Aminoglycosides

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Regio- and Chemoselective 6′-N-Derivatization of Aminoglycosides: Bisubstrate Inhibitors as Probes To Study Aminoglycoside 6′-*N*-Acetyltransferases**

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Aminoglycosides are among the most commonly used broad-spectrum antibiotics.[1] The biological activity relies on their high affinity for the major groove of bacterial 16S rRNA,[2] thereby impeding protein synthesis. A number of aminoglycosides also display antiviral activity owing to specific interactions with viral RNA.[3] The rapid emergence of aminoglycoside resistance in the treatment of infections, however, is a serious threat.[4] In clinical isolates of aminoglycoside-resistant strains, the most frequently observed cause of resistance is the expression of *N*-acetyltransferases.[5] For example, aminoglycoside 6′-*N*-acetyltransferase type Ii (AAC(6′)-Ii) is chromosomally encoded in *Enterococcus faecium*, which is one of the leading causes of hospital-acquired infections.[6] Studies by Wright and co-workers suggest that catalysis by AAC(6′)-Ii occurs through an ordered bi-bi mechanism in which acetyl coenzyme A (Ac-CoA) must bind the enzyme before the aminoglycoside.[5,7] Next, attack of the aminoglycoside 6′-NH_2 at the thioester of Ac-CoA is believed to generate a tetrahedral intermediate that collapses to yield an 6′-*N*-acetylaminoglycoside and CoA. Although crystal structures have been reported for complexes of AAC(6′)-Ii with either Ac-CoA or CoA, crystallization experiments of enzyme–aminoglycoside complexes have not been successful.[8] It was envisaged that either 6′-*N*-(S-CoA)aminoglycoside derivatives or bisubstrates (Scheme 1) would facilitate the study of this important class of enzymes. Bisubstrate analogues have exhibited inhibition of serotonin

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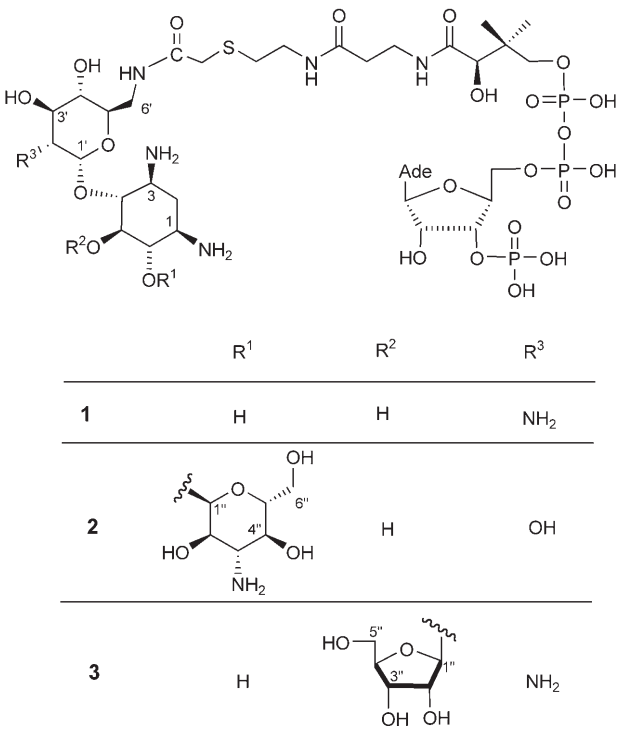
Supporting information (including experimental procedures, characterization data, some NMR spectra and HPLC traces) for this article is available on the WWW under http://www.angewandte.org or from the author.

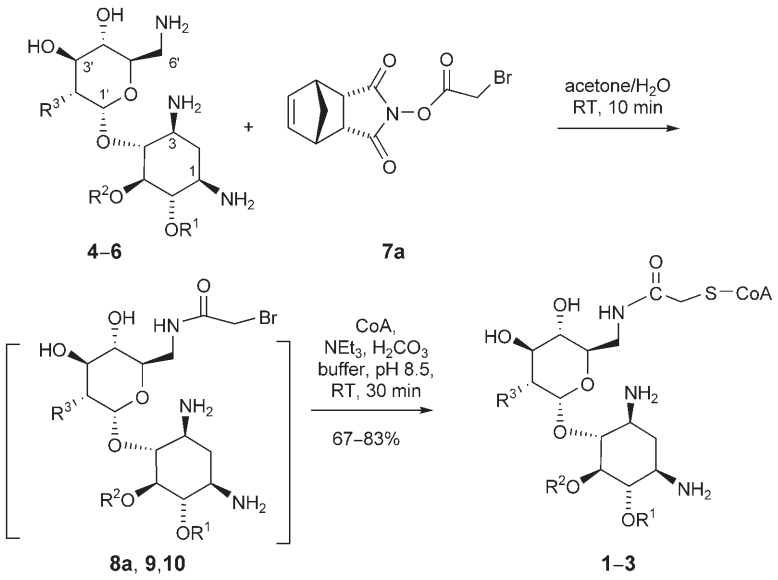


Scheme 1. Target aminoglycoside–CoA bisubstrates **1**–**3**. Ade = adenine.

acetyltransferase,[9] GCN5 histone acetyltransferase,[10] and carnitine acetyltransferases.[11] 3-*N*-(2-*S*-CoA-acetyl)gentamicin C_{1a}, the only aminoglycoside-CoA derivative reported so far, was prepared enzymatically by using AAC(3)-I (3 mg) to yield the desired product (0.89 mg),[12] which was subsequently found to inhibit AAC(3)-I with high affinity. To date, however, there are no reports of chemical syntheses of CoA-aminoglycoside derivatives. Naturally occurring aminoglycosides are complex molecules, and their regioselective modification remains challenging. Currently, the use of judicious functional-protection chemistry is common; however, the overall yields are low.[13] Herein we report an efficient procedure for the regioselective derivatization of unprotected aminoglycosides at the 6′-NH_2 position. This strategy was shown to proceed with high chemoselectivity towards the assembly of CoA-aminoglycoside derivatives **1**–**3** and **11a**–**c** (Scheme 2 and Scheme 3). Activity assays of these bisubstrates reveal novel nanomolar tight-binding competitive inhibition of AAC(6′)-Ii.

The target bisubstrates **1**–**3** were designed based on the proposed tetrahedral intermediate that results from the attack of the aminoglycoside 6′-NH_2 on the thioester carbonyl of Ac-CoA in the active site of the enzyme.[5] As the crystal structures of AAC(6′)-Ii[8] did not reveal a potential oxyanion hole, it was envisaged that a bisubstrate containing an amide-based linker could mimic the intermediate. The targets were built from neamine (**4**), kanamycin A (**5**), and ribostamycin (**6**), which are examples of nonsubstituted, 4,6-substituted, and 4,5-substituted aminoglycosides, respectively, and are AAC(6′)-Ii substrates. The key step in the synthesis of **1**–**3** from **4**–**6** is the preparation of the bromide intermediates **8a**, **9**, and **10**, respectively (Scheme 2). The trifluoroacetic acid (TFA) salt of intermediate **8a** has been synthesized previously by using orthogonal protection in four steps, and with a resultant very low yield (11.6%).[14] As a more-efficient alternative to orthogonal protection/deprotection schemes, we envisaged the use of *N*-(2-bromoacetyl)oxy-5-norbornene-*endo*-2,3-dicarboximide (bromoacetyl-NBD ester, **7a**) to transfer regioselectively a bromoacetyl group to the 6′-NH_2 of the aminoglycosides. The Boc-NBD ester has been reported to effect regioselective Boc protection of aminoglycosides;[15] however, NBD esters have not been employed for direct aminoglycoside derivatization.

Remarkably, simply mixing the free base neamine and reagent **7a** in either acetone/H_2O or acetonitrile/H_2O (1:1) at room temperature and in a vial open to the air for a few minutes (monitored by ESI MS) was sufficient to complete the reaction. In contrast, extended reaction times yielded complicated mixtures, possibly arising from the nucleophilic attacks of amino groups at the newly formed bromide. The desired *N*-6′-bromoacetylneamine (**8a**) was isolated in good yield (70%) by quenching of the reaction mixture with TFA



Scheme 2. Synthesis of the bisubstrate analogues **1**–**3**; see Scheme 1 for R^1, R^2, R^3; **4**: neamine; **5**: kanamycin A; **6**: ribostamycin; intermediates **9** and **10** were not isolated.

after 10 min and subsequent purification by reversed-phase HPLC. To avoid the isolation step and potential decomposition, a one-pot synthesis of **1** from **4** and CoA through **8a**, was evaluated. Bisubstrate **1** was obtained in excellent yield (83%) after purification by HPLC (the crude sample was more than 85% pure (Figure 1a)). The reaction proceeded equally well with the aminoglycosides **5** and **6** (72 and 67%, respectively). In spite of their significant structural differences, kinetic studies revealed that **1**–**3** showed nanomolar

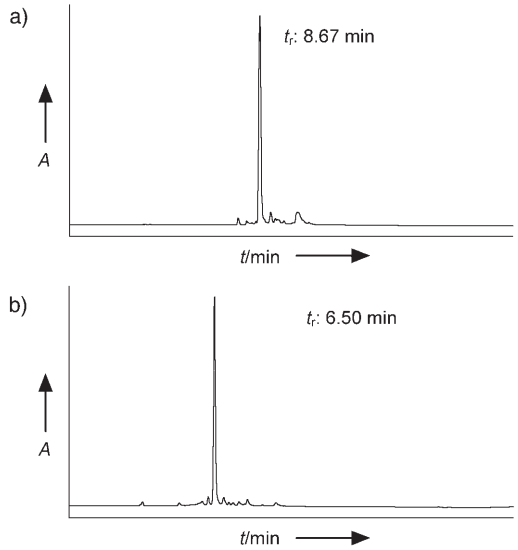


Figure 1. HPLC chromatograms for the purification of bisubstrate analogues: a) **1** and b) **11a**.

tight-binding competitive inhibition of AAC(6′)-Ii (Table 1). This observation is consistent with the fact that AAC(6′)-Ii has a broad substrate specificity.[5]

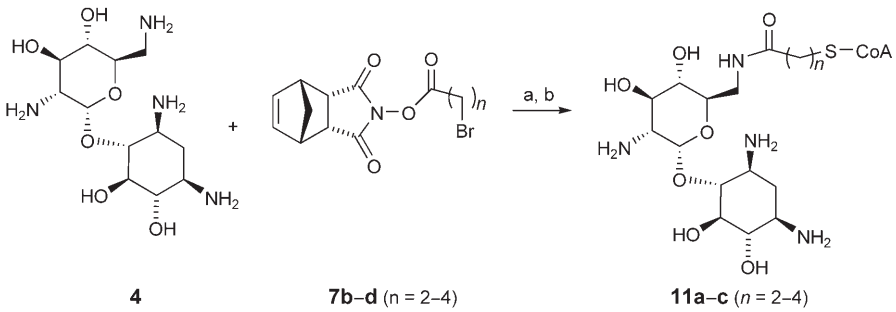
Table 1: The AAC(6′)-Ii-inhibition constants (K_i) for the bisubstrates.

Inhibitor	K_i [nM]	Inhibitor	K_i [nM]
1	76 ± 25	**11a**	43 ± 23
2	111 ± 28	**11b**	161 ± 98
3	119 ± 14	**11c**	7990 ± 2663

The size and geometry of linkers in serotonin-CoA bisubstrates have been reported to show important effects on the inhibition of serotonin *N*-acetyltransferase.[9,16] A similar effect was expected for AAC(6′)-Ii and was investigated by using bisubstrates **1** and **11a–c** (Scheme 3). Unfortunately, the procedure described herein for the synthesis of bisubstrates **1–3** was not easily applicable to the preparation of **11a–c**. The major products observed when synthesis of **11a** was attempted were: an ammonium bromide (likely from the addition of triethylamine to 6′-*N*-(3-bromo-*n*-propanoyl)neamine); 6′-*N*-acryloyl neamine (the elimination product); and a propanoyl-conjugated neamine dimer (either through S_N2 substitution at the bromide by a second neamine function or by Michael addition of a second neamine group to 6′-*N*-acryloyl neamine). Similar products resulted during the initial efforts to prepare **11b** and **11c**. A difference in the reactivities of these longer linkers was expected.[17] Indeed, the 3-bromo-*n*-propanoyl, 4-bromo-*n*-butyroyl, and 5-bromovaleroyl neamine derivatives (**8b–d**, from the reaction of neamine with **7b–d**) are much less electrophilic than **8a**. Moreover, triethylamine may be too nucleophilic and used in too large an amount, which may favor *N* alkylation over *S* alkylation. As most reported chemoselective *S*-/*N*-alkylation procedures[17] are not compatible with our reagents, we reasoned that a less-nucleophilic base at a lower concentration may favor *S* alkylation. Model studies were performed by using *N*-acetylcysteine as a mimic of CoA, and an array of bases were screened, including $KHCO_3$, $NaHCO_3$, diisopropylethylamine (DIPEA), 1,4-diazabicyclo[2.2.2]octane (DABCO), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), and 4-dimethylaminopyridine (DMAP). Excellent yields resulted when DIPEA (20 equiv) was used and the mixture was ultrasonicated (5 min). When applied to CoA, these conditions allowed the preparation of **11a**, **11b**, and **11c** in moderate to excellent yields (91, 52, and 93%, respectively). The HPLC trace of crude **11a** is shown in Figure 1b.

AAC(6′)-Ii-inhibition assays showed that **11a** is the most potent bisubstrate inhibitor of this series. Remarkably, the enzyme binds **11a** ≈200-fold tighter than its natural substrate Ac-CoA ($K_m = 9.6$ μM). A further increase in the length of the linker, however, rapidly leads to a decrease in activity (Table 1). To demonstrate the usefulness of these inhibitors as structural and mechanistic probes, they were studied by X-ray crystallographic analysis. Although previous crystallization experiments of AAC(6′)-Ii–aminoglycoside complexes had not been successful,[8b,c] bisubstrates **1–3** and **11a–c** crystallized well with the enzyme, providing X-ray diffraction data to ≈2.0-Å resolution. Preliminary analysis of the diffraction data for the complex with bisubstrate **11a** (Figure 2) suggests that the conformation of the aminoglycoside bound to AAC(6′)-Ii is very different from that reported for AAC(6′)-Iy,[18] even though both enzymes catalyze the same reaction. Detailed structural and mechanistic analysis of these structures will be reported elsewhere.

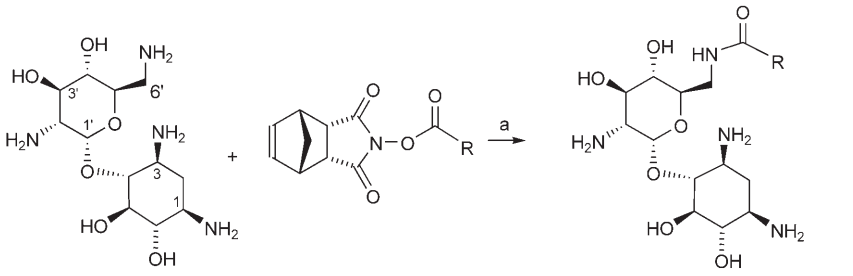
After the success of this methodology in the preparation of CoA-aminoglycoside bisubstrates, its general applicability to acylation was investigated (Scheme 4). Most of the acyl groups tested were transferred regioselectively to neamine with excellent yields. NBD esters of highly hindered acyl groups such as 2-methylbenzoyl and 2,6-dichlorobenzoyl, however, did not react at all. The regioselectivity of the transfer to kanamycin A, ribostamycin, and neomycin was tested with benzoyl-NBD and showed excellent selectivity for *N*-6′-acylation.



4 **7b–d** (n = 2–4) **11a–c** (*n* = 2–4)

Scheme 3. Reagents and conditions: a) acetone/H_2O (1:1), room temperature, 10 min; b) CoA, DIPEA (20 equiv), sonicate 5 min; then room temperature, 1 h; **11a**: 91%; **11b**: 52%; **11c**: 93%.

Figure 2. Crystallographically determined structure of the *E. faecium* AAC(6′)-Ii dimer (blue and red) when complexed to the bisubstrate inhibitor **11a** (stick representation, C: light blue, N: dark blue, O: red, P: gray, S: orange.



	e	f	g	h	i	j	k	l	m
R:	Me	*n*Pr	*n*-$C_{11}H_{23}$	Ph	Bn	Naph	2-ClC_6H_4	2-MeC_6H_4	2,6-$Cl_2C_6H_3$
yield [%]	60	70	70	80	75	85	90	0	0

Scheme 4. Regioselective *N*-6′-acylation of neamine. a) acetone/H_2O (1:1), room temperature, 30 min. Bn = benzyl, Naph = naphthyl

In conclusion, we have described a highly efficient synthetic strategy for the regioselective acylation of aminoglycosides at the 6′-NH_2 group. This process was successfully applied to the one-pot synthesis of 6′-*N*-(*S*-CoA)aminoglycoside analogues. Most of these bisubstrates were nanomolar tight-binding competitive inhibitors of AAC(6′)-Ii, an important enzyme leading to antibiotic resistance. The high potency of these bisubstrate inhibitors and the crystal structure of enzyme-bound **11a** suggest that they may be good mimics of one of the enzymatic reaction intermediates.[5] The bisubstrates reported here have allowed crystallization of AAC(6′)-Ii with aminoglycoside derivatives. The resulting structures should provide valuable guidance in further studies of this enzyme and other members of this family.

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