Supporting Information

for

Engineering the Mycomembrane of Live Mycobacteria with an Expanded Set of Trehalose Monomycolate Analogues

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I. Supplementary Results

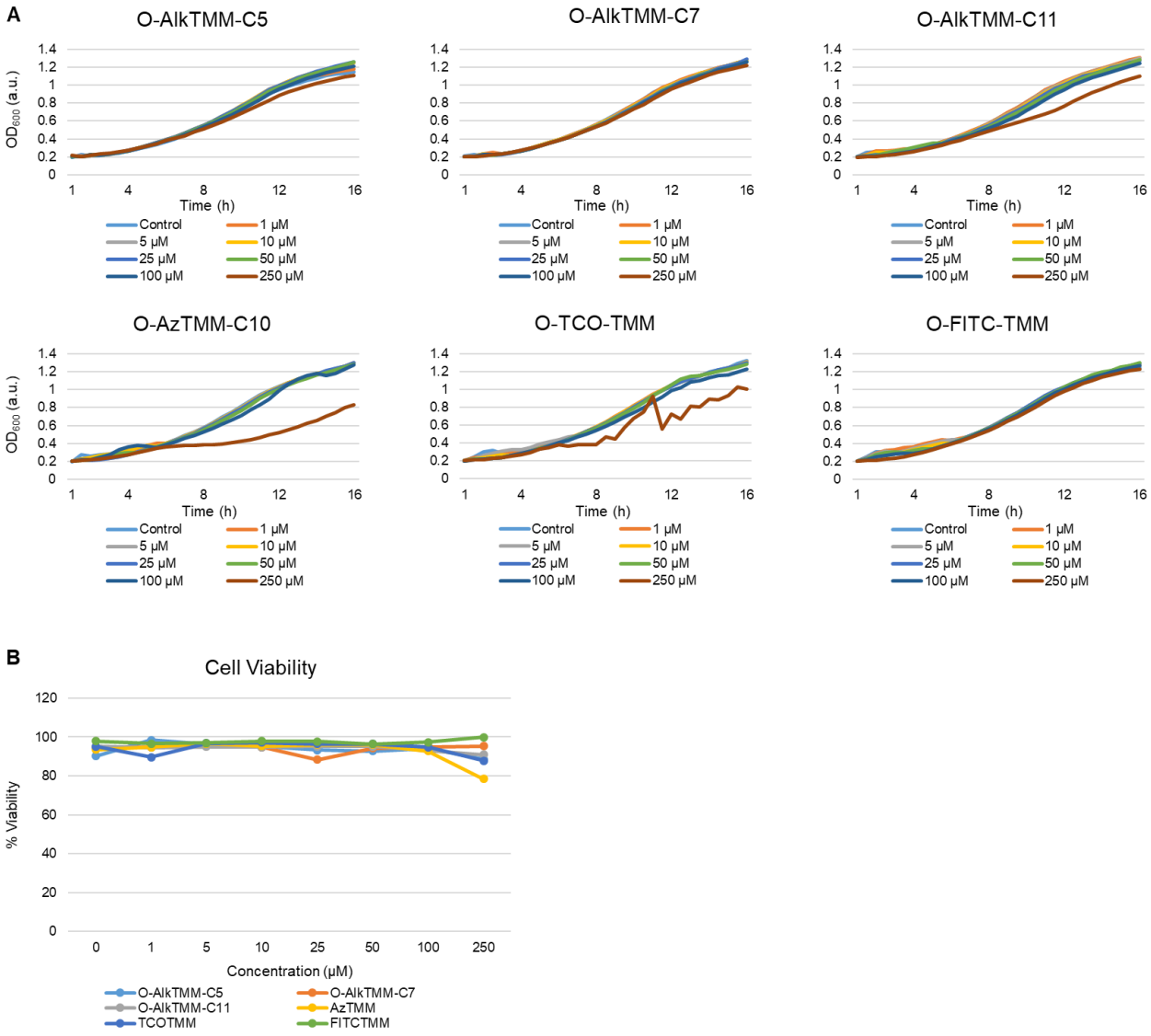

Figure S1. TMM analogue growth inhibition and cell viability evaluation in *M. smegmatis*. (A) Optical density at 600 nm was monitored for 16 h while bacteria were cultured at 37 ºC in the presence of 0–250 μM of compounds **1**–**6** containing a final DMSO concentration of 1%. (B) At the end of 16 h, cellular viability was assessed using the resazurin assay. Values shown are the mean of the two replicate experiments. A.u., absorbance units.

Table S1. *Primers used to generate deletion fragment and screen for Δcg0413 mutation.*

No.	Primer Name	Sequence
A	Cg0413UPfor	AATTTAAGCCCGGGAGCAGGCGTTACTCTTATGGAG
B	Cg0413UPRev	CGCACGAGCGAAGGTGGTAATGGTGGACATCGCAATTCC
C	Cg0413Dwnfor	GGAATTGCGATGTCCACCATTACCACCTTCGCTCGTGCG
D	Cg0413DwnRev	TCCTTCCGTCTAGAGCTTTGGTACTCGTGCACC
E	Cg0413FOR_UPselect	TTAGTTCAGCGCAATTTCCA
F	Cg0413Rev_DWNselect	CGGAGGCAAGAAGAAAAATG

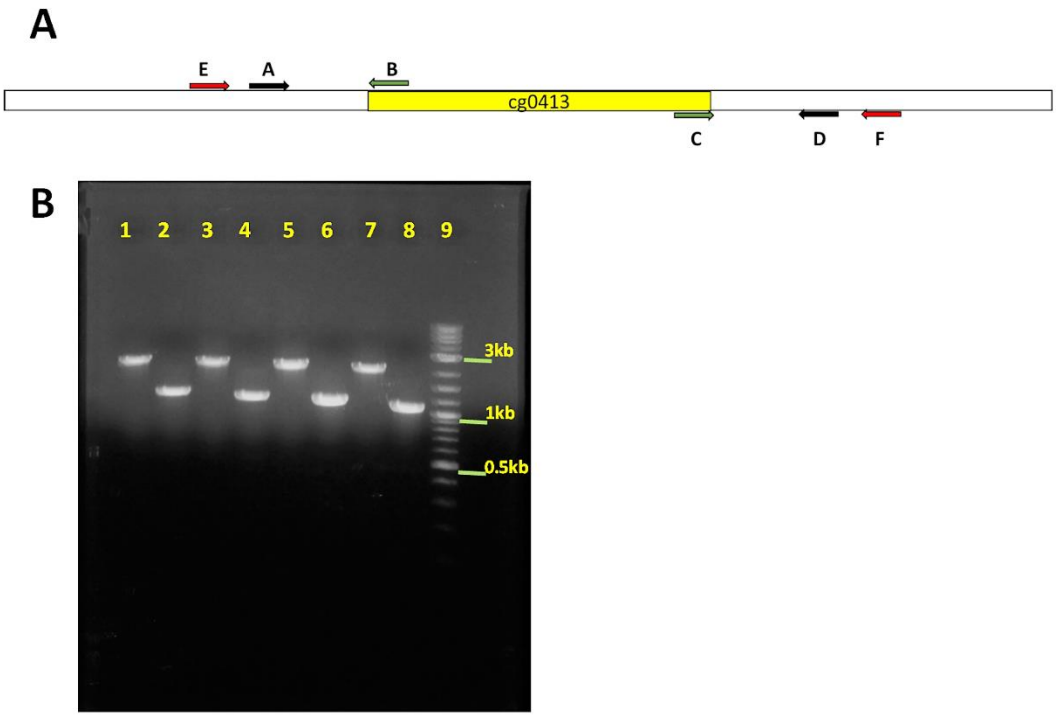

Figure S2. Generation of deletion fragment and identification of Δ*cg0413* mutant. (A) Graphic representation of deletion strategy. Primer pairs A-B and C-D were respectively used to generate products upstream and downstream of *cg0413*. The resulting products and primer pair A-D pair were used to generate the deletion fragment by overlapping PCR. (B) Confirmation of the Δ*cg0413* mutant. After screening the first and second recombination events, colonies were screened for the desired deletion by PCR using the primer pair E-F. Lanes 1, 3, 5, and 7 are products generated from wildtype template DNA. Lanes 2, 4, 6, and 8 are products generated from Δ*cg0413* mutant DNA. Lane 9 is the 2logDNA ladder (New England Biolabs, Ipswitch, MA) as a standard. The primer pairs used are as follows: lanes 1 and 2, E- F; lanes 3 and 4, E-D; lanes 5 and 6, A-F; lanes 7 and 8; A-D.

II. Supplementary Methods

Synthesis of TMM reporters

General Methods for Synthesis. Materials and reagents were obtained from commercial sources without further purification unless otherwise noted. Anhydrous solvents were obtained either commercially or from an alumina column solvent purification system. All reactions were carried out in oven-dried glassware under inert gas unless otherwise noted. Analytical TLC was performed on glass-backed silica gel 60 Å plates (thickness 250 μm) and detected by charring with 5% H_2SO_4 in EtOH. Column chromatography was performed using flash-grade silica gel 32–63 μm (230–400 mesh). ^{1}H NMR spectra were recorded at 500 MHz with chemical shifts in ppm (δ) referenced to solvent peaks. ^{13}C NMR spectra were recorded at 125 MHz. NMR spectra were obtained on a Varian Inova 500 instrument. Coupling constants (*J*) are reported in hertz (Hz). High-resolution electrospray ionization (HR ESI) mass spectra were obtained using a Waters LCT Premier XE using raffinose as the lock mass.

General procedure for the two-step synthesis of TMM analogues 1–4 from 2,3,4,2',3',4'-hexakis-*O*-(trimethylsilyl)-α,α-trehalose. An oven-dried round-bottom flask was charged with DCC (107 mg, 0.52 mmol) and DMAP (0.236 g, 1.94 mmol). After drying the reagents under high vacuum and placing the flask under a nitrogen atmosphere, anhydrous CH_2Cl_2 (2 mL) was added and the mixture was cooled to 0 °C. To the stirring solution was added carboxylic acid (0.258 mmol) followed by slow, dropwise addition of a freshly prepared solution of 2,3,4,2',3',4'-hexakis-*O*-(trimethylsilyl)-α,α-trehalose (**7**, prepared as described by Sarpe et al,[1] 0.200 g, 0.258 mmol) in anhydrous CH_2Cl_2 (2 mL). The reaction mixture was stirred and gradually allowed to warm to room temperature. After TLC (hexanes/ethyl acetate 4:1) showed generation of the monoester as the major product (approximately 4 h), the reaction was quenched by addition of excess CH_3OH and concentrated by rotary evaporation. After resuspension of the crude product in CH_2Cl_2, the insoluble byproduct DCU was removed by filtration. The filtrate containing crude product was concentrated by rotary evaporation and purified by silica gel chromatography (hexanes/ethyl acetate containing 1% Et_3N) to give the monoester intermediate as a pale yellow syrup. The intermediate was dissolved in a mixture of anhydrous CH_3OH (12 mL) and anhydrous CH_2Cl_2 (5 mL) and placed under a nitrogen atmosphere. Dowex 50WX8-400 H^+ ion-exchange resin was added and the reaction was stirred for 30 min at room temperature, after which TLC (CH_2Cl_2/CH_3OH 2:1) indicated that the reaction was complete. After the ion-exchange resin was filtered off, the filtrates were concentrated by rotary evaporation and filtered to give the desired TMM analogue as a white solid.

6-*O*-(4-pentynoyl)-α,α-D-trehalose (O-AlkTMM-C5, compound **1**). From 200 mg of compound **7**, obtained 40 mg of compound **1** (36% over two steps). ^{1}H NMR (500 MHz, D_2O): δ 5.16 (d, *J* = 3.0 Hz, 1 H, H-1'), 5.15 (d, *J* = 3.5 Hz, 1 H, H-1), 4.42 (dd, *J* = 2.0, 12 Hz, 1 H, H-6a' or 6b'), 4.35 (dd, *J* = 5.0, 12 Hz, 1 H, H-6a' or 6b'), 4.01 (ddd, *J* = 2.0, 4.5, 10.5 Hz, 1 H, H-5'), 3.85-3.78 (m, 4 H, H-3, 3', 5, 6a or 6b), 3.73 (dd, *J* = 5.0, 12 Hz, 1 H, H-6a or 6b), 3.63 (t, *J* = 11 Hz, 1 H, H-2'), 3.62 (t, *J* = 11.5 Hz, 1 H, H-2), 3.51 (t, *J* = 10 Hz, 1 H, H-4'), 3.42 (t, *J* = 9.0 Hz, 1 H, H-4), 2.66 (t, *J* = 7.0 Hz, 2 H, α-CH_2), 2.51 (dt, *J* = 2.5, 7.0 Hz, 2 H, propargylic CH_2), 2.36 (t, *J* = 2.5 Hz, 1 H, terminal alkyne H). ^{13}C NMR (125 MHz, CD_3OD): δ 173.6, 95.4, 95.2, 83.5, 74.7,

74.6, 74.0, 73.3, 73.2, 72.0, 71.5, 70.4, 64.9, 62.7, 34.5, 30.3, 15.1. HR ESI MS negative mode: calcd. for $C_{17}H_{25}O_{12}$ $[M–H]^–$: 421.1346, found: 421.1356.

6-*O*-(6-heptynoyl)-α,α-D-trehalose (O-AlkTMM-C7, compound **2**). Synthesized using the procedure reported by Foley et al.[2]

6-*O*-(10-undecynoyl)-α,α-D-trehalose (O-AlkTMM-C11, compound **3**). From 200 mg of compound **7**, obtained 50 mg of compound **3** (38% over two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 5.01 (d, *J* = 3.0 Hz, 1 H, H-1'), 4.98 (d, *J* = 4.0 Hz, 1 H, H-1), 4.27 (dd, *J* = 2.0, 12 Hz, 1 H, H-6a' or 6b'), 4.11 (dd, *J* = 5.0, 12 Hz, 1 H, H-6a' or 6b'), 3.93 (ddd, *J* = 2.0, 5.0, 10 Hz, 1 H, H-5'), 3.75–3.66 (m, 4 H, H-3, 3', 5, 6a or 6b), 3.58 (dd, *J* = 5.5, 12 Hz, 1 H, H-6a or 6b), 3.39 (t, *J* = 5.0 Hz, 1 H, H-2'), 3.37 (t, *J* = 4.0 Hz, 1 H, H-2), 3.25 (t, *J* = 9.5 Hz, 1 H, H-4'), 3.23–3.18 (m, 1 H, H-4), 2.25 (*t*, J = 7.0 Hz, 2 H, α-CH_2), 2.09–2.05 (m, propargylic CH_2 and terminal alkyne H), 1.53 (pent, *J* = 7.5 Hz, 2 H, β-CH_2), 1.41 (pent, *J* = 7.0 Hz, 2 H, homopropargylic CH_2), 1.33–1.23 (m, 8 H, CH_2s). ^{13}C NMR (125 MHz, CD_3OD): δ 175.6, 95.3, 95.2, 85.2, 74.7, 74.6, 74.0, 73.3, 73.2, 72.0, 72.0, 71.5, 69.5, 64.5, 62.7, 35.1, 30.4, 30.3, 30.2, 29.9, 29.8, 26.2, 19.1. HR ESI MS negative mode: calcd. for $C_{23}H_{37}O_{12}$ $[M–H]^–$: 505.2285, found: 505.2292.

6-*O*-(10-azidodecanoyl)-α,α-D-trehalose (O-AzTMM-C10, compound **4**). From 200 mg of compound **7**, obtained 48 mg of compound **6** (44% over two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 5.01 (d, *J* = 3.5 Hz, 1 H, H-1'), 4.99 (d, *J* = 3.5 Hz, 1 H, H-1), 4.30 (dd, *J* = 2.0, 12 Hz, 1 H, H-6a' or 6b'), 4.12 (dd, *J* = 5.0, 11.5 Hz, 1 H, H-6a' or 6b'), 3.94 (ddd, *J* = 2.0, 5.0, 10 Hz, 1 H, H-5'), 3.77–3.67 (m, 4 H, H-3, 3', 5, 6a or 6b), 3.64 (dd, *J* = 5.0, 7.0 Hz, 1 H, H-6a or 6b), 3.40–3.37 (m, 2 H, H-2, 2'), 3.27–3.22 (m, 2 H, H-4, 4'), 3.19 (t, *J* = 7.0 Hz, 2 H, CH_2-N_3), 2.31 (t, *J* = 7.0 Hz, 2 H, α-CH_2), 1.55–1.47 (m, 4 H, CH_2s), 1.31–1.22 (m, 10 H, CH_2s). ^{13}C NMR (125 MHz, CD_3OD): δ 175.4, 95.2, 95.1, 74.6, 74.4, 73.9, 73.2, 73.1, 71.9, 71.8, 71.4, 64.4, 62.6, 52.4, 35.0, 30.5, 30.3, 30.2, 30.1, 29.9, 27.8, 26.0. HR ESI MS negative mode: calcd. for $C_{23}H_{40}N_3O_{14}$ $[M+CHO_2^–]^–$: 582.2510, found: 582.2529.

6-*O*-(10-aminodecanoyl)-α,α-D-trehalose (compound **8**). To a solution of compound **4** (51 mg, 0.095 mmol) in CH_2Cl_2/CH_3OH (2:1) under an argon atmosphere was added Pd/C (35 mg). A hydrogen-filled balloon was connected to the reaction flask and the argon atmosphere was exchanged for hydrogen. After stirring under a hydrogen atmosphere at room temperature overnight, the reaction mixture was filtered through celite and the filtrate was concentrated by rotary evaporation to give the reduced product **8** (48 mg, 99%) as a white solid. ^{1}H NMR (500 MHz, D_2O): δ 5.16 (d, *J* = 4.0 Hz, 1 H, H-1'), δ 5.14 (d, *J* = 4.0 Hz, 1 H, H-1), 4.42 (dd, *J* = 2.0, 12 Hz, 1 H, H-6'a or b), δ 4.30 (dd, *J* = 5.0, 12 Hz, 1 H, H-6'a or b), 4.01 (ddd, *J* = 2.0, 5.0, 10 Hz, 1 H, H-5'), 3.86–3.78 (m, 4 H, H-3', 3, 5, 6a or 6b), 3.74 (dd, *J* = 5.0, 12 Hz, 1 H, H6a or b), 3.63 (dd, *J* = 4.0, 9.5 Hz, 1 H, H-2'), 3.61 (dd, *J* = 4.0, 10 Hz, 1 H, H-2), 3.48 (t, *J* = 10 Hz, 1 H, H-4'), 3.42 (t, *J* = 10 Hz, 1 H, H-4), 2.97 (t, *J* = 8.0 Hz, 2 H, CH_2-NH_2), 2.42 (t, *J* = 7.5 Hz, 2 H, α-CH_2), 1.67–1.59 (m, 4 H, CH_2s), 1.38–1.26 (m, 10 H, CH_2s). ^{13}C NMR (125 MHz, D_2O): 177.9, 94.7, 94.6, 73.9, 73.7, 73.5, 72.4, 72.3, 71.3, 71.1, 71.0, 64.3, 61.9, 40.8, 35.1, 29.7, 29.6, 29.5, 29.4, 28.0, 26.9, 25.6. HR ESI MS positive mode: calcd. for $C_{22}H_{42}NO_{12}$ $[M+H]^+$: 512.2707, found: 512.2699.

6-*O*-(10-{*N*-[(*E*)-cylcooct-4-en1yloxy]carbonyl}aminodecanoyl)-α,α-D-trehalose (O-TCO-TMM, compound **5**). To a 20 mL glass scintillation vial containing compound **8** (23 mg, 0.045 mmol) stirring in CH_3OH (1.0 mL) was added a solution of (*E*)-cyclooct-4-enyl 2,5-dioxo-1-pyrrolidinyl carbonate (TCO-NHS, 11.5 mg, 0.0435 mmol) and Et_3N (7 μL, 0.05 mmol) dissolved in *N*,*N*-dimethylformamide (DMF) (2.5 mL). After stirring for 20 h, the reaction mixture was concentrated by rotary evaporation and purified using a Biotage Isolera One automated flash chromatography system (10 g C18 column; 20% CH_3CN in H_2O → 100% CH_3CN) to give product **5** (18.5 mg, 62%). ^{1}H NMR (500 MHz, CD_3OD) δ 5.57–5.35 (m, 2 H, TCO vinyl-CH), 5.00 (d, *J* = 3.5 Hz, 1 H, H-1'), 4.98 (d, *J* = 3.0 Hz, 1 H, H-1), 4.27 (dd, *J* = 2.0, 12 Hz, 1 H, H-6a' or 6b'), 4.22–4.16 (m, 1 H, TCO CH), 4.10 (dd, *J* = 5.0, 12 Hz, 1 H, H-6a' or 6b'), 3.95–3.89 (m, 1 H, H-5'), 3.74–3.66 (m, 4 H, H-3, 3', 5, 6a or 6b), 3.58 (dd, *J* = 5.5, 11 Hz, 1 H, H-6a or 6b), 3.40–3.35 (m, 2 H, H-2, 2'), 3.27–3.20 (m, 2 H, H-4, 4'), 2.96 (t, *J* = 5.5 Hz, 2 H, CH_2-N), 2.27–2.19 (m, 4 H, α-CH_2, TCO allylic CH_2), 1.92–1.77 (m, 4 H, TCO CH_2s), 1.61 (pent, *J* = 12.5 Hz, 2 H, TCO CH_2), 1.56–1.46 (m, 4 H, β-CH_2, TCO CH_2), 1.37–1.35 (m, 2 H, CH_2), 1.26–1.16 (m, 10 H, CH_2s). ^{13}C NMR (125 MHz, CD_3OD): δ 174.0, 172.5, 134.6, 132.3, 93.7, 93.6, 80.1, 73.2, 73.0, 72.5, 71.8, 71.7, 70.5, 70.4, 70.0, 62.9, 61.2, 40.8, 40.2, 38.2, 33.8, 33.6, 32.1, 30.7, 29.6, 29.1, 29.0, 28.9, 28.7, 26.4, 24.6. HR ESI MS positive mode: m/z calcd. for $C_{31}H_{54}NO_{14}$ $[M+H]^+$: 664.3544, found: 664.3521.

6-*O*-(10-[(fluorescein-5-yl)thioureido]decanoyl)-α,α-D-trehalose (O-FITC-TMM, compound **6**). To a 20 mL glass scintillation vial containing compound **8** (15.7 mg, 0.0306 mmol) stirring in CH_3OH (0.5 mL) was added a solution of fluorescein isothiocyanate (FITC, 12.4 mg, 0.316 mmol) and Et_3N (7 μL, 0.05 mmol) dissolved in *N*,*N*-dimethylformamide (DMF) (1.5 mL). After stirring for 20 h, the reaction mixture was concentrated by rotary evaporation and purified using a Biotage Isolera One automated flash chromatography system (10 g C18 column; 30% CH_3CN in H_2O → 70% CH_3CN in H_2O) to give product **6** (23.4 mg, 85%) as a yellow solid. ^{1}H NMR (500 MHz, 10% $CDCl_3$ in CD_3OD) δ 8.18 (s, broad, 1 H, FITC Ar-CH), 7.86 (dd, *J* = 2.0, 8.5 Hz, 1 H, FITC Ar-CH), 7.16 (d, *J* = 8.5 Hz, 1 H, FITC Ar-CH), 6.71–6.69 (m, 4 H, FITC Ar-CH), 6.56 (dd, *J* = 2.5, 9.0 Hz, 2 H, FITC Ar-CH), 5.14 (d, *J* = 4.0 Hz, 1 H, H-1'), 5.11 (d, *J* = 3.5 Hz, 1 H, H-1), 4.38 (dd, *J* = 2.0, 12 Hz, 1 H, H-6a' or 6b'), 4.26 (dd, *J* = 5.5, 12.5 Hz, 1 H, H-6a' or 6b'), 4.03 (ddd, *J* = 2.0, 4.5, 10 Hz, 1 H, H-5'), 3.85–3.78 (m, 4 H, H-3, 3', 5, 6a or 6b), 3.71 (dd, *J* = 6.0, 12 Hz, 1 H, H-6a or 6b), 3.67–3.60 (m, 2 H, CH_2-N), 3.55 (dd, *J* = 3.5, 10 Hz, 1 H, H-2'), 3.51 (dd, *J* = 4.0, 10 Hz, 1 H, H-2), 3.42–3.36 (m, 2 H, H-4, 4'), 2.37 (t, *J* = 7.5 Hz, 2 H, α-CH_2), 1.70–1.63 (m, 4 H, CH_2s), 1.44–1.30 (m, 10 H, CH_2s). ^{13}C NMR (125 MHz, CD_3OD): δ 181.2, 175.2, 173.4, 170.9, 153.8, 142.0, 130.0, 113.4, 111.1, 103.3, 94.6, 94.5, 74.1, 73.9, 73.2, 72.6, 72.5, 71.4, 71.3, 70.8, 63.9, 62.3, 34.8, 30.2, 30.1, 30.0, 29.9, 29.6, 27.7, 25.7. HR ESI MS positive mode: m/z calcd. for $C_{43}H_{53}N_2O_{17}S$ $[M+H]^+$: 901.3065, found: 901.3084.

10-[(fluorescein-5-yl)thioureido]decanoic acid (compound **9**). To a 20 mL scintillation vial containing 10-aminodecanoic acid (40.1 mg, 0.214 mmol) dissolved in methanol (1.3 mL) and DMF (4 mL) was added FITC (99.8 mg, 0.256 mmol) and Et_3N (17.9 μL). The vial was wrapped in aluminum foil and the reaction mixture was stirred at 37 °C overnight, then the crude product was concentrated by rotary evaporation and purified a Biotage Isolera One automated flash chromatography system (10 g C18 column; gradient elution using 0 % CH_3CN in H_2O to 100 % CH_3CN in H_2O) to give compound **9** (50 mg, 43 %) as a yellow solid. ^{1}H NMR (500 MHz, CD_3OD) δ 8.13 (s, broad, 1 H, FITC Ar-CH), 7.73 (m, 1 H, FITC Ar-CH), 7.13 (d, *J* = 8.6 Hz, 1 H, FITC Ar-CH), 6.70–6.64 (m, 4 H, FITC Ar-CH), 6.53 (dd, *J* = 2.5, 8.8 Hz, 2 H, FITC Ar-CH), 3.32–

3.30 (m, 2 H, CH_2-N), 2.27 (t, J = 7.5 Hz, 2 H, α-CH_2), 1.71–1.58 (m, 4 H, CH_2s), 1.42–1.27 (m, 10 H, CH_2s). ^{13}C NMR (125 MHz, CD_3OD) δ 178.42, 171.71, 162.10, 154.72, 130.85, 114.27, 112.00, 104.04, 35.55, 30.99, 30.85, 30.70, 30.41, 28.49, 26.61. HR ESI MS positive mode: m/z calcd. for $C_{31}H_{33}N_2O_7S$ $[M+H]^+$: 577.2009, found: 577.2031.

Metabolic Labeling and Analysis of Bacteria

Bacterial strains, media, and reagents. The bacterial strains used in this work included *Msmeg* mc^2155 wild type, *Corynebacterium glutamicum* 534, *Escherichia coli* K12 MG1655, and *Bacillus subtilis* 168. *Msmeg* wild type stocks were from the Swarts lab. *C. glutamicum*, *E. coli*, and *B. subtilis* stocks were from the Siegrist lab (University of Massachusetts, Amherst). *Msmeg* was cultured in Middlebrook 7H9 liquid medium supplemented with ADC (albumin, dextrose, and catalase), 0.5% glycerol, and 0.05% Tween-80. *C. glutamicum*, *E. coli*, and *B. subtilis* were cultured in LB liquid medium. All bacteria were cultured at 37 °C, except *C. glutamicum*, which was cultured at 30 °C.

Stock solutions of O-AlkTMM O-AzTMM analogues (**1**–**4**) were prepared in phosphate-buffered saline (PBS) at concentrations of 25 mM, sterile-filtered (0.2 μm), and stored at –20 °C. Prior to usage in labeling experiments, stock solutions of compounds **1**–**4** were diluted to the desired concentration with PBS and appropriate culture medium. O-TCO-TMM (**7**), and O-FITC-TMM (**8**) probe stocks were prepared in dimethylsulfoxide (DMSO) at 25 mM and stored at –20 °C. Prior to usage in labeling experiments, stock solutions of compounds **7**–**9** were diluted to the desired concentrations with DMSO and appropriate culture medium. Other reagent stocks included: azide-modified carboxyrhodamine 110 (Az488, Click Chemistry Tools, 1 mM in DMSO, stored at –20 °C); alkyne-modified carboxyrhodamine 110 (Alk488, Click Chemistry Tools, 1 mM in DMSO, stored at –20 °C); DBCO-488 (Click Chemistry Tools, 1 mM in DMSO, stored at –20 °C); methyltetrazine-Cy3 or tetrazine-Cy3 (Click Chemistry Tools, 1 mM in DMSO, stored at –20 °C); sodium ascorbate (60 mM in H_2O, always freshly prepared); tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) ligand for CuAAC reactions (Click Chemistry Tools, 6.4 mM in *tert*-BuOH/DMSO 4:1, stored at –20 °C); $CuSO_4$ (50 mM in H_2O, stored at –20 °C).

General procedures for bacterial labeling. Starter cultures of bacteria were generated by inoculating a single colony from a freshly streaked LB agar plate into 3 mL liquid medium in a culture tube. Starter cultures were incubated at 37 °C (or 30 °C for *C. glutamicum*) with shaking until reaching mid-logarithmic phase and then diluted with liquid medium to the desired density for initiating experiments.

Labeling experiments were performed either in 96-well plate format or in aerated culture tubes. For experiments in 96-well plate format, bacteria were mixed with liquid medium and probe stock solution in sterile flat-bottom 96-well plates to achieve the desired cell density and probe concentration at a final volume of 200 μL. The final DMSO concentration for probes stocked in DMSO (and there corresponding controls) was 1%. Plates were incubated at 37 °C (or 30 °C for *C. glutamicum*) with shaking in a Tecan plate reader (Infinite F200 PRO operated by Tecan iControl software) until the desired end-point (typical culture time 4 h).

For secondary labeling of bacteria with a fluorophore, suspensions of alkyne-, or azide-, or TCO-labeled cells (200 μL) were transferred to a v-bottom 96 well plate, centrifuged (3,600 rpm, 5 min, room temperature) and washed with PBS 1x containing 0.5% bovine serum albumin (PBSB) three times. Depending on which reaction the reporter required (CuAAC, SPAAC or

tetrazine ligation), the cells were treated appropriately. For **CuAAC**, cells were first fixed with 4% paraformaldehyde in PBS for 20 min and washed three times with PBSB. Then, the CuAAC reaction was carried out by resuspension of cells in PBSB (138 μL) and sequential addition of stock solutions of 1 mM Az488 (or Alk488) (3 μL), 60 mM sodium ascorbate (3 μL), 6.4 mM TBTA (3 μL), and 50 mM $CuSO_4$ (3 μL) to give a final reaction volume of 150 μL and the following final reagent concentrations: Az488 (or Alk488), 20 μM; sodium ascorbate, 1.2 mM; TBTA, 128 μM; $CuSO_4$, 1 mM. After thorough mixing, reactions were incubated in the dark at room temperature for 30 min. Finally, cells were washed with PBSB three times and prepared for analysis by flow cytometry or fluorescence microscopy as described below. For **SPAAC**, cells were resuspended in PBSB (171 μL), then typically 9 μL of a stock solution of 1 mM DBCO-488 was added to give a final reaction volume of 180 μL and a final DBCO-488 concentration of 50 μM. After thorough mixing, reactions were incubated in the dark at room temperature for 30 min. Finally, cells were washed with PBSB, fixed, and prepared for analysis by flow cytometry or fluorescence microscopy as described below. For the **tetrazine ligation**, cells were resuspended in PBSB (171 μL), then typically 9 μL of a stock solution of 1 mM tetrazine-Cy3 or methyltetrazine-Cy3 was added to give a final reaction volume of 180 μL and a final reagent concentration of 50 μM. After thorough mixing, reactions were incubated in the dark at room temperature for 30 min. Finally, cells were washed with PBSB, fixed, and prepared for analysis by flow cytometry or fluorescence microscopy as described below. For **O-FITC-TMM** labeling experiments, no secondary labeling was required. In this case, O-FITC-TMM-labeled cells were fixed with 4% paraformaldehyde in PBS for 20 min, washed three times with PBSB, and prepared for flow cytometry or fluorescence microscopy. For the SPAAC vs. tetrazine ligation cell-surface reaction kinetics comparison, experiments were carried out as described above with minor modifications. DBCO-Cy3 and tetrazine-Cy3 were used at final concentrations of 20 μM each. To vary the cell-surface reaction times, cells were incubated with secondary labeling reagent for the indicated period of time, then immediately centrifuged and washed to remove unreacted reagent.

Flow cytometry. After fluorescent labeling of bacteria according to the above general procedure, bacteria were transferred to 5 mL polystyrene Falcon tubes (BD Biosciences) and analyzed by flow cytometry. Flow cytometry was performed on a BD Biosciences FACSAria II flow cytometer. Fluorescence data was collected for 50,000 cells at an event rate of 500–1,000 events/sec and processed using BD FACSDIVA 8.0.1. All flow cytometry experiments were performed with three replicate samples, and data shown were representative of at least two independent experiments. Scatter-gated fluorescence analysis was used to obtain mean fluorescence intensities with doublet discrimination.

Fluorescence microscopy. 10 μL of bacterial sample in PBS were spotted onto a microscope slide, lightly spread into a thin layer using the edge of a coverslip, and allowed to air dry in the dark. Fluoromount-G mounting medium (SouthernBiotech) was applied, then cover slips were placed over the sample and immobilized with adhesive. Microscopy was carried out using an EVOS FL (Life Technologies) inverted microscope equipped with a 100 × 1.4 numerical aperture Plan-Apochromat oil immersion lens. Fluorescence imaging was performed using GFP (maximum excitation/emission = 470/510 nm) and RFP (maximum excitation/emission = 531/593 nm) LED light cubes. Images were captured with a Sony ICX445 CCD camera and processed using the FIJI distribution of ImageJ. Image acquisition and processing were performed identically for all test

and control samples being compared. Imaging data shown were representative of at least two independent experiments.

Labeling distribution of TMM reporters in AGM-containing and extractable lipids fractions. 10 mL *Msmeg* cultures (starting OD_{600} of 0.6) were grown in 7H9 liquid medium in the presence of O-AzTMM-C10 (25 μM), O-TCO-TMM (25 μM), O-FITC-TMM (100 μM), compound **9** (100 μM), or left untreated in 50 mL conical tubes. Cells were incubated with shaking for 4 h at 37 °C, then pelleted by centrifugation and washed with PBSB three times as described above. The cells were re-suspended in 500 μL PBS-B, followed by transferring into amber scintillation vials. For O-AzTMM-C10- and O-TCM-TMM-labeled cells and their untreated controls, the above-described ligation procedures were carried out with Alk488 and tetrazine-Cy3, respectively, at reaction volumes of 500 μL. After the reactions, cells were pelleted by centrifugation and washed with PBSB three times as described above. Cell pellets were re-suspended in 10 mL of chloroform/methanol (1:2, v/v) in a 20 mL scintillation vial, and stirred overnight at room temperature while protected from light to accomplish separation of the soluble lipids from the insoluble AGM-containing material. The chloroform/methanol suspension was centrifuged at 3,200 xg at 25 °C for 10 min. The supernatant was saved and the pellet was re-suspended in 10 mL chloroform/methanol (2:1, v/v) and stirred overnight at room temperature, followed by centrifugation at 3,200 xg at 25 °C for 10 min. The pellet (AGM-containing fraction) was saved, and allowed to dry at 37°C. Supernatants from both extraction steps were combined in a 100 mL round bottom flask and the solvents were removed by rotary evaporation. Both fractions (chloroform/methanol extract and the AGM fractions) were then resuspended in 1 mL of 0.1 M NaOH containing 0.1% SDS in amber glass screw-cap vials, sealed and then stirred for 1 hour at 100 °C to saponify and solubilize the samples. Next, the samples were cooled to room temperature and 200 μL aliquots were transferred to a black flat-bottom 96-well plate. Fluorescence analysis of samples versus a 0.1 M NaOH, 0.1% SDS blank control was carried out using a Tecan plate reader (Infinite F200 PRO operated by Tecan iControl software) using excitation/emission wavelengths for GFP or Cy3 using the optimal gain setting.

C. glutamicum **mutant generation, protein labeling & analysis**

Disruption of **cg0413** ***in*** **C. glutamicum.** To remove the *cg0413* gene, the deletion fragment was constructed by overlapping PCR (Figure S2A). This fragment was then cloned into the pK18*mobsacB* plasmid.[3] The resulting plasmid was sequenced and electroporated into *C. glutamicum* as previously described.[4] Recombinants were obtained by antibiotic selection followed by sucrose counterselection.[3] Colonies were screened for the *cg0413* deletion by PCR (Figure S2B), and positive colonies were verified by sequencing. Primers are listed in Table S1.

Protein labeling and analysis in **C. glutamicum** ***and the Δ*****cg0413** ***mutant with O-AlkTMM-C7.*** The procedure for O-AlkTMM-C7 protein labeling was performed essentially as we previously described as a positive control.[5] 100 mL cell cultures of wild-type *C. glutamicum* and the Δ*cg0413* mutant growing in LB liquid medium at early log phase (OD_{600} of ~0.2) were treated with O-AlkTMM-C7 (100 μM) or left untreated. Cells were incubated overnight in LB liquid medium at 30 °C, then harvested by centrifugation at 6,000xg for 10 min using a Thermo Scientific Sorvall LYNX 4000 Centrifuge. An aliquot of each sample was removed and subjected to CuAAC reaction with Az488 and flow cytometry analysis as described above in General Procedures for

Bacterial Labeling (giving the graph in Figure 6A). The cell pellets were washed sequentially with 150 mL chloroform/methanol (2:1) and methanol/chloroform (2:1), then air-dried and re-suspended in 1 mL of 0.5% SDS/0.05% LDAO buffer. The samples were transferred to a 1.5 mL screw cap vial containing 0.25 mL zirconia/silica beads (BioSpec Products), then subjected to bead beating at 5.5 m/s for 20 s (three times) using a FastPrep-24 bead beater to mechanically disrupt the cells. The lysates were transferred to a 1.5 mL microcentrifuge tube and spun down at 3,900 rpm for 5 min, then transferred to a new tube and spun down at 10,000xg for 10 min to give a clear supernatant. 360 μL of each protein sample was subjected to CuAAC click as follows: 10 μL each of stock solutions of 1 mM Az488, 6.4 mM TBTA, 50 mM sodium ascorbate, and 60 mM $CuSO_4$ were added to the protein samples in amber vials and the mixtures were stirred at 37 °C overnight. The samples were then concentrated by speedvac and the proteins were precipitated and collected three times with 1 mL of chloroform/methanol (2:1) at −20 °C for 1 hour to remove the excess unreacted fluorophore. The proteins were recovered by centrifugation at 3,200xg and prepared for SDS-PAGE analysis.

Protein samples were mixed with 6x sample loading buffer (250 mM Tris-HCl, 8% w/v SDS, 0.02% w/v bromophenol blue, 30% glycerol) and loaded into the gel. Next, the proteins were resolved by gel electrophoresis at a maximum voltage of 150V and a maximum current of 80 mA for 90 min or until loading dye exited the gel on Mini-PROTEAN TGX precast Tris-Tricene gels (4–20% cross-linked; Bio-Rad) using Tris-Tricene-SDS (Bio-Rad) as the running buffer. The running buffer was kept cold using an ice pack. In-gel fluorescence was detected with a Typhoon FLA 7000 (GE Healthcare Life Sciences) using fluorescein excitation/emission filters. For Coomassie blue staining, the same gel was fixed in 40% ethanol and 10% acetic acid for 10 min and then washed with 100 mL of reverse osmosis (RO) water and stained with QC colloidal Coomassie stain (Bio-Rad) overnight with gentle agitation. The gel was then washed in 100 mL RO water for 3 h, changing the water after each hour. The Coomassie-stained gel was imaged using a ChemiDoc Touch Imaging System (Bio-Rad) and processed by Image Lab software version 6.0 (Bio-Rad).

Protein labeling and analysis in* C. glutamicum *and the Δ*cg0413 *mutant with compounds 4–6. 100 mL cell cultures of wild-type *C. glutamicum* and the Δ*cg0413* mutant growing in LB liquid medium at early log phase (OD_{600} of ~0.2) were treated with O-AzTMM-C10 (100 μM), O-TCO-TMM (100 μM), O-FITC-TMM (100 μM), compound **9** as a control for O-FITC-TMM (100 μM), or left untreated. Cells were incubated overnight in LB liquid medium at 30 °C, then harvested by centrifugation at 6,000xg for 10 min using a Thermo Scientific Sorvall LYNX 4000 Centrifuge. The cell pellets were air-dried and re-suspended in 2 mL of 2 mg/mL lysozyme and 1 mM phenylmethylsulfonyl fluoride (PMSF) and the solution was warmed at 37 °C for 2 hours. The cells were then transferred to a 1.5 mL screw cap vial containing 0.25 mL zirconia/silica beads (BioSpec Products) and subjected to bead beating at 5.5 m/s for 20 s (three times) using a FastPrep-24 bead beater to mechanically disrupt the cells. The lysates were transferred into 20 mL scintillation vials and SDS was added to 2% and heated at 60 °C for 2 hours. The samples were transferred into 15 mL conical tubes and the proteins were collected by centrifugation at 3,200xg for 10 min. For the O-FITC-TMM-treated samples (and corresponding controls treated with compound **9**), no further processing was done. For O-AzTMM-C10-labeled proteins, 10 μL each of stock solutions of 1 mM Alk488, 6.4 mM TBTA, 50 mM sodium ascorbate, and 60 mM $CuSO_4$ were added to 360 μL of the protein samples in an amber vial. For O-TCO-TMM-labeled proteins, 20 μL of 1 mM tetrazine-Cy3 was added to 380 μL of protein samples. For CuAAC and tetrazine

ligations, the reaction mixtures were stirred at 37 °C overnight. The samples were then concentrated by speedvac and the proteins were precipitated and collected three times with 1 mL of chloroform/methanol (2:1) at −20 °C for 1 hour to remove the excess unreacted fluorophore. The proteins were recovered by centrifugation at 3,200xg and analyzed by SDS-PAGE as described above.

Growth curves and cellular viability assays. *Msmeg* cultures were prepared for growth inhibition and cellular viability assays essentially as described above for the labeling experiments. Starter cultures of *Msmeg* were mixed with liquid 7H9 medium and TMM analogue stock solutions in sterile flat-bottom 96-well plates to achieve the desired cell density (OD_{600} ~0.2) and probe concentration at a final volume of 200 μL. The final DMSO concentration for all analogue-treated and control samples 1%. Plates were incubated at 37 °C with shaking in a Tecan plate reader (Infinite M200 PRO operated by Tecan iControl software) over 16 h and optical density at 600 nm (OD_{600}) was monitored continuously. Experiments were performed in duplicate, and the mean values from each data point were used to construct growth curves plotted as OD_{600} versus time. Following 16 h of incubation, 90 μL of cells were transferred into another 96-well plate and 10 μL of 10x alamarBlue (Thermo Scientific) were added and incubated for 4 h. Absorbance at 570 nm with 600 nm reference was measured in a Tecan plate reader (Infinite M200). Percentage cell viability was calculated as the difference between the probe-treated and untreated cells using the following equation: [(O2 x A1 – O1 x A2)/(O2 X P1 – O1 x P2)] x 100. Where O1 and O2 are the molar extinction coefficients of resazurin at 570 nm and 600 nm, respectively, A1 and A2 are the absorbance of probe-treated cells at 570 nm and 600 nm, respectively, and P1 and P2 are the absorbance of untreated cells at 570 nm and 600 nm, respectively. Experiments were performed in duplicate, and the mean values from each data point were used to construct cell viability plots.

III. References

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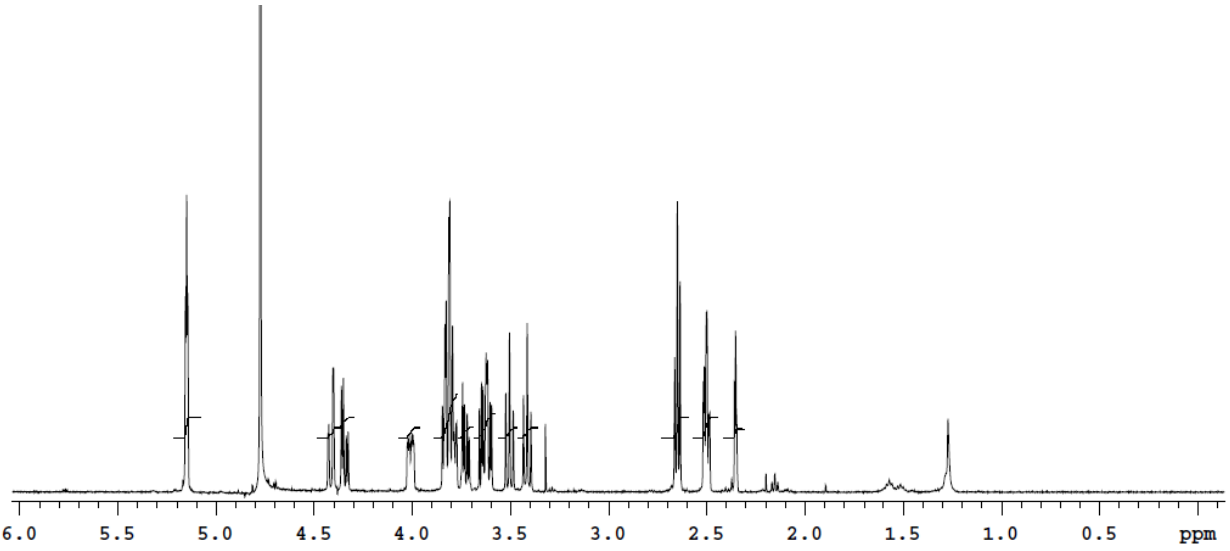
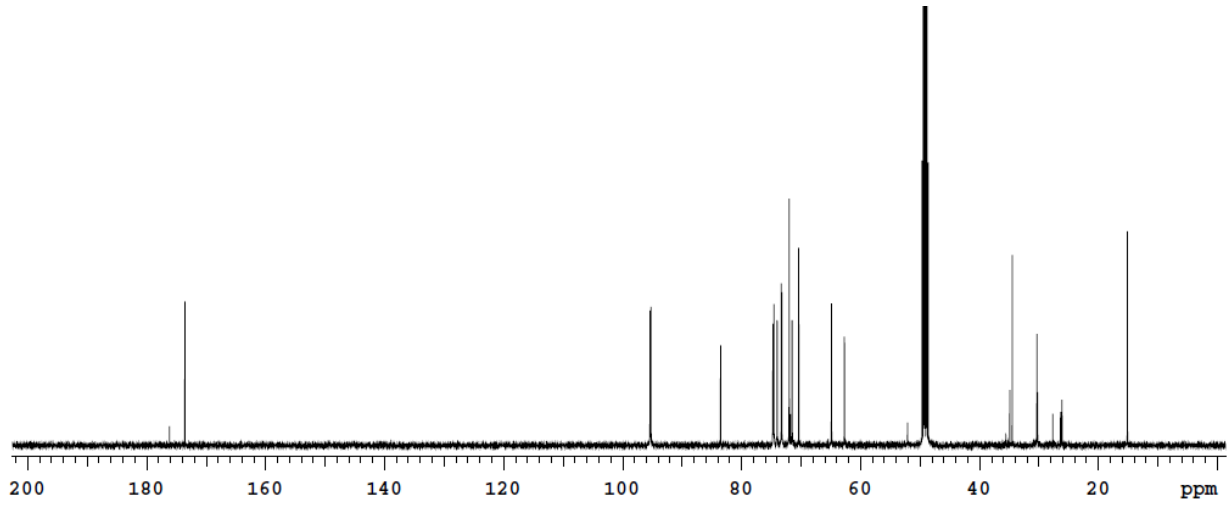
III. NMR Spectra

O-AlkTMM-C5 (**1**) ^{1}H NMR (500 MHz)



O-AlkTMM-C5 (**1**) ^{13}C NMR (125 MHz)



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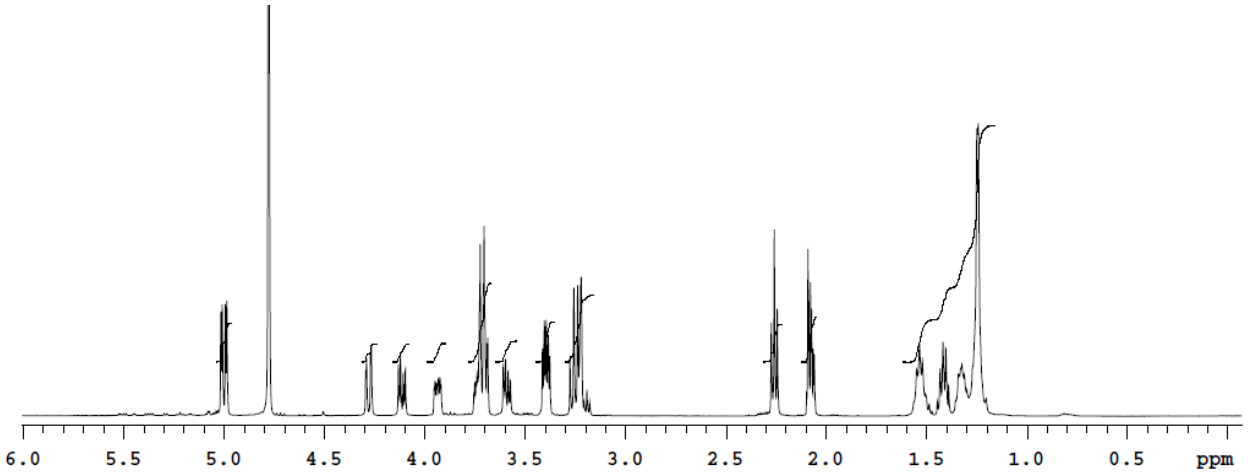

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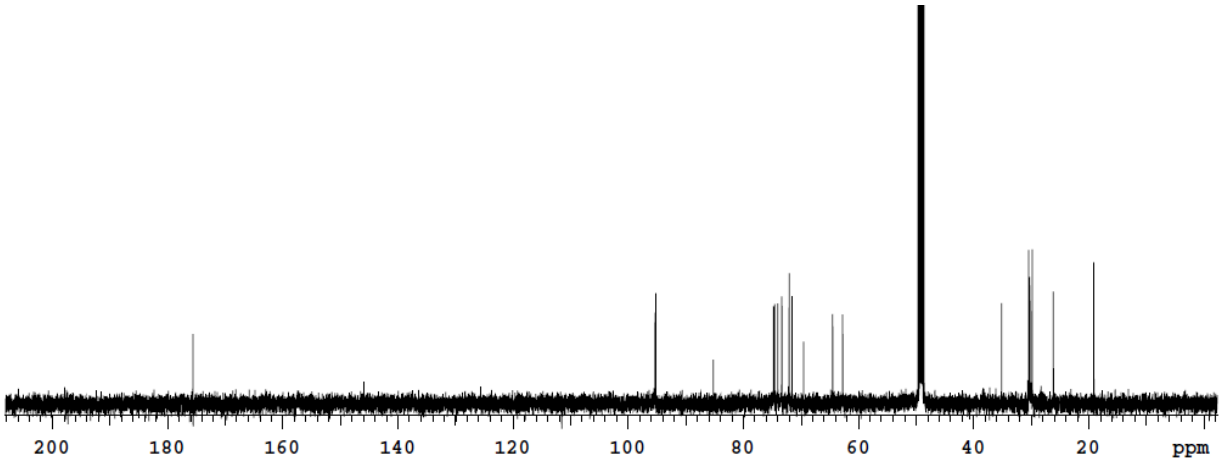

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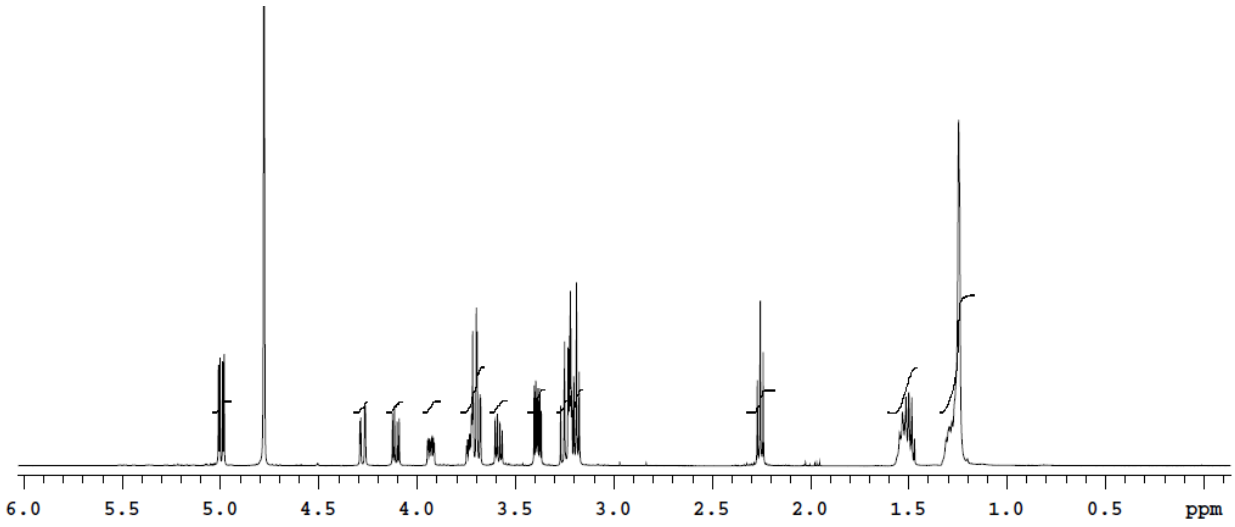

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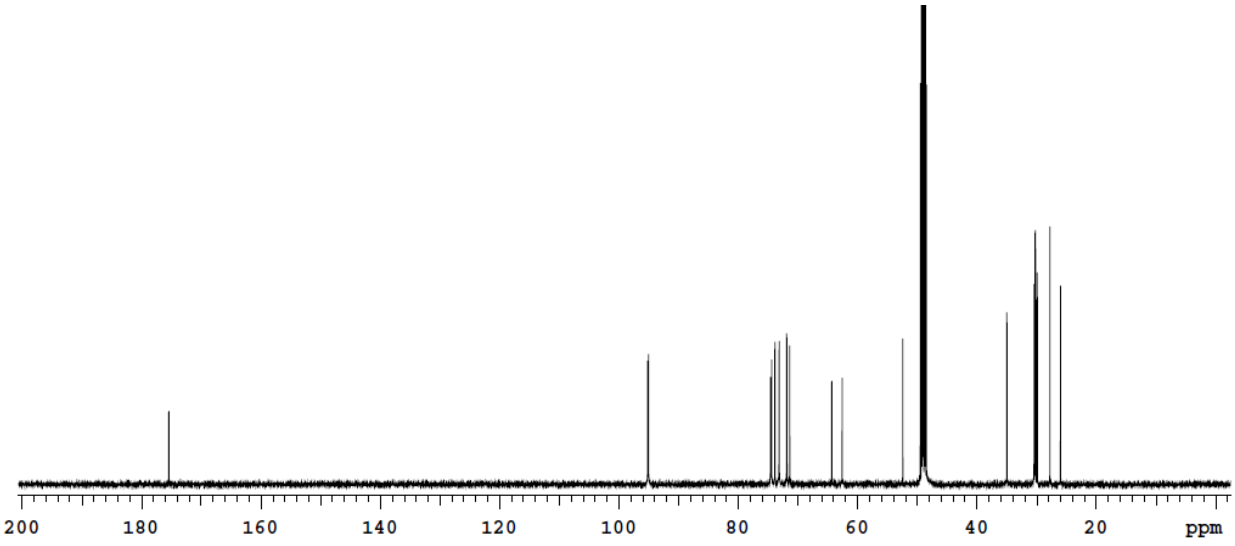
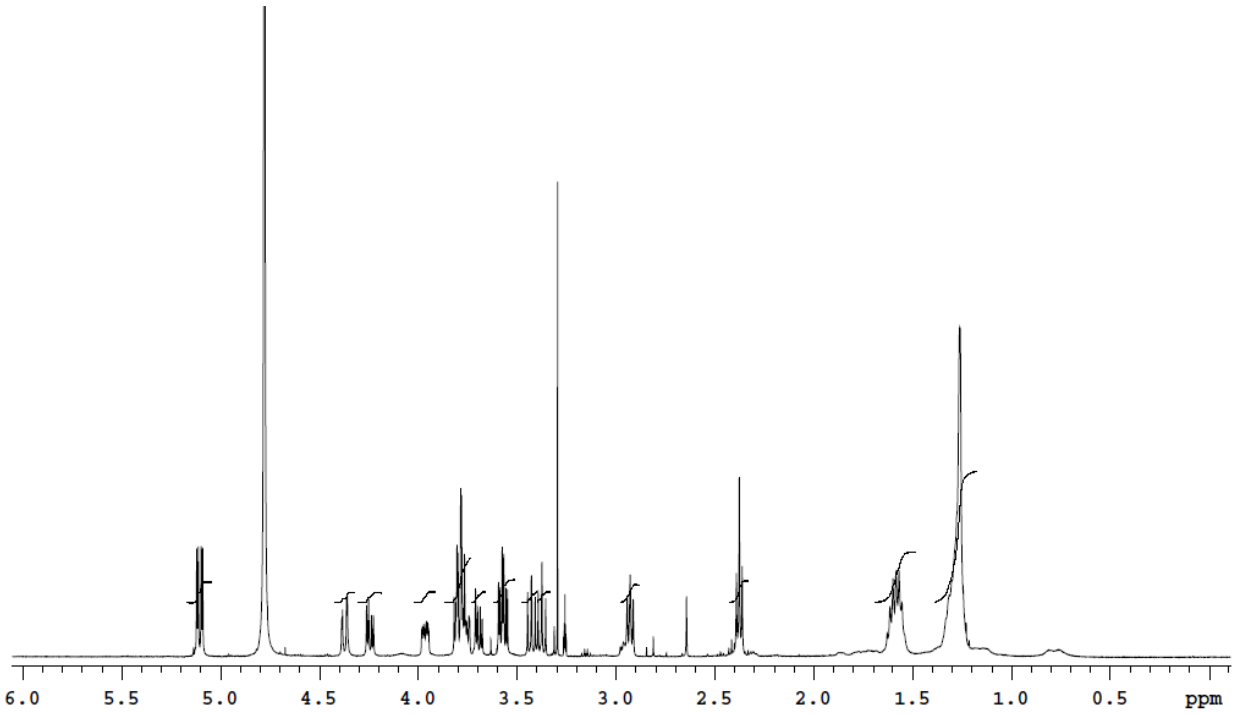


Compound **8** ^{1}H NMR (500 MHz)



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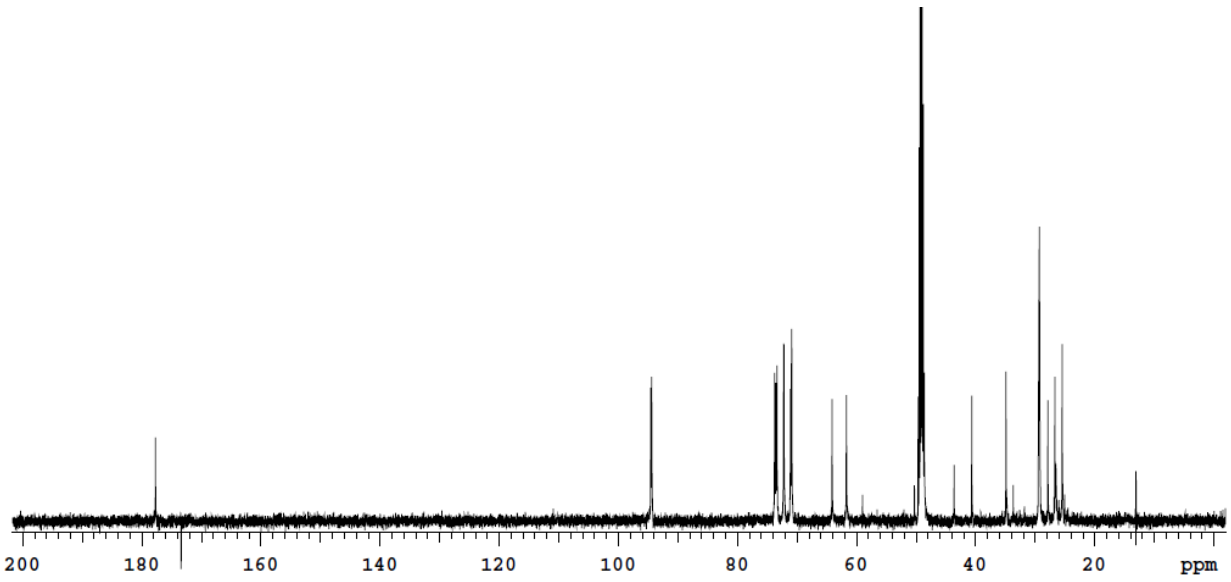
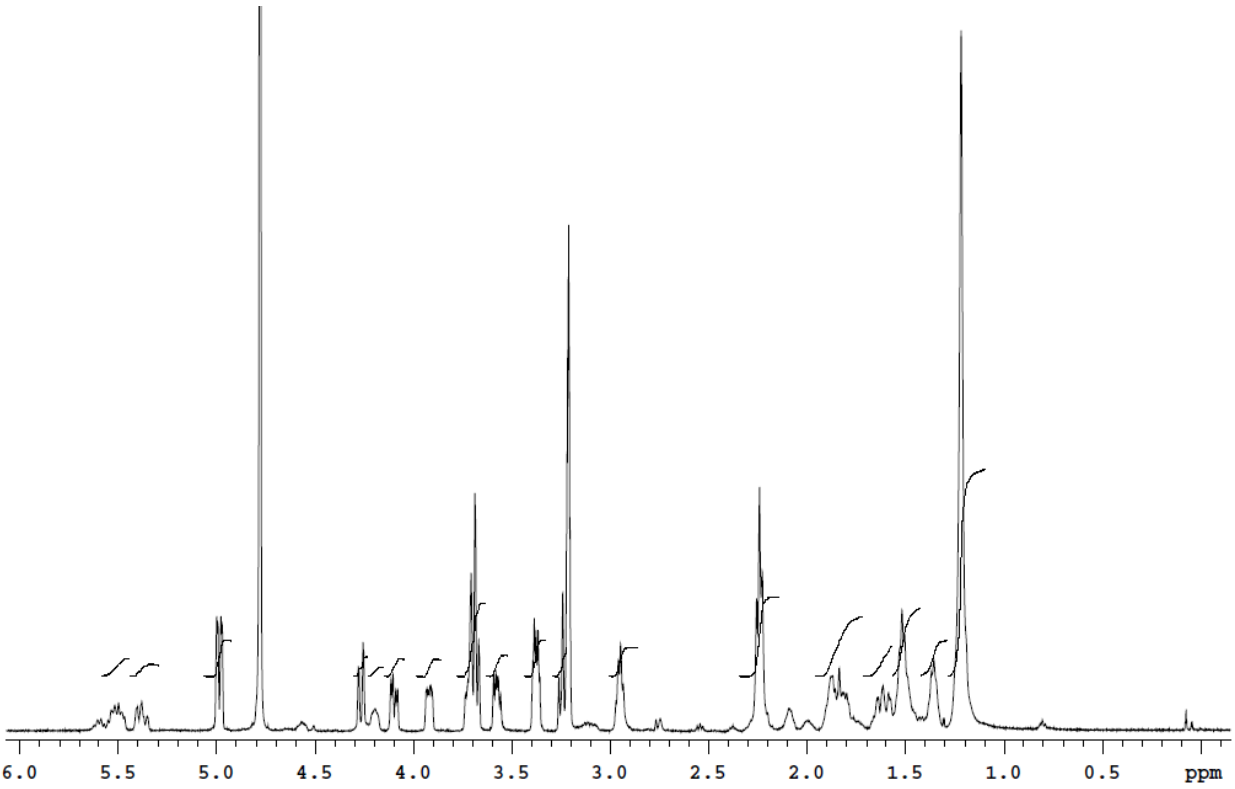
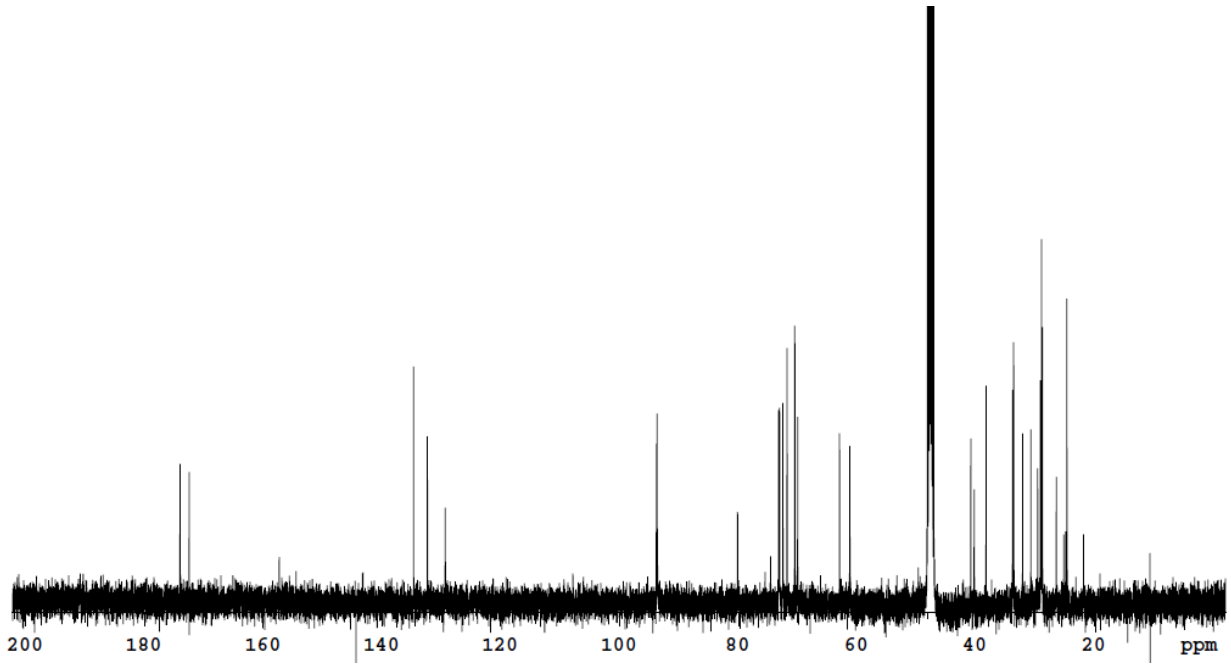


O-TCO-TMM (**5**) ^{1}H NMR (500 MHz)



O-TCO-TMM (**5**) ^{13}C NMR (125 MHz)



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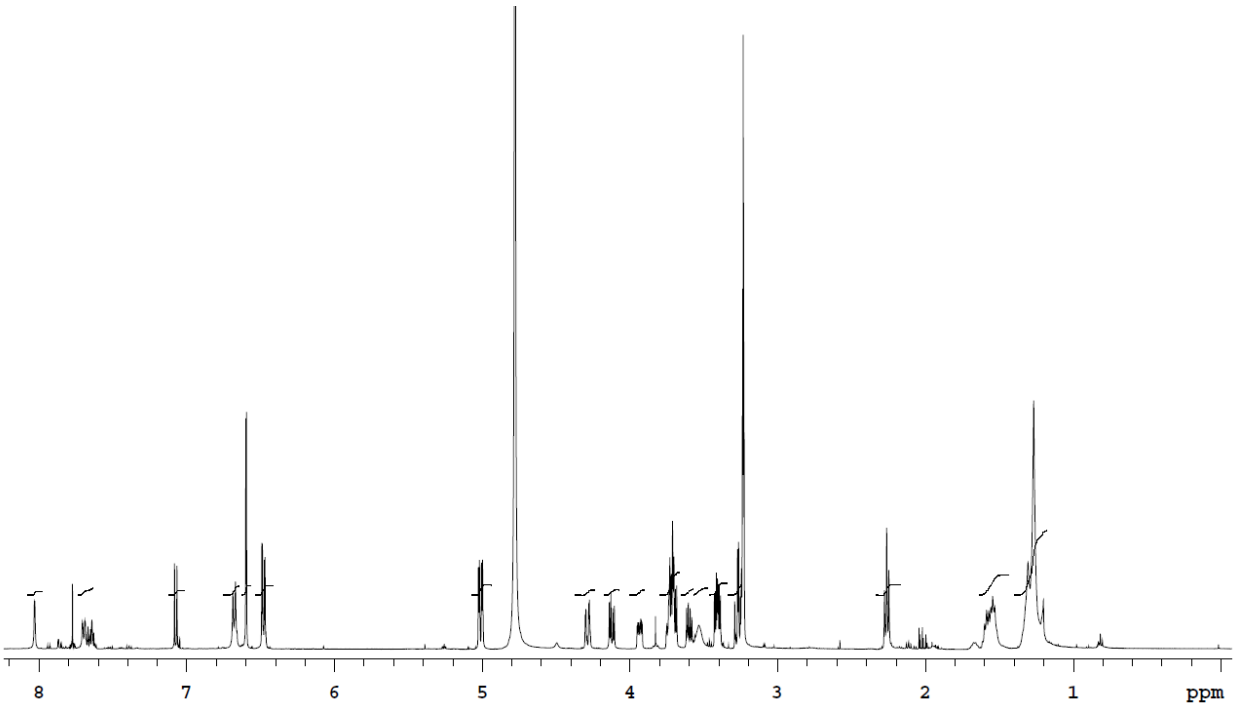
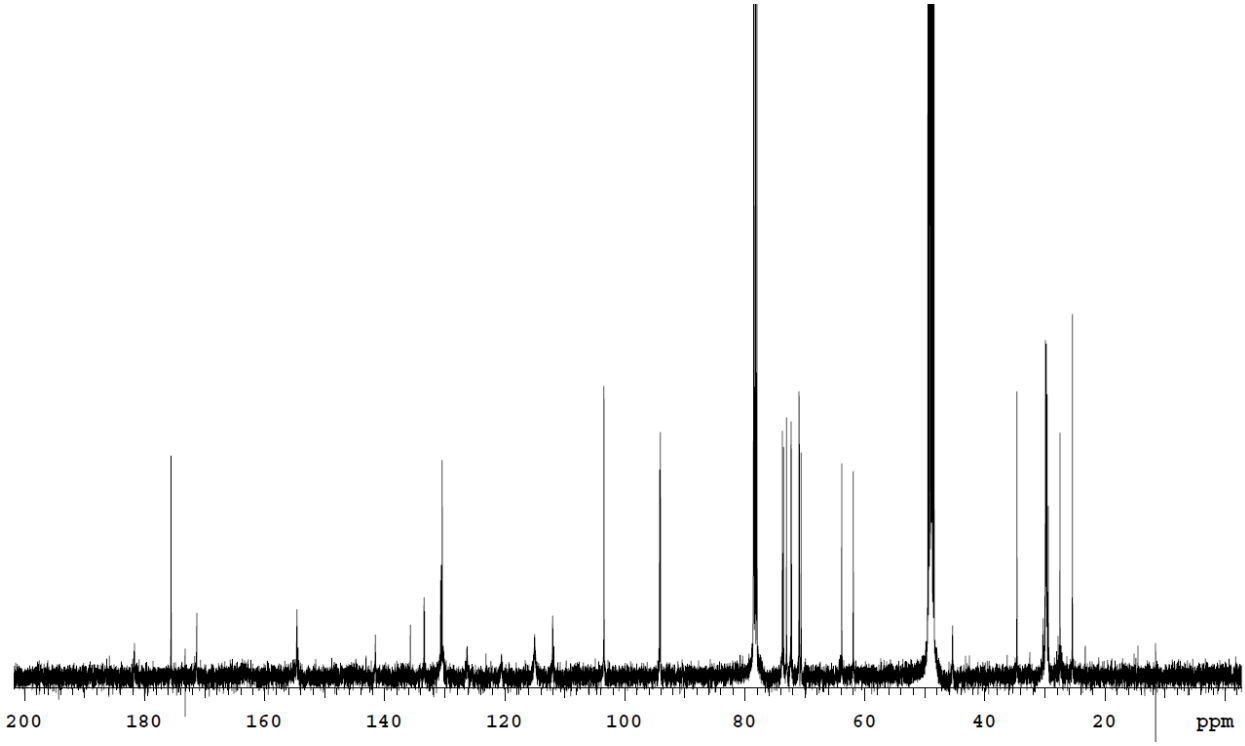
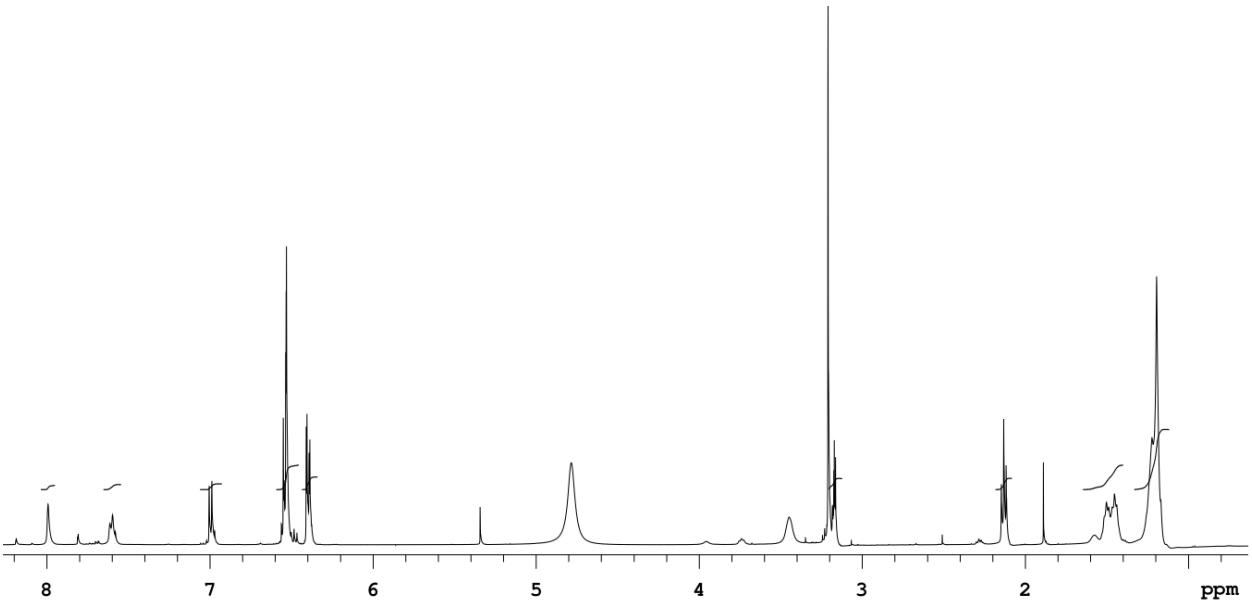


O-FITC-TMM (**6**) ^{13}C NMR (125 MHz)



Compound **9** ^{1}H NMR (500 MHz)



Compound **9** ^{13}C NMR (125 MHz)