NMR spectroscopy (^{1}H, ^{13}C and ^{19}F) and HPLC with Chemiluminescent Nitrogen Detector (CLND) indicated exceptional purities in excess of 90% (see Experimental Section for NMR spectra and HPLC reports, pages 228-253).

Scheme 5.2. Synthesis of tetradeoxy neomycins with N1-HABAs of a range of γ-N pK_a values.

The pK_a of the aminoglycoside analogs could not be derived by titrations using shifts in the ^{13}C-NMR[18] or natural-abundance ^{15}N-NMR spectra,[50] due to the obscuring prevalence of two slowly equilibrating conformers upon neutralization, a typical quality of the neomycin/paromomycin family. The pK_a of similar primary amines has been well characterized in the literature, which suggests γ-amine pK_a values of 8.8-9.0 for **5.11**,[26,18] 7.3-7.5 for **5.12**,[26] 9.3-9.5 for **5.13**[26] and 10.0-10.7 for **5.10**,[26,18] assuming that charge repulsion with the relatively distant sugar amines produces minor alterations.

5.5 - Analysis of Antibacterial Activity

As discussed in the previous chapter, 3',4'-deoxygenated-N1-HABA neomycins, such as the 'Frankenmycin' analog **4.34** are powerful antibiotics with broad-spectrum activity, and the same is true for compound **5.10** (Table 5.1). It has been shown in our group that further deoxygenations on ring D do not significantly perturb the antibacterial activity, yet simplify importantly the semisynthetic functionalization of the N1 position (Chapter 4).

At first glance, all six novel analogs **5.11** to **5.16** were good antibiotics against the susceptible and quality control strains (Table 5.1). Larger MIC differences between the diastereomeric β-substituted N1-HABA tetradeoxy neomycins become apparent only in resistant strains. It is noteworthy that the weakest antibiotic, (*S*)-β,β-difluoro-N1-HABA analog **5.15**, remains similar to N1-HABA paromomycin (H-Par), a 5-amine aminoglycoside. Gratifyingly, in the series of natural C_α-stereochemistry series, the lowest pK_a analog (*R*)-β,β-difluoro-HABA **5.12** has suffered only slight loss of antibiotic potency across the vast range of resistant isolates expressing modifying enzymes, comparing well with congeners **5.10**, **5.11** or **5.13**. In fact, all three are excellent antibiotics evading the actions of the problematic APH(3')s, ANT(4')s, AAC(3)s and several AAC(6')s (Chapter 3 and 4). The effluxing strains are more difficult to contest, but remained unable to impart complete resistance.

These outstanding results validate the importance of devising isosteric modifications when drastic reductions of charge potential are introduced on aminoglycosides. The region of the scaffold which is modified is also crucial, since previous experience by our group and others has demonstrated that 6'''-substitutions which remove the charge at this position lead to a dramatic loss of potency.[48,51,52]

Table 5.1. Antibacterial assesment of N1-β-substituted-HABA tetradeoxy neomycins and controls. Minimum inhibitory concentration (μg/mL).

		MIC (μg/mL)											
		controls					'natural' C_{α}-stereochem.				opposite C_{α}-stereochem.		
Bacterium	**Description/Phenotype**	Amk	Gent	NeoB	Par	H-Par	**5.10** (H)	**5.11** (F)	**5.12** (diF)	**5.13** (OH)	**5.14** (F)	**5.15** (diF)	**5.16** (OH)
Wild Type Strains													
S. aureus	ATCC 29213 (QC)	2	0.5	0.5	2	2	0.125	0.5	0.5	0.5	0.5	1	0.5
E. coli	ATCC 25922 (QC)	2	0.5	2	4	2	0.5	0.5	1	1	1	4	2
K. pnuemoniae	ATCC 10031	0.5	0.25	0.5	2	1	0.25	0.5	0.5	0.5	0.5	2	1
P. aeruginosa	ATCC 27853 (QC)	2	0.5	32	>64	8	0.5	0.5	1	0.5	1	4	1
P. aeruginosa	Wild type pump	2	2	8	>64	4	0.5	0.5	1	0.5	1	4	2
A. baumannii	Susceptible	2	2	1	4	2	0.5	0.5	0.5	0.5	0.5	2	1
Efflux Strains													
P. aeruginosa	MexXY upregulated	4	2	16	>64	8	1	1	2	1	2	16	2
A . baumannii	ATCC 19606	16	16	4	16	16	4	4	16	8	8	64	8
P. aeruginosa	Efflux system	16	4	32	>64	32	2	4	8	4	8	32	8
P. aeruginosa	Efflux system	16	8	64	>64	64	4	8	32	8	8	>64	16
Modifying Enzymes and Methytransferase													
Providencia stuartii	AAC(2')-I	4	64	32	>64	>64	16	16	64	32	64	>64	64
E. coli	APH(3')-Ib	0.5	0.25	64	>64	2	0.25	0.25	0.25	0.5	1	4	2
S. aureus	APH(3')-III	8	0.5	>64	>64	32	1	1	1	1	1	4	2
A. baumannii	AAC(3)-I + APH(3')-VI + ANT(2')-I	>64	>64	>64	>64	64	1	1	2	2	2	8	2
Acinetobacter spp.	APH(3')-VI	>64	0.5	16	>64	32	0.5	0.5	2	0.5	1	4	1
S. aureus	ANT(4')-I	64	0.5	>64	>64	>64	1	1	2	1	1	4	2
P. aeruginosa	ANT(4')-II	32	2	8	>64	4	1	1	2	1	2	16	2
S. marcescens	AAC(6')-I	32	4	8	4	8	16	4	32	32	64	>64	>64
P. aeruginosa	AAC(6')-I	>64	64	>64	>64	16	>64	>64	>64	>64	>64	>64	>64
P. aeruginosa	AAC(6')-II	4	32	8	>64	8	2	1	2	1	2	16	2
E. coli	ANT(2")-I	4	64	2	4	8	2	2	2	2	2	8	8
E. cloacae	ANT(2")-I + AAC(6') + APH(3')-I	64	32	>64	>64	8	2	2	2	2	4	32	16
S. aureus	AAC(6')/APH(2")	64	>64	>64	>64	32	2	2	4	2	8	16	8
P. aeruginosa	AAC(3)-I	4	32	8	>64	8	2	2	4	2	4	32	4
E. coli	AAC(3)-IV	2	16	2	8	4	1	1	1	1	4	8	2
E. coli	ArmA	>64	>64	1	4	4	0.5	0.5	1	1	2	4	2

5.6 - Analysis of HK2 Cell Line Nephrotoxicity Models

Mammalian kidney cell lines are well-known models for the study of mode-of-action of aminoglycoside antibiotics (Chapter 3).[53] In particular, Achaogen Inc. has adapted a proprietary luciferase-coupled apoptosis assay of caspase-3/7 activation for luminescence measurements on microtiter plates,[54,55] thus requiring only milligram amount of aminoglycosides (procedure detailed in reference 54). The Human Kidney-2 (HK2) cell line has been preferred over the porcine LLC-PK1 line,[53] because it showed a somewhat better correlation between apoptosis and blood-urea-nitrogen measurements in rat models for several antibiotics.[54,47] These assays cannot be claimed to predict preclinical toxicity outcomes; however, they assess the relative impact of structurally similar antibiotics on pertinent kidney cells. In this study, the HK2 cell line was challenged with the novel β-substituted-HABAs on the known tetradeoxy neomycin scaffold,[49] using gentamicin and amikacin as controls.[54]

Measurements provided by Achaogen Inc. were carried out in duplicate and are grouped for the diastereomeric β-substituted N1-HABA series, each including controls and analog **5.10** (Figure 5.6). Caspase activation was measured at 31 h of incubation, at aminoglycoside concentrations ranging from 4 to 250 μg/mL (Figure 5.6).[54] All analogs displayed sigmoidal dose-response curves, which were analyzed by area under the curve (Figure 5.7) and their half-maximal effective concentration (EC_{50}), which was derived from non-linear curve fitting (Table 5.2).[54,56] The overall cellular viability, including apoptotic and necrotic pathways, was assessed in parallel measurements (Figure 5.8).[57]

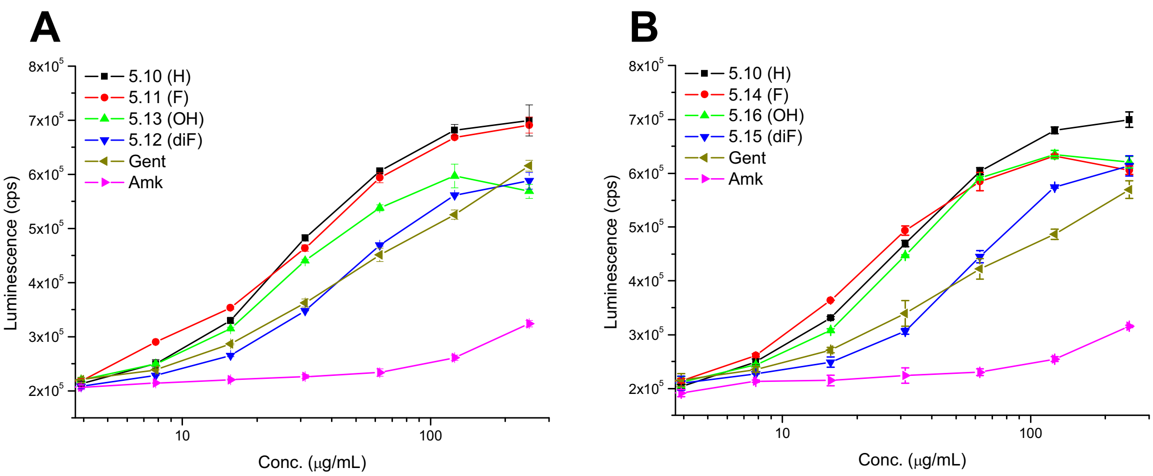

Figure 5.6. Luminescence caspase-3/7 assay at 31 h incubation for β-substituted HABA analogs (A) **5.11** – **5.13** and (B) **5.14** – **5.16**, each with analog **5.10**, gentamicin and amikacin controls.

Table 5.2. EC_{50} values from nonlinear fit analysis of Figure 5.7 for assays of β-substituted N1-HABA analogs (A) 5.11 – 5.13, (B) 5.14 – 5.16 and controls. See Experimental Section for OriginPro 8 nonlinear fitting reports, pages 254-261.[56]

Assay A		
Compound	EC_{50} (μg/mL)	R^2
5.10	28.1	0.999
5.11	28.3	0.989
5.12	**46.9**	0.999
5.13	26.1	0.998
Gent	49.0	0.994
Amk	>250	-

Assay B		
Compound	EC_{50} (μg/mL)	R^2
5.10	29.1	0.999
5.14	20.4	0.996
5.15	**58.9**	0.999
5.16	30.2	0.998
Gent	75.0	0.997
Amk	>250	-

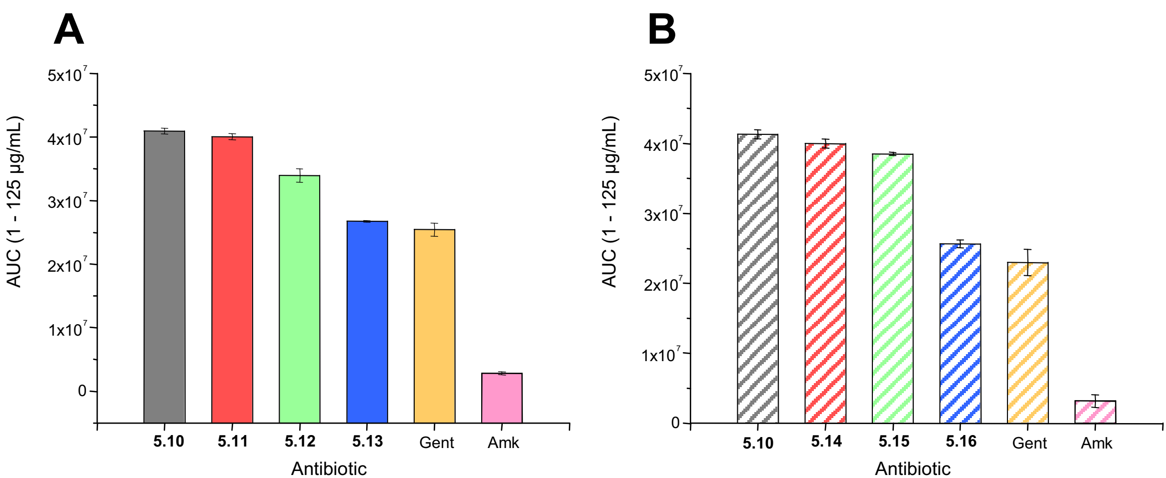

Figure 5.7. Area under the curve analysis of Figure 5.6, for β-substituted N1-HABA analogs (A) **5.11** – **5.13** and (B) **5.14** – **5.16**, each with analog **5.10**, gentamicin and amikacin controls.

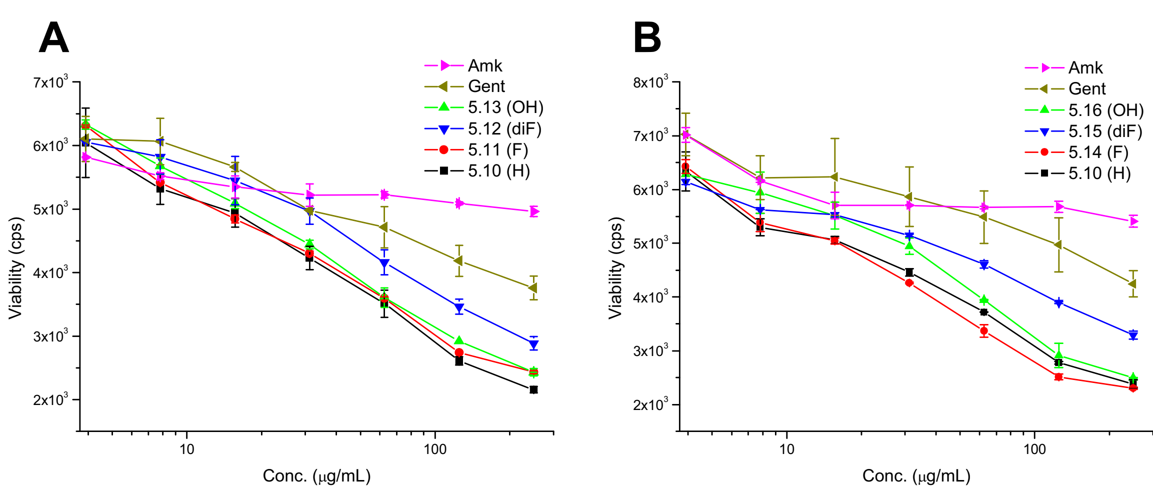

Figure 5.8. Luminescence viability assay at 31 h incubation with β-substituted HABA analogs (A) **5.11** – **5.13** and (B) **5.14** – **5.16**, each with analog **5.10**, gentamicin and amikacin controls.

At first glance, both diastereomeric series of β-substituted N1-HABA analogs of tetradeoxy neomycin display similar cellular toxicity trends in the respective assays, as expected since the mechanism of toxicity is unrelated to interference with the mitochondrial or eukaryote ribosomes (Chapter 3). In both series, the β,β-difluoro substituted analogs **5.12** and **5.15** clearly stand out from their congeners and the control unsubstituted N1-HABA analog **5.10**. In comparison, the apoptotic responses to β,β-difluoro analogs were overall lower (AUC) and elicited with 1.5- to 2-fold higher EC_{50} than for **5.10**, as well as the *mono*-substituted congeners **5.11** and **5.14** (Figures 5.6, 5.7 and Table 5.2). Likewise, the overall loss of viability of the cells, encompassing apoptotic and minor necrotic mechanisms, stands a cut above for the β,β-difluorinated analogs relative to their congeners (Figure 5.8).

Taken together, these *in vitro* measurements corroborate that pK_a modulation can indeed significantly affect the interaction of aminoglycosides with mammalian cells, provided that the values attained are within the same order of magnitude range of physiological pH. Future *in vivo* evaluation of **5.12** or similar analogs of the neomycin framework containing N1-(*R*)-β,β-difluoro-HABA will be required to fully assess the magnitude of these developments.

5.7 - Conclusions

This chapter presented the conception and asymmetric synthesis of *mono* and *bis* β-substituted HABA groups for γ-amine pK_a modulation of neomycin family antibiotics. Antimicrobial testing and preliminary *in vitro* toxicology structure-activity relationships of these N1-analogs have uncovered promising results for the application of the novel isosteric (*R*)-β,β-difluoro-HABA side chain (*R*)-**5.6**, setting the stage for *in vivo* preclinical assessment of next-generation neomycin antibiotics with improved therapeutic properties.

The bulk of this work has been published in patent WO 2010/030704.[49]

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Chapter 5 - Experimental Section

General Procedures

All reactions were carried out under an inert atmosphere of argon with dry solvents, using anhydrous conditions unless otherwise stated. Dry dichloromethane (DCM) and tetrahydrofuran (THF) were obtained from a solvent delivery system with activated alumina columns. Methanol (MeOH) was distilled from CaH_2 under argon. Reagents were purchased at the highest commercial quality and used without further purification. Flash chromatography was performed with silica gel from SilicaFlash P60, particle size 40-63 μm, 230-400 mesh and distilled hexanes, ethyl acetate (EtOAc) or DCM. Free-base deprotected aminoglycosides were purified with homogeneous solvent systems consisting of $CHCl_3$/MeOH/$NH_4OH_{(aq)}$ in ratios ranging from 2:3:0.5 to 2:3:2, freshly prepared with 28% ammonia liquor before use. Yields refer to chromatographically and spectroscopically homogeneous material. Low temperature experiments conducted for longer than 3 h used a Cryocool apparatus with an acetone bath. Reactions were monitored by direct-injection low resolution mass spectrometry (LRMS) and thin layer chromatography (TLC) carried out on 0.25 mm E. Merck silica precoated plates (60F-254), visualized under UV light and developed with acidified ammonium molybdate/cerium sulfate and heat. NMR spectra were recorded on Bruker ARX-400, AV-400, AV-500 or AV-700 instruments and are calibrated using residual undeuterated solvent as an internal reference. The following abbreviations were used to explain multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Low resolution mass spectra were recorded on a Thermo Finnigan Surveyor MSQ and high resolution mass spectra (HRMS) were recorded on an Agilent Technologies LC-MSD TOF mass spectrometer by electrospray ionization in positive mode, and either protonated molecular ions $[M+H]^+$ or sodium adducts $[M+Na]^+$ were used for empirical formula confirmation, unless otherwise stated. IR spectra were recorded on a Perkin-Elmer Spectrum One FT-IR using. Optical rotations were recorded in a 1 dm cell at ambient temperature, on a Perkin-Elmer 343 polarimeter. Analytical HPLC was performed in Achaogen Inc. using mobile phases with 0.1% HFBA, column Sunfire C18, 3x50mm, 2.5 μm, flow of 0.5 mL/min at 40 ºC and Chemiluminiescent Nitrogen Detection (CLND), water/MeOH gradient 30 to 75% in 30 min. Analytical chiral GC was performed on Supelco Beta Dex 120 fused silica capillary column, 30 m x 0.25 μm film thickness, 120 °C isothermal flow.

Compound Index

(2*S*, 3*R*)-1,2-Epoxy-3-benzyloxy-4-pentene ((*R*,*S*)-**5.1**) 189
(2*S*,3*R*)-1-Azido-3-benzyloxy-4-penten-2-ol ((*R*,*S*)-**5.2**) 191
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General procedure for global hydrogenative deprotection for **5.S2** – **5.S7** 198
1-*N*-((2''''*R*,3''''R)-4''''-Azido-2''''-benzyloxy-3''''-fluorobutanoyl)-3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-*per*-*N*-Cbz-neomycin (**5.S2**) 199
1-*N*-((*R*)-4''''-Azido-2''''-benzyloxy-3'''',3''''-difluorobutanoyl)-3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-*per*-*N*-Cbz-neomycin (**5.S3**) 200
1-*N*-((2''''*S*,3''''*S*)-4''''-Azido-2'''',3''''-bisbenzyloxybutanoyl)-3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-*per*-*N*-Cbz-neomycin (**5.S4**) 201
1-*N*-((2''''*S*,3''''*S*)-4''''-Azido-2''''-benzyloxy-3''''-fluorobutanoyl)-3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-*per*-*N*-Cbz-neomycin (**5.S5**) 202
1-*N*-((*S*)-4''''-Azido-2''''-benzyloxy-3'''',3''''-difluorobutanoyl)-3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-*per*-*N*-Cbz-neomycin (**5.S6**) 203
1-*N*-((2''''*R*,3''''*R*)-4''''-Azido-2'''',3''''-bisbenzyloxybutanoyl)-3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-*per*-*N*-Cbz-neomycin (**5.S7**) 204
1-*N*-((2''''*R*,3''''*R*)-4''''-Amino-3''''-fluoro-2''''-hydroxybutanoyl)-3',4',3''',4'''-tetradeoxy-neomycin (**5.11**) 205
1-*N*-((*R*)-4''''-Amino-3'''',3''''-difluoro-2''''-hydroxybutanoyl)-3',4',3''',4'''-tetradeoxy-neomycin (**5.12**) 206

1-*N*-((2''''*S*,3''''*S*)-4''''-Amino-2'''',3''''-bishydroxybutanoyl)-3',4',3''',4'''-tetradeoxy-neomycin (**5.13**) 207
1-*N*-((2''''*S*,3''''*S*)-4''''-Amino-3''''-fluoro-2''''-hydroxybutanoyl)-3',4',3''',4'''-tetradeoxy-neomycin (**5.14**) 208
1-*N*-((*S*)-4''''-Amino-3'''',3''''-difluoro-2''''-hydroxybutanoyl)-3',4',3''',4'''-tetradeoxy-neomycin (**5.15**) 209
1-*N*-((2''''*R*,3''''*R*)-4''''-Amino-2'''',3''''-bishydroxybutanoyl)-3',4',3''',4'''-tetradeoxy-neomycin (**5.16**) 210
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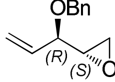


5.1

(2*S*,3*R*)-1,2-Epoxy-3-benzyloxy-4-pentene ((*R*,*S*)-5.1).

Penta-1,4-dienol was desymmetryzed using the modification of Sharpless' procedure[1,2] followed by benzylation.[3] Molecular sieves (4 Å, 4 g) were dried with a Bunsen burner under high vacuum, allowed to cool, and mixed with DCM (100 mL). The suspension of sieves was cooled to -35 to -40 °C with a Cryocooler apparatus, and titanium tetraisopropoxide (1.75 mL, 5.95 mmol) and (*R,R*)-(-)-diisopropyl tartrate (1.65 mL, 7.75 mmol) were added to the cooled mixture, which was stirred for 30 min. Penta-1,4-dienol (5.0 g, 59.4 mmol) followed by excess cumene hydroperoxide (80%, 17.5 mL) were added in small portions to the cooled mixture, and stirring was continued at -35 to -40 °C for 48 h. With rapid stirring, the reaction was quenched by adding sat. Na_2SO_4 (5 mL), immediately followed by Et_2O (50 mL). The mixture was allowed to warm to RT with stirring for 2 h, filtered through Celite™, and washed with copious amounts of Et_2O. The volume of the filtrate and washings was reduced under vacuum *without heating*, resulting in approximately 30 mL of a yellow solution. Excess cumene alcohol and hydroperoxide were

removed by silica gel chromatography (40% Et_2O/hexanes). Evaporation of the collected fractions under vacuum *without heating* yielded an inseparable mixture of (2*S*,3*R*)-1,2-epoxy-4-penten-3-ol ((*R*,*S*)-**5.S1**, R_f = 0.47, 1:1 EtOAc/hexanes) and diisopropyl tartrate (R_f = 0.6), which was used without further purification. Pure epoxide (*R*,*S*)-**5.S1** may be obtained by short path vacuum distillation (Kugelroh apparatus).[1,2]

The mixture of epoxide (*R*,*S*)-**5.S1** and tartrate was diluted in THF (100 mL), cooled to -15 °C in an ice/acetone bath under an argon atmosphere, treated with tetrabutylammonium iodide (2.2 g, 5.96 mmol) and benzyl bromide (8.6 mL, 71.9 mmol), followed by small portions of sodium hydride (60% in mineral oil, 2.65 g, 66.1 mmol), and stirred overnight allowing the mixture to warm to RT. The reaction was quenched with MeOH, filtered through Celite™, washed with copious amounts of Et_2O, and the filtrate was evaporated to an oily residue under vacuum. Purification by column chromatography (5 → 10% Et_2O/hexanes) yielded 5.3 g (47%, 27.9 mmol) of title the compound (*R*,*S*)-**5.1**, as a clear high boiling liquid. The enantiomeric series was derived identically using (*S*,*S*)-(+)-diisopropyl tartrate to produce (*S*,*R*)-**5.1**.

R_f = 0.69 in 1:4 EtOAc/hexanes.
HRMS (ESI) calcd. for $C_{12}H_{14}O_2$, M + H^+ = 191.1067 found 191.1064 (-1.21 ppm).
For (*R*,*S*)-**5.1** $[\alpha]^{22}_D$ -36.7° (*c* 1.52, $CHCl_3$) and for (*S*,*R*)-**5.1** $[\alpha]^{22}_D$ 35.7° (*c* 1.76, $CHCl_3$).
^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.38-7.33 (m, 5H), 5.92-5.78 (m, 1H), 5.41-5.39 (m, 1H), 5.37-5.33 (m, 1H), 4.66 (d, *J* = 11.95 Hz, 1H), 4.49 (d, *J* = 11.96 Hz, 1H), 3.83 (dd, *J* = 7.34, 4.20 Hz, 1H), 3.10 (dt, *J* = 4.07, 4.06, 2.70 Hz, 1H), 2.79 (dd, *J* = 5.21, 4.00 Hz, 1H), 2.70 (dd, *J* = 5.23, 2.64 Hz, 1H).
^{13}C NMR ($CHCl_3$, 100 MHz) δ 138.3, 134.7, 128.6 (2C), 127.9 (2C), 127.8, 119.7, 79.5, 70.8, 53.4, 45.0.

The enantiomeric excess was determined to be >99% for both (*R*,*S*)-**5.1** and (*S*,*R*)-**5.1** by chiral GC: Supelco Beta Dex 120 fused silica capillary column 30 m x 0.25 µm film thickness, 120 °C isothermal flow. For (*R*,*S*)-**5.1** R_t = 47.5 min and (*S*,*R*)-**5.1** R_t = 48.6 min, 2 mg/mL hexanes solutions, 5 µL injections.

OBn
(S)
(R)
N_3
OH

5.2

(2*S*,3*R*)-1-Azido-3-benzyloxy-4-penten-2-ol ((*R*,*S*)-5.2).

A mixture of NaN_3 (3.38 g, 52 mmol) and NH_4Cl (2.78 g, 52 mmmol) in 10 mL of H_2O was heated until the solids had dissolved. The azide solution was added dropwise to a solution of epoxide **2** (3.3 g, 17.4 mmol) in MeOH (100 mL), and stirred for 4 days. The volatiles were evaporated under vacuum with gentle heating, and the aqueous layer was extracted three times with DCM. The combined organic layers were dried over Na_2SO_4, filtered and evaporated to a residue under vacuum. Purification by column chromatography (10 → 20% Et_2O/hexanes) yielded 2.66 g of azide (*R*,*S*)-**5.2** (66%, 11.4 mmol), as a clear high boiling liquid, which was stored at -20 ºC, yet was not explosive to shock nor flame challenges. Differential scanning calorimetry performed at Achaogen Inc. revealed low exotherms consistent with a decomposition pathway involving azide-olefin cycloaddition to the triazoline and further rearrangements.[4,5]

.

R_f = 4.8, 1:4 EtOAc/hexanes.

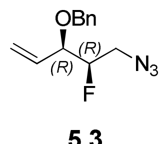
HRMS (ESI) calcd. for $C_{12}H_{15}N_3O_2$, M + Na^+ = 256.1056 found 256.1057 (0.09 ppm).

For (*R*,*S*)-**5.2** $[\alpha]^{22}_D$ -46.3° (*c* 1.50, $CHCl_3$) and for (*S*,*R*)-**5.2** $[\alpha]^{22}_D$ 47.3° (*c* 1.30, $CHCl_3$).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.42-7.28 (m, 5H), 5.91-5.76 (m, 1H), 5.46 (dd, *J* = 17.16, 1.42 Hz, 1H), 5.42 (dd, *J* = 24.00, 1.37 Hz, 1H), 4.65 (d, *J* = 11.67 Hz, 1H), 4.39 (d, *J* = 11.67 Hz, 1H), 3.88-3.80 (m, 2H), 3.44-3.40 (m, 2H), 2.22 (d, *J* = 3.60 Hz, 1H).

^{13}C NMR ($CHCl_3$, 100 MHz) δ 137.9, 134.6, 128.7 (2C), 128.1 (2C), 128.1, 121.4, 81.4, 72.6, 70.7, 53.0.

FTIR (NaCl): 3435, 2870, 2102, 1642, 1454, 1070 cm^{-1}.



(3*R*,4*R*)-5-Azido-4-fluoro-3-benxyloxy-pent-1-ene ((*R*,*R*)-5.3).

A solution of DAST (900 μL, 6.87 mmol) in 3.2 mL of benzene and 400 μL of pyridine in a plastic container was cooled to -10 °C in a Cryocooler apparatus. Alcohol (*R*,*S*)-**5.2** (750 mg, 3.21 mmol) was added to the mixture in small portions, and stirred at -10 ºC for 48 h, followed by 6 h at RT. The solution was poured slowly into a stirred ice-cold sat. $NaHCO_3$ solution over 5 min. The mixture was partitioned with one volume of DCM, which was washed with 2 N HCl and sat. $NaHCO_3$, dried over Na_2SO_4, filtered and evaporated to a residue under vacuum. Purification by column chromatography (1% Et_2O/hexanes) eluted first a mixture of unidentified byproducts (133 mg, Rf ~ 0.75 in 1:9 EtOAc/Hex) followed by 128 mg of the title compound (*R*,*R*)-**5.3** (17%, 0.544 mmol), as a clear high boiling liquid.

R_f = 0.63 in 1:9 EtOAc/hexanes.
For (*R*,*R*)-**5.3** $[\alpha]^{22}_{D}$ -11.9° (*c* 1.50, $CHCl_3$) and for (*S*,*S*)-**5.3** $[\alpha]^{22}_{D}$ 10.7° (*c* 1.50, $CHCl_3$).
^{1}H NMR ($CDCl_3$, 400 MHz) δ7.44-7.29 (m, 5H), 4.63 (dddd, *J* = 47.64, 7.07, 4.99, 3.32 Hz, 1H), 5.49-5.42 (m, 2H), 4.70 (d, *J* = 11.95 Hz, 1H), 4.57 (ddd, *J* = 7.07, 4.99, 3.32 Hz, 1H), 4.44 (d, *J* = 11.90 Hz, 1H), 4.03 (ddd, *J* = 16.87, 7.57, 5.04 Hz, 1H), 3.64-3.52 (m, 1H), 3.45 (ddd, *J* = 27.45, 13.63, 3.27 Hz, 1H).
^{19}F NMR ($CHCl_3$, 282 MHz) δ -196.66 (dddd, J = 47.27, 27.08, 19.84, 16.89 Hz).
^{13}C NMR ($CHCl_3$, 100 MHz) δ 137.8, 133.1 (d, *J* = 5.30 Hz), 128.7 (2C), 128.1 (3C), 121.0, 93.3 (d, *J* = 181.54 Hz), 79.1 (d, *J* = 20.39 Hz), 70.9, 51.5 (d, *J* = 22.25 Hz).
FTIR (NaCl):2930, 2104, 1643, 1454, 1281, 1115, 1069 cm^{-1}.

OBn
(R)
N_3
F F
5.5

(*R*)-5-Azido-4,4-difluoro-3-benxyloxy-pent-1-ene ((*R*)-5.5).

To a solution of DMSO (690 µL, 9.65 mmol) in dry DCM (25 mL) at -78 °C, oxalyl chloride (2.0 M in DCM, 3.21 mL, 6.43 mmol) was added and stirred for 1 h. Alcohol (*R,S*)-**5.2** (750 mg, 3.21 mmol) in DCM (1 mL) was added dropwise to the -78 ºC solution, which was stirred for 1 h, treated dropwise with *N*-methyl morpholine (1.41 mL, 12.9 mmol), and warmed in a -15 °C ice/acetone bath for 2 h. The reaction was quenched with one volume of phosphate buffer (0.1 M, pH 6.0). The organic layer was separated, washed three times with phosphate buffer, dried over Na_2SO_4, filtered and evaporated under vacuum. The resulting brown residue contained the major base-sensitive ketone product (R_f = 0.71 in 1:4 EtOAc/hexanes), with traces of unreacted alcohol **5.2** and minimum contamination by an isomerized conjugated by-product (R_f = 0.62, UV-active). The residue was taken up in Et_2O, dried over $MgSO_4$, filtered through cotton, evaporated to a residue under vacuum and used immediately without further purification. A solution of DAST (2 mL, 15.3 mmol) in DCM (3 mL) in a plastic container at -25 °C was added dropwise to a solution of the crude mixture of ketone in DCM (1 mL). The mixture was allowed to warm slowly to RT and stirred for 48 h. The solution was poured into an ice-cold stirred sat. $NaHCO_3$ solution over 5 min, and the mixture was extracted with DCM. The combined organic layers were dried over Na_2SO_4, filtered and evaporated to a residue under vacuum. Purification by column chromatography (1% Et_2O/hexanes) and evaporation of the collected fractions gave 219 mg of the title compound (*R*)-**5.5** contaminated with minor unidentified by-products. Preparative TLC (0.5 mm layer, 5% Et_2O/hexanes) yielded 193 mg of the title compound (*R*)-**5.5** (23% for two steps, 0.762 mmol), as a clear high boiling liquid.

R_f = 0.72, 1:4 EtOAc/hexanes.

For (*R*)-**5.5** $[\alpha]^{22}_D$ -23.8° (*c* 1.52, $CHCl_3$) and for (*S*)-**5.5** $[\alpha]^{22}_D$ 27.9° (*c* 3.14, $CHCl_3$).

^{1}H NMR ($CDCl_3$, 300 MHz) δ7.44-7.31 (m, 5H), 5.89 (dddd, *J* = 16.88, 10.61, 7.11, 0.62 Hz, 1H), 5.59-5.56 (m, 1H), 5.53 (d, *J* = 10.74 Hz, 1H), 4.71 (d, *J* = 11.67 Hz, 1H), 4.50

(d, J = 11.66 Hz, 1H), 4.14 (td, J = 14.25, 7.13, 7.13 Hz, 1H), 3.64 (tq, J = 13.67, 13.67, 13.67, 11.19, 11.19 Hz, 2H).

^{19}F NMR ($CHCl_3$, 282 MHz) δ -116.63 (dtd, J = 257.62, 13.91, 13.90, 8.72 Hz), -111.27 (dtd, J = 257.59, 16.18, 16.16, 7.04 Hz).

^{13}C NMR ($CHCl_3$, 75 MHz) δ 137.1, 130.3 (t, J = 3.06, 3.06 Hz), 128.7 (2C), 128.3, 128.2 (2C), 122.8, 120.7 (dd, J = 249.89, 246.83 Hz), 78.9 (dd, J = 30.35, 25.35 Hz), 71.5 (d, J = 0.48 Hz), 51.5 (dd, J = 30.26, 25.92 Hz).

FTIR (NaCl): 2928, 2108, 1455, 1292, 1091 cm^{-1}.

OBn
(S)
(R)
N_3
OBn

5.7

(3*R*,4*S*)-5-Azido -3,4-bisbenxyloxy-pent-1-ene ((*R*,*S*)-5.7).

A mixture of alcohol (*R,S*)-**5.2** (250 mg, 1.07 mmol), tetrabutylammonium iodide (42 mg, 0.11 mmol) and benzyl bromide (155 µL, 1.27 mmol) in THF (50 mL), was cooled in an ice bath under an argon atmosphere. The solution was then added sodium hydride in small portions (60% in mineral oil, 47 mg, 1.18 mmol), and stirred overnight allowing to warm to RT. The reaction was quenched with MeOH, filtered through Celite^{TM}, and washed with copious amounts of Et_2O. The volatiles were evaporated to give an oily residue, which was purified by column chromatography (2% Et_2O/hexanes), to yield 237 mg of the title compound (*R,S*)-**5.7** (68%, 0.734 mmol), as a clear high boiling liquid.

R_f = 0.62 in 1:4 EtOAc/hexanes.

For (*R,S*)-**5.7** $[\alpha]^{22}_D$ -6.1° (c 1.50, $CHCl_3$) and for (*S,R*)-**5.7** $[\alpha]^{22}_D$ 7.9° (c 1.50, $CHCl_3$).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.35-7.24 (m, 10H), 5.81 (ddd, J = 17.15, 10.58, 7.45 Hz, 1H), 5.37 (ddd, J = 5.70, 1.65, 0.86 Hz, 1H), 5.33 (ddd, J = 12.07, 1.44, 0.81 Hz, 1H), 4.63 (s, 2H), 4.61 (d, J = 11.87 Hz, 1H), 4.35 (d, J = 11.78 Hz, 1H), 3.90 (tdd, J = 7.37, 5.65, 0.79, 0.79 Hz, 1H), 3.60 (ddd, J = 6.39, 5.69, 3.64 Hz, 1H), 3.43 (dd, J = 12.93, 6.42 Hz, 1H), 3.35 (dd, J = 12.93, 3.60 Hz, 1H).^{13}C NMR ($CHCl_3$, 75 MHz) δ 138.3, 138.0, 135.4, 128.6 (4C), 128.3 (2C), 128.0, 128.0 (2C), 127.9, 120.0, 80.8, 80.2, 73.3, 70.8, 51.7.

FTIR (NaCl): 2867, 2100, 1606, 1454, 1286, 1095, 1073.

General procedure for ozonolysis and Pinnick oxidation of 5.3, 5.5 and 5.7.

The olefin substrate (0.5 to 0.75 mmol) was dissolved in DCM (30 mL), and cooled to -78 °C. Ozone in oxygen was produced in a Welsbach ozone generator (8 psi O_2, 75 V, approx. 7 mmol/h) and bubbled through the olefin solution until a light blue color persisted (3 to 5 min). After stirring at -78 °C for 1 h, the solution was purged with argon bubbles over 10 minutes to remove excess ozone. Dimethyl sulfide (10 equiv.) was added to the -78 ºC mixture, which was allowed to warm to RT over 30 min. The volatiles were evaporated under vacuum, followed by high-vacuum for 10 min. The aldehyde intermediates were not purified to avoid HF elimination and epimerization, and were oxidized immediately. Crude aldehyde intermediate was dissolved in 10 mL of a solvent mixture of THF, *t*BuOH and water (3:3:2), treated with NaH_2PO_4 (4 equiv.) and 2-methyl-2-butene (10 equiv.), followed by sodium chlorite (2 equiv.), and stirred for 4 h. The mixture was treated with sat. NaCl (10 mL) and extracted three times with DCM. The combined organic layers were dried over Na_2SO_4, filtered and evaporated to a residue under vacuum. Purification by column chromatography (0 → 0.5 or 1% MeOH/DCM) provided the desired acids. Additional procedures for each substrate are described below.

OBn
HO (R) (R) N3
O F

5.4

(2*R*,3*R*)-4-Azido-2-benzyloxy-3-fluorobutanoic acid ((*R,R*)-5.4).

The product obtained from the ozonolysis and Pinnick oxidation procedure, followed by purification by chromatography, was recrystallized twice from hot hexanes producing fine needles. X-ray quality crystals were produced from hot hexanes containing several drops of DCM, allowing to cool slowly to RT. From (*R,R*)-**5.3** (128 mg, 0.543 mmol), 120 mg of (*R,R*)-**5.4** (87%, 0.474 mmol) was obtained. From (*S,S*)-**5.3** (75 mg, 0.32 mmol), 59 mg of (*S,S*)-**5.4** (73%, 0.233 mmol) was obtained.

For (*R,R*)-**5.4** $[\alpha]^{22}_{D}$ -56.9° (*c* 0.68, $CHCl_3$) and for (*S,S*)-**5.4** $[\alpha]^{22}_{D}$ 58.6° (*c* 0.73, $CHCl_3$). HRMS (ESI negative mode) calcd. for $C_{11}H_{12}FN_3O_3$, M - H^+ = 252.0790, found 252.0782 (-3.09 ppm).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 10.55 (s, 1H), 7.46-7.34 (m, 5H), 4.98 (dddd, *J* = 46.40, 7.57, 4.91, 2.92 Hz, 1H), 4.94 (d, *J* = 11.47 Hz, 1H), 4.55 (d, *J* = 11.51 Hz, 1H), 4.17 (dd, *J* = 27.26, 2.86 Hz, 1H), 3.77 (dt, *J* = 13.89, 13.66, 7.27 Hz, 1H), 3.42 (ddd, *J* = 24.28, 13.20, 4.92 Hz, 1H).

^{19}F NMR ($CDCl_3$, 376 MHz) δ -198.36 (dddd, *J* = 46.28, 27.22, 24.46, 14.15 Hz).

^{13}C NMR ($CHCl_3$, 100 MHz) δ 174.6 (d, *J* = 4.21 Hz), 136.4, 129.2 (2C), 129.1, 129.0 (2C), 91.5 (d, *J* = 182.59 Hz), 76.4 (d, *J* = 19.90 Hz), 74.0 (s), 50.9 (d, *J* = 25.13 Hz).

FTIR (NaCl): 3151, 2098, 1753, 1407, 1283, 1112 cm^{-1}.

OBn
HO (R) N3
O F F

5.6

(*R*)-4-Azido-2-benzyloxy-3,3-difluorobutanoic acid ((*R*)-5.6).

The oily product obtained from the ozonolysis and Pinnick oxidation procedure, followed by purification by chromatography, was triturated thee times with cold hexanes at -20 °C, to produce a clear yellow oil. From (*R*)-**5.5** (193 mg, 0.762 mmol), 139.5 mg of (*R*)-**5.6** (67%, 0.514 mmol) was obtained. From (*S*)-**5.5** (75.5 mg, 0.298 mmol), 34.8 mg of (*S*)-**5.6** (43%, 0.128 mmol) was obtained.

For (*R*)-**5.6** $[\alpha]^{22}_D$ -32.4° (*c* 0.80, $CHCl_3$) and for (*S*)-**5.6** $[\alpha]^{22}_D$ 36.4° (*c* 0.80, $CHCl_3$).

HRMS (ESI negative mode) calcd. for $C_{11}H_{11}F_2N_3O_3$, M - H^+ = 270.0696, found 270.0692 (-1.24 ppm).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.46-7.32 (m, 5H), 6.48 (s, 1H), 4.84 (d, *J* = 11.30 Hz, 1H), 4.67 (d, *J* = 11.30 Hz, 1H), 4.37 (dd, *J* = 12.23, 9.78 Hz, 1H), 3.75 (dd, *J* = 14.67, 12.35 Hz, 2H).

^{19}F NMR ($CDCl_3$, 376 MHz) δ -112.61 (qd, *J* = 260.95, 12.30, 12.29, 12.29 Hz), -109.68 (dtd, *J* = 260.79, 14.75, 14.68, 9.94 Hz).

^{13}C NMR ($CHCl_3$, 100 MHz) δ 170.8, 135.5, 129.0, 128.9 (2C), 128.8 (2C), 119.6 (t, *J* = 251.58, 251.58 Hz), 76.6 (dd, *J* = 29.86, 27.24 Hz), 74.3, 51.6 (dd, *J* = 28.94, 26.76 Hz).

FTIR (NaCl): 3337, 2929, 2112, 1738, 1455, 1292, 1210, 1119 cm^{-1}.

5.8

(2*S*,3*S*)-4-Azido-2,3-bisbenzyloxybutanoic acid ((*S,S*)-5.8).

From (*R,S*)-**5.7** (237 mg, 0.733 mmol), 187.7 mg of (*S,S*)-**5.8** (75%, 0.550 mmol) was obtained, as a clear oil. From (*S,R*)-**5.7** (178 mg, 0.550 mmol), 144 mg of (*R,R*)-**5.8** (77%, 0.422 mmol) was obtained, as a clear oil.

For (*S,S*)-**5.8** $[\alpha]^{22}_{D}$ -15.1° (*c* 1.05, $CHCl_3$) and for (*R,R*)-**5.8** $[\alpha]^{22}_{D}$ 15.2° (*c* 0.81, $CHCl_3$).
HRMS (ESI negative mode) calcd. for $C_{18}H_{19}N_3O_4$, M - H^+ = 340.1303, found 340.1296 (-1.97 ppm).
^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.24 (s, 1H), 7.38-7.33 (m, 10H), 4.79 (d, *J* = 11.61 Hz, 1H), 4.66 (s, 2H), 4.56 (d, *J* = 11.61 Hz, 1H), 4.20 (d, *J* = 4.24 Hz, 1H), 3.98 (td, *J* = 6.56, 4.30, 4.30 Hz, 1H), 3.58 (dd, *J* = 13.04, 6.62 Hz, 1H), 3.42 (dd, *J* = 13.04, 4.31 Hz, 1H).
^{13}C NMR ($CHCl_3$, 75 MHz) δ 175.6, 137.9, 137.3, 129.4 (2C), 129.4 (2C), 129.2, 129.0 (2C), 129.0 (2C), 128.9, 79.7, 77.7, 74.0, 73.9, 51.7.
FTIR (NaCl): 3000, 2918, 2103, 1722, 1455, 1284, 1110 cm^{-1}.

General procedure for amide coupling of acids 5.4, 5.6 and 5.8

A solution of acid substrate (0.060 mmol) and *N*-hydroxysuccinamide (7.4 mg, 63 μmol) in EtOAc (2 mL), was treated with *N,N'*-dicyclohexylcarbodiimide (12.4 mg, 60 μmol) and stirred for 1 h. The resulting white suspension was filtered through a tightly-packed cotton pad, washed with EtOAc. Filtrate was evaporated under vacuum to a residue of active ester, which was dissolved in THF (2 mL), treated with $NaHCO_3$ (15 mg, 0.18 mmol) and N1-free-base neomycin intermediate **5.9** (see below, 50 mg, 41 μmol), and stirred for 24 h, when LRMS showed molecular ions corresponding to coupled product and indicated disappearance of the free-amine. The solution was treated with sat. $NaHCO_3$ and extracted three times with DCM. The combined organic layers were dried over Na_2SO_4, filtered and evaporated to a residue under vacuum. Purification by column chromatography (1 → 1.5% MeOH/DCM) yielded the coupled products **5.S2** – **5.S7**, which were obtained as white amorphous solids.

3',4',3''',4'''-Didehydro-3',4',3''',4'''-tetradeoxy-3,2',6',2''',6'''-*N*-Cbz-neomycin (5.9) was obtained from bulk preparations at Achaogen Inc. following procedures developed by colleagues in the Hanessian laboratory.[6] This compound was characterized:

R_f = 0.1-0.2 streak, 20:5:3 $CHCl_3$/EtOAc/MeOH

HRMS (ESI) calcd. for $C_{63}H_{72}N_6O_{19}$, M + H^+ = 1217.4925, found 1217.4912 (-1.07 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.37-7.16 (m, 25H), 5.79-5.72 (m, 2H), 5.49-5.41 (m, 2H), 5.19 (s, 1H), 5.15-5.06 (m, 1H), 5.06-4.93 (m, 10H), 4.75-4.65 (m, 1H), 4.33 (dd, J = 3.5, 3.1 Hz, 1H), 4.30-4.24 (m, 1H), 4.24-4.19 (m, 2H), 4.17-4.09 (m, 2H), 4.02-3.91 (m, 1H), 3.70-3.52 (m, 4H), 3.48 (dd, J = 9.4, 8.6 Hz, 1H), 3.40-3.28 (m, 2H), 3.21 (dd, J = 13.9, 3.7 Hz, 1H), 3.18-3.09 (m, 2H), 2.77 (dt, J = 11.1, 10.9, 3.5 Hz, 1H), 2.03-1.93 (m, 1H), 1.42 (dd, J = 12.6, 12.5 Hz, 1H). ^{13}C NMR (CD_3OD, 100 MHz) δ 159.6 - 158.5 (5C), 138.4 - 138.3 (5C), 131.7, 129.7 - 128.7 (25C), 127.0, 126.6, 109.1, 100.2, 96.9, 85.8, 83.5, 78.5, 78.1, 75.8, 75.4 (2C), 70.0, 67.9, 67.7 - 67.6 (6C), 63.0, 52.1, 51.9, 48.8, 48.4, 45.5, 34.4.

General procedure for global hydrogenative deprotection for 5.S2 – 5.S7.

The protected aminoglycoside (25 to 35 μmol) was dissolved in a minimum THF (0.5 mL), and treated with MeOH (2 mL) and 5 drops of AcOH (10 drops for **5.S4** and **5.S7**). The mixture was stirred vigorously and treated with approx. 50 mg wet palladium hydroxide (20% wt. Pd, 50% water) and connected to a H_2 atmosphere. After 1 h, water (2 mL) was added to the mixture, which was stirred overnight under a H_2 atmosphere, when LRMS showed molecular ions of product indicating complete deprotection of the carboxybenzyl groups and more resilient benzyl group(s). The black suspension was filtered through a 0.45 μm syringe filter, and the filtrate was neutralized with a few drops of $NH_4OH_{(aq)}$ and freeze-dried. The resulting residue was dissolved in $CHCl_3$/MeOH/NH_4OH (2:3:1) and purified by column chromatography using the same solvent system (10 → 30% NH_4OH). The fractions containing aminoglycoside were identified by TLC, collected and evaporated under vacuum to furnish a wet residue, which was freeze-dried. The dry residue obtained was redissolved in 1 mL of water, at which point insoluble traces of silica were generally observed, and were removed by filtration of the solution through a 0.45 μm syringe filter. Finally, freeze-drying the filtrate yielded the aminoglycoside analog as the

free base fluffy powder. For characterization purposes, the aminoglycoside was redissolved in a minimum volume of water, treated with AcOH (50 μL) and freeze-dried to provide the aminoglycoside acetate salts, which were obtained as light yellow solids.

5.S2

1-*N*-((2''''*R*,3''''R)-4''''-Azido-2''''-benzyloxy-3''''-fluorobutanoyl)-3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-*per*-*N*-Cbz-neomycin (5.S2).

The general coupling and chromatographic purification procedure yielded 51.1 mg of **5.S2** (85.6%, 35.2 μmol).

R_f = 0.6, 20:5:3 $CHCl_3$/EtOAc/MeOH

HRMS (ESI) calcd. for $C_{74}H_{82}FN_9O_{21}$, M + Na^+ = 1474.5502, found 1474.5450 (-3.49 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.34-7.15 (m, 30H), 5.76-5.69 (m, 2H), 5.46 (d, *J* = 2.9 Hz, 1H), 5.42 (s, 1H), 5.19-5.15 (m, 1H), 5.11-5.04 (m, 2H), 5.04-5.00 (m, 2H), 5.00-4.92 (m, 7H), 4.68-4.63 (m, 1H), 4.61 (d, *J* = 11.7 Hz, 1H), 4.45 (d, *J* = 11.7 Hz, 1H), 4.31 (dd, *J* = 3.8, 3.1 Hz, 1H), 4.25 (dd, *J* = 8.0, 4.4 Hz, 1H), 4.22-4.11 (m, 3H), 4.09 (s, 1H), 4.01-3.89 (m, 2H), 3.71-3.60 (m, 2H), 3.60-3.41 (m, 6H), 3.40-3.23 (m, 3H), 3.22-3.06 (m, 3H), 1.92-1.82 (m, 1H), 1.40 (q, *J* = 12.4, 12.3, 12.3 Hz, 1H).

^{19}F NMR (CD_3OD, 282 MHz) δ -198.68 (dtd, *J* = 26.82, 22.78, 22.66, 18.13 Hz, 1C).

^{13}C NMR (CD_3OD, 100 MHz) δ 171.1 (d, *J* = 5.12 Hz) , 159.6 - 158.5 (5C), 138.4 - 138.3 (6C), 131.7, 129.9 - 128.9 (30C), 128.7, 127.1, 126.6, 126.6, 109.5, 100.1, 96.9, 93.3 (d, *J* = 181.53 Hz), 86.4, 83.4, 80.4 (d, *J* = 19.71 Hz), 78.9, 78.2, 75.4, 75.0, 74.7, 69.8, 68.0 - 67.6 (6C), 63.4, 52.3 (d, *J* = 22.53 Hz), 52.0, 51.4, 48.9, 48.4, 45.6, 34.6.

FTIR (NaCl): 3332, 2919, 2477, 2105, 1695, 1531, 1454, 1245, 1114, 1048, 753, 698 cm^{-1}.

5.S3

1-*N*-((*R*)-4''''-Azido-2''''-benzyloxy-3'''',3''''-difluorobutanoyl)-3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy *per*-*N*-Cbz-neomycin (5.S3).

The general coupling and chromatographic purification procedure yielded 45.1 mg of **5.S3** (75%, 30.7 μmol).

R_f = 0.6, 20:5:3 $CHCl_3$/EtOAc/MeOH

HRMS (ESI) calcd. for $C_{74}H_{81}F_2N_9O_{21}$, M + Na^+ = 1492.5407, found 1492.5343 (-4.32 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.35-7.14 (m, 30H), 5.76-5.70 (m, 2H), 5.45 (d, *J* = 2.8 Hz, 1H), 5.41 (s, 1H), 5.20-5.13 (m, 1H), 5.12-5.04 (m, 2H), 5.02 (d, *J* = 4.3 Hz, 2H), 5.00-4.91 (m, 7H), 4.68-4.64 (m, 1H), 4.63 (d, *J* = 11.9 Hz, 1H), 4.51 (d, *J* = 11.7 Hz, 1H), 4.33-4.27 (m, 1H), 4.27-4.21 (m, 1H), 4.21-4.07 (m, 5H), 3.92 (dd, *J* = 8.7, 4.8 Hz, 1H), 3.75-3.59 (m, 4H), 3.58-3.41 (m, 5H), 3.29 (dd, *J* = 14.0, 5.9 Hz, 1H), 3.18 (dd, *J* = 13.9, 3.7 Hz, 1H), 3.15-3.06 (m, 2H), 1.91-1.79 (m, 1H), 1.38 (dd, *J* = 24.7, 11.8 Hz, 1H).

^{19}F NMR (CD_3OD, 282 MHz) δ -112.56 (dtd, *J* = 26.54, 15.07, 14.87, 10.32 Hz), -114.62 (dtd, *J* = 24.89, 12.69, 12.68, 9.51 Hz).

^{13}C NMR (CD_3OD, 100 MHz) δ 168.2, 159.6 - 158.5 (5C), 138.4 - 137.7 (6C), 131.7, 129.9 - 128.9 (30C), 128.7, 127.0, 126.6, 121.4 (t, *J* = 249.42, 249.42 Hz), 109.4, 100.1, 96.9, 86.4, 83.5, 79.5 (t, *J* = 27.20, 27.20 Hz), 78.8, 78.2, 75.4, 75.1, 74.7, 69.8, 67.9 - 67.6 (7C), 63.3, 52.6 (t, *J* = 26.17, 26.17 Hz), 52.0, 51.4, 48.9, 48.4, 45.6, 34.6.

FTIR (NaCl): 3412, 2938, 2476, 2110, 1693, 1497, 1428, 1428, 1356, 1163, 1111, 1047, 753, 697 cm^{-1}.

5.S4

1-*N*-((2''''*S*,3''''*S*)-4''''-Azido-2'''',3''''-bisbenzyloxybutanoyl)-3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy -*per*-*N*-Cbz-neomycin (5.S4).

The general coupling and chromatographic purification procedure yielded 37.9 mg of **5.S4** (60%, 24.6 μmol).

R_f = 0.7, 20:5:3 $CHCl_3$/EtOAc/MeOH

HRMS (ESI) calcd. for $C_{81}H_{89}N_9O_{22}$, M + Na^+ = 1562.6014, found 1562.5939 (-0.61 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.33-7.16 (m, 35H), 5.74-5.69 (m, 2H), 5.47-5.40 (m, 2H), 5.18-5.14 (m, 1H), 5.11-5.04 (m, 2H), 5.04-5.00 (m, 2H), 5.01-4.90 (m, 7H), 4.64 (d, *J* = 1.5 Hz, 1H), 4.60-4.50 (m, 2H), 4.46-4.39 (m, 2H), 4.30 (dd, *J* = 3.7, 3.0 Hz, 1H), 4.24 (dd, *J* = 7.9, 4.4 Hz, 1H), 4.21-4.13 (m, 2H), 4.13-4.06 (m, 2H), 3.98 (d, *J* = 5.9 Hz, 1H), 3.92 (dd, *J* = 8.3, 4.9 Hz, 1H), 3.83 (dt, *J* = 5.9, 5.9, 3.0 Hz, 1H), 3.73 (dt, *J* = 11.1, 11.1, 3.8 Hz, 1H), 3.66-3.58 (m, 1H), 3.57-3.41 (m, 5H), 3.41-3.33 (m, 2H), 3.31-3.25 (m, 1H), 3.21-3.06 (m, 3H), 1.78-1.67 (m, 1H), 1.28 (dd, *J* = 24.5, 12.2 Hz, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 172.6, 159.6 - 158.4 (5C), 139.2 - 138.3 (7C), 131.7, 129.7 - 128.9 (35C), 128.7, 127.0, 126.6, 109.5, 100.1, 96.9, 86.5, 83.5, 81.0, 79.8, 78.9, 78.2, 75.4, 74.0, 73.8, 69.8, 68.0 - 67.6 (8C), 63.4, 52.0, 51.9, 51.0, 48.9, 48.4, 45.5, 34.8.

FTIR (NaCl): 3330, 2918, 2479, 2102, 1698, 1530, 1454, 1245, 1157, 1111, 1047, 752, 697 cm^{-1}.

1-*N*-((2''''*S*,3''''*S*)-4''''-Azido-2''''-benzyloxy-3''''-fluorobutanoyl)-3',4',3''',4'''-tetradehydro-*per*-*N*-Cbz-neomycin (5.S5).

The general coupling and chromatographic purification procedure yielded 54.2 mg of **5.S5** (91%, 37.3 µmol).

R_f = 0.6, 20:5:3 $CHCl_3$/EtOAc/MeOH

HRMS (ESI) calcd. for $C_{74}H_{82}FN_9O_{21}$, M + Na^+ = 1474.5502, found 1474.5449 (-1.77 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.39-7.18 (m, 30H), 5.82-5.74 (m, 2H), 5.52-5.42 (m, 2H), 5.25-5.18 (m, 1H), 5.17-5.09 (m, 2H), 5.08-5.04 (m, 2H), 5.04-4.95 (m, 7H), 4.77-4.68 (m, 2H), 4.45 (d, *J* = 11.4 Hz, 1H), 4.37-4.31 (m, 1H), 4.31-4.26 (m, 1H), 4.26-4.19 (m, 2H), 4.18-4.10 (m, 2H), 4.04-3.89 (m, 2H), 3.84-3.70 (m, 1H), 3.70-3.46 (m, 7H), 3.40-3.26 (m, 3H), 3.23 (dd, *J* = 14.1, 3.7 Hz, 1H), 3.19-3.09 (m, 2H), 2.02-1.90 (m, 1H), 1.40 (dd, *J* = 23.9, 11.8 Hz, 1H).

^{19}F NMR (CD_3OD, 282 MHz) δ -196.28 (dtd, *J* = 43.03, 26.62, 26.46, 15.93 Hz).

^{13}C NMR (CD_3OD, 100 MHz) δ 171.2 (d, *J* = 4.44 Hz), 159.6 - 158.5 (5C), 138.5 - 138.2 (6C), 131.7, 130.4 - 128.9 (30C), 128.7, 127.0, 126.6, 109.0, 100.2, 96.8, 93.3 (d, *J* = 181.17 Hz), 86.6, 83.5, 80.3 (d, *J* = 19.39 Hz), 78.6, 78.3, 75.5, 75.4, 75.1, 74.7, 69.8, 68.0 - 67.6 (6C), 63.1, 52.3 (d, *J* = 23.30 Hz), 52.0, 51.2, 48.9, 48.4, 45.6, 34.7.

FTIR (NaCl): 3384, 2931, 2478, 2105, 1697, 1523, 1498, 1430, 1356, 1163, 1048, 752, 698 cm^{-1}.

5.S6

1-*N*-((*S*)-4''''-Azido-2''''-benzyloxy-3'''',3''''-difluorobutanoyl)-3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-*per*-*N*-Cbz-neomycin (5.S6).

The general coupling and chromatographic purification procedure yielded 31.2 mg of **5.S6** (52%, 21.2 μmol).

R_f = 0.6, 20:5:3 $CHCl_3$/EtOAc/MeOH

HRMS (ESI) calcd. for $C_{74}H_{81}F_2N_9O_{21}$, M + Na^+ = 1492.5407, found 1492.5348 (-0.64 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.40-7.17 (m, 30H), 5.81-5.74 (m, 2H), 5.51-5.42 (m, 2H), 5.25-5.18 (m, 1H), 5.17-5.08 (m, 2H), 5.08-5.04 (m, 2H), 5.04-4.95 (m, 7H), 4.73 (d, *J* = 11.5 Hz, 1H), 4.73-4.70 (m, 1H), 4.56 (d, *J* = 11.3 Hz, 1H), 4.34 (td, *J* = 7.3, 3.4, 3.4 Hz, 1H), 4.31-4.26 (m, 1H), 4.26-4.18 (m, 2H), 4.18-4.10 (m, 3H), 3.98 (dd, *J* = 8.8, 4.9 Hz, 1H), 3.83-3.62 (m, 4H), 3.63-3.46 (m, 5H), 3.36-3.27 (m, 2H), 3.23 (dd, *J* = 14.0, 3.8 Hz, 1H), 3.19-3.10 (m, 1H), 1.99-1.91 (m, 1H), 1.41 (dd, *J* = 24.2, 12.2 Hz, 1H).

^{19}F NMR (CD_3OD, 282 MHz) δ -113.13 (dtd, *J* = 256.95, 14.17, 14.05, 10.22 Hz), -114.17 (dtd, *J* = 256.88, 13.49, 13.19, 10.96 Hz).

^{13}C NMR (CD_3OD, 100 MHz) δ 168.4, 159.6 - 158.5 (5C), 138.5 - 137.7 (6C), 131.7, 130.0 - 128.9 (30C), 128.7, 127.1, 126.6, 121.4 (t, *J* = 25.99, 25.99 Hz), 109.1, 100.3, 96.9, 86.6, 83.6, 79.5 (t, *J* = 25.99, 25.99 Hz), 78.6, 78.4, 75.5, 75.4, 75.0 (2C), 69.9, 68.0 - 67.6 (6C), 63.1, 52.8 (t, *J* = 27.32, 27.32 Hz), 52.0, 51.3, 49.0, 48.5, 45.6, 34.6.

FTIR (NaCl): 3417, 2932, 2477, 2110, 2694, 1497, 1428, 1357, 1164, 1107, 1048, 753, 698 cm^{-1}.

5.S7

1-*N*-((2''''*R*,3''''*R*)-4''''-Azido-2'''',3''''-bisbenzyloxybutanoyl)-3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-*per*-*N*-Cbz-neomycin (5.S7).

The general coupling and chromatographic purification procedure yielded 37.9 mg of **5.S7** (60%, 24.6 μmol).

R_f = 0.7, 20:5:3 $CHCl_3$/EtOAc/MeOH

HRMS (ESI) calcd. for $C_{81}H_{89}N_9O_{22}$, M + Na^+ = 1562.6014.

^{1}H NMR (CD_3OD, 400 MHz) δ 7.36-7.11 (m, 35H), 5.76-5.68 (m, 2H), 5.47-5.37 (m, 2H), 5.20-5.12 (m, 1H), 5.12-5.04 (m, 2H), 5.04-4.89 (m, 10H), 4.65 (d, *J* = 9.9 Hz, 1H), 4.59 (d, *J* = 13.4 Hz, 1H), 4.33-4.26 (m, 1H), 4.26-4.21 (m, 1H), 4.21-4.15 (m, 2H), 4.14-4.06 (m, 2H), 3.99 (d, *J* = 4.1 Hz, 1H), 3.96-3.89 (m, 1H), 3.86-3.79 (m, 1H), 3.74-3.58 (m, 2H), 3.57-3.43 (m, 6H), 3.43-3.37 (m, 1H), 3.32-3.28 (m, 1H), 3.28-3.22 (m, 2H), 3.21-3.06 (m, 3H), 1.88-1.79 (m, 1H), 1.34 (dd, *J* = 24.5, 11.8 Hz, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 172.6, 159.6 - 158.5 (m, 5C), 139.2 - 138.3 (m, 7C), 131.7, 129.8 - 129.1 (m, 35C), 128.7, 127.0, 126.6, 109.1, 100.2, 96.9, 86.6, 83.5, 80.9, 80.3, 78.6, 78.2, 75.4, 75.0, 74.2, 73.8, 69.8, 68.0 - 67.6 (8C), 63.1, 52.0, 51.3, 48.7, 48.4, 45.6, 34.7.

FTIR (NaCl): 3384, 2928, 2479, 2103, 1695, 1524, 1497, 1454, 1429, 1356, 1165, 1110, 1048, 753, 698 cm^{-1}.

1-*N*-((2''''*R*,3''''*R*)-4''''-Amino-3''''-fluoro-2''''-hydroxybutanoyl)-3',4',3''',4'''-tetradeoxy-neomycin (5.11).

From intermediate **5.S2** (51.1 mg, 35.2 μmol), the hydrogenation and purification procedures yielded 29.9 mg of acetate salt **5.11** (82.5%, 29.0 μmol).

R_f = 0.5, 2:3:2 $CHCl_3$/MeOH/NH_4OH.

$[\alpha]^{22}_D$ 33.9º (*c* 1.5, H_2O).

HRMS (ESI) calcd. for $C_{27}H_{52}FN_7O_{11}$, M + H^+ = 670.3782, found 670.3773 (-1.25 ppm).

^{1}H NMR (D_2O, 500 MHz) δ 5.88 (d, *J* = 3.12 Hz, 1H), 5.39 (s, 1H), 5.26 (dd, *J* = 47.47, 8.72 Hz, 1H), 5.00 (s, 1H), 4.48-4.45 (m, 1H), 4.42 (d, *J* = 22.70 Hz, 1H), 4.36 (d, *J* = 4.21 Hz, 1H), 4.21-4.16 (m, 1H), 4.16-4.10 (m, 1H), 4.01 (dd, *J* = 10.94, 9.21 Hz, 1H), 3.98-3.92 (m, 2H), 3.90 (dd, *J* = 12.56, 2.26 Hz, 1H), 3.86 (t, *J* = 9.26, 9.26 Hz, 1H), 3.72 (dd, *J* = 12.24, 4.94 Hz, 1H), 3.67-3.60 (m, 2H), 3.56-3.50 (m, 1H), 3.50-3.42 (m, 4H), 3.42-3.33 (m, 1H), 3.25 (ddd, *J* = 18.82, 13.48, 2.57 Hz, 1H), 3.14 (dd, *J* = 13.42, 8.63 Hz, 1H), 3.08 (dd, *J* = 13.38, 7.41 Hz, 1H), 2.23 (td, *J* = 12.75, 3.84, 3.84 Hz, 1H), 2.19-2.06 (m, 2H), 2.06-1.96 (m, 2H), 1.91 (s, 18H AcOD), 1.81-1.73 (m, 1H), 1.73-1.66 (m, 1H), 1.58 (ddd, *J* = 12.86, 12.61, 4.51 Hz, 1H), 1.48 (ddd, *J* = 13.99, 13.73, 5.08 Hz, 1H).

^{19}F NMR (D_2O, 282 MHz) δ -206.50 (dtd, *J* = 48.34, 31.64, 31.44, 16.89 Hz).

^{13}C NMR (D_2O, 125 MHz) δ 181.2 (AcOD), 172.2 (d, *J* = 2.49 Hz), 110.5, 97.6, 94.4, 89.7 (d, *J* = 177.04 Hz), 86.0, 81.2, 76.0, 74.7, 73.7, 73.5, 72.6, 71.0 (d, *J* = 19.49 Hz), 65.9, 60.3, 49.2, 49.1, 48.7, 47.7, 42.6, 42.5, 40.4 (d, *J* = 20.90 Hz), 30.4, 25.5, 24.0, 23.3 (AcOD), 20.9, 20.5.

LC/CAD, water/acetonitrile gradient 30 to 75% in 30 min, R_t = 16.7 min, 94.4% purity.

5.12

1-*N*-((*R*)-4''''-Amino-3'''',3''''-difluoro-2''''-hydroxybutanoyl)-3',4',3''',4'''-tetradeoxy-neomycin (5.12).

From intermediate **5.S3** (45.1 mg, 30.7 μmol), the hydrogenation and purification procedures yielded 28.1 mg of acetate salt **5.12** (87%, 26.7 μmol).

R_f = 0.5, 2:3:2 $CHCl_3$/MeOH/NH_4OH.
$[\alpha]^{22}_D$ 38.0º (*c* 1.41, H_2O).
HRMS (ESI) calcd. for $C_{27}H_{51}F_2N_7O_{11}$, M + H^+ = 688.3688, found 688.3687 (0.02 ppm).
^1H NMR (D_2O, 400 MHz) δ 5.88 (d, *J* = 3.48 Hz, 1H), 5.39 (d, *J* = 2.00 Hz, 1H), 5.01 (d, *J* = 1.11 Hz, 1H), 4.62 (t, *J* = 11.77, 11.77 Hz, 1H), 4.46 (dd, *J* = 6.61, 5.02 Hz, 1H), 4.36 (dd, *J* = 4.73, 2.13 Hz, 1H), 4.18 (ddd, *J* = 7.06, 5.17, 3.15 Hz, 1H), 4.16-4.10 (m, 1H), 4.04-3.93 (m, 3H), 3.90 (dd, *J* = 12.65, 3.15 Hz, 1H), 3.86 (t, *J* = 9.22, 9.22 Hz, 1H), 3.74 (d, *J* = 5.08 Hz, 1H), 3.73-3.66 (m, 1H), 3.71 (d, *J* = 5.12 Hz, 1H), 3.68-3.59 (m, 3H), 3.53 (ddd, *J* = 11.24, 5.29, 3.88 Hz, 1H), 3.45 (ddd, *J* = 12.79, 10.45, 3.83 Hz, 1H), 3.27 (dd, *J* = 13.52, 3.19 Hz, 1H), 3.23 (dd, *J* = 13.62, 3.52 Hz, 1H), 3.14 (dd, *J* = 13.49, 8.62 Hz, 1H), 3.08 (dd, *J* = 13.47, 7.39 Hz, 1H), 2.23 (td, *J* = 12.46, 3.91, 3.91 Hz, 1H), 2.19-2.07 (m, 2H), 2.06-1.97 (m, 2H), 1.92 (s, 18H AcOD), 1.75 (dd, *J* = 25.03, 12.45 Hz, 1H), 1.73-1.67 (m, 1H), 1.59 (ddd, *J* = 12.08, 12.04, 4.81 Hz, 1H), 1.48 (ddd, *J* = 26.31, 13.35, 4.97 Hz, 1H).
^{19}F NMR (D_2O, 282 MHz)δ -114.91 (tdd, *J* = 21.94, 19.84, 12.25, 12.25 Hz.
^{13}C NMR (D_2O, 125 MHz) δ 181.1 (AcOD), 169.2, 118.9 (t, *J* = 249.79, 249.79 Hz), 110.5, 97.7, 94.4, 86.0, 81.3, 75.9, 74.7, 73.7, 73.5, 72.6, 71.2 (t, *J* = 25.52, 25.52 Hz), 65.9, 60.3, 49.2, 49.0, 48.9, 48.7, 47.7, 42.5, 41.2 (t, *J* = 24.02, 24.02 Hz), 30.3, 25.5, 24.0, 23.2 (AcOD), 20.9, 20.5.
LC/CAD, water/acetonitrile gradient 30 to 75% in 30 min, R_t = 16.8 min, 97.5% purity.

1-*N*-((2''''*S*,3''''*S*)-4''''-Amino-2'''',3''''-bishydroxybutanoyl)-3',4',3''',4'''-tetradeoxy-neomycin (5.13).

From intermediate **5.S4** (37.9 mg, 24.6 μmol), the hydrogenation and purification procedures yielded 14.3 mg of acetate salt **5.13** (56.5%, 13.9 μmol).

R_f = 0.5, 2:3:2 $CHCl_3$/MeOH/NH_4OH.

$[\alpha]^{22}_D$ 32.9° (*c* 0.72, H_2O).

HRMS (ESI) calcd. for $C_{27}H_{53}N_7O_{12}$, M + Na^+ = 690.3644, found 690.3654 (1.33 ppm).

^{1}H NMR (D_2O, 400 MHz) δ 5.88 (d, *J* = 3.17 Hz, 1H), 5.39 (d, *J* = 1.24 Hz, 1H), 5.03 (s, 1H), 4.50 (dd, *J* = 6.63, 5.02 Hz, 1H), 4.39 (dd, *J* = 4.49, 1.49 Hz, 1H), 4.30 (d, *J* = 4.16 Hz, 1H), 4.21-4.10 (m, 3H), 4.08-3.83 (m, 5H), 3.74 (dd, *J* = 12.27, 5.01 Hz, 1H), 3.70-3.61 (m, 2H), 3.58-3.50 (m, 1H), 3.44 (ddd, *J* = 12.42, 10.06, 3.35 Hz, 1H), 3.26 (dt, *J* = 13.97, 13.80, 2.57 Hz, 2H), 3.19-3.05 (m, 4H), 2.27-2.19 (m, 1H), 2.18-2.08 (m, 2H), 2.08-1.92 (m, 3H), 1.92 (s, 8H AcOD), 1.81-1.66 (m, 2H), 1.59 (ddd, *J* = 12.47, 12.21, 5.16 Hz, 1H), 1.48 (ddd, *J* = 26.21, 12.91, 5.37 Hz, 1H).

^{13}C NMR (D_2O, 100 MHz) δ 181.5 (AcOD), 173.6, 110.8, 97.9, 94.8, 86.4, 81.5, 76.3, 74.9, 74.1, 73.9, 73.7, 73.0, 69.0, 66.2, 60.7, 49.5, 49.1 (2C), 48.1, 42.9 (2C), 40.9, 30.8, 25.9, 24.3, 23.5 (AcOD), 21.3, 20.8.

LC/CAD, water/acetonitrile gradient 30 to 75% in 30 min, R_t = 16.8 min, 96.5% purity.

5.14

1-*N*-((2''''*S*,3''''*S*)-4''''-Amino-3''''-fluoro-2''''-hydroxybutanoyl)-3',4',3''',4'''-tetradeoxy-neomycin (5.14).

From intermediate **5.S5** (31.0 mg, 21.3 μmol), the hydrogenation and purification procedures yielded 15.7 mg of acetate salt **5.14** (71.4%, 15.2 μmol).

R_f = 0.5, 2:3:2 $CHCl_3$/MeOH/NH_4OH.

$[\alpha]^{22}_D$ 41.9º (*c* 0.79, H_2O).

HRMS (ESI) calcd. for $C_{27}H_{52}FN_7O_{11}$, M + H^+ = 670.3782, found 670.3780 (-0.29 ppm).

^1H NMR (D_2O, 400 MHz) δ 5.87 (d, *J* = 3.29 Hz, 1H), 5.39 (d, *J* = 1.87 Hz, 1H), 5.27 (dddd, *J* = 47.57, 8.85, 2.54, 1.70 Hz, 1H), 5.00 (d, *J* = 0.97 Hz, 1H), 4.46 (dd, *J* = 6.44, 5.07 Hz, 1H), 4.44 (dd, *J* = 31.70, 1.56 Hz, 1H), 4.35 (dd, *J* = 4.42, 1.86 Hz, 1H), 4.21-4.08 (m, 2H), 4.04-3.96 (m, 2H), 3.96-3.82 (m, 3H), 3.72 (dd, *J* = 12.26, 5.04 Hz, 1H), 3.68-3.59 (m, 2H), 3.56-3.32 (m, 4H), 3.24 (dt, *J* = 14.53, 14.13, 2.96 Hz, 2H), 3.13 (dd, *J* = 13.72, 8.79 Hz, 1H), 3.07 (dd, *J* = 13.35, 7.17 Hz, 1H), 2.19 (td, *J* = 12.65, 3.96, 3.96 Hz, 1H), 2.16-2.06 (m, 2H), 2.06-1.96 (m, 2H), 1.91 (s, 17H AcOD), 1.75 (dd, *J* = 23.92, 11.23 Hz, 1H), 1.72-1.65 (m, 1H), 1.58 (ddd, *J* = 12.28, 11.99, 4.93 Hz, 1H), 1.47 (ddd, *J* = 13.38, 13.24, 5.37 Hz, 1H).

^{19}F NMR (D_2O, 376 MHz) δ -206.62 (dtd, *J* = 48.19, 32.12, 32.00, 16.33 Hz).

^{13}C NMR (D_2O, 100 MHz) δ 181.5 (AcOD), 172.7 (d, *J* = 2.33 Hz), 110.7, 98.0, 94.8, 90.3 (d, *J* = 176.74 Hz), 86.2, 81.6, 76.3, 75.1, 74.3, 74.0, 73.0, 71.3 (d, *J* = 19.37 Hz), 66.2, 60.7, 49.5, 49.4, 49.1, 48.1, 42.9, 42.9, 40.7 (d, *J* = 20.76 Hz), 30.8, 25.9, 24.3, 23.5 (AcOD), 21.2, 20.8.

LC/CAD, water/acetonitrile gradient 30 to 75% in 30 min, R_t = 16.6 min, 89.6% purity.

5.15

1-*N*-((*S*)-4''''-Amino-3'''',3''''-difluoro-2''''-hydroxybutanoyl)-3',4',3''',4'''-tetradeoxy-neomycin (5.15).

From intermediate **5.S6** (54.0 mg, 36.7 μmol), the hydrogenation and purification procedures yielded 20.2 mg of acetate salt **5.14** (52.5%, 19.3 μmol).

R_f = 0.5, 2:3:2 $CHCl_3$/MeOH/NH_4OH.

$[\alpha]^{22}_D$ 41.0º (*c* 1.01, H_2O).

HRMS calcd. for $C_{27}H_{51}F_2N_7O_{11}$, M + Na^+ = 688.3680, found 688.3687 (-1.13 ppm).

^{1}H NMR (D_2O, 400 MHz) δ 5.88 (d, *J* = 3.51 Hz, 1H), 5.39 (d, *J* = 2.18 Hz, 1H), 5.01 (d, *J* = 1.37 Hz, 1H), 4.62 (t, *J* = 12.00, 12.00 Hz, 1H), 4.46 (dd, *J* = 6.59, 4.99 Hz, 1H), 4.36 (dd, *J* = 4.76, 2.25 Hz, 1H), 4.18 (ddd, *J* = 6.93, 5.26, 3.04 Hz, 1H), 4.13 (ddd, *J* = 6.65, 6.52, 2.86 Hz, 1H), 4.04-3.97 (m, 2H), 3.97-3.83 (m, 3H), 3.72 (dd, *J* = 12.25, 5.09 Hz, 1H), 3.72-3.58 (m, 4H), 3.53 (ddd, *J* = 11.32, 5.29, 3.87 Hz, 1H), 3.45 (ddd, *J* = 12.67, 10.25, 3.61 Hz, 1H), 3.27 (dd, *J* = 13.52, 3.21 Hz, 1H), 3.23 (dd, *J* = 13.55, 3.44 Hz, 1H), 3.14 (dd, *J* = 13.51, 8.62 Hz, 1H), 3.08 (dd, *J* = 13.50, 7.38 Hz, 1H), 2.25-2.19 (m, 1H), 2.19-2.06 (m, 2H), 2.06-1.96 (m, 2H), 1.92 (s, 17H AcOD), 1.75 (dd, *J* = 25.39, 12.65 Hz, 1H), 1.72-1.67 (m, 1H), 1.58 (ddd, *J* = 12.40, 12.23, 4.96 Hz, 1H), 1.48 (ddd, *J* = 26.26, 13.61, 4.98 Hz, 1H).

^{19}F NMR (D_2O, 282 MHz) δ -114.43 (dtd, *J* = 251.64, 20.55, 11.53, 11.53 Hz), -115.68 (dtd, *J* = 253.12, 21.63, 12.03, 12.03 Hz).

^{13}C NMR (D_2O, 100 MHz) δ 181.1 (AcOD), 169.3, 119.0 (t, *J* = 248.05, 248.05 Hz), 110.4, 97.7, 94.4, 85.9, 81.3, 76.0, 74.8, 73.9, 73.5, 72.6, 71.2 (t, *J* = 25.65, 25.65 Hz), 65.9, 60.4, 49.2, 49.1, 48.9, 48.7, 47.7, 42.6, 41.2 (t, *J* = 23.94, 23.94 Hz), 30.4, 25.5, 24.0, 23.2 (AcOD), 20.9, 20.5.

LC/CAD, water/acetonitrile gradient 30 to 75% in 30 min, R_t = 16.8 min, 97.9% purity.

1-*N*-((2''''*R*,3''''*R*)-4''''-Amino-2'''',3''''-bishydroxybutanoyl)-3',4',3''',4'''-tetradeoxy-neomycin (5.16).

From intermediate **5.S7** (37.9 mg, 24.6 μmol), the hydrogenation and purification procedures yielded 15.4 mg of acetate salt **5.16** (61.0%, 15.0 μmol).

R_f = 0.5, 2:3:2 $CHCl_3$/MeOH/NH_4OH.

$[\alpha]^{22}_D$ 44.0º (*c* 0.77, H_2O).

HRMS (ESI) calcd. for $C_{27}H_{53}N_7O_{12}$, M + Na^+ = 690.3644, found 690.3641 (-0.46 ppm).

^1H NMR (D_2O, 400 MHz) δ 5.82 (d, *J* = 2.13 Hz, 1H), 5.33 (s, 1H), 4.96 (s, 1H), 4.43 (dd, *J* = 6.29, 4.47 Hz, 1H), 4.32 (d, *J* = 3.89 Hz, 1H), 4.24 (d, *J* = 3.51 Hz, 1H), 4.16-4.04 (m, 3H), 4.01-3.76 (m, 5H), 3.67 (dd, *J* = 12.15, 4.59 Hz, 1H), 3.62-3.53 (m, 2H), 3.51-3.43 (m, 1H), 3.39 (dd, *J* = 11.13, 8.89 Hz, 1H), 3.26-3.14 (m, 2H), 3.13-2.97 (m, 4H), 2.20-2.12 (m, 1H), 2.12-2.01 (m, 2H), 2.01-1.91 (m, 2H), 1.86 (s, 14H AcOD), 1.76-1.60 (m, 2H), 1.60-1.48 (m, 1H), 1.42 (dq, *J* = 13.19, 12.94, 12.94, 5.69 Hz, 1H).

^{13}C NMR (D_2O, 100 MHz) δ 180.8 (AcOD), 172.8, 109.9, 97.1, 94.0, 85.5, 80.7, 75.5, 74.1, 73.6, 73.0, 72.7, 72.1, 68.4, 65.4, 59.8, 48.7, 48.4, 48.3, 47.3, 42.1, 42.1, 40.1, 30.1, 25.1, 23.5, 22.7 (AcOD), 20.4, 20.0.

LC/CAD, water/acetonitrile gradient 30 to 75% in 30 min, R_t = 16.8 min, 94.7% purity.

Experimental references

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"Compound 5.4 (JPM5-129)" 2 1 D: Hanessian

10.552

7.442
7.438
7.434
7.422
7.415
7.404
7.398
7.386
7.376
7.365
7.360

5.052
5.045
5.040
5.033
5.027
5.022
5.014
4.951
4.936
4.922
4.911
4.906
4.898
4.564
4.535
4.209
4.202
4.141
4.134
3.815
3.797
3.781
3.763
3.747
3.728
3.477
3.464
3.444
3.431
3.416
3.404
3.383
3.371

OBn
HO
(R)
(R)
N_3
O
F
5.4

1.088
5.063
0.498
1.511
1.000
1.008
1.015
1.010

10 8 6 4 2 [ppm]

[rel] 8 6 4 2 0

"Compound 5.4 (JPM5-129)" 6 1 D: Hanessian

174.390

136.138

128.913
128.837
128.750

92.202
90.387

76.363
76.065
73.723

50.752
50.502

OBn
HO
(R)
(R)
N_3
O
F

5.4

[rel]

10
8
6
4
2
0

200
150
100
50
0
[ppm]

"Compound 5.4 (JPM5-129)" 1 1 D: Hanessian

-198.193
-198.231
-198.258
-198.266
-198.296
-198.303
-198.316
-198.330
-198.354
-198.368
-198.381
-198.389
-198.419
-198.426
-198.453
-198.491

OBn
HO
(R)
(R)
N_3
O
F

5.4

[rel]

25
20
15
10
5
0

-160
-180
-200
-220
-240
[ppm]

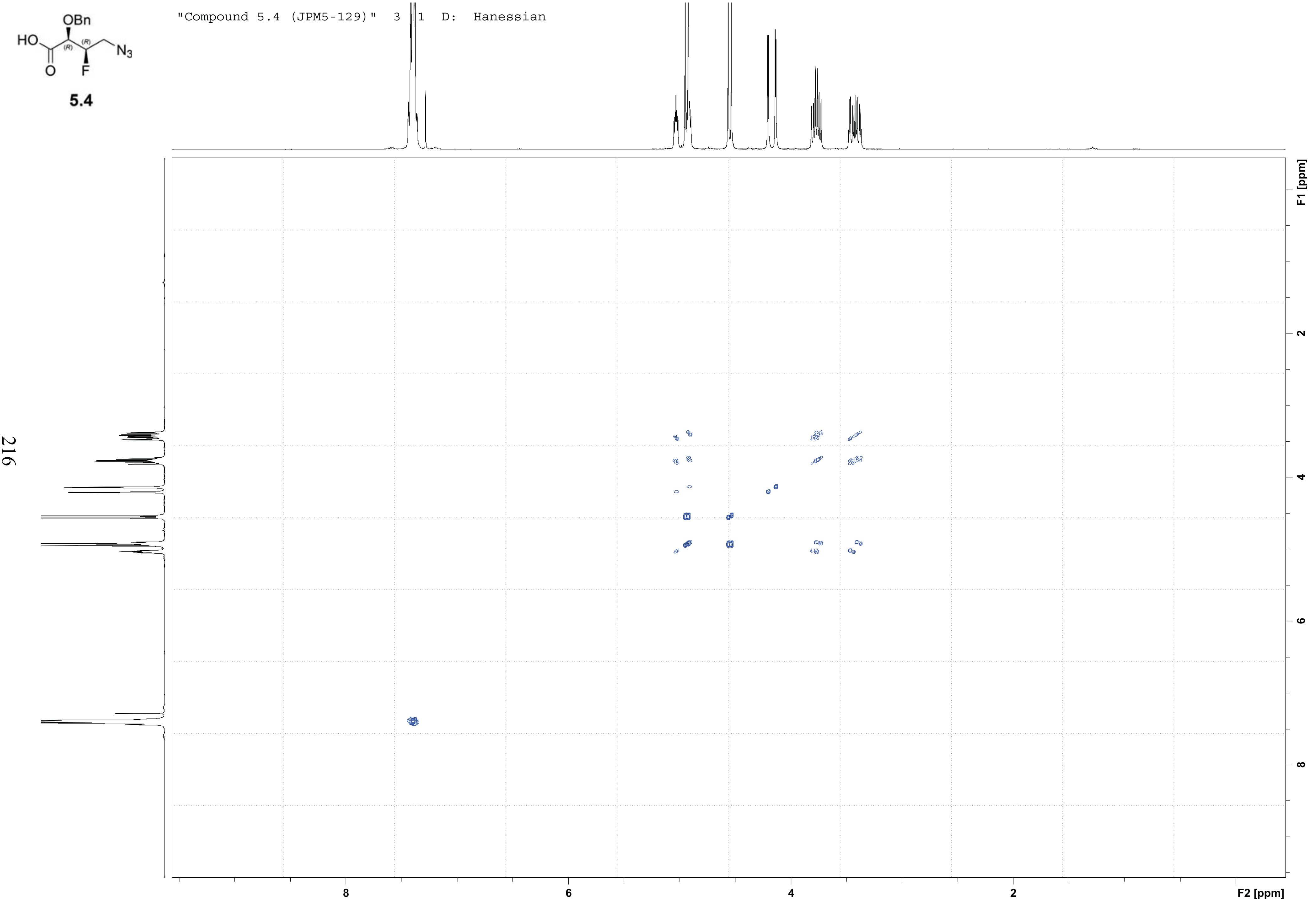
"Compound 5.4 (JPM5-129)" 1 1 D: Hanessian

-198.193
-198.231
-198.258
-198.266
-198.296
-198.303
-198.316
-198.330
-198.354
-198.368
-198.381
-198.389
-198.419
-198.426
-198.453
-198.491

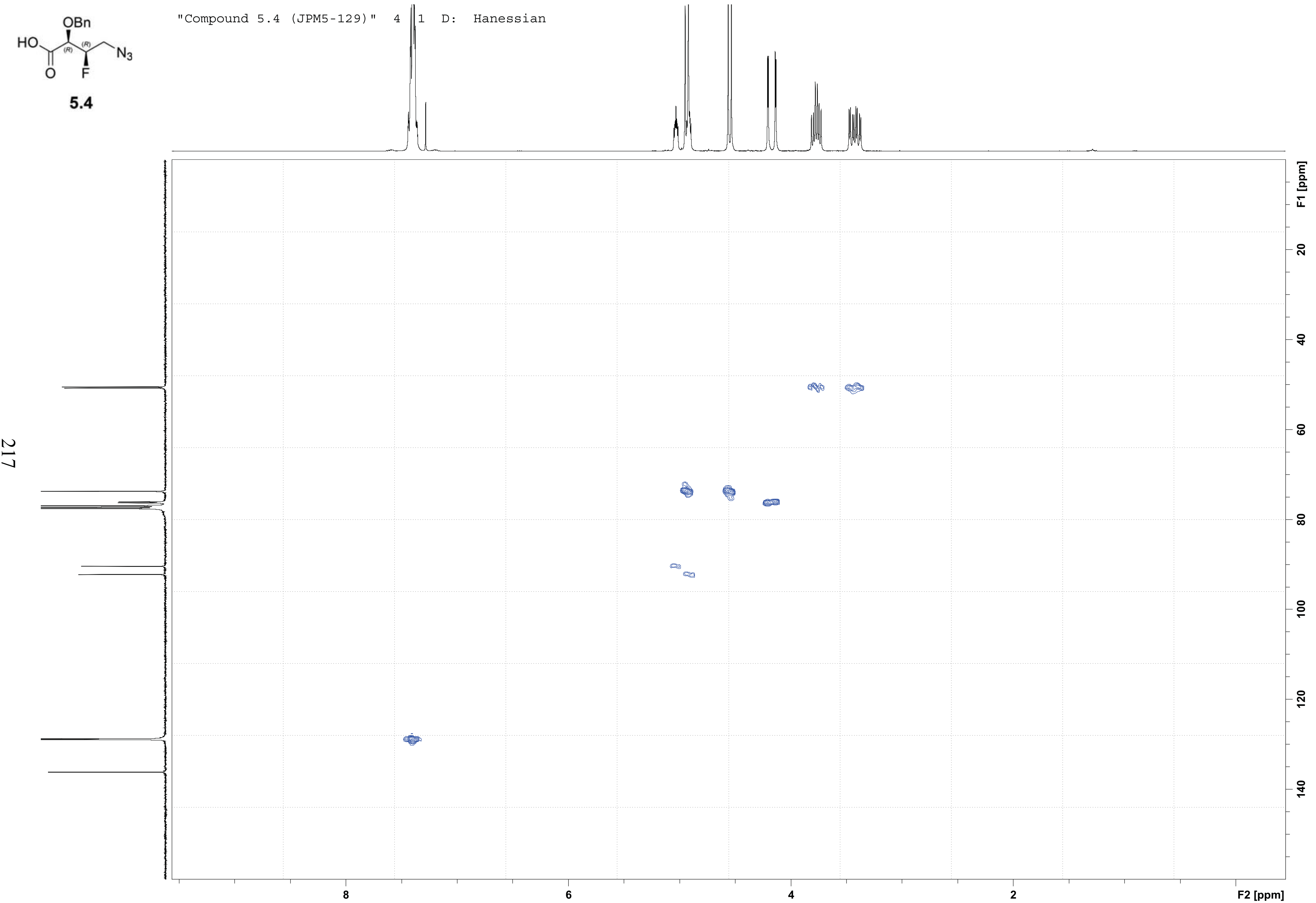
OBn
HO
(R)
(R)
N_3
O
F
5.4

20 [rel]
15
10
5
0

- 198.0
- 198.2
- 198.4
- 198.6
- 198.8
[ppm]



OBn
HO
(R)
(R)
N3
O
F
5.4
"Compound 5.4 (JPM5-129)" 3 1 D: Hanessian
F1 [ppm]
2
4
6
8
F2 [ppm]



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OBn
HO
(R)
(R)
N3
O
F
5.4
F1 [ppm]
20
40
60
80
100
120
140
8
6
4
2
F2 [ppm]

"Compound 5.6 (JPM5-137)" 2 1 D: Hanessian

7.416
7.411
7.398
7.392
7.381
7.374

6.474

4.849
4.821
4.680
4.651
4.400
4.376
4.370
4.345

3.784
3.754
3.748
3.717

OBn
HO
(R)
N_3
O
F F

5.6

5.475
1.646
1.062
1.045
1.042
2.097

[rel]

8
6
4
2
0

10
8
6
4
2
[ppm]

"Compound 5.6 (JPM5-137)" 6 1 D: Hanessian

"Compound 5.6 (JPM5-137)" 1 1 D: Hanessian

-109.273
-109.299
-109.312
-109.337
-109.350
-109.377
-109.965
-109.991
-110.004
-110.030
-110.043
-110.069
-112.207
-112.239
-112.272
-112.304
-112.899
-112.932
-112.964
-112.997

OBn
HO
(R)
N3
O
F F

5.6

[rel]
20
15
10
5
0

-80 -90 -100 -110 -120 -130 -140 [ppm]

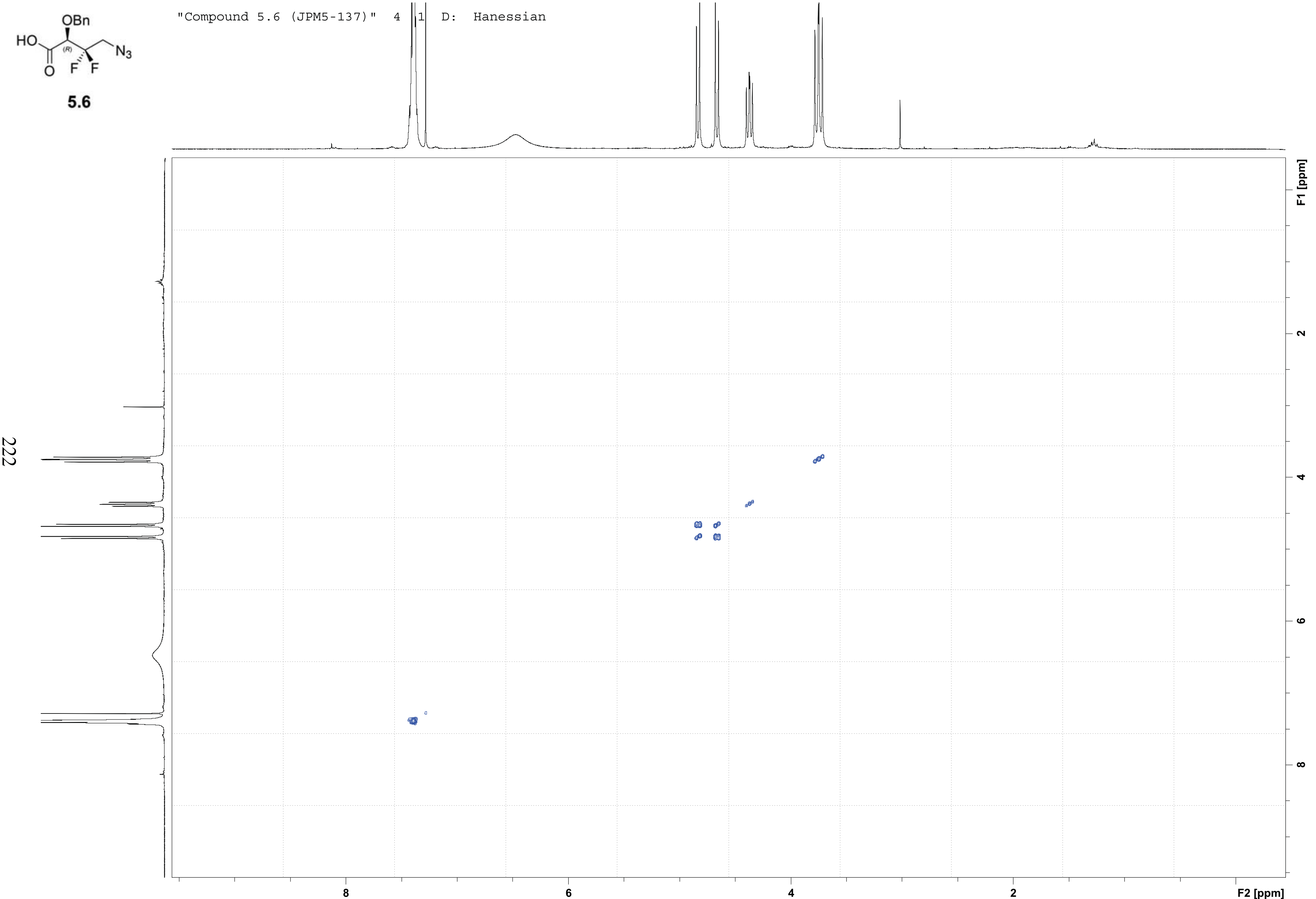
"Compound 5.6 (JPM5-137)" 1 1 D: Hanessian

-109.273
-109.299
-109.312
-109.337
-109.350
-109.377
-109.965
-109.991
-110.004
-110.030
-110.043
-110.069
-112.207
-112.239
-112.272
-112.304
-112.899
-112.932
-112.964
-112.997

OBn
HO
(R)
N_3
O
F F
5.6

[rel]
15
10
5
0

- 108 - 109 - 110 - 111 - 112 - 113 - 114 [ppm]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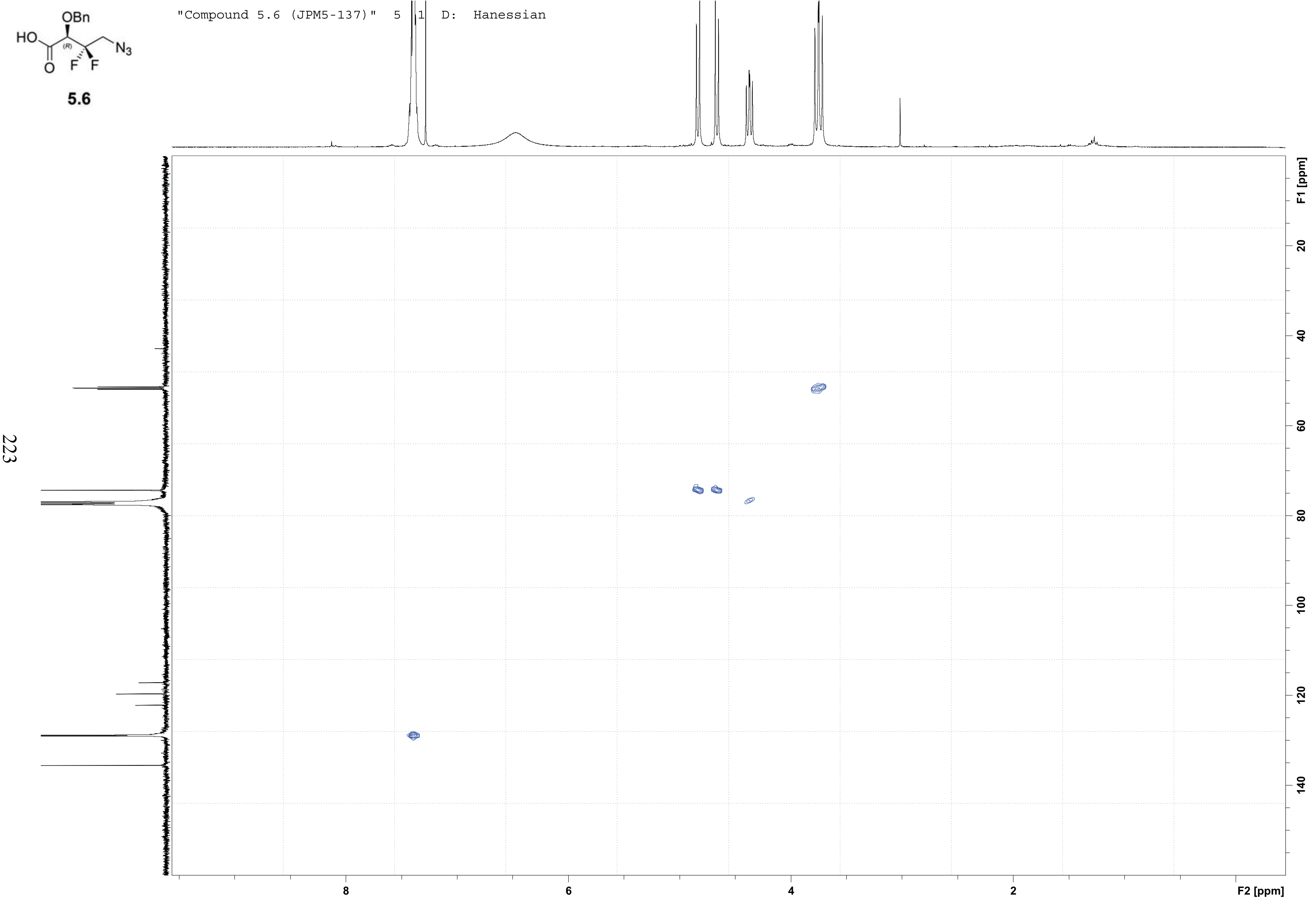
OBn
HO
(R)
N_3
O
F F
5.6
F1 [ppm]
F2 [ppm]
8
6
4
2



OBn
HO
(R)
N3
O
F
F
5.6
"Compound 5.6 (JPM5-137)" 5 1 D: Hanessian
F1 [ppm]
20
40
60
80
100
120
140
8
6
4
2
F2 [ppm]

"Compound 5.8 (JPM5-127)" 1 1 D: Hanessian

OBn
HO
(S)
(S)
N3
O
OBn

5.8

7.334
7.311
7.301

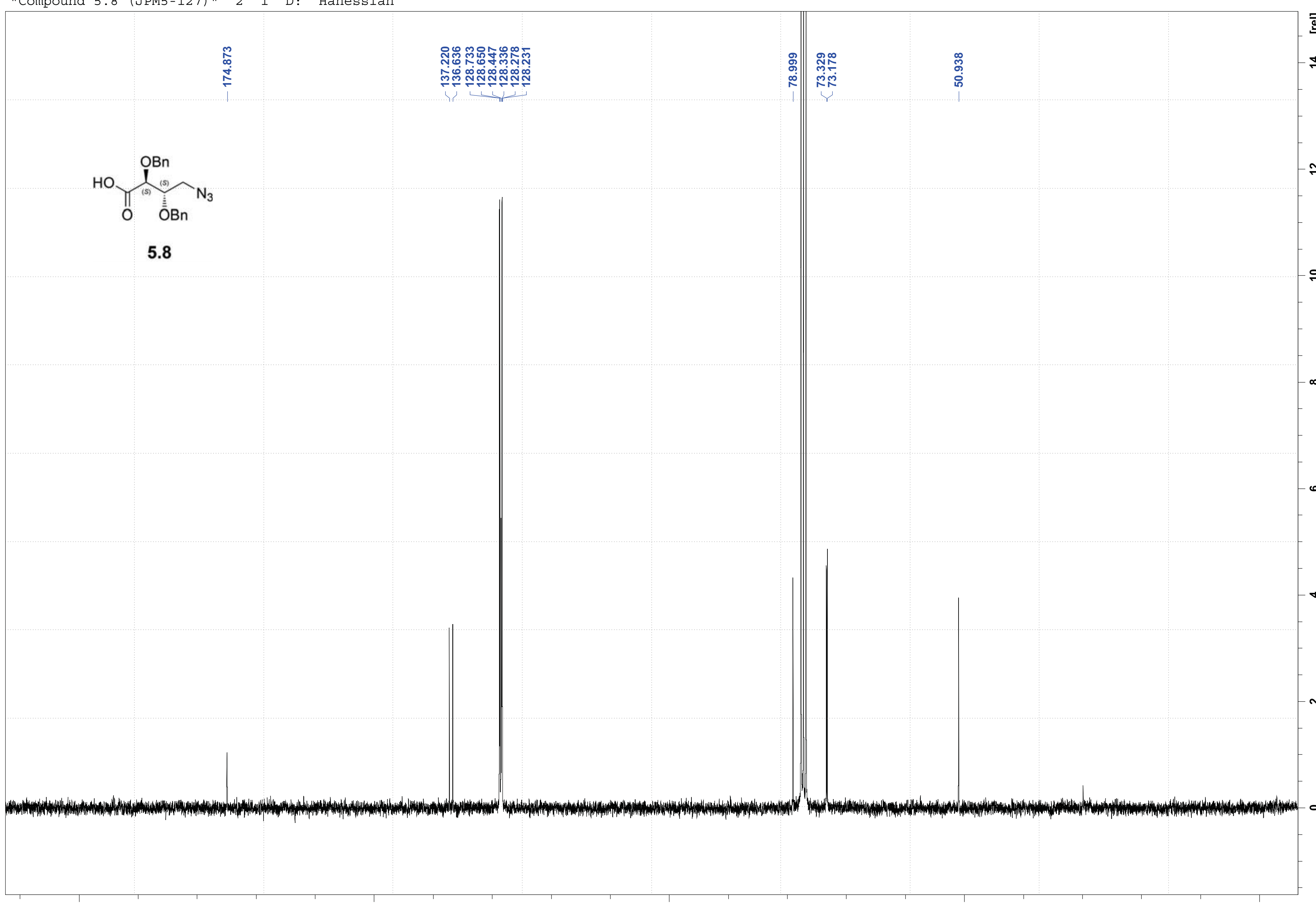
4.778
4.740
4.634
4.549
4.510
4.179
4.165
3.976
3.962
3.954
3.947
3.940
3.925
3.579
3.557
3.536
3.514
3.418
3.404
3.374
3.360

11.903

1.011
2.026
1.040
1.022
1.026
1.000
1.027

10
8
6
4
2
[ppm]

[rel]
10
8
6
4
2
0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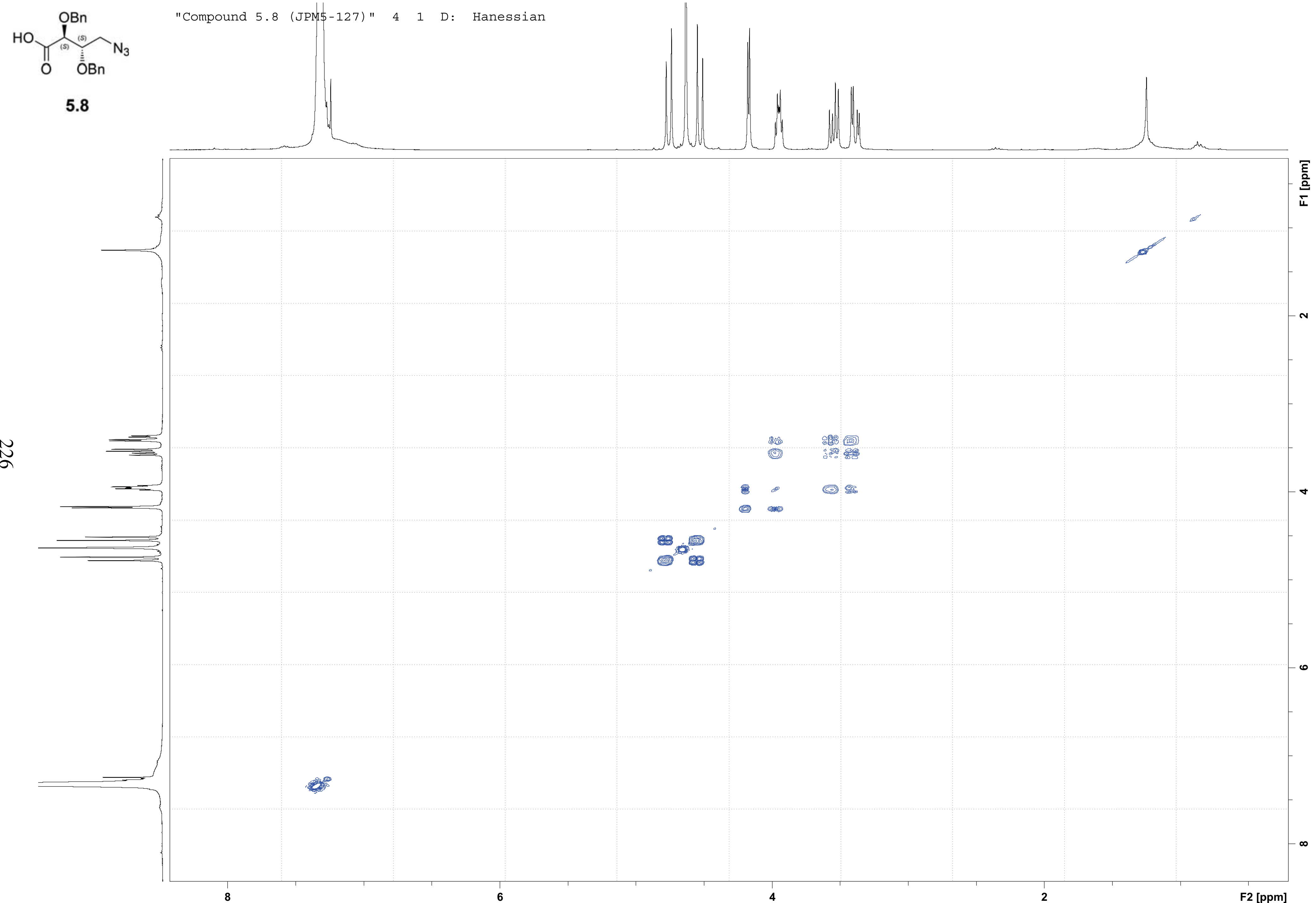
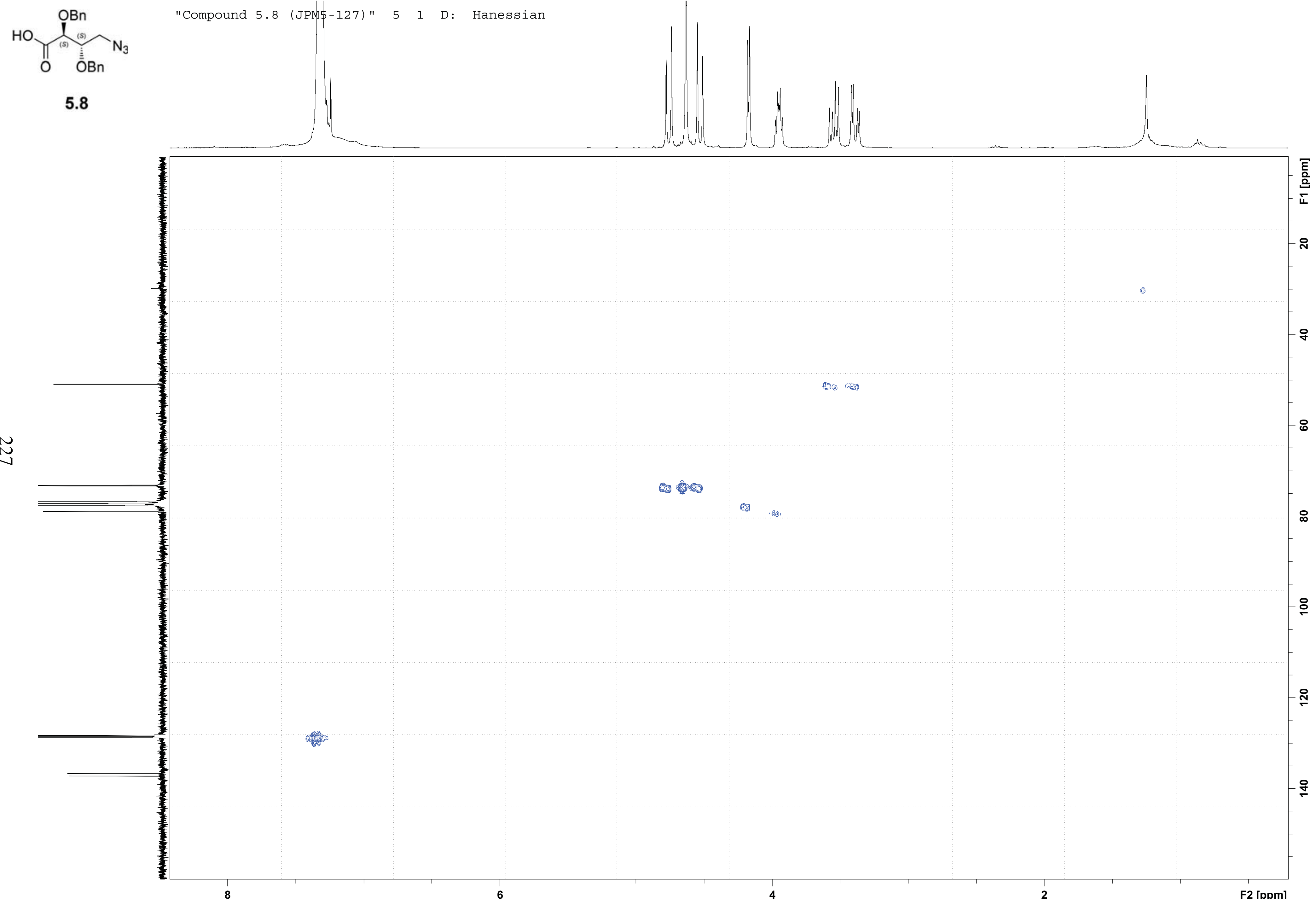
137.220
136.636
128.733
128.650
128.447
128.336
128.278
128.231
78.999
73.329
73.178
50.938
OBn
HO
(S)
(S)
N3
O
OBn
5.8
[rel]
14
12
10
8
6
4
2
0
200
150
100
50
[ppm]


"Compound 5.8 (JPM5-127)" 4 1 D: Hanessian
OBn
HO
(S)
(S)
N_3
O
OBn
5.8
F1 [ppm]
2
4
6
8
F2 [ppm]
8
6
4
2


OBn
HO
(S)
(S)
N3
O
OBn
5.8
"Compound 5.8 (JPM5-127)" 5 1 D: Hanessian
F1 [ppm]
20
40
60
80
100
120
140
8
6
4
2
F2 [ppm]

"Compound 5.11 (JPM5-177)" 1 1 D: Hanessian

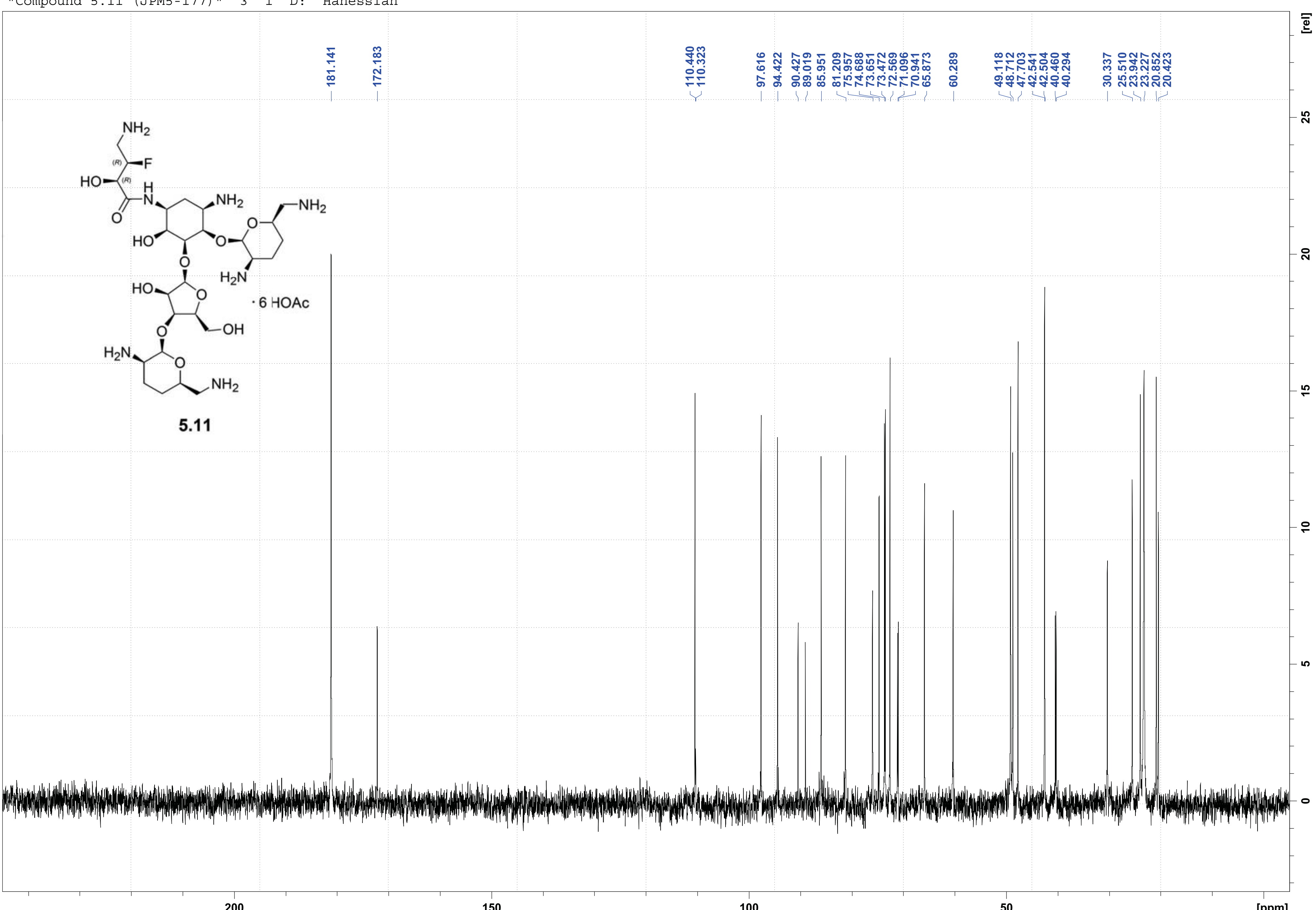
5.302, 5.224, 5.207, 5.003, 4.462, 4.447, 4.402, 4.360, 4.354, 4.178, 4.148, 4.142, 4.133, 4.132, 4.129, 4.120, 4.112, 4.026, 4.004, 3.985, 3.978, 3.959, 3.940, 3.913, 3.908, 3.888, 3.883, 3.879, 3.859, 3.841, 3.737, 3.727, 3.713, 3.703, 3.647, 3.638, 3.617, 3.544, 3.535, 3.520, 3.513, 3.503, 3.489, 3.471, 3.458, 3.452, 3.441, 3.424, 3.388, 3.361, 3.338, 3.283, 3.278, 3.256, 3.250, 3.239, 3.218, 3.212, 3.160, 3.143, 3.133, 3.116, 3.099, 3.084, 3.072, 3.058, 2.248, 2.240, 2.232, 2.223, 2.215, 2.208, 2.170, 2.142, 2.124, 2.096, 2.066, 2.040, 2.016, 2.000, 1.914, 1.798, 1.786, 1.772, 1.747, 1.712, 1.683, 1.623, 1.614, 1.598, 1.591, 1.572, 1.565, 1.550, 1.541, 1.519, 1.510, 1.492, 1.484, 1.466, 1.459, 1.441, 1.431

0.972, 1.002, 0.993, 1.043, 1.982, 1.009, 1.062, 0.984, 2.903, 2.101, 1.031, 2.043, 1.145, 2.900, 2.023, 1.017, 0.985, 0.967, 2.115, 2.142, 17.847, 2.106, 1.027, 1.000

[rel]

6 5 4 3 2 1 0

8 6 4 2 [ppm]


"Compound 5.11 (JPM5-177)" 3 1 D: Hanessian
181.141
172.183
110.440
110.323
97.616
94.422
90.427
89.019
85.951
81.209
75.957
74.688
73.651
73.472
72.569
71.096
70.941
65.873
60.289
49.118
48.712
47.703
42.541
42.504
40.460
40.294
30.337
25.510
23.942
23.227
20.852
20.423
NH2
(R)
F
HO
(R)
H
N
O
NH2
NH2
O
HO
O
O
H2N
HO
O
· 6 HOAc
O
OH
H2N
O
NH2
5.11
[rel]
25
20
15
10
5
0
200
150
100
50
[ppm]

"Compound 5.11 (JPM5-177)" 4 1 D: Hanessian

-206.246
-206.304
-206.359
-206.416
-206.473
-206.526
-206.586
-206.636
-206.698

5.11

· 6 HOAc

[rel]

30

20

10

0

- 140

- 160

- 180

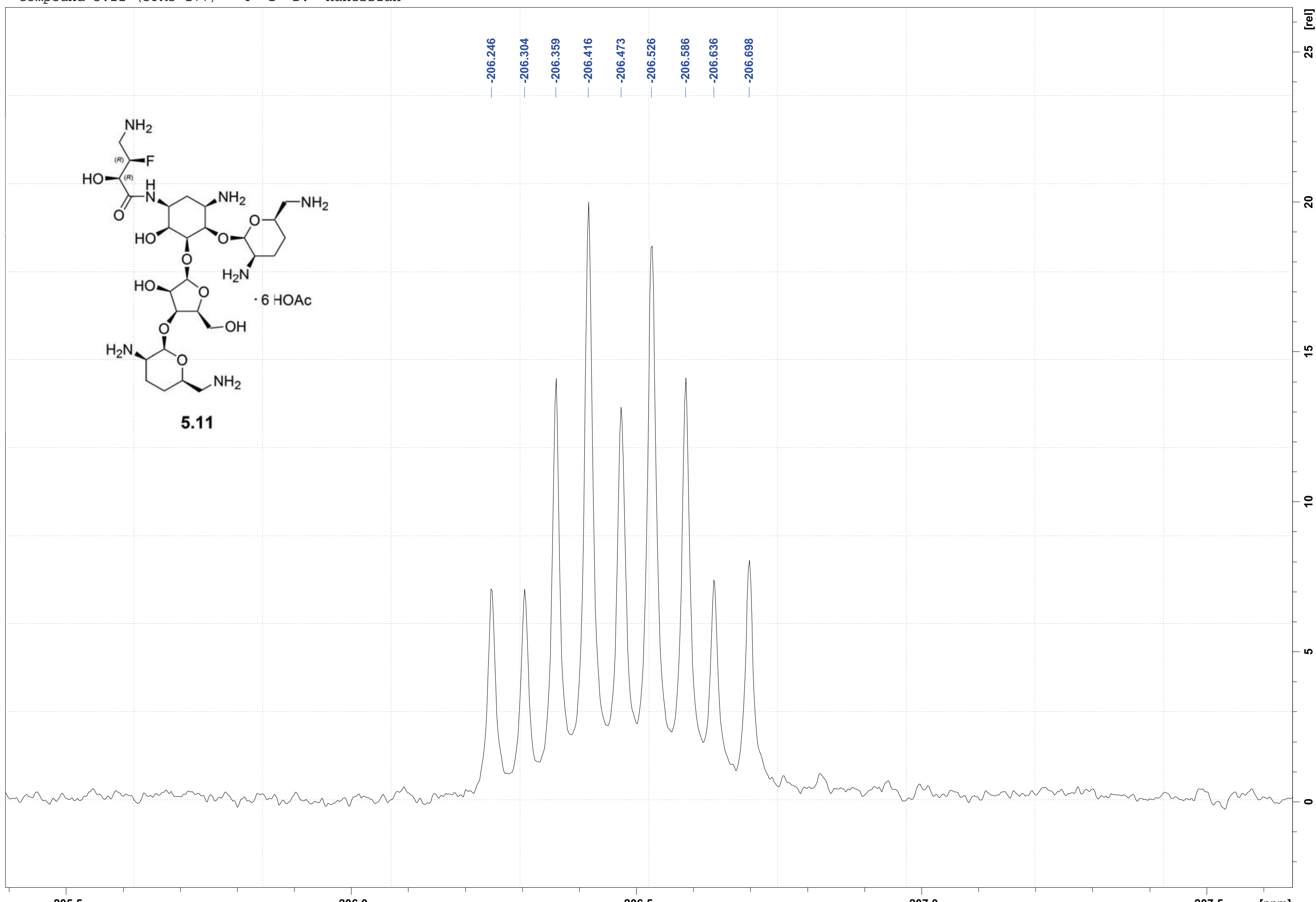
- 200

- 220

- 240

- 260

[ppm]



"Compound 5.11 (JPM5-177)" 4 1 D: Hanessian
-206.246
-206.304
-206.359
-206.416
-206.473
-206.526
-206.586
-206.636
-206.698
[rel]
25
20
15
10
5
0
- 205.5
- 206.0
- 206.5
- 207.0
- 207.5
[ppm]
NH2
F
HO
H
N
O
NH2
O
NH2
HO
O
H2N
O
HO
O
· 6 HOAc
OH
O
H2N
O
NH2
5.11

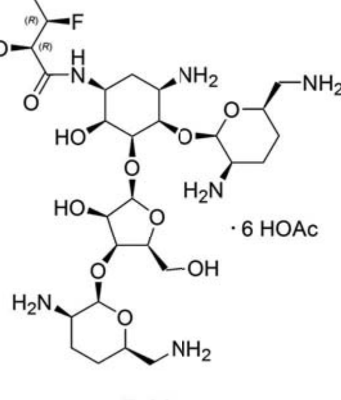
Data File C:\CHEM32\1\DATA\20090720RD-AG\680\RD-AG000018.D
Sample Name: JPM-5-177

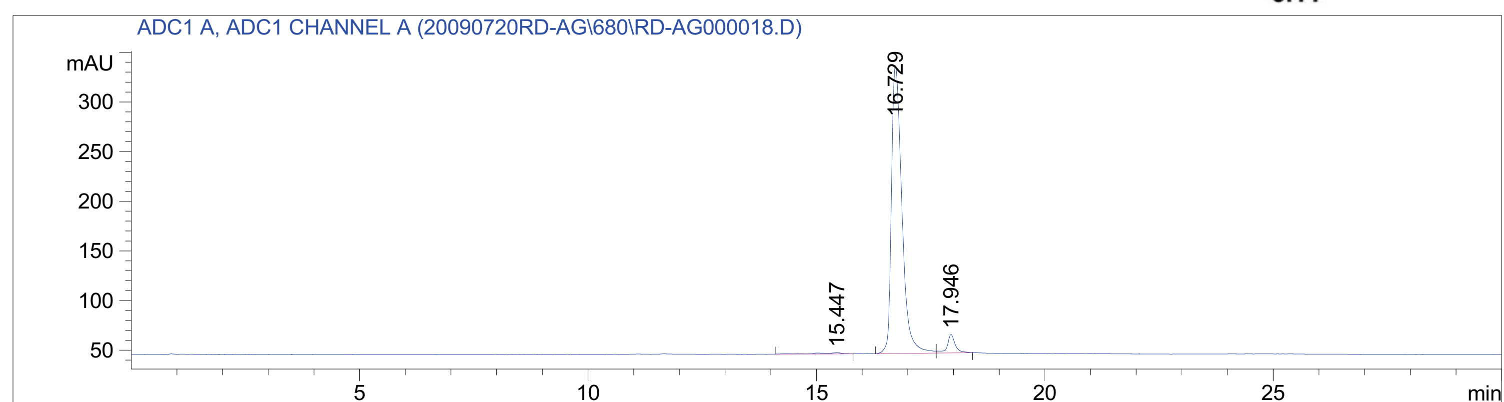
===

Acq. Operator	: rd	Seq. Line :	18
Acq. Instrument	: Chemstation 8	Location :	Vial 27
Injection Date	: 7/20/2009 9:12:11 PM	Inj :	1
		Inj Volume :	20 µl
Different Inj Volume from Sequence !		Actual Inj Volume :	5 µl

Acq. Method : C:\Chem32\1\DATA\20090720RD-AG\680\05-30-75B_30M.M
Last changed : 6/3/2009 4:40:11 PM by rd
Analysis Method : C:\CHEM32\1\METHODS\INT_30MIN.M
Last changed : 7/22/2009 10:16:51 AM by rd
(modified after loading)
Method Info : Integration method.

Sample Info : 20x dilution





===

Area Percent Report

===

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: ADC1 A, ADC1 CHANNEL A

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	15.447	MM	0.6713	46.90804	1.16453	0.9562
2	16.729	MF	0.2652	4632.53711	291.16046	94.4355
3	17.946	FM	0.2025	226.05675	18.60634	4.6082

Totals : 4905.50189 310.93133

===

*** End of Report ***

"Comopund 5.12 (JPM5-175)" 1 1 D: Hanessian

5.887 5.880 5.392 5.388 5.010 5.008 4.642 4.618 4.594 4.474 4.464 4.461 4.450 4.370 4.366 4.361 4.356 4.197 4.191 4.187 4.182 4.174 4.168 4.151 4.144 4.136 4.130 4.122 4.114 4.030 4.026 4.013 4.008 3.998 3.983 3.964 3.953 3.944 3.917 3.911 3.892 3.883 3.865 3.847 3.742 3.732 3.717 3.707 3.672 3.653 3.645 3.626 3.606 3.551 3.540 3.532 3.518 3.510 3.471 3.464 3.445 3.426 3.288 3.282 3.261 3.255 3.250 3.243 3.223 3.216 3.166 3.148 3.138 3.121 3.105 3.090 3.078 3.063 2.248 2.240 2.232 2.223 2.214 2.207 2.151 2.146 2.142 2.128 2.120 2.109 2.102 2.094 2.070 2.052 2.027 2.021 2.012 2.004 1.920 1.791 1.760 1.735 1.716 1.711 1.688 1.601 1.595 1.577 1.569 1.497 1.489 1.472 1.463

NH_2 F F HO (R) H N O NH_2 NH_2 O HO O O H_2N HO O · 6 HOAc O OH H_2N O NH_2

5.12

0.975 1.006 1.034 1.014 1.002 0.995 1.006 1.011 2.989 2.050 1.988 2.878 1.043 0.986 1.989 1.005 0.976 0.994 2.096 2.114 17.394 2.098 1.016 1.000

8 6 4 2 [ppm]

[rel] 5 4 3 2 1 0

"Comopund 5.12 (JPM5-175)" 3 1 D: Hanessian

181.097
169.224
120.905
118.920
116.933
97.636
94.403
85.966
81.237
75.900
74.708
73.692
73.482
72.572
71.363
71.161
70.957
65.885
60.305
49.133
48.952
48.701
47.707
42.516
41.341
41.152
40.957
30.268
25.492
23.942
23.198
20.852
20.422

[rel]

25
20
15
10
5
0

200
150
100
50
[ppm]

NH_2
F
F
HO
(R)
H
N
O
NH_2
NH_2
O
HO
O
O
H_2N
HO
O
· 6 HOAc
OH
O
H_2N
O
NH_2

5.12

"Comopund 5.12 (JPM5-175)" 4 1 D: Hanessian

-114.776
-114.820
-114.844
-114.862
-114.889
-114.898
-114.931
-114.942
-114.967
-115.010

[rel]

30

20

10

0

- 50

- 100

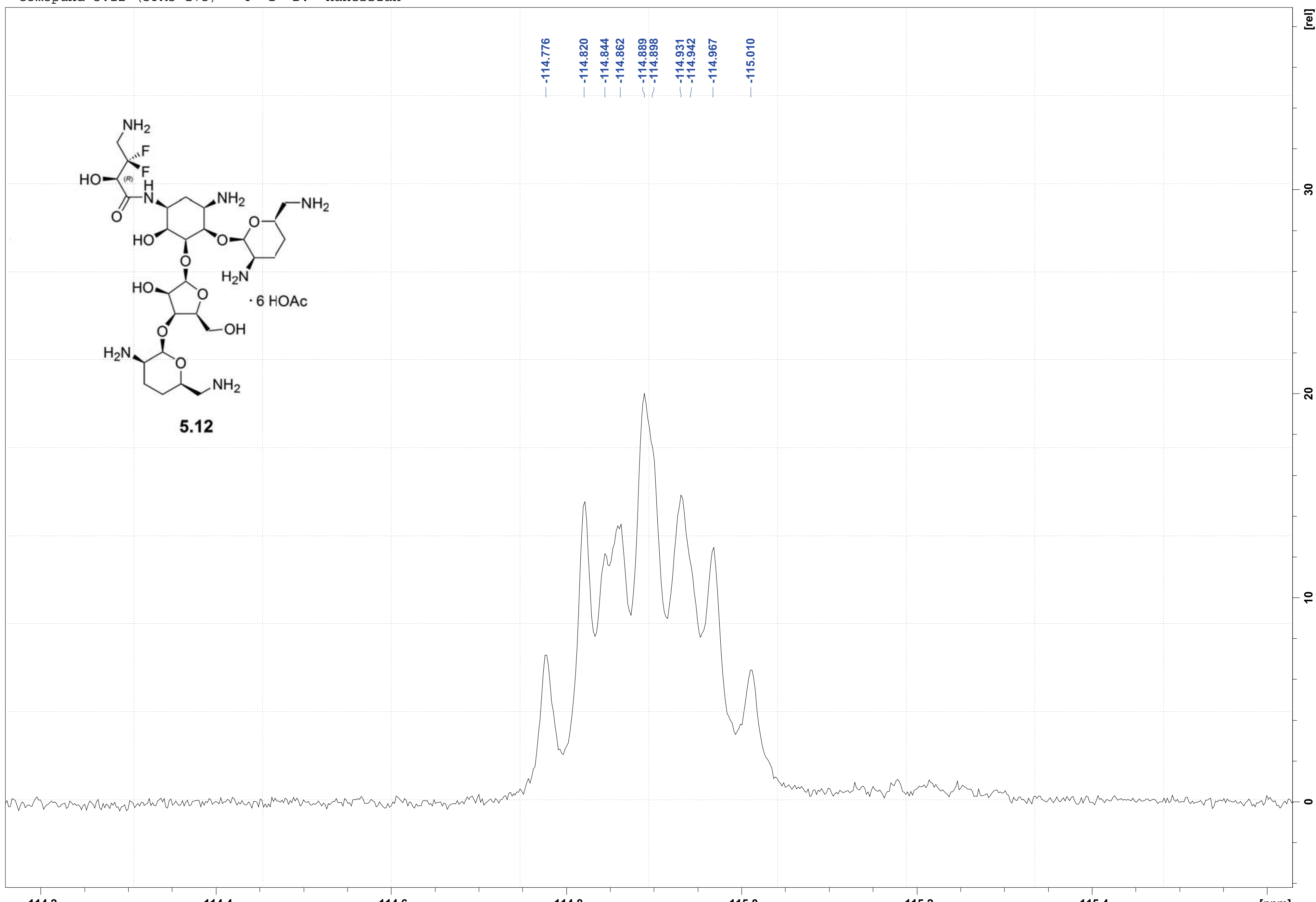
- 150

- 200

[ppm]

NH2
F
F
HO
(R)
H
N
O
NH2
NH2
O
HO
O
O
H2N
HO
O
· 6 HOAc
O
OH
H2N
O
NH2

5.12



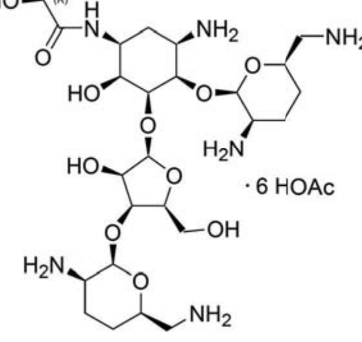
"Comopund 5.12 (JPM5-175)" 4 1 D: Hanessian
-114.776
-114.820
-114.844
-114.862
-114.889
-114.898
-114.931
-114.942
-114.967
-115.010
[rel]
30
20
10
0
- 114.2
- 114.4
- 114.6
- 114.8
- 115.0
- 115.2
- 115.4
[ppm]
NH2
F
F
HO
(R)
H
N
O
NH2
NH2
O
HO
O
O
H2N
HO
O
· 6 HOAc
OH
O
H2N
O
NH2
5.12

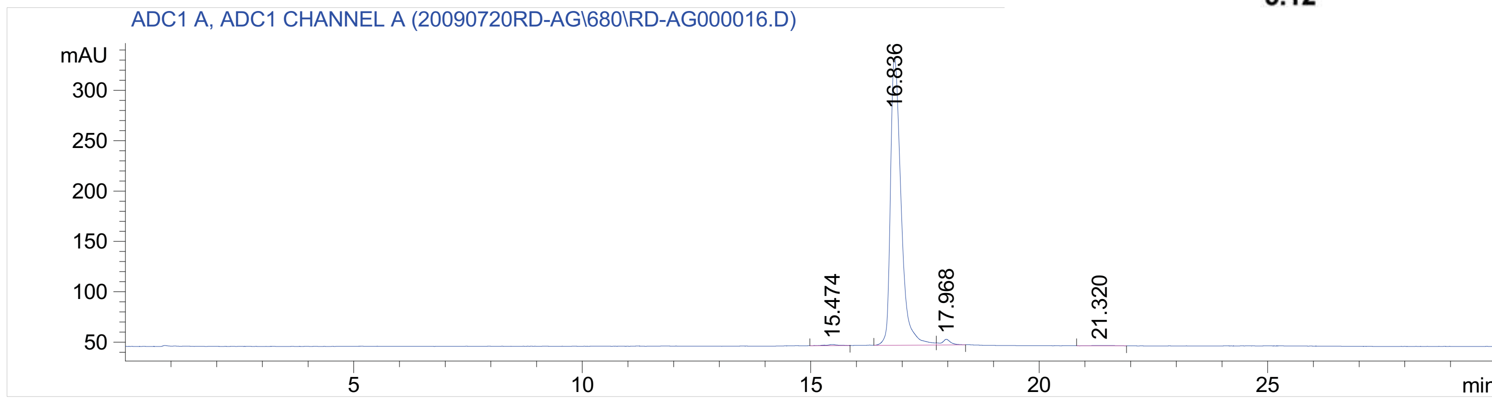
Data File C:\CHEM32\1\DATA\20090720RD-AG\680\RD-AG000016.D
Sample Name: JPM-5-175

===

Acq. Operator : rd Seq. Line : 16
Acq. Instrument : Chemstation 8 Location : Vial 26
Injection Date : 7/20/2009 8:10:03 PM Inj : 1
Inj Volume : 20 µl
Different Inj Volume from Sequence ! Actual Inj Volume : 5 µl
Acq. Method : C:\Chem32\1\DATA\20090720RD-AG\680\05-30-75B_30M.M
Last changed : 6/3/2009 4:40:11 PM by rd
Analysis Method : C:\CHEM32\1\METHODS\INT_30MIN.M
Last changed : 7/22/2009 10:16:51 AM by rd
(modified after loading)
Method Info : Integration method.

Sample Info : 20x dilution





===

Area Percent Report

===

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

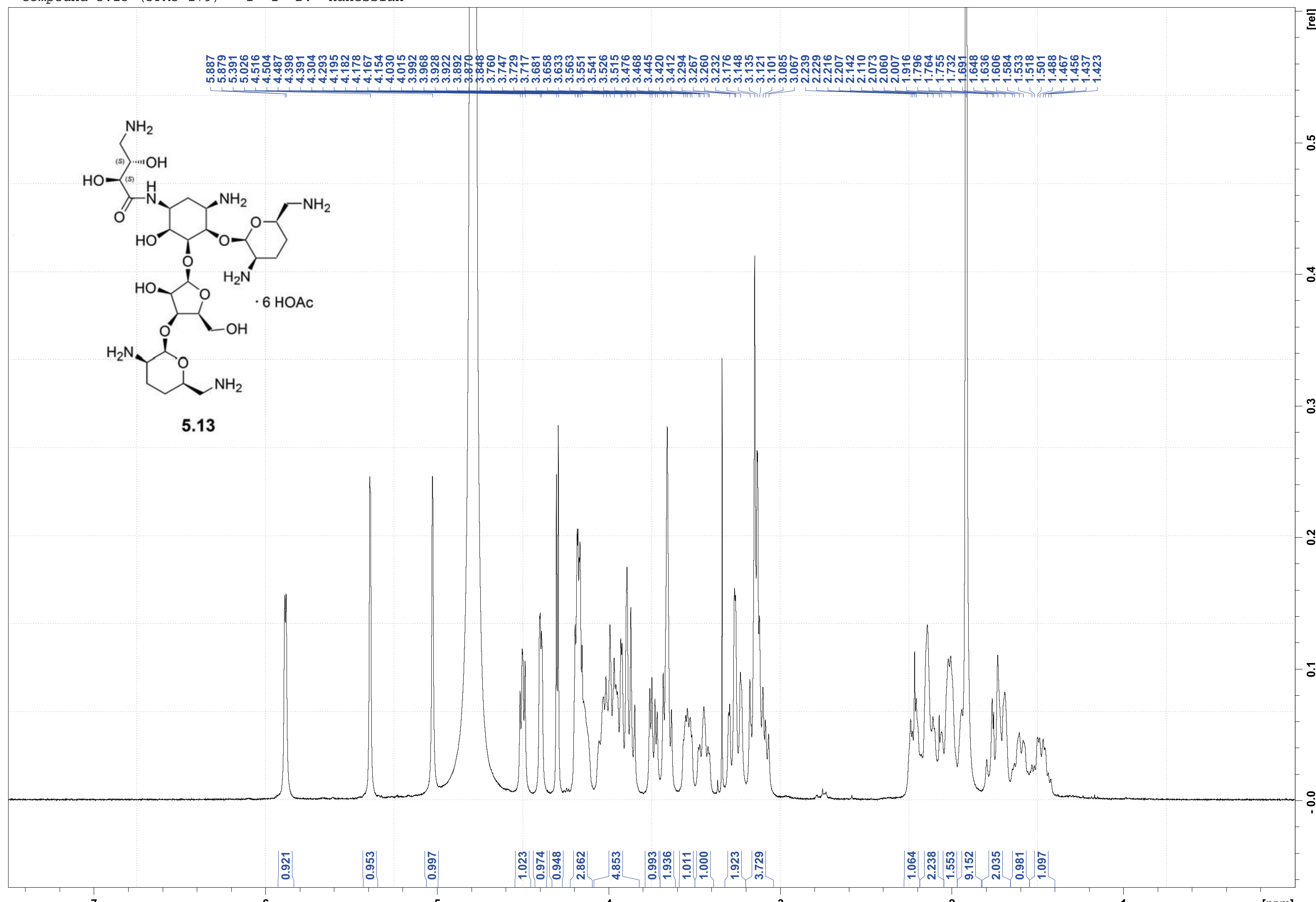
Signal 1: ADC1 A, ADC1 CHANNEL A

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	15.474	MM	0.3494	24.63910	1.17544	0.5217
2	16.836	MF	0.2682	4607.08398	286.30731	97.5469
3	17.968	FM	0.2321	78.56721	5.64193	1.6635
4	21.320	MM	0.5934	12.65054	3.55318e-1	0.2679

Totals : 4722.94083 293.48000

===

*** End of Report ***


"Compound 5.13 (JPM5-179)" 1 1 D: Hanessian
5.887 5.879 5.391 5.026 4.516 4.504 4.487 4.398 4.391 4.304 4.293 4.195 4.182 4.178 4.167 4.154 4.030 4.015 3.992 3.968 3.928 3.922 3.892 3.870 3.848 3.760 3.747 3.729 3.717 3.681 3.658 3.633 3.563 3.551 3.541 3.526 3.515 3.476 3.468 3.445 3.420 3.412 3.294 3.267 3.260 3.232 3.176 3.148 3.135 3.121 3.101 3.085 3.067 2.239 2.229 2.216 2.207 2.142 2.110 2.073 2.060 2.007 1.916 1.796 1.764 1.755 1.732 1.691 1.648 1.636 1.606 1.584 1.533 1.518 1.501 1.488 1.467 1.456 1.437 1.423
NH2
(S)
OH
HO
(S)
H
N
NH2
NH2
O
O
HO
O
O
H2N
HO
O
· 6 HOAc
O
OH
H2N
O
NH2
5.13
0.921
0.953
0.997
1.023
0.974
0.948
2.862
4.853
0.993
1.936
1.011
1.000
1.923
3.729
1.064
2.238
1.553
9.152
2.035
0.981
1.097
7
6
5
4
3
2
1
[ppm]
[rel]
0.5
0.4
0.3
0.2
0.1
-0.0

"Compound 5.13 (JPM5-179)" 2 1 D: Hanessian

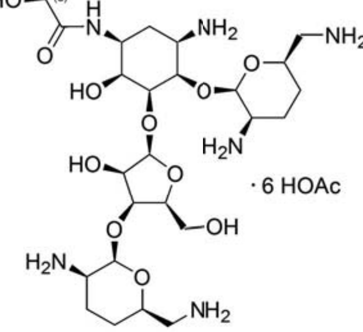
181.525, 173.586, 110.800, 97.885, 94.829, 86.346, 81.486, 76.321, 74.924, 74.113, 73.834, 73.658, 72.931, 68.982, 66.213, 60.671, 49.523, 49.092, 48.101, 42.911, 40.884, 30.802, 25.862, 24.323, 23.496, 21.252, 20.806

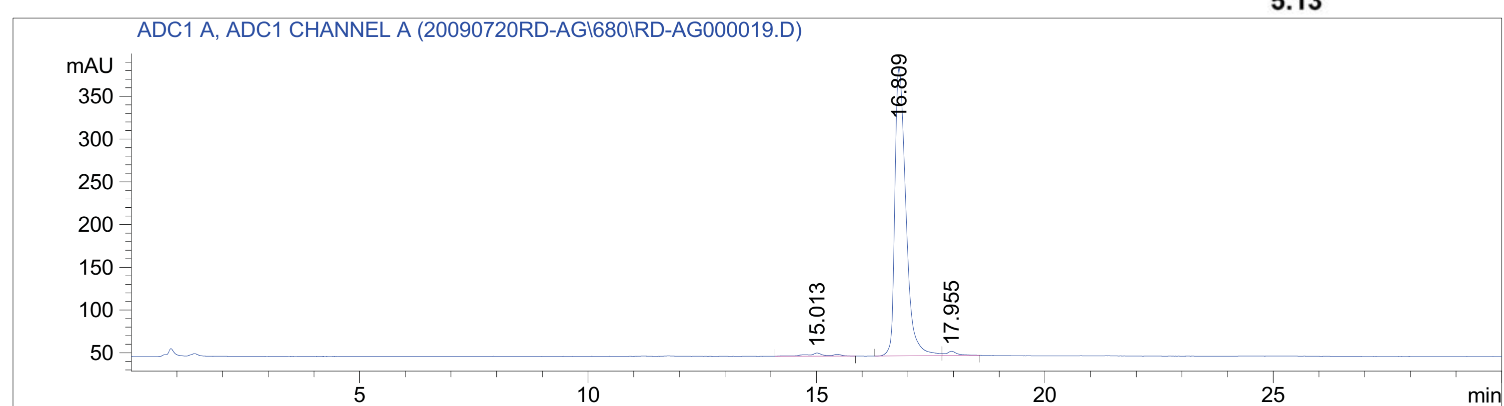
Data File C:\CHEM32\1\DATA\20090720RD-AG\680\RD-AG000019.D
Sample Name: JPM-5-179

===

Acq. Operator : rd   Seq. Line : 19
Acq. Instrument : Chemstation 8   Location : Vial 28
Injection Date : 7/20/2009 9:43:15 PM   Inj : 1
Inj Volume : 20 µl
Different Inj Volume from Sequence !   Actual Inj Volume : 5 µl
Acq. Method : C:\Chem32\1\DATA\20090720RD-AG\680\05-30-75B_30M.M
Last changed : 6/3/2009 4:40:11 PM by rd
Analysis Method : C:\CHEM32\1\METHODS\INT_30MIN.M
Last changed : 7/22/2009 10:16:51 AM by rd
(modified after loading)
Method Info : Integration method.

Sample Info : 20x dilution





===

Area Percent Report

===

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: ADC1 A, ADC1 CHANNEL A

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	15.013	MM	0.5230	116.93092	3.72629	1.9854
2	16.809	MF	0.2806	5681.50732	337.45233	96.4659
3	17.955	FM	0.2955	91.21282	5.14437	1.5487

Totals : 5889.65107 346.32299

===

*** End of Report ***

"Compound 5.14 (JPM5-151)" 2 1 D: Hanessian

"Compound 5.14 (JPM5-151)" 4 1 D: Hanessian

181.467
172.688
110.701
98.008
94.816
91.138
89.381
86.224
81.621
76.281
75.063
74.327
73.871
72.935
71.393
71.201
66.221
60.635
49.427
49.069
48.067
42.868
40.818
40.612
30.818
25.877
24.309
23.505
23.479
21.219
20.788

[rel]

25
20
15
10
5
0

NH2
(S)
F
HO
(S)
H
N
NH2
NH2
O
O
HO
O
O
H2N
HO
O
· 6 HOAc
O
OH
H2N
O
NH2

5.14

200
150
100
50
[ppm]

"Compound 5.14 (JPM5-151)" 1 1 D: Hanessian

-206.426
-206.468
-206.511
-206.553
-206.596
-206.638
-206.680
-206.721
-206.766

[rel]

25 20 15 10 5 0

NH2 F HO H N NH2 O HO O NH2 O H2N HO O · 6 HOAc O OH H2N O NH2

5.14

-180 -200 -220 -240 [ppm]

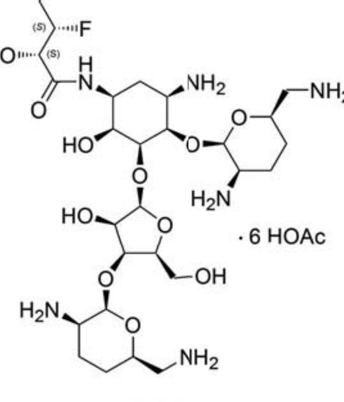
"Compound 5.14 (JPM5-151)" 1 1 D: Hanessian

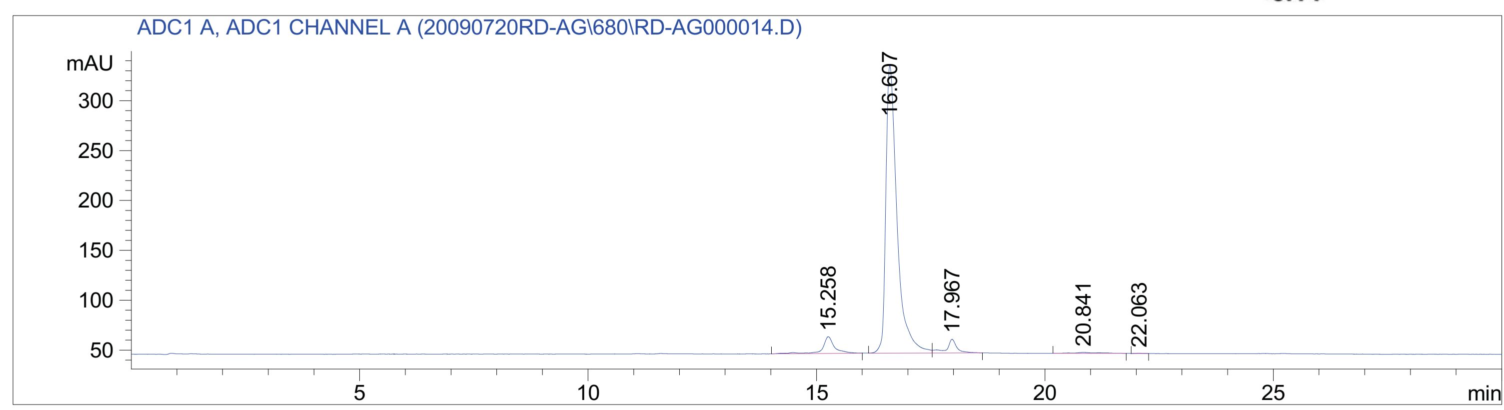
Data File C:\CHEM32\1\DATA\20090720RD-AG\680\RD-AG000014.D
Sample Name: JPM-5-151

===

Acq. Operator : rd   Seq. Line : 14
Acq. Instrument : Chemstation 8   Location : Vial 24
Injection Date : 7/20/2009 7:07:57 PM   Inj : 1
Inj Volume : 20 µl
Different Inj Volume from Sequence !   Actual Inj Volume : 5 µl
Acq. Method : C:\Chem32\1\DATA\20090720RD-AG\680\05-30-75B_30M.M
Last changed : 6/3/2009 4:40:11 PM by rd
Analysis Method : C:\CHEM32\1\METHODS\INT_30MIN.M
Last changed : 7/22/2009 10:16:51 AM by rd
(modified after loading)
Method Info : Integration method.

Sample Info : 20x dilution





===

Area Percent Report

===

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: ADC1 A, ADC1 CHANNEL A

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	15.258	MM	0.3037	306.18039	16.80083	5.5798
2	16.607	MF	0.2830	4914.58496	289.43253	89.5632
3	17.967	FM	0.2625	218.36775	13.86411	3.9795
4	20.841	MM	0.7193	43.64676	1.01128	0.7954
5	22.063	MM	0.2028	4.50189	3.69911e-1	0.0820

Totals : 5487.28175 321.47865

===

*** End of Report ***

"Compound 5.15 (JPM5-154)" 1 1 D: Hanessian

5.883 5.876 5.391 5.386 5.008 5.005 4.646 4.622 4.598 4.471 4.461 4.458 4.448 4.365 4.360 4.356 4.351 4.196 4.190 4.186 4.183 4.181 4.178 4.173 4.167 4.142 4.134 4.129 4.120 4.113 4.028 4.015 4.011 4.006 3.993 3.990 3.986 3.982 3.970 3.960 3.940 3.913 3.907 3.888 3.882 3.864 3.846 3.739 3.729 3.714 3.704 3.677 3.652 3.646 3.626 3.618 3.606 3.548 3.540 3.537 3.529 3.526 3.517 3.514 3.507 3.452 3.447 3.442 3.286 3.279 3.259 3.252 3.241 3.220 3.214 3.162 3.145 3.136 3.118 3.102 3.087 3.075 3.060 2.230 2.222 2.214 2.205 2.197 2.148 2.144 2.139 2.135 2.126 2.100 2.092 2.024 2.018 2.010 2.002 1.918 1.789 1.759 1.734 1.719 1.714 1.709 1.690 1.686 1.681 1.593 1.575 1.493 1.484 1.469

NH2 F F HO (S) H N O NH2 O NH2 HO O O H2N O HO O OH O H2N O NH2 · 6 HOAc

5.15

0.957 0.984 1.017 1.040 0.979 0.975 0.986 0.995 2.029 0.922 1.994 1.948 2.929 1.013 0.980 1.950 0.988 0.962 1.002 2.115 2.058 17.131 2.087 1.015 1.000

7 6 5 4 3 2 1 [ppm]

[rel] 0 1 2 3

"Compound 5.15 (JPM5-154)" 3 1 D: Hanessian

"Compound 5.15 (JPM5-154)" 4 1 D: Hanessian

-113.897
-113.934
-113.975
-114.010
-114.042
-114.793
-114.834
-114.866
-114.904
-114.946
-115.134
-115.176
-115.215
-115.249
-115.292
-116.030
-116.071
-116.110
-116.146
-116.189

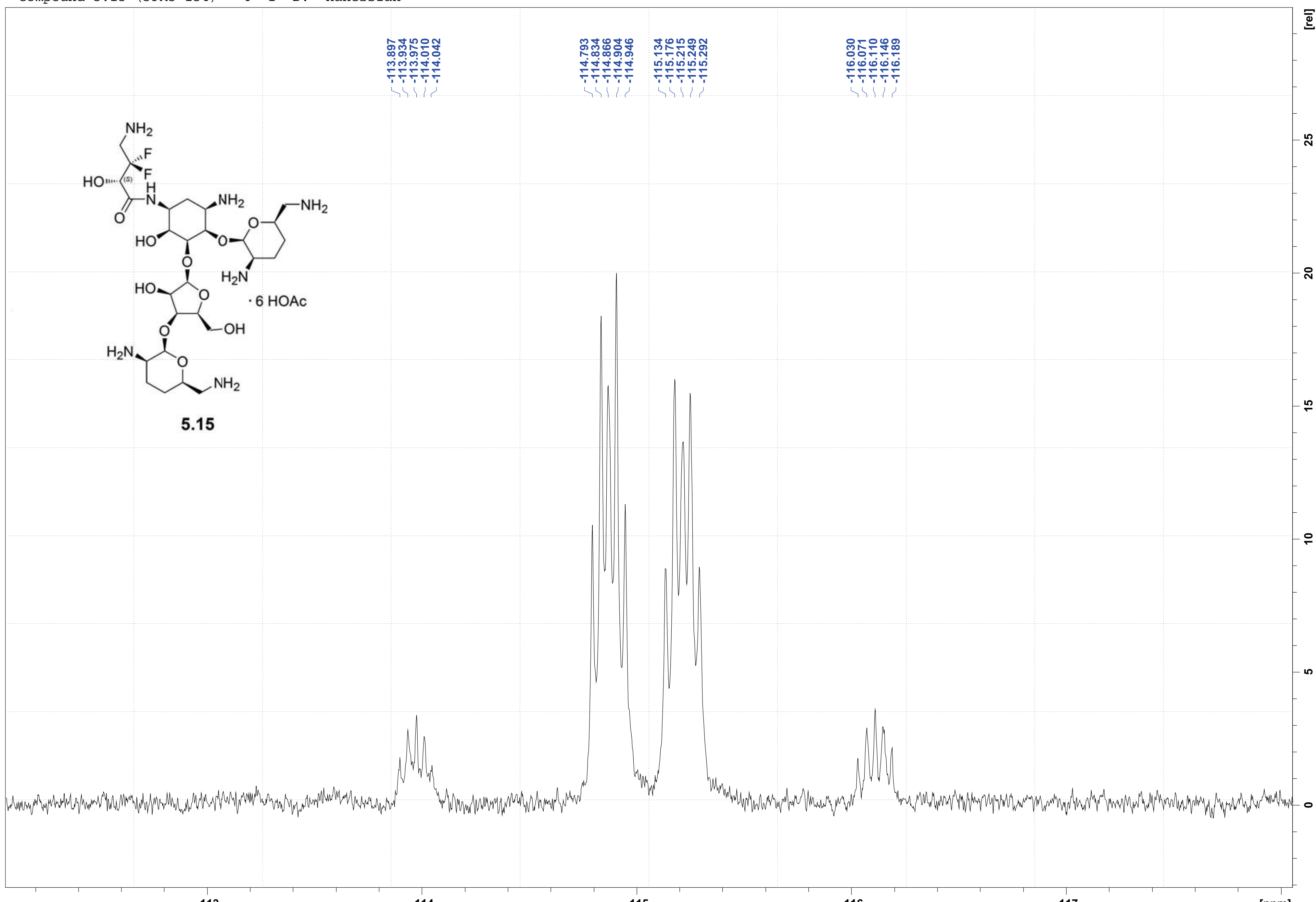
NH2, F, F, HO, (S), H, N, NH2, O, NH2, O, HO, O, O, H2N, HO, O, · 6 HOAc, OH, O, H2N, O, NH2

5.15

[rel]

25 20 15 10 5 0

- 40 - 60 - 80 - 100 - 120 - 140 - 160 - 180 [ppm]



"Compound 5.15 (JPM5-154)" 4 1 D: Hanessian
-113.897
-113.934
-113.975
-114.010
-114.042
-114.793
-114.834
-114.866
-114.904
-114.946
-115.134
-115.176
-115.215
-115.249
-115.292
-116.030
-116.071
-116.110
-116.146
-116.189
· 6 HOAc
5.15
[rel]
[ppm]

Data File C:\CHEM32\1\DATA\20090720RD-AG\680\RD-AG000015.D
Sample Name: JPM-5-154

===

Acq. Operator : rd   Seq. Line : 15
Acq. Instrument : Chemstation 8   Location : Vial 25
Injection Date : 7/20/2009 7:38:59 PM   Inj : 1
Inj Volume : 20 µl
Different Inj Volume from Sequence ! Actual Inj Volume : 5 µl
Acq. Method : C:\Chem32\1\DATA\20090720RD-AG\680\05-30-75B_30M.M
Last changed : 6/3/2009 4:40:11 PM by rd
Analysis Method : C:\CHEM32\1\METHODS\INT_30MIN.M
Last changed : 7/22/2009 10:16:51 AM by rd
(modified after loading)
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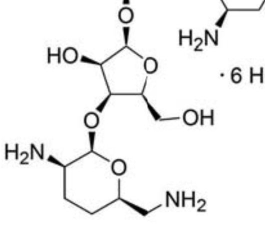
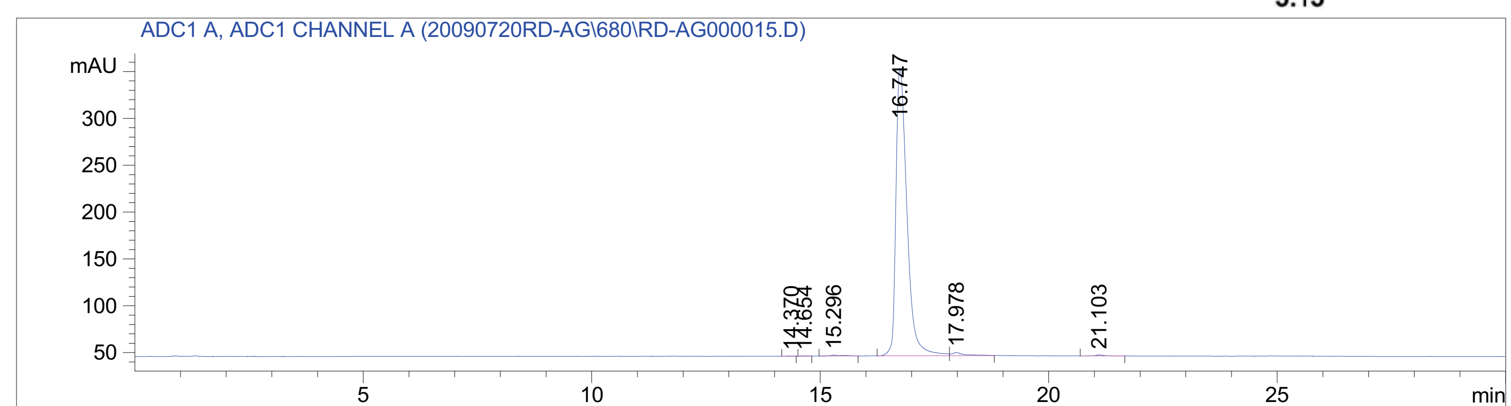
Sample Info : 20x dilution





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Area Percent Report

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Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: ADC1 A, ADC1 CHANNEL A

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	14.370	MM	0.1714	1.87630	1.82448e-1	0.0356
2	14.654	MM	0.1760	2.66441	2.52376e-1	0.0506
3	15.296	MM	0.3664	21.96153	9.99039e-1	0.4167
4	16.747	MF	0.2795	5161.20850	307.72284	97.9206
5	17.978	FM	0.3309	67.04270	3.37640	1.2720
6	21.103	MM	0.2527	16.05596	1.05881	0.3046

Totals : 5270.80940 313.59191

===

*** End of Report ***

"Compound 5.16 (JPM5-149)" 1 1 D: Hanessian

5.824 5.820 5.331 4.957 4.439 4.426 4.412 4.320 4.313 4.244 4.235 4.121 4.109 4.086 4.066 3.984 3.962 3.934 3.909 3.885 3.865 3.831 3.808 3.786 3.695 3.683 3.664 3.653 3.594 3.570 3.546 3.480 3.470 3.456 3.409 3.385 3.362 3.233 3.199 3.164 3.110 3.082 3.067 3.054 3.033 3.017 2.999 2.158 2.136 2.081 2.046 2.016 1.996 1.956 1.943 1.859 1.738 1.703 1.672 1.629 1.570 1.542 1.518 1.491 1.472 1.458 1.438 1.408 1.376 1.363

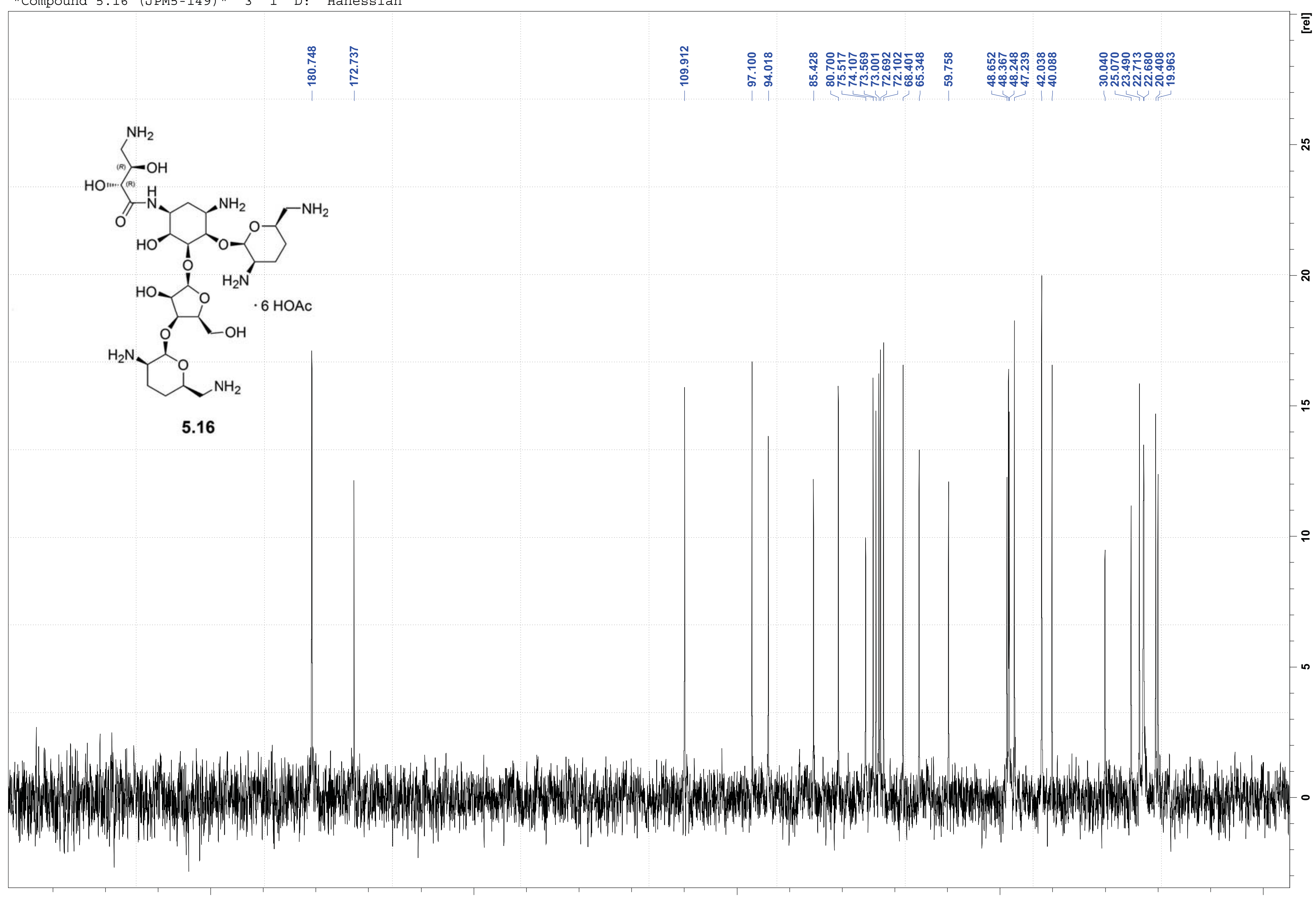
NH_2, OH, HO, (R), (R), H, N, O, NH_2, NH_2, O, HO, O, O, H_2N, HO, O, · 6 HOAc, O, OH, H_2N, O, NH_2

5.16

0.911 0.986 1.070 1.000 0.995 1.021 2.920 4.943 1.025 1.999 1.069 1.028 1.931 3.681 2.377 2.597 14.258 2.055 2.057

7 6 5 4 3 2 1 [ppm]

[rel] 0.5 0.4 0.3 0.2 0.1 -0.0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75.517
74.107
73.569
73.001
72.692
72.102
68.401
65.348
59.758
48.652
48.367
48.248
47.239
42.038
40.088
30.040
25.070
23.490
22.713
22.680
20.408
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150
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50
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NH2
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HO
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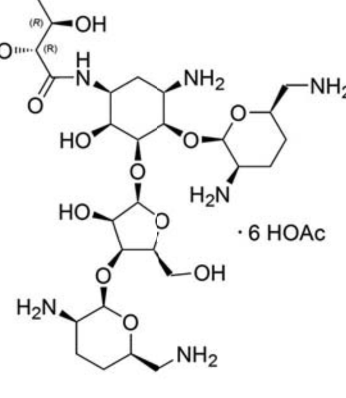
N
NH2
O
NH2
HO
O
H2N
HO
· 6 HOAc
OH
H2N
NH2
5.16

Data File C:\CHEM32\1\DATA\20090720RD-AG\680\RD-AG000012.D
Sample Name: JPM-5-149



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Acq. Instrument : Chemstation 8                     Location : Vial 23
Injection Date  : 7/20/2009 6:05:50 PM                   Inj :   1
                                                  Inj Volume : 20 µl
Different Inj Volume from Sequence !       Actual Inj Volume : 5 µl
Acq. Method     : C:\Chem32\1\DATA\20090720RD-AG\680\05-30-75B_30M.M
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Last changed    : 7/22/2009 10:16:51 AM by rd
                  (modified after loading)
Method Info     : Integration method.

Sample Info     : 20x dilution
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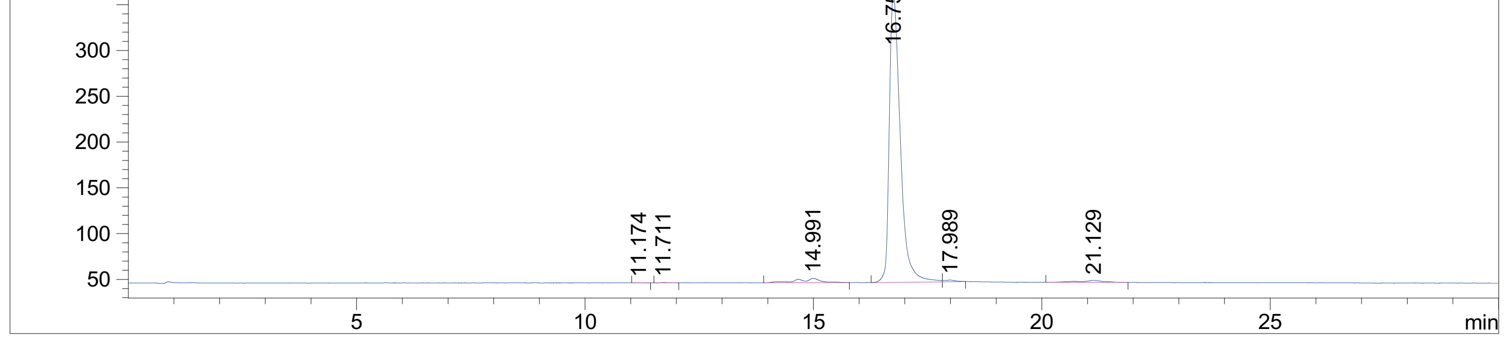

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Area Percent Report

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Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs
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Signal 1: ADC1 A, ADC1 CHANNEL A

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	11.174	MM	0.2023	2.59703	2.13988e-1	0.0467
2	11.711	MM	0.2642	5.41364	3.41519e-1	0.0974
3	14.991	MM	0.5720	164.35103	4.78857	2.9557
4	16.751	MF	0.2783	5267.21338	315.49460	94.7261
5	17.989	FM	0.2848	34.01289	1.99023	0.6117
6	21.129	MM	0.6513	86.87753	2.22310	1.5624
Totals :				5560.46549	325.05200	

===

*** End of Report ***

NL Fit (DoseResp) (6/25/2010 03:57:02)

Notes

Description	NL Fit
User Name	Pablo
Operation Time	6/25/2010 03:57:02
Model	DoseResp
Equation	y = A1 + (A2-A1)/(1 + 10^((LOGx0-x)*p))
Report Status	New Analysis Report
Multi-Data Fit Mode	Independent Fit - Consolidated Report

Parameters

		Value	Standard Error
5.10 (H) - C003932	A1	204192.84387	5399.54435
	A2	699764.46914	16423.45327
	LOGx0	1.44824	0.02151
	p	1.81813	0.1199
	EC50	28.06954	
5.11 (F) - C004675	A1	239496.60787	19502.09868
	A2	690975.5	0
	LOGx0	1.45209	0.05004
	p	1.61753	0.23002
	EC50	28.31951	
5.12 (diF) - C004674	A1	210539.5489	1687.50767
	A2	625563.45629	5961.00641
	LOGx0	1.67099	0.00842
	p	1.73401	0.05612
	EC50	46.88016	

All datasets were fitted successfully.
Some parameter values were fixed.

Statistics

	5.10 (H) - C003932	5.11 (F) - C004675	5.12 (diF) - C004674
Number of Points	8	8	8
Degrees of Freedom	4	5	4
Reduced Chi-Sqr	1.41584	31.70456	1.13534
Residual Sum of Squares	5.66335	158.52281	4.54137
Adj. R-Square	0.99945	0.98935	0.99998
Fit Status	Succeeded(100)	Succeeded(100)	Succeeded(100)

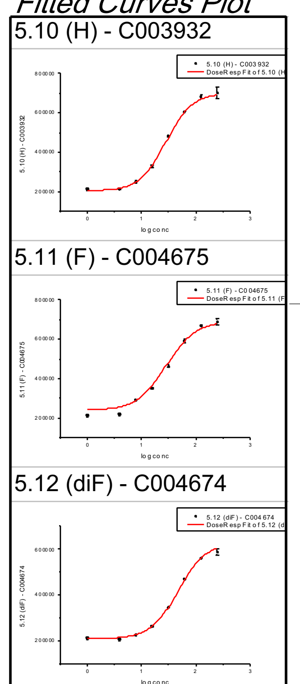
Fit Status Code :
100 : Fit converged

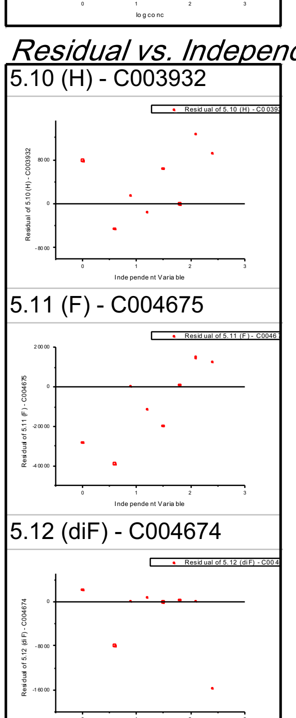
ANOVA

		DF	Sum of Squares	Mean Square	F Value	Prob>F
5.10 (H) - C003932	Regression	4	1.81625E12	4.54063E11	3.20702E11	0
	Residual	4	5.66335	1.41584		
	Uncorrected Total	8	1.81625E12			
	Corrected Total	7	18085.95701			
5.11 (F) - C004675	Regression	3	1.79344E12	5.97814E11	1.88558E10	0
	Residual	5	158.52281	31.70456		
	Uncorrected Total	8	1.79344E12			
	Corrected Total	7	20841.06038			
5.12 (diF) - C004674	Regression	4	1.21371E12	3.03429E11	2.67258E11	0
	Residual	4	4.54137	1.13534		
	Uncorrected Total	8	1.21371E12			

NL Fit (DoseResp) (6/25/2010 03:57:02)

Fitted Curves Plot





NL Fit (DoseResp) (6/25/2010 03:59:40)

Notes

Description	NL Fit
User Name	Pablo
Operation Time	6/25/2010 03:59:40
Model	DoseResp
Equation	y = A1 + (A2-A1)/(1 + 10^((LOGx0-x)*p))
Report Status	New Analysis Report
Multi-Data Fit Mode	Independent Fit - Consolidated Report

Parameters

		Value	Standard Error
5.13 (OH) - C004676	A1	214970.32544	4180.21725
	A2	596812	0
	LOGx0	1.41719	0.00842
	p	1.96505	0.07763
	EC50	26.13328	
Gentamicin	A1	196635.23655	9646.15352
	A2	651208.19995	41325.15821
	LOGx0	1.69046	0.07621
	p	1.24645	0.17856
	EC50	49.03	
Amikacin	A1	209148.83637	4713.2375
	A2	3.73056E8	1.08391E12
	LOGx0	6.48091	1461.98103
	p	0.865	0.43847
	EC50	3.02629E6	

Fitting failed on some of the datasets.
Some parameter values were fixed.

Statistics

	5.13 (OH) - C004676	Gentamicin	Amikacin
Number of Points	8	8	8
Degrees of Freedom	5	4	4
Reduced Chi-Sqr	1.32014	3.67143	2.36441
Residual Sum of Squares	6.60068	14.68571	9.45763
Adj. R-Square	0.99828	0.99415	0.9649
Fit Status	Succeeded(100)	Succeeded(100)	Reduced chi-sq(1)

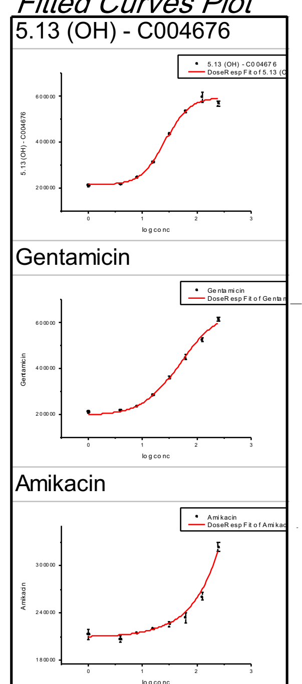
Fit Status Code :
100 : Fit converged
1 : Chi-sqr is reduced.

ANOVA

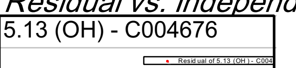
		DF	Sum of Squares	Mean Square	F Value	Prob>F
5.13 (OH) - C004676	Regression	3	1.41831E12	4.72771E11	3.58123E11	0
	Residual	5	6.60068	1.32014		
	Uncorrected Total	8	1.41831E12			
	Corrected Total	7	5369.67704			
Gentamicin	Regression	4	1.22321E12	3.05802E11	8.32924E10	0
	Residual	4	14.68571	3.67143		
	Uncorrected Total	8	1.22321E12			
	Corrected Total	7	4393.62396			
Amikacin	Regression	4	4.61716E11	1.15429E11	4.88194E10	0
	Residual	4	9.45763	2.36441		
	Uncorrected Total	8	4.61716E11			

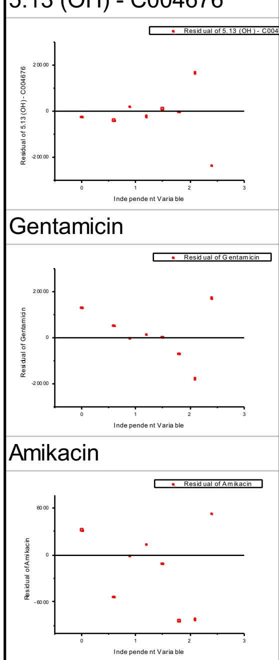
NL Fit (DoseResp) (6/25/2010 03:59:40)

Fitted Curves Plot



Residual vs. Independ





NL Fit (DoseResp) (6/25/2010 03:41:42)

Notes

Description	NL Fit
User Name	Pablo
Operation Time	6/25/2010 03:41:42
Model	DoseResp
Equation	y = A1 + (A2-A1)/(1 + 10^((LOGx0-x)*p))
Report Status	New Analysis Report
Multi-Data Fit Mode	Independent Fit - Consolidated Report

Parameters

		Value	Standard Error
5.10 (H) - C003932	A1	188599.60256	6105.79392
	A2	723536.72848	12003.41954
	LOGx0	1.46446	0.01696
	p	1.62524	0.0884
	EC50	29.1381	
5.14 (F) - C004672	A1	199052.05129	13692.02953
	A2	637213.10101	11091.68475
	LOGx0	1.30922	0.0153
	p	1.89708	0.26693
	EC50	20.38081	
5.15 (diF) - C004673	A1	222081.82241	1787.24061
	A2	643143.0918	21596.46492
	LOGx0	1.76976	0.03268
	p	2.1612	0.20775
	EC50	58.85125	

All datasets were fitted successfully.

Statistics

	5.10 (H) - C003932	5.14 (F) - C004672	5.15 (diF) - C004673
Number of Points	8	8	8
Degrees of Freedom	4	4	4
Reduced Chi-Sqr	1.16765	5.30064	0.97621
Residual Sum of Squares	4.67062	21.20258	3.90482
Adj. R-Square	0.99885	0.99601	0.99984
Fit Status	Succeeded(100)	Succeeded(100)	Succeeded(100)

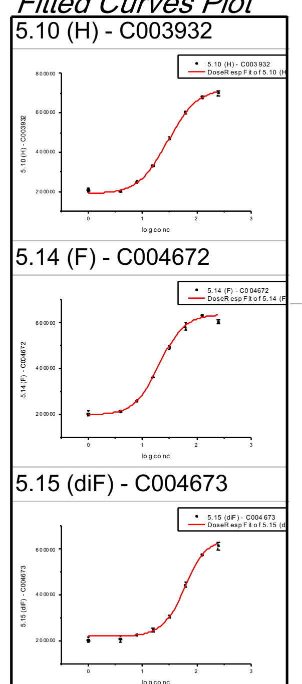
Fit Status Code :
100 : Fit converged

ANOVA

		DF	Sum of Squares	Mean Square	F Value	Prob>F
5.10 (H) - C003932	Regression	4	1.79214E12	4.48035E11	3.83705E11	0
	Residual	4	4.67062	1.16765		
	Uncorrected Total	8	1.79214E12			
	Corrected Total	7	7136.12239			
5.14 (F) - C004672	Regression	4	1.6406E12	4.10149E11	7.73772E10	0
	Residual	4	21.20258	5.30064		
	Uncorrected Total	8	1.6406E12			
	Corrected Total	7	9292.72475			
5.15 (diF) - C004673	Regression	4	1.19846E12	2.99616E11	3.06919E11	0
	Residual	4	3.90482	0.97621		
	Uncorrected Total	8	1.19846E12			

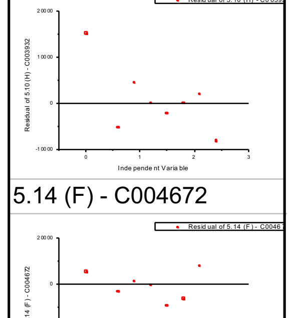
NL Fit (DoseResp) (6/25/2010 03:41:42)

Fitted Curves Plot

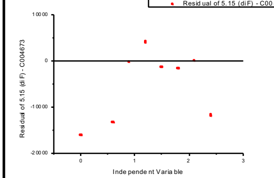


Residual vs. Independ

5.10 (H) - C003932



5.15 (diF) - C004673



NL Fit (DoseResp) (6/25/2010 03:50:59)

Notes

Description	NL Fit
User Name	Pablo
Operation Time	6/25/2010 03:50:59
Model	DoseResp
Equation	y = A1 + (A2-A1)/(1 + 10^((LOGx0-x)*p))
Report Status	New Analysis Report
Multi-Data Fit Mode	Independent Fit - Consolidated Report

Parameters

		Value	Standard Error
5.16 (OH) - C004671	A1	225700.83255	7854.34252
	A2	655510.40962	15782.79632
	LOGx0	1.48069	0.01354
	p	2.30975	0.23462
	EC50	30.24766	
Gentamicin	A1	198098.64076	6693.26525
	A2	663389.39987	43359.15768
	LOGx0	1.87514	0.087
	p	1.06634	0.10603
	EC50	75.01302	
Amikacin	A1	209872.52394	6847.79374
	A2	3.09274E7	1.65419E10
	LOGx0	4.45326	198.50185
	p	1.1978	1.5282
	EC50	28395.89832	

Fitting failed on some of the datasets.

Statistics

	5.16 (OH) - C004671	Gentamicin	Amikacin
Number of Points	8	8	8
Degrees of Freedom	4	4	4
Reduced Chi-Sqr	5.70338	0.36076	2.50386
Residual Sum of Squares	22.81354	1.44305	10.01545
Adj. R-Square	0.99799	0.99742	0.9891
Fit Status	Succeeded(100)	Succeeded(100)	Reduced chi-sq(1)

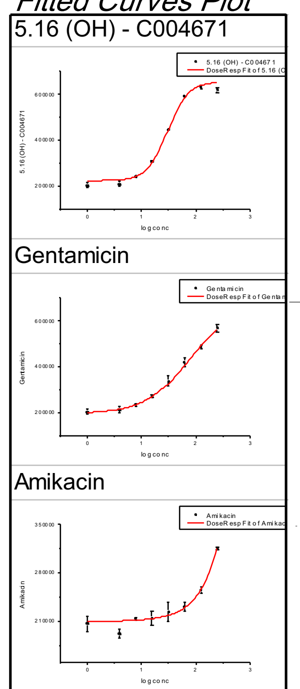
Fit Status Code :
100 : Fit converged
1 : Chi-sqr is reduced.

ANOVA

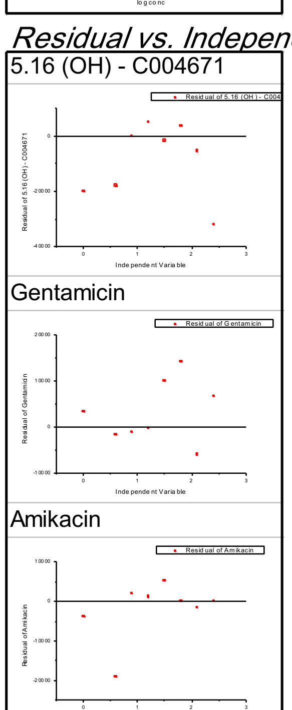
		DF	Sum of Squares	Mean Square	F Value	Prob>F
5.16 (OH) - C004671	Regression	4	1.5795E12	3.94876E11	6.92353E10	0
	Residual	4	22.81354	5.70338		
	Uncorrected Total	8	1.5795E12			
	Corrected Total	7	19899.24456			
Gentamicin	Regression	4	1.07317E12	2.68292E11	7.43678E11	0
	Residual	4	1.44305	0.36076		
	Uncorrected Total	8	1.07317E12			
	Corrected Total	7	980.22355			
Amikacin	Regression	4	4.38856E11	1.09714E11	4.38179E10	0
	Residual	4	10.01545	2.50386		
	Uncorrected Total	8	4.38856E11			

NL Fit (DoseResp) (6/25/2010 03:50:59)

Fitted Curves Plot



Residual vs. Independ



Chapter 6

Biomimetic Synthesis, Structural Refinement and Examination of Self-Assembly of the Macrocyclic Dimer Aminoglycoside 66-40C

6.1 - Biosynthetic Origins of Aminoglycoside 66-40C

Aminoglycoside 66-40C (**6.1**), is a remarkable C_2-symmetric 16-membered macrocyclic dimer, consisting of anti-parallel α,β-unsaturated imine systems bridging two sisomicin-derived scaffolds.[1] It was discovered at Schering-Plough Corporation during the detailed account of the minor components in the fermentation broth of *Micromonospora inyoensis*, the producer of the antibiotic sisomicin (Figure 6.1).[2,3]

Figure 6.1. Aminoglycoside *66-40C* (**6.1**), sisomicin and their hypothetical biosynthetic relationship through a 6'-aldehyde monomer precursor (**6.2**).

The characteristics of *aminoglycoside 66-40C* set it apart from all similar classes as it appears to defy the definitions of an aminoglycoside (Chapter 1).[2] It is constructed of meta-stable functional groups arranged in a C_2-symmetric macrocycle clearly unfit to target the A-site H44 helix of the bacterial ribosome, and unable to produce antimicrobial activity.[1] Moreover, it is the only example among minor fermentation products which appears to require additional transformations on the parent antibiotic (Figure 6.1). The usual by-products from aminoglycoside producers consist of immature intermediates of the main biosynthetic pathway.[2,4,5] The intriguing origin of this macrocyclic dimer remained unknown for over four decades, and likewise, its potential biological functions remain still more mysterious.

We hypothesized that *aminoglycoside 66-40C* could potentially self-assemble from 6'-aldehyde monomers (**6.2**) (Figure 6.1), reacting sequentially toward transient C6'-N3-aminohydrin or imine intermediates, the second of which could benefit from a proximity effect owing to the rigidity conferred by the anomeric effect and the 4',5'-unsaturation, thereafter locking the macrocyclic structure. This hypothesis implied that the putative aldehyde monomer **6.2** would be self-selective and self-assemble to produce the macrocyclic azadiene bridges exclusively at N3, defying hydrolysis in aqueous solution.

Self-assembling natural products are a relatively rare subgroup (reviewed in references 6 and 7) which would include *aminoglycoside 66-40C* in a category far more exclusive than the traditional C_2-symmetric or macrocyclic classifications.[8] In many cases, it has been demonstrated that self-assembling dimeric or pseudodimeric natural products owe their structural complexity to simpler precursors whose intrinsic properties predispose them for spontaneous intermolecular attacks, condensations, rearrangements, cyclo-additions or electrocyclic reactions (Figure 6.2).[6] Synthetic chemists have long profited and validated biomimetic strategies to construct such dimeric or pseudodimeric natural products. Classic examples are dimeric pyrazines, such as palythazin, constructed by spontaneous condensation and aromatization of precursor 1,2-amino-ketones (Figure 6.2 A).[9] More complex examples of self-assembly have also been demonstrated by biomimetic syntheses in the last decade (Figure 6.1 B-G), including C_2-symmetric trichodimerol by Corey,[10] and the related pseudodimeric bisorbicillinols by Nicolaou[11,12] (Figure 6.1 B). The renowned biomimetic synthesis of torreyanic acid was accomplished by J. A. Porco's group (Figure 6.1 C).[13] J. E. Baldwin's group developed an elegant and expedient biomimetic route to panepophenanthrin (Figure 6.1 D).[14] The bisanthraquinones and their biomimetic “cytoskyrin cascade” were studied by Nicolaou’s group (Figure 6.1 D).[15] Lastly, the biomimetic dimerization of stephacidin B was demonstrated by the groups of Baran,[16] Myers,[17] and later Williams[18] from the uncommon 3-alkylidene indole N-oxide electrophilic core of the biologically active monomer avrainvillamide (Figure 6.2 G). Unlike *aminoglycoside 66-40C* (**6.1**), these latter examples have in common the use of reactions which form thermodynamically stable bonds between the monomers, such as aldol, Michael and oxa-Michael additions, as well as Diels-Alders cycloadditions.

A Jarglis *et al*, *Angew. Chem.* **1982**, *94* (2), 140-1.

B Corey *et al*, *Org. Lett.* **1999**, *1* (9), 1503-4.
Nicolaou *et al*, *Angew. Chemie Int. Ed.* **1999**, *38* (23), 3555-9.

C Porco *et al*, *J. Am. Chem. Soc.* **2003**, *125* (17), 5095-106.

D Baldwin *et al*, *Org. Lett.* **2003**, *5* (17), 2987-8.

E Nicolaou *et al*, *J. Am. Chem. Soc.* **2007**, *129* (13), 4001-13; and

F Baran *et al*, *Angew. Chem. Int. Ed.* **2005**, *44* (25), 3892-5; and Myers *et al*, *J. Am. Chem. Soc.* **2005**, *127* (15), 5342-4.

Figure 6.2. Examples of biomimetic self-assembly of dimeric or pseudodimeric natural products.

On the other hand, macrocyclic meta-stable imine functions are more common in the context of dynamic combinatorial libraries (DCL)[19] and supramolecular chemistry.[7] The value of imines in such studies stems from the rapid exchange between monomers, such that the outcome of a mixture of imines can be displaced and selected,[19,20] using for example host-guest templating,[19] kinetic trapping (i.e. reductive amination),[21,22] and metal chelation (e.g. salen ligands)[23] (Figure 6.3). However, unsubstituted alkyl imines are unstable in the presence of water, usually requiring dehydrating agents, azeotropic removal of water or metal chelation to drive such condensations to completion.[19,24] The condensation equilibrium may be favorably displaced by N-substitution with electron-withdrawing groups, such as in anilines, acyl-hydrazones and oximes (Figure 6.3).[19,20] Aromatic aldehydes give typically more stable adducts and are used extensively. In certain examples of bifunctional monomers, single dimeric or trimeric macrocycles have been consistently obtained. Although this lack of diversity und unresponsiveness are often considered a drawback in dynamic combinatorial libraries, such examples serve to illustrate abiotic self-assembling dimers and trimers (Figure 6.3).[19,25]

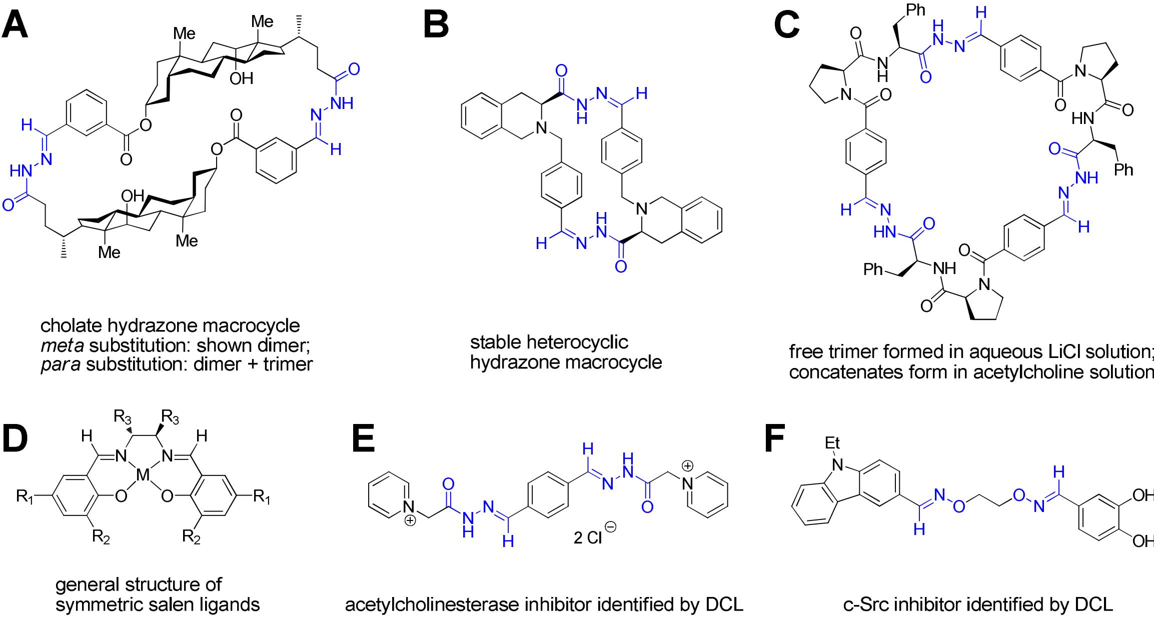


Figure 6.3. Stable *N*-substituted imines, hydrazides and oximino ethers. (A-C) examples of abiotic self-assembling stable dimers and trimers,[25] (D) general structure of salen ligands used in several organic reactions,[23] (E and F) examples of enzyme inhibitors identified from combinatorial dynamic libraries.[26,27]

Unique features of *aminoglycoside 66-40C* make it an intriguing synthetic target and a prime subject for the study of self-assembly in natural products. In particular, the reported stability of a single dimeric isomer in aqueous media is quite striking, because the imine defies conventional substitution patterns. Another salient feature of dimer *66-40C* is that each monomer contains several reactive amine groups which may be able to undergo condensations which in principle could produce a vast stochastic oligomeric library (8 possible linear dimers, 128 linear trimers, *etc*.). Careful consideration of the experimental data reported by the researchers of Schering-Plough Corporation indicated the dimeric structure of *aminoglycoside 66-40C*.[1] Critical evidence included descriptions of $^{1}H/^{13}C$-NMR spectra, each bearing a single peak assigned to the 6'-imine, meticulous osmometric molecular weight measurements and the aqueous acid degradation of **6.1** to a hydrated mixture of aldehyde **6.2**. Finally, the bulk of isolated *aminoglycoside 66-40C* served as substrate for reductive amination toward sisomicin, antibiotic *G-52* (6'*N*-methyl sisomicin) and for accessing several 6'*N*-substituted analogs.[28] However, the configuration of the *bis*-azadiene groups linking the 16-membered macrocycle remained unquestioned since the original report. Furthermore, little evidence substantiated imine formation specifically on N3, leaving the overall structure of this dimer unresolved and based on the framework of the parent sisomicin (Figure 6.1).

6.2 - Biomimetic Synthesis of Aminoglycoside 66-40C

Validation of the proposed biomimetic synthesis required unmasking of the aldehyde functionality in the presence of the unprotected aminoglycoside framework. To this end, we applied a procedure for selective oxidation of the 6'-position of azido sisomicins, which was recently reported by our group.[28] This chapter presents the first synthetic effort, which involved protection of sisomicin to tetra-azido sisomicin **6.3**, which was converted to 3'''*N*-Cbz analogue **6.4** (Scheme 6.1).[29] A similar synthetic sequence using 3'''*N*-Fmoc protection was later developed, which allowed convenient one-pot amine deprotection by treatment with piperidine and a phosphine.[30]

Selenium dioxide allylic oxidation of the 6'-azide group of **6.4** (Scheme 6.1) was effected in excellent yield by means of our previously reported conditions employing the sacrificial additive dihydro-[2H]-pyran.[28] This inexpensive additive can significantly boost the yield when oxidizing these particular cyclic enol-ether substrates, possibly by out-competing starting material for electrophilic species in the oxidizing mixture preventing detrimental side-reactions. Subsequently, protection of **6.5** as the acid-sensitive dimethylacetal intermediate **6.6** provided the key latent form of the 6'-aldehyde. Concomitant cleavage of the *N*-Cbz group and reduction of the azides under optimized Birch conditions produced the crucial masked biomimetic precursor **6.7** (Scheme 6.1). The reaction produced clean **6.7** only when *t*BuOH was omitted from the Birch procedure.

Finally, exposure of dimethylacetal **6.7** to an acidic solution readily liberated the desired aldehyde precursor **6.2**. The preferred acid/base procedure involved treatment of **6.7** with dilute H_2SO_4, followed by neutralization with saturated $Ba(OH)_2$ and removal of the $BaSO_4$ precipitate. Gratifyingly, the appearance and accumulation of *aminoglycoside 66-40C* **6.1** upon neutralization was confirmed by mass spectrometry. With further standing or freeze-drying of this solution only *aminoglycoside 66-40C* **6.1** prevailed. Under optimized pH and temperature, the acid treatment and neutralization transformations could be directly witnessed by ^{1}H-NMR spectroscopy over the course of several hours, owing to the downfield signals of the functionalities involved (Figure 6.4). These experiments clearly demonstrated the self-assembly of the macrocyclic dimer *aminoglycoside 66-40C* **6.1** from its biosynthetic precursor aldehyde **6.2**, undergoing complete conversion without the accumulation of intermediates. The NMR studies also revealed that the rate of dimerization is strictly dependent on pH. Only at pH >8, the self-assembly to produce *aminoglycoside 66-40C* becomes appreciable within minutes. This behavior is consistent with the requirement of N3 in its nucleophilic form,[2,31] and with previous reports of reactions with imine intermediates having optimum rate in mildly alkaline buffers.[24,32]

sisomicin sulfate
1) TfN_3, Na_2CO_3
2) CbzCl, $NaHCO_3$
(59%)
6.3 R = H
6.4 R = Cbz
SeO_2, DCM, DHP
(92%)
6.5
$(MeO)_3CH$, TFA, MeOH
(82%)
6.6
Na, NH_3(liq.), -78 °C;
AcOH quench
(75%)
6.7 · 4 AcOH
0.5 N H_2SO_4
6.2
$Ba(OH)_2$ neutralization
(69% two steps)
key HMBC correlations
nOe's
aminoglycoside 66-40C (**6.1**) (refined structure)

Scheme 6.1 Synthesis and biomimetic self-assembly of *aminoglycoside 66-40C* (**6.1**), the refined structure, key nOe (blue arrows) and HMBC (red arrows) correlations are shown.

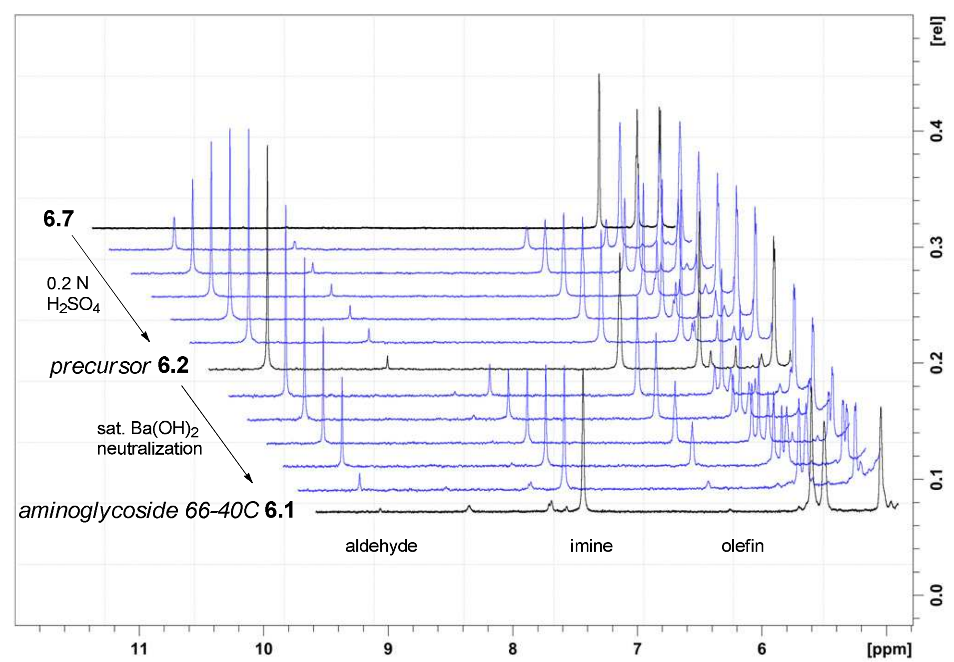


Figure 6.4. ^{1}H-NMR spectra of the evolution of a D_2O solution of dimethylacetal **6.7** (top), acidified to 0.2 N H_2SO_4 until aldehyde **6.2** prevailed, then neutralized with sat. $Ba(OH)_2$. See experimental section for further details on the procedure (page 297). Spectra are truncated prior to the HOD peak for clarity (4.8 ppm). Acquisitions in the first section are 5 min apart, and after the neutralization 10 min apart.

Further purification of *aminoglycoside 66-40C* **6.1** from the crude traces of salts was performed by column chromatography under alkaline conditions which maximise its stability, using the common solvent system for aminoglycoside TLC: mixtures of methanol, chloroform and conc. ammonium hydroxide in proportions varying from 2:3:0.25 to 2:3:1. *Aminoglycoside 66-40C* **6.1** was eluted as the free base, usually with a trace of silica (<5% weight), which was removed by filtration after lyophilization and reconstitution in water. Assessment by NMR spectroscopy proved excellent purity and stability in D_2O solutions (see Experimental Section for NMR spectra, pages 313-318). Synthetic *aminoglycoside 66-40C* exhibited physical and spectroscopic characteristics identical to those previously disclosed (see Tables 6.1 and 6.2 for ^{1}H- and ^{13}C-NMR comparison).

Subsequently, two-dimensional homo- and heteronuclear shift correlation experiments, in particular HMBC, attested to the imine location on N3 of the deoxystreptamine ring B as originally deduced from hand-held models (Scheme 6.1). In addition, nuclear Overhauser effect (nOe) spectroscopy revealed spatial proximity between the 6'-imine proton and both the vinylic 4'-H and axial proton 3-H_a of ring B, an in-line arrangement only possible for a *trans,trans* azadiene configuration on both macrocycle bridges, the refined structure is shown in Scheme 6.1. See Experimental Section for complete spectral data, pages 313-318.

Exposure of *aminoglycoside 66-40C* to acid in D_2O, as done in the isolation report,[1] led to slow hydrolysis of the dimer to monomer aldehyde **6.2** (Figure 6.5), also producing a matching ^{13}C-NMR spectrum (Table 6.3). The half-life of *aminoglycoside 66-40C* was approximately 30 minutes in 0.2 N H_2SO_4 at room temperature. Such a rate of hydrolysis can be considered slow, but is not uncommon for imine hydrolysis, known to vary over 4 orders of magnitude depending on the substrate.[33] The rate limiting step of imine hydrolysis under acidic conditions is the collapse of the hydrated tetrahedral intermediate, and hence pH independent.[33] Sequential observations of ^{1}H-NMR spectra for *aminoglycoside 66-40C* titrated with H_2SO_4/D_2O solutions revealed that hydrolysis begins upon addition of the third equivalent of acid, once N3''', N2' and N1 are protonated (in this order). The hydrolysis rate is similar with 4 equivalents of H_2SO_4 or in 0.2 N H_2SO_4, indicating pH independence once the neighboring amines are protonated, as is the case for traditional imine substrates.[33]

Table 6.1. ^{1}H-NMR shift comparison of natural and synthetic *aminoglycoside 66-40C* (**6.1**)

Natural *66-40C* (100 MHz)[1]				Synthetic *66-40C* (700 MHz)					
Assign.[1]	δ_H (ppm)	Mult.	J (Hz)	Assign.[a]	δ_H (ppm)[b]	Mult.	J_1 (Hz)	J_2 (Hz)	J_3 (Hz)
6'-H	7.56	s	-	6'-H	7.44	s	-		
4'-H	5.50	d	2	4'-H	5.49	d	2.3		
1'-H	5.48	m	-	1'-H	5.48	d	2.1		
1"-H	5.15	d	4	1"-H	5.13	d	4.0		
5"-H_e	4.10	d	12.5	5"-H_e	4.09	d	12.6		
2"-H	3.84	dd	11	2"-H	3.88	dd	10.8	3.9	
n/a	n/a	-	-	4-H	3.71	dd	9.2	9.2	
n/a	n/a	-	-	5-H	3.65	dd	9.3	9.3	
n/a	n/a	-	-	6-H	3.38	dd	9.6	9.6	
5"-H_a	3.36	d	12.5	5"-H_a	3.33	d	12.6		
n/a	n/a	-	-	3-H	3.18	ddd	11.9	9.6	4.8
2'-H	2.95	m	-	2'-H/1-H	2.95	m	-		
n/a	2.61	d	-	3"-H	2.75	d	10.8		
3"-NCH_3	2.52	s	-	3"-NCH_3	2.59	s	-		
3'-H	2.26	m	-	3'-H_e	2.26	ddd	17.5	6.1	6.0
n/a	n/a	-	-	3'-H_a	2.20	ddd	17.8	11.9	2.1
2-H	1.81	ddd	12.5	2-H_e	1.92	ddd	12.8	4.2	3.9
				2-H_a	1.65	ddd	12.6	12.6	12.6
4"-CH_3	1.22	s	-	4"-CH_3	1.23	s	-		

a) based on COSY, HSQC and HMBC; b) slight shift variations are attributed to concentration or pH differences.

Table 6.2. ^{13}C-NMR shift comparison of natural and synthetic *aminoglycoside 66-40C* (**6.1**)

Natural *66-40C* (25 MHz)[1]		Synthetic *66-40C* (176 MHz)	
Assign.[1]	δ_C (ppm)	Assign.[a]	δ_C (ppm)
6'	161.0	6'	161.22
5'	146.4	5'	145.90
4'	115.5	4'	115.86
1"	101.3	1"	101.00
1'	99.8	1'	99.48
6	87.4	6	86.75
4	80.8	4	80.61
5	76.7	5	76.19
4"	73.1	4"	72.44
2"	70.0	2"	69.26
5"	68.6	5"	68.13
3	65.9	3	66.40
3"	64.3	3"	64.03
1	51.3	1	51.15
2'	47.1	2'	46.83
3"-NMe	37.8	3"-NMe	37.01
2	36.0	2	35.61
3'	25.6	3'	24.88
4"-Me	22.7	4"-Me	21.95

a) based on COSY, HSQC and HMBC.

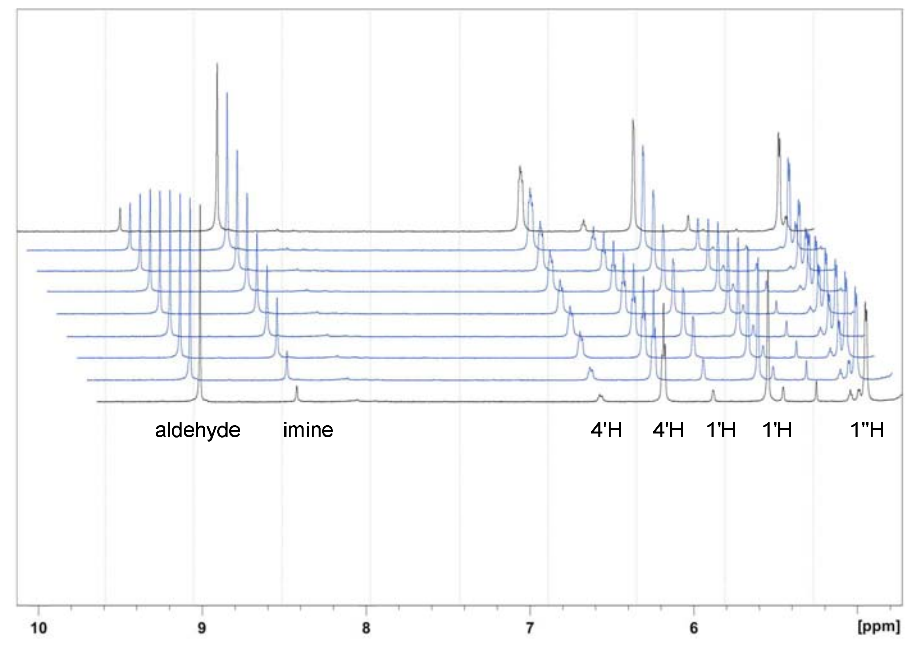


Figure 6.5. ^{1}H-NMR spectra of the evolution of a D_2O solution of *aminoglycoside 66-40C* **6.1** (top), acidified to 0.2 N H_2SO_4. Acquisitions are 10 min apart. Spectra are cut before the HOD peak (4.8 ppm) for clarity.

Table 6.3. Comparison of ^{13}C-NMR shifts of naturally-derived and synthetic **6.2** in acid.

Naturally-derived 6.2 in D_2O/HCl (25 MHz)[1]		Synthetic 6.2 in D_2O/H_2SO_4 (176 MHz)	
Assign.[1]	δ_C (ppm)	Assign.[a]	δ_C (ppm)
6'	189.9	6'	189.33[b]
5'	149.5	5'	149.38[c]
6'-$(OH)_2$	123.8	4'	123.52[c]
4' and 1"	102.0	1"	101.98
1'	97.7	1'	97.70[c]
6	83.9	6	83.99
4	80.5	4	80.33
5	74.6	5	74.22
4"	70.8	4"	70.70
5"	68.6	5"	68.44
2"	67.2	2"	67.07
3"	64.2	3"	64.09
1	50.6	1	50.57
3	49.0	3	48.81
2'	46.8	2'	46.68[c]
3"-NMe	35.4	3"-NMe	35.23
2	28.3	2	28.29
3'	24.8	3'	24.99[d]
4"-Me	21.8	4"-Me	21.61

a) based on COSY and HMQC; b) the ratio of aldehyde:hydrate observed in these conditions was 7:1 (lit.,[1] 1:2); c) the aldehyde gives a 1:1 mixture of conformers splitting these peaks; d) position 3' becomes slowly deuterated with extended exposure. No ^{1}H-NMR characterization is available for comparison.[1]

6.3 - Computer-Generated Model of Aminoglycoside 66-40C

The two-dimensional drawing in Scheme 6.1 for the refined structure of *aminoglycoside 66-40C* (**6.1**) depicts hardly the parallel relationship of the *s-trans-bis*-azadiene bridges and the spatial arrangement of rings A and B. Although a crystal structure would be highly desirable, crystalls of **6.1** could not be obtained. A computer model of the core rings A and B was generated. The initial model was drawn accounting for the proximity of 6'H, 4'H and $3H_a$ established by nOe, and thereafter semi-empirical geometry optimizations and molecular dynamic calculations were run with the Amber99 parameter set in Hyperchem 8 suite.[34] Ring C was then modeled on this structure in the conformation observed for gentamicinin in the A-site model co-crystal structure, followed by optimization (Figure 6.6).[34]

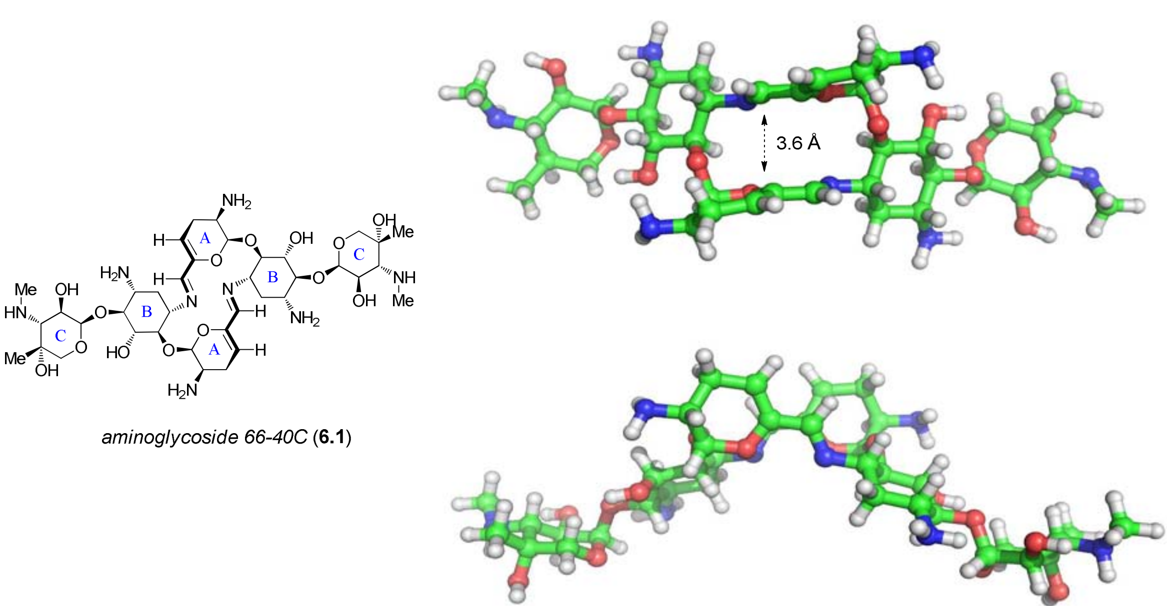


Figure 6.6. Computer model of a*minoglycoside 66-40C* (top and side views), generated on Hyperchem 8 suite, semi-empirical geometry optimizations and molecular dynamic calculations were run using Amber99 parameter set.[34] Ring C was modeled in the conformation observed for gentamicin in the A-site oligonucleotide model co-crystal structure.[34] Images generated on PyMol.[35]

Pair fitting was performed between this model and paromomycin in the A-site oligonucleotide model co-crystal,[36] also believed to be the lowest energy conformation in solution (Figure 6.7).[37] Considering all 16 sp^3 atoms in the core rings (ring A: C1'-3', N2', O1' and ring B: C1-6, N1, N3, O3-5) an average root mean square (RMS) distance of 0.23 Å was calculated. This close structural match suggests the innate conformation of aminoglycoside rings A and B, predispose the spontaneous self-selection and self-assembly of the 6'-aldehyde precursor **6.2**, leading to little distortion over the lowest energy conformation in the prevalent macrocyclic motif of *aminoglycoside 66-40C*. This conformation is dominated by the stabilizing anomeric effects of the α-glycosidic linkage, comprising the *endo* effect *n*O1'→σ*C1'-O4 which favors the axial conformer of the glycosidic bond and the *exo* effect *n*O4→σ*C1'-O1' which orients ring B (Figure 6.7).[38,39]

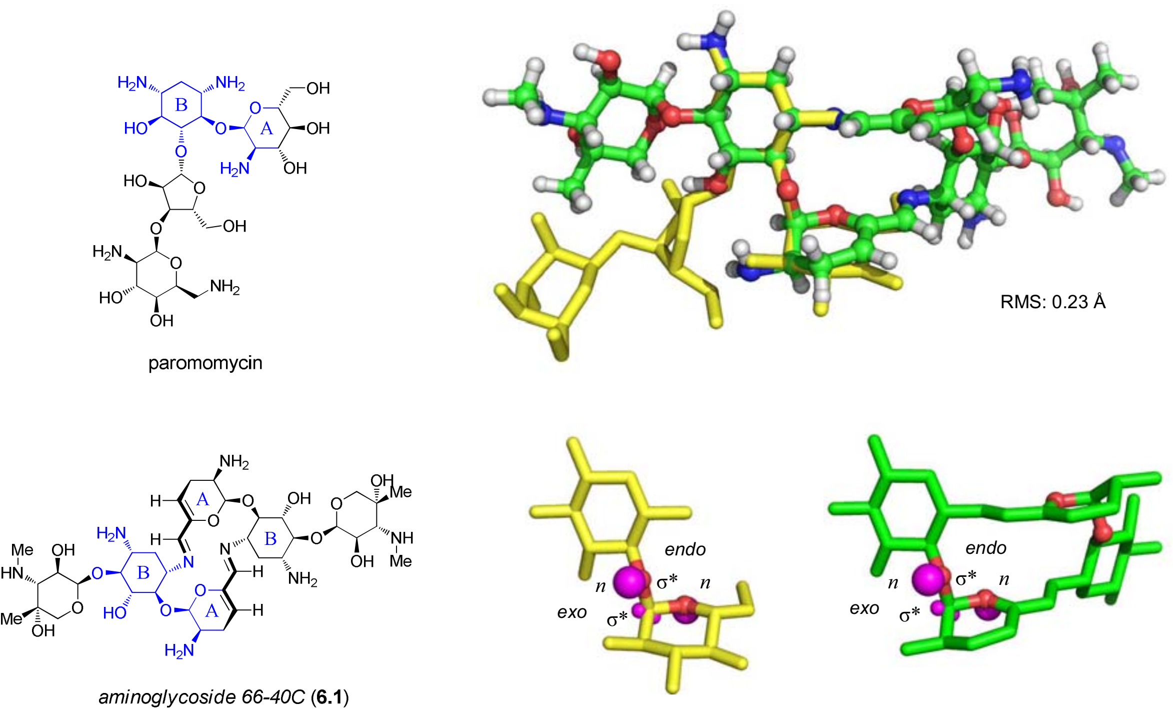


Figure 6.7. Top: model of *aminoglycoside 66-40C* (green), overlaid on the skeleton of paromomycin (yellow) from the A-site oligonucleotide co-crystal structure.[36] Pair fitting was performed using all 16 sp^3 matching atoms highlighted in blue. The calculated average root mean square (RMS) distance was 0.23 Å. Bottom: comparison of the *exo*- and *endo*-anomeric effects in the frameworks of paromomycin (yellow) and the *aminoglycoside 66-40C* macrocycle (green). The involved *n* and σ* orbitals are highlighted in magenta. Images rendered on PyMol.[35]

6.4 - Synthesis of the Sisamine Dimer Macrocyclic Core

The self-assembling properties of *aminoglycoside 66-40C* (**6.1**) are therefore assigned to its core structure of rings A and B, leaving ring C as an observer appendage. The generality of the self-assembling dimerization process was demonstrated with three analogs: a nominal macrocyclic core, a macrocycle congener based on the 4,5-disubstituted 2-deoxystreptamine family, a cross-over experiment, and finally an expanded 20-membered macrocycle dimer.

Scheme 6.2. Degradation of tetra-azido sisomicin ring C and synthesis of the nominal macrocyclic dimer motif (**6.12**), key nOe and HMBC correlations are shown.

In order to access the nominal macrocyclic core of *aminoglycoside 66-40C*, a periodate and Barry degradation procedure for removal of ring C was applied to *per*-azido sisomicin intermediate **6.3** producing tetraazido sisamine **6.8** as a versatile model of the rings A and B (Scheme 6.2).[40] Allylic oxidation to aldehyde **6.9** and acetal protection of position 6' in **6.10** was carried out uneventfully. Global azide reduction to 6'-dimethylacetal sisamine **6.11** could be effected either through Birch reduction, or otherwise with 10 equivalents of trimethylphosphine in MeOH at room temperature. Subsequently, the acidic unmasking of the 6'-aldehyde, followed by self-assembly of the macrocycle during neutralization was in all respects equal to *aminoglycoside 66-40C*, affording sisamine dimer **6.13** (Scheme 6.2). Identical HMBC and nOe correlations confirmed the similar structure of the macrocycle. See Experimental Section for NMR spectral data (pages 319-314).

6.5 - Synthesis of the 4',5'-Unsaturated Paromomycin Macrocyclic Dimer

As presented in Chapter 4, the Tsuji deoxygenation of paromomycin to access sisomicin hybrids led conveniently to intermediates with the analogous α,β-unsaturated aldehyde required for macrocondensation. The product of Tsuji-deoxygenation **4.18** was exposed to mild sodium methoxide, which removed the methylcarbonate groups in presence of the 6'-aldehyde, which was then protected as dimethylacetal **6.14** (Scheme 6.3). Subsequent deprotections and dimerization were carried out uneventfully, leading to the self-assembly of the novel paromomycin macrocyclic dimer **6.15**. Analysis of this particular dimer required transformation to its acetate salt to simplify the NMR spectrum to a single conformer as usually associated with ring D. The typical structural characteristics observed for the preceding macrocyclic dimers were likewise observed for **6.15** (Scheme 6.3). See Experimental Section for NMR spectra, pages 325-330.

Although the intermediacy of a 6'-aldehyde during the biosynthetic conversion of paromamine to neamine (see Chapter 2) has been evidenced by glucosamine C6-deuterium labeling studies,[2,41,42] dimers of this type have not been reported in the fermentations of any other aminoglycoside class except the 4',5'-unsaturated sisomicin.

Scheme 6.3. Synthesis of the 4',5'-unsaturated paromomycin macrocyclic dimer **6.14** from intermediate **4.18** presented in Chapter 4. Key nOe and HMBC correlations are shown.

6.6 - Synthesis of the Expanded Macrocyclic Dimer Core

The predicted conformation of the macrocyclic core positions the anti-parallel azadiene bridges facing each other, approximately 3.5 to 3.7 Å apart, a situation potentially stabilized by non-covalent interactions between the two 5-atom π-systems (Figure 6.6). This arrangement implied that concomitant extension of each π-system should not be detrimental for the self-assembly of the macrocycle. To challenge this proposal, the sisamine 6'-aldehyde intermediate **6.9** was homologated by two carbons using the classic Horner-Wadsworth-Emmons olefination procedure (Scheme 6.4). A *trans*-olefin was selected over a *cis* configuration because the latter would poise the system for possible Nazarov cyclizations in subsequent acid-catalyzed transformations. The required stereoselectivity was achieved employing a stabilized ylide to extend aldehyde to the α,β-unsaturated ester, which was reduced *in situ* with DIBAL to afford allylic alcohol **6.16**, as a single isomer. Subsequently, the allylic alcohol was oxidized with manganese oxide to aldehyde **6.17**, which was protected as the corresponding dimethylacetal **6.18**. The Staudinger reaction was used to deprotect the homologated masked monomer **6.18** (Scheme 6.4), because even with the 6'-aldehyde masked as a dimethylacetal, the remaining conjugated olefins would be liable to reduction under Birch conditions.

Scheme 6.4. Synthesis of the 20-membered macrocycle (**6.18**) by two-carbon homologation of the sisamine 6'-aldehyde monomer. Key nOe and HMBC correlations are shown.

Gratifyingly, self-assembly of 6'-aldehyde **6.19** led to macrocycle **6.20** as predicted under the standard conditions. The usual analysis of homo- and heteronuclear two-dimensional shift correlations as well as Overhauser effects indicated that the azatriene bridges had assumed *trans,trans,trans*-configurations in this 20-membered macrocycle, as expected from the original proposal (Scheme 6.4). See Experimental Section for complete NMR spectral data, pages 331-338.

6.7 - Examination of the Dynamics of Macrocycle Self-Assembly

The sharp proton and carbon signals in NMR spectra of *aminoglycoside 66-40C* and its analogs indicated that the imine form of the dimers was prevalent and stable, and the equilibration to the aldehyde monomer was apparently insignificant. Considering the relaxation times and detection limits of NMR spectroscopy, a dynamic equilibrium through fleeting monomeric or open intermediates could not, however, be ruled out.

With sisamine macrocycle **6.13** in hand, a cross-over experiment with *aminoglycoside 66-40C* (**6.1**) was designed (Scheme 6.5). In the event of cross-over, LCMS would discern between all three dimers based on their mass and polarity (Figure 6.8).

aminoglycoside 66-40C (**6.1**) + sisamine dimer (**6.13**) ⇌ (see Figure 6.8) crossover dimer (**6.21**)

Scheme 6.5. Dynamic equilibrium challenge of *aminoglycoside 66-40C* (**6.1**) and dimer **6.13**.

Equimolar solutions of *aminoglycoside 66-40C* (**6.1**) and sisamine dimer **6.13** in D_2O were subjected to a range of pH conditions, then incubated for 72 hours at room temperature and sequentially analyzed by LCMS as shown in Figure 6.8. See Experimental Section for further details on the procedures and incubation conditions, page 312.

A positive control was produced by exposing an equimolar mixture to 0.2 N H_2SO_4 in D_2O overnight, until aldehyde monomers were the only species observed by ^{1}H-NMR spectroscopy. Neutralization with sat. $Ba(OH)_2$ produced the statistical cross-over mixture (positive control incubation, Figure 6.8 F). Three incubations served as negative controls, *aminoglycoside 66-40C* (**6.1**) alone, dimer **6.13** alone, and a sample of discrete cross-over product **6.21**, which was purified by preparative TLC using $MeOH/CHCl_3/NH_4OH_{(aq)}$ in 2:3:1 proportion (Figure 6.8 panels A, B and G, respectively). Although most traditional imine substrates would undergo significant scrambling catalyzed by 0.1% formic acid present in the elution solvents,[19,24] the slow rate of acid hydrolysis of the *bis*-azadiene macrocycle allowed LCMS analysis under routine conditions (Figure 6.5).

Notably, the mixtures of the macrocycle dimers **6.1** and **6.13** in their free-base state or as neutral acetate salts did not undergo significant cross-over after 72 hours in solution (Figure 6.8 C and D, respectively). In the presence of slight excess acetic acid (2 equiv. per amine, pH ~4), slow hydrolysis of the dimers was observed during the 72 h incubation (25% by H-NMR integration of aldehyde and imine signals). Surprisingly, upon removal of excess acetic acid by lyophilization of this mixture, LCMS analysis showed incomplete scrambling (Figure 6.8 E). In spite of the appreciable hydrolysis, a dynamic equilibrium was not established, implying the hydrolysis reaction is irreversible due to N3 protonation.

These experiments demonstrate that *aminoglycoside 66-40C* (**6.1**) can be considered a self-assembling and stable entity over pH ranges of relevance in extra- or intracellular media.

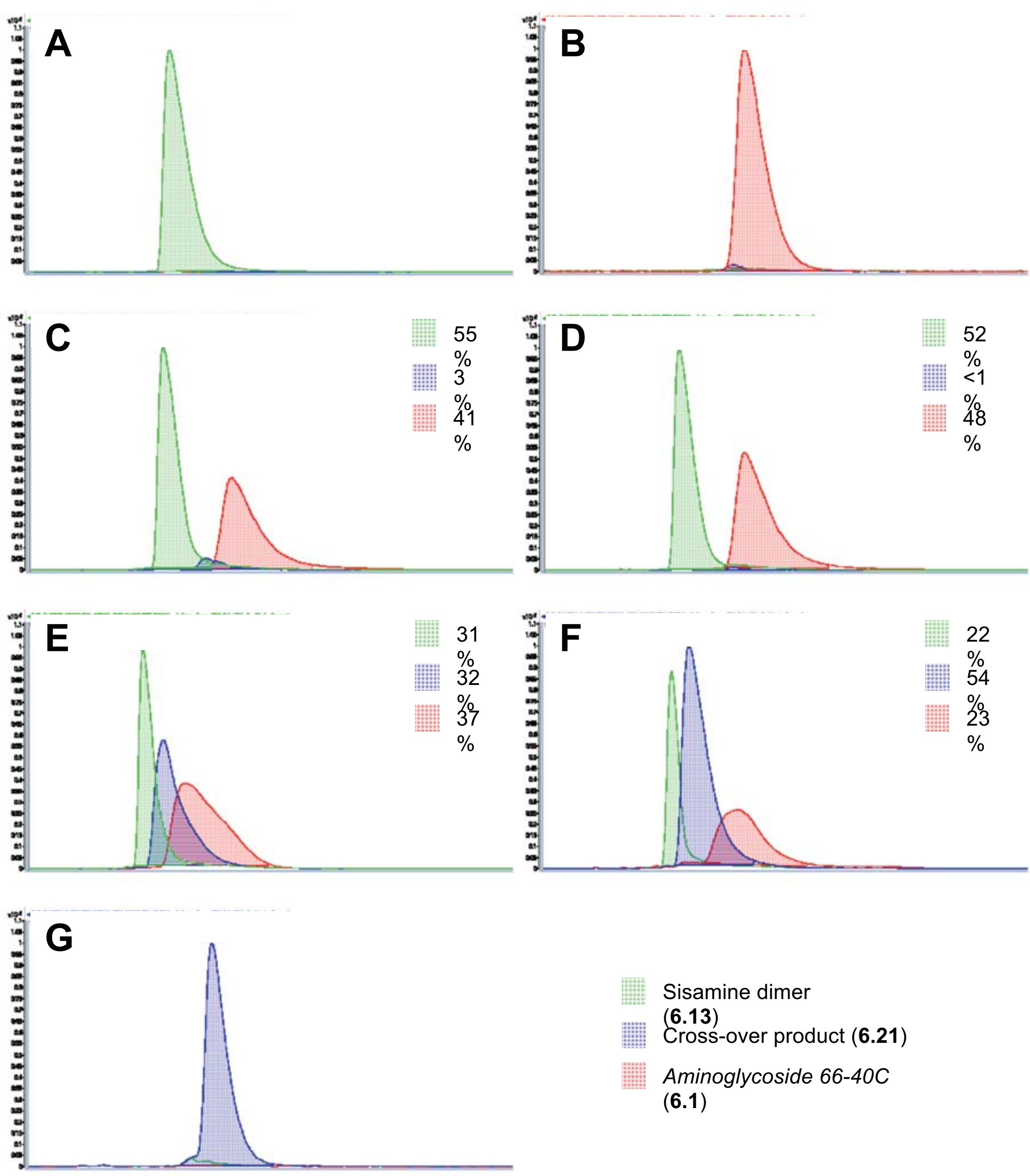


Figure 6.8. Sequential LCMS injections of aqueous solutions incubated 72 h in the following conditions: (A) sisamine dimer **6.13** alone, (B) *aminoglycoside 66-40C* **6.1** alone, (C) 1:1 mixture of free bases, (D) 1:1 mixture of lyophilized acetate salts, (E) 1:1 mixture in pH 4 excess acetic acid, then lyophilized, (F) positive control, (G) cross-over product **6.21** isolated by preparative TLC. See text and Experimental Section for details, page 312.

6.8 - Conclusions

The proposal and validation of the biomimetic self-assembly of *aminoglycoside 66-40C*, permitting spectroscopic confirmation and refinement of the originally proposed structure of the *bis*-azadiene macrocycle was presented in this chapter. The concise synthesis was demonstrated in amounts amply sufficient for future investigation of the potential biological functions of *aminoglycoside 66-40C* and continued exploration of its remarkable properties.

The generality and specificity of the self-assembly of this macrocyclic motif was asserted by the self-assembly of structural analogs, which challenged substitution and extension of the core macrocyclic motif. The formation of the specific N3-*bis*-azadiene macrocycle is apparently predisposed by the structural features of the core aminoglycoside rings A and B, dominated by the stabilizing anomeric effects of their α-glycoside linkage. Furthermore, *aminoglycoside 66-40C* was examined under cross-over conditions which revealed the unexpected stability and non-dynamic properties that the polyamine macrocyclic structure imparts to the imine functional groups. These experiments demonstrated that *aminoglycoside 66-40C* is a natural product capable of self-assembly to a conformationally distinct and stable macrocyclic *trans,trans-bis*-azadiene in aqueous solution. Furthermore, the biomimetic synthesis described herein opens a new avenue of research, connecting the field of aminoglycosides with supramolecular chemistry.

The bulk of this work has been published in *Chem. Commun. (Camb)* **2010,** *46* (12), 2013-5, reference [29] and highlighted as a hot article.[43]

6.9 - References

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Chapter 6 - Experimental Section

General Procedures

All reactions were carried out under an inert atmosphere of nitrogen or argon with dry solvents, using anhydrous conditions unless otherwise stated. Dry dichloromethane (DCM) and tetrahydrofuran (THF) were obtained from a solvent delivery system with activated alumina columns. Methanol (MeOH) was distilled from CaH_2 under argon. Reagents were purchased at the highest commercial quality and used without further purification. Flash chromatography was performed with silica gel from SilicaFlash P60, particle size 40-63 μm, 230-400 mesh and distilled hexanes, ethyl acetate (EtOAc) or DCM. Free amines were purified with DCM or $CHCl_3$ applying gradients of ammoniacal MeOH (referring to a 1:9 solution which was prepared fresh with ammonia liquor before use). Deprotected aminoglycosides were purified with homogeneous solvent systems consisting of $CHCl_3$/MeOH/$NH_4OH_{(aq)}$ in ratios ranging from 2:3:0.5 to 2:3:2.5. Yields refer to chromatographically and spectroscopically homogeneous material. Reactions were monitored by direct-injection low resolution mass spectrometry (LRMS) and thin layer chromatography (TLC) carried out on 0.25 mm E. Merck silica precoated plates (60F-254), visualized under UV light and developed with acidified ammonium molybdate/cerium sulfate and heat. NMR spectra were recorded on Bruker ARX-400, AV-400 or AV-700 instruments and are calibrated using residual undeuterated solvent as an internal reference. The following abbreviations are used to explain multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Low resolution mass spectra (LRMS) were recorded on a Thermo Finnigan Surveyor MSQ and high resolution mass spectra (HRMS) were recorded on an Agilent Technologies LC-MSD TOF mass spectrometer by electrospray ionization in positive mode. Either protonated molecular ions $[M+H]^+$ or sodium adducts $[M+Na]^+$ were used for empirical formula confirmation. Optical rotations were recorded in a 1 dm cell at ambient temperature, on a Perkin-Elmer 343 polarimeter. Analytical LCMS was performed using a Luna Hilic column 3 μ 100 x 3.0 mm, SN 413454-5 on an Agilent Technologies LC-MSD TOF mass spectrometer. Chromatograms were analyzed with MassHunter Workstation Software version B.03.01, Agilent Technologies.

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6.3

1,3,2',6'-Tetraazido-sisomicin (6.3)

Sodium azide (7.6 g, 117 mmol) was dissolved in a minimum volume of water (25 mL) and an equal volume of DCM (25 mL) was added. The resulting biphasic mixture was cooled to 0 °C, and treated dropwise with a solution of Tf_2O (10 mL, 59.4 mmol) in DCM (10 mL) over 1 h with vigorous stirring. The mixture was warmed to RT, stirred for 2 h at this temperature, and treated with sat. $NaHCO_3$ (50 mL). The organic layer was separated and the aqueous layer was extracted with DCM (10 mL, 2 times). The organic layers were combined to give a solution of TfN_3 which was kept at 0 ºC until needed. Note: this DCM solution should be treated as a hazardous material, kept behind a shield and not evaporated under any circumstance due to the potential explosiveness of TfN_3.

In a separate flask, sisomicin sulfate (3.0 g, 4.33 mmol), $NaHCO_3$ (7.6 g, 81 mmol) and $CuSO_4$ (119 mg, 0.75 mmol) were dissolved in H_2O (50 mL) and MeOH (100 mL), cooled to at 0 ºC, treated with the freshly prepared TfN_3 solution (~45 mL), and the mixture was stirred vigorously and allowed to warm to RT. After 4 h, the major ion observed by LRMS corresponded to the product **6.3**, and the excess TfN_3 was quenched with *n*-butylamine (5.9 mL, 60 mmol). The solution was evaporated under vacuum and the residual mixture was extracted three times with DCM. The organic layer was separated, dried over Na_2SO_4, filtered and evaporated to a residue. Purification by column chromatography (3 → 5 → 7% ammoniacal MeOH in DCM), yielded 1.60 g of the title compound **6.3** (66%, 2.90 mmol), as the N3''' free-base and a light yellow amorphous solid.[1]

R_f = 0.4, 20% ammoniacal MeOH/$CHCl_3$.

HRMS (ESI) calcd. for $C_{19}H_{29}N_{13}O_7$, M + H^+ = 552.2386, found 552.2396 (1.93 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 5.87 (d, *J* = 2.2 Hz, 1H), 5.22 (d, *J* = 3.7 Hz, 1H), 4.97 (dd, *J* = 4.9, 1.84 Hz, 1H), 4.08 (d, *J* = 12.2 Hz, 1H), 3.78 (d, *J* = 13.9 Hz, 1H), 3.72-3.58 (m, 5H), 3.50-3.38 (m, 2H), 3.34-3.28 (m, 1H), 3.25 (d, *J* = 12.2 Hz, 1H), 2.56 (s, 3H),

2.54 (s, 1H), 2.43 (dd, J = 16.0, 11.3 Hz, 1H), 2.34-2.21 (m, 2H), 1.38 (ddd, J = 12.6, 12.6, 12.6 Hz, 1H), 1.17 (s, 3H).

^{13}C NMR (CD_3OD, 100 MHz) δ 147.16, 100.0, 99.4, 98.3, 82.3, 81.2, 76.2, 72.9, 70.4, 69.2, 65.4, 61.9, 61.6, 56.0, 53.3, 38.4, 34.0, 23.5, 22.2.

6.4

1,3,2',6'-Tetraazido-3''-*N*-Cbz-sisomicin (6.4)

Tetra-azido sisomicin N3''' free-base **6.3** (450 mg, 0.816 mmol) was dissolved in MeOH (7 mL), treated with $NaHCO_3$ (150 mg, 1.8 mmol) and benzyl chloroformate (130 μL, 0.91 mmol), and stirred overnight. The solution was evaporated to a slurry under vacuum, diluted with EtOAc and washed with water. The organic layer was evaporated to a residue, which was purified by column chromatography (1 → 2% MeOH in DCM) to yield 550 mg of 1,3,2',6'-tetraazido-3''-*N*-Cbz-sisomicin **6.4** (98%, 0.802 mmol), as a light yellow amorphous solid.

R_f = 0.4, 20:5:3, $CHCl_3$/EtOAc/MeOH.

HRMS (ESI) calcd. for $C_{27}H_{35}N_{13}O_9$, M + H^+ = 686.2754, found 686.2775 (3.09 ppm).

Two 3'''-*N*MeCbz conformers approx. 5:2 ratio in CD_3OD:

Major conformer ^{1}H NMR (CD_3OD, 400 MHz) δ 7.39-7.25 (m, 5H), 5.88 (d, J = 2.4 Hz, 1H), 5.43 (d, J = 3.8 Hz, 1H), 5.15 (d, J = 1.9 Hz, 1H), 4.97 (dd, J = 5.0, 2.0 Hz, 1H), 4.29 (d, J = 11.4 Hz, 1H), 4.21 (d, J = 12.2 Hz, 1H), 4.16-4.10 (m, 1H), 3.78 (d, J = 13.9 Hz, 1H), 3.71-3.59 (m, 4H), 3.50-3.27 (m, 4H), 3.20 (d, J = 12.1 Hz, 1H), 3.05 (s, 3H), 2.49-2.38 (m, 1H), 2.34-2.20 (m, 2H), 1.40 (ddd, J = 12.6, 12.6, 12.6 Hz, 1H), 1.03 (s, 3H).

^{13}C NMR (CD_3OD, 100 MHz) δ 160.1, 147.2, 138.4, 129.6 (2C), 129.0, 129.0, 128.7, 100.1, 99.4, 98.4, 81.0, 80.8, 76.1, 75.1, 70.5, 68.5, 66.0, 62.0, 61.7, 59.9, 56.0, 53.3, 34.0, 30.9, 22.1, 22.1.

Minor conformer ^{1}H NMR (CD_3OD, 400 MHz) δ 7.39-7.25 (m, 5H), 5.84 (d, *J* = 2.3 Hz, 1H), 5.31 (d, *J* = 3.9 Hz, 1H), 5.15 (d, *J* = 1.9 Hz, 1H), 4.97 (dd, *J* = 5.0, 2.1 Hz, 1H), 4.29 (d, *J* = 11.2 Hz, 1H), 4.17 (d, *J* = 7.4 Hz, 1H), 4.16-4.10 (m, 1H), 3.77 (d, *J* = 14.0 Hz, 1H), 3.71-3.59 (m, 4H), 3.50-3.27 (m, 4H), 3.20 (d, *J* = 12.1 Hz, 1H), 3.04 (s, 3H), 2.49-2.38 (m, 1H), 2.34-2.21 (m, 2H), 1.38 (dd, *J* = 25.1, 12.5 Hz, 1H), 0.99 (s, 3H).

^{13}C NMR (CD_3OD, 100 MHz) δ 159.7, 147.2, 138.3, 129.6 (2C), 129.1, 129.0, 128.7, 100.6, 99.4, 98.4, 83.0, 81.3, 76.0, 74.8, 70.6, 68.4, 65.9, 61.8, 61.5, 60.0, 56.1, 53.3, 34.0, 31.1, 22.5, 22.3.

6.5

6'-Aldehydo-1,3,2'-triazido-3''-*N*-Cbz-sisomicin (6.5)

Azide **6.4** (345 mg, 0.503 mmol) was dissolved in DCM (3 mL), treated with 3,4-dihydro-[2*H*]-pyran (135 μL, 1.5 mmol),[1] SeO_2 (280 mg, 2.5 mmol), and stirred vigorously. After 36 h, the red solids were filtered and washed with DCM (20 mL). The filtrate and washings were washed with 2 M HCl and satd. $NaHCO_3$, dried over Na_2SO_4, filtered and evaporated to a residue under vacuum. Purification by silica gel chromatography (1 → 2% MeOH in DCM) yielded 303 mg of 6'-aldehydo-1,3,2'-triazido-3''-*N*-Cbz-sisomicin **6.5** (92%, 0.460 mmol), as a light yellow amorphous solid.

R_f = 0.6, 20:5:3, $CHCl_3$/ EtOAc/MeOH.

HRMS (ESI) calcd. for $C_{27}H_{34}N_{10}O_{10}$, M + H^+ = 659.2532, found 659.2536 (0.62 ppm).

Two 3'''-*N*MeCbz conformers approx. 5:2 ratio in CD_3OD:

Major conformer ^{1}H NMR (CD_3OD, 400 MHz) δ 9.24 (s, 1H), 7.44-7.29 (m, 5H), 6.24 (dd, *J* = 4.7, 3.2 Hz, 1H), 6.01 (d, *J* = 2.4 Hz, 1H), 5.47 (d, *J* = 3.7 Hz, 1H), 5.19 (d, *J* = 1.9 Hz, 1H), 4.33 (d, *J* = 11.4 Hz, 1H), 4.26 (d, *J* = 12.1 Hz, 1H), 4.20-4.15 (m, 1H), 3.75-3.60 (m, 3H), 3.58-3.50 (m, 1H), 3.49-3.31 (m, 3H), 3.24 (d, *J* = 12.1 Hz, 1H), 3.09 (s, 3H), 2.72-2.62 (m, 2H), 2.27 (ddd, *J* = 12.8, 4.4, 4.4 Hz, 1H), 1.45 (dd, *J* = 24.0, 11.4 Hz, 1H), 1.07 (s, 3H).

^{13}C NMR (CD_3OD, 100 MHz) δ 188.1, 160.1, 150.2, 138.4, 129.6 (2C), 129.0, 129.0, 128.7, 122.7, 100.1, 98.0, 81.1, 80.8, 76.0, 75.1, 70.5, 68.5, 66.0, 62.0, 61.5, 59.9, 55.5, 33.7, 30.9, 23.2, 22.1.

Minor conformer ^1H NMR (CD_3OD, 400 MHz) δ 9.23 (s, 1H), 7.45-7.28 (m, 5H), 6.24 (dd, J = 4.6, 3.4 Hz, 1H), 5.97 (d, J = 2.4 Hz, 1H), 5.35 (d, J = 3.8 Hz, 1H), 5.19 (d, J = 1.9 Hz, 1H), 4.34 (d, J = 11.2 Hz, 1H), 4.24-4.20 (m, 1H), 4.20-4.15 (m, 1H), 3.75-3.60 (m, 3H), 3.58-3.50 (m, 1H), 3.49-3.31 (m, 3H), 3.24 (d, J = 12.1 Hz, 1H), 3.08 (s, 3H), 2.71-2.63 (m, 2H), 2.27 (ddd, J = 12.8, 4.4, 4.4 Hz, 1H), 1.42 (dd, J = 24.6, 12.3 Hz, 1H), 1.03 (s, 3H).

^{13}C NMR (CD_3OD, 100 MHz) δ 188.1, 159.7, 150.2, 138.3, 129.6 (2C), 129.1, 129.0, 128.7, 122.7, 100.6, 97.9, 82.9, 81.4, 75.9, 74.8, 70.6, 68.4, 65.9, 61.7, 61.4, 60.0, 55.6, 33.8, 31.1, 23.3, 22.5.

Me OH N3 OMe
CbzN O N3 OMe
O
Me O HO O
OH N3

6.6

1,3,2'-Triazido-3''-*N*-Cbz-6',6'-dimethoxy-sisomicin (6.6)

Aldehyde **6.5** (200 mg, 0.304 mmol) was dissolved in anhydrous MeOH (3 mL), treated with trimethylorthoformate (830 μL, 7.60 mmol) and TFA (45 μL, 0.61 mmol), and stirred at RT for 5 h, when no UV-active starting material was observed on TLC, the reaction was neutralized with Et_3N (93 μL, 0.67 mmol). The volatiles were removed under vacuum to a residue. Purification by ammoniacal silica gel chromatography to avoid acetal cleavage (1 → 2 % ammoniacal MeOH in DCM) yielded 175 mg of 1,3,2'-triazido-3''-*N*-Cbz-6',6'-dimethoxy-sisomicin **6.6** (82%, 0.248 mmol), as a white amorphous solid.

R_f = 0.65, 20:5:3 $CHCl_3$/EtOAc/MeOH.

HRMS (ESI) calcd. for $C_{29}H_{40}N_{10}O_{11}$, M + Na^+ = 727.27702, found 727.27790 (1.21 ppm).

Two 3'''-NMeCbz conformers approx. 5:2 ratio in CD_3OD:

Major conformer ^1H NMR (CD_3OD, 400 MHz) δ 7.31 (m, 5H), 5.85 (d, J = 2.2 Hz, 1H), 5.42 (d, J = 3.8 Hz, 1H), 5.17-5.13 (m, 2H), 4.72 (s, 1H), 4.29 (d, J = 11.4 Hz, 1H), 4.21

(d, J = 12.1 Hz, 1H), 4.13 (dd, J = 11.5, 3.4 Hz, 1H), 3.69-3.58 (m, 3H), 3.48-3.39 (m, 2H), 3.39-3.27 (m, 2H), 3.36 (s, 3H), 3.26 (s, 3H), 3.19 (d, J = 12.1 Hz, 1H), 3.05 (s, 3H), 2.48-2.36 (m, 1H), 2.34-2.20 (m, 2H), 1.41 (ddd, J = 12.5, 12.5, 12.5 Hz, 1H), 1.02 (s, 3H).
^{13}C NMR (CD_3OD, 100 MHz) δ 160.2, 146.7, 138.5, 129.7 (2C), 129.1, 129.1, 128.8, 101.9, 100.2, 99.0, 98.2, 81.1, 81.0, 76.2, 75.2, 70.6, 68.6, 66.1, 62.2, 61.8, 60.0, 56.4, 55.1, 52.3, 34.0, 31.0, 22.3, 22.1.
Minor conformer ^{1}H NMR (CD_3OD, 400 MHz) δ 7.31 (m, 5H), 5.80 (d, J = 2.2 Hz, 1H), 5.31 (d, J = 3.8 Hz, 1H), 5.18-5.13 (m, 2H), 4.71 (s, 1H), 4.29 (d, J = 11.2 Hz, 1H), 4.19-4.15 (m, 1H), 4.15-4.10 (m, 1H), 3.69-3.58 (m, 3H), 3.48-3.39 (m, 2H), 3.39-3.27 (m, 2H), 3.36 (s, 3H), 3.26 (s, 3H), 3.19 (d, J = 12.1 Hz, 1H), 3.03 (s, 3H), 2.41 (ddd, J = 11.7, 7.0, 6.2 Hz, 1H), 2.27 (tdd, J = 12.7, 8.8, 5.0, 5.0 Hz, 2H), 1.39 (dd, J = 24.9, 12.4 Hz, 1H), 0.98 (s, 3H).
^{13}C NMR (CD_3OD, 100 MHz) δ 159.78, 146.68, 138.38, 129.66 (2C), 129.20, 129.14, 128.83, 101.93, 100.67, 99.03, 98.19, 83.0, 81.3, 76.2, 74.9, 70.7, 68.5, 66.0, 61.9, 61.6, 60.1, 56.5, 55.0, 52.4, 34.1, 31.2, 22.6, 22.3.

6',6'-Dimethoxy-sisomicin (6.7)

Approx. 5 to 7 mL of ammonia was condensed into a two-neck flask equipped with a cold finger condenser at -78 °C. Approx. 20 to 30 mg (~ 1 mmol) of sodium metal were added to the ammonia solution, which was stirred vigorously at -78 °C until the reaction turned deep blue and the sodium fully dissolved. A solution of acetal intermediate **6.6** (87.2 mg, 123.7 μmol) in anhydrous THF (1 mL) was transferred dropwise into the blue solution. Note: *t*BuOH was not used. After 15 minutes, LRMS analysis showed the product molecular ion indicating complete removal of the protecting groups, and the reaction was quenched with excess AcOH (100 μL). The ammonia was slowly evaporated by bubbling argon at RT, to obtain a white residue of salts, which was dissolved with 10% ammoniacal MeOH in $CHCl_3$, and purified by column chromatography using the same solvent system

(12 → 15 → 20% ammoniacal MeOH). The fractions containing aminoglycoside were identified by TLC, collected and evaporated under vacuum to furnish a wet residue, which was dissolved in a minimum volume of water and freeze-dried. The dry residue obtained was redissolved in 1 mL of water, at which point insoluble traces of silica were generally observed, and were removed by filtration of the solution through a 0.45 μm syringe filter. Finally, freeze-drying the filtrate yielded 68.3 mg of 6',6'-dimethoxy-sisomicin acetate salt **6.7** (75%, 9.32 μmol), as an amorphous solid.

R_f = 0.2, 40% ammoniacal MeOH/$CHCl_3$.
$[\alpha]^{22}_D$ 101.7° (*c* 0.3 in H_2O).
HRMS (ESI) calcd. for $C_{21}H_{40}N_4O_9$, M + H^+ = 493.2868, found 493.2876 (1.60 ppm).
^1H NMR (D_2O, 400 MHz) δ 5.42 (d, *J* = 1.3 Hz, 1H), 5.12 (dd, *J* = 3.6, 3.6 Hz, 1H), 4.93 (d, *J* = 3.7 Hz, 1H), 4.61 (s, 1H), 4.04 (dd, *J* = 10.9, 3.6 Hz, 1H), 3.86 (d, *J* = 12.9 Hz, 1H), 3.72 (dd, *J* = 9.6, 9.6 Hz, 1H), 3.65 (ddd, *J* = 6.9, 6.5, 1.7 Hz, 1H), 3.54 (ddd, *J* = 19.0, 9.1, 9.1 Hz, 2H), 3.35-3.30 (m, 2H), 3.29 (s, 3H), 3.28-3.17 (m, 3H), 3.26 (s, 3H), 2.75 (s, 3H), 2.45 (ddd, *J* = 17.5, 5.0, 5.0 Hz, 1H), 2.31-2.16 (m, 2H), 1.74 (s, 12H AcOD), 1.64 (ddd, *J* = 12.7, 12.7, 12.6 Hz, 1H), 1.18 (s, 3H).
^{13}C NMR (D_2O, 100 MHz) δ 180.9 (AcOD), 144.0, 101.8, 100.6, 99.4, 96.3, 83.6, 80.4, 73.1, 69.5, 67.2, 65.9, 63.1, 54.7, 54.5, 49.5, 47.9, 46.0, 34.1, 28.4, 22.8 (AcOD), 21.1, 20.5.

6.1

***Aminoglycoside 66-40C* (6.1)**

6',6'-Dimethyl acetal sisomicin intermediate **6.7** (68.1 mg, 9.3 μmol) was dissolved in 1 mL 0.5 N H_2SO_4 and stirred for 15 min to liberate aldehyde **6.2**. The solution was neutralized with 10 mL of sat. $Ba(OH)_2$ (approx. 0.05 N, freshly prepared by heating 315 mg of the octahydrate salt in 20 mL H_2O at reflux for 20 min). The resulting white

suspension of $BaSO_4$ was filtered through a 0.45 μm syringe filter and freeze-dried to generate a white fluffy cotton solid. ^{1}H-NMR analysis in D_2O showed only signals belonging to dimer *66-40C* (**6.1**). The residue was dissolved in $CHCl_3$/MeOH/NH_4OH (2:3:0.5) and purified by silica gel column chromatography using the same solvent system, increasing ammonia gradually to a 2:3:1 mixture. The fractions containing aminoglycoside were identified by TLC, collected and evaporated under vacuum to furnish a wet residue, which was dissolved in a minimum volume of water and freeze-dried. The dry residue obtained was redissolved in 1 mL of water, at which point insoluble traces of silica were generally observed, and were removed by filtration of the solution through a 0.45 μm syringe filter. Finally, freeze-drying the filtrate yielded 27.4 mg of *aminoglycoside 66-40C* **6.1** (69%, 3.20 μmol), as the free-base fluffy cotton solid.

R_f = 0.3, 2:3:2 $CHCl_3$/MeOH/NH_4OH.
$[\alpha]^{22}_D$ 111.0° (*c* 0.4 in H_2O) (lit.,[2] +112.5 (*c* 0.3 in H_2O)).
ν_{max}/cm^{-1} 3333, 1653, 1632 and 1017 (lit.,[2] 3300, 1670, 1630 and 1025).
HRMS (ESI) calcd. for $C_{38}H_{64}N_8O_{14}$, M + H^+ = 857.4615, found 857.4603 (-1.40 ppm).
^{1}H NMR (D_2O, 700 MHz) δ 7.44 (s, 2H), 5.49 (d, *J* = 2.3 Hz, 2H), 5.48 (d, *J* = 2.1 Hz, 2H), 5.13 (d, *J* = 4.0 Hz, 2H), 4.09 (d, *J* = 12.6 Hz, 2H), 3.88 (dd, *J* = 10.8, 3.9 Hz, 2H), 3.71 (dd, *J* = 9.2, 9.2 Hz, 2H), 3.65 (dd, *J* = 9.3, 9.3 Hz, 2H), 3.38 (dd, *J* = 9.6, 9.6 Hz, 2H), 3.33 (d, *J* = 12.6 Hz, 2H), 3.18 (ddd, *J* = 11.9, 9.6, 4.8 Hz, 2H), 2.95 (dddd, *J* = 18.9, 13.4, 8.0, 3.2 Hz, 4H), 2.59 (s, 6H), 2.26 (ddd, *J* = 17.5, 6.1, 6.0 Hz, 2H), 2.20 (ddd, *J* = 17.8, 11.9, 2.1 Hz, 2H), 1.92 (ddd, *J* = 12.8, 4.2, 3.9 Hz, 2H), 1.65 (ddd, *J* = 12.6, 12.6, 12.6 Hz, 2H), 1.23 (s, 6H).
^{13}C NMR (D_2O, 175 MHz) δ 161.2, 145.9, 115.9, 101.0, 99.5, 86.8, 80.6, 76.2, 72.4, 69.3, 68.1, 66.4, 64.0, 51.2, 46.8, 37.0, 35.6, 24.9, 22.0.

Procedure for ^{1}H-NMR observation of dimerization of *aminoglycoside 66-40C* (6.1)

Dimethyl acetal **6.7** (6.0 mg, 12.2 μL) was dissolved in 600 μL of a chilled 0 °C 0.1 N H_2SO_4 solution in D_2O, and H-NMR (400 MHz) acquisitions were started within 5 min. Under these conditions acquisition for 1 min every 5 min clearly showed conversion to sisomicin 6'-aldehyde (**6.2**) within 1 h. The solution was transferred to a vial, cooled to 0 °C and carefully neutralized with approx. 1.2 mL D_2O sat. $Ba(OH)_2$ at 0 °C, and a portion was immediately filtered through a 0.45 μm syringe filter into an NMR tube. Sequential ^{1}H-NMR acquisitions revealed a mixture approx. 20:1 of aldehyde **6.2** and imine **6.1** which advanced slowly (pH ~7). A small drop of sat. $Ba(OH)_2$ was added every 10 min until the dimerization proceed at a convenient rate (pH ~8). Under these conditions (approx. 7 mM for aldehyde **6.2**) acquisition for 2 min every 10 min showed conversion of sisomicin 6'-aldehyde **6.2** to *aminoglycoside 66-40C* (**6.1**) within 2 h, without evidence of other imine intermediates.

6.2

Procedure for ^{1}H-NMR observation of hydrolysis of *aminoglycoside 66-40C* (6.1) to 6'-aldehydo-sisomicin (6.2)

Aminoglycoside 66-40C (**6.1**, 15 mg, 17.5 μmol) was dissolved in chilled 0 ºC 0.2 N H_2SO_4 in D_2O (0.6 mL).[2] ^{1}H-NMR (400 MHz) acquisitions were started within 5 min. Under these conditions acquisition for 1 min every 20 min showed complete hydrolysis within 2 h to sisomicin 6'-aldehyde (**6.2**), which was observed as a 7:1 mixture with its 6'-hydrate (lit.,[2] 1:2). The spectrum was identical to aldehyde produced from dimethyl acetal **6.7**. Neutralization of this mixture as previously described reformed *aminoglycoside 66-40C* (**6.1**).

HRMS (ESI) calcd. for $C_{19}H_{34}N_4O_8$, M + H^+ = 447.24494, found 447.24652 (3.54 ppm).
^{1}H NMR (D_2O/0.2 N H_2SO_4, 700 MHz) δ 8.97 (s, 1H), 6.16-6.13 (m, 1H), 5.51 (s, 1H), 4.91 (d, *J* = 3.7 Hz, 1H), 4.01 (dd, *J* = 10.9, 3.6 Hz, 1H), 3.86 (dd, *J* = 9.9, 9.0 Hz, 1H), 3.83-3.79 (m, 2H), 3.65-3.55 (m, 2H), 3.41-3.33 (m, 2H), 3.30 (d, *J* = 11.0 Hz, 1H), 3.28

(d, J = 12.9 Hz, 1H), 2.77 (dd, J = 15.3, 12.1 Hz, 1H*), 2.71 (s, 3H), 2.51 (ddd, J = 20.2, 4.3, 4.3 Hz, 1H*), 2.37 (ddd, J = 12.5, 4.3, 4.3 Hz, 1H), 1.74 (ddd, J = 12.6, 12.6, 12.6 Hz, 1H), 1.14 (s, 3H).

^{13}C NMR (D_2O/0.2 N H_2SO_4, 175 MHz) δ 189.3, 149.4/149.4 (1:1 ratio), 123.6/123.5 (1:1 ratio), 102.0, 97.7/97.7 (1:1 ratio), 84.0, 80.3, 74.2, 70.7, 68.4, 67.1, 64.1, 50.6, 48.8, 46.7/46.6 (1:1 ratio), 35.2, 28.3, 25.0*, 21.6. *Note: protons slowly exchange with D_2O.

N_3 HO HO N_3 O O N_3 N_3

6.8

1,3,2',6'-Tetraazido-sisamine (6.8)

Tetra-azido sisomicin N3''' free-base **6.3** (400 mg, 0.727 mmol) was dissolved in MeOH (15 mL), treated with $NaIO_4$ (470 mg, 2.20 mmol), stirred vigorously for 16 h, diluted with a volume of EtOH, stirred 30 min and filtered, washing with EtOH. The filtrate and washings were evaporated to a slurry under vacuum, the solids were redissolved in EtOH and refiltered through a thin pad of Celite^{TM}. The filtrate was evaporated to a residue, dissolved with EtOAc, washed with sat. NaCl, dried over Na_2SO_4, filtered and evaporated. The resulting residue was dissolved in EtOH (15 mL), treated with NMe_2-NH_2 (500 μL, 6.60 mmol) and 2 mL of AcOH, and heated to reflux for 24 h. The mixture was cooled and evaporated under vacuum to approx. 2 mL of a yellow residue, which was dissolved in EtOAc (20 mL), washed successively with 2 N HCl twice and sat. $NaHCO_3$, dried over $MgSO_4$, filtered and evaporated to a residue. Purification by column chromatography (20 → 30% EtOAc in hexanes) yielded 151 mg of 1,3,2',6'-tetraazido-sisamine **6.8** (53%, 0.385 mmol), as a light yellow amorphous solid.

R_f = 0.6, 1:1 EtOAc/hexanes.

HRMS (ESI) calcd. for $C_{12}H_{16}N_{12}O_4$, M + H^+ = 393.1490, found 393.1505 (3.69 ppm).

^1H NMR (CD_3OD, 400 MHz) δ 5.67 (d, J = 1.9 Hz, 1H), 4.96 (d, J = 4.2 Hz, 1H), 3.88 (s, 1H), 3.75 (d, J = 13.9 Hz, 1H), 3.67 (d, J = 13.4 Hz, 1H), 3.65-3.51 (m, 4H), 3.45-3.31 (m,

3H), 2.47 (dd, J = 15.4, 12.2 Hz, 1H), 2.32 (ddd, J = 16.2, 5.9, 5.9 Hz, 1H), 2.24 (ddd, J = 12.8, 3.6, 3.6 Hz, 1H), 1.41 (ddd, J = 12.6, 12.5, 12.5 Hz, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 145.5, 98.1, 96.7, 80.8, 76.1, 75.7, 60.0, 59.8, 55.3, 52.3, 32.4, 20.9.

6.9

6'-Aldehydo-1,3,2'-triazido-sisamine (6.9)

Compound **6.8** (150 mg, 0.382 mmol) was dissolved in DCM (5 mL), and treated with 3,4-dihydro-[2*H*]-pyran (160 μL, 1.80 mmol) and SeO_2 (380 mg, 3.42 mmol) in 3 portions 12 h apart. After 48 h, the red solids were filtered and washed with DCM (20 mL). The filtrate and washings were combined, washed with 2 M HCl and satd. $NaHCO_3$, dried over Na_2SO_4, filtered and evaporated to a residue under vacuum. Purification by silica gel chromatography (30% EtOAc/hexanes) yielded 137 mg of 6'-aldehydo-1,3,2'-triazido-sisamine **6.9** (98%, 0.375 mmol), as a yellow amorphous solid.

R_f = 0.3, 1:1 EtOAc/hexanes.

HRMS (ESI) calcd. for $C_{12}H_{15}N_9O_5$, M + Na^+ = 388.1088, found 388.1103 (3.73 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 9.21 (s, 1H), 6.08 (dd, J = 5.3, 2.6 Hz, 1H), 5.93 (d, J = 2.1 Hz, 1H), 3.75 (dd, J = 9.5, 9.5 Hz, 1H), 3.64 (dd, J = 9.0, 9.0 Hz, 1H), 3.56 (ddd, J = 11.0, 6.5, 2.1 Hz, 1H), 3.47-3.30 (m, 3H), 2.72 (ddd, J = 18.1, 11.5, 2.6 Hz, 1H), 2.62 (ddd, J = 18.4, 6.1, 6.1 Hz, 1H), 2.23 (ddd, J = 13.3, 4.3, 4.3 Hz, 1H), 1.41 (ddd, J = 12.6, 12.6, 12.6 Hz, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 185.8, 148.6, 120.3, 96.3, 80.3, 76.1, 75.9, 60.0, 59.8, 54.5, 32.3, 22.3.

6.10

1,3,2'-Triazido-6',6'-dimethoxy-sisamine (6.10)

Aldehyde **6.9** (135 mg, 0.370 mmol) was dissolved in anhydrous MeOH (5 mL), treated with trimethylorthoformate (1.0 mL, 9.3 mmol) and TFA (57 μL, 0.74 mmol), and stirred at RT for 5 h, when no UV-active starting material was observed on TLC, the reaction was neutralized with Et_3N (115 μL, 0.82 mmol). The volatiles were removed under vacuum to a residue. Purification by chromatography on silica gel which was previously neutralized with 3% Et_3N/hexanes and thoroughly washed with the elution solvent (20 → 40% EtOAc/hexanes), yielded 110 mg of 1,3,2'-tri-azido-6',6'-dimethoxy-sisamine **6.10** (72%, 0.267 mmol), as a white amorphous solid.

R_f = 0.3 (EtOAc/hexanes, 1:1).

HRMS (ESI) calcd. for $C_{14}H_{21}N_9O_6$, M + Na^+ = 343.1507, found 343.1513 (1.36 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 5.73 (d, *J* = 2.3 Hz, 1H), 5.19 (dd, *J* = 5.6, 1.8 Hz, 1H), 4.71 (s, 1H), 4.09-3.95 (m, 2H), 3.63 (dd, *J* = 17.6, 8.3 Hz, 1H), 3.59-3.55 (m, 1H), 3.49 (ddd, *J* = 11.3, 6.3, 2.3 Hz, 1H), 3.42-3.31 (m, 3H), 3.39 (s, 3H), 3.30 (s, 3H), 2.52-2.41 (m, 1H), 2.31 (ddd, *J* = 16.3, 6.0, 6.0 Hz, 1H), 2.21 (ddd, *J* = 13.0, 3.9, 3.9 Hz, 1H), 1.44-1.30 (m, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 144.8, 100.5, 97.8, 96.1, 80.2, 76.1, 75.9, 59.9, 59.6, 55.4, 54.8, 52.1, 32.4, 20.6.

6',6'-Dimethoxy-sisamine (6.11)

The conditions for Birch reduction with Na/liq. NH_3 were identical to those described for deprotection of compound **6.6** to **6.7**, utilizing compound **6.10** (49 mg, 0.119 mmol). Once the ammonia was evaporated, the salt residue was suspended using 10% ammoniacal MeOH in $CHCl_3$ and purified by column chromatography using the same solvent system (12 → 15 → 20% ammoniacal MeOH). Evaporation, filtration (0.45 μm) and freeze-drying of the collected fractions yielded 41.6 mg of 6',6'-dimethoxy-sisamine acetate salt **6.11** (68%, 81.1 μmol), as a fluffy cotton solid.

R_f = 0.2, 30% ammoniacal MeOH/$CHCl_3$.

$[\alpha]^{22}_D$ 71.5º (*c* 0.30 in MeOH).

HRMS (ESI) calcd. for $C_{14}H_{27}N_3O_6$, M + Na^+ = 356.1792, found 356.1806 (4.04 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 5.29 (d, *J* = 1.7 Hz, 1H), 4.96 (dd, *J* = 4.6, 2.5 Hz, 1H), 4.43 (s, 1H), 3.40 (dd, *J* = 9.5, 9.5 Hz, 1H), 3.31-3.16 (m, 3H), 3.17 (s, 3H), 3.16 (s, 3H), 2.91-2.77 (m, 2H), 2.22 (ddd, *J* = 16.7, 5.5, 5.5 Hz, 1H), 2.14-2.01 (m, 2H), 1.71 (s, 9H AcOD), 1.41 (ddd, *J* = 12.4, 12.4, 12.4 Hz, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 179.4 (AcOD), 146.7, 103.1, 99.8, 99.1, 85.3, 76.7, 74.9, 55.0, 54.9, 51.9, 50.7, 48.2, 31.8, 23.6, 23.2 (AcOD).

6.13

3-6'-*Bis*-imino-sisamine dimer (6.13)

Dimer **6.13** was generated under identical conditions as *aminoglycoside 66-40C* (**6.2**). Using compound **6.11** (37.6 mg, 73.3 μmol), incubating with 1 mL 0.5 N H_2SO_4 for 15 min liberated sisamine 6'-aldehyde **6.12** (HRMS (ESI) calcd. for $C_{12}H_{22}N_3O_5$, M + H^+ = 288.15540, found 288.15604 (2.23 ppm)). Neutralization with sat. $Ba(OH)_2$, filtration and freeze-drying gave a fluffy cotton of crude dimer **6.13**. The residue was dissolved in $CHCl_3$/MeOH/NH_4OH (2:3:0.5) and purified by silica gel column chromatography using the same solvent system, increasing ammonia liquor gradually to a 2:3:0.75 mixture. Evaporation, filtration (0.45 μm) and freeze-drying of the collected fractions yielded 17.7 mg of 3-6'-*bis*-imino-sisamine dimer **6.13** (90%, 33.0 μmol), as the free-base fluffy cotton solid.

R_f = 0.8, 2:3:2 $CHCl_3$/MeOH/NH_4OH.

$[\alpha]^{22}_D$ -10.8° (*c* 0.3 in H_2O).

HRMS (ESI) calcd. for $C_{24}H_{38}N_6O_8$, M + H^+ = 539.28239, found 539.28433 (-3.60 ppm).

^{1}H NMR (D_2O, 700 MHz) δ 7.45 (s, 2H), 5.50 (dd, *J* = 6.3, 2.6 Hz, 2H), 5.48 (d, *J* = 2.7 Hz, 2H), 3.69 (dd, *J* = 9.3, 9.3 Hz, 2H), 3.56 (dd, *J* = 9.3, 9.3 Hz, 2H), 3.37 (dd, *J* = 9.7, 9.7 Hz, 2H), 3.21 (ddd, *J* = 11.9, 9.6, 4.7 Hz, 2H), 3.02 (ddd, *J* = 11.8, 6.1, 2.6 Hz, 2H), 2.92 (ddd, *J* = 12.7, 10.1, 4.0 Hz, 2H), 2.29 (ddd, *J* = 17.6, 6.1, 6.1 Hz, 2H), 2.23 (ddd, *J* = 17.9, 11.8, 2.5 Hz, 2H), 2.01 (ddd, *J* = 13.0, 4.3, 4.3 Hz, 2H), 1.72 (ddd, *J* = 12.5, 12.5, 12.3 Hz, 2H).

^{13}C NMR (D_2O, 175 MHz) δ 160.8, 145.2, 115.2, 98.4, 79.5, 76.9, 75.6, 65.5, 50.0, 46.2, 33.7, 23.8.

6.S1

6'-Aldehydo-4',5'-dehydro-3',4'-dideoxy-*per*-*N*-Cbz-paromomycin (6.S1)

Sodium methoxide in MeOH was prepared by addition of a piece of sodium to anhydrous MeOH. The resulting alkaline solution was diluted with anhydrous MeOH to approx. pH 8 to 9 (Accutint pH paper roll). Intermediate 6'-aldehydo-4',5'-dehydro-3'-deoxy-6,3',2'',5'',3''',4'''-hexa-*O*-methylcarbonate-*per*-*N*Cbz-paromomycin **4.18** (see Chapter 4, 300 mg, 0.195 mmol) was dissolved with the dilute NaOMe solution (5 mL) and stirred 5 h, monitoring by TLC and LRMS, when the observed molecular ions corresponding to the product indicated complete deprotection. The solution was neutralized with a few drops of AcOH and the solution was evaporated to a residue under vacuum. Purification by silica gel column chromatography (2 → 4 % MeOH in DCM) yielded 186.5 mg of the title compound **6.S1** (76%, 0.149 mmol), as a white amorphous solid.

R_f = 0.5, 20:5:3 $CHCl_3$/EtOAc/MeOH.

HRMS (ESI) calcd. for $C_{63}H_{71}N_5O_{22}$, M + Na^+ = 1272.4488, found 1272.4488 (-3.72 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 8.75 (s, 1H), 7.45-6.97 (m, 25H), 5.75 (s, 1H), 5.60 (s, 1H), 5.20-4.92 (m, 12H), 4.90 (s, 1H), 4.25-4.13 (m, 2H), 3.97 (s, 1H), 3.94-3.86 (m, 2H), 3.83 (s, 1H), 3.70-3.22 (m, 11H), 2.37-2.26 (m, 1H), 2.03-1.83 (m, 1H), 1.33 (dd, *J* = 24.3, 12.2 Hz, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 188.4, 159.2-158.0 (5C), 149.8, 138.3-138.0 (5C), 129.6-128.5 (25C), 124.2, 110.7, 100.3, 96.8, 87.4, 83.7, 78.9, 78.5, 75.8, 75.7, 74.7, 71.6, 69.3, 68.0, 67.6 (2C), 67.5 (2C), 63.3, 54.2, 52.8, 51.0, 48.2, 42.6, 35.1, 24.7.

4',5'-Dehydro-3',4'-dideoxy-6',6'-dimethoxy-*per*-*N*-Cbz-paromomycin (6.14)

Aldehyde **6.S1** (186 mg, 0.149 mmol) was dissolved in anhydrous MeOH (5 mL), treated with trimethylorthoformate (400 µL, 3.7 mmol) and TFA (23 µL, 0.3 mmol), and stirred at RT for 5 h, when no UV-active starting material was observed on TLC, the solution was neutralized with Et_3N (46 µL, 0.33 mmol). The volatiles were removed under vacuum to a residue, which was purified by ammoniacal silica gel chromatography to avoid acetal cleavage (4 → 6 % ammoniacal MeOH in DCM), yielding 162 mg of the title compound **6.14** (84%, 0.125 mmol), as a white amorphous solid.

R_f = 0.4, 20:5:3 $CHCl_3$/EtOAc/MeOH.

HRMS (ESI) calcd. for $C_{65}H_{77}N_5O_{23}$, M + Na^+ = 1318.4902, found 1318.4858 (3.34 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.29 (ddd, *J* = 17.9, 16.0, 4.3 Hz, 25H), 5.64 (s, 1H), 5.16-4.92 (m, 11H), 4.90 (s, 1H), 4.45 (s, 1H), 4.22-4.14 (m, 2H), 4.01-3.94 (m, 1H), 3.94-3.86 (m, 2H), 3.86-3.75 (m, 2H), 3.70-3.50 (m, 4H), 3.50-3.38 (m, 3H), 3.37-3.25 (m, 4H), 3.21 (s, 6H), 2.15-1.93 (m, 3H), 1.40-1.17 (m, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 159.2-158.4 (5C), 145.5, 138.3-138.1 (5C), 129.6-128.5 (25C), 110.8, 103.0, 101.2, 100.3, 96.5, 87.2, 83.7, 79.0, 77.1, 75.9, 75.5, 74.6, 71.6, 69.3, 68.0, 67.7, 67.6 (2C), 67.5, 63.4, 54.9, 54.2, 53.8, 52.8, 51.2, 47.2, 42.6, 35.7, 23.3.

6.S2

4',5'-Dehydro-3',4'-dideoxy-6',6'-dimethoxy-paromomycin (6.S2)

The conditions for Birch reduction with Na/liq. NH_3 were identical to those described for deprotection of compound **6.6** to **6.7**, utilizing intermediate **6.14** (100 mg, 77 µmol). Once the ammonia was evaporated, the residue of salts was dissolved in $CHCl_3/MeOH/NH_4OH$ (2:3:0.5) and purified by silica gel column chromatography using the same solvent system, increasing ammonia gradually to a 2:3:1 mixture. Evaporation, filtration (0.45 µm) and freeze-drying of the collected fractions yielded 43.2 mg of 4',5'-dehydro-3'-deoxy-6',6'-dimethoxy-paromomycin **6.S2** (90%, 69 µmol) as the free-base fluffy cotton solid. For characterization purposes, a portion of this solid (5.2 mg, 8.3 µmol) was redissolved in a minimum volume of water, treated with AcOH (30 µL) and freeze-dried to provide the aminoglycoside acetate salt (quant.).

R_f = 0.2, 2:3:1 $CHCl_3/MeOH/NH_4OH$.

$[\alpha]^{22}_D$ 71.5º (*c* 0.30 in MeOH).

HRMS (ESI) calcd. for $C_{25}H_{47}N_5O_{13}$, M + Na^+ = 626.3243, found 626.3255 (1.84 ppm).

^{1}H NMR (D_2O, 400 MHz) δ 5.61 (d, *J* = 1.81 Hz, 1H), 5.25-5.22 (m, 2H), 5.22 (d, *J* = 2.08 Hz, 1H), 4.73 (s, 1H), 4.46 (dd, *J* = 6.40, 5.02 Hz, 1H), 4.34 (dd, *J* = 4.85, 2.19 Hz, 1H), 4.26 (ddd, *J* = 6.27, 3.96, 1.34 Hz, 1H), 4.18 (t, *J* = 3.01, 3.01 Hz, 1H), 4.13 (ddd, *J* = 6.43, 5.59, 3.23 Hz, 1H), 3.98 (dd, *J* = 10.20, 9.29 Hz, 1H), 3.86-3.81 (m, 2H), 3.78-3.77 (m, 1H), 3.75 (t, *J* = 9.24, 9.24 Hz, 1H), 3.68 (dd, *J* = 12.09, 5.44 Hz, 1H), 3.60 (dd, *J* = 10.26, 9.40 Hz, 1H), 3.54-3.52 (m, 1H), 3.42-3.36 (m, 2H), 3.39 (s, 3H), 3.37 (s, 3H), 3.32 (dd, *J* = 13.66, 3.87 Hz, 1H), 3.27 (ddd, *J* = 12.37, 10.99, 4.23 Hz, 1H), 2.59 (ddd, *J* = 17.88, 5.72, 4.23 Hz, 1H), 2.40 (td, *J* = 12.43, 4.33, 4.33 Hz, 1H), 2.32 (ddd, *J* = 17.93, 6.17, 3.67 Hz, 1H), 1.87 (s, 15H AcOD), 1.77 (q, *J* = 12.71, 12.52, 12.52 Hz, 1H).

^{13}C NMR (D_2O, 100 MHz) δ 181.0 (AcOD), 144.5, 110.4, 102.0, 99.7, 96.1, 95.4, 83.9, 81.1, 78.4, 75.7, 73.3, 72.0, 70.1, 67.6, 67.3, 60.9, 55.0, 54.9, 50.8, 49.7, 48.3, 46.0, 40.4, 28.1, 22.9 (AcOD), 21.9.

6.15

3-6'-*Bis*-imino-(4',5'-dehydro-3',4'-dideoxy-paromomycin) dimer (6.15)

The 4',5'-dehydro-3'-deoxy-paromomycin dimer **6.15** was generated under identical conditions as *aminoglycoside 66-40C* (**6.2**). Using compound **6.S2** (42.2 mg, 67 μmol), incubating with 1 mL 0.5 N H_2SO_4 for 15 min liberated aldehyde **6.S3**: HRMS (ESI) calcd. for $C_{25}H_{47}N_5O_{13}$, M + Na^+ = 580.2824, found 580.2824 (2.76 ppm). Neutralization with sat. $Ba(OH)_2$, filtration and freeze-drying gave a fluffy cotton of crude dimer **6.15**. The residue was dissolved in $CHCl_3$/MeOH/NH_4OH (2:3:1) and purified by silica gel column chromatography using the same solvent system, increasing ammonia gradually to a 2:3:2.5 mixture. Evaporation, filtration (0.45 μm) and freeze-drying of the collected fractions yielded 21.1 mg of the title compound **6.15** (56%, 18.7 μmol), as the free-base fluffy cotton solid.

R_f = 0.1, 2:3:2.5 $CHCl_3$/MeOH/NH_4OH.

$[\alpha]^{22}_D$ 14.4º (*c* 0.8 in H_2O).

HRMS (ESI) calcd. for $C_{25}H_{47}N_5O_{13}$, M + Na^+ = 1123.5365, found 1123.5325 (-3.54 ppm).

^1H NMR (D_2O, 700 MHz) δ 7.45 (s, 2H), 5.74 (d, *J* = 2.67 Hz, 2H), 5.54-5.51 (m, 2H), 5.31 (d, *J* = 0.60 Hz, 2H), 5.20 (d, *J* = 1.47 Hz, 2H), 4.47 (dd, *J* = 7.26, 4.68 Hz, 2H), 4.41 (dd, *J* = 4.50, 0.88 Hz, 2H), 4.23 (ddd, *J* = 6.02, 3.95, 1.17 Hz, 2H), 4.15-4.11 (m, 4H),

3.85 (dd, J = 12.24, 2.81 Hz, 2H), 3.83-3.78 (m, 4H), 3.73 (td, J = 2.93, 1.20, 1.02 Hz, 2H), 3.67 (dd, J = 12.30, 5.68 Hz, 2H), 3.62 (dd, J = 10.16, 9.07 Hz, 2H), 3.50-3.44 (m, 4H), 3.33 (dd, J = 13.71, 6.42 Hz, 2H), 3.30-3.24 (m, 4H), 3.21 (ddd, J = 13.04, 10.66, 3.96 Hz, 2H), 2.49-2.39 (m, 4H), 2.08 (ddd, J = 12.71, 4.55, 3.56 Hz, 2H), 1.82 (s, 18H), 1.86-1.78 (m, 2H).

^{13}C NMR (D_2O, 100 MHz) δ 180.8, 160.6, 145.2, 112.9, 110.5, 95.0, 93.9, 86.2, 80.7, 77.7, 75.1, 73.5, 72.8, 70.0, 67.6, 67.2, 64.8, 60.4, 50.7, 50.2, 46.2, 40.3, 31.0, 22.7, 20.3.

6.16

1,3,2'-Triazido-6'-propenol-sisamine (6.16)

Triethylphosphonoacetate (60 µL, 0.3 mmol) was dissolved in anhydrous THF (5 mL), cooled to 0 ºC, treated with *n*BuLi (2.0 M in cyclohexane, 152 µL, 0.3 mmol), followed by aldehyde **6.9** (100 mg, 0.274 mmol), and stirred 15 min, when TLC indicated complete consumption of **6.9** (R_f = 0.6 *vs* 0.9, 2:1, EtOAc/hexanes). The solution was then treated with DIBAL (1.0 M in hexanes, 1.1 mL, 1.1 mmol), and stirred until TLC indicated disappearance of the intermediate UV-active ester (R_f = 0.9 *vs* 0.5, 2:1, EtOAc/hexanes). When no UV-active material was observed on TLC, the reaction was quenched by neutralization with a few drops of AcOH, diluted with EtOAc and washed twice with sat. Rochelle's salt solution and sat. NaCl, dried over Na_2SO_4, filtered and evaporated to a residue under vacuum. Purification by column chromatography (40 % EtOAc in hexanes), yielded 73.2 mg of the title compound **6.16** (68%, 0.186 mmol), as a white amorphous solid.

R_f = 0.3, 1:1 EtOAc/hexanes.

HRMS (ESI) calcd. for $C_{14}H_{19}N_9O_6$, M + H^+ = 394.1582, found 394.1584 (0.52 ppm).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.17 (td, J = 15.44, 5.43, 5.43 Hz, 1H), 6.03 (d, J = 15.57 Hz, 1H), 5.60 (d, J = 2.32 Hz, 1H), 4.91 (dd, J = 5.44, 2.41 Hz, 1H), 4.27-4.18 (m, 2H),

3.77 (s, 1H), 3.66-3.60 (m, 1H), 3.60-3.58 (m, 1H), 3.58-3.54 (m, 1H), 3.44-3.36 (m, 2H), 3.36-3.33 (m, 1H), 3.33-3.27 (m, 1H), 2.53 (dd, J = 16.18, 11.56 Hz, 1H), 2.39 (td, J = 16.82, 6.07, 6.07 Hz, 1H), 2.26-2.18 (m, 1H), 1.83-1.75 (m, 1H), 1.40 (dd, J = 25.04, 12.53 Hz, 1H).

^{13}C NMR ($CDCl_3$, 100 MHz) δ 146.6, 128.1, 124.8, 99.6, 97.0, 82.1, 76.1, 75.6, 63.0, 60.0, 59.6, 56.0, 32.5, 21.9.

6.17

6'-Acrylaldehydo-1,3,2'-triazido-sisamine (6.17)

Compound **6.16** (53.2 mg, 0.137 mmol) was dissolved in anhydrous DCM (5 mL), treated with MnO_2 (5 μ ~85%, 330 mg, 3.82 mmol) and stirred at RT for 1 hr. The reaction was filtered through Celite™, washing with 1% MeOH/EtOAc. The filtrate and washings were combined, and evaporated to a residue under vacuum. Purification by chromatography (20 → 30 % EtOAc in DCM) yielded 38.6 mg of the title compound **6.17** (73%, 98.6 μmol), as a white amorphous solid.

R_f = 0.5, 1:1 EtOAc/hexanes, UV-active.

HRMS (ESI) calcd. for $C_{14}H_{17}N_9O_6$, M + Na^+ = 414.1245, found 414.1243 (-0.56 ppm).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.60 (d, J = 7.96 Hz, 1H), 6.82 (d, J = 15.39 Hz, 1H), 6.46 (dd, J = 15.37, 7.94 Hz, 1H), 5.71 (d, J = 2.43 Hz, 1H), 5.46 (dd, J = 5.64, 3.01 Hz, 1H), 3.65 (ddd, J = 11.16, 6.67, 2.55 Hz, 1H), 3.60 (d, J = 3.35 Hz, 1H), 3.59-3.56 (m, 1H), 3.41-3.38 (m, 1H), 3.38-3.35 (m, 1H), 3.35-3.29 (m, 1H), 2.87 (s, 1H), 2.63 (ddd, J = 17.72, 11.29, 3.00 Hz, 1H), 2.54 (dd, J = 15.05, 9.07 Hz, 1H), 2.23 (td, J = 13.24, 4.27, 4.27 Hz, 1H), 1.61 (s, 1H), 1.39 (dd, J = 25.27, 12.50 Hz, 1H).

^{13}C NMR ($CDCl_3$, 100 MHz) δ 193.5, 146.3, 145.1, 127.7, 110.0, 96.8, 81.7, 76.1, 75.7, 60.1, 59.7, 55.2, 32.4, 22.7.

6.S4

1,3,2'-Triazido-6',6'-dimethoxypropenyl-sisamine (6.S4)

Aldehyde **6.17** (47 mg, 0.120 mmol) was dissolved in anhydrous MeOH (5 mL), treated with trimethylorthoformate (380 μL, 3.47 mmol) and TFA (18.5 μL, 0.24 mmol), and stirred at RT for 3 h, when no UV-active starting material remained on TLC, the reaction was neutralized with Et_3N (37 μL, 0.265 mmol). The solvents were removed under vacuum to a residue. Purification by chromatography on silica gel, which was neutralized with Et_3N (20 → 30 % EtOAc in hexanes, 1% Et_3N). The collected fractions were evaporated to a minimum volume, which was added dropwise to stirring pentane. The precipitate was collected and dried under vacuum, to yield 44 mg of the title compound **6.S4** (84%, 0.101 mmol), as a white amorphous solid.

R_f = 0.8, 1:1 EtOAc/hexanes.

HRMS (ESI) calcd. for $C_{16}H_{23}N_9O_6$, M + Na^+ = 460.1664, found 460.1666 (0.63 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 6.12 (d, J = 15.64 Hz, 1H), 5.86 (dd, J = 15.71, 4.84 Hz, 1H), 5.82 (d, J = 2.16 Hz, 1H), 4.98 (dd, J = 4.99, 2.90 Hz, 1H), 4.83 (s, 1H), 3.58 (t, J = 9.46, 9.46 Hz, 1H), 3.49 (t, J = 9.20, 9.20 Hz, 1H), 3.42 (m, 2H), 3.34 (ddd, J = 12.30, 9.80, 4.47 Hz, 1H), 3.27 (s, 6H), 3.22 (dd, J = 9.67, 9.18 Hz, 1H), 2.47 (ddd, J = 16.69, 10.63, 2.36 Hz, 1H), 2.34 (td, J = 17.17, 5.76, 5.76 Hz, 1H), 2.08 (td, J = 12.98, 4.46, 4.46 Hz, 1H), 1.20 (q, J = 12.54, 12.54, 12.46 Hz, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 147.7 129.4, 125.9, 103.9, 103.2, 98.4, 81.9, 78.0, 77.8, 62.0, 61.8, 56.2, 53.0 (2C), 33.9, 23.2.

6.18

6',6'-Dimethoxypropenyl-sisamine (6.18)

Dimethoxy acetal **6.S4** (30 mg, 68.6 μmol) was dissolved in MeOH (5 mL), treated with trimethylphosphine (1 M in THF, 1 mL, 1.0 mmol) and 28% ammonia liquor (50 μL), and stirred overnight, when LRMS showed the molecular ion corresponding to the product indicating complete reduction of azide groups. The solvent was evaporated under vacuum to a residue. Purification by column chromatography (10 → 20% ammoniacal MeOH/$CHCl_3$) yielded 22.9 mg of the title compound **6.18** (93%, 63.7 μmol), as a free-base white amorphous solid.

R_f = 0.6, 40% ammoniacal MeOH/$CHCl_3$.

$[\alpha]^{22}_D$ 0.95º (*c* 1.1 in MeOH).

HRMS (ESI) calcd. for $C_{16}H_{29}N_3O_6$, M + Na^+ = 382.1949, found 382.1948 (-0.16 ppm).

^{1}H NMR (CD_3OD, 700 MHz) δ 6.10 (dd, *J* = 15.70, 0.74 Hz, 1H), 5.81 (dd, *J* = 15.71, 4.89 Hz, 1H), 5.33 (d, *J* = 2.50 Hz, 1H), 4.99 (dd, *J* = 5.55, 2.70 Hz, 1H), 4.80 (d, *J* = 4.86 Hz, 1H), 3.31-3.28 (m, 2H), 3.25 (s, 3H), 3.25 (s, 3H), 3.06-3.03 (m, 1H), 3.01 (ddd, *J* = 10.99, 6.31, 2.51 Hz, 1H), 2.60 (ddd, *J* = 12.11, 10.02, 3.95 Hz, 1H), 2.56 (ddd, *J* = 12.62, 9.35, 4.24 Hz, 1H), 2.22 (td, *J* = 17.49, 5.93, 5.93 Hz, 1H), 2.10 (ddd, *J* = 17.50, 11.07, 2.31 Hz, 1H), 1.95 (td, *J* = 12.93, 4.18, 4.18 Hz, 1H), 1.18 (q, *J* = 12.28, 12.28, 12.26 Hz, 1H).

^{13}C NMR (CD_3OD, 175 MHz) δ 147.0, 129.7, 125.6, 105.3, 104.0, 101.9, 88.1, 78.9, 77.9, 53.2, 53.2, 52.5, 51.6, 48.5, 36.8, 27.0.

6.20

3-6'-*Bis*-propenimino-sisamine dimer (6.20)

The expanded sisamine dimer (**6.20**) was generated under identical conditions as *aminoglycoside 66-40C* (**2**). Using compound **6.18** (22.9 mg, 63.7 μmol), incubating with 1 mL 0.5 N H_2SO_4 for 15 min liberated sisamine 6'-acrylaldehyde **6.19**: HRMS (ESI) calcd. for $C_{14}H_{23}N_3O_5$, M + H^+ = 314.1710, found 314.17158 (1.69 ppm). The solution was neutralized with sat. $Ba(OH)_2$, filtered and lyophilized to give a fluffy cotton of crude dimer **6.20**. The residue was dissolved in $CHCl_3$/MeOH/NH_4OH (2:3:0.25) and purified by silica gel column chromatography using the same solvent system, increasing ammonia liquor gradually to a 2:3:0.5 mixture. Evaporation, filtration (0.45 μm) and freeze-drying of the collected fractions yielded 18.7 mg of 3-6'-*bis*-propenimino-sisamine dimer **6.20** (97%, 63.3 μmol), as a free-base fluffy cotton solid.

R_f = 0.8, 2:3:2 $CHCl_3$/MeOH/NH_4OH.

$[\alpha]^{22}_D$ -373.6° (*c* 0.4 in H_2O).

HRMS (ESI) calcd. for $C_{28}H_{42}N_6O_8$, M + Na^+ = 613.2956, found 613.2950 (-1.01 ppm).

^{1}H NMR (D_2O, 700 MHz) δ 7.75 (d, *J* = 9.37 Hz, 2H), 6.29 (d, *J* = 15.48 Hz, 2H), 6.16 (dd, *J* = 15.50, 9.38 Hz, 2H), 5.34 (d, *J* = 2.54 Hz, 2H), 5.21 (dd, *J* = 6.16, 2.78 Hz, 2H), 3.66 (dd, *J* = 9.44, 8.97 Hz, 2H), 3.56 (dd, *J* = 9.53, 9.04 Hz, 2H), 3.44 (dd, *J* = 10.11, 9.54 Hz, 2H), 3.29-3.26 (m, 2H), 3.25 (ddd, *J* = 11.78, 9.47, 4.90 Hz, 2H), 3.06 (ddd, *J* = 12.66, 10.26, 3.99 Hz, 2H), 2.36 (td, *J* = 17.63, 6.17, 6.17 Hz, 2H), 2.26 (ddd, *J* = 17.51, 11.52, 1.96 Hz, 2H), 2.02 (td, *J* = 12.93, 4.31, 4.31 Hz, 2H), 1.70 (q, *J* = 12.56, 12.56, 12.56 Hz, 2H).

^{13}C NMR (D_2O, 175 MHz) δ 166.2, 145.7, 138.5, 125.1, 107.1, 99.3, 85.0, 76.1, 73.9, 65.8, 50.2, 46.5, 32.9, 23.1.

Cross-over experiments between *aminoglycoside 66-40C* (6.1) and sisamine dimer 6.13

Solutions of *aminoglycoside 66-40C* **6.1** (10.6 mg, 12 µmol in 1.6 mL H_2O) and dimer **6.13** (6.7 mg, 12 µmol in 1.6 mL H_2O) were prepared and treated as follows:

- *Incubation C)* 300 µL aliquot of each solution was mixed and incubated for 72 h.
- *Incubation D)* 300 µL aliquot of each solution was added 2 µL AcOH and immediately lyophilized, each was redissolved in 300 µL H_2O, mixed and incubated for 72 h.
- *Incubation E)* 300 µL aliquot of each solution was mixed, added 3 µL AcOH and incubated for 72 h. The sample was then lyophilized and redissolved in 600 µL H_2O.
- *Incubation F)* 300 µL aliquot of each solution was mixed, added 100 µL 0.5 N H_2SO_4 and incubated for 12 h, then neutralized with sat. $Ba(OH)_2$ as previously described.

A sample of each incubation was analyzed by LCMS to determine the ratio of parent homodimers to crossover product 3-6'-*bis*-imino-sisamine/sisomicin heterodimer **6.21**: HRMS (ESI) calcd. for $C_{31}H_{51}N_7O_{11}$, M + H^+ = 698.37193, found 698.36943 (-3.58 ppm), a sample of which was purified by preparative TLC (0.5 mm, $CHCl_3$/MeOH/NH_4OH, 2:3:1) and treated as control *incubation G*. LCMS: column Luna Hilic 3 µ 100 x 3.0 mm, SN 413454-5 on an Agilent Technologies LC-MSD TOF mass spectrometer, using acetonitrile/water with 0.1% FA, gradient 60 → 40%, 0.5 mL/min, 50 °C. Chromatograms were analyzed with MassHunter Workstation Software version B.03.01, Agilent Technologies, extracting sodium adduct counts of dimmers **6.1**, **6.13** and **6.21** (single and double H^+ adduct counts gave unreliable results due to pH and ionization differences).

Experimental references

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"Aminoglycoside 66-40 C (6.1)" 1 1 D: Hanessian

7.436

5.490 5.480 5.128 5.123 4.100 4.082 3.886 3.881 3.870 3.865 3.721 3.708 3.694 3.658 3.645 3.632 3.396 3.382 3.368 3.344 3.326 3.194 3.187 3.177 3.164 3.158 2.974 2.962 2.958 2.948 2.931 2.926 2.760 2.744 2.588 2.280 2.272 2.263 2.255 2.247 2.218 2.200 2.177 1.932 1.927 1.921 1.914 1.908 1.903 1.682 1.664 1.646 1.628

1.227

NH_2 OH OH Me O H O O H H_2N H N H O NH Me OH Me HN OH O H N H NH_2 O O HO O H Me OH H_2N

aminoglycoside 66-40C (**6.1**)

2.000 4.104 1.888 2.095 1.979 2.264 1.948 1.945 2.086 2.031 3.942 1.900 6.056 4.027 1.879 1.938 6.178

8 6 4 2 [ppm]

[rel] 1.5 1.0 0.5 0.0

"Aminoglycoside 66-40 C (6.1)" 3 1 D: Hanessian

161.221, 145.896, 115.854, 100.996, 99.473, 86.736, 80.602, 76.176, 72.426, 69.246, 68.116, 66.393, 64.016, 51.142, 46.820, 36.997, 35.592, 24.861, 21.937

[rel]

15
10
5
0

NH$_2$, OH, Me, O, H, H$_2$N, N, NH, HN, HO, NH$_2$, OH

aminoglycoside 66-40C (**6.1**)

200 150 100 50 0 [ppm]

"Aminoglycoside 66-40 C (6.1)" 13 1 D: Hanessian

7.436

5.491
5.486
5.480
5.474

3.200
3.190
3.181
3.176
3.173
3.168
3.158
3.148

aminoglycoside 66-40C (**6.1**)

1.000 0.116 0.137

8 6 4 2 [ppm]

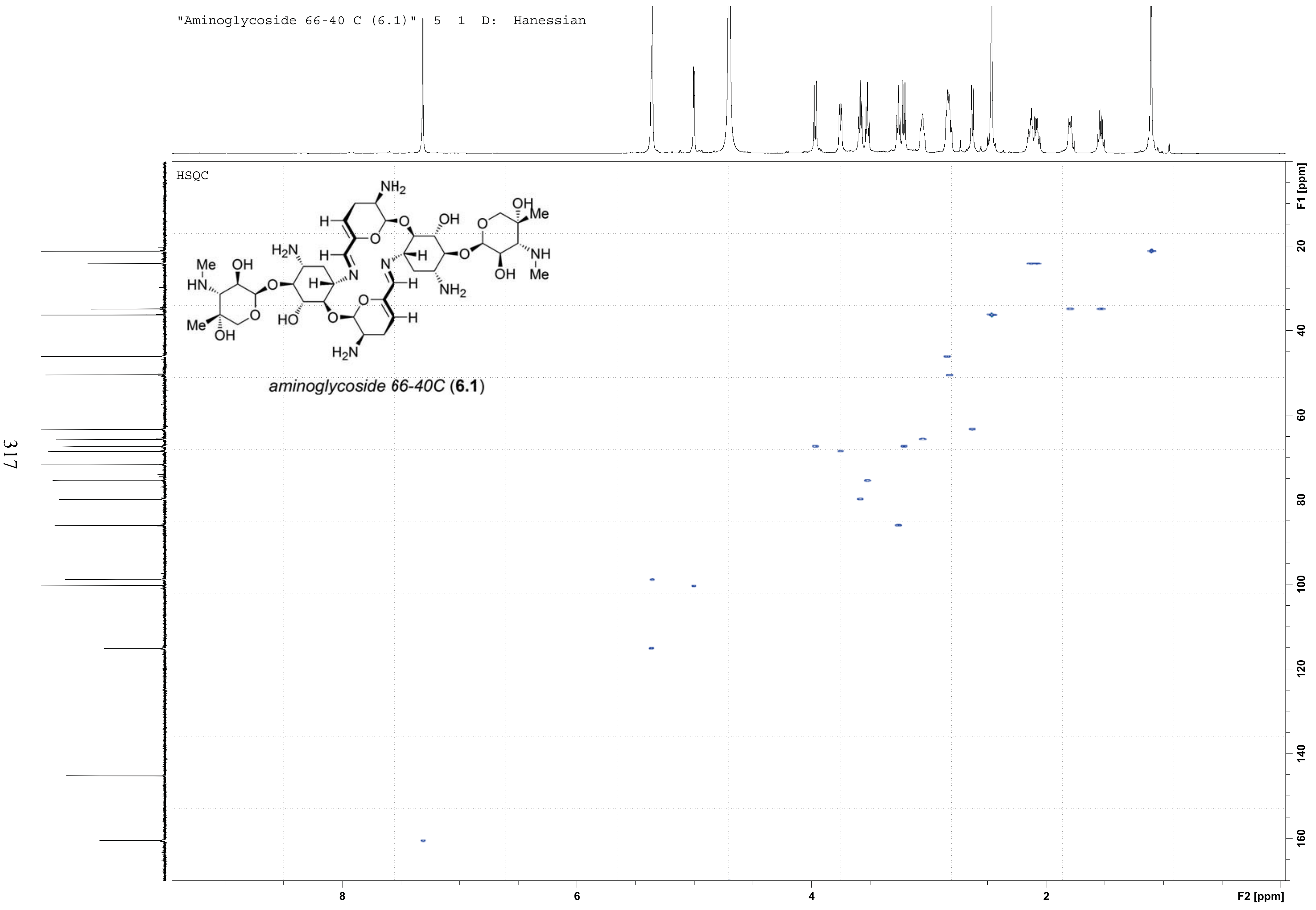
[rel] 15 10 5 0

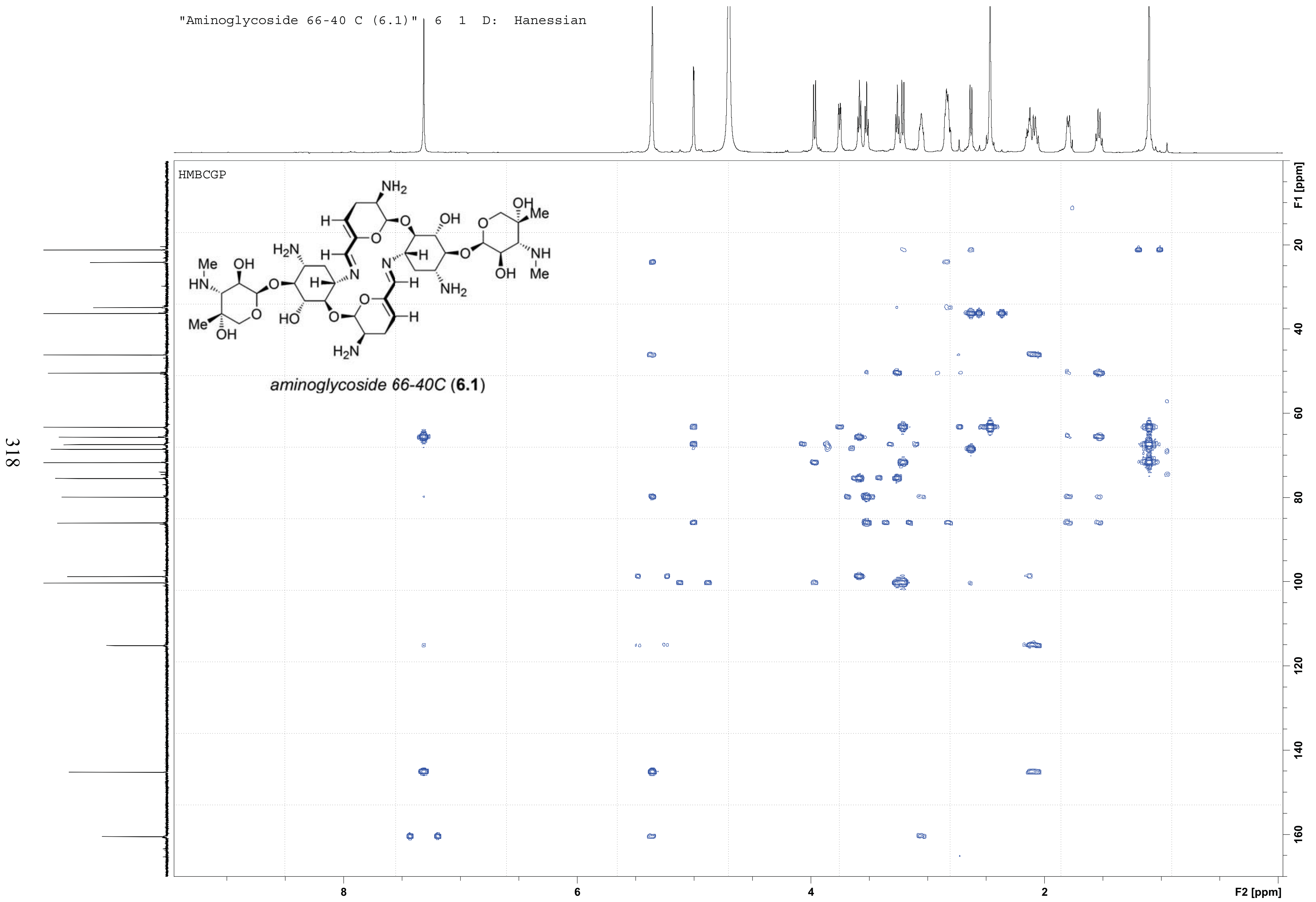
"Aminoglycoside 66-40 C (6.1)" 7 1 D: Hanessian

aminoglycoside 66-40C (**6.1**)

F1 [ppm]

F2 [ppm]


"Aminoglycoside 66-40 C (6.1)" 5 1 D: Hanessian
HSQC
aminoglycoside 66-40C (6.1)
F1 [ppm]
F2 [ppm]
8
6
4
2
20
40
60
80
100
120
140
160


"Aminoglycoside 66-40 C (6.1)" 6 1 D: Hanessian
HMBCGP
aminoglycoside 66-40C (6.1)
F1 [ppm]
20
40
60
80
100
120
140
160
F2 [ppm]
8
6
4
2

"Compound 6.13 (JPM6-19)" 1 1 D: Hanessian

7.453

5.510 5.507 5.502 5.498 5.482 5.478 3.701 3.688 3.675 3.577 3.563 3.550 3.381 3.367 3.353 3.230 3.224 3.216 3.214 3.211 3.207 3.200 3.193 3.039 3.035 3.030 3.026 3.022 3.019 3.013 3.010 2.941 2.936 2.926 2.923 2.922 2.918 2.909 2.904 2.311 2.302 2.293 2.285 2.276 2.268 2.252 2.249 2.235 2.232 2.227 2.224 2.210 2.206 2.028 2.022 2.016 2.010 2.004 1.998 1.743 1.726 1.708 1.690

NH_2, H_2N, HO, HO, H, H, O, O, N, N, OH, OH, NH_2, H_2N

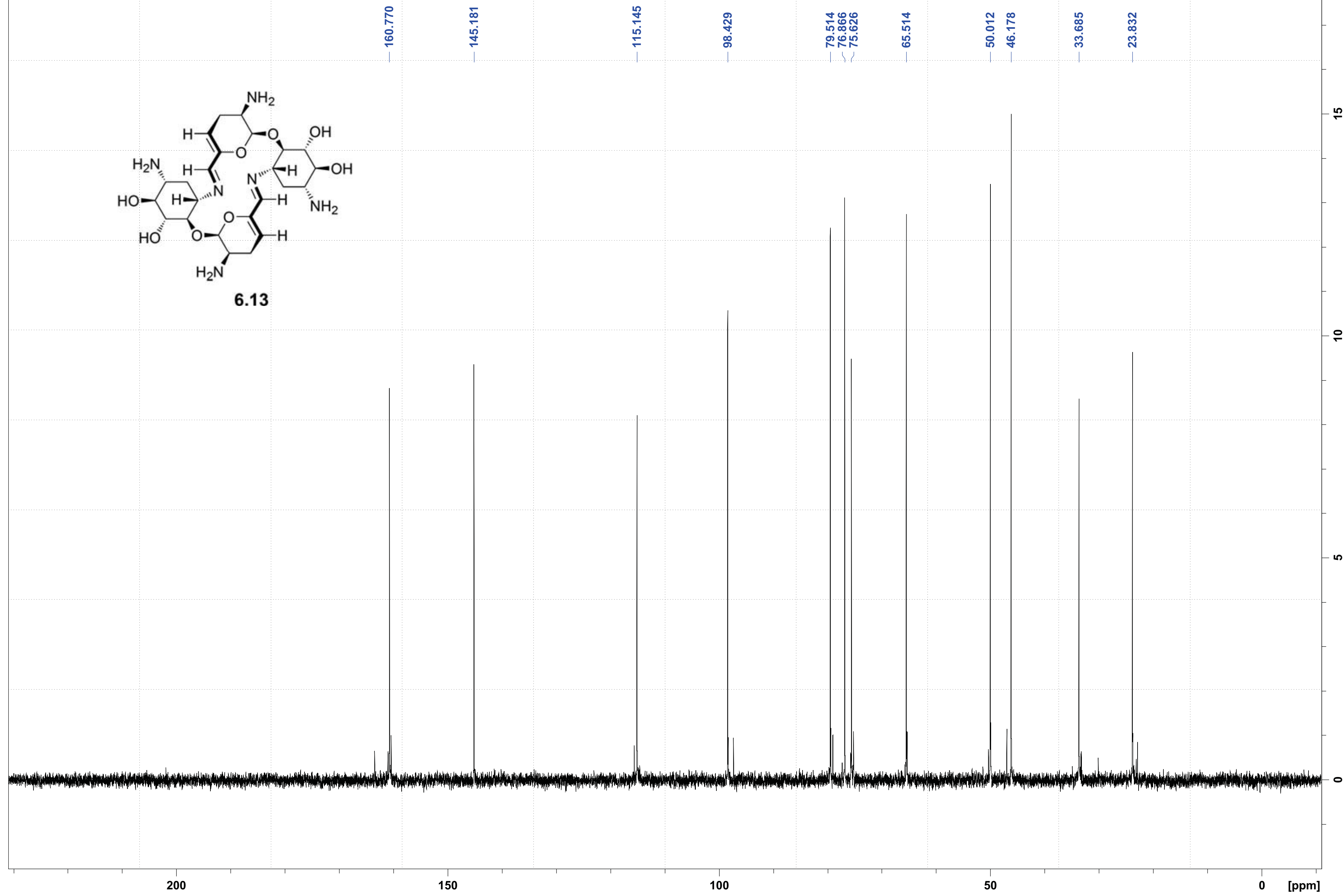
6.13

[rel]: 0.5, 0.4, 0.3, 0.2, 0.1, -0.0

2.000 4.112 2.080 1.895 1.900 2.217 1.995 1.860 2.405 1.908 1.907 1.923

8 6 4 2 [ppm]

"Compound 6.13 (JPM6-19)" 3 1 D: Hanessian



"Compound 6.13 (JPM6-19)" 8 1 D: Hanessian

7.453

5.511
5.507
5.502
5.498

3.230
3.224
3.217
3.214
3.210
3.207
3.200
3.193

[rel]

0.5
0.4
0.3
0.2
0.1
-0.0

1.000
0.051
0.059

8
6
4
2
[ppm]

NH2
H
O
OH
H2N
H
N
O
H
OH
HO
H
N
H
NH2
O
HO
O
H
H2N
6.13

"Compound 6.13 (JPM6-19)" 4 1 D: Hanessian

NH_2 OH OH H_2N HO H N O NH_2 HO H_2N

6.13

F1 [ppm]

2 4 6 8

8 6 4 2

F2 [ppm]

"Compound 6.13 (JPM6-19)" 5 1 D: Hanessian

HSQC

NH$_2$ H O OH H$_2$N H O N H OH HO H N N H NH$_2$ O HO O H H$_2$N

6.13

F1 [ppm]

20 40 60 80 100 120 140 160

8 6 4 2 F2 [ppm]

"Compound 6.13 (JPM6-19)" 6 1 D: Hanessian

HMBCGP

6.13

F1 [ppm]: 20, 40, 60, 80, 100, 120, 140, 160

F2 [ppm]: 8, 6, 4, 2

"Compound 6.15 (JPM6-23)" 1 1 D: Hanessian

7.450 5.744 5.740 5.532 5.528 5.524 5.519 5.309 5.206 5.204 4.484 4.477 4.473 4.466 4.418 4.411 4.241 4.235 4.227 4.137 4.134 4.130 4.126 4.120 4.115 3.861 3.857 3.844 3.840 3.824 3.812 3.800 3.787 3.731 3.729 3.727 3.715 3.713 3.684 3.676 3.667 3.659 3.638 3.623 3.610 3.491 3.488 3.472 3.466 3.455 3.452 3.347 3.338 3.328 3.318 3.295 3.289 3.282 3.275 3.270 3.252 3.246 3.234 3.227 3.217 3.215 3.209 3.200 3.194 2.487 2.478 2.468 2.462 2.453 2.441 2.427 2.424 2.402 2.399 2.089 2.082 2.077 2.070 1.847 1.823 1.812 1.811 1.794

H₂N OH OH O HO NH₂ O O NH₂ OH H O O H₂N H OH H N N H HO H NH₂ O O O H HO H₂N O O H₂N O HO O OH HO NH₂

6.15

[rel]

0.25 0.20 0.15 0.10 0.05 -0.00

2.000 1.896 1.980 1.810 1.941 1.956 1.954 2.019 4.459 2.227 4.167 2.081 2.113 2.498 4.426 2.294 4.507 2.481 3.810 1.977 18.426

8 6 4 2 [ppm]

"Compound 6.15 (JPM6-23)" 8 1 D: Hanessian

180.738, 160.542, 145.176, 112.895, 110.458, 95.012, 93.889, 86.131, 80.702, 77.663, 75.090, 73.518, 72.812, 70.008, 67.533, 67.212, 64.745, 60.354, 50.687, 50.130, 46.162, 46.103, 40.297, 30.980, 22.719, 20.230

6.15

[rel]: 0, 5, 10, 15

200, 150, 100, 50, 0 [ppm]

"Compound 6.15 (JPM6-23)" 13 1 D: Hanessian

7.450

5.533
5.529
5.525
5.520
5.516

3.283
3.276
3.264
3.259
3.253
3.246

2.479
2.463
2.454
2.442
2.424
2.403

1.000 0.167 0.197 0.052

10 8 6 4 2 [ppm]

[rel] 1.5 1.0 0.5 -0.0

H_2N OH OH O HO NH_2 O NH_2 O OH H O O H H N H OH H_2N N HO H H NH_2 O O H HO O H_2N O H_2N O OH HO O HO NH_2

6.15

"Compound 6.15 (JPM6-23)" 2 1 D: Hanessian

6.15

F1 [ppm]

F2 [ppm]

"Compound 6.15 (JPM6-23)" 4 1 D: Hanessian

6.15

F1 [ppm]

50

100

150

8

6

4

2

F2 [ppm]

"Compound 6.15 (JPM6-23)" 5 1 D: Hanessian

6.15

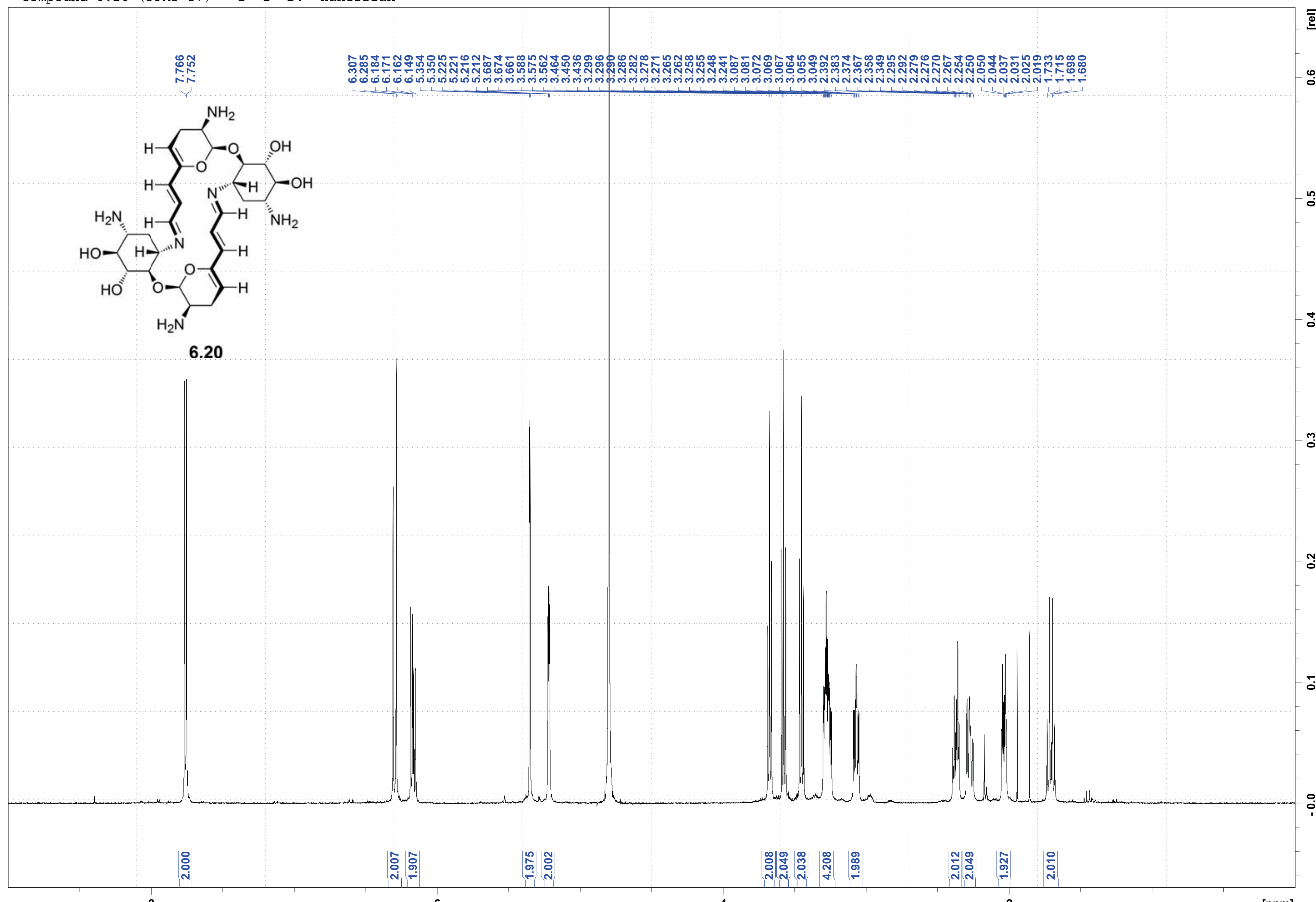
F1 [ppm]

50

100

150

10 8 6 4 2 F2 [ppm]


"Compound 6.20 (JPM5-57)" 1 1 D: Hanessian
7.766
7.752
6.307
6.285
6.184
6.171
6.162
6.149
5.354
5.350
5.225
5.221
5.216
5.212
3.687
3.674
3.661
3.588
3.575
3.562
3.464
3.450
3.436
3.299
3.296
3.290
3.286
3.282
3.278
3.271
3.265
3.262
3.258
3.255
3.248
3.241
3.087
3.081
3.072
3.069
3.067
3.064
3.055
3.049
2.392
2.383
2.374
2.367
2.358
2.349
2.295
2.292
2.279
2.276
2.270
2.267
2.254
2.250
2.050
2.044
2.037
2.031
2.025
2.019
1.733
1.715
1.698
1.680
NH2
OH
OH
NH2
H2N
HO
HO
H2N
6.20
2.000
2.007
1.907
1.975
2.002
2.008
2.049
2.038
4.208
1.989
2.012
2.049
1.927
2.010
8
6
4
2
[ppm]
[rel]
0.6
0.5
0.4
0.3
0.2
0.1
-0.0

"Compound 6.20 (JPM5-57)" 2 1 D: Hanessian

166.216, 145.636, 138.523, 125.094, 107.112, 99.277, 84.978, 76.086, 73.920, 65.787, 50.140, 46.449, 32.885, 23.044

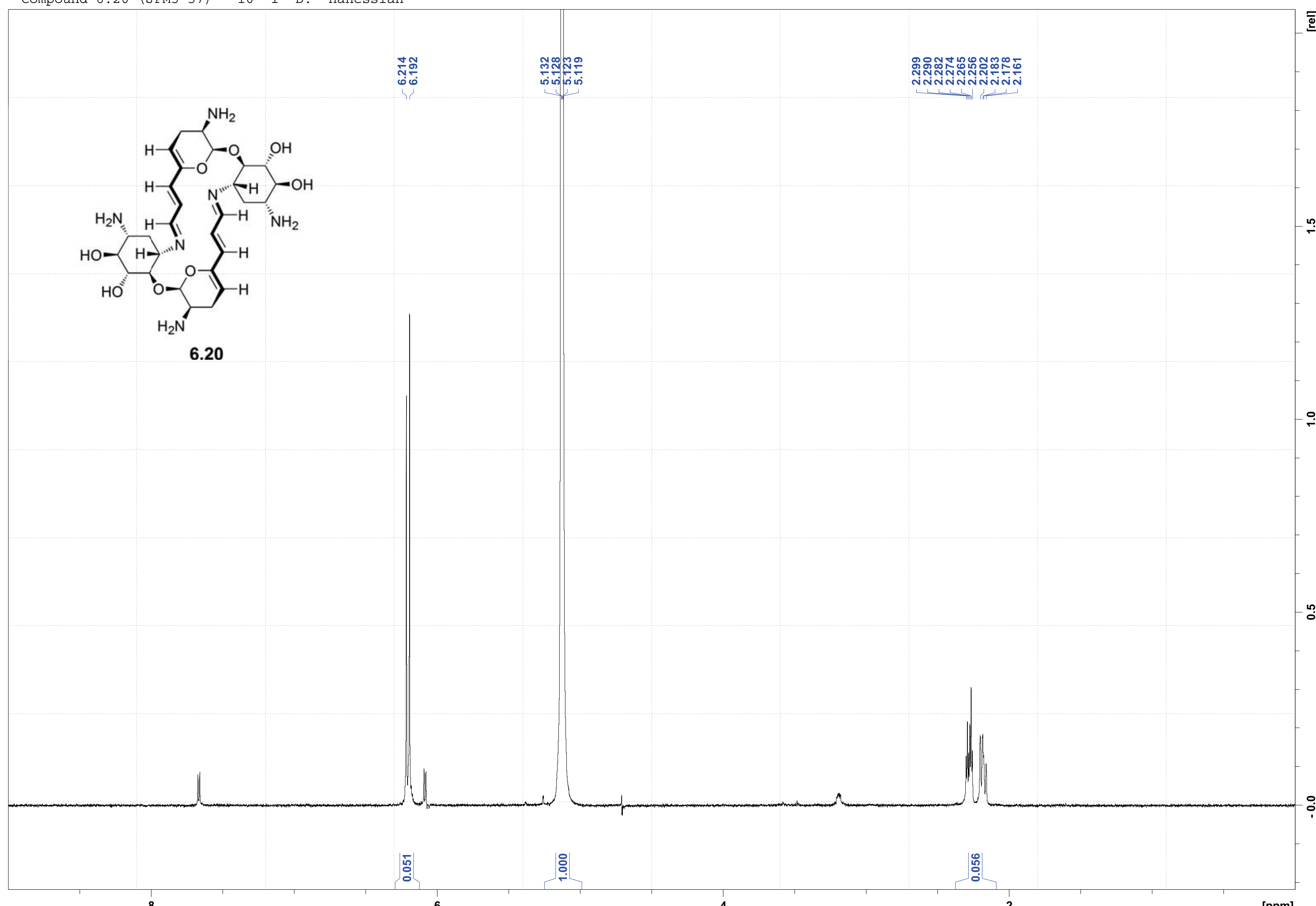
[rel]

15 10 5 0

200 150 100 50 0 [ppm]

NH2, OH, OH, NH2, H2N, HO, HO, H2N, O, N, H

6.20


"Compound 6.20 (JPM5-57)" 10 1 D: Hanessian
6.214
6.192
5.132
5.128
5.123
5.119
2.299
2.290
2.282
2.274
2.265
2.256
2.202
2.183
2.178
2.161
NH2
H
O
O
OH
H
N
H
OH
H2N
H
H
NH2
HO
H
N
H
O
HO
O
H
H2N
6.20
0.051
1.000
0.056
8
6
4
2
[ppm]
[rel]
1.5
1.0
0.5
-0.0

"Compound 6.20 (JPM5-57)" 12 1 D: Hanessian

"Compound 6.20 (JPM5-57)" 11 1 D: Hanessian

7.672
7.659

6.214
6.192
6.091
6.078
6.069
6.055

3.185
3.178
3.172
3.168
3.165
3.162
3.155
3.148

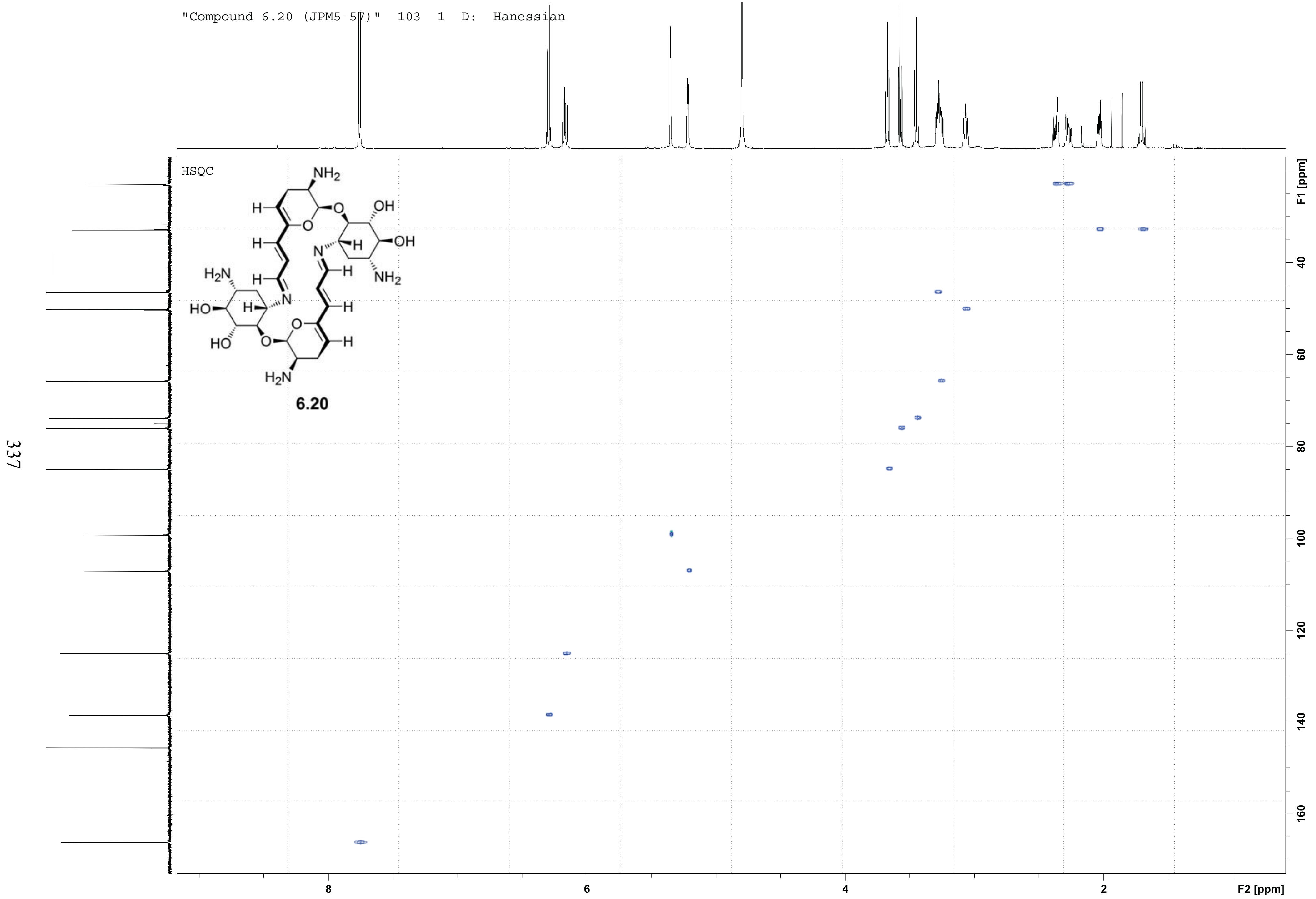
NH2
H
O
OH
O
H
OH
N
H
H2N
H
NH2
H
N
HO
H
H
O
HO
O
H
H2N
6.20

1.000
0.058
0.016
0.085

8
6
4
2
[ppm]

1.2
1.0
0.8
0.6
0.4
0.2
-0.0
[rel]

"Compound 6.20 (JPM5-57)" 102 1 D: Hanessian


"Compound 6.20 (JPM5-57)" 103 1 D: Hanessian
HSQC
NH2
OH
OH
NH2
H2N
HO
HO
H2N
6.20
8
6
4
2
F2 [ppm]
40
60
80
100
120
140
160
F1 [ppm]

"Compound 6.20 (JPM5-57)" 104 1 D: Hanessian

HMBC

NH2 H OH O H OH N H NH2 H2N H N HO H N H HO O O H H2N

6.20

F1 [ppm]

40

60

80

100

120

140

160

8

6

4

2

F2 [ppm]

Annex 1

Crystallography Reports

CRYSTAL AND MOLECULAR STRUCTURE OF

C15 H19 N O5 COMPOUND (HAN451)

Equipe Hanessian

Département de chimie, Université de Montréal,

C.P. 6128, Succ. Centre-Ville, Montréal, Québec, H3C 3J7 (Canada)

Compound 4.9

Structure solved and refined in the laboratory of X-ray diffraction Université de Montréal by Benoît Deschênes Simard.

Table 1. Crystal data and structure refinement for C15 H19 N O5.

Identification code	HAN451
Empirical formula	C15 H19 N O5
Formula weight	293.31
Temperature	150(2)K
Wavelength	1.54178 Å
Crystal system	Orthorhombic
Space group	P212121
Unit cell dimensions	a = 4.7348(2) Å α = 90° b = 10.7188(5) Å β = 90° c = 28.7574(12) Å γ = 90°
Volume	1459.48(11)$Å^3$
Z	4
Density (calculated)	1.335 g/cm^3
Absorption coefficient	0.837 mm^{-1}
F(000)	624
Crystal size	0.20 x 0.08 x 0.06 mm
Theta range for data collection	3.07 to 72.61°
Index ranges	$-5 \leq h \leq 5$, $-12 \leq k \leq 13$, $-35 \leq \ell \leq 35$
Reflections collected	19773
Independent reflections	2875 [R_{int} = 0.039]
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.95 and 0.92
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	2875 / 0 / 193
Goodness-of-fit on F^2	1.070
Final R indices [I>2sigma(I)]	R_1 = 0.0337, wR_2 = 0.0870
R indices (all data)	R_1 = 0.0381, wR_2 = 0.0892
Absolute structure parameter	-0.07(19)

Extinction coefficient	0.0034(4)
Largest diff. peak and hole	0.145 and -0.176 e/$Å^3$

Table 2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$ x 10^3) for C15 H19 N O5.

U_{eq} is defined as one third of the trace of the orthogonalized Uij tensor.

	x	y	z	U_{eq}
O(1)	9297(3)	-734(1)	277(1)	34(1)
O(2)	9929(3)	-257(1)	-714(1)	42(1)
O(3)	6714(3)	-460(1)	965(1)	35(1)
O(4)	12534(2)	2952(1)	1108(1)	32(1)
O(5)	8913(2)	3408(1)	1595(1)	31(1)
N(1)	8233(3)	2007(1)	1042(1)	28(1)
C(1)	6887(4)	-611(2)	-27(1)	33(1)
C(2)	5674(4)	674(2)	-25(1)	30(1)
C(3)	6548(3)	1559(2)	263(1)	27(1)
C(4)	8822(3)	1318(1)	615(1)	27(1)
C(5)	9083(4)	-83(2)	702(1)	30(1)
C(6)	7812(5)	-1025(2)	-508(1)	40(1)
C(7)	7076(5)	-1654(2)	1174(1)	46(1)
C(8)	10090(3)	2793(2)	1236(1)	25(1)
C(9)	10744(4)	4293(2)	1830(1)	35(1)
C(10)	8926(4)	4998(2)	2168(1)	31(1)
C(11)	9042(4)	4762(2)	2643(1)	42(1)
C(12)	7356(5)	5432(2)	2947(1)	52(1)
C(13)	5561(5)	6340(2)	2784(1)	51(1)
C(14)	5433(4)	6578(2)	2311(1)	47(1)
C(15)	7097(4)	5906(2)	2006(1)	37(1)

Table 3. Hydrogen coordinates (x 10^4) and isotropic displacement parameters ($Å^2$ x 10^3) for C15 H19 N O5.

	x	y	z	U_{eq}
H(2)	9174	386	-826	63
H(1)	6587	1901	1179	33
H(1A)	5393	-1199	83	39
H(2A)	4211	867	-239	36
H(3)	5715	2364	246	33
H(4)	10654	1624	485	32
H(5)	10829	-242	888	36
H(6A)	8540	-1889	-488	48
H(6B)	6139	-1037	-715	48
H(7A)	7600	-2264	935	70
H(7B)	5304	-1912	1322	70
H(7C)	8571	-1609	1409	70
H(9A)	11605	4872	1602	42
H(9B)	12274	3849	1996	42
H(11)	10283	4140	2759	51
H(12)	7441	5263	3271	63
H(13)	4417	6801	2994	61
H(14)	4198	7204	2196	56
H(15)	6985	6070	1682	45

Table 4. Anisotropic parameters ($Å^2 \times 10^3$) for C15 H19 N O5.

The anisotropic displacement factor exponent takes the form:

$$-2 \pi^2 [h^2 a^{*2} U_{11} + ... + 2 h k a^* b^* U_{12}]$$

	U11	U22	U33	U23	U13	U12
O(1)	40(1)	35(1)	27(1)	-4(1)	-2(1)	9(1)
O(2)	42(1)	51(1)	33(1)	3(1)	5(1)	9(1)
O(3)	45(1)	30(1)	31(1)	7(1)	7(1)	2(1)
O(4)	23(1)	39(1)	34(1)	-5(1)	3(1)	-1(1)
O(5)	28(1)	37(1)	30(1)	-9(1)	5(1)	-3(1)
N(1)	23(1)	36(1)	25(1)	-4(1)	6(1)	-3(1)
C(1)	35(1)	33(1)	30(1)	-2(1)	-2(1)	-2(1)
C(2)	26(1)	39(1)	25(1)	2(1)	0(1)	1(1)
C(3)	27(1)	31(1)	24(1)	3(1)	2(1)	2(1)
C(4)	25(1)	32(1)	23(1)	-1(1)	4(1)	1(1)
C(5)	32(1)	36(1)	23(1)	-1(1)	1(1)	4(1)
C(6)	50(1)	35(1)	35(1)	-6(1)	-2(1)	2(1)
C(7)	65(1)	33(1)	41(1)	9(1)	-4(1)	-3(1)
C(8)	24(1)	28(1)	23(1)	2(1)	2(1)	2(1)
C(9)	29(1)	38(1)	38(1)	-10(1)	0(1)	-5(1)
C(10)	32(1)	30(1)	30(1)	-3(1)	1(1)	-6(1)
C(11)	48(1)	48(1)	31(1)	0(1)	-5(1)	-2(1)
C(12)	65(2)	64(1)	29(1)	-8(1)	6(1)	-14(1)
C(13)	48(1)	55(1)	49(1)	-25(1)	17(1)	-13(1)
C(14)	43(1)	37(1)	60(1)	-8(1)	5(1)	5(1)
C(15)	42(1)	36(1)	33(1)	1(1)	2(1)	-1(1)

Table 5. Bond lengths [Å] and angles [°] for C15 H19 N O5

O(1)-C(5)	1.4105(18)	C(8)-N(1)-C(4)	122.84(13)
O(1)-C(1)	1.4438(19)	O(1)-C(1)-C(2)	112.71(14)
O(2)-C(6)	1.427(2)	O(1)-C(1)-C(6)	107.30(14)
O(3)-C(5)	1.4116(19)	C(2)-C(1)-C(6)	112.67(14)
O(3)-C(7)	1.425(2)	C(3)-C(2)-C(1)	122.88(15)
O(4)-C(8)	1.2262(18)	C(2)-C(3)-C(4)	121.49(15)
O(5)-C(8)	1.3465(18)	N(1)-C(4)-C(3)	110.07(13)
O(5)-C(9)	1.4513(19)	N(1)-C(4)-C(5)	112.06(12)
N(1)-C(8)	1.339(2)	C(3)-C(4)-C(5)	109.74(13)
N(1)-C(4)	1.4607(18)	O(1)-C(5)-O(3)	112.28(13)
C(1)-C(2)	1.492(2)	O(1)-C(5)-C(4)	110.52(12)
C(1)-C(6)	1.518(2)	O(3)-C(5)-C(4)	107.72(13)
C(2)-C(3)	1.325(2)	O(2)-C(6)-C(1)	114.33(15)
C(3)-C(4)	1.501(2)	O(4)-C(8)-N(1)	125.65(14)
C(4)-C(5)	1.527(2)	O(4)-C(8)-O(5)	123.57(15)
C(9)-C(10)	1.502(2)	N(1)-C(8)-O(5)	110.78(13)
C(10)-C(15)	1.384(2)	O(5)-C(9)-C(10)	106.70(13)
C(10)-C(11)	1.390(2)	C(15)-C(10)-C(11)	118.85(17)
C(11)-C(12)	1.386(3)	C(15)-C(10)-C(9)	119.70(15)
C(12)-C(13)	1.374(3)	C(11)-C(10)-C(9)	121.44(17)
C(13)-C(14)	1.386(3)	C(12)-C(11)-C(10)	120.30(19)
C(14)-C(15)	1.381(3)	C(13)-C(12)-C(11)	120.45(17)
		C(12)-C(13)-C(14)	119.53(18)
C(5)-O(1)-C(1)	115.00(12)	C(15)-C(14)-C(13)	120.16(19)
C(5)-O(3)-C(7)	112.80(15)	C(14)-C(15)-C(10)	120.70(17)
C(8)-O(5)-C(9)	115.44(13)		

Table 6. Torsion angles [°] for C15 H19 N O5.

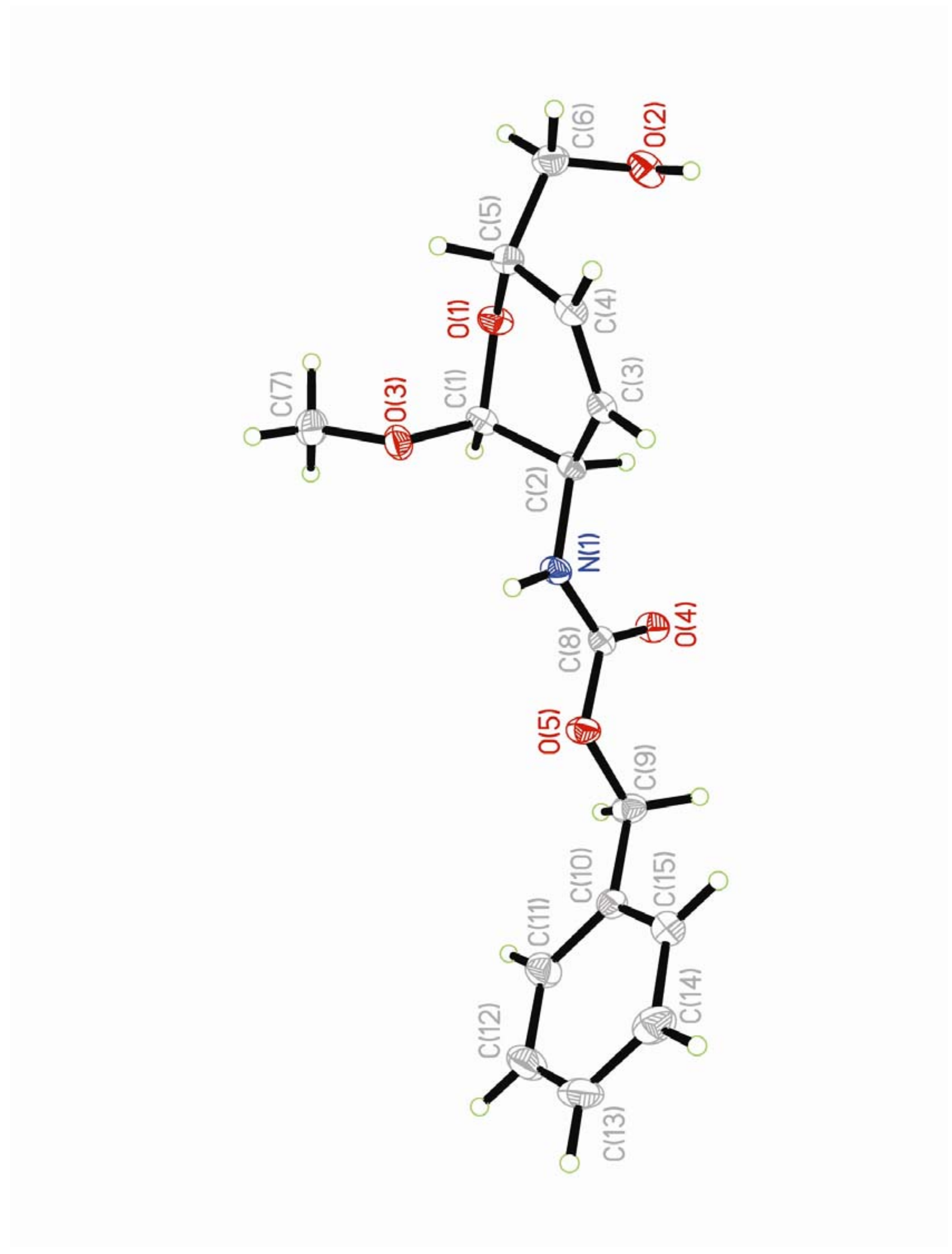
Torsion	Angle
C(5)-O(1)-C(1)-C(2)	38.87(18)
C(5)-O(1)-C(1)-C(6)	163.48(14)
O(1)-C(1)-C(2)-C(3)	-6.7(2)
C(6)-C(1)-C(2)-C(3)	-128.27(18)
C(1)-C(2)-C(3)-C(4)	-0.8(2)
C(8)-N(1)-C(4)-C(3)	-125.35(16)
C(8)-N(1)-C(4)-C(5)	112.23(17)
C(2)-C(3)-C(4)-N(1)	-144.10(15)
C(2)-C(3)-C(4)-C(5)	-20.3(2)
C(1)-O(1)-C(5)-O(3)	58.49(17)
C(1)-O(1)-C(5)-C(4)	-61.81(18)
C(7)-O(3)-C(5)-O(1)	74.08(16)
C(7)-O(3)-C(5)-C(4)	-164.00(13)
N(1)-C(4)-C(5)-O(1)	172.66(13)
C(3)-C(4)-C(5)-O(1)	50.05(17)
N(1)-C(4)-C(5)-O(3)	49.66(16)
C(3)-C(4)-C(5)-O(3)	-72.95(15)
O(1)-C(1)-C(6)-O(2)	-64.55(19)
C(2)-C(1)-C(6)-O(2)	60.1(2)
C(4)-N(1)-C(8)-O(4)	-6.6(3)
C(4)-N(1)-C(8)-O(5)	173.14(13)
C(9)-O(5)-C(8)-O(4)	0.0(2)
C(9)-O(5)-C(8)-N(1)	-179.76(13)
C(8)-O(5)-C(9)-C(10)	171.96(13)
O(5)-C(9)-C(10)-C(15)	-75.22(19)
O(5)-C(9)-C(10)-C(11)	105.06(19)
C(15)-C(10)-C(11)-C(12)	-0.2(3)
C(9)-C(10)-C(11)-C(12)	179.55(17)
C(10)-C(11)-C(12)-C(13)	-0.3(3)
C(11)-C(12)-C(13)-C(14)	0.4(3)
C(12)-C(13)-C(14)-C(15)	0.0(3)
C(13)-C(14)-C(15)-C(10)	-0.5(3)
C(11)-C(10)-C(15)-C(14)	0.6(3)
C(9)-C(10)-C(15)-C(14)	-179.13(17)

Table 7. Bond lengths [Å] and angles [°] related to the hydrogen bonding for C15 H19 N O5.

D-H	..A	d(D-H)	d(H..A)	d(D..A)	<DHA
O(2)-H(2)	O(4)#1	0.84	2.11	2.9441(18)	176.4
N(1)-H(1)	O(4)#2	0.88	2.23	2.8885(18)	130.9

Symmetry transformations used to generate equivalent atoms:

#1 x-1/2,-y+1/2,-z   #2 x-1,y,z



ORTEP view of the C15 H19 N O5 compound with the numbering scheme adopted. Ellipsoids drawn at 30% probability level. Hydrogen atoms are represented by sphere of arbitrary size.

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CRYSTAL AND MOLECULAR STRUCTURE OF

C11 H12 F N3 O3 COMPOUND (bent32)

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Compound 5.4

Structure solved and refined in the laboratory of X-ray diffraction Université de Montréal by Benoît Deschênes Simard.

Table 1. Crystal data and structure refinement for C11 H12 F N3 O3.

Identification code	bent32
Empirical formula	C11 H12 F N3 O3
Formula weight	253.24
Temperature	150K
Wavelength	1.54178 Å
Crystal system	Orthorhombic
Space group	P212121
Unit cell dimensions	a = 5.4945(1) Å α = 90° b = 10.4751(2) Å β = 90° c = 21.0798(3) Å γ = 90°
Volume	1213.26(4)$Å^3$
Z	4
Density (calculated)	1.386 g/cm^3
Absorption coefficient	0.965 mm^{-1}
F(000)	528
Crystal size	0.21 x 0.18 x 0.05 mm
Theta range for data collection	4.19 to 72.33°
Index ranges	$-6 \le h \le 6$, $-12 \le k \le 12$, $-25 \le \ell \le 25$
Reflections collected	14268
Independent reflections	2387 [R_{int} = 0.037]
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.9529 and 0.8008
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	2387 / 0 / 165
Goodness-of-fit on F^2	1.076
Final R indices [I>2sigma(I)]	R_1 = 0.0268, wR_2 = 0.0686
R indices (all data)	R_1 = 0.0278, wR_2 = 0.0694

Absolute structure parameter	0.03(12)
Extinction coefficient	0.0082(6)
Largest diff. peak and hole	0.146 and -0.207 e/$Å^3$

Table 2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$ x 10^3) for C11 H12 F N3 O3.

U_{eq} is defined as one third of the trace of the orthogonalized Uij tensor.

	x	y	z	U_{eq}
F(1)	4405(1)	8115(1)	1707(1)	30(1)
O(1)	6514(2)	6126(1)	2737(1)	35(1)
O(2)	3656(2)	4940(1)	2242(1)	29(1)
O(3)	3091(2)	8004(1)	2964(1)	27(1)
N(1)	-82(2)	9049(1)	1202(1)	39(1)
N(2)	1550(2)	9766(1)	1012(1)	38(1)
N(3)	2882(3)	10431(1)	769(1)	57(1)
C(1)	4491(2)	5978(1)	2531(1)	26(1)
C(2)	2495(2)	6994(1)	2547(1)	25(1)
C(3)	2189(2)	7549(1)	1883(1)	26(1)
C(4)	233(2)	8560(1)	1853(1)	30(1)
C(5)	2597(3)	7727(1)	3625(1)	34(1)
C(6)	3477(2)	8853(1)	3999(1)	31(1)
C(7)	5574(3)	8786(1)	4365(1)	40(1)
C(8)	6457(3)	9869(2)	4666(1)	49(1)
C(9)	5265(3)	11023(2)	4597(1)	48(1)
C(10)	3161(3)	11092(1)	4245(1)	44(1)
C(11)	2258(3)	10011(1)	3948(1)	36(1)

Table 3. Hydrogen coordinates (x 10^4) and isotropic displacement parameters ($Å^2$ x 10^3) for C11 H12 F N3 O3.

	x	y	z	U_{eq}
H(2)	4783	4402	2208	44
H(2A)	930	6594	2684	30
H(3)	1787	6847	1579	31
H(4A)	-1325	8195	2004	36
H(4B)	673	9274	2138	36
H(5A)	3465	6943	3758	41
H(5B)	830	7598	3692	41
H(7)	6408	7996	4408	48
H(8)	7882	9818	4919	58
H(9)	5898	11769	4793	58
H(10)	2326	11882	4205	53
H(11)	797	10062	3708	44

Table 4. Anisotropic parameters ($Å^2 \times 10^3$) for C11 H12 F N3 O3.

The anisotropic displacement factor exponent takes the form:

$$-2 \pi^2 [h^2 a^{*2} U_{11} + ... + 2 h k a^* b^* U_{12}]$$

	U11	U22	U33	U23	U13	U12
F(1)	21(1)	32(1)	36(1)	3(1)	3(1)	-1(1)
O(1)	25(1)	32(1)	49(1)	2(1)	-5(1)	2(1)
O(2)	28(1)	22(1)	39(1)	-2(1)	2(1)	2(1)
O(3)	30(1)	24(1)	28(1)	-1(1)	2(1)	-3(1)
N(1)	32(1)	39(1)	46(1)	8(1)	-11(1)	-5(1)
N(2)	44(1)	33(1)	38(1)	2(1)	-9(1)	-6(1)
N(3)	71(1)	57(1)	43(1)	11(1)	-12(1)	-26(1)
C(1)	24(1)	24(1)	30(1)	4(1)	4(1)	0(1)
C(2)	20(1)	22(1)	33(1)	-2(1)	2(1)	-3(1)
C(3)	20(1)	25(1)	32(1)	-2(1)	0(1)	-3(1)
C(4)	22(1)	30(1)	38(1)	2(1)	-2(1)	0(1)
C(5)	42(1)	30(1)	31(1)	2(1)	7(1)	-2(1)
C(6)	35(1)	33(1)	27(1)	0(1)	7(1)	0(1)
C(7)	38(1)	47(1)	35(1)	-2(1)	2(1)	8(1)
C(8)	40(1)	72(1)	34(1)	-8(1)	1(1)	-6(1)
C(9)	64(1)	46(1)	35(1)	-10(1)	12(1)	-16(1)
C(10)	66(1)	32(1)	34(1)	-2(1)	10(1)	4(1)
C(11)	42(1)	36(1)	32(1)	-1(1)	4(1)	5(1)

Table 5. Bond lengths [Å] and angles [°] for C11 H12 F N3 O3

Bond	Length	Angle	Value
F(1)-C(3)	1.4043(13)	N(3)-N(2)-N(1)	171.25(14)
O(1)-C(1)	1.2034(15)	O(1)-C(1)-O(2)	126.17(11)
O(2)-C(1)	1.3278(15)	O(1)-C(1)-C(2)	124.40(11)
O(3)-C(2)	1.4149(13)	O(2)-C(1)-C(2)	109.41(10)
O(3)-C(5)	1.4487(14)	O(3)-C(2)-C(3)	108.15(9)
N(1)-N(2)	1.2359(17)	O(3)-C(2)-C(1)	111.61(9)
N(1)-C(4)	1.4756(17)	C(3)-C(2)-C(1)	108.97(9)
N(2)-N(3)	1.1331(18)	F(1)-C(3)-C(4)	108.07(9)
C(1)-C(2)	1.5286(15)	F(1)-C(3)-C(2)	107.89(9)
C(2)-C(3)	1.5248(16)	C(4)-C(3)-C(2)	112.59(9)
C(3)-C(4)	1.5099(16)	N(1)-C(4)-C(3)	111.43(10)
C(5)-C(6)	1.4990(18)	O(3)-C(5)-C(6)	106.74(9)
C(6)-C(7)	1.388(2)	C(7)-C(6)-C(11)	119.17(12)
C(6)-C(11)	1.3905(18)	C(7)-C(6)-C(5)	121.32(12)
C(7)-C(8)	1.388(2)	C(11)-C(6)-C(5)	119.38(12)
C(8)-C(9)	1.383(2)	C(6)-C(7)-C(8)	120.15(14)
C(9)-C(10)	1.376(2)	C(9)-C(8)-C(7)	120.12(15)
C(10)-C(11)	1.385(2)	C(10)-C(9)-C(8)	120.01(14)
		C(9)-C(10)-C(11)	120.12(14)
C(2)-O(3)-C(5)	113.92(8)	C(10)-C(11)-C(6)	120.39(14)
N(2)-N(1)-C(4)	115.25(11)		

Table 6. Torsion angles [°] for C11 H12 F N3 O3.

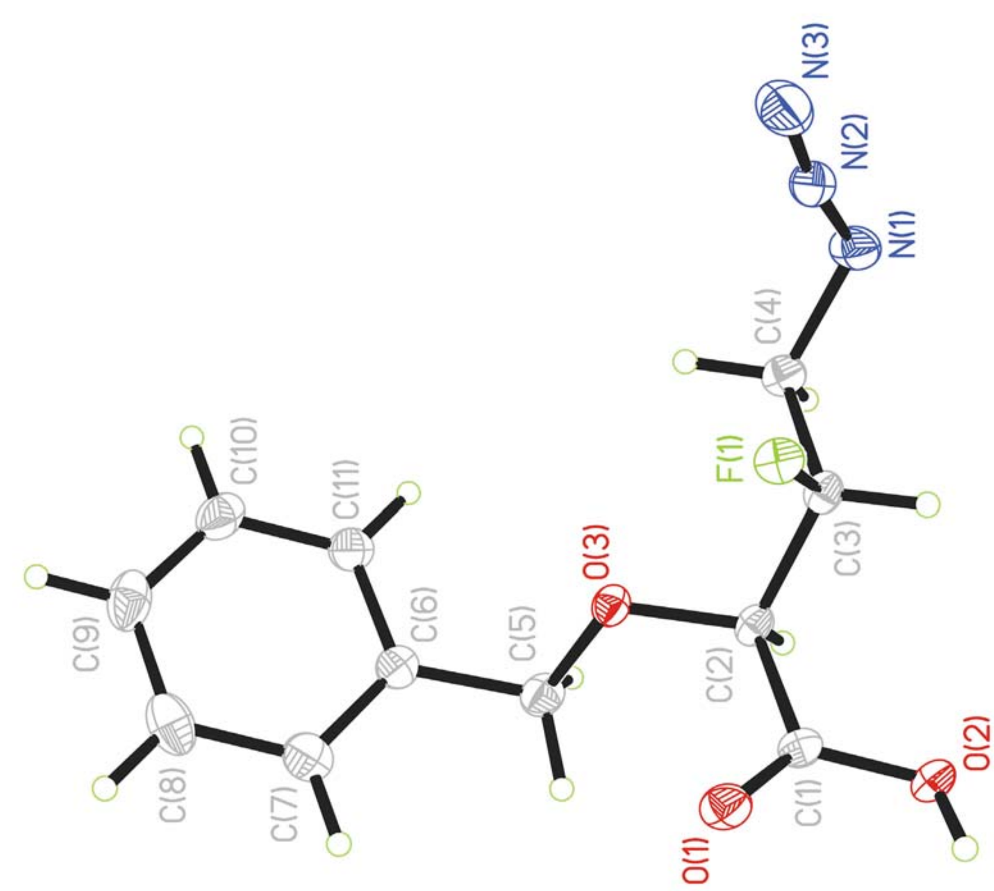
Atoms	Angle	Atoms	Angle
C(5)-O(3)-C(2)-C(3)	159.17(10)	C(2)-O(3)-C(5)-C(6)	176.42(10)
C(5)-O(3)-C(2)-C(1)	-80.98(12)	O(3)-C(5)-C(6)-C(7)	-106.92(13)
O(1)-C(1)-C(2)-O(3)	-14.47(16)	O(3)-C(5)-C(6)-C(11)	68.91(15)
O(2)-C(1)-C(2)-O(3)	167.21(9)	C(11)-C(6)-C(7)-C(8)	-1.00(19)
O(1)-C(1)-C(2)-C(3)	104.89(13)	C(5)-C(6)-C(7)-C(8)	174.83(13)
O(2)-C(1)-C(2)-C(3)	-73.43(11)	C(6)-C(7)-C(8)-C(9)	-0.7(2)
O(3)-C(2)-C(3)-F(1)	60.31(11)	C(7)-C(8)-C(9)-C(10)	1.9(2)
C(1)-C(2)-C(3)-F(1)	-61.18(11)	C(8)-C(9)-C(10)-C(11)	-1.2(2)
O(3)-C(2)-C(3)-C(4)	-58.86(12)	C(9)-C(10)-C(11)-C(6)	-0.5(2)
C(1)-C(2)-C(3)-C(4)	179.65(9)	C(7)-C(6)-C(11)-C(10)	1.65(19)
N(2)-N(1)-C(4)-C(3)	-73.97(14)	C(5)-C(6)-C(11)-C(10)	-174.27(12)
F(1)-C(3)-C(4)-N(1)	62.87(12)		
C(2)-C(3)-C(4)-N(1)	-178.07(9)		

Table 7. Bond lengths [Å] and angles [°] related to the hydrogen bonding for C11 H12 F N3 O3.

D-H	..A	d(D-H)	d(H..A)	d(D..A)	<DHA
O(2)-H(2)	O(3)#1	0.84	1.91	2.7379(11)	169.1

Symmetry transformations used to generate equivalent atoms:

#1 -x+1,y-1/2,-z+1/2



ORTEP view of the C11 H12 F N3 O3 compound with the numbering scheme adopted. Ellipsoids drawn at 30% probability level. Hydrogen atoms are represented by sphere of arbitrary size.

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CRYSTAL AND MOLECULAR STRUCTURE OF

C11 H12 F N3 O3 COMPOUND (bent27)

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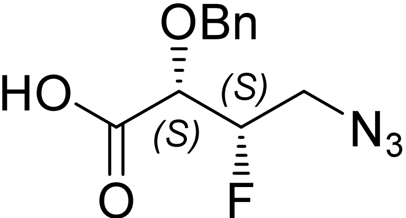


Compound *ent*-5.4

Structure solved and refined in the laboratory of X-ray diffraction Université de Montréal by Benoît Deschênes Simard.

Table 1. Crystal data and structure refinement for C11 H12 F N3 O3.

Identification code	bent27
Empirical formula	C11 H12 F N3 O3
Formula weight	253.24
Temperature	150K
Wavelength	1.54178 Å
Crystal system	Orthorhombic
Space group	P212121
Unit cell dimensions	a = 5.5106(2) Å α = 90° b = 10.4989(4) Å β = 90° c = 21.1010(9) Å γ = 90°
Volume	1220.80(8)$Å^3$
Z	4
Density (calculated)	1.378 g/cm^3
Absorption coefficient	0.959 mm^{-1}
F(000)	528
Crystal size	0.22 x 0.18 x 0.05 mm
Theta range for data collection	4.19 to 72.38°
Index ranges	$-6 \leq h \leq 6$, $-12 \leq k \leq 12$, $-26 \leq \ell \leq 26$
Reflections collected	16014
Independent reflections	2389 [R_{int} = 0.038]
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.9532 and 0.7983
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	2389 / 3 / 165
Goodness-of-fit on F^2	1.057
Final R indices [I>2sigma(I)]	R_1 = 0.0299, wR_2 = 0.0780
R indices (all data)	R_1 = 0.0312, wR_2 = 0.0792

Absolute structure parameter	-0.05(14)
Extinction coefficient	0.0109(8)
Largest diff. peak and hole	0.140 and -0.209 e/$Å^3$

Table 2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$ x 10^3) for C11 H12 F N3 O3.

U_{eq} is defined as one third of the trace of the orthogonalized Uij tensor.

	x	y	z	U_{eq}
F(1)	5599(1)	1889(1)	8293(1)	34(1)
O(1)	3483(2)	3878(1)	7265(1)	39(1)
O(2)	6345(2)	5063(1)	7757(1)	33(1)
O(3)	6904(2)	2002(1)	7034(1)	31(1)
N(1)	10084(2)	953(1)	8797(1)	43(1)
N(2)	8465(3)	237(1)	8986(1)	43(1)
N(3)	7133(3)	-430(2)	9229(1)	61(1)
C(1)	5507(3)	4027(1)	7468(1)	30(1)
C(2)	7500(2)	3011(1)	7452(1)	29(1)
C(3)	7813(2)	2457(1)	8116(1)	30(1)
C(4)	9768(3)	1445(1)	8145(1)	34(1)
C(5)	7386(3)	2277(1)	6373(1)	38(1)
C(6)	6512(3)	1150(1)	5999(1)	36(1)
C(7)	4413(3)	1216(2)	5634(1)	44(1)
C(8)	3525(3)	130(2)	5334(1)	52(1)
C(9)	4727(4)	-1019(2)	5403(1)	52(1)
C(10)	6826(4)	-1089(2)	5754(1)	48(1)
C(11)	7735(3)	-5(1)	6050(1)	40(1)

Table 3. Hydrogen coordinates (x 10^4) and isotropic displacement parameters ($Å^2$ x 10^3) for C11 H12 F N3 O3.

	x	y	z	U_{eq}
H(2)	5227	5604	7788	50
H(2A)	9060	3410	7314	34
H(3)	8214	3157	8420	36
H(4A)	9328	733	7859	41
H(4B)	11321	1810	7994	41
H(5A)	9146	2408	6305	46
H(5B)	6514	3057	6240	46
H(7)	3582	2003	5590	53
H(8)	2098	178	5082	63
H(9)	4099	-1764	5206	62
H(10)	7656	-1877	5795	57
H(11)	9195	-54	6288	48

Table 4. Anisotropic parameters ($Å^2 \times 10^3$) for C11 H12 F N3 O3.

The anisotropic displacement factor exponent takes the form:

$$-2 \pi^2 [h^2 a^{*2} U_{11} + ... + 2 h k a^* b^* U_{12}]$$

	U11	U22	U33	U23	U13	U12
F(1)	26(1)	35(1)	40(1)	3(1)	3(1)	-1(1)
O(1)	29(1)	35(1)	54(1)	1(1)	-6(1)	2(1)
O(2)	31(1)	25(1)	44(1)	-2(1)	1(1)	2(1)
O(3)	34(1)	27(1)	34(1)	-2(1)	2(1)	-3(1)
N(1)	35(1)	42(1)	51(1)	8(1)	-11(1)	-4(1)
N(2)	48(1)	36(1)	45(1)	2(1)	-10(1)	-7(1)
N(3)	74(1)	59(1)	51(1)	11(1)	-12(1)	-28(1)
C(1)	28(1)	27(1)	34(1)	4(1)	4(1)	-1(1)
C(2)	23(1)	24(1)	39(1)	-3(1)	2(1)	-3(1)
C(3)	24(1)	27(1)	38(1)	-2(1)	0(1)	-4(1)
C(4)	26(1)	33(1)	43(1)	2(1)	-2(1)	0(1)
C(5)	46(1)	33(1)	36(1)	2(1)	6(1)	-1(1)
C(6)	38(1)	37(1)	32(1)	0(1)	6(1)	0(1)
C(7)	41(1)	50(1)	41(1)	-2(1)	1(1)	7(1)
C(8)	43(1)	75(1)	38(1)	-9(1)	1(1)	-7(1)
C(9)	66(1)	49(1)	41(1)	-10(1)	12(1)	-16(1)
C(10)	68(1)	36(1)	39(1)	-3(1)	9(1)	3(1)
C(11)	45(1)	38(1)	36(1)	-1(1)	3(1)	5(1)

Table 5. Bond lengths [Å] and angles [°] for C11 H12 F N3 O3

Bond	Length	Angle	Value
F(1)-C(3)	1.4089(15)	N(3)-N(2)-N(1)	171.49(16)
O(1)-C(1)	1.2050(17)	O(1)-C(1)-O(2)	126.24(13)
O(2)-C(1)	1.3299(16)	O(1)-C(1)-C(2)	124.41(12)
O(3)-C(2)	1.4178(15)	O(2)-C(1)-C(2)	109.31(11)
O(3)-C(5)	1.4490(16)	O(3)-C(2)-C(3)	108.21(10)
N(1)-N(2)	1.2328(18)	O(3)-C(2)-C(1)	111.6(1)
N(1)-C(4)	1.4812(19)	C(3)-C(2)-C(1)	109.04(10)
N(2)-N(3)	1.1365(19)	F(1)-C(3)-C(4)	107.96(10)
C(1)-C(2)	1.5317(17)	F(1)-C(3)-C(2)	107.87(10)
C(2)-C(3)	1.5266(18)	C(4)-C(3)-C(2)	112.61(11)
C(3)-C(4)	1.5140(18)	N(1)-C(4)-C(3)	111.45(12)
C(5)-C(6)	1.501(2)	O(3)-C(5)-C(6)	106.88(11)
C(6)-C(7)	1.390(2)	C(7)-C(6)-C(11)	119.26(14)
C(6)-C(11)	1.392(2)	C(7)-C(6)-C(5)	121.26(14)
C(7)-C(8)	1.394(2)	C(11)-C(6)-C(5)	119.35(14)
C(8)-C(9)	1.384(3)	C(6)-C(7)-C(8)	120.24(15)
C(9)-C(10)	1.376(3)	C(9)-C(8)-C(7)	119.81(16)
C(10)-C(11)	1.391(2)	C(10)-C(9)-C(8)	120.33(16)
		C(9)-C(10)-C(11)	120.10(16)
C(2)-O(3)-C(5)	114.05(10)	C(10)-C(11)-C(6)	120.23(15)
N(2)-N(1)-C(4)	115.36(12)		

Table 6. Torsion angles [°] for C11 H12 F N3 O3.

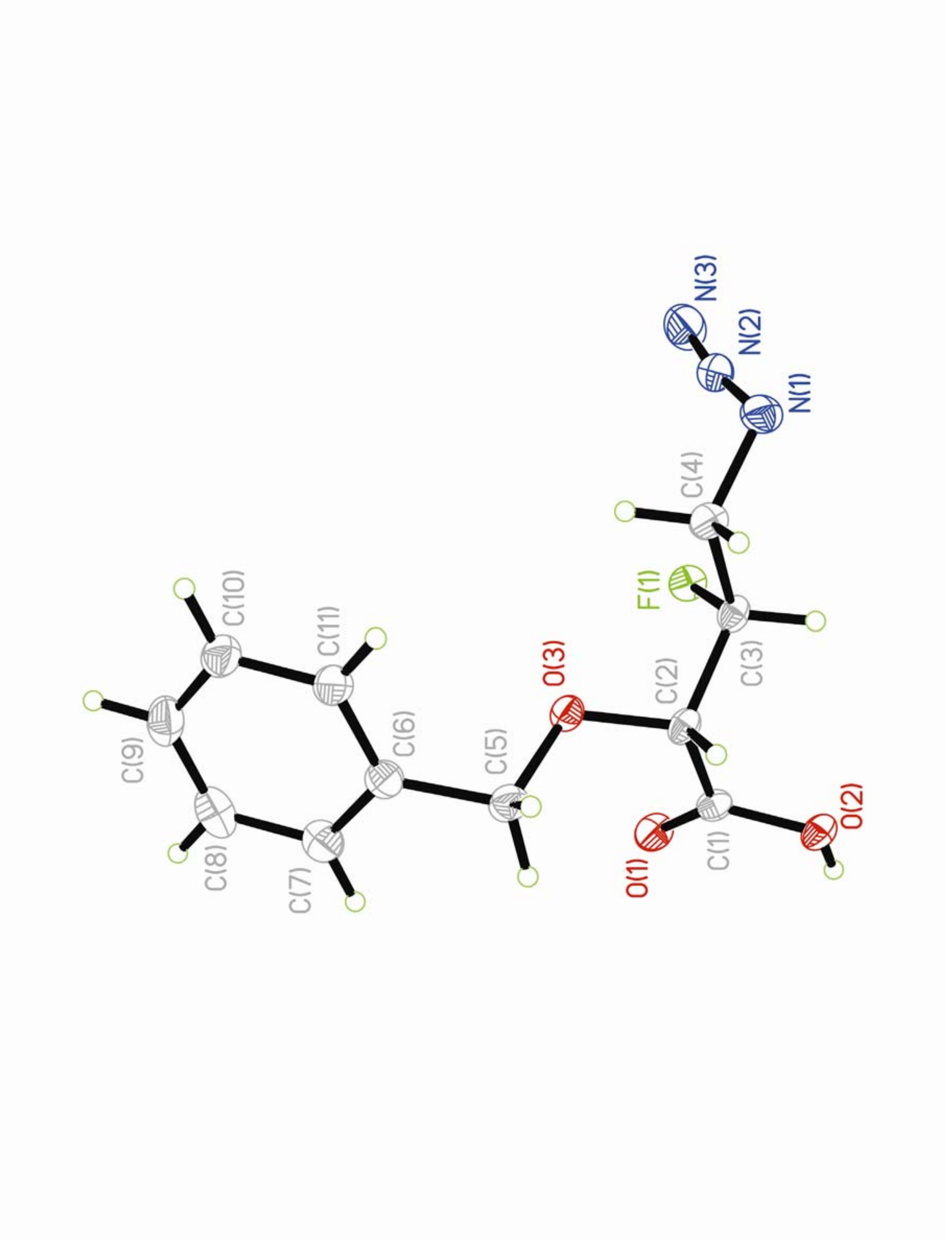
Torsion	Angle	Torsion	Angle
C(5)-O(3)-C(2)-C(3)	-159.25(11)	C(2)-C(3)-C(4)-N(1)	178.25(10)
C(5)-O(3)-C(2)-C(1)	80.78(13)	C(2)-O(3)-C(5)-C(6)	-176.53(11)
O(1)-C(1)-C(2)-O(3)	14.82(18)	O(3)-C(5)-C(6)-C(7)	106.98(15)
O(2)-C(1)-C(2)-O(3)	-167.21(10)	O(3)-C(5)-C(6)-C(11)	-68.76(17)
O(1)-C(1)-C(2)-C(3)	-104.66(15)	C(11)-C(6)-C(7)-C(8)	1.1(2)
O(2)-C(1)-C(2)-C(3)	73.31(13)	C(5)-C(6)-C(7)-C(8)	-174.62(14)
O(3)-C(2)-C(3)-F(1)	-60.27(12)	C(6)-C(7)-C(8)-C(9)	0.5(2)
C(1)-C(2)-C(3)-F(1)	61.29(12)	C(7)-C(8)-C(9)-C(10)	-1.6(3)
O(3)-C(2)-C(3)-C(4)	58.76(13)	C(8)-C(9)-C(10)-C(11)	0.9(2)
C(1)-C(2)-C(3)-C(4)	-179.68(10)	C(9)-C(10)-C(11)-C(6)	0.8(2)
N(2)-N(1)-C(4)-C(3)	74.07(16)	C(7)-C(6)-C(11)-C(10)	-1.8(2)
F(1)-C(3)-C(4)-N(1)	-62.77(14)	C(5)-C(6)-C(11)-C(10)	174.06(13)

Table 7. Bond lengths [Å] and angles [°] related to the hydrogen bonding for $C_{11}H_{12}FN_3O_3$.

D-H	..A	d(D-H)	d(H..A)	d(D..A)	<DHA
O(2)-H(2)	O(3)#1	0.84	1.92	2.7458(13)	169

Symmetry transformations used to generate equivalent atoms:

#1 -x+1,y+1/2,-z+3/2



ORTEP view of the C11 H12 F N3 O3 compound with the numbering scheme adopted. Ellipsoids drawn at 30% probability level. Hydrogen atoms are represented by sphere of arbitrary size.

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Annex 2
Supplementary Website Material on Antibiotic Resistance

The following website materials, which were referenced in Chapter 3, were annexed for preservation by request of the dissertation jury, because of the importance of their content:

1. *IDSA Report: Bad Bugs, No Drugs: As Antibiotic Discovery Stagnates, a Public Health Crisis Brews.* July 19, 2004. (PDF) http://www.idsociety.org/WorkArea/linkit.aspx?LinkIdentifier=id&ItemID=5554, also freely available at http://www.idsociety.org/10x20.htm (accessed June 2, 2010).
2. *Statement of the Infectious Diseases Society of America Before the Food and Drug Administration Part 15 Hearing Panel on Antimicrobial Resistance*. Apr. 28, 2008. (PDF) http://www.idsociety.org/WorkArea/linkit.aspx?LinkIdentifier=id&ItemID=11150, also freely available at http://www.idsociety.org/10x20.htm (accessed June 2, 2010).
3. *ECDC/EMEA JOINT TECHNICAL REPORT. The bacterial challenge: time to react.* Stockholm, September 2009. (PDF). http://www.ema.europa.eu/pdfs/human/antimicrobial_resistance/EMEA-576176-2009.pdf. ISBN 978-92-9193-193-4 (accessed June 2, 2010).
4. *IDSA's 10 X '20 INITIATIVE: ID Experts Call for 10 Novel Antibacterial Drugs by 2020 - Letter to President Barack Obama and Prime Minister Fredrik Reinfeldt.* Nov. 20, 2009. (PDF). http://www.idsociety.org/WorkArea/linkit.aspx?LinkIdentifier=id&ItemID=15752, also available at http://www.idsociety.org/10x20.htm (accessed June 2, 2010).

Bad Bugs, No Drugs

As Antibiotic Discovery Stagnates ... A Public Health Crisis Brews

July 2004

About IDSA

The Infectious Diseases Society of America (IDSA) represents more than 7,500 physicians, scientists, and other health professionals who specialize in infectious diseases in the United States and internationally.

Acknowledgements

IDSA is grateful to the many individuals who provided expert advice and assistance to the Society in the development of this paper. Most notably, we wish to express our sincere appreciation to the members of IDSA's Task Force on Antimicrobial Availability, who provided enormous contributions to our work over the past year. We also are grateful to the many government and industry officials and others who met with and advised IDSA leaders on the unique technical and business aspects of antibiotic research, development, evaluation, and review.

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The Next Epidemic Begins...

Day 1 A 34-year-old New Hampshire expectant mother visits her doctor's office complaining of severe stomach pain, vomiting, diarrhea, fever, and chills. She is diagnosed with an intestinal infection, given intravenous fluids and a prescription for a fluoroquinolone—an antibiotic—and is sent home.

Day 2 At a Massachusetts hospital's emergency room, a 2-year-old boy with a severe case of diarrhea, vomiting, dehydration, and fever is given fluids and administered a cephalosporin, another type of antibiotic, and is admitted to the hospital.

Day 4 The boy's lab results come back identifying the cause of his illness as *Salmonella*, a common foodborne bacterial infection, but, in this instance, the "bug" is highly resistant to the antibiotics commonly used to treat such infections, including cephalosporins and fluoroquinolones.

The baby boy dies of dehydration and bloodstream infection. As for the 34-year-old woman, the *Salmonella* infection results in a miscarriage of an otherwise normal baby followed by the woman's death.

Day 5 325 people are dead. Thousands—many of them children, the elderly, and other vulnerable individuals—jam emergency rooms across the Northeast complaining of similar symptoms. Cases have been reported in 15 states along the East Coast and in the Mid-Atlantic region. Isolated cases are reported in other states, including Texas and California. Fourteen cases are reported in Mexico and 27 cases in Canada.

Day 6 1,730 deaths and 220,000 illnesses have occurred in the United States. The epidemic expands in other countries.

Canada, Mexico, and Europe close their borders to U.S. food imports, and travel initiated from the United States is banned around the globe. Economic losses to the U.S. and global economies soon reach tens of billions of dollars.

The Food and Drug Administration and Centers for Disease Control and Prevention identifiy the source of the infections as a milk distribution facility located in New York state. They confirm that the *Salmonella* not only causes severe illness, but also is resistant to all available antibiotics. Doctors can only provide supportive care, not specific, antibiotic treatment.

Day 7 The number of deaths and illnesses continues to climb.

Think it can't happen? Think again. In 1985, milk contaminated with *Salmonella typhimurium* infected 200,000 people across the Midwest. What distinguishes that case from our scenario is the development of a fully antibiotic-resistant strain of the bacteria as compared to the one that is only partially drug-resistant. Such "bad bugs" are evolving. Some are already here.

Had bioterrorism prompted this scenario, infection rates could have been significantly higher, as several sources could have been intentionally contaminated. The toll on human lives and the U.S. economy would have been substantially worse.

Can we avert this catastrophe? If we act now, the answer is yes.

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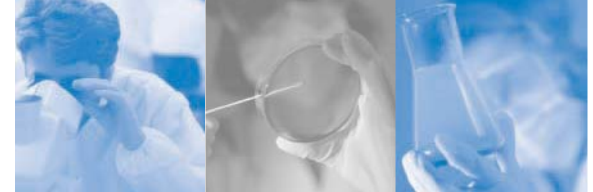


Executive Summary

Antibiotic-Resistant Bacterial Pathogens: Why We Are Concerned

Antibiotics and other antimicrobial drugs have saved millions of lives and eased patients' suffering. Although they have been dubbed "miracle drugs," antibiotics are not always effective. Over time, bacteria can develop resistance to existing drugs, making infections difficult if not impossible to treat.

A multi-pronged approach is needed to limit the impact of antibiotic resistance on patients and the public. These efforts include educating physicians, patients, and parents about the appropriate use of antibiotics, developing and applying infection control and immunization policies and practices to prevent transmission, surveying clinical and prescription data, and developing safer alternatives to antibiotic uses in agriculture.

The purpose of this document, however, is to call attention to a frightening twist in the antibiotic resistance problem that has not received adequate attention from federal policymakers: The pharmaceutical pipeline for new antibiotics is drying up.

Until recently, research and development (R&D) efforts have provided new drugs in time to treat bacteria that became resistant to older antibiotics. That is no longer the case. Unfortunately, both the public and private sectors appear to have been lulled into a false sense of security based on past successes. The potential crisis at hand is the result of a marked decrease in industry R&D, government inaction, and the increasing prevalence of resistant bacteria. Infectious diseases physicians are alarmed by the prospect that effective antibiotics may not be available to treat seriously ill patients in the near future.

Why Policymakers Should be Concerned Too

Policymakers already have recognized the urgent need to spur R&D related to biodefense. While this concern is appropriate, it is important to keep things in perspective. There has not been a single case of smallpox anywhere on the planet since the 1970s, but drug-resistant bacterial infections kill tens of thousands of Americans every year, and an epidemic could harm millions.

Why should policymakers care about antibiotic resistance and the lack of new antibiotics to treat resistant infections?

- Infections caused by resistant bacteria can strike anyone—the young and the old, the healthy and the chronically ill. Antibiotic resistance is a particularly serious problem for patients whose immune systems are compromised, such as people with HIV/AIDS and patients in critical care units.
- About 2 million people acquire bacterial infections in U.S. hospitals each year, and 90,000 die as a result. About 70 percent of those infections are resistant to at least one drug. The trends toward increasing numbers of infection and increasing drug resistance show no sign of abating.
- Resistant pathogens lead to higher health care costs because they often require more expensive drugs and extended hospital stays. The total cost to U.S. society is nearly $5 billion annually.
- The pipeline of new antibiotics is drying up. Major pharmaceutical companies are losing interest in the antibiotics market because these drugs simply are not as profitable as drugs that treat chronic (long-term) conditions and lifestyle issues.
- Drug R&D is expensive, risky, and time-consuming. An aggressive R&D program initiated today would likely require 10 or more years and an investment of $800 million to $1.7 billion to bring a new drug to market.
- Resistant bacterial infections are not only a public health problem; they have national and global security implications as well.
- The Institute of Medicine and federal officials have identified antibiotic resistance and the dearth of antibiotic R&D as increasing threats to U.S. public health.

IDSA's Investigation

IDSA has investigated the decline in new antibiotic R&D for more than a year, interviewing stakeholders from all sectors. Society leaders have met with officials from the Food and Drug Administration (FDA), the National Institute of Allergy and Infectious Diseases (NIAID), the Centers for Disease Control and Prevention (CDC), congressional members and staff, executives from leading pharmaceutical and biotechnology companies, representatives from public-private partnerships that are focused on infectious diseases-related product development, patients, and other stakeholders. Each stakeholder has an important role in furthering future antibiotic discovery and development and limiting the impact of antibiotic resistance. However, based upon past successes, the pharmaceutical and biotechnology industries are clearly best situated to take the lead in developing the new antibiotics needed to treat bacterial diseases. As such, industry action must become the central focus of an innovative federal public health effort designed to stimulate antibiotic R&D.

IDSA's investigation has revealed that the incentives most likely to spur R&D within major pharmaceutical companies include those that provide financial benefits prior to a drug's approval (e.g., tax credits for R&D), commence at the time of approval (e.g., wild-card patent extension), reduce the costs of clinical trials (e.g., FDA flexibility concerning the evidence necessary to demonstrate safety and efficacy; NIAID-sponsored research to develop rapid diagnostics tests, etc.), and reduce companies' risks (e.g., liability protections). R&D at smaller biotechnology companies also could be stimulated through statutory and administrative changes. Finally, new funding for critical federal public health programs, and public and private research efforts, would help to ensure progress as well as limit the public health impact of antibiotic resistance.

Following is a list of specific potential legislative solutions, administrative recommendations, and funding requests:

Potential Legislative Solutions To Fuel Innovation

Congress and the Administration must work together to enact statutory incentives that stimulate the discovery and development of new antibiotics to treat drug-resistant and other dangerous infections. Critical priority incentives that will have the greatest impact are indicated.

Commission to Prioritize Antimicrobial Discovery

CRITICAL PRIORITY

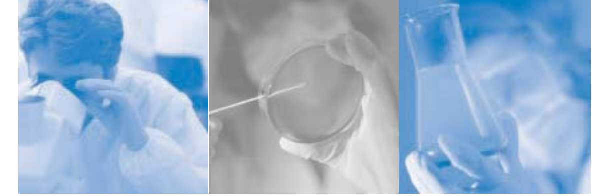
Establish and empower an independent Commission to Prioritize Antimicrobial Discovery to decide which infectious pathogens to target using these legislative R&D incentives and administrative solutions:

Supplemental intellectual property protections:

- "Wild-card patent extension." ***CRITICAL PRIORITY*** A company that develops and receives approval for a priority antibiotic could extend the market exclusivity period of another FDA-approved drug as long as the company commits to invest a portion of the profits derived during the extension period back into antibiotic R&D.
- Restoration of all patent time lost during FDA's review of priority antibiotics
- Extended market exclusivity similar to what has been successfully implemented for pediatric and orphan drugs

Other potential statutory incentives:

- Tax incentives for R&D of priority antibiotics ***CRITICAL PRIORITY***
- Measured liability protections ***CRITICAL PRIORITY***
- Additional statutory flexibility at FDA regarding approval of antibiotics, as needed
- Antitrust exemptions for certain company communications
- A guaranteed market



In 2002, out of 89 new drugs, no new antibiotics were approved.

Establish similar statutory incentives to spur R&D for rapid diagnostic tests for targeted pathogens, which will help to reduce the cost of clinical trials

Potential statutory incentives of interest to small biopharmaceutical companies:

- Waive FDA supplemental application user fees for priority antibiotics
- Tax credits specifically targeting this segment of the industry
- Small business grants

In addition to enacting statutory incentives to spur antibiotic R&D, Congress should work with the Administration to implement administrative recommendations at FDA and NIAID.

July 1997. A 7-year-old girl from urban Minnesota was admitted to a hospital with an infected right hip joint. Doctors drained the infected joint and treated the girl with the antibiotic cefazolin. On the third day of her hospital stay, tests showed the girl was infected with methicillin-resistant *Staphylococcus aureus* (MRSA), and the doctors changed her antibiotic to vancomycin, but it was too late: The infection had already invaded too deeply into her lungs. The girl suffered respiratory failure that day and was placed on a ventilator. After five weeks in the hospital, she died from a lung hemorrhage. This girl was previously healthy with no recent hospitalizations.

Food and Drug Administration Recommendations

FDA is a pivotal and constructive partner in the process of antibiotic development. In order to effectively implement FDA's plan, *Innovation or Stagnation: Challenge and Opportunity on the Critical Path to New Medical Products*, modifications to existing policy, procedures, and guidelines are necessary. Each of the following recommendations is a critical priority: ***CRITICAL PRIORITIES***

- Accelerate the publication of updated guidelines for antibiotic clinical trials to provide needed clarity, and revisit existing guidelines as appropriate to ensure their relevance
- Encourage imaginative clinical trial designs that lead to a better understanding of drug efficacy against resistant bacterial pathogens
- Provide a clear definition of acceptable surrogate markers as end points for clinical trials of bacterial infections
- Explore and, when appropriate, encourage the use of animal models of infection, in vitro technologies, and valid microbiologic surrogate markers to reduce the number of efficacy studies required for each additional indication while maintaining safe and effective drug dose regimens
- Explore with NIAID all opportunities to streamline antibiotic drug development
- Grant priority antibiotics accelerated review status

Drug-resistant infections can strike anyone, even healthy children.

National Institute of Allergy and Infectious Diseases Recommendations

NIAID could play a central role in the R&D process. To do so, NIAID should implement the following recommendations. Each is a critical priority: **CRITICAL PRIORITIES**

- Aggressively encourage translational (bench to bedside) research as described in NIH's *Roadmap for Medical Research*
- Remove roadblocks to antibiotic R&D that may exist in NIAID's structure and guidelines, including any unnecessary restrictions affecting companies' intellectual property rights
- Increase the number and size of grants that support discovery of new drugs that treat targeted pathogens
- Develop and expand collaborations with industry and the infectious diseases research community
- Sufficiently fund and rapidly launch NIAID's newly established Drug Discovery and Mechanisms of Antimicrobial Resistance Study Section
- Engage outside experts in research planning and ensure more transparent decision-making
- Explore with FDA all opportunities to streamline antibiotic drug development
- Encourage research on topics directly related to conduct of clinical trials
- Sponsor research into new rapid diagnostic tests for bacterial infections that, when available, could reduce the cost of clinical trials
- Encourage research on antibiotic use and resistance development
- Fund placebo-controlled trials to evaluate the necessity of antibiotic therapy for selected diseases

New Funding Needed

The increasing threat of drug resistance, concomitant with decreasing antibiotic R&D, requires a dramatic increase in public funding for CDC, FDA, NIAID, and public-private research efforts. At a minimum, Congress and the Administration must work together to invest ***new*** resources (i.e., not shift funds from other public health efforts) into the following critical program areas:

- Double CDC's antimicrobial resistance program funding to $50 million in 2005 and continue to increase it by $25 million increments until 2009 to a total of $150 million
- Increase FDA's funding by $25 million to support implementation of the *Critical Path* plan (which would help decrease the cost of antibiotic development), the development of new antibiotic guidelines, and to speed antibiotic reviews
- Significantly increase NIAID's translational and antibiotic resistance research efforts
- Support synergistic public/private partnerships that focus on infectious diseases medicines

Conclusion

Without innovative public policy and additional financial support, fewer and fewer antibiotics will be available to treat the increasing number of drug-resistant and dangerous microbes that threaten Americans and the global community. The proposals advanced in this document are intended to ensure a sustainable supply of safe and effective antibiotics to protect the public's health.

We urge policymakers to act quickly.

Bad Bugs, No Drugs

As Antibiotic Discovery Stagnates ...
A Public Health Crisis Brews

"Infectious diseases physicians are alarmed by the prospect that effective antibiotics may not be available to treat seriously ill patients in the near future. There simply aren't enough new drugs in the pharmaceutical pipeline to keep pace with drug-resistant bacterial infections, so-called 'superbugs.'"

Joseph R. Dalovisio, MD
IDSA President

Resistance on the Rise

Antibiotics* have saved millions of lives and eased the suffering of patients of all ages for more than 60 years. These "wonder drugs" deserve much of the credit for the dramatic increase in life expectancy in the United States and around the world in the 20th century. They prevent amputations and blindness, advance our ability to perform surgery, enable new cancer treatments to be used, and protect the lives of our military men and women. A famous infectious disease expert once noted that the discovery of penicillin in the early 1940s gave more curative power to a lone provider than the collective talent of all the physicians in New York City at that time. Unfortunately, it is inevitable that, over time, bacteria develop resistance to existing antibiotics, making infections more difficult to treat.

Antibiotic resistance is not a new phenomenon. National surveillance data and independent studies show that drug-resistant, disease-causing bacteria have multiplied and spread at alarming rates in recent decades. A diverse range of patients is affected. The Institute of Medicine (IOM), Centers for Disease Control and Prevention (CDC), National Institutes of Health (NIH), and the Food and Drug Administration (FDA) warn that drug-resistant bacteria are a serious public health threat, especially considering that there are few novel drugs in the pipeline to combat them.

Infections that were once easily curable with antibiotics are becoming difficult, even impossible, to treat, and an increasing number of people are suffering severe illness—or dying—as a result. This year, nearly 2 million people in the United States will acquire bacterial infections while in the hospital, and about 90,000 of them will die, according to CDC estimates. More than 70 percent of the bacteria that cause these infections will be resistant to at least one of the drugs commonly used to fight them. (See Table 1.) In a growing and frightening number of cases, these bacteria are resistant to many approved drugs, and patients have to be treated with new, investigational compounds or older, toxic alternatives. For many patients, there simply are no drugs that work.

The resistance problem "has probably been smoldering for years, but recently it's almost like a switch got triggered," medical professor Stuart H. Cohen, MD, of the University of California, Davis, recently told the *Wall Street Journal*.

"Antibiotic resistance is increasing too quickly and in too many organisms," said Harvard Medical School pediatric infectious disease specialist Jonathan Finkelstein, MD, in the same article.

Table 1: Estimated Cases of Hospital-Acquired Infections Caused by Selected Resistant Bacteria in the United States in 2002

Antibiotic-Resistant Bacteria	Estimated Cases
Methicillin/*S. aureus*	102,000
Methicillin/CNS	130,000
Vancomycin/enterococci	26,000
Ceftazidime/*P. aeruginosa*	12,000
Ampicillin/*E. coli*	65,000
Imipenem/*P. aeruginosa*	16,000
Ceftazidime/*K. pneumoniae*	11,000

Source: Centers for Disease Control and Prevention, Division of Healthcare Quality Promotion

These preliminary estimates were extrapolated by CDC staff from data collected from hospitals that participate in the National Nosocomial Infections Surveillance System. NNIS hospitals are disproportionately large, urban, and affiliated with medical schools and are more likely to have more seriously ill patients. As such, these estimates should be interpreted cautiously.
CNS=Coagulase-negative staphylococci

*Antibiotics are a type of antimicrobial, a broad term used to describe any agent that inhibits the growth of microorganisms, including bacteria, viruses, fungi, yeast, protozoa, and parasites. Antibiotics target bacteria—the "bad bugs" addressed in this paper. Bacteria are by far the most common cause of infectious diseases-related deaths in the United States.

According to IOM and FDA, only two new classes of antibiotics have been developed in the past 30 years, and resistance to one class emerged even before FDA approved the drug. (See Table 2.)

Furthermore, some strains of resistant bacteria are no longer confined to hospitals and are occurring in otherwise healthy individuals in communities across the United States and other countries.

As resistant bacteria multiply, so does the burden they place on our health care system. The economic cost has reached billions of dollars annually in the United States, according to estimates from IOM and the former Congressional Office of Technology Assessment. The human cost in terms of pain, grief, and suffering, however, is incalculable.

Table 2: History of Antibiotic Discovery and Approval

Year Introduced	Class of Drug
1935	Sulfonamides
1941	Penicillins
1945	Cephalosporins
1944	Aminoglycosides
1949	Chloramphenicol
1950	Tetracyclines
1952	Macrolides/ Lincosamides/ Streptogramins
1956	Glycopeptides
1957	Rifamycins
1959	Nitroimidiazoles
1962	Quinolones
1968	Trimethoprim
2000	Oxazolidinones
2003	Lipopeptides

Source: Food and Drug Administration (modified)

Presented by John H. Powers, MD, at April 15-16, 2004 "Antimicrobial Drug Development Workshop," co-sponsored by FDA, IDSA, and the International Society of Anti-Infective Pharmacology.

Fast-Moving Targets

To understand how quickly disease-causing bacteria can develop resistance to antibiotics, take the example of *Staphylococcus aureus* (staph), a common cause of hospital infections that can spread to the heart, bones, lungs, and bloodstream with fatal results. Penicillin, introduced in the early 1940s, once kept staph bacteria at bay. However, penicillin-resistant staph bacteria were identified as early as 1942. By the late 1960s, more than 80 percent of staph bacteria were penicillin-resistant. Methicillin was introduced in 1961 to combat resistant staph bacteria, but reports of methicillin-resistant *Staphylococcus aureus* (MRSA) rapidly followed. In 1974, 2 percent of the staph bacteria found in U.S. hospitals were methicillin-resistant. By 2002, that figure had jumped to 57.1 percent, according to CDC data. (See Chart 1 and Table 3.)

Staph infections have acquired resistance to many other drugs in addition to penicillin and methicillin. In fact, according to CDC, about half of the identified MRSA strains in U.S. hospitals are resistant to all but a few antibiotics. Causing even greater alarm, staph bacteria partially resistant to vancomycin, a drug of last resort in the treatment of several resistant infections, were discovered in patients in the late 1990s. Two cases of fully vancomycin-resistant *Staphylococcus aureus* (VRSA) were reported in 2002 and a third in 2004.

MRSA is no longer a problem confined to hospitals. One ongoing study of children with community-acquired staph infections at the University of Texas has found nearly 70 percent infected with MRSA. In a 2002 outbreak, 235 MRSA infections were reported among military recruits at a training facility in the southeastern United States. In addition, a total of 12,000 cases of community-acquired MRSA were found in three correctional facilities (Georgia, California, and Texas) between 2001 and 2003.

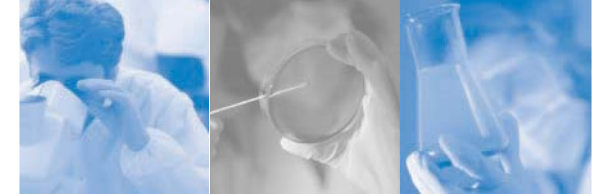
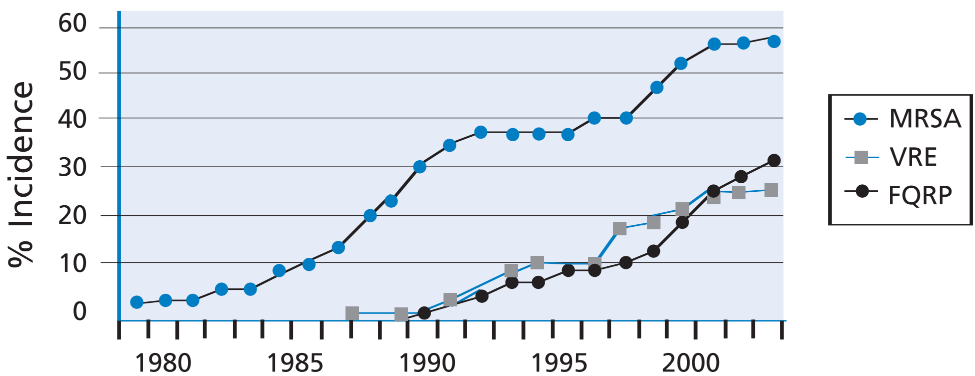


Chart 1: Resistant Strains Spread Rapidly



Source: Centers for Disease Control and Prevention

This chart shows the increase in rates of resistance for three bacteria that are of concern to public health officials: methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and fluoroquinolone-resistant *Pseudomonas aeruginosa* (FQRP). These data were collected from hospital intensive care units that participate in the National Nosocomial Infections Surveillance System, a component of the CDC.

Other resistant bacterial infections also are raising significant public health concerns:

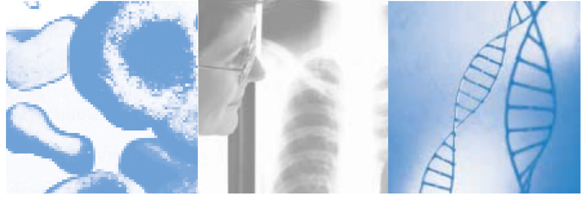
- In 1998, IOM reported an alarming rise in the incidence of infections due to a bacterium called enterococcus, which causes wound infections, infections in blood, the urinary tract and heart, and life-threatening infections acquired in hospitals. Vancomycin has been a core treatment for enterococci. The percentage of enterococci resistant to vancomycin (VRE) has been increasing dramatically since the late 1980s, according to CDC. In 2002, more than 27 percent of tested enterococci samples from intensive care units were resistant to vancomycin. (See Chart 1 and Table 3.)
- The percentage of *Pseudomonas aeruginosa* bacteria resistant to either ciprofloxacin or ofloxacin, two common antibiotics of the fluoroquinolone class (FQRP), has increased dramatically from the late 1980s to the present. Recent CDC data show that in 2002, nearly 33 percent of tested samples from intensive care units were resistant to fluoroquinolones. *P. aeruginosa* causes infections of the urinary tract, lungs, and wounds and other infections commonly found in intensive care units. (See Chart 1 and Table 3.)

Table 3: Percent of Drug Resistance in Hospital-Acquired Infections in 2002

Drug/Pathogen	Resistance (%)
Methicillin/*S. aureus*	57.1
Vancomycin/enterococci	27.5
Quinolone/*P. aeruginosa*	32.8
Methicillin/CNS	89.1
3rd-gen. Ceph./*E. coli*	6.3
3rd-gen. Ceph./*K. pneumoniae*	14.0
Imipenem/*P. aeruginosa*	22.3
3rd-gen. Ceph./*P. aeruginosa*	30.2
3rd-gen. Ceph./*Enterobacter spp.*	32.2
Penicillin/*S. pneumoniae*	11.3

Source: CDC National Nosocomial Infections Surveillance System, August 2003 for all, except penicillin resistant *Streptococcus pneumoniae*, which is the Active Bacterial Core Surveillance of the Emerging Infections Network.

This table provides a snapshot of selected drug-resistant pathogens associated with hospital infections in intensive care unit patients during 2002. CNS=Coagulase-negative staphylococci; 3rd Ceph=resistance to 3rd generation cephalosporins (either ceftriaxone, cefotaxime, or ceftazidime); Quinolone=resistance to either ciprofloxacin or ofloxacin.



- *Streptococcus pneumoniae* is the most feared bacterium that causes pneumonia. *S. pneumoniae* strains that are resistant to penicillin and other drugs are emerging rapidly in the United States. Up to 40 percent of infections caused by this bacterium are resistant to at least one drug, and 15 percent are resistant to three or more drugs, the CDC reports. Aside from 100,000 cases of pneumonia each year, this bacterium causes childhood ear infections (6 million per year), meningitis (3,300 per year), and sinusitis (thousands of cases).

- Multidrug-resistant *Acinetobacter*, a type of bacteria that has caused stubborn wound infections in U.S. soldiers and civilians stationed in Iraq, has been increasingly reported worldwide. Pneumonia due to *Acinetobacter* infections is now considered one of the most difficult hospital-acquired infections to control and treat, according to a recent study in *Clinical Infectious Diseases (CID)*. An international surveillance study, also reported in *CID*, tested hundreds of *Acinetobacter* samples and found various levels of resistance to 15 drugs. Some *Acinetobacter* strains are resistant to virtually every available drug with the exception of one toxic antibiotic that causes substantial side effects.

- Salmonellosis, a common foodborne infection that causes diarrhea, can cause serious illness and death. Nationally, the incidence of *Salmonella* bacteria resistant to cephalosporins, an antibiotic commonly used to treat severe salmonellosis, rose nearly fivefold (from 0.5 percent to 2.4 percent) between 1998 and 2001, according to a study published in the *Journal of Infectious Diseases*. In Massachusetts during the same time period, the prevalence of drug-resistant *Salmonella* rose from 0 percent to 53 percent.

- Tuberculosis (TB) is becoming increasingly difficult to treat. The World Health Organization estimates that up to 50 million people worldwide may be infected with drug-resistant strains of TB. Treatment for resistant TB strains can take up to 24 months, as opposed to the six months generally required to treat non-resistant strains.

Since 2000, CDC has reported a new phenomenon—community-acquired outbreaks of MRSA among athletes, including college football players in Pennsylvania, wrestlers in Indiana, and a fencing club in Colorado. Public health officials believe that physical contact and the sharing of clothing or equipment probably leads to the spread of infection in these otherwise healthy people. In September of 2003, this issue was brought to national attention when MRSA broke out in Florida among the Miami Dolphins, sending two players to the hospital for treatment.

Many athletes have developed methicillin-resistant Staphylococcus aureus (MRSA), which can infect the heart, bones, lungs, and bloodstream.

The Human Toll

Statistics cannot convey the human toll that resistant organisms take on their victims. Throughout this paper are stories of previously healthy people who became seriously ill or died as a result of drug-resistant infections. These examples, reported by the CDC, the media, and infectious diseases physicians, show that resistant infection can strike anyone, at any time. They serve as examples of what an increasing number of Americans could face as a result of the impending public health crisis.

The Economic Burden

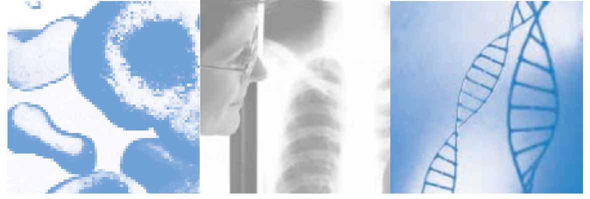
Drug-resistant bacteria impose an economic burden on the United States on the order of billions of dollars annually, according to several authoritative analyses. Drug-resistant infections are significantly more expensive to treat than non-resistant infections because of longer hospitalizations, extra physician visits, the higher cost of alternative antibiotics, more post-hospital care, lost work days, and deaths. For example, resistant TB strains are as much as 100 times more expensive to treat than non-resistant strains, according to Lee B. Reichman, MD, MPH, director of the New Jersey Medical School National Tuberculosis Center. MRSA infections cost an average of $31,400 per case to treat compared to $27,700 per case for non-resistant infections, according to a study cited in the IOM report *Antimicrobial Resistance: Issues and Options (1998)*.

The same IOM report estimated that the total cost to U.S. society of antimicrobial resistance was at least $4 billion to $5 billion annually. A 1995 cost analysis by the former Congressional Office of Technology Assessment (OTA) provided similar dollar estimates when factors such as the costs of lost work days and costs for post-hospital care are considered. OTA went further to say that "these costs can be expected to increase rapidly as the numbers of antibiotic resistant bacteria increase."

A multi-pronged approach is essential to limit the impact of antibiotic resistance on patients and public health. Good antibiotic stewardship, infection control and prevention efforts, increased surveillance, and limits on agricultural uses of antibiotics are extremely important. But a more pressing concern is that, as the number of resistant pathogens continues to grow, the pipeline of antibiotics used to treat these "bad bugs" is quickly drying up.

Patients with Compromised Immune Systems at Greater Risk

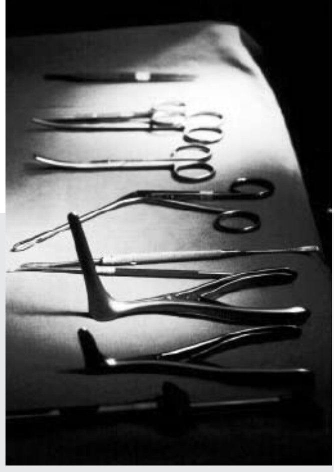
Antibiotic resistance is a serious problem for people with compromised immune systems, including patients in hospital critical care units and the 40 million people living with HIV/AIDS in the United States and globally. Their weakened immune systems make these patients particularly vulnerable to drug-resistant and other bacterial infections. A recent study published in *Clinical Infectious Diseases* has shown that the very patients most vulnerable to the devastating impact of resistant infections—those with compromised immune systems—also are more likely than other patients to be infected with resistant pathogens. Furthermore, in many areas of the world, patients infected with HIV are more likely to die as a result of bacterial infections, such as tuberculosis, than of the underlying HIV infection. A wider array of antibiotics that treat bacterial infections—particularly drug-resistant strains—could offer significant hope to people with compromised immune systems.



The Pipeline of New Antibiotics Is Drying Up

In spite of the pressing need for new drugs to treat resistant infections, there simply are not enough new antibiotics in the pharmaceutical pipeline to keep pace. Major pharmaceutical companies with the R&D "muscle" to make progress are losing interest in the antibiotics market, even as they increase their overall R&D budgets. Of greatest concern is the dearth of resources being invested in drug discovery.

The trend started more than 10 years ago. In 1990, half of the large pharmaceutical companies in the United States and Japan reported that they had halted or significantly decreased their antibiotic discovery efforts. That same year, several companies attempted to get back into the market, spurred on by worsening problems with MRSA and a VRE outbreak. But the enthusiasm was short-lived. In 2000, Roche announced that it was spinning off its anti-infective discovery division. In 2002, Bristol-Myers Squibb Company, Abbott Laboratories, Eli Lilly and Company, and Wyeth all halted or substantially reduced their anti-infective discovery efforts, and Aventis announced plans to spin off its anti-infectives division. Procter & Gamble also appears to be withdrawing from new antibiotic R&D. Other companies appear to have decreased the number of employees assigned to antibiotic discovery and development.



April 2004. A 46-year-old Maryland man received a transplant and was sent to the intensive care unit. His blood cultures grew *Acinetobacter* that was resistant to all antibiotics except colistin, a drug rarely used because it is very toxic. He died.

A growing number of drug companies appear to be withdrawing from new antibiotic research and development.

An article in the January-February 2004 issue of *Health Affairs* described the impact of these reductions on the ability of pharmaceutical companies to develop new drugs to target antibiotic resistance: "Today there are few champions for the study of infectious diseases mechanisms, and few within the industry are able to interpret the epidemiological data in a way that translates into business decisions."

Companies' efforts to downsize antibiotic R&D activities have had a notable impact on the number of antibiotics moving through the pipeline.

A recent analysis published in *Clinical Infectious Diseases* found only five new antibiotics in the R&D pipeline out of more than 506 drugs in development.* The authors evaluated the websites or 2002 annual reports of 15 major pharmaceutical companies with a track record in antibiotic development and seven major biotechnology companies.** Their analysis revealed four new antibiotics being developed by pharmaceutical companies, and only one antibiotic being developed by a biotech company. By comparison, the analysis found that the pharmaceutical companies were developing 67 new drugs for cancer,

*"Development" in this context refers to phases 2 and 3 of human testing—the later stages of the R&D process.

**Pharmaceutical companies examined were Merck & Co., Johnson & Johnson, Pfizer, GlaxoSmithKline, Bristol-Myers Squibb, Aventis, Pharmacia, Novartis, F. Hoffman-La Roche, AstraZeneca, Abbott Laboratories, Wyeth, Eli Lilly & Company, Schering-Plough, and Bayer. Biotech companies were Amgen, Genentech, Applera, Genzyme, Serono, Chiron, and Biogen. The authors' list of new drugs in the pipeline also included telithromycin, which was subsequently approved by FDA.

33 for inflammation/pain, 34 for metabolic/endocrine disorders, and 32 for pulmonary disease. The biotech companies were developing 24 drugs for inflammation/ immunomodulators, 14 drugs for metabolic/endocrine disorders, and 13 for cancer.

The end result of the decline in antibiotic discovery research is that FDA is approving few new antibiotics. Since 1998, only 10 new antibiotics have been approved, two of which are truly novel—i.e., defined as having a new target of action, with no cross-resistance with other antibiotics. In 2002, among 89 new medicines emerging on the market, none was an antibiotic.

IOM's 2003 report on microbial threats reinforces the point, noting that although at first glance the situation with respect to antibiotics currently in clinical development looks encouraging, not one *new class* of antibiotics is in late-stage development. "Rather these 'new' antibiotics belong to existing classes, including macrolides and quinolones, that have been used to treat humans for years," IOM said.

Infectious disease experts are particularly concerned about the dearth of new "narrow-spectrum" agents—that is, drugs that fight a specific infectious organism. Many of the antibiotics in development today are "broad-spectrum"—meaning they are intended to work against a wide range of organisms—which are more likely to contribute to the development of resistance.

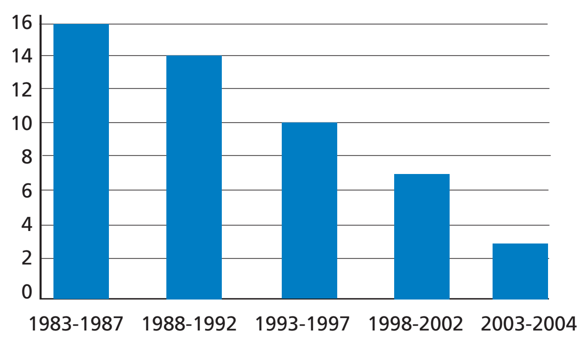
Only about five new antibiotics are in the drug pipeline, out of more than 506 agents in development.

Table 4: New Antibacterial Agents Approved Since 1998

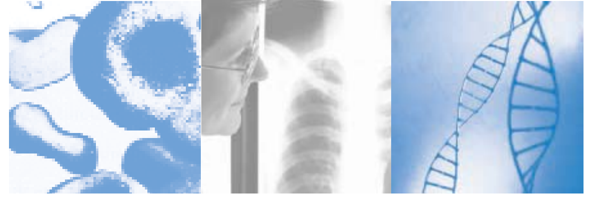
Antibacterial	Year	Novel
rifapentine	1998	No
quinupristin/dalfopristin	1999	No
moxifloxacin	1999	No
gatifloxacin	1999	No
linezolid	2000	Yes
cefditoren pivoxil	2001	No
ertapenem	2001	No
gemifloxacin	2003	No
daptomycin	2003	Yes
telithromycin	2004	No

Source: Spellberg et al., *Clinical Infectious Diseases*, May 1, 2004 (modified)

Chart 2: Antibacterial Agents Approved, 1983-2004



Source: Spellberg et al., *Clinical Infectious Diseases*, May 1, 2004 (modified)



Medical Need Versus Market Realities

There is a growing disconnect between the medical need perceived by those who practice infectious diseases medicine and the market as assessed by the pharmaceutical industry. Infectious diseases physicians see a significant need for new antibiotics to treat a growing number of bacterial infections from which their patients suffer—but antibiotic R&D does not add up from a business perspective. The costs outweigh the benefits to a company's bottom line.

Are Small Biotechnology Companies Engaged?

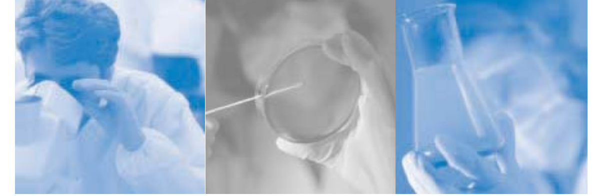
If major pharmaceutical companies are exiting the field, what about smaller biopharmaceutical companies? Indeed, several smaller companies are focusing on the development of antibiotic compounds (e.g., Cubist Pharmaceuticals, Basilea, Paratek, Vicuron Pharmaceuticals, and Oscient). However, a substantial number of other small companies simply are pursuing development of drugs that have been licensed from the major companies—i.e., most are not involved in basic discovery research. While some smaller companies are funding antibiotic discovery programs, it remains to be seen whether they can be successful in the absence of the financial support and expertise available at larger companies. In order to advance new classes of antibiotics from discovery to development, they may need the financial support of larger companies or other backers to fund late-stage clinical trials and commercialization. For the economic reasons described in this paper, it is not apparent that such support will be forthcoming.

The pharmaceutical industry, like all other publicly traded industries, must deliver for its shareholders in order to justify their continued investment. The unique nature of antibiotics makes securing investments challenging. Because antibiotics work so well and so fast, they produce a weak return on investment for manufacturers. Antibiotics are commonly prescribed for seven to 14 days. Even for the most serious of infections, these drugs are rarely needed for more than four to six weeks.

Understandably, pharmaceutical and biotechnology companies and their investors are drawn to develop products that provide greater returns on investments. The favored drugs include those that patients take for life, like insulin for diabetes, statins for elevated cholesterol, and drugs that treat hypertension and arthritis. Although these drugs do address significant medical needs, other drugs—like those used to treat impotence, baldness, and other lifestyle issues—have little to no medical benefit at all but are likely to reap huge profits.

Experts in industry, government, and academia understand the problem and have acknowledged it for years:

- "Product development in areas crucial to public health goals, such as antibiotics, has slowed significantly during the past decade." (U.S. Food and Drug Administration. *Innovation/Stagnation: Challenge and Opportunity on the Critical Path to New Medical Products.* March 2004.)
- "To describe drug research in trendy terms: chronic disease medications are in; anti-infectives are out." When it comes to annual sales potential, antibiotics don't measure up. An industry representative speaking at a scientific conference noted that a musculoskeletal drug is worth about $1.150 billion, a neuroscience treatment is rated at $720 million, and a medicine for resistant Gram-positive cocci is worth only $100 million. (Sellers, LJ. Big pharma bails on anti-infectives research. *Pharmaceutical Executive.* December 2003, 22.)



- "As a consumer, you want a drug [that] you don't have to take very long and works very well. But that isn't the most profitable type of drug. … [I]n some cases the economics and the public health imperative do not match up." (Mark Goldberger, acting deputy director of FDA's Center for Drug Evaluation and Research, quoted in Service, RF. Orphan drugs of the future? *Science.* March 19, 2004, Vol. 303, 1798.)
- U.S. demographics shifting toward an increasingly older population will lure even more investors and companies to the chronic diseases market. As generics compete with existing products, companies face additional pressure to develop new blockbusters, which account for most of their revenue. (Health Care Industry Market Update: Pharmaceuticals, Centers for Medicare and Medicaid Service. January 10, 2003.)

Limiting Resistance—and Profitability, Too

Antibiotic resistance—and public health measures to combat resistance—also pose unique challenges to securing investment in antibiotic R&D. Resistance limits the effectiveness of antibiotics over time and therefore decreases a drug's long-term profitability. Antibiotics and other antimicrobials are the only drugs where extensive use leads to loss of benefit.

In addition, infectious diseases physicians and other public health experts often hold new antibiotics in reserve, hoping to avoid fostering the rapid emergence of resistant bacteria and saving them for when they are most needed. This unusual practice is unique to anti-infective drugs. From a public health perspective, the strategy is sensible. However, in pharmaceutical industry terms, this practice translates into a "slow commercial uptake" that limits the potential market for new antibiotics. Drug company representatives have said that physicians' efforts to preserve antibiotics for the treatment of resistant infections serve as a disincentive to antibiotic discovery and development.

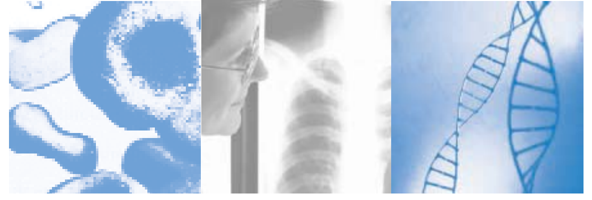
August 2003. A 7-year-old Texas boy came down with a fever of nearly 103 degrees and complained of severe pain in his leg. He was taken to Hermann Children's Hospital in Houston, where doctors discovered that a virulent, drug-resistant staph infection was causing a potentially fatal blood clot in the boy's leg. Fortunately, in this case, surgery was life-saving.

Infectious disease doctors often hold new antibiotics in reserve because of concerns about resistance.

Technical Hurdles

In addition to the lack of effective market incentives, antibiotic R&D is hampered by technical challenges as well. As IOM's microbial threats report noted, "the discovery of new antibiotics is not as easy as was once believed."

Until the early 1990s, pharmaceutical companies tended to develop new infectious diseases drugs by randomly screening natural products to identify those demonstrating antimicrobial activity. New technologies in use since then, such as combinational chemistry, X-ray crystallography, high throughput screening, and molecular modeling, have not been as successful in identifying new antibiotics as might have been hoped.



Moreover, industry representatives speaking about these challenges at a recent scientific meeting said that genomic data have "failed to deliver the expected flood of novel targets."

Assuming one has a novel target of action within the bacterium, there is still the challenge of finding a chemical entity that can reach the target site and inhibit growth, without being too highly toxic to patients. "The technical hurdles, coupled with competition for resources within pharmaceutical companies from other significant medical needs with larger market opportunities, have led to reduced investment in or, in the case of most companies, elimination of antibiotic drug discovery programs," concluded IOM.

Additional Hurdles for Clinical Trials of New Antibiotics

In addition to market and technical challenges, industry representatives cite scientific and regulatory hurdles as impediments to antibiotic approvals.

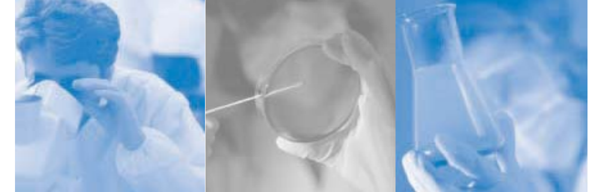
Because antibiotics are used to treat various types of infection (e.g., pneumonia, urinary tract infection, skin and soft tissue infection), the drug approval process requires clinical trials for each of these indications (one trial or often more per indication), with enrollment of large numbers of patients to ensure an understanding of a drug's safety and effectiveness against specific bacterial pathogens.

Finding enough patients to enroll in clinical trials of new drugs to treat resistant pathogens is no easy task. By contrast, when enrolling patients in a clinical trial to test a new cancer drug, researchers know from the start whether a specific patient has the specific type of cancer they are targeting. With antibiotic clinical trials, that is not necessarily the case. For many resistant pathogens, there are no rapid diagnostic tests available to help researchers to identify patients who would be eligible for their studies.

As one industry consultant explained, in order to test a drug that is intended to treat resistant strains, "You have to wait for epidemics to break out in hospital wards, and you can't predict when that will happen. It may take five years to complete a clinical study."

One company's experience in trying to develop a new drug to treat vancomycin-resistant enterococci (VRE) illustrates some of the challenges. Researchers used entry criteria that were developed in consensus with FDA and academia. With 54 research sites open for two years, only three patients enrolled in the study—it was closed for insufficient enrollment. When a second study was launched, only 45 subjects enrolled over a period of 18 months. This does not mean that there are few VRE infections; indeed, according to CDC, there are estimated to be 26,000 hospital-acquired cases each year in the United States. (See Table 1.) The problem is in the ability to anticipate their presence and to enroll critically ill patients in clinical trials.

July 2001. An 11-year-old boy struck by a resistant staph infection first spent seven weeks in the hospital, two of those weeks in intensive care, and then underwent 12 surgeries over the next two years to excise the infection and repair the damage it inflicted on his thigh bone. After two years of operations, body casts, wheelchairs, and crutches, this boy is finally able to walk and run again, although with a limp because his previously infected leg is now shorter than the other.



Updated FDA guidance documents defining the investigational approaches for each type of infection, some of which are currently in review, will bring needed clarity to drug development teams within industry. Such guidance would provide a better understanding about the type of safety and efficacy data that FDA could find to be scientifically compelling and acceptable when evaluating new antibiotic applications.

Lengthy, Costly, and Risky Process

As with any other drug, antibiotic R&D is a lengthy, costly, and risky process.

According to a September 2003 review by the Tufts Center for the Study of Drug Development, the median time from the beginning of clinical testing through FDA review for new antibiotics and similar drugs was just over six years (55.8 months in the clinical phase; 18.6 months in the review phase).* Preclinical identification and testing of potential candidate drugs may add several more years to the process.

During the pre-approval phases of drug discovery and development, a product's patent clock is ticking away. Most patents are filed during the pre-clinical phase, which means that the effective patent life of a new compound once it is brought to market is less (sometimes substantially so) than the 20 years provided by law. Although current law allows for restoration of some patent time lost during FDA's period of review, not all lost time is restored.

> Because antibiotics work so well and so fast, they produce a weak return on investment for manufacturers.

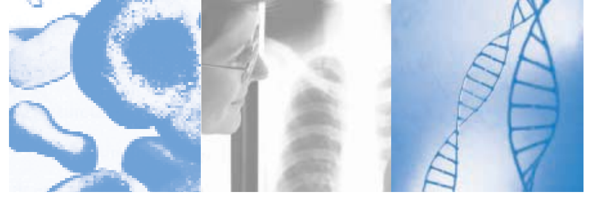
The 2003 IOM report acknowledged this challenge, noting that "the development of an antibiotic is an expensive and risky process; no guarantee can be made that the antibiotic will remain effective and the investment will be regained before the patent period has ended." As for the cost, according to a recent FDA report, bringing a new drug to market can cost $800 million to $1.7 billion.

The pharmaceutical industry's risks are high. According to the Pharmaceutical Research and Manufacturers of America, only five in 5,000 compounds that enter preclinical testing make it to human testing, and only one of these five is approved. If a product is not going to produce strong profits, then other products with greater market potential will get the "green light" for the next phase of development.

Pharmaceutical Charity Helps, But Is Not the Solution

The pharmaceutical industry participates in many areas of public health and provides many good works *pro bono*. Some examples include Merck & Co.'s efforts related to River Blindness; efforts by Bristol-Myers Squibb, Pfizer, and other drug companies related to global AIDS; and GlaxoSmithKline's malaria and AstraZeneca's TB drug discovery initiatives. Nevertheless, industry cannot alter its fundamental business strategy in any way that would place its bottom line at risk. Policymakers and the public should have no illusions that future pharmaceutical charity will be sufficient to address the existing and emerging pathogens that threaten public health.

*The study looked at small molecule anti-infectives approved between 1982 and 2001.



The Federal Government's Response

Much has been written about antibiotic resistance and the decline in R&D. Many groups have supported strengthening the U.S. and international governments' response to this growing public health crisis, including IOM, the World Health Organization, the Congressional Office of Technology Assessment, the American Society for Microbiology, and the Alliance for the Prudent Use of Antibiotics.

To date, the U.S. government's action has been inadequate to address the brewing crisis, but the Administration and Congress recently have announced several proposals, which, if successfully and fully implemented, could make a difference.

• NIH's Roadmap for Medical Research

NIH's *Roadmap*, issued in September 2003, outlines a series of initiatives to "speed the movement of research discoveries from the bench to the bedside." After decades of investment in basic biomedical research, the *Roadmap* is intended to widen NIH's mission to include *translational research*—i.e., translating basic discoveries from concept into clinical evaluation, focusing on specific diseases or therapies.

• FDA's Innovation/Stagnation: Challenge and Opportunity on the Critical Path to New Medical Products

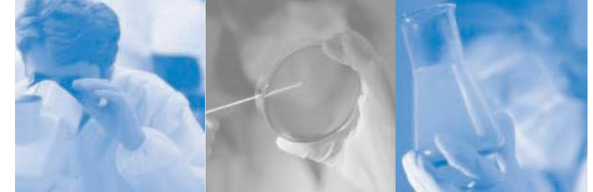
In March 2004, FDA issued its *Critical Path* report to complement the NIH *Roadmap* initiative. In FDA's view "applied sciences have not kept pace with the tremendous advances in basic sciences." The *Critical Path* plan is FDA's attempt to encourage the creation of new tools to get fundamentally better answers about how the safety and effectiveness of new drugs can be demonstrated, in faster time frames, with more certainty, and at lower costs. FDA's report has been called "timely and significant" and "courageous" by

National Security and Antibiotic Resistance

Antibiotic resistance not only threatens public health, but may have national and global security implications as well. Virtually all of the antibiotic-resistant pathogens that exist naturally today can be bio-engineered through forced mutation or cloning. In addition, genetic manipulation of existing pathogens could render them resistant to currently available antibiotics. A better understanding of the mechanisms related to drug resistance and tools that could be derived from such research may help U.S. public health officials as they monitor and respond to any future bioterrorism episodes that involve genetically engineered resistant pathogens. Moreover, antibiotic resistance may limit the effectiveness of antibiotics during future bioterrorism events, outbreaks, and other emergencies.

Members of Congress are beginning to see the connection and to understand our vulnerability. In their reports on Project Bioshield in 2003, both the House Government Reform Committee and the Energy and Commerce Committee linked *natural conditions*, including antimicrobial resistance and dangerous viruses, to national security concerns. The Energy and Commerce Report stated "advancing the discovery of new antimicrobial drugs to treat resistant organisms … may well pay dividends for both national security and public health."

[See also the report, *Beyond Anthrax: Confronting the Biological Weapons Threat*, issued May 4, 2004, by the Democrats of the House Select Committee on Homeland Security simultaneously with the introduction of the Rapid Pathogen Identification to Delivery of Cures Act (H.R. 4258).]



industry leaders who have praised the report for "recognizing the serious problems that are preventing new, innovative drugs and biologics from getting to the patients who need them."

• Project Bioshield

Following the 2001 anthrax attacks, the Administration and congressional leaders moved rapidly to introduce the Project Bioshield Act.* The legislation is intended to spur R&D of new drugs, vaccines, and diagnostics for use against potential bioterrorism agents by establishing a guaranteed market for these products with the federal government serving as purchaser. Project Bioshield focuses on the six category A bioterrorism agents of greatest concern (smallpox, anthrax, botulism, tularemia, viral hemorrhagic fevers, and plague).

The legislation does not include incentives to spur R&D of new antibiotics to treat drug-resistant infections that threaten public health, despite IDSA's pleas that they be included.

• Public Health Service Action Plan to Combat Antimicrobial Resistance

In January 2001, a federal interagency task force including CDC, FDA, NIH, and other agencies published the *Public Health Service Action Plan to Combat Antimicrobial Resistance.* The action plan is a comprehensive strategy that includes efforts to reverse the stagnation in antibiotic R&D. Other key action items target antimicrobial resistance surveillance, prevention and control, and research. Due to limited appropriations, the Administration's implementation of the plan thus far has been slow, not well coordinated, and incomplete.

*Although not enacted at the time this paper went to press, the Act likely will have been enacted by its publication date.

• General Accounting Office Study

In May 2003, Senators Judd Gregg (R-NH) and Jack Reed (D-RI) asked the General Accounting Office (GAO) to study the antimicrobial availability problem. The senators stated:

> "With the threat of bioterrorism, the growing number of microorganisms resistant to drug therapy, the reemergence of previously deadly infectious diseases, such as tuberculosis, and the emergence of new infectious diseases in the United States, such as severe acute respiratory syndrome and West Nile virus, there is an urgent need for new antimicrobials."

A year later, GAO has yet to begin the study, and their analysis of the many challenges to antibiotic R&D may be years away. ... **The time for studying the problem is over.**

January 1999. A 13-year-old girl from rural Minnesota was brought to a local hospital with fever and respiratory distress. She was coughing up blood. A chest X-ray revealed fluid in the lungs. The girl was treated with the antibiotics ceftriaxone and nafcillin. Within five hours of arriving at the hospital, the girl's blood pressure dropped, and she was transferred to a pediatric hospital, intubated, and treated with vancomycin and cefotaxime. Despite intensive medical care, the girl's health deteriorated, and she died on the seventh hospital day from multiple organ failures and excessive fluid and swelling in the brain. An autopsy and tests revealed that MRSA had destroyed her left lung. The girl had no chronic medical conditions and no recent hospitalizations.

Innovative Federal Policy and Immediate Action Are Needed

The federal government must take decisive action now. Primarily, policymakers must focus on adopting incentives to stimulate investment in this area of discovery by pharmaceutical and biotechnology companies. Any antibiotic R&D plan that does not include industry action at its core will yield hollow promises. Government-sponsored research and refinement of existing regulations, policies, and guidance can help to address the overall problem of antibiotic resistance, fill in some of the gaps in research, and reduce the cost of antibiotic discovery and development. But industry must take the lead to ensure success. Industry decision-making is not perfect from a public health perspective, but the focus on financial incentives has made industry successful in the past, and new incentives can lead to future successes.

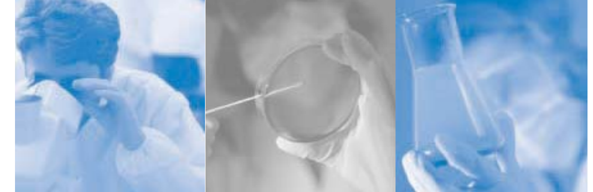
The past two decades of antibiotic development clearly have demonstrated that we no longer can rely on existing market forces to keep companies engaged in this area of drug discovery and development. Should additional companies' antibiotic R&D infrastructures be dismantled, it will take years to establish new programs—or this expertise could simply be lost forever. Moreover, given the 10-year time gap that it takes for new antibiotics to move from concept to market, time for action is running out.

Creative thinking and innovative policy will solve both the antibiotic R&D and antibiotic resistance problems. IDSA has explored with industry, government officials, academics, patient representatives, and congressional staff the long-term value of many potential solutions. Our investigation has revealed that the incentives most likely to spur R&D within major pharmaceutical companies include those that provide financial benefits prior to a drug's approval (e.g., tax credits for R&D), commence at the time of approval (e.g., wild-card patent extension), reduce the costs of clinical trials (e.g., FDA flexibility concerning the evidence necessary to demonstrate safety and efficacy; National Institute of Allergy and Infectious Diseases [NIAID] sponsored research to foster the development of rapid diagnostics tests, etc.); and reduce companies' risks (e.g., liability protections). R&D at smaller companies also could be stimulated through statutory and administrative changes. Finally, new funding could help to ensure a better understanding about biological mechanisms related to antibiotic resistance, limit the public health impact of antibiotic resistance, and spur public-private R&D efforts.

IDSA does not claim to possess all of the answers, but a combination of the solutions listed in the next section will help. Policymakers should use these recommendations to shape a framework for governmental action.

April 2004. A 52-year-old Maryland man, previously healthy, was hospitalized complaining of cough, fever, and shortness of breath. His sputum culture grew MRSA. A chest X-ray showed pneumonia involving almost all segments of the lung. He was treated aggressively with antibiotics, transferred to the intensive care unit, and placed on a ventilator but died on the second hospital day.

Resistant infections can lead to longer hospital stays.



RECOMMENDATIONS FOR CONGRESS

Legislative action is necessary to stem the tide of pharmaceutical company departures from antibiotic R&D and to stimulate the involvement of non-active companies. Critical priorities that will have the greatest impact are indicated.

Commission to Prioritize Antimicrobial Discovery

CRITICAL PRIORITY

To begin to address the "bad bugs, no drugs" problem, Congress should establish and empower an independent Commission to Prioritize Antimicrobial Discovery (CPAD). CPAD's specific focus would be to identify the targeted pathogens that are (or are likely to become) a significant threat to public health due to drug resistance and other factors. The statutory R&D incentives that follow would apply to drugs that treat these pathogens. CPAD's decision-making would be based on an analysis of risks as well as benefits to public health.

An expert independent commission is needed to address the public health and R&D issues unique to antimicrobial R&D. Similar entities in other areas of medicine include the National Vaccine Advisory Committee and the National Cancer Advisory Board.

CPAD would make recommendations directly to the Secretary of Health and Human Services (HHS) and would be comprised of experts from the infectious diseases medical and research communities, representatives from relevant government agencies (CDC, FDA, NIH), and representatives from industry and relevant patient advocacy groups.

Companies would register with HHS to become eligible for the incentives. Once HHS certified a company as eligible, it could receive tax credits (R&D, capital formation, etc.). When a company successfully developed a product that met HHS predetermined specifications, it would become eligible for other incentives (intellectual property, liability, etc.).

Proposed Statutory Incentives

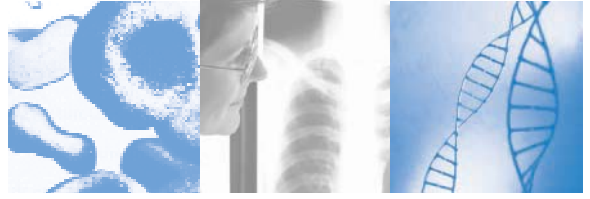
Congress must enact a robust set of statutory incentives to stimulate private sector investment and innovation. Unless such incentives are established, Americans will be at even greater risk from infectious disease threats in the future.

The Project Bioshield Act and pending legislation, such as the Biological, Chemical, and Radiological Weapons Countermeasures Research Act (S. 666), introduced by Senators Lieberman and Hatch in 2003, provide good starting points for congressional discussions about what incentives are appropriate. Like Project Bioshield, S. 666 includes progressive ideas to spur R&D for bioterrorism countermeasures. S. 666 goes further, however, providing tax credits, special intellectual property incentives, and antitrust and indemnification provisions.

Existing law offers other models to consider. The Best Pharmaceuticals for Children Act, for example, provides an additional six months of market exclusivity for new or already-marketed drugs and priority review status for pediatric supplements to a drug application, if the holder of an approved application undertakes studies of these drugs in children. Under the Orphan Drug Act,* qualifying drugs receive seven years of market exclusivity protection against generics and innovator drugs, tax incentives (up to 50 percent for clinical research), and research grants.

Following is a list of potential statutory incentives for Congress to consider:

*Orphan diseases or conditions must affect fewer than 200,000 individuals in the United States or provide no reasonable expectation that the sales of the drug will recover the costs of development.



Recommendations for Congress (continued)

1. Supplemental intellectual property protections for companies that invest in R&D for priority antibiotics

- **Establishment of a "wild-card patent extension" linked to R&D for antibiotics to treat targeted pathogens** *CRITICAL PRIORITY*

The original concept of a wild-card patent extension is provided in S. 666. Under this proposal, a company that receives approval for a new antibiotic, or a new indication for an existing antibiotic, that treats a targeted pathogen would be permitted to extend the market exclusivity period for another of the company's FDA-approved drugs. S. 666 supports a patent extension of two years.

The wild-card incentive may not be acceptable to all policymakers. For that reason, Congress should explore the feasibility of modifying the wild-card concept to require that the company commit a substantive portion (10 percent-20 percent) of the profits derived from the patent extension to additional targeted antibiotic R&D. This incentive is unlikely to help small biopharmaceutical companies, but would be a significant lure to major pharmaceutical firms.

- **Restoration of all patent time lost during FDA's review of applications for antibiotics that treat targeted pathogens**

FDA's review time for new antibiotic applications can vary, but the mean time is as long as 18 months. Although some of the patent time lost during FDA's review may be restored under current law, the specter of losing any patent time can have dramatic implications for companies' decision-making. S.666 permits a company to select either this incentive or the wild-card patent extension incentive, but not both. Because the profit potential of most antibiotics is not very high and is likely to decline as the patent runs out, this is unlikely to be a very strong incentive in most cases.

- **Extension of market exclusivity for antibiotics that treat targeted pathogens similar to what has been successfully implemented for pediatric and orphan drugs**

Extended periods of market exclusivity can be an incentive to the original sponsor of a drug, as generic copies of the drug may not be approved or marketed during this time. Lengths of market exclusivity used or proposed in the past include six months under the Best Pharmaceuticals for Children Act (BPCA), seven years under the Orphan Drug Act, and 10 years under S. 666. Several pharmaceutical companies have indicated that an additional six months of market exclusivity would not provide a sufficient draw for them to invest in the development of new antibiotics or to seek a new indication for an existing antibiotic. For that reason, new legislation should include the longer periods of exclusivity as available under the Orphan Drug Act or as proposed in S. 666.

The fundamental principle behind the passage of BPCA and the Orphan Drug Act is that the government has a public health interest in spurring the discovery of new treatments to assist vulnerable populations. This same principle should prompt Congress to address the problem of drug-resistant infections.

Because the profit potential of most antibiotics is not high and is likely to decline over time, this profit is unlikely to be a very strong incentive in most cases.

2. Other potential statutory incentives to spur antibiotic R&D

- **Provide tax incentives** (as provided in S. 666). The company seeking to fund research would be eligible to elect among the following tax incentives:

  – Claim tax credits for R&D of antibiotics that treat targeted pathogens *CRITICAL PRIORITY*

– Allow R&D limited partnerships to conduct research on drugs to treat targeted pathogens. The partnerships would pass through all business deductions and credits to the partners.
– Issue a special class of stock for the entity to conduct the research. The investors would be entitled to a zero capital gains tax rate on any gains realized on the stock.
– Receive a special tax credit for research conducted at a non-profit and academic research institution

- **Provide FDA with additional statutory flexibility to approve antibiotics that treat targeted pathogens as opposed to types of infection (e.g., resistant *S. aureus* vs. pneumonia) and encourage the agency to use that authority**

- **Create a guaranteed market with the federal government as purchaser and sufficient appropriations to stimulate R&D for antibiotics that treat targeted pathogens** (as provided for biodefense in Project Bioshield and S. 666)

The "bad bugs, no drugs" problem highlights the need for an open and flowing pipeline of antibiotics to treat patients on a daily basis in hospitals and communities across the United States. A guaranteed market that prompts stockpiling of drugs is unlikely to have much applicability in this regard.

3. Establish similar statutory incentives (as listed previously) to spur R&D for rapid diagnostic tests to identify targeted pathogens, which will help to reduce the cost of clinical trials

Policymakers should consider applying the incentives outlined above as potential solutions to encourage R&D for rapid diagnostic tests. New rapid diagnostics would greatly reduce the cost and time needed to conduct clinical trials for new antibiotics. For many resistant pathogens, there currently are no rapid diagnostic tests available to assist in identifying eligible patients for clinical trials. Cutting costs and time will serve as incentives for greater investment in and more speedy approval of targeted antibiotics. In addition, new rapid diagnostics will permit physicians to diagnose specific bacterial infections in their patients. This will enable physicians to prescribe the most appropriate antibiotics, which will slow the evolution of new resistance.

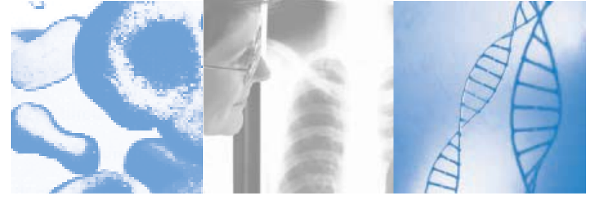
4. Potential statutory incentives of interest to small biopharmaceutical companies that have far less up-front capital to invest in R&D for antibiotics that treat targeted pathogens

- **Provide tax incentives to form capital from investors and retained earnings for biopharmaceutical companies that cannot use tax credits, because they have no tax liability, or permit the small company to save or sell its credits** (as provided in S. 666)

- **Significantly increase the number and amount of Small Business Innovation Research (SBIR) grants that NIH can provide for these antibiotics**

- **Waive user fees for supplemental new drug applications submitted to FDA for the treatment of targeted pathogens**

Currently, companies can submit supplemental applications for new indications of drugs that have already been approved by FDA—for example, if an existing drug is found to be effective in treating a different bacterial infection or the same infection located in a different area of the body. Under current law, the user fee is waived for the original new drug application that an eligible "small company" submits to FDA for review. However, the company is charged a user fee for supplemental applications submitted for each new indication even if the new indication will treat an organism that threatens public health.



Recommendations for Congress (continued)

5. Liability protections afforded to companies that receive FDA approval for antibiotics that treat targeted pathogens (as provided in S. 666)

CRITICAL PRIORITY

For obvious reasons, the pharmaceutical company representatives with whom IDSA met each saw government indemnification, similar to what has been afforded childhood vaccines, as a powerful incentive to develop new antibiotics. IDSA's recommendation is limited to antibiotics as they are being used to treat pathogens targeted by the Commission to Promote Antimicrobial Discovery.

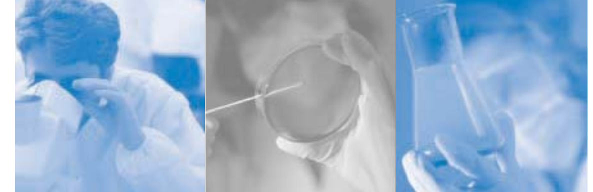
6. Limited antitrust exemptions for companies that seek to work together to expedite research on targeted antibiotics (as provided in S. 666)

February 2004. A 34-year-old Maryland woman had the flu and went to an emergency room where a chest X-ray showed pneumonia. Laboratory studies confirmed it was due to MRSA. She developed shock and required a ventilator and tracheostomy to support breathing. As a complication of shock, both legs were amputated. She remained in the hospital for more than two months.

Next Steps for Congress

Hearings should be scheduled as soon as possible to highlight the human consequences of the "bad bugs, no drugs" problem and to determine which combination of incentives are most appropriate. The Senate and House leadership should work together in a bipartisan manner to enact sufficient statutory incentives to stimulate new antibiotic R&D. Congress should work cooperatively with the Administration to encourage greater antibiotic R&D and to limit the public health impact of antibiotic resistance.

Congress must act now to encourage pharmaceutical and biotech companies to invest in the antibiotics market.



Recommendations for FDA

The Food and Drug Administration's (FDA) high standards for evaluating antibiotics' safety and efficacy must be maintained. However, avenues must be explored to better address the unique nature of antibiotic discovery and stimulate industry-sponsored antibiotic R&D. As FDA implements its new *Critical Path* plan, the agency should implement the following recommendations. Each of the recommendations should be considered a critical priority:

CRITICAL PRIORITIES

- **Publish updated guidelines for clinical trials of anti-infectives.** Industry is understandably hesitant to initiate new clinical trials in areas where the standards for safety and efficacy are unclear. FDA should issue, as soon as possible, guidelines for resistant pathogens, bacterial meningitis, acute bacterial sinusitis, acute bacterial otitis media, and acute exacerbation of chronic bronchitis. These guidelines have been in revision or development for some time. FDA also should move quickly to identify additional areas of uncertainty in antibiotic drug development and develop or update guidelines in those areas as well. Review of these guidance documents at appropriate intervals also would be extremely useful in ensuring their continued relevance and accuracy.

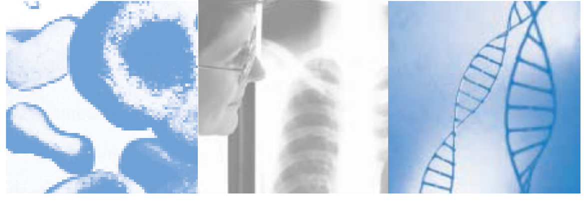
- **Encourage imaginative clinical trial designs that lead to a better understanding of drug efficacy against resistant pathogens.** For example, clinical trial data on resistant pathogens are time-consuming and costly to accrue. FDA could define ways in which an antibiotic's efficacy against drug-sensitive types of bacteria could be used to extrapolate efficacy against drug-resistant strains.

- **Provide a clear definition of acceptable surrogate markers as endpoints for clinical trials of bacterial infections.** In other words, FDA needs to define new ways to determine an antibiotic's effectiveness, such as clearing bacteria from blood or other body sites (e.g., hip and knee implants) or resolving fever. This concept has been accepted for antiviral agents, but has had limited application to bacterial infections.

- **Explore, and when appropriate encourage, the use of animal models of infection, in vitro technologies (e.g., test tube), and valid microbiologic surrogate markers (e.g., clearance of bacteremia) to reduce the number of efficacy studies required for each additional indication.** These data are easier and less costly to obtain than full results of safety and efficacy testing in human subjects, and therefore, when appropriate, could result in a more timely and efficient approval process. Of course, safe and effective drug dose regimens must be maintained.

- **Explore with NIAID all opportunities to streamline antibiotic drug development.** (See examples outlined under NIAID recommendations.)

- **Grant accelerated approval status for antibiotics that treat targeted pathogens.** This regulatory pathway allows FDA to grant approval prior to completion of full human testing, based upon a demonstration of efficacy using surrogate endpoints with a commitment for post-approval human testing to confirm the effect on disease outcomes. Moving beyond the current scenario, FDA could give provisional approval for antibiotics that treat targeted pathogens followed by a post-approval study of the drug by a select group of investigators certified to treat patients with the drug. The certified investigators would collect additional efficacy data needed to lead to a full approval, while providing patients with earlier access to the drug. Health care payers would offset the costs of the clinical trials, which may prompt companies to pursue candidate drugs that they otherwise might not.



RECOMMENDATIONS FOR NIAID

NIH has shown leadership in developing the *Roadmap* initiative. The true test is still to come as the plan is implemented. The National Institute of Allergy and Infectious Diseases (NIAID) has primary responsibility for implementing the *Roadmap* in the infectious diseases arena. To achieve success, NIAID should implement the following recommendations. Each of these recommendations should be considered a critical priority:

CRITICAL PRIORITIES

- **Move aggressively to expand the translational (bench to bedside) research concepts contained in the *Roadmap* to strengthen antibiotic R&D, remove roadblocks that may exist in NIAID's structure and guidelines, and accelerate antibiotic resistance research activities**

- **Increase the number and size of grants to small businesses, academic institutions, and non-profit organizations that focus on R&D of antibiotics to treat targeted pathogens**

- **Seek greater opportunities to work with pharmaceutical and biotechnology companies to advance antibiotic R&D, and ensure that NIAID staff who oversee technology-transfer efforts understand industry's motivations and goals**

- **Engage more aggressively the infectious diseases research community in research planning efforts and create a more transparent decision-making process**

- **Sufficiently fund and rapidly implement NIAID's newly launched Drug Discovery and Mechanisms of Antimicrobial Resistance Study Section**

- **Encourage research on topics directly related to the implementation of clinical trials (e.g., surrogate endpoints of response to therapy, animal models, and analytical methods)**

- **Sponsor research into new rapid diagnostic tests for bacterial infections that, when available, could reduce the cost of clinical trials**

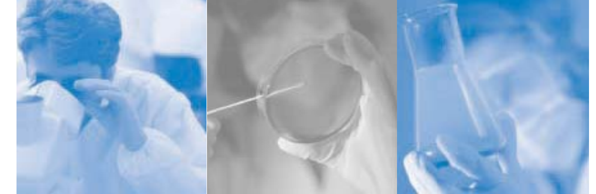
- **Re-examine NIH's 1999 research tool guidelines and modify or waive the guidelines where necessary.** NIH's guidelines have been criticized for unnecessarily restricting companies' intellectual property rights and revenue generation where research tools have been developed in conjunction with federally funded research. Critics believe the guidelines should be modified to breathe new life into research tool development, particularly to help fight emerging infectious pathogens. Research tools include cell lines, drug delivery technologies, laboratory animals, clones and cloning tools, databases, and other technologies.

- **Develop a fellowship curriculum designed for clinician investigators to provide expertise in clinical trials of new antibiotics.** FDA and the National Cancer Institute (NCI) announced an analogous program for anti-cancer drugs in 2003.

- **Explore joint programs with FDA to streamline antibiotic drug development similar to programs initiated by NCI and FDA in 2003.** The NCI/FDA programs are intended to inform and harmonize all phases of cancer drug discovery, development, and regulatory review.

- **Encourage research on antibiotic use patterns and their impact on resistance, specifically the impact of use restrictions on newly approved antibiotics**

- **Fund placebo controlled trials to determine if certain diseases require antibiotic therapy (e.g., acute otitis media, acute exacerbation of chronic bronchitis, and acute bacteria sinusitis).** There is reasonable concern that antibiotics frequently are prescribed to treat diseases that are not caused by bacteria (e.g., are viral in origin). This inappropriate use of antibiotics promotes antibiotic resistance with no benefit to patients. Definitive placebo-controlled studies are needed to elucidate this point.



New Funding Needed

Public and private efforts that target the growing problem of drug resistance and lack of antibiotic R&D are drastically under-funded. An infusion of new resources (i.e., not shifting funds from other public health efforts) in several critical program areas will go a long way toward assuring Americans that they will soon be protected from dangerous and drug-resistant pathogens.

- **Double CDC's antimicrobial resistance program to $50 million in 2005 and continue to increase it by $25 million increments until 2009 to a total of $150 million**

  CDC is the primary coordinator of much of the *Public Health Service Action Plan to Combat Antimicrobial Resistance*. Increasing CDC's funding will enable the agency to expand its surveillance of clinical and prescribing data that are associated with drug-resistant infections, which would assist the Commission to Prioritize Antimicrobial Discovery (referenced above), CDC, and other public health agencies in setting priorities. Funding also is needed to educate physicians and parents about the need to protect the long-term effectiveness of antibiotics as well as to strengthen infection control activities across the United States. Finally, broadening the number of CDC's extramural grants targeting applied research at academic-based centers would harness the brainpower of our nation's researchers and assist the agency in developing practical and successful antimicrobial resistance prevention and control strategies.

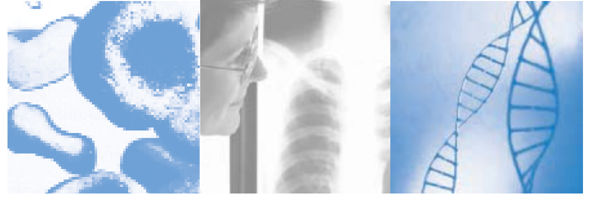
- **Increase by $25 million funding for FDA's programs that support antibiotic development and reduce the costs of clinical trials**

  New funding will enable the anti-infective review group within FDA's Center for Drug Evaluation and Review to begin to implement the *Critical Path* plan, including funding research efforts envisioned under the plan and creating guidelines that clarify for industry the standards FDA will apply to antibiotic R&D. New funding also would strengthen the anti-infective review group's ability to evaluate antibiotics for the treatment of targeted pathogens, by permitting them to contract with companies that provide national, real-time microbiological data related to relevant antibiotics and all clinically relevant strains of bacteria. This information is not available through government sources. New funding also would enhance the Center for Devices and Radiological Health's ability to support the review of rapid diagnostics to detect resistant microorganisms.

- **Significantly increase NIAID's critical translational and antibiotic resistance research efforts**

  IDSA and other organizations have called for a 10 percent across-the-board funding increase for NIH in 2005. Such funding is necessary to allow NIAID to move aggressively to implement the *Roadmap* initiative in the area of antibiotic R&D as well as to support research that will lead to a better understanding of mechanisms related to antibiotic resistance.

February 1999. A 12-month-old boy from rural North Dakota was admitted to a hospital with vomiting, dehydration, and inflammation of his airway. He had a temperature of slightly more than 105 degrees. Tests and X-rays revealed an infection in his right lung. Doctors transferred the boy to the intensive-care unit, inserted a chest tube, and treated him with the antibiotics vancomycin and cefuroxime. The next day the boy developed severe respiratory distress and falling blood pressure, and he died. The boy had not been hospitalized since birth and had no known medical problems. However, his 2-year-old sister had been treated for a culture-confirmed MRSA infection three weeks earlier.



New Funding Needed (continued)

- **Support Synergistic Public/Private Solutions**

A growing number of international public-private partnerships are focusing on the discovery of medicines to treat infectious diseases in the United States and globally. Initiatives like the International AIDS Vaccine Initiative (formed in 1996), the Medicines for Malaria Venture (1999), and the Global Alliance for TB Drug Development (2000) offer promising opportunities to advance product R&D in areas that have languished in the past. Public-private partnerships have adopted business models that exploit the venture capital approach to investment in new product R&D. Such initiatives receive the bulk of funding from the public and philanthropic sectors. They involve for-profit partners by seeking in-kind contributions from industry. The commitment of U.S. public dollars for these and similar initiatives would take advantage of the entrepreneurial spirit possessed by many researchers and humanitarians.

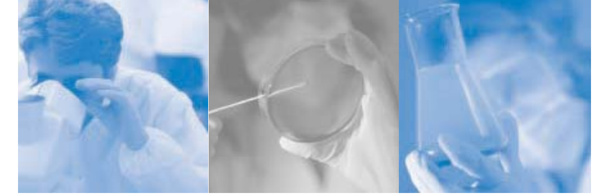
In addition to funding public-private partnerships, policymakers should seriously consider ways to prompt companies to inventory their shelves for promising drug candidates that could be donated to the partnerships for development. Such candidates exist, and companies recently have shown some interest in donating them. This is not a current priority for companies, however, because the resources required would have to be diverted from other efforts.

Emerging and Re-Emerging Infections

Robust research and development programs are needed to respond successfully to existing infectious diseases as well as new threats on the horizon.

More than three dozen new infectious diseases have been identified since the 1970s that have impacted the United States and more vulnerable countries. The list includes HIV/AIDS, severe acute respiratory syndrome (SARS), Lyme disease, hepatitis C, a new form of cholera, waterborne disease due to *Cryptosporidium*, foodborne disease caused by *E. coli* 0157:H7, and a plethora of neglected diseases that primarily affect patients in the developing world.

Some of these diseases have no treatment except for supportive care. For diseases that do have effective treatments, complacency can stifle new research and allow us to be caught off guard when current treatments become less effective due to resistance. This has been the case with tuberculosis (TB). It has been 30 years since a new class of antibiotic was approved to treat TB despite the fact that it is the second most common microbial cause of death in the world. Doctors also are concerned about the rapid rate at which other bacterial infections, such as gonorrhea and syphilis, are becoming resistant to drugs. Finally, for diseases such as TB, AIDS, and malaria, which have notoriously complex and sometimes toxic treatment regimens, there is a substantial need for new drugs that are not only more effective but easier to deliver to the patient so that greater drug adherence and, ultimately, successful care and treatment will be achieved.



Conclusion

The time for talk has passed—it's time to act. The "bad bugs, no drugs" problem is growing more severe, and patients are suffering. Government-sponsored research and refinement of existing regulations, policies, and guidance can help to address the overall problem of antibiotic resistance, fill in some of the gaps in drug development, and help reduce the cost of drug discovery and development. However, industry action must remain policymakers' central focus. Incentives that encourage pharmaceutical companies to remain active in this area of discovery or stimulate additional investment by inactive pharmaceutical and biotechnology companies must be a critical part of any solution.

New drugs are desperately needed to treat serious as well as common infections (e.g., blood, heart, and urinary tract infections; pneumonia; childhood middle-ear infections; boils; food poisoning; gonorrhea; sore throat, etc.). The bacteria that cause these infections are becoming increasingly resistant to the antibiotics that for years have been considered standard of care, and the list of resistant pathogens keeps growing. It is not possible to predict when an epidemic of drug-resistant bacteria will occur—but we do know it will happen.

Congress and the Administration have a window of opportunity to act now—*before a catastrophe occurs*—to spur both R&D of antibiotics to treat dangerous and drug-resistant infections and to promote a better understanding of antibiotic resistance and its implications for both public health and national and global security. Time is running out. Even if all of the incentives outlined in this paper were implemented today, it likely would take 10 or more years for companies to move safe and effective new drugs to market.

Federal officials have worked tirelessly over the past few years to help improve U.S. defenses against, and treatments for, bioterrorism agents. Although this work is needed and appropriate, it also is necessary to keep risks in perspective. Drug-resistant bacterial infections kill tens of thousands of Americans every year and a growing number of individuals are succumbing to community-acquired infections. An epidemic may harm millions. Unless Congress and the Administration move with urgency to address these infections now, there is a very good chance that U.S. patients will suffer greatly in the future.

January 1998. A 16-month-old girl from rural North Dakota was taken to a local hospital with a temperature of over 105 degrees. She was suffering from seizures and was in shock. Doctors treated her with the antibiotic ceftriaxone, but the girl died within two hours of heart and lung failure. An autopsy and tests revealed that MRSA had spread to her brain, heart, liver, and kidneys. One month earlier, the patient had been treated with amoxicillin for otitis media (an ear infection). Neither the girl nor her family members had been hospitalized during the previous year.

Drug-resistant infections are more difficult to treat.

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STATEMENT OF THE
INFECTIOUS DISEASES SOCIETY OF AMERICA
BEFORE THE FOOD AND DRUG ADMINISTRATION
PART 15 HEARING PANEL ON
ANTIMICROBIAL RESISTANCE

APRIL 28, 2008

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The Infectious Diseases Society of America (IDSA) appreciates the opportunity to provide this statement before the U.S. Food and Drug Administration's (FDA) Part 15 panel on the critically important issue of antimicrobial resistance.

IDSA represents more than 8,000 infectious diseases physicians and scientists devoted to patient care, education, research, prevention, and public health. Our members care for patients of all ages with serious and life-threatening infections, including meningitis, pneumonia, tuberculosis, antimicrobial drug-resistant infections, and those with cancer or transplants who have life-threatening infections caused by unusual microorganisms, HIV/AIDS, and other emerging infections.

Antimicrobial resistance is a serious patient safety and burgeoning public health problem. The resistant infections about which Infectious Diseases specialists and other physicians are most concerned at this time are caused by bacteria. For this reason, our statement will primarily focus on antibacterial resistance. IDSA's statement includes a discussion of the antibacterial resistance problem and why Infectious Diseases and other physicians are concerned, the critical need for new products to detect, treat and prevent resistant infections as well as 12 priority recommendations that require FDA's immediate consideration.

Antibacterial-Resistant Infections: Why IDSA Is Concerned

Bacterial infections affect hundreds of thousands of Americans and cause tens of thousands of deaths each year, perhaps more. Resistant infections are painful, difficult to treat, and cost many billions of dollars to the U.S. health care system annually. These "bad bugs" have become a silent epidemic in communities and hospitals across the United States as well as around the world. And yet, an astoundingly diminutive amount of federal resources are being committed to address this staggering problem. In fiscal year (FY) 2006, FDA spent only $24 million on its collective antimicrobial resistance activities, the Centers for Disease Control and Prevention (CDC) spent only $17.2 million, and the National Institutes of Health (NIH) spent only $220 million ($194.5 million of which was spent at the National Institutes of Allergy and Infectious Diseases (NIAID)—4.4% of NIAID's total budget. $90 million of NIAID's antimicrobial resistance research budget was dedicated to antibacterial resistance research—2.1% of NIAID's total budget).

The bacteria of greatest concern include multi-drug-resistant *Staphylococcus aureus* (MRSA), resistant *Escherichia coli (E. coli), Acinetobacter baumannii* (which is

threatening soldiers returning from Iraq and Afghanistan), resistant *Klebsiella species* (which appear to have originated in or near Brooklyn and now are spreading across the East Coast and into the Midwest), extensively drug-resistant tuberculosis (XDR-TB), *Clostridium difficile, Enterococcus faecium, Enterobacter species,* and resistant *Pseudomonas aeruginosa.* There are many others.

Although primarily affecting ill people in hospitals, a growing number of the victims of drug-resistant bacteria, such as MRSA, are people in the community and outside hospitals, including healthy athletes and children. A recent study in the Journal of the American Medical Association (October 17, 2007) demonstrates that more than 94,000 people are infected and nearly 19,000 die annually from MRSA alone around the country – more deaths than those caused by emphysema, HIV/AIDS, Parkinson's disease and homicide. New national surveillance data from the CDC demonstrate that an incredible 80% of *E. faecium* associated with device-related healthcare-associated infections (HAIs) were resistant to vancomycin. More broadly, these new data demonstrate high rates of antimicrobial resistance in the gram-negative pathogens associated with device-related HAIs. Disturbingly, analysis of national data indicated that twenty-six to thirty-seven percent of *A. baumannii* were carbapenem-resistant, as was ten percent of *K. pneumoniae.* Thirty percent of *P. aeruginosa* were resistant to fluoroquinolones.

Antibacterial Drug Portfolio: Why IDSA Is Concerned

Of serious concern, Infectious Diseases physicians are alarmed by the prospect that effective antibacterial drugs may not be available to treat seriously ill patients in the near future. Since their discovery, antibacterial drugs have dramatically reduced the morbidity and mortality associated with bacterial diseases, saving millions of lives and easing patients' suffering. However, over time, certain classes of drugs are losing their effectiveness to increasingly resistant infections. Until recently, the pharmaceutical industry's research and development (R&D) efforts counteracted this phenomenon by developing new drugs in time to treat bacteria that became resistant to older antibacterial drugs. However, that is no longer the case. Unfortunately, both the public and private sectors appear to have been lulled into a false sense of security based on past successes.

The potential crisis at hand is the result of a marked decrease in industry R&D, government inaction, and the increasing prevalence of resistant bacteria. It takes 8 years to develop a new antibacterial drug. Thus, today we should be planning for our pharmaceutical needs of 2012-2015. Given the state of the current pipeline, our future looks bleak. To call attention to the resistance problem and the diminishing pipeline, in 2004, IDSA launched its "Bad Bugs, No Drugs" advocacy campaign by issuing a landmark report and several subsequent articles to highlight the brewing patient safety and public health crisis. (www.idsociety.org/badbugsnodrugs).

FDA has acknowledged there is problem in the antibacterial drug pipeline. In the agency's March 2004 Critical Path report "Innovation/Stagnation: Challenge and Opportunity on the Critical Path to New Medical Products", the agency reported that "product development in areas crucial to public health goals, such as antibiotics, has

slowed significantly during the past decade." However, since 2004, the agency has dedicated insufficient time and resources to solve the resistance or pipeline problems. In fact, if anything, the Agency's recent actions have produced greater cause for worry as growing uncertainty about FDA's anti-infective drug review process mounts.

It is frustrating to see patients with life-threatening infections and the toll it takes on their lives and on their families and friends, or to see children and adults die when we have the resources and the tools to reduce the impact of these diseases. We cannot stop the development of antibacterial resistance – bacteria will continue to mutate in response to antibacterial drug use. However, we can slow the pace of the rise in antibacterial resistance, if we have the will to act.

The Path Forward

Antibacterial resistance and the diminishing antibacterial pipeline are complex and multi-dimensional problems. Multi-pronged solutions are required to sufficiently limit the impact of antibacterial resistance on patients and the public and to spur the development of products to address antibacterial resistant infections. Such efforts include:

- removing disincentives in the antibacterial drug review process by reestablishing consistency, predictability and timeliness;
- providing economic and other incentives to spur the development of new products in this area (e.g., antibacterial drugs, relevant diagnostic tests, and vaccines);
- significantly strengthening federal research in new, relevant rapid diagnostics as a means to reduce the number of patients needed for antibacterial drug clinical trials;
- creating an antimicrobial resistance strategic research plan that establishes priorities and significantly strengthens collaborations between FDA, NIH, CDC, the Departments of Agriculture, Veteran Affairs, Defense and the Environmental Protection Administration.
- educating physicians, patients, and parents about the appropriate use of antibacterial drugs;
- developing and applying infection control and immunization policies and practices to prevent transmission;
- improving our collection of data regarding clinical, veterinary and human antibacterial use, and other data;
- protecting antibiotics of importance to human health from being used in agriculture;
- improving surveillance efforts to detect and monitor the emergence of resistance; and
- developing safer alternatives to antibacterial drug uses in agriculture.

Implementing many of these solutions will require significant attention, resources, and, in some cases, substantial political will to overcome special interests in favor of public health and patient safety.

In 2001, the Administration recognized the gravity of the problem and developed a federal "Action Plan to Combat Antimicrobial Resistance," an effort led by CDC, FDA, and NIH. Unfortunately, very limited progress has been made toward implementing the Action Plan's 13 Top Priority Action Items, let alone the remainder of the 84 action items. Moreover, in 2000, FDA's own Task Force on Antimicrobial Resistance issued a report (http://www.fda.gov/oc/antimicrobial/taskforce2000.html) containing nine (9) recommendations which, if implemented, could have helped significantly to address the antimicrobial resistance problem. Unfortunately, the Agency has made little progress toward implementing these recommendations since 2000.

Below are twelve additional recommendations that IDSA believes will go far toward addressing the antimicrobial resistance and pipeline problems. Within these recommendations are several critical steps that FDA can take immediately.

The United States Government, including FDA, can no longer take a business-as-usual approach toward addressing the antimicrobial resistance problem. Following today's Part 15 hearing, it is imperative that the agency dedicate sufficient resources to implement the Action Plan's recommendations, FDA's own recommendations from 2000 as well as the additional recommendations that IDSA outlines below.

IDSA's Twelve Recommendations For FDA's Immediate Consideration

[The following recommendations are not listed by priority ranking. They are listed chronologically as they will be presented by IDSA's presenters.]

Recommendation # 1 ***FDA Should Work Aggressively To Re-Establish Consistency, Predictability, and Timeliness In The Antibacterial Drug Review Process.***

Significant uncertainty exists within the agency's antibacterial human drug review process, which we fear is shaking the foundation of the nation's antibacterial pharmaceutical industry. The agency must move quickly to reestablish consistency, predictability, and timeliness to this process which IDSA believes requires the attention and leadership of the director of the Center for Drug Evaluation and Review as well as an infusion of additional staff and resources. IDSA has heard from many pharmaceutical industry representatives that FDA has lost credibility by throwing out existing policies without having new policies available to replace the old, rejecting prior agreements with companies, and employing endless delays including in the issuance of clinical trial guidances. Time delays are costing millions and threatening bankruptcy for some companies. FDA must move rapidly to restore trust by making public its plan to resolve internal disincentives to antibacterial drug discovery and development. The plan should include the agency's priorities for action.

Recommendation # 2 ***FDA Should Strengthen Critical Path And Other Research, Establish Research Priorities With NIH, CDC, USDA, and Other Agencies And Collaborate More Often With These Agencies On Antibacterial Resistance and Drug Pipeline-Supportive Research.***

In 2000, FDA's Task Force on Antimicrobial Resistance reported "In hearing about the efforts of multiple centers, it also became clear that coordination of antimicrobial resistance research, both within FDA and with sister government agencies and academia, is currently largely informal." In IDSA's estimation, little has changed to formalize the U.S. antimicrobial resistance research agenda and to establish and publicize U.S. priorities. For this reason, in June 2007 IDSA proposed the creation of an antimicrobial resistance strategic research plan to strengthen existing epidemiological, interventional, clinical, translational and basic research efforts in a letter to Drs. Anthony Fauci, NIAID Director, and Julie Gerberding, CDC Director, (http://www.idsociety.org/WorkArea/showcontent.aspx?id=4642). FDA has a critical role to play in that effort, and we urge FDA, NIH, CDC, the U.S. Department of Agriculture (USDA), and other relevant agencies to move immediately to establish a Blue Ribbon Panel to establish U.S. research priorities and create a strategic research plan.

Even without such a strategic plan, FDA should act now to support research that can help lead to a reduction in the number of patients necessary for antibacterial clinical trials. Such research should include validation of endpoints such as quality of life, length of stay, duration of fever, time to elimination of pathogen, patient-reported outcomes, etc. as well as the development of new, rapid diagnostics. The agency should seek and strengthen collaborations with NIH, CDC, USDA and other agencies on studies that will address resistance and support the antibacterial drug pipeline. Finally, FDA should publish a summary of the antimicrobial resistance/pipeline research efforts it currently has underway under its Critical Path and other initiatives.

Recommendation # 3 ***FDA Should Take Immediate Action To Update Antibacterial Clinical Susceptibility Concentrations.***

Physicians need accurate information on antibacterial clinical susceptibility concentrations ("breakpoints") to use antibacterial drugs wisely. Patients' safety and lives are on the line. Given a new requirement included in the FDA Amendments Act, it is incumbent upon the agency to establish workable processes for expeditiously updating antibacterial breakpoints as well as to publish its methodology for setting breakpoints. FDA staff's recent inventory of antibacterial labels has uncovered that out of the more than 100 currently approved antibacterial labels more than 70 contain breakpoints that are out-of-date.

Prior to 2006, FDA's Center for Devices and Radiological Health (CDRH) allowed susceptibility test device manufacturers to include both FDA-established breakpoints listed in package inserts at the time of the drug's approval as well as the more up-to-date breakpoint recommended by the Clinical Laboratory Standards Institute (CLSI) (formerly NCCLS). CLSI, a 501(c)(3) non profit organization, is a recognized standard setting body comprised of experts in infectious diseases and clinical microbiology. CLSI is not affiliated with IDSA. In 2006, FDA began to require that CLSI submit citizen petitions to the agency when recommending updated breakpoints. In IDSA's estimation, the new

process has been a failure as FDA has taken limited definitive action on the five CLSI petitions submitted to date.

The agency should explore several options available to it for updating breakpoints including:

- establishing a less-cumbersome process for reviewing and accepting CLSI's recommendations;
- creating FDA's own scientific board of external experts comprised of infectious diseases clinicians, microbiologists, pharmacologists, and others who understand the clinical impact of changes in the interpretations of the MIC. The board could help the agency update breakpoints and/or review and approve breakpoints recommended by CLSI or other appropriate entities;
- contracting out some of the activities needed to update breakpoints through organizations such as CLSI. Infusing some of the agency's patient safety funding into CLSI's effort could help to more quickly address out-of-date breakpoints.

Specifically, IDSA urges FDA to:

- review and update antibacterial drug breakpoints on a regular basis as clinical need dictates, but no less frequently than every 5 years
- return to purchasing from private vendors the critically needed susceptibility data used to update breakpoints;
- require pharmaceutical sponsors (both pioneer and generic) to provide any such additional data as may be needed to update breakpoints quickly; and
- immediately publish its methodology for setting breakpoints, which previously has not been made public. IDSA members have heard statements from FDA officials that such methods are "not written down anywhere". This is unacceptable and only helps to demonstrate the lack of attention and sufficient resources the agency has devoted to this critical patient safety responsibility over the last several decades.

Recommendation # 4 ***FDA, Working With Other Agencies Represented On The Interagency Task Force On Antimicrobial Resistance, Should Establish A Public Health Antimicrobial Advisory Board Of Outside Experts To Advise The Task Force In Its Efforts.***

Antimicrobial resistance is a complex, multi-dimensional problem. Addressing the problem will require expertise from the infectious diseases, medical (including hospital and community-based physicians), veterinary, public health, research, pharmacoeconomic, and international health communities. The private sector provides incredible expertise in each of these fields which would be extremely valuable to the Interagency Task Force on Antimicrobial Resistance in setting and maintaining priorities and in carryout out its responsibilities. For this reason, IDSA strongly recommends that FDA, NIH and CDC create an advisory board to advise the existing Interagency Task Force on Antimicrobial Resistance. Such an advisory board should meet with Task Force

members on a regular (biannual) basis. For a list of the advisory board's potential duties and responsibilities see H.R. 3697/S. 2313, the "Strategies to Address Antimicrobial Resistance Act".

<u>Recommendation # 5</u> ***FDA Should Reevaluate And Strengthen Regulatory Requirements On Data Collection Of Antibacterial Use In Humans.***

FDA should reevaluate (including by holding a public workshop) the manner by which it collects antibacterial drug human use data from pharmaceutical companies to determine if it may be collected in a manner and format that is reliable and comparable and which best ensures it is of maximum value to the study of antibacterial resistance development (e.g., using defined daily doses, collected by calendar year). In addition, the agency should seek an agreement with IMS Health or another private vendor to obtain antibacterial drug human use data in a manner whereby such data may be shared with the Interagency Task Force on Antimicrobial Resistance as well as with members of the advisory board proposed in Recommendation #4. Of course, members of the Task Force and advisory board will need to sign confidentiality agreements.

<u>Recommendation # 6</u> ***FDA's Center For Veterinary Medicine Should Move Quickly To Issue Its Long-Delayed Draft Regulation And Guidance #146 On Collection Of Data On Antibacterial Use In Animals.***

FDA's Center for Veterinary Medicine (CVM) should move forward immediately to issue for public comment its draft Guidance #146 and draft regulation developed in 2001 and 2002, which were intended to redefine the type of antibacterial distribution data that animal drug manufacturers must provide to the agency on antibacterial drug use in animals. The draft regulation and guidance have been held up within the agency since 2002 for unknown reasons. It is extremely important that animal antibacterial drug use data be collected in a manner and format that is reliable and comparable and which best ensures it is of maximum value to the study of antibacterial resistance development (e.g., collection using "defined daily doses," by species and indication, and based on a calendar year model as opposed to the anniversary date of the product's approval). The collection of such use data will make information currently collected under the National Antimicrobial Resistance Monitoring System of greater relevance as it can be used to show where there may be correlations between antibacterial use and the development of resistance. This likely will be one of those instances where the Administration will need to muster substantial political will to overcome special interests in favor of public health and patient safety.

<u>Recommendation # 7</u> ***FDA/CVM Should Update Guidance #152 To Include Missing Criteria Concerning The Relative Importance Of Antibacterials In Human Medicine.***

FDA's CVM should hold a public workshop to bring together experts in infectious diseases human and veterinary medicine as well as other key stakeholders to reassess the criteria currently contained in CVM's Guidance #152 with regard to their potential

impact on resistance patterns in human drugs. Guidance #152 is the framework document used for the approval of antibacterial drugs for use in animals. In particular, the agency should reconsider the criteria used to categorize antibacterial drugs as "critically important" and "highly important" to human health and whether the scope of such criteria should be broadened beyond enteric pathogens. Additionally, the agency immediately should change the classification of cefepime to "critically important" to be consistent with the World Health Organization's classification of this drug. Cefepime is the only 4th generation cephalosporin in use in the United States in humans, and IDSA and other expert organizations have emphasized the threat posed both to cefepime and to 3rd generation cephalosporins by the prospect of 4th generation cephalosporin use in livestock.

Recommendation # 8 ***FDA Should Move Forward Immediately To Implement Its Own Recommendations On Antimicrobial Resistance.***

In December 2000, FDA's Task Force on Antimicrobial Resistance issued a report (http://www.fda.gov/oc/antimicrobial/taskforce2000.html) containing priority recommendations the agency was to implement to address the antimicrobial resistance and pipeline problems. Few of these recommendations have been implemented to date. Such inaction can only be interpreted as demonstrating the Agency's past lack of commitment to a brewing crisis that Infectious Diseases physicians believe should be a significant priority of the U.S. Government. IDSA hopes that along with the Agency's desire to hold today's Part 15 hearing, we are witnessing a turning point in the Agency's thinking and a demonstration of it's desire to tackle these critical patient safety and public health problems. To confirm this, the Agency should act immediately to dedicate the necessary resources necessary to implement its own Task Force's recommendations as well as the additional recommendations that IDSA is proposing today.

Recommendation # 9 ***FDA Should Require That Antibacterial Impact Statements And Management Plans Intended To Predict And Limit Resistance Development Be Included In Human Drug Applications.***

FDA should require antibacterial drug sponsors (both pioneer and generic) to submit as part of their drug applications a resistance impact statement that attempts to predict how approval and use of such antibacterial drug may impact upon the development of resistance. Pioneer and generic sponsors of human drugs also should be required to submit antibacterial use management plans intended to be used to limit the development of resistance associated with the drug's use. Such impact statements and management plans should be made public so that researchers may use each to study and strengthen our understanding of the science of predicting resistance development as well as how to prevent and control its development. Neither the impact statement nor management plan should be used for enforcement purposes.

<u>Recommendation #10</u> ***FDA Should Commission A Study Through The Tufts Center On The Study Of Drug Development (Or Another Appropriate Entity) To Obtain Expert Recommendations As To Which Incentives Are Necessary To Strengthen The Antibacterial Drug, And Relevant Diagnostics And Vaccine Pipelines.***

New products (antibacterial drugs, vaccines, diagnostic tests) are critically needed to treat and prevent serious and life-threatening antibacterial resistant infections as well as to rapidly detect the organism causing that infection. New rapid diagnostic tests will be particularly useful in helping to reduce the numbers of patients needed to demonstrate the safety and effectiveness of antibacterial drugs in clinical trials.

Tax credits for R&D, extensions on periods of market exclusivity, strengthening of intellectual property rights, priority FDA review vouchers, grants, prizes and other incentives have all been offered as potential incentives. IDSA does not have all of the answers as to which combination of incentives will ultimately be successful.

Within the federal Interagency Task Force's Action Plan to Combat Antimicrobial Resistance issued to 2001, FDA was designated to take the lead on implementing two of the thirteen "Top Priority Action Items." These include:

"Top Priority Action Item *— Create an Interagency AR Product Development Working Group to identify and publicize priority public health needs in human and animal medicine for new AR products (e.g., innovative drugs, targeted spectrum antibiotics, point-of-care diagnostics, vaccines and other biologics, anti-infective medical devices, and disinfectants)."*

"Top Priority Action Item *— Identify ways (e.g., financial and/or other incentives or investments) to promote the development and/or appropriate use of priority AR products, such as novel compounds and approaches, for human and veterinary medicine for which market incentives are inadequate."*

To date, FDA has done nothing to address either Top Priority Action Item.

Arguably, neither FDA nor any federal agency is well-placed to examine and make recommendations about the type of incentives that will promote antibacterial drug development. However, the Tufts Center for the Study of Drug Development commonly studies pharmaceutical pipelines and the pharmacoeconomic and non-economic factors that spur drug development. For this reason, IDSA strongly urges FDA to commission a study through the Tufts Center (or some other similar entity, if one exists) seeking a report on strengths and weaknesses in the antibacterial and related diagnostics and vaccine R&D pipelines with a particular emphasis on products needed to treat, detect, and prevent serious and life-threatening infections. The study also should provide recommendations as to what combination of incentives, considering each phase of product development, will work to spur greater R&D of such products among the biotechnology, pharmaceutical, vaccine, and diagnostics industries as well as within academic settings.

Recommendation #11 ***FDA Should Immediately Clarify Its Policies Under The Orphan Drug Act Relating To Serious and Life-Threatening Infectious Diseases Associated With Antibacterial Resistant Organism.***

FDA should immediately clarify how it calculates prevalence of cases under the Orphan Drug Act related to serious and life-threatening infectious diseases associated with antibacterial resistant organisms. Agency officials have provided contradictory messages on this point, which has had a negative impact on IDSA's ability to advocate for the adoption of new statutory incentives to spur R&D on products that will protect patient safety and public health.

Recommendation #12 ***FDA Should Effectively Communicate Its Professional Judgment As To The Funding It Needs To Sufficiently Address The Antimicrobial Resistance Problem.***

The Department of Health and Human Services (HHS), including FDA, NIH, and CDC need to effectively communicate the resources the department and agencies need to address antimicrobial resistance. Last year, two members of Congress [Reps. Jim Matheson (D-UT) and James McGovern (D-MA)] asked Secretary Leavitt during an HHS' budget hearing for FDA's, NIH's, and CDC's professional judgments concerning the amount of funding each agency needs to implement their respective responsibilities under the federal Action Plan to Combat Antimicrobial Resistance. It is unclear whether FDA ever submitted its professional judgment request to HHS. However, it is clear that HHS did not submit any professional judgments to the Congressmen.

TECHNICAL REPORT

The bacterial challenge: time to react

A call to narrow the gap between multidrug-resistant bacteria in the EU and the development of new antibacterial agents

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ECDC/EMEA JOINT TECHNICAL REPORT

The bacterial challenge: time to react

A call to narrow the gap between multidrug-resistant bacteria in the EU and the development of new antibacterial agents

ECDC/EMEA Joint Working Group

The Mandate (full document in Annex A):
'The ECDC/EMEA Joint Working Group is agreed by the ECDC and the EMEA to oversee and to facilitate, follow-up and be part of the work aimed at producing a report on the gap between the increasing prevalence of multidrug resistant bacteria and antibacterial drug development aimed at treating such infections.'

- The Working Group was given this task on 28 February 2008.
- The Working Group included two members nominated by the Committee for Medicinal Products for Human Use (CHMP) and Paediatric Committee (PDCO) at the European Medicines Agency (EMEA), respectively; two members from the Advisory Forum at the European Centre for Disease Prevention and Control (ECDC), and a member of staff from the EMEA and the ECDC, respectively. In addition, two co-opted independent experts, including one member from 'Action on Antibiotic Resistance' (ReAct), were selected for their clinical/microbiological expertise in the fields of interest.
- The EMEA/CHMP adopted the Technical Report at its meeting on 23 July 2009 and again circulated for information on 20 August 2009. The draft report was presented to the ECDC Advisory Forum on 13 May 2009 and input from the Advisory Forum on the final report was sought through a written procedure on 20 August 2009.

Contributors to the report

Members and co-opted members of the Joint Working Group:

- **Ragnar Norrby** chaired the Joint Working Group. He was Director-General of the Swedish Institute of Infectious Disease Control from January 2001 to May 2009. He was appointed by the Advisory Forum at ECDC.
- **Mair Powell** was the vice-chair of the Joint Working Group. She is a Senior Clinical Assessor at the Medicines and Healthcare products Regulatory Agency in the UK. She was appointed by EMEA/CHMP.
- **Bo Aronsson** is a Principal Scientific Administrator at EMEA. He was responsible for the 'Pipeline analysis' part of this report.
- **Dominique L. Monnet** is Senior Expert and Coordinator of the Programme on Antimicrobial Resistance and Healthcare-Associated Iinfections at ECDC. He was responsible for the 'Trends and burden' part of this report.
- **Irja Lutsar** is Professor of Medical Microbiology at the University of Tartu, Estonia. She was appointed by EMEA/PDCO.
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Observers: European Commission's DG Enterprise, DG Research and DG SANCO C3; European Society for Clinical Microbiology and Infectious Diseases (ESCMID)

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Acknowledgements: Additional participants in the pipeline study: Anthony So and Chris Manz, the Strategic Policy Unit of ReAct at Duke University, North Carolina US, contributed to the search of the Pharmaprojects database. Murat Akova, Gunnar Kahlmeter, Hartmut Lode, Johan W. Mouton and Carl Erik Nord contributed to the assessment of agents in the pipeline.

Financial support: Financial support was provided by ECDC, EMEA and ReAct.

Declarations of interest: Declarations of interest for members of the working group and external reviewers are available from EMEA upon request.

In this report, the terms 'antibiotics' and 'antibacterial agents' have been used interchangeably.

EMEA doc. ref. EMEA/576176/2009

Stockholm, September 2009

ISBN 978-92-9193-193-4
doi 10.2900/2518

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Abbreviations and acronyms

AMR	Antimicrobial resistance
CHMP	Committee for Medicinal Products for Human Use at EMEA
DG ENTR	Directorate-General for Enterprise and Industry
DG RTD	Research Directorate-General
DG SANCO	Directorate-General for Health and Consumers
EC	European Commission
ECDC	European Centre for Disease Prevention and Control
EMEA	European Medicines Agency
ENB	*Enterobacteriaceae*
EphMRA	European Pharmaceutical Market Research Association
ESBL	Extended-spectrum beta-lactamase
ESCMID	European Society for Clinical Microbiology and Infectious Diseases
EU	European Union
GNB	Gram-negative bacilli
IDSA	Infectious Diseases Society of America
IV	Intravenously
MESH	Medical Subject Headings
MRSA	Methicillin-resistant *Staphylococcus aureus*
PBP	Penicillin-binding protein
PDCO	Paediatric Committee at EMEA
PO	Per os, by mouth
PRSP	Penicillin-resistant *Streptococcus pneumoniae*
ReAct	Action on Antibiotic Resistance
VISA	Vancomycin-intermediate *Staphylococcus aureus*
VRSA	Vancomycin-resistant *Staphylococcus aureus*
WHO	World Health Organization

Foreword

The introduction of antibacterial agents (commonly referred to as antibiotics) led to a revolution in the management of bacterial infections. Today, emerging and increasing resistance to antibiotics has become a threat to public health in Europe and globally. Only 70 years after their introduction, we are now facing the possibility of a future without effective antibiotics for several types of bacteria that cause infections in humans.

In 2001, the European Commission presented its 'Community Strategy against Antimicrobial Resistance'. It proposed 15 actions in the areas of surveillance, prevention, international cooperation and research and development of new antibacterial agents. Later in the same year, European Union (EU) Health Ministers adopted a Council Recommendation on the prudent use of antimicrobial agents in human medicine[1].

While surveillance of resistance, infection control measures and strategies to prevent the occurrence of infections are central to combating antibacterial resistance trends, patients still get infected and there is a particular lack of antibacterial agents to treat infections caused by bacteria that are resistant to many of the available treatments (i.e. multidrug-resistant bacteria).

In 2004, a report from the World Health Organization on 'Priority Medicines for Europe and the World'[2] identified infections caused by resistant bacteria as the number one disease requiring priority medicines based on the potential public health impact if effective new antibiotics were not developed. The report suggested that Europe should play a global leadership role in this area.

In 2007, the European Centre for Disease Prevention and Control (ECDC), the European Medicines Agency (EMEA) and the international network Action on Antibiotic Resistance (ReAct) entered into a discussion on the need to produce a report that reviewed and documented the gap between infections caused by multidrug-resistant bacteria in the EU and the development of new antibiotics to treat them. An ECDC/EMEA Joint Working Group was established in 2008 to prepare this report.

The objective of this report is to give an account of facts and figures that would allow reasonable predictions of the gap between bacterial resistance in the EU and the likely availability of new treatments that would be effective against multidrug-resistant bacteria in the near future. As such, this technical report is made available to the European Commission, and particularly to DG SANCO, DG ENTR and DG RTD, for consideration. The report will also serve as a basis for discussions at the expert conference on 'Innovative Incentives for Effective Antibacterials' scheduled for 17 September 2009, as part of the Swedish EU Presidency.

We note with satisfaction the timely availability of the final report endorsed by the main scientific Committees in the two agencies and would like to thank the working group for its achievement.

Zsuzsanna Jakab, ECDC Director — Thomas Lönngren, EMEA Executive Director

[1] Council Recommendation of 15 November 2001 on the prudent use of antimicrobial agents in human medicine (2002/77/EC). Available from: http://antibiotic.ecdc.europa.eu/PDFs/l_03420020205en00130016.pdf

[2] http://whqlibdoc.who.int/HQ/2004/WHO_EDM_PAR_2004.7.pdf

Executive summary

Main findings:

There is a gap between the burden of infections due to multidrug-resistant bacteria and the development of new antibiotics to tackle the problem.

- Resistance to antibiotics is high among Gram-positive and Gram-negative bacteria that cause serious infections in humans and reaches 25% or more in several EU Member States.
- Resistance is increasing in the EU among certain Gram-negative bacteria such as recently observed for *Escherichia coli*.
- Each year, about 25 000 patients die in the EU from an infection with the selected multidrug-resistant bacteria.
- Infections due to these selected multidrug-resistant bacteria in the EU result in extra healthcare costs and productivity losses of at least EUR 1.5 billion each year.
- Fifteen systemically administered antibacterial agents with a new mechanism of action or directed against a new bacterial target were identified as being under development with a potential to meet the challenge of multidrug resistance. Most of these were in early phases of development and were primarily developed against bacteria for which treatment options are already available.
- There is a particular lack of new agents with new targets or mechanisms of action against multidrug-resistant Gram-negative bacteria. Two such agents with new or possibly new targets and documented activity were identified, both in early phases of development.
- A European and global strategy to address this gap is urgently needed.

In 2007, the European Centre for Disease Prevention and Control (ECDC), the European Medicines Agency (EMEA) and the international network Action on Antibiotic Resistance (ReAct) entered into a discussion on the need to document the gap between the frequency of multidrug-resistant bacterial infections in the EU and the development of new antibiotics. As a result, an ECDC/EMEA Joint Working Group was established in 2008 to give an account of facts and figures that would allow reasonable predictions of the extent of the gap in the coming years.

The following antibiotic-resistant bacteria were selected because they frequently are responsible for bloodstream infections and because the associated antibiotic resistance trait is, in most cases, a marker for multiple resistance to antibiotics:

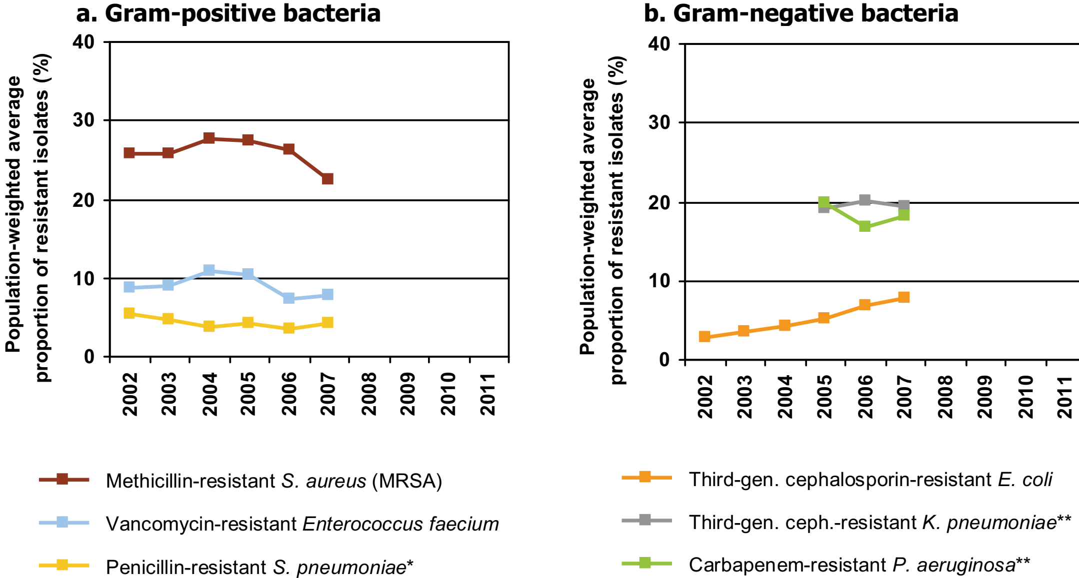
- *Staphylococcus aureus,* methicillin resistance (MRSA);
- *S. aureus*, vancomycin intermediate resistance and vancomycin resistance (VISA/VRSA);
- *Enterococcus* spp. (e.g. *Enterococcus faecium*), vancomycin resistance (VRE);
- *Streptococcus pneumoniae*, penicillin resistance (PRSP);
- *Enterobacteriaceae* (e.g. *Escherichia coli*, *Klebsiella pneumoniae*), third-generation cephalosporin resistance;
- *Enterobacteriaceae* (e.g. *K. pneumoniae*), carbapenem resistance; and
- Non-fermentative Gram-negative bacteria (e.g. *Pseudomonas aeruginosa*), carbapenem resistance.

Trends and burden of infections due to multidrug-resistant bacteria in the EU

Data on these selected antibiotic-resistant bacteria in invasive infections (mainly bloodstream infections) were available from the European Antimicrobial Resistance Surveillance System (EARSS) for EU Member States, Iceland and Norway for each year during the period 2002–2007.

The trends in the proportion of antibiotic-resistant isolates among blood isolates of the selected bacteria frequently responsible for bloodstream infections in Europe are shown in Figure E1.

Figure E1. Population-weighted, average proportion of resistant isolates among blood isolates of bacteria frequently responsible for bloodstream infections, EU Member States, Iceland and Norway, 2002–2007.



* S. pneumoniae*: excluding Greece, which did not report data on this bacterium to EARSS.*

** K. pneumoniae *and* P. aeruginosa*: excluding Belgium and Slovakia, which did not report data on these bacteria to EARSS.*

In 2007, the average proportion of *Staphylococcus aureus* blood isolates that showed resistance to methicillin (% MRSA) was the highest proportion of antibiotic-resistant isolates among the selected bacteria frequently responsible for bloodstream infections in the European Union. However, this proportion has been decreasing in recent years (Figure E1). This is due to decreasing MRSA trends in several Member States, likely due to action plans at national level as documented for France, Slovenia and United Kingdom. The average proportion of MRSA has reached a level close to that of the selected antibiotic-resistant Gram-negative bacteria.

The proportion of *S. aureus* blood isolates that showed intermediate resistance to vancomycin (VISA) was very low (less than 0.1%) in EU Member States, Iceland and Norway. No vancomycin-resistant *S. aureus* isolates were reported to EARSS in 2007 (data not presented on Figure E1).

In contrast, the average proportion of *Escherichia coli* – the most common Gram-negative bacteria responsible for infections in humans – blood isolates showing resistance to third-generation cephalosporins has been rising steadily.

At the same time, there is no sign of decreasing resistance to third-generation cephalosporins in *Klebsiella pneumoniae* or to carbapenems in *Pseudomonas aeruginosa* (Figure E1).

In 2007, the proportion of *K. pneumoniae* blood isolates from EU Member States, Iceland and Norway that showed resistance to carbapenems was, in general, very low (median=0%) with the exception of Greece, where it reached 42% (data not presented on Figure E1).

The human and economic burden of antibiotic-resistant bacteria could only be estimated for the following five antibiotic-resistant bacteria: MRSA, vancomycin-resistant *Enterococcus faecium*, third-generation cephalosporin-resistant *E. coli* and *K. pneumoniae* and carbapenem-resistant *P. aeruginosa*.

The study confirmed that MRSA was the most common, single, multidrug-resistant bacterium in the European Union. However, the sum of cases of common, antibiotic-resistant Gram-positive bacteria (mostly MRSA and vancomycin-resistant *Enterococcus faecium*) was comparable to that of common, antibiotic-resistant Gram-negative bacteria (third-generation cephalosporin-resistant *E. coli* and *K. pneumoniae*, and carbapenem-resistant *P. aeruginosa*).

Overall, it was estimated that in 2007 approximately 25 000 patients died from an infection due to any of the selected five antibiotic-resistant bacteria in the European Union, Iceland and Norway. In addition, infections due to any of the selected antibiotic-resistant bacteria resulted in approximately 2.5 million extra hospital days and extra in-hospital costs of more than EUR 900 million.

Subsequently, an estimate was made of loss of productivity due to these infections. Based on 2007 data, outpatient care costs were estimated at about EUR 10 million and productivity losses due to absence from work of infected patients were estimated at more than EUR 150 million, each year. Productivity losses due to patients who died from their infection were estimated at about EUR 450 million each year. Overall, societal costs of infections due to the selected antibiotic-resistant bacteria were estimated at about EUR 1.5 billion each year.

There are many reasons (e.g. limited range of included bacteria, outpatient infections not being considered, average cost of hospital care which does not take into account special patient care such as intensive care) to support a conclusion that these figures correspond to an underestimate of the human and economic burden of infections due to antibiotic-resistant bacteria.

Research and development pipeline of antibacterial agents

In order to assess the state of the antibacterial drug development pipeline, two commercial databases (Adis Insight R&D and Pharmaprojects) were queried for antibacterial agents in clinical development worldwide. It was decided not to perform an in-depth exploration of agents that had not yet reached clinical trials due to the high attrition rate during preclinical testing and the scarcity of data available for review.

Whenever possible, agents identified by the search were assessed for their antibacterial activity against the selected bacteria based on actual data available in the databases or in the literature. In the absence of actual *in vitro* data, reviewers also took into account reasonable assumptions of the activity of some agents based on the properties of similar agents (i.e. of the same class or with a common mechanism of action) in order to construct a 'best-case scenario'.

Additionally, for each agent, reviewers were requested to indicate whether it was of a new class or belonged to an existing class of antibiotics and to indicate whether it:

- acted on the same target and in the same way as that of at least one previously licensed antibacterial agent;
- acted through a known mechanism of action on a new target; or
- acted through a new mechanism of action.

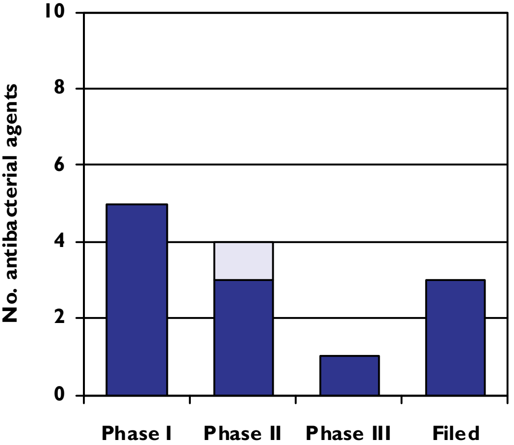
The main results from this analysis were as follows:

- Of 167 agents identified by the searches, there were 90 antibacterial agents with *in vitro* activity in a best-case scenario (based on actual data or assumed based on class properties of mechanism of action) against at least one organism in the panel of bacteria selected for their public health importance.
- Of these 90 agents, 24 were new presentations of licensed antibacterial agents and 66 were new active substances.
- Of the 66 new active agents, only 27 were assessed as having either a new target or a new mechanism of action, thus potentially offering a benefit over existing antibiotics.
- Of these 27 agents, there were 15 that could be systemically administered.
- Of the 15 agents with systemic administration, eight were judged to have activity against at least one of the selected Gram-negative bacteria.
- Of the eight with activity against Gram-negative bacteria, four had activity based on actual data and four had assumed activity based on known class properties or mechanisms of action.
- Of the four with activity against Gram-negative bacteria based on actual data, two acted on new or possibly new targets and none via new mechanisms of action.

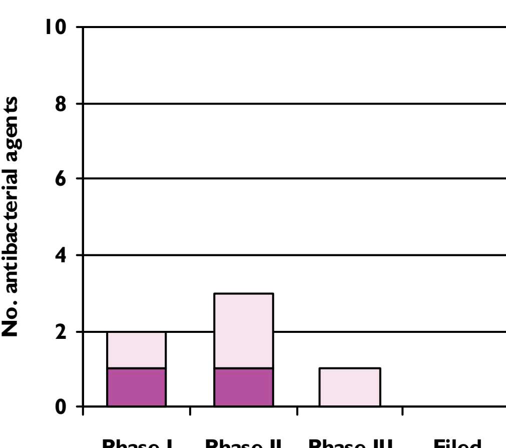
Figure E2 shows the information on these 15 antibacterial agents. Notably, only five of these agents had progressed to clinical trials to confirm clinical efficacy (Phase 3 or later of clinical development).

Figure E2. **New systemic antibacterial agents with a new target or new mechanism of action and *in vitro* activity based on actual data (dark colour bars) or assumed *in vitro* activity based on class properties or mechanisms of action (light colour bars) against the selected bacteria (best-case scenario), by phase of development (n=15).**

a. Gram-positive bacteria



b. Gram-negative bacteria*



Note: In vitro *activity based on actual data is depicted at the bottom of each column in darker colour. Assumed in vitro activity based on class properties or mechanisms of action (where applicable) is depicted in a lighter colour at the top of each column.*

** Two carbapenems have been omitted from Figure E2b since they are no more active than earlier carbapenems against Gram-negative bacteria. The relative novelty of these agents was based on a better profile of activity against antibiotic-resistant Gram-positive bacteria and are therefore included in Figure E2a.*

The burden of bacterial resistance in the EU is already substantial and is likely to increase. Based on current data, it is expected that particular problems will arise in the coming years due to resistance among Gram-negative bacteria.

At the same time, there are very few antibacterial agents with new mechanisms of action under development to meet the challenge of multidrug resistance. There is a particular lack of new agents to treat infections due to multidrug-resistant Gram-negative bacteria.

This report has identified a gap between the burden of infections due to multidrug-resistant bacteria and the development of new antibacterial agents to tackle the problem. A European and global strategy to address the gap is urgently needed. Measures that spur drug development need to be put in place.

1 Introduction

1.1 Multidrug resistant bacteria: an increasing concern

1.1.1 What is antibacterial resistance?

Antibacterial agents inhibit the growth of bacteria and may rapidly kill them by disrupting one or more of their essential cellular functions. For example, depending on the type of antibacterial agent, the mechanism of activity may result in:

- inhibition of the production of proteins or cell wall materials;
- inhibition of DNA replication;
- disruption of cell membrane activities that maintain chemical balance.

Bacteria are usually grouped according to various attributes such as the structure of their outer coverings and their metabolic functions. The primary classification of bacteria is based on their staining properties, which, for almost all types of bacteria, divides them into Gram-positive or Gram-negative groups. Those called Gram-positive have a cell membrane plus a thick layer of cell wall material (peptidoglycan) lying outside the membrane. In contrast, Gram-negative bacteria have a cell membrane, a relatively thin layer of peptidoglycan and then an outer membrane. These major structural differences result in different patterns of susceptibility to antibacterial agents because the outer coverings of the bacteria affect access to the sites where they exert their activity. Therefore, each group of bacteria is usually susceptible to the actions of only a limited range of antibacterial agents and show inherent (i.e. normal) resistance to the actions of others.

Moreover, bacteria have the ability to acquire resistance to one or more antibacterial agents to which they would normally be susceptible. Acquired resistance can arise by mutations that can occur during replication or by gaining genes encoding a mechanism of resistance from other bacteria [1]. The ease with which resistance can be acquired varies between bacterial types. Unfortunately, some of the types of bacteria that are normally not susceptible to many antibacterial agents are also easily able to acquire resistance to others. The result is multidrug resistance. In extreme cases, bacteria can show resistance to most or all of the agents that would commonly be used to treat them.

In addition, each acquired mechanism of resistance may render the bacterium resistant to many or all antibacterial agents of the same type (class) and sometimes confers resistance to agents from many classes. This is called cross-resistance. The genes encoding some mechanisms of resistance are sometimes linked in such a way that they are transferred all together between organisms. This is often referred to as co-resistance.

Each time an antibacterial agent is used to treat an infection, there is a risk that the agent will select, in the population of infecting bacteria, for bacteria that are resistant to it, thus causing unresolved infection in the patient who was treated. The agent will also select for resistant bacteria in the patient's commensal flora, thus resulting in colonisation by resistant bacteria, which may subsequently be responsible for another infection at the same or another body site. In both cases, these resistant bacteria will have the possibility to spread to other patients, especially within hospitals. Thus, increasing rates of resistance to an antibacterial agent and to all other agents that are rendered inactive by common mechanisms of resistance is an inevitable consequence of its use. In the last 10–20 years, multidrug resistance has emerged in many frequently encountered pathogenic bacteria. In extreme cases, these bacteria are not susceptible to any licensed antibacterial agent or are susceptible only to those that are more toxic to the patient than the more commonly used drugs.

1.1.2 What are the consequences of resistance and multidrug resistance?

Multidrug-resistant bacteria represent a major threat to the success of almost all branches of medical practice. Some patients are especially vulnerable to acquiring multidrug-resistant bacterial infections as a consequence of treatments for underlying illnesses, such as organ transplant patients, haemodialysis patients and those with various types of cancer [2-7].

Bacterial resistance potentially complicates the management of every infection, no matter how mild it may be at the time of first presentation. For example, bladder infections in young women should be very easy to treat with commonly used antibacterial agents but the appearance of multidrug resistance among organisms often associated with these infections means that physicians have to resort to other agents that may not be so well tolerated and may even have to be given intravenously when usually oral agents are efficient.

Physicians in the EU are increasingly faced with infections for which antibacterial treatment options are very limited. However, the overall burden of infections caused by multidrug-resistant bacteria is not well documented in the EU. There is a lack of data on the morbidity and mortality attributable to antibacterial resistance, including the economic impact on individuals as well as on healthcare systems and societies.

Multidrug resistance among bacteria is a global problem and organisms are easily carried across international boundaries. All regions of the world [8] are already experiencing the effects of multidrug resistance on clinical practise. Therefore, stimulating the development of new antibiotics has far-reaching potential benefits.

1.1.3 Antibacterial resistance and the response from the pharmaceutical industry

The launch of every antibiotic has been and will be followed by resistance in the targeted bacteria. Therefore, there is a constant need to develop new agents to keep up with the acquisition of resistance among pathogenic bacteria.

For approximately four decades (from the 1940s up to the 1970s) the pharmaceutical industry provided a steady flow of new antibiotics, including several with new mechanisms of action that circumvented the problems caused by bacterial resistance to earlier agents. Since then, only three systemically-administered antibiotics (quinupristin-dalfopristin, linezolid and daptomycin), including two from new classes (oxazolidinones and lipopeptides,) have been marketed in the EU to treat infections caused by multidrug-resistant Gram-positive bacteria. The other systemically-administered antibiotics that have reached the EU market during this period belong to existing classes of antibiotics and are not efficacious against the majority of organisms already resistant to other agents in the same class.

Figure 1. Discovery of new classes of antibiotics.

Trimethoprim
Streptogramins
Quinolones
Lincosamides
Chloramphenicol
Tetracyclines
Macrolides
Glycopeptides
Aminoglycosides
Beta-lactams*
Sulfonamides
Lipopeptides
Oxazolidinones
1930s 1940s 1950s 1960s 1970s 1980s 1990s 2000s

Source: [4, 9-10]

** Penicillins were the first beta-lactams. Other frequently used agents of the beta-lactam class include cephalosporins and carbapenems, developed in the 1960s and 1980s, respectively.*

Meanwhile, multidrug resistance among Gram-negative bacteria has been increasing relentlessly. International and local surveillance networks such as the European Antimicrobial Resistance Surveillance System (EARSS)[3], as well as numerous reports in the literature [11-13] provide evidence that the frequency of infections caused by multidrug-resistant Gram-negative bacteria is escalating in many countries. In some Gram-negative bacteria, acquired resistance to three or more classes of antibiotics that are commonly used to treat infections is often reported [14]. Therefore, there is particular concern regarding the paucity of new agents with activity against Gram-negative bacteria that have reached the market in the last decade. Those that have been marketed do not show efficacy against Gram-negative bacteria with resistance to most or all beta-lactam drugs.

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[3] http://www.rivm.nl/earss/result/Monitoring_reports/

1.2 Time to react

The growing gap between the increasing frequency of infections caused by multidrug-resistant bacteria and the decline in research and development of new antibiotics is now threatening to take us back to the pre-antibiotic era. Strategies to curtail the spread of multidrug-resistant bacteria have met with limited success. While effective implementation of these strategies may reduce the rate of increase in infections caused by multidrug-resistant bacteria, a reversal of the existing problems cannot be expected. The continued development of effective antibiotics must be considered as a 'common good' [15-16]. An analysis of the antibacterial agents currently under development in view of current resistance patterns and trends is a starting point for discussing incentives for the development of urgently needed new treatments.

1.3 The response from ECDC and EMEA

One of the aims of the EMEA Road Map 2010 is to foster research and innovation in the pharmaceutical industry across the European Union. In this context, an 'EMEA/CHMP think-tank group on innovative drug development' was set up in 2006. The purpose was to offer stakeholders the possibility to present and discuss informally their views on evolving strategies in drug development. The report from the think-tank[4] describes the technical and scientific highlights of all these consultations, incorporates reflections and draws recommendations from the think-tank group. In this process, the paucity of new antibacterial agents, which has been the subject of several reports, including the *Antibiotic Innovation Study*[5] from the international network Action on Antibiotic Resistance (ReAct) in 2005, attracted considerable attention. During this EMEA/CHMP think-tank discussion with industry and academia, the idea of an analysis of the gap between the frequency of infections caused by multidrug-resistant bacteria in the EU and the development of new antibiotics was raised.

ECDC was established in 2005 with the mission to identify, assess and communicate current and emerging threats to human health posed by infectious diseases. In its first Annual Epidemiological Report, published in 2007[6], ECDC identified antimicrobial resistance as one of the most serious public health problems, globally and in Europe. Antimicrobial resistance, together with healthcare-associated infections, was consequently selected as one of the priority work areas in the *ECDC Strategic Multi-annual Programme 2007–2013*[7] with the objective of significantly contributing to the scientific knowledge base on antimicrobial resistance and its health consequences, its underlying determinants, the methods for its prevention and control, and the design characteristics that enhance effectiveness and efficiency of its prevention and control programmes.

In 2007, ECDC, EMEA and ReAct entered into a discussion on the need to provide a comprehensive technical report to the European Commission on the pipeline of antibacterial medicinal products in development. In particular, to describe the frequency, trends and burden of disease associated with multidrug-resistant bacteria in the European Union and to assess the pipeline of new agents in development that might have clinically useful activity against them. Production of a joint report was included as a priority project for 2008 and 2009 in the ECDC's programme on antimicrobial resistance and healthcare-associated infections and for EMEA. The assessment of the pipeline of antibacterial drug development was performed in co-operation with ReAct and was conducted under a memorandum of understanding between Duke University, EMEA and ReAct.

An ECDC-EMEA joint working group was established in 2008 with a mandate to produce the joint report. The mandate, composition, meetings, roles and responsibilities of the joint working group are presented in Annex A and its detailed composition is shown on the verso of the title page.

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[4] http://www.emea.europa.eu/pdfs/human/itf/12731807en.pdf

[5] http://soapimg.icecube.snowfall.se/stopresistance/Innovation_report.pdf

[6] http://ecdc.europa.eu/en/publications/Publications/0706_SUR_Annual_Epidemiological_Report_2007.pdf

[7] http://ecdc.europa.eu/en/aboutus/Key%20Documents/07-13_KD_Strategic_multiannual_programme.pdf

2 Trends and burden of infections due to multidrug-resistant bacteria in the EU

Most relevant findings:

- Resistance to antibiotics is high among Gram-positive and Gram-negative bacteria that cause serious infections in humans and reaches 25% or more in several EU Member States.
- Resistance is increasing in the EU among certain Gram-negative bacteria such as recently observed for *Escherichia coli*.
- Each year, about 25 000 patients die in the EU from an infection with the selected multidrug-resistant bacteria.
- Infections due to these selected multidrug-resistant bacteria in the EU result in extra healthcare costs and productivity losses of at least EUR 1.5 billion each year.

2.1 Introduction

Antibiotic resistance is not by itself a disease entity. It encompasses many types of infections, bacteria and antibiotic resistance traits. Although the global nature of the problem is known, the lack of overview of the size and the consequences of multidrug-resistant bacteria means that this public health threat is not fully appreciated and often ignored by policymakers and the public.

Data on antibiotic resistance in various bacteria are available from many countries [17], but summarising the situation for the whole European Union in a simple manner remains a challenge. Additionally, there are studies showing that infections due to antibiotic-resistant bacteria result in higher mortality and extra hospital costs [18,19]. However, there currently is no estimate of the burden imposed by multidrug-resistant bacteria on the EU.

The purpose of this study was to give an overview of the trends of antibiotic resistance in bacteria frequently responsible for infections in humans, as well as estimating the human and economic burden associated with multidrug-resistant bacteria, in the EU, Iceland and Norway.

2.2 Materials and methods

2.2.1 Selection of bacteria

The study focused on bacteria most frequently isolated from blood cultures in Europe [20]. For each bacterium, the resistance traits, which in most cases are markers of multiple resistance to antibiotics, were listed (Table 1). Although they are frequently isolated from blood cultures, coagulase-negative staphylococci, beta-haemolytic and viridans streptococci, *Enterobacter spp.* and *Acinetobacter spp.* were excluded from the study because reliable resistance data were not available.

Table 1. Bacteria frequently responsible for bloodstream infections and resistances used as markers for resistance to multiple antibiotics.

Bacteria[a]	Resistance used as a marker of multiple resistance to antibiotics
Gram-positive bacteria	
Staphylococcus aureus	Methicillin resistance (MRSA)
	Vancomycin-intermediate resistance and resistance (VISA/VRSA)
Enterococcus spp. (e.g., *Enterococcus faecium*)	Vancomycin resistance (VRE)
Streptococcus pneumoniae	Penicillin resistance[b]
Gram-negative bacteria	
Enterobacteriaceae	
Escherichia coli	Third-generation cephalosporin resistance[c,d]
	Carbapenem resistance[e]
Klebsiella spp.	Third-generation cephalosporin resistance[c,d]
	Carbapenem resistance[e]
Non-fermentative Gram-negative bacteria	
Pseudomonas aeruginosa	Carbapenem resistance[e]

[a] *Coagulase-negative staphylococci, beta-haemolytic and viridans streptococci,* Enterobacter spp. *and* Acinetobacter spp. *are among the list of the 10 bacteria most frequently isolated from blood cultures [20], but were excluded from the study because reliable resistance data are not available for these bacteria.*

[b] *Most fully penicillin-resistant* Streptococcus pneumoniae *isolates are resistant to both penicillin and macrolides.*

[c] *Resistance to cefotaxime or ceftriaxone or ceftazidime (as in the European Antimicrobial Resistance Surveillance System, EARSS).*

[d] *Mostly extended-spectrum beta-lactamase (ESBL)-producing isolates.*

[e] *Resistance to imipenem or meropenem (as in EARSS).*

2.2.2 Data source

The European Antimicrobial Resistance Surveillance System (EARSS) is the preferred source of data for multidrug-resistant bacteria in Europe because it includes ongoing surveillance data on antibiotic resistance in bacteria responsible for invasive infections (mostly bloodstream infections) such as *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecium*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* causing invasive infections.

EARSS is a network of national antimicrobial resistance surveillance systems in European countries coordinated by the Dutch National Institute of Public Health and the Environment (RIVM). EARSS collects comparable and validated antibacterial susceptibility data for public health action. In 2007, routine data for major indicator bacteria were submitted by more than 900 laboratories serving more than 1 400 hospitals in 31 countries [17].

2.2.3 Assessment of the situation in 2007 and of trends of selected antibiotic-resistant bacteria

Data on the proportion of isolates resistant to antibiotics among selected bacteria responsible for invasive infections (mainly bloodstream infections) in each EU Member State, Iceland and Norway and each year during the period 2002–2007 were extracted from the EARSS interactive database[8]. This proportion represents the percentage of bloodstream infection cases in which, based on *in vitro* laboratory data, the antibiotic (or antibiotic group) would be inactive to treat an infection due to this bacteria.

[8] http://www.rivm.nl/earss/database/

Such data were available for methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-intermediate and -resistant *S. aureus* (VISA/VRSA), vancomycin-resistant *Enterococcus faecium*, penicillin-resistant *Streptococcus pneumoniae* and third-generation cephalosporin-resistant *Escherichia coli*, carbapenem-resistant *E. coli* for the period 2002–2007, and for third-generation cephalosporin-resistant *Klebsiella pneumoniae*, carbapenem-resistant *K. pneumoniae* and carbapenem-resistant *Pseudomonas aeruginosa* for the period 2005–2007.

Mid-year population data for each EU Member State, Iceland and Norway and each year during the period 2002–2007 were obtained from Eurostat[9].

To give an overview of the situation for each selected antibiotic-resistant bacteria, data were presented on maps, as well as plotted on graphs where each square represented one country. The trends in the proportion of resistant isolates in each country for the period 2005–2007 were assessed by the Chi-square test for trend (Epi Info™ Version 3.3.2, Statcalc).

Additionally, for each year in the study period 2002–2007 and for each bacteria and antibiotic included in the survey, a population-weighted average proportion (percentage) of resistant isolates was calculated. These data were plotted on two graphs. When data were not available for a particular year, data for the closest available year were used. Data were not available for the whole study period for *S. pneumoniae* in Greece and for *K. pneumoniae* and *P. aeruginosa* in Belgium and Slovakia.

2.2.4 Assessment of the human burden of infections caused by the selected antibiotic-resistant bacteria in 2007

For estimating the burden of antibiotic-resistant bacteria, data were only available on the following five antibiotic-resistant bacteria: MRSA, vancomycin-resistant *Enterococcus faecium*, third-generation cephalosporin-resistant *Escherichia coli* and *Klebsiella pneumoniae* and carbapenem-resistant *Pseudomonas aeruginosa*.

Number of infections

Data on the number of isolates resistant to antibiotics among bacteria responsible for invasive infections (mainly bloodstream infections) in each EU Member State, Iceland and Norway in 2007 were extracted from the EARSS interactive database[10].

Data on the estimated population covered by EARSS for each type of bacteria were obtained directly from country representatives in the EARSS network. For each country, the number of invasive infections (mainly bloodstream infections) due to the selected antibiotic-resistant bacteria was estimated from this reported population coverage. Data for Belgium and Slovakia on *K. pneumoniae* and *P. aeruginosa* were not available. They were replaced by values based on the median incidence of invasive infections due to these bacteria multiplied – for Belgium with the percentage of resistance in 2005–2007 from national surveillance of nosocomial septicaemia[11], and for Slovakia with the average percentage of resistance for the EU, Iceland and Norway.

The number of infections due to the selected antibiotic-resistant bacteria (with the exception of vancomycin-resistant *E. faecium* and penicillin-resistant *S. pneumoniae*) from the three other main body sites (respiratory tract, skin and soft tissue and urine), was estimated by applying correction factors corresponding to the relative distribution of infections from these body sites compared to bloodstream, as reported in published literature [21-22].

For third-generation cephalosporin-resistant *E. coli*, the same relative distributions as for third-generation cephalosporin-resistant *K. pneumoniae* were used [22]. For vancomycin-resistant *E. faecium*, the three other main body sites considered were: abdomen (abdominal infections), skin and soft tissue (wounds) and urine [23]. For penicillin-resistant *S. pneumoniae*, the only other body site considered was respiratory tract [24]. Parameters used to estimate the number of infections are shown in Annex B1.

The total number of infections due to the selected antibiotic-resistant bacteria was obtained by adding the number of invasive infections (mainly bloodstream) and of infections from the three other main body sites (respiratory tract, skin and soft tissue and urine).

Number of extra deaths due to these infections

Attributable mortality corresponds to the percentage of deaths that are attributable to infection with an antibiotic-resistant isolate of a given bacteria as compared with infection with an antibiotic-susceptible isolate of the same

[9] http://epp.eurostat.ec.europa.eu

[10] http://www.rivm.nl/earss/database/

[11] http://www.iph.fgov.be/nsih/surv_sep/beschrijving_fr.asp

bacteria when all other possible causes of deaths have been controlled for. It was calculated with the formula: *attributable mortality = ((relative risk -1)/relative risk) × crude mortality*. For each selected antibiotic-resistant bacteria, data for calculating attributable mortality of bloodstream infections were obtained from published studies [19,22-23,25-26]. Such data were not available for penicillin-resistant *S. pneumoniae*. When only attributable mortality of bloodstream infections was available from published studies, attributable mortality was estimated by applying correction factors corresponding to the relative mortality of nosocomial infections from these body sites compared to nosocomial bloodstream infection [27]. Parameters used to estimate the number of extra deaths are shown in Annex B1.

The number of extra deaths due to the selected antibiotic-resistant bacteria was estimated by applying attributable mortality to each of the estimates of the number of infections described above.

Number of extra hospital days due to these infections

Extra days spent in a hospital are a direct, short-term effect of infections due to antibiotic-resistant bacteria. For each selected antibiotic-resistant bacteria, the extra length of hospital stay for each infection was estimated as the difference between the average length of hospital stay in patients infected with an antibiotic-resistant isolate of a given bacteria to the average length of hospital stay in patients infected with an antibiotic-susceptible isolate of the same bacteria, as reported in published studies selected because they controlled for other factors affecting length of hospital stay such age, sex, comorbidities, severity of underlying diseases, antibiotic therapy and appropriateness of antibiotic therapy [18,23,25,28-29]. For carbapenem-resistant *P. aeruginosa*, the extra length of hospital stay for bloodstream infections was used for all infections [25]. Such data were not available for penicillin-resistant *S. pneumoniae*. Parameters used to estimate the number of extra hospital days are shown in Annex B1.

The number of extra hospital days due to the selected antibiotic-resistant bacteria was estimated by applying the extra length of hospital stay to each of the estimates of the number of infections described above.

2.2.5 Assessment of the economic burden of infections caused by the selected antibiotic-resistant bacteria in 2007

Principles of cost calculations

Cost-of illness analyses involve the identification, measurement and valuing of resources related to an illness.

The principal features of this study were:

- Time frame: Year (2007)
- Perspective: Societal
- Methodology: Standard prevalence-based
- Approach: Bottom-up

An annual time frame was considered whereby all costs within the most recent year for which data were available were measured. The reference year was 2007, the most recent year for which EARSS data were available. The most recently reported year was used for those few instances where 2007 data were not available.

A societal perspective was adopted considering direct and indirect healthcare costs, as well as productivity losses from absence from work due to illness or premature death.

A prevalence-based study was performed to estimate annual costs. In such studies, costs are measured during one period, usually a year, regardless of the date of onset of illness.

A bottom-up approach was used because only aggregated data on the number of infections due to the selected antibiotic-resistant bacteria were available. This approach estimates costs by multiplying the number of cases of an illness by the unit cost of treatment of this illness.

Publications and websites from international organisations, national ministries, bodies and statistical institutes, as well as published literature, were consulted for epidemiological and healthcare utilisation data. If no data were found for a specific country, extrapolations were performed from data from similar countries based on gross domestic product, population and geographical location.

Hospital inpatient care and outpatient care were included in cost calculations for healthcare services. Activities aiming at the prevention of patient-to-patient transmission of antibiotic-resistant bacteria such as the search-and-destroy approach for MRSA that is actually being performed in some EU countries, e.g. the Netherlands, but is not routine in all EU Member States, were not included.

Non-health service costs include productivity losses, informal care costs, patient travel costs and out-of-pocket expenses. Little data is available on informal care, patient travel and out-of-pocket expenses. As a consequence, only productivity losses from absence from work due to illness or premature death were estimated.

Hospital inpatient care costs

Total hospital inpatient care costs were estimated by multiplying the number of extra hospital days, as calculated above, by an average cost for a hospital day in the EU in 2007 of EUR 366. The average cost of a hospital day was obtained from the European Commission [30] and converted to 2007 prices using the health component of the harmonised index of consumer prices (HICP)[12].

Outpatient care costs

In this study, outpatient care corresponded to one consultation with a general practitioner after hospital discharge. An estimate of the cost of this consultation was obtained from published literature [31]. These data were not available for Bulgaria and Romania, Iceland and Norway, for which costs of similar countries were used. All costs were converted to 2007 prices using the health component of the harmonised index of consumer prices (HICP)[13].

Productivity losses

Productivity losses include the foregone earnings from absence from work due to illness or premature death.

For each country, productivity losses due to absence from work were estimated by multiplying the number of days being absent from work due to infection with an antibiotic-resistant bacteria by the daily earnings and employment rates in 2007, assuming that the number of days being absent from work was equal to the number of extra hospital days due to the infection. A friction period, i.e. the period until another worker from the pool of unemployed has fully replaced the worker who is absent due to illness, was not taken into account because absence from work due to infection is generally not long enough for a worker to be replaced.

Productivity losses from premature deaths from infection due to antibiotic-resistant bacteria correspond to the likely earnings that patients who died would otherwise have received from paid employment. For each country, they were estimated by calculating age-specific products of the following:

- estimated number of extra deaths attributable to antibiotic-resistant bacteria in 2007;
- population distribution in 2007, by age;
- probability of dying, by age;
- number of remaining work years at time of death, by age;
- average annual gross earning in 2007; and
- employment rate in 2007[14].

Because these productivity losses will be incurred in the future, earnings were discounted using a 3.5% annual rate to obtain present values [32]. Additionally, since the age distribution of patients with an infection due to antibiotic-resistant bacteria is different from that of the general population and skewed towards older age, a correction factor of 0.37 was applied, based on the percentage of individuals aged less than 65 years in the general population and among patients with a healthcare-associated infection as reported in a national prevalence survey[15].

Total productivity losses were obtained by adding productivity losses for each selected type of antibiotic-resistant bacteria and for each country.

2.3 Results

2.3.1 Antibiotic resistance situation in 2007 and trends

The population-weighted, average proportions (percentages) of resistant isolates among the selected bacteria are presented in Figure 2. This is an attempt to summarise the general antibiotic resistance situation in the EU, Iceland and Norway. However, for each selected antibiotic-resistant bacteria, there were large variations between countries from less than 1% to more than 50% resistant isolates in many instances. These maps, as well as graphs presenting the distribution of country data, are presented in Figures 3 and 4.

Methicillin-resistant Staphylococcus aureus *(MRSA)*

Overall, the average proportion of MRSA in the EU, Iceland and Norway was high (22%), although it has been decreasing in recent years (Figure 2a). There were large intercountry variations, from less than 1% in Denmark,

[12] http://epp.eurostat.ec.europa.eu/portal/page/portal/hicp/data

[13] http://epp.eurostat.ec.europa.eu/portal/page/portal/hicp/data

[14] Eurostat. http://epp.eurostat.ec.europa.eu/portal/page/portal/eurostat/home

[15] http://www.invs.sante.fr/publications/2009/enquete_prevalence_infections_nosocomiales/enquete_prevalence_infections_nosocomiales_vol1.pdf

Iceland, Norway and Sweden to more than 25% in 10 countries (Figure 3a). Between 2005 and 2007, the proportion of MRSA significantly decreased in eight EU Member States (Figure 3a). These decreasing trends are likely due to increased prevention and control at national level, as documented for France, Slovenia and United Kingdom [33-35].

Vancomycin-intermediate and vancomycin-resistant S. aureus *(VISA/VRSA)*

The proportion of *S. aureus* isolates that showed intermediate resistance to vancomycin (VISA) was very low in the EU, Iceland and Norway. Overall, it represented less than 0.1% of *S. aureus* bloodstream isolates reported to EARSS by these countries, corresponding to only four confirmed isolates, reported by France (n=1), Ireland (n=1) and the Netherlands (n=2). No vancomycin-resistant *S. aureus* isolates was reported to EARSS in 2007.

Vancomycin-resistant Enterococcus faecium

The average proportion of *Enterococcus faecium* isolates that showed resistance to vancomycin was below 8% in the EU, Iceland and Norway (Figure 2a). There was a large intercountry variation, from less than 1% in 14 countries to more than 25% in Ireland, Greece and Portugal, with very few significant variations over the period 2005–2007 (Figure 3b).

Penicillin-resistant Streptococcus pneumoniae

The average proportion of *S. pneumoniae* isolates in the EU, Iceland and Norway, that showed full resistance to penicillin was 4% in 2007 (Figure 2a). Intercountry variation showed a much narrower range than for other bacteria, with most countries reporting a proportion below 10% (Figure 3c). Only a few countries showed an increasing or decreasing trend over the period 2005–2007 (Figure 3c).

Third-generation cephalosporin-resistant Escherichia coli

The average proportion of third-generation cephalosporin-resistant isolates among *Escherichia coli* – the most common Gram-negative bacterium responsible for infections in humans – is rising steadily in the EU, Iceland and Norway and reached 8% in 2007 (Figure 2b). Indeed, 13 countries showed a significant increase in this proportion during the period 2005–2007 (Figure 4a). There was a large intercountry variation in the proportion reported in 2007, from 1–5% in 12 countries to more than 25% in Romania (Figure 4a).

Third-generation cephalosporin-resistant Klebsiella pneumoniae

The average proportion of third-generation cephalosporin-resistant isolates among *K. pneumoniae* in the EU, Iceland and Norway remained high (19%) in 2007 (Figure 2b). There was a large intercountry variation, from less than 5% in Estonia, Finland, Iceland, Norway and Sweden to more than 25% in 11 countries, but only a few countries showed increasing or decreasing trends over the period 2005–2007 (Figure 4b).

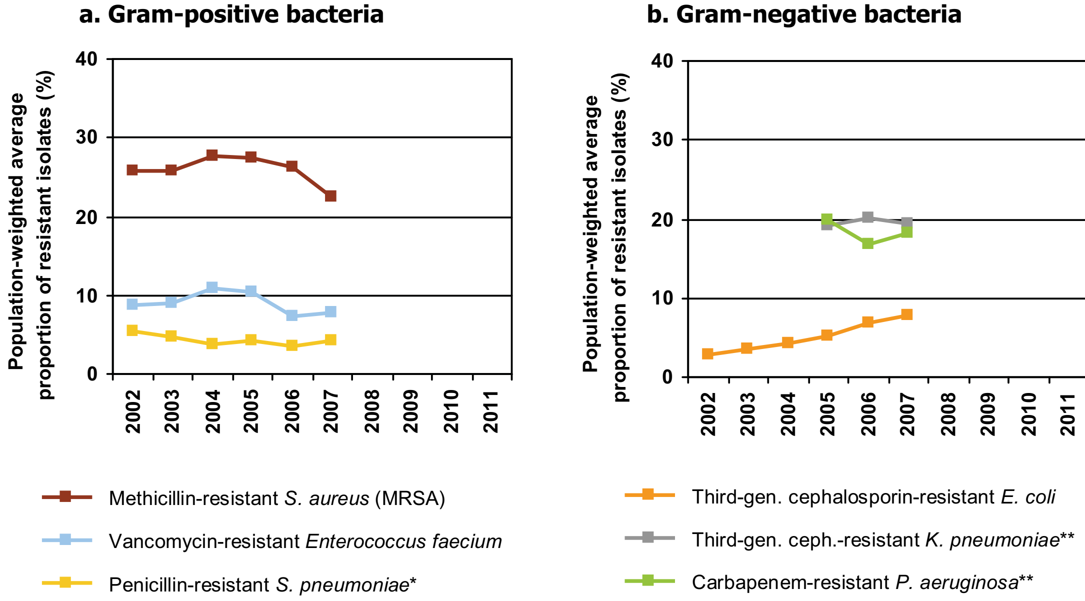
Carbapenem-resistant K. pneumoniae

Carbapenem resistance was still absent from *K. pneumoniae* isolates from blood cultures in most EU Member States in 2007 and only six countries reported such isolates. Five of these countries reported only a few isolates: Cyprus (n=1; 3%), France (n=1; <1%), Germany (n=3; 2%), Italy (n=4; 1%) and United Kingdom (n=1; <1%). Greece was a notable exception with 410 reported carbapenem-resistant *K. pneumoniae* isolates, which corresponded to 42% of reported *K. pneumoniae* isolates. The situation in Greece has been attributed to the spread of a hyperepidemic, carbapenemase-producing clone, as well as the spread of the bla_{VIM-1} resistance gene cassette and ecological pressure due to antibiotic use [13,36].

Carbapenem-resistant Pseudomonas aeruginosa

The average proportion of carbapenem-resistant isolates among *P. aeruginosa* in the EU, Iceland and Norway remained high (18%) in 2007 (Figure 2b). There was a large intercountry variation, from less than 5% in Denmark, Iceland and the Netherlands to more than 25% in the Czech Republic, Greece, Italy and Lithuania, but only a few countries showed increasing or decreasing trends over the period 2005–2007 (Figure 4c).

Figure 2. **Population-weighted, average proportion of resistant isolates among blood isolates of bacteria frequently responsible for bloodstream infections, EU Member States, Iceland and Norway, 2002–2007.**

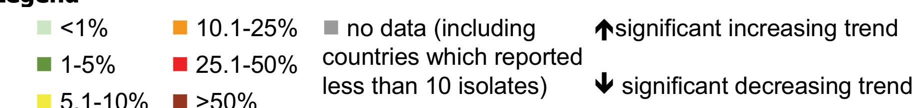


* S. pneumoniae*: excluding Greece, which did not report data on this bacterium to EARSS.*

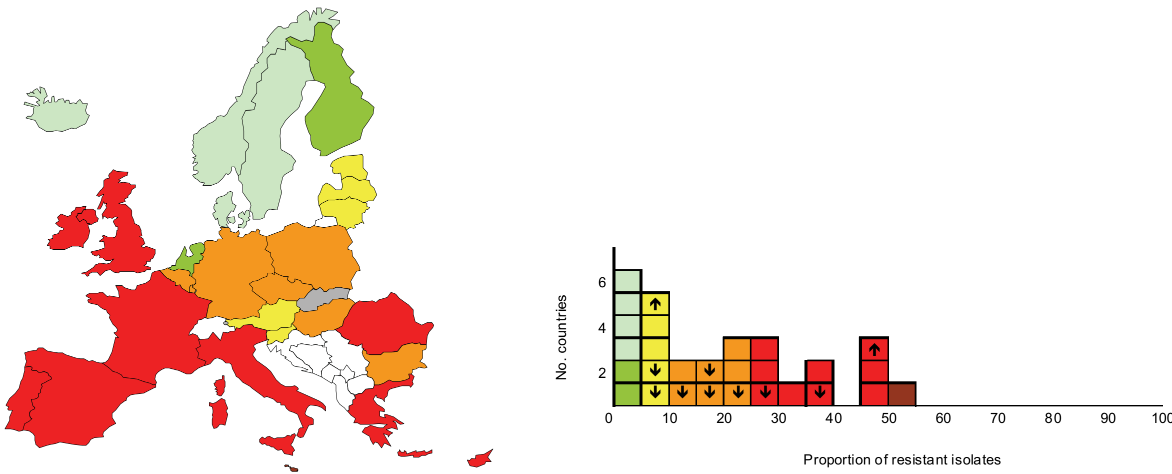
** K. pneumoniae *and* P. aeruginosa*: excluding Belgium and Slovakia, which did not report data on these bacteria to EARSS.*

Figure 3. **Proportion of resistant isolates among blood isolates of Gram-positive bacteria frequently responsible for bloodstream infections, EU Member States, Iceland and Norway, 2007 and trends for 2005–2007.**

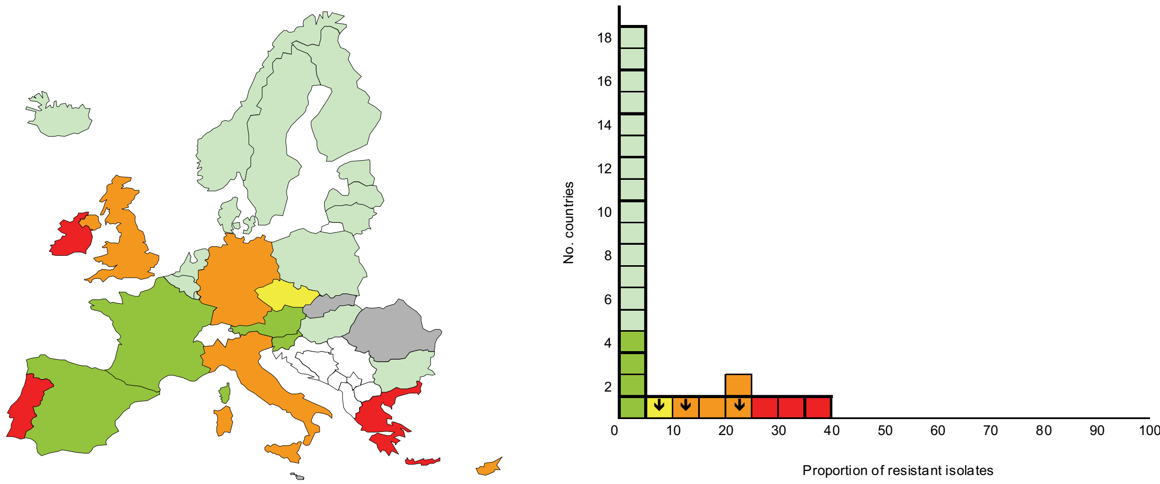
Legend



a. Methicillin-resistant *Staphylococcus aureus* (MRSA)



b. Vancomycin-resistant *Enterococcus faecium*



c. Penicillin-resistant *Streptococcus pneumoniae*

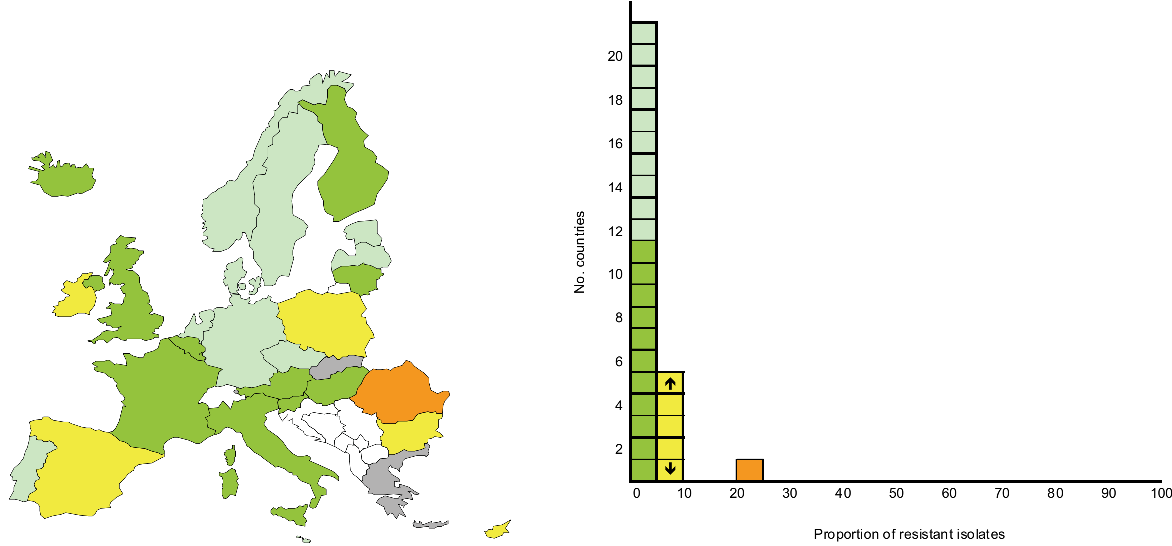
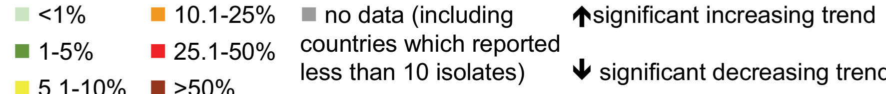


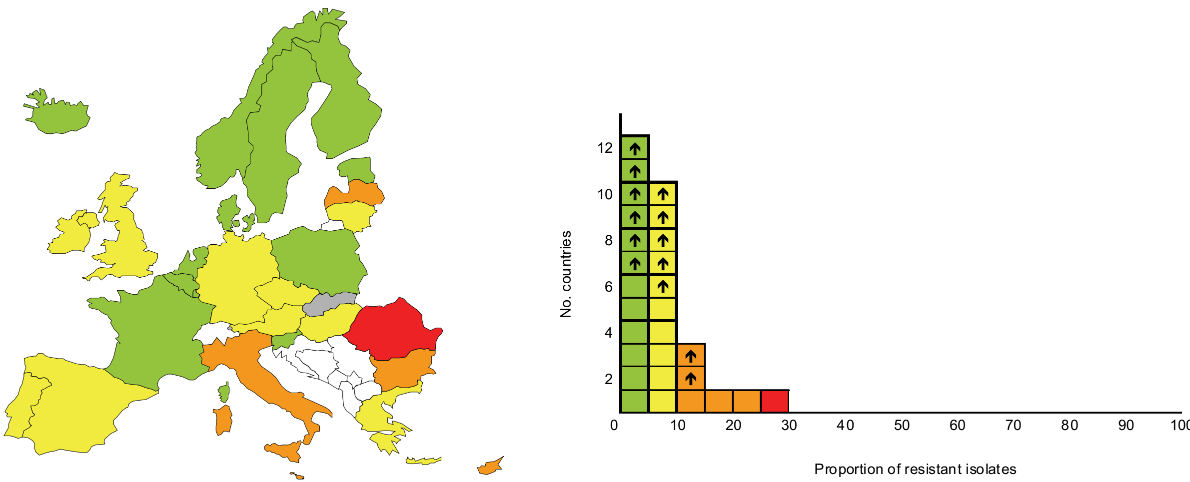
Figure 4. Proportion of resistant isolates among blood isolates of Gram-negative bacteria frequently responsible for bloodstream infections, EU Member States, Iceland and Norway, 2007 and trends for 2005–2007.



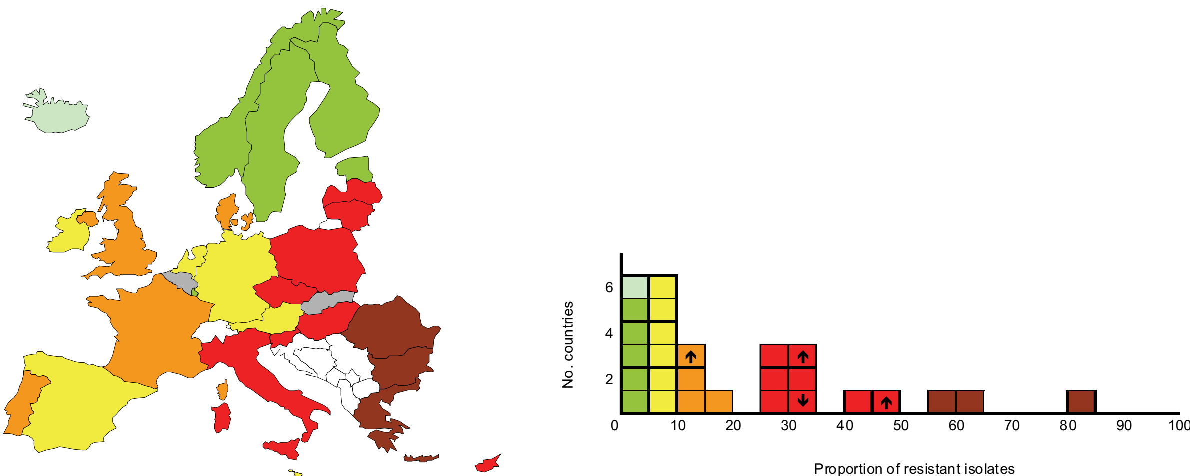
Legend

<1% | 10.1-25% | no data (including countries which reported less than 10 isolates) | significant increasing trend
1-5% | 25.1-50% | | significant decreasing trend
5.1-10% | >50%

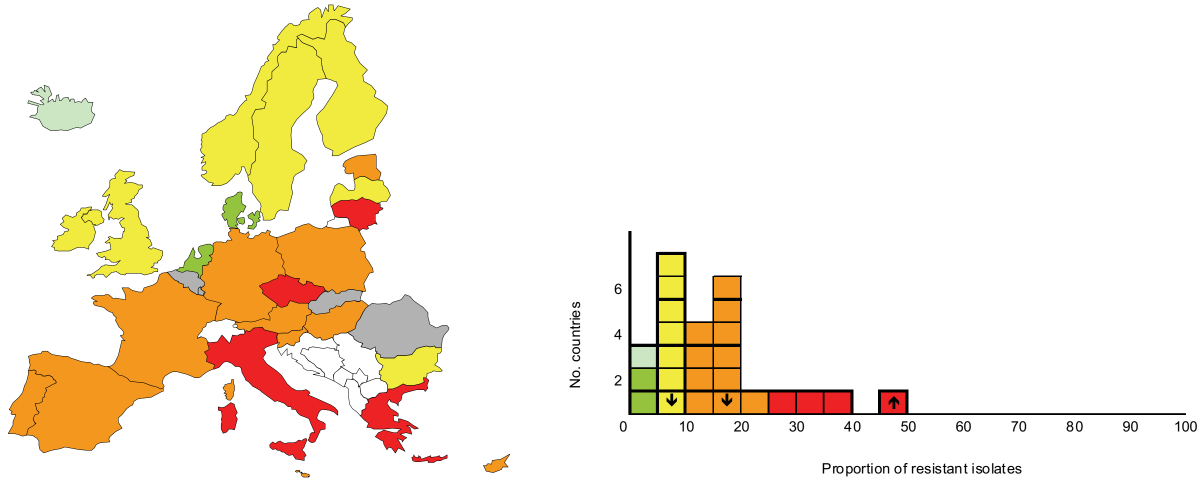
a. Third-generation cephalosporin-resistant *Escherichia coli*



b. Third-generation cephalosporin-resistant *Klebsiella pneumoniae*



c. Carbapenem-resistant *Pseudomonas aeruginosa*



2.3.2 Human burden of antibiotic resistance

The estimated human burden of infections due to the selected antibiotic-resistant bacteria is presented in Table 2.

The study confirmed that MRSA was, in 2007, the most common, single, multidrug-resistant bacterium in the EU as per the estimated number of cases of infection due to this bacterium. However, the sum of cases of antibiotic-resistant Gram-positive bacteria (mostly MRSA and vancomycin-resistant *Enterococcus faecium*) was comparable to that of antibiotic-resistant Gram-negative bacteria (third-generation cephalosporin-resistant *E. coli* and *K. pneumoniae*, and carbapenem-resistant *P. aeruginosa*).

Overall, it was estimated that in 2007 approximately 25 000 patients died from an infection due to any of the selected frequent antibiotic-resistant bacteria in the EU, Iceland and Norway. Notably, about two thirds of these deaths were caused by infections due to Gram-negative bacteria. In addition, infections due to any of the selected antibiotic-resistant bacteria resulted in approximately 2.5 million extra hospital days.

2.3.3 Economic burden of antibiotic resistance

The estimated economic burden of infections due to the selected antibiotic-resistant bacteria is presented in Table 3.

Based on the number of extra hospital days, extra in-hospital costs in 2007 were estimated at more than EUR 900 million in the EU, Iceland and Norway.

Based on 2007 data, outpatient care costs were estimated at about EUR 10 million and productivity losses due to absence from work of infected patients were estimated at more than EUR 150 million, each year. Productivity losses due to patients who died from their infection were estimated at about EUR 450 million each year. Overall, societal costs of infections due to the selected antibiotic-resistant bacteria were estimated at about EUR 1.5 billion each year.

There are many reasons to suggest that these figures correspond to an underestimate of the human and economic burden of infections due to the selected antibiotic-resistant bacteria. These reasons are developed in the discussion section of this chapter.

Table 2. Estimated yearly human burden of infections due to the selected antibiotic-resistant bacteria and percentage of this burden due to bloodstream infections, EU Member States, Iceland and Norway, 2007.

Antibiotic-resistant bacteria[a]	No. cases of infection (four main types)[b] (% bloodstream infections)	No. extra deaths (% from bloodstream infections)	No. extra hospital days (% from bloodstream infections)
Antibiotic-resistant Gram-positive bacteria			
Methicillin-resistant *Staphylococcus aureus* (MRSA)	171 200 (12%)	5 400 (37%)	1 050 000 (16%)
Vancomycin-resistant *Enterococcus faecium*	18 100 (9%)	1 500 (28%)	111 000 (22%)
Penicillin-resistant *Streptococcus pneumoniae*[c]	3 500 (27%)	–[f]	–
Sub-total	*192 800 (12%)*	*6 900 (35%)*	*1 161 000 (16%)*
Antibiotic-resistant Gram-negative bacteria			
Third-generation cephalosporin-resistant *Escherichia coli*[d]	32 500 (27%)	5 100 (52%)	358 000 (27%)
Third-generation cephalosporin-resistant *Klebsiella pneumoniae*	18 900 (27%)	2 900 (52%)	208 000 (27%)
Carbapenem-resistant *Pseudomonas aeruginosa*[e]	141 900 (3%)	10 200 (7%)	809 000 (3%)
Sub-total	*193 300 (9%)*	*18 200 (27%)*	*1 375 000 (13%)*
Total	386 100 (11%)	25 100 (29%)	2 536 000 (14%)

[a]*Data on antimicrobial resistance for* Klebsiella sp. *other than* K. pneumoniae*,* Enterobacter spp. *and* Acinetobacter spp. *were not available from EARSS. Although coagulase-negative staphylococci as well as beta-haemolytic and viridans streptococci are among the 10 most common bacteria isolated from blood cultures [20], they were excluded from the study because reliable resistance data are not available for these bacteria.*

[b]*Bloodstream infections, lower respiratory tract infections, skin and soft tissue infections and urinary tract infections.*

[c]*Most fully penicillin-resistant* Streptococcus pneumoniae *isolates are resistant to both penicillin and macrolides.*

[d]*Resistant to cefotaxime or ceftriaxone or ceftazidime.*

[e]*Resistant to imipenem or meropenem.*

[f] *-, could not be calculated*

Table 3. Estimated yearly economic burden of infections (four main types[a]) due to the selected antibiotic-resistant bacteria, EU Member States, Iceland and Norway, 2007.

Antibiotic-resistant bacteria[b]	Extra in-hospital costs (EUR)	Extra outpatient costs[c] (EUR)	Productivity losses due to absence from work (EUR)	Productivity losses due to patients who died from their infection (EUR)	Overall costs (EUR)
Antibiotic-resistant Gram-positive bacteria	*424 700 000*	*5 500 000*	*91 100 000*	*145 600 000*	*666 900 000*
Antibiotic-resistant Gram-negative bacteria	*503 100 000*	*4 500 000*	*59 300 000*	*300 300 000*	*867 200 000*
Total	927 800 000	10 000 000	150 400 000	445 900 000	1 534 100 000

[a]*Bloodstream infections, lower respiratory tract infections, skin and soft tissue infections and urinary tract infections.*

[b]*Gram-positive bacteria: methicillin-resistant* Staphylococcus aureus *(MRSA), vancomycin-resistant* Enterococcus faecium. *Data for penicillin-resistant* Streptococcus pneumoniae *were not available. Gram-negative bacteria: third-generation cephalosporin-resistant* Escherichia coli *and* Klebsiella pneumoniae *(i.e., resistant to cefotaxime or ceftriaxone or ceftazidime) and carbapenem-resistant* Pseudomonas aeruginosa *(i.e., resistant to imipenem or meropenem).*
Data on antimicrobial resistance for Klebsiella sp. *other than* K. pneumoniae*,* Enterobacter spp. *and* Acinetobacter spp. *were not available from EARSS. Although coagulase-negative staphylococci as well as beta-haemolytic and viridans streptococci are among the 10 most common bacteria isolated from blood cultures [20], they were excluded from the study because reliable resistance data are not available for these bacteria.*

[c]*Visit to general practitioner.*

2.4 Discussion

This is the first study that provides an overview of overall trends in antibiotic resistance, as well as estimates of the human and economic burden of infections due to antibiotic-resistant bacteria in the EU, Iceland and Norway. For the study, certain antibiotic-resistant bacteria were selected because they represent markers for resistance to multiple antibiotics. Multidrug-resistant bacteria represent a challenge for therapy since the number of antibiotics that remain active and can be used for treatment is limited.

The study showed that the average proportion of MRSA among *S. aureus* from bloodstream infections, although on average high, has levelled out and even decreased in several countries; a phenomenon that has already been reported by EARSS [17]. Since, in EARSS, the proportion of MRSA is correlated with the incidence of MRSA bloodstream infections [17], this suggests that the incidence of MRSA bloodstream infections is currently decreasing in these countries. Despite this trend, the study also indicated that MRSA was the most common single multidrug-resistant bacterium in the EU, Iceland and Norway. Other common antibiotic-resistant Gram-positive bacteria contributed to a much smaller fraction of the burden of antibiotic-resistant bacteria, although there were variations between countries.

The study also showed that the average proportion of antibiotic-resistant Gram-negative bacteria was high or increasing in the case of third-generation cephalosporin-resistant *E. coli*. These findings corroborate those of independent analyses from EARSS and other reports in the literature showing that infections caused by multidrug-resistant Gram-negative bacteria are becoming increasingly frequent in Europe [11-13,17,37]. Considering this current trend, it is likely that the human and economic burden caused by antibiotic-resistant Gram-negative bacteria will outweigh that of antibiotic-resistant Gram-positive bacteria such as MRSA and will represent a major challenge to appropriate therapy, prevention and control in the foreseeable future.

The number of deaths attributable to infections due to the selected antibiotic-resistant bacteria in the EU, Iceland and Norway was estimated at approximately 25 000 each year; two-thirds being due to Gram-negative bacteria. As a comparison, each year in the EU, about 48 000 persons are killed in a road accident[16], about 37 000 patients die as a direct consequence of a hospital-acquired infection and an additional 111 000 die as an indirect consequence of the hospital-acquired infection [38].

For the United States, the Centers for Disease Control and Prevention made similar estimates of about 99 000 deaths associated with a healthcare-associated infection [39] and 12 000 deaths associated with either MRSA, VRE or *Clostridium difficile* each year [40]. Although they are within the same range, data from the EU and the United States are not immediately comparable since different bacteria were included (e.g. *Clostridium difficile* was not included in the EU study) and US data include cases where antibiotic-resistant bacteria directly and indirectly contributed to patient death whereas this EU study only considered directly attributable deaths.

This study has several limitations. Although EARSS provides the most comprehensive database on antibiotic resistance in Europe, the system itself has some limitations. EARSS does not centrally test bacterial isolates. Efforts are made by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and EU Member States to standardise antimicrobial susceptibility testing in Europe. EARSS organises regular external quality assessment exercises to foster improvement of antimicrobial susceptibility testing in laboratories that participate in EARSS. Nevertheless, EARSS relies on data as reported by Member States according to the EARSS protocol. For some Member States, population coverage is low and EARSS data are not yet geographically representative of the country. Updated data on the estimated population covered by EARSS for each type of bacteria were obtained directly from country representatives in the EARSS network. These data, however, often represent a broad estimate ('best estimate') of population coverage by the EARSS network in each country. Data were missing for only a few countries in the EARSS database. For this study, missing data were replaced by data from the closest available year or by an estimate based on an EU median or average. The number of antibiotic-resistant bacteria included in this study was limited to those bacteria included in EARSS. In particular, EARSS does not perform surveillance of extensively drug-resistant or pandrug-resistant bacteria, i.e. bacteria that are almost totally or totally resistant to antibiotics, which are currently emerging in the EU [14]. Finally, many parameters used in the study were extracted from published literature and may not exactly reflect the value of these parameters in each EU Member State, Iceland and Norway in 2007.

The costs of infections due to the selected antibiotic-resistant bacteria for the EU, Iceland and Norway were estimated at about EUR 1.5 billion each year, with more than EUR 900 million corresponding to hospital costs. Because these costs are based on many assumptions, a nomogram is provided in Annex B1, which allows to calculate yearly in-hospital costs using other values for the total number of infections, the average extra length of

[16] http://epp.eurostat.ec.europa.eu/portal/page/portal/eurostat/home

hospital stay per infection and the average cost per hospital day, thus providing a means of testing the sensitivity of the estimates in this study.

In the US, the US Office of Technology Assessment estimated the hospital costs for five major groups of hospital-acquired infections due to antibiotic-resistant bacteria at USD 1.3 billion (in 1992 dollars) [41]. More recently, Spellberg et al. [42] estimated the societal costs of infections due to one single type of antibiotic-resistant bacteria, i.e. multidrug-resistant *P. aeruginosa*, at USD 2.7 billion each year in the US. However, cost comparisons with the US should be made with caution since healthcare is more costly in the US than in the EU [43].

Despite the limitations described earlier, there are many reasons to believe that the human and economic burden of antimicrobial resistance for the EU from this study corresponds to an underestimate. Firstly, population coverage data obtained directly from country representatives in the EARSS network may be overestimated because, in many countries, catchment populations of participating hospitals frequently overlap, which leads to underestimating the total number of infections from these population coverage data. Secondly, data on infections in outpatients are not reported to EARSS and could not be included. This, in particular, includes bacteria such as *Neisseria gonorrhoeae,* for which resistance to first-line agents is increasing in Europe. Thirdly, although the study focused on selected antibiotic-resistant bacteria, there are several other antibiotic-resistant bacteria, e.g. multidrug-resistant *Enterobacter* spp., *Acinetobacter* spp. and coagulase-negative staphylococci that are often responsible for healthcare-associated infection and for which data were not available from EARSS. Fourthly, the study only considered the four main body sites of infection (bloodstream, lower respiratory tract, skin and soft tissue and urinary tract), thus slightly underestimating the number of infections for each of the selected antibiotic-resistant bacteria.

In addition, there are several reasons, other than the above stated underestimation of the number of infections, to believe that the results of the economic burden analysis correspond to an underestimate. Many patients with an infection due to antibiotic-resistant bacteria require intensive care and incur substantially higher hospital costs, since the cost of a day in an intensive care unit is more than twice that of the average cost for a hospital day considered in this study [44]. Infections due to antibiotic-resistant bacteria generally require antibiotics that are more costly than for infections with susceptible bacteria, and these antibiotic costs were not considered. Moreover, in the absence of rapid point-of-care diagnostic tests for multidrug-resistant bacteria, these costly antibiotics are also used empirically to treat many patients with a suspected infection with a multidrug-resistant type of bacteria. These costs were not included. Indirect costs after discharge from the hospital – such as informal care, patient travel and out-of-pocket expenses – were not considered. The costs related to possible disabilities following the infection were also not considered. Finally, the costs of infection control and prevention strategies, such as the search-and-destroy approach for MRSA, were not considered.

In conclusion, and despite its limitations, this study showed that overall, antibiotic resistance in the EU, Iceland and Norway is high, sometimes increasing, and its human and economic consequences are serious. Considering current trends, it is likely that the burden of antibiotic-resistant bacteria will soon shift towards an increasing prevalence of antibiotic-resistant Gram-negative bacteria such as third-generation cephalosporin-resistant *Enterobacteriaceae* and carbapenem-resistant non-fermentative Gram-negative bacteria.

3 Analysis of the research and development pipeline of antibacterial agents

Most relevant findings

- Fifteen systemically administered antibacterial agents with a new mechanism of action or directed against a new bacterial target were identified as being under development with a potential to meet the challenge of multidrug resistance. Most of these were in early phases of development and were primarily developed against bacteria for which treatment options are already available.
- There is a particular lack of new agents with new targets or mechanisms of action against multidrug-resistant Gram-negative bacteria. Two such agents with new or possibly new targets and documented activity were identified, both in early phases of development.

3.1 Introduction

Recent reports suggest that drug development will not adequately address the problems posed by the increasing frequency of antibiotic resistance among common bacterial pathogens [4,45-46]. In contrast, there are other reports that paint a more optimistic picture of the future availability of new antibacterial agents [47-48].

Hence, the aim of this study was to document and characterise the activity of those antibacterial agents that had entered clinical development as accurately and as comprehensively as possible based on information in the public domain. The focus was on antibacterial agents with potential to be clinically active against at least one of the selected panel of antibiotic-resistant bacteria of public health interest. Special emphasis was placed on agents being developed for systemic administration that also appeared to have a new bacterial target and/or a new mechanism of action.

3.2 Methods

3.2.1 Selection of bacteria

In accordance with the trends and burden analysis (see Chapter 2), the same panel of antibiotic-resistant bacteria was selected for the pipeline analysis:

- Methicillin-resistant *Staphylococcus aureus* (MRSA)
- Vancomycin-intermediate and vancomycin-resistant *S. aureus* (VISA/VRSA)
- Vancomycin-resistant *Enterococcus* spp. (VRE)
- Penicillin-resistant *Streptococcus pneumoniae* (PRSP)
- Third-generation cephalosporin-resistant *Enterobacteriaceae* (ENB)
- Carbapenem-resistant *Enterobacteriaceae*
- Carbapenem-resistant non-fermentative Gram-negative bacteria

3.2.2 Pipeline database search

In a joint undertaking between EMEA (London, United Kingdom) and the Strategic Policy Unit of ReAct at Duke University (Durham, North Carolina, United States), a pipeline search was carried out on 14 March 2008.

Selection of databases

Three commercial databases were identified for the analysis of the R&D pipeline: Pharmaprojects (T&F Informa UK Limited, London, UK), Adis Insight R&D (Wolters Kluwer Health, Amsterdam, NL) and BioPharm Insight (Infinata, Norwood, MA-USA). A pilot sensitivity analysis was performed, following a two-step approach, which compared Pharmaprojects with Adis Insight R&D and Pharmaprojects with BioPharm Insight. The first step consisted of a search for antibacterial agents that had reached phase II of clinical development for any given indication. The second step consisted in evaluating the results obtained in a search for antibacterial compounds in phases I-III of development. The combination of Pharmaprojects with Adis Insight R&D was chosen based on the higher yield provided by this search (see results). The criteria of inclusion into these databases are described in Annex B2.

Search strategy

The database searches followed the Anatomical Therapeutic Chemical (ATC) classification systems of either Pharmaprojects or EphMRA (European Pharmaceutical Market Research Association) for Adis Insight R&D. Both databases were searched for antibacterial agents that had reached phase I, II or III clinical trials. Due to differences in the classification of the databases consulted, the search for antibacterial agents in Adis Insight R&D database had to be extended to include topical antibacterial agents.

The search also included agents for which an application to at least one regulatory agency had already been made. Agents with a status of 'no development reported' or 'discontinued' according to the database definitions were excluded. Agents that had reached clinical trials but were reported as suspended (i.e. put on hold rather than definitely discontinued) were considered to be still under active development, in accordance with the definition by Pharmaprojects (Janet Beal, personal communication) and were therefore included in the search. Details on the relevant definitions used by each of the pipeline database companies can be found in Annex B2.

Pooled dataset

The results produced by the database searches were matched by compound name, synonyms and originator in order to avoid duplicate entries and to highlight any inconsistencies (e.g. misclassifications) in the dataset. If differences on the development phase of the agent were found between the databases, the most advanced phase reported was included in the analysis. Where compounds were marked as 'discontinued' or 'no development reported' in one of the databases, but not in the other, these were considered as still being under active development. Agents reported as 'suspended' in one database but under a clinical phase of development on the other were included in the pooled dataset as being under clinical development.

Sensitivity analysis

To check the completeness of the data, PubMed was searched for literature relevant to the topic, published from January 2006 through January 2009, using the following Boolean combinations of Medical Subject Headings (MeSH) terms, as well as the search terms previously described by Talbot *et al.* [7]: (((("Anti-Bacterial Agents/therapeutic use"[Mesh] AND "Bacteria/drug effects"[Mesh]) AND "Bacterial Infections/drug therapy"[Mesh]) AND "Drug Resistance, Bacterial"[Mesh]) OR ("Anti-Bacterial Agents"[Mesh] AND "Drugs, Investigational"[Mesh])) AND "Humans"[Mesh] AND anti-bacterial agents[Substance Name] OR "antimicrobial drug development" OR "investigational antimicrobials" OR "novel antimicrobials". Only PubMed-designated reviews published in English were examined. Agents identified through this search were then checked for fulfilment of the inclusion criteria in the Adis Insight R&D database.

3.2.3 Assessment strategy

Scope and inclusion criteria

The agents identified by the searches were divided into two categories: new active substances or new presentations of licensed antibacterial agents, as defined below:

New active substances – All unlicensed (anywhere in the world, to the knowledge of the working group of this report) antibacterial chemical and biological agents with a direct antibacterial effect on at least one of the selected bacteria were considered for further analysis. Agents which had a mechanism of action involving only immunomodulation, vaccines and monoclonal antibodies were excluded.

New presentations of licensed antibacterial agents – Unlicensed presentations of approved active agents were considered for further analysis if there were data to suggest that the new presentation might be active against at least one of the selected bacteria.

Agents in both of the above categories were excluded from the analysis if they were being developed only to treat bacteria not included in the target list (e.g. those that were apparently under development only to treat tuberculosis, *Helicobacter pylori*, *Chlamydia trachomatis* or non-bacterial pathogens such as *Plasmodium spp.*).

Outcome parameters – best case scenario

The two outcome parameters considered for the assessment were the spectrum of *in vitro* activity and novelty of the agent using the approaches and definitions given below. Any information available in the databases or found in the public domain was taken into account.

In vitro activity of each agent against the selected bacteria was assigned based on the following approaches:

- Actual data on *in vitro* activity were reviewed whenever available. If actual data on *in vitro* activity were not reported for an agent against any of the selected pathogens then assumptions were made regarding likely activity based on the properties of the antibiotic class or of the mechanism of action involved.
- The assessment of *in vitro* activity disregarded any known potential for cross-resistance and co-resistance.

- While *in vitro* activity alone cannot predict *in vivo* efficacy, it was decided not to take into account any available pharmacokinetic data or PK/PD analyses when scoring the antibacterial activity of agents since the amount of data available was very variable.
- However, if there was already information available on non-clinical or clinical efficacy, these data were factored into the assessment.
- In the case of new agents intended for topical or inhalational administration and new presentations and/or routes of administration of licensed antibacterial agents, the assessment took into account the possibility that very high local concentrations of drug might occur. In the case of licensed agents, the antibacterial spectrum was sometimes considered to be possibly extended beyond that associated with systemic administration of the licensed product.

The assignment of *in vitro* activity, which took into account available data together with assumptions based on class properties or mechanisms of action as well as the route of administration, took the most optimistic view of what the new agent might be able to achieve and represents a 'best case scenario'.

Novelty was rated according to the following:

a) Substance that acts on the same target as that of at least one previously licensed antibacterial agent;
b) Substance with a known mechanism of action that likely acts on a new target. Agents displaying a broader range of activity than earlier agents from the same class, implying different target range, were also included here, e.g. beta-lactam agents with activity against MRSA were assumed to be able to bind to PBP 2' (PBP 2a). In some cases it was acknowledged that activity reported against organisms resistant to earlier agents from the same class might not actually represent a different target range but could be due only to evasion of resistance mechanisms by the new agent. However, in the absence of information to allow for differentiation, these agents have been counted in this category. In addition, beta-lactamases that appeared to inhibit enzymes not inhibited by licensed inhibitors were also included in this category;
c) Substance with a new mechanism of action known or very likely.

Assessment procedure

Anti-infective compounds identified by the searches were divided into five batches and each batch was allocated to a team of two reviewers, including one from the working group and one external reviewer selected for their experience in the field. Reviewers were unaware of the identity of their team counterparts. Each reviewer independently assessed their allotted list of agents and assigned to each an antibacterial spectrum of activity and a level of novelty using the approaches and definitions detailed above. All assessments were discussed in the ECDC/EMEA Joint Working Group in order to resolve any discrepancies between reviewers' opinions.

3.3 Results

3.3.1 Selection of databases based on pilot sensitivity studies

In the first step of the sensitivity analysis (i.e. based upon antibacterial agents that had reached at least phase II of clinical development), combining the results from the Pharmaprojects and Adis Insight R&D databases resulted in an increase in the number of identified agents by 10%. In the second step of the sensitivity analysis, the addition of information from the BioPharm Insight database into the Pharmaprojects database provided no additional information.

3.3.2 Pooled dataset

The results from the searches for antibacterial agents, including topical agents, in phases I, II, III and pre-registration were pooled and matched as described above. In total 167 agents were identified through the searches and were examined by the reviewers.

3.3.3 Sensitivity analysis

The search for information on antibacterial agents in development yielded 320 PubMed-designated review articles of which 29 were considered relevant and were subsequently analysed. The only extra agent that potentially fulfilled the study inclusion criteria was the novel efflux-pump inhibitor MP-601,205 [49]. However, this agent does not possess any direct antibacterial activity by itself and at the time of the data search, no clinical study involving co-administration of this efflux pump inhibitor with an antibacterial agent had commenced. Therefore it was excluded from the analysis.

3.3.4 Overall findings

After completion of the assessment by the reviewers, 90 out of 167 agents in the pooled dataset were considered to fulfil the inclusion criteria for the analysis. Of these 90 agents, 24 were new presentations of licensed antibacterial agents and 66 were new active substances (see also flow chart Annex B2).

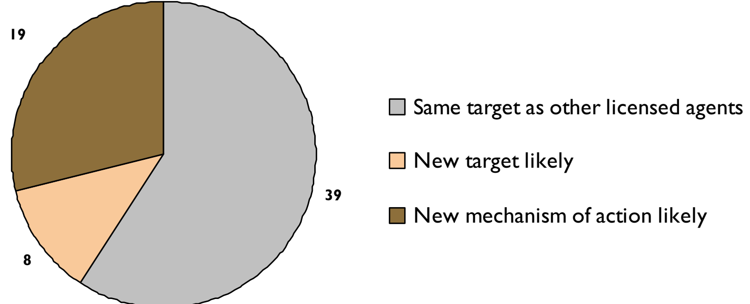
3.3.5 New presentations of licensed antibacterial agents

Of the 24 new presentations of licensed antibacterial agents, 11 were assigned *in vitro* activity that went beyond the known spectrum of activity of the licensed presentation based on optimistic assumptions of what might be achieved by using a different route of administration. These 11 agents comprised topical or inhalational presentations of ciprofloxacin, tobramycin or amikacin, mainly being developed for *P. aeruginosa* infection. They were assessed as possibly having activity against PRSP based on the higher concentrations that could be achieved in the eye or in the respiratory tract. A list of those 11 agents is provided in Annex B2 (List A).

3.3.6 New active substances

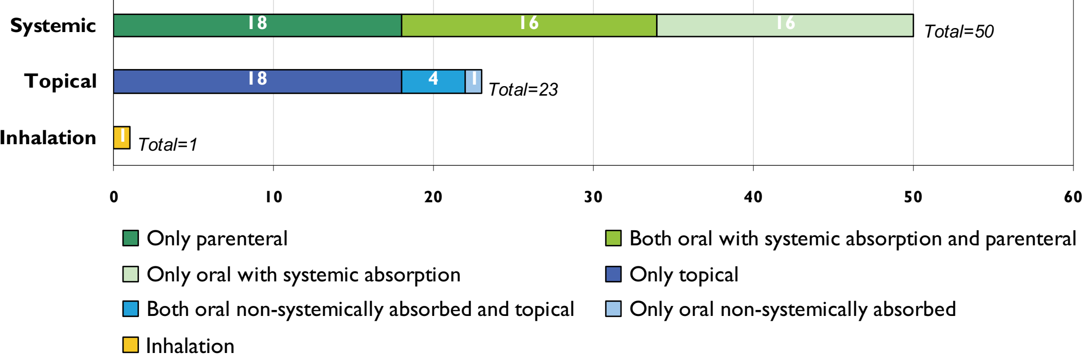
Of the 66 new active substances, 30 (45%) were in phase I of development, 16 (24%) in phase II, nine (14%) in phase III, eight (12%) had been filed with a regulatory agency and three (5%) were reported to have been suspended from further development. Twenty-seven (41%) of these 66 compounds were assessed as having either a new target or a new mechanism of action, thus displaying some degree of novelty (Figure 5). A list of those 39 agents that were assessed as acting on the same target via the same mechanism of action as that of at least one previously licensed antibacterial agent is provided in Annex B2 (List B).

Figure 5. Novelty of new antibacterial agents which, in a best-case scenario (*in vitro* activity based on actual data and assumed *in vitro* activity based on known class properties or mechanisms of action), could have activity against the selected bacteria (n=66, as of 14 March 2008).



An analysis by route of administration (Figure 6) showed that, at the time of the search, 50 of these 66 agents were formulated for systemic administration (34 for oral and 33 for parenteral administration).

Figure 6. Route of administration[a] of new antibacterial agents which, in a best-case scenario (*in vitro* activity based on actual data and assumed *in vitro* activity based on known class properties or mechanisms of action), could have activity against the selected bacteria (n=66, as of 14 March 2008).



[a] *Some agents have several possible routes of administration.*

A list of agents with new mechanism of action or new target and topical administration can be found in Annex B2 (List C). Agents that have a new mechanism of action or a new target and that can be systemically administered are shown in Figure 7. It should be noted that the 15 agents in this figure result from adopting the best-case scenario approach described above, i.e. taking into account the agents with actual data available and also those with the likelihood of activity based on known class properties or mechanisms of action.

Figure 7. New systemic antibacterial agents with new target or new mechanism of action and *in vitro* activity against selected bacteria based on actual data (● green) or assumed activity based on known class properties or mechanisms of action (● grey), by phase of development (n=15, as of 14 March 2008). Total represents the number of agents active against each of the selected bacteria in a best-case scenario.

Name of agent	Gram-positive bacteria				Gram-negative bacteria			Phase of development
	MRSA	VISA/VRSA	PRSP	VRE	3rd Gen Cep. R ENB	Carb. R ENB	Carb. R NF GNB	
WAP 8294A2	● green							I
PZ-601*	● green	● green	● green	● green	● green			I
ME 1036*	● green	● green	● green		● green			I
NXL 101	● green	● green	● green	● green				I
Friulimicin B	● green	● green	● green	● green				I
Oritavancin	● green	● green	● green	● green				Filed
Telavancin	● green	● green	● green	● green				Filed
Ceftobiprole medocaril†	● green	● green	● green					Filed
Ceftaroline fosamil †	● green	● green	● green					III
Tomopenem‡	● green	● grey	● green		● green	● green	● green	II
hLF1-11	● green	● green			● grey	● grey	● grey	II
Lactoferrin	● green	● green			● grey	● grey	● grey	I
Talactoferrin-alfa	● grey	● grey			● grey	● grey	● grey	II
Opebacan					● grey	● grey	● grey	III
NXL 104/ceftazidime §					● green	● green	● green	I
● green	12	9	8	5	3	2	2	
● grey	1	3	1	1	4	4	4	
Total	**13**	**12**	**9**	**6**	**7**	**6**	**6**	

Abbreviations:
– *3rd Gen Cep. R ENB: Third-generation cephalosporin-resistant* Enterobacteriaceae
– *Carb. R ENB: Carbapenem-resistant* Enterobacteriaceae
– *Carb. R NF GNB: Carbapenem-resistant non-fermentative Gram-negative bacilli*

** Are no more active than earlier carbapenems against Gram-negative bacteria. The relative novelty of these agents was based on a better profile of activity against antibiotic-resistant Gram-positive bacteria.*

† Reported MRSA activity suggests a different binding profile to PBPs than currently licensed cephalosporins.

‡ Reported activity against bacteria resistant to earlier carbapenems might not actually represent a different target range but could be due only to evasion of resistance mechanisms by the new agent.

§ Ceftazidime is a licensed cephalosporin. Only the beta-lactamase inhibitor NXL104 displays additional enzyme inhibition resulting in a broader range of activity than earlier agents.

Note: Phase of development refers to the highest phase of development, regardless of indication.

Table 4 describes the individual characteristics of the antibacterial agents presented in Figure 7.

Table 4. New systemic antibacterial agents with new target or new mechanism of action and in vitro activity based on actual data or assumed based on known class properties or mechanisms of action against the selected bacteria (n=15, as of 14 March 2008).

Name of agent	Mechanism of action (MoA)	Degree of novelty	Route of administration*
WAP 8294A2	Membrane integrity antagonist	New MoA	IV, Top
PZ-601	Cell wall synthesis inhibitor	New target	IV
ME 1036	Cell wall synthesis inhibitor	New target	IV
NXL 101	DNA gyrase inhibitors / DNA topoisomerase inhibitor	New MoA	IV, PO
Friulimicin B	Cell wall synthesis inhibitor	New MoA	IV
Oritavancin	Cell wall synthesis inhibitor Membrane integrity antagonist	New target	IV, PO
Telavancin	Cell wall synthesis inhibitor Membrane integrity antagonist	New target	IV
Ceftobiprole medocaril	Cell wall synthesis inhibitor	New target	IV
Ceftaroline fosamil	Cell wall synthesis inhibitor	New target	IV
Tomopenem	Cell wall synthesis inhibitor	New target	IV
hLF1-11	Chelating agent / immunomodulation	New MoA	IV, PO
Lactoferrin	Chelating agent / immunomodulation	New MoA	IV, PO
Talactoferrin-alfa†	Chelating agent / immunomodulation	New MoA	PO, Top
Opebacan†	Membrane permeability enhancer/immunomodulation	New MoA	IV
NXL104/ ceftazidime	Beta-lactamase inhibitor + cell-wall synthesis inhibitor	New target	IV

** Information on routes of administration is uncertain in early drug development.*

† Agents with only assumed in vitro *activity.*

3.4 Discussion

This study is believed to be the first systematic review of available commercial databases that compile publicly-available information on antibacterial agents in clinical development.

The focus of the study was to give a detailed description of agents with some degree of novelty. These agents may have the potential to become useful in the treatment of infections due to the selected multidrug-resistant bacteria. They may also have the potential to become useful in the treatment of other bacteria of public health importance that were not included in this study such as *Neisseria gonorrhoeae* or fluoroquinolone-resistant Gram-negative bacteria.

A decision was made to take an optimistic approach to the identification of agents potentially active against the selected panel of antibiotic-resistant bacteria. For example, the pooled dataset was built taking into consideration the most optimistic phase of development reflected in the databases used (i.e. the highest phase of clinical development was taken; and reports on clinical development were preferred over those of suspension or discontinuation of studies). The possibility of cross- and co-resistance was not taken into account when assessing *in vitro* activities. Also, assumptions were made on *in vitro* activity based on class properties in the absence of data in order to present a best-case scenario. All of these approaches could have lead to the pipeline looking 'healthier' than it actually is.

Based on this optimistic approach, the main results from the analysis conducted by the ECDC/EMEA Working Group were as follows:

- Of 167 agents identified by the searches, there were 90 antibacterial agents with *in vitro* activity in a best-case scenario (based on actual data or assumed based on known class properties or mechanisms of action) against at least one organism in the panel of bacteria selected for their public health importance.
- Of these 90 agents, 24 were new presentations of licensed antibacterial agents and 66 were new active substances.

- Of the 66 new active agents, 27 were assessed as having either a new target or a new mechanism of action, thus potentially offering a benefit over existing antibiotics.
- Of these 27 agents, there were 15 that could be systemically administered. These 15 agents included 13 for which actual data indicated *in vitro* activity against at least one of the selected bacteria, and two additional agents for which activity was assumed due to known class properties or mechanisms of action.
- Of the 15 agents with systemic administration, eight were judged to have activity against at least one of the selected Gram-negative bacteria.
- Of the eight with activity against Gram-negative bacteria, four had activity based on actual data and four had assumed activity based on known class properties or mechanisms of action.
- Of the four with activity against Gram-negative bacteria based on actual data, two acted on new or possibly new targets and none via new mechanisms of action.

The data search was done on 14 March 2008. These results therefore represent the state of the antibacterial drug pipeline at the search date. Since this date, development was discontinued for several agents. Other agents moved from preclinical to clinical development.

Overall, these findings corroborate earlier reports [3,45-46] on the lack of antibacterial drug development to tackle multidrug resistance. In particular, the results of the current analysis indicate that there is a general lack of agents that act on new targets or possess new mechanisms of action.

The IDSA has also attempted to give a systematic account of what is in the antibacterial pipeline, restricted to agents in phase II of clinical development. These reports used the following sources to identify drug candidates: the Pharmaceutical Research and Manufacturers Association survey of medicines in development for treatment of infectious diseases abstracts from the Interscience Conferences on Antimicrobial Agents and Chemotherapy 2002–2004; the websites of the 15 major pharmaceutical and the seven largest biotechnology companies identified by Spellberg *et al.* [6] and literature referenced in the PubMed database from January 2003 to December 2007. In contrast, this study takes also into account investigational agents in phase I of clinical trials. In addition, the databases used in the present analysis state screening all of the sources used by IDSA plus considering additional specialised literature as well as having regular direct communication with companies (see Annex B2).

There are many reasons for the current situation, including difficulties encountered in identifying new bacterial targets and the possibility that the majority of targets amenable to antibacterial activity have already been identified [50]. It is no surprise then that the majority of the investigational agents identified by the searches were directed against the same target and had the same mechanism of action as at least one licensed agent. Almost a third of those with activity against the selected panel of bacteria were new presentations of licensed antibacterial agents. Only 11 out of the 24 new presentations of licensed agents were thought likely to possess an extended spectrum of activity (and only against penicillin-resistant *S. pneumoniae*) as a result of the new route of administration.

It could be argued that there are a number of agents in preclinical development that could improve the gloomy picture presented here. However, it was decided not to include an in-depth exploration of the preclinical pipeline given the high attrition rate of compounds during this phase of development and also due to the scarcity of data available for review. Moreover, it should be noted that the databases used excluded information on agents that were, so far, under development only by academic groups. The search criteria contained EphMRA or EphMRA-derived ATC codes, which are assigned by the database companies and could be subject to variability. Both of these limitations were minimised by performing the literature search for reviews on PubMed and by selecting broader criteria for the main search as described previously.

Multidrug-resistant Gram-negative bacteria constitute a major challenge for the future [45]. Therefore, the lack of systemically administered agents with activity against Gram-negative bacteria displaying new mechanisms of action found in this study is particularly worrisome, and more so when the high attrition rates for agents in early stages of clinical development [50] is taken into consideration. In fact, it is unclear if any of these identified agents will ever reach the market, and if they do, they may be indicated for use in a very limited range of infections. Even if a public health driven approach for R&D of antibacterial agents is commenced in the near future the burden of resistance will inevitably increase during the next years. Therefore, a European and global strategy to address this serious problem is urgently needed, and measures that spur new antibacterial drug development need to be put in place.

4 Conclusions

- There is a gap between the burden of infections due to multidrug-resistant bacteria and the development of new antibiotics to tackle the problem.
- Resistance to antibiotics is high among Gram-positive and Gram-negative bacteria that cause serious infections in humans.
- Resistance is increasing among certain Gram-negative bacteria.
- Infections caused by multidrug-resistant bacteria are associated with excess morbidity and mortality.
- Infections caused by multidrug-resistant bacteria are associated with substantial extra costs.
- Very few antibacterial agents with new mechanisms of action are under development to meet the challenge of multidrug resistance.
- There is a particular lack of new agents to treat infections due to multidrug-resistant Gram-negative bacteria.
- A European and global strategy to address this gap is urgently needed.

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Glossary

Antimicrobial agents: medicinal products that kill or stop the growth of living microorganisms and include **antibacterial agents** (more commonly referred to as **antibiotics**), which are active against bacterial infections.

Antibacterial (antibiotic) resistance: is the ability of a bacterium to survive and even replicate during a course of treatment with a specific antibiotic. Failure to resolve an infection with the first course of antibiotic treatment may mean that the infection may spread, may become more severe and may be more difficult to treat with the next antibiotic that is tried.

- **Intrinsic resistance:** natural resistance of bacteria to certain antibiotics.
- **Acquired resistance:** normally susceptible bacteria have become resistant as a result of adaptation through genetic change.
- **Multidrug resistance:** corresponds to resistance of a bacterium to multiple antibiotics.

Attrition rate: the number of antibacterial agents moving out of development over a specific period of time.

Bacteria are microorganisms and can be divided into categories according to several criteria. One way to classify bacteria is based on staining them using a method that divides most bacteria into two groups – **Gram-positive** and **Gram-negative** – according to the properties of their cell walls.

Bloodstream infection: presence of bacteria in the blood, in quantities that allow isolation from blood samples in the laboratory.

Burden of disease: refers to the overall impact of disabling clinical or public health conditions at the individual level, or at the societal level or to the economic costs of diseases.

Carbapenemase: enzyme produced by some bacteria causing resistance to carbapenems, a class of antibiotics.

Cephalosporins: a class of antibiotics. The class is often divided into generations to indicate incremental increase in spectrum of antibacterial activity. Third-generation cephalosporins, for example, have a broad spectrum of activity and further increased activity against Gram-negative bacteria as compared to previous generations of cephalosporins.

Clinical development of antibacterial agents: see annex B.

Clinical trial: a research activity that involves the administration of a test regimen to humans to evaluate its efficacy and safety.

Commensal flora: the natural bacteria that live on and in a healthy person.

Comorbidities: the presence of one or more diseases or disorders in addition to a primary disease or disorder.

Drug (antibiotic) formulation: the composition of a dosage form, including the characteristics of its raw materials and the operations required to process it. Examples are **oral formulation** (by mouth), **intravenous formulation** (by infusion into a vein).

Extended-spectrum beta-lactamase (ESBL): enzyme produced by Gram-negative bacteria causing resistance to most beta-lactams, including most penicillin and cephalosporins.

***Enterobacteriaceae*:** a family of Gram-negative bacteria. Examples of common *Enterobacteriaceae* are *Escherichia coli* and *Klebsiella pneumoniae*.

Gram-positive bacteria: bacteria that are stained purple or violet by Gram staining.

Gram-negative bacteria: bacteria that cannot retain the purple stain of Gram staining and are stained pink as a result of Gram staining.

***In vitro* activity:** activity tested outside the living body and in an artificial environment.

Morbidity: any departure, subjective or objective, from a state of physiological or psychological well-being.

Mortality rate: an estimate of the portion of a population that dies during a specified period.

Multidrug resistance: occurs when a bacterium is resistant to the action of many types of antibiotics. This severely limits the choice of antibiotics that would be suitable for treatment.

Non-fermentative bacteria: bacteria that do not ferment sugars, which distinguishes them from fermentative bacteria.

Nosocomial (hospital-acquired) infection: an infection occurring in a hospital or another healthcare facility, when the infection was not present or incubating at time of admission.

Pharmacokinetics: study of the rate of drug action, particularly with respect to the variation of drug concentrations in tissues with time, and the absorption, metabolism and excretion of drugs and metabolites (i.e. what the body does to the drug).

Pharmacodynamics: study of the physiological effects of drugs on the body (or on microorganisms within or on the body), the mechanisms of drug action and the relationship between drug concentration and effect (i.e. what the drug does to the body or microorganisms).

PK/PD: Pharmacokinetics/pharmacodynamics.

Phases of clinical trials of antibiotics: see annex B.

Preclinical development of antibiotics: see annex B.

Priority medicines: those medicines which are needed to meet the priority healthcare needs of the population ('essential medicines') but which have not yet been developed. In this Report, a 'priority' medicine for a priority disease is by definition also a significant improvement over already marketed agents.

Soft tissue: tissues that connect, support, or surround other structures and organs of the body, e.g. tendons, ligaments, muscles, fibrous tissue.

Systemic (or systemically administered) antibiotics: compounds administered parenterally (e.g. intravenously) or systemically absorbed after oral administration.

Systemic infection: an infection in which the pathogen is distributed throughout the body rather than concentrated in one area.

Topical antibiotics: antibiotic applied to body surfaces, e.g. to treat skin infections.

Annex A: Mandate, composition, meetings, roles and responsibilities

Mandate

The ECDC/EMEA Joint Working Group is agreed by the ECDC and the EMEA to oversee, facilitate, follow-up and be part of the work aimed at producing a report on the gap between the increasing prevalence of multidrug-resistant bacteria and antibacterial drug development aimed at treating such infections. The Scientific Committees of the ECDC and the EMEA will finally adopt the Technical Report prior to publication.

Composition and meetings

1. Core members of the Joint Working Group

- Two members appointed by each of the Scientific Committees from ECDC and EMEA, respectively.
- One representative from the administrative staff of EMEA and ECDC, respectively.
- Two co-opted independent experts. They will be selected by the working group for their clinical/microbiological expertise in the field of interest.
- The paid consultant employed to run the project.

2. Observers

- One observer from each of DG Enterprise, DG SANCO, DG Research and ESCMID[17].

3. Invitation of additional experts to attend a working group meeting

- Invitation of additional experts is made on a case-by-case basis according to the expertise required to provide advice. The Chairperson, together with the members of the implementation group, will call for additional experts to attend working group meetings or other ad hoc technical meetings that may become necessary.
- The working group may decide to invite representatives of interested parties (e.g. industry organisations) to address and discuss issues of common interest, such as aspects on the drug development pipeline.

Chairperson and Vice-Chairperson

The Chairperson – and in his absence, the Vice-Chairperson – is in charge of the efficient conduct of the business of the working group meetings and shall in particular:

- Plan the work of the working group meetings together with the implementation group and paid consultant.
- Ensure the fulfilment of the mandate of the working group.
- Be in charge of the conduct and running of the meetings.
- Seek confirmation from working group members that no conflict of interest exists in relation with topics raised during meetings.

The Vice-Chairperson will deputise for the Chairperson when the latter is unable to chair either all or part of the working group meeting. On such occasions, the Chairperson will seek the agreement of the Vice-Chairperson as early as possible, prior to the meeting and the implementation group shall be informed immediately.

Election of Chairperson and Vice-Chairperson

Core members of the working group shall elect one of the core members to act as Chairperson and one to act as Vice-Chairperson.

Organisation of meetings

1. Dates for working group meetings will preliminary be set for 2008 with additional ad hoc meetings to be decided as appropriate (see point 7).

[17] European Society of Clinical Microbiology and Infectious Diseases

2. The working group shall meet at the ECDC in Stockholm. Ad hoc meetings may also take place at the EMEA in London.
3. The meetings will be held and minuted in English and sent to EMEA and ECDC Scientific Committees for information.
4. The draft agenda for every meeting shall be circulated, together with the relating documents, by the implementation group/paid consultant, in consultation with the Chairperson, at least seven calendar days before the meeting.
5. When a member of the working group is unable to participate in a meeting, or part of a meeting, he/she must inform the paid consultant/implementation group in advance, in writing.
6. A minimum of five core members are required to attend the working group meeting or the meeting will have to be rescheduled.
7. The proposal for an ad hoc working group meeting and the conduct and objectives of such meeting shall be proposed by the Chairperson in collaboration with the implementation group. The implementation group/paid consultant shall inform the working group on the need for an ad hoc meeting as early as possible.

Roles and responsibilities of the working group

In accordance with its mandate to oversee, facilitate, follow-up and advise on the work aimed at producing a report on the gap analysis, the working group and its Chairman will regularly be kept updated on the progress of the project as set out in this document.

- The main role of the working group will be to advise the implementation group with regard to:
  - definitions of objectives and main output, i.e. the gap analysis and technical reports;
  - overall strategy;
  - definitions of the individual components of the gap analysis;
  - proposals for improvements to reach the objectives;
  - discussions on the scientific methodology for the individual project;
  - content of the technical reports; and
  - giving support to the implementation group and paid consultant as far as meeting the objectives, including the final reports.

- The members shall provide declarations of interest in the area of antibiotic drug development by filling in an agreed form provided by EMEA/ECDC. In addition, members shall declare any conflict of interest as appropriate before or during the working group meetings. At the discretion of the Chairman, the member may be prevented from active participation on certain specific issues.
- The members shall commit to active participation of the activities of the group. Should a member fail to attend two consecutive meetings, replacement of the member will be considered by any of the appointing bodies.

Roles and responsibilities of the implementation group

Under the authority of the working group, the implementation group shall closely oversee and lead the work of the paid consultant responsible for the daily running of the individual projects.

- Provide technical and scientific lead to the paid consultant.
- Provide legal and regulatory lead to the paid consultant.

The composition of the implementation group includes EMEA and ECDC staff representatives, one of the co-opted members and the paid consultant.

Responsibilities of the paid consultant

The overall projects will be run and monitored by a paid consultant. The responsibilities of the paid consultant include the close monitoring of the ongoing projects to ensure timely feedback of the work in accordance with the agreed timetable. The paid consultant will be part of the implementation group and will produce:

1. A regular report on the progress of the different projects, which includes:
   - monthly written updates to the implementation and working groups members; and
   - regular telephone discussions and agreements with members of the implementation group and the working group Chairperson.

2. In consultation with the Chairperson and the implementation group, the relevant documents to be conveyed to the working group, i.e. timely invitations to meetings, provision of agendas, documents and presentations, as appropriate.
3. In liaison with the different study contractors to convey technical and scientific steer to meet the objectives of each project and thereby to ensure high quality output from the projects.
4. The overarching technical report and reports on the subprojects in liaison with the working group members and implementation group.

Observers and contractors

In addition to the observers mentioned above, the working group may admit additional representatives of international organisations, EU scientific committees or political bodies with interests in the issues of antimicrobial resistance as observers during working group meetings.

In addition, contractors of the scientific projects may be invited to discuss and give presentations to the working group.

General provisions

The members of the working group, as well as observers and all experts, shall not disclose any information, which, by its nature, must be covered by professional secrecy (i.e. not to divulge any of the materials discussed at the meetings until such time that this material becomes published, unless otherwise sanctioned by the working group).

Annex B: Additional information on the study on the burden of infections due to multidrug-resistant bacteria and pipeline analysis

B1 Burden study

B1.1 Table of parameters used

Parameter	Unit	Value	Reference
No. MRSA[a] from LRTI[b] / from BSI[b]	Ratio	1.25	[21]
No. MRSA from SSTI[b] / from BSI	Ratio	5.25	[21]
No. MRSA from UTI[b] / from BSI	Ratio	0.75	[21]
Attributable mortality of MRSA BSI	%	9.8	[26]
Attributable mortality of MRSA LRTI	%	7.0	[26-27]
Attributable mortality of MRSA SSTI	%	1.4	[26-27]
Attributable mortality of MRSA UTI	%	0.2	[26-27]
Extra length of hospital stay for MRSA BSI	Days	8	[29]
Extra length of hospital stay for MRSA LRTI	Days	9.4	[29]
Extra length of hospital stay for MRSA SSTI	Days	5	[29]
Extra length of hospital stay for MRSA UTI	Days	6.1	[28]
No. VRE[c] from wound infection / from BSI	Ratio	4.67	[23]
No. VRE from intra-abdominal infection / from BSI	Ratio	1.89	[23]
No. VRE from UTI / from BSI	Ratio	3.44	[23]
Attributable mortality of VRE BSI	%	25	[23]
Attributable mortality of VRE wound infection	%	6	[23]
Attributable mortality of VRE intra-abdominal infection	%	3	[23]
Attributable mortality of VRE UTI	%	9	[23]
Extra length of hospital stay for VRE BSI	Days	15	[23]
Extra length of hospital stay for VRE wound infection	Days	6.2	[23]
Extra length of hospital stay for VRE intra-abdominal infection	Days	2.6	[23]
Extra length of hospital stay for VRE UTI	Days	5.4	[23]
No. penicilllin-resistant *Streptococcus pneumoniae* from respiratory tract infection/ from BSI	Ratio	2.7	[24]
No. third-generation cephalosporin-resistant *Klebsiella pneumoniae* from LRTI / from BSI[d]	Ratio	1.19	[22]
No. third-generation cephalosporin-resistant *K. pneumoniae* from SSTI / from BSI[d]	Ratio	0.33	[22]
No. third-generation cephalosporin-resistant *K. pneumoniae* from UTI / from BSI[d]	Ratio	1.19	[22]
Attributable mortality of third-generation cephalosporin resistant *E. coli* and *K. pneumoniae* BSI	%	30	[19]
Attributable mortality of third-generation cephalosporin resistant *E. coli* and *K. pneumoniae* LRTI	%	21	[19, 27]

Parameter	Unit	Value	Reference
Attributable mortality of third-generation cephalosporin resistant *E. coli* and *K. pneumoniae* SSTI	%	4	[19, 27]
Attributable mortality of third-generation cephalosporin resistant *E. coli* and *K. pneumoniae* UTI	%	1	[19, 27]
Extra length of hospital stay for third-generation cephalosporin resistant *E. coli* and *K. pneumoniae* infection	Days	11	[18-19]
No. carbapenem-resistant *Pseudomonas aeruginosa* from LRTI / from BSI	Ratio	16.0	[22]
No. carbapenem-resistant *P. aeruginosa* from SSTI / from BSI	Ratio	4.67	[22]
No. carbapenem-resistant *P. aeruginosa* from UTI / from BSI	Ratio	11.3	[22]
Attributable mortality of carbapenem-resistant *P. aeruginosa* BSI	%	17.7	[25]
Attributable mortality of carbapenem-resistant *P. aeruginosa* LRTI	%	12.7	[25, 27]
Attributable mortality of carbapenem-resistant *P. aeruginosa* SSTI	%	2.6	[25, 27]
Attributable mortality of carbapenem-resistant *P. aeruginosa* UTI	%	0.4	[25, 27]
Extra length of hospital stay for carbapenem-resistant *P. aeruginosa* BSI[e]	Days	5.7	[25]

[a] *Methicillin-resistant* Staphylococcus aureus *(MRSA)*

[b] *BSI, bloodstream infection; LRTI, lower respiratory tract infection; SSTI, skin and soft tissue infection (including wounds and surgical site infections); UTI, urinary tract infection*

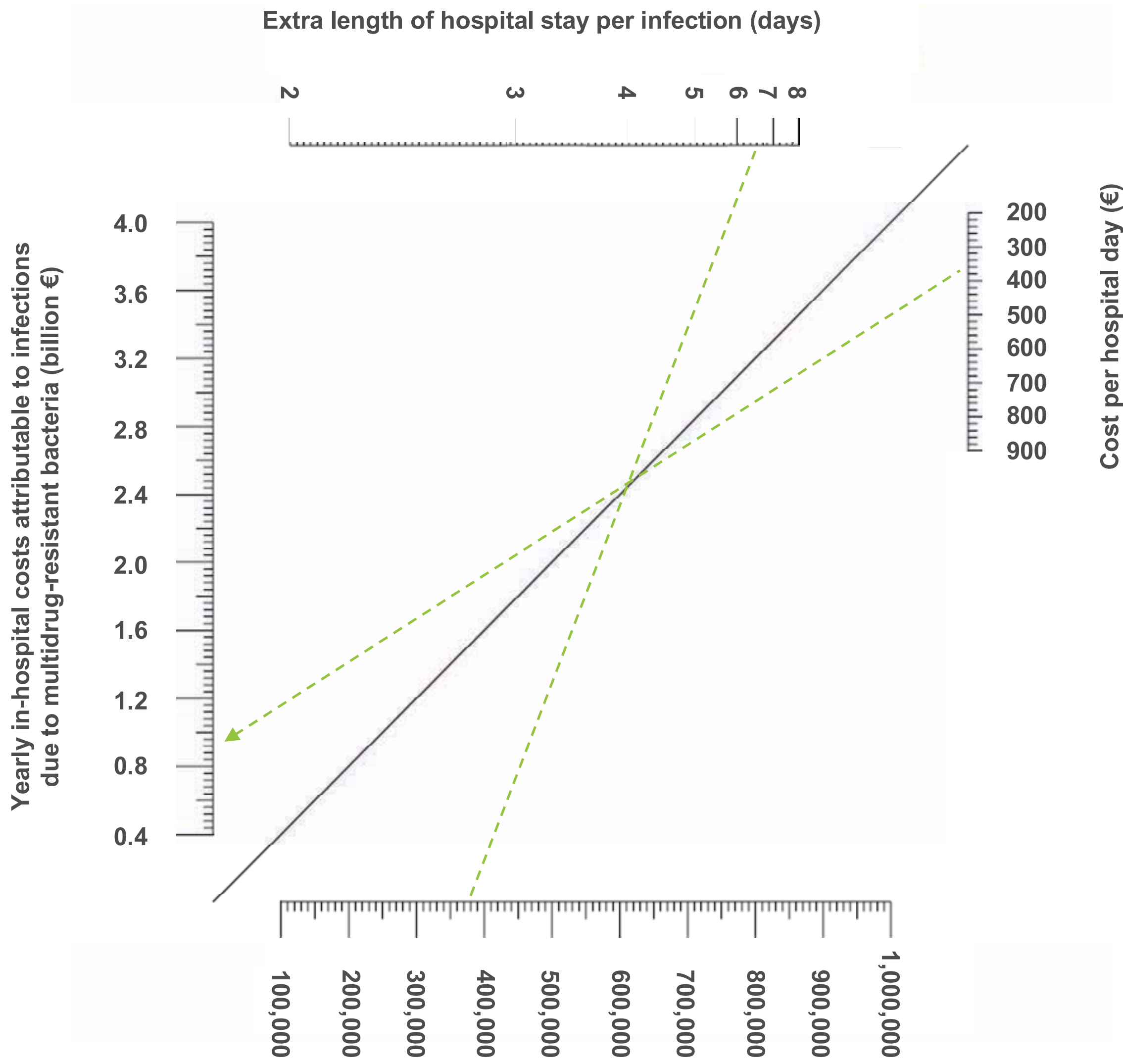
[c] *Vancomycin-resistant enterococci (VRE)*

[d] *These ratios were also used for third-generation cephalosporin resistant* Escherichia coli*.*

[e] *This extra length of hospital stay was also used for LRTI, SSTI and UTI.*

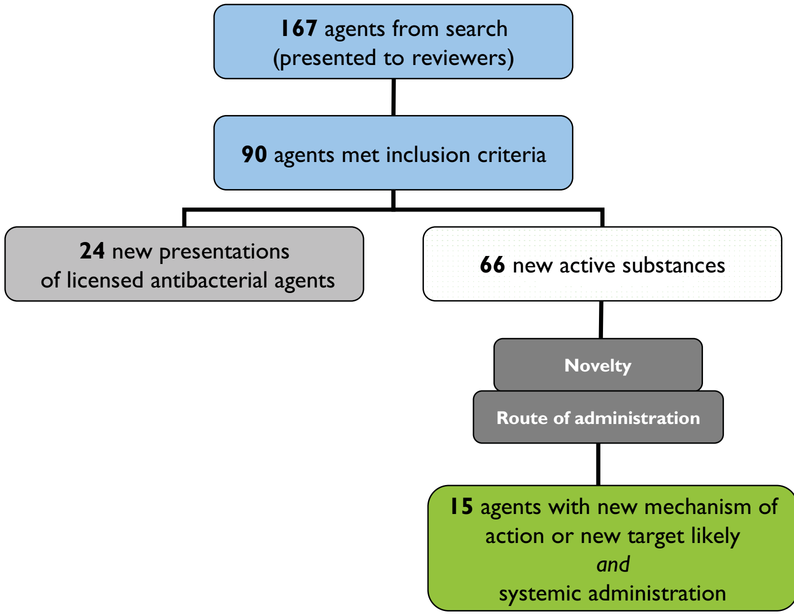
B1.2 Nomogram

This nomogram can be used to calculate yearly in-hospital costs attributable to infections due to multidrug-resistant bacteria with various values for the total number of infections, the average extra length of hospital stay per infection and the average cost per hospital day.



B2 Pipeline analysis

B2.1 Flow-chart of the pipeline analysis



B2.2 Agents with same target as previously licensed agents.

List A. New presentations of licensed antibacterial agents with assigned *in vitro* activity that goes beyond the known spectrum of activity of the licensed presentation, based on optimistic assumptions of the activity that might be achieved by using a different route of administration (n=11, search date 14 March 2008).

Amikacin inhalation

Amikacin sustained release

Ciprofloxacin inhalation

Research programme: liposomal ciprofloxacin inhalation

SPRC AB01

Tobramycin liposomal

Tobramycin inhalational

Tobramycin/Dexamethasone

Tobramycin/fosfomycin

Tobramycin/prednisolone acetate

Ciprofloxacin otic solution

List B. Agents that were assessed as acting on the same target via the same mechanism of action as that of at least one previously licensed antibacterial agent (n=39, search date 14th March 2008).

AFN 1252	E 5065	RX 1741
AR 709	EDP 420	Sulopenem
BC-3205	Faropenem medoxomil	Tebipenem pivoxil
BC-7013	Finafloxacin	WCK 1152
CBR 2092	Iclaprim	WCK 771A
Cetefloxacin	MCB 3837	Zabofloxacin
Cethromycin	Rifalazil	TD 1792
CS 834	MK 2764	WQ 3034
TR 701	Nemonoxacin	Clinafloxacin
DC 159a	NXL 103	Dalbavancin
DW 286	Ozenoxacin	Trospectomycin
DX 619	PF 3709270	Besifloxacin
E 4767	Ranbezolid	NPI-32101

List C. New topical* antibacterial agents with new target or new mechanism of action and in vitro activity based on actual data or assumed based on known class properties or mechanisms of action against the selected bacteria (n=12, search date 14 March 2008).

AN 0128

Bacteriophage, pseudomonal

Iseganan†

Lysostaphin cream

Lysostaphin (topical)

NVC 422

Omiganan

OPT 80

Pexiganan

Ramoplanin

REP 8839

XOMA 629

** Oral non-systemically absorbed agents have been also counted in this category.*

† In addition, Iseganan can also be given via inhalation.

B3. Technical information provided by the pipeline database companies

B3.1 Adis Insight R&D (Wolters Kluwer Health)

Internal processes

Information Sources

Proceedings from 150+ major scientific meetings are monitored routinely and pertinent data presented as posters and abstracts are included in R&D Insight within 10 days of each meeting. News from media releases is added to the database daily from PR Newswire, Business Wire, Canada Newswire, Hugin Online, Japan CNN and direct from company websites that do not use these services.

Additional sources are:

- Direct contact with pharmaceutical company representatives to verify information.
- Company reports and regulatory filings are routinely checked for new and updated information. More than 3 500 companies are monitored.
- Information from ongoing clinical trials is incorporated from international media releases and more than 20 clinical trial registries, such as www.clinicaltrials.gov.
- Scientific journals: more than 1 400 journals are monitored routinely for inclusion

All information sources are evaluated by expert staff for relevance to *R&D Insight*. All staff are provided with comprehensive training by experienced senior editors on the selection of relevant material.

Inclusion and exclusion criteria

All information on drugs being developed by pharmaceutical companies and biotechnology companies, either alone or in collaboration with non-commercial institutions, is entered into the database for all countries, all therapeutic areas and all indications. Information about the following is excluded:

- Medical devices (unless in combination with a drug).
- Generic drugs, unless undergoing reformulation and regulation as new drugs, and biosimilars.
- Drugs launched in all major markets prior to 1995 that do not have new development since launch.

Company profiles – Inclusion criteria

Adis *R&D Insight* contains links to more than 400 company profiles, detailing information about each company's subsidiaries, history, R&D expenditure, licensing agreements, mergers and acquisitions, as well as links to the complete R&D pipeline for each company.

To qualify for the addition of a Company Profile to *R&D Insight* a company must have:

- 10 or more active drugs in development;
- be amongst the top 50 biotechnology companies according to MedAd News (Engel Publishing);
- be a client of Wolters Kluwer Health with five or more products in development; or
- made a specific request to have a company profile included.

Timeliness

- Client queries and requests: response within 24 hours of receipt.
- Media releases: three-day turnaround.
- Scientific conference processing: completion 10 working days post conference.
- Company reports: annual, quarterly and half-yearly reports completed throughout each year as they become available;
- Clinical trial data: incorporated when the data is made available.
- All relevant scientific data added for completeness in advance of regulatory submission (phase III).

EPhMRA codes

These are codes used by the European Pharmaceutical Market Research Association (EPhMRA) classification system. These codes mainly classify products according to their indications and use. Therefore, the same compound may be found in several classes, depending on the product. For example, Naproxen tablets can be classified in M1A (anti-rheumatic), N2B (analgesic) and G2C (if indicated for gynaecological conditions only). The main purpose of the EPhMRA classification system is to satisfy the marketing needs of pharmaceutical companies.

Phase of development – Definitions

Development phase	Definition
Research	The early development of a drug research programme, including lead screening and lead validation. Used when a company has identified a small number of candidates and is conducting early research to identify or optimise lead compounds for further *in vitro* and *in vivo* testing.
Preclinical	The drug is being tested *in vitro* (cells, test tubes) or *in vivo* (animals). The developer applies for permission to go into clinical testing. The procedure for applying for permission will depend on the country. For example, in the USA, an Investigational New Drug (IND) application must be granted before clinical trials can begin.
Phase 0	Purpose: the drug is being tested in first-in-human trials conducted in accordance with US FDA 2006 Guidance on Exploratory Investigational New Drug (IND) studies. These studies are designed to speed up development of promising drugs by establishing very early on whether the agent behaves in human subjects as was anticipated from preclinical studies. Studies will include the administration of single subtherapeutic doses of the study drug to a small number of subjects (10–15) to gather preliminary data on the agent's pharmacokinetics and pharmacodynamics.
Phase I	Purpose: to identify adverse events and determine efficacy and initial pharmacokinetics. These trials of a new drug or therapy are usually conducted in normal male volunteers. Patients may be evaluated instead of volunteers in phase I trials in order to treat immediately life-threatening and serious conditions for which there is no comparable or satisfactory alternative therapy available. In addition, expanded access programmes allow patients for whom standard therapy is ineffective or contraindicated, and who are ineligible to enter trials, to receive investigational drugs in parallel with controlled trials.
Phase I/II	Purpose: to establish the maximum tolerated dose (phase I) and drug tolerability (phase II) in patients. In life threatening and serious conditions, the second part may confirm preliminary efficacy.
Phase II	Purpose: to provide a measure of efficacy in addition to short-term tolerability and safety. Phase II studies are conducted in patients who have the disease or condition that the drug is intended to treat. Other phase II study objectives include determining the minimum dose that is maximally effective, or that is sufficiently effective without undue toxicity. For the purposes of using R&D Insight, phase II includes **phase IIa** pilot or feasibility trials, and **phase IIb** well controlled, pivotal trials.
Phase II/III	Purpose: to address within a single trial objective what is normally addressed through separate trials in phases IIb and III. The aim of having the seamless phase II/III trial design is so that data can be used more efficiently, which may lead to a reduction in the duration of drug development. The trial is designed to assess efficacy and safety of the test drug and most are designed with parallel treatment groups rather than crossover.
Phase III	Purpose: to confirm efficacy and monitor adverse reactions from long-term use. In phase III studies, a drug is tested under conditions more closely resembling those under which the drug would be used if approved for marketing. The goal is to gather additional information about efficacy and tolerability that is needed to evaluate the overall benefit-risk relationship of the drug and to provide an adequate basis for physician labelling. NB. Approval/disapproval decisions are based on the results of adequate and well-controlled (pivotal) studies. To be considered pivotal, a study must meet at least the following four FDA-defined criteria; they must be: (1) controlled – using placebo or a standard therapy; (2) double-blind – when such a design is practical and ethical; (3) randomised; (4) of adequate size – study sample size is a common clinical trial design flaw. For the purposes of using R&D Insight, phase III includes **phase IIIa** and **phase IIIb** trials. Phase IIIb trials are usually those undertaken after a regulatory dossier has been submitted.
Pre-registration	All the necessary clinical trials have been completed and the drug is waiting for registration or approval for use by a governing body. For example, a New Drug Application (NDA) has been filed with the FDA in the USA.
Registered	The drug has been registered or approved for use in a particular country, or group of countries such as the European Union countries.
Launched	The drug has been launched and is now marketed in a particular country, or group of countries.
Discontinued	The company has chosen to stop development. This term is usually qualified by the phase at which development was discontinued, for example, discontinued (preclinical).

Development phase	Definition
No development reported	If there has been no activity associated with a drug (no commercial information released, no recently published studies) for 18 months to two years, the term 'no development reported' is assigned. The time frame depends on the last phase of the drug. This is the term used until a drug is confirmed as discontinued, withdrawn or suspended, or activity is resumed.
Withdrawn	The drug has been withdrawn from the market. This term applies to drugs that have been launched but subsequently withdrawn from the market.
Suspended	This term is used when a company has suspended development of a drug, often in order to focus on the development of some other drug. Development has not been discontinued.
Clinical (Phase unknown)	This option is only used when the clinical phase of development is unclear.
Phase groupings	
Active	This group will include all active phases: those which are not discontinued, suspended, withdrawn, or have a no development reported status.
Inactive	This group will include all inactive phases: those which are discontinued, suspended, withdrawn, or have a no development reported status.
Clinical	This group will include all active, clinical phases from phase 0 to launched.
Preclinical	This group will include active, preclinical and research phases.

B3.2 Pharmaprojects (T&F Informa UK Ltd.)

Development status

The following development stages are used throughout Pharmaprojects:

Preclinical	All stages of preclinical investigation including discovery, research, lead optimisation. This is also used where the developmental status is unknown.
Phase I clinical trial	Human pharmacokinetic and volunteer studies.
Phase II clinical trial	Early clinical studies to demonstrate activity in patients.
Phase III clinical trial	Multicentre clinical trials to obtain data for registration.
Clinical trial	In clinical trials, stage unknown
Pre-registration (also known as pre-approval)	Registration documents submitted but not yet approved.
Registered (also known as approved)	Registration obtained but marketing not yet started.
Launched	Product available in at least one market.
Suspended	Development suspended with the possibility of restarting.
Discontinued	Development terminated.
No development reported	No evidence of continuing development reported.
Withdrawn	Withdrawn from marketing.

How the drug profiles are updated

As a drug advances and new information becomes available, its profile is amended and updated. Where there is controversy surrounding important information, this is verified with the developing company before a profile is amended.

The Pharmaprojects editorial staff are continually reviewing worldwide information on new drug development. In addition, a significant amount of new information derives from the work of the editorial team of Pharmaprojects' sister publication Scrip World Pharmaceutical News.

Much of the Pharmaprojects data comes directly from the companies themselves, with extensive reference to company websites, reports and press releases.

There is continual two-way communication between Pharmaprojects staff and their contacts in the pharmaceutical and biotechnology industries; both to gather new data and importantly, to verify information obtained from other sources.

Every company with an entry in Pharmaprojects is asked to verify, at least annually, the information relating to its development pipeline.

Pharmaprojects editorial staff attend the major international medical and scientific congresses to gather information often entering the public arena for the first time. Editors use these opportunities to question company personnel attending the congress to ascertain the companies' development plans for any products reported. This ensures that, as far as possible, all new compounds entered into the database are true development candidates.

International research literature is scanned for new developments; however, there is less dependence on journals since, by definition, research information is 'old' by the time it is published.

Criteria for addition to Pharmaprojects of new drug candidates

Here, we look at the criteria used to decide when to add a new product entry on to the database – and, just as importantly, when not to.

The aim of Pharmaprojects is to provide the most accurate picture possible of what is really going on in pharmaceutical research and development worldwide. A vital part of this is deciding whether or not a preclinical compound is a genuine candidate for development as a new drug. Now more than ever, in the days of combinatorial chemistry and mass screening, there are thousands more compounds synthesised than are development candidates. If we added every compound which we came across onto the database, the Pharmaprojects active database would be huge and just as full of inactive compounds as if we did not perform the 'No Development Reported' procedure (see later). So we have to be selective. One of the questions we get asked most often is what are the criteria our editors use to decide whether or not to add a new compound to the active database. Some databases, particularly those that rely heavily on patent applications as data sources, add on many more preclinical drugs than are ever seriously considered as drug development candidates. While it can be useful to alert those in the industry to early research areas in which companies have interests, the downside is that it can give a badly distorted view of what is really in company's portfolios. At Pharmaprojects, we make strenuous efforts to discover whether or not a drug is a serious candidate for development before adding it on to the database to keep ours the most accurate reflection of genuine pipelines.

So how do we do this? Certain data sources themselves can be regarded as confirmation of active development; for example, if a drug appears on the pipeline section of a company's website. Often companies will contact us themselves and provide us with details of new drugs that they wish to see included in their Pharmaprojects pipeline, particularly if the drugs are available for licensing. Company press releases or R&D portfolio presentations are also reliable sources. It gets more difficult if the first appearance of a compound is not in such a source. In such cases where it is not explicitly stated that a compound is in development, we will generally contact the company concerned to see if it is a pipeline compound. However, many companies decline to comment on early development compounds. It is in these cases that we have to use other criteria to decide whether or not to add the compound to the database.

Although we do use a set series of criteria to evaluate the likelihood of a product being in development, we do not use a 'points' system or a formula; rather we combine our analysis on a number of fronts with the years of experience our editorial team has accrued. However, here are a number of points that they will consider.

Firstly, the name of the compound will be considered. If it is obviously an INN, it is more likely to be a serious development candidate. If it is given a more spurious name, for instance one based on the research institute where it was synthesised or discovered, it might be treated with more caution. A lot of information can be gathered from a compound's lab code. For instance, Merck & Co's research compounds begin with an L- code; when they are chosen for development, they are rechristened with an MK- number. The same applies with Abbott and A- codes becoming ABT- codes. Thus an editor coming across an MK- or ABT- code would be inclined to treat this as a potentially serious development candidate. Also, as most companies label their lab codes sequentially, we can get a good idea of the age of a compound, with a higher numbered code more likely to be a new candidate.

A very important consideration is the published source of the data. Most drugs that we have to decide whether or not to include will be those presented at conferences or appearing in journals. The identity of the journal thus has

a bearing. For instance, the Japanese Journal of Antibiotics is a very important source of useful information for us, but it also includes reports on many antibiotics that have been newly isolated, but are not serious candidates for development as medicines. Thus, we have to be careful.

One of the ways in which we are aided in our decision is by actually looking at the scientific data provided and, in particular, its activity. For instance, in the case of an antibiotic, a good rule of thumb is that reasonable activity is indicated by an MIC of less than 1mg/ml. Higher MICs may thus indicate a less active compound that is therefore less likely to be taken forward. However, this would be organism-dependent, with a higher MIC for a multidrug-resistant organism being looked at more seriously.

We can also apply our judgement on other benefits a new drug has or does not have against existing therapies. These may include a better side-effect profile, easier dosing regimen, or a more convenient route of administration. A serious development candidate would be expected to have advantages in at least one area.

Finally, we are more likely to add in a new profile if the drug is in a new therapeutic area for the company involved. In other words, we may feel it is more important to alert to the fact that a company has moved into the analgesic area with its first compound in that field than we would to inform that a 14th preclinical analgesic had been reported by a company. In the latter case, we would probably wish to ascertain from the company whether the drug was a serious lead rather than just another in a series that they have synthesised.

So the combination of good contacts, entry criteria and, above all, editorial experience come together in deciding whether a drug mentioned in the literature merits inclusion in Pharmaprojects. These procedures, along with others such as the 'No Development Reported' programme enable us to provide what we believe is the most accurate picture of what is really in development at the world's pharmaceutical companies.

Keeping our pipelines accurate using the No Development Reported status

The challenge for Pharmaprojects is to bring you the most accurate picture of what is really happening in pharmaceutical R&D. To do this, we must not only add to the active section of the database all compounds that enter development, but we must remove all of those whose development ceases. The latter task is not as easy as it might seem. Although the discontinuation of products in more advanced stages of development is often high-profile news, companies are usually unwilling to make announcements about drug failures at earlier stages. It is perfectly normal for many compounds not to make it past the early stages of development, but quite naturally, a company is not going to send out a press release every time it drops an early drug candidate.

At Pharmaprojects, we will only list a drug as discontinued if this has positively been confirmed by the company. Therefore, we needed to devise a programme to weed out other drugs whose development is not continuing. This involves contacting companies to ask them about drugs that we suspect have been dropped, and having a way to deal with such drugs that the companies decline to comment on. This is where the 'No Development Reported' (NDR) status comes in.

The first stage of the process is to identify which drugs may have dropped out of development. To do this, we look at how long it is since we last obtained new information on a drug. With our extensive contacts at companies, our wide-range of published information and our series of stringent checks to keep our data up-to-date, if nothing new has been heard on a development project for over a year, we begin to suspect that it has halted. Thus, each month, we produce from our internal database a list of compounds that have not been updated for some time, typically 14 to 18 months (it varies slightly due to our publishing schedule). We then get in touch with all of the companies involved, using our extensive network of contacts built up over 25 years, to enquire about the development status of the programmes. In some cases, the companies will confirm that development is ongoing; in some, they will confirm that development has been dropped. But in quite a large number of cases, they decline to comment at all.

The reasons why companies decline to respond to our questioning are many. In some cases, it is company policy never to comment on early development projects. Some companies do not comment on 'negatives', such as lapses of development. In a small number of cases, although the compound has been reported as a development candidate at a meeting, it may never have been a serious candidate, so the company's Investor Relations department or whichever department deals with our queries may have no information on it.

We now have to decide what to do with these compounds whose development appears to have stalled. The NDR category was created to apply to those compounds that are believed to have been dropped, but for which the companies involved have not confirmed discontinuation. Thus these entries can be listed as 'No Development Reported'. They immediately become part of the Ceased data set and do not appear as part of a company's R&D pipeline any more. The passing of a drug to NDR is recorded as a Major Event. In fact, there is a little more editorial discretion than the above would suggest. Compounds in phase II and beyond are often investigated further before being moved to NDR, as since phase II and III trials can take more than 18 months, there may be nothing unusual about the lack of new data reported. Preclinical and phase I drugs are much more likely to be

switched to NDR after the first inquiry, but even here, each one is looked at on a case-by-case basis. For instance, if the text of an entry reads 'Company A and Company B have entered into a 3yr agreement to investigate COX-2 inhibitors', it does not make much sense to mark up the entry as ceased after only 18 months!

Of course, if Pharmaprojects subsequently uncovers evidence that a project marked as NDR is indeed proceeding, it is brought back into active development and 'Development Continuing' is recorded to alert subscribers to this fact. Around 91% of records marked up as NDR never return to active development. Of those that do, most do so within a year of being marked up as NDR. If a profile has been listed as NDR for a year or more, you can thus be 97-98% certain that its development has ceased.

The importance of the 'No Development Reported' process in keeping our company R&D pipelines accurate cannot be overstated. Without this process, we would be giving a totally distorted view of the company's development programme.

At Pharmaprojects, we are committed to reporting only what is really in development at the pharmaceutical companies across the world. This involves much more than just scanning the literature and reporting every compound mentioned as a development drug. This is a complex process that involves vetting which compounds are added and close liaison with all of the pharmaceutical companies. The 'No Development Reported' process is just one of the methods that we employ to provide a truer picture of today's drug R&D.

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November 20, 2009

President Barack Obama
The White House
1600 Pennsylvania Avenue NW
Washington D.C. 20500

Prime Minister Fredrik Reinfeldt
On behalf of the EU Presidency
Swedish Government Offices
SE-103 33 Stockholm, Sweden

Dear President Obama and Prime Minister Reinfeldt:

I write on behalf of the Infectious Diseases Society of America (IDSA), a medical society comprised of more than 9,000 infectious diseases physicians and scientists based in the United States (U.S.) and globally, to applaud your mutual decision to establish a Transatlantic Task Force ("Task Force") to address antimicrobial resistance, an urgent and growing problem that threatens patient safety and public health worldwide. Your commitment to address this critical problem, during the November 2-3 U.S./European Union (EU) summit, provided the necessary gravitas that has been missing from past U.S. and global drug resistance action plans.

Bad Bugs, No Drugs: An 'Impending Disaster'

The increasing number of multi-antibacterial drug-resistant infections worldwide and the diminishing number of new antibacterial drugs in development with the potential to treat these infections represent one of the world's greatest health threats. The World Health Organization (WHO) has supported this premise, identifying antimicrobial resistance as one of the three greatest threats to human health. Two recent reports—one by IDSA[1] and another by the European Centre for Disease Prevention and Control (ECDC) and the European Medicines Agency (EMEA)[2]—demonstrate that there are few candidate drugs in the pipeline to treat infections due to highly-drug-resistant bacteria. The ECDC/EMEA report, for example, found only 15 antibacterial drugs with systemic administration in the development pipeline and only five of these had progressed to clinical trials to confirm clinical efficacy (Phase III or later). Unfortunately, based on past experience, we know that few of these drugs are likely to make it to market. Resistance to the current library of antibacterial drugs is a serious problem in all parts of the world including the Asia-Pacific region, Latin America, Europe and North America. Accordingly, the disincentives for financial commitment to antibacterial drug development are a global problem.

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[1] IDSA Report on Development Pipeline: "Bad Bugs, No Drugs, No ESKAPE" CID 2009:48 (1 January 2009), Boucher, et al. (http://www.idsociety.org/WorkArea/linkit.aspx?LinkIdentifier=id&ItemID=13478)

[2] ECDC/EMEA Joint Technical Report: "The Bacterial Challenge: Time to React", September 2009 (http://www.emea.europa.eu/pdfs/human/antimicrobial_resistance/EMEA-576176-2009.pdf)

Global Commitment to Develop 10 Novel Antibacterial Drugs by 2020 (10 X '20)
The time has come for a ***"Global Commitment to Develop New Antibacterial Drugs****" to address the emerging disaster caused by the confluence of increasing bacterial resistance and a stagnant antibacterial drug pipeline.* Despite the good faith efforts of many individuals, professional societies, governmental agencies, and philanthropic groups, the looming crisis has only worsened over the past decade. The problem only can be solved by bringing together global political, scientific, industry, economic, intellectual property, policy, medical and philanthropic leaders to discuss and commit to a sustainable antibacterial drug research enterprise. In IDSA's opinion, our immediate goal should be the development of ***"10 novel antibacterial drugs by 2020"***. Key to advancing antibacterial drug development is the concomitant need to advance the development of improved diagnostic tests specific to multi-drug-resistant infections.

Global stakeholders must capitalize on each other's strengths to create a long-term, sustainable research and development (R&D) infrastructure model that provides incentives across the spectrum of the antibacterial drug and related diagnostics research enterprises. Success would be of immense benefit to the health of the citizens of the world. Further, the sustained infrastructure created to achieve this goal would help to recreate the highly skilled scientific workforce that was lost over the past two decades as many companies abandoned antibacterial drug development and would otherwise provide the necessary incentives for perpetual antibacterial drug discovery and development. Microbial evolution causing antibiotic resistance is constant; our collective efforts at antibiotic discovery must be constant, or we risk being permanently overtaken by the microbes.

The discovery of antibacterial drugs in the 1930s and 1940s represented a transformative moment in human history. One of the leading physicians of the 20th century, who bore witness to the pre- and post-antibiotic era, has described the discovery and development of antibacterial drugs as an "awesome acquisition of power" for physicians and their patients.[3] Now, 70 years later, in the U.S., EU, and around the world, the challenges posed by infections caused by the multiply-drug resistant pathogens continue to escalate, causing patient morbidity and mortality, as well as increasing health care costs. As a global society, we have a moral obligation to ensure, in perpetuity, that the treasure of antibiotics is never lost and that no infant, child or adult dies unnecessarily of a bacterial infection caused by the lack of effective and safe antibiotic therapies.

Lending your imprimatur to the creation of a Task Force that will focus on "strategies for improving the pipeline of new antimicrobial drugs," among other important objectives, is a critically important first step. Only after establishing a global commitment to address the antibiotic pipeline problem can global multifaceted solutions be instituted. In 1961, U.S. President John F. Kennedy declared that it was possible for humans to walk on the moon. Many thought the statement was only political and impossible to achieve. History proved Kennedy's dream was possible in 1969—*less than 10 years after the President first committed to act.*

Antibacterial Drug Pipeline Work Group ("Work Group")
To make the Task Force most effective and to achieve our ***"10 novel antibacterial drugs by 2020"*** *dream, we strongly recommend that the U.S. and EU Task Force establish an Antibacterial Drug Pipeline Work Group ("Work Group") as a component of the Task Force.* The Work Group would focus specifically on the antibiotic drug pipeline problem as this problem requires expertise not essential to the Task Force's other responsibilities.

[3] McDermott W & Rodgers DE. Social ramifications of control of microbial disease., Johns Hopkins Med J. 1982 151:302-12.

In our vision, the Work Group would:

1. *be established as a public/private entity including experts from the national and international scientific, industry (including small, medium and large pharmaceutical, biotechnology and medical diagnostic companies), medical, economic, intellectual property, reimbursement and other policy, public health, philanthropic and governmental communities. Only through the direct involvement of such experts within and without government can true progress be made in this critical area;*

2. *be co-established by the U.S. and EU, with the U.S. activities being administered from within the White House as a component of or working in close collaboration with the U.S. President's Council of Advisors on Science and Technology (PCAST) and engaging and relying upon the expertise of the White House's National Council of Economic Advisors and with the EU activities being administered from within the European Commission (EC);*

3. *be co-chaired on both sides by either a former or current political leader who has the capacity to assist in turning recommendations into actions or respected non-governmental and international, scientific/philanthropic leaders with an excellent understanding of the economics of the pharmaceutical, biotechnology and medical diagnostic industries;*

4. *include among its members the heads of the U.S. National Institute for Allergy and Infectious Diseases (NIAID), U.S. Food and Drug Administration (FDA), and U.S. Biomedical Advanced Research and Development Authority (BARDA) as well as their EU counterparts within the European Commission's (EC) Directorates General for Research (DG Research), Health and Consumers (DG SANCO), and Enterprise (DG Enterprise), including the EMEA. NIAID and FDA are co-leads of the existing U.S. Interagency Task Force on Antimicrobial Resistance, along with the U.S. Centers for Disease Control and Prevention (CDC). Moving forward, NIAID, FDA, BARDA, and the EC Directorates General and EMEA will be integrally involved in developing and advancing pipeline solutions;*

5. *include CDC, ECDC, and WHO representation for public and global health expertise as well as representation from the U.S. Department of Commerce and its EU counterpart, the EC's DG Enterprise, for expertise in intellectual property rights and the economics of the pharmaceutical, biotechnology, and medical diagnostics industries;*

6. *explore and identify recommendations across a broad spectrum of policy options including those which address regulatory and financial disincentives that negatively impact the antibacterial drug and related diagnostics pipeline. A review of the findings of a new EU-commissioned report titled "Policies and Incentives for Promoting Innovation in Antibiotic Research," drafted by a team from the London School of Economics and Political Science (LSEPS), will be helpful in this regard. A draft version[4] of the report was released at an EU conference held in Stockholm, Sweden in September 2009; the final report is likely to be published by the end of this year. The LSEPS report provides a good starting point for discussion about the kinds of incentives that will be needed to reach the* ***"10 novel antibacterial drugs by 2020"*** *goal. Related to this, the U.S. will need to commission a similar report that addresses the United States' own unique regulatory and economic environments;*

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[4] http://www.se2009.eu/polopoly_fs/1.16814!menu/standard/file/LSE-ABI%20F-Final.pdf

7. *identify scientific challenges that need to be addressed and consider new research opportunities that the U.S. and EU should fund to advance antibacterial drug and related diagnostics discovery and development;*

8. *immediately examine U.S. and EU funding levels specific to antibacterial drug and related diagnostics discovery and development and recommend supplemental funding targets in this area consistent with the urgent needs; and*

9. *be a transparent process—Work Group meetings/calls must be open to the public and meeting materials and transcripts must be made publicly available.*

The Task Force's Other Critical Responsibilities: Appropriate Use and Infection Prevention

At the same time the Work Group focuses on the drug and related diagnostics pipelines, the U.S., EU, WHO and global community must continue to work towards attenuating the serious problem of drug resistance. We strongly believe that aspects of recommendations 1 through 9 (above) also are relevant to the Task Force's responsibilities related to: (a) the appropriate uses of antibacterial drugs in the medical and veterinary communities, and (b) prevention of both health care- and community-associated drug-resistant infections. For example, we believe the Task Force, as a whole, should be a public/private initiative with non-governmental experts directly represented, its focus should be global, and its processes should be transparent. Also, of great importance, as with the drug pipeline problem, numerous components of the U.S. and EU governments will be necessary to tackle the appropriate use and prevention issues. Indeed, the United States' own Interagency Task Force on Antimicrobial Resistance has representation from eleven (11) different agencies representing six (6) U.S. departments. For this reason, the idea of nesting the appropriate use and infection prevention efforts under a single department or directorate general likely will not take full advantage of the U.S. and EU governments' breadth of expertise and abilities to respond effectively and would most likely result in an insufficient response. To address these concerns, we believe the U.S. and EU Task Force activities, as a whole, should be administered from within the White House, perhaps in conjunction with PCAST, and the European Commission, respectively.

Conclusion

As the new Transatlantic Task Force gets underway, IDSA seeks a ***"Global Commitment to Develop New Antibacterial Drugs"*** from U.S., EU and other global leaders to take all necessary actions to ensure that 10 novel systemically administered antibacterial drugs will be brought to market by 2020 and to compose the new Task Force in a way that makes this ***10 X '20*** commitment a reality. Naysayers will immediately discount the ***10 X '20*** commitment as radical, impossible, and unacceptable to political leaders in the U.S. and EU, industry, academe, governmental experts, and the international scientific and medical communities. Objections are inevitable, but easily nullified by recognition of the magnitude of the problem and the moral imperative incumbent upon all stakeholders to make it happen. Without a global commitment to create and maintain the necessary sustainable infrastructure, the inventory of safe and effective antibiotics will inevitably shrink as the bacteria grow ever more resistant. This need not happen, if we all work together to make the ***10 X '20*** commitment a priority.

As President Kennedy forecast, we can walk on the moon within 10 years, if we commit to this goal.

IDSA stands ready and willing to work with the U.S. and EU governments and the Task Force's members as this extremely important initiative advances. Please contact Robert J. Guidos, JD, IDSA's vice president for public policy and government relations, at [redacted] or by phone at 703-299-0202 should you have any questions or comments.

Sincerely,

[redacted]

Richard Whitley, MD, FIDSA
President

cc: Kathleen Sebelius, MPA, Secretary, U.S. DHHS
Anthony Fauci, MD, FIDSA, Director, U.S. NIAID
Margaret Hamburg, MD, Commissioner, U.S. FDA
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Senator Tom Harkin, Chair, U.S. Senate Health, Education, Labor and Pensions Committee