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PAPER

Assessment of 6'- and 6'''-N-acylation of aminogly cosides as a strategy to overcome bacterial resistance \dagger

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Amongst the many synthetic aminoglycoside analogues that were developed to regain the efficacy of this class of antibiotics against resistant bacterial strains, the 1-N-acylated analogues are the most clinically used. In this study we demonstrate that 6'-N-acylation of the clinically used compound tobramycin and 6'''-N-acylation of paromomycin result in derivatives resistant to deactivation by 6'-aminoglycoside acetyltransferase (AAC(6')) which is widely found in aminoglycoside resistant bacteria. When tested against AAC(6')- or AAC(3)-expressing bacteria as well as pathogenic bacterial strains, some of the analogues demonstrated improved antibacterial activity compared to their parent antibiotics. Improvement of the biological performance of the N-acylated analogues was found to be highly dependent on the specific aminoglycoside and acyl group. Our study indicates that as for 1-N-acylation, 6'- and 6'''-N-acylation of aminoglycosides offer an additional promising direction in the search for aminoglycosides capable of overcoming infections by resistant bacteria.

Introduction

Aminoglycosides are broad-spectrum antibiotics that interfere with protein translation by binding to the 16S ribosomal RNA, which ultimately leads to bacterial cell death.¹ The extensive clinical use of aminoglycosides enhanced the evolution of bacterial resistance to this important class of antibitotics. Bacterial resistance may result from the alteration of the bacterial outer membrane, active efflux systems, alteration of the aminoglycoside binding site in the bacterial ribosome, and deactivation of aminoglycosides by aminoglycoside-modifying enzymes (AMEs).^{2,3} The widespread emergence of bacterial resistance has led to an urgent need to continuously develop new ideas to regain the efficacy of these important antibacterials.⁴ One successful solution that resulted in novel clinically used semi-synthetic aminoglycosides is the development of N-acylated aminoglycosides. Inspired by naturally occurring N-acylated aminoglycosides, amikacin was developed by 1-N-acylation of kanamycin A with the (S)- α -hydroxy- γ amino-n-butyryl (AHB) group (Fig. 1).5 Other members of the *N*-acylated aminoglycoside subfamily include the semi-synthetic



Fig. 1 Structures of representative N-acylated aminoglycosides.

1-*N*-AHB-containing arbekacin and butirosin B, as well as the *N*-glycinyl-containing sporaricin A, astromicin, and dactimicin, which are isolated from bacterial sources. These *N*-acylated aminoglycosides display improved broad-spectrum antibacterial activity when compared their non-*N*-acylated counterparts.⁵⁻⁷ As a result of *N*-acylation, aminoglycoside acetyltransferases (AACs), which confer resistance to aminoglycosides, are blocked from modifying the already *N*-acylated position. Therefore, these compounds retain efficacy against a variety of resistant bacterial strains.⁷

In view of the high potential of discovering potent new aminoglycosides by exploring novel families of their *N*-acylated

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analogues, we recently developed a chemoenzymatic methodology that utilized AACs and unnatural acyl-CoA derivatives for the small-scale preparation of N-acylated aminoglycosides.⁸ Using the bifunctional aminoglycoside acetyltransferase-aminoglycoside phosphotransferase AAC(6')-APH(2") from Staphylococcus aureus, which exhibited relaxed substrate and cosubstrate specificity, a variety of 6'-N-acylated aminoglycosides were prepared using acyl-CoA analogues. When qualitatively tested for antibacterial activity using a bio-TLC protocol (silica gel TLC onto which a bacterial strain is overlayed after migration of the compounds followed by growth and visualization for zones of inhibition), some of the chemoenzymatically prepared 6'-N-acylated aminoglycosides demonstrated bacterial growth inhibition. These results suggested that further development and exploration of 6'-Nacylated aminoglycosides in search of antibiotics with superior activity against resistant bacterial strains is a promising direction.

Several 6'-N-acylated aminoglycoside derivatives have previously been designed to inhibit the catalytic activity of the AAC(6') family of AMEs as a strategy to overcome bacterial resistance.9,10 However, the antibacterial activity potential of such analogues was proposed to be poor on the basis of X-ray crystallographic data obtained for 6'-N-acetyl kanamycin A bound to its target rRNA. The structure indicated that the 6'-Nacetylation of kanamycin A would block the hydrogen bonding of the 6'-amine of the parent drug to the target rRNA and confer a high-level of drug resistance.^{11,12} This structural infomation suggests that 6'-N-acylation might not be useful for the discovery of kanamycin A analogues with improved antibacterial activity against aminoglycoside resistant bacterial strains such as AAC(6') expressing bacteria. However, since aminoglycoside structures are highly variable, we postulated that the deactivating effect of 6'-N-acylation is not general and is highly dependent on both the aminoglycoside scaffold and on the specific acyl group. Indeed, 6'-N-acetyl tobramycin has been shown to maintain significant antibacterial activity, indicating that while still deactivating, 6'-N-acylation of this aminoglycoside scaffold has a more limited effect on the drug efficacy.¹³ In the case of the 4,5-substituted 2deoxystreptamine paromomycin that lacks a 6'-amine (Scheme 1), the 6"'-amine has no specific interactions with the target rRNA thereby making it an attractive position for the development of 6"'-N-acylated paromomycin analogues resistant to chemoenzymatic inactivation by AAC(6') enzymes.14 We therefore suggested that replacing the 6'- or 6'''-amine groups of tobramycin and paromomycin, respectively, with acyl groups such as those found on other naturally occurring as well as semi-synthetic Nacylated aminoglycosides may prove to be a useful strategy to overcome AAC(6')-based bacterial resistance to aminoglycosides while still maintaining good antibacterial activity. There have been no previous reports on the potential loss or removal of N-acyl moieties in the literature, which suggests that the N-acyl groups introduced should be maintained on the aminoglycoside scaffolds inside the bacterial cell.

6'- and 6'''-N-acylated aminoglycosides can be readily made in a single step from the commercially available parent drugs by exploiting the special chemical features of the 6'- and 6'''amine of several aminoglycoside scaffolds. The 6'-and 6'''-amine positions are significantly more reactive as nucleophiles compared to other amine groups of aminoglycosides. An explanation for this phenomenon can be derived from the previously reported



Scheme 1 General synthetic scheme for the preparation of 6'-*N*-acylated tobramycin derivatives **1a–d** and 6'''-*N*-acylated paromomycin derivatives **2a–d**.

¹⁵N NMR of nebramine and neamine analogues. Analysis of the ¹⁵N NMR spectra revealed that the 6'-N signal is at a significantly higher field compared to that of the ¹⁵N signals of all other amine groups on the molecule.15 This observation suggests that the higher reactivity of the 6'-amine results not only from the fact that this position is less sterically hindered, but also from the more electron rich environment of the 6'-nitrogen that increases its nucleophilicity. Based on the special features of the 6'-amine, several examples for its chemoselective activation have been previously reported in the literature. 6'-N-AHB-kanamycin A was obtained in good yields by the direct acylation of kanamycin A with the N-hydroxysuccinymyl (NHS) ester of AHB.⁵ A set of 6'-N-acylated neamine analogues as inhibitors of AAC(6') was obtained by direct acylation of neamine.9 Finally, kanamycin A is chemoselectively converted to its 6'-N-Cbz protected derivative using NHS carboxybenzoyl esters as the first step of the synthetic path for the preparation of amikacin.5

Results and discussion

To test the antibacterial potential of acylated tobramycin and paromomycin analogues, we prepared 6'-N-glycinyl and 6'-N-AHB tobramycin (**1b** and **1d**, respectively) and 6'''-N-glycinyl and 6'''-N-AHB paromomycin (**2b** and **2d**, respectively). Acylation was performed on the commercially available tobramycin sulfate (**1**) and paromomycin sulfate (**2**), which were dissolved in a mixture of water and acetonitrile, and added the NHS ester and potassium carbonate as base catalyst (Scheme 1). NHS esters of azidoacetate and CbzAHB were prepared according to previously reported procedures.^{5,16} In all cases a major product that was confirmed to be the 6'-N-acylated tobramycin or 6'''-N-acylated paromomycin, and only traces of additional products

were formed. Flash chromatography gave the desired products (**1a**, **1c**, **2a**, and **2c**) in moderate to good yields, 57–91%. The final deprotected *N*-AHB and *N*-glycinyl analogues (**1b**, **1d**, **2b**, and **2d**) were obtained by catalytic hydrogenation of the azide and Cbz protecting groups with no additional purification required after the deprotection step in yields ranging from 82–95%. The position of acylation, purity, and structure identification were confirmed by ¹H, ¹³C, 1D-TOCSY, DEPT, and HMQC NMR as well as HR-ESI-MS (Figs. S1–S12, ESI[†]).

To test the effect of 6'- and 6'"-N-acylation on the activity of AMEs that have the most prevalent mode of resistance amongst aminoglycoside-resistant bacterial strains, AAC(6')-APH(2") and AAC(3)-IV were studied. When biochemically tested with AAC(6')-APH(2"), none of the synthetic analogues served as substrates for AAC(6') (Fig. 2 and S13A, B⁺). Even though this is not too surprising as the 6'-position is already occupied, these results are promising as it has been previously demonstrated that AAC(6')-APH(2") displays relaxed substrate specificity and is, in some cases, capable of catalyzing the O-acetylation of aminoglycosides.¹⁷ When tested for APH(2") activity, different trends were observed for the various analogues (Fig. S13E, F[†]). For the tobramycin series, the APH(2") activity was barely affected, whereas in the case of the paromomycin series phosphorylation was in some cases achieved more efficiently and in others less efficiently. It is important to note that all glycinylated compounds were found to behave in a similar manner to that of their corresponding parent drugs when reacted with APH(2"). To study if the structural modification caused by the 6'- and 6'''acylation has any effect on the ability of other AACs to confer resistance, we tested compounds 1a-2d with AAC(3)-IV (Fig. 2 and S13C, D[†]).¹⁸ As expected based on previous studies,^{8,19} compounds 1a and 2b-d were found to readily undergo 3-Nacetylation, however analogues 1b-d served as significantly poorer substrates of the enzyme. The catalytic efficiency of acetyl transfer at the 3-position for compound **2b** $[k_{cat}/K_m 0.019 \ \mu M^{-1} \ s^{-1}]$ was found to be similar to that of 2 $[k_{cat}/K_m 0.035 \ \mu M^{-1} \ s^{-1}]$.⁸ We are currently in the process of determining the rules governing multiple sequential modifications of aminoglycosides.



Fig. 2 Representative spectrophotometric assay plots monitoring the acetylation reaction of tobramycin (1) (square) and 6'-N-glycinyl tobramycin (1b) (triangle) with acetyl-CoA using AAC(6') (green) and AAC(3)-IV (red). For 1b 6'-acetylation is completely abolished, while 3-acetylation is significantly reduced.

To study the correlation between the *in vitro* study of the tested enzymes and their effect on the *N*-acylated analogues in bacterial

Table 1 Antibacterial activity. MIC values ($\mu g m L^{-1}$) of novel 6'- and6'''-N-acylated aminoglycosides compared to their parent drugs

Bacterial strain ^b	AG ^a					
	1	1b	1d	2	2b	2d
A	4	64	64	2	64	4
В	0.5	8	16	1	2	4
С	15.6	15.6	>125	31.3	62.5	125
D	62.5	3.9	>125	31.3	62.5	>125
Е	62.5	7.8	>125	62.5	62.5	>125
F	3.9	31.3	62.5	7.8	7.8	31.3
G	15.6	7.8	>125	62.5	62.5	125
Н	4	16	62.5	16	8	62.5
I	16	>250	>250	62.5	62.5	125

^{*a*} AG = Aminoglycoside. ^{*b*} MIC values were determined against *S. epidermis* ATCC12228 (A), *B. subtilis* ATCC6633 (B), *E. coli* BL21 (DE3) (C), *E. coli* BL21 (DE3) with AAC(6')-APH(2'')-pET22b (D), *E. coli* BL21 (DE3) with AAC(3)-IV-Int-pET19b-pps (E), *B. subtilis* 168 (F), *B. subtilis* 168 AAC(6')-APH(2'')-pRB374 (G), *E. coli* ToIC (H), and *S. aureus* NorA (I). Strains were tested by using the double-dilution method, with 1024 μ g mL⁻¹ starting concentration with strains A and B, 125 μ g mL⁻¹ starting concentration with strains C–G, and 250 μ g mL⁻¹ starting concentration with strains were performed in triplicates and analogous results were obtained in two different sets of experiments.

systems, we chose strains harboring no resistance enzymes and compared the activity of compounds 1, 1b, 1d, 2, 2b, and 2d to their activity on the same bacterial strains, which were transformed with one of our tested AMEs (Table 1). The following Gramnegative bacterial strains were tested: *E. coli* TolC (H), *E. coli* BL21 (DE3) (C) and the corresponding recombinant resistant strains *E. coli* BL21 (DE3) with AAC(6')-APH(2'') (D) and *E. coli* BL21 (DE3) with AAC(3)-IV (E). *S. epidermis* ATCC12228 (A), *B. subtilis* ATCC6633 (B), *S. aureus* NorA (I), *B. subtilis* 168 (F), and *B. subtilis* 168 with AAC(6')-APH(2'') (G) as the corresponding resistant strain were the Gram-positive bacterial strains utilized.

The best antibacterial activity results were observed for 6'-Nglycinyl tobramycin (1b). With E. coli BL21 (DE3) (C), the parent drug tobramycin (1) exhibited good antibacterial activity (MIC = 15.6 µg mL⁻¹). However, a significant loss of activity for the parent antibiotic 1 was observed in the two corresponding Gram-negative resistant strains (62.5 μ g mL⁻¹ for strains **D** and **E**). Interestingly, 1b maintained good antibacterial activity against both of these resistant strains (3.9 and 7.8 μ g mL⁻¹, respectively). As with most N-acylated compounds generated, 1b did not maintain good antibacterial activity against S. aureus (strain I). With the Grampositive B. subtilis 168 (F), 1 exhibited good antibacterial activity while **1b** displayed moderate potency (3.9 and 31.3 μ g mL⁻¹, respectively). However, when the AAC(6')-APH(2'')-resistant B. subtilis 168 (G) was tested, the MIC value for 1 increased (15.6 µg mL⁻¹), whereas 1b exhibited better potency against the resistant strain (7.8 μ g mL⁻¹). In both the tobramycin and paromomycin series, we observed that N-glycinyl is a better choice of an Nacylating group than the N-AHB.

Conclusions

In summary, we have demonstrated that 6'- and 6'''-N-acylation of aminoglycosides is a promising direction to search for novel aminoglycosides with potency against resistant bacterial strains. However, 6'-N-acylation cannot be used as a general method to

overcome aminoglycoside resistance and is heavily dependent on the specific aminoglycoside scaffold, the acyl group, and the tested bacterial strains. The semi-synthetic N-acylated analogues did not go through N-acetylation by the bifunctional-modifying enzyme AAC(6')-APH(2"). The AAC(6') site of the enzyme did not catalyze N- or O-acetylation on other positions of the analogues. Moreover, the 6'- and 6'''-N-acylation was found to affect other AACs. Significant drops in the catalytic efficacy were observed for some of the analogues when tested as substrates for AAC(3)-IV hence, demonstrating that 6'-N-acylation may reduce the efficacy of additional AMEs. In resistant bacteria expressing either of the tested AACs as their mode of aminoglycoside resistance, the effect of some of the analogues was more dramatic and good antibacterial activity was retained while the activity of the parent drug was compromised. The results of this study demonstrate that, like the clinically used 1-N-acylated aminoglycosides, some 6'- and 6'''-N-acylated aminoglycoside scaffolds maintain potent antibacterial activity against several aminoglycoside resistant as well as pathogenic bacterial strains. 6'- and 6"'-N-acylation therefore represents an additional direction for the development of aminoglycosides which cannot be modified by several AMEs, and as such, maintain their antibacterial activity against aminoglycoside resistant bacteria in which resistance is conferred by such enzymes.

Experimental details

Bacterial strains, plasmids, materials, and instrumentation

The bacterial strains utilized in this study were obtained from various sources. Escherichia coli TolC and Staphylococcus aureus were a gift from Prof. David H. Sherman (University of Michigan). S. epidermis ATCC12228 and Bacillus subtilis ATCC6633 were bought from the American Type Culture Collection (Manassas, VA, USA). The B. subtilis 168 utilized for preparation of the B. subtilis containing the AAC(6')-APH(2") resistance gene was obtained from the Bacillus Genetic Stock Center (Columbus, OH, USA). The AAC(3)-IV and AAC(6')-APH(2") enzymes were purified as previously described.8 The APH(2") (amino acids 175-479 of the bifunctional AAC(6')-APH(2")) was cloned into pET22b using primers TGTTATCATATGGAATATAGATATGATG with NdeI restriction site shown in bold and ATTATA-CTCGAGATCTTTATAAGTCCTTTTATAAATTTC with XhoI restriction site shown in bold, and purified and overexpressed using the same protocol as the one utilized for the bifunctional enzyme. The pRB374 plasmid harboring the AAC(6')-APH(2") gene for cloning into B. subtilis 168 was a generous gift from Prof. Gerard D. Wright (McMaster University, ON, Canada).¹⁷ DTDP, pyruvate kinase lactic dehydrogenase mix (cat # P0294), phosphoenolpyruvate (cat # P0564), NADH, GTP, acetyl-CoA and paromomycin (cat # P9297) were bought from Sigma-Aldrich and used without any further purification. Tobramycin (cat # 02865) was bought from Chem-Impex International (Wood Dale, IL, USA). The aminoglycosides were used without further purification. MTT was purchased from TCI America (Portland, OR, USA). The acylating reagent O-azidoacetyl-N-hydroxysuccinimide (3) and N-hydroxysuccinimyl ester of L-(–)- γ -benzyloxycarbonylamino- α -hydroxybutyric acid (4) were prepared as previously described.^{20,21} Analyses by UV-Vis assays were done on a multimode SpectraMax M5 plate reader using 96-well plates (Fisher Scientific). Reactions were monitored by TLC (Merck, Silica gel 60 F₂₅₄). Visualization was achieved using a cerium-molybdate stain $((NH_4)_2Ce(NO_3)_6$ (5 g), (NH₄)₆Mo₇O₂₄·4H₄O (120 g), H₂SO₄ (80 mL), H₂O (720 mL)). Compounds were purified by SiO₂ flash chromatography (Merck, Kieselgel 60). ¹H NMR spectra (including DEPT, 2D-COSY, 1D-TOCSY, HMQC, and HMBC) and ¹³C NMR spectra were recorded on Bruker Avance[™] 400 and 500 spectrometers. Low-resolution electron spray ionization (LR-ESI) mass spectra were measured on a Waters 3100 mass detector. High-resolution electron spray ionization (HR-ESI) mass spectra were measured on a Waters Synapt instrument. Analytical HPLC analyses of compounds 1b, 1d, 2b, and 2d were performed on a Merck Hitachi instrument equipped with a diode array detector using an Alltech Apollo C18 reversed-phase column (5 μ , 4.6 \times 250 mm). The solvents used were $A = H_2O (0.1\% \text{ TFA})$ and B = acetonitrile(0.1% TFA). The method used was 0-20% B over 20 min at a flow rate of 1 mL min⁻¹. Product elution was monitored at 204 nm.

6'-N-Azidoacetyl tobramycin (1a)

To tobramycin sulfate (1) (350 mg, 0.62 mmol) dissolved in MeCN:H₂O/2:1 (4 mL) were added K₂CO₃ (120 mg, 0.87 mmol) and azidoacetyl-N-hydroxysuccinimiyl ester (3) (172 mg, 0.89 mmol). The reaction mixture was stirred at rt for 16 h. The progress of the reaction was monitored by TLC (MeOH:NH₄OH/3:2, R_f 0.75). The reaction mixture was concentrated under reduced pressure and further purified by flash chromatography (SiO₂, MeOH \rightarrow MeOH:Et₃N/90:10). The fractions containing the pure product were concentrated under reduced pressure. The residue was dissolved in a minimal volume of H₂O and freeze-dried to afford the pure product **1a** (194 mg, 57%) as a white powder: ¹H NMR (500 MHz, D_2O) δ 3.88–3.93 (m, 2H, CH₂ of azidoacetyl), **Ring I:** 1.54 (q, 1H, J = 11.9 Hz, H3ax), 1.95 (ddd, 1H, $J_1 = 4.3$, $J_2 = 9.1$, $J_3 = 11.5$ Hz, H3eq), 2.95 (ddd, 1H, $J_1 = 4.0$, $J_2 = 7.8$, $J_3 = 12.6$ Hz, H2), 3.27–3.33 (m, 1H, H6), 3.34-3.44 (m, 1H, H4), 3.45-3.55 (m, 1H, H6'), 3.57-3.69 (m, 1H, H5), 4.98 (d, 1H, J = 3.4 Hz, H1), **Ring II:** 1.11–1.17 (m, 1H, H2ax), 1.87 (ddd, 1H, $J_1 = 4.1$, $J_2 = 8.4$, $J_3 = 13.1$ Hz, H2eq), 2.75 (ddd, 1H, J₁ = 4.2, J₂ = 9.6, J₃ = 12.2 Hz, H3), 2.85 (ddd, 1H, $J_1 = 4.0, J_2 = 9.9, J_3 = 12.3$ Hz, H1), 3.13–3.25 (m, 2H, H4 and H6), 3.45–3.55 (m, 1H, H5), **Ring III:** 2.90 (dd, 1H, $J_1 = J_2 = 10.0$ Hz, H3ax), 3.13-3.25 (m, 1H, H4), 3.41 (dd, 1H, $J_1 = 3.7$, $J_2 =$ 10.3 Hz, H2), 3.57-3.69 (m, 2H, H6 and H6'), 3.75-3.81 (m, 1H, H5), 4.90 (d, 1H, J = 3.9 Hz, H1); ¹³C NMR (100 MHz, D₂O) δ 33.5, 34.6, 39.8, 46.3, 48.6, 49.1, 51.5, 53.9, 60.0, 65.7, 68.7, 71.2, 71.5, 71.9, 74.0, 86.6, 87.2, 99.2 (anomeric C), 99.5 (anomeric C), 170.2 (C=O); ESI-MS m/z calcd for C₂₀H₃₉N₈O₁₀ 551.2789, found 551.2782 [M + H]⁺.

6'-N-Glycinyl tobramycin (1b)

Compound **1a** (110 mg, 0.20 mmol) was dissolved in MeOH: $H_2O/1:1$ (3 mL) and catalytically hydrogenated using H_2 and 5% Pd/C (20 mg) as the catalyst at rt for 2 h. The reaction mixture was filtered through Celite and concentrated under reduced pressure. The residue was dissolved in minimal

volume of H₂O and freeze-dried to afford the pure product 1b (80 mg, 84%) as a white powder (MeOH:NH₄OH/3:2, $R_{\rm f}$ 0.54): ¹H NMR (500 MHz, D_2O) δ 3.71–3.77 (m, 2H, CH₂ of *N*-glycinyl), **Ring I:** 1.82–1.97 (m, 1H, H3ax), 2.15 (ddd, 1H, $J_1 = 4.2$, $J_2 = 8.2$, $J_3 = 12.8$ Hz, H3eq), 3.43-3.49 (m, 1H, H6), 3.49-3.61 (m, 2H, H2) and H5), 3.63-3.78 (m, 2H, H4 and H6'), 5.45 (d, 1H, J = 2.8 Hz, H1), **Ring II:** 1.85–2.00 (m, 1H, H2ax), 2.44 (ddd, 1H, $J_1 = 4.3$, $J_2 = 8.4, J_3 = 12.5$ Hz, H2eq), 3.38-3.52 (m, 1H, H3), 3.48-3.52(m, 1H, H1), 3.67-3.79 (m, 2H, H4 and H6), 3.84-3.90 (m, 1H, H5), Ring III: 3.35-3.43 (m, 1H, H3ax), 3.55-3.61 (m, 1H, H4), 3.63-3.68 (m, 1H, H6), 3.70-3.75 (m, 1H, H6'), 3.77-3.83 (m, 1H, H5), 3.78–3.90 (m, 1H, H2), 5.03 (d, 1H, J = 3.4 Hz, H1) (Fig. S1[†]); ¹³C NMR (100 MHz, D₂O) δ 34.7, 35.7, 40.7, 44.1, 49.7, 50.2, 51.0, 54.9, 61.0, 66.6, 69.7, 72.2, 72.6, 72.9, 75.1, 87.7, 88.4, 100.4 (anomeric C), 100.7 (anomeric C), 175.9 (C=O) (Fig. S2⁺); ESI-MS m/z calcd for C₂₀H₄₀N₆O₁₀Na 547.2704, found 547.2695 $[M + Na]^+$; Retention time on HPLC: 3.53 min.

6'-N-CbzAHB tobramycin (1c)

To tobramycin sulfate (1) (300 mg, 0.53 mmol) dissolved in MeCN: $H_2O/2$: 1 (5 mL) were added K_2CO_3 (165 mg, 1.26 mmol) and L-(-)- γ -benzyloxycarbonylamino- α -hydroxybutyric acid (4) (660 mg, 1.89 mmol). The reaction mixture was stirred at rt for 16 h. The progress of the reaction was monitored by TLC (MeOH:NH₄OH/3:2, R_f 0.73). The reaction mixture was concentrated under reduced pressure and further purified by flash chromatography (SiO₂, MeOH \rightarrow MeOH:Et₃N/90:10). The fractions containing the pure product were concentrated under reduced pressure. The residue was dissolved in a minimal volume of H₂O and freeze-dried to afford the pure product 1c (340 mg, 91%) as a white powder: ¹H NMR (500 MHz, D_2O) δ 1.63–1.68 (m, 1H, AHB), 1.80–1.87 (m, 1H, AHB), 3.15–3.22 (m, 2H, AHB), 4.06 (dd, 1H, $J_1 = 4.0$, $J_2 = 8.0$ Hz, AHB), 4.97 (m, 2H, benzyl), 7.23-7.33 (m, 5H, aromatic), Ring I: 1.59-1.72 (m, 1H, H3ax), 2.02 (ddd, 1H, $J_1 = 4.2$, $J_2 = 9.1$, $J_3 = 11.5$ Hz, H3eq), 3.14–3.23 (m, 1H, H2), 3.29-3.36 (m, 1H, H6), 3.37-3.44 (m, 1H, H6'), 3.40-3.45 (m, 1H, H4), 3.59-3.65 (m, 1H, H5), 5.11 (s, 1H, H1), **Ring II:** 1.23 (q, 1H, J = 11.9 Hz, H2ax), 1.95 (ddd, 1H, $J_1 = 4.1$, $J_2 = 9.4, J_3 = 13.0$ Hz, H2eq), 2.77–2.85 (m, 1H, H1), 2.95–3.03 (m, 1H, H3), 3.23–3.32 (m, 2H, H4 and H6), 3.49–3.56 (m, 1H, H5), Ring III: 3.00–3.10 (m, 1H, H3ax), 3.30–3.38 (m, 1H, H4), 3.52-3.58 (m, 1H, H2), 3.61-3.64 (m, 1H, H6), 3.66-3.68 (m, 1H, H6'), 3.75-3.80 (m, 1H, H5), 4.91 (d, 1H, J = 3.8 Hz, H1); ${}^{13}C$ NMR (100 MHz, D₂O) δ 23.9, 32.5, 34.1, 37.2, 40.2, 49.4, 49.9, 55.2, 59.7, 60.9, 65.9, 67.5, 68.5, 69.8, 71.0, 72.9, 73.2, 74.9, 86.1, 87.2, 98.0 (anomeric C), 100.8 (anomeric C), 128.3, 129.0, 129.5, 159.0 (C=O), 177. 5 (C=O); ESI-MS m/z calcd for $C_{30}H_{51}N_6O_{13}$ 703.3514, found 703.3510 [M + H]+.

6'-N-AHB tobramycin (1d)

Compound 1c (34 mg, 0.05 mmol) was dissolved in MeOH: $H_2O/1:1$ (2 mL) and catalytically hydrogenated using H_2 and 5% Pd/C (20 mg) as the catalyst at rt for 2 h. The reaction mixture was filtered through Celite and concentrated under reduced pressure. The residue was dissolved in minimal volume of H_2O and freeze-dried to afford the pure product 1d (27 mg, 95%) as a white powder (MeOH:NH₄OH/3:2, R_f 0.38):

¹H NMR (500 MHz, D₂O) δ 1.75–1.88 (m, 1H, AHB), 2.04 (m, 1H, AHB), 3.00–3.16 (m, 2H, AHB), 3.93–4.01 (q, 1H, J = 4.0 Hz, AHB), **Ring I:** 1.84–1.95 (m, 1H, H3ax), 2.17 (ddd, 1H, $J_1 = 3.9$, $J_2 = 8.4$, $J_3 = 12.8$ Hz, H3eq), 3.37-3.45 (m, 1H, H6'), 3.49-3.57(m, 2H, H5 and H6), 3.49-3.69 (m, 1H, H2), 3.74-3.80 (m, 1H, H4), 5.43 (d, 1H, J = 2.7 Hz, H1), **Ring II:** 1.80–1.95 (m, 1H, H2ax), 2.41-2.45 (m, 1H, H2eq), 3.36-3.53 (m, 2H, H1 and H3), 3.68-3.79 (m, 2H, H4 and H6), 3.78-3.87 (m, 1H, H5), Ring III: 3.35-3.44 (m, 1H, H3ax), 3.55-3.64 (m, 1H, H4), 3.64-3.70 (m, 1H, H6), 3.70-3.77 (m, 1H, H6'), 3.78-3.87 (m, 1H, H5), 3.79-3.87 (m, 1H, H2), 5.03 (d, 1H, J = 3.0 Hz, H1) (Fig. S4[†]); ¹³C NMR (100 MHz, D₂O) δ 28.4, 29.9, 31.6, 37.3, 47.4, 48.6, 49.3, 50.4, 55.6, 59.2, 60.5, 64.8, 66.1, 68.8, 70.1, 73.5, 74.5, 79.3, 84.2, 95.3 (anomeric C), 101.2 (anomeric C), 176.7 (C=O) (Fig. S5⁺); ESI-MS m/z calcd for C₂₂H₄₅N₆O₁₁ 569.3146, found 569.3146 [M + H]⁺; Retention time on HPLC: 4.89 min.

6^{'''}-N-Azidoacetyl paromomycin (2a)

To paromomycin sulfate (2) (200 mg, 0.28 mmol) dissolved in MeCN:H₂O/2:1 (5 mL) were added K₂CO₃ (80 mg, 0.56 mmol) and azidoacetyl-N-hydroxysuccinimiyl ester (3) (82 mg, 0.42 mmol). The reaction mixture was stirred at rt for 16 h. The progress of the reaction was monitored by TLC (MeOH:NH₄OH/3:2, R_f 0.64). The reaction mixture was concentrated under reduced pressure and further purified by flash chromatography (SiO₂, MeOH \rightarrow MeOH:Et₃N/90:10). The fractions containing the pure product were concentrated under reduced pressure. The residue was dissolved in a minimal volume of H₂O and freeze-dried to afford the pure product 2a (109 mg, 67%) as white powder: ¹H NMR (500 MHz, D₂O) δ 3.45–3.51 (m, 2H, CH₂ of azidoacetyl), Ring I: 3.34 (m, 1H, H2), 3.36–3.39 (m, 1H, H4), 3.60-3.66 (m, 1H, H6), 3.69-3.71 (m, 1H, H5), 3.80-3.90 (m, 2H, H3 and H6'), 5.74 (d, 1H, J = 3.3 Hz, H1), Ring II: 1.83 $(ddd, 1H, J_1 = J_2 = J_3 = 12.7 Hz, H2ax), 2.43 (dt, 1H, J_1 = 4.1, J_2 =$ 12.4 Hz, H2eq), 3.28-3.30 (m, 1H, H1), 3.45-3.51 (m, 1H, H3), 3.69-3.71 (m, 1H, H5), 3.80-3.90 (m, 1H, H6), 3.97-4.01 (m, 1H, H4), Ring III: 3.60–3.66 (m, 1H, H5), 3.80–3.90 (m, 1H, H5'), 4.12 (m, 1H, H4), 4.25 (m, 1H, H2), 4.32 (t, 1H, J = 5.2 Hz, H3), 5.31 (s, 1H, H1), **Ring IV:** 3.45–3.51 (m, 1H, H2), 3.57–3.60 (m, 1H, H4), 3.69-3.71 (m, 1H, H6), 3.80-3.90 (m, 1H, H6'), 3.97-4.01 (m, 1H, H5), 4.16 (m, 1H, H3), 5.12 (s, 1H, H1); ¹³C NMR (100 MHz, D₂O) δ 28.8, 40.4, 49.5, 50.4, 51.7, 52.6, 54.6, 59.2, 61.1, 67.1, 68.4, 69.4, 70.0, 73.0, 73.3, 74.3, 74.5, 76.5, 77.9, 82.3, 84.9, 96.4 (anomeric C), 96.7 (anomeric C), 110.5 (anomeric C), 171.4 (C=O); ESI-MS m/z calcd for C₂₅H₄₆N₈O₁₅Na 721.3004, found 721.2994 [M + Na]⁺.

6^{'''}-N-Glycinyl paromomycin (2b)

Compound **2a** (35 mg, 0.05 mmol) was dissolved in MeOH: H₂O/1:1 (2 mL) and catalytically hydrogenated using H₂ and 5% Pd/C (20 mg) as the catalyst at rt for 2 h. The reaction mixture was filtered through Celite and concentrated under reduced pressure. The residue was dissolved in minimal volume of H₂O and freeze-dried to afford the pure product **2b** (27 mg, 82%) as a white powder (MeOH:NH₄OH/3: 2, R_f 0.34): ¹H NMR (500 MHz, D₂O) δ 3.48–3.51 (m, 2H, CH₂ of *N*-glycinyl), **Ring I:** 3.35 (dd, 1H, $J_1 = 3.7$, $J_2 = 10.9$ Hz, H2), 3.41 (d, 1H,

J = 8.8 Hz, H4), 3.64–3.76 (m, 1H, H5), 3.78–3.89 (m, 2H, H3 and H6), 3.99–4.05 (m, 1H, H6'), 5.73 (d, 1H, J = 3.7 Hz, H1), Ring **II:** 1.85 (ddd, 1H, $J_1 = J_2 = J_3 = 12.7$ Hz, H2ax), 2.43 (dt, 1H, $J_1 =$ 4.0, $J_2 = 12.5$ Hz, H2eq), 3.24–3.32 (m, 1H, H1), 3.48–3.54 (m, 1H, H3), 3.64-3.76 (m, 1H, H6), 3.78-3.89 (m, 1H, H5), 3.99-4.05 (m, 1H, H4), **Ring III:** 3.64–3.76 (m, 1H, H5), 3.79–3.89 (m, 1H, H5'), 3.99–4.05 (m, 1H, H4), 4.28 (m, 1H, H2), 4.37 (t, 1H, J = 5.7 Hz, H3), 5.30 (s, 1H, J = 2.1 Hz, H1), Ring IV: 3.48–3.54 (m, 2H, H2 and H6), 3.64–3.76 (m, 1H, H4), 3.72–3.76 (m, 1H, H6'), 3.97-4.05 (m, 1H, H5), 4.16 (m, 1H, H3), 5.14 (s, 1H, H1) (Fig. S7[†]); ¹³C NMR (125 MHz, D₂O) δ 27.5, 39.2, 40.0, 48.4, 49.2, 50.6, 53.4, 59.8, 59.9, 65.8, 66.7, 67.1, 68.3, 68.8, 71.6, 72.0, 73.1, 73.3, 75.4, 77.0, 83.6, 95.3 (anomeric C), 95.5 (anomeric C), 109.3 (anomeric C), 166.9 (C=O) (Fig. S8^{\dagger}); ESI-MS m/z calcd for $C_{25}H_{48}N_6O_{15}Na$ 695.3075, found 695.3080 [M + Na]⁺; Retention time on HPLC: 3.55 min.

6^{'''}-N-CbzAHB paromomycin (2c)

To paromomycin sulfate (2) (200 mg, 0.28 mmol) dissolved in MeCN: $H_2O/2$: 1 (4 mL) were added K_2CO_3 (80 mg, 0.56 mmol) and L-(-)- γ -benzyloxycarbonylamino- α -hydroxybutyric acid (4) (150 mg, 0.42 mmol). The reaction mixture was stirred at rt for 16 h. The progress of the reaction was monitored by TLC (MeOH:NH₄OH/3:2, R_f 0.72). The reaction mixture was concentrated under reduced pressure and further purified by flash chromatography (SiO₂, MeOH \rightarrow MeOH:Et₃N/90:10). The fractions containing the pure product were concentrated under reduced pressure. The residue was dissolved in a minimal volume of H₂O and freeze-dried to afford the pure product 2c (179 mg, 75%) as a white powder: ¹H NMR (500 MHz, D_2O) δ 1.68 (m, 1H, AHB), 1.84 (m, 1H, AHB), 3.10-3.12 (m, 2H, AHB), 4.06 (dd, 1H, $J_1 = 4.0$, $J_2 = 7.8$ Hz, AHB), 4.97 (m, 2H, benzyl), 7.29 (m, 5H, aromatic), Ring I: 2.71-2.73 (m, 1H, H2), 3.20-3.27 (m, 1H, H4), 3.49–3.58 (m, 2H, H3 and H6), 3.59–3.66 (m, 1H, H5), 3.66–3.70 (m, 1H, H6'), 5.25 (d, 1H, J = 3.5 Hz, H1), Ring II: 1.13-1.15 (m, 1H, H2ax), 1.87-1.90 (m, 1H, H2eq), 2.71-2.74 (m, 1H, H1), 3.20–3.27 (m, 1H, H5), 3.34–3.36 (m, 1H, H6), 3.48 (m, 1H, H3), 3.59-3.66 (m, 1H, H4), Ring III: 3.50-3.58 (m, 1H, H5), 3.70-3.72 (m, 1H, H5'), 3.99 (m, 1H, H4), 4.09 (m, 1H, H2), 4.19 (t, 1H, J = 5.5 Hz, H3), 5.20 (d, 1H, J = 2.8 Hz, H1), Ring IV: 2.94(m, 1H, H2), 3.47 (m, 1H, H4), 3.50-3.53 (m, 1H, H6), 3.55-3.59 (m, 1H, H6'), 3.70-3.72 (m, 1H, H5), 3.91 (m, 1H, H3), 4.80 (s, 1H, H1); ¹³C NMR (125 MHz, D₂O) δ 22.6, 32.7, 35.9, 39.1, 49.5, 49.7, 51.6, 54.4, 58.3, 60.0, 60.5, 66.2 (benzyl), 67.2, 68.8, 69.1, 69.3, 71.6, 72.1, 72.5, 73.0, 74.3, 75.1, 75.5, 81.1, 81.8, 83.6, 98.0 (anomeric C), 98.1 (anomeric C), 108.0 (anomeric C), 127.1, 127.8, 128.2, 135.9, 157.0 (C=O), 176.0 (C=O); ESI-MS m/z calcd for $C_{35}H_{59}N_6O_{18}$ 851.3886, found 851.3891 [M + H]⁺.

6^{'''}-N-AHB paromomycin (2d)

Compound **2c** (32 mg, 0.04 mmol) was dissolved in MeOH: $H_2O/1:1$ (2 mL) and catalytically hydrogenated using H_2 and 5% Pd/C (20 mg) as the catalyst at rt for 2 h. The reaction mixture was filtered through Celite and concentrated under reduced pressure. The residue was dissolved in minimal volume of H_2O and freeze-dried to afford the pure product **2d** (25 mg, 92%) as a white powder (MeOH:NH₄OH/3:2, R_f 0.45):

¹H NMR (500 MHz, D_2O) δ 1.75–1.88 (m, 1H, AHB), 2.04 (m, 1H, AHB), 3.00-3.16 (m, 2H, AHB), 3.93-4.01 (m, 1H, AHB), **Ring I:** 3.32 (t, 1H, J = 4.6 Hz, H2), 3.35–3.38 (m, 1H, H4), 3.57– 3.64 (m, 2H, H5 and H6), 3.75-3.85 (m, 2H, H3 and H6'), 5.66 (d, 1H, J = 4.9 Hz, H1), Ring II: 1.75–1.88 (m, 1H, H2ax), 2.37 (dt, 1H, $J_1 = 5.1$, $J_2 = 15.5$ Hz, H2eq), 3.22 (m, 1H, H1), 3.41–3.47 (m, 1H, H3), 3.59-3.62 (m, 1H, H6), 3.78-3.80 (m, 1H, H5), 3.85-4.01 (m, 1H, H4), Ring III: 3.57-3.64 (m, 1H, H5), 3.75-3.85 (m, 1H, H5'), 4.06–4.09 (m, 1H, H4), 4.19 (dd, 1H, $J_1 = 3.5$, $J_2 = 6.0$ Hz, H2), 4.29 (t, 1H, J = 6.5 Hz, H3), 5.26 (d, 1H, J = 3.5 Hz, H1), Ring IV: 3.44 (m, 1H, H2), 3.59 (m, 1H, H4), 3.59–3.62 (m, 1H, H6), 3.69 (m, 1H, H6'), 3.75–3.82 (m, 1H, H5), 4.11 (t, 1H, J = 7.2 Hz, H3), 5.10 (d, 1H, J = 1.8 Hz, H1) (Fig. S10[†]); ¹³C NMR (125 MHz, D₂O) δ 28.7, 31.6, 37.3, 41.0, 49.6, 50.9, 51.7, 54.5, 61.0, 61.2, 67.2, 68.3, 69.5, 70.0, 70.1, 72.8, 73.1, 73.8, 75.9, 76.7, 78.2, 82.5, 84.7, 96.5 (anomeric C), 96.7 (anomeric C), 110.5 (anomeric C), 176.5 (C=O) (Fig. S11^{\dagger}); ESI-MS m/z calcd for C₂₇H₅₃N₆O₁₆ 717.3518, found 717.3519 [M + H]⁺; Retention time on HPLC: 5.42 min.

Transformation of AAC(6')-APH(2") into B. subtilis 168

Competent B. subtilis 168 cells were prepared by growing spores in SPI medium (Spizizen salts ((NH₄)₂SO₄ (15 mM), K₂HPO₄ (80 mM), KH₂PO₄ (44 mM), Na citrate (3.5 mM)) with 0.5% glucose, 0.2% yeast extract, and 0.25% Casamino acids) (30 °C, overnight, 200 rpm). SPI medium (3 mL) was then inoculated with the overnight culture (3 µL) and grown (37 °C, 200 rpm, 5 h). The entire 3 mL culture was added to SPII medium (SPI medium with an additional CaCl₂ (0.5 mM) and MgSO₄ (2.5 mM)) (27 mL) and grown (37 °C, 200 rpm, 90 min). Cells were pelleted and the supernatant was removed, reserving 1.6 mL for resuspension. Cells were resuspended in the saved supernatant supplemented with 50% glycerol (400 µL). The pRB374 plasmid harboring the AAC(6')-APH(2") gene (10 µL) was transformed into freshly prepared competent B. subtilis 168 cells (100 µL) by incubating the cells with SPII medium containing EGTA (2 mM) (100 µL) (37 °C, 20 min, gentle rocking).

Determination of MIC values of the *N*-acylated aminoglycoside analogues

MIC values reported in Table 1 were determined against *S. epidermis* ATCC12228 (A), *B. subtilis* ATCC6633 (B), *E. coli* BL21 (DE3) (C), *E. coli* BL21 (DE3) with AAC(6')-APH(2'')-pET22b (D), *E. coli* BL21 (DE3) with AAC(3)-IV-Int-pET19b pps (E), *B. subtilis* 168 (F), *B. subtilis* 168 AAC(6')-APH(2'')-pRB374 (G), *E. coli* TolC (H), and *S. aureus* NorA (I). Strains were tested by using the double-dilution method, with 1024 μ g mL⁻¹ starting concentration with strains A and B, 125 μ g mL⁻¹ starting concentration with strains C–G, and 250 μ g mL⁻¹ starting concentration with strains H and I. All experiments were performed in triplicate and analogous results were obtained in two different sets of experiments.

Determination of AAC and APH activity on the *N*-acylated aminoglycoside analogues

To determine the reactivity of our novel *N*-acylated aminoglycoside derivatives with acetyl-CoA and AAC(6')-APH(2")(CHis)

and AAC(3)-IV(NHis) from Int-pET19b-pps plasmid, reactions were monitored for AAC activity using the previously described spectrophotometric assay.8 Briefly, reactions (200 µL) containing MES buffer pH 6.6 (50 mM), DTDP (2 mM), acetyl-CoA (80 µM), and enzyme (0.5 µM for AAC(6')-APH(2") and 0.25 µM for AAC(3)-IV or 6 μ g and 1.5 μ g, respectively) were initiated by addition of the aminoglycoside derivative (40 µM). The reactions were incubated at 37 °C for AAC(6')-APH(2") and 25 °C for AAC(3)-IV and were monitored for CoASH production by taking readings at 324 nm every 30 s for 30 min. The reactivity of the aminoglycoside derivatives was also tested for APH activity using a well-established enzyme-coupled assay.17 Briefly, reactions (250 µL) containing HEPES pH 8.0 (50 mM), MgCl₂ (10 mM), KCl (40 mM), NADH (0.5 mg mL⁻¹), phosphoenolpyruvate (2.5 mM), GTP (1 mM), lactic dehydrogenase (0.9–1.4 U mL⁻¹), pyruvate kinase (0.6-1 U mL⁻¹), were initiated by addition of APH(2") (2.5 µM). Reactions were incubated at 37 °C and monitored at 340 nm for NADH consumption taking measurements every 30 s for 30 min. Results are represented in Fig. S13[†].

Determination of kinetic parameters for 2b

The kinetic parameters for compound **2b** were determined in reactions (200 µL) containing acetyl-CoA (80 µM), aminoglycoside **2b** (0, 3.1, 6.3, 12.5, 25, and 50 µM), DTDP (2 mM), and AAC(3)-IV (0.125 µM) at 25 °C in MES pH 6.6 (50 mM). Reactions were initiated by the addition of the aminoglycoside derivative and were carried out in triplicate. The kinetic parameters, $K_{\rm m}$ (45.0±1.1µM) and $k_{\rm cat}$ (0.86±0.09 s⁻¹) were determined using Lineweaver–Burke plots (not shown).

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References

- 1 J. L. Houghton, K. D. Green, W. Chen and S. Garneau-Tsodikova, *ChemBioChem*, 2010, **11**, 880–902.
- 2 J. Davies, Rev. Infect. Dis., 1979, 1, 23-29.
- 3 S. Shakil, R. Khan, R. Zarrilli and A. U. Khan, J. Biomed. Sci., 2008, 15, 5–14.
- 4 G. De Pascale and G. D. Wright, *ChemBioChem*, 2010, **11**, 1325–1334.
- 5 H. Kawaguchi, T. Naito, S. Nakagawa and K. I. Fujisawa, J. Antibiot. (Tokyo), 1972, 25, 695–708.
- 6 D. Ikeda, T. Tsuchiya, S. Umezawa and H. Umezawa, J. Antibiot. (Tokyo), 1972, 25, 741–742.
- 7 T. Deushi, I. Watanabe, A. Iwasaki, K. Kamiya, T. Mizoguchi, M. Nakayama, M. Okuchi, H. Itoh and T. Mori, *J. Antibiot. (Tokyo)*, 1981, **34**, 811–817.
- 8 K. D. Green, W. Chen, J. L. Houghton, M. Fridman and S. Garneau-Tsodikova, *ChemBioChem*, 2010, 11, 119–126.
- 9 X. Yan, F. Gao, S. Yotphan, P. Bakirtzian and K. Auclair, *Bioorg. Med. Chem.*, 2007, **15**, 2944–2951.
- 10 F. Gao, X. Yan, O. M. Baettig, A. M. Berghuis and K. Auclair, Angew. Chem., Int. Ed., 2005, 44, 6859–6862.
- 11 S. N. Hobbie, P. Pfister, C. Brull, E. Westhof and E. C. Bottger, Antimicrob. Agents Chemother., 2005, 49, 5112–5118.
- 12 Q. Vicens and E. Westhof, Chem. Biol., 2002, 9, 747-755
- 13 S. Sicsic, J. F. Le Bigot, C. Vincent, C. Cerceau and F. Le Goffic, J. Antibiot. (Tokyo), 1982, 35, 574–579.
- 14 Q. Vicens and E. Westhof, Structure, 2001, 9, 647-658.
- 15 L. Chen, M. Hainrichson, D. Bourdetsky, A. Mor, S. Yaron and T. Baasov, *Bioorg. Med. Chem.*, 2008, 16, 8940–8951.
- 16 H. Yu, R. Karpel and X. Chen, Bioorg. Med. Chem., 2004, 12, 6427– 6435.
- 17 D. M. Daigle, D. W. Hughes and G. D. Wright, *Chem. Biol.*, 1999, 6, 99–110.
- 18 E. Chaslus-Dancla, J. L. Martel, C. Carlier, J. P. Lafont and P. Courvalin, Antimicrob. Agents Chemother., 1986, 29, 239– 243.
- 19 M. L. Magalhaes and J. S. Blanchard, *Biochemistry*, 2005, 44, 16275– 16283.
- 20 F. Wojciechowski, M. Suchy, A. X. Li, H. A. Azab, R. Bartha and R. H. Hudson, *Bioconjugate Chem.*, 2007, 18, 1625–1636.
- 21 T. Naito, N. Susumu and M. Oka, Patent #3,860,574, USA.