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Bioorganic & Medicinal Chemistry 13 (2005) 6252–6263
Bioorganic & Medicinal Chemistry

Discovery of non-carbohydrate inhibitors of aminoglycoside-modifying enzymes

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Received 6 January 2005; revised 24 June 2005; accepted 24 June 2005
Available online 1 September 2005

Abstract—Chemical modification and inactivation of aminoglycosides by many different enzymes expressed in pathogenic bacteria are the main mechanisms of bacterial resistance to these antibiotics. In this work, we designed inhibitors that contain the 1,3-diamine pharmacophore shared by all aminoglycoside antibiotics that contain the 2-deoxystreptamine ring. A discovery library of molecules was prepared by attaching different side chains to both sides of the 1,3-diamine motif. Several of these diamines showed inhibitory activity toward two or three different representative aminoglycoside-modifying enzymes (AGMEs). These studies yielded the first non-carbohydrate inhibitor *N*-cyclohexyl-*N*′-(3-dimethylamino-propyl)-propane-1,3-diamine (Compound **G**,**H**) that is competitive with respect to the aminoglycoside binding to the enzyme aminoglycoside-2″-nucleotidyltransferase-Ia (ANT(2″)). Another diamine molecule *N*-[2-(3,4-dimethoxyphenyl)-ethyl]-*N*′-(3-dimethylamino-propyl)-propane-1,3-diamine (Compound **H**,**I**) was shown to be a competitive inhibitor of two separate enzymes (aminoglycoside-3′-phosphotransferase-IIIa (APH(3′)) and ANT(2″)) with respect to metal–ATP. Thermodynamic and structural-binding properties of the complexes of APH(3′) with substrates and inhibitor were shown to be similar to each other, as determined by isothermal titration calorimetry and NMR spectroscopy.

1. Introduction

The therapeutic use of aminoglycoside antibiotics (Fig. 1) began in the mid-1940s when streptomycin, isolated from *Streptomyces griseus*, was developed as the first effective treatment for tuberculosis.[1,2] Since then, many new aminoglycosides have been isolated and synthetic derivatives prepared. These antibiotics are the weapons of choice against certain gram-negative bacilli, including *Pseudomonas*. However, as with many other antibiotics, resistance to these drugs has developed and now compromises their usage.[3,4] Resistance to aminoglycosides is caused primarily by plasmid-encoded enzymes that covalently modify certain functional groups and prevent the aminoglycosides from interacting with their target—the small subunit of the bacterial ribosome.[5,6] Three types of covalent modifications: acetylation, nucleotidylation, and phosphorylation reactions are achieved by many different aminoglycoside-modifying enzymes (AGMEs).[7,8] Thus, inhibition of AGMEs may allow aminoglycosides to regain their antibiotic efficacy.

Carbohydrate-based inhibitors of AGMEs, such as those resulting from the removal of selected hydroxyl or amino groups of the aminoglycoside scaffold, are known. More recently, various inhibitors based on neamine dimers,[9,10] glycodiversification strategy,[11] pseudo-pentasaccharide analogs of neomycin,[12] amikacin derivatives,[13,14] 2-deoxystreptamine (DOS) derivatives,[15] and cyclic aminoglycosides[16] have been prepared. Fewer efforts are directed toward non-carbohydrate aminoglycoside mimetics, such as small-molecule RNA binders,[17] benzimidazoles,[18] and cationic peptides.[19]

Most clinically utilized aminoglycosides have a DOS as their aglycone. In general, these aminoglycosides are

Keywords: Aminoglycosides; Drug design; Inhibitors; Bacterial resistance.
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doi:10.1016/j.bmc.2005.06.059

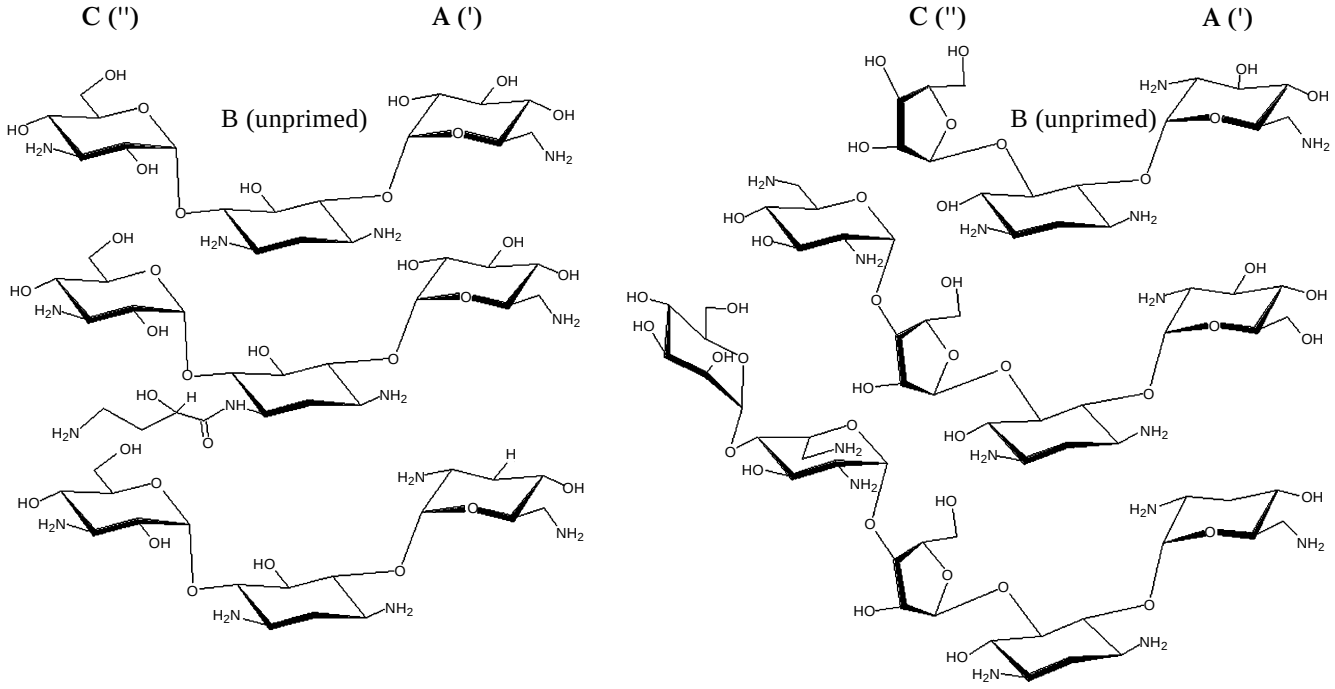


Figure 1. Aminoglycosides with 4,6-(left panel) and 4,5-(right panel) disubstituted 2-deoxystreptamine ring. Left panel (from top): kanamycin A, amikacin, and tobramycin. Right panel (from top): ribostamycin, paromomycin, and lividomycin A. Letter and prime notations for the first three rings are also shown on top.

4,5- or 4,6-substituted at the DOS ring and can be pseudotri-, tetra-, or pentasaccharides (Fig. 1). Aminoglycosides with a 4,5- or 4,6-substituted DOS ring can also be classified as neomycin- and kanamycin-type antibiotics, respectively. In this work, we selected the simplest common pharmacophore to aminoglycosides bearing a 2-DOS aglycone: the 1,3 diamine motif (Fig. 2) and designed an exploratory library of non-carbohydrate aminoglycoside analogs to evaluate the potential of this functional group in producing molecules that recognize, bind, and inhibit multiple AGMEs. These analogs contain a 1,3-diamine unit that is flanked by a diverse array of side-chain functionality. This proof-of-concept study yielded the first non-carbohydrate inhibitor that is competitive with the aminoglycoside substrates of an aminoglycoside nucleotidyltransferase (the aminoglycoside-2″-nucleotidyltransferase-Ia ANT(2″)).

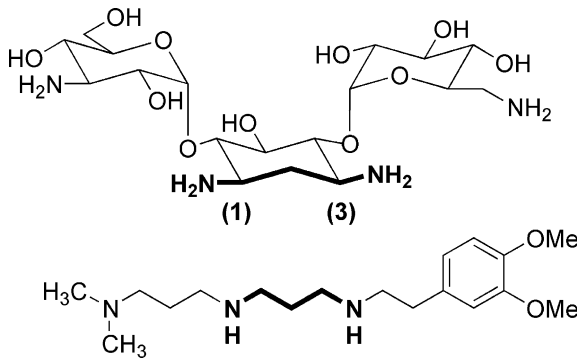
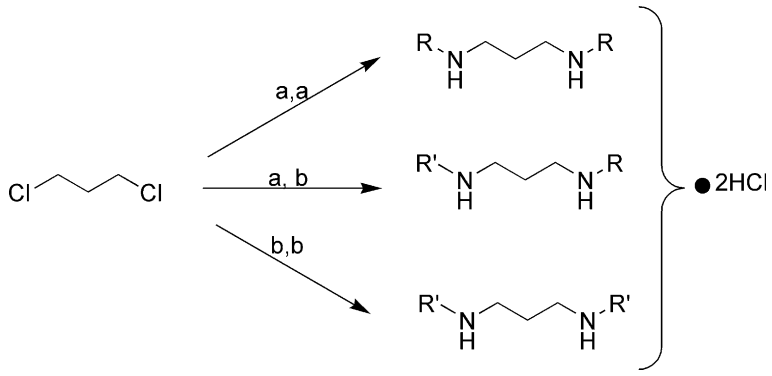


Figure 2. The 1,3 diamine motifs of kanamycin A (top) and the compound **H**,**I** (bottom).

2. Results and discussion

The diamines were prepared by the matrix synthesis using a one-pot reaction (Scheme 1). Two equivalents of primary amines **A**–**I** (Fig. 3) were reacted with one equivalent of 1,3-dichloropropane in a solvent-free reaction at an elevated temperature for 48 h[20] Two S_N2 reactions took place to yield one or three diamines, based upon starting materials, with the product secondary amines salting out as dihydrochlorides. Thus, in one step, we were quickly able to produce 45 compounds containing the 1,3-x motif. The reaction mixtures were easily deconvoluted by testing three related samples, that is, **A**,**A**; **A**,**B**; and **B**,**B**. The chemistry of this synthetic route was complicated by the tendency of amine **H** to undergo side reactions. Amine **H** contains a tertiary nitrogen atom that can act as an internal strong base to desalt the product secondary amine and allow it to react with another molecule of 1,3-dichloropropane. This process leads to the formation of undesired high molecular weight adducts. Several test wells containing amine **H** were active in our primary screen; therefore, an alternate synthetic route was needed to validate hits resulting from these wells.

The alternate synthetic devised route is shown in Scheme 2. The first amine is added to acryloyl chloride, yielding an α,β-unsaturated amide containing one of the desired side-chains. The second amine is subsequently added by a Michael addition to the α,β-unsaturated amide by the



Scheme 1. Synthesis of compounds **A**,**A**–**I**,**I**. (a) R-NH_2 (amines **A**–**I**); (b) R′-NH_2 (amines **A**–**I**).

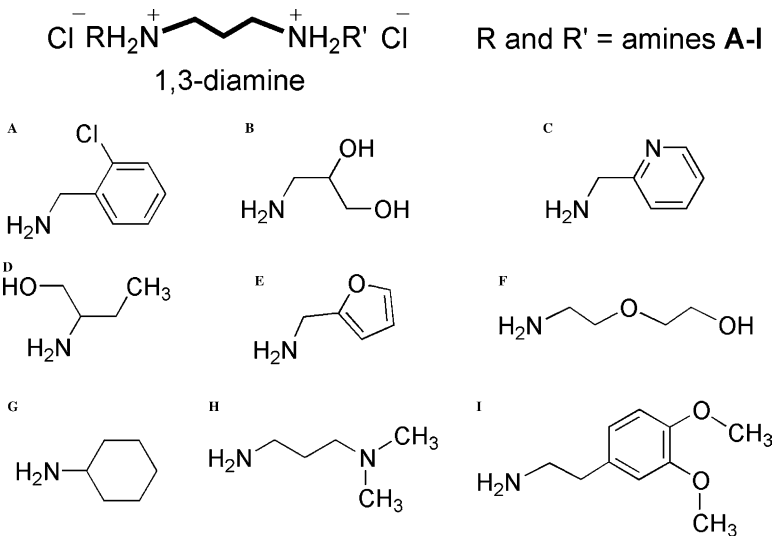
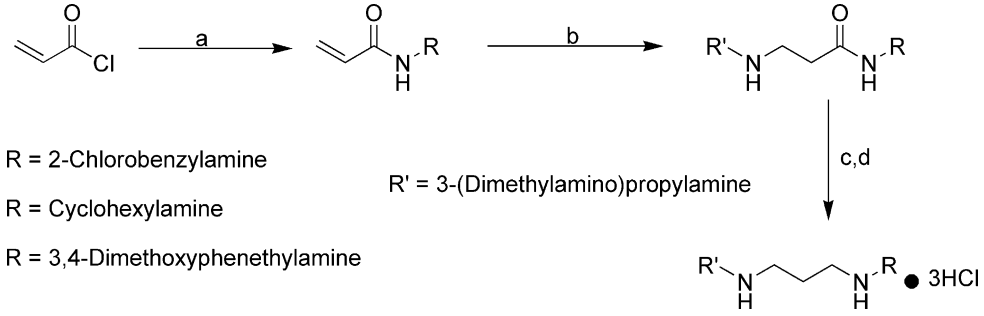


Figure 3. Diamine compounds that showed inhibitory activity to one or more AGME.



Scheme 2. Synthesis of compounds **A**,**H**; **G**,**H**; and **H**,**I**. Reagents: (a) R-NH_2, pyridine, DCM; (b) R′-NH_2, EtOH; (c) THF·BH_3 complex, THF; and (d) 6 N HCl, MeOH.

second amine. The resulting compound is purified by flash chromatography and then reduced with borane in tetrahydrofuran to afford the desired diamine. One advantage of this synthesis over the previous one is the greater control of reactivity. Another is the production of a single diamine, as opposed to a mixture of up to three compounds.

Three representative AGMEs[7] were chosen to evaluate the activity of these compounds. Aminoglycoside 3′-phosphotransferase-IIIa (APH(3′)) transfers a phosphate group from ATP to the 3′-hydroxyl group of appropriately functionalized aminoglycosides. The 2″-nucleotidyltransferase-Ia (ANT(2″)) catalyzes the transfer of adenosine monophosphate from ATP to the 2″-hydroxyl group of appropriately functionalized aminoglycoside antibiotics. Aminoglycoside 3-acetyltransferase-IIIb (AAC(3)) catalyzes the transfer of an acetyl group from acetyl CoA to the 3-amino group of aminoglycosides containing a 2-deoxystreptamine ring.

These enzymes represent three distinct covalent modifications, two different co-substrates (Metal–ATP with nucleotidyl- and phosphotransferases and acetylCoA with the acetyltransferase), and different levels of aminoglycoside substrate specificity. Moreover, they also vary in the distance between the site of covalent modification and the 1,3-diamine unit of the 2-deoxystreptamine ring.

Enzymatic screening of the synthetic diamines proceeded as follows: all crude reaction mixtures were screened for inhibition of aminoglycoside modification with APH(3′). A smaller set of samples, more than half of the total number of those tested with APH(3′), were screened with AAC(3). From these, a subset of compounds were chosen for inhibitory activity and ease of purification, and these compounds were HPLC purified and retested with AAC(3), ANT(2″), and APH(3′). The most potent inhibitor of all three enzymes was tested to determine the type of inhibition. Percent inhibition for diamines that inhibit more than one AGME is shown in Table 1.

The AGME that was most affected by these compounds was AAC(3). Eighteen of the 45 compounds tested were inhibitors of this enzyme. Compounds containing amine **H** (3-(dimethylamino)propylamine) were inhibitors of AAC3, decreasing the rate of catalysis to less than 30% of the uninhibited rate.

In contrast, a smaller number of compounds retarded the rate of catalysis of APH(3′). There was not as strong a trend in the chemical structures of the active compounds as there was for AAC(3); however, all compounds that inhibited APH(3′) contained amine **H** (3-(dimethylamino)propylamine), amine **I** (3,4-dimethoxyphenethylamine), or both. The common theme for inhibitors of APH(3′) was an aromatic or cyclohexyl ring plus a dimethylamino group or diol.

Even fewer among these compounds seem to be inhibitors of ANT(2″). Only five of the purified diamines tested showed significant inhibition of this enzyme. Similar to APH(3′), all the compounds that inhibit ANT(2″) contain either amine **H**, amine **I**, or both.

Compounds **G**,**H** and **H**,**I** were the most potent compounds identified by the primary screen and were subsequently resynthesized by the second route in greater scale and purity (Scheme 2). These compounds were tested to determine the nature of inhibition with APH(3′) and ANT(2″). The results of these assays indicate a difference in the action of the two compounds. Compound **G**,**H** is competitive with the aminoglycoside substrate for ANT(2″) but not for APH(3′). Data shown in Figure 4A yielded a K_i of 540 ± 32 μM for this inhibition.

Table 1. Inhibition of enzymatic activity by ~0.5 mM diamine compounds (% inhibition with respect to full activity. Errors ~10%)[a]

Diamine	APH(3′)	AAC(3)	ANT(2″)
B,**I**	18	21	23
C,**H**	13	58	28
C,**I**	13	27	16
D,**H**	21	62	0
F,**H**	21	68	0
G,**H**	28	77	22
H,**I**	54	63	42

[a] Substrate and enzyme concentrations used were: 50 μM kanamycin A with 2 μ APH(3′) or 1 μM AAC(3) and 100 μM tobramycin with 0.24 μM ANT(2″).

2.1. Compound G,H is the first non-carbohydrate inhibitor of an AGME that is competitive with the aminoglycoside substrate

Conversely, compound **H**,**I** is competitive with metal–ATP for both enzymes that require it for catalysis. The K_i values for **H**,**I** are comparable—160 ± 30 μM for APH(3′) and 125 ± 24 μM for ANT(2″). Competitive inhibition of ANT(2″) by **H**,**I** is shown in Figure 4B. Due to the low solubility of AAC(3) and ANT(2″) and availability of X-ray structure for APH(3′), further structural studies were performed with APH(3′) and complexes of APH(3′) with ATP or compound **H**,**I** by isothermal titration calorimetry (ITC), NMR, and docking calculations. These studies are described in the following paragraphs.

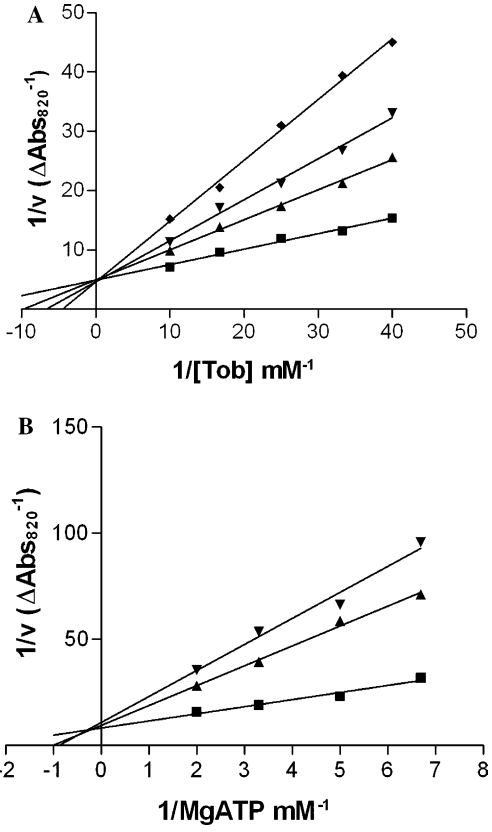


Figure 4. Inhibition of enzymatic activity of ANT(2″) by compounds **G**,**H** (A) and **H**,**I** (B). (A) Competitive inhibition with respect to tobramycin: (■) No inhibitor, (▲) 0.625 mM, (▼) 1.25 mM, and (♦) 2.5 mM **G**,**H**. (B) Competitive inhibition with respect to MgATP: (■) No inhibitor, (▲) 0.625 mM, and (▼) 1.25 mM **H**,**I**. In both plots, the rate is shown as the change in absorbance per unit time and data are shown with linear regression lines.

Table 2. ITC-derived thermodynamic parameters for CaATP and **H**,**I** Binding to APH(3′)-IIIa[a]

	N	K_D (μM)	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)	ΔG (kcal/mol)
CaATP	0.93	35	−3.5	−2.9	−6.4
H,**I**	0.98	47	−8.6	2.5	−6.1

[a] Determined at 303 K. Standard errors: N, 15%; K_D, 10%; ΔH, 8%; $T\Delta S$, 16%; and ΔG, 1%.

Table 3. ITC-derived thermodynamic parameters for kanamycin A binding to APH(3′)-IIIa and its complexes with CaATP and **H**,**I**[a]

	N	K_D (μM)	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)	ΔG (kcal/mol)
APH(3′)-IIIa	0.8	5.4	−31.9	24.4	−7.5
APH(3′)-IIIa–CaATP	0.9	1.0	−11.3	2.8	−8.5
APH(3′)-IIIa–**H**,**I**	0.9	1.3	−9.4	1.2	−8.2

[a] Determined at 310 K. Standard errors: N, 9%; K_D, 8%; ΔH, 7%; $T\Delta S$, 16%; and ΔG; 1%.

The use of isothermal titration calorimetry allowed for a direct thermodynamic characterization of various complexes of APH(3′), substrates, and inhibitor. Binding of CaATP and compound **H**,**I** to APH(3′) takes place with similar affinity (Table 2). Binding of both compounds to APH(3′) shows a very similar free energy change; however, the binding of compound **H**,**I** to the enzyme is entropically disfavored, which is compensated by a large enthalpic term. This is not unexpected since compound **H**,**I** is much more flexible in solution than free ATP and loss of rotational and translational freedom of molecule on binding to the enzyme is entropically more disfavored than it is with ATP. Similarities in the binding of compound **H**,**I** and CaATP also extend to the ternary enzyme–CaATP/**H**,**I**–kanamycin A complexes. As shown in Figure 5, titration of APH(3′)–CaATP or APH(3′)–**H**,**I** with kanamycin A showed that affinity of kanamycin A to both complexes was identical and was also enhanced when compared to its affinity to the apoenzyme (Table 3). These observations are consistent with the complexes of APH(3′)–**H**,**I** and APH(3′)–CaATP having similar active site organizations. When the competitive nature of compound **H**,**I** inhibition with respect to ATP is considered, it is possible that APH(3′) active site undergoes similar conformational changes in the complexes of the enzyme with **H**,**I** and CaATP.

Further evidence for similar binding patterns of a metal–ATP complex and compound **H**,**I** was obtained by NMR spectroscopy. Figures 6 and 7 show a set of ^{15}N–^{1}H HSQC spectra obtained by using a uniformly enriched ^{15}N-APH(3′). The spectra acquired with the binary complexes of enzyme–CaATP or enzyme–**H**,**I** are similar to each other but different from that acquired with the enzyme–kanamycin A complex. Figure 6 shows the full spectra obtained with apoenzyme, enzyme–CaATP, enzyme–**H**,**I**, and enzyme–kanamycin A complexes. These spectra also showed that the enzyme is quite flexible in solution, even with bound CaATP or **H**,**I**, and some of the resonances of this 31 kDa protein are broadened under these conditions. This is not surprising because this enzyme can modify many structurally different aminoglycosides; hence, it must have a flexible structure in solution to accommodate these substrates. Contrary to this, a significant increase in the dispersion of cross-peaks is visible in the spectrum of the binary enzyme–kanamycin A complex, suggesting the formation of a 'firmer' complex with this substrate. This

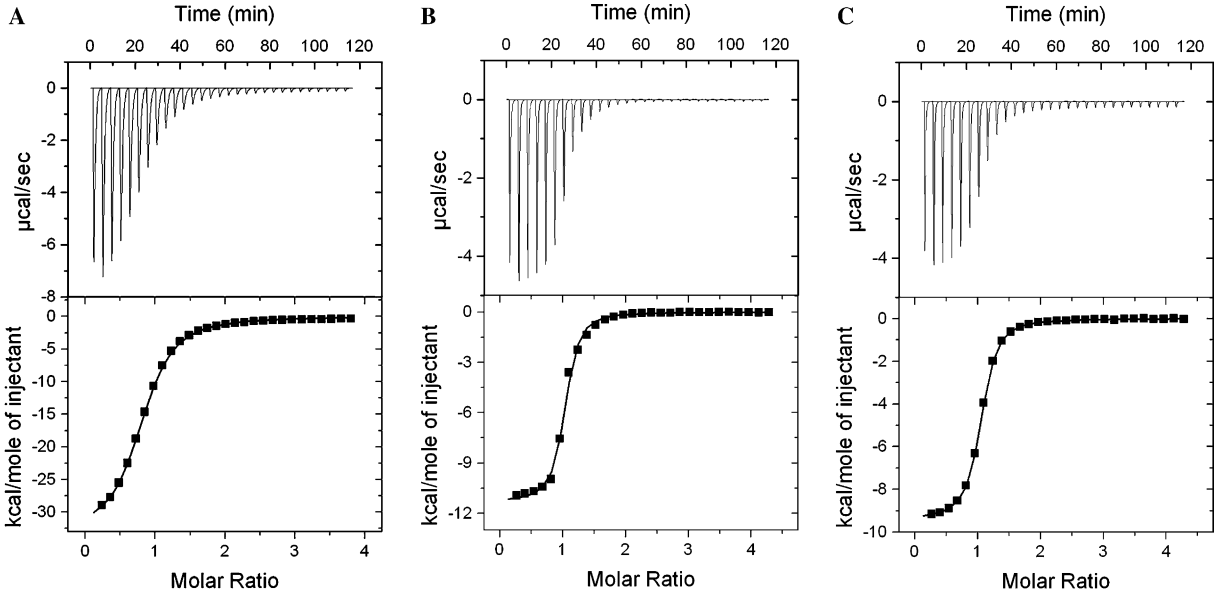


Figure 5. Titration of APH(3′) (A), APH(3′)–CaATP (B), and APH(3′)–**H**,**I** (C) with kanamycin A. Top panels show the raw ITC data, while the bottom panels show the best fits that yielded parameters shown in Table 2.

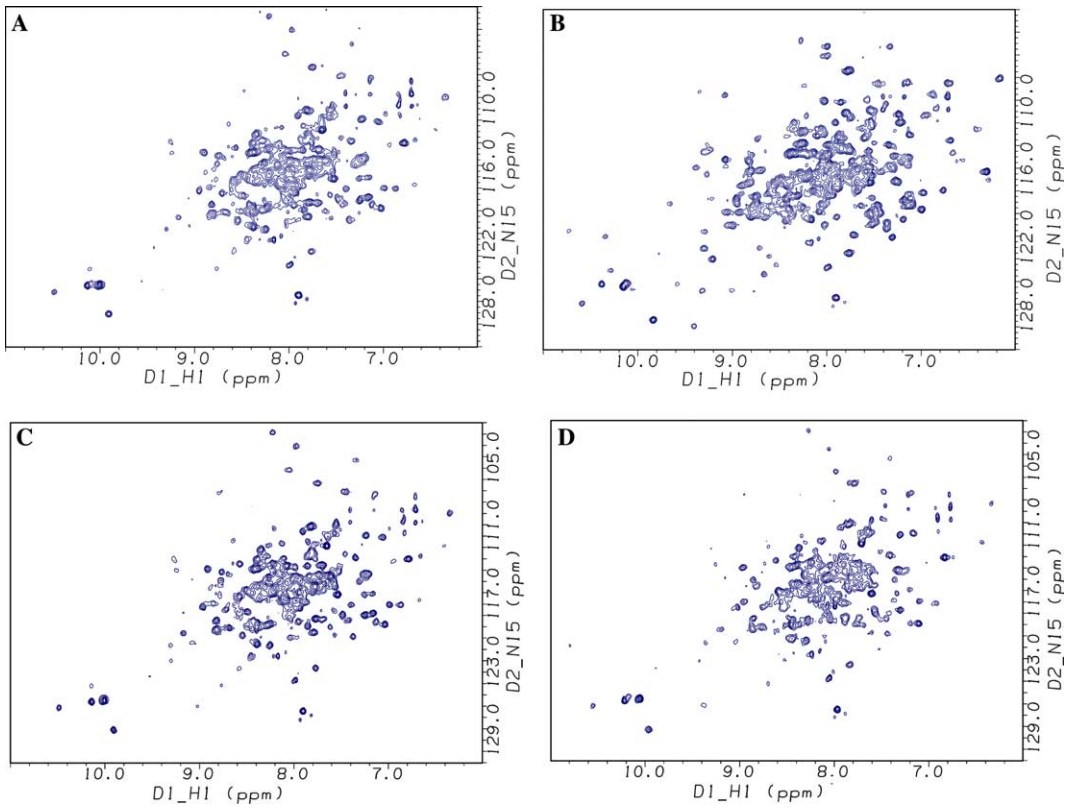


Figure 6. ^{15}N–^{1}H HSQC spectra acquired with APH(3′) alone (A), APH(3′)–kanamycin A (B), APH(3′)–CaATP (C), and APH(3′)–**H**,**I** (D).

is in concert with the observed dissociation constants of the binary enzyme–ligand complexes, which indicate that kanamycin A has the tightest binding to the enzyme. In Figure 7, the region of each spectrum showing the tryptophan side-chain nitrogen–proton correlations is displayed as an expanded plot. Of the five tryptophanes of APH(3′), only W159 is located near the aminoglycoside-binding site,[21] while the ATP-binding region of APH(3′) does not contain any tryptophan residues. Therefore, tryptophan resonances can be used as probes to confirm the presence or absence of a substrate in the aminoglycoside-binding site; the binding of ATP (or metal–ATP) is not expected to cause any changes in chemical shifts of tryptophan residues. The expanded regions clearly show that, as expected, the spectra of binary enzyme–CaATP and enzyme–**H**,**I** complexes are similar to that obtained with the apoenzyme. However, this region is significantly different in the binary enzyme–kanamycin A spectrum, indicating changes in the environment of W159 and other tryptophan residues. These observations are again consistent with the binding of compound **H**,**I** to the ATP site.

Docking calculations were used to predict the conformation of compound **H**,**I** in the active site of APH(3′), as well as to predict the contacts made by **H**,**I** in the active site. The docked conformation of compound **H**,**I** in the active site of APH(3′) is shown in Figure 8. Compound **H**,**I** contains a set of key features that are required for binding to the nucleotide-binding pocket of APH(3′). First, one of the methoxy groups on the aromatic ring forms a hydrogen bond with the backbone NH group of A93. A hydrogen bond with the backbone NH of A93 is also important for ATP binding to APH(3′); and a similar interaction has been found for isoquinoline sulfonamide kinase inhibitors bound to protein kinases[22] The hydrogen bond between **H**,**I** and A93 promotes stacking of the 3,4-dimethoxyphenyl ring in an antiparallel fashion with the side-chain of Y42. In addition, there are three potential interactions between the amino groups of compound **H**,**I** and the active site of APH(3′). These interactions take place, with D22, E24, and D190. This final contact provides an indication of the relatively larger size of **H**,**I** as compared to ATP, D190 is part of the aminoglycoside subpocket and is believed to act as the catalytic base.[23] Figure 9 shows a structural comparison of the diamines containing an aromatic ring and amine H. Thereby offering an explanation for the observed lower levels of inhibition afforded by compounds **A**,**H** (green); **C**,**H** (blue); and **F**,**H** (orange). These compounds would not be able to maintain contact with Y42 by aromatic stacking and one or more of the charge-based contacts simultaneously. Similarly, although **C**,**H** and **F**,**H** contain hydrogen bond acceptor groups, they are not situated in such a way as to form the same network of enzyme–ligand interactions available to **H**,**I** (shown in red).

Figure 10 shows the requirements for binding of diamines to three different AGMEs based on 55 compounds tested in this work. It appears that the amine **H** moiety (colored blue for all three structures) is a key feature for recognition by these enzymes, as the most active diamines against all three enzymes contain amine **H**.

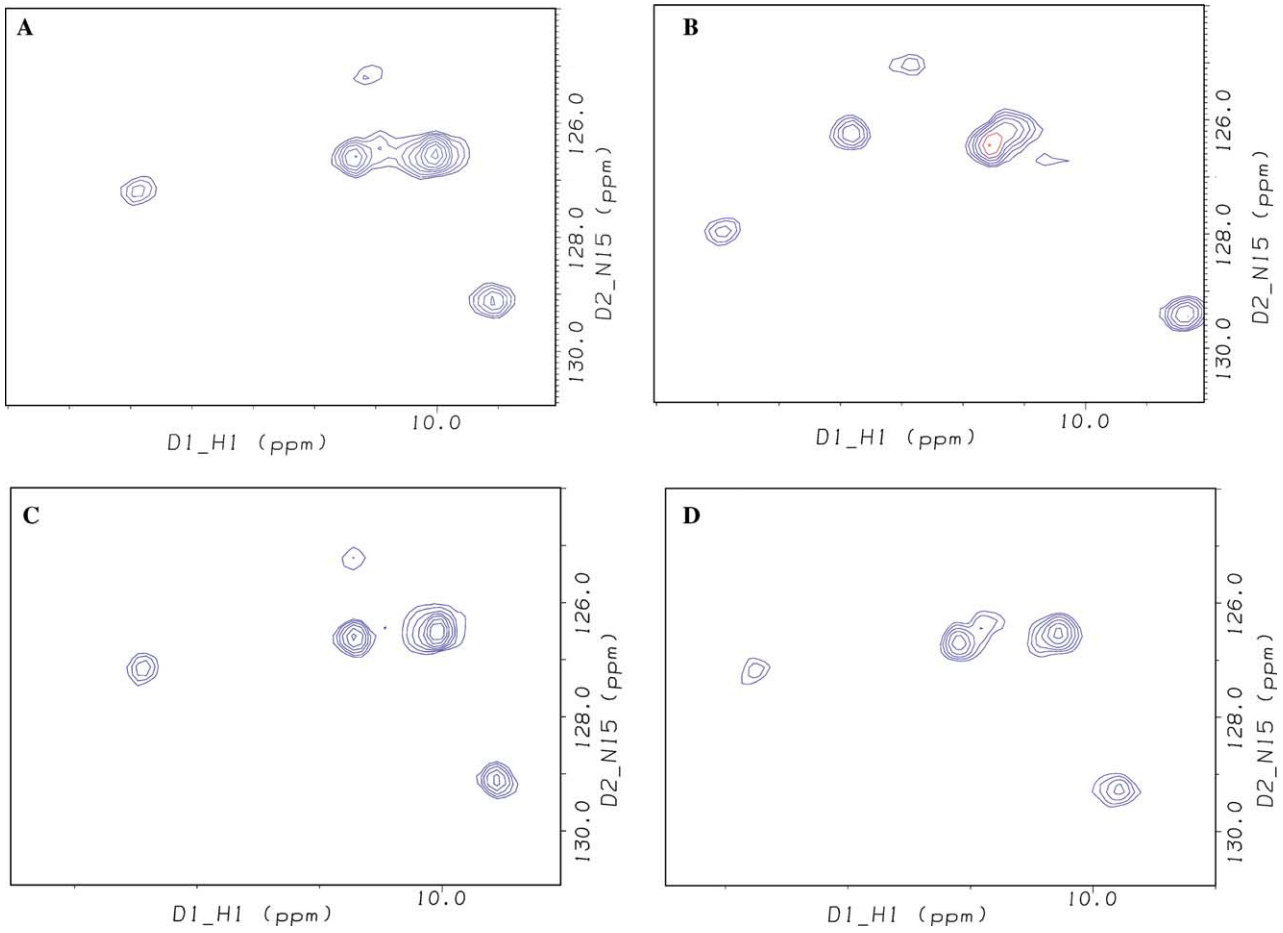


Figure 7. Expanded regions of the HSQC spectra shown in Figure 6; apoenzyme (A), APH(3′)–kanamycin A (B), APH(3′)–CaATP (C), and APH(3′)–**H**,**I** (D).

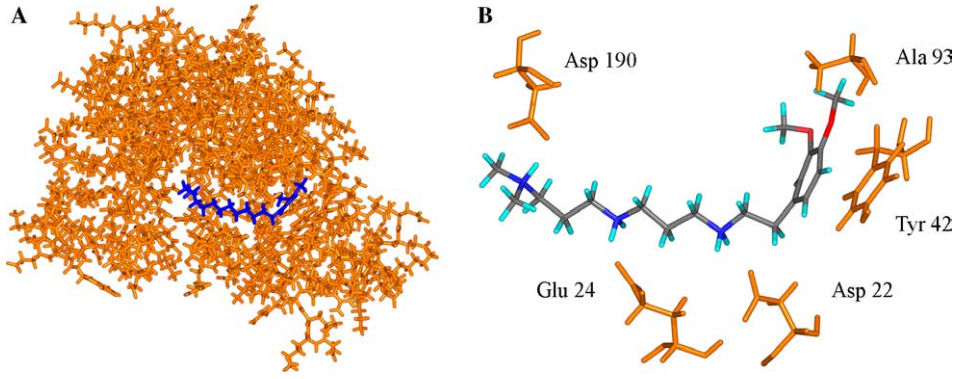


Figure 8. The compound **H**,**I** docked into the ATP-binding site of APH(3′) (A). Close-up of the ATP-binding site with docked **H**,**I** (B). This figure was created with PyMol. (DeLano, W. L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, San Carlos, CA, USA. http://www.pymol.org.)

Compound **H**,**I** (top) is competitive with ATP for APH(3′) and ANT(2″). The proposed structure of **H**,**I** in the complex with APH(3′) suggests that binding to the ATP site of APH(3′) is mediated by one or more of the amino groups along with a hydrogen bond between the phenoxy hydrogen bond donor and A93, and a possible aromatic stacking interaction. Compounds **A**,**H**, **C**,**H**, and **F**,**H**, which lack a full complement of enzyme–ligand interactions available to **H**,**I**, are not competitive with ATP when assayed with APH(3′). We note that conclusions about the structural requirements for binding of inhibitors to these three enzymes are based on a small library of compounds. Thus, they may represent only a small fraction of structural units that can be recognized by these enzymes.

The relationship between inhibitory activity of diamine compounds and multiple amino groups is not surprising,

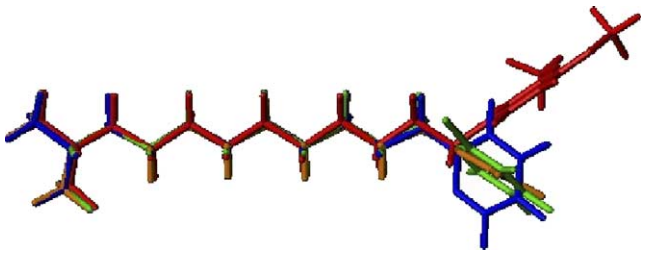


Figure 9. Superpositioning of compounds **A**,**H** (green), **C**,**H** (blue), **F**,**H** (orange), and **H**,**I** (red) docked into the active site of aminoglycoside-3′-phosphotransferase-IIIb.

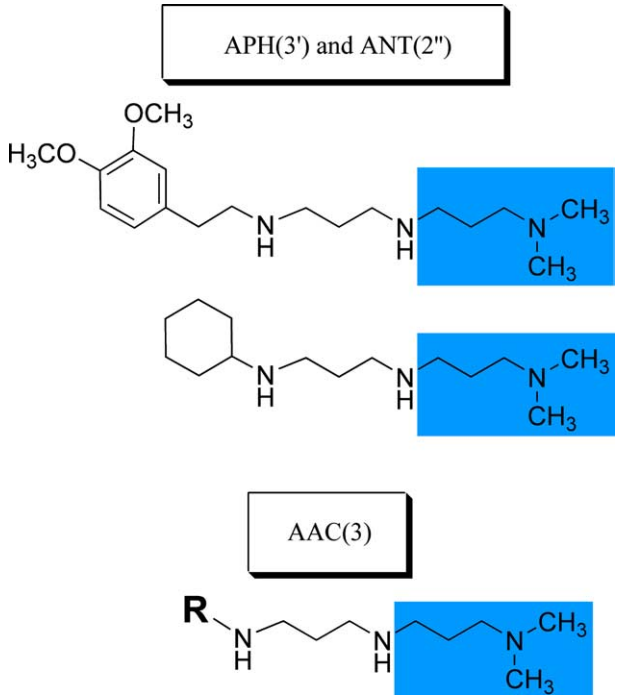


Figure 10. Requirements for binding of diamines to aminoglycoside-modifying enzymes. Shared motifs among the three different enzymes are shown in blue.

however, as the amino groups of aminoglycosides are key recognition elements for RNA, as well as AGMEs. What is somewhat surprising is the wide variety of functionality that was tolerated in the second side-chain. Specifically, compound **G**,**H**, which inhibited all three enzymes, has neither hydrogen bond donors nor polar groups of any kind on the amine **G** side-chain. Compound **G**,**H** is a competitive inhibitor of ANT(2″) with respect to the aminoglycoside substrate ($K_i = 540 \pm 32$). Thus, compound **G**,**H** highlighted the differences in the aminoglycoside-binding pockets of APH(3′) and ANT(2″). Interestingly, compound **G**,**H** was also the most potent inhibitor of in vivo growth of *Escherichia coli* AGME transformants. Compound **G**,**H** inhibited cell growth for all three transformants at both concentrations. At 10 mM, growth was 50% to 75% that of the control wells for the *E. coli* transformants producing AAC(3) and APH(3′). Cell growth was inhibited to 30% of the absorbance of the growth control for the wells containing the ANT(2″) *E. coli* transformants.

3. Conclusions

A study directed at finding novel, non-carbohydrate inhibitors of aminoglycoside-modifying enzymes yielded several molecules that inhibit multiple aminoglycoside-modifying enzymes. A stepwise synthesis was developed to prepare larger amounts of the most active compounds. Multiple experimental techniques, including ITC and NMR, were used to characterize inhibitor binding to one of the AGMEs.

Of the diamines tested, compound **H**,**I** has been identified as an inhibitor of all three AGMEs studied and is a competitive inhibitor of ATP binding for two of the enzymes (APH(3′) and ANT(2″)). It binds to APH(3′) as in a similar fashion to ATP, as evidenced by comparison of the APH(3′)–CaATP–kanamycin A and APH(3′)–**H**,**I**–kanamycin A ternary complexes using ITC and NMR. The presence of a hydrogen bond donor and positively charged nitrogen atoms are predicted to be the key factors in binding. This pattern is reminiscent of the isoquinoline sulfonamide class of protein kinase inhibitors.[22]

A common theme for the active compounds is the presence of amine **H**, which contains a propyldimethylamino side-chain. This functionality is reminiscent of aminoglycosides, which contain multiple amino groups. Despite the fact that compound **G**,**H** lacks a hydrogen bond donor on the cyclohexyl ring, it inhibited all three enzymes tested and is the first competitive inhibitor of ANT(2″) with respect to the aminoglycoside substrate. Thus, compound **G**,**H** demonstrated the potential of this conceptually simple approach to develop non-carbohydrate aminoglycoside analogs. This compound will be used as a starting point for the design of more potent, non-carbohydrate inhibitors of AGMEs.

4. Materials and methods

4.1. General

All chemicals used in enzyme preparation and assays were purchased from Sigma (St. Louis, MO) unless otherwise noted. Deuterated solvents were purchased from Cambridge Isotope Laboratories (Andover, MA).

All starting materials for synthesis of diamines were purchased from Aldrich Chemical Company (Milwaukee, WI). All reagent-grade solvents used for purification of the diamines were purchased from Fischer Scientific (Suwanee, GA). Product purification was performed by HPLC on a Gilson 215 HPLC (Middletown, WI) equipped with an Xterra® Prep MS C18 5 μm, 19 × 100 mm column, Waters (Milford, MA). Select product identifications and characterizations were performed first by ESI MS on a Bruker Esquire LCMS, Bruker Daltonics (Billerica, MA). Further

characterization of select compounds was performed by ^{1}H NMR recorded on a Varian INOVA-500 NMR spectrometer. ^{1}H–^{15}N HSQC TROSY spectra of various enzyme–ligand complexes were recorded on a Varian INOVA-600 NMR spectrometer, equipped with triple resonance probe, using uniformly enriched ^{15}N-APH(3′).

4.2. Protein preparation and purification

APH(3′) was overexpressed in *E. coli*, isolated, and purified according to the procedure described by McKay et al.,[24] with the modifications described in Özen and Serpersu.[25] Briefly, cells were grown in Luria broth (LB) media in New Brunswick (Edison, NJ) BioFlo 110 fermentor vessel at 37 °C. Cells were harvested five hours after induction with 1 mM IPTG and were then lysed by passage through French press in 50 mM Tris, 5 mM EDTA, 200 mM NaCl, 1 mM PMSF, and 0.2 mM DTT solution. Cell debris was removed by centrifugation and the supernatant was diluted with buffer A (50 mM Tris, 1 mM EDTA), pH 8.0, before loading it onto a strong anion exchanger POROS 20HQ column (250 × 4.6 mm) attached to BIOCAD 700E perfusion chromatography workstation from Applied Biosystems. A linear gradient of 0–20% with buffer B in buffer A was applied through five column volumes to elute contaminant proteins. The pure APH(3′)-IIIa was then eluted with two column volumes of 20% buffer B in buffer A. Pooled fractions were dialyzed against buffer A, freeze-dried, and stored at −80 °C. AAC(3)-IIIb and ANT(2″)-Ia were also overexpressed in *E. coli*, isolated, and purified according to the procedures described earlier,[26,27] respectively.

4.3. Preparation of diamine samples for testing

Most of the samples prepared from crude reaction mixtures were dissolved in 50% DMSO–water. All samples that were not soluble under these conditions were dissolved in 100% DMSO. In the first round enzymatic screen (reaction mixtures), the concentrations of the diamine samples ranged from 0.2 to 3 mM. In enzymatic assays of purified diamines, the concentration of diamine used in the assay was 0.625–2.5 mM depending upon the enzyme tested.

4.4. Enzyme assays

Inhibition pattern of enzymes by diamines was determined by Lineweaver–Burk plots of data (Fig. 4). The lines were fitted by the least-squares analysis. Below are given the assay conditions for each enzyme.

APH(3′)—The assay for ATP-dependent phosphorylation of kanamycin A was performed, as described in McKay et al.[24] The assays were performed at 27 °C. The assay mix was adjusted to contain 10% hexylene glycol for compounds with low water solubility. Enzymatic activity of APH(3′) was not affected in the presence of 10% hexylene glycol. For the kinetic assays, the concentrations of kanamycin A were in the range of 50–400 μM, while those of ATP in the range of 25–400 μM.

AAC(3)—The assays were performed according to the procedure used to determine the activity of other acetyltransferases[28,29] with modifications described in Owston and Serpersu.[26] The assay was based on the determination of product pyridine-4-thiolate formed as the reaction product CoA reacted with 4,4′-dithio-dipyridine, present in the assay mixture. Thus, the amount of CoA formation was continuously monitored by measuring the absorbance at 324 nm using an extinction coefficient of 19,800 M^{-1} cm^{-1} for the product pyridine-4-thiolate.

ANT(2″)—The assays were performed according to the procedure reported from this laboratory.[27,30] The assay was based on the determination of inorganic phosphate after hydrolysis of the reaction product pyrophosphate by a pyrophosphatase enzyme. For the kinetic assays, the concentrations of ATP were in the range of 250–800 μM.

4.5. General procedure for preparation of diamines A,A–I,I in parallel

Synthesis of diamine compounds **A**,**A**–**I**,**I** was carried out in a parallel array in 13 × 100 mm screw-capped tubes, whereby nine primary amines were selected and each given a letter designation **A**–**I**, respectively. This array resulted in a total of 45 individual reactions using a 1:1:1 ratio of reactants, where a 1 mmol of each of two primary amines was reacted with 1 mmol of 1,3-dichloropropane (95 μL). The labeling, name, and quantity of each amine used were as follows: (**A**) 2-Chlorobenzylamine (121 μL); (**B**) 3-amino-1,2-propanediol (78 μL); (**C**) 2-(aminomethyl)pyridine (103 μL); (**D**) 2-amino-1-butanol (94.5 μL); (**E**) furfurylamine (88.4 μL); (**F**) 2-(2-aminoethoxy)ethanol (100 μL); (**G**) cyclohexylamine (114.4 μL); (**H**) 3-(dimethylamino)propylamine (126 μL); and (**I**) 3,4-dimethoxyphenethylamine (169 μL). The disubstitution reactions were carried out under neat conditions at 100 °C for 48 h. The reaction mixtures were gently agitated by hand, intermittently to ensure complete mixing of all reactants. Included as part of the 45 reactions, compounds **A**,**A**; **B**,**B**; **C**,**C**; **D**,**D**; **E**,**E**; **F**,**F**; **G**,**G**; **H**,**H**; and **I**,**I** were all synthesized. This was done to provide a control for those compounds, as it was realized that each of those compounds would be formed as a product homologous addition in each of the reactions for which they were involved. Initially, these reaction mixtures were tested in crude form. The most active compounds from the initial screen (**B**,**I**; **C**,**H**; **C**,**I**; **D**,**H**; **F**,**H**; **G**,**H**; and **H**,**I**) were then partially purified by HPLC and again screened for enzyme inhibition activity (Table 1).

4.6. General procedure for discrete preparation of diamines A,H; G,H; and H,I

A discrete synthesis for compounds ***A***,***H***; ***G***,***H***; *and* ***H***,***I*** was carried out to provide compounds as trihydrochloride salts.

Step 1 (formation of acrylamides). The reactions were carried out in three discrete, previously dried 100 mL reaction vessels. To a stirred solution of acryloyl chloride (4.9 mmol, 400 μL for compound **A**,**H** and 11 mmol, 894 μl for compounds **G**,**H** and **H**,**I**) in dichloromethane (50 mL) at −70 °C was added dropwise each of the three amines (**A** 4 mmol, 500 μl; **G** 10 mmol, 1.66 mL; or **I** 10 mmol, 1.14 mL) to one of each of the reaction vessels, followed by the addition of pyridine (12 mmol, 970 μL for compound **A**,**H** and 30 mmol, 2.43 mL for **G**,**H** and **H**,**I**) to each of the reaction vessels. The reaction mixtures were allowed to slowly warm up to ambient temperature and stirred for 12 h. Each reaction mixture was then washed with aqueous $NaHCO_3$ three times, followed by washing once with saturated brine. The organic layers from each reaction mixture were then dried over anhydrous Na_2SO_4 and evaporated in vacuo to complete dryness. Each of the acrylamide intermediates was obtained in approximately 80–90% yield based on crude weight. The compounds produced were sufficiently purified by TLC and NMR analysis to be used directly for the next reaction with no further purification.

*Step 2 (Michael addition of amine **H**)*. Each of the acrylamide intermediates was dissolved in ethanol (5 mL for compound **A**,**H** and 10 mL for compounds **G**,**H** and **H**,**I**) and transferred to three discrete Radley's carousel reaction vessels. Following it, three equivalents of amine **H** were added to each of the reaction vessels (9.44 mmol, 1.2 mL for compound **A**,**H** and 27 mmol, 3.4 mL for compounds **G**,**H** and **H**,**I**) and the reaction mixtures were stirred at 80 °C for 48 h. They were then evaporated in vacuo under high heat and pressure to remove ethanol and excess amine **H**. The oily residues were purified by flash chromatography using a linear gradient from 10% to 50% methanol in chloroform.

4.6.1. *N*-(2-Chlorobenzyl)-3-(3-dimethylamino-propylamino)-propionamide (A,H amide intermediate). (840 mg, 78%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.12 (br s, 1H), 7.31–7.26 (m, 2H), 7.18–7.12 (m, 2H), 4.42 (d, J = 5.9 Hz, 2H), 3.01(t, J = 6.1 Hz, 2H), 2.90 (t, J = 6.1 Hz, 2H), 2.61 (t, J = 5.9 Hz, 2H), 2.44 (t, J = 6.4 Hz, 2H), 2.17 (s, 6H), 1.72 (qin, J = 6.1 Hz, 2H); (ESI, Pos.) m/z 298.3 $(M+H)^+$.

4.6.2. *N*-Cyclohexyl-3-(3-dimethylamino-propylamino)-propionamide (G,H amide intermediate). (970 mg, 38%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.66 (br s, 1H), 3.73–3.64 (m, 1H), 2.80 (t, J = 6.1 Hz, 2H), 2.60 (t, J = 6.8 Hz, 3H), 2.27 (t, J = 7.1 Hz, 4H), 2.15 (s, 6H), 1.83–1.77 (m, 2H), 1.65–1.56 (m, 4H), 1.55–1.48 (m, 1H), 1.35–1.24 (m, 2H), 1.17–1.04 (m, 3H); (ESI, Pos.) m/z 256.2 $(M+H)^+$.

4.6.3. *N*-[2-(3,4-Dimethoxyphenyl)-ethyl]-3-(3-dimethylamino-propylamino)-propionamide (H,I amide intermediate). (1.89, 56%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (t, J = 4.9 Hz, 1H), 6.74–6.69 (m, 1H), 6.68–6.62 (m, 2H), 3.77 (d, J = 5.9 Hz, 6H), 3.40 (q, J = 7.1 Hz, 2H), 2.73 (t, J = 6.1 Hz, 2H), 2.67 (t, J = 7.3 Hz, 2H), 2.49 (t, J = 7.1 Hz, 2H), 2.23 (t, J = 5.6 Hz, 2H), 2.18 (t, J = 7.1 Hz, 2H), 2.11 (s, 6H), 2.11–2.10 (br s, 1H), 1.49 (qin, J = 7.1 Hz, 2H); (ESI, Pos.) m/z 338.6 $(M+H)^+$.

Step 3 (reduction of the amides). Each of the corresponding amides (840 mg, 2.8 mmol **A**,**H**; 970 mg, 3.8 mmol **G**,**H**; and 1.89 g, 5.6 mmol **H**,**I**) was dissolved in THF (15 mL for compound **A**,**H** and 40 mL for compounds **G**,**H** and **H**,**I**). To this, a solution of THF/BH_3 complex was added (6 mmol for compound **A**,**H** and 18 mmol for compounds **G**,**H** and **H**,**I**) and the reactions were refluxed for 48 h. *Note.* The reduction of the amide intermediate for compound **A**,**H** was attempted first. The reduction procedure for **A**,**H** was repeated a second time, as it was realized that the reduction was incomplete. For this reason, a large excess of THF/BH_3 complex was used (3–5 equiv) in the subsequent two amide reductions for compounds **G**,**H** and **H**,**I**. The reaction mixtures were cooled to ambient temperature and then quenched with the drop wise addition of 6 N HCl in methanol. The reaction mixtures were allowed to stir for an additional 3 h, and then evaporated to dryness in vacuo. Each of the dried reaction mixtures was redissolved in acidic methanol (50 mL) and again evaporated to dryness. The products were obtained as white crystalline solids by recrystallization from ethanol/chloroform (1:3).

4.6.4. *N*-[3-(2-Chlorobenzylamino)-propyl]-*N*′,*N*′-dimethyl-propane-1,3-diamine trihydrochloride (A,H). The product **A**,**H** (323 mg, 21%) was obtained as a fine, white crystalline powder, mp 210–213 °C. ^{1}H NMR (500 MHz, D_2O) δ 7.47 (t, J = 7.6 Hz, 2H), 7.39 (t, J = 7.8 Hz, 1H), 7.34 (t, J = 7.6 Hz, 1H), 4.35 (s, 2H), 3.21–3.13 (m, 4H), 3.09 (q, J = 8.1 Hz, 4H), 2.83 (s, 6H), 2.13–2.03 (m, 4H); ^{13}C NMR (75 MHz, D_2O) δ 133.8, 131.7, 131.2, 129.6, 127.7, 127.4, 53.8, 48.1, 44.2, 44.0, 43.8, 42.4 (2C), 22.1, 20.7; (ESI, Pos.) m/z 284.2 $(M+H)^+$.

4.6.5. *N*-Cyclohexyl-*N*′-(3-dimethylamino-propyl)-propane-1,3-diamine trihydrochloride (G,H). The product **G**,**H** (334 mg,10%) was obtained as a fine, white crystalline powder, mp 239–242 °C. ^{1}H NMR (500 MHz, D_2O) δ 3.16 (t, J = 8.1 Hz, 2H), 3.12–2.98 (m, 7H), 2.82 (s, 6H), 2.14–1.90 (m, 6H), 1.80–1.66 (br s, 2H), 1.56 (d, J = 12.9 Hz, 1H), 1.30–1.15 (m, 4H), 1.14–1.01 (m, 1H); ^{13}C NMR (75 MHz, D_2O) δ 57.0, 53.8, 44.3, 44.0, 42.4 (2C), 40.7, 28.3 (2C), 24.0, 23.4(2C), 22.4, 20.7; (ESI, Pos.) m/z 242.4 $(M+H)^+$; CHN Anal. Calcd for $C_{14}H_{34}Cl_3N_3{\cdot}2H_2O$: C, 43.47; H, 9.90; N, 10.86. Found: C, 43.55; H, 9.92; N, 10.80.

4.6.6. *N*-[2-(3,4-Dimethoxyphenyl)-ethyl]-*N*′-(3-dimethylamino-propyl)-propane-1,3-diamine trihydrochloride (H,I). The product **H**,**I** (720 mg, 17%) was obtained as a fine, white crystalline powder, mp 254–256 °C. ^{1}H NMR (500 MHz, D_2O) δ 6.92 (d, J = 7.8 Hz, 1H), 6.89 (s, 1H), 6.82 (d, J = 8.3 Hz, 1H), 3.8 (d, J = 7.8 Hz, 6H), 3.24 (t, J = 7.6 Hz, 2H), 3.20–3.14 (m, 2H), 3.12–3.04 (m, 6H), 2.89 (t, J = 7.6 Hz, 2H), 2.83 (s, 6H), 2.12–1.98 (m, 4H); ^{13}C NMR (75 MHz, D_2O) δ 147.9, 146.9, 128.9, 121.0, 112.0, 111.9, 55.3 (2C),

53.8, 48.3, 44.2, 44.0, 43.9, 42.7 (2C), 30.7, 22.0, 20.7; (ESI, Pos.) *m/z* 324.5 $(M+H)^+$; CHN Anal. Calcd for $C_{18}H_{36}Cl_3N_3O_2$: C, 49.95; H, 8.38; N, 9.71. Found: C, 49.82; H, 8.54; N, 9.70.

4.7. Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) experiments were performed at 37 °C using a VP-ITC calorimeter from Microcal, Inc. (Northampton, MA). The enzyme and ligand solutions were prepared in 50 mM Tris buffer, pH 7.5, containing 50 mM KCl. The enzyme solution was extensively dialyzed against this buffer and ligand solutions were prepared in the final dialysate. Enzyme and ligand solutions were degassed before titrations. Sample cell and syringe contained 90 μM APH(3′) and 1.5 mM kanamycin A, respectively, in all titrations. In ternary complex experiments, kanamycin A solution containing 1.5 mM Ca^{2+} and 1.0 mM ATP or 2.0 mM diamine **H**,**I** was titrated into an enzyme solution containing matched concentrations of Ca^{2+} and ATP or diamine **H**,**I**. Under these conditions, more than 95% of APH(3′) was present either in enzyme–CaATP or enzyme–**H**,**I** complex during the onset of titrations. Binding data were corrected by performing appropriate ligand- to buffer-dilution titrations. For all experiments, injection volume was set to 10 μL and injections were separated by 240 s. Cell stirring speed was 300 rpm. One set of binding site(s) model of Origin software package was used for data fitting to obtain binding parameters N (stoichiometry), K_A (association constant), and ΔH (enthalpy change). The dissociation constant, free energy of binding (ΔG) and entropy change (ΔS) were obtained using Eqs. 1–3.

$$K_D = 1/K_A, \tag{1}$$

$$\Delta G = -RT \ln K_A, \tag{2}$$

$$\Delta G = \Delta H - T\Delta S, \tag{3}$$

CaATP, a competitive inhibitor of the enzyme,[31] was used, instead of the catalytically required cofactor MgATP, to prevent product formation in ternary complex titrations.

4.8. NMR spectroscopy

All proton spectra for the characterization of compounds **A**,**A** to **I**,**I** were acquired on a 500 MHz Varian INOVA spectrometer. All the spectra with enzyme–substrate and enzyme–inhibitor complexes were acquired on a 600 MHz Varian INOVA spectrometer using uniformly ^{15}N-enriched APH(3′). NMR samples contained 219 μM APH(3′) in 25 mM Tris–HCl, pH 7.5, containing 25 mM KCl. When present, kanamycin A was 450 μM, Ca^{2+} and ATP were each 0.5 mM, and **H**,**I** was 1.0 mM. A sensitivity-enhanced ^1H–^{15}N HSQC spectrum,[32] with the TROSY option,[33] from the BioPack set of pulse sequences for the Varian spectrometer, was acquired in the phase-sensitive mode using the States-Haberkorn method for quadrature detection in the indirect dimension.[34] Datasets were obtained with a spectral width of 8000 Hz in the ^1H dimension and 2127.55 Hz in the ^{15}N dimension and 128 scans of 512 real time points for each of 80 t_1 increments were recorded. The data were processed using the Felix processing software package (Accelerys, San Diego, CA). The ^{15}N dimension was zero-filled to 256 pts, with the sensitivity enhancement option selected on the left half of the spectrum.

4.9. Docking calculations

The docking calculations were performed using the Flexidock program within the SYBYL® 6.9 software package (Tripos, Inc., St. Louis, MO). The crystal structure of aminoglycoside phosphotransferase 3′-IIIa cocrystallized with AMPPNP (PDB #1J7U) was used for all docking calculations.[35] The enzyme-binding site was based upon the position of the bound AMPPNP; and the binding site was prepared for calculations according to the Flexidock setup dialog. No water molecules or metal ions were included in the calculation. Residues within three angstroms of the ANPPNP that contained a carboxylate group were selected as containing hydrogen bond acceptors. Alanine 93 was selected as containing a hydrogen bond donor. The diamines were built using the Sketch module of SYBYL 6.9. All the torsion angles in the chain were set to 180° and then the energy was minimized to give the starting structure for the docking calculations. All nitrogen atoms in each diamine molecule were modeled as positively charged, and Gasteiger–Huckel charges were calculated for each of the diamines. Two docking calculations were performed on each diamine, representing two different alignments in the binding pocket that were 180° apart. The first orientation aligned the aromatic ring of the diamine with the adenine ring system of ANPPNP, with the rest of the diamine oriented to loosely match the positioning of the sugar phosphate backbone. The second orientation aligned the amino terminal group of the diamine (the tertiary amino group of amine **H**) with the NH in the adenine ring of AMPPNP that forms a hydrogen bond with the backbone carbonyl of alanine 93. The rest of the molecule was oriented to loosely match the positioning of the sugar phosphate moiety. The charged amino groups were selected as hydrogen bond donors and oxygen atoms with available lone pairs were identified as hydrogen bond acceptors. The maximum number of generations was raised to 30,000 and each calculation produced 20 docked structures. Diamine **H**,**I**, the only compound proven to be a competitive inhibitor of APH(3′) with respect to MgATP, was the only one to yield an energy minimum inside the ATP-binding site.

4.10. Whole-cell assays

Whole-cell assays were performed with the purified diamines and the *E. coli* transformants used to produce the AGMEs. The assays were performed in 96-well plates with selected diamine compounds. Diamines **B**,**I**; **C**,**H**; **C**,**I**; **D**,**H**; **E**,**H**; **G**,**H**; and **H**,**I** were assayed at concentrations of 10 and 50 mM. The 96-well plates were inoculated with the transformants to yield an initial absorbance of

0.03 U/well. Cell growth was monitored using a Labsystems Multiskan MCC/340 Spectrophotometer manufactured by Fisher Scientific. Absorbance was compared with those of control cultures on the same plate every 8 h for 36 h by reading the absorbance at 600 nM.

Acknowledgments

This research was supported by Grants MCB 01110741 from the National Science Foundation (E.H.S.) and from the National Institutes of Health AI057836 (R.E.L.) and AI053796 (R.E.L.).

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