First access ta a mycolic acid-based bioorthogonal reporter for the study of mycomembrane and mycoloyltransferases in Corynebacteria

Emilie Lesur,[a] Aurélie Baron,[b] Christiane Dietrich,[c] Marie Buchotte,[a] Gilles Doisneau,[a] Dominique Urban,[a] Jean-Marie Beau,[ab] Nicolas Bayan,[c] Boris Vauzeilles,[ab] Dominique Guianvarc'h*[a] and Yann Bourdreux*[a]

[a]*Institut de Chimie Moléculaire et des Matériaux d'Orsay (ICMMO), UMR 8182, Université Paris-Sud, Université Paris-Saclay, CNRS, F-91405 Orsay, France*
[b]*Institut de Chimie des Substances Naturelles, CNRS UPR2301, Université Paris-Sud, Université Paris-Saclay, 1 av. de la Terrasse, F-91198 Gif-sur-Yvette, France*
[c]*Institute for Integrative Biology of the Cell (I2BC), Université Paris-Sud, Université Paris-Saclay, CEA, CNRS, Gif-sur-Yvette Cedex, France*

Supporting Information

Table of Contents:

I. Abbreviations in the manuscript and supporting information

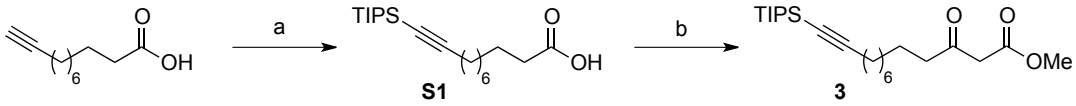
AG, Arabinogalactan
AGM, Arabinogalactan-linked mycolate
BHI, Brain Heart Infusion
Binap, 2,2'-bis(Diphenylphosphino)-1,1'-binaphthyl
CDI, Carbonyldiimidazole
CgMyt, *Corynebacterium glutamicum* mycoloyltransferase
Cod, 1,5-Cyclooctadiene
CR110-Az, 5/6-Carboxyrhodamine 110-PEG3-Azide
CuAAC, Copper(I)-catalyzed azide-alkyne cycloaddition
DMAP, 4-Dimethylaminopyridine
DMSO, Dimethylsulfoxide
EDCI, *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride
Fbp, Fibronectin binding protein
HMPA, Hexamethylphosphoramide
LB, Lysogeny broth
MFI, Mean fluorescence intensity
MM, Mycomembrane
MTPA, α-Methoxy-α-trifluoromethylphenylacetic acid
Myt, Mycoloyl transferase
p-NPP, *p*-Nitrophenyl palmitate
PBS, Phosphate-buffered saline
Pks, Polyketide synthase
PorA, Porin protein A
PorH, Porin protein H
PorB, Porin protein B
PTM, Post-translational modification
TBAF, *n*-Tetrabutylammonium fluoride
TDM, Trehalose dimycolate
TES, Triethylsilyl
TGTA, Tris[(1-glucosyl-1*H*-1,2,3-triazol-4-yl)methyl]amine
THF, Tetrahydrofuran
TIPS, Triisopropylsilyl
TMM, Trehalose monomycolate
TMS, Trimethylsilyl

II. Methods

1. Chemical Synthesis

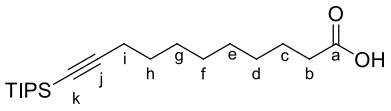
General methods:
All air sensitive reactions were carried out in oven-dried glassware under a slight positive pressure of argon. Solvents were dried by standard methods. THF was distilled from sodium benzophenone ketyl. TLC (Silica Gel 60 F_{254}) were visualized under UV (254 nm) and by staining either in 5% ethanolic sulfuric acid or orcinol or phosphomolybdic acid. Silica gel SDS 60 ACC 35-70 μm was used for column chromatography. Melting points were measured on a Stuart SMP10 apparatus and are uncorrected. NMR spectra were recorded on Bruker DRX 300 or AV 360 spectrometers. Chemical shifts (in ppm) were determined relative to residual undeuterated solvent as an internal reference. Abbreviations of multiplicity were as follows: s (singlet), d (doublet), dd (doublet of doublet), t (triplet), at (apparent triplet), m (multiplet), b (broad). Coupling constants in hertz (Hz) were measured from one-dimensional spectra. High-resolution mass spectra (positive or negative mode ESI) were performed on a Bruker Daltonics micrOTOF-QII spectrometer. Optical rotations were measured on an Anton Paar MCP 150 polarimeter (*c* in g / 100 mL).

Synthesis of β-ketoester 3
Compound **3** was prepared starting from commercially available 10-undecynoic acid as described as below:



Scheme S1: synthesis of β-ketoester **3**. Reagents and conditions: a) (i) *n*-BuLi, - 78 °C, THF, 30 min; (ii) TIPSCl, THF, - 78 °C to rt, 2 h, 40%; b) (i) CDI, THF, rt, 6 h; (ii) $Mg(MeO_2CCH_2CO_2)_2$, rt, overnight, 55%.

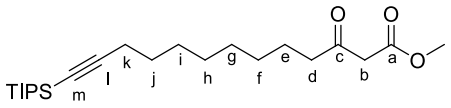
11-Triisopropylsilyl-undec-10-ynoic acid S1[1]



n-BuLi (9.6 mL, 24.0 mmol, 2.5 M in hexane, 2.19 equiv) was added dropwise to a solution of commercially available undec-10-ynoic acid (2.0 g, 10.97 mmol) in dry THF (120 mL) at -78°C. The solution was stirred for 30 min at this temperature and TIPSCl (5.88 mL, 27.44 mmol, 2.5 equiv) was then added slowly. The reaction mixture was stirred for 2 h at room temperature. The reaction was quenched by addition of saturated aqueous NaCl solution (20 mL). The mixture was extracted three times with Et_2O and the combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was dissolved in THF/MeOH/H_2O (50 mL, 3:1:1) and K_2CO_3 (7.6 g, 54.9 mmol, 5 equiv) was added. The solution was stirred at room temperature for 30 min. The reaction mixture was diluted with saturated aqueous NaCl solution (100 mL) and CH_2Cl_2 (40 mL) and then acidified with HCl 1M (pH ≈ 1). The reaction mixture was extracted three times with CH_2Cl_2, and the organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash silica gel chromatography (cyclohexane/EtOAc, 98:2 to 80:20) to give the desired product **S1** (1.48 g, 40%) as a colourless oil. Analytical data were in

agreement with those previously reported.[2] R_f = 0.51 (cyclohexane/EtOAc, 75:25). ^{1}H NMR ($CDCl_3$, 360 MHz) δ (ppm): 2.35 (t, 2H, *J* = 7.5 Hz, H_b), 2.24 (t, 2H, *J* = 6.8 Hz, H_i), 1.70-1.25 (m, 12H, H_c-H_h), 1.05 (m, 21H, Si(*i*-Pr)$_3$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 180.5 (C_a), 109.2 (C_k), 80.0 (C_j), 34.1 (C_b), 29.1, 28.9, 28.8, 28.7, 28.5, 24.6 (6C, C_c-C_h), 19.8 (C_i), 18.6 (Si(CH(**C**H$_3$)$_2$)$_3$), 11.3 (Si(**C**H(CH$_3$)$_2$)$_3$). HRMS (ESI): calcd for $C_{20}H_{38}NaO_2Si$ $[M+Na]^+$ 361.2533, found 361.2545.

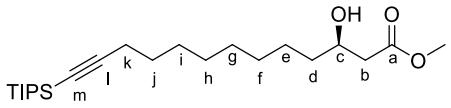
Methyl 13-triisopropylsilyl-3-oxo-tridec-12-ynoate 3



Compound **3** was synthetized according to Masamune's procedure.[3] Carbonyldiimidazole (575 mg, 3.54 mmol, 1.2 equiv) was added to a solution of 11-triisopropylsilyl-undec-10-ynoic acid **S1** (1.0 g, 2.95 mmol) in dry THF (30 mL). After stirring at room temperature for 6 h, the magnesium salt of monomethylmalonate (990 mg, 3.83 mmol, 1.3 equiv) was added. The reaction mixture was stirred for 18 h at room temperature and was then acidified with an aqueous HCl solution (1M, pH ≈ 1). The reaction mixture was extracted three times with EtOAc, and the combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash silica gel chromatography (cyclohexane/EtOAc, 98:2) to give compound **3** (646 mg, 55%) as a colourless oil. R_f = 0.54 (cyclohexane/EtOAc, 9:1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 3.75 (s, 3H, OCH_3), 3.46 (s, 2H, H_b), 2.53 (t, 2H, *J* = 7.4 Hz, H_d), 2.24 (t, 2H, *J* = 6.8 Hz, H_k), 1.70-1.25 (m, 12H, H_e-H_j), 1.07 (m, 21H, Si(*i*-Pr)$_3$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 202.8 (C_c), 167.7 (C_a), 109.2 (C_m), 80.0 (C_l), 52.4 (OCH_3), 49.0 (C_b), 43.1 (C_d), 29.3, 29.0, 28.9, 28.8, 28.6, 23.4 (6C, C_e-C_j), 19.8 (C_k), 18.6 (Si(CH(**C**H$_3$)$_2$)$_3$), 11.3 (Si(**C**H(CH$_3$)$_2$)$_3$). HRMS (ESI): calcd for $C_{23}H_{42}NaO_3Si$ $[M+Na]^+$ 417.2795, found 417.2782.

Synthesis of probes 1 and 2

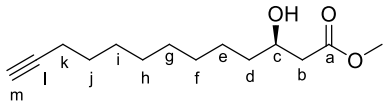
Methyl (*R*)-3-hydroxy-13-triisopropylsilyl-tridec-12-ynoate 4



The (*R*)-BinapRuBr$_2$ complex was prepared under argon according to a reported procedure.[4] To a solution of (*R*)-Binap (32.2 mg, 0.052 mmol, 0.024 equiv) and (Cod)Ru(2-methylallyl)$_2$ (15.1 mg, 0.047 mmol, 0.022 equiv) in anhydrous and degassed acetone, were added 632 µL of a methanolic HBr solution (0.15 M, 0.095 mmol, 0.044 equiv). The reaction mixture was stirred for 30 min at room temperature and then concentrated under vacuum. A solution of β-ketoester **3** (850 mg, 2.15 mmol) in degassed and dry MeOH (2.8 mL) was added *via* cannula to the catalyst. The mixture was purged three times with dihydrogen and vigorously stirred overnight at 50 °C under 1 atm of dihydrogen. The solvent was evaporated under vacuum and the residue was purified by silica gel chromatography (cyclohexane/EtOAc, 9:1) to give the desired product **4** (839 mg, 98%) as a colourless oil. R_f = 0.5 (cyclohexane/EtOAc 75:25). ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 4.02 (m, 1H, H_c), 3.74 (s, 3H, OCH_3), 2.54 (dd, 1H, *J* = 16.5 Hz, *J* = 3.2 Hz, H_b), 2.43 (dd, 1H, *J* = 16.5 Hz, 8.8 Hz, $H_{b'}$), 2.26 (t, 2H, *J* = 6.8 Hz, H_k), 1.60-1.20 (m, 14H, H_d-H_j), 1.07 (m, 21H, Si(*i*-Pr)$_3$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 173.7 (C_a), 109.4 (C_m), 80.1 (C_l), 68.1 (C_c), 51.9 (OCH_3), 41.2 (C_b), 36.6 (C_d), 29.6, 29.1,

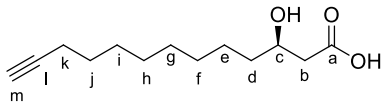
28.9, 28.8, 25.6 (6C, C_e-C_j), 20.0 (C_k), 18.8 (Si(CH(**C**H_3)$_2$)$_3$), 11.4 (Si(**C**H(CH$_3$)$_2$)$_3$). HRMS (ESI): calcd for $C_{23}H_{44}NaO_3Si$ $[M+Na]^+$ 419.2952, found 419.2935. $[\alpha]_D^{20}$ = -8 (*c* 1.2, $CHCl_3$).

Methyl (*R*)-3-hydroxy-tridec-12-ynoate 6



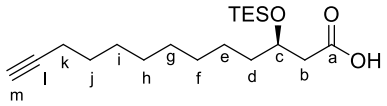
To a solution of compound **4** (103 mg, 0.26 mmol) in THF (2.7 mL) at 0 °C was added a solution of TBAF (1.0 M in THF, 0.5 mL, 0.52 mmol, 2 equiv). The reaction mixture was stirred overnight at room temperature and then concentrated under vacuum. The residue was purified by flash silica gel chromatography (cyclohexane/EtOAc, 98:2 to 90:10) to give the expected product **6** (39 mg, 63%) as a colourless oil. R_f = 0.24 (cyclohexane/EtOAc, 85/15). ^{1}H NMR ($CDCl_3$, 360 MHz) δ (ppm): 4.00 (m, 1H, H_c), 3.71 (s, 3H, CH_3), 2.85 (d, 1H, *J* = 4 Hz, OH), 2.52 (dd, 1H, *J* = 16.6 Hz, 3.5 Hz, H_b), 2.41 (dd, 1H, *J* = 16.6 Hz, 8.7 Hz, $H_{b'}$), 2.18 (td, 2H, *J* = 7.1 Hz, *J* = 2.7 Hz, H_k), 1.93 (t, 1H, *J* = 2.7 Hz, H_m), 1.59-1.25 (m, 14H, H_d-H_j). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 173.6 (C_a), 84.8 (C_l), 68.2, 68.1 (2C, C_c, C_m), 51.8 (CH_3), 41.3 (C_b), 36.6 (C_d), 29.5, 29.4, 29.1, 28.8, 28.6, 25.5 (6C, C_e-C_j), 18.5 (C_k). HRMS (ESI): calcd for $C_{14}H_{24}NaO_3$ $[M+Na]^+$ 263.1618, found 263.1615. $[\alpha]_D^{20}$ = -16 (*c* 1, $CHCl_3$).

(*R*)-3-Hydroxy-tridec-12-ynoic acid 8



A 10% aqueous solution of NaOH (2.5 mL) was added to a solution of compound **6** (77.9 mg, 0.32 mmol) in MeOH (2.5 mL). The reaction mixture was stirred overnight at 45 °C and then acidified to pH ≈ 1 with an aqueous HCl solution (1M). The mixture was extracted with EtOAc, the combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash silica gel chromatography (cyclohexane/EtOAc, 1:1) to give derivative **8** (71.4 mg, 98%) as a colourless syrup. R_f = 0.15 (cyclohexane/EtOAc, 1:1). ^{1}H NMR ($CDCl_3$, 360 MHz) δ (ppm): 4.03 (m, 1H, H_c), 2.58 (dd, 1H, *J* = 16.5 Hz, *J* = 3.4 Hz, H_b), 2.49 (dd, 1H, *J* = 16.5 Hz, 9.0 Hz, $H_{b'}$), 2.18 (td, 2H, *J* = 7.0 Hz, *J* = 2.7 Hz, H_k), 1.94 (t, 1H, *J* = 2.7 Hz, H_m), 1.70-1.20 (m, 14H, H_d-H_j). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 177.9 (C_a), 84.9 (C_l), 68.2 (2C, C_c, C_m), 41.2 (C_b), 36.7 (C_d), 29.5, 29.2, 28.8, 28.6, 25.6 (6C, C_e-C_j), 18.5 (C_k). HRMS (ESI): calcd for $C_{13}H_{21}O_3$ $[M-H]^-$ 225.1496, found 225.1495. $[\alpha]_D^{20}$ = -9 (*c* 0.9, $CHCl_3$).

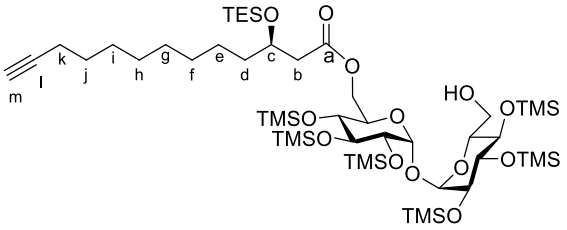
(*R*)-3-Triethylsilyloxy-tridec-12-ynoic acid 10



To a solution of compound **8** (126 mg, 0.56 mmol) in dry pyridine (2 mL) was added dropwise TESCl (467 µL, 2.78 mmol, 5 equiv). The reaction mixture was stirred overnight at room temperature and was then diluted with a saturated aqueous NaCl solution. The mixture was extracted three times with EtOAc, the combined organic layers were dried over Na_2SO_4

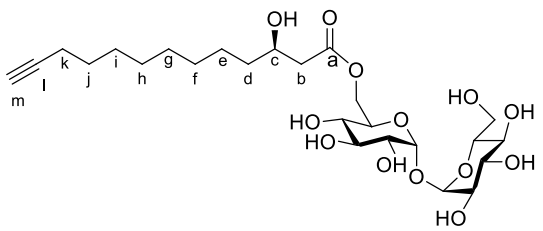
and concentrated under reduced pressure. The residue was purified by flash silica gel chromatography (cyclohexane/EtOAc, 95:5 to 50:50) to give compound **10** (104 mg, 55%) as a colourless syrup. R_f = 0.51 (cyclohexane/EtOAc, 8:2). ^{1}H NMR ($CDCl_3$, 360 MHz) δ (ppm): 4.01 (m, 1H, H_c), 2.61 (dd, 1H, *J* = 15.8 Hz, *J* = 5.1 Hz, H_b), 2.52 (dd, 1H, *J* = 15.8 Hz, 4.9 Hz, $H_{b'}$), 2.20 (td, 2H, *J* = 7.0 Hz, *J* = 2.8 Hz, H_k), 1.95 (t, 1H, *J* = 2.8 Hz, H_m), 1.60-1.25 (m, 14H, H_d-H_j), 0.99 (t, 9H, *J* = 8.0 Hz, $Si(CH_2C\mathbf{H}_3)_3$), 0.66 (q, 6H, *J* = 8.0 Hz, $Si(C\mathbf{H}_2\text{-}CH_3)_3$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 176,5 (C_a), 84.9 (C_l), 69.5, 68.2 (2C, C_c, C_m), 42.2 (C_b), 37.5 (C_d), 29.9, 29.7, 29.1, 28.8, 28.6, 25.3 (6C, C_e-C_j), 18.5 (C_k), 6.9 ($Si(CH_2\mathbf{C}H_3)_3$), 5.0 ($Si(\mathbf{C}H_2\text{-}CH_3)_3$). HRMS (ESI): calcd for $C_{19}H_{36}NaO_3Si$ $[M+Na]^+$ 363.2326, found 363.2314. $[\alpha]_D^{20}$ = -1 (*c* 1.1, $CHCl_3$).

6-*O*-((*R*)-3-Triethylsilyloxy-tridec-12-ynoyl)-2,3,4,2',3',4',-hexa-*O*-(trimethylsilyl)-α,α-D-trehalose S2



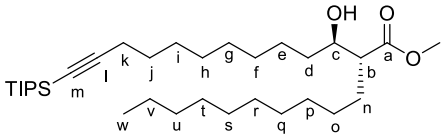
A solution of 2,3,4,2',3',4'-hexa-*O*-(trimethylsilyl)-α,α-D-trehalose[5,6] (185 mg, 0.24 mmol, 2 equiv) in CH_2Cl_2 (1.5 mL) was added to compound **10** (40.5 mg, 0.12 mmol). EDCI (45.6 mg, 0.24 mmol, 2 equiv) and DMAP (29 mg, 0.24 mmol, 2 equiv) were added and the reaction mixture was stirred at 37 °C for 8 h. Saturated aqueous NaCl solution was added and the reaction mixture was extracted three times with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash silica gel chromatography (cyclohexane/EtOAc, 98:2 to 90:10) to give the esterified derivative **S2** (77 mg, 58%) as a colourless oil. R_f = 0.43 (cyclohexane/EtOAc 9/1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 4.91 (d, 1H, *J* = 3.0 Hz, H_1 or $H_{1'}$), 4.88 (d, 1H, *J* = 3.0 Hz, $H_{1'}$ or H_1), 4.28 (dd, 1H, *J* = 11.6 Hz, *J* = 1.8 Hz, H_{6a}), 4.14 (m, 1H, H_c), 4.10-3.95 (m, 2H, H_{6b}, H_5), 3.94-3.80 (m, 3H, H_3, $H_{3'}$, $H_{5'}$), 3.73-3.64 (m, 2H, $H_{6'a}$, $H_{6'b}$), 3.51-3.38 (m, 4H, H_2, $H_{2'}$, H_4, $H_{4'}$), 2.53 (dd, 1H, *J* = 15.3 Hz, *J* = 6.6 Hz, H_b), 2.44 (dd, 1H, *J* = 15.3, *J* = 6.3, $H_{b'}$), 2.18 (td, 2H, *J* = 7.0 Hz, *J* = 2.7 Hz, H_k), 1.93 (t, 1H, *J* = 2.7 Hz, H_m), 1.72 (t, 1H, *J* = 5.9 Hz, OH), 1.58-1.20 (m, 14H, H_d-H_j), 0.94 (t, 9H, *J* = 8.0 Hz, $Si(CH_2C\mathbf{H}_3)_3$), 0.59 (q, 6H, *J* = 8.0 Hz, $Si(C\mathbf{H}_2CH_3)_3$), 0.19-0.07 (m, 54H, $6Si(CH_3)_3$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 171.7 (C_a), 94.5, 94.3 (2C, C_1, $C_{1'}$), 84.8 (C_l (alkyne)), 73.4, 73.3 (2C, C_3, $C_{3'}$), 72.9 ($C_{5'}$), 72.8, 72.6, 72.0, 71.4 (4C, C_2, $C_{2'}$, C_4, $C_{4'}$), 70.7 (C_5), 69.2 (C_c), 68.1 (C_m), 63.4 (C_6), 61.7 ($C_{6'}$), 42.6 (C_b), 37.6 (C_d), 29.6, 29.4, 29.0, 28.7, 28.5, 25.1 (6C, C_e-C_j), 18.4 (C_k), 6.9 ($Si(CH_2\mathbf{C}H_3)_3$), 4.9 ($Si(\mathbf{C}H_2CH_3)_3$), 1.1, 1.0, 0.9, 0.8, 0.2, 0.1 (6 $Si(CH_3)_3$). HRMS (ESI): calcd for $C_{49}H_{104}NaO_{13}Si_7$ $[M+Na]^+$ 1119.5754, found 1119.5716. $[\alpha]_D^{20}$ = +76 (*c* 0.9, $CHCl_3$).

6-*O*-((*R*)-3-Hydroxy-tridec-12-ynoyl)-α,α-D-trehalose 1



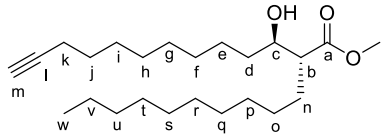
To a solution of compound **S2** (70.3 mg, 0.064 mmol) in a MeOH/CH_2Cl_2 mixture (4 mL, 3:1), were added 300 mg of Dowex 50WX8 (H^+ form). The reaction mixture was stirred at room temperature for 1 h, filtered, washed three times with MeOH and concentrated under reduced pressure. The residue was purified by flash silica gel chromatography (EtOAc/MeOH, 95:5 to 90:10) to give derivative **1** (30.6 mg, 87%) as a colourless oil. R_f = 0.20 (EtOAc/MeOH, 10 mL, 7:3 +2 drops of water). ^{1}H NMR ($CD_3OD/CDCl_3$, 360 MHz) δ (ppm): 5.08 (d, 1H, *J* = 3.7 Hz, H_1 or $H_{1'}$), 5.05 (d, 1H, *J* = 3.7 Hz, $H_{1'}$ or H_1), 4.44 (dd, 1H, *J* = 11.9 Hz, *J* = 2.0 Hz, H_{6a}), 4.18 (dd, 1H, *J* = 11.9 Hz, *J* = 5.8 Hz, H_{6b}), 4.04 (ddd, 1H, *J* = 10.1 Hz, *J* = 5.8 Hz, *J* = 2.0 Hz, H_5), 3.99 (m, 1H, H_c), 3.84-3.73 (m, 4H, H_3, $H_{3'}$, $H_{6a'}$, $H_{5'}$), 3.67 (dd, 1H, *J* = 12.0 Hz, *J* = 5.3 Hz $H_{6b'}$), 3.51 (dd, 1H, *J* = 9.7 Hz, *J* = 3.7 Hz, H_2 or $H_{2'}$), 3.47 (dd, 1H, *J* = 9.9 Hz, *J* = 3.7 Hz, $H_{2'}$ or H_2), 3.35-3.27 (m, 2H, H_4, $H_{4'}$), 2.50 (dd, 1H, *J* = 15.5 Hz, *J* = 3.6 Hz, H_b), 2.39 (dd, 1H, *J* = 15.5 Hz, *J* = 8.7 Hz, $H_{b'}$), 2.14 (td, 2H, *J* = 7.1 Hz, *J* = 2.6 Hz, H_k), 2.00 (t, 1H, *J* = 2.6 Hz, H_m), 1.54-1.20 (m, 14H, H_d-H_j). ^{13}C NMR (CD_3OD, 75 MHz) δ (ppm): 173.6 (C_a), 95.5, 95.3 (2C, C_1, $C_{1'}$), 85.2 (C_l (alkyne)), 74.7, 74.6, 74.0 (3C, C_3, C_3', C_5), 73.4, 73.3 (2C, C_2, C_2'), 72.1, 72.0 (2C, C_4, C_4'), 71.5 (C_5'), 69.6, 69.5 (2C, C_c, C_m), 64.8 (C_6), 62.8 ($C_{6'}$), 43.6 (C_b), 38.2 (C_d), 30.9, 30.8, 30.3, 29.9, 29.8, 26.8 (6C, C_d-C_j), 19.2 (C_k). HRMS (ESI): calcd for $C_{25}H_{42}NaO_{13}$ $[M+Na]^+$ 573.2517, found 573.2496. $[\alpha]_D^{20}$ = +95 (*c* 0.9, $CHCl_3$/MeOH 1/3).

Methyl (2*R*, 3*R*)-2-decyl-3-hydroxy-13-triisopropylsilyl-tridec-12-ynoate 5



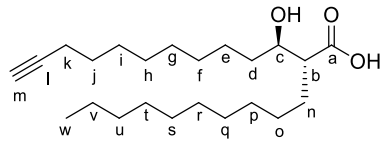
To a solution of dry diisopropylamine (742 µL, 5.29 mmol, 3.5 equiv) in THF (4.4 mL) cooled to - 40 °C were added dropwise 2.84 mL of *n*-butyllithium (4.54 mmol, 1.6 M in THF, 3 equiv). After stirring for 40 min at - 40 °C, the mixture was cooled to - 78 °C and a solution of β-hydroxyester **4** (600 mg, 1.51 mmol) in dry THF (11 mL) was added *via* a cannula. The reaction mixture was stirred for 1h30 at - 78 °C then 1-iododecane (968 µL, 4.54 mmol, 3 equiv) and HMPA (315 µL, 1.81 mmol, 1.2 equiv) were added slowly. The mixture was warmed slowly to - 10 °C and stirred overnight at this temperature. The mixture was diluted with a saturated aqueous NH_4Cl solution and extracted three times with EtOAc. The combined organic layers were washed with an aqueous NaCl solution, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash silica gel chromatography (cyclohexane/EtOAc, 98:2 to 95:5) to give compound **5** (287 mg, 35%) as a colourless oil. R_f = 0.56 (cyclohexane/EtOAc, 85:15). ^{1}H NMR ($CDCl_3$, 360 MHz) δ (ppm): 3.71 (s, 3H, OCH_3), 3.65 (m, 1H, H_c), 2.43 (m, 1H, H_b), 2.24 (t, 2H, *J* = 6.8 Hz, H_k), 1.80-1.20 (m, 32H, H_d-H_j, H_n-H_v), 1.10-0.98 (m, 21H, Si(*i*-Pr)$_3$), 0.88 (t, 3H, *J* = 6.7 Hz, H_w). ^{13}C NMR ($CDCl_3$, 90 MHz) δ (ppm): 176.4 (C_a), 109.4 (C_m), 80.1 (C_l), 72.5 (C_c), 51.7 (OCH_3), 51.1 (C_b), 35.9 (C_d), 32.1, 29.8, 29.7, 29.6, 29.5, 29.4, 29.1, 29.0, 28.8, 27.6, 25.9, 22.8 (15C, C_e-C_j, C_n-C_v), 20.0 (C_k), 18.8 (Si(CH(**C**H$_3$)$_2$)$_3$), 14.3 (C_w), 11.5 (Si(**C**H(CH$_3$)$_2$)$_3$). HRMS (ESI): calcd for $C_{33}H_{64}NaO_3Si$ $[M+Na]^+$ 559.4517, found 559.4490. $[\alpha]_D^{20}$ = + 3 (*c* 1, $CHCl_3$).

Methyl (2*R*, 3*R*)-2-decyl-3-hydroxy-tridec-12-ynoate 7



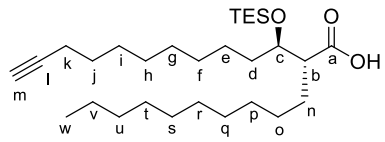
To a solution of compound **5** (100 mg, 0.186 mmol) in THF (2 mL) at 0 °C was added a solution of TBAF (1.0 M in THF, 300 µL, 0.30 mmol, 1.6 equiv). The reaction mixture was stirred overnight at room temperature. A saturated aqueous NaCl solution was added and the mixture was extracted three times with EtOAc. The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash silica gel chromatography (cyclohexane/EtOAc, 98:2 to 95:5) to give derivative **7** (60 mg, 85%) as a colourless oil. R_f = 0.43 (cyclohexane/EtOAc, 85:15). ^{1}H NMR ($CDCl_3$, 360 MHz) δ (ppm): 3.71 (s, 3H, OCH_3), 3.65 (m, 1H, H_c), 2.43 (m, 1H, H_b), 2.18 (td, 2H, *J* = 7.0 Hz, *J* = 2.7 Hz, H_k), 1.93 (t, 1H, *J* = 2.7 Hz, H_m), 1.76-1.18 (m, 32H, H_d-H_j, H_n-H_v), 0.88 (t, 3H, *J* = 6.9 Hz, H_w). ^{13}C NMR ($CDCl_3$, 90 MHz) δ (ppm): 176.4 (C_a), 84.9 (C_l), 72.4 (C_c), 68.2 (C_m), 51.7 (OCH_3), 51.1 (C_b), 35.8 (C_d), 32.1, 29.8, 29.7, 29.6, 29.5, 29.4, 29.2, 28.9, 29.6, 27.6, 25.9, 22.8 (15C, C_e-C_j, C_n-C_v), 18.5 (C_k), 14.3 (C_w). HRMS (ESI): calcd for $C_{24}H_{44}NaO_3$ $[M+Na]^+$ 403.3182, found 403.3183. $[\alpha]_D^{20}$ = + 9 (*c* 1, $CHCl_3$).

(2*R*, 3*R*)-2-Decyl-3-hydroxy-tridec-12-ynoic acid 9



A 10% aqueous NaOH solution (5.3 mL) was added to compound **7** (149 mg, 0.39 mmol) in MeOH (5.3 mL). The reaction mixture was stirred overnight at 45 °C and then acidified to pH ≈ 1 with an aqueous HCl solution (1M). The mixture was extracted three times with EtOAc, the combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash silica gel chromatography (cyclohexane/EtOAc, 1:1) to give compound **9** (133 mg, 93%) as a colourless syrup. R_f = 0.51 (cyclohexane/EtOAc, 1:1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 3.78-3.65 (m, 1H, H_c), 2.51-2.40 (m, 1H, H_b), 2.18 (td, 2H, *J* = 7.0 Hz, *J* = 2.7 Hz, H_k), 1.94 (t, 1H, *J* = 2.7 Hz, H_m), 1.80-1.16 (m, 32H, H_d-H_j, H_n-H_v), 0.88 (t, 3H, *J* = 6.9 Hz, H_w). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 180.3 (C_a), 84.8 (C_l), 72.1 (C_c), 68.1 (C_m), 51.0 (C_b), 35.5 (C_d), 31.9, 29.6, 29.5, 29.4, 29.3, 29.0, 28.7, 28.5, 27.4, 25.7, 22.7 (15C, C_e-C_j, C_n-C_v), 18.4 (C_k), 14.2 (C_w). HRMS (ESI): calcd for $C_{23}H_{42}NaO_3$ $[M+Na]^+$ 389.3026, found 389.3008.$[\alpha]_D^{20}$ = + 11 (*c* 0.9, $CHCl_3$).

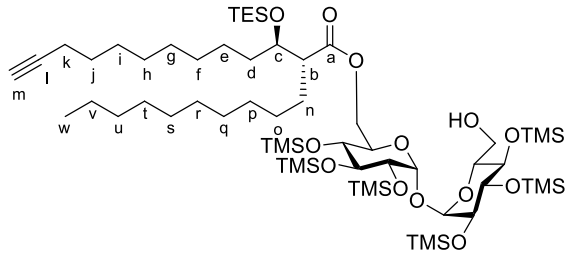
(2*R*, 3*R*)-2-Decyl-3-triethylsilyloxy-tridec-12-ynoic acid 11



To a solution of compound **9** (102 mg, 0.28 mmol) in dry pyridine (2 mL) was added dropwise TESCl (910 µL, 1.39 mmol, 5 equiv). The reaction mixture was stirred overnight at 60 °C and was then diluted with a saturated aqueous NaCl solution. The reaction mixture was

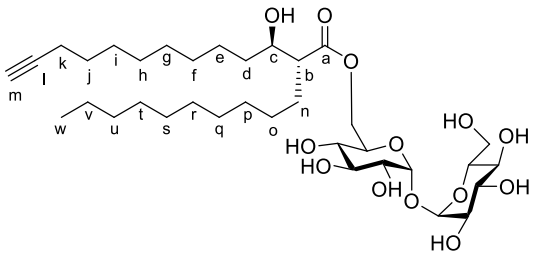
extracted three times with EtOAc. The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash silica gel chromatography (cyclohexane/EtOAc, 95:5 to 50:50) to give the desired product **11** (83 mg, 62%) as a colourless syrup. R_f = 0.57 (cyclohexane/EtOAc, 8:2). ^{1}H NMR ($CDCl_3$, 360 MHz) δ (ppm): 3.89-3.82 (m, 1H, H_c), 2.50 (m, 1H, H_b), 2.18 (td, 2H, *J* = 7.0 Hz, *J* = 2.6 Hz, H_k), 1.94 (t, 1H, *J* = 2.6 Hz, H_m), 1.75-1.16 (m, 32H, H_d-H_j, H_n-H_v), 0.97 (t, 9H, *J* = 7.9 Hz, $Si(CH_2CH_3)_3$), 0.87 (t, 3H, *J* = 6.9 Hz, H_w), 0.65 (q, 6H, *J* = 7.9 Hz, $Si(CH_2CH_3)_3$). ^{13}C NMR ($CDCl_3$, 90 MHz) δ (ppm): 176.3 (C_a), 84.9 (C_l), 73.9 (C_c), 68.2 (C_m), 50.7 (C_b), 35.7 (C_d), 32.0, 29.8, 29.7, 29.6, 29.5, 29.4, 29.1, 28.8, 28.6, 27.7, 25.2, 22.8 (15C, C_e-C_j, C_n-C_v), 18.5 (C_k), 14.3 (C_w), 6.9 ($Si(CH_2CH_3)_3$), 5.1 ($Si(CH_2CH_3)_3$). HRMS (ESI): calcd for $C_{29}H_{56}NaO_3Si$ $[M+Na]^+$ 503.3891, found 503.3885. $[\alpha]_D^{20}$ = +5 (*c* 0.9, $CHCl_3$).

6-*O*-((2*R*, 3*R*)-2-Decyl-3-triethylsilyloxy-tridec-12-ynoyl)-2,3,4,2',3',4',-hexa-*O*-(trimethylsilyl)-α,α-D-trehalose S3



A solution of 2,3,4,2',3',4'-hexa-*O*-(trimethylsilyl)-α,α-D-trehalose[5,6] (128 mg, 0.16 mmol, 2 equiv) in CH_2Cl_2 (1.5 mL) was added to compound **11** (40 mg, 0.08 mmol). EDCI (31 mg, 0.16 mmol, 2 equiv) and DMAP (20 mg, 0.16 mmol, 2 equiv) were then added and the reaction mixture was stirred at 37 °C for 8 h. Saturated aqueous NaCl solution was added and the reaction mixture was extracted three times with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash silica gel chromatography (cyclohexane/EtOAc, 95:5) to give the desired product **S3** (43 mg, 42%) as a colourless oil. R_f = 0.58 (petroleum ether/Et_2O, 95:5). ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 4.90 (d, 1H, *J* = 3.1 Hz, H_1 or $H_{1'}$), 4.84 (d, 1H, *J* = 3.1 Hz, $H_{1'}$ or H_1), 4.36 (dd, 1H, *J* = 11.6 Hz, *J* = 2.0 Hz, H_{6a}), 4.05 (dd, 1H, *J* = 11.6 Hz, *J* = 3.8 Hz H_{6b}), 4.01-3.80 (m, 5H, H_c, H_3, $H_{3'}$, H_5, $H_{5'}$), 3.74-3.65 (m, 2H, $H_{6a'}$, $H_{6b'}$), 3.50 (at, 1H, *J* = 9.0 Hz, H_4 or $H_{4'}$), 3.47 (at, 1H, *J* = 9.0 Hz, $H_{4'}$ or H_4), 3.42 (dd, 1H, *J* = 8.7 Hz, *J* = 3.1 Hz, H_2 or $H_{2'}$), 3.38 (dd, 1H, *J* = 9.1 Hz, *J* = 3.1 Hz, $H_{2'}$ or H_2), 2.54 (m, 1H, H_b), 2.17 (td, 2H, *J* = 7.0 Hz, *J* = 2.6 Hz, H_k), 1.93 (t, 1H, *J* = 2.6 Hz, H_m), 1.72 (t, 1H, *J* = 6.4 Hz, OH), 1.66-1.16 (m, 32H, H_d-H_j, H_n-H_v), 0.96 (t, 9H, *J* = 7.8 Hz, $Si(CH_2CH_3)_3$), 0.88 (t, 3H, *J* = 6.6 Hz, CH_3), 0.60 (q, 6H, *J* = 7.8 Hz, $Si(CH_2CH_3)_3$), 0.20-0.03 (m, 54H, 6 $Si(CH_3)_3$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 174.1 (C_a), 94.7, 94.6 (2C, C_1, $C_{1'}$), 84.9 (C_l (alkyne)), 73.6, 73.5, 73.1, 73.0, 72.9 (6C, C_3, $C_{3'}$, C_5, $C_{5'}$, C_2, $C_{2'}$), 72.0, 71.5 (2C, C_4, $C_{4'}$), 70.9 (C_c), 68.3 (C_m), 62.6 (C_6), 61.8 ($C_{6'}$), 52.5 (C_b), 33.6 (C_d), 32.1, 30.0, 29.9, 29.8, 29.7, 29.6, 29.5, 29.2, 28.9, 28.6, 28.3, 26.2, 26.1, 25.4, 22.8 (15C, C_e-C_j, C_n-C_v), 18.5 (C_k), 14.3 (C_w), 7.1 ($Si(CH_2CH_3)_3$), 5.2 ($Si(CH_2CH_3)_3$), 1.2, 1.1, 1.0, 0.3, 0.2 (6 $Si(CH_3)_3$). HRMS (ESI): calcd for $C_{59}H_{124}NaO_{13}Si_7$ $[M+Na]^+$ 1259.7319, found 1259.7250. $[\alpha]_D^{20}$ = +57 (*c* 1.2, $CHCl_3$).

6-*O*-((2*R*, 3*R*)-2-Decyl-3-hydroxy-tridec-12-ynoyl)-*α*,*α*-D-trehalose 2



To a solution of compound **S3** (40 mg, 0.032 mmol) in a MeOH/CH_2Cl_2 mixture (3 mL, 3:1) were added 300 mg of Dowex 50WX8 (H^+ form). The reaction mixture was stirred at room temperature for 1 h, then filtered, washed three times with MeOH and concentrated under reduced pressure. The residue was purified by flash silica gel chromatography (EtOAc/MeOH, 95:5 to 90:10) to give the desired product **2** (19 mg, 85%) as a colourless oil. R_f = 0.51 (EtOAc/MeOH, 10 mL, 7:3 + 2 drops of water). ^{1}H NMR ($CD_3OD/CDCl_3$, 360 MHz) δ (ppm): 5.08 (d, 2H, *J* = 3.7 Hz, H_1 and $H_{1'}$), 4.47 (dd, 1H, *J* = 11.8 Hz, *J* = 1.9 Hz, H_{6a}), 4.17 (dd, 1H, *J* = 11.8 Hz, *J* = 5.4 Hz H_{6b}), 4.06 (ddd, 1H, *J* = 10.1 Hz, *J* = 5.4 Hz, *J* = 1.9 Hz, H_5) 3.85-3.74 (m, 4H, $H_{5'}$, $H_{6a'}$, H_3, $H_{3'}$), 3.71-3.63 (m, 2H, $H_{6b'}$, H_c), 3.49 (dd, 1H, *J* = 9.7 Hz, *J* = 3.7 Hz, H_2 or $H_{2'}$), 3.48 (dd, 1H, *J* = 9.7 Hz, *J* = 3.7 Hz, $H_{2'}$ or H_2), 3.37-3.30 (m, 2H, H_4, $H_{4'}$), 2.43 (m, 1H, H_b), 2.15 (td, 2H, *J* = 6.8 Hz, *J* = 2.6 Hz, H_k), 2.10 (t, 1H, *J* = 2.6 Hz, H_m), 1.67-1.18 (m, 32H, H_d-H_j, H_n-H_v), 0.88 (t, 3H, *J* = 6.8 Hz, CH_3). ^{13}C NMR (CD_3OD, 75 MHz) δ (ppm): 176.2 (C_a), 95.3, 95.2 (2C, C_1, $C_{1'}$), 85.1 (C_l (alkyne)), 74.5, 74.4, 73.9 (3C, C_3, $C_{3'}$, $C_{5'}$), 73.6 (C_c), 73.2 (2C, C_2, $C_{2'}$), 72.0, 71.9 (2C, C_4, $C_{4'}$), 71.4 (C_5), 69.4 (C_m), 64.4 (C_6), 62.6 ($C_{6'}$), 54.2 (C_b), 35.6, 33.1, 30.7, 30.6, 30.5, 30.4, 30.2, 29.8, 29.7, 28.6, 26.6, 23.7 (16C, C_d-C_j, C_n-C_v), 19.0 (C_k), 14.5 (C_w). HRMS (ESI): calcd for $C_{35}H_{62}NaO_{13}$ $[M+Na]^+$ 713.4083, found 713.4056. $[\alpha]_D^{20}$ = +95 (*c* 0.3, $CHCl_3$/MeOH, 3:1).

Determination of enantiomeric excess and absolute configuration of compound (*R*)-4 using Mosher's method: [7,8,9]

Synthesis of MTPA-esters of (*R*)-4: Dry pyridine (50 µL, 0.62 mmol, 24.6 equiv) and (*S*)-(+)-MTPA-Cl (7 µL, 0.037 mmol, 1.5 equiv) were added at 0 °C to a solution of compound (*R*)-**4** (10.0 mg, 0.025 mmol) in dry CH_2Cl_2 (200 µL). The reaction mixture was stirred for 4 h at room temperature and then diluted with CH_2Cl_2 (5 mL) and saturated aqueous NaCl solution (5 mL). The aqueous layer was extracted twice with CH_2Cl_2 and the combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash silica gel chromatography (cyclohexane/EtOAc, 98:2 to 95:5) to give the (*R*)-MTPA-ester of (*R*)-**4** (12.5 mg, 81%). The same protocol was used starting from *rac*-**4** (reduction of **3** with $NaBH_4$) to obtain the (*R*)-MTPA-ester of *rac*-**4**; and with (*R*)-(-)-MTPA-Cl to obtain the (*S*)-MTPA-ester of (*R*)-**4**.

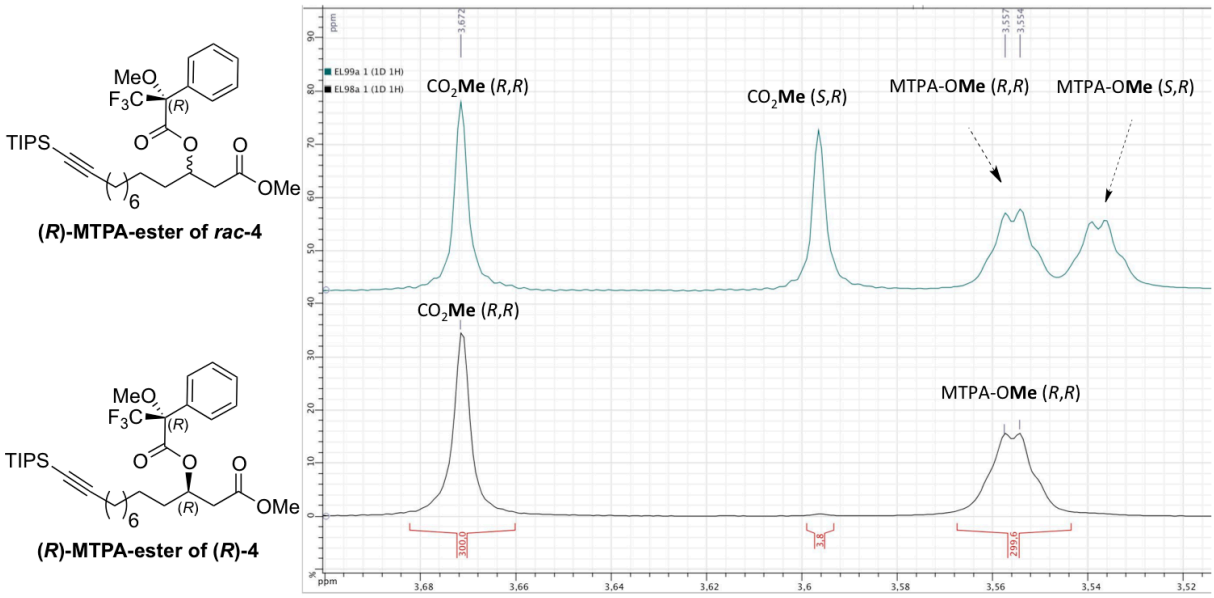


Figure S1: ^{1}H NMR (360 MHz, $CDCl_3$) analysis of the CO_2Me and MTPA-OMe regions. Optical purity was determined by integration of the CO_2**Me** signals. The calculated >95% *de* for the (*R*)-MTPA-ester of (*R*)-**4** corresponds to a >95% *ee* for (*R*)-**4.**

The absolute configuration of the stereogenic center of (*R*)-**4**, assumed to be *R* as predicted by Noyori asymmetric reduction[10] using (*R*)-Binap-$RuBr_2$, was assigned using Mosher's model.[7,8,9] See Table S1 and Figure S2.

Table S1: Selected ^{1}H NMR ($CDCl_3$, 360 MHz) data for (*S*)- and (*R*)-MTPA esters of (*R*)-**4**, and $\Delta\delta^{SR}$ values allowing the determination of the absolute configuration

			$\Delta\delta^{SR}$ = δ (*S*) - δ (*R*)	
	δ (*S*)-MTPA	δ (*R*)-MTPA	ppm	Hz
CO_2Me	3.59	3.67	-0.08	-28.8
H_{2a}	2.66	2.71	-0.05	-18.0
H_{2b}	2.58	2.61	-0.03	-10.8
H_4	1.72	1.63	+0.09	+32.4

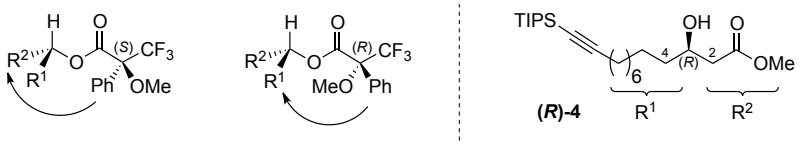
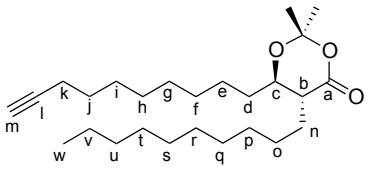


Figure S2: (*S*)-MTPA ester: the phenyl group shields R^2; (*R*)-MTPA ester: the phenyl group shields R^1. As a result $\Delta\delta^{SR} > 0$ for R^1 and $\Delta\delta^{SR} < 0$ for R^2.

Determination of the relative *anti* relationship for compound 5.

The *anti* stereochemistry of β-hydroxyester **5** was defined after conversion of derivative **9** to its corresponding acetonide showing a large observed H_b-H_c coupling constant ($J_{Hb\text{-}Hc}$ = 10.2 Hz).[11] The same approach has been used for the relative configuration determination in the synthesis of mycolate derivatives.[12]

(5*R*,6*R*)-6-(Dec-9-yn-1-yl)-5-decyl-2,2-dimethyl-1,3-dioxan-4-one S4



2-Methoxypropene (31 μL, 0.33 mmol, 4.1 equiv) and PPTS (1 mg, 0.004 mmol, 0.05 equiv) were added at room temperature to a solution of compound **9** (30 mg, 0.08 mmol) in dry CH_2Cl_2 (2 mL). The reaction mixture was stirred for 30 min at room temperature and $NaHCO_3$ (6.4 mg, 0.076 mmol, 0.95 equiv) was then added. The mixture was stirred for 5 min and concentrated under reduced pressure. The residue was purified by flash silica gel chromatography (cyclohexane/EtOAc, 9:1 + 1% Et_3N) to give acetonide **S4** (20 mg, 61%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 3.91 (ddd, 1H, J = 10.2 Hz, J = 8.7 Hz, J = 2.1 Hz, H_c), 2.33 (ddd, 1H, J = 10.2 Hz, J = 5.3 Hz, J = 4.5 Hz, H_b), 2.19 (td, 2H, J = 7.0 Hz, J = 2.6 Hz, H_k), 1.94 (t, 1H, J = 2.6 Hz, H_m), 1.81 (m, 1H, 1H of H_n), 1.72-1.19 (m, 31H, 1H of H_n, H_o-H_v, H_d-H_j), 1.57, 1.56 (2s, 6H, $C(CH_3)_2$), 0.88 (t, J = 6.2 Hz, 3H, CH_3). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 171.2 (C_a), 105.4 (**C**$(CH_3)_2$), 84.9 (C_l), 70.9 (C_c), 68.2 (C_m), 45.6 (C_b), 33.9, 32.0, 30.1, 29.7, 29.5, 29.4, 29.2, 28.8, 28.6, 27.5, 26.7, 22.8 (16C, C_d-C_j, C_n-C_v), 25.4, 25.3 (2C, C(**C**$H_3)_2$), 18.5 (C_k), 14.3 (C_w). HRMS (ESI): calcd $C_{26}H_{47}O_3$ for $[M+H]^+$ 407.3520, found 407.3504. $[\alpha]_D^{20}$ = +2 (*c* 0.8, $CHCl_3$).

2. Labeling of bacteria with TMM analogues

Bacterial strains, growth conditions and reagents

Dimethylsulfoxide (DMSO) was purchased from Sigma-Aldrich. Phosphate-buffered saline solution (PBS), Gene Frame seals, Microscope Slides and Microscope Coverslips were purchased from Thermo Fisher Scientific. 5/6-Carboxyrhodamine 110-PEG_3-Azide (CR110-Az) was purchased from Jena Bioscience. Tris((1-(β-D-glucopyranosyl)-1H-1,2,3-triazol-4-yl)methyl)amine (TGTA) was synthesized according to a reported procedure.[13]

Solutions stocks of organic and inorganic compounds were prepared as followed: Sodium ascorbate: 50 mM in PBS 1X; TGTA: 40 mM in PBS 1X; Copper sulfate ($CuSO_4$): 20 mM in H_2O; CR110-Az: 2 mM in DMSO. Stock solutions of TMM analogues were prepared in milliQ water. Bacteria (*Corynebacterium glutamicum* ATCC13032 and *Escherichia coli* K12 *Bacillus subtilis* NCIB 3610) were inoculated into their appropriate growth medium (LB for *E. coli* and *B. subtilis*, and BHI for *C. glutamicum*) and cultured at 37 °C for *E. coli* and *B. subtilis*, and at 30 °C for *C. glutamicum*, under agitation (180 rpm).

General procedure for labeling *C. glutamicum*

C. glutamicum was inoculated (1:100) in BHI medium (final volume 900 µL). Bacteria were cultured until mid-log phase growth (7 h, OD ≈ 6) at which point probe **1** or **2** (5 or 50 µM) was added (control experiments were treated with probe vehicle i.e. water) and the growth was pursued for 1 h at 30 °C.

Then, 350 µL of these suspensions were transferred in a 1.5 mL microtube and the cells were harvested by centrifugation at 3200 rpm for 5 min. The pellets were washed three times with PBS buffer (200 µL, 3200 rpm, 5 min, rt). The pellets were re-suspended in 200 µL of the "click" solution (0.05 mM CR110-Az, 2.5 mM sodium ascorbate, 2.0 mM TGTA and 1.0 mM copper sulfate pentahydrate in PBS/DMSO (98:2) buffer) or in 200 µL of PBS/DMSO (97:3) buffer, and transferred in a 2.0 mL microtube for a better agitation. This suspension was vigorously shaken (550 rpm) in the dark for 30 min at room temperature, then transferred back in a 1.5 mL microtube and the cells were harvested by centrifugation (3200 rpm, 5 min, rt). Bacterial pellets were washed one times with PBS/DMSO (97:3) buffer (200 µL, 3200 rpm, 5 min, rt) and two times with PBS buffer (200 µL, 3200 rpm, 5 min, rt). The pellets were re-suspended in PBS buffer (200 µL) and kept at 4 °C between 1 and 3 hours in the dark before being analyzed.

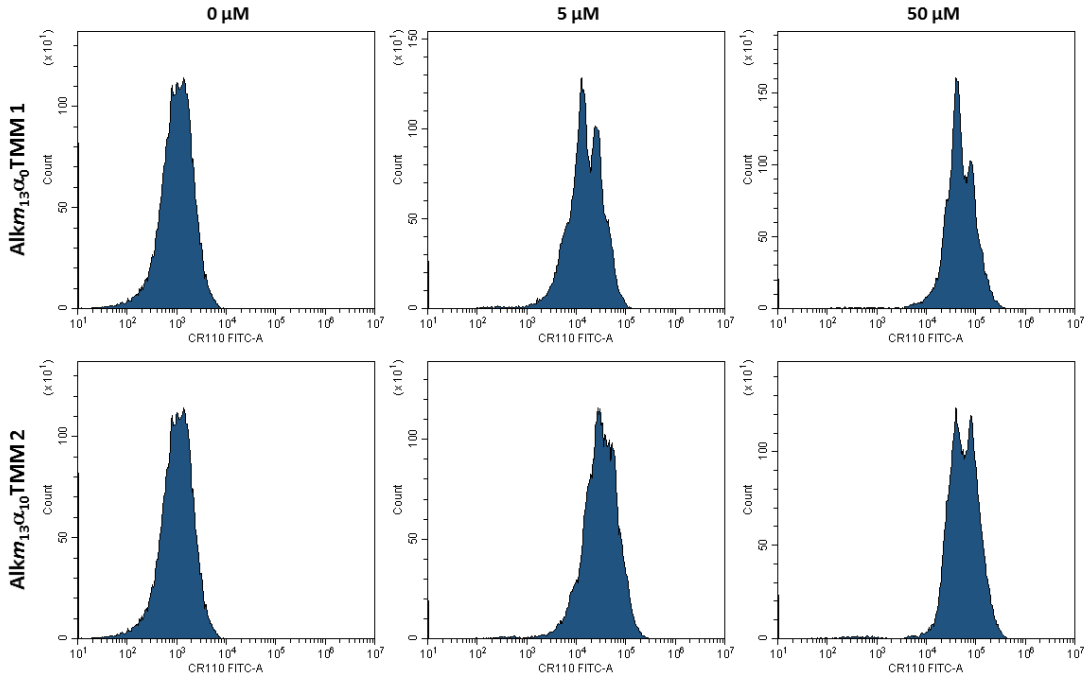
General procedure for labeling *E. coli* and *B. subtilis.*

Bacteria were inoculated (1:100) in LB medium (final volume 900 µL) containing or not, probe **1** or **2** (50 µM; control experiments were treated with probe vehicle i.e. water). Bacteria were cultured for 16 h at 37 °C under agitation (180 rpm for *E. coli* and 220 rpm for *B. subtilis*). Then, 350 µL of these suspensions were transferred in a 1.5 mL microtube and the cells were harvested by centrifugation (*E. coli*: 12000 rpm for 1 min; *B. subtilis*: 5000 rpm for 5 min). The pellets were washed three times with PBS buffer (200 µL; 12000 rpm, 1 min for *E. coli*; 5000 rpm, 5 min for *B. subtilis*; rt). The pellets were re-suspended in 200 µL of the "click" solution (0.05 mM CR110-Az, 2.5 mM sodium ascorbate, 2.0 mM TGTA and 1.0 mM copper sulfate pentahydrate in PBS/DMSO (98:2) buffer) and transferred in a 2.0 mL microtube for a better agitation. This suspension was vigorously shaken (550 rpm) in the dark for 30 min at room temperature, then transferred back in a 1.5 mL microtube and the cells were harvested by centrifugation (*E. coli*: 12000 rpm, 1 min; *B. subtilis*: 5000 rmp, 5 min; rt). Bacterial pellets were washed one times with PBS/DMSO (95:3) buffer (200 µL; 12000 rpm, 1 min for *E. coli*; 5000 rpm, 5 min for *B. subtilis*; rt) and two times with PBS buffer (200 µL; 12000 rpm, 1 min for *E. coli*; 5000 rpm, 5 min for *B. subtilis*; rt). The pellets were re-

suspended in PBS buffer (1.0 mL) and samples of 2 µL were deposited on agar (1%) pads placed on glass slides and then covered by coverslips.

Flow cytometry quantification

Fluorescence of the samples (diluted 100 times in PBS buffer) were analyzed using a Cytoflex flow cytometer (Beckman-Coulter). A minimum of 20000 events were counted for each data set (10 µL/min). Data were analyzed using CytExpert software (Beckman-Coulter). Representative histograms for *C. glutamicum* experiments labeling with probes **1** and **2** at 5 and 50 µM, followed by reaction with azide functionalized carboxyrhodamine 110 (CR110-Az) using CuAAC are shown below.



General procedures for confocal imaging of labeled bacteria

Samples of 2 µL were deposited on agar (1%) pads placed on glass slides and then covered by coverslips. Cell morphology was observed with bright field light. Fluorescence microscopy experiments were performed on a confocal straight Leica SP8 microscope (DM 6000), using a 63x PLAN APO oil immersion lens (Leica), an argon laser and a PMT detector (Hamamatsu). CR110 were excited at 488 nm and collected with a 510-540 nm band pass filter. The experiments were imaged with identical exposure setting. The microscope was operated with LAS-X program. Images were visualized and montages were constructed with ImageJ using Fiji without further processing.

3. *C. glutamicum* cell growth assays

C. glutamicum strain was grown in BHI medium overnight at 30 °C. The culture was then diluted in BHI medium to a final absorbance at 600 nm of 0.9. Sample aliquots (9 µL) of this diluted cell culture and 181 µL of BHI medium were placed in wells of a microtiter plate. The probe solution (10 µL) at various concentrations was then added to the wells and the plate was incubated at 30 °C for 16 h in a microtiter plate reader (MultiskanEX system microplate reader from Labsystem). Optical density readings (600 nm) were taken at 60 min intervals. The plate was shaken for 30 s before each reading.

4. *E. coli* and *B. subtilis* cells growth assays

Bacteria were grown in LB medium overnight at 37 °C. The culture was then diluted in LB medium to a final absorbance at 600 nm of 0.04 and 190 µL were placed in the wells of a microtiter plate. The probe solution (10 µL) at various concentrations was then added to the wells and the plate was incubated at 37 °C for 16 h in a microtiter plate reader (MultiskanEX system microplate reader from Labsystem). Optical density readings (600 nm) were taken at 60 min intervals. The plate was shaken for 30 s before each reading.

5. Purification of *MytAhis*

mytAhis encoding gene cloned in pCGL482 under its own promoter and signal sequence was introduced in the Δ*aftB* strain in which exported recombinant proteins are released in the culture supernatant and easily purified.[14]

For large scale purification of MytAhis, cells from 1.2 L overnight cultures (BHI, 30 °C) were recovered by centrifugation at 7000 x g at 10 °C. The cleared supernatant was further centrifuged at 35000 rpm (45 Ti) for 2 h at 10 °C to get rid of membrane fragments that are released by the Δ*aftB* strain as reported previously.[14] Proteins were then precipitated from the supernatant by adding ammonium sulfate to 70% saturation. Incubation was carried out for 1h at 4 °C with constant shaking. After centrifugation at 7000 x g for 15 min at 4 °C, the protein-containing pellet was resuspended in 40 mL of 100 mM Phosphate buffer pH 8.0 (purification buffer). The solution was dialyzed for 24 h at 4 °C against the purification buffer (Spectrum, Spectra/Por MWCO 10 kDa) before loading on a 2 mL Ni-NTA column at 1 $mL.min^{-1}$. The flow through was recovered and loaded one more time on the column. Proteins weakly associated with the Ni-NTA resin were washed off by running 40 mL of purification buffer. Elution was finally performed with 5 column volumes of purification buffer containing imidazole at 250 mM. Fractions containing MytA were pooled and immediately injected on a Sephadex 75 (GE Healthcare Life Science) column at 1 $mL.min^{-1}$. The protein eluted as a single symmetrical peak and MytAhis containing fractions were pooled and concentrated to 8-12 $mg.mL^{-1}$ by ultrafiltration on Vivaspin 20 (Sartorius, MWCO 5 kDa). The whole purification procedure (Ni-NTA, Sephadex 75 and ultrafiltration) was always done on the same day and aliquots of MytA preparations were conserved at - 20 °C. The purification was controlled by running SDS PAGE gels after ammonium sulfate, Ni-NTA, Sephadex 75 and ultrafiltration steps. The concentration of MytA (ε_{280} = 140 260 $M^{-1}cm^{-1}$) was determined using absorbance at 280 nm.

6. MytA esterase activity assay

The esterase activity of MytA was assayed by using the *p*-nitrophenyl palmitate (*p*-NPP) as a substrate. Stock solution of *p*-NPP 5 mM was prepared in DMSO. A standard reaction mixture (300 µL) consisted of 250 µM *p*-NPP (so that the final concentration of DMSO in the assay did not exceed 5% (v:v)) and 2 µM MytA in reaction buffer (20 mM TES (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonicacid), 0.05% Triton X-100, 20 mM NaCl, pH 7.5). A premixture without the enzyme was incubated at 37 °C for 5 min, and the reaction was initiated by the addition of MytA. All experiments were performed in duplicate in microplates at 37 °C and formation of *p*-nitrophenolate was monitored by measurement of the absorbance at 405 nm every 20 min on a microplate photometer (Thermo Scientific Multiskan EX). For the competition assays, 100 µM TMM, probe **1** or **2** was added with *p*-NPP.

III. Supplementary figures

Figure S3. Growth curves of *C. glutamicum* treated with probe **1** (A) and probe **2** (B) at 7 different concentrations (C) treated with probe **2** and TMM. For high concentration of TMM alone, a background absorbance was observed due to turbidity of the solution and the OD values for growth curves in the presence of TMM 1 mM were thus corrected. Experiments were performed in duplicate and data represent an average of two independent assays with error bars indicating standard deviations.

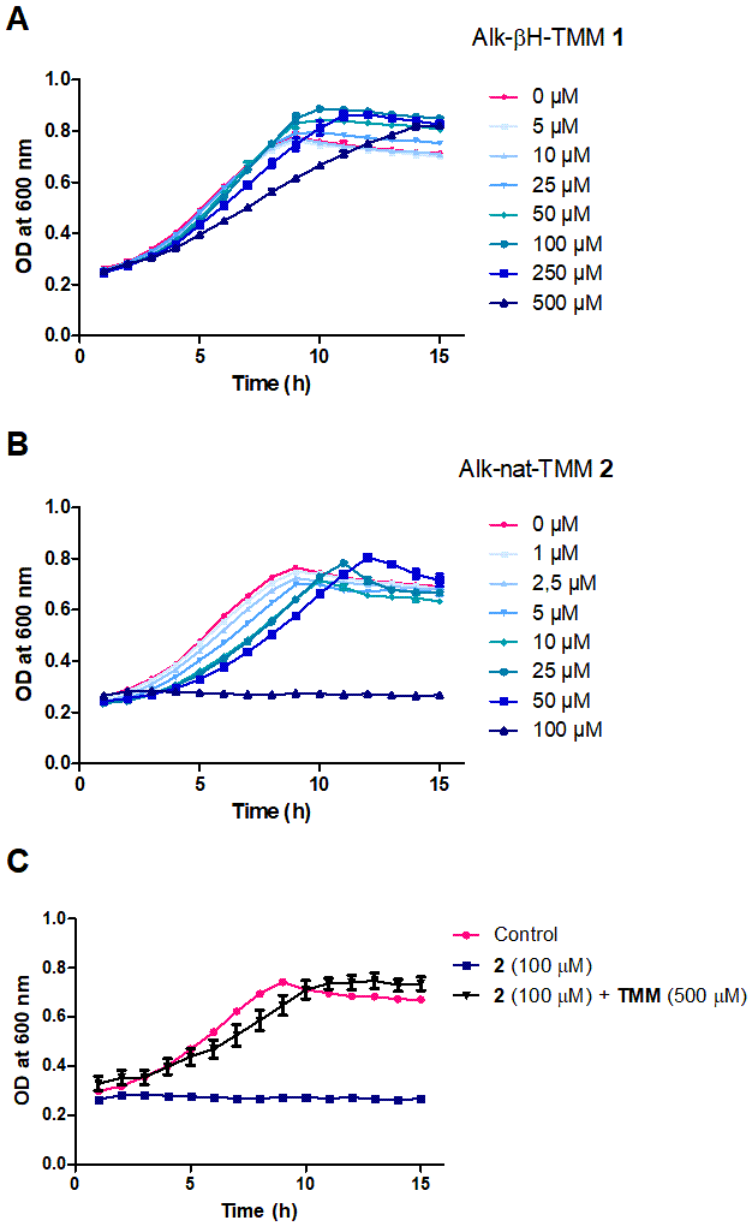


Figure S4. Growth curves of *E. coli* treated with probe **1** (A) and probe **2** (B) at 3 different concentrations. Experiments were performed in duplicate and data represent an average of two independent assays with error bars indicating standard deviations.

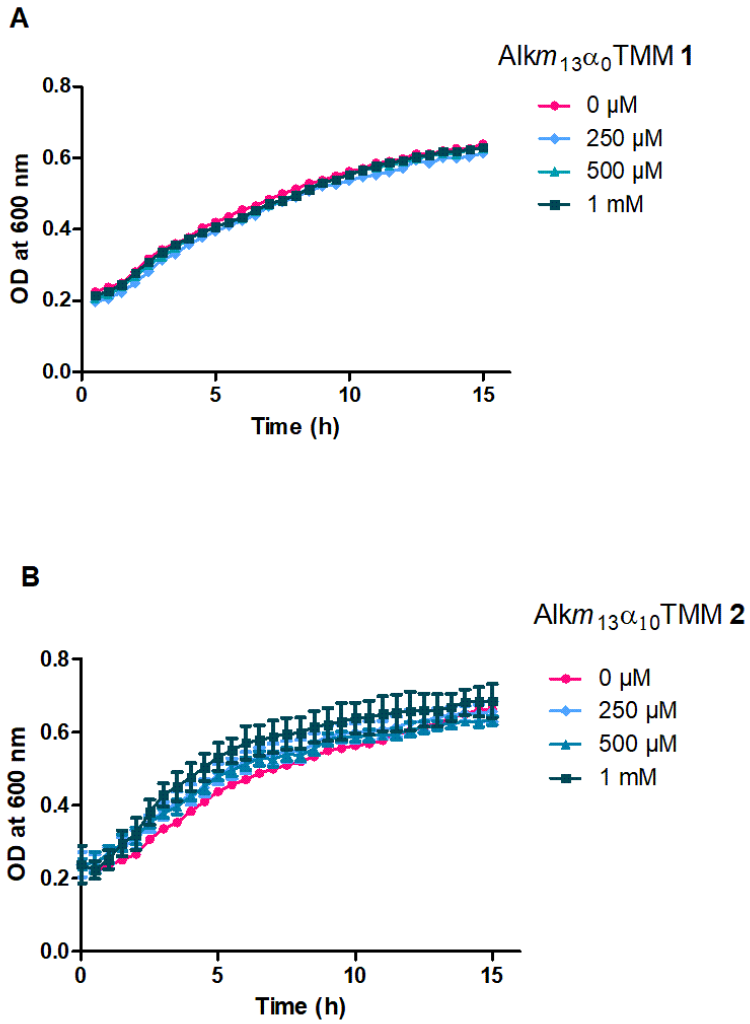


Figure S5. Growth curves of *B. subtilis* treated with probe **1** (A) and probe **2** (B) at 2 different concentrations. Experiments were performed in duplicate and data represent an average of two independent assays with error bars indicating standard deviations.

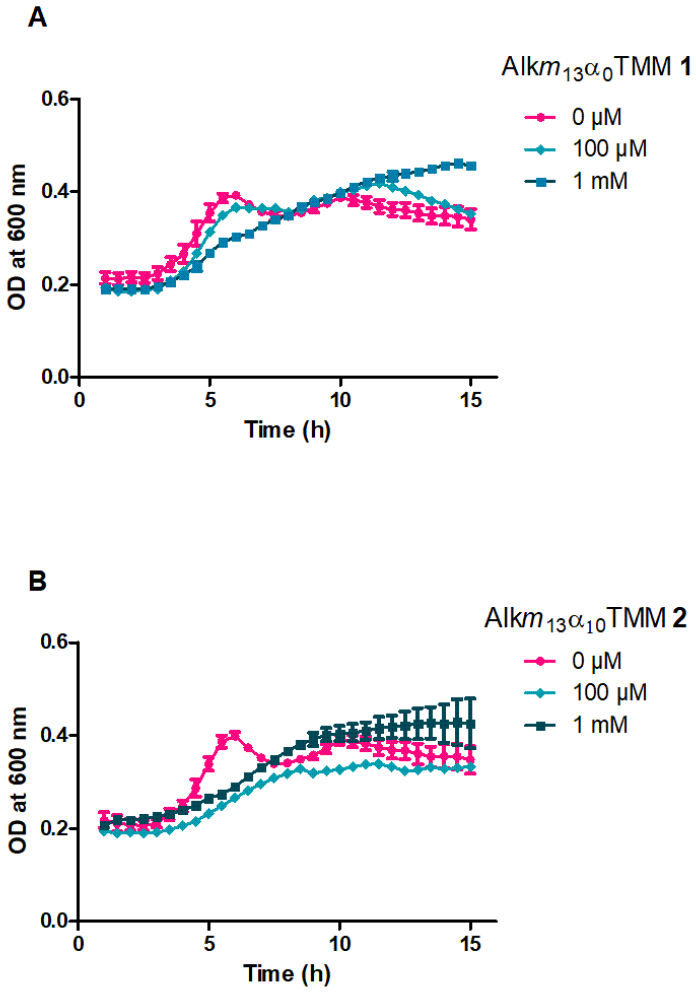


Figure S6. Hydrolysis of *p*-NPP catalyzed by MytA at 37 °C with an initial substrate concentration of 250 μM. Measuring the absorbance at 405 nm allows direct monitoring of the change in *p*-nitrophenolate production. The means of three independent experiments were plotted with error bars indicating standard deviations.

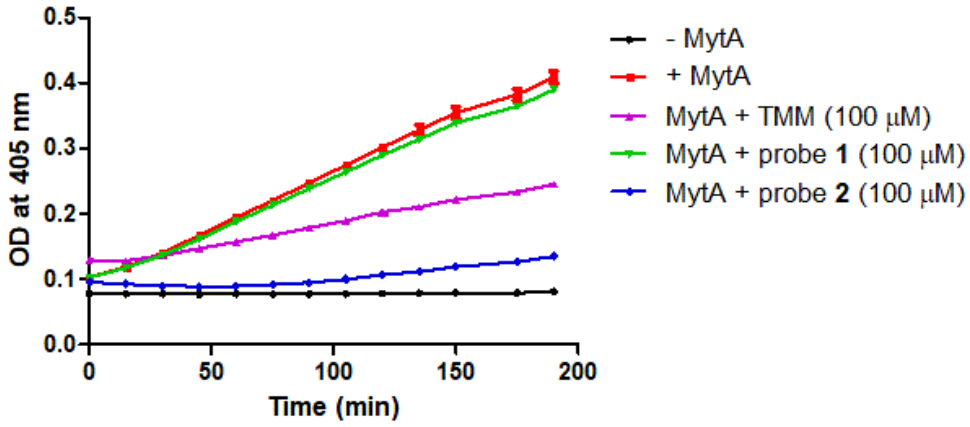


Figure S7. A. *C. glutamicum* labeled with 50 µM of probe **1** or probe **2** for 1 h (control experiments were treated with probe vehicle i.e. water) followed by bioorthogonal reaction with CR110-Az. Fluorescence microscopy with bright field images on top. Scale bars = 5 µm. B. *C. glutamicum* labeled with 50 µM and 5 µM of probe **1** or probe **2** for 1 h followed by bioorthogonal reaction with CR110-Az. Scale bars = 2 µm. Arrows indicate the septa (white) and the poles (red).

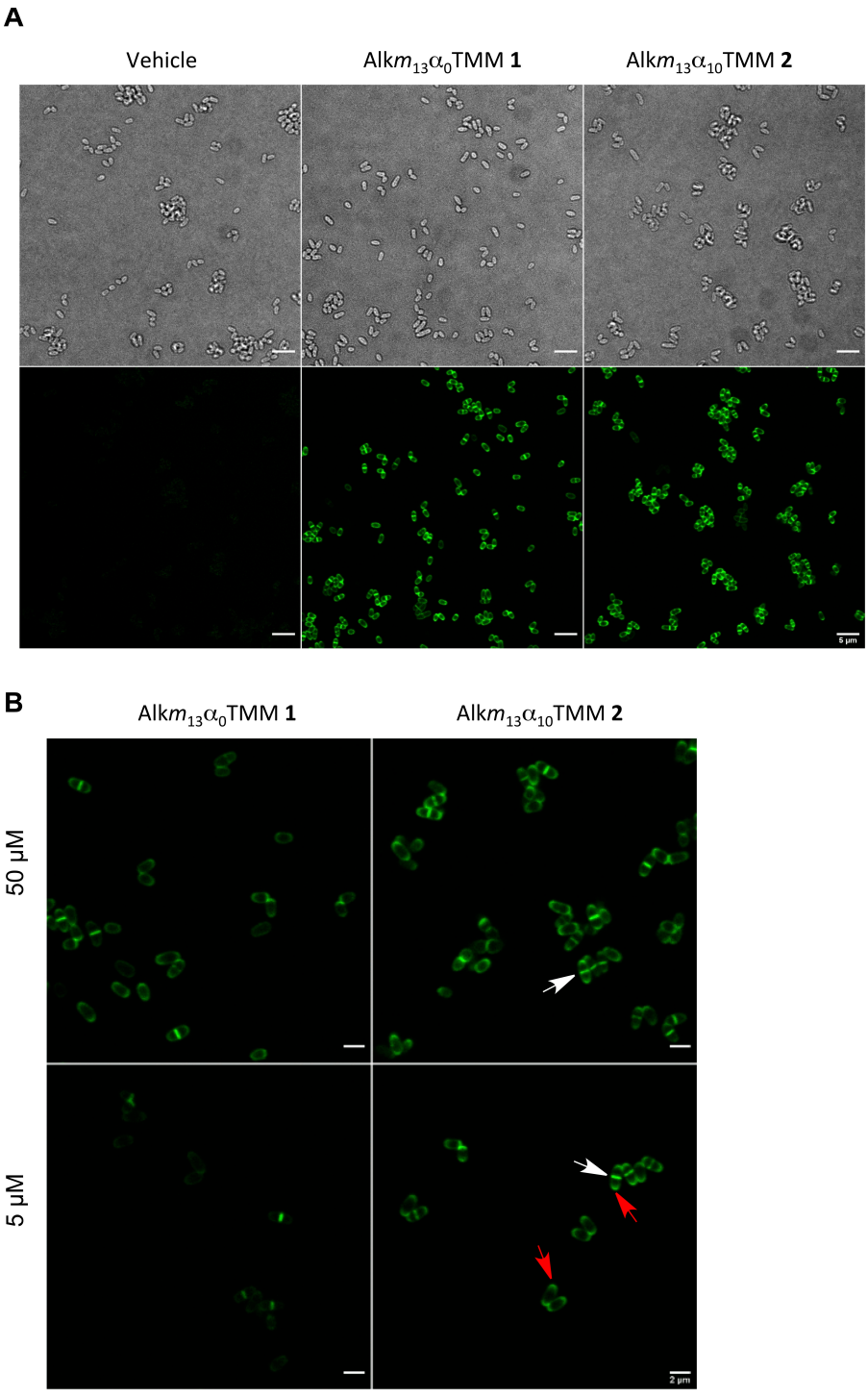


Figure S8. Fluorescence microscopy of *E. coli* cultured in the presence of probes **1** or **2** (control experiments were treated with probe vehicle i.e. water) then reacted with CR110-Az with bright field images on top and fluorescence bellow. Scale bars = 5 μm.

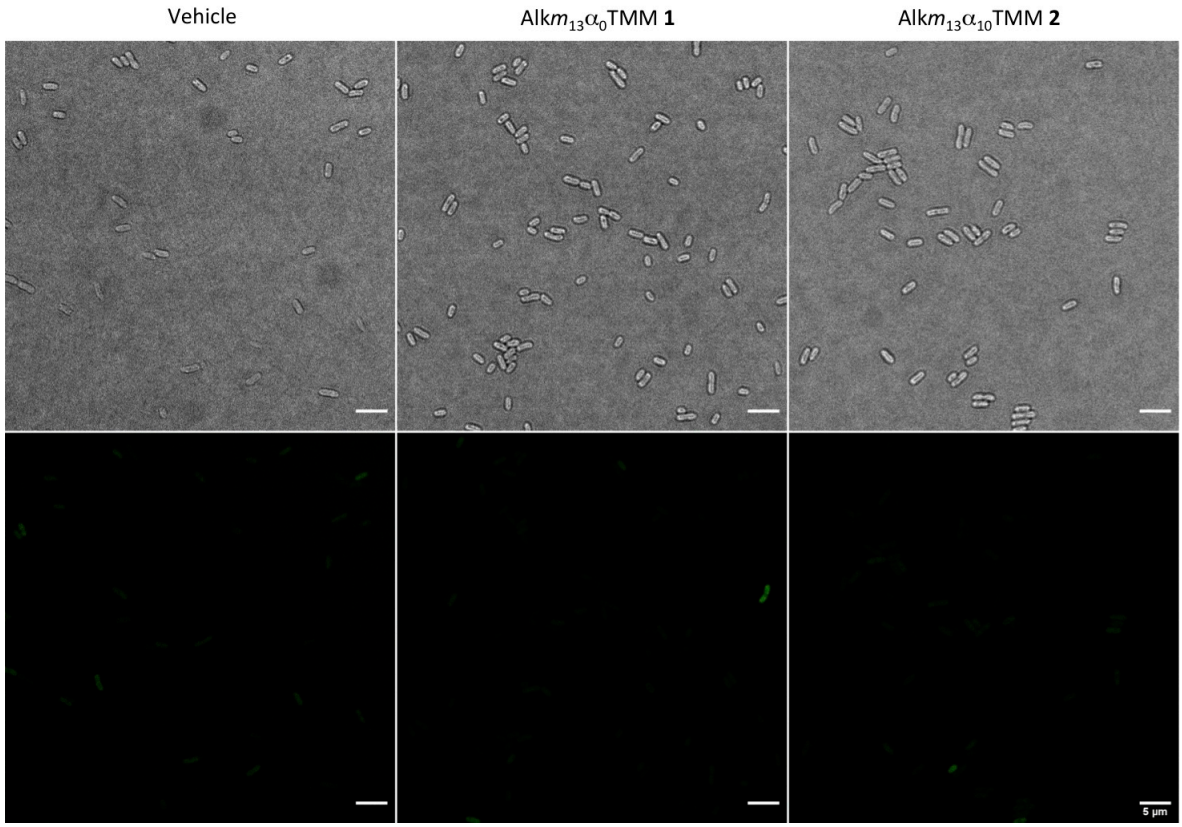
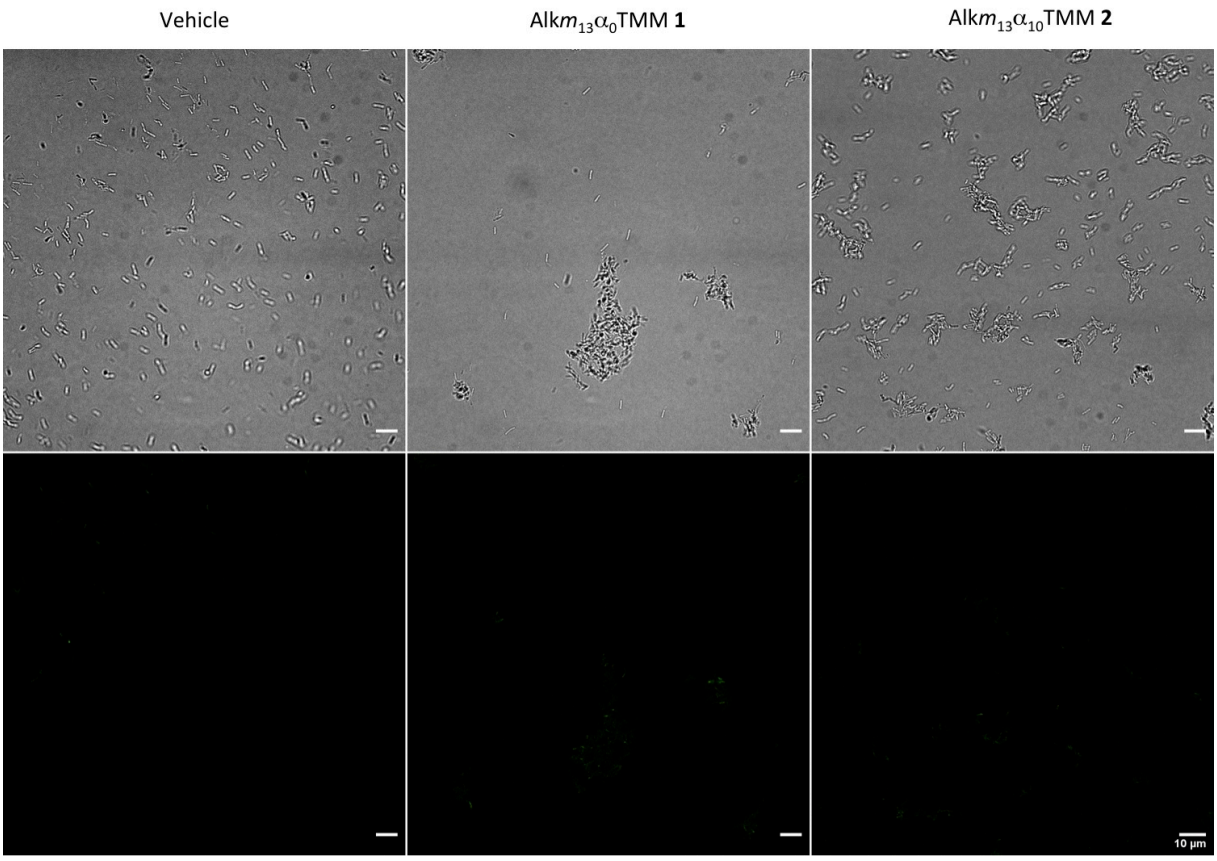
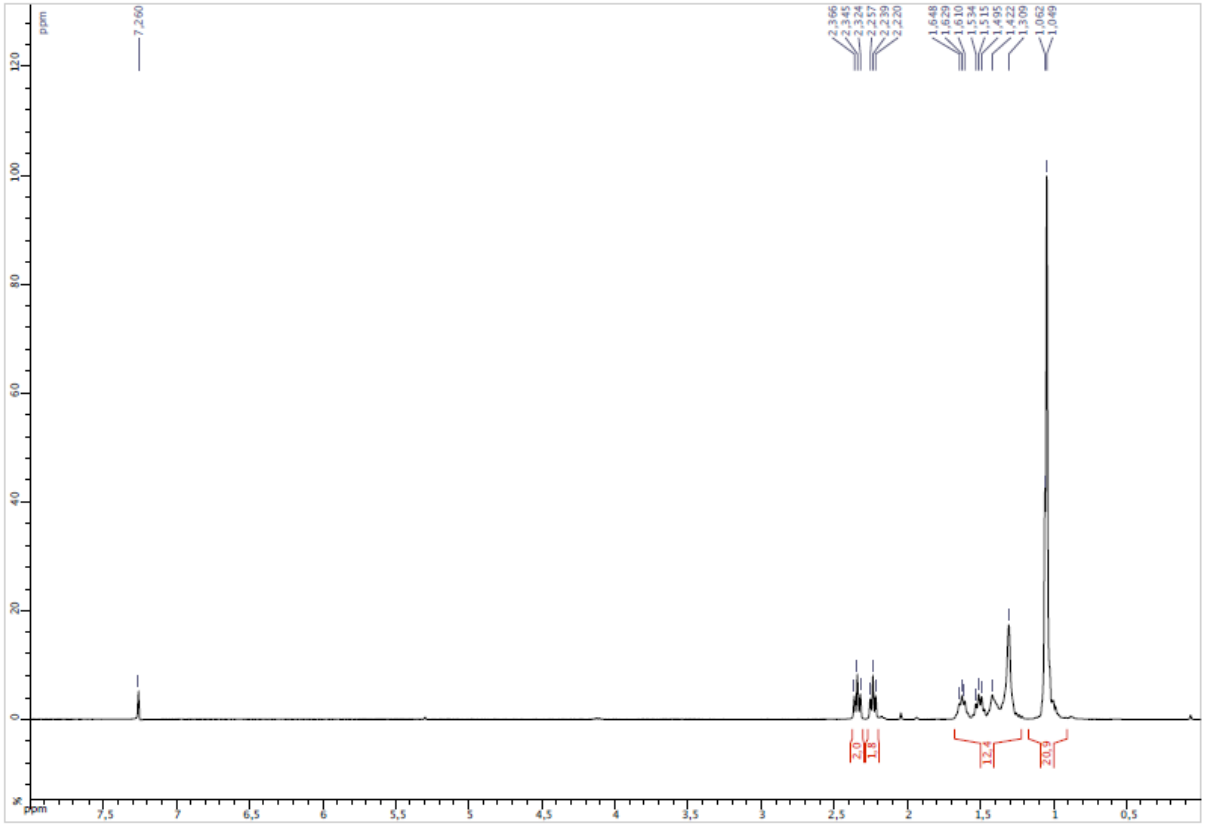


Figure S9. Fluorescence microscopy of *B. subtilis* cultured in the presence of probes **1** or **2** (control experiments were treated with probe vehicle i.e. water) then reacted with CR110-Az with bright field images on top and fluorescence bellow. Scale bars = 10 μm.

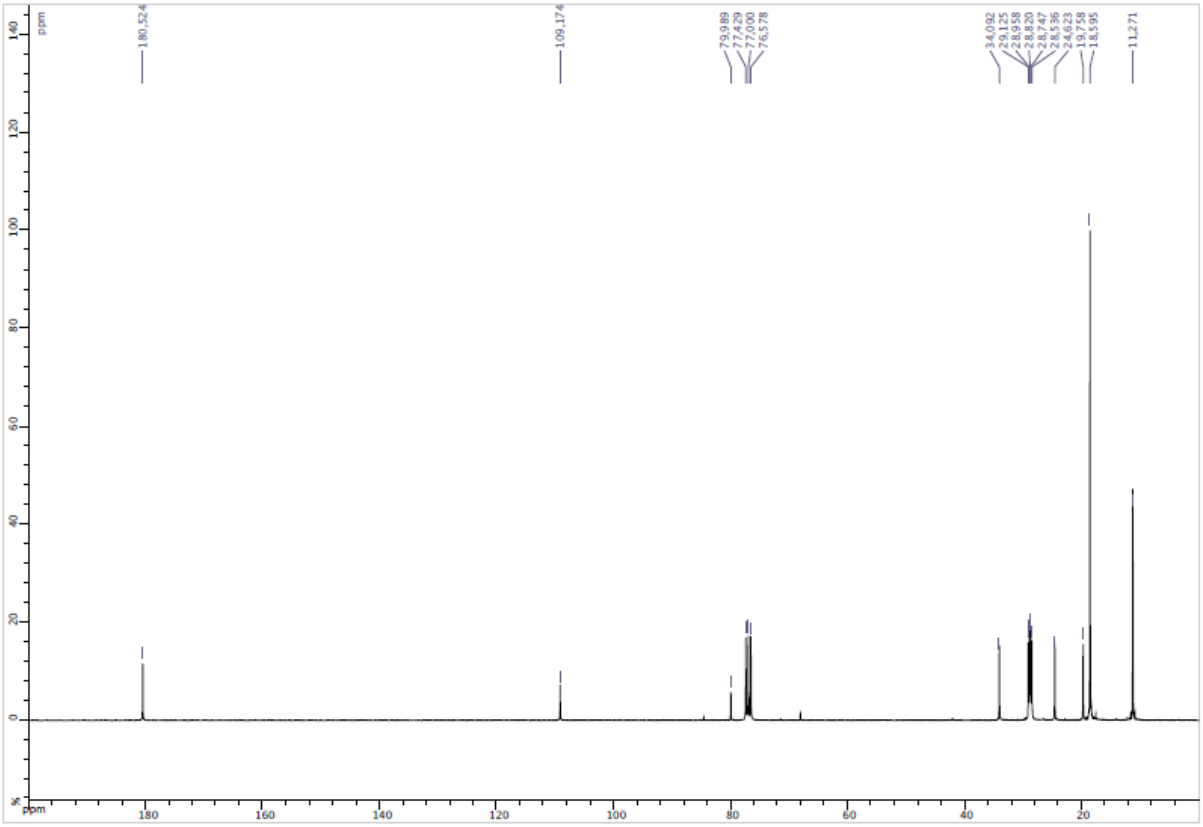


III. NMR spectra

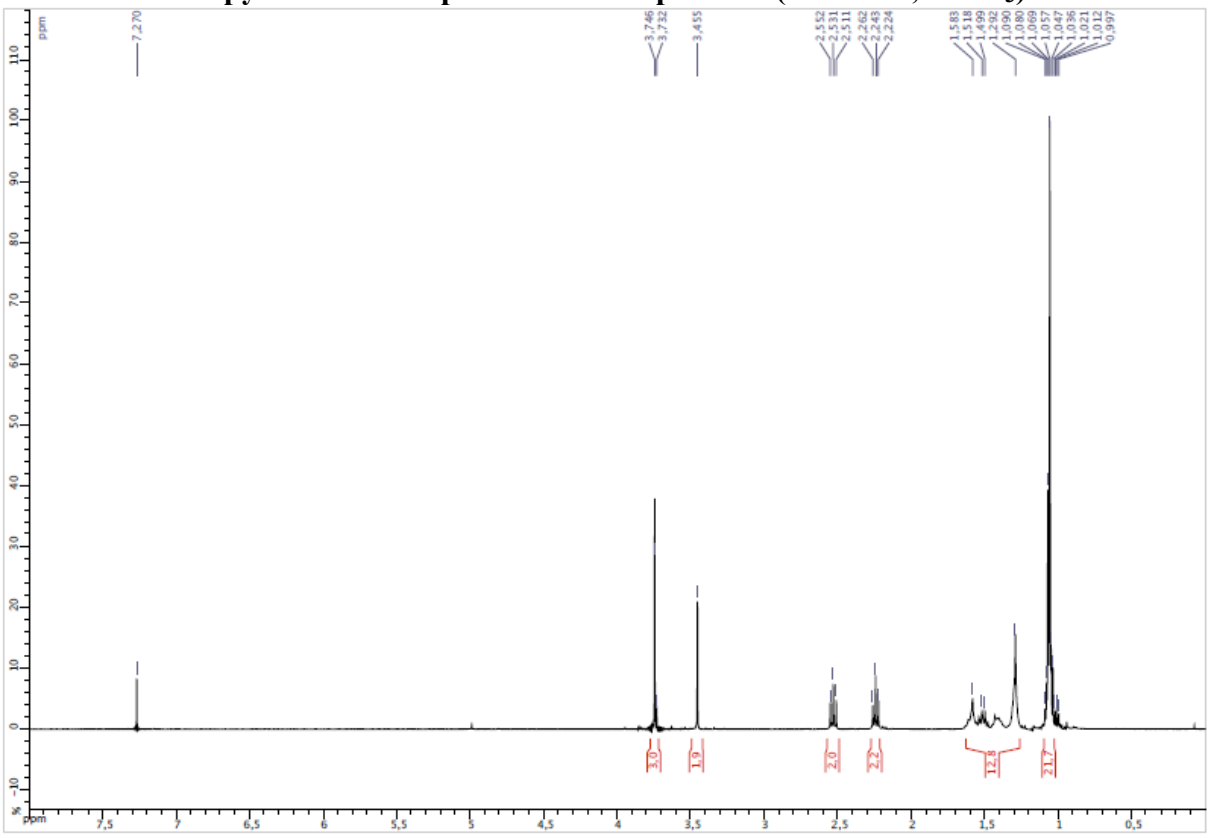
Copy of ^{1}H NMR spectrum of compound S1 (360 MHz, $CDCl_3$)



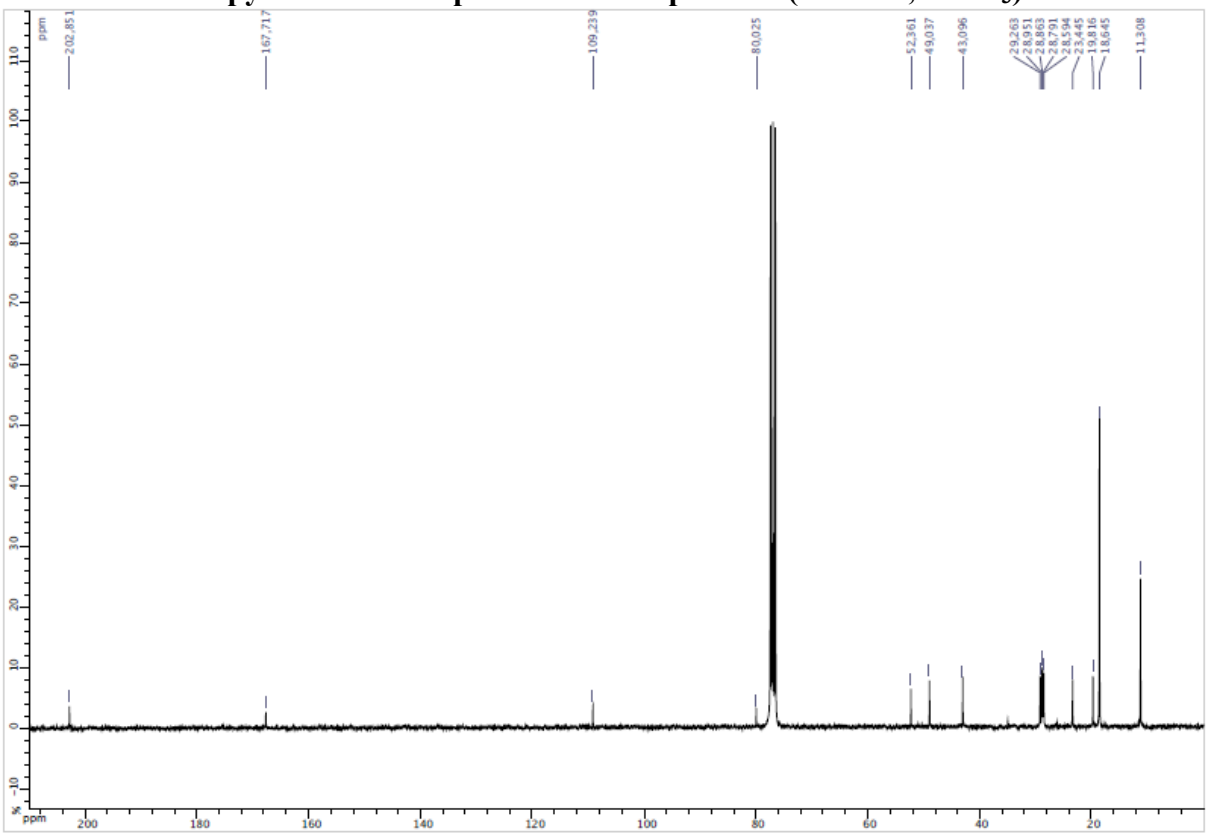
Copy of ^{13}C NMR spectrum of compound S1 (75 MHz, $CDCl_3$)



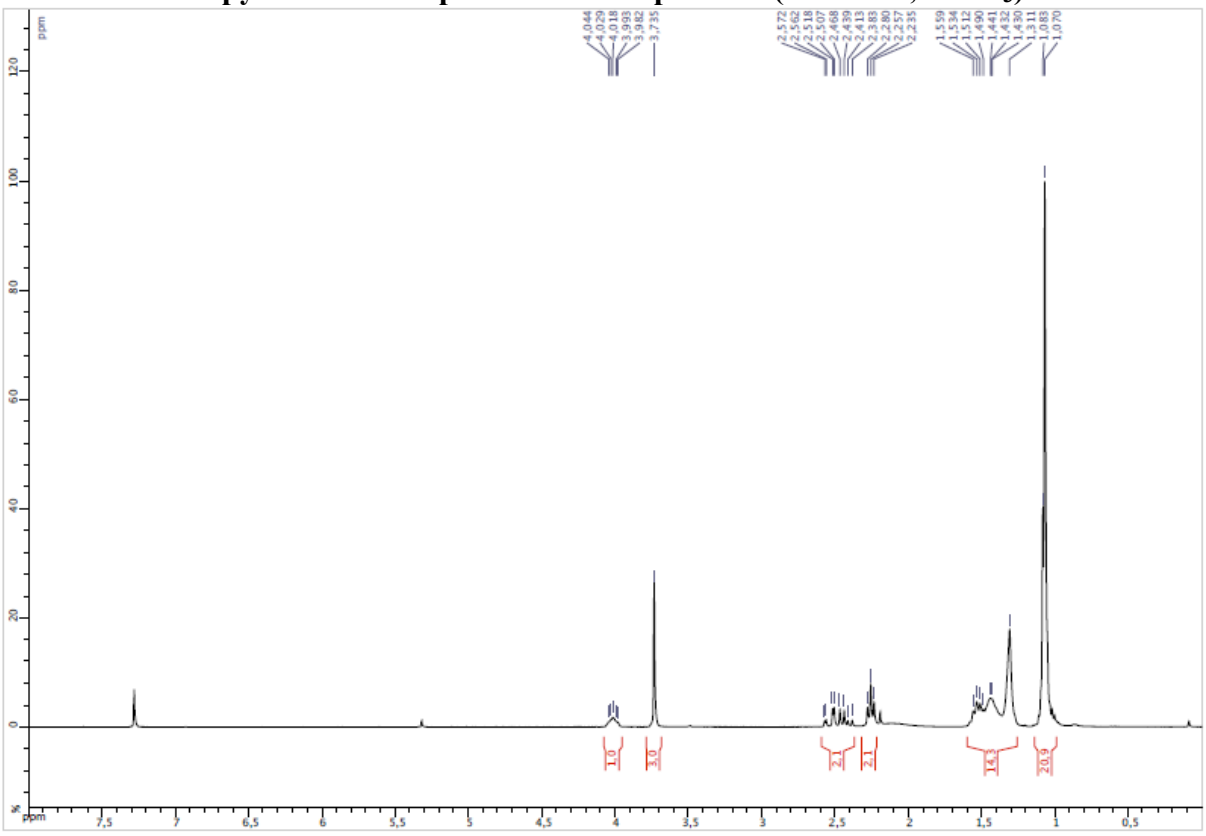
Copy of ^{1}H NMR spectrum of compound 3 (300 MHz, $CDCl_3$)



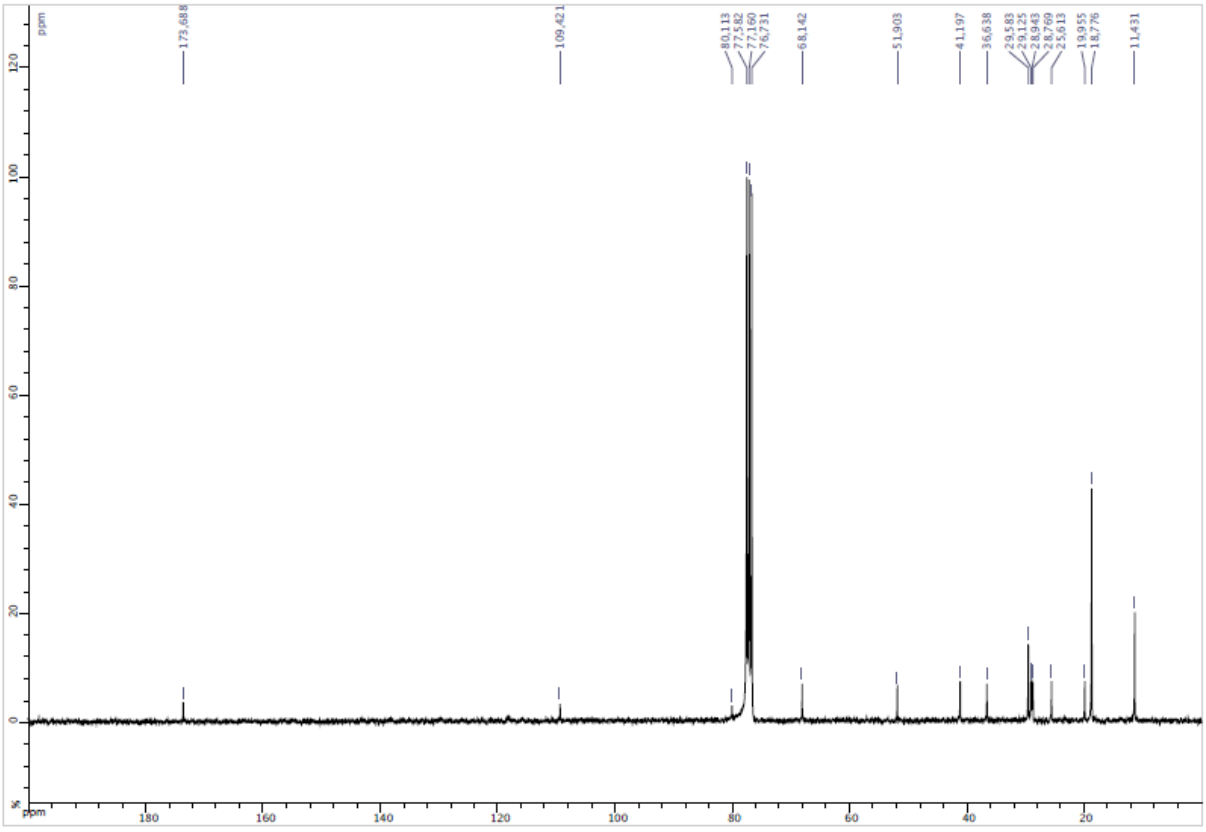
Copy of ^{13}C NMR spectrum of compound 3 (75 MHz, $CDCl_3$)



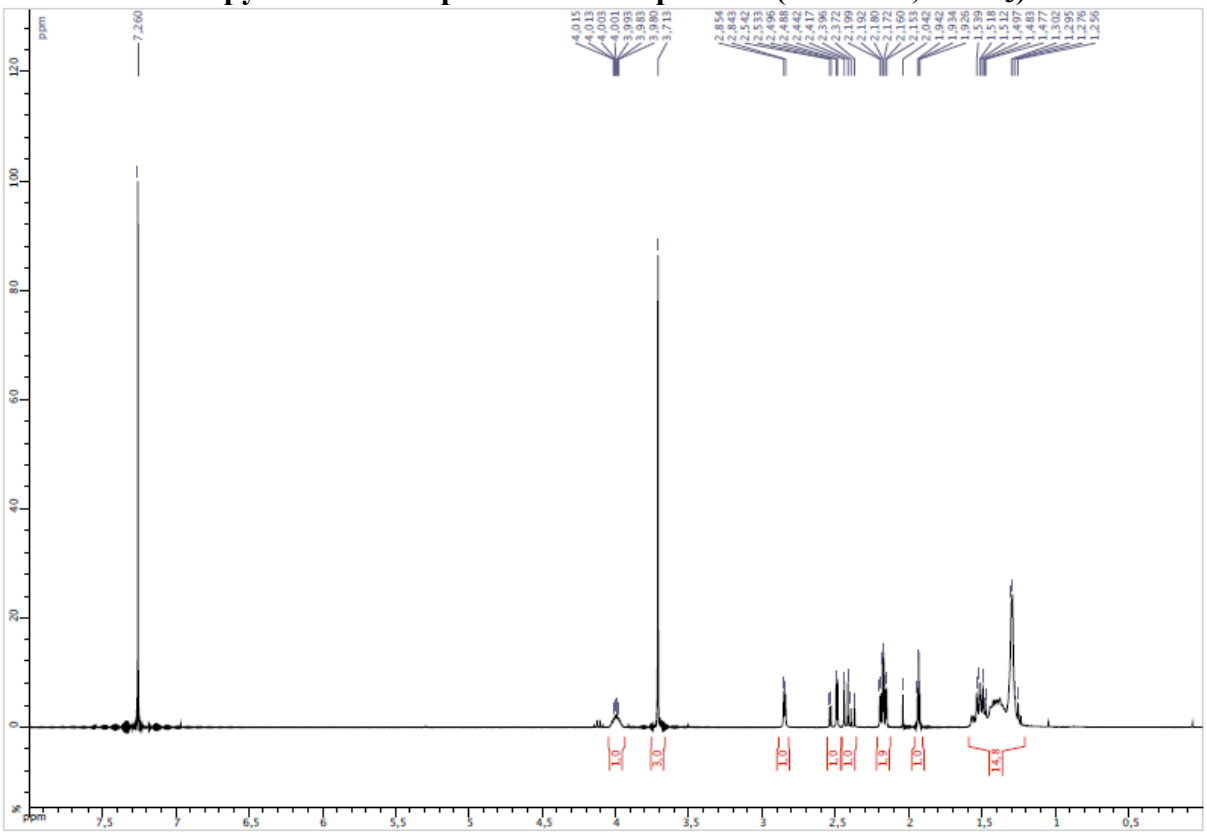
Copy of ^{1}H NMR spectrum of compound 4 (300 MHz, $CDCl_3$)



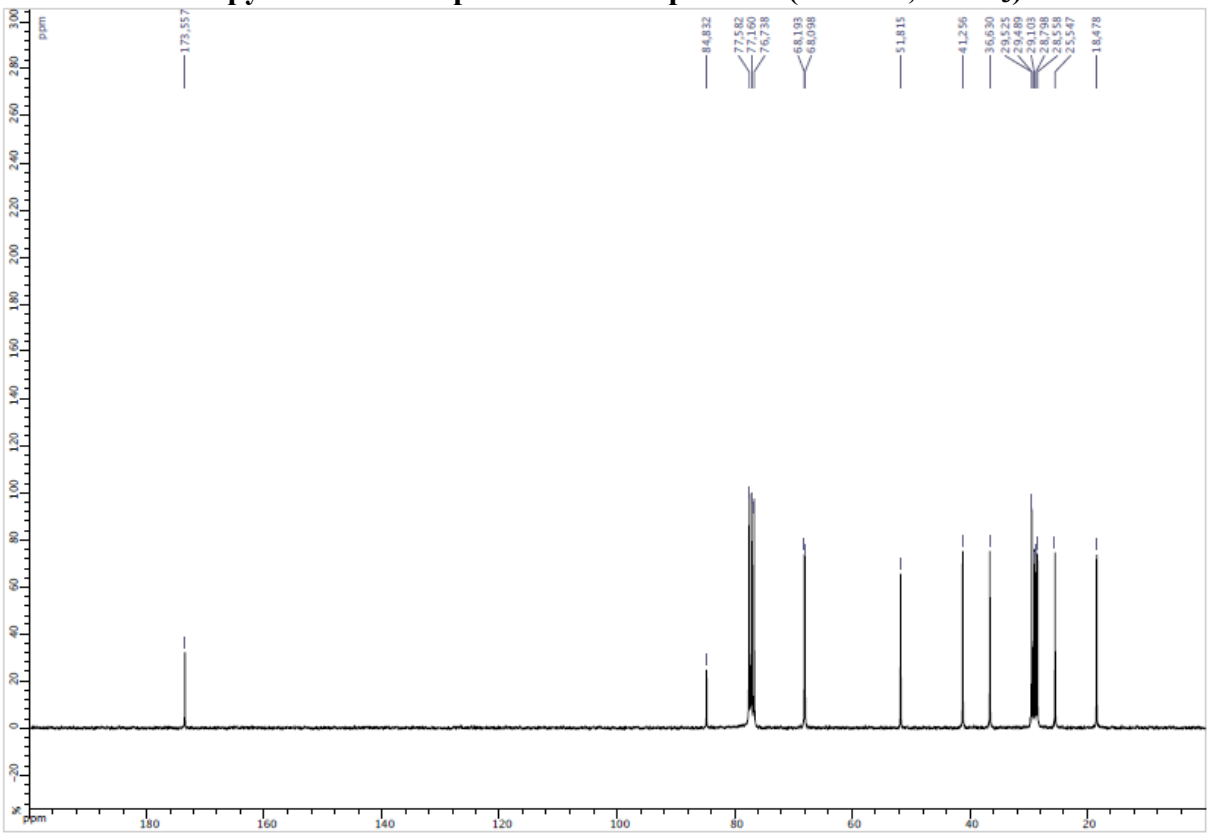
Copy of ^{13}C NMR spectrum of compound 4 (75 MHz, $CDCl_3$)



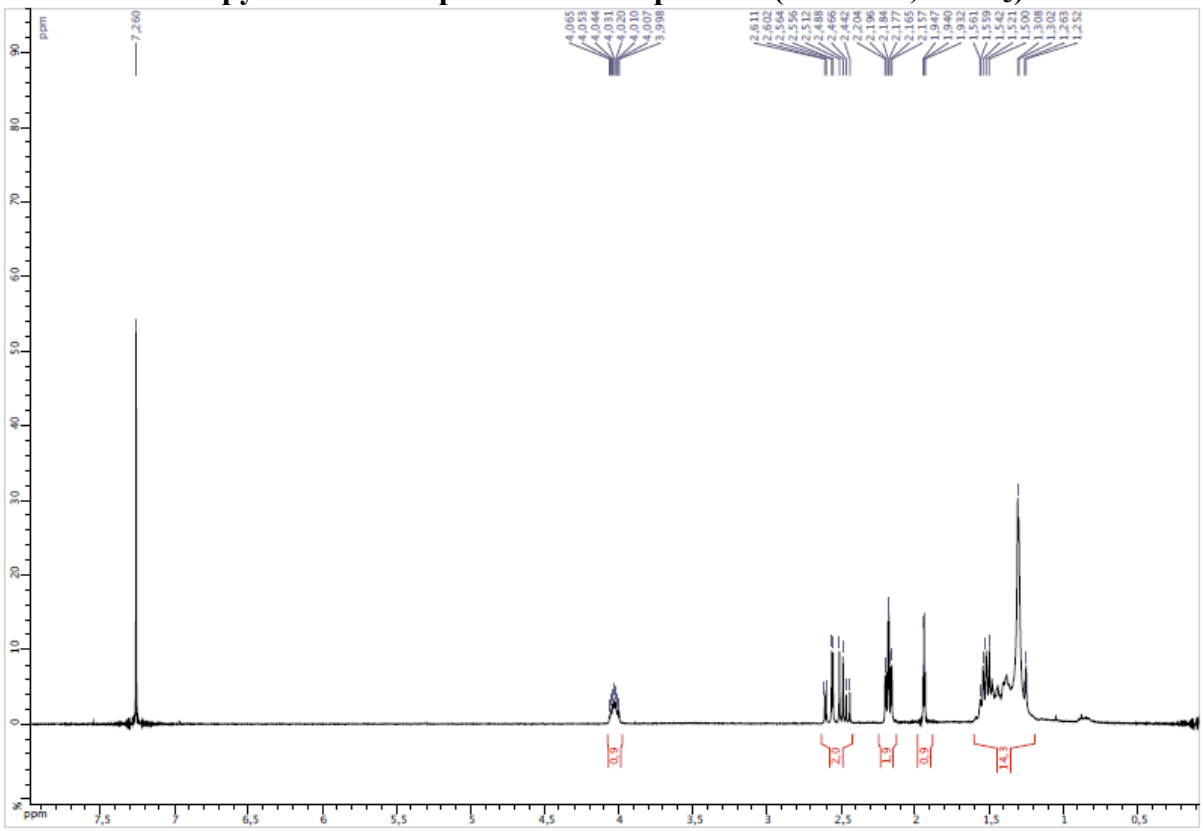
Copy of ^{1}H NMR spectrum of compound 6 (360 MHz, $CDCl_3$)



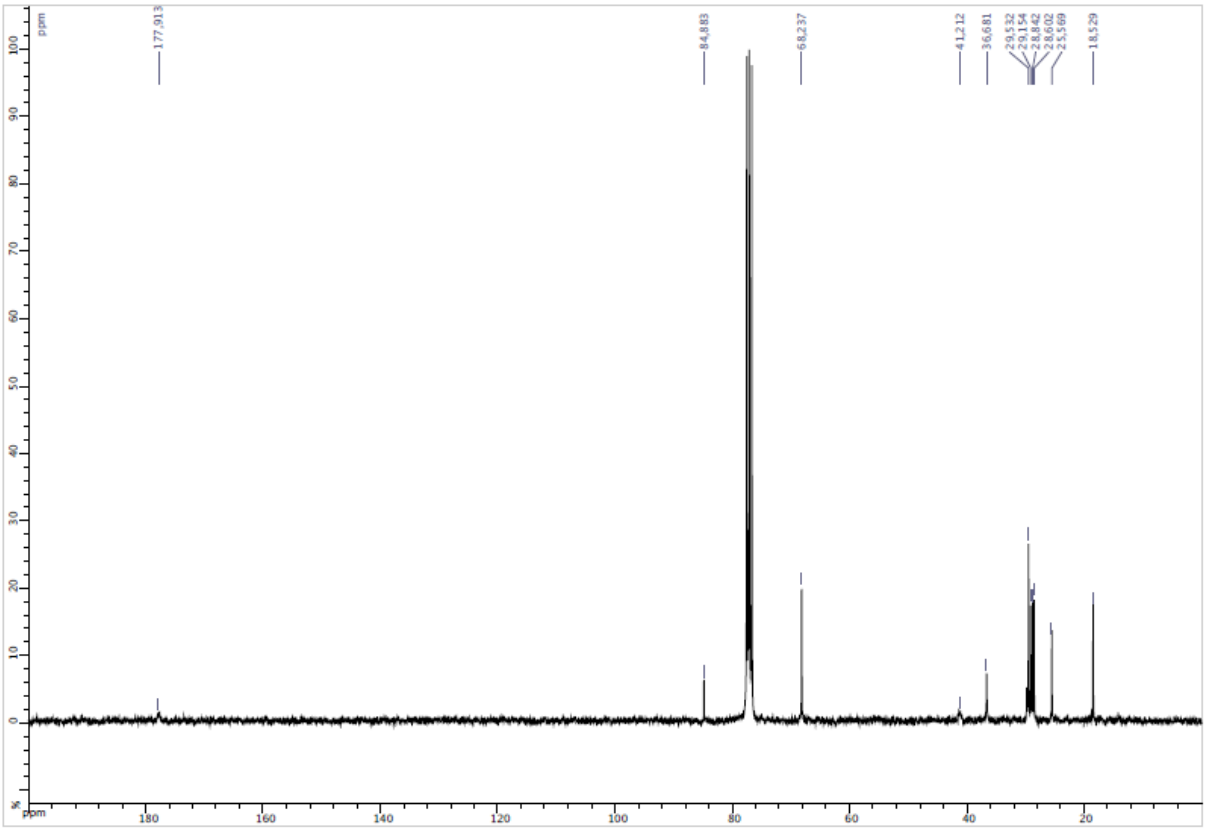
Copy of ^{13}C NMR spectrum of compound 6 (75 MHz, $CDCl_3$)



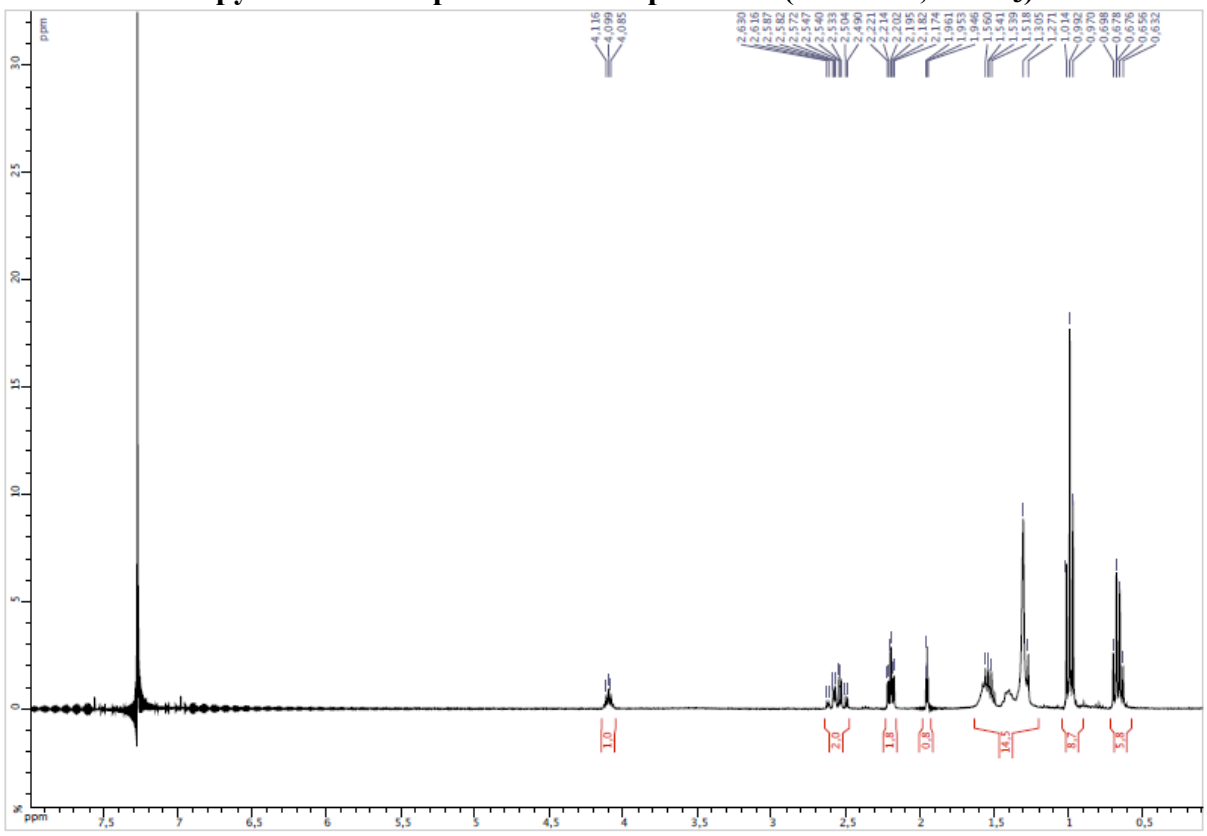
Copy of ^{1}H NMR spectrum of compound 8 (360 MHz, $CDCl_3$)



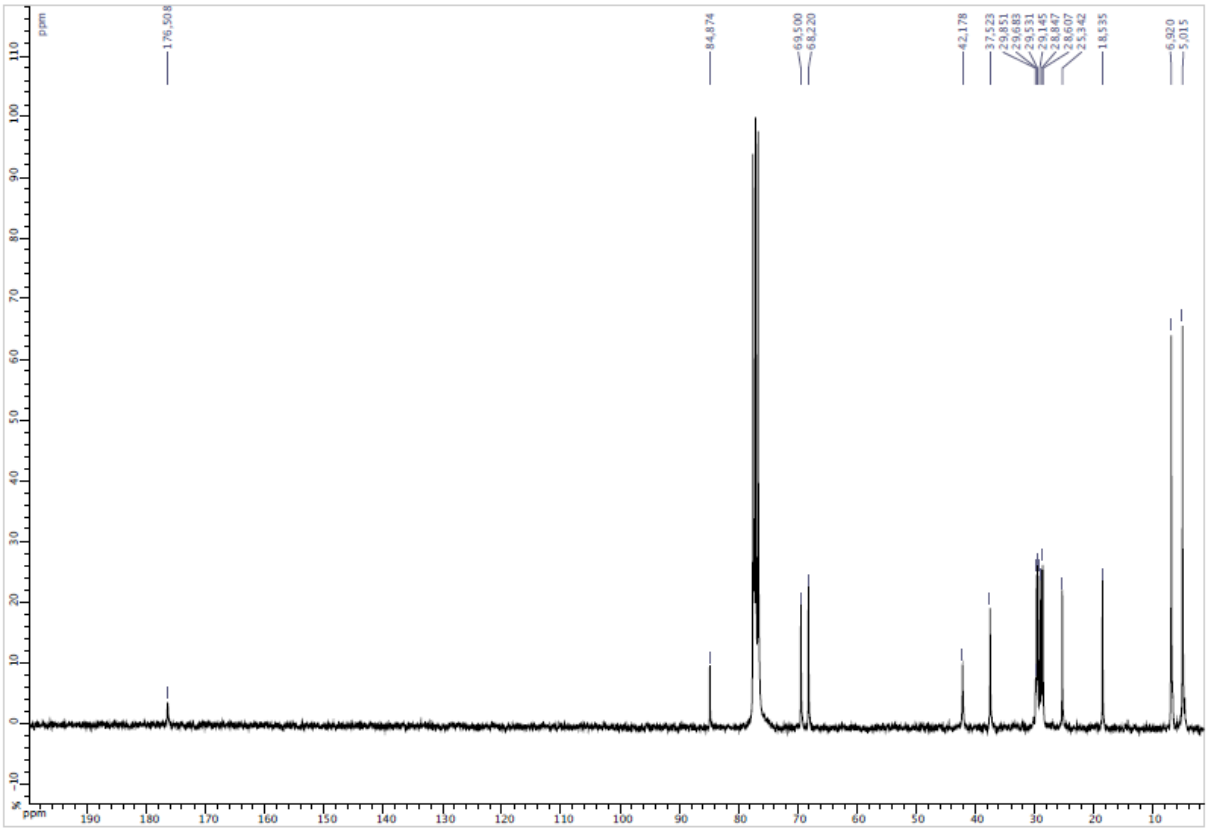
Copy of ^{13}C NMR spectrum of compound 8 (75 MHz, $CDCl_3$)



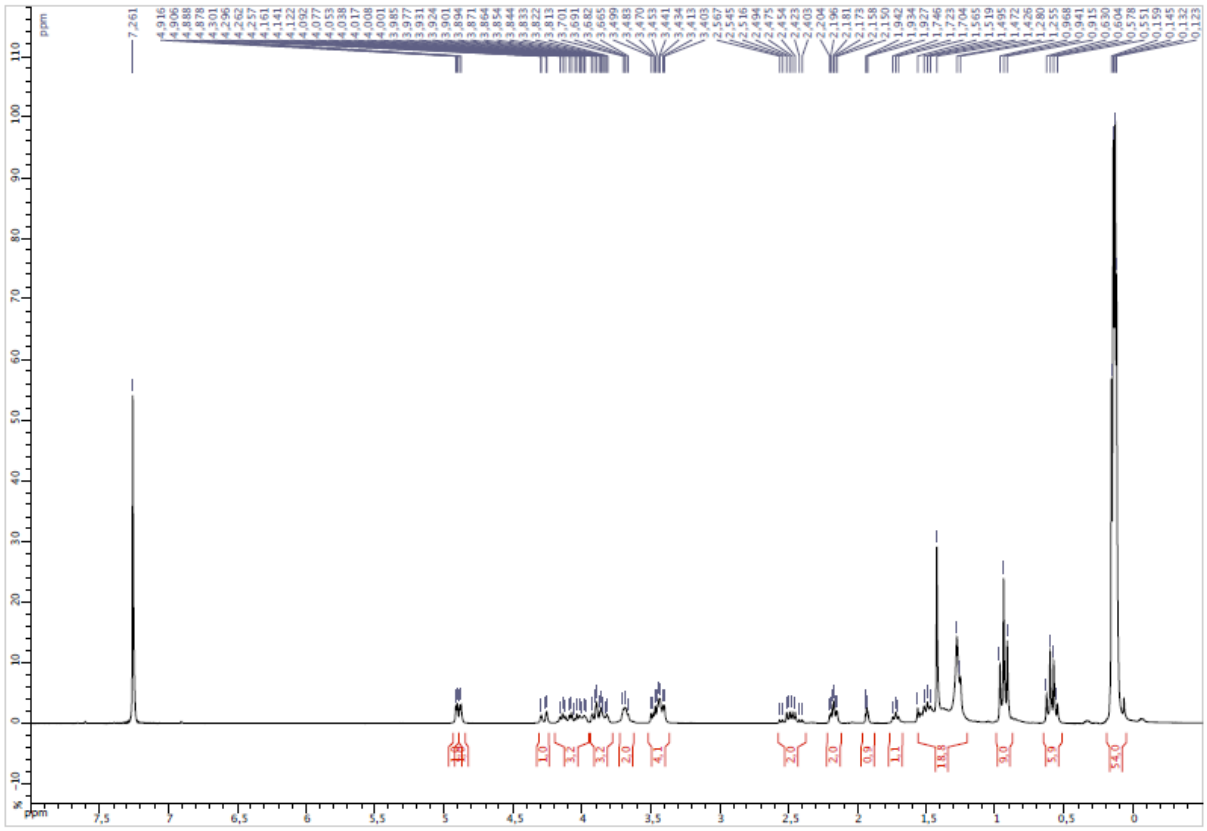
Copy of ^{1}H NMR spectrum of compound 10 (360 MHz, $CDCl_3$)



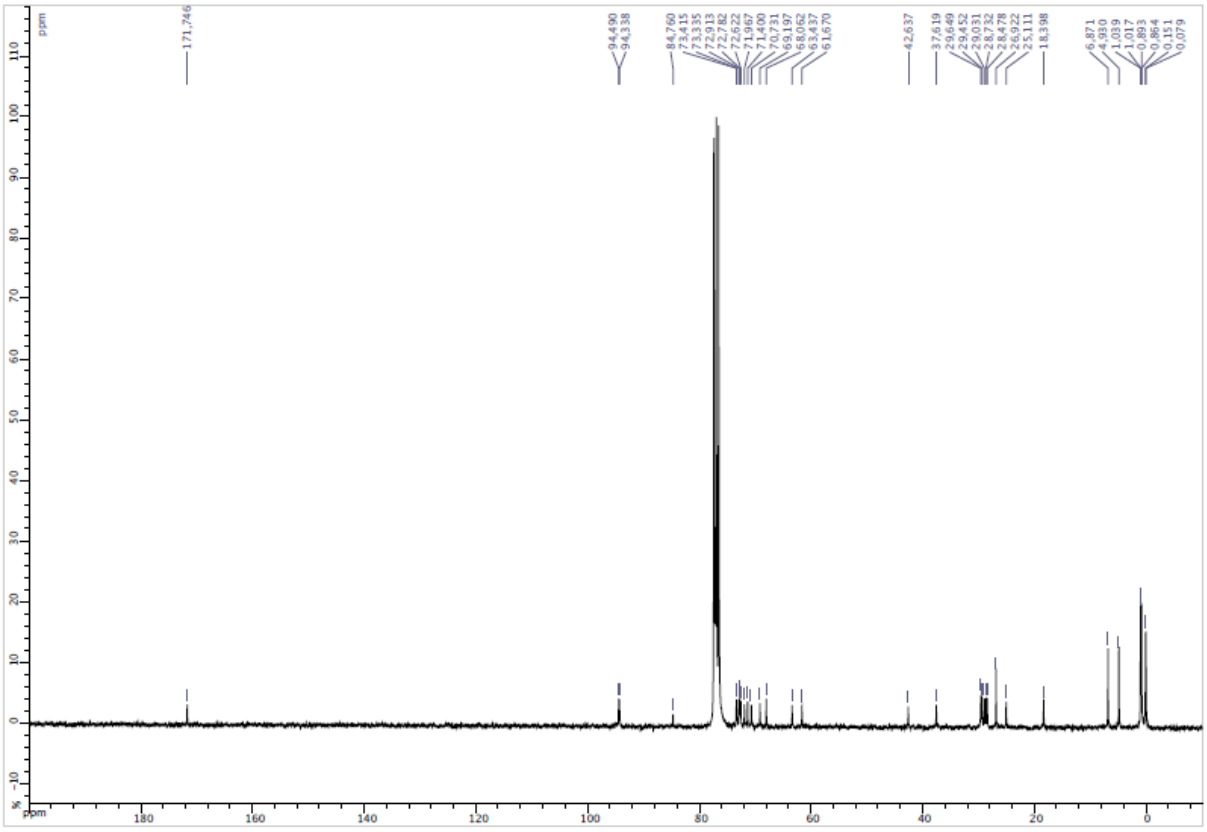
Copy of ^{13}C NMR spectrum of compound 10 (75 MHz, $CDCl_3$)



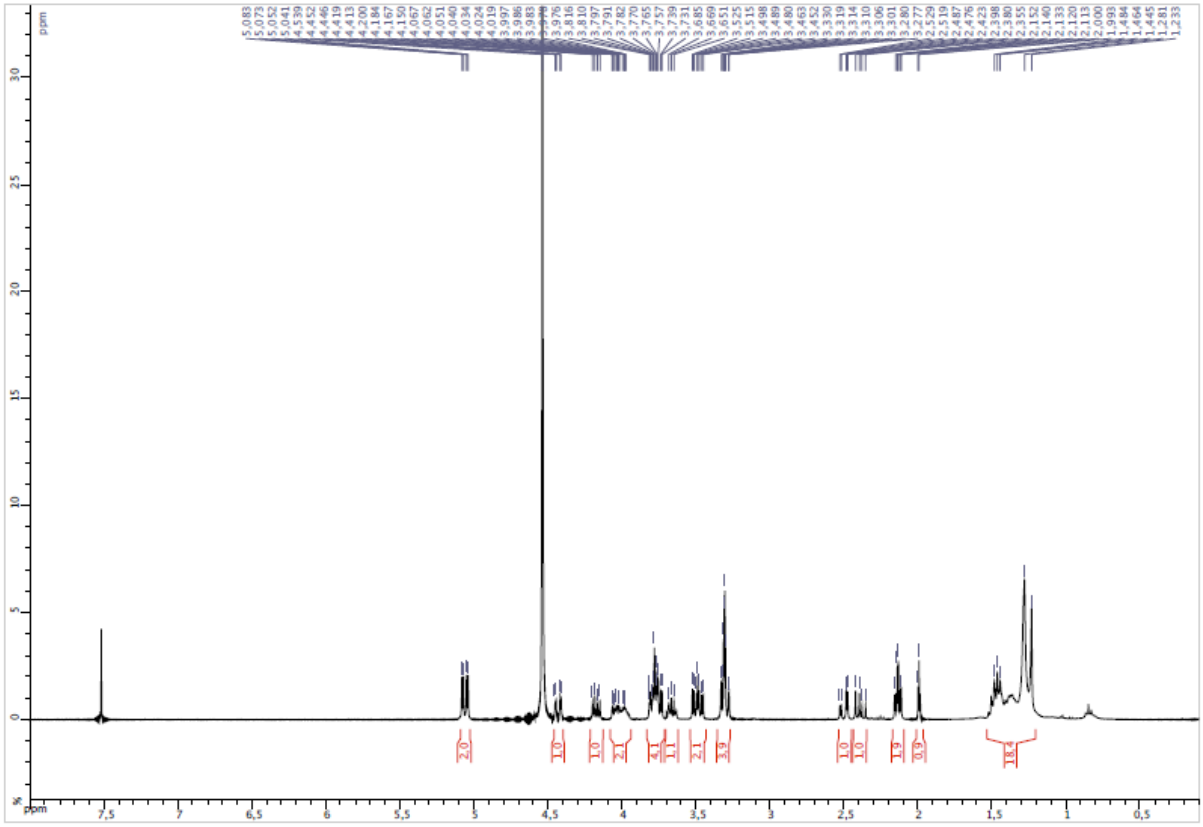
Copy of ^{1}H NMR spectrum of compound S2 (300 MHz, $CDCl_3$)



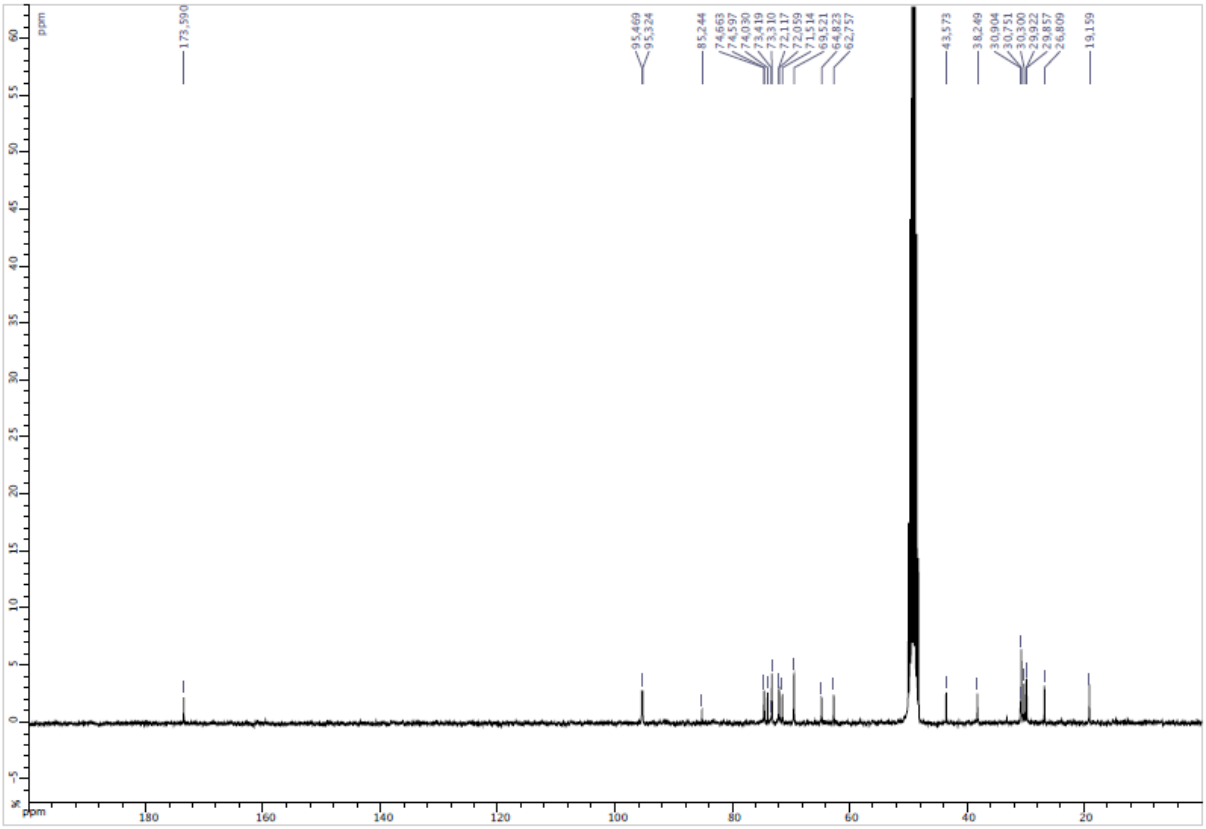
Copy of ^{13}C NMR spectrum of compound S2 (75 MHz, $CDCl_3$)



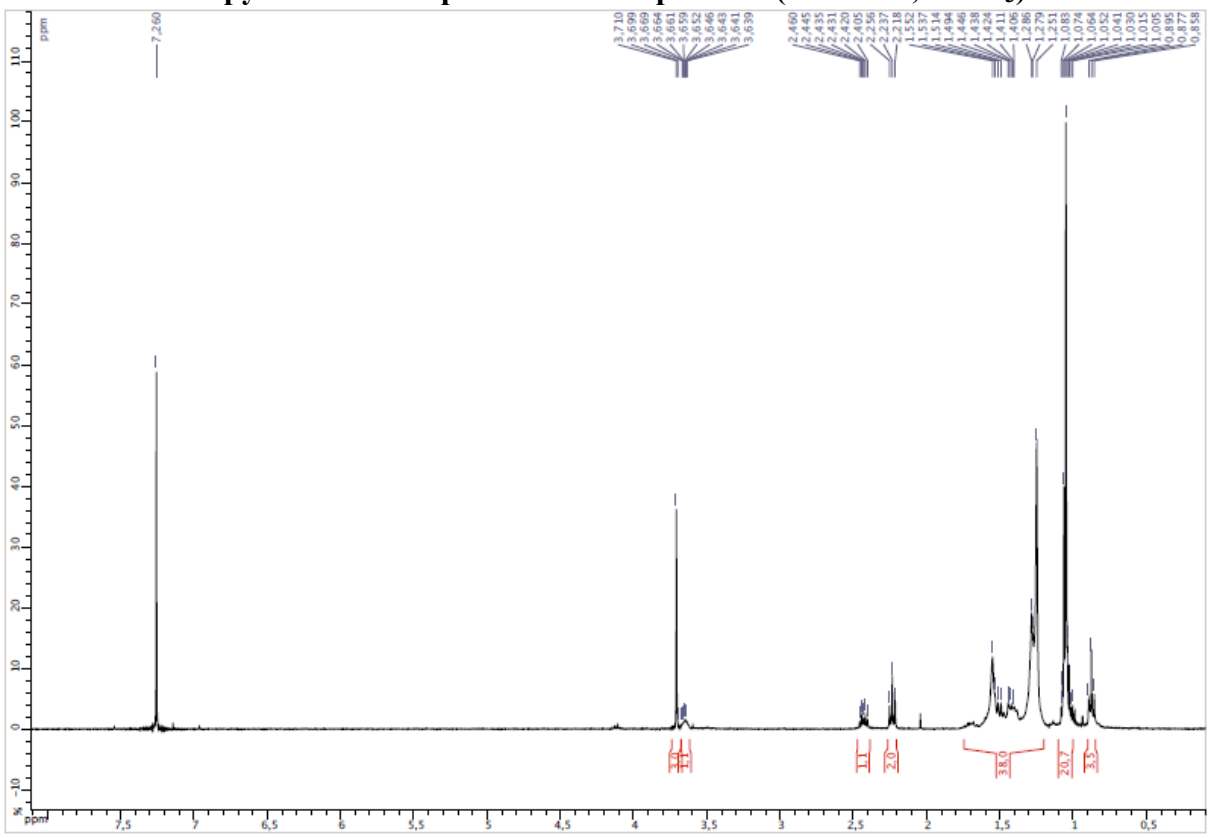
Copy of ^{1}H NMR spectrum of compound 1 (360 MHz, $CD_3OD/CDCl_3$)



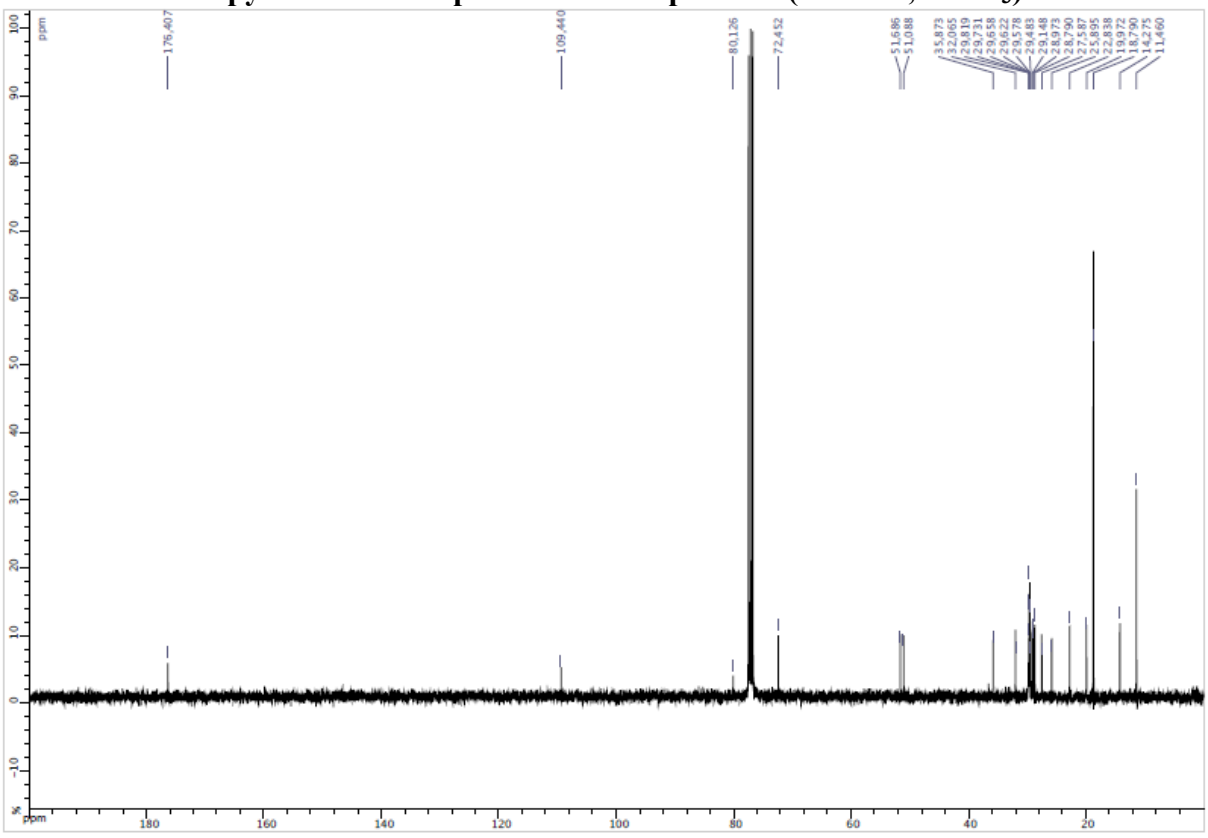
Copy of ^{13}C NMR spectrum of compound 1 (75 MHz, CD_3OD)



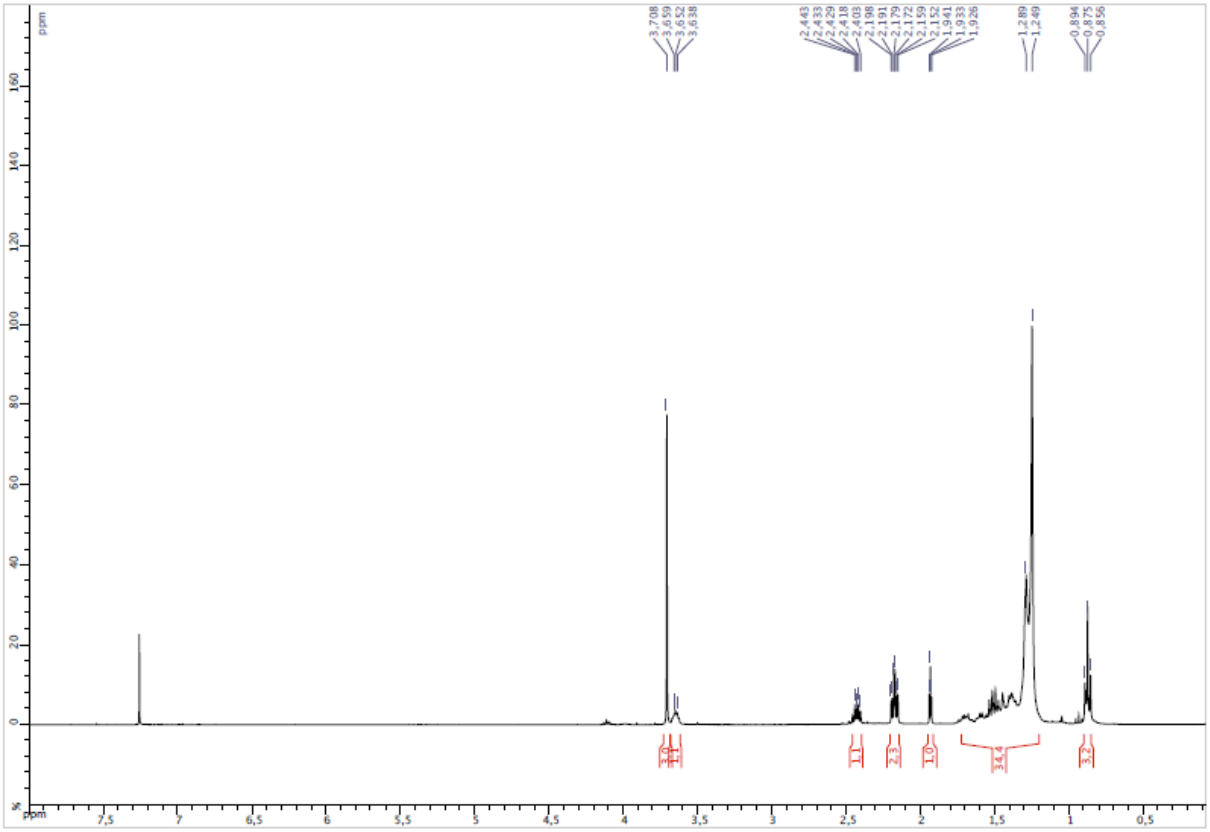
Copy of ^{1}H NMR spectrum of compound 5 (360 MHz, $CDCl_3$)



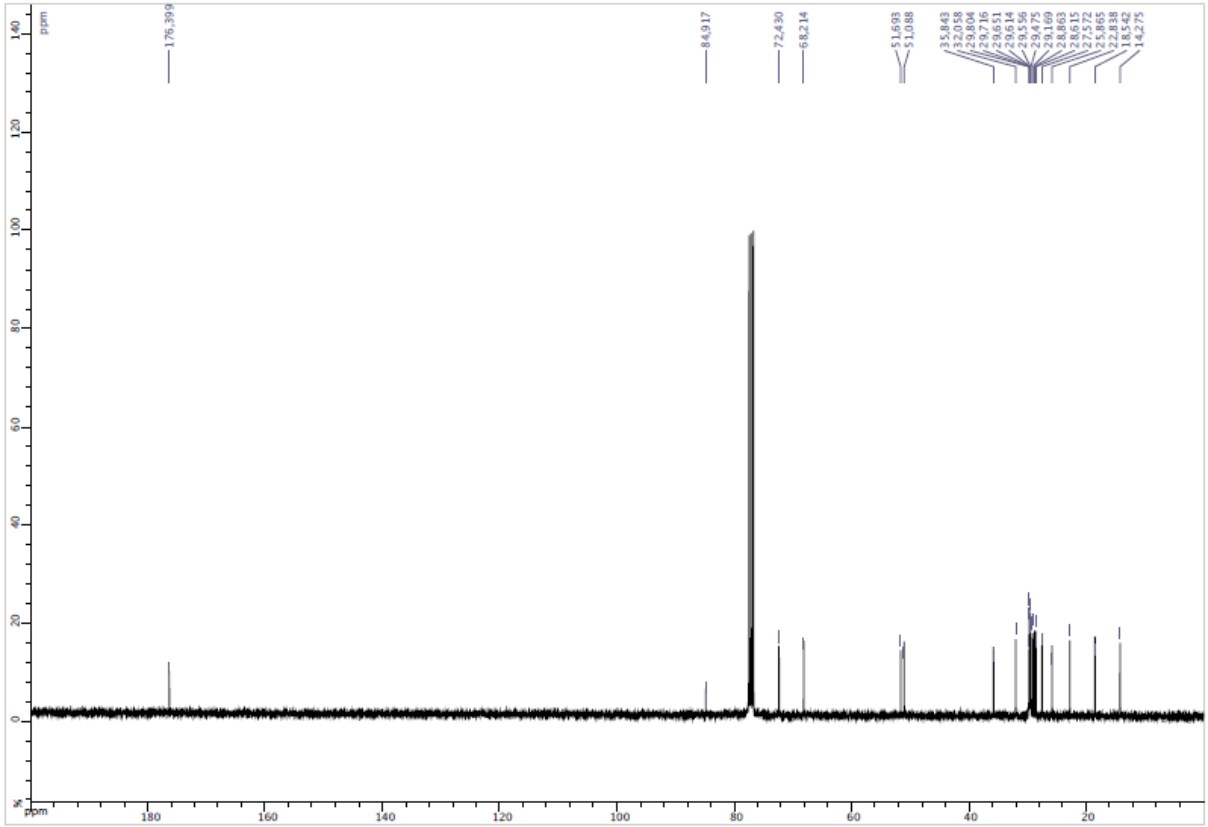
Copy of ^{13}C NMR spectrum of compound 5 (90 MHz, $CDCl_3$)



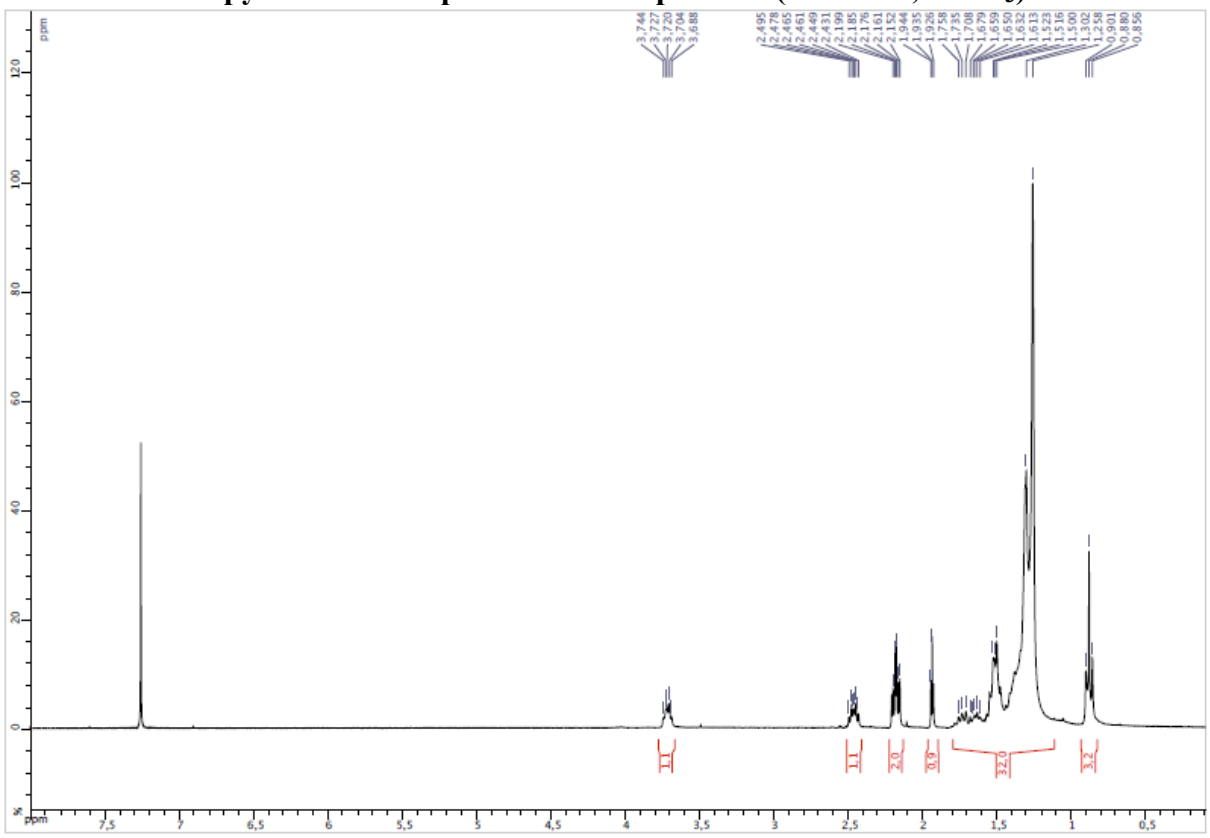
Copy of ^{1}H NMR spectrum of compound 7 (360 MHz, $CDCl_3$)



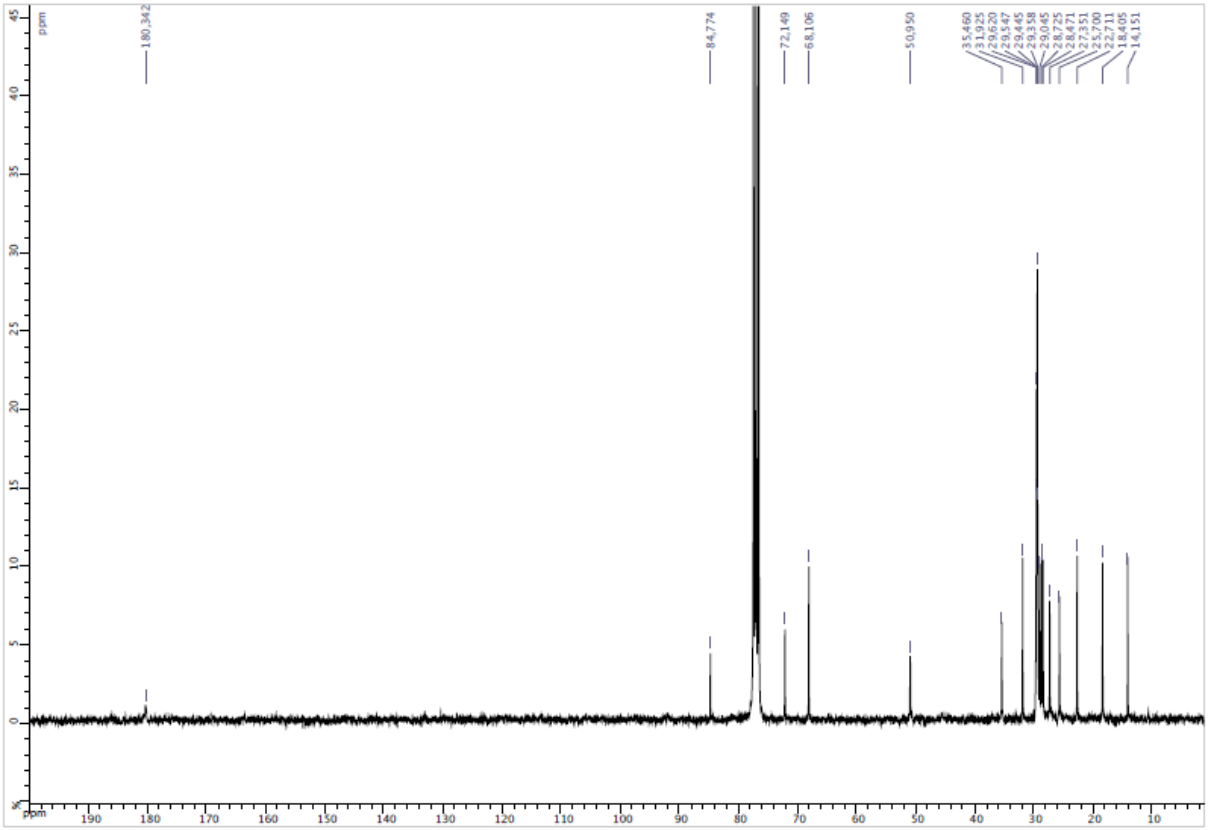
Copy of ^{13}C NMR spectrum of compound 7 (90 MHz, $CDCl_3$)



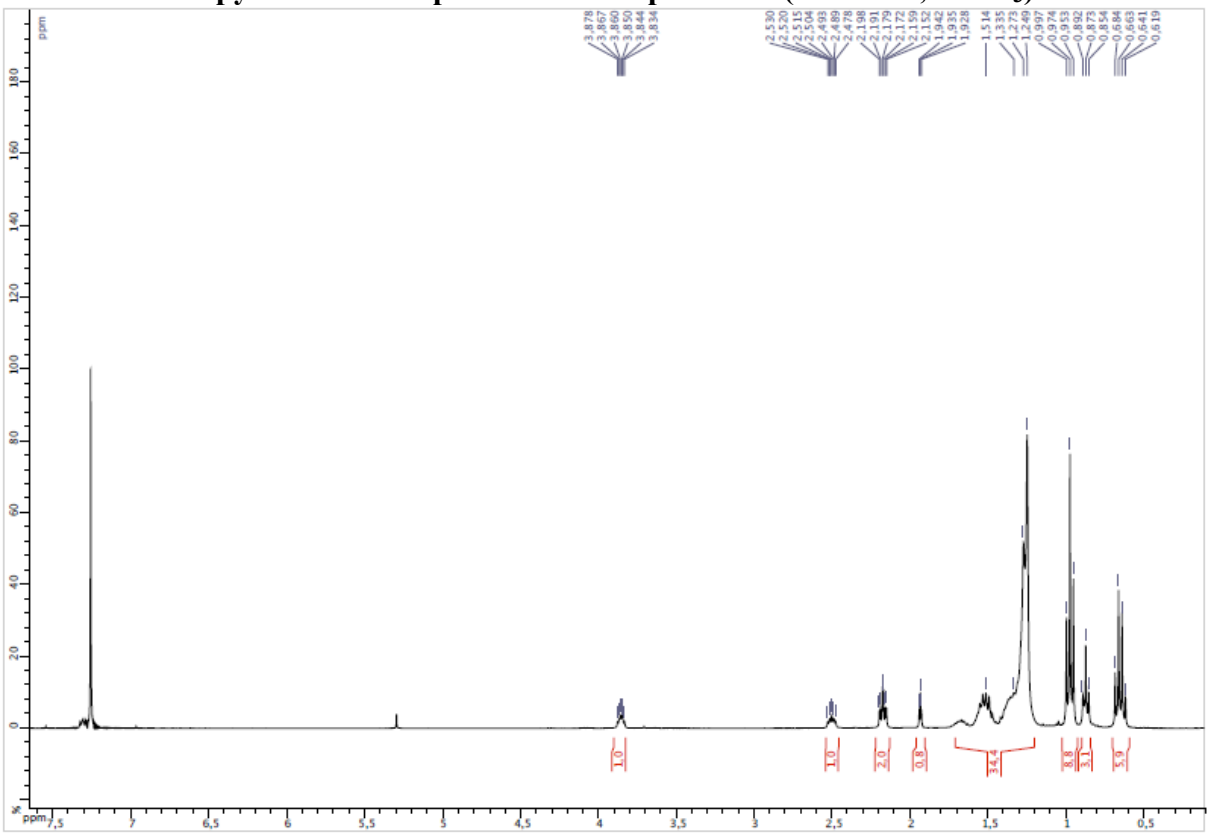
Copy of ^{1}H NMR spectrum of compound 9 (300 MHz, $CDCl_3$)



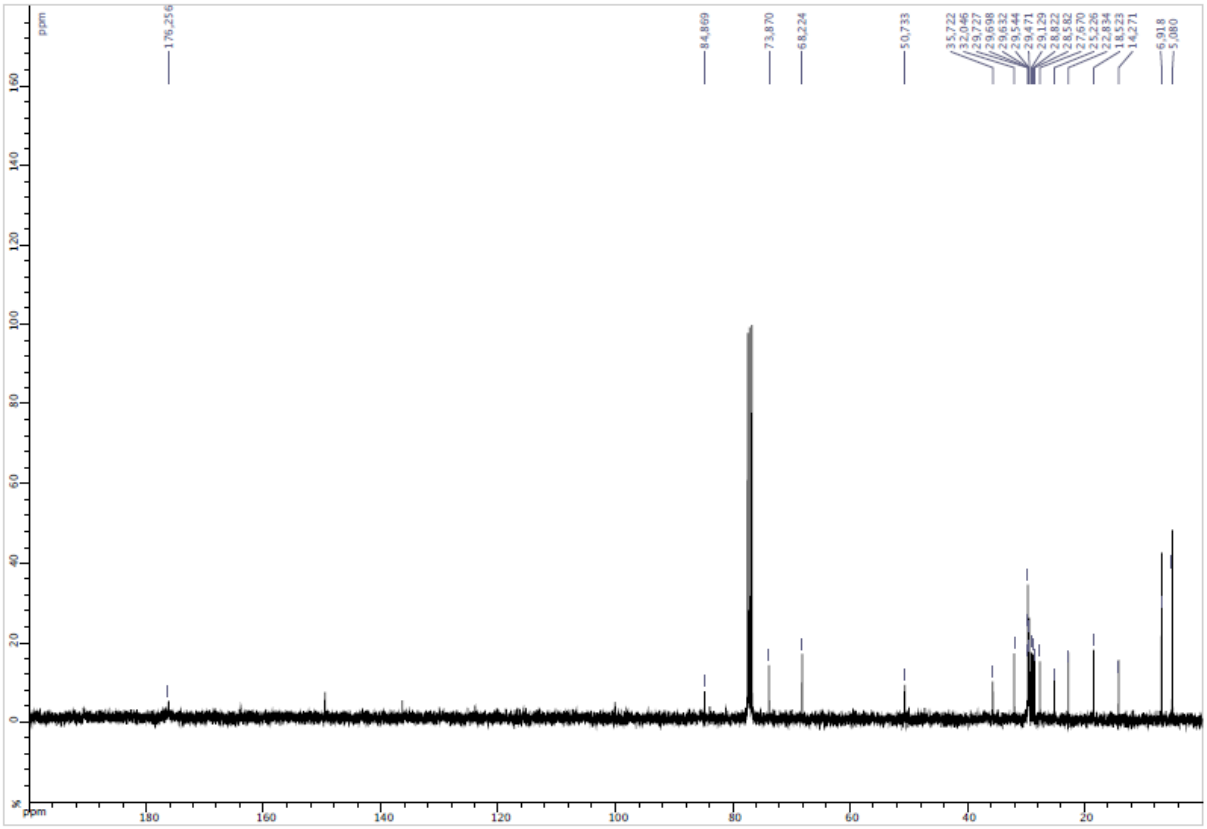
Copy of ^{13}C NMR spectrum of compound 9 (75 MHz, $CDCl_3$)



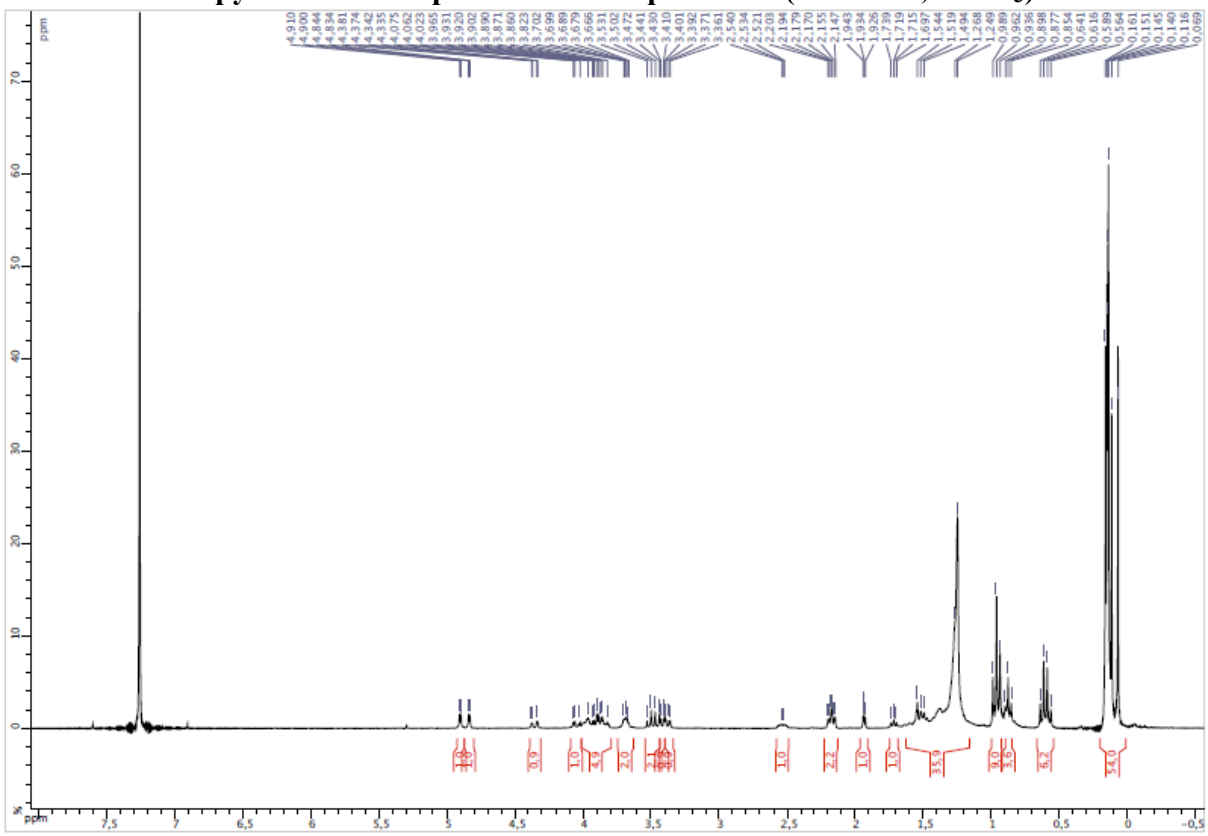
Copy of ^{1}H NMR spectrum of compound 11 (360 MHz, $CDCl_3$)



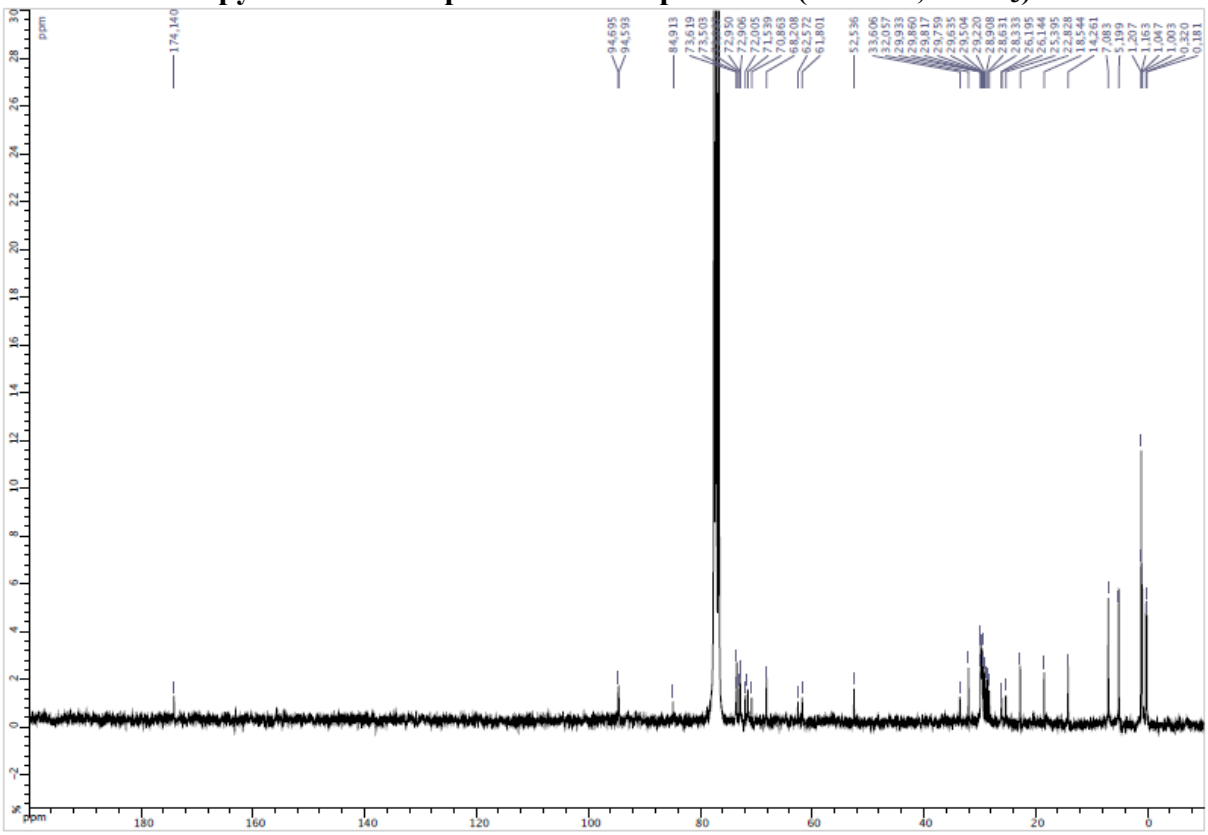
Copy of ^{13}C NMR spectrum of compound 11 (90 MHz, $CDCl_3$)



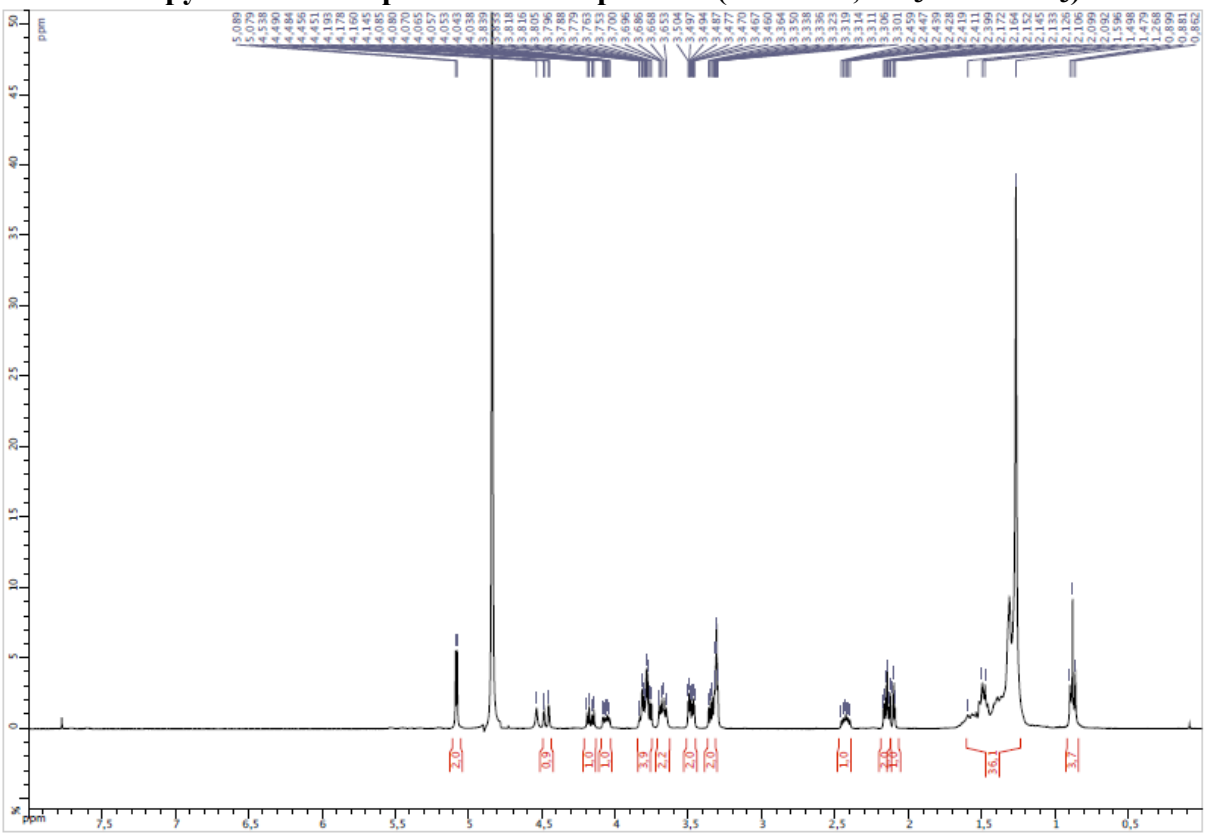
Copy of ^{1}H NMR spectrum of compound S3 (300 MHz, $CDCl_3$)



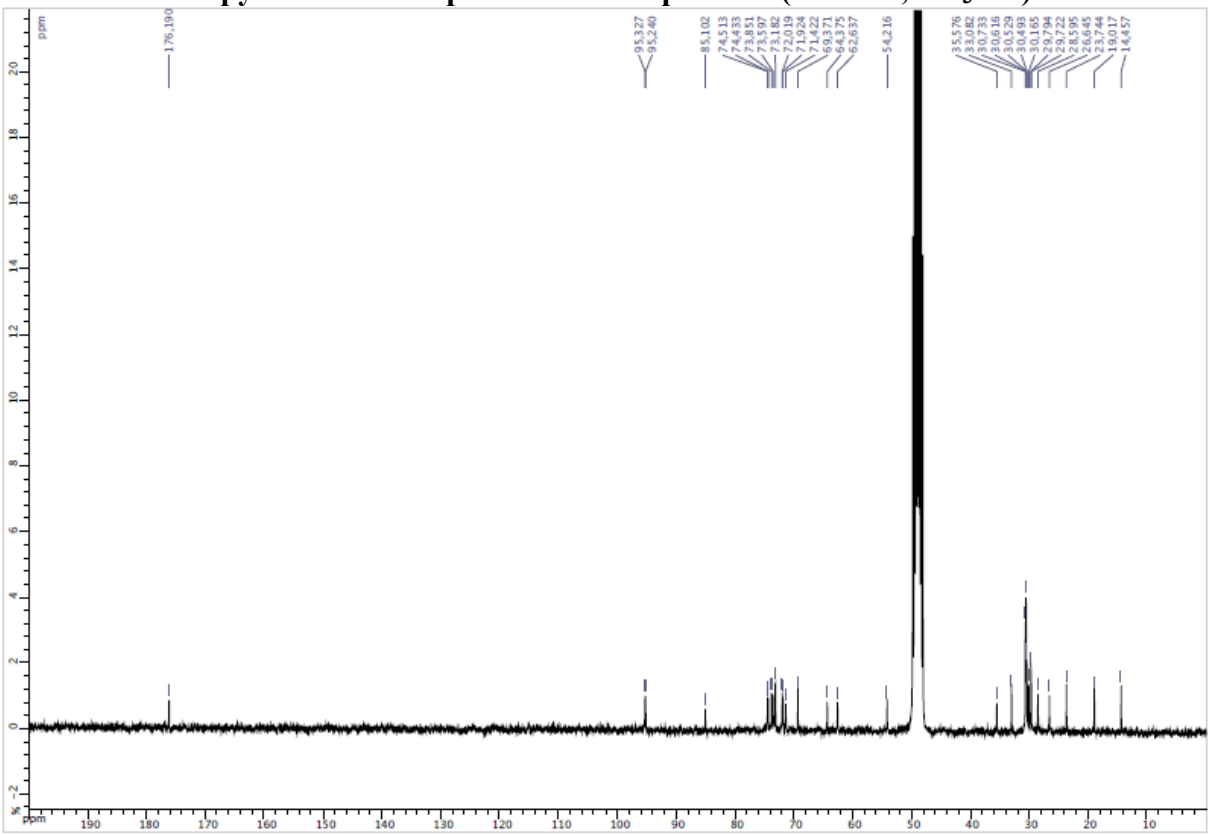
Copy of ^{13}C NMR spectrum of compound S3 (75 MHz, $CDCl_3$)



Copy of ^{1}H NMR spectrum of compound 2 (360 MHz, $CD_3OD/CDCl_3$)



Copy of ^{13}C NMR spectrum of compound 2 (75 MHz, CD_3OD)



IV. References in the supporting information

1 Adapted from: S. P. Breazzano, Y. B. Poudel and D. L. Boger, *J. Am. Chem. Soc.*, 2013, **135**, 1600–1606.

2 Y. M. Lee, C. Lim, H. S. Lee, Y. K. Shin, K.-O. Shin, Y.-M. Lee and S. Kim, *Bioconjug. Chem.*, 2013, **24**, 1324–1331.

3 D. W. Brooks, L. D.-L. Lu and S. Masamune, *Angew. Chem. Int. Ed. Engl.*, 1979, **18**, 72–74.

4 J. P. Genêt, C. Pinel, V. Ratovelomanana-Vidal, S. Mallart, X. Pfister, M. C. C. De Andrade and J. A. Laffitte, *Tetrahedron: Asymmetry*, 1994, **5**, 665–674.

5 R. Toubiana, B. C. Das, J. Defaye, B. Mompon and M.-J. Toubiana, *Carbohydr. Res.*, 1975, **44**, 308–312.

6 V. A. Sarpe and S. S. Kulkarni, *J. Org. Chem.*, 2011, **76**, 6866–6870.

7 J. A. Dale, D. L. Dull and H. S. Mosher, *J. Org. Chem.*, 1969, **34**, 2543–2549.

8 J. A. Dale and H. S. Mosher, *J. Am. Chem. Soc.*, 1973, **95**, 512–519.

9 T. R. Hoye, C. S. Jeffrey and F. Shao, *Nat. Protoc.*, 2007, **2**, 2451–2458.

10 R. Noyori, T. Ohkuma, M. Kitamura, H. Takaya, N. Sayo, H. Kumobayashi and S. Akutagawa, *J. Am. Chem. Soc.*, 1987, **109**, 5856–5858.

11 Z. Yang, X. Jin, M. Guaciaro, B. F. Molino, U. Mocek, R. Reategui, J. Rhea and T. Morley, *Org. Lett.*, 2011, **13**, 5436–5439.

12 P. L. van der Peet, C. Gunawan, S. Torigoe, S. Yamasaki and S. J. Williams, *Chem. Commun.*, 2015, **51**, 5100–5103.

13 A. Baron, Y. Blériot, M. Sollogoub and B. Vauzeilles, *Org. Biomol. Chem.*, 2008, **6**, 1898–1901.

14 R. Bou Raad, X. Meniche, C. de Sousa-d'Auria, M. Chami, C. Salmeron, M. Tropis, C. Labarre, M. Daffe, C. Houssin and N. Bayan, *J. Bacteriol.*, 2010, **192**, 2691–2700.