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CuAAC-mediated diversification of aminoglycoside–arginine conjugate mimics by non-reducing di- and trisaccharides

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ABSTRACT

Di- and triguanidinylation of trehalose, sucrose, and melizitose has been achieved via a Huisgen-cycloaddition approach. They can serve as aminoglycoside–arginine conjugate mimics, which has been demonstrated by their biological profiles in assays against *Bacillus subtilis*. For comparative studies, tetraguanidinylated neamine and kanamycin derivatives have also been synthesized and evaluated.

1. Introduction

The search for new strategies to face bacterial infections is an everlasting battle due to the ability of these organisms to efficiently adjust to current antibiotic treatments. Among the therapeutics which inhibit the prokaryotic protein biosynthesis, polycationic aminoglycoside antibiotics (e.g., neamine **1**, kanamycin **2**, streptomycin **4**, neomycin **5**, and paromomycin **8**, see Fig. 1) are among the most well established ones.[1] Computational and crystallographic studies suggest that the aminoglycosides bind to the highly negatively charge RNA regions and prevent the productive binding of divalent metal ions (Fig. 2A).[2] Other aminoglycoside targets are RNA/RNA binding protein (RBP) complexes, which play a vital role in regulative processes such as nuclear RNA-export, RNA-localization, translation, and degradation.[3] The binding of the RBP to its holoenzyme, which differs in large for pro- and eukaryotic systems, occurs at arginine-rich sequences. This recognition can thus be inhibited very efficiently by the polycationic aminoglycosides (see, Fig. 2B). In this context it could be shown, that arginine–aminoglycoside conjugates (AACs) can enhance the inhibition of prokaryotic transfer RNA maturation by large extent, preventing the recognition of the peptidic and RNA-subdomain.[4,5]

In a seminal paper series by Lapidot and Litovchik it was demonstrated that guanidinylated aminoglycosides **3**, **6**, and **7** can discriminate between different pro- and eukaryotic RNAs making them suitable for inhibiting the prokaryotic counterpart exclusively.[6] Among these, the arginine-modified congeners **3** and **7** displayed better inhibition rates compared to **6**, which has been ascribed to the higher flexibilities of the guanidinylated side arms.

In order to diversify these AACs, we set out to explore a two-directional strategy for the production of AAC mimics. To this end, we explored the possibility of employing disaccharide and trisaccharide scaffolds as core units for the attachment of guanidinylated side chains. The attachment of the cationic side chains was envisioned to be accomplished by a copper-catalyzed azide-alkyne cycloaddition (CuAAC) approach starting from suitable azide-containing aminoglycosides and alkyne-modified guanidine (Fig. 3). To the best of our knowledge, this approach has not yet been applied in the diversification of AACs. Our strategy entailed the use of two non-reducing disaccharides (i.e., sucrose and trehalose), a non-reducing trisaccharide (melizitose), and aminoglycosides neamine (**1**) and kanamycin **2**. To validate the activity of these modified AACs, a preliminary evaluation against *Gram*-positive and *Gram*-negative bacteria was performed.

2. Results and discussion

As shown by various research groups, the scaffold of the natural occurring aminoglycosides (e.g., neamine **1** resp. neomycin **5**, and kanamycin **2**) can be significantly changed while retaining their biological activities.[7] In 2000, Lapidot and Litovchik were able to demonstrate that arginine-modified aminoglycosides can increase

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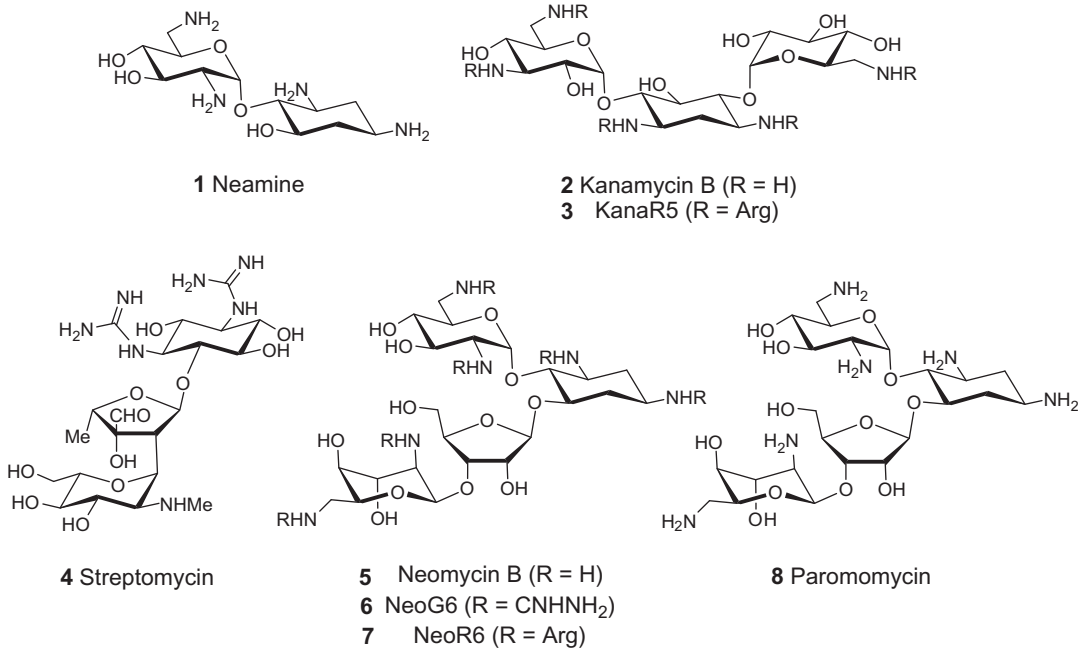


Figure 1. Structures of commonly used aminoglycoside antibiotics.

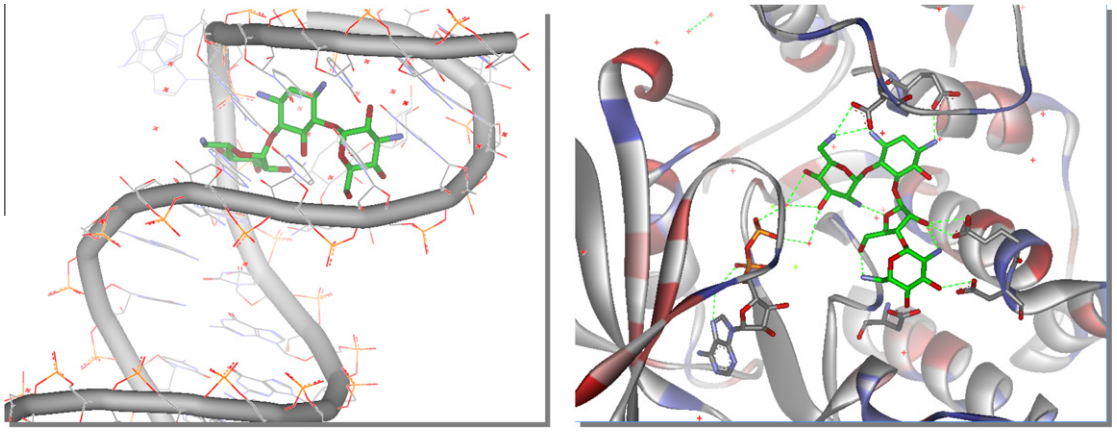


Figure 2. (A) Left: complex between kanamycin A (green) and the 16S-rRna A Site (gray). PDB-ID: 2ESI[5] (B) right: Crystal structure of aminoglycoside phosphotransferase IIIa (gray) with ADP and neomycin B (green) complexed. PDB-ID: 2B0Q[5]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

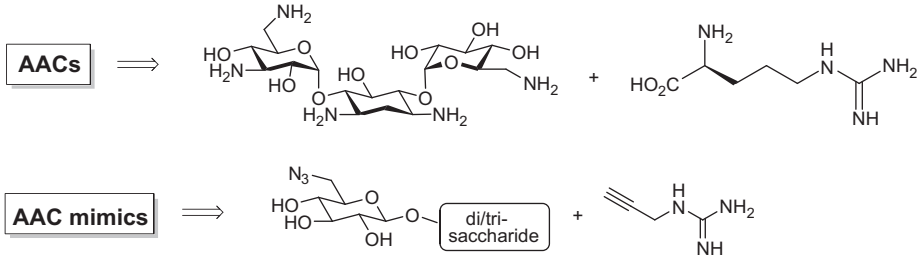
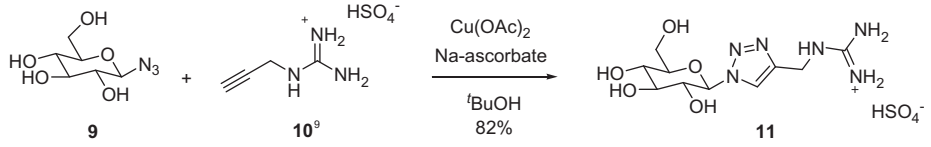


Figure 3. Strategy for the synthesis of AAC mimics.

the antibiotic activity up to 500 fold.[6b] Utilizing peptide coupling conditions up to six arginine residues could be attached to the tetracyclic framework of neomycin (**5**) yielding hexa-argininylated neomycin **7** (NeoR6). Prompted by these results, we envisioned the application of non-reducing di- and trisaccharide scaffolds for the attachment of multiple guanidine residues. The CuAAC reaction was envisioned as preferred ligation method for the coupling of the different building blocks, as a result of the generally obtained high yields, as well as the formation of a triazole ring which is considered an isostere for an amide group.[8] To establish a suitable



9 10[9] 11

Scheme 1. Synthesis of guanidinylated glucose **11**.

protocol with a minimum amount of protection/deprotection steps we commenced with the formation of triazole **11** starting from 1-azido glucose 9 and alkyne **10** (Scheme 1).[9]

The reaction went smoothly under classical CuAAC conditions, to afford the coupled product **11** in 82% yield. This encouraging result forecasts that this protocol can most likely be used for the straightforward synthesis of ACC mimics circumventing laborious protection and deprotection steps of both the carbohydrate and the guanidine moiety.

Continuing our studies, we used non-reducing trehalose **12**, sucrose **17**, and melizitose **21** as carbohydrate moieties, which required no further treatment of the anomeric center. The primary hydroxyl groups of these carbohydrates can be readily transformed into the corresponding di- and triazides **13**, **18**, and **22**, respectively, by direct azidation of the unprotected carbohydrates with $PPh_3/CCl_4/NaN_3$ according to the published protocols (Scheme 2).[10] Surprisingly, no substitution of the primary (neopentyl) hydroxyl groups to the corresponding azides at C-1 of the fructose moieties of sucrose and melizitose could be detected.

An initial attempt to react alkyne **10** with trehalose diazide **13** turned out to be sluggish, leading to the formation of multiple products according to TLC-analysis. To our satisfaction, changing to the Boc-protected guanidine derivative **14**,[11] resulted in the desired triazole **15** in a good yield (91%). Subsequent deprotection of compound **15** with trifluoroacetic acid afforded the bis-guanidine compound **16** which could be easily isolated as its trifluoroacetate salt in 79% yield.

This protocol could also be applied for the synthesis of the protected di- and tri-guanidinylated sucrose and melizitose derivatives **19** (quant.) and **23** (73%), respectively. Therefore, this protocol can be considered robust for a variety of carbohydrate scaffolds. The deprotection of the Boc groups on compounds **19** and **23** was carried out under similar conditions as described above. The overall yields for these two-step processes were 61% and 63% for **20** and **24**, respectively.

In order to compare the biological data of known AACs with the here reported AAC mimics we additionally prepared aminoglycoside derivatives **27** and **30** (Scheme 3) which allows for the direct comparison with aminoglycosides **1** and **3**. To this end, we used tetraazides **26** and **29**, which can be efficiently generated from neomycin **5** and kanamycin **2**.[12,13] Following the two step CuAAC/deprotection protocol, as described above, the corresponding tetraguanidinylated derivates **27** and **30** could be obtained in reasonable to good overall yields.

Biological evaluation of the guanidinylated compounds revealed no activity against *Gram*-negative bacteria (*Vibrio fischeri* assay[14]). However, pronounced activities could be observed against *Gram*-positive bacteria (*Bacillus subtilis* assay[15]), as depicted in Table 1. The activity against the *Gram*-positive bacterial strain *Bacillus subtilis* was measured by the percentage of growth inhibition. All AAC mimics were examined at concentrations of 1 and 10 μM using kanamycin as the standard.

As can be deduced from the obtained data, at a substrate concentration of 10 μM all AAC mimics show a lower activity as compared to unsubstituted kanamycin (**5**). Moreover, at a concentration of 1 μM, AAC mimics **27** (neamine-based), **16**(trehalose-based), and **24** (melizitose-based) all showed significantly lower activity compared to kanamycin. Surprisingly, at a substrate concentration of 1 μM, both the kanamycin-based AAC mimic **30** and the sucrose-based AAC mimic **20**, showed slightly increased activities in comparison with kanamycin. This might suggest a different mode of binding for the AAC mimics compared to the aminoglycosides. In addition, these results indicate that different carbohydrates can be utilized as suitable backbones for the orientation of the guanidine groups, however, the biological activity is not dependent on the number of guanidine groups attached. Further synthetic efforts to provide new AAC derivatives with increased activities are currently underway. Moreover, modeling studies to gain more insights into the orientation of the guanidine groups on the AACs are currently being conducted in our laboratories.

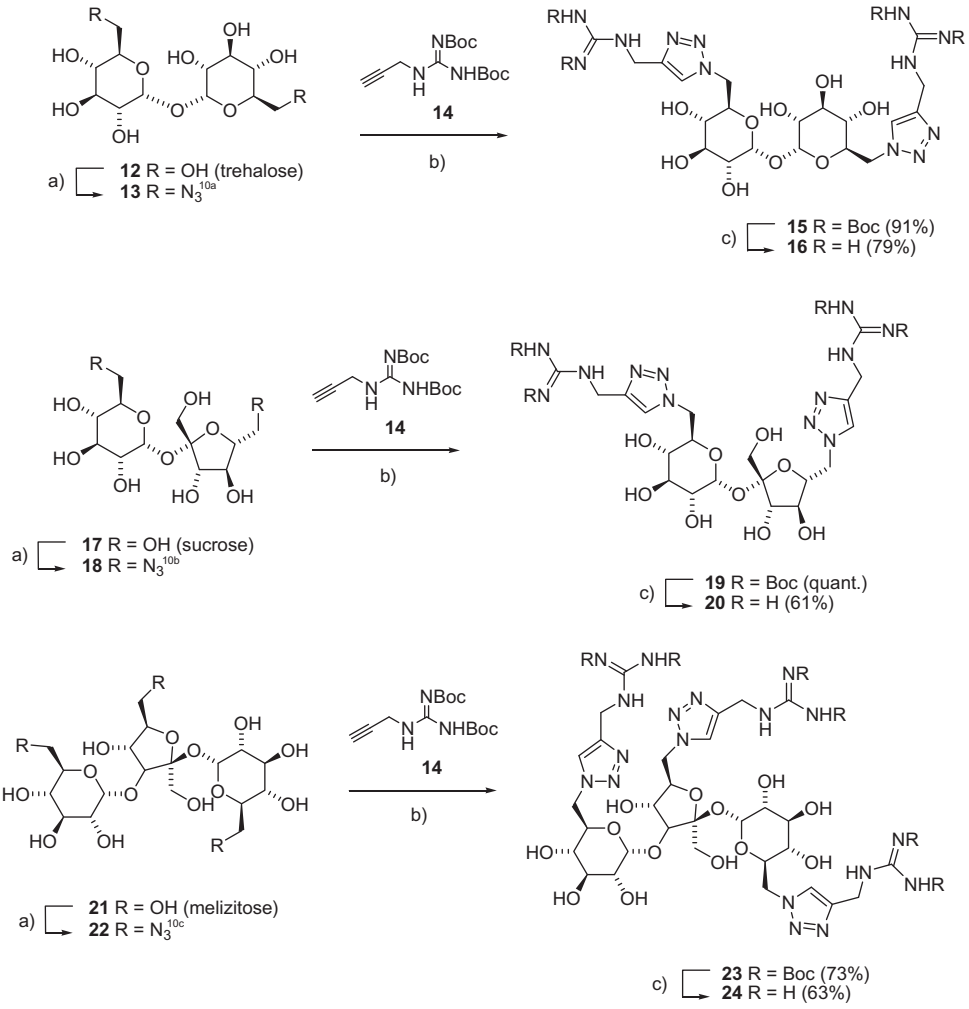
3. Experimental section

3.1. General experimental information

Unless otherwise stated all chemicals and solvents were obtained from commercial sources and were used without further purification. All ^1H and ^{13}C NMR spectra were recorded in either $CDCl_3$, methanol-d_4, or D_2O on either a 300 MHz Varian MERCURY-VX 300 apparatus (300 MHz for 1H NMR and 75 MHz for ^{13}C NMR, respectively) or on a 400 MHz Varian MERCURY-VX 400 apparatus (400 MHz for 1H NMR and 100 MHz for ^{13}C NMR, respectively). Chemical shifts are reported in δ values (ppm) with tetramethylsilane (TMS) as internal standard. ESI-MS spectra were obtained from an API-150EX spectrometer. HRMS spectra were recorded on an FT-ICR Bruker Apex III 70e mass spectrometer. Purification of the crude products by column chromatography was performed on silica gel 60 (230–400 mesh, 0.040–0.063 mm), Merck, Germany. TLC identification of products and reactants was performed on silica gel coated aluminum foil (silica gel 60 F254 with fluorescence indicator), Merck, Germany. The antibacterial activity against *Bacillus subtilis* was determined using a fluorescence-based antibacterial growth inhibition assay. The fluorescence was measured on a GENios Pro plate reader (Fa. Tecan, excitation, 510 nm; emission, 535 nm). The *Bacillus subtilis* strain 168 (P_{AbrB}-IYFP) [9] was maintained on TY medium (tryptone-yeast extract) supplemented with 1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCl, and chloramphenicol (5 μg ml^{-1}).

3.1.1. *tert*-Butyl {(1*E*)-{[(1-{[(2*R*,3*S*,4*S*,5*R*,6*R*)-6-({(2*R*,3*R*,4*S*,5*S*,6*R*)-6-[(4-{[*N*′,*N*″-bis(*tert*-butoxycarbonyl)carbamimidamido]methyl}-1*H*-1,2,3-triazol-1-yl)methyl]-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl}oxy)-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl]methyl}-1*H*-1,2,3-triazol-4-yl)methyl]amino}[(*tert*-butoxycarbonyl)amino] methylidene}carbamate (15) *General Procedure A (GP-A)*

Diazide **13** (98 mg, 0.20 mmol), *N*,*N*′-di-(*tert*-butyloxycarbonyl)guanidine **14** (148 mg, 0.50 mmol), a freshly prepared 0.08 M aq sodium ascorbate solution (2.00 mL, 0.16 mmol), and a 0.04 M aq copper(II) acetate solution (2.00 mL, 0.08 mmol) in *tert*-butanol (10 mL) were combined and stirred vigorously at room temperature. After completion of the reaction indicated by



Scheme 2. Synthesis of AAC mimics (**16**, **20**, and **24** isolated as trifluoroacetate salts). Reagents: (a) NaN_3, PPh_3, CCl_4; (b) Na-ascorbate, $Cu(OAc)_2$, ^tBuOH; (c) TFA, DCM.

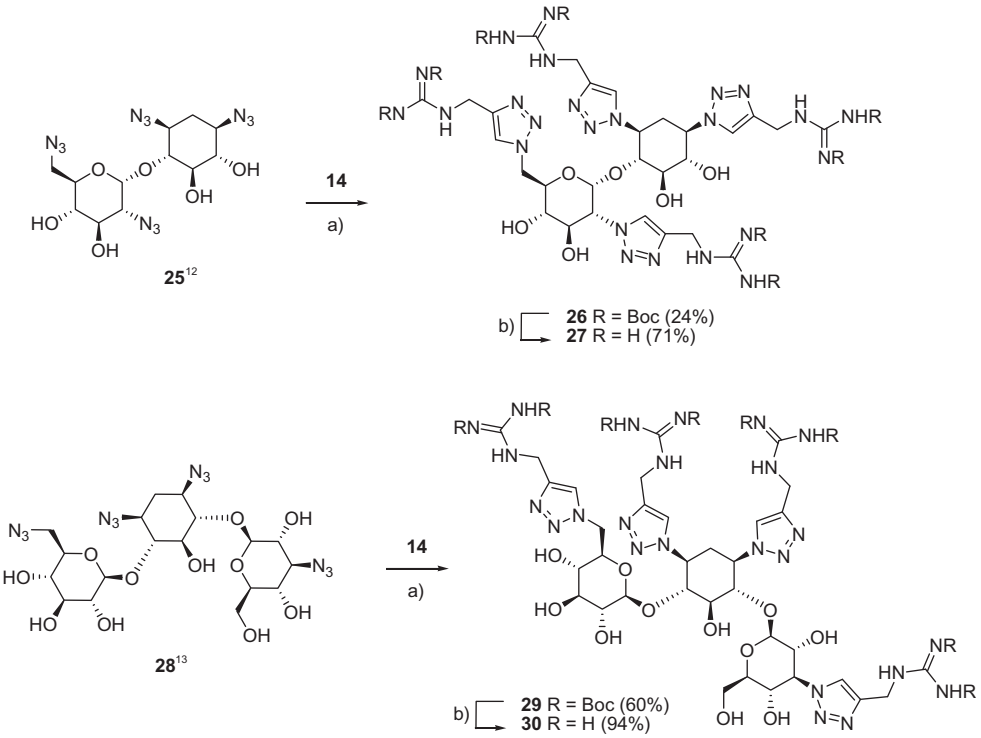
TLC analysis all volatiles were removed in vacuo. Purification by column chromatography using a gradient of ethyl acetate/ethanol/water 70:30:0 > 70:15:8 afforded **15** as colorless solid (180 mg, 91%). Mp >248 °C (decomposition); R_F = 0.40 (ethyl acetate/ethanol/water = 70:15:8); $[\alpha]_D^{23}$ +42.6 (*c* = 0.15, MeOH); ^{1}H NMR (300 MHz, methanol-d_4) δ = 1.41–1.54 (m, 36H), 3.08 (t, *J* = 9.3 Hz, 2H), 3.31 (dt, *J* = 3.3, 1.6 Hz, 4H), 3.35 (dd, *J* = 9.5, 3.7 Hz, 2H), 3.71 (t, *J* = 9.3 Hz, 2H), 4.22 (t, *J* = 7.7 Hz, 2H), 4.46–4.57 (m, 2H), 4.64 (m, 4H), 4.69–4.79 (m, 4H), 7.99 (s, 2H) ppm; ^{13}C NMR (75 MHz, methanol-d_4) δ = 28.3, 28.6, 37.0, 52.4, 71.8, 72.8, 74.5, 80.5, 84.7, 96.1, 154.0, 157.4, 164.4 ppm; ESI-MS *m*/*z*: 987.6 $[M+H]^+$, 1009.8 $[M+Na]^+$; HRMS (ESI) calcd for $C_{40}H_{66}N_{12}O_{17}$-Na 1009.4561 $[M+Na]^+$, found 1009.4569.

3.1.2. 6-[4-({[Amino(iminio)methyl] amino}methyl)-1*H*-1,2,3-triazol-1-yl]-6-deoxy-α-D-glucopyranosyl 6-[4-({[amino(iminio)methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]-6-deoxy-α-D-glucopyranoside bis(trifluoroacetate) (16) *General Procedure B (GP-B)*

Compound **15** (170 mg, 0.17 mmol) was treated with dichloromethane/trifluoracetic acid (5:1 v/v, 6 mL) and was stirred at room temperature until TLC analysis indicated completion of the reaction. All volatiles were removed under reduced pressure. Diguanidinylated **16** was obtained as white solid (110 mg, 79%) after purification by column chromatography (RP18, H_2O). M.p. 132-134 °C; $[\alpha]_D^{27}$ +70.8 (*c* = 0.12, MeOH); ^{1}H NMR (300 MHz, methanol-d_4) δ = 3.09 (t, *J* = 9.3 Hz, 2H), 3.31 (dt, *J* = 3.3, 1.6 Hz, 4H), 3.73 (t, *J* = 9.3 Hz, 2H), 4.13–4.22 (m, 2H), 4.49 (s, 4H), 4.55 (dd, *J* = 14.5, 7.5 Hz, 2H), 4.69 (d, *J* = 3.7 Hz, 2H), 4.71–4.79 (m, 2H), 7.97 (s, 2H) ppm; ^{13}C NMR (75 MHz, methanol-d_4) δ = 37.5, 52.3, 71.9, 72.8, 74.4, 95.9, 111.4, 125.5, 144.1, 158.8 ppm; ESI-MS *m*/*z*: 294.0 $[M+2H]^{2+}$, 587.3 $[M+H]^+$; HRMS (ESI) calcd for $C_{20}H_{35}N_{12}O_9$ 587.2644 $[M+H]^+$, found 587.2652.

3.1.3. *tert*-Butyl {(1*E*)-{[(1-{[(2*R*,3*S*,4*S*,5*R*,6*R*)-6-{[(2*S*,3*S*,4*S*,5*R*)-5-[(4-{[*N*′,*N*″-bis(*tert*-butoxycarbonyl)carbamimidamido] methyl}-1*H*-1,2,3-triazol-1-yl)methyl]-3,4-dihydroxy-2-(hydroxymethyl)tetrahydrofuran-2-yl]oxy}-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl]methyl}-1*H*-1,2,3-triazol-4-yl)methyl]amino}[(*tert*-butoxycarbonyl)amino] methylidene}carbamate (19)

Diazide **18** (98 mg, 0.25 mmol), alkyne **14** (0.186 mg, 0.63 mmol), 0.08 M aq sodium ascorbate solution (2.50 mL, 0.20 mmol), and 0.04 M aq copper(II) acetate solution (2.50 mL, 0.10 mmol) in *tert*-butanol (10 mL) were reacted according to GP-A. Purification by column chromatography using a gradient of ethyl acetate/ethanol/water 70:30:0 > 70:20:10 afforded compound **19** as colorless oil (252 mg, quant.). R_F = 0.37 (ethyl acetate/ethanol/water = 70:20:10); $[\alpha]_D^{27}$ +18.6 (*c* = 0.13, MeOH); ^{1}H NMR (400 MHz, methanol-d_4) δ = 1.44–1.54 (m, 36H), 3.16 (t, *J* = 9.5 Hz, 1H), 3.31 (dt, *J* = 3.3, 1.6 Hz, 2H), 3.41–3.47 (m, 2H), 3.50–3.56 (m, 1H), 3.68–3.76 (m, 2H), 3.80 (t, *J* = 7.9 Hz, 1H),



Scheme 3. Synthesis of guanidyl–aminoglycoside conjugates (**27** and **30** isolated as trifluoroacetate salts). Reagents: (a) Na-ascorbate, $Cu(OAc)_2$, ^{t}BuOH; (b) TFA, DCM.

Table 1
Activity of AAC-mimics against *Bacillus subtilis*

	Inhibition (%) (c = 1 μM)	Inhibition (%) (c = 10 μM)
Kanamycin	40.9 (±18.1)	99.3 (±9.4)
16	16.4 (±7.7)	30.9 (±9.6)
20	48.1 (±14.8)	54.1 (±11.7)
24	28.1 (±8.6)	49.4 (±12.6)
27	24.9 (±5.4)	36.7 (±10.3)
30	50.2 (±10.3)	57.9 (±7.9)

3.90–3.98 (m, 1H), 4.05–4.08 (m, 1H), 4.31 (dd, J = 14.1, 2.3 Hz, 1H), 4.35–4.42 (m, 1H), 4.45–4.57 (m, 3H), 4.67 (s, 2H), 4.84 (dd, J = 14.4, 2.1 Hz, 2H), 5.32 (d, J = 3.5 Hz, 1H), 7.86 (s, 1H), 7.93 (s, 1H) ppm; ^{13}C NMR (75 MHz, methanol-d_4) δ = 28.3, 28.6, 36.7, 37.1, 53.2, 54.2, 63.6, 72.8, 73.0, 73.3, 74.1, 77.5, 78.7, 80.5, 81.3, 84.6, 93.7, 105.9, 125.3, 126.0, 145.2, 145.4, 154.0, 157.4; 164.4, 164.5 ppm; ESI-MS m/z: 985.9 $[M-H]^-$, 987.7 $[M+H]^+$, 1009.9 $[M+Na]^+$; HRMS (ESI) calcd for $C_{40}H_{66}N_{12}O_{17}Na$ 1009.4561 $[M+2H]^{2+}$, found 1009.4555.

3.1.4. 6-[4-({[Amino(iminio)methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]-6-deoxy-α-D-fructofuranosyl 6-[4-({[amino(iminio)methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]-6-deoxy-α-D-glucopyranoside bis(trifluoroacetate) (20)

Compound **19** (50 mg, 50.7 μmol) was treated with dichloromethane/trifluoracetic acid (5:1 v/v, 6 mL) as described in GP-B. After purification by column chromatography (RP18, H_2O), Deprotected compound **20** was obtained as a colorless oil (21 mg, 61%). $[\alpha]_D^{28}$ +14.1 (c = 0. 30, MeOH); ^{1}H NMR (300 MHz, D_2O) δ = 3.29 (t, J = 9.4 Hz, 1H), 3.45–3.63 (m, 3H), 3.81 (t, J = 8.8 Hz, 2H), 4.01–4.12 (m, 1H), 4.16 (d, J = 8.5 Hz, 1H), 4.27–4.39 (m, 2H), 4.47 (s, 2H), 4.53–4.58 (m, 3H), 4.62 (dd, J = 11.4, 8.2 Hz, 1H), 4.69 (d, J = 7.9 Hz, 1H), 5.37 (d, J = 3.5 Hz, 1H), 7.96 (s, 1H), 8.08 (s, 1H) ppm; ^{13}C NMR (75 MHz, D_2O) δ = 37.1, 37.2, 52.7, 53.6, 62.2, 71.7, 72.2, 72.4, 73.0, 76.5, 77.0, 79.7, 93.2, 105.2, 115.4, 119.3, 125.5, 126.1, 144.4, 158.0, ppm; ESI-MS m/z: 294.4 $[M+2H]^{2+}$; HRMS (ESI) calcd for $C_{20}H_{35}N_{12}O_9$ 587.2644 $[M+H]^+$, found 587.2642.

3.1.5. *tert*-Butyl {(1*Z*)-{[(1-{[(2*R*,3*S*,4*S*,5*R*,6*R*)-6-{[(2*S*,4*R*,5*R*)-5-[(4-{[*N*′,*N*″-bis(*tert*-butoxy carbonyl)carbamimidamido]methyl}-1*H*-1,2,3-triazol-1-yl)methyl]-3-({(2*R*,4*S*,5*S*,6*R*)-6-[(4-{[*N*′,*N*″-bis(*tert*-butoxycarbonyl)carbamimidamido]methyl}-1*H*-1,2,3-triazol-1-yl)methyl]-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl}oxy)-4-hydroxy-2-(hydroxymethyl)tetrahydrofuran-2-yl]oxy}-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl]methyl}-1*H*-1,2,3-triazol-4-yl)methyl]amino}[(*tert*-butoxycarbonyl)amino] methylidene}carbamate (23)

Triazide **22** (115 mg, 0.20 mmol), alkyne **14** (208 mg, 0.70 mmol), 0.08 M aq sodium ascorbate solution (3.00 mL, 0.24 mmol), and 0.04 M aq copper(II) acetate solution (3.00 mL, 0.12 mmol) in *tert*-butanol (20 mL) were reacted according to GP-A. Purification by column chromatography using a gradient of ethyl acetate/ethanol/water 70:30:0 > 70:15:8 afforded **23** as white solid (215 mg, 73%). Mp >275 °C (decomposition); R_F = 0.52 (ethyl acetate/ethanol/water = 70:15:8); $[\alpha]_D^{27}$ +23.8 (c = 0.13, MeOH); ^{1}H NMR (300 MHz, methanol-d_4) δ = 1.35–1.63 (m, 54H), 3.06 (t, J = 9.5 Hz, 1H), 3.18 (t, J = 9.3 Hz, 1H), 3.27–3.33 (m, 6H), 3.34–3.52 (m, 3H), 3.71 (t, J = 9.3 Hz, 1H), 3.77–4.04 (m, 3H), 4.12 (d, J = 6.2 Hz, 1H), 4.24 (d, J = 9.5 Hz, 1H), 4.31–4.53 (m, 4H), 4.53–4.70 (m, 4H), 4.75 (br. s., 2H), 4.82 (br. s., 1H), 5.09 (d, J = 3.7 Hz, 1H), 5.34 (d, J = 3.7 Hz, 1H), 7.81–8.08 (m, 3H) ppm; ^{13}C NMR (75 MHz, methanol-d_4) δ = 28.2, 28.3, 28.6, 36.8, 37.0, 37.1, 52.1, 53.1, 53.9, 64.0, 72.0, 72.4, 72.8, 73.3, 74.5, 74.6, 76.8, 80.5, 82.4, 84.3, 84.5, 84.6, 93.7, 101.1, 106.8, 154.0, 157.4, 164.4 ppm; ESI-MS m/z: 1494.3 $[M+Na]^+$; HRMS (ESI) calcd for $C_{60}H_{98}N_{18}O_{25}Na$ 1493.6842 $[M+Na]^+$, found 1493.6806.

3.1.6. (2ξ)-6-[4-({[Amino(iminio)methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]-6-deoxy-α-D-*arabino*-hexopyranosyl-(1→3)-(3ξ)-6-[4-({[amino(iminio)methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]-6-deoxy-α-D-*erythro*-hex-2-ulofuranosyl 6-[4-({[amino(iminio) methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]-6-deoxy-α-D-glucopyranoside tris(trifluoroacetate) (24)

Compound **23** (180 mg, 0.18 mmol) was treated with dichloromethane/trifluoracetic acid (5:1 v/v, 12 mL) as described in GP-B. After purification by column chromatography (RP18, H_2O), deprotected compound **24** was obtained as white foam (139 mg, 63%). Mp 133–135 °C; $[\alpha]_D^{27}$ +31.3 (c = 0.14, MeOH); ^{1}H NMR (300 MHz, D_2O) δ = 3.11–3.22 (m, 3H), 3.30 (t, J = 9.5 Hz, 1H), 3.52 (dd, J = 10.2, 3.7 Hz, 1H), 3.56–3.62 (m, 1H), 3.72 (t, J = 9.3 Hz, 1H), 3.80 (t, J = 9.5 Hz, 1H), 3.87–3.97 (m, 1H), 3.97–4.18 (m, 4H), 4.27–4.44 (m, 2H), 4.47–4.57 (m, 6H), 4.62–4.72 (m, 1H), 4.73–4.77 (m, 1H), 4.85–4.92 (m, 2H), 5.16 (d, J = 3.7 Hz, 1H), 5.33 (d, J = 3.7 Hz, 1H), 7.98 (s, 1H), 8.03 (s, 1H), 8.09 (s, 1H) ppm; ^{13}C NMR (75 MHz, D_2O) δ = 37.1, 37.2, 49.9, 51.9, 52.6, 53.4, 62.5, 71.5, 71.7, 72.2, 73.5, 75.8, 79.9, 83.6, 93.1, 101.0, 105.2, 111.6, 115.4, 119.3, 123.2, 125.5, 126.2, 126.5, 144.1, 144.3, 144.4, 157.9, 158.0 ppm; ESI-MS m/z: 291.2 $[M+3H]^{3+}$, 436.7 $[M+2H]^{2+}$, 871.5 $[M+H]^+$; HRMS (ESI) calcd for $C_{30}H_{52}N_{18}O_{13}$ 436.1975 $[M+2H]^{2+}$, found 436.1971.

3.1.7. *tert*-Butyl {(1*E*)-{[(1-{(2*R*,3*R*,4*R*,5*S*,6*R*)-2-{[(1*R*,2*R*,3*S*,4*R*,6*S*)-4,6-bis(4-{[*N*′,*N*″-bis(*tert*-butoxycarbonyl)carbamimidamido]methyl}-1*H*-1,2,3-triazol-1-yl)-2,3-dihydroxy-cyclohexyl]oxy}-6-[(4-{[*N*′,*N*″-bis(*tert*-butoxycarbonyl)carbamimidamido] methyl}-1*H*-1,2,3-triazol-1-yl)methyl]-4,5-dihydroxytetrahydro-2*H*-pyran-3-yl}-1*H*-1,2,3-triazol-4-yl)methyl]amino}[(*tert*-butoxycarbonyl)amino]methylidene}carbamate (26)

Tetraazido neamine **25** (188 mg, 0.44 mmol), alkyne **14** (594 mg, 2.00 mmol), 0.08 M aq sodium ascorbate solution (9.2 mL, 0.72 mmol), and 0.04 M aq copper(II) acetate solution (9.2 mL, 0.36 mmol) in *tert*-butanol (20 mL) were reacted according to GP-A. Purification by column chromatography using a gradient of dichloromethane/methanol 100:0 > 95:5) afforded compound **26** as white solid (170 mg, 24%). Mp >315 °C (decomposition); R_F = 0.18 (dichloromethane/methanol = 95:5); $[\alpha]_D^{25}$ +21.7 (c = 0.13, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.34–1.56 (m, 73H), 2.12–2.34 (m, 1H), 2.51–2.62 (m, 1H), 2.80–3.04 (m, 1H), 3.19–3.32 (m, 1H), 3.88–4.00 (m, 1H), 4.10–4.43 (m, 5H), 4.44–4.87 (m, 10H), 5.82–5.94 (m, 1H), 7.60–7.95 (m, 4H), 8.68–8.93 (m, 4H), 11.34–11.50 (m, 4H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ = 28.0, 28.3, 79.4, 79.5, 79.6, 80.2, 83.3, 83.5, 83.6, 152.9, 153.0, 155.9, 156.0, 156.1, 156.2, 163.1, 163.3 ppm; ESI-MS m/z: 1617.0 $[M+H]^+$, 1638.0 $[M+Na]^+$; HRMS (ESI) calcd for $C_{68}H_{110}N_{24}O_{22}Na$ 1637.8119 $[M+Na]^+$, found 1637.8105.

3.1.8. (1*R*,2*R*,3*S*,4*R*,6*S*)-4,6-Bis[4-({[amino(iminio)methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]-2,3-dihydroxycyclohexyl 2,6-bis[4-({[amino(iminio)methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]-2,6-dideoxy-α-D-glucopyranoside tetrakis(trifluoroacetate) (27)

Compound **26** (160 mg, 0.10 mmol) was treated with dichloromethane/trifluoracetic acid (5:1 v/v, 6 mL) as described in GP-B. After purification by column chromatography (RP18, MeOH/H_2O = 30:70), deprotected compound **27** was obtained as a white solid (90 mg, 71%). Mp 135–136 °C; $[\alpha]_D^{27}$ +20.6 (c = 0.12, MeOH); ^{1}H NMR (300 MHz, methanol-d_4) δ = 1.95–2.09 (m, 1H), 2.47 (d, J = 13.2 Hz, 1H), 3.01 (t, J = 9.5 Hz, 2H), 3.27–3.33 (m, 8H), 3.82 (t, J = 9.7 Hz, 1H), 3.92–4.04 (m, 1H), 4.11 (t, J = 9.7 Hz, 1H), 4.33–4.69 (m, 12H), 5.86–6.01 (m, 1H), 8.04 (br s, 2H), 8.27 (br s, 2H) ppm; ^{13}C NMR (75 MHz, methanol-d_4) δ = 34.7, 37.5, 37.7, 37.8, 60.5, 61.9, 65.6, 70.9, 71.0, 72.0, 76.2, 78.4, 97.6, 158.9 ppm; ESI-MS m/z: 408.2 $[M+2H]^{2+}$; HRMS (ESI) $C_{28}H_{48}O_6N_{24}$ calcd 408.2089, found 408.2086.

3.1.9. *tert*-Butyl {(1*E*)-[({1-[(2*R*,3*R*,4*S*,5*S*,6*R*)-2-{[(1*S*,2*R*,3*R*,4*S*,6*R*)-4,6-bis(4-{[*N*′,*N*″-bis(*tert*-butoxycarbonyl)carbamimidamido]methyl}-1*H*-1,2,3-triazol-1-yl)-3-({(2*S*,3*R*,4*S*,5*S*,6*R*)-6-[(4-{[*N*′,*N*″-bis(*tert*-butoxycarbonyl)carbamimidamido]methyl}-1*H*-1,2,3-triazol-1-yl)methyl]-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl}oxy)-2-hydroxycyclohexyl]oxy}-3,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-4-yl]-1*H*-1,2,3-triazol-4-yl}methyl)amino][(*tert*-butoxycarbonyl)amino]methylidene} carbamate (29)

Tetraazido kanamycin **28** (88 mg, 0.15 mmol), alkyne **14** (199 mg, 0.67 mmol), 0.08 M aq sodium ascorbate solution (3.00 mL, 0.24 mmol), and 0.04 M aq copper(II) acetate solution (3.00 mL, 0.12 mmol) in *tert*-butanol (10 mL) were reacted according to GP-A. Purification by column chromatography using a gradient of ethyl acetate/ethanol/water 70:30:0 > 70:15:8 afforded compound **29** as a white solid (160 mg, 60%). Mp >295 °C (decomposition); R_F = 0.65 (ethyl acetate/ethanol/water = 70:15:8); $[\alpha]_D^{27}$ +28.4 (c = 0.13, MeOH); ^{1}H NMR (300 MHz, methanol-d_4) δ = 1.37–1.56 (m, 72H), 2.48 (d, J = 3.3 Hz, 1H), 2.69 (t, J = 9.5 Hz, 1H), 2.82–3.04 (m, 1H), 3.13–3.22 (m, 1H), 3.26–3.33 (m, 8H), 3.44–3.56 (m, 1H), 3.63–3.70 (m, 1H), 3.70–3.81 (m, 1H), 3.81–3.91 (m, 1H), 3.91–4.16 (m, 4H), 4.17–4.36 (m, 2H), 4.37–4.54 (m, 2H), 4.56–4.75 (m, 8H), 5.07–5.13 (m, 1H), 7.74–8.09 (m, 4H) ppm; ^{13}C NMR (75 MHz, methanol-d_4) δ = 28.2, 28.3, 28.6, 28.7, 31.1, 37.0, 58.3, 60.8, 61.3, 61.5, 61.7, 68.7, 69.4, 70.8, 70.9, 71.5, 73.4, 74.0, 74.4, 80.4, 80.5, 84.5, 84.6, 84.9, 154.0, 157.3, 157.4, 157.5, 164.4 ppm; ESI-MS m/z: 889.8 $[M+2H]^{2+}$, 1779.0 $[M+H]^+$; HRMS (ESI) calcd for $C_{74}H_{120}N_{24}O_{27}Na_2$ 911.4270 $[M+2Na]^{2+}$, found 911.4256.

3.1.10. (1*S*,2*R*,3*R*,4*S*,6*R*)-4,6-Bis[4-({[amino(iminio)methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]-3-({6-[4-({[amino(iminio)methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]-6-deoxy-β-D-glucopyranosyl}oxy)-2-hydroxycyclohexyl 3-[4-({[amino(iminio)methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]-3-deoxy-β-D-glucopyranoside tetrakis(trifluoroacetate) (30)

Compound **29** (120 mg, 67.5 μmol) was treated with dichloromethane/trifluoracetic acid (5:1 v/v, 6 mL) as described in GP-B. After purification by column chromatography (RP18, H_2O), deprotected compound 30 was obtained as a white solid (91 mg, 94%). Mp 140–142 °C; $[\alpha]_D^{26}$ +33.0 (c = 0.12, MeOH); ^{1}H NMR (300 MHz, methanol-d_4) δ = 2.40–2.51 (m, 1H), 2.54–2.65 (m, 1H), 2.76 (t, J = 9.3 Hz, 1H), 3.09–3.21 (m, 1H), 3.31 (dt, J = 3.3, 1.6 Hz, 8H), 3.41–3.55 (m, 1H), 3.64–3.71 (m, 1H), 3.71–3.81 (m, 2H), 3.86 (dd, J = 11.0, 3.7 Hz, 1H), 3.92–4.07 (m, 2H), 4.13–4.23 (m, 2H), 4.24–4.30 (m, 1H), 4.30–4.35 (m, 1H), 4.36–4.62 (m, 11H), 5.31 (d, J = 3.7 Hz, 1H), 7.88 (s, 1H), 7.99 (s, 1H), 8.04–8.08 (m, 1H), 8.14–8.20 (m, 1H) ppm; ^{13}C NMR (75 MHz, methanol-d_4) δ = 34.6, 37.2, 37.3, 49.9, 51.2, 60.1, 60.8, 61.0, 66.7, 68.2, 69.9, 70.3, 70.5, 70.8, 72.0, 72.9, 73.2, 73.4, 76.4, 81.1, 81.2, 99.1, 99.2, 116.2, 118.5, 125.2, 125.8, 126.3, 144.3, 144.4, 157.9, 158.0, 163.6, 163.9, 164.2, 164.5 ppm; ESI-MS m/z: 326.9 $[M+3H]^{3+}$, 489.7 $[M+2H]^{2+}$; HRMS (ESI) calcd for $C_{34}H_{58}N_{24}O_{11}$ 489.2353 $[M+2H]^{2+}$, found 489.2357.

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