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Supporting Information

An Advanced Apralog with Increased in vitro and in vivo Activity toward Gram-negative Pathogens and Reduced ex vivo Cochleotoxicity

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General Experimental

All experiments were carried out under a dry argon atmosphere unless otherwise specified. All reagents and solvents were purchased from commercial suppliers and were used without further purification unless otherwise specified. Chromatographic purifications were carried over silica gel (230-400 mesh). Thin layer chromatography was performed with precoated glass backed plates (w/UV 254). TLC plates were visualized by UV irradiation (254 nm) and by charring with sulfuric acid in ethanol (20:80, v/v) or with ceric ammonium molybdate solution [$Ce(SO_4)_2$: 4 g, $(NH_4)_6Mo_7O_{24}$: 10 g, H_2SO_4: 40 mL, H_2O: 360 mL]. Optical rotations were measured at 589 nm and 21 ºC on a digital polarimeter with a path length of 10 cm. ^1H and ^{13}C NMR spectra of all compounds were recorded using at 600 MHz unless otherwise specified and assignments made with the help of COSY, HMBC, and HSQC spectra. ESIHRMS were recorded using a time-of-flight mass spectrometer fitted with an electrospray source.

Synthesis and Characterization

5-Azido-3-*O*-(2-azidoethyl)-5-deoxy-1,2-*O*-isopropylidene-α-D-ribofuranose (6): To a stirred solution of **5** (1.31 g, 6.07 mmol) in tetrahydrofuran (12 mL) at 0 °C was added NaH (1.02 g, 25.4 mmol, 60% dispersion in mineral oil). After 10 mins, a solution of 2-azidoethyltosylate (6.13 g, 25.4 mmol) in tetrahydrofuran (11 mL) was added and the reaction mixture was warmed to room temperature. After 17 h, the reaction was quenched with saturated ammonium chloride and diluted with ethyl acetate. The organic layer was washed with 1N HCl and brine, dried over Na_2SO_4, and concentrated to dryness. The residue was purified via silica gel chromatography, eluting with a gradient of 5%-50% ethyl acetate in hexanes (Rf = 0.3 in 20% ethyl acetate/hexanes) to give the desired compound **6** as a colorless oil (796 mg, 46%). $[\alpha]_D^{21}$ = +73.4 (*c* 0.4, $CHCl_3$); ^1H NMR (600 MHz, $CDCl_3$) δ 5.84 (d, *J* = 3.7 Hz, 1H, H-1), 4.68 (t, *J* = 4.0 Hz, 1H, H-2), 4.18 (ddd, *J* = 8.9, 3.8, 2.7 Hz, 1H, H-4), 3.93 (ddd, *J* = 10.2, 5.6, 3.4 Hz, 1H, CH_2O), 3.81 (dd, *J* = 8.9, 4.4 Hz, 1H, H-3), 3.75 (dd, *J* = 13.6, 2.7 Hz, 1H, H-5), 3.70 (ddd, *J* = 10.2, 7.6, 3.3 Hz, 1H, CH_2O), 3.52 (ddd, *J* = 13.4, 7.6, 3.4 Hz, 1H, C*H*$_2$CH$_2$O), 3.41 – 3.32 (m, 2H, H-5, C*H*$_2$CH$_2$O), 1.60 (s, 3H, CH_3), 1.39 (s, 3H, CH_3); ^{13}C NMR (150 MHz, $CDCl_3$) δ 113.4 (*C*$(CH_3)_2$), 104.2 (C-1), 79.3 (C-3), 77.4 (C-2), 77.1 (C-4), 69.4 (CH_2O), 50.6 (*C*H_2CH$_2$O), 50.4 (C-5), 26.7 (CH_3), 26.59 (CH_3); ESI-HRMS: m/z calcd. for $C_{10}H_{16}N_6O_4Na$ $[M+Na]^+$ 307.1125; found, 307.1112.

1,2-Di-*O*-acetyl-5-azido-3-*O*-(2-azidoethyl)-5-deoxy-D-ribofuranose (7): Compound **6** (360 mg, 1.3 mmol) was dissolved in aqueous acetic acid (80%, 5 mL) and heated to reflux. After 3.5 h, the reaction mixture was concentrated to dryness and co-evaporated thrice with pyridine. The crude mixture was dissolved in acetic anhydride (1.3 mL) and pyridine (1.3 mL) followed by addition of DMAP (52 mg, 0.43 mmol). After 2 h, the reaction mixture was quenched with methanol and diluted in ethyl acetate. The organic layer was washed with 1N HCl, saturated aqueous $NaHCO_3$, and brine, dried over Na_2SO_4, and concentrated to dryness. The residue was purified via silica gel chromatography, eluting with a gradient of 0%-50% ethyl acetate in hexanes (Rf = 0.35 in 20% ethyl acetate/hexanes) to give a mixture of anomers **7** (0.25:1 α:β, 348 mg, 82% over 2 steps) as a yellow oil that was used without further purification. ESI-HRMS: m/z calcd. for $C_{11}H_{16}N_6O_6Na$ $[M+Na]^+$ 351.1024; found, 351.1008. α: ^1H NMR (600 MHz, $CDCl_3$) δ 6.43 (d, *J* = 4.5 Hz, 1H, H-1), 5.17 (dd, *J* = 6.5, 4.5 Hz, 1H, H-2), 4.36 (d, *J* = 4.2 Hz, 1H, H-4), 4.03 (dd, *J* = 6.5, 4.7 Hz, 1H, H-3), 3.79 – 3.73 (m, 1H, CH_2O), 3.70 – 3.62 (m, 2H, CH_2O, H-5), 3.48 (d, *J* = 3.8 Hz, 1H, H-5), 3.39 – 3.32 (m, 2H, C*H*$_2$CH$_2$O), 2.18 (s, 3H, CH_3CO), 2.14 (s, 3H, CH_3CO); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.0 (CH_3*C*O), 169.8 (CH_3*C*O), 94.5 (C-1), 82.3 (C-4), 77.1 (C-3), 71.0 (C-2),

70.7 (CH_2O), 51.9 (C-5), 50.8 (CH_2CH_2O), 21.00 (CH_3CO), 20.5 (CH_3CO); β: ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.18 (s, 1H, H-1), 5.34 (d, *J* = 4.1 Hz, 1H, H-2), 4.26 (dd, *J* = 8.3, 4.2 Hz, 1H, H-3), 4.22 (dt, *J* = 8.3, 3.1 Hz, 1H, H-4), 3.84 – 3.79 (m, 1H, CH_2O), 3.79 – 3.73 (m, 1H, H-5), 3.70 – 3.62 (m, 1H, CH_2O), 3.39 – 3.32 (m, 2H, CH_2CH_2O), 3.28 (dd, *J* = 13.7, 3.1 Hz, 1H, H-5), 2.20 (s, 3H, CH_3CO), 2.14 (s, 3H, CH_3CO). ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.8 (CH_3CO), 169.0 (CH_3CO), 98.1 (C-1), 80.5 (C-4), 77.2 (C-3), 73.3 (C-2), 70.4 (CH_2O), 50.72 (CH_2CH_2O), 50.68 (C-5), 21.03 (CH_3CO), 20.7 (CH_3CO).

5-*O*-[2‴-*O*-Acetyl-5‴-azido-3‴-*O*-(2-azidoethyl)-5’’’-deoxy-β-D-ribofuranosyl]- 1,3,2′,4″-tetra(desamino)-1,3,2′,4″-tetraazido-6,2″,3″,6″-tetra-*O*-benzoyl-6′,7′-oxazolidino-apramycin (9): To a stirred solution of **7** (364 mg, 1.11 mmol) and **8** (508 mg, 0.47 mmol) in dichloromethane (10.5 mL) at room temperature with oven-dried 4Å molecular sieves was added $BF_3{\cdot}OEt_2$ (0.33 mL, 2.67 mmol). Additional $BF_3{\cdot}OEt_2$ (0.8 mL, 6.48 mmol) was added over the course of the reaction whenever progress stopped. After 23 h, the reaction was quenched with excess triethylamine. The reaction mixture was filtered through Celite®, diluted with ethyl acetate, washed with saturated aqueous sodium bicarbonate and brine, dried over Na_2SO_4, and concentrated to dryness. The residue (an 0.3:1 α:β mixture) was purified via silica gel chromatography, eluting with a gradient of 0.3-1.0% dichloromethane in methanol (Rf = 0.3 in 1.8% methanol in dichloromethane) to give the desired anomer **9** as a white solid (335 mg, 44%). $[\alpha]_D^{21}$ = +54.5 (*c* 0.47, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.21 – 8.16 (m, 2H, Ar), 8.13 – 8.08 (m, 2H, Ar), 8.08 – 8.03 (m, 2H, Ar), 8.01 – 7.96 (m, 2H, Ar), 7.65 (ddq, *J* = 9.3, 5.8, 1.3 Hz, 2H, Ar), 7.61 – 7.56 (m, 2H, Ar), 7.54 (tdd, *J* = 7.4, 4.3, 1.7 Hz, 4H, Ar), 7.48 – 7.41 (m, 4H, Ar), 6.02 (t, *J* = 10.1 Hz, 1H, H-3’’), 5.73 (d, *J* = 3.7 Hz, 1H, H-1’’), 5.52 (d, *J* = 3.6 Hz, 1H, H-1’), 5.31 (s, 1H, H-1’’’), 5.23 – 5.17 (m, 2H, H-6, H-2’’), 4.99 (d, *J* = 4.4 Hz, 1H, H-2’’’), 4.84 (d, *J* = 5.2 Hz, 1H, H-8’), 4.77 (dd, *J* = 7.1, 3.6 Hz, 1H, H-6’), 4.70 (dd, *J* = 12.1, 2.5 Hz, 1H, H-6’’), 4.65 (dd, *J* = 12.1, 5.4 Hz, 1H, H-6’’), 4.27 (dd, *J* = 10.3, 3.6 Hz, 1H, H-5’), 4.10 (ddd, *J* = 10.6, 5.4, 2.5 Hz, 1H, H-5’’), 3.99 (ddd, *J* = 7.9, 5.6, 3.2 Hz, 1H, H-4’’’), 3.90 (t, *J* = 10.1 Hz, 1H, H-4’’), 3.83 (t, *J* = 9.1 Hz, 1H, H-5), 3.80 – 3.76 (m, 2H, H-4, H-7’), 3.65 (dd, *J* = 7.9, 4.4 Hz, 1H, H-3’’’), 3.60 (ddd, *J* = 12.5, 10.2, 4.4 Hz, 1H, H-1), 3.55 (dd, *J* = 11.0, 4.3 Hz, 1H, H-4’), 3.50 – 3.42 (m, 2H, H-3, H-5’’’), 3.34 (dd, *J* = 13.2, 5.7 Hz, 1H, H-5’’’), 3.16 (dt, *J* = 13.1, 4.2 Hz, 1H, H-2’), 3.03 (ddd, *J* = 13.2, 5.5, 4.2 Hz, 1H, CH_2CH_2O), 3.00 – 2.95 (m, 1H, CH_2CH_2O), 2.94 (s, 3H, N-CH_3), 2.92 (m, 2H, CH_2O), 2.51 (dt, *J* = 13.2, 4.6 Hz, 1H, H-2eq), 1.86 (s, 3H, CH_3CO), 1.83 – 1.78 (m, 1H, H-3’eq), 1.72 (q, *J* = 12.6 Hz, 1H, H-2ax), 1.36 (dt, *J* = 12.8, 11.5 Hz, 1H, H-3’ax); ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.9 (CH_3CO), 166.1, 1656.0, 165.7, 164.7 (Ph*C*O), 157.0 (NC=O), 133.9, 133.8, 133.7, 130.3, 130.0, 129.8, 129.3, 129.0, 128.8, 128.8, 128.7, 128.7, 128.6, 128.5 (Ar), 107.2 (C-1’’’), 99.5 (C-8’), 96.7 (C-1’), 95.5 (C-1’’), 80.1 (C-5), 80.0 (C-4’’’), 77.9 (C-4), 77.8 (C-3’’’), 74.6 (C-6), 73.3 (C-2’’’), 71.4 (C-6’, C-2’’), 70.5 (C-3’’), 69.8 (C-5’’), 69.4 (CH_2O), 66.4 (C-5’), 65.8 (C-4’), 63.4 (C-6’’), 61.0 (C-4’’), 60.3 (C-7’), 58.9 (C-1), 58.3 (C-3), 56.2 (C-2’), 52.3 (C-5’’’), 50.4 (CH_2CH_2O), 31.5 (C-2), 30.2 (N-CH_3), 29.0 (C-3’), 20.30 (CH_3CO); ESI-HRMS: m/z calcd. for $C_{59}H_{59}N_{19}O_{20}Na$ $[M+Na]^+$ 1376.4076; found, 1376.4038. Although not isolated pure, the minor α-anomer was identified in the crude reaction mixture by the following diagnostic signals: δ 4.88 (d, *J* = 3.6 Hz, 1H), 4.66 (t, *J* = 2.7 Hz, 1H), 2.83 (s, 3H).

5-*O*-[5-Amino-3-*O*-(2-aminoethyl)-5-deoxy-β-D-ribofuranosyl] apramycin heptaacetate salt (4): To a stirred solution of **9** (130 mg, 0.097 mmol) in 1,4-dioxane (5 mL) was added aqueous NaOH (3N, 5 mL), and the reaction mixture was heated to reflux. After 6 h, the reaction mixture was cooled to 55 °C and trimethylphosphine (1M in THF, 0.5 mL) was added. After 4 h, the reaction mixture was neutralized with glacial acetic acid and concentrated to dryness. The crude residue was dissolved in minimal aqueous acetic

acid (10%) and purified using a CM Sephadex C-25 column, eluting with 0.1-1.2% NH_4OH in water to give **4** as a white solid (58 mg, 53%); $[\alpha]_D^{21}$ = +72.5 (*c* 0.7, H_2O); ^1H NMR (900 MHz, D_2O) δ 5.75 (d, *J* = 3.8 Hz, 1H, H-1'), 5.43 (d, *J* = 4.0 Hz, 1H, H-1''), 5.37 (d, *J* = 1.5 Hz, 1H, H-1'''), 5.13 (d, *J* = 8.6 Hz, 1H, H-8'), 4.51 (t, *J* = 2.8 Hz, 1H, H-6'), 4.38 (dd, *J* = 4.6, 1.5 Hz, 1H, H-2'''), 4.16 (td, *J* = 7.7, 4.0 Hz, 1H, H-4'''), 4.01 (dd, *J* = 7.3, 4.6 Hz, 1H, H-3'''), 3.92 – 3.85 (m, 4H, H4, H5, H5'', H4'), 3.83 (t, *J* = 10.0 Hz, 1H, H-3''), 3.81 – 3.77 (m, 2H, CH_2O, H-6''), 3.77 – 3.74 (m, 1H CH_2O), 3.72 (dd, *J* = 12.5, 4.7 Hz, 1H, H-6''), 3.66 – 3.61 (m, 3H, H-5', H-2'', H-6), 3.56 (dt, *J* = 12.8, 4.3 Hz, 1H, H-2'), 3.31 (m, 2H, H-3, H-5'''), 3.28 (dd, *J* = 8.6, 2.8 Hz, 1H, H-7'), 3.23 (ddd, *J* = 12.5, 10.5, 4.1 Hz, 1H, H-1), 3.17 (m, 4H, CH_2CH_2O, H-5''', H-4''), 2.70 (s, 3H, N-CH_3), 2.32 – 2.28 (m, 2H, H-3'eq, H-2eq), 1.95 (q, *J* = 11.9 Hz, 1H, H-3ax), 1.85 (s, 21H, H_3CCO), 1.68 (q, *J* = 12.6 Hz, 1H, H-2ax); ^{13}C NMR (226 MHz, D_2O) δ 181.0 (H_3CCO), 109.0 (C-1'''), 94.5 (C-1'), 93.3 (C-1''), 93.0 (C-8'), 83.4 (C-5), 79.2 (C-3'''), 77.14 (C-4'''), 75.5 (C-4), 72.8 (C-2'''), 72.33 (C-6), 70.3 (C-2''), 69.9 (C-5''), 69.6 (C-5'), 68.8 (C-3''), 66.06 (C-4'), 66.03 (CH_2O), 62.8 (C-6'), 60.4 (C-6''), 59.7 (C-7'), 52.1 (C-4''), 50.3 (C-1), 48.9 (C-3), 47.9 (C-2'), 42.2 (C-5'''), 39.3 (CH_2CH_2O), 30.2 (N-CH_3), 29.3 (C-2), 27.5 (C-3'), 22.98 (H_3CCO); ESI-HRMS: m/z calcd. for $C_{28}H_{56}N_7O_{14}$ $[M+H]^+$ 714.3885; found, 714.3868.

Biological Testing

Cell-free translation inhibition assays. The S30 fraction of *Mycobacterium smegmatis* cell extracts was used for bacterial cell-free translation inhibition assays as described previously.[1] Inhibition of mammalian ribosomes has been assessed with a commercial Rabbit Reticulocyte Lysate System (Promega) as described previously.[1] Firefly luciferase mRNA was used as reporter to monitor translation activity. Luminescence was measured using a luminometer Flx800 (Bio-Tek Instruments).

Analysis of aminoglycoside-A site interactions with 70S ribosomes by quantitative footprinting. Ribosomes were isolated from *E. coli* MRE 600 using the sucrose gradient sedimentation method.[2] Briefly, a 3 mL volume of LB broth was inoculated with MRE 600 and grown overnight with 250 rpm shaking at 37 °C. Then, 1 L of LB broth was inoculated with 1 mL of overnight culture (1:1000 dilution) and growth continued with 250 rpm shaking at 37 °C. The optical density at 600 nm (OD_{600}) was measured at 30 min intervals and the growth was stopped by placing the culture on ice when the OD_{600} reached 0.1. After cooling the cultures on ice for 20 min, the cells were pelleted by centrifugation at 6000 g, for 30 min at 4 °C. The supernatant was removed, and the cell pellet was resuspended in 4 mL of lysis buffer (20 mM HEPES-KOH, pH 7.5, 10 mM $MgCl_2$, 100 mM NH_4Cl, 4.6 mM 2-mercaptoethanol, and 0.5 mM EDTA). The solution was passed through a French Press twice to lyse cells using a 3/8-inch diameter piston to create 18,000 psi of pressure. The lysate was collected dropwise to ensure the complete lysis of cells. Then, DNaseI was added to the lysed sample to give a final concentration of 5 µg/mL and incubated on ice for 5 min. The lysate was then centrifuged at 15,000 rpm for 30 min at 4 °C. The upper 2/3 of the supernatant was carefully decanted into a sterile tube and the NH_4Cl was adjusted to a final concentration of 0.2 M. Then, the crude ribosome pelleting was initiated by centrifugation at 42,000 rpm for 4 h at 4 °C. After removing the supernatant, the crude ribosomes were dissolved in the ribosome buffer (20 mM HEPES-KOH; pH 7.5, 6 mM $MgCl_2$, 30 mM NH_4Cl, and 4.6 mM 2-mercaptoethanol). The Gradient Master program with time 2.25 min., angle 76, and speed 25 was used to prepare the sucrose gradient using 10% and 30% sucrose solutions in sterile Beckman tubes. Then, the OD_{260} of the dissolved ribosome samples was adjusted to 30 per sample and loaded on top of the sucrose gradient without disrupting it. Then, the tubes were centrifuged at 18,000 rpm for 18 h

at 4 °C. The separated ribosomes were then fractionated using the absorbance detector, monitoring the peak corresponding to 70S ribosomes. The isolated ribosomes were centrifuged at 24,000 rpm, for 24 h. After removing the supernatant, the purified ribosomes were stored in ribosome buffer (20 mM HEPES-KOH; pH 7.5, 6 mM $MgCl_2$, 30 mM NH_4Cl). The concentration was determined such that 1 unit of OD_{260} is equal to 23.5 pmol of 70S ribosomes.[1] The purity of the isolated ribosomes was checked by 0.8% agarose gel electrophoresis.

Ten picomoles of full ribosomes (final conc. of 0.2 mM) were activated by incubating at 37 °C for 15 min in the activation buffer (20 mM HEPES-KOH, pH 7.5, 6 mM $MgCl_2$, and 100 mM NH_4Cl). Activated ribosomes were then incubated with the probing buffer (80 mM HEPES-KOH, pH 7.0, 6 mM $MgCl_2$, and 100 mM NH_4Cl) at 37 °C for 10 min. Then, the DMS reaction was initiated by incubating the "no-drug control" with 2 µL DMS (final conc. of 25 mM), the "no-DMS control" with 2 µL of ddH_2O, and "test" samples with 2 µL of corresponding AG concentration series at 37 °C for 10 min. The DMS reaction of the "no-drug control" sample was quenched by adding 10 µL of stop buffer (3 M mercaptoethanol, 100 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$) to the "no-drug control" sample and to the "no-DMS control" sample. "Test" samples were also incubated with DMS (final conc. of 25 mM) at 37 °C for 10 min. DMS reactions of "test samples" were quenched by adding 10 µL of stop buffer. All the samples were subjected to ethanol precipitation followed by phenol-chloroform extraction to isolate reacted rRNA. A 2.5 V of ice-cold 100% ethanol and 0.1 V of 3 M NaOAc (pH 5.2) were added to rRNA and kept at -80 °C for 45 min. The pellet was collected by spinning down at 14,000 rpm for 20 min at 4 °C and removing the supernatant. The pellet was washed twice with ice-cold 70% ethanol followed by brief drying in the speed-vac. The pellet was dissolved in 50 µL of 1 M Tris-EDTA. Then, 1 V of phenol: chloroform: isoamylalcohol (PCI), 25: 24: 1 mixture was added and mixed the contents thoroughly. Samples were spun down at 12,000 rpm for 10 min at 4 °C and the upper aqueous layer was carefully decanted in to a new tube. The PCI extraction was repeated one more time. Then, 1 V of chloroform was\added, and the sample was vortexed thoroughly followed by spinning down at 12,000 rpm for 10 min at 4 °C and the upper aqueous layer was decanted into a new tube. The chloroform extraction was also repeated one more time. The ethanol precipitation was carried out and the pellet was dried using speed-vac. The purified RNA was used in the reverse transcription by primer extension using radio-labeled DNA primer.

The primer DNA (5'-GTTAAGCTACCTACTTCT) was selected to probe the region of interest (A1408) in the decoding region of bacterial ribosomes. For radio-labeling, 50 pmol of DNA (Integrated DNA technologies, Coralville, IA) was mixed with 3 µL of 10× T4 polynucleotide kinase (PNK) buffer (New England Biolabs, Ipswich, MA), 10 µCi of fresh [γ-^{32}P]-ATP (Perkin Elmer), and PNK enzyme (New England Biolabs, Ipswich, MA) in a total reaction volume of 30 µL. The reaction was incubated at 37 °C for 45 min, followed by 70 °C for 10 min to inactivate the PNK enzyme. The labeled DNA was desalted by ethanol precipitation, and the resulting pellet was dried and then dissolved in 50 µL of nuclease-free water. Five hundred ng of purified RNA was mixed with ~1 x 10^5 CPM of radio-labeled primer and incubated at 80 °C for 3 min. Then, the sample was allowed to come to room temperature and transferred to ice. Next, a reverse transcription mixture containing reverse transcriptase (Promega, Madison, WI), reaction buffer (Promega, Madison, WI), $MgCl_2$ (final concentration of 5 mM) and deoxynucleotide triphosphates (GenScript, Piscataway, NJ; final concentration of 0.5 mM) was added. For the sequencing reactions, each of the four dideoxynucleotide triphosphate (Roche Diagnostics, Indianapolis, IN), ddATP, ddGTP, ddCTP, and ddTTP was added to four separate reactions for a final concentration of 2 mM. The total volume was adjusted to 5 µL using deionized water. Samples were incubated at 43 °C for 1 h. The reverse transcription reaction was quenched by adding 2 µL of denaturing dye followed by boiling for 3 min and quickly transferring to ice. The radioactivity of the resulting cDNA product was measured by scintillation counting. The cDNA product was resolved on a 10% denaturing polyacrylamide gel by loading

the same number of counts of each sample. The gel was run at 1300 V in 1× Tris-boric-EDTA buffer (pH 8.3) and an image was obtained with the Typhoon FLA 9500 (GE Healthcare, Chicago, IL) with reader settings for phosphor imaging and a PMT value of 800. Quantification of the bands was carried out using Image Quant 5.1 (Amersham). Background correction was performed for each band. For normalization of the DMS-modification-specific primer extension stop at A1408, the band corresponding to a base-dependent primer extension stop band (C1400) was selected. The percent DMS reactivity and percent protection at N1 of A1408 was calculated as shown below.

Eq. (1) --------- $background\ corrected\ intensity\ (I) = I_{obs} - I_{background}$

Eq. (2) --------- $normalized\ I_{A1408} = \frac{I_{A1408}}{I_{c1400}}$

Eq. (3) --------- $\%\ DMS\ rectivity = (1 - normalized\ I_{A1408}) * 100$

Eq. (4) --------- $\%\ protection = (100 - \%\ DMS\ rectivity)$

Antibacterial inhibition assays. The minimal inhibitory concentrations (MIC) of synthesized compounds were determined by broth microdilution assays according to CLSI reference methodology M07[3] as described previously.[4] A summary of bacterial strains used in this study is provided in Table S1. Clinical bacterial isolates were obtained from the diagnostic laboratories of the Institute of Medical Microbiology, University of Zurich. Whole genome sequencing of the bacterial isolates and bioinformatic annotation of resistance genes was done as described previously.[4]

Cochlear Explant Study

Animals: FVB mice were purchased as breeder pairs from the Jackson Laboratory. Mice were housed in the animal facility of the Children's Research Institute at the Medical University of South Carolina (MUSC) and kept at 22 ± 1 °C under a standard 12:12h light-dark cycle with free access to water and a regular mouse diet (Irradiated Lab Diet #5V75). All research protocols were approved by the Institutional Animal Care and Use Committee at MUSC. Animal care was under the supervision of the Division of Laboratory Animal Resources at MUSC.

Cochlear Explants. Compounds were screened for toxicity to hair cells in cochlear explants from FVB mice on postnatal day 3. The explants were placed on collagen-coated 10-millimeter round coverslips (Microscopy Products for Science and Industry, cat# 260367), submerged in a four-well dish containing 1 mL of serum-free Basal Medium Eagle plus serum-free supplement (Invitrogen), 1% BSA, 2 mM glutamine, and 5 mg/mL glucose, and incubated (37 °C, 5% CO_2) for 4 h. Subsequentlyan additional 1 mL of culture medium was added for an overnight incubation. The medium was then exchanged for new medium containing the drugs, and incubation continued for 72 h. Cultures were fixed overnight in 4% (vol/vol) paraformaldehyde at 4 °C, and then permeabilized for 30 min with 3% (vol/vol) Triton X-100 in PBS. Following incubation at room temperature for 1 h with Alexa-Flour-594-phalloidin (ThermoFisher Scientific) and at least three final washes with PBS, the cover slip with the explant was removed from the well plate and 8 µL of mounting agent (Fluoro-gel with Tris buffer, Electron Microscopy Sciences, #17985-10) were added. The mounted explant was covered with another round coverslip, the edges of the sandwich were sealed with nail polish and the sample placed on microscope slides. Hair cell

presence was determined by fluorescent microscopy of the phalloidin-stained stereociliary bundles and circumferential F-actin rings on the cuticular plate.

In-vivo Efficacy studies. All animal efficacy studies were performed by Evotec International GmbH under UK Home Office Licensure P2BC7D240 and with local ethical committee clearance. All studies were performed by technical staff who have completed parts A, B and C of the UK Home Office Personal License course and hold current personal licenses. All experiments were performed in dedicated Biohazard 2 facilities (this site holds a Certificate of Designation). Male mice used in these studies were supplied by Charles River UK and were specific pathogen free. The strain of mouse used was Hsd:ICR (CD-1®), which is a well characterized outbred strain. Mice were 11-15 g on receipt and were allowed to acclimatize for minimum of 7 days prior to infection. Mice were approximately 30 g at the start of the study. Mice were housed in sterile individual ventilated cages exposing animals at all times to HEPA filtered sterile air. Mice had free access to food and water (sterile) and had sterile aspen chip bedding. The room temperature was 22 °C ± 1 °C, with a relative humidity of 50-60% and maximum background noise of 56dB. Mice were exposed to 12 hour light/dark cycles with dawn/dusk phases.

Neutropenic thigh infection model. All mice were rendered neutropenic by immunosuppression with cyclophosphamide at 150 mg/kg 4 days before infection and 100 mg/kg 1 day before infection by intraperitoneal injection. The immunosuppression regime leads to neutropenia starting 24 hours post administration of the first injection, which continues throughout the study. Mice were infected approximately 24 hours after the second dose of immunosuppressive agent with an inoculum of 2.17x106 CFU/thigh *E. coli* ATCC25922 in phosphate buffered saline (PBS). Mice were infected by intramuscular injection of 50 µL inoculum into both lateral thigh muscles under inhaled anaesthesia using 2.5% isofluorane in 97.5% oxygen. Whilst still under anaesthesia mice were administered a single dose of buprenorphine (0.03 mg/kg) subcutaneously for pain relief. One hour post infection, animals were administered subcutaneously at 6 mg/kg with **4** for injection. Five hours post infection all remaining animals were euthanized by a pentobarbitone overdose. Thigh samples were homogenized in ice cold sterile phosphate buffered saline using a Precellys bead beater; the homogenates were quantitatively cultured onto CLED agar and incubated at 37 °C for 18 – 24 hours before colonies were counted. All mice survived the infection in the study period.

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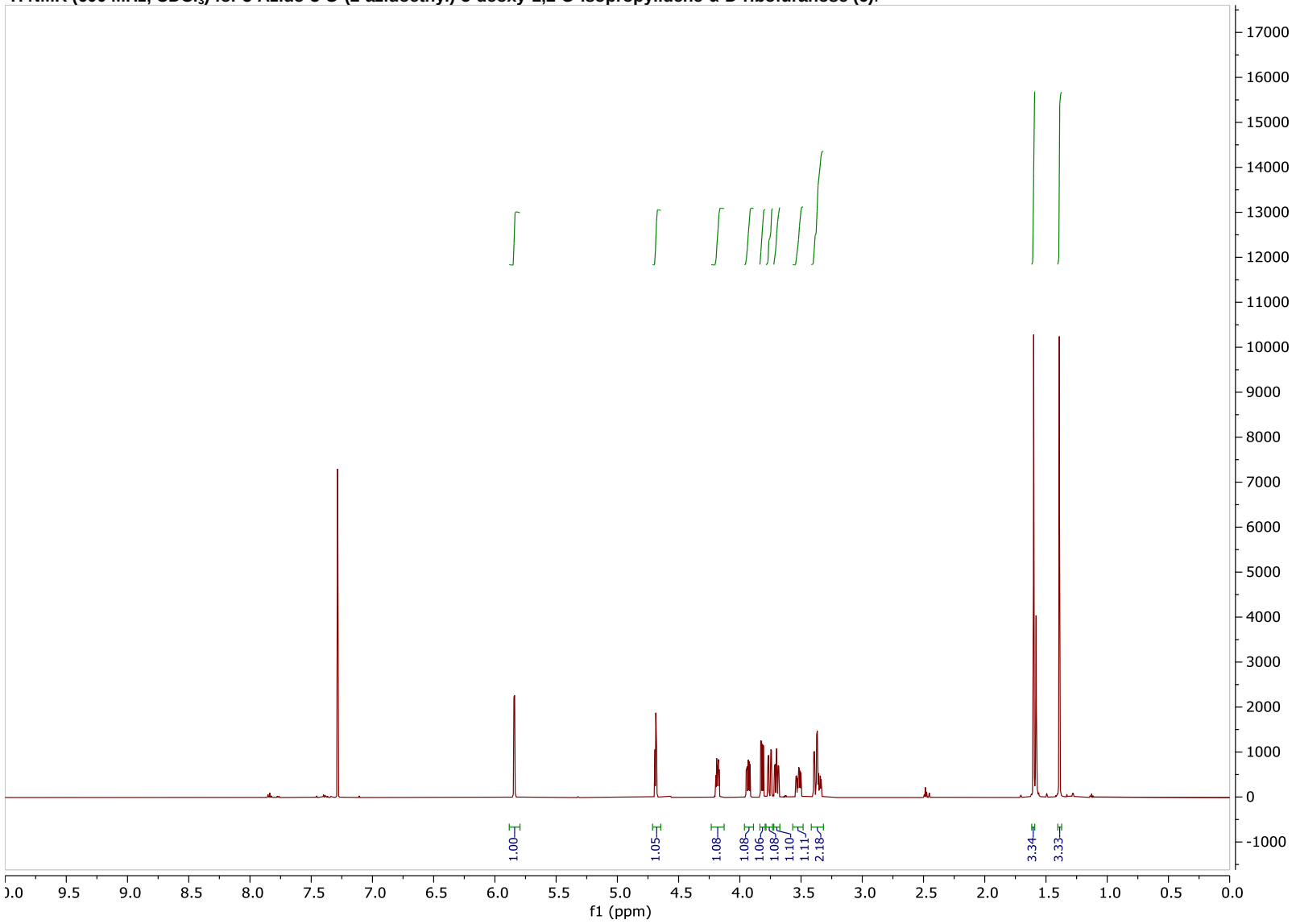
Author Contributions

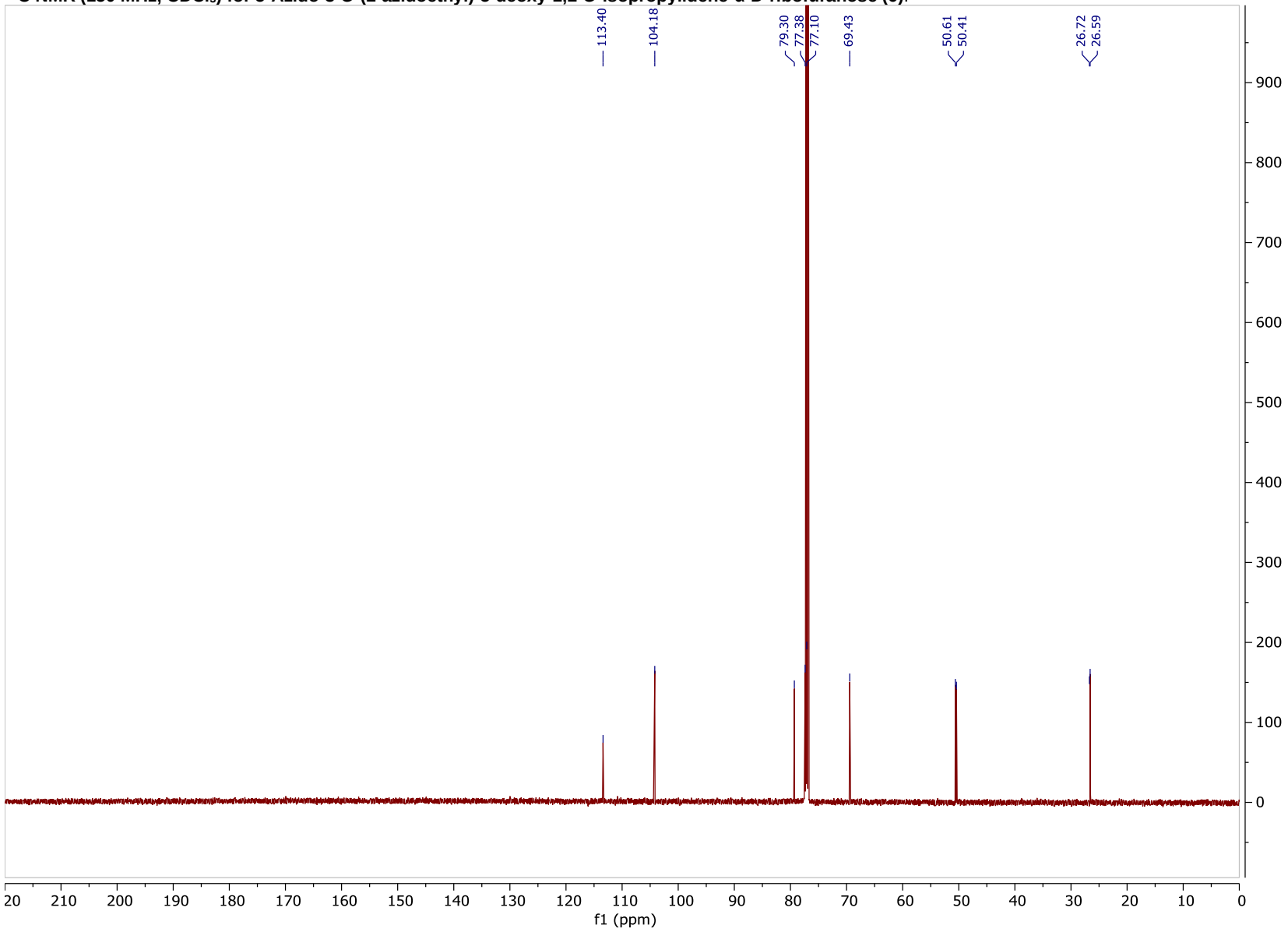
AV, DC and ECB designed the project. DC and ECB secured funding for the project. SNH and ECB designed and oversaw the microbiological screening. CSC designed the footprinting experiments. JS and SHS designed and oversaw the studies with cochlear explants. AS and JCKQ designed and conducted the chemical synthesis. PW conducted the footprinting studies. TJ, MG, and KH conducted the microbiological assays and SX carried out the studies with cochlear explants. The manuscript was written by DC and ECB with input from all authors.

NMR Spectra

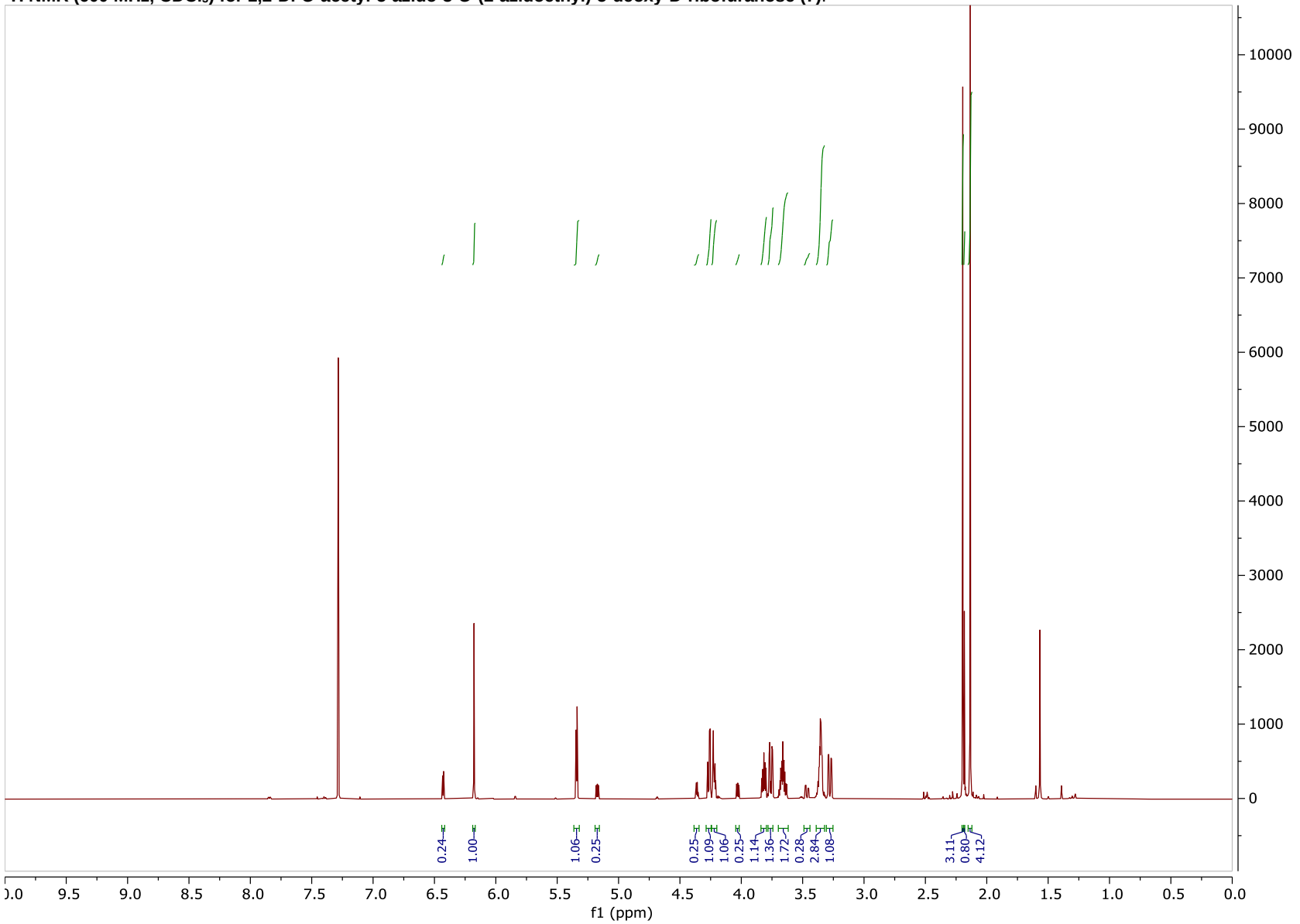
^{1}H NMR (600 MHz, $CDCl_3$) for 5-Azido-3-*O*-(2-azidoethyl)-5-deoxy-1,2-*O*-isopropylidene-α-D-ribofuranose (6):



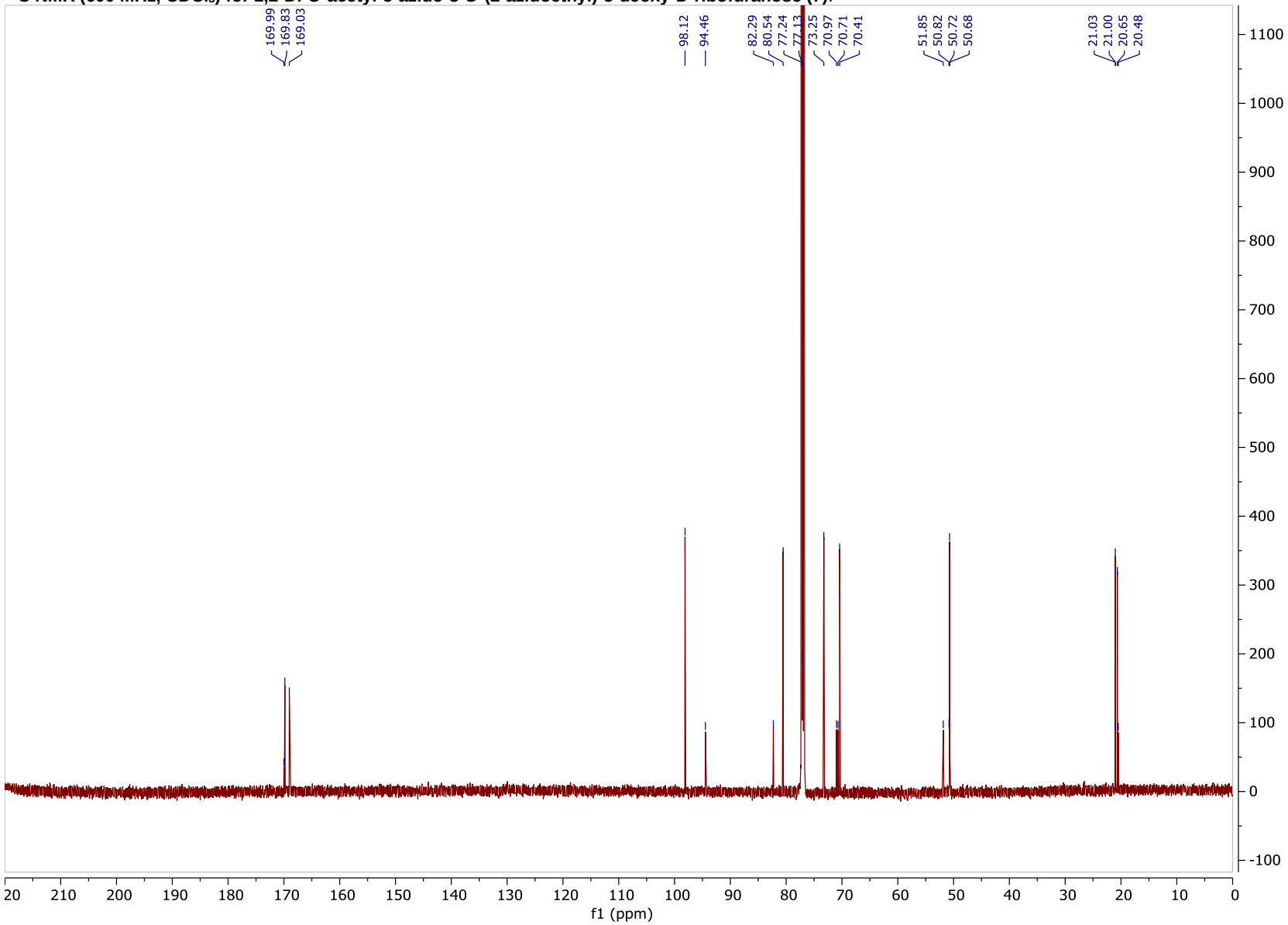
^{13}C NMR (150 MHz, $CDCl_3$) for 5-Azido-3-*O*-(2-azidoethyl)-5-deoxy-1,2-*O*-isopropylidene-α-D-ribofuranose (6):



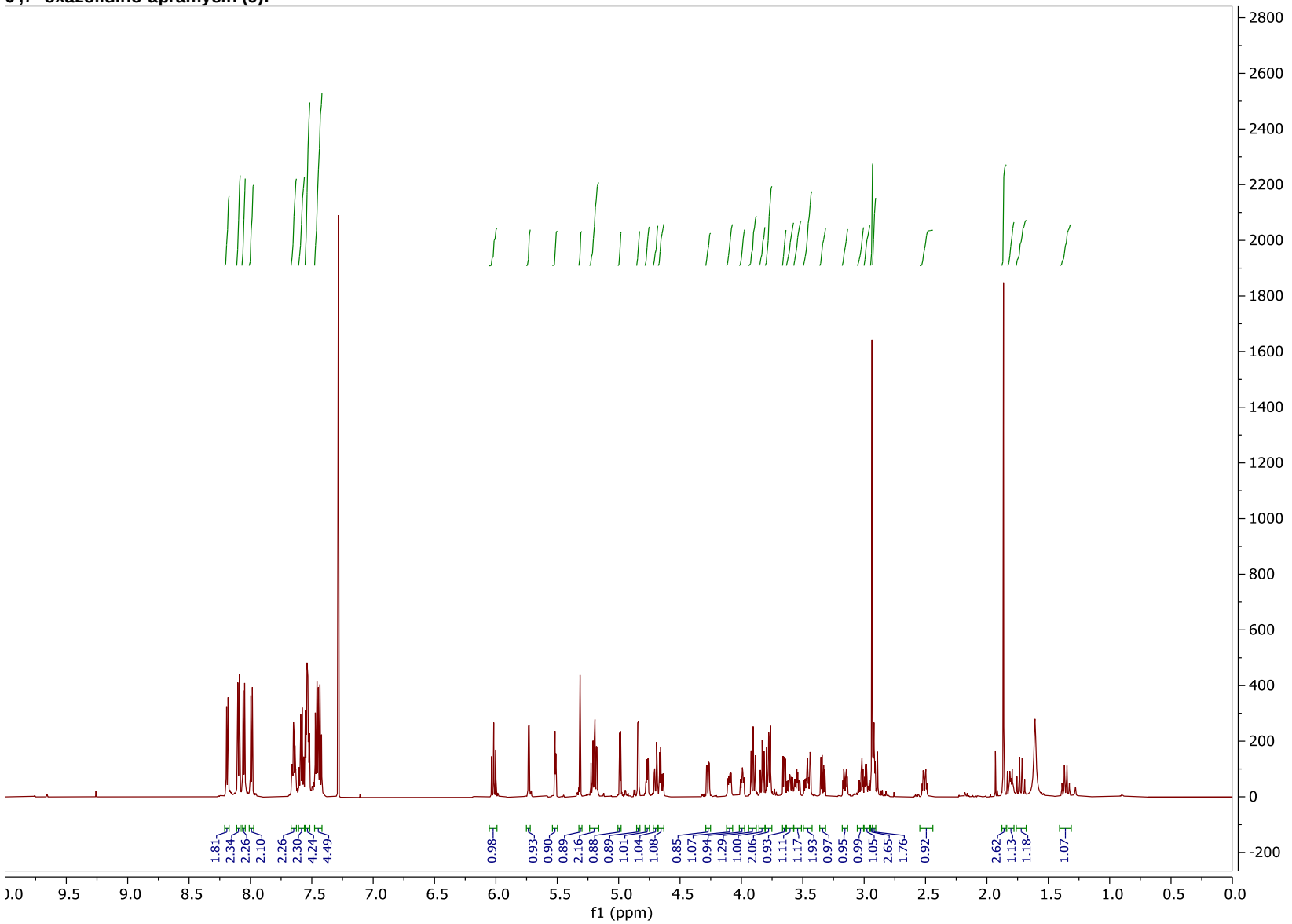
^{1}H NMR (600 MHz, $CDCl_3$) for 1,2-Di-*O*-acetyl-5-azido-3-*O*-(2-azidoethyl)-5-deoxy-D-ribofuranose (7):



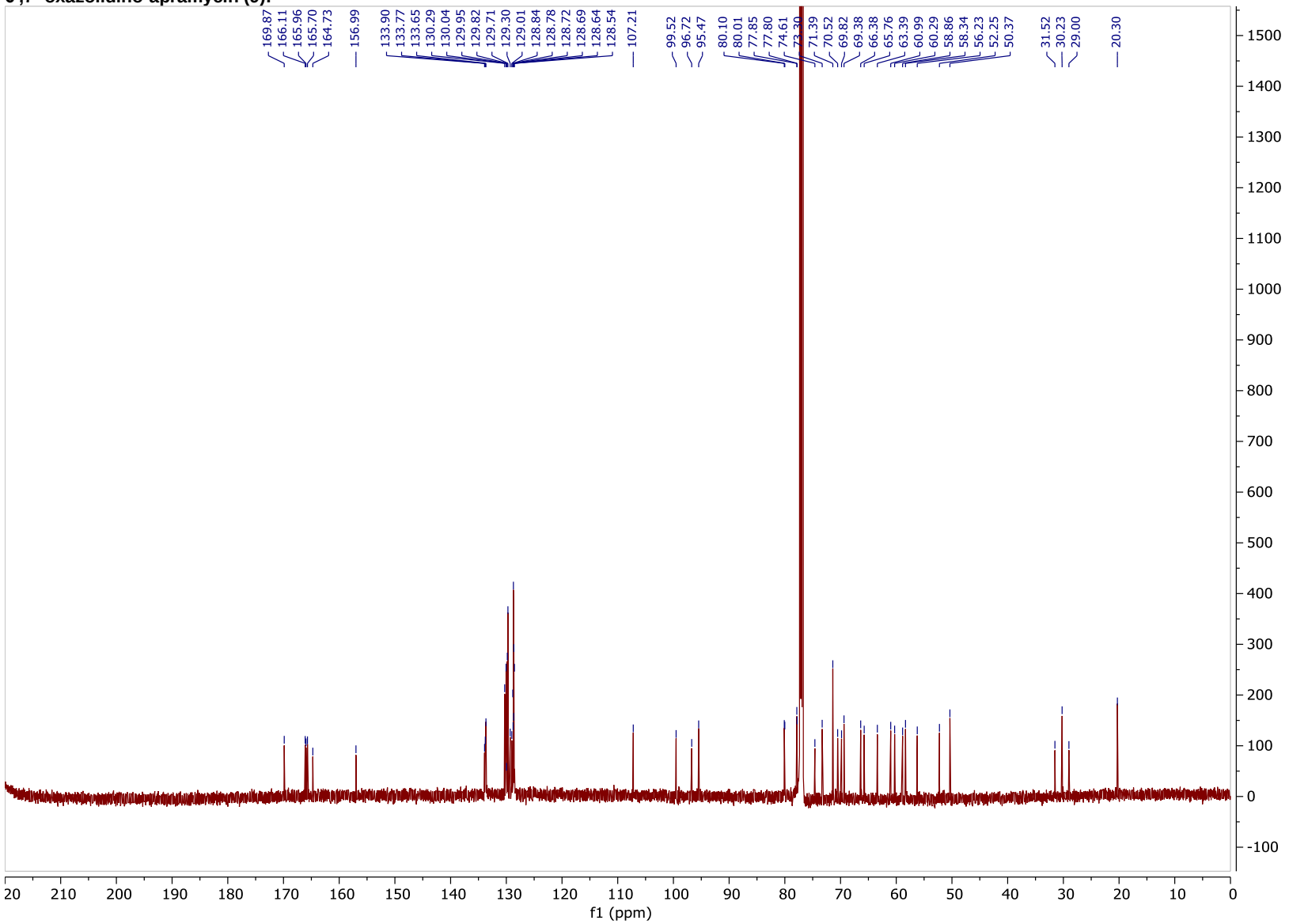
^{13}C NMR (600 MHz, $CDCl_3$) for 1,2-Di-*O*-acetyl-5-azido-3-*O*-(2-azidoethyl)-5-deoxy-D-ribofuranose (7):



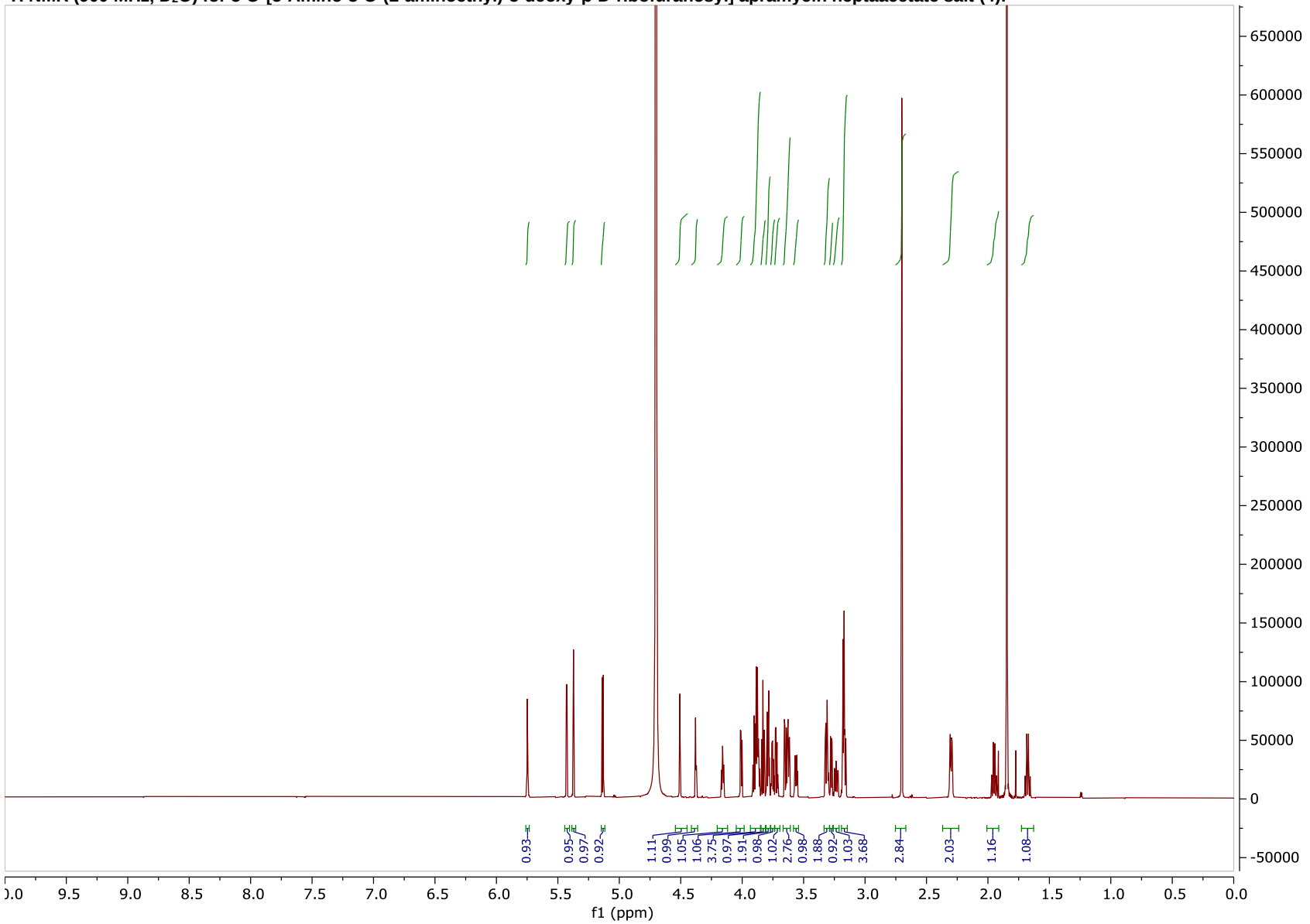
^{1}H NMR (600 MHz, $CDCl_3$) for 5-*O*-[2‴-*O*-Acetyl-5‴-azido-3‴-*O*-(2-azidoethyl)-5‴-deoxy-β-D-ribofuranosyl]- 1,3,2′,4″-tetra(desamino)-1,3,2′,4″-tetraazido-6,2″,3″,6″- tetra-*O*-benzoyl-6′,7′-oxazolidino-apramycin (9):



^{13}C NMR (150 MHz, $CDCl_3$) for 5-*O*-[2‴-*O*-Acetyl-5‴-azido-3‴-*O*-(2-azidoethyl)-5‴-deoxy-β-D-ribofuranosyl]- 1,3,2′,4″-tetra(desamino)-1,3,2′,4″-tetraazido-6,2″,3″,6″- tetra-*O*-benzoyl-6′,7′-oxazolidino-apramycin (9):



^{1}H NMR (900 MHz, D_2O) for 5-*O*-[5-Amino-3-*O*-(2-aminoethyl)-5-deoxy-β-D-ribofuranosyl] apramycin heptaacetate salt (4):



^{13}C NMR (225 MHz, D_2O) for 5-*O*-[5-Amino-3-*O*-(2-aminoethyl)-5-deoxy-β-D-ribofuranosyl] apramycin heptaacetate salt (4):