The Amido-Pentadienoate-Functionality of the Rakicidins is a Thiol Reactive Electrophile – Development of a General Synthetic Strategy

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SUPPORTING INFORMATION

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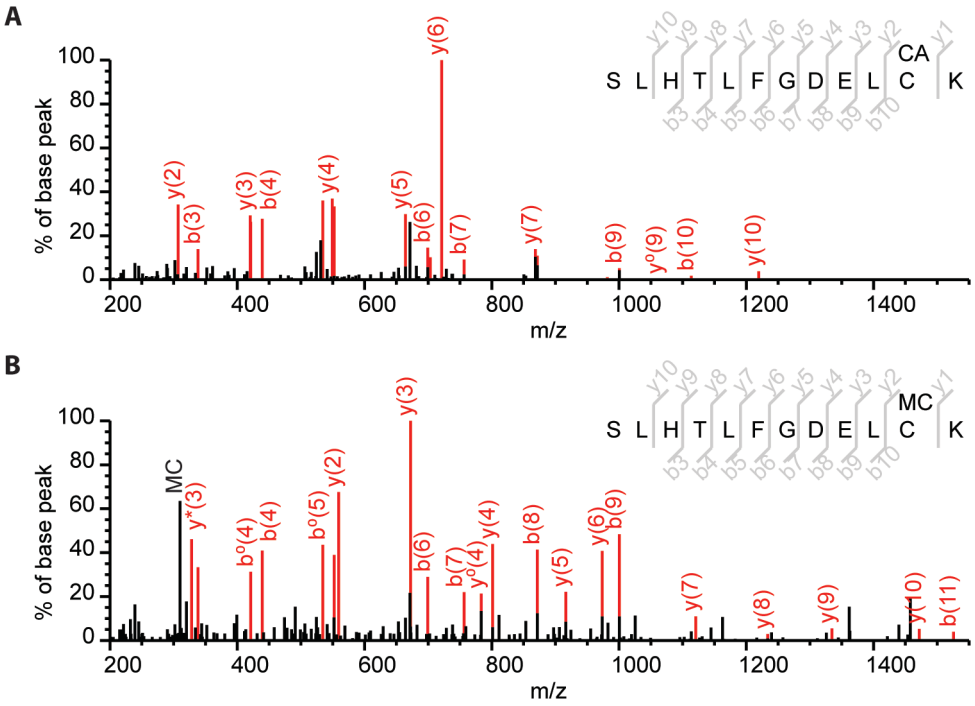


Figure S1

LC-MSMS data of the tryptic peptide SLHTLFGDELCK modified with either iodoacetamide (CA) or the macrocyclic thiopeptide (MC). A) The fragmentations of the 473.9 m/z peptides match the SLHTLFGDELCK peptide with an iodoacetamide (CA) modified cysteine. B) The fragment ions of the 557.9 m/z match the same peptide with a macrocyclic thiopeptide at the cysteine. Furthermore, the macrocyclic thiopeptide generates a fragment (MC), confirming the presence of the modification. For a complete overview of modified peptides, see Table S1.

Synthetic Chemistry – General Procedures and Instrumentation

All reactions with air- and moisture sensitive compounds were conducted in flame-dried glassware under an atmosphere of argon. Dichloromethane, MeCN, THF and toluene were dried over aluminium oxide via an MBraun SPS-800 solvent purification system. Dry DMF were purchased and used as received. *n*-BuLi was titrated with diphenyl acetic acid to determine concentration before use, according to the procedure by Kofron and Baclawski.[1] Reagents were used as received from commercial suppliers. Concentration *in vacuo* was performed using a rotary evaporator with the water bath temperature at 40 °C followed by further concentration using a high vacuum pump. TLC analysis was carried out on silica coated aluminum foil plates (Merck Kieselgel 60 F_{254}). The TLC plates were visualized by UV light and/or by staining with either CAM stain ($(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (10 g), Cerric ammonium sulfate (4 g), 10% H_2SO_4 (aq., 400 mL)), ninhydrin stain (ninhydrin (12 g) and AcOH (12 mL) in butanol (400 mL)) or $KMnO_4$ stain ($KMnO_4$ (5.0 g), 5% NaOH (aq., 8.3 mL) and K_2CO_3 (33.3 g) in H_2O (500 mL)). Flash column chromatography was carried out using silica gel (230-400 mesh particle size, 60 Å pore size) as stationary phase.

NMR spectra were recorded on a Varian Mercury or a Bruker BioSpin GmbH spect 400 MHz spectrometer. Chemical shifts (δ) are reported in ppm relative to the residual solvent signals adjusted according to Gottlieb *et al*.[2] Mass spectra were recorded on a Bruker Daltonics MicrOTOF time-of-flight spectrometer with positive electrospray ionization. The masses were calibrated by internal standard of sodium formate clusters. Melting points were measured on a Büchi B-540 instrument and are uncorrected. Infrared spectra were acquired on a PerkinElmer Spektrum TwoTM UATR. Optical rotation was measured on an ADP 440+ spectrometer and the concentration is reported in 10 mg/1 mL. LC-MS and HPLC analysis and purification were performed using a Gilson HPLC system and a PerkinElmer Flexar SQ 300 MS detector.

Proteomics – Sample Preparation and Analysis

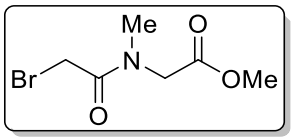
A protein-containing sample with reduced cysteins was produced by incubating bovine serum albumin (BSA) in 6 M Urea, 50 mM Tris-HCL, pH 8.0 and 5 mM DTT. After 30 min the 5 mM DTT was removed by buffer exchange using Zeba Spin Desalting Columns (Thermo Scientific) according to manufactures description. Macrocycle **1** was added to a final concentration of 100 µM and incubated at 37°C for 2 hours. After 2 hrs, any free cysteine residues were alkylated by adding 15 mM iodoacetamide. The sample was diluted 3 times with 50 mM Tris-HCl and treated with trypsin for 16 hours at 37 °C. The resulting peptides were micro purified and analyzed by LC-MSMS.[3]

nLCI-MS/MS analyses were performed on an EASY-nLC II system (ThermoScientific) connected to a TripleTOF 5600 mass spectrometer (AB Sciex) equipped with a NanoSpray III source (AB Sciex) operated under Analyst TF 1.5.1 control. The micropurified sample was suspended in 0.1% formic acid, injected, trapped and desalted on a 2 cm x 100 µm Trap column packed in-house with RP ReproSil-Pur C18-AQ 3 µm resin (Dr. Marisch GmbH, Ammerbuch-Entringen, Germany). The peptides were eluted from the trap column and separated on a 15-cm analytical column (75 µm i.d.) packed in-house in a pulled emitter with RP ReproSil-Pur C18-AQ 3 µm resin (Dr. Marisch GmbH, Ammerbuch-Entringen, Germany). Peptides were separated using a 20 min gradient from 5% to 35% phase B (0.1% formic acid and 90% acetonitrile) and a flow rate of 250 nl/min. The collected MS files were converted to Mascot generic format

(MGF) using the AB SCIEX MS Data Converter beta 1.1 (AB SCIEX) and the "proteinpilot MGF" parameters. The generated peak lists were searched against the swiss-prot database (bovine) using the Mascot search engine (matrix science). Search parameters were allowing Macrocyclic-thiopeptides, Macrocyclic-thiopeptides+H_2O and iodoacetamide as variable modifications with peptide tolerance and MS/MS tolerance set to 10 ppm and 0.1 Da respectively. Peptide information was extracted using MDM.[4]

Synthetic Protocols and Characterization Data

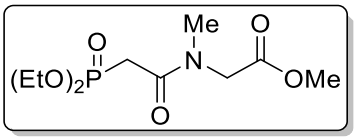
Methyl N-(2-bromoacetyl)-N-methylglycinate (2)



Sarcosine methyl ester hydrochloride (2.094 g, 14.999 mmol) was dissolved in CH_2Cl_2 (90 mL) under argon. Triethylamine (6.3 mL, 45 mmol, 3.0 equiv.) was added, and the mixture was cooled to -78 ˚C. A solution of bromoacetyl bromide (1.96 mL, 22.5 mmol, 1.5 equiv.) in CH_2Cl_2 (30 mL) was added dropwise over ca. 10 min. The reaction was maintained at -78 ˚C for 30 min and was then allowed to warm to -5 ˚C over a period of 140 min. The reaction was diluted with CH_2Cl_2 (100 mL), and washed with 1 M HCl (aq., 110 mL). The aqueous phase was back-extracted with CH_2Cl_2 (2 x 60 mL). The combined organic phases were washed with brine (75 mL), and the aqueous phase was back-extracted with CH_2Cl_2 (75 mL). The combined organics were dried (Na_2SO_4) and concentrated *in vacuo* to give a brown oil that was further purified by flash column chromatography (pentane:EtOAc 1:1 → 0:1) to give the product (2.768 g, 82%) as yellow oil.

R_f 0.47 (1:1 pentane:EtOAc, $KMnO_4$ stain). HRMS calc.: $C_6H_{10}BrNNaO_3^+$: 245.9736; found: 245.9738 $[M + Na]^+$. Two rotamers were observed, NMR data is reported for the major rotamer. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.02 (s, 2H), 3.83 (s, 2H), 3.63 (s, 3H), 3.07 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.9, 167.1, 52.0, 49.5, 37.1, 25.8. IR (neat) ν_{max} /cm^{-1} 2954, 1743, 1649, 1206.

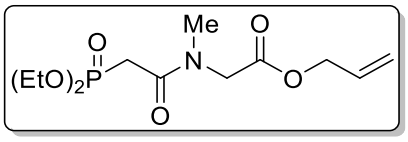
Methyl N-(2-(diethoxyphosphoryl)acetyl)-N-methylglycinate (3)



Bromide **2** (0.854 g, 3.81 mmol) was dissolved in anhydrous 1,2-dichlorethane (3.6 mL) under argon while stirred. $P(OEt)_3$ (1.00 mL, 5.75 mmol, 1.5 equiv.) was added in one portion and the reaction was heated to reflux. After 22 h the reaction mixture was allowed to cool to rt and concentrated *in vacuo*. Flash column chromatography (EtOAc/MeOH 98:2 → 90:10) then gave **3** (0.76 g, 71%) as a clear yellow oil.

R_f 0.12 (EtOAc, $KMnO_4$ stain). HRMS calc.: $C_{10}H_{20}NNaPO_5^+$: 304.0920; found: 304.0926 $[M + Na]^+$. Two rotamers were observed, NMR data is reported for the major rotamer. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.22 – 4.08 (m, 6H), 3.73 (s, 3H), 3.19 – 3.08 (m, 4H), 1.34 (t, J = 7.0, Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.3, 165.6, 62.7 (d, J = 6.4 Hz), 52.35 (d, J = 14.9 Hz), 49.6, 37.8, 33.8 (d, J = 132.5), 16.3 (d, J = 6.2 Hz). IR (neat) ν_{max} /cm^{-1} 2984, 1746, 1649, 1207, 1018.

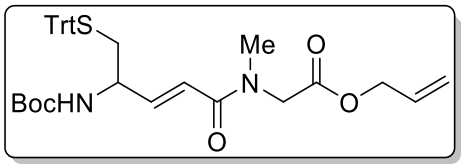
Allyl N-(2-(diethoxyphosphoryl)acetyl)-N-methylglycinate (4)



Methyl ester **3** (2.5 g, 8.89 mmol) was dissolved in allylic alcohol (1.8 mL) in a flame dried flask under argon and $OSnBu_2$ (2.43 g, 9.78 mmol, 1.1 equiv.) was added. The reaction mixture was heated to 85 ˚C and stirred at that temperature for 24 h. The reaction mixture was concentrated and the crude product purified by flash column chromatography (5% MeOH/CH_2Cl_2) to give the product (2.03 g, 74%) as an oil.

R_f 0.28 (5% MeOH/EtOAc, ninhydrin stain); HRMS (ESI): Calc. $C_{12}H_{23}NPO_6^+$: 308.1258; found: 308.1263 $[M + H]^+$. Two rotamers were observed, NMR data is reported for the major rotamer. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.95 – 5.81 (m, 1H), 5.37 – 5.19 (m, 2H), 4.60 (dt, J = 5.8, 1.4 Hz, 2H), 4.30 – 4.08 (m, 6H), 3.18 (s, 3H), 3.09 (d, J = 22.2 Hz, 2H), 1.31 (t, J = 7.1 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.6, 165.8 (d, J = 5.5 Hz), 131.7, 118.8, 65.9, 62.8 (d, J = 6.4 Hz), 49.8, 37.9, 33.4 (d, J = 133.6 Hz), 16.4 (d, J = 6.1 Hz). IR (neat) ν_{max} / cm^{-1} 2963, 1746, 1650, 1191, 1021, 956.

Allyl (E)-N-4-((tert-butoxycarbonyl)amino)-5-(tritylthio)pent-2-enoyl)-N-methyl glycinate (6b)

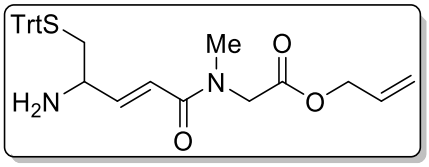


Phosphonate ester **4** (1.39 g, 4.52 mmol, 3.8 equiv.) was dissolved in anhydrous THF (8.6 mL) and cooled to -78 ˚C. *n*-BuLi (1.3 M in hexanes, 3.8 mL, 4.97 mmol, 4.2 equiv.) was added dropwise and the reaction mixture stirred 60 min before aldehyde **5**[5] (528 mg, 1.18 mmol, 1.0 equiv.), dissolved in anhydrous THF (23 mL), was added dropwise. The reaction mixture was then stirred at -78 ˚C for 18 h before it was quenched with brine, warmed to rt and extracted with EtOAc. The organic phase was then dried (Na_2SO_4) and concentrated. The crude product was purified by flash column chromatography (30 → 40% EtOAc/pentane) to give the product as a white amorphous solid (447 mg, 1:1 mixture of cis and trans isomers, 63%).

Tributylphosphine (1.5 equiv., 1.12 mmol, 280 µL) was added to the solution of a 1:1 mixture of **6a** and **6b** (744 µmol, 447 mg) in anhydrous toluene (6.7 mL) and stirred 19 h at rt until NMR showed full conversion of the cis to the trans isomer. The reaction mixture was purified by flash column chromatography (30 → 40% EtOAc/pentane) to give the pure trans isomer **6b** (384 mg, 86%) as a white amorphous solid.

R_f 0.50 (40% EtOAc/pentane, UV and CAM stain); HRMS (ESI): Calc. $C_{35}H_{40}N_2O_5SNa^+$: 623.2550; found: 623.2560 $[M + Na]^+$. Two rotamers were observed, NMR data is reported for the major rotamer. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (d, J = 7.7 Hz, 6H), 7.28 (t, J = 7.6 Hz, 6H), 7.23 – 7.18 (m, 3H), 6.59 (dd, J = 14.6, 5.6 Hz, 1H), 6.27 (d, J = 15.1 Hz, 1H), 5.97 – 5.81 (m, 1H), 5.31 (d, J = 17.3 Hz, 1H), 5.23 (d, J = 10.5 Hz, 1H), 4.69 – 4.58 (m, 3H), 4.23 – 4.23 (m, 1H), 4.17 (s, 2H), 3.09 (s, 3H), 2.49 – 2.34 (m, 2H), 1.42 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.9, 168.6, 166.5, 154.9, 144.4, 131.7, 129.6, 128.1, 126.9, 120.3, 118.8, 79.8, 67.1, 65.8, 51.1, 49.7, 36.7, 36.4, 28.4. IR (neat) ν_{max} /cm^{-1} 3314, 3057, 2976, 1746, 1709, 1663, 1621, 1487, 742, 699.

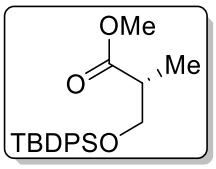
Allyl (E)-N-(4-amino-5-(tritylthio)pent-2-enoyl)-N-methylglycinate (7)



TFA (0.2 mL) was added to a solution of carbamate **6b** (56 mg, 92.8 µmol) in CH_2Cl_2 (1 mL) and stirred at rt for 45 min. The reaction mixture was then concentrated, dissolved in EtOAc, washed with $NaHCO_3$ and brine before it was dried (Na_2SO_4) and concentrated to give the product (46 mg, 99%) as a yellow oil.

R_f 0.48 (10% MeOH/EtOAc, UV and CAM stain); HRMS (ESI): Calc. $C_{30}H_{33}N_2O_3S^+$: 501.2201; found: 501.2212 $[M + H]^+$. Two rotamers were observed, NMR data is reported for the major rotamer. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 – 7.39 (m, 6H), 7.30 – 7.25 (m, 6H), 7.23 – 7.18 (m, 3H), 6.63 (dd, *J* = 15.1, 5.7 Hz, 1H), 6.30 (d, *J* = 15.1 Hz, 1H), 5.95 – 5.83 (m, 1H), 5.35 – 5.27 (m, 1H), 5.26 – 5.20 (m, 1H), 4.65 – 4.57 (m, 2H), 4.15 (s, 2H), 3.11 – 2.97 (m, 1H), 3.09 (s, 3H), 2.42 – 2.34 (m, 2H), 2.21 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.1, 166.9, 148.4, 144.8, 131.8, 129.7, 128.1, 126.9, 119.0, 118.9, 67.1, 65.9, 52.3, 49.8, 39.7, 36.8. IR (neat) ν_{max} /cm^{-1} 2931,1746, 1660, 1621, 1487, 1444, 1399, 1186, 742, 699.

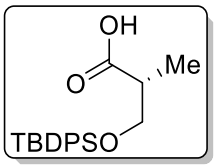
Methyl (R)-3-((tert-butyldiphenylsilyl)oxy)-2-methylpropanoate (9)[6]



Methyl (*R*)-(-)-3-hydroxy-2-methylpropionate **8** (0.94 mL, 8.47 mmol) was dissolved in anhydrous CH_2Cl_2 (35 mL). Imidazole (1.79 g, 26.24 mmol, 3.1 equiv.) and TBDPSCl (2.3 mL, 8.89 mmol, 1.05 equiv.) were added and the reaction mixture was stirred at rt. After 19 h additional TBDPSCl (0.66 mL, 2.54 mmol, 0.3 equiv.) was added. After stirring an additional 45 min at rt, the reaction was quenched by slow addition of NH_4Cl (sat., aq.). The phases were separated and the aqueous phase was extracted with $CHCl_3$ and the combined organic phases were then dried (Na_2SO_4), filtered and concentrated. The resulting colorless oil was purified by flash column (0% → 1% → 5% → 10% EtOAc/pentane) to give the product (2.96 g, 98%) as a colorless oil.

R_f 0.59 (5% EtOAc/pentane, UV and $KMnO_4$ stain); $[\alpha]_D^{300.4K}$ -20.3 (*c* 1.0, $CHCl_3$). HRMS (ESI): Calc. $C_{21}H_{28}O_3SiNa^+$: 379.1700; found: 379.1701 $[M + Na]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 – 7.63 (m, 4H), 7.47 – 7.36 (m, 6H), 3.83 (dd, *J* = 9.7, 6.9 Hz, 1H), 3.72 (dd, *J* = 9.8, 5.8 Hz, 1H), 3.69 (s, 3H), 2.72 (h, *J* = 6.8 Hz, 1H), 1.16 (d, *J* = 7.0 Hz, 3H), 1.03 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.5, 135.7, 133.6, 133.6, 129.8, 127.8, 66.0, 51.6, 42.5, 26.8, 19.4, 13.6. IR (neat) ν_{max} /cm^{-1} 2932, 2858, 1739, 1428, 1105, 1084, 700, 503.

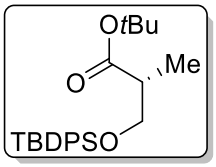
(R)-3-((tert-butyldiphenylsilyl)oxy)-2-methylpropanoic acid (10)[6]



Methyl ester **9** (2.06 g, 5.79 mmol) was dissolved in THF/H_2O (80 mL, 2.5:1) and cooled to 0 ˚C. Then $LiOH \cdot H_2O$ (607 mg, 14.48 mmol, 2.5 equiv.) was added and the reaction mixture was stirred 57 h at rt. The reaction mixture was concentrated *in vacuo* and redissolved in HCl (10%, aq.). The aqueous phase was extracted with chloroform, and the organic phase was dried (Na_2SO_4) and concentrated to give the product (1.91 g, 96%) as a yellow oil.

R_f 0.75 (1:1 EtOAc:pentane, UV and $KMnO_4$ stain); $[\alpha]_D^{301.2K}$ – 17.6 (*c* 0.5, $CHCl_3$). HRMS (ESI): Calc. $C_{20}H_{26}O_3SiNa^+$: 365.1543; found: 365.1533 $[M + Na]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.69 – 7.63 (m, 4H), 7.47 – 7.36 (m, 7H), 3.82 (dd, *J* = 10.0, 7.2 Hz, 1H), 3.76 (dd, *J* = 9.9, 5.5 Hz, 1H), 2.79 – 2.67 (m, 1H), 1.18 (d, *J* = 7.0 Hz, 3H), 1.04 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.5, 135.7, 133.2, 133.2, 129.9, 127.9, 65.7, 42.0, 26.9, 19.4, 13.4. IR (neat) ν_{max} /cm^{-1} 2932, 2858, 1705, 1472, 1427, 1105, 700, 503, 485.

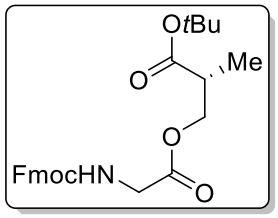
tert-Butyl (R)-3-((tert-butyldiphenylsilyl)oxy)-2-methylpropanoate (11)



To a solution of acid **10** (1.03 g, 3.01 mmol) and $(Boc)_2O$ (1.31 g, 6.02 mmol, 2.0 equiv.) in *t*-BuOH (6.2 mL) was added DMAP (110 mg, 903 µmol, 0.3 equiv.). The reaction mixture was stirred at rt for 90 min before it was diluted with CH_2Cl_2 and washed with HCl (1 M, aq.), dried (Na_2SO_4) and concentrated. The crude product was purified by flash column chromatography (0.5% → 0.75% → 1% Et_2O/pentane) to give the product (935 mg, 78%) as a colorless oil.

R_f 0.34 (0.5% Et_2O/pentane, UV and $KMnO_4$ stain); $[\alpha]_D^{296.8K}$ – 0.6 (*c* 1.0, $CHCl_3$). HRMS (ESI): Calc. $C_{24}H_{34}SiO_3Na^+$: 421.2169; found: 421.2173 $[M + Na]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.71 – 7.67 (m, 4H), 7.47 – 7.36 (m, 6H), 3.83 (dd, *J* = 9.7, 7.0 Hz, 1H), 3.70 (dd, *J* = 9.7, 5.5 Hz, 1H), 2.60 (pd, *J* = 7.0, 1.3 Hz, 1H), 1.48 (s, 9H), 1.11 (d, *J* = 7.0 Hz, 3H), 1.06 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.4, 135.7, 135.7, 133.8, 133.7, 129.7, 127.8, 80.2, 66.3, 43.6, 28.3, 26.9, 19.4, 13.7. IR (neat) ν_{max} /cm^{-1} 3071, 3050, 2962, 2932, 2858, 1730, 1156, 1106, 503.

tert-Butyl (R)-3-(((((9H-fluoren-9-yl)methoxy)carbonyl)glycyl)oxy)-2-methylpropanoate (13)

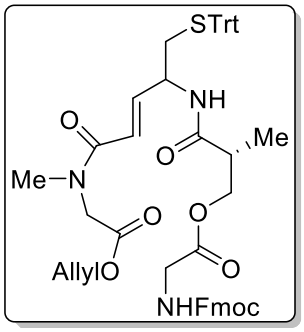


Silyl ether **11** (2.26 mmol, 900 mg) was dissolved in anhydrous THF (24 mL) and TBAF (4.5 mL, 1.0 M/THF, 4.52 mmol, 2.0 equiv.) was added. The reaction mixture was stirred 4 h 15 min at rt and then diluted with Et_2O and washed with

brine. The aqueous phase was back-extract with Et_2O, and the combined organic phases were dried (Na_2SO_4) and carefully concentrated under reduced pressure at no lower than 300 mbar at 40 ˚C, since the free hydroxyl **12** is volatile. The crude product was purified by flash column chromatography (30% Et_2O/pentane) and the product **12** was isolated as a colorless oil (2.02 mmol, when corrected for residues of Et_2O, 90%). To a solution of alcohol **12** (2.02 mmol) in anhydrous DMF (9.5 mL) was added DIPEA (1.6 mL, 10.11 mmol, 5.0 equiv.), *N*-Fmoc-glycine (1.50 g, 5.06 mmol, 2.5 equiv.), and HOAt (826 mg, 6.07 mmol, 3.0 equiv.). The reaction mixture was then cooled to 0 ˚C and HATU (2.30 g, 6.066 mmol, 3.0 equiv.) was added portion-wise. The reaction mixture was stirred at 0 ˚C for 90 min. The solution was diluted with EtOAc, washed with $NaHCO_3$ (sat., aq.) and brine, dried (Na_2SO_4) and concentrated. Flash column chromatography (30% Et_2O/pentane) then gave the product **13** (730 mg, 82%) as a white solid.

m.p. 66.5-67.4 °C. R_f 0.33 (30% Et_2O/pentane, UV and $KMnO_4$ stain); $[\alpha]_D^{295.7K}$ – 10.0 (*c* 1.0, $CHCl_3$). HRMS (ESI): Calc. $C_{25}H_{29}NO_6Na^+$: 462.1887; found: 462.1893 $[M + Na]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, *J* = 7.6 Hz, 2H), 7.60 (d, *J* = 7.5 Hz, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.31 (td, *J* = 7.4, 1.2 Hz, 2H), 5.30 (t, *J* = 5.4 Hz, 1H), 4.41 (d, *J* = 7.1 Hz, 2H), 4.31 (dd, *J* = 10.7, 7.4 Hz, 1H), 4.27 – 4.18 (m, 2H), 4.00 (d, *J* = 5.6 Hz, 2H), 2.70 (h, *J* = 7.1 Hz, 1H), 1.45 (s, 9H), 1.17 (d, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.9, 169.9, 156.3, 143.9, 141.4, 127.8, 127.2, 125.2, 120.1, 81.1, 67.4, 66.8, 47.2, 42.8, 40.1, 28.1, 13.9. IR (neat) ν_{max} /cm^{-1} 3359, 2977, 1722, 1190, 1149, 759, 738.

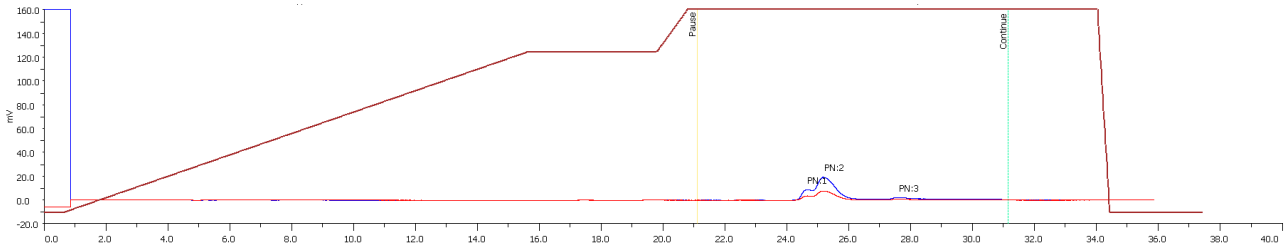
Allyl N-((E)-4-((R)-3-(((((9H-fluoren-9-yl)methoxy)carbonyl)glycyl)oxy)-2-methylpropanamido)-5-(tritylthio)pent-2-enoyl)-N-methylglycinate (15)



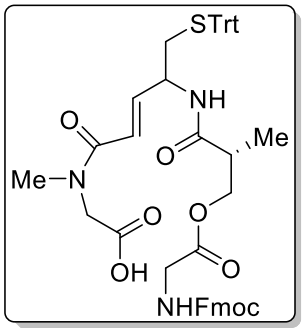
TFA (0.35 mL) was added to a solution of the *tert*-butyl ester **13** (77 mg, 175 µmol) in CH_2Cl_2 (1.85 mL) and stirred at rt for 16 h. The solution was then diluted with Et_2O and the organic phase was washed with brine, dried (Na_2SO_4) and concentrated to give the free acid 1**4** as a colorless oil. The acid **14** (175 µmol, 1.5 equiv.) and the amine **7** (111 µmol) were combined and azeotroped with anhydrous toluene. The mixture was then dissolved in a mixture of anhydrous CH_2Cl_2/DMF (4:1, 1.2 mL) and cooled to 0 ˚C before DIPEA (77 µL, 0.444 mmol, 4.0 equiv.), HOAt (27 mg, 0.20 mmol, 1.8 equiv.) and EDCI·HCl (38 mg, 0.20 mmol, 1.8 equiv.) were added. The reaction mixture was then stirred at rt for 19 h, diluted with EtOAC and washed with $NaHCO_3$ (sat., aq.) and brine, dried (Na_2SO_4) and concentrated. The crude product was purified by flash colum chromatography (60% → 65% → 70% EtOAc/pentane) to give **15** (43 mg, 63%) as a white amorphous solid HPLC-analysis (analytical Kinetex-C18 column, 4.6x25 mm, ACN/H_2O gradient, 1.0 mL/min).

R_f 0.47 and 0.50 (5% MeOH/CH_2Cl_2, CAM stain); HRMS (ESI): Calc. $C_{51}H_{51}N_3O_8SNa^+$: 888.3289; found: 888.3310 $[M + Na]^+$. Mixture of diastereoisomers. Two rotamers were observed, NMR data is reported for the major rotamer. ^1H NMR (400 MHz, $CDCl_3$) δ 7.80 – 7.73 (m, 2H), 7.63 – 7.59 (m, 2H), 7.44 – 7.27 (m, 14H), 7.25 – 7.16 (m, 5H), 6.57 – 6.46

(m, 1H), 6.29 (d, *J* = 15.1 Hz, 0.5H), 6.23 (d, *J* = 15.1 Hz, 0.5H), 5.93 – 5.81 (m, 1H), 5.64 (m, 1H), 5.34 – 5.19 (m, 2H), 4.63 – 4.57 (m, 2H), 4.54 – 4.46 (m, 1H), 4.46 – 4.34 (m, 2H), 4.29 – 4.03 (m, 6H, H1), 3.92 – 3.70 (m, 2H), 3.08 (s, 1.5H), 3.07 (s, 1.5H), 2.57 – 2.34 (m, 3H), 1.13 (d, *J* = 6.1 Hz, 1.5H), 1.11 (d, *J* = 6.1 Hz, 1.5H). ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 172.9, 172.7, 170.0, 169.9, 168.9, 166.9, 166.5, 156.8, 156.5, 144.4, 144.4, 144.0, 143.9, 143.9, 143.6, 143.4, 141.4, 131.7, 131.6, 129.6, 129.6, 128.2, 128.1, 127.8, 127.8, 127.8, 127.2, 127.0, 127.0, 125.4, 125.3, 125.2, 121.5, 121.0, 120.1, 120.0, 120.0, 118.9, 118.9, 67.3, 67.2, 67.2, 65.9, 65.9, 50.0, 49.9, 49.8, 49.7, 47.2, 42.9, 42.8, 40.4, 36.8, 36.8, 35.9, 35.7, 14.1, 13.8. IR (neat) ν_{max} /cm^{-1} 3301, 2935, 1748, 1660, 1618, 1186, 740, 700.



N-((E)-4-((R)-3-(((((9H-Fluoren-9-yl)methoxy)carbonyl)glycyl)oxy)-2-methylpropanamido)-5-(tritylthio)pent-2-enoyl)-N-methylglycine (16)

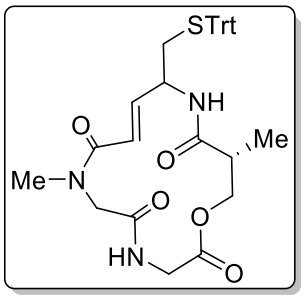


Allyl ester **15** (305 µmol, 264 mg) was dissolved in anhydrous CH_2Cl_2 (19 mL) and $Pd(PPh_3)_4$ (106 mg, 91.5 µmol, 0.3 equiv.) was added followed by *N*-methylaniline (83 µL, 763 µmol, 2.5 equiv.). The reaction mixture was then stirred 2.5 h at rt, before additional $Pd(PPh_3)_4$ (35 mg, 30.2 µmol, 0.1 equiv.) was added. The reaction mixture was then stirred for 1 h before it was diluted with CH_2Cl_2 and washed with HCl (1 M, aq.). The aqueous phase was back-extracted twice with CH_2Cl_2 and the combined organic phases were washed with brine. The aqueous phase was back-extracted twice with CH_2Cl_2 and the combined organic phases were dried (Na_2SO_4) and concentrated. The crude acid was purified by flash column chromatography (EtOAc then 1% AcOH/EtOAc) to give the product (230 mg, 91%) as a yellow amorphous solid.

R_f 0.42 and 0.58 (15% MeOH/CH_2Cl_2, UV and CAM stain); HRMS (ESI): Calc. $C_{48}H_{47}N_3O_8SNa^+$: 848.2976; found: 848.2981 $[M + Na]^+$. Mixture of diastereoisomers. ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 7.79 – 7.70 (m, 2H), 7.58 (d, *J* = 7.0 Hz, 2H), 7.40 – 7.16 (m, 19H), 6.56 – 6.38 (m, 1H), 6.28 – 5.91 (m, 2H), 4.50 – 3.71 (m, 11H), 3.05 – 2.92 (m, 3H), 2.57 – 2.28 (m, 3H), 1.12 – 1.02 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 173.3, 173.2, 171.6, 170.1, 167.3, 167.1, 157.3, 157.0, 144.4, 143.9, 143.8, 141.4, 141.4, 129.6, 128.2, 127.9, 127.2, 127.0, 125.3, 125.2, 120.1, 120.1, 67.5, 67.4, 67.3, 67.3, 67.1, 66.9,

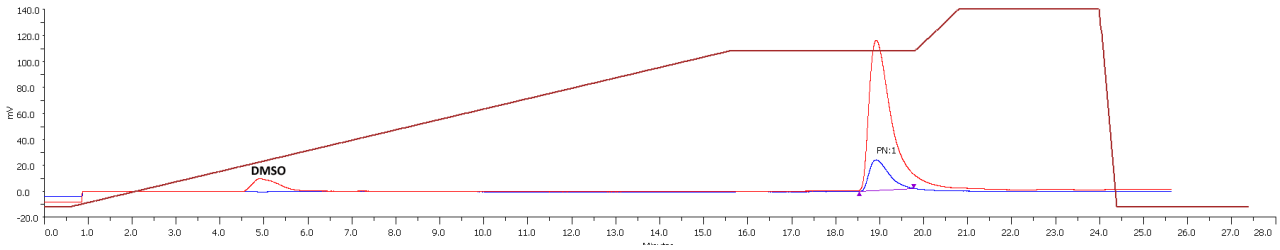
52.0, 50.4, 50.0, 47.1, 42.9, 42.8, 40.4, 37.1, 35.8, 35.6, 14.1, 13.9. IR (neat) ν_{max} /cm^{-1} 3300, 3058, 2921, 2850, 1721, 1657, 1186, 738, 699.

(14R,E)-7,14-Dimethyl-11-((tritylthio)methyl)-1-oxa-4,7,12-triazacyclopentadec-9-ene-2,5,8,13-tetraone (18a)

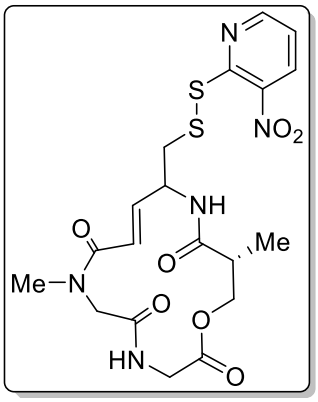


The Fmoc protected amine **16** (41.7 mg, 50.5 µmol) was dissolved in MeCN (525 µL) and Et_2NH (525 µL) and stirred at rt for 1 h and 20 min. The reaction mixture was then concentrated and washed with pentane (0.5 mL, 9 times) to remove most of the dibenzofulvene derived byproduct. The product was then azeotroped twice with toluene and CH_2Cl_2 and dried under high vacuum overnight to give the amino acid **17**, which was advanced to the next reaction without further purification.

The amino acid **17** (50.5 µmol) was dissolved in anhydrous CH_2Cl_2/DMF (4:1, 58 mL) and cooled to 0 ˚C. DEPBT (121 mg, approx. 75%, 606 µmol, 6 equiv.) and DIPEA (8 equiv., 404 µmol, 35 µL) were added and stirring was continued 48 h at rt. The mixture was washed with $NaHCO_3$ (sat., aq.) and back-extracted with CH_2Cl_2, then washed with brine and back-extracted with CH_2Cl_2. The combined organic phases were then dried (Na_2SO_4) and concentrated. Flash column chromatography (0% → 2.5% → 5% → 6.5% *i*-PrOH/CH_2Cl_2) then gave the product (15 mg, 51%) as a yellow amorphous solid. HPLC-analysis (analytical Kinetex-C18 column, 4.6x25 mm, ACN/H_2O gradient, 1.0 mL/min) R_f 0.48 and 0.63 (15% *i*PrOH/CH_2Cl_2, UV and CAM stain); HRMS (ESI): Calc. $C_{33}H_{35}N_3O_5SNa^+$: 608.2190; found: 608.2194 $[M + Na]^+$. Mixture of diastereoisomers. ^{1}H NMR (400 MHz, acetone-d_6) δ 8.02 (s, 1H), 7.43 – 7.38 (m, 6H), 7.32 (t, J = 7.6 Hz, 6H), 7.24 (t, J = 7.2 Hz, 3H), 6.59 (d, J = 15.3 Hz, 0.5H), 6.49 (d, J = 15.3 Hz, 0.5H), 6.14 (d, J = 15.2 Hz, 0.5H), 6.07 (d, J = 15.1 Hz, 0.5H), 4.66 – 3.72 (m, 8H), 2.93 (s, 3H), 2.81 – 2.62 (m, 1.5H), 2.52 (dd, J = 12.3, 8.3 Hz, 0.5H), 2.39 (dd, J = 12.9, 5.6 Hz, 0.5H), 2.34 (dd, J = 12.6, 4.6 Hz, 0.5H), 1.20 (d, J = 7.2 Hz, 1.5H), 1.08 (d, J = 6.9 Hz, 1.5H). ^{13}C NMR (100 MHz, acetone-d_6) δ 173.8, 170.0, 169.8, 169.7, 169.5, 167.0, 145.7, 145.6, 144.5, 143.6, 130.4, 128.8, 127.7, 121.9, 121.3, 67.9, 67.6, 67.5, 53.8, 53.8, 51.5, 50.3, 42.1, 41.9, 40.8, 40.3, 36.5, 35.8, 35.5, 15.0, 13.9. IR (neat) ν_{max} /cm^{-1} 3287, 3058, 2930, 1737, 1633, 1538, 742, 699.



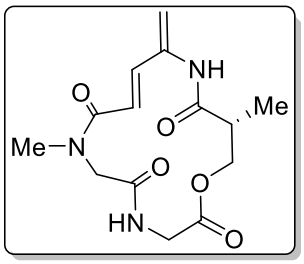
(14R,E)-7,14-Dimethyl-11-(((3-nitropyridin-2-yl)disulfanyl)methyl)-1-oxa-4,7,12-triazacyclopentadec-9-ene-2,5,8,13-tetraone (19)



Macrocycle **18a** (5.3 mg, 9 µmol, 1 equiv.) was dissolved in anhydrous CH_2Cl_2 (0.3 mL) under Ar and cooled to -30 ^oC before Npys-Cl (2 equiv., 18 µmol, 3.4 mg) was added and the mixture was stirred for 18 h. The mixture was allowed to reach room temperature and was directly loaded on a flash column and eluted with (0% → 5% → 20% *i*-PrOH/EtOAc) to furnish the product (3.4 mg, 76%) as a yellow amorphous solid. The product was directly advanced to the next step.

R_f 0.50 and 0.65 (10% *i*PrOH/CH_2Cl_2, UV and $KMnO_4$ stain); Mixture of diastereoisomers. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.91-8.86 (m, 1H), 8.58-8.50 (m, 1H), 7.67 – 7.60 (br s, 0.5H), 7.56 – 7.51 (br s, 0.5H), 7.46 (dd, *J* = 8.2 and 4.6 Hz, 0.5H), 7.41 (dd, *J* = 8.2 and 4.6 Hz, 0.5H), 7.34 (d, *J* = 7.8 Hz, 0.5H), 6.82 (dd, *J* = 15.1 and 3.1 Hz, 0.5H), 6.75 (dd, *J* = 15.2 and 3.1 Hz, 0.5H), 6.71 (d, *J* = 7.6 Hz, 0.5H), 6.20 (dd, *J* = 15.0 and 2.0 Hz, 0.5H), 6.11 (dd, *J* = 15.1 and 2.2 Hz, 0.5H), 4.55 – 4.45 (m, 0.5H), 4.32 – 3.66 (m, 7H), 3.38 – 3.28 (m, 0.5H), 3.22 – 3.10 (m, 1H), 3.01 (s, 3H), 2.80 – 2.71 (m, 0.5H), 2.67 – 2.57 (m, 0.5H), 1.32 (d, *J* = 7.2 Hz, 1.5H), 1.15 (d, *J* = 7.0 Hz, 1.5H).

Rakicidin Macrocycle (1) ((R,E)-7,14-dimethyl-11-methylene-1-oxa-4,7,12-triazacyclo-pentadec-9-ene-2,5,8,13-tetraone)[6]

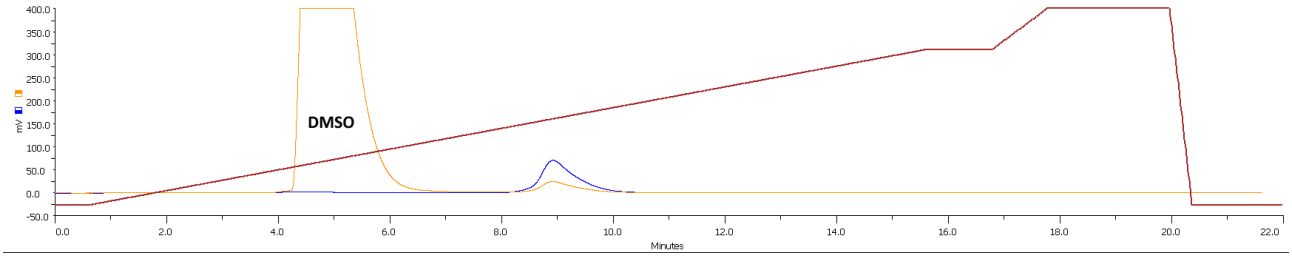


The Npys-disulfide **19** (8 mg, 16.1 µmol, 1 equiv.) was dissolved in anhydrous CH_2Cl_2 (1 mL) and DBU (8 equiv., 0.123 mmol, 24 µL) was added at ambient temperature. After 1 h of stirring TLC analysis showed full conversion. The mixture was directly loaded onto a prep-TLC plate and developed twice eluting with 30% *i*PrOH/EtOAc. The part of silica containing the product was scrapped off the plate into a beaker and was dissolved in 10% *i*PrOH/DCM. Filtration through a short pad of sand and concentration* gave the desired final product (3.3 mg, 67%) as a white solid. (*NOTE: All compounds comprising the sensitive mc-APD functionality have been observed at times to decompose during concentration under reduced pressure in organic solvents. Therefore, before concentration, DMSO (1 mL) was added

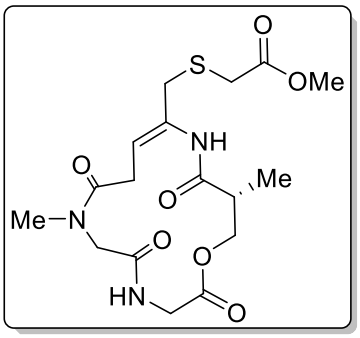
to the solution, the volatile solvents were removed at the rotavap and the remaining solution was freeze-dried to obtain the pure final product).

The analytic data of product **1** was identical to that reported from our 1st generation synthesis.[6]

HPLC-analysis (analytical Kinetex-C18 column, 4.6x25 mm, ACN/H_2O gradient, 1.0 mL/min)



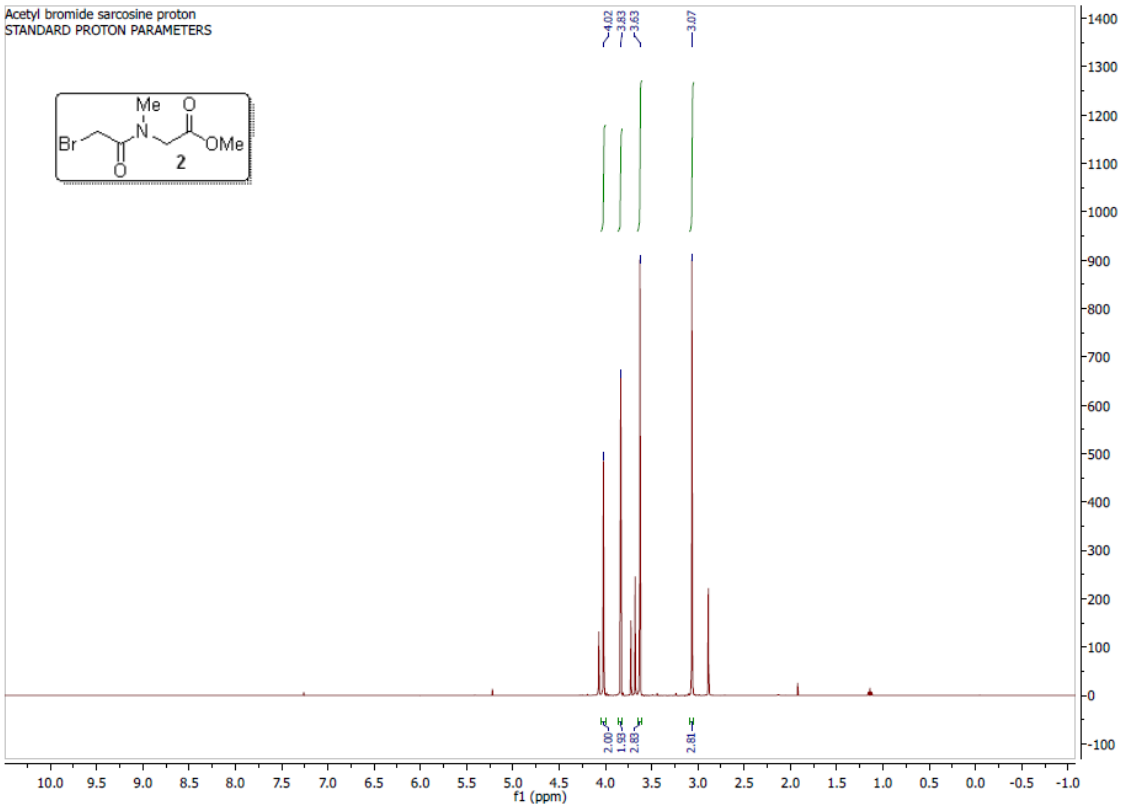
Methyl 2-((((14R,E)-7,14-dimethyl-2,5,8,13-tetraoxo-1-oxa-4,7,12-triazacyclopenta-dec-9-en-11-yl)methyl)thio)acetate (20)



A solution of rakicidin macrocycle **1** (2.3 mg, 7.4 µmol) and methyl thioglycolate (10.0 equiv., 6.6 µL, 74.3 µmol) in DMSO-d_6 (200 µL) was stirred under Ar at ambient temperature for 72 hours. The solvent and the excess of methyl thioglycolate (bp 42-43 ^{o}C) were removed by lyophilization, to afford the product of purity allowing for full characterization. Further purification was achieved by HPLC (semi-prep C18 column, 10 mL/min, 5% → 80% MeCN/H_2O over 15 min, 210 and 254 nm, R_t = 6.047 min) delivering the product as a white solid.

HRMS (ESI): Calc. $C_{17}H_{25}N_3O_7SNa^+$: 438.1305; found: 438.1308 $[M + Na]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.86 (s, 1H), 8.58 (t, *J* = 6.1 Hz, 1H), 5.47 (dd, *J* = 7.6, 5.7 Hz, 1H), 4.21 (dd, *J* = 10.6, 3.7 Hz, 1H), 4.00 – 3.75 (m, 5H), 3.63 (s, 3H), 3.46 (d, *J* = 13.9 Hz, 1H), 3.27 – 3.20 (m, 3H), 2.90 (dd, *J* = 17.9, 5.6 Hz, 1H), 2.81 – 2.73 (m, 4H), 2.69 – 2.60 (m, 1H), 1.02 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) *δ* 171.7, 170.9, 170.4, 169.4, 168.7, 129.7, 119.5, 66.4, 52.3, 52.0, 51.9, 41.4, 36.2, 35.3, 32.5, 31.2, 13.8.

Spectra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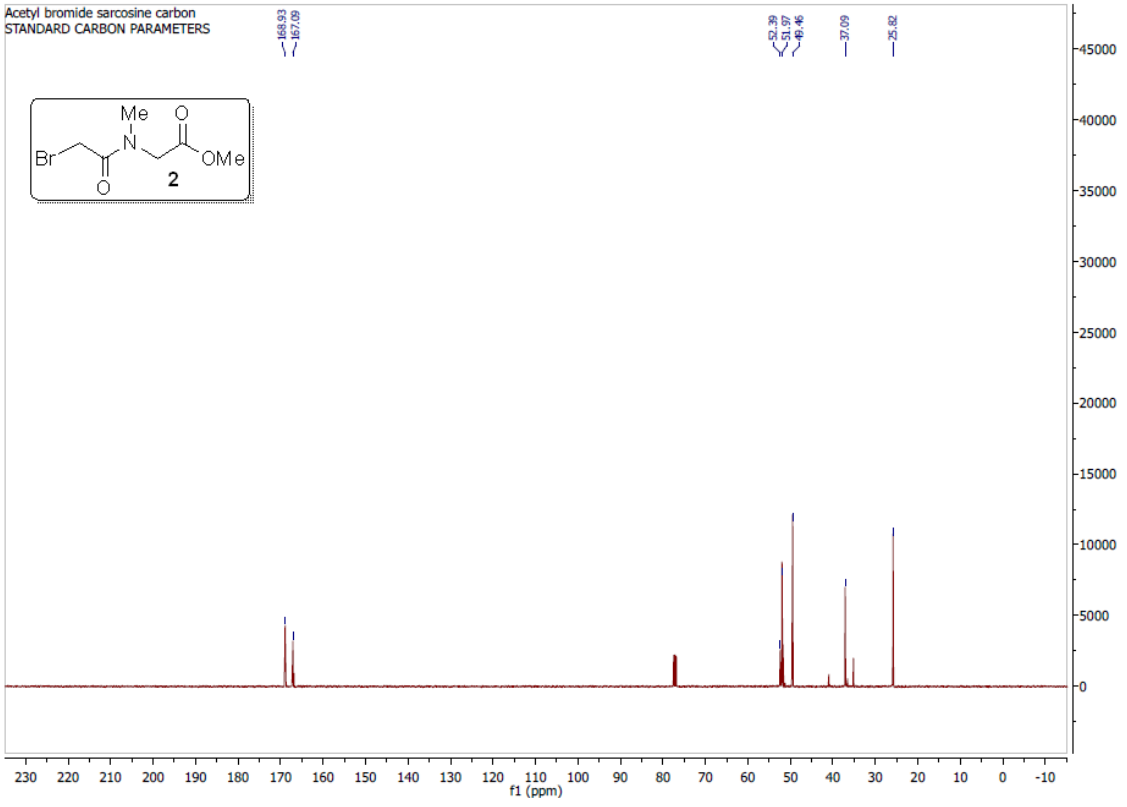
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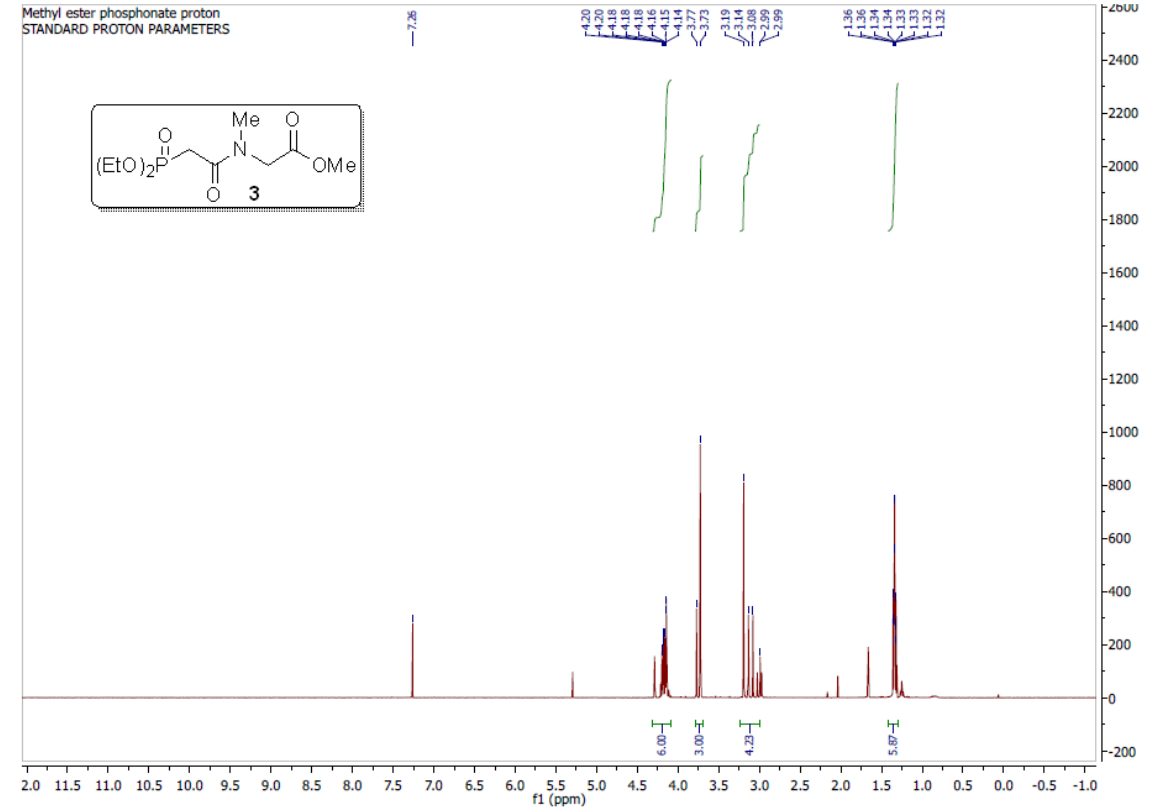
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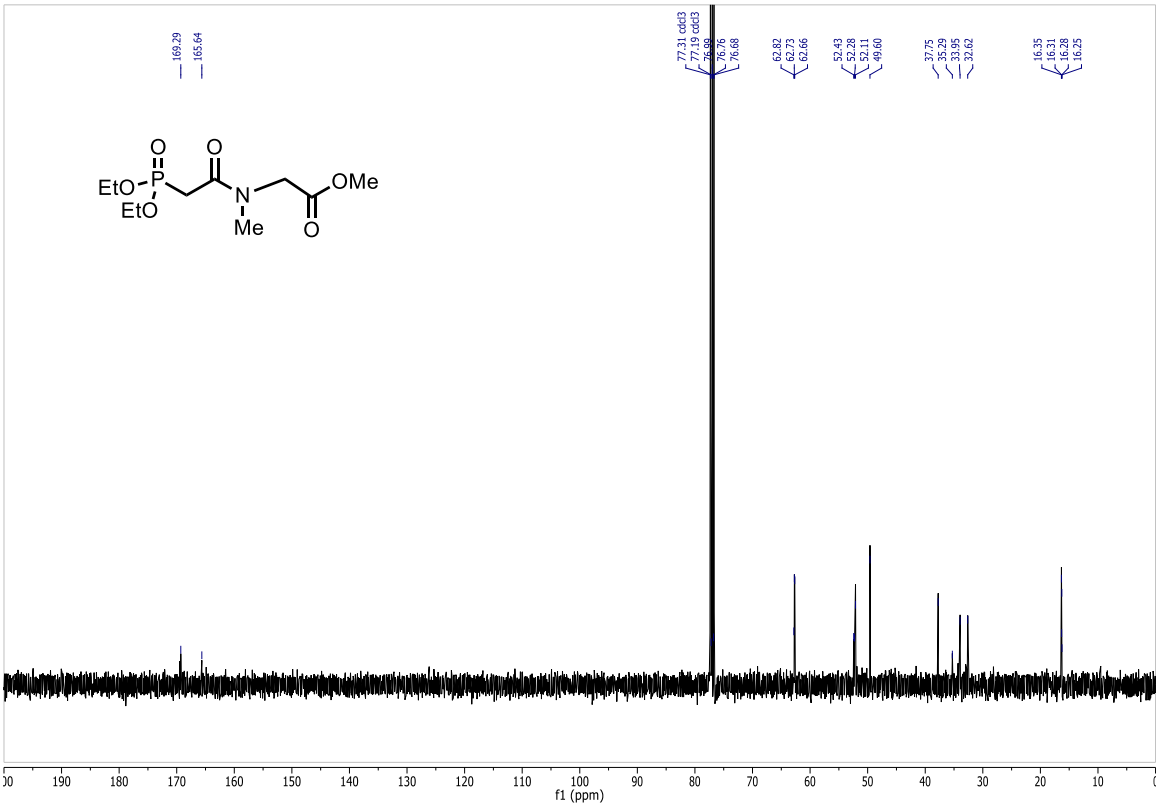
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EtO
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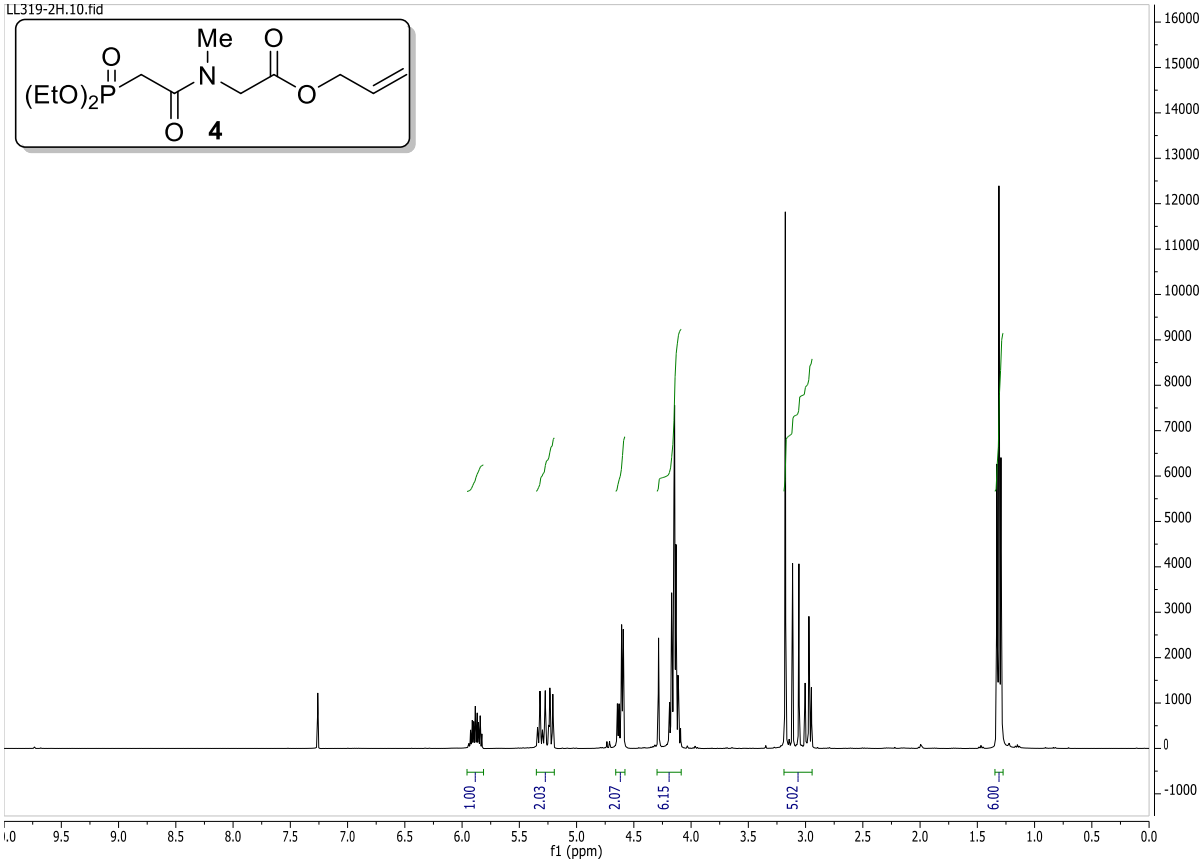
f1 (ppm)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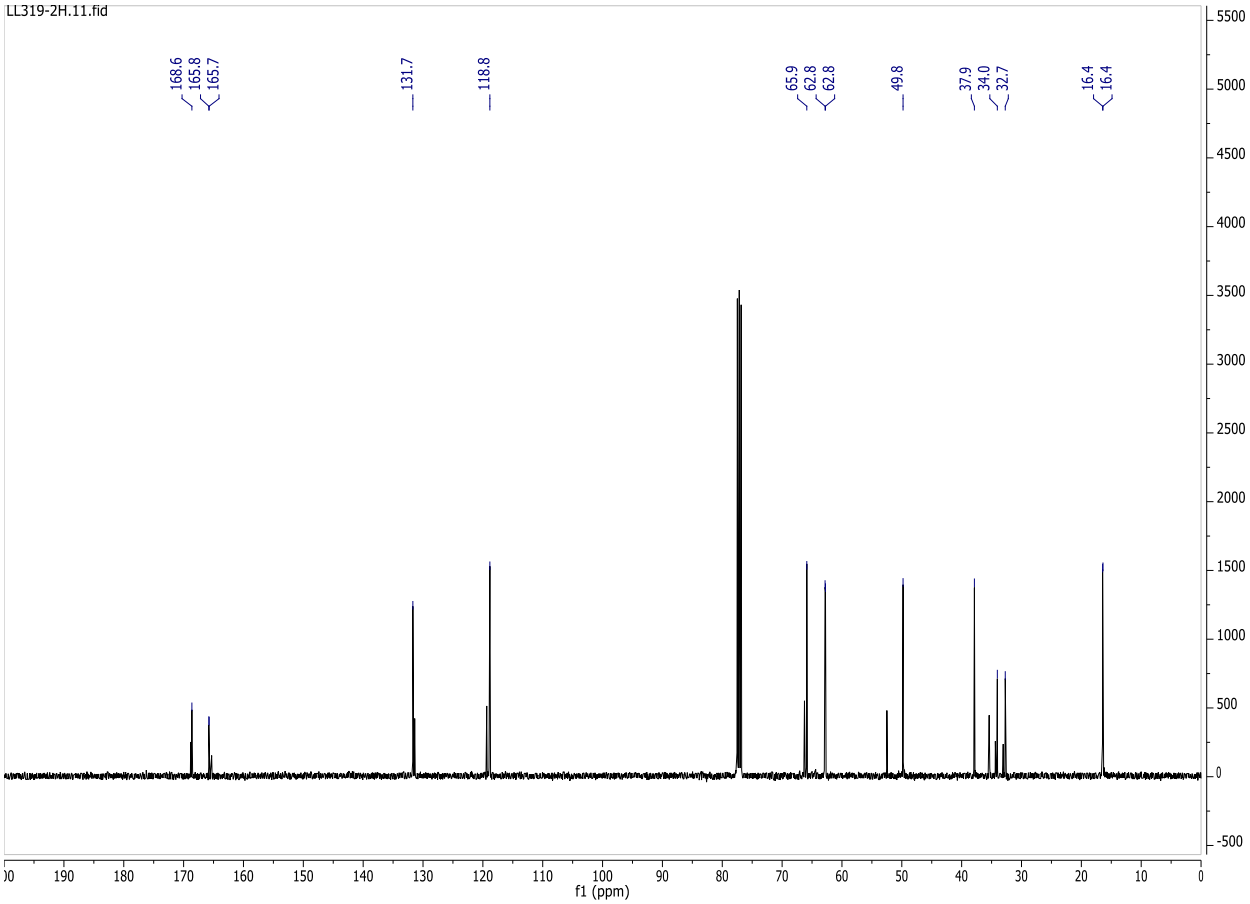
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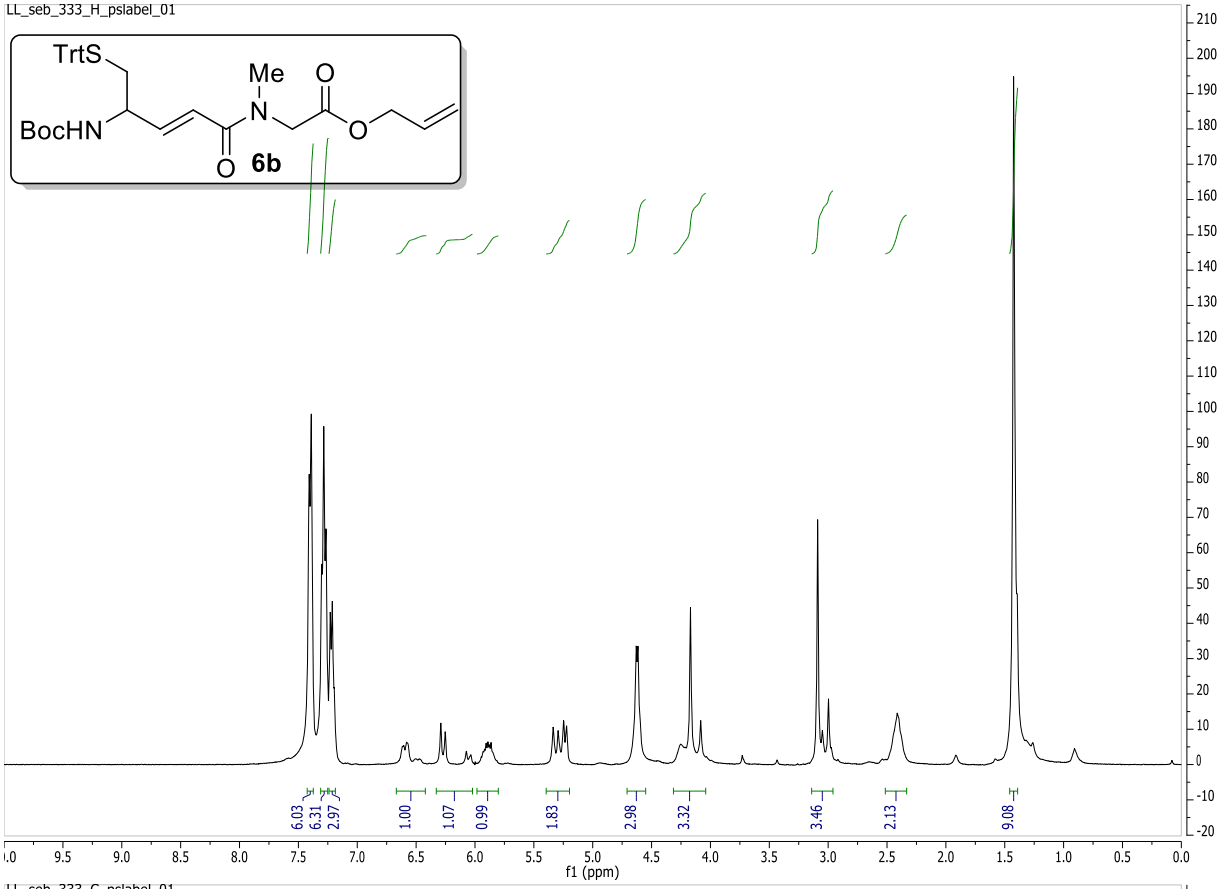
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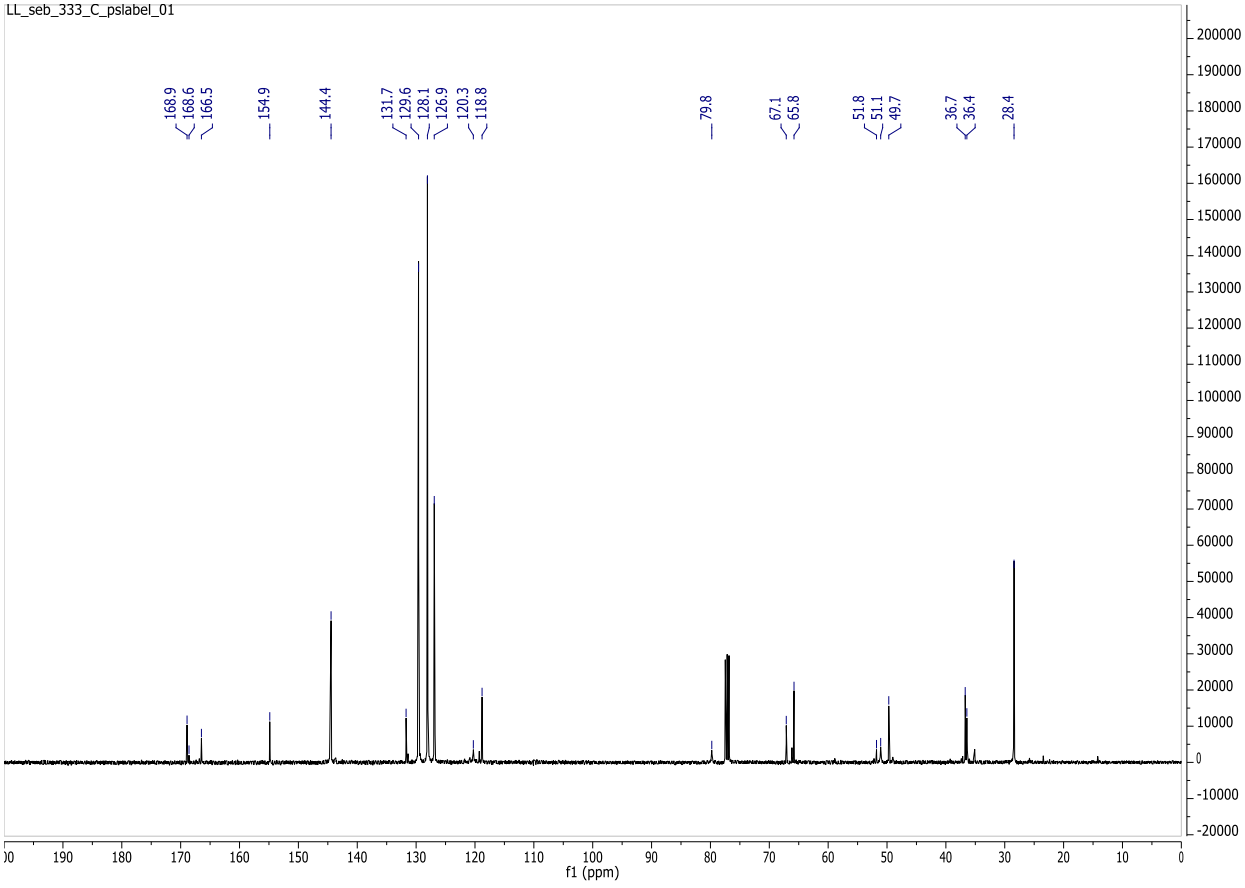
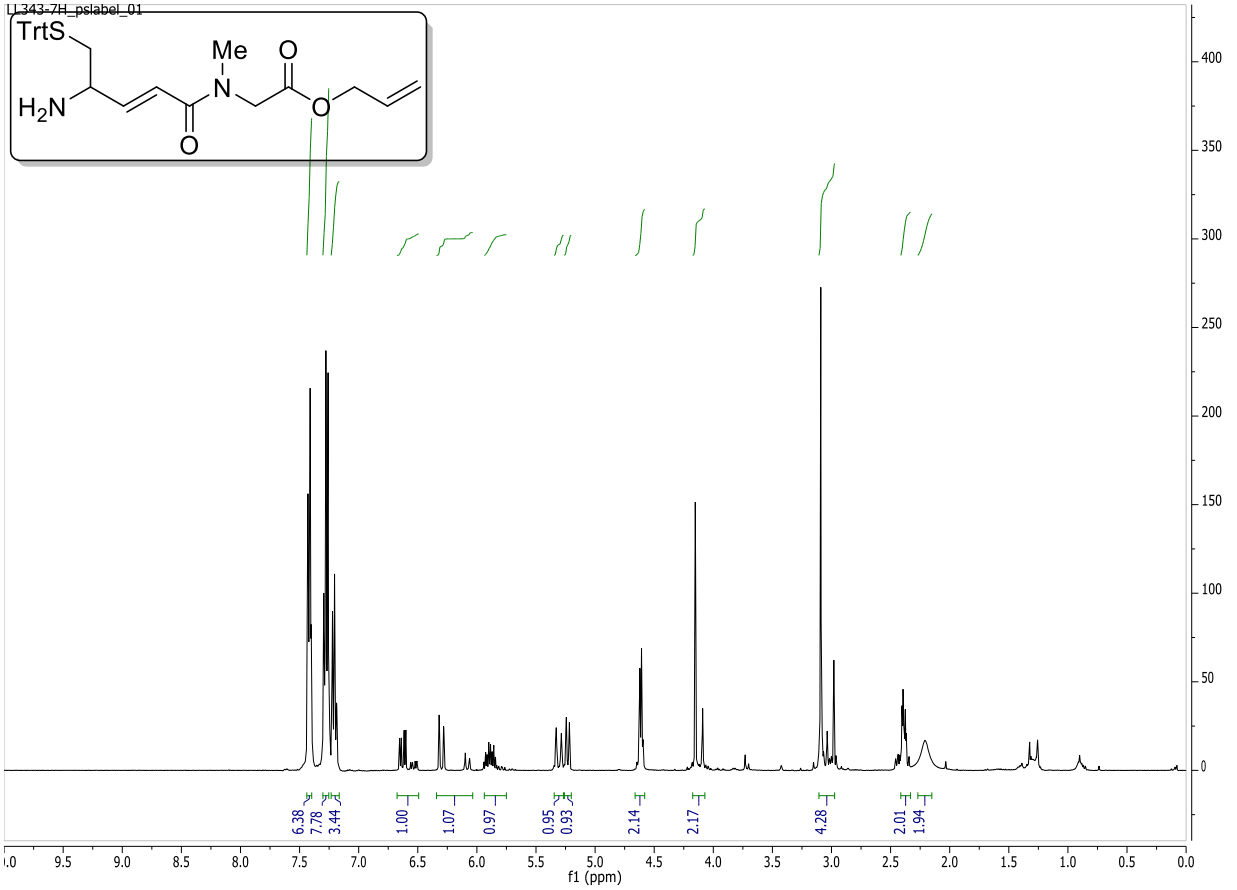
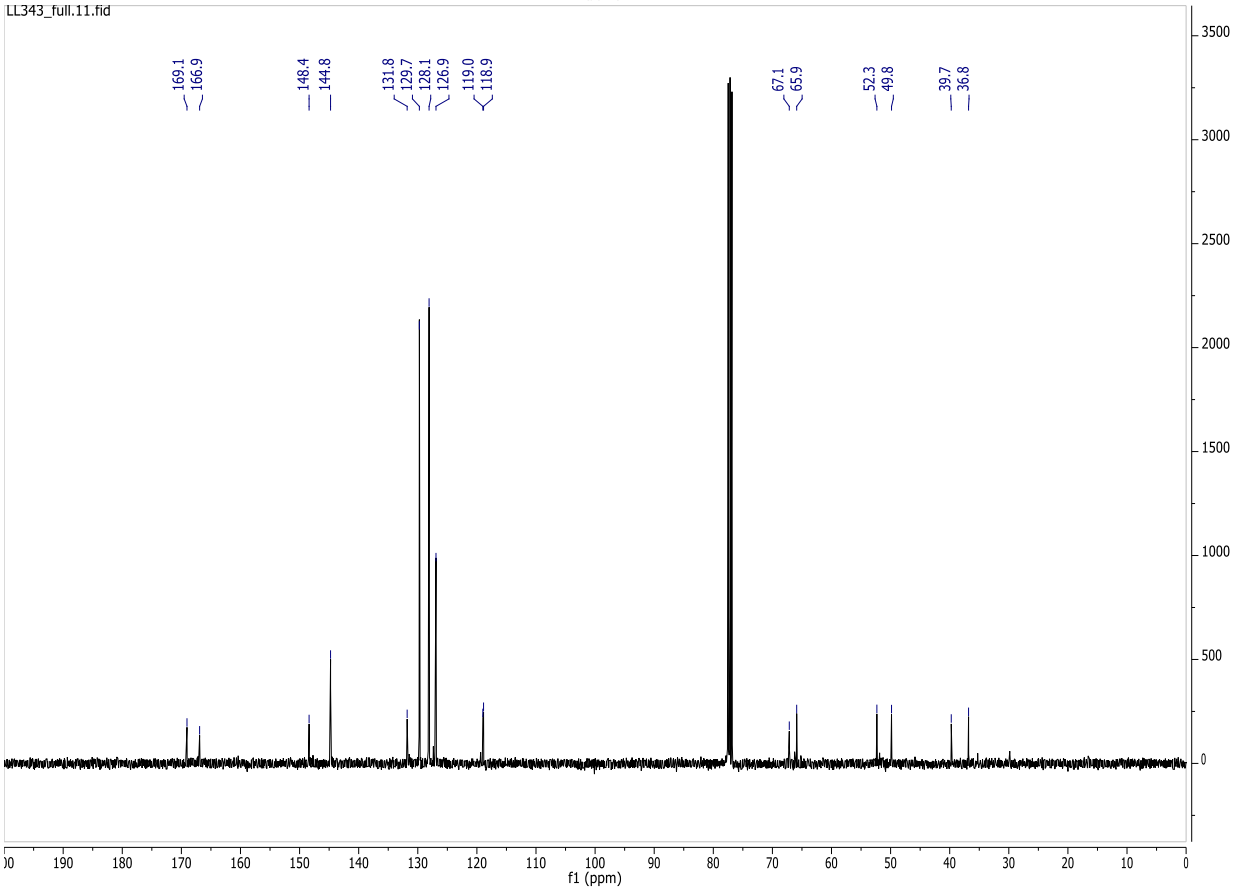
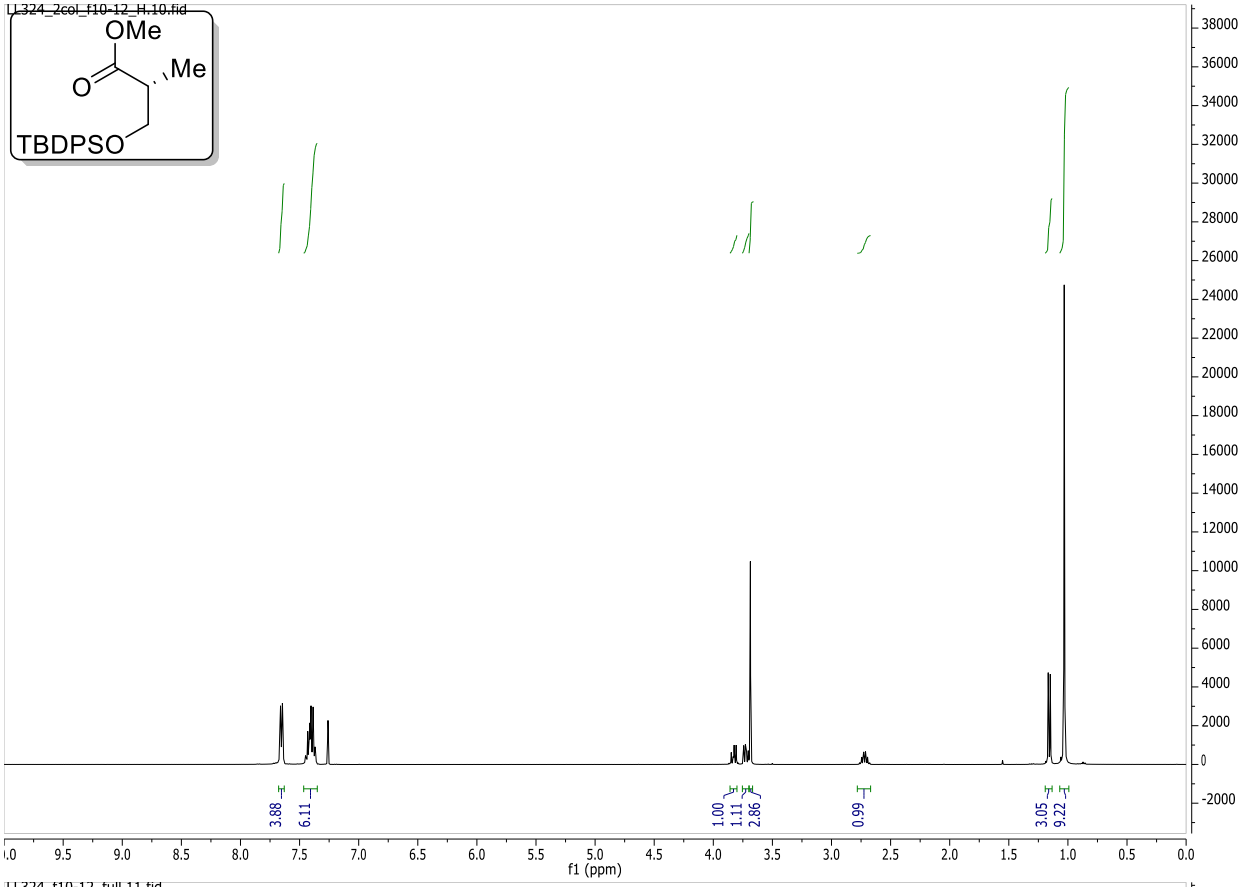
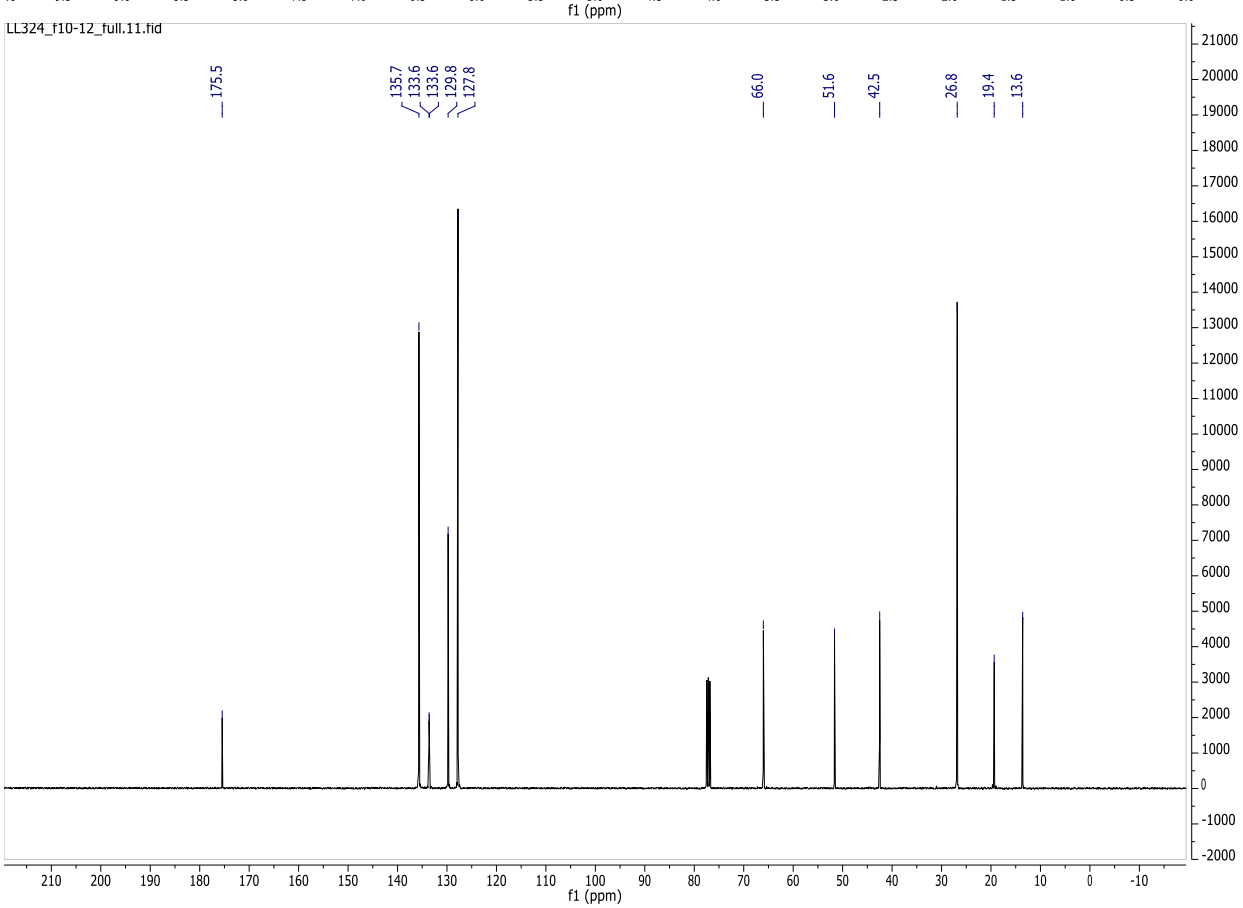
0.99
1.83
2.98
3.32
3.46
2.13
9.08
f1 (ppm)

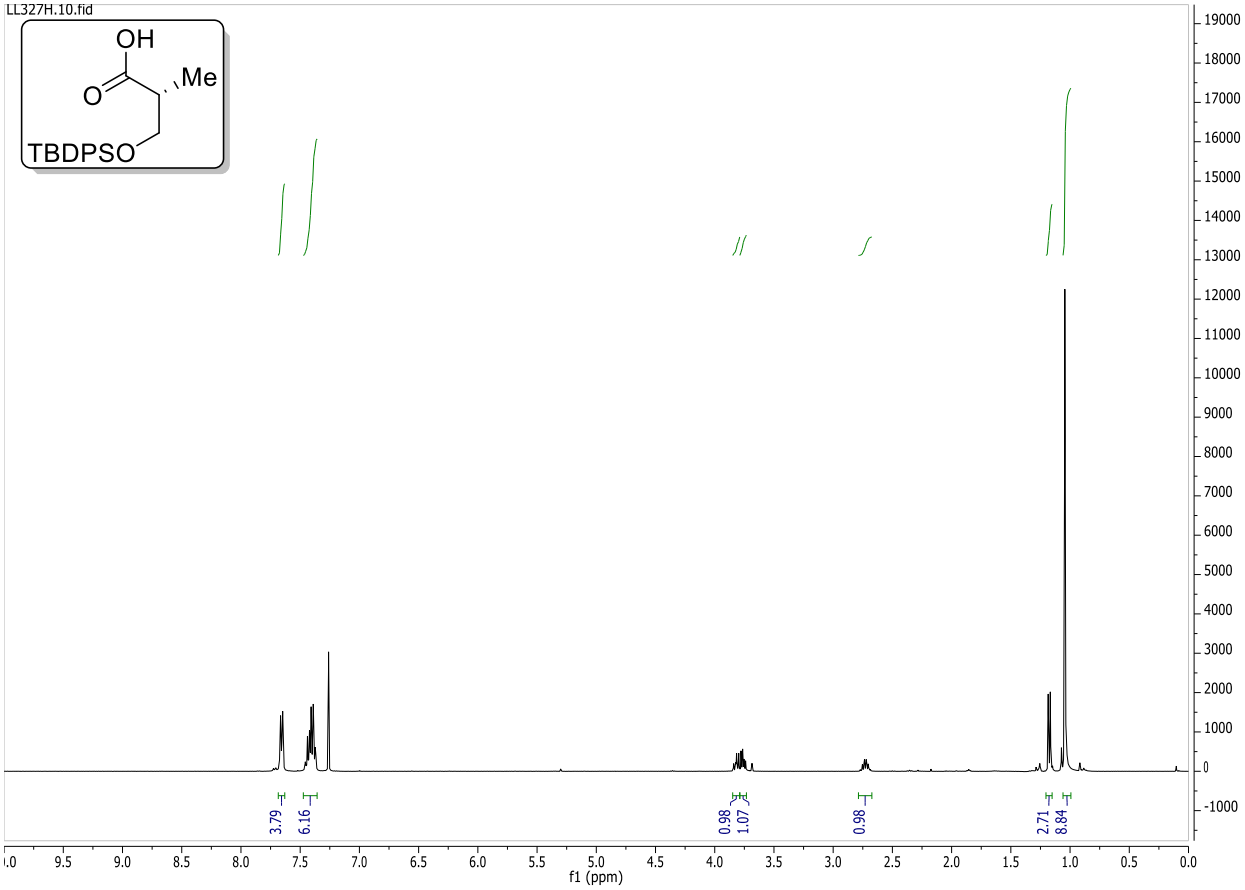

LL_seb_333_C_pslabel_01
168.9
168.6
166.5
154.9
144.4
131.7
129.6
128.1
126.9
120.3
118.8
79.8
67.1
65.8
51.8
51.1
49.7
36.7
36.4
28.4
f1 (ppm)

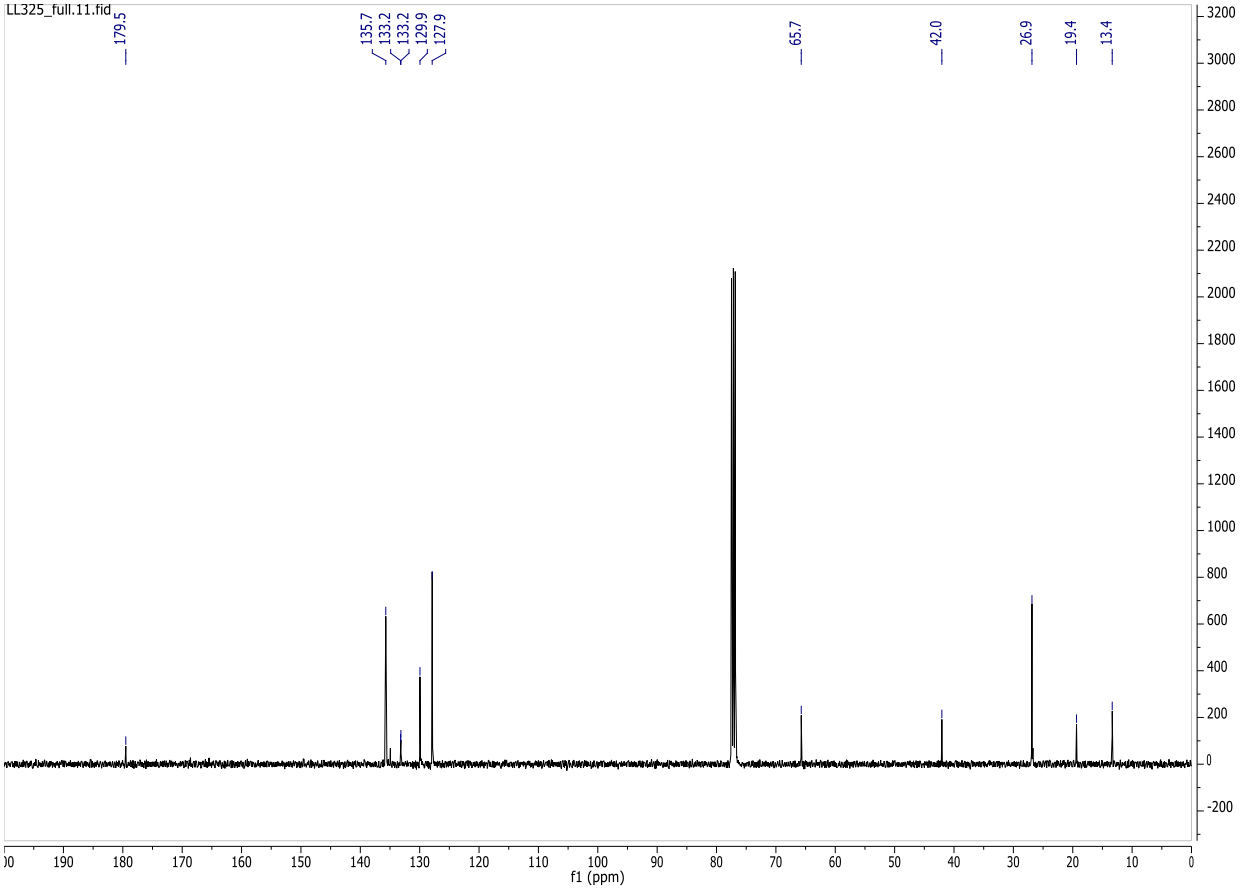

LL343-7H_pslabel_01
TrtS
Me
O
N
H_2N
O
O
6.38
7.78
3.44
1.00
1.07
0.97
0.95
0.93
2.14
2.17
4.28
2.01
1.94
f1 (ppm)

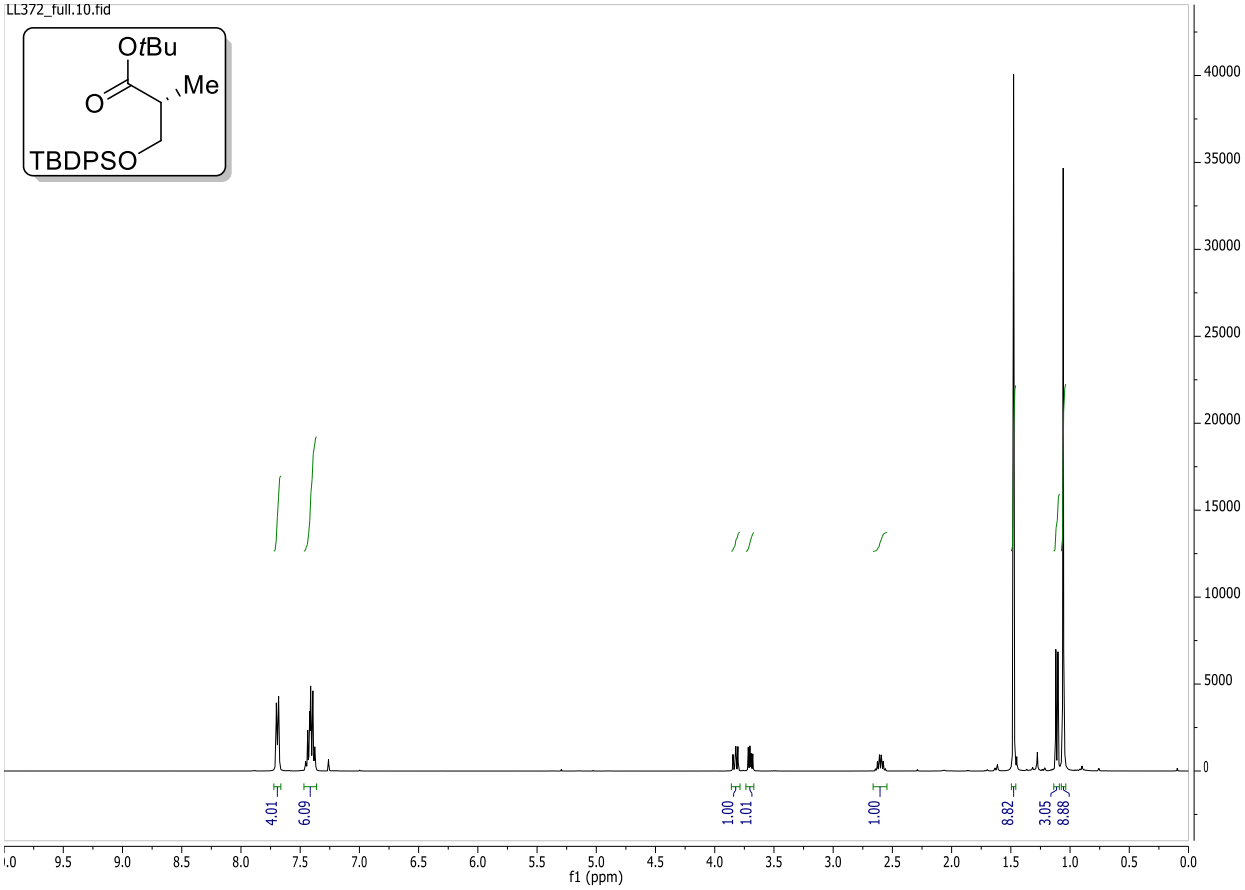

LL343_full.11.fid
169.1
166.9
148.4
144.8
131.8
129.7
128.1
126.9
119.0
118.9
67.1
65.9
52.3
49.8
39.7
36.8
f1 (ppm)

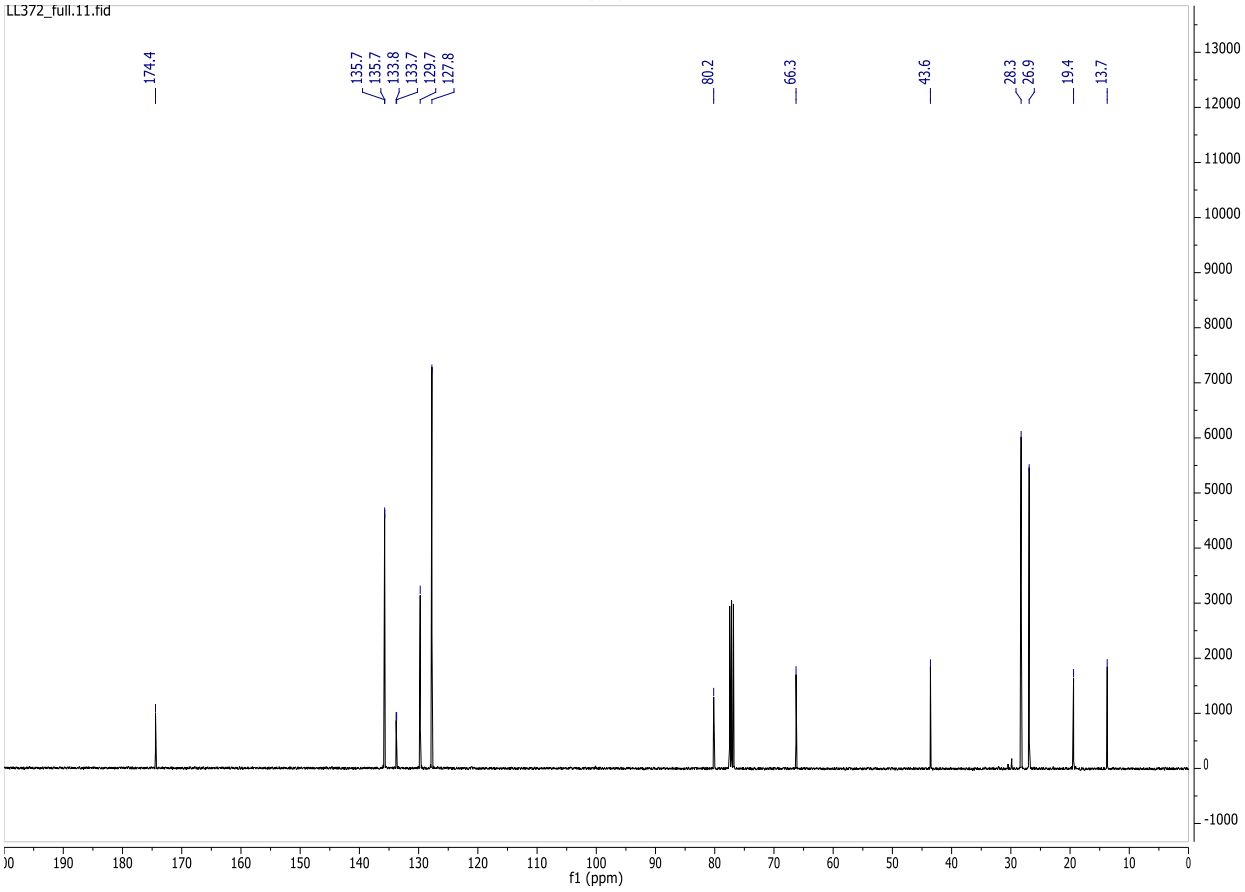

LL324_2col_f10-12_H.10.fid
OMe
Me
O
TBDPSO
3.88
6.11
1.00
1.11
2.86
0.99
3.05
9.22
f1 (ppm)

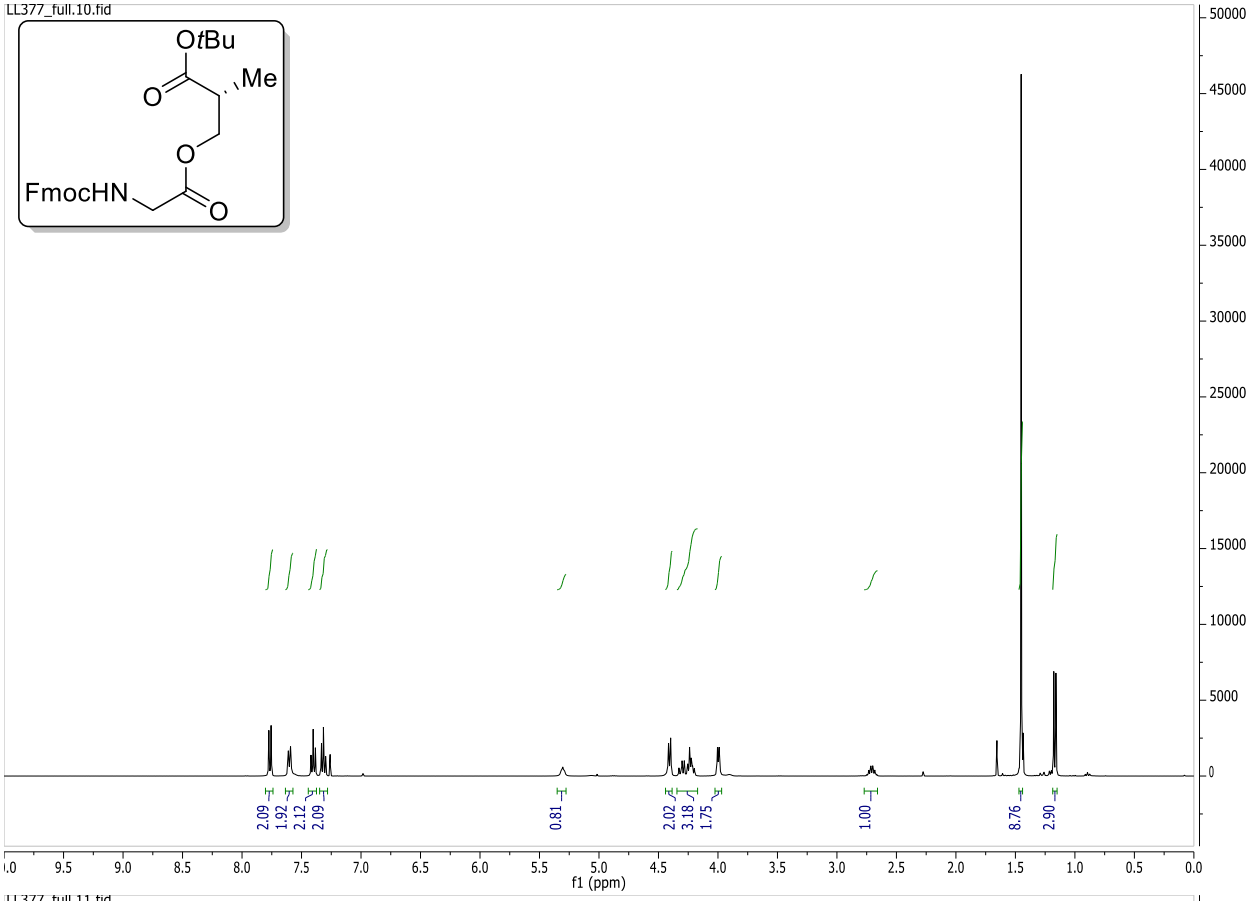

LL324_f10-12_full.11.fid
175.5
135.7
133.6
133.6
129.8
127.8
66.0
51.6
42.5
26.8
19.4
13.6
f1 (ppm)

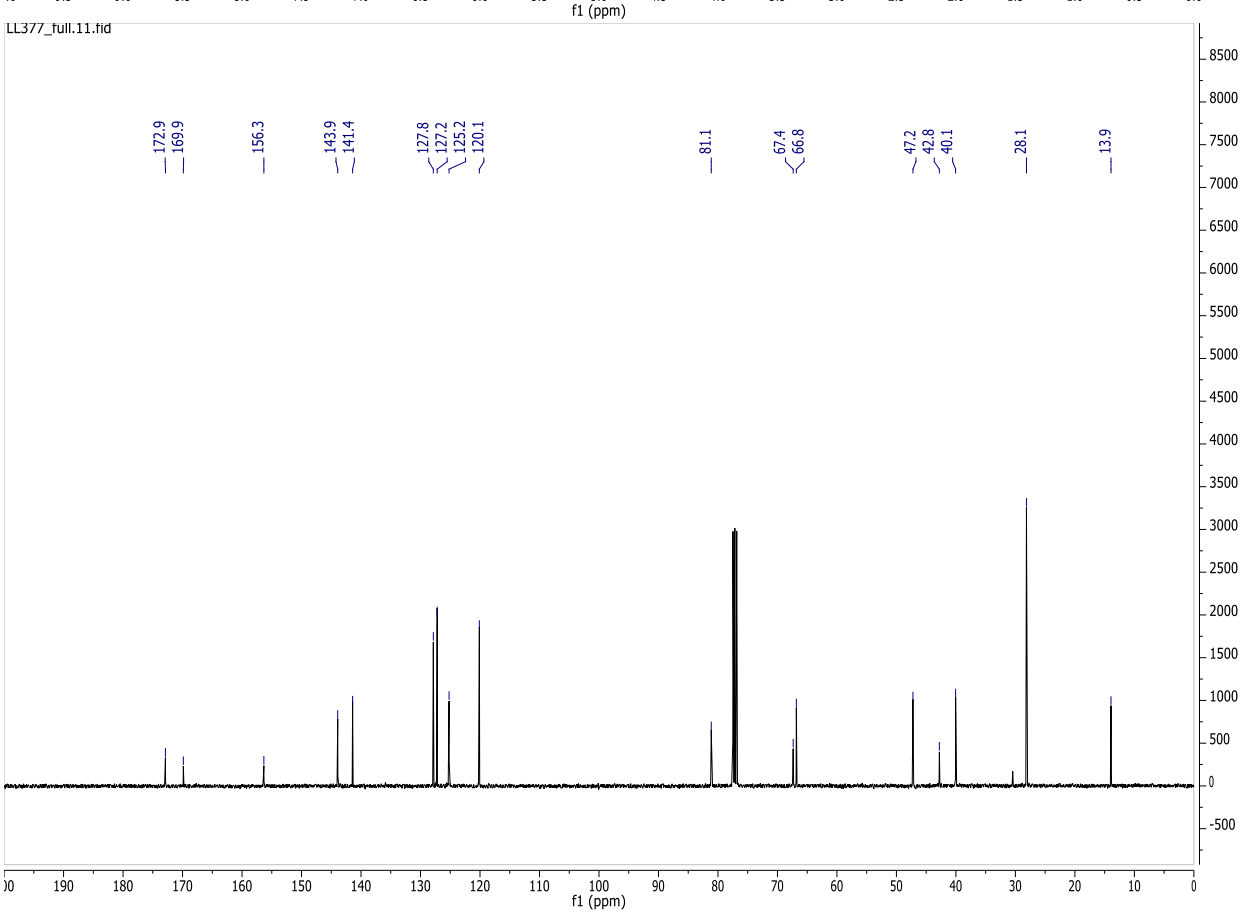

LL327H.10.fid
OH
O
Me
TBDPSO
3.79
6.16
0.98
1.07
0.98
2.71
8.84
f1 (ppm)

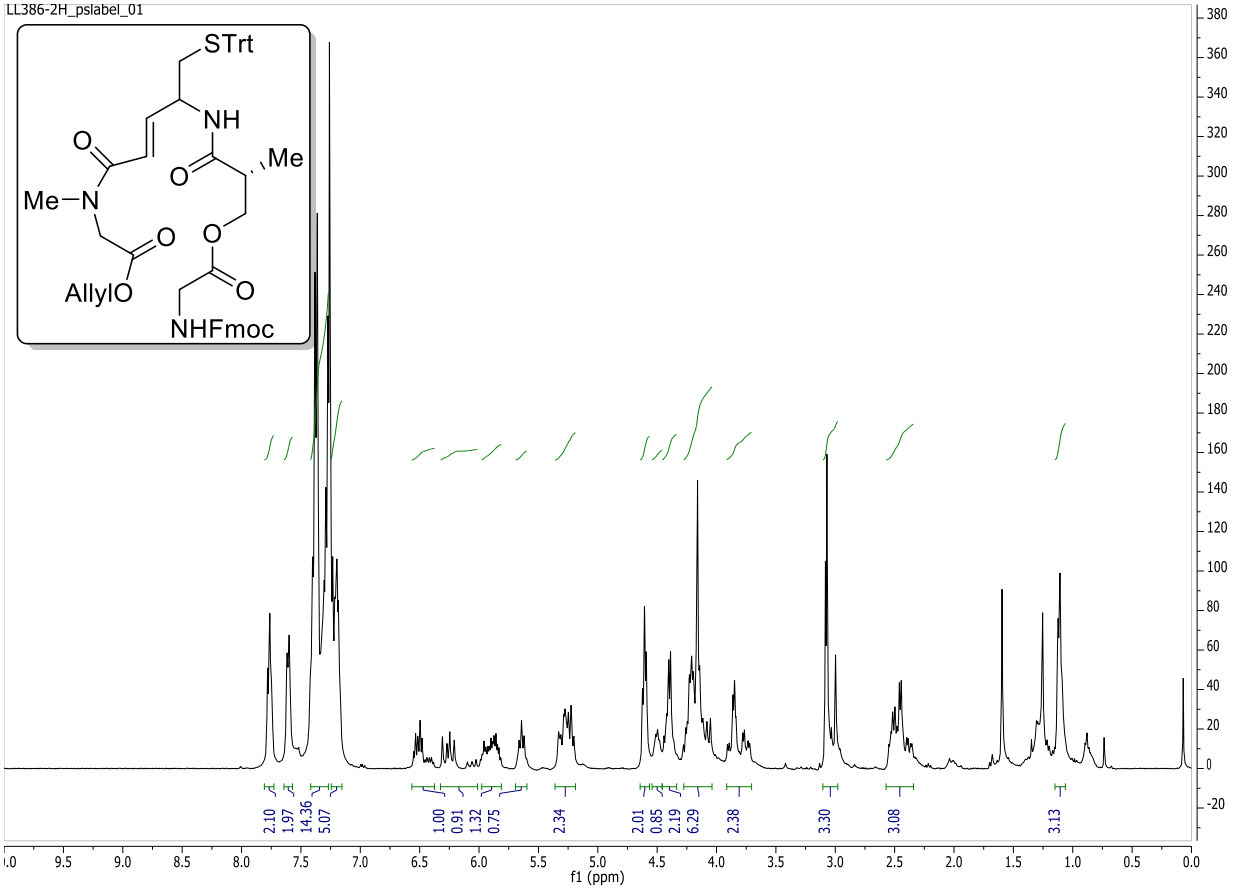

LL325_full.11.fid
179.5
135.7
133.2
133.2
129.9
127.9
65.7
42.0
26.9
19.4
13.4
f1 (ppm)

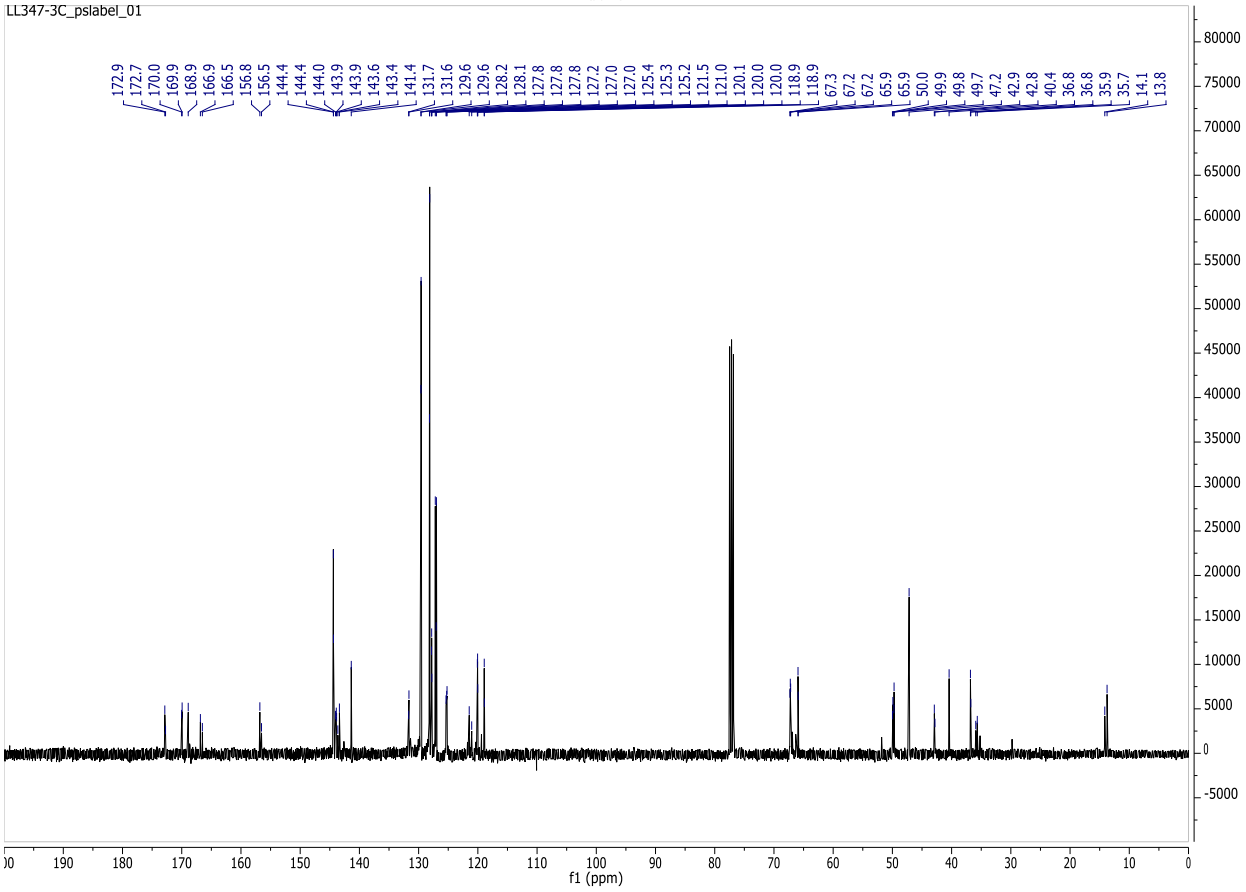

LL372_full.10.fid
OtBu
Me
O
TBDPSO
4.01
6.09
1.00
1.01
1.00
8.82
3.05
8.88
f1 (ppm)

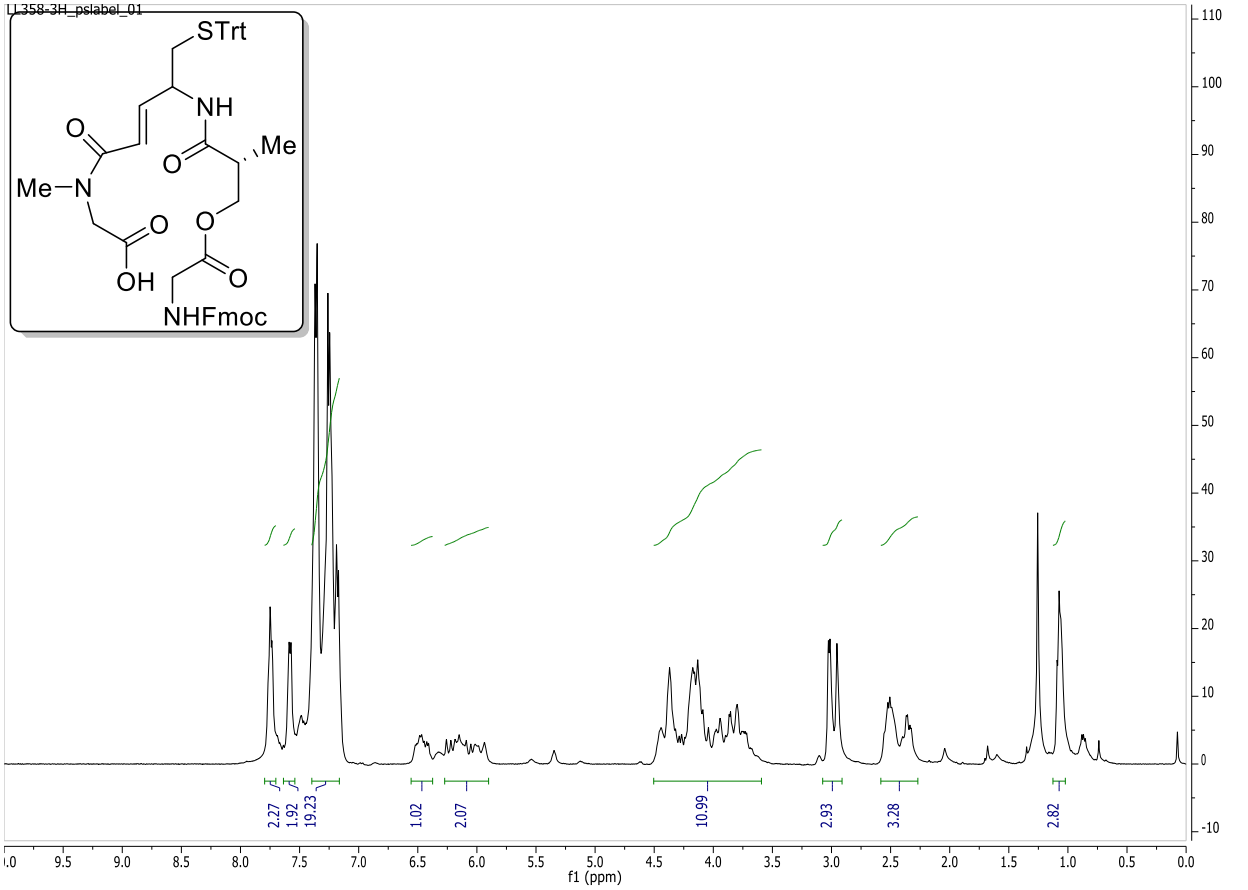

LL372_full.11.fid
174.4
135.7
135.7
133.8
133.7
129.7
127.8
80.2
66.3
43.6
28.3
26.9
19.4
13.7
f1 (ppm)

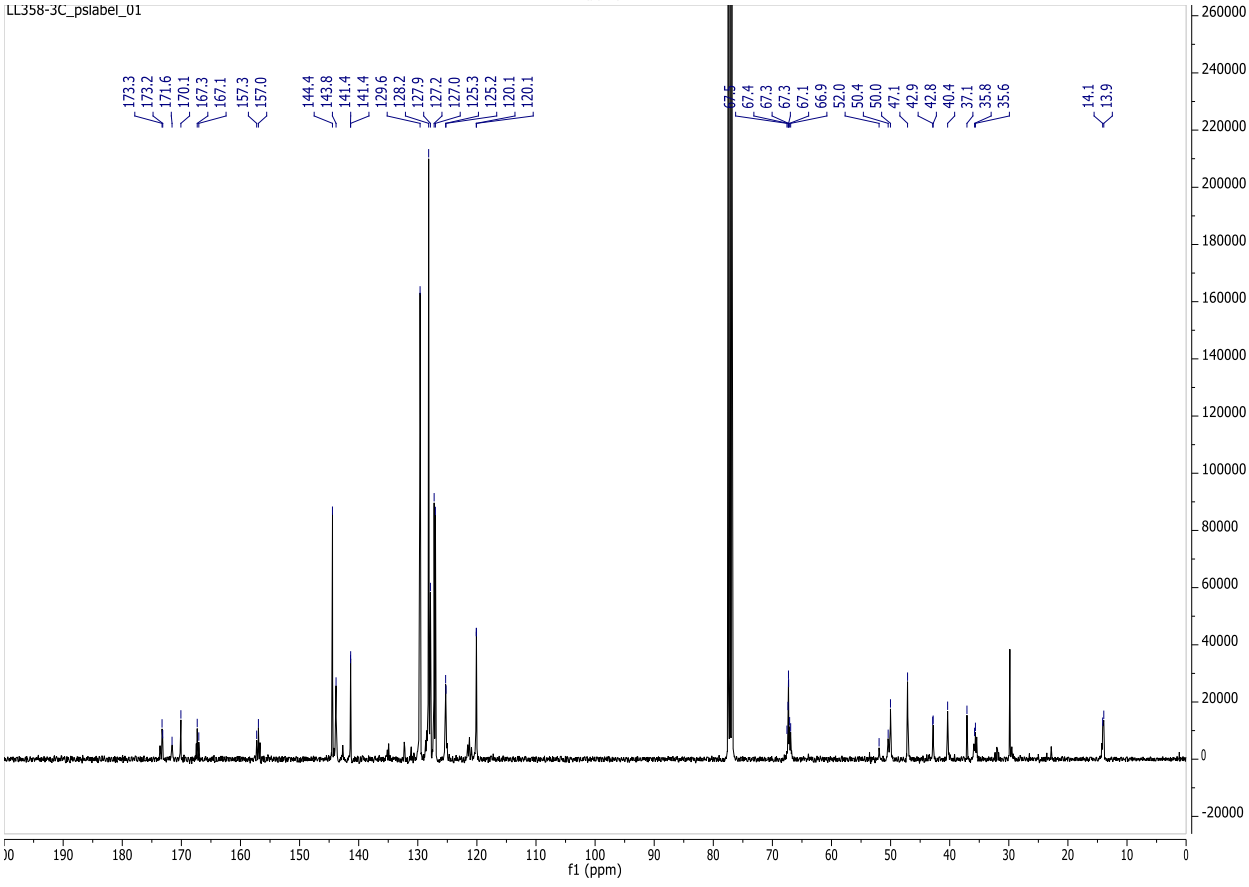

LL377_full.10.fid
OtBu
Me
O
O
FmocHN
O
2.09
1.92
2.12
2.09
0.81
2.02
3.18
1.75
1.00
8.76
2.90
f1 (ppm)

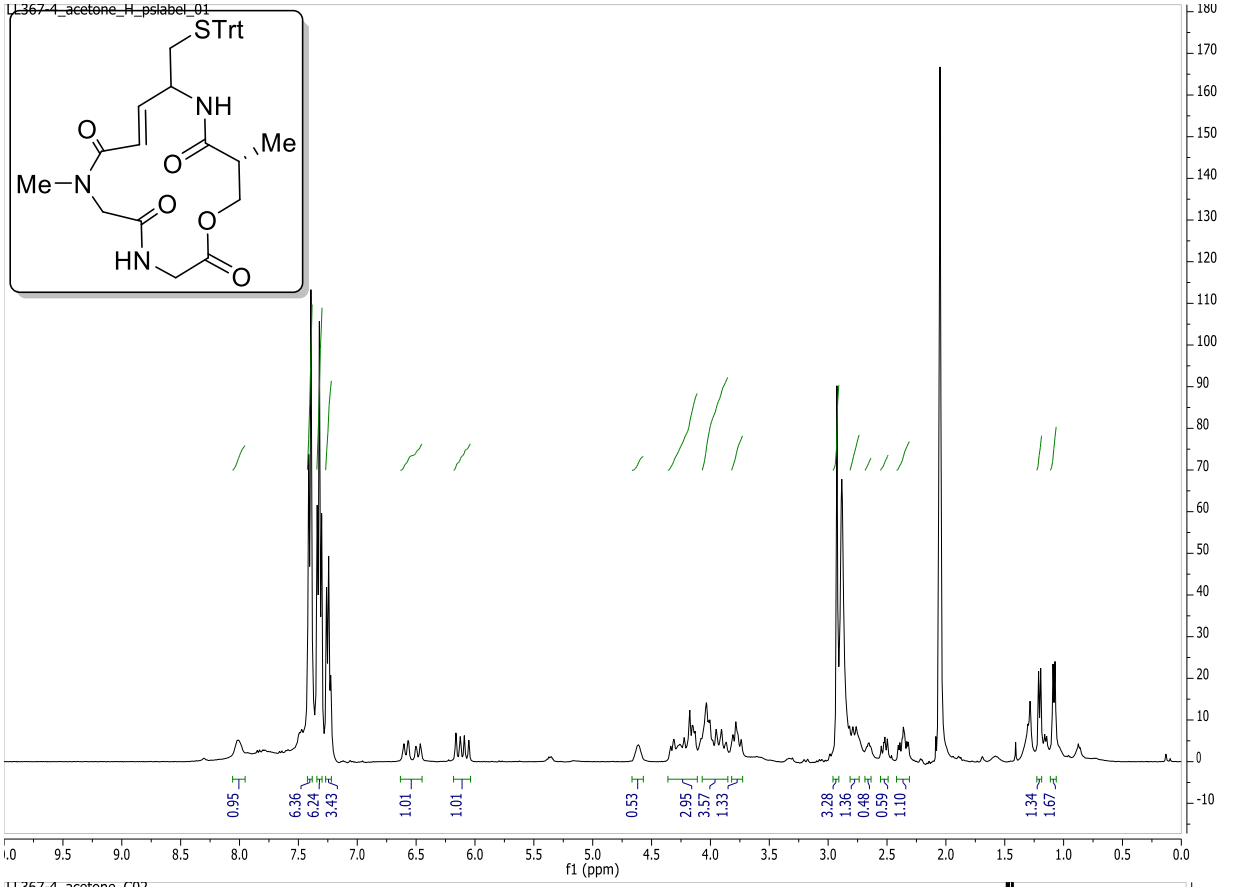

LL377_full.11.fid
172.9
169.9
156.3
143.9
141.4
127.8
127.2
125.2
120.1
81.1
67.4
66.8
47.2
42.8
40.1
28.1
13.9
f1 (ppm)

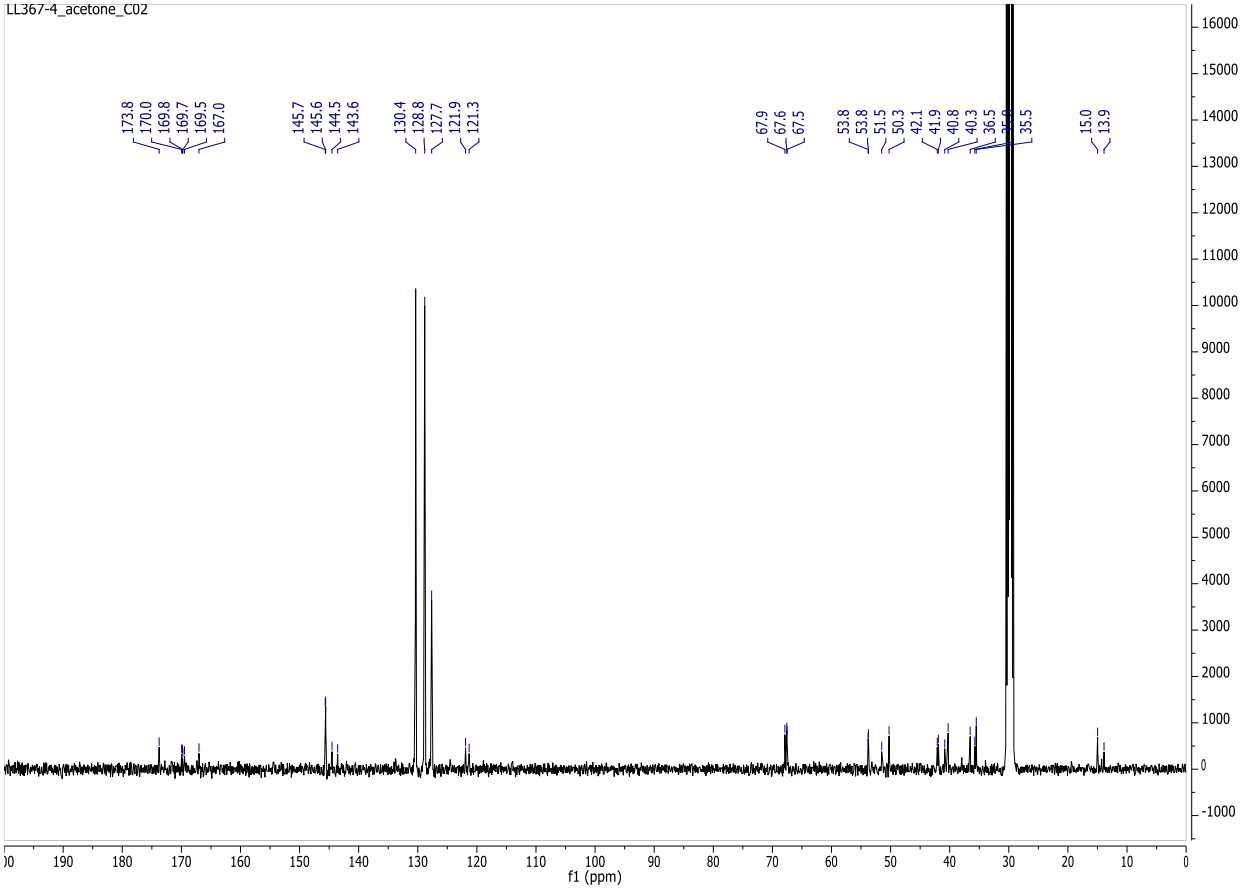

LL386-2H_pslabel_01
STrt
NH
O
O
Me
Me—N
O
O
O
AllylO
O
NHFmoc
2.10
1.97
14.36
5.07
1.00
0.91
1.32
0.75
2.34
2.01
0.85
2.19
6.29
2.38
3.30
3.08
3.13
f1 (ppm)

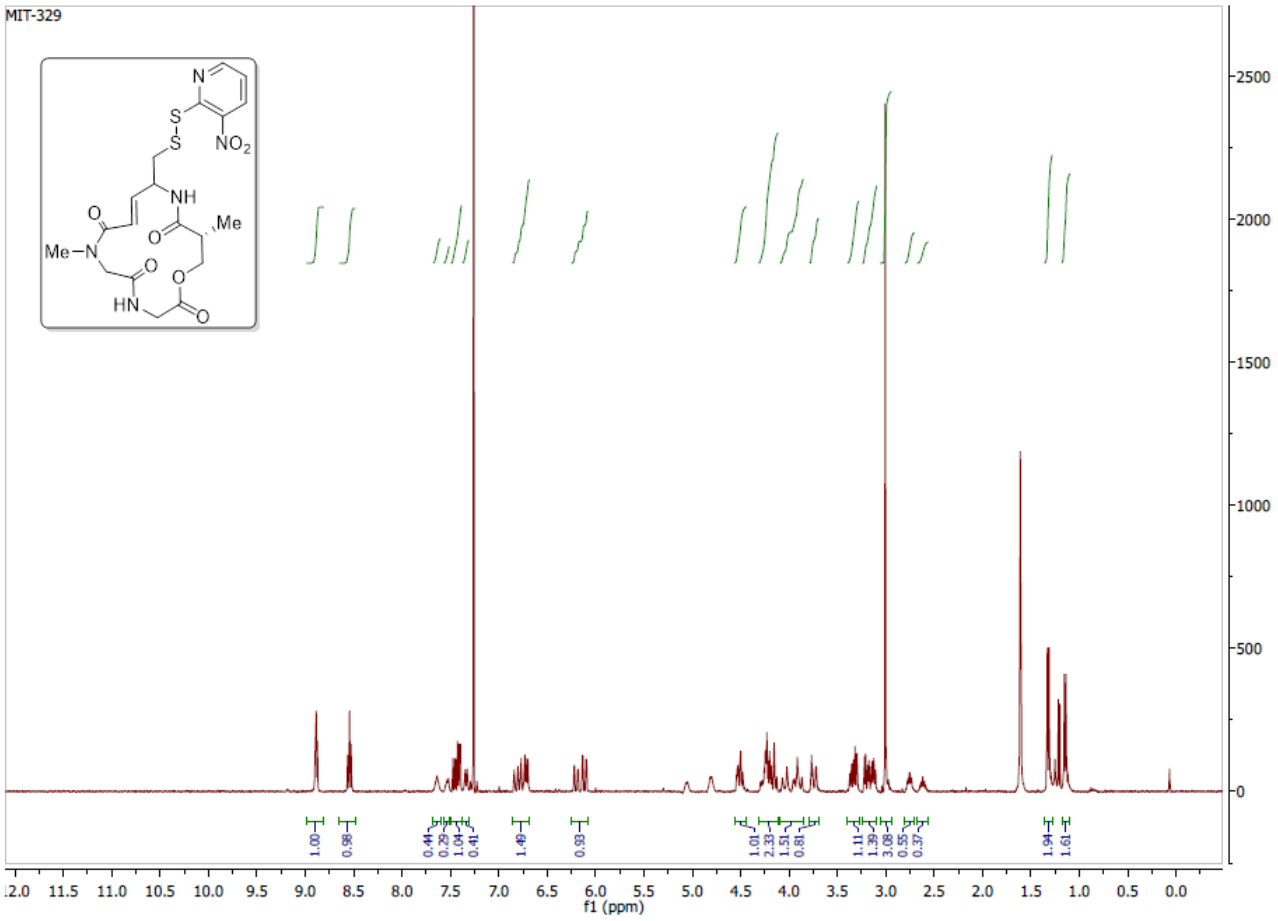

LL347-3C_pslabel_01
172.9
172.7
170.0
169.9
168.9
166.9
166.5
156.8
156.5
144.4
144.4
144.0
143.9
143.9
143.6
143.4
141.4
131.7
131.6
129.6
129.6
128.2
128.1
127.8
127.8
127.8
127.2
127.0
127.0
125.4
125.3
125.2
121.5
121.0
120.1
120.0
120.0
118.9
118.9
67.3
67.2
67.2
65.9
65.9
50.0
49.9
49.8
49.7
47.2
42.9
42.8
40.4
36.8
36.8
35.9
35.7
14.1
13.8
f1 (ppm)

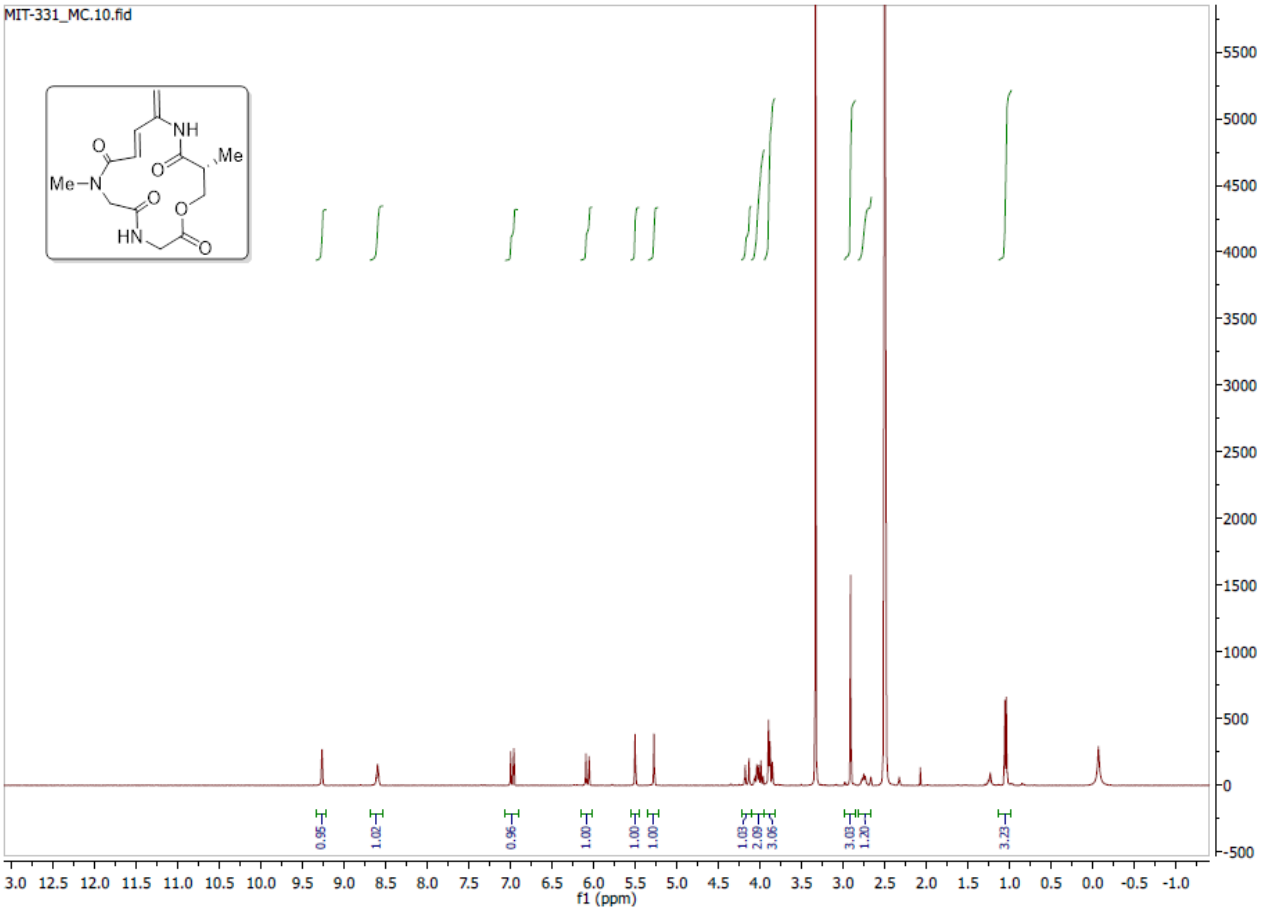

LL358-3H_pslabel_01
STrt
NH
O
O
Me
Me—N
O
O
O
OH
O
NHFmoc
2.27
1.92
19.23
1.02
2.07
10.99
2.93
3.28
2.82
f1 (ppm)

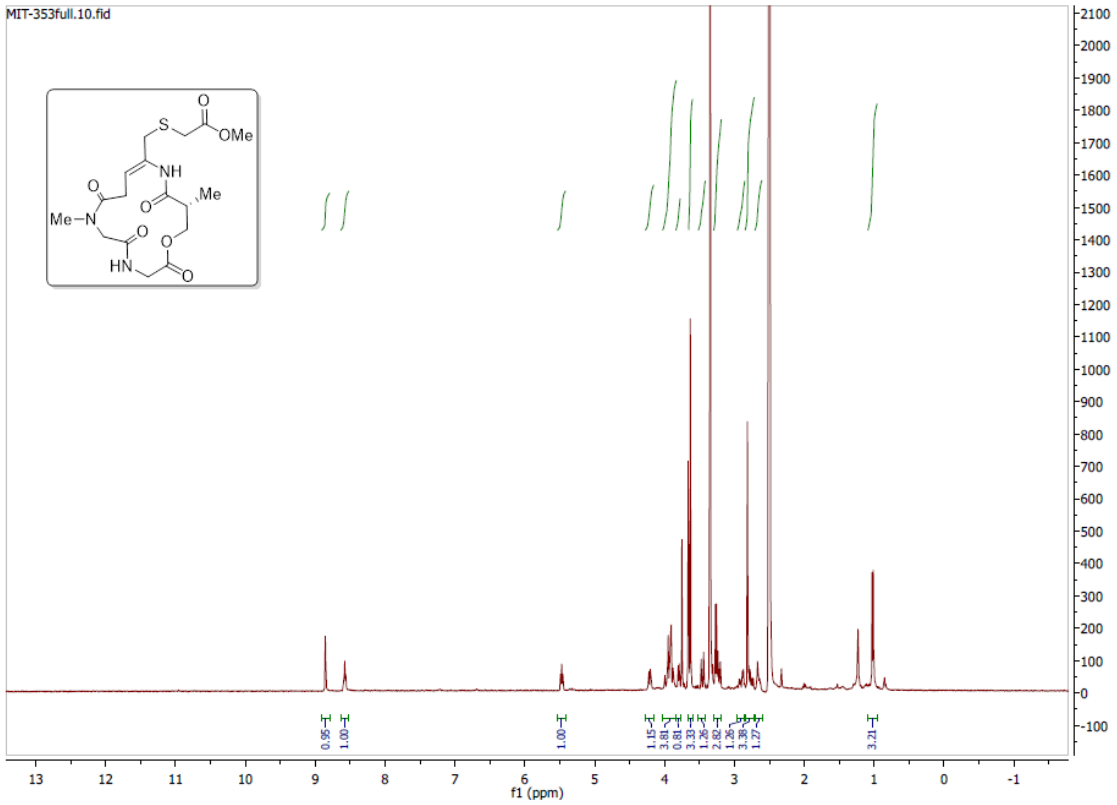

LL358-3C_pslabel_01
173.3
173.2
171.6
170.1
167.3
167.1
157.3
157.0
144.4
143.8
141.4
141.4
129.6
128.2
127.9
127.2
127.0
125.3
125.2
120.1
120.1
67.5
67.4
67.3
67.3
67.1
66.9
52.0
50.4
50.0
47.1
42.9
42.8
40.4
37.1
35.8
35.6
14.1
13.9
f1 (ppm)

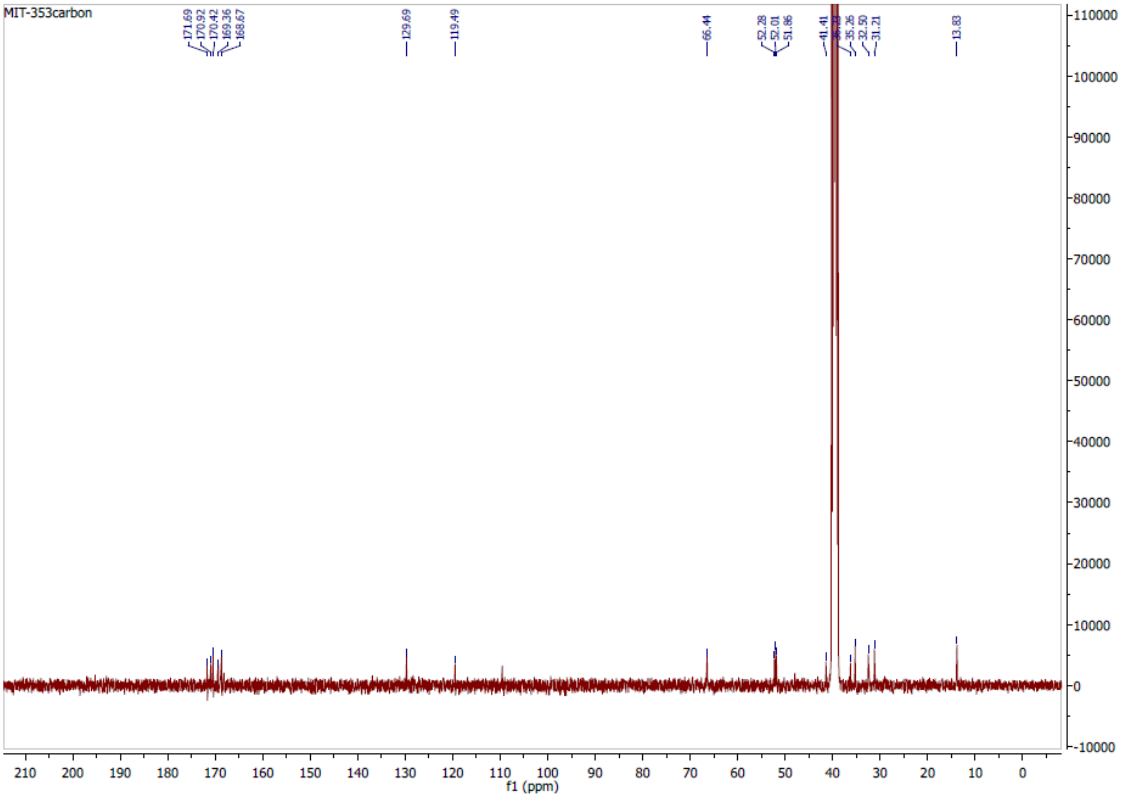

LL367-4_acetone_H_pslabel_01
STrt
NH
O
O
Me
Me—N
O
O
HN
O
O
0.95
6.36
6.24
3.43
1.01
1.01
0.53
2.95
3.57
1.33
3.28
1.36
0.48
0.59
1.10
1.34
1.67
f1 (ppm)


LL367-4_acetone_C02
173.8
170.0
169.8
169.7
169.5
167.0
145.7
145.6
144.5
143.6
130.4
128.8
127.7
121.9
121.3
67.9
67.6
67.5
53.8
53.8
51.5
50.3
42.1
41.9
40.8
40.3
36.5
35.8
35.5
15.0
13.9
f1 (ppm)

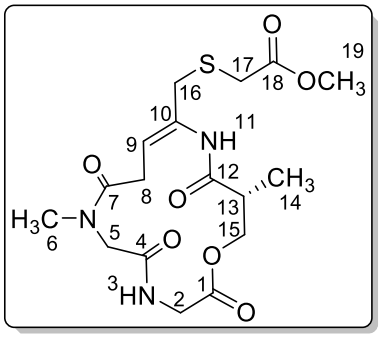

MIT-329
N
S
S
NO2
NH
O
O
Me
Me–N
O
O
HN
O
2500
2000
1500
1000
500
0
1.00
0.98
0.44
0.29
1.04
0.41
1.49
0.93
1.01
2.33
1.51
0.81
1.11
1.39
3.08
0.55
0.37
1.94
1.61
12.0
11.5
11.0
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)


MIT-331_MC.10.fid
NH
O
O
Me
Me–N
O
O
HN
O
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
-500
0.95
1.02
0.96
1.00
1.00
1.00
1.03
2.09
3.06
3.03
1.20
3.23
3.0 12.5 12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0
f1 (ppm)


MIT-353full.10.fid
f1 (ppm)

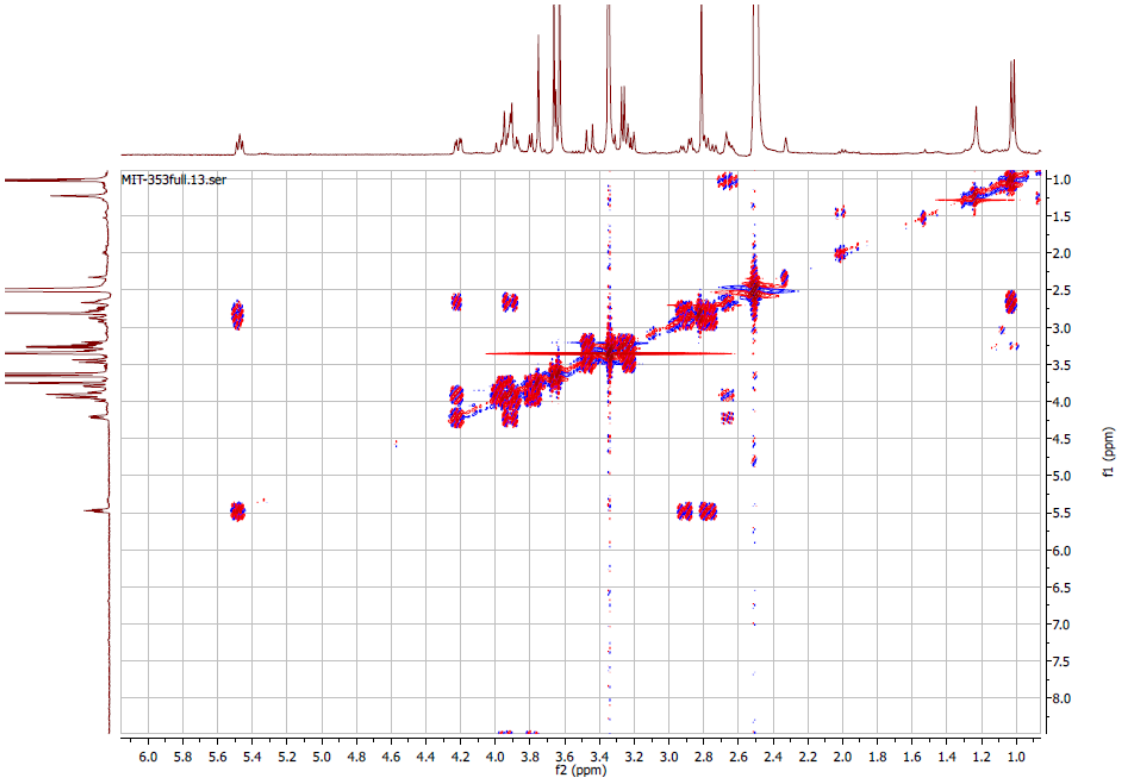

MIT-353carbon
171.69
170.92
170.42
169.36
168.67
129.69
119.49
66.44
52.28
52.01
51.86
41.41
35.26
32.50
31.21
13.83
f1 (ppm)

Full characterization of compound 20

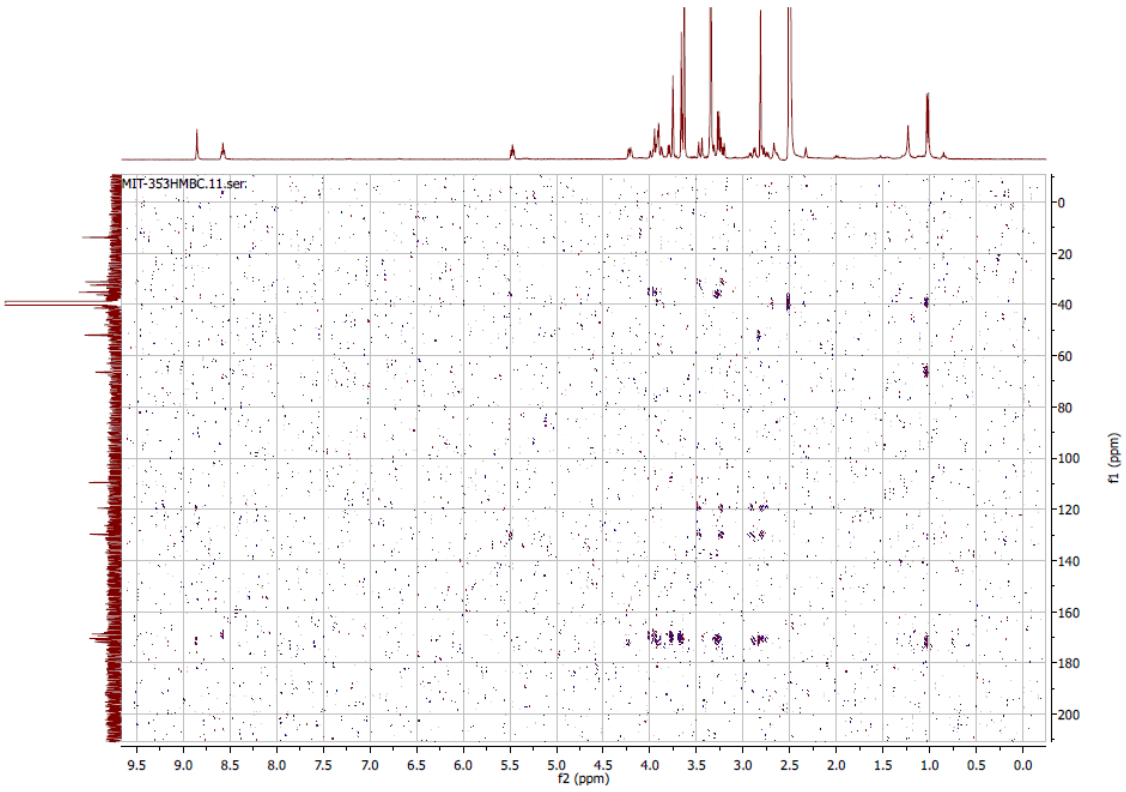


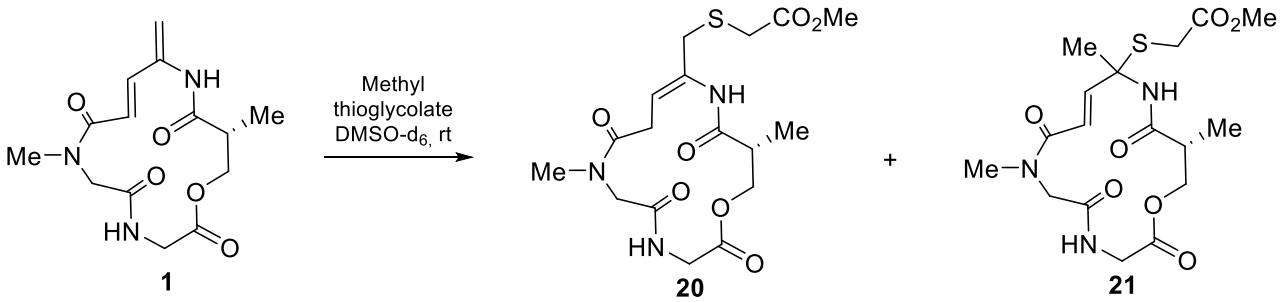
Assignment	^{13}C (ppm)	^{1}H (ppm)
C1, CO	169.4	-
C2, CH_2	52.3	3.75-4.00
3-NH	-	8.58
C4, CO	168.7	-
C5, CH_2	52.0	3.75-4.00
C6, CH_3	35.3	2.73-2.81
C7, CO	170.4	-
C8, CH_2	32.5	2.73-2.81, 2.90
C9, CH	119.5	5.47
C10, C	129.7	-
11-NH	-	8.86
C12, CO	171.7	-
C13, CH	41.4	2.60-2.69
C14, CH_3	13.8	1.02
C15, CH_2	66.4	3.75-4.00
C16, CH_2	36.2	3.20-3.27, 3.46
C17, CH_2	31.2	3.20-3.27
C18, CO	170.9	-
C19, CH_3	52.0	3.63

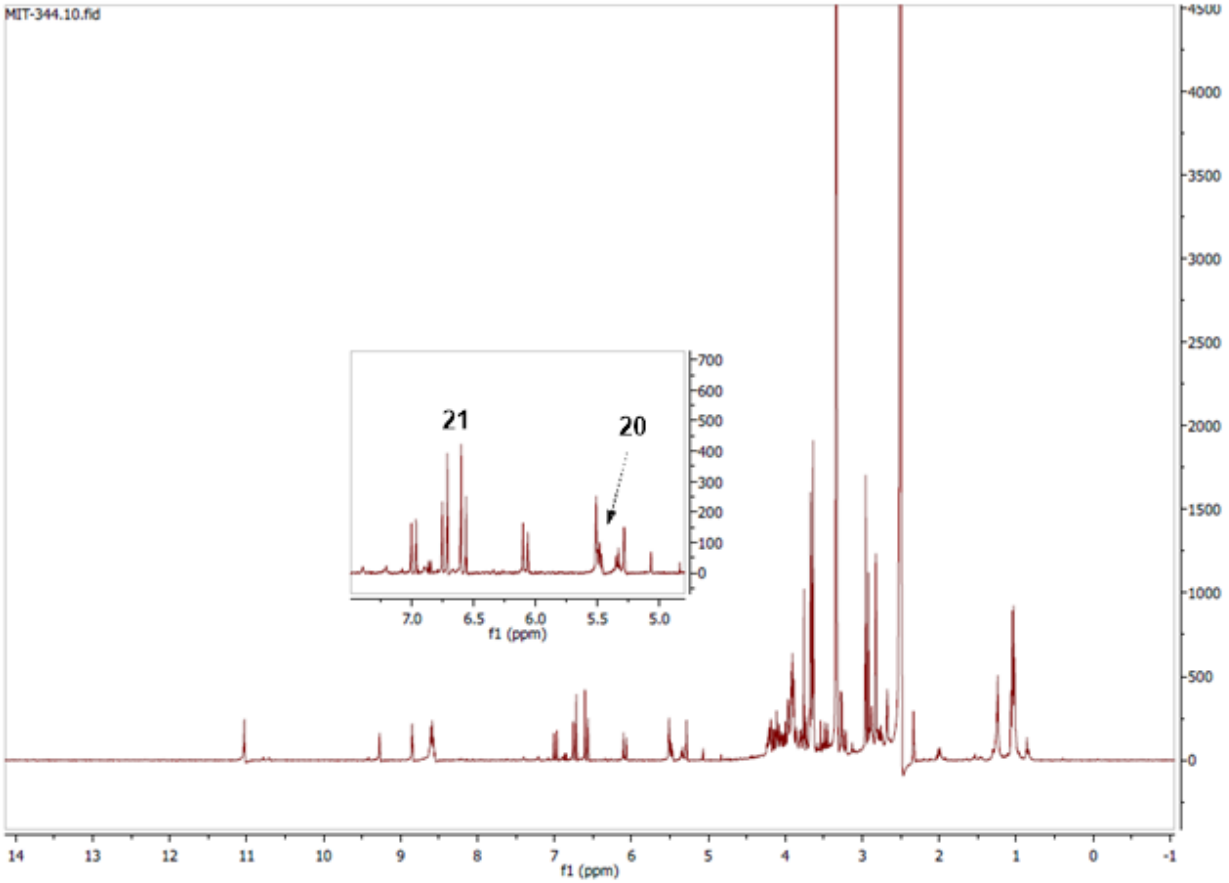
COSY (DMSO- d_6)



HMBC (DMSO-d_6)




Methyl
thioglycolate
DMSO-d6, rt
1
20
21


MIT-344.10.fid
21
20
f1 (ppm)
f1 (ppm)

References

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[2] H. E. Gottlieb, V. Kotlyar, and A. Nudelman, *J. Org. Chem.*, 1997, **62**, 7512-7515.

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[4] T. F. Dyrlund, E. T. Poulsen, C. Scavenius, K. W. Sanggaard, and J. J. Enghild, *Proteomics*, 2012, **12**, 2792-2796.

[5] T. Doi, Y. Numajiri, A. Munakata, and T. Takahashi, *Org. Lett.*, 2006, **8**, 531-534.

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