Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

A Convergent Total Synthesis of the Death Cap Toxin α-Amanitin

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Abstract: The toxic bicyclic octapeptide α-amanitin is mostly found in different species of the mushroom genus *Amanita*, with the death cap (*Amanita phalloides*) as one of the most prominent members. Due to its high selective inhibition of RNA polymerase II which is directly linked to its high toxicity, particularly to hepatocytes, α-amanitin received an increased attention as a toxin-component of antibody-drug conjugates (ADC) in cancer research. Furthermore, the isolation of α-amanitin from mushrooms as the sole source severely restricts compound supply as well as further investigations, as structure-activity relationship (SAR) studies. Based on a straightforward access to the non-proteinogenic amino acid dihydroxyisoleucine we herein present a robust total synthesis of α-amanitin providing options for production at larger scale as well as future structural diversifications.

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1. Experimental Procedures

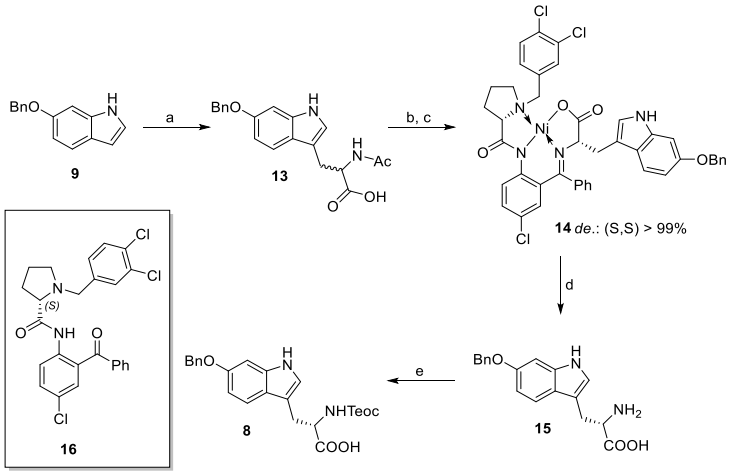
1.1. General experimental details

Commercially available reagents (Acros, Geel, Belgien; Carl Roth GmbH and Co KG, Karlsruhe, Germany; Sigma-Aldrich, Taufkirchen, Germany; Alfa Aesar, Karlsruhe, Germany; Iris Biotech GmbH, Marktredwitz, Germany; ABCR, Karlsruge, Germany) were used without further purification. If necessary the reaction was carried out under nitrogen or argon atmosphere and dry solvents. **Column chromatography** was carried out on silica gel 40 – 63 µm purchased from GRACE DAVISON (Deerfield, USA) using pressurized air. **^{1}H-NMR spectra and ^{13}C-NMR spectra** were measured on Bruker DRX 500 and AM400 instruments. Chemical shifts δ are reported in *parts per million* (ppm) and relative to the undeuterated solvent residual signals of C*D*Cl$_3$. (^{1}H: δ = 7.26 ppm; ^{13}C: δ = 77.16 ppm) and DMSO-d_6 (^{1}H: δ = 2.5 ppm; ^{13}C: δ = 39.5 ppm). Coupling constants *J* are given in Hertz (Hz) and refer to h-H-coupling. Integrals are in accordance with assignments. Muliplicities: s = singlet, d = doublet, t = triplet, q = quartet, dd doublet of doublet. When the multiplicity could not be identified, the chemical shift range of the signal was given (m = multiplet). ^{13}C-NMR spectra are proton decoupled. **HPLC-ESI-MS coupled measurements** were performed on an Orbitrap XL-mass spectrometer from *Thermo Scientific* (Waltham, MA, USA) coupled with 1200-HPLC from *Agilent Technologies* using a hypersil 100-C18-column from *Thermo Scientific* (solvent A: H_2O + 0.1 % HCOOH, solvent B: AcN + 0.1% HCOOH; flow rate 1.3 mL/min). Xcalibur (*Thermo Scientific*) was used for the evaluation of the spectra. **Flash-Chromatographie** was performed with a Combiflash Rf system from Teledyne ISCO (Lincoln, CA, USA) using a Redi*Sep* Rf Gold column (C18 Aq, size: 150 g, particle size: 20-40 µM, pore size: 100 Å) from Teledyne ISCO. **Preparative HPLC** was performed with a 1260 Infinity from *Agilent Technologies* using Sunfire Prep C18 OBD 10 µm, 50 x 150 mm column (partical size 10 µm x 100 Å) from *Agilent Technologies* (solvent A: H_2O + 0.1% HCOOH, solvent B: AcN + 0.1% HCOOH, flow rate 60 mL/min). **Analytical HPLC** was performed with a 1100 Infinity from Agilent Technologies using an Agilent Eclipse XDB column (4.0 x 50 mm, 5 µm) from Agilent Technologies (solvent A: H_2O + 0.1% HCOOH, solvent B: AcN + 0.1% HCOOH, flow rate 2 mL/min). **Chiral GC-MS** was measured with 5975C from *Agilent Technologies*, using a CS EnantioSELECT GC column (30 m, 250 µm x 0.3 µm, CS Chromatographie Service GmbH, Langerwehe, Germany). The temperature gradient (44 min) started with an initial hold for 2 min at 70°C followed by an increase of 4°C/min up to 240°C. Scans were performed in electron impact (EI) mode (MS source: 300°C, MS Quad: 150°C, Emission: 108.8 µÅ, Energy: 50 eV) with a flow of 1.2 mL/min using helium as carrier gas. **Chiral HPLC** was performed with a LaChrom system (Hitachi, Tokyo, Japan) equipped with a Chiralpak® Daicel-polysaccharide-column (250 x 4.6 mm, particle size 5 µm, Chiral Technologies Europe - Daicel Group, Illkirch, France). **CD-spectroscopy** was performed using a Jasco J-815 spectrophotomether (Jaso, Groß-Umstadt). Cuvette path length: 1 mm, accumulation: 3 spectra, scan speed: 20 nm/min, resolution: 0.1 nm, wavelength: 200-300 nm.

Abbreviations: AcN: acetonitrile; BF_3°Et_2O: boron trifluoride etherate; Bn: benzyl; Boc: *ter*t-butyloxycarbonyl; Cbz: benzyloxycarbonyl; COMU: (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium-hexafluorophosphate; Dhil: dihydroxyisoleucine; DIPEA: N,N-diisopropylethylamine Fm: 9-Fluorenylmethyl; Fmoc: fluorenylmethyloxycarbonyl; Fmoc-OSu: 9-Fluorenylmethyl *N*-succinimidyl carbonate; HATU: 1-[*Bis*(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium ; Hyp: *trans*-4-hydroxy-proline; LHMDS: lithium *bis*(trimethylsilyl)amide; NMO: 4-methylmorpholine 4-oxide; T3P: 2,4,6-tripropyl-1,3,5,2,4,6-trioxatriphosphorinane-2,4,6-trioxide; TBAF: tetra-n-butylammonium fluoride; TBS: tert.-butyldimethylsilyl; Teoc: 2-(Trimethylsilyl)ethoxycarbonyl; TFA: trifluoroacetic acid; TMSOTf: trimethylsilyl trifluoromethanesulfonate

1.2 Synthesis of amino acid building blocks:

1.2.1 Preparation of *N*-Teoc-6-benzyloxy-L-tryptophan (8)



Scheme S1: Synthesis of *N*-Teoc-6-benzyloxy-L-tryptophan (**8**) by dynamic kinetic resolution: a) L-serine, Ac_2O, AcOH, 75°C, 2 h, 82%, b) 40% NaOH, MeOH/H_2O, c) chiral ligand **16**, $Ni(NO_3)_2$ $6H_2O$, MeOH, reflux, 16 h, 84% over two steps, d) 6 M HCl, MeOH, 70°C, 2 h, e) TeocOSu, Et_3N, DMF, 60°C, quant.

3-(6-Benzyloxy-1H-indol-3-yl)-2-acetylaminopropionic acid (13)[1]

L-Serine (942 mg, 8.96 mmol, 4.00 eq.) and Ac_2O (4.02 mL, 42.6 mmol, 9.50 eq.) in AcOH (22 mL, 5 mL/mmol) were stirred at r.t. for 16 h. 6-Benzyloxyindol (**9**, 500 mg, 2.24 mmol, 1.00 eq.) was added and the mixture was stirred at 75°C for additional 2 h. The solvent was removed under reduced pressure and the residue was dissolved in H_2O (20 mL). After the solution was adjusted to pH = 11 and washed with MTBE (2 x 20 mL), the mixture was acidified to pH = 3, extracted with EtOAc (3 x 50 mL), dried over Na_2SO_4 and the solvent was removed *in vacuo*. Recrystallization in MeOH led to the *rac*-6-benzyloxytryptophan **13** (647 mg, 1.84 mmol) in 82% yield.

^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) = 1.79 (s, 3 H), 2.92 (dd, *J*=14.68, 8.66 Hz, 1 H), 3.09 (dd, *J*=14.68, 4.89 Hz, 1 H), 4.42 (td, *J*=8.22, 5.14 Hz, 1 H), 5.09 (s, 2 H), 6.72 (d, *J*=6.27 Hz, 1 H), 6.89 (d, *J*=2.01 Hz, 1 H), 6.98 (d, *J*=2.01 Hz, 1 H), 7.27 - 7.33 (m, 1 H), 7.35 - 7.41 (m, 3 H), 7.42 - 7.48 (m, 2 H), 8.11 (d, *J*=7.78 Hz, 1 H), 10.64 (s, 1 H).

^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) = 22.4, 27.2, 53.0, 69.5, 96.0, 109.2, 110.0, 118.8, 121.9, 122.3, 127.5, 127.6, 128.4, 136.7, 137.7, 154.5, 169.2, 173.6.

HRMS (ESI): *m/z* calc for $C_{20}H_{20}N_2O_4$ $[M+H]^+$ 353.1496 found 353.1503.

Nickel(II)-(*S*)-*N*-(2-benzoyl-4-chlorophenyl)-1-(3,4-dichlorobenzyl)pyrrolidine-2-carboxamide/ (*S*)-3-amino-3-(6-(benzyloxy)-1H-indol-3-yl)propanoic acid Schiff Base Complex (14)[2,3]

A suspension of **13** (1.03 g**,** 3.32 mmol, 1.00 eq.) in 40% NaOH (H_2O/MeOH v/v = 1:1, 14 mL/mmol) was stirred at 110°C for 4 h, neutralized with conc. HCl to pH =7 and the solvent was removed *in vacuo*. The crude product was dissolved in MeOH (66.4 mL, 20 mL/mmol) and $Ni(OAc)_2$*6 H_2O (966 mg, 3.32 mmol, 1.00 eq.) and **16** (1.78 g, 3.65 mmol, 1.10 eq.) were added followed by K_2CO_3 (2.09 g, 15.1 mmol, 4.50 eq.). The resulting mixture was refluxed for 16 h and the precipitate was filtered of and washed with DCM. The solvent of the filtrate was removed *in vacuo* and the crude product was suspended in DCM (50 mL), washed with H_2O (50 mL) and dried with Na_2SO_4. After removal of the solvent *in vacuo* the crude product was purified with DCM/MeOH (v/v = 40:1) to give the product

14 (major diastereomer) (2.44 g, 84%) and the minor diastereomer (10 mg) as an orange solids. The major diastereomer was obtained with *de* of >99%.

^{1}H-NMR (500 MHz, C*D*Cl$_3$): δ (ppm) = 1.37 - 1.48 (m, 1 H), 1.69 - 1.83 (m, 2 H), 1.84 - 1.96 (m, 1 H), 2.08 - 2.19 (m, 1 H), 2.88 (br. s., 1 H), 2.94 (dd, *J* = 14.65, 5.04 Hz, 1 H), 3.00 (d, *J* = 12.51 Hz, 1 H), 3.07 (dd, *J* = 10.15, 7.10 Hz, 1 H), 3.31 (dd, *J* = 14.65, 3.97 Hz, 1 H), 4.09 (d, *J* = 12.51 Hz, 1 H), 4.28 (t, *J* = 4.65 Hz, 1 H), 5.08 (s, 2 H), 6.63 (d, *J* = 2.44 Hz, 1 H), 6.70 - 6.80 (m, 2 H), 6.87 (s, 1 H), 6.96 (d, *J* = 1.83 Hz, 1 H), 7.11 (dd, *J* = 9.31, 2.44 Hz, 1 H), 7.20 (d, *J* = 8.70 Hz, 1 H), 7.24 - 7.41 (m, 7 H), 7.45 (s, 2 H), 7.49 (s, 1 H), 7.53 - 7.59 (m, 1 H), 7.62 (dd, *J* = 8.16, 1.75 Hz, 1 H), 8.18 (s, 1 H), 8.46 (br. s., 1 H), 8.88 (d, *J* = 1.68 Hz, 1 H).

^{13}C-NMR (126 MHz, C*D*Cl$_3$): δ (ppm) = 22.5, 30.6, 58.3, 63.1, 70.7, 71.4, 96.7, 109.7, 110.5, 120.3, 123.1, 123.3, 123.9, 125.5, 127.2, 127.3, 127.5, 127.7, 127.8, 128.5, 129.0, 129.3, 129.8, 130.1, 130.9, 132.3, 133.2, 133.7, 134.9, 137.2, 137.5, 141.0, 155.9.

HRMS (ESI): *m/z* calc for $C_{43}H_{35}Cl_3N_4NiO_4$ $[M+H]^+$ 835.1150 found 835.1155.

(*S*)-2-amino-3-(6-(benzyloxy)-1H-indol-3-yl)propanoic acid (15)

A solution of complex **14** (129 mg, 0.154 mmol, 1 eq.) in 6 M HCl (0.8 mL, 5.6 mL /mmol) and MeOH (3 mL, 20 mL/mmol) was stirred for 30 min at 70 °C. The reaction mixture was concentrated *in vacuo*, adjusted to pH 9 with sodium hydroxide solution (5 M) and washed with DCM (3 x 15 mL). The organic layer was dried over Na_2SO_4, evaporated *in vacuo* and recrystallized with EtOH to afford the ligand **16**. The aqueous layer was evaporated *in vacuo* and the crude product was used in the next step without further purification.

^{1}H-NMR (500 MHz, C*D*O*D*$_3$): δ (ppm) = 3.80 (s, 2 H), 4.30 (dd, *J* = 7.63, 5.34 Hz, 1 H), 5.10 (s, 2 H), 6.83 (dd, *J* = 8.70, 2.29 Hz, 1 H), 6.99 (d, *J* = 2.14 Hz, 1 H), 7.06 (s, 1 H), 7.26 - 7.32 (m, 1 H), 7.33 - 7.39 (m, 2 H), 7.41 (d, *J* = 8.70 Hz, 1 H), 7.43 - 7.46 (m, 2 H).

^{13}C-NMR (126 MHz, C*D*O*D*$_3$): 26.2, 52.2, 53.2, 70.1, 96.1, 106.1, 118.1, 123.0, 127.1, 127.3, 128.1, 137.6, 147.7, 155.6.

HRMS (ESI): *m/z* calc for $C_{24}H_{30}N_2O_5Si$ $[M+H]^+$ 311.1390 found 311.1382.

(*S*)-3-(6-(benzyloxy)-1H-indol-3-yl)-2-((2-(trimethylsilyl)ethyl)amino)propanoic acid (8)

A suspension of **15** (947 mg, 3.05 mmol, 1.00 eq.) in DMF (15 mL, 5 mL/mmol) was treated with triethylamine (1.11 g, 10.9 mmol, 3.60 eq.) and TeocOSu (2.37 g, 9.16 mmol, 3.00 eq.) and was stirred at 60°C until the TLC indicated complete conversion. The suspension was filtered and the solvent was removed *in vacuo*. The crude product was purified with C18 reverse phase chromatography (MeOH/H_2O 20% to 100%, 30 min) to give compound **8** as a green oil (1.38 g, 3.05 mmol, quant. over 2 steps).

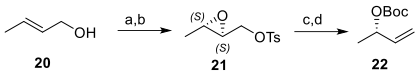
^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) = -0.15 - 0.11 (m, 9 H), 0.81 - 0.96 (m, 2 H), 2.83 - 3.02 (m, 1 H), 3.11 (dd, *J* = 14.56, 4.27 Hz, 1 H), 3.89 - 4.07 (m, 2 H), 4.13 - 4.26 (m, 1 H), 5.11 (s, 2 H), 6.74 (dd, *J* = 8.53, 2.01 Hz, 1 H), 6.92 (d, *J* = 2.01 Hz, 1 H), 7.01 (s, 1 H), 7.22 (d, *J* = 8.03 Hz, 1 H), 7.33 (d, *J* = 7.28 Hz, 1 H), 7.36 - 7.44 (m, 3 H), 7.47 (d, *J* = 7.03 Hz, 2 H), 10.64 (br. s., 1 H).

^{13}C-NMR (101 MHz, DMSO-d_6): δ (ppm) = 18.8, 28.4, 56.2, 63.2, 71.0, 97.5, 110.7, 111.6, 120.2, 123.2, 123.9, 128.9, 129.1, 129.8, 138.2, 139.1, 155.9, 157.6, 175.3.

HRMS (ESI): *m/z* calc for $C_{24}H_{30}N_2O_5Si$ $[M+H]^+$ 455.1997 found 455.1997.

1.2.2. Preparation of protected (2*S*,3*R*,4*R*)-4,5-L-dihydroxyisoleucine (6)

1.2.2.1 Preparation of but-3-en-2-yl *tert*-butyl carbonate (22)[4,5]



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Scheme S2: Synthesis of but-3-en-2-yl *tert*-butyl carbonate (**22**) a) (+)-DIPT, Ti(O*i*Pr)$_4$, TBHP, DCM, -20°C, 4 h; b) TsCl, Et$_3$N, DMAP, DCM, -10°C, 30 h, 67%; c) NaI, Zn(Cu), THF, 70°C, 2 h; d) Boc$_2$O, NaH, THF, 0°C to r.t., 16 h, 75%.

Synthesis of ((2*S*,3*S*)-3-methyloxiran-2-yl)methyl 4-methylbenzenesulfonate (21)

A flame dried flask was charged with 10 g of crushed activated 3 Å molecular sieves and flushed with nitrogen for several minutes. Then, DCM (200 ml) was added and the flask was cooled to -20°C. (+)-Diisopropyl tartrate (DIPT) (1.75 g, 7.49 mmol, 0.06 eq), crotyl alcohol (9.00 g, 125 mmol, 1.00 eq) and Ti(O*i*Pr)$_4$ (1.77 g, 6.24 mmol, 0.05 eq) were added sequentially at this temperature. The resulting mixture was stirred for 15 min at -20°C, then a solution of *tert*-butyl hydroperoxide (TBHP, 5 M in DCM) (50.0 ml, 150 mmol, 2.00 eq) was added dropwise. The reaction mixture was stirred for 4 h at this temperature. Careful quenching of the excess TBHP was carried out by careful addition of trimethyl phosphite (22.0 mL, 187 mmol, 1.50 eq) at -20°C after which triethyl amine (26.1 mL, 187 mmol, 1.50 eq), DMAP (1.83 g, 15.0 mmol, 0.12 eq) and a solution of *p*-toluenesulfonyl chloride (23.8 g, 125 mmol, 1.00 eq) in DCM (100ml) was added sequentially. The temperature was raised to -10°C and the reaction mixture stirred for 30 h. Afterwards the mixture was filtered through a pad of Celite and washed with DCM. The filtrate was then washed with 10 % tartaric acid (2 x 100 mL), saturated $NaHCO_3$ (2 x 100 mL) and brine (2 x 100 mL). The organic phase was dried over $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel (hexane/EtOAc, 2:1) and recrystallized (Et_2O/hexane) in order to afford the tosylate (**21**) as white needles (20.2 g, 67%).

^{1}H NMR ($CDCl_3$-*d*^{1}, 400 MHz): δ = 1.30 (d, *J*=5.27 Hz, 3 H), 2.46 (s, 3 H), 2.87 – 2.94 (m, 1 H), 2.92 (s, 1 H), 3.98 (dd, *J*=11.42, 5.90 Hz, 1 H), 4.18 (dd, *J*=11.42, 3.89 Hz, 1 H), 7.36 (d, *J*=8.28 Hz, 2 H), 7.80 ppm (d, *J*=8.53 Hz, 2 H).

^{13}C NMR ($CDCl_3$-*d*^{1}, 100 MHz): δ = 17.0, 21.6, 52.8, 55.4, 70.0, 127.9, 129.9, 132.7, 145.1 ppm.

HRMS (ESI): m/z calculated: $C_{11}H_{14}O_4S$ $(M+H)^+$ 243.0686, found 243.0678.

Synthesis of (*S*)-but-3-en-2-yl *tert*-butyl carbonate (22)

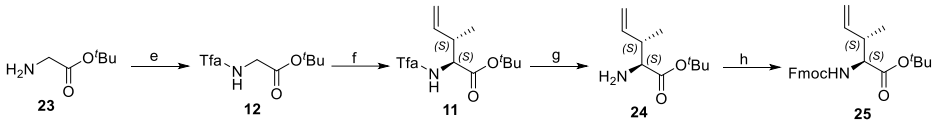
A solution of tosylate **23** (12.0 g, 49.5 mmol, 1.00 eq) in dry THF (20 mL) was added to a suspension of Zinc-Copper-couple (6 g) and dry NaI (22.3 g, 149 mmol, 3.00 eq) in dry THF (150 mL). The resulting suspension was stirred for 2 h at 70°C, cooled to room temperature and filtered through a pad of silica. Afterwards the THF-butenol mixture was distilled under reduced pressure (200 mbar, 100°C), then cooled to 0°C and NaH (5.82 g, 145 mmol, 3.00 eq) was carefully added in small portions. Then, Boc_2O (11.7 g, 53.4 mmol, 1.10 eq) was added portion-wise over 10 min under vigorous stirring at this temperature. The reaction mixture was allowed to warm to room temperature overnight and then diluted with Et_2O after 16 h. Excess sodium hydride was quenched by the slow addition of water at 0°C. The resulting mixture was extracted with diethyl ether (3 x 50 mL). The combined organic extracts were washed with brine (1 x 50 mL), dried over $MgSO_4$ and concentrated under reduced pressure. After purification by column chromatography on silica gel (hexane/EtOAc, 20:1) the product **22** was obtained as a colourless liquid (6.25 g, 75%).

^{1}H NMR ($CDCl_3$-*d*^{1}, 400 MHz): δ = 1.36 (d, *J*=6.53 Hz, 3 H), 1.49 (s, 9 H), 5.13 - 5.17 (m, 2 H), 5.25 - 5.31 (m, 1 H), 5.83 – 5.91 ppm (m, 1 H).

^{13}C NMR ($CDCl_3$-*d*^{1}, 100 MHz): δ = 19.7, 27.5, 73.7, 81.6, 115.8, 137.8, 137.2, 152.5 ppm.

HRMS (ESI): m/z calculated: $C_9H_{16}O_3$ $(M+H)^+$ 173.1172, found 173.1171.

1.2.2.2 Preparation of Fmoc-4,5-didehydroisoleucine-O*t*Bu (25)[6,7]



Scheme S3: Synthesis of Fmoc-4,5-didehydroisoleucine-O*t*Bu (**10**) e) ethyl trifluoroacetate, NEt_3, MeOH, r.t., 16 h, quant.; f) LHMDS, $ZnCl_2$, PPh_3,(*p*-Cymene)$RuCl_2]_2$, **22**, THF, -72°C to r.t., 16 h, 88%; g) $NaBH_4$, MeOH, r.t., 1 h; h) FmocOSu, Et_3N, dioxane, r.t., 4 h, 82%.

Synthesis of *tert*-butyl (2,2,2-trifluoroacetyl)glycinate (12)

To a solution of glycine *tert*-butyl ester hydrochloride (**23**) (10.0 g, 137 mmol, 1.00 eq) in MeOH (15 mL) triethylamine (17.4 mL, 125 mmol, 2.10 eq) was added dropwise. After stirring for 5 min ethyl trifluoroacetate (16.4 mL, 137 mmol, 2.30 eq) was added and the mixture was stirred for 16 h at room temperature during which time a clear solution formed. Then, the reaction mixture was concentrated under reduced pressure and the resulting residue acidified with 2 N HCl before being extracted with EtOAc (3 x 100 mL). The organic layers were combined and washed with sat. $NaHCO_3$ (2x100 mL), distilled H_2O (2 x 100 mL) and brine (2 x 100 mL), then dried over $MgSO_4$. The solvent was removed *in vacuo* to give the product (**12**) as a yellow oil (13.5 g, quant.).

^{1}H NMR ($CDCl_3$-d^1, 400 MHz): δ = 1.50 (s, 9 H), 4.02 (d, *J*=5.02 Hz, 2 H), 6.89 ppm (br s, 1 H).

^{13}C NMR ($CDCl_3$-d^1, 100 MHz): δ = 27.9, 41.9, 83.5, 115.6 (q, *J*=287.28 Hz), 157.1 (q, *J*=39.60 Hz), 167.3 ppm.

HRMS (ESI): m/z calculated: $C_8H_{12}F_3NO_3$ $(M-H)^-$ 226.0686, found 226.0693.

Synthesis of *tert*-butyl (2*S*,3*S*)-3-methyl-2-(2,2,2-trifluoroacetamido)pent-4-enoate (11)

LHMDS (1 M in THF, 11 mmol, 2.5 eq) was added slowly to a solution of *tert*-butyl (2,2,2-trifluoroacetyl)glycinate (**12**, 1.0 g, 4.4 mmol, 1.5 eq) in dry THF (12 mL) at -78°C. After stirring for 10 min a solution of dried $ZnCl_2$ (720 g, 5.30 mmol, 1.20 eq) in dry THF (6 mL) was added at this temperature and stirring was continured for another 30 min at -78°C. Meanwhile, a solution was prepared from [(*p*-cymene)$RuCl_2]_2$ (37 mg, 0.06 mmol, 0.02eq) and triphenylphosphine (32 mg, 0.12 mmol, 0.04 eq) in dry THF (6 mL) and stirred for 10 min at room temperature. Then, allylic carbonate **22** (517 mg, 3.00 mmol, 1 00eq) was added to the catalyst-ligand mixture and the resulting solution was added to the chelated enolate at -78°C. The reaction mixture was allowed to warm to room temperature overnight. After diluting with Et_2O (100 mL) the reaction mixture was hydrolyzed by addition of 10% $KHSO_4$–solution until the precipitate was fully dissolved in the organic layer. Then, the layers were separated, the aqueous layer was extracted with diethyl ether (3 x 50 mL) and the combined organic layers were washed with brine (100 mL) and dried over $MgSO_4$. The solvent was removed *in vacuo* and the crude product was purified by column chromatography on silica gel (hexane/EtOAc, 20:1), which afforded the product (**11**) as a colourless oil (740 mg, 88%).

^{1}H NMR ($CDCl_3$-d^1, 400 MHz): δ = 1.10 (d, *J*=7.03 Hz, 3 H), 1.49 (s, 9 H), 2.82 – 2.89 (m, 1 H), 4.48 - 4.54 (m, 1 H), 5.07 - 5.20 (m, 2 H), 5.70 (s, 1 H), 6.70 ppm (d, *J*=7.53 Hz, 1 H).

^{13}C NMR ($CDCl_3$-d^1, 100 MHz): δ = 15.75, 27.97, 40.21, 56.71, 83.35, 117.15 (q, *J*=273.2 Hz), 117.46, 136.78, 157.03 (q, *J*=39.60 Hz), 168.89 ppm.

Synthesis of *tert*-butyl (2*S*,3*S*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylpent-4-enoate (25)

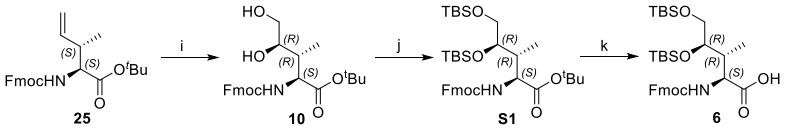
$NaBH_4$ (1.18 g, 31.3 mmol, 4.00 eq) was added to solution of *tert*-butyl (2*S*,3*S*)-3-methyl-2-(2,2,2-trifluoroacetamido)pent-4-enoate (**11**, 2.2 g, 7.8 mmol, 1.0 eq) in absolute EtOH (32 mL) under a nitrogen atmosphere at 0°C, then warmed to rt and stirred for 1 h. Afterwards, excess $NaBH_4$ was quenched with acetone and the resulting mixture slowly acidified to pH 4 by careful addition of 1 M HCl at 0°C. Then, saturated $NaHCO_3$ solution was added and the slightly basic (pH 8) solution was extracted with EtOAc (3 x30 mL). The combined organic extracts were diluted with dioxane (90 mL), then Et_3N (1.36 mL, 9.78 mmol, 1.25 eq) and Fmoc-OSu (2.64 g, 7.83 mmol, 1.00 eq) were added. After stirring for 2 h the reaction mixture was diluted with 100 ml water and evaporated under reduced pressure. The mixture was then acidified to pH 2 with 1 M HCl and extracted with EtOAc (3 x 50 mL). Afterwards, the combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (hexane/EtOAc, 15:1) to obtain the fully protected 4,5-didehydroisoleucine **25** as a colourless oil (2.6 g, 82% over 2 steps).

^{1}H NMR ($CDCl_3$-d^1, 400 MHz): δ = 1.11 (d, *J*=6.78 Hz, 3 H), 1.49 (s, 9 H), 2.73 - 2.86 (m, 1 H), 4.26 (t, *J*=7.28 Hz, 1 H), 4.29 (q, *J*=4.50 Hz, 1 H), 4.34 - 4.44 (m, 2 H), 5.08 - 5.18 (m, 2 H), 5.25 (d, *J*=9.03 Hz, 1 H), 5.68 - 5.80 (m, 1 H), 7.33 (t, *J*=7.28 Hz, 2 H), 7.41 (t, *J*=7.53 Hz, 2 H), 7.61 (d, *J*=7.53 Hz, 2 H), 7.78 ppm (d, *J*=7.53 Hz, 2 H).

^{13}C NMR ($CDCl_3$-d^1, 100 MHz): δ = 15.7, 27.8, 40.1, 46.9, 58.0, 66.7, 81.9, 116.3, 119.6, 124.8, 126.7, 127.4, 137.5, 141.0, 143.5, 155.9, 170.3 ppm.

HRMS (ESI): m/z calculated: $C_{25}H_{29}NO_4$ $(M+H)^+$ 430.1989, found 430.1982.

1.2.2.3 Preparation of (2*S*,3*R*,4*R*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4,5-bis((*tert*-butyldimethylsilyl)oxy)-3-methyl pentanoic acid (6)



Scheme S4: Synthesis of (2*S*,3*R*,4*R*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4,5-bis((*tert*-butyldimethylsilyl)oxy)-3-methyl pentanoic acid (**6**): i) K_2OsO_4*H_2O, NMO, $CHCl_3$/H_2O (4:1), 6 h, 40%; i) TBSCl, pyridine/DMF(1:9), 24 h, 95%; k) TMSOTf, 2,6-lutidine, 0°C, 2 h, 90%.

Synthesis of *tert*-butyl (2*S*,3*R*,4*R*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4,5-dihydroxy-3-methylpentanoate (10)

N-methylmorpholine-*N*-oxide (1.6 g, 13.7 mmol, 1.30 eq) was added to a stirring solution of **25** (4.30 g, 10.6 mmol, 1.00 eq) and potassium osmate dihydrate (194 mg, 527 µmol, 0.05 eq) in 120 mL of a 4:1 mixture of $CHCl_3$ and water and stirred for 6 h at rt. Afterwards, the mixture was diluted with DCM (100 mL) and saturated sodium metabisulfite solution (20 mL) was added. After vigorous stirring for 20 min the mixture was extracted with DCM (3x50 mL). The combined organic phases were dried over $NaSO_4$ and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel (2:1 hexane/ ethyl acetate – 4:3 hexane/ethyl acetate gradient) to obtain the product (**10**) as a white solid (1.9 g, 40%).

^{1}H NMR ($CDCl_3$-d^1, 700 MHz): δ = 0.95 (d, *J* = 6.87 Hz, 3 H), 1.50 (s, 9 H), 2.24 - 2.34 (m, 1 H), 3.50 - 3.66 (m, 2 H), 3.77 (d, *J* = 10.22 Hz, 1 H), 4.24 (t, *J* = 6.94 Hz, 1 H), 4.42 (d, *J* = 4.73 Hz, 2 H), 4.50 (d, *J* = 6.41 Hz, 1 H), 5.64 (d, *J* = 8.09 Hz, 1 H), 7.30 - 7.35 (m, 2 H), 7.41 (t, *J* = 7.32 Hz, 2 H), 7.62 (d, *J* = 6.87 Hz, 2 H), 7.78 (d, *J* = 7.63 Hz, 2 H).

^{13}C NMR ($CDCl_3$-d^1, 125 MHz): δ = 170.9, 156.4, 143.8, 141.3, 127.7, 127.06, 125.05, 82.7, 73.6, 67.0, 64.7, 56.4, 47.2, 38.9, 28.0, 134.4.

HRMS (ESI): m/z calculated: $C_{25}H_{31}NO_6$ $(M+H)^+$ 442.2224, found 442.2222.

Synthesis of *tert*-butyl (2S,3R,4R)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4,5-bis((tert-butyldimethylsilyl)oxy)-3-methylpentanoate (S1)

tert-Butyldimethylsilyl chloride (6.5 g, 4.3 mmol, 10.0 eq) was added to a stirring solution of dihydroxyisoleucine derivative **10** (1.9 g, 4.3 mmol, 1.00 eq) in 40 mL of a 9:1 mixture of dry DMF and pyridine. The resulting mixture was stirred for 24 h at rt. Afterwards, the reaction mixture was diluted with 80 mL EtOAc and washed with 1 M HCl (3 x 10 mL) and brine (2 x 20mL), dried over $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel (hexane/EtOAc, 19:1) to obtain the fully protected (2*S*,3*R*,4*R*)-4,5-dihydroxyisoleucine derivative **S1** as a colourless oil (2.7 g, 95%).

^{1}H NMR ($CDCl_3$-d^1, 500 MHz): δ (ppm) = 0.06 - 0.13 (m, 12 H), 0.91 - 0.94 (m, 18 H), 1.07 (d, *J* = 7.28 Hz, 3 H), 1.47 - 1.51 (m, 9 H), 2.36 - 2.45 (m, 1 H), 3.53 - 3.65 (m, 2 H), 3.69 - 3.74 (m, 1 H), 4.25 (t, *J* = 7.03 Hz, 1 H), 4.33 (dd, *J* = 8.66, 5.14 Hz, 1 H), 4.37 - 4.44 (m, 2 H), 6.35 (d, *J* = 8.53 Hz, 1 H), 7.28 - 7.33 (m, 2 H), 7.40 (t, *J* = 7.53 Hz, 2 H), 7.63 (dd, *J* = 7.03, 4.52 Hz, 2 H), 7.76 (d, *J* = 7.53 Hz, 2 H).

^{13}C NMR ($CDCl_3$-d^1, 126 MHz): -5.4, -5.3, -4.7, -4.3, 13.8, 18.1, 18.4, 25.8, 26.0, 28.1, 38.7, 47.3, 56.8, 64.9, 66.8, 75.1, 81.6, 119.9, 125.2,127.0, 127.6, 141.3, 144.0, 144.2, 156.5, 171.1.

HRMS (ESI): m/z calculated: $C_{37}H_{59}NO_6Si_2$ $(M+H)^+$ 670.3953, found 670.3945.

Synthesis of (2*S*,3*R*,4*R*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4,5-bis((*tert*-butyldimethylsilyl)oxy)-3- methyl pentanoic acid (6)

A solution of fully protected 4,5-dihydroxyisoleucine derivative **S1** (2.3 g, 3.4 mmol, 1.0 eq) was dissolved in 30 mL dry DCM, treated with 2,6 lutidine (4.0 mL, 34.3 mmol, 10.0 eq) and cooled to 0°C. Then, TMSOTf (3.11 mL, 17.2 mmol, 5.0 eq) was added and the reaction mixture was stirred at r.t. for 2 h. The solution was diluted with Et_2O (100 mL) followed by the addition of Sørensen phosphate buffer (pH 7; 5 mL). Afterwards, the pH of the mixture was adjusted to pH 2 by the dropwise addition of $KHSO_4$-solution (10%). The phases were separated and the aqueous phase was extracted with Et_2O (3 x 50mL). The combined organic phases were washed with brine, dried over $NaSO_4$ and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel (DCM/MeOH 2%/AcOH 0.5%) to furnish the final 4,5-dihydroxyisoleucine derivative **6** as a pale yellow solid (1.9 g, 90%).

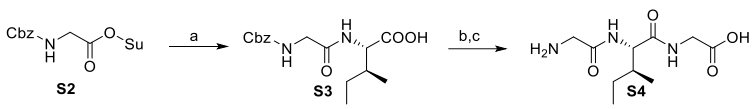
^{1}H NMR ($CDCl_3$-d^1, 500 MHz): δ (ppm) = 0.08 - 0.17 (m, 12 H), 0.88 - 0.95 (m, 18 H), 1.08 (d, *J* = 7.17 Hz, 3 H), 2.44 - 2.52 (m, 1 H), 3.73 - 3.78 (m, 1 H), 3.78 - 3.85 (m, 2 H), 4.23 (t, *J* = 7.02 Hz, 1 H), 4.35 - 4.44 (m, 2 H), 4.67 (dd, *J* = 6.71, 2.90 Hz, 1 H), 6.19 (d, *J* = 6.71 Hz, 1 H), 7.32 (t, *J* = 7.02 Hz, 2 H), 7.40 (t, *J* = 7.48 Hz, 2 H), 7.62 (dd, *J* = 7.32, 3.66 Hz, 2 H), 7.77 (d, *J* = 7.48 Hz, 2 H).

^{13}C NMR ($CDCl_3$-d^1, 126 MHz): δ (ppm) = -5.5, -5.4, -4.7, -4.1, 11.07, 18.0, 18.3, 25.8, 25.9, 37.0, 47.2, 57.8, 64.7, 67.0, 74.0, 120.0, 125.1, 125.1, 127.0, 127.6, 141.3, 143.9, 156.4.

HRMS (ESI): m/z calculated: $C_{33}H_{51}NO_6Si_2$ $(M+H)^+$ 614.3328, found 614.3324.

1.3. Preparation of monocyclic building block 5 (monocyclic pentapeptide)

1.3.1 Preparation of H-Gly-Ile-Gly-OH (S4)



Scheme S5. Synthesis of tripeptide **S4**: a) isoleucine, $NaHCO_3$, acetone/H_2O, r.t., 3 h 96%; b) HCl°NH_2-Gly-OBn, COMU, DIPEA, DMF, 0°C, 16 h; c) H_2, Pd/C, THF/water, r.t., 16 h, 74%.

Synthesis of ((benzyloxy)carbonyl)glycyl-L-isoleucine (**S3**)

To a solution of Cbz-glycine (**S2**), 10.0 g, 32.7 mmol, 1.00 eq.) in acetone (100 mL) was added a suspension of L-isoleucine (4.71 g, 35.9 mmol, 1.10 eq.) and $NaHCO_3$ (8.23 g, 87.9 mmol, 3.00 eq.) in water (100 mL). The reaction mixture was stirred at r.t. for 3 h and concentrated under reduced pressure .The aqueous layer was carefully acidified to pH = 4 by dropwise addition of 1 M HCl and extracted with EtOAc (3 x150 mL). The organic phase was then washed with brine (2 x 100 mL), dried over Na_2SO_4 and evaporated under reduced pressure to afford the product **S3** as a colourless oil (10.1 g, 96%).

^{1}H NMR ($CDCl_3$-*d*^{1}, 400 MHz): δ (ppm) = 0.79 - 0.97 (m, 6 H), 1.16 (s, 4 H), 1.43 (m, *J*=11.92, 6.40 Hz, 4 H), 1.91 (br. s., 3 H), 3.89 (dd, *J*=15.56, 4.02 Hz, 4 H), 4.00 (dd, *J*=16.81, 5.27 Hz, 1 H), 4.57 (s, 4 H), 5.11 (br. s., 2 H), 5.95 (br. s., 1 H), 7.08 (d, *J*=7.78 Hz, 1 H), 7.28 - 7.39 (m, 5 H).

^{13}C NMR ($CDCl_3$-*d*^{1}, 101 MHz): δ (ppm) =11.5, 15.3, 25.0, 37.5, 44.3, 56.8, 67.5, 128.1, 128.3, 128.6, 136.0, 157.2, 170.5, 174.6.

HRMS (ESI): *m/z* calc. for $C_{16}H_{22}N_2O_5$ $(M+H)^+$ 323.1601, found 323.1606.

Synthesis of glycyl-L-isoleucineglycine (S4)

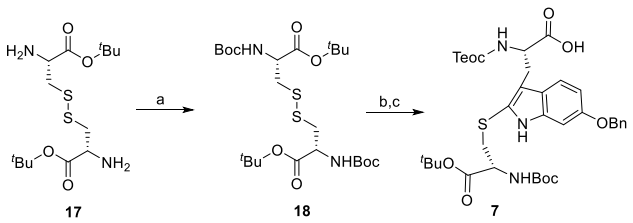
Dipeptide **S3** (10.1 g, 31.3 mmol, 1.00 eq.) and benzyl glycinate (8.21 g, 40.7 mmol, 1.30 eq.) were dissolved in dry DMF (125 mL). Then, COMU (17.4 g, 40.7 mmol, 1.30 eq.) and DIPEA (12.6 mL, 72.1 mmol, 3.00 eq.) were added at 0°C. The reaction mixture was allowed to warm to r.t. overnight and diluted with EtOAc (300 mL) afterwards. After washing with a solution of 10% $KHSO_4$-solution (2 x 100 mL) the fully protected tripeptide precipitated in the organic phase. The organic phase was cooled to 4°C for 4 h, then the precipitate was filtered and washed with cold EtOAc. The precipitate was redissolved in a 1:1 mixture of water and THF (260 mL). Pd/C (1 g) was added to the solution after degassing with N_2 for 30 min. Then, the reaction mixture was degassed with hydrogen for 1 h. After vigorous stirring at room temperature under 1.0 atm of hydrogen overnight, the catalyst was filtered through a pad of Celite. Afterwards, the mixture was concentrated under reduced pressure to obtain the product **S4** as a white solid (5.71 g, 74%).

^{1}H NMR (D_2O-*d*^{2}, 400 MHz): δ (ppm) = 1.16 (t, *J*=7.40 Hz, 3 H), 1.20 (d, *J*=7.03 Hz, 3 H), 1.39 - 1.50 (m, 3 H), 1.69 - 1.80 (m, 1 H), 2.11 - 2.22 (m, 1 H), 2.30 - 2.33 (m, 2 H), 3.96 (d, *J*=17.32 Hz, 4 H), 4.05 (d, *J*=17.57 Hz, 1 H), 4.11 (d, *J*=2.01 Hz, 2 H), 4.54 (d, *J*=7.03 Hz, 1 H).

^{13}C NMR (D_2O-*d*^{2}, 101 MHz): δ (ppm) = 10.4, 14.7, 24.1, 36.2, 40.2, 43.0, 58.3, 166.8, 172.1.

HRMS (ESI): *m/z* calc. for $C_{10}H_{19}N_3O_4$ $(M+H)^+$ 246.1448, found 246.1440.

1.3.2 Preparation of tryptathionine 7[8]



Scheme S6. Synthesis of tryptathionine **7**: a) Boc_2O, $NaHCO_3$, dioxane/H_2O, r.t., 16 h, 99%; b) SO_2Cl_2, $CHCl_3$, r.t., 1 h; c) **8**, $NaHCO_3$, $CHCl_3$, 0°C to r.t., quant.

Synthesis of (*N*-Boc)$_2$-cystine-(O*t*Bu)$_2$ 18

A solution of L-cystine-(OtBu)$_2$ (**17**, 10 g, 24 mmol, 1.0 eq.) in a 1:1 mixture of H_2O/dioxane (240 mL) was treated with $NaHCO_3$ (8.06 g, 96.0 mmol, 4.00 eq.) and Boc_2O (10.1 mL, 47.0 mmol, 2.00 eq.) and the reaction mixture was stirred for 16 h at r.t. The reaction mixture was concentrated under reduced pressure and the aqueous layer was extracted with EtOAc (3 x 120 mL). The organic layer was washed with brine (100 mL), dried over Na_2SO_4 and the solvent was removed under reduced pressure to afford **18** (13.2 g, 24.0 mmol, quant.) as a pale yellow solid.

^{1}H-NMR (400 MHz, $CDCl_3$-*d*^{1}): δ (ppm) = 5.35 (d, *J*=7.03 Hz, 2 H), 4.34 - 4.60 (m, 2 H), 3.01 - 3.30 (m, 4 H), 1.48 (s, 36 H).

^{13}C-NMR (101 MHz, $CDCl_3$-*d*^{1}): δ (ppm) = 27.6, 28.0, 41.7, 53.4, 79.6, 82.4, 154.7, 169.3.

HRMS (ESI): *m/z* calc for $C_{24}H_{44}N_2O_8S_2$ $(M+H)^+$ 553.2612, found 553.2615.

Synthesis of thioether building block 7

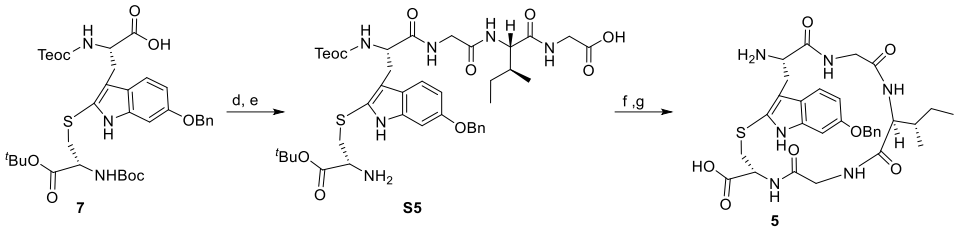
To a solution of (*N*-Boc)$_2$-L-Cystine-(OtBu)$_2$ (**18**, 506 mg, 0.92 mmol, 1.00 eq.) in $CHCl_3$ (9.2 mL, 10 mL/mmol) was added SO_2Cl_2 (223 µL, 2.76 mmol, 3.00 eq.). After the reaction mixture was stirred for 1 h at r.t. The solvent was removed under reduced pressure. The residue was redissolved in $CHCl_3$ (16.3 mL) and cooled to 0°C. The solution was added dropwise to an ice cold solution of **8** (500 mg, 1.10 mmol, 1.20 eq.) and $NaHCO_3$ (231 mg, 2.76 mmol, 3.00 eq.) in $CHCl_3$ (9.9 mL, 9 mL/mmol) over a periode of 10 min. Afterwards the reaction mixture was stirred for 15 min at 0°C and 1 h at r.t. The organic layer was washed with H_2O (5 mL) and 10% $KHSO_4$ solution (5 mL). After drying of the organic layer with Na_2SO_4 and removal of the solvent under reduced pressure the crude product was purified by C18 reverse phase chromatography (AcN/H_2O 30% to 100%, 30min) to give compound **7** as a yellow solid (810 mg, quant).

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) = -0.10 - 0.01 (m, 9 H), 0.77 - 0.88 (m, 2 H), 1.31 - 1.44 (m, 18 H), 2.90 - 3.07 (m, 2 H), 3.08 - 3.25 (m, 2 H), 3.78 - 3.87 (m, 1 H), 3.91 (q, *J* = 8.28 Hz, 1 H), 4.17 (d, *J* = 5.27 Hz, 1 H), 5.11 (s, 2 H), 6.73 (dd, *J* = 8.78, 2.26 Hz, 1 H), 6.83 (d, *J* = 2.01 Hz, 1 H), 7.01 (d, *J* = 8.28 Hz, 1 H), 7.23 (d, *J* = 8.28 Hz, 1 H), 7.29 - 7.35 (m, 1 H), 7.35 - 7.42 (m, 3 H), 7.42 - 7.49 (m, 2 H), 11.04 (s, 1 H), 12.56 (br. s., 1 H).

^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) = -1.5, 17.2, 27.5, 28.1, 36.8, 54.1, 54.8, 61.7, 69.5, 78.4, 80.9, 95.2, 109.6, 116.6, 119.5, 121.9, 123.5, 127.4, 127.7, 128.4, 137.5, 155.3, 156.0, 170.0, 173.5.

HRMS (ESI): *m/z* calc for $C_{36}H_{51}N_3O_9SSi$ $[M+H]^+$ 730.3183 found 730.3188.

1.3.3 Preparation of monocyclic pentapeptide 5



Scheme S7. d) DSC, 2,4,6-collidine, glycyl-L-isoleucineglycine (**S4**), r.t., 3 h, 90%; e) PTSA, THF, 5 h, r.t., 70% over 2 steps; f) T3P, DIPEA, DMF/DCM, r.t., 16 h, 70%; g) TFA/H_2O (7:3), r.t., 2 h, quant.

Synthesis of pentapeptide S5

A solution of thioether building block **7** (800 mg, 1.10 mmol, 1.00 eq.) in AcN (5.5 mL) was treated with collidine (318 µL, 2.41 mmol, 2.2 eq) and *N,N'*-disuccinimidyl carbonate (365 mg, 1.42 mmol, 1.30 eq.) and stirred for 2 h at r.t.. A solution of tripeptide **S4** (537 mg, 2.20 mmol, 2.00 eq) in a 1:4 mixture of AcN/H_2O (5 mL) was added and the reaction mixture was stirred for 2 h at r.t. Afterwards, the mixture was diluted with EtOAc (100 mL), 10% $KHSO_4$-solution (20 mL) was added and the aqueous layer was extracted with EtOAc (2 x 20 mL). The organic layer was washed with brine (2 x 20 mL), dried over Na_2SO_4 and evaporated under reduced pressure. The residue was dissolved in THF (5.7 mL), treated with PTSA (2.0 g, 11 mmol) and stirred for 5 h at r.t. The pentapeptide was precipitated in Et_2O and The crude product was purified by C18 reverse phase chromatography (AcN/H_2O 40% to 100%, 30 min) to afford pentapeptide **S5** as a yellow solid (626 mg, 70%).

^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) = 11.12 (s, 1 H), 8.27 (br. s., 1 H), 8.12 - 8.20 (m, 1 H), 7.73 (d, *J*=9.03 Hz, 1 H), 7.44 - 7.51 (m, 3 H), 7.39 (t, *J*=7.40 Hz, 2 H), 7.29 - 7.35 (m, 1 H), 7.12 (d, *J*=7.78 Hz, 1 H), 6.95 (d, *J*=8.03 Hz, 1 H), 6.83 (d, *J*=2.01 Hz, 1 H), 6.72 (dd, *J*=8.66, 1.88 Hz, 1 H), 5.11 (s, 2 H), 4.20 (dt, *J*=16.12, 8.12 Hz, 2 H), 3.90 (dd, *J*=9.54, 6.53 Hz, 2 H), 3.58 - 3.82 (m, 5 H), 3.40 (br. s., 1 H), 3.15 (dd, *J*=14.18, 4.64 Hz, 1 H), 2.90 - 3.08 (m, 3 H), 1.75 (br. s., 1 H), 1.33 - 1.41 (m, 9 H), 1.03 - 1.16 (m, 1 H), 0.73 - 0.89 (m, 8 H), -0.03 (s, 9 H).

^{13}C-NMR (101 MHz, DMSO-d_6): δ (ppm) = -1.1, 11.5, 15.9, 17.6, 24.5, 28.0, 42.7, 54.9, 57.0, 62.3, 69.9, 81.2, 95.6, 110.0, 119.1, 122.6, 124.5, 125.9, 127.8, 128.1, 128.5, 128.8, 137.8, 137.9, 155.5, 164.3, 167.1, 168.9, 171.6.

HRMS (ESI): *m/z* calc. for $C_{46}H_{68}N_6O_{12}SSi$ $(M+H)^+$ 857.3934, found 857.3914.

Synthesis of monocyclic pentapeptide 5

Pentapeptide **S5** (151 mg, 0.180 mmol, 1.00 eq.) was dissolved in a mixture of DCM/DMF (9:1, 36 mL), then DIPEA (60.2 µL, 354 µmol, 2.00 eq.) and T3P (50% in EtOAc, 210 µL, 354 µmol, 0.34 eq.) were added. After the solution was stirred for 16 h at r.t. 2/3 of the the solvent was concentrated under reduced pressure. The organic phase was washed with 10% $KHSO_4$-solution (20 mL), sat. $NaHCO_3$-solution (20 mL), water (20 mL) and brine (20 mL). The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude product was purified by C18 reverse phase chromatography (AcN/H_2O 50% to 100% gradient), then redissolved in a mixture of TFA/water (7:3) and stirred for 16 h. After precipitation in Et_2O monocyclic pentapeptide **5** was afforded as a white powder (57.5 mg, 70% over 2 steps)

^{1}H-NMR (500 MHz, DMSO-d_6): δ (ppm): 0.79 - 0.88 (m, 6 H), 1.04 - 1.15 (m, 1 H), 1.43 - 1.52 (m, 1 H), 1.87 - 1.97 (m, 1 H), 2.77 - 2.88 (m, 2 H), 3.38 - 3.47 (m, 2 H + water), 3.74 (d, *J*=4.88 Hz, 2 H), 3.77 - 3.86 (m, 2 H), 3.87 - 3.93 (m, 1 H), 4.00 (t, *J*=8.47 Hz, 1 H), 4.06

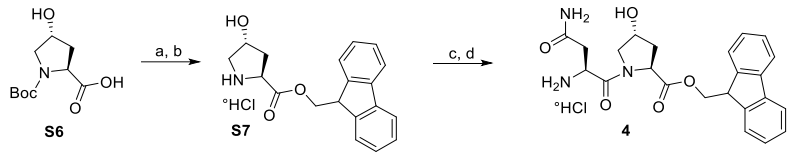
- 4.13 (m, 1 H), 5.15 (s, 2 H), 6.81 (dd, *J*=8.70, 2.29 Hz, 1 H), 6.91 (d, *J*=2.29 Hz, 1 H), 7.33 (t, *J*=7.17 Hz, 1 H), 7.40 (t, *J*=7.48 Hz, 2 H), 7.47 (d, *J*=7.02 Hz, 2 H), 7.77 (d, *J*=8.70 Hz, 1 H), 7.86 (t, *J*=4.58 Hz, 1 H), 7.93 (br. s., 2 H), 8.17 (d, *J*=8.39 Hz, 1 H), 8.36 (d, *J*=7.78 Hz, 1 H), 9.09 (t, *J*=5.11 Hz, 1 H), 11.27 (s, 1 H).

^{13}C-NMR (176 MHz, DMSO-d_6): δ (ppm) = 10.8, 15.5, 24.5, 27.5, 35.5, 36.2, 42.5, 43.5, 50.4, 52.5, 59.1, 69.5, 95.4, 109.8, 114.4, 116.3, 118.0, 120.0, 121.2, 123.9, 127.4, 128.4, 137.5, 138.3, 155.5, 157.8, 158.0, 168.2, 168.7, 169.4, 171.1, 172.1.

HRMS (ESI): *m/z* calc. for $C_{31}H_{38}N_6O_7S$ $(M+H)^+$ 639.2595, found. 639.2603.

1.4. Preparation of α-amanitin (1)

1.4.1 Preparation of dipeptide building block



Scheme S8. a) 9-fluorenemethanol, EDC*HCl, DMAP, DMF, r.t., 2 h; b) HCl (4 M in Dioxane), r.t., 30 min, 56%; c) Boc-Asn-OH, EDC*HCl, HOBt*H_2O, DMF, r.t., 16 h; d) HCl (4 M in Dioxane), r.t., 30 min, 72%.

Synthesis of (9H-fluoren-9-yl)methyl (2*S*,4*R*)-4-hydroxypyrrolidine-2-carboxylate (S7)

A solution of *N*-Boc-protected (2*S*,4*R*)-4-hydroxyproline **S6** (5.0 g, 22 mmol, 1.0 eq.) in DMF (20 mL) was added dropwise to a solution of 9-fluorenemethanol (8.5 mg, 43 mmol, 2.0 eq.), EDC*HCl (8.3 g, 43 mmol, 2.0 eq.) and DMAP (396 mg, 3.24 mmol, 0.150 eq.) in DCM (220 mL). The reaction mixture was stirred at r.t. for 2 h. Then, 10% $KHSO_4$ solution (50 mL) was added. The organic phase was washed with brine (50 mL) and dried over $NaSO_4$. Afterwards, the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (hexane/ethyl acetate = 1:1) and treated with 4 M HCl in dioxane for 30 min. Evaporation of the solvent under reduced pressure afforded the product **S7** as a white solid (3.7 g, 56%).

^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) = 1.81 (ddd, *J*=13.30, 10.79, 4.52 Hz, 1 H), 2.02 – 2.07 (m, 1 H), 3.06 (d, *J*=12.05 Hz, 1 H), 3.30 (dd, *J*=12.17, 4.39 Hz, 1 H), 4.34 – 4.37 (m, 2 H), 4.47 (dd, *J*=10.79, 7.28 Hz, 1 H), 4.55 (dd, *J*=10.54, 6.00 Hz, 1 H), 4.66 (dd, *J*=10.79, 6.78 Hz, 1 H), 5.57 (br. s., 1 H), 7.36 (q, *J*=7.03 Hz, 2 H), 7.44 (t, *J*=7.53 Hz, 2 H), 7.70 (dd, *J*=18.32, 7.28 Hz, 2 H), 7.92 (d, *J*=7.78 Hz, 2 H).

^{13}C-NMR (101 MHz, DMSO-d_6): δ (ppm) = 36.9, 46.2, 53.1, 57.4, 67.0, 68.3, 120.2, 125.2, 127.2, 127.9, 140.9, 143.1, 143.5, 188.7.

HRMS (ESI): *m/z* calc. for $C_{19}H_{19}NO_3$ $(M+H)^+$ 310.1438, found 310.1426.

Synthesis of (9H-fluoren-9-yl)methyl (2*S*,4*R*)-1-(L-asparaginyl)-4-hydroxypyrrolidine-2-carboxylate (4)

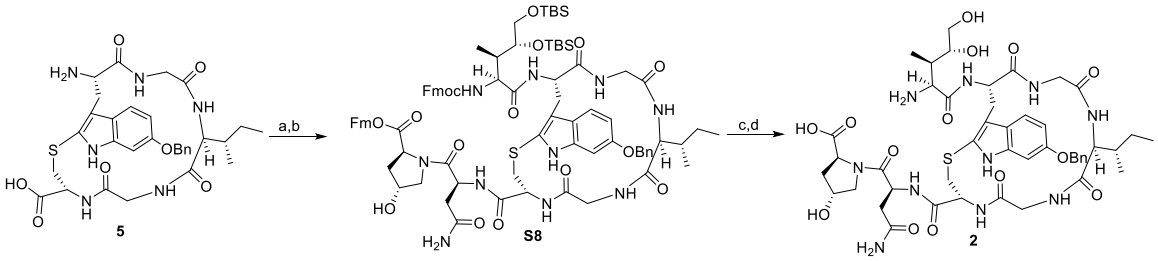
Boc-L-asparagine (1.7 g, 7.3 mmol, 1.0 eq.), **S7** (1.7 g, 4.8 mmol, 1.0 eq.), EDC°HCl (1.4 g, 7.3 mmol, 1.5 eq.) and HOBt*H_2O (1.5 g, 9.7 mmol, 2.0 eq.) were dissolved in DMF (72 mL) and stirred at r.t. for 16 h. The reaction mixture was diluted with EtOAc (200 mL). Then, 10% $KHSO_4$ solution (50 mL) was added. The organic phase was washed with 10% $KHSO_4$ solution (50 mL) and brine (2 x 50 mL). After drying over $NaSO_4$ and removal of the solvent under reduced pressure the crude product was redissolved in 4 M HCl and stirred for 1 h. After removal of the solvent under reduced pressure the crude product was purified by C18 reversed phase chromatography (ACN/H_2O 10% to 70%, 30 min) to afford dipeptide **4** as a white powder (1.6 g, 72%).

^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) = 1.42 – 1.48 (m, 1 H), 1.93 – 1.99 (m, *J*=8.78 Hz, 1 H), 3.41 (d, *J*=10.79 Hz, 1 H), 3.52 (d, *J*=11.54 Hz, 1 H), 4.23 (s, 1 H), 4.28 – 4.31 (m, 1 H), 4.34 – 4.37 (m, 2 H), 4.51 – 4.53 (m, 2 H), 5.29 (br. s, 1 H), 7.27 (br. s., 1 H), 7.33 – 7.37 (m, 2 H), 7.40 – 7.45 (m, 2 H), 7.65 (d, *J*=7.53 Hz, 1 H), 7.68 – 7.70 (m, 2 H), 7.90 (d, *J*=6.53 Hz, 2 H), 8.21 (br. s., 3 H).

^{13}C-NMR (101 MHz, DMSO-d_6): δ (ppm) = 34.2, 36.7, 46.5, 47.9, 54.6, 57.7, 65.5, 68.8, 120.1, 125.0, 127.2, 124.7, 140.8, 143.3, 143.6, 167.4, 169.7, 171.02.

HRMS (ESI): *m/z* calc. for $C_{23}H_{25}N_3O_5$ $(M+H)^+$ 424.1866, found 424.1858.

1.4.2 Preparation of the fully deprotected octapeptide 2[9,10]



Scheme S9. Fragment couplings towards the fully deprotected octapeptide **2**: a) Fmoc-Dhil(OTBS)$_2$-OH (**6**), COMU, MSA, DIPEA, 0°C to r.t., 3 h, 95%; b) HCl°Asn-Hyp-OFm (**4**), 0°C to r.t., 30 min; c) Et_2NH, DMF, r.t., 1 h, 90%; d) TBAF (1 M in THF), THF, r.t., 2 h, 77%.

Synthesis of monocyclic octapeptide S8

Fully deprotected monocyclic pentapeptide **5** (100 mg, 0.16 mmol, 1.00 eq.) was dissolved in DMA (1 mL) and MSA (28 µL, 0.17 mmol, 1.1 eq.) was added. The solution was stirred for 2 h at 50°C. A solution of Fmoc-Dhil(TBS)$_2$-OH (**6**, 105 mg, 0.17 mmol, 1.10 eq.), COMU (74 mg, 0.17 mmol, 1.1 eq.) and DIPEA (71 µL, 0.41 mmol, 2.60 eq.) in DMA (0.4 mL) was stirred for 30 min at 0°C.The silylated monocyclic pentapeptide was added to the preactivated amino acid and stirred for 1 h. Then, dipeptide **4** (107 mg, 0.230 mmol, 1.50 eq.) and HATU (89.2 mg, 0.230 mmol, 1.50 eq.) were added to the reaction mixture at 0°C. DIPEA (81.7 mL, 0.230 mmol, 1.50 eq.) was added and the reaction mixture was stirred for 2 h, then diluted with EtOAc (50 mL) and washed with 10% citric acid (2 x 10mL) and sat. $NaHCO_3$ (2 x 10 mL). The organic phase was washed with brine (2 x 20 mL), dried over $NaSO_4$ and evaporated under reduced pressure. The crude product **S8** was submitted to the next step without any further purification.

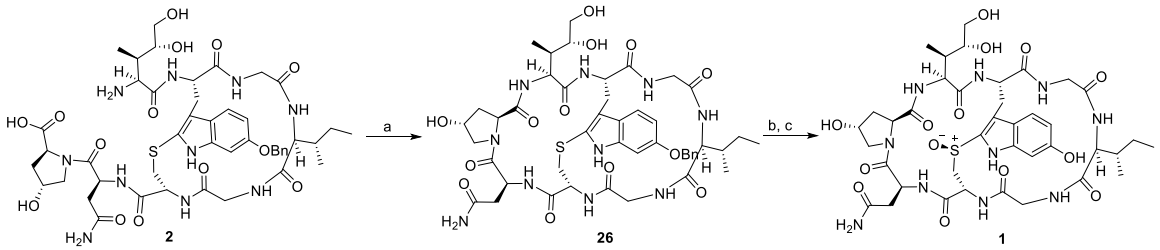
HRMS (ESI): *m/z* calc. for $C_{87}H_{110}N_{10}O_{16}SSi_2$ $(M+H)^+$ 1639.7433, found 1639.7404.

Synthesis of monocyclic octapeptide 2

Monocyclic octapeptide **S8** (250 mg, 152.0 µmol, 1.00 eq.) was dissolved in DMF (5 mL). Et_2NH (563 µL, 1.52 mmol, 10 eq.) was added and stirred for 1 h at r.t. The solvent was removed under reduced pressure and the precipitate was redissolved in THF (3 mL). Then, a solution of TBAF in THF (1 M, 1.5 mL, 10 eq) was added and the reaction mixture was stirred for 2 h at r.t. The solvent was evaporated *in vacuo* and the crude product purified by C18 reverse phase chromatography (AcN/H_2O 5% to 70%, 30 min) to afford the product **2** as a white solid (121 mg, 77%).

HRMS (ESI): *m/z* calc. for $C_{46}H_{62}N_{10}O_{14}S$ $(M+H)^+$ 1011.4240, found 1001.4241.

1.4.3 Final preparation of α-amanitin (1)[11,12]



Scheme S10. Final cyclization and sulfide oxidation reaction affording α-amanitin (**1**): a) HATU, DIPEA, DMF, r.t., 16 h, 68%; b) BF_3°OEt_2, EtSH, r.t., 2 h, c) *m*CPBA, *i*PrOH/EtOH 2:1, r.t. 30 min, 35 % over 2 steps.

Synthesis of thioether α-amanitin precursor 26

Monocyclic octapeptide **2** (20.0 mg, 19.2 µmol, 1.00 eq.) was dissolved in DMF (19 mL). Then, DIPEA (6.71 µL, 38.5 µmol, 2.00 eq.) and HATU (4.98 mg, 38.5 µmol, 2.00 eq) was added at 0°C. The reaction mixture was allowed to warm to r.t. overnight and concentrated under reduced pressure. The crude product was purified using preparative HPLC (0-30 min 5-40% B, 30-35 min 40-100% B, 35-40 min 100% B, 40-45 min 100-5% B) to afford bicyclic octapeptide **26** (13 mg, 68%) as a white powder.

^{1}H-NMR (700 MHz, DMSO-d_6): δ (ppm) = 0.79 (d, *J*=6.83 Hz, 1 H), 0.83 (t, *J*=7.37 Hz, 2 H), 0.88 (m, *J*=7.05 Hz, 2 H), 1.12 (m, *J*=7.90 Hz, 1 H), 1.56 (m, *J*=12.49, 5.66 Hz, 1 H), 1.88 (td, *J*=11.90, 3.10 Hz, 1 H), 2.20 (m, *J*=6.83 Hz, 1 H), 2.76 (dd, *J*=10.89, 3.20 Hz, 1 H), 2.94 (dd, *J*=15.91, 4.38 Hz, 1 H), 2.96 (m, 1H), 3.01 (m, *J*=11.42, 11.42 Hz, 1 H), 3.24 (m, *J*=13.45 Hz, 1 H), 3.40 (m, *J*=4.27 Hz, 1 H), 3.43 (m, *J*=5.34 Hz, 1 H), 3.50 (m, *J*=4.91 Hz, 1 H), 3.71 (dd, *J*=8.22, 4.38 Hz, 1 H), 3.74 (d, *J*=10.68 Hz, 1 H), 3.81 (d, *J*=8.33 Hz, 1 H), 3.91 (dd, *J*=17.19, 7.58 Hz, 1 H), 4.18 (dd, *J*=18.68, 8.22 Hz, 1 H), 4.28 (dd, *J*=11.42, 6.94 Hz, 1 H), 4.41 (m, *J*=5.12 Hz, 1 H), 4.45 (dd, *J*=9.39, 5.98 Hz, 1 H), 4.56 (m, *J*=3.42 Hz, 1 H), 4.71 (d, *J*=3.84 Hz, 1 H), 4.83 (d, *J*=5.34 Hz, 1 H), 4.93 (m, *J*=13.88 Hz, 1 H), 5.08 - 5.13 (m, 1 H), 5.24 (br. s., 1 H), 6.77 (dd, *J*=8.65, 2.24 Hz, 1 H), 6.81 (d, *J*=2.14 Hz, 1 H), 7.33 (m, *J*=7.47 Hz, 1 H), 7.40 (t, *J*=7.58 Hz, 1 H), 7.46 (m, *J*=7.47 Hz, 2 H), 7.88 (d, *J*=7.90 Hz, 1 H), 7.96 (d, *J*=9.61 Hz, 1 H), 8.00 (s, 0 H), 8.08 (d, *J*=8.54 Hz, 1 H), 8.14 (br. s., 1 H), 8.42 (br. s., 1 H), 8.51 (br. s., 1 H), 8.83 (m, *J*=6.41 Hz, 1 H), 11.04 (s, 1 H).

^{13}C-NMR (175 MHz, DMSO-d_6): δ (ppm) = 10.9, 13.8, 15.0, 25.3, 30.1, 34.4, 34.8, 37.5, 37.9, 38.2, 38.4, 41.7, 42.4, 50.7, 52.5, 53.5, 55.4, 55.9, 59.3, 62.0, 68.8, 69.5, 72.8, 98.7, 114.1, 125.3, 131.6, 131.8, 132.6.

HRMS (ESI): *m/z* calc. for $C_{46}H_{60}N_{10}O_{13}S$ $(M+H)^+$ 993.4135, found 993.4145.

Synthesis of α-amanitin (1)

Bicyclic octapeptide **26** (8 mg, 8.9 µmol, 1.00 eq.) was dissolved in EtSH (1 mL) and treated with BF_3°OEt_2 (23 µL, 178 µmol, 20.0 eq.) under vigorous stirring for 2 h after which the fully deprotected bicyclic octapeptide was precipitated in Et_2O. The white powder was dissolved in 1 mL of a mixture of *i*PrOH/EtOH (2:1). Then, a solution of mCPBA (0.7 eq) in *i*PrOH/EtOH (2:1) was added dropwise. The resulting solution of was stirred for 30 min after which the reaction was terminated by the addition of water (1 mL). The crude product was purified *via* preparative HPLC (0-30 min 5-11% B, 30-35 min 11-100% B, 35-40 min 100% B, 40-45 min 100-5% B) which afforded α-amanitin (**1**, 2.9 mg, 35%) as a white powder.

^{1}H-NMR (700 MHz, DMSO-d_6): δ (ppm): 0.76 - 0.91 (m, 9 H), 1.07 - 1.15 (m, 1 H), 1.55 (d, *J*=14.09 Hz, 2 H), 1.81 - 1.89 (m, 1 H), 2.01 (dd, *J*=15.48, 7.79 Hz, 1 H), 2.08 - 2.14 (m, 1 H), 2.19 (dd, *J*=11.85, 6.73 Hz, 1 H), 2.73 (m, *J*=14.31 Hz, 1 H), 2.96 (d, *J*=9.39 Hz, 2 H), 3.08 (t, *J*=13.24 Hz, 1 H), 3.19-3.22 (m 1 H), 3.28 (m, 2 H), 3.32 (m, 1 H), 3.43 (m, 1 H), 3.50 (m, 1 H), 3.67 (d, *J*=5.12 Hz, 2 H), 3.74 (d, *J*=10.04 Hz, 1 H), 3.80 (d, *J*=8.75 Hz, 1 H), 3.87 - 3.96 (m, 1 H), 4.22 - 4.33 (m, 2 H), 4.35 - 4.43 (m, 2 H), 4.65 (br. s., 1 H), 4.87 - 4.98 (m, 2 H), 6.60 (dd,

J=8.54, 1.92 Hz, 1 H), 6.75 (d, *J*=1.92 Hz, 1 H), 7.44 (d, *J*=8.54 Hz, 1 H), 7.60 (br. s., 1 H), 7.80 (d, *J*=9.39 Hz, 1 H), 7.90 (d, *J*=7.69 Hz, 1 H), 7.98 (d, *J*=9.18 Hz, 1 H), 8.30 (d, *J*=10.03 Hz, 1 H), 8.39 (br. s., 1 H), 8.42 - 8.47 (m, 1 H), 8.52 (br. s., 1 H), 8.78 (br. s., 1 H), 9.28 (d, *J*=4.06 Hz, 1 H), 11.26 (s, 1 H).

^{13}C-NMR (175 MHz, DMSO-d_6):: 11.0, 13.6, 15.1, 25.6, 28.8, 33.6, 35.0, 33.6, 33.7, 37.7, 38.3, 41.2, 42.8, 50.2, 51.0, 53.1, 55.5, 56.0, 59.1, 59.6, 62.3, 63.5, 68.9, 72.4, 96.8, 111.1, 111.9, 120.9, 122.6.

HRMS (ESI): *m/z* calc. for $C_{39}H_{54}N_{10}O_{14}S$ $(M+H)^+$ 919.3614, found 919.3618.

2. Analytical Data

2.1 Chiral GC-MS analysis

GC-MS chromatogram of the asymmetric ruthenium catalyzed allylic alkylation product (**11**) after methylation of the carboxylic group:

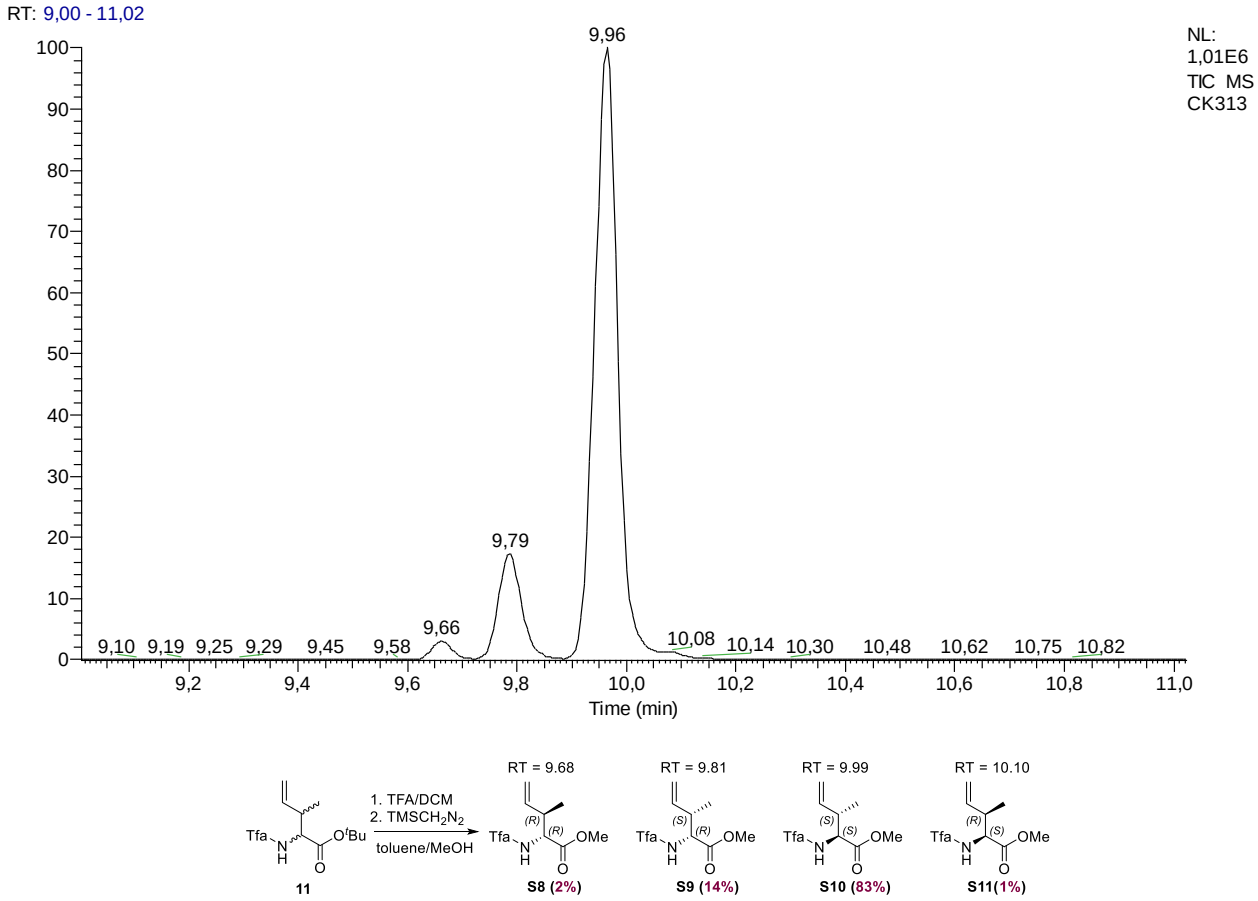


Figure S1: Chiral GC-MS chromatogram of the methylated and Tfa-protected 4,5-didehydroisoleucine **11** resulting from the asymmetric ruthenium-catalyzed allylic alkylation reaction.

2.2 NMR spectra

2.2.1 Comparison of NMR spectra of synthetic and natural α-amanitin

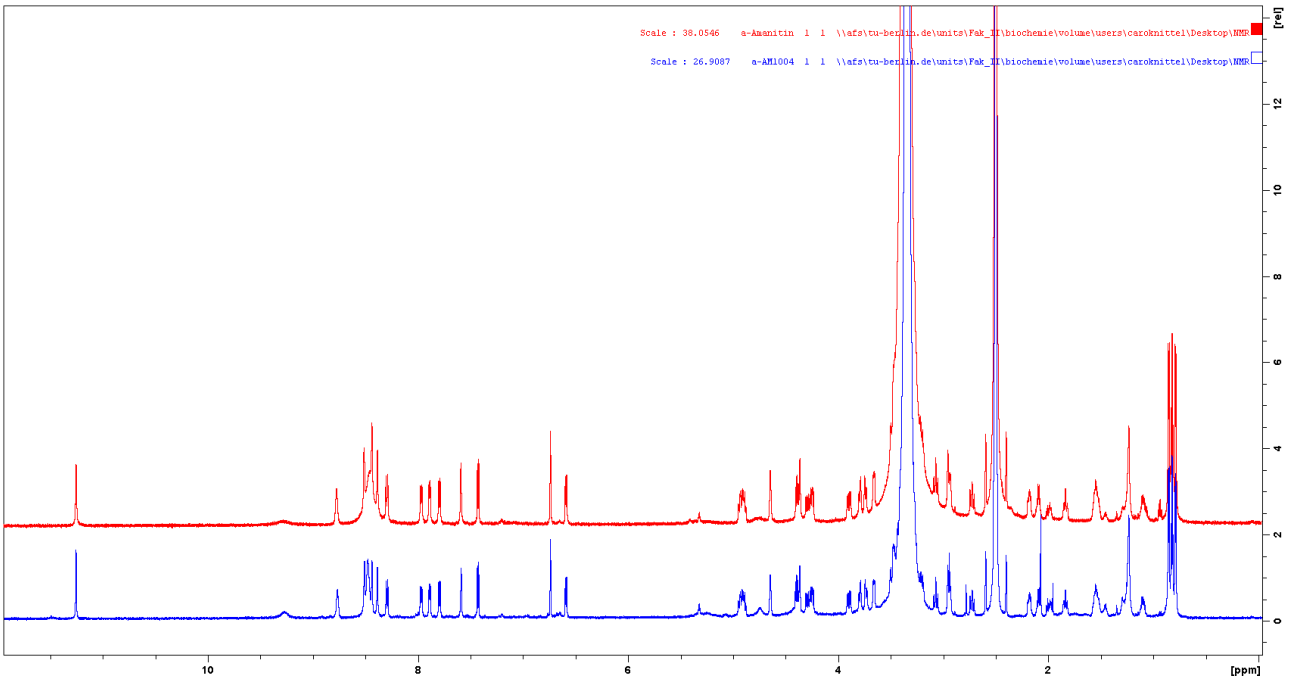


Figure S2: Overlay of ^{1}H NMR spectra of synthetic α-amanitin (**1**, blue) and natural α-amanitin (red, purchased from Sigma Aldrich). Both compounds were subjected to the same HPLC-purification conditions (0-30 min 5-11% B, 30-35 min 11-100% B, 35-40 min 100% B, 40-45 min 100-5% B) prior to NMR measurement (700 MHz). ^{1}H NMR chemical shifts are summarized in Table S1 on page 44.

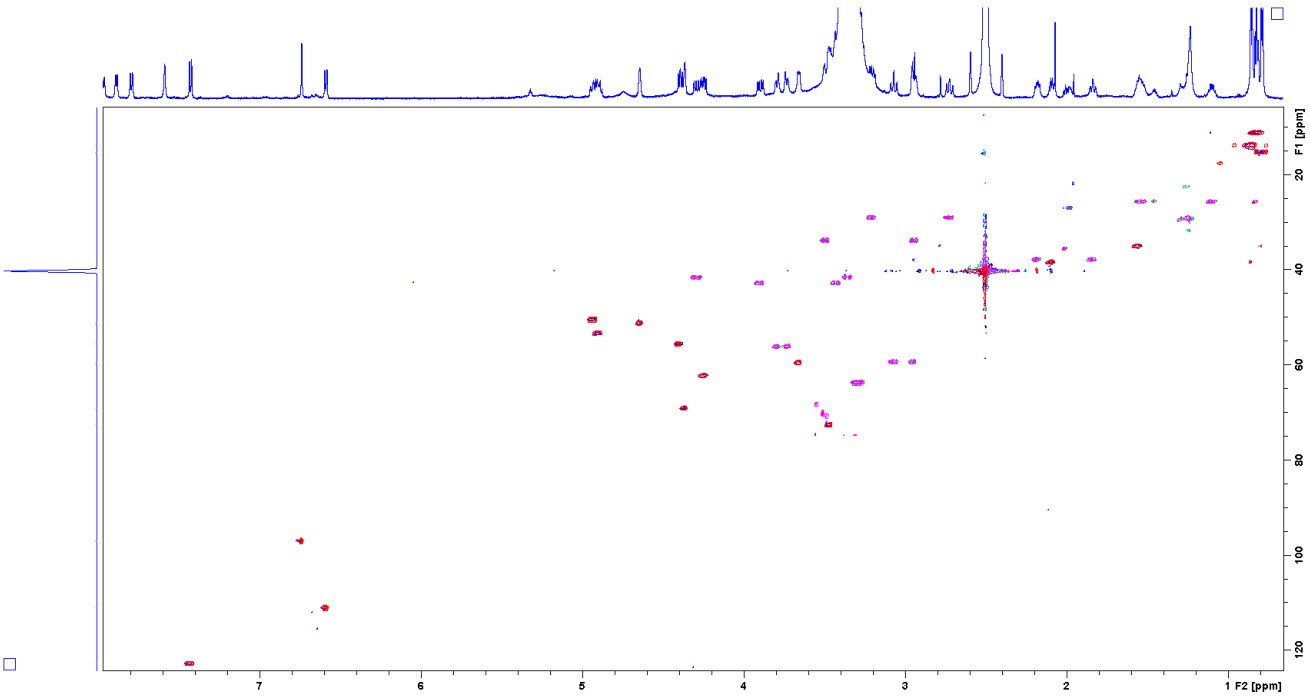
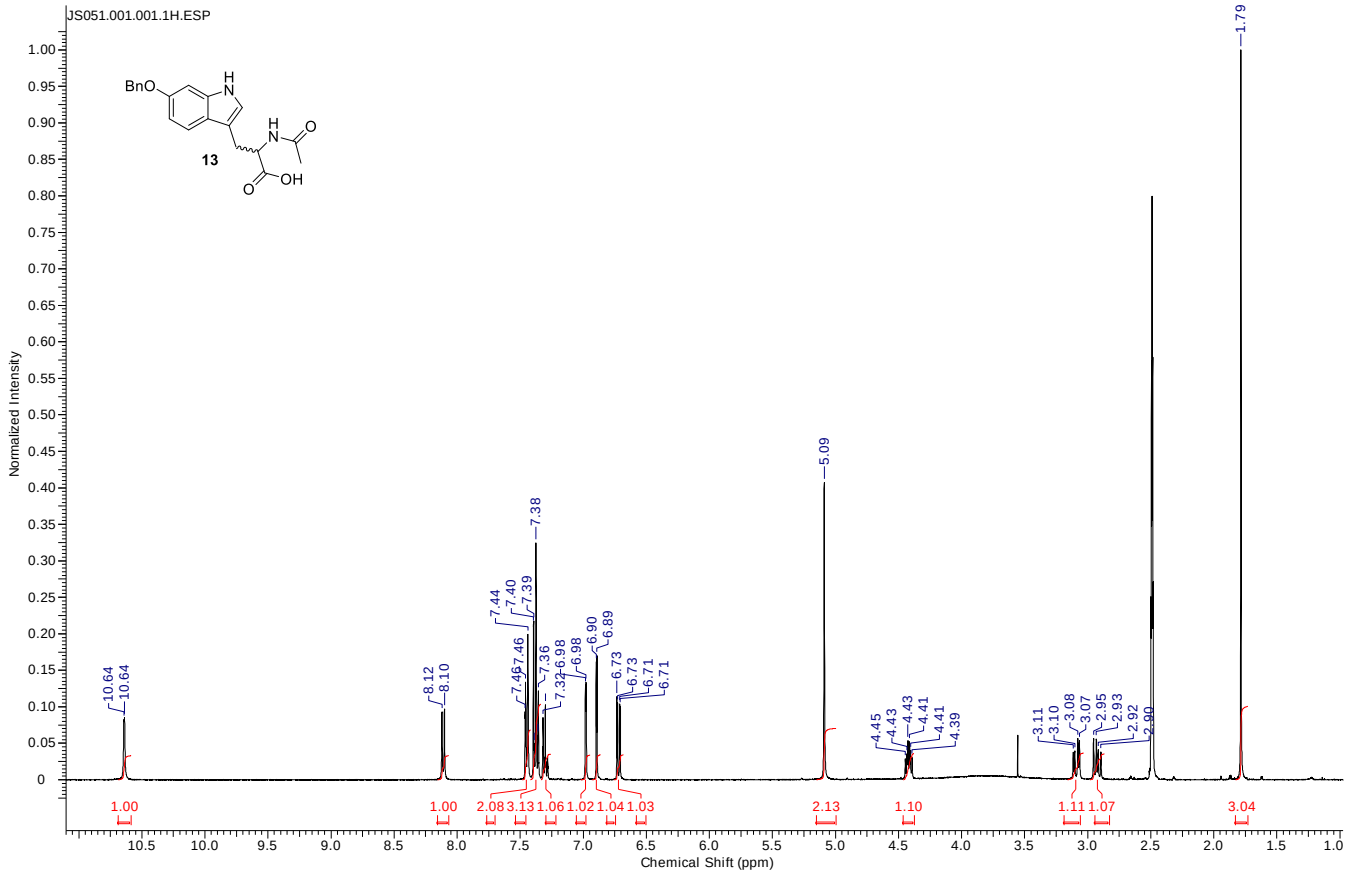
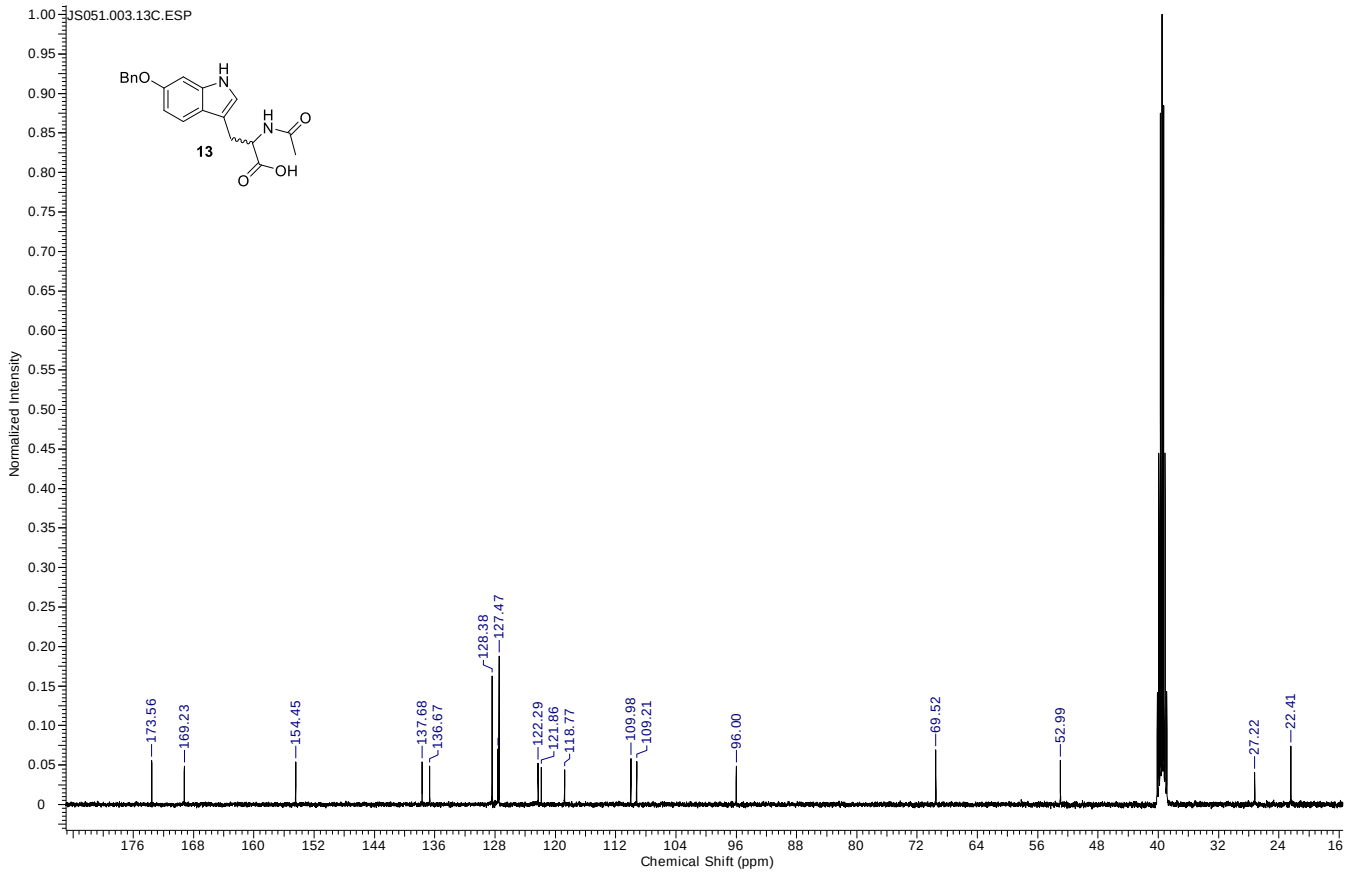
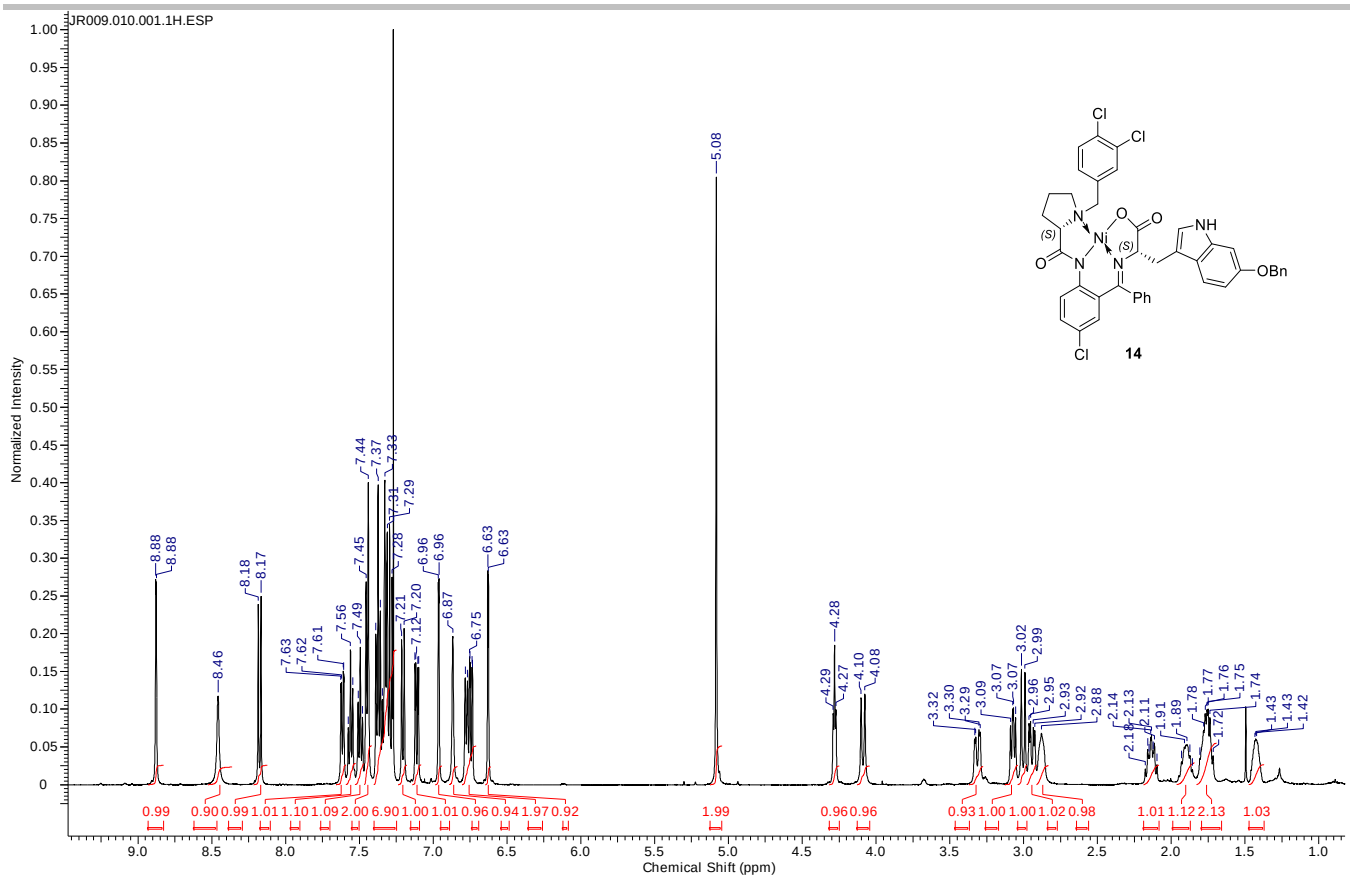


Figure S3: Overlay of HSQC spectra of synthetic α-amanitin (**1**, blue/green) and natural α-amanitin (red/violet, purchased from Sigma Aldrich).

2.2.2 NMR spectra of synthetic intermediates



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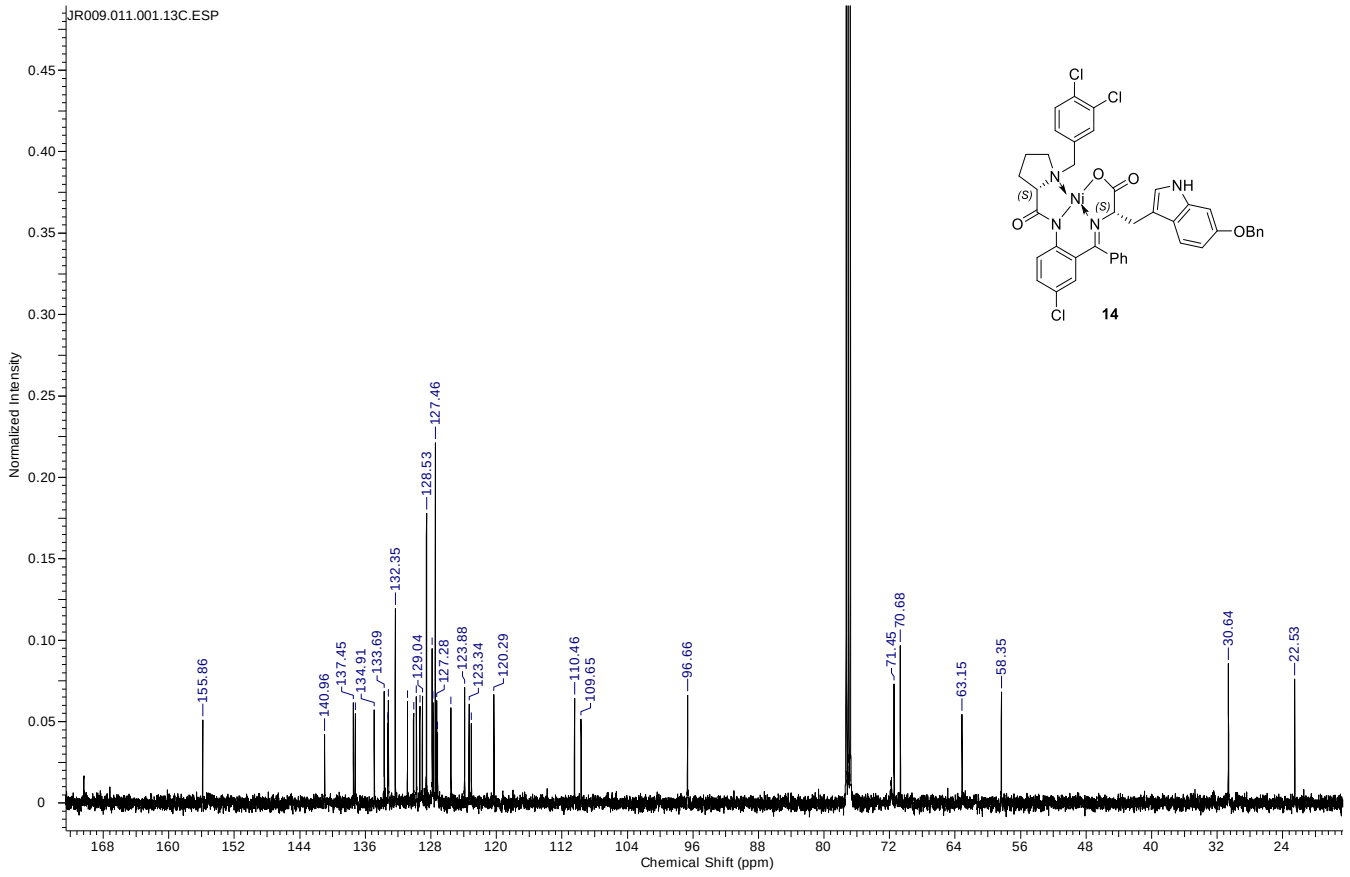
Normalized Intensity
Chemical Shift (ppm)
14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Chemical Shift (ppm)
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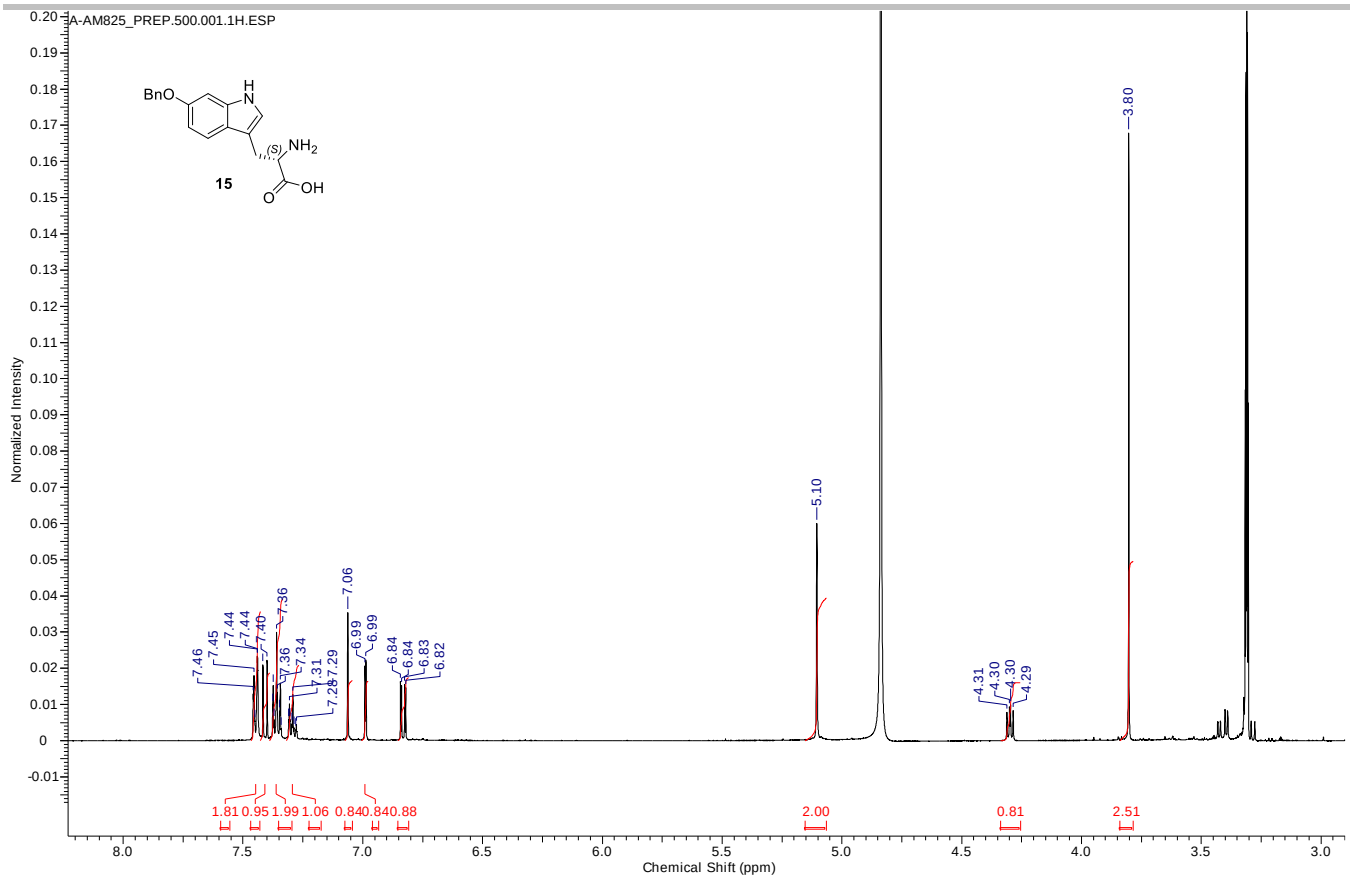
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N
(S)
NH2
O
OH
15
Normalized Intensity
Chemical Shift (ppm)
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7.44
7.44
7.40
7.36
7.36
7.34
7.31
7.29
7.28
7.06
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6.99
6.84
6.84
6.83
6.82
5.10
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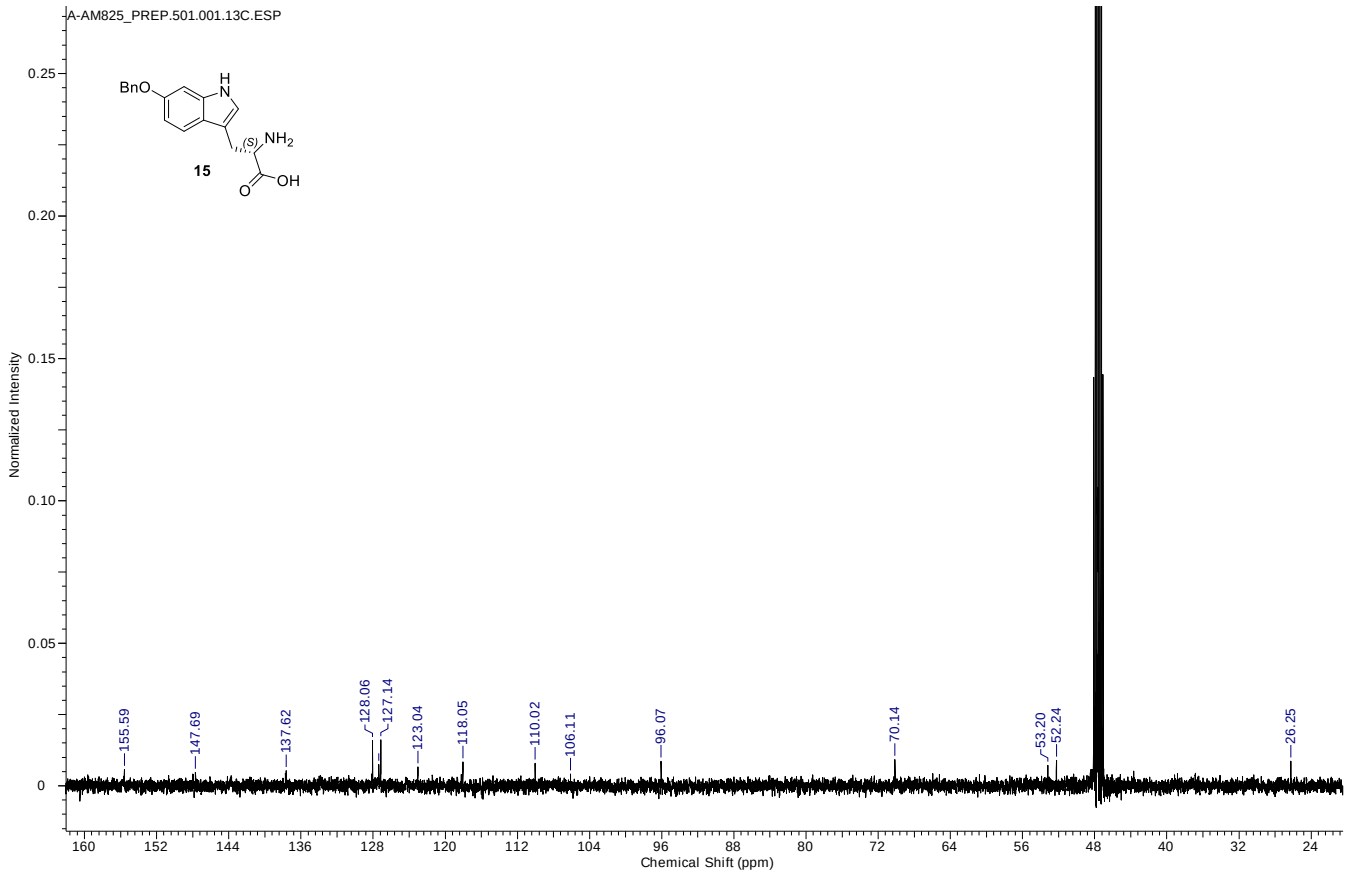
2.51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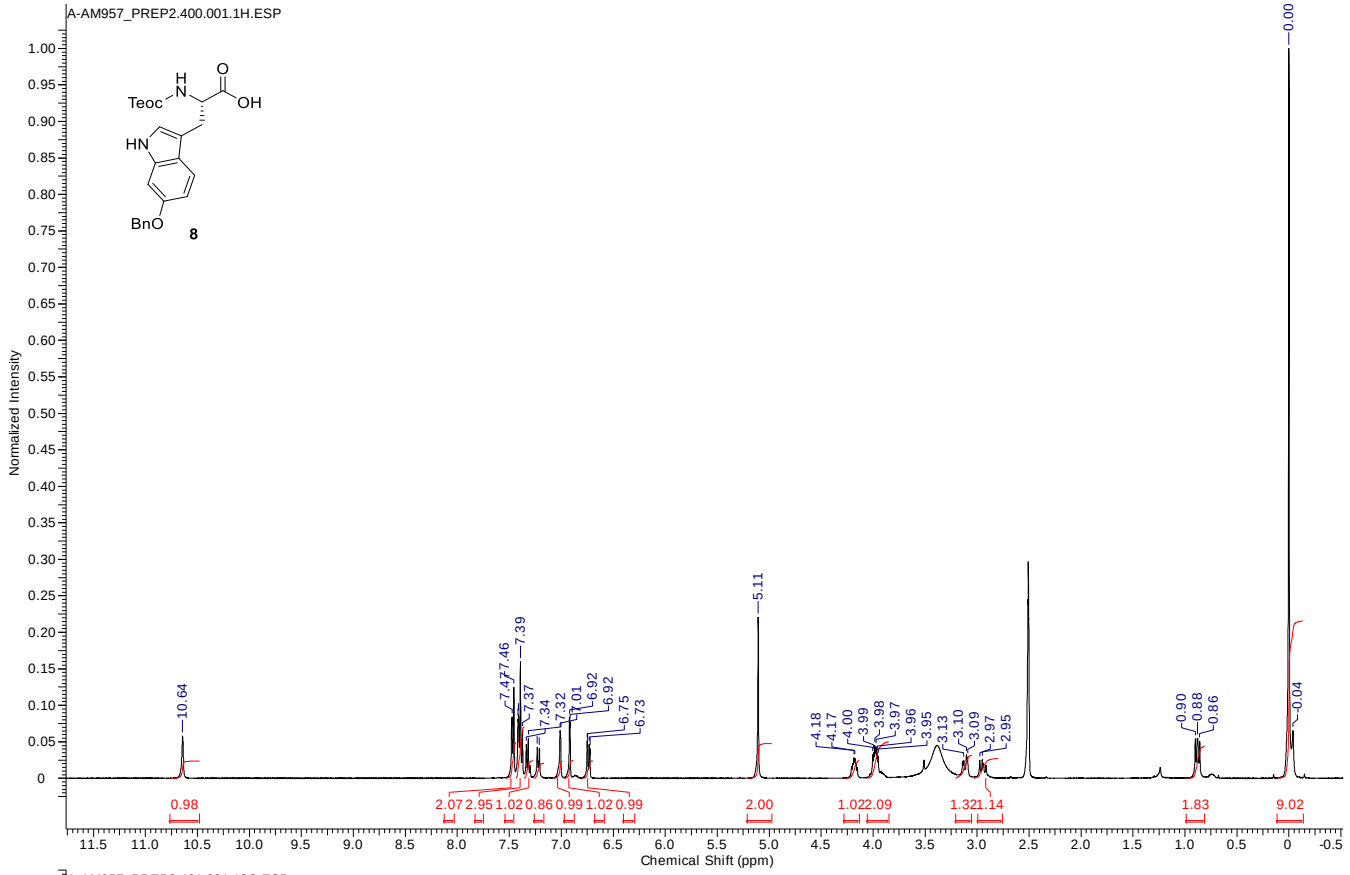
52.24
26.25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HN
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7.32
7.01
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6.92
6.75
6.73
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4.17
4.00
3.99
3.98
3.97
3.96
3.95
3.13
3.10
3.09
2.97
2.95
0.90
0.88
0.86
0.00
0.04
0.98
2.07
2.95
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2.09
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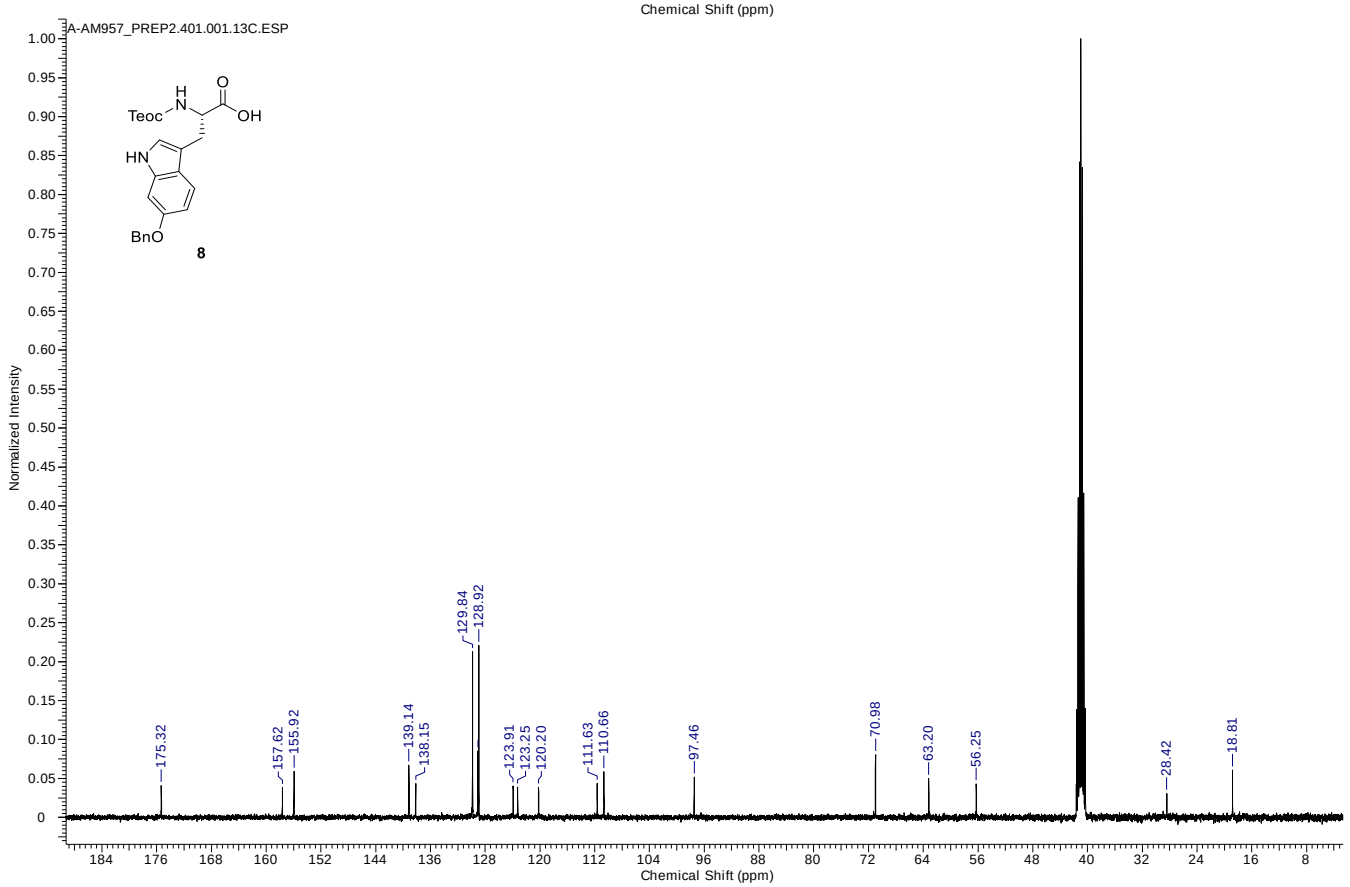
9.02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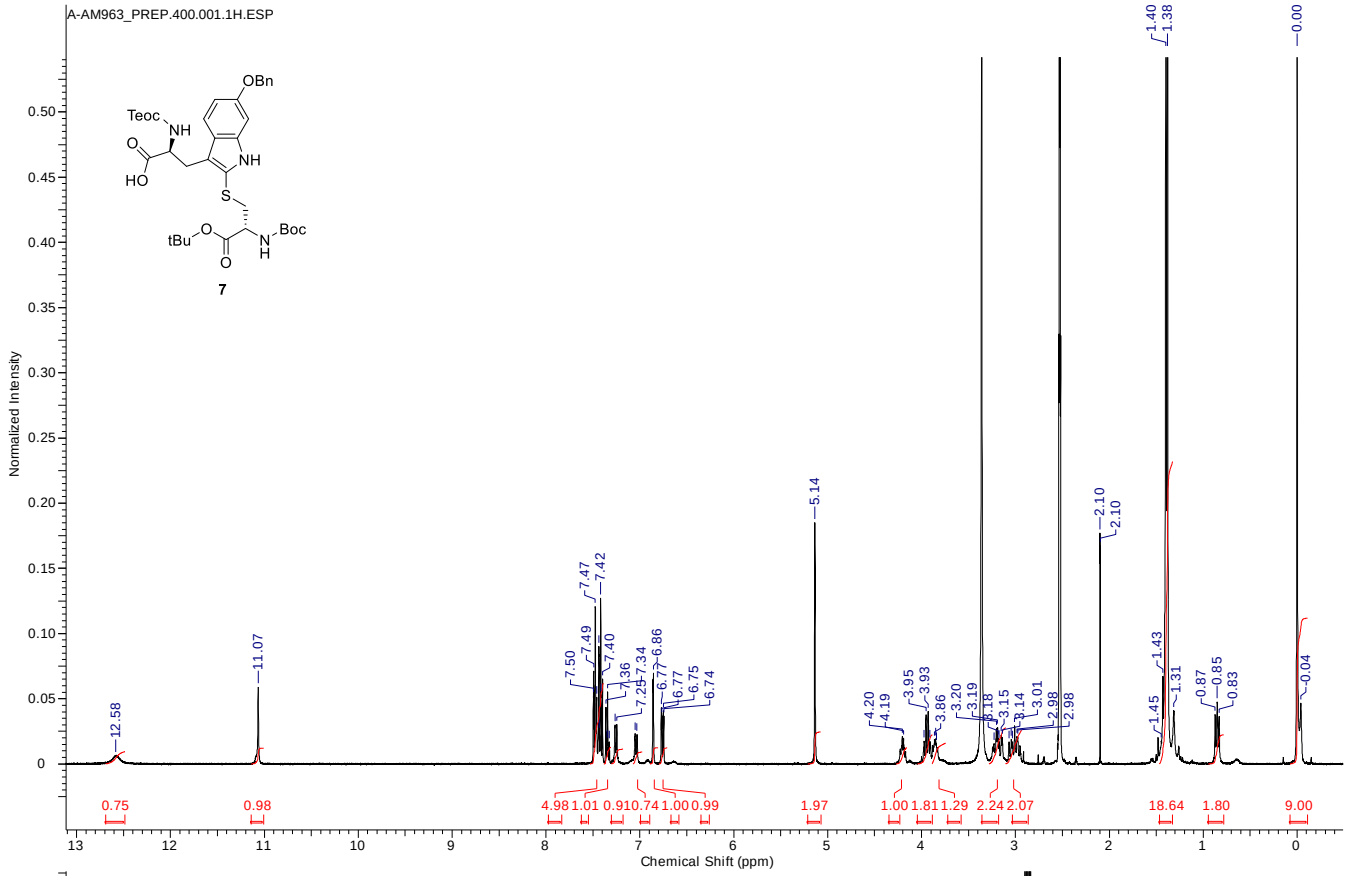
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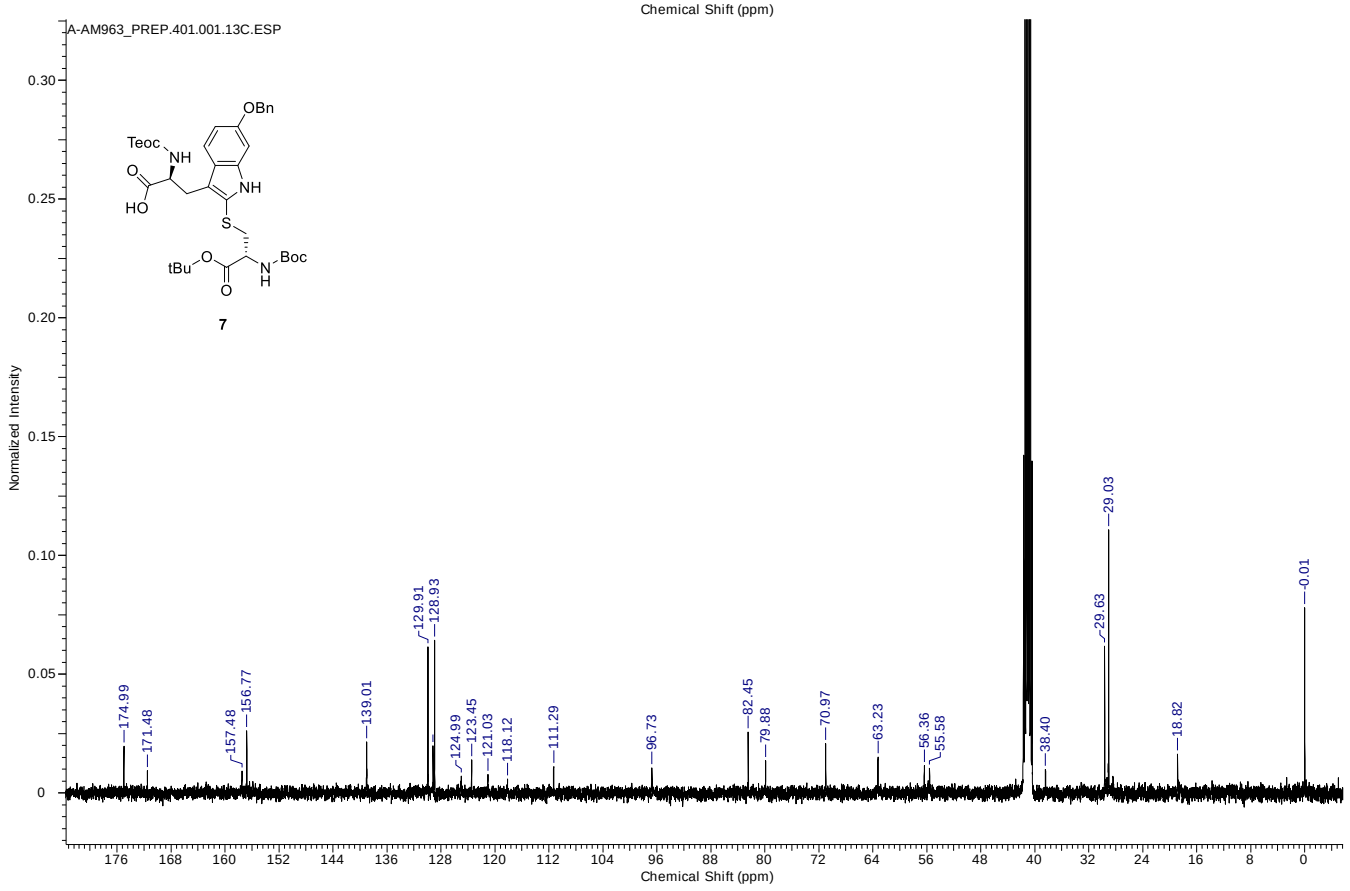
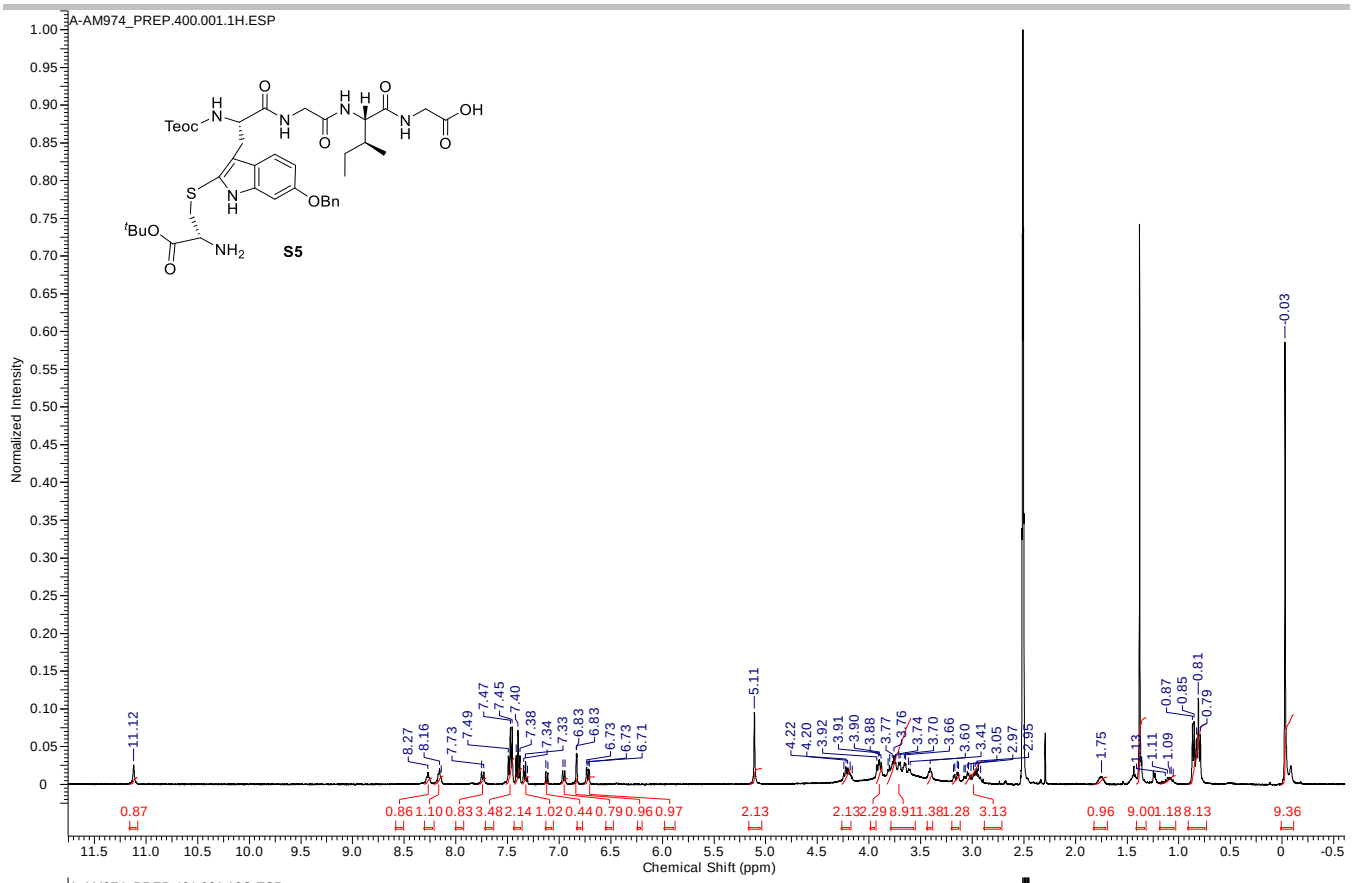
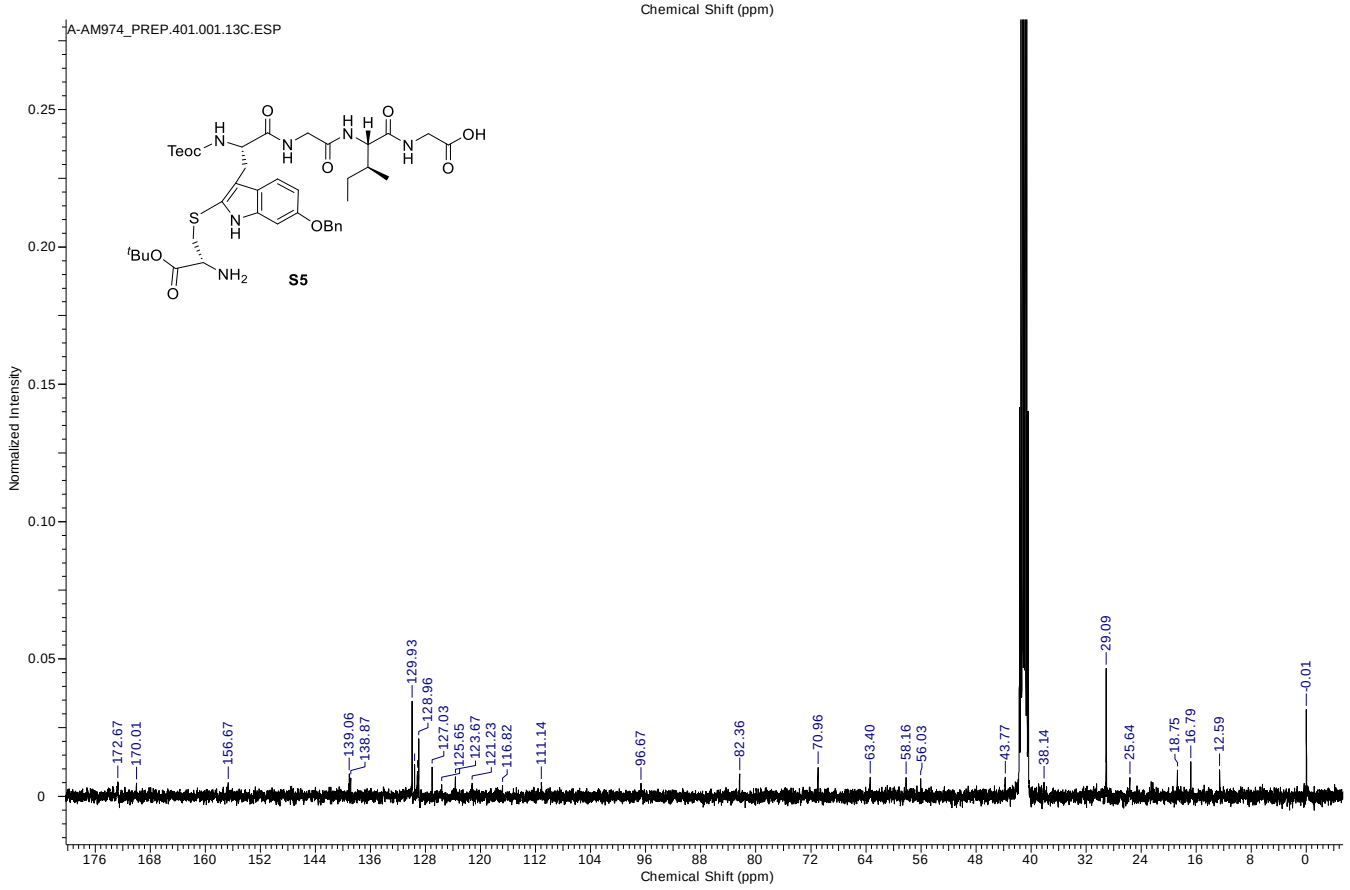
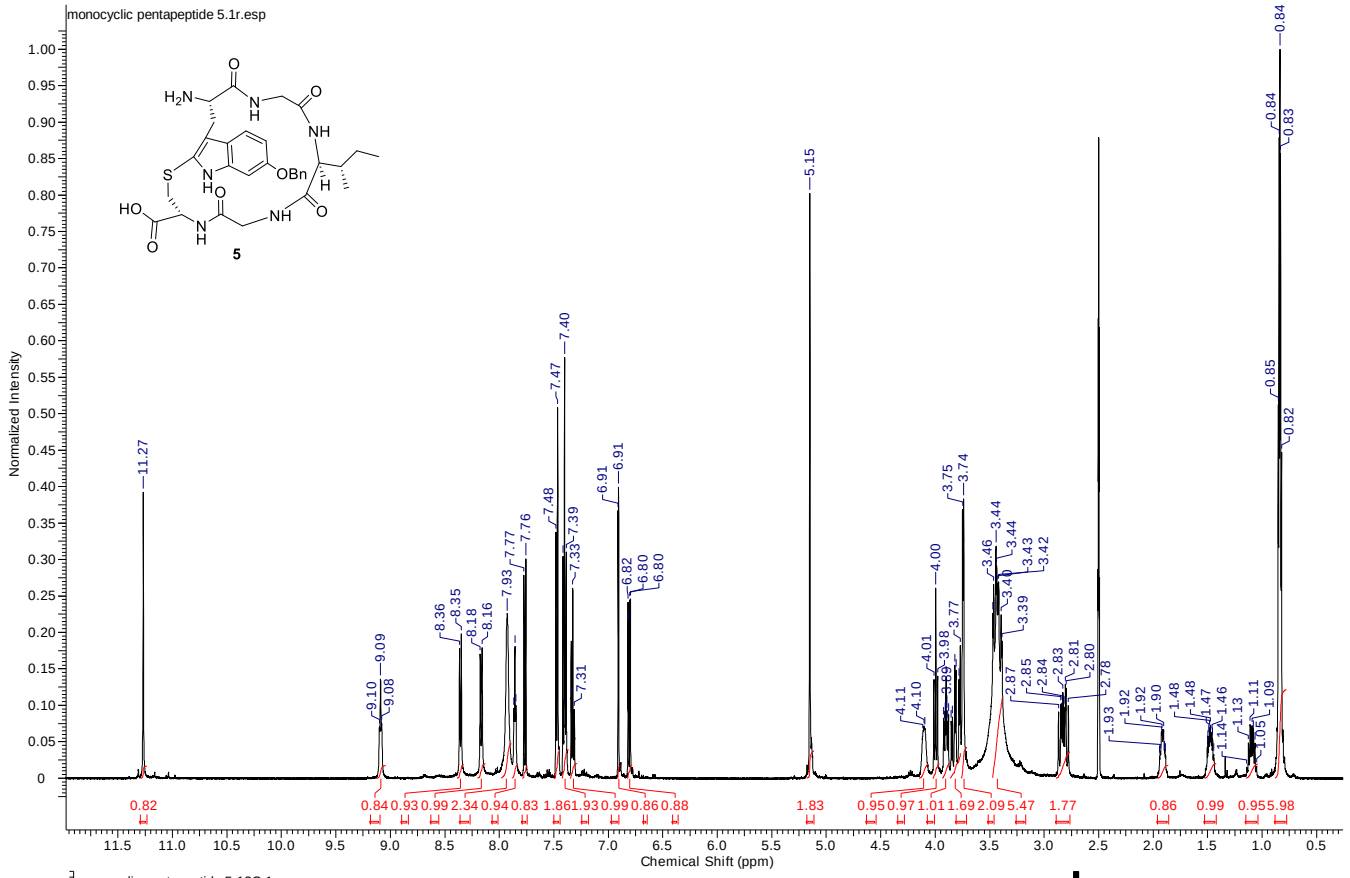
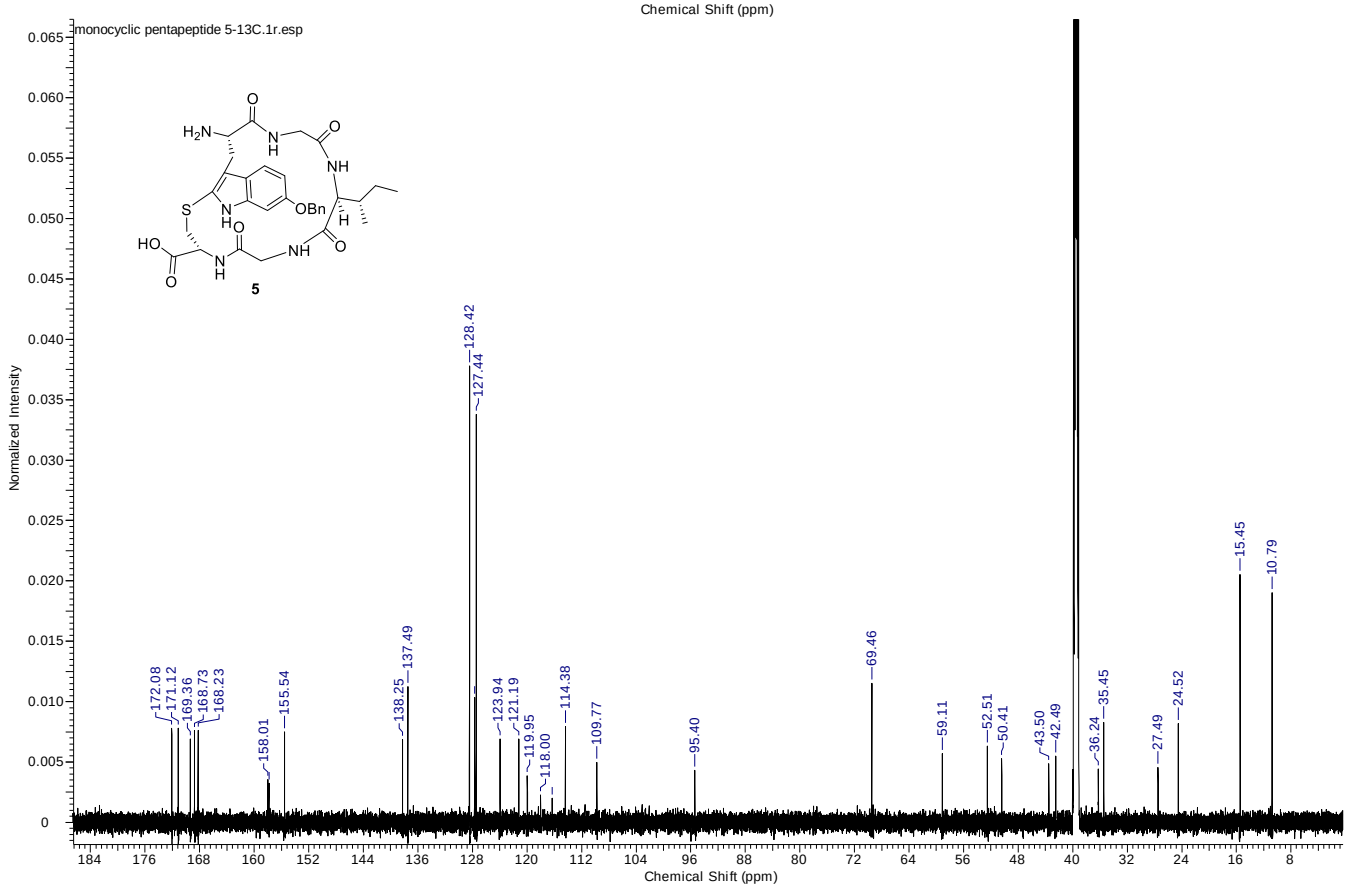
Teoc
NH
O
HO
NH
S
tBu
O
N
Boc
H
O
7
Normalized Intensity
Chemical Shift (ppm)
12.58
11.07
7.50
7.49
7.47
7.42
7.40
7.36
7.34
6.86
6.77
6.77
6.75
6.74
5.14
4.20
4.19
3.95
3.93
3.86
3.20
3.19
3.18
3.15
3.14
3.01
2.98
2.98
2.10
2.10
1.43
1.40
1.38
1.31
1.45
0.87
0.85
0.83
0.04
0.00
0.75
0.98
4.98
1.01
0.91
0.74
1.00
0.99
1.97
1.00
1.81
1.29
2.24
2.07
18.64
1.80
9.00

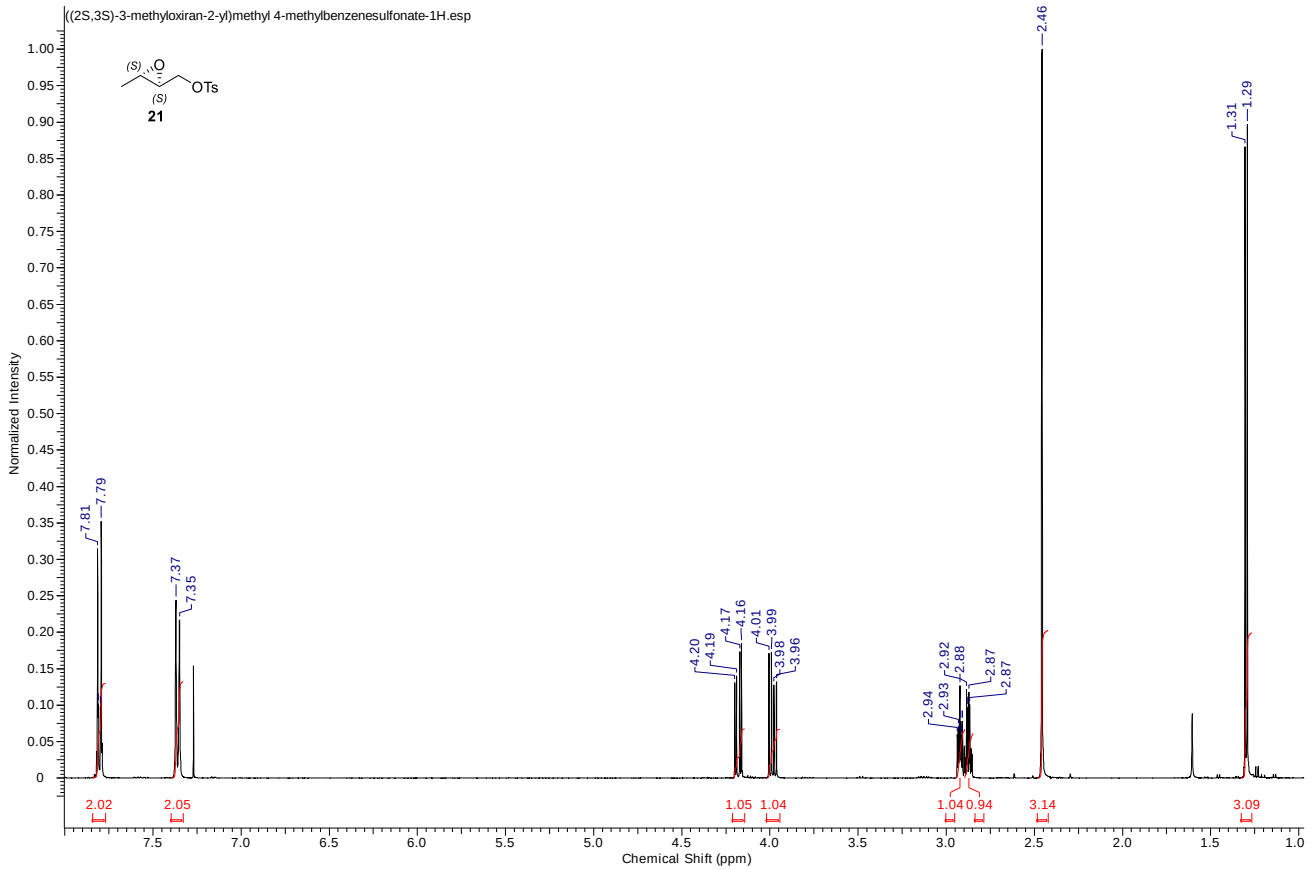

A-AM963_PREP.401.001.13C.ESP
OBn
Teoc
NH
O
HO
NH
S
tBu
O
N
Boc
H
O
7
Normalized Intensity
Chemical Shift (ppm)
174.99
171.48
157.48
156.77
139.01
129.91
128.93
124.99
123.45
121.03
118.12
111.29
96.73
82.45
79.88
70.97
63.23
56.36
55.58
38.40
29.03
29.63
18.82
0.01

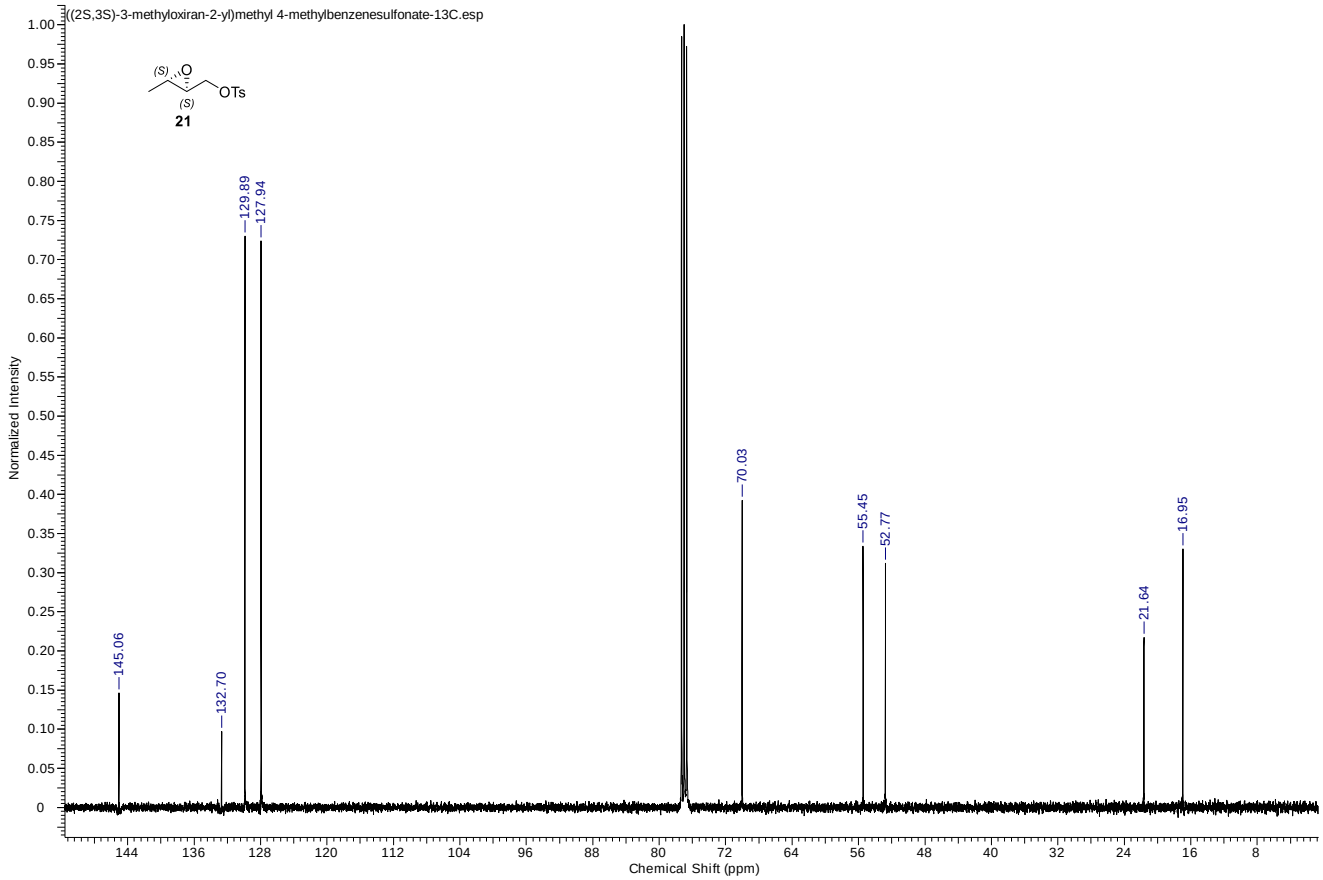

A-AM974_PREP.400.001.1H.ESP
Normalized Intensity
Chemical Shift (ppm)
S5

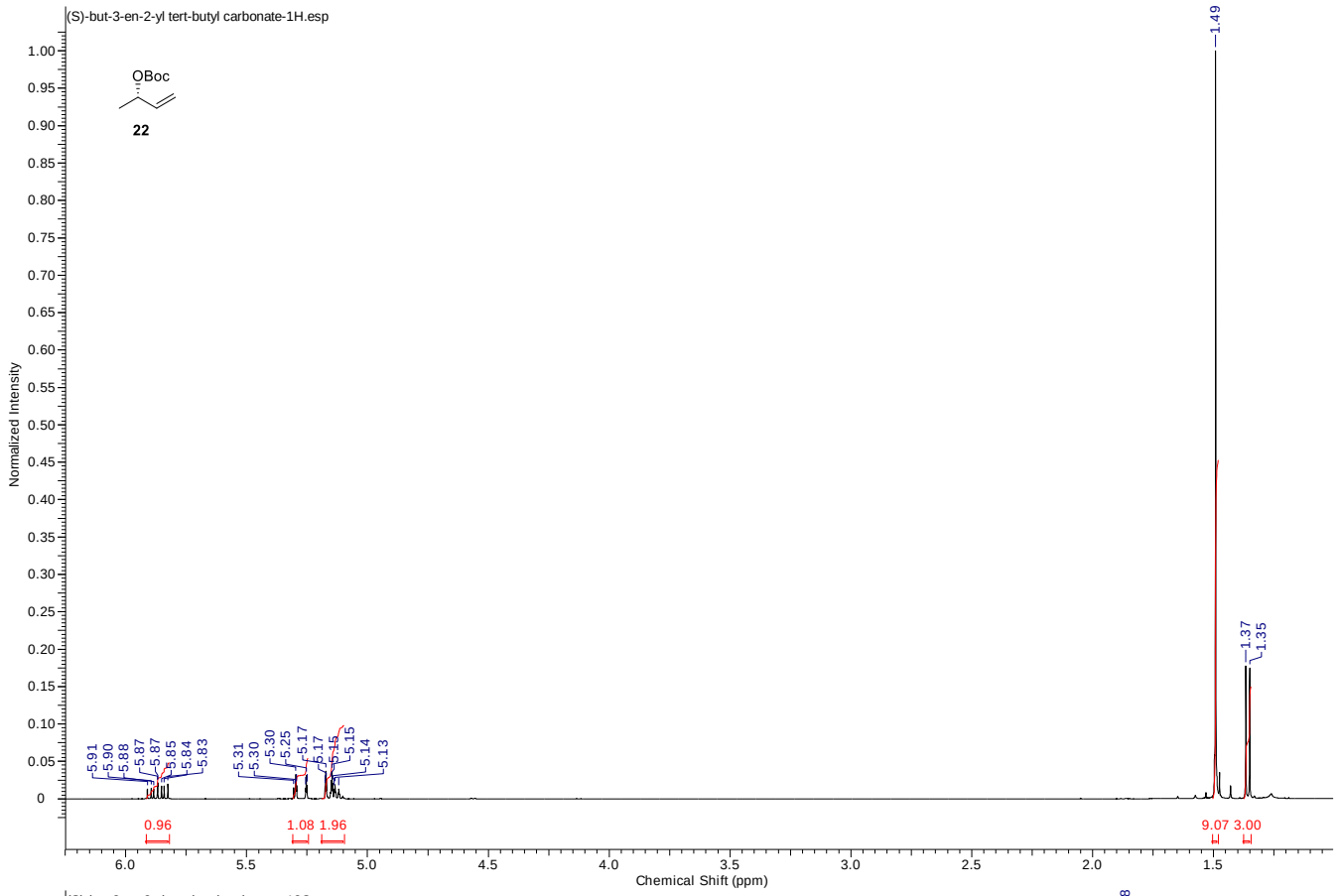

A-AM974_PREP.401.001.13C.ESP
Normalized Intensity
Chemical Shift (ppm)
S5

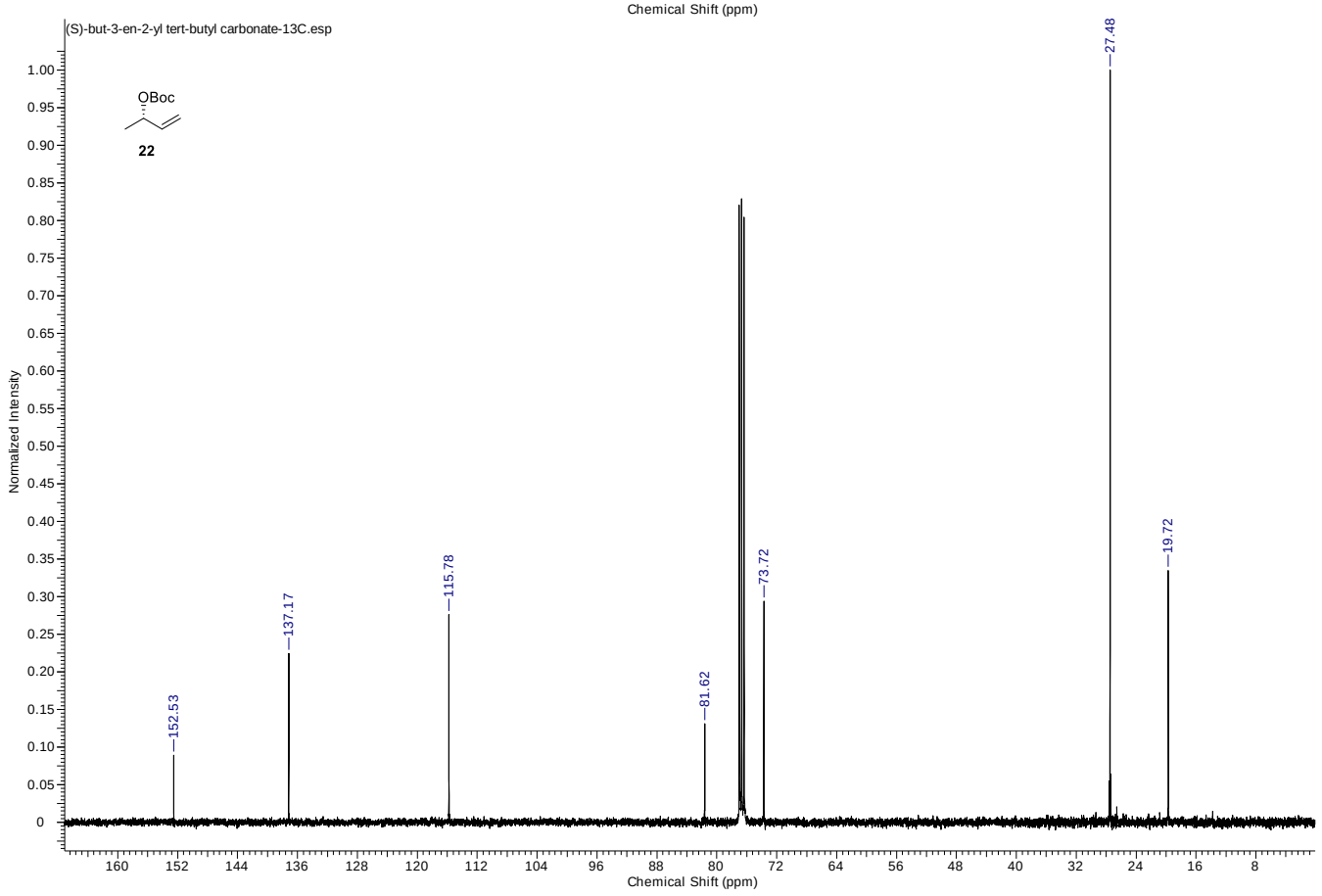

monocyclic pentapeptide 5.1r.esp
Normalized Intensity
Chemical Shift (ppm)
5

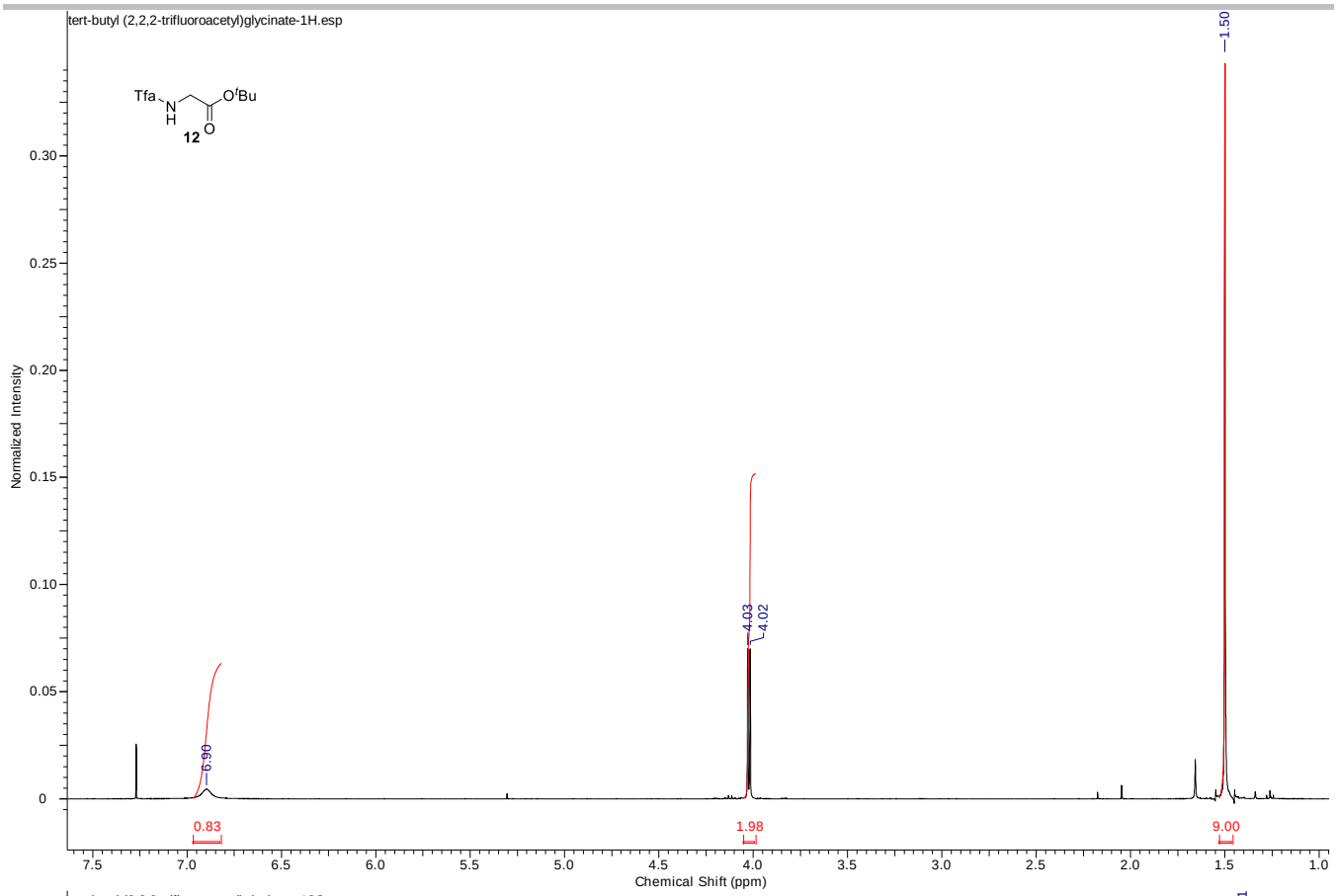

monocyclic pentapeptide 5-13C.1r.esp
Normalized Intensity
Chemical Shift (ppm)
5

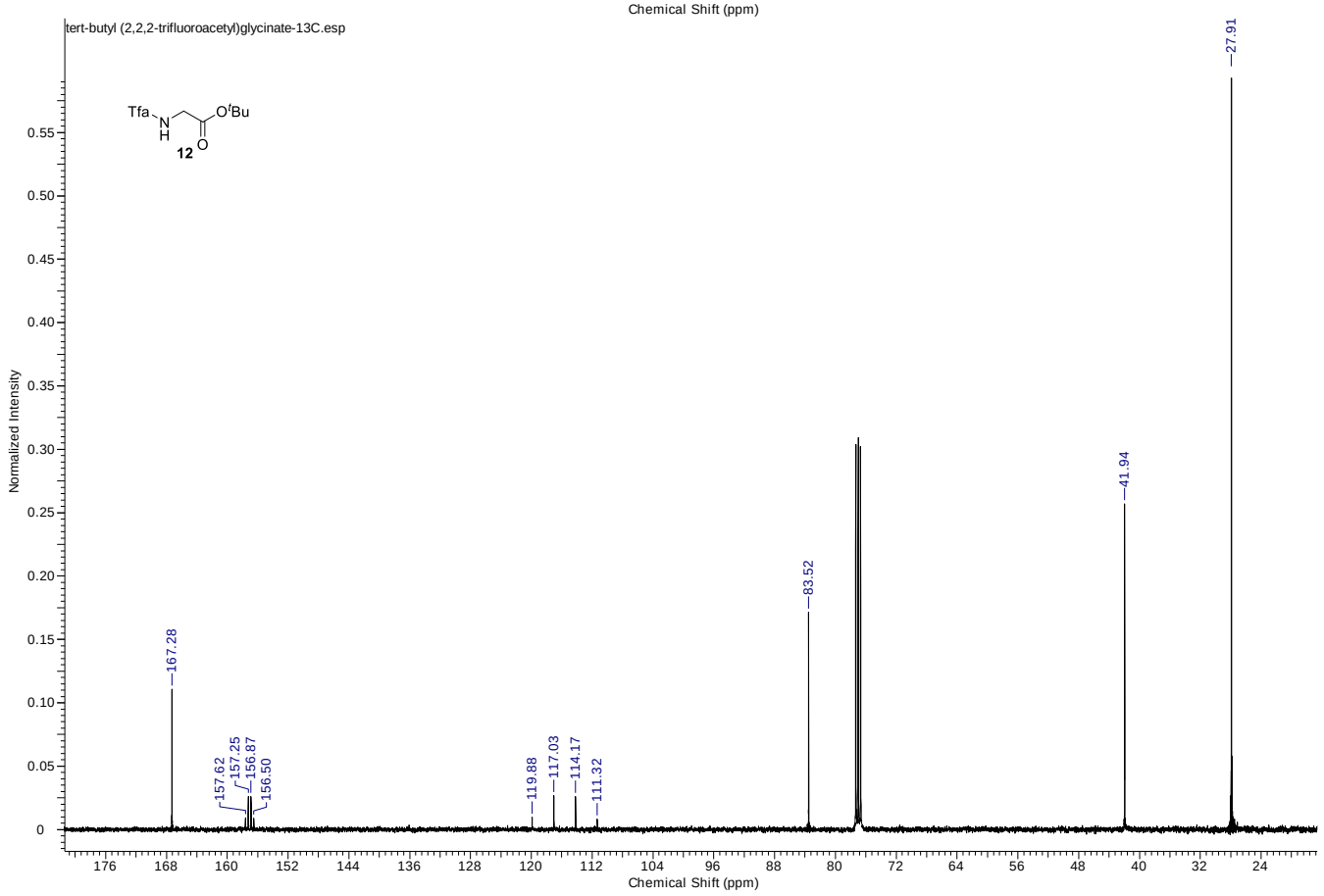

((2S,3S)-3-methyloxiran-2-yl)methyl 4-methylbenzenesulfonate-1H.esp
(S)
(S)
OTs
21
Normalized Intensity
Chemical Shift (ppm)
7.81
7.79
7.37
7.35
4.20
4.19
4.17
4.16
4.01
3.99
3.98
3.96
2.94
2.93
2.92
2.88
2.87
2.87
2.46
1.31
1.29
2.02
2.05
1.05
1.04
1.04
0.94
3.14
3.09

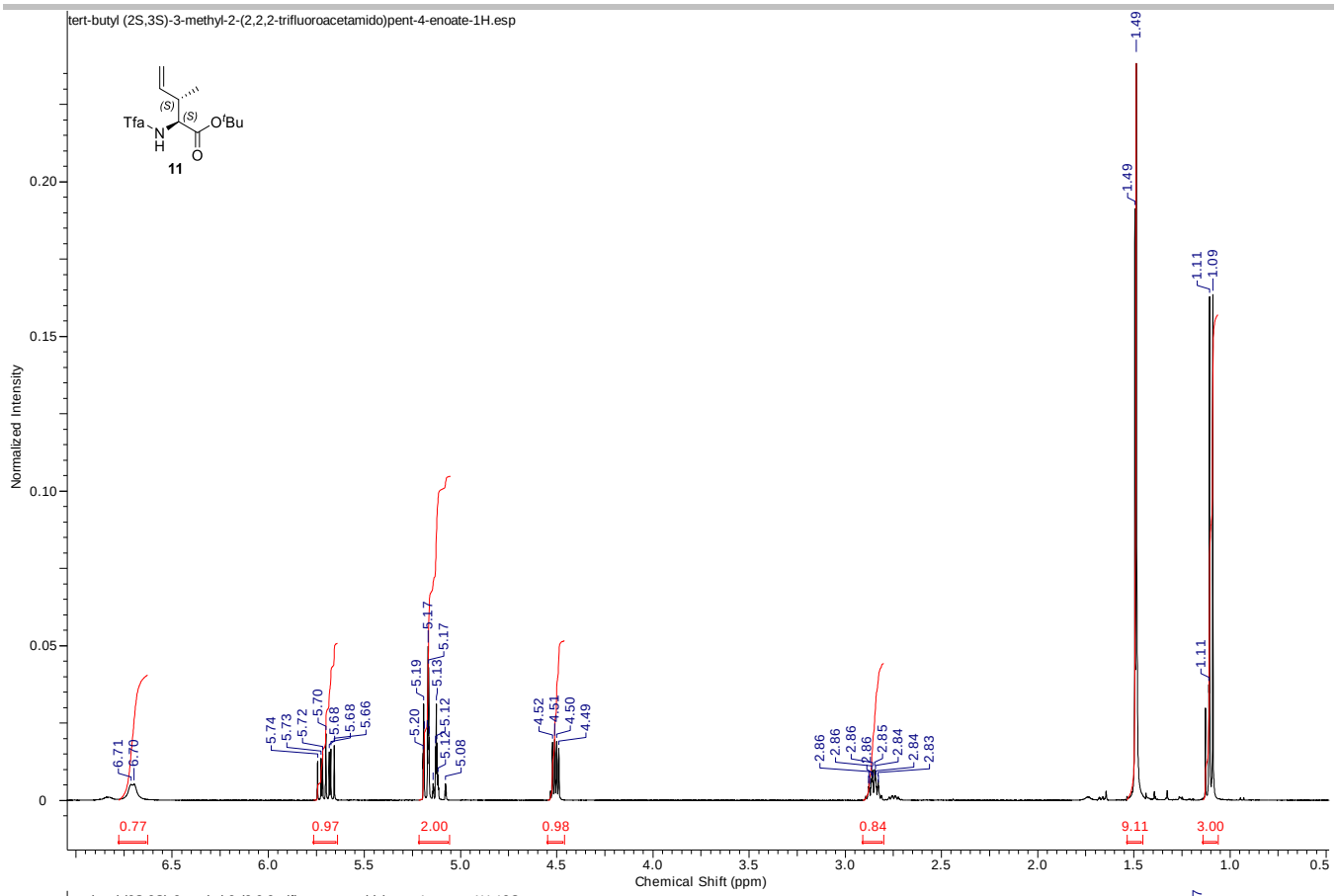

((2S,3S)-3-methyloxiran-2-yl)methyl 4-methylbenzenesulfonate-13C.esp
(S)
(S)
OTs
21
Normalized Intensity
Chemical Shift (ppm)
145.06
132.70
129.89
127.94
70.03
55.45
52.77
21.64
16.95

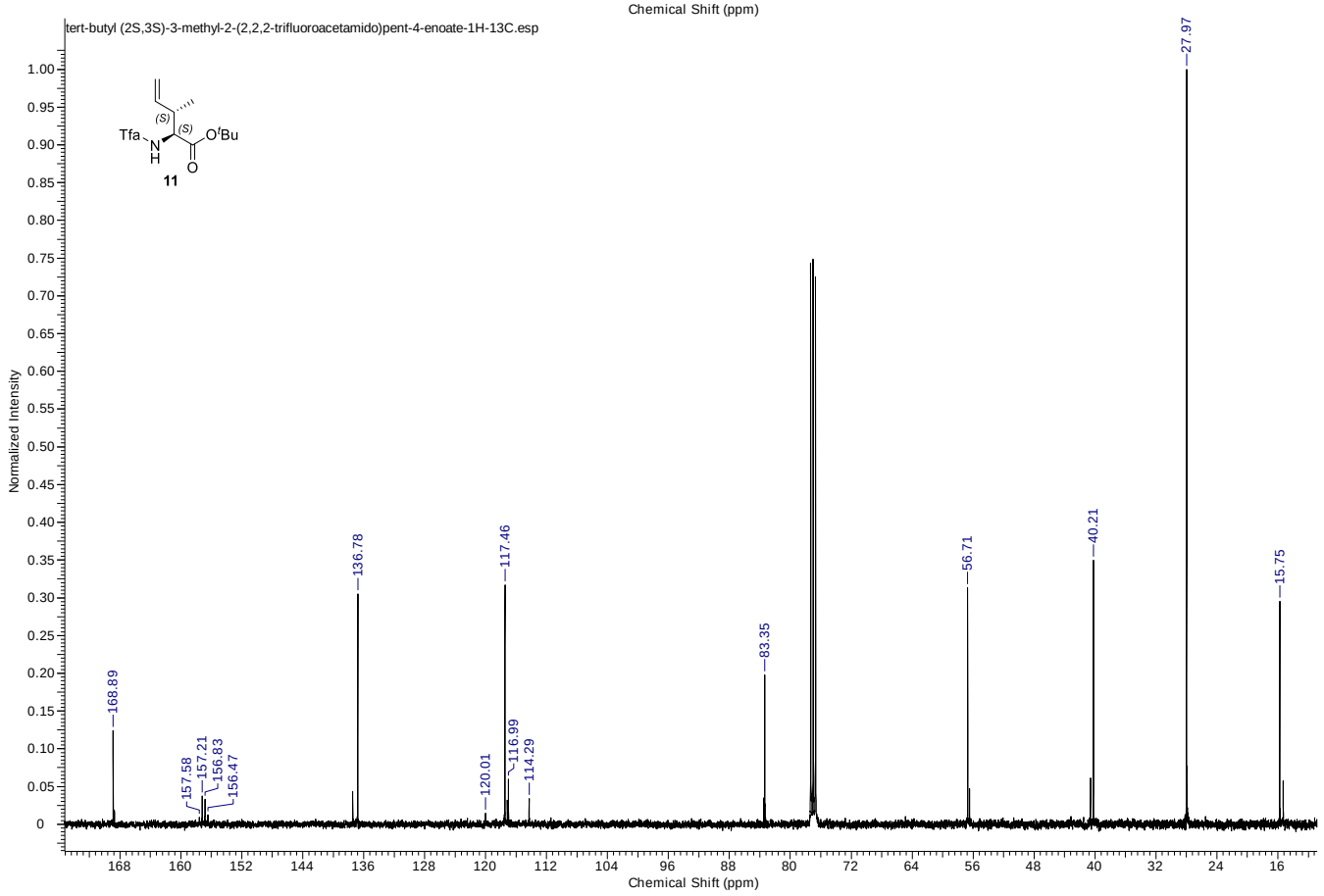

(S)-but-3-en-2-yl tert-butyl carbonate-1H.esp
OBoc
22
Normalized Intensity
Chemical Shift (ppm)
1.49
1.37
1.35
5.91
5.90
5.88
5.87
5.87
5.85
5.84
5.83
5.31
5.30
5.30
5.25
5.17
5.17
5.15
5.15
5.14
5.13
0.96
1.08
1.96
9.07
3.00

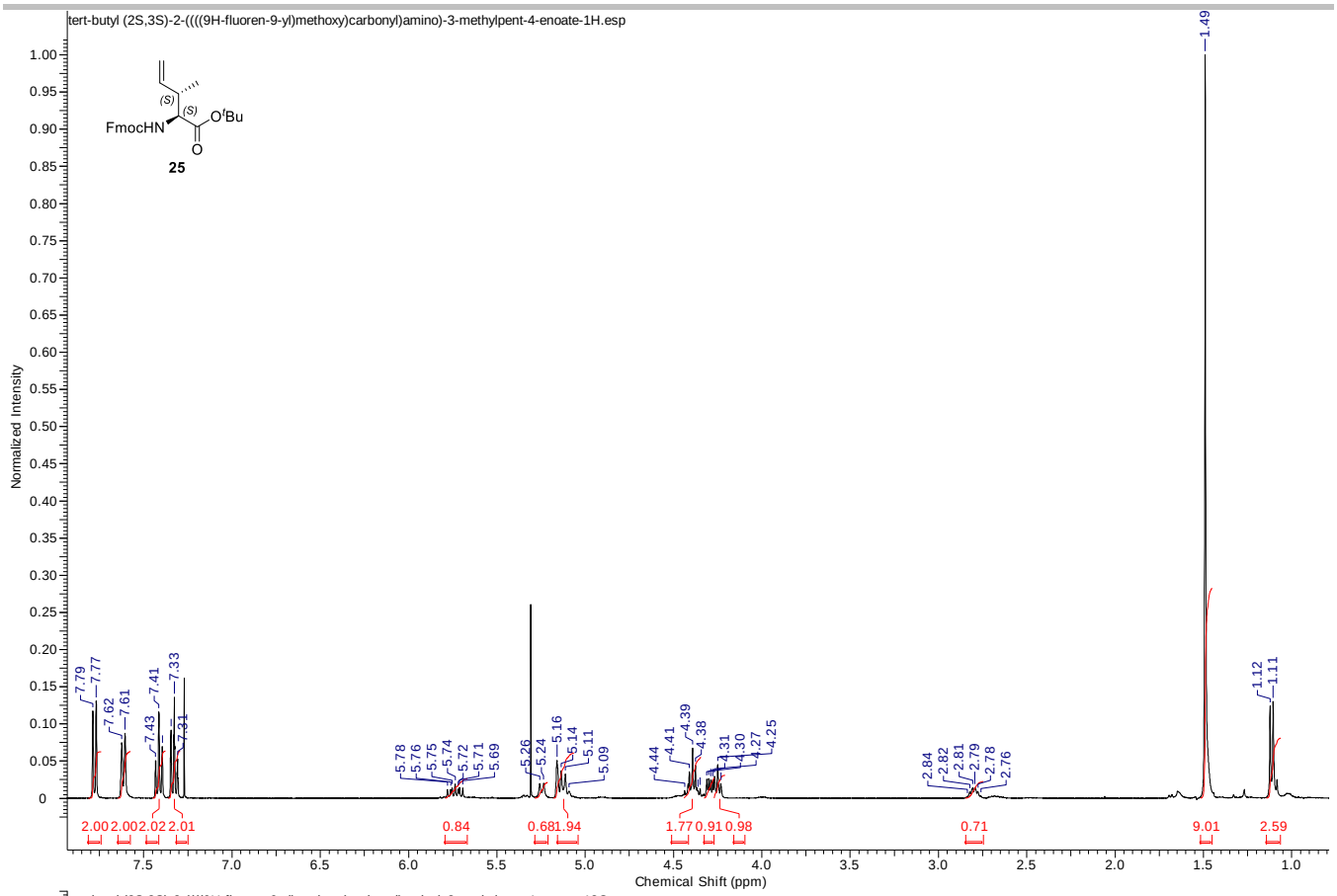

(S)-but-3-en-2-yl tert-butyl carbonate-13C.esp
OBoc
22
Normalized Intensity
Chemical Shift (ppm)
27.48
152.53
137.17
115.78
81.62
73.72
19.72

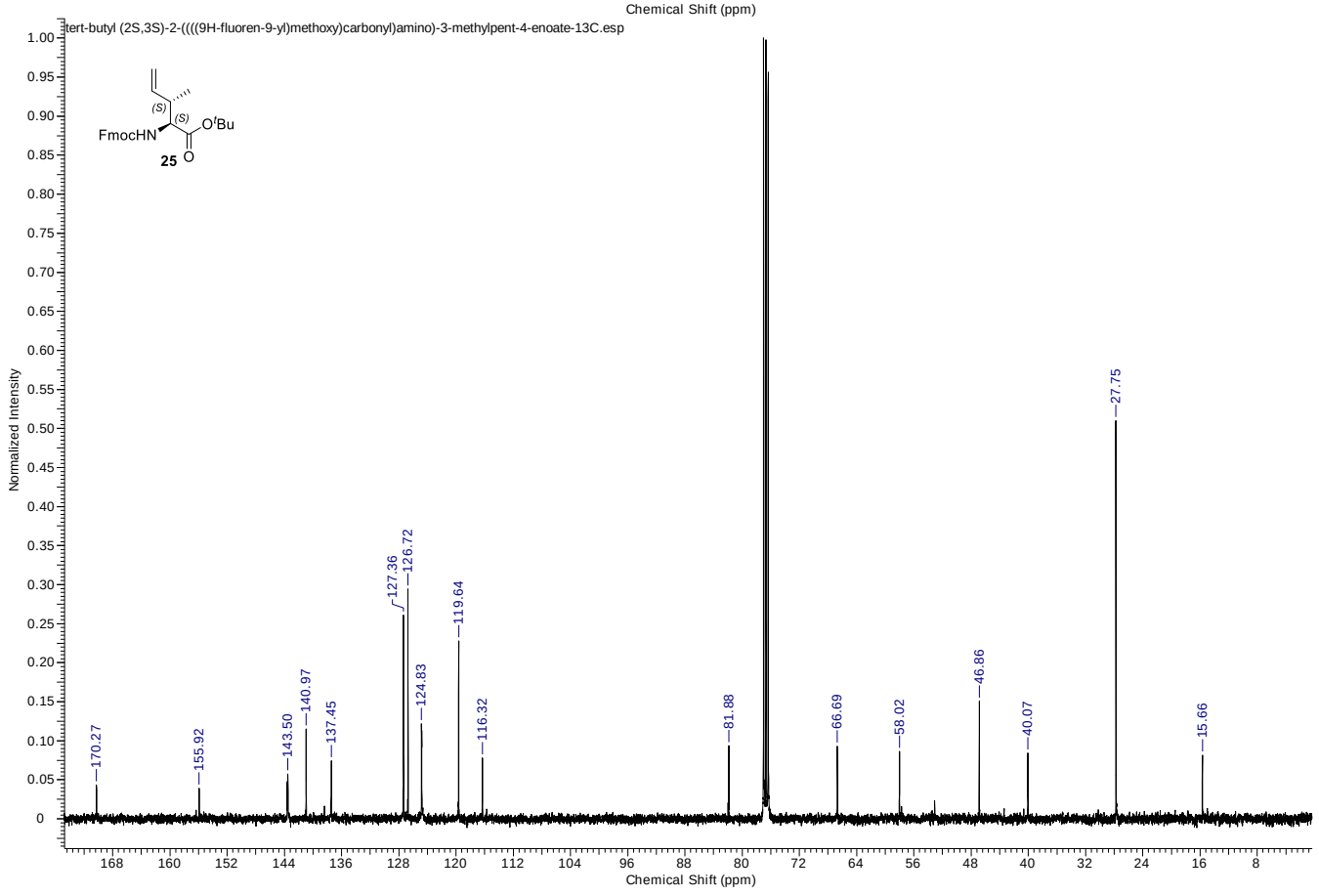

tert-butyl (2,2,2-trifluoroacetyl)glycinate-1H.esp
Tfa
N
H
O
O'Bu
12
1.50
4.03
4.02
6.90
0.83
1.98
9.00
Normalized Intensity
Chemical Shift (ppm)

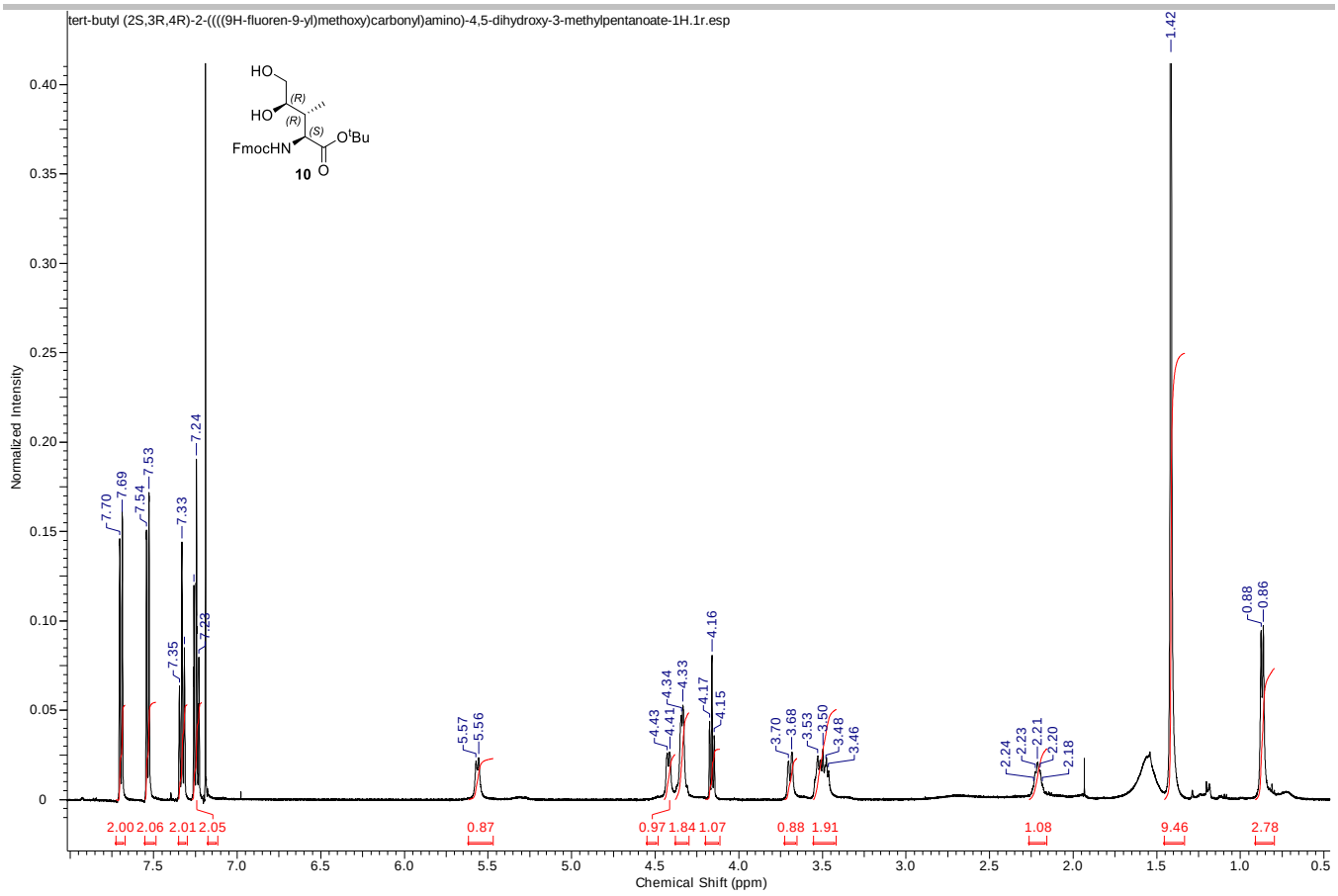

tert-butyl (2,2,2-trifluoroacetyl)glycinate-13C.esp
Tfa
N
H
O
O'Bu
12
27.91
41.94
83.52
167.28
157.62
157.25
156.87
156.50
119.88
117.03
114.17
111.32
Normalized Intensity
Chemical Shift (ppm)

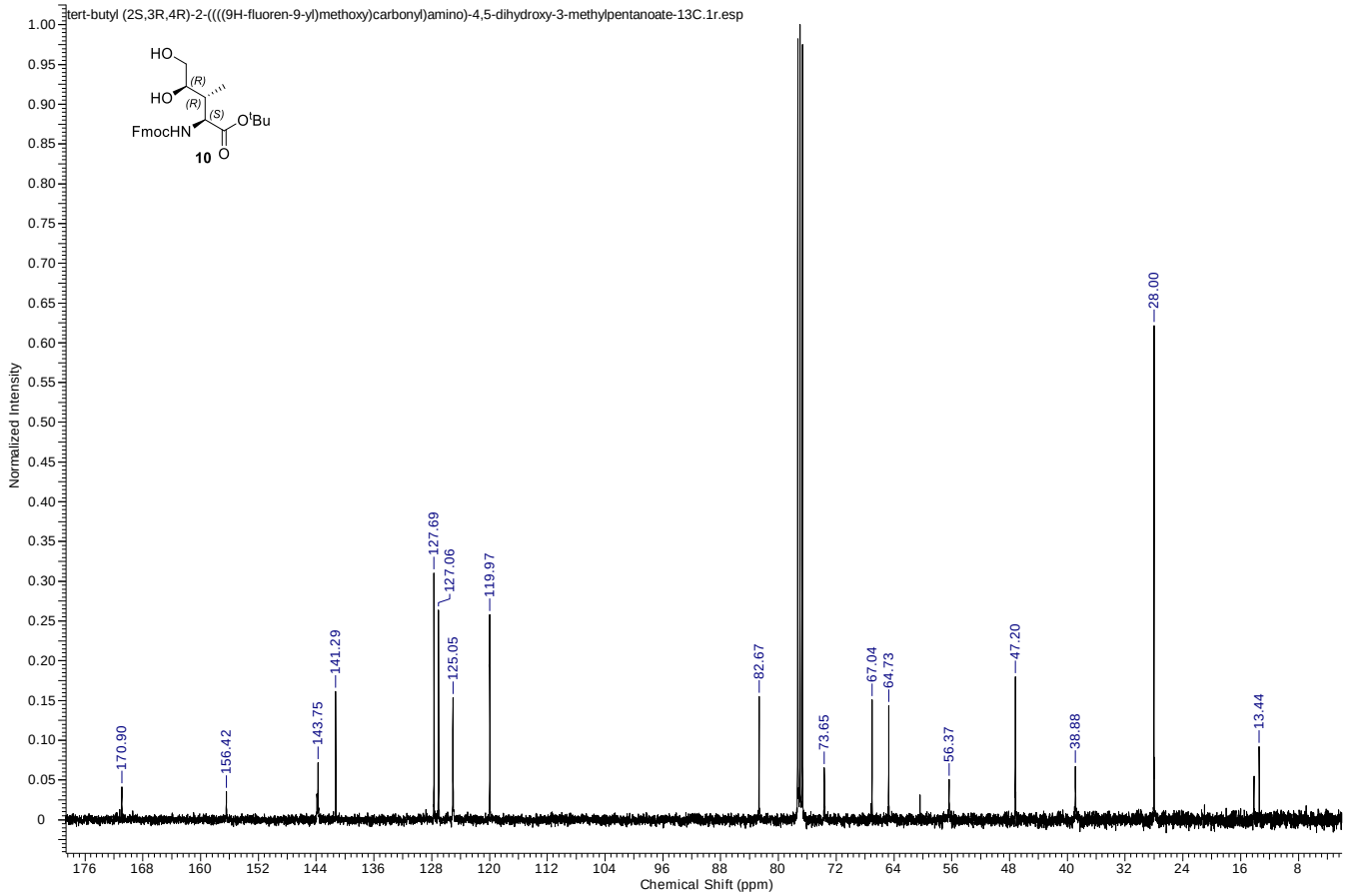

tert-butyl (2S,3S)-3-methyl-2-(2,2,2-trifluoroacetamido)pent-4-enoate-1H.esp
Tfa
N
H
(S)
(S)
O^{t}Bu
O
11
Normalized Intensity
Chemical Shift (ppm)
1.49
1.11
1.09
6.71
6.70
5.74
5.73
5.72
5.70
5.68
5.66
5.20
5.19
5.17
5.13
5.12
5.08
4.52
4.51
4.50
4.49
2.86
2.85
2.84
2.83
0.77
0.97
2.00
0.98
0.84
9.11
3.00

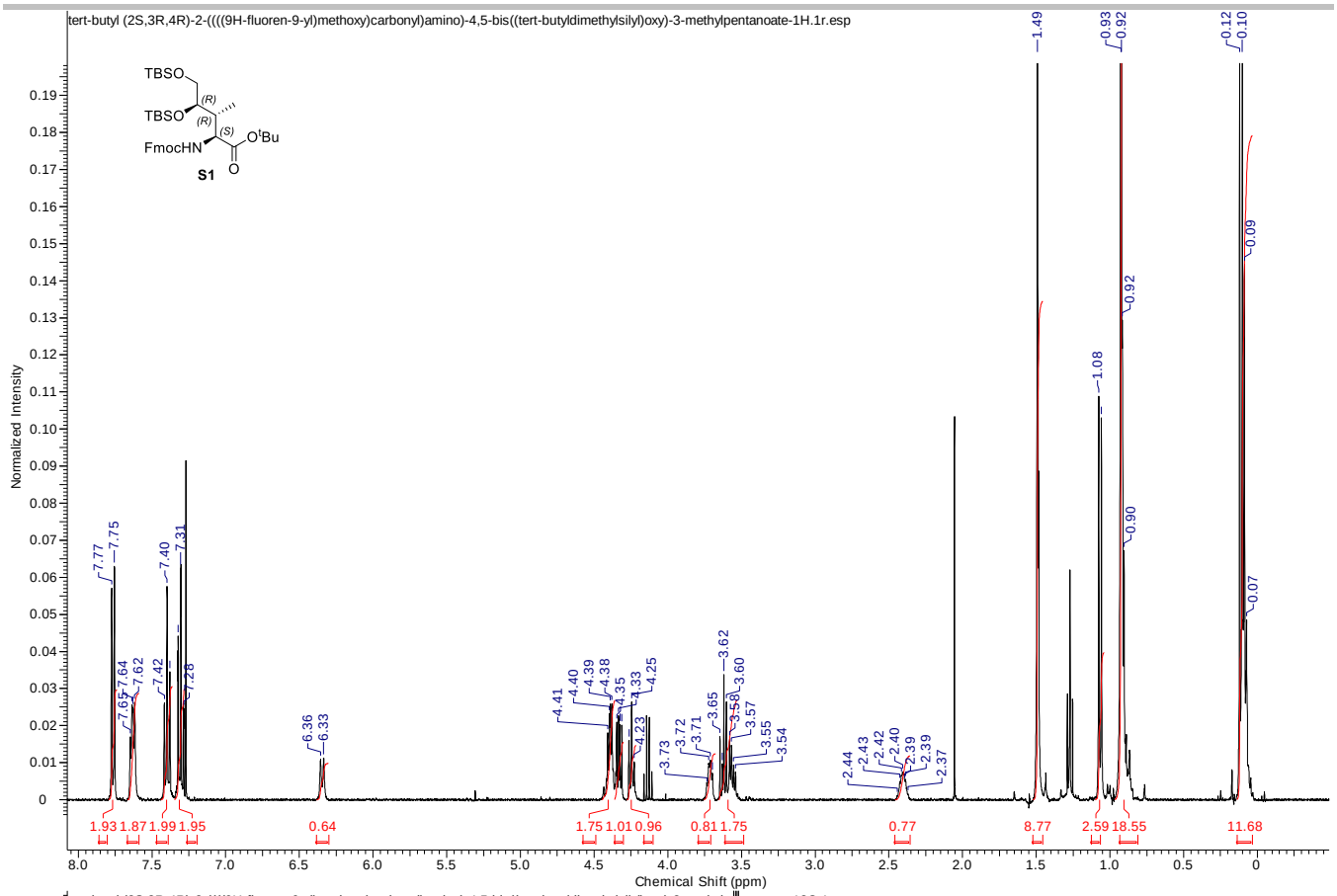

tert-butyl (2S,3S)-3-methyl-2-(2,2,2-trifluoroacetamido)pent-4-enoate-1H-13C.esp
Tfa
N
H
(S)
(S)
O^{t}Bu
O
11
Normalized Intensity
Chemical Shift (ppm)
168.89
157.58
157.21
156.83
156.47
136.78
120.01
117.46
116.99
114.29
83.35
56.71
40.21
27.97
15.75

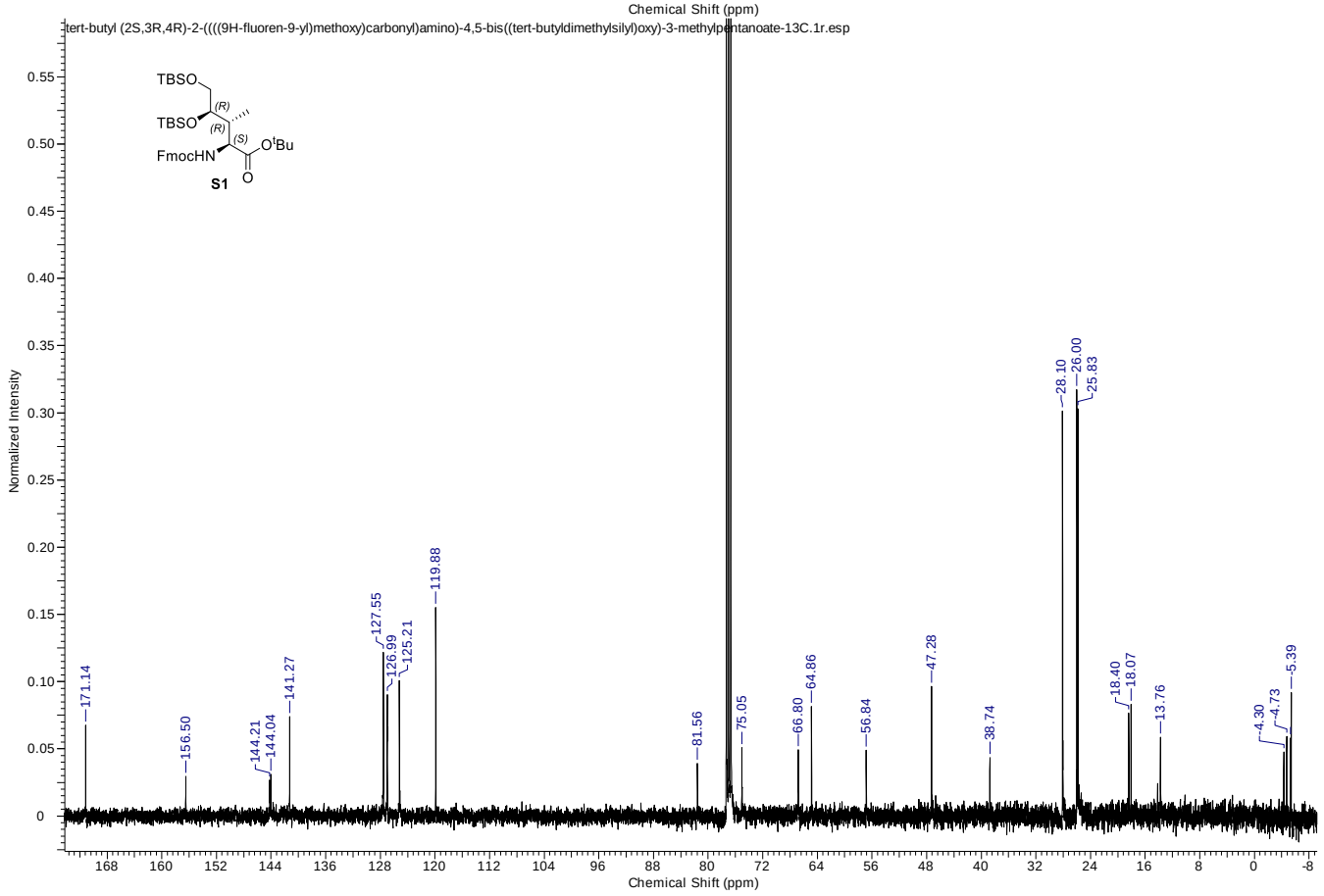

tert-butyl (2S,3S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylpent-4-enoate-1H.esp
FmocHN
O'Bu
25
Normalized Intensity
Chemical Shift (ppm)
7.79
7.77
7.62
7.61
7.43
7.41
7.33
7.31
5.78
5.76
5.75
5.74
5.72
5.71
5.69
5.26
5.24
5.16
5.14
5.11
5.09
4.44
4.41
4.39
4.38
4.31
4.30
4.27
4.25
2.84
2.82
2.81
2.79
2.78
2.76
1.49
1.12
1.11
2.00
2.00
2.02
2.01
0.84
0.68
1.94
1.77
0.91
0.98
0.71
9.01
2.59

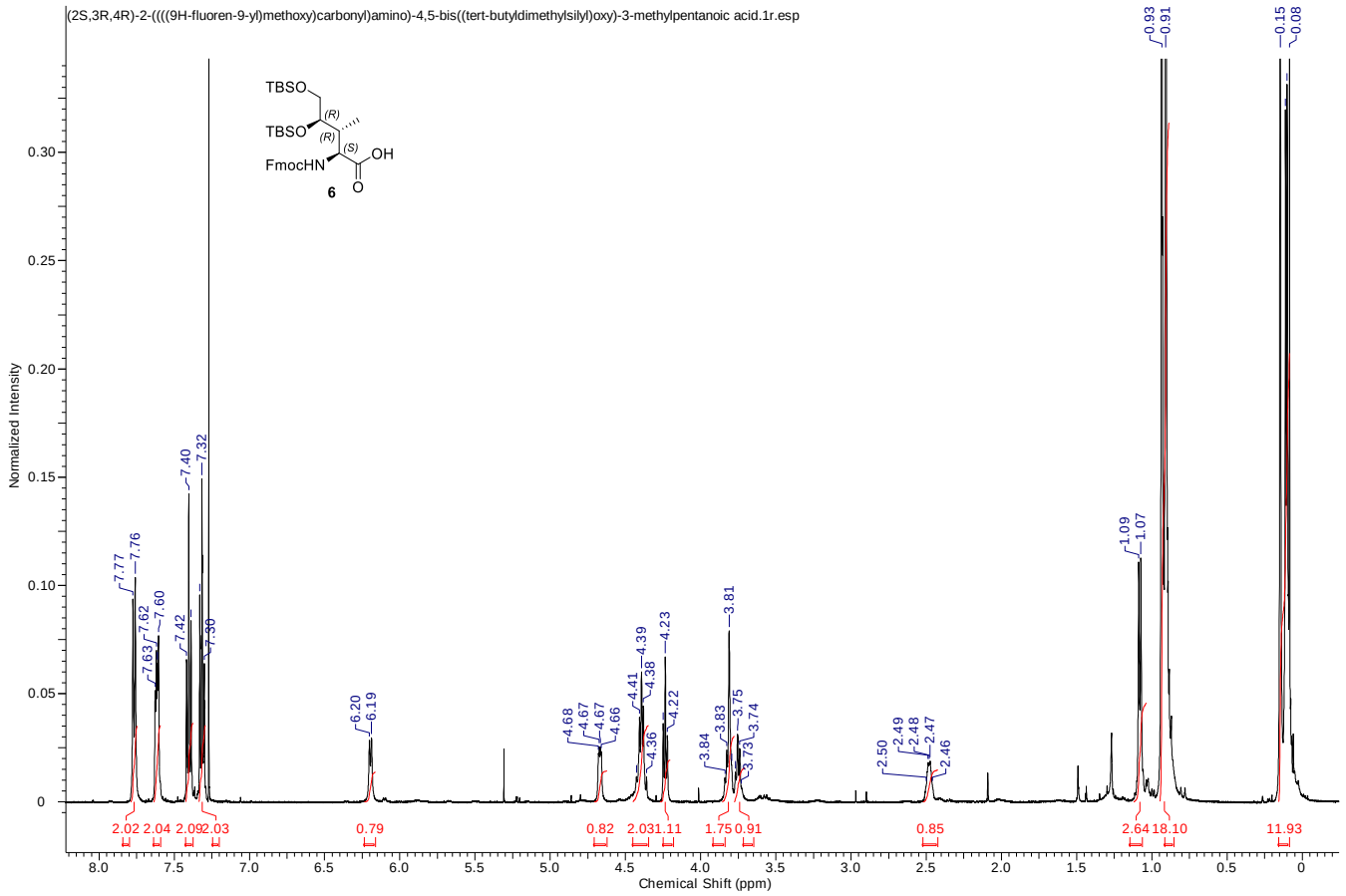

tert-butyl (2S,3S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylpent-4-enoate-13C.esp
FmocHN
O'Bu
25
Normalized Intensity
Chemical Shift (ppm)
170.27
155.92
143.50
140.97
137.45
127.36
126.72
124.83
119.64
116.32
81.88
66.69
58.02
46.86
40.07
27.75
15.66

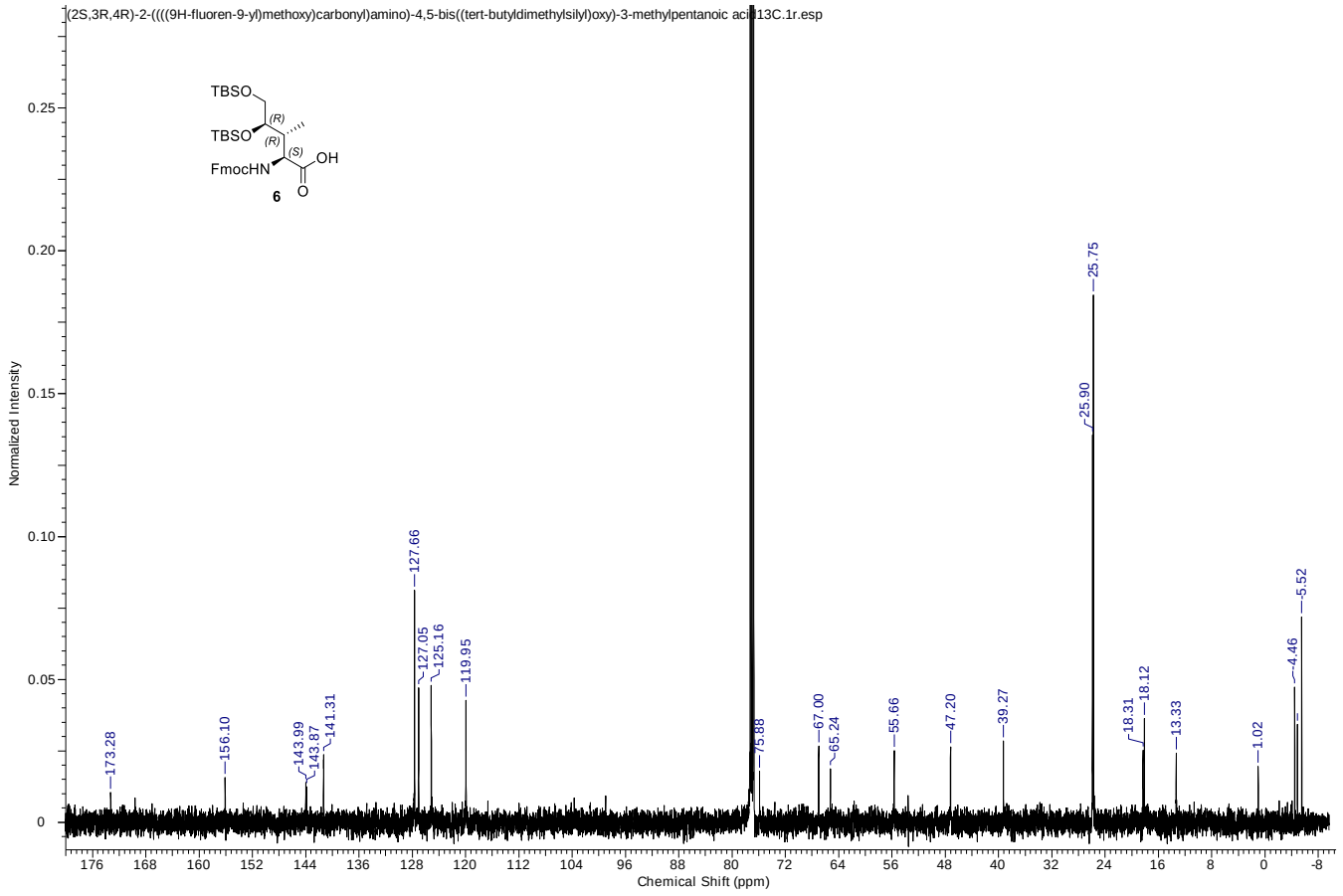

tert-butyl (2S,3R,4R)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4,5-dihydroxy-3-methylpentanoate-1H.1r.esp
HO
(R)
HO
(R)
(S)
FmocHN
O^{t}Bu
10
O
Normalized Intensity
Chemical Shift (ppm)
7.70
7.69
7.54
7.53
7.35
7.33
7.24
7.23
5.57
5.56
4.43
4.41
4.34
4.33
4.17
4.16
4.15
3.70
3.68
3.53
3.50
3.48
3.46
2.24
2.23
2.21
2.20
2.18
1.42
0.88
0.86
2.00 2.06 2.01 2.05
0.87
0.97 1.84 1.07
0.88 1.91
1.08
9.46
2.78

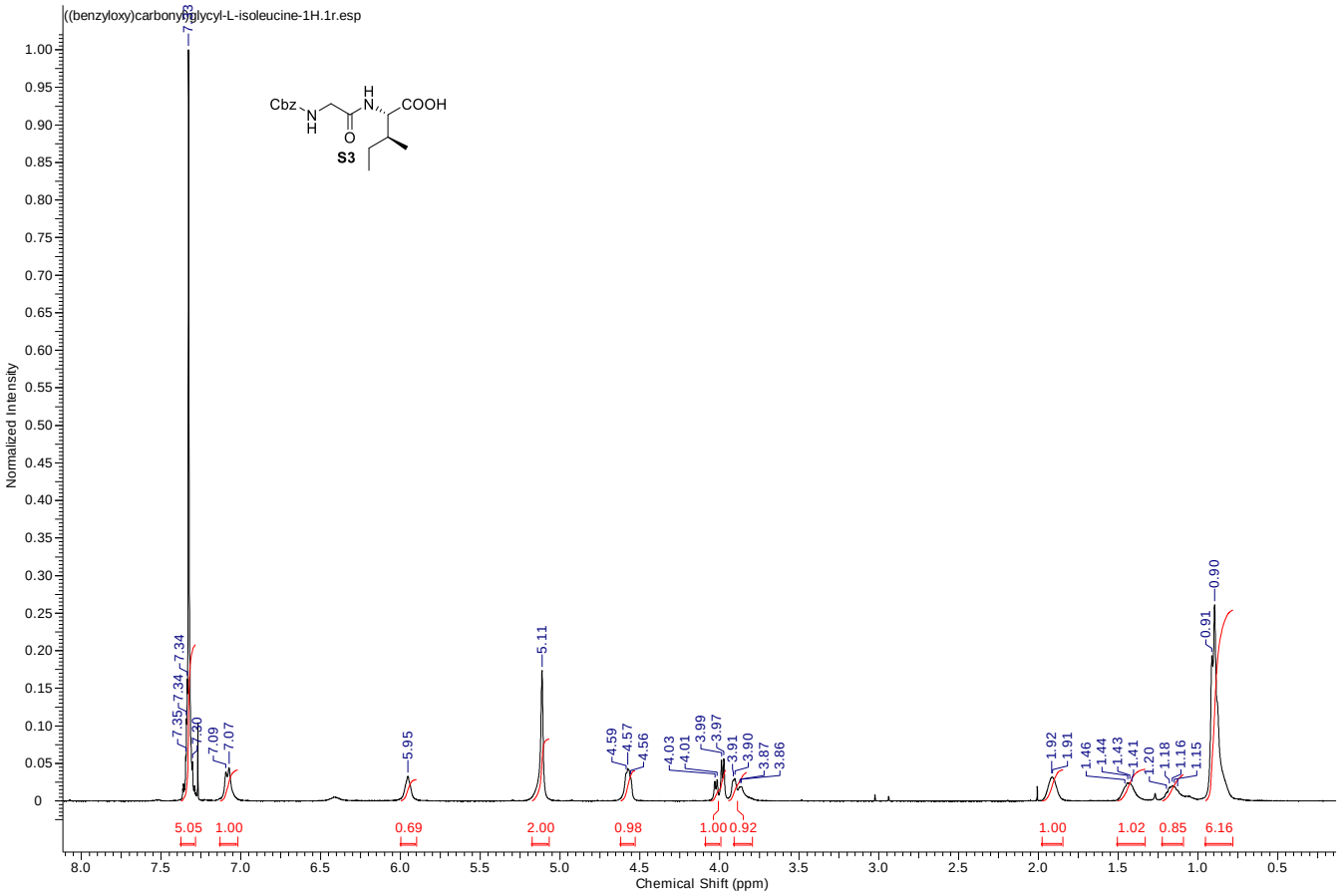

tert-butyl (2S,3R,4R)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4,5-dihydroxy-3-methylpentanoate-13C.1r.esp
HO
(R)
HO
(R)
(S)
FmocHN
O^{t}Bu
10
O
Normalized Intensity
Chemical Shift (ppm)
170.90
156.42
143.75
141.29
127.69
127.06
125.05
119.97
82.67
73.65
67.04
64.73
56.37
47.20
38.88
28.00
13.44

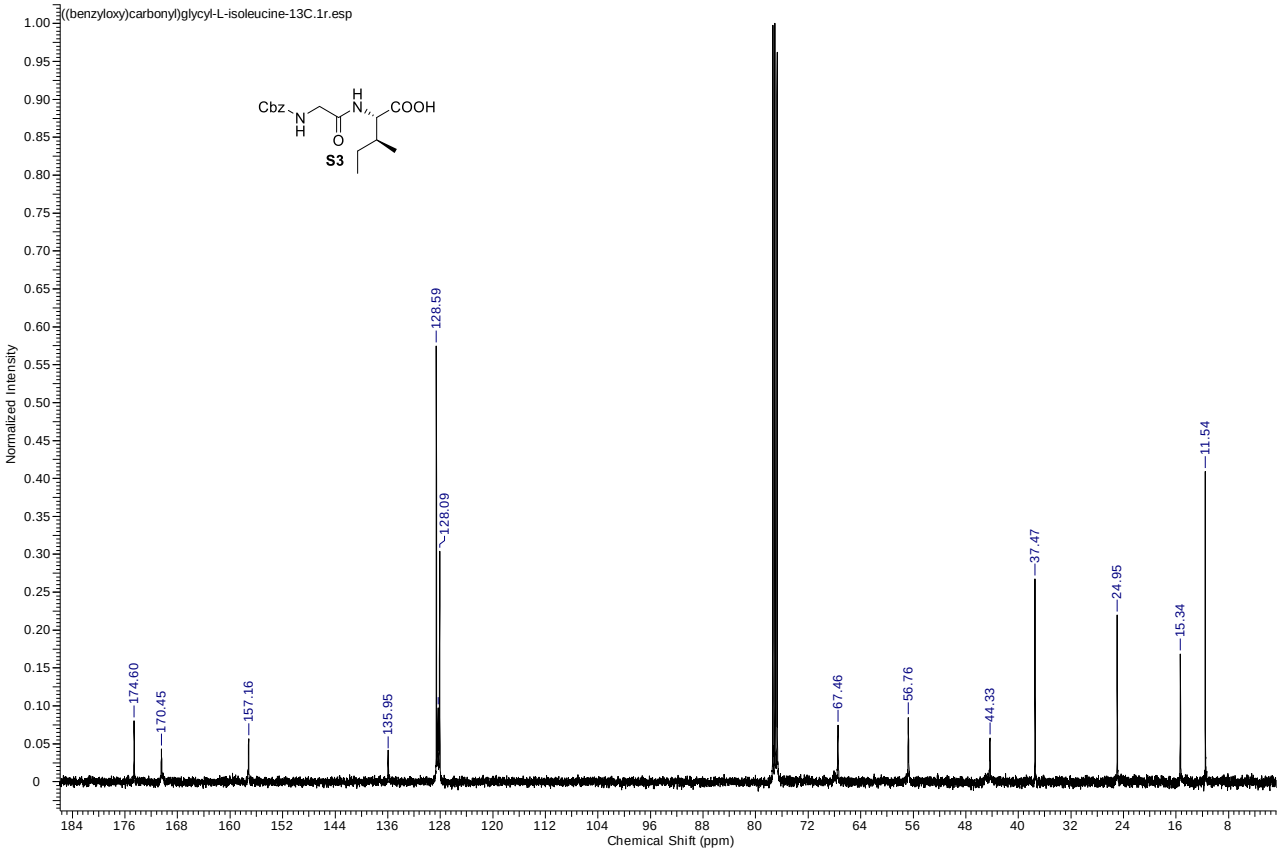

tert-butyl (2S,3R,4R)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4,5-bis((tert-butyldimethylsilyl)oxy)-3-methylpentanoate-1H.1r.esp
TBSO
TBSO
FmocHN
O^{t}Bu
S1
Normalized Intensity
Chemical Shift (ppm)
1.93 1.87 1.99 1.95
0.64
1.75 1.01 0.96
0.81 1.75
0.77
8.77
2.59 18.55
11.68

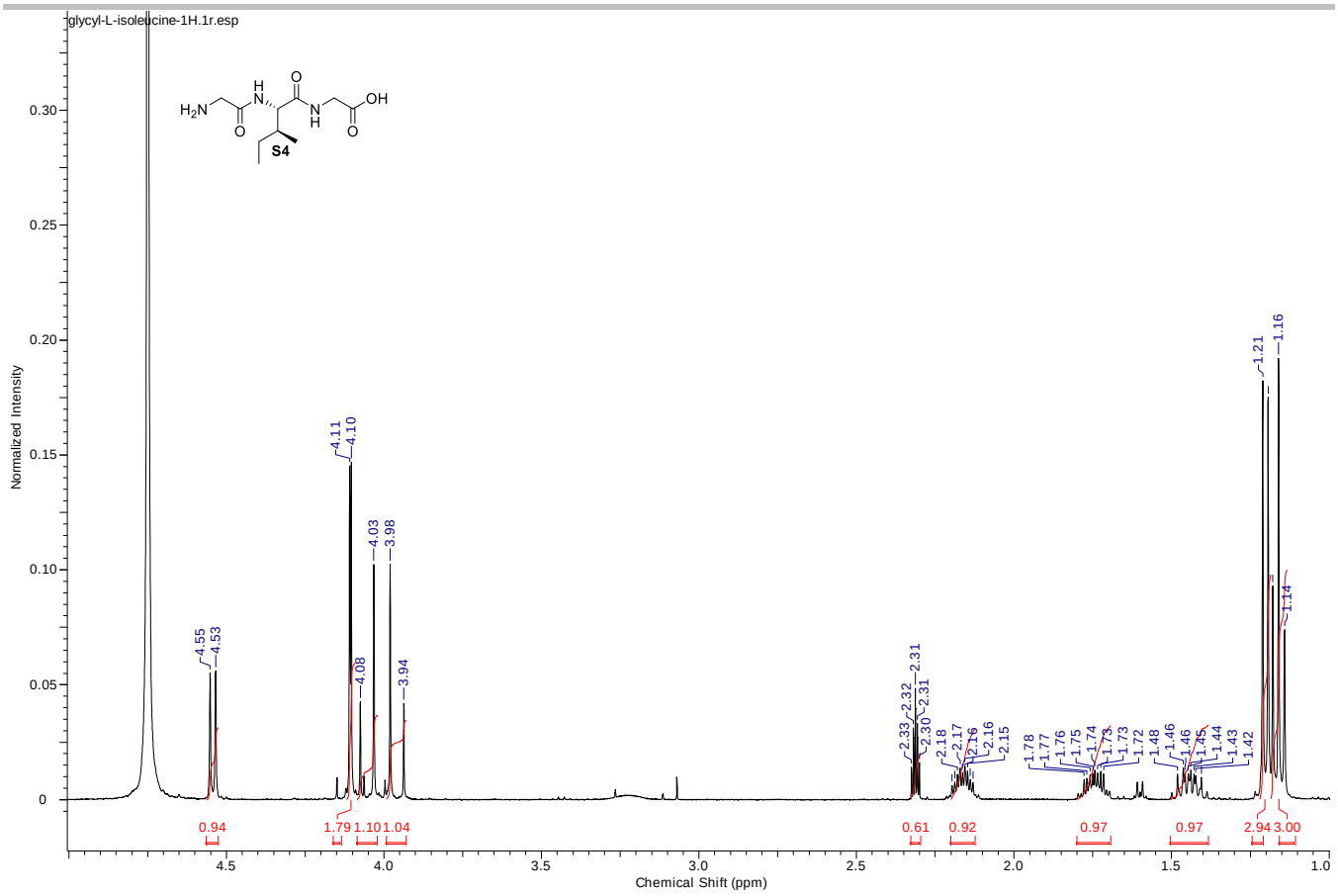

tert-butyl (2S,3R,4R)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4,5-bis((tert-butyldimethylsilyl)oxy)-3-methylpentanoate-13C.1r.esp
TBSO
TBSO
FmocHN
O^{t}Bu
S1
Normalized Intensity
Chemical Shift (ppm)

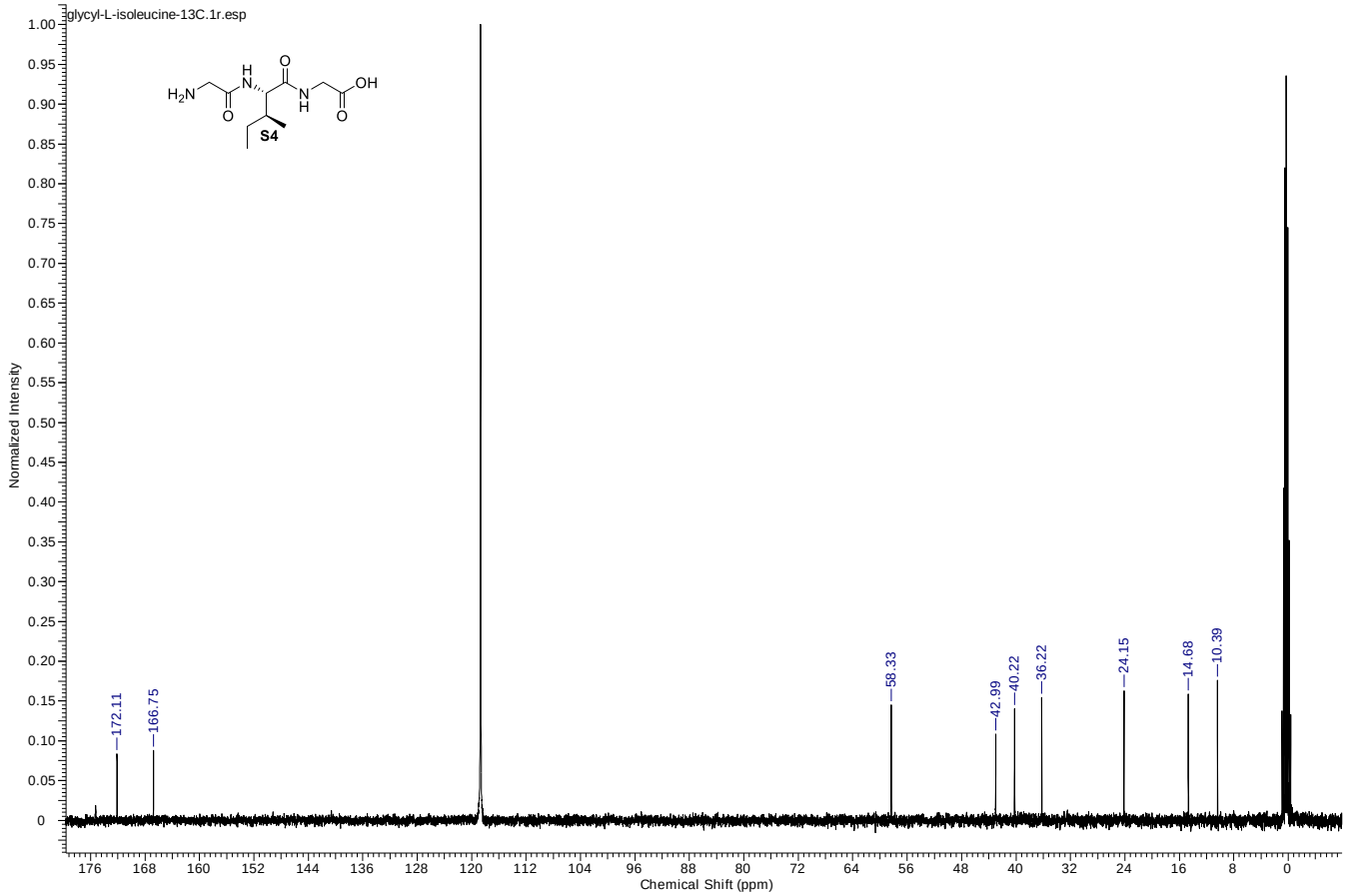

(2S,3R,4R)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4,5-bis((tert-butyldimethylsilyl)oxy)-3-methylpentanoic acid.1r.esp
TBSO
TBSO
FmocHN
OH
6
Normalized Intensity
Chemical Shift (ppm)


(2S,3R,4R)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4,5-bis((tert-butyldimethylsilyl)oxy)-3-methylpentanoic acid.13C.1r.esp
TBSO
TBSO
FmocHN
OH
6
Normalized Intensity
Chemical Shift (ppm)

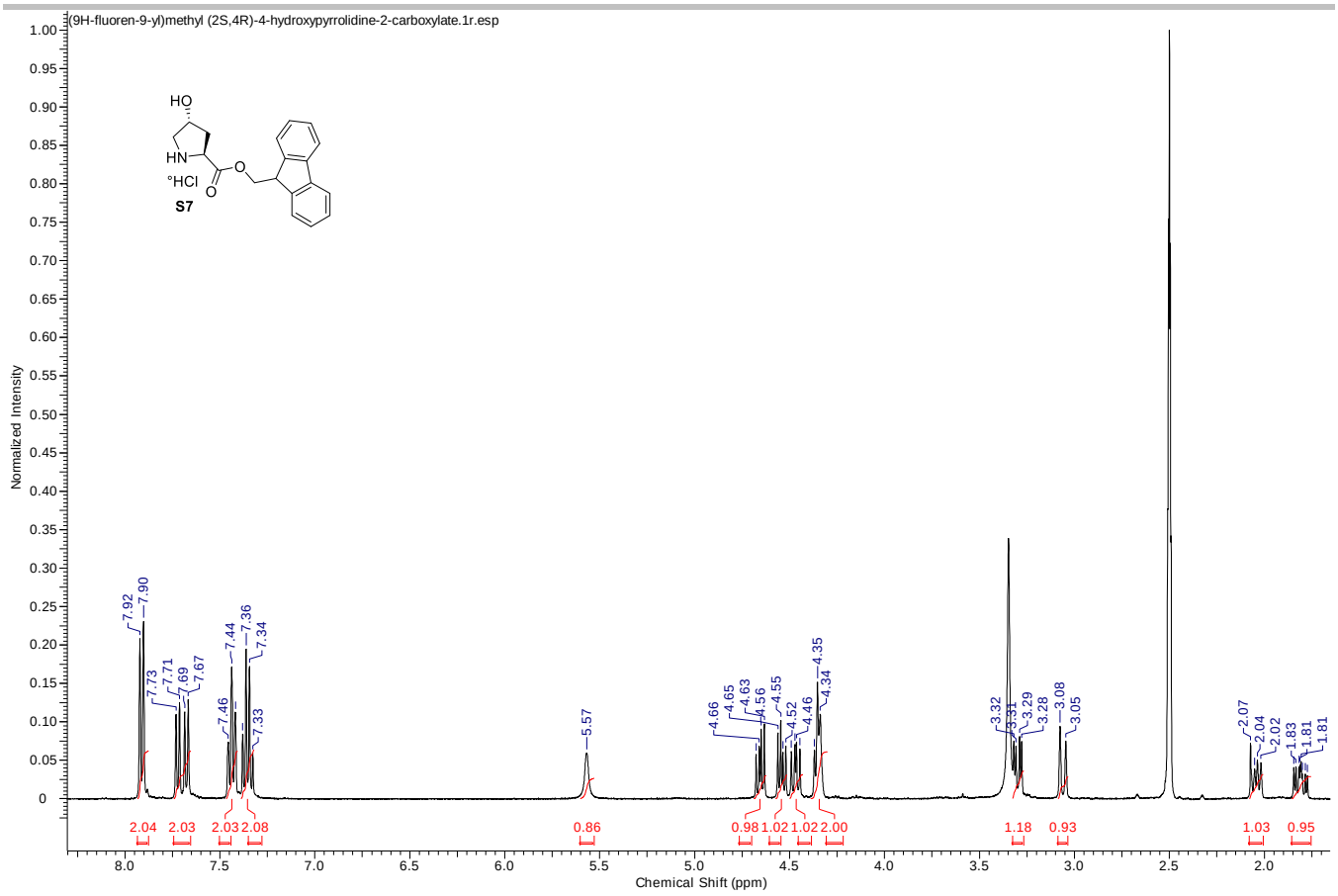

((benzyloxy)carbonyl)glycyl-L-isoleucine-1H.1r.esp
Cbz
N
H
H
N
O
COOH
S3
Normalized Intensity
Chemical Shift (ppm)
7.63
7.35
7.34
7.34
7.30
7.09
7.07
5.95
5.11
4.59
4.57
4.56
4.03
4.01
3.99
3.97
3.91
3.90
3.87
3.86
1.92
1.91
1.46
1.44
1.43
1.41
1.20
1.18
1.16
1.15
0.91
0.90
5.05
1.00
0.69
2.00
0.98
1.00
0.92
1.00
1.02
0.85
6.16

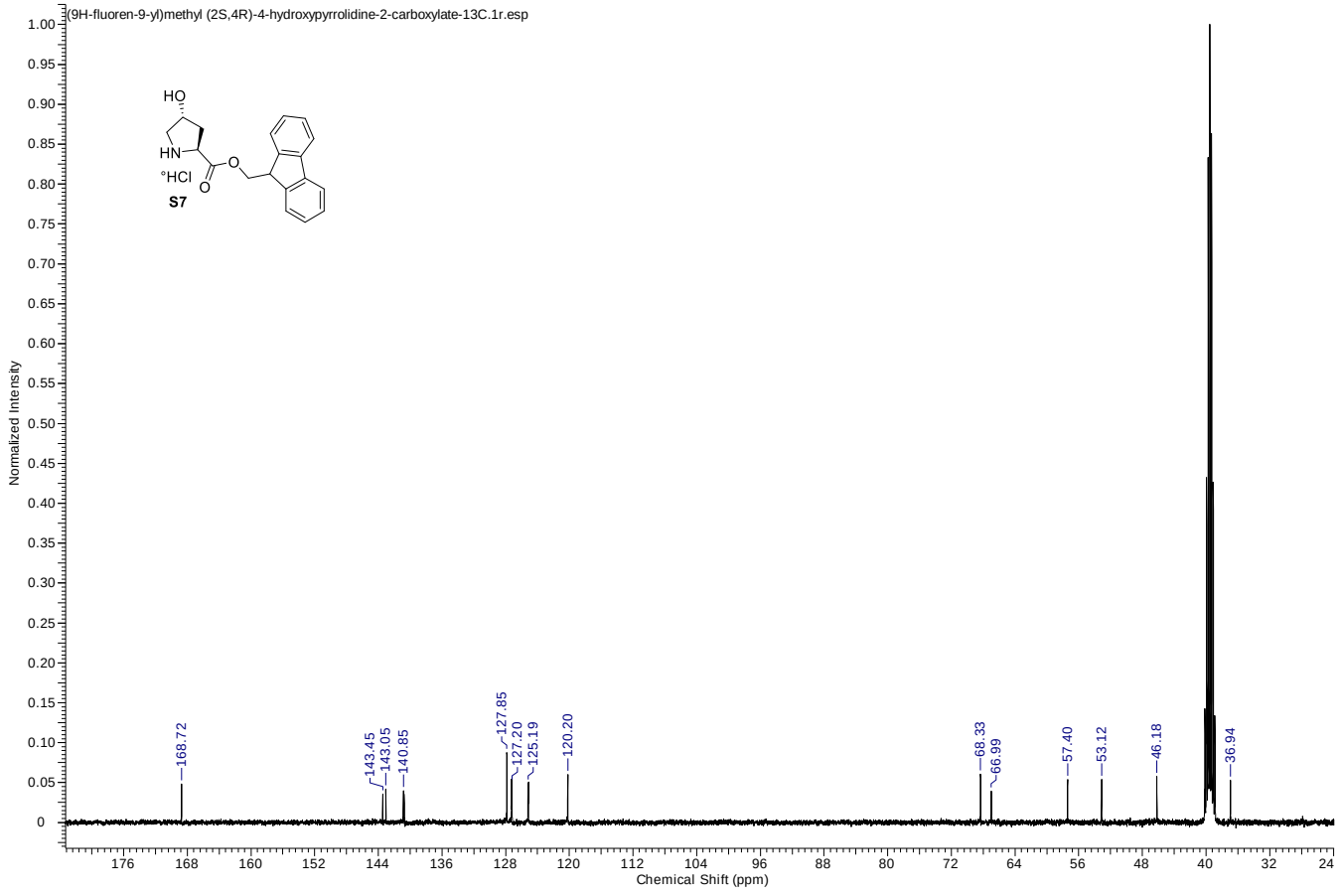

((benzyloxy)carbonyl)glycyl-L-isoleucine-13C.1r.esp
Cbz
N
H
H
N
O
COOH
S3
Normalized Intensity
Chemical Shift (ppm)
174.60
170.45
157.16
135.95
128.59
128.09
67.46
56.76
44.33
37.47
24.95
15.34
11.54

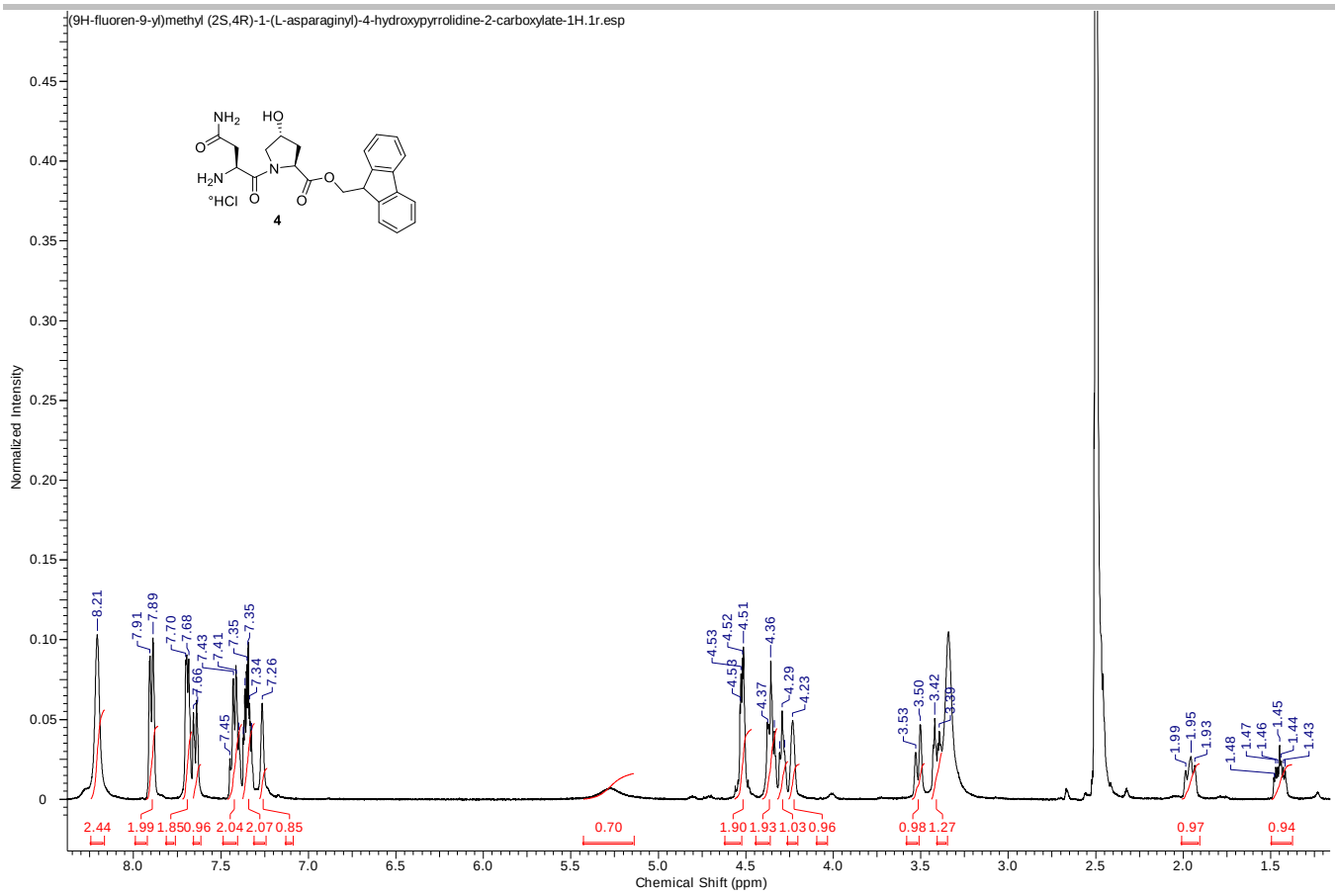

glycyl-L-isoleucine-1H.1r.esp
S4
Normalized Intensity
Chemical Shift (ppm)
4.55
4.53
4.11
4.10
4.08
4.03
3.98
3.94
2.33
2.32
2.31
2.30
2.18
2.17
2.16
2.15
1.78
1.77
1.76
1.75
1.74
1.73
1.72
1.48
1.46
1.45
1.44
1.43
1.42
1.21
1.16
1.14
0.94
1.79
1.10
1.04
0.61
0.92
0.97
0.97
2.94
3.00

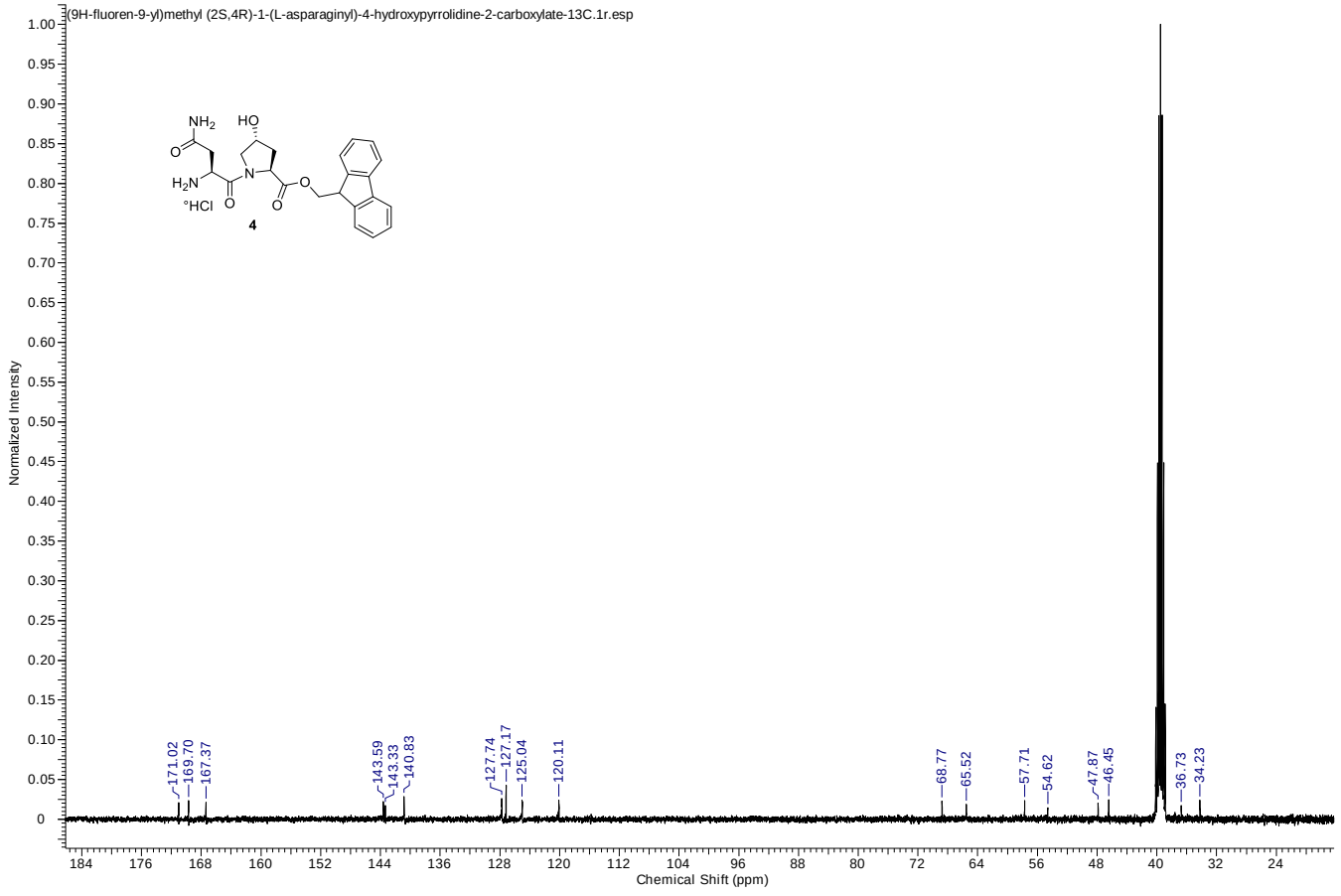

glycyl-L-isoleucine-13C.1r.esp
S4
Normalized Intensity
Chemical Shift (ppm)
172.11
166.75
58.33
42.99
40.22
36.22
24.15
14.68
10.39

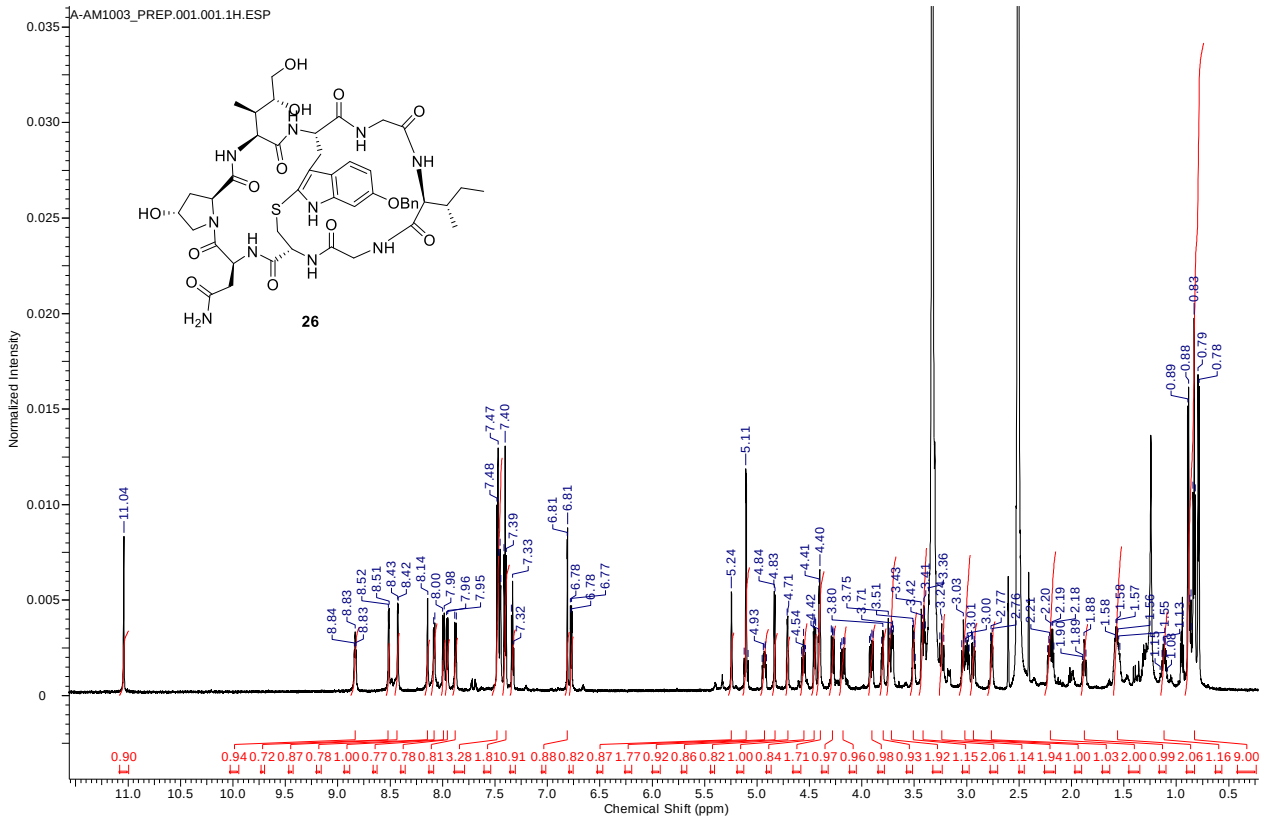
SUPPORTING INFORMATION



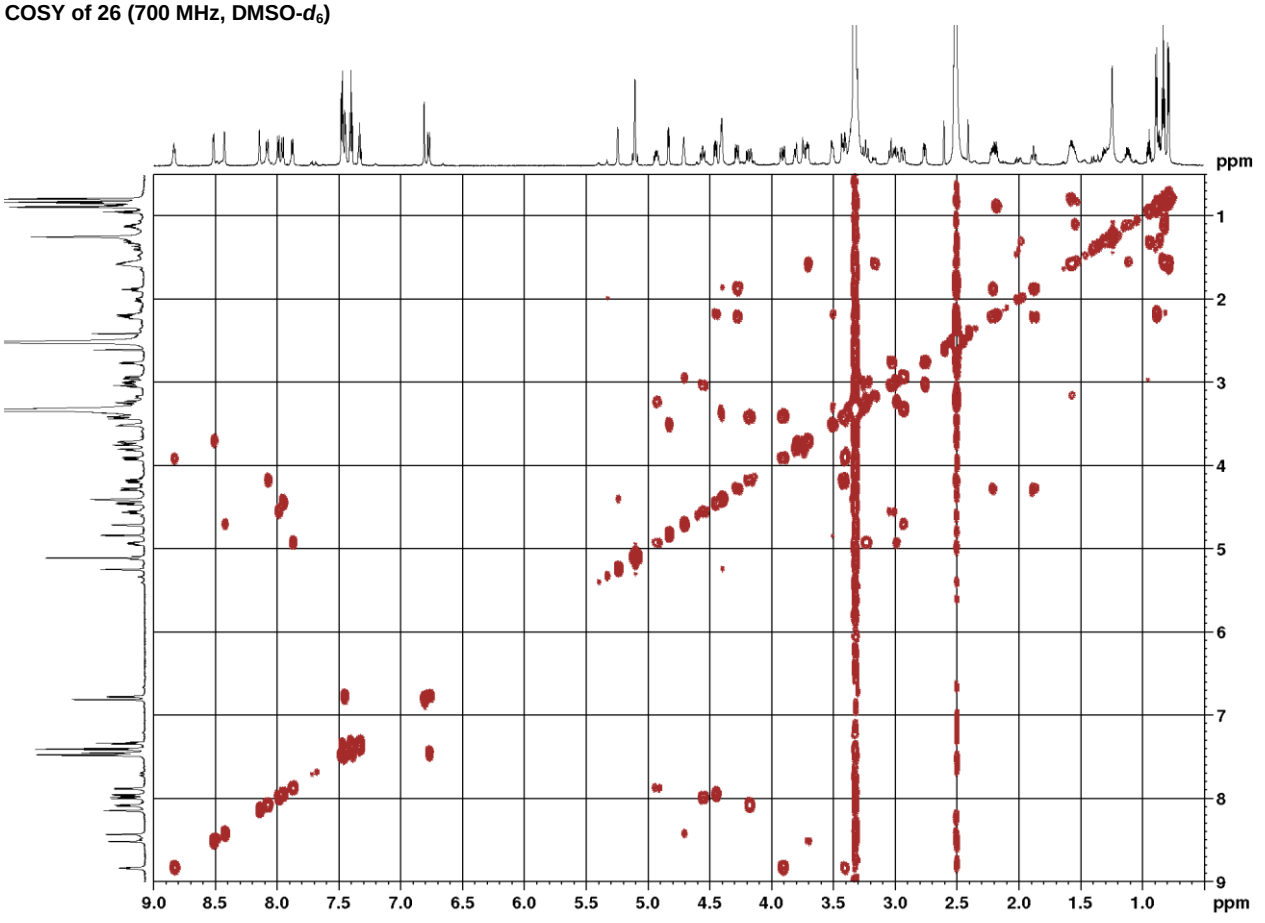



(9H-fluoren-9-yl)methyl (2S,4R)-1-(L-asparaginyl)-4-hydroxypyrrolidine-2-carboxylate-1H.1r.esp
Normalized Intensity
Chemical Shift (ppm)
4


(9H-fluoren-9-yl)methyl (2S,4R)-1-(L-asparaginyl)-4-hydroxypyrrolidine-2-carboxylate-13C.1r.esp
Normalized Intensity
Chemical Shift (ppm)
4



COSY of 26 (700 MHz, DMSO-d_6)



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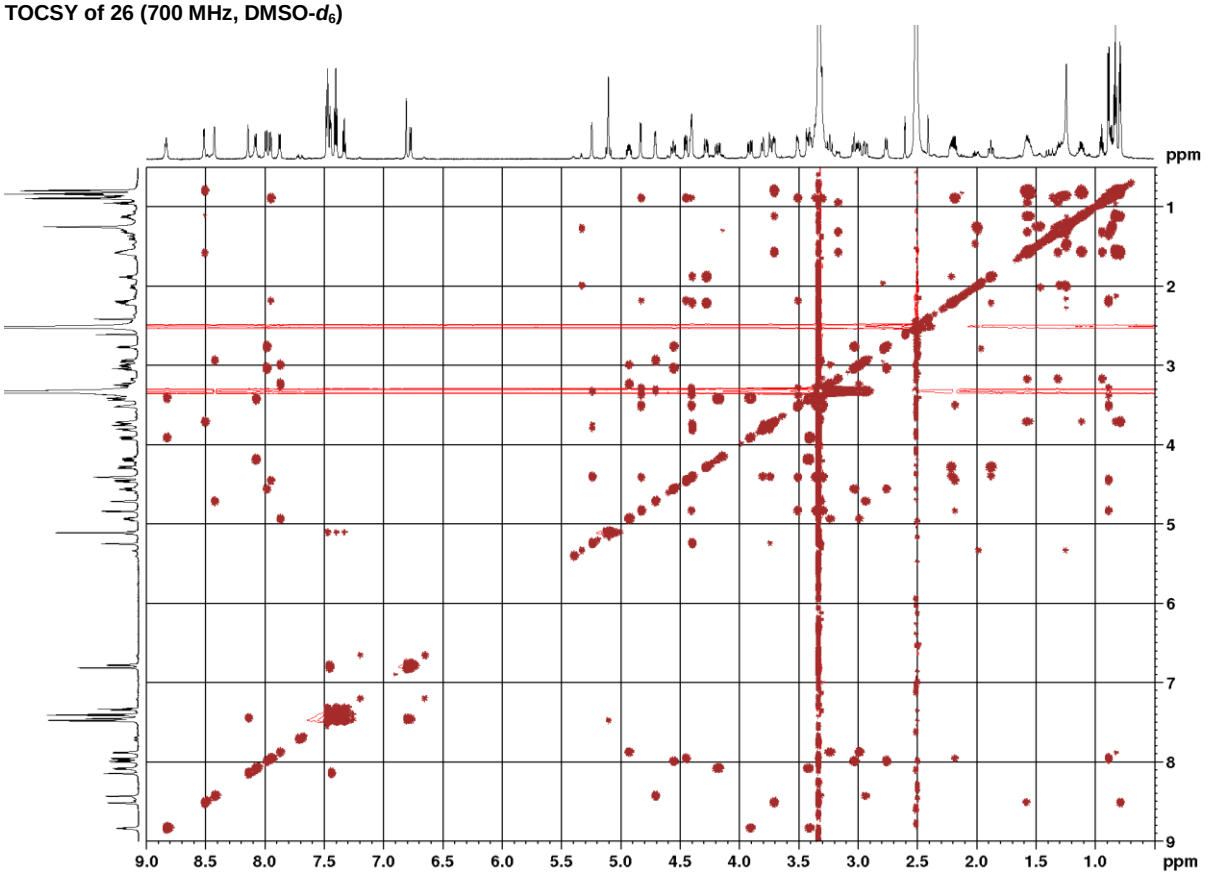

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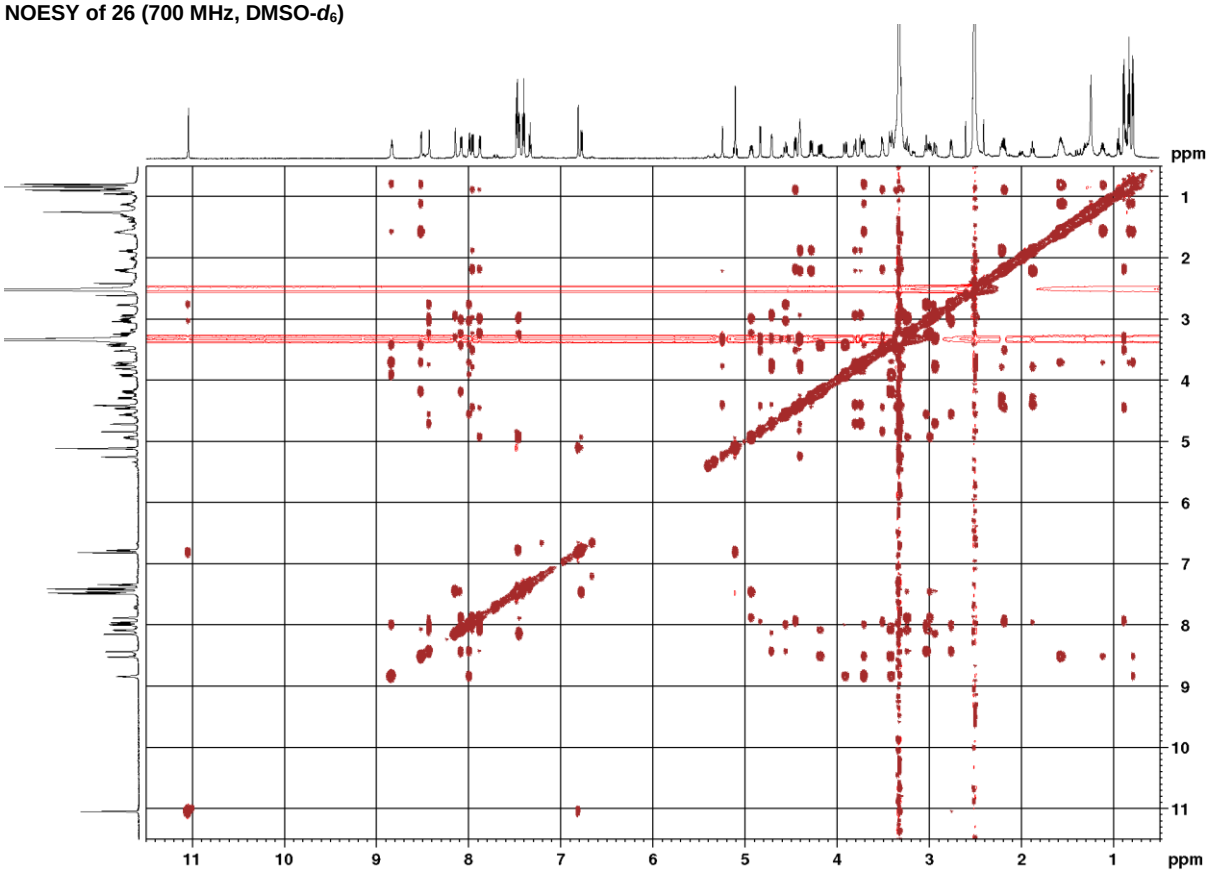
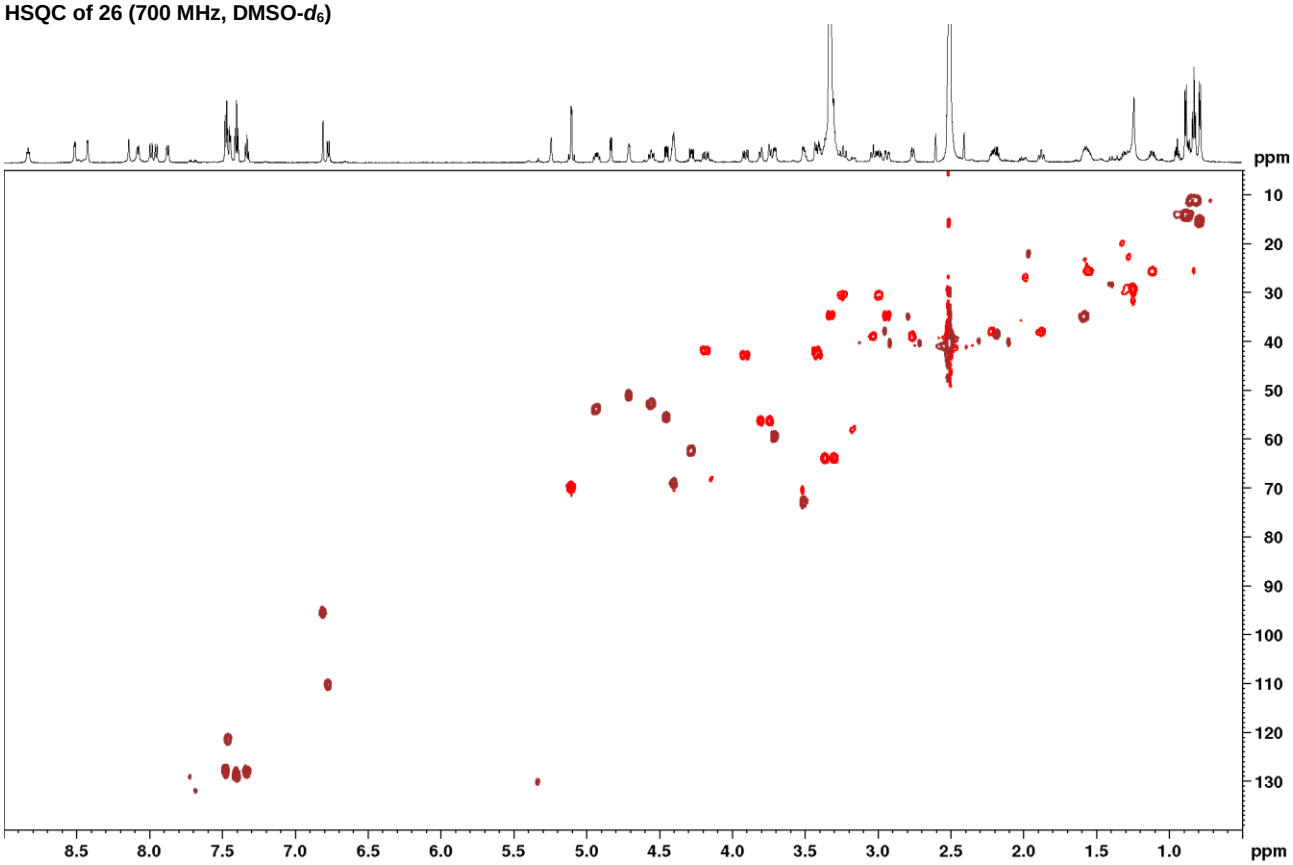
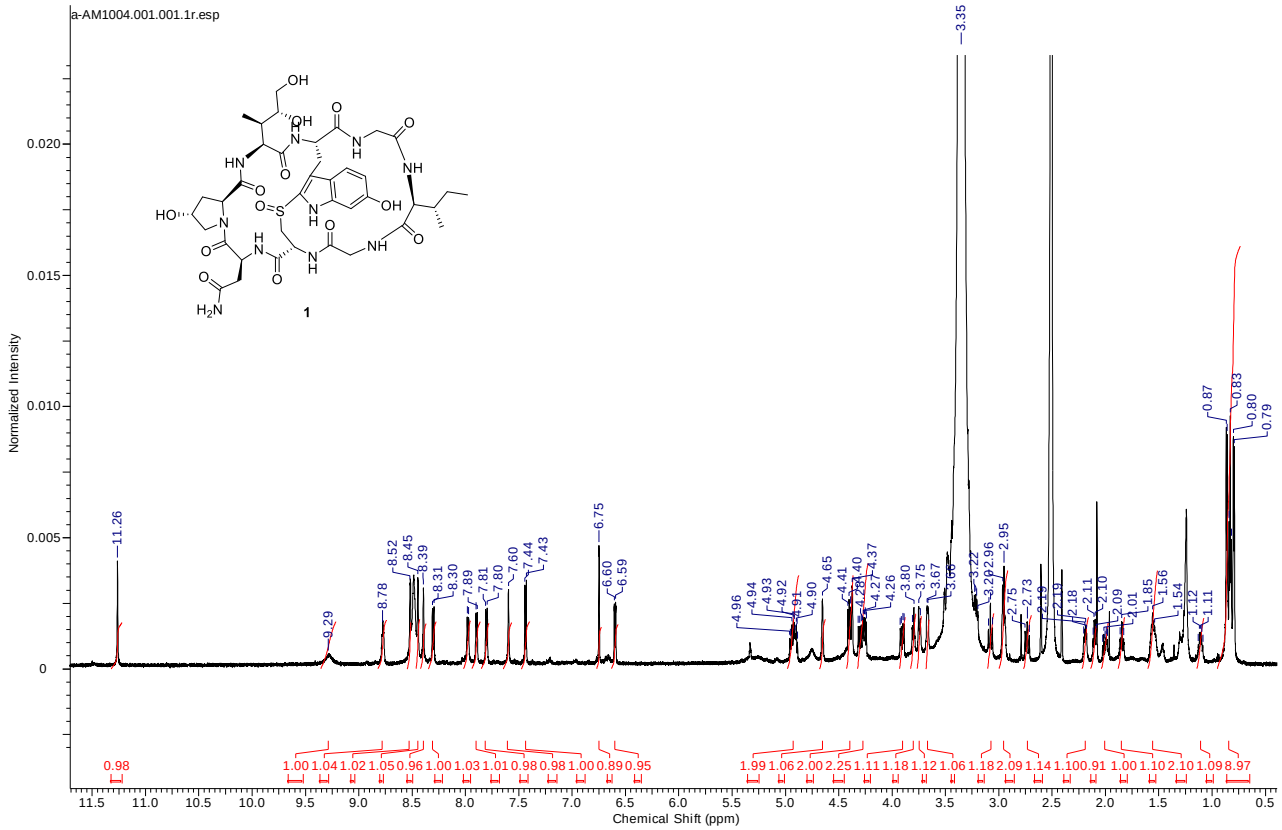


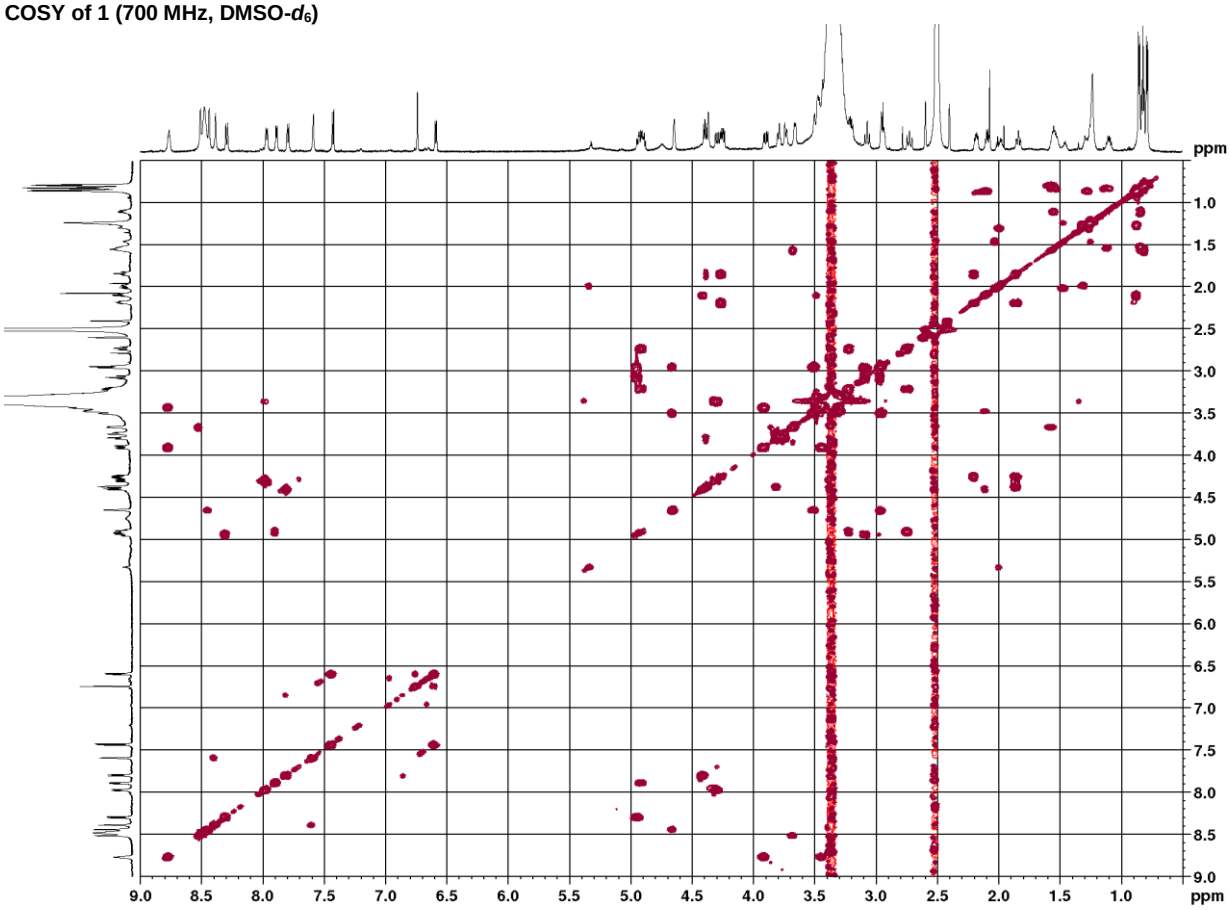
HSQC of 26 (700 MHz, DMSO-d_6)



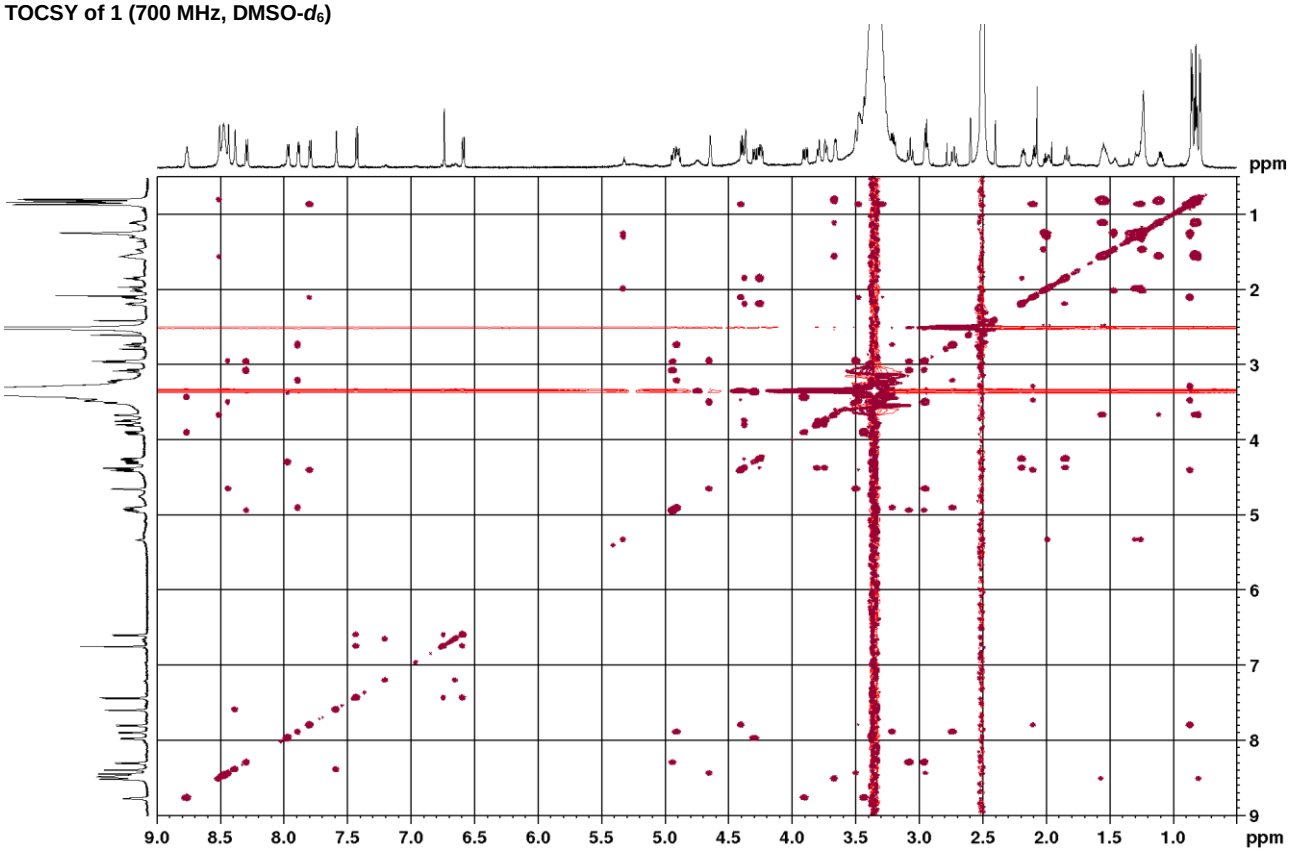
2.2.3 NMR spectra of synthetic α-amanitin (1)



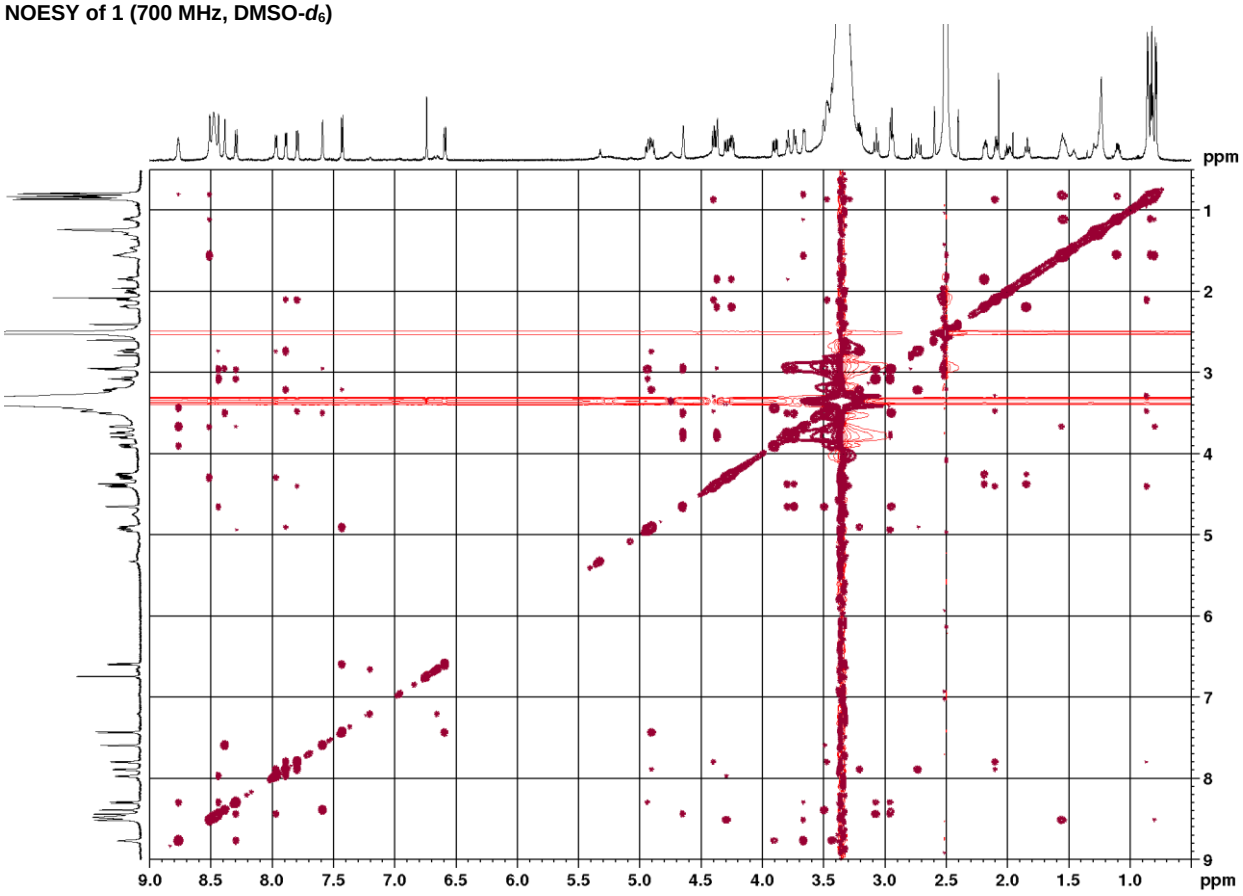
COSY of 1 (700 MHz, DMSO-d_6)



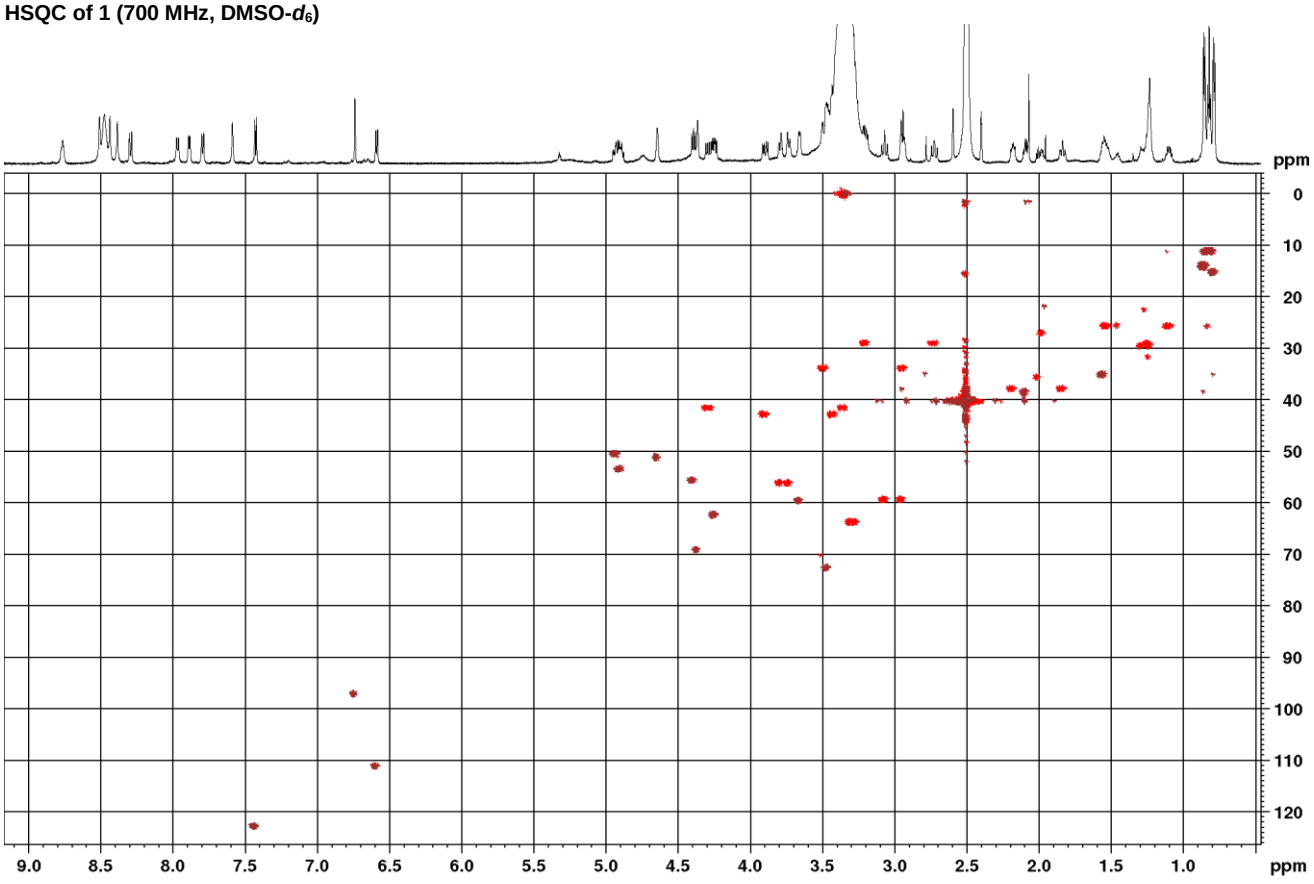
TOCSY of 1 (700 MHz, DMSO-d_6)

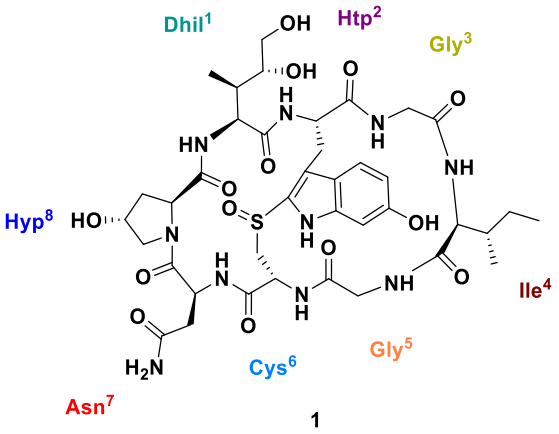


NOESY of 1 (700 MHz, DMSO-d_6)



HSQC of 1 (700 MHz, DMSO-d_6)





1

^{1}H chemical shifts for various samples of α-amanitin: 1a (synthetic α-amanitin), 1b (natural α-amanitin purchased from Sigma), 1c (synthetic α-amanitin from Perrin *et al.*),[12] 1d (natural α-amanitin from Shoham *et al.*).[13] Chemical shifts that could not be observed in the 1H-NMR due to the H_2O signal were analyzed using COSY-experiment.

Residue	Proton	1a	1b	1c	1d
Dhlle[1]	HN	7.80	7.80	-	7.78
	HC^{α}	4.39	4.39	4.39	4.43
	HC^{β}	2.09	2.09	2.09	2.11
	HC^{γ}	3.43	3.43	3.54	3.52
	$H´C^{\delta}$	3.28	3.28	-	3.30
	$H´´C^{\delta}$	3.28	3.28	-	3.30
	CH_3	0.86	0.86	0.82	0.87
	HO^{γ}	-	-	-	4.64
	HO^{δ}	-	-	-	4.28
Htp[2]	HN	8.44	8.44	-	7.83
	HC^{α}	4.65	4.65	4.89	4.92
	$H´C^{\beta}$	2.95	2.95	2.75	2.74
	$H´´C^{\beta}$	3.50	3.50	3.18	3.20
	H-4´	7.43	7.43	7.43	7.43
	H-5´	6.59	6.59	6.58	6.59
	H-7´	6.74	6.74	6.73	6.75
	HN (Indol)	11.25	11.25	11.21	11.18
	OH	9.27	9.27	-	9.12
Gly[3]	HN	7.97	7.97	7.88	7.94
	$H´C^{\alpha}$	4.29	4.29	4.33	4.31
	$H´´C^{\alpha}$	3.32	3.32	-	3.39
Ile[4]	HN	8.51	8.51	8.39	8.44
	HC^{α}	3.66	3.66	3.67	3.67
	HC^{β}	1.56	1.56	1.55	1.56
	$H´C^{\gamma}$	1.52	1.52	1.54	1.50
	$H´´C^{\gamma}$	1.10	1.10	1.10	1.11
	CH_3^{β}	0.79	0.79	0.77	0.80
	CH_3^{γ}	0.82	0.82	0.82	0.83

Gly[5]	HN	8.77	8.77	8.82	8.69
	H´C$^{\alpha}$	3.43	3.43	3.48	3.44
	H´´C$^{\alpha}$	3.90	3.90	3.91	3.90
Cys[6]	HN	8.30	8.30	8.28	8.25
	HC$^{\alpha}$	4.94	4.94	4.93	4.94
	HC$^{\beta}$	2.95	2.95	2.88	2.96
	H´´C$^{\beta}$	3.07	3.07	3.03	3.08
Asn[7]	HN	7.90	7.90	8.44	8.41
	HC$^{\alpha}$	4.90	4.90	4.64	4.64
	H´C$^{\beta}$	2.73	2.73	2.95	2.95
	H´´C$^{\beta}$	3.21	3.21	3.55	3.50
	NH_2	8.39, 7.59	8.39, 7.59	-	8.32, 7.51
Hyp[8]	HC$^{\alpha}$	4.25	4.25	4.20	4.28
	H´C$^{\beta}$	1.84	1.84	1.81	1.85
	H´´C$^{\beta}$	2.18	2.18	2.16	2.19
	HC$^{\gamma}$	4.37	4.37	4.37	4.37
	H´C$^{\delta}$	3.73	3.73	3.80	3.80
	H´´C$^{\delta}$	3.80	3.80	3.80	3.80
	HO	-	-	-	5.12

Table S1: ^{1}H chemical shifts for some samples of synthetic and natural α-amanitin.

2.3 CD-spectra of natural and synthetic α-amanitin (1)

Sample preparation: Natural (purchased from Sigma, 1 mg) and synthetic α-amanitin (**1**, 1 mg) were dissolved in 1 mL H_2O and diluted to a concentration of 75 µM. A quartz cuvette (1 mm path-length) was used. CD spectra were measured as described in the General experimental details section.

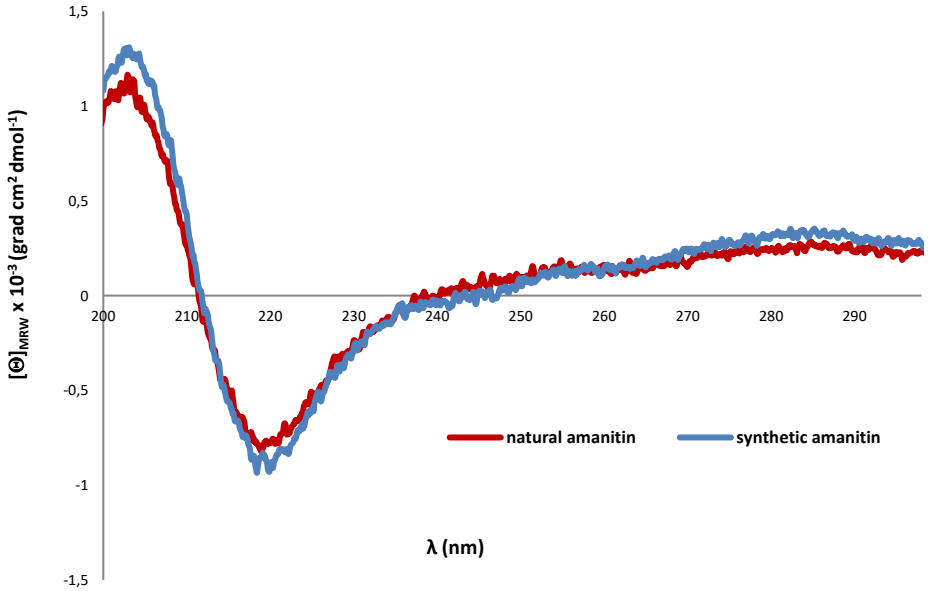


Figure S4: Comparative CD-spectra of synthetic α-amanitin (**1**, blue) and natural α-amanitin (red, purchased from Sigma Aldrich)

2.4 Co-Injection of natural and synthetic α-amanitin (1)

Gradient D: 0-30 min: 6% to 18% AcN + 0.1% HCO_2H, 30-34 min: 18% AcN to 100% AcN + 0.1% HCO_2H, 34-37 min: 100% AcN to 6% AcN+ 0.1% HCO_2H, 39-44 min: 6% AcN**.** Flowrate: 0.2 mL/min. Absorption: 305 nm.

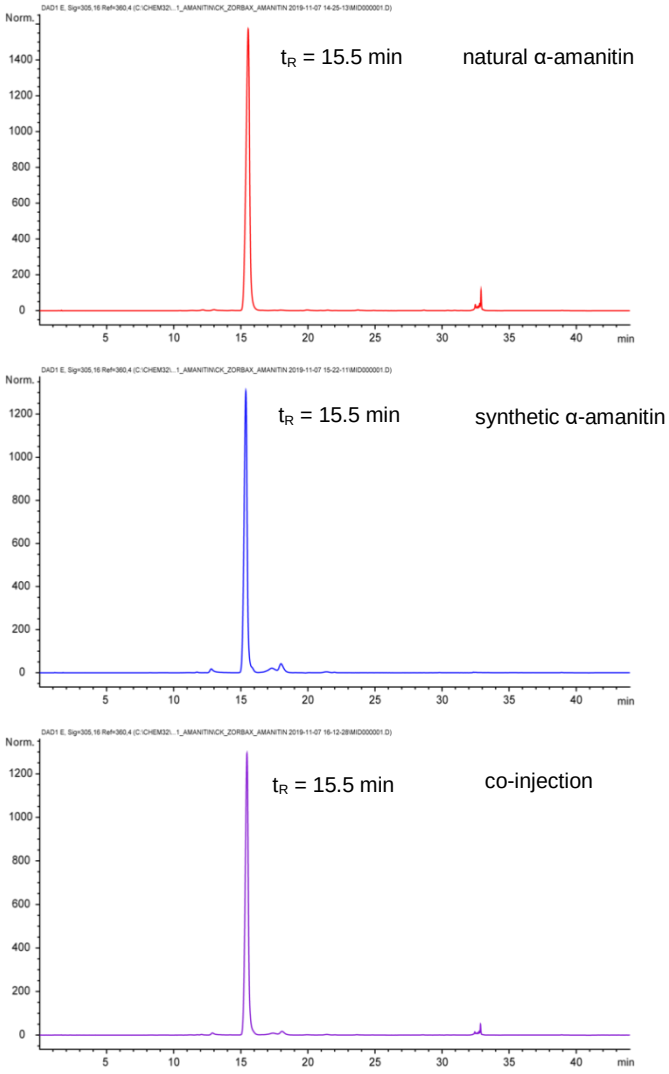


Figure S5: Comparative co-injection of natural and synthetic α-amanitin (**1**).

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