Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications

ELECTRONIC SUPPLEMENTARY INFORMATION

Design of membrane targeting tobramycin-based cationic amphiphiles with reduced hemolytic activity

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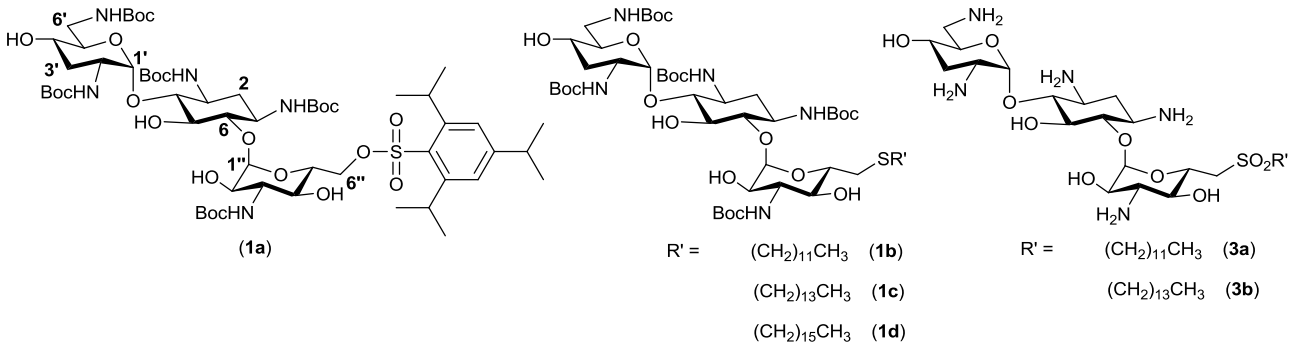
Content:

1. General information.

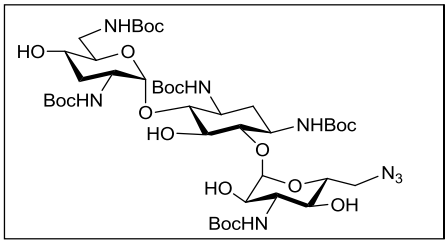
^{1}H NMR spectra (including 1D-TOCSY) were recorded on a Bruker Avance™ 400 spectrometer, and chemical shifts reported (in ppm) were calibrated to CD_3OD (δ = 3.31) with CD_3OD as the solvent, and to HOD (δ =4.63) with D_2O as the solvent. ^{13}C NMR spectra were recorded on a Bruker Avance™ 400 spectrometer at 100.6 MHz. Multiplicities are reported using the following abbreviations: br = broad, s = singlet, d = doublet, dd = doublet of doublets, ddd = doublet of doublet of doublets, t = triplet, dt = doublet of triplets, app. q = appears quartet, m = multiplet, and td = triplet of doublets. Coupling constants (J) are given in Hertz. 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. Chemical reactions were monitored by TLC (Merck, Silica gel 60 F_{254}). Visualization was achieved using a cerium-molybdate stain ($(NH_4)_2Ce(NO_3)_6$ (5 g), $(NH_4)_6Mo_7O_{24}\bullet 4H_2O$ (120 g), H_2SO_4 (80 mL), H_2O (720 mL)). All reactions were carried out under an argon atmosphere with anhydrous solvents, unless otherwise noted. All chemicals unless otherwise stated, were obtained from commercial sources. Tobramycin was bought from Tzamal D-Chem Laboratories Ltd. Compound **1a** was prepared as previously described.[1]

Compounds **1b, 1c, 1d, 3a** and **3b** (Figure S1) were prepared as previously reported.[2] Compounds were purified flash chromatography (SiO_2, Merck, Kieselgel 60). *S. pyogenes* serotype M12 (strain MGAS9429) (**A**), methicillin-resistant *S. aureus* (MRSA) (**B**), and vancomycin-resistant *Enterococcus* (VRE) (**D)** were a gift from Prof. Itzhak Ofek (Faculty of Medicine, Tel Aviv University). *S. mutans* UA159 (**C**) was a gift from Prof. Doron Steinberg (Faculty of Dental Medicine, The Hebrew University of Jerusalem). *E. faecalis* ATCC29212 (**E**), *S.aureus* ATCC9144 Oxford strain (**F**), *S. epidermidis* ATCC35984 (**G)**, *S. epidermidis* ATCC12228 (**H),** and *P. aeruginosa* ATCC33347 (**I**) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *S. sonnei* clinical isolate 6831(O-antigen positive) (**J**), and *S. sonnei* clinical isolate 6831 (O-antigen negative) (**K**) were a gift from Prof. Dani Cohen (School of Public Health, Tel Aviv University).

Figure S1: Tobramycin-based precursors and ring numbering system demonstrated for compound **1a**.

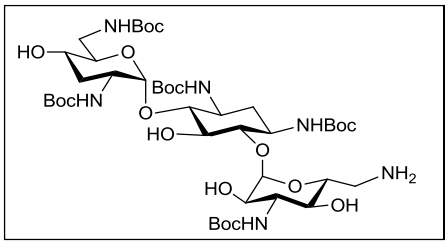


2. Synthetic procedures.

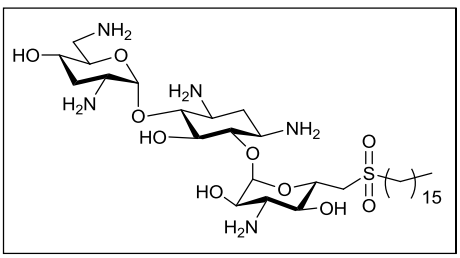


Compound 1e: Compound **1e** was prepared as previously reported[3] with the following changes: Compound **1a** (2.3 gr, 1.9 mmol) in DMF (12 mL) was added sodium azide (197 mg, 3.0 mmol) and the reaction was stirred under argon at 75°C overnight. Propagation of the reaction was monitored by TLC (EtOAc/petroleum ether: 7/3). Upon completion, the solvent was removed by evaporation under reduced pressure; the crude was re-

dissolved in EtOAc (80 mL), washed twice with brine (2x60 mL), dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification by flash column chromatography (SiO_2, EtOAc: petroleum ether) afforded compound **1e** as a white solid (1.68, 91%); LR-ESI *m/z* calc'd for $C_{43}H_{76}N_8O_{18}$ 992.53, found 994.07 $[M+H]^+$.

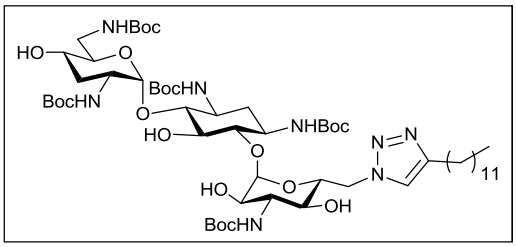


Compound 1f: Compound **1e** (470 mg, 0.47 mmol) was dissolved in THF (2 mL), NaOH 0.01M (100 µL) and stirred at ambient temperature for 10 minutes after which PMe_3 (1M solution in THF, 0.5 mL, 0.50 mmol) was added to the reaction mixture. Propagation of the reaction was monitored by TLC (EtOAc/petroleum ether: 7/3). Upon completion, the solvent was removed by evaporation under reduced pressure, and the crude was purified by flash column chromatography (SiO_2, MeOH: CH_2Cl_2) to afford compound **1f** (366 mg, 80%) as a white solid: ^{1}H NMR (400 MHz, D_2O) (Fig. S2) δ 5.10 (br s, 2H, H-1', H-1"), 3.95 (ddd, J_1 = 9.8 Hz, J_2 = 7.0 Hz, J_3 = 3.0 Hz, 1H, H-5"), 3.69 (t, J = 10.2Hz, 1H, H-3"), 3.65-3.35 (m, 11H, H-1, H-3, H-4, H-5, H-6, H-2', H-4', H-5', H-6'(2H), H-2"), 3.18 (t, J = 9.8 Hz, 1H, H-4"), 3.02 (br d, J = 13.0 Hz, 1H, H-6"), 2.70 (dd, J_1 = 13.5 Hz, J_2 = 6.9 Hz, 1H, H-6"), 2.10 (br d, J = 10.6 Hz, 1H, H-2eq), 2.01 (m, 1H, H-3'eq), 1.65 (app. q, J_1 = J_2 = J_3 = 12.1 Hz, 1H, H-3'ax), 1.52-1.40 (m, 46H, H-2ax, 5x$CO_2C(C\underline{H}_3)_3$); ^{13}C NMR (100 MHz, CD_3OD) (Fig. S3) δ 159.5, 159.3, 157.9, 157.8, 99.4 (anomeric C), 99.3 (anomeric C), 82.8, 80.7, 80.5, 80.4, 80.2, 76.9, 74.1, 73.5, 72.1, 71.5, 66.4, 57.2, 51.6, 51.1, 51.0, 43.5, 42.0, 35.9, 34.3, 28.9, 28.8; LR-ESI *m/z* calc'd for $C_{43}H_{78}N_6O_{18}$ 966.54, found 967.94 $[M+H]^+$.



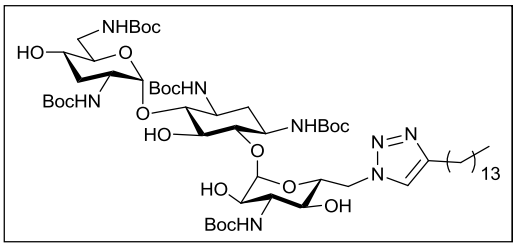
Compound 3c: Compound **1d** (100 mg, 0.08 mmol) in $CHCl_3$ (3 mL) was treated with *m*-chloroperbenzoic acid (70-75%) (54 mg, ~0.31 mmol), and stirred at ambient temperature under argon atmosphere. Propagation of the reaction was monitored by ESI-

MS by following the disappearance of the starting material ($[M+H]^+$, *m/z* 1209.43) and the formation of the corresponding sulfone ($[M+H]^+$, *m/z* 1241.15). Upon completion, the reaction mixture was diluted with $CHCl_3$ (15 mL), washed twice with a 1M water solution of KOH (5 mL), concentrated under reduced pressure, and treated with 99% TFA (1.0 mL) for 3 min after which reaction mixture was evaporated under reduced pressure. The product was dissolved in a minimal volume of H_2O and freeze-dried to afford the TFA salt of compound **3c** as a white foam (89 mg, 82%). ^{1}H NMR (400 MHz, D_2O) (Fig. S4) δ 5.57 (d, *J* = 3.4 Hz, 1H, H-1'), 4.97 (d, *J* = 3.2 Hz, 1H, H-1''), 4.28 (t, *J* = 9.2 Hz, 1H, H-5''), 3.91-3.76 (m, 4H, H-4, H-5, H-5', H-2''), 3.67 (t, *J* = 9.0 Hz, 1H, H-6), 3.60-3.35 (m, 7H, H-1, H-3, H-2', H-4', H-3'', H-4'', H-6''), 3.33-3.23 (m, 2H, H-6', H-6''), 3.08 (m, 2H, $SO_2C\underline{H}_2(CH_2)_{14}CH_3$), 3.02 (dd, J_1 = 13.5 Hz, J_2 = 7.8 Hz, 1H, H-6'), 2.43 (m, 1H, H-2eq), 2.16 (m, 1H, H-3'eq), 1.86 (m, 2H, H-2ax, H-3'ax), 1.62 (m, 2H, $SO_2CH_2(C\underline{H}_2)_{14}CH_3$), 1.26 (m, 2H, $SO_2CH_2(C\underline{H}_2)_{14}CH_3$), 1.22-1.07 (m, 24H, $SO_2CH_2(C\underline{H}_2)_{14}CH_3$), 0.74 (t, 3H, *J* = 6.9 Hz, $SO_2CH_2(CH_2)_{14}C\underline{H}_3$); ^{13}C NMR (100 MHz, D_2O) (Fig. S5) δ 162.8 (q, *J* = 35 Hz, $CF_3\underline{C}O_2H$), 116.4 (q, *J* = 290 Hz, $\underline{C}F_3CO_2H$), 100.6 (anomeric C), 94.8 (anomeric C), 84.4, 77.6, 74.6, 70.2, 67.9, 67.7, 64.9, 54.52, 54.46, 52.4, 49.3, 48.2, 48.0, 40.2, 31.7, 29.9, 29.52, 29.49, 29.3, 29.2, 29.1, 28.7, 28.5, 27.9, 27.8, 22.5, 21.7, 13.7; HRESI-MS *m/z* calc'd for $C_{34}H_{69}N_5O_{10}SNa$ 762.4663, found 762.4659 $[M+Na]^+$.



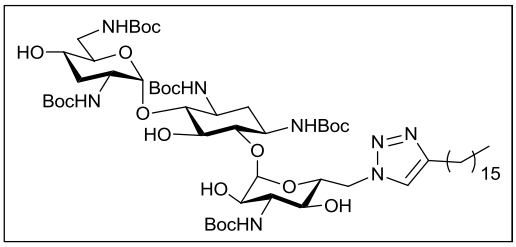
***NH*Boc-protected 4a:** Compound **1e** (151 mg, 0.15 mmol), in DMF (1 mL), was added $CuSO_4 \cdot 5H_2O$ (3.6 mg, 0.01 mmol), sodium ascorbate (5.0 mg, 0.03 mmol) and 1-tetradecyne 90% (75 µL, 0.30 mmol). The reaction mixture was irradiated by a microwave for 4 min. Propagation of the reaction was monitored by TLC (EtOAc/petroleum ether: 7/3) and upon completion, reaction mixture was diluted with EtOAc (10 mL) and the organic layer was washed twice with brine (2x20 mL). The aqueous layer was extracted again with EtOAc (10 mL) and the combined organic layers were dried over anhydrous $MgSO_4$ and

concentrated under reduced pressure. Purification by flash column chromatography (SiO_2, EtOAc: petroleum ether) afforded the corresponding *NH*Boc-protected **4a** as a white solid (171 mg, 95%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.75 (s, 1H, triazole ring), 5.11 (d, *J* = 3.3 Hz, 1H, H-1"), 5.06 (br s, 1H, H-1'), 4.68 (br d, *J* = 14.0 Hz, 1H, H-6"), 4.55 (m, 1H, H-6"), 4.42 (m, 1H, H-5"), 3.74 (t, *J* = 10.0 Hz, H-3"), 3.64-3.27 (m, 11H, H-1, H-3, H-4, H-5, H-6, H-2', H-4', H-5', H-6' (2H), H-2"), 2.99 (t, *J* = 9.8 Hz, 1H, H-4"), 2.68 (t, *J* = 7.7 Hz, 2H, $C_2HN_3C\underline{H}_2(CH_2)_{10}CH_3$), 2.04 (m, 2H, H-2eq, H-3'eq), 1.66 (m, 3H, H-3'ax, $C_2HN_3CH_2(C\underline{H}_2)_{10}CH_3$), 1.47-1.28 (m, 64H, H-2ax, $5xCO_2C(C\underline{H}_3)_3$, $C_2HN_3CH_2(C\underline{H}_2)_{10}CH_3$), 0.90 (t, *J* = 7.0 Hz, 3H, $C_2HN_3CH_2(CH_2)_{10}C\underline{H}_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 159.4, 159.2, 157.9, 157.7, 149.0 (triazole ring), 124.6 (triazole ring), 99.8 (anomeric C), 98.8 (anomeric C), 83.2, 81.7, 80.7, 80.5, 80.4, 80.2, 76.5, 73.6, 71.8, 71.7, 70.4, 66.5, 57.1, 51.7, 51.3, 42.1, 36.1, 34.3, 33.1, 31.8, 30.8, 30.6, 30.5, 30.4, 28.9, 28.84, 28.80, 26.4, 23.7, 14.4; LR-ESI *m/z* calc'd for $C_{57}H_{102}N_8O_{18}$ 1186.73, found 1188.27 $[M+H]^+$.

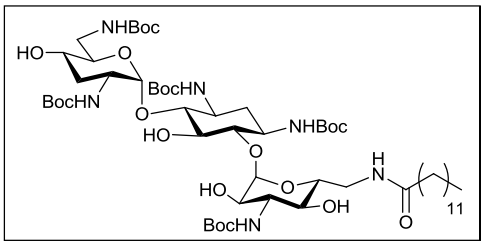


***NH*Boc-protected 4b:** Compound **1e** (200 mg, 0.20 mmol), $CuSO_4 \cdot 5H_2O$ (5 mg, 0.02 mmol), sodium ascorbate (9 mg, 0.05 mmol), DMF (1.5 mL), 1-hexadecyne 90% (120 μL, 0.43 mmol). Purification by flash column chromatography (SiO_2, EtOAc:petroleum ether) afforded the corresponding *NH*Boc-protected **4b** as a white solid (212 mg, 87%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.75 (s, 1H, triazole ring), 5.11 (d, *J* = 3.3 Hz, 1H, H-1"), 5.06 (br s, 1H, H-1'), 4.69 (br d, *J* = 13.9 Hz, 1H, H-6"), 4.55 (m, 1H, H-6"), 4.42 (m, 1H, H-5"), 3.74 (t, *J* = 9.9 Hz, H-3"), 3.64-3.27 (m, 11H, H-1, H-3, H-4, H-5, H-6, H-2', H-4', H-5', H-6' (2H), H-2"), 2.99 (t, *J* = 9.8 Hz, 1H, H-4"), 2.68 (t, *J* = 7.8 Hz, 2H, $C_2HN_3C\underline{H}_2(CH_2)_{12}CH_3$), 2.04 (m, 2H, H-2eq, H-3'eq), 1.66 (m, 3H, H-3'ax, $C_2HN_3CH_2(C\underline{H}_2)_{12}CH_3$), 1.47-1.28 (m, 68H, H-2ax, $5xCO_2C(C\underline{H}_3)_3$, $C_2HN_3CH_2(C\underline{H}_2)_{12}CH_3$), 0.90 (t, *J* = 7.0 Hz, 3H, $C_2HN_3CH_2(CH_2)_{12}C\underline{H}_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 159.4, 159.2, 157.8, 157.7, 149.0 (triazole ring), 124.6 (triazole ring), 99.8 (anomeric C),

98.8 (anomeric C), 83.1, 81.8, 80.7, 80.4, 80.3, 80.2, 76.5, 73.6, 71.8, 71.6, 70.4, 66.5, 57.0, 51.7, 51.2, 42.1, 36.0, 34.3, 33.0, 31.8, 30.8, 30.6, 30.5, 30.4, 30.3, 28.9, 28.84, 28.80, 26.4, 23.7, 14.5; LR-ESI *m/z* calc'd for $C_{59}H_{106}N_8O_{18}$ 1214.76, found 1216.27 $[M+H]^+$.

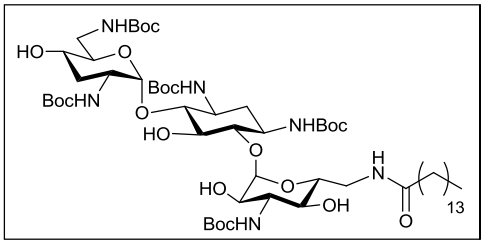


***NH*Boc-protected 4c:** Compound **1e** (150 mg, 0.15 mmol), $CuSO_4 \cdot 5H_2O$ (2 mg, 0.01 mmol), sodium ascorbate (3 mg, 0.02 mmol), DMF (1 mL), 1-octadecyne >95% (100 μL, 0.32 mmol). Purification by flash column chromatography (SiO_2, EtOAc:petroleum ether) afforded the corresponding *NH*Boc-protected **4c** as a white solid (170 mg, 90%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.75 (s, 1H, triazole ring), 5.11 (d, *J* = 3.3 Hz, 1H, H-1"), 5.06 (br s, 1H, H-1'), 4.69 (br d, *J* = 13.9 Hz, 1H, H-6"), 4.55 (m, 1H, H-6"), 4.42 (m, 1H, H-5"), 3.74 (t, *J* = 10.0 Hz, H-3"), 3.66-3.26 (m, 11H, H-1, H-3, H-4, H-5, H-6, H-2', H-4', H-5', H-6' (2H), H-2"), 2.99 (t, *J* = 9.9 Hz, 1H, H-4"), 2.68 (t, *J* = 7.8 Hz, 2H, $C_2HN_3C\underline{H}_2(CH_2)_{14}CH_3$), 2.04 (m, 2H, H-2eq, H-3'eq), 1.66 (m, 3H, H-3'ax, $C_2HN_3CH_2(C\underline{H}_2)_{14}CH_3$), 1.48-1.26 (m, 72H, H-2ax, $5xCO_2C(C\underline{H}_3)_3$, $C_2HN_3CH_2(C\underline{H}_2)_{14}CH_3$), 0.90 (t, *J* = 7.0 Hz, 3H, $C_2HN_3CH_2(CH_2)_{14}C\underline{H}_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 159.4, 159.2, 157.8, 157.7, 148.9 (triazole ring), 124.6 (triazole ring), 99.8 (anomeric C), 98.8 (anomeric C), 83.1, 81.8, 80.7, 80.4, 80.3, 80.2, 76.5, 73.6, 71.8, 71.6, 70.4, 66.5, 57.0, 51.7, 51.2, 42.0, 36.0, 34.3, 33.0, 31.8, 30.8, 30.6, 30.5, 30.4, 30.3, 28.9, 28.84, 28.80, 26.4, 23.7, 14.5; LR-ESI *m/z* calc'd for $C_{61}H_{110}N_8O_{18}$ 1242.79, found 1243.89 $[M+H]^+$.



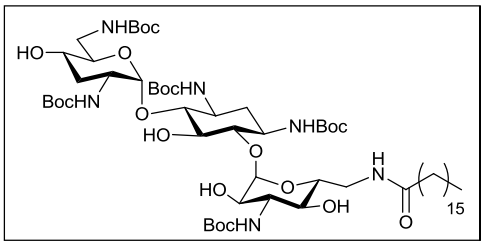
***NH*Boc-protected 5a:** Tridecanoic acid 98% (36 mg, 0.17 mmol) and *N*,*N*-diisopropylethylamine (DIEA) (83 μL, 0.50 mmol) in dry DMF (2 mL), was added HBTU (77 mg, 0.20 mmol) and stirred at ambient temperature for 15 min under argon atmosphere. The mixture was then cooled in an ice-bath, added with compound **1f** (81 mg, 0.08

mmol) and allowed to reach ambient temperature. Propagation of the reaction was monitored by TLC (EtOAc: petroleum ether/7:3), and upon completion, the reaction mixture was diluted with EtOAc (50 mL) and the organic layer was washed three times with brine (3x40 mL). The aqueous layer was extracted again with EtOAc (50 mL) and the combined organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification by flash column chromatography (SiO_2, EtOAc:petroleum ether) gave the corresponding *NH*Boc-protected **5a** (84 mg, 86%) as a white solid.: ^{1}H NMR (400 MHz, CD_3OD) δ 5.13 (br s, 1H, H-1'), 5.04 (br d, *J* = 3.4 Hz, 1H, H-1''), 4.04 (m, 1H, H-5''), 3.69-3.31 (m, 14H, H-1, H-3, H-4, H-5, H-6, H-2', H-4', H-5', H-6' (2H), H-2'', H-3'', H-6'' (2H)), 3.16 (t, *J* = 9.9 Hz, 1H, H-4''), 2.21 (t, *J* = 7.7 Hz, 2H, NHCOC$\underline{H}_2$(CH_2)$_{10}$$CH_3$), 1.98-2.12 (m, 2H, H-2eq, H-3'eq), 1.65 (app. q, *J* = 12.1 Hz, 1H, H-3'ax), 1.62-1.25 (m, 66H, H-2ax, 5x$CO_2C(C\underline{H}_3)_3$, NHCOC$H_2$($C\underline{H}_2$)$_{10}$$CH_3$), 0.90 (t, *J* = 7.0 Hz, 3H, NHCOCH_2(CH_2)$_{10}$$C\underline{H}_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 177.0 (NH$\underline{C}$O), 159.3, 159.2, 158.0, 157.8, 157.6, 99.8 (anomeric C), 99.4 (anomeric C), 83.2, 82.6, 80.7, 80.5, 80.3, 80.1, 77.0, 73.5, 72.6, 72.1, 70.7, 66.5, 57.0, 51.7, 51.0, 42.1, 41.5, 37.1, 34.3, 33.1, 31.8, 30.8, 30.7, 30.5, 28.8, 27.0, 23.7, 14.5; LR-ESI *m/z* calc'd for $C_{56}H_{102}N_6O_{19}$ 1162.72, found 1163.76 $[M+H]^+$.

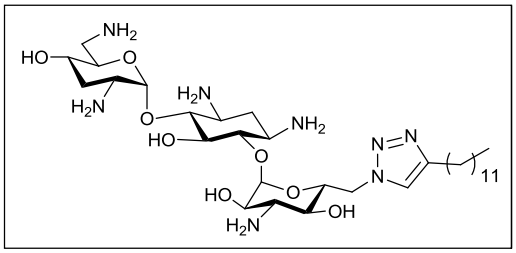


***NH*Boc-protected 5b:** Pentadecanoic acid 99% (40 mg, 0.17 mmol), DIEA (82 μL, 0.49 mmol), dry DMF (2 mL), HBTU (75 mg, 0.20 mmol), **1f** (80 mg, 0.08 mmol). Purification by flash column chromatography (SiO_2, EtOAc:petroleum ether) gave the corresponding *NH*Boc-protected **5b** as a white solid (77 mg, 78%). ^{1}H NMR (400 MHz, CD_3OD) δ 5.13 (br s, 1H, H-1'), 5.04 (br d, *J* = 3.6 Hz, 1H, H-1''), 4.03 (m, 1H, H-5''), 3.67-3.30 (m, 14H, H-1, H-3, H-4, H-5, H-6, H-2', H-4', H-5', H-6' (2H), H-2'', H-3'', H-6'' (2H)), 3.16 (t, *J* = 9.9 Hz, 1H, H-4''), 2.21 (t, *J* = 7.8 Hz, 2H, NHCOC$\underline{H}_2$(CH_2)$_{12}$$CH_3$), 2.13-1.98 (m, 2H, H-2eq, H-3'eq), 1.66 (app. q, *J* = 12.2 Hz, 1H, H-3'ax), 1.62-1.28 (m, 70H, H-2ax, 5x$CO_2C(C\underline{H}_3)_3$, NHCOC$H_2$($C\underline{H}_2$)$_{12}$$CH_3$),

0.90 (t, *J* = 7.0 Hz, 3H, NHCOCH$_2$(CH$_2$)$_{12}$C<u>H</u>$_3$); ^{13}C NMR (100 MHz, CD$_3$OD) δ 177.0 (NH<u>C</u>O), 159.3, 159.2, 158.0, 157.8, 157.6, 99.9 (anomeric C), 99.4 (anomeric C), 83.2, 82.7, 80.7, 80.5, 80.4, 80.2, 77.0, 73.5, 72.7, 72.1, 70.7, 66.6, 57.0, 51.8, 51.1, 42.0, 41.6, 37.1, 35.9, 34.3, 33.1, 31.8, 30.8, 30.7, 30.5, 28.9, 28.83, 28.81, 27.0, 23.7, 14.4; LR-ESI *m/z* calc'd for $C_{58}H_{106}N_6O_{19}$ 1190.75, found 1191.83 $[M+H]^+$.

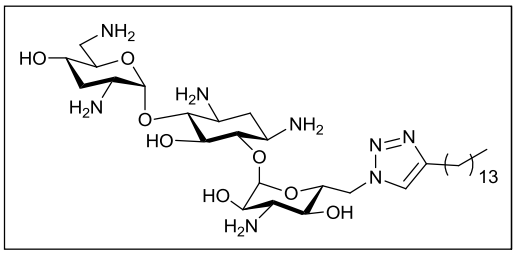


***NH*Boc-protected 5c.** Heptadecanoic acid >98% (45 mg, 0.17 mmol), DIEA (83 μL, 0.50 mmol), dry DMF (2 mL), HBTU (75 mg, 0.20 mmol), **1f** (80 mg, 0.08 mmol). Purification by flash column chromatography (SiO_2, EtOAc:petroleum ether) gave the corresponding *NH*Boc-protected **5c** as a white solid (72 mg, 71%). ^{1}H NMR (400 MHz, CD$_3$OD) δ 5.13 (br s, 1H, H-1'), 5.04 (br d, *J* = 3.6 Hz, 1H, H-1''), 4.03 (m, 1H, H-5''), 3.69-3.30 (m, 14H, H-1, H-3, H-4, H-5, H-6, H-2', H-4', H-5', H-6' (2H), H-2'', H-3'', H-6'' (2H)), 3.16 (t, *J* = 9.9 Hz, 1H, H-4''), 2.21 (t, *J* = 7.8 Hz, 2H, NHCOC<u>H</u>$_2$(CH$_2$)$_{14}$CH$_3$), 2.13-1.98 (m, 2H, H-2eq, H-3'eq), 1.66 (app. q, *J* = 12.2 Hz, 1H, H-3'ax), 1.62-1.27 (m, 74H, H-2ax, 5xCO$_2$C(C<u>H</u>$_3$)$_3$, NHCOCH$_2$(C<u>H</u>$_2$)$_{14}$CH$_3$), 0.90 (t, *J* = 7.0 Hz, 3H, NHCOCH$_2$(CH$_2$)$_{14}$C<u>H</u>$_3$); ^{13}C NMR (100 MHz, CD$_3$OD) δ 177.0 (NH<u>C</u>O), 159.3, 159.2, 158.0, 157.8, 157.6 99.9 (anomeric C), 99.4 (anomeric C), 83.2, 82.7, 80.7, 80.5, 80.4, 80.1, 77.0, 73.5, 72.7, 72.1, 70.7, 66.6, 57.0, 51.8, 51.1, 42.0, 41.6, 37.1, 34.3, 33.1, 31.8, 30.8, 30.5, 28.9, 28.8, 27.0, 23.7, 14.4; LR-ESI *m/z* calc'd for $C_{60}H_{110}N_6O_{19}$ 1218.78, found 1219.71 $[M+H]^+$.



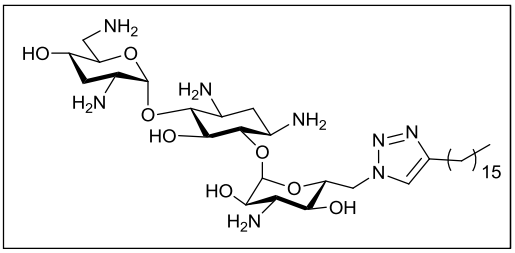
Compound 4a: *NH*Boc-protected **4a** (42 mg, 0.04 mmol) was treated with 99% TFA (2mL) at ambient temperature for 3 min. The TFA was then removed under reduced pressure, the product was re-dissolved in a minimal volume of H_2O and freeze-dried to afford **4a** as a white foam (42 mg, 94%). ^{1}H NMR (400 MHz, D_2O) (Fig. S6) δ 7.60

(s, 1H, triazole ring), 5.69 (d, J = 2.9 Hz, 1H, H-1'), 4.91 (d, J = 3.5 Hz, 1H, H-1''), 4.62 (m, 1H, H-6''), 4.46 (dd, J_1 = 13.8 Hz, J_2 = 7.0 Hz, 1H, H-6''), 4.21 (ddd, J_1 = 9.1 Hz, J_2 = 7.0 Hz, J_3 = 2.1 Hz, 1H, H-5''), 3.84 (t, J = 9.8 Hz, 1H, H-4), 3.79-3.72 (m, 2H, H-5', H-2''), 3.65 (t, J = 8.9 Hz, 1H, H-5), 3.63-3.52 (m, 3H, H-6, H-2', H-4'), 3.47-3.28 (m, 5H, H-1, H-3, H-6', H-3'', H-4''), 3.08 (m, 1H, H-6'), 2.53 (t, J = 6.3 Hz, 1H, $C_2HN_3C\underline{H}_2(CH_2)_{10}CH_3$), 2.40 (dt, J_1 = 12.6 Hz, J_2 = J_3 = 4.1 Hz, 1H, H-2eq), 2.20 (dt, J_1 = 12.1 Hz, J_2 = J_3 = 4.4 Hz, 1H, H-3'eq), 1.88 (app. q, J_1 = J_2 = J_3 = 11.6 Hz, 1H, H-3'ax), 1.78 (app. q, J_1 = J_2 = J_3 = 12.6 Hz, 1H, H-2ax), 1.43 (m, 2H, $C_2HN_3CH_2(C\underline{H}_2)_{10}CH_3$), 1.18-1.06 (m, 18H, $C_2HN_3CH_2(C\underline{H}_2)_{10}CH_3$), 0.65 (t, 3H, J = 6.8 Hz, $C_2HN_3CH_2(CH_2)_{10}C\underline{H}_3$); ^{13}C NMR (100 MHz, D_2O) (Fig. S7) δ 162.9 (q, J = 35 Hz, $CF_3\underline{C}O_2H$), 148.6 (triazole ring), 124.9 (triazole ring), 116.4 (q, J = 290 Hz, $\underline{C}F_3CO_2H$), 100.9 (anomeric C), 93.8 (anomeric C), 84.0, 76.7, 74.4, 70.9, 70.2, 67.9, 66.6, 64.7, 54.8, 50.3, 49.6, 48.4, 47.8, 40.1, 31.3, 29.9, 29.6, 29.4, 28.9, 28.7, 28.6, 28.3, 27.7, 24.5, 22.1, 13.5; HRESI-MS *m/z* calc'd for $C_{32}H_{62}N_8O_8Na$ 709.4588, found 709.4583 $[M+Na]^+$.

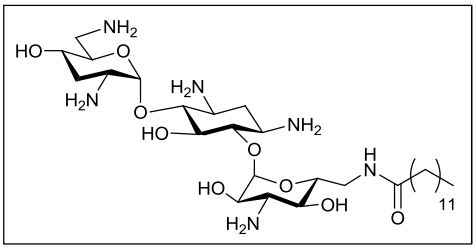


Compound 4b: *NH*Boc-protected **4b** (59 mg, 0.05 mmol) was treated with 99% TFA (2mL) at ambient temperature for 3 min. The TFA was removed under reduced pressure, the product was re-dissolved in a minimal volume of H_2O and freeze-dried to afford **4b** as a white foam (58 mg, 93%). ^{1}H NMR (400 MHz, D_2O) (Fig. S8) δ 7.47 (s, 1H, triazole ring), 5.72 (d, J = 3.2 Hz, 1H, H-1'), 4.89 (d, J = 3.1 Hz, 1H, H-1''), 4.58(m, 1H, H-6''), 4.40 (dd, J_1 = 14.5 Hz, J_2 = 5.8 Hz, 1H, H-6''), 4.21 (m, 1H, H-5''), 3.87 (t, J = 9.6 Hz, 1H, H-4), 3.77 (td, J_1 = J_2 = 8.6 Hz, J_3 = 3.4 Hz, 1H, H-5'), 3.71 (dd, J_1 = 10.7 Hz, J_2 = 3.1 Hz, 1H, H-2''), 3.68-3.56 (m, 2H, H-5, H-6), 3.55-3.24 (m, 7H, H-1, H-3, H-2', H-4', H-6', H-3'', H-4''), 3.01 (dd, J_1 = 13.4 Hz, J_2 = 7.8 Hz, 1H, H-6'), 2.48 (t, J = 7.4 Hz, 2H, $C_2HN_3C\underline{H}_2(CH_2)_{12}CH_3$), 2.40 (dd, J_1 = 12.2 Hz, J_2 = 3.6 Hz, 1H, H-2eq), 2.19 (dd, J_1 = 7.3 Hz, J_2 = 4.5 Hz, 1H, H-3'eq), 1.91 (app. q, J_1 = J_2 = J_3 = 11.4 Hz, 1H, H-3'ax), 1.83 (app. q, J_1 = J_2 = J_3 = 12.6 Hz, 1H, H-2ax), 1.44 (m, 2H, $C_2HN_3CH_2(C\underline{H}_2)_{12}CH_3$),

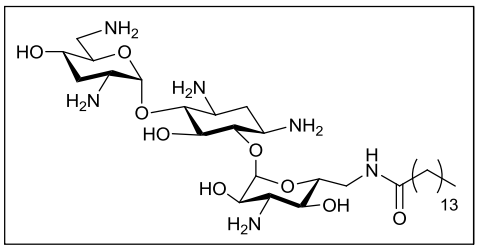
1.56-1.04 (m, 22H, $C_2HN_3CH_2(C\underline{H}_2)_{12}CH_3$), 0.68 (t, 3H, J = 6.8 Hz, $C_2HN_3CH_2(CH_2)_{12}C\underline{H}_3$); ^{13}C NMR (100 MHz, D_2O) (Fig. S9) δ 162.7 (q, J = 35 Hz, $CF_3\underline{C}O_2H$), 148.5 (triazole ring), 124.4 (triazole ring), 116.4 (q, J = 290 Hz, $\underline{C}F_3CO_2H$), 100.9 (anomeric C), 93.7 (anomeric C), 84.1, 76.7, 74.4, 70.7, 70.2, 67.9, 66.5, 64.9, 54.8, 50.2, 49.7, 48.4, 47.9, 40.3, 31.7, 30.7, 29.8, 29.6, 29.4, 29.3, 29.1, 29.0, 28.8, 27.7, 24.7, 22.4, 13.6; HRESI-MS *m/z* calc'd for $C_{34}H_{67}N_8O_8$ 715.5082, found 715.5089 $[M+H]^+$.



Compound 4c: *NH*Boc-protected **4c** (46 mg, 0.04 mmol) was treated with 99% TFA (2mL) at ambient temperature for 3 min. The TFA was removed under reduced pressure, the product was re-dissolved in a minimal volume of H_2O and freeze-dried to afford **4c** as a white foam (48 mg, 99%). ^{1}H NMR (400 MHz, D_2O) (Fig. S10) δ 7.43 (s, 1H, triazole ring), 5.73 (d, J = 3.2 Hz, 1H, H-1'), 4.88 (d, J = 3.0 Hz, 1H, H-1''), 4.57 (m, 1H, H-6''), 4.38 (dd, J_1 = 14.2 Hz, J_2 = 5.4 Hz, 1H, H-6''), 4.21 (m, 1H, H-5''), 3.88 (t, J = 9.6 Hz, 1H, H-4), 3.77 (td, $J_1 = J_2$ = 8.5 Hz, J_3 = 3.4 Hz, 1H, H-5'), 3.71 (dd, J_1 = 10.7 Hz, J_2 = 3.1 Hz, 1H, H-2''), 3.68-3.56 (m, 2H, H-5, H-6), 3.54-3.28 (m, 6H, H-1, H-3, H-2', H-4', H-6', H-3''), 3.25 (t, J = 9.9 Hz, 1H, H-4''), 2.99 (dd, J_1 = 13.3 Hz, J_2 = 7.9 Hz, 1H, H-6'), 2.47 (t, J = 7.2 Hz, 2H, $C_2HN_3C\underline{H}_2(CH_2)_{14}CH_3$), 2.40 (dd, J_1 = 8.2 Hz, J_2 = 3.6 Hz, 1H, H-2eq), 2.19 (dd, J_1 = 7.4 Hz, J_2 = 4.4 Hz, 1H, H-3'eq), 1.92 (app. q, $J_1 = J_2 = J_3$ = 11.5 Hz, 1H, H-3'ax), 1.84 (app. q, $J_1 = J_2 = J_3$ = 12.6 Hz, 1H, H-2ax), 1.43 (m, 2H, $C_2HN_3CH_2(C\underline{H}_2)_{14}CH_3$), 1.19-1.03 (m, 26H, $C_2HN_3CH_2(C\underline{H}_2)_{14}CH_3$), 0.69 (t, 3H, J = 6.9 Hz, $C_2HN_3CH_2(CH_2)_{14}C\underline{H}_3$); ^{13}C NMR (100 MHz, D_2O) (Fig. S11) δ 162.7 (q, J = 35 Hz, $CF_3\underline{C}O_2H$), 148.5 (triazole ring), 124.2 (triazole ring), 116.4 (q, J = 290 Hz, $\underline{C}F_3CO_2H$), 100.9 (anomeric C), 93.7 (anomeric C), 84.1, 76.7, 74.4, 70.7, 70.2, 67.9, 66.5, 65.0, 54.9, 50.2, 49.7, 48.4, 47.9, 40.3, 31.8, 30.8, 29.8, 29.6, 29.4, 29.3, 29.2, 29.0, 28.9, 27.7, 24.8, 22.5, 13.7; HRESI-MS *m/z* calc'd for $C_{36}H_{70}N_8O_8Na$ 765.5214, found 765.5216 $[M+Na]^+$.

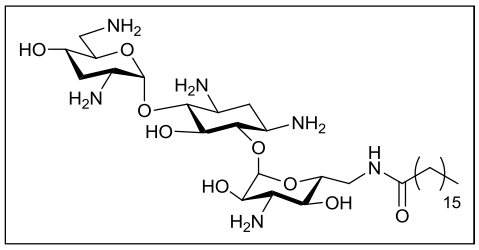


Compound 5a: *NH*Boc-protected **5a** (23.8 mg, 0.02 mmol) was treated with 99% TFA (1mL) at ambient temperature for 3 min. The TFA was then removed under reduced pressure, the residue was re-dissolved in a minimal volume of H_2O and freeze-dried to afford **5a** as a white foam (21.5 mg, 85%). ^{1}H NMR (400 MHz, D_2O) (Fig. S12) δ 5.67 (d, *J* = 3.5 Hz, 1H, H-1'), 4.91 (d, *J* = 3.5 Hz, 1H, H-1"), 3.88-3.73 (m, 4H, H-4, H-5', H-2", H-5"), 3.70 (t, *J* = 9.0 Hz, 1H, H-5), 3.65-3.26 (m, 10H, H-1, H-3, H-6, H-2', H-4', H-6', H-3", H-4", H-6"(2H)), 3.12 (dd, J_1 =13.6 Hz, J_2 = 7.0 Hz, 1H, H-6'), 2.40 (dt, J_1 = 12.5 Hz, J_2 = J_3 = 4.2 Hz, 1H, H-2eq), 2.17 (m, 1H, H-3'eq), 2.11 (t, *J* = 7.5 Hz, 2H, NHCOC$\underline{H}_2(CH_2)_{10}CH_3$), 1.89 (app. q, J_1 = J_2 = J_3 = 11.0 Hz, 1H, H-3'ax), 1.79 (app. q, J_1 = J_2 = J_3 = 12.6 Hz, 1H, H-2ax), 1.43 (m, 2H, $NHCOCH_2(C\underline{H}_2)_{10}CH_3$), 1.18-1.06 (m, 18H, $NHCOCH_2(C\underline{H}_2)_{10}CH_3$), 0.70 (t, 3H, *J* = 7.0 Hz, $NHCOCH_2(CH_2)_{10}C\underline{H}_3$); ^{13}C NMR (100 MHz, D_2O) (Fig. S13) δ 178.2 (NH$\underline{C}$O), 162.9 (q, *J* = 35 Hz, $CF_3\underline{C}O_2H$), 116.4 (q, *J* = 290 Hz, $\underline{C}F_3CO_2H$), 100.9 (anomeric C), 94.1 (anomeric C), 83.6, 77.3, 74.2, 71.0, 70.4, 68.1, 66.6, 64.4, 54.7, 49.8, 48.4, 47.8, 39.8, 38.9, 35.8, 31.2, 29.3, 28.8, 28.7, 28.6, 28.5, 28.4, 28.3, 27.8, 25.4, 22.0, 13.4; HRESI-MS *m/z* calc'd for $C_{31}H_{63}N_6O_9$ 663.4657, found 663.4648 $[M+H]^+$.



Compound 5b: *NH*Boc-protected **5b** (24.0 mg, 0.02 mmol) was treated with 99% TFA (1mL) at ambient temperature for 3 min. The TFA was removed under reduced pressure, the residue was dissolved in a minimal volume of H_2O and freeze-dried to afford **5b** as a white foam (24.9 mg, 98%). ^{1}H NMR (400 MHz, D_2O) (Fig. S14) δ 5.66 (d, *J* = 3.5 Hz, 1H, H-1'), 4.92 (d, *J* = 3.6 Hz, 1H, H-1"), 3.86-3.73 (m, 4H, H-4, H-5', H-2", H-5"), 3.69 (t, *J* = 9.0 Hz, 1H, H-5), 3.65-3.26 (m, 10H, H-1, H-3, H-6, H-2', H-4', H-6', H-3", H-4", H-6"(2H)), 3.11 (dd, J_1 = 13.6 Hz, J_2 = 7.1 Hz, 1H, H-6'), 2.39 (dt, J_1 = 12.5 Hz, J_2 = J_3 = 4.2 Hz, 1H, H-2eq), 2.19-2.09 (m, 3H,

H-3'eq, NHCOC<u>H</u>$_2$(CH$_2$)$_{12}$CH$_3$), 1.89 (app. q, J_1 = J_2 = J_3 = 12.0 Hz, 1H, H-3'ax), 1.78 (app. q, J_1 = J_2 = J_3 = 12.6 Hz, 1H, H-2ax), 1.43 (m, 2H, NHCOCH$_2$(C<u>H</u>$_2$)$_{12}$CH$_3$), 1.17-1.08 (m, 22H, NHCOCH$_2$(C<u>H</u>$_2$)$_{12}$CH$_3$), 0.71 (t, 3H, J = 7.0 Hz, NHCOCH$_2$(CH$_2$)$_{12}$C<u>H</u>$_3$); ^{13}C NMR (100 MHz, D$_2$O) (Fig. S15) δ 178.1 (NH<u>C</u>O), 162.9 (q, J = 35 Hz, CF$_3$<u>C</u>O$_2$H), 116.4 (q, J = 290 Hz, <u>C</u>F$_3$CO$_2$H), 100.9 (anomeric C), 94.0 (anomeric C), 83.7, 77.6, 74.2, 71.0, 70.3, 68.1, 66.6, 64.5, 54.7, 49.8, 48.4, 47.8, 39.9, 38.9, 35.8, 31.3, 29.8, 29.4, 28.9, 28.7, 28.6, 28.5, 28.4, 28.0, 25.4, 22.1, 13.5; HRESI-MS *m/z* calc'd for C$_{33}$H$_{67}$N$_6$O$_9$ 691.4970, found 691.4963 [M+H]$^+$.



Compound 5c: *NH*Boc-protected **5c** (33.2 mg, 0.03 mmol) was treated with 99% TFA (1.3mL) at ambient temperature for 3 min. The TFA was removed under reduced pressure, the product was re-dissolved in a minimal volume of H$_2$O and freeze-dried to afford **5c** as a white foam (33.8 mg, 96%). ^{1}H NMR (400 MHz, D$_2$O) (Fig. S16) δ 5.67 (d, J = 3.1 Hz, 1H, H-1'), 4.90 (d, J = 2.8 Hz, 1H, H-1''), 3.86 (t, J = 9.3 Hz, 1H, H-4), 3.82-3.75 (m, 3H, H-5', H-2'', H-5''), 3.68 (t, J = 8.8 Hz, 1H, H-5), 3.62 (t, J = 9.1 Hz, 1H, H-6), 3.57-3.20 (m, 9H, H-1, H-3, H-2', H-4', H-6', H-3'', H-4'', H-6''(2H)), 3.04 (dd, J_1 = 13.4 Hz, J_2 = 7.7 Hz, 1H, H-6'), 2.38 (m, 1H, H-2eq), 2.18-2.04 (m, 3H, H-3'eq, NHCOC<u>H</u>$_2$(CH$_2$)$_{14}$CH$_3$), 1.89 (app. q, J_1 = J_2 = J_3 = 11.9 Hz, 1H, H-3'ax), 1.81 (app. q, J_1 = J_2 = J_3 = 12.4 Hz, 1H, H-2ax), 1.40 (m, 2H, NHCOCH$_2$(C<u>H</u>$_2$)$_{14}$CH$_3$), 1.18-1.06 (m, 26H, NHCOCH$_2$(C<u>H</u>$_2$)$_{14}$CH$_3$), 0.71 (t, 3H, J = 6.8 Hz, NHCOCH$_2$(CH$_2$)$_{14}$C<u>H</u>$_3$); ^{13}C NMR (100 MHz, D$_2$O) (Fig. S17) δ 177.2 (NH<u>C</u>O), 162.5 (q, J = 35 Hz, CF$_3$<u>C</u>O$_2$H), 116.0 (q, J = 290 Hz, <u>C</u>F$_3$CO$_2$H), 100.5 (anomeric C), 93.5 (anomeric C), 83.3, 77.0, 73.8, 70.8, 70.3, 67.8, 66.1, 64.4, 54.2, 49.5, 48.0, 47.5, 39.7, 38.5, 35.5, 31.3, 29.4, 29.1, 28.9, 28.8, 28.6, 28.5, 27.5, 25.1, 22.1, 13.3; HRESI-MS *m/z* calc'd for C$_{35}$H$_{70}$N$_6$O$_9$Na 741.5102 found 741.5101 [M+Na]$^+$.

3. Minimal Inhibitory Concentration (MIC) test.

Starter cultures were incubated for 24 hours (37 °C, 5% CO_2, aerobic conditions), and diluted in fresh broth medium to obtain an optical density of 0.004 (OD_{655}). All strains were tested using a double-dilution method starting at 128 μg/mL in 96-well plates (Sarstedt, Newton, NC). After 24 hours of incubation, MTT reagent (50 μL of a 1 mg/mL solution in H_2O) was added to each well followed by additional incubation at 37°C for 2 hours. MIC values (μg/mL) were determined as the lowest concentration at which no bacterial growth was observed. Results were obtained from two independent experiments and each experiment was done in triplicate. Tested strains: **Gram-positive:** *Streptococcus pyogenes* M12 (strain MGAS9429) (**A**), *Methicillin-resistant Staphylococcus aureus* (MRSA) (**B**), *Streptococcus mutans* UA 159 (**C**), *vancomycin-resistant enterococci* (VRE) (**D**), *Enterococcus faecalis* ATCC 29212 (**E**), *Staphylococcus aureus* (Oxford strain ATCC9144) (**F**), *Staphylococcus epidermidis* ATCC 35984, and *Staphylococcus epidermidis* ATCC 12228. **Gram-negative:** *Pseudomonas aeruginosa* ATCC33347 (**I**), *Shigella sonnei* clinical isolate 6831(*O*-antigen positive) (**J**), and *Shigella sonnei* clinical isolate 6831 (*O*-antigen negative) (**K**). All strains were grown in Brain Heart Infusion broth (BHI) (BBL Microbiology Systems, Cockeysville, MD) with the exception of *Shigella sonnei* that was grown in Trypticase Soy Broth (TSB).

4. Minimal Biofilm Inhibitory Concentration (MBIC) test.

S. epidermidis ATCC35984 (**G**) and *S. mutans* UA 159 (**C**) were grown in biofilms in Trypticase Soy Broth (TSB) (BBL Microbiology Systems, Cockeysville, MD) supplemented with glucose 1%, and in BHI supplemented with sucrose 2%, respectively with presence of the tested analogues. Strains were tested using a double-dilution starting at 128 μg/mL, aerobically at 37 °C, 5% CO_2, 96-well plates. After 24 hours of growth, the plates were vigorously washed three times with phosphate-buffered saline (PBS) to remove any unattached bacteria and then dried for 1 hour at 60 °C. The air-dried wells were stained with 0.1% crystal violet (200 μL) for 30 min, and the plate was rinsed with PBS. The air-

dried plates were added with 200 µL of acetic acid 30%, and the OD at 570 nm was measured by microtiter plate reader (Tecan). Experiments were performed in triplicate.

5. Red blood cells (RBCs) hemolysis assay.

Rat RBC solution (2% w/w) was incubated with the amphiphilic 6"-tobramycin analogues using the double dilution method starting at concentration of 256 µg/mL for 1 hour at 37 °C, 5% CO_2. Negative control was PBS and positive control was 1% w/v solution of Triton X100 (100% hemolysis). Following centrifugation (2,000 rpm, 10 min, ambient temperature), the supernatant was drawn off and its absorbance measured at 550 nm using a microplate reader (Genios, TECAN). The results were expressed as percentage of hemoglobin released relative to the positive control (Triton X100).

This experiment was preformed in triplicate, and the results are an average of two different blood samples.

6. Abbreviations.

BHI, brain heart infusion; BOC, *tert*-butoxycarbonyl; DIEA, *N*,*N*-Diisopropylethylamine; DMF, dimethylformamide; EtOAc, ethyl acetate; HBTU, *O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate; MBIC, minimal biofilm inhibitory concentration; MIC, minimum inhibitory concentration; MTT, thiazolyl blue tetrazolium bromide; PBS, phosphate buffered saline; RBC, red blood cells; rt, room temperature; TFA, trifluoroacetic acid; TLC, thin layer chromatography; TSB, trypticase soy broth.

7. References.

1. K. Michael, H. Wang and Y. Tor, *Bioorgan. Med. Chem.*, 1999, **7**, 1361-1371.

2. I. M. Herzog, K. D. Green, Y. Berkov-Zrihen, M. Feldman, R. R. Vidavski, A. Eldar-Boock, R. Satchi-Fainaro, A. Eldar, S. Garneau-Tsodikova and M. Fridman, *Angew. Chem. Int. Ed.*, 2012, **51**, 5652-5656.

3. M. D. Disney and O. J. Barrett, *Biochemistry*, 2007, **46**, 11223-11230.

8. NMR spectra: Figures S2-S17.

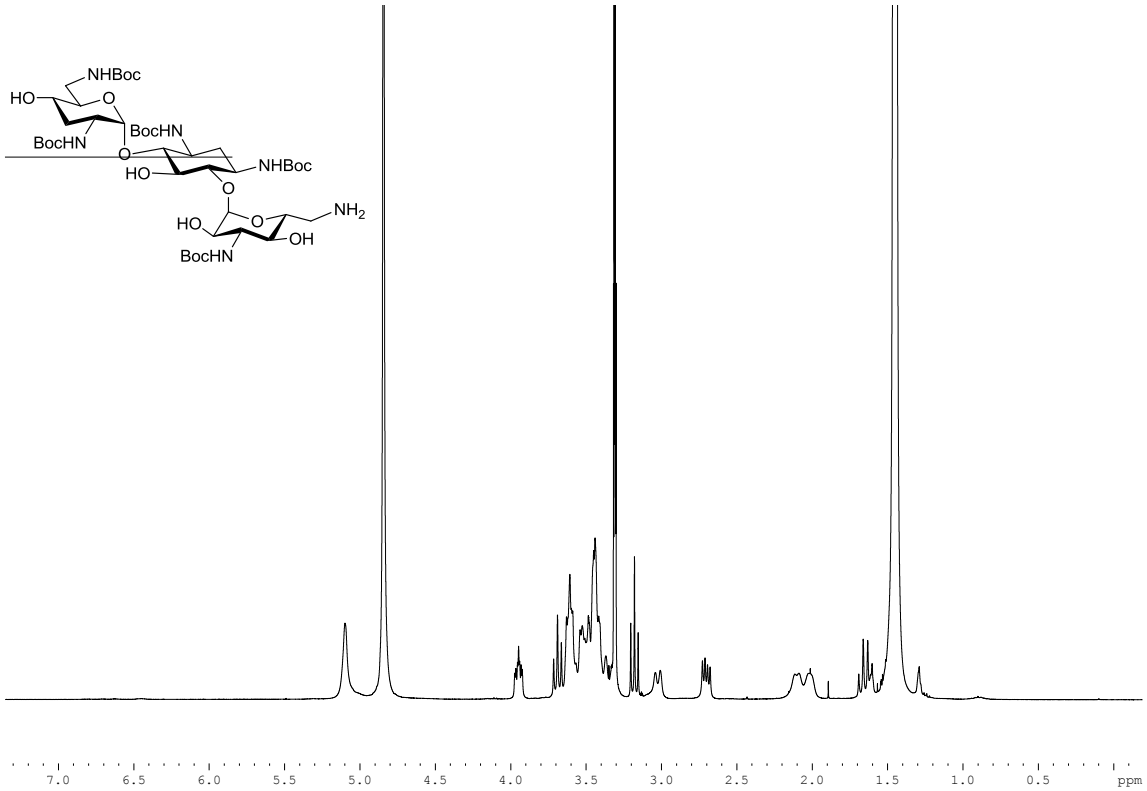


Fig. S2. ^{1}H NMR for 6"-amine tobramycin analogue **1f**

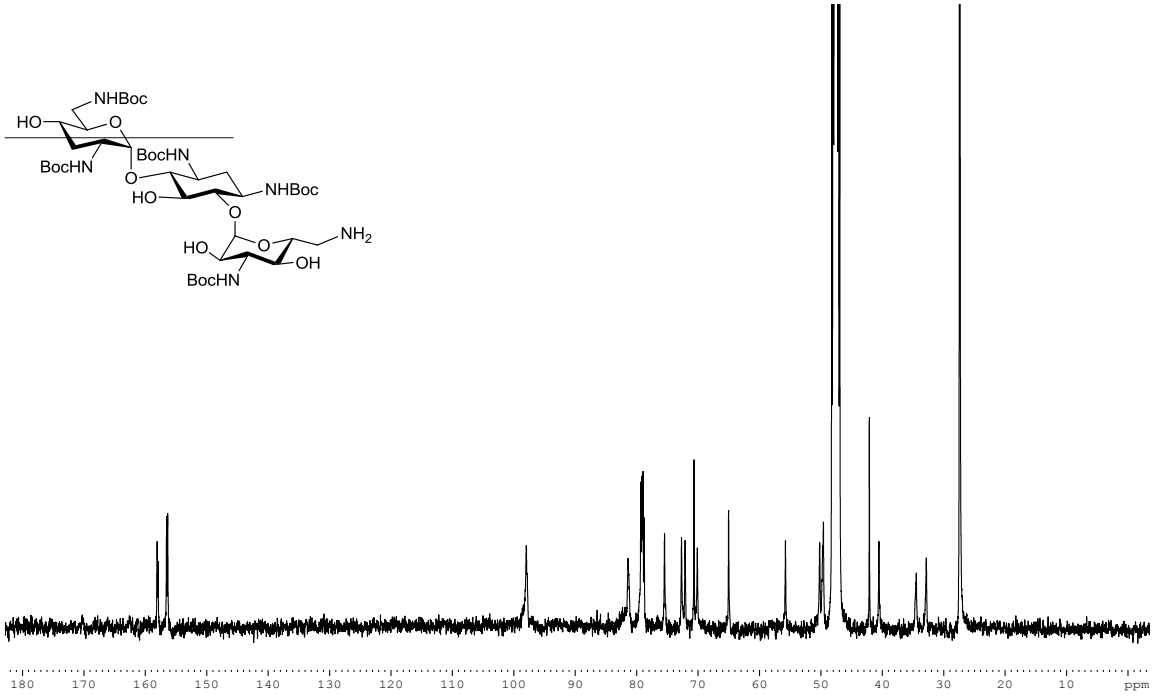


Fig. S3. ^{13}C NMR for 6"-amine tobramycin analogue **1f**

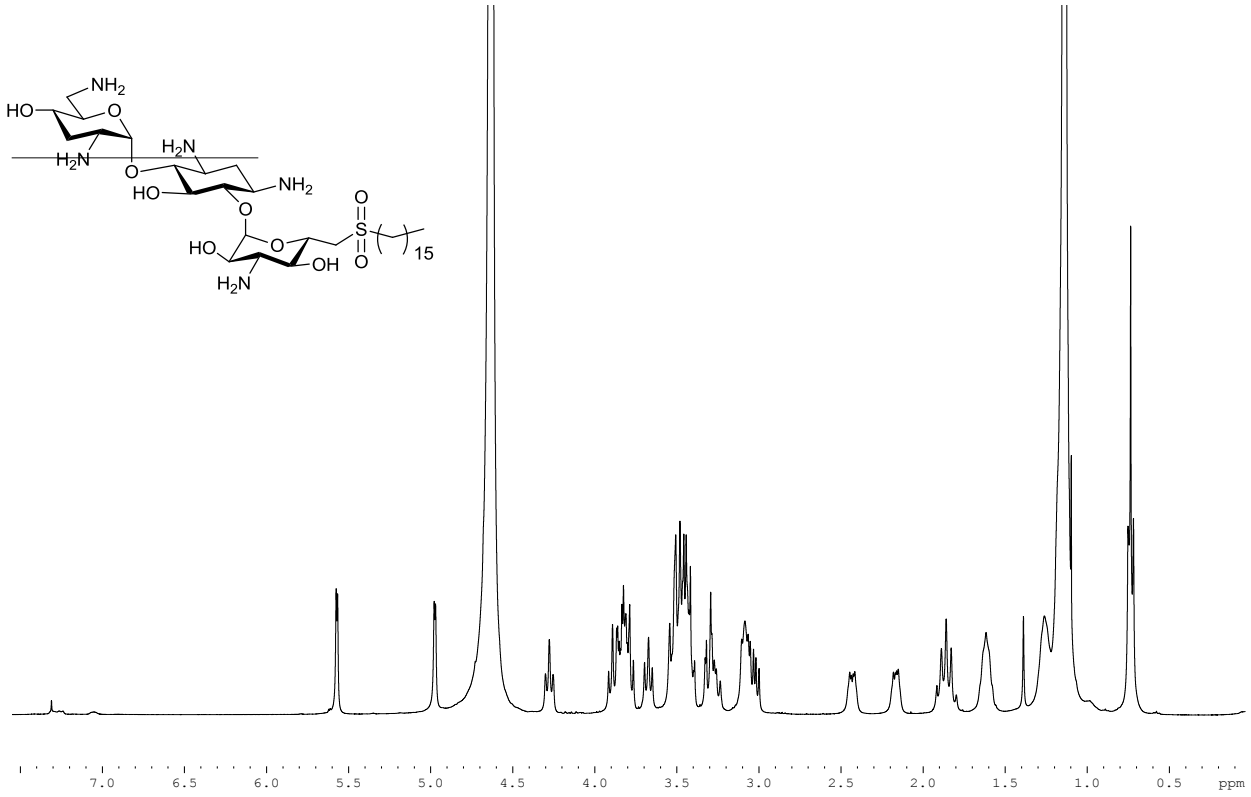


Fig. S4. ^{1}H NMR for 6"-sulfone tobramycin analogue **3c**.

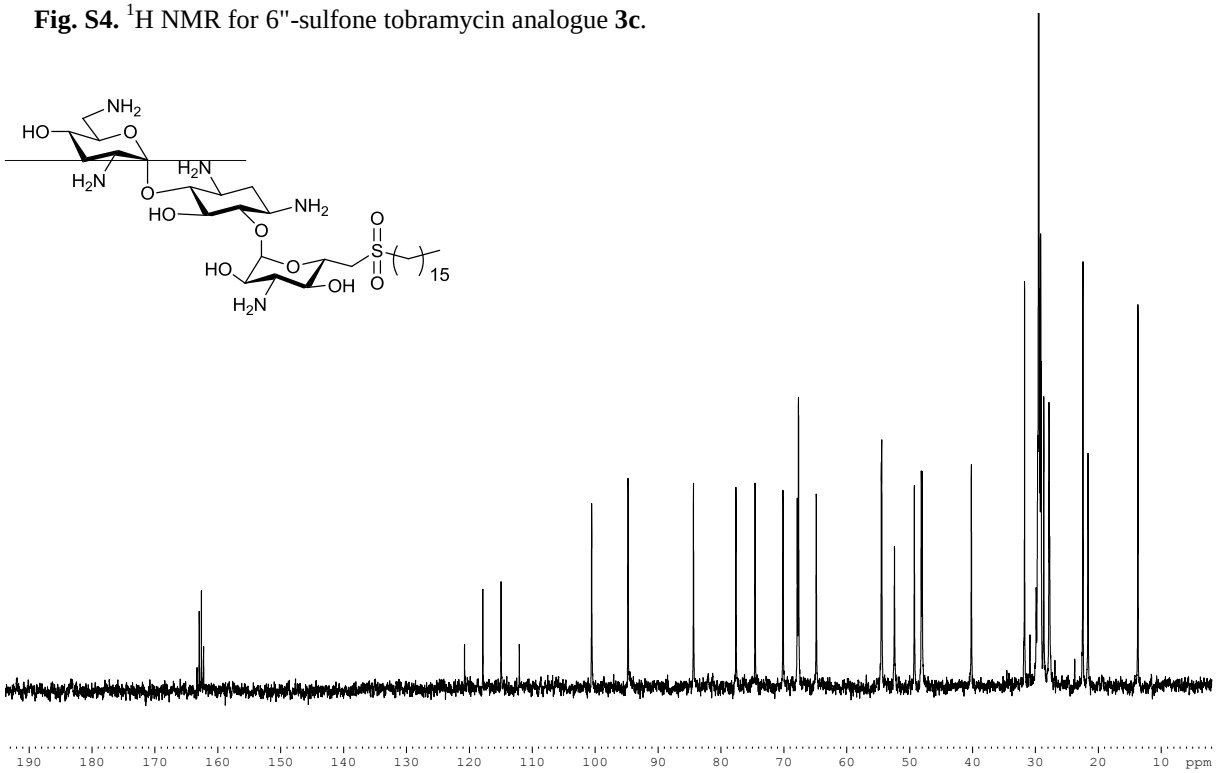


Fig. S5. ^{13}C NMR for 6"-sulfone tobramycin analogue **3c**.

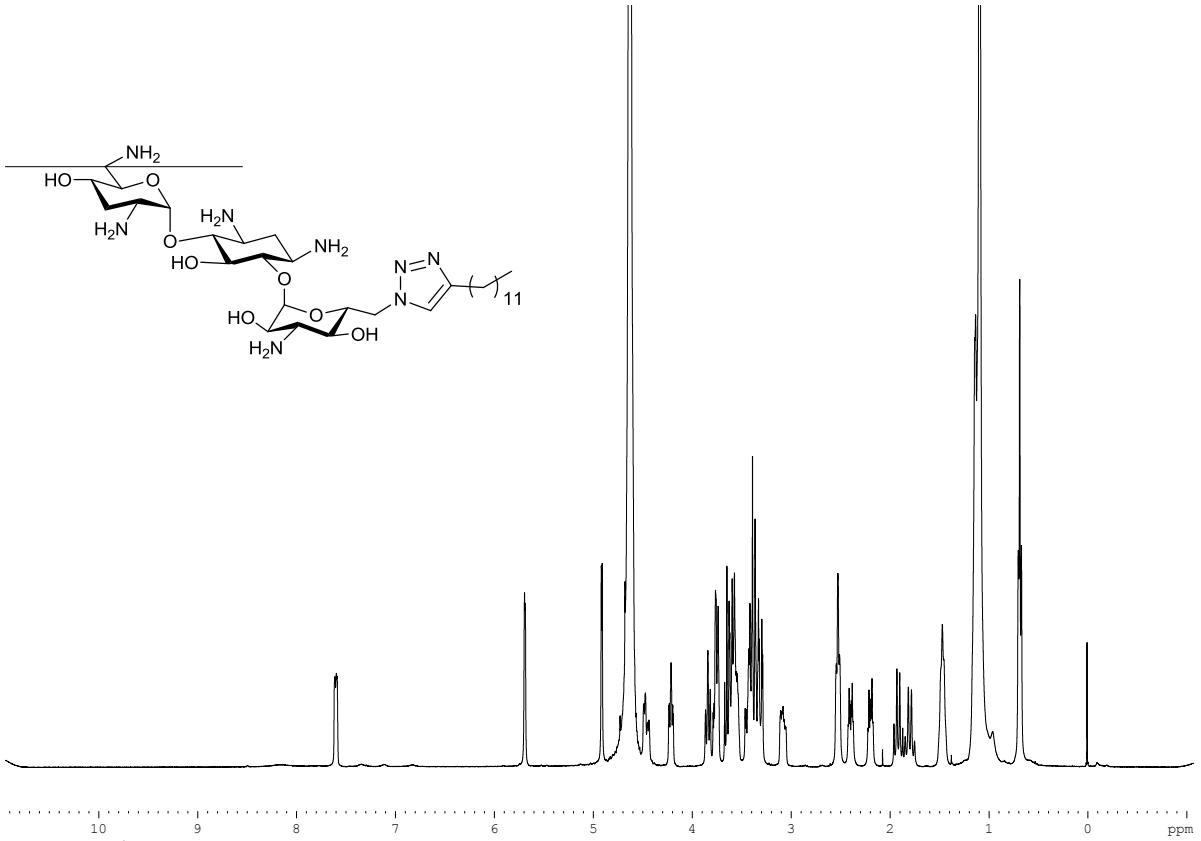


Fig. S6. ^{1}H NMR for 6"-triazole tobramycin analogue **4a**.

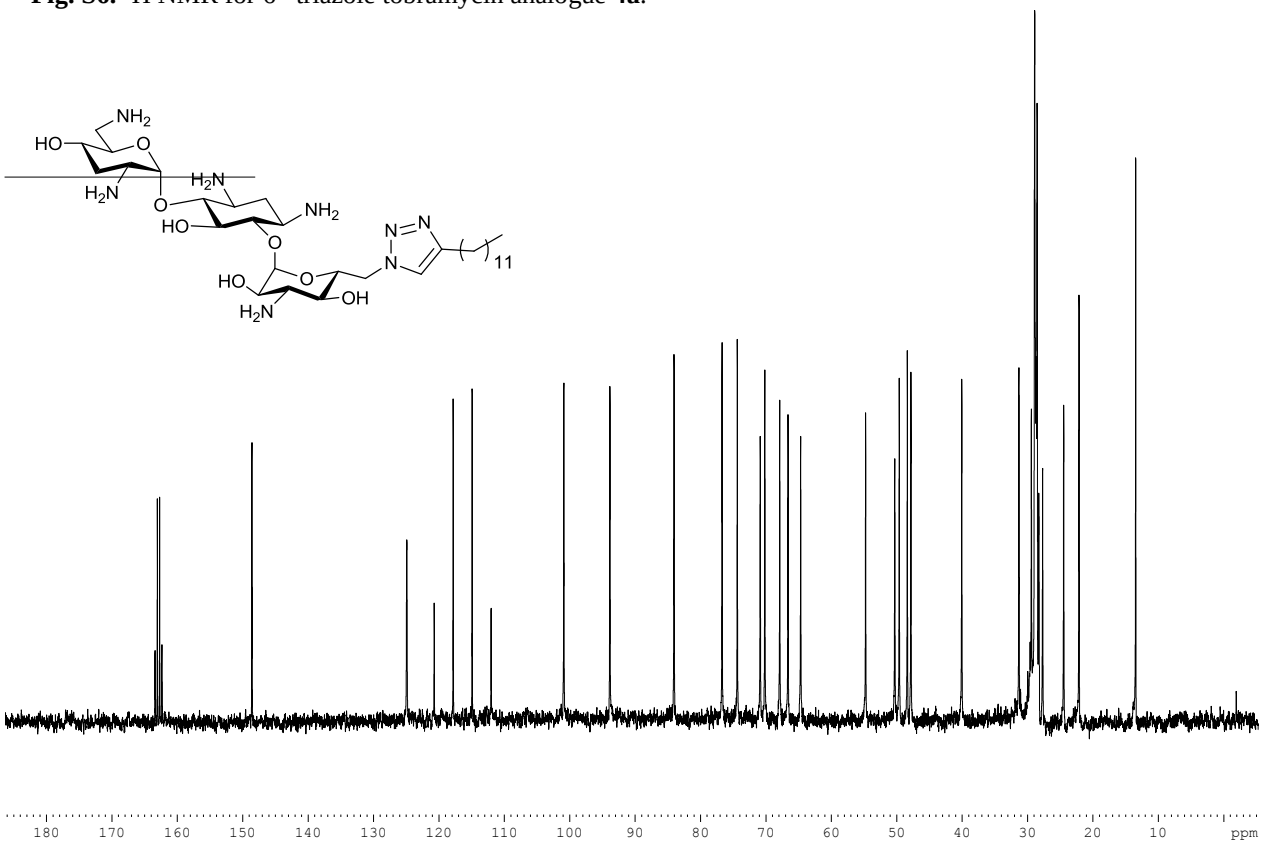


Fig. S7. ^{13}C NMR for 6"-triazole tobramycin analogue **4a.**

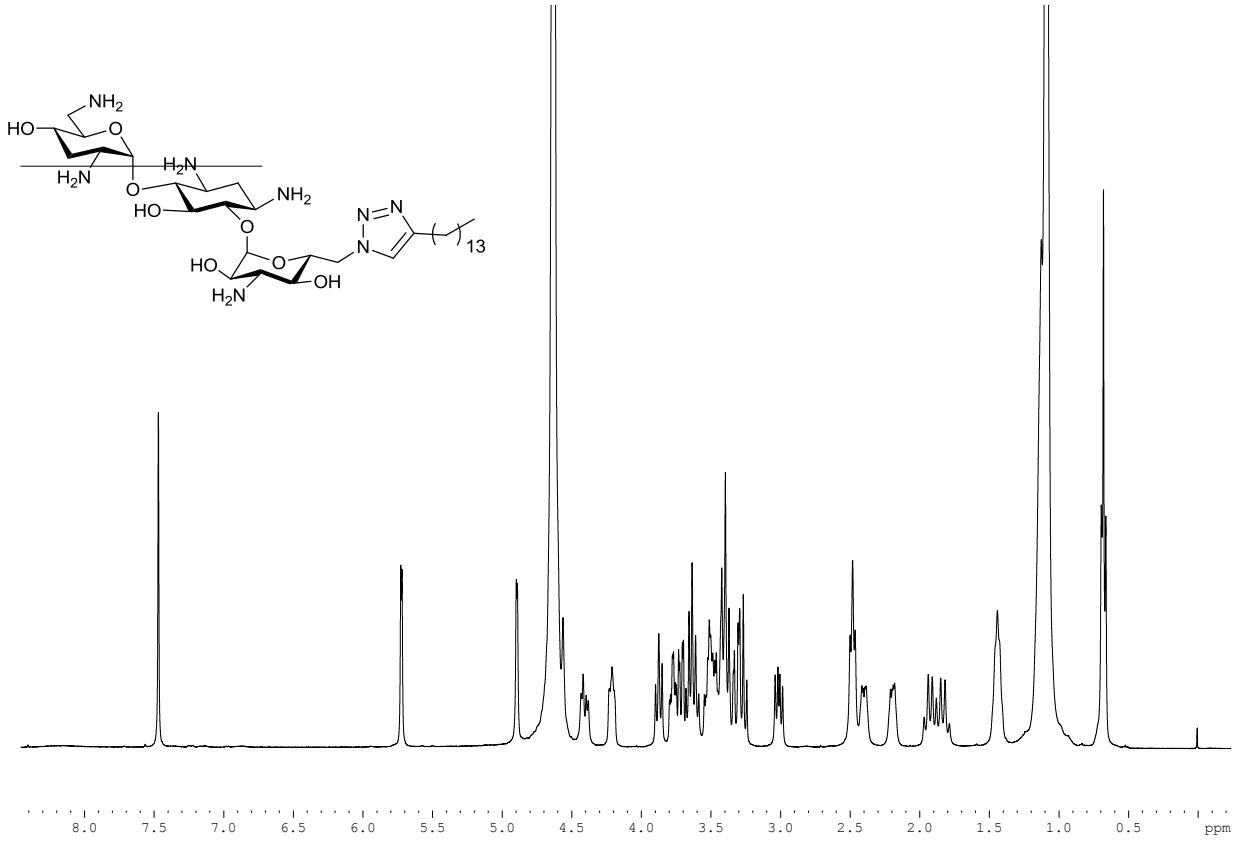


Fig. S8. ^{1}H NMR for 6''-triazole tobramycin analogue **4b**.

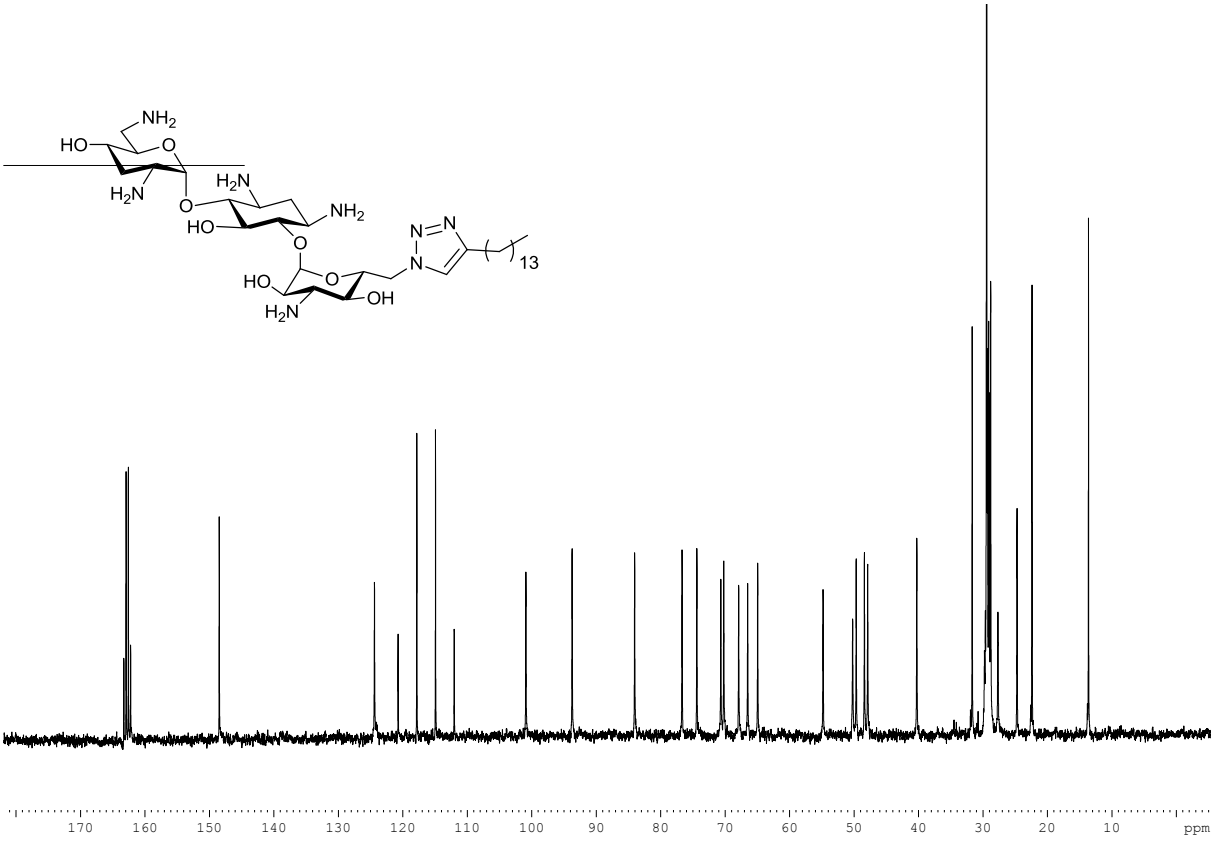


Fig. S9. ^{13}C NMR for 6''-triazole tobramycin analogue **4b.**

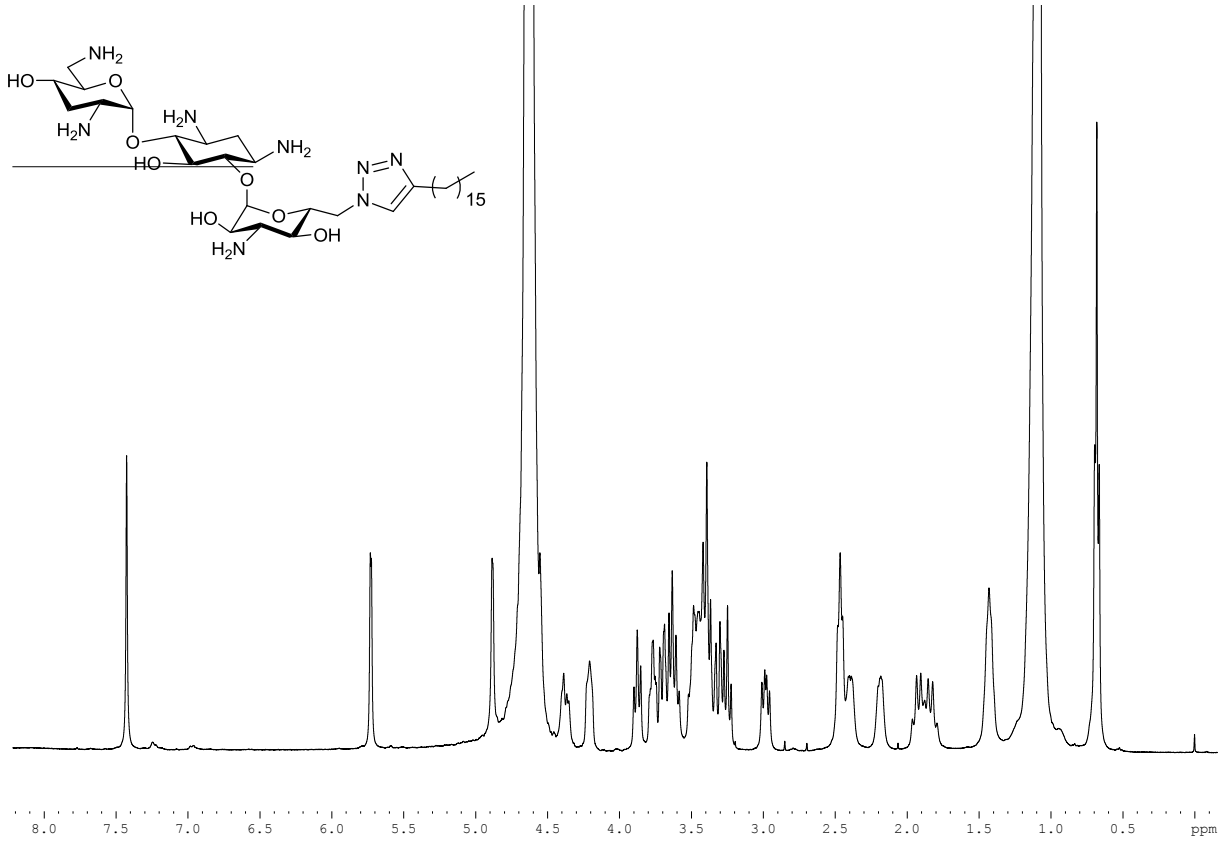


Fig. S10. ^{1}H NMR for 6"-triazole tobramycin analogue **4c**.

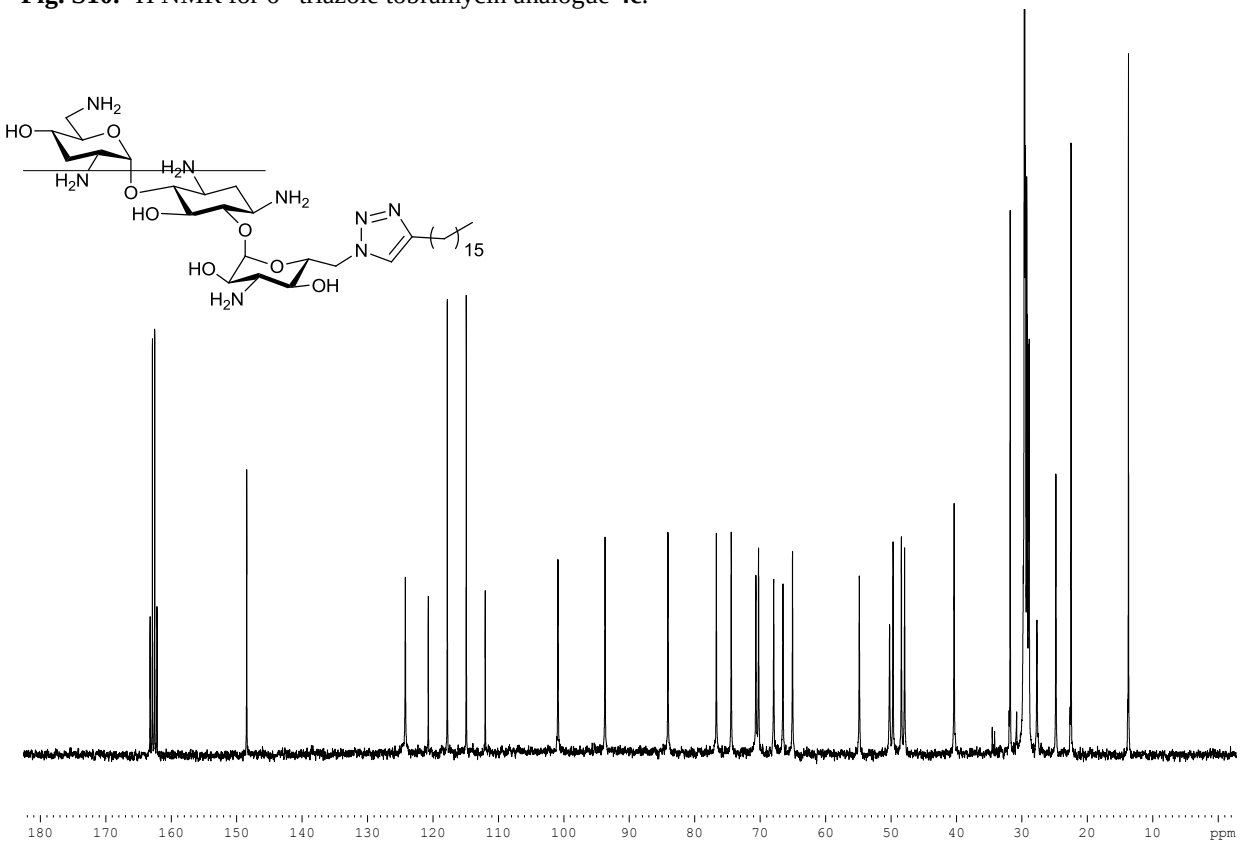


Fig. S11. ^{13}C NMR for 6"-triazole tobramycin analogue **4c.**

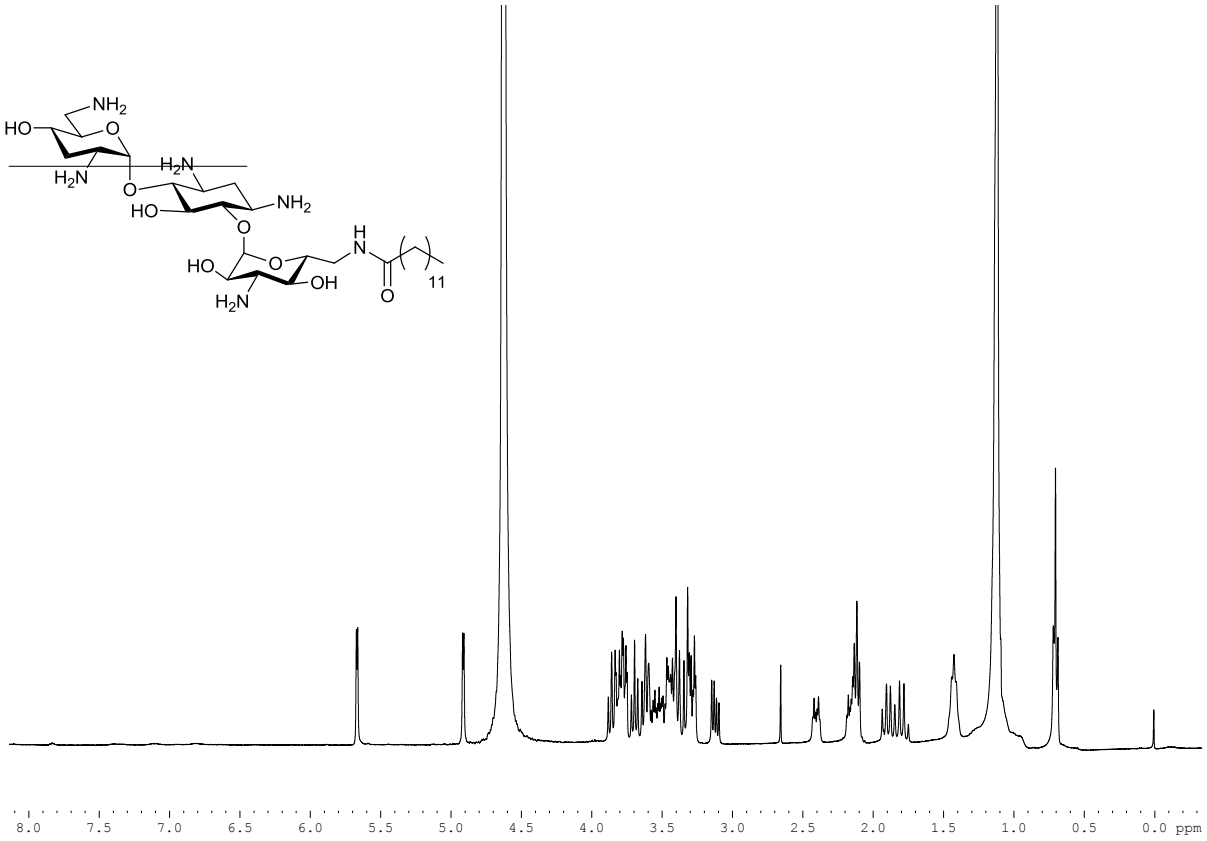


Fig. S12. ^{1}H NMR for 6"-amide tobramycin analogue **5a**.

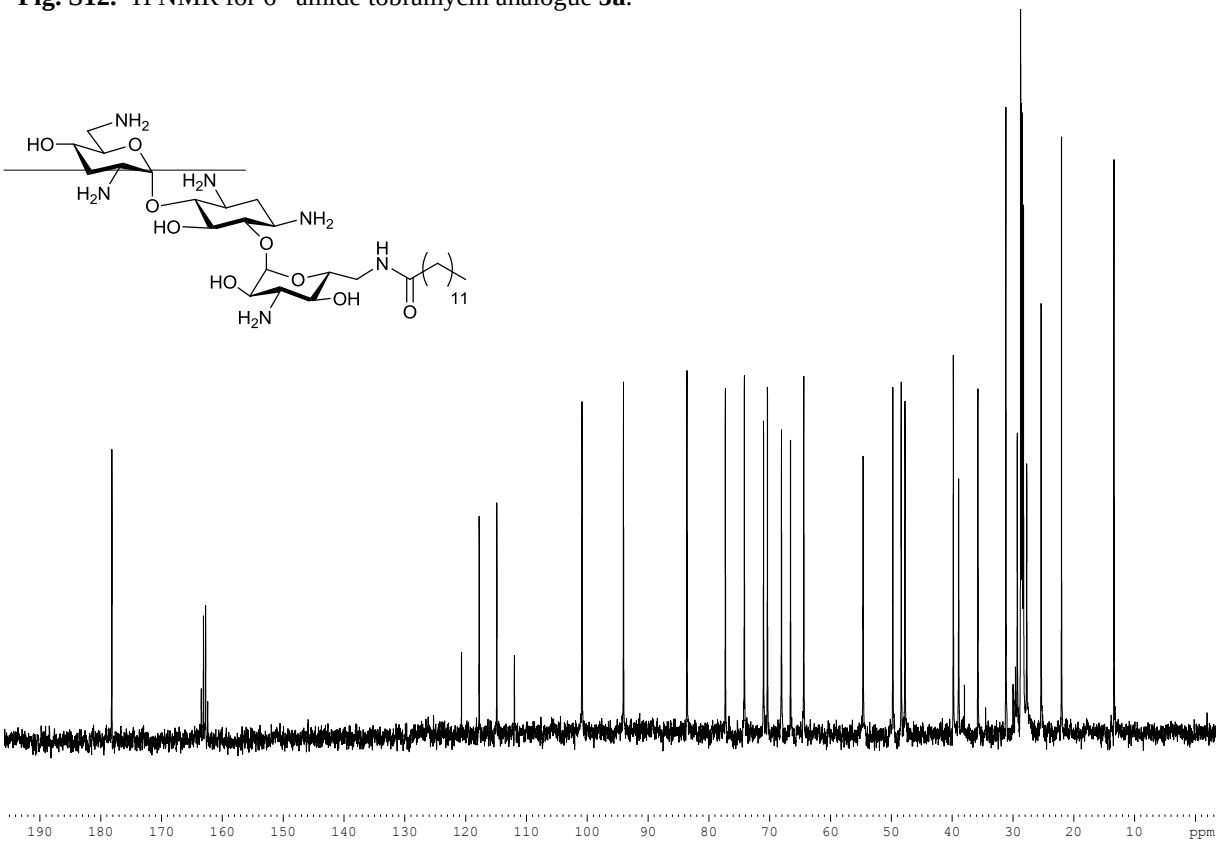


Fig. S13. ^{13}C NMR for 6"-amide tobramycin analogue **5a.**

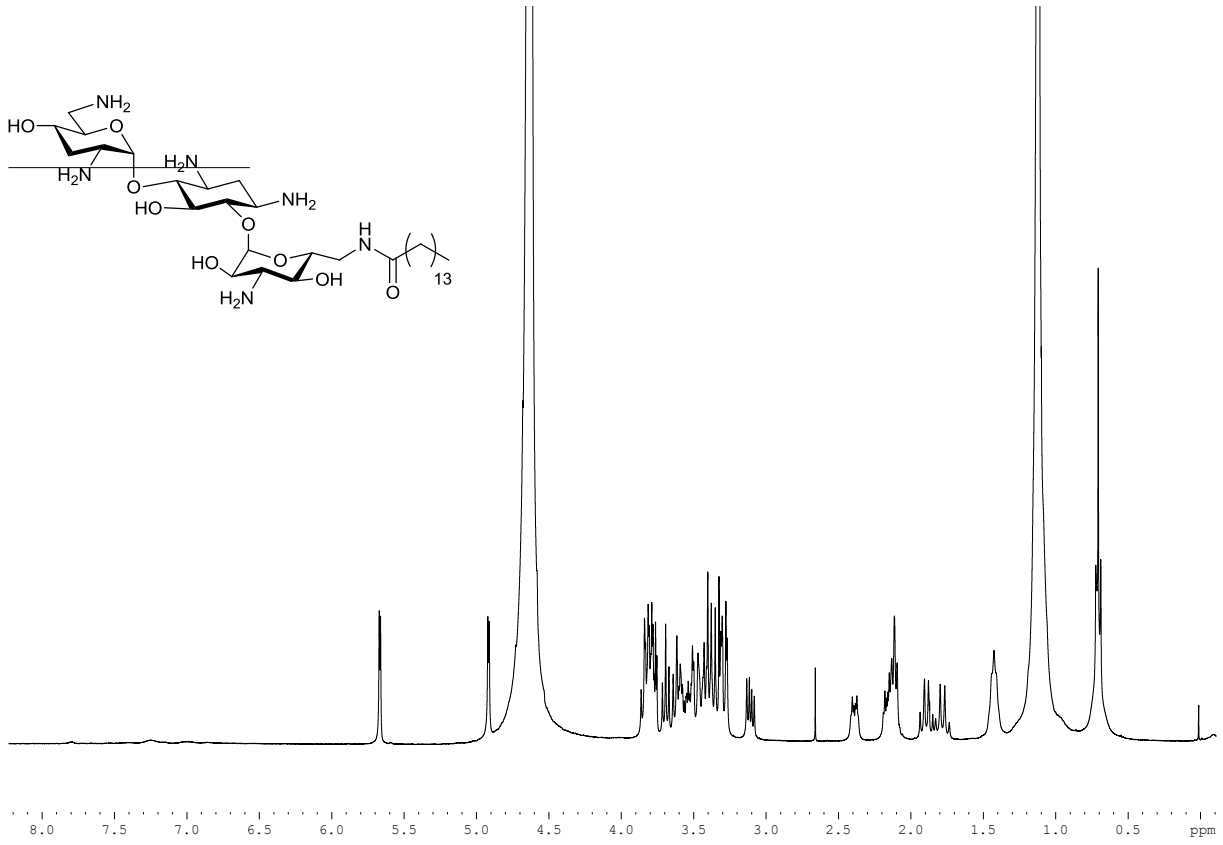


Fig. S14. ^{1}H NMR for 6"-amide tobramycin analogue **5b**.

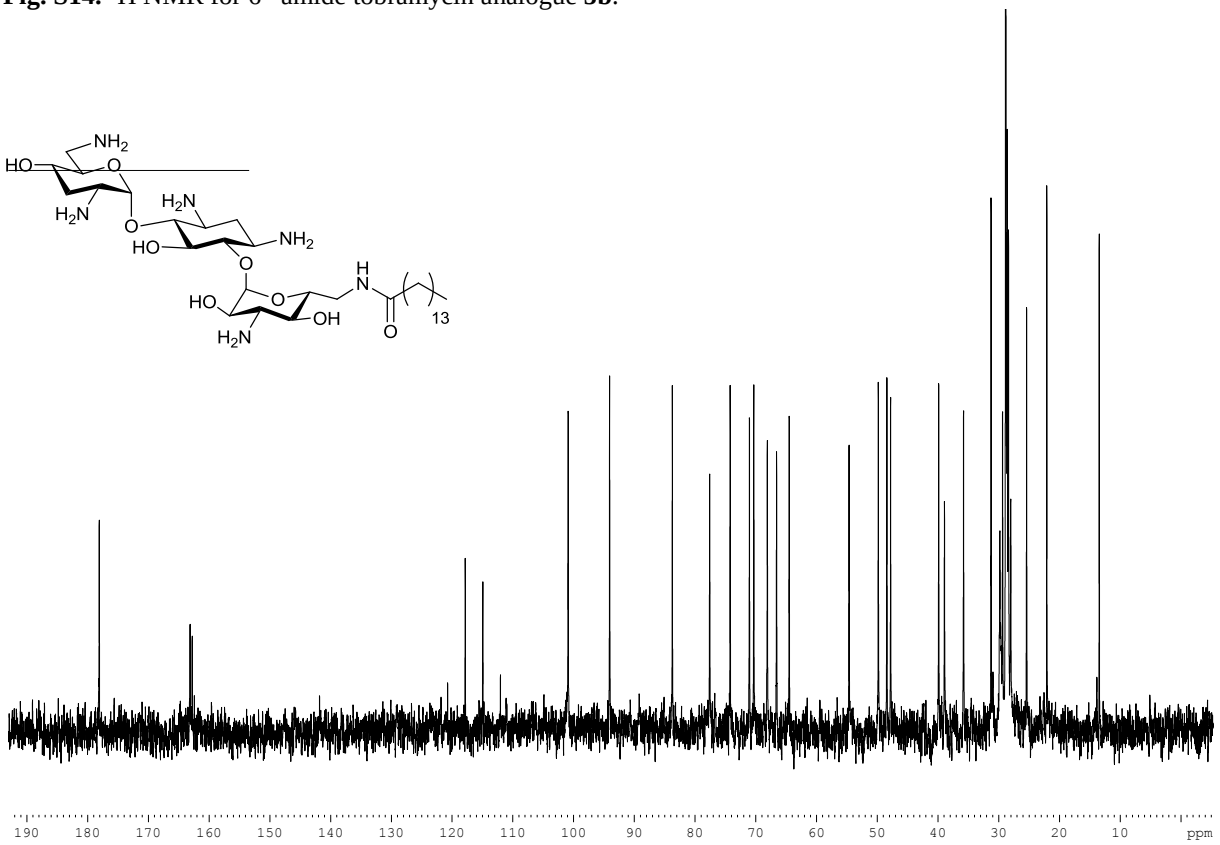


Fig. S15. ^{13}C NMR for 6"-amide tobramycin analogue **5b.**

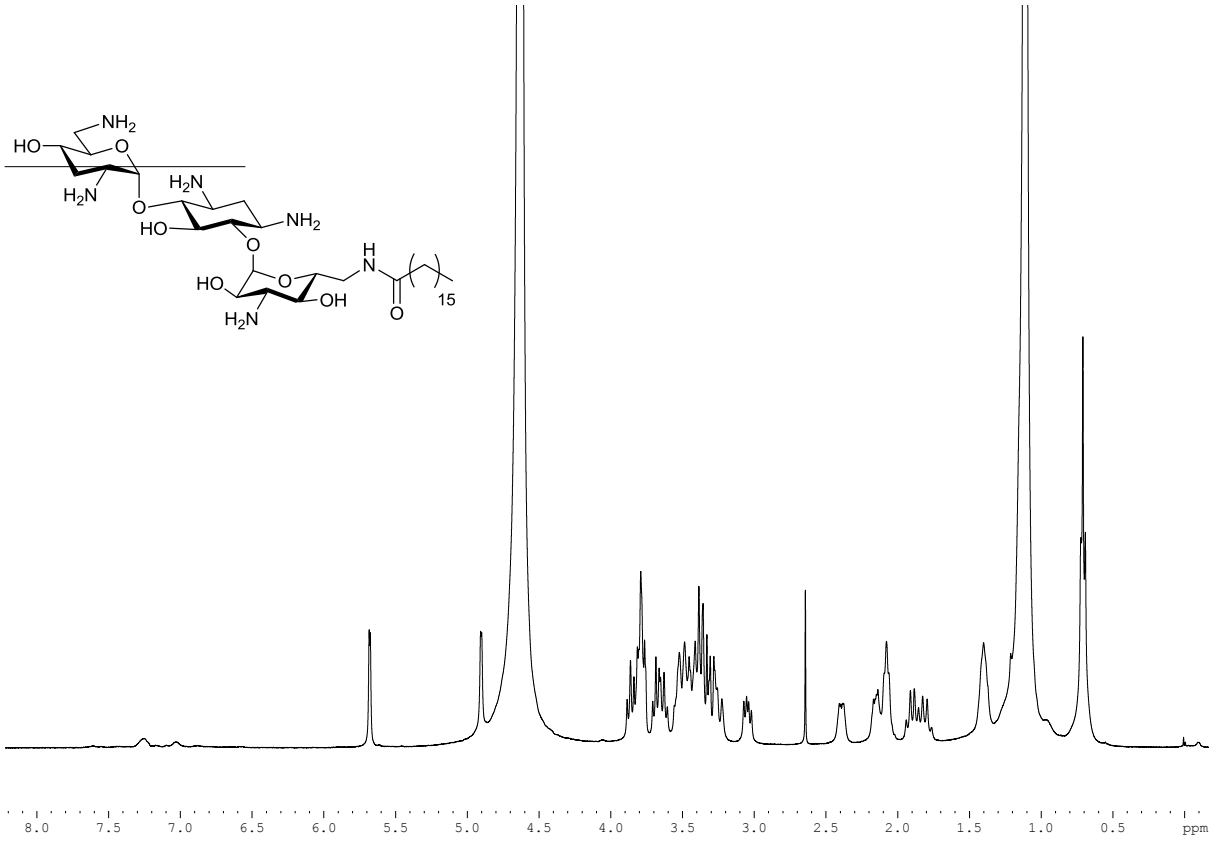


Fig. S16. ^{1}H NMR for 6"-amide tobramycin analogue **5c**.

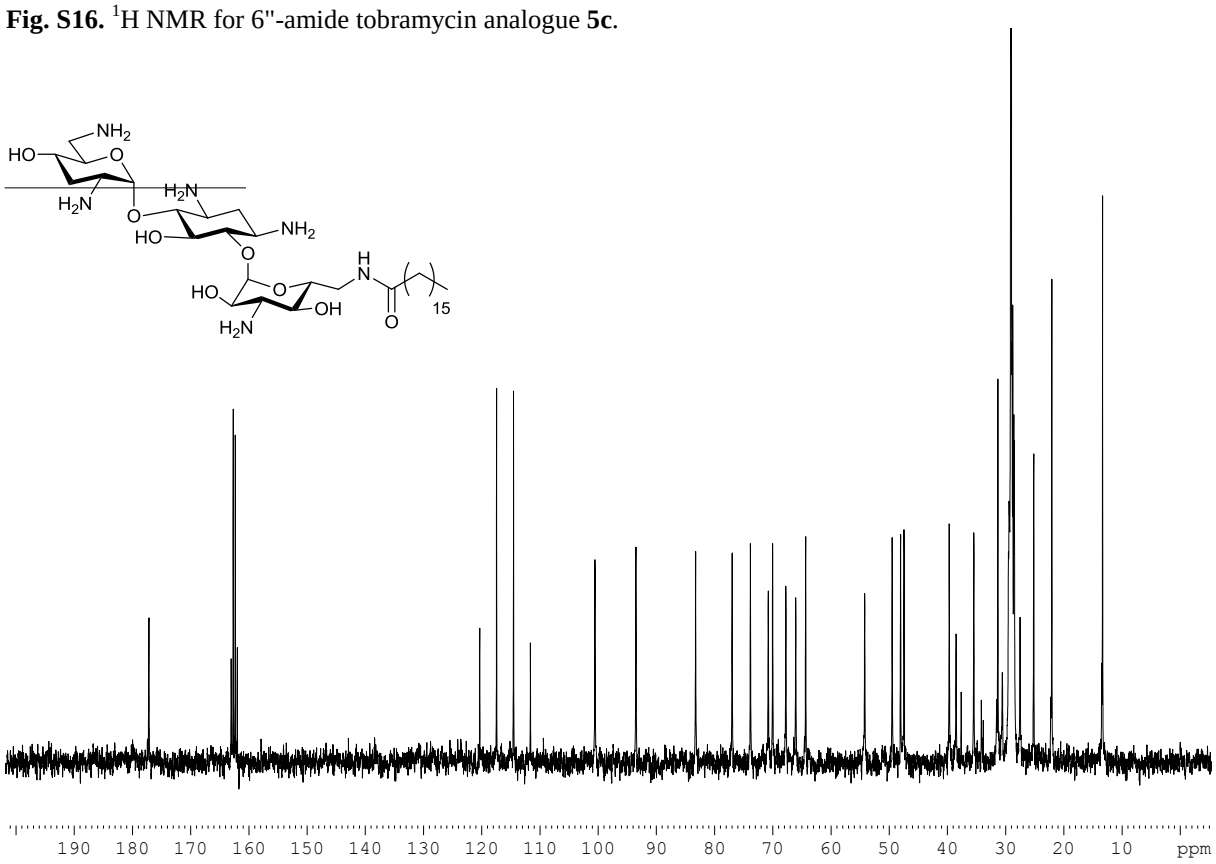


Fig. S17. ^{13}C NMR for 6"-amide tobramycin analogue **5c.**