Supporting Information

for

Design, Multigram Synthesis, and in Vitro and in Vivo Evaluation of Propylamycin: A Semisynthetic 4,5-Deoxystreptamine Class Aminoglycoside for the Treatment of Drug-Resistant Enterobacteriaceae and Other Gram-Negative Pathogens

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Chemical Synthesis

1,2′,2‴,3,6‴-Pentaazido-2″,3′,3‴,4‴,5″,6,6′-hepta-*O*-benzyl-1,2′,2‴,3,6‴-pentadeamino-4′-deoxy-4′-propylthio-paromomycin (**7**). To a stirred solution of (**6**)[1] (315 mg, 209 μmol) in *N*,*N*-dimethylformamide (3.15 mL) was added a solution of sodium ethylthiolate (26.3 mg, 230 μmol) in ethanol:tetrahydrofuran (1:1, *v*/*v*, 575 μL) dropwise in an ice bath. After 30 min of stirring in an ice bath, the reaction solution was diluted with ethyl acetate:hexane (4:1, 10 mL), washed with brine (1×10 mL), and dried over $MgSO_4$. After concentration of the filtrate under reduced pressure, the residue was purified by flash column chromatography on silica gel (eluent: hexane:ethyl acetate = 7:1 to 2:1) to afford **7** (247 mg, 83%). $[\alpha]_D^{23}$ = +80.0 (c = 0.8, chloroform). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.48 – 7.43 (m, 2H: aromatic), 7.39 – 7.11 (m, 33H: aromatic), 6.19 (d, *J* = 3.5 Hz, 1H: H1′), 5.65 (d, *J* = 5.7 Hz, 1H: H1″), 4.99 (d, *J* = 10.2 Hz, 1H: PhC*H*$_2$O-C3′), 4.97 (d, *J* = 10.7 Hz, 1H: PhC*H*$_2$O-C6), 4.85 (d, *J* = 10.0 Hz, 1H: PhC*H*$_2$O-C3′), 4.84 (d, *J* = 1.8 Hz, 1H: H1‴), 4.69 – 4.53 (m, 6H: PhC*H*$_2$O-C6, PhC*H*$_2$O-C6′, PhC*H*$_2$O-C4‴, PhC*H*$_2$O-C6′, PhC*H*$_2$O-C2″, PhC*H*$_2$O-C5″), 4.48 – 4.38 (m, 3H: PhC*H*$_2$O-C2″, PhC*H*$_2$O-C5″, PhC*H*$_2$O-C3‴), 4.31 (d, *J* = 11.9 Hz, 1H: PhC*H*$_2$O-C3‴), 4.28 (q, *J* = 2.8 Hz, 1H: H4″), 4.27 – 4.21 (m, 2H: PhC*H*$_2$O-C4‴, H3″), 4.16 (ddd, *J* = 11.5, 4.3, 1.8 Hz, 1H: H5′), 4.02 – 3.88 (m, 5H: H6a′, H2″, H5, H3′, H6′b), 3.81 – 3.71 (m, 4H: H5″a, H3‴, H5‴), 3.68 (t, *J* = 9.4 Hz, 1H: H4), 3.63 – 3.54 (m, 2H: H6‴a, H5″b), 3.45 (ddd, *J* = 10.7, 8.9, 3.5 Hz, 1H: H3), 3.40 (ddd, *J* = 14.6, 8.8, 3.9 Hz, 1H: H1), 3.33 (t, *J* = 2.7, 1H: H2‴), 3.23 (t, *J* = 9.3 Hz, 1H: H6), 3.12 (t, *J* = 2.3 Hz, 1H: H4‴), 3.10 (dd, *J* = 10.0, 3.6 Hz, 1H: H2′), 2.91 (dd, *J* = 12.9, 4.2 Hz, 1H: H6b‴), 2.73 (t, *J* = 10.9 Hz, 1H: H4′), 2.68 – 2.56 (m, 2H: CH_3CH_2), 2.21 (dt, *J* = 13.2, 4.6 Hz, 1H: $H2_{eq}$), 1.33 (q, *J* = 12.7 Hz, 1H: $H2_{ax}$), 1.19 (t, *J* = 7.4 Hz, 3H: CH_3CH_2). ^{13}C NMR (150 MHz, $CDCl_3$) δ 138.4, 138.3, 138.1, 137.9, 137.6, 137.0, 137.0, 128.7, 128.5, 128.4, 128.4, 128.4, 128.3, 128.3, 128.3, 128.2, 128.2, 127.9, 127.8, 127.75, 127.6, 127.5, 127.5, 127.5, 127.4, 106.1 (C1″), 98.7 (C1‴), 96.0 (C1′), 84.1 (C6), 82.3 (C2″), 82.0 (C4″), 81.9 (C5), 79.0 (C3′), 76.0 (Ph*C*H$_2$O-C3′), 75.6 (C3″), 75.0 (Ph*C*H$_2$O-C6), 74.6 (C4), 74.2 (C5‴), 73.3 (Ph*C*H$_2$O-C6′, Ph*C*H$_2$O-C2″), 73.2 (Ph*C*H$_2$O-C5″), 72.9 (C3‴), 72.4 (Ph*C*H$_2$O-C3‴), 71.9 (C5′), 71.7 (Ph*C*H$_2$O-C4‴), 71.5 (C4‴), 70.0 (C5″), 69.9 (C6′), 64.4 (C2′), 60.4 (C1), 60.2 (C3), 57.3 (C2‴), 51.0 (C6‴), 48.2 (C4′), 32.6 (C2), 27.0 (CH_3CH_2), 15.0 (CH_3CH_2). ESI-HRMS: *m*/*z* calcd for $C_{74}H_{81}N_{15}O_{13}SNa$ $[M+Na]^+$ 1442.5757, found 1442.5758.

4′-Deoxy-4′-ethylthio-paromomycin pentaacetate (**4**)**.** To a stirred solution of **7** (62.2 mg, 43.8 μmol) in THF (450 μL) were added a solution of 1 M trimethylphosphine in tetrahydrofuran (543 μL, 543 μmol) and 0.1 M NaOH (225 μL) at room temperature. After 19 h of stirring at 50 °C, the reaction mixture was concentrated under reduced pressure and the residue was purified by flash chromatography (stepwise elution with chloroform, chloroform:2-propanol:25% aqueous ammonia = 5:2:0.1, and chloroform:2-propanol:25% aqueous ammonia = 5:3:0.2) to give a colorless syrup (47.3 mg). A part of the resulting compound (36.8 mg) was taken in a mixed solution of water/MeOH/acetic acid (2:1:0.1, *v*/*v*/*v*, 3.1 mL), and $Pd(OH)_2$/C (20% loading, 187 mg) was added. After 22 h of stirring under H_2 atmosphere (48 psi) at room temperature, the reaction mixture was filtered through Celite, neutralized with Amberlite IRA-400

(OH^- form), and concentrated under reduced pressure. The crude product was purified by flash chromatography (stepwise elution with chloroform:methanol:25% aqueous ammonia = 3:3:1 and chloroform:methanol:25% aqueous ammonia = 2:2:1) and subsequent CM Sephadex C-25 ion exchange column (stepwise elution with water, 0.125% ammonia, and 0.375% ammonia) to give **4** (6.1 mg), which was dissolved in water (1 mL) and acetic acid (3.2 μL) and lyophilized *in vacuo* to afford the corresponding pentaacetate salt (6.6 mg, 20%). $[\alpha]_D^{23}$ = +54.9 (c = 0.4, water). ^{1}H NMR (600 MHz, D_2O) δ 5.61 (d, *J* = 3.9 Hz, 1H: H1′), 5.21 (d, *J* = 2.6 Hz, 1H: H1″), 5.13 (d, *J* = 1.8 Hz, 1H: H1‴), 4.36 (dd, *J* = 6.6, 5.0 Hz, 1H: H3″), 4.20 (dd, *J* = 5.0, 2.6 Hz, 1H: H2″), 4.15 (ddd, *J* = 6.1, 4.1, 1.6 Hz, 1H: H5‴), 4.07 (t, *J* = 3.1 Hz, 1H: H3‴), 4.04 (ddd, *J* = 7.0, 4.5, 2.9 Hz, 1H: H4″), 3.95 – 3.89 (m, 1H: H6′a), 3.79 – 3.67 (m, 6H: H5″a, H4′, H6′b, H3′, H4, H5), 3.66 (dt, *J* = 3.0, 1.3 Hz, 1H: H4‴), 3.62 (dd, *J* = 12.4, 4.6 Hz, 1H: H5″b), 3.53 – 3.46 (m, 1H: H6), 3.42 (dt, *J* = 2.9, 1.3 Hz, 1H: H2‴), 3.30 – 3.16 (m, 4H: H6‴a, H2′, H6‴b, H3), 3.13 (ddd, *J* = 12.7, 10.4, 4.2 Hz, 1H: H1), 2.56 – 2.47 (m, 2H: H5′, CH_3CH_2), 2.22 (dt, *J* = 12.9, 4.3 Hz, 1H: $H2_{eq}$), 1.75 (s, 15H: CH_3CO_2H), 1.57 (q, *J* = 12.6 Hz, 1H: $H2_{ax}$), 1.07 (t, *J* = 7.4 Hz, 3H: CH_3CH_2). ^{13}C NMR (150 MHz, D_2O) δ 181.1 (CH_3CO_2H), 109.9 (C1″), 96.3 (C1′), 95.4 (C1‴), 84.4 (C5), 81.3 (C4″), 79.1 (C4), 75.1 (C3″), 74.0 (C4′), 73.3 (C2″), 72.6 (C6), 70.2 (C5‴), 67.6 (C3′), 67.5 (C3‴), 67.3 (C4‴), 61.2 (C6′), 60.0 (C5″), 55.0 (C2′), 50.8 (C2‴), 49.9 (C1), 48.9 (C3), 48.0 (C5′), 40.4 (C6‴), 29.4 (C2), 26.0 (CH_3CH_2), 23.1 (CH_3CO_2H), 14.2 (CH_3CH_2). ESI-HRMS: *m*/*z* calcd for $C_{25}H_{50}N_5O_{13}S$ $[M+H]^+$ 660.3126, found 660.3129.

1,2′,2‴,3,6‴-Pentaazido-2″,3′,3‴,4‴,5″,6,6′-hepta-*O*-benzyl-1,2′,2‴,3,6‴-pentadeamino-4′-propylthio-paromomycin (**8**). To a stirred solution of (**6**)[1] (109 mg, 72 μmol) in *N*,*N*-dimethylformamide (0.5 mL) was added a solution of sodium propylthiolate (0.74 M in *N*,*N*-dimethylformamide, 100 μL) at room temperature. After 15 min of stirring, the reaction solution was diluted with brine (15 mL) and water (1 mL), extracted with hexane:ethyl acetate (1:4, 5×10 mL), and dried over $MgSO_4$. The crude product was purified by flash chromatography (hexane:ethyl acetate = 7:1) to afford **8** (72.1 mg, 70%). $[\alpha]_D^{23}$ = +72.9 (c = 1.0, dichloromethane). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.48 – 7.43 (m, 2H: aromatic), 7.44 – 7.11 (m, 33H: aromatic), 6.20 (d, *J* = 3.5 Hz, 1H: H1′), 5.66 (d, *J* = 5.8 Hz, 1H: H1″), 5.00 (d, *J* = 10.2 Hz, 1H: PhCH_2O-C3′), 4.97 (d, *J* = 10.6 Hz, 1H: PhCH_2O-C6), 4.86 (d, *J* = 10.1 Hz, 1H: PhCH_2O-C3′), 4.84 (d, *J* = 1.9 Hz, 1H: H1‴), 4.70 – 4.50 (m, 6H: PhCH_2O-C6, PhCH_2O-C5″, PhCH_2O-C4‴, PhCH_2O-C2″, PhCH_2O-C5″, PhCH_2O-C6′), 4.52 – 4.37 (m, 3H: PhCH_2O-C2″, PhCH_2O-C6′, PhCH_2O-C3‴), 4.34 – 4.20 (m, 4H: PhCH_2O-C3‴, H4″, PhCH_2O-C4‴, PhCH_2O-C3″), 4.17 (ddd, *J* = 11.5, 4.3, 1.8 Hz, 1H: H5′), 4.02 – 3.88 (m, 5H: H6′a, H2″, H5, H3′, H6′b), 3.81 – 3.71 (m, 3H: H5″a, H3‴, H5‴), 3.69 (t, *J* = 9.4 Hz, 1H: H4), 3.63 – 3.55 (m, 2H: H6‴a, H5″b), 3.43 (m, 2H: H3, H1), 3.33 (t, *J* = 2.4 Hz, 1H: H2‴), 3.24 (t, *J* = 9.4 Hz, 1H: H6), 3.14 – 3.07 (m, 2H: H4‴, H2′), 2.90 (dd, *J* = 12.9, 4.2 Hz, 1H: H6‴b), 2.73 (t, *J* = 10.9 Hz, 1H: H4′), 2.62 – 2.52 (m, 2H: $CH_3CH_2CH_2$S-C4′), 2.21 (dt, *J* = 13.1, 4.6 Hz, 1H: $H2_{eq}$), 1.55 (p, *J* = 7.3 Hz, 2H: $CH_3CH_2CH_2$S-C4′), 1.33 (q, *J* = 12.7 Hz, 1H: $H2_{ax}$), 0.90 (t, *J* = 7.3 Hz, 3H: $CH_3CH_2CH_2$S-C4′). ^{13}C NMR (150 MHz, $CDCl_3$) δ 138.3, 138.3, 138.0, 137.9, 137.6, 137.0, 137.0, 128.7, 128.5, 128.4, 128.4, 128.4, 128.3, 128.3, 128.2, 128.2, 127.9, 127.8, 127.8, 127.7, 127.6, 127.5, 127.5, 106.1 (C1″), 98.7 (C1‴), 96.0 (C1′), 84.1 (C6), 82.3 (C2″), 82.0 (C4″), 81.9 (C5), 79.0 (C3′), 76.1 (PhCH_2O-C3′), 75.6 (C3″), 75.1 (PhCH_2O-C6), 74.5 (C4), 74.2 (C5‴), 73.3 (PhCH_2O-C2″), 73.3 (PhCH_2O-C5″), 73.2 (PhCH_2O-C6′), 72.9 (C3‴), 72.4 (PhCH_2O-C3‴), 71.9 (C5′), 71.7 (PhCH_2O-C4‴), 71.4 (C4‴), 70.0 (C6′), 69.9 (C5″), 64.5 (C2′), 60.4 (C1), 60.2 (C3), 57.3 (C2‴), 51.0 (C6‴),

48.4 (C4′), 34.9 ($CH_3CH_2\mathit{C}H_2S$-C4′), 32.6 (C2), 23.2 ($CH_3\mathit{C}H_2CH_2S$-C4′), 13.5 ($\mathit{C}H_3CH_2CH_2S$-C4′). ESI-HRMS: *m*/*z* calcd for $C_{75}H_{83}N_{15}O_{13}SNa$ $[M+Na]^+$ 1456.5913, found 1456.5938.

4′-Deoxy-4′-*S*-propylthio-paromomycin (**15**). To a stirred solution of **8** (72.1 mg, 50.3 μmol) in tetrahydrofuran (1 μL) were added a solution of 1 M trimethylphosphine in tetrahydrofuran (402 μL, 402 μmol) and 0.1 M NaOH (508 μL) at room temperature. After 10 h of stirring at 50 °C, the reaction mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography on silica gel (stepwise elution with chloroform, chloroform:ethanol = 5:3, and chloroform:ethanol:25% aqueous ammonia =5:3:0.2). The resulting compound (49.0 mg) was taken in a mixed solution of water/methanol/acetic acid (2:1:0.3, *v*/*v*/*v*, 1.5 mL), and $Pd(OH)_2$/C (20% loading, 245 mg) was added. After 15 h of stirring under H_2 atmosphere (48 psi) at room temperature, the reaction mixture was filtered through a Celite pad and the filtrate was concentrated by blowing air. The crude product was purified by CM Sephadex C-25 ion exchange column (stepwise elution with water, 0.1% ammonia, and 0.5% ammonia) and lyophilized *in vacuo*. To a stirred solution of the resultant white amorphous powder (9.1 mg) in water (200 μL) were added K_2CO_3 (24.5 mg, 178 μmol), methanol (200 μL), imidazole-1-sulfonyl azide hydrochloride[2-4] (13.7 mg, 65 μmol), and $CuSO_4$ (0.07 mg, 0.47 μmol) at room temperature. After 8 h of stirring, the reaction mixture was neutralized with 1 M HCl in an ice bath and added solid NaCl at room temperature up to saturation. The mixture was extracted with THF (3 × 1 mL) and dried over $MgSO_4$. The crude product was purified by flash column chromatography on silica gel (stepwise elution with chloroform:methanol = 30:1, 20:1, and 15:1) and subsequent semi-preparative RP-HPLC to give 1,2′,2‴,3,6‴-pentaazide-1,2′,2‴,3,6‴-pentadeamino-4′-*S*-propyl-4′-thio-paromomycin (3.3 mg, 4.1 μmol). To a solution of the pentaazide in tetrahydrofuran (500 μL) were added a solution of 1 M trimethylphosphine in tetrahydrofuran (33 μL, 33 μmol) and 0.1 M NaOH (91 μL) at room temperature. After 14 h of stirring at 50 °C, the reaction mixture was concentrated under reduced pressure and the residue was purified by CM Sephadex C-25 ion exchange column (stepwise elution with water, 0.1% aqueous ammonia, and 0.5% aqueous ammonia) to give **15** (2.8 mg). This was dissolved in a mixed solution of water (1 mL) and acetic acid (2.8 μL) and lyophilized *in vacuo* to afford the corresponding pentaacetate salt (3.3 mg, 3.4 μmol, 7%). $[\alpha]_D^{23}$ = +38.4 (c = 1.0, water). ^{1}H NMR (600 MHz, D_2O) δ 5.58 (d, *J* = 3.9 Hz, 1H: H1′), 5.18 (d, *J* = 2.5 Hz, 1H: H1″), 5.11 (d, *J* = 1.7 Hz, 1H: H1‴), 4.34 (dd, *J* = 6.7, 5.0 Hz, 1H: H3″), 4.18 (dd, *J* = 5.1, 2.6 Hz, 1H: H2″), 4.15 – 4.10 (m, 1H: H5‴), 4.04 (t, *J* = 3.2 Hz, 1H: H3‴), 4.03 – 3.99 (m, 1H: H4″), 3.90 (m, 1H: H6′a), 3.77 – 3.64 (m, 6H: H6′b, H4′, H5″a, H3′, H4, H5), 3.64 – 3.62 (m, 1H: H4‴), 3.59 (dd, *J* = 12.4, 4.6 Hz, 1H: H5″b), 3.47 (m, 1H: H6), 3.40 (dd, *J* = 3.0, 1.6 Hz, 1H: H2‴), 3.29 – 3.14 (m, 2H: H2′, H3), 3.14 – 3.07 (m, 2H: H1), 2.51 – 2.43 (m, 3H: H5′, $CH_3CH_2\mathit{C}H_2S$-C4′), 2.19 (dt, *J* = 12.7, 4.3 Hz, 1H: $H2_{eq}$), 1.73 (s, 15H: $\mathit{C}H_3CO_2H$), 1.54 (q, *J* = 12.6 Hz, 1H: $H2_{ax}$), 1.46 – 1.37 (m, 2H: $CH_3\mathit{C}H_2CH_2S$-C4′), 0.77 (t, *J* = 7.4 Hz, 3H: $\mathit{C}H_3CH_2CH_2S$-C4′). ^{13}C NMR (150 MHz, D_2O) δ 181.0 ($CH_3\mathit{C}O_2H$), 109.9 (C1″), 96.3 (C1′), 95.3 (C1‴), 84.4 (C5), 81.1 (C4′), 78.9 (C4), 75.0 (C3″), 74.0 (C4′), 73.3 (C2″), 72.6 (C6), 70.1 (C5‴), 67.6 (C4‴), 67.4 (C3′), 67.2 (C3‴), 61.1 (C6′), 59.8 (C5″), 54.9 (C2′), 50.7 (C2‴), 49.8 (C1), 48.8 (C3), 48.1 (C5′), 40.3 (C6‴), 33.9 ($CH_3CH_2\mathit{C}H_2S$-C4′), 29.3 (C2), 23.0 ($\mathit{C}H_3CO_2H$), 22.6 ($CH_3\mathit{C}H_2CH_2S$-C4′), 12.5 ($\mathit{C}H_3CH_2CH_2S$-C4′). ESI-HRMS: *m*/*z* calcd for $C_{26}H_{52}N_5O_{13}S$ $[M+H]^+$ 674.3282, found 674.3279.

1,2′,2‴,3,6‴-Pentaazido-2″,3′,3‴,4‴,5″,6,6′-hepta-*O*-benzyl-1,2′,2‴,3,6‴-pentadeamino-4′-deoxy-4′-isopropylthio-paromomycin (**9**). To a stirred solution of 6[1] (90.8 mg, 60.2 μmol) in *N*,*N*-dimethylformamide (908 μL) was added a solution of sodium isopropylthiolate (66.2 μmol) in tetrahydrofuran:2-propanol (1:1, 166 μL) dropwise and the mixture was stirred for 1 h in an ice bath. The mixture was diluted with ethyl acetate/hexane (4:1, 20 mL), washed with water and saturated NaCl solution (1×20 mL, each), dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by flash column chromatography on silica gel (hexane:ethyl acetate = 9:1 to 5:1) to afford **9** (64.9 mg, 75%). $[\alpha]_D^{23}$ = +79.5 (c = 1.0, chloroform). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.46 – 7.11 (m, 35H: aromatic), 6.20 (d, *J* = 3.5 Hz, 1H: H1′), 5.65 (d, *J* = 5.7 Hz, 1H: H1″), 5.02 (d, *J* = 10.3 Hz, 1H: PhC*H*$_2$O-C3′), 4.97 (d, *J* = 10.7 Hz, 1H: PhC*H*$_2$O-C6), 4.88 – 4.83 (m, 2H: PhC*H*$_2$O-C3′, H1‴), 4.71 – 4.53 (m, 7H: PhC*H*$_2$O-C6, PhC*H*$_2$O-C6′, PhC*H*$_2$O-C4‴, PhC*H*$_2$O-C2″, PhC*H*$_2$O-C6′, PhC*H*$_2$O-C5″), 4.48 – 4.39 (m, 3H: PhC*H*$_2$O-C2″, PhC*H*$_2$O-C5″, PhC*H*$_2$O-C3‴), 4.32 (d, *J* = 12.0 Hz, 1H: PhC*H*$_2$O-C3‴), 4.29 (q, *J* = 2.8 Hz, 1H: H4″), 4.27 – 4.22 (m, 2H: PhC*H*$_2$O-C4‴, H3″), 4.13 (ddd, *J* = 11.5, 4.3, 1.9 Hz, 1H: H5′), 4.00 – 3.85 (m, 5H: H6′a, H2″, H5, H6′b, H3′), 3.78 (dd, *J* = 10.4, 2.4 Hz, 1H: H5″a), 3.74 (dt, *J* = 8.7, 2.7 Hz, 2H: H3‴, H5‴), 3.69 (t, *J* = 9.4 Hz, 1H: H4), 3.63 – 3.55 (m, 2H: H5‴a, H5″b), 3.42 (m, 2H: H3, H1), 3.34 (t, *J* = 2.6 Hz, 1H: H2‴), 3.23 (t, *J* = 9.4 Hz, 1H: H6), 3.12 (t, *J* = 2.5 Hz, 1H: H4‴), 3.08 (dd, *J* = 10.0, 3.6 Hz, 1H: H2′), 3.00 (hept, *J* = 6.6 Hz, 1H: C*H*(CH$_3$)$_2$), 2.91 (dd, *J* = 12.9, 4.2 Hz, 1H: H5‴b), 2.75 (t, *J* = 10.9 Hz, 1H: H4′), 2.20 (dt, *J* = 13.1, 4.6 Hz, 1H: H2$_{eq}$), 1.31 (q, *J* = 12.7 Hz, 1H: H2$_{ax}$), 1.22 (d, *J* = 6.7 Hz, 3H: CH(C*H*$_3$)$_2$), 1.20 (d, *J* = 6.7 Hz, 3H: CH(C*H*$_3$)$_2$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 138.3, 138.1, 137.9, 137.6, 137.0, 137.0, 128.7, 128.5, 128.4, 128.4, 128.3, 128.3, 128.3, 128.2, 128.2, 127.9, 127.8, 127.8, 127.7, 127.6, 127.6, 127.5, 127.4, 106.1 (C1″), 98.7 (C1‴), 96.0 (C1′), 84.1 (C6), 82.3 (C2″), 82.0 (C4″), 81.9 (C5), 79.5 (C3′), 76.2 (Ph*C*H$_2$O-C3′), 75.6 (C3″), 75.0 (Ph*C*H$_2$O-C6), 74.6 (C4), 74.2 (C5‴), 73.3 (Ph*C*H$_2$O-C2″), 73.2 (Ph*C*H$_2$O-C6′), 73.2 (Ph*C*H$_2$O-C5″), 72.9 (C3‴), 72.4 (Ph*C*H$_2$O-C3‴), 72.2 (C5′), 71.7 (Ph*C*H$_2$O-C4‴), 71.4 (C4‴), 70.0 (C5″), 69.8 (C6′), 64.5 (C2′), 60.4 (C1), 60.2 (C3), 57.3 (C2‴), 51.0 (C6‴), 47.4 (C4′), 37.1 (*C*H(CH$_3$)$_2$), 32.6 (C2), 24.0 (CH(*C*H$_3$)$_2$), 23.7 (CH(*C*H$_3$)$_2$). ESI-HRMS: *m*/*z* calcd for $C_{75}H_{83}N_{15}O_{13}SNa$ $[M+Na]^+$ 1456.5913, found 1456.5889.

4′-Deoxy-4′-isopropylthio-paromomycin pentaacetate (**16**)**.** To a stirred solution of **9** (64.9 mg, 45.2 μmol) in tetrahydrofuran (1.35 mL) were added a solution of 1 M trimethylphosphine in tetrahydrofuran (362 μL, 362 μmol) and 0.1 M NaOH (457 μL) at room temperature. After 14 h of stirring at 50 °C, the reaction mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography on silica gel (stepwise elution with chloroform, chloroform:ethanol = 5:2, and chloroform:ethanol:25% aqueous ammonia =5:2:0.1). The resulting compound (52.6 mg) was taken in a mixed solution of water/1,4-dioxane/acetic acid (2:1:0.1, *v*/*v*/*v*, 1.5 mL), and $Pd(OH)_2$/C (20% loading, 263 mg) was added. After 14 h of stirring under H_2 atmosphere (48 psi) at room temperature, the reaction mixture was filtered through a Celite pad and the filtrate was concentrated by blowing air. The crude product was purified by CM Sephadex C-25 ion exchange column (stepwise elution with water, 0.1% aqueous ammonia, and 0.5% aqueous ammonia) and lyophilized *in vacuo* to give **16** (16.2 mg). This was dissolved in a mixed solution of water (0.5 mL) and acetic acid (8.3 μL), and lyophilized *in vacuo* to afford the corresponding pentaacetate salt (23.6 mg, 60%). $[\alpha]_D^{23}$ = +49.0 (c = 1.46, water). ^{1}H NMR (600

MHz, D_2O) δ 5.62 (d, *J* = 4.0 Hz, 1H: H1′), 5.18 (d, *J* = 2.5 Hz, 1H: H1′′), 5.10 (d, *J* = 1.7 Hz, 1H: H1′′′), 4.33 (dd, *J* = 6.7, 5.0 Hz, 1H: H3′′), 4.18 (dd, *J* = 5.0, 2.6 Hz, 1H: H2′′), 4.11 (ddd, *J* = 6.4, 4.1, 1.4 Hz, 1H: H5′′′), 4.05 – 3.98 (m, 2H: H3′′′, H4′′), 3.94 – 3.86 (m, 1H: H6′a), 3.79 (t, *J* = 9.6 Hz, 1H: H4), 3.75 – 3.64 (m, 5H: H5′′a, H5, H3′, H6′b, H5′), 3.62 (dt, *J* = 3.1, 1.3 Hz, 1H: H4′′′), 3.58 (dd, *J* = 12.4, 4.6 Hz, 1H: H5′′b), 3.49 (dd, *J* = 10.5, 9.2 Hz, 1H: H6), 3.39 (dt, *J* = 2.9, 1.3 Hz, 1H: H2′′′), 3.29 (ddd, *J* = 12.5, 10.1, 4.2 Hz, 1H: H3), 3.26 – 3.19 (m, 2H: H2′, H6′′′a), 3.19 – 3.08 (m, 2H: H6′′′a, H1), 2.88 (p, *J* = 6.7 Hz, 1H: C*H*$(CH_3)_2$), 2.53 (t, *J* = 10.6 Hz, 1H: H4′), 2.26 (dt, *J* = 12.8, 4.4 Hz, 1H: $H2_{eq}$), 1.73 (s, 15H, C*H*$_3CO_2H$), 1.62 (q, *J* = 12.6 Hz, 1H: $H2_{ax}$), 1.08 (d, *J* = 6.7 Hz, 3H: CH(C*H*$_3)_2$), 1.06 (d, *J* = 6.7 Hz, 3H: CH(C*H*$_3)_2$). ^{13}C NMR (150 MHz, D_2O) δ 180.5 (CH_3*C*O_2H), 109.9 (C1′′), 96.2 (C1′), 95.3 (C1′′′), 84.2 (C5), 81.2 (C4′′), 77.7 (C4), 75.0 (C3′′), 74.4 (C5′), 73.3 (C2′′), 72.2 (C6), 70.1 (C5′′′), 67.7 (C3′), 67.5 (C3′′′), 67.2 (C4′′′), 61.1 (C6′), 59.8 (C5′′), 54.7 (C2′), 50.7 (C2′′′), 49.6 (C1), 48.7 (C3), 47.2 (C4′), 40.3 (C6′′′), 36.9 (*C*H$(CH_3)_2$), 28.3 (C2), 23.2 (CH(*C*H$_3)_2$), 22.7 (CH(*C*H$_3)_2$). ESI-HRMS: *m*/*z* calcd for $C_{26}H_{52}N_5O_{13}S$ $[M+H]^+$ 674.3282, found 674.3281.

1,2′,2′′′,3,6′′′-Pentaazido-2′′,3′,3′′′,4′′′,5′′,6,6′-hepta-*O*-benzyl-1,2′,2′′′,3,6′′′-pentadeamino-4′-deoxy-4′-isobutylthio-paromomycin (**10**). To a stirred solution of **6**[1] (109 mg, 72 μmol) in *N*,*N*-dimethylformamide (0.5 mL) was added a solution of sodium isobutylthiolate (0.74 M in *N*,*N*-dimethylformamide, 100 μL) at room temperature. After 15 min of stirring, the reaction solution was diluted with saturated NaCl solution (15 mL) and water (1 mL), extracted with hexane:ethyl acetate (1:4, 5×10 mL), and dried over $MgSO_4$. The crude product was purified by flash column chromatography on silica gel (hexane:ethyl acetate = 8:1) to afford **10** (81.6 mg, 78%). $[\alpha]_D^{23}$ = +75.0 (c = 1.0, dichloromethane). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.48 – 7.43 (m, 2H: aromatic), 7.40 – 7.11 (m, 33H: aromatic), 6.21 (d, *J* = 3.6 Hz, 1H: H1′), 5.66 (d, *J* = 5.8 Hz, 1H: H1′′), 5.01 (d, *J* = 10.2 Hz, 1H: PhC*H*$_2$O-C3′), 4.97 (d, *J* = 10.7 Hz, 1H: PhC*H*$_2$O-C6), 4.86 (d, *J* = 10.2 Hz, 1H: PhC*H*$_2$O-C3′), 4.84 (d, *J* = 1.9 Hz, 1H: H1′′′), 4.71 – 4.53 (m, 6H: PhC*H*$_2$O-C6, PhC*H*$_2$O-C6′, PhC*H*$_2$O-C4′′′, PhC*H*$_2$O-C6′, PhC*H*$_2$O-C2′′, PhC*H*$_2$O-C5′′), 4.55 – 4.39 (m, 3H: PhC*H*$_2$O-C2′′, PhC*H*$_2$O-C5′′, PhC*H*$_2$O-C3′′′), 4.34 – 4.20 (m, 4H: PhC*H*$_2$O-C3′′′, H4′′, PhC*H*$_2$O-C4′′′, H3′′), 4.17 (ddd, *J* = 11.4, 4.4, 1.8 Hz, 1H: H5′), 4.02 – 3.85 (m, 5H: H6′a, H2′′, H5, H6′b, H3′), 3.81 – 3.72 (m, 3H: H5′′a, H3′′′, H5′′′), 3.69 (t, *J* = 9.4 Hz, 1H: H4) 3.63 – 3.54 (m, 2H: H6′′′a, H5′′b), 3.43 (m, 2H: H3, H1), 3.34 (d, *J* = 2.4 Hz, 1H: H2′′′), 3.24 (t, *J* = 9.4 Hz, 1H: H6), 3.12 (d, *J* = 2.4 Hz, 1H: H4′′′), 3.09 (dd, *J* = 10.0, 3.6 Hz, 1H: H2′), 2.93 – 2.87 (m, 1H: H6′′′b), 2.72 (t, *J* = 10.9 Hz, 1H: H4′), 2.51 (dd, *J* = 25.6, 11.9 Hz, 1H: $(CH_3)_2$CHC*H*$_2$O-C4′), 2.50 (dd, *J* = 25.7, 11.9 Hz, 1H: $(CH_3)_2$CHC*H*$_2$O-C4′), 2.21 (dt, *J* = 13.1, 4.6 Hz, 1H: $H2_{eq}$), 1.75 (m, *J* = 6.8, 6.7 Hz, 1H: $(CH_3)_2$C*H*CH$_2$O-C4′), 1.34 (q, *J* = 12.7 Hz, 1H: $H2_{ax}$), 0.91 (d, *J* = 6.7 Hz, 3H: (C*H*$_3)_2$CHCH$_2$O-C4′), 0.90 (d, *J* = 6.7 Hz, 3H: (C*H*$_3)_2$CHCH$_2$O-C4′). ^{13}C NMR (150 MHz, $CDCl_3$) δ 138.3, 138.3, 138.0, 137.9, 137.6, 137.0, 137.0, 128.7, 128.5, 128.4, 128.4, 128.4, 128.3, 128.3, 128.3, 128.2, 128.2, 127.9, 127.8, 127.8, 127.7, 127.6, 127.5, 127.5, 106.1 (C1′′), 98.7 (C1′′′), 96.0 (C1′), 84.1 (C6), 82.3 (C2′′), 82.0 (C4′′), 81.9 (C5), 76.0 (Ph*C*H$_2$O-C3′), 75.6 (C3′′), 75.1 (Ph*C*H$_2$O-C6), 74.5 (C4), 74.2 (C5′′′), 73.3 (Ph*C*H$_2$O-C6′), 73.3 (Ph*C*H$_2$O-C2′′), 73.2 (Ph*C*H$_2$O-C5′′), 72.9 (C3′′′), 72.4 (Ph*C*H$_2$O-C3′′′), 72.0 (C5′), 71.7 (Ph*C*H$_2$O-C4′′′), 71.4 (C4′′′), 70.0 (C5′′), 69.9 (C6′), 64.5 (C2′), 60.4 (C1), 60.2 (C3), 57.3 (C2′′′), 51.0 (C6′′′), 48.8 (C4′), 41.9 (($CH_3)_2$CH*C*H$_2$O-C4′), 32.6 (C2), 28.9 ((CH$_3)_2$*C*HCH$_2$O-C4′), 22.0 ((CH$_3)_2$CHCH$_2$O-C4′). ESI-HRMS: *m*/*z* calcd for $C_{76}H_{85}N_{15}O_{13}SNa$ $[M+Na]^+$ 1470.6070, found 1470.6045.

4′-Deoxy-4′-isobutylthio-paromomycin (**17**)**.** To a stirred solution of **10** (81.6 mg, 56.3 μmol) in tetrahydrofuran (1 mL) were added a solution of 1 M trimethylphosphine in tetrahydrofuran (451 μL, 451 μmol) and 0.1 M NaOH (569 μL) at room temperature. After 8 h of stirring at 50 °C, the reaction mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography on silica gel (stepwise elution with chloroform, chloroform:ethanol = 5:3, and chloroform:ethanol:25% aqueous ammonia =5:3:0.2). The resulting compound (41.1 mg) was taken in a mixed solution of water/methanol/acetic acid (2:1:0.3, *v*/*v*/*v*, 1.5 mL), and $Pd(OH)_2/C$ (20% loading, 206 mg) was added. After 12 h of stirring under H_2 atmosphere (48 psi) at room temperature, the reaction mixture was filtered through a Celite pad and the filtrate was concentrated by blowing air. The crude product was purified by CM Sephadex C-25 ion exchange column (stepwise elution with water, 0.1% aqueous ammonia, and 0.5% aqueous ammonia) and lyophilized *in vacuo*. To a stirred solution of the resultant white amorphous powder (11.2 mg) in water (200 μL) were added K_2CO_3 (42.3 mg, 306 μmol), methanol (200 μL), imidazole-1-sulfonyl azide hydrochloride[2-4] (23.6 mg, 113 μmol), and $CuSO_4$ (0.13 mg, 0.8 μmol) at room temperature. After 7 h of stirring, the reaction mixture was neutralized with 1 M HCl in an ice bath and added solid NaCl at room temperature up to saturation. The mixture was extracted with tetrahydrofuran (3×1 mL) and dried over $MgSO_4$. The crude product was purified by flash column chromatography on silica gel (stepwise elution with chloroform:methanol = 30:1, 20:1, and 15:1) and subsequent semi-preparative RP-HPLC to give 1,2′,2‴,3,6‴-pentaazide-1,2′,2‴,3,6‴-pentadeamino-4′-deoxy-4′-isobutylthio-paromomycin (4.0 mg, 4.9 μmol). To a solution of the pentaazide in tetrahydrofuran (800 μL) were added a solution of 1 M trimethylphosphine in tetrahydrofuran (37 μL, 37 μmol) and 0.1 M NaOH (96 μL) at room temperature. After 8 h of stirring at 50 °C, the reaction mixture was concentrated under reduced pressure and the residue was purified by CM Sephadex C-25 ion exchange column (stepwise elution with water, 0.1% aqueous ammonia, and 0.5% aqueous ammonia) to give **17** (4.3 mg). This was dissolved in a mixed solution of water (1 mL) and acetic acid (3 μL), and lyophilized *in vacuo* to afford the corresponding pentaacetate salt (6.1 mg, 6.2 μmol, 11%). $[\alpha]_D^{23}$ = +42.1 (c = 1.1, water). ^{1}H NMR (600 MHz, D_2O) δ 5.58 (d, *J* = 3.9 Hz, 1H: H1′), 5.18 (d, *J* = 2.6 Hz, 1H: H1″), 5.10 (d, *J* = 1.8 Hz, 1H: H1‴), 4.34 (dd, *J* = 6.7, 5.0 Hz, 1H: H3″), 4.18 (dd, *J* = 4.9, 2.6 Hz, 1H: H2″), 4.15 – 4.09 (m, 1H: H5‴), 4.04 (t, *J* = 3.1 Hz, 1H: H3‴), 4.02 (dq, *J* = 7.2, 4.1, 3.4 Hz, 1H: H4″), 3.90 (d, *J* = 10.1 Hz, 1H: H6′a), 3.79 – 3.64 (m, 6H: H5″a, H6′b, H4′, H3′, H4, H5), 3.63 (dd, *J* = 3.1, 1.6 Hz, 1H: H4‴), 3.59 (dd, *J* = 12.5, 4.6 Hz, 1H: H5″b), 3.50 – 3.44 (m, 1H: H6), 3.40 (d, *J* = 2.7 Hz, 1H: H2‴), 3.27 – 3.14 (m, 4H: H6‴a, H2′, H3, H6‴b), 3.14 – 3.07 (m, 1H: H1), 2.47 (t, *J* = 10.5 Hz, 1H: H5′), 2.38 (d, *J* = 6.9 Hz, 2H: $(CH_3)_2CHCH_2$O-C4′), 2.20 (dt, *J* = 12.5, 4.2 Hz, 1H: $H2_{eq}$), 1.73 (s, 15H: CH_3CO_2H), 1.62 – 1.50 (m, 2H: $(CH_3)_2CHCH_2$O-C4′, $H2_{ax}$), 0.77 (dd, *J* = 6.7, 2.9 Hz, 6H $(CH_3)_2CHCH_2$O-C4′). ^{13}C NMR (150 MHz, D_2O) δ 181.03 (CH_3CO_2H), 109.88 (C1″), 96.23 (C1′), 95.28 (C1‴), 84.37 (C5), 81.12 (C4″), 78.67 (C4), 74.91 (C3″), 74.01 (C4′), 73.23 (C2″), 72.51 (C6), 70.11 (C5‴), 67.55 (C3′), 67.40 (C3‴), 67.16 (C4‴), 61.13 (C6′), 59.81 (C5″), 54.95 (C2″), 50.71 (C2‴), 49.80 (C1), 48.80 (C3), 48.50 (C5′), 40.78 ($(CH_3)_2CHCH_2$O-C4′), 40.27 (C6‴), 29.17 ($(CH_3)_2CHCH_2$O-C4′), 28.19 (C2), 22.97 (CH_3CO_2H), 20.99 ($(CH_3)_2CHCH_2$O-C4′), 20.90 ($(CH_3)_2CHCH_2$O-C4′). ESI-HRMS: *m*/*z* calcd for $C_{27}H_{54}N_5O_{13}S$ $[M+H]^+$ 688.3439, found 688.3428.

1,2′,2‴,3,6‴-Pentaazido-2″,3′,3‴,4‴,5″,6,6′-hepta-*O*-benzyl-1,2′,2‴,3,6‴-pentadeamino-4′-deoxy-4′-acetylthio-paromomycin (**11**). To a solution of **6**[1] (74.8 mg, 49.6 μmol) in *N*,*N*-dimethylformamide (100 μL) was added potassium thioacetate (12.5 mg, 109 μmol) and the mixture was stirred for 1 h in an ice bath. The mixture was diluted with hexane/ethyl acetate (1:4, 10 mL), washed with water and saturated NaCl solution (1×10 mL, each), dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (hexane:ethyl acetate = 6:1 to 3:1) to afford **11** (41.8 mg, 59%). $[\alpha]_D^{23}$ = +77.7 (c = 1.0, CH_2Cl_2). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.39 – 7.11 (m, 10H: aromatic), 6.23 (d, *J* = 3.6 Hz, 1H: H1′), 5.65 (d, *J* = 6.1 Hz, 1H: H1″), 4.96 (d, *J* = 10.6 Hz, 1H: PhC*H*$_2$O-C6), 4.87 (d, *J* = 1.9 Hz, 1H: H1‴), 4.78 (d, *J* = 10.8 Hz, 1H: PhC*H*$_2$O-C3′), 4.68 – 4.54 (m, 6H: PhC*H*$_2$O-C3′, PhC*H*$_2$O-C6, PhC*H*$_2$O-C4‴, PhC*H*$_2$O-C6′, PhC*H*$_2$O-C2″, PhC*H*$_2$O-C6′), 4.52 (d, *J* = 11.8 Hz, 1H: PhC*H*$_2$O-C5″), 4.47 (d, *J* = 11.6 Hz, 2H: PhC*H*$_2$O-C2″), 4.44 (d, *J* = 11.8 Hz, 1H: PhC*H*$_2$O-C5″), 4.41 (d, *J* = 12.0 Hz, 1H: PhC*H*$_2$O-C3‴), 4.31 (d, *J* = 12.0 Hz, 4H: PhC*H*$_2$O-C3‴), 4.29 – 4.22 (m, 1H: H4″, H5′, H3″, PhC*H*$_2$O-C4‴), 3.98 – 3.90 (m, 3H: H2″, H3′, H5), 3.81 – 3.73 (m, 3H: H5″a, H5‴, H3‴), 3.72 – 3.57 (m, 5H: H4, H6′a, H6′b, H6‴a, H4′), 3.55 (dd, *J* = 10.4, 3.1 Hz, 1H: H5″b), 3.45 (ddd, *J* = 12.5, 9.8, 4.5 Hz, 1H: H3), 3.40 (ddd, *J* = 12.5, 9.7, 4.6 Hz, 1H: H1), 3.33 (t, *J* = 2.5 Hz, 1H: H2‴), 3.19 (t, *J* = 9.4 Hz, 1H: H6), 3.12 (d, *J* = 2.5 Hz, 1H: H4‴), 3.06 (dd, *J* = 9.8, 3.6 Hz, 1H: H2′), 2.89 (dd, *J* = 12.9, 4.1 Hz, 1H: H6‴b), 2.25 (s, 3H: C*H*$_3$CO), 2.18 (dt, *J* = 13.2, 4.6 Hz, 1H: $H2_{eq}$), 1.26 (q, *J* = 13.0 Hz, 1H: $H2_{ax}$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 193.3 (CH_3*C*O), 138.4, 138.1, 137.9, 137.5, 137.0, 137.0, 128.7, 128.5, 128.4, 128.3, 128.3, 128.3, 128.2, 128.2, 128.2, 127.9, 127.8, 127.8, 127.8, 127.7, 127.6, 127.5, 127.5, 127.4, 106.0 (C1″), 98.7 (C1‴), 95.7 (C1′), 84.2 (C6), 82.4 (C2″), 82.1 (C4″), 81.9 (C5), 77.0 (C3′), 75.6 (C3″), 75.1 (Ph*C*H$_2$O-C3′), 75.0 (Ph*C*H$_2$O-C6), 74.6 (Ph*C*H$_2$O-C4), 74.2 (C5‴), 73.4 (Ph*C*H$_2$O-C5″), 73.3 (Ph*C*H$_2$O-C2″), 73.1 (Ph*C*H$_2$O-C6′), 72.9 (C3‴), 72.4 (Ph*C*H$_2$O-C3‴), 71.7 (Ph*C*H$_2$O-C4‴), 71.4 (C4‴), 70.8 (C5′), 70.1 (C6′), 70.0 (C5″), 63.9 (C2′), 60.4 (C1), 60.2 (C3), 57.3 (C2‴), 51.0 (C6‴), 45.6 (C4′), 32.6 (C2), 30.6 (*C*H$_3$CO). ESI-HRMS: *m*/*z* calcd for $C_{74}H_{79}N_{15}O_{14}SNa$ $[M+Na]^+$ 1456.5549, found 1456.5554.

1,2′,2‴,3,6‴-Pentaazido-2″,3′,3‴,4‴,5″,6,6′-hepta-*O*-benzyl-1,2′,2‴,3,6‴-pentadeamino-4′-deoxy-4′-(2-fluoroethylthio)-paromomycin (14). To a solution of **11** (75.4 mg, 50.7 μmol) in *N*,*N*-dimethylformamide (0.75 mL) was added hydrazine acetate (5.6 mg, 61 μmol) in an ice bath and the mixture was stirred for 30 min at room temperature. The mixture was diluted with hexane/ethyl acetate (1:4, 50 mL) and washed with water (1×50 mL). The aqueous layer was extracted with hexane/ethyl acetate (1:4, 3×25 mL). The combined organic layer was dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting compound (ESI-HRMS: *m*/*z* calcd for $C_{72}H_{77}N_{15}O_{13}SNa$ $[M+Na]^+$ 1414.5444, found 1414.5435) was dissolved in *N*,*N*-dimethylformamide (0.70 mL). NaH (60% in mineral oil, 6.6 mg, 165 μmol) and 2-fluoroethyl trifluoromethanesulfonate (39.8 mg, 203 μmol) were added and the mixture was stirred for 3 h in an ice bath. Saturated NH_4Cl solution (2 mL) was added. The mixture was diluted with hexane/ethyl acetate (1:4, 25 mL) and washed with water (1×25 mL). The aqueous layer was extracted with hexane/ethyl acetate (1:4, 2×25 mL) and the combined organic layer was dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (hexane:ethyl acetate = 7:1) to afford **14** (27.1 mg, 37%). $[\alpha]_D^{23}$ = +105.7 (c = 1.0, dichloromethane). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.50 – 7.12 (m, 35H: aromatic), 6.20 (d, *J* = 3.6 Hz, 1H:

H1′), 5.65 (d, *J* = 5.6 Hz, 1H: H1′′), 4.96 (d, *J* = 10.6 Hz, 1H: PhC*H*$_2$O-C6), 4.93 (d, *J* = 10.2 Hz, 1H: PhC*H*$_2$O-C3′), 4.87 (d, *J* = 10.3 Hz, 1H: PhC*H*$_2$O-C3′), 4.85 (d, *J* = 1.9 Hz, 1H: H1′′′), 4.69 (d, *J* = 10.6 Hz, 1H: PhC*H*$_2$O-C6), 4.65 (d, *J* = 12.0 Hz, 1H: PhC*H*$_2$O-C6′), 4.62 (d, *J* = 12.0 Hz, 1H: PhC*H*$_2$O-C4′′′), 4.59 – 4.53 (m, 3H: PhC*H*$_2$O-C2′′, PhC*H*$_2$O-C6′, PhC*H*$_2$O-C5′′), 4.47 – 4.35 (m, 5H: FC*H*$_2$CH$_2$, PhC*H*$_2$O-C5′′, PhC*H*$_2$O-C2′′, PhC*H*$_2$O-C3′′′, FC*H*$_2$CH$_2$), 4.35 – 4.23 (m, 4H: PhC*H*$_2$O-C3′′′, H4′′, PhC*H*$_2$O-C4′′′, H3′′), 4.15 – 4.10 (m, 1H: H5′), 4.03 (dd, *J* = 10.8, 3.9 Hz, 1H: H6′a), 3.96 (t, *J* = 5.3 Hz, 1H: H2′′), 3.93 (t, *J* = 8.9 Hz, 1H: H5), 3.90 – 3.83 (m, 2H: H3′, H6′b), 3.81 – 3.73 (m, 3H: H5′′a, H3′′′, H5′′′), 3.67 (t, *J* = 9.4 Hz, 1H: H4), 3.64 – 3.55 (m, 2H: H6′′′a, H5′′b), 3.48 – 3.37 (m, 2H: H3, H1), 3.34 (t, *J* = 2.4 Hz, 1H: H2′′′), 3.24 (t, *J* = 9.4 Hz, 1H: H6), 3.15 – 3.11 (m, 1H: H4′′′), 3.04 (dd, *J* = 10.0, 3.6 Hz, 1H: H2′), 2.94 – 2.81 (m, 2H: H6′′′b, FCH$_2$C*H*$_2$), 2.75 (t, *J* = 10.9 Hz, 1H: H4′), 2.21 (dt, *J* = 13.2, 4.7 Hz, 1H: H2$_{eq}$), 1.34 (q, *J* = 12.8 Hz, 1H, H2$_{ax}$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 138.3, 138.1, 137.9, 137.9, 137.6, 137.0, 137.0, 128.7, 128.6, 128.5, 128.4, 128.4, 128.3, 128.3, 128.2, 128.2, 127.9, 127.8, 127.8, 127.8, 127.7, 127.7, 127.6, 127.5, 127.5, 106.2 (C1′′), 98.6 (C1′′′), 96.1 (C1′), 84.1 (C6), 82.3 (C2′′), 82.2 (d, *J* = 171.2 Hz: F*C*H$_2$CH$_2$), 82.0 (C4′′), 81.9 (C5), 79.2 (C3′), 75.6 (Ph*C*H$_2$O-C3′), 75.1 (C3′′), 74.8 (Ph*C*H$_2$O-C6), 74.2 (C4), 73.4 (C5′′′), 73.3 (Ph*C*H$_2$O-C6′), 73.2 (Ph*C*H$_2$O-C5′′), 73.0 (Ph*C*H$_2$O-C2′′), 72.4 (Ph*C*H$_2$O-C3′′′), 71.9 (C5′), 71.8 (Ph*C*H$_2$O-C4′′′), 71.5 (C4′′′), 70.1 (C5′′), 69.6 (C6′), 64.4 (C2′), 60.4 (C1), 60.2 (C3), 57.3 (C2′′′), 51.0 (C6′′′), 48.7 (C4′), 32.8 (d, *J* = 21.3 Hz: FCH$_2$*C*H$_2$), 32.5 (C2). ESI-HRMS: *m/z* calcd for $C_{74}H_{80}N_{15}O_{13}SNa$ $[M+Na]^+$ 1460.5662, found 1460.5640.

4′-Deoxy-4′-(2-fluoroethylthio)-paromomycin pentaacetate (**18**)**.** To a solution of **14** (27.1 mg, 18.8 μmol) in tetrahydrofuran (1 mL) were added a solution of 1 M trimethylphosphine in tetrahydrofuran (144 μL, 144 μmol) and 0.1 M NaOH (181 μL) at room temperature. After 9 h of stirring at 50 °C, the reaction mixture was concentrated under reduced pressure and the residue was purified by flash chromatography (stepwise elution with chloroform, chloroform:2-propanol = 5:2, and chloroform:2-propanol:25% aqueous ammonia = 5:2:0.1). The resulting compound (21.2 mg) was dissolved in a solution of water/methanol/acetic acid (2:1:0.3, *v*/*v*/*v*, 990 μL) and $Pd(OH)_2$/C (20% loading, 42.4 mg) was added. After 15 h of stirring under H_2 atmosphere (48 psi) at room temperature, the mixture was filtered through a Celite pad and the filtrate was concentrated by blowing air. The crude product was purified by CM Sephadex C-25 ion exchange column (stepwise elution with water, 0.1% aqueous ammonia, and 0.5% aqueous ammonia) and lyophilized *in vacuo* to give **18** (3.4 mg, 27%). This was dissolved in a mixed solution of water (300 μL) and acetic acid (3.0 μL), and lyophilized *in vacuo* to afford the corresponding pentaacetate salt (5.4 mg, 29%). ^{1}H NMR (600 MHz, D_2O) δ 5.60 (d, *J* = 3.9 Hz, 1H: H1′), 5.21 (d, *J* = 2.6 Hz, 1H: H1′′), 5.13 (d, *J* = 1.8 Hz, 1H: H1′′′), 4.48 (dt, *J* = 46.8, 5.8 Hz, 1H: FC*H*$_2$CH$_2$), 4.36 (dd, *J* = 6.7, 4.9 Hz, 1H: H3′′), 4.21 (dd, *J* = 5.0, 2.6 Hz, 1H: H2′′), 4.15 (t, *J* = 5.1 Hz, 1H: H5′′′), 4.07 (t, *J* = 3.1 Hz, 1H: H3′′′), 4.04 (q, *J* = 4.6 Hz, 1H: H4′′), 3.91 (d, *J* = 10.4 Hz, 1H: H6′a), 3.82 – 3.73 (m, 3H: H6′b, H4′, H5′′a), 3.64 (m, 5H: H5, H3′, H4′′′, H5′′b, H4), 3.47 (t, *J* = 9.7 Hz, 1H: H6), 3.43 – 3.36 (m, 1H: H2′′′), 3.27 (dd, *J* = 13.6, 6.7 Hz, 1H: H6′′′a), 3.24 – 3.17 (m, 2H: H2′, H6′′′b), 3.14 – 3.05 (m, 2H: H3, H1), 2.84 (ddt, *J* = 24.5, 10.8, 5.8 Hz, 2H: FCH$_2$C*H*$_2$), 2.61 (t, *J* = 10.7 Hz, 1H: H5′), 2.17 (dt, *J* = 11.1, 3.5 Hz, 1H: H2$_{eq}$), 1.75 (s, 15H: C*H*$_3$CO$_2$H), 1.51 (q, *J* = 12.7 Hz, 1H: H2$_{ax}$).^{13}C NMR (150 MHz, D_2O) δ 180.1 (CH_3CO_2H), 110.0 (C1′′), 96.1(C1′), 95.3(C1′′′), 84.3 (C5), 83.41 (d, *J* = 168.5 Hz: F*C*H$_2$CH$_2$), 81.3 (C4′′), 77.6 (C4), 75.1 (C3′′), 74.0 (C4′), 72.3 (C6), 70.2 (C5′′′), 67.6 (C3′′′), 67.2 (C3′), 63.0 (C4′′′), 61.1 (C6′), 59.9 (C5′′), 54.8 (C2′), 50.8

(C2‴), 49.6 (C1), 48.7 (C3), 48.5 (C5′), 40.3 (C6‴), 32.0 (d, *J* = 19.2 Hz: FCH_2CH_2), 28.3 (C2), 22.5 (CH_3CO_2H). ESI-HRMS: *m*/*z* calcd for $C_{25}H_{49}N_5O_{13}S$ $[M+H]^+$ 678.3032, found 678.3034.

1,2′,2‴,3,6‴-Pentaazido-2″,3′,3‴,4‴,5″,6,6′-hepta-*O*-benzyl-1,2′,2‴,3,6‴-pentadeamino-4′-deoxy-4′-ethylsulfinyl-paromomycin (**12**). Compound **7** (126 mg, 89 µmol) was dissolved in dichloromethane (1.3 mL) and the solution was cooled to -78°C. After 10 min of stirring, 3-chloroperoxybenzoic acid (20 mg, 8.9 µmol) was added and the mixture was stirred for 5 h. Saturated $Na_2S_2O_3$ solution (2 mL) was added and the mixture was stirred for 10 min. After warming to room temperature, the mixture was diluted with dichloromethane (3 mL) and water (3 mL). The organic layer was separated, washed with saturated $NaHCO_3$ solution and saturated NaCl solution (1 × 5 mL, each), dried over MgSO4, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (hexane:ethyl acetate = 3:1 to 1:2) to afford **12** (106 mg, 83%). $[\alpha]_D^{23}$ = +69.7 (c = 0.39, methanol). ^{1}H NMR (600 MHz, $CDCl_3$, major isomer) δ 7.42 – 7.11 (m, 35H, aromatic), 6.20 (d, *J* = 3.4 Hz, 1H: H1′), 5.64 (d, *J* = 5.5 Hz, 1H: H1″), 4.95 (d, *J* = 10.7 Hz, 1H: PhC*H*$_2$O-C6), 4.88 (d, *J* = 10.2 Hz, 1H: PhC*H*$_2$O-C3′), 4.85 (d, *J* = 1.8 Hz, 1H: H1‴), 4.75 – 4.68 (m, 1H: PhC*H*$_2$O-C6), 4.68 – 4.59 (m, 4H: PhC*H*$_2$O-C3′, PhC*H*$_2$O-C6′, H5′, PhC*H*$_2$O-C4‴), 4.59 – 4.50 (m, 3H: PhC*H*$_2$O-C2″, PhC*H*$_2$O-C5″, PhC*H*$_2$O-C6′), 4.45 – 4.37 (m, 4H: PhC*H*$_2$O-C5″, PhC*H*$_2$O-C2″, PhC*H*$_2$O-C3‴, H3′), 4.34 – 4.24 (m, 4H: PhC*H*$_2$O-C3‴, H4″, H3″, PhC*H*$_2$O-C4‴), 4.05 (dd, *J* = 11.2, 3.4 Hz, 1H: H6′a), 3.97 – 3.84 (m, 3H: H2″, H5, H6′b), 3.83 – 3.74 (m, 3H: H6″a, H5‴, H3‴), 3.68 (t, *J* = 9.3 Hz, 1H: H4), 3.65 – 3.55 (m, 2H: H6‴a, H6″b), 3.48 – 3.38 (m, 2H: H3, H1), 3.37 – 3.32 (m, 1H: H2‴), 3.26 (t, *J* = 9.5 Hz, 1H: H6), 3.16 – 3.11 (m, 1H: H4‴), 3.07 (t, *J* = 10.8 Hz, 1H: H4′), 3.02 (dd, *J* = 9.7, 3.5 Hz, 1H: H2′), 2.92 (dd, *J* = 12.8, 4.3 Hz, 1H: H6‴b), 2.79 (dq, *J* = 13.0, 7.5 Hz, 1H: CH_3CH_2), 2.69 (dq, *J* = 13.0, 7.5 Hz, 1H: CH_3CH_2), 2.29 – 2.18 (m, 1H, $H2_{eq}$), 1.46 – 1.31 (m, 1H: $H2_{ax}$), 1.19 – 1.13 (m, 3H: CH_3CH_2). ^{13}C NMR (150 MHz, $CDCl_3$, major isomer) δ 138.3, 138.1, 137.9, 137.6, 137.3, 137.0, 136.9, 128.7, 128.5, 128.4, 128.4, 128.4, 128.3, 128.3, 128.3, 128.3, 128.2, 128.2, 128.0, 127.8, 127.8, 127.7, 127.7, 127.7, 127.6, 127.5, 127.5, 127.5, 127.5, 127.4, 106.4 (C1″), 98.6 (C1‴), 96.2 (C1′), 84.0 (C6), 82.2 (C2″), 82.0 (C5), 81.9 (C4″), 75.6 (C3′), 75.5 (C3″), 75.4 (Ph*C*H$_2$O-C3′), 75.3 (C4), 75.1 (Ph*C*H$_2$O-C6), 74.2 (C5‴), 73.3 (Ph*C*H$_2$O-C6′), 73.2 (Ph*C*H$_2$O-C2″), 73.2 (Ph*C*H$_2$O-C5″), 73.0 (C3‴), 72.4 (Ph*C*H$_2$O-C3‴), 71.8 (Ph*C*H$_2$O-C4‴), 71.6 (H4‴), 70.3 (C6′), 70.1 (C5″), 66.9 (C5′), 64.0 (C2′), 60.5 (C1), 60.2 (C3), 57.5 (C4′), 57.3 (C2‴), 51.1 (C6‴), 47.1 (CH_3CH_2), 32.5 (C2), 7.4 (CH_3CH_2). ^{1}H NMR (600 MHz, $CDCl_3$, minor isomer) δ 7.42 – 7.11 (m, 35H: aromatic), 6.14 (d, *J* = 3.6 Hz, 1H: H1′), 5.62 (d, *J* = 5.1 Hz, 1H, H1″), 5.02 (d, *J* = 10.1 Hz, 1H: PhC*H*$_2$O-C3′), 4.92 (d, *J* = 10.7 Hz, 1H: PhC*H*$_2$O-C6), 4.85 (d, *J* = 1.8 Hz, 1H: H1‴), 4.83 (d, *J* = 10.1 Hz, 1H: PhC*H*$_2$O-C3′), 4.75 – 4.68 (m, 1H: PhC*H*$_2$O-C6), 4.68 – 4.59 (m, 3H: PhC*H*$_2$O-C6′, H5′, PhC*H*$_2$O-C4‴), 4.59 – 4.50 (m, 4H: PhC*H*$_2$O-C2″, PhC*H*$_2$O-C5″, H3′, PhC*H*$_2$O-C6′), 4.48 – 4.37 (m, 3H: PhC*H*$_2$O-C5″, PhC*H*$_2$O-C2″, PhC*H*$_2$O-C3‴), 4.35 – 4.23 (m, 4H: PhC*H*$_2$O-C3‴, H4″, H3″, PhC*H*$_2$O-C4‴), 4.05 (dd, *J* = 11.2, 3.4 Hz, 1H: H6′a), 3.97 – 3.84 (m, 3H: H2″, H5, H6′b), 3.83 – 3.74 (m, 3H: H6″a, H5‴, H3‴), 3.68 (t, *J* = 9.3 Hz, 1H: H4), 3.65 – 3.55 (m, 2H: H6‴a, H6″b), 3.48 – 3.38 (m, 2H: H3, H1), 3.37 – 3.32 (m, 1H: H2‴), 3.26 (t, *J* = 9.5 Hz, 2H: H6, H2′), 3.23 – 3.18 (m, 1H, CH_3CH_2), 3.16 – 3.11 (m, 1H: H4‴), 2.98 (dd, *J* = 11.8, 10.1 Hz, 1H: H4′), 2.95 – 2.89 (m, 1H: H6‴b), 2.52 (dq, *J* = 12.8, 7.6 Hz, 1H, CH_3CH_2), 2.29 – 2.18 (m, 1H: $H2_{eq}$), 1.46 – 1.31 (m, 1H: $H2_{ax}$), 1.19 – 1.13 (m, 3H, CH_3CH_2). ^{13}C NMR (150 MHz, $CDCl_3$, minor isomer) δ 138.3, 137.9, 137.7, 137.7, 137.6, 137.0, 136.9, 128.7, 128.5, 128.4, 128.4, 128.4, 128.3, 128.3, 128.3, 128.3, 128.2, 128.2, 128.0, 127.8, 127.8, 127.7, 127.7, 127.7, 127.6, 127.5, 127.5,

127.5, 127.5, 127.4, 106.5 (C1′′), 98.5 (C1′′′), 96.5 (C1′), 83.9 (C6), 82.1 (C2′′), 82.0 (C5), 81.9 (C4′′), 75.5 (C3′′), 75.4 (Ph*C*H_2O-C3′), 75.3 (C4), 75.0 (Ph*C*H_2O-C6), 74.1 (C5′′′), 73.4 (Ph*C*H_2O-C6′), 73.2 (Ph*C*H_2O-C2′′), 73.2 (Ph*C*H_2O-C5′′), 73.1 (C3′′′), 72.4 (Ph*C*H_2O-C3′′′), 71.8 (Ph*C*H_2O-C4′′′), 71.6 (C4′′′), 71.2 (C3′), 70.2 (C6′), 69.8 (C5′′), 68.1 (C5′), 65.0 (C2′), 60.3 (C1), 59.8 (C3), 59.3 (C4′), 57.4 (C2′′′), 51.1 (C6′′′), 45.7 (CH_3*C*H_2), 32.3 (C2), 8.1 (*C*H_3CH$_2$). ESI-HRMS: *m*/*z* calcd for $C_{74}H_{81}N_{15}O_{14}SNa$ $[M+Na]^+$ 1458.5706, found 1458.5699.

4′-Deoxy-4′-ethylsulfinyl-paromomycin (**19**)**.** To a solution of **12** (116 mg, 81.0 μmol) in tetrahydrofuran (11.6 mL) were added a solution of 1 M trimethylphosphine in tetrahydrofuran (648 μL, 648 μmol) and 0.1 M NaOH (818 μL) at room temperature. After 23 h of stirring at 50 °C, the reaction mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography on silica gel (chloroform:2-propanol:25% aqueous ammonia = 5:3:0.2). The resulting compound (120 mg) was taken in a mixed solution of water/methanol/acetic acid (2:1:0.3, *v*/*v*/*v*, 5 mL) and $Pd(OH)_2$/C (20% loading, 240 mg) was added. After 11 h of stirring under H_2 atmosphere (balloon) at room temperature, the reaction mixture was filtered through a Celite pad and the filtrate was concentrated by blowing air. The crude product was purified by CM Sephadex C-25 ion exchange column (stepwise elution with water, 0.1% ammonia, and 0.5% ammonia) and lyophilized *in vacuo*. To a stirred solution of the residue in water (1.5 mL) were added K_2CO_3 (325 mg, 2.35 mmol), methanol (1.5 mL), imidazole-1-sulfonyl azide hydrochloride (182 mg, 867 μmol), and $CuSO_4$ (1.0 mg, 6.2 μmol) at room temperature. After 12 h of stirring, the reaction mixture was neutralized with 1 M HCl in an ice bath and solid NaCl was added at room temperature up to saturation. The mixture was extracted with tetrahydrofuran (3×10 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (chloroform:methanol = 5:1) and subsequent semi-preparative RP-HPLC. The resulting compound was taken in tetrahydrofuran (720 μL). A solution of 1 M trimethylphosphine in tetrahydrofuran (71 μL, 71 μmol) and 0.1 M NaOH (90 μL) were added to the solution and the mixture was stirred for 5 h at room temperature. After concentration under reduced pressure, the resiude was purified by CM Sephadex C-25 ion exchange column (stepwise elution with water, 0.1% aqueous ammonia, and 0.5% aqueous ammonia) to give **19** (5.4 mg, 10%) as a mixture of diastereomers at sulfur. This was dissolved in a mixed solution of water (1 mL) and acetic acid (3 μL), and lyophilized *in vacuo* to afford the corresponding pentaacetate salt (4.4 mg, 6%). $[\alpha]_D^{23}$ = +37.9 (c = 0.29, water). ^{1}H NMR (600 MHz, D_2O, major diastereomer) δ 5.70 (d, *J* = 3.7 Hz, 1H: H1′), 5.27 – 5.21 (m, 1H: H1′′), 5.27 – 5.21 (m, 1H: H1′′′), 4.44 – 4.31 (m, 2H: H3′, H3′′), 4.25 – 4.22 (m, 1H: H2′′), 4.19 – 4.15 (m, 1H: H5′′′), 4.11 – 4.02 (m, 3H: H3′′′, H5′′, H5′′′), 3.87 – 3.70 (m, 5H: H6′a, H5′′a, H4, H5, H6′b), 3.70 – 3.66 (m, 1H: H4′′′), 3.66 – 3.58 (m, 1H: H5′′b), 3.46 – 3.36 (m, 3H: H2′′′, H2′, H6), 3.35 – 3.25 (m, 2H: CH_3C*H*$_2$, H6′′′a), 3.26 – 3.14 (m, 3H: H6′′′b, H3, H1), 3.07 – 2.94 (m, 2H: H4′, CH_3C*H*$_2$), 2.32 – 2.20 (m, 1H: $H2_{eq}$), 1.78 (s, 15H: C*H*$_3$CO$_2$H), 1.65 – 1.52 (m, 1H: $H2_{ax}$), 1.22 – 1.14 (m, 3H: C*H*$_3$CH$_2$). ^{13}C NMR (150 MHz, D_2O, major isomer) δ 181.0 (CH_3*C*O$_2$H), 109.9 (C1′′), 96.1 (C1′), 95.4 (C1′′′), 84.4 (C5), 81.3 (C4′′), 75.2 (C3′′), 73.4 (C2′′), 71.1 (C6), 70.2 (C5′′′), 69.7 (C5′), 67.6 (C3′′′), 67.3 (C4′′′), 60.8 (C3′), 60.1 (C6′), 60.0 (C5′′), 57.4 (C4′), 55.0 (C2′), 50.8 (C2′′), 49.9 (C1), 48.9 (C3), 45.3 (CH_3*C*H$_2$), 40.4 (C6′′′), 29.6 (C2), 23.1 (*C*H$_3$CO$_2$H), 7.4 (*C*H$_3$CH$_2$). ^{1}H NMR (600 MHz, D_2O, minor diastereomer) δ 5.68 (d, *J* = 3.8 Hz, 1H: H1′), 5.27 – 5.21 (m, 1H: H1′′), 5.27 – 5.21 (m, 1H: H1′′′), 4.44 – 4.31 (m, 2H: H3′, H3′′), 4.25 – 4.22 (m, 1H: H2′′), 4.19 – 4.15 (m,

1H: H5‴), 4.11 – 4.02 (m, 3H: H3‴, H5″, H5‴), 3.87 – 3.70 (m, 5H: H6′a, H5″a, H4, H5, H6′b), 3.70 – 3.66 (m, 1H: H4‴), 3.66 – 3.58 (m, 1H: H5″b), 3.46 – 3.36 (m, 2H: H2‴, H6), 3.35 – 3.25 (m, 2H: H2′, H6‴a), 3.26 – 3.14 (m, 3H: H6‴b, H3, H1), 3.07 – 2.94 (m, 2H: H4′, CH_3CH_2), 2.94 – 2.87 (m, 1H: CH_3CH_2), 2.32 – 2.20 (m, 1H: $H2_{eq}$), 1.78 (s, 15H: CH_3CO_2H), 1.65 – 1.52 (m, 1H: $H2_{ax}$), 1.22 – 1.14 (m, 3H: CH_3CH_2). ^{13}C NMR (150 MHz, D_2O, minor isomer) δ 181.0 (CH_3CO_2H), 110.0 (C1″), 95.9 (C1′), 95.1 (C1‴), 84.5 (C5), 81.2 (C4″), 75.4 (C3″), 73.4 (C2″), 71.1 (C6), 70.2 (C5‴), 69.7 (C5′), 67.6 (C3‴), 67.2 (C4‴), 61.0 (C3′), 60.1 (C6′), 59.9 (C5″), 57.7 (C4′), 54.6 (C2′), 50.8 (C2″), 49.8 (C1), 48.8 (C3), 45.6 (CH_3CH_2), 40.4 (C6‴), 29.4 (C2), 23.1 (CH_3CO_2H), 6.7 (CH_3CH_2). ESI-HRMS: *m*/*z* calcd for $C_{25}H_{50}N_5O_{14}S$ $[M+H]^+$ 676.3075, found 676.3060.

1,2′,2‴,3,6‴-Pentaazido-2″,3′,3‴,4‴,5″,6,6′-hepta-*O*-benzyl-1,2′,2‴,3,6‴-pentadeamino-4′-deoxy-4′-ethylsulfonyl-paromomycin (**13**). To a solution of **7** (76.2 mg, 53.6 μmol) in dichloromethane (1.5 mL) was added 3-chloroperoxybenzoic acid (26.4 mg, 118 μmol) in an ice bath and the mixture was stirred for 4 h at room temperature. 0.1 M solution of $Na_2S_2O_3$ in water (1 mL) was added and the mixture was stirred for 10 min. The mixture was diluted with dichloromethane (3 mL) and water (3 mL). The organic layer was separated, washed with saturated NaCl solution (1 × 2 mL), dried over MgSO4, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (hexane:ethyl acetate = 5:1 to 1:1) to afford **13** (62.7 mg, 81%). $[\alpha]_D^{23}$ = +80.0 (c = 0.83, chloroform). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44 – 7.15 (m, 35H: aromatic), 6.17 (d, *J* = 3.4 Hz, 1H: H1′), 5.64 (d, *J* = 5.3 Hz, 1H: H1″), 4.96 (d, *J* = 10.6 Hz, 1H: PhCH_2O-C6), 4.93 (d, *J* = 10.0 Hz, 1H: PhCH_2O-C3′), 4.89 (d, *J* = 1.9 Hz, 1H: H1‴), 4.81 (d, *J* = 10.1 Hz, 1H: PhCH_2O-C3′), 4.79 – 4.72 (m, 2H: H5′, PhCH_2O-C6), 4.65 (d, *J* = 12.1 Hz, 1H: PhCH_2O-C4‴), 4.62 (d, *J* = 11.6 Hz, 1H: PhCH_2O-C6′), 4.60 – 4.50 (m, 4H: PhCH_2O-C5″, PhCH_2O-C2″, PhCH_2O-C6′, H3′), 4.50 – 4.39 (m, 3H: PhCH_2O-C5″, PhCH_2O-C3‴, PhCH_2O-C2″), 4.38 – 4.24 (m, 5H: PhCH_2O-C3‴, H4″, H3″, PhCH_2O-C4‴, H6′a), 3.96 (t, *J* = 5.1 Hz, 1H: H2″), 3.93 (t, *J* = 8.9 Hz, 1H: H5), 3.87 – 3.77 (m, 4H: H5″a, H6′b, H5‴, H3‴), 3.71 – 3.56 (m, 5H: H6‴a, H4, H5″b, H4′), 3.52 – 3.41 (m, 2H: H3, H1), 3.41 – 3.37 (m, 1H: H2‴), 3.28 (t, *J* = 9.3 Hz, 1H: H6), 3.17 (t, *J* = 2.2 Hz, 1H: H4‴), 3.12 (dd, *J* = 9.7, 3.4 Hz, 1H: H2′), 2.94 (tdd, *J* = 9.1, 6.8, 2.9 Hz, 3H: H6‴b, CH_3CH_2), 2.29 (dt, *J* = 13.2, 4.6 Hz, 1H: $H2_{eq}$), 1.44 (q, *J* = 12.7 Hz, 1H: $H2_{ax}$), 1.23 (t, *J* = 7.4 Hz, 3H: CH_3CH_2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.3, 138.2, 137.9, 137.6, 137.4, 137.0, 136.9, 128.7, 128.6, 128.5, 128.4, 128.4, 128.4, 128.3, 128.3, 128.2, 128.2, 128.0, 127.9, 127.9, 127.8, 127.8, 127.8, 127.7, 127.6, 127.6, 127.5, 106.5 (C1″), 98.6 (C1‴), 95.8 (C1′), 84.0 (C6), 82.2 (C2″), 82.1 (C5), 81.9 (C4″), 75.4 (C3″), 75.2 (C4), 75.1 (PhCH_2O-C6), 74.9 (PhCH_2O-C3′), 74.4 (C3′), 74.3 (C5‴), 73.5 (PhCH_2O-C6′), 73.3 (C3‴), 73.2 (PhCH_2O-C5″), 72.9 (PhCH_2O-C2″), 72.4 (PhCH_2O-C3‴), 71.8 (PhCH_2O-C4‴), 71.5 (C4‴), 70.3 (C6′), 70.2 (C5″), 66.9 (C5′), 64.0 (C2′), 60.6 (C4′), 60.4 (C1), 60.0 (C3), 57.3 (C2‴), 51.6 (CH_3CH_2), 51.1 (C6‴), 32.5 (C2), 6.0 (CH_3CH_2). ESI-HRMS: *m*/*z* calcd for $C_{74}H_{81}N_{15}O_{14}SNa$ $[M+Na]^+$ 1474.5655, found 1474.5647.

4′-Deoxy-4′-ethylsulfonyl-paromomycin (**20**)**.** To a solution of **13** (63.7 mg, 43.9 μmol) in tetrahydrofuran (5 mL) were added a solution of 1 M trimethylphosphine in tetrahydrofuran (450 μL, 450 μmol) and 0.1 M NaOH (125 μL) at room temperature. After 21 h of stirring at 50 °C, the reaction

mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography on silica gel (chloroform:2-propanol:25% aqueous ammonia = 5:2:0.1). The resulting material was taken in a mixed solution of water/methanol/acetic acid (2:1:0.1, *v*/*v*/*v*, 3.1 mL) and $Pd(OH)_2/C$ (20% loading, 77.4 mg) was added. After 7 h of stirring under H_2 atmosphere (balloon) at room temperature, the reaction mixture was filtered through a Celite pad and the cake was washed with water. The combined filtrate was concentrated by blowing air. The crude product was purified by CM Sephadex C-25 ion exchange column (stepwise elution with water, 0.1% aqueous ammonia solution, and 0.5% aqueous ammonia solution) to give **20** (12.4 mg, 41%). This was dissolved in water (1.5 mL) and acetic acid (5.5 µl), and lyophilized *in vacuo* to afford the corresponding pentaacetate salt (17.2 mg). $[\alpha]_D^{23}$ = +40.1 (c = 1.15, water). ^{1}H NMR (600 MHz, D_2O) δ 5.59 (d, *J* = 3.6 Hz, 1H: H1′), 5.20 (d, *J* = 2.5 Hz, 1H: H1′′), 5.12 (t, *J* = 1.9 Hz, 1H: H1′′′), 4.38 – 4.29 (m, 2H: H3′, H3′′), 4.25 – 4.16 (m, 2H: H2′′, H5′), 4.14 (ddd, *J* = 7.0, 3.7, 1.6 Hz, 1H: H5′′′), 4.06 (t, *J* = 3.1 Hz, 1H: H2′′′), 4.03 (ddd, *J* = 6.5, 4.9, 3.1 Hz, 1H: H4′′), 3.88 – 3.71 (m, 4H: H6′a, H6′b, H4, H5′′a), 3.71 – 3.62 (m, 2H: H5, H4′′′), 3.62 – 3.53 (m, 2H: H5′′b, H4′), 3.48 (dd, *J* = 10.4, 8.6 Hz, 1H: H6), 3.43 – 3.39 (m, 1H: H2′′′), 3.31 – 3.09 (m, 7H: H6′′′a, H2′, H3, H6′′′b, CH_3CH_2, H1), 2.18 (dt, *J* = 13.0, 4.3 Hz, 1H: $H2_{eq}$), 1.75 (s, 15H: CH_3CO_2H), 1.52 (q, *J* = 12.6 Hz, 1H: $H2_{ax}$), 1.20 (t, *J* = 7.3 Hz, 1H: CH_3CH_2). ^{13}C NMR (150 MHz, D_2O) δ 181.1 (CH_3CO_2H), 109.7 (C1′′), 95.7 (C1′′′) 95.5 (C1′), 84.1 (C5), 81.3 (C4′′), 79.2 (C4), 75.4 (C3′′), 73.3 (C2′′), 72.6 (C6), 70.2 (C5′′′), 68.5 (C5′), 67.7 (C3′′′), 67.3 (C4′′′), 64.6 (C3′), 62.0 (C6′), 61.1 (C4′), 60.3 (C5′′), 54.5 (C2′), 51.2 (C6′′′), 50.8 (C2′′′), 50.0 (C1), 49.0 (C3), 40.4 (CH_3CH_2), 29.8 (C2), 23.2 (CH_3CH_2), 5.1 (CH_3CH_2). ESI-HRMS: *m*/*z* calcd for $C_{25}H_{50}N_5O_{15}S$ $[M+H]^+$ 692.3024, found 692.3029.

1,3,2′,2′′′,6′′′-Penta-*N*-trifluoroacetyl paromomycin (21). Trifluoroacetic anhydride (760 mL, 5.4 mol) was added dropwise with shaking to an ice-cooled mixture of paromomycin hydrogensulfate **1**[i] (100 g, 0.106 mol) and potassium acetate (200 g, 2.04 mol). The resultant slurry was kept 0.5 h in an ice bath, then was heated to 65 °C for 8 h, with monitoring by ESIMS, resulting in a clear homogeneous solution. After cooling to room temperature the reaction mixture was concentrated under the reduced pressure and held under vacuum until all the remaining trifluoroacetic anhydride and trifluoroacetic acid was removed.[ii] The residual mixture was diluted with ethyl acetate (2.0 L) and was washed with saturated aqueous sodium bicarbonate (1.5 L). The aqueous phase was extracted with ethyl acetate (500 mL x 3) and the combined organic phase was washed with water (500 mL) and brine (500 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated to dryness under vacuum. The resultant reside was dissolved in 2M ethanolic acetic acid (2.5 L) and was heated to 75 °C, with monitoring by ESIMS, for 10 h. The reaction mixture was cooled to ambient temperature and then concentrated to dryness under vacuum. The residue was taken up in ethyl acetate (500 mL) and the solution was poured to the hexane (4.0 L) to give a precipitate that was collected by suction filtration.

[i] As determined by ^{1}H NMR with acetonitrile as internal standard.

[ii] Complete removal of TFA and remaining TFAA is necessary for successful extraction. Any remaining TFA results in a poor phase separation in the extraction with loss of a significant amount of the compound in the aqueous layer.

The white precipitate of **21** (110 g 0.10 mol, 94%) was dried under reduced pressure at room temperature and used in the next reaction without further purification.

4′,6′-*O*-Benzylidene-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl paromomycin (22). Benzaldehyde dimethylacetal (30.0 mL, 0.20 mol) and then camphorsulfonic acid (5.80 g, 0.025 mol) were added with stirring to a solution of the precipitate **21** (110 g 0.10 mol) in acetonitrile (1.1 L) at ambient temperature. After stirring at room temperature for 12 h, with monitoring by ESIMS, the reaction was quenched with triethylamine (10 mL) then was concentrated under reduced pressure. The residue was dissolved in ethyl acetate (1.0 L) and washed with water (500 mL). The aqueous phase was extracted with ethyl acetate (500 mL) and the combined organic layer was washed with 0.1 N aqueous hydrochloric acid (500 mL), water (500 mL), saturated sodium bicarbonate (500 mL), water (500 mL) and brine (500 mL), and dried over sodium sulfate. After filtration the solution was concentrated under reduced pressure and the reside was dissolved in ethyl acetate (500 mL) and poured into hexane (4.0 L) to give a precipitate of **S11** that was collected by filtration, dried and was used in the next reaction without further purification (120 g, 0.101 mol, 101%).[iii]

6,3′,2″,5″,3‴,4‴-Hexa-*O*-benzoyl-4′,6′-*O*-benzylidene-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl paromomycin (23). To a stirred solution of precipitate **22** (120 g, 0.101 mol) in pyridine (1.2 L) was added at room temperature benzoic anhydride[iv] (206.7 g, 0.910 mol) and *N,N*-dimethyl-4-aminopyridine (2.47 g, 0.020 mol). Stirring was maintained at room temperature for 24 h, with monitoring by ESIMS and by silica gel TLC (toluene:ethyl acetate 3:1, R_f = 0.45), then the reaction mixture was concentrated under reduced pressure and was co-evaporated with toluene (500 mL x 2). The residue was dissolved in ethyl acetate (2.0 L) and washed with 1.0 L of water (1.0 L), and the aqueous phase was extracted with ethyl acetate (500 mL). The combined organic layer was washed with 0.1 N hydrochloric acid (500 mL), water (500 mL), saturated aqueous sodium bicarbonate (1.0 L), water (1.0 L) and brine (1.0 L), and dried over sodium sulfate. After filtration and concentration under vacuum the residue was dissolved in ethyl acetate (700 mL) and then poured into hexane (4.0 L). The so-obtained precipitate was collected by filtration and was used in the next reaction without further purification (189 g, 0.101 mol, 103%). An aliquot of **23** was purified by chromatography over silica gel (eluent: hexane: ethyl acetate 9:1 to 3:2, R_f=0.25 eluting with 2:1 hexane:ethyl acetate) and had the following physical characteristics: $[\alpha]_D^{23}$ = +31.5 (c = 1.0, methanol). ^{1}H NMR (600 MHz, CD_3OD) δ 8.23 (d, *J* = 7.5 Hz, 2H), 8.14 (d, *J* = 7.5 Hz, 2H), 8.04 (d, *J* = 7.7 Hz, 2H), 7.91 (d, *J* = 7.5 Hz, 2H), 7.75 (d, *J* = 7.5 Hz, 2H), 7.71 (t, *J* = 7.4 Hz, 1H), 7.67 (t, *J* =

[iii] ESIMS of the precipitate indicated the presence of a small amount of a di-*O*-benzylidene adduct, presumably the 4′,6′;4‴,6‴-di-*O*-benzylidene derivative.

[iv] The use of benzoyl chloride as benzoylation reagent gave a messy reaction. Benzoyl cyanide gave a clean reaction that easy monitored by mass spectrometry and which was faster than benzoic anhydride. In the benzoic anhydride case, the reaction was clean but mass spectrometry showed several unexplained signals. The large amount of cyanide generated using benzoyl cyanide was judged to be less than favorable. Overall, benzoic anhydride is the best solution and works well practically.

7.4 Hz, 1H), 7.58 (dd, *J* = 12.5, 7.5 Hz, 5H), 7.53 (t, *J* = 7.4 Hz, 1H), 7.46 (*d*, J = 7.6 Hz, 2H), 7.41 (dt, *J* = 15.6, 7.8 Hz, 5H), 7.35 – 7.31 (m, 2H), 7.26 – 7.22 (m, 3H), 7.19 (t, *J* = 7.8 Hz, 2H), 7.03 (t, *J* = 7.3 Hz, 1H), 6.93 (t, *J* = 7.7 Hz, 2H), 6.13 (d, *J* = 3.8 Hz, 1H), 5.49 (t, *J* = 10.1 Hz, 1H), 5.44 – 4.41 (m, 2H), 5.32 (t, *J* = 9.9 Hz, 1H), 5.27 (t, *J* = 2.8 Hz, 1H), 5.20 – 5.13 (m, 3H), 4.73 – 4.66 (m, 2H), 4.60 (dd, J_1 = 12.4, J_2 = 3.3 Hz, 1H), 4.55 (dd, J_1 = 10.6, J_2 = 4.0 Hz, 1H), 4.42 (t, *J* = 6.9 Hz, 1H), 4.38 (t, *J* = 8.9 Hz, 1H), 4.36 – 4.24 (m, 3H), 4.23 – 4.20 (m, 1H), 4.12 (dd, J_1 = 10.2, J_2 = 4.7 Hz, 1H), 4.03 (t, *J* = 9.3 Hz, 1H), 3.91 (br s, 1H), 3.82 (td, J_1 = 9.8, J_2 = 5.1 Hz, 1H), 3.56 (t, *J* = 9.6 Hz, 1H), 3.50 (t, *J* = 10.3 Hz, 1H), 3.44 (dd, *J* = 13.8, 6.6 Hz, 1H), 3.36 (dd, *J* = 13.8, 6.9 Hz, 1H), 2.15 (q, *J* = 12.8 Hz, 1H), 2.01 (dt, J_1 = 12.9 Hz, J_1 = 4.3 Hz, 1H). ^{13}C NMR (150 MHz, CD_3OD) δ 166.7, 166.0, 165.8, 165.0, 164.8, 164.1, 158.5 – 156.5 (5 X $COCF_3$), 137.3, 133.6, 133.5, 133.2, 133.0, 129.8, 129.8, 129.6, 129.5, 129.3, 129.2, 128.9, 128.6, 128.5, 128.4, 128.2, 128.1, 127.9, 127.8, 127.6, 126.1, 119.5 – 111.5 (5 X CF_3), 109.6, 101.6, 97.2, 96.1, 85.7, 79.4, 78.9, 76.1, 75.5, 75.4, 74.6, 72.2, 70.00, 68.2, 67.8, 66.1, 63.1, 62.5, 51.81, 49.0, 48.7, 48.2, 39.0, 30.6. ESI-HRMS: *m*/*z* calcd for $C_{82}H_{68}N_5O_{25}F_{15}Na$ $[M+Na]^+$ 1830.3862, found 1830.3796.

6,3′,2″,5″,3‴,4‴-Hexa-*O*-benzoyl-1,3,2‴,2‴,6‴-penta-*N*-trifluoroacetyl paromomycin (24). A solution of precipitate **23** (189 g, 0.101 mol) in 80% of aqueous acetic acid (1.5 L, prepared by mixing 1.2 L of glacial acetic acid and 0.3 L of water) was heated to 65 ^{o}C for 14 h, with monitoring by ESIMS. After cooling to ambient temperature, the solution was concentrated and the residue was co-evaporated with toluene (500 mL x 3). The residue was dissolved in ethyl acetate (700 mL) and poured into hexane (4.0 L). The so-obtained precipitate was collected by filtration, dried under vacuum, and used in the next reaction without further purification (164 g, 0.095 mol, 94%). An aliquot of **24** was purified by chromatography over silica gel (eluent: hexane:ethyl acetate 9:1 to 1:1, R_f=0.35 eluting with 1:1 hexane:ethyl acetate) and had the following physical characteristics: $[\alpha]_D^{23}$ = +52.7 (c = 1.0, methanol) ^{1}H NMR (600 MHz, CD_3OD) δ 8.0 (d, *J* = 7.2 Hz, 2H), 8.15 (d, *J* = 7.3 Hz, 2H), 8.04 (d, *J* = 7.3 Hz, 2H), 7.95 (d, *J* = 7.3 Hz, 2H), 7.76 (d, *J* = 7.3 Hz, 2H), 7.72 (t, *J* = 7.4 Hz, 1H), 7.65 (t, *J* = 7.4 Hz, 1H), 7.62 – 7.54 (m, 6H), 7.51 (d, J = 7.2 Hz, 2H), 7.46 – 7.41 (m, 5H), 7.21 (t, *J* = 7.8 Hz, 2H), 7.06 (t, *J* = 7.4 Hz, 1H), 6.95 (t, *J* = 7.8 Hz, 2H), 6.10 (d, *J* = 4.0 Hz, 1H), 5.43 (br s, 1H), 5.34 – 5.26 (m, 2H), 5.26 (t, *J* = 3.0 Hz, 1H), 5.17 (d, *J* = 4.7 Hz, 1H), 5.14 (br s, 1H) 5.12 (d, *J* = 1.7 Hz, 1H), 4.65 (dd, J_1 = 12.4 Hz, J_2 = 4.1 Hz, 1H), 4.58 (m, 2H), 4.44 – 4.24 (m, 6H), 4.23 – 4.18 (m, 1H), 4.12 (dd, J_1 = 10.1, J_2 = 8.6 Hz, 1H), 3.86 (br s, 1H), 3.83 (dd, J_1 = 11.3, J_2 = 1.9 Hz, 1H), 3.76 – 3.72 (m, 1H), 3.61 (dd, J_1 = Hz, J_2 = 5.8 Hz, 1H), 3.49 (t, *J* = 9.6 Hz, 1H), 3.45 (dd, J_1 = 13.9, J_2 = 6.5 Hz, 1H), 3.32 (dd, *J* = 13.9, 7.0 Hz, 1H), 2.09 (q, *J* = 12.7 Hz, 1H), 2.02 (dt, J_1 = 12.8, J_2 = 4.3 Hz, 1H). ^{13}C NMR (150 MHz, CD_3OD) δ 166.8, 166.4, 165.9, 165.0, 164.9, 164.1, 158.5 – 156.5 (5 X $COCF_3$), 133.6, 133.5, 133.2, 133.2, 133.0, 132.8, 129.8, 129.7, 129.6, 129.6, 129.5, 129.3, 129.2, 128.9, 128.5, 128.5, 128.4, 128.2, 128.1, 128.0, 127.9, 127.8, 119.5 – 111.5 (5 X CF_3), 109.3, 97.2, 95.6, 85.2, 79.4, 75.8, 75.5, 75.3, 75.0, 73.6, 72.6, 72.2, 68.3, 68.2, 66.0, 62.8, 61.1, 51.8, 49.1, 48.7, 48.1, 39.0, 30.9. ESI-HRMS: *m*/*z* calcd for $C_{75}H_{64}N_5O_{25}F_{15}Na$ $[M+Na]^+$ 1742.3549, found 1742.3524.

6,3′,6′,2″,5″,3‴,4‴-Hepta-*O*-benzoyl-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl paromomycin (25). A stirred solution of precipitate **24** (164 g, 0.095 mol) in acetonitrile (1.6 L) was cooled to 0 ^{o}C in an ice bath and

treated with triethylamine (19.9 mL, 0.14 mol) followed by drop wise addition of a solution of benzoyl cyanide (12.46 g, 0.095 mol) in acetonitrile (310 mL).[v] After stirring for a further 2.5 h in an ice bath, with monitoring by ESIMS, the reaction was quenched with methanol (10 mL). The reaction mixture was concentrated under reduced pressure and the residue was dissolved in ethyl acetate (2.0 L) and washed with 0.1 N aqueous hydrochloric acid (500 mL), water (500 mL), saturated sodium bicarbonate (500 mL), and brine (2 x 500 mL). The organic phase was dried over anhydrous sodium sulfate and filtered. The filtrate was concentrated under the vacuum, and the residue was dissolved in ethyl acetate (700 mL) and poured into hexane (4.0 L). The precipitate was collected by filtration, dried under reduced pressure, and was used in the next reaction without further purification (165 g, 0.091 mol, 95%). An aliquot of **25** was purified by chromatography over silica gel (eluent: hexane:ethyl acetate 9:1 to 3:2, R_f=0.23 eluting with 2:1 hexane:ethyl acetate) and had the following physical characteristics: $[\alpha]_D^{23}$ = +73.5 (c = 1.0, methanol). ^{1}H NMR (600 MHz, CD_3OD) δ 8.14 (d, *J* = 7.5 Hz, 2H), 8.02 (t, *J* = 8.9 Hz, 4H), 7.99 (d, *J* = 7.9 Hz, 2H), 7.99 (d, *J* = 7.6 Hz, 2H), 7.74 (d, *J* = 7.6 Hz, 2H), 7.70 (t, *J* = 7.4 Hz, 1H), 7.62 – 7.54 (m, 5H), 7.48 – 7.40 (m, 9H), 7.37 (t, *J* = 7.4 Hz, 1H), 7.28 (t, *J* = 7.6 Hz, 2H), 7.19 (t, *J* = 7.6 Hz, 1H), 6.93 (t, *J* = 7.6 Hz, 2H), 6.19 (d, *J* = 3.8 Hz, 1H), 5.45 – 5.40 (m, 2H), 5.33 ((t, *J* = 9.9 Hz, 1H)), 5.25 (t, *J* = 2.8 Hz, 1H), 5.17 – 5.13 (m, 2H), 5.09 (br s, 1H), 4.72 – 4.61 (m, 3H), 4.52 (dd, J_1 = 10.9 Hz, J_2 = 3.8 Hz, 1H), 4.48 (dd, J_1 = 11.9 Hz, J_2 = 1.7 Hz, 1H), 4.43 (t, *J* = 8.8 Hz, 1H), 4.39 – 4.30 (m, 3H), 4.29 – 4.22 (m, 1H), 4.16 – 4.11 (m, 1H), 3.99 – 3.89 (m, 3H), 3.37 (dd, J_1 = 13.6 Hz, J_2 = 7.2 Hz, 1H), 3.12 (dd, J_1 = 13.7 Hz, J_2 = 6.5 Hz, 1H), 2.21 (q, *J* = 12.9 Hz, 1H), 2.03 (dt, J_1 = 12.9, J_2 = 4.2 Hz, 1H). ^{13}C NMR (150 MHz, CD_3OD) δ 166.6, 166.5, 166.3, 166.0, 165.0, 164.7, 164.1, 158.5 – 156.5 (5 X $COCF_3$), 133.6, 133.5, 133.2, 133.0, 132.9, 129.8, 129.7, 129.6, 129.6, 129.5, 129.4, 129.3, 129.2, 128.9, 128.5, 128.4, 128.2, 128.1, 128.0, 127.9, 127.9, 127.7, 119.5 – 111.5 (5 X CF_3), 109.3, 97.1, 95.8, 85.8, 79.2, 76.2, 75.5, 75.3, 74.2, 73.5, 71.9, 70.2, 68.2 67.7, 65.9, 62.5, 61.7, 51.8, 49.4, 48.7, 48.2, 38.7, 30.5. ESI-HRMS: *m/z* calcd for $C_{82}H_{68}N_5O_{26}F_{15}Na$ $[M+Na]^+$ 1846.3811, found 1830.3807.

6,3′,6′,2″,5″,3‴,4‴-Hepta-*O*-benzoyl-4′-deoxy-4′-iodo-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl-4′-epi-paromomycin (26). A stirred solution of the precipitate **25** (165 g, 0.091 mol) in dichloromethane (1.6 L) was treated under ice cooling with added pyridine (55.0 mL, 0.68 mol) and then drop wise with freshly distilled trifluoromethanesulfonic anhydride (45.9 mL, 0.27 mol). The reaction mixture then was stirred for 6 h under ice cooled conditions before methanol (10 mL) was added and the mixture concentrated under reduce pressure at 4 ^{o}C (ice cooled bath) and dried under vacuum. The so-obtained yellow oil[vi] was dissolved in acetone (1.6 L), treated with sodium iodide (139.4 g, 0.91 mol) and heated with stirring to 65 ^{o}C for 16 h, with monitoring by ESIMS. After cooling to ambient temperature the solvent was removed under reduced pressure, and the residue was partitioned between ethyl acetate (1.0 L) and water (1.0 L). The aqueous layer was extracted ethyl acetate (2 x 500 mL) and the combined organic layer was washed with 0.1 N hydrochloric acid (500 mL), water (500 mL), saturated aqueous sodium bicarbonate (500 mL), and brine (500 mL). The organic phase was dried over anhydrous sodium sulfate

[v] To avoid over reaction the benzoyl cyanide must be added as a solution in acetonitrile, dropwise with ice cooling.
[vi] The yellow color could not be removed by column chromatography at this stage.

and filtered and the filtrate was concentrated under the vacuum. The residue was dissolved in toluene (600 mL) and charged to a silica gel column made up of 870 g of silica gel slurried in hexane. Gradient elution with hexane:acetone (4:1 to 2:1) gave a series of fractions containing the product that were collected, concentrated and charged to a second silica gel column (870 g SiO_2 slurried in hexane). Elution with hexane:ethyl acetate (2:1) gave **26** in the form of a white foam that was used in the next reaction without further purification. R_f SiO_2 0.25 (hexane:ethyl acetate 2:1) (72.2 g, 0.036 mol, 39 % from compound **25**).[vii] An aliquot of **26** was purified by chromatography over silica gel (eluent: hexane:acetone 4:1 to 2:1, R_f=0.25 eluting with hexane:ethyl acetate 2:1) and had the following physical characteristics: $[\alpha]_D^{23}$ = +59.6 (c = 1.0, methanol). ^{1}H NMR (600 MHz, CD_3OD) δ 8.20 (d, *J* = 7.2 Hz, 2H), 8.16 (d, *J* = 7.2 Hz, 2H), 8.05 (d, *J* = 7.3 Hz, 2H), 8.01 (d, *J* = 7.2 Hz, 2H), 7.97 (d, *J* = 7.2 Hz, 2H), 7.76 – 7.41 (m, 3H), 7.65 (t, *J* = 7.4 Hz, 1H), 7.62 – 7.58 (m, 4H), 7.54 (t, *J* = 7.8 Hz, 2H), 7.51 – 7.41 (m, 10H), 7.20 (t, *J* = 7.8 Hz, 2H), 7.02 (t, *J* = 7.4 Hz, 1H), 6.93 (t, *J* = 7.7 Hz, 2H), 6.26 (d, *J* = 3.6 Hz, 1H), 5.46 (br s, 1H), 5.34 (t, *J* = 9.9 Hz, 1H), 5.27 (t, *J* = 2.9 Hz, 1H), 5.22 (d, *J* = 1.6 Hz, 1H), 5.19 (d, *J* = 4.5 Hz, 1H), 5.10 (br s, 1H), 5.02 (d, *J* = 2.1 Hz, 1H), 4.92 (dd, J_1 = 11.0 Hz, J_2 = 3.8 Hz, 1H), 4.76 (dd, J_1 = 7.9 Hz, J_2 = 4.6 Hz, 1H), 4.69 (br s, 1H), 4.58 (dd, J_1 = 11.0 Hz, J_2 = 3.7 Hz, 1H), 4.46 – 4.29 (m, 5H), 4.26 (dd, J_1 = 10.6 Hz, J_2 = 5.2 Hz, 1H), 4.18 – 4.15 (m, 1H), 4.02 (dd, J_1 = 10.2 Hz, J_2 = 3.7 Hz, 1H), 3.96 – 3.89 (m, 2H), 3.56 – 3.51 (m, 1H), 3.40 (dd, J_1 = 13.7 Hz, J_2 = 7.6 Hz, 1H), 3.19 (dd, J_1 = 13.7, J_2 = 6.4 Hz, 1H), 2.14 (q, *J* = 12.8 Hz, 1H), 2.03 (dt, J_1 = 12.8 Hz, J_2 = 4.3 Hz, 1H). ^{13}C NMR (150 MHz, CD_3OD) δ 166.6, 165.9, 165.5, 165.3, 165.0, 164.7, 164.1, 158.5 – 156.5 (5 X $COCF_3$), 133.6, 133.5, 133.5, 133.2, 133.2, 133.1, 133.0, 129.9, 129.7, 129.7, 129.6, 129.5, 129.2, 129.1, 129.07, 129.0, 128.8, 128.7, 128.5, 128.3, 128.3, 128.3, 128.2, 128.0, 127.9, 127.9, 127.7, 119.5 – 111.5 (5 X CF_3), 109.4, 97.1, 95.7, 85.6, 79.1, 76.1, 75.4, 75.3, 73.9, 71.9, 69.0, 68.2, 66.1, 65.9, 65.7, 61.8, 50.2, 48.9, 48.7, 48.2, 38.7, 36.3, 30.5. ESI-HRMS: *m/z* calcd for $C_{82}H_{67}N_5O_{25}F_{15}INa$ $[M+Na]^+$ 1956.2828, found 1956.2896.

4′-Allyl-4′-deoxy-6,3′,6′,2″,5″,3‴,4‴-hepta-*O*-benzoyl-1,3,2″,2‴,6‴-penta-*N*-trifluoroacetyl paromomycin (27). A gently stirred solution of iodide **26** (72.2 g, 0.036 mol) in α,α,α-trifluorotoluene (480 mL) was treated with allylphenyl sulfone (26.2 g, 0.144 mol) and cooled in an ice bath. A solution of 1.0 M triethylborane in hexane solution (72.0 mL, 0.072 mol) was then added dropwise with gentle stirring under the ambient laboratory atmosphere keeping the internal reaction temperature under below 5 °C, with monitoring by ESIMS[viii] After 3 h another portion of 1.0 M triethylborane in hexane (72.0 mL, 0.072 mol) was added under ice cooled conditions after which stirring was maintained for 8 h at 0 °C The reaction mixture was concentrated under reduced pressure and the residue was purified by silica gel column chromatography (SiO_2 500 g, eluent: hexane : ethyl acetate 2:1, R_f = 0.25)[ix]. The

[vii] The first hexane:acetone column gives a better separation from and removes aminoglycoside-based impurities. The second hexane:ethyl acetate column removes the yellow colored impurity carried forward from the previous step. It is essential that this yellow impurity is removed at this stage, otherwise it impedes the smooth operation of the subsequent radical step.

[viii] Vigorous stirring results in the uptake of too much oxygen causing the reaction to stop.

[ix] Chromatography is needed to remove other odorous sulfur-containing byproducts that otherwise prevent effective hydrogenation in the next step.

fractions were concentrated and the resultant **27** in the form of a light yellow foam was used in the next reaction without further purification (61.2 g, 0.033 mol, 92%). This product contains a major byproduct, 5-benzenesulfonyl-4-(benzenesulfonylmethyl)pentene **29** that could not be removed at this stage. An aliquot of **27** was purified by chromatography over silica gel (eluent: hexane:ethyl acetate 4:1 to 2:1, Rf=0.25 eluting with 2:1 hexane:ethyl acetate) and had the following physical characteristics: $[\alpha]_D^{23}$ = +72.5 (c = 1.0, methanol). ^{1}H NMR (600 MHz, CD_3OD) δ 8.15 (d, *J* = 7.3 Hz, 2H), 8.03 (t, *J* = 7.0 Hz, 4H), 7.97 – 7.92 (m, 4H), 7.75 – 7.69 (m, 3H), 7.62 – 7.56 (m, 5H), 7.49 – 7.40 (m, 9H), 7.37 (t, *J* = 7.4 Hz, 1H), 7.27 (t, *J* = 7.8 Hz, 2H), 7.20 (t, *J* = 7.8 Hz, 2H), 7.02 (t, *J* = 7.4 Hz, 1H), 6.92 (t, *J* = 7.8 Hz, 2H), 6.22 (d, *J* = 4.0 Hz, 1H), 5.75 – 5.65 (m, 1H), 5.43 (t, *J* = 10.8 Hz, 1H), 5.40 (br s, 1H), 5.30 (t, *J* = 9.8 Hz, 1H), 5.24 (t, *J* = 3.0 Hz, 1H), 5.16 (d, *J* = 1.8 Hz, 1H), 5.14 (d, *J* = 4.5 Hz, 1H), 5.07 (br s, 1H), 4.92 (dd, J_1 = 17.0 Hz, J_2 = 1.3 Hz, 1H), 4.80 (d, *J* = 10.6 Hz, 1H), 4.71 – 4.62 (m, 3H), 4.49 (dd, J_1 = 10.6 Hz, J_2 = 4.0 Hz, 1H), 4.45 (dd, J_1 = 12.6, J_2 = 2.3 Hz, 1H), 4.42 (t, *J* = 8.7 Hz, 1H), 4.39 – 4.23 (m, 4H), 4.13 – 4.05 (m, 2H), 3.98 (d, *J* = 10.7 Hz, 1H), 3.92 (br s, 1H), 3.36 (dd, J_1 = 13.8 Hz, J_2 = 7.5 Hz, 1H), 3.10 (dd, J_1 = 13.7 Hz, J_2 = 6.4 Hz, 1H), 2.31 (ddt, *J* = 15.7 Hz, J_2 = 10.6 Hz, J_3 = 4.8 Hz, 1H), 2.2 0 – 2.11 (m, 3H), 2.01 (dt, J_1 = 12.8 Hz, J_2 = 3.9 Hz, 1H). ^{13}C NMR (150 MHz, CD_3OD) δ 166.6, 166.3, 166.2, 165.9, 164.9, 164.6, 164.1, 159.0– 156.5 (5 X $COCF_3$), 134.1, 133.5, 133.4, 133.2, 133.0, 133.0, 132.9, 129.7, 129.6, 129.6, 129.6, 129.5, 129.4, 129.3, 129.2, 129.0, 128.5, 128.4, 128.3, 128.2, 128.2, 128.0, 127.9, 127.9, 127.7, 119.5 – 111.5 (5 X CF_3), 116.3, 109.2, 97.1, 95.8, 85.9, 79.1, 76.3, 75.6, 75.3, 74.0, 71.8, 71.2, 69.5, 68.2, 65.9, 63.5, 61.7, 52.7, 49.4, 48.6, 48.1, 41.2, 38.7, 31.2, 30.7, 23.8. ESI-HRMS: *m/z* calcd for $C_{85}H_{72}N_5O_{25}F_{15}Na$ $[M+Na]^+$ 1870.4175, found 1870.4109.

4′-Deoxy-4′-propyl-6,3′,6′,2″,5″,3‴,4‴-hepta-*O*-benzoyl-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl paromomycin (28). Palladium hydroxide (3.06 g) was added at room temperature to stirred solution of the alkene **27** (61.2 g, 0.033 mol) in methanol (610 mL) and the mixture was stirred under 1 atm of hydrogen at room temperature for 38 h, with monitoring by ESIMS. The reaction mixture was passed through a Celite pad and concentrated under vacuum. The residue was purified by silica gel column chromatography (eluent: dichloromethane:methanol 10:0.5, R_f = 0.25). The fractions were concentrated and used in the next reaction without further purification (32.5 g, 0.018 mol, 53%). This preparation contains 1-benzenesulfonyl-2-(benzenesulfonylmethyl)pentane **30**resulting from the hydrogenation of 5-benzenesulfonyl-4-(benzenesulfonylmethyl)pentene **29** present in the starting material.[x] An aliquot of **28** was purified by chromatography over silica gel (eluent: dichloromethane:methanol 10:0 to 9:1, R_f = 0.25 eluting with 10:0.5 dichloromethane:methanol) and had the following physical characteristics: $[\alpha]_D^{23}$ = +68.4 (c = 0.85, methanol). ^{1}H NMR (600 MHz, CD_3OD) δ = 8.14 (d, *J* = 7.7 Hz, 2H), 8.03 (t, *J* = 7.5 Hz, 6.5 Hz, 3H), 8.00 -7.90 (m, 3H), 7.75-7.69 (m, 3H), 7.64-7.55 (m, 6H), 7.50-7.40 (m, 9H), 7.32 (t, *J* = 7.7 Hz, 1H), 7.30, (m, 1H), 7.29, (t, *J* = 8.07 Hz, 7.7 Hz, 1H), 7.19 (t, *J* = 7.7 Hz, 7.5 Hz, 3H), 7.03 (t, *J* = 7.3 Hz, 7.3 Hz, 1H), 6.93 (t, *J* = 7.7 Hz, 7.5 Hz, 2H), 6.23 (d, *J* = 3.3 Hz, 1H), 5.42 (t, *J* = 11.0 Hz, 10.6 Hz, 1H), 5.41 (br s, 1H), 5.32 (t, *J* = 10.2 Hz, 9.5 Hz, 1H), 5.23 (t, *J* = 2.9 Hz, 1H), 5.15, (d, *J* = 1.8 Hz, 1H) 5,14 (d, *J* = 2.1 Hz,

[x] This material can be removed by careful chromatography but is best carried forward and removed after saponification of the benzoate esters when it is easy removed.

1H), 5.07 (s, 1H), 4.71 – 4.62 (m, 3H), 4.46 (dd, *J* = 10.6 Hz, 2.6 Hz, 1H), 4.42 (t, *J* = 9.1 Hz, 1H), 4.41 – 4.23 (m, 5H), 4.12 (br d, *J* = 8.4 Hz, 1H), 4.08 (dd, *J* = 9.9 Hz, 8.4 Hz, 1H), 3.98 (d, *J* = 11.0 Hz, 1H), 3.91 (br s, 1H), 3.36 (dd, *J* = 13.5 Hz, 7.3 Hz, 1H), 3.10 (dd, J_1 = 13.6 Hz, 6.3 Hz, 1H), 2.20-2.10 (br m, 2H), 2.04-1.98 (br m, 1H), 1.41-1.08 (m, 4H), 0.69 (t, *J* = 7.1 Hz, 6.9 Hz, 3H). ^{13}C NMR (150 MHz, CD_3OD) δ 166.6, 166.4, 166.3, 165.9, 165.0, 164.7, 164.1, 158.2– 156.5 (5 C=O coupled with ^{19}F), 133.6, 133.5, 133.2, 133.1, 133.1 133.0, 129.7, 129.6, 129.6, 129.6, 129.5, 129.4, 129.3, 129.2, 129.0, 128.5, 128.4, 128.3, 128.2, 128.2, 128.0, 127.9, 127.9, 127.7, 120.0 – 110.0 (5 CF_3 coupled with ^{19}F), 109.4, 97.1, 95.9, 85.8, 79.2, 76.1, 75.6, 75.3, 74.1, 71.8, 71.8, 70.6, 69.6, 68.1, 65.9, 63.7, 61.8, 52.7, 49.4, 48.6, 48.1, 41.2, 38.7, 30.7, 28.95, 19.31, 13.24. ESI-HRMS: *m*/*z* calcd for $C_{85}H_{74}N_5O_{25}F_{15}Na$ $[M+Na]^+$ 1872.4331, found 1872.4326.

4′-Deoxy-4′-propyl paromomycin pentaacetate (5). Compound **28** (46.3 g 0.025 mol) was dissolved in anhydrous methanol (460 mL) and treated with stirring under an inert atmosphere at room temperature with 6-10% magnesium methoxide in methanol (322 mL, 0.18-0.30 mol). After stirring for 48 h at ambient temperature with monitoring by ESIMS, the reaction mixture was concentrated and under reduced pressure and used in the next reaction without further purification.[xi,xii] The residue was taken up in water (460 mL), stirred and treated with barium hydroxide (92 g 0.54 mol) at room temperature. The solution was heated to 80 °C for 24 h with monitoring by ESIMS. The reaction mixture was cooled to 0 °C with an ice bath, then dry ice was added portion wise until pH = 8-9. The precipitate was filtered off and concentrated the filtrate dried under reduced pressure at room temperature. The residue was dissolved in water (800 mL), and neutralized with glacial acetic acid (7.0 mL). Any remaining barium salts were removed by addition of saturated aqueous ammonium sulfate (added dropwise with stirring until no more precipitation occurred) and filtration of the resulting precipitate.[xiii] The filtrate was directly loaded onto a Sephadex C-25 column (100 g, packed in water) and was eluted with gradually increasing concentrations of ammonium hydroxide up to 0.36%. The fractions containing the title compound, detected with 0.8% ethanolic ninhydrin, were combined and concentrated (11.2 g, 0.017 mol).[xiv] The residue was dissolved in water (50 mL) and treated with glacial acetic acid (7.0 mL 0.12 mol) then lyophilized to give the pentaacetate salt of **5**[xv] as a white amorphous solid. (15.1 g, 0.016 mol, 64 %). $[\alpha]_D^{23}$ = +51.2 (c = 1.10, water). δ= 5.38 (d, *J* = 3.7 Hz, 1H, H1′), 5.07 (d, *J* = 2.2 Hz, 1H, H1′′), 5.00 (br s, 1H, H1′′′), 4.23 (t, *J* = 5.8 Hz, 5.5 Hz, 1H, H3′′), 4.06 (dd, *J* = 4.0Hz, 2.6 Hz, 1H, H2′′), 4.02 (t, *J* = 4.8 Hz, 4.4 Hz, 1H, H5′′′), 3.94 (t, *J* = 2.9 Hz, 1H, H3′′′), 3.91 (m, 1H, H4′′), 3.67 (t, *J* = 9.5 Hz, 1H, H3′), 3.68 – 3.59 (m, 5H,

[xi] It is essential that the methanol be removed, otherwise a gel is formed in the final step.

[xii] 1-Benzenesulfonyl-2-(benzenesulfonylmethyl)pentane **S19** carried forward from the previous step elutes in front of the title compound and is readily removed at this stage.

[xiii] In large scale reactions, Ba ions and Mg ions are difficult to remove completely. Also excess CO_2 results in conversion of $BaCO_3$ to $Ba(HCO_3)_2$ which is again water soluble. Consequently, the Ba ions are best removed with $(NH_4)_2SO_4$.

[xiv] The reaction mixture takes on a yellow color during the deprotection steps. This color can be difficult to remove by Sephadex C-25 chromatography. In such cases stirring the free base of the aminoglycoside **S17** (5.6 g) with activated charcoal (5.0 g) in water (200 mL) at ambient temperature before Sephadex chromatography is advantageous.

[xv] As determined by ^{1}H NMR spectroscopy.

H5''a, H5', H6'a, H4, H5), 3.52 (br s, 1H, H4'''), 3.48 (dd, *J* = 12.5 Hz, 4.4 Hz, 1H, H5''b), 3.41 (t, *J* = 9.9 Hz, 9.5 Hz, 1H, 6H), 3.37 (dd, *J* = 12.5 Hz, 5.4 Hz, 1H, H6'b), 3.29 (br s, 1H, H2'''), 3.21 (m, 1H, H3), 3.13 (dd, *J* = 13.2 Hz, 6.6 Hz, 1H, H6'''a), 3.14-3.04 (m, 3H, H6'''b,H2', H1), 2.27 (dt, *J* = 12.8, 4.3 Hz, 3.7 Hz, 1H, H2eq), 1.67 (s, 15H, acetic acid), 1.54 (q, *J* = 12.8 Hz, 1H, H2ax), 1.34 (m 1H, H4'), 1.18 (br s, 2H, $CH_3CH_2CH_2$-C4'), 1.15 – 0.99 (m, 2H, $CH_3CH_2CH_2$-C4'), 0.59 (t, *J* = 7.0 Hz, 3H, $CH_3CH_2CH_2$-C4'). ^{13}C NMR (150 MHz, D_2O) δ 181.1, 109.8, 96.6, 95.3, 84.2, 81.1, 79.1, 74.9, 73.8, 73.2, 72.4, 70.1, 67.5, 67.1, 66.7, 61.0, 59.8, 55.3, 50.7, 49.7, 48.9, 42.2, 40.2, 29.0, 27.8, 23.1, 18.5, 13.6. ESI-HRMS: *m/z* calcd for $C_{26}H_{52}N_5O_{13}$ $[M+H]^+$ 642.3562, found 642.3574.

Allyl phenyl sulfone. A stirred solution of thiophenol (100 mL, 0.97 mol) in methanol (1.0 L) was cooled in an ice bath and treated with sodium methoxide (68 g, 1.26 mol). Allyl bromide (93 mL, 1.10 mol) was then added under ice cooled conditions and the resulting solution allowed to come to room temperature and stirred 12 h. The reaction was monitored by TLC on silica gel (eluent: hexane : ethyl acetate 5:1 R_f = 0.65). The reaction mixture was concentrated under reduced pressure, and the residue was diluted with hexane (1.0 L) and washed with water (2 x 500 mL). The hexane layer was dried over sodium sulfate, filtered, and concentrated to give allylphenyl sulfide (150 g, 1.0 mol, 103%). A stirred solution of llylphenyl sulfide (150 g, 1.0 mol) in acetonitrile (1.5 L) and was treated at room temperature with manganese(II) sulfate monohydrate (1.7 g, 0.01 mol) and a mixture of 30% hydrogen peroxide (562 mL, 5.50 mol) and 0.2 M aqueous sodium bicarbonate (1.5 L) was carefully and slowly added to the reaction mixture at ambient temperature. The reaction mixture was stirred 16 h. The reaction was monitored by silica gel TLC (eluent: hexane : ethyl acetate 3:1, R_f = 0.25). The solvent was removed under reduced pressure, and the residue was diluted with ethyl acetate (1.0 L) and washed with water (2 x 500 mL). The organic phase was dried over sodium sulfate, filtered, concentrated and dried under reduced pressure to give the title compound(170 g, 0.94 mol, 97%), which was used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ= 7.85 (d, *J* = 7.8 Hz, 2H), 7.63 (t, *J* = 7.8 Hz, *J* = 7.3Hz, 1H), 7.53 (t, *J* = 7.8 Hz, 2H), 5.77 (m, 1H), 5.30 (d, *J* = 10.3 Hz, 1H), 5.12 (d, *J* = 17.1 Hz, 1H), 3.79 (d, *J* = 7.8 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 138.2, 133.8, 129.0, 128.4, 124.7, 124.6, 60.8.

5-Benzenesulfonyl-4-(benzenesulfonylmethyl)pentene (29). ^{1}H NMR (600 MHz, $CDCl_3$) δ= 7.83 (d, *J* = 7.7 Hz, 4H), 7.67 (t, *J* = 7.7 Hz, *J* = 7.3Hz, 2H), 7.56 (t, *J* = 8.0 Hz, 7.7 Hz, 4H), 5.47 (m, 1H), 5.06 (dd, *J* = 10.3 Hz, 0.7 Hz, 1H), 5.00 (dd, *J* = 17.2 Hz, 1.5 Hz, 1H), 3.46 (dd, *J* = 14.3 Hz, 6.2 Hz, 2H), 3.20 (dd, *J* = 14.7 Hz, 5.5 Hz, 2H), 2.54-2.45 (m, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 133.9, 133.2, 129.4, 128.0, 119.5, 57.2, 37.2, 29.1. ESI-HRMS: *m/z* calcd for $C_{18}H_{20}O_4S_2Na$ $[M+Na]^+$ 387.0701, found 387.0692.

1-Benzenesulfonyl-2-(benzenesulfonylmethyl)pentane (30). ^{1}H NMR (600 MHz, $CDCl_3$) δ= 7.85 (d, *J* = 7.7 Hz, 4H), 7.67 (t, *J* = 7.3 Hz, *J* = 7.2Hz, 2H), 7.56 (t, *J* = 7.3 Hz, 4H), 3.51 (dd, *J* = 14.7 Hz, 6.6 Hz, 2H), 3.18 (dd, *J* = 14.3 Hz, 4.8 Hz, 2H), 2.47 (m, 1H), 1.67 (q , *J* = 7.7 Hz, 2H), 1.15 (m, 2H), 0.75 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ= 133.9, 129.4, 128.0, 57.8, 35.1, 29.4, 19.1, 13.4. ESI-HRMS: *m/z* calcd for $C_{18}H_{22}O_4S_2Na$ $[M+Na]^+$ 389.0857, found 389.0865.

4′-Deoxy-4′-(2-hydroxyethyl)-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl-6,3′,6′,2‴,5‴,3‴″,4‴″-hepta-*O*-benzoyl paromomycin (31). Compound **27** (214 mg, 0.11 mmol) was dissolved in a mixture of dichloromethane (8 mL) and methanol (2 mL) and cooled to – 78 ^{o}C. Ozone was bubbled through the reaction mixture for 10 min until a permanent blue color was formed. On completion, as indicated ESIMS, argon was bubbled through the reaction mixture to disperse residual ozone. $NaBH_4$ (7 mg, 0.17 mmol) was then added and the mixture stirred under argon for 1 h before it was warmed to room temperature and stirred for 3.5 h. The reaction mixture was concentrated to dryness under reduced pressure. The residue was dissolved in EtOAc (6 mL) and washed with 1M HCl (3 mL) then saturated aqueous $NaHCO_3$ (2 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated to dryness under reduced pressure. The crude product was purified by column chromatography on silica gel (eluent: gradient of 10% to 40% EtOAc in hexane) to give **31** (50 mg, 26%) as a white solid. R*f*= 0.5 (45% EtOAc in Hexane). $[\alpha]_D^{23}$ = +52.7 (*c* = 1.0, methanol). ^{1}H NMR (600 MHz, CD_3OD)δ 8.15 (d, 2H, CO*Ph*), 8.04 – 7.97 (m, 9H, CO*Ph*), 7.76 – 7.72 (m, 3H, CO*Ph*), 7.64 – 7.56 (m, 6H, CO*Ph*), 7.48 – 7.43 (m, 7H, CO*Ph*), 7.28 (t, *J* = 7.7 Hz, 2H, CO*Ph*), 7.20 (t, *J* = 7.9 Hz, 2H, CO*Ph*), 6.93 (t, *J* = 7.8 Hz, 2H, CO*Ph*), 6.24 (d, *J* = 4.0 Hz, 1H, H1′), 5.44 – 5.40 (m, 2H, H3′, H1″), 5.35 – 5.29 (m, 1H, H6), 5.23 (t, *J* = 3.1 Hz, 1H, H3‴), 5.15 (d, *J* = 2.0 Hz, 1H, H1‴), 5.14 (s, 1H, H2‴), 5.06 (s, 1H, H4‴), 4.70 – 4.60 (m, 3H, H3″, H5′a, H5′b), 4.46 (dd, *J* = 10.7, 4.0 Hz, 1H, H2′), 4.41 (d, *J* = 2.8 Hz, 1H, H5), 4.40 – 4.38 (m, 1H, H6′b), 4.36 (ddd, *J* = 8.1, 6.8, 1.9 Hz, 1H, H5‴), 4.30 (ddd, *J* = 10.5, 7.3, 2.7 Hz, 2H, H1, H6′b), 4.28 – 4.21 (m, 1H, H3), 4.12 (dt, *J* = 8.1, 2.5 Hz, 1H, H4″), 4.07 (d, *J* = 2.3 Hz, 1H, H4), 3.99 (dt, *J* = 11.0, 2.5 Hz, 1H, H5′), 3.91 (s, 1H, H2‴), 3.44 (t, *J* = 7.4 Hz, 2H, OH*CH*$_2$CH$_2$-C4′), 3.36 (dd, *J* = 13.8, 7.5 Hz, 1H, H6‴b), 3.09 (dd, *J* = 13.7, 6.5 Hz, 1H, H6‴a), 2.23 (dq, *J* = 10.9, 5.7, 5.2 Hz, 1H, H4′), 2.21 – 2.15 (m, 1H, H2a), 2.02 (dt, *J* = 12.9, 4.3 Hz, 1H, H2b), 1.60 (ddtd, *J* = 41.2, 14.6, 7.3, 4.5 Hz, 2H, OHCH$_2$*CH*$_2$-C4′). ^{13}C NMR (151 MHz, CD_3OD) δ 166.6 (*CO*Ph), 166.4 (*CO*Ph), 166.3 (*CO*Ph), 166.0 (*CO*Ph), 165.0 (*CO*Ph), 164.7 (*CO*Ph), 164.1 (*CO*Ph), 158.2 (*CO*CF$_3$), 157.9 (*CO*CF$_3$), 157.7 (*CO*CF$_3$), 157.2 (*CO*CF$_3$), 156.9 (*CO*CF$_3$), 133.6 (CO*Ph*), 133.4 (CO*Ph*), 133.1 (CO*Ph*), 133.1 (CO*Ph*), 132.9 (CO*Ph*), 129.7 (CO*Ph*), 129.7 (CO*Ph*), 129.6 (CO*Ph*), 129.6 (CO*Ph*), 129.5 (CO*Ph*), 129.5 (CO*Ph*), 129.4 (CO*Ph*), 129.3 (CO*Ph*), 129.2 (CO*Ph*), 129.0 (CO*Ph*), 128.6 (CO*Ph*), 128.5 (CO*Ph*), 128.2 (CO*Ph*), 128.2 (CO*Ph*), 128.1 (CO*Ph*), 128.0 (CO*Ph*), 127.9 (CO*Ph*), 127.7 (CO*Ph)*, 117.0 (CO*CF$_3$*), 116.7 (CO*CF$_3$*), 116.6 (CO*CF$_3$*), 116.3 (CO*CF$_3$*), 115.1 (CO*CF$_3$*), 109.4 (C1″), 97.1 (C1‴), 95.8 (C1′), 85.8 (C5), 79.2 (C4″), 76.0 (C4), 75.6 (C6), 75.3 (C2″), 74.1 (C3″), 72.4 (C3′), 71.8 (C5‴), 69.9 (C5′), 68.1 (C3‴), 65.9 (C4‴), 63.8 (C6′a,b), 61.7 (C5′a,b), 59.5 (HO*CH$_2$*CH$_2$-C4′), 52.7 (C2′), 49.2 (C3), 48.6 (C1), 38.79 (C6‴a,b), 38.67(C4′), 30.58(C2a,b), 30.37 (HOCH$_2$*CH$_2$*-C4′). ESI-HRMS: *m/z* calcd for $C_{84}H_{72}F_{15}N_5O_{26}$[M+Na]$^+$ 1874.4146, found 1874.4124.

4′-Deoxy-4′-(2-iodoethyl)-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl-6,3′,6′,2‴,5‴,3‴″,4‴″-hepta-*O*-benzoyl paromomycin (32). A solution of compound **31** (50 mg, 0.03 mmol) in amixture of benzene (1 mL) and acetonitrile (0.5 mL) was treated with PPh_3 (16 mg, 0.06 mmol) and imidazole (8.1 mg, 0.12 mmol) and then portion-wise with iodine (11.4 mg, 0.95 mmol) before it was heated to 60 ^{o}C with stirring under argon for 1 h. After cooling to room temperature, the reaction mixture was diluted with ethyl acetate (2 mL) and stirred with saturated aqueous sodium thiosulfate (1 mL). The organic layer was separated, diluted with hexane (2 mL), washed with brine, dried over anhydrous sodium sulfate and concentrated

to dryness. The crude product was purified by column chromatography on silica gel (eluent: gradient of 10% to 25% EtOAc in hexane) to give **32** (30 mg, 51%) as a white solid. R*f*= 0.6 (30% EtOAc in Hexane). $[\alpha]_D^{23}$ = 74.2 (*c* = 1.0, methanol). ^{1}H NMR (600 MHz, CD_3OD) δ 8.16 (dd, *J* = 8.2, 1.4 Hz, 2H, CO*Ph*), 8.07 – 8.00 (m, 4H, CO*Ph*), 7.97 (dtd, *J* = 18.3, 8.4, 1.4 Hz, 6H, CO*Ph*), 7.75 – 7.71 (m, 3H, CO*Ph*), 7.65 – 7.56 (m, 6H, CO*Ph*), 7.49 – 7.41 (m, 10H, CO*Ph*), 7.24 – 7.18 (m, 2H, CO*Ph*), 6.93 (t, *J* = 7.7 Hz, 2H CO*Ph*), 6.21 (d, *J* = 4.1 Hz, 1H, H1′), 5.47 (t, *J* = 10.8 Hz, 1H, H3′), 5.40 (s, 1H, H1′′), 5.35 – 5.30 (m, 1H, H6), 5.23 (t, *J* = 3.1 Hz, 1H, H3′′′), 5.16 (d, *J* = 2.1 Hz, 1H, H1′′′), 5.13 (d, *J* = 4.5 Hz, 1H, H2′′), 5.05 (t, *J* = 2.4 Hz, 1H, H4′′′), 4.65 (d, *J* = 4.2 Hz, 3H, H3′′, H5′a, H5′b), 4.47 (dd, *J* = 10.7, 4.0 Hz, 1H, H2′), 4.40 (t, *J* = 8.8 Hz, 1H, H5), 4.38 – 4.32 (m, 2H, H6′b, H5′′′), 4.33 – 4.24 (m, 2H, H1, H6′a), 4.26 – 4.21 (m, 1H, H3), 4.12 (dt, *J* = 8.0, 2.5 Hz, 1H, H4′′), 4.04 (dd, *J* = 10.2, 8.3 Hz, 1H, H4), 3.97 (dd, *J* = 13.0, 10.2 Hz, 1H,H5′), 3.90 (s, 1H, H2′′′), 3.37 (dd, *J* = 13.8, 7.6 Hz, 1H, H6′′′b), 3.17 (td, *J* = 9.1, 6.4 Hz, 1H, I*CH*$_{2a}$CH$_2$-C4′), 3.12 (dd, *J* = 13.8, 6.5 Hz, 1H, H6′′′a), 3.04 (td, *J* = 9.3, 8.8, 6.7 Hz, 1H, I*CH*$_{2b}$CH$_2$-C4′), 2.23 (d, *J* = 4.2 Hz, 1H, H4′), 2.20 – 2.12 (m, 1H, H2a), 2.05 – 1.95 (m, 3H, H2b, ICH$_2$*CH*$_2$-C4′).^{13}C NMR (151 MHz, CD_3OD) δ 166.6 (*CO*Ph), 166.2 (*CO*Ph), 166.2 (*CO*Ph), 165.9 (*CO*Ph), 165 0 (*CO*Ph), 164.6 (*CO*Ph), 164.1 (*CO*Ph), 157.9 (*CO*CF$_3$), 157.7 (*CO*CF$_3$), 157.6 (*CO*CF$_3$), 157.23 (*CO*CF$_3$), 157.20 (*CO*CF$_3$), 133.6 (CO*Ph*), 133.4 (CO*Ph*), 133.3 (CO*Ph*), 133.2 (CO*Ph*), 133.1 (CO*Ph*),132.9 (CO*Ph*), 133.0 (CO*Ph*), 129.7 (CO*Ph*), 129.64 (CO*Ph*), 129.62 (CO*Ph*), 129.5 (CO*Ph*), 129.4 (CO*Ph*), 129.2 (CO*Ph*), 129.0 (CO*Ph*), 128.6 (CO*Ph*), 128.5 (CO*Ph*), 128.2 (CO*Ph*), 128.2 (CO*Ph*), 128.1 (CO*Ph*), 127.9 (CO*Ph*), 127.7 (CO*Ph*), 117.0 (CO*CF$_3$*), 116.7 (CO*CF$_3$*), 116.6 (CO*CF$_3$*), 116.3 (CO*CF$_3$*) 115.1 (CO*CF$_3$*), 109.4 (C1′′), 97.1 (C1′′′), 95.8 (C1′), 85.8 (C5), 79.2 (C4′′), 76.2 (C4), 75.5 (C6), 75.3 (C2′′), 74.1 (C3′′), 71.8 (C3′), 71.6 (C5′′′), 69.1 (C5′), 68.1 (C3′′′), 65.9 (C4′′′), 63.6 (C6′a,b), 61.8 (C5′a,b), 52.5 (C2′), 49.3 (C3), 48.8 (C1), 42.7 (C4), 38.7 (C6′′′a,b), 32.0 (ICH$_2$*CH*$_2$-C4′), 31.6 (C2a,b), 0.71 (I*CH*$_2$CH$_2$-C4′). ESI-HRMS: *m*/*z* calcd for $C_{84}H_{71}F_{15}IN_5O_{25}[M+K]^+$ 1874.4146, found 1874.4124.

4′-Deoxy-4′-(ethyl)-1,3,2′,2′′′,6′′′-penta-*N*-trifluoroacetyl-6,3′,6′,2′′′,5′′′,3′′′′,4′′′′-hepta-*O*-benzoyl paromomycin (33). Compound **32** (40mg, 0.006 mmol) was dissolved in ethyl acetate (1 mL), triethylamine (10 µL, 0.03 mmol) and Pd-C (20 mg) were added, and mixture was stirred under 1 atm of hydrogen at room temperature for 12 h. The reaction mixture was filtered through Celite, and concentrated to dryness. The crude product was purified by column chromatography on silica gel (eluent: gradient of 10% to 25% ethyl acetate in hexane) to give **33** (31 mg, 86%) as a white solid. R*f*= 0.6 (35% EtOAc in Hexane). $[\alpha]_D^{23}$ = 51.3 (c = 1.1, methanol). ^{1}H NMR (600 MHz, CD_3OD) δ 8.16 (dd, *J* = 8.2, 1.4 Hz, 2H, CO*Ph*), 8.07 – 8.00 (m, 4H, CO*Ph*), 7.97 (dtd, *J* = 18.3, 8.4, 1.4 Hz, 6H, CO*Ph*), 7.75 – 7.71 (m, 3H, CO*Ph*), 7.65 – 7.56 (m, 6H, CO*Ph*), 7.49 – 7.41 (m, 10H, CO*Ph*), 7.24 – 7.18 (m, 2H, CO*Ph*), 6.93 (t, *J* = 7.7 Hz, 2H, CO*Ph*), 6.21 (d, *J* = 4.1 Hz, 1H, H1′), 5.47 (t, *J* = 10.8 Hz, 1H, H3′), 5.40 (s, 1H, H1′′), 5.35 – 5.30 (m, 1H, H6), 5.23 (t, *J* = 3.1 Hz, 1H, H3′′′), 5.16 (d, *J* = 2.1 Hz, 1H, H1′′′), 5.13 (d, *J* = 4.5 Hz, 1H, H2′′), 5.05 (t, *J* = 2.4 Hz, 1H, H4′′), 4.71 – 4.61 (m, 3H, H3′′, H5′a, H5′b), 4.47 (dd, *J* = 10.7, 4.1 Hz, 1H, H2′), 4.42 (d, *J* = 8.7 Hz, 1H, H5), 4.42 – 4.38 (m, 1H, H6′b), 4.37 (d, *J* = 7.5 Hz, 1H, H5′′′), 4.35 – 4.31 (m, 2H, H6′a, H1), 4.28 (d, *J* = 2.1 Hz, 1H, H3), 4.13 – 4.07 (m, 2H, H4′′, H4), 4.03 (d, *J* = 10.8 Hz, 1H, H5), 3.91 (s, 1H, H2′′′), 3.36 (dd, *J* = 13.8, 7.6 Hz, 1H, H6′′′b), 3.10 (dd, *J* = 13.7, 6.4 Hz, 1H, H6′′′a), 2.18 – 2.11 (m, 2H, H4′, H2a), 2.05 – 1.99 (m, 1H, H2b), 1.67 – 1.35 (m, 2H, CH$_3$*CH*$_2$-C4′), 0.83 (t, *J* = 7.6 Hz, 3H, (*CH*$_3$CH$_2$-C4′). ^{13}C NMR (151 MHz, CD_3OD) δ 166.6 (*CO*Ph), 166.3 (*CO*Ph), 166.2 (*CO*Ph), 166.0 (*CO*Ph), 165.0 (*CO*Ph), 164.7 (*CO*Ph), 164.1 (*CO*Ph), 158.2 (*CO*CF$_3$), 157.9 (*CO*CF$_3$), 157.1 (*CO*CF$_3$), 133.5 (*CO*CF$_3$), 133.4 (*CO*CF$_3$), 133.1

(CO*Ph*), 132.9 (CO*Ph*), 129.7 (CO*Ph*), 129.64 (CO*Ph*), 129.61 (CO*Ph*), 129.5 (CO*Ph*), 129.4 (CO*Ph*), 129.3 (CO*Ph*), 129.2 (CO*Ph*), 128.9 (CO*Ph*), 128.6 (CO*Ph*), 128.5 (CO*Ph*), 128.2 (CO*Ph*), 128.2 (CO*Ph*), 128.1 (CO*Ph*), 127.9 (CO*Ph*), 127.7 (CO*Ph*), 117.0 (COCF_3), 116.7 (COCF_3), 116.6 (COCF_3), 116.3 (COCF_3), 115.7 (COCF_3) 109.3 (C1''), 97.1 (C1'''), 96.0 (C1'), 85.9 (C5), 79.1 (C4''), 76.3 (C4), 75.6 (C6), 75.3 (C2''), 74.1 (C3''), 71.8 (C3'), 70.9 (C5'''), 69.1 (C5'), 68.1 (C3'''), 65.9 (C4'''), 63.7 (C6'a,b), 61.8 (C5'a,b), 61.4 (C2'), 52.8 (C3), 51.5 (C1), 49.5 , 42.4 (C4'), 38.7 (C6'''a,b), 29.0 (C2a,b), 19.0 (CH_3CH_2-C4'), 9.2 (CH_3CH_2-C4'). ESI-HRMS: *m*/*z* calcd for $C_{84}H_{72}F_{15}N_5O_{26}[M+Na]^+$ 1874.3982, found 1874.3944.

4'-Deoxy-4'-ethyl paromomycin (35). A stirred solution of compound **33** (20 mg, 0.006 mmol) in anhydrous MeOH (200 μL) was treated under an inert atmosphere at room temperature with 6% $Mg(OMe)_2$ in MeOH (0.3 mL, 0.05 mmol). After stirring for 58 h at room temperature, the reaction mixture was concentrated under reduced pressure. The residue was taken up in water (0.6 mL), treated at room temperature with barium hydroxide (80 mg, 0.47 mmol), and heated to 80 °C for 24 h. After cooling to 0 °C with an ice bath, dry ice was added portion-wise until the pH of the solution reached 8-9. The precipitate was filtered off and the filtrate was concentrated under reduced pressure at room temperature. The residue was dissolved in water (2 mL) and neutralized with glacial acetic acid (5 μL). Saturated aqueous ammonium sulfate was added dropwise until no further precipitate was formed. The precipitate was filtered off and the resulting filtrate was directly loaded onto a Sephadex C-25 column, from which the target compound was eluted with gradually increasing concentrations of ammonium hydroxide from 0.1% to 0.6%. After concentration under reduced pressure the residue was dissolved in water (1 mL) and glacial acetic acid (5 μL) was added. Lyophilization then gave **35**as a white amorphous solid (8 mg, 58%). $[\alpha]_D^{23}$ = 51.3 (c = 1.1, methanol).^{1}H NMR (600 MHz, D_2O) δ 5.52 (d, *J* = 4.0 Hz, 1H, H1'), 5.21 (d, *J* = 2.9 Hz, 1H, H1''), 5.14 (d, *J* = 4.4 Hz, 1H, H1'''), 4.37 (q, *J* = 5.8 Hz, 1H, H3''), 4.19 (dd, *J* = 5.1, 2.9 Hz, 1H, H2''), 4.16 (t, *J* = 5.4 Hz, 1H, H5'''), 4.07 (t, *J* = 3.1 Hz, 1H, H3'''), 4.05 (s, 1H, H4''), 3.81 – 3.69 (m, 4H, H4, H3', H6'a, H5''), 3.67 (s, 1H, H5), 3.63 (dd, *J* = 12.5, 4.5 Hz, 1H, H4'''), 3.57 – 3.50 (m, 2H, H6'b, H5'b), 3.44 (s, 1H, H2'''), 3.36 (d, *J* = 13.0 Hz, 2H, H3, H6'''a), 3.27 (dd, *J* = 13.7, 6.5 Hz, 1H, H3), 3.21 (ddd, *J* = 21.5, 11.5, 5.4 Hz, 4H, H6'''a, H6'''b, H2', H1), 2.34 – 2.28 (m, 1H, H2), 1.85 (d, *J* = 5.5 Hz, 15H, acetic acid), 1.66 (q, *J* = 12.6 Hz, 1H, H2ax), 1.48 (t, *J* = 3.8 Hz, 1H, H4'), 1.47 – 1.34 (m, 2H, CH_3CH_2-C4'), 0.74 (t, *J* = 7.6 Hz, 3H, CH_3CH_2-C4').^{13}C NMR (151 MHz, D_2O) δ 180.9, 110.0 (C1'''), 96.5 (C1'), 95.4 (C1'''), 84.3 (C5''), 81.2 (C4''), 78.3 (C4) 75.1 (C3''), 74.1 (C3'), 73.3 (C2''),70.2 (C5''')67.6 (C3'''), 67.4 (C3'), 67.2 (C4), 61.2 (C2'''), 59.9 (C4'''), 59.5 (C6'a,b), 55.2 (C2'), 50.8 (C2'''), 49.8 (C1), 48.9 (C3), 40.3 (C6'''a,b), 28.9 (C2a,b), 22.9 (CH_3CH_2-C4'), 8.7 (CH_3CH_2-C4'). ESI-HRMS: *m*/*z* calcd for $C_{25}H_{79}N_5O_{23}[M+H]^+$ 628.3405, found 628.3405.

4'-Deoxy-4'-*C*-(2-hydroxyethyl) paromomycin (36). A stirred solution of compound **31** (50 mg, 0.03 mmol) in anhydrous MeOH (500 μL) was treated under an inert atmosphere at room temperature with 6% $Mg(OMe)_2$ in MeOH (0.45 mL, 0.25 mmol). After stirring for 48 h at room temperature, the reaction mixture was concentrated under reduced pressure. The residue was taken up in water (0.7 mL), treated at room temperature with barium hydroxide (100 mg, 0.58 mmol), and heated to 80 °C for 24 h. After cooling to 0 °C with an ice bath, dry ice was added portion-wise until the pH of the solution reached 8-9.

The precipitate was filtered off and the filtrate was concentrated under reduced pressure at room temperature. The residue was dissolved in water (2 mL) and neutralized with glacial acetic acid (5 µL). Saturated aqueous ammonium sulfate was added dropwise until no further precipitate was formed. The precipitate was filtered off and the resulting filtrate was directly loaded onto a Sephadex C-25 column, from which the target compound was eluted with gradually increasing concentrations of ammonium hydroxide from 0.1% to 0.4%. After concentration under reduced pressure the residue was dissolved in water (1 mL) and glacial acetic acid (5 µL) was added. Lyophilization then gave **36** as a white amorphous solid (10 mg, 66%). $[\alpha]_D^{23}$ = +46.8 (c = 1.0, water). ^{1}H NMR (600 MHz, D_2O) δ 5.57 (d, J = 4.0 Hz, 1H, H1'), 5.19 (d, *J* = 2.6 Hz, 1H, H1''), 5.12 (d, *J* = 1.8 Hz, 1H, H1'''), 4.35 (dd, *J* = 6.7, 5.0 Hz, 1H, H3''), 4.19 (dd, *J* = 5.0, 2.6 Hz, 1H, H2''), 4.13 (ddd, *J* = 6.8, 4.0, 1.6 Hz, 1H, H5'''), 4.05 (t, *J* = 3.1 Hz, 1H, H3'''), 4.03 (ddd, *J* = 7.1, 4.6, 2.9 Hz, 1H, H4''), 3.75 – 3.71 (m, 4H, H4, H3', H6'a, H5''), 3.68 (d, *J* = 9.0 Hz, 1H, H5), 3.64 (dt, *J* = 3.1, 1.3 Hz, 1H, H4'''), 3.60 (dd, *J* = 12.4, 4.6 Hz, 1H, H6'b), 3.52 (dt, *J* = 12.5, 6.3 Hz, 1H, H5''b), 3.50 – 3.46 (m, 2H, HO*CH*$_2$CH$_2$-C4'), 3.41 (dt, *J* = 3.1, 1.3 Hz, 1H, H2'''), 3.30 – 3.22 (m, 2H, H3, H6'''a), 3.21 – 3.12 (m, 3H, H6'''b, H2', H1), 2.25 (dt, *J* = 12.7, 4.3 Hz, 1H, H2eq), 1.74 (s, 15H, acetic acid), 1.65 – 1.59 (m, 1H,H2ax), 1.56 (m, 1H, H4'), 1.55 – 1.48 (m, 2H, HOCH$_2$C*H*$_2$-C4'). ^{13}C NMR (151 MHz, D_2O) δ 180.9, 110.0 (C1'''), 96.5 (C1'), 95.4 (C1'''), 84.3 (C5''), 81.2 (C4''), 78.3 (C4) 75.1 (C3''), 74.1 (C3'), 73.3 (C2''), 72.4 (HO*CH*$_2$CH$_2$-C4'), 70.2 (C5'''), 67.6 (C3'''), 67.4 (C3'), 67.2 (C4), 61.2 (C2'''), 59.9 (C4'''), 59.5 (C6'a,b), 55.2 (C2'), 50.8 (C2'''), 49.8 (C1), 48.9 (C3), 40.3 (C6'''a,b), 28.9 (C2a,b), 22.9 (HOCH$_2$*CH*$_2$-C4'). ESI-HRMS: *m/z* calcd for $C_{35}H_{49}N_5O_{24}[M+H]^+$ 644.3372, found 644.3354.

4'-Deoxy-4'-(2,3-dihydroxypropyl)-1,3,2',2''',6'''-penta-*N*-trifluoroacetyl-6,3',6',2''',5''',3'''',4''''-hepta-*O*-benzoyl paromomycin (34). A stirred solution of **27** (108 mg, 0.06 mmol) in a mixture of THF (4 mL) and water (1 mL) was treated at room temperature with 4-methylmorpholine *N*-oxide (38 mg, 0.32 mmol) followed by dropwise addition of OsO_4. THF 20% w/w (7 mg, 0.03 mmol). The reaction mixture was stirred for 23 h, then was diluted with EtOAc (5 mL) and washed with saturated aqueous $NaHCO_3$ (3 mL). The organic layer was concentrated under reduced pressure at room temperature and the crude product was purified by column chromatography on silica gel (eluent: gradient of 0.5% to 1% MeOH in DCM) to give **34**. (92 mg, 49%) as a white solid. R*f*= 0.6 (5% MeOH in DCM). The compound is mixture of isomers in 1:1 ratio. Isomer 1: ^{1}H NMR (600 MHz, CD_3OD) 6.24 (d, *J* = 3.9 Hz, 1H, H1'), 4.46 – 4.43 (m, 1H, H2'), 3.33 (s, 1H, H6'''b). Isomer 2: ^{1}H NMR (600 MHz, CD_3OD) 6.21 (d, *J* = 4.0 Hz, 1H, H1'), 4.48 (dd, *J* = 10.6, 4.0 Hz, 1H, H2'), 3.40 – 3.36 (m, 1H, H6'''b). Common signals: ^{1}H NMR (600 MHz, CD_3OD) δ 8.18 – 8.14 (m, 4H, CO*Ph*), 8.06 – 8.02 (m, 5H, CO*Ph*), 7.98 (ddd, *J* = 8.6, 4.5, 1.6 Hz, 11H, CO*Ph*), 7.74 (ddd, *J* = 4.8, 2.7, 1.4 Hz, 5H, CO*Ph*), 7.64 – 7.57 (m, 12H, CO*Ph*), 7.48 – 7.42 (m, 20H, CO*Ph*), 7.21 (t, *J* = 7.8 Hz, 5H, CO*Ph*), 7.07 – 7.01 (m, 2H, CO*Ph*), 6.94 (dd, *J* = 8.0, 2.2 Hz, 5H, CO*Ph)*, 5.43 (dd, *J* = 10.7, 5.5 Hz, 2H, H3'), 5.40 (d, *J* = 3.6 Hz, 2H, H1''), 5.31 (dd, *J* = 10.5, 9.3 Hz, 2H, H6), 5.22 (t, *J* = 3.1 Hz, 2H, H3'''), 5.16 – 5.12 (m, 4H, H1''', H2''), 5.05 (s, 2H, H4'''), 4.70 – 4.61 (m, 6H, H3'', H5'a, H5'b), 4.40 (d, *J* = 2.7 Hz, 2H, H5), 4.38 – 4.31 (m, 8H, H6'b, H5''', H1, H6'a), 4.29 – 4.22 (m, 2H, H3), 4.10 (d, *J* = 8.3 Hz, 2H, H4''), 4.09 – 4.02 (m, 2H, H4), 3.92 (d, *J* = 11.1 Hz, 2H, H5'), 3.89 (q, *J* = 3.2, 2.6 Hz, 2H, H2'''), 3.59 (dq, *J* = 9.3, 5.2 Hz, 2H, HOCH$_2$HO*CH*CH$_2$-C4'), 3.28 – 3.24 (m, 2H, HOCH$_{2a}$HOCHCH$_2$-C4'), 3.09 (ddd, *J* = 20.5, 12.5, 6.5 Hz, 4H, HOCH$_{2b}$HOCHCH$_2$-C4', H6'''a), 2.41 (dd, *J* = 13.7, 8.9 Hz, 2H, H4'), 2.18 (td, *J* = 12.8, 3.5 Hz, 2H, H2a), 2.04

– 1.98 (m, 2H, H2b), 1.57 – 1.51 (m, 4H, $HOCH_2HOCH\mathit{CH_2}$-C4′). ^{13}C NMR (151 MHz, CD_3OD) δ 166.6 (*CO*Ph), 166.5 (*CO*Ph), 166.4 (*CO*Ph), 165.9 (*CO*Ph), 164.9 (*CO*Ph), 164.6 (*CO*Ph), 164.0 (*CO*Ph), 158.1 ($\mathit{CO}CF_3$), 157.9 ($\mathit{CO}CF_3$), 157.6 ($\mathit{CO}CF_3$), 156.8 ($\mathit{CO}CF_3$), 133.5 ($\mathit{CO}CF_3$), 133.4 (CO*Ph*), 133.1 (CO*Ph*), 133.1 (CO*Ph*), 132.9 (CO*Ph*), 132.7 (CO*Ph*), 129.9 (CO*Ph*), 129.7 (CO*Ph*), 129.6 (CO*Ph*), 129.5 (CO*Ph*), 129.5 (CO*Ph*), 129.4 (CO*Ph*), 129.3 (CO*Ph*), 129.3 (CO*Ph*), 129.2 (CO*Ph*), 129.0 (CO*Ph*), 128.9 (CO*Ph*), 128.6 (CO*Ph*), 128.5 (CO*Ph*), 128.4 (CO*Ph*), 128.2 (CO*Ph*), 128.1 (CO*Ph*), 128.0 (CO*Ph*), 127.9 (CO*Ph*), 127.9 (CO*Ph*), 127.7 (CO*Ph*), 127.6 (CO*Ph*), 117.0 ($CO\mathit{CF_3}$), 116.7 ($CO\mathit{CF_3}$), 116.6 ($CO\mathit{CF_3}$), 116.3 ($CO\mathit{CF_3}$), 114.7 ($CO\mathit{CF_3}$), 109.4 (C1′′), 97.1 (C1′′′), 95.9 (C1′), 85.8 (C5), 79.2 (C4′′), 79.1 (C4), 76.2 (C6), 75.9 (C2′′), 75.6 (C3′′), 75.3 (C3′), 74.1 (C5′′′), 73.6 (C5′), 72.3 (3′′′), 72.2 (C4′′′), 71.8 (C6′a,b), 68.1 ($HOCH_2HO\mathit{C}HCH_2$-C4′), 64.3 ($HO\mathit{C}H_2HOCHCH_2$-C4′), 63.9 (C5′a,b), 52.8 (C2′), 52.6 (C3), 49.5 (C1) , 38.8 (C6′′′a,b), 38.6 (C4′), 29.3 (C2a,b), 29.0 ($HOCH_2HOCH\mathit{C}H_2$-C4′). ESI-HRMS: *m*/*z* calcd for $C_{85}H_{72}F_{15}N_5O_{27}[M+Na]^+$ 1904.4216, found 1904.4225.

4′-Deoxy-4′-(2,3-dihydroxypropyl) paromomycin (37). A stirred solution of **34** (42 mg, 0.02 mmol) in anhydrous MeOH (420 μL) was treated under an inert atmosphere at room temperature with 6% $Mg(OMe)_2$ in MeOH (0.01 mL, 0.10 mmol). After stirring for 58 h at room temperature, the reaction mixture was concentrated under reduced pressure. The residue was taken up in water (0.4 mL), treated at room temperature with barium hydroxide (80 mg, 0.47 mmol), and heated to 80 °C for 24 h. After cooling to 0 °C with an ice bath, dry ice was added portion-wise until the pH of the solution reached 8-9. The precipitate was filtered off and the filtrate was concentrated under reduced pressure at room temperature. The residue was dissolved in water (2 mL) and neutralized with glacial acetic acid (10 μL). Saturated aqueous ammonium sulfate was added dropwise until no further precipitate was formed. The precipitate was filtered off and the resulting filtrate was directly loaded onto a Sephadex C-25 column, from which the target compound was eluted with gradually increasing concentrations of ammonium hydroxide from 0.1% to 0.6%. After concentration under reduced pressure the residue was dissolved in water (1 mL) and glacial acetic acid (5 μL) was added. Lyophilization then gave **37** as a white amorphous solid (16 mg, 74%). $[\alpha]_D^{23}$ = +61.7 (*c* = 0.9, water). ^{1}H NMR (600 MHz, D_2O) δ 5.60 (d, *J* = 3.9 Hz, 1H, H1′), 5.21 (d, *J* = 2.7 Hz, 1H, H1′′), 5.13 (d, *J* = 1.8 Hz, 1H, H1′′′), 4.36 (t, *J* = 5.9 Hz, 1H, H3′′), 4.23 – 4.19 (m, 1H, H2′′), 4.14 (t, *J* = 5.6 Hz, 1H, H5′′′), 4.06 (t, *J* = 3.2 Hz, 1H, H3′′′), 4.05 – 4.03 (m, 1H, H4′′), 3.83 – 3.67 (m, 5H,H4, H3′, H6′a, H5′,$HOCH_2\mathit{CH}OHCH_2$-C4′), 3.65 (d, *J* = 3.3 Hz, 1H, H4′′′), 3.61 (dd, *J* = 12.6, 4.7 Hz, 2H, H6′b, $HO\mathit{CH_{2o}}CHOHCH_2$-C4′), 3.57 – 3.49 (m, 2H, H5′′b, $HO\mathit{CH_{2b}}CHOHCH_2$-C4′), 3.45 – 3.37 (m, 1H, H2′′′), 3.29 (td, *J* = 11.4, 4.5 Hz, 2H, H3, H6′′′a), 3.24 – 3.13 (m, 3H, H6′′′b, H21′, H1), 2.30 – 2.24 (m, 1H, H2eq), 1.76 (s, 15H, acetic acid), 1.65 – 1.61 (m, 2H, H2ax, H4′), 1.47 (m, *J* = 15.2, 9.0, 4.5 Hz, 2H, $HOCH_2CHOH\mathit{CH_2}$-C4′). ^{13}C NMR (151 MHz, D_2O) δ 180.7, 110.0 (C1′′′), 96.4 (C1′), 95.4 (C1′′′), 84.4 (C4′′), 81.2 (C4), 75.1 (C3′′), 74.9 (C3′), 74.4 (C2′′), 70.6 ($HOCH_2\mathit{CH}OHCH_2$-C4′), 70.2 (C5′′′), 69.8 (C3′′′), 66.0 ($HO\mathit{CH_2}CHOHCH_2$-C4′), 65.4 (C3′), 61.2 (C4), 60.0 (C2′′′), 59.9 (C4′′′), 55.2 (C6′a,b), 55.1 (C2′), 50.8 (C2′′′), 49.7 (C1), 48.9 (C3), 40.3 (C6′′′a,b), 28.7 (C2a,b), 22.8 ($HOCH_2CHOH\mathit{CH_2}$-C4′). ESI-HRMS: *m*/*z* calcd for $C_{26}H_{51}N_5O_{15}[M+H]^+$ 674.3480, found 674.3460.

Ribosome inhibition assays. IC_{50} values were determined by cell-free translation inhibition assays with bacterial S30 extracts (University of Zurich) and rabbit reticulocyte lysate (Promega) as described previously.[5] 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.[6] 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.[6] 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} = I_{A1408} / I_{C1400}

Eq. (3) ---------- % DMS reactivity = (1 – normalized I_{A1408}) × 100

Eq. (4) ---------- % DMS protection = (100 – % DMS reactivity)

Crystallographic structure determination of Propylamycin 5 in complex with the bacterial ribosome. First, the ribosome-mRNA-tRNA complex was pre-formed by programming 5 µM 70S *Tth* ribosomes with 10 µM mRNA and incubation at 55 °C for 10 minutes, followed by addition of 20 µM P-site ($tRNA_i^{Met}$) and 20 µM A-site ($tRNA^{Val}$) substrates, with minor changes from the method of Steitz and coworkers.[7] Each of these two steps was allowed to reach equilibrium for 10 minutes at 37 °C in the buffer containing 5 mM HEPES-KOH (pH 7.6), 50 mM KCl, 10 mM NH_4Cl, and 10 mM $Mg(CH_3COO)_2$, Then, **5** dissolved in the same buffer was added to a final concentration of 250 µM to the pre-formed ribosome-mRNA-tRNA complex. Crystals were grown by vapor diffusion in sitting drop crystallization trays at 19 °C. Initial crystalline needles were obtained by screening around previously published ribosome crystallization conditions.[8-10] The best-diffracting crystals were obtained by mixing 2-3 µL of the ribosome-**5** complex with 3-4 µL of a reservoir solution containing 100 mM Tris-HCl (pH 7.6), 2.9% (w/v) PEG-20K, 7-12% (v/v) MPD, 100-200 mM arginine, 0.5 mM β-mercaptoethanol.[7] Crystals appeared within 3-4 days and grew up to 150 × 150 × 1600 µm in size within 10-12 days. Crystals were cryo-protected stepwise using a series of buffers with increasing MPD concentrations until reaching the final concentration of 40% (v/v) MPD, in which they were incubated overnight at 19 °C. In addition to MPD, all stabilization buffers contained 100 mM Tris-HCl (pH 7.6), 2.9% (w/v) PEG-20K, 50 mM KCl, 10 mM NH_4Cl, 10 mM $Mg(CH_3COO)_2$ and 6 mM β-mercaptoethanol. Propylamycin 5 was added to the final cryo-protection solution. After stabilization, crystals were harvested and flash frozen in a nitrogen cryo-stream at 80 K.

Diffraction data were collected on the beam Line 24ID-C at the Advanced Photon Source (Argonne National Laboratory, Argonne, IL). A complete dataset for each ribosome complex was collected using 0.979 Å wavelength at 100 K from multiple regions of the same crystal using 0.3° oscillations. The raw data were integrated and scaled using the XDS software package.[11] All crystals belonged to the primitive orthorhombic space group $P2_12_12_1$ with approximate unit cell dimensions of 210 Å x 450 Å x 620 Å and contained two copies of the 70S ribosome per asymmetric unit. Each structure was solved by molecular replacement using PHASER from the CCP4 program suite.[12] The search model was generated from the previously published structure[7] of the *T. thermophilus* 70S ribosome with bound mRNA and tRNAs (PDB entry 4Y4P). The initial molecular replacement solutions were refined by rigid body refinement with the ribosome split into multiple domains, followed by 10 cycles of positional and individual B-factor refinement using PHENIX.[13] Non-crystallographic symmetry restraints were applied to 4 domains of the 30S ribosomal subunit (head, body, spur, helix 44), and 4 domains of the 50S subunit (body, L1-stalk, L10-stalk, C-terminus of the L9 protein).

An atomic model of **5** was generated from its known chemical structure using PRODRG online software,[14] which was also used to generate restraints based on idealized 3D geometry. Atomic model and restraints were used to fit/refine **5** into the obtained unbiased electron density. The final model of

the 70S ribosome in complex with **5** and mRNA/tRNAs was generated by multiple rounds of model building in COOT,[15] followed by refinement in PHENIX.[13] The statistics of data collection and refinement are compiled in Table S1. The figures showing atomic models were generated using PYMOL (www.pymol.org).

Table S1. X-ray data collection and refinement statistics

Crystals		**70S complex with A-, P- and E-tRNAs and Dirithromycin**
Diffraction data		
Space Group		$P2_12_12_1$
Unit Cell Dimensions, Å (a x b x c)		209.02 x 447.76 x 618.39
Wavelength, Å		0.9795
Resolution range (outer shell), Å		309-2.75 (2.82-2.75)
I/σI (outer shell with I/σI=1)		6.97 (0.80)
Resolution at which I/σI=1, Å		2.80
Resolution at which I/σI=2, Å		2.98
CC(1/2) at which I/σI=1, %		13.9
CC(1/2) at which I/σI=2, %		43.3
Completeness (outer shell), %		99.5 (99.8)
R_{merge} (outer shell)%		21.4 (195.9)
No. of crystals used		1
No. of Reflections Used:	Observed	6,267,855
	Unique	1,475,480
Redundancy (outer shell)		4.25 (4.33)
Wilson B-factor, $Å^2$		52.4
Refinement		
R_{work}/R_{free}, %		23.5/28.4
No. of Non-Hydrogen Atoms		
RNA		200,295
Protein		90,976
Ions (Mg, K, Zn, Fe)		2,786
Waters		4,700
Ramachandran Plot		
Favored regions, %		94.13
Allowed regions, %		5.22
Outliers, %		0.64
Deviations from ideal values (RMSD)		
Bond, Å		0.003
Angle, degrees		0.668
Chirality		0.035
Planarity		0.004
Dihedral, degrees		14.927
Average B-factor (overall), $Å^2$		53.3

$R_{merge} = \Sigma |I - \langle I \rangle| / \Sigma I$, where I is the observed intensity and <I> is the average intensity from multiple measurements.
$R_{work} = \Sigma|F_{obs} - F_{calc}| / \Sigma F_{obs}$. For calculation of R_{free}, 5% of the truncated dataset was excluded from the refinement.

Antimicrobial susceptibility testing. MIC values were determined by broth microdilution assays as described previously.[5] Bacterial strains and clinical isolates were obtained from the diagnostic department at the Institute of Medical Microbiology, University of Zurich. Engineered *E. coli* strains with defined resistance mechanisms were kindly provided by Patrice Courvalin from the Pasteur institute.

Animal 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).

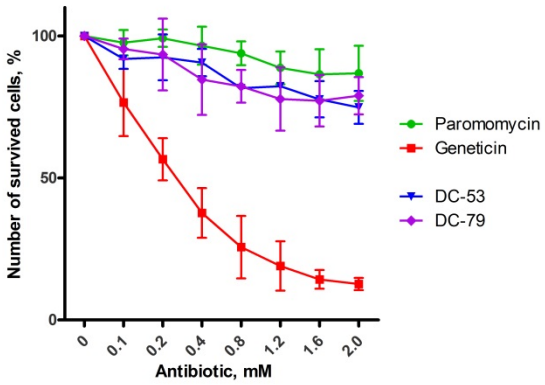
Male mice used in these studies were supplied by Charles River UK and were specific pathogen free. The strain of mouse used was Hsd:ICR (CD-1®), which is a well characterized outbred strain. Mice were 11-15 g on receipt and were allowed to acclimatize for minimum of 7 days prior to infection. Mice were 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 2.17×10^6 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 10 mL/kg with paromomycin or **5** diluted in saline 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.

Peritoneal infection model. All mice were rendered neutropenic by immunosuppression with cyclophosphamide at 200 mg/kg 4 days before infection and 150 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 IP inoculum of $\sim 1.77 \times 10^8$ CFU /mouse *E. coli* AF45 in PBS. One hour post infection, animals were administered subcutaneously at 10 mL/kg with paromomycin or **5** diluted in water for injection. Five hours post infection all remaining animals were euthanized by a pentobarbitone overdose. As soon as the mice

were deeply unconscious they were exsanguinated by cardiac puncture using a heparinized syringe and needle. The blood was stored on ice until culture. Following confirmation of death the peritoneal cavity was washed with 2 mL of PBS. Approximately 1 mL of this wash was recovered and stored on ice until culture. The blood and IP wash were quantitatively cultured onto CLED agar and incubated at 37 °C for 18 to 24 hours before colonies were counted.

Cytotoxicity. Cytotoxicity was assessed in mouse fibroblasts (NIH3T3) cells (mean ± SD; n = 3) (Supplementary Figure S14) that were grown in 96-well plates (4000 cells/well) in DMEM medium containing 10% FBS and 1% glutamine (100 µL/well) at 37 °C and 5% CO_2. Following overnight incubation, serial dilutions of compound dissolved in DMEM medium were added (20 µL per well), and the cells were incubated for an additional 72 h. Cell viability was assessed using Alamar Blue fluorimetric assay (Life Technologies) according to the manufacturer's instructions. Fluorescence was measured using an FLx800 plate reader (Bio-Tek Instruments). Cell viability was calculated as the ratio between the numbers of living cells in cultures grown in the presence of the tested compounds and those in cultures grown under the identical conditions without the tested compound. Paromomycin and geneticin were used as negative and positive controls accordingly. The concentration LC_{25} values, *i.e.*, the drug concentration at which 25% of the cells in culture are non-viable, were calculated from fitting concentration-response curves to the data of at least three independent experiments using PRISM 5 software.



	LC_{25} (µM)
Geneticin	97±11
Paromomycin	>2000
4 (DC53)	>2000
5 (DC79)	>2000

Figure S1. Cytotoxicity of Paromomycin, Geneticin, **4** (DC53), and **5** (DC79) in Mouse Fibroblasts (NIH3T3) Cells (mean ± SD; n = 3). Paromomycin (green) geneticin (red), **4** (DC-53) (blue), **5** (DC-79) (lilac). The concentration LC_{25} values, *i.e.*, the drug concentration at which 25% of the cells in culture are non-viable, were calculated from fitting concentration-response curves to the data of at least three independent experiments using PRISM 5 software.

In-vivo ototoxicity

Auditory brain stem responses. Pigmented male guinea pigs of about 200 g at purchase (Elm Hill Laboratories) were maintained on a 12 h light/12 h dark schedule and had free access to water and a regular diet. They were acclimated for one week prior to experiments. Experimental protocols were approved by the University of Michigan Committee on the Use and Care of Animals and animal care was under the supervision of the University of Michigan's Unit for Laboratory Animal Medicine.

Gentamicin and propylamycin were dissolved in saline, and the animals received once-daily subcutaneous injections for 14 days at dosages indicated in the figure legends. Body weight was measured before each injection and the drug dose adjusted accordingly. Saline injections of the same volume served as controls.

For auditory brainstem responses (ABR), guinea pigs were anesthetized with intramuscular injections of xylazine (2.4 mg/kg body weight), ketamine (58.8 mg/kg), and acepromazine (1.2 mg/kg). Body temperature was maintained near 37 °C with a heating pad. ABRs were measured at 8, 16 and 32 kHz in a sound-isolated and electrically shielded booth (Acoustic Systems, Austin, TX). Sub-dermal electrodes were inserted at the vertex of the skull, under the left ear, and under the right ear (ground). Tucker Davis Technology System III hardware and SigGen/Biosig software were used to present the stimuli (15 ms duration tone bursts with 1 ms rise-fall time) through a Beyer earphone and to average up to 1024 responses for each stimulus level. Thresholds (the lowest stimulus level with a reproducible response) were determined by reducing the intensity in 10 dB steps and in 5 dB close to threshold.

Hair cell counts. Following final ABR recordings, animals were sacrificed under xylazine and ketamine anesthesia. The temporal bones were immediately removed and placed in 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS, pH 7.4). The otic capsules and the tectorial membranes of the cochleae were removed and the remaining tissues stained with Phalloidin Alexa Fluor 568 (Thermo Fisher Scientific). Phalloidin outlines the hair cells as well as the scars that replace them after their death. After staining, cochleae were further dissected into four to eight segments representing apex, middle turns 3 and 2, and base. Each turn was mounted separately as a surface preparation on a glass slide with Fluoromount (EMS Inc.) and a coverslip was placed above. Slides were stored at 4 ^{o}C until examination.

Hair cells were counted under epifluorescence optics on a Leica fluorescent microscope using a 50x objective and a 0.19 mm reticule in the eyepiece. Each successive 0.19 mm field was evaluated for the absence of inner and outer hair cells, starting at the apex and moving basally until the entire length of the cochlear spiral had been assessed. The percentage of missing hair cells was calculated by comparison with a normative data base and plotted as a function of distance from the apical turn of the explant.

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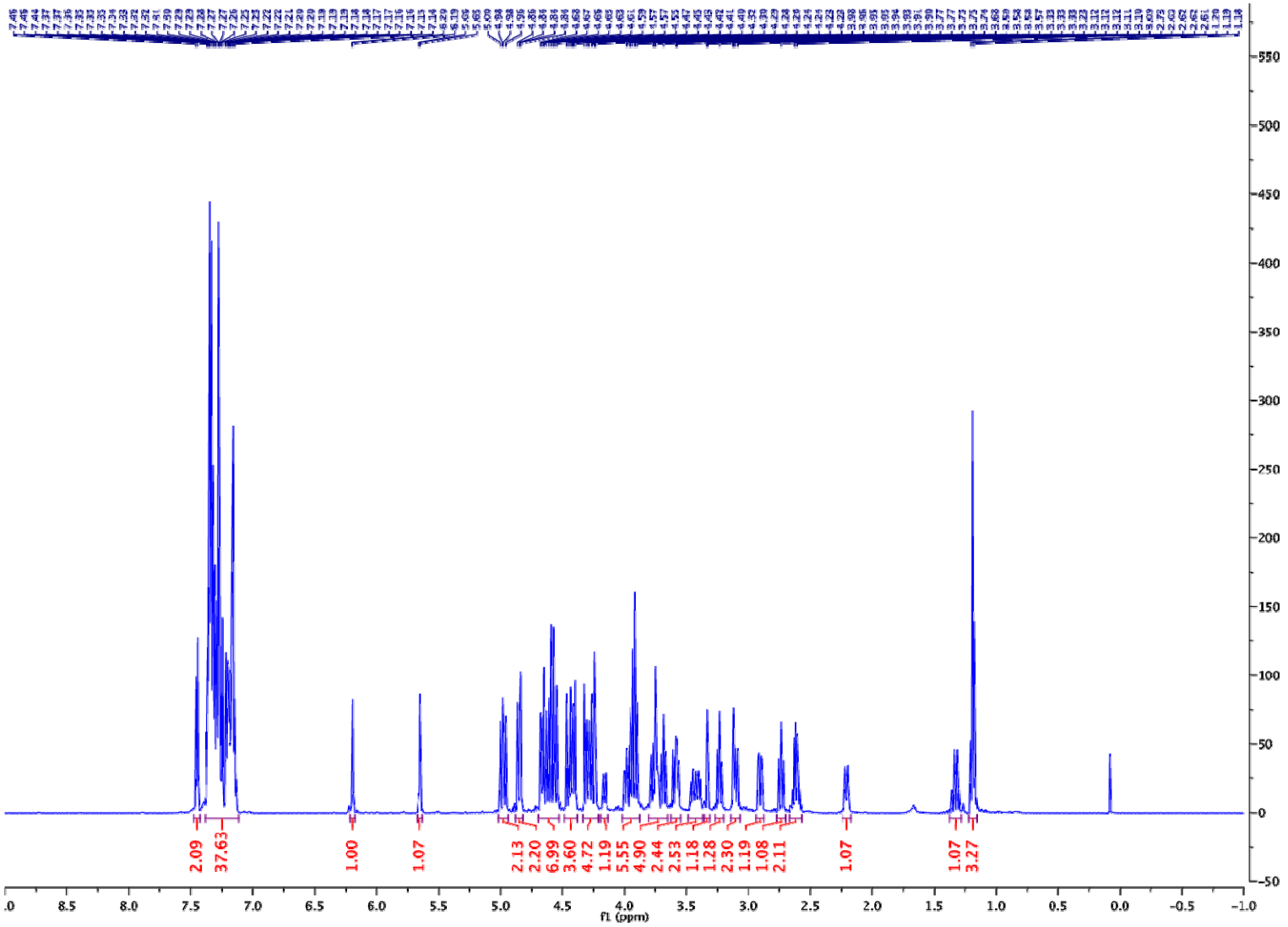
1,2′,2‴,3,6‴-Pentaazido-2″,3′,3‴,4‴,5″,6,6′-hepta-*O*-benzyl-1,2′,2‴,3,6‴-pentadeamino-4′-deoxy-4′-propylthio-paromomycin (7) ^{1}H NMR (600 MHz, $CDCl_3$)



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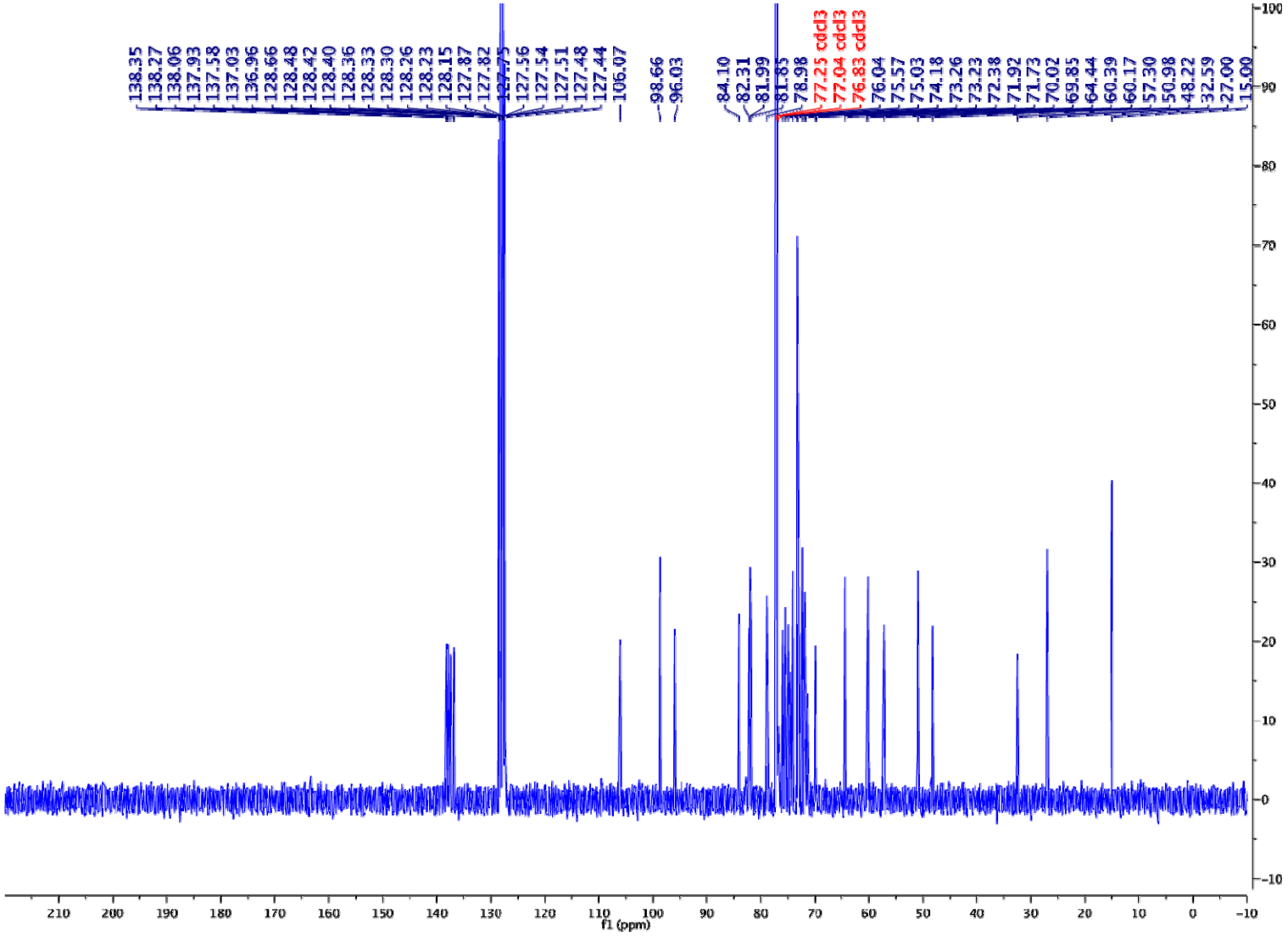

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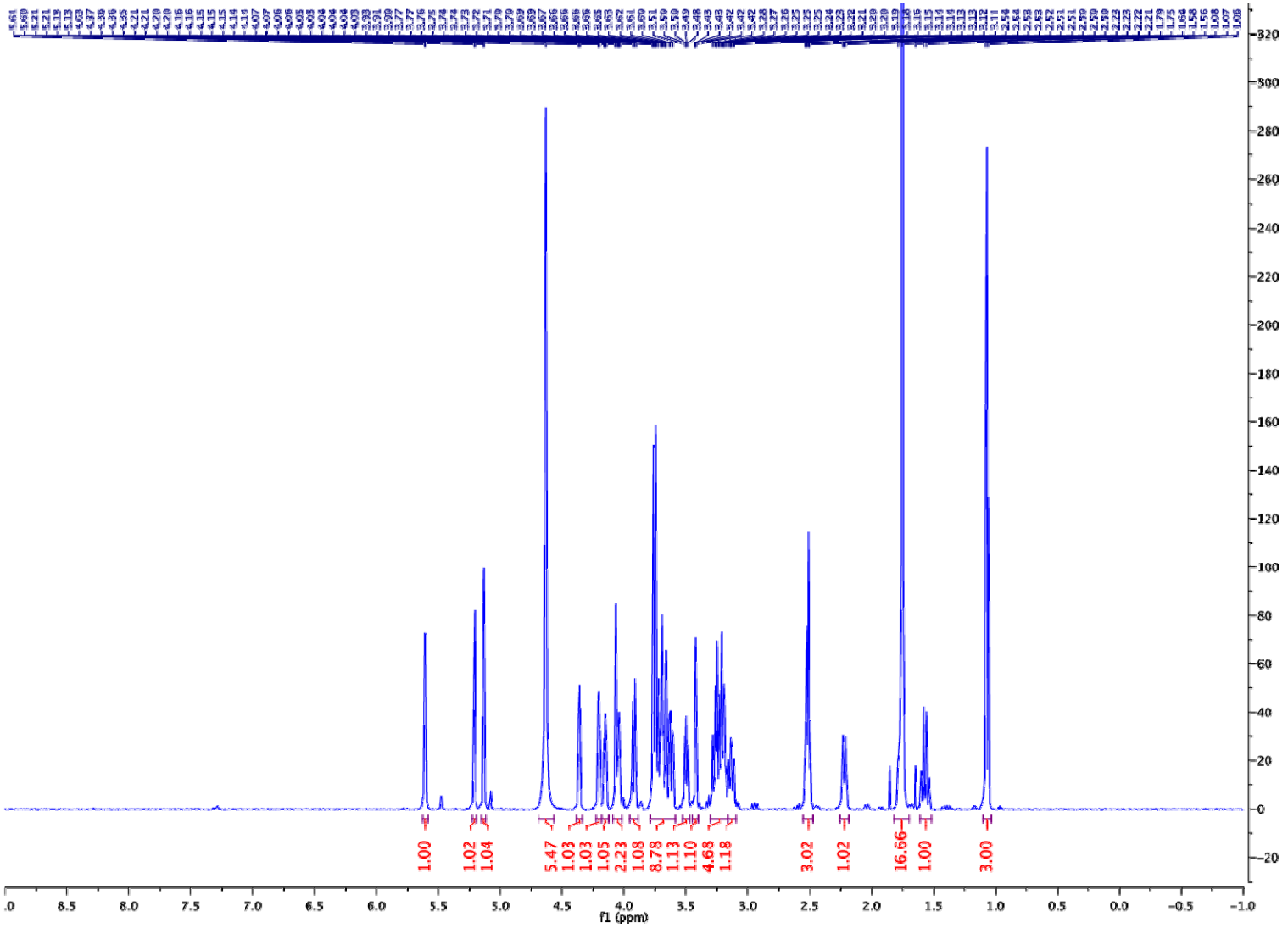

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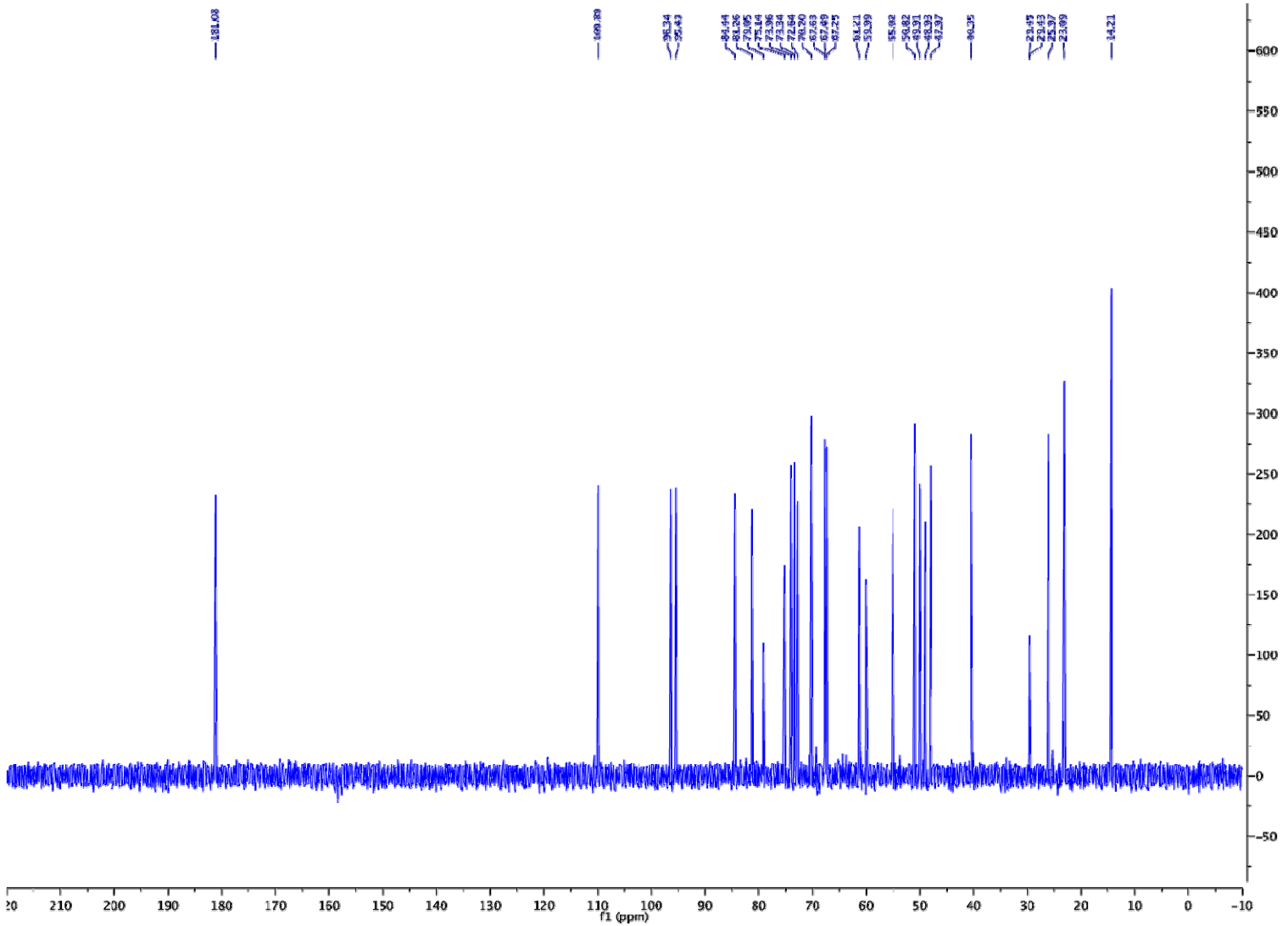

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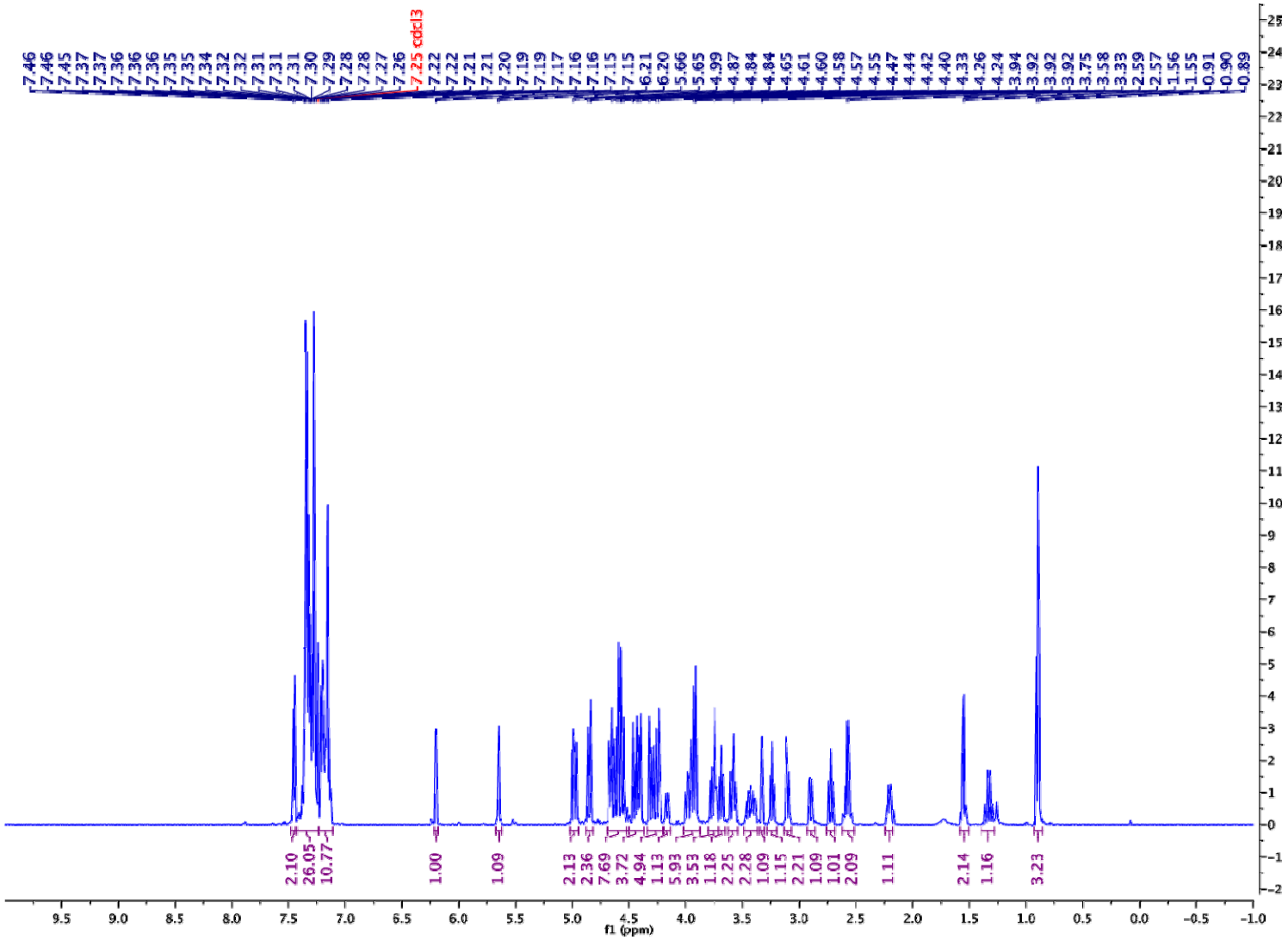

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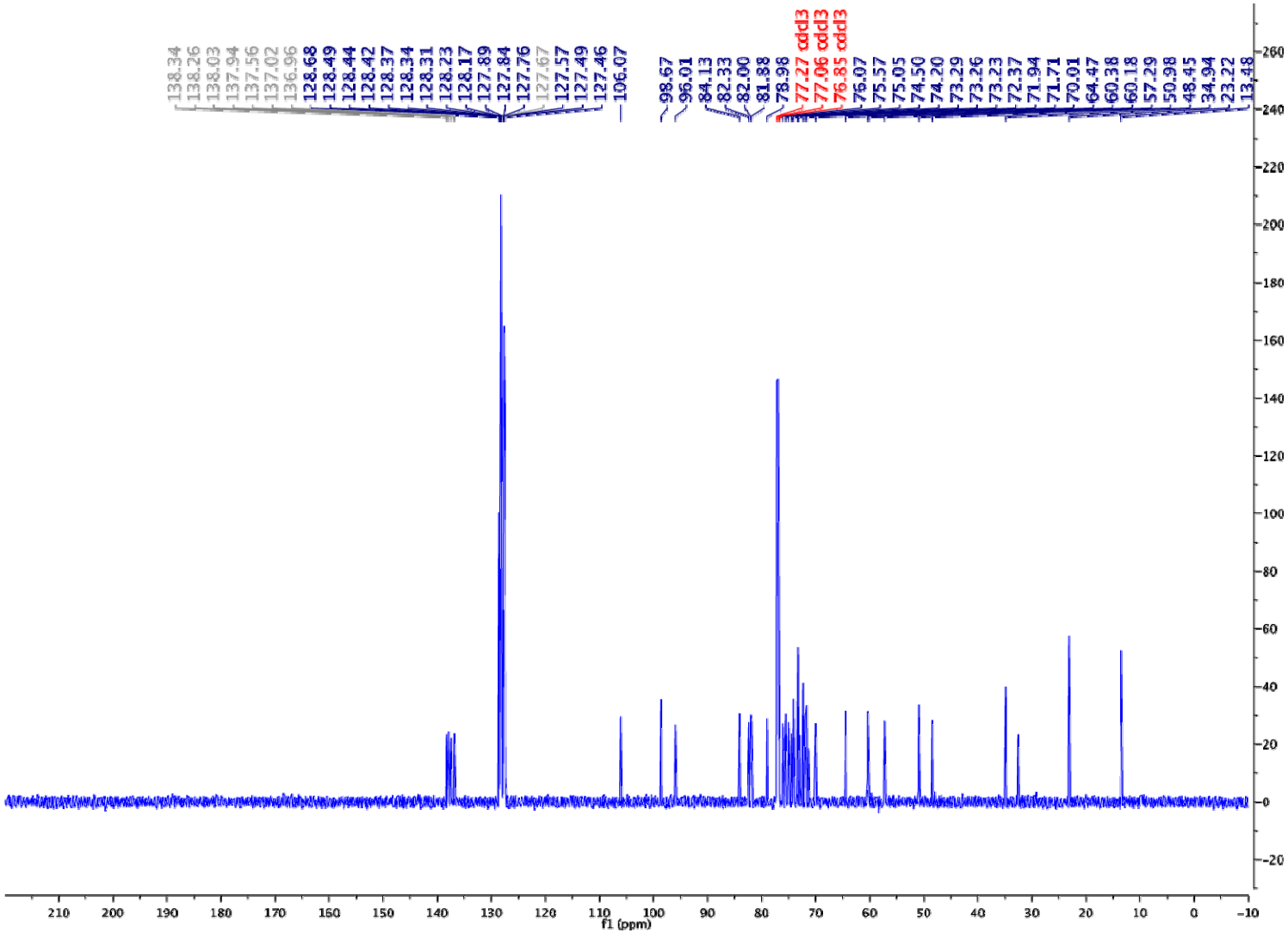

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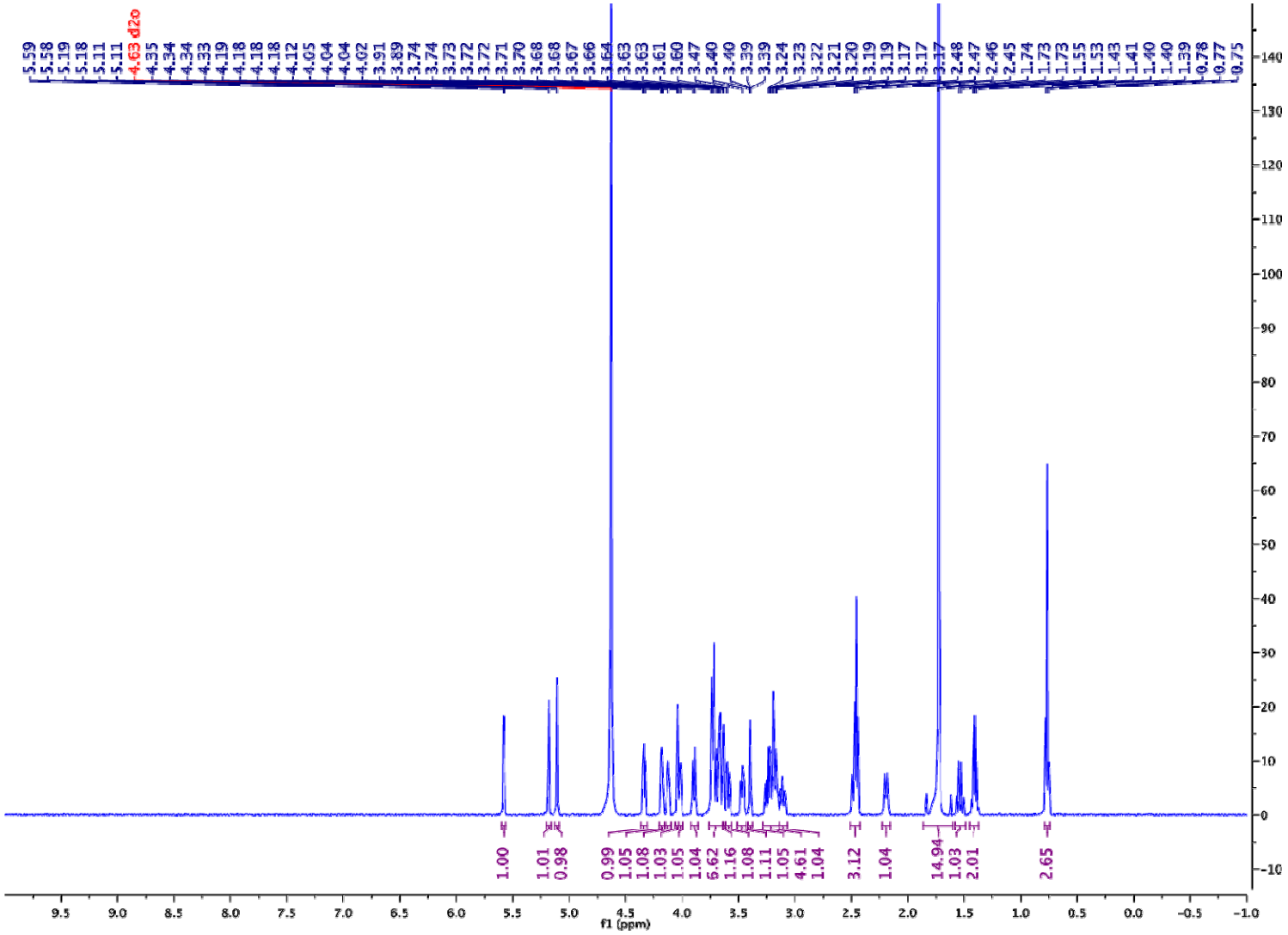

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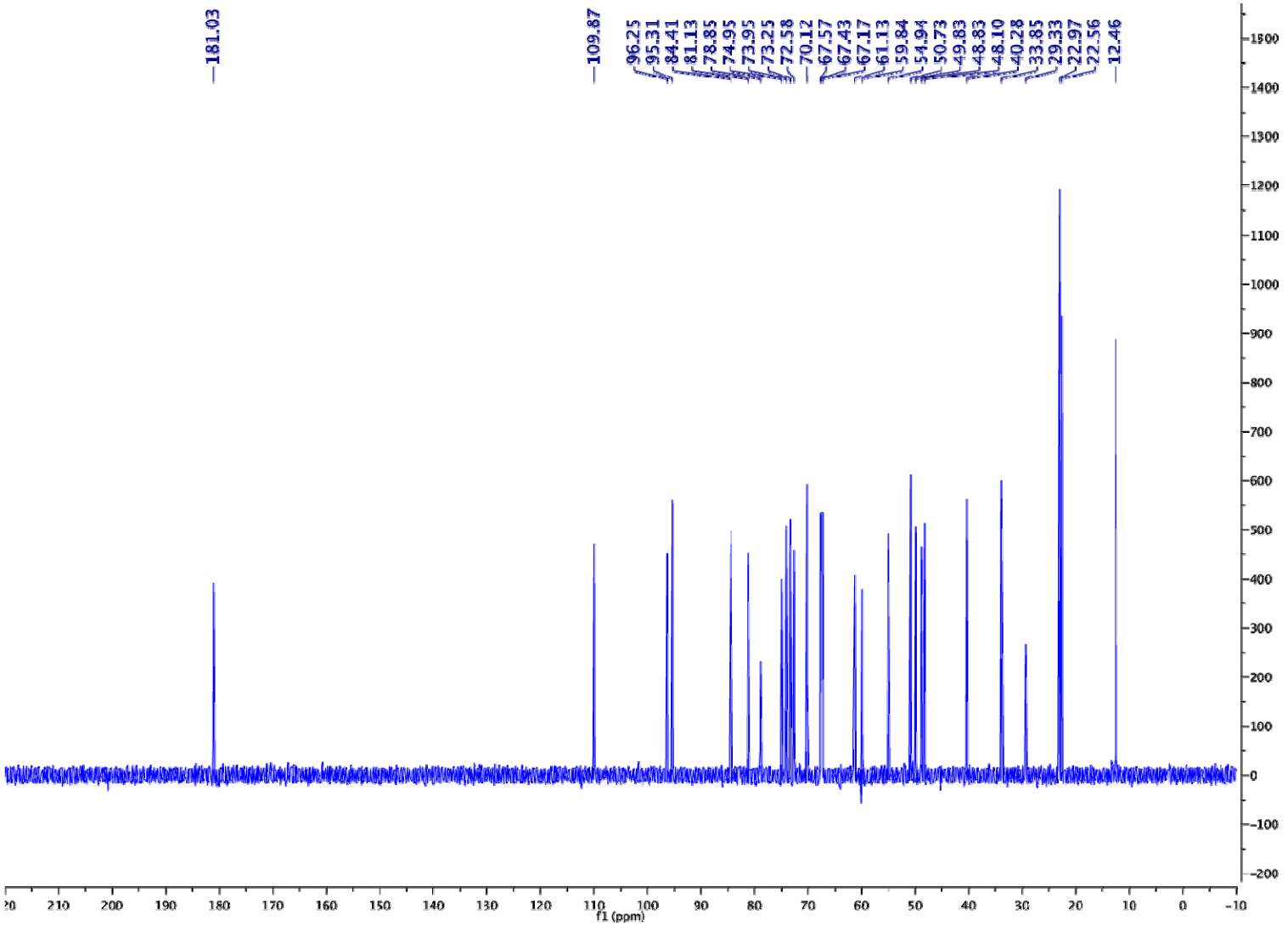

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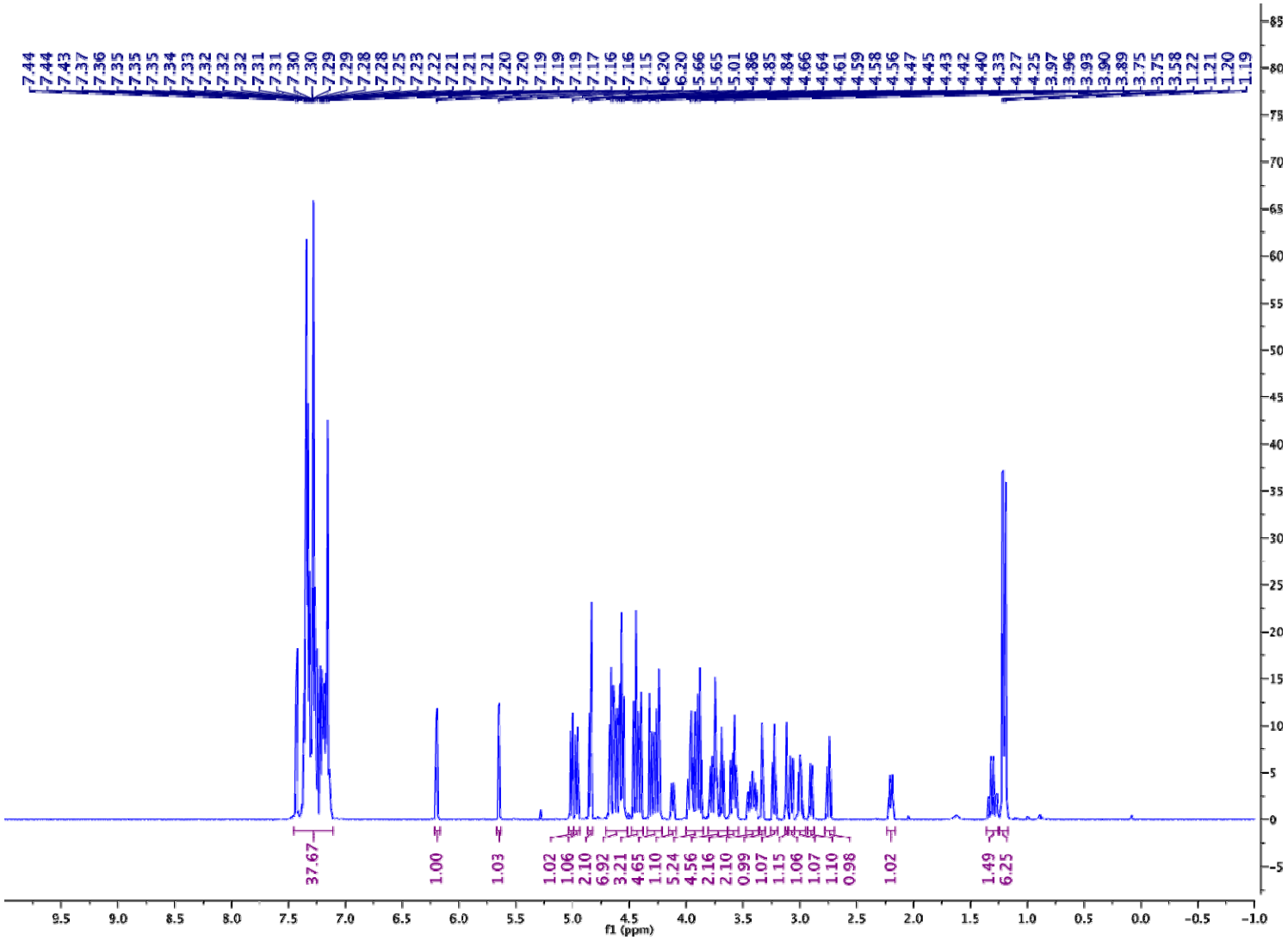

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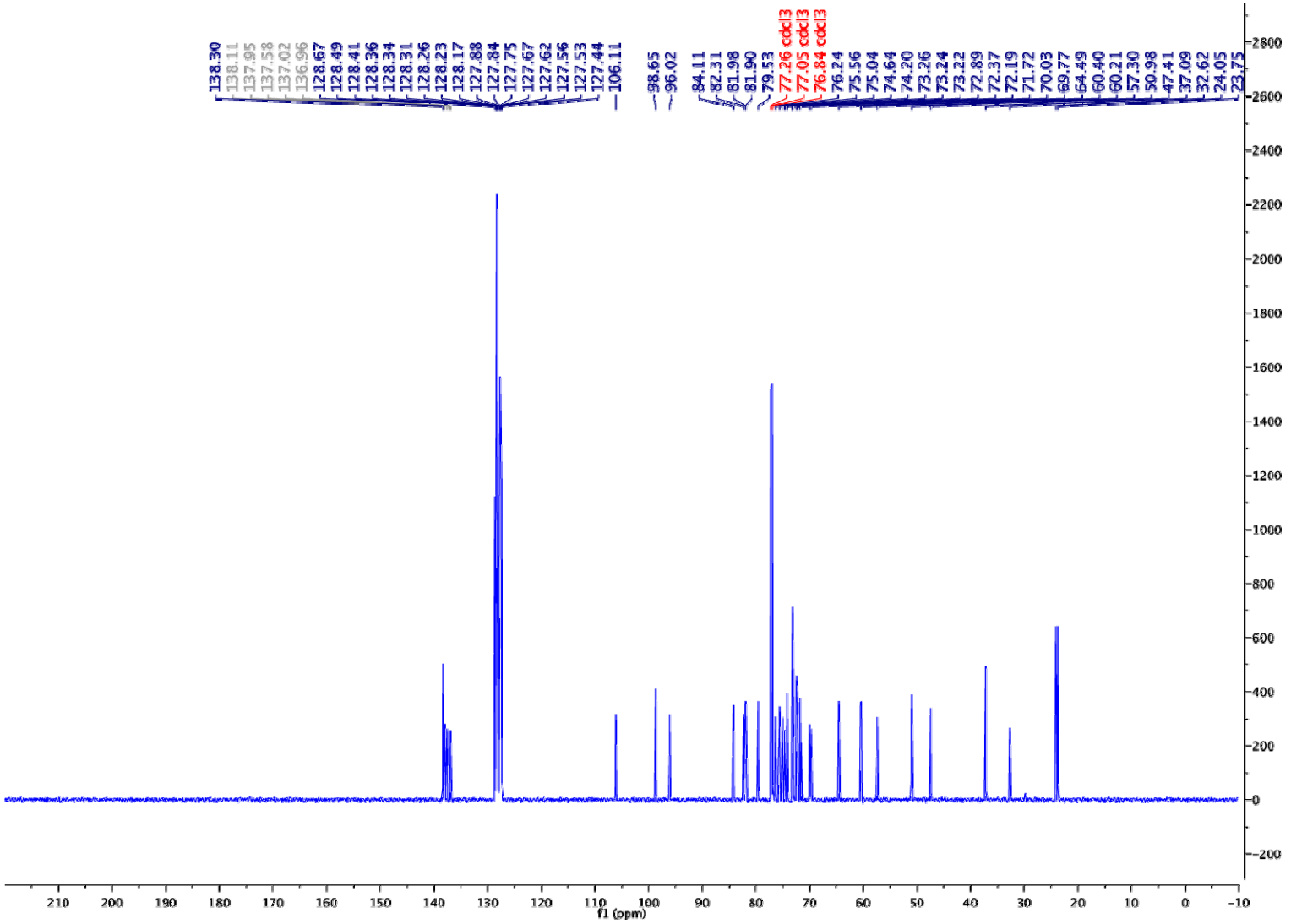

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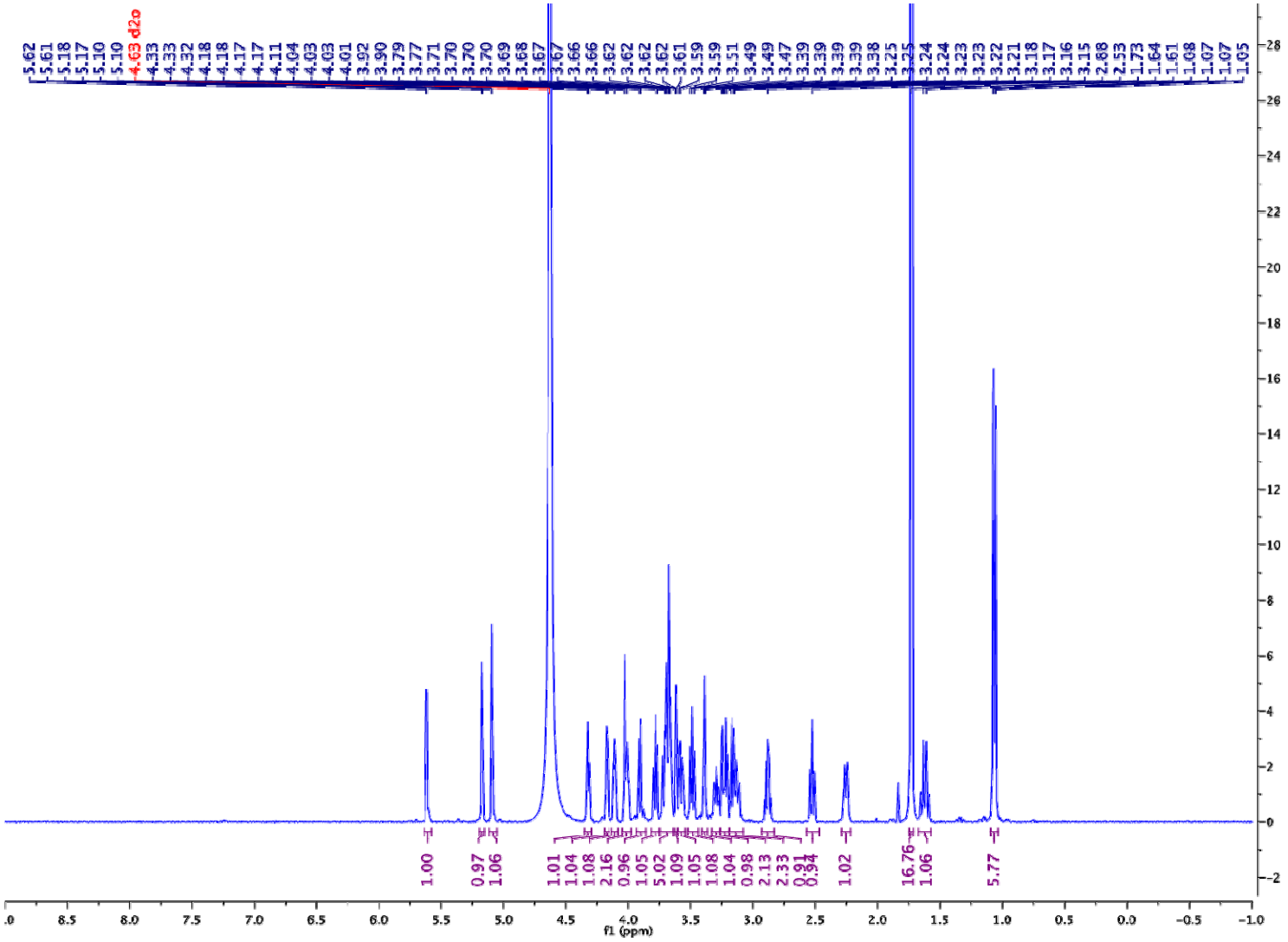

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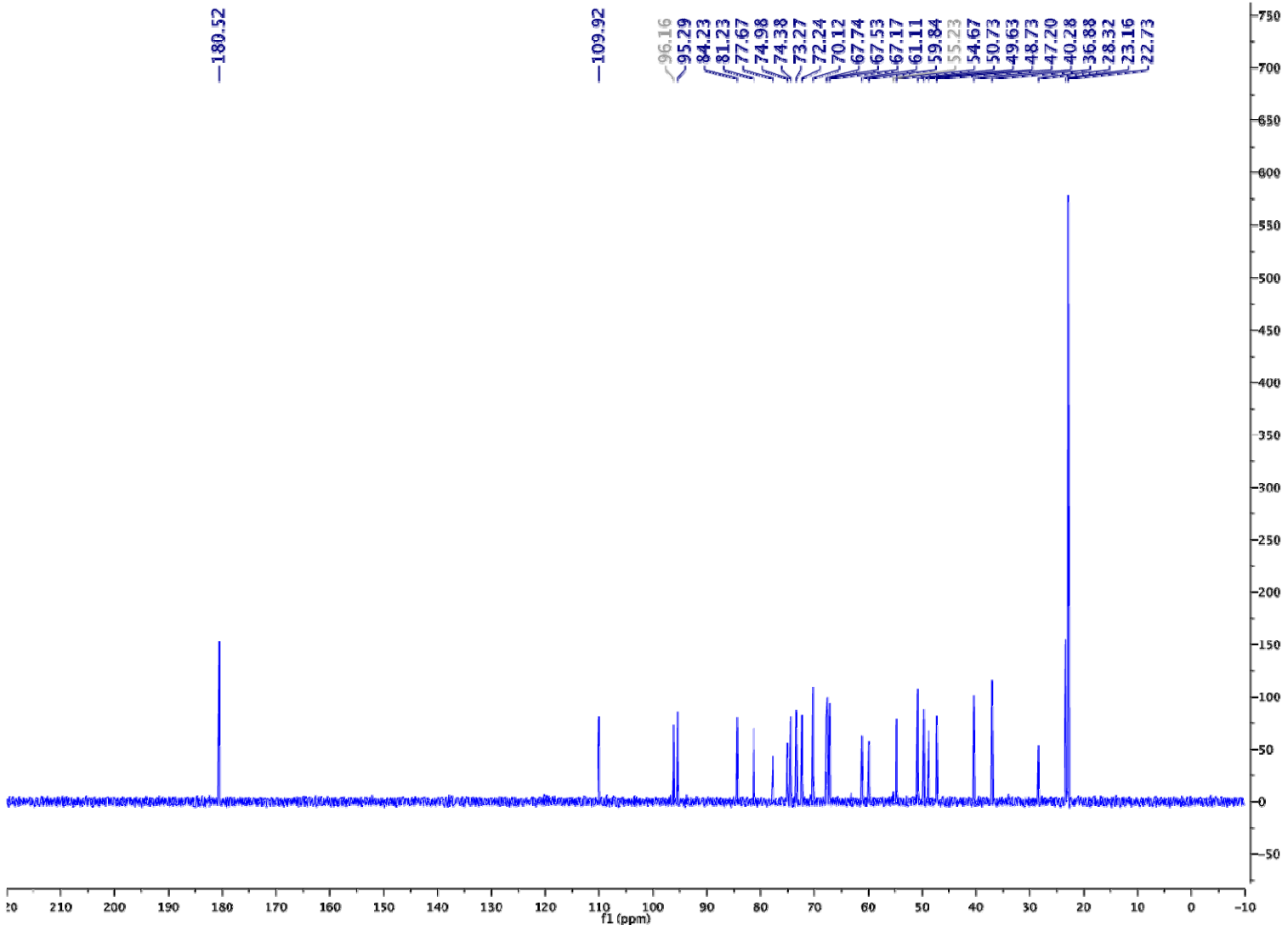

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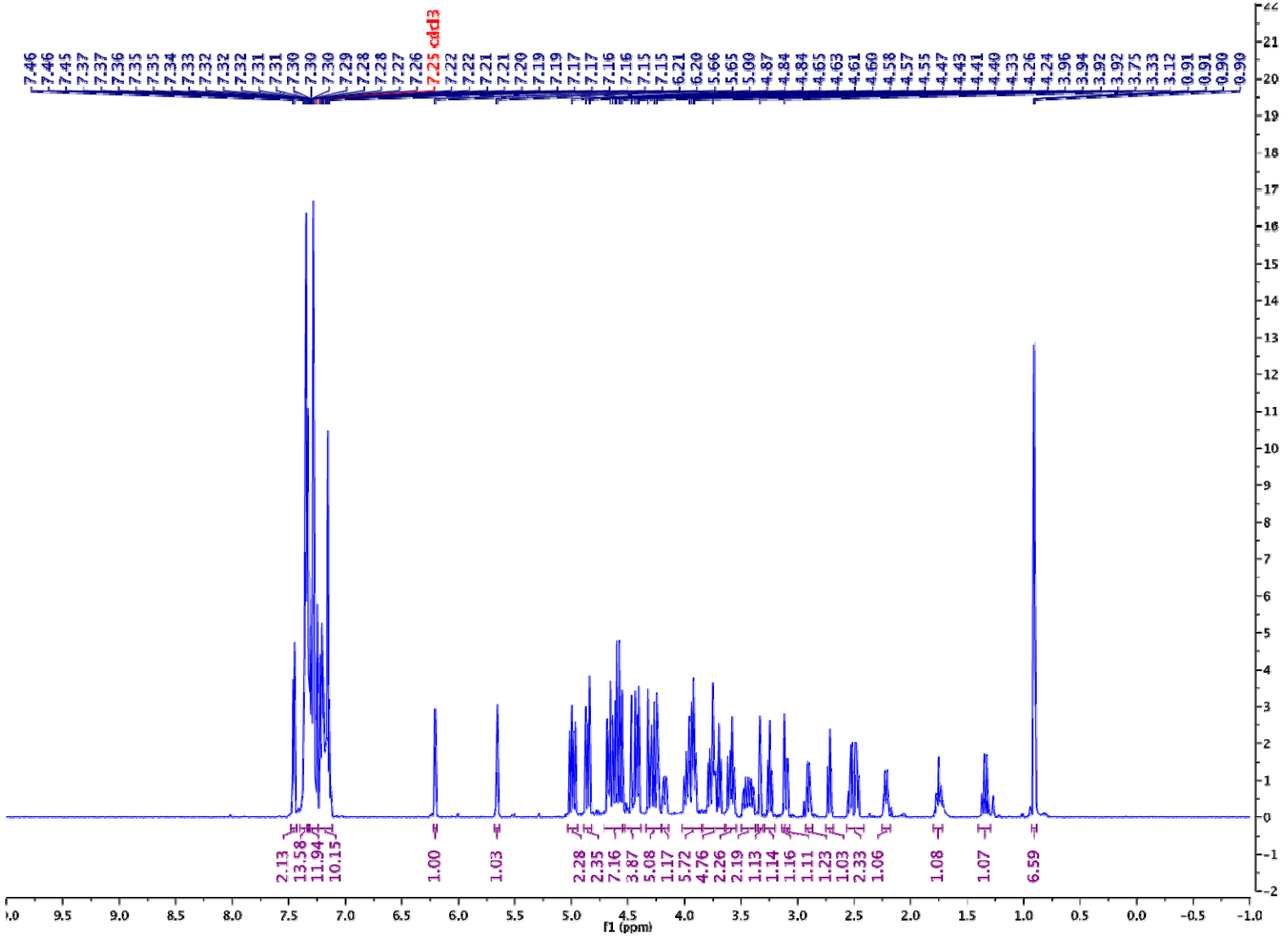

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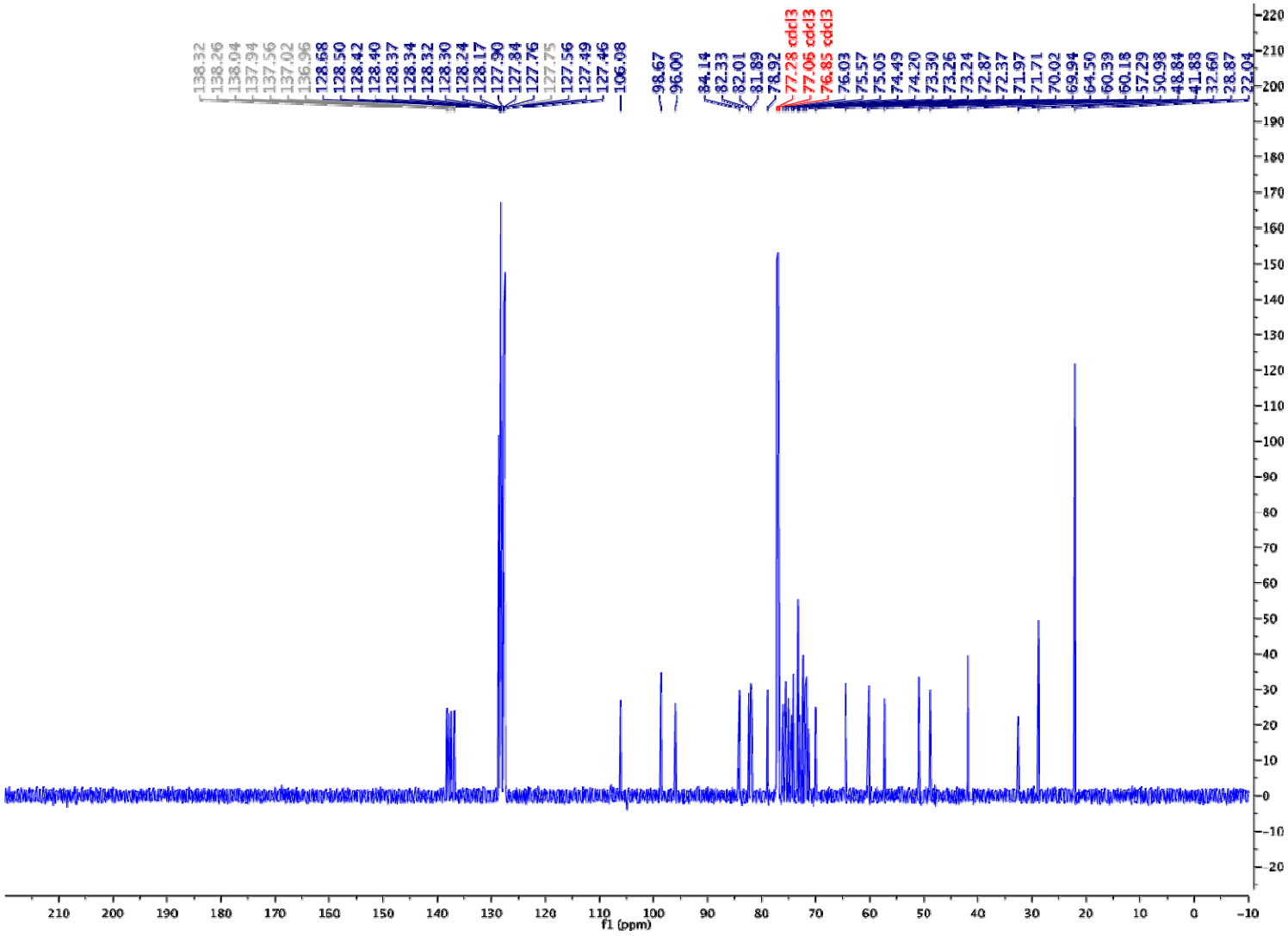

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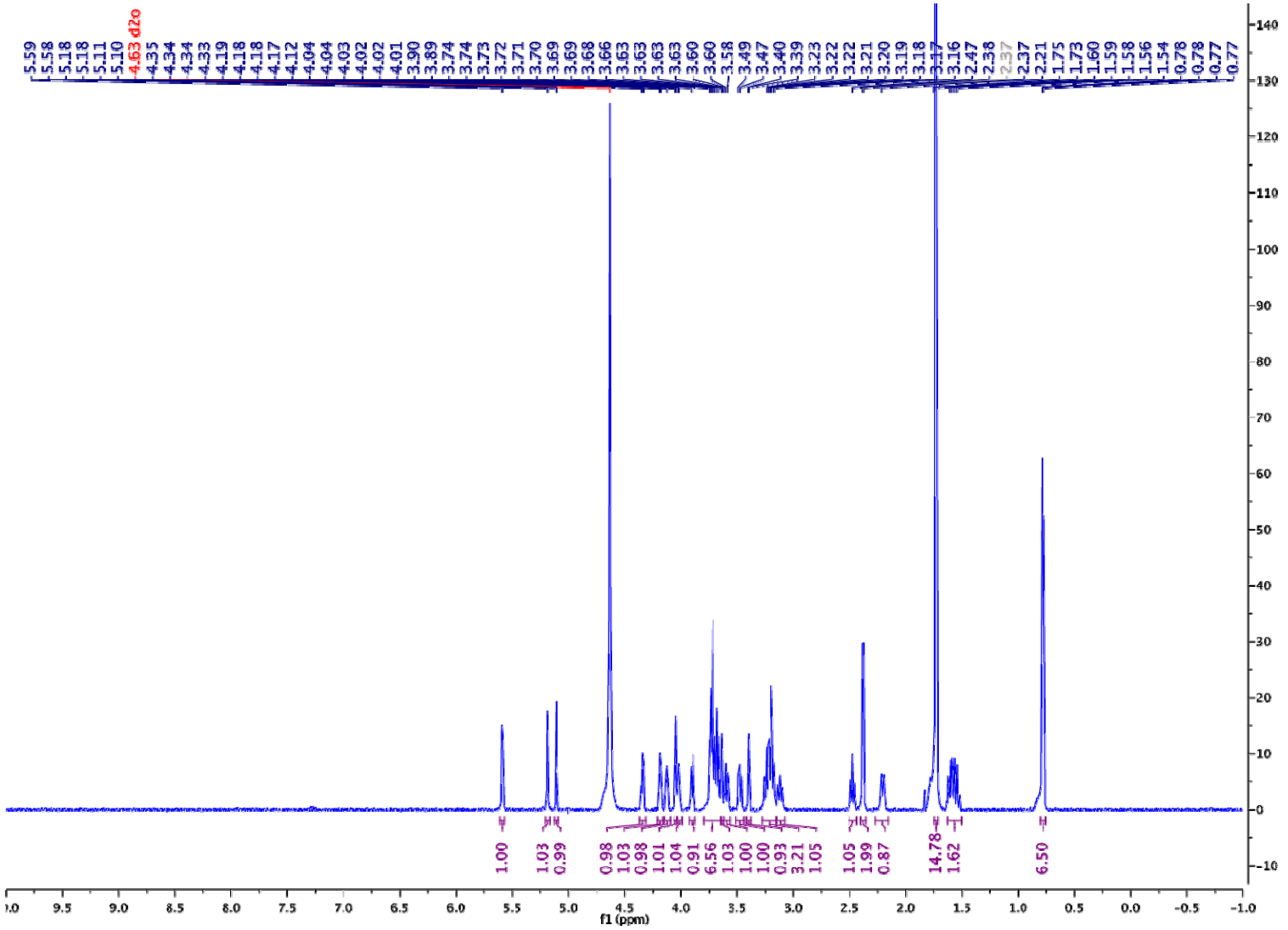
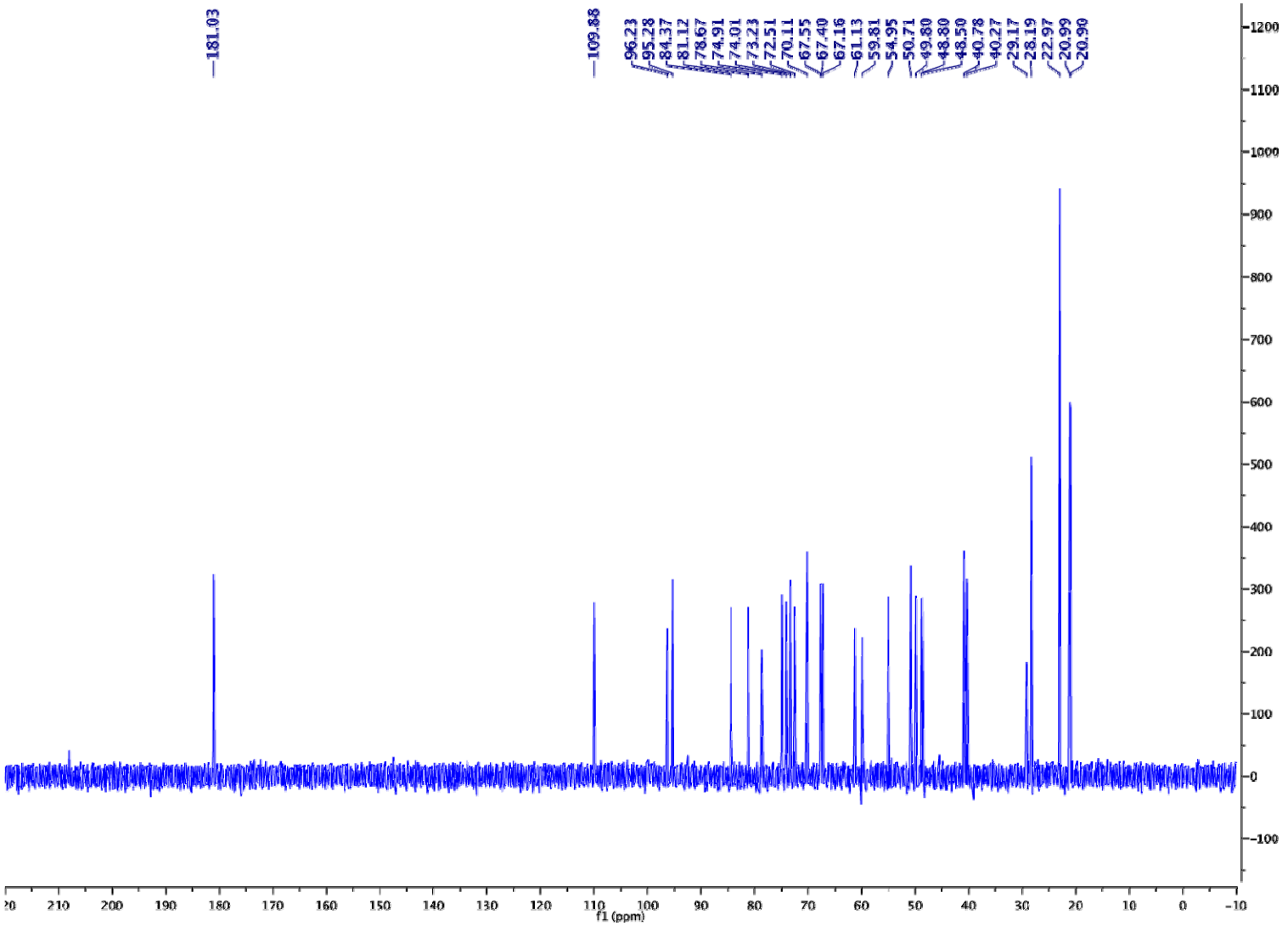


4′-Deoxy-4′-isobutylthio-paromomycin (17) ^{13}C NMR (150 MHz, D_2O)



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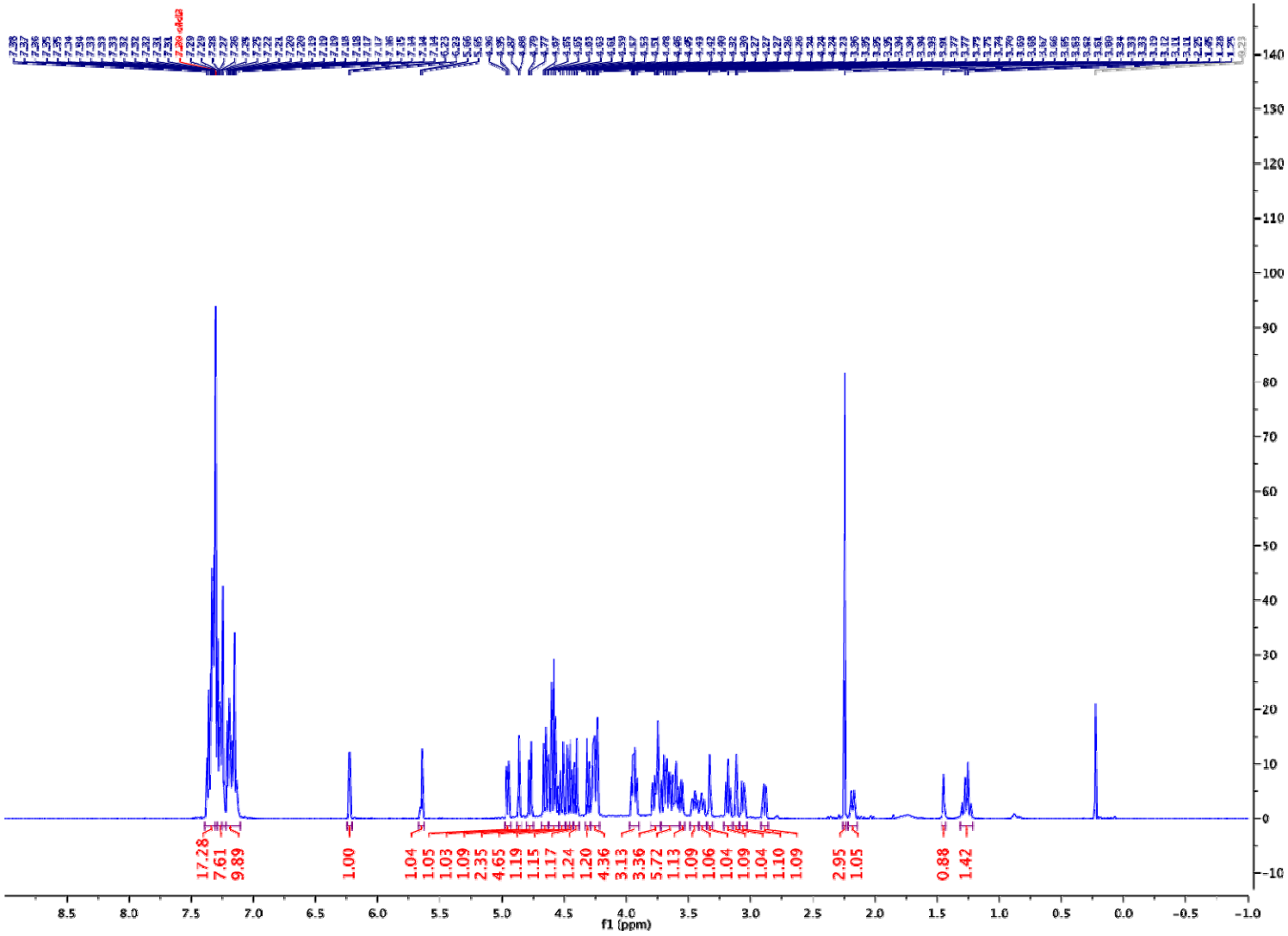

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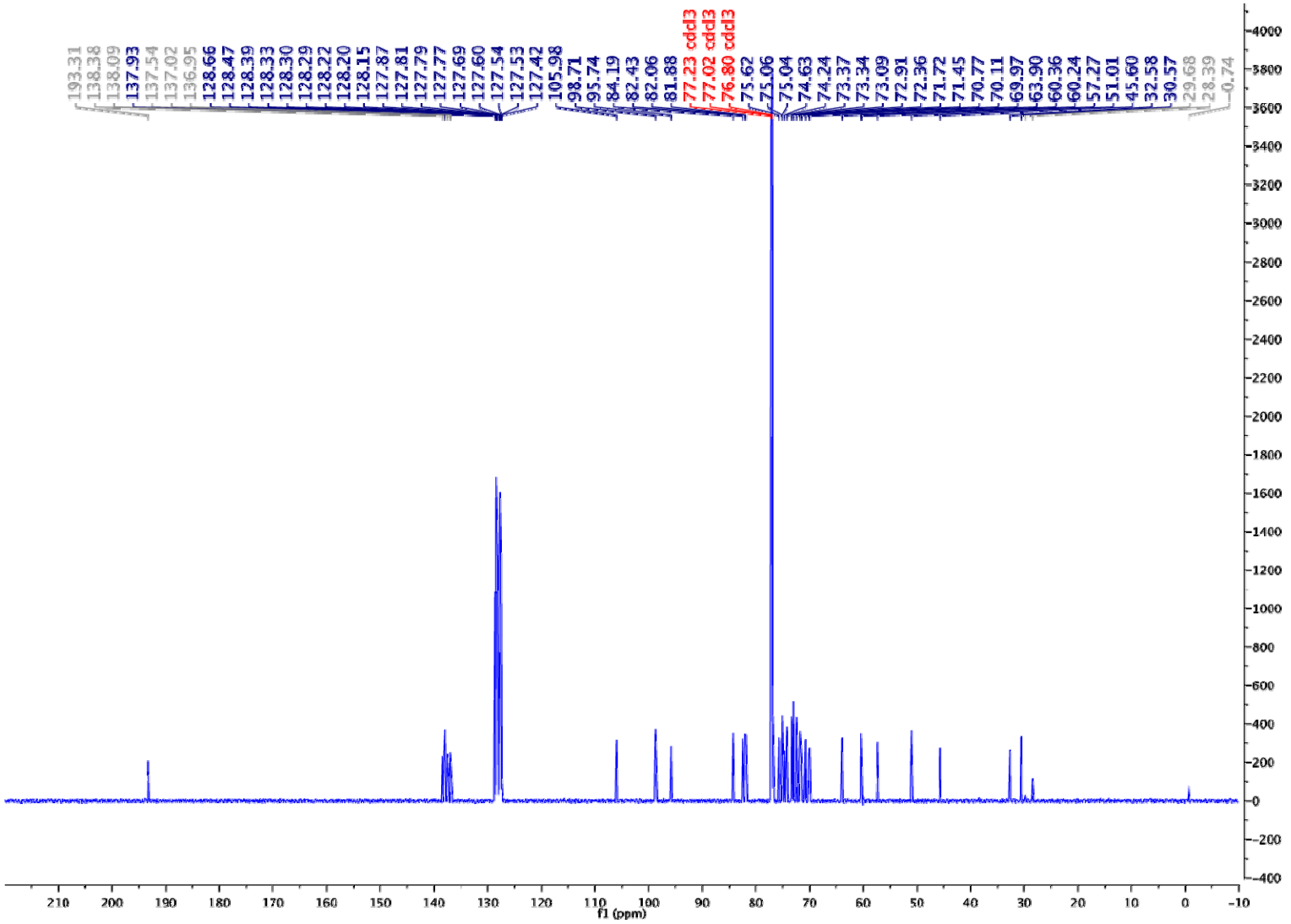

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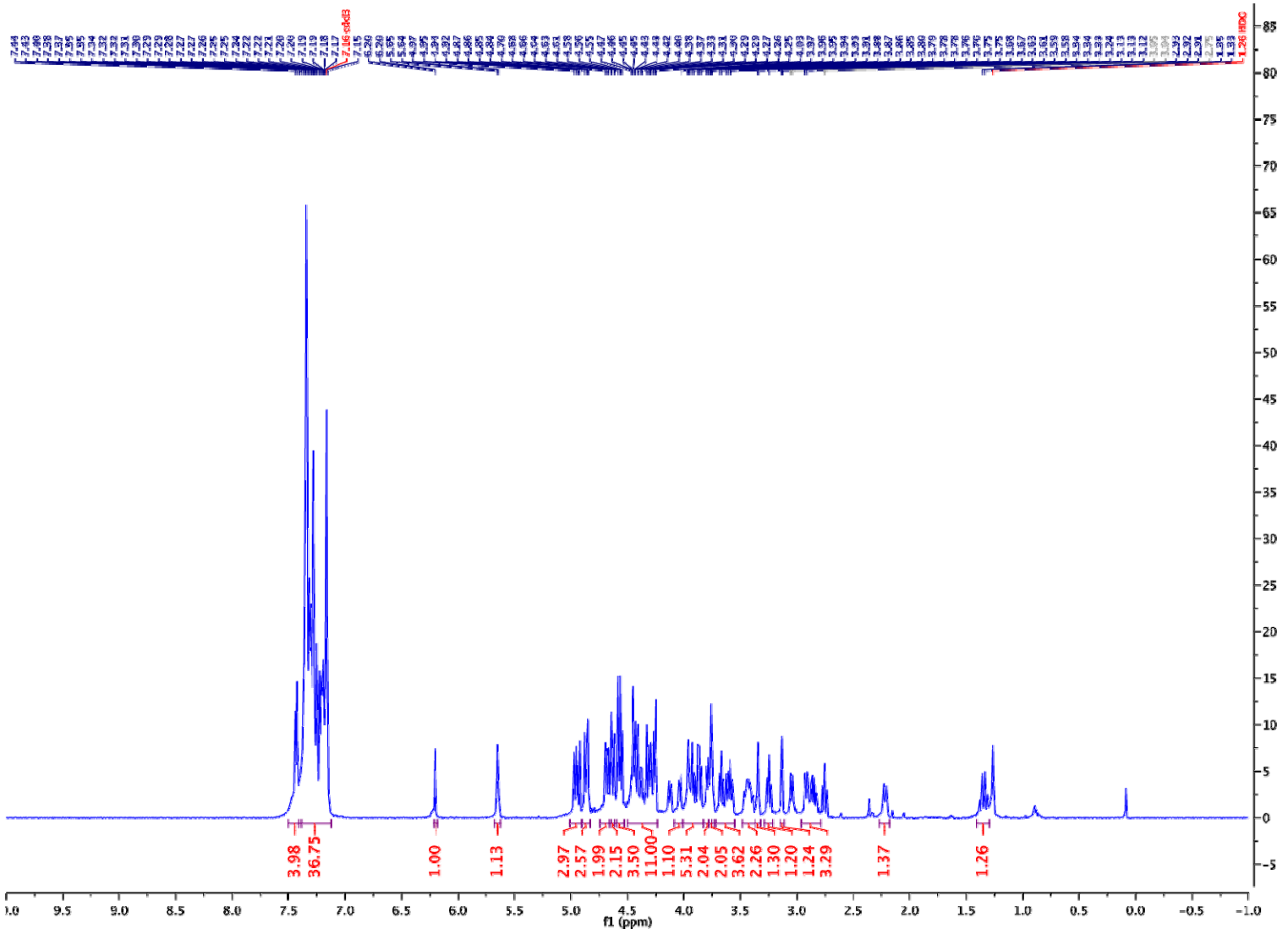

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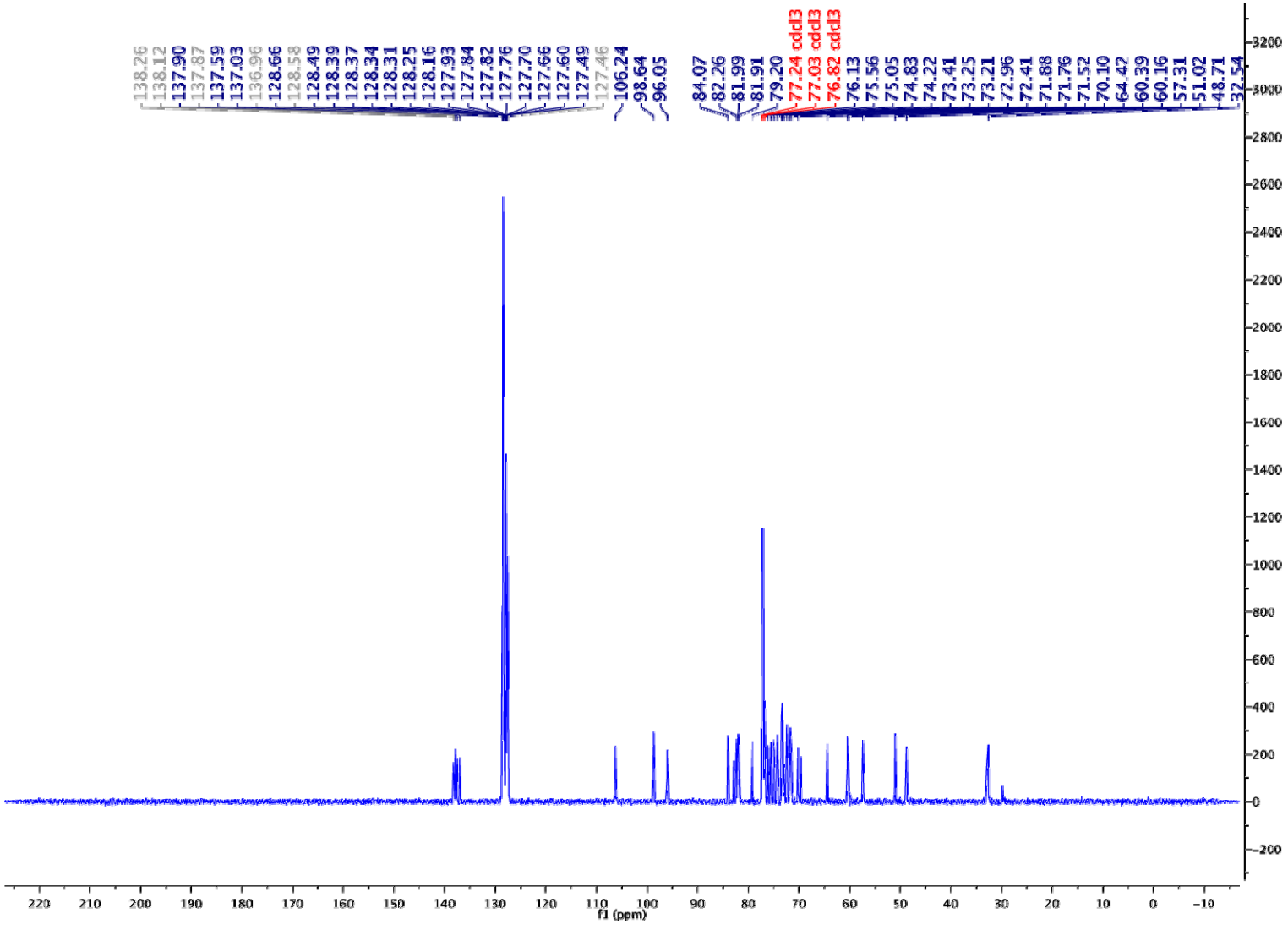

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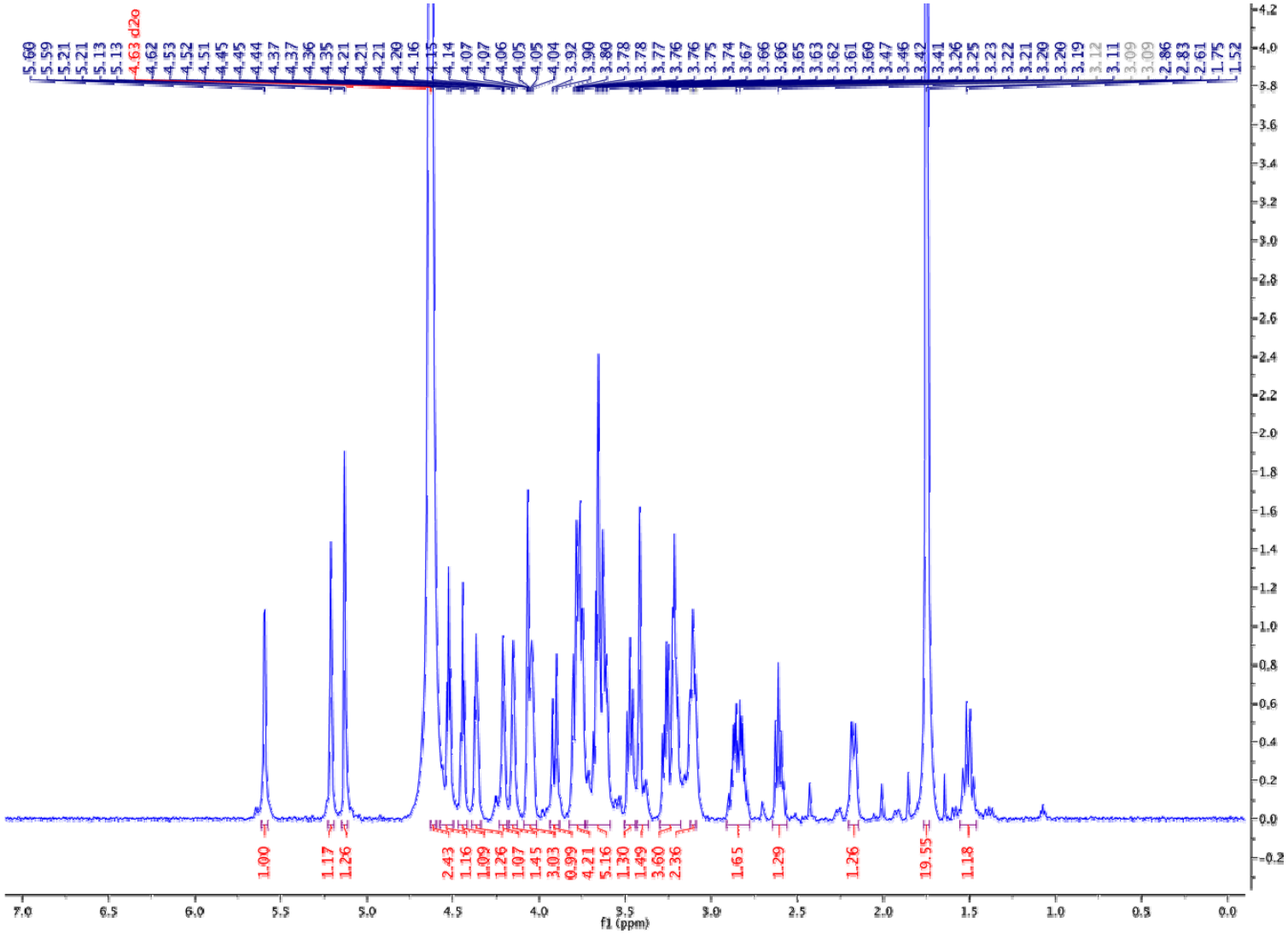

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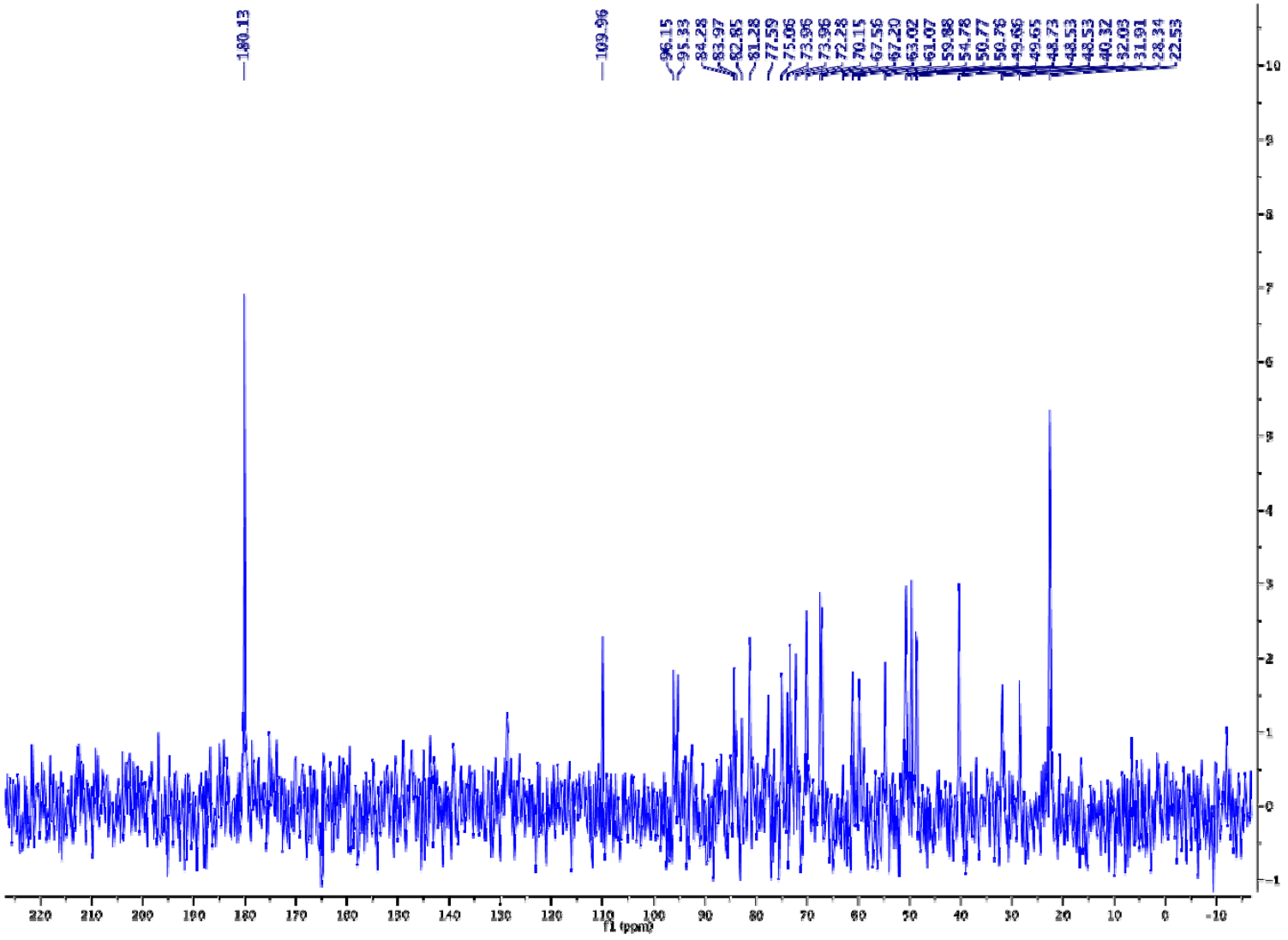

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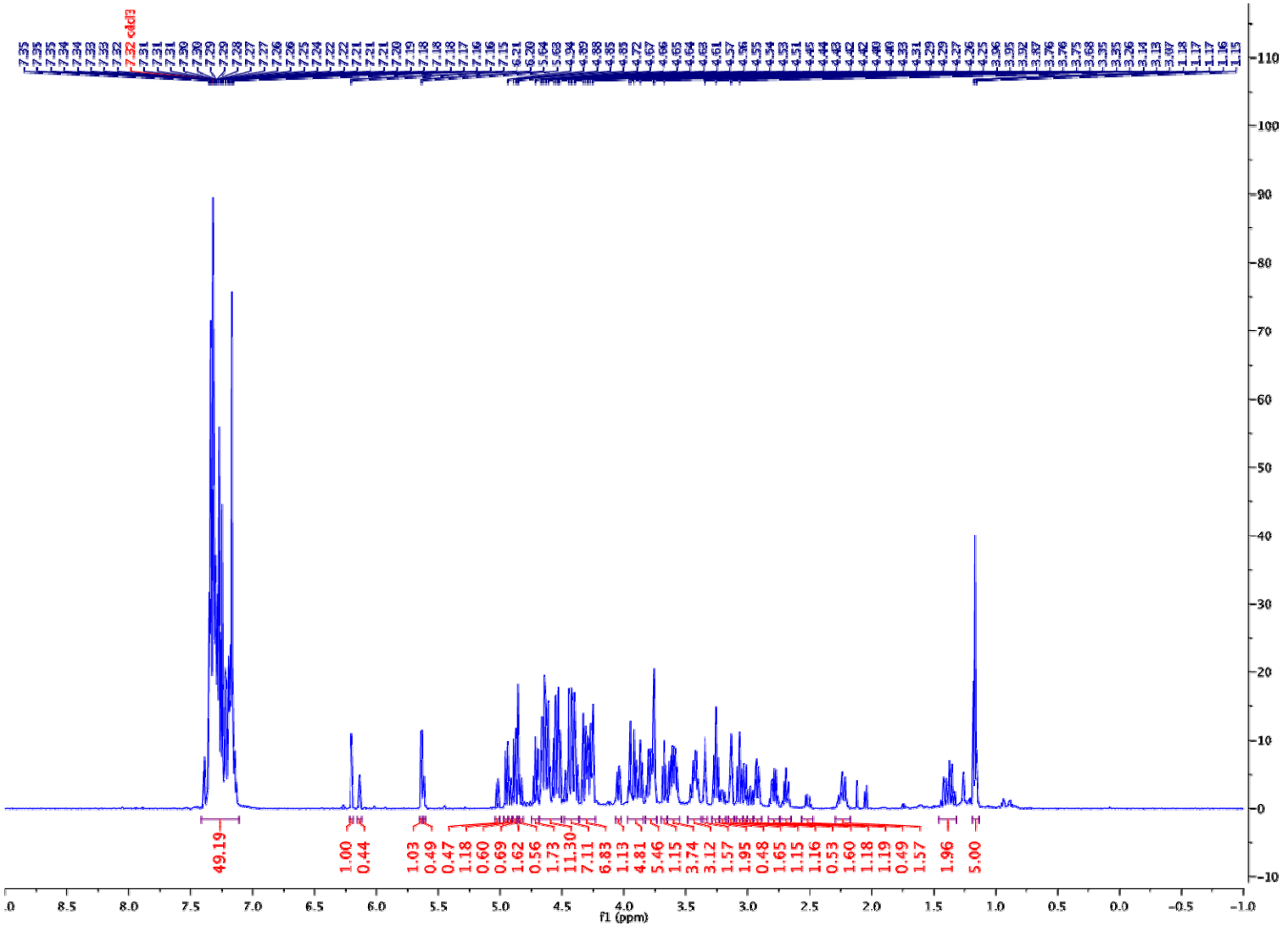

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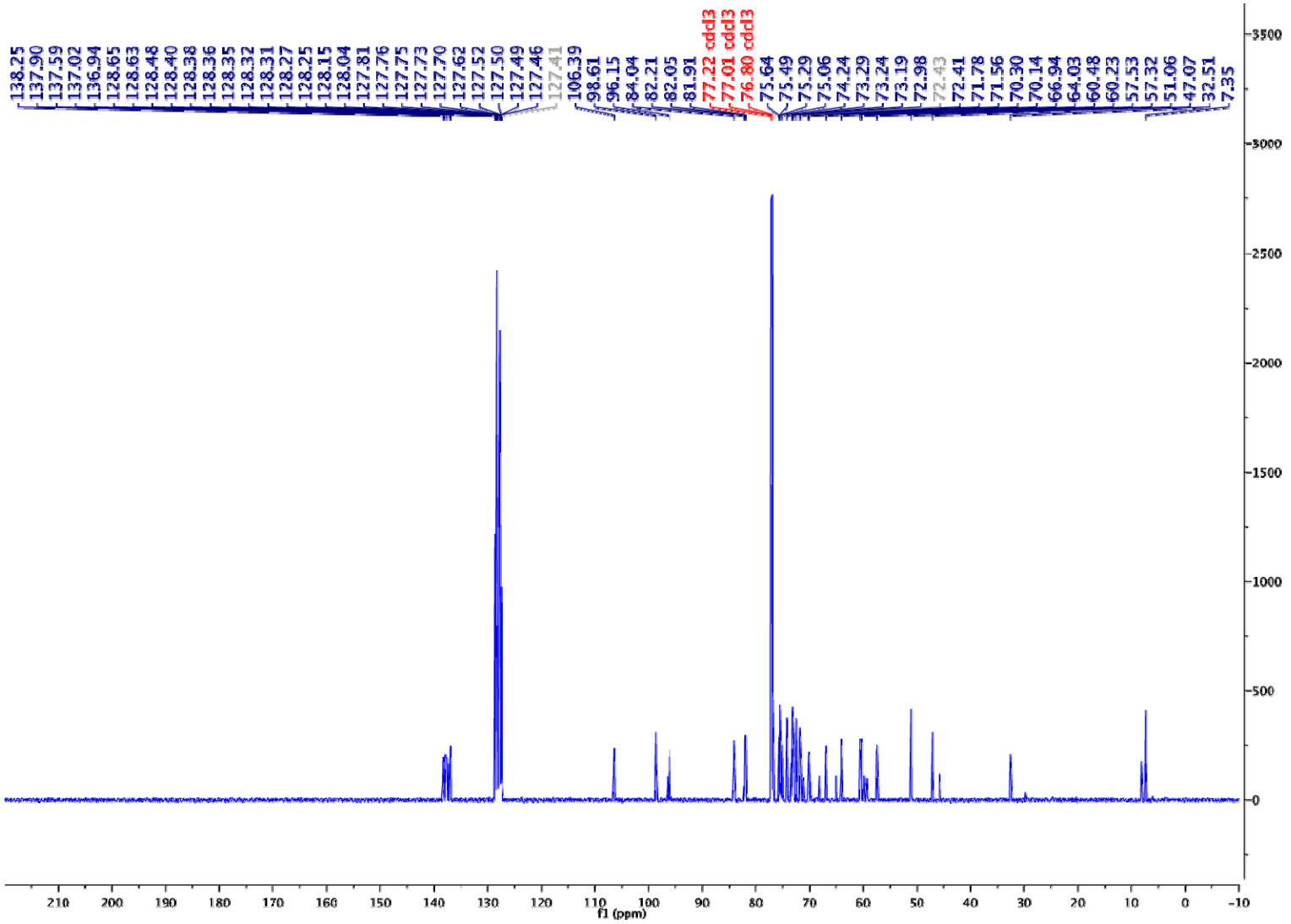

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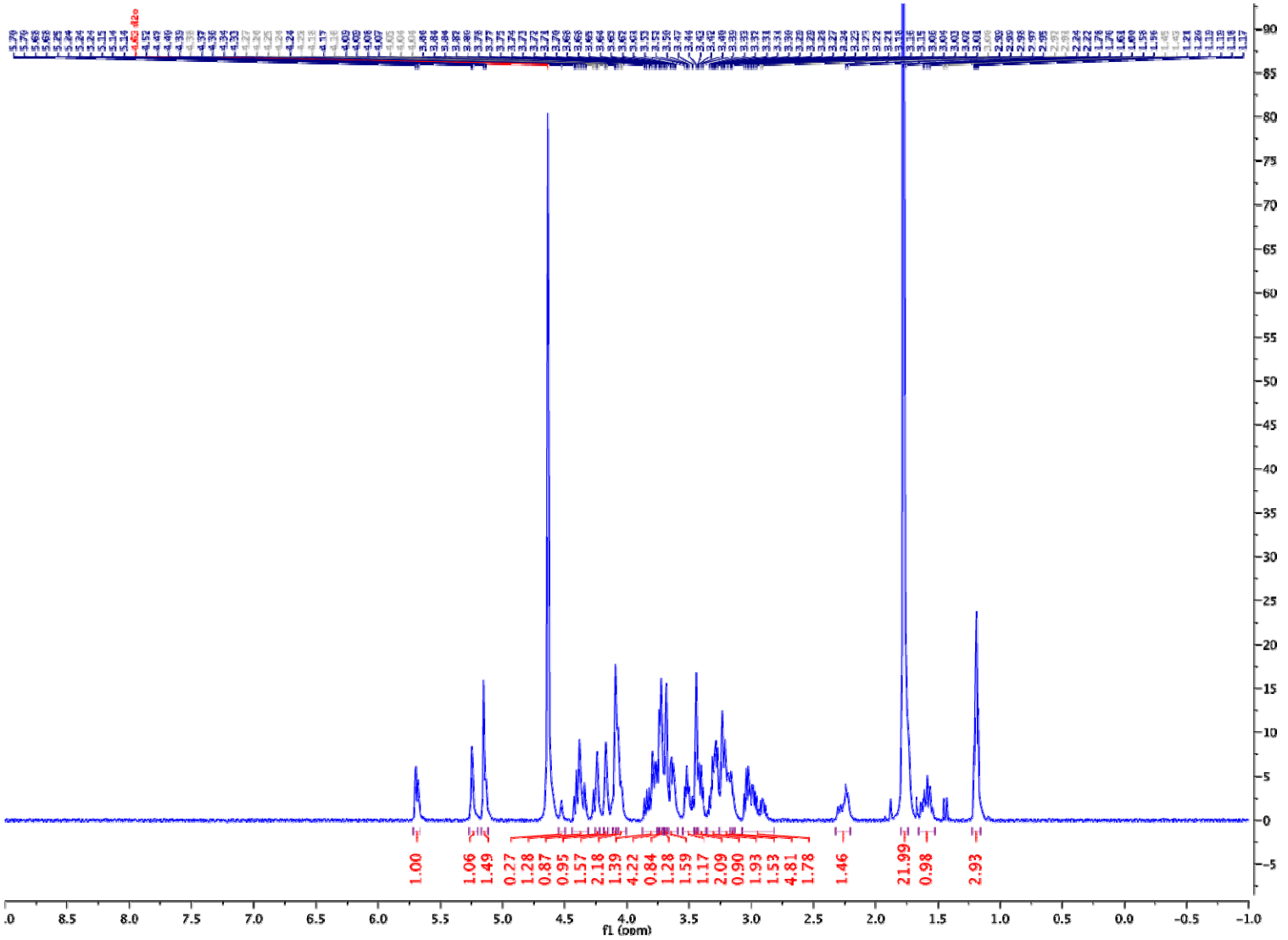
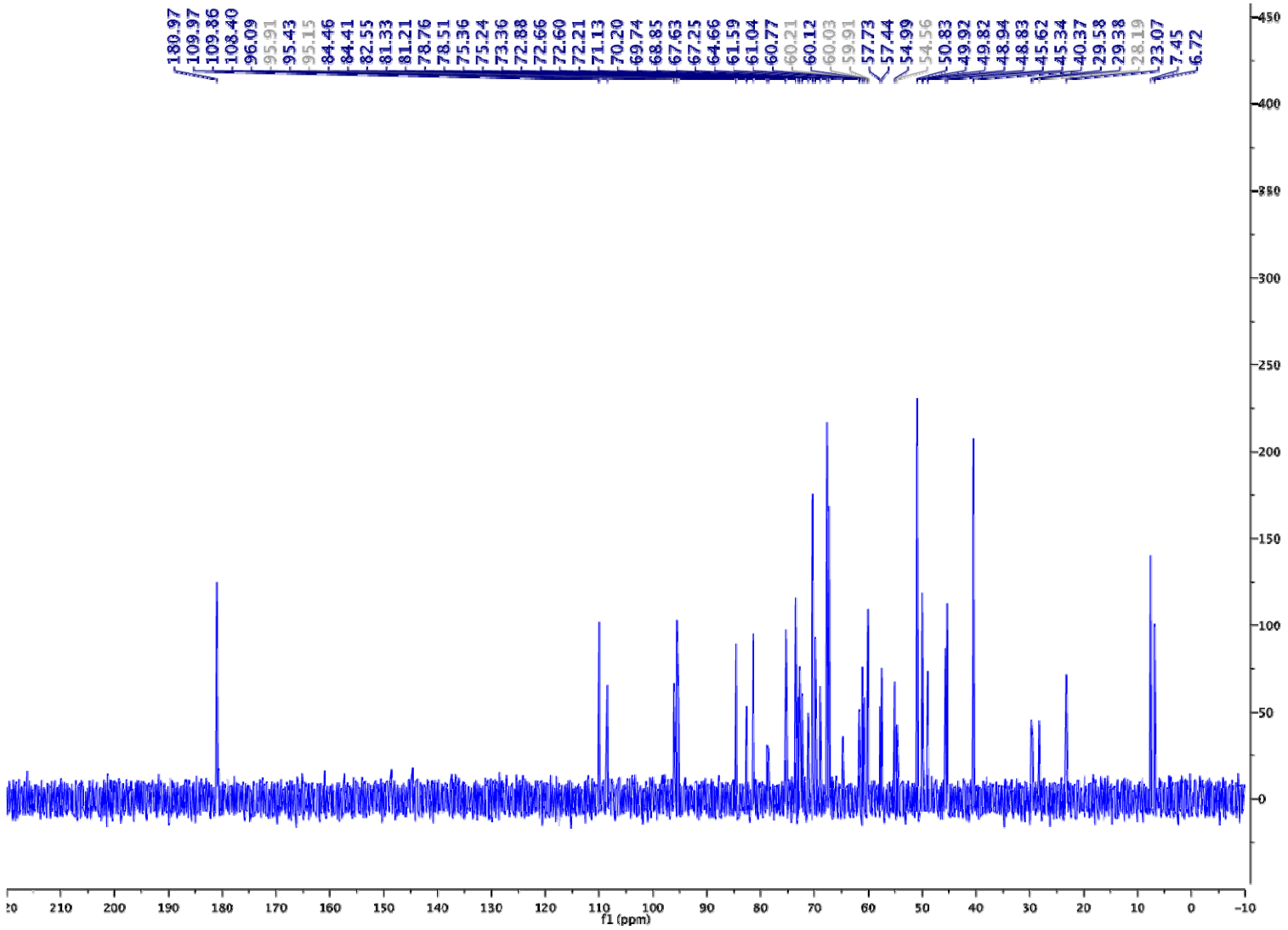


4′-Deoxy-4′-ethylsulfinyl-paromomycin (19) ^{13}C NMR (150 MHz, D_2O)



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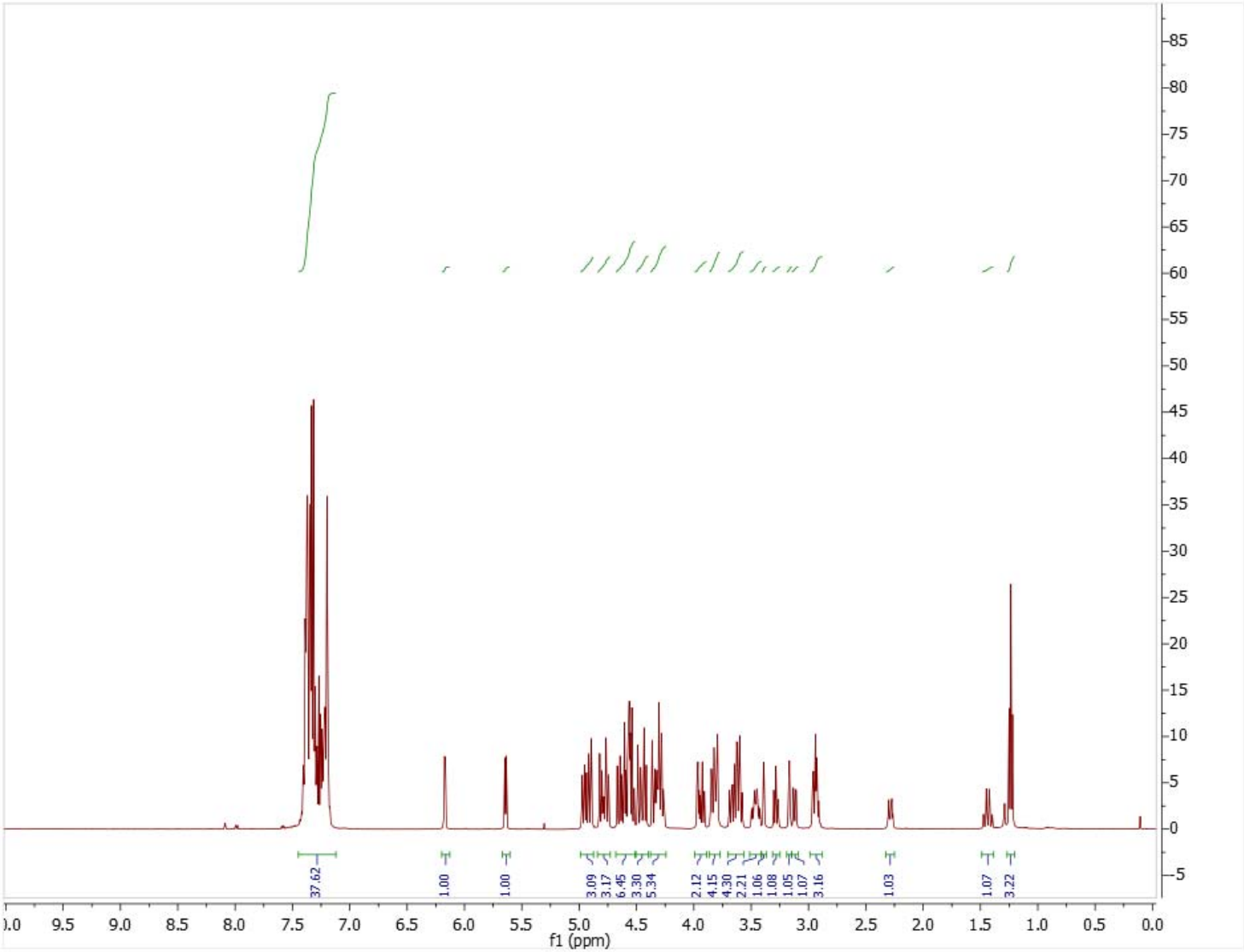

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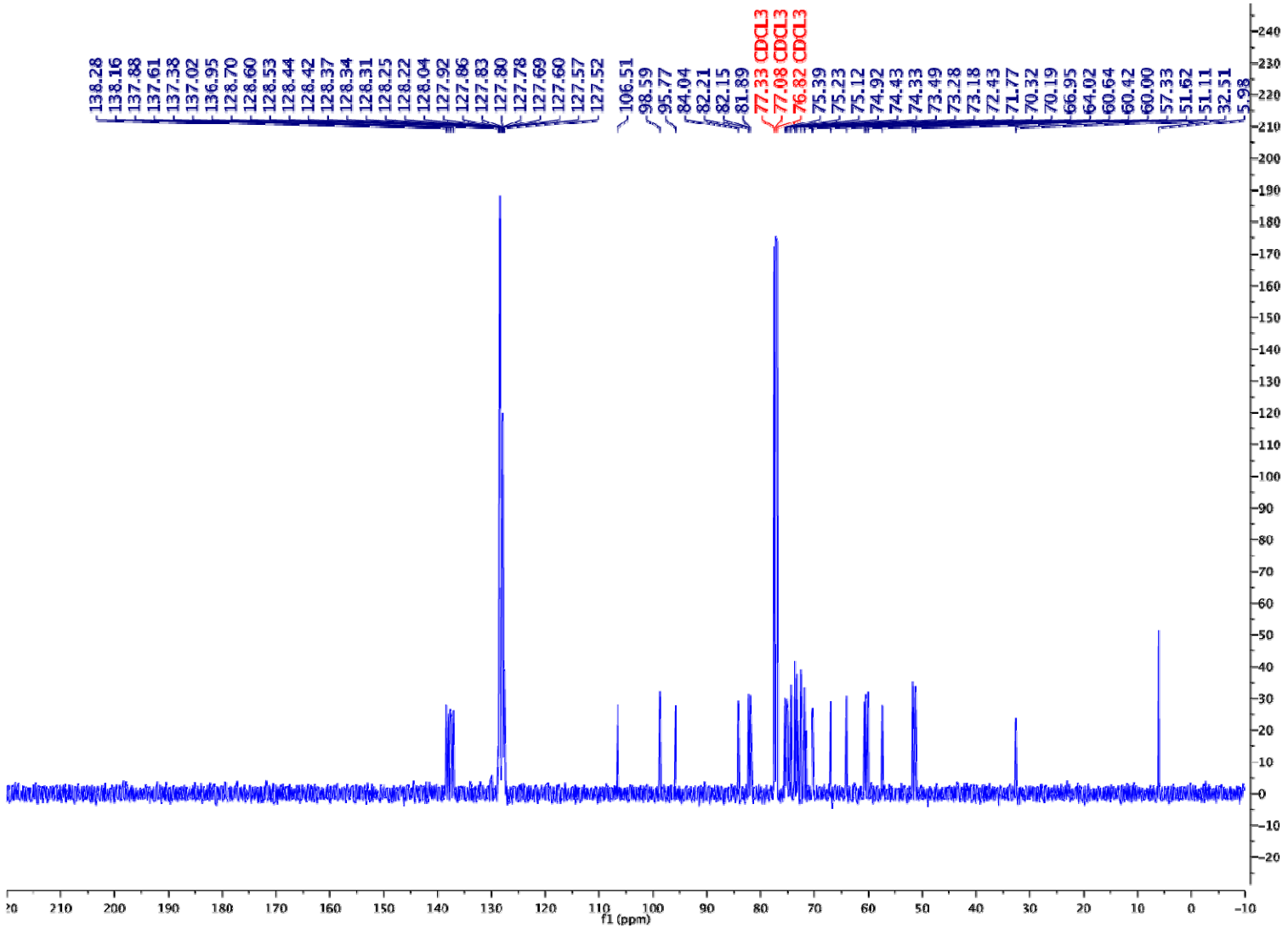

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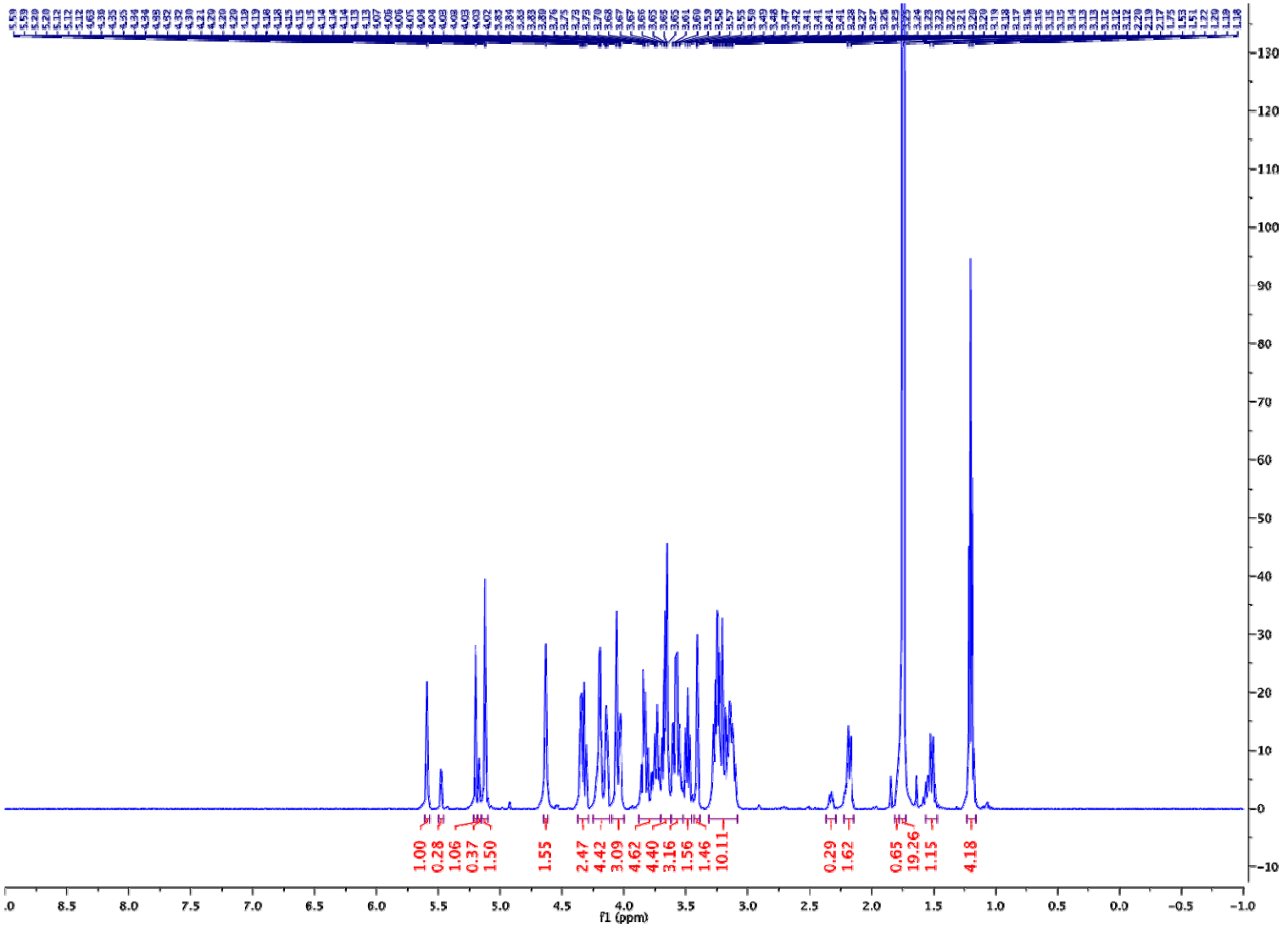

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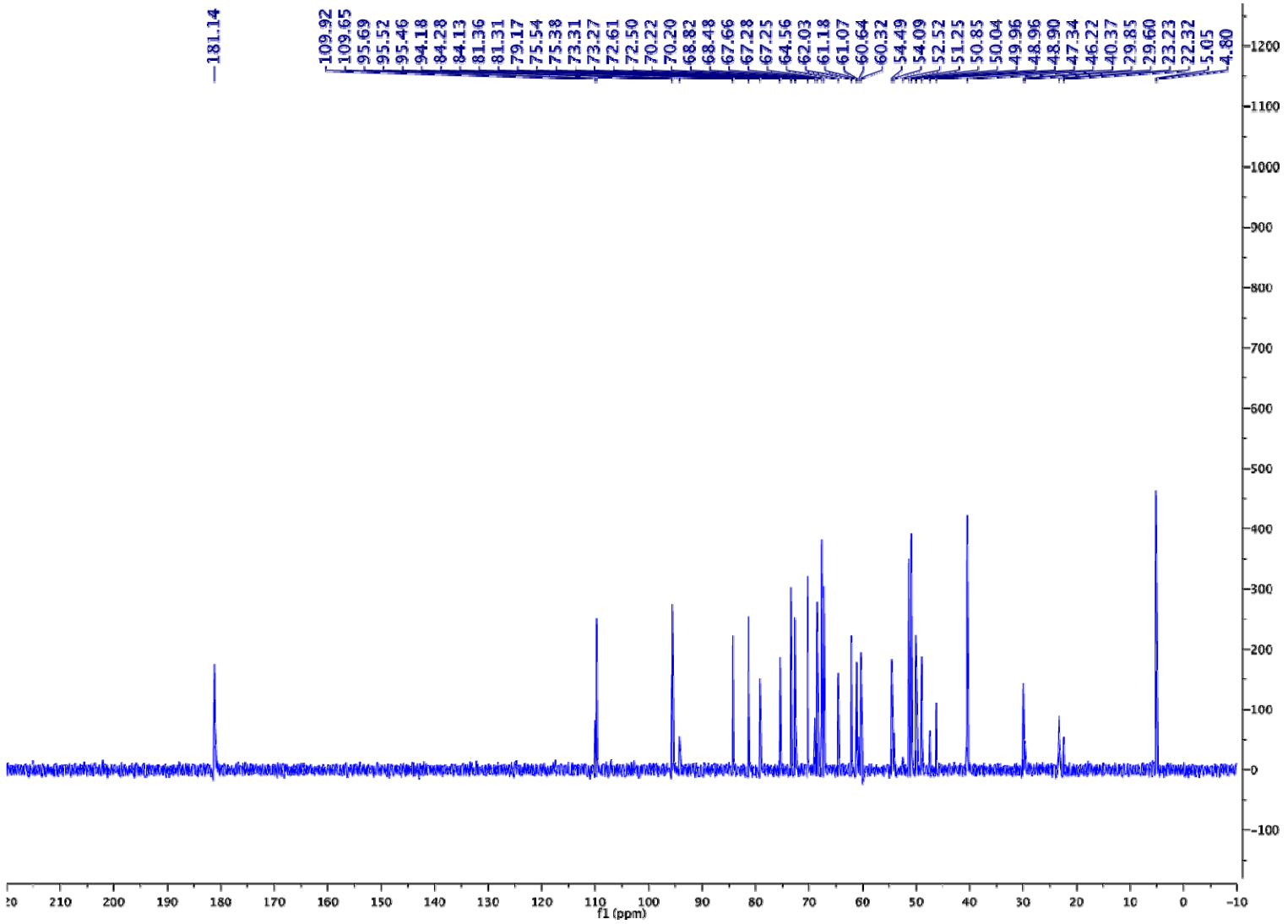
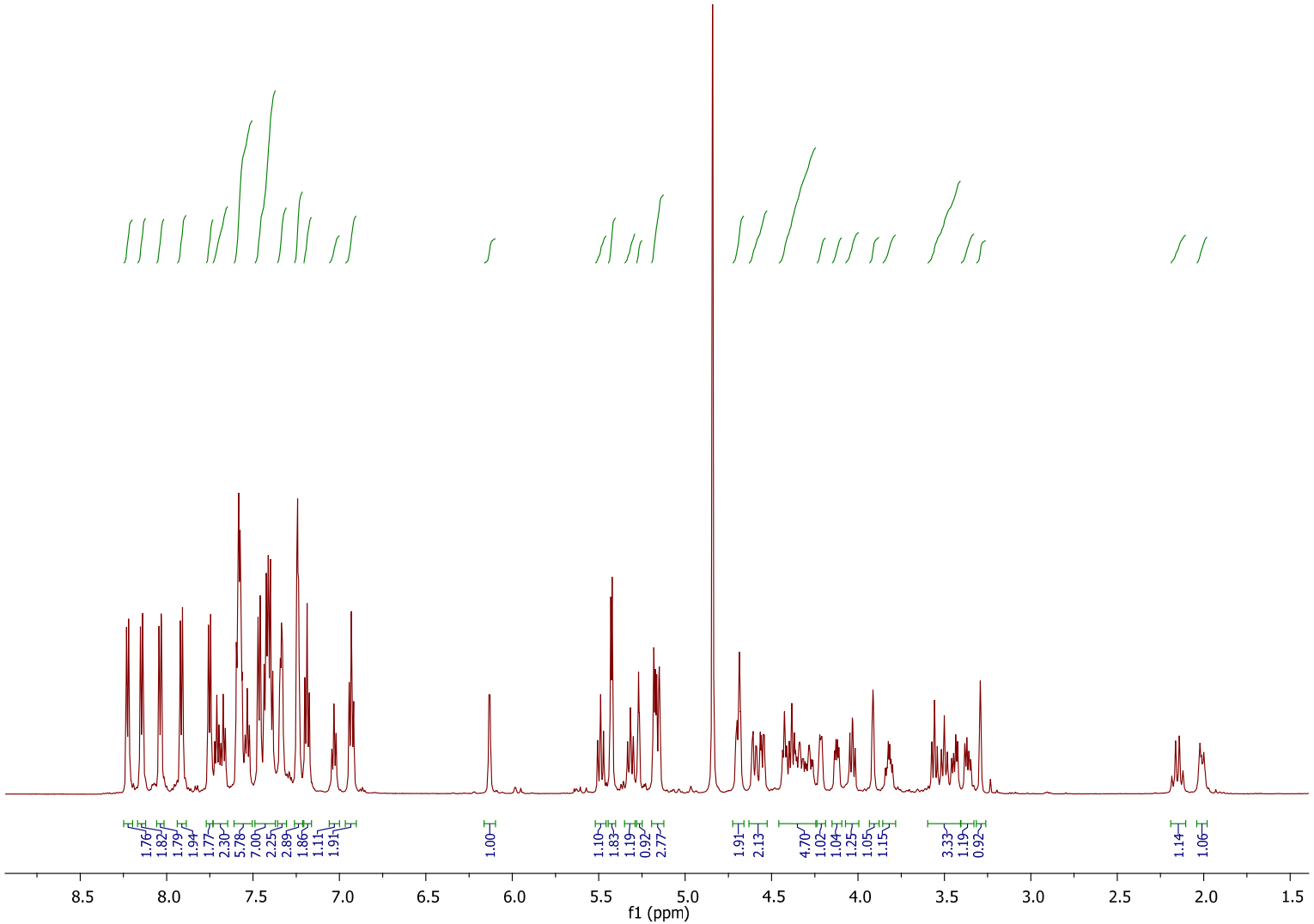
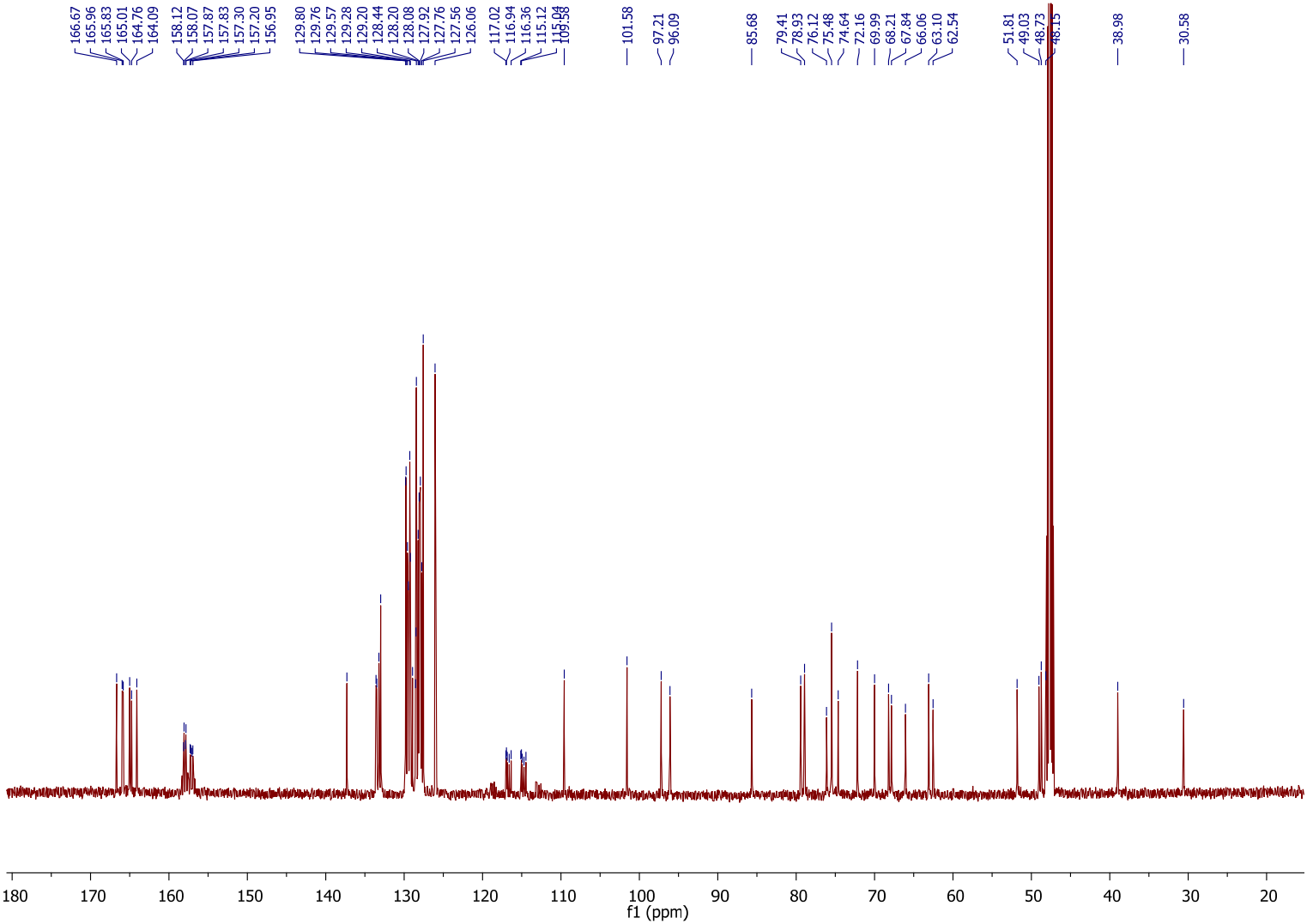


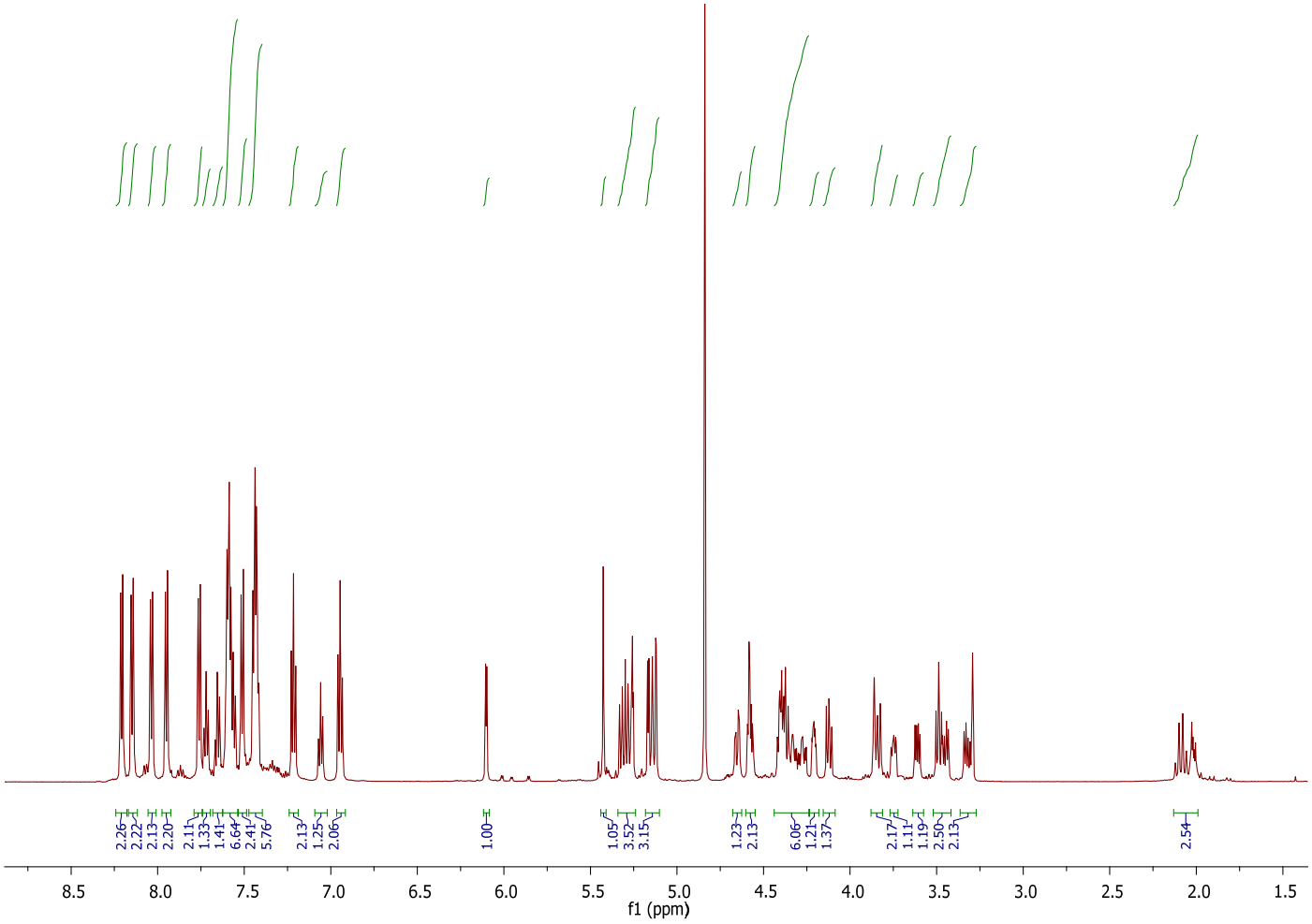
6,3′,2″,5″,3‴,4‴-Hexa-*O*-benzoyl-4′,6′-*O*-benzylidene-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl paromomycin (23) ^{1}H NMR (600 MHz, CD_3OD)



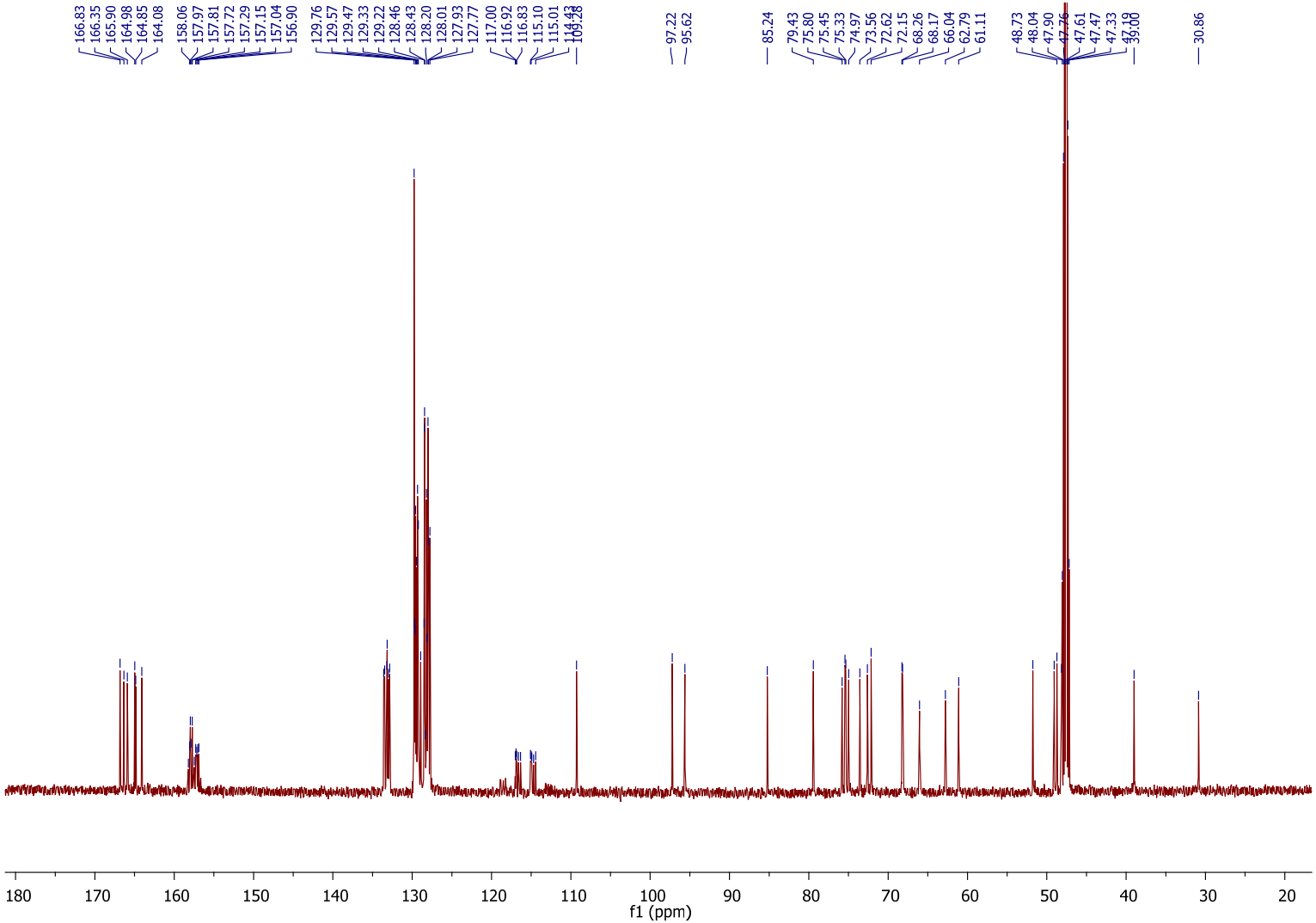
6,3′,2″,5″,3‴,4‴-Hexa-*O*-benzoyl-4′,6′-*O*-benzylidene-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl paromomycin (23) ^{13}C NMR (150 MHz, CD_3OD)



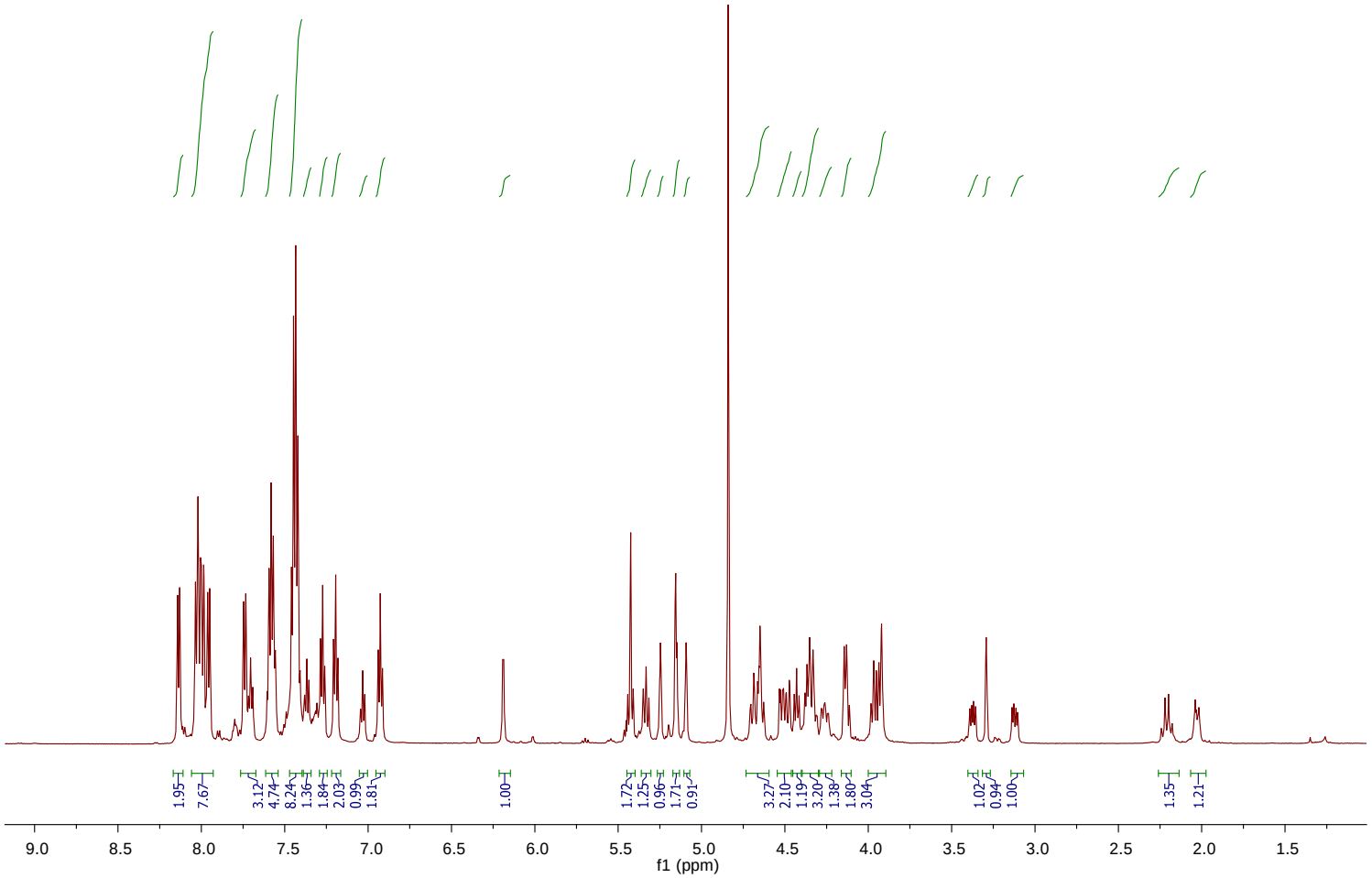
6,3′,2″,5″,3‴,4‴-Hexa-*O*-benzoyl-1,3,2‴,2‴,6‴-penta-*N*-trifluoroacetyl paromomycin (24) ^{1}H NMR (600 MHz, CD_3OD)



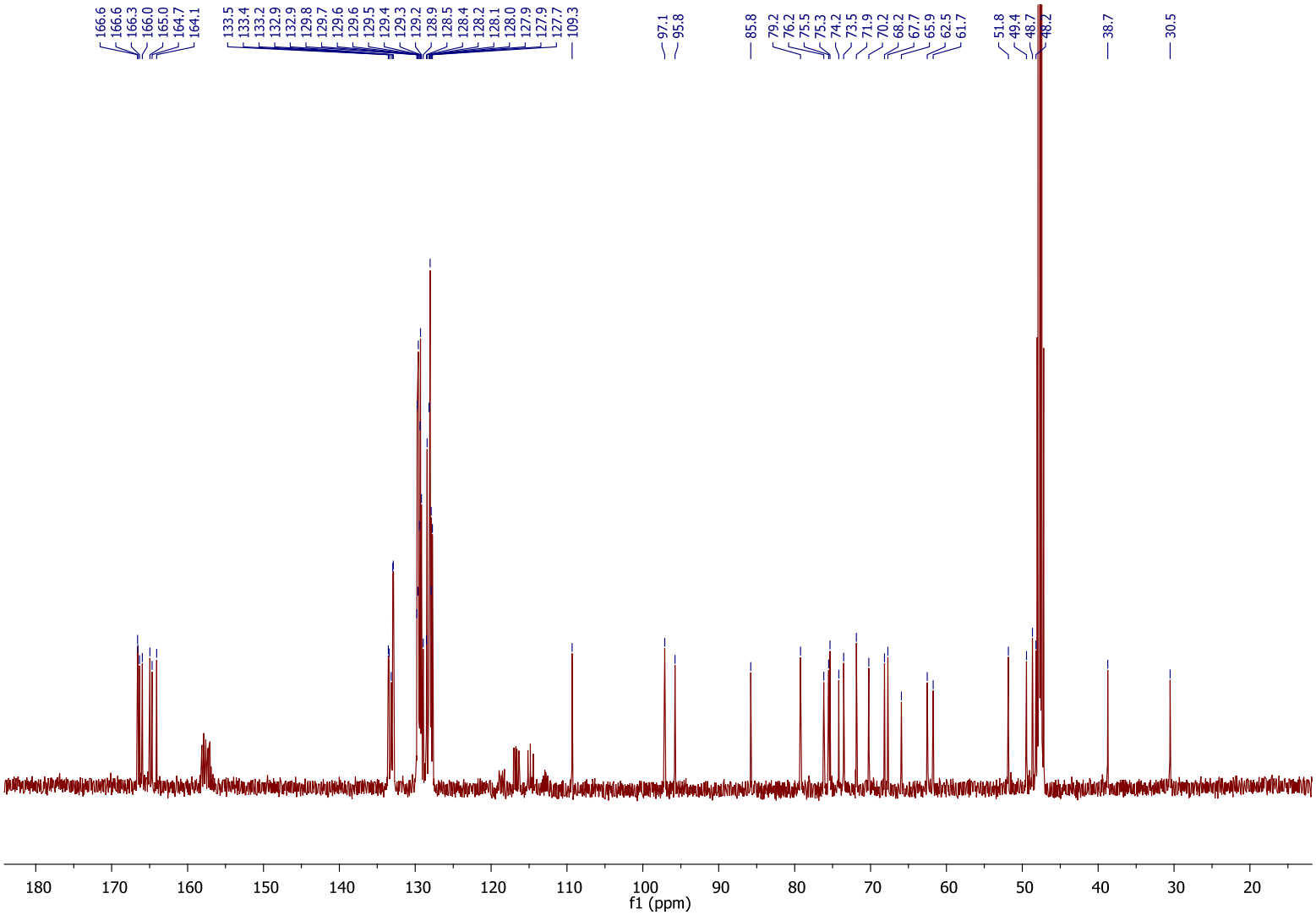
6,3′,2″,5″,3‴,4‴-Hexa-*O*-benzoyl-1,3,2‴,2‴,6‴-penta-*N*-trifluoroacetyl paromomycin (24) ^{13}C NMR (150 MHz, CD_3OD)



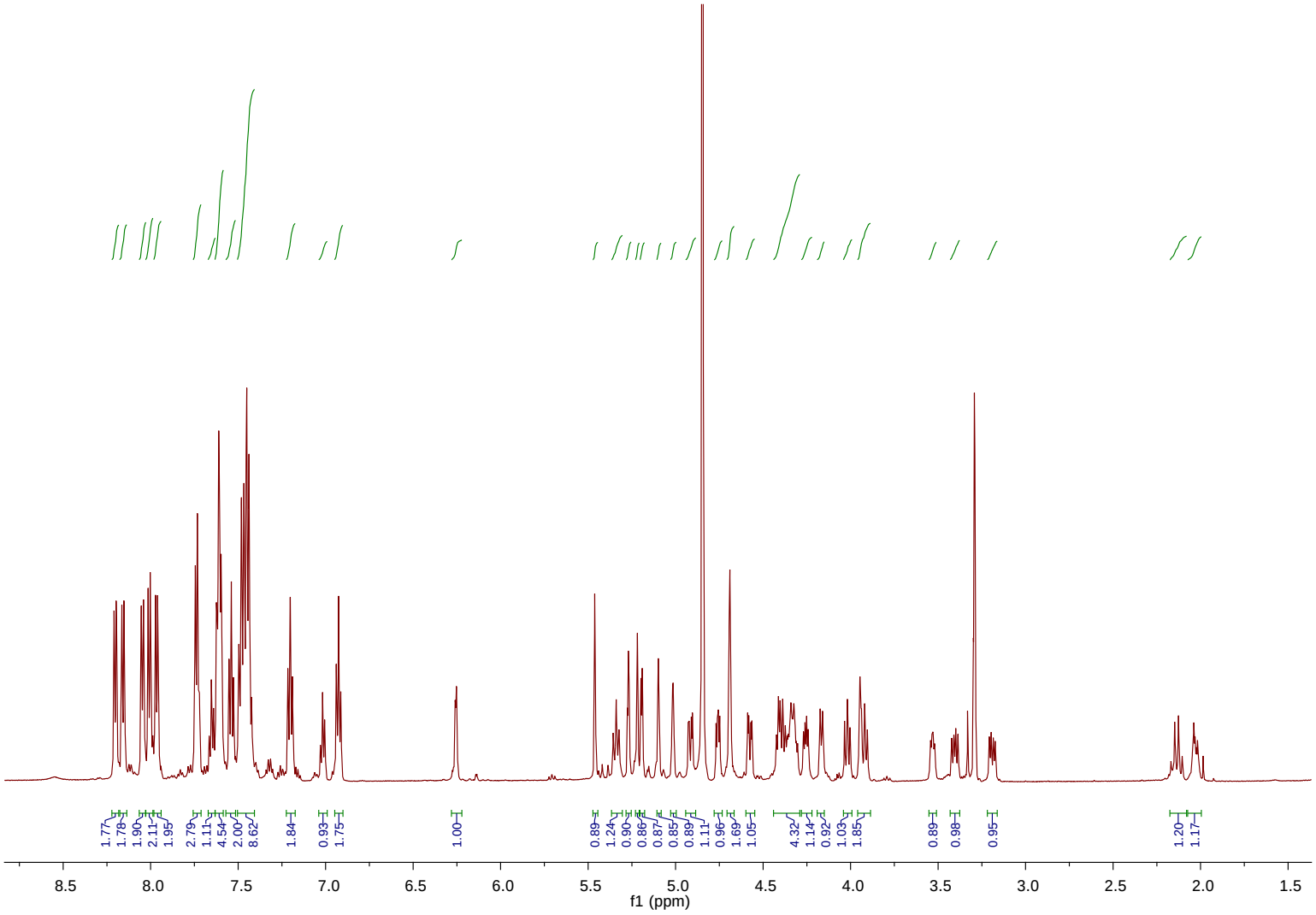
6,3′,6′,2″,5″,3‴,4‴-Hepta-*O*-benzoyl-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl paromomycin (25) ^{1}H NMR (600 MHz, CD_3OD)



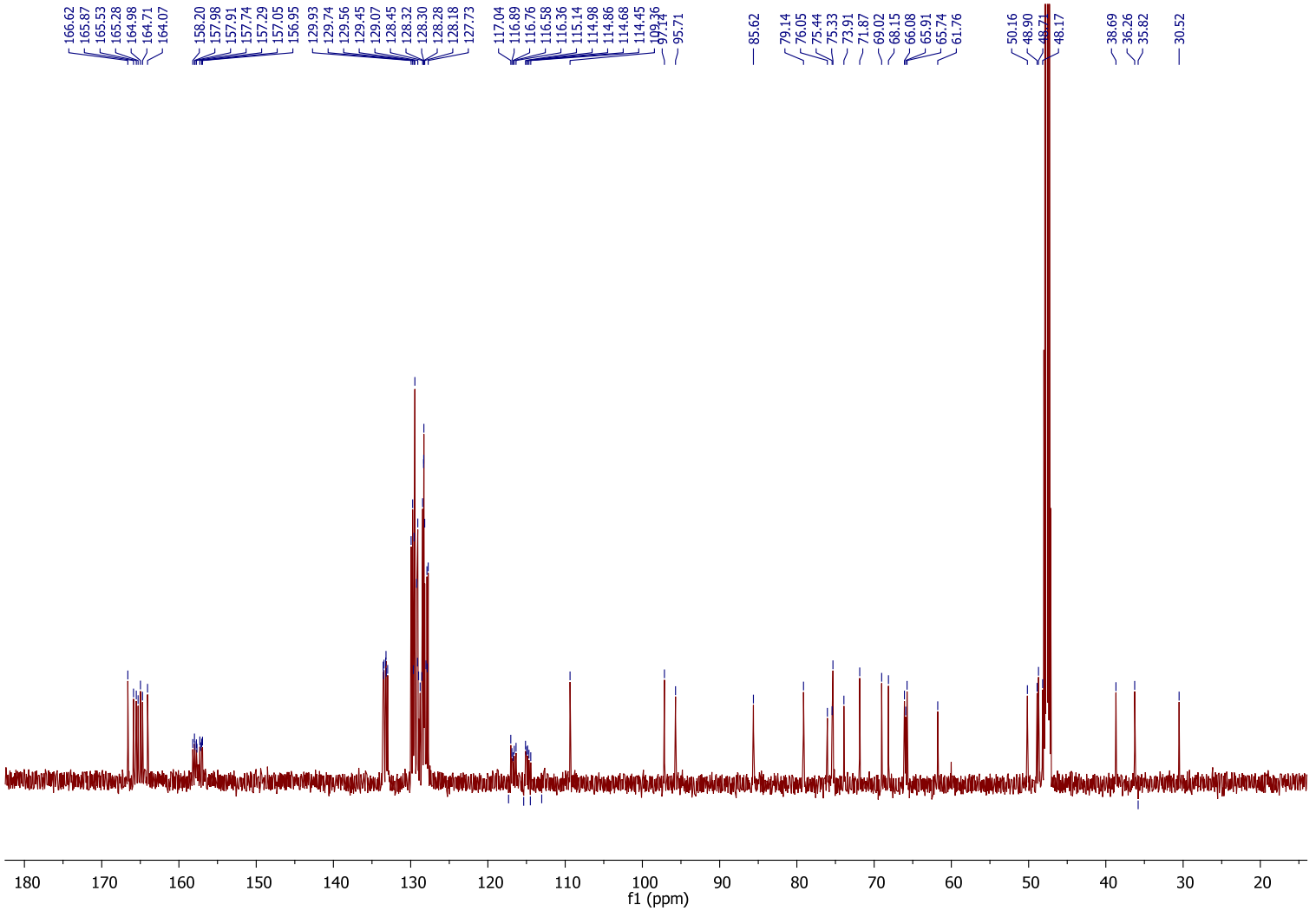
6,3′,6′,2″,5″,3‴,4‴-Hepta-*O*-benzoyl-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl paromomycin (25) ^{13}C NMR (150 MHz, CD_3OD)



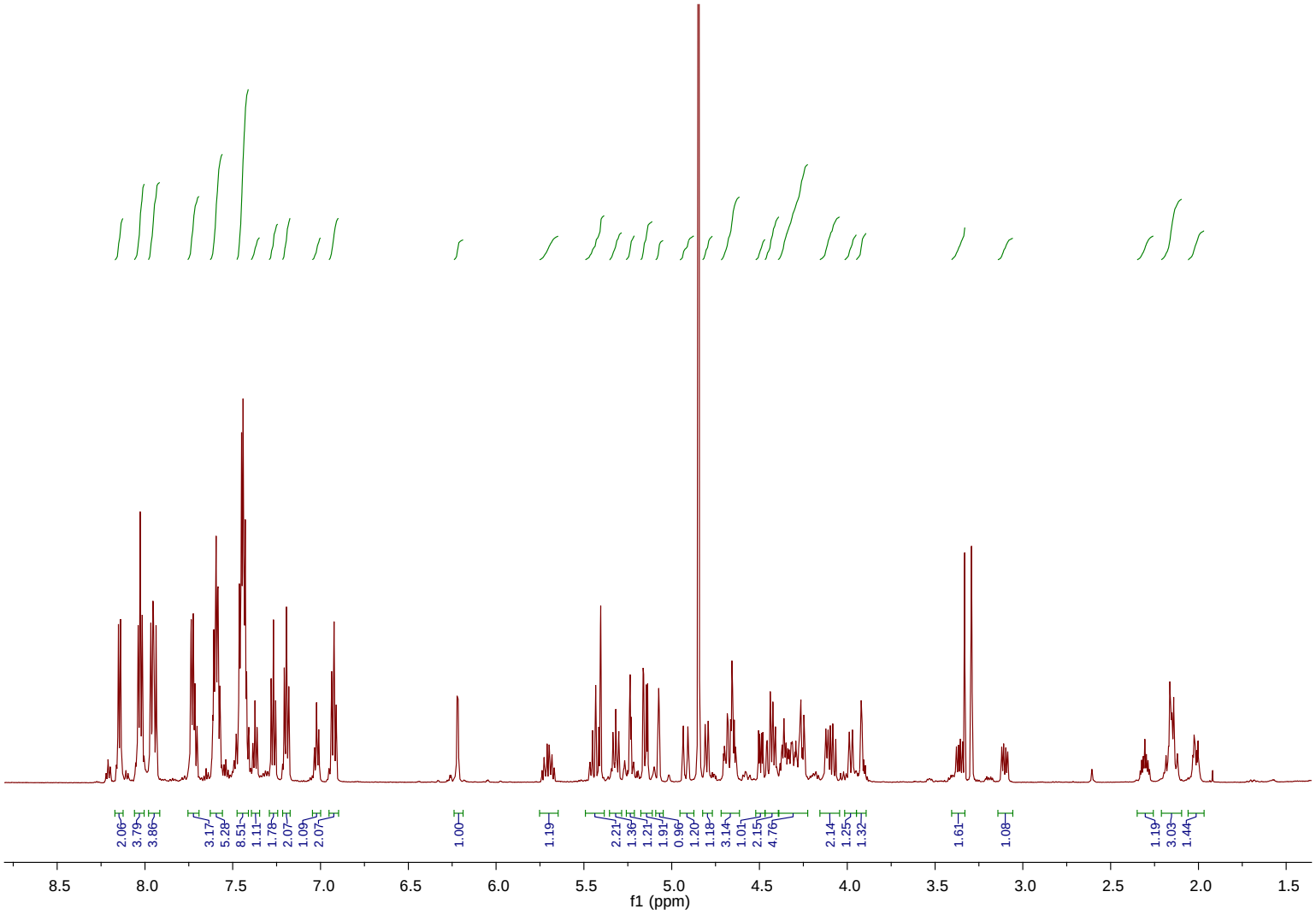
6,3′,6′,2″,5″,3‴,4‴-Hepta-*O*-benzoyl-4′-deoxy-4′-iodo-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl-4′-epi-paromomycin (26) ^{1}H NMR (600 MHz, CD_3OD)



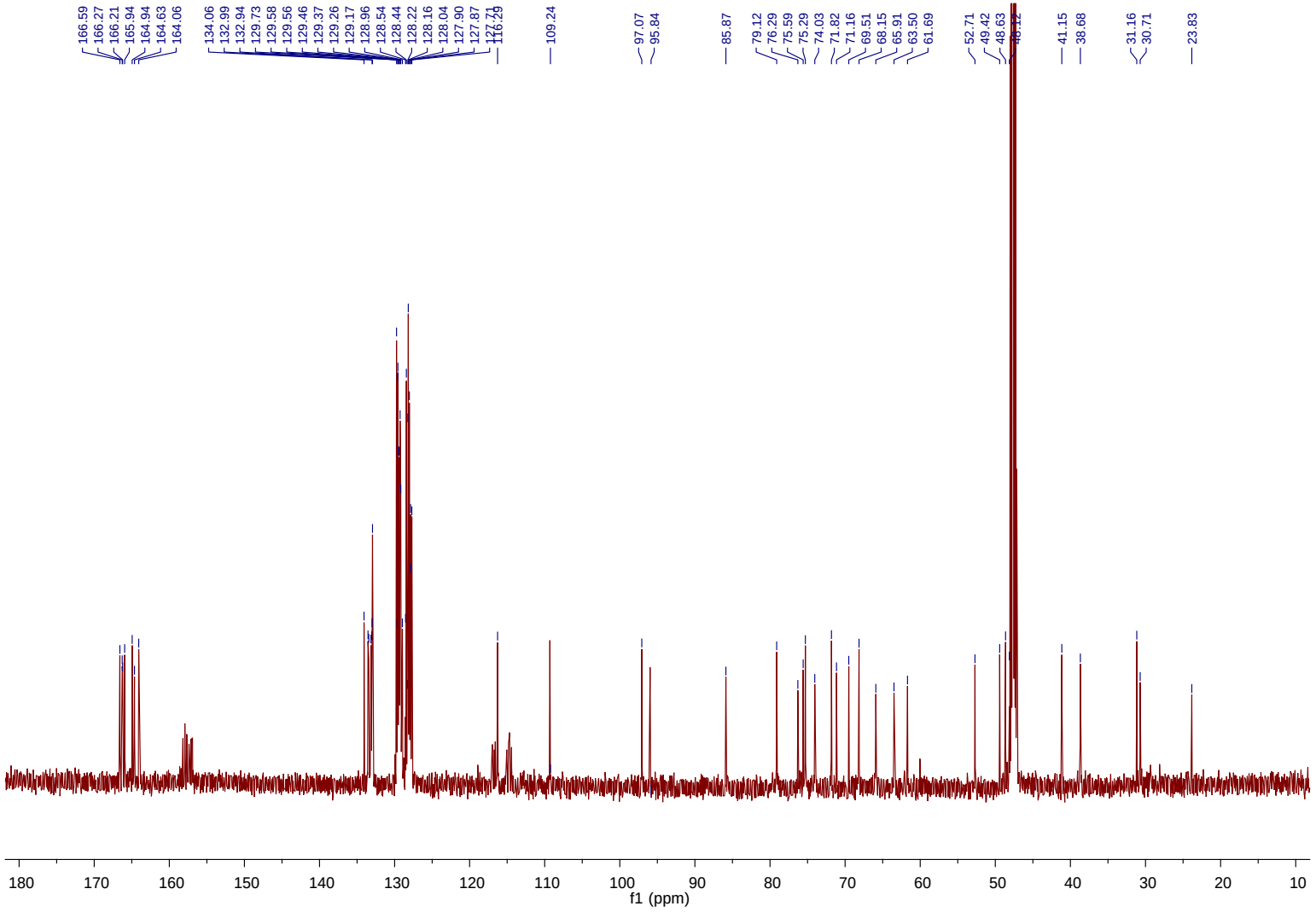
6,3′,6′,2″,5″,3‴,4‴-Hepta-*O*-benzoyl-4′-deoxy-4′-iodo-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl-4′-epi-paromomycin (26) ^{13}C NMR (150 MHz, CD_3OD)



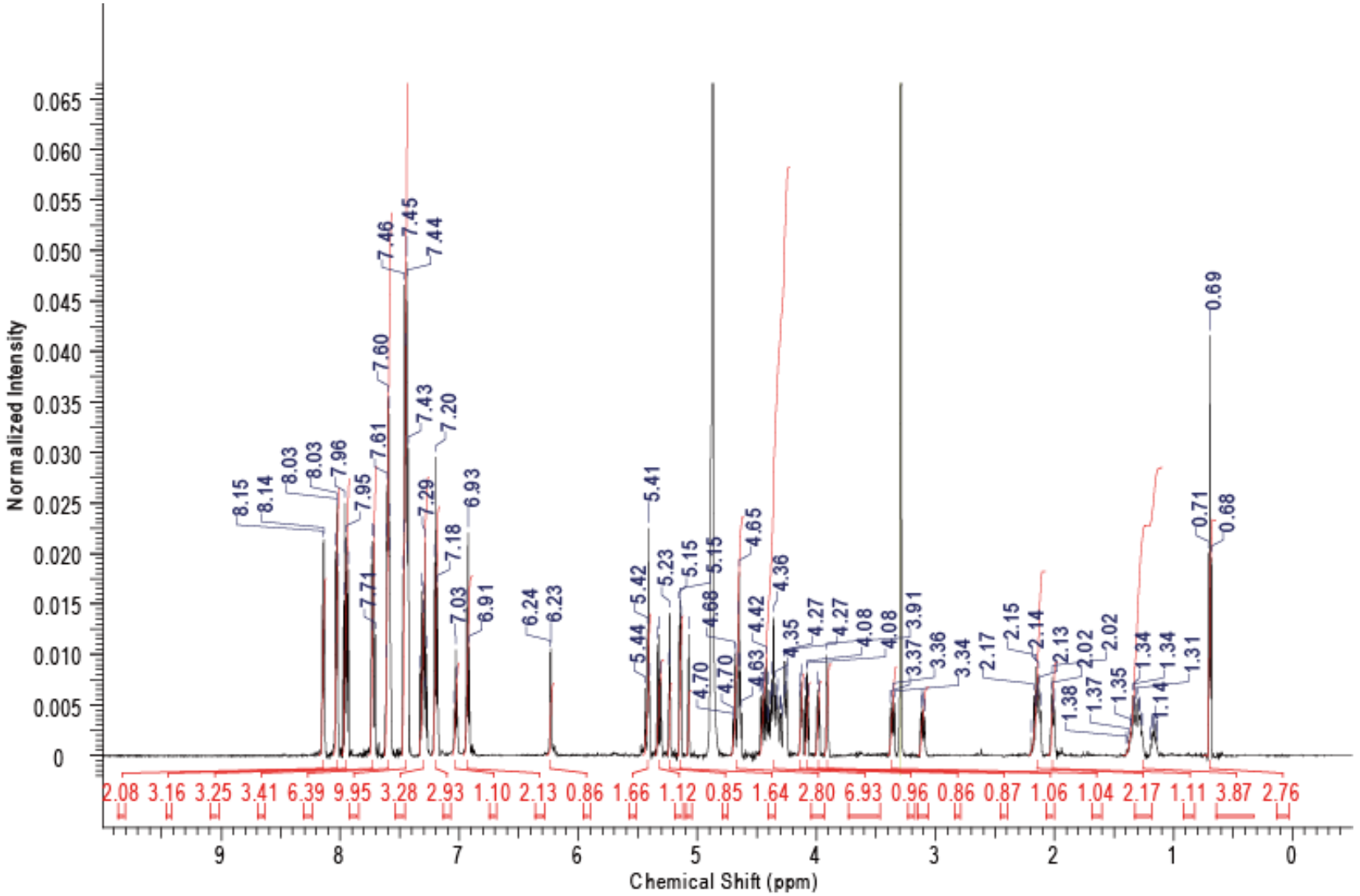
4′-Allyl-4′-deoxy-6,3′,6′,2″,5″,3‴,4‴-hepta-*O*-benzoyl-1,3,2″,2‴,6‴-penta-*N*-trifluoroacetyl paromomycin (27) ^{1}H NMR (600 MHz, CD_3OD)



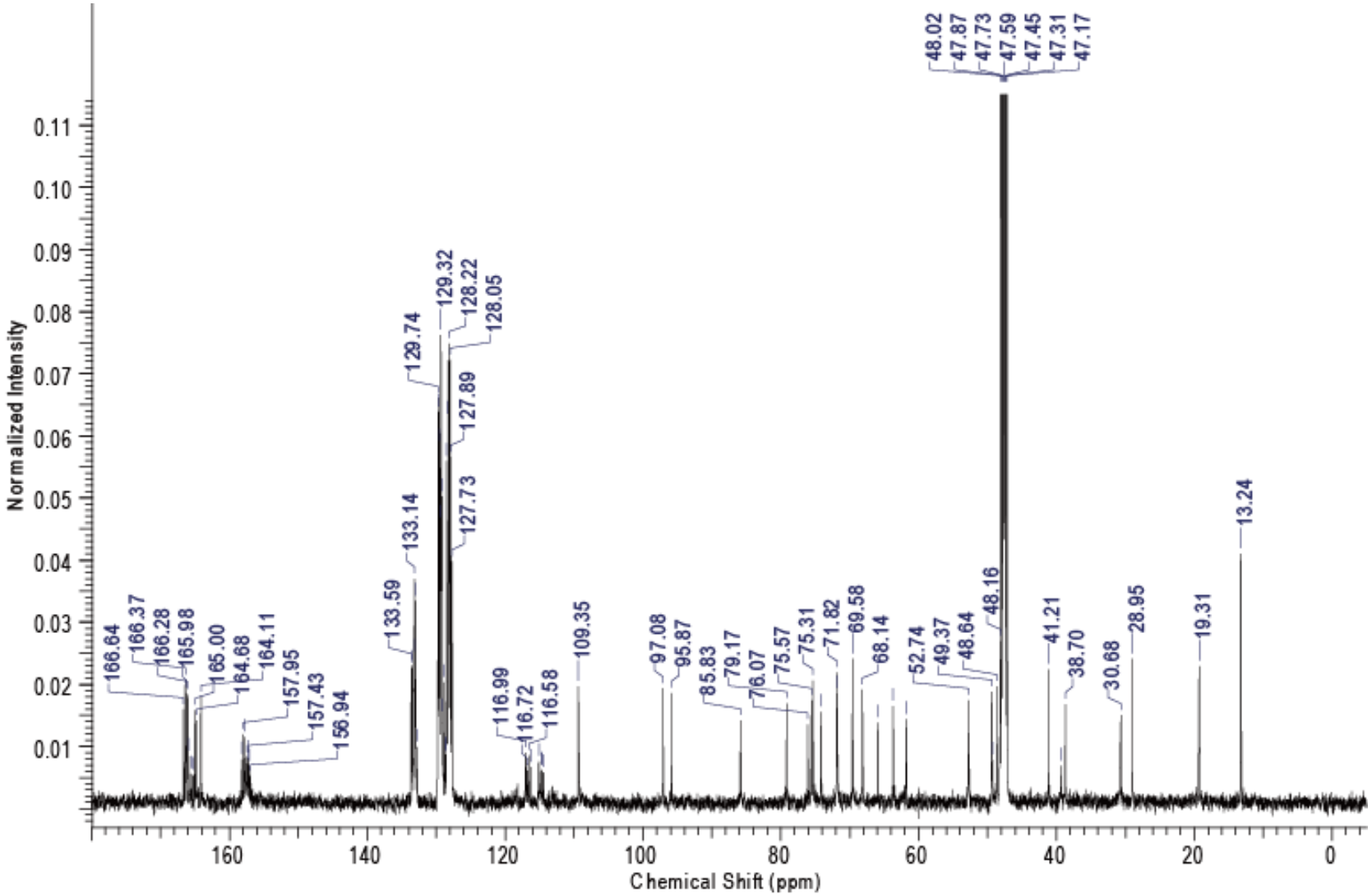
4′-Allyl-4′-deoxy-6,3′,6′,2″,5″,3‴,4‴-hepta-*O*-benzoyl-1,3,2″,2‴,6‴-penta-*N*-trifluoroacetyl paromomycin (27) ^{13}C NMR (150 MHz, CD_3OD)



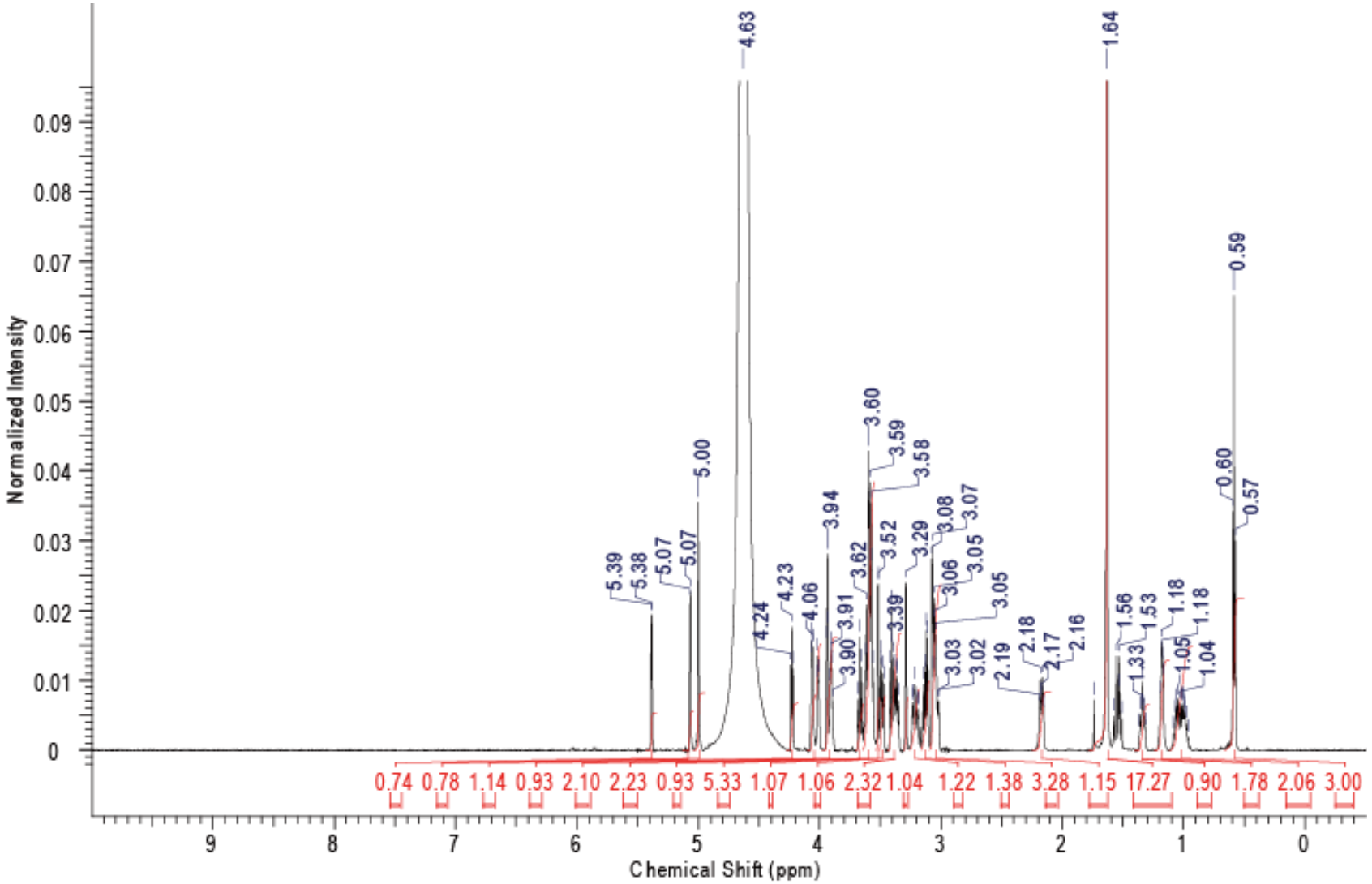
4′-Deoxy-4′-propyl-6,3′,6′,2″,5″,3‴,4‴-hepta-*O*-benzoyl-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl paromomycin (28) ^{1}H NMR (600 MHz, CD_3OD)



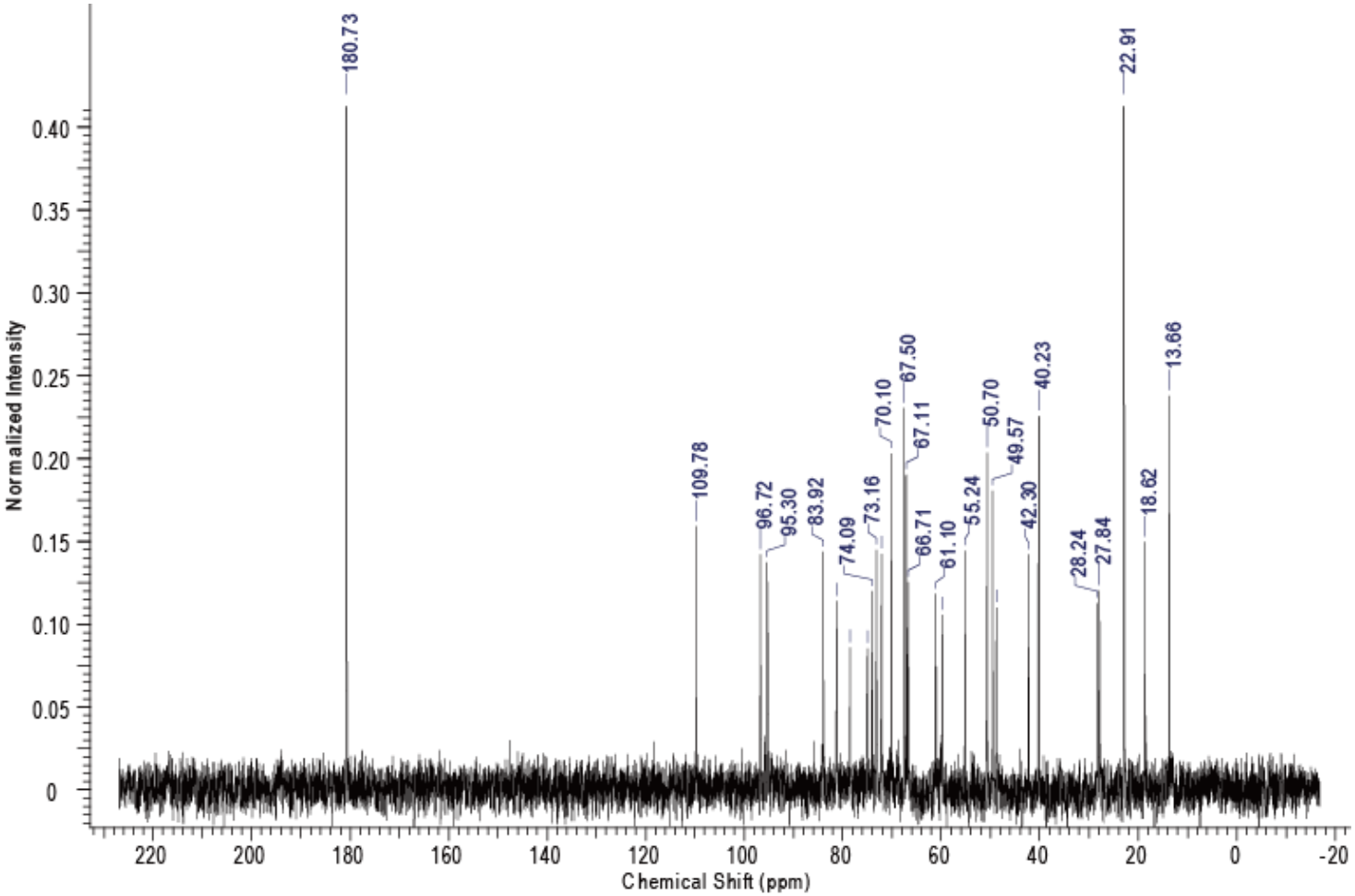
4′-Deoxy-4′-propyl-6,3′,6′,2″,5″,3‴,4‴-hepta-*O*-benzoyl-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl paromomycin (28) ^{13}C NMR (150 MHz, CD_3OD)



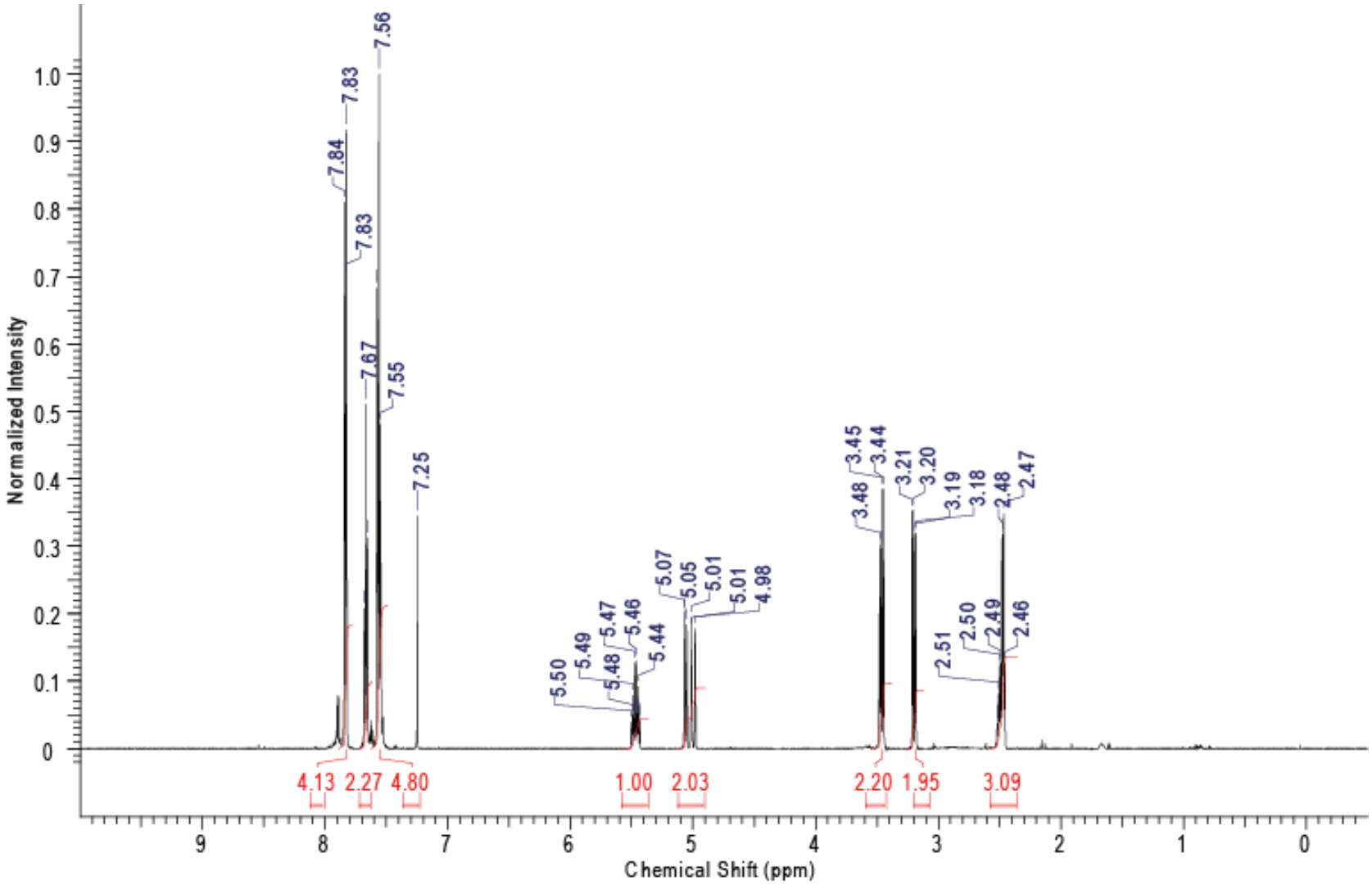
4′-Deoxy-4′-propyl paromomycin pentaacetate (5) ^{1}H NMR (600 MHz, D_2O)



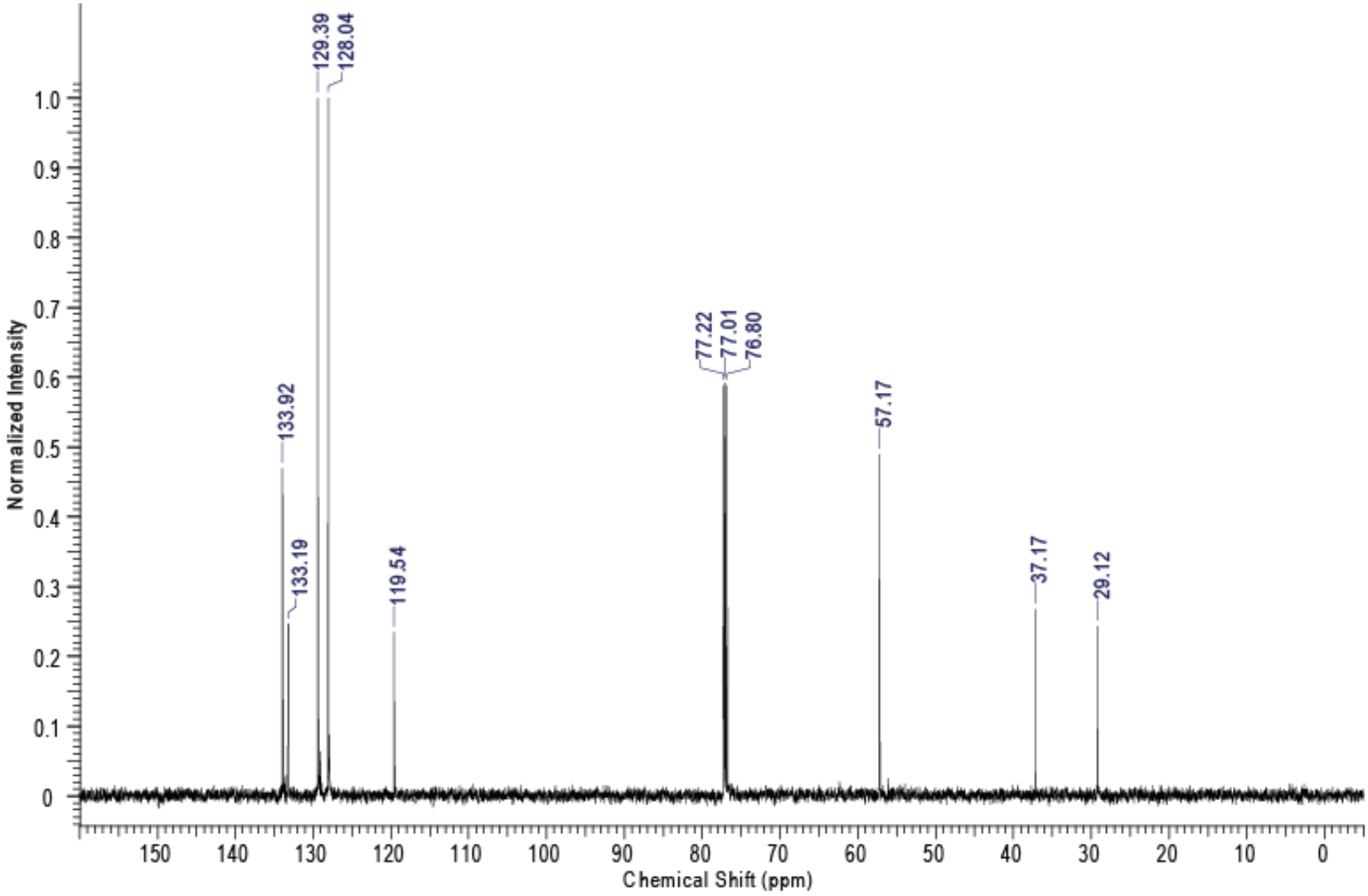
4′-Deoxy-4′-propyl paromomycin pentaacetate (5) ^{13}C NMR (150 MHz, D_2O)



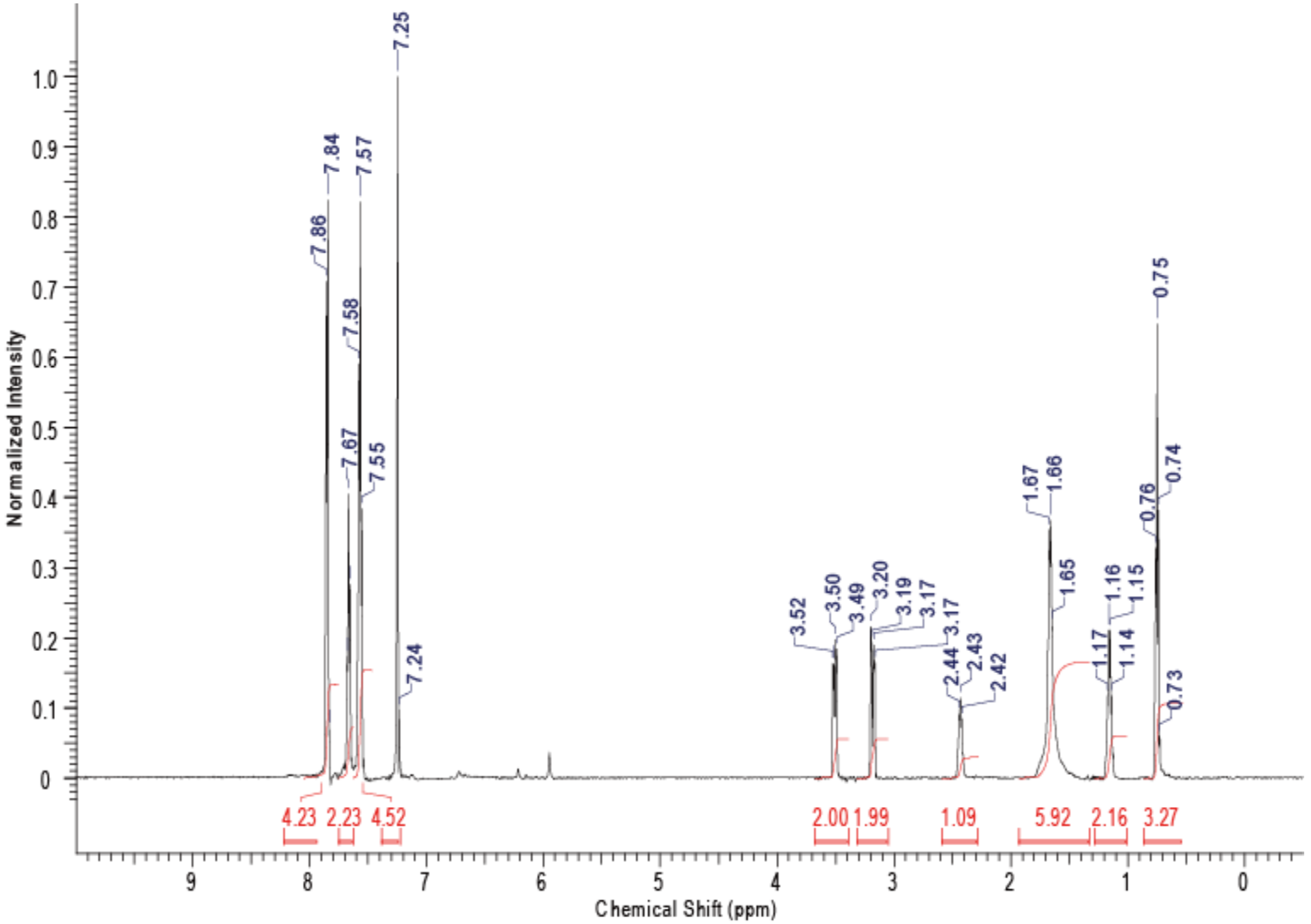
5-Benzenesulfonyl-4-(benzenesulfonylmethyl)pentene (29) ^{1}H NMR (600 MHz, $CDCl_3$)



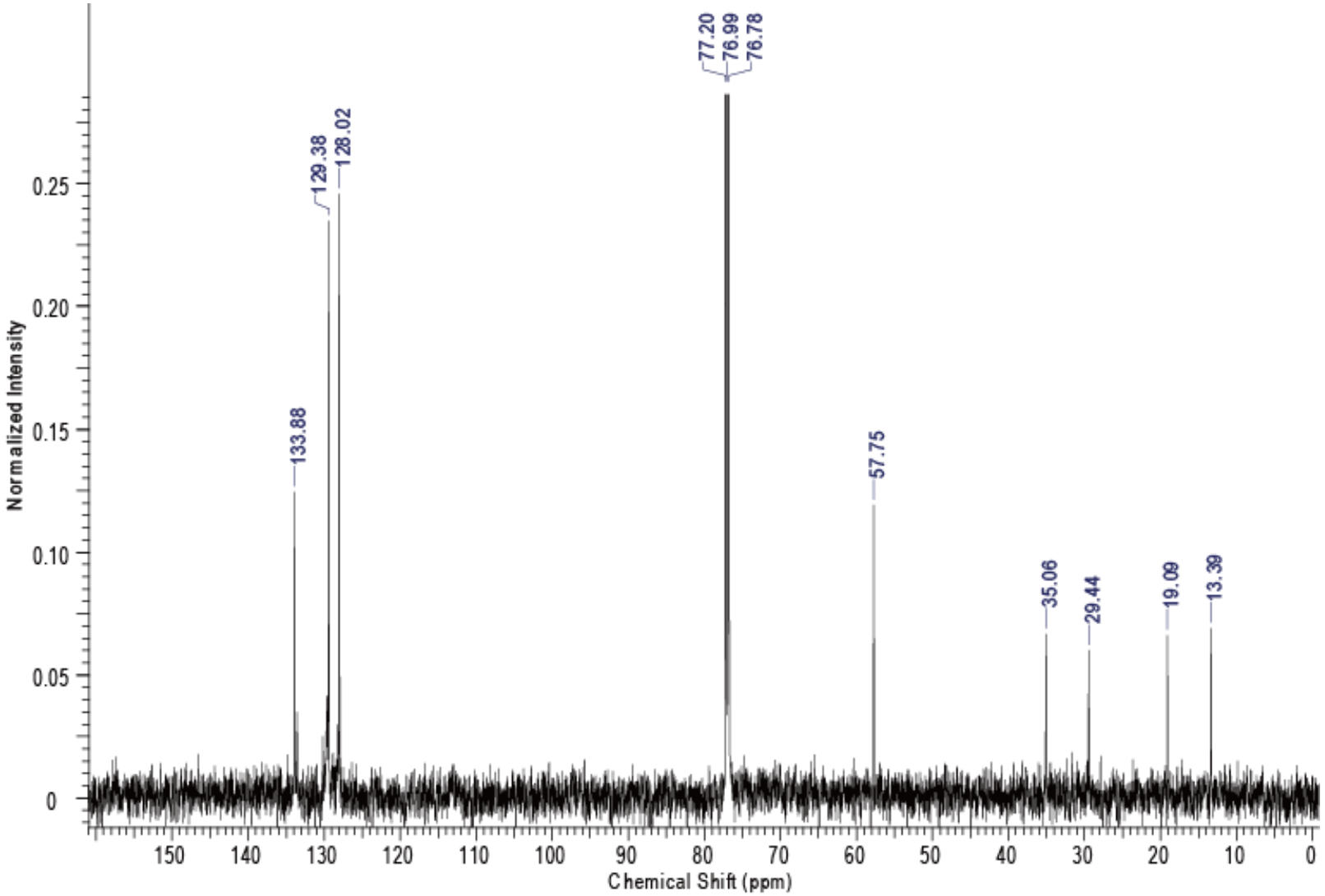
5-Benzenesulfonyl-4-(benzenesulfonylmethyl)pentene (29) ^{13}C NMR (150 MHz, $CDCl_3$)



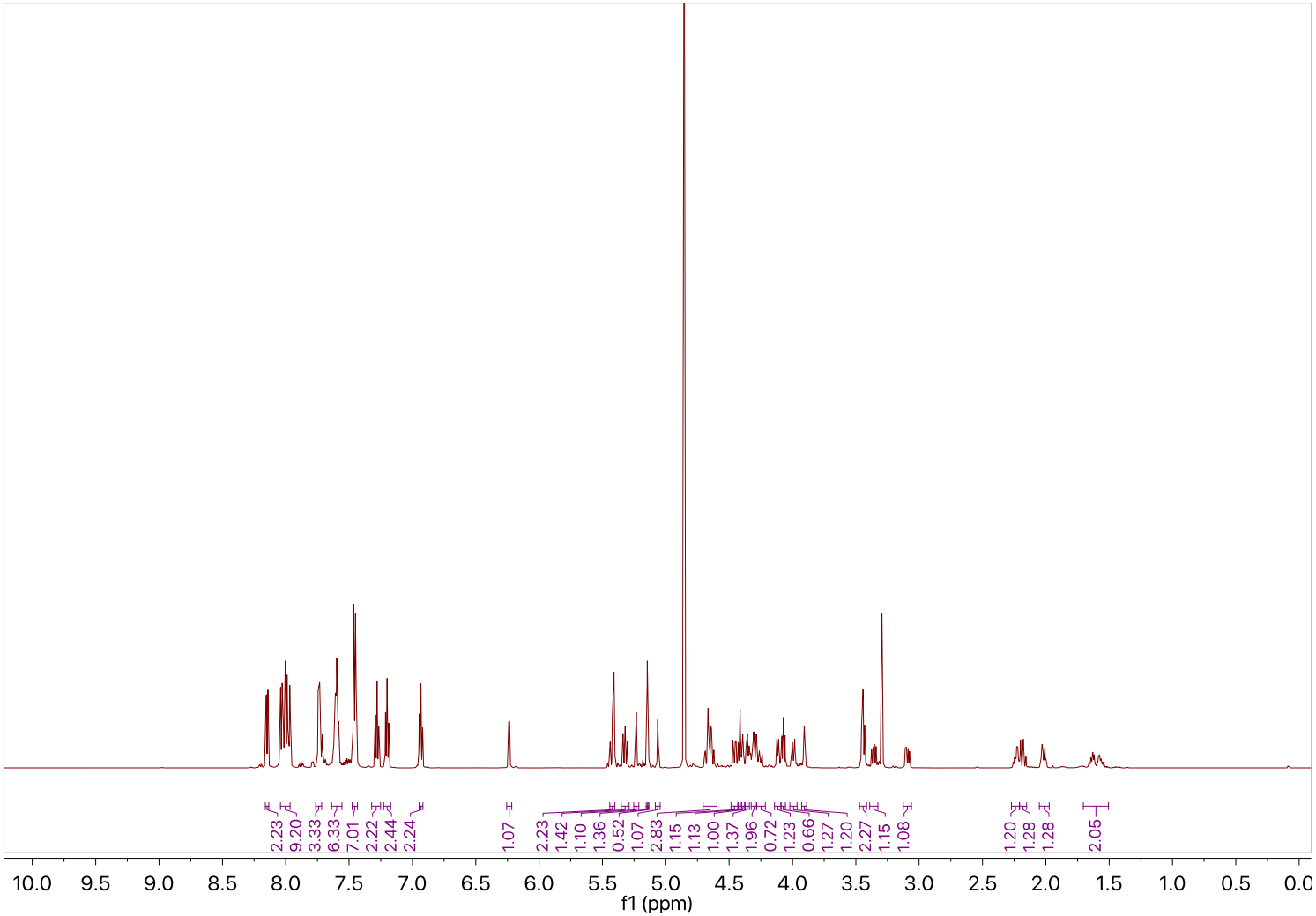
1-Benzenesulfonyl-2-(benzenesulfonylmethyl)pentane (30) ^{1}H NMR (600 MHz, $CDCl_3$)



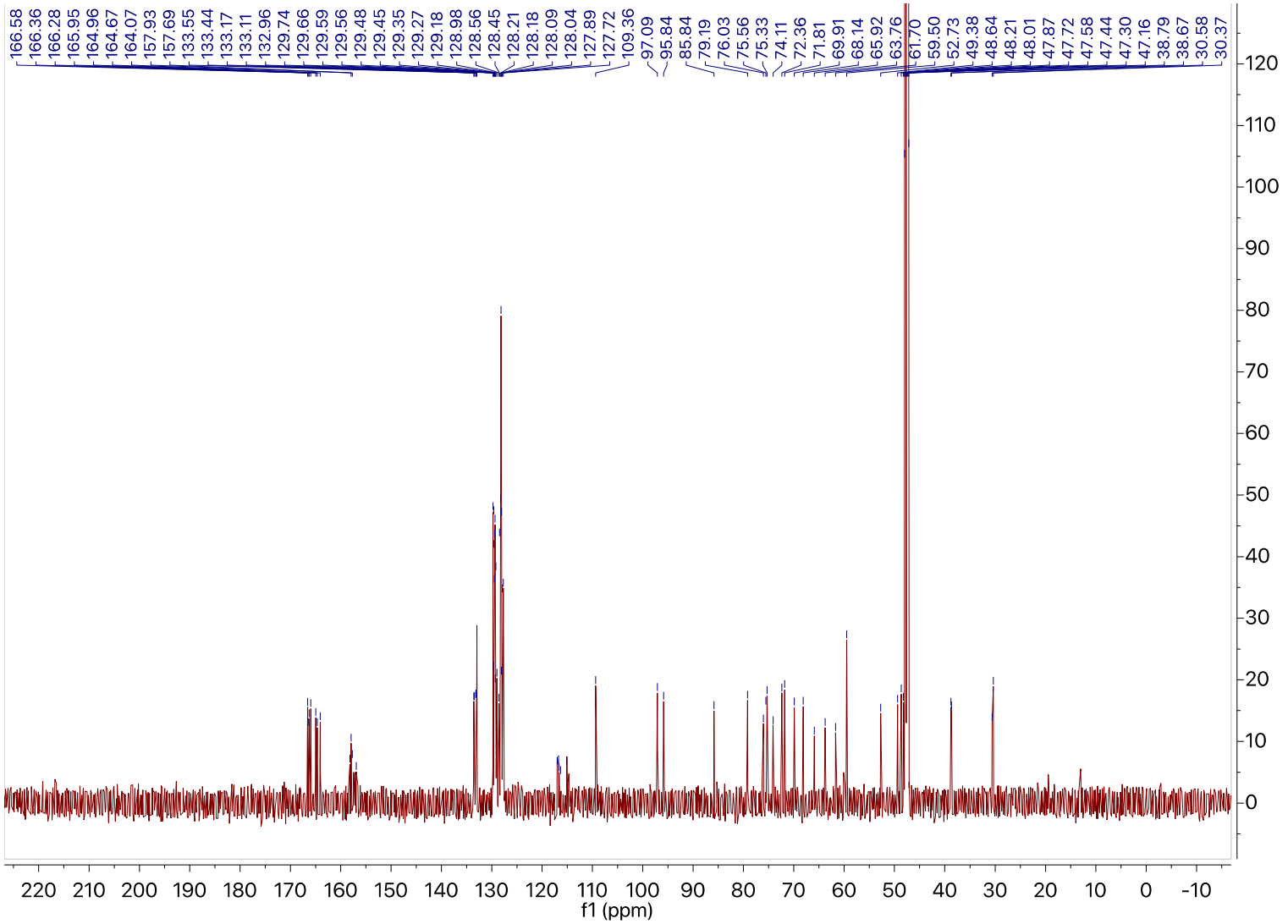
1-Benzenesulfonyl-2-(benzenesulfonylmethyl)pentane (30) ^{13}C NMR (150 MHz, $CDCl_3$)



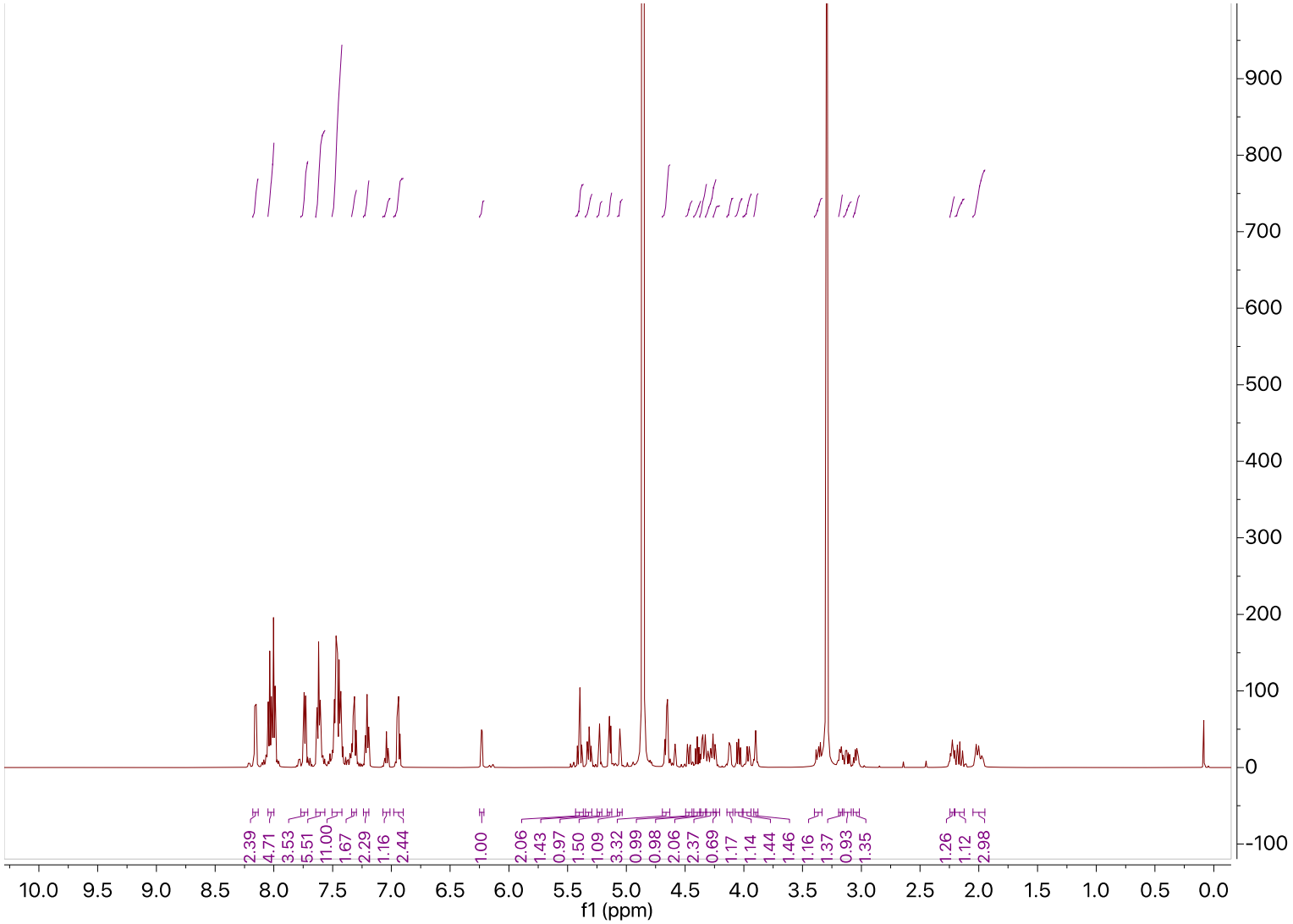
4′-Deoxy-4′-(2-hydroxyethyl)-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl-6,3′,6′,2‴,5‴,3‴″,4‴″-hepta-*O*-benzoyl paromomycin (31) ^{1}H NMR (600 MHz, CD_3OD)



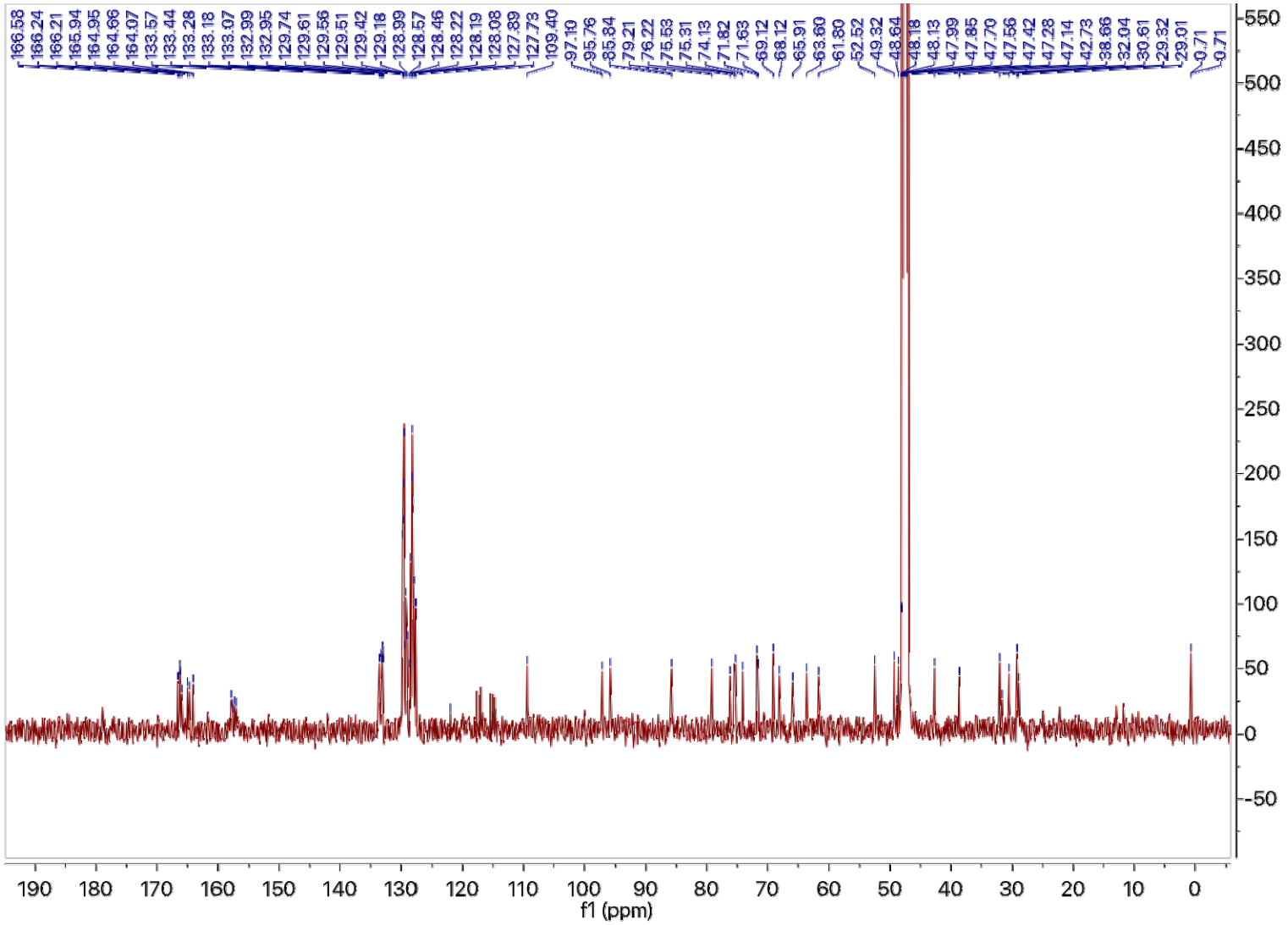
4′-Deoxy-4′-(2-hydroxyethyl)-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl-6,3′,6′,2‴,5‴,3‴″,4‴″-hepta-*O*-benzoyl paromomycin (31) ^{13}C NMR (150 MHz, CD_3OD)



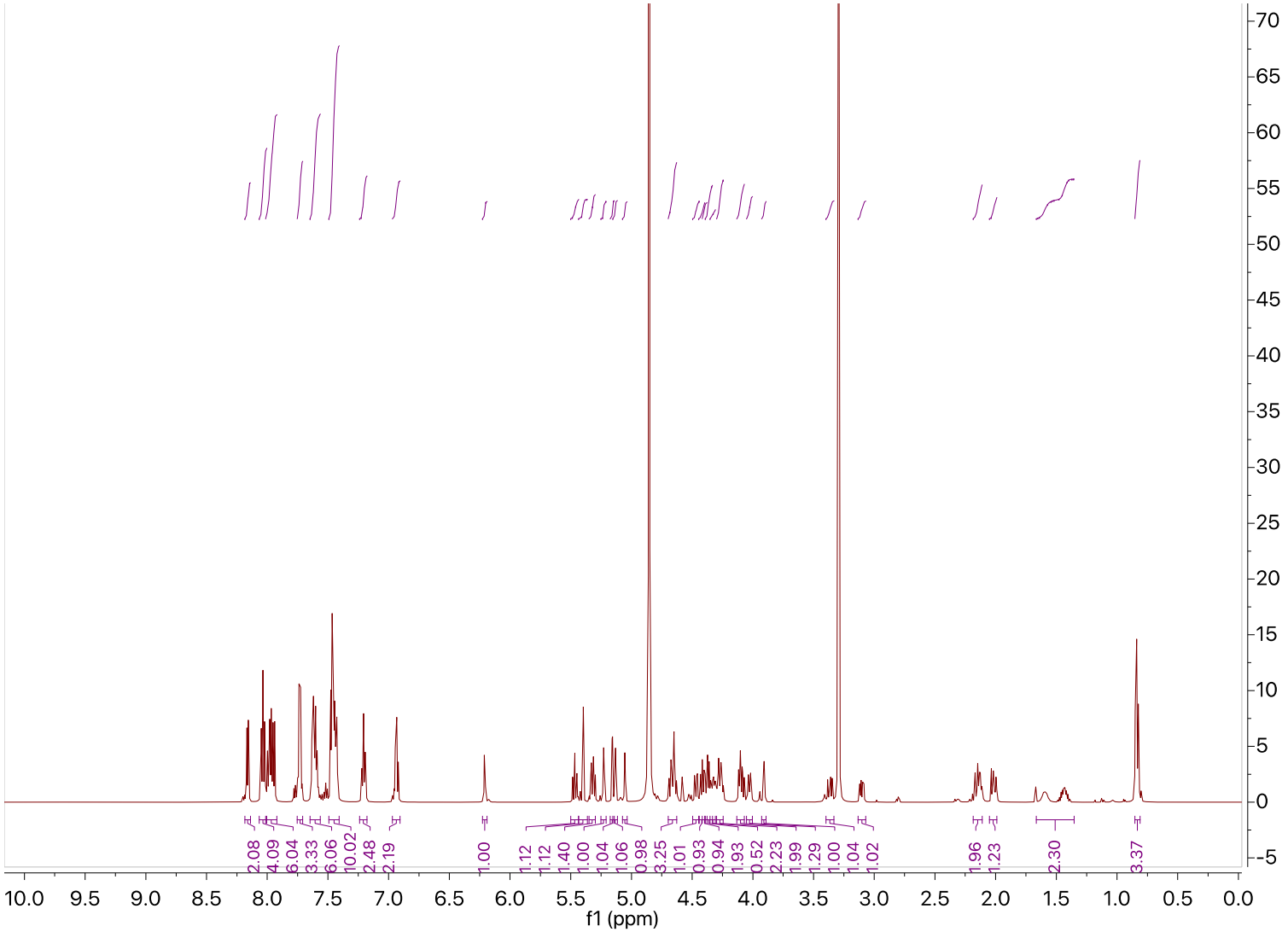
4′-Deoxy-4′-(2-iodoethyl)-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl-6,3′,6′,2‴,5‴,3‴″,4‴″-hepta-*O*-benzoyl paromomycin (32) ^{1}H NMR (600 MHz, CD_3OD)



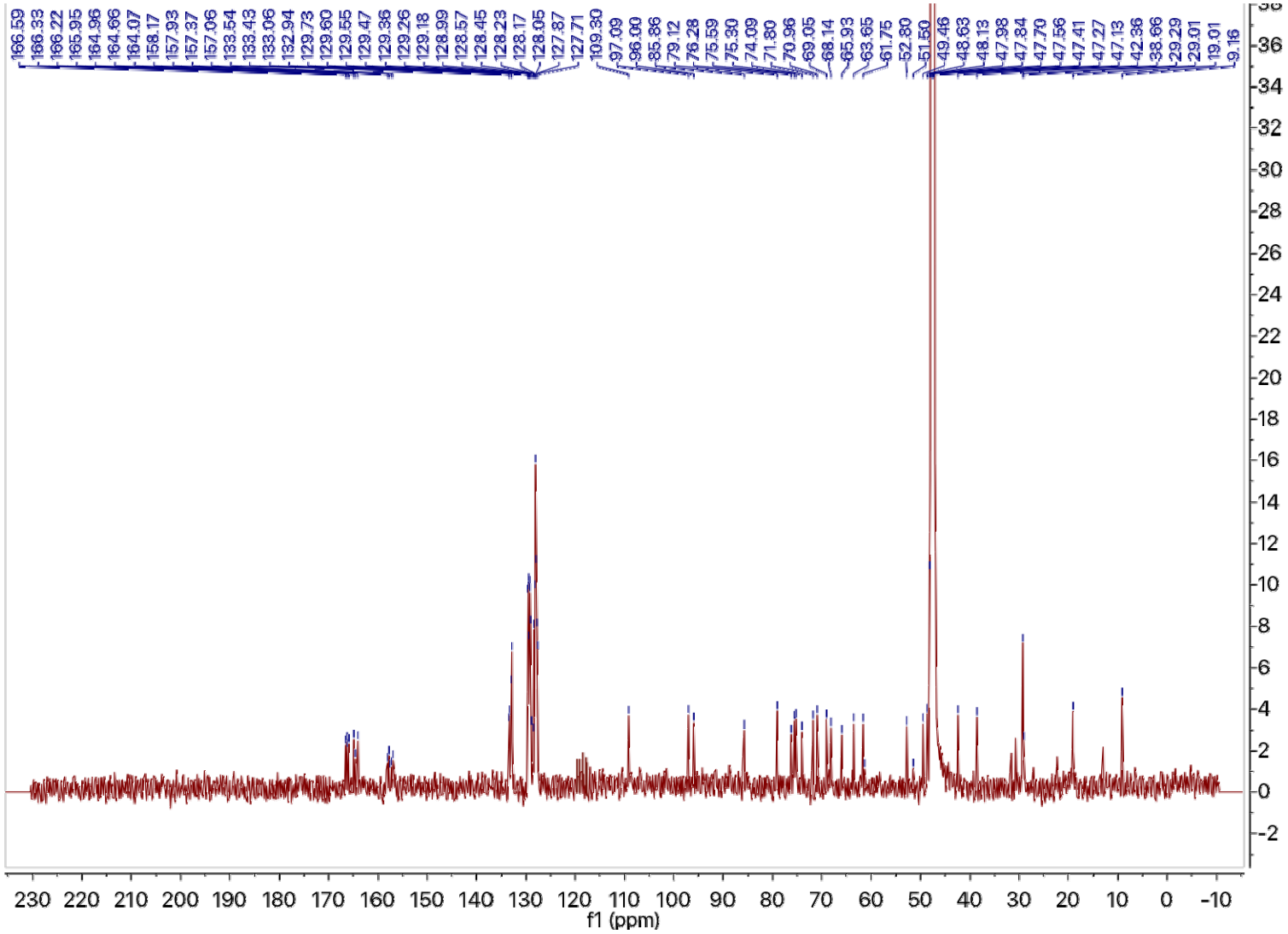
4′-Deoxy-4′-(2-iodoethyl)-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl-6,3′,6′,2‴,5‴,3⁗,4⁗-hepta-*O*-benzoyl paromomycin (32) ^{13}C NMR (150 MHz, CD_3OD)



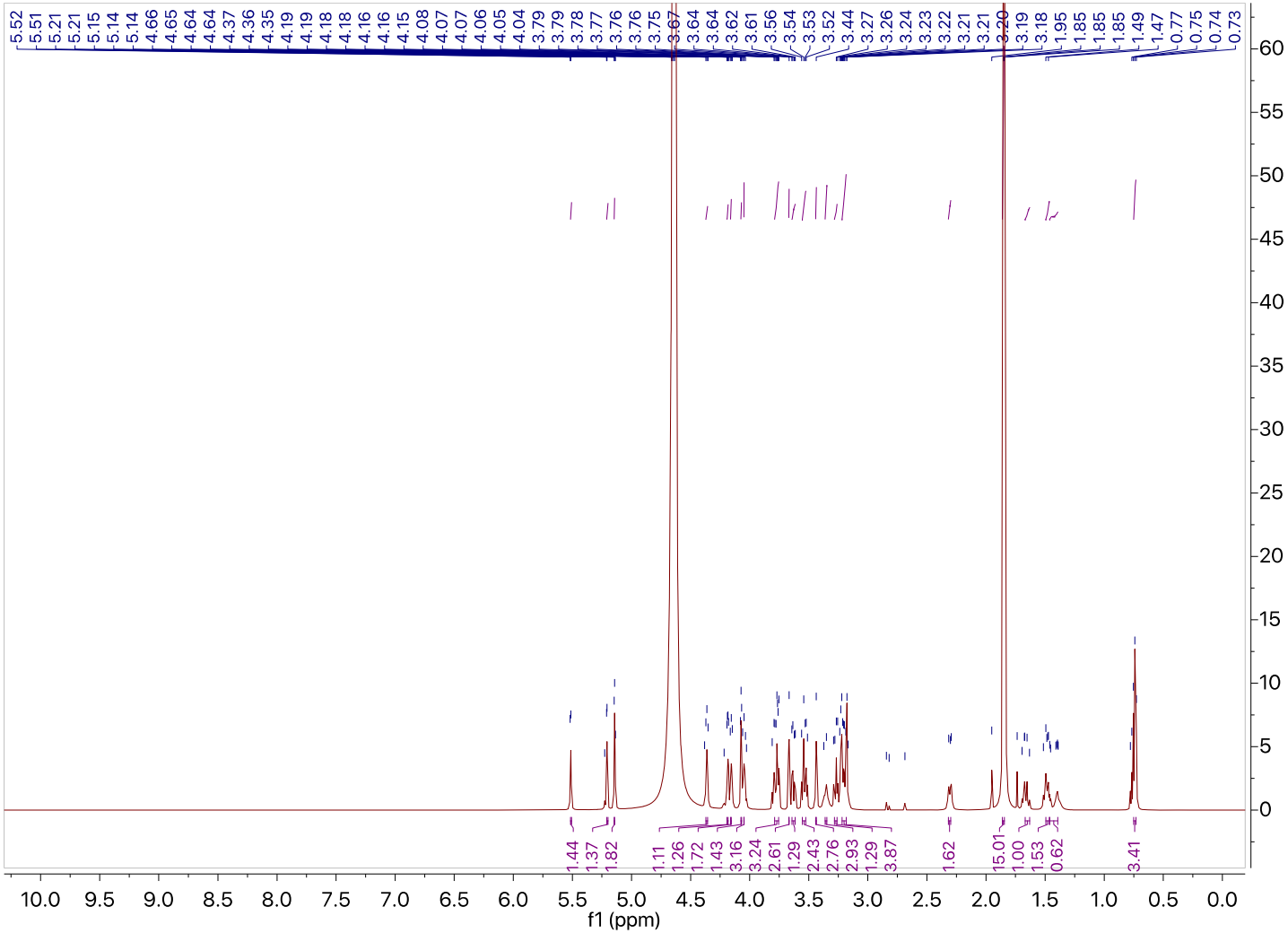
4′-Deoxy-4′-(ethyl)-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl-6,3′,6′,2‴,5‴,3‴″,4‴″-hepta-*O*-benzoyl paromomycin (33) ^{1}H NMR (600 MHz, CD_3OD)



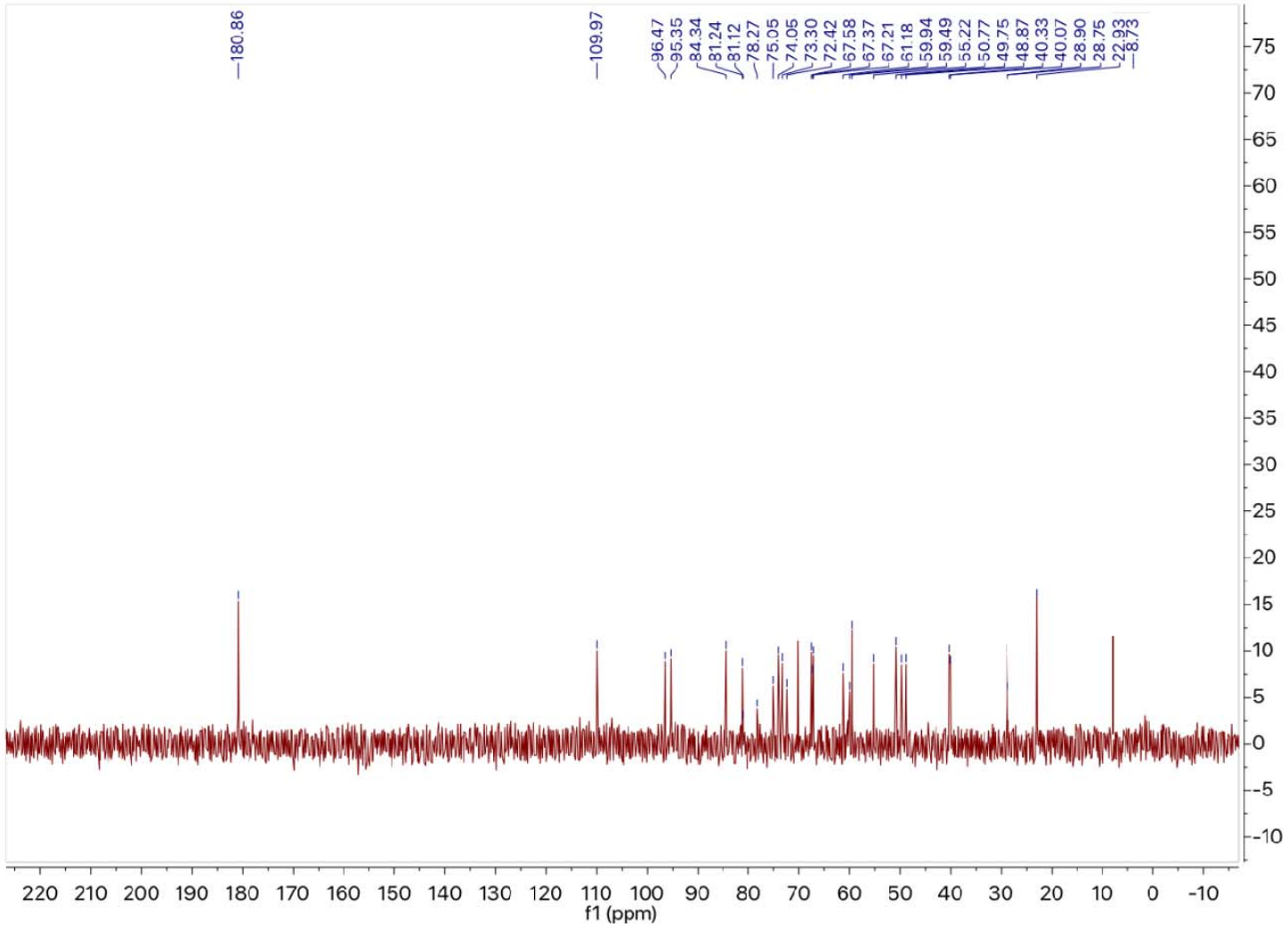
4′-Deoxy-4′-(ethyl)-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl-6,3′,6′,2‴,5‴,3‴″,4‴″-hepta-*O*-benzoyl paromomycin (33) ^{13}C NMR (150 MHz, CD_3OD)



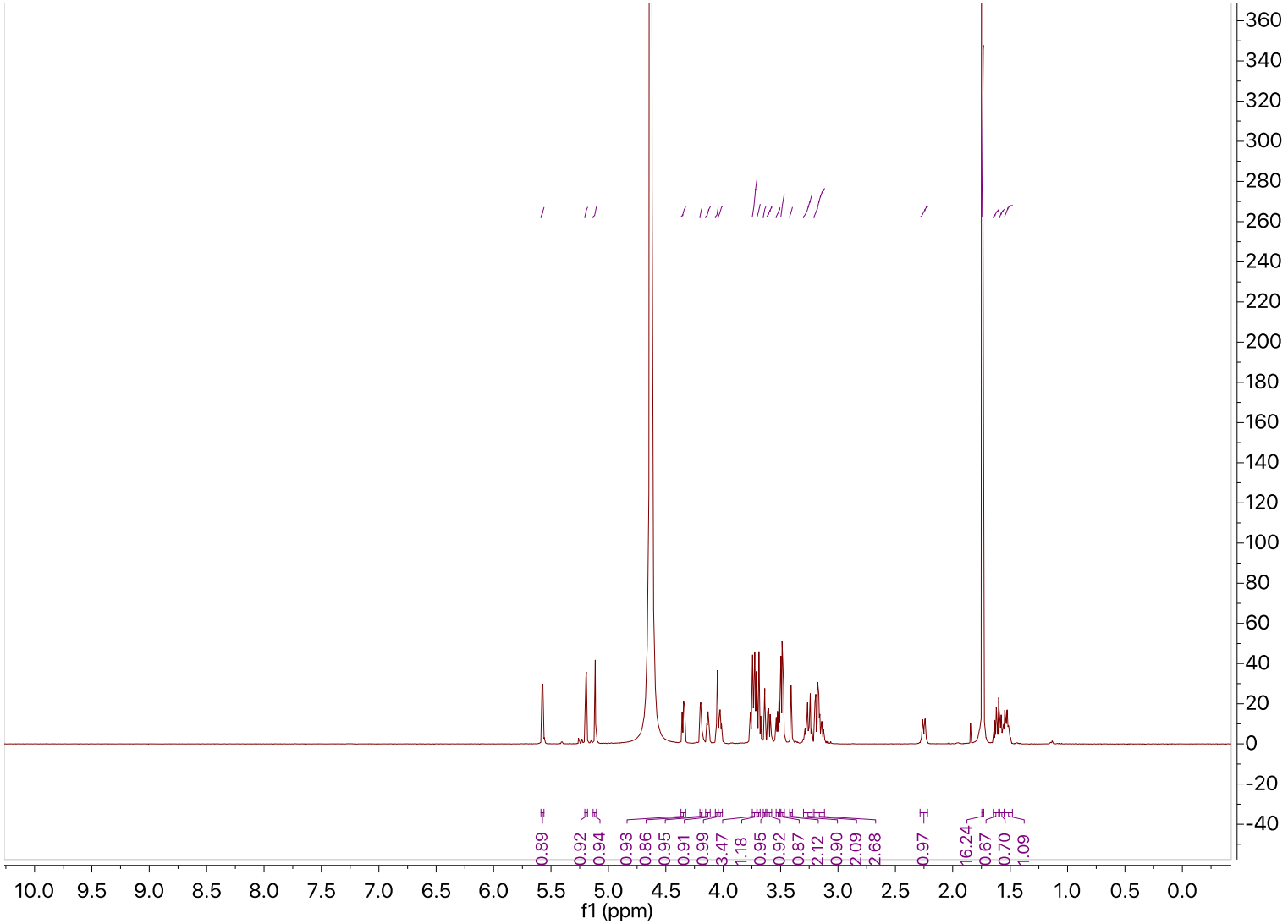
4′-Deoxy-4′-ethyl paromomycin (35) ^{1}H NMR (600 MHz, D_2O)



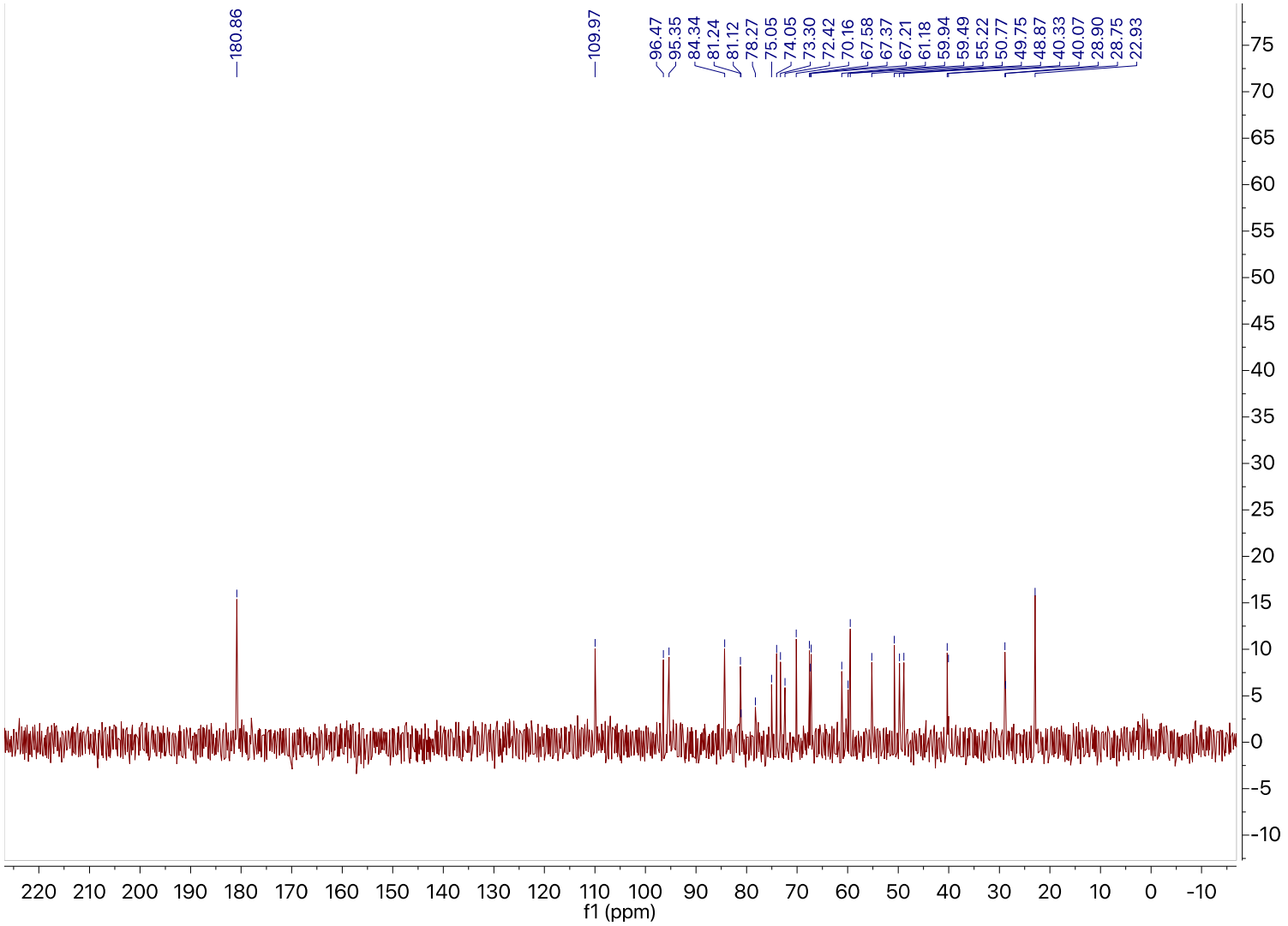
4′-Deoxy-4′-ethyl paromomycin (35) ^{13}C NMR (150 MHz, D_2O)



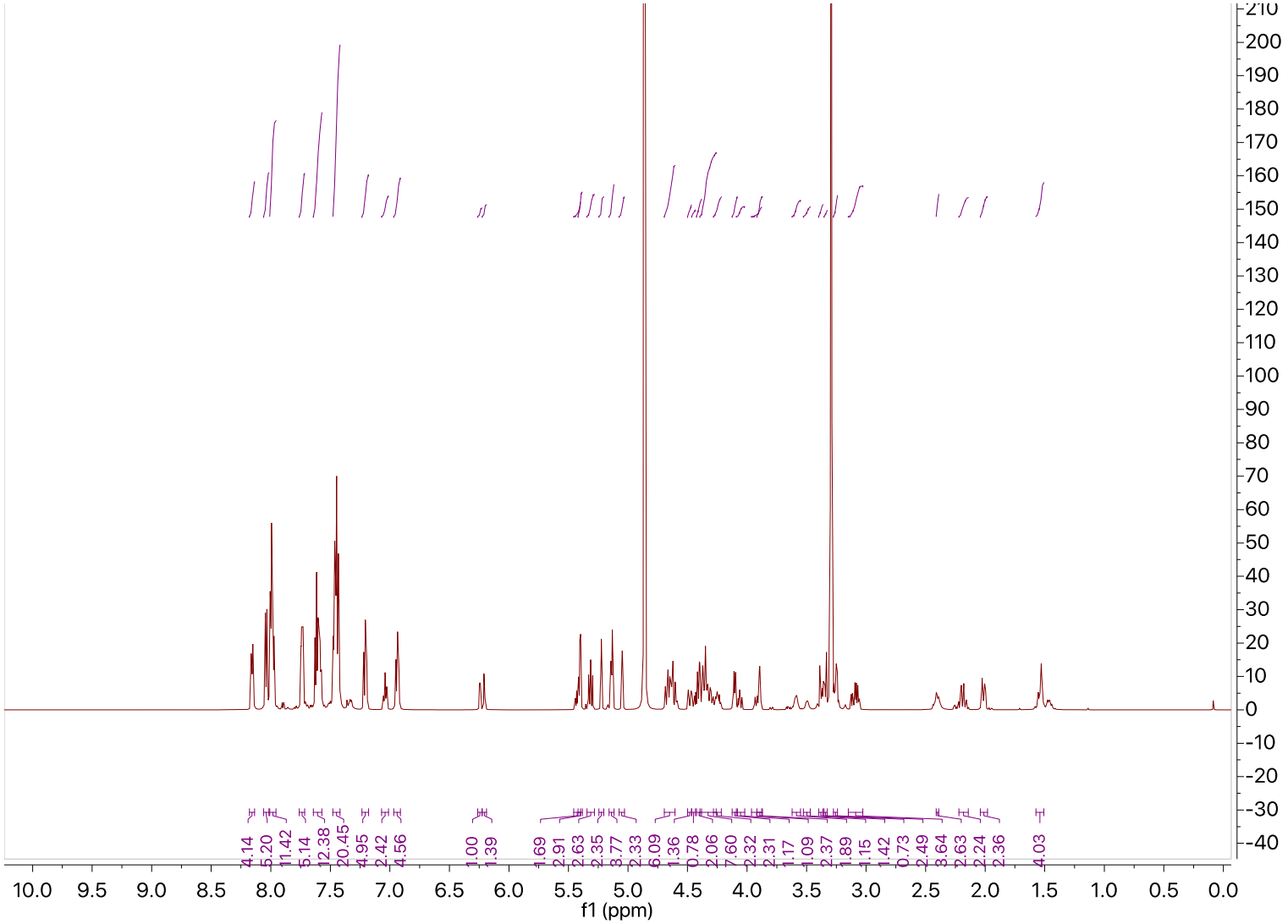
4′-Deoxy-4′-*C*-(2-hydroxyethyl) paromomycin (36) ^{1}H NMR (600 MHz, D_2O)



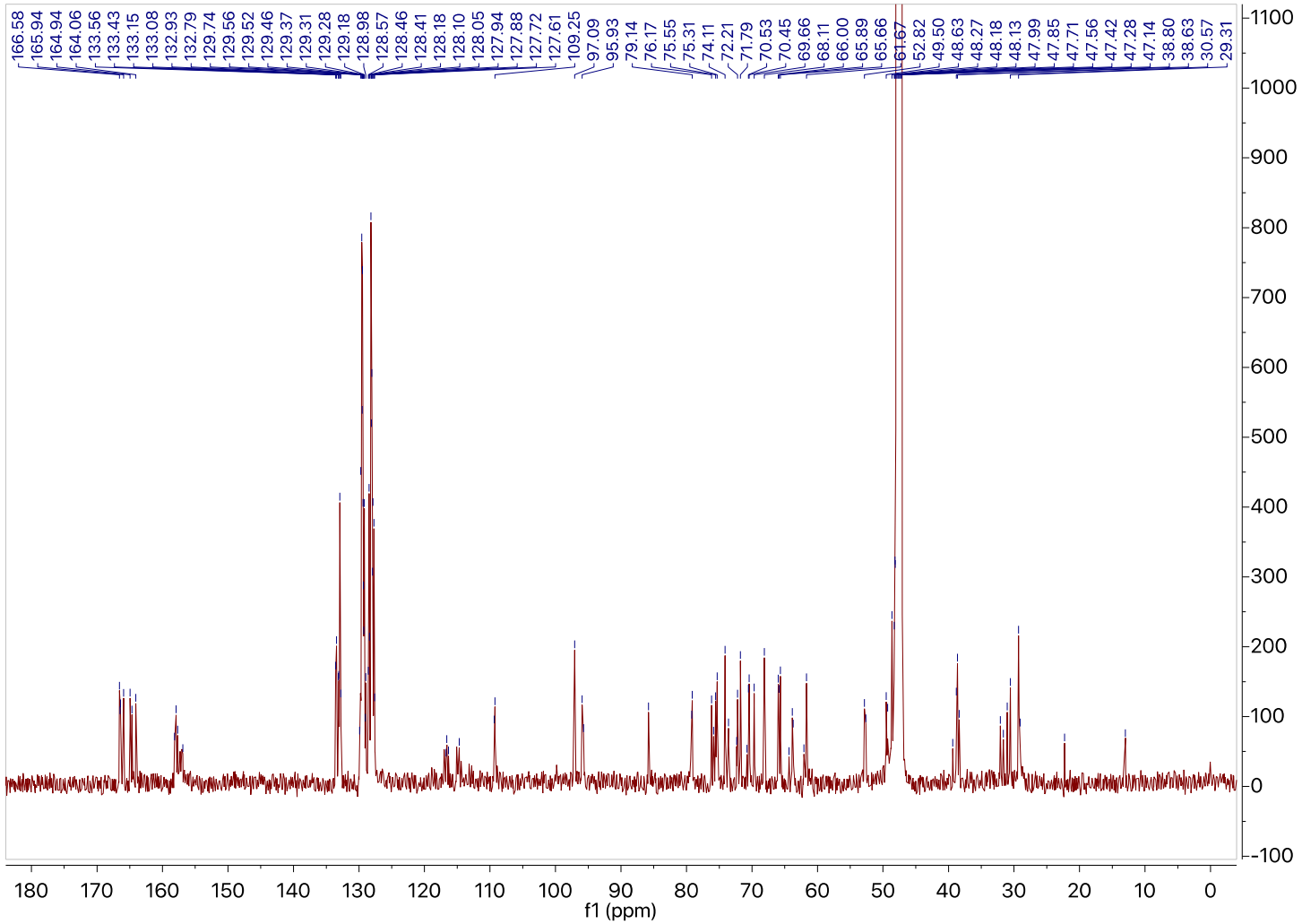
4′-Deoxy-4′-*C*-(2-hydroxyethyl) paromomycin (36) ^{13}C NMR (150 MHz, D_2O)



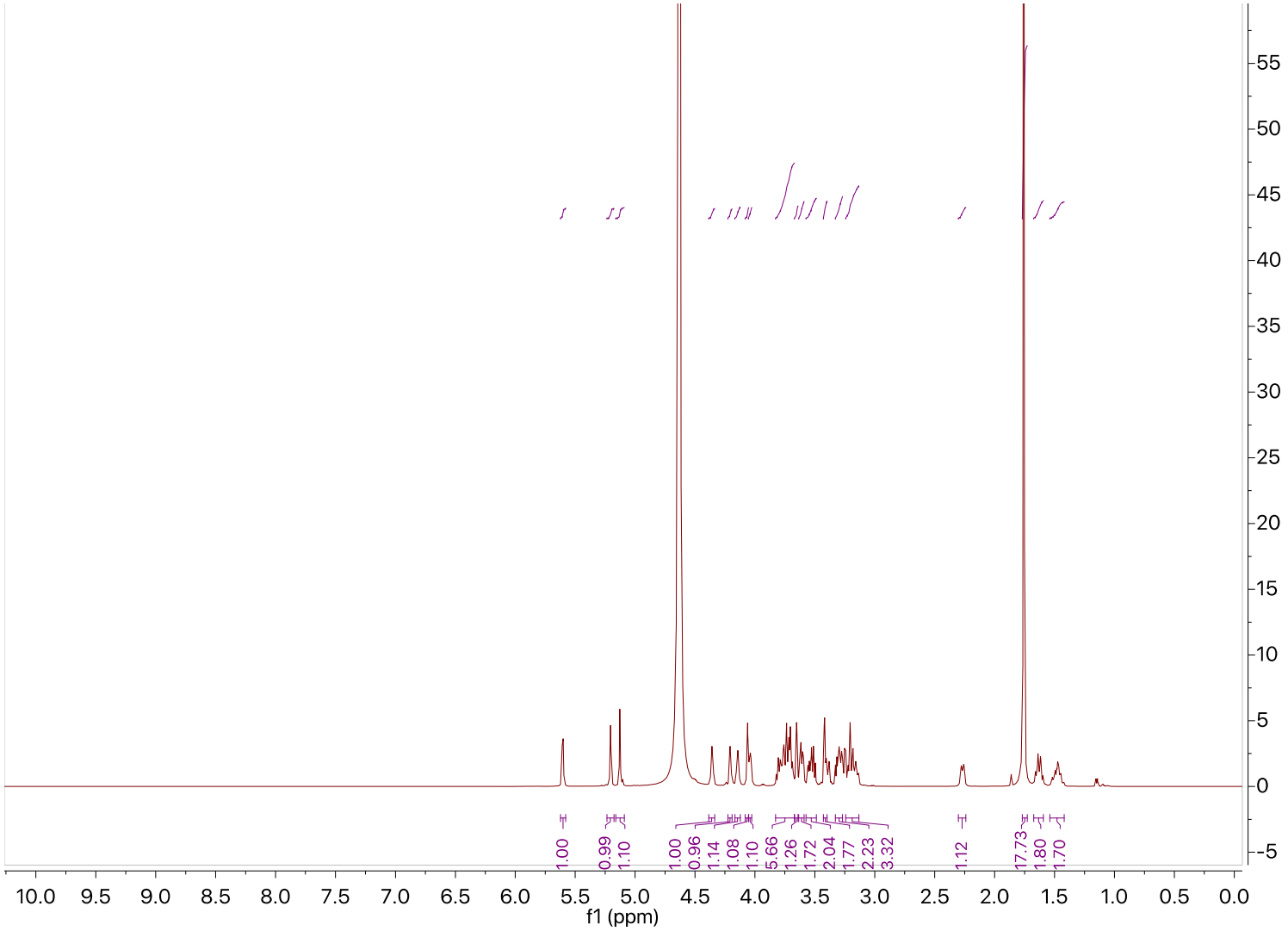
4′-Deoxy-4′-(2,3-dihydroxypropyl)-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl-6,3′,6′,2‴,5‴,3‴″,4‴″-hepta-*O*-benzoyl paromomycin (34) ^{1}H NMR (600 MHz, CD_3OD)



4′-Deoxy-4′-(2,3-dihydroxypropyl)-1,3,2′,2‴,6‴-penta-*N*-trifluoroacetyl-6,3′,6′,2‴,5‴,3‴″,4‴″-hepta-*O*-benzoyl paromomycin (34) ^{13}C NMR (150 MHz, CD_3OD)



4′-Deoxy-4′-(2,3-dihydroxypropyl) paromomycin (37) ^{1}H NMR (600 MHz, D_2O)



4′-Deoxy-4′-(2,3-dihydroxypropyl) paromomycin (37) ^{13}C NMR (150 MHz, D_2O)