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Combined Chemical-Enzymatic Assembly of Aminoglycoside Derivatives with N-1-AHB Side Chain

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Dedicated to the 60th birthday of Professor Chi-Huey Wong.

Supporting information for this article is available on the WWW under http://asc.wiley-vch.de/home/.

Abstract: A series of unprotected pseudo-disaccharides and pseudo-trisaccharides of 2-deoxystreptamine-containing aminoglycosides have been selectively acylated at the N-1 position with the valuable (*S*)-4-amino-2-hydroxybutanoyl (AHB) pharmacophore by using the recombinant BtrH and BtrG enzymes from butirosin biosynthesis in combination with a synthetic acyl donor. The process was optimized by performing two enzymatic steps in a sequential manner without purification of the intermediate product.

Keywords: aminoglycosides; aminoglycosides toxicity; biosynthesis of butirosin; chemoenzymatic synthesis; resistance to aminoglycosides; stop codon readthrough

2-Deoxystreptamine (2-DOS) aminoglycosides are highly potent, broad-spectrum antibiotics that exert their bactericidal activity by selectively binding to the decoding aminoacyl site (A-site) of the bacterial 16S rRNA, which leads to the disruption of protein synthesis by interfering with translational fidelity and translocation.[1] The rapid spread of antibiotic resistance towards this family of antibiotics[2] and their relative toxicity[3] to mammals are critical problems that largely limit the intensive clinical use of these drugs. To overcome these problems, a wide variety of aminoglycoside derivatives – along with various modification methods – have been documented during the last few decades.[4] Among these methods, a selective *N*-acylation of the 2-DOS ring (ring II) at the N-1 position with the (*S*)-4-amino-2-hydroxybutanoyl (AHB) group, turned out to be one of the most effective modifications of the natural aminoglycosides, restoring the activity of both the kanamycin and neomycin family of antibiotics against selected resistant bacteria.[5] Earlier investigations in this direction yielded semisynthetic drugs such as amikacin (the N1-AHB-derivative of kanamycin A)[5a] and arbekacin (the N1-AHB-derivative of 3′,4′-dideoxykanamycin B)[5d] that are largely unaffected by a number of common resistance enzymes and were introduced in clinical practice in the 1970s and 1990s.

The motivation in exploring the effect of the selective introduction of the AHB side chain at the N-1 position of different aminoglycosides was largely triggered by the discovery of butirosin.[6] This aminoglycoside, first isolated from *Bacillus circulans*, bears the AHB side chain at N-1 of its 2-DOS moiety, and exhibits improved antibiotic activity compared to that of its parent molecule ribostamycin against several aminoglycoside-resistant bacteria. Furthermore, it is of note that butirosin is also about two times less toxic than ribostamycin (LD_{50} values in mg kg^{-1} of butirosin = 520, ribostamycin = 260).[7] Importantly, introduction of the N-1-AHB moiety to several other aminoglycosides has also been shown to be effective in lowering the acute toxicity of the resulting derivative relative to that of the parent molecule [neamine LD_{50} = 125 *vs.* N-1-AHB-neamine LD_{50} = 260; dibekacin LD_{50} = 71 *vs.* arbekacin (N-1-AHB-dibekacin) LD_{50} = 118].[8] Numerous structural and biochemical studies

have revealed the impact of the AHB group on the improved antibacterial activity of aminoglycosides. X-ray analyses of the bacterial A site in a complex with amikacin[9] and some other N-1-AHB-modified aminoglycosides[10] have demonstrated that the AHB group can increase binding affinity of aminoglycosides to the A site through making additional direct contacts. In parallel, detailed kinetic analysis of amikacin and of other synthetic derivatives with several resistance determinant enzymes have revealed that the AHB side chain can perturb molecular recognition by resistance enzymes.[11]

Interestingly, in the last several years, aminoglycosides have come into the focus of numerous investigations that rely on their rather unique ability to induce mammalian ribosomes to readthrough disease-causing premature stop codon mutations and generate full-length functional proteins in several genetic disorders.[12] However, while these studies provided compelling proof of the concept, the high toxicity of clinical aminoglycosides, along with their reduced readthrough activity at subtoxic doses, largely limits the therapeutic utility of the existing aminoglycosides to treat genetic diseases. Recently, we have reported a new series of aminoglycoside derivatives that were designed for better readthrough activity performance.[13] In this study, the pseudo-disaccharide paromamine **1** was selected as a common scaffold, to which either ribose (compounds **5**, **7**, and **9**) or 5-aminoribose (compounds **6**, **8**, and **10**) were attached at the 3′, 5, and 6 positions (Figure 1). One of these structures, compound **8** (also named NB30), showed markedly higher stop codon readthrough activity in cultured mammalian cells[13] and lower toxicity[14] compared to those of gentamicin (the only aminoglycosides tested to date in patients) and paromomycin.

Encouraged by the observed data on NB30, we sought to further improve both its readthrough activity and toxicity. For this purpose, we decided to explore the effect of an AHB group at the N-1 position of the pseudo-trisaccharides **5**–**10**. In parallel to this direction of research, we also strived towards the design of new derivatives of aminoglycosides with improved antibiotic performance. For this purpose, we designed the new pseudo-disaccharide scaffold **4** (also named NB23)[15] and its pseudo-trisaccharide derivatives **11**–**14** (Figure 1). These structures contain a 3′,4′-methylidene moiety as a 'defense' against the action of various aminoglycoside phosphotransferase [APH(3′)] and aminoglycoside nucleotidyltransferase [ANT(4′)] resistance enzymes. We reasoned that the installation of an AHB group at the N-1 positions of **11**–**14** may further increase the affinity of the resulting derivatives to the bacterial A site and, consequently, improve their antibacterial activity and toxicity.

Since the regioselective attachment of the AHB moiety on the aminoglycoside structure frequently requires long protection schemes[5] and the efficiency of a particular strategy is generally dependent on the structure of the parent aminoglycoside, we wanted to probe a shorter enzymatic approach as an alternative method. In this context, we have recently reported[16] that the biosynthesis of butirosin from ribostamycin

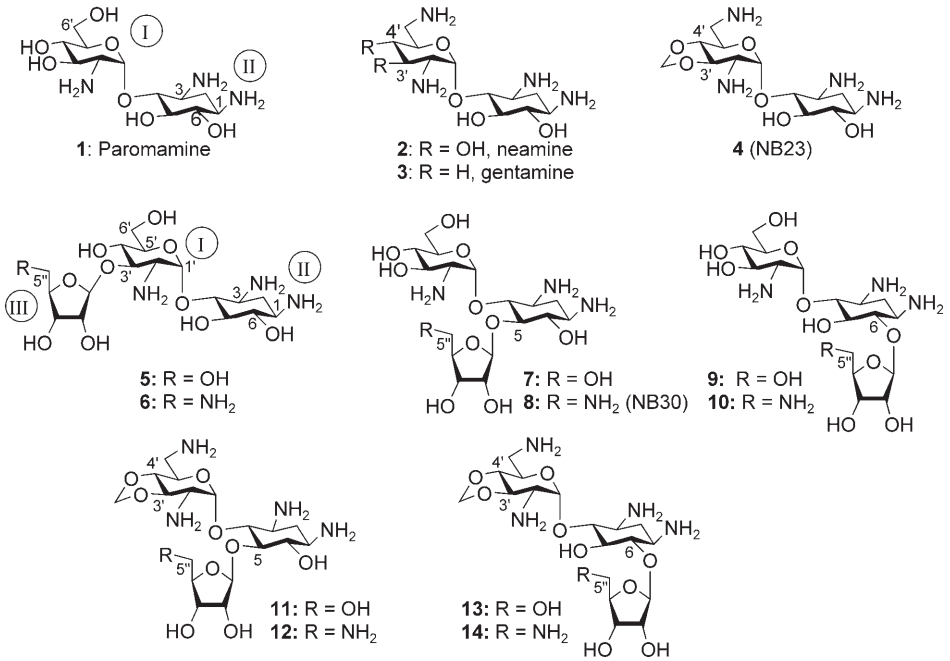
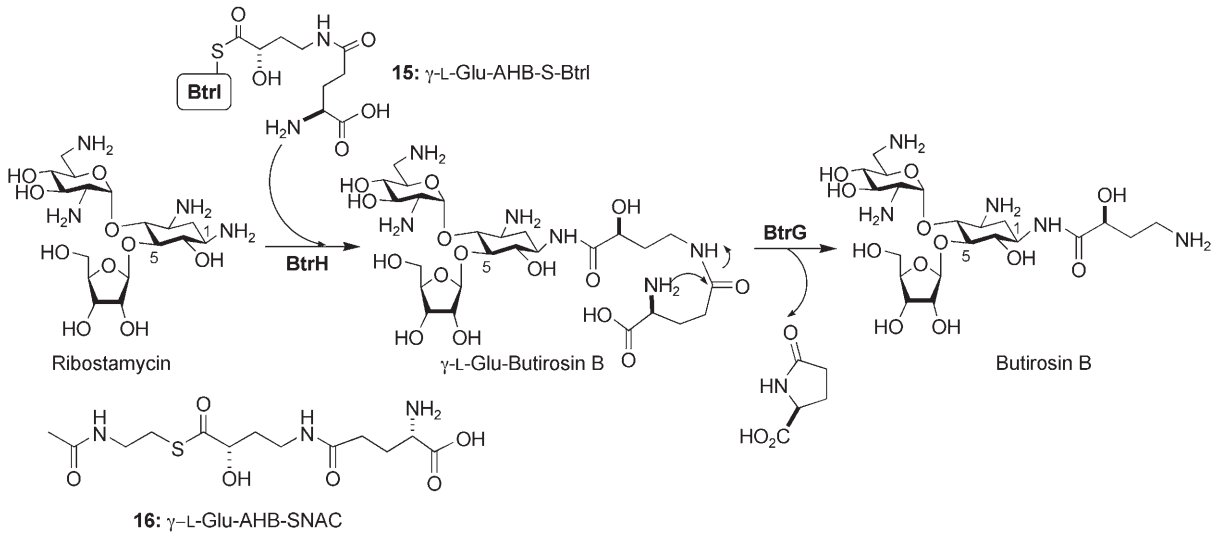


Figure 1. Sets of 2-DOS-containing pseudo-disaccharides (compounds **1**–**4**) and pseudo-trisaccharides (compounds **5**–**14**) that were tested for the tolerance toward the enzymatic addition of AHB moiety.



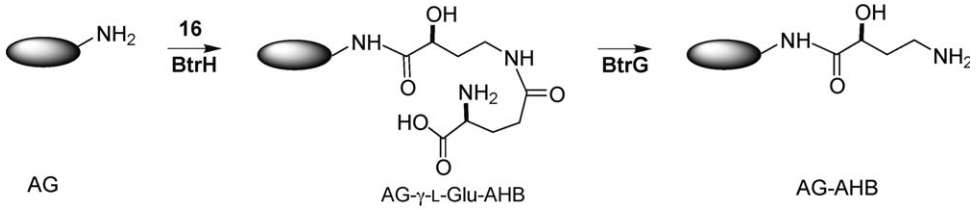
Scheme 1. Biosynthetic pathway for the conversion of ribostamycin to butirosin B.

involves two sequential enzymatic steps (Scheme 1). The AHB moiety is first transferred from the acyl carrier protein BtrI to the precursor aminoglycoside ribostamycin as a γ-glutamylated dipeptide by the acyltransferase enzyme BtrH to yield γ-L-Glu-butirosin B; the protective γ-glutamyl group is then cleaved by the BtrG enzyme *via* an intramolecular transamidation mechanism. The application of this method to the combined chemical-enzymatic production of a variety of novel N-1-AHB-bearing aminoglycosides was particularly attractive because the recombinant BtrH and BtrG enzymes are easily accessible as N-terminally His_6-tagged proteins,[16] and that the native acyl donor γ-L-Glu-AHB-S-BtrI (**15**, Scheme 1), which is rather difficult to produce in large quantities, can be very efficiently replaced by the synthetic *N*-acetylcysteamine thioester γ-L-Glu-AHB-SNAC (**16**, Scheme 1).[17] In addition, preliminary tests of this system's tolerance for alternative aminoglycoside acceptors indicated that, in addition to ribostamycin, related native aminoglycosides such as neomycin and paromomycin could also be efficiently converted to the corresponding N-1-AHB derivatives.[17]

Pseudo-disaccharides **1**–**4** along with pseudo-trisaccharides **5**–**14** were tested as alternative substrates for purified recombinant BtrH in the presence of **16** as an acyl donor. The reactions were easily monitored by TLC and the products were analyzed by LC-ESI-MS/MS (Table 1 and Supporting Information). Satisfyingly, all the 3′- and 5-substituted pseudo-trisaccharides (compounds **5**–**8**, and **11**–**12**) were essentially completely converted to the corresponding γ-L-Glu-AHB-derivatives after 6 h incubation at 20 °C. Except the paromamine **1**, which gave only 65% conversion, all the pseudo-disaccharides NB23 (96%), neamine **2** (90%), and gentamine **3** (92%) were also almost completely converted under the same conditions. Extension of the incubation time resulted in complete conversion of paromamine as well. The 6-substituted pseudo-trisaccharides, however, are acylated with moderate (compound **10**, ~50%; compound **13**, ~39%) to poor (compound **9**, 19%; compound **14**, ~27%) yields. Similar low conversion of the native 4,6-disubstituted aminoglycosides kanamycin A and gentamicin C_{1A} under the same conditions was previously observed.[17] The observed modest activity of BtrH with 6-substituted pseudo-trisaccharides, whether the substitution is a pyranose (kanamycin and gentamicin) or furanose ring (compounds **9**–**10**, and **13**–**14**), suggests that the 6-OH group of the 2-DOS moiety may play an important role for the proper recognition of the aminoglycoside substrate and catalysis by BtrH enzyme. Except for the modifications at 6-OH, all the modifications that have been tested so far, either on the pseudo-disaccharide scaffold (neamine **2**) of the native substrate ribostamycin or on ribostamycin itself are well tolerated by BtrH. The collective data indicate that BtrH, in combination with the synthetic acyl donor **16**, possesses broad substrate tolerance. Furthermore, all the γ-Glu-AHB-derivatives generated by a BtrH-assisted enzymatic step turned out to be excellent substrates for the BtrG; they were quantitatively deglutamylated in the next step with the purified recombinant BtrG to yield the corresponding AHB-derivatives (Table 1). Since the synthetic dipeptide **16** also functions as a good substrate of BtrG, the experiments were performed in a stepwise manner; first incubation with BtrH and **16** followed by incubation with BtrG.

Using this method, a number of synthetic compounds (**4**, **8**, **11** and **12**), were converted to the corresponding AHB-derivatives in several milligrams scale, and the purified products were characterized by NMR and MS analyses. The combination of standard

Table 1. Selective introduction of the AHB moiety to a wide variety of aminoglycosides (AG) by stepwise incubation with BtrH and **16** followed by incubation with BtrG.[a]



AG	Yield [%] AG-γ-L-Glu-AHB (Incubation 6 h)	AG-AHB (Incubation 24 h)	AG	Observed mass $[M+H]^+$ AG-γ-L-Glu-AHB product	AG-AHB product
RB[b]	100	100	455.17	685.26	556.20
1	65	100	324.11	554.21	425.12
2	90	100	323.19	553.20	424.12
3	92	100	291.07	521.16	392.08
4	96	100	335.10	565.14	436.09
5	97	100	456.14	686.20	557.16
6	99	100	454.92	685.22	556.16
7	99	100	456.15	686.21	557.15
8	99	100	455.33	685.24	556.18
9	19	100	456.12	686.24	557.19
10	52	99	455.11	685.26	556.21
11	100	99	467.01	697.18	568.12
12	100	91	466.14	696.22	567.19
13	39	96	467.07	697.19	568.13
14	27	97	466.09	696.23	567.16

[a] The reactions were performed in a total volume of 50 μL (with the AG concentration of 1.2 mM) and the percentages of conversion (% yield) for each step were determined by LC-MS analysis of the reaction mixtures as described in Experimental Section. The observed average mass differences of 230.1 Da between the AG-γ-L-Glu-AHB products and AG substrates are consistent with the attachment of γ-L-Glu-AHB ($[M+H]^+$ calculated at 230.10) moiety for each aminoglycoside substrate tested. The observed average mass differences of 101.1 Da between the AG-AHB products and AG substrates are consistent with the attachment of AHB ($[M+H]^+$ calculated at 101.06) moiety for each aminoglycoside substrate tested.

[b] The natural substrate of BtrH, ribostamycin (RB), was used as a positive control.

NMR experiments (including ^1H, ^{13}C, ^1H-^{13}C HMQC and HMBC, 2D COSY, and 1D selective TOCSY) allowed unambiguous assignment of all the hydrogen atoms present in each structure. In addition, for each product, the chemical shift comparisons for 1-H and 3-H protons of the 2-DOS ring with those in the parent compound unequivocally secured the regioselective attachment of the AHB side chain at the N-1 position; the 1-H resonance in the product is significantly de-shielded relative to that of the same proton in the parent compound due to the acyl substitution at the N-1 position. As a representative example, Figure 2 traces **A** and **B** illustrate the partial 2D COSY spectra of the chemoenzymatically produced N-1-AHB-**4** and its parent pseudo-disaccharide **4**, respectively. Both 2-H_{eq} and 2-H_{ax} protons (2-DOS ring) correlate to the neighbouring 1-H and 3-H protons in both compounds; while the resonance of the 3-H proton is almost unchanged (3.38–3.43 ppm), the resonance of 1-H in **4** ($\delta = 3.13$–3.14 ppm) is strongly shifted to the downfield region ($\delta = 3.77$–3.79 ppm) in the product N-1-AHB-**4** due to the presence of AHB.

To provide full confirmation of the preservation of N-1 regioselectivity by BtrH with the synthetic substrates, the N-1-AHB-**8** (compound NB54, Scheme 2)[18] was chemically synthesized as a representative example from paromamine **1** as a starting material and its analytical data were compared to those of a sample prepared by the combined chemical-enzymatic method. As expected, all the 1D and 2D NMR data of the enzymatic product, under the same pH and counterion conditions, are identical to those of the synthetic compound (see Supporting Information). Furthermore, preliminary tests of NB54 for *in vitro* readthrough activity of the TGA stop codon, demonstrated that it exhibits significantly higher stop codon readthrough activity than its parent NB30 (**8**) and the natural drug paromomycin (Figure 2 **C**). Further biological tests of NB54 along with other N-1-AHB derivatives prepared in this

study are underway and will be reported in due course.

In conclusion, we have developed an efficient chemoenzymatic method for the preparation of a wide variety of 2-DOS-containing aminoglycosides with the valuable AHB pharmacophore by using the BtrH/BtrG catalytic system with the synthetic acyl donor **16**. Since aminoglycosides are polycationic, water-soluble substances, the addition of the AHB side chain is done in the final steps of the synthesis and, as such, the presented method significantly shortens and simplifies the chemical strategies that necessitate long protection schemes for this purpose. The observed broad substrate tolerance of the BtrH enzyme for the synthetic substances tested so far, along with the easy accessibility of the recombinant BtrH and BtrG enzymes also make this method attractive for high throughput synthesis of a library of 2-DOS-containing aminoglycosides to discover valuable hits with potent biomedical relevance. Further investigations in this direction, along with research to develop a suitable assay for detailed kinetic and mechanistic analysis of the BtrH-catalyzed reaction with synthetic substrates are currently underway.

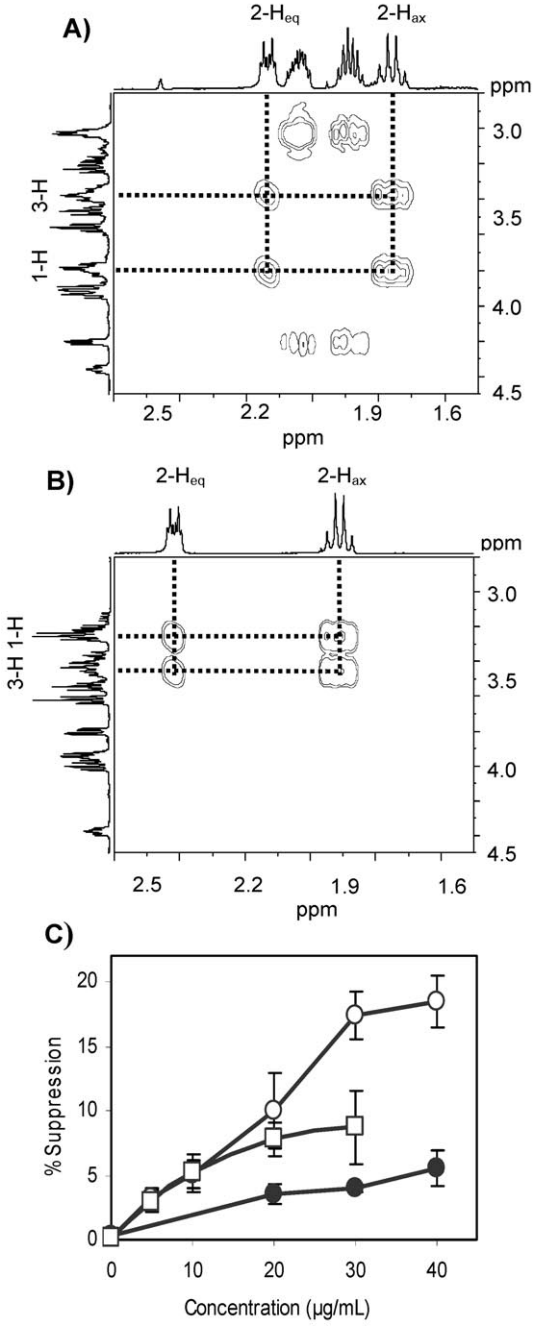


Figure 2. Partial 2D ^{1}H-^{1}H COSY spectra of the chemoenzymatically produced N-1-AHB-**4** (**A**) and of its parent compound **4** (**B**). The dashed lines show correlations between 2-H_{ax} and 2-H_{eq} protons with 1-H and 3-H protons of the 2-DOS ring, highlighting strong downfield shift of the 1-H proton in N-1-AHB-**4** *versus* 1-H proton in the parent compound **4**. Panel **C** shows the levels of *in vitro* suppression of a TGA C nonsense mutation by compound N-1-AHB-**8** (○), by compound **8** (●) and by natural drug paromomycin (□).

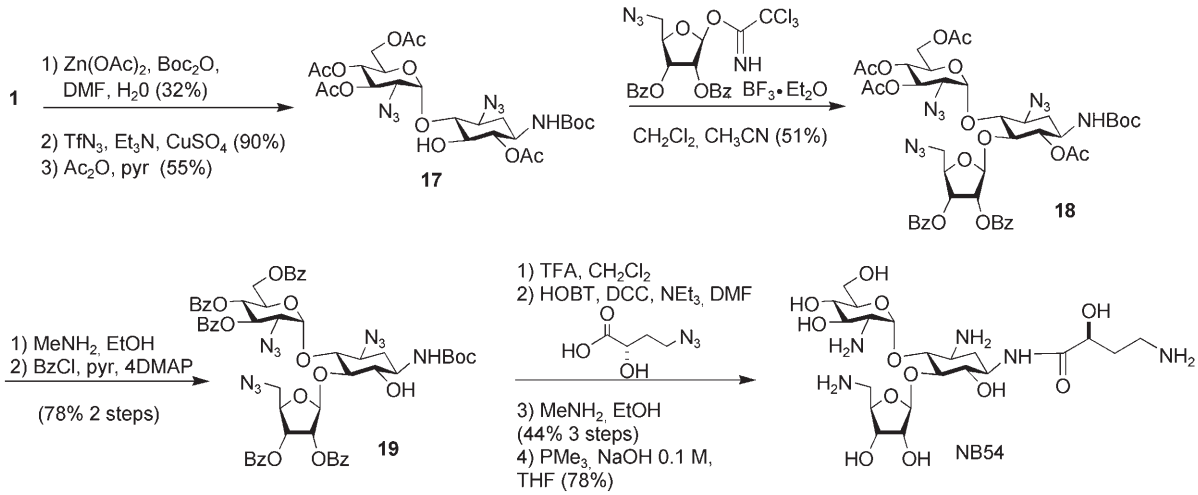
Experimental Section

General Procedure for the Introduction of the AHB Side Chain

To the reaction mixture (total volume of 50 μL) containing HEPES buffer (50 mM, pH 7.9), synthetic acyl donor **16** (5 mM) and an aminoglycoside (1.2 mM) was added the purified BtrH enzyme (125 μg) and the mixture was incubated at 20 °C for 6 h. The protein was removed by addition of 20 μL chloroform followed by vortexing and centrifugation (13,000 rpm, 5 min). The clear aqueous layer was taken for the next enzymatic step without further purification. A small aliquot (about 1 μL) of this solution was taken for LC-ESI-MS/MS analysis to determine the percentage of conversion of the aminoglycoside substrate to the desired product (Table 1 and Supporting Information). To the aqueous layer from the previous step was added the purified BtrG enzyme (18 μg) and the mixture was incubated at 20 °C for 24 h. The protein was removed as above and the aqueous layer was taken for LC-ESI-MS/MS analysis using an Agilent HP1100 HPLC system coupled to a Thermo-Finnigan LCQ ion-trap mass spectrometer equipped with an electrospray ionization (ESI) source.

Samples after enzymatic reactions were separated on a 2.0×250 mm Luna 5μ C18(2) column (Phenomenex) by the following gradient at a flow rate of 0.3 mL min^{-1} and

The p2Luc plasmid containing a TGA C nonsense mutation in a polylinker between renilla and firefly luciferase genes[20] was transcribed and translated using the TNT® Reticulocyte Lysate Quick Coupled Transcription/Translation System (Promega™). Luciferase activity was determined following 90 min incubation using the Dual Luciferase Reporter Assay System (Promega™) and the stop codon suppression efficiency was calculated as described previously[20]. The results are averages of at least three independent experiments.



Scheme 2. Chemical transformation of paromamine **1** into NB54.[18]

column temperature of 40 °C: 0–20 min 10%–50% B, 20–21 min 50%–10% B, 21–25 min 10% B [buffer A: 0.1% pentafluoropropionic acid (PFPA) in H_2O; buffer B: 0.1% PFPA in MeCN]. Mass spectra were acquired from 250 to 1000 Da. MS/MS was carried out on target ions with 20% relative collision energy (helium as collision gas).

Preparative scale reactions were performed as above but in a total volume of 10–15 mL, and with the addition of 1.5 mg BtrH and 1.0 mg BtrG. The incubation time for both enzymatic steps was also extended to 24 h. The aqueous layer, after removal of BtrG, was loaded onto Dowex 50W (NH_4^+ form) 15×80 mm ion-exchange column. The column was washed with water (50 mL) followed by elution with 1% NH_4OH in water. Fractions containing product [TLC: CH_2Cl_2/MeOH/H_2O/$MeNH_2$ (33% solution in EtOH), 10:15:6:15] were combined and evaporated to dryness. The residue was dissolved in water, the pH was adjusted to 3.5 by H_2SO_4 (0.05 M) and lyophilized to afford the sulfate salt which was used for all the spectral analyses.

The recombinant BtrH and BtrG enzymes were isolated as homogeneous N-terminally His_6-tagged proteins according to the previously reported procedures.[16] The synthetic acyl donor **16** was synthesized as previously described.[17] The pseudo-disaccharides **1**,[13] **2**, **3** and **4**[15] along with the pseudo-trisaccharides **5**–**10**[13] and **11**–**14**[15] were prepared by reported strategies.

Supporting Information

A supplementary table including R_f values of TLC analysis and retention time data of LC-MS analysis of all the aminoglycosides tested (**1**–**14**) and of their γ-L-Glu-AHB and AHB products, along with the complete characterization of the intermediate structures **17**, **18**, **19** and of the N-1-AHB products of **4**, **8**, **11** and **12** can be found in the Supporting Information.

Acknowledgements

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[18] For the chemical installation of AHB at the N-1 position of NB30, we choose an approach that utilizes direct regioselective differentiation of the aminoglycoside amino groups by metal-mediated chelation[19] as illustrated in Scheme 2. Treatment of paromamine with $Zn(OAc)_2$ and Boc_2O afforded the selectively *N*-1-Boc-protected paromamine (32% isolated yield), which after azidation (TfN_3) and regioselective acetylation (Ac_2O at low temperature) gave the acceptor **17**. Glycosidation of **17** with the trichloroacetimidiate donor under Lewis acid conditions furnished the desired pseudotrisaccharide **18**. Compound **18** was then subjected to a sequential two-step procedure for the installation of the AHB moiety: treatment with TFA to remove the Boc group and the reaction of the resulted N-1 amine with (*S*)-2-hydroxy-4-azidobutyric acid (HOBT, DCC, Et_3N) afforded the acyl migration product from the 6 position to N-1 amine as a main product (75%). To avoid this limitation, **17** was first converted to the corresponding 6-hydroxy compound **19** in two successive steps (78%), which after the same two steps for the installation of AHB followed by treatment with methylamine and the Staudinger reaction furnished the desired product NB54 bearing the AHB moiety at the N-1 position.
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