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ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 2944–2951
Bioorganic & Medicinal Chemistry

The use of aminoglycoside derivatives to study the mechanism of aminoglycoside 6′-*N*-acetyltransferase and the role of 6′-NH_2 in antibacterial activity

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Received 5 December 2006; revised 30 January 2007; accepted 8 February 2007
Available online 11 February 2007

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.

1. Introduction

Aminoglycosides are frequently prescribed broad spectrum antibiotics. They act by binding to the 16S rRNA A-site causing mistranslation and blocking protein synthesis.[1,2] Their use is however threatened by the rapid spread of resistance. Aminoglycoside resistance occurs mainly via drug modifications[3–6] such as acetylation, adenylation, or phosphorylation. Aminoglycoside 6′-*N*-acetyltransferase (AAC(6′)) is one of the most common determinants of resistance to this family of antibiotics.

One strategy to overcome aminoglycoside resistance is to develop inhibitors of resistance-causing enzymes.[7–13] Northrop and coworkers reported the first nanomolar inhibitor of an AAC, 3-*N*-(2-*S*-CoA-acetyl)gentamicin C1a.[12] This bisubstrate 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.[13–16] We recently developed an effective one-pot regio- and chemo-selective method for the direct N-6′-derivatization of unprotected aminoglycosides in high yield.[7] 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.[8] This also led to a derivative able to block aminoglycoside modification in cells.

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.[15,17–21] 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 preparation using our novel synthetic methodology, their assay with AAC(6′)-Ii, and their antibacterial activity.

2. Results

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[20,22–29] reveal an important hydrogen bond

Keywords: Antibiotic; A-site; Neamine; Regioselective; Resistance; Aminoglycoside; Acetyltransferase; Antibacterial; RNA.
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doi:10.1016/j.bmc.2007.02.009

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 the 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.[30] 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 N-6′-linked aminoglycoside–CoA bisubstrates.[7] The structures of AAC(6′)-Ii with different bisubstrate inhibitors provide valuable information about the effect of extra carbons near N-6′. Unexpectedly, the most potent bisubstrate inhibitor of AAC(6′)-Ii reported so far has two extra carbons compared to the proposed tetrahedral intermediate (Fig. 1). According to the X-ray structure, the methylene groups of this bisubstrate labeled as C1 and C2 are surrounded mostly by hydrophobic residues. On the other hand, the reported structures for aminoglycosides in complex with RNA[20,22–29] 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 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-stacking interactions. Thus, targets **2b**–**2c** were designed with aromatic groups near the 6′ position and expected to stack with A1408.

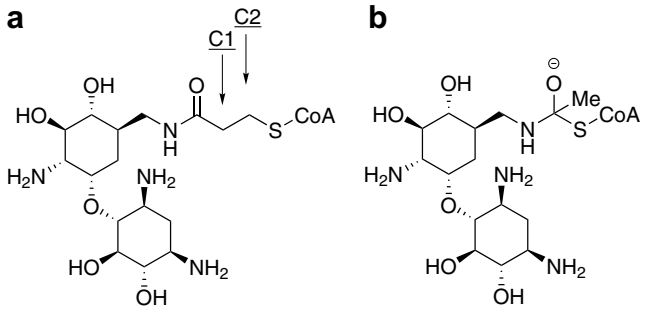


Figure 1. (a) Previously reported nanomolar inhibitor of AAC(6′)-Ii.[7] This bisubstrate is two carbon longer than the putative enzymatic tetrahedral intermediate (b).

Synthesis of the targets was achieved using our previously reported regioselective strategy.[7,8] 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. With derivatives **2a**–**f** however, reverse phase HPLC was necessary. Standard deprotection protocols generated target derivatives **3g**–**k** in yields ranging from 20% to 60% (unoptimized). To maximize the purity, large amounts of impure products had to be sacrificed during purification on silica gel, thus greatly reducing the yields.

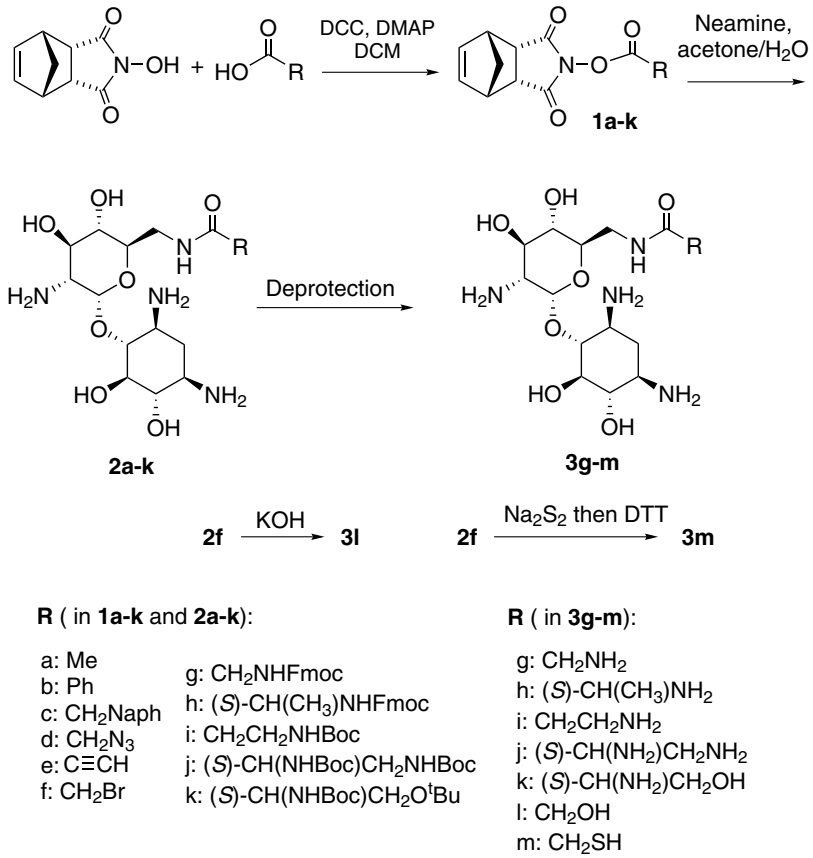
Compound **2f** was an intermediate in the preparation of targets **3l**–**m**. Shortly treating **2f** with KOH yielded **3l** (61%). Compound **2f** was also separately treated with Na_2S_2 (generated by mixing Na_2S with S_8 in boiling water) to afford a disulfide intermediate that was further reduced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

2.2. Transformation of 2a–e and 3g–m by the resistance-causing enzyme AAC(6′)-Ii

The neamine derivatives **2a**–**e** and **3g**–**m** were first tested for inhibition of AAC(6′)-Ii. No reduction in activity was observed. The molecules were next tested as substrates and found to be insignificantly transformed (UV detection after in situ derivatization of the CoA produced). Compound **3g** was selected for further analysis of the transformation by AAC(6′)-Ii. No acetylation products were detected (detection limit: 10^{-8} M) after HPLC separation and ESI-MS analysis. As a control experiment, the acetylation product of neamine was clearly recognized (10^{-4} M). This verifies our assumption that the neamine derivatives (**2a**–**2e** and **3g**–**3m**) are very poor or not substrates for AAC(6′)-Ii. It also confirms that N-6′ is the position acetylated by this enzyme.

2.3. Antibacterial activity in cells

The RNA binding of derivatives **2a**–**2e** and **3g**–**3m** was next evaluated indirectly via antibacterial cell-based activity assays on paper discs and in solution. In the assays, *Escherichia coli* strains JR66, JR67, pETAAC wt, and pSF815A-1 were kanamycin-resistant. *E. coli* strain JR88 was resistant to gentamicin. *E. coli* strain MG1655 was not resistant to aminoglycosides and the *Staphylococcus aureus* strain was sensitive to all usual antibiotics. 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. 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 activity.



Scheme 1. Synthesis of neamine derivatives **2a**–**f** and **3g**–**m**.

Table 1. Results of antimicrobial activity assay using the dilution method

Compound	MIC[a] ($\mu g\,mL^{-1}$)
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] Minimum inhibitory concentration.
[b] Below detection.

3. 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 antimicrobials are vital. The antibacterial activity of aminoglycosides is due to their high affinity for the prokaryotic A site of 16S 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 resistance-causing AAC(6′)-Ii and the role of the N-6′ of aminoglycosides in antibacterial activity. Using the available crystal structures of aminoglycosides complexed to either RNA[22] or AAC(6′)s,[7,30] 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 to regioselectively modify aminoglycosides, and efficiently synthesized a series of neamine derivatives acylated at N-6′ (**2a**–**f** and **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 in an overall yield of 8.8%,[31,32] whereas our methodology allows its synthesis in 68% overall yield.

As predicted, none of derivatives **2a**–**e** or **3g**–**m** were significantly transformed by AAC(6′)-Ii. Based on

the crystal structures of bisubstrates–AAC(6′)-Ii complexes,[7] 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**, **3 l**, and **3m**) combined with our previous studies with bisubstrate inhibitors[7] 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 4-carbon linkers at N-6′ were potent inhibitors of AAC(6′)-Ii, and those with 3-carbon linkers near N-6′ were more potent inhibitors than 2-carbon linkers. This is surprising since the latter more closely resemble the tetrahedral enzymatic intermediate (1-carbon). The better binding of the bisubstrate containing a 3-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 three 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.[33] 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 or translation interference. Further studies would be required to settle this issue and confirm cell permeability. 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 with these derivatives suggests that the aryl groups could not reach far enough to stack with any 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 was more than three atoms long (**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. Since the addition of acyl chains of less than three atoms should not cause steric clashes, this confirms our initial hypothesis that acetylation affects RNA affinity by reducing the hydrogen bonding ability at N-6′. The ~4-fold decrease in antibacterial activity observed for **3g** and **3l** suggests that the newly added hydrogen bond donors/acceptors may not be positioned properly.

In conclusion, our results not only demonstrate once again 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.

4. Experimental

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 (Que., 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 T 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.

4.2. Synthesis

4.2.1. General procedure for the synthesis of compounds 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 vacuum. When higher purity was needed, a silica gel chromatography column was used for the purification.

4.2.2. General procedure for the preparation of compounds 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 compounds **2g**–**2k**, however, were purified by flash chromatography.

Deprotection of Boc, Fmoc, and *tert*-butyl ether groups was performed using standard procedures.[34]

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); ^1H 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.

Compound **1a**. TLC $R_f = 0.53$ (Hex/EtOAc 1/1); yield: 90%; ^1H 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, 238.2.

Compound **1b**. TLC $R_f = 0.65$ (Hex/EtOAc 1:1); yield: 94%; ^1H 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; MS for $C_{16}H_{13}NO_4$(M+H) calcd 284.1, found: 284.1.

Compound **1c**. TLC $R_f = 0.55$ (Hex/EtOAc 1:1); yield: 99%; ^1H 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; MS for $C_{21}H_{17}NO_4$ (M+H) calcd 348.1, found: 348.1.

Compound **1d**. TLC $R_f = 0.35$ (Hex/EtOAc 1:1); yield: 80%; ^1H 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.

Compound **1e**. TLC $R_f = 0.47$ (Hex/EtOAc 1:1); yield: 94%; ^1H 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.

Compound **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 mp 90–92 °C; ^1H 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; HRMS for $C_{11}H_{10}NO_4$ calcd 298.98 (100) and 300.98 (97), found: 299.00 (100) and 301.00 (97).

Compound **1g**. TLC $R_f = 0.66$ (Hex/EtOAc 1:1). The starting material Fmoc-Gly-OH was used as the limiting reagent; yield: 94%; ^1H 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).

Compound **1h**. TLC $R_f = 0.38$ (Hex/EtOAc 1:1). The starting material Fmoc-Ala-OH was the limiting reagent; Yield: 76%; ^1H 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).

Compound **1i**. $R_f = 0.38$ (Hex/EtOAc, 1:1). The starting material Boc-β-Ala-OH was the limiting reagent; yield: 84%; ^1H 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).

Compound **1j**. $R_f = 0.33$ (Hex/EtOAc, 2:1). The starting material di-Boc-2,3-diaminopropionic acid was the limiting reagent; yield 52%; ^1H 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).

Compound **1k**. $R_f = 0.68$ (Hex/EtOAc, 1:4). The starting material Fmoc-Ser (^{t}Bu)-OH was the limiting reagent; yield 89%; ^1H 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).

Compound **2a**. Yield 60%; ^{1}H NMR (D_2O, 400 MHz, pD = 4.0, presat.): 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 387.20, found: 387.20.

Compound **2b**. Yield 80%; ^{1}H NMR (D_2O, 400 MHz, pD = 4.0, presat.): 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 439.21, found: 439.11.

Compound **2c**. Yield 85%; ^{1}H NMR (D_2O, 400 MHz, presat.): 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.

Compound **2d**. Yield 75%; ^{1}H NMR (D_2O, 400 MHz, pD = 4.0, presat.): 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.

Compound **2e**. Yield 77%; ^{1}H NMR (D_2O, 400 MHz, pD = 4.0, presat.): 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.

Compound **2f**. Yield 70%; ^{1}H NMR (D_2O, 400 MHz, pD = 4.0, presat.): 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 $C_{14}H_{27}BrN_4O_7$ (M+H) calcd 442.11, 444.10, found: 442.20 (100), 444.20 (98).

Compound **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.

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

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

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

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

Compound **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 requires 380.2, found: 380.1.

Compound **3h**. Yield 17%; ^{1}H NMR (D_2O, 400 MHz, pD = 10, presat.): 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.

Compound **3i**. Yield 33%; ^{1}H NMR (D_2O, 400 MHz, pD = 10, presat.): 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.

Compound **3j**. Yield 60%; ^{1}H NMR (D_2O, 400 MHz, pD = 10, presat.): 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.

Compound **3k**. Yield 22%; ^{1}H NMR (D_2O, 400 MHz, pD = 10, presat.): 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.

Compound **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, pD = 4.0, presat.): 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.

Compound **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 h 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, pD = 4.0, presat.): 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.

4.3. AAC(6′)-Ii inhibition assays

AAC(6′)-Ii was expressed and purified as previously described elsewhere.[7] Enzyme activity was monitored using a procedure reported earlier.[35]

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 37 °C, and the reaction mixture was initiated by the addition of AAC(6′)-Ii (3.6 μM). The initial reaction velocities ($\varepsilon_{324\ nm}$ (thiopyridine) = 19, 800 $M^{-1}\,cm^{-1}$) obtained at various concentrations of neamine derivatives were fit to the Michaelis–Menten equation.

4.5. 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, CA). 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.

4.6. Anti-microbial activity test

Escherichia coli strains JR66, JR67, pETAACWT, and pSF815A-1 were kanamycin-resistant. *E. coli* strain JR88 was resistant to gentamicin. 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 OD at 600 nm.

Acknowledgments

The authors thank Karen Lu and Prof. Julian E. Davies for performing the antimicrobial activity assays. This work was supported by the Natural Science and Engineering Research Council of Canada (NSERC).

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