Supporting Information

for

Substrate Tolerance of Bacterial Glycosyltransferase MurG: Novel Fluorescence-based Assays

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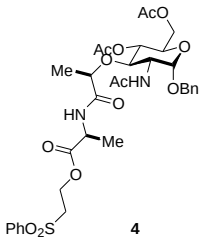
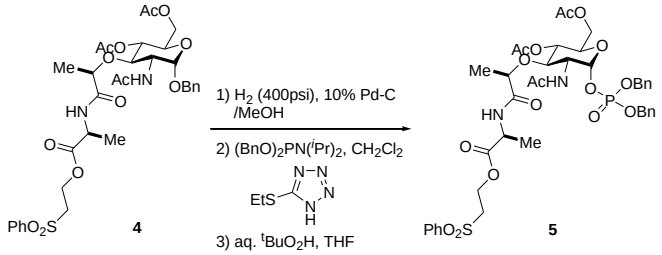
General

All chemicals were purchased from commercial sources and used without further purification unless otherwise noted. THF, CH_2Cl_2, and DMF were purified via Innovative Technology's Pure-Solve System. All reactions were performed under an Argon atmosphere. All stirring was performed with an internal magnetic stirrer. Reactions were monitored by TLC using 0.25 mm coated commercial silica gel plates (EMD, Silica Gel $60F_{254}$). TLC spots were visualized by UV light at 254 nm, or developed with ceric ammonium molybdate or anisaldehyde or copper sulfate or ninhydrin solutions by heating on a hot plate. Reactions were also monitored by using SHIMADZU LCMS-2020 with solvents: A: 0.1% formic acid in water, B: acetonitrile. Flash chromatography was performed with SiliCycle silica gel (Purasil 60 Å, 230-400 Mesh). Proton magnetic resonance (^{1}H-NMR) spectral data were recorded on 400, and 500 MHz instruments. Carbon magnetic resonance (^{13}C-NMR) spectral data were recorded on 100 and 125 MHz instruments. For all NMR spectra, chemical shifts (δH, δC) were quoted in parts per million (ppm), and *J* values were quoted in Hz. ^{1}H and ^{13}C NMR spectra were calibrated with residual undeuterated solvent ($CDCl_3$: δH = 7.26 ppm, δC = 77.16 ppm; CD_3CN: δH = 1.94 ppm, δC = 1.32ppm; CD_3OD: δH =3.31 ppm, δC =49.00 ppm; DMSO-d_6: δH = 2.50 ppm, δC = 39.52 ppm; D_2O: δH = 4.79 ppm) as an internal reference. The following abbreviations were used to designate the multiplicities: s = singlet, d = doublet, dd = double doublets, t = triplet, q = quartet, quin = quintet, hept = heptet, m = multiplet, br = broad. Infrared (IR) spectra were recorded on a Perkin-Elmer FT1600 spectrometer. HPLC analyses were performed with a Shimadzu LC-20AD HPLC system. HR-MS data were obtained from a Waters Synapt G2-Si (ion mobility mass spectrometer with nanoelectrospray ionization).



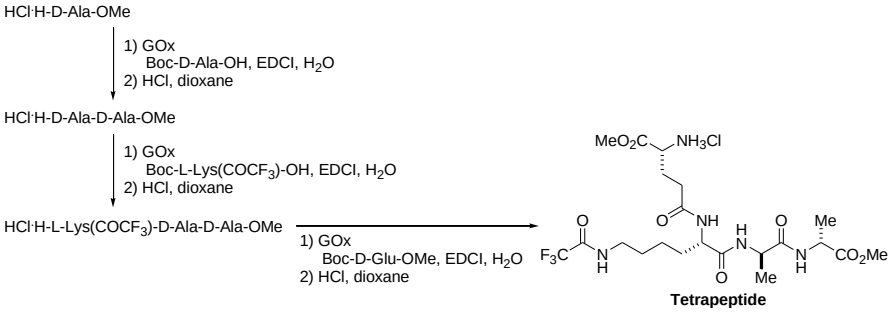
4

2-(Phenylsulfonyl)ethyl ((*R*)-2-(((2*S*,3*R*,4*R*,5*S*,6*R*)-3-acetamido-5-acetoxy-6-(acetoxymethyl)-2-(benzyloxy)tetrahydro-2*H*-pyran-4-yl)oxy)propanoyl)-*L*-alaninate (4). The title compound was synthesized according to the reported procedure [1-3]: ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.92 (dd, *J* = 8.4, 1.2 Hz, 2H), 7.68 (tt, *J* = 7.4, 1.3 Hz, 1H), 7.61 – 7.56 (m, 2H), 7.41 – 7.34 (m, 3H), 7.34 – 7.30 (m, 2H), 6.86 (d, *J* = 6.9 Hz, 1H), 5.87 (d, *J* = 9.5 Hz, 1H), 5.06 (dd, *J* = 10.3, 9.2 Hz, 1H), 4.88 (d, *J* = 3.6 Hz, 1H), 4.69 (d, *J* = 11.7 Hz, 1H), 4.49 (d, *J* = 11.8 Hz, 1H), 4.43 (hex, *J* = 6.0 Hz, 2H), 4.36 (ddd, *J* = 10.3, 9.5, 3.7 Hz, 1H), 4.20 (dd, *J* = 12.3, 4.5 Hz, 1H), 4.13 (t, *J* = 7.1 Hz, 1H), 4.03 (dd, *J* = 12.3, 2.4 Hz, 1H), 3.95 (q, *J* = 7.0 Hz, 1H), 3.91 (ddd, *J* = 10.2, 4.6, 2.4 Hz, 1H), 3.64 (dd, *J* = 10.4, 9.2 Hz, 1H), 3.44 (qt, *J* = 6.3, 6.2 Hz, 2H), 2.11 (s, 3H), 2.07 (s, 3H), 1.88 (s, 3H), 1.30 (d, *J* = 6.8 Hz, 3H), 1.29 (d, *J* = 7.3 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.02, 171.60, 170.77, 170.22, 169.32, 139.08, 136.48, 134.07, 129.44 (2C), 128.74 (2C), 128.50, 128.33 (2C), 128.08 (2C), 97.02, 78.67, 78.45, 70.20, 69.30, 68.49, 62.06, 58.00, 54.87, 52.89, 47.95, 23.33, 20.85, 20.78, 18.56, 16.85; HRMS (ESI+) *m*/*z* calcd for $C_{33}H_{42}N_2NaO_{13}S$ [M + Na] 729.2305, found: 729.2320.

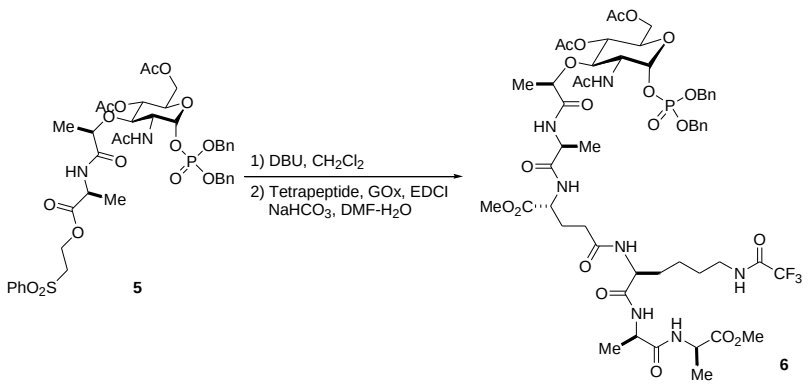


2-(Phenylsulfonyl)ethyl ((*R*)-2-(((2*R*,3*R*,4*R*,5*S*,6*R*)-3-acetamido-5-acetoxy-6-(acetoxymethyl)-2-((bis(benzyloxy)phosphoryl)oxy)tetrahydro-2*H*-pyran-4-yl)oxy)propanoyl)-*L*-alaninate (5). To a stirred solution of **4** (0.33 g, 0.46 mmol) in a 9:5:1 mixture of MeOH, formic acid and H_2O (15 mL) was added Pd-C (0.65 g). The reaction solution was stirred under hydrogen atmosphere (400 psi) for 17 h. The reaction mixture was filtrated and the residue was concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (EtOAc/MeOH = 90/10) to afford the free-alcohol (0.26 g, 91%). To a stirred solution of the anomeric-free alcohol (0.21 g, 0.34 mmol) and 5-(ethylthio)-1*H*-tetrazole (0.13 g, 1.0 mmol) in CH_2Cl_2 (3.4 mL) was added dibenzyl *N*,*N*-diisopropylphosphoramidite (0.29 mL, 0.86 mmol) at 0 °C. After 2 h at 0 °C, the reaction was quenched with aq. saturated $NaHCO_3$ and extracted with $CHCl_3$. The combined organic solution was dried over Na_2SO_4 and concentrated *in vacuo*. To a stirred solution of the crude mixture and $NaHCO_3$ (58 mg, 0.69 mmol) in THF (3.4 mL), 70% aq. *tert*-butyl hydroperoxide (0.48 mL, 3.4 mmol) was added at 0 °C. After 30 min. at r.t., the reaction

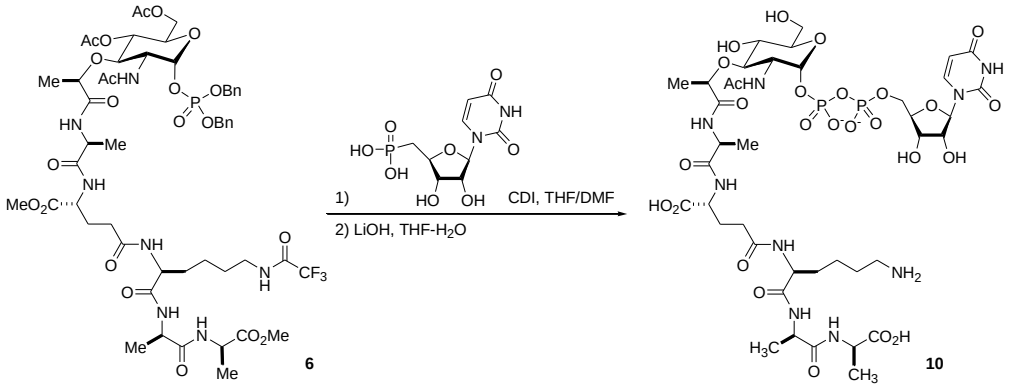
was quenched with aq. $Na_2S_2O_3$ and extracted with $CHCl_3$. The combined organic solution was dried over Na_2SO_4 and concentrated *in vacuo*. The crude mixture was purified by silica gel column chromatography (EtOAc/MeOH = 90/10) to afford 0.28 g (93% for 2 steps) of **5**: ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.92 (dd, *J* = 8.4, 1.3 Hz, 2H), 7.68 (tt, *J* = 7.4, 1.3 Hz, 1H), 7.61 – 7.56 (m, 2H), 7.39 – 7.32 (m, 10H), 6.73 (d, *J* = 7.0 Hz, 1H), 6.12 (d, *J* = 9.0 Hz, 1H), 5.62 (dd, *J* = 5.8, 3.2 Hz, 1H), 5.14 – 4.97 (m, 6H), 4.45 (td, *J* = 6.2, 1.3 Hz, 2H), 4.33 (ddt, *J* = 10.6, 9.0, 3.1 Hz, 1H), 4.19 (t, *J* = 7.2 Hz, 1H), 4.10 (dd, *J* = 13.1, 4.6 Hz, 1H), 3.92 (d, *J* = 13.8 Hz, 1H), 3.89 (d, *J* = 9.8 Hz, 1H), 3.50 – 3.41 (m, 3H), 2.07 (s, 3H), 2.01 (s, 3H), 1.75 (s, 3H), 1.30 (d, *J* = 6.9 Hz, 3H), 1.29 (d, *J* = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.93, 171.51, 170.63, 170.60, 169.12, 139.05, 134.11 (2C), 129.47 (2C), 129.04 (2C), 128.80 (4C), 128.16 (2C), 128.08 (4C), 96.85, 96.78, 78.37, 70.15, 70.03, 69.98, 68.65, 61.45, 58.07, 54.84, 52.92, 52.85, 47.92, 22.97, 20.79, 20.65, 18.62, 17.00; ^{31}P NMR (162 MHz, $CDCl_3$) δ -2.39 ; HRMS (ESI+) *m*/*z* calcd for $C_{40}H_{49}N_2NaO_{16}PS$ [M + Na] 899.2438, found: 899.2412.



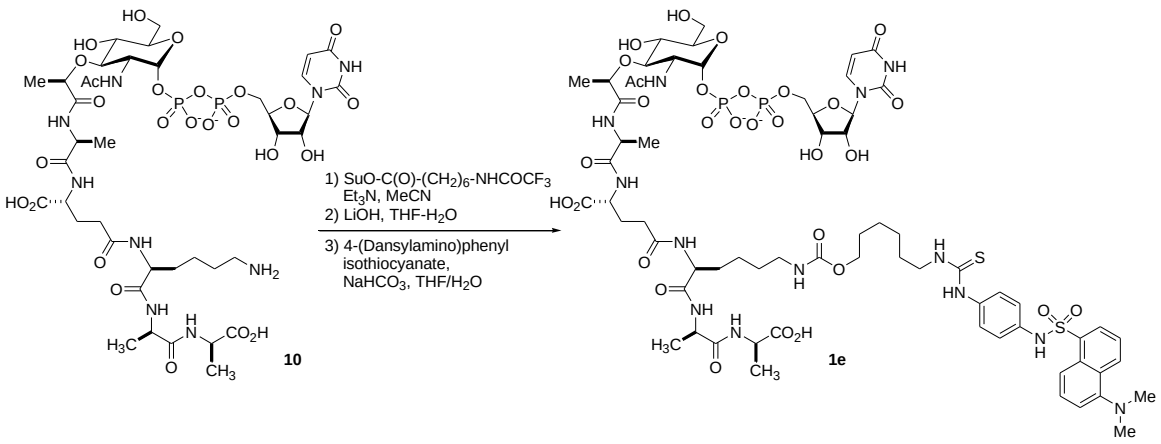
Tetrapeptide. Tetrapeptide was synthesized according to the reported procedure [4-6]: ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.45 (t, *J* = 5.8 Hz, 1H), 8.62 (s, 3H), 8.27 (d, *J* = 7.0 Hz, 1H), 8.23 (d, *J* = 8.1 Hz, 1H), 8.21 (d, *J* = 7.8 Hz, 1H), 4.34 – 4.25 (m, 1H), 4.26 – 4.18 (m, 2H), 3.99 (t, *J* = 6.7 Hz, 1H), 3.72 (s, 3H), 3.60 (s, 3H), 2.33 (ddt, *J* = 37.5, 15.2, 7.6 Hz, 2H), 2.05 – 1.94 (m, 2H), 1.64 – 1.40 (m, 6H), 1.29 (d, *J* = 7.2 Hz, 3H), 1.27 – 1.20 (m, 2H), 1.19 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, DMSO) δ 173.87, 173.08, 172.26, 171.81, 170.74, 157.10 (q, *J* = 35.9 Hz), 116.96 (q, *J* = 288.3 Hz), 53.72, 53.62, 52.81, 52.45, 49.54, 48.58, 48.55, 32.49, 31.23, 28.89, 26.97, 23.51, 19.05, 17.72; HRMS (ESI+) *m*/*z* calcd for $C_{21}H_{35}F_3N_5O_8$ [M + H] 542.2438, found: 542.2457.



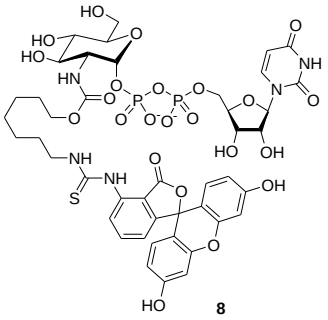
Methyl N^2-(((*R*)-2-(((2*R*,3*R*,4*R*,5*S*,6*R*)-3-acetamido-5-acetoxy-6-(acetoxymethyl)-2-((bis(benzyloxy)phosphoryl)oxy)tetrahydro-2*H*-pyran-4-yl)oxy)propanoyl)-*L*-alanyl)-N^5-((*S*)-1-(((*R*)-1-(((*R*)-1-methoxy-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxo-6-(2,2,2-trifluoroacetamido)hexan-2-yl)-*D*-glutaminate (6). To a stirred solution of **5** (78 mg, 0.089 mmol) in CH_2Cl_2 (0.45 mL) was added DBU (15 μL, 0.098 mmol). After being stirred for 1 h at r.t., the reaction was quenched with 1 M aq. HCl and extracted with EtOAc. The combined organic solution was dried over Na_2SO_4 and concentrated *in vacuo*. To a stirred solution of the crude mixture, tetrapeptide (0.10 g, 0.18 mmol) and $NaHCO_3$ (38 mg, 0.45 mmol) in 25:1 solution of DMF and H_2O (1.0 mL), GOx (41 mg, 0.18 mmol) and EDCI (34 mg, 0.18 mmol) were added. After being stirred for 2 h at r.t., the reaction was quenched with aq. saturated $NaHCO_3$ and extracted with EtOAc. The combined organic solution was dried over Na_2SO_4 and concentrated *in vacuo*. The crude mixture was purified by silica gel column chromatography (EtOAc/MeOH/Et_3N = 93/7/0.5 - 90/10/0.5) to afford 99 mg (90% for 2 steps) of **6**. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.75 (t, *J* = 5.8 Hz, 1H), 7.60 (d, *J* = 7.7 Hz, 1H), 7.38 – 7.30 (m, 10H), 7.15 (d, *J* = 5.8 Hz, 1H), 7.10 (d, *J* = 8.5 Hz, 1H), 6.88 (d, *J* = 8.7 Hz, 1H), 5.64 (dd, *J* = 5.9, 3.2 Hz, 1H), 5.06 (ddd, *J* = 9.2, 8.4, 3.0 Hz, 6H), 4.54 – 4.30 (m, 7H), 4.24 (quin, *J* = 6.9 Hz, 1H), 4.15 – 4.09 (m, 2H), 3.91 (d, *J* = 10.6 Hz, 2H), 3.70 (s, 3H), 3.68 (s, 3H), 3.69 – 3.66 (m, 1H), 3.59 (t, *J* = 9.9 Hz, 1H), 3.31 (q, *J* = 6.5 Hz, 2H), 3.10 (q, *J* = 7.3 Hz, 1H), 2.38 – 2.13 (m, 4H), 2.07 (s, 3H), 1.99 (s, 3H), 1.78 (s, 3H), 1.58 (tt, *J* = 13.5, 6.2 Hz, 2H), 1.43 (d, *J* = 7.0 Hz, 3H), 1.38 (d, *J* = 7.4 Hz, 3H), 1.37 (d, *J* = 7.3 Hz, 3H), 1.29 (d, *J* = 6.6 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.32, 173.24, 172.73, 172.60, 172.18, 172.02, 171.94, 171.05, 170.57, 169.26, 157.45 (q, *J* = 36.8 Hz), 129.00 (2C), 128.76 (4C), 128.63, 128.56, 128.09 (4C), 70.02, 69.98, 68.63, 61.46, 53.82, 53.09, 53.01, 52.48, 52.29, 50.76, 50.27, 49.12, 48.03, 45.85, 39.42, 31.40, 31.16, 29.66, 28.16, 27.58, 22.78, 22.36, 20.79, 20.61, 18.54, 17.82, 17.64, 17.22, 8.57; ^{31}P NMR (162 MHz, $CDCl_3$) δ -2.72; HRMS (ESI+) *m*/*z* calcd for $C_{53}H_{74}F_3N_7O_{21}P$ [M + H] 1232.4628, found: 1232.4646.



Park's nucleotide (10). To a stirred solution of **6** (7.5 mg, 6.1 μmol) in MeOH (10 mL) was added Pd-C (15 mg). After being stirred under hydrogen atmosphere (1 atm) for 1 h at r.t., Et_3N (0.5 mL) was added to the reaction mixture. After being stirred for 1 h at r.t., the catalyst was filtered through a pad of Celite. The filtrate solution of pentapeptide was concentrated *in vacuo* and the resulting product was dried in high vacuum. To a stirred solution of uridyl phosphate (62 mg, 0.15 mmol) in 1:1 solution of THF and DMF (2.0 mL) was added CDI (74 mg, 0.46 mmol). After being stirred for 14 h at r.t., MeOH (0.2 mL) was added to the reaction mixture. After being stirred for 1 h, the solution was concentrated *in vacuo* and the resulting product was dried in high vacuum. To a stirred solution of the crude product of pentapeptide in 4:1 solution of THF and DMF (2.0 mL) was added the crude product of uridyl phosphate. After being stirred for 48 h at r.t., the reaction mixture was filtered and concentrated *in vacuo*. To a stirred solution of the crude product in THF (1.7 mL) was added 1.7 mL of aq. LiOH (43.2 mg, 1.03 mmol). After being stirred for 2.5 h at r.t., the reaction mixture was filtered and concentrated *in vacuo*. The filtrate was purified by reverse phase HPLC [column: HYPERSIL GOLD™ (175 Å, 12 μm, 150 x 20 mm), solvents: 0 : 100 CH_3CN : 0.05 M aq. NH_4HCO_3 for 5 min then 5 : 95 CH_3CN : 0.05 M aq. NH_4HCO_3 for 10 min then 10 : 90 CH_3CN : 0.05 M aq. NH_4HCO_3 for 10 min, flow rate: 4.0 mL/min, UV: 254nm] to afford **10** (4.7 mg, 68% overall, retention time: 18.7 min): ^{1}H NMR (400 MHz, Deuterium Oxide) δ 7.95 (d, *J* = 8.1 Hz, 1H), 5.99 – 5.94 (m, 2H), 5.46 (dd, *J* = 7.2, 3.2 Hz, 1H), 4.37 – 4.34 (m, 2H), 4.31 (d, *J* = 7.2 Hz, 1H), 4.29 – 4.09 (m, 11H), 3.94 (ddd, *J* = 10.1, 2.3, 2.1 Hz, 1H), 3.89 – 3.81 (m, 2H), 3.78 (dd, *J* = 10.2, 9.0 Hz, 1H), 3.63 (dd, *J* = 10.2, 9.0 Hz, 1H), 2.99 (t, *J* = 7.5 Hz, 2H), 2.30 (t, *J* = 7.6 Hz, 2H), 2.16 (ddd, *J* = 12.8, 7.7, 4.8 Hz, 1H), 2.00 (s, 3H), 1.94 – 1.84 (m, 1H), 1.83 – 1.75 (m, 2H), 1.68 (dd, *J* = 8.6, 6.1 Hz, 2H), 1.43 (d, *J* = 7.3 Hz, 3H), 1.39 (d, *J* = 6.9 Hz, 3H), 1.36 (d, *J* = 6.9 Hz, 3H), 1.33 (d, *J* = 7.1 Hz, 3H); HRMS (ESI+) *m*/*z* calcd for $C_{40}H_{66}N_9O_{26}P_2$ [M + H] 1150.3594, found: 1150.3581.



Park's nucleotide-N^{ε}-C_6-dansyl (1e). To a stirred solution of **10** (6.3 mg, 5.5 μmol) and SuO-C(O)O-$(CH_2)_6$-$NHCOCF_3$ (5.8 mg, 0.017 mmol) in MeCN (0.5 mL) was added Et_3N (3.9 μL, 0.028 mmol). After being stirred for 12 h at r.t., the reaction was concentrated *in vacuo*. To a stirred solution of the crude product in THF (0.5 mL) was added 0.2 mL of aq. LiOH (2.3 mg, 0.055 mmol). After being stirred for 3 h at r.t., the reaction mixture was filtered and concentrated *in vacuo*. The crude product was purified by reverse phase HPLC [column: HYPERSIL GOLD™ (175 Å, 12 μm, 150 x 20 mm), solvents: 0 : 100 CH_3CN : 0.05 M aq. NH_4HCO_3 for 5 min then 5 : 95 CH_3CN : 0.05 M aq. NH_4HCO_3 for 10 min then 10 : 90 CH_3CN : 0.05 M aq. NH_4HCO_3 for 10 min, flow rate: 4.0 mL/min, UV: 254nm]. To a stirred solution of the product and $NaHCO_3$ (4.6 mg, 0.055 mmol) in a 4 : 1 solution of THF and H_2O (0.5 mL) was added 4-(dansylamino)phenyl isothiocyanate (10.5 mg, 0.028 mmol). After being stirred for 4 h at r.t., the reaction mixture was filtered and concentrated *in vacuo*. The filtrate was purified by reverse phase HPLC [column: Phenomenex Luna (100 Å, 10 μm, C18, 250 x 10 mm), solvents: 10 : 90 CH_3CN : 0.05 M aq. NH_4HCO_3 for 5 min then 20 : 80 CH_3CN : 0.05 M aq. NH_4HCO_3 for 10 min then 30 : 70 CH_3CN : 0.05 M aq. NH_4HCO_3 for 10 min, flow rate: 3.0 mL/min, UV: 350nm] to afford **1e** (6.4 mg, 70% overall, retention time: 24.7 min): ^{1}H NMR (400 MHz, Deuterium Oxide) δ 8.55 (d, *J* = 8.2 Hz, 1H), 8.30 (d, *J* = 8.2 Hz, 1H), 8.15 (d, *J* = 7.4 Hz, 1H), 7.87 (d, *J* = 8.1 Hz, 1H), 7.32 (d, *J* = 7.2 Hz, 1H), 7.07 (s, 1H), 6.87 (s, 4H), 5.93 (ddd, *J* = 3.8, 1.6, 0.9 Hz, 1H), 5.89 (d, *J* = 8.0 Hz, 1H), 5.41 (dd, *J* = 7.7, 3.2 Hz, 1H), 4.30 (d, *J* = 3.3 Hz, 3H), 4.28 – 4.01 (m, 10H), 3.92 – 3.87 (m, 1H), 3.82 – 3.77 (m, 2H), 3.74 – 3.70 (m, 1H), 3.61 – 3.57 (m, 1H), 3.39 – 3.32 (m, 2H), 2.98 – 2.90 (m, 2H), 2.80 (s, 6H), 2.24 – 2.18 (m, 4H), 2.14 – 2.09 (m, 3H), 1.95 (s, 3H), 1.80 – 1.76 (m, 1H), 1.62 – 1.43 (m, 4H), 1.37 (d, *J* = 7.5 Hz, 3H), 1.34 (d, *J* = 7.1 Hz, 3H), 1.28 (d, *J* = 7.0 Hz, 3H), 1.26 (d, *J* = 6.9 Hz, 3H), 1.17 – 1.11 (m, 4H), 0.86 – 0.78 (m, 4H); HRMS (ESI+) *m*/*z* calcd for $C_{66}H_{96}N_{13}O_{30}P_2S_2$ [M + H] 1676.5303, found: 1676.5322.



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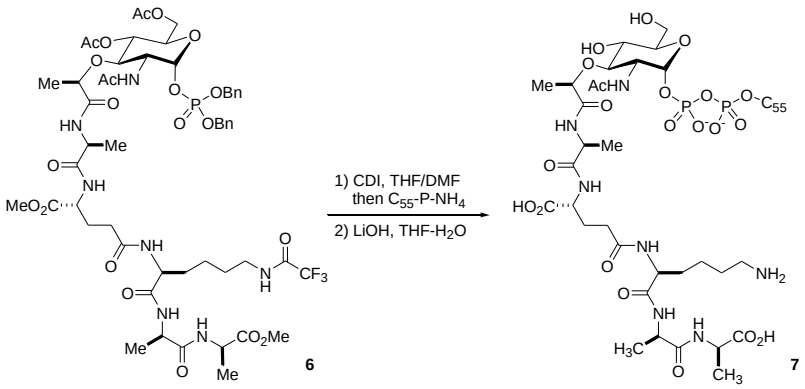
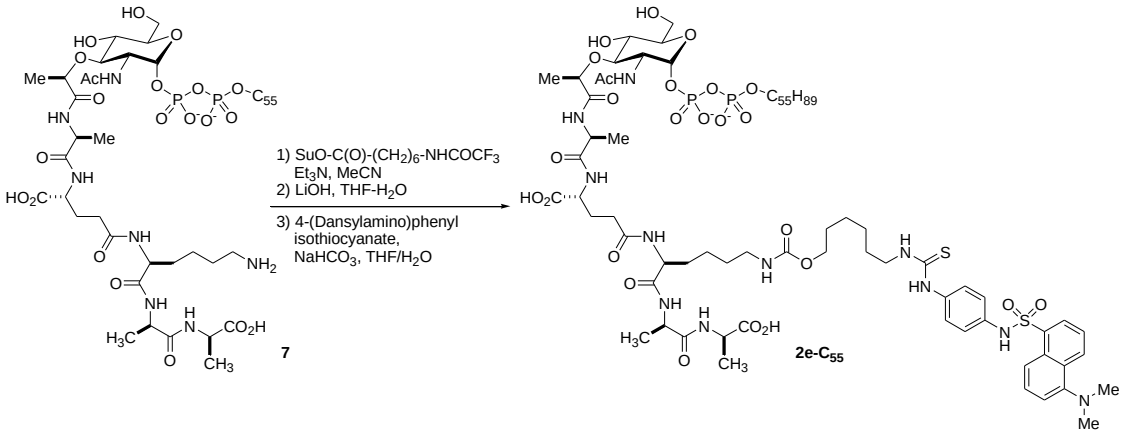
UDP-GlcN-C_6-FITC (8). The title compound was synthesized according to the reported procedure [7]: ^{1}H NMR (400 MHz, Deuterium Oxide) δ 7.90 (d, *J* = 8.1 Hz, 1H), 7.71 – 7.63 (m, 1H), 7.61 – 7.51 (m, 1H), 7.37 – 7.30 (m, 3H), 7.30 – 7.20 (m, 4H), 5.99 – 5.85 (m, 2H), 5.52 (d, *J* = 6.3 Hz, 1H), 4.35 – 4.27 (m, 3H), 4.26 – 4.13 (m, 2H), 4.12 – 4.00 (m, 2H), 3.92 – 3.84 (m, 1H), 3.80 (dd, *J* = 16.2, 3.2 Hz, 1H), 3.76 – 3.69 (m, 2H), 3.62 – 3.56 (m, 2H), 3.54 – 3.47 (m, 1H), 3.45 – 3.37 (m, 1H), 1.70 – 1.57 (m, 4H), 1.46 – 1.33 (m, 4H); HRMS (ESI+) *m/z* calcd for $C_{44}H_{51}N_5NaO_{23}P_2S$ [M + H] 1134.2069, found: 1134.2084.



6 7

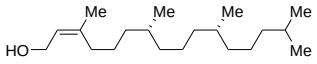
Lipid I (7). To a stirred solution of **6** (9.2 mg, 7.5 μmol) in MeOH (10 mL) was added Pd-C (18 mg). After being stirred under hydrogen atmosphere (1 atm) for 1 h at r.t., Et_3N (0.5 mL) was added to the reaction mixture. After being stirred for 1 h at r.t., the catalyst was filtered through a pad of Celite. The filtrate solution was concentrated *in vacuo* and the resulting product was dried in high vacuum. To a stirred solution of the crude product in 3:1 solution of THF and DMF (0.5 mL) was added CDI (3.6 mg, 0.023 mmol). After being stirred for 3 h at r.t., MeOH (20 μL) was added to the reaction mixture. After being stirred for 30 min, the solution was concentrated *in vacuo* and the resulting product was dried in high vacuum. To a stirred solution of the crude product in 3:1 solution of THF and DMF (0.5 mL) was added C_{55}-P-NH_4 (5.3 mg, 6.0 μmol). After being stirred for 48 h at r.t., the reaction mixture was filtered and concentrated *in vacuo*. To a solution of the crude product in THF (0.5 mL) was added 0.05 mL of aq. LiOH (3.2 mg, 0.075 mmol). After being stirred for 3 h at r.t., the reaction mixture was filtered and concentrated *in vacuo*. The filtrate was purified by reverse phase HPLC [column: Phenomenex Luna (100 Å, 10 μm, C18, 250 x 10 mm), solvents: a gradient elution of 85 : 15 to 100 : 0 MeOH : 0.05 M aqueous

NH_4HCO_3 over 30 min, flow rate: 3.0 mL/min, UV: 220nm] to afford 7.5 mg (60% overall) of **7** (retention time: 24.3 min). ^{1}H NMR (400 MHz, Methanol-d_4) δ 5.57 – 5.51 (m, 1H), 5.48 – 5.42 (m, 1H), 5.18 – 5.06 (m, 11H), 4.55 – 4.48 (m, 2H), 4.41 – 4.10 (m, 6H), 4.03 – 3.96 (m, 1H), 3.88 (q, *J* = 10.3 Hz, 2H), 3.74 (dd, *J* = 12.3, 5.1 Hz, 1H), 3.53 (t, *J* = 9.5 Hz, 1H), 2.99 – 2.88 (m, 2H), 2.79 (s, 1H), 2.31 (s, 3H), 2.14 – 2.03 (m, 31H), 1.99 (q, *J* = 7.8, 7.1 Hz, 8H), 1.90 – 1.78 (m, 2H), 1.74 (s, 3H), 1.68 (s, 21H), 1.63 – 1.58 (m, 10H), 1.47 (d, *J* = 7.2 Hz, 3H), 1.41 (t, *J* = 6.7 Hz, 6H), 1.36 (d, *J* = 7.2 Hz, 3H), 1.30 (d, *J* = 7.6 Hz, 8H), 0.89 (d, *J* = 8.1 Hz, 2H); HRMS (EI) calcd for $C_{86}H_{144}N_7O_{21}P_2$ ([M + H]$^+$): 1672.9891, found: 1672.9908.



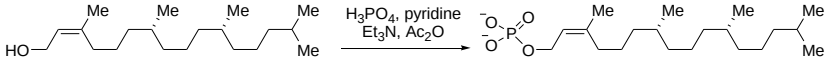
C_{55}-Lipid I-N^{ε}-C_6-dansyl (2e-C_{55}). To a stirred solution of **7** (4.4 mg, 2.6 μmol) and SuO-C(O)O-$(CH_2)_6$-$NHCOCF_3$ (4.6 mg, 0.013 mmol) in MeCN (0.5 mL) was added Et_3N (3.6 μL, 0.026 mmol). After being stirred for 12 h at r.t., the solution was concentrated *in vacuo*. To a stirred solution of the crude product in THF (0.5 mL) was added 0.05 mL of aq. LiOH (1.1 mg, 0.026 mmol). After being stirred for 1 h at r.t., the reaction mixture was filtered and concentrated *in vacuo*. The crude product was purified by reverse phase HPLC [column: Phenomenex Luna (100 Å, 10 μm, C18, 250 x 10 mm), solvents: a gradient elution of 85 : 15 to 100 : 0 MeOH : 0.05 M aqueous NH_4HCO_3 over 30 min, flow rate: 3.0 mL/min, UV: 220nm]. To a stirred solution of the product and $NaHCO_3$ (2.2 mg, 0.026 mmol) in 4 : 1 solution of THF and H_2O (0.5 mL) was added 4-(dansylamino)phenyl isothiocyanate (5.0 mg, 0.013 mmol). After being stirred for 4 h at r.t., the reaction mixture was filtered and concentrated *in vacuo*. The filtrate was purified by reverse phase HPLC [column: Phenomenex Luna (100 Å, 10 μm, C18, 250 x 10 mm), solvents: a gradient elution of 85 : 15 to 100 : 0 MeOH : 0.05 M aqueous NH_4HCO_3 over 30 min, flow rate: 3.0 mL/min, UV: 350nm] to afford **2e-C_{55}** (4.6 mg, 80% overall, retention time: 23.4 min): ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.97 (s, 1H), 7.76 – 7.71 (m, 1H), 7.58 (d, *J* = 8.9 Hz, 1H), 7.32 (t, *J* = 7.7 Hz, 1H), 7.24 (d, *J* = 8.3 Hz, 1H), 7.02 (d, *J* = 7.5 Hz, 1H), 5.49 – 5.42 (m, 1H), 5.39 (t, *J* = 5.7 Hz, 1H), 5.31 – 5.25 (m, 1H), 5.12 – 4.99 (m, 11H), 4.46 (t, *J* = 6.4 Hz, 2H), 4.34 – 4.05 (m, 6H), 4.00 – 3.88 (m, 3H), 3.82 (d, *J* = 11.8 Hz, 1H), 3.61 (td, *J* = 14.4, 13.4, 6.5 Hz, 3H), 3.54 (s, 1H), 3.50 – 3.37 (m, 2H), 3.12 (q, *J* = 7.3 Hz, 2H), 3.04 – 2.94 (m, 1H), 2.29 – 2.15 (m, 4H), 2.07 – 1.88 (m, *J* = 8.8, 7.9 Hz, 41H), 1.66 (s,

3H), 1.61 (s, 21H), 1.55 – 1.51 (m, 10H), 1.46 – 1.40 (m, 3H), 1.33 (dt, *J* = 18.1, 7.1 Hz, 12H), 1.22 (d, *J* = 5.0 Hz, 18H).



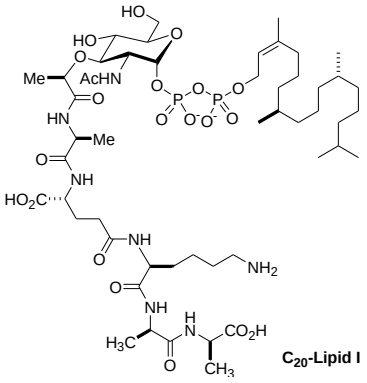
(2*Z*)-Phytol

(2*Z*)-Phytol was purchased as an *E*/*Z* mixture, and used after purification by silica gel column chromatography (hexanes/EtOAc = 95/5): ^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.41 (td, *J* = 7.1, 1.4 Hz, 1H), 4.13 (t, *J* = 3.4 Hz, 2H), 2.09 – 2.01 (m, 2H), 1.74 (d, *J* = 1.1 Hz, 3H), 1.56 – 1.46 (m, 1H), 1.45 – 1.21 (m, 14H), 1.16 – 1.01 (m, 4H), 0.89 – 0.82 (m, 12H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 140.66, 123.97, 59.16, 39.39, 37.44, 37.37, 37.30, 36.86, 32.80, 32.70, 32.21, 27.99, 25.71, 24.80, 24.47, 23.45, 22.72, 22.63, 19.75, 19.69; HRMS (EI) calcd for $C_{20}H_{41}O$ ($[M + H]^+$): 297.3157, found: 297.3172.

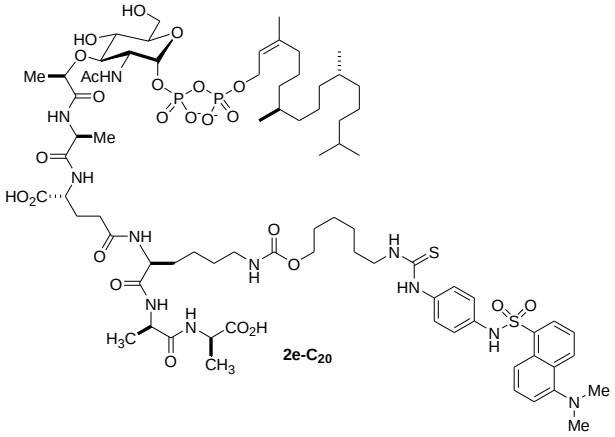


(2*Z*)-Phytol **(2*Z*)-Phytyl phosphate**

(2*Z*)-Phytyl phosphate. To a stirred solution of phosphoric acid (9.80 mg, 0.10 mmol), pyridine (40.4 μL, 0.50 mmol), and (2*Z*)-phytol **S1** (126 mg, 0.42 mmol) was added Et_3N (27.9 μL, 0.20 mmol). After being stirred for 30 min, Ac_2O (18.9 μL, 0.20 mmol) was added to the reaction mixture. After being stirred for 24 h at 80 °C, the reaction mixture was cooled to r.t., and the reaction was quenched with water (1 mL). After being stirred for 1 h at 80 °C, the reaction mixture was cooled to r.t., and the aqueous solution was washed with ether. The aqueous solution was lyophilized, and the crude mixture was purified by DOWEX 50WX8 afforded **(2*Z*)-phytyl phosphate** (24.5 mg, 65%): ^{1}H NMR (400 MHz, Methanol-d_4) δ 5.41 (td, *J* = 7.0, 1.5 Hz, 1H), 4.42 (td, *J* = 6.7, 1.2 Hz, 2H), 3.20 (q, *J* = 7.3 Hz, 2H), 2.09 (ddd, *J* = 8.3, 6.5, 3.4 Hz, 2H), 1.73 (d, *J* = 1.3 Hz, 3H), 1.54 (dp, *J* = 13.1, 6.6 Hz, 1H), 1.46 – 1.01 (m, 16H), 0.95 – 0.78 (m, 12H); ^{13}C NMR (101 MHz, MeOD) δ 141.20, 123.10, 123.02, 63.10, 63.05, 40.54, 38.52, 38.50, 38.39, 37.95, 33.94, 33.22, 29.14, 26.70, 25.88, 25.51, 23.55, 23.08, 22.99, 20.18, 20.11, 9.19; ^{31}P NMR (162 MHz, MeOD) δ 1.08; HRMS (EI) calcd for $C_{20}H_{42}O_4P$ ($[M + H]^+$): 377.2821, found: 377.2838.



C20-Lipid I. The title compound was synthesized according to the procedure described for Lipid I (**7**): ^{1}H NMR (400 MHz, Methanol-d_4) δ 5.54 (d, J = 7.3 Hz, 1H), 5.43 (q, J = 6.6 Hz, 1H), 4.54 (t, J = 6.6 Hz, 2H), 4.41 – 4.09 (m, 6H), 4.02 – 3.95 (m, 1H), 3.92 – 3.79 (m, 2H), 3.74 (dd, J = 12.1, 5.1 Hz, 1H), 3.53 (t, J = 9.5 Hz, 1H), 2.98 – 2.89 (m, 2H), 2.32 (s, 3H), 2.13 – 2.05 (m, 1H), 2.05 – 1.97 (m, 5H), 1.91 – 1.77 (m, 2H), 1.69 (s, 3H), 1.60 – 1.50 (m, 4H), 1.47 (d, J = 7.2 Hz, 4H), 1.45 – 1.35 (m, 14H), 1.35 – 1.22 (m, 10H), 1.19 – 1.04 (m, 4H), 0.88 (t, J = 6.4 Hz, 12H); HRMS (EI) calcd for $C_{51}H_{94}N_7O_{21}P_2$ ([M + H]$^+$): 1202.5978, found: 1202.5995.



C20-Lipid I-N^{ε}-C6-dansyl (2e-C20). The title compound was synthesized according to the procedure described for C_{55}-Lipid I-N^{ε}-C_6-dansyl **(2e-C55)**: ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.55 (d, J = 8.2 Hz, 1H), 8.36 (d, J = 8.7 Hz, 1H), 8.19 (d, J = 7.4 Hz, 1H), 7.76 (d, J = 8.2 Hz, 1H), 7.57 (q, J = 7.5 Hz, 1H), 7.27 (d, J = 7.2 Hz, 1H), 6.96 (s, 4H), 5.41 – 5.21 (m, 2H), 4.88 – 4.81 (m, 2H), 4.38 – 4.29 (m, 2H), 4.12 (tdd, J = 19.8, 11.3, 5.6 Hz, 6H), 4.01 (dd, J = 10.9, 5.4 Hz, 1H), 3.91 (t, J = 6.5 Hz, 2H), 3.85 – 3.78 (m, 2H), 3.74 – 3.68 (m, 1H), 3.54 (dt, J = 10.4, 7.1 Hz, 2H), 3.50 – 3.46 (m, 1H), 3.35 (d, J = 1.2 Hz, 2H), 3.27 – 3.21 (m, 1H), 3.15 – 3.11 (m, 1H), 2.90 – 2.77 (m, 8H), 2.61 – 2.53 (m, 2H), 2.39 – 2.29 (m, 4H), 2.26 – 2.22 (m, 2H), 2.15 (t, J = 7.6 Hz, 2H), 2.06 (d, J = 5.5 Hz, 3H), 2.02 (d, J = 1.2 Hz, 3H), 1.89 (d, J = 1.2 Hz, 4H), 1.61 (q, J = 7.9 Hz, 2H), 1.53 (q, J = 6.4, 5.9 Hz, 2H), 1.41 (d, J = 1.2 Hz, 2H), 1.31 (dd, J = 9.1, 5.0 Hz, 26H), 0.90 (t, J = 6.6 Hz, 12H); HRMS (ESI+) m/z calcd for $C_{77}H_{124}N_{11}O_{25}P_2S_2$ [M + H] 1728.7687, found: 1728.7669.

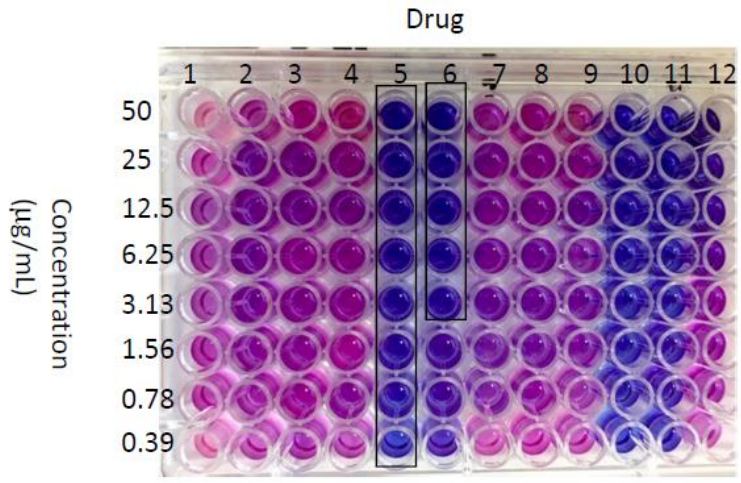
Bacterial strains and growth of bacteria

Mycobacterium smegmatis (ATCC 607), *Staphylococcus aureus* (BAA-1683), *Bacillus subtilis* (ATCC 6051), and *E. coli* (ATCC 10798) were obtained from American Type Culture Collection (ATCC). A single colony of *Mycobacterium smegmatis* was obtained on Difco Middlebrook 7H10 nutrient agar enriched with with albumin, dextrose, and catalase (ADC). Single colonies of *S.aureus* and *E. coli* were grown on tryptic soy agar for 24 h at 37ºC in a static incubator and cultured in tryptic soy broth until log phase to be an optical density (OD) of 0.2-0.5. The OD was monitored at 600 and 570 nm using a 96-well microplate reader. A single colony of *C. difficile* was obtained on a BHI agar plate and incubated at 37 ºC under anaerobic conditions for 48 h (Gas: 10% hydrogen, 5% carbon dioxide and 85% nitrogen mixture was used. Chambers: Plas Labs™ Model 855 Anaerobic Chambers was used.). The other bacteria were cultured in the recommended conditions by ATCC.

MIC assays

Minimum inhibitory concentrations were determined by broth dilution microplate alamar blue assay or by OD measurement. All compounds were stored in DMSO or water (1 mg/100 μL concentration). This concentration was used as the stock solution for all MIC studies. Each compound from stock solution was placed in the first well of a sterile 96 well plate and a serial dilution was conducted with the culturing broth (total volume of 10 μL). The bacterial suspension at log phase (190 μL) was added to each well (total volume of 200 μL). *M. smegmatis, S. aureus, E. coli,* and the aerobic bacteria were incubated for 24 h (48-72 h for *M. smegmatis*) at 37 ºC. 20 μL of resazurin (0.02%) was added to each well and incubated for 4 h for *Mycobacterium spp.*, and 1 h for the other bacteria (National Committee for Clinical Laboratory Standards (NCCLS) method (pink = growth, blue = no visible growth)).The OD measurements were performed for all experiments prior to colorimetric The anaerobic bacteria were applied to a microplate reader. The MIC values were determined according to the colorimetric assays using resazurin. The absorbance of each well was also measured at 570 and 600 nm via UV-Vis. If necessary, CFU method was applied to confirm bactericidal activity. MIC against *M. tuberculosis* was performed at UTHSC RBL. Due to the compliance, images acquired in the BSL-3 in RBL are prohibited to show. The MIC data were recorded on laminated paper.

Figure S1. MIC assay against *M. smegmatis* (ATCC607).



	MIC (μg/mL)
1. Polyoxin D	>50
2. Nikkomycin Z	>50
3. ToXa-1	>50
4. PyDT-1	>50
5. Ristocetin A	**<0.39**
6. Ramoplanin A2	3.13
7. DMSO	>100
8. DyDT-1 + polymixin nonapeptide	>100
9. Toxa-1 + polymixin nonapeptide	>100
10. Ristocetin A + polymixin nonapeptide	<0.39
11. Ramoplanin A2 + polymixin nonapeptide	<0.39
12. vancomycin	12.5-25.0

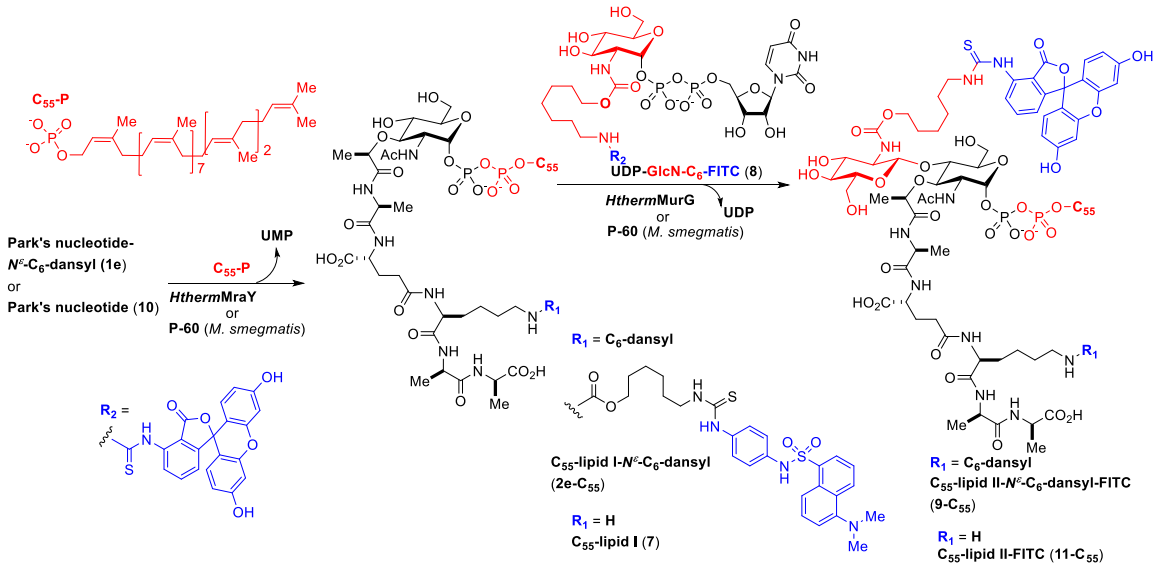
Tunicamycin was not included in this plate.
Average MIC of tunicamycin is 6.25 μg/mL.

Preparation of membrane fraction P-60 containing MraY and MurG

M. smegmatis cells were harvested by centrifugation (4700 rpm) at 4 ºC followed by washing with 0.9% saline solution (thrice). The washed cell pellets were washed with homogenization buffer (containing 50 mM K_2HPO_4, 5 mM $MgCl_2$, 5 mM 1,4-dithio-DL-threitol, and 10% glycerol, pH = 7.2) (thrice), and approximately 5 g of pellet (wet weight) was collected. The washed cell pellets were suspended in homogenization buffer and disrupted by probe sonication on ice (5 s on and 2 s off for 1 min, then cool down for 1.5 min, 5 cycles, cool down for 15 min, and then, 5 s on and 2 s off for 1 min, cool down for 1.5 min, 5 cycles). The resulting suspension was centrifuged at 4,700 x g for 15 min at 4 ºC to remove unbroken cells. The lysate was centrifuged at 25,000 x g for 20 min at 4 ºC. The supernatant was subjected to ultracentrifugation at 60,000 x g for 1 h at 4 ºC. The supernatant was discarded, and the membrane fraction containing MurG enzyme (P-60) was suspended in the Tris-HCl buffer (pH = 7.5). Total protein concentrations were approximately 8 to 10 mg/mL. Aliquots were stored in Eppendorf tubes at -80 ºC. Similarly, the membrane fractions containing WecA enzyme (P-60) were prepared from *E. coli*.

MurG assay.

MurG assay substrates, Park's nucleotide-N^{ε}-C_6-dansyl, UDP-GlcN- N^{ε}-C_6-FITC, were chemically synthesized according to the reported procedures.



Protocol A:

Park's nucleotide-N^{ε}-C_6-dansylthiourea (**1e**) (2 mM stock solution, 1.88 µL), CHAPS (20%, 1.25 µL), β-mercaptoethanol (50 mM, 5 µL), $MgCl_2$ (0.5 M, 5 µL), KCl (2 M, 5 µL), and C_{55}-phosphate dissolved in $NaHCO_3$ (50 mM) : DMSO (1 : 4) (4 mM, 2.81 µL) were placed in a 1.5 mL Eppendorf tube. To a stirred reaction mixture, *Hy*MraY (4.18 mg/mL, 1 µL) was added (total volume of reaction mixture: 50 µL adjust with Tris buffer (50 mM, pH = 8.0)). The reaction mixture was incubated for 1 h at 37 °C. To a reaction mixture, inhibitor molecule (0 - 100 µg/mL in Tris buffer), UDP-GlcNAc (10 mM stock solution, 1.88 µL), and P60 (1 mg/µL, 30 µL) or *Hy*MurG (5.2 mg/mL, 5 µL) were added. The reaction mixture was incubated for 1 h at 37 °C, and quenched with water saturated n-butanol (150µL). Two phases were mixed via vortex for 2 min and centrifuged at 25,000 xg for 10 min. The upper n-butanol phase was assayed via reverse-phase HPLC. The n-butanol phase (30 µL) was injected into HPLC (solvent: a gradient elution of CH_3OH/0.05 M aq. NH_4HCO_3 = 70 : 30 to 100 : 0 over 30 min; UV: 350 nm; flow rate: 1.0 mL/min; column: Luna 5µm C_8, 100 A, 250 x 4.60 mm), and the area of the peak for C_{55}-lipid II-N^{ε}-C_6-dansyl-FITC was quantified to obtain the IC_{50} value. The IC_{50} values were calculated from plots of the percentage product inhibition versus the inhibitor concentration. All inhibition curves, Michaelis-Menten plots and Lineweaver-Burk plots were obtained by using GraphPad Prism 7.04.

Protocol B:

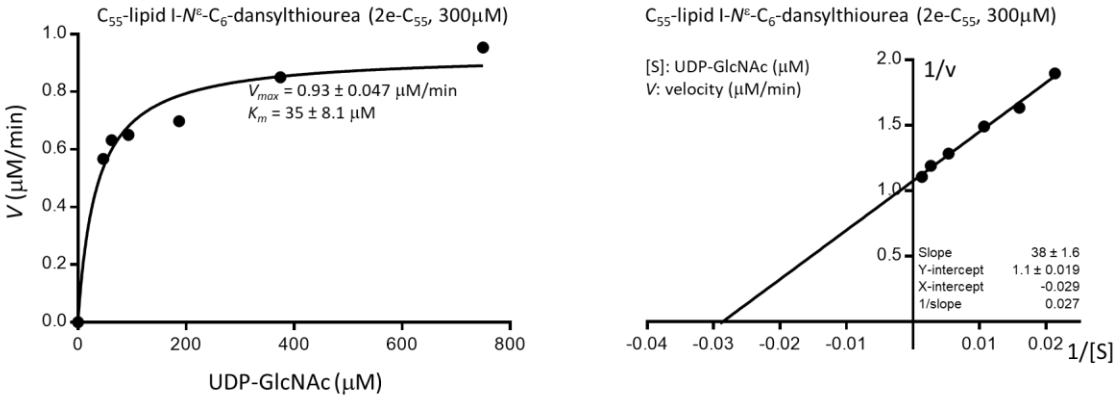
Park's nucleotide-N^{ε}-C_6-dansylthiourea (**1e**) (2 mM stock solution, 1.88 µL), CHAPS (20%, 1.25 µL), β-mercaptoethanol (50 mM, 5 µL), $MgCl_2$ (0.5 M, 5 µL), KCl (2 M, 5 µL), and C_{55}-phosphate dissolved in $NaHCO_3$ (50 mM) : DMSO (1 : 4) (4 mM, 2.81 µL) were placed in a 1.5 mL Eppendorf tube. To a stirred reaction mixture, P60 (1 mg/µL, 30 µL) was added (total volume of reaction mixture: 60 µL adjust with Tris buffer (50 mM, pH = 8.0)). The reaction mixture was incubated for 1 h at 37 °C. To a reaction mixture, tunicamycin (10

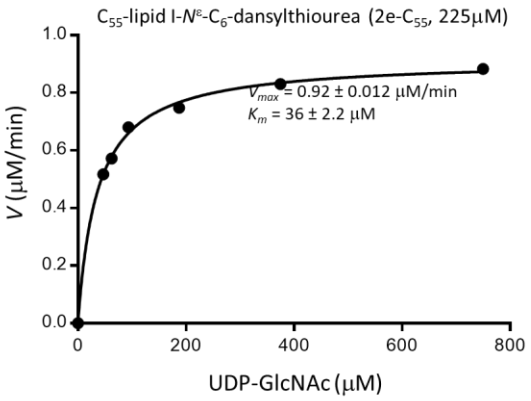
mg/mL stock solution, 0.25 μL) was added, then inhibitor molecule (0 - 100 μg/mL in Tris buffer) and UDP-GlcNAc (10 mM stock solution, 1.88 μL) were added. The reaction mixture was incubated for 1 h at 37 °C, and quenched with water saturated n-butanol (150μL). Two phases were mixed via vortex for 2 min and centrifuged at 25,000 xg for 10 min. The upper n-butanol phase was assayed via reverse-phase HPLC. The n-butanol phase (30 μL) was injected into HPLC (solvent: a gradient elution of CH_3OH/0.05 M aq. NH_4HCO_3 = 70 : 30 to 100 : 0 over 30 min; UV: 350 nm; flow rate: 1.0 mL/min; column: Luna 5μm C_8, 100 A, 250 x 4.60 mm), and the area of the peak for C_{55}-lipid II-N^{ε}-C_6-dansyl-FITC was quantified to obtain the IC_{50} value. The IC_{50} values were calculated from plots of the percentage product inhibition versus the inhibitor concentration. All inhibition curves, Michaelis-Menten plots and Lineweaver-Burk plots were obtained by using GraphPad Prism 7.04.

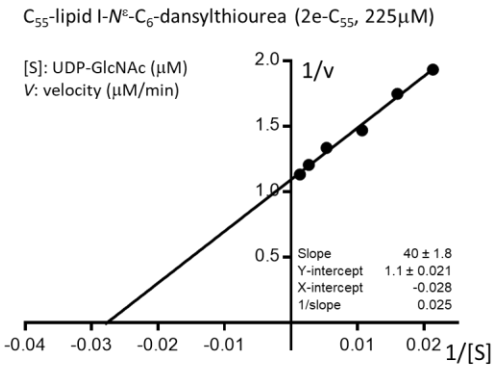
UV/VIS spectroscopy-based assay

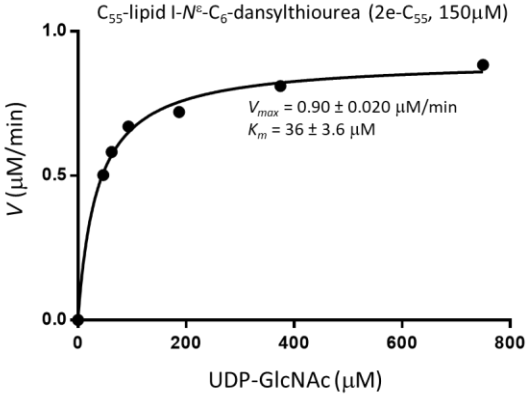
Park's nucleotide-N^{ε}-C_6-dansylthiourea (**1e**) (2 mM stock solution, 1.88 μL), CHAPS (20%, 1.25 μL), β-mercaptoethanol (50 mM, 5 μL), $MgCl_2$ (0.5 M, 5 μL), KCl (2 M, 5 μL), and C_{55}-phosphate dissolved in $NaHCO_3$ (50 mM) : DMSO (1 : 4) (4 mM, 2.81 μL) were placed in a 1.5 mL Eppendorf tube. To a stirred reaction mixture, *Hy*MraY (4.18 mg/mL, 1 μL) was added (total volume of reaction mixture: 50 μL adjust with Tris buffer (50 mM, pH = 8.0)). The reaction mixture was incubated for 1 h at 37 °C. To a reaction mixture, inhibitor molecule (0 - 100 μg/mL in Tris buffer), UDP-GlcN-C_6-FITC (10 mM stock solution, 1.88 μL), and P60 (1 mg/μL, 30 μL) or *Hy*MurG (5.2 mg/mL, 5 μL) were added. The reaction mixture was incubated for 1 h at 37 °C, and quenched with water saturated n-butanol (150μL). Two phases were mixed via vortex for 2 min and centrifuged at 25,000 xg for 10 min. The upper n-butanol phase was washed with n-butanol saturated 1 : 1 mixture of saline/0.2 M mannitol (50 μL, thrice) and then, the washed n-butanol phase (20 μL) was transferred to a 384 well black plate and fluorescence was measured at an excitation of 485 nm and emission of 528 nm. The IC_{50} values were calculated from plots of the percentage product inhibition versus the inhibitor concentration. All inhibition curves, Michaelis-Menten plots and Lineweaver-Burk plots were obtained by using GraphPad Prism 7.04.

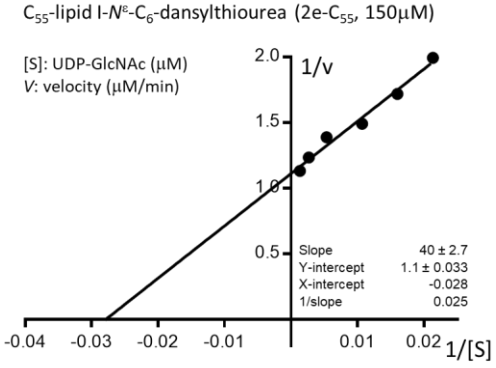
Figure S2. Michaelis-Menten plots and Lineweaver-Burk plots for 2e-C_{55} at the different concentrations of UDP-GlcNAc.

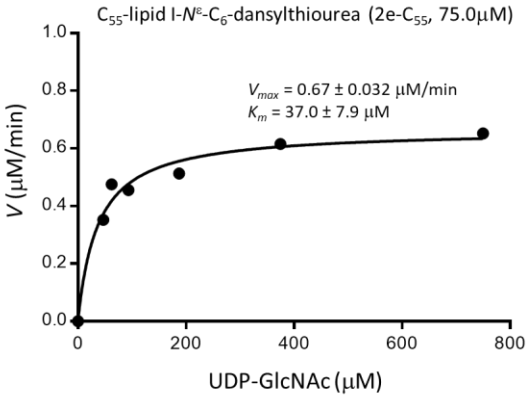


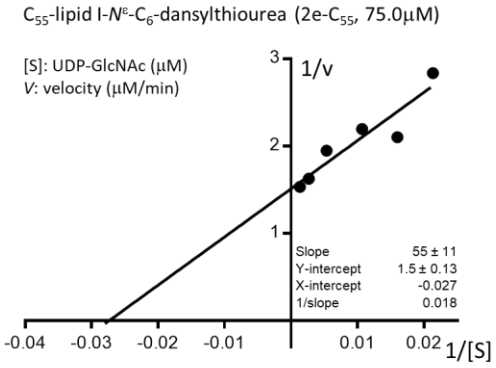

C55-lipid I-Nε-C6-dansylthiourea (2e-C55, 225μM)
Vmax = 0.92 ± 0.012 μM/min
Km = 36 ± 2.2 μM
V (μM/min)
UDP-GlcNAc (μM)


C55-lipid I-Nε-C6-dansylthiourea (2e-C55, 225μM)
[S]: UDP-GlcNAc (μM)
V: velocity (μM/min)
1/v
Slope 40 ± 1.8
Y-intercept 1.1 ± 0.021
X-intercept -0.028
1/slope 0.025
1/[S]


C55-lipid I-Nε-C6-dansylthiourea (2e-C55, 150μM)
Vmax = 0.90 ± 0.020 μM/min
Km = 36 ± 3.6 μM
V (μM/min)
UDP-GlcNAc (μM)


C55-lipid I-Nε-C6-dansylthiourea (2e-C55, 150μM)
[S]: UDP-GlcNAc (μM)
V: velocity (μM/min)
1/v
Slope 40 ± 2.7
Y-intercept 1.1 ± 0.033
X-intercept -0.028
1/slope 0.025
1/[S]


C55-lipid I-Nε-C6-dansylthiourea (2e-C55, 75.0μM)
Vmax = 0.67 ± 0.032 μM/min
Km = 37.0 ± 7.9 μM
V (μM/min)
UDP-GlcNAc (μM)


C55-lipid I-Nε-C6-dansylthiourea (2e-C55, 75.0μM)
[S]: UDP-GlcNAc (μM)
V: velocity (μM/min)
1/v
Slope 55 ± 11
Y-intercept 1.5 ± 0.13
X-intercept -0.027
1/slope 0.018
1/[S]

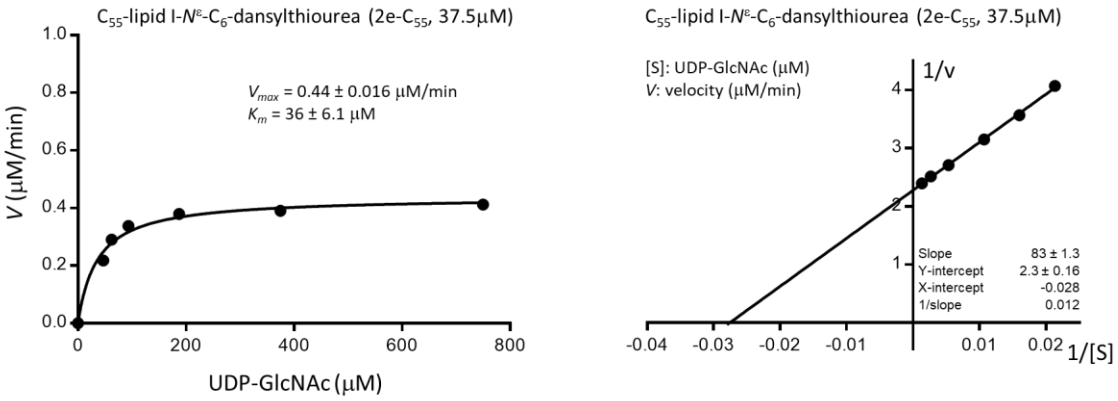


Figure S3. Superimposed Lineweaver-Burk plots for 2e-C_{55} at the different concentrations of UDP-GlcNAc.

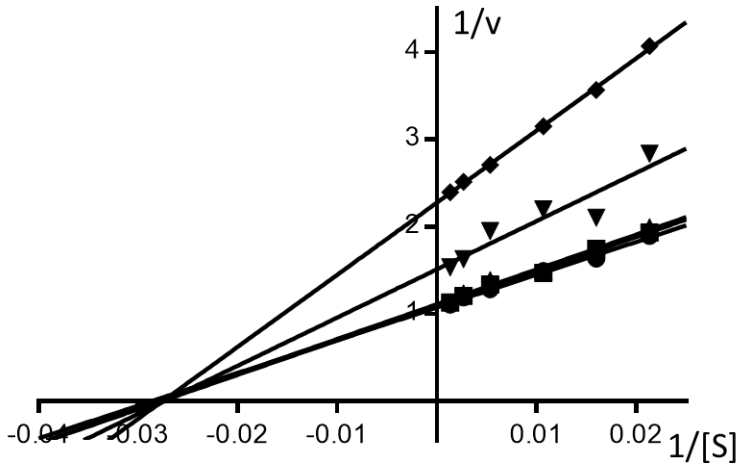
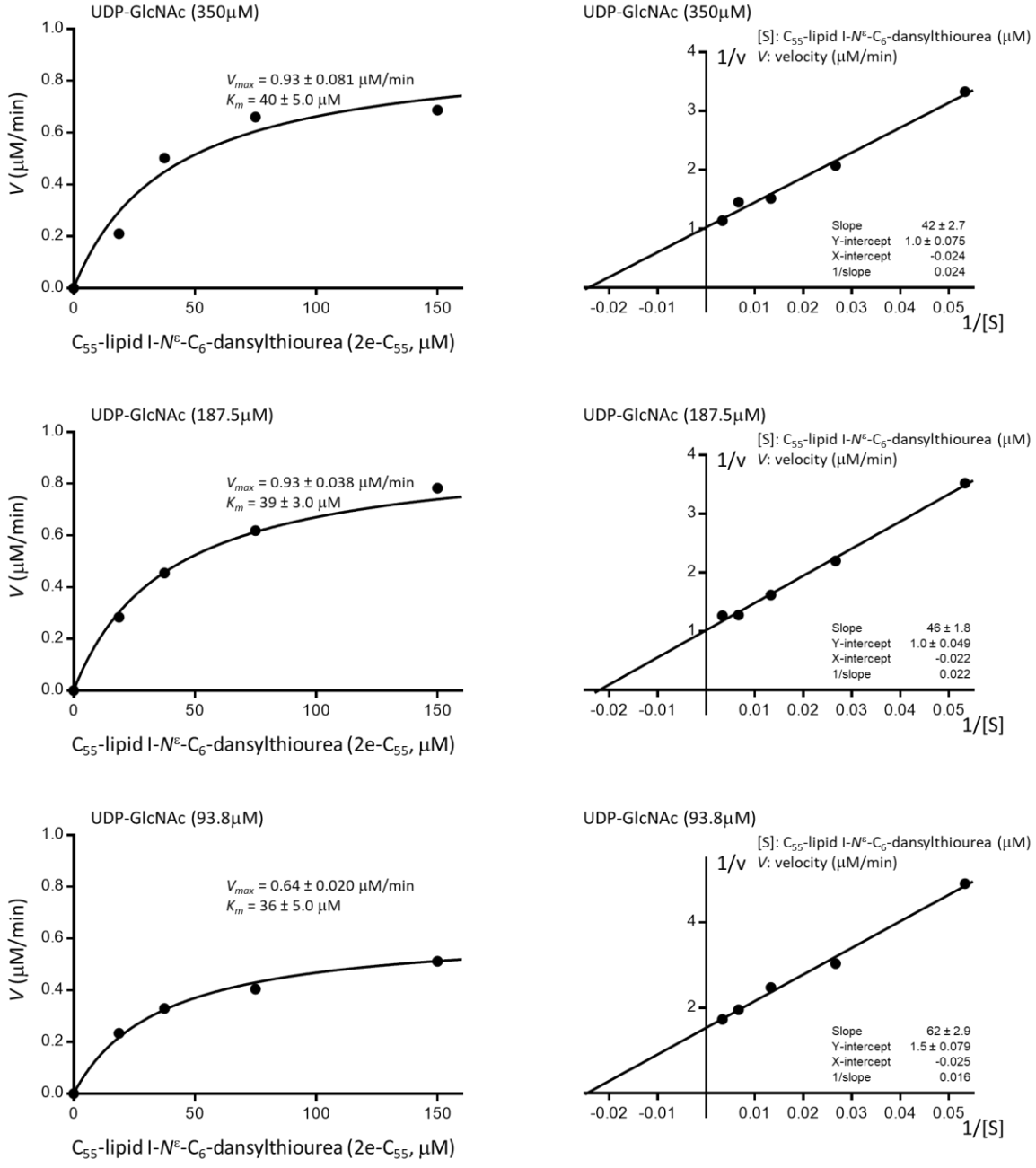


Figure S4. Michaelis-Menten plots and Lineweaver-Burk plots for UDP-GlcNAc at the different concentrations of 2e-C_{55}.



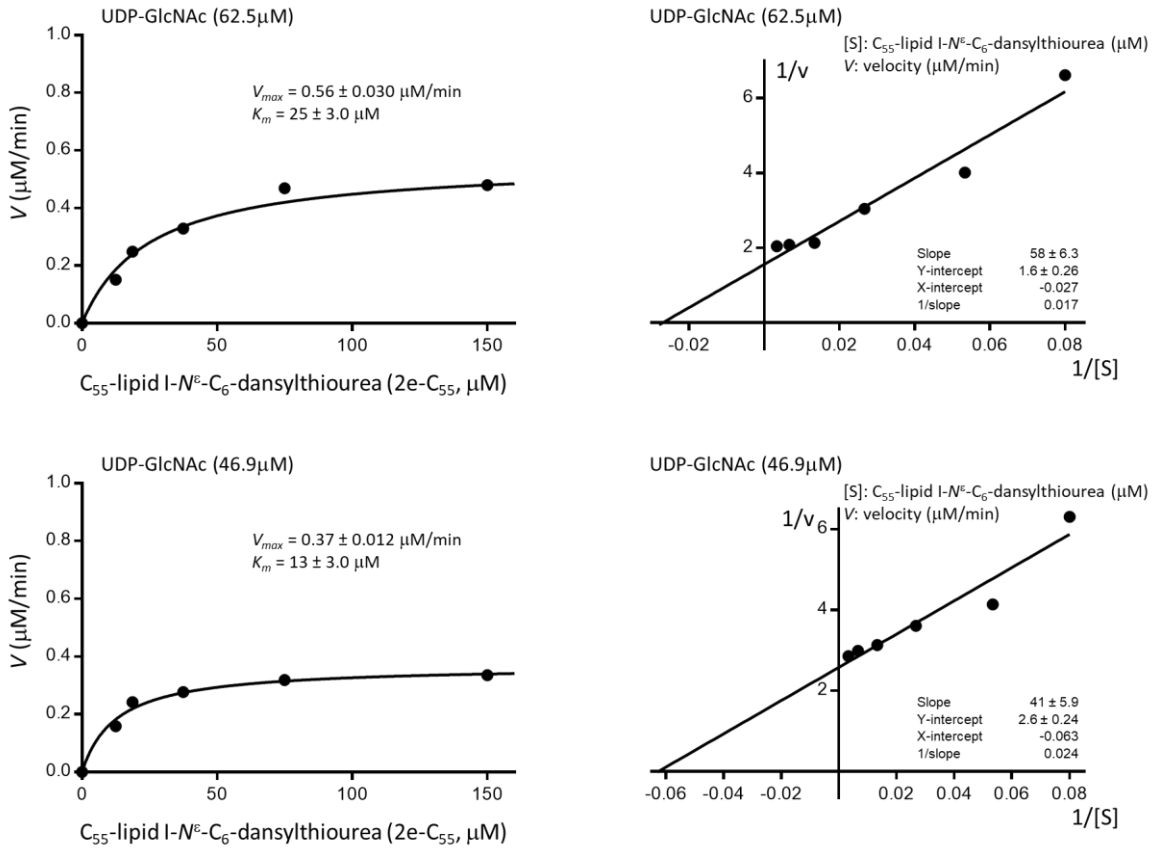


Figure S5. Superimposed Lineweaver-Burk plots for UDP-GlcNAc at the different concentrations of 2e-C_{55} (93.8-350 μM)

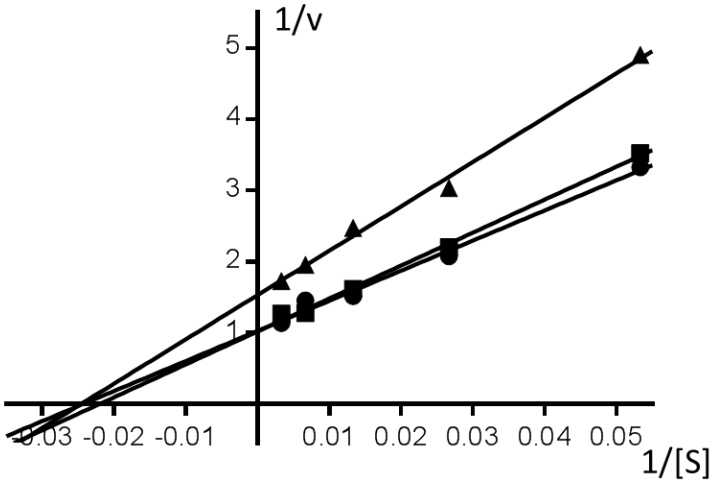


Figure S6. Inhibition curve for 2e-C_{55} by restocetin A against MurG.

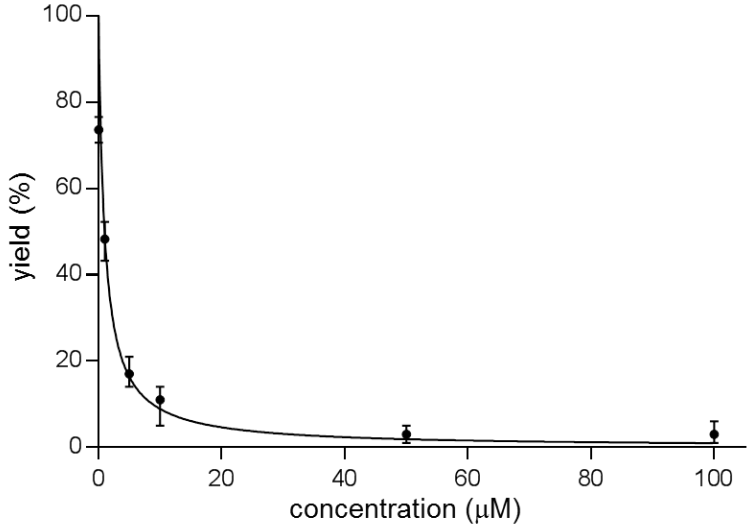


Figure S7. Inhibition curve for 1e by restocetin A against MraY (IC_{50} 0.81 μM against *Msmeg*MraY).

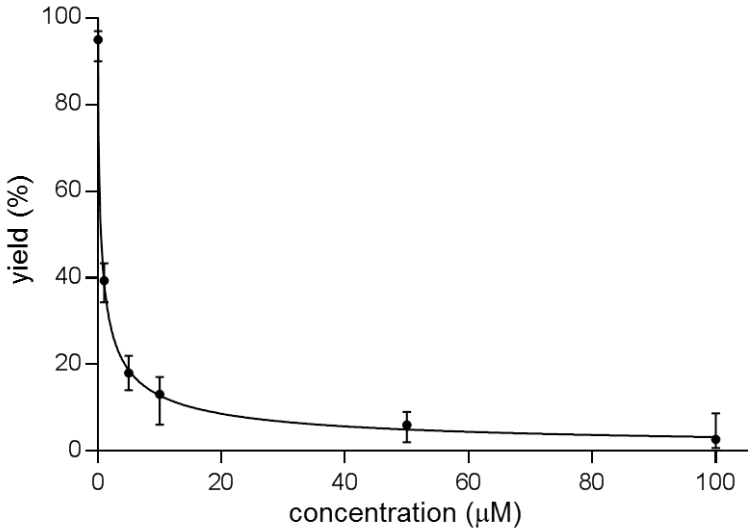


Figure S8. *MsmegMraY* kinetics in the presence of ristocetin A at different concentrations of 1e.

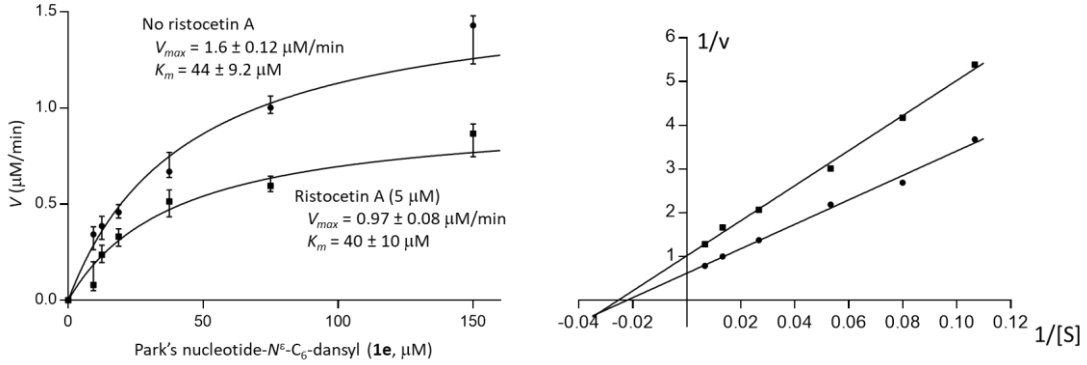


Figure S9. *MsmegMraY* kinetics in the presence of ristocetin A at different concentrations of C_{55}-P.

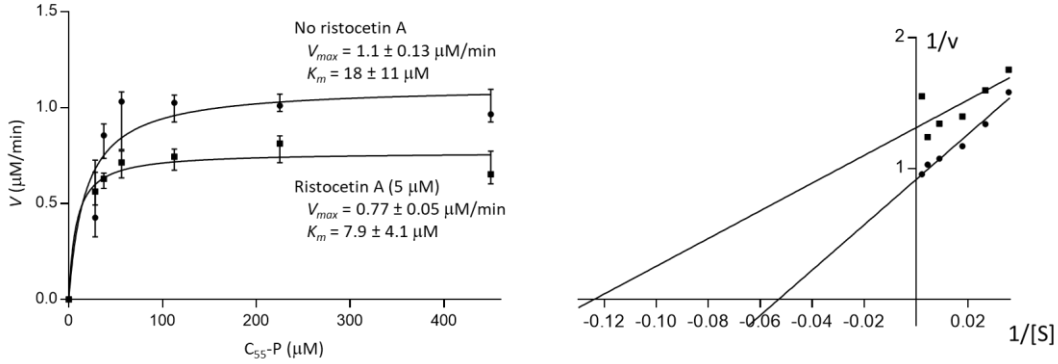


Figure S10. Product-time curve for the MurG-catalyzed reactions (2e-C_{55} (75 μM) UDP-GlcNAc (375 μM).

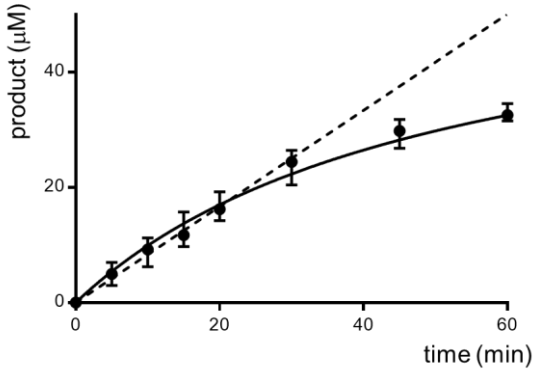


Figure S11. Effect of DMSO on the MurG activity.

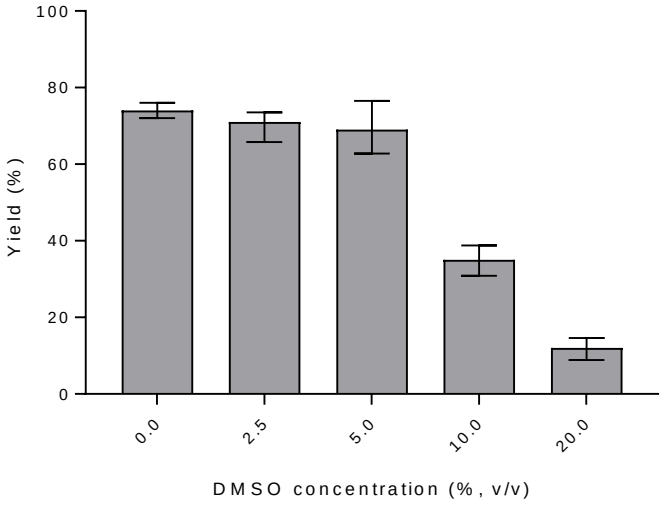


Table S1. MraY, WecA and AglH assays [7], [8] against 15 and 16.

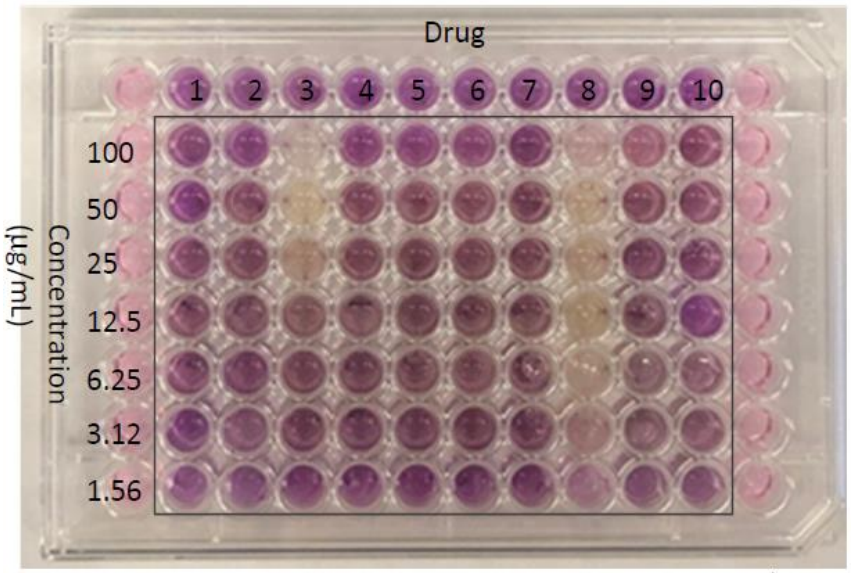
entry	Compound	*Hy*MraY inhibition (%)			WecA inhibition (%)			*Mj*AglH inhibition (%)		
		1 μM	10 μM	100 μM	1 μM	10 μM	100 μM	1 μM	10 μM	100 μM
1	ToXa-1 (**15**)	0	0	0	0	0	0	0	0	0
2	PyDT-1 (**16**)	0	0	0	0	0	13	0	0	9

Each experiment was performed two-three times. Analyses: HPLC-based method

Determination of cytotoxicity in Vero Cells

Selected molecules were tested for cytotoxicity (IC50) in Vero cells via MTT colorimetric assay. Vero cell line was cultured in Complete eagle`s minimum essential growth medium (EMEM) containing L-glutamine, sodium pyruvate, minimum essential amino acids, penicillin-streptomycin and 10% fetal bovine serum. Inoculating number of cells were 400,000 cells/mL and a final 40,000 cells/well. After 48h of exposure of molecules to this cell line at concentrations ranging from 0.78 to 100 μg/mL, 10 μL of MTT, yellow tetrazolium dye; was added to each well and the assay was terminated by removing the media and addition of 100 μL of DMSO. The Viability was assessed on the basis of cellular conversion of MTT into a purple formazan product. The absorbance of the colored formazan product was measured at 570 nm by BioTek Synergy HT Spectrophotometer. Linearity of the MTT response to the cell number was determined.

Figure S12. MTT assay in Vero cells.



Purple: viable cells remained
Clear-Pink: cells were killed

	IC_{50} (μg/mL)
1. Polyoxin D	>100
2. Nikkomycin Z	>100
3. ToXa-1	12.5
4. PyDT-1	>100
5. Ristocetin A	**>100**
6. Ramoplanin A2	>100
7. Vancomycin	>100
8. Tunicamycin	1.56
9. Rifampicin	>100
10. Ristocetin A + polymixin nonapeptide	>100

Structural comparison between MurG proteins.

Hydrogenivirga sp. (128-5-R1-1) and *M. tuberculosis* (H37Rv): 26% identity /42% similarity

```
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E. coli (K-12) and *M. tuberculosis* (H37Rv): 39% identity /53% similarity

References

[1] Li, K., Kurosu, M. (2008) Synthetic Studies on Mycobacterium tuberculosis Specific Fluorescent Park's Nucleotide Probe. *Hetrocycles 76*, 445-469.

[2] Van Niieuwenhze, M. S., Mauldin, S. C., Zia-Ebrahimi, M., Winger, B. E., Hornback, W. J., Saha, S. L., Aikins, J. A., Blaszack, L. C. (2002) The first total synthesis of lipid II: The final monomeric intermediate in bacterial cell wall biotynthesis. *J. Am. Chem. Soc. 124*, 3656-3660.

[3] Schwartz, B., Markwalder, J. A., Wang, Y. (2001) Lipid II: Total synthesis of the bacterial cell wall precursor and utilization as a substrate for glycosyltransfer and transpeptidation by penicillin binding protein (PBP) 1b of Escherichia coli. *J. Am. Chem. Soc*. *12*, 11638-11643.

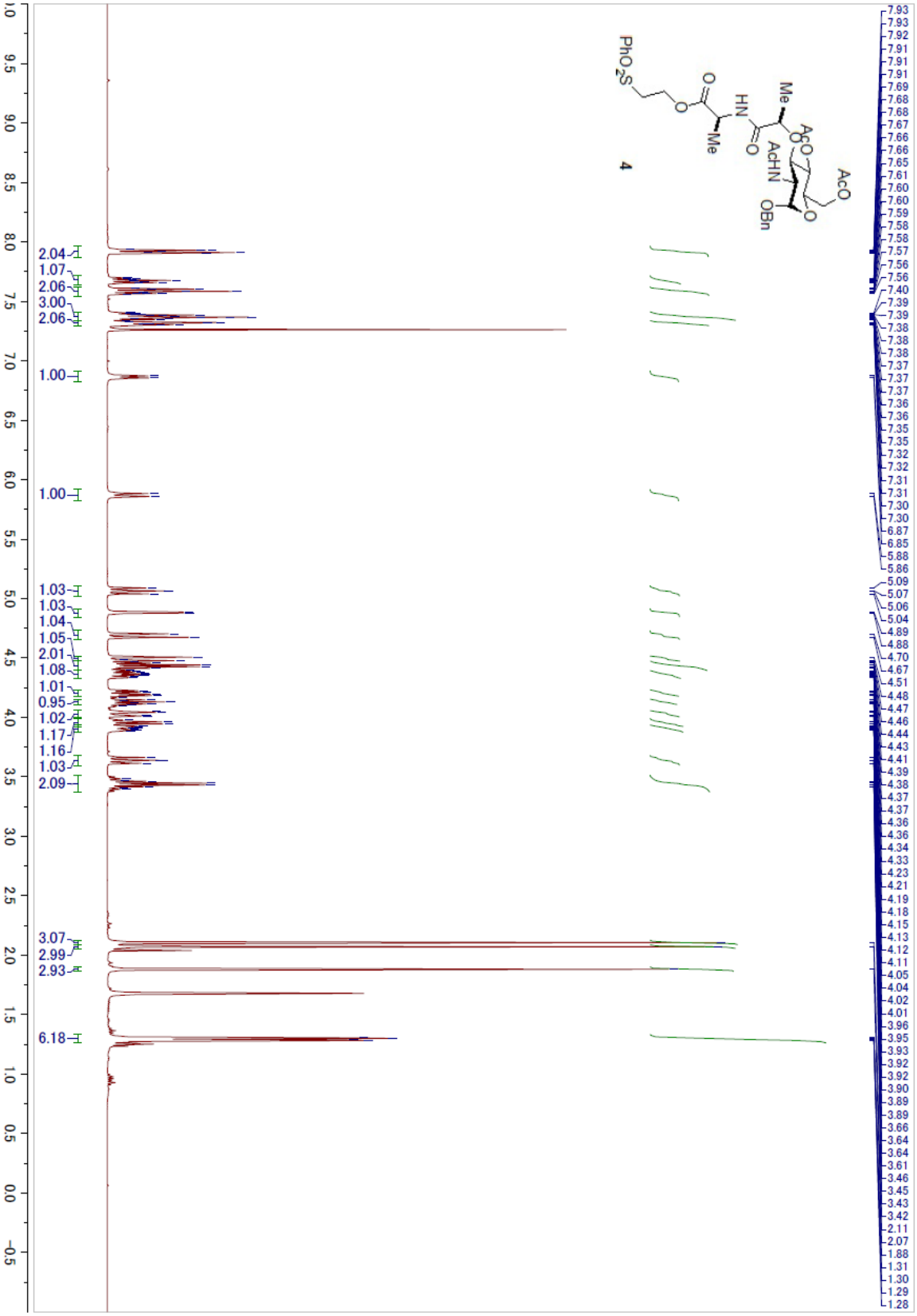
[4] Wang, Q., Wang, Y., Kurosu, M. (2012) A new Oxyma derivative for nonracemizable amide-forming reactions in water. *Org. Lett*. *14*, 3372-3375.

[5] Kurosu, M., Mahapatra, S., Narayanasamy, P., Crick, D. C. (2007) Chemoenzymatic synthesis of Park's nucleotide: toward the development of high-throughput screening for MraY inhibitors. *Tetradedron Lett*. *48*, 799-803.

[6] Li, K., Kurosu, M. (2008) Synthetic Studies on Mycobacterium tuberculosis Specific Fluorescent Park's Nucleotide Probe. *Heterocycles 76*, 455-469.

[7] Mitachi, K., Siricilla, S., Yang, D., Kong, Y., Skorupinska-Tudek, K., Swiezewska, E., Franzblau, S. G., Kurosu, M. (2016) Fluorescence-based assay for polyprenyl phosphate-GlcNAc-1-phosphate transferase (WecA) and identification of novel antimycobacterial WecA inhibitors. *Anal. Biochem*. *512*, 78-90.

[8] Mitachi, K., Yun, H-G., Kurosu, S. M., Eslamimehr, S., Lemieux, M. R., Klaić, L., Clemons, W. M., Kurosu, M. (2018) Novel FR-900493 analogues that inhibit the outgrowth of Clostridium difficile spores. *ACS Omega*, *3*, 1726-1739.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1.03
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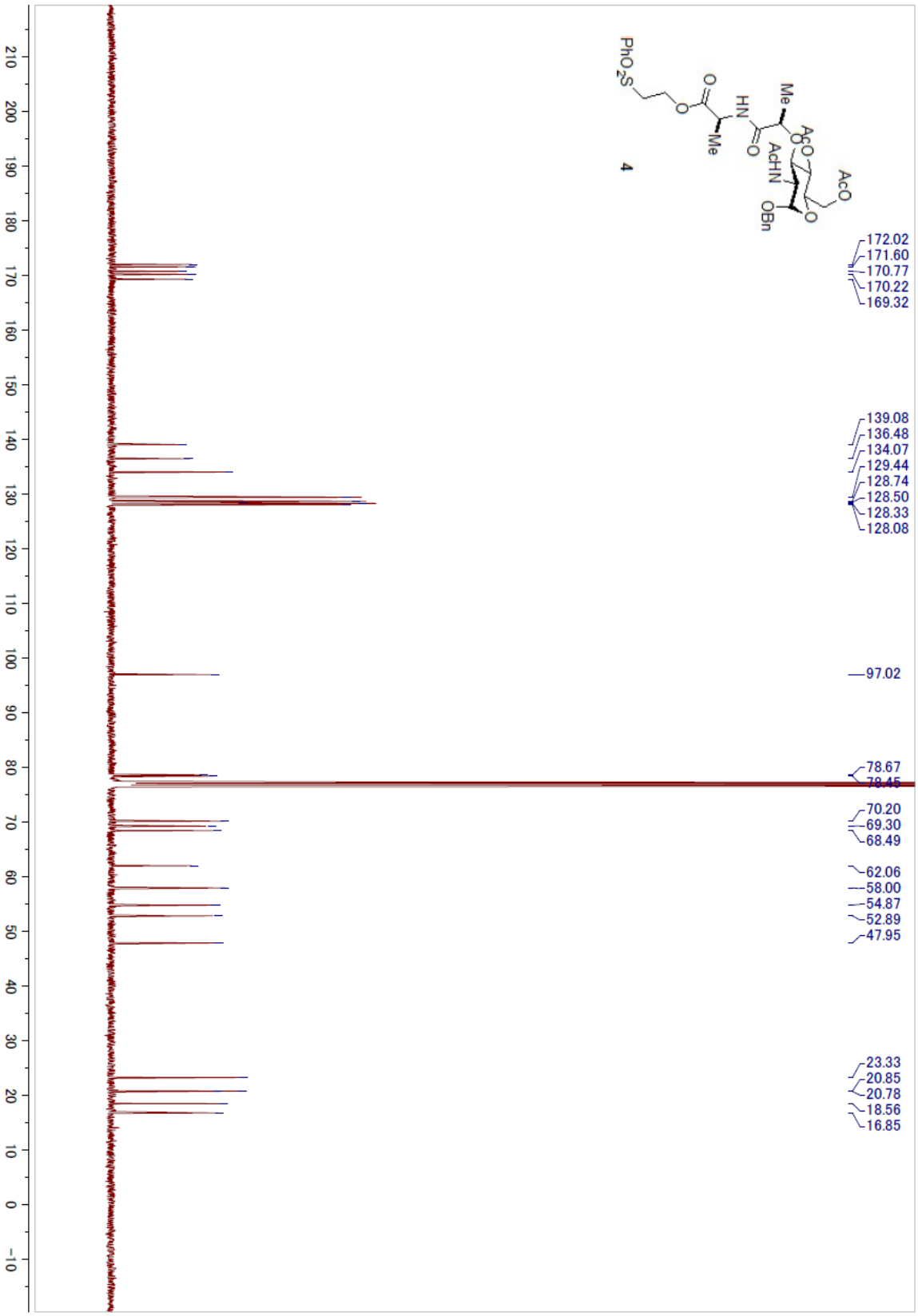
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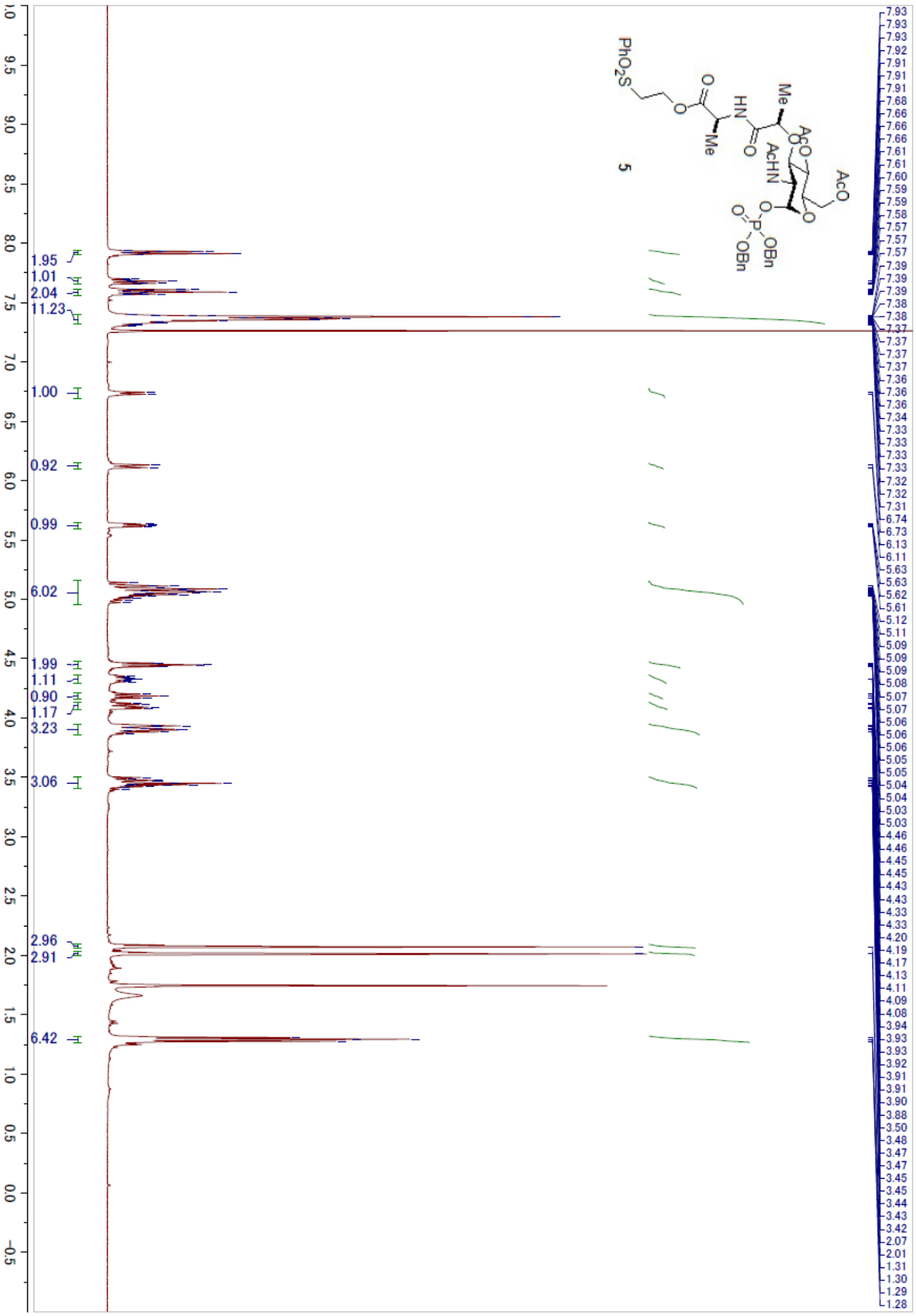
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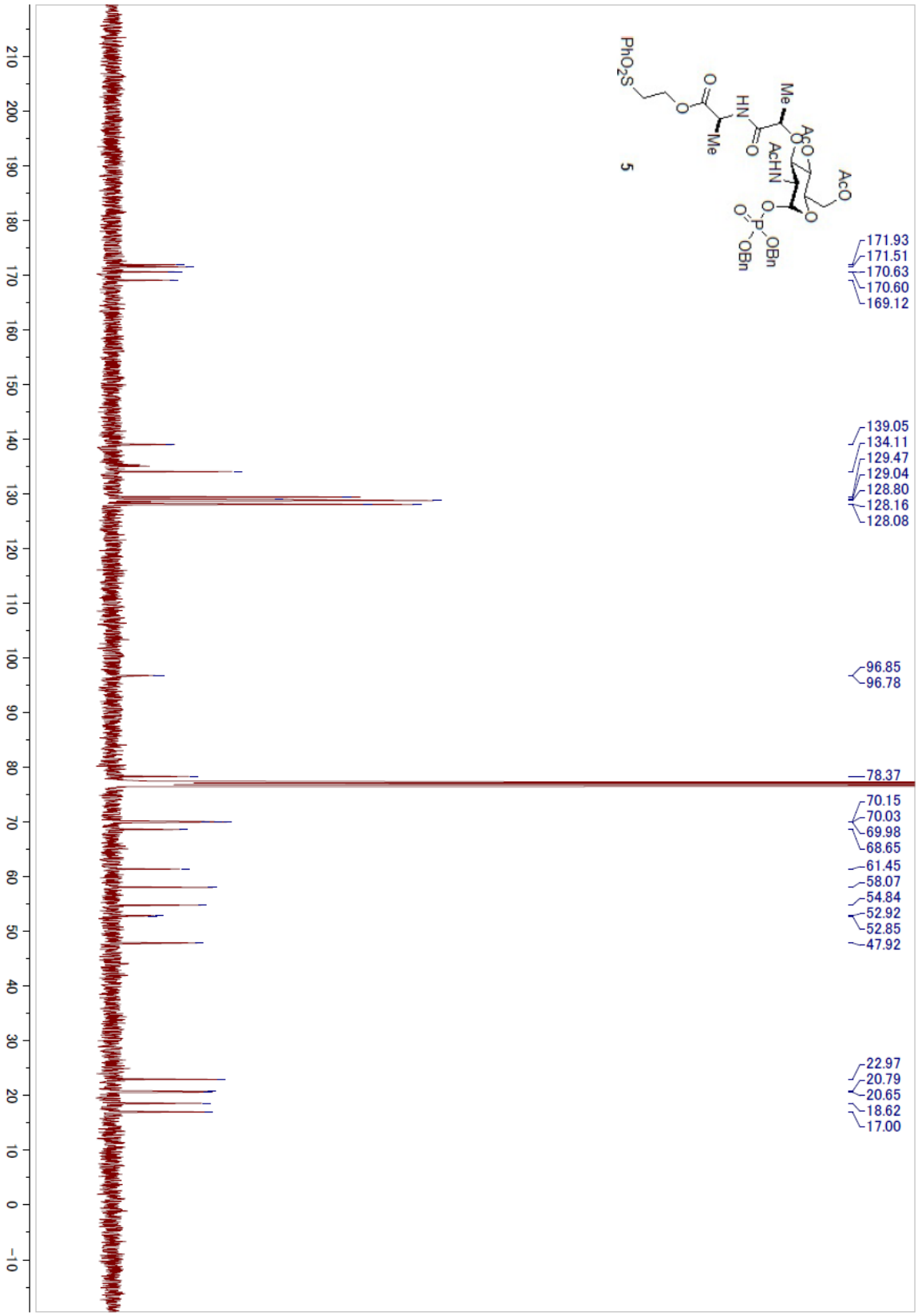
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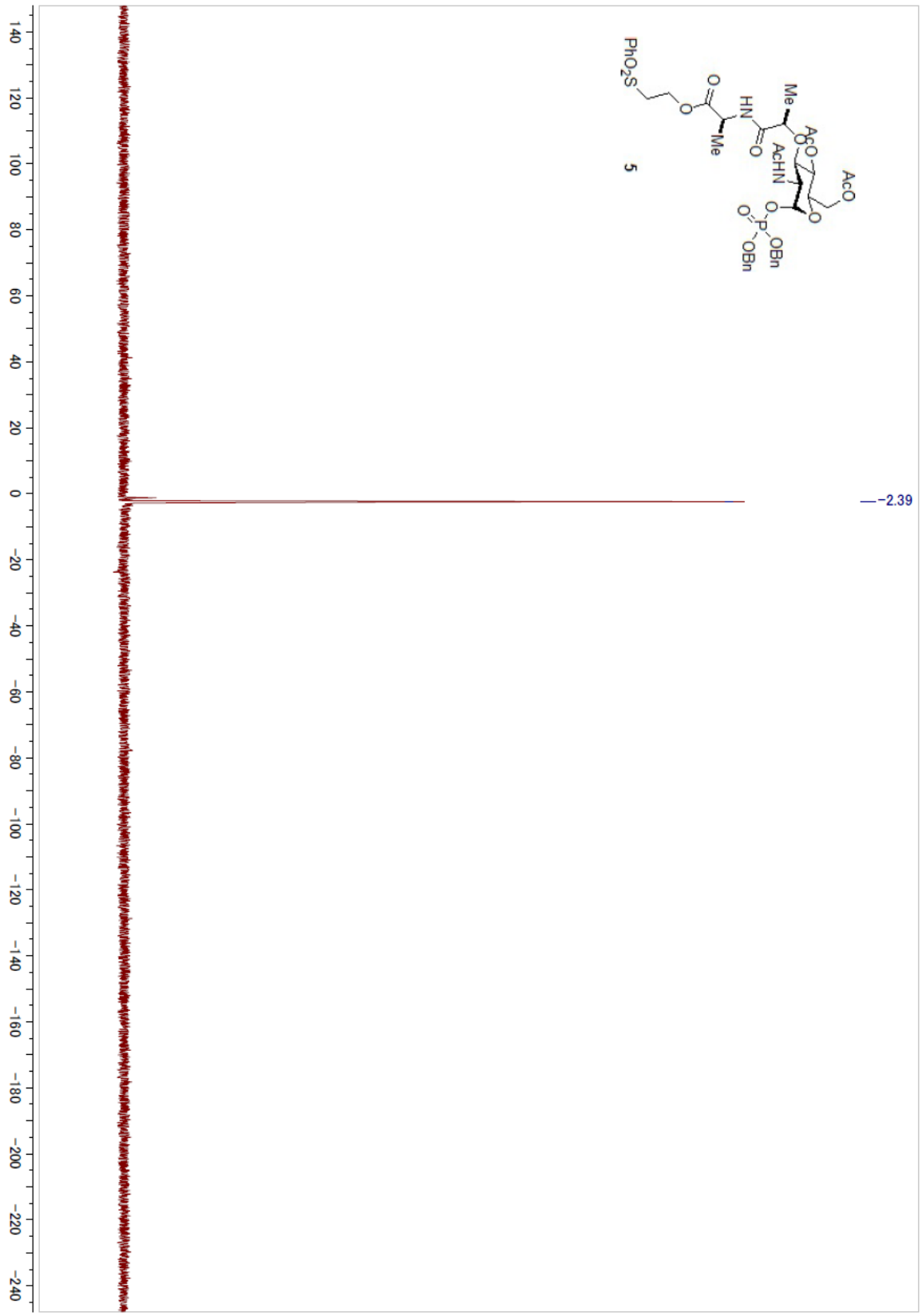
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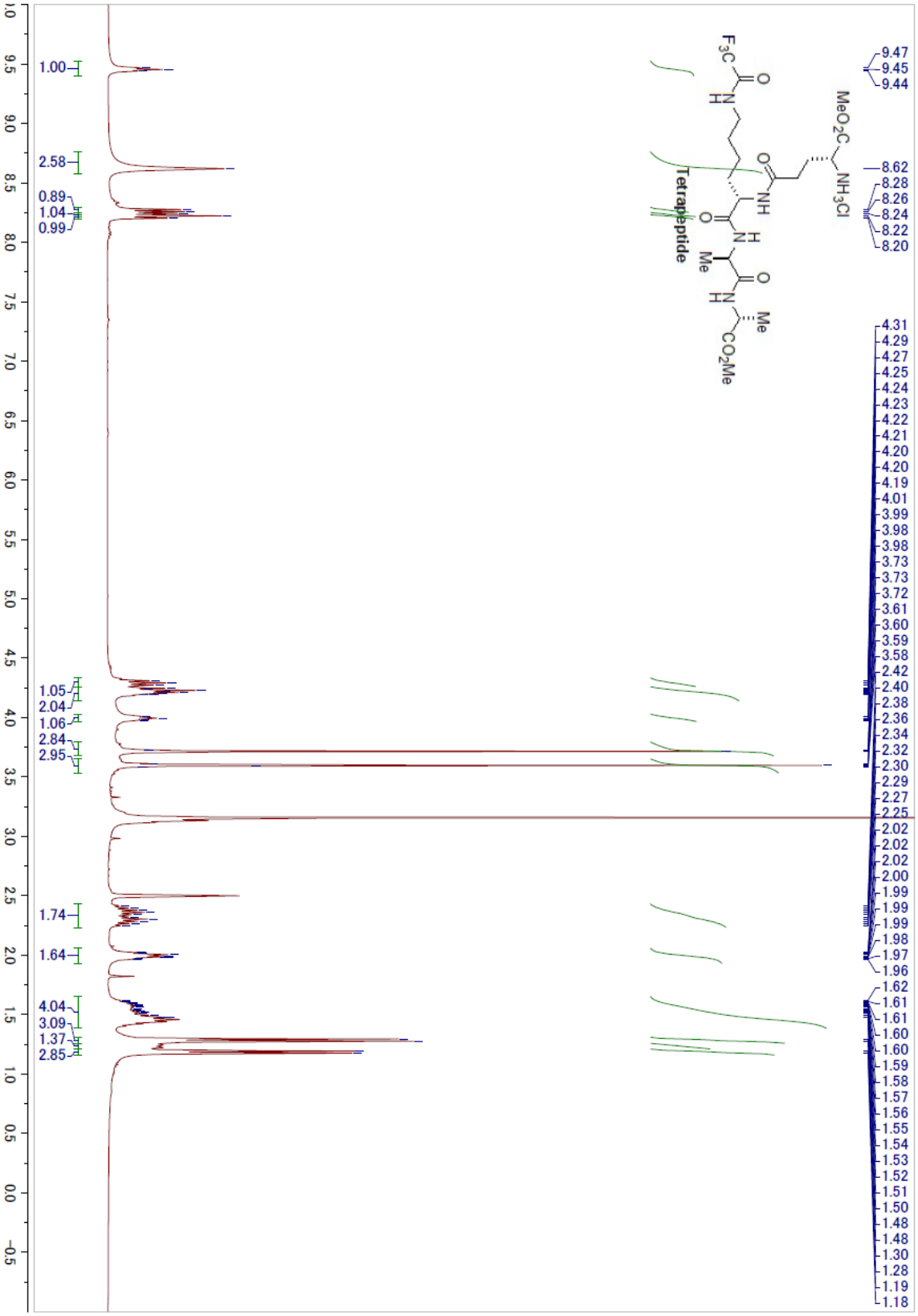
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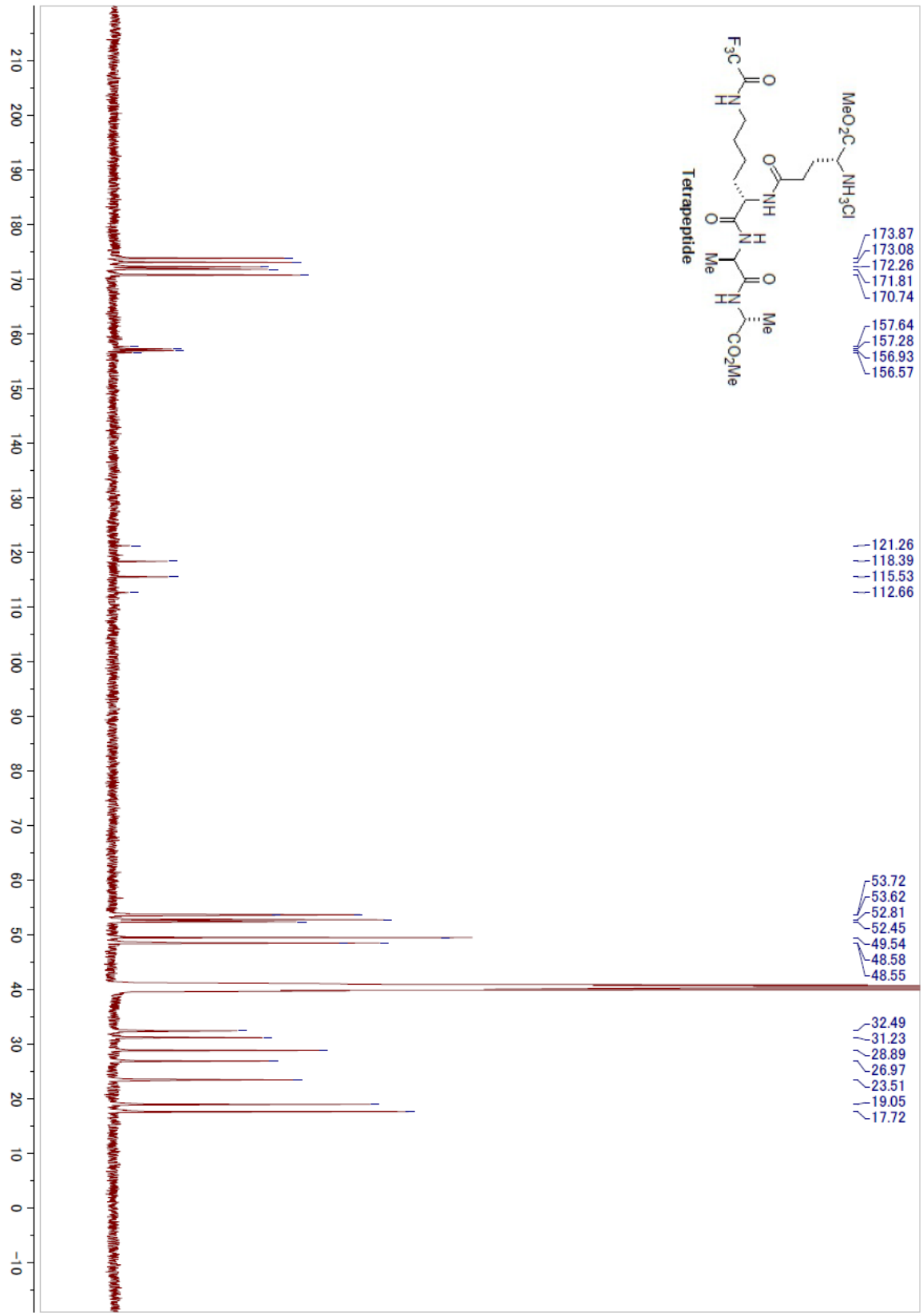
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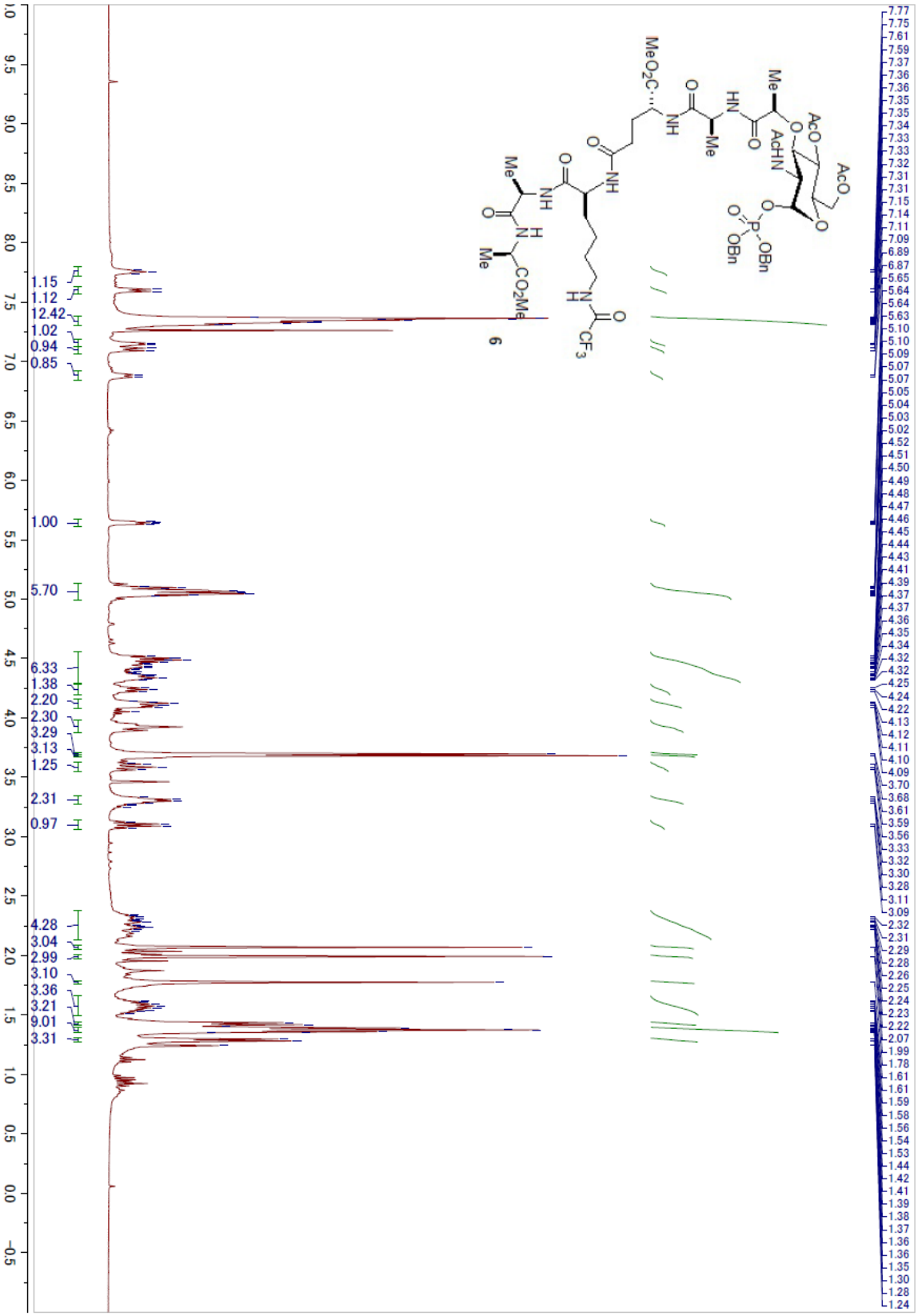

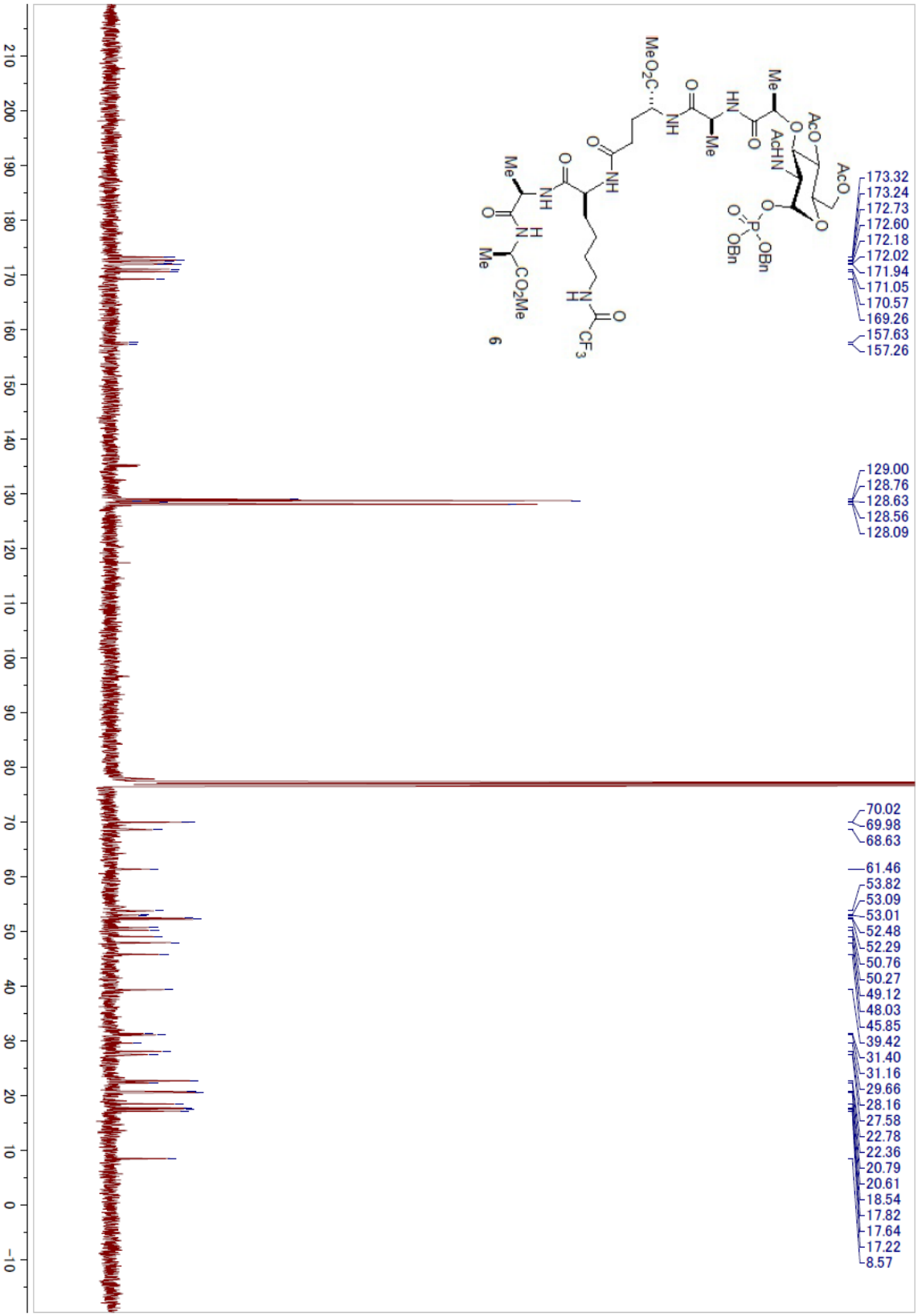
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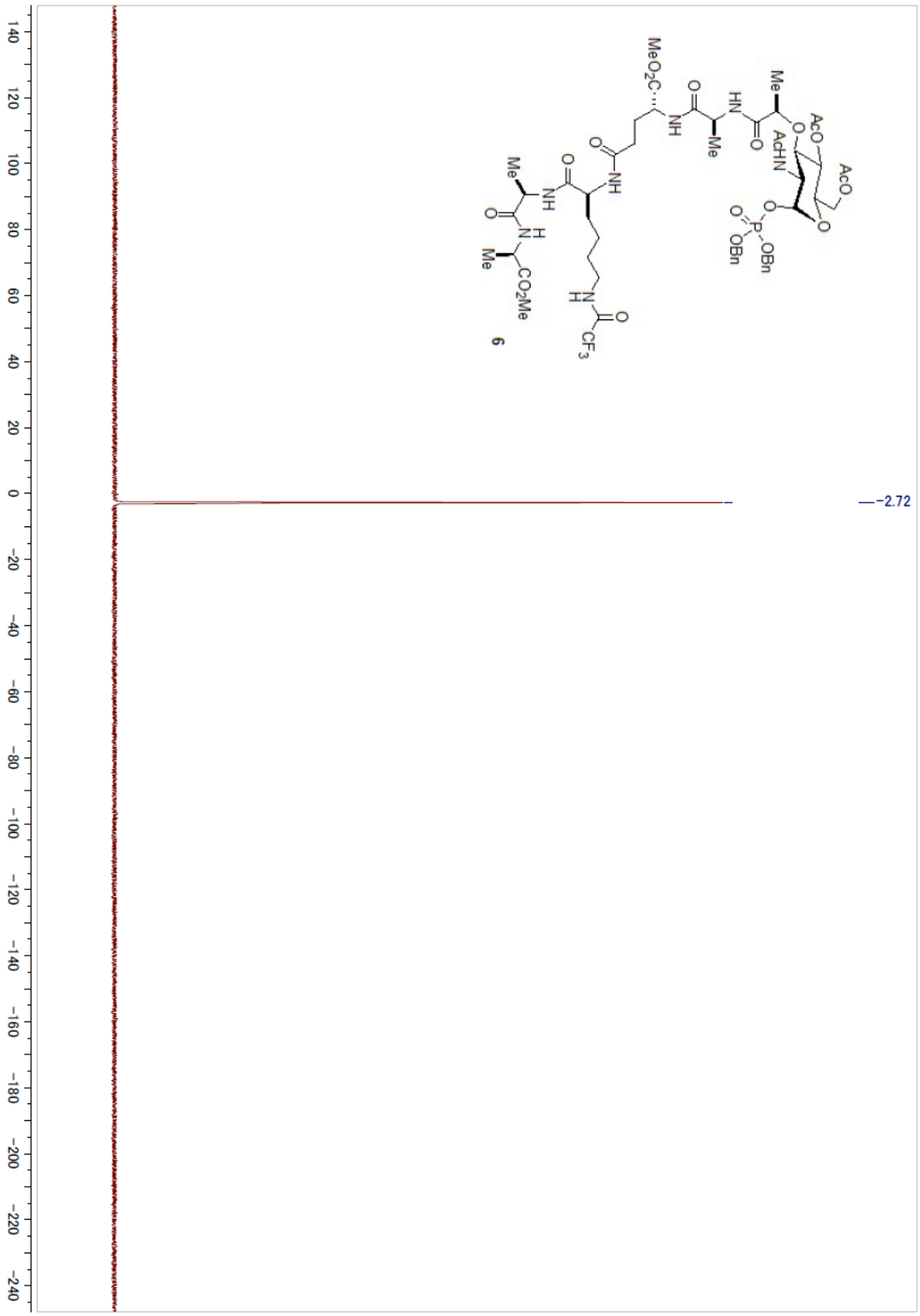
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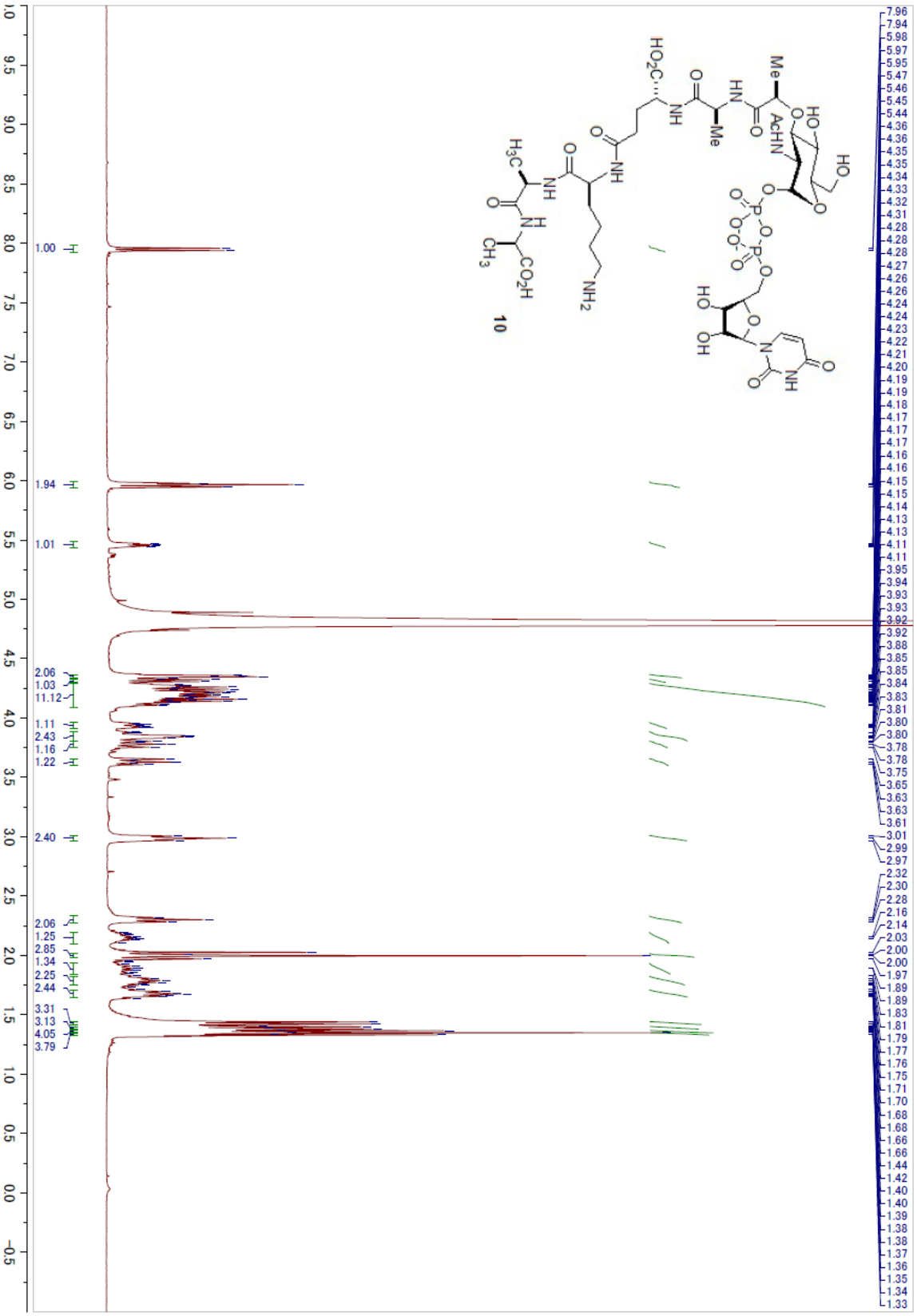
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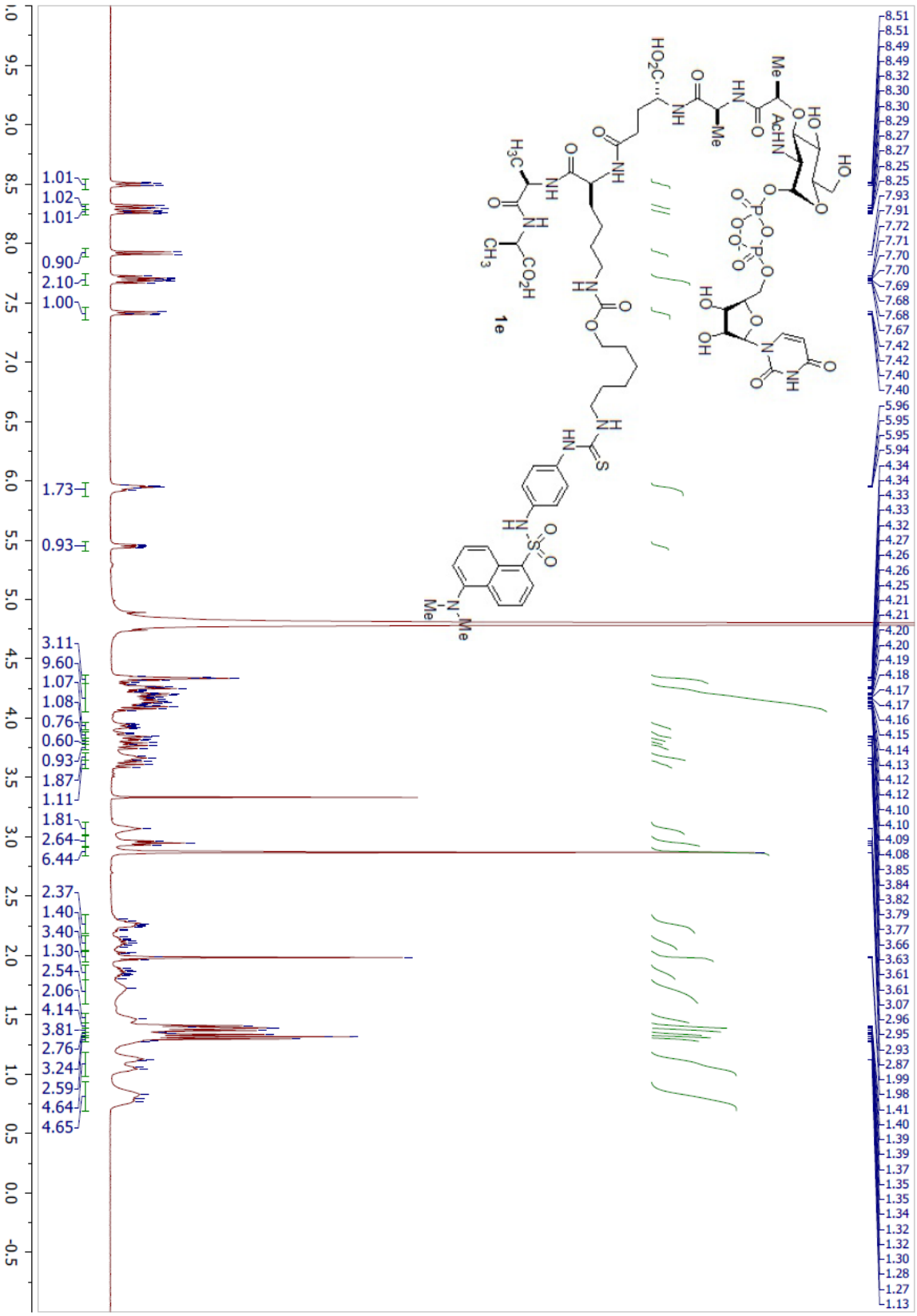
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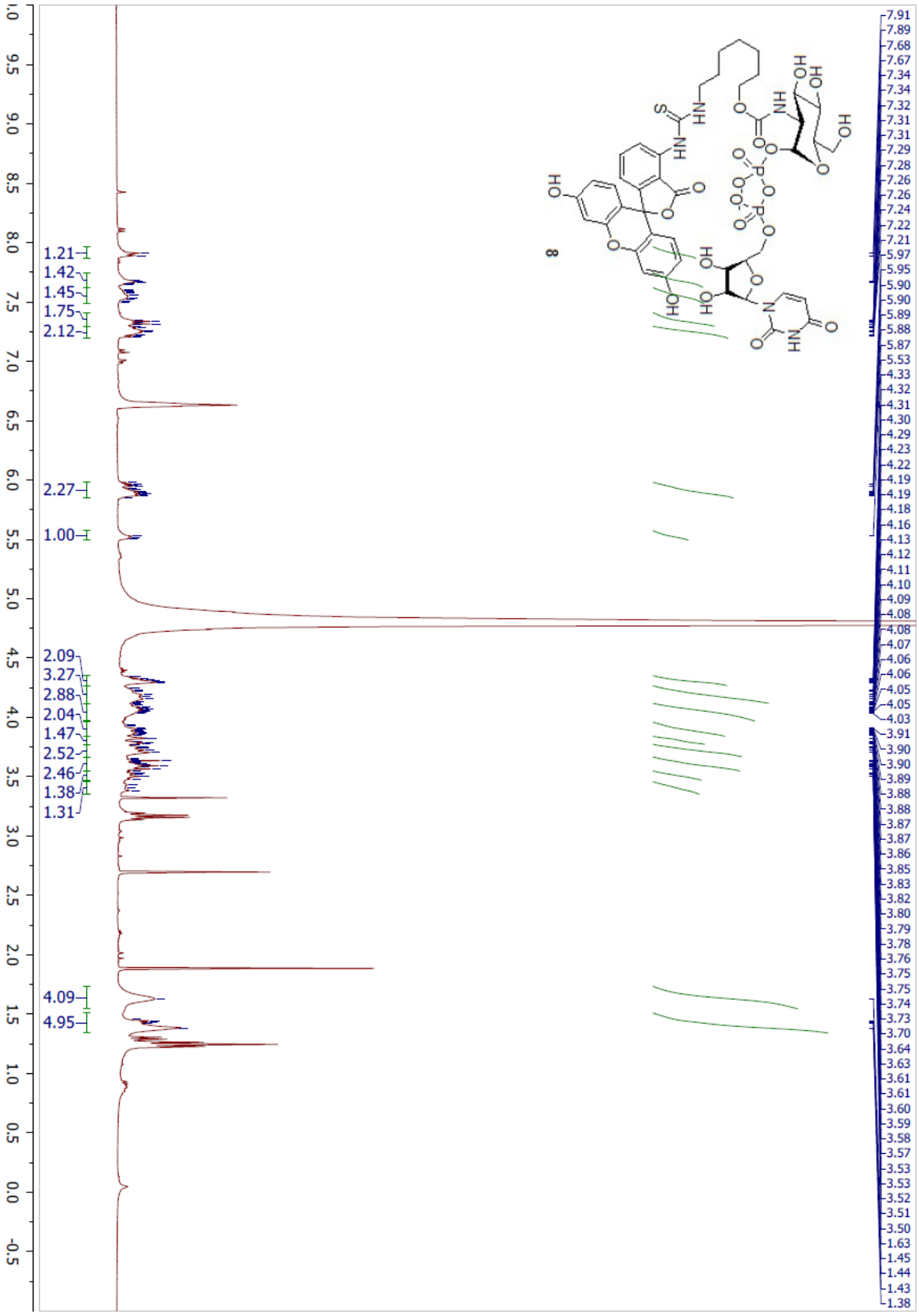
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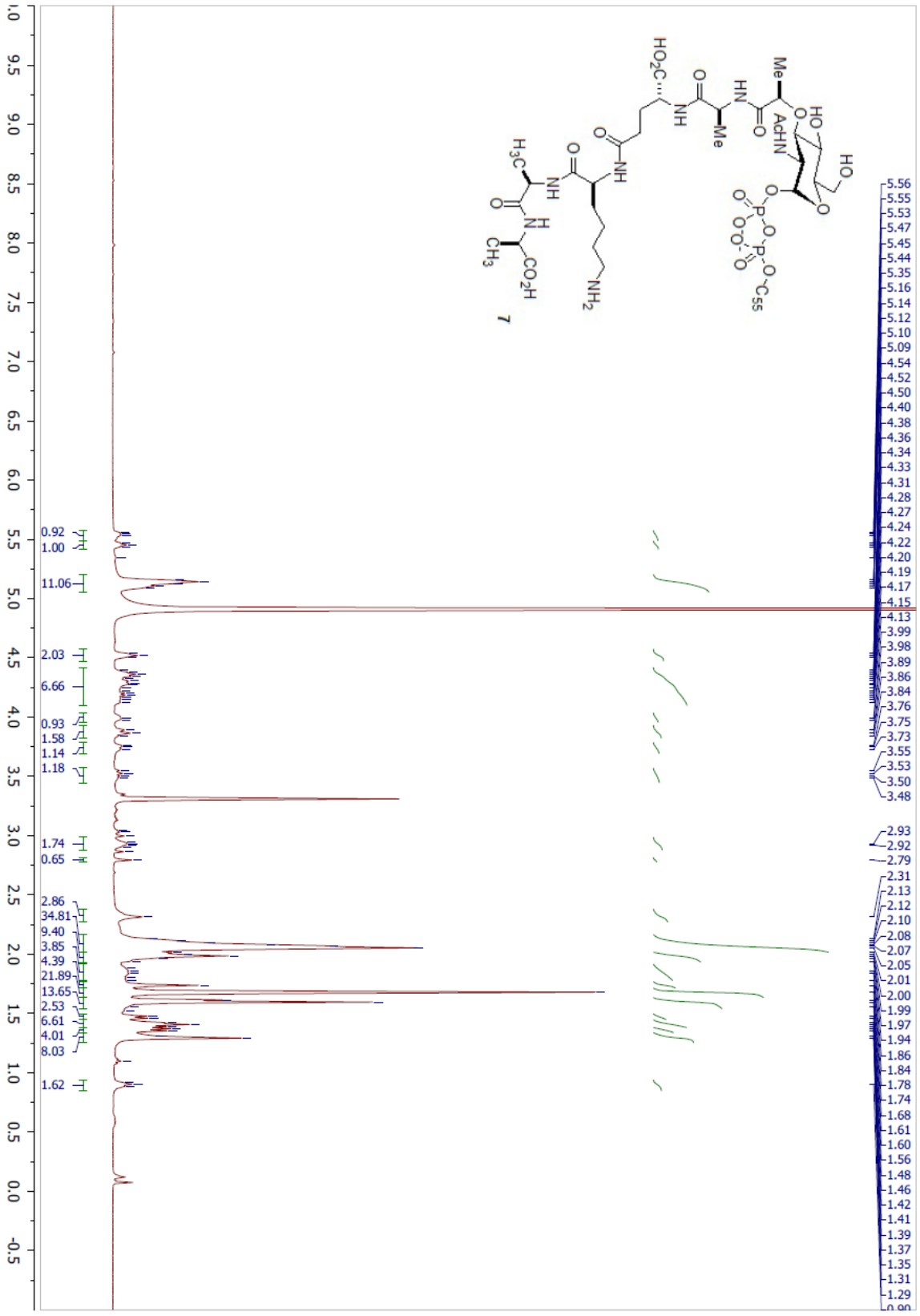

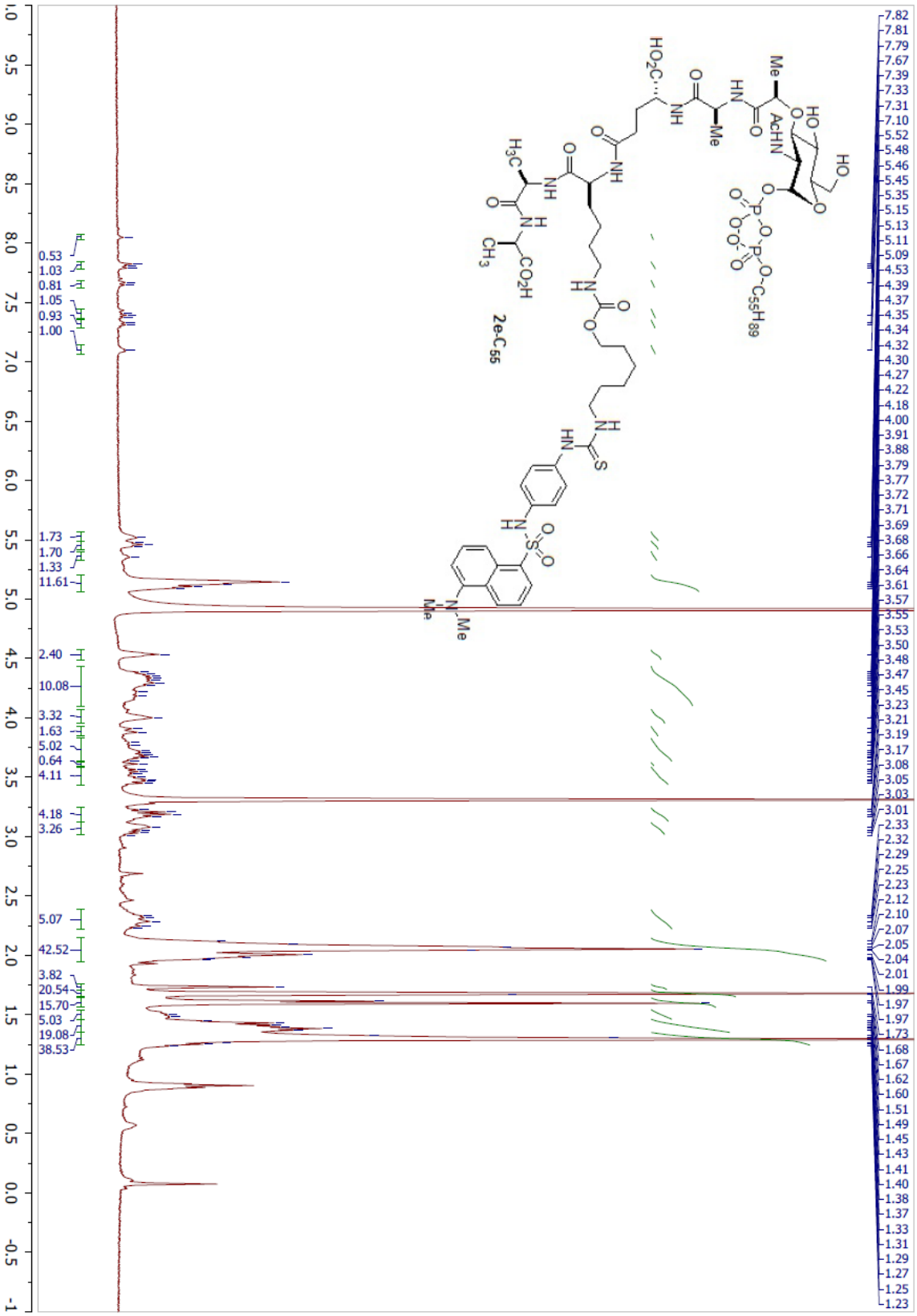

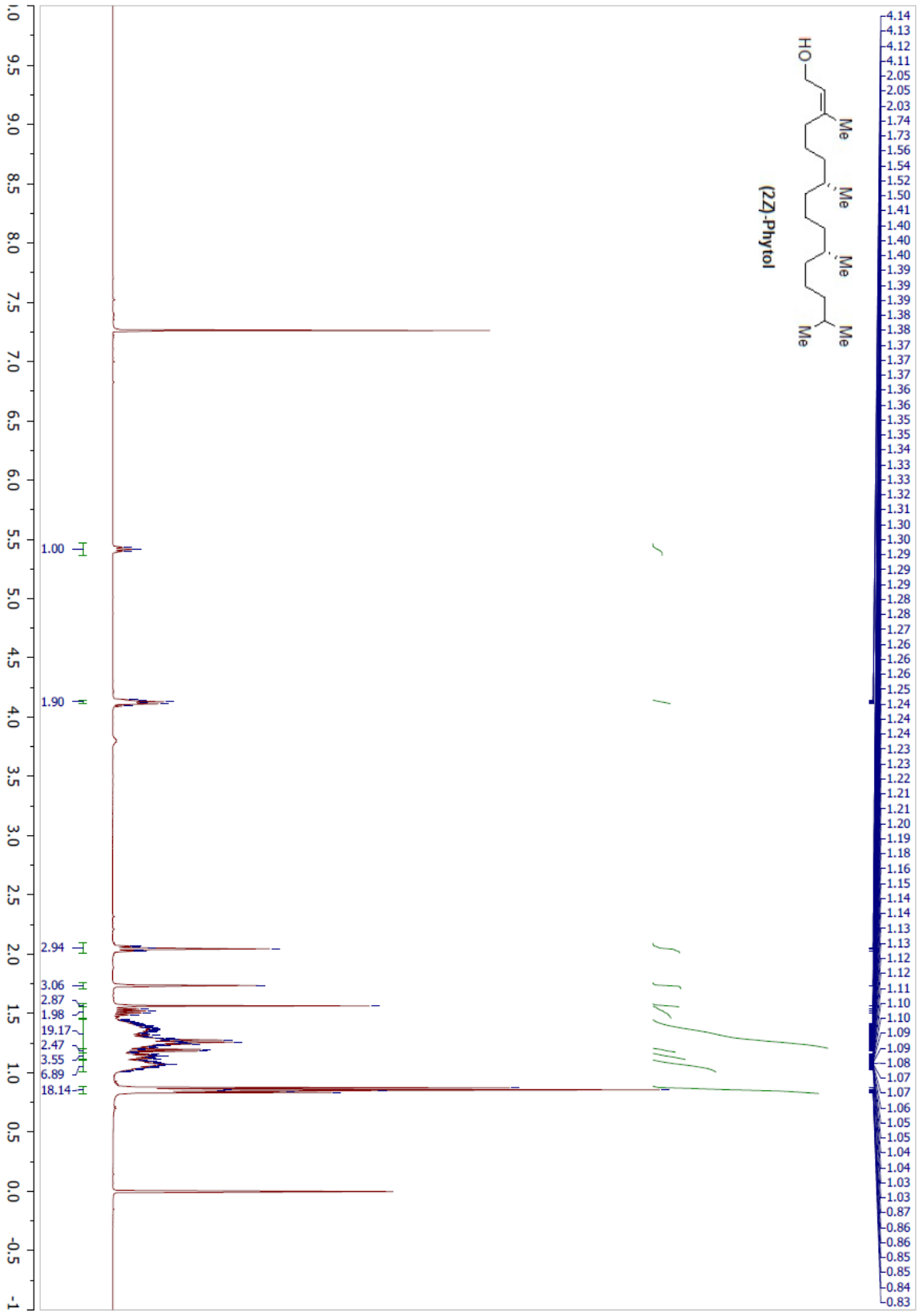
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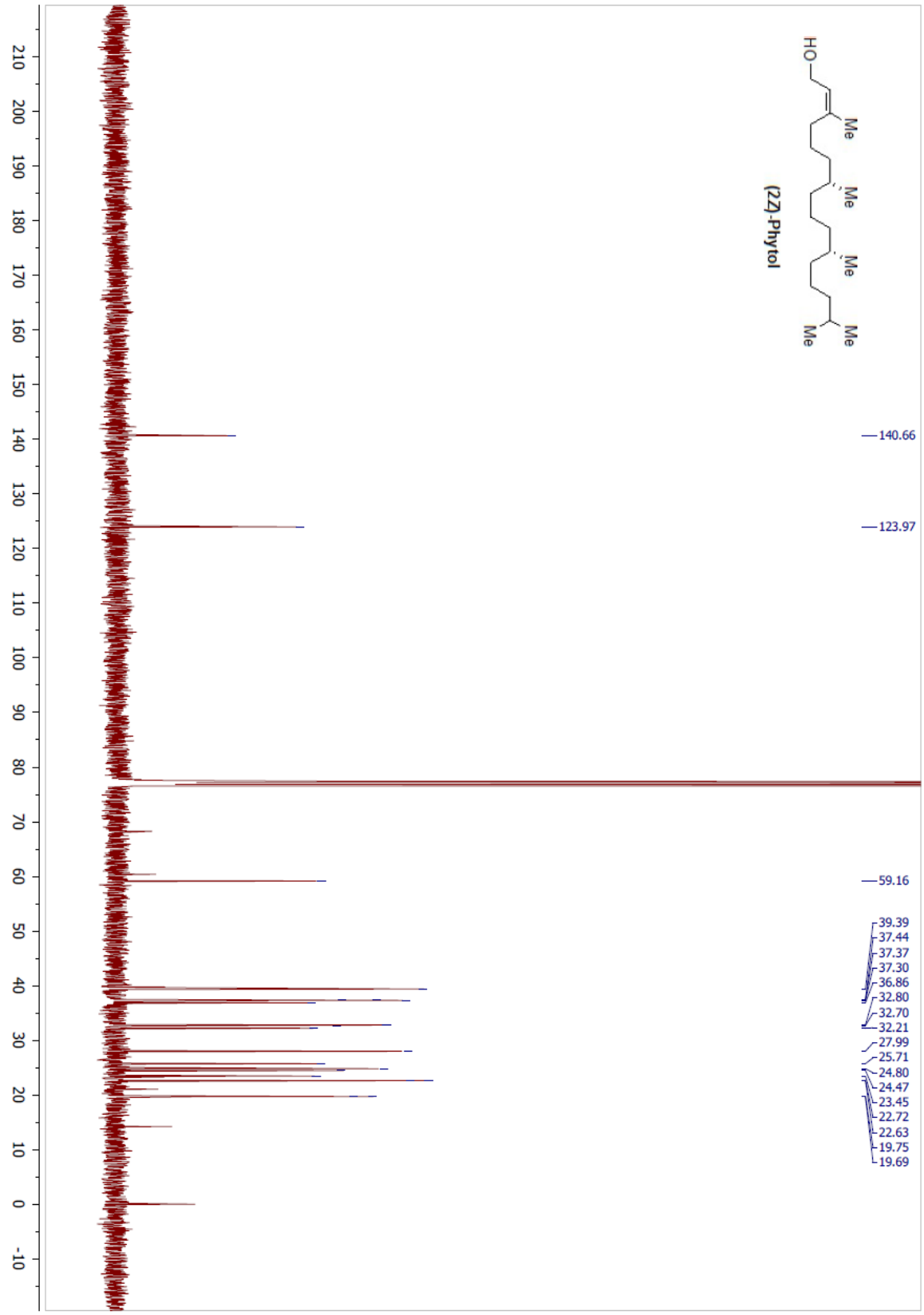
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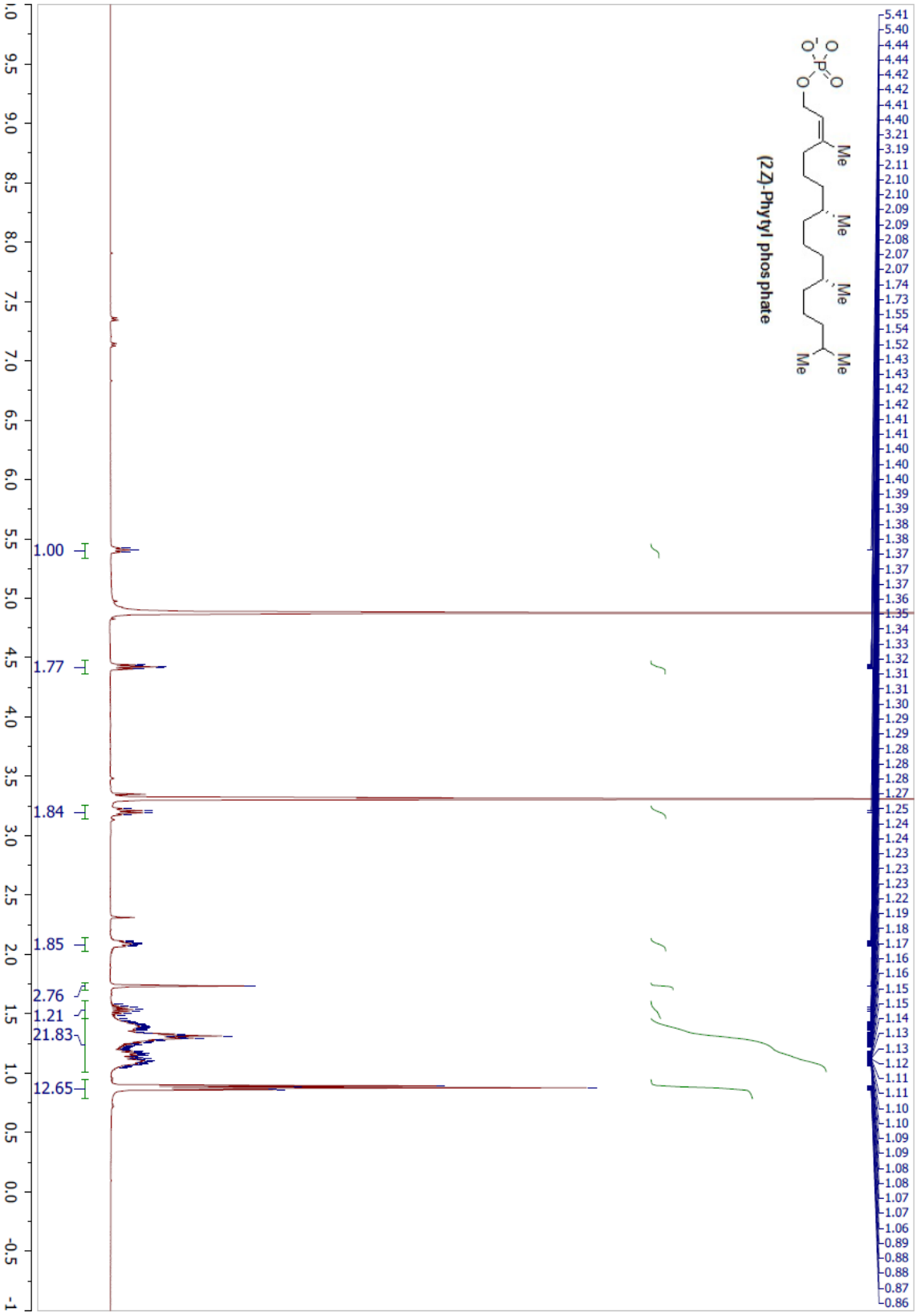
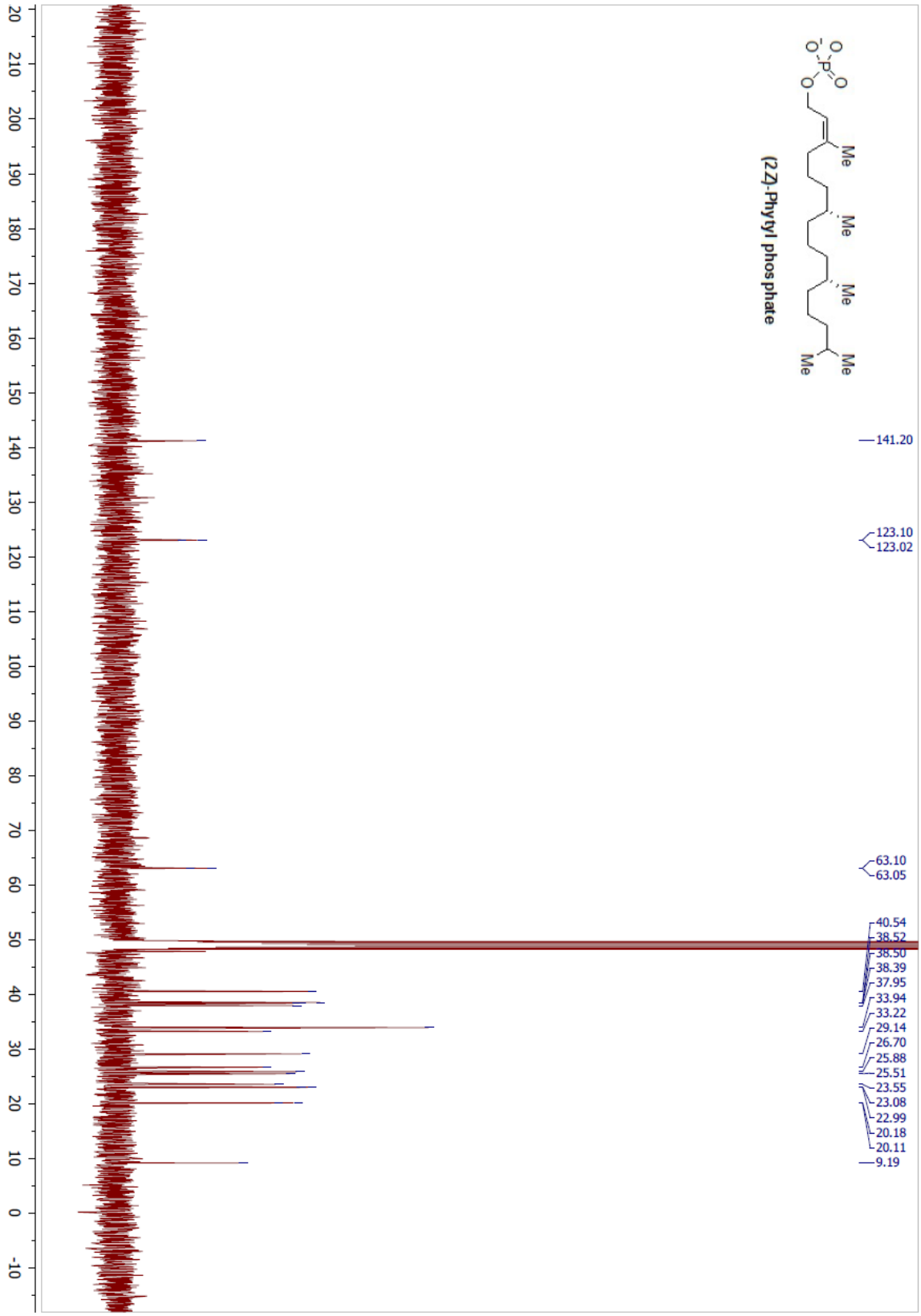
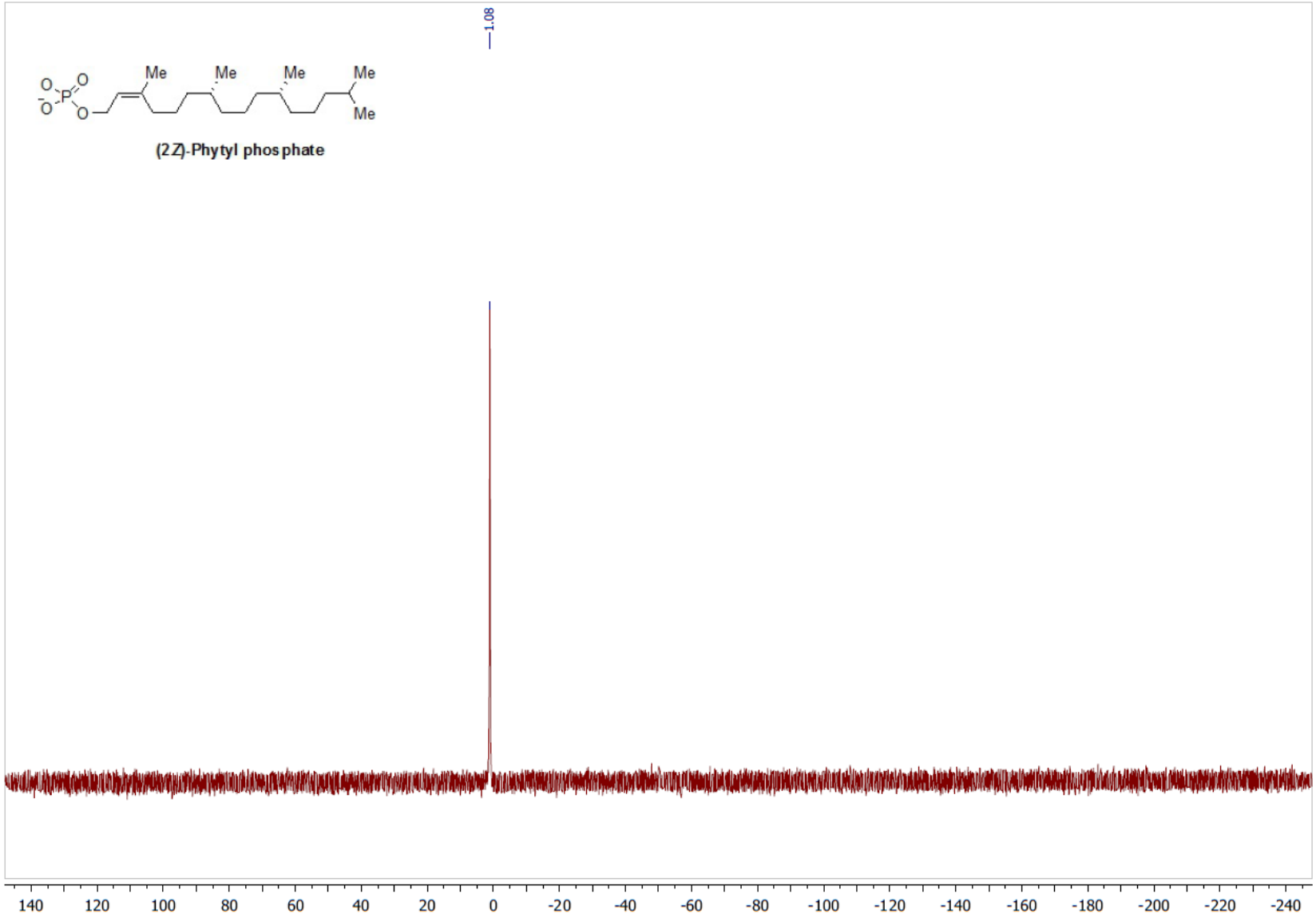
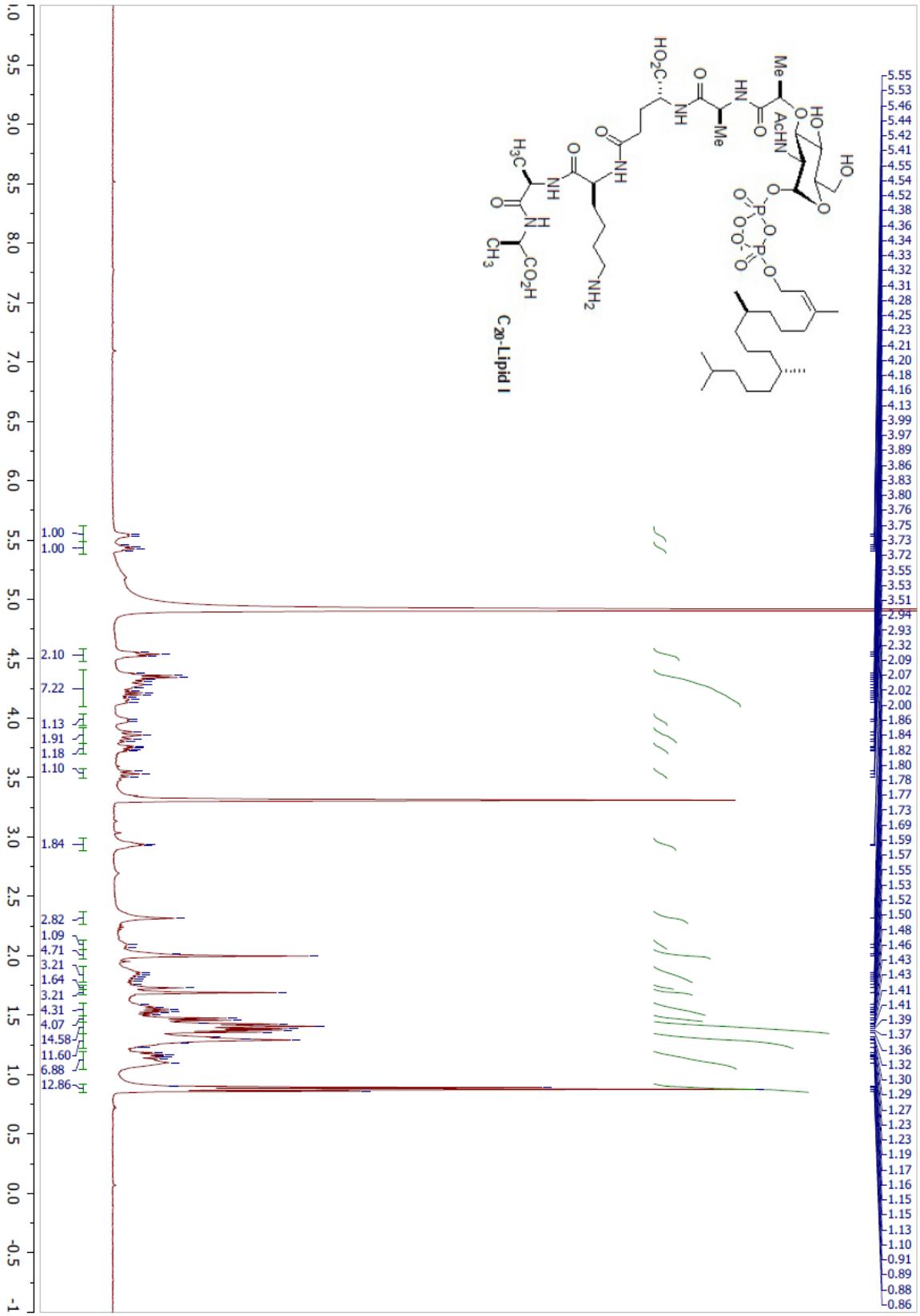
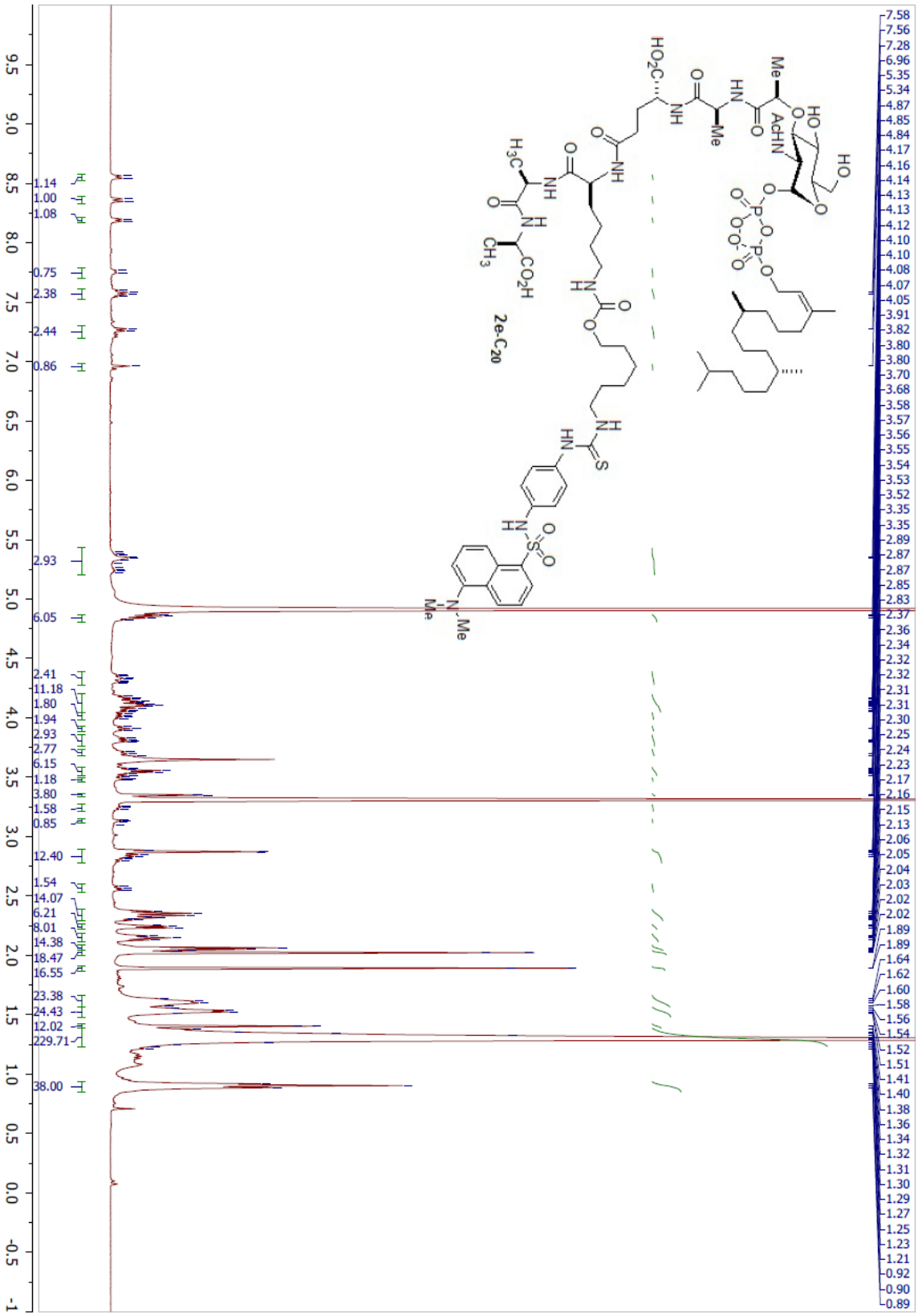
50
40
30
20
10
0
-10


(2Z)-Phytyl phosphate
Me
Me
Me
Me
1.00
1.77
1.84
1.85
2.76
1.21
21.83
12.65


(2Z)-Phytyl phosphate
141.20
123.10
123.02
63.10
63.05
40.54
38.52
38.50
38.39
37.95
33.94
33.22
29.14
26.70
25.88
25.51
23.55
23.08
22.99
20.18
20.11
9.19


1.08
Me
Me
Me
Me
Me
O
O
P
O
O
(2Z)-Phytyl phosphate
140
120
100
80
60
40
20
0
-20
-40
-60
-80
-100
-120
-140
-160
-180
-200
-220
-240


C20-Lipid I


2e-C20