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Synthesis and Biological Activity of Mono- and Di-*N*-acylated Aminoglycosides

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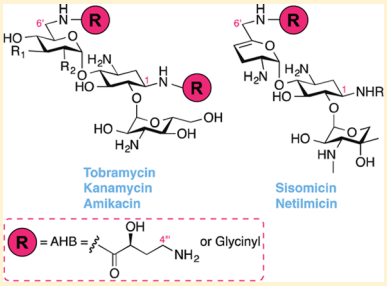
Supporting Information

ABSTRACT: Despite issues with oto/nephrotoxicity and bacterial resistance, aminoglycosides (AGs) remain an effective and widely used class of antibacterial agents. For decades now, efforts toward the development of novel AGs with potential to overcome some of these problems have been major research focuses. 1-*N*-Acylation, especially γ-amino-β-hydroxybutyrate (AHB) derivatization, has proven to be one of the most successful strategies for improving the overall properties of AGs, including their ability to avoid certain resistance mechanisms. More recently, 6′-*N*-acylation arose as another possible strategy to improve the properties of these drugs. In this study, we report on the glycinyl, carboxybenzyl, and AHB mono- and diderivatization at the 1-, 6′-, and/or 4‴-amines of the AGs amikacin, kanamycin A, netilmicin, sisomicin, and tobramycin. We also present the antibacterial activities and the reduced reactivity of AG-modifying enzymes (AMEs) toward these new AG derivatives, and identify the AMEs present in the bacterial strains tested.



KEYWORDS: *Acylation, aminoglycoside antibiotics, bacterial resistance, drug development, drug-modifying enzymes*

Aminoglycosides (AGs) are a potent class of natural product antibiotics, targeting the bacterial ribosome, that are effective in the treatment of Gram-positive and Gram-negative bacterial infections, including those that accompany other diseases such as cancer and HIV.[1] More recently, AGs have also been investigated as potential antifungal agents and as a treatment option for genetic disorders associated with premature termination codons.[2,3]

The manifestation of a number of resistance mechanisms that reduce the efficacy of AGs is a major obstacle in the development of novel members of this family of antibiotics. Examples of resistance mechanisms include increased efflux or decreased uptake of AGs by bacterial cells,[4] modifications of the 16S rRNA,[5] and acquisition of AG-modifying enzymes (AMEs) by the bacteria.[6] AMEs, the most common of these mechanisms of resistance, tend to be specific toward particular AGs. The chemical modifications of AGs by AMEs, including AG *N*-acetyltransferase (AACs), AG *O*-phosphotransferases (APHs), or AG *O*-nucleotidyltransferase (ANTs), lead to a decreased affinity of the modified AGs for the ribosome. While we have shown that a single chemical modification does not necessarily remove all antibacterial activity,[7,8] the existence of bifunctional AMEs, such as AAC(3)-Ib/AAC(6′)-Ib′[9] and AAC(6′)-30/AAC(6′)-Ib′[10] from *Pseudomonas aeruginosa*, AAC(6′)-Ie/APH(2″)-Ia from *Staphylococcus aureus*,[11] and ANT(3″)-Ii/AAC(6′)-IId from *Serratia marcescens*,[12] capable of performing two chemical modifications on AG scaffolds, and that of the enhanced intracellular survival (Eis)[13,14] enzyme capable of multiple acetylations increase the need for new AGs capable of evading the action of AMEs.

Two main strategies for circumventing the activity of AMEs on AGs consist of (1) developing inhibitors of AMEs to be used in conjunction with currently approved AGs, and (2) making small modifications on the scaffolds of known AGs to yield products that remain active against bacteria, but are no longer modified by AMEs. Inhibitors of AMEs have been identified,[15,16] and some of them were found capable of preventing deactivation of AGs,[17−19] but biological testing and methods of coadministration are still under investigation. The development of semisynthetic AGs has proven a successful strategy for overcoming, to some extent, the AG resistance problem. In particular, the incorporation of a γ-amino-β-hydroxybutyric acid (AHB), exemplified by plazomicin[20] and amikacin (AMK), or a glycinyl group to the scaffold of known AGs has been one successful strategy used to circumvent AMEs while retaining or improving upon their antibacterial properties.[21]

The synthesis of complex carbohydrates like AGs, which comprise two to five individual sugars, is often a daunting task, and their total synthesis from basic chemical building blocks is rarely an efficient means of developing novel, effective compounds. Additionally, the numerous protecting group

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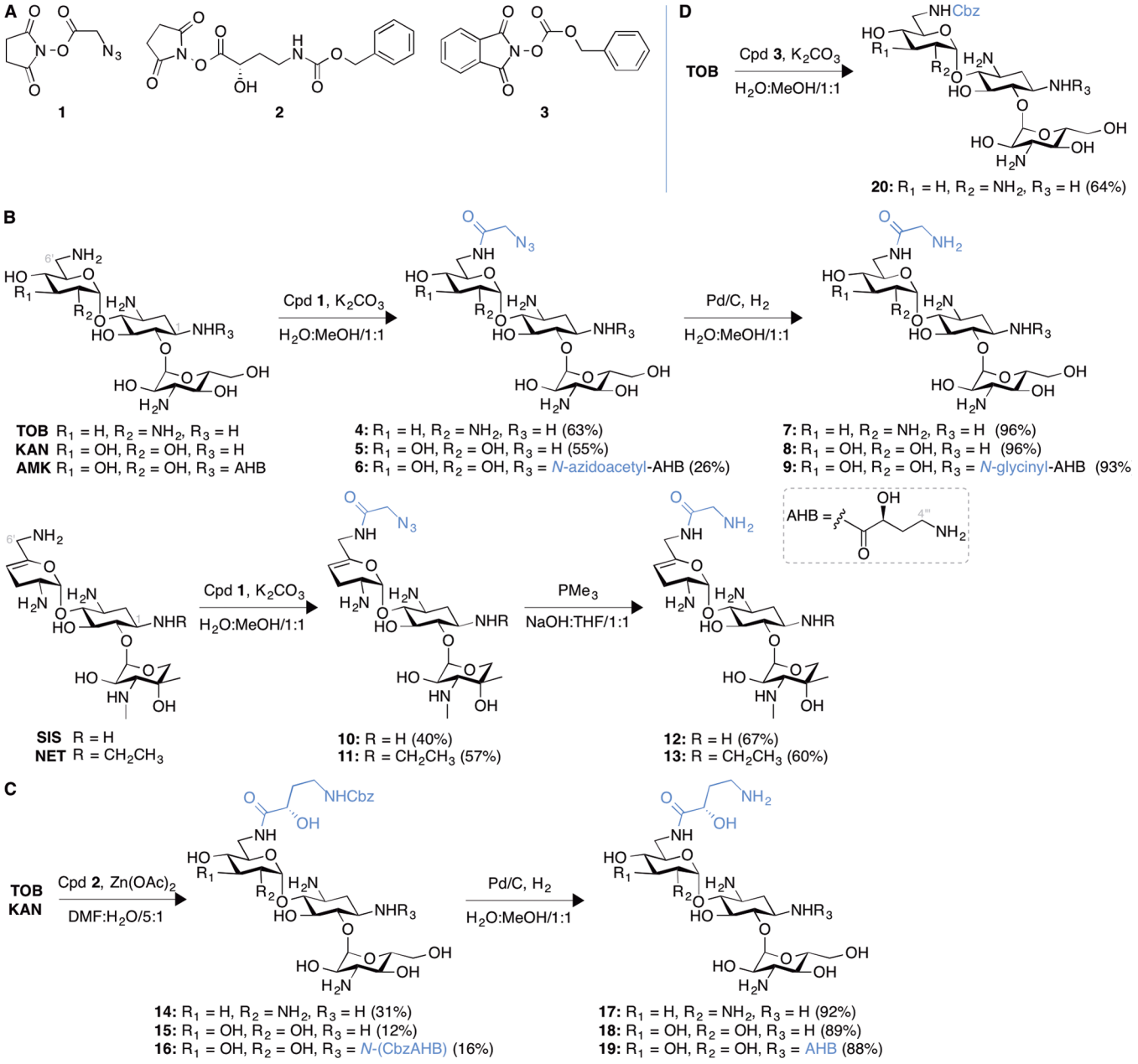
Scheme 1[a]



[a]A. Structures of coupling reagents (**1**–**3**) used in this study. B. Synthetic scheme illustrating the preparation of the 6′-*N*-azidoacetyl compounds (**4**–**6** and **10**–**11**), the conversion of 6′-*N*-azidoacetyl compounds **4**–**6** to the respective 6′-*N*-glycinyl compounds **7**–**9** by catalytic hydrogenation, and the conversion of 6′-*N*-azidoacetyl compounds **10** and **11** to the respective 6′-*N*-glycinyl compounds **12** and **13** by Staudinger reaction. C. Synthetic scheme illustrating the preparation of the 6′- and di-*N*-(CbzAHB) compounds (**14**–**16**) from TOB or KAN followed by the conversion of 6′- and di-*N*-(CbzAHB) compounds **14**–**16** to the respective 6′- and di-*N*-(AHB) compounds **17**–**19** by catalytic hydrogenation. D. Synthetic scheme illustrating the preparation of the 6′-*N*-Cbz TOB (**20**).

manipulations required to selectively modify known AGs in a predictable manner is also quite difficult owing to the large number of amine and hydroxyl groups with similar reactivity. In most cases, the overall yields of syntheses are rather low, requiring large quantities of starting materials and many steps to achieve a single product. With the challenges in synthesizing AGs from scratch, several approaches have targeted modifying currently approved AGs including modifications at various positions such as the 1-, 6′-,[21] 2″-,[22] 5″-,[23] and 6″-positions,[3,24,25] dimerization,[26,27] and covalent attachment to other antibiotics,[23,28] which have all had varying degree of success. Herein, we report on the facile syntheses of AMK, kanamycin A (KAN), netilmicin (NET), sisomicin (SIS), and tobramycin (TOB) mono- and dimodified at the 1-, 6′-, and/or 4‴-amines by glycinyl, carboxybenzyl, and AHB moieties (Scheme 1). We establish the antibacterial activities and the reduced reactivity of AMEs toward these new AG derivatives. We also identify the AMEs comprised in the bacterial strains tested.

We have previously shown that it is possible to acylate, for example, the 3-*N* position of some AGs and retain activity against some bacterial strains while reducing their susceptibility

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Table 1. Antibacterial Activity of AGs against Various Bacterial Strains[a]

	strain									
	Gram-positive							Gram-negative		*Mycobacteria*
compd	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
AMK	16	9.4	37.5	9.4	1	0.5	32	37.5	2.3	≤0.25
9	>128	>150	>150	>150	>128	>128	>128	>150	>150	64−128
19	>128	150	>150	150	128	>128	>128	>150	75	8−16
KAN	>128	9.4	>150	4.7	0.5	>128	>128	9.4	37.5	>128
8	2	150	>150	150	16	>128	>128	150	>150	>128
18	4	>150	>150	>150	32	32−64	>128	>150	150	2
NET	≤0.25	4.7	4.7	2.3	≤0.25	≤0.25	≤0.25	2.3	2.3	≤0.25
13	0.5	75	37.5	18.8	0.5	32	1−2	37.5	37.5	1
SIS	≤0.25	9.4	4.7	2.3	≤0.25	≤0.25	≤0.25	4.7	1.2	≤0.25
12	≤0.25	75	37.5	18.8	1	8	2	37.5	18.8	0.5
TOB	>128	9.4	9.4	2.3	0.5	≤0.25	>128	4.7	0.6	≥128
7	0.5−1	18.8	75	18.8	8	4−8	128	75	18.8	4
17	2−4	>150	>150	>150	8	16	>128	>150	>150	8−16
20	64−128	>150	>150	>150	128	>128	>128	75	>150	16

[a]Minimum inhibitory concentration (MIC) values (μg/mL) determined by the double dilution method. **A** = *B. anthracis* str. Sterne; **B** = *B. cereus* ATCC 17788; **C** = *B. subtilis* 168; **D** = *L. monocytogenes* ATCC 19115; **E** = *S. aureus* ATCC 29213; **F** = MRSA1; **G** = MRSA2; **H** = *H. influenzae* ATCC 51907; **I** = *P. aeruginosa* PA01; **J** = *M. smegmatis* MC2-155.

to modification by AMEs,[7] while in other cases acylation at this position is deleterious to antibacterial activity. Those results indicated that acylation can be a good methodology to generate novel active AG variants.[8] We also previously reported that adding a glycinyl group at the 6′-position of TOB results in a derivative with good antibacterial activity[21] and decided, in the current study, to expand this approach to other functional groups and AGs. We modified the reported methodology by slowing down the rate of addition of the acylating reagent and by adjusting the solvent gradient during the purification process, which resulted in improved overall yields.

We first prepared a series of 6′-*N*-glycinylated AG derivatives (**7**−**8** and **12**−**13**) and 6′,4‴-di-*N*-glycinyl-AMK (**9**) by using an activated ester in the form of *O*-azidoacetyl-*N*-hydroxysuccinimide (**1**) to selectively append an α-azidoacetyl group, which was followed by reduction of the azide into the corresponding amine (Scheme 1B). Compound **1** was prepared via the *N*,*N*′-dicyclohexylcarbodiimide (DCC) mediated coupling of α-azidoacetic acid and *N*-hydroxysuccinimide in THF (Scheme 1A). The reaction of TOB, KAN, AMK, SIS, or NET with **1** using potassium carbonate as a base in a 1:1 mixture of H_2O and MeOH afforded the 6′-*N*-azidoacetylated compounds **4**−**5** and **10**−**11** as well as 6′,4‴-di-*N*-azidoacetyl-AMK (**6**) in yields ranging from 26 to 63% after purification (Scheme 1B). The 6′-*N*-azidoacetylated derivative was the major product in each of the reactions and was easily isolated in pure form for use in the following synthetic step. In the case of AMK, the acylation reaction occurred at a relatively equal rate at the 6′- and 4‴-amine. For this reason, compound **6** was prepared in the same fashion as all other 6′-*N*-azidoacetylated compounds, but with an additional molar equivalent of the acylating reagent **1** to generate 6′,4‴-di-*N*-azidoacetyl-AMK (**6**). In all reactions, some side-products were also generated, including di-*N*-azidoacetylated AGs as well as a mixture of other mono-*N*-azidoacetylated compounds (*i.e.*, 6′-, 3-, or 1-*N*-azidoacetyl), but isolation of these other minor compounds in pure form was not achievable. 6′-*N*-azidoacetylated compounds **4**−**5** and **10**−**11** as well as the 6′,4‴-di-*N*-azidoacetyl-AMK (**6**) were all isolated as single spots on TLC and used directly in the following chemical reaction. For compounds **4**−**6**, the azide functionalities were then converted to the free amines to afford compounds **7**−**9** via catalytic hydrogenation (Scheme 1B), and after removal of the catalyst by filtration through Celite, no further purification was necessary. The 6′-*N*-azidoacetylated compounds **10** and **11** both contain an alkene that is not compatible with catalytic hydrogenation, and for that reason they were converted to their respective 6′-*N*-glycinylated counterparts using a Staudinger reaction. Treatment of compounds **10** and **11** with trimethylphosphine in a 1:1 mixture of aqueous NaOH (1 mM) and THF yielded compounds **12** and **13**, in 67% and 60% yield, respectively (Scheme 1B).

Since the incorporation of an AHB group has proven to be a successful strategy in the development of novel AGs, we next attempted to expand the current methodology to selectively incorporate the AHB group at the 1- and 6′-positions of TOB and KAN. Reaction conditions similar to that for the preparation of the glycinylated AG derivatives, in which **1** was replaced by *O*-γ-benzyloxycarbonylamino-α-hydroxybutyrate-*N*-hydroxysuccinimide (**2**, Cbz-AHB), were used in our attempts to prepare compounds with AHB groups. The use of chelation with zinc diacetate has been shown to work well to incorporate protecting groups at the 1-position of KAN.[29] However, when zinc diacetate was used in combination with Cbz-AHB (**2**) in a 5:1 mixture of DMF and H_2O, only the 6′-*N*-modified TOB (**14**) or KAN (**15**) were generated. Adding additional equivalents of **2** led to the synthesis of the 1,6′-di-*N*-modified KAN (**16**). In the case of TOB, even in the presence of multiple equivalents of **2**, only the 6′-*N*-modified TOB (**14**) was observed. It is important to note that the reaction of TOB with **2**, using potassium carbonate as a base in a 1:1 mixture of H_2O and MeOH, as for the preparation of compounds **4**−**6**, **10**, and **11** also efficiently afforded compound **14** (Scheme 1C). Catalytic hydrogenation of compounds **14**−**16** (Scheme 1C) provided the respective *N*-AHB products, **17**−**19**.

Finally, to investigate the importance of the presence of an amine functionality in the group added to AG scaffolds, we protected the 6′-*N* of TOB with a Cbz group (Scheme 1D). *O*-Cbz-*N*-hydroxyphthalimide (**3**) was synthesized from *N*-hydroxyphthalimide and benzylchloroformate using Et_3N as a

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base in dichloromethane for use as the acylating reagent (Scheme 1A). 6′-*N*-Cbz-TOB (**20**) was prepared from the reaction of TOB and compound **3**, using potassium carbonate as a base in a 1:1 mixture of H_2O and MeOH. It is important to note that an attempt at using *O*-Cbz-*N*-hydroxysuccinimide instead of compound **3**, did not yield any of the desired compound **20**.

With our AG derivatives generated, we next examined their antibacterial properties against ten bacterial strains: seven Gram-positive, two Gram-negative, and one *mycobacteria* (Table 1). In general, with the exception of compound **19** displaying moderate activity (MIC = 8−16 μg/mL) against *Mycobacterium smegmatis* MC2−155 (**J**), both AMK derivatives lost antibacterial activity. However, when exploring KAN derivatives, we observed that 6′-*N*-glycinyl-KAN (**8**) and 6′-*N*-AHB-KAN (**18**) had at least a 64- and 32-fold decrease in MIC value, respectively, when compared to KAN when tested against *Bacillus anthracis* str. Sterne (**A**). Compounds **8** (MIC = 16 μg/mL) and **18** (MIC = 2 μg/mL, at least a 64-fold reduction in MIC when compared to the parent KAN) were also found to moderately and effectively inhibit the growth of *Staphylococcus aureus* ATCC 29213 (**E**) and *M. smegmatis* MC2-155 (**J**), respectively. Most interestingly, 6′-*N*-glycinyl-NET (**13**) and 6′-*N*-glycinyl-SIS (**12**) were the two best antibacterial agents that we generated. Compounds **12** and **13** were found to be particularly active (MIC values ranging from ≤0.25 to 2 μg/mL, values that are similar to that of the parent AGs) against *B. anthracis* str. Sterne (**A**), *S. aureus* ATCC 29213 (**E**), a methicillin-resistant *S. aureus* strain (MRSA2, **G**), and *M. smegmatis* MC2-155 (**J**). These SIS and NET derivatives, especially the SIS derivative **12**, were also found to be moderately active (MIC values ranging from 8 to 18.8 μg/mL) against *Listeria monocytogenes* ATCC 19115 (**D**), MRSA1 (**F**), and *Pseudomonas aeruginosa* PA01 (**I**). The 6′-*N*-glycinyl-TOB (**7**) and 6′-*N*-AHB-TOB (**17**) demonstrated exceptional antibacterial activity. They displayed 128- and 32-fold decrease in MIC values, respectively, when compared to TOB against *B. anthracis* str. Sterne (**A**). They displayed ≥32- and ≥16-fold decrease in MIC values, respectively, when tested against *M. smegmatis* MC2-155 (**J**). They were also moderately active (MIC = 8 μg/mL) against *S. aureus* ATCC 29213 (**E**). Testing of the 6′-*N*-Cbz-TOB (**20**) suggested that having an amine in the group added to the parent AG scaffold is greatly beneficial as **20** was found to be inactive against almost all bacterial strains tested.

Having established the antibacterial properties of our AG derivatives, we next tested their ability to potentially evade the action of AMEs (Figure 1). We tested derivatives **7**−**9**, **12**−**13**, and **17**−**20** against the *N*-acetyltransferases AAC(3)-IV from *E. coli*,[30] AAC(6′)-Ie by using the bifunctional AAC(6′)-Ie/APH(2″)-Ia from *S. aureus*,[11] and Eis from *M. tuberculosis*,[13] as well as against the *O*-phosphotransferase APH(3′)-Ia from *E. coli*. These enzymes were chosen based on the positions modified in nature, and the abundance of enzymes modifying those selected positions. Eis was added to the list due to its ability to acetylate the 3″-, 6′-, 1-, and 4‴-positions of AGs.[14] It is of importance to note that of the five AG scaffolds tested (AMK, KAN, NET, SIS, and TOB), only the KAN derivatives **8** and **18** were modified by APH(3′)-Ia at 60% and 5%, respectively, compared to KAN itself. This suggests that, while the glycinyl group of compound **8** slightly hinders the phosphorylation of the 3′-hydroxyl of the KAN scaffold, the larger AHB moiety of compound **18** almost completely

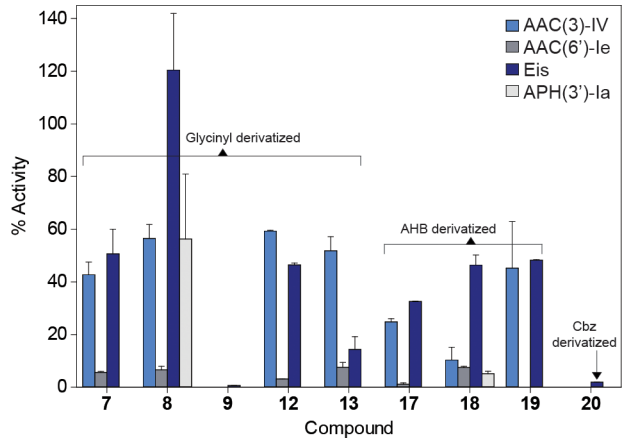

Figure 1. Bar graph displaying AME activity in the presence of AG derivatives generated in this study.

prevents APH(3′)-Ia from modifying this KAN derivative. All other AGs either did not contain a 3′-hydroxyl or were not substrates of the phosphotransferase. When exploring the activity of AACs on our compounds, we found that, in general, all AG derivatives were modified at a lower rate than their parent counterpart, with the exception of compound **8** where a 1.2-fold increase in activity was observed with Eis only. Since Eis can multiacetylate AGs, it is not surprising that this enzyme has the potential to acetylate 6′-*N*-glycinyl-KAN (**8**) at a higher rate than KAN. Interestingly, all of our derivatives resisted the activity of AAC(6′)-Ie, one of the most common types of AAC. AAC(3)-IV modified the derivatized AGs, on average, at 50% of the rate observed with the parent AGs. Overall, it appears that the AHB derivatization is slightly better at preventing acetylation and phosphorylation than the glycinyl modification.

Finally, to understand a subset of the resistance mechanisms involved in the bacterial strains that we tested, we probed these strains using PCR, for specific AME genes: *aac(6′)-Ib*, *aac(3)-IV*, and *aph(3′)-Ia* (Figure 2). After gel analysis we found that five strains, *B. anthracis* str. Sterne (**A**), *B. cereus* ATCC 17788 (**B**), *B. subtilis* 168 (**C**), *L. monocytogenes* ATCC 19115 (**D**), and *H. influenzae* ATCC 51907 (**H**), contained the *aph(3′)-Ia* gene, two strains, *B. cereus* ATCC 17788 (**B**) and *B. subtilis* 168 (**C**), contained the *aac(3)-IV* gene, two strains, *B. cereus* ATCC 17788 (**B**) and MRSA1 (**F**), contained the *aac(6′)-Ib* gene, and

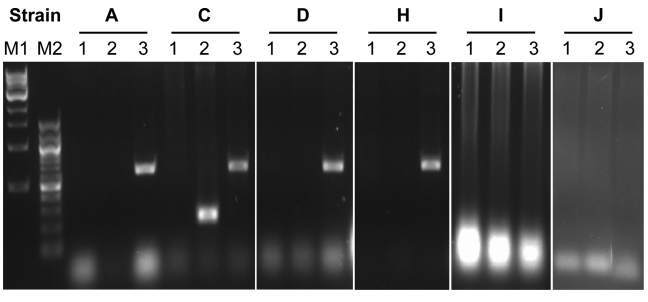

Figure 2. Agarose gels (1.5%) used in determining the AMEs present in the bacterial strains tested in this study. Genes probed for included *aac(6′)-Ib* (lanes 1, 482 bp), *aac(3)-IV* (lanes 2, 230 bp), and *aph(3′)-Ia* (lanes 3, 624 bp). Bacterial strains **A** = *B. anthracis* str. Sterne; **C** = *B. subtilis* 168; **D** = *L. monocytogenes* ATCC 19115; **H** = *H. influenzae* ATCC 51907; **I** = *P. aeruginosa* PA01; **J** = *M. smegmatis* MC2-155. Molecular weight markers M1 (1 kb) and M2 (100 bp) were from New England BioLabs.

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four strains, *S. aureus* ATCC 29213 (**E**), MRSA2 (**G**), *P. aeruginosa* PA01 (**I**), and *M. smegmatis* MC2-155 (**J**), contained no genes for which we probed. PCR analysis was previously performed for strains **B** and **E**−**G**.[26] While this data does not confirm that the proteins associated with these genes are actively expressed under the conditions tested, it does inform us that inactivity of compounds on strains that did not have a positive hit in the probing experiments may have a means of deactivating AGs other than AMEs.

In sum, a series of novel AMK, KAN, NET, SIS, and TOB mono- and diderivatized at the 1-, 6′-, and 4‴-positions with glycinyl, carboxybenzyl, and AHB groups has been synthesized and tested against a panel of Gram-positive and Gram-negative bacterial strains as well as a strain of *mycobacteria*. Overall, we found that in the case of KAN, the 6′-amine is likely important for most of its antibacterial activity and therefore its affinity for the ribosome target. The same is true of the combination of the 6′-amine and terminal amine of AHB from AMK. The best compounds, in terms of overall MIC values, were compounds **12** and **13**, 6′-*N*-glycinyl-SIS and -NET derivatives, respectively (Table 1). SIS and NET both showed the overall best antibacterial activity in these studies, they also were minimally modified by the AMEs tested in this study, so it is not necessarily surprising that this was the case. Therefore, SIS and NET derivatives appear to be promising scaffolds for further investigation. Achaogen has already demonstrated that a SIS derivative, plazomicin, is nontoxic and can evade the action of most AMEs. Work in our group is currently underway to further derivatize NET.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.5b00255.

(i) Materials and methods, (ii) all experimental protocols for the synthesis of compounds and their characterization, (iii) experimental protocols for determination of AME activity by UV−vis assays, MIC values determination, and identification of AMEs in bacterial strains, and (iv) all ^{1}H and ^{13}C NMR spectra for the final compounds (PDF)

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Notes

The authors declare no competing financial interest.

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