# Synthesis of Aminoglycoside Derivatives to Combat Bacterial Resistance

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# Abstract

Aminoglycosides are broad spectrum antibiotics that act by binding to 16S rRNA of bacteria. The wide spread of aminoglycoside resistance threatens the use of these important medicines. Two general approaches can be used to address the aminoglycoside resistance problem. One is to derive the existing aminoglycoside antibiotics; the other is to develop inhibitors blocking resistance pathways.

We developed a novel methodology to regio- and chemo-selectively derivatize unprotected aminoglycosides at the *N*-6′ position, and used this method to prepare a series of amide-linked aminoglycoside-CoA bisubstrate analogs. These analogs are the first reported nanomolar inhibitors of AAC(6′)-Ii, an aminoglycoside resistance-causing enzyme. They have been proved useful as mechanistic and structural probes to investigate the molecular mechanism of the catalysis by AAC(6′)-Ii.

Although the aminoglycoside-CoA bisubstrates are nanomolar inhibitors of AAC(6′)-Ii, they are not active in cells due to their size and negative charges. A series of truncated aminoglycoside-CoA bisubstrates were next synthesized. These derivatives were used to determine key structure-activity relationships. One analog is discovered active against resistant strain in cells.

Bisubstrate inhibitors containing sulfonamide, sulfone and sulfoxide linkers were synthesized and used as mechanistic probes to study mechanism of AAC(6′)-Ii. Our results support the suggestion that AAC(6′)-Ii may catalyze acetyltransfer without stabilization of the tetrahedral intermediate. Surprisingly, sulfide oxidation of the amide-linked bisubstrate dramatically improved inhibition of AAC(6′)-Ii.

Bisubstrates with linkers containing phosphoryl group (P=O) were proposed and synthesized, the biological results are under investigation. These molecules will facilitate investigations of the potential stabilization of the tetrahedral intermediate by the enzyme. Our efforts in this project also improved our

chemical knowledge of phosphorus chemistry. For example, attempts to synthesize such bisubstrates by adapting our 6′-regioselective acylation methodology to phosphorylation led to the discovery of a rapid rearrangement of *N*-[(phosphino)oxy] amines to phosphoramidic or phosphorodiamidic acid triesters. The *N*-[(phosphino)oxy] amine intermediate is generated *in situ* from the reaction of hydroxylamines with chlorophosphites or chlorophosphoramidites and with rearrangement via cleavage of the weaker N-O bond to generate a more stable P=O bond. The rearrangement proceeds spontaneously in excellent yield when the hydroxylamine is electron poor. Various substituents on the phosphorus are well tolerated.

An alternative approach to combat bacterial resistance is to modify the existing antibiotics yielding derivatives active against resistant strains. Available crystallographic data of aminoglycoside-RNA complexes together with our crystallographic data of the AAC(6′)-Ii–bisubstrate complex were used to design a series of neamine-*N*-6′-derivatives able to bind to RNA but not to AAC(6′)-Ii. These derivatives allowed us to determine more structure-activity relationships.

# Résumé

Les aminoglycosides sont des antibiotiques à large spectre. Ils agissent en se fixant à l′ARN ribosomal 16S des bactéries et perturbent la synthèse protéique. L'extension rapide du phénomène de résistance aux aminoglycosides menace cependant leur efficacité. Parmi les différents mécanismes de résistance aux aminoglycosides, le plus important est la production d'enzymes inactivant l'antibiotique. Trois types d'enzymes impliquées dans ce mécanisme ont été identifiés, à savoir les aminoglycoside-N-acétyltransférases (AACs), les aminoglycoside-O-phosphoryltransférases (APHs) et les aminoglycoside-O-nucléotidyltransférases (ANTs). Deux approches peuvent être envisagées pour combattre ou contourner le phénomène de résistance. La première consiste à créer de nouveaux antibiotiques en modifiant des antibiotiques existant. L'autre implique le développement d'inhibiteurs capables de bloquer la résistance.

Une nouvelle méthodologie pour la dérivatisation régio- et chimio-sélective d'aminoglycosides non protégés à la position *N*-6′ a été développée. Cette méthode a ensuite été appliquée à la synthèse d'une série de bisubstrats aminoglycoside-coenzyme A. Ces analogues se sont avérés être de puissants inhibiteurs (resistance d'inhibition de l′ordre de la nanomole) de l′enzyme aminoglycoside 6′-*N* acétyltransférase Ii (AAC(6′)-Ii), impliquée dans la résistance à de nombreux antibiotiques aminoglycosides. Plus particulièrement, ces molécules ont pu être co-cristallisées avec AAC(6′)-Ii par nos collaborateurs, qui n'étaient à ce jour jamais parvenus à cristalliser cette enzyme avec un aminoglycoside. Les structures 3D ainsi obtenues ont permis d′obtenir de précieuses informations sur le site de fixation des aminoglycosides.

Une deuxième génération d'inhibiteurs de l′AAC(6′)-Ii a ensuite été synthétisée. Elle consistait en une série d′analogues tronqués de la première génération d′inhibiteurs. Ces analogues ont permis de déterminer d′importantes relations structure-activité, et ont conduit à la découverte d'une molécule capable

d′inhiber la résistance aux aminoglycosides dans des cellules exprimant AAC(6′)-Ii.

Le remplacement du lien amide des inhibiteurs bisubstrats par un lien sulfonamide a résulté en une diminution de leur pouvoir inhibiteur. Ces résultats suggèrent soit que l′enzyme AAC(6′)-Ii catalyse le transfert d′acétyle par un effet de proximité, soit que le lien sulfonamide n′imite pas bien l′intermédiaire tétraédrique. Par la suite, bien que notre tentative de synthétiser des bisubstrats contenant un lien phosphoryl ait échoué, l′extension de notre acylation sélective pour *N*-6′ à une phosphorylation a conduit à la découverte d'un important réarrangement de *N*-[(phosphino)oxy]amines en triesters phosphoramidiques ou triesters d′acide phosphorodiamidique. Ce réarrangement est en particulier très efficace en présence d'hydroxylamines pauvres en électrons.

Enfin, l′étude des structures d′inhibiteurs bisubstrats complexés à AAC(6′)-Ii et du site A de l′ARN 16S complexé à la néamine a permis l′élaboration d'une librairie de dérivés d′aminoglycosides, sensés conserver l′activité antibiotique sans être modifiés par les enzymes AAC(6′)s. Ces dérivés, bien que possédant une activité antibiotique moindre, ont en effet un $K_m$ plus élevé et un $k_{cat}$ moins élevé envers AAC(6′)-Ii.

## Acknowledgements

I have been very fortunate to work with and learn from so many wonderful people at McGill University during the past four years. It would be impossible for me to complete this thesis without their teaching, advice, help and supports.

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I am indebted to my wife Mei, and would like to dedicate this thesis to her. I thank her and my son for being extremely patient with my long hours on the bench. I am also grateful to my parents for their constant supports.

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## Contributions of co-authors

This thesis consists of one introduction, three publications (Chapters 2, 3 and 6) and three drafts (Chapters 4, 5 and 7). Chapter 4 will be submitted for publication shortly and Chapter 7 has already been accepted for publication. All the work described in these manuscripts was carried out as part of my research for the degree of Doctor of Philosophy in organic chemistry.

All the manuscripts have co-authors, their contributions are described below. Professor Karine Auclair was my supervisor throughout my doctoral degree and a co-author for each manuscript.

Chapter 2: I synthesized all compounds, Xuxu Yan, a graduate student in our lab, carried out all enzymatic tests and Oliver Baettig (Professor Albert Berghuis' lab) performed the crystallography experiments.

Chapter 3: I synthesized all the compounds, Xuxu Yan performed enzymatic tests, Tusher Shakya (Professor Gerard D. Wright's lab) carried out bacterial test, Samia Ait-Mohand-Brunet, a research associate in our lab, provided partial help on synthesis of compound **5a**, and Oliver Baettig purified the enzyme.

Chapter 4: I synthesized all the compounds, Xuxu Yan carried out enzymatic tests, Lee Freiburger, a graduate student in our lab, purified the enzyme.

Chapter 5: I performed all experiments reported.

Chapter 6: I performed all experiments reported.

Chapter 7: I synthesized all the compounds except for **3g-j**, which was synthesized by Sarilata Yotphan, a summer student in our group. Xuxu Yan and Parseh Bakirtzian (an undergraduate student) carried out enzymatic tests, Lee Freiburger purified the enzyme.

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# Abbreviations

| | |
|---|---|
| Å | Ångstrom |
| AAC(6′)-Ii | aminoglycoside *N*-6′-acetyltransferase type Ii |
| AAC(6′)-Iy | aminoglycoside *N*-6′-acetyltransferase type Iy |
| AAC(6′)-APH(2″) | aminoglycoside *N*-6′-acetyl *O*-2″-phosphoryltransferase |
| Ac | acetate |
| AcCoA | acetyl coenzyme A |
| Ade | adenine |
| AHB | (S)-4-amino-2-hydroxybutyryl |
| AHP | (S)-3-amino-2-hydroxypropionyl |
| AIBN | *N,N′*-azoisobutyronitrile |
| APCI | atmospheric pressure chemical ionization |
| APH(3′) | aminoglycoside *O*-3′-phosphotransferase |
| Asp or D | aspartic acid |
| *B. cereus* | *Bacillus cereus* |
| *B. subtilis* | *Bacillus subtilis* |
| [BMIM][PF6] | 1-butyl-3-methylimidazonium hexafluorophosphate |
| n-BuLi | n-butyl lithium |
| Bn | benzyl |
| Boc | *tert*-butoxycarbonyl |
| bs | broad singlet |
| Bz | benzoyl |
| Cys or C | cytosine |
| $^{o}$C | celsius |
| Cbz | benzyloxycarbonyl |
| CDI | *N,N′*-Carbonyldiimidazole |
| CHDK | cyclohexanone dimethyl ketal |
| CoA | coenzyme A |
| COSY | correlation spectroscopy |

| | |
|---|---|
| *m*CPBA | *meta*-chloroperbenzoic acid |
| δ | chemical shift |
| Dabco | 1,4-diazabicyclo[2,2,2]octane |
| DBU | 1,8-diazabicyclo[5,4,0]undec-7-ene |
| DCC | 1,3-dicyclohexylcarbodiimide |
| DCM | dichloromethane |
| *de* | diastereomeric excess |
| DIAD | diisopropyl azodicarboxylate |
| DIBAL | diisobutylaluminium hydride |
| DIPEA/Hünig base | diisopropylethylamine |
| DMAP | *N*,*N*-dimethylpyridine |
| DMF | *N,N*-dimethylformamide |
| DMSO | dimethylsulphoxide |
| 2-DOS | 2-deoxystreptamine |
| DTDP | 4,4′-dithiodipyridine |
| DTT | dithiothreitol |
| $EC_{50}$ | the concentration of a compound where 50% of its maximal effect is observed |
| *ee* | enantiomeric excess |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylenediaminetetraactic acid |
| Et | ethyl |
| ESI-MS | electrospray ionization mass spectrometry |
| EtOAc | ethyl acetate |
| Fmoc | 9-fluorenylmethoxycarbonyl |
| G | guanine |
| Glu or E | glutamic acid |
| h | hour(s) |
| HATU | 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium |
| HEPES | [4-(2-hydroxyethyl)-1-piperizine]ethanesulfonic acid |

| | |
|---|---|
| His or H | histidine |
| HMQC | heteronuclear multiple quantum correlation spectroscopy |
| HMBC | heteronuclear multiple bond correlation spectroscopy |
| HRMS | high resolution mass spectrometry |
| HSQC | heteronuclear single quantum correlation spectroscopy |
| Hz | hertz |
| $IC_{50}$ | the concentration of an inhibitor that is required for 50% inhibition of the biological target |
| $J$ | coupling constant |
| $k_{cat}$ | turnover number |
| $K_d$ | dissociation constant |
| $K_i^{app}$ | inhibition constant |
| $K_m^{app}$ | Michaelis-Menten constant |
| LDA | lithium diisopropylamide |
| Leu or L | leucine |
| m | multiplet |
| MIC | minimum inhibitory concentration |
| min | minute(s) |
| MMPP | magnesium monoperoxyphthalate |
| MRSA | methicillin-resistant *Staphylococcus aureus* |
| MS | mass spectrum |
| *m/z* | mass-to-charge ratio |
| Ms | methanesulphonyl |
| NHS | *N*-hydroxylsuccinimide |
| NBS | *N*-bromosuccinimide |
| NBD | *endo*-*N*-hydroxy-5-norbornene-2,3-dicarboximide |
| NIS | *N*-iodosuccinimide |
| NMR | nuclear magnetic resonance |
| Nu | nucleophile |
| Oxone® | potassium peroxymonosulfate |
| *P. aeruginosa* | *Pseudomonas aeruginosa* |

| | |
|---|---|
| pH | negative logarithm of hydrogen ion concentration |
| pKa | negative logarithm of equilibrium constant for association |
| ppm | parts per million |
| Py | pyridine |
| q | quartet |
| $R_f$ | the retention factor, is defined as the distance traveled by the compound divided by the distance traveled by the solvent |
| rRNA | ribosomal RNA |
| RT | room temperature |
| s | singlet |
| sec | second(s) |
| *S* as in 16*S* | Svedberg, a unit for the sedimentation coefficient, equivalent to $10^{-13}$ s |
| *S. aureus* | *Staphylococcus aureau* |
| *S. epidermidis* | *Staphylococcus epidermidis* |
| t | triplet |
| *t*-Bu | *tert*-butyl |
| TBDMS | *tert*-butyldimethylsilyl |
| TEA | triethylamine |
| TEMPO | 2,2,6,6-tetramethyl-1-piperidinyloxy free radical |
| $Tf_2O$ | trifluoromethanesulfonic anhydride |
| Tf | trifluoromethanesulfonyl |
| TFA | trifluoroacetic acid |
| THF | tetrahydrofuran |
| Thr or T | threonine |
| TLC | thinlayer chromatography |
| $t_R$ | retention time (HPLC) |
| Trp or W | tryptophan |
| TsCl | toluenesulfonyl chloride |
| Tyr or Y | tyrosine |

| | |
|---|---|
| U | uracil |
| Δ | reflux |
| $v$ | initial velocity |
| $V_{max}$ | maximum velocity |
| VRE | vancomycin-resistant *Enterococcus* |
| VRSA | vancomycin-resistant *Staphylococcus aureus* |
| XPhos | 2-dicyclohexylphosphino-2′,4′, 6′-triisopropyl biphenyl |

# Chapter One

## Aminoglycoside: Action, Resistance and Rejuvenation

### 1.1 Aminoglycoside: structure and action

The 1952 Nobel Prize in Physiology and Medicine was awarded to Selman Waksman for his discovery of streptomycin, the first aminoglycoside antibiotic used and the first medicine effective against tuberculosis. This was followed by the isolation of over 150 naturally occurring aminoglycosides from culture broths of actinomycetes and bacterial strains.[1] Fig. 1.1 and 1.2 show the structures of some important aminoglycoside antibiotics. They consist of a six-membered carbacyclic ring, the aminocyclitol, which consists of either streptidine (streptomycins), actinamine (spectinomycins), fortamine (fortimicins) or 2-deoxystreptamine with a varying number of sugar substituents (Fig. 1.1). The most therapeutically relevant aminoglycoside antibiotics fall into two classes, the 4,5-linked (neomycins, Fig. 1.2, A) and the 4,6-linked 2-deoxystreptamines (kanamycins/gentamicins, Fig. 1.2, B).

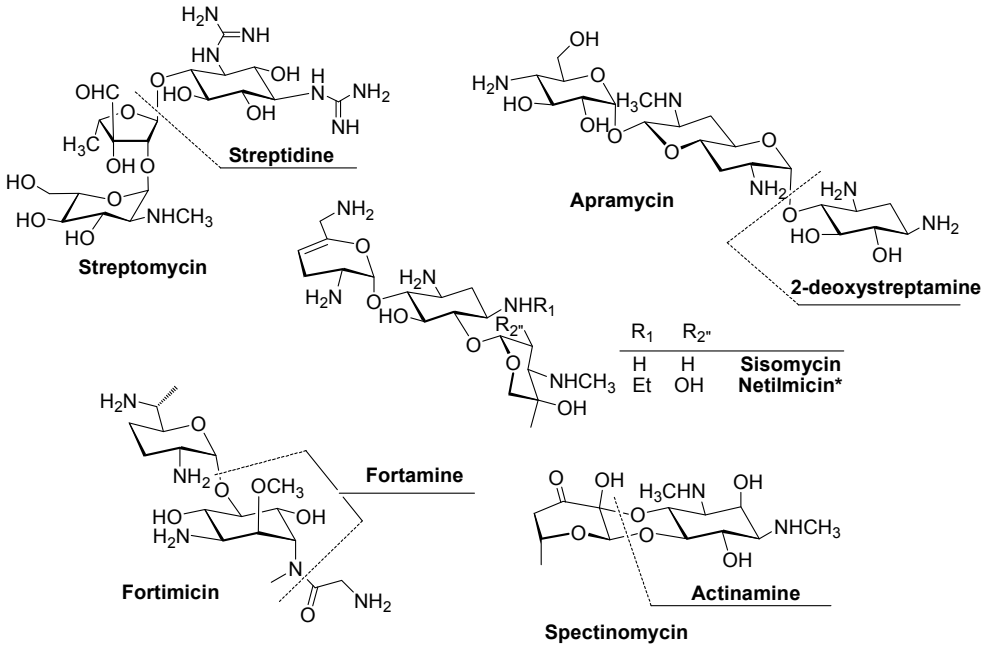
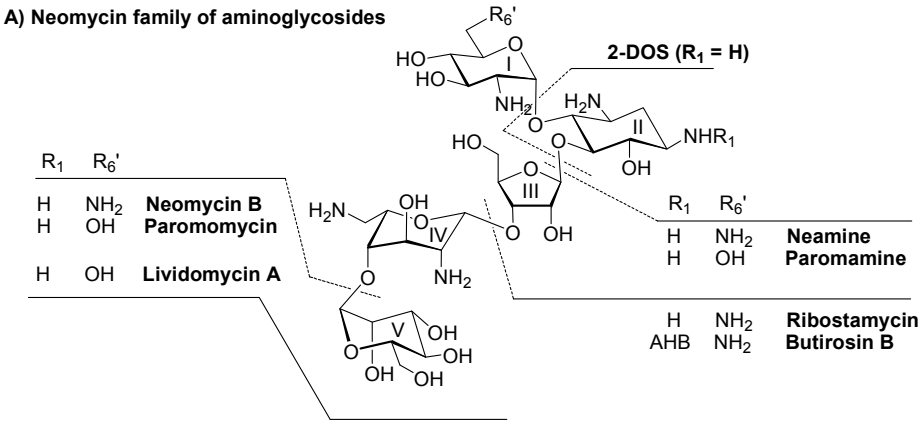
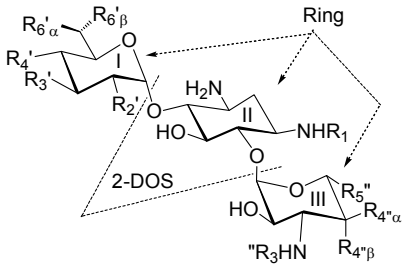



***Figure 1.1***. Naturally occurring aminoglycosides.

Aminoglycoside antibiotics exhibit a broad antimicrobial spectrum and bactericidal activity towards Gram-positive *mycobacteria* and *staphylococci* as well as Gram-negative *pseudomonads*, *salmonella* and *Escherichia coli*. They display a synergistic effect when used in combination with β-lactam antibiotics.[2]




**B) Kanamycins/gentamicins family of aminoglycosides**




| Aminoglycoside | $R_1$ | $R_2'$ | $R_3'$ | $R_4'$ | $R_6'_\alpha$ | $R_6'_\beta$ | $R_3''$ | $R_4''_\alpha$ | $R_4''_\beta$ | $R_5''$ |
|---|---|---|---|---|---|---|---|---|---|---|
| **Gentamicin $C_1$** | H | $NH_2$ | H | H | $NHCH_3$ | $CH_3$ | $CH_3$ | $CH_3$ | OH | H |
| **Gentamicin $C_2$** | H | $NH_2$ | H | H | $NH_2$ | $CH_3$ | $CH_3$ | $CH_3$ | OH | H |
| **Gentamicin $C_{1a}$** | H | $NH_2$ | H | H | $NH_2$ | H | $CH_3$ | $CH_3$ | OH | H |
| **Geneticin** | H | $NH_2$ | OH | OH | OH | $CH_3$ | $CH_3$ | $CH_3$ | OH | H |
| **Kanamycin A** | H | OH | OH | OH | $NH_2$ | H | H | OH | H | $CH_2OH$ |
| **Kanamycin B** | H | $NH_2$ | OH | OH | $NH_2$ | H | H | OH | H | $CH_2OH$ |
| **Tobramycin** | H | $NH_2$ | H | OH | $NH_2$ | H | H | OH | H | $CH_2OH$ |

***Figure 1.2.*** Clinically important aminoglycoside antibiotics: A) neomycin family; and B) gentamicins/kanamycins family. 2-DOS: 2-deoxystreptamine.

Remarkably, it was more than 50 years after the discovery of aminoglycosides that the 3D-structures of an aminoglycoside in complex with the target, RNA, were reported.[3-9] Aminoglycosides selectively bind to the A site of eubacterial 16S ribosomal RNA via multiple ionic and H-bond interactions (Fig. 1.3). The number of contacts between RNA and an aminoglycoside varies

between 10 and 30. Eight direct hydrogen bonds between rings I and II of the neamine moiety are conserved (Fig. 1.3).[9] The puckered glucosamine ring (I) is inserted into the A site helix by stacking with G1491 (*E. coli* numbering) and forms a pseudo base pair via two H-bonds with the Watson-Crick sites of the universally conserved A1408. Overall binding of aminoglycosides to RNA favors a bulged-out conformation for A1492 and A1493 (Fig. 1.4).

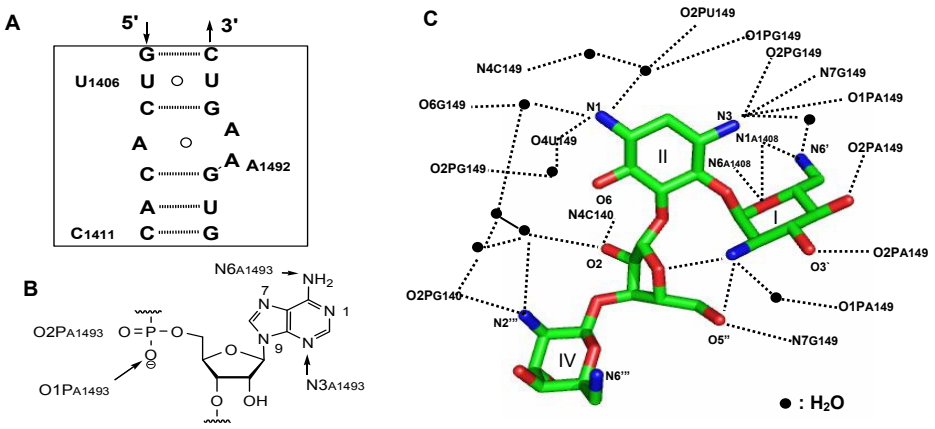



***Figure 1.3***. A) A site 16S rRNA; B) atom numbering of nucleic acid as shown for adenine 1493 (A1493); C) interactions between neomycin B and a RNA fragment. Ionic and hydrogen bonds are shown as dashed lines. Neomycin is represented with sticks and colored by atoms (N: blue, C: green, O: red).

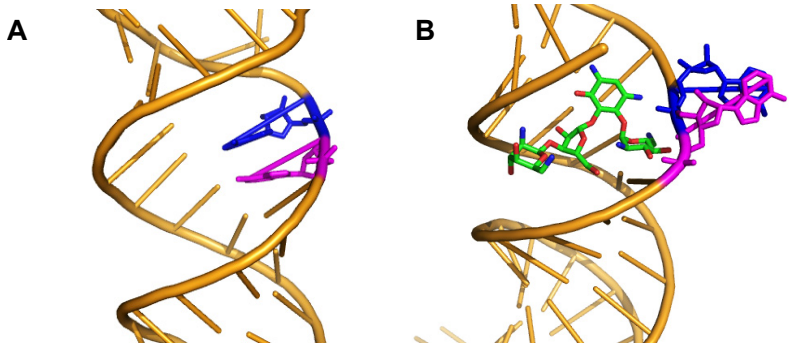



***Figure 1.4***. Schematic representation of the A site 16S rRNA structure in the absence of aminoglycoside (A) and in complex with the aminoglycoside neomycin B (B). A1492 is colored as magenta, A1493 is colored as blue and neomycin B is represented with sticks and colored by elements (C: green, O: red, N: blue). This figure (PDB: 2F4S) is prepared using PyMol.

This conformational change leads to mistranslation, as explained in Fig. 1.5.[10] Bases A1492 and A1493 are the proofreading base pairs of the A site. They

are in conformational equilibria between intrahelical and extrahelical states, with the intrahelical states being favored in the absence of aminoglycosides. When the intrahelical states are adopted, the translation is off. In the extrahelical states, A1492 and A1493 turn on the translation through interaction with the minor groove of the cognate tRNA anticodon-mRNA codon minihelix (Fig. 1.5, A). Upon aminoglycoside binding, the conformational equilibria shift towards the extrahelical states. As a result, the interactions between A1492 and A1493 and the codon-anticodon double helix are favored, even when the anticodon is noncognate (Fig. 1.5, B). Accordingly, the translation machinery remains turned on. The production of mistranslated and/or misfolded proteins is believed to compromise cellular integrity, eventually leading to cell death.

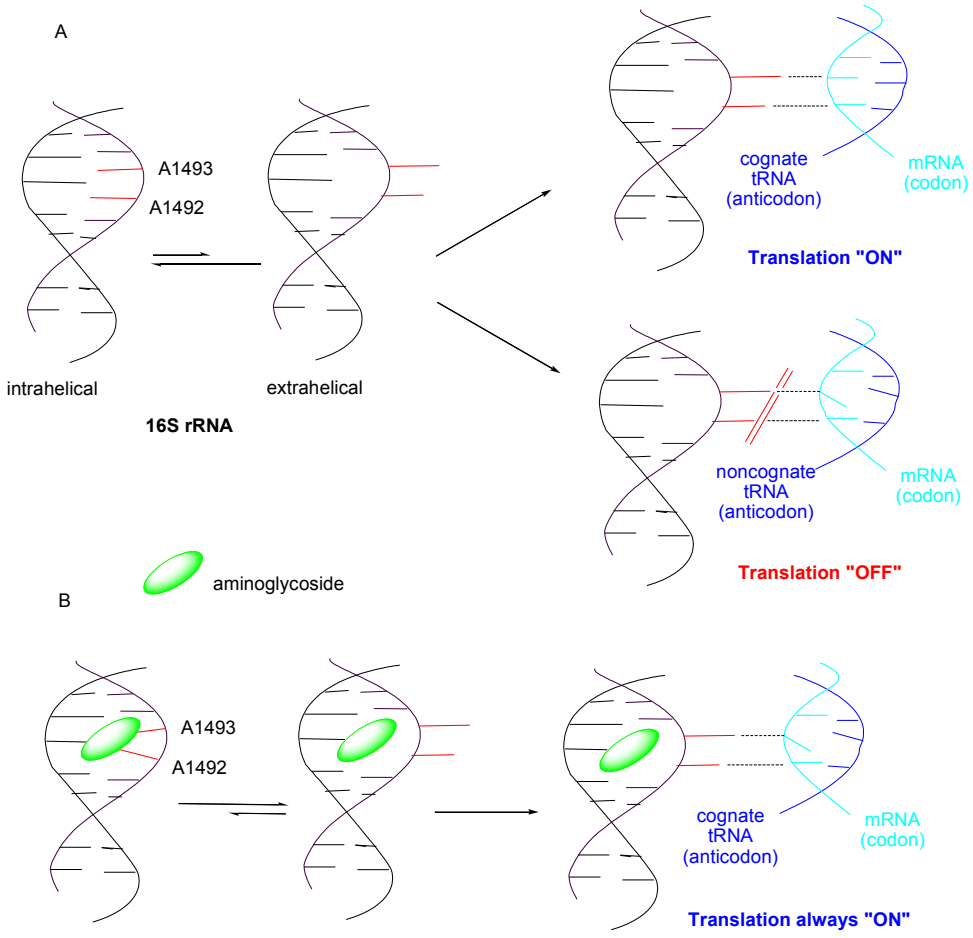



***Figure 1.5***. Schematic representation of aminoglycoside binding to 16S rRNA causing mistranslation (see text).

## 1.2 Aminoglycoside resistance

Unfortunately, the use of aminoglycosides as antibacterial agents has been threatened by the emergence of resistance. The mechanisms of bacterial resistance to aminoglycosides have been the subject of numerous reviews.[11-13] These mechanisms include efflux-mediated processes, ribosomal methylation and drug modification by resistance-causing enzymes.[1]

Energy-dependant drug efflux is recognized as a major cause of antibiotic resistance,[14] nevertheless this mechanism is not observed frequently with aminoglycosides.[14-18]

Mutation of the A site has not been reported to date. This is in part due to the central function played by rRNA in protein biosynthesis and its high conservation across genera. In addition, all organisms have multiple copies of the genes that encode rRNA. In order to generate rRNA resistant to a given antibiotic, multiple genes would have to be mutated simultaneously, and the probability of occurrence for such an event is virtually nonexistent. Moreover, it is easier for an organism either to produce resistance-causing enzymes or to modify the rRNA target post-translationally. The enzymatic methylation of either *N*1 of A1408 or *N*7 of G1405 has recently been observed in pathogenic bacteria and found to result in aminoglycoside resistance.[19-21]

### Aminoglycoside-modifying enzymes

Clinically, the most relevant mechanism of resistance to aminoglycosides is enzymatic inactivation. Three types of resistance-causing enzymes, namely aminoglycoside nucleotidyltransferases (ANTs), aminoglycoside acetyltransferases (AACs), and aminoglycoside phosphotransferases (APHs), have been reported. Within each type, the enzymes are conventionally named after the resistance genes that encode the enzymes.[22] The first three letters indicate the type of enzyme, followed by a bracketed number that indicates the specific site modified by the enzyme (Fig. 1.6). The roman numerial is the subclass and a

lowercase character is used to identify the various isoforms. For example, AAC(6′)-Ii is the isoform i of a type I aminoglycoside *N*-6′-acetyltransferase,[23] AAC(6′)-Iy is another type I aminoglycoside *N*-6′-acetyltransferase.[24]

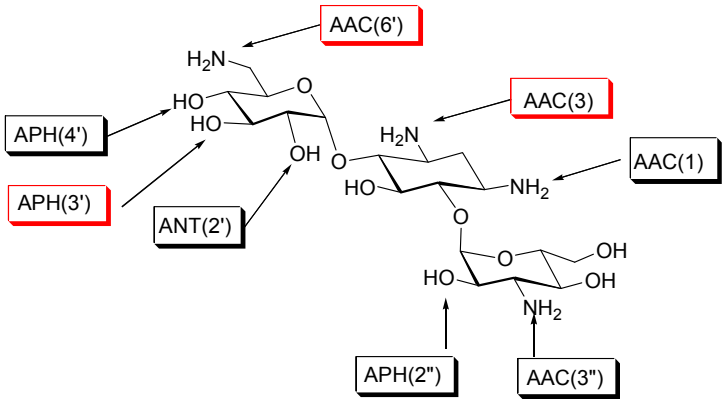



***Figure 1.6***. Designation of the sites attacked by aminoglycoside resistance-causing enzymes. AAC, aminoglycoside acetyltransferase; ANT, aminoglycoside nucleotidyltransferase; APH, aminoglycoside phosphotransferase.

There are five known classes of aminoglycoside nucleotidyltransferases: ANT(6),[25] ANT(9),[26, 27] ANT(4′),[28, 29] ANT(2″),[30-33] and ANT(3″);[34, 35] four reported classes of aminoglycoside acetyltransferases: AAC(1),[36, 37] AAC(3),[38-41] AAC(2′)[42, 43] and AAC(6′);[23, 44-46] and seven known classes of aminoglycoside phosphotransferases: APH(4),[47, 48] APH(6),[49] APH(9),[50] APH(3′),[51-53] APH(2″),[54, 55] APH(3″),[56, 57] and APH(7″).[58] There are also two reported bifunctional enzymes: AAC(6′)-Ie/APH(2″)-Ia[59-62] and AAC(6′)-IId/ANT(3″)-Ii.[63]

The most prominent aminoglycoside nucleotidyltransferases are ANT(4′) and ANT(2″). Mechanistic and structural studies are available for these enzymes.[64-67] The most common APHs are APH(3′)-IIIa and APH(2″)-Ia in Gram-positive bacteria, and APH(3′)-Ia and APH(3′)-IIa in Gram-negative organisms. These enzymes have a broad substrate profile, and efficiently modify both 4,5- and 4,6-disubstituted aminoglycosides. The kinetic, mechanistic and structural studies reported for these enzymes have previously been reviewed,[12, 68-70] and will not be repeated here in the interest of brevity.

**Aminoglycoside acetyltransferases**

AACs are the largest group of aminoglycoside resistance-causing enzymes with close to 50 unique enzymes identified from both Gram-positive and Gram-negative bacteria. Most AACs are *N*-acetyltransferases but *O*-acetyltransferase activity has been shown in the mycobacterial AAC(2′)-Ic, and in the AAC portion of the bifunctional enzyme AAC(6′)-APH(2″).[71]

AAC(6′) is the largest subclass of AACs and the most relevant in clinics. Twenty-seven unique members of this class have been identified from a wide variety of pathogenic bacteria. The prevalence of aminoglycoside resistance via 6′-*N*-acetylation renders routine β-lactam/aminoglycoside therapy ineffective. This failure of commonplace treatments increases the reliance on other drugs such as the glycopeptides and fluoroquinolones, and creates a pressure to generate multiresistant strains. For example, *aac(6′)* genes have been found in resistance gene cassettes conferring both vancomycin resistance (VRSA),[72, 73] methicillin resistance to *Staphylococcus aureus* (MRSA),[74] and ciprofloxacin resistance (a fluoroquinolone drug).[75]

AAC(6′)s specifically catalyze the transfer of acetyl to the 6′-amino groups of most aminoglycosides (Fig. 1.7, A). Among AAC(6′)s, three enzymes have extensively been studied, the chromosomally encoded AAC(6′)-Iy from *Salmonella enterica,*[24, 76] the plasmid-encoded bifunctional AAC(6′)-APH(2″) from *Enterococcus*,[25] *Staphylococcus*,[55] and *Streptococcus agalactiae,*[77] and chromosomally encoded AAC(6′)-Ii from *E. faecium.*[23, 45, 78]

The AAC(6′)-Iy isoform confers broad aminoglycoside resistance.[79] The purified recombinant AAC(6′)-Iy was expressed in *E. coli* and shown to exist as a dimer in solution. Steady-state kinetics studies indicated that the enzyme displays a sequential kinetic mechanism with the release of Coenzyme A being rate-limiting.[24]

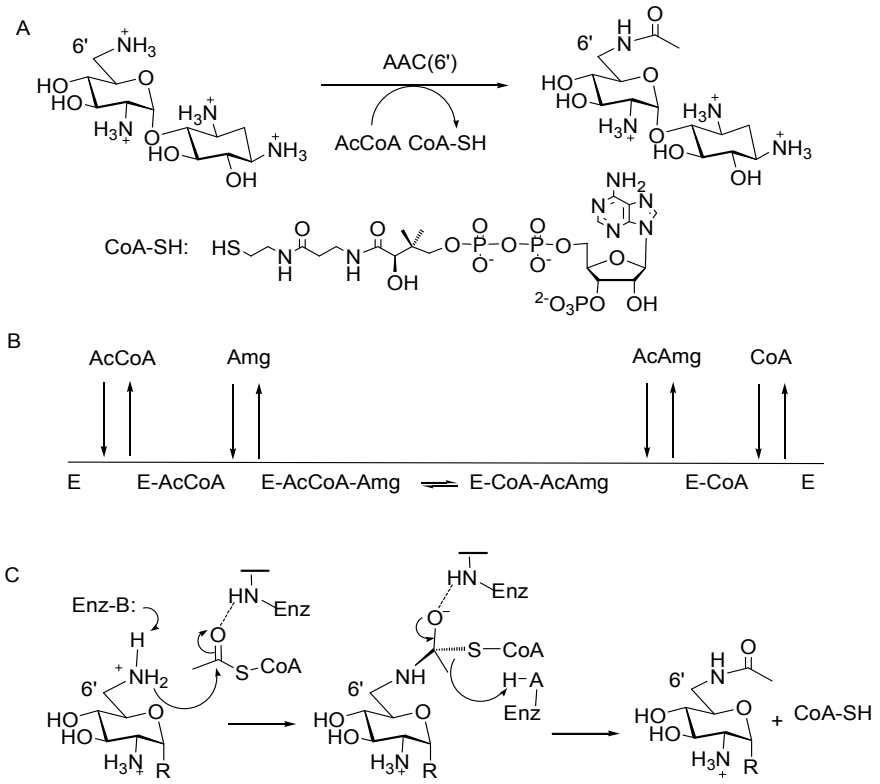



***Figure 1.7.*** Acetyltransfer reaction by AAC(6′)s. (A) Typical aminoglycoside (shown neamine) modification by AAC(6′)s. (B) Schematic representation of the ordered bi-bi kinetic mechanism proposed for AAC(6′)-Ii. (C) Details of the reaction pathway for AAC(6′)-Ii. Abbreviations: AcCoA, acetyl-CoA; Amg, aminoglycoside; AcAmg, *N*-6′-acetylated aminoglycosid; E or Enz, enzyme.

The bifunctional AAC(6′)-Ie-APH(2″)-Ia enzyme is the most clinically relevant determinant of aminoglycoside resistance.[80, 81] This enzyme is monomeric in solution and confers high-level aminoglycoside resistance.[59, 80, 82] Unlike other AAC(6′)s, the acetyltransfer domain of this bifunctional enzyme AAC(6′)-Ie can catalyze both *N*- and *O*-acetylation. The corresponding gene is generally found on transposable elements, which accounts for the inter-genus transfer of this resistance determinant, originally isolated from *E. faecalis*.[25]

Initial characterization of purified AAC(6′)-Ii by Wright and Ladak[23] revealed that the protein is a homodimer in solution with a broad substrate specificity for aminoglycosides, including both 4,5- and 4,6-disubstituted deoxystreptamine. The regiospecificity of acetyl transfer was neatly demonstrated by the fact that two aminoglycosides which substitute a hydroxyl group for an

amino group at the 6′ position, lividomycin A and paromomycin, were not substrates. Turnover rates ($k_{cat}$) for all substrates were relatively low (0.1 to 1.1 $s^{-1}$), while the Michaelis-Menten constants ($K_m$s) were all in the low micromolar range. This results in specificity constants ($k_{cat}/K_m$) on the order of $10^4$ $M^{-1}$ $s^{-1}$,[23] well below the diffusion controlled rates of $10^8$ to $10^9$ $M^{-1}$ $s^{-1}$ and below values for other aminoglycoside-inactivating enzymes, such as type IIIa aminoglycoside phosphotransferase [APH(3′)-IIIa] ($10^6$ $M^{-1}$ $s^{-1}$)[83] and APH(3′)-Ia and –IIa ($10^6$ to $10^8$ $M^{-1}$ $s^{-1}$)[84-86] Product and dead-end inhibition studies revealed that AAC(6′)-Ii follows an ordered bi-bi mechanism with acetyl coenzyme A (AcCoA) binding first and CoA released last (Fig. 1.7, B).[87] The rate-limiting steps of the reaction were explored by solvent viscosity and solvent isotope effects. The results suggest that diffusion-controlled events (substrate binding and/or product release) are rate-limiting rather than the chemistry.[87] The chemical reaction pathway of acetyl transfer by AAC(6′)-Ii is believed to involve a possible general acid/base catalysis and stabilization of the tetrahedral intermediate by the enzyme. Crystal structure of AAC(6′)-Ii in complex with AcCoA revealed that the amide NH group of Leu76 is H-bonded with the acetyl carbonyl of AcCoA, which suggests that the amide NH of Leu76 might be involved in the stabilization of the tetrahedral intermediate of the catalysis (Fig. 1.7, C).[88, 89] Yet, site-directed mutagenesis experiments could not identify specific amino acid residues positioned to act as the proposed general acid and/or base, either stabilization of the tetrahedral intermediate.[89]

**Structural studies of AACs**

To date, four crystal structures of AACs in complex with CoA have been solved (Fig. 1.8). Among them two, namely AAC(3)-Ia (Fig. 1.8, B)[90] and AAC(6′)-Ii (Fig. 1.8, D)[78, 88] were reported in complex with CoA or AcCoA; the other two AAC(2′)-Ic (Fig. 1.8, A)[91] and AAC(6′)-Iy (Fig. 1.8, C)[92] were reported in complex with both a substrate and CoA.

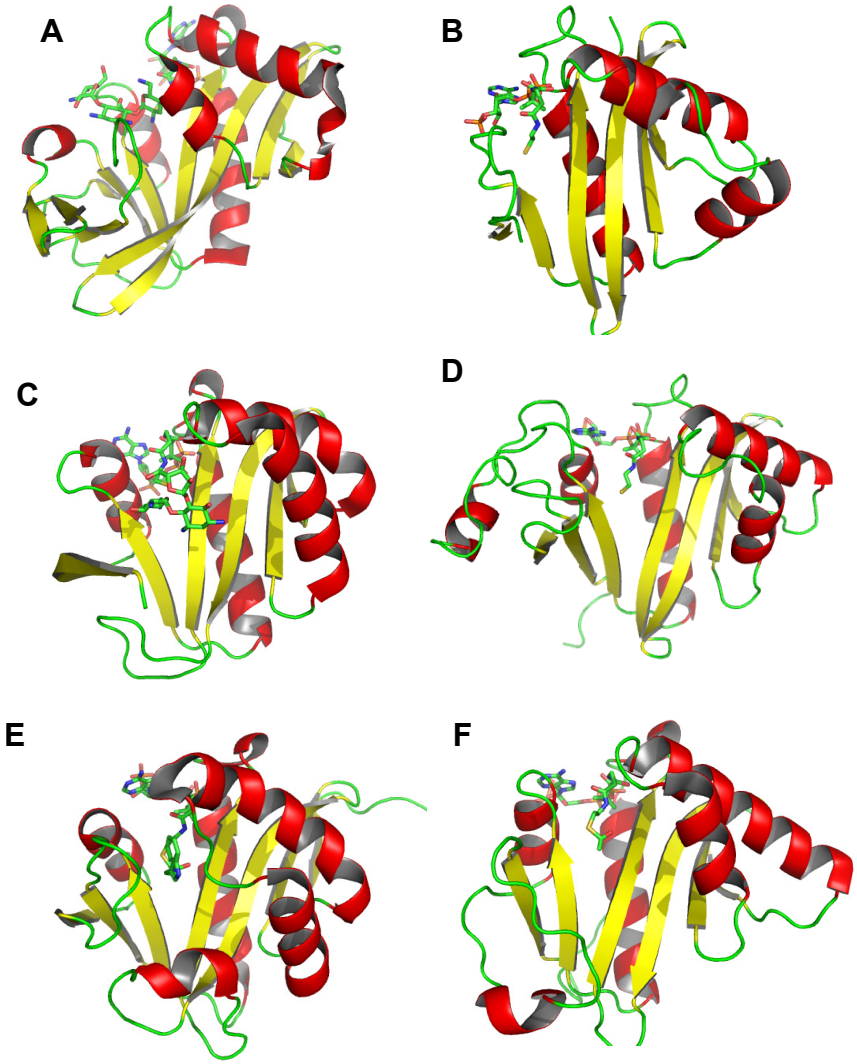



***Figure 1.8***. 3D structures for AACs and other acetyltransferases with the same overall fold. A) AAC(2′)-Ic from *Mycobacterium tuberculosis* in complex with tobramycin and CoA (1M4D.pdb);[93] B) AAC(3)-Ia from *Serratia marcescens* in complex with CoA (1BO4.pdb);[94] C) AAC(6')-Iy from *Salmonella enterica* in complex with ribostamycin and CoA (1S3Z.pdb);[92] D) AAC(6′)-Ii from *Enteroccocus faecium* in complex with CoA (1N71.pdb);[78] E) *Tetrahymena* GCN5 histone *N*-acetyltransferse in complex with AcCoA and a peptide (1QSR.pdb);[95] and F) serotonin *N*-acetyltransferase in complex with a bisubstrate analog (1L0C.pdb).[96] The proteins are shown as cartoon and colored by secondary structure (helix, red; b-sheet, yellow; random coil, green); CoA, AcCoA, the substrates and the bisubstrate analog are shown in stick representation and colored by atom type (C, green; N, blue; O, red; P, orange; S, yellow). Coordinates were obtained from the Protein Data Bank, and the images were prepared using PyMol.

All AACs share a common overall fold that classifies them as members of the GCN5-related *N*-acetyltransferase (GNAT) superfamily.[97, 98] This superfamily includes histone acetyltransferases (Fig. 1.8, E),[66, 95, 99-101] *N*-myristoyl-transferases,[102] serotonin *N*-acetyltransferases (Fig. 1.8, F),[96, 103-105] and carnitine acetyltransferase (structures are not shown).[106,107]

There are no conserved residues or mechanisms for acetyl transfer by enzymes of the GNAT family, but the CoA binding site is highly conserved, including an *N*-terminal α-helix, a central, antiparallel three-stranded β-sheet, and a helix-sheet-helix at the C-terminus of the fold (Fig. 1.8). This fold serves to bind and orient the phosphopantetheinyl arm of AcCoA, while fewer interactions are observed between the enzyme and the adenine ring.

Biochemical and structural studies reveal that the known AACs all have very broad substrate specificity. As discussed earlier, only two AACs (AAC(2′)-Ic and AAC(6′)-Iy) have been crystallized in the presence of aminoglycosides. The structures determined show that the interactions between aminoglycosides and the enzymes are diversified. In AAC(2′)-Ic the majority of interactions between the enzyme and the aminoglycoside occur at acidic residues in the active site (D35, D40, E82, D152, D179) through electrostatic or H-bond interactions, sometimes via water molecules.[90] In AAC(6′)-Iy, on the other hand, electrostatic and H-bond interactions between aminoglycoside and acidic residues (E79, D115, and E136) are still important but not sufficient, the 2-DOS ring of the aminoglycoside is also found stacked with aromatic residues (W22 and Y66).[92]

### 1.3 Rejuvenation: solutions to the aminoglycoside resistance

In spite of rising resistance, aminoglycoside antibiotics remain indispensable for the treatment of serious infections.[2] For example, opportunistic pathogenic *Pseudomonas aeruginosa*, a Gram-negative bacterium, is intrinsically resistant to a wide variety of antibacterial agents including penicillins, cephalosporins, tetracyclins, quinolones, and chloramphenicol. Aminoglycosides such as

tobramycin and gentamicin, on the other hand, are effective against this organism.[108]

With the advancement of knowledge related to resistance mechanisms and aminoglycoside-RNA interactions, recent efforts have focused on rejuvenating aminoglycosides. In spite of the challenge of designing molecules that can differentiate the A site of RNA and the binding site of resistance-causing enzymes, a number of research groups worldwide have committed a great deal of efforts to prepare derivatives of natural aminoglycosides. Alternatively, research has also focused on the development of inhibitors to block resistance pathways.

### 1.3.1 Derivatization of aminoglycosides

Derivatization of existent antibiotics has been the main approach to combat bacteria resistance. For example, many new generations of β-lactam drugs have been developed (Fig. 1.9).

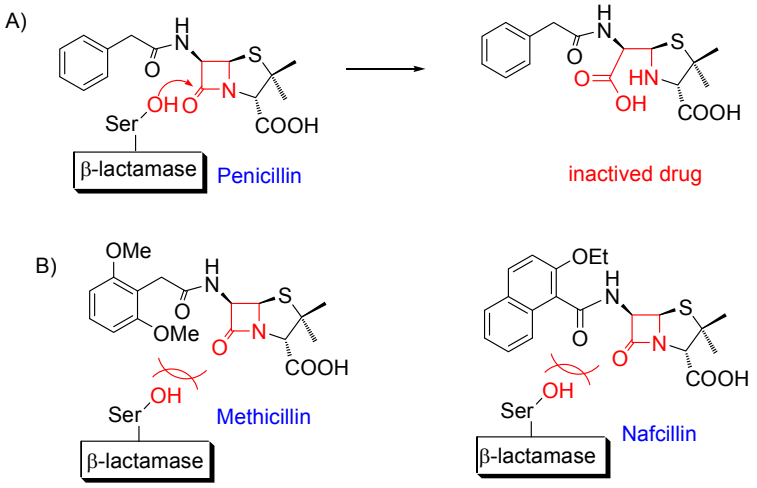
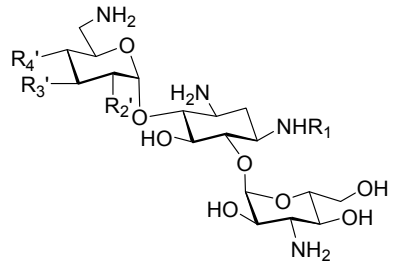



***Figure 1.9***. Penicillin inactivation by β-lactamase and a successful strategy to overcome resistance. A) proposed mechanism of inactivation of penicillin by β-lactamase; B) two examples of semisynthetic penicillin derivatives that have bulky group adjacent to the β-lactam to block β-lactamase accessibility.

As evidenced by the success of semisynthetic penicillin derivatives as antibacterial agents, an important aspect of antibiotic research is the

understanding of the molecular mechanisms of resistance. For example, the discovery of regiospecific phosphorylation of aminoglycosides by APH(3′)s in the 1960s' enabled the development of aminoglycosides lacking the sites of inactivation, e.g., tobramycin (3′-deoxykanamycin B) and dibekacin (3′,4′-dideoxykanamycin B) (Fig. 1.9).[109] Subsequently, amikacin, and isepamicin were developed by introducing (*S*)-4-amino-2-hydroxybutyryl (AHB) and (*S*)-3-amino-2-hydroxypropionyl (AHP) side chains (Fig. 1.10) at the 1-amino group of kanamycin and gentamicin B, respectively. These side chains are believed to block the access of aminoglycoside-modifying enzymes to the target sites. Moreover, the incorporated AHB or AHP groups contain a 1,3- or 1,2-hydroxylamine moiety that binds to phosphodiesters and to the Hoogsten face of guanosine of the A site of 16S rRNA. The latest semisynthetic aminoglycoside in clinical use is arbekacin commercialized in 1990s (Fig. 1.10). It is active against methicillin-resistant *Staphylococcus aureus* (MRSA).[110]



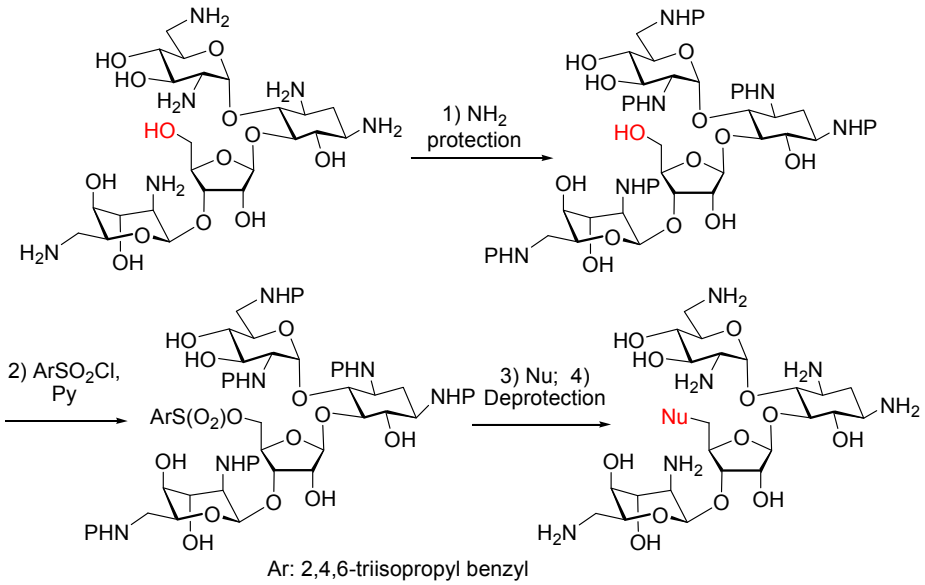

| Aminoglycoside | $R_1$ | $R_2'$ | $R_3'$ | $R_4'$ |
|---|---|---|---|---|
| **Tobramycin** | H | $NH_2$ | H | OH |
| **Amikacin** | AHB | OH | OH | OH |
| **Arbekacin** | AHB | $NH_2$ | H | H |
| **Dibekacin** | H | $NH_2$ | OH | H |
| **Isepamicin** | AHP | OH | OH | OH |

***Figure 1.10.*** Semisynthetic aminoglycosides. AHB: (*S*)-4-amino-2-hydroxy-butyryl; AHP: (*S*)-3-amino-2-hydroxypropionyl.

Recently, numerous novel aminoglycoside derivatives have been reported. The synthesis and biological activity of these derivatives are summarized in the next few sections. They are organized based on the structure of the parent aminoglycosides.

**Neomycin B derivatives**

Neomycin B derivatives with substituents at C5′′-OH were prepared without complicated protection/deprotection steps. The derivatization procedure usually followed a general approach shown in Scheme 1.1. First, the amino groups of neomycin B were protected as either carbamates or azides; second, the least hindered C5′′-OH was regio-selectively transformed into a better leaving group (e.g. 2,4,6- trimethylphenylsulfonate) without the need for protection of other hydroxyl groups. Next, a nucleophile (usually a thiol or an amine) was used to displace the leaving group at O-5′′ positions; and finally, removal of the amino protecting groups afforded the desired neomycin B derivatives in a relatively efficient manner.




***Scheme 1.1***. General synthetic approach to prepare neomycin B derivatives with substituents at C5′′-OH position.

Two research groups have reported using 2′,5′′-tethered constrained neomycin derivative (Fig. 1.11, **1a** and **1b**) to evade aminoglycoside resistance-causing enzymes yet maintain RNA binding affinity.[111-113] Comparison of the structures of RNA-aminoglycoside complexes with those of resistance-causing enzymes in complex with aminoglycosides suggests that the aminoglycoside ring conformations recognized by enzymes are not the same as the ones recognized by RNA. Thus aminoglycoside derivatives physically locked into an RNA

recognizable conformation such as **1a** and **1b** (Fig. 1.11) were expected to be resistant to enzymatic modification by the resistance-causing enzymes. Derivatives **1a** and **1b** were tested with three classes of aminoglycoside resistance-causing enzymes, *Staphylococcus aureus* ANT(4′), *Mycobacterium tuberculosis* AAC(2′) and *Enterococcus faecalis* APH(3′). They were found significantly resistant to these enzymes either in pure form or in cells, while maintaining significant antibacterial activities.[111-113]

Targeting multiple binding sites that may coexist on a large RNA molecule was explored for enhancing the binding affinity between aminoglycosides and RNA. Covalently 5″,5″-dimerized neomycins (Fig. 1.11, **2a-b**) were synthesized for this purpose. Relatively long and conformationally flexible linkers were selected to allow the molecules to 'scan' the conformational space in the search for a second RNA binding site. Dimer **2b** was found to inhibit the ribozyme function 20-fold more effectively than neomycin B.[114, 115]

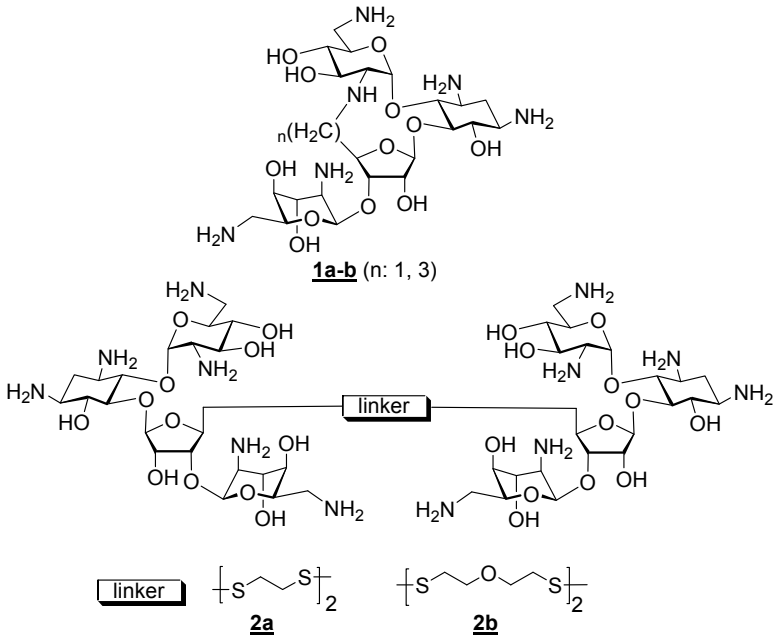



***Figure 1.11.*** Constrained neomycin B derivatives and neomycin B dimers.

5″-*O*-Glycosylated neomycin derivatives (Fig. 1.12, **3a-d**) represent a defined class of branched aminoglycoside antibiotics. They show antibacterial

activity comparable to that of neomycin B (Table 1.1), yet they demonstrate poor substrate activity with APH(3′)-IIIa.[116-118]

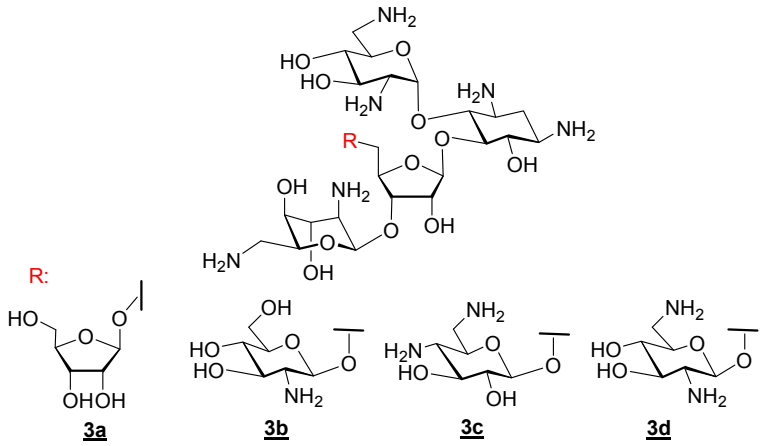



***Figure 1.12***. Structure of 5′′-*O*-glycosylated aminoglycoside antibiotics.[116-118]

**Table 1.1.** MIC (μg/mL) of 5′′-*O*-glycosylated aminoglycoside antibiotics.[116-118]

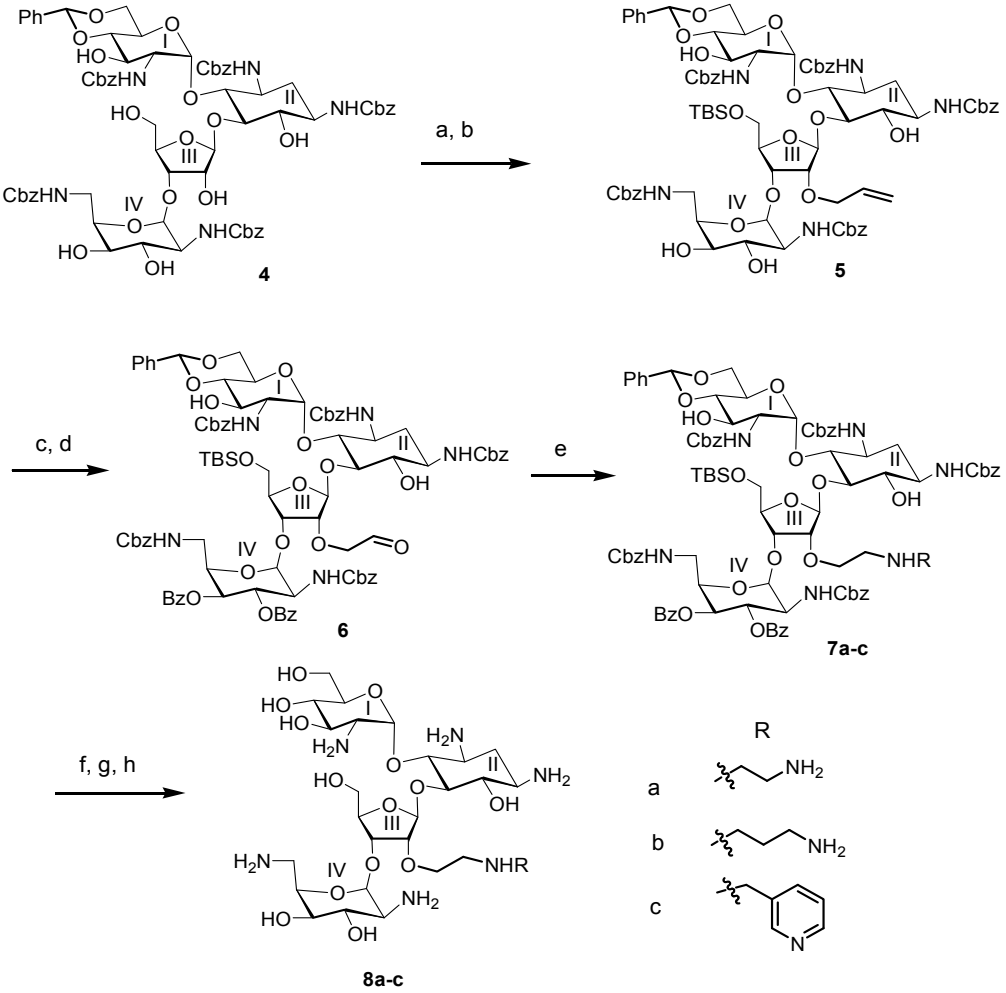
| Compounds | Neo* | **3a** | **3b** | **3c** | **3d** |
|---|---|---|---|---|---|
| *E. coli* (ATCC 25922) | 8-10 | 10-11 | 25-30 | 40-50 | 95 |
| *E. coli* (APH(3′)) | 50-60 | 35-45 | >200 | >200 | >200 |
| *S. epidermidis* (ATCC 12228) | 0.3-0.4 | 0.2-0.4 | 1.4-1.8 | 1.5-1.8 | 5.5-7 |
| *Bacillus. subtilis* (ATCC 6633) | 0.8-0.9 | 0.6-0.8 | 1.4-1.8 | 3.5-4 | 8.5-10 |
| *S. virchow* (APH(3′)) | 200-250 | 75-125 | >1250 | >1250 | >1250 |
| *P. aeruginosa* (ATCC 27853) | 55-60 | 60-65 | 40-50 | 30-35 | 120 |

* Neo: neomycin B

**Paromomycin derivatives**

The synthesis of paromomycin derivatives **8a-c** (Scheme 1.2) represents a new paradigm in aminoglycoside modification by successfully employing a structure-based design approach.[119] Examination of the crystal structure of the paromomycin complex[120] reveals that the hydroxy group at C2′′ of ring III is favorably disposed for appropriate functional diversification. The readily available paromomycin derivative **4**[121] was silylated at C5′′ and the product was subjected to *O*-allylation. Remarkably, after TBS protection of the C5′′ hydroxy group, a highly regioselective allylation of the C2′′ hydroxy group led to **5** in good yield. Protection of the hydroxy groups on rings II and IV by benzoylation

and oxidative cleavage of the allyl group afforded aldehyde **6**. Reductive amination with three representative alkyl amines gave the protected aminoalkyl ether analogues **7a-c**. Treatment with a catalytic amount of sodium methoxide in methanol afforded the corresponding polyols. Hydrolysis of the *O*-benzylidene acetal and the TBS ether with aqueous acetic acid, followed by catalytic hydrogenation gave the paromomycin derivatives **8a-c**.




***Scheme 1.2***. Synthesis of paromomycin derivatives **8a-c**.[119] Reagents and conditions: a) TBSOTf, 2,4,6-collidine, $CH_2Cl_2$, 75%; b) $CH_2$=$CHCH_2I$, KHMDS, THF, 70%; c) BzCl, pyridine, DMAP, 95%; d) 1. $O_3$, $CH_2Cl_2$, -78°C; 2. $Ph_3P$, -78°C→RT, 80%; e) amine, $NaBH_3CN$, MeOH/AcOH (30:1), 90%; f) NaOMe, MeOH, 80%; g) AcOH, 80%, 60°C, 2 h; h) Pd(OH)2/C, $H_2$, AcOH 80%, 70% (2 steps). Bz: benzoyl, Cbz: benzyloxycarbonyl, KHMDS: potassium hexamethyldisilylamide, TBS: *tert*-butyldimethylsilyl.

The paromomycin derivatives **8a-c** maintained similar activity against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria to the parent aminoglycoside, paromomycin.[119] Most interesting was the 3-(aminomethyl)pyridyl analogue **8c**, which was slightly more potent than the parent antibiotic paromomycin against the Gram-positive *S. aureus* strain. It was envisaged that the incorporation of a large side chain could prevent resistance enzymes from modifying the compounds and rendering them inactive, which is a key element in resistance to aminoglycosides. This hypothesis was partially supported by the activity (MIC 25–50 mm) of **8c** against a multidrug-and methicillin resistant *S. aureus* (MRSA) strain (ATCC BAA-44). In contrast, paromomycin, neomycin, kanamycin, tobramycin, gentamicin, sisomicin, and streptomycin were completely ineffective against MRSA. These results suggest that a C2″ substituent with an aromatic terminal group in 4,5-disubstituted aminoglycosides offers distinct advantages in the fight against the emergence of drug-resistant bacteria. However, it remains to be seen if this type of modification does in fact hinder interaction with one or more resistance enzymes, while allowing strong binding to the target RNA.

**Tobramycin derivatives**

Also based on the structures of aminoglycoside-RNA complexes, it was observed that rings I and II of both paromomycin and tobramycin (Fig. 1.13) bind to the same site of the 16S rRNA A site and with nearly superimposable conformations.[4, 120, 122-125] Rings III and IV of paromomycin interact with the lower stem of the A-site RNA while the ring III of tobramycin binds to the upper stem. Thus, it was reasoned that the introduction of ether-linked basic groups at *O*-5 of tobramycin would bind to RNA the way that ring III of paromomycin does and improve the affinity. A library of tobramycin analogs containing various functionalities at *O*-5 was prepared (Fig. 1.13).[126] In general, these analogs (**9a-j**) were less active than tobramycin against susceptible *E. coli* and *S. aureus* (Table 1.2); however, derivatives **9d** and **9g** showed activity against resistant strain *P. aeruginosa* (ATCC 27853) with a MIC of 12.5 μg/mL.[126]

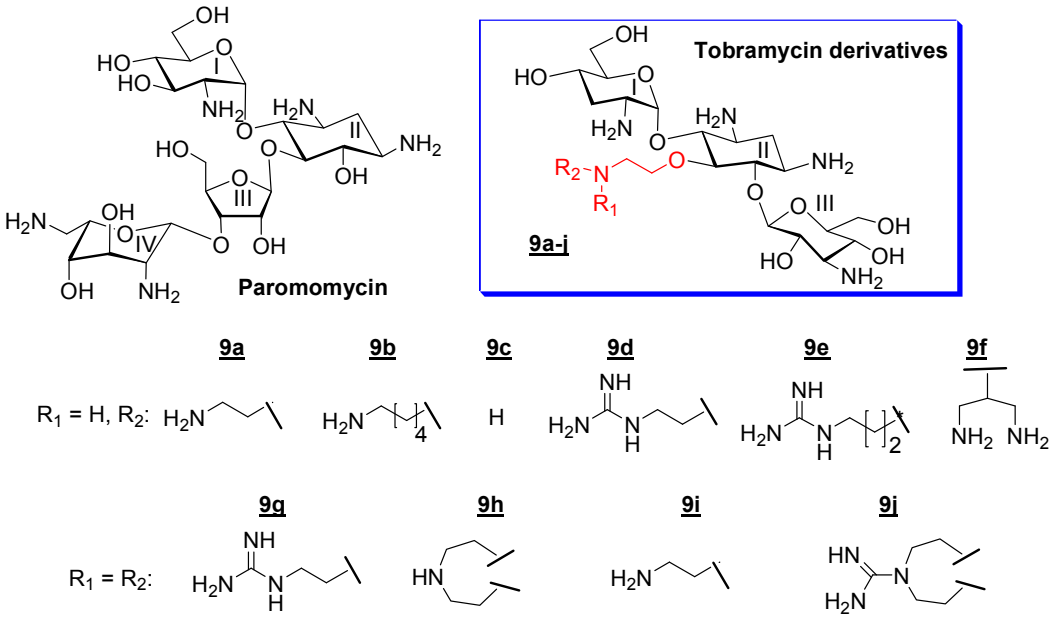



***Figure 1.13***. 5-*O*-Tobramycin derivatives.[126]

**Table 1.2.** MIC (μg/mL) of tobramycin derivatives[126]

| Compound | *E. coli* (ATCC 25922) | *S. aureus* (ATCC 13709) |
|---|---|---|
| Kanamycin A | 2.5-5 | 1.2-2.5 |
| Kanamycin B | 1.2-2.5 | 0.3-0.6 |
| Tobramycin | 0.6-1.2 | 0.3-0.6 |
| Amikacin | 1.2-2.5 | 1.2-2.5 |
| Paromomycin | 2.4-5 | 1.2-2.5 |
| Neamine | >10 | >10 |
| **9a** | 2.5-5 | >10 |
| **9c** | 5-10 | >10 |
| **9d** | 2.5-5 | >10 |
| **9f** | 1-2 | 5-10 |
| **9g** | 0.6-1.2 | 2-5 |
| **9h** | 1-2 | 5-10 |
| **9i** | 1-2 | 5-10 |
| **9j** | 2.5-5 | >10 |

**Neamine and paromamine derivatives**

Structural studies of RNA-aminoglycoside complexes reveal that neamine is the minimal structure to specifically bind to the 16S rRNA A site.[9] Biological studies have also confirmed that neamine is necessary yet sufficient to cause

mistranslation in bacteria.[127] Neamine or paromamine themselves are poor antibiotic and not clinically useful, yet many of their synthetic derivatives have been reported. The general derivatization strategy involves multiple protection/deprotection manipulations to generate one free hydroxyl group. Since the electrostatic interactions between the amino groups of aminoglycosides and RNA are critical, introduction of derivatives is generally more productive at hydroxyls than at amino groups, and many derivatives have been prepared where one or more hydroxyls are substituted with amino-containing groups (Fig. 1.14). To this end, after selective protection, the desired hydroxyl function is allylated. Ozonolysis is next used to generate an aldehyde, which undergoes reductive amination to incorporate multiple amino groups (Scheme 1.2, steps 6-10).

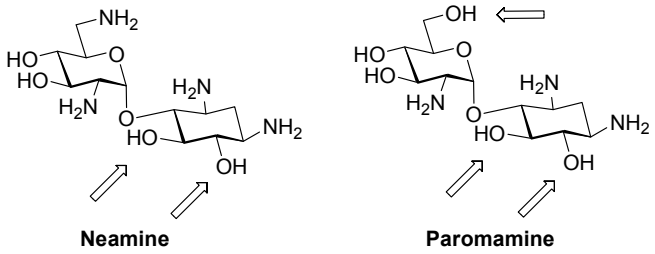
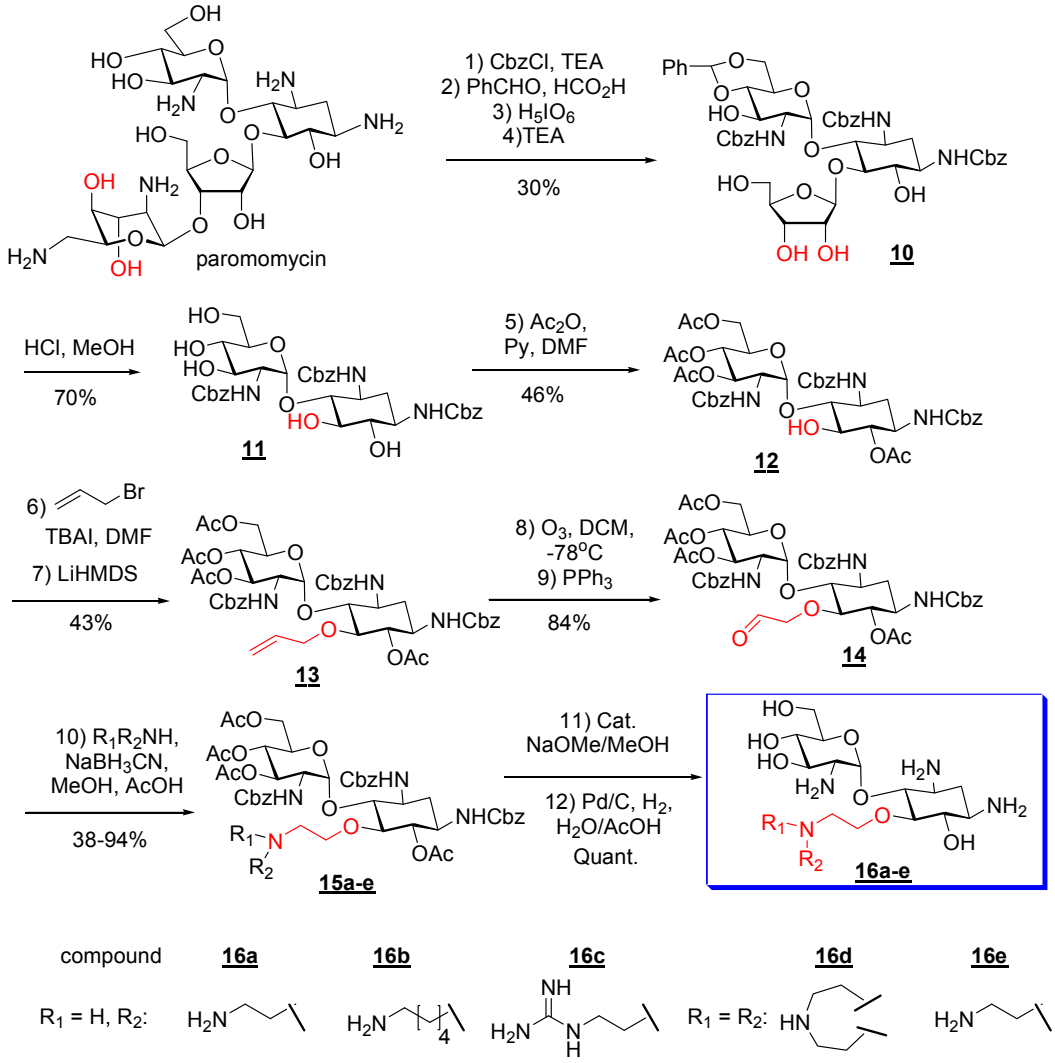



***Figure 1.14***. Derivatization sites of neamine and paromamine.

Paromamine derivatives (Scheme 1.3, **16a-e)** were prepared from paromomycin (Scheme 1.3).[121,128] The amino groups and the 4′,6′-diol of paromomycin were first protected. Oxidation with periodic acid cleaved the 3‴,4‴-diol of the idose (ring IV) to generate a dialdehyde, which underwent β-elimination when treated with TEA to generate **10**.[121] Compound **10** was subjected to methanolysis to afford Cbz-protected paromamine **11**. It was observed that the C5-OH was resistant towards acetylation, thus treating **11** with acetic anhydride provided compound **12**. At this point, only one free hydroxyl group (C5-OH) was left. The general transformation strategy described above was applied. Thus, *O*-allylation of **12** afforded **13**, followed by ozonolysis of **13** to **14**, which upon reductive coupling with a variety of *N*-protected primary alkylamines, secondary amines, and alkylguanidines provided fully protected intermediates **15a-e** in good yields. Deprotection of compounds **15a-e** with catalytic NaOMe

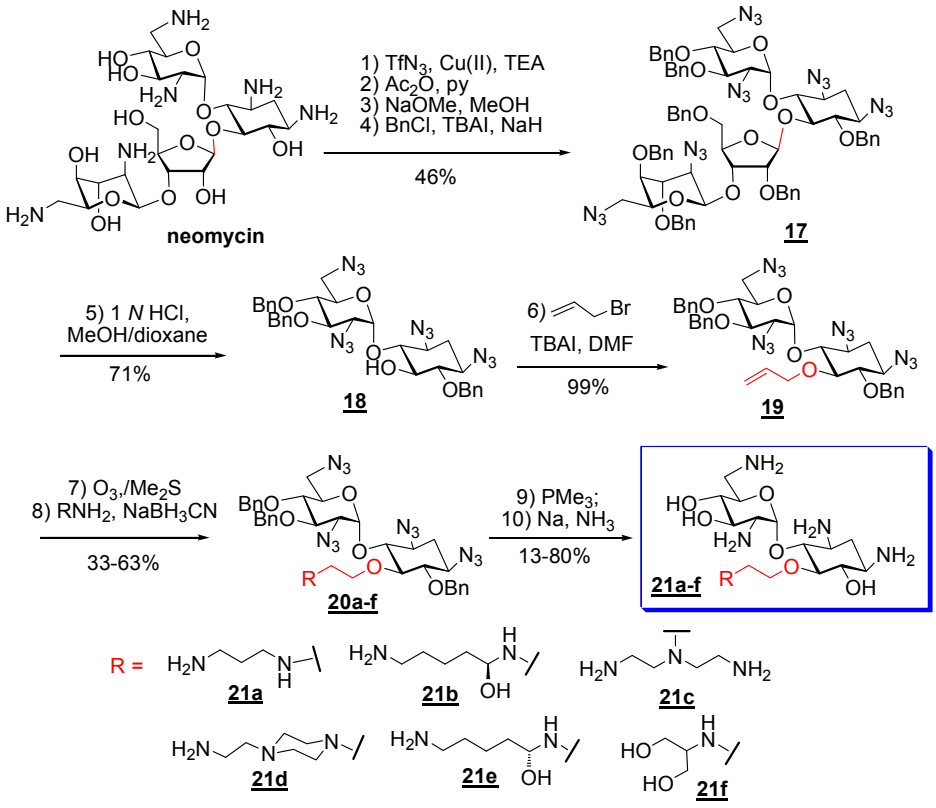
followed by hydrogenolysis afforded derivatives **16a-e** (Scheme 1.3).[128] Disappointingly, none of analogs **16a-e** exhibited any antibacterial activity when tested with a panel of resistant and sensitive strains of *P. aeruginosa*, *S. aureus*, *E. coli,* and *E. faecalis* at 50 μg/mL.




***Scheme 1.3.*** *O*-5-derivatized paromamine analogs.[128]

Derivatives of neamine (Scheme 1.4, **21a-f**) were prepared via a comparable approach, starting from neomycin. Protection of the amino groups as azides using triflic azide[129-131] was followed by peracetylation for a facilitated separation of the product. The peracetylated product was hydrolyzed again and followed by benzylation to afford protected neomycin **17** in 46% overall yield. The β-ribofuranosyl bond of neomycin was selectively cleaved with 1 *N* HCl in

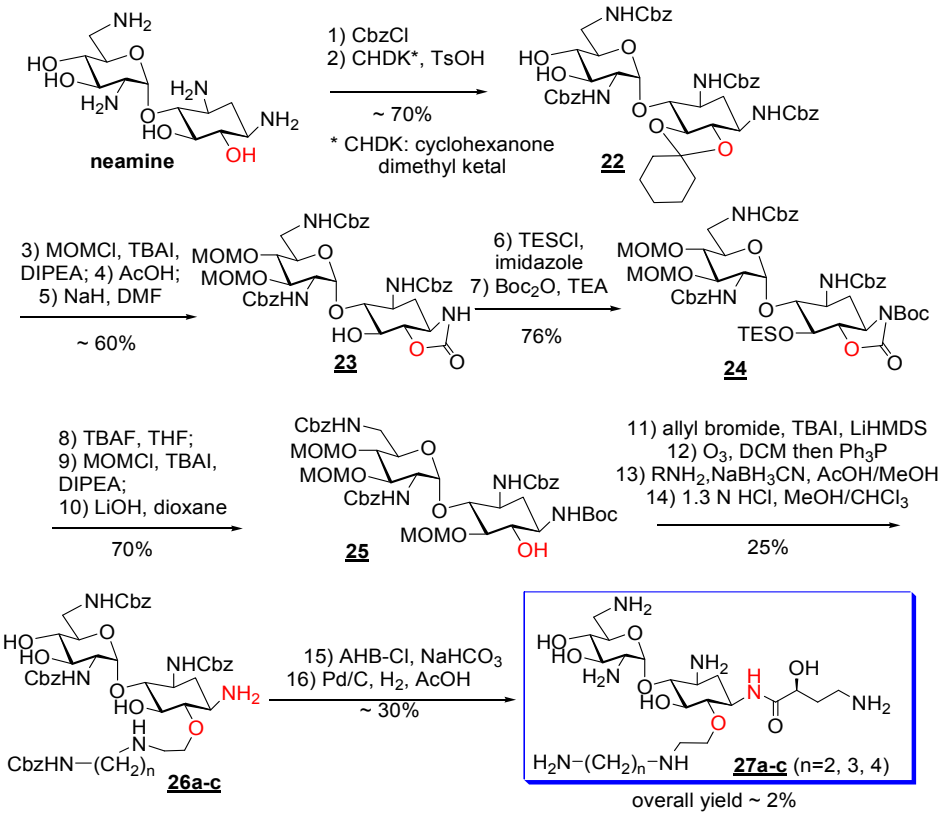
methanol/dioxane, to yield building block **18** in 71% yield after recrystallization. Allylation of **18** at C5-OH provided **19**, which was subjected to ozonolysis and reductive amination of the resultant aldehyde, providing compounds **20a-f**. Reduction of the azides using Staudinger reaction was followed by debenzylation to afford derivatives **21a-f** (Scheme 1.3).[132,133] Derivatives **21c** and **21e** showed the most potent activity with MICs of 5-12 μM against *E.coli* (ATCC 25922), i.e., about 2-4-fold more potent than neamine.[132,133]




***Scheme 1.4.*** *O*-5-derivatized neamine analogs.[132,133]

Based on molecular docking from a partial structure of the 16S rRNA A site, a library of *N*-1,*O*-6-derivatized neamine analogs were designed and each was synthesized in 16 sequential steps with 2% overall yield (Scheme 1.4).[134] Treatment of neamine with benzyl chloroformate in the presence of sodium carbonate afforded the tetra-*N*-Cbz-protected neamine, which was reacted with cyclohexanone dimethyl ketal (CDMK) to provide the mono-cyclohexylidene-protected neamine derivative **22**. Protection of the 3′- and 4′-hydroxyl groups as

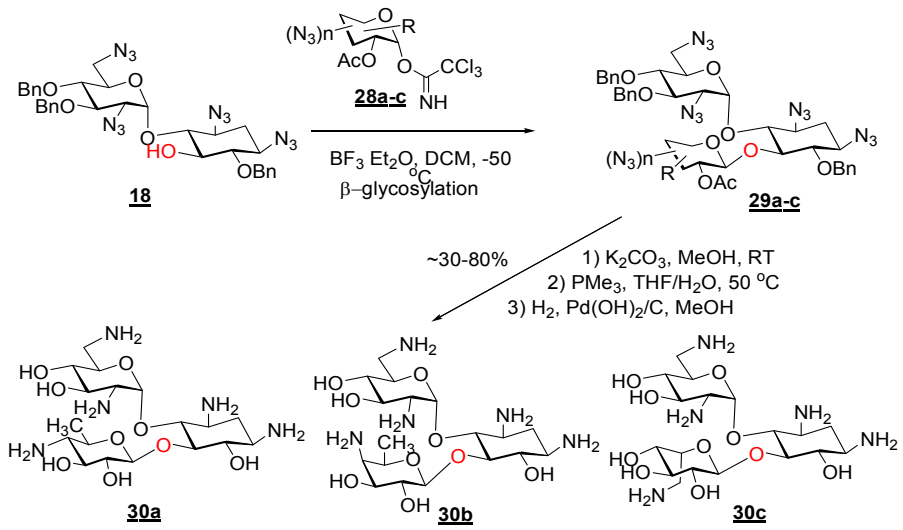
methoxymethyl (MOM) ethers followed by cyclohexylidene deprotection with acetic acid afforded an intermediate which was treated with NaH in DMF to furnish the cyclic carbamate **23**. Protection of the C5-OH of **23** using triethylsilyl chloride (TESCl) followed by treatment with di-*tert*-butyl dicarbonate ($Boc_2O$) in the presence of triethylamine and *N,N*-dimethylaminopyridine (DMAP) afforded compound **24** in high yield. The TES group of **24** was removed using tetrabutyl ammonium fluoride (TBAF) and protected as a MOM ether. This intermediate was treated with 0.5 *N* aqueous lithium hydroxide to give **25**. At this point, the C6-OH was the only free hydroxyl group. Alkylation with allyl bromide was followed by ozonolysis, and reductive amination with mono-*N*-Cbz-protected diaminoalkanes. Deprotection of the Boc and MOM groups with 1.3 *N* methanolic HCl afforded the aminated products **26a-c,** with the *N*-1 group free for installation of AHB. Treatment of these compounds **26a-c** with an activated ester of *N*-Cbz-protected AHB was followed by deprotection of the Cbz to afford the final *N*-1, *O*-6 derivatized neamine analogs **27a-c**.[134] All the derivatives showed improved activity compared to the parent compound neamine against a variety of sensitive and resistant bacterial strains harboring the *aph(3′)*-I or *aac(6′)/aph(2″)* genes.[134]




***Scheme 1.5.*** *O*-6, *N*-1-derivatives of neamine.[134]

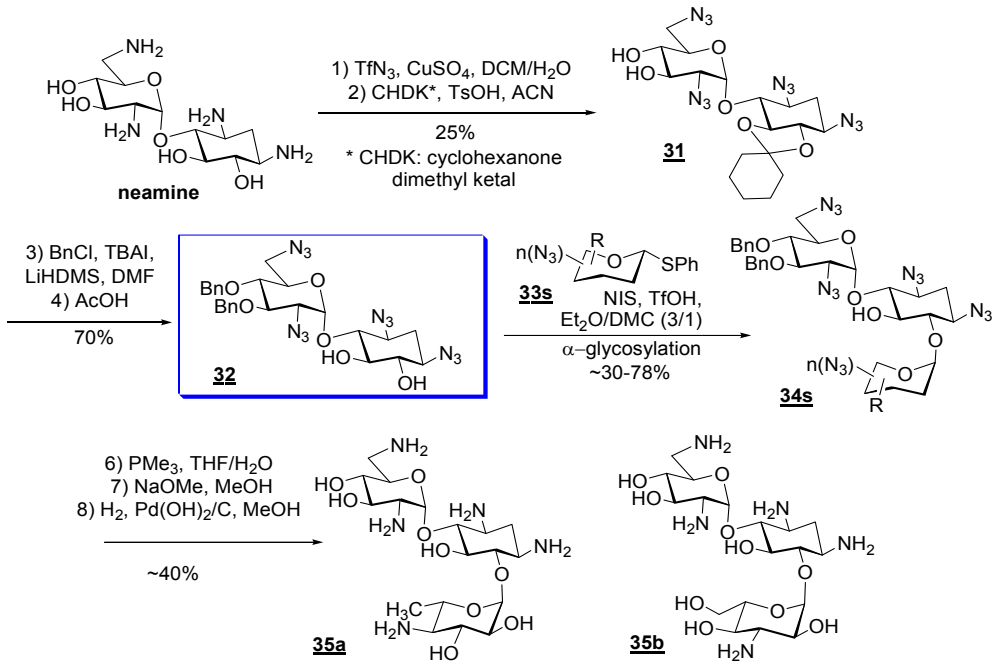
## Glycosylated neamine derivatives

More recently a new strategy termed "glycodiversification" was developed to synthesize libraries of pyranmycins (Scheme 1.5, *O*-5-glycosylated neamine)[135, 136] and kanamycin analogs (Scheme 1.6, *O*-6-glycosylated neamine).[137, 138] This approach uses glycosyl trichloroacetimidate donors **28a-c** to favor the formation of β-glycosidic bonds and afford protected pyranmycins **29a-c**. The acetyl groups were hydrolyzed and the azido groups and benzyl groups were reduced to give pyranmycins **30a-c** (Scheme 1.6). These three pyranmycins showed moderate activity against both susceptible and resistant strains of *E. coli*. [135, 136]



***Scheme 1.6***. Synthesis of *O*-5-glycosylated neamine derivatives.[135,136]

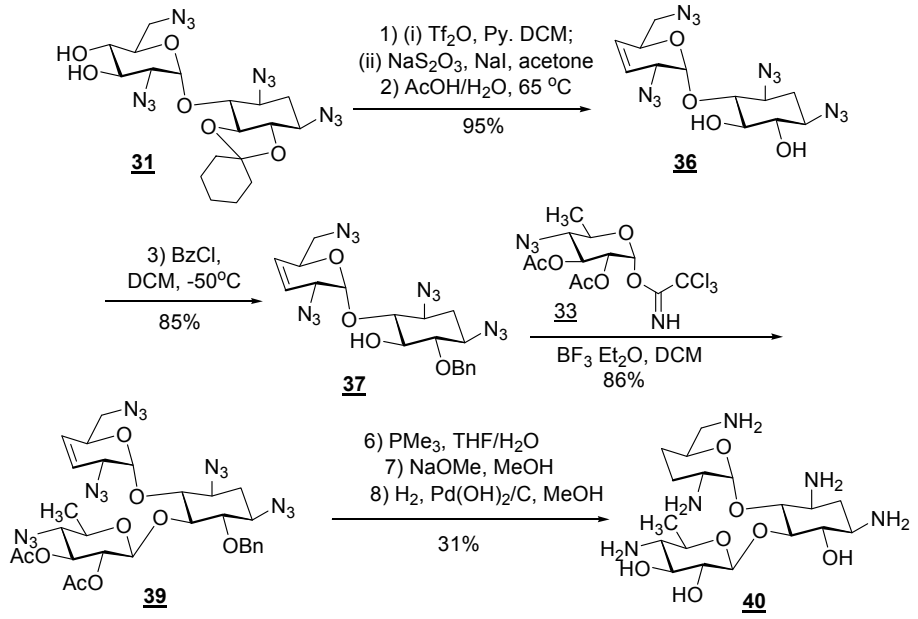
When phenylthioglycosyl donors **33s** were used, the formation of α-glycosidic bonds was favored (Scheme 1.7).[137, 138] The structure-activity relationship (SAR) studies with this library of kanamycin analogs revealed two lead compounds, **35a** and **35b** (Scheme 1.7), with MICs ranging from 2 to 12 μg/mL against susceptible strains *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923).



***Scheme 1.7***. General synthetic approach involving *O*-6-glycosylation of neamine to generate kanamycin analogs.[137, 138]

### 3′,4′-Dideoxygenated pyranmycin derivatives

3′,4′-Dideoxygenation has been reported to be an effective strategy to escape from APH(3′)s.[139] A mild procedure has been developed for this transformation.[140] As shown with compound **31**, it involves triflation, followed by treatment with $Na_2S_2O_3$ and a catalytic amount of NaI, to yield compound **36** after hydrolysis of the acetal (Scheme 1.8). Regioselective benzoylation of 6-OH followed by glycosylation with donor **38** afforded compound **39**. Staudinger reaction and hydrogenation yielded pyranmycin **40**. This derivative was found to be more active than ribostamycin but less active than butirosin, a neamine derivative containing a AHB group at the *N*-1 position (Table 1.3).[140]

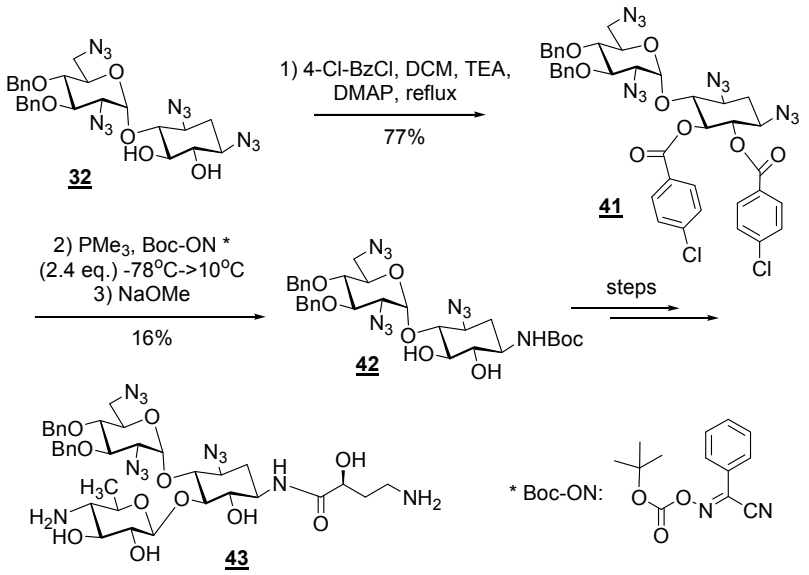



***Scheme 1.8.*** Synthesis of 3′,4′-dideoxypyranmycin derivatives.[140]

### *N*-1 AHB pyranmycin derivatives

Derivatization at the *N*-1 position of the 2-deoxysteptamine has been shown to be an effective method to avoid resistance.[134, 141-145] The finding that electron-withdrawing protecting groups will enhance the reactivity of vicinal azido groups toward the Staudinger reaction[130] has led to an expedient protocol to synthesize *N*-1-derivatized pyranmycin (Scheme 1.9).[146] Thus, addition of a 4-chlorobenzoyl

group at *O*-6 of **41** favored regioselective reduction of the *N*-1 azido group. Using this approach, compound **43**, containing AHB at *N*-1, proved a promising antibacterial agent (Table 1.3).




***Scheme 1.9.*** General synthetic approach for the preparation of *N*-1-derivatized pyranmycins.[146]

**Table 1.3.** MIC (μg/mL) of natural and synthetic aminoglycosides[146]

| Compound | *E. coli* (TGI)[a] | *E. coli* (pSF815)[b] | *E. coli* (pTZ19U-3)[c] |
|---|---|---|---|
| Ribostamycin | 2 | 16 | inactive |
| Butirosin | 0.5 | 0.5 | 0.5 |
| **40** | 8 | 4 | 4 |
| **43** | 4 | 4 | 4 |

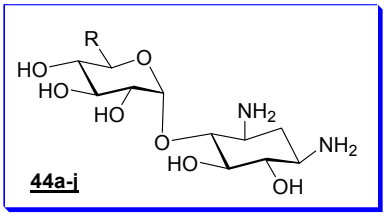
[a]: susceptible strain; [b]: strain expressing AAC(6′)-APH(2′′); [c]: strain expressing APH(3′)-Ia

### *N*-6′ Paromamine derivatives

Structural data from NMR[4, 122, 123] and X-ray crystallography[120, 124, 147] along with molecular modeling studies[148] suggested that extension at the 6′-position of paromamine might allow for additional H-bond interactions with the decoding-site RNA. Paromamine derivatives **44a-j** (Table 1.4) containing various

functional groups at the 6′-position were synthesized and tested as inhibitors of protein translation in bacteria (Table 1.4).[149] The observed SARs for the paromamine 6′-derivatives reveal an exquisite sensitivity of the 6′-position towards substitution, which emphasized the pivotal role of the 6′-OH/$NH_2$ for RNA target recognition.

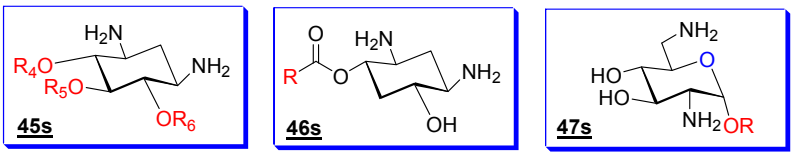
**Table 1.4.** Inhibition of translation by *N*-6′-paromamine derivatives[149]



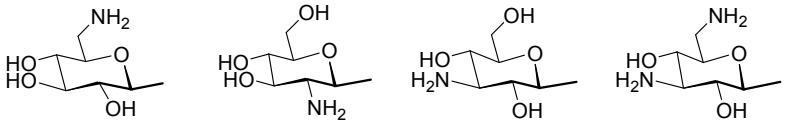

| Derivatives | R | $IC_{50}$ (mM) |
|---|---|---|
| Paromamine | $CH_2OH$ | 3.9 |
| **44a** | COOH | 620 |
| **44b** | $CONH_2$ | >1000 |
| **44c** | $CH_2CONH_2$ | 380 |
| **44d** | $CH_2CH_2OH$ | 170 |
| **44e** | $CH_2CH(OH)CH_3$ | 97 |
| **44f** | $CH(OH)CH_3$ | 23 |
| **44g** | $CH(OH)CH_2OH$ | 24 |

**Aminoglycoside derivatives containing modified 2-DOS rings**

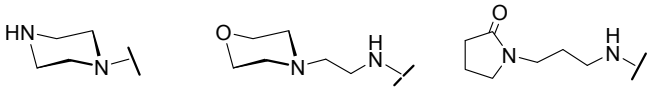
Aminoglycoside analogs obtained by glycosylation at various positions of 2-deoxystreptamine (2-DOS) have been synthesized (Fig. 1.15, **45s)**.[150] A series of 2,5-dideoxystreptamine derivatives were also reported (Fig. 1.15, **46s**) and showed 50-fold decreased antibacterial activity compared to neamine.[151] Acyclic deoxystreptamine mimetics (Fig. 1.15, **47s**) were also reported to be much weaker antibacterial agents than the natural aminoglycosides.[152] Similarly, the replacement of 2-DOS with glucosamine resulted in decreased antibacterial activity. It was concluded that the rigidity of the 2-DOS scaffold is essential to maintain antibacterial activity.




For **45s**, $R_n$: natural or synthetic aminosugar, some examples are given below



For **46s**, R: cyclic amino substituents, some examples are given below



For **47s**, R: amino substituents, some examples are given below

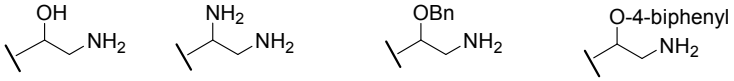
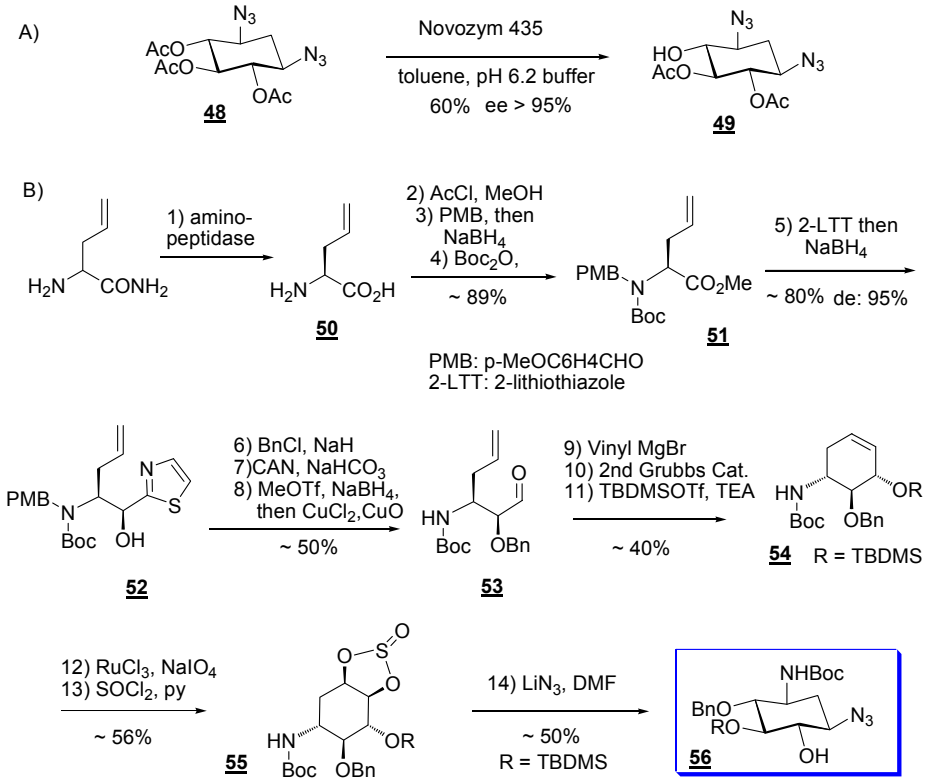


***Figure 1.15.*** Examples of aminoglycoside derivatives containing modified 2-DOS.[150-152]

A key step to generate the library **45s** (Fig. 1.15) involves the enzymatic desymmetrization of 2-DOS (Scheme 1.10, A).[153] The *meso*-2-DOS is usually prepared from the degradation of neomycin,[154, 155] and requires desymmetrisation as well as numerous protective group manipulations before incorporation into new aminoglycoside entities. Synthesis of the orthogonally protected, enantiopure 2-DOS derivative **56** (Scheme 1.10) has only been reported recently.[156] This compound serves as a suitable scaffold for the generation of new aminoglycoside derivatives. The synthesis of **56** starts with resolution of racemic allylglycine using aminopeptidase to afford D-allylglycine. The *N*-doubly protected (PMB, Boc) methyl ester of D-allylglycine **51** was next treated with 2-lithiothiazole (2-LTT), and followed by $NaBH_4$ reduction of the resulting ketone to afford **52** in good yield and excellent *de*. Benzylation of **52** followed by removal of PMB and the thiazole yielded an aldehyde **53**. Compound **53** was treated with vinylmagnesium bromide and followed by ring-closing metathesis, and silylation to afford **54** in a moderate overall yield. The double bond of **54** was dihydroxy-

lated and converted to the five-membered cyclic sulfate **55**. Opening of the cyclic sulfate with $LiN_3$ proceeded regioselectively to give, after sulfate hydrolysis, the protected 2-DOS derivative in enantiomerically pure form in an overall yield of 6.1%.[156]



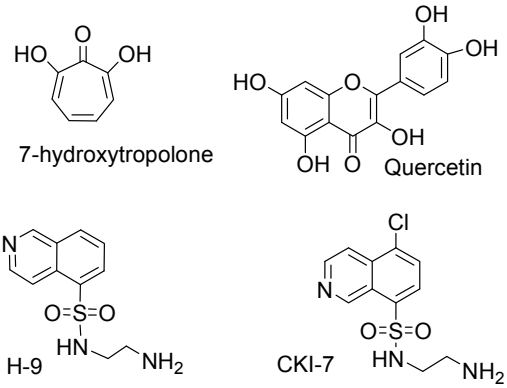

***Scheme 1.10***. Total synthesis of orthogonally protected 2-DOS.[156]

### 1.3.2 Development of inhibitors of aminoglycoside resistance-causing enzymes

The development of novel antibiotics active against resistant strains is one strategy to fight resistance. Another important approach uses inhibitors of the corresponding biological pathways. The inhibitors may be co-administered with the antibiotics, therefore rescuing the antimicrobial activity of the drug.

**Competitive inhibitors**

A few competitive inhibitors for aminoglycoside resistance-causing enzymes have been reported (Fig. 1.16).[157-159] The nucleotide-binding site of ATP-dependent aminoglycoside resistance-causing enzymes was explored towards inhibitor design. For example, the natural product 7-hydroxytropolone (Fig. 1.16) shows competitive (versus ATP) inhibition of ANT(2″).[157] Protein kinases inhibitors isoquinolone-sulfonamides H-9, CKI-7 and quercetin (Fig. 1.16) are high micromolar inhibitors of APH(3′)-IIIa and APH(2′) (Table 1.6).[158] The bovine antimicrobial cationic peptide indolicidin and the lysine/triptophan rich peptide analog CP10A (Fig. 1.16) display micromolar inhibition of AAC(6′)-Ii.[159]




$H_2N$-Ile-Leu-Pro-Trp-Lys-Trp-Pro-Trp-Trp-Pro-Trp-Arg-Arg-$CONH_2$

Indolicidin

$H_2N$-Ile-Leu-Ala-Trp-Lys-Trp-Ala-Trp-Trp-Ala-Trp-Arg-Arg-$CONH_2$

CP10A

***Figure 1.16***. Selected inhibitors for aminoglycoside resistance-causing enzymes.

**Table 1.5.** Michaelis-Menten constant of substrates for aminoglycoside resistance-causing enzymes

| Substrate | $K_m$ (μM) | | |
|---|---|---|---|
| | APH(3′)-IIIa[160] | AAC(6′)-APH(2″)[59] | AAC(6′)-Ii[23] |
| Kanamycin A | 13 ± 3 | 4.5 ± 0.1- 24 ± 2 | 20 ± 9 |
| Kanamycin B | | | 19 ± 5 |
| Ribostamycin | | | 9 ± 2 |
| AcCoA | | 23 ± 1 | 24 ± 4 |
| ATP | 28 ± 4 | 58 ± 3 | |

**Table 1.6.** Inhibition constants reported for inhibitors of aminoglycoside resistance-causing enzymes

| Inhibitor | Variable substrate* | $K_i$ (μM) APH(3′)-IIIa | AAC(6′)-APH(2″) | AAC(6′)-Ii |
|---|---|---|---|---|
| H-9 | kanamycin A | 155 ± 26 | 998 ± 307 | |
| | ATP | 138 ± 40 | 63 ± 19 | |
| Quecertin | ATP | 126 ± 22 | no inhibition | |
| CKI-7 | ATP | 66 ± 7 | 87 ± 18 | |
| CP10A | kanamycin A | 30 ± 5 | | |
| | ATP | 14 ± 1 | | |
| | kanamycin B | | | 2 ± 1 |
| | AcCoA | | | 5 ± 1 |
| Indolicidin | kanamycin A | 15 ± 2 | | |
| | ATP | 10 ± 1 | | |
| | kanamycin B | | | 4 ± 1 |
| | AcCoA | | | 38 ± 2 |

* The Michaelis-Menten constant ($K_m$) of these substrates are listed in Table 1.5.

The synthesis and evaluation of 4′,4′-difluorokanamycin A and 4′,4′-difluoroneamine derivatives (Fig. 1.17) have been reported.[161] The strong electron withdrawing effect of the fluorine substituents adjacent to the 3′-hydroxyl group results in a significant decrease of its nucleophilicity. The turnover number for the 3′-phosphorylation of 4′,4′-difluoroaminoglycosides are decreased by almost 3 orders of magnitude. 4′,4′-Difluoration of aminoglycosides is a good strategy to overcome resistance by tuning aminoglycosides into poorer substrates of APH(3′)s.

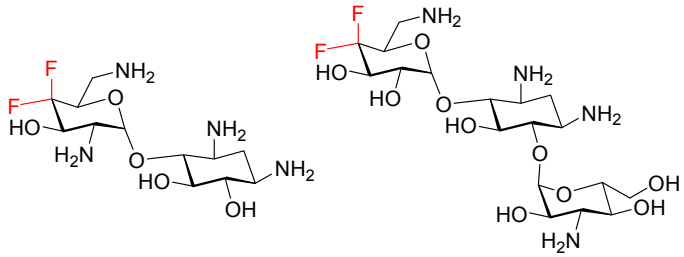



***Figure 1.17***. 4′,4′-Difluoroneamine and 4′,4′-difluorokanamycin A.[161]

**Mechanism-based inhibitor**

Suicide or mechanism-based inhibitor (MBI) is competitive inhibitor that is transformed by the enzyme into a reactive chemical entity, usually an electrophile. This transient product is subsequently attacked by the enzyme or by the cofactor, leading to enzyme inactivation.

MBIs have been successfully used to overcome resistance to the penicillinases (type 2 class of β-lactamases, Fig. 1.18). A mechanism-based inhibitor of β-lactamases, clavulanic acid, is co-administered with amoxicillin and marketed as Augmentin® by the pharmaceutical company GlaxoSmithKline with a total sale of $1.2 billion in 2002.

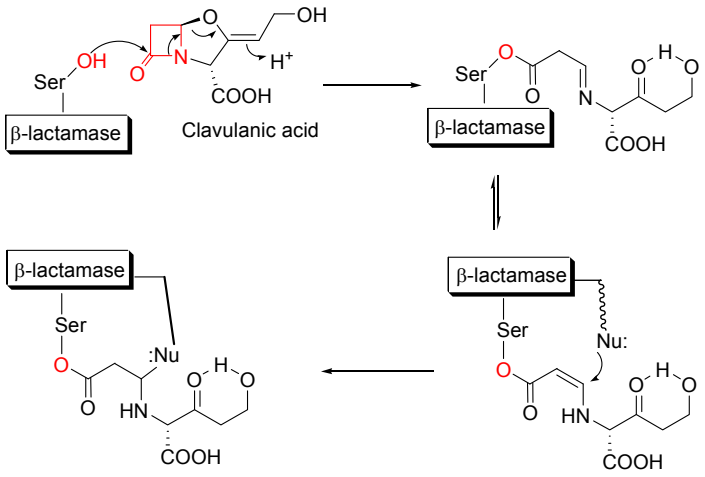
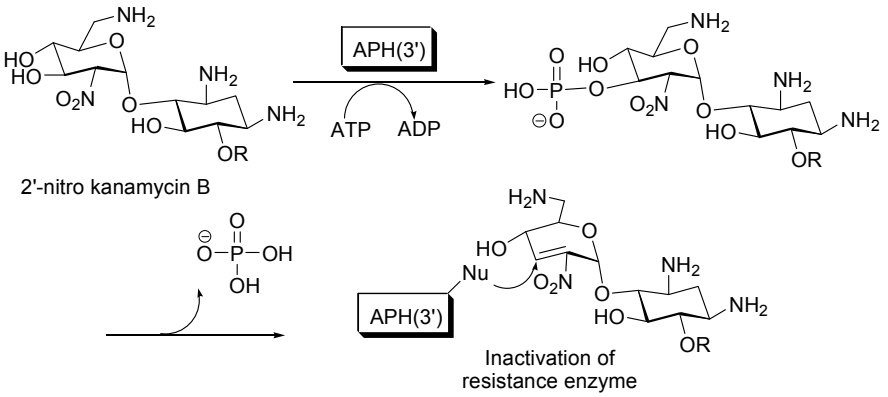



***Figure 1.18.*** Mechanism-based inhibition of β-lactamase by clavulanic acid.

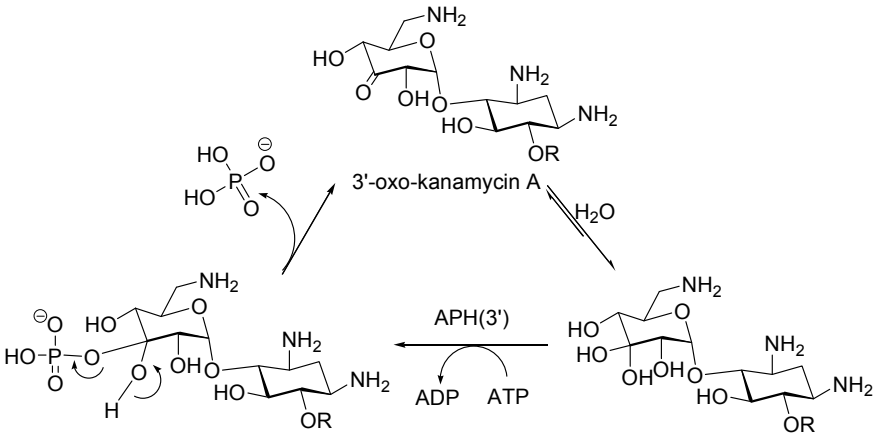
Given the success of the β-lactamases inbibitors, expanding this strategy to other antibiotics may be a promising strategy to recover the effectiveness of aminoglycosides towards resistant strains. Mobashery and co-workers have attempted to use MBIs of APH(3′) to block resistance. They synthesized 2′-nitroneamine and 2′-nitrokanamycin B (Scheme 1.11). Once phosphorylated by the enzyme, these molecules undergo elimination to generate nitroalkenes, which are excellent Michael addition acceptors and rapidly attacked by a nucleophilic residue of the active-site, resulting in irreversible inactivation of APH(3′).[162]

These inhibitors were tested with bacteria harboring the *aph(3′)*-IIa gene but no significant synergistic effects were observed with aminoglycosides.[162]




***Scheme 1.11***. Mechanism-based inhibitors of APH(3′).[162]

Another clever example from the same group used a 3′-oxo analogue of kanamycin A (Scheme 1.12).[160] 3′-Oxo-kanamycin A exists in equilibrium with the hydrated ketone. When phosphorylated by APH(3′), it generates an unstable intermediate that undergoes β-elimination to regenerate the 3′-oxo-kanamycin A. Thus the molecule remains active yet inhibits the enzyme and consumes cofactors. The 3′-oxo-kanamycin A is less effective than its parent compound kanamycin A against susceptible strains, but 4- to 8-fold more effective than kanamycin A against resistant strains of *E. coli* expressing APH(3′)-Ia.




***Scheme 1.12***. 3′-Oxo-kanamycin A consumes APH(3′) in a catalytic cycle.[160]

**Bisubstrate inhibitors**

One problem with inhibitors that compete with ATP or AcCoA is that they may inhibit host proteins and cause side effects. A safer strategy is to design inhibitors that bind to both the aminoglycoside and the cofactor pockets, resulting in higher specificity and tighter binding compared to single substrate analog inhibitors. To date, bisubstrate inhibitors have been used as probes for numerous enzymes especially for protein kinases,[163] but also for aminoglycoside resistance-causing enzymes. Bisubstrate analogs **57a-d** (Fig. 1.19, A) are prepared by covalently linking adenosine to the 3′-hydroxyl of neamine via all-methylene tethers of 5-8 carbons (Fig. 1.19, B).[164] They displayed micromolar inhibition against APH(3′)s (Table 1.7).

**Table 1.7.** Inhibition constants for bisubstrate inhibitors of APH(3′)s

| Inhibitor | variable substrate* | $K_i$ (μM) APH(3′)-Ia | $K_i$ (μM) APH(3′)-IIa |
|---|---|---|---|
| **57a** | kanamycin A | 558 ± 65 | 10 ± 4 |
| | ATP | 32 ± 5 | 18 ± 11 |
| **57b** | kanamycin A | 10 ± 8 | 3 ± 2 |
| | ATP | 3 ± 2 | 5 ± 3 |
| **57c** | kanamycin A | 22 ± 14 | 6 ± 5 |
| | ATP | 9 ± 1 | 17 ± 4 |
| **57d** | kanamycin A | 35 ± 26 | 9 ± 5 |
| | ATP | 224 ± 61 | 14 ± 2 |

*: The $K_m$ for kanamycin A and ATP when tested with APH(3′)-Ia are 3.0 ± 0.3 μM and 77 ± 4 μM, respectively. The $K_m$ for kanamycin A and ATP when tested with APH(3′)-IIa are 3 ± 0.5 μM and 69 ± 13 μM, respectively.[164]

Dimerized neamine derivative **58** (Scheme 1.13, B) was shown to bind to an A-site RNA fragment with high affinity ($K_d$ = 40 nM) and displayed antibacterial activity (MIC = 6.25 μM against *E. coli*).[165] This compound was a poor substrate for AAC(6′)-Ii, and a powerful inhibitor of and AAC(6′)-APH(2″) ($K_i$ = 0.7 μM).[165]

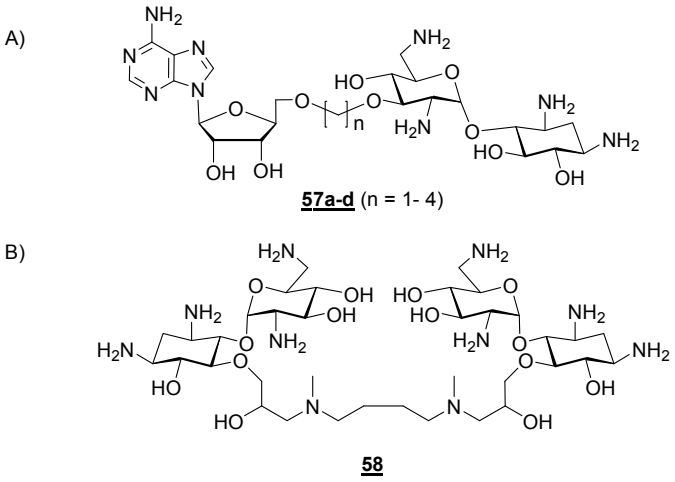
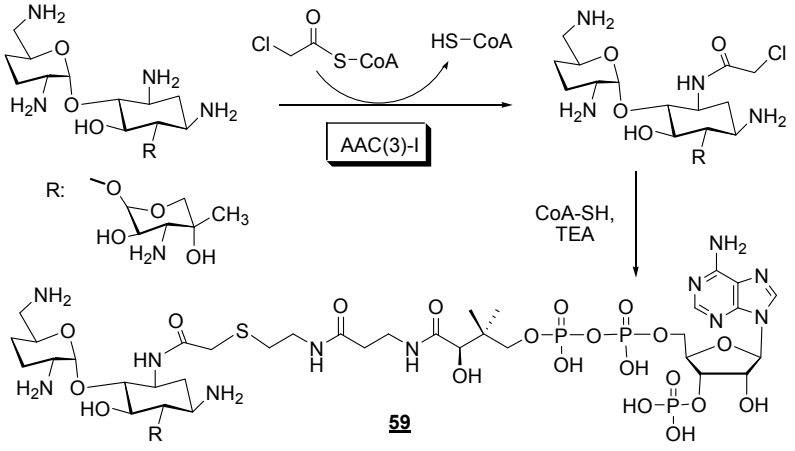



***Figure 1.19***. Bisubstrate inhibitors of aminoglycoside resistance-causing enzymes

The first aminoglycoside-CoA bisubstrate **59** was synthesized enzymatically (Scheme 1.13).[166] The reported $K_i$ for this bisubstrate inhibitor was 9 nM when tested with gentamicin AAC(3)-I, but showed no activity when tested against resistant *E. coli,* presumably due to its inability to cross cell membranes.[166] Although the bisubstrate is not drug-like, it is useful for mechanistic and structural enzymatic studies.




***Scheme 1.13.*** The first aminoglycoside-coenzyme A bisubstrate.

No inhibitors or new aminoglycoside derivatives have reached clinical trials since 1990s. This may be explained by the synthetic challenges associated with these targets and the complex nature of the resistance mechanisms.

## 1.4 Overview of the project

The discovery of antibiotics was a major incentive to the foundation of many pharmaceutical companies. Ironically, a few decades later, most of them have dropped their antibacterial research programs for economical reasons. Unfortunately, the drying-out pipeline of new antibiotics and the increasing frequency of multi-drug resistance raise fears of a fallback to the pre-antibiotic era.[167, 168]

Two strategies, derivatization of existing antibiotics and development of resistance inhibitors, are generally used to address the resistance problem. However, neither of these can be achieved without a good understanding the mechanisms of resistance. As discussed above, the most prevalent mechanisms conferring aminoglycoside resistance in bacteria are enzymatic transformations of the drug, i.e., phosphorylation, adenylation and acetylation. *N*-6′-acetylation is one of the most relevant determinants of resistance, clinically. When we embarked on this project, no crystal structures were available for AAC(6')s in complex with aminoglycosides. Furthermore, very few inhibitors had been reported for resistance-causing enzymes, none of which were active in cells. The first aim of this thesis was to synthesize aminoglycoside-CoA bisubstrate inhibitors, which were expected to be tight binders of AAC(6′)s and useful mechanistic and structural probes for these enzymes. The next goal was to synthesize truncated bisubstrate inhibitors to investigate structure-activity relationships. Thirdly, it was envisaged that a new generation of bisubstrates that better mimic the proposed tetrahedral intermediate would be more potent. Finally, based on the crystal structures of both RNA in complex with aminoglycosides, and of the resistance-causing enzyme AAC(6′)-Ii in complex with AcCoA and

bisubstrates, we intended to design and synthesize novel aminoglycosides that would not bind to AAC(6′)-Ii but maintain their antibacterial activity.

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# Chapter Two

The rapid spread of antibiotic resistance poses a serious threat for medical facilities around the world. Without intense research, this problem may reverse medicine to a pre-antibiotic era. Aminoglycoside *N*-6′-acetyltransferases (AAC(6′)s) are clinically relevant aminoglycoside resistance determinants. Understanding resistance mechanisms is essential to combat this problem. To delineate the mechanism of AAC(6′)-Ii, Professor Berghuis and co-workers have reported the 3D structures of AAC(6′)-Ii in complex with both AcCoA and CoA, but did not succeed in obtaining a crystal structure of this enzyme with an aminoglycoside, in spite of numerous years of work. We proposed to synthesize aminoglycoside-CoA bisubstrates as useful mechanistic probes to facilitate crystallization of the enzyme with aminoglycosides. The synthesis of aminoglycoside-CoA derivatives poses a serious synthetic challenge because of the high level of functionalization and the water solubility of both starting materials, which require judicious protection/deprotection chemistry. In this chapter, a novel method for the regioselective *N*-6′-derivatization of unprotected aminoglycosides was reported and used to prepare six aminoglycoside-CoA bisubstrates in a very efficient manner.

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Contributions of co-authors

This chapter is a copy of a published communication and is reproduced with permission from the journal. It is cited as Feng Gao, Xuxu Yan, Oliver M. Baettig, Albert M. Berghuis, and Karine Auclair, "Regio- and Chemoselective 6′-*N*-Derivatization of Aminoglycosides: Bisubstrate Inhibitors as Probes to Study Aminoglycoside 6′-*N*-Acetyltransferases" *Angewandte Chemie-International Edition* **2005**, 44, 6859-6682. I carried out the synthesis of all the compounds and wrote the article. Xuxu Yan, a graduate student in our lab performed the enzymatic tests. Oliver M. Baettig (a graduate student in Professor Berghuis' lab) purified the enzyme and carried out crystallography experiments.

# Regio- and Chemo-Selective *N*-6′-Derivatization of Aminoglycosides: Bisubstrate Inhibitors and Probes to Study Aminoglycoside *N*-6′-Acetyltransferases

**Abstract**

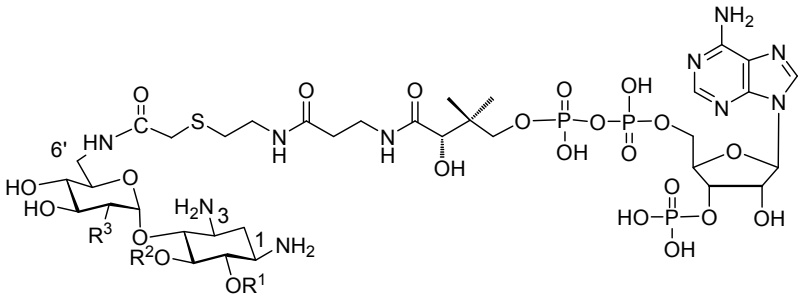
A novel methodology of regio- and chemo-selective derivatization of unprotected aminoglycosides at *N*-6′ position was developed. This method was used to synthesize a series of aminoglycoside-Coenzyme A bisubstrate analogs. These bisubstrate analogs are the first reported nanomolar inhibitors of aminoglycoside 6′-*N* acetyltransferase Ii (AAC(6′)-Ii). They have been used to facilitate co-crystallization with AAC(6′)-Ii to harvest ternary complex crystals of the protein, providing for the first time, useful information about the aminoglycoside binding site of this enzyme.

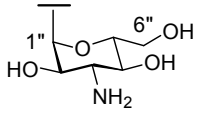
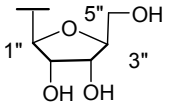
## 2.1 Communication

Aminoglycosides are among the most commonly used broad-spectrum antibiotics.[1-3] Their biological activity relies on their high affinity for the major groove of 16S rRNA on the 30S ribosome of prokaryotic cells,[4-7] thereby impeding the synthesis of bacterial proteins. A number of aminoglycoside derivatives also display antiviral activity due to specific interactions with viral RNA.[8-10] The rapid emergence of aminoglycoside resistance in the treatment of infections however, is a serious threat.[11-14] In clinical isolates of aminoglycoside-resistant strains, the most frequently observed cause of resistance is the expression of *N*-acetyltransferases.[15, 16] For example, aminoglycoside *N*-6′-acetyltransferase type Ii (AAC(6′)-Ii) is chromosomally encoded in *Enterococcus faecium* which is one of the leading causes of hospital-acquired infections.[17] Studies by Wright and coworkers, suggest that catalysis by AAC(6′)-Ii occurs via an ordered bi-bi mechanism where acetyl coenzyme A (AcCoA) must bind the enzyme before the

aminoglycoside.[16, 18, 19] Next, attack of the aminoglycoside 6′-$NH_2$ at the thioester of AcCoA is believed to proceed via a tetrahedral intermediate which collapses to yield an *N*-6′-acetylaminoglycoside and CoA. Although crystal structures have been reported for complexes of AAC(6′)-Ii with AcCoA or CoA, crystallization experiments of enzyme-aminoglycoside complexes have not been successful.[20-22] It was envisaged that *N*-6′-(*S*-CoA)aminoglycoside derivatives (Scheme 2.1), or bisubstrates, would facilitate the study of this important class of enzymes. Bisubstrate analogs have shown inhibition of serotonin acetyltransferase,[23, 24] GCN5 histone acetyltransferase,[25, 26] and carnitine acetyltransferases.[27] *N*-3-(2*S*-CoA-acetyl)gentamicin $C_{1a}$, the only reported aminoglycoside-CoA derivative reported so far, has been prepared enzymatically using AAC(3)-I (3 mg) to yield the desired bisubstrate analog (0.89 mg),[28] which was subsequently found to inhibit AAC(3)-I with nanomolar affinity ($K_i^{app}$ = 9 $\pm$ 7 nM). To date however, there are no reports of chemical synthesis of CoA-aminoglycoside derivatives. Naturally occurring aminoglycosides are complex molecules and their regioselective modifications remain challenging. Currently, the use of judicious functional protection chemistry is the common.[29-37]

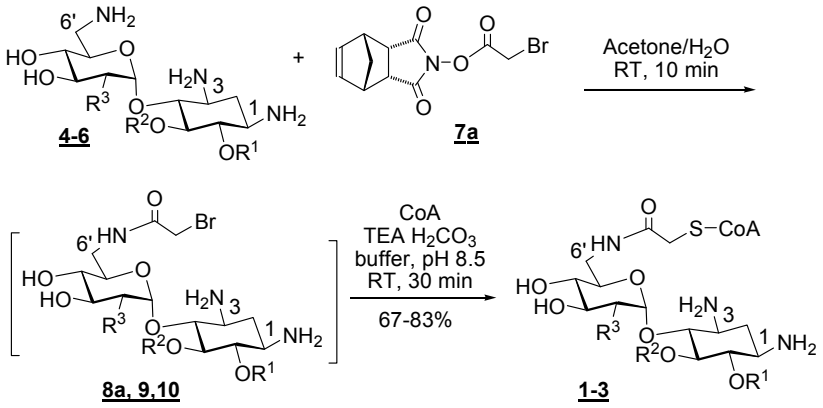
We report here an efficient procedure to regioselectively derivatize unprotected aminoglycosides at the 6′-$NH_2$ position. This strategy is shown to proceed with high chemoselectivity towards the assembly of *N*-6′-(*S*-CoA)aminoglycoside derivatives **1-3** and **11a-c** (Schemes 2.2 and 2.3). Inhibition assays with these bisubstrates reveal novel nanomolar competitive inhibitors of AAC(6′)-Ii.



| | $R^1$ | $R^2$ | $R^3$ |
|---|---|---|---|
| **1** | H | H | $NH_2$ |
| **2** |  | H | OH |
| **3** | H |  | $NH_2$ |

***Scheme 2.1.*** Target aminoglycoside-CoA bisubstrates **1**-**3**.

The target bisubstrates were built from neamine (**4**), kanamycin A (**5**) and ribostamycin (**6**) which are examples of non-substituted, 4,6-substituted, and 4,5-substituted aminoglycosides. The key step in the synthesis of **1-3** from **4-6** is to prepare bromide intermediates **8a, 9** and **10** respectively (Scheme 2.2). The trifluoroacetic acid salt of intermediate **8a** has previously been synthesized using orthogonal protection in four steps with very low yield (11.6%).[31] As a more efficient alternative to orthogonal protection/deprotection schemes, we envisaged the use of *N*-(2-bromoacetyl)oxy-5-norbornene-*endo*-2,3-dicarboximide (bromoacetyl-NBD ester, **7a**) to regioselectively transfer a bromoacetyl group to 6′-$NH_2$ of aminoglycosides. BOC-NBD ester has been reported to achieve regioselective BOC protection of aminoglycosides[38] however, NBD esters have not been employed for aminoglycoside derivatization.




***Scheme 2.2.*** Synthesis of bisubstrate analogs **1-3**; $R^1$, $R^2$ and $R^3$: refer to Scheme 2.1; **4**: neamine; **5**: kanamycin A; **6**: ribostamycin; intermediates **9** and **10** were not isolated

Remarkably, simply mixing free base neamine and reagent **7a** in acetone/$H_2O$ (1/1) at RT in a vial open to air for a few min (monitored by ESI-MS), suffices to complete the reaction. In contrast, extended reaction times yield complicated mixtures, possibly arising from nucleophilic attack of amino groups at the newly formed bromide. The desired *N*-6′-bromoacetylneamine (**8a**) can be isolated in good yield (70%) by TFA quench of the reaction mixture after 10 min and purification by reverse phase HPLC. To avoid the isolation step and potential decomposition, a one-pot synthesis of **1** from **4** and CoA via **8a** was evaluated. Bisubstrate **1** was obtained in excellent yield (83%) after HPLC purification (Figure 2.1, **a**).

The reaction proceeded equally well with the aminoglycosides **5** or **6** (72% and 67% respectively). In spite of their significant structural differences, kinetic studies reveal that **1-3** show nanomolar competitive inhibition of AAC(6′)-Ii (Table 2.1). This observation is consistent with the fact that AAC(6′)-Ii has a broad substrate specificity.[16]

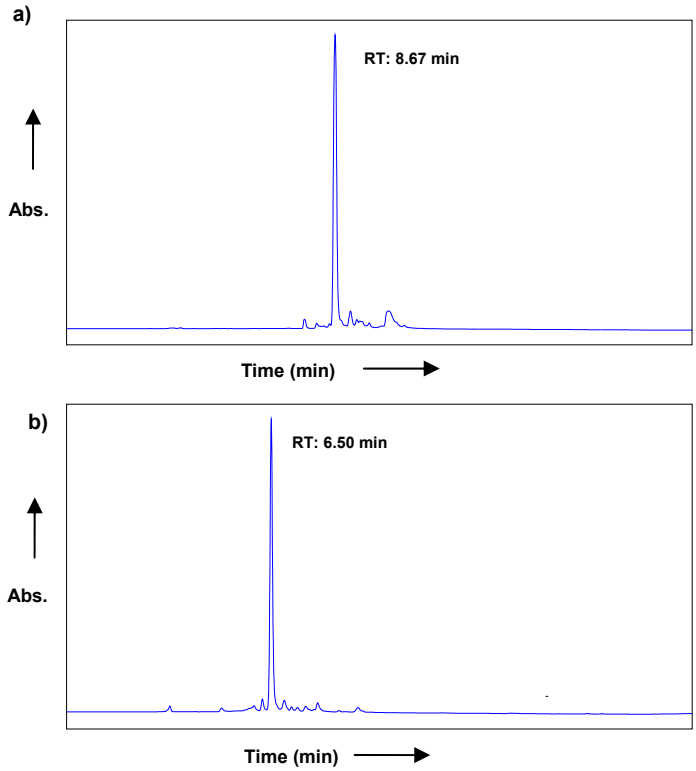
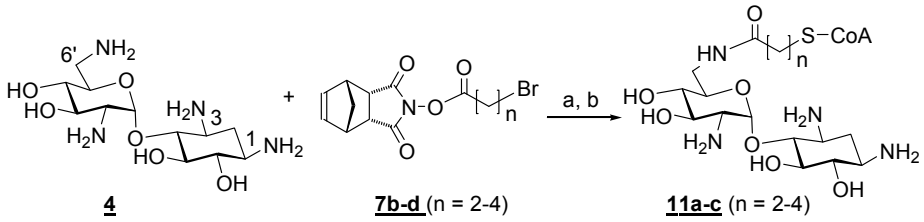



***Figure 2.1***. HPLC chromatogram from purification of bisubstrate analogs **1** (a) and **11a** (b).

Size and geometry of linkers in serotonin-CoA bisubstrates have been reported to show important effects on the inhibition of serotonin *N*-acetyltransferase.[23, 24, 26, 39-41] A similar effect was expected for AAC(6′)-Ii. This was investigated by varying the length of the tether between the aminoglycoside and CoA of the bisubstrates (Scheme 2.3). Unfortunately, the procedure described above for the synthesis of bisubstrates **1-3** was not easily applicable to the preparation of **11a-c**. The major products observed when synthesis of **11a** was attempted were: a triethylammonium bromide from addition of triethylamine to *N*-6′-(3-bromo-*n*-propanoyl)neamine; *N*-6′-acryloyl neamine (elimination product); and a propanoyl-conjugated neamine dimer (either via $S_{N2}$ substitution at the bromide of *N*-6′-(3-bromo-*n*-propanoyl)neamine by a second neamine or by Michael addition of a second neamine to *N*-6′-acryloyl neamine). Similar products resulted in the initial efforts to prepare **11b** and **11c**. A difference of reactivity of these longer linkers was expected.[42-44] First, the 3-bromo-*n*-propanoyl-, 4-bromo-

*n*-butyroyl-, and 5-bromovaleroyl-neamine derivatives (**8b-d** from the reaction of neamine with **7b-d**) are much less electrophilic than *N*-6′-(2-bromoacetyl)neamine (**8a**). Second, triethylamine may be too nucleophilic and used in high concentration that may favor *N*-alkylation over *S*-alkylation. Since most reported chemoselective *S-/N*-alkylation procedures[42-44] are not compatible with our reagents, we reasoned that a less nucleophilic base at lower concentration may favor *S*-alkylation. Model studies were carried using *N*-acetylcysteine as a mimic of CoA and an array of bases were screened including $KHCO_3$, $NaHCO_3$, DIPEA, DABCO, DBU, and DMAP. DIPEA provided excellent yields when used at 20-equivalent and with 5 min of ultrasonication of the mixture. When applied to CoA, these conditions allowed preparation of **11a**, **11b**, and **11c** in excellent to moderate yields (91%, 52% and 93% respectively). The HPLC trace of crude **11a** is shown on Figure 2.1, b.




***Scheme 2.3***. Reagents and conditions: a) acetone/$H_2O$ (1/1), RT, 10 min, b) CoA-SH, 20 eq. DIPEA, sonicate 5 min then stirring 1 hr RT; Yields: 91% (**11a**); 52% (**11b**); 93% (**11c**).

AAC(6′)-Ii inhibition assays show that **11a** is the most potent bisubstrate inhibitors of this series. Remarkably, the enzyme binds **11a** ~ 200-fold tighter than its natural substrate Ac-CoA ( $K_m^{app}$ = 9.6 μM).[16] A further increase in the length of the linker, however, rapidly leads to a decrease in activity (Table 2.1). To demonstrate the usefulness of these inhibitors as structural and mechanistic probes, they were studied by X-ray crystallographic analysis. Although previous crystallization experiments of AAC(6′)-Ii-aminoglycoside complexes had not been successful,[20-22] bisubstrates **1-3** and **11a-c** crystallized well with the enzyme, providing X-ray diffraction data to ~ 2.0 Å resolution. Preliminary analysis of the diffraction data for the complex with bisubstrate **11a** (Figure 2.2) suggests that the

conformation of the aminoglycoside bound to AAC(6′)-Ii is very different from that reported for AAC(6′)-Iy,[45] even though both enzymes catalyze the same reaction. Detailed structural and mechanistic analysis of these structures will be reported in due course.

**Table 2.1.** AAC(6′)-Ii inhibition constants ( $K_i^{app}$ ) for the bisubstrates

| Inhibitor | $K_i^{app}$ [nM] | Inhibitor | $K_i^{app}$ [nM] |
|---|---|---|---|
| **1** | 76 ± 25 | **11a** | 43 ± 23 |
| **2** | 111 ± 28 | **11b** | 161 ± 98 |
| **3** | 119 ± 14 | **11c** | 7990 ± 2663 |

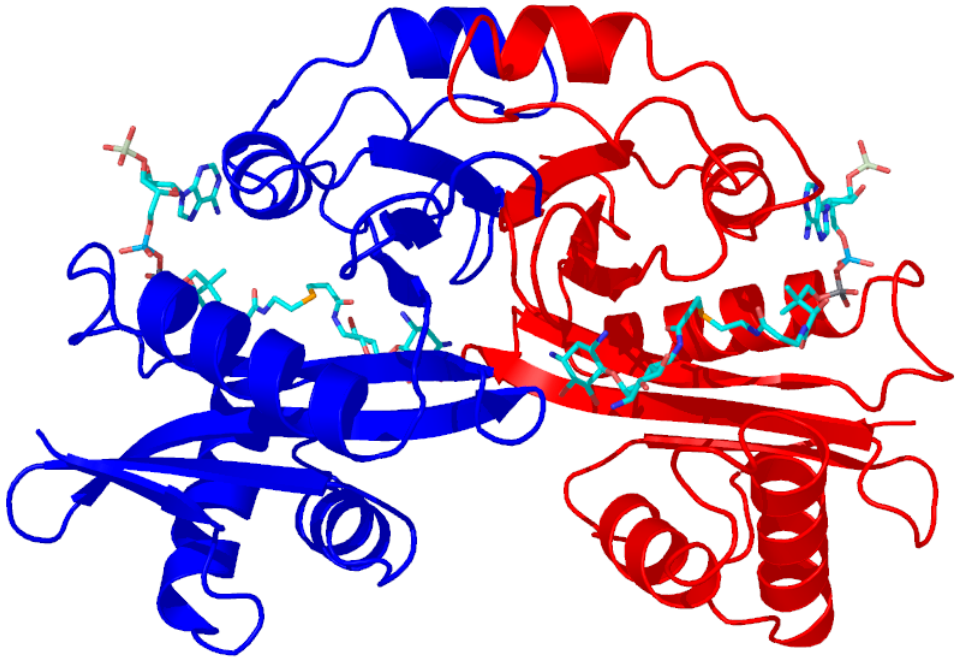
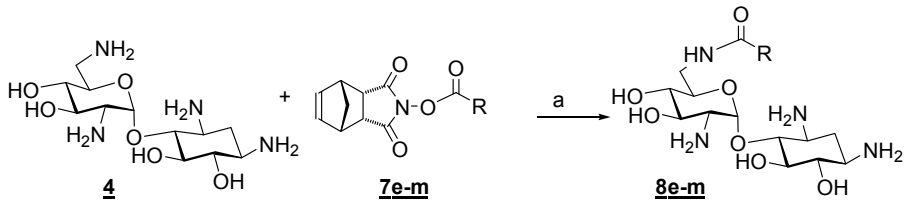


***Figure 2.2***. Crystallographically determined structure of the *E. faecium* AAC(6')-Ii dimer (blue and red) when complexed to the bisubstrate inhibitor **11a** (stick representation, C: light blue, N: dark blue, O: red, P: gray, S: orange.

After the success of this methodology for the preparation of bisubstrates, its general applicability to acylation was investigated (Scheme 2.4). Most acyl groups tested were transferred regioselectively to neamine with excellent yields. NBD esters of highly hindered acyl groups such as 2-methylbenzyl and 2,6-dichlorobyzoyl however, did not react at all. The regioselectivity of transfer to kanamycin A, ribstamycin and neomycin was tested with benzoyl-NBD and showed excellent selectivity for 6′-*N*-acylation.




| | e | f | g | h | i | j | k | l | m |
|---|---|---|---|---|---|---|---|---|---|
| R: | Me | *n*Pr | $nC_{11}H_{23}$ | Ph | Bn | Naph | 2-chloro-phenyl | 2-methyl-phenyl | 2,6-dichloro-phenyl |
| % yield | 60 | 70 | 70 | 80 | 75 | 85 | 90 | 0 | 0 |

***Scheme 2.4***. Regioselective *N*-6′-acylation of neamine. a) acetone/$H_2O$ (1/1), RT, 30 min; Bn: benzyl, Naph: 1-naphthylmethyl.

In conclusion, we have described a highly efficient synthetic strategy to regioselectively acylate aminoglycosides at *N*-6′. This process was successfully applied to the one-pot synthesis of *N*-6′-(S-CoA)aminoglycoside analogs. All of these bisubstrates were nanomolar competitive inhibitors of AAC(6′)-Ii, an important enzyme leading to resistance to aminoglycoside antibiotics. The high potency of these bisubstrates suggests that they may be good mimics of the putative tetrahedral enzymatic reaction intermediate.[16, 18] The bisubstrates reported here have allowed, for the first time, crystallization of AAC(6′)-Ii with aminoglycoside derivatives. The resulting 3D structures should provide valuable guidance in further studies of this enzyme and other members of this family.

**Acknowledgements**

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# Chapter 2 - Appendix

**Experimental section (published as Supporting Information)**

**General.** All commercial reagents were used without further purification unless otherwise specified. Anhydrous THF was prepared freshly by distillation over sodium. Acetonitrile and dichloromethane were distilled form calcium hydride under inert atmosphere. Bromoacetic acid, 1-bromopropionic acid, 1-bromobutanoic acid and 1-bromovaleric acid were recrystallized from hexane. Neamine hydrochloride was prepared by methanolysis of neomycin B using the procedure reported before.[37] Kanamycin A, ribostamycin, AcCoA, DTDP, EDTA, and HEPES were purchased from Sigma-Aldrich (St. Louis, MO). All aminoglycoside free bases were prepared by neutralization using the anionic exchange resin Amberlite IRA-400(OH).

Melting points were not corrected. HRMS of compounds **1-3**, **11a-c** were analyzed by direct infusion electrospray ionization from a solution in 90:10 methanol:50 mM aqueous ammonium hydroxide at 2 μL/min in an IonSpec 7 Tesla FTICR instrument at a resolving power of approximately 80,000. Other HRMS samples were analyzed using a Kratos MS 25RFA mass spectrometer at a source temperature of 200°C and 70 eV. LRMS was performed using a Finnigan LCQDUO mass spectrometer with either ESI or APCI without fragmentation. Routine $^{1}H$ and $^{13}C$ NMR spectra were recorded using Varian mercury 400 or 300 or Unity 500 spectrometers. The chemical shifts (δ) were reported in parts per million (ppm) relative to the internal standard TMS (0 ppm). The peak patterns are indicated as follows: s, singlet; d, doublet; t, triplet; dt, doublet of triplet; ddd, doublet of doublet of doublet; td, triplet of doublet; m, multiplet; q, quartet; br s, broad singlet, etc. All $^{1}H$ NMR and correlation spectra including COSY, HMBC and HSQC of aminoglycoside coenzyme bisubstrates and aminoglycosides were recorded with solutions of pD = 4 unless otherwise stated. $^{1}H$ and $^{13}C$ NMR assignments were confirmed by HSQC, HMQC, and HMBC.

Bisubstrate analogs **1-3, 11a-c,** and neamine 6′-N derivatives **8a and 8e-k** were purified by reversed-phase HPLC using an Agilent Zorbax SB-CN column (4.6 × 250 mm, 5 μ) on an Agilent 1100 system with diode array UV detector. Samples were eluted at a flow rate of 3 ml/min, using the linear gradients shown in Table 2.2.

**Table 2.2**. Gradient profile for HPLC purification

| Time (min) | % A (0.05% TFA in $H_2O$) | % B (0.04% TFA in ACN ) |
|---|---|---|
| 0 | 98 | 2 |
| 20 | 60 | 40 |
| 25 | 0 | 100 |
| 28 | 0 | 100 |

**General procedure for the synthesis of 1-3**

To a solution of **7a** (0.052 mmol) dissolved in deoxygenated ACN (1.5 ml) is added a solution of free base aminoglycoside (**4, 5,** or **6**, 0.104 mmol) in deoxygenated $H_2O$ (1.5 ml). This mixture is next transferred into a solution of the sodium salt of coenzyme A (18 mg, 0.026 mmol, in 3 ml TEA/$H_2CO_3$ buffer at pH = 8.5 stirred for 10 min before use). The reaction is monitored by ESI-MS until disappearance of coenzyme A (766.1 in negative mode). The mixture is then evaporated *in vacuo* to ~1 ml and acidified to pH = 4 using TFA. The solution is diluted with water (4 ml) and purified by reversed phase HPLC. The desired product is collected and lyophilized to yield a white fluffy powder.

**General procedure for the synthesis of 7a-m**

*N*-hydroxy-5-norbornene-*endo*-2,3-dicarboximide (3 mmol) and the desired carboxylic acid (3 mmol) are dissolved in DCM (20 ml). A few ml of THF was sometimes required to completely dissolve the carboxylic acid. DCC (1.05 eq.) was next added followed by a catalytic amount of DMAP (ca. 5 mg). Usually, a few min after addition of DCC, a white solid (DCU) precipitated out and the reaction was most often complete in 2 hours, as judged by TLC. DCU was filtered

out and the filtrate was evaporated to dryness yielding the desired product as a pure solid (>95% purity from NMR). When higher purity was needed, the crude product was dissolved in 35:65 EtOAc:Hex (50 ml) and filtered again to obtain a product of >99% purity after evaporation.

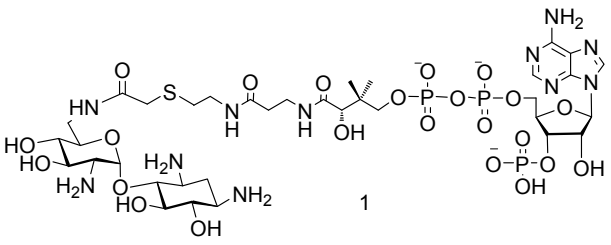
**General procedure for the synthesis of 8a, 12e-k**

The NBD ester, **7a**, **7e-k** (0.058 mmol) and neamine free base (0.12 mmol) are dissolved in $H_2O$:ACN 1:1 (8 ml). The solution is sonicated for 30 × 2 seconds and stirred for 30 min. The reaction mixture is concentrated *in vacuo* to ~1 ml, diluted in water (30 ml), acidified to pH = 2 using TFA and washed with diethyl ether. The aqueous layer is concentrated to 4 ml and the product is purified by reversed phase HPLC.

**General procedure for the synthesis of 11a-c**

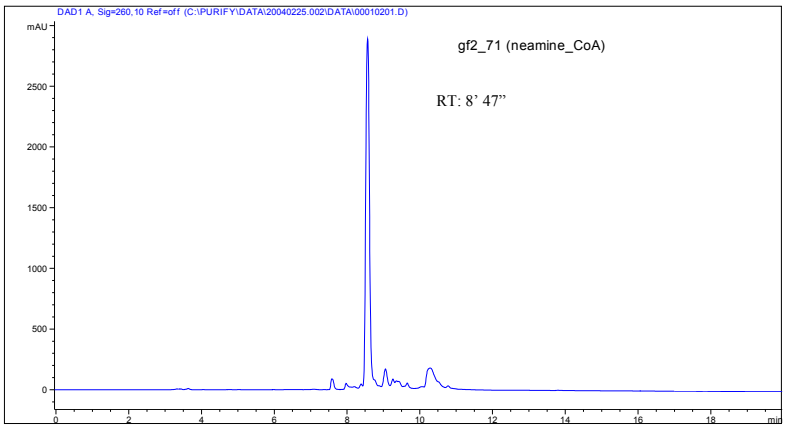
To a solution of **7b-d** (0.052 mmol) dissolved in deoxygenated acetone (1.5 ml) is added a solution of free base neamine (**4**, 0.104 mmol) dissolved in deoxygenated $H_2O$ (1.5 ml). This mixture is next transferred into a solution of the sodium salt of coenzyme A (18 mg, 0.026 mmol, in 3 ml acetone:$H_2O$ 2:3 containing 0.3 % w/v DTT and stirred for 10 min before use). The reaction is monitored by ESI-MS for disappearance of coenzyme A (766.1 in negative mode). The reaction mixture is then evaporated *in vacuum* to ~1 ml. The residue is diluted in $H_2O$ (30 ml) and acidified to pH = 4 using TFA. The solution is washed with diethyl ether and the aqueous phase is lyophilized to afford the crude product (white fluffy powder). The crude product is dissolved in $H_2O$ (4 ml) and purified by reversed phase HPLC. The desired product is collected and lyophilized to yield a white fluffy powder.

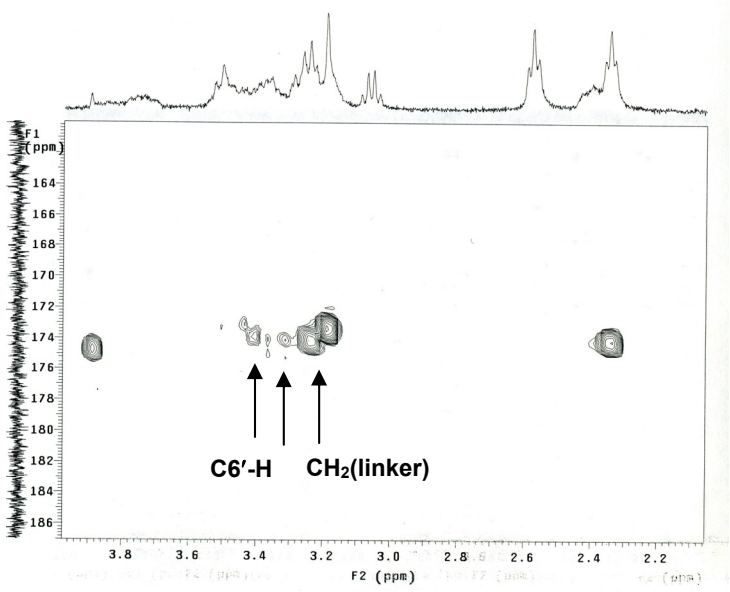
**Neamine-CoA bisubstrate analog 1:**



1

HPLC chromatogram of purification:



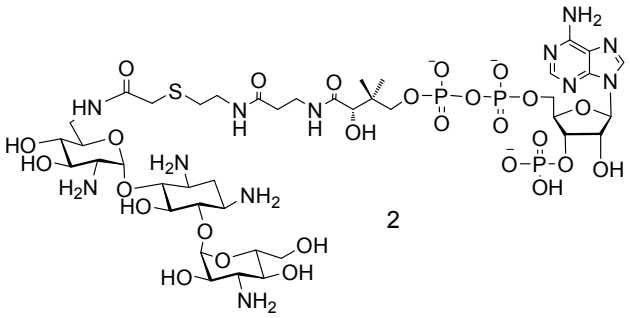




$^{13}$C-$^{1}$H HMBC shows the linker amide carbonyl correlates with both the linker methylene protons and C6′ protons.

Yield: 83%. **$^{1}$H NMR** ($D_2O$, 500 MHz): δ 8.48 (s, 1H), 8.26 (s, 1H), 6.05 (d, *J* = 4.5 Hz, 1H), 5.57 (d, *J* = 3.5 Hz, 1H), 4.44 (br s, 1H), 4.11 (m, 2H), 3.86 (s, 1H), 3.81 (t, *J* = 9.2 Hz, 1H), 3.76-3.67 (m, 4H), 3.48-3.38 (m, 6H), 3.38-3.28 (m, 4H), 3.25-3.18 (m, 5H), 2.53 (t, *J* = 6.5 Hz, 2H), 2.35 (m, 1H), 2.30 (t, *J* = 6.0 Hz, 2H), 1.80 (q, *J* = 12.5 Hz, 1H), 0.76 (s, 3H), 0.66 (s, 3H); **$^{13}$C NMR** ($D_2O$, 125 MHz, by HSQC and HMBC): δ 177.9, 174.9, 173.7, 150.2, 149.1, 144.8, 142.3, 118.7, 97.1, 88.0, 83.9, 79.8, 79.7, 75.8, 75.1, 74.0, 73.0, 72.8, 72.0, 71.9,

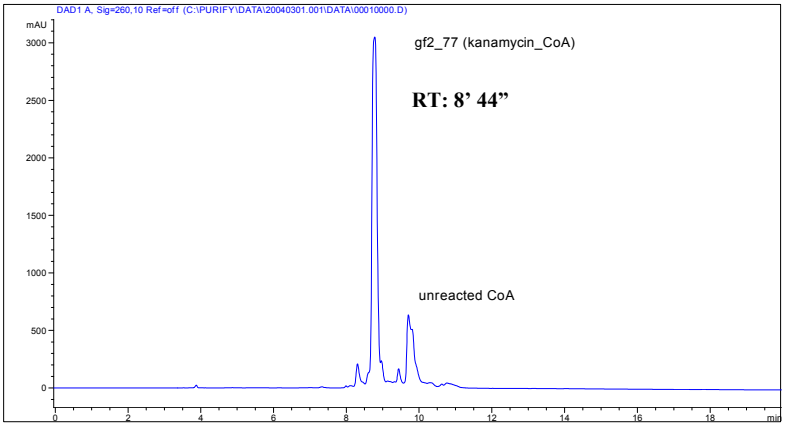
71.8, 69.6, 66.0, 54.0, 50.2, 48.9, 40.0, 38.7, 35.9, 35.8, 34.8, 31.6, 28.3, 21.2, 18.8; **HRMS** for $C_{35}H_{62}N_{11}O_{23}P_3S$ $[M+H]^+$ calcd. 1130.3034, found 1130.3035.

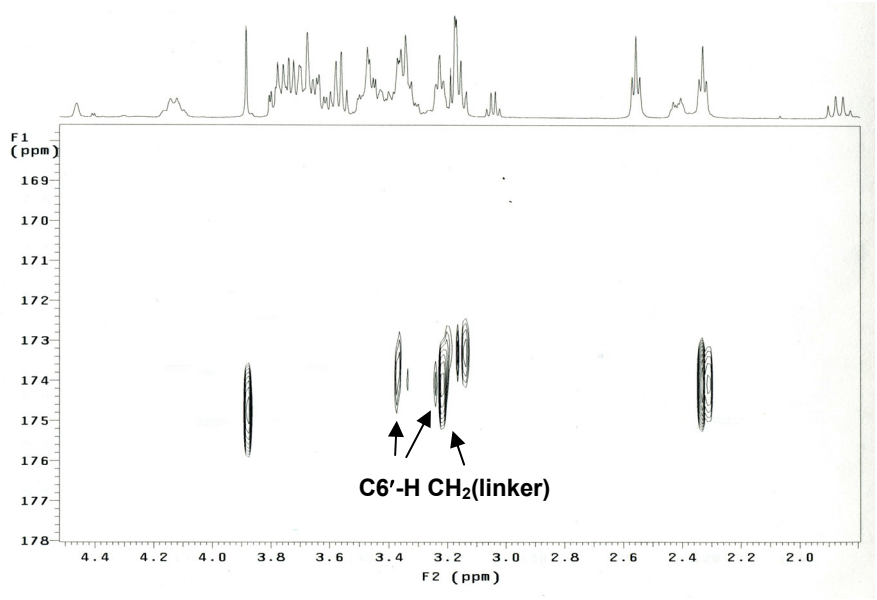
**Kanamycin-CoA bisubstrate analog 2:**



2

HPLC chromatogram of purification:

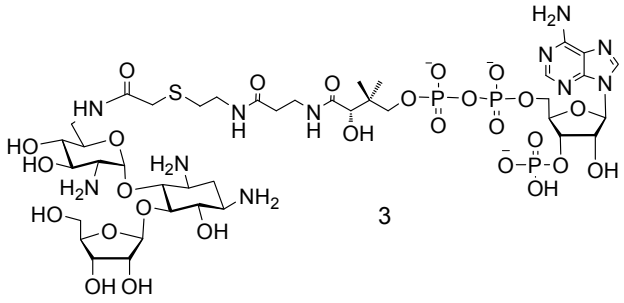


$^{13}C$-$^{1}H$ HMBC shows the linker amide carbonyl correlates with both the linker methylene protons and C6′ protons.

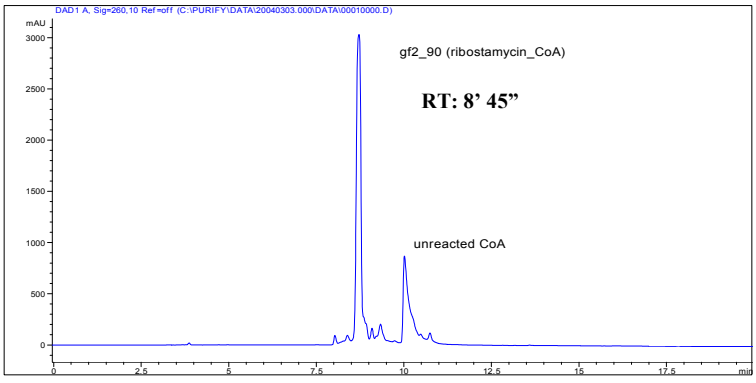
Yield: 72%. **$^{1}H$ NMR** ($D_2O$, 500 MHz): δ 8.49 (s, 1H), 8.27 (s, 1H), 6.06 (d, *J* = 4.5 Hz, 1H), 5.32 (d, *J* = 4.5 Hz, 1H), 4.96 (d, *J* = 3.5 Hz, 1H ), 4.44 (br s, 1H), 4.10 (m, 2H), 3.86 (s, 1H), 3.78-3.61 (m, 9H), 3.58-3.52 (m, 3H), 3.46-3.40 (m, 4H), 3.36-3.28 (m, 5H), 3.18 (m, 2H), 3.14 (m, 4H), 2.53 (t, *J* = 6.5 Hz, 2H), 2.40 (m, 1H), 2.30 (t, *J* = 6.5 Hz, 2H), 1.74 (q, *J* = 12.5 Hz, 1H), 0.77 (s, 3H), 0.66 (s, 3H); **$^{13}C$ NMR** ($D_2O$, 125 MHz, by HSQC and HMBC): δ 174.8, 174.0, 173.2, 150.4, 149.5, 144.6, 142.0, 118.5, 100.8, 98.1, 87.5, 83.8, 83.6, 79.4, 74.4, 74.0, 73.6, 72.8, 72.2, 72.1, 72.0, 71.3, 71.2, 70.6, 68.0, 65.5, 65.3, 55.0, 50.0, 48.6, 46.7, 40.0, 39.9, 38.4, 38.3, 37.8, 37.7, 31.8, 28.4, 22.0, 20.0; **HRMS** for $C_{41}H_{72}N_{11}O_{28}P_3S$ $[M+H]^+$ calcd. 1291.6264, found 1291.6267.

**Ribostamycin-CoA bisubstrate analog 2:**



3

HPLC chromatogram of purification:

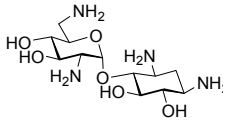



Yield: 67%. **$^{1}H$ NMR** ($D_2O$, 400 MHz): δ 8.51 (s, 1H), 8.29 (s, 1H), 6.09 (d, *J* = 4.2, 1H), 5.74 (d, *J* = 4.0, 1H), 5.20 (s, 1H ), 4.46 (br s, 1H), 4.14 (m, 2H), 4.08 (d, *J* = 6.0 Hz, 1H), 4.03 (t, *J* = 7.2 Hz, 1H), 3.96 (t, *J* = 9.6 Hz, 1H), 3.89

(s, 2H), 3.82-3.71 (m, 4H), 3.67 (dt, *J* = 9.6, 4.0 Hz, 1H), 3.59 (t, *J* = 9.2 Hz, 1H), 3.54-3.48 (m, 4H), 3.36 (m, 4H), 3.28-3.18 (m, 8H), 2.56 (t, *J* = 6.5 Hz, 2H), 2.39 (m, 1H), 2.34 (t, *J* = 6.0 Hz, 1H), 1.87 (q, *J* = 12.0 Hz, 1H), 0.81 (s, 3H), 0.71 (s, 3H); **$^{13}$C NMR** ($D_2O$, 125 MHz, by HSQC and HMBC): δ 175.0, 174.0, 173.0, 151.0, 149.4, 142.8, 139.6, 118.6, 110.4, 96.8, 87.8, 84.3, 83.6, 82.2, 76.0, 75.6, 74.5, 74.0, 72.5, 72.0, 71.9, 71.5, 70.3, 69.0, 68.5, 65.2, 61.8, 54.0, 50.0, 49.0, 39.9, 39.6, 38.5, 35.8, 35.6, 34.7, 31.2, 28.5, 21.8, 18.6; **HRMS** for $C_{40}H_{70}N_{11}O_{28}P_3S$ $[M+H]^+$ calcd. 1261.3440, found: 1262.3445.

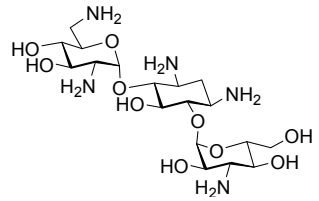
**Neamine (4)**

The $^1$H NMR spectrum of neamine varied with the pH/pD value of the solution. Both the free base and the TFA salt of neamine were fully characterized by NMR. In general, the free base produces a cleaner $^1$H NMR spectrum than the TFA salt. Enhanced solvation of the salt form yields much shorter relaxation time and more overlapping chemical shifts.

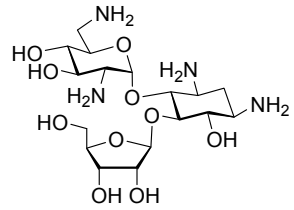



**Neamine free base**. **$^1$H NMR** ($D_2O$, 400 MHz): δ 5.15 (d, *J* = 4.0 Hz, H1′), 3.63 (br t, *J* ~ 8 Hz, H5′), 3.42 (t, *J* = 9.2 Hz, H3′), 3.36 (m, H5), 3.16 (t, *J* = 9.2 Hz, H4′), 3.13 (t, *J* = 9.2 Hz, H4), 3.00 (t, *J* = 9.6 Hz, H6), 2.89 (dd, *J* = 13.4, 2.6 Hz, H6′), 2.72-2.52 (m, H3, H6′, H2′, H1), 1.83 (td, *J* = 12.4, 4.0 Hz, H2eq), 1.06 (q, *J* = 12.4 Hz, H2ax). **$^{13}$C NMR** ($D_2O$, 75 MHz) δ 101.09, (C1′), 87.21 (C4), 77.75 (C6), 76.34 (C5), 73.74 (C3′), 72.79 (C5′), 71.61 (C4′), 55.54 (C2′), 50.18 (C1), 49.63 (C3), 41.60 (C6′), 35.79 (C2).

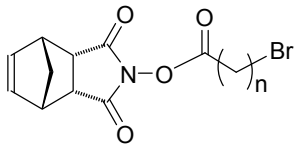
**Neamine HCl salt**. **$^1$H NMR** ($D_2O$, 400 MHz): δ 5.85 (d, *J* = 3.5 Hz, H1′), 3.95 (m, H6), 3.90 (m, H4), 3.87 (m, H5), 3.62 (t, *J* = 5.6 Hz, H4′), 3.50 (m, H3′) 3.47 (m, H3), 3.45 (m, H5), 3.42 (m, H6′), 3.39 (m, H2′), 3.28 (m, H1), 3.22 (m, H6′), 2.37 (td, *J* = 13.2, 4.0 Hz, H2eq), 1.76 (q, *J* = 13.2 Hz, H2ax). **$^{13}$C NMR** ($D_2O$, 75 MHz) δ 96.21 (C1′), 77.89 (C5′), 75.37 (C4′), 72.69 (C3′), 70.84 (C5), 69.40 (C6), 68.39 (C4), 53.71 (C2′), 49.90 (C1), 48.62 (C3), 40.32 (C6′), 28.52 (C2).



**Kanamycin A (5). $^{1}$H NMR** ($D_2O$, 500 MHz): δ 5.35 (d, *J* = 3.5 Hz, H1′), 4.89 (d, *J* = 3.5 Hz, H1″), 3.81 (dt, *J* = 9.5, 3.5 Hz, H5″), 3.74 (dt, *J* = 9.5, 3.5 Hz, H5′), 3.60 (br s, H6″), 3.57-3.54 (m, H3′, H5), 3.51 (dd, *J* = 10.5, 3.5 Hz, H2′), 3.43 (dd, *J* = 10.5, 3.5 Hz, H2″), 3.35-3.29 (m, H4, H4″ ), 3.26 (t, *J* = 9.5 Hz, H4′), 3.20 (m, H4), 3.16 (m, H6′), 3.03 (t, *J* = 10.5 Hz, H3″), 3.02-2.89 (m, H1, H3, H6′), 1.95 (br d, *J* = 12.5 Hz, H2eq), 1.27 (q, *J* = 12.5 Hz, H2ax); **$^{13}$C NMR** ($D_2O$, 125 MHz, by HSQC): δ 100.5 (C1″), 97.5 (C1′), 86.4 (C5′), 82.8 (C5″), 73.6 (C5), 72.3 (C2″), 72.0 (C4′), 71.3 (C4), 71.0 (C3′), 70.2 (C4″), 69.0 (C2′), 67.4 (C6), 60.0 (C6″), 54.6 (C3″), 50.5 (C1), 48.7 (C3), 40.8 (C6′), 33.1 (C2).



**Ribostamycin (6). $^{1}$H NMR** ($D_2O$, 500 MHz): δ 5.80 (d, *J* = 4.0 Hz, H1′), 5.19 (br s, H1″), 4.06 (br d, H2″), 4.01 (t, *J* = 7.0 Hz, H3″), 3.86 (m, H4″, H5′), 3.81 (t, *J* = 10.0 Hz, H4′), 3.74 (dd, *J* = 12.5, 2.0 Hz, H5″), 3.68 (m, H6, H5), 3.52 (dd, *J* = 12.5, 5.5 Hz, H5″), 3.47 (t, *J* = 7.5 Hz, H4), 3.30 (dd, *J* = 12.5, 3.0 Hz, H6′), 3.25 (t, *J* = 10.0 Hz, H3′), 3.21 (dd, *J* = 10.0, 4.0 Hz, H2′), 3.15-3.03 (m, H1, H3, H6′), 2.15 (td, *J* = 12.5, 4.0 Hz, H2eq), 1.56 (q, *J* = 12.5 Hz, H2ax); **$^{13}$C NMR** ($D_2O$, 125 MHz, by HSQC): δ 110.8 (C1″), 95.3 (C1′), 85.7 (C4), 82.8 (C4″), 78.3 (C5′), 75.8 (C2″), 73.7 (C6), 71.8 (C3′), 69.2 (C3″), 69.0 (C4′), 68.8 (C5), 61.4 (C5″), 54.6 (C2′), 50.7 (C1), 49.1 (C3), 41.1 (C6′), 30.8 (C2).



**7a-d** (n=1-4)

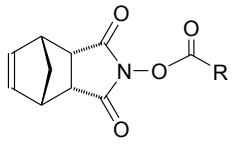
**7a**. TLC $R_f$ = 0.47 in EtOAc/Hex (1/1). Yield: 99%, the crude product was recrystallized using hexane to give earth-red crystals, m.p. 90-92 °C. **$^{1}$H NMR** ($CDCl_3$, 400 MHz): δ 6.20 (br s, 2H), 4.04 (s, 2H), 3.46 (br s, 2H), 3.34 (br s, 2H),

1.80 (d, *J* = 8.8 Hz, 1H), 1.54 (d, *J* = 8.8 Hz, 1H). **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 169.5, 135.0, 51.6, 45.1, 43.7, 21.9. **MS** for $C_{11}H_{10}{}^{79}BrNO_4$ $[M+H]^+$ calcd. 299.0, found 299.0.

**7b**. TLC $R_f$ = 0.60 in EtOAc/Hex (1/1). Yield: >99%, the crude product was recrystallized using hexane to give yellowish crystals, m.p. 118-120 °C. **$^1$H NMR** ($CDCl_3$, 400 MHz): δ 6.20 (br s, 2H), 3.58 (t, *J* = 7.2 Hz, 2H), 3.46 (br s, 2H), 3.34 (br s, 2H), 3.17 (t, *J* = 7.2 Hz, 2H), 1.80 (d, *J* = 8.8 Hz, 1H), 1.54 (d, *J* = 8.8 Hz, 1H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 169.8, 135.0, 51.6, 45.1, 43.6, 35.0, 24.0. **MS** for $C_{12}H_{12}{}^{79}BrNO_4$ $[M+H]^+$ calcd. 313.0, found 313.0.

**7c**. TLC $R_f$ = 0.40 in EtOAc/Hex (1/2). Yield: 97%, the crude product was recrystallized using hexane to give white crystals, m.p. 65-66 °C. **$^1$H NMR** ($CDCl_3$, 400 MHz): δ 6.19 (br s, 2H), 3.49 (t, *J* = 6.4 Hz, 2H), 3.45 (br s, 2H), 3.33 (br s, 2H), 2.76 (t, *J* = 6.8 Hz, 2H), 2.25 (tt, *J* = 6.8, 6.4 Hz, 2H), 1.79 (d, *J* = 8.8 Hz, 1H), 1.54 (d, *J* = 8.8 Hz, 1H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 170.0, 135.0, 51.6, 45.0, 43.6, 31.9, 29.8, 27.8. **MS** $[M+H]^+$ for $C_{13}H_{14}{}^{79}BrNO_4$ calcd. 326.9, found 327.0.

**7d**. TLC $R_f$ = 0.36 in EtOAc/Hex (1/2). Yield: 95%, the crude product was recrystallized using hexane to give white crystals, m.p. 51-52 °C. **$^1$H NMR** ($CDCl_3$, 400 MHz): δ 6.18 (t, *J* = 2.0 Hz, 2H), 3.44 (m, 4H), 3.30 (m, 2H), 2.57 (t, *J* = 7.2 Hz, 2H), 2.05-1.83 (m, 4H), 1.78 (td, *J* = 9.0, 2.0 Hz, 1H), 1.53 (d, *J* = 9.0 Hz, 1H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 170.1, 134.9, 51.6, 45.0, 43.5, 33.0, 31.7, 30.3, 23.5. **MS**for $C_{14}H_{16}{}^{79}BrNO_4$ $[M+H]^+$ calcd. 340.9, found 340.9.




7e-m

| | e | f | g | h | i | j | k | l | m |
|---|---|---|---|---|---|---|---|---|---|
| R: | Me | *n*Pr | $nC_{11}H_{23}$ | Ph | Bn | Naphthe-nyl-$CH_2$ | 2-chloro-phenyl | 2-methyl-phenyl | 2,6-dichloro -phenyl |

**7e**. TLC $R_f$ = 0.15 in EtOAc/Hex (1/1). Yield: 90%. **$^1$H NMR** ($CDCl_3$, 400 MHz): δ 6.18 (br s, 2H), 3.45 (br s, 2H), 3.33 (br s, 2H), 2.25 (s, 3H), 1.79 (d, *J* = 8.8 Hz, 1H), 1.54 (d, *J* = 8.8 Hz, 1H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 169.5, 135.0, 51.6, 45.1, 43.7, 17.2. **MS** for $C_{11}H_{11}NO_4$ $[M+H]^+$ calculated 222.1, found 222.0.

**7f**. TLC $R_f$ = 0.33 in EtOAc/Hex (1/2). Yield: 91%. **$^1$H NMR** ($CDCl_3$, 400 MHz): δ 6.19 (br s, 2H), 3.45 (br s, 2H), 3.32 (br s, 2H), 2.52 (t, *J* = 7.6 Hz, 2H), 1.77 (m, 3H), 1.54 (d, *J* = 8.8 Hz, 1H), 1.02 (m, 3H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz): δ 169.9, 134.9, 51.4, 45.1, 43.6, 31.2, 16.3, 13.6; **MS** for $C_{13}H_{15}NO_4$ $[M+H]^+$ calculated 250.1, found 250.0.

**7g**. TLC $R_f$ = 0.60 in EtOAc/Hex (1/2). Yield: 90%. **$^1$H NMR** ($CDCl_3$, 400 MHz): δ 6.18 (br s, 2H), 3.44 (br s, 2H), 3.32 (br s, 2H), 2.53 (t, *J* = 7.6 Hz, 2H), 1.75 (m, 3H), 1.52 (m, 3H), 1.25 (br s, 14H), 0.88 (t, *J* = 6.8 Hz, 3H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz): δ 169.9, 134.7, 51.5, 45.4, 43.7, 33.0, 29.6, 29.4, 29.3, 29.1, 29.0, 28.5, 28.5, 23.5, 22.2, 14.1. **MS** for $C_{21}H_{31}NO_4$ $[M+H]^+$ calculated 362.2, found 362.2.

**7h**. TLC $R_f$ = 0.65 in EtOAc/Hex (1/1). Yield: 94%. **$^1$H NMR** ($CDCl_3$, 400 MHz): δ 8.09 (d, *J* = 8.0 Hz, 2H), 7.65 (t, *J* = 8.0 Hz, 1H), 7.48 (t, *J* = 8.0 Hz, 2H), 6.28 (br s, 2H), 3.50 (br s, 2H), 3.40 (br s, 2H), 1.83 (d, *J* = 8.8 Hz, 1H), 1.58 (d, *J* = 8.8 Hz, 1H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz): δ 170.2, 134.9, 134.7, 130.7, 130.4, 128.9, 51.7, 45.2, 43.5; **MS** for $C_{16}H_{13}NO_4$ $[M+H]^+$ calculated 284.1, found 284.1.

**7i**. TLC $R_f$ = 0.66 in EtOAc/Hex (1/1). Yield: 100%. **$^1$H NMR** ($CDCl_3$, 400 MHz): δ 7.31 (m, 5H), 6.19 (br s, 2H), 3.87 (s, 2H), 3.44 (br s, 2H), 3.32 (br s, 2H), 1.80 (d, *J* = 8.8 Hz, 1H), 1.58 (d, *J* = 8.8 Hz, 1H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz): δ 170.0, 135.1, 135.0, 129.5, 129.0, 127.9, 51.5, 45.0, 43.4, 37.2; **MS** for $C_{17}H_{15}NO_4$ $[M+H]^+$ calculated 298.1, found 298.0.

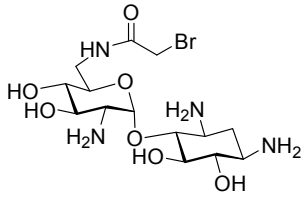
**7j**. TLC $R_f$ = 0.55 in EtOAc/Hex (1/1). Yield: 99%. **$^1$H NMR** ($CDCl_3$, 400 MHz): δ 7.93 (d, *J* = 8.0 Hz, 1H), 7.86 (d, *J* = 8.0 Hz, 1H), 7.82 (d, *J* = 8.0 Hz,

1H), 7.58 (t, *J* = 8.0 Hz, 1H), 7.50 (t, *J* = 8.0 Hz, 1H), 7.45 (m, 2H), 6.15 (br s, 2H), 4.30 (s, 2H), 3.41 (br s, 2H), 3.27 (br s, 2H), 1.74 (br s, 1H), 1.47 (d, *J* = 8.8 Hz, 1H). **$^{13}$C NMR** ($CDCl_3$, 75 MHz): δ 170.0, 166.4, 150.2, 138.2, 134.9, 127.2, 125.4, 125.0, 124.8, 124.8, 123.8, 122.5, 113.3, 51.2, 44.7, 43.2, 37.8; **MS** for $C_{21}H_{17}NO_4$ $[M+H]^+$ calcd. 348.1, found 348.1.

**7k**. TLC $R_f$ = 0.77 in EtOAc/Hex (1/1). Yield: 93%. **$^1$H NMR** ($CDCl_3$, 400 MHz): δ 8.05 (d, *J* = 7.6 Hz, 1H), 7.48 (t, *J* = 8.0 Hz, 1H), 7.27 (t, *J* = 8.0 Hz, 1H), 7.25 (d, *J* = 7.6 Hz, 1H), 6.26 (br s, 2H), 3.49 (br s, 2H), 3.39 (br s, 2H), 2.59 (s, 3H), 1.82 (d, *J* = 8.8 Hz, 1H), 1.56 (d, *J* = 8.8, 1H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz): δ 169.9, 136.2, 134.8, 133.3, 132.4, 130.7, 127.76, 125.8, 51.4, 44.9, 43.3, 21.3. **MS** for $C_{17}H_{15}NO_4$ $[M+H]^+$ calcd. 298.1, found 298.0.

**7l**. TLC $R_f$ = 0.53 in EtOAc/Hex (1/1). Yield: 96%. **$^1$H NMR** ($CDCl_3$, 400 MHz): δ 8.04 (d, *J* = 7.6 Hz, 1H), 7.52 (m, 2H), 7.36 (m, 1H), 6.27 (br s, 2H), 3.50 (br s, 2H), 3.39 (br s, 2H), 1.82 (d, *J* = 8.8 Hz, 1H), 1.57 (d, *J* = 8.8 Hz, 1H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz): δ 169.9, 136.5, 134.7, 130.1, 129.0, 127.5, 126.7, 51.2, 44.4, 43.1; **MS** for $C_{16}H_{12}ClNO_4$ $[M+H]^+$ calcd. 318.0, found 318.0.

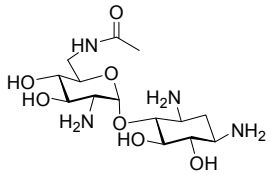
**7m**: TLC $R_f$ = 0.49 in EtOAc/Hex (1/1). Yield: 97%. **$^1$H NMR** ($CDCl_3$, 400 MHz): δ 7.38 (d, *J* = 8.4 Hz, 2H), 7.26 (t, *J* = 8.4 Hz, 1H), 6.25 (br s, 2H), 3.50 (br s, 2H), 3.39 (br s, 2H), 1.81 (d, *J* = 7.2 Hz, 1H), 1.57 (d, *J* = 7.2, 1H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz): δ 169.9, 134.8, 133.5, 129.6, 128.5, 127.8, 51.2, 44.4, 43.0; **ESI-MS** for $C_{16}H_{11}Cl_2NO_4$ $[M+H]^+$ calcd. 351.0, found 351.0.



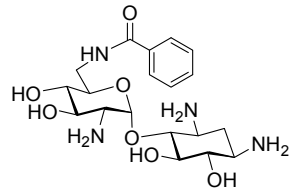

**8a**. Yield: 70%. **$^1$H NMR** ($D_2O$, 400 MHz, pD = 4.0, TFA salt): 5.50 (d, *J* = 4.0 Hz, H1'), 3.92 (s, 2H), 3.70-3.61 (m, H3′, H4, H6), 3.43 (t, *J* = 8.8 Hz, H4′), 3.38-3.31 (m, H5′, H3, H6′), 3.21-3.16 (m, H5, H2′), 3.10 (td, *J* = 10, 3.8 Hz, H1), 2.31 (td, *J* = 8.8, 4.0 Hz, H2eq), 1.66 (q, *J* = 8.8 Hz, H2ax); **$^{13}$C NMR** ($D_2O$, 75 MHz): δ 169.6, 96.8 (C1′), 79.0 (C3′), 75.0 (C4′), 72.4 (C5′), 71.8 (C6), 70.2 (C5), 68.3 (C4), 54.0

(C2′), 50.7 (-CO***C***$H_2$Br), 49.8 (C1), 48.2 (C3), 39.2 (C6′), 28.8 (C2); **ESI-MS** for $C_{14}H_{27}{}^{79}BrN_4O_7$ $[M+H]^+$ calcd. 442.1, found 442.2.

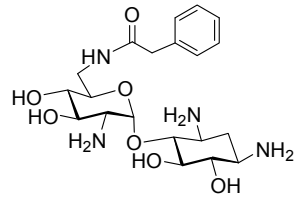
**8b-d, 9, 10** not isolated**.**



**8e.** Yield: 60%. **$^1$H NMR** ($D_2O$, 400 MHz): 5.50 (d, *J* = 4.0 Hz, H1′), 3.72-3.63 (m, H3′, H4, H6), 3.46 (t, *J* = 8.8 Hz, H4′), 3.40-3.32 (m, H5′, H3, H6′), 3.24-3.19 (m, H5, H2′), 3.13 (td, *J* = 10, 3.8 Hz, H1), 2.32 (td, *J* = 8.8, 4.0 Hz, H2eq), 1.82 (s, -COC*H*$_3$), 1.67 (q, *J* = 8.8 Hz, H2ax); **$^{13}$C NMR** ($D_2O$, 75 MHz): δ 169.6, 96.8 (C1′), 79.0 (C3′), 75.0 (C4′), 72.4 (C5′), 71.8 (C6), 70.2 (C5), 68.3 (C4), 54.0 (C2′), 49.8 (C1), 48.2 (C3), 39.2 (C6′), 28.8 (C2), 21.9 (-CO***C***$H_3$); **ESI-MS** for $C_{14}H_{28}N_4O_7$ $[M+Na]^+$ calcd. 387.2, found 387.2.

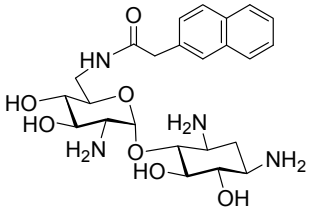


**8h.** Yield: 80%. **$^1$H NMR** ($D_2O$, 400 MHz, pD = 4.0, TFA salt): 7.59 (d, *J* = 8.0 Hz, ArH2, ArH6), 7.45 (t, *J* = 6.8 Hz, ArH4), 7.35 (t, *J* = 8.0Hz, ArH3 ArH5), 5.55 (d, *J* = 4.0 Hz, H1′), 3.81-3.70 (m, H3′, H6, H4), 3.62 (m, H6′), 3.49 (t, H4′), 3.40-3.30 (m, H5′, H5, H3), 3.26 (br d, *J* = 13.2 Hz, H2′), 3.13 (td, *J* = 10.0, 4.0 Hz, H1), 2.32 (td, *J* = 12.0, 4.0 Hz, H2eq), 1.67 (q, *J* = 12 Hz, H2ax); **$^{13}$C NMR** ($D_2O$, 75 MHz) δ 167.7, 135.4, 132.2, 128.7, 126.5, 97.0 (C1′), 79.0 (C3′), 75.2 (C4′), 72.8 (C5′), 72.0 (C6), 71.0 (C5), 69.0 (C4), 54.0 (C2′), 50.0 (C1), 48.6 (C3), 40.0 (C6′), 28.5 (C2); **ESI-MS** for $C_{19}H_{30}N_4O_7$ $[M+Na]^+$ calcd. 439.2, found 439.1.



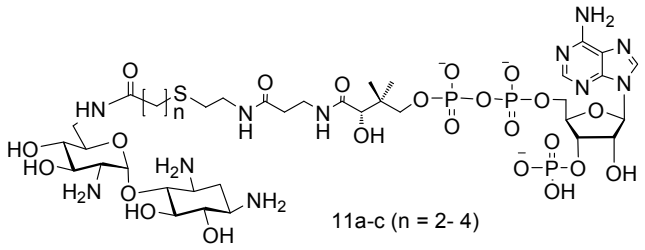
**8i.** Yield: 75%. **$^1$H NMR** ($D_2O$, 400 MHz): δ 7.24-7.14 (m, 5H), 5.48 (d, *J* = 4.4, H1′), 3.73-3.65 (m, H3′, H4, H6), 3.47 (s, 2H, CO-$CH_2$-Ph), 3.45-3.40 (m, H6′, H4′), 3.40-3.30 (m, H5′, H6′, H3)**,** 3.17-3.07 (m, H5, H1, H2′), 2.32 (td, *J* = 12.0, 4.0 Hz, H2eq), 1.67 (q, *J* = 12.0 Hz, H2ax); **$^{13}$C NMR** ($D_2O$, 75 MHz) δ 169.8, 135.1, 131.8, 130.0, 127.6, 97.2 (C1′), 79.4 (C3′), 75.6 (C4′), 72.8 (C5′), 72.0 (C6), 70.2 (C5), 69.1 (C4),

54.0 (C2′), 50.0 (C1), 48.9(C3), 42.6 (*C*$H_2$Ph), 38.0 (C6′), 28.4 (C2); **ESI-MS** for $C_{20}H_{32}N_4O_7$ $[M+H]^+$ calcd. 441.2, found 441.2.

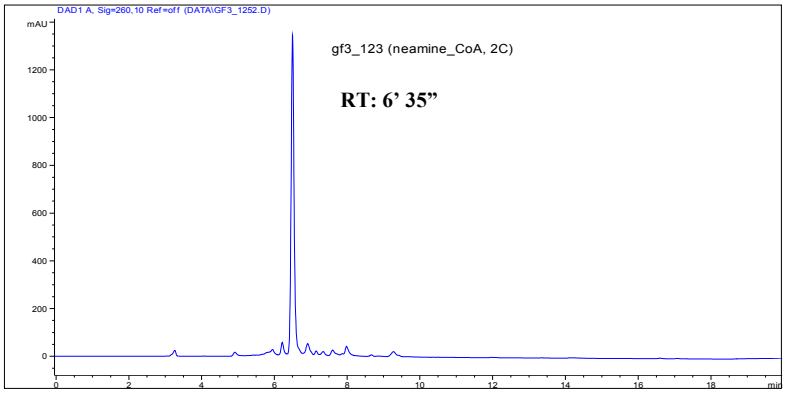


**8j**. Yield: 85%. **$^1$H NMR** ($D_2O$, 400 MHz): δ 7.84 (t, *J* = 8.4 Hz, H5″, H8″), 7.79 (d, *J* = 8.0 Hz, H2″), 7.47(m, H6″, H7″), 7.40 (t, *J* = 8.0 Hz, H3″), 7.36 (d, *J* = 8.0 Hz, H4″), 5.33 (d, *J* = 4.0 Hz, H1′), 3.98 (s, CO-$CH_2$-Ar), 3.64-3.58 (m, H3′, H4, H6), 3.48-3.35 (m, H4′, H6′, H5′), 3.32 (m, H3), 3.15 (dt, *J* = 11.0, 4.0 Hz, H1), 2.88 (t, *J* = 9.6 Hz, H5), 2.69 (dd, *J* = 11.2, 4.0 Hz, H2′), 2.33 (td, *J* = 12.4, 4.8 Hz, H2eq), 1.67 (q, *J* = 12.8 Hz, H2ax); **$^{13}$C NMR (**$D_2O$, 100 MHz): δ 163.3 (CO), 133.8 (C10″), 131.8 (C9″), 131.1 (C1″), 129.1 (C4″), 128.9 (C2″), 128.5 (C8″), 126.9 (C3″), 126.4 (C6″), 126.2 (C5″), 123.7 (C7″), 96.7 (C1′), 79.0 (C3′), 75.2 (C4′), 72.7 (C5′), 71.9 (C6), 70.0 (C5), 68.6 (C4), 53.9 (C2′), 49.9 (C1), 48.7 (C3), 40.5 ($CH_2$), 38.9 (C6′), 28.6 (C2); **ESI-MS** for $C_{24}H_{34}N_4O_7$ $[M+Na]^+$ calcd. 513.2, found 513.2;

**Neamine-CoA bisubstrate 11a-c.**

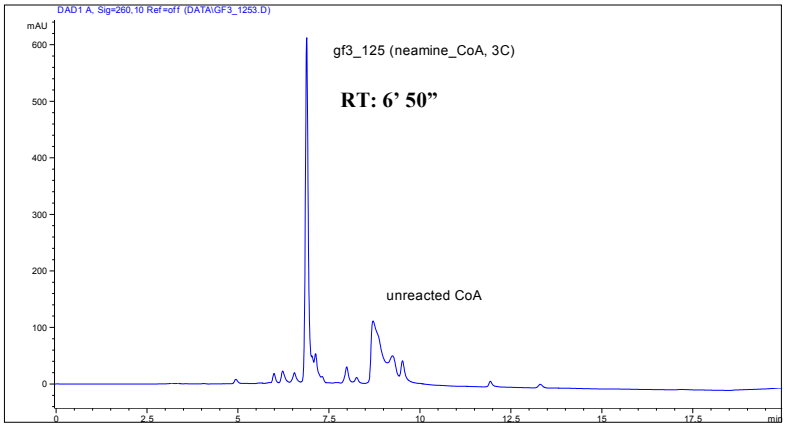



**11a**: HPLC chromatogram of purification.




Yield: 91%. **$^{1}$H NMR** ($D_2O$, 500 MHz): δ 8.61 (s, 1H), 8.41 (s, 1H), 6.18 (d, *J* = 5.0 Hz, 1H), 5.69 (d, *J* = 4.5 Hz, 1H), 4.56 (br s, 1H), 4.24 (m, 2H), 3.97 (s, 1H), 3.91 (m, 1H), 3.87 (t, *J* = 10.5 Hz, 1H), 3.82-3.80 (m, 2H), 3.60-3.57 (m, 4H), 3.53-3.29 (m, 11H), 2.76 (t, *J* = 6.5 Hz, 2H), 2.62 (t, *J* = 6.5 Hz, 2H), 2.53 (t, *J* = 6.5 Hz, 2H), 2.48 (dt, *J* = 12.0, 4.0 Hz, 1H), 2.42 (t, *J* = 6.5 Hz, 2H), 1.80 (q, *J* = 12.5 Hz, 1H), 0.89 (s, 3H), 0.78 (s, 3H); **$^{13}$C NMR** ($D_2O$, 125 MHz by HSQC and HMBC): δ 177.7, 175.0, 173.5, 150.0, 149.1, 145.1, 142.5, 119.2, 96.8, 88.7, 83.9, 79.2, 78.6, 75.6, 74.7, 74.0, 73.0, 72.8, 72.0, 71.9, 70.8, 69.0, 65.8, 54.2, 50.0, 49.1, 39.9, 38.8, 36.0, 35.9, 35.8, 30.9, 28.4, 27.6, 22.0, 19.7; **HRMS** for $C_{36}H_{64}N_{11}O_{23}P_3S$ $[M+H]^+$ calcd. 1144.3183, found 1144.3188.

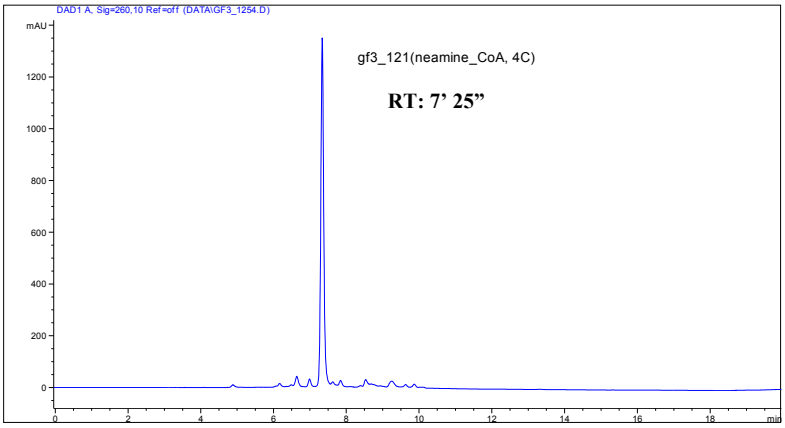
**11b**: HPLC chromatogram of purification.




Yield: 52%. **$^{1}$H NMR** ($D_2O$, 500 MHz): δ 8.60 (s, 1H), 8.38 (s, 1H), 6.18 (d, *J* = 5.5 Hz, 1H), 5.69 (d, *J* = 4.0 Hz, 1H), 4.57 (br s, 1H), 4.34 (m, 2H), 3.97 (s, 1H), 3.93 (m, 1H), 3.87 (t, *J* = 9.0 Hz, 1H), 3.84-3.77 (m, 2H), 3.61-3.55 (m, 5H),

3.52-3.43 (m, 4H), 3.38-3.28 (m, 6H), 2.59 (t, $J$ = 6.5 Hz, 2H), 2.49 (m, 3H), 2.42 (t, $J$ = 6.5 Hz, 2H), 2.33 (t, $J$ = 6.5 Hz, 2H), 1.92 (q, $J$ = 12.5 Hz, 1H), 1.79 (br t, $J$ = 7.0 Hz, 2H), 0.88 (s, 3H), 0.77 (s, 3H); **$^{13}$C NMR** ($D_2O$, 125 MHz, by HSQC and HMBC): δ 176.6, 174.8, 174.1, 151.0, 149.5, 146.0, 142.0, 118.0, 96.7, 87.8, 83.4, 79.2, 75.0, 74.4, 73.5, 72.5, 72.2, 71.7, 71.5, 70.6, 68.5, 65.0, 54.0, 49.2, 48.5, 38.9, 37.8, 36.8, 35.5, 35.3, 34.8, 30.5, 30.2, 28.0, 25.4, 20.8, 17.6; **HRMS** for $C_{37}H_{66}N_{11}O_{23}P_3S$ $[M+H]^+$ calcd. 1158.3346, found 1158.3345.

**11c:** HPLC chromatogram of purification




Yield: 93%. **$^1$H NMR** ($D_2O$, 500 MHz): δ 8.61 (s, 1H), 8.40 (s, 1H), 6.18 (d, $J$ = 5.0 Hz, 1H), 5.69 (d, $J$ = 4.0, 1H), 4.56 (br s, 1H), 4.23 (m, 2H), 3.97 (s, 1H), 3.93 (br t, $J$ = 9.2 Hz, 1H), 3.87 (t, $J$ = 9.0 Hz, 1H), 3.81 (m, 2H), 3.61-3.55 (m, 5H), 3.52-3.43 (m, 4H), 3.38-3.28 (m, 6H), 2.59 (t, $J$ = 6.5 Hz, 2H), 2.50 (m, 3H), 2.42 (t, $J$ = 6.5 Hz, 2H), 2.25 (t, $J$ = 6.5 Hz, 2H), 1.90 (q, $J$ = 12.5 Hz, 1H), 1.50 (m, 2H), 1.51 (m, 2H), 0.88 (s, 3H), 0.77 (s, 3H); **$^{13}$C NMR** ($D_2O$, 125 MHz, by HSQC and HMBC): δ 178.0, 175.2, 173.6, 149.8, 148.8, 145.0, 142.4, 118.8, 97.0, 87.9, 83.6, 79.2, 75.0, 74.4, 73.5, 72.6, 72.3, 71.9, 71.6, 70.8, 68.8, 65.2, 53.8, 49.9, 48.4, 39.0, 38.8, 38.4, 35.5, 35.3, 35.0, 30.5, 30.2, 28.2, 27.8, 24.4, 21.0, 18.4; **HRMS** for $C_{38}H_{68}N_{11}O_{23}P_3S$ $[M+H]^+$ calcd. 1172.3496, found 1172.3501.

**Expression and purification of AAC(6′)-Ii**.

AAC(6′)-Ii was obtained using a protocol previously described elsewhere.[1] The *Escherichia coli* strain BL21 was transformed with a pET22b expression plasmid containing the AAC(6')-Ii gene. The bacteria were grown in Luria-Bertani (LB) media at 37°C containing ampicillin (100 μg/mL). Expression of the protein was induced using isopropyl-β-D-thiogalactoside (IPTG). After harvesting the cells by centrifugation and washing them with a 0.85% NaCl solution, the cells were lysed by sonication. AAC(6′)-Ii was purified in a two-step process: first, the lysate was ran through a Q-Sepharose ion exchange column (GE Healthsciences); second, the AAC(6′)-Ii containing fractions were further purified using a Gentamicin agarose affinity column (BioRad).

**AAC(6′)-Ii inhibition assay**

Enzyme activity was monitored using a procedure described elsewhere.[2] Thus reaction mixtures in HEPES (1 mM, pH = 7.5) and containing 4,4′-dithiodipyridine (DTDP, 2 mM), aminoglycoside (200 μM), and AAC(6′)-Ii (25 μg/mL) were prepared with varying concentrations of AcCoA. Reaction volumes were typically 400 μl. The assay mixtures were preincubated for 3 min at 37ºC. The initial velocities $V$ in absence of inhibitor were fit to Equation S1, where [S] is the concentration of AcCoA, $K_m^{app}$ is the Michaelis-Menten constant, and $V_m$ is the maximal velocity. The calculated $K_m^{app}$ for AcCoA is 9.56 μM.

$$[S]/V=[S]/V_m+K_m^{app}/V_m \qquad \textbf{(S1)}$$

The initial reaction velocity (steady-state) obtained at various concentrations of inhibitor were fit to Hanes-Wolff plot. Equation S2 for competitive inhibition, Equation S3 for noncompetitive inhibition, or Equation S4 for uncompetitive inhibition, where [I] is the concentration of inhibitor and $K_i^{app}$ is the inhibition constant.

$$[S]/V = [S]/V_m+(1+[I]/K_i^{app})\ K_m^{app}/V_m \qquad \textbf{(S2)}$$

$$[S]/V = (1+[I]/K_i^{app})[S]/V_m+K_m^{app}/V_m \qquad \textbf{(S3)}$$

$$[S]/V = (1+[I]/K_i^{app})[S]/V_m + (1+[I]/K_i^{app} K_i^{app}) K_m^{app}/V_m \qquad \textbf{(S4)}$$

All the experimental data fit Equation S2 much better than S3 or S4, which suggested competitive inhibition. However, these results imply that the bisubstrates are tight binding inhibitors ($K_i^{app}$ is less than 1000 times the concentration of AAC(6′)-Ii). Thus Equations S5, S6 and S7 were applied for tight binding competitive inhibition, tight binding noncompetitive inhibition, or tight binding uncompetitive inhibition respectively.[3]

$$IC_{50} = K_i^{app} (1+[S]/K_m^{app}) + [E]/2 \qquad \textbf{(S5)}$$

$$IC_{50} = K_i^{app} + [E]/2 \qquad \textbf{(S6)}$$

$$IC_{50} = K_i^{app} (1+ K_m^{app}/[S]) + [E]/2 \qquad \textbf{(S7)}$$

The $IC_{50}$ was determined from Equation S8 where $v_i$ is the initial velocity in the presence of inhibitor at concentration [I] and $v_0$ is the initial velocity in the absence of inhibitor.

$$v_i/v_0 = 1/(1+[I]/IC_{50}) \qquad \textbf{(S8)}$$

The experimental data fit Equation S5 much better than S6 or S7, confirming that the bisubstrates are tight binding competitive inhibitors.

**References**

[1] Wright, G. D.; Ladak, P., Overexpression and characterization of the chromosomal aminoglycoside 6′-*N*-acetyltransferase from *Enterococcus faecium*. *Antimicrobial Agents & Chemtherapy* 1997, 41, 956-960.

[2] Williams, J. W.; Northrop, D., *Journal of Biology Chemistry* **1978**, *253*, 5902.

[3] Copeland, R.A. *Enzymes*, 2nd edition; Wiley-VCH, 2000

# Chapter Three

Although the bisubstrate analogs described in the previous chapter proved useful structural and mechanistic probes for aminoglycoside 6′-*N*-acetyltransferases (AAC(6′)s), they are not likely to cross cell membranes because of the negative charges of the CoA portion. We next aimed to synthesize inhibitors with lower molecular weight and without negative charges. These new inhibitors were truncated derivatives of the first generation of bisubstrate inhibitors. One of them was able to block aminoglycoside resistance in cells. Furthermore, these inhibitors allowed us to determine important structure-activity relationships for the binding sites of AAC(6′)-Ii.

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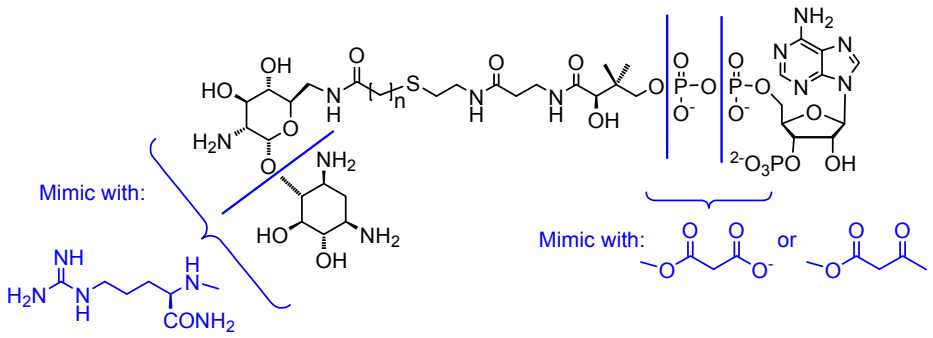
Contributions of co-authors

This chapter is a copy and is reproduced with permission from the Journal of Medicinal Chemistry. This article is cited as Feng Gao, Xuxu Yan, Tushar Shakya, Oliver M. Baettig, Samia Ait-Mohand-Brunet, Albert M. Berghuis, Gerard D. Wright, and Karine Auclair. "Synthesis and Structure-activity Relationships of Truncated Bisubstrate Inhibitors of Aminoglycoside 6′-*N*-Acetyltransferases". *Journal of Medicinal Chemistry* **2006**, 49, 5273-5281. I carried out the synthesis of all the compounds and wrote the article. Xuxu Yan, a graduate student in our lab performed the enzymatic tests. Oliver M. Baettig (Berghuis' lab) purified the enzyme. Tushar Shakya (Wright's lab) performed bacterial tests. Samia Ait-Mohand-Brunet (a research associate in our lab) participated in the synthesis of **4a**.

# Synthesis and Structure-activity Relationships of Truncated Bisubstrate Inhibitors of Aminoglycoside 6′-*N*-Acetyltransferases

**Table of Contents graphic:**




## Abstract

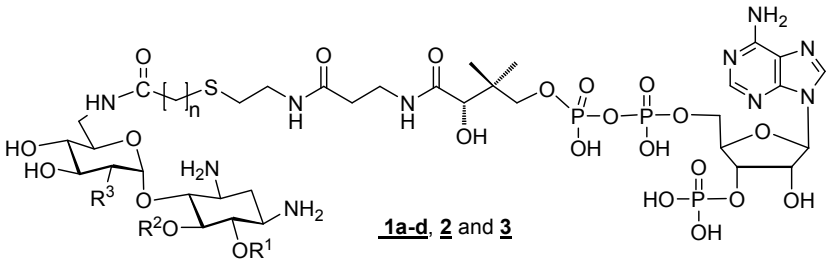
Truncated aminoglycoside-coenzyme A bisubstrate analogs were efficiently prepared using a convergent approach where the amine and the thiol are coupled in one pot with the addition of a linker, without the need for protecting groups. These derivatives were tested for their effects on the activity of the resistance-causing enzyme aminoglycoside 6′-*N*-acetyltransferase Ii and key structure-activity relationships are reported. Moreover, one of the inhibitors is able to block aminoglycoside resistance in cells expressing this enzyme.

## 3.1 Introduction

Aminoglycosides are potent antibiotics particularly active against aerobic, gram-negative bacteria.[1, 2] Unfortunately, the emergence of aminoglycoside resistance is increasingly restricting their use as antibacterials.[3, 4] The most common mechanism of resistance to aminoglycosides is enzymatic modification by acetyltransferases, phosphoryltransferases or adenylyltransferases.[5-7] Of these, the aminoglycoside 6′-*N*-acetyltransferase (AAC(6′)) family is one of the most widespread determinants of aminoglycoside resistance. *Enterococcus faecium* AAC(6′)-Ii was selected for this study based on its clinical importance as the reason for loss of β-lactam/aminoglycoside synergy in this important pathogen, and because of the availability of detailed mechanistic and structural information for the enzyme.[8-14] Investigations by Wright and coworkers suggest that AAC(6′)-Ii proceeds via an ordered bi bi mechanism where AcCoA binds first to the enzyme, followed by the aminoglycoside.[11, 12] Crystallographic[8, 9, 14] and mutagenic[12] studies have failed to reveal specific amino acid side chains available to stabilize the proposed tetrahedral intermediate, suggesting that the role of this enzyme may be to bind and orient the two substrates in a geometry that favors the acyl transfer, without direct stabilization of an aminoglycoside intermediate.

In our efforts to study the catalytic mechanism of AAC(6′)s, we have developed a regio- and chemo-selective method for the direct *N*-6′-derivatization of unprotected aminoglycosides.[15, 16] This facilitated the preparation of the first generation of synthetic AAC(6′)-Ii inhibitors, the bisubstrate analogs **1a-c**, **2** and **3** (Fig. 3.1). These nanomolar inhibitors have allowed crystallization of AAC(6′)-Ii with aminoglycoside derivatives for the first time, though they lacked biological activity.[16] After the success of these molecules as structural and mechanistic probes of AAC(6′)s, we envisaged the preparation of a second generation of AAC(6′) inhibitors of smaller size to determine structure-activity relationships. Bisubstrate analogs **1a**, **2** and **3** show similar $K_i$*s* against AAC(6′)-Ii, suggesting that aminoglycoside rings III and IV are not essential for inhibition. Bisubstrate

**1a** was therefore used as the starting point in the design of our second generation of AAC(6′) inhibitors. Thus the role of the different functionalities of **1a** for binding to AAC(6′)-Ii was investigated with the preparation of shorter analogs.



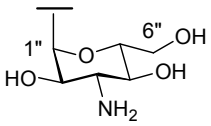
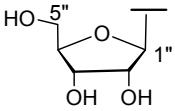

| Inhibitor | n | $R^1$ | $R^2$ | $R^3$ | $K_i$ (nM) |
|---|---|---|---|---|---|
| **1a** | 1 | OH | OH | $NH_2$ | 76 ± 25 |
| **1b** | 2 | OH | OH | $NH_2$ | 43 ± 23 |
| **1c** | 3 | OH | OH | $NH_2$ | 161 ± 98 |
| **1d** | 4 | OH | OH | $NH_2$ | 8000 ± 2700 |
| **2** | 1 |  | OH | OH | 110 ± 28 |
| **3** | 1 | OH |  | $NH_2$ | 119 ± 14 |

***Figure 3.1***. First generation of bisubstrate inhibitors and $K_i$ values against AAC(6′)-Ii.

### 3.2 Results and discussion

The synthesis of aminoglycoside-CoA bisubstrates is a challenging task; in part because of the complex protection/deprotection schemes often required for aminoglycoside derivatization, and because of the water solubility of aminoglycosides and coenzyme A (CoA) derivatives. It was envisaged that our previously reported method to assemble aminoglycoside-CoA bisubstrates in one

pot would be a great asset in the synthesis of truncated derivatives.[16] The syntheses described below all converge to the one pot chemo- and regio-selective coupling of an amine to a thiol with incorporation of an acetyl linker. From there, retrosynthetic analyses are reduced to the preparation of the corresponding amines and thiols, with no need for protecting groups. The first target, **4a** (Fig. 3.2), is an analog of **1a** lacking the 2-deoxystreptamine ring (2-DOS, ring II of the aminoglycoside). The amine moiety of target **4a** was prepared from *N*-acetylglucosamine (Scheme 3.1). Thus after glycosylation to methyl 2-acetamido-α-D-glucopyranoside (**6a**),[17] the *N*-6′-amine was introduced by selective *O*-6′-mesylation and azidation, followed by reduction to methyl 2-acetamido-6-amino-α-D-glucopyranoside (**6d**). The *N*-2-acetyl group was removed using $Ba(OH)_2$ to yield **7**. This step could not be reported to later because of reagent incompatibility with the bromoacetyl group. As reported for aminoglycosides,[16] the use of *endo*-*N*-hydroxy-5-norbornene-2,3-dicarboximide bromoacetate (NBD-bromoacetate), followed by addition of CoA afforded **4a** in a one-pot reaction. Interestingly, the removal of ring II from the aminoglycoside moiety of **1a** led to a 50-fold decrease in affinity for AAC(6′)-Ii (Table 3.1). This result emphasizes the strength of the salt bridges and hydrogen bonds observed between the 2-DOS of **1a** and the enzyme in the crystal structure of the **1a**-AAC(6′)-Ii complex.[18]

**Table 3.1.** Patterns of inhibition and inhibition constants for **4a-f** and **5a-e** measured against AAC(6′)-Ii.

| Inhibitor[a] | Inhibition pattern | $K_i$ (μM)[b] |
|---|---|---|
| **4a** | Competitive | 3.4 ± 2.0 |
| **4b** | Competitive | ~ 130 |
| **4c** | Competitive | 1.2 ± 0.2 |
| **4d** | Competitive | 3.4 ± 1.7 |
| **4e** | Competitive | 3.6 ± 0.4 |
| **4f** | Competitive | 7.4 ± 2.9 |
| **5a** | No inhibition | ≥ 200 |

| | | |
|---|---|---|
| **5b** | Competitive | 12 ± 8 |
| **5c** | No inhibition | ≥ 1000 |
| **5d** | Competitive | 2.2 ± 1.1 |
| **5e** | Competitive | 11 ± 6 |

[a]All molecules were tested as the TFA salt. [b]For comparison, $K_m$ and $k_{cat}$ were determined for neamine, kanamycin A and ribostamycin. They were comparable to those reported in the literature.[13]

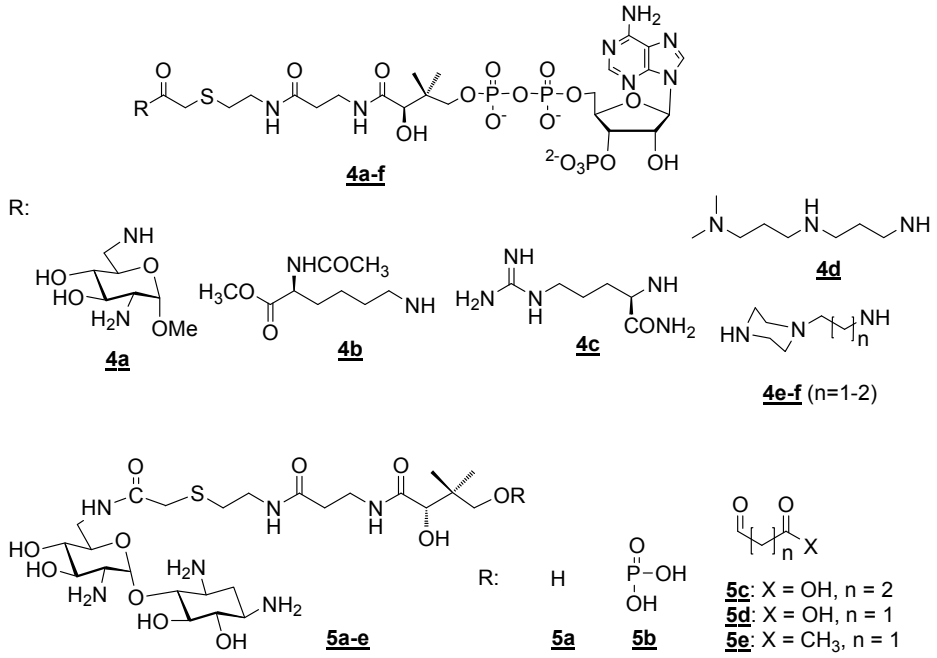



***Figure 3.2.*** Second generation of AAC(6') inhibitors.

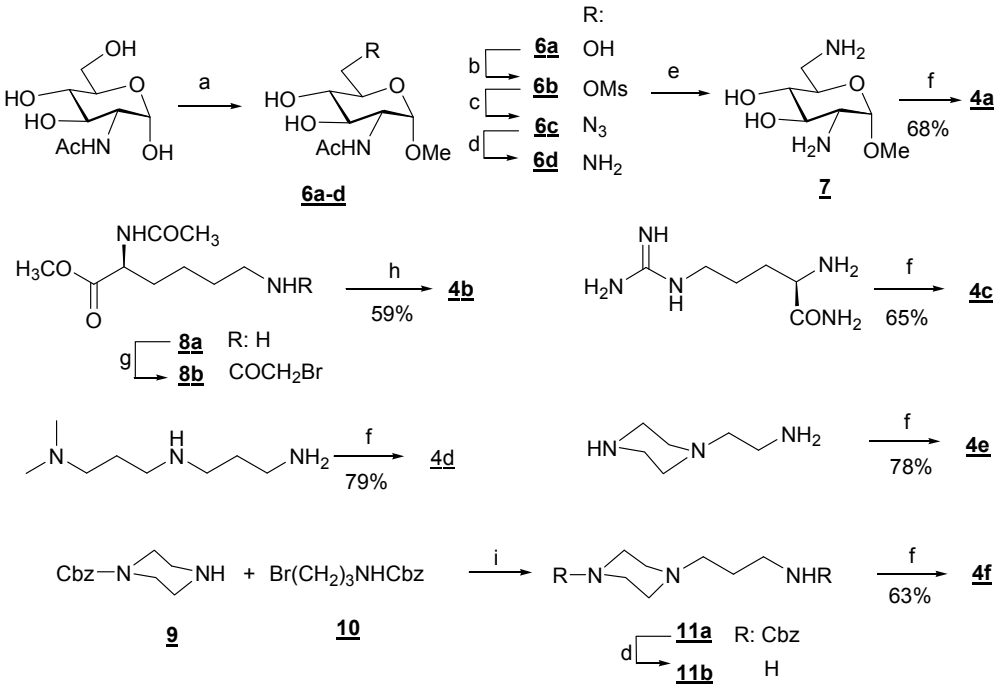
AAC(6′)-Ii has been shown to acetylate poly-lysines and histones at the ε-$NH_2$.[13] To better understand the specificity of AAC(6′)-Ii for aminoglycosides, the neamine moiety of **1a** was replaced with lysine (target **4b**). Commercial $N^{\alpha}$-acetyl-L-lysine methyl ester (**8a**) was selected to better mimic peptidic lysines. Compound **8a** was bromoacetylated to **8b**, before reaction with CoA to yield **4b** (Scheme 3.1). To our surprise, **4b** showed 1700-fold reduced inhibition against AAC(6′)-Ii compared to **1a**. This result was attributed to the lack of positive charges available to interact with the negatively charged aminoglycoside binding

site of this enzyme. To further confirm this assumption, we next prepared an arginine-CoA analog. The use of NBD-bromoacetate, here to selectively bromoacetylate arginine at the α-$NH_2$, followed by addition of CoA led to the formation of **4c** in one-pot (Scheme 3.1). Remarkably, the arginine-CoA analog **4c** was at least two orders of magnitude more potent than the lysine-CoA analog **4b**, and even slightly more potent than **4a** (Table 3.1). This result confirms the importance of positive charges for binding the aminoglycoside pocket of AAC(6′)-Ii, consistent with the electrostatic properties of the active site.[14]

Propylenediamine derivatives have been shown to imitate well the diamine portion of 2-DOS in the design of non-carbohydrate mimics of aminoglycosides.[19] This concept was used to conceive target **4d**, which includes one more positive charge compared to **4c**. Compound **4d** was assembled from commercial *N, N*-dimethyldipropylenetriamine without the need for protection, using NBD-bromoacetate and CoA as described above (Scheme 3.1). In spite of the extra positive charge, **4d** shows comparable inhibition to that observed for **4c** (Table 3.1). The larger entropic penalty needed for binding the polyamine may compensate for the higher enthalpy of binding expected with an additional electrostatic interaction.

To reduce the flexibility of the linear polyamine, it was next envisaged to prepare piperazine derivatives **4e** and **4f**. Indeed, piperazine is a common pharmacophore that may resemble 2-DOS.[20] Compound **4e** was synthesized directly from commercially available 1-(2-amino)piperazine (Scheme 3.1). Synthesis of **4f** began with a reaction between benzyl 1-piperazinecarboxylate (**9**) and benzyl 3-bromopropylcarbamate (**10**) in the presence of $K_2CO_3$ to form **11a** in quantitive yield (Scheme 3.1). Global Cbz deprotection of **11a** to **11b,** was followed by our standard one-pot reaction with NBD-bromoacetate and CoA to yield **4f**. Inhibition studies with **4e** and **4f** revealed similar potency compared to **4c** and **4d**. The longer chain of **4f** is somewhat detrimental. Overall these results not only confirm the importance of the positive charges for interaction with the aminoglycoside pocket of AAC(6′)-Ii, but also the necessity of proper orientation

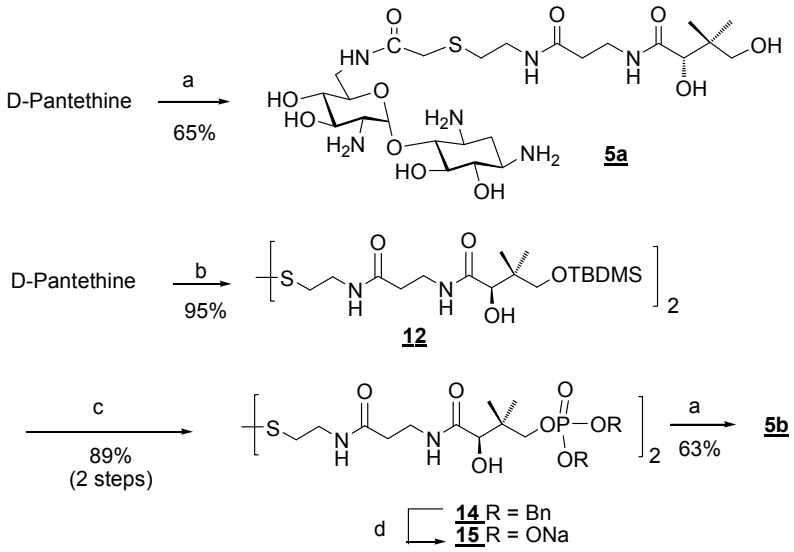
of the amino groups. Our results are also in agreement with a previous report suggesting that neamine is the minimal substrate of AAC(6′)-Ii.[13] Retaining neamine in the inhibitor is rather convenient because of the low cost of this aminoglycoside, the ease of derivatization using our one-pot methodology, and the known active transport of aminoglycosides across the cell membranes of most bacterial strains.[1] Neamine is also the smallest aminoglycoside able to cause mistranslation in bacterial protein synthesis.[21]




***Scheme 3.1.*** Synthetic route to inhibitors **4a-f**. Reagents and conditions: (a) Amberlite IR-120 (H), MeOH, reflux 16 h (89%); (b) MsCl, py, 0°C 6 h (73%); (c) $NaN_3$, acetone/$H_2O$, reflux 16 h (78%); (d) $H_2$/$Pd(OH)_2$, MeOH, RT 16 h (94%); (e) $Ba(OH)_2$, MeOH/$H_2O$, reflux 16 h (81%); (f) 0.95 eq. NBD-bromoacetate, acetone/$H_2O$, RT 10 min, then add 0.9 eq. CoA in TEA-$H_2CO_3$ buffer pH 8.5, RT 1 h; (g) 1.05 eq. bromoacetic acid, DCC, DMAP, DCM, RT 3 h (78%); (h) 0.95 eq. CoA, TEA-$H_2CO_3$ buffer pH 8.5, RT 1 h; (i) $K_2CO_3$, DMF, RT 16 h (97%).

After modifications at the neamine moiety of **1a** (targets **4a-f**) we next envisaged to truncate the CoA end of **1a** (targets **5a-e**, Figure 3.2). The multiple negative charges of CoA are believed to prevent bisubstrates **1-4** from crossing bacterial cell membranes. Inhibitors that can penetrate cells are desirable not only for cell-based experiments but also for potential use as pharmaceuticals.

Acetyltransferases adopt a variety of folds near AcCoA, and the cofactor is either bent or linear. Binding to the pantetheinyl portion of AcCoA is however relatively conserved.[22-26] We hypothesized that with an intact pantetheinyl group, the molecules would more likely be general AAC(6′) inhibitors. Target **5a** was prepared in one-pot by regioselective *N*-6′-derivatization of neamine in the presence of NBD-bromoacetate and D-pantetheine (Scheme 3.2). D-pantetheine was produced *in situ* by reduction of commercial D-pantethine using dithiothreitol (DTT). Compound **5a** was next tested at concentrations up to 200 μM for its effect on the activity of AAC(6′)-Ii but did not show any significant inhibition. A considerable reduction in affinity was expected based on the crystal structures of AAC(6′)-Ii,[8, 9, 14] which reveal that of the hydrogen bonds formed between the enzyme and AcCoA, five are between the phosphate groups and the protein. These hydrogen bonds likely serve as strong anchoring points for the cofactor. The absence of inhibition is likely also explained by the high flexibility of the pantetheinyl group.



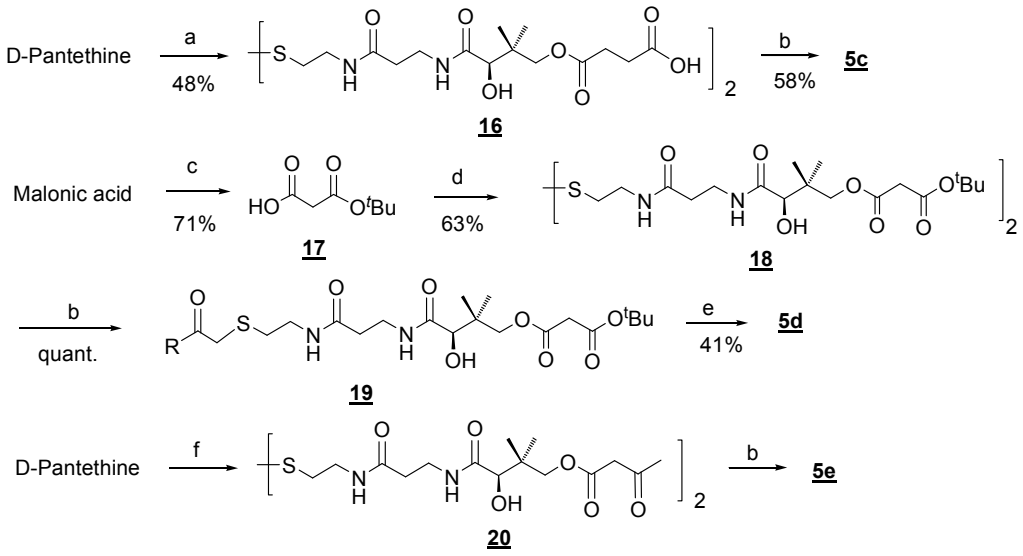

***Scheme 3.2.*** Synthetic route to inhibitors **5a-b**. Reagents and conditions: (a) *N*-6'-bromoacetylneamine, acetone/$H_2O$, DTT, 20 eq. DIPEA, RT 1 h; (b) *tert*-butyldimethylsilyl chloride, imidazole, DMF, RT 16 h (99%); (c) dibenzylchlorophosphate (**13**), CsF (2.5 eq.), py, RT 3 h (97%); (d) Na, naphthalene, THF, -40$^{o}$C to RT 2 h (91%).

Target **5b** was next prepared to examine the importance of the pantetheinyl 4′-phosphate group. Synthesis of **5b** began with the preparation of known dibenzylphosphoryl chloride (**13**) using a modification of the reported procedure.[27] Direct phosphorylation of D-pantethine using **13** under conditions described previously for other alcohols[28] was not successful. This may be due to the low nucleophilicity of the 4′-hydroxyl group of D-pantethine, likely resulting from an intramolecular hydrogen bond with the nearby hydroxyl group. Thus a silyl ether was formed to temporarily break this hydrogen bond. Cleavage of the silyl ether with CsF to generate the alcohol *in-situ* facilitated the reaction with **13** to afford **14** (Scheme 3.2). Intermediate **14** was reduced using sodium naphthalenide[29] to remove the benzyl groups and to generate the free thiol. With the desired thiol in hand, our one-pot methodology was next applied using neamine and NBD-bromoacetate to afford **5b** in 63% yield after HPLC purification. To our surprise, the simple addition of one phosphate group was sufficient to recover low micromolar inhibition.

The relatively good activity of **5b** encouraged us to add a second phosphate group; however, phosphates have negative charges which reduce their applicability. Two strategies may be envisaged to overcome this issue. One is to temporarily mask the phosphoric acids as esters or amides (e.g. as in some pro-drugs).[30] A second approach is to use a mimic of the phosphate group.[31] The crystal structures reveal that the interactions between the CoA pyrophosphate group and the enzyme involve mainly hydrogen bonds as opposed to salt bridges.[8, 9, 14] This implies that it may be possible to mimic the pyrophosphate moiety with two carbonyl groups, as in targets **5c**, **5d** and **5e**. Synthesis of **5c** first involved the coupling of succinic anhydride to the 4′-hydroxyl group of D-pantethine to yield **16** (Scheme 3.3). Compound **16** was transformed into **5c** in one-pot in the presence of DTT (*in situ* reduction of the disulfide), neamine and NBD-bromoacetate. The succinate moiety turned out to be a bad mimic of the pyrophosphate group as suggested by the absence of AAC(6′)-Ii inhibition in the presence of **5c**. Synthesis of **5d** began with the coupling of *tert*-butylmalonate (**17**)

and D-pantethine to yield **18** (Scheme 3.3). Removal of the *tert*-butyl protecting group at this point was not successful, but proceeded more easily when carried after disulfide reduction and reaction with NBD-bromoacetate and neamine. We were pleased to see that **5d** is a more potent inhibitor of AAC(6′)-Ii than **5b**, which contained a phosphate group on 4′-OH. This result suggests that malonate is a good mimic of the pyrophosphate. The two carbonyl groups of succinate are likely too far apart to mimic the pyrophosphate group, as suggested by the absence of inhibition observed for **5c**. The malonate group of **5d** on the other hand, seems to properly position the two carbonyls for interaction with the enzyme. It is interesting to note that truncation of ring II of neamine (see **4a**) has the same effect on the inhibition as the deletion of the entire ADP moiety of CoA (see **5b** and **5d**). This was unexpected based on the $K_m s$ of neamine (6 μM) and CoA (9 μM) for this enzyme.[13] Next, **5e** was prepared and tested to verify the importance of the negative charge of the malonate group (the methyl malonate derivative was prepared but found to be unstable). Synthesis of **5e** involved the coupling of acetoacetate to pantethine, followed by disulfide reduction and reaction with NBD-bromoacetate and neamine as before (Scheme 3). We were pleased to find that replacement of a negatively charged oxygen with a methyl group did not cause a considerable reduction in the affinity for AAC(6′)-Ii. This result confirms that, for this enzyme, the pyrophosphate moiety of the CoA substrate can be conveniently mimicked with a β-dicarbonyl group.

Molecules **5d** and **5e** were tested in combination with the aminoglycoside kanamycin (a substrate for AAC(6′)s) against *E. faecium* C238 harboring the AAC(6′)-Ii encoding gene. Compound **5d** did not show any activity, whereas **5e** displayed a moderate synergistic effect with kanamycin A (Figure 3.3). This supports our hypothesis that the negative charge of the carboxylate group may prevent cell penetration.




***Scheme 3.3.*** Synthetic route to inhibitors **5c-e**. R = *N*-6'-neamine. Reagents and conditions: (a) succinic anhydride, py, RT 16 h; (b) *N*-6'-bromoacetylneamine, acetone/$H_2O$, DTT, 20 eq. DIPEA, RT 1 h; (c) MsCl, py, *tert*-butanol, THF, 0°C 2 h, RT 1 h; (d) D-pantethine, DCC, DMAP, THF, RT 16 h; (e) TFA/DCM/anisole, RT 16 h; (f) lithium acetoacetate, HATU, 2 eq. DIPEA, DMF, RT 16 h.

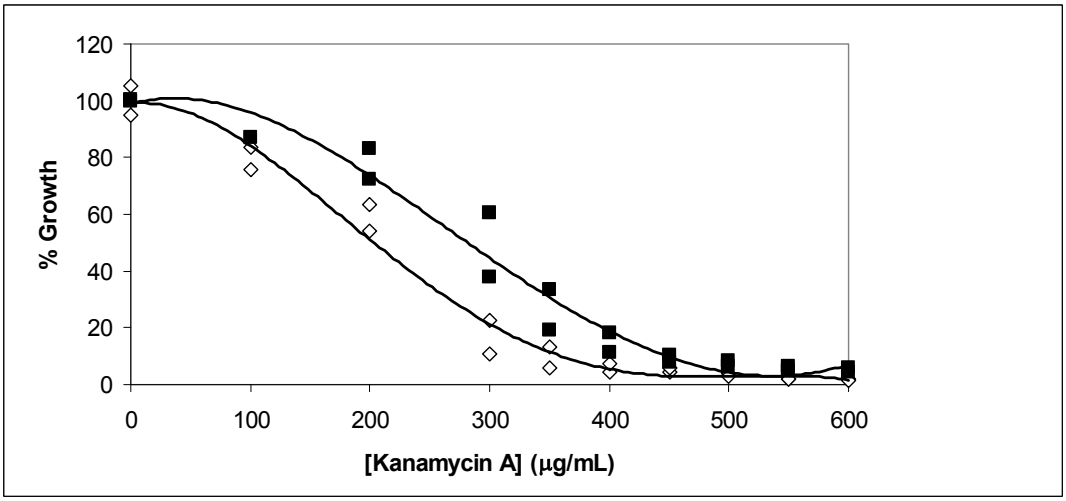



***Figure 3.3***. Potentiation of kanamycin antibiotic activity by compound **5e**. Growth of *E. faecium* C238 in the absence (solid square) and presence of 512 µg/mL **5e**.

## 3.3 Conclusions

In conclusion, we have demonstrated that aminoglycoside-CoA bisubstrate analogs can be rapidly and efficiently assembled using NBD-bromoacetate

directly with unprotected amines and truncated CoA analogs. Our methodology is chemo- and regio-selective, efficient and applicable to water soluble molecules. Using this method, 11 analogs of bisubstrate **1a** were rapidly synthesized. These molecules were tested for inhibition of the aminoglycoside resistance-causing enzyme AAC(6′)-Ii. This enzyme represents a good model not only for AAC(6′)s but also for the numerous other acetyltransferases which adopt the same fold and/or proceed via a similar catalytic mechanism (e.g. some histone acetyltransferases involved in gene regulation and cancer, [26, 32-34] serotonine acetyltransferase involved in circadian rhythm,[35, 36] and spermidine/spermine *N*-1-acetyltransferase involved in cancer and HIV infection[37]).

The bisubstrate analogs were tested *in vitro* for their effect on the activity of AAC(6′)-Ii. This led to the elucidation of several structure-activity relationships. Our results indicate that inhibition by aminoglycoside-CoA bisubstrates is more sensitive to truncation at the aminoglycoside than at the CoA end. The smaller neamine is however, as efficient as larger aminoglycosides to maintain the affinity of the bisubstrate for AAC(6′)-Ii. This was expected based on the reported $K_m$ for various AAC(6′)-Ii substrates.[13] Our results with analogs **4a-e** have also emphasized the importance of properly oriented positively charged amino groups for interaction with the aminoglycoside binding pocket of AAC(6′)-Ii.

Truncation of the CoA moiety of bisubstrate **1a** revealed that the pantetheinyl group is not sufficient for recognition by the enzyme (likely too flexible) and at least one phosphate group is needed. More potent inhibitors are obtained when the pyrophosphate moiety of CoA is mimicked with β-dicarbonyl groups such as malonate or acetoacetate. To our knowledge, this is the first time that a pyrophosphate group is successfully mimicked with acetoacetate. Compound **5e** is also the first inhibitor of an aminoglycoside resistance-causing enzyme active in cells. This provides a proof of concept for the pharmaceutical potential of AAC(6′) inhibitors.

These structure-activity relationships, combined with our structural studies of bisubstrate-AAC(6′)-Ii complexes,[16] provide crucial information for the design of AAC(6′) inhibitors and novel antibacterials effective against resistant strains. Compound **5e** represents a new lead in the development of pharmaceuticals able to block aminoglycoside resistance caused by AAC(6′)s.

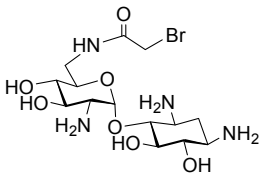
## 3.4 Experimental section

### 3.4.1 General

All reagents were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario) except for protected lysine **8a** which was purchased from Chem-Impex International Inc (Wood Dale, Illinois) and HATU was purchased from GL Biochem Ltd. (Shanghai, China). Coenzyme A was purchased as the trilithium salt (~95%). Reagents and solvents were used without further purification unless otherwise stated. Flash chromatography and analytical TLCs (F-254) were performed with 60 Å silica gel from Silicycle (Quebec, Canada). All the target molecules were purified by reversed-phase HPLC using an Agilent 1100 modular system equipped with an autosampler, a quaternary pump system, a photodiode array detector, a thermostatted column compartment and a ChemStation (for LC 3D A.09.03) data system. The column used was a semi-preparative Zorbax SB-CN of 4.6 × 250 mm and 5 μm (Agilent, Palo Alto, California). Samples were eluted at a flow rate of 3 mL/min, using a combination of mobile phase A (0.05% TFA in water at pH ~ 3.5) and mobile phase B (acetonitrile containing 0.05% TFA). After 2 min at 1% B, the eluent was brought to 40% B in a linear gradient over 20 min, the detector was set to 260 nm and 214 nm. The purity of the targets was evaluated by HPLC using the same equipment and column described above but using an isocratic elution with ratios of phase A/phase B of either 65/35 (method A) or 55/45 (method B). The purity of all target molecules used in the biological assays was >95%. HRMS were obtained by direct infusion electrospray ionization from a solution of 50 mM formic acid:methanol 90:10 at 2 μL/min in an IonSpec 7 Tesla FTICR instrument. The resolving power was approximately

80,000. The low resolution MS of all intermediates were obtained on a Kratos MS 25RFA mass spectrometer at a source temperature of 200°C and 70 eV. ESI-MS was performed using a Finnigan LCQDUO mass spectrometer. $^{31}$P NMR spectra were acquired on Varian Mercury 200 using broadband gated decoupling ($^{1}$H) and chemical shifts are listed relative to 85% phosphoric acid. $^{1}$H and $^{13}$C NMR spectra were recorded using Varian mercury 400 or 300, or Unity 500 spectrometers. For all $^{1}$H NMR spectra of target molecules, presaturation (presat.) is used to suppress the water peak. The chemical shifts (δ) were reported in parts per million (ppm) and are referenced to either the internal standard TMS (when $CDCl_3$ is used) or the deuterated solvent used. The peak patterns are indicated as follows: s, singlet, d, doublet, t, triplet, dt, doublet of triplet, ddd, doublet of doublet of doublet, td, triplet of doublet, m, multiplet, q, quartet, p, pentet, and br s, broad singlet. The coupling constants, *J* are reported in Hertz (Hz). The NMR spectra were obtained from their TFA salt of **4a-f** and **5a-e** in $D_2O$ (pD ~ 4). Reactions with air or moisture sensitive reagents and solvents were carried out under an atmosphere of argon. All the reactions were carried out in round bottom flasks capped with a septum unless otherwise stated.

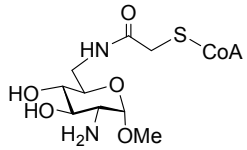
### 3.4.2 Synthetic procedures and compound characterization.



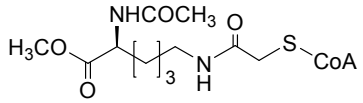

***N*-6′-Bromoacetylneamine solution**. Neamine free base (32 mg, 0.1 mmol) was dissolved in water (2 mL) in a vial and *endo*-*N*-hydroxy-5-norbornene-2,3-dicarboximide bromoacetate (18 mg, 0.06 mmol) was dissolved in acetone (2 mL) in another vial, the two solutions were mixed, sonicated for 1 min, and stirred for 15 min. This solution is referred to as the *N*-6′-bromoacetylneamine solution.

**General procedure for the preparation of 4a, 4c-f:** The general procedure for the preparation of **4a, 4c-f** is exemplified with the following procedure for **4a**. Compound **7** (20 mg, 0.1 mmol) was dissolved in water (2 mL) in a vial. *endo*-*N*-Hydroxy-5-norbornene-2,3-dicarboximide bromoacetate[16] (18 mg, 0.06 mmol)

was dissolved in acetone (2 mL) in another vial. The two solutions were mixed, sonicated for 1 min and stirred for an extra 15 min, before addition of CoA-SH (15 mg, 0.02 mmol) in TEA $H_2CO_3$ buffer (4 mL, pH 8.5). The mixture was stirred for another hour and then evaporated to dryness under vacuum. The crude powder was dissolved in a 0.05% solution of TFA in water and purified by HPLC.

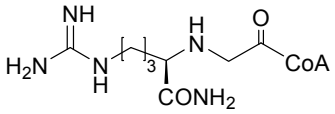



**4a**. Yield: 11 mg, 68%. **$^1$H NMR** ($D_2O$, 400 MHz, presat.): δ 8.51 (s, 1H), 8.29 (s, 1H), 6.06 (d, *J* = 5.0, 1H), 4.83 (d, *J* = 3.5, 1H), 4.78-4.71 (m, 1H), 4.46 (br s, 1H), 4.14 (m, 2H), 3.88 (s, 3H), 3.73 (m, 1H), 3.66 (t, *J* = 10.0, 1H), 3.56 (m, 1H), 3.51-3.46 (m, 2H), 3.33 (m, 3H), 3.26 (s, 3H), 3.21 (t, *J* = 6.0, 2H), 3.18 (dd, *J* = 30, 9.0, 1H), 3.15 (s, 3H), 2.54 (t, *J* = 6.5, 2H), 2.33 (t, *J* = 6.5, 2H), 0.80 (s, 3H), 0.68 (s, 3H). **$^{13}$C NMR** ($D_2O$, 100 MHz): δ 174.9, 174.1, 173.1, 150.0, 144.9, 142.6, 118.7, 117.6, 109.5, 103.5, 96.2, 87.7, 83.6, 74.4, 74.0, 72.2, 71.2, 70.3, 69.9, 65.3, 55.4, 54.1, 40.2, 38.4, 35.6, 35.5, 34.8, 31.4, 21.0, 18.5. **HRMS** for $C_{30}H_{52}N_9O_{21}P_3S$ $[M+2H]^{2+}$ calcd. 500.6178, found 500.6177. Purity, 98% (method A, $t_R$: 3.13 min; method B, $t_R$: 3.00 min).

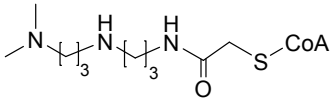



**4b**. Compound **8b** (16 mg, 0.05 mmol) and CoA (25 mg, ~0.03 mmol of the lithium salt) were dissolved in triethylammonium bicarbonate buffer (pH ~ 8.5, 4 mL). The mixture was stirred overnight at RT and evaporated to dryness. The resulting white powder was dissolved in water containing 0.05% TFA and purified by HPLC to yield **4b** as white powder (yield: 19.8 mg, 59%). **$^1$H NMR** ($D_2O$, 400 MHz): δ 8.31 (s, 1H), 8.13 (s, 1H), 6.05 (d, *J* = 5.0, 1H), 4.46 (s, 1H), 4.20 (dd, *J* = 8.8, 3.6, 1H), 4.13 (s, 2H), 3.91 (s, 1H), 3.68 (d, *J* = 9.2, 1H), 3.64 (s, 3H), 3.44 (d, *J* = 9.2, 1H), 3.35 (t, *J* = 6.4, 2H), 3.25 (t, *J* = 6.4, 2H), 3.14 (s, 2H), 3.08 (t, *J* = 6.4, 2H), 2.56 (t, *J* = 6.4, 2H), 2.35 (t, *J* = 6.4, 2H), 1.93 (s, 3H), 1.81 (m, 1H), 1.61 (m, 1H), 1.56 (m, 2H), 1.40 (p, *J* = 7.2, 2H), 1.24 (m, 2H), 0.76 (s, 3H), 0.63 (s, 3H). **$^{13}$C NMR** ($D_2O$, 100 MHz): δ 174.7, 174.4, 174.3, 174.2, 174.0, 154.1, 150.8, 149.3, 140.5, 119.2, 95.4, 86.8, 84.0, 74.2, 74.0, 73.8, 72.1, 71.9, 68.0, 67.6, 65.7, 53.5, 52.9, 39.7, 38.2, 35.8, 34.6, 31.4, 27.5, 21.3,

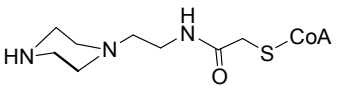
20.4, 18.2. **HRMS** for $C_{32}H_{54}N_9O_{20}P_3S$ $[M+H]^+$ calcd. 1010.2423, found 1010.2425. Purity, 98% (method A, $t_R$: 3.44 min; method B, $t_R$: 3.29 min).



**4c**. Yield: 10.2 mg, 65%. **$^1$H NMR** ($D_2O$, 400 MHz): δ 8.49 (d, *J* = 1.6, 1H), 8.26 (s, 1H), 6.05 (d, *J* = 5.2, 1H), 4.74 (m, 1H), 4.43 (br s, 1H), 4.11 (m, 3H), 3.87 (s, 1H), 3.71 (m, 1H), 3.46 (br d, *J* = 9.6, 1H), 3.32 (t, *J* = 6.4, 2H), 3.20 (t, *J* = 6.4, 2H), 3.16 (s, 2H), 3.01 (t, *J* = 6.4 , 3H), 2.54 (t, *J* = 6.4, 2H), 2.31 (t, *J* = 6.4, 2H), 1.75-1.65 (m, 1H), 1.65-1.55 (m, 1H), 1.55-1.40 (m, 2H), 0.79 (s, 3H), 0.68 (s, 3H). **$^{13}$C NMR** ($D_2O$, 100 MHz): δ 175.8, 174.4, 174.2, 174.0, 158.2, 154.4, 151.2, 149.7, 141.0, 118.9, 86.8, 84.0, 74.2, 73.8, 72.1, 71.9, 67.6, 65.7, 53.5, 52.9, 39.5, 38.2, 35.8, 34.6, 31.4, 27.5, 21.3, 20.4, 18.0. **HRMS** for $C_{29}H_{51}N_{12}O_{18}P_3S$ $[M+H]^+$ calcd. 981.2451, found 981.2475. Purity, 98% (method A, $t_R$: 3.13 min; method B, $t_R$: 3.00 min).

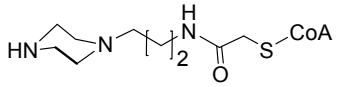


**4d**. Yield: 13 mg, 78%. **$^1$H NMR** ($D_2O$, 400 MHz): δ 8.52 (s, 1H), 8.33 (s, 1H), 6.09 (d, *J* = 5.0, 1H), 4.78 (m, 1H), 4.48 (br s, 1H), 4.16 (m, 2H), 3.89 (s, 1H), 3.74 (m, 1H), 3.65-3.50 (m, 12H), 3.41-3.32 (m, 4H), 3.26-3.19 (m, 4H), 2.57 (t, *J* = 6.4, 2H), 2.34 (t, *J* = 6.4, 2H), 0.81 (s, 3H), 0.71(s, 3H). **$^{13}$C NMR** ($D_2O$, 100 MHz): δ 174.6, 174.2, 173.9, 150.0, 149.3, 145.0, 143.1, 119.5, 88.6, 82.8, 74.0, 73.8, 72.0, 70.2, 67.9, 56.8, 49.3, 42.0, 41.4, 39.0, 36.1, 36.0, 35.2, 34.7, 32.1, 21.2, 18.8. **HRMS** for $C_{29}H_{51}N_{10}O_{17}P_3S$ $[M+H]^+$ calcd. 937.2367, found 937.2456. Purity, 96% (method A, $t_R$: 3.11 min; method B, $t_R$: 2.98 min).

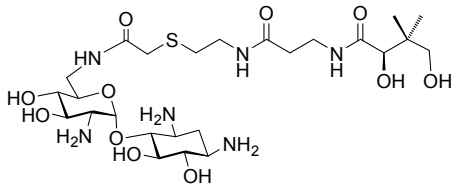


**4e**. Yield: 9.7 mg, 63%. **$^1$H NMR** ($D_2O$, 400 MHz): δ 8.55 (s, 1H), 8.35 (s, 1H), 6.11 (d, *J* = 5.0, 1H), 4.79 (m, 1H), 4.46 (br s, 1H), 4.19 (m, 2H), 3.92 (s, 1H), 3.79 (m, 3H), 3.70-3.50 (m, 6H), 3.50-3.38 (m, 4H), 3.28-3.15 (m, 8H), 2.61 (t, *J* = 6.4, 2H), 2.39 (t, *J* = 6.4, 2H), 1.94 (m, 2H) 0.81 (s, 3H), 0.71 (s, 3H). **$^{13}$C NMR** ($D_2O$, 100 MHz): δ 174.5, 174.0, 173.6, 150.1, 149.4, 145.0, 143.2, 119.5, 88.0, 83.1, 74.2, 73.8, 72.2, 69.5, 64.8, 54.7, 46.6, 40.4, 39.8, 38.4, 36.4, 35.3, 35.1, 34.7, 31.2, 23.4, 20.9, 18.3. **HRMS** for $C_{30}H_{53}N_{10}O_{17}P_3S$ $[M+H]^+$ calcd.

951.2524, found 951.2528. Purity, 97% (method A, $t_R$: 3.27 min; method B, $t_R$: 3.08 min).

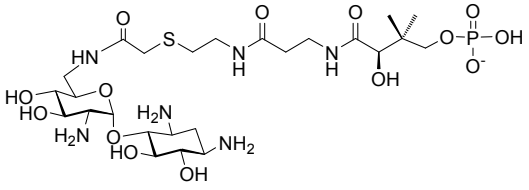


**4f**. Yield: 13 mg, 79%. **$^1$H NMR** ($D_2O$, 400 MHz): δ 8.47 (s, 1H), 8.25 (s, 1H), 6.04 (d, *J* = 5.0, 1H), 4.41 (br s, 1H), 4.16-4.03 (m, 2H), 3.83 (s, 1H), 3.67 (dd, *J* = 9.6, 4.4, 1H), 3.43 (dd, *J* = 9.6, 4.4, 1H), 3.30 (m, 2H), 3.19-3.09 (m, 5H), 3.09-3.02 (m, 3H), 3.02-2.98 (m, 2H), 2.98-2.88 (m, 4H), 2.72 (s, 6H), 2.50 (t, *J* = 6.8, 2H), 2.29 (t, *J* = 6.8, 2H), 2.03-1.95 (m, 2H), 1.80-1.71 (m, 2H), 0.76 (s, 3H), 0.65 (s, 3H). **$^{13}$C NMR** ($D_2O$, 100 MHz): δ 174.6, 174.0, 173.0, 150.0, 148.5, 147.4, 144.8, 142.5, 118.7, 118.33, 87.1, 83.5, 74.5, 74.2, 72.2, 65.3, 54.5, 46.9, 44.5, 43.0, 38.6, 36.7, 35.6. 35.1, 31.5, 25.8, 23.4, 21.6, 21.2, 18.7. **HRMS** for $C_{31}H_{57}N_{10}O_{17}P_3S$ $[M+H]^+$ calcd. 967.2827, found 967.2917. Purity, 98% (method A, $t_R$: 3.32 min; method B, $t_R$: 3.11 min).

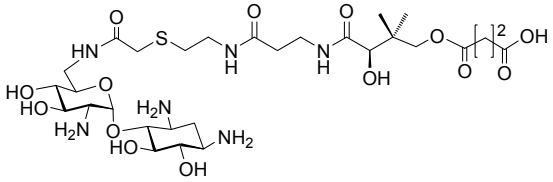


**5a**. In a vial, D-pantethine (15 mg, 0.03 mmol), DTT (5 mg, 0.03 mmol) and DIPEA (1 mL, 5 mmol) were mixed in acetone/$H_2O$ (2 mL, 1/1 v/v) and sonicated for 2 min. This solution was transferred to the *N*-6'-bromoacetylneamine solution (0.06 mmol, 4 ml) and the resulting mixture was sonicated for another 2 min then stirred for 1 h. After evaporation of the solvent under vacuum, the residue was dissolved in 0.1% aqueous TFA (10 mL) and washed with ethyl acetate (2 × 3 mL). The aqueous layer was evaporated to dryness under vacuum and redissolved in 0.05% TFA water (3 mL) before purification by HPLC to give **5a** (22 mg, 65% based on D-pantethine). In general the final work-up process and purification for **5b-d** are the same as described here for **5a**. **$^1$H NMR** ($D_2O$, 400 MHz): δ 5.56 (d, *J* = 3.2, 1H), 3.82 (s, 1H), 3.74 (m, 3H), 3.52-3.30 (m, 10H), 3.30-3.12 (m, 6H), 2.58 (t, *J* = 6.4, 2H), 2.35 (m, 3H), 1.73 (q, *J* = 12.4, 1H), 0.76 (s, 3H), 0.73 (s, 3H). **$^{13}$C NMR** ($D_2O$, 100 MHz): δ 174.4, 173.9, 173.7, 96.8, 79.0, 75.9, 75.0, 72.6, 71.5, 70.5, 68.8, 68.5, 53.8, 49.7, 48.5, 40.0, 39.5, 38.3, 35.4, 35.3, 34.7, 31.2, 28.3, 20.5, 19.2. **HRMS** for

$C_{25}H_{48}N_6O_{11}S$ $[M+H]^+$ calcd. 641.3175, found 641.3167. Purity, 95% (method A, $t_R$: 3.25 min; method B, $t_R$: 3.06 min).

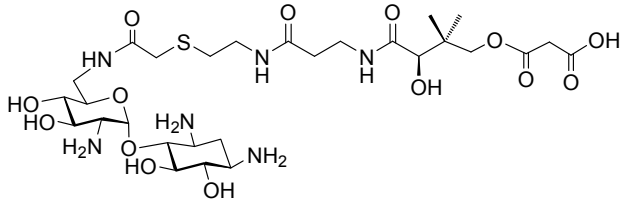


**5b**. Compound **14** (25 mg, 0.05 mmol) and DIPEA (1 mL, 5 mmol) were mixed in acetone/$H_2O$ (2 mL, 1/1 v/v) and sonicated for 2 min. This solution was transferred into the N-6'-bromoacetylneamine solution (0.06 mmol, 4 ml). The resulting mixture was sonicated for another 2 min and stirred for 1 h. Work up as described for **5a** was followed by HPLC to give **5b** (24 mg, 63% based on **14**). **$^1$H NMR** ($D_2O$, 400 MHz): δ 5.60 (br s, 1H), 3.90 (br s, 1H), 3.70-3.85 (m, 3H), 3.70-3.60 (m, 1H), 3.52-3.39 (m, 9H), 3.39-3.19 (m, 6H), 2.60 (t, *J* = 6.5, 2H), 2.38 (m, 3H), 1.78 (q, *J* = 13.2, 1H), 0.85 (s, 3H), 0.78 (s, 3H). **$^{13}$C NMR** ($D_2O$, 100 MHz): δ 177.4, 175.0, 173.7, 96.1, 78.2, 77.1, 75.4, 72.7, 70.8, 70.2, 69.5, 68.4, 53.8, 49.9, 48.6, 41.5, 40.6, 39.7, 36.0, 33.1, 30.4, 29.1, 28.4, 21.0, 18.6. **$^{31}$P NMR** ($D_2O$, 81 MHz): δ -0.05. **HRMS** for $C_{25}H_{49}N_6O_{14}PS$ $[M+H]^+$ calcd. 721.2838, found 721.2846. Purity, 97% (method A, $t_R$: 3.10 min; method B, $t_R$: 2.99 min).

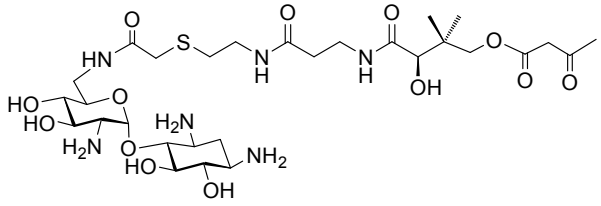


**5c**. Compound **15** (25 mg, 0.03 mmol), DTT (5 mg, 0.03 mmol) and DIPEA (1 mL, 5 mmol) were mixed in acetone/$H_2O$ (3 mL, 1/1 v/v). The mixture was sonicated 2 min. and stirred overnight. This solution was transferred to the *N*-6′-bromoacetylneamine solution (4 mL). The resulting mixture was sonicated 2 min and stirred for 1 h. Work up as described for **5a** was followed by HPLC to give **5d** (26 mg, 58%). **$^1$H NMR** ($D_2O$, 400 MHz): δ 5.57 (d, *J* = 3.5, 1H), 3.89 (d, *J* = 10.5, 1H), 3.83 (s, 1H), 3.79-3.70 (m, 4H), 3.51 (d, *J* = 9.0, 1H), 3.48 (t, *J* = 4.0, 1H), 3.45 (t, *J* = 4.0, 1H), 3.43-3.40 (m, 1H), 3.39 (m, 1H), 3.38-3.34 (m, 3H), 3.28-3.22 (m, 4H), 3.17 (m, 2H), 2.56 (m, 6H), 2.40-2.32 (m, 3H), 2.06 (s, 2H), 1.73 (q, *J* = 13.2, 1H), 0.81 (s, 3H), 0.79 (s, 3H). **$^{13}$C NMR** ($D_2O$, 100 MHz): δ 174.4, 173.2, 172.2, 171.6, 170.6, 96.9, 79.3, 75.3, 75.2, 72.7,

71.8, 70.7, 70.6, 70.4, 68.9, 54.0, 48,8, 41.8, 39.8, 39.4, 38.5, 37.3, 35.0, 31.5, 30.5, 29.1, 28.5, 20.7, 19.6. **HRMS** for $C_{29}H_{52}N_6O_{14}S$ $[M+H]^+$ calcd. 741.3335, found 741.3333. Purity, 98% (method A, $t_R$: 3.55 min; method B, $t_R$: 3.34 min).

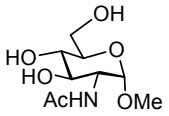


**5d**. Crude **18** (80 mg, 0.10 mmol) was dissolved in TFA/DCM/ anisole (3 mL, 90/9/1 v/v) in a plastic bottle and stirred overnight. After work up as described for **5a** and evaporation of the TFA and DCM, the residue was purified by HPLC to give **5c** (18 mg, 41%). **$^1$H NMR** ($D_2O$, 500 MHz): δ 5.57 (d, *J* = 3.5, 1H), 3.94 (d, *J* = 10.5, 1H), 3.84 (s, 1H), 3.80 (d, *J* = 10.5, 1H), 3.79-3.70 (m, 3H), 3.54-3.30 (m, 9H), 3.30-3.22 (m, 4H0, 3.21-3.13 (m, 3H), 2.56 (t, *J* = 6.4, 2H), 2.40-2.30 (m, 3H), 1.72 (q, *J* = 13.2, 1H), 0.82 (s, 3H), 0.79 (s, 3H). **$^{13}$C NMR** ($D_2O$, 125 MHz): δ 174.4, 173.2, 172.2, 171.6, 170.6, 97.2, 79.3, 75.5, 75.2, 72.6, 72.0, 71.2, 70.6, 69.0, 54.0, 50.0, 48.8, 40.1, 39.4, 38.5, 35.6, 35.5, 35.0, 34.8, 31.5, 28.5, 20.7, 20.0. **HRMS** for $C_{28}H_{50}N_6O_{14}S$ $[M+H]^+$ calcd. 727.3246, found 727.3237. Purity, 98% (method A, $t_R$: 3.47 min; method B, $t_R$: 3.25 min).

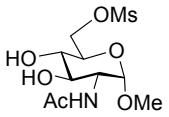


**5e**. D-pantethine (0.27 g, 0.5 mmol) and lithium acetoacetate (0.18 g, 1.5 mmol) were dissolved in DMF (10 ml). The mixture was stirred for 10 min followed by the addition of HATU (0.38 g, 1.0 mmol) and DIPEA (0.4 ml, 2.0 mmol). The mixture was stirred for 16 h at RT before evaporation under vacuum. The residue was triturated with dichloromethane (2 × 10 mL) to yield a paste. This paste was dissolved in $CHCl_3$/MeOH (30 mL, 10/1, v/v) and washed with $H_2O$ (2 × 10 mL). The organic layer was dried over sodium sulfate and evaporated to dryness to give **20** as a yellowish powder. Compound **20** (80 mg, ~ 0.1 mmol), DTT (15 mg, 0.1 mmol) and DIPEA (1 mL, 5 mmol) were mixed in

acetone/$H_2O$ (5 mL, 1/1 v/v). The mixture was sonicated 2 min. and stirred overnight. This solution was transferred to the *N*-6′-bromoacetylneamine solution (4 mL). The resulting mixture was sonicated 2 min and stirred for 1 h. Work up was carried out as described for **5a** and followed by HPLC purification to afford **5e** (4.6 mg, 10%). **$^1$H -NMR** ($D_2O$, 400 MHz): δ 5.57 (s, 1H), 3.87 (s, 1H), 3.79 (t, *J* = 9.6, 2H), 3.69 (t, *J* = 9.6, 1H), 3.53-3.19 (m, 18 H), 2.62 (t, *J* = 6.0, 2 H), 2.41-2.34 (m, 3H), 2.16 (s, 3H), 1.71 (q, *J* = 13.2, 1H), 0.80 (s, 3H), 0.77 (s, 3H). **$^{13}$C NMR** ($D_2O$, 100 MHz): δ 215.2, 174.4, 174.0, 173.8, 173.5, 98.9, 88.5, 84.8, 76.2, 75.8, 73.2, 73.0, 72.0, 71.8, 69.3, 69.0, 55.8, 53.7, 51.2, 50.0, 48.8, 40.0, 39.6, 36.0, 35.8, 35.0, 32.0, 21.1, 19.3. **HRMS** for $C_{29}H_{53}N_6O_{13}S$ $[M+H]^+$ calcd. 741.3386, found 741.3384. Purity, 98% (method A, $t_R$: 3.35 min; method B, $t_R$: 3.21 min).

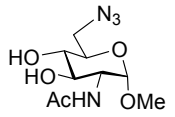



**6a**. *N*-Acetyl-D-glucosamine (3g, 13.5 mmol) was dissolved in methanol (70 mL) and Amberlite IR 120 (H) resin (5g) was added. The mixture was refluxed overnight, cooled to RT and filtered to remove the resin. The filtrate was evaporated to dryness to give **6a** as a white powder (2.83 g, 89%, highly hygroscopic). **$^1$H NMR** (400 MHz, DMSO-$d_6$): δ 7.71 (d, *J* = 4.0, 1H), 4.6 (br s, 3H), 4.50 (d, *J* = 3.0, 1H), 3.62 (m, 2H), 3.38-3.46 (m, 2H), 3.28 (m, 1H), 3.22 (s, 3H), 3.10 (m, 1H), 1.82 (s, 3H). **$^{13}$C NMR** (100 MHz, DMSO-$d_6$): δ 169.9, 98.6, 73.5, 71.6, 71.5, 61.3, 55.0, 54.5, 23.5. **ESI-MS** $[M+H]^+$ for $C_9H_{17}NO_6$, calcd. 236.1, found 236.3.

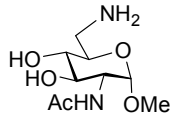



**6b**. Compound **6a** (1 g, 4.25 mmol) was dissolved in pyridine (15 mL), the mixture was cooled to -40°C and methanesulfonyl chloride (0.35 mL, 4.5 mmol) was added. The mixture was stirred at -40°C for 2 h and at RT for 10 h. After evaporation of the solvent, the residue was purified by flash chromatography ($R_f$: 0.40, $CHCl_3$/MeOH 99/1) to give **6b** as a white powder (0.97 g, 73%). **$^1$H NMR** (DMSO-$d_6$, 400 MHz): δ 7.77 (d, *J* = 5.6, 1H), 5.36 (d, *J* = 5.6, 1H), 4.88 (d, *J* = 6.0, 1H), 4.54 (d, *J* = 3.6, 1H), 4.38 (d, *J* = 9.8, 1H), 4.27 (dd, *J* =10.8, 5.6, 1H), 3.66 (td, *J* = 10.8, 3.6, 1H), 3.57 (dd, *J* = 10.8, 5.6, 1H), 3.34 (s, 3H), 3.43 (m, 1H), 3.23 (s, 3H), 3.15 (m, 1H),

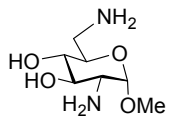
1.81 (s, 3H). **$^{13}$C NMR** (DMSO-$d_6$, 100 MHz): δ 170.0, 98.8, 79.9, 71.2, 71.0, 70.4, 55.4, 54.3, 37.5, 23.4. ESI-MS $[M+H]^+$ for $C_{10}H_{19}NO_8S$ calcd. 314.3, found 314.2.



**6c**. Compound **6b** (0.63 g, 2.11 mmol) and $NaN_3$ (0.68 g, 10.55 mmol) were mixed in acetone/$H_2O$ (30 mL, 3/2 v/v). The mixture was refluxed for 16 h. After evaporation of the solvent, the residue was purified by flash chromatography ($R_f$ : 0.66, $CHCl_3$/MeOH, 95/5) to give **6c** as a yellowish powder (0.43 g, 78%). **$^1$H NMR** (DMSO-$d_6$, 300 MHz): δ 7.76 (d, *J* = 5.6, 1H), 5.26 (d, *J* = 5.6, 1H), 4.85 (d, *J* = 5.6, 1H), 4.54 (d, *J* = 3.6, 1H), 3.66 (td, *J* = 11.2, 5.4, 1H), 3.38-3.49 (m, 4H), 3.26 (s, 3H), 3.12 (m, 1H), 1.82 (s, 3H). **$^{13}$C NMR** (DMSO-$d_6$, 100 MHz): δ 170.0, 98.8, 72.3, 71.9, 71.1, 55.3, 54.3, 52.0, 23.4. **ESI-MS** $[M+H]^+$ for $C_9H_{16}N_4O_5$: 261.1, found 261.2.

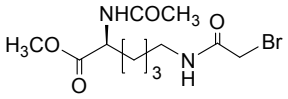


**6d**. Compound **6c** (0.33 g, 1.27 mmol) and $Pd(OH)_2$/C (10%, 25 mg) were mixed in methanol (20 mL) and hydrogenolysis was performed under 1 atm of $H_2$ overnight. The mixture was ran through a short pad of Celite and purified by flash chromatography ($R_f$: 0.28, $CHCl_3$/MeOH, 10/1 containing 2% TEA) to give **6d** as a yellowish paste (0.30 g, quant.). **$^1$H NMR** (DMSO-$d_6$, 400 MHz): δ 7.76 (d, *J* = 5.4, 1H), 5.38 (br s, 1H), 4.90 (br s, 1H), 4.52 (d, *J* = 3.6, 1H), 4.38 (d, *J* = 9.8, 1H), 4.27 (dd, *J* =10.2, 5.6, 1H), 3.40-3.60 (m, 3H), 3.00-3.40 (m, 6H), 1.80 (s, 3H). **$^{13}$C NMR** (DMSO-$d_6$, 75 MHz): δ 170.0, 99.0, 74.6, 71.2, 71.0, 55.4, 54.3, 38.1, 23.4. **ESI-MS** $[M+Na]^+$ for $C_9H_{18}N_2O_5$: 257.1, found 257.2.

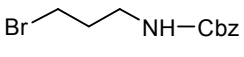


**7**. Compound **6d** (0.28 g, 1.20 mmol) was dissolved in MeOH/$H_2O$ (20 mL, 1/1 v/v). $Ba(OH)_2$ (1.71g, 10 mmol) was added and the mixture was refluxed for 12 h before concentration to ca. 10 mL. Following slow addition of 10% $H_2SO_4$ until pH ~ 3, the solution was left in the fridge overnight. The next day, the solution was filtered to remove the precipitate ($BaSO_4$). The filtrate was evaporated to dryness to afford **7** as a white foamy powder (0.20 g, 81%). **$^1$H NMR** ($D_2O$, 400 MHz): δ 4.62 (d, *J* = 3.4, 1H), 3.63 (m, 1H), 3.40 (t, *J* = 9.6, 1H), 3.23 (s, 3H), 3.17 (dd, *J*

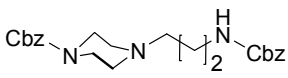
= 13.2, 3.6, 1H), 3.13 (t, *J* = 9.6, 1H), 2.92 (dd, *J* =13.2, 8.8, 1H), 2.72 (dd, *J* = 9.6, 3.2, 1H). **$^{13}$C NMR** ($D_2O$, 75 MHz): δ 99.3, 72.8, 71.7, 68.4, 55.7, 54.7, 40.8. **HRMS** $[M+H]^+$ for $C_7H_{16}N_2O_4$, calcd. 193.1110, found 193.1102.



**8b**. Compound **8a** (0.95 g, 4.72 mmol) and bromoacetic acid (0.69 g, 4.96 mmol) were dissolved in dry $CH_2Cl_2$ (40 mL). With stirring, DCC (1.02 g, 4.96 mmol) and DMAP (20 mg) were added and the mixture was stirred overnight at RT. The mixture was filtered to remove DCU, and the filtrate was evaporated to dryness before purification by flash chromatography ($R_f$: 0.30, $CHCl_3$/MeOH, 95/5 v/v) to yield **8b** as a white and very fine powder (0.96 g, 78%). **$^1$H NMR** ($CDCl_3$, 400 MHz): δ 6.30 (br s, NH), 6.09 (br s, NH), 4.55 (m, 1H), 3.86 (s, 2H), 3.74 (s, 3H), 3.28 (m, 2H), 2.03 (s, 3H), 1.88 (m, 1H), 1.75 (m, 1H), 1.56 (m, 2H), 1.36 (m, 2H); **$^{13}$C NMR** ($CDCl_3$, 100 MHz), δ 173.0, 170.3, 165.9, 52.9, 52.0, 39.9, 32.3, 29.6, 28.9, 23.6, 22.6; **HRMS** for $C_{11}H_{19}BrN_2O_4$ $[M+H]^+$ calcd. 322.0528 (100%) and 324.0508 (97%), found 322.0520 (100%) and 324.0502 (97%).

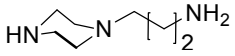


**10**. 3-Bromopropylamine hydrobromide (2.5 g, 11.4 mmol) was dissolved in 15% aqueous NaOH (40 mL). After cooling to 0$^o$C, benzyl chloroformate (3.26 mL, 22.8 mmol) was added with vigorous stirring. The mixture was stirred overnight at RT. The precipitate was collected, washed with warm water (200 mL) and purified by flash chromatography ($R_f$: 0.35, ethyl acetate/hexane, ¼, v/v) to afford **10** as a colorless oil (3.4 g, 98%). **$^1$H NMR** ($CDCl_3$, 300 MHz) δ 7.31(m, 5H), 5.42 (br s, 1H), 5.06 (s, 2H), 3.38 (t, *J* = 6.5, 2H), 3.28 (m, 2H), 2.01 (m, 2H). **$^{13}$C NMR** ($CDCl_3$,75 MHz) δ 156.8, 146.4, 128.7, 128.3, 127.6, 67.0, 39.8, 32.9, 31.2. ESI-MS $[M+Na]^+$ for $C_{11}H_{14}BrNO_2$, calcd. 294.0, found, 294.2 (100%), 296.2 (97%).

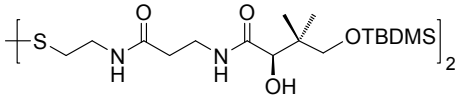


**11a**. Compound **9** (1.1 g, 5.0 mmol), **10** (1.36 g, 5.0 mmol) and $K_2CO_3$ (1.38 g, 10 mmol) were mixed in DMF (20 mL). The mixture was stirred at 90$^o$C for 5 h and then evaporated to

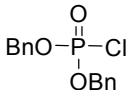
dryness. The residue was dissolved in water (100 mL) and extracted with ethyl acetate (3 × 50 mL) to yield **11a** as a colorless oil (1.45 g, 70%). **$^1$H NMR** ($CDCl_3$, 400 MHz) δ 7.34 (m, 10H), 5.62 (br s, 1H), 5.19 (s, 2H), 5.08 (s, 2H), 3.49 (t, *J* = 5.2, 4H), 3.26 (m, 2H), 2.39 (m, 6H), 1.68 (p, *J* = 5.2, 2H). **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 155.6, 155.3, 136.9, 136.8, 128.7, 128.6, 128.2, 128.0, 127.6, 127.1, 67.4, 66.8, 57.0, 53.2, 44.1, 40.7, 26.6. **ESI-MS** for $C_{23}H_{29}N_3O_4$ $[M+H]^+$ calcd. 412.2, found 412.5.



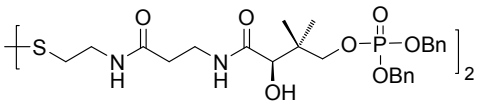
**11b**. Compound **11a** (1.40 g, 3.4 mmol) was dissolved in methanol (10 mL). $Pd(OH)_2/C$ (10% Pd, 0.15 g) was added and hydrogenolysis was performed under 1 atm of hydrogen at RT for 12 h. The mixture was filtered and evaporated to give a yellowish oil (0.52 g) which was dissolved in water (10 mL). The aqueous solution was filtered through cotton and lyophilized to yield **11b** as a white powder (0.46 g, 94%). **$^1$H NMR** ($D_2O$, 300 MHz) δ 2.56-2.64 (m, 6H), 2.20-2.30 (m, 6H), 1.54 (p, *J* = 5.4, 2H). **$^{13}$C NMR** ($D_2O$, 75 MHz) δ 55.6, 52.6, 44.2, 38.8, 25.8. **ESI-MS** for $C_7H_{17}N_3$, $[M+H]^+$calcd. 144.1, found 144.3.



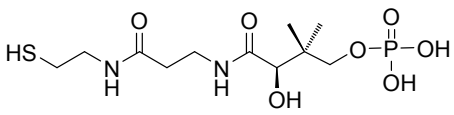
**12**. D-pantethine (0.22 g, 0.4 mmol) was dissolved in dry DMF (10 mL). Imidazole (75 mg, 1.1 mmol) and *tert*-butyldimethylchlorosilane (140 mg, 1.0 mmol) were added. The mixture was stirred overnight at RT. DMF was evaporated under vacuum. The resulting viscous liquid was extracted with $CHCl_3/H_2O$ to yield **13** as a transparent glass-like solid (0.30 g, 95%). **$^1$H NMR** ($CDCl_3$, 400 MHz) δ 7.28 (t, *J* = 6.8, 1H), 6.94 (t, *J* = 6.8, 1H), 4.72 (br s, 1H), 3.99 (s, 1H), 3.53 (m, 6H), 2.80 (t, *J* = 6.8, 2H), 2.48 (t, *J* = 6.8, 2H), 0.95 (s, 3H), 0.94 (s, 3H), 0.90 (s, 9H), 0.08 (s, 6H). **$^{13}$C NMR** ($CDCl_3$, 100 MHz) δ 172.9, 171.7, 79.1, 73.3, 40.0, 38.8, 38.7, 38.1, 36.9, 25.4, 22.1, 19.8, 18.5, -5.2. **HRMS**for $C_{34}H_{70}N_4O_8S_2Si_2$ $[M+H]^+$ calcd. 783.4174, found 783. 4166.



**13**. NCS (0.30 g, 2.2 mmol) was dissolved in dry toluene (10 mL). Benzylphosphite (0.47 mL, 2.0 mmol) was added and the mixture was stirred for 2 h at RT. After filtration to remove the precipitate, the filtrate was evaporated to yield **13** as a colorless oil (0.58 g, 98%). **$^{1}$H NMR** ($CDCl_3$, 400 MHz) δ 7.36 (br s, 10H), 5.19 (d, $J^3_{PH}$ = 10.2, 4H). **$^{13}$C NMR** ($CDCl_3$, 100 MHz) δ 141.1, 132.2, 130.8, 128.1, 70.1. **$^{31}$P NMR** ($CDCl_3$, 81 MHz) δ 6.04. **ESI-MS** for $C_{14}H_{15}ClO_3P$ $[M+H]^+$ calcd. 297.0, found 297.2.

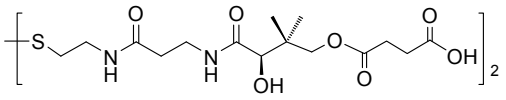


**14**. Compound **13** (0.23 g, 0.29 mmol) was dissolved in dry THF (10 mL). CsF (0.22 g, 1.45 mmol) was added and the mixture was refluxed for 1 h before cooling to RT. To this solution, freshly prepared dibenzylchlorophosphate (**13**, 0.19 g, 0.65 mmol) was added and the mixture was stirred overnight at RT. This mixture was evaporated to dryness under high vacuum to afford a fine powder which was triturated with $CHCl_3$/MeOH (5 × 20 mL, 10/1, v/v). The combined solutions were evaporated to dryness to yield **14** as a fine white powder (0.29 g, ~ 97%). This crude product was used directly in the Birch reduction to prepare **16**. **$^{1}$H NMR** ($CDCl_3$, 400 MHz) δ 7.36 (br s, 10H), 7.28 (t, $J$ = 6.8, 1H), 7.01 (t, $J$ = 6.8, 1H), 5.12 (d, $J^3_{PH}$ = 10.2, 4H), 4.75 (br s, 1H), 4.01 (s, 1H), 3.54 (m, 6H), 2.81 (t, $J$ = 6.8, 2H), 2.50 (t, $J$ = 6.8, 2H), 0.93 (s, 3H), 0.88 (s, 3H). **$^{13}$C NMR** ($CDCl_3$, 100 MHz) δ 173.2, 171.9, 141.4, 132.5, 131.1, 128.4, 79.3, 73.5, 70.3, 39.8, 38.4, 38.0, 37.3, 35.6, 22.0, 19.2. **$^{31}$P NMR** ($CDCl_3$, 81 MHz) δ 0.85. **ESI-MS** for $C_{50}H_{68}N_4O_{14}P_2S_2$ $[M+H]^+$ calcd. 1075.4, found 1075.3.

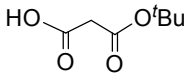


**15**. Sodium metal (0.12 g, 5 mmol, washed with hexane, THF, methanol and THF) and naphthalene (0.67 g, 5.2 mmol) were dissolved in dry THF (20 mL) at -40$^{o}$C. The color of the mixture changed from clear to blue after stirring for 10 min, and then to black. This mixture was stirred for a total of 1 h before slow transfer (by canula) to into a solution of **14** (0.25 g, 0.23 mmol) in THF (10 mL) until the solution turned pink (transfer was

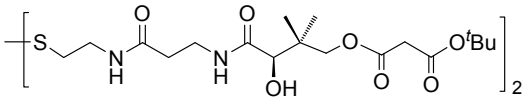
stopped after ~ 15 mL). This mixture was stirred for another hour and then quenched with water (2 mL). After evaporation to dryness the residue was triturated with water (100 mL). The aqueous solution was washed with DCM (2 × 20 mL) and ether (1 × 20 mL), and lyophilized to yield crude product **15** (0.31 g, ~ 91%). **$^{31}$P NMR** ($D_2O$, 81 MHz) δ 5.27; **ESI-MS** for $C_{11}H_{21}N_2O_7PSNa_2$ $[M+H]^+$calcd. 403.1, found 403.3.



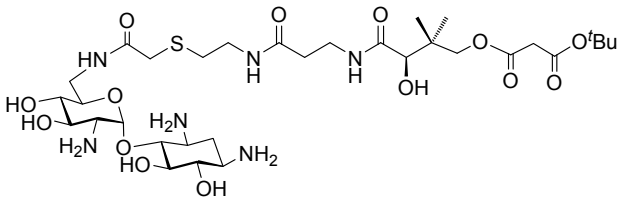
**16**. D-pantethine (0.275 g, 0.5 mmol), succinic anhydride (0.11 g, 1.1 mmol) and DMAP (25 mg) were dissolved in pyridine (10 mL). The solution was stirred at 60°C overnight. After evaporation of the pyridine, the residue was purified by flash chromatography ($R_f$: 0.22, $CHCl_3$/MeOH, 10/1 v/v) to give **16** as a white powder (0.18 g, 48%). **$^1$H NMR** ($D_2O$, 300 MHz) δ 3.86 (d, *J* = 11, 1H), 3.84 (s, 1H), 3.71 (d, *J* =11, 1H), 3.33 (m, 4H), 2.66 (t, *J* = 6.4, 2H), 2.47 (t, *J* = 6.4, 2H), 2.33 (m, 4H), 0.81 (s, 3H), 0.76 (s, 3H). **$^{13}$C NMR** ($D_2O$, 75 MHz) δ 175.9, 175.1, 174.8, 174.0, 74.8, 70.4, 38.2, 37.8, 36.7, 35.6, 35.5, 31.6, 30.6, 20.8, 19.5. **HRMS** for $C_{30}H_{50}N_4O_{14}S_2$, $[M+H]^+$ calcd. 755.2766, found 755.2759.



**17**. Malonic acid (11.88 g, 114 mmol) was dissolved in dry THF (70 mL). Pyridine (20 mL) and *tert*-butanol (20 mL, 209 mmol) were added with stirring. The mixture was cooled to 0°C and methanesulfonyl chloride (9 mL, 116 mmol) was added over 30 min. The mixture was stirred at RT for 3 h and then filtered to remove the pyridine hydrochloride salt. The filtrate was concentrated to ca. 20 mL and diluted in water (200 mL). This solution was brought to pH ~ 11 using 4 N NaOH and washed with DCM (2 × 25 mL). The aqueous layer was acidified to pH ~ 3 with conc. HCl and extracted with DCM (4 × 50 mL) before evaporation to give **17** as a colorless oil **(**13.5 g, 71%). **$^1$H NMR** ($CDCl_3$, 400 MHz) δ 11.12 (br s, 1H), 3.34 (s, 2H), 1.47 (s, 9H). **$^{13}$C NMR** ($CDCl_3$, 100 MHz) δ 172.2, 166.4, 82.8, 42.4, 28.3. **ESI-MS** for $C_7H_{12}O_4$ $[M+H]^+$calcd. 161.1, found 161.3.



**18**. D-pantethine (0.55 g, 1 mmol) and **17** (0.32 g, 2 mmol) were dissolved in THF/DCM (50 mL, 4/1 v/v). DCC (0.4 g, 2 mmol) and DMAP (25 mg) were added and the mixture was stirred for 36 h at 60°C. After filtration to remove DCU, the filtrate was evaporated to dryness and purified by flash chromatography ($R_f$: 0.35, $CHCl_3$/MeOH 10/1) to yield **18** as a white powder (0.53 g, 63%). **$^1$H NMR** ($CDCl_3$, 400 MHz) δ 7.48 (t, *J* = 5.6, 1H), 7.32 (t, *J* = 5.6, 1H), 4.58 (br s, 1H), 4.10 (d, *J* = 9.2, 1H), 3.88 (s, 2H), 3.56-3.41 (m, 4H), 3.30 (s, 2H), 2.76 (t, *J* = 6.8, 2H), 2.46 (t, *J* = 6.8, 2H), 1.44 (s, 9H), 1.00 (s, 3H), 0.93 (s, 3H). **$^{13}$C NMR** ($CDCl_3$, 100 MHz) δ 173.1, 172.2, 167.2, 166.3, 82.8, 75.2, 71.6, 43.2, 38.9, 38.8, 38.0, 36.1, 35.6, 28.4, 21.6, 20.6. **HRMS** for $C_{36}H_{62}N_4O_{14}S_2$ $[M+H]^+$ calcd. 839.3722, found 839. 3712.



**19**. Compound **18** (55 mg, 0.065 mmol), DTT (10 mg, 0.065 mmol) and DIPEA (1 mL, 5 mmol) were mixed in acetone (5 mL). The mixture was sonicated for 2 min and vigorously stirred overnight. ESI-MS revealed the disappearance of **18** and the formation of a product with peaks at 421.2 and 443.3 (m/e), corresponding to $[M+H]^+$ and $[M+Na]^+$ of reduced **18** (free thiol). This mixture was added to the *N*-6′-bromoacetylneamine solution (0.06 mmol, 4 ml). The resulting mixture was sonicated for 2 min, stirred for 1 h and evaporated to dryness to give crude **19** as a white powder (106 mg, quant.).

### 3.4.3 AAC(6′)-Ii inhibition assays.

AAC(6′)-Ii was expressed and purified as previously described elsewhere.[13] Enzyme activity was monitored using a procedure reported earlier.[38] Thus the assays were performed in HEPES buffer (25 mM, pH 7.5) containing EDTA (1 mM), 4,4′-dithiodipyridine (DTDP, 400 μM), neamine (100 μM) and various concentrations of AcCoA. Reaction volumes were typically 400 μL. The assay

mixtures were preincubated for 3 min at 37ºC, and the reaction was initiated by the addition of AAC(6′)-Ii (3.6 μM). The initial reaction velocities obtained at various concentrations of inhibitor were fit to Equation S1 for competitive inhibition, Equation S2 for noncompetitive inhibition, or Equation S3 for uncompetitive inhibition, where [*S*] is the concentration of AcCoA, $K_m$ is the Michaelis-Menten constant, $V_{max}$ is the maximal velocity, [*I*] is the concentration of inhibitor and $K_i$ is the inhibition constant.

$$v = \frac{V_{max}[S]}{[S] + K_m(1 + \frac{[I]}{K_i})} \quad \textbf{(S1)}$$

$$v = \frac{V_{max}[S]}{([S] + K_m)(1 + \frac{[I]}{K_i})} \quad \textbf{(S2)}$$

$$v = \frac{V_{max}[S]}{[S](1 + \frac{[I]}{K_i}) + K_m} \quad \textbf{(S3)}$$

For all molecules tested Equation S1 provided a much better fit than S2 or S3, which suggests competitive inhibition. However, the low $K_i$s obtained imply that these molecules are tight binding inhibitors ($K_i$ of less than 1000 times the concentration of AAC(6′)-Ii).[39] Thus Equations S4, S5 and S6 were applied for tight binding competitive inhibition, tight binding noncompetitive inhibition, or tight binding uncompetitive inhibition respectively.[39]

$$K_i^{app} = K_i(1 + \frac{[S]}{K_m}) \quad \textbf{(S4)}$$

$$K_i^{app} = K_i \quad \textbf{(S5)}$$

$$K_i^{app} = K_i(1 + \frac{K_m}{[S]}) \quad \textbf{(S6)}$$

The $K_i^{app}$ was determined from Morrison equation S7 where [*E*] is AAC(6′)-Ii concentration;[39] $v_i$ is the initial velocity in the presence of inhibitor at concentration [*I*] and $v_0$ is the initial velocity in the absence of inhibitor, respectively and.

$$\frac{v_i}{v_0} = 1 - \frac{([E]+[I]+K_i^{app}) - \sqrt{([E]+[I]+K_i^{app})^2 - 4[E][I]}}{2[E]} \quad \textbf{(S7)}$$

The experimental data fit Equation S4 much better than S5 or S6, confirming that the aminoglycoside derivatives are tight binding competitive inhibitors. Calculated kinetic constants are listed in Table 3.1.

### 3.4.4 Evaluation of biological activity of compound 5e.

A two-dimensional checkerboard MIC assay was conducted with **5e** and kanamycin A (Bioshop Canada) versus *Enterococcus faecium* C238 in Brain Heart Infusion media (Difco) using NCCLS protocols in duplicate. Compound **5e** was diluted using a two-fold dilution to create gradient from 8 μg/mL to 512 μg/mL and the range of concentrations for kanamycin A was 100 μg/mL to 600 μg/mL. The cultures were grown at 37°C for 16 h in 96-well microtiter U-bottom MIC plates and growth was monitored in a SPECTRAmax Plus plate reader at $OD_{625}$. The data from each set were averaged and normalized against the mean of the sterility controls and reported as percentage of growth with respect to the mean of the positive growth controls.

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# Chapter 3 – Appendix

## Attempted Synthesis of Truncated Bisubstrate Analogs without Negative Charges

### 3A.1 Introduction

The truncated bisubstrate analog **1** (Fig. 3A.1) showed no significant inhibition of the enzyme AAC(6′)-Ii.[1] This suggests that the pantetheine moiety of CoA is not sufficient for CoA recognition by AAC(6′)-Ii. On the other hand, the addition of a 1,3-diketone group to mimic the pyrophosphate was able to recover binding affinity of the bisubstrate analog **2** at the low micromolar level.[1] Although bisubstrates containing the full CoA moiety are extremely potent inhibitors of AAC(6′)-Ii, they are not active in cells due to the negative charges preventing them crossing cell membranes. Thus, it was reasoned that bisubstrate analogs having all the functional groups of CoA but without the negative phosphates would be more potent inhibitors of AAC(6′)-Ii, and have a chance to be active against resistant strains.

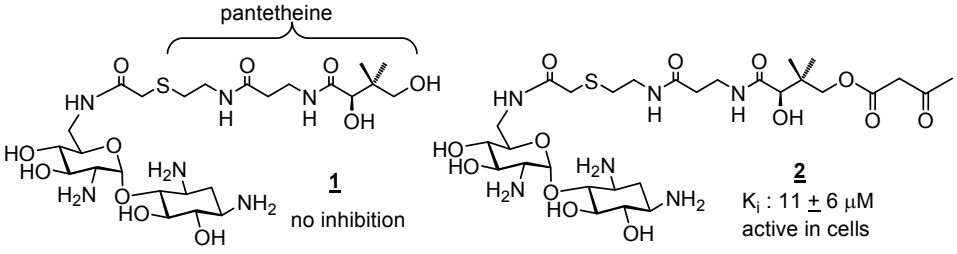



***Figure 3A.1***. Truncated bisubstrate analogs.

Crystal structures of AAC(6′)-Ii in complex with both AcCoA and CoA reveal a few hydrogen bonds between the adenine of CoA and the protein (Fig. 3A.2 and 3A.3). One between residue Glu141 and the 6-amino group of adenine is observed. A second is formed between the 6-amino group of adenine and the

carbonyl of the β-alanyl (pantetheine of CoA) via a water molecule (Fig. 3A.2 and 3A.3). Lastly, N7 of adenine is close enough to potentially form another hydrogen bond with the hydroxyl group of the pantetheinyl moiety of CoA. Moreover, it was noted that the ribose phosphate group of CoA forms very few interactions with the enzyme. Overall, the CoA molecule is folded into a cyclized conformation.

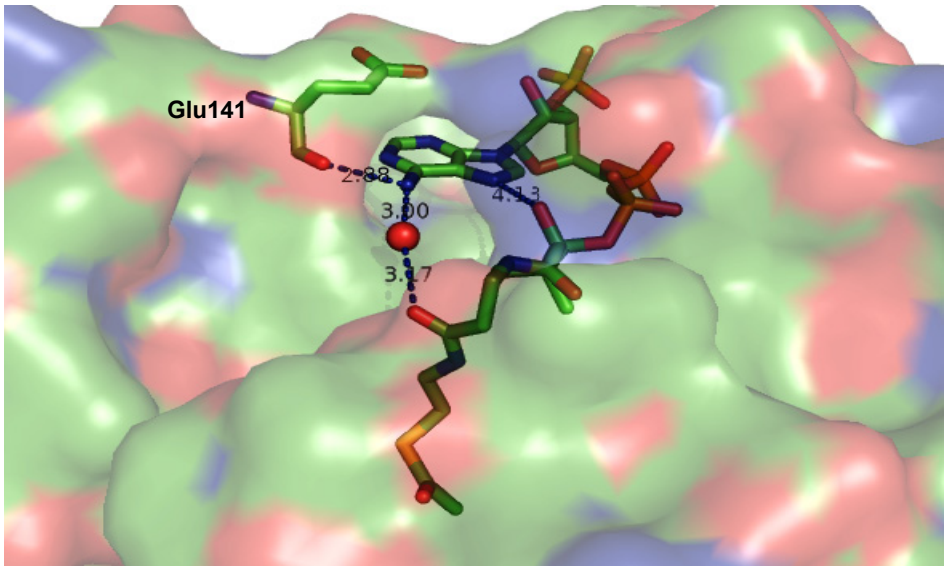



***Figure 3A.2*** The conformation of AcCoA bound to AAC(6′)-Ii (1B87.pdb).[2] The protein is represented by surface contour, AcCoA is represented with sticks (C: green, O: red, N: blue, S: yellow and P: orange), key oxygen atoms are represented with spheres (red), *N*7 and *N*6 of adenine are highlighted as spheres (blue). The figure was produced using PyMol. The chemical structure of AcCoA is shown in Fig. 3A.3.

Based on this structural information, we designed bisubstrates containing 5 different linkers to replace the pyrophosphate and the ribose phosphate. The optimal linker would favor the overall conformation of the enzyme-bound CoA. A few factors had to be controlled to achieve this goal. First, by using malonate to mimic pyrophosphate, the interactions between the phosphate oxygens and protein backbone amides would be preserved. Second, the use of ring structures was envisaged to control the geometry of the molecule and to decrease the entropy of binding to the enzyme. Lastly, the potential to form an intramolecular

H-bond within the linker could help align the adenine as observed in the crystal structure.

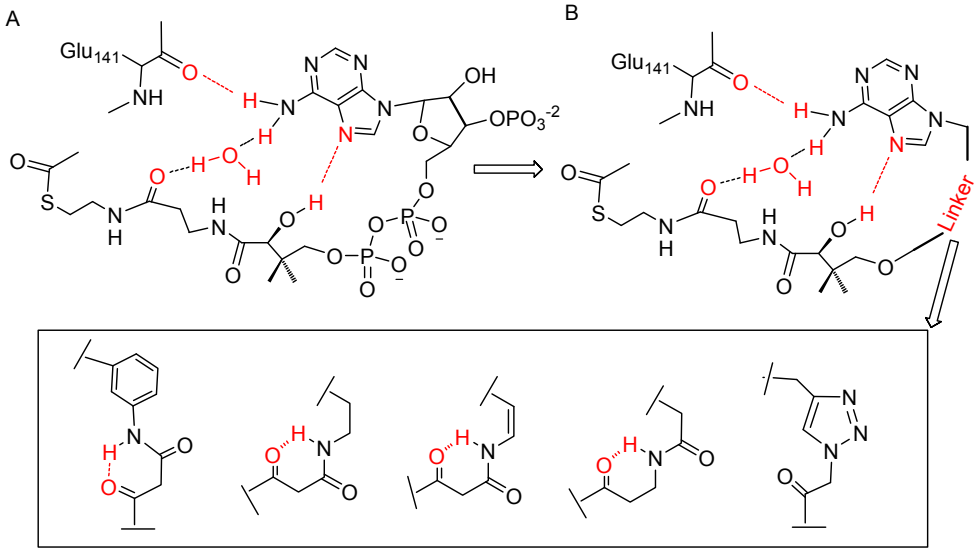



***Figure 3A.3***. Strategy for the design of the CoA mimetics containing no negative charges. A, structure of AcCoA bound to AAC(6′)-Ii, highlighting key H-bonds; B, structures of the proposed linkers to replace ribose phosphate.

The target molecules **3a-e** described here are shown in Fig. 3A.4. The retrosynthetic analysis converges to two key steps: the first one involving an ester bond formation between 4′-OH of pantetheine and the carboxylic acids **4a-e**; and the second a regioselective conjugation of the thiol with *N*-6′-bromoacetyl neamine, as previously developed by us.[3] A rough retrosynthetic analysis for preparation of the carboxylic acids needed is also shown in Fig. 3A.4.

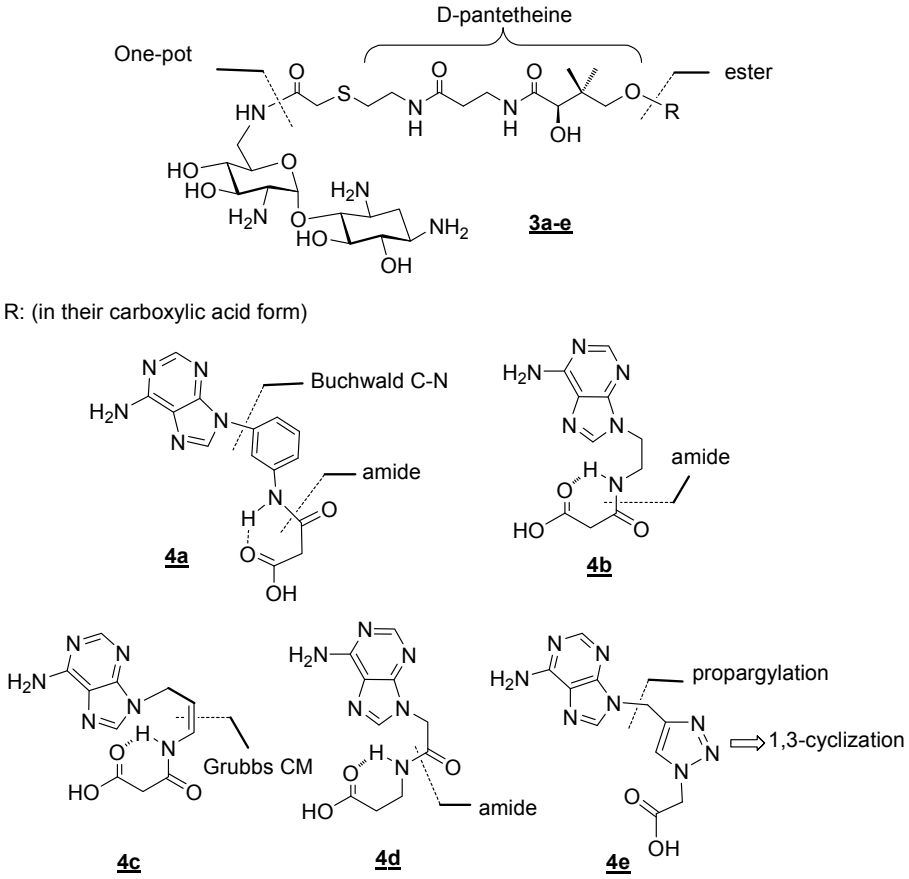
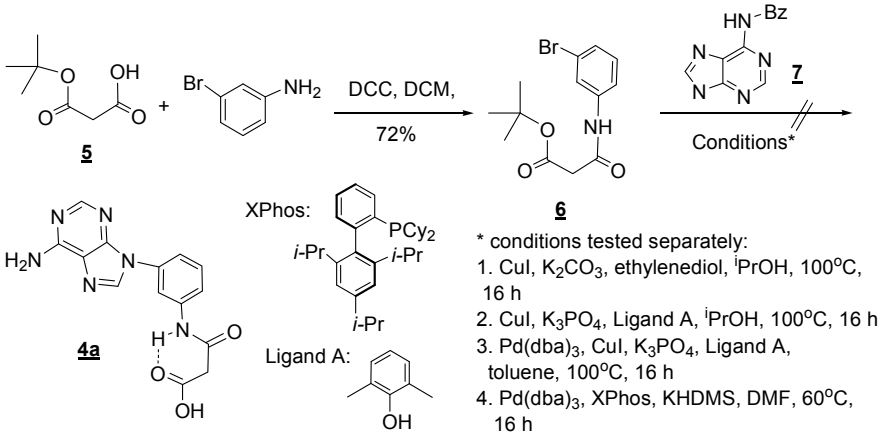



***Figure 3A.4***. Target molecules designed to mimic the neamine-CoA bisubstrates without the negative charges.

### 3A.2 Synthesis and biological tests

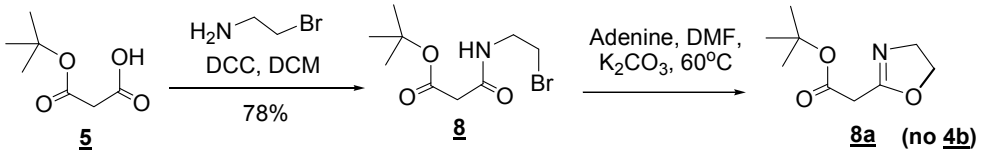
The synthesis of the first target molecule **3a** was initiated with the preparation of **6** (Scheme 3A.1), which was synthesized via a standard amide coupling reaction. We hoped that the Buchwald C-N bond formation method[4] could be used to install the adenyl group. Even though this method is quite robust and versatile (a variety of aryl halides have previously been coupled with both aliphatic and aromatic amines),[5-8] examples with adenine, with or without protection of the 6-$NH_2$, were not reported. Various conditions were applied without success (Scheme 3A. 1). It is likely that the acidic methylene protons of diketone (compound 6) are easily deprotonated to generate carbanion, which would poison the catalyst. Using 2-(tert-butoxycarbonyl)-2-methylpropanoic acid

instead **5** as building block for synthesis of **4a** may be a solution to this problem. As well, the acidic protons of amides **6** and **7** could potentially pose same problem, which essentially led us discard this target.



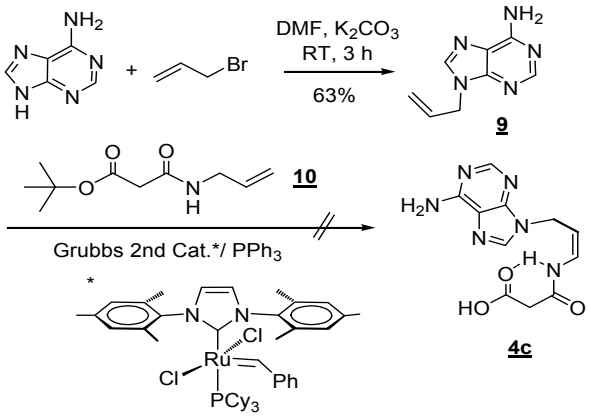

***Scheme 3A.1***. Attempted synthesis of **3a**.

We attempted next to prepare target molecule **3b** via conventional *N*-alkylation of adenine to form the C-N bond (Scheme 3A.2). *tert*-Butyl monomalonate was coupled with 2-bromoethylamine to afford **8** in good yield. However, **8** did not react with adenine at room temperature yet led to a intramolecular *O*-alkylation product **8a** (yield 65%) when the temperature was raised (60°C).



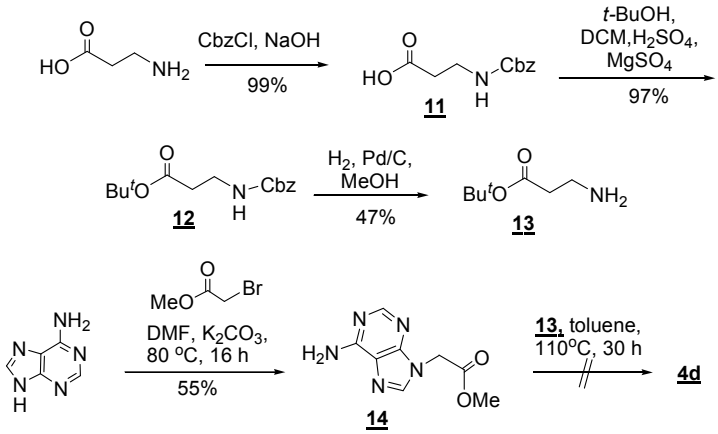

***Scheme 3A.2***. Attempted synthesis of **4b**.

Milder conditions were next attempted to avoid deprotonation of malonate. Addition of an allyl group at N-7 of adenine and one on the malonate group, followed by olefin cross-metathesis[9] was attempted to prepare carboxylic acid **4c** (Fig. 3A.4). The second generation Grubbs catalyst was reported to have excellent tolerance towards a variety of functionalities.[10] We thus hoped that olefins **9** and **10** (Scheme 3A.3) would react to yield **4c** in spite of the presence of a free amino group. Unfortunately, this reaction did not produce any of the desired product (with or without phosphine ligands).




***Scheme 3A.3***. Attempted synthesis of **4c**.

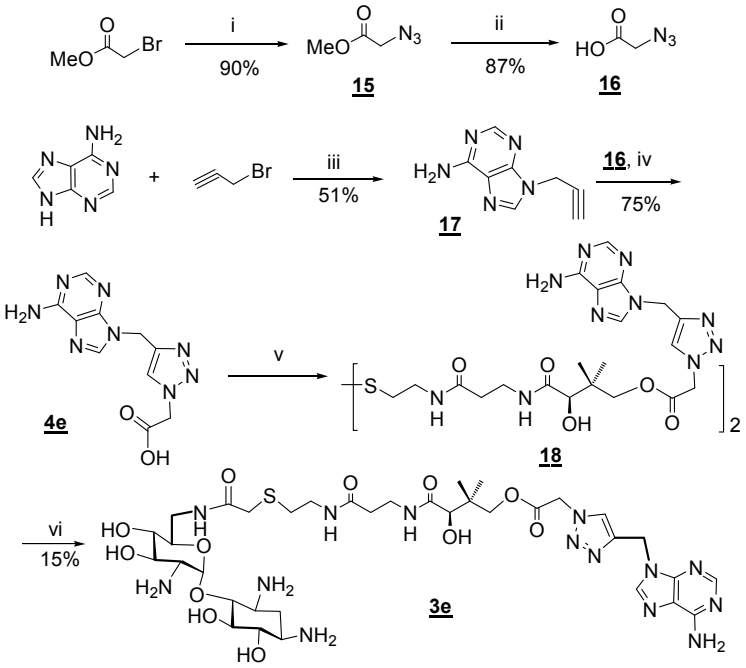
Next we envisaged to replace the malonate with β-alanine (target **3d**, Fig. 3A.4). Spontaneous intramolecular H-bonding was expected to rigidify the conformation of **3d**. Thus β-alanine was treated with benzyloxycarbonyl chloride (CbzCl) in a basic aqueous solution to afford *N*-Cbz-β-alanine (**11**) in excellent yield (Scheme 3A.4). Treating **11** with *tert*-butanol under acidic conditions yielded fully protected β-alanine (**12**), which was hydrogenated to afford **13**. Attempts to hydrolyze compound **14** to a carboxylic acid (to be coupled with **13**) yielded a solid material that did not dissolve in any of the common solvents. A direct ester-amide exchange reaction between **14** and **13** did not generate the desired product either (Scheme 3A.4).




***Scheme 3A.4***. Attempted synthesis of **4d**.

The last target molecule (**3e)** was assembled via an azide-alkyne cycloaddition reaction[11] (Scheme 3A.5). 1,3-Dipolar cycloadditions have proven

effective to connect molecules bearing a wide variety of functional groups.[12, 13] The triazole ring also serves as a rigid structure to decrease the conformational freedom. Thus, 2-azidoacetic acid (**16**) was reacted with alkyne **17** (Scheme 3A.5) in the presence of a catalytic amount of CuI in aqueous solvent to afford the corresponding 1,4-adduct. This carboxylic acid was coupled with D-pantethine using HATU to afford disulfide **18**, which was reduced to the free thiol using dithiothreitol (DTT). The thiol was next reacted with *N*-6′-(2-bromoacetyl)neamine (prepared in situ) to finally yield the target molecule **3e** in low yield.




***Scheme 3A.5***. Synthesis of **3e**. Reagents and conditions: i) $NaN_3$, acetone, RT, 16 h; ii) LiOH, THF/$H_2O$, RT, 3 h; iii) DMF, $K_2CO_3$, RT, 4 h; iv) CuI, MeOH/$H_2O$, RT, 16 h; v) D-pantethine, HATU, DIPEA, DMF, RT, 16 h; vi) DTT, MeOH, then *N*-6′-bromoacetylneamine, DIPEA, acetone/$H_2O$, RT, 1 h.

Disappointedly, the bisubstrate analog **3e** showed almost no inhibition towards AAC(6′)-Ii. This result reflects the sensitiveness of the CoA binding site of AAC(6′)-Ii.

### 3A.3 Experimental section

**General.** Refer to same experimental section of chapter 3.

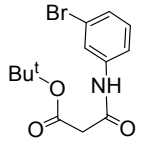
**General procedure for amide coupling.**

Compound **6**, **8**, **12** were prepared using this procedure. Thus, *tert*-butyl monomalonic ester (**5**, 1.40 g, 10 mmol) and the corresponding amines (10 mmol) were dissolved in DCM (20 mL). DCC (2.06 g, 10 mmol) and a catalytic amount of DMAP were added into the reaction mixture with stirring. The reaction mixture was stirred at RT for 6 h. Filtration removes DCU, and the filtrate was purified on silica gel.

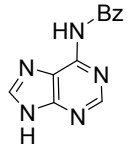
**General procedure for *N*-9-alkylation of adenine.**

Compound **9**, **14**, **17** were prepared using the following procedure. Adenine (1.35 g, 10 mmol) and $K_2CO_3$ (2.5 g, 18 mmol) were dissolved in DMF (20 mL), and the corresponding bromide (10 mmol) was added dropwise with vigorously stirring. The reaction mixture was stirred for 3 h at RT. Filtration removes the salt, and the filtrate was purified on silica gel.

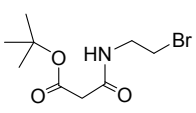
**5.** It was prepared as described in Chapter 3.




**6**. $R_f$: 0.60 (EtOAc/Hex, 1/1). Yield: 72%. **$^1$H NMR** ($CDCl_3$, 400 MHz) δ 9.46 (bs, 1H), 7.79 (s, 1H), 7.45 (d, *J* = 6.6, 1H), 7.18 (m, 2H), 3.35 (s, 2H), 1.45 (s, 9H). **$^{13}$C NMR** ($CDCl_3$, 100 MHz) δ 169.2, 163.8, 139.1, 130.4, 127.5, 123.1, 122.7, 118.7, 83.8, 42.7, 28.4. **ESI-MS** for $C_{13}H_{16}{}^{79}BrNO_3$ $[M+H]^+$ calcd. 314.1, found 314.2.

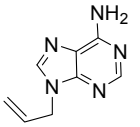



**7**. It was prepared according to a previously reported procedure.[14] Yield: 68%. **$^1$H NMR** (DMSO-$d_6$, 400 MHz) δ 12.36 (bs, 1H), 11.52 (bs, 1H), 8.71 (s, 1H, *H*2), 8.48 (s, 1H, *H*8), 8.10 (d, *J* = 6.8, 2H), 7.65 (t, *J* = 7.2, 2H), 7.56 (t, *J* = 6.8, 1H); 3.83 (s, 2H), 3.73 (s, 3H). **$^{13}$C NMR** (DMSO-$d_6$, 100 MHz) δ 167.2, 161.7, 151.7, 146.4, 145.4, 133.5, 133.3, 129.2, 129.1, 116.4. **ESI-MS** for $C_{12}H_9N_5O$ $[M+H]^+$ calcd. 240.1, found 240.2.

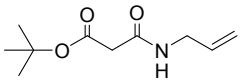



**8**. $R_f$: 0.68 (EtOAc/Hex, 1/1). Yield: 58%. **$^1$H NMR** ($CDCl_3$, 400 MHz) δ 7.59 (bs, 1H), 3.70 (q, *J* = 6.5, 2H); 3.47 (t, *J* = 6.5,

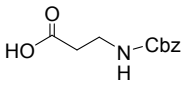
2H), 3.33 (s, 2H), 1.49 (s, 9H). **$^{13}$C NMR** ($CDCl_3$, 100 MHz) δ 168.8, 166.0, 82.9, 42.3, 41.4, 32.0, 28.2. **ESI-MS** for $C_9H_{16}{}^{79}BrNO_3$ $[M+H]^+$ calcd. 266.0, found 266.1.



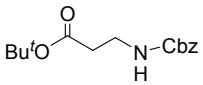
**9**. $R_f$: 0.55 ($CHCl_3$/MeOH, 10/1). Yield: 63%. **$^1$H NMR** ($CDCl_3$, 400 MHz) δ 8.33 (s, 1H), 7.77 (s, 1H), 6.55 (bs, 2H), 6.00 (m, 1H), 5.27 (d, *J* = 10.4, 1H); 5.16 (d, *J* = 17.2, 1H), 4.78 (d, *J* = 6.0, 2H). **$^{13}$C NMR** ($CDCl_3$, 100 MHz) δ 156.0, 153.2, 150.0, 140.3, 132.0, 119.7, 119.0, 46.1. **ESI-MS** for $C_8H_9N_5$ $[M+H]^+$ calcd. 176.1, found 176.1.



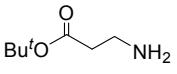
**10**. $R_f$: 0.47 (EtOAc/Hex, 1/2). Yield: 58%. **$^1$H NMR** (MeOH-$d_4$, 400 MHz) δ 5.89 (m, 1H), 5.36 (d, *J* = 17.2, 1H), 5.25 (d, *J* = 10.2, 1H), 4.07 (m, 2H), 3.40 (s, 2H), 1.46 (s, 9H). **$^{13}$C NMR** (MeOH-$d_4$, 100 MHz) δ 172.0, 165.2, 131.4, 119.0, 82.7, 42.2, 41.6, 28.3. **ESI-MS** for $C_{10}H_{17}NO_3$ $[M+H]^+$ calcd. 200.1, found 200.1



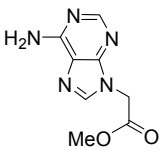
**11**. β-Alanine (8.9 g, 0.1 mol) was dissolved in $H_2O$ (100 mL) and NaOH (12 g, 0.3 mol) was added. The resulting solution was cooled to 0°C. CbzCl (16 mL, 0.11 mmol) was added dropwise and the reaction mixture was stirred for 2 h. The reaction mixture was washed with ether (3 × 100 mL), and the separated aqueous solution was neutralized with 1 *N* HCl. The resulting precipitate was collected and washed by water to yield the desired compound **11** (22 g, 99%). **$^1$H NMR** ($CDCl_3$, 400 MHz) δ 7.34 (m, 5H), 5.34 (bs, 1H), 5.09 (s, 2H), 3.47 (t, *J* = 5.6, 2H), 2.61 (t, *J* = 5.6, 2H). **$^{13}$C NMR** ($CDCl_3$, 100 MHz) δ 177.7, 156.4, 136.4, 128.7, 128.3, 128.1, 67.2, 36.7, 34.6. **ESI-MS** for $C_{11}H_{13}NO_4$ $[M+Na]^+$ calcd. 246.2, found 246.2.



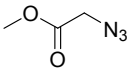
**12**. It was prepared using a previously reported procedure.[15] Yield: 97%. **$^1$H NMR** ($CDCl_3$, 400 MHz) δ 7.34 (m, 5H), 5.24 (bs, 1H), 5.09 (s, 2H), 3.43 (t, *J* = 5.6, 2H), 2.46 (t, *J* = 5.6, 2H), 1.44 (s, 9H). **$^{13}$C NMR** ($CDCl_3$, 100 MHz) δ 177.7, 177.1, 136.4, 128.7, 128.4, 128.0, 83.9, 81.5, 66.9, 36.7, 34.7, 28.5. **ESI-MS** for $C_{15}H_{21}NO_4$ $[M+H]^+$calcd. 279.2, found 279.2.



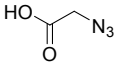
**13**. Hydrogenation (Pd/C, $H_2$, MeOH) afforded this compound. Yield: 47%. **$^1$H NMR** ($CDCl_3$, 400 MHz) δ 3.07 (bs, 2H), 2.90 (t, *J* = 5.6, 2H), 2.37 (t, *J* = 5.6, 2H), 1.40 (s, 9H). **$^{13}$C NMR** ($CDCl_3$, 100 MHz) δ 171.8, 81.8, 38.3, 37.8, 28.5. **ESI-MS** $[M+Na]^+$ for $C_7H_{15}NO_2$ calcd. 146.1, found 146.2.



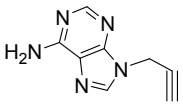
**14**. $R_f$: 0.32 ($CHCl_3$/MeOH, 8/1). Yield: 55%. **$^1$H NMR** (DMSO-$d_6$, 400 MHz) δ 8.10 (s, 1H), 8.09 (s, 1H), 7.28 (s, 2H), 5.07 (s, 2H); 3.69 (s, 3H). **$^{13}$C NMR** (DMSO-$d_6$, 100 MHz) δ 169.0, 156.5, 153.2, 150.3, 141.8, 118.9, 53.3, 44.6. **ESI-MS** $[M+H]^+$ for $C_8H_9N_5O_2$ calcd. 208.1, found 208.1.



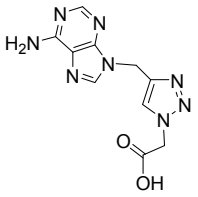
**15**. Methylbromoacetate (12 g, 78.4 mmol) and $NaN_3$ (16 g, 246 mmol) were disolved in acetone (100 mL). The mixture was stirred overnight at RT and filtered to remove the insoluble salt. The filtrate was evaporated to afford the desired product (8.1 g, 90%). **$^1$H NMR** ($CDCl_3$, 400 MHz) δ 3.83 (s, 2H), 3.73 (s, 3H). **$^{13}$C NMR** ($CDCl_3$, 100 MHz) δ 168.9, 52.8, 50.4. **ESI-MS** $[M+H]^+$ for $C_3H_5N_3O_2$ calcd. 116.2, found 116.2.



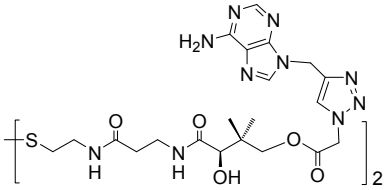
**16**. Methyl azidoacetate **(**4.5 g, 39 mmol) LiOH monohydrate (3 g) were dissolved in mixture of THF/$H_2O$ (30 mL, 3/2, v/v). The mixture was refluxed for 2 h and then evaporated to dryness. The solid was dissolved in water (50 ml) and washed with EtOAc (2 × 25 ml). The aqueous solution was acidified (1 *N* HCl) to pH ~ 2, followed by extraction with EtOAc (4 × 30 ml) to give the titled product as colorless oil (3.6 g). Yield: 89%. **$^1$H NMR** ($CDCl_3$, 300 MHz) δ 11.37 (bs, 1H), 3.93 (s, 2H). **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 173.9, 50.3. **ESI-MS** $[M+H]^+$ for $C_2H_3N_3O_2$ calcd. 101.2, found 101.2.



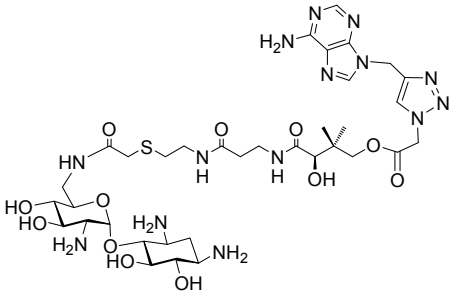
**17**. $R_f$: 0.30 ($CHCl_3$/MeOH, 10/1). Yield: 54%. **$^1$H NMR** ( DMSO-$d_6$, 400 MHz) δ 8.17 (s, 1H), 8.15 (s, 1H), 7.28 (bs, 2H), 5.10 (d, *J* = 2.8, 2H), 3.47 (t, *J* = 2.8, 1H). **$^{13}$C NMR** (DMSO-$d_6$, 100 MHz) δ 156.6, 153.3, 149.7, 140.7, 119,1, 79.1, 76.6, 33.1. **ESI-MS** for $C_8H_7N_5$ $[M+H]^+$ calcd. 174.2, found 174.2.



**4e**. *N*-9-propargyladenine (**17**, 0.17 g, 1 mmol), azidoacetic acid (**16**, 0.11 g, 1.1 mmol) and CuI (50 mg) were mixed in MeOH (20 mL). The mixture was stirred overnight and filtered to collect the grey precipitate. This precipitate was slowly dissolved in 0.1 M NaOH (pH ~ 11). The solution was filtered to remove $Cu(OH)_2$. The filtrate was acidified with to pH ~ 3 (1 *N* HCl) and the resulting solution was placed in a fridge overnight. After filtration, the desired product was collected as a grey crystalline powder (0.22 g, 81%). **$^1$H NMR** (DMSO-$d_6$, 400 MHz) δ 9.93 (bs, 1H), 8.40 (s, 1H), 8.24 (s, 1H), 8.08 (s, 1H), 7.31 (bs, 2H), 5.45 (bs, 2H), 5.22 (bs, 2H). **$^{13}$C NMR** (DMSO-$d_6$, 100 MHz) δ 169.2, 156.5, 155.3, 153.0, 143.7, 141.8, 126.0, 121.2, 50.4, 41.2. **HR-MS** for $C_{10}H_{10}N_8O_2$ $[M+H]^+$ calcd. 275.0935, found 275.0912.



**18**. Compound **4e** (29 mg 0.1 mmol), D-pantethine (27.5 mg, 0.05 mmol), HATU (40 mg, 0.1 mmol) and DIPEA (0.1 ml, 0.5 mmol) were mixed in dry DMF (10 mL). The mixture was stirred overnight at RT, and then evaporated to dryness. The residue was dissolved in $H_2O$ (30 ml) and washed with ether (3 × 10 mL). The aqueous solution was acidified to pH ~ 3 (TFA) and washed with ethyl acetate (3 × 10 mL). The aqueous solution was lyophilized to give crude **14 (**51 mg) as a yellowish powder which was not purified further.



**3e.** Compound **18** (40 mg, ca. 0.03 mmol), DTT (5 mg, 0.03 mmol) and DIPEA (1 mL, 5 mmol) were mixed in acetone/$H_2O$ (3 mL, 1/1, v/v). The mixture was sonicated for 2 min and stirred overnight. This solution was transferred to a *N*-6′(2-bromoacetyl)neamne solution and the resulting solution was sonicated for 2 min and stirred 1 h. RP-HPLC was used for purification and yielded 7.3 mg of **3e** (14%) **$^1$H NMR** ($D_2O$, 400 MHz): δ 8.36 (s, 1H), 8.29 (s, 1H), 8.12(s, 1H), 5.56 (d, $J$ = 3.2 Hz, 1H), 5.12

(s, 2H), 4.60 (s, 2H), 3.92 (d, *J* = 10.5, 1H), 3.85 (s, 1H), 3.81 (d, *J* = 10.5, 1H), 3.74 (m, 3H), 3.50-3.17 (m, 14H), 2.57 (t, *J* = 6.4, 2H), 2.32 (m, 3H), 1.71 (q, *J* = 13.2, 1H), 0.81 (s, 3H), 0.78 (s, 3H). **$^{13}$C NMR** ($D_2O$, 100 MHz): δ 173.2, 172.3, 171.7, 167.4, 154.0, 150.3, 143,4, 139.2, 123.7, 119.1, 113.3, 97.1, 79.4, 75.5, 75.4, 72.7, 71.9, 70.8, 70.6, 70.4, 69.1, 54.2, 48,8, 47.4, 41.6, 39.8, 39.4, 38.5, 38.0, 31.5, 30.5, 29.1, 28.4, 20.8, 19.7. **HRMS** for $C_{35}H_{56}N_{14}O_{12}S$ $[M+H]^+$ calcd. 897.3941, found 897.3932.

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# Chapter Four

In the previous chapters we have demonstrated that aminoglycoside-CoA bisubstrate analogs with amide linkers show nanomolar inhibition towards aminoglycoside *N*-6′-acetyltransferase Ii (AAC(6′)-Ii), an enzyme causing antibiotic resistance. Others have suggested that this enzyme catalyzes *N*-6′-acetylation via a proximity effect, i.e., by bringing the reacting groups in proximity. It is also possible that AAC(6′)-Ii stabilizes the tetrahedral intermediate. In this regard, we envisaged to synthesize sulfonamide-, sulfoxide- and sulfone-containing bisubstrates that better mimic the tetrahedral intermediate. Synthesis and biological activity of these new bisubstrates are reported in this chapter.

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Contributions of coauthors

This chapter is reproduced from a manuscript that will be submitted for publication shortly. I carried out the synthesis of all compounds reported and wrote the article under the supervision of Dr. Karine Auclair. Xuxu Yan, a graduate student in our group performed the enzyme assays and Lee Freiburger, another graduate student in our group, purified the enzyme, AAC(6′)-Ii.

# Use of Sulfonamide-, Sulfoxide-, or Sulfone-containing Aminoglycoside-CoA Bisubstrate as Mechanistic Probes for Aminoglycoside *N*-6′-Acetyltransferase

**Abstract**

Aminoglycoside *N*-6′-acetyltransferase Ii (AAC(6′)-Ii) has been proposed to catalyze acetylations via a promixity effect, without stabilization of the tetrahedral intermediate. We previously reported that amide-linked aminoglycoside-CoA bisubstrates are nanomolar inhibitors of this enzyme. It was envisaged that bisubstrates containing linkers that better mimic the tetrahedral intermediate would help investigate the catalytic role of this enzyme. We report here the synthesis and biological activity of five new aminoglycoside-CoA bisubstrates containing sulfonamide, sulfoxide, or sulfone groups. Interestingly, the sulfonamide-linked bisubstrate, which was expected to best mimic the tetrahedral intermediate, did not show improved inhibition when compared with amide-linked bisubstrates. On the other hand, sulfide oxidation of the bisubstrate containing an amide linker dramatically improved inhibition of AAC(6′)-Ii.

## 4.1 Introduction

Aminoglycosides are broad spectrum antimicrobials.[1] Unfortunately, widespread resistance to aminoglycosides threatens the use of this important class of antibiotics, alone or in synergistic combination with β-lactams. Resistance to aminoglycosides occurs mostly via drug modifications by enzymes such as aminoglycoside *N*-6′-acetyltransferases (AAC(6′)s).[2-7] Wright and coworkers have shown that catalysis by AAC(6′)-Ii proceeds via an ordered bi-bi mechanism in which acetyl coenzyme A (AcCoA) binds before the aminoglycoside.[9] It was hypothesized that a general base residue might be involved in the deprotonation of

the 6′-$NH_3^+$. Attack of the aminoglycoside 6′-$NH_2$ at the thioester of AcCoA is believed to generate a tetrahedral intermediate, which subsequently collapses to yield a 6′-*N*-acetylaminoglycoside and CoA (Fig. 4.1).[8-10] Extensive mutagenic studies[9,10] and examination of the crystal structures of AAC(6′)-Ii[17,18]have not allowed the identification of a residue that may stabilize the tetrahedral intermediate.

We recently reported the use of amide-linked aminoglycoside-CoA bisubstrate inhibitors as mechanistic and structural probes of this enzyme. An effective regio- and chemo-selective protocol for the direct *N*-6′-derivatization of unprotected aminoglycosides was used to synthesize these inhibitors in one-pot (Fig. 4.1, **1a-b**).[11, 12] These molecules exhibited nanomolar inhibition towards AAC(6′)-Ii and allowed crystallization of AAC(6′)-Ii in complex with an aminoglycoside derivative for the first time.[11]

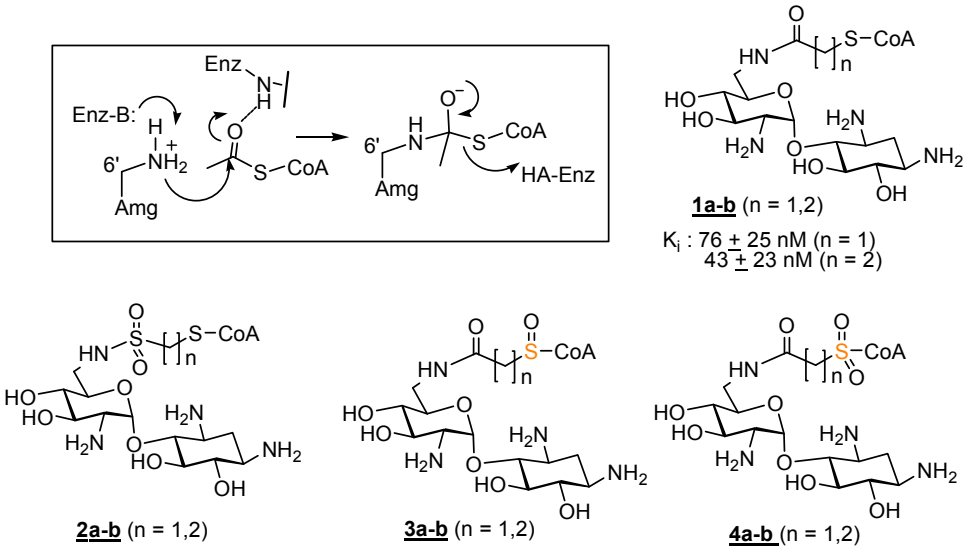



***Figure 4.1***. Proposed chemical steps catalyzed by AAC(6′)-Ii (top). Previously reported amide-linked aminoglycoside-CoA bisubstrate inhibitors (**1a-b**) and proposed the new bisubstrate inhibitors with sulfonamide-based linkers (**2a-b**, **3a-b** and **4a-b**).

To improve inhibition and to investigate whether AAC(6′)-Ii stabilizes the tetrahedral intermediate, we envisaged to prepare a second generation of bisubstrates containing either a sulfonamide, sulfoxide or sulfone, expected to

better mimic the tetrahedral intermediate (Fig. 4.1, **2a-b**, **3a-b** and **4a-b**). We hypothesized that if stabilization of this intermediate is important, a better mimetic would lead to increased affinity for the enzyme. Oxidized sulfides were selected for their ease of preparation, the higher polarizability of the S=O bond compared to a carbonyl, and the tetrahedral geometry at the sulfur atom. Sulfonamides have previously been used to mimic the tetrahedral intermediates involved in enzymatic catalysis by proteases[13] arginase,[14] dihydroorotase,[15] and isoleucyl tRNA synthetase ($K_i$ = 0.04 nM).[16]

The crystal structures of AAC(6′)-Ii in complex with AcCoA (Fig. 4.2),[17] CoA[18] or one of our bisubstrate inhibitors[11] all reveal the presence of two hydroxyl groups, Tyr147-OH (3.71Å away) and Thr111-OH (4.31 Å away), near the CoA sulfur atom. We reasoned that oxidization of the sulfur atom of bisubstrates **1a-b** into sulfoxides **3a-b** or sulfones **4a-b** may also increase the affinity for the enzyme by allowing two extra H-bonds between the oxygen of S=O and these two hydrogen donors.

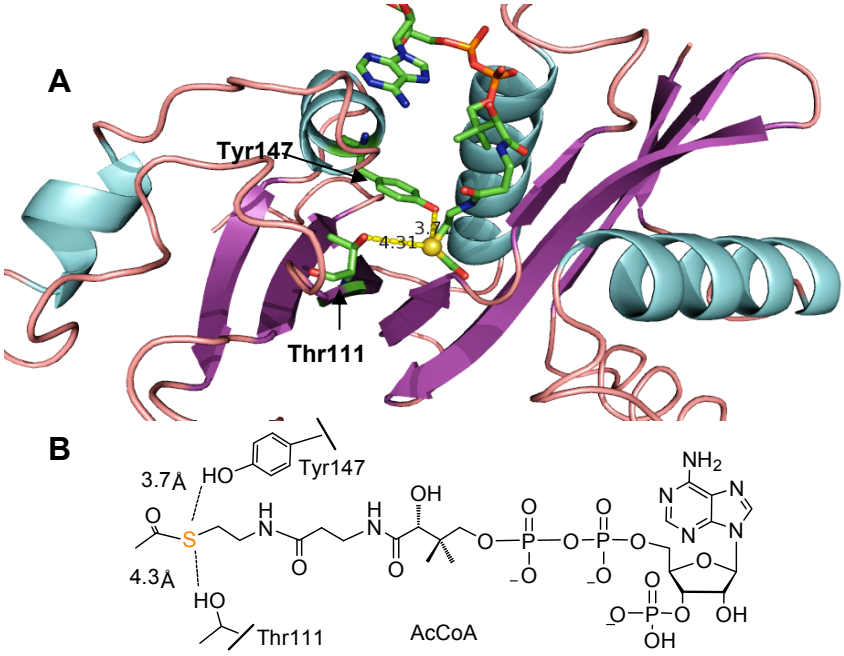



***Figure 4.2***. A) Crystal structure of AAC(6′)-Ii in complex with AcCoA (1B87.pdb)[17]. The protein is represented with cartoon. AcCoA is represented with line and colored by C (green), O (red), N (blue), P (purple), the sulfur atom of AcCoA is represented with a yellow sphere. B) Chemical structure of AcCoA and

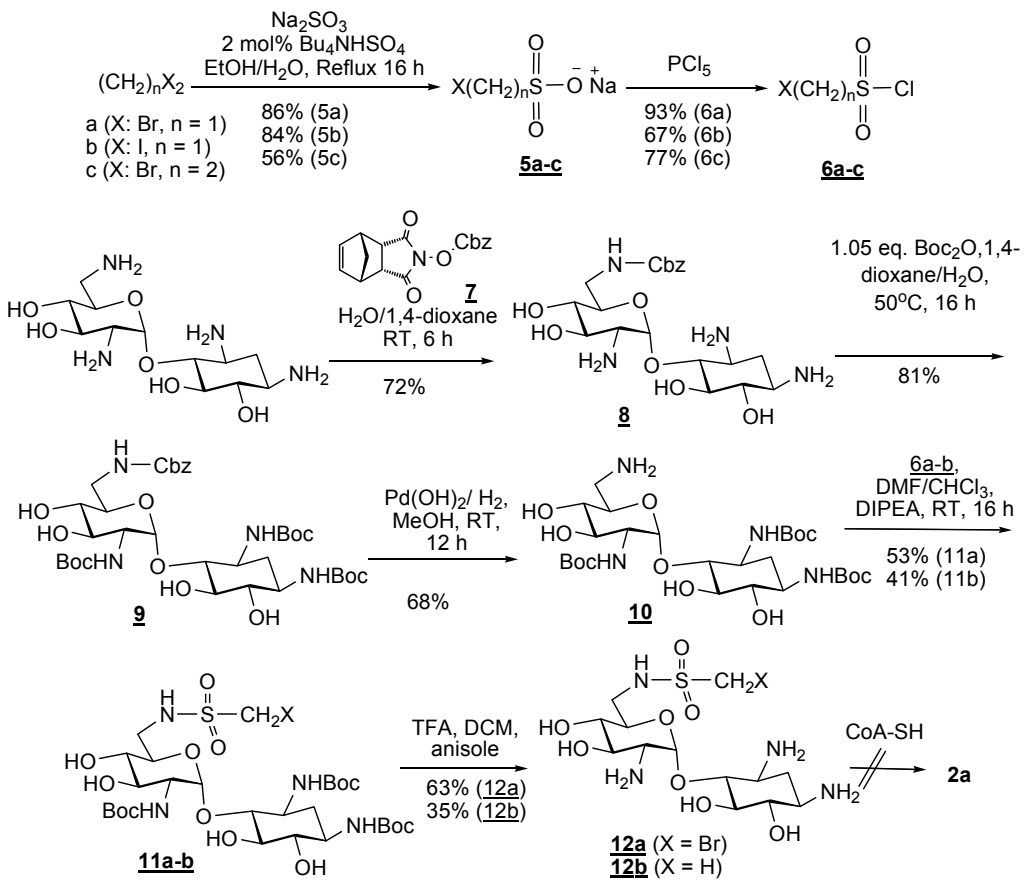
the distance between the sulfur and two amino acid residues. The figure was reproduced using PyMol.

We report here the synthesis of bisubstrates **2a-b**, **3a-b**, **4a-b** and their effect on the activity of AAC(6′)-Ii.

## 4.2 Results and Discussion

### 4.2.1 Synthesis

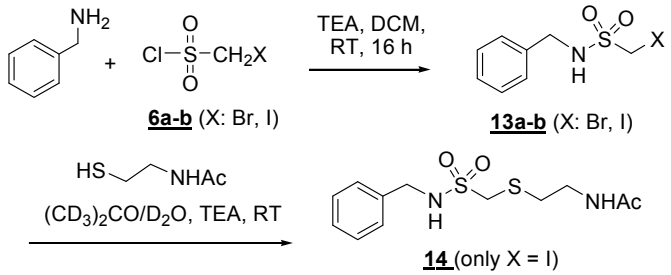
Bromomethanesulfonyl chloride (**6a**) was used as the main building block for the assembly of **1a-b**. Compound **6a** was prepared by treatment of sodium bromomethylsulfonate (**5a**) with $PCl_5$ a previously reported.[19] Compound **5a** was prepared using a reported procedure with some modifications.[20] Thus, sodium sulfite and dibromomethane were refluxed in a mixture of ethanol and water in the presence of a catalytic amount of tetraammonium hydrogen sulfate (2 mol%), to afford crystalline product **5a** (Scheme 4.1). 1,3,2′-tri-*N*-(*tert*-Butoxycarbonyl)neamine (**10**) was prepared using a known procedure.[21] *N*-Benzyloxycarbonyloxy-5-norbornene-*endo*-2,3-dicarboximide (**7**) was used to regioselectively protect the 6′-$NH_2$ of neamine and generate **8** in good yield. Treatment of **8** with di-*tert*-butyl dicarbonate (Boc anhydride) protected all the remaining amino groups of neamine to yield **9**, which was debenzylated to afford **10**. Compound **10** reacted with **6a** to afford **11a**, which was deprotected to give **12a**, *N*-6′-bromomethylsulfonyl neamine (Scheme 4.1).




***Scheme 4.1***. First synthetic to prepare bisubstrate analog **2a.** Abbreviations: CbzCl, *N*-benzyloxycarbonyl chloride; CoA-SH, coenzyme A; DCM, dichloromethane; DIPEA, diisopropylethylamine; TFA: trifluoroacetic acid.

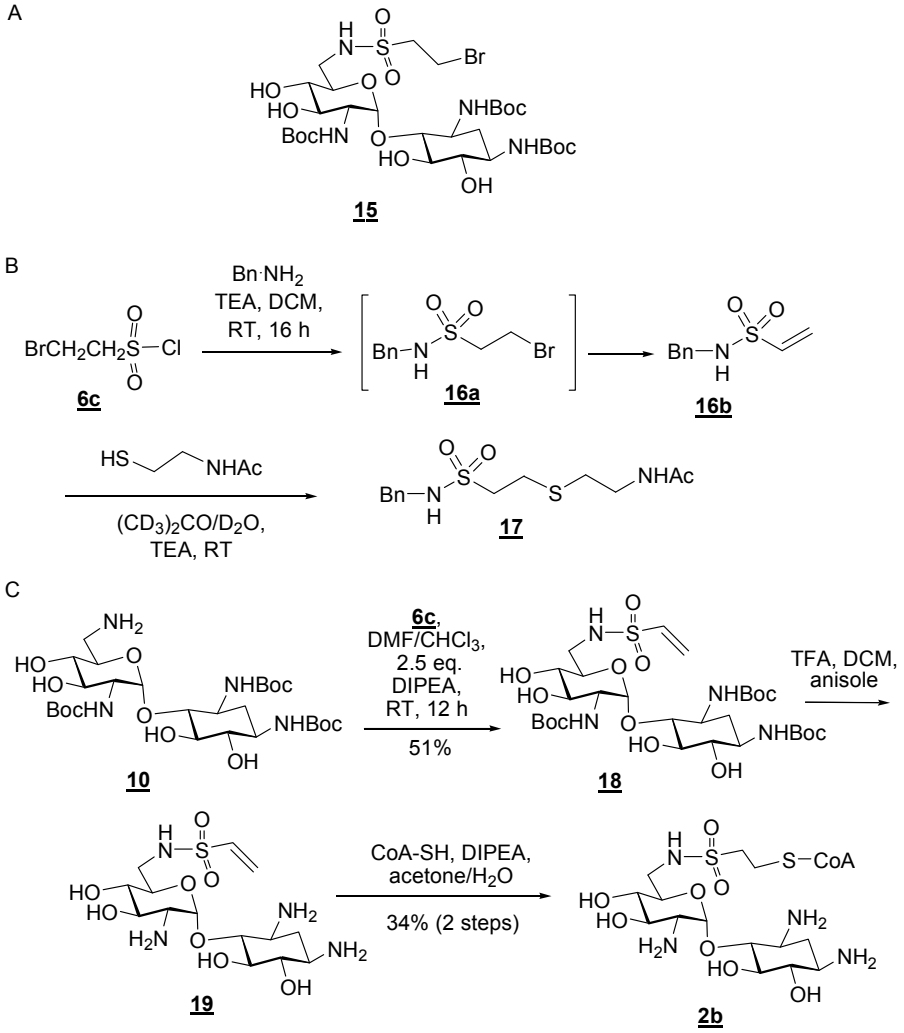
Unfortunately, bromide **12a** did not react with CoA. We suspected that this lack of reactivity was due to the low electrophilicity of the bromomethylene carbon. To confirm this hypothesis, we carried out two model reactions (Scheme 4.2) to compare the reactivity of bromomethyl sulfonamide and iodomethanesulfonamide toward sulfhydryl nucleophile. Indeed, when *N*-benzyl bromomethanesulfonamide (Scheme 4.2, **13a**) was treated with *N*-acetylcysteamine (a surrogate for CoA) in an aqueous solvent for two days at RT, no product (**14**) was detectable. The iodomethanesulfonamide **13b**, however, reacted with *N*-acetylcysteamine to yield product **14** under the same conditions after 2 days at RT. Encouraged by this, we set out to prepare the corresponding iodide **12b** (Scheme 4.1). A synthetic pathway similar to that described for **12a** was used. Surprisingly, removal of the Boc group followed by chromatography on

silica gel led to reduction of the product to 6′-*N*-methanesulfonylneamine (**12b)**. Reduction of the iodomethanesulfonamide was also observed in the model reaction but only as a minor product.[22]




***Scheme 4.2*** Model reaction for thiol attack at halomethanesulfonamides.

Target **2a** was abandoned and synthesis of bisubstrate **2b** (Fig. 4.1) was initiated. Retrosynthetic analysis suggests compound **15** (Scheme 4.3, A) as a reasonable intermediate. Again, a model reaction was used to evaluate the feasibility of this synthetic approach (Scheme 4.3, B). Not surprisingly, the β-bromosulfinamide **16a** easily eliminated to give a mixture of **16a** and the vinylsulfonamide **16b**. With 2.5 equivalents of base, **16b** was the only isolated product. Vinylsulfinamides are known to be susceptible to Michael addition by sulfhydryl groups.[23-26] To optimize the reaction conditions, vinylsulfinamide **16b** was reacted with *N*-acetyl cysteamine (Scheme 4.3, B). In the presence of triethylamine (TEA), the reaction was complete within 30 min and afforded adduct **17**. Next, 2-bromoethanesulfonyl chloride (**6c**) was reacted with protected neamine **10** to yield the vinylsulfinamide **18** after spontaneous bromide elimination (Scheme 4.3, C). Deprotection of **18** yielded **19** as the trifluoroacetate salt. Attempts to purify this intermediate on silica gel led to decomposition of the product. Crude **19** was therefore used directly in a reaction with CoA, which yielded bisubstrate **2b** in 34% yield after reverse phase HPLC purification.




***Scheme 4.3***. Synthesis of bisubstrate **2b**. Abbreviations: Bn, benzyl; CoA-SH, coenzyme A; DCM, dichloromethane; DIPEA, diisopropylethylamine; TFA: trifluoroacetic acid.

It was decided to prepare bisubstrates **3a-b** and **4a-b** by direct oxidation of the known sulfides **1a-b**. Selective oxidation of sulfides to sulfoxides has interested chemists for many years.[27-35] Magnesium monoperoxyphthalate (MMPP) was reported to selectively oxidize glycosyl sulfides to sulfoxides[31, 36] or sulfones[37] in good yields. Thus MMPP appeared as the most suitable oxidant for our purpose. Unfortunately, the oxidation of **1b** with MMPP was very slow. Half of the starting material remained unchanged after one hour at RT in the presence of 3 equivalents of MMPP. Furthermore, only the sulfoxide product was observed

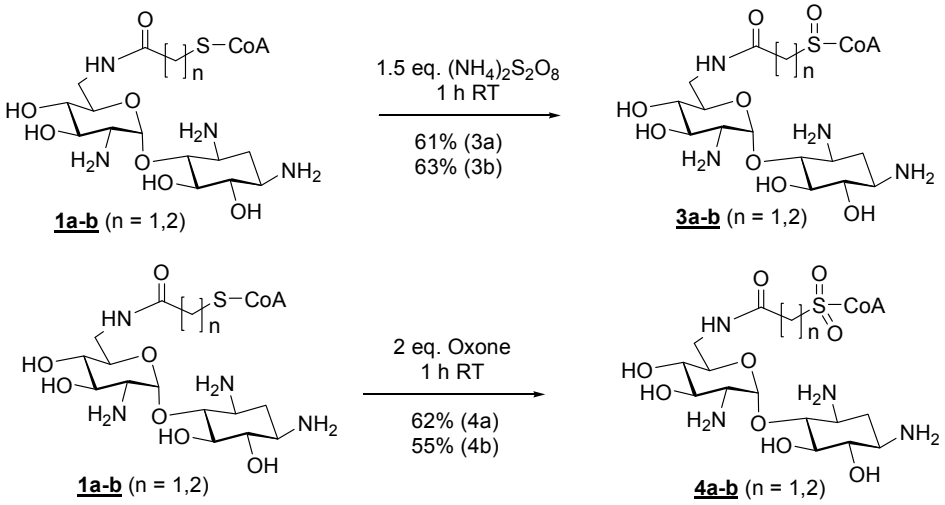
under these conditions. Longer reaction times led to decomposition of the reactant and product. *m*-Chloroperbenzoic acid *(m*-CPBA),[38-40] sodium periodate,[41-44] *tert*-butylhydroperoxide (TBHP),[34, 45-47] Oxone[34, 48-51] *N*-sulfonyloxaziridine,[33] and dioxirane[50] have also been used for the selective oxidation of sulfides to sulfoxides. Except for *N*-sulfonyloxaziridines, all of these reagents, and more, were explored for the oxidation of **1b**. The results are summarized in Table 4.1.

**Table 4.1.** Screening of oxidants for the selective sulfide oxidation of **1b**

| Entry | Oxidant[a] | Equiv. | Results[b] | | |
|---|---|---|---|---|---|
| | | | Sulfide | Sulfoxide | Sulfone |
| 1 | *m*CPBA | 3 | 60% | 40% | 0 |
| 2 | TBHP | 3 | 90% | 10% | 0 |
| 3 | $H_2O_2$ | 3 | 60% | 40% | 0 |
| 4 | $H_2O_2$ Urea | 3 | 100% | | |
| 5 | $H_2O_2$ $Na_2CO_3$ | 3 | | decompose | |
| 6 | $H_2O_2$ $Na_2BO_2$ | 3 | | decompose | |
| 7 | $NaIO_4$ | 3 | | decompose | |
| 8 | $NaIO_4$ | 1 | 50% | 50% | 0 |
| 9 | $(NH_4)_2S_2O_8$ | 1 | 50% | 50% | 0 |
| 10 | $(NH_4)_2S_2O_8$ | 2 | 0 | 100% | 0 |
| 11 | Oxone[c] | 1 | 0 | 60% | 40% |
| 12 | Oxone | 2 | 0 | 0 | 100% |

[a] The reaction time is 1 h at RT in water; [b] composition was normalized according to corresponding peak area from LC-MS; [c] potassium monopersulfate as in $2KHSO_5 \cdot KHSO_4 \cdot K_2SO_4$.

Ammonium persulfate was the best oxidant for the selective oxidation of the sulfide **1a** and **1b** to the corresponding sulfoxides **3a** and **3b**. The reactions were complete within one hour when two equivalents of oxidant were used. To our knowledge, this is the first time that $(NH_4)_2S_2O_8$ is successfully used for the selective oxidation of a sulfide containing multiple functionalities, to a sulfoxide, under aqueous conditions. Bisubstrate **3a-b** were purified by reverse phase HPLC as a mixture of two diastereoisomerisomers (>90%). As for the oxidation of the sulfides **1a** and **1b** to sulfones **4a** and **4b** respectively, oxone appeared to be the most efficient oxidant (Scheme 4.4).




***Scheme 4.4***. Optimized conditions for the syntheses of bisubstrates **3a-b** and **4a-b** via the selective oxidation of the sulfides **1a-b**.

### 4.2.2 Biological activity

The bisubstrates **2b**, **3a-b** and **4a-b** were tested for inhibition of AAC(6′)-Ii. The results are shown in Table 4.2. All were potent competitive inhibitors with $K_i$s ranging from low micromolar to picomolar. Surprisingly, the bisubstrate with a sulfonamide linker (**2b**) showed a decreased inhibition compared to the corresponding amide-linked bisubstrate (**1b)**. This result suggests either that the enzyme may not stabilize the tetrahedral intermediate or that **2b** is a poor mimic of the tetrahedral intermediate.

Remarkably, the two bisubstrates containing sulfone groups (**4a-b**) were better inhibitors than those containing sulfoxides (**3a-b**). This may be explained by the formation of two new H-bonds as opposed to only one with the sulfoxide, or by improper orientation of the one S=O bond of **3a-b**. Bisubstrate **4a** was the most potent inhibitor, with an estimated $K_i^{app}$ in the picomolar range.

**Table 4.2**. AAC(6′)-Ii inhibition constants ($K_i^{app}$) for bisubstrate inhibitors **2b**, **3a-b** and **4a-b**

| Inhibitor | 2b | 3a | 3b | 4a | 4b |
|---|---|---|---|---|---|
| Inhi. pattern | competitive | competitive | competitive | N.A. | competitive |
| $K_i$ (μM) | 2.20 ± 0.10 | 0.70 ± 0.05 | 5.50 ± 1.80 | < 0.08 | 0.084 ± 0.004 |

## 4.3 Conclusions

In conclusion, report here the synthesis of 5 new bisubstrates containing sulfonamide, sulfoxide or sulfone groups. We demonstrate for the first time the utility of $(NH_4)_2S_2O_8$ for selective oxidize of highly functionalized sulfides to sulfoxides under aqueous conditions. Although sulfonamides are expected to better mimic tetrahedral intermediates than amides, sulfonamide-linked bisubstrate **2b** showed poorer inhibition of AAC(6′)-Ii than amide-linked inhibitor **1b**. This supports the hypothesis that AAC(6′)-Ii may catalyze the reaction mainly via proximity effects. On the other hand, sulfide oxidation of the bisubstrates containing an amide linker (**1a-b**) to the corresponding sulfones **4a-b** dramatically improved inhibition of AAC(6′)-Ii. Evaluation of the reported crystal structures of AAC(6′)-Ii suggests that this gain in affinity may be the result of extra hydrogen bonds formed between the new S=O bonds and Tyr147-OH and Thr111-OH. Bisubstrate **4a** is the most potent inhibitors known for this enzyme to date.

## 4.4 Experimental section

**Materials**. All commercial reagents were used without further purification unless otherwise specified. Neamine hydrochloride was prepared by methanolysis of neomycin B using a previously reported procedure.[52] Bisubstrate analog **1a** and **1b** were prepared as reported before.[11] AcCoA, DTDP, EDTA, and HEPES were purchased from Sigma-Aldrich (St. Louis, MO). HRMS of compounds **2b**, **3a-b, 4a-b** were performed by direct infusion electrospray ionization from a solution in

90:10 methanol:50 mM aqueous ammonium hydroxide at 2 μL/min in an IonSpec 7 Tesla FTICR instrument at a resolving power of approximately 80,000. Other HRMS samples were analyzed using a Kratos MS 25RFA mass spectrometer at a source temperature of 200°C and 70 eV. LRMS was performed using a Finnigan LCQDUO mass spectrometer with either ESI or APCI without fragmentation. Routine $^{1}H$ and $^{13}C$ NMR spectra were recorded using Varian Mercury 400 or 300 or Unity 500 spectrometers. The chemical shifts (δ) were reported in parts per million (ppm) relative to the internal standard TMS (0 ppm). The peak patterns are indicated as follows: s, singlet; d, doublet; t, triplet; dt, doublet of triplet; ddd, doublet of doublet of doublet; td, triplet of doublet; m, multiplet; q, quartet; br s, broad singlet, etc. All $^{1}H$ NMR and correlation spectra including COSY, HMBC and HSQC were recorded with solutions at pD = 4.5 unless otherwise stated. $^{1}H$ and $^{13}C$ NMR assignments were confirmed by HSQC, HMQC, and HMBC.

Bisubstrate analogs **2b, 3a-b,** and **4a-b** were purified by reversed-phase HPLC using an Agilent Zorbax SB-C8 column (4.6 × 250 mm, 5 μ) on an Agilent 1100 system with diode array UV detector. Samples were eluted at a flow rate of 3 mL/min, using the linear gradients shown in Table 4.2. The purity of the bisubstrate inhibitors was evaluated by HPLC with the same equipment and column but using isocratic eluent with a ratio of phase A/phase B of either 85/15 (method A) or 64/40 (method B). The purity of all the bisubstrate inhibitors used in the enzymatic inhibition assays was > 95%.

**Table 4.2**. Gradient profile for HPLC purification

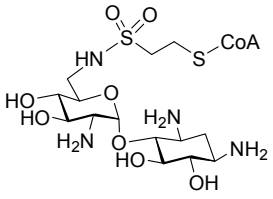
| Time (min) | % A (0.05% TFA in $H_2O$) | % B (0.04% TFA in ACN ) |
|---|---|---|
| 0 | 99 | 1 |
| 20 | 60 | 40 |
| 30 | 99 | 1 |

**General procedure for the syntheses of 3a-b and 4a-b.** The trifluroacetate salts of the bisbustrate **1a** or **1b** in (15 mg, ca. 0.01 mmol) was dissolved in $H_2O$ (3 mL). A solution of the oxidant (1 mL of 0.01 M; $(NH_4)_2S_2O_8$

for **3a-b**; Oxone for **4a-b**) was added. The mixture was sonicated for 30 seconds and stirred for 30 min at RT. The solvent was evaporated *in vacuo* and the residue was purified by reverse phase HPLC to give the desired products.

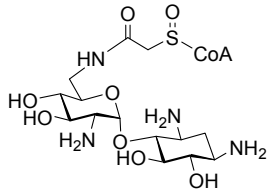
**General procedure for the syntheses of 5a-c.** The procedure reported by Brienne et al.[20] was modified as follows. A mixture of sodium sulfite (22 g, 0.175 mol), the desired alkyl halide (0.175 mol), tetrabutylammonium hydrogen sulfate (1 g, 3 mmol), and a mixture of EtOH/$H_2O$ (1/2, v/v, 100 mL) was refluxed for 16 h with vigorous stirring. The solvent was evaporated *in vacuo* and the resulting solid residue was dissolved in a warm mixture of EtOH/$H_2O$ (5/1, v/v, 120 mL, 45°C). After filtration of the insoluble material, the filtrate was cooled to -20°C overnight. The crystalline sodium sulfonate salt was collected by filtration.

**General procedure for the syntheses of 6a-c.** A mixture of the desired sodium sulfonate (**5a-c**, 30 mmol) and $PCl_5$ (6.6 g, 30 mmol) was vigorously stirred until the mixture became a yellowish slurry (exothermic reaction for ca. 5 min). The mixture was heated at 130°C for 30 min and then at 70°C for another 30 min. It was then poured into ice-cold water (70 mL) with vigorous stirring. The desired sulfonyl chloride was extracted in methylene chloride (3 × 40 mL). The combined methylene chloride extracts were washed with water (100 mL), ice-cold 5% sodium bicarbonate (2 × 50 mL), and water again (50 mL). The organic layer was dried over sodium sulfate. Evaporation of the solvent yielded a yellowish residue, which was distilled to give the sulfonyl chloride as a clear liquid.

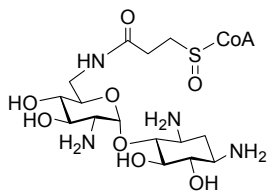



**2b**. Crude product **19** (30 mg, ca. 0.05 mmol) was dissolved in $H_2O$ (6 mL), the solution was filtered through a microfilter (0.26 μ) to remove insoluble impurities. CoA-SH (lithium salt, 25 mg, 0.03 mmol) was added and the solution was sonicated for one minute and then cooled to 4°C. Next, TEA (50 μL, 0.3 mmol dissolved in 1 mL of acetone) was added and the mixture was sonicated again for 2 periods of one minute. The reaction mixture was stirred at 4°C for 12 h. Evaporation of all the volatiles yielded the crude product as a white powder, which was purified by

reverse phase HPLC. Yield: 34%. **$^1$H NMR** ($D_2O$, 500 MHz): δ 8.49 (s, 1H), 8.30 (s, 1H), 6.07 (d, *J* = 4.5 Hz, 1H), 5.53 (d, *J* = 4.0 Hz, 1H), 4.42 (br s, 1H), 4.13 (m, 2H), 3.86-3.72 (m, 6H), 3.49-3.16 (m, 16H), 2.76 (t, *J* = 7.6 Hz, 2H), 2.56 (t, *J* = 7.6 Hz, 2H), 2.32 (m, 3H), 1.88 (q, *J* = 12.0 Hz, 1H), 0.79 (s, 3H), 0.68 (s, 3H); **$^{13}$C NMR** ($D_2O$, 125 MHz by HSQC and HMBC): δ 174.8, 174.3, (C=O), 150.2, 148.8, 144.9, 142.8, 118.4 (aromatic), 97.3 (C1′), 87.9, 83.4, 80.2, 75.9, 75.1, 74.6, 73.9, 72.8, 72.6, 72.4, 70.4, 69.1, 65.4, 54.1, 51.7, 50.0, 49.0, 43.2, 40.3, 38.6, 35.7, 35.6, 30.9, 28.4, 24.4, 21.0, 18.7; **HRMS** for $C_{35}H_{64}N_{11}O_{24}P_3S_2$ $[M+H]^+$ calcd. 1180.2644, found 1180.2632. Purity, 95% (method A, $t_R$ : 3.91 min; method B, 3.54 min).

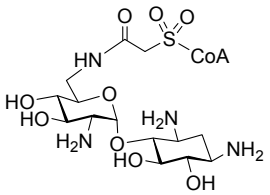



**3a.** Yield: 61% **$^1$H NMR** ($D_2O$, 500 MHz): δ 8.48 (s, 1H), 8.27 (s, 1H), 6.07 (d, *J* = 4.5 Hz, 1H), 5.60 (d, *J* = 3.5 Hz, 1H), 4.70 (br s, 1H), 4.44 (br s, 1H), 4.12 (m, 2H), 3.86-3.10 (m, 23H), 2.33 (m, 3H), 1.82 (q, *J* = 12.5 Hz, 1H), 0.78 (s, 3H), 0.69 (s, 3H); **$^{13}$C NMR** ($D_2O$, 125 MHz, by HSQC and HMBC): δ 175.0, 174.0, 164.0 150.4, 148.4, 142.6, 142.2, 118.8, 96.6, 87.8, 83.2, 78.6, 75.1, 74.8, 74.0, 72.2, 72.0, 71.8, 70.4, 68.4, 65.2, 58.6, 53.8, 52.2, 49.6, 48.8, 43.6, 41.0, 39.8, 35.4, 35.2, 32.4, 28.2, 21.0, 18.8; **HRMS** for $C_{35}H_{63}N_{11}O_{24}P_3S$ $[M+H]^+$, calcd. 1146.2998, found 1146.2976. Purity, 95% (method A, $t_R$ : 3.76 min; method B, 3.50 min).

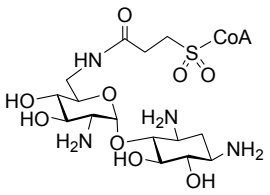



**3b**. Yield: 63%. **$^1$H NMR** ($D_2O$, 500 MHz): δ 8.57 (s, 1H), 8.36 (s, 1H), 6.15 (d, *J* = 4.5 Hz, 1H), 5.66 (d, *J* = 3.5 Hz, 1H), 4.81 (br s, 1H), 4.46 (m, 1H), 4.20 (m, 2H), 3.93-3.02 (m, 23H), 2.73 (t, *J* = 6.5 Hz, 2H), 2.41 (m, 3H), 1.84 (q, *J* = 12.5 Hz, 1H), 0.86 (s, 3H), 0.75 (s, 3H); **$^{13}$C NMR** ($D_2O$, 125 MHz by HSQC and HMBC): δ 177.6, 175.2, 173.4, 150.2, 149.2, 145.0, 142.4, 119.2, 96.8, 88.0, 83.7, 79.4, 75.0, 74.4, 74.0, 72.3, 71.6, 70.8, 68.8, 65.2, 53.8, 51.8, 51.4, 49.5, 48.8, 46.8, 46.4, 40.0, 39.8, 35.6, 35.4, 33.0, 29.0, 28.2, 20.8,

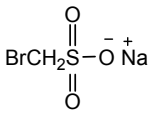
18.6; **HRMS** for $C_{36}H_{65}N_{11}O_{24}P_3S$ $[M+H]^+$ calcd. 1160.3142, found 1160.3132. Purity, 95% (method A, $t_R$ : 3.78 min; method B, 3.52 min).



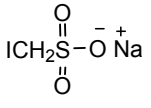
**4a.** Yield: 62%. **$^1$H NMR** ($D_2O$, 500 MHz): δ 8.50 (s, 1H), 8.29 (s, 1H), 6.07 (d, *J* = 4.5 Hz, 1H), 5.61 (d, *J* = 4.0 Hz, 1H), 4.72 (br s, 1H), 4.44 (br s, 1H), 4.11 (m, 2H), 3.86-3.00 (m, 23H), 2.33 (m, 3H), 1.82 (q, *J* = 12.5 Hz, 1H), 0.77 (s, 3H), 0.68 (s, 3H); **$^{13}$C NMR** ($D_2O$, 125 MHz, by HSQC and HMBC): δ 175.0, 173.7, 166.0, 150.2, 149.0, 145.2, 142.0, 118.8, 96.6, 87.8, 83.6, 78.8, 75.5, 74.8, 74.0, 72.0, 71.8, 71.0, 68.6, 65.4, 56.0, 54.0, 51.6, 50.0, 48.7, 48.6, 46.4, 40.4, 39.8, 37.4, 37.2, 33.4, 28.2, 21.4, 18.8; **HRMS** for $C_{35}H_{63}N_{11}O_{25}P_3S$ $[M+H]^+$, calcd. 1162.2924, found 1162.2917. Purity, 95% (method A, $t_R$ : 3.80 min; method B, 3.52 min).



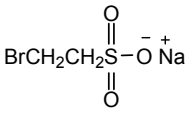
**4b**. Yield: 55%. **$^1$H NMR** ($D_2O$, 500 MHz): δ 8.57 (s, 1H), 8.37 (s, 1H), 6.15 (br s, 1H), 5.66 (br s, 1H), 4.81 (br s, 1H), 4.53 (br s 1H), 4.20 (m, 2H), 3.93-3.25 (m, 23H), 2.75 (t, *J* = 6.5 Hz, 2H), 2.41 (m, 3H), 1.88 (q, *J* = 12.5 Hz, 1H), 0.86 (s, 3H), 0.76 (s, 3H); **$^{13}$C NMR** ($D_2O$, 125 MHz by HSQC and HMBC): δ 175.4, 174.3, 172.6, 150.2, 149.2, 144.6, 142.2, 118.8, 96.8, 88.7, 83.4, 79.2, 75.2, 75.0, 74.2, 74.0, 72.6, 72.0, 71.8, 71.0, 68.6, 65.5, 54.0, 52.7, 52.0, 50.0, 49.0, 39.8, 39.2, 35.8, 35.4, 32.8, 28.2, 27.8, 21.0, 18.8; **HRMS** for $C_{36}H_{65}N_{11}O_{25}P_3S$ $[M+H]^+$ calcd. 1176.3075, found 1144.3081. Purity, 95% (method A, $t_R$ : 3.82 min; method B, 3.53 min).



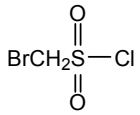
**5a**. Yield: 86% (175 mmol scale, 29.5 g). **$^1$H NMR** ($D_2O$, 300 MHz): δ 4.33 (s, 2H); **$^{13}$C NMR** ($D_2O$, 75 MHz) δ 40.7. **ESI-MS** for $^{79}BrCH_2SO_3Na$ $[M+H]^+$ cald. 196.9, found 197.1.



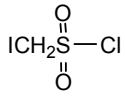
**5b**. Yield: 84% (70 mmol scale, 14.6 g). **$^1$H NMR** ($D_2O$, 300 MHz): δ 4.30 (s, 2H); **$^{13}$C NMR** ($D_2O$, 75 MHz) δ 12.0. **ESI-MS** for $ICH_2SO_3Na$ $[M+H]^+$ cald. 244.9, found 245.2.



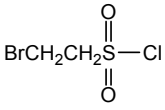
**5c**. Yield: 56% (175 mmol scale, 20.7 g). **$^1$H NMR** ($D_2O$, 300 MHz): δ 3.59 (t, 2H, *J* = 8.1), 3.36 (t, 2H, *J* = 8.1); **$^{13}$C NMR** ($D_2O$, 75 MHz) δ 53.4, 24.3. **ESI-MS** for $^{79}BrCH_2CH_2SO_3Na$ $[M+H]^+$ 212.9, found 213.1.



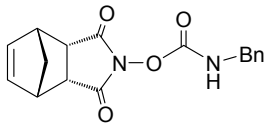
**6a**. Yield: 93% (30 mmol scale, 5.5 g). **$^1$H NMR** ($CDCl_3$, 300 MHz): δ 4.97 (s, 2H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 50.9. **LRMS** for $^{79}BrCH_2SO_2{}^{35}Cl$ (m/z) calcd. 193.9, found 193.8.



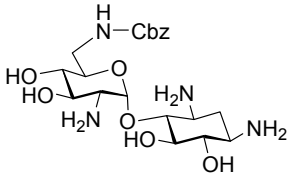
**6b**. Yield: 67% (20 mmol scale, 3.2 g). **$^1$H NMR** ($CDCl_3$, 300 MHz): δ 5.12 (s, 2H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 21.0. **LRMS** for $ICH_2SO_2{}^{35}Cl$ (m/z) cald. 239.8, found 239.8.



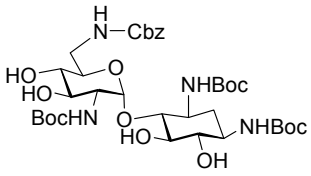
**6c**. Yield: 78% (28 mmol scale, 4.6 g). **$^1$H NMR** (CDCl3, 300 MHz): δ 4.12 (t, 2H, J = 7.2), 3.75 (t, 2H, J = 7.2); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 66.0, 20.4. **LRMS** for $^{79}BrCH_2CH_2SO_2{}^{35}Cl$ (m/z) cald. 207.9, found 207.8.



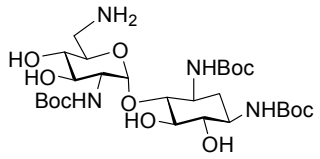
**7**. *N*-Hydroxy-5-norbornene-*endo*-2,3-dicarboximide (5.4 g, 30 mmol) and TEA (6 mL, 42 mmol) were dissolved in DCM (150 mL). The mixture was cooled to 0$^o$C and *N*-benzyloxycarbonyl chloride (5.12 g, 30 mmol) was added dropwise with stirring into this solution. The mixture was stirred for 12 h and evaporated to ca. 30 mL, filtered to remove the triethylamine hydrogen chloride salt. The filtrate was washed with water (2 × 30 mL), and the organic phase was dried over $Na_2SO_4$ before evaporation of the solvent. A white crystalline powder resulted. Yield: 8.8 g, 93%. **$^1$H NMR** ($CDCl_3$, 300 MHz): δ 7.36 (s, 5H), 6.15 (br s, 2H), 5.26 (s, 2H), 3.44 (br s, 2H), 3.30 (br s, 2H), 1.76 (d, *J* = 8.8 Hz, 1H), 1.50 (d, *J* = 8.8 Hz, 1H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 169.8, 166.3, 135.0, 133.6, 129.4, 129.0, 128.8, 72.7, 51.4, 45.0, 43.3. **ESI-MS** for $C_{17}H_{16}N_2O_4$ $[M+H]^+$ 313.1, found 313.1.



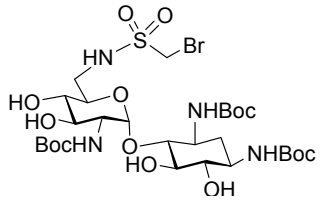
**8**. This compound was prepared using a slightly modified version of a procedure reported before.[21] *N*-Benzyloxy-carbonyloxy-5-norbornene-*endo*-2,3-dicarboximide (**7**, 3.2 g, 10 mmol) was dissolved in dioxane (125 mL) and added dropwise to a solution of neamine (free base, 3.3 g, 10.1 mmol) in dioxane/$H_2O$ (1/l, 250 mL). The mixture was stirred for 6 h at RT. Subsequently, the solvent was evaporated *in vacuo,* and the oily residue was purified on silica gel ($R_f$: 0.4, $CHC1_3$/MeOH/$NH_4OH$, 3/2/1) to yield product **8** (3.3 g, 72%). **$^1$H NMR** (DMSO-$d_6$, 400 MHz) δ 7.33 (m, 6H), 5.00 (s, 2H), 4.74 (bs, 1H), 3.49-2.85 (m, 20H), 2.53 (m, 2H), 1.71 (bs, 1H), 0.88 (bs, 1H); **$^{13}$C NMR** (DMSO-$d_6$, 75 MHz) δ 156.7 (C=O), 137.7, 134.6, 128.7, 126.2 ($CH_2$***C***$_6H_5$), 102.3(C1′), 91.4 (C4), 77.2 (C6), 76.3 (C5), 73.7 (C3′), 72.6 (C5′), 71.9 (C4′), 65.6 (O***C***$H_2$Ph), 51.7 (C1), 50.6 (C3), 42.7 (C6′), 37.6 (C2). **ESI-MS** for $C_{22}H_{32}N4O_8$ $[M+H]^+$ 457.2, found 457.2.



**9**. Compound **8** (1.8 g, 3.95 mmol) was dissolved in of dioxane/$H_2O$ (3/l, 100 mL), followed by the addition of di-*tert*-butyldicarbonate (2.62 g, 12 mmol). The mixture was stirred overnight at 50$^o$C. The solvent was evaporated *in vacuo*, and the residue was purified on silica gel ($R_f$: 0.7, $CHCl_3$/MeOH/$NH_4OH$, 4/l/0.l) to give the desired product (4.3 g, 86%). **$^1$H NMR** spectrum (DMSO-*$d_6$*, 300 MHz) δ 7.26 (m, 5H), 6.89 (bs, 1H), 6.63 (bs, 1H), 6.50 (bs, 1H), 6.18 (bs, 1H), 5.02 (s, 2H), 4.74 (bs, 1H), 3.58-3.15 (m, 14H), 1.78 (bs, 1H), 1.36 (s, 27H), 1.17 (m, 1H); **$^{13}$C NMR** (DMSO-*$d_6$*, 75 MHz) δ 157.0, 156.2, 155.7, 155.3 (C=O), 137.7, 134.8, 128.7, 128.2 (***C***$_6H_5CH_2$), 99.0 (C1′), 81.7 (C4), 78.1, 77.9, 77.6 [O***C***$(CH_3)_3$], 77.5 (C6), 74.2 (C5), 71.8 (C3′), 71.0 (C5′), 66.8 (C4′), 65.7 (O***C***$H_2$Ph), 55.8 (C2′), 51.2 (C1), 50.0 (C3), 42.4 (C6′), 35.8 (C2), 28.6 [OC(***C***$H_3)_3$]; ESI-MS for $C_{35}H_{56}N_4O_{14}$ $[M+H]^+$ cacld. 757.3, found 757.2.

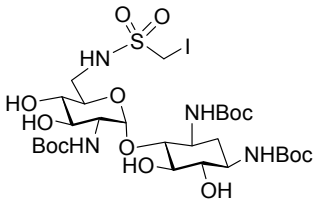


**10**. Compound **9** (1.34 g, 0.18 mmol) was dissolved in MeOH/DMF (10/1, 50 mL), the mixture was quickly degassed under vacuum (house vacuum for 15 sec) and saturated with argon. To this mixture was added 10% $Pd(OH)_2/C$ (0.22 g) before degassing again and saturating with hydrogen gas. The mixture was vigorously stirred in the presence of hydrogen for 12 h. The catalyst was subsequently removed by filtration through a layer (thickness ca. 2 cm) of Celite, and the filtrate was evaporated to give a white residue which was purified on silica gel ($R_f$: 0.35, $CHC1_3$/MeOH/$NH_4OH$, 5/l/0.l) to yield the product (0.75 g, 68%). **$^1$H NMR** (DMSO-$d_6$, 300 MHz): δ 6.39 (bs, 1H), 6.18 (bs, 1H), 6.10 (bs, 1H) 5.12 (s, 1H), 4.66 (bs, 2H), 3.58-3.15 (m, 14H), 1.78 (bd, 1H), 1.36 (s, 27H), 1.24 (m, 1H); **$^{13}$C NMR** (DMSO-$d_6$, 75 MHZ) δ 156.4, 155.9, 155.3 (C=O), 99.1 (C1′), 81.7 (C4), 78.1, 77.9, 77.6 [O***C***$(CH_3)_3$], 77.4 (C6), 74.3 (C5), 72.8 (C3′), 72.4 (C5′), 71.4 (C4'), 56.1 (C2′), 51.2 (C1), 50.0 (C3), 43.1 (C6′), 35.8 (C2), 28.7, 28.6 [OC(***C***$H_3)_3$]; **ESI-MS** for $C_{27}H_{50}N_4O_{12}$ $[M+H]^+$ calcd. 623.3, found, 623.1.

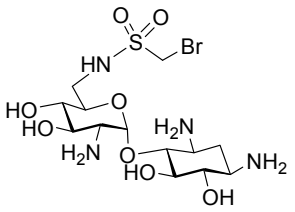


**11a**. Compound **10** (0.28 g, 0.45 mmol) and DIPEA (0.2 mL, 1.0 mmol) were mixed in anhydrous DMF (20 mL). To this mixture a solution of bromomethanesulfonyl chloride (**6a**, 88 mg, 0.45 mmol) in chloroform (5 mL) was added dropwise. The mixture was stirred overnight at RT and then evaporated *in vacuo*. The residue was purified on silica gel ($R_f$: 0.38, $CHCl_3$/MeOH/$NH_4OH$, 10/1/0.1) to give the desired product *(*0.44 g, 53%). **$^1$H NMR** (DMSO-$d_6$, 300 MHz): δ 7.39 (bs, 1H), 6.66 (bs, 1H), 6.63 (bs, 1H), 6.21 (bs, 1H) 5.12 (s, 1H), 4.66 (bs, 2H), 3.58-3.15 (m, 14H), 1.78 (bd, 1H), 1.36 (s, 27H), 1.24 (m, 1H); **$^{13}$C NMR** (DMSO-$d_6$, 75 MHz) δ 156.4, 155.9, 155.6 (C=O), 99.3 (C1′), 82.0 (C4), 78.6, 78.4, 78.2 [O***C***$(CH_3)_3$], 77.9 (C6), 77.5 (C5), 74.5 (C3′), 71.4 (C5′), 71.2 (C4′), 55.9 (C2′), 51.4 (C1), 50.1 (C3), 44.2 ($SO_2$***C***$H_2$Br), 43.5 (C6′), 36.1 (C2), 28.9,

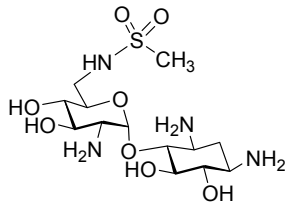
28.8 [OC(***C***$H_3$)$_3$]; **ESI-MS** for $C_{28}H_{51}BrN_4O_{14}S$ $[M+Na]^+$ calcd. 803.2 (100), 801.2 (98), found 803.2 (100), 801.2 (98).



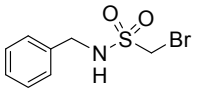

**11b**. Compound **10** (0.38 g, 0.61 mmol) and DIPEA (0.2 mL, 1.0 mmol) were mixed in anhydrous DMF (20 mL). To this mixture a solution of iodomethanesulfonyl chloride (**6b**, 148 mg, 0.61 mmol) in chloroform (5 mL) was added dropwise. The mixture was stirred overnight at RT and then evaporated *in vacuo*. The residue was purified on silica gel ($R_f$ 0.40, $CHCl_3$/MeOH, 7/l) to give the desired product (0.21 g, 41%). **$^1$H-NMR** (DMSO-*$d_6$*, 300 MHz) δ 7.32 (bs, 1H), 6.66 (bs, 1H), 6.63 (bs, 1H), 6.21 (bs, 1H) 5.02 (s, 1H), 4.09 (s, 2H), 3.58-3.15 (m, 14H), 1.78 (bd, 1H), 1.31 (s, 27H), 1.24 (m, 1H); **$^{13}$C-NMR** (DMSO-*$d_6$*, 75 MHz) δ 156.7, 156.1, 155.7 (C=O), 99.4 (C1′), 81.5 (C4), 78.6, 78.4, 78.0 [O***C***$(CH_3)_3$], 77.9 (C6), 77.6 (C5), 74.5 (C3′), 71.4 (C5′), 71.3 (C4′), 55.9 (C2′), 51.4 (C1), 50.0 (C3), 44.4 (C6′), 35.6 (C2), 28.8, 28.7 [OC(***C***$H_3$)$_3$]; **ESI-MS** for $C_{28}H_{51}IN_4O_{14}S$ $[M+Na]^+$ cacld. 849.2, found 849.1.



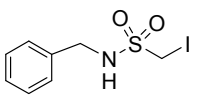

**12a**. Compound **11a** (0.14 g, 0.18 mmol) was dissolved DCM (5 mL) containing 3 drops of anisole. The mixture was sonicated for 2 min followed by addition of TFA (3 mL). The mixture immediately became clear and was stirred for 1 h at RT. Evaporation of the solvent yielded a white powder, which was purified on silica gel ($CHCl_3$/MeOH/$NH_4OH$, 4/3/1 then 3/2/1, $R_f$: 0.36 in the latter eluent) to give the desired product as a white powder (54 mg, 63%). **$^1$H NMR** ($D_2O$, 400 MHz) δ 5.13 (br d, 1H); 4.68 (s, 2H, $SO_2C$***H***$_2$Br), 3.76 (m, 1H), 3.44 (m, 3H), 3.26 (m, 4H), 2.75 (m, 3H), 1.82 (br td, 1H), 1.06 (q, 1H, *J* = 12.4); **$^{13}$C NMR** ($D_2O$, 75 MHz) δ 100.5 (C1′), 86.7 (C4), 76.0 (C6), 75.5 (C5), 73.1 (C3'), 71.8 (C5′), 70.7 (C4'), 55.0 (C2′), 50.3 (C1), 49.3 (C3), 43.7 (***C***$H_2$Br), 41.4 (C6′), 33.9 (C2); **ESI-MS** for $C_{13}H_{27}{}^{79}BrN_4O_8S$ $[M+H]^+$ calcd. 481.1, found 481.1. This compound is not stable under basic conditions and decomposes if left at RT overnight.



**12b**. Compound **11b** (0.14 g, 0.17 mmol) was dissolved in DCM (5 mL) containing 3 drops of anisole. The mixture was sonicated for 2 min followed by addition of TFA (3 mL). The mixture immediately became clear and was stirred for 1 h at RT. Evaporation of the solvent yielded a white powder, which was purified on silica gel ($CHCl_3$/MeOH/$NH_4OH$, 4/3/1 then 3/2/1, $R_f$ 0.32 in the latter eluent) to give the desired product as awhite powder (24 mg, 35%). **$^1$H NMR** ($D_2O$, 400 MHz) δ 5.14 (d, *J* = 3.5, 1H), 3.73 (m, 1H), 3.44 (m, 4H), 3.26 (m, 6H), 2.75 (m, 3H), 1.98 (br td, 1H), 1.25 (q, 1H, *J* = 12.4); **$^{13}$C NMR** ($D_2O$, 75 MHz) δ 100.8 (C1′), 86.7 (C4), 75.8 (C6), 75.4 (C5), 73.3 (C3'), 72.1 (C5′), 71.0 (C4'), 55.3 (C2′), 50.7 (C1), 49.5 (C3), 44.2 (C6′), 38.7 (***C***$H_3$), 33.7 (C2); **ESI-MS** for $C_{13}H_{28}N_4O_8S$ $[M+H]^+$ calcd. 401.3, found 481.1 (100) and 401.2.

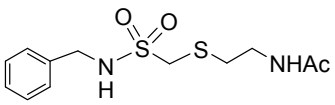


**13a**. Benzylamine (0.22 g, 2 mmol) and bromomethanesulfonyl chloride (**6a**, 0.39 g, 2 mmol) were dissolved in DCM (30 mL). The mixture was cooled to 0$^o$C. TEA (0.3 mL, 2.1 mmol) was added dropwise with stirring. The reaction mixture was warmed to RT and stirred overnight. Filtration was used to remove the precipitated salt and the filtrate was evaporated. The residue was purified on silica gel ($R_f$: 0.42, EtOAc/Hex, 1/3) to give the desired compound as a white crystalline powder (0.39 g, 74%). **$^1$H NMR** ($CDCl_3$, 400 MHz) δ 7.77 (s, 1H), 7.31 (m, 5H), 4.45 (s, 2H), 4.14 (d, *J* = 5.0, 2H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 137.0, 130.0, 128.4, 127.0, 47.1, 44.3. **ESI-MS** for $C_8H_{10}{}^{79}BrNO_2S$ $[M+H]^+$ calcd. 264.0, found 264.1.

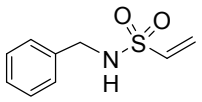


**13b**. Benzyleamine (0.22 g, 2 mmol) and iodomethanesulfonyl chloride (**6b**, 0.48 g, 2 mmol) were dissolved in DCM (30 mL). The mixture was cooled to 0$^o$C. TEA (0.3 mL, 2.1 mmol) was added dropwise with stirring. The reaction mixture was warmed up to RT and stirred overnight. There was no salt formed. The reaction mixture was evaporated and the residue was purified on silica gel ($R_f$:

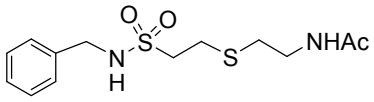
0.38, EtOAc/Hex, 1/3) to give the desired compound as a white crystalline powder (0.32 g, 52%). **$^1$H-NMR** (DMSO-$d_6$, 400MHz) δ 8.0 (s, 1H), 7.30 (m, 5H), 4.54 (s, 2H), 4.15 (d, $J$ = 5.4, 2H); **$^{13}$C-NMR** ($CDCl_3$, 75 MHz) δ 136.8, 129.0, 128.3, 127.9, 47.0, 15.3. **ESI-MS** for $C_8H_{10}INO_2S$ $[M+H]^+$ calcd. 312.0, found 312.0.



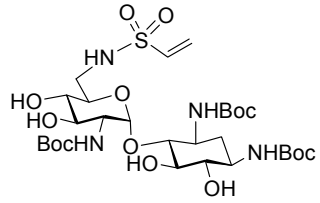

**14**. *N*-benzyl iodomethane-sulfonamide (**13b,** 60 mg, 0.19 mmol) and *N*-acetylcysteamine (21 mg, 0.2 mmol) were mixed in acetone-$d_6$ and $D_2O$ (v/v, 1/1, to mimic the solvents to be used with CoA-SH) in an NMR tube (5 mm). TEA (60 μL, 0.4 mmol) was added and the mixture was sonicated for 1 min. The reaction was followed by NMR. After two days, conversion of the starting material **13b** to product **14** was about 50%. The crude product was purified on silica gel ($R_f$: 0.43, $CHCl_3$/MeOH, 5/1) to give the desired product (26 mg, 40%). **$^1$H NMR** (DMSO-$d_6$, 300 MHz) δ 7.32 (m, 5H, Ph*H*), 6.25 (s, 1H, N*H*), 5.18 (s, 1H), 4.26 (d, $J$ = 5.2, 2H, PhC$H_2$-), 4.15 (s, 2H), 3.39 (m, 2H), 2.81 (t, $J$ = 6.4, 2H), 1.87 (s, 3H, C$H_3$CO); **$^{13}$C-NMR** (DMSO-$d_6$, 75 MHz) δ 172.0 (C=O), 138.8, 128.8, 128.2, 127.8 (Ph), 47.2, 46.5, 38.9, 37.7, 22.3; **ESI-MS** for $C_{12}H_{18}N_2O_3S_2$ $[M+H]^+$ calcd. 303.1, found 303.1.



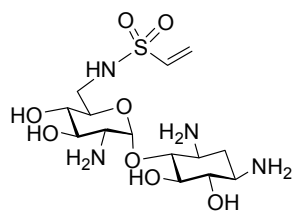

**16b**. Benzylamine (0.22 g, 2 mmol) and bromomethanesulfonyl chloride (**6c**, 0.42 g, 2 mmol) were dissolved in DCM (30 mL). The mixture was cooled to 0$^o$C. TEA (0.6 mL, 4.2 mmol) was added dropwise with stirring. The reaction mixture was warmed to RT and stirred for another 3 h. The reaction mixture was evaporated to ca. 15 mL. The white salt ($Et_3N$ HBr) that precipitated out was filtered off. The filtrate was purified on silica gel ($R_f$: 0.68, EtOAc/Hex, 1/1) to afford the desired product as a colorless oil (0.28 g, 70%). **$^1$H NMR** ($CDCl_3$, 300 MHz) δ 7.31 (m, 5H), 6.44 (dd, $J$ = 16.5, 9.6, 1H), 6.18 (d, $J$ = 16.5, 2H), 5.87 (d, $J$ = 9.6, 1H), 5.07 (bs, 1H), 4.16 (d, $J$ = 6.0); **$^{13}$C-NMR** ($CDCl_3$, 75 MHz) δ 136.9, 136.3, 136.1, 128.9, 128.2, 126.8, 47.2. **ESI-MS** for $C_9H_{11}NO_2S$ $[M+H]^+$ calcd. 198.1, found 198.1.



**17**. *N*-benzyl vinyl-sulfonamide (**16b,** 40 mg, 0.20 mmol) and *N*-acetylcysteamine (21 mg, 0.2 mmol) were mixed in acetone-$d_6$ and $D_2O$ (v/v, 1/1) in an NMR tube (5 mm). TEA (60 μL, 0.4 mmol) was added and the mixture was sonicated for 1 min. After 15 min, some crystals formed. The crystalline product was collected via filtration (30 mg, 85%). **$^1$H NMR** (acetone-$d_6$, 300 MHz) δ 7.38-7.30 (m, 6H), 6.76 (bs, 1H, N*H*), 4.32 (d, *J* = 5.2, 2H, PhC$H_2$-), 3.37 (m, 2H), 3.22 (t, *J* = 6.9, 2H), 2.82 (t, *J* = 6.4), 2.59 (t, *J* = 6.4), 1.84 (s, 3H); **$^{13}$C-NMR** (acetone-$d_6$, 75 MHz) δ 169.4 (C=O), 138.6, 128.7, 128.1, 127.6 (Ph), 52.8, 46.8, 38.9, 31.3, 24.9, 22.2; **ESI-MS** for $C_{13}H_{20}N_2O_3S_2$ $[M+H]^+$ calcd. 317.1, found 317.2.



**18**. 1,3,2'-Tri-*N*-(*tert*-butoxy-carbonyl)neamine **10** (0.31 g, 0.5 mmol) was dissolved in dry DMF (30 mL), followed by the addition of DIPEA (0.3 mL, 1.5 mmol), and a solution of vinylsulfinyl chloride (**6c**, 0.10 g, 0.5 mmol) in chloroform (10 mL) was next added dropwise at RT. The mixture was stirred overnight at RT. The solvent was evaporated *in vacuo*, and the residue was purified on silica gel ($CHCl_3$/MeOH/$NH_4OH$, 15/1/0.1 then 10/1/0.1, $R_f$ :0.36 in the latter eluent) to give the desired product (0.18 g, 51%). **$^1$H NMR** (DMSO-$d_6$, 300 MHz) δ 6.67 (dd, *J* = 18.5, *J* = 10.0, 1H), 6.02 (d, *J* = 18.5, 1H), 5.91 (d, *J* = 10.0, 1H); 1.81 (td, 1H, *J* = 6.4, *J* =12.5). **$^{13}$C NMR** (DMSO-$d_6$, 75 MHz) δ 156.4, 156.0, 155.7 (C=O), 137.7, 125.6 ($SO_2$***C***H=***C***$H_2$), 98.9 (C1′), 81.6 (C4), 78.5, 78.2, 77.9 [O***C***$(CH_3)_3$], 77.2 (C6), 74.6 (C5), 71.4 (C3'), 71.0 (C5′), 56.0 (C4'), 54.2 (C2′), 51.4 (C1), 50.2 (C3), 42.3 (C6′), 36.0 (C2), 28.9 [OC(***C***$H_3)_3$]; **ESI-MS** for $C_{29}H_{52}N_4O_{14}S$ $[M+Na]^+$ calcd. 735.2, found 735.5.



**19**. 1,3,2'-Tri-*N*-(*tert*-butoxycarbonyl)-6′-*N*-vinylsulfinylneamine (**18**, 75 mg, 0.1 mmol ) was mixed in DCM (5 mL) containing 3 drops of anisole.

TFA (2 mL) was added dropwise into the mixture with stirring, The mixture immediately became homogeneous. TLC showed the reaction was complete within 30 min. The solvent was evaporated *in vacuo* to yield the product as a yellowish powder (68 mg, ca. 90%). **ESI-MS** for this compound $C_{14}H_{28}N_4O_8S$ $[M+H]^+$ calcd. 413.2, found 413.2. This compound decomposed on silica gel, it was therefore used as crude for the preparation of **2b**.

**AAC(6′)-Ii inhibition assays**

The inhibition assays were carried out and processed using same procedure as described in Chapter 3.4.3.

**4.5 References**

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22. Note, *1H NMR (DMSO-d6, 300 MHz) d 8.00 (s, 1H), 7.30 (m, 5H), 4.14 (d, J = 5.4, 2H), 3.32 (s, 3H); 13C NMR (DMSO-d6, 75 MHz) d 136.8, 129.0, 128.3, 127.8, 47.3, 40.4.*

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# Chapter Five

In Chapter 2, we have demonstrated that amide-linked aminoglycoside-CoA bisubstrates were nanomolar inhibitors of aminoglycoside *N*-6′-acetyltransferase AAC(6′)-Ii. In Chapter 4, to further investigate whether this enzyme catalyzes the acetyltransfer via stabilization of the tetrahedral intermediate, we synthesized sulfonamide-, sulfoxide- and sulfone-containing bisubstrates, one of which showed substantially improved inhibition. Phosphonamidates have been used for many years as tetrahedral intermediate mimetics; we envisaged that bisubstrates with phosphonamidate-based linkers would be more potent inhibitors and better molecular probes to investigate the mechanism of this enzyme. This Chapter summarizes the attempted synthetic experiments towards such bisubstrates.

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Contributions of co-authors

I carried out all the experiments described under the supervision of Professor Auclair.

# Synthesis of Bisubstrate Analogs with Linkers Containing a Phosphoryl (P=O) Group


## Abstract

Aminoglycoside *N*-6′-acetyltransferases (AAC(6′)s) are important determinants of antibiotic resistance. A good mechanistic understanding of these enzymes is essential to overcome aminoglycoside resistance. We have attempted the synthesis of bisubstrates **1**, **2** and **3a-b** (Fig. 5.1), which represent useful probes for studying catalysis process by AAC(6′)s. The linkers of these bisubstrates contain a phosphoryl group to mimic the tetrahedral intermediate proposed for catalysis by AAC(6′)s. The synthesis of these targets poses a number of challenges due to the water solubility of the starting materials and incompatibility of P(III) chemistry with water. Attempted synthetic studies are summarized.

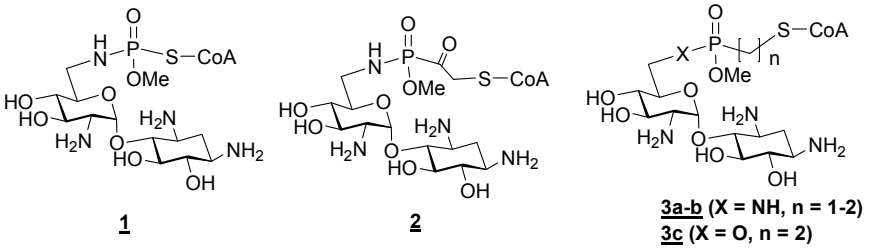




***Figure 5.1***. Proposed bisubstrate targets with different phosphoryl-containing linkers to mimic the tetrahedral intermediate proposed for catalysis by AAC(6′)s.

## 5.1 Introduction

Aminoglycosides are useful antibiotics acting by binding to the 16S RNA A site of bacteria. The ever-rising bacterial resistance, however, threatens the use of these important medicines. The aminoglycoside resistance is mainly caused by three types of enzymes, nucleotidyltransferases (ANTs), phosphortransferases (APHs) and acetyltransferases (AACs). Among them, aminoglycoside *N*-6′-

acetyltransferase (AAC(6′)) is one of the most clinically relevant resistance determinants. AAC(6′)s use acetyl coenzyme A as a cofactor to transfer an acetyl group to 6′-$NH_2$ of most aminoglycosides. The acetylated aminoglycosides display a greatly reduced affinity for bacterial RNA. It is proposed that a tetrahedral intermediate is formed during the acetyltransfer process by these enzymes. We have reported the use of amide-linked aminoglycoside-CoA bisubstrates as mechanistic probes for these enzymes. Most bisubstrates were nanomolar inhibitors of AAC(6′)-Ii and successfully crystallized with the enzyme.[1] To further investigate whether this enzyme catalyzes the acetyltransfer via stabilization of the tetrahedral intermediate, we synthesized sulfonamide-, sulfoxide- and sulfone-containing bisubstrates. To our surprise, the bisubstrate analog with sulfonamide linker was expected to best mimic the tetrahedral intermediate, but showed 50 fold decreased inhibition compared to the one with an amide linker (Fig. 5.2, **4a** vs. **4b**, for details see Chapter 2 & 4). These results support the earlier suggestion by Wright and co-workers that AAC(6′)-Ii may catalyse the acetyltransfer reaction mainly via proximity effects.[2, 3] Alternatively, sulfonamides may not be good mimics of the enzymatic intermediate.

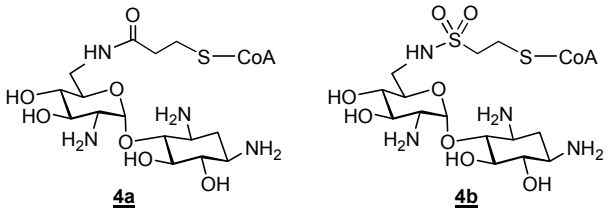



***Figure 5.2.*** Comparison of inhibition constants of amide-linked and sulfonamide-linked bisubstrates.

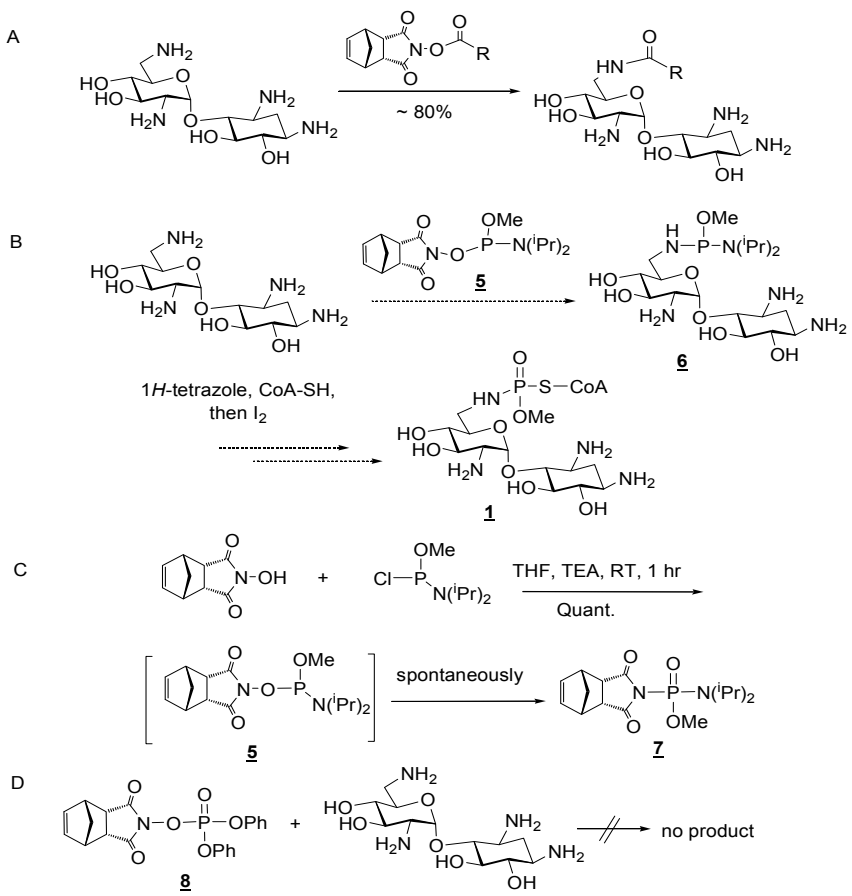
Indeed, very few examples of the use of sulfonamides as the tetrahedral intermediate have been reported (see introduction of Chapter 4). Phosphoryl groups, on the hand, have been extensively used as mimics for tetrahedral intermediates.[4-17] Bisubstrates that mimic tetrahedral intermediate better than amide-linked bisubstrates represent useful probes to clarify the mechanism of AAC(6)s. We therefore embarked on the synthesis of bisubstrate **1-3** (Fig. 5.1).

The synthesis of these targets poses a number of challenges. Both starting materials have highly dense functional groups; regio- and chemo-selective modifications require judicious and laborious protection/deprotection schemes. CoA-SH, one of the starting materials, is only soluble in water, while the very efficient P(III) chemistry is not compatible with aqueous solutions. Moreover, the functional groups of CoA-SH can not withstand the harsh conditions employed for protection/deprotection of aminoglycosides. Finally, CoA-SH is expensive and only available in small amounts. Our attempted syntheses of **1-3** are reported here.

## 5.2 Synthesis

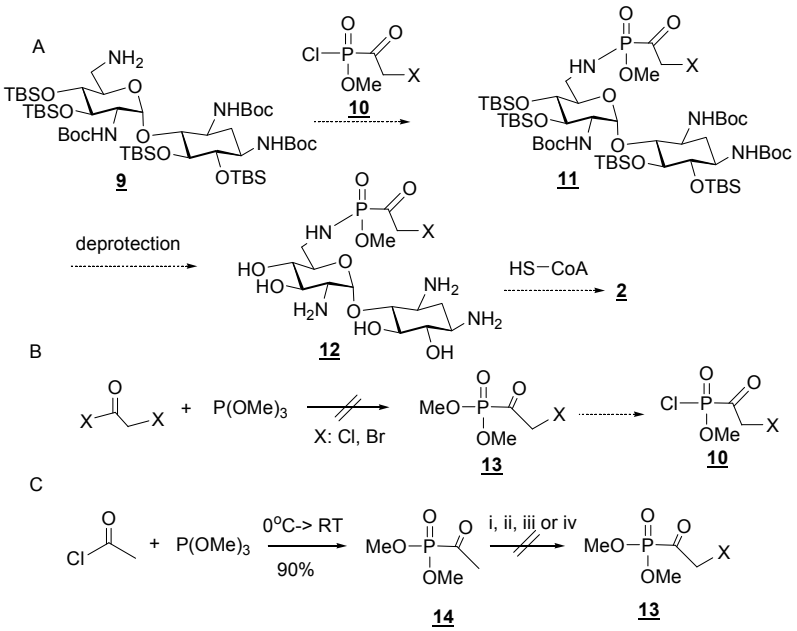
We envisaged to assemble the first target, phosphorothioamidate **1** via a regioselective approach, expanding on the regio-selective acylation of unprotected aminoglycosides previously reported by us (Scheme 5.1, A).[1] We hoped to achieve regio-selective *N*-6′-phosphoramidite transfer (Scheme 5.1, B). The resulting phosphorodiamidite **6** would next be reacted with CoA-SH in the presence of tetrazole as the activating agent, followed by $I_2$ promoted oxidation to yield target bisubstrate **1**.[18] Thus, commercially available *endo*-*N*-hydroxy-5-norbornene-2,3-dicarboximide was used to react with methyl *N,N*-diisopropyl-chlorophosphoramidite to prepare the phosphoramidite-transfer reagent **5** (Scheme 5.1, C). Surprisingly, phosphorodiamidite **5** spontaneously underwent a rearrangement to give phosphorodiamidate **7** in high yield (for characterization of this rearrangement, refer to Chapter 6).

Based on this result, phosphate **8** was next tested as a regio-selective phosphoramidyl-transfer reagent (Scheme 5.1, D). When reacted with neamine under various conditions, it did not afford any detectable amount of the desired product.




***Scheme 5.1***. Synthetic plan and first attempt to prepare bisubstrate **1**.

It was envisaged to assemble target **2** via attack of CoA-SH at the α-halocarbonyl electrophile **12** (Scheme 5.2, A). Michaelis-Arbuzov-type reaction of acyl halides with trialkyl phoshites are well documented.[19] Thus acylphosphonyl **10** represents a logical building block for installing the linker. Dimethyl acylphosphonate **13** would be the precursor to prepare **10**. Unexpectedly, treatment of trimethylphosphite with either 2-chloroacetyl chloride or 2-bromoacetyl bromide yielded inseparable mixtures (Scheme 5.2, B). Alternatively, α-bromination of acetyl dimethylphosphonate (**14**) was attempted to prepare **13**, but was not successful (Scheme 5.2, C). Instead acylphosphonate **14** decomposed rapidly in whether acidic or basic aqueous solutions. Target **2** seemed unrealistic and was not pursued further.




***Scheme 5.2***. Synthetic routes attempted for the synthesis of bisubstrate **2**. Reagents and conditions: i) NBS, THF; NCS, $CHCl_3$; iii) $Br_2$, MeOH; iv) $PBr_3$, $CCl_4$. Abbreviations NBS: *N*-bromosuccinimide; NCS: *N*-chlorosuccinimide.

Bisubstrates **3a-b** were designed for their ease of synthesis. The extra carbons between the phosphoryl and the sulfur groups were not expected to compromise inhibition. This assumption was supported by our previous results with the bisubstrate inhibitor (Fig. 5.3) containing 2 carbons between the amide carbonyl and the sulfur atom, yet the most potent known inhibitor of AAC(6′)-Ii.[1]

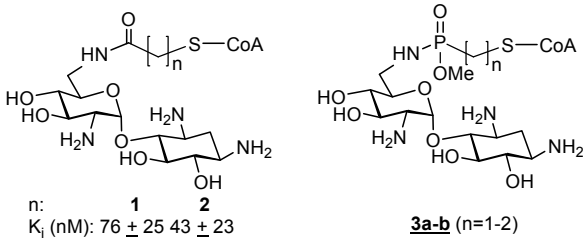
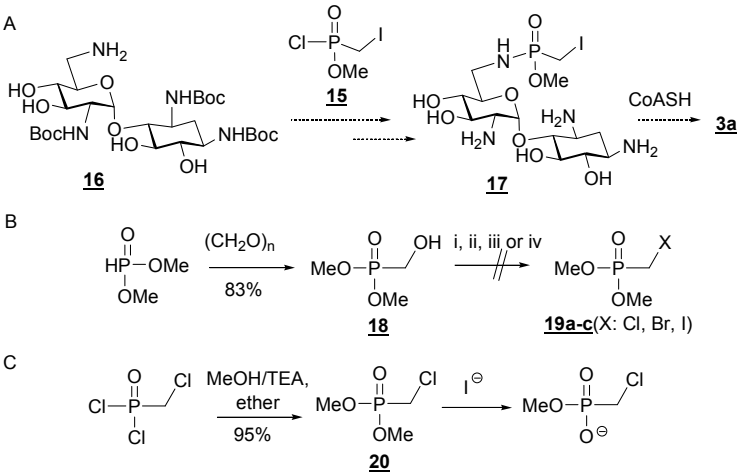



***Figure 5.3.*** Amide-linked bisubstrates and phosphoramidate-linked bisubstrates.

The key intermediate in the synthesis of bisubstrate analog **3a** was iodomethylphosphonyl chloride **15** (Scheme 5.3), which may be derived from dimethyl iodomethylphosphonate **19c**. α-Hydroxymethylphosphonate **18** was used in numerous attempts to prepare the corresponding α-

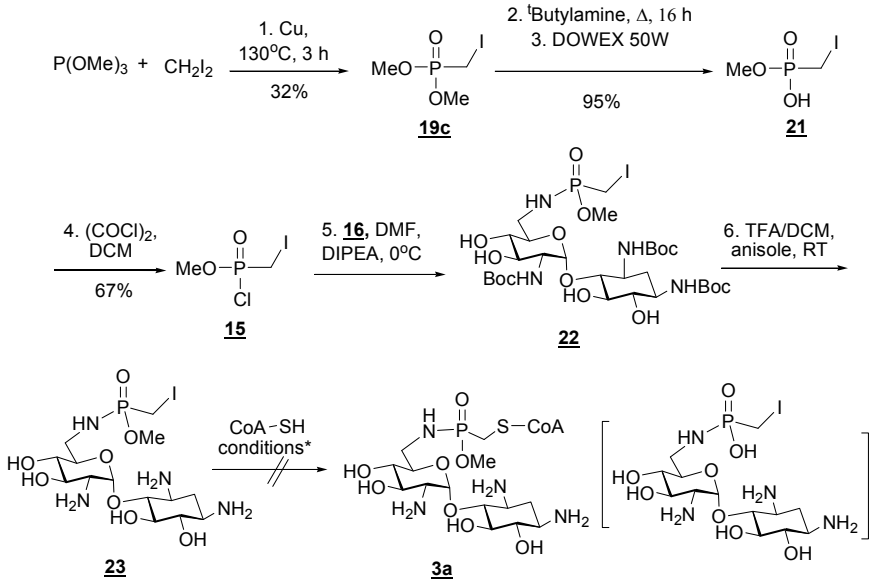
halomethylphosphonate **19a-c**, without success (Scheme 5.3, B). Conventional halogenation procedures led to complex mixtures, even with very mild chlorination reagents such as trichlorocyanuric acid.[20] Alternatively, attempts to convert chloromethyl dimethylphosphonate (**20**) into iodomethyl dimethylphosphonate via the Finkelstein reaction yielded the demethylation product (Scheme 5.3, C).




***Scheme 5.3***. First synthetic plan for the preparation of bisubstrate **3a**. Reagents and conditions: i) NBS, $PPh_3$; ii) $PBr_3$, $CCl_4$; iii) $I_2$, $PPh_3$; d) trichlorocyanuric acid, DMF.

As an alternative route, the Michaelis-Arbuzov reaction of trimethylphosphite and diiodomethane was attempted but proceeded with an extremely low yield due to the decomposition of diiodomethane and the rearrangement of trimethylphosphite to dimethyl methylphosphonate.[21-23] To optimize this reaction, various conditions and catalysts were tested. The addition of copper powder, reaction at a lower temperature (130°C, literature reported 160°C[21]) and for a shorter reaction time (3 h instead of 12 h) afforded iodomethyl dimethylphosphonate (**19c**) in ca. 30% yield. The copper is believed to slow down the decomposition of diiodomethane (Scheme 5.4). A typical two-step chlorination procedure[10, 24] was adopted to prepare phosphonyl chloride **15** (Scheme 5.4). A number of nucleophilic reagents such as NaOH/MeOH,[10, 11] $LiN_3$/DMF[25], TMSBr/Py[26], *tert*-butylamine[24], thiophenol/TEA[27], and NaI/acetone[28, 29] were reported to cleave dimethyl phosphonates to the mono-

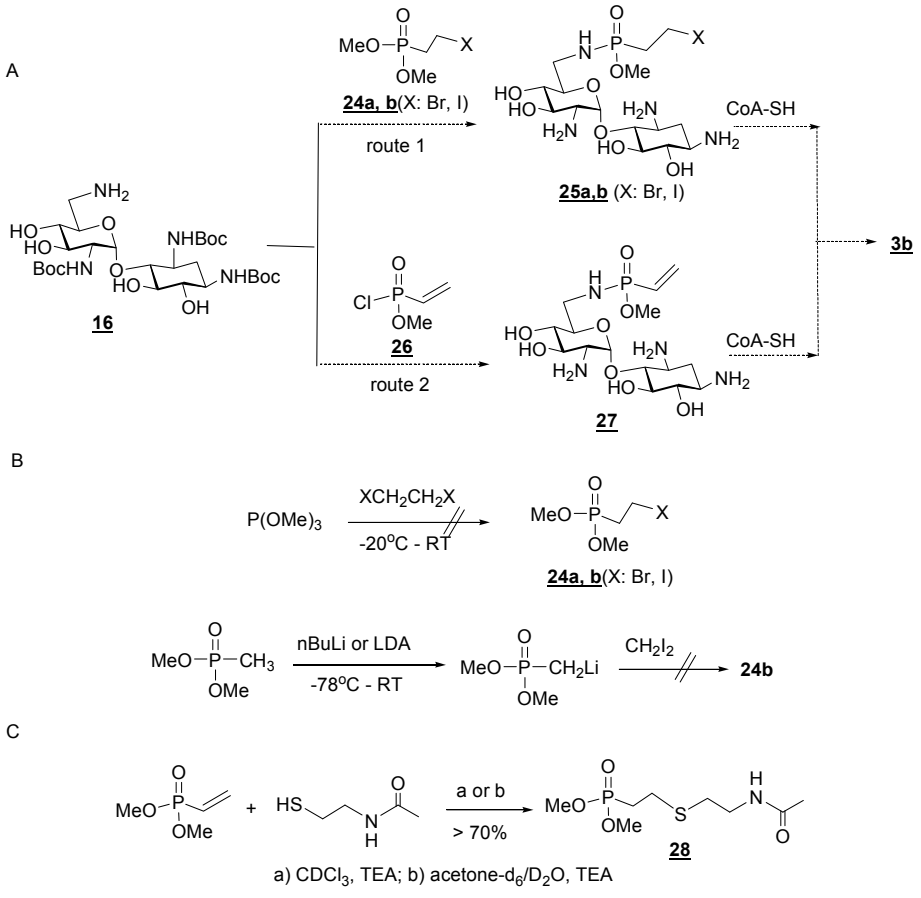
phosphonic acids. We found that *tert*-butylamine worked the best to convert **19c** to **21**. The phosphonic acid **21** was next converted to phosphonyl chloride **15** in good yield using oxalyl choride. Treatment of 1,3,2'-tri-*N*-(*tert*-butoxycarbonyl)neamine **16,** prepared as described in Chapter 4) with phosphonyl chloride **15** in the presence of Hunig's base afforded the *N*-6′-phosphonamidate intermediate **22**, which yielded phosphonamidate **23** after removal of Boc groups. The iodomethylphosphonamidate **23** was reacted with CoA-SH under various conditions, none of which yielded the desired product; instead the demethylated product was observed (Scheme 5.4). It was reasoned that the resulting negative charge may repulse the approaching thiol.




***Scheme 5.4.*** Attempted synthesis of bisubstrate **3a**. Conditions tested separately: i) TEA/$H_2CO_3$ buffer, pH ~ 8.4; ii) $H_2O$/acetone, DIPEA; iii) $H_2O$/acetone, TEA; iv) $H_2O$, 0.1 N NaOH

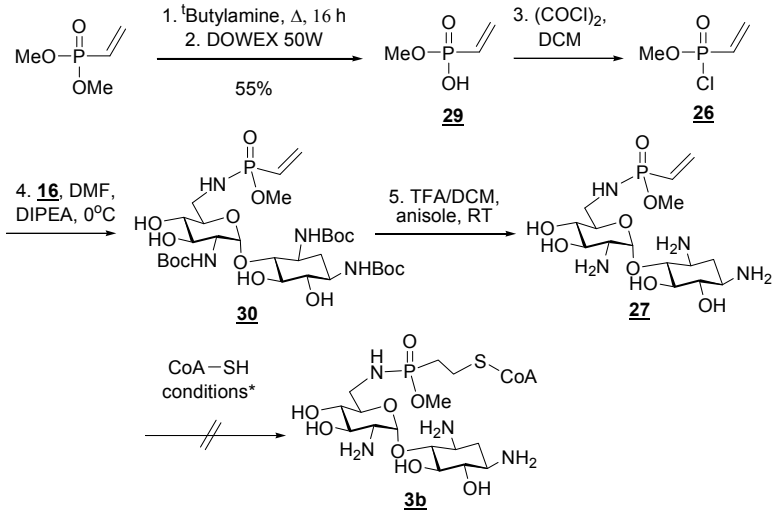
The lack of reaction between iodomethylphosphonamidate **23** and CoA-SH thiol may also be explained by a significant hindrance at the iodomethylene carbon. To avoid this problem, we decided to lengthen the distance between the phosphorus and the iodomethylene carbon with an extra methylene group. Therefore, bisubstrate **3b** was the next target. Two reasonable synthetic routes were envisaged to prepare this bisubstrate: *S*-alkylation of halide **25a-b**, or Michael addition onto vinylphosphonate **27** (Scheme 5.5, A).

The Michaelis-Arbuzov reaction of trimethylphosphite with 1,2-dibromoethane or 1,2-diiodoethane yielded a complex mixture (Scheme 5.6, B). Nucleophilic monosubstitution of diiodomethane with lithium dimethyl methyl phosphonate did not succeeded either.[30, 31] Thus route 2, which involves a Michael addition of the CoA-SH thiol onto vinylphosphonate **27** was tested (Scheme 5.5, route 2). *S*-alkylations of vinylphosphonates have been reported to proceed either via a radical process,[32] or via a Michael addition with vinylphosphonates having α-electron-withdrawing groups (EWGs).[33] To investigate the feasibility of this scheme, model reactions were carried out using dimethyl vinylphosphonate and *N*-acetylcysteamine (as a surrogate for CoA-SH) (Scheme 5.5, C). Surprisingly, the addition was complete within one hour at RT in both organic and aqueous solutions.




***Scheme 5.5***. Synthetic plan for the preparation of bisubstrate **3b**.

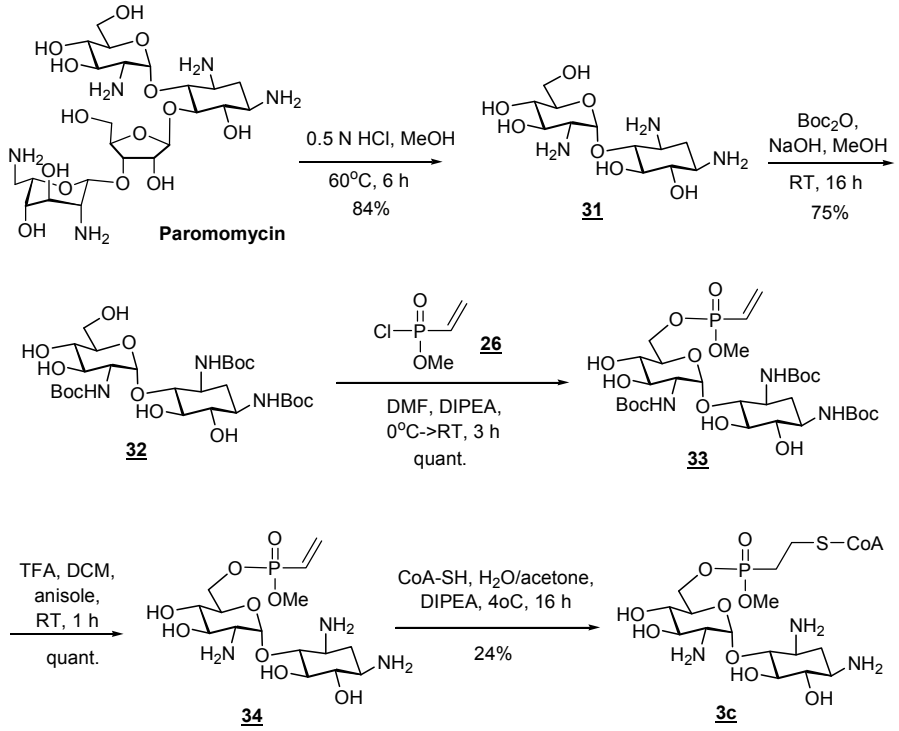
Thus, the synthesis of bisubstrate **3b** was initiated as shown in Scheme 5.6. Mono demethylation of dimethyl vinylphosphonate (commercially available) yielded phosphonic acid **29**, which was converted to phosphonyl chloride **26**. Treatment of tri-*N*-(*tert*-butoxycarbonyl)neamine (**16**) with phosphonyl chloride **26** in the presence of Hunig base afforded the *N*-6′-phosphonamidate intermediate **30**, which yielded phosphonamidate **27** after removal of Boc groups. The Michael addition of CoA-SH onto vinylphosphonamidate **27** was not successful under the conditions tested (Scheme 5.6). As described for the reaction between iodomethylphosphonamidate **23** and CoA-SH (Scheme 5.4), the demethylated product was observed. Alternatively, radical reactions were also tested but did not produce the desired product either.




***Scheme 5.6***. Attempted synthesis of bisubstrate **3b**. Conditions tested separately: i) TEA $H_2CO_3$ buffer, pH ~ 8.4; ii) $H_2O$/acetone, TEA; iii) $H_2O$/acetone, DIPEA; iv) $H_2O$, 0.1 N NaOH; v) $H_2O$/CAN, AIBN, 50°C.

The difficulty of addition of thiol CoA-SH onto vinyl phosphonamidate **27** was surprising and unexpected. However the vinyl dimethylphosphonate reacts with *N*-acetyl cysteamine (Scheme 5.5, C) produced the desired adduct product in good yields. This fact prompted us to changed our target molecule into **3c** (Fig. 5.1), in which paromamine was used for substrate instead of neamine (Scheme 5.7). We reasoned that the more electronegative O (vs.N) with stronger electron-

withdrawing would make vinyl phosphonate **34** (Scheme 5.7) a better Michael addition receptor than **27** (Scheme 5.6). Thus, methanolysis of paromomycin sulfate gave paromamine **31** in good yield.[34] Treatment of **31** with Boc anhydride provided 1,3,2'-tri-*N*-(*tert*-butoxycarbonyl) paromamine **32**, which was reacted with **26** to yield **33**. Removal of Boc groups of **33** generated **34**, which was reacted with CoA-SH under basic aqueous solution to produce the bisubstrate **3c** as two diasteromers in 24% yield (based on CoA-SH). The regiochemistry site of phosphorylation is supported by $^{31}$P-$^{1}$H HMBC correlation spectrum, in which the 6′-$CH_2$ are correlated to linker phosphorus $^{31}$P.




Scheme 5.7. Synthesis of bisubstrate **3c** containing phosphonate linker.

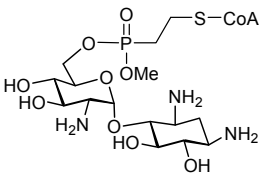
## 5.3 Conclusion

Phosphonates are better mimetics for tetrahedral intermediates. Various synthetic approaches were attempted to prepare bisubstrates with linkers containing a phosphoryl group. In spite of the challenges associated with the incompatibility of P(III) chemistry in aqueous solutions, we successfully synthesized paromamine-CoA bisubstrates (diasteromers) containing a

phosphonate linker **3c** (Scheme 5.8). The biological activities of these two bisubstrates are under investigation.

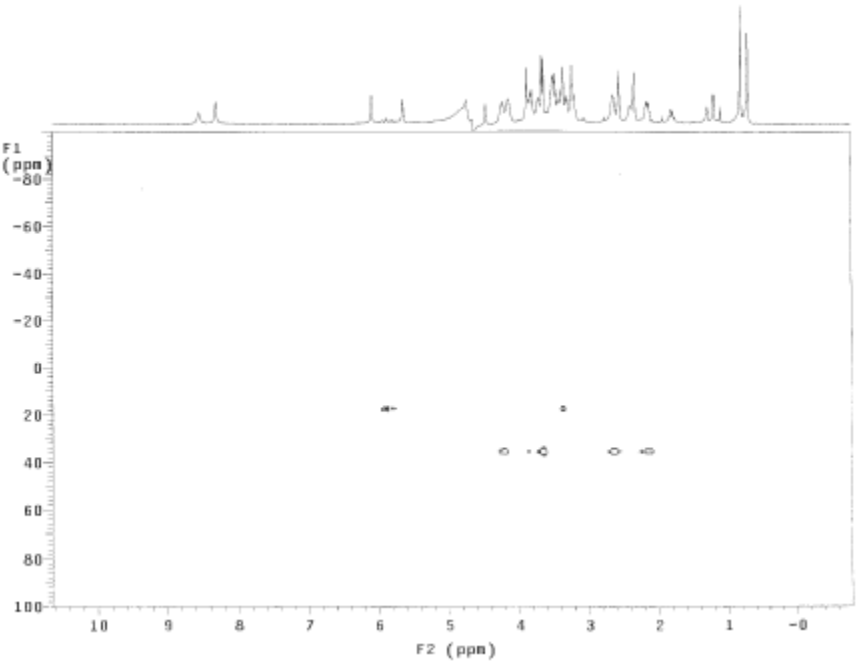
## 5.4 Experimental section

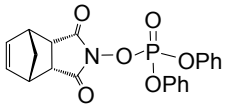
**General**: refer to experimental section of Chapter 3.



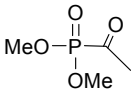
**3c**. Crude product **33** (55 mg, ca. 0.06 mmol) was dissolved in a mixed solvent of water (5 mL) and acetone (1 mL), CoA-SH (sodium salt, 15 mg ) was directly added into this solution and followed addition of DIPEA (0.25 mL). The mixture was sonicated for 10 min. and then stirred for 16 h at 4°C. The solution was then poured into water (30 mL) and washed with EtOAc ( 10 mL × 2). The aqueous solution was separated and freeze dried to product a yellowish powder, which was further purified by reverse phase HPLC to yield the bisubstrate **3c** as two diasteromers (5.4 mg, yield: 24.2%). The HPLC purification procedure was same as the one described in Chapter 3. The two diasteromers have retention time at 13.30 and 13.75 min, respectively. The purity of the two diasteromers was evaluated by HPLC using an isocratic elution with ratios of phase A/phase B of either 65/35 (method A) or 55/45 (method B), showing purity > 95%. **$^{1}$H NMR** ($D_2O$, 500 MHz) δ 8.55 (s, 1H), 8.31 (s, 1H), 6.08 (d, $J$ = 4.0, 1H), 5.64 (br s, 1H), 4.49 (bs, 1H), 4.24 (bs, 2H), 4.16 (bs, 2H), 3.90 – 3.20 (m, 21H), 2.66 (m, 2H), 2.54 (t, $J$ = 6.5 Hz, 2H), 2.34 (m, 3H), 2.17 (m, 2H), 1.79 (q, J = 12.5, 1H), 0.78 (bs, 3H), 0.70 (bs, 3H) ; **$^{13}$C NMR** ($D_2O$, 400 MHz by HMQC and HMBC): δ 174.7, 174.4 (C=O of CoA), 150.2, 148.7, 144.9, 142.7, 118.5 (aromatic carbons of adenine), 96.7, 87.5, 83.3, 79.3, 74.9, 74.3, 73.8, 72.2, 71.8, 71.6, 68.6, 68.5, 65.1, 64.7, 53.8, 53.7, 53.2, 51.3, 39.9, 38.2, 35.4, 35.3, 30.2, 27.6, 24.8, 23.4, 23.3, 20.8, 18.4; **$^{31}$P NMR** ($D_2O$, 81 MHz) δ 35.7, 35.6 (diasteromers); **ESI-MS** for $C_{36}H_{66}N_{10}O_{25}P_4S$ $[M+H]^+$ calcd. 1195.23, found 1195.09.

$^{31}$P-$^{1}$H HMBC:

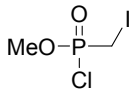


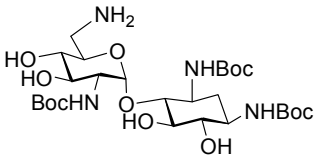

**8**. *Endo-N*-hydroxy-5-norbornene-2,3-dicarboximide (1.79 g, 10 mmol) and diphenyl phosphorochloridate (2.75 g, 11 mmol) were dissolved in DCM (50 mL). TEA (2 mL, 14 mmol) was added dropwise. The mixture was stirred for 6 h at RT. The salt was filtered off and the filtrate was washed with 1 N $NaHCO_3$ (3 × 10 mL). The organic phase was dried over $Na_2SO_4$ and evaporated to afford **9** (3.97 g, 96%). **$^{1}$H NMR** ($CDCl_3$, 300 MHz) δ 7.46-7.22 (m, 10H), 6.16 (bs, 2H), 3.47 (s, 2H), 3.31 (s, 2H), 1.79 (d, *J* = 8.8, 1H), 1.53 (d, *J* = 8.8, 1H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 170.4, 156.9, 135.0, 130.0, 126.2, 120.4, 51.4, 45.2, 43.1; **$^{31}$P NMR** ($CDCl_3$, 81 MHz) δ -10.8; **ESI-MS** for $C_{21}H_{18}NO_6P$ $[M+Na]^+$ calcd. 434.2, found 434.2.



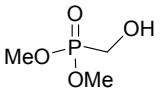

**14**. This compound was prepared using a procedure reported before.[35] **$^{1}$H NMR** ($CDCl_3$, 200 MHz): δ 3.73 (d, *J* = 10.6, 6H), 2.35 (d, *J* = 6.6, 3H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz): δ 208.2 (d, *J* =150), 54.2 (d, *J* = 5.6), 31.0; **$^{31}$P NMR** ($CDCl_3$, 81 MHz): δ -0.60; **ESI-MS** for $C_4H_9O_4P$ $[M+H]^+$ calcd. 153.2, found 153.2.



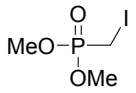
**15**. Compound **21** (0.33 g, 1.4 mmol) and DMF (2.5 μL, 0.03 mmol) were dissolved in dry DCM (10 mL). Oxalyl chloride (0.15 mL, 2 mmol) was added dropwise. The solution was stirred at 0°C for 20 min, warmed to RT, and stirred for one hour. The reaction mixture was concentrated to give phosphonochloridate **15** as a yellowish oil, which was used immediately in reaction with the 1,3,2′-tri-*N*-(*tert*-butoxycarbonyl)neamine. **$^{1}$H NMR** ($CDCl_3$, 300 MHz): δ 3.96 (d, *J* = 10.8, 3H), 3.46 (d, *J* = 8.8, 2H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 54.7 (d, *J* = 5.8), -9.3 (d, *J* = 155); **$^{31}$P NMR** ($CDCl_3$, 81 MHz): δ 34.0; **ESI-MS** for $C_2H_5ClIO_2P$ $[M+H]^+$ calcd. 254.9, found 254.9.



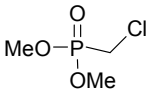
**16**. This compound is prepared as described for compound **10** in Chapter 4.



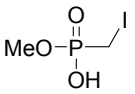
**18**. This compound was prepared using a previous reported procedure.[36] Yield: 76%. **$^{1}$H NMR** ($CDCl_3$, 200 MHz): δ 4.89 (bs, 1H), 3.96 (d, *J* = 5.8, 2H), 3.82 (d, *J* = 10.6, 6H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz): δ 54.3 (d, *J* = 5.6), 62.0 (d, *J* = 155); **$^{31}$P NMR** ($CDCl_3$, 81 MHz): δ 27.9; **ESI-MS** for $C_3H_9O_4P$ $[M+H]^+$ calcd. 141.1, found 141.1.



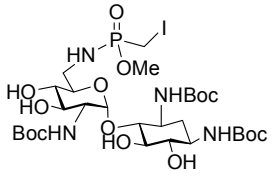
**19c**. Diiodomethane (16 g, 60 mmol) and copper beads (1 g, diameter < 0.5 mm) were mixed and heated to 130°C. Trimethyl phosphate (7.7 g, 60 mmol) was added through an automatic syringe pump at speed of 5 mL/h. The mixture was refluxed for 3 h. After distillation of the by-product dimethyl methylphosphonate (house vacuum at about 70°C), the remaining yellowish liquid afforded the desired compound in low yield (32%, purity > 95% by NMR). **$^{1}$H NMR** ($CDCl_3$, 200 MHz) δ 3.70 (d, *J* = 11.0, 6H), 2.95 (d, *J* = 10.2, 2H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 54.1 (d, *J* = 5.8), -15.8 (d, *J* = 155); **$^{31}$P NMR** ($CDCl_3$, 81 MHz): δ 23.9; **MS** for $C_3H_8IO_3P$ $[M+H]^+$ calcd. 250.9334, found 250.9329.



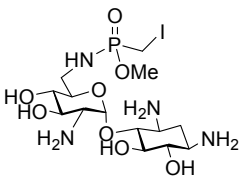
**20**. This compound was prepared using a previously reported procedure.[37] Yield: 62%. **$^1$H NMR** ($CDCl_3$, 200 MHz) δ 3.71 (d, *J* = 10.8, 6H), 3.46 (d, *J* = 10.6, 2H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 53.9 (d, *J* = 5.6), 32.7 (d, *J* = 156); **$^{31}$P NMR** ($CDCl_3$, 81 MHz): δ 22.2; **LRMS** for $C_3H_8{}^{35}ClO_4P$ [m/z] calcd. 158.0, found 158.0.



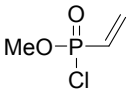
**21**. Compound **22c** (2.50 g, 1 mmol) was dissolved in *tert*-butylamine (20 mL) and the resulting solution was refluxed for 16 h. The reaction mixture was concentrated (10 mL) and filtered to collect the crystalline methyl iodomethylphosphonate *tert*-butylamine salt (3.13 g, quantitative yield). This salt (2.0 g, 6.5 mmol) was dissolved in chloroform (60 mL) and Dowex 8-100 resin (10 g, 200-400 mesh, wet) was added. After 30 min of shaking, the resin was removed by filtration, and the filtrate was dried over $Na_2SO_4$. After evaporation of the solvent, the desired compound was obtained as a yellowish oil (1.1 g, 72%). **$^1$H NMR** ($CDCl_3$, 300 MHz) δ 11.60 (bs, 1H), 3.81 (d, *J* = 10.8, 3H), 3.00 (d, *J* = 8.8, 2H); **$^{31}$P NMR** ($CDCl_3$, 81 MHz) δ 24.1; **ESI-MS** for $C_2H_6IO_3P$ $[M+H]^+$ calcd. 236.9, found 236.9.



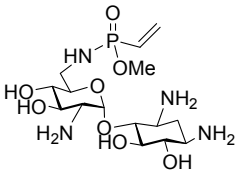
**22**. Compound **16** (125 mg, 0.2 mmol) and diisopropyl ethylamine (50 μL, 0.25 mmol) were dissolved in anhydrous DMF (20 mL). The mixture was cooled to 0$^o$C, and methyl iodomethylphosphonate chloride (50 mg, 0.2 mmol) was added dropwise. The reaction mixture was stirred for one hour at 0$^o$C and another two hours at RT . Reaction completion was monitored by TLC, $R_f$ = 0.40 (tailing, $CHCl_3$/MeOH/$NH_4OH$, 4/1/0.1) and **ESI-MS** for $C_{29}H_{54}IN_4O_{14}P$ $[M+Na]^+$ calcd. 863.2, found 863.2. The reaction mixture was evaporated to dryness to give a white powder. This solid was triturated with water (20 mL) to remove the ammonium salt. The remaining solid material was dried to afford the crude product as a white powder (138 mg, 82%). **$^{31}$P NMR** (DMSO-$d_6$, 81 MHz) 28.3 and 28.9 (diasteromers). Attempted purification on silica gel led to decomposition. It was therefore used as a crude sample in the next step.



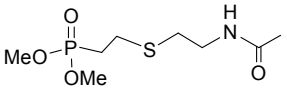
**23**. Crude **25** (80 mg, ca. 0.06 mmol) was mixed with DCM (10 mL) containing 1 drop of anisole. To the mixture, TFA (2 mL) was added and the mixture became homogeneous immediately. The reaction was complete after 1 h at RT as monitored by TLC and ESI-MS. Evaporation of the volatiles afforded a white salt, which was dissolved in water (20 mL) and washed with chloroform (3 × 5 mL). The aqueous phase was lyophilized to afford the product as a white powder (48 mg, ca, 60%). **$^{31}$P NMR** ($D_2O$, 81 MHz) δ 26.4, 27.2. **ESI-MS** for $C_{14}H_{30}IN_4O_8P$ $[M+H]^+$ calcd. 541.1, found 541.0. This compound is also prone to decomposition on silica gel; it was directly used in the next step.



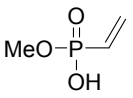
**26**. Compound **30** (250 mg, 2 mmol) and dry DMF (25 μL) were dissolved in dry DCM (15 mL). The mixture was cooled to 0$^o$C, to which oxalyl chloride (0.26 mL, 3 mmol) was added dropwise. The reaction mixture was stirred for 30 min and then for another hour at RT. All the volatiles were evaporated under vacuum (45$^o$C, 15 mmHg) to yield the desired product as yellowish oil (0.32 g). This crude product was directly used in the next reaction. **$^1$H NMR** ($CDCl_3$, 200 MHz) δ 6.80-6.22 (m, 3H), 3.95 (d, $J$ = 10.8, 3H); **$^{31}$P-NMR** ($CDCl_3$, 81 MHz) δ 29.9.



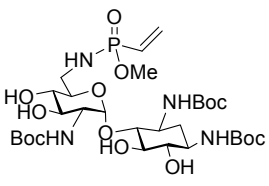
**27**. Crude **31** (75 mg) was mixed with DCM (10 mL) containing 1 drop of anisole. To the mixture, TFA (2 mL) was added and the mixture became homogeneous immediately. The reaction was completed after 1 h at RT as monitored by TLC and ESI-MS. Evaporation of the volatiles afforded a white salt, which was dissolved in water (20 mL) and washed with chloroform (3 × 5 mL). The aqueous phase was lyophilized to afford the crude product as a white powder (36 mg, ca. 34%). **$^{31}$P NMR** ($D_2O$, 81 MHz) δ 24.2, 24.8. **ESI-MS** for $C_{15}H_{31}N_4O_8P$ $[M+H]^+$ calcd. 427.2, found 427.2. Attempts to purify this compound by silica gel led to decomposition, therefore the crude product was used for the conjugation of CoA-SH.



**28**. Dimethyl vinylphosphonate (68 mg, 0.5 mmol) and *N*-acetylcysteamine (60 mg, 0.5 mmol) were dissolved in a mixture of acetone-$d_6$/$D_2O$ (1 mL, 1/1). The reaction was monitored by NMR. There was no reaction before addition of TEA. The reaction was, however, complete within 2 h after addition of TEA (100 μL, 0.7 mmol). The mixture was purified on silica gel ($R_f$: 0.43, $CHCl_3$/MeOH, 10/1) to yield the desired product (89 mg, 70%). **$^1$H NMR** ($CDCl_3$, 200 MHz) δ 6.28 (bs, 1H), 3.60 (d, *J* = 10.6, 6H), 3.31 (q, *J* = 6.4, 2H), 2.90 (t, *J* = 6.8, 2H), 2.85 (d, *J* = 6.4, 2H), 1.95 (s, 3H), 1.80 (m, 2H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 176.0, 54.2 (d, *J* = 5.2), 40.5, 33.2, 30.6, 29.2 (d, *J* = 10.2), 22.5; **$^{31}$P NMR** ($CDCl_3$, 81 MHz) δ 34.1; **ESI-MS** for $C_8H_{18}NO_4PS$ $[M+H]^+$ calcd. 256.1, found 256.1.

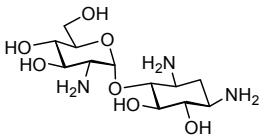


**29**. Dimethyl vinylphosphonate (4.0 g, 29.4 mmol) was dissolved in *tert*-butylamine (20 mL) and refluxed for 16 h at 50°C. The reaction mixture was concentrated to ca. 10 mL and filtered to collect the methyl vinylphosphonate *tert*-butylaminium salt as a white crystal (3.2 g, yield: 56%). **$^1$H NMR** ($CDCl_3$, 200 MHz) δ 8.59 (bs, 3H), 6.20-5.60 (m, 3H), 3.52 (d, *J* = 10.8, 3H); **$^{31}$P NMR** ($CDCl_3$, 81 MHz) δ 13.8. This salt (1.95 g, 10 mmol) was dissolved in chloroform (60 mL) and Dowex 8-100 resin (8 g, 200-400 mesh, wet) was added. The heterogeneous mixture was shaken for 30 min. The resin was removed by filtration, the filtrate was dried over $Na_2SO_4$ and evaporated the solvents under vacuum to give the desired compound as a yellowish oil (0.81 g, 66%). **$^1$H NMR** ($CDCl_3$, 200 MHz) δ 11.99 (bs, 1H), 6.30-5.98 (m, 3H), 3.71 (d, *J* = 10.8, 3H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 127.0, 124.5, 52.3 (d, *J* = 5.8); **$^{31}$P NMR** ($CDCl_3$, 81 MHz) δ 21.1; **ESI-MS** for $C_2H_6IO_3P[M+H]^+$ calcd. 236.9, found 236.9.

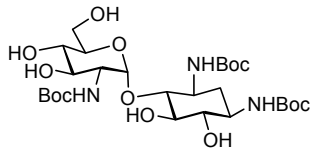


**30**. Compound **15** (125 mg, 0.2 mmol) and diisopropyl ethyl amine (50 μL, 0.25 mmol) were dissolved in anhydrous DMF (20 mL). The mixture was cooled to 0°C, and methyl vinylphosphonyl chloride (28 mg, 0.2 mmol) was added. The reaction mixture was stirred for one hour at 0°C and for another

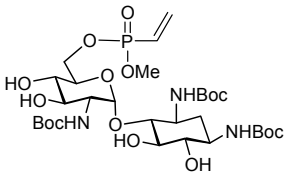
two hours at RT. Reaction completion was monitored by TLC, $R_f$ = 0.38 ($CHCl_3$/MeOH/$NH_4OH$, 4/1/0.1) and **ESI-MS** for $C_{30}H_{55}N_4O_{14}P$ $[M+Na]^+$ calcd. 749.3, found 749.2. **$^{31}$P NMR** (DMSO-$d_6$, 81 MHz) 25.1 and 25.7.



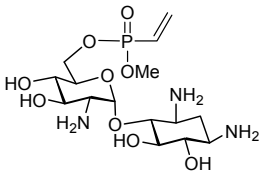
**31**. Paromomycin sulfate (5 g, 7.0 mmol) was dissolved in MeOH (100 mL), to the mixture conc. HCl (12 N, 4 mL) was added. The mixture was refluxed for 6 h, and evaporated to a volume of 20 mL. Ether (50 mL) was added and the mixture was put in a fridge for 2 h. Filtration to collect the product, paromamine hydrogen chloride salt as white crystal (3.65 g, yield: 84%). **$^1$H NMR** ($D_2O$, 400 MHz) δ 5.52 (d, *J* = 4.0, 1H), 3.82-3.69 (m, 4H), 3.62 (dd, *J* = 6.8, 5.2, 1H), 3.55 (t, *J* = 8.8, 1H), 3.46 (m, 2H), 3.40-3.32 (m, 2H), 3.21 (m, 1H), 2.38 (td, *J* = 12.0, 4.0, 1H); 1.75 (q, *J* = 12.0, 1H); **$^{13}$C NMR** ($D_2O$, 75 MHz) δ 97.6 (C1′), 80.6 (C5′), 74.7 (C4′), 74.3 (C3′), 73.6 (C5), 69.6 (C6), 68.8 (C4), 60.3 (C6′), 54.1 (C2′), 49.4 (C1), 48.9 (C3), 28.2 (C2); **ESI-MS** for $C_{12}H_{25}N_3O_7$ $[M+H]^+$ calcd. 323.2, found 323.2.



**32**. Paromomycin hydrogen chloride salt (compound **32**, 0.86 g, 2 mmol) and NaOH (0.60 g, 15 mmol) were dissolved in MeOH (80 mL). The mixture was stirred for a few minutes into a homogeneous solution. Boc anhydride (2.0 g, 9.2 mmol) was added in three batches. The mixture was stirred for 16 h and then evaporated solvent. The residue was trituated with petroleum (60 mL × 3) and washed by water (50 mL × 2). The white solid material is the desired product (0.93 g, yield: 75%). **$^1$H NMR** (DMSO-$d_6$, 400 MHz) δ 6.63 (d, *J* = 7.5, 1H), 6.46 (bs, 1H), 6.30 (d, J = 7.5, 1H), 4.97 (bs, 1H), 4.82 (bs, 1H), 4.73 (bs, 1H), 4.65 (bs, 1H), 4.58 (bs, 1H), 4.18 (bs, 1H), 3.63-3.52 (m, 3H), 3.35-3.04 (m, 8H), 1.75 (bs, 1H), 1.37 (s, 27H), 1.18 (q, *J* = 10.5, 1H); **$^{13}$C NMR** (DMSO-$d_6$, 75 MHz) δ 156.5, 155.9, 155.6, 99.8, 82.6, 78.4, 78.2, 77.7, 74.4, 73.2, 71.8, 70.6, 61.0, 56.3, 51.4, 50.3, 36.0, 29.0, 28.8; **ESI-MS** for $C_{27}H_{49}N_3O_{13}$ $[M+H]^+$ calcd. 624.3, found 624.3.



**33**. Into a flame-dried flask, compound **32** (0.32 g, 0.5 mmol) was dissolved in anhydrous DMF (20 mL), activated 4 Å molecular sieve (3 g) was added. The mixture was stand for 2 h and then cannula to another dry flask. Freshly prepared solution of compound **29** (67 mg, 0.48 mmol) in DCM (10 mL) was added dropwise into above solution at 0°C. The reaction completed after 3 h monitored by TLC ($R_f$: 0.42, $CHCl_3$/MeOH/$NH_4OH$, 5/1/0.1). Evaporation of solvent under vacuum produced the crude product as white solid powder. **ESI-MS** gave the correct mass as for $C_{30}H_{54}N_3O_{15}$ P $[M+Na]^+$ calcd. 750.3, found 750.2. Purification using silica column was not reported possible due to the decomposition, thus the crude product was used directly for next step.



**34**. Crude compound **33** (0.16 g) was mixed with DCM (5 mL) and 3 drops of anisole. TFA (2 mL) was added into this mixture and the mixture became homogeneous immediately. The mixture was stirred for 1 h at RT and evaporation of the solvent yielded the crude product **34** (200 mg, yield: 100%). **$^{31}$P-NMR** ($D_2O$, 81 MHz): δ 23.9, 23.8; **ESI-MS** for $C_{15}H_{30}N_3O_9P$ $[M+H]^+$ calcd. 428.3, found 428.1.

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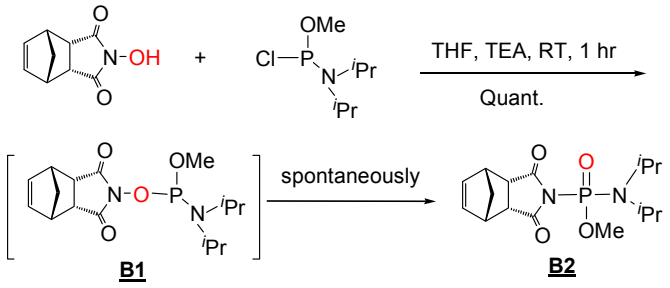
# Chapter Six

As described in Chapter 5, we tried to expand our regioselective *N*-6′-acylation of unprotected aminoglycoside to *N*-6′-phosphorylation. In trying to prepare the corresponding reagent *N*-((phosphino)oxy)amine **B1**, the phosphorodiamidate **B2** was obtained instead, as a clean product (Scheme 6b.1).




***Scheme 6b.1***. The course for the discovery of P(III) to P(V) rearrangement.

Detailed investigation of this reaction led to the discovery of this oxidative P(III) to P(V) rearrangement. This rearrangement completed within minutes and proceeded in quantitative yield at RT. It was envisioned that this rearrangement could be very useful to prepare a variety of organophosphorus compounds under mild conditions. The scope of rearrangement was investigated and summarized.

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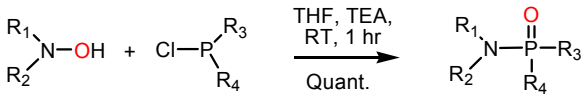
Contributions of co-authors

This chapter is a copy of a published article and is reproduced with permission from the journal, *Phosphorus, Sulfur, and Silicon.* It is cited as Feng Gao, Karine Auclair, " Highly Efficient P(III) to P(V) Oxidative Rearrangement" *Phosphorus, Sulfur, and Silicon* 2006, **181**, 159-165. I carried out all the experiments and wrote the article under the supervision of Dr. Auclair.

# Highly Efficient P(III) to P(V) Oxidative Rearrangement


## Abstract




A rapid and efficient P(III) to P(V) intramolecular rearrangement of N-[(phosphino)oxy] amines is reported. This intermediate is generated *in situ* from the reaction of hydroxylamines with chlorophosphites or chlorophosphoramidites and with rearrangement *via* cleavage of the weaker N-O bond to generate a more stable P=O bond. The reaction proceeds spontaneously in excellent yield when the hydroxylamine is electron poor. Various substituents on the phosphorus are well tolerated.


## 6.1 Introduction

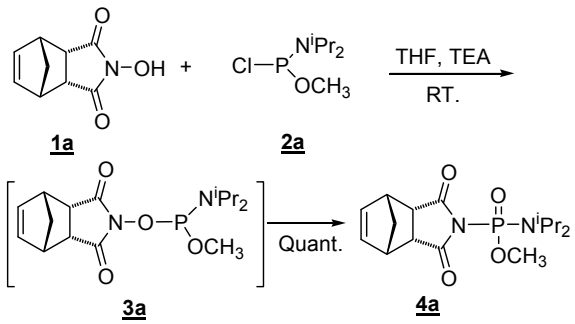
P(III)-containing molecules are good nucleophiles due to the great polarizability of the phosphorus lone pair electrons. This is especially true for di- or tri- alkyl/aryl/alkoxy substituted phosphines and favors rearrangement of P(III) to P(V).[1, 2] A number of P(III) to P(V) transformations have been reported, the most common of which is the Arbusov reaction.[1, 3-5] This reaction is believed to proceed in two steps *via* nucleophilic attack of the phosphorus on an alkyl halide to generate an alkylated phosphonium intermediate. This is followed by cleavage of one of the alkoxy C-O bonds to form a phosphoryl linkage (P=O). Owing to the relative high strength of C-O bonds, the second step is normally rate-determining and the rearrangement usually requires high temperature yet offers moderate yields.[1]

It should be emphasized that most P(III) to P(V) rearrangements reported involve the formation of a P=O bond at the expense of C-O bond cleavage. We report here a highly efficient P(III) to P(V) rearrangement of *N*-[(phosphino)oxy] amines where a P=O bond is formed at the expense of N-O bond. Similar rearrangements were reported in 1976 with cyclic aminophosphines[5] and in 1989 with chlorophosphites[4] however, either the yields were low and/or a complex mixture was obtained. Bailly and Burgada reported a more extensive study of this rearrangement but only tested cyclic phosphines.[3] More recently, Sekine and coworkers have suggested that phosphites and H-phophonates may be oxidized by 1-hydroxybenzotriazole (HOBt) derivatives *via* an analogous rearrangement.[6] This oxidation was shown to be of use in the preparation of oligonucleotides using the N-unprotected approach.[7] We recently encountered a similar rearrangement between electron-poor hydroxylamines and various chlorophosphines. The high efficiency of our reaction prompted us to investigate its scope. It was envisioned that this P(III) to P(V) rearrangement could be used to synthesize a wide range of organophosphorus compounds such as phosphoramidates, phosphorodiamidates, phosphonamidates, phosphonic diamides, phosphinic amide, and phosphoric triamides. These organophosphorus compounds are often important synthetic reagents or biologically active molecules.[1, 8, 9] For example, phosphonates and their derivatives are important analogs of peptides,[10, 11] nucleic acid[12] and lipids.[13]

## 6.2 Results and Discussion

In our initial experiment, the reaction of substituted hydroxylamine **1a** with chlorophosphoramidite **2a** in the presence of triethylamine proceeded rapidly and quantitatively to give the phosphorodiamidic acid ester **4a**, without detection of the expected phosphoramidite **3a** (Scheme 6.2). Although **3a** and **4a** cannot be differentiated by mass spectrometry, $^{31}$P-NMR clearly revealed the difference. Trivalent phosphorus-containing molecules such as phosphite **3a** are expected to show $^{31}$P chemical shifts around 140 ppm relative to 85% phosphoric acid,

whereas the chemical shift of pentavalent phosphorus compounds like **4a** is generally at higher field (*ca.* 1 ppm).[14-16] The expected range of $^{31}P$ chemical shifts for the products were confirmed using commercially available material. Formation of the intermediate phosphoramidite **3a** was observed when the reaction was monitored over time by $^{31}P$ NMR ($\delta$ =141.56 ppm). It was apparent for only about 5 minutes at room temperature before rearranging to the thermodynamically more stable product **4a** (Figure 6.1). To further confirm the structure of **4a**, *tert*-butylhydroperoxide and *m*-chloroperbenzoic acid were allowed to react separately with the product and as expected no changes in $^{31}P$ chemical shift were apparent (data not shown).




***Scheme 6.1***. Observed P(III) to P(V) rearrangement.

The rearrangement was complete within a hour. Surprisingly, the reaction was very clean and no chromatography was needed for purification. Acidic and basic aqueous washes of the crude product were sufficient to yield product of > 95% purity. Compound **4a** was quite stable, even when exposed to air at room temperature. These data strongly and the strong IR absorbance around 1270 $cm^{-1}$ suggest conversion of P(III) to P(V).

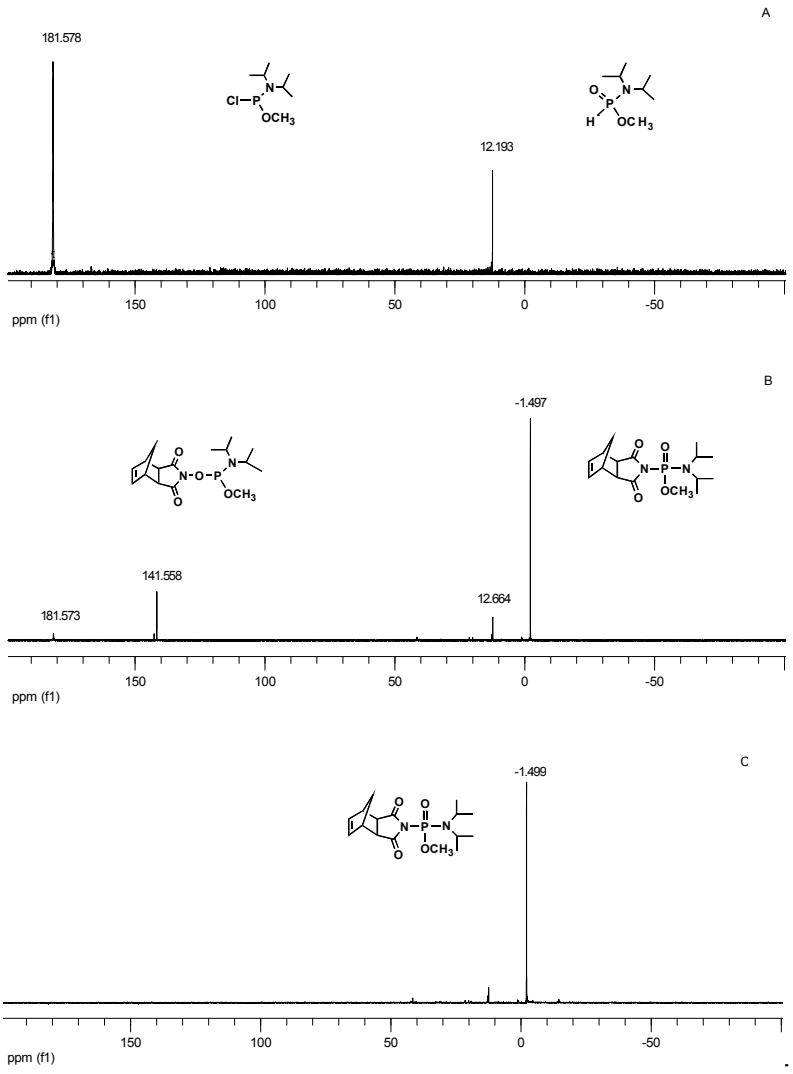
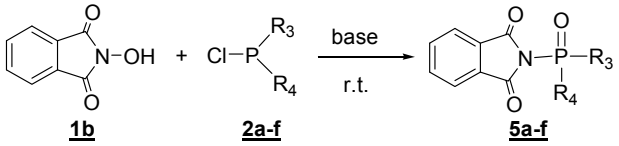



***Figure 6.1.*** $^{31}$P NMR spectra at different time points for the reaction shown in Scheme 6.1. (A) spectrum of **2a** (0.15 M) in $CD_2Cl_2$ before starting the reaction; (B) 5 minutes after addition of **1a** (0.15 M) to **2a**; (C) 1 h after starting the reaction.

When *N*-hydroxyphthalimide (**1b**) was used instead of **1a** (Scheme 6.2), the rearrangement proceeded to > 92% for different chlorophosphites or chlorophosphoramidites (entries 1-5, Table 6.1) except for bis(diisopropylamino)-chlorophosphoramidite (entry 6, Table 6.1). This reduced yield may be explained by steric hindrance caused by the two diisopropylamino groups.




**1b** **2a-f** **5a-f**

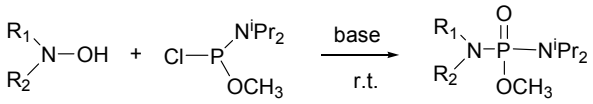
***Scheme 6.2***. P(III) to P(V) rearrangement with various chlorophosphines.

**Table 6.1.** Results for the reactions of Scheme 6.2

| Entry | $R_3$ | $R_4$ | Product | Yield[a,b] | $\delta$ $^{31}$P (ppm) |
|---|---|---|---|---|---|
| 1 | $OCH_3$ | $N[CH(CH_3)_2]_2$ | **5a** | 98% | 1.73 |
| 2 | $OCH_2CH_2CN$ | $N[CH(CH_3)_2]_2$ | **5b** | 96% | 0.56 |
| 3 | $OCH_2CH_3$ | $OCH_2CH_3$ | **5c** | 95% | -7.55 |
| 4 | $CH(CH_3)_2$ | $CH(CH_3)_2$ | **5d** | 97% | 63.40 |
| 5 | Ph | Ph | **5e** | 92% | 17.93 |
| 6 | $N[CH(CH_3)_2]_2$ | $N[CH(CH_3)_2]_2$ | **5f** | 31% | 6.99 |

[a]all reactions were carried at room temperature for 2 h in dichloromethane and using the base diisopropylethylamine. No chromatography was required to purify except for 5f. [b]isolated yield.

THF, acetonitrile or dichloromethane could be used without any significant effect on the reaction yields but polar solvents increased the rate of the rearrangement process significantly. Whether the transformation was carried out at –20°C or at room temperature also did not affect the results. However the reaction appeared to be less tolerant towards substitutions on the hydroxylamine moiety. The results are summarized in Scheme 6.3 and Table 6.2.




**1a-h** **2a** **4a, 5a, 6a-f**

***Scheme 6.3***. P(III) to P(V) rearrangement with various *N*-hydroxyamines.

**Table 6.2.** Results for the reactions of Scheme 6.3

| Entry | *N*-hydroxyl-amines | $R_1$ and $R_2$ | Product | Yield[a] | $\delta$ $^{31}$P (ppm) |
|---|---|---|---|---|---|
| 7 | **1a** | see 1a | **4a** | 95%[b] | 1.38 |
| 1 | **1b** | see 1b | **5a** | 98%[b] | 1.73 |
| 8 | **1c** | $PhSO_2$- and H | **6a** | 58%[b] | 3.75 |
| 9 | **1d** | PhCO- and H | **6b** | ≤ 30%[c] | 2.31 |
| 10 | **1e** | iPr and H | **6c** | ≤ 30%[c] | 13.55;12.41;12.12 |
| 11 | **1f** | Et and Et | **6d** | ≤ 30%[c] | 19.86; 5.78 |
| 12 | **1g** | $(CH_3)_2$= | **6e** | ≤ 50%[c] | 7.50 |
| 13 | **1h** | $Ph_2$= | **6f** | ≤ 30%[c] | 2.90 |

[a]all reactions were carried at room temperature for 2 h in dichloromethane and using the base diisopropylethylamine except for **4a** where triethylamine was used. [b]isolated yield. [c]by $^{31}$P NMR (data not shown).

Although formation of the intermediate remains extremely rapid (based on NMR studies), the efficiency of the rearrangement appears to be significantly dependent on the electron density at the nitrogen atom of the hydroxylamine. Electron-poor hydroxylamine derivatives undergo rapid and efficient (92-99%) rearrangement within a few hours (entries 1-5 in Table 6.1 and entry 7 in Table 6.2). This suggests a weakening of the hydroxylamine N-O bond by electron-withdrawing groups. Greater electron density around the nitrogen atom, *e.g.* with a single carbonyl or sulfonyl substituent (entries 8 and 9), does not affect the formation of the P-O-N-containing intermediate but lead to a slower rearrangement.

This confirms earlier data for the reaction between *N*-methyl-*N*-*p*-tolyl-hydroxylamine and chlorophosphites, chlorophosphonites or chlorophosphinites (with phenyl, ethoxy or ethylenedioxy substituents) for which multiple non-recoverable products were reported in addition to the desired rearrangement product.[4] Based on CIDNP NMR studies, the authors suggested that the rearrangement proceeded by a radical mechanism. The observed reactivity trend reported here as well as experiments using 2,2,6,6-tetramethyl-1-piperidinyloxy

free radical (TEMPO), suggest that hydroxylamines with two electron-withdrawing substituents (e.g. **1a** or **1b**) may not undergo the rearrangement via a radical process. Table 5.2 demonstrates that the presence of an *N*-benzosulfonyl (**1c**) substituent on the hydroxylamine moiety accelerates the rearrangement more than that of a *N*-benzocarbonyl (**1d**) group. Finally, in the absence of any electron-withdrawing substituents the rearrangement is even slower and yields a complex mixture (entries 10 and 11).

Others have previously reported that cyclic aminophosphines react almost as efficiently as cyclic chlorophosphines in similar rearrangements,[3] hence aminophosphines were not tested here. The use of oximes instead of hydroxylamine derivatives have also been reported and were briefly tested here. Acetone oxime and benzophenone oxime proceeded with moderate to low yield as expected from a previous report[8] (entries 12 and 13) whereas *syn*-benzaldehyde oxime proceeded *via* a Beckman rearrangement[17, 18] to yield benzonitrile and *N,N*-diisopropyl-methyl-phosphonamidic acid ester as the major products. These results are consistent with the reactivity trend observed for hydroxylamine derivatives.

## 6.3 Conclusions

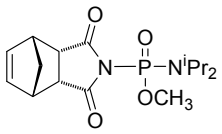
In summary, we have explored the scope of the P(III) to P(V) rearrangement of phosphoramidites prepared from hydroxylamines and chlorophosphites or chlorophophoramidites. Our results demonstrate that various substituents are well tolerated on the phosphorus atom, and when electron-poor hydroxylamines are used, the reaction proceeds very rapidly (< 2 hours) and cleanly (> 95% purity) to give excellent yields (> 90%) of rearranged product.

## 6.4 Experimental section

**Materials and general procedures.** Unless mentioned otherwise, all reactions were performed at room temperature under an atmosphere of argon and in flame-dried flasks capped with rubber septa. All commercial reagents were

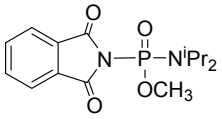
used without further purification unless otherwise specified. Anhydrous THF was prepared freshly by distillation over sodium. Acetonitrile and dichloromethane were distilled form calcium hydride under inert atmosphere. Abbreviations used include THF, tetrahydrofuran; TEA, triethylamine; Hex, hexane; EtOAc, ethyl acetate, DIPEA, diisopropylethylamine. Analytical HPLC analyses were performed using a VYDAC C4 column. Samples were eluted at a flow rate of 1 ml/min, using the following gradient of water (A) in acetonitrile (B): 0-20 min: 98 to 40% A; 21-30 min: 40 to 2% A; post-run time: 10 min. Chemical shifts are all in ppm were adjusted relative to the solvent used. $^{31}$P NMR spectra were acquired using broadband gated decoupling ($^{1}$H) and chemical shifts are listed relative to 85% phosphoric acid.

**Experimental procedures and characterization for all new compounds.**

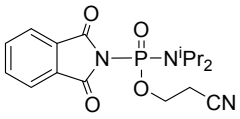



**4a**. *endo-N*-hydroxy-5-norbornene-2,3-dicarboximide (95 mg, 0.514 mmol) was evacuated under vacuum for 2 minutes and purged with argon before addition of freshly distilled THF (10 ml) and methyl *N,N*-diisopropylchlorophosphoramidite (100 µl, 0.490 mmol). After 5 minutes, triethylamine (75 µl, 0.54 mmol) was added and the mixture was stirred for 2 h. TLC showd one spot ($R_f$ : 0.66, $CHCl_3$/Hex/TEA, 100/20/1). The solvent was evaporated and the residue was re-dissolved in ethyl acetate (25 ml), washed with 5% $NaHCO_3$ and brine, dried over $Na_2SO_4$ and filtered. The filtrate was evaporated to give compound **5a** as a white powder (0.158 g, 95%). **IR** (KBr) 2910 (br, s), 1786 (s), 1715 (s), 1274 (s, P=O), 1026 (br, s) cm$^{-1}$; **HPLC:** 16.45 min; **$^{31}$P NMR** ($CDCl_3$, 81 MHz) δ 1.385; **$^{1}$H NMR** ($CDCl_3$, 400 MHz) δ 6.20 (br s, 2H, -C*H*=C*H*-), 3.71 (d, 3H, $^{3}J_{P-H}$ = 11.6 Hz, OC$H_3$), 3.50 (m, 2H), 3.42 (s, 2H, C*H*-CO-), 3.32 (t, 2H, $^{3}J_{H-H}$ = 3.2 Hz), 1.65 (br d, 1H), 1.53 (br d, 1H), 1.22 (dd, 12H, $^{4}J_{P-H}$ = 11.6 Hz, $^{3}J_{H-H}$ = 6.4 Hz, -N(CH(C$H_3$)$_2$)$_2$); **$^{13}$C NMR** ($CDCl_3$, 100 MHz) δ 177.3, 177.2, 135.1 (d, $^{5}J_{P-C}$ = 2.5 Hz), 53.0 (d, $^{2}J_{P-C}$ = 5.7 Hz), 52.8 (***C***H$_2$), 47.6 (d, $^{2}J_{P-C}$ = 5.4 Hz), 47.5 (d, $^{2}J_{P-C}$ = 5.4 Hz), 46.8 (d, $^{3}J_{P-C}$ = 5.4 Hz), 46.0 (d, $^{4}J_{P-C}$ = 4.3 Hz), 22.9 (d, $^{3}J_{P-C}$ = 13.2 Hz); **HRMS** for $C_{16}H_{25}N_2O_4P$ [*m/z*]

calcd. 340.1552; Found: 340.1559; **Elem. Anal.**: Calcd.: C: 56.46%, H: 7.40%, N: 8.23%; Found: C: 56.45%, H: 7.58%, N: 8.03%.

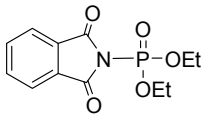


**5a**. *N*-hydroxyphthalimide (82 mg, 0.489 mmol) was dissolved in freshly distilled THF (10 ml), followed by the addition of methyl *N,N*-diisopropylchlorophosphoramidite (100 µl, 0.489 mmol). After 5 minutes, diisopropylethylamine (85 µl, 0.538 mmol) was added. The mixture was stirred for 2 h. TLC showed one spot ($R_f$ : 0.20, EtOAc/Hex, 1/1). The solvent was evaporated and the resulting residue was re-dissolved in ethyl acetate (25 ml), washed with 5% $NaHCO_3$ and brine, dried over $Na_2SO_4$ and filtered. The filtrate was evaporated to dryness to afford the product **5a** as a white powder (0.16 g, 98%). **IR** (KBr) 2967 (s), 1789 (s), 1730 (s), 1707 (s), 1291 (s, P=O), 1264 (s, P=O), 1040 (br, s) $cm^{-1}$; **HPLC:** 17.68 min; **$^{31}$P NMR** ($CDCl_3$, 81 MHz) δ 1.728; **$^{1}$H NMR** ($CDCl_3$, 400 MHz) δ 7.88 (m, 2H, Ar***H***), 7.77 (m, 2H, Ar***H***), 3.87 (d, 3H, $^{3}J_{P\text{-}H}$ = 11.6 Hz, P-OC***H***$_3$), 3.60 (m, 2H, N(C*H*$(CH_3)_2)_2$), 1.29 (d, 6H, $^{3}J_{H\text{-}H}$ = 7.2 Hz), 1.25 (d, 6H, $^{3}J_{H\text{-}H}$ = 7.2 Hz); **$^{13}$C-NMR** ($CDCl_3$, 75 MHz) δ 167.8, 134.9, 132.6 (d, $^{2}J_{C\text{-}P}$ = 7.17), 124.1, 53.3 (d, $^{2}J_{C\text{-}P}$ = 5.73), 47.0 (d, $^{2}J_{C\text{-}P}$ = 5.81), 23.0, 22.8; **HRMS** for $C_{15}H_{21}N_2O_4P$ [*m/z*] calcd. 324.1239, Found: 324.1241; **Elem. Anal.** Calcd.: C: 55.55%, H: 6.53%, N: 8.64%; Found: C: 55.55%, H: 6.57%, N: 8.32%.

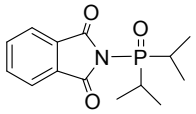


**5b**. *N*-hydroxyphthalimide (151 mg, 0.854 mmol) was dissolved in freshly distilled acetonitrile (25 ml) before addition of 2-cyano-1-ethyl *N,N*-diisopropylchlorophosphoramidite (200 µl, 0.845 mmol). After 5 minutes diisopropylethylamine (165 µl, 0.929 mmol) was added. The mixture was stirred for 2 h after which the TLC showed one spot ($R_f$ : 0.22, EtOAc/Hex, 1/1). The solvent was evaporated and the resulting residue was re-dissolved in ethyl acetate (50 ml), washed with 5% $NaHCO_3$ and brine, dried over $Na_2SO_4$ and filtered. The filtrate was evaporated to dryness to yield **5b** as yellowish crystals (0.2993 g, 96%). **IR** (KBr) 2979 (m), 2937 (m), 1784 (s), 1728 (s), 1277 (s, P=O), 1032 (br, s) $cm^{-1}$; **HPLC:** 17.85 min; **$^{31}$P NMR** ($CDCl_3$, 81 MHz) δ 0.556; **$^{1}$H NMR**

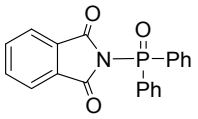
($CDCl_3$, 400 MHz) δ 7.88 (m, 2H), 7.78 (m, 2H), 4.39 (m, 2H), 3.55 (m, 2H), 2.88 (t, 2H, $^3J_{H-H}$ = 6.0 Hz), 1.32 (d, 6H, $^3J_{H-H}$ = 6.4 Hz), 1.27 (d, 6H, $^3J_{H-H}$ = 6.4 Hz); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 167.6, 135.2, 132.4 (d, $^2J_{C-P}$ = 6.8 Hz), 124.2, 117.0, 61.5, 47.4, 22.8, 20.3; **HRMS** for $C_{17}H_{22}N_3O_4P$ [*m/z*] calcd. 363.1339, Found: 363.1342.



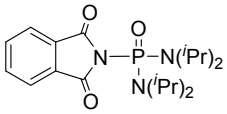
**5c**. *N*-hydroxyphthalimide (0.23 g, 1.364 mmol) was dissolved in anhydrous THF (30 ml). Diethyl chlorophosphite (200 µl, 1.364 mmol) was added, followed by addition of diisopropylethylamine (240 µl, 1.568 mmol). The mixture was stirred for 2 h. TLC showed one spot ($R_f$ : 0.15, $CHCl_3$/Hex/TEA, 50/50/1). The solvent was evaporated and the residue was re-dissolved in ethyl acetate (75 ml), washed with 5% $NaHCO_3$ and brine. The organic phase was dried over $Na_2SO_4$, and evaporation of solvent afforded the desired product as yellowish oil (0.38 g, 95% yield). **HPLC**: 14.44 min; **$^{31}$P NMR** ($CDCl_3$, 81 MHz) δ -7.547; **$^1$H NMR** ($CDCl_3$, 400 MHz) δ 7.88 (m, 2H), 7.77 (m, 2H), 4.34 (m, 4H), 1.37 (t, 6H, $^3J_{H-H}$ = 6.9 Hz); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 166.8, 134.7, 132.4, 124.3, 65.6, 16.5; **HRMS** for $C_{12}H_{14}NO_5P$ [*m/z*] calcd. 263.0610, found: 283.0650.



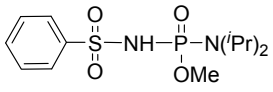
**5d**. Chlorodiisopropyl phosphine (300 µl, 1.81 mmol), *N*-hydroxyphthalimide (320 mg, 1.90 mmol) and diisopropylethylamine (350 µl, 2.10 mmol) were mixed in acetonitrile. Same work-up procedure as described for **5c** was applied to yield the desired compound **5d** as yellowish crystals (0.492 g, 97%). **HPLC:** 16.88 min; **$^{31}$P NMR** ($CDCl_3$, 81 MHz) δ 64.39; **$^1$H NMR** ($CDCl_3$, 400 MHz) δ 7.82 (m, 2H), 7.73 (m, 2H), 2.70 (m, 2H), 1.54 (dd, 6H, $^3J_{H-H}$ = 6.8 Hz, $^3J_{P-H}$ = 17.6 Hz), 1.27 (dd, 6H, $^3J_{H-H}$ = 6.8 Hz, $^3J_{P-H}$ = 17.6 Hz); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 168.9, 135.1, 132.6 (d, $^3J_{P-C}$ = 3.4 Hz), 124.2, 28.1, 27.1, 16.2 (d, $^3J_{P-C}$ = 3.65 Hz), 15.7 (d, $^3J_{P-C}$ = 2.03 Hz); **HRMS** for $C_{14}H_{18}NO_3P$ [*m/z*] calcd. 279.1024, found 279.1019.



**5e**. *N*-hydroxyphthalimide (0.294 g, 1.70 mmol) was dissolved in freshly distilled THF (30 ml). Diphenylchlorophosphine (300 µl, 1.59 mmol) was added slowly, followed by diisopropylethylamine (0.5 ml, 3.17 mmol). The mixture was stirred for 1 h and evaporated to dryness. The residue was triturated with $H_2O$ and ether to yield **5e** as a white solid product (0.48 g, 92%). **HPLC**: 17.72 min; **$^{31}$P NMR** ($CDCl_3$, 81 MHz) δ 17.93; **$^{1}$H NMR** ($CDCl_3$, 400 MHz) δ 7.90 (m, 4H), 7.84 (m, 2H), 7.76 (m, 2H), 7.61 (m, 2H), 7.52 (m, 4H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 167.9, 135.3, 133.1 (d, $^{1}J_{P\text{-}C}$ = 2.8 Hz), 132.2, 132.0, 128.9, 128.8 124.4 **HRMS** for $C_{20}H_{14}NO_3P$ [*m/z*] calcd. 347.0711; found: 347.0707.



**5f**. *N*-hydroxyphthalimide (0.15 g, 0.892 mmol) was dissolved in freshly distilled acetonitrile (30 ml). Bis(diisopropylamine)chloro-phosphine (0.238 g, 0.892 mmol) was added slowly, followed by diisopropylethylamine (173 µl, 0.98 mmol). The mixture was stirred for 1 h. After evaporation of the solvent, the residue was purified on silica-gel ($R_f$ ; 0.33, EtOAc/Hex/TEA, 15/85/1) to yield a yellowish paste (109 mg, 31%). **$^{31}$P NMR** ($CDCl_3$, 81 MHz) δ 6.99; **$^{1}$H NMR** ($CDCl_3$, 400 MHz) δ 7.86 (m, 2H), 7.38 (m, 2H), 3.66 (m, 4H), 1.36 (d, 12H, $^{3}J_{H\text{-}H}$ = 6.3 Hz), 1.29 (d, 12H, $^{3}J_{H\text{-}H}$ = 6.3 Hz); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 169.0, 134.6, 132.7, 123.8, 47.6, 24.0, 23.1; **HRMS** for $C_{20}H_{32}N_3O_3P$ [m/z] calcd. 393.2181, found 393.2175.



**6a**. *N*-hydroxy-benzene-sulfonamide (90 mg, 0.515 mmol) was dissolved in freshly distilled acetonitrile (10 ml), followed by methyl *N,N*-diisopropylchlorophosphoramidite (100 µl, 0.489 mmol). After 5 minutes, diisopropylethylamine (90 µl, 0.569 mmol) was added and the mixture was stirred for 1 h. After evaporation of the solvent, the residue was purified on silica-gel ($R_f$ ; 0.25, $CH_2Cl_2$/Hex/TEA, 75/25/1) to yield the titled compound as a white solid (123 mg, 58%). **HPLC**: 16.14 min; **$^{31}$P NMR** ($CDCl_3$, 81 MHz) δ 4.43; **$^{1}$H NMR** ($CDCl_3$, 400 MHz) δ 8.25 (1H, N*H*), 7.98 (d, 2H, $^{3}J_{H\text{-}H}$

= 3.2 Hz, Ar*H*), 7.32 (m, 3H, Ar*H*), 3.52 (d, 3H, $^{3}J_{P\text{-}H}$ = 12.0 Hz, P-OC$H_3$), 3.40 (m, 2H, -N(C*H*$(CH_3)_2)_2$), 1.12 (dd, 12H, $^{4}J_{P\text{-}H}$ = 11.6 Hz, $^{3}J_{H\text{-}H}$ = 6.4 Hz); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 129.8, 128.0, 128.0, 126.1, 52.1, 45.9, 23.1, 23.0; **HRMS** for $C_{13}H_{23}N_2O_4PS$ [*m/z*] calcd. 334.1116, found: 334.1108.

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# Chapter Seven

Although the development of inhibitors for resistance biological pathways is an important strategy to overcome bacterial resistance. An alternative approach is to modify the existing antibiotics to yield derivatives active against resistant strains. In both cases, it requires a good understanding of resistance mechanisms and of the mode of action of antibiotics. Crystallographic data of aminoglycoside-RNA complexes are increasingly available, providing insights for the design of new aminoglycoside derivatives. Together with our crystallographic data of the AAC(6′)-Ii–bisubstrate complex, we synthesized a series of neamine-*N*-6′-derivatives designed to bind to RNA but not to AAC(6′)-Ii. The syntheses were facilitated by the previous developed method for *N*-6′-derivatization of unprotected aminoglycosides. These molecules were tested against aminoglycoside-resistant strains. The results and implications are summarized in this chapter.

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Contributions of co-authors

This chapter is reproduced from an article published in journal *Bioorganic and Medicinal Chemistry,* It is cited as Yan, X., Gao, F., Yotphan, S., Bakirtzian, P., Auclair, K. "The use of aminoglycoside derivatives to study the mechanism of aminoglycoside 6'-N-acetyltransferase and the role of 6'-NH2 in antibacterial activity". *Bioorg. Med. Chem.* 2007, 15, 2944-2951. I synthesized all the compounds except for **3g-j**, which were synthesized by Sarilata Yotphan, a summer student in our group. Xuxu Yan and Parseh Bakirtzian (an undergraduate student) carried out enzymatic tests, Lee Freiburger (a graduate student in our lab) purified the enzyme. Karen Liu (a gradudate student in Professor Julian E. Davies' lab) carried out bacterial test.

# The Use of Aminoglycoside Derivatives to Study the Mechanism of Aminoglycoside 6′-*N*-Acetyltransferase and the Role of 6′-$NH_2$ in Antibacterial Activity


## Abstract

Aminoglycoside antibiotics act by binding to 16S rRNA. Resistance to these antibiotics occurs via drug modifications by enzymes such as aminoglycoside 6′-*N*-acetyltransferases (AAC(6′)s). We report here the regioselective and efficient synthesis of *N*-6′-acylated aminoglycosides and their use as probes to study AAC(6′)-Ii and aminoglycoside-RNA complexes. Our results emphasize the central role of *N*-6′ nucleophilicity for transformation by AAC(6′)-Ii and the importance of hydrogen bonding between 6′-$NH_2$ and 16S rRNA for antibacterial activity.


## 7.1 Introduction

Aminoglycosides are frequently prescribed broad spectrum antibiotics. They act by binding to the 16S ribosomal RNA causing mistranslation and blocking protein synthesis.[1-4] Their use is however threatened by the rapid spread of resistance. Aminoglycoside resistance occurs mainly via drug modifications[5, 6, 7] such as acetylation, adenylation, or phosphorylation.[8,9] Aminoglycoside 6′-*N*-acetyltransferase (AAC(6′)) is one of the most common determinant of resistance to this family of antibiotics.

One strategy to overcome aminoglycoside resistance is to develop inhibitors of resistance-causing enzymes.[10-14] Northrop and coworkers reported the first nanomolar inhibitor of an AAC, 3-*N*-(2-*S*-CoA-acetyl)gentamicin C1a.[15] This bisubstrate inhibitor was prepared enzymatically using its target, AAC(3)-I. The

chemical synthesis of aminoglycoside-coenzyme A (CoA) bisubstrates is challenging in part because of the judicious functional protection chemistry needed with aminoglycosides and CoA, and the water solubility of the starting materials. We recently developed an effective one-pot regio- and chemo-selective method for the direct *N*-6′-derivatization of unprotected aminoglycosides in high yield.[13] This facilitated the preparation of the first generation of synthetic AAC nanomolar inhibitors. Using the same strategy we prepared a second generation of AAC(6′) inhibitors to determine structure-activity relationships.[14] This also led to a derivative able to block aminoglycoside modification in cells.[14]

Besides inhibition, a more direct approach to counter aminoglycoside resistance is to design antibacterials active against resistant strains. Such a venture however requires a good understanding of resistance mechanisms.[16-21] We have designed a series of *N*-6′-acylated aminoglycoside derivatives to no longer bind to AAC(6′)-Ii but still interact with bacterial 16S rRNA. We report their synthesis using our novel synthetic methodology, their assay with aminoglycoside-AAC(6′)-Ii, and their antibacterial activity. Structural-activity relationships (SARs) are derived for the consequences of aminoglycoside acetylation by AAC(6′)s and for the role of 6′-$NH_2$ in antibacterial activity.

## 7.2 Results and Discussion

### 7.2.1 Design and synthesis of neamine derivatives

The 6′-amino group of aminoglycosides is protonated at physiological pH and may thus interact with RNA via electrostatic or hydrogen bond interactions. The reported crystal structures of aminoglycoside-RNA complexes[19, 22] reveal an important hydrogen bond between 6′-$NH_2$ and N1 of A1408. Moreover, the antibacterial activity of aminoglycosides containing a 6′-OH suggests that hydrogen bonding at the 6′ position may be more important than the electrostatic binding to RNA.

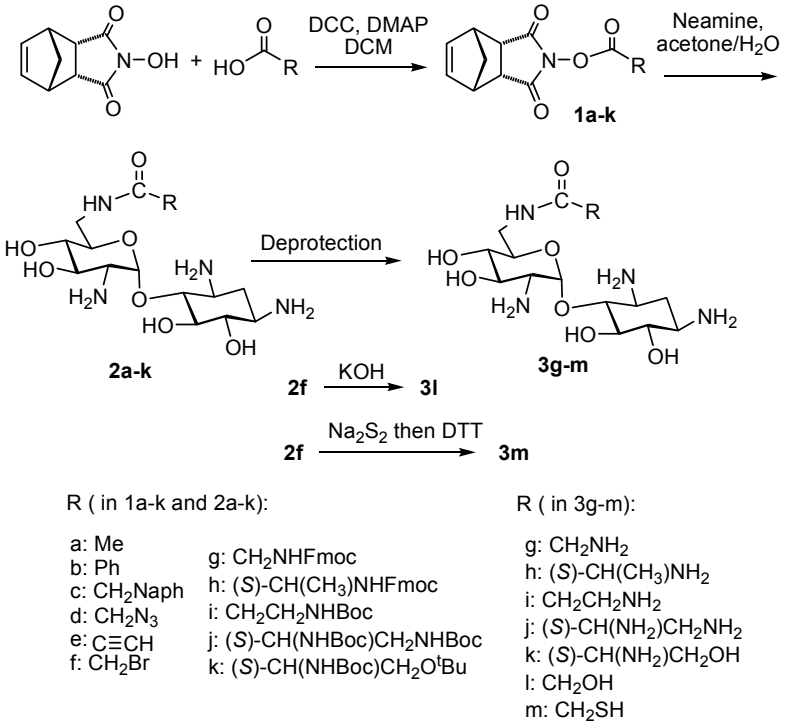
*N*-6′-acetylation by resistance-causing AAC(6′)s decreases the affinity of

aminoglycosides for RNA. This may be the result of steric hindrance, loss of hydrogen bonds, and/or loss of electrostatic interactions. As explained above, disruption of a salt bridge by acetylation is not likely to be a major factor affecting the interaction of aminoglycosides with RNA. Two AAC(6′)s have been successfully crystallized in the presence of aminoglycoside derivatives. Blanchard and coworkers have published a crystal structure of the AAC(6′)-Iy-ribostamycin complex.[23] Some of us in collaboration with the group of Berghuis have reported the crystal structure of AAC(6′)-Ii in complex with a series of aminoglycoside-CoA bisubstrates.[13] The structures of AAC(6′)-Ii with different bisubstrate inhibitors provide valuable information about the effect of extra carbons near N-6′. On the other hand, the reported structures for aminoglycosides in complex with RNA[19, 22] reveal a much more polar environment near 6′-$NH_2$. Such differences are useful in the design of aminoglycosides that bind to RNA but not to AAC(6′)s.

Compounds **3g-3m** (Scheme 7.1) were designed to investigate the importance of the hydrogen bonds of 6′-$NH_2$ and their specific location. Acylation at 6′-$NH_2$ of neamine decreases the hydrogen bonding strength of the nitrogen, yet the presence of amino and/or hydroxyl functionalities in the acyl group is expected to compensate for this loss, if positioned correctly. We also envisaged to take advantage of π-stack interactions. Thus, targets **2b-2c** were designed with aromatic groups near the 6′ position and expected to stack with A1408. Synthesis of the targets was achieved using our previously reported regioselective strategy.[13] The required *N*-hydroxyl-5-norbornene-*endo*-2,3-decarboximide esters (NBD esters, **1a-k**), were prepared by standard amide coupling with 1,3-dicyclohexylcarbodiimide in high yields (>90%). The NBD esters were used as regioselective reagents to deliver the acyl groups to the 6′-amino group of neamine and yield molecules **2a-k** (73-92%). Boc or Fmoc protected amino acids were used (with **2g-k**) to allow purification on silica gel. Reverse phase HPLC chromatography was used to purify derivatives **2a-f**. Standard deprotection protocols were used to generate target derivatives **3g-k** in yields ranging from

20% to 60% (unoptimized).

Compound **2f** was an intermediate in the preparation of target compound **3l-m**. Shortly treating **2f** with KOH yielded **3l** (61%). Compound **2f** was also separately treated with $Na_2S_2$ (prepared by mixing $Na_2S$ with $S_8$ in boiling water) to afford a disulfide intermediate that was further reduced by dithiothreitol to generate **3m** in 68% yield.




***Scheme 7.1***. Synthesis of neamine derivatives **2a-f** and **3g-m**.

### 7.2.2 Transformation of 2a-f and 3g-m by the resistance-causing enzyme AAC(6′)-Ii

The neamine derivatives **2a-f** and **3g-m** were first tested for inhibition of AAC(6′)-Ii. No inhibition was observed. The molecules were next tested as substrates and found to be much poorer substrates than neamine. Compound **3g** was selected for further analysis of the transformation by AAC(6′)-Ii. No acetylation products were detected (detection limit: $5\times10^{-8}$ M) after HPLC separation and ESI-MS analysis. As a control experiment, the acetylation product of neamine was clearly recognized. This verifies our assumption that the neamine

derivatives (**2a-2f, 3g-3m**) are very poor or not substrates for AAC(6′)-Ii.

### 7.2.3 Antibacterial activity in cells

The RNA binding of these derivatives was next investigated indirectly via antibacterial cell-based activity assays on paper discs and in solution. At 30 μg/disc, none of the derivatives showed clear growth inhibition of any of the strains JR66, JR67, JR88, pETAAC wt, *S. aureus*, or pSF815A-1. Partial inhibition was observed with MG1655, a strain not resistant to aminoglycosides. Minimum inhibitory concentrations (MICs) against MG1655 were next determined using standard dilution methods (Table 1). Compared to the parent compound neamine, none of the derivatives displayed significant activity, except **3g** and **3l** which showed reduced antibacterial ativity.

**Table 7.1.** Results of anti-microbial activity tested via the dilution method

| Compounds | MIC,[a] μg $mL^{-1}$ |
|---|---|
| **2b** | N/A[b] |
| **2c** | N/A |
| **2d** | N/A |
| **2e** | N/A |
| **3g** | <50 |
| **3h** | N/A |
| **3i** | N/A |
| **3j** | N/A |
| **3k** | N/A |
| **3l** | <25 |
| **3m** | N/A |
| Neamine | <6.25 |

[a] MIC = minimum inhibitory concentration;[b] below detection

### 7.2.4 Discussion

With the advent of antibiotics and the spread of their use in the late 1960s, the battle against bacterial infections was considered won. The increasing incidence of antibiotic resistance is alarming and threatens to turn back the clock.

Investigations of resistance mechanisms and searches for new antibacterials are vital. The antibacterial activity of aminoglycosides is due to their high affinity for the prokaryotic A site of rRNA. Acetylation of aminoglycosides at the 6′-amino group is sufficient to increase the MIC to a clinically ineffective level. We report here the efficient synthesis of *N*-6′-acylated aminoglycosides and their use to probe the mechanism of aminoglycoside 6′-*N*-acetyltransferase Ii and the role of the *N*-6′ of aminoglycosides in antibacterial activity. Using the available crystal structures of aminoglycosides complexed to either RNA[18, 22] or AAC(6')s,[24, 25] we designed a series of derivatives expected to be poor substrates of AAC(6′)s yet maintain binding to RNA. Such targets also have the potential to become lead compounds in the development of antibacterials active against resistant strains. We expected that acylation at *N*-6′ would prevent transformation by AAC(6′)-Ii yet the addition of an amino and/or alcohol groups in the acyl group would rescue the hydrogen bonding ability necessary for RNA affinity.

We used our previously reported methodology[13] to regioselectively modify aminoglycosides, and efficiently synthesized a series of neamine derivatives acylated at *N*-6′ (**2a-f**, **3g-m**). Regular protection-deprotection strategies would have required at least four more steps for each target and greatly reduced the overall yield. For example, compound **2f** was previously prepared by Mobashery and coworkers in an overall yield of 8.8%,[26, 27] whereas our methodology allows its synthesis in 68% overall yield.

As predicted, none of derivatives **2a-f** or **3g-m** were significantly transformed by AAC(6′)-Ii. Based on the crystal structures of bisubstrates-AAC(6′)-Ii complexes,[13] steric hindrance likely explains the reduced affinity observed when large acyl groups are added to neamine (**2b**, **2f**, **3h**, **3i**, **3j**, and **3k**). On the other hand, the results obtained with shorter acyl groups (**2a**,**2d**,**2e**, **3g**, **3l**, and **3m**) combined with our previous studies with bisubstrate inhibitors[13] suggest that *N*-6′-acylation affects AAC(6′)-Ii catalysis by reducing the nucleophilicity of *N*-6′ as opposed to steric hindrance. Indeed, bisubstrates with 3-carbon linkers at

*N*-6′ were potent inhibitors of AAC(6′)-Ii, and those with 2-carbon linkers near *N*-6′ were more potent inhibitors than 1-carbon linkers. This is surprising since the latter more closely resemble the tetrahedral enzymatic intermediate (0-carbon). The better binding of the bisubstrate containing a 2-carbon linker indicates that enough space is available to accommodate extra carbons near *N*-6′ such as in compounds **2a**,**2d**,**2e**, **3g**, **3l**, and **3m**. Therefore, the negative effect of aminoglycoside acylation with chains of 3 atoms or less on AAC(6′)-Ii-catalysis cannot be attributed to steric hindrance to binding but rather to a reduction in the nucleophilicity of *N*-6′. This supports the earlier suggestion by Wright and coworkers that AAC(6′)-Ii catalysis uses mainly a proximity effect.[28] In light of our results we can also conclude that the lower nucleophilicity of alcohols compared to that of free amines is responsible for the absence of AAC(6′)-Ii-catalysis with aminoglycosides containing a 6′-OH.

Based on their similarity to currently used aminoglycoside antibiotics, we expected our derivatives to penetrate cells. The antibacterial activity measured was thus expected to correlate with RNA affinity and/or disturbance. As with AAC(6′)-Ii, the reduced hydrogen bonding ability at *N*-6′ was expected to decrease the affinity of the aminoglycosides for RNA. To compensate, compounds **2b** and **2c** were designed to take advantage of possible π-stacking with A1408. The absence of antibacterial activity observed suggest that the aryl groups could not reach far enough to stack with the bases.

As an alternative strategy to offset the amidation of *N*-6′, amino- hydroxyl- and/or thio- groups were added on the acyl group (**3g-m**). The use of a poor hydrogen bond acceptor/donor such as a thiol (**3m**) abolished activity. When the acyl group had more than 2 carbons (**3h, 3i**, **3j**, and **3k**), the antibacterial activity decreased below the detection limit, likely due to steric hindrance (based on crystal structures). With shorter acyl groups however (**3g** and **3l**), some activity was detected, but it was reduced by ~4 fold compared to that of the parent compound neamine. This confirms our initial hypothesis that acetylation affects RNA affinity by reducing the hydrogen bonding ability at *N*-6′. The addition of

acyl chains of more than 3 atoms also causes steric clashes. The decreased antibacterial activity observed for **3g** and **3l** suggests that the newly added hydrogen bond donors/acceptors may not be positioned properly.

## 7.3 Conclusions

In conclusion, our results not only demonstrate the utility of our *N*-6′ regioselective synthetic methodology to derivatize aminoglycosides, but also show the central role of *N*-6′ nucleophilicity for transformation by AAC(6′)-Ii, and the importance of hydrogen bonding between 6′-$NH_2$ and RNA for antibacterial activity.

## 7.4 Experimental section

### 7.4.1 General.

Unless mentioned otherwise, all reagents were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario). Reagents and solvents were used without further purification. Flash chromatography and TLC analysis (F-254) were performed with 60 Å silica gel from Silicycle (Quebec, Canada). Compounds **2a-2f**, **3l** and **3m** were purified by reversed-phase HPLC using an Agilent 1100 modular system equipped with an autosampler, a quaternary pump system, a photodiode array detector, a thermostatted column compartment and a ChemStation (for LC 3D A.09.03) data system. The column used was a semi-preparative Zorbax SB-CN of 4.6 × 250 mm and 5 μm (Agilent, Palo Alto, CA). Samples were eluted at a flow rate of 3 mL/min, using a combination of mobile phase A (0.05% TFA in water at pH ~ 3.5) and mobile phase B (acetonitrile containing 0.05% TFA). After 2 min at 1% B, the eluent was brought to 40% B in a linear gradient over 20 min. The detector was set to 214 nm. HRMS spectra were obtained by direct infusion electrospray ionization from a solution of 50 mM formic acid:methanol 90:10 at 2 μL/min in an IonSpec 7 Tesla FTICR instrument. The resolving power was approximately 80,000. LRMS

was performed using a Finnigan LCQDUO mass spectrometer with ESI without fragmentation. $^{1}H$ and $^{13}C$ NMR spectra were recorded using Varian mercury 400 or 300 spectrometers. For all $^{1}H$ NMR spectra of target molecules, presaturation (presat.) was used to suppress the water peak. The chemical shifts (δ) were reported in parts per million (ppm) and are referenced to either the internal standard TMS (when $CDCl_3$ is used) or the deuterated solvent used. The peak patterns are indicated as follows: s, singlet, d, doublet, t, triplet, dt, doublet of triplet, ddd, doublet of doublet of doublet, td, triplet of doublet, m, multiplet, q, quartet, p, pentet, and br s, broad singlet. The coupling constants, *J,* are reported in Hz. Reactions with air or moisture sensitive reagents were carried out under an atmosphere of argon.

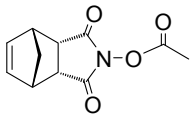
### 7.4.2 Synthesis

**General Procedure for the synthesis of compound 1a-1k from carboxylic acids.** *Endo-N*-hydroxy-5-norbornene-2,3-dicarboximide (NBD) (0.538 g, 3 mmol) and the desired carboxylic acid (3 mmol, protected at other functional groups if necessary) were dissolved in dichloromethane (20 mL). When necessary a small amount of THF (ca. 5 mL) was added to dissolve the carboxylic acid. DCC (0.619 g, 3 mmol) was added to the mixture, followed by a catalytic amount of DMAP (5 mg). A few minutes after addition of DCC, a white solid (DCU) precipitated out. The reaction was stirred under argon at room temperature overnight. The presence of the desired NBD ester (**1a-1k)** was monitored by TLC. The solid DCU was removed by filtration and the filtrate was evaporated to dryness. EtOAc (100 mL) was added to dissolve the product. The solution was extracted several times with 10% $Na_2CO_3$ (15 mL). The organic layer was dried over anhydrous $MgSO_4$ and filtered. The filtrate was evaporated to near dryness and the NBD ester was dried under the vacuum. When higher purity was needed, a silica gel chromatography column was used for the purification.

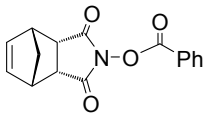
**General procedure for the preparation of compound 2a-2k.** The general procedure can be exemplified with the preparation of compound **2f**. Neamine (32 mg, 0.1 mmol) was dissolved in water (2 mL) in a vial. *endo*-*N*-hydroxy-5-norbornene-2,3-dicarboximide bromoacetate (NBD ester **1f**, 24 mg, 0.08 mmol) was dissolved in acetone (2 mL) in another vial. The two solutions were mixed, sonicated for 1 min and stirred for one hour before quenching with formic acid to pH = 4. Purification by reverse phase HPLC afforded the desired product **2f** as a fluffy white powder (24 mg, 70%). Compounds **2a-2f** were purified by reverse phase HPLC and compound **2g-2k**, however, were purified by flash chromatography.

Deprotection of Boc Fmoc, and $^{t}$Butyl ether groups was performed under standard procedures.[29]

**Methyl azidoacetate**: methylbromoacetate (3 g, 20 mmol) and sodium azide (2.5 g, 20 mmol) were mixed in acetone (30 mL). The mixture was refluxed overnight. The mixture was evaporated to dryness and the residue was extracted with EtOAc to give methyl azidoacetate (2.18 g, 95%) as a yellowish liquid. **IR**: 2110 cm $^{-1}$ (azide absorption); **$^{1}$H NMR** ($CDCl_3$, 400 MHz): δ 3.89 (s, 2H), 3.80 (s, 3H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 170.2, 49.2, 52.2.

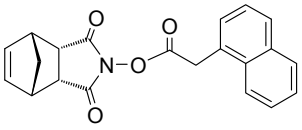


**1a**. TLC $R_f$ = 0.53 (Hex/EtOAc 1/1); Yield: 90%; **$^{1}$H NMR** ($CDCl_3$, 400 MHz): δ 6.18 (br s, 2H), 3.45 (br s, 2H), 3.33 (br s, 2H), 2.25 (s, 3H), 1.79 (d, *J* = 8.8, 1H), 1.54 (d, *J* = 8.8, 1H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 169.5, 135.0, 51.6, 45.1, 43.7, 17.2; **ESI-MS** for $C_{11}H_{11}NO_4$ $[M+H]^+$ calcd. 222.1, found 222.0.

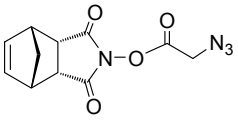


**1b**. TLC $R_f$ = 0.65 (Hex/EtOAc 1/1); Yield: 94%; **$^{1}$H NMR** ($CDCl_3$, 400 MHz): δ 8.09 (d, *J* = 8.0, 2H), 7.65 (t, *J* = 8.0, 1H), 7.48 (t, *J* = 8.0, 2H), 6.28 (br s, 2H), 3.50 (br s, 2H), 3.40 (br s, 2H), 1.83 (d, *J* = 8.8, 1H), 1.58 (d, *J* = 8.8, 1H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz): δ 170.2, 134.9,

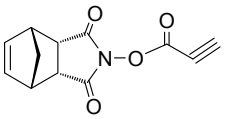
134.7, 130.7, 130.4, 128.9, 51.7, 45.2, 43.5; **ESI-MS** for $C_{16}H_{13}NO_4$ $[M+H]^+$ calcd. 284.1, found 284.1.



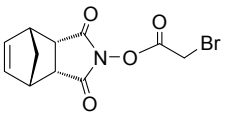
**1c**. TLC $R_f$ = 0.55 (Hex/EtOAc 1/1); Yield: 99%; **$^1$H NMR** ($CDCl_3$, 400 MHz): δ 7.93 (d, *J* = 8.0, 1H), 7.86 (d, *J* = 8.0, 1H), 7.82 (d, *J* = 8.0, 1H), 7.58 (t, *J* = 8.0, 1H), 7.50 (t, *J* = 8.0, 1H), 7.45 (m, 2H), 6.15 (br s, 2H), 4.30 (s, 2H), 3.41 (br s, 2H), 3.27 (br s, 2H), 1.74 (br s, 1H), 1.47 (d, *J* = 8.8, 1H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz): δ 170.0, 166.4, 150.2, 138.2, 134.9, 127.2, 125.4, 125.0, 124.8, 124.8, 123.8, 122.5, 113.3, 51.2, 44.7, 43.2, 37.8; **ESI-MS** for $C_{21}H_{17}NO_4$ $[M+H]^+$ calcd. 348.1, found 348.1.



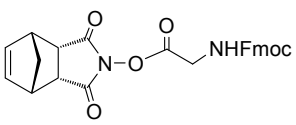
**1d**. TLC $R_f$ = 0.35 (Hex/EtOAc 1/1); Yield: 80%; **$^1$H NMR** ($CDCl_3$, 400 MHz): δ 6.20 (br s, 2H), 4.17 (s, 2H), 3.46 (s, 2H), 3.35 (br s, 2H), 2.25 (s, 3H), 1.80 (d, *J* = 8.8, 1H), 1.55 (d, *J* = 8.8, 1H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 169.6, 135.0, 51.6, 48.2, 45.1, 43.7; **MS** for $C_{11}H_{10}N_4O_4$ $[M+H]^+$ calcd. 263.1, found 263.2.



**1e**. TLC $R_f$ = 0.47 (Hex/EtOAc 1/1); Yield: 94%; **$^1$H NMR** ($CDCl_3$, 400 MHz): δ 6.17 (br s, 2H), 3.45 (s, 2H), 3.34 (br s, 2H), 3.27 (s, 1H), 1.80 (d, *J* = 8.8, 1H), 1.53 (d, *J* = 8.8, 1H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 169.3, 135.0, 81.9, 70.6, 51.5, 45.1, 43.6; **MS** for $C_{12}H_9NO_4$ $[M+H]^+$ calcd. 232.2, found 232.3.

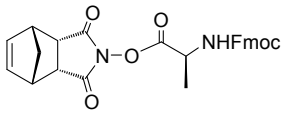


**1f**. TLC $R_f$ = 0.47 (Hex/EtOAc 1/1); Yield: 95%; The crude product was recrystallized using hexane to yield earth-red crystals of m.p. 90-92 °C; **$^1$H NMR** ($CDCl_3$, 400 MHz): δ 6.20 (br s, 2H), 4.04 (s, 2H), 3.46 (br s, 2H), 3.34 (br s, 2H), 1.80 (d, *J* = 8.8, 1H), 1.54 (d, *J* = 8.8, 1H); **$^{13}$C NMR** ($CDCl_3$, 75 MHz) δ 169.5, 135.0, 51.6, 45.1, 43.7, 21.9; **ESI-MS** for $C_{11}H_{10}{}^{79}BrNO_4$ $[M+H]^+$ calcd. 300.0, found 300.1.

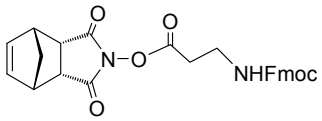


**1g**. TLC $R_f$ = 0.66 (Hex/EtOAc 1/1); The starting material Fmoc-Gly-OH was used as the limiting

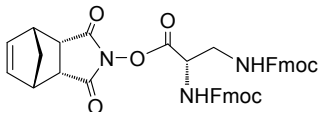
reagent; Yield: 94 %; **$^1$H NMR** (300 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.4, 2H), 7.57 (d, *J* = 4.0, 2H), 7.35 (m, 4H), 6.18 (br s, 2H), 5.30 (m, 1H), 4.43 (d, *J* = 7.4, 2H), 4.29 (m, 3H), 3.45 (m, 2H), 3.34 (br s, 2H), 1.78 (d, *J* = 8.8, 1H), 1.53(d, *J* = 8.8, 1H). **ESI-MS** for $C_{26}H_{22}N_2O_6$ $[M+H]^+$ calcd. 459.2, found 459.1.



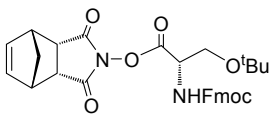

**1h**. TLC $R_f$ = 0.38 (Hex/EtOAc 1/1); The starting material Fmoc-Ala-OH was the limiting reagent; Yield: 76 %; **$^1$H NMR** (200 MHz, $CDCl_3$) δ 7.74 (d, *J* = 7.1, 2H), 7.56 (d, *J* = 7.1, 2H), 7.32 (m, 4H), 6.18 (s, 2H), 5.26 (d, *J* = 7.6, 1H), 4.71 (t, *J* = 7.6, 1H), 4.38 (m, 2H), 4.23 (m, 1H), 3.44 (s, 2H), 3.32 (m, 2H), 1.76 (d, *J* = 8.8, 1H), 1.57 (d, *J* = 7.2, 3H), 1.51 (d, *J* = 8.8, 1H). **ESI-MS** for $C_{27}H_{24}N_2O_6$ $[M+H]^+$ calcd. 473.2, found 473.1.



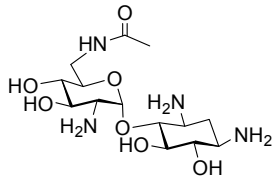

**1i**. $R_f$ = 0.38 (Hex/EtOAc, 1/1); The starting material Boc-β-Ala-OH was the limiting reagent; Yield: 84%; **$^1$H NMR** (200 MHz, $CDCl_3$) δ 6.155 (br s, 2H), 5.12 (br s, 1H), 3.46 (br s, 2H), 3.43 (br s, 2H), 3.31 (m, 2H), 1.75 (br d, 1H), 1.54 (br d, 1H), 1.41 (s, 9H). **ESI-MS** for $C_{27}H_{24}N_2O_6$ $[M+H]^+$ calcd. 473.2, found 473.1.



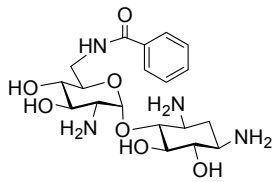

**1j**. $R_f$ = 0.33 (Hex/EtOAc, 2/1); The starting material di-Boc-2,3-diaminopropionic acid was the limiting reagent; Yield 52%; **$^1$H NMR** (200 MHz, $CDCl_3$) δ 6.132(s, 2H), 5.71 (br, 1H), 5.39 (t, *J* = 7.4, 1H), 4.61 (m, 1H), 3.66 (m, 1H), 3.51 (m, 1H), 3.39 (s, 2H), 3.30 (s, 2H), 1.73 (d, *J* = 8.8 Hz, 1H), 1.48 (d, *J* = 8.8, 1H), 1.38 (s, 18H). **ESI-MS** for $C_{42}H_{35}N_3O_8$ $[M+H]^+$ calcd. 710.2, found 710.1.



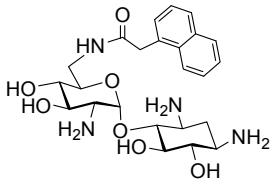

**1k**. $R_f$ = 0.68 (Hex/EtOAc, 1/4); The starting material Fmoc-Ser (tBu)-OH was the limiting reagent; Yield 89%; **$^1$H NMR** (300 MHz, $CDCl_3$) δ 8.30 (m, 1H), 7.75 (d, *J* = 11, 3H), 7.50 (m, 2H), 7.30 (m, 4H), 6.58 (d, *J* = 2, 1H), 6.20 (s, 1H), 6.08 (s, 2H), 4.33 (m, 4H), 3.90 (m, 1H), 3.67 (m, 1H), 3.36 (m, 2H), 3.13 (m, 2H), 1.78 (m, 1H), 1.30 (s, 9H). **ESI-MS** for $C_{31}H_{32}N_2O_7$ $[M+H]^+$ calcd. 545.2, found 545.1.



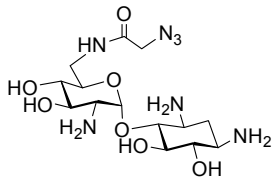
**2a**. Yield 60%; **$^1$H NMR** ($D_2O$, 400 MHz, pD = 4.0): 5.50 (d, *J* = 4.0, 1H), 3.72-3.63 (m, 3H), 3.46 (t, *J* = 8.8, 1H), 3.40-3.32 (m, 3H), 3.24-3.19 (m, 2H), 3.13 (td, *J* = 10, 4.0, 1H), 2.32 (td, *J* = 12.0, 4.0 , 1H), 1.82 (s, 3H), 1.67 (q, *J* = 12.0, 1H); **$^{13}$C NMR** ($D_2O$, 75 MHz): δ 169.6, 96.8 (C1′), 79.0 (C3′), 75.0 (C4′), 72.4 (C5′), 71.8 (C6), 70.2 (C5), 68.3 (C4), 54.0 (C2′), 49.8 (C1), 48.2 (C3), 39.2 (C6′), 28.8 (C2), 21.9 (-CO***C***$H_3$); **ESI-MS** for $C_{14}H_{28}N_4O_7$ $[M+Na]^+$ calcd 387.2, found 387.2.



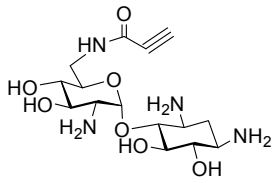
**2b**. Yield 80%; **$^1$H NMR** ($D_2O$, 400 MHz): 7.59 (d, *J* = 8.0, 2H), 7.45 (t, *J* = 6.8, 1H), 7.35 (t, *J* = 8.0, 2H), 5.55 (d, *J* = 4.0, 1H), 3.81-3.70 (m, 3H), 3.62 (m, 1H), 3.49 (t, 1H), 3.40-3.30 (m, 3H), 3.26 (br d, *J* = 13.2, 1H), 3.13 (td, *J* = 10.0, 4.0, 1H), 2.32 (td, *J* = 12.0, 4.0, 1H), 1.67 (q, *J* = 12, 1H); **$^{13}$C NMR** ($D_2O$, 75 M) δ 167.7, 135.4, 132.2, 128.7, 126.5, 97.0 (C1′), 79.0 (C3′), 75.2 (C4′), 72.8 (C5′), 72.0 (C6), 71.0 (C5), 69.0 (C4), 54.0 (C2′), 50.0 (C1), 48.6 (C3), 40.0 (C6′), 28.5 (C2); **ESI-MS** for $C_{19}H_{30}N_4O_7$ $[M+Na^+]$ calcd 439.2, found 439.1.



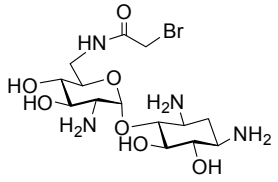
**2c**. Yield 85%; **$^1$H NMR** ($D_2O$, 400 MHz): δ 7.84 (t, *J* = 8.4, 2H), 7.79 (d, *J* = 8.0, 1H), 7.47(m, 2H), 7.40 (t, *J* = 8.0, 1H), 7.36 (d, *J* = 8.0, 1H), 5.33 (d, *J* = 4.0, 1H), 3.98 (s, 2H), 3.64-3.58 (m, 3H), 3.48-3.35 (m, 3H), 3.32 (m, 1H), 3.15 (dt, *J* = 11.0, 4.0, 1H), 2.88 (t, *J* = 9.6, 1H), 2.69 (dd, *J* = 11.2, 4.0, 1H), 2.33 (td, *J* = 12.4, 4.8, 1H), 1.67 (q, *J* = 12.8, 1H); **$^{13}$C NMR (**$D_2O$, 100 MHz, presat.): δ 163.3 (CO), 133.8, 131.8, 131.1, 129.1, 128.9, 128.5, 126.9, 126.4, 126.2, 123.7, 96.7 (C1′), 79.0 (C3′), 75.2 (C4′), 72.7 (C5′), 71.9 (C6), 70.0 (C5), 68.6 (C4), 53.9 (C2′), 49.9 (C1), 48.7 (C3), 40.5 ($CH_2$), 38.9 (C6′), 28.6 (C2); **MS (ESI)** for $C_{24}H_{34}N_4O_7$ $[M+Na]^+$ calcd 513.2, found 513.2.



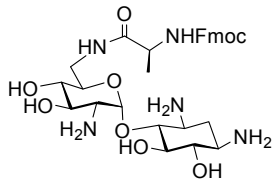
**2d**. Yield 75%; **$^1$H NMR** ($D_2O$, 400 MHz, pD = 4.0): 5.57 (d, *J* = 4.0, 1H), 3.91 (s, 2H), 3.73 (dt, *J* = 8.8, 4.0, 1H), 3.56 (d, *J* = 12.4, 1H), 3.43 (t, *J* = 7.6, 1H), 3.35 (m, 2H), 3.14 (t, *J* = 9.2, 1H), 3.10 (t, *J* = 9.2, 1H), 3.00 (t, *J* = 9.2, 1H), 2.55 (dt, *J* = 9.2, 3.6, 1H), 1.83 (td, *J* = 12.0, 4.0, 1H), 1.05 (q, *J* = 12.0, 1H); **$^{13}$C NMR** ($D_2O$, 75 MHz): δ 163.5, 101.4, 88.2, 77.4, 75.0, 73.7, 72.9, 72.6, 55.6, 52.0, 50.2, 49.6, 40.3, 35.9; **ESI-MS** for $C_{14}H_{27}N_7O_7$ $[M+H]^+$ calcd 406.2, found 406.1.



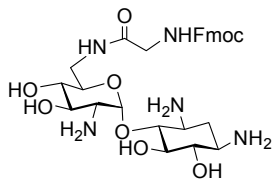
**2e**. Yield 77%; **$^1$H NMR** ($D_2O$, 400 MHz): 5.57 (d, *J* = 4.0, 1H), 3.79-3.64 (m, 3H), 3.55–3.40 (m, 6H), 3.32-3.28 (m, 2H), 3.20 (m, 1H), 2.32 (td, *J* = 12.0, 4.0 , 1H), 1.67 (q, *J* = 12.0 , 1H); **$^{13}$C NMR** ($D_2O$, 75 MHz): δ 155.0, 97.1, 79.4, 77.3, 75.8, 75.1, 72.6, 71.6, 70.6, 68.9, 53.9, 49.8, 48.7, 39.9, 28.5; **ESI-MS** for $C_{15}H_{26}N_4O_7$ $[M+H]^+$ calcd 375.2, found 375.1.



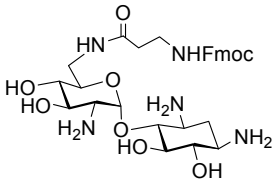
**2f.** Yield 70%; **$^1$H NMR** ($D_2O$, 400 MHz): 5.50 (d, *J* = 4.0, 1H), 3.92 (s, 2H), 3.70-3.61 (m, 3H), 3.43 (t, *J* = 8.8, 1H), 3.38-3.31 (m, 3H), 3.21-3.16 (m, 2H), 3.10 (td, *J* = 10, 3.8, 1H), 2.31 (td, *J* = 8.8, 4.0, 1H), 1.66 (q, *J* = 8.8, 1H); **$^{13}$C NMR** ($D_2O$, 75 MHz): δ 169.6, 96.8 (C1′), 79.0 (C3′), 75.0 (C4′), 72.4 (C5′), 71.8 (C6), 70.2 (C5), 68.3 (C4), 54.0 (C2′), 50.7 (-CO***C***$H_2$Br), 49.8 (C1), 48.2 (C3), 39.2 (C6′), 28.8 (C2); **ESI-MS** for $C_{14}H_{27}{}^{79}BrN_4O_7$ $[M+H]^+$ calcd 442.1 found 442.2.



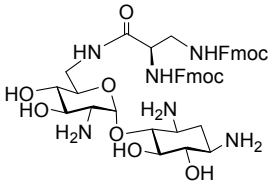
**2g**. $R_f$ = 0.65 ($CHCl_3$/MeOH/$NH_4OH$, 4/3/1); Yield 89%; **ESI-MS** (MeOH): $[M+H]^+$ calcd 602.2, found 602.2.



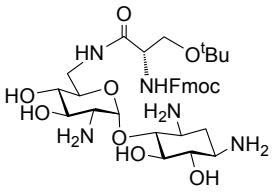
**2h**. $R_f$ = 0.38 ($CHCl_3$/MeOH/$NH_4OH$, 4/3/1); Yield 91%; **ESI-MS**: $[M+H]^+$ calcd 616.2, found 616.2.



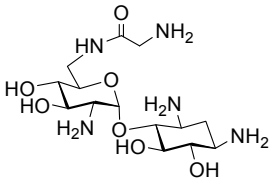
**2i**. $R_f$ = 0.42 ($CHCl_3$/MeOH/$NH_4OH$, 4/3/1); Yield 87%; **ESI-MS** (MeOH): [M+$H^+$] calcd 493.2 and found 493.2.



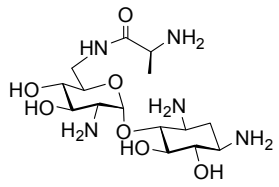
**2j**. $R_f$ = 0.45 ($CHCl_3$/MeOH/$NH_4OH$, 4/3/1); Yield 92%; **ESI-MS** (MeOH): [M+$H^+$] calcd 609.2 and found 609.2.



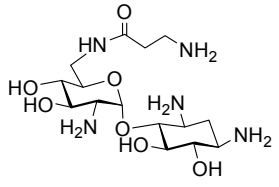
**2k**. $R_f$ = 0.62 ($CHCl_3$/MeOH/$NH_4OH$, 4/3/1); Yield 79%; **ESI-MS** (MeOH): [M+$H^+$] calcd 688.3 and found 688.3.



**3g.** Yield 27%; **$^1$H NMR** ($D_2O$, 400 MHz, pD = 10, presat.): 5.03 (d, *J* = 4.0, 1H), 3.72 (m, 1H), 3.52–3.18 (m, 3H), 3.18 (s, 2H), 3.14–3.07 (m, 4H), 2.96 (t, *J* = 9.2, 1H), 2.67 (m, 2H), 1.82 (m, 1H), 1.76 (q, *J* = 12.0, 1H); **$^{13}$C NMR** ($D_2O$, 75 MHz): δ 175.8, 101.2, 88.0, 77.2, 75.8, 73.3, 71.3, 71.2, 55.1, 50.2, 49.3, 43.6, 40.0, 35.4; **ESI-MS** for $C_{14}H_{29}N_5O_7$ [M+$H^+$] calcd. 380.2, found 380.1.

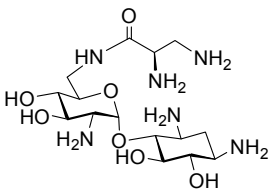


**3h**. Yield 17%; **$^1$H NMR** ($D_2O$, 400 MHz): 5.05 (d, *J* = 4.0, 1H), 3.71 (dt, *J* = 9.2, 4.0, 1H), 3.47–3.28 (m, 4H), 3.15–2.94 (m, 4H), 2.78 (m, 3H), 1.82 (m, 1H), 1.18 (d, *J* = 6.8, 3H), 1.04 (q, *J* = 12.0, 1H); **ESI-MS** for $C_{15}H_{31}N_5O_7$ [M+$H^+$] calcd 394.2, found 394.2.

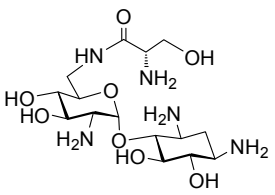


**3i**. Yield 33%; **$^1$H NMR** ($D_2O$, 400 MHz): 5.05 (d, *J* = 4.0, 1H), 3.71 (dt, *J* = 9.2, 4.0, 1H), 3.47–3.28 (m, 4H), 3.15–2.94 (m, 4H), 2.82 (t, *J* = 6.9, 1H), 2.68–2.50 (m, 3H), 2.33 (t, *J* = 6.9, 2H), 1.82 (m, 1H), 1.04 (q, *J* =

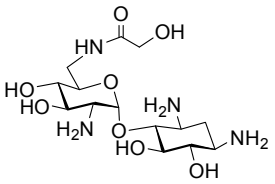
12.0, 1H); **$^{13}$C NMR** ($D_2O$, 75 MHz): δ 174.8, 101.4, 88.2, 77.5, 76.1, 73.6, 71.7, 71.5, 55.4, 50.5, 49.6, 40.3, 37.2, 37.0, 35.8; **ESI-MS** for $C_{15}H_{31}N_5O_7$ [M+H$^+$] calcd 394.2, found 394.2.



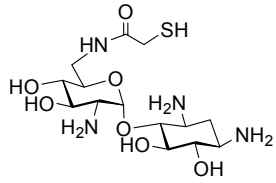
**3j**. Yield 60%; **$^1$H NMR** ($D_2O$, 400 MHz): 5.50 (d, *J* = 4.0, 1H), 3.73–2.91 (m, 11H), 2.62–2.50 (m, 2H), 1.76 (m, 1H), 1.00 (m, 2H); **$^{13}$C NMR** ($D_2O$, 75 MHz): δ 173.4, 101.2, 87.0, 77.5, 76.2, 73.5, 71.8, 63.7, 61.5, 56.0, 55.0, 50.2, 49.6,45.4, 35.3; **ESI-MS** for $C_{15}H_{31}N_5O_8$ [M+H$^+$] calcd 410.2, found 410.2.



**3k**. Yield 22%; **$^1$H NMR** ($D_2O$, 400 MHz): 5.05 (d, *J* = 4.0, 1H), 3.73 (m, 1H), 3.47–3.08 (m, 8H), 2.78 (m, 5H), 1.82 (m, 1H), 1.04 (q, *J* = 12.0, 1H); **$^{13}$C NMR** ($D_2O$, 75 MHz): δ 175.2, 101.2, 87.6, 77.5, 76.1, 73.6, 71.4, 71.3, 56.6, 55.4, 50.6, 49.6, 45.2, 40.1, 35.7; **ESI-MS** for $C_{15}H_{32}N_6O_7$ [M+H$^+$] calcd 409.2, found 409.2.



**3l**. Compound **2f** (TFA salt, 36 mg, 0.05 mmol) was dissolved in $H_2O$ (3 mL), KOH (120 mg, 2 mmol) was added, and the mixture was refluxed overnight. The mixture was run through a short column of neutralized Amberlite IRA-67 and the collected crude product was purified by reverse phase HPLC. Yield: 61%; **$^1$H NMR** ($D_2O$, 400 MHz): 5.50 (d, *J* = 4.0, 1H), 3.88 - 3.58 (m, 5H), 3.55–3.28 (m, 5H), 3.19 (m, 2H), 2.38 (td, *J* = 12.0, 4.0, 1H), 1.76 (q, *J* = 12.0, 1H); **$^{13}$C NMR** ($D_2O$, 75 MHz): δ 166.7, 96.4, 78.1, 75.4, 72.7, 70.8, 69.5, 68.9, 53.6, 49.9, 48.7, 40.3, 28.5; **ESI-MS** for $C_{14}H_{28}N_4O_8$ [M+Na$^+$] calcd 403.2, found 403.2.



**3m**. The $Na_2S_2$ solution was prepared by mixing $Na_2S$ (39 mg, 0.5 mmol) and $S_8$ (16 mg, 0.5 mmol) in $H_2O$ (2 mL) in a glass tube, and gently boiled using a heat gun

for a few minutes until the solution turns clear yellow. Compound **2f** (free base, 23 mg, 0.05 mmol) was dissolved in $H_2O$ (3 mL), the $Na_2S_2$ solution (0.2 mL, 0.05 mmol) was added and the mixture was stirred for 3 hours at room temperature. Dithiothreitol (8 mg, 0.05 mmol) was added and the mixture was stirred overnight at room temperature. After acidification to pH = 3 using 20% aqueous TFA the product was purified by HPLC to afford compound **3m** as a white fluffy powder. Yield: 68%; **$^1$H NMR** ($D_2O$, 400 MHz): δ 5.55 (d, *J* = 4.0, 1H), 3.83-3.79 (m, 3H), 3.52–3.10 (m, 10H), 2.34 (td, *J* = 12.0, 4.0, 1H), 1.70 (q, *J* = 12.0, 1H); **$^{13}$C NMR** ($D_2O$, 75 MHz): δ 172.3, 96.9, 78.9, 75.2, 72.7, 71.8, 70.8, 69.4, 68.5, 54.0, 49.9, 48.8, 40.0, 28.5; **ESI-MS** for $C_{14}H_{28}N_4O_7S$ [$M+H^+$] calcd 397.2, found 397.1.

### 7.4.3 AAC(6′)-Ii inhibition assays

AAC(6′)-Ii was expressed and purified as previously described elsewhere.[13] Enzyme activity was monitored using a procedure reported earlier.[30]

### 7.4.4 Substrate tests.

The substrate tests were performed in HEPES buffer (25 mM, pH 7.5) containing EDTA (1 mM), 4,4′-dithiodipyridine (DTDP, 400 μM), AcCoA (100 μM) and various concentrations of the molecule tested as a potential substrate (10, 20, 40, and 80 μM). Reaction volumes were typically 400 μL. The assay mixtures were preincubated for 3 min at 37ºC, and the reaction was initiated by the addition of AAC(6')-Ii (3.6 μM). The initial reaction velocities ($\varepsilon_{324nm}$ (thiopyridine) = 19, 800 $M^{-1}$ $cm^{-1}$) obtained at various concentrations of neamine derivatives were fit to the Michaelis-Menten Equation.

**Enzymatic transformation and detection of the acetylated neamine derivatives.**

The reaction mixtures (total volume of 2 mL) consisted of EDTA (1 mM), AcCoA (0.7 mM), the molecule tested (4 mM), and AAC(6')-Ii (0.4 mg) in HEPES (25 mM, pH 7.5). It was incubated at room temperature with orbital

shaking at 200 rpm. Additional portions of enzyme (0.1 mg) were added every 2 h for 10 h, and the mixture was left stirring at room temperature overnight. The enzyme was separated from the reaction mixture using an Amicon ultrafiltration system (10K MWCO membrane, filter code: YM10), and the filtrate was concentrated by rotary evaporation. The residue was suspended in water (1 mL) and applied to HPLC for separation. The column used was a semi-preparative Zorbax SB-CN of 4.6 × 250 mm and 5 μm (Agilent, Palo Alto, California). The sample was eluted at a flow rate of 2 mL/min, using a combination of mobile phase A (0.05% TFA in water at pH ~ 3.5) and mobile phase B (acetonitrile containing 0.05% TFA). After 10 min at 0% B, the eluent was brought to 40% B in a linear gradient over 30 min. The detector was set to 214 nm. After collection, the fractions were analyzed by ESI-MS. A control reaction was performed with neamine.

### 7.4.5 Anti-microbial activity test.

*E.coli* strains JR66, JR67, pETAACWT, and pSF815A-1 were kanamycin-resistant. *E. coli* strain JR88 was resistant to gentamycin. The *S. aureus* strain was sensitive to all usual antibiotics. The tests on solid medium were carried out at 30 μg/disc on LB. Standard dilution assays were performed in overnight cultures of MG1655 in LB (3 mL) diluted 100 times. The readings were O.D. at 600nm.

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# Chapter 8

## Summary, conclusions and future work

Aminoglycosides are widely prescribed antibiotics. They act by binding to the 16S rRNA A site of prokaryotes, thus causing mistranslation which eventually leads to cell death. The rising resistance threatens the use of these important medicines. Two general approaches have been used to address the aminoglycoside resistance problem. One is to derive the existing aminoglycoside antibiotics; the other is to develop inhibitors blocking resistance.

Aminoglycoside *N*-6′-acetyltransferase (AAC(6′) is one of most clinically relevant determinants of aminoglycoside resistance. We developed a novel methodology to regio- and chemo-selective derivatize unprotected aminoglycosides at the *N*-6′ position, and used this method to prepare a series of amide-linked aminoglycoside-CoA bisubstrate analogs. These analogs are the first reported nanomolar inhibitors of AAC(6′)-Ii. They have proved useful as mechanistic and structural probes to investigate the molecular mechanism of catalysis by AAC(6′)-Ii. These bisubstrates have been successfully used in co-crystallization experiments with AAC(6′)-Ii, carried out by our collaborators. The resulting 3D structures provided, *for the first time*, useful information about the aminoglycoside binding site of this enzyme.

Although the above amide-linked aminoglycoside-CoA bisubstrates are nanomolar inhibitors of AAC(6′)-Ii, they are not likely active in cells due to the negative charges of CoA, preventing these molecules crossing cell membranes. We next synthesized a series of truncated aminoglycoside-CoA bisubstrates using a convergent approach where the amine and the thiol are coupled in one pot with the addition of a linker, without the need for protecting groups. These derivatives were tested for their effect on the activity of the resistance-causing enzyme AAC(6′)-Ii and key structure-activity relationships were revealed. Our results

indicate that inhibition by aminoglycoside-CoA bisubstrates is more sensitive to truncation at the aminoglycoside than at the CoA end. The smaller neamine is however, as efficient as larger aminoglycosides to maintain the affinity of the bisubstrate for AAC(6′)-Ii. Our results have also emphasized the importance of properly oriented positively charged amino groups for interaction with the aminoglycoside binding pocket of AAC(6′)-Ii.

Truncation of the CoA moiety of amide-linked aminoglycoside-CoA bisubstrates revealed that the pantetheinyl group is not sufficient for recognition by the enzyme and at least one phosphate group is needed. More potent inhibitors were obtained when the pyrophosphate moiety of CoA was mimicked with β-dicarbonyl groups such as malonate or acetoacetate. To our knowledge, this is the first time that a pyrophosphate group is successfully mimicked with acetoacetate. The resulting bisubstrate analog is the first inhibitor of an aminoglycoside resistance-causing enzyme active in cells. This provides a proof of concept for the pharmaceutical potential of AAC(6′) inhibitors.

To improve inhibition and better mimic the proposed tetrahedral intermediate, bisubstrate inhibitors containing sulfonamide, sulfone and sulfoxide linkers were synthesized and used as mechanistic probes for AAC(6′)-Ii. Our results support the suggestion that AAC(6′)-Ii may catalyse acetyltransfer without stabilization of the tetrahedral intermediate. Interestingly, sulfide oxidation of the bisubstrate containing an amide linker dramatically improved inhibition of AAC(6′)-Ii. This inhibitor is the most potent one known for an aminoglycoside resistance-causing enzyme. The structure-activity relationships derived from this thesis are summarized in Fig. 8.1.

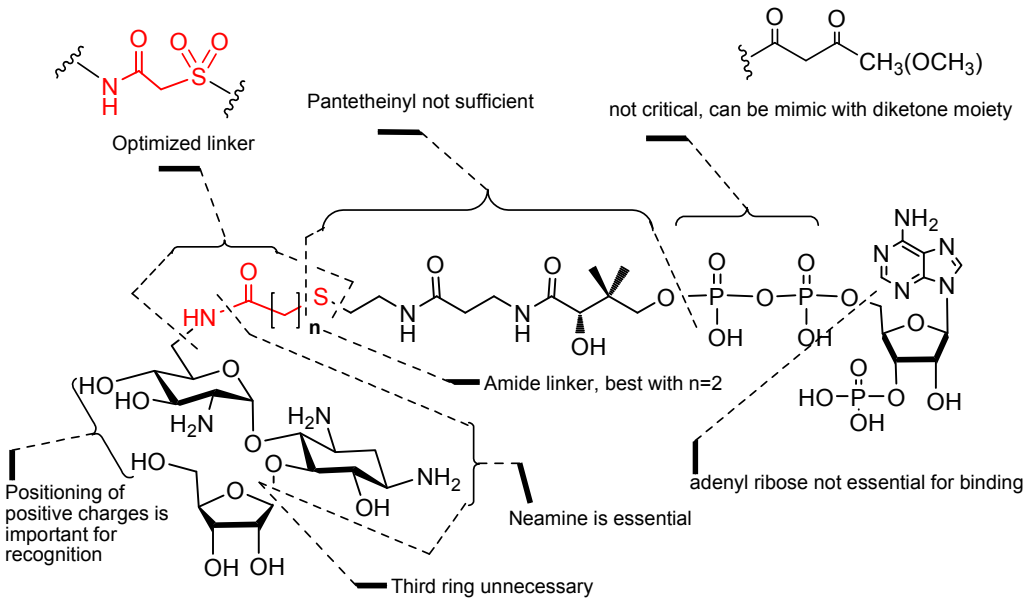


***Fig. 8.1***. Structure-activity relationship study of bisubstrate inhibitors of AAC(6′)-Ii.

Bisubstrates with linkers containing phosphoryl group (P=O) were proposed and synthesized, the biological results are under investigation. These molecules will facilitate investigations of the potential stabilization of the tetrahedral intermediate by the enzyme. Our efforts in this project also improved our chemical knowledge of phosphorus chemistry. For example, attempts to synthesize such bisubstrates by adapting our 6′-regioselective acylation methodology to phosphorylation led to the discovery of a rapid rearrangement of *N*-[(phosphino)oxy] amines to phosphoramidic or phosphorodiamidic acid triesters. The *N*-[(phosphino)oxy] amine intermediate is generated *in situ* from the reaction of hydroxylamines with chlorophosphites or chlorophosphoramidites and with rearrangement via cleavage of the weaker N-O bond to generate a more stable P=O bond. The rearrangement proceeds spontaneously in excellent yield when the hydroxylamine is electron poor. Various substituents on the phosphorus are well tolerated.

An alternative approach to combat bacterial resistance is to modify the existing antibiotics yielding derivatives active against resistant strains. Crystallographic data of aminoglycoside-RNA complexes are increasingly

available, providing insights for the design of new aminoglycoside derivatives. Together with our crystallographic data of the AAC(6′)-Ii–bisubstrate complex, these structures were used to design a series of neamine-*N*-6′-derivatives able to bind to RNA but not to AAC(6′)-Ii. The syntheses were facilitated by the previous developed method for *N*-6′-derivatization of unprotected aminoglycosides. These derivatives showed greatly increased $K_m^{app}$ and reduced $k_{cat}$ (data not shown) towards AAC(6′)-Ii, but had diminished antibacterial activity as well. Although none of our derivatives have the potential to become antibiotics, our results emphasize the central role of *N*-6′ nucleophilicity for transformation by AAC(6′)-Ii and the importance of hydrogen bonding between 6′-$NH_2$ and 16S rRNA for antibacterial activity.

For the future work, it will shed more lights on the catalysis mechanism of AAC(6′)s if these bisubstrate analogs synthesized in chapter 4 and 5 can be used as structural probes to carry out crystallographic study of the AAC(6′)s. Fortunately we have on-going collaborations with a few structural biology labs, the results will be reported on due course. It is worthy to develop pro-drug type inhibitors, for example, phosphate ester capped bisubstrate **5b** (chapter 3), which focuses on shielding of negative charges of phosphate therefore, enhance the chance of these bisubstrate inhibitor passing cell membrane. Truncated bisubstrate analog **5a** showed enhanced activation of AAC(6′)-Ii, would this molecule has similar effect on other AAC(6′)s? The detailed study of this activation mechanism would be very interesting and important as well in order to fully understand the aminoglycoside resistance-causing enzyme AAC(6′)s.

## Contribution to knowledge

I. We have developed a methodology for regio- and chemo-selective derivatization at 6′-$NH_2$ of unprotected aminoglycosides. We have also demonstrated that this novel method is very used to efficiently prepare amide-linked aminoglycoside-CoA bisubstrate inhibitors.

II. We have shown that amide-linked aminoglycoside-CoA bisubstrates are nanomolar inhibitors of aminoglycoside *N*-6′-acetyltransferase AAC(6′)-Ii, they have been proven useful structural and mechanistic probes to study the catalysis mechanism of AAC(6′)s. In collaboration with others, these bisubstrates were successfully employed to provide ternary complex crystals, which for the first time, provided the structural information of the aminoglycoside binding site of AAC(6′)-Ii. The results are published in *Angew. Chem. Int. Ed.* **2005**, 44, 6859-6862

III. We have demonstrated that the neamine is a minimal substrate for effective binding of AAC(6′)-Ii via structure-activity relationship studies of truncated bisubstrates. Positive charges of substrate is essential, yet the correct orientation of positive charges is optional. Our results suggest that the negative charges from the CoA moiety prevent bisubstrates from crossing cell membranes. For the first time, we have shown that a β-diketone chemical entity can be used to mimic the pyrophosphate of CoA and the resulting bisubstrate analog is active in cell. The results are published in *J. Med. Chem.* **2006**, 49, 5273-5281 and the molecules are the subject of a patent submitted by McGill.

IV. We have demonstrated for the first time, that ammonium persulfate can be used to selectively oxidize a sulfide containing multiple functionalities to a sulfoxide in aqueous solution. Oxidation of the sulfide of amide-linked bisubstrates led to significantly improved inhibition of AAC(6′)-Ii, possibly due to extra H-bond interactions between the sulfone and two amino acid residues, Tyr141 and Thr111 of the enzyme.

V. We have demonstrated that replacement of the amide linker of bisubstrates with a sulfonamide leads to a significant loss of AAC(6′)-Ii inhibition. As previously suggested by others, this results suggests that AAC(6′)-Ii may catalyze the acetyltransfer reaction without the stabilization of the tetrahedral intermediate.

VI. We discovered a rapid rearrangement of *N*-[(phosphino)oxy] amines to phosphoramidic or phosphorodiamidic acid triesters. This P(III) to P(V) rearrangement proceeds via cleavage of the weaker N-O bond to generate a more stable P=O bond. The rearrangement takes place spontaneously in excellent yield and is useful to prepare a variety of organophosphorus compounds. This led to a publication in *Phosphorus, Sulfur and Silicon and the Related Elements*, **2006**, 181, 159-165.

VII. We have shown that a library of *N*-6′-aminoglycoside derivatives can be synthesized efficiently without protection of the aminoglycosides. Biological activity with these derivatives demonstrates the importance of the *N*-6′ nucleophilicity for transformation of aminoglycosides by AAC(6′)-Ii and for antibacterial activity. This was published in journal *Bioorg. Med. Chem*. 2007, 15, 2944-2951.

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To Whom It May Concern:

We have no objection the following unpublished papers, of which we are a co-author, being included in the Ph.D. thesis of Feng Gao entitled "Synthesis of aminolgycoside derivative to combat antibacterial resistance":

"The Use of Aminoglycoside Derivatives to Study the Mechanism of Aminoglycoside 6′-*N*-Acetyltransferase and the Role of 6′-$NH_2$ in Antibacterial Activity

"Use of Sulfonamide-, Sulfoxide-, or Sulfone-containing Aminoglycoside-CoA Bisubstrate as Mechanistic Probes for Aminoglycoside N-6′-Acetyltransferase"

Professor Karine Auclair

Signature:  Date: 31 Jan 2007

Student Xuxu Yan

Signature:  Date: 31 Jan 2007

Student Lee Freiburger

Signature:  Date: 1 Feb 2007

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Thank you in advance for your assistance on this matter.

Sincerely,

Feng Gao
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