Supporting Information

Synthesis of Constrained Tetracyclic Peptides by Consecutive CEPS, CLiPS and Oxime Ligation

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Contents

1 General information

1.1 Amino acid and scaffold synthesis

Unless stated otherwise, reactions were performed without special precautions like drying or N_2/Argon atmosphere. Dried CH_2Cl_2 and CH_3CN were obtained by distilling these solvents with CaH_2 as drying agent. Dried THF was obtained by distillation with sodium. All dried solvents were stored under N_2 atmosphere. Dry DMF on 4 Å molecular sieves was obtained from Sigma-Aldrich and stored under N_2 atmosphere. Reagents were purchased with the highest purity (usually >98%) from Sigma Aldrich, Bachem and Fluorochem and used as received. Reactions were monitored with thin layer chromatography (TLC) carried out on 0.25 mm E. Merck silica gel plates (60F-254). SilaFlash® P60 (particle size 40-63 µm) was used for silica column chromatography. NMR spectra were recorded on Bruker DRX-500, 400 and 300 MHz instruments and calibrated on residual undeuterated solvent signals as internal standard. The 1H-NMR multiplicities were abbreviated as followed: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet. High resolution mass spectra (HRMS) were recorded on an AccuTOF GC v 4g, JMS-T100GCV Mass spectrometer (JEOL, Japan). FD/FI probe equipped with FD Emitter, Carbotec or Linden (Germany), FD 10 µm. Current rate 51.2 mA/min over 1.2 min machine using field desorption (FD) and ESI as ionization methods. Melting points were recorded on a Wagner & Munz Polytherm A melting point apparatus and are uncorrected. IR spectra were recorded on a Bruker Alpha FTIR machine.

1.2 Peptide synthesis and reaction analysis

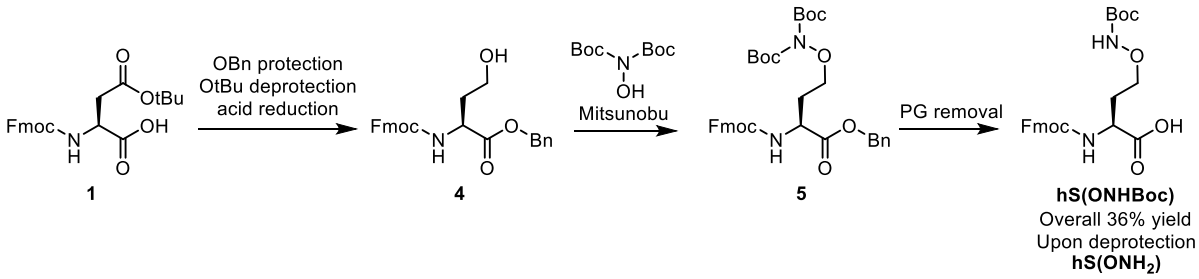
The peptide purity and identity was assessed using an Agilent 1260 Infinity HPLC system coupled with an Agilent 6130 quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA) to determine the peptide mass. Separation was performed using a Waters XSelect® CSH C18 column (2.5 µm, 3.0 x 150 mm. Waters Corporation, Milford, MA, USA) column, eluting with 0.05% (v/v) MSA in a water/ACN gradient, with a flow rate of 1 mL min-1 and a column temperature of 50°C. As mobile phase a binary mixture of solvent A (water + 0.05% (v/v) MSA) and solvent B (ACN + 0.05% (v/v) MSA) was used. A linear gradient from 5-60 % B in 7.5 min, followed by isocratic 95% solvent B for 3 min was used by default. The purity of peptides was determined by automatically integrating product and impurity peaks of the relevant HPLC spectrum (λ= 220 nm).

CLIPS and oxime ligation reaction mixtures were measured on a UPLC-ESMS system (3 min, 5-80%B , where B=MeCN, column temperature of 50°C), Acquity UPLC Peptide BEH C18 Column, 130Å, 1.7 µm, 2.1x50 mm with UV detection (λ = 215 nm) and positive ion current for MS analysis, unless stated otherwise.

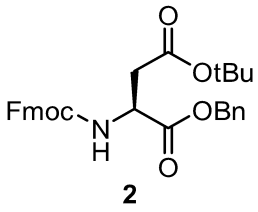
2 Amino Acid Synthesis

2.1 hS(ONH_2)

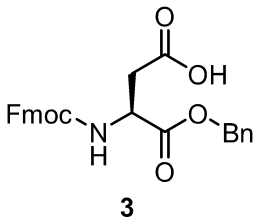
hS(ONH_2) is prepared for SPPS, with an N-terminal Fmoc-protecting group and a Boc-group protecting the aminooxy ONH_2. As such, it is synthesized as **hS**(ONHBoc).



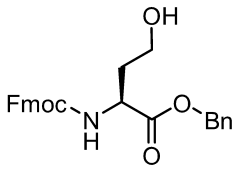
Scheme 1: Synthesis of **hS**(ONHBoc). Steps are detailed in procedures.



In a flame-dried flask, under Ar-pressure, Fmoc-Asp(OtBu)-OH (**1**) (43.106 g, 106.12 mmol, 1 equiv) was suspended in 400 ml of anhydrous MeOH. Cs_2CO_3 (17.288 g, 53.06 mmol, 0.5 equiv) was added and the mixture immediately becomes a colorless solution, which was subsequently stirred for 45 min. The volatiles were removed under reduced pressure, yielding a while solid. The residue is dissolved in 500 ml anhydrous MeCN and benzyl bromide (37.86 ml, 318.36 mmol, 3 equiv) was added. The mixture was stirred for 3 hours at rt. during which a white precipitate forms. The volatiles were removed and the remaining solid was washed with water and EtOH twice, yielding the desired product as a white solid, in quantitative yield. **^{1}H NMR** (400 MHz, Chloroform-*d*) δ 7.78 (d, *J* = 7.5 Hz, 2H), 7.62 (d, *J* = 6.9 Hz, 2H), 7.42 (t, *J* = 7.4 Hz, 2H), 7.33 (dd, *J* = 15.3, 7.5 Hz, 7H), 5.93 (d, *J* = 8.5 Hz, 1H), 5.33 – 5.14 (m, 2H), 4.70 (dt, *J* = 8.4, 4.1 Hz, 1H), 4.50 – 4.40 (m, 1H), 4.40 – 4.30 (m, 1H), 4.26 (t, *J* = 7.1 Hz, 1H), 3.01 (dd, *J* = 17.0, 4.5 Hz, 1H), 2.83 (dd, *J* = 16.9, 4.2 Hz, 1H), 1.45 (s, 9H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 170.70, 169.85, 155.89, 143.81, 143.59, 141.17, 135.15, 128.47, 128.31, 128.13, 127.60, 126.97, 125.08, 125.03, 119.87, 81.74, 67.34, 67.17, 50.55, 46.98, 37.62, 27.89. Spectroscopic data are in accordance with those reported in literature.[1]

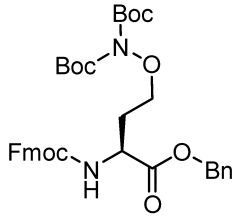


Fmoc-Asp(OtBu)-OBn (**2**) (1.604 g, 3.197 mmol) was dissolved in 15 ml of freshly distilled CH_2Cl_2. 15 ml HCOOH was added to the solution and the mixture was stirred overnight at rt, after which TLC showed full conversion of the starting material. The volatiles were removed under reduced pressure and the remnants of HCOOH were removed by co-evaporation with CH_2Cl_2, yielding Fmoc-Asp(OH)-OBn as a white solid (1.310 g, 2.94 mmol, 92%).**^{1}H NMR** (400 MHz, Chloroform-*d*) δ 11.15 (s, 1H), 7.80 (d, *J* = 7.5 Hz, 2H), 7.66 (d, *J* = 7.4 Hz, 2H), 7.44 (t, *J* = 7.4 Hz, 2H), 7.36 (d, *J* = 7.4 Hz, 7H), 6.16 (d, *J* = 8.5 Hz, 1H), 5.26 (s, 2H), 4.83 (dt, *J* = 8.6, 4.4 Hz, 1H), 4.50 (dd, *J* = 10.4, 7.4 Hz, 1H), 4.46 – 4.38 (m, 1H), 4.26 (t, *J* = 7.1 Hz, 1H), 3.18 (dd, *J* = 17.4, 4.6 Hz, 1H), 3.01 (dd, *J* = 17.4, 4.2 Hz, 1H) **^{13}C NMR** (100 MHz, $CDCl_3$) δ 175.36, 170.34, 156.04, 143.53, 143.39, 141.02, 134.84, 128.34, 128.20, 127.94, 127.51, 126.87, 124.90, 119.76, 67.44, 67.21, 50.18, 46.79, 36.12. Spectroscopic data are in accordance with those reported in literature.[2]



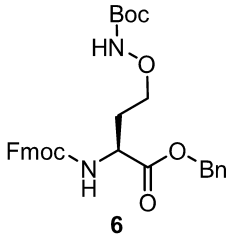
4

In a flame-dried flask, under N_2 flow, Fmoc-Asp(OH)-OBn (**3**) (11.11 g, 25 mmol) was dissolved in 175 ml of freshly distilled THF. The reaction mixture was cooled to 0 °C, after which BH_3•SMe_2 (11.85 ml, 125 mmol, 5 equiv) is added dropwise over 1 hour. The mixturewas stirred on ice for 2h, and subsequently warmed to rt and stirred overnight, after which TLC showed full consumption of the starting material. The mixture was cooled on ice, and carefully quenched with sat. NH_4Cl solution and extracted with EtOAc (3x). The organic phase was washed with 1M $KHSO_4$, brine and dried over Na_2SO_4. The volatiles were removed under reduced pressure, after which **4** crystallized as a white solid (10.74 g, 24.86 mmol, 99%). **^{1}H NMR** (500 MHz, Chloroform-*d*) δ 7.81 – 7.73 (m, 2H), 7.61 (d, *J* = 6.6 Hz, 2H), 7.41 (t, *J* = 7.4 Hz, 2H), 7.33 (dd, *J* = 15.3, 7.4 Hz, 8H), 5.93 – 5.81 (m, 1H), 5.26 – 5.09 (m, 2H), 4.64 – 4.54 (m, 1H), 4.44 (p, *J* = 10.5 Hz, 3H), 4.22 (t, *J* = 6.6 Hz, 1H), 3.74 – 3.65 (m, 1H), 3.65 – 3.52 (m, 1H), 3.16 (s, 1H), 2.18 (td, *J* = 9.3, 4.6 Hz, 1H), 1.77 (td, *J* = 9.6, 4.7 Hz, 1H). **^{13}C NMR** (125 MHz, $CDCl_3$) δ 172.28, 156.64, 143.68, 143.57, 143.52, 143.47, 141.17, 135.03, 128.50, 128.38, 128.13, 127.62, 126.97, 124.93, 124.90, 119.88, 119.86, 67.25, 67.04, 58.20, 51.29, 47.03, 35.23, 29.97. Spectroscopic data are in accordance with those reported in literature.[3]



5

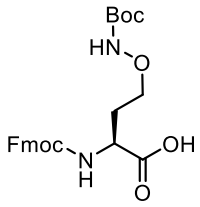
In a flame-dried flask, under N_2, Fmoc-*homo*Ser-OBn (**4**) (7.01 g, 16.21 mmol) was dissolved in 125 ml of anhydrous THF. Subsequently Boc_2NOH (prepared according to Jacobson *et al.*[4]) (3.97 g, 17.02 mmol, 1.05 equiv) and PPh_3 (4.46 g, 17.02 mmol, 1.05 equiv) were added, and the flask was cooled on an ice bath. DIAD (4.29 ml, 17.02 mmol, 1.05 equiv) was added dropwise *via* a syringe pump (4.4 ml/h). The mixture was warmed to rt, and stirred overnight. The volatiles were removed under reduced pressure, after which the mixture was immobilized in silica. Column chromatography (6:2:1 – petroleum ether: CH_2Cl_2:EtOAc) provided the product **5** as a white solid (7.514 g, 11.60 mmol, 72%). **^{1}H NMR** (500 MHz, Chloroform-*d*) δ 7.77 (d, *J* = 7.5 Hz, 2H), 7.67 (d, *J* = 7.3 Hz, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.37 – 7.33 (m, 2H), 7.33 – 7.24 (m, 5H), 6.63 (d, *J* = 8.6 Hz, 1H), 5.20 (s, 2H), 4.65 (dt, *J* = 10.3, 5.5 Hz, 1H), 4.42 (dd, *J* = 10.2, 7.5 Hz, 1H), 4.38 – 4.29 (m, 1H), 4.24 (t, *J* = 7.2 Hz, 1H), 4.16 – 4.07 (m, 1H), 4.03 – 3.93 (m, 1H), 2.29 (q, *J* = 10.2, 6.0 Hz, 2H), 2.23 (dd, *J* = 12.8, 8.1 Hz, 1H), 1.55 (s, 18H).**^{13}C NMR** (125 MHz, $CDCl_3$) δ 171.63, 156.42, 150.34, 144.08, 143.95, 141.28, 135.52, 128.54, 128.31, 128.17, 127.66, 127.65, 127.07, 127.06, 125.32, 125.31, 119.92, 84.41, 72.54, 67.18, 67.14, 52.04, 47.21, 29.64, 28.10. **IR** (cm^{-1}) 3349, 2979, 2044, 1969, 1953, 1789, 1746, 1715, 1608, 1524, 1477, 1540, 1392, 1368, 1345, 1270, 1246, 1145, 1110, 1001, 911, 846, 793, 755, 736. **HR-MS** FD m/z [M^+] calcd for $C_{36}H_{42}N_2O_9$: 646.2890, found 646.2866. **mp** 31-34 °C**.**



6

Bis-boc amino-oxy amino acid **5** (11.32 g, 17.51 mmol) was dissolved in 150 ml of CH_2Cl_2. TFA (2.14 ml, 27.94 mmol, 1.6 equiv). It was stirred overnight, after which NMR showed incomplete conversion. 0.9 ml (11.75 mmol, 0.67 equiv) of TFA was added and the reaction mixture was again stirred overnight. The volatiles were removed, after which the mono-Boc compound was purified *via* column chromatography (6:4:1–petroleum ether: CH_2Cl_2:EtOAc) yielding **6** as a white solid 5.17 g (9.22 mmol, 53%). **^{1}H NMR** (500 MHz, Chloroform-*d*) δ 7.79 (d, *J* = 7.6 Hz, 2H),7.69 (d, *J* = 7.5 Hz, 2H), 7.42 (t, *J* = 7.5 Hz, 3H), 7.40 – 7.27 (m, 9H), 6.57 (s, 1H), 5.22 (s, 2H), 4.64 (q, *J* = 6.5 Hz, 1H), 4.42 (tt, *J* = 17.9, 8.9 Hz, 2H), 4.26 (t, *J* = 7.4 Hz, 1H), 4.01 (ddd, *J* = 11.4, 7.4, 4.3 Hz, 1H), 3.93 (dt, *J* = 10.5, 5.3 Hz, 1H), 2.20 (tq, *J* = 15.4, 9.8, 9.2 Hz, 2H), 1.52 (s, 9H**). ^{13}C NMR** (125 MHz, $CDCl_3$) δ 171.96, 157.13, 156.32, 143.98,

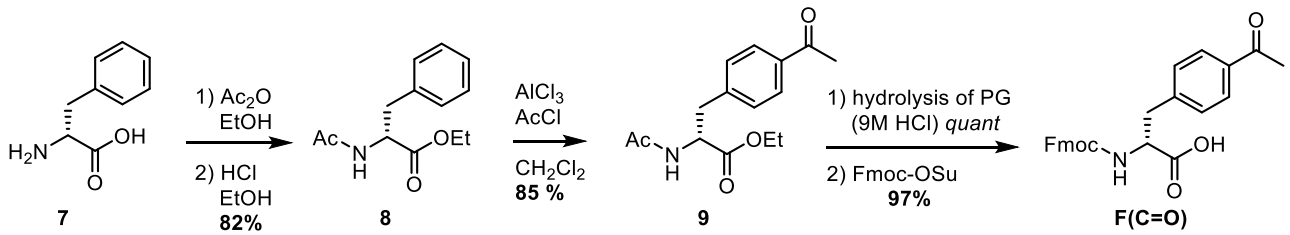
143.79, 141.21, 135.33, 128.49, 128.30, 128.19, 127.59, 127.00, 125.21, 119.85, 82.05, 72.89, 67.16, 67.05, 51.81, 47.11, 29.92, 28.14. **IR** (cm^{-1}) 3285, 3065, 2977, 2933, 1702, 1529, 1477, 1449, 1391, 1367, 1337, 1248, 1214, 1159, 1104, 1080, 1057, 1003, 909, 853, 757, 737. **HR-MS** FD m/z [M^+] calcd for $C_{31}H_{34}N_2O7$: 546.2336, found 546.2366. **mp** 43 °C.



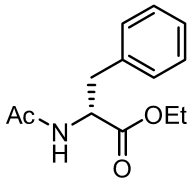
hS(ONHBoc)

In a flame-dried flask, benzyl-ester **6** (5.17 g, 9.22 mmol) was dissolved in 200 ml EtOH. The flask was degassed and Pd/C (10 wt% loading, 256 mg) was added. The flask was evacuated and purged with H_2 three times and the reaction mixture was stirred under H_2 pressure (balloon) for 4h at rt. TLC showed full conversion of the starting material and the reaction flask was purged with N_2. The mixture was filtered over Celite and eluted with EtOH. The volatiles were evaporated under reduced pressure, yielding the product **hS**(ONHBoc) as a white solid (4.20 g, 9.21 mmol, 99%). **^{1}H NMR** (500 MHz, Chloroform-*d*) δ 7.91 (s, 1H), 7.75 (d, *J* = 7.2 Hz, 2H),7.65 (t, *J* = 6.1 Hz, 2H), 7.38 (t, *J* = 7.0 Hz, 3H), 7.29 (t, *J* = 7.3 Hz, 3H), 6.68 (s, 1H), 4.58 (q, J = 6.1 Hz, 1H), 4.38 (d, *J* = 6.8 Hz, 2H), 4.23 (t, *J* = 6.7 Hz, 1H), 4.07 – 3.91 (m, 2H), 2.18 (d, *J* = 3.7 Hz, 2H), 1.49 (s, 9H**). ^{13}C NMR** (125 MHz, $CDCl_3$) δ 175.70, 157.66, 156.61, 143.82, 143.65, 141.11, 127.55, 126.97, 125.18, 119.78, 82.32, 72.93, 67.15, 51.61, 46.95, 29.67, 28.07. **IR** (cm^{-1}) 3272, 3065, 2977, 1695, 1524, 1477, 1448, 1393, 1368, 1336, 1249, 1158, 1105, 1080, 1051, 940, 907, 850, 758, 733. **HR-MS** FD m/z [M^+] calcd for $C_{24}H_{28}N_2O_7$: 456.1897 found 456.1896. **mp** 46.2 °C

2.2 F(C=O)

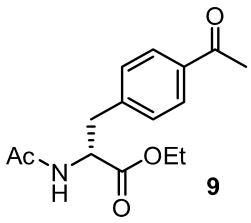


Scheme 2: The synthesis of **F**(C=O). Procedures are detailed below.



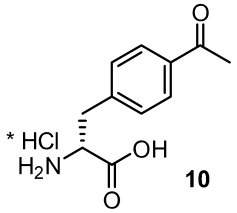
8

H-Phe-OH (**7**) (33.073 g, 200 mmol, 1 equiv) was added to a flask equipped with a with reflux condenser, and suspended in 170 ml EtOH. Ac_2O (52 ml, 540 mmol, 2.7 equiv) was added and the solution was stirred at reflux overnight. The volatiles (acetic acid remnants) were removed under reduced pressure, yielding a yellowish sticky oil. The mixture was redissolved in 170 ml of EtOH and concentrated HCl (4 ml, cat) was added. The mixture was heated to reflux and stirred overnight. The volatiles were removed under reduced pressure. The yellow oil was redissolved in EtOAc and washed with a 1M $KHSO_4$ solution, sat. $NaHCO_3$ solution and brine, and subsequently dried over Na_2SO_4. The volatiles were removed under reduced pressure, yielding Ac-Phe-OEt (**8**) as an off-white solid (38.89 g, 165.31 mmol, 82%). **^{1}H NMR** (500 MHz, Chloroform-d) δ 7.28 (dt, J = 15.6, 7.0 Hz, 3H), 7.13 (d, J = 6.9 Hz, 2H), 6.16 (d, J = 7.2 Hz, 1H), 4.87 (q, J = 6.0 Hz, 1H), 4.17 (q, J = 7.1 Hz, 2H), 3.12 (tt, J = 13.8, 7.0 Hz, 2H), 1.99 (s, 3H), 1.25 (t, J = 7.1 Hz, 3H). **^{13}C NMR** (125 MHz, CDCl3) δ 171.69, 169.61, 135.95, 129.26, 128.45, 127.00, 61.43, 53.16, 37.90, 23.04, 14.06. **IR** (cm^{-1}) 3312, 3025, 3002, 2973, 2932, 2908, 1946, 1728, 1641, 1530, 1493, 1480, 1444, 1398, 1374, 1345, 1319, 1259, 1220, 1198, 1156, 1129, 1075, 1033, 1021, 966, 929, 909, 867, 824, 813, 764, 745. **mp** 67 °C. Spectroscopic data are in accordance with those reported in literature.[5]



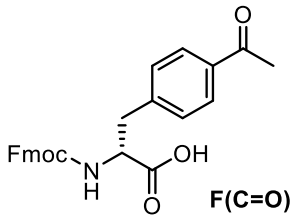
9

To a flame-dried flask, under N_2 flow and at 0 °C, $AlCl_3$ (12.40 g, 93.00 mmol, 5.5 equiv) was added, followed by the dropwise addition of AcCl (7.2 ml, 101.27 mmol, 6.0 equiv). To the chuncky suspension, a solution of Ac-Phe-OEt (**8**) (4.00 g, 17.00 mmol, 1 equiv) in 18 ml of CH_2Cl_2 was added dropwise. The dark orange solution was stirred for 30 min on ice, then the mixture was warmed to rt and stirred overnight. The mixture was crashed onto ice, containing 10% 1M HCl solution. The product was extracted with CH_2Cl_2, and the organic phase was washed twice with a sat. $NaHCO_3$ solution and water. After drying over Na_2SO_4, the volatiles were removed under reduced pressure to yield a dark brown oil. Flash column chromatography (1:2 – petroleum ether:EtOAc) provided the product **9** as a yellowish oil, which crystallizes upon standing (4.26 g, 15.36 mmol, 90%). **^{1}H NMR** (400 MHz, Chloroform-*d*) δ 7.84 (d, *J* = 8.2 Hz, 2H), 7.19 (d, *J* = 8.2 Hz, 2H), 6.17 (d, *J* = 7.5 Hz, 1H), 4.86 (dd, *J* = 19.7, 7.7 Hz, 1H), 4.14 (q, *J* = 6.2 Hz, 2H), 3.19 (dd, *J* = 13.8, 6.1 Hz, 1H), 3.10 (dd, *J* = 13.8, 5.9 Hz, 1H), 2.54 (s, 3H), 1.95 (s, 3H), 1.21 (t, *J* = 7.1 Hz, 3H). **^{13}C NMR** (100 MHz, CDCl3) δ 197.50, 171.19, 169.48, 141.59, 135.78, 129.39, 128.32, 61.51, 52.79, 37.74, 26.38, 22.90, 13.94. Spectroscopic data are in accordance with those reported in literature.[6]



10

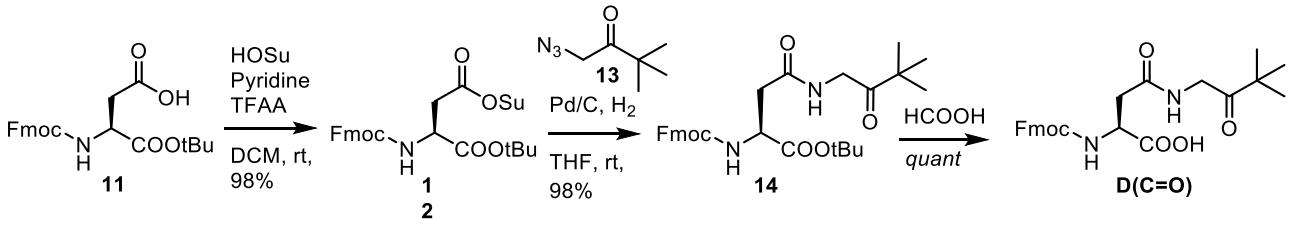
To a flask, equipped with a reflux condenser, **9** (7.00 g, 25.24 mmol, 1 equiv) was added. A 9M HCl solution was added (100 ml, excess) and the slight orange mixture was heated to 90°C and stirred for 6h. The mixture was cooled to rt, yielding a precipitate. This precipitate was filtered and washed with acetone and Et_2O to yield the product **10** as fine, slightly brown needles (3.769 mg, 15.41 mmol, 61%). The remaining solution was evaporated to dryness, yielding a yellow solid, which was washed with acetone and Et_2O. Filtration yielded the second batch of the product as a pale-yellow solid (2.32 g, 9.47 mmol, 37%). **^{1}H NMR** (500 MHz, Deuterium Oxide) δ 7.89 (d, J = 8.1 Hz, 2H), 7.36 (d, J = 8.1 Hz, 2H), 4.28 – 4.20 (m, 1H), 3.26 (ddd, J = 58.6, 14.5, 6.7 Hz, 2H), 2.55 (s, 3H). **^{13}C NMR** (125 MHz, D2O) δ 203.57, 171.59, 140.59, 135.99, 129.81, 129.29, 54.09, 35.75, 26.31. Spectroscopic data are in concurrence with literature.[6]



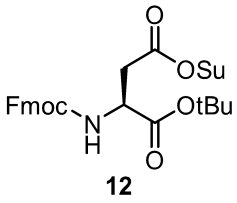
F(C=O)

The free H-*p*-AcPhe-OH (1.00 g, 4.09 mmol, 1 equiv) was dissolved in 11 ml of 1,4-dioxane, after which 15 ml of an aqueous saturated $NaHCO_3$ solution was added, and the solution was subsequently cooled to 0 °C. A solution of Fmoc-OSu (1.45 g, 4.29 mmol, 1.05 equiv) in 10 ml of acetone was added in a dropwise fashion. The flask was warmed to rt, and the solution was stirred overnight. The volatiles were removed under reduced pressure and the remaining solution was diluted with EtOAc. The organic phase was washed with an 1M $KHSO_4$ solution (8 times) followed by brine. After drying over Na_2SO_4, the volatiles were vaporized under reduced pressure, yielding pF, or **F**(C=O) as an off-white solid (1.72 g, 3.99 mmol, 97%). **^{1}H NMR** (500 MHz, Methanol-d4) δ 7.89 (d, J = 8.1 Hz, 2H), 7.78 (d, J = 7.5 Hz, 2H), 7.57 (dd, J = 11.1, 7.7 Hz, 2H), 7.38 (t, J = 6.4 Hz, 4H), 7.28 (q, J = 7.0 Hz, 2H), 7.20 (d, J = 7.3 Hz, 1H), 4.50 (dd, J = 9.6, 4.6 Hz, 1H), 4.30 (dd, J = 10.5, 7.2 Hz, 1H), 4.22 (dd, J = 10.4, 7.1 Hz, 1H), 4.12 (t, J = 6.9 Hz, 1H), 3.31 (d, J = 4.5 Hz, 1H), 3.03 (dd, J = 13.7, 9.8 Hz, 1H), 2.52 (s, 3H). **^{13}C NMR** (125 MHz, MeOD) δ 198.79, 173.70, 156.85, 143.75, 143.52, 141.09, 135.40, 129.28, 128.16, 127.31, 126.68, 124.83, 124.74, 119.44, 66.49, 55.01, 46.89, 37.15, 25.20. **IR** (cm^{-1}) 3311, 1679, 1606, 1538, 1448, 1267, 1084, 1048, 826, 759. **HR-MS** ESI+, for $C_{26}H_{23}NO_5$ calc 430.1654, found 430.1663. **mp** 155-160 °C.

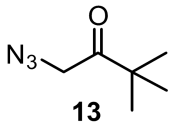
2.3 D(C=O)



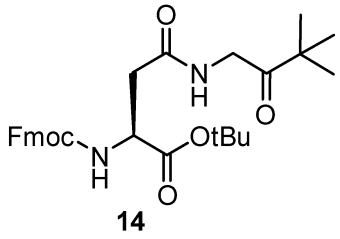
Scheme 3: The synthesis of **D**(C=O). Synthesis steps are detailed below.



In a flame-dried flask, Fmoc-Asp(OH)-OtBu (**11**) (10.00 g, 24.30 mmol) was dissolved in 120 ml of anhydrous CH_2Cl_2. HOSu (5.87 g, 51.03 mmol, 2.1 equiv) and pyridine (9.78 ml, 121.50 mmol, 5 equiv) were added and the mixture was cooled on ice. TFAA (6.76 ml, 48.60 mmol, 2 equiv) was added dropwise. Once addition was finished, the mixture was warmed to rt and stirred overnight. The organic phase was washed 3x with 1M $KHSO_4$solution, water and brine, and dried over Na_2SO_4. The solvent was removed under reduced pressure, yielding the activated ester **12** as an off-white foam (12.06 g, 23.71 mmol, 98%). **^{1}H NMR** (300 MHz, Chloroform-*d*) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.62 (d, *J* = 7.4 Hz, 2H), 7.40 (t, *J* = 7.3 Hz, 2H), 7.31 (t, *J* = 7.4 Hz, 2H), 5.90 (d, *J* = 7.8 Hz, 1H), 4.65 (dt, *J* = 8.4, 4.5 Hz, 1H), 4.38 (dd, *J* = 7.2, 4.5 Hz, 2H), 4.25 (t, *J* = 7.3 Hz, 1H), 3.26 (dd, *J* = 8.7, 4.5 Hz, 2H), 2.84 (s, 4H), 1.49 (s, 9H). **^{13}C NMR** (125 MHz, $CDCl_3$) δ 168.73, 168.37, 166.32, 155.90, 143.81, 141.27, 127.71, 127.12, 125.31, 125.25, 119.95, 83.62, 67.45, 50.56, 47.09, 34.21, 27.77, 25.57. **IR** (cm^{-1}) 3341, 2976, 2944, 1814, 1784, 1735, 1650, 1631, 1612, 1563, 1511, 1477, 1449, 1427, 1394, 1368, 1340, 1292, 1202, 1152, 1062, 1045, 992, 879, 842, 811, 759. **HR-MS** FD m/z [M^+] calcd for $C_{27}H_{28}N_2O_8$: 508.1846, found 508.1845. **mp** 69-72 °C.

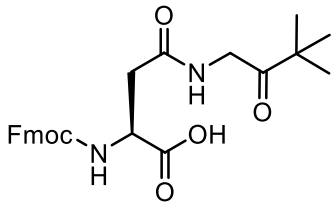


In a flask, 1-bromo-3,3-dimethylbuta*N*-2-one (6.73 ml, 50 mmol) was dissolved in 40 ml of acetone, after which NaN_3 (4.23 g, 65 mmol, 1.3 equiv) was added. The suspension was stirred overnight at rt. The suspension was filtered over a Celite pad and eluted with acetone. The volatiles were removed under rotary evaporation, where the flask was kept under 40 °C. The resulting oil was further dried under high vacuum, yielding the desired azide **13** as a yellowish oil (6.95 g, 49.23 mmol, 98%). **^{1}H NMR** 400 MHz, Chloroform-d) δ 4.10 (s, 2H), 1.20 (s, 9H). Spectroscopic data are in concurrence with those reported in literature.[7]



In a flame-dried flask, Fmoc-Asp(OSu)-OtBu (**12**) (11.65 g, 22.91 mmol) was dissolved in 200 ml of freshly distilled THF. Azide **13** (5.18 g, 36.52 mmol, 1.6 equiv) was added, after which the mixture was degassed and purged with N_2. Pd/C (10 wt% loading, 3 mol%, 760 mg) was added, and the reaction vessel was evacuated and purged with H_2 (repeated trice). The reaction mixture was stirred under H_2 pressure (balloon). The reaction was monitored *via* TLC, and upon consumption of the starting material, the reaction was flushed with N_2. The mixture was filtered over a Celite pad, and eluted with CH_2Cl_2. The volatiles were removed under reduced pressure. The remaining oil was dissolved in EtOAc and the organic phase was washed with 1M $KHSO_4$, water, and brine. After drying over Na_2SO_4, the volatiles were removed under

reduced pressure, yielding **14** as a white fluffy solid (11.67 g, 22.9 mmol, 99%). **^{1}H NMR** (300 MHz, Chloroform-*d*) δ 7.75 (d, *J* = 7.4 Hz, 2H), 7.61 (d, *J* = 7.3 Hz, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.30 (t, *J* = 7.4 Hz, 2H), 6.40 (s, 1H), 6.00 (d, *J* = 8.1 Hz, 1H), 4.51 (dt, *J* = 8.3, 4.2 Hz, 1H), 4.44 – 4.31 (m, 2H), 4.27 (d, *J* = 4.2 Hz, 2H), 4.26 – 4.17 (m, 2H), 2.97 (dd, *J* = 15.7, 4.2 Hz, 1H), 2.79 (dd, *J* = 15.6, 4.3 Hz, 1H), 1.47 (s, 9H), 1.18 (s, 9H). **^{13}C NMR** (125 MHz, $CDCl_3$) δ 210.32, 169.93, 156.13, 143.94, 141.27, 127.67, 127.06, 125.24, 119.94, 82.40, 67.17, 51.36, 47.15, 44.77, 43.10, 37.86, 27.91, 26.34. **IR** (cm^{-1}) 3368, 3064, 2976, 2925, 1714, 1671, 1555, 1507, 1476, 1466, 1448, 1395, 1366, 1342, 1287, 1247, 1215, 1167, 1113, 1077, 1049, 1013, 1000, 989, 955, 943, 906, 849, 825, 776, 759, 734. **HR-MS** FD m/z [M^+] calcd for $C_{29}H_{36}N_2O_6$: 508.2573, found 508.2553. **mp** 186 °C



D(C=O)

The *tert*-butyl ester **14** (11.66 g, 22.92 mmol) was dissolved in CH_2Cl_2: HCOOH (1:2, 300 ml). The reaction mixture goes from yellowish to red to dark green over 36h, after which TLC shows the deprotection is complete. The volatiles were removed under reduced pressure and the resulting oil is dissolved in EtOAc. The organic phase is washed with 1M $KHSO_4$ solution (3x), water (2x) and brine (2x). The remaining product is very bright yellow. The organic phase is dried over Na_2SO_4 while stirring for 15 min. To remove the discoloration, some activated carbon powder is added, and stirred for 5 min. After filtration, the volatiles were removed under reduced pressure, yielding the product amino acid **F**(C=O) as a pale-yellow solid (10.40 g, 22.9 mmol, *quant*). The product amino acid is used in peptide synthesis without further purification, even though some impurities are noticeable on ^{1}H-NMR. Peptides are of good quality and no detrimental effect of any impurities is recorded. Column purification was performed for analytical data: EtOAc: CH_2Cl_2 1:1, after first spots are eluted, 0.05% HOAc is added, to elute the acid **D**(C=O). **^{1}H-NMR** (400 MHz, Chloroform-d) δ 10.21 (s, 1H), 7.76 (d, J = 7.4 Hz, 2H), 7.62 (d, J = 6.4 Hz, 2H), 7.39 (t, J = 7.3 Hz, 2H), 7.35 – 7.25 (m, 3H), 7.01 (s, 1H), 6.35 (d, J = 7.3 Hz, 1H), 4.70 – 4.59 (m, 1H), 4.37 (d, J = 6.3 Hz, 2H), 4.30 (s, 2H), 4.22 (t, J = 7.2 Hz, 1H), 3.09 (d, J = 13.3 Hz, 1H), 2.92 (dd, J = 15.7, 5.4 Hz, 1H), 1.17 (s, 9H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 210.32, 173.43, 171.12, 156.20, 143.68, 143.63, 141.14, 127.60, 126.99, 125.12, 119.84, 67.29, 50.51, 46.94, 44.94, 43.02, 37.41, 26.17. **IR (cm^{-1})** 3325, 3066, 2967, 1709, 1646, 1519, 1477, 1448, 1416, 1367, 1328, 1244, 1155, 1105, 1049, 994, 940, 912, 874, 845, 758, 733. **HR-MS** ESI+ for $C_{25}H_{28}N_2O_6$ calc 453.2026, found 453.2017. **mp** 101 °C

3 Peptide synthesis

Amino acids are indicated by single-letter codes. Special amino acids used are: *homo*-Serine aminooxy derivative (**hS**(ONH_2)), para-acetyl phenylalanine (**F**(C=O)), Aspartic acid tert-butyl ketone derivative (**D**(C=O)), O-carboxyamidomethyl is abbreviated as Cam.

3.1 General procedure for solid-phase peptide synthesis of C-terminal Cam-ester peptides

Fmoc-L-Wang resin (0.2 mmol) was washed with DCM (3x) and DMF (3x) and Fmoc-deprotected using piperidine/DMF (20% (v/v), 2x 8 min). After washing with DMF (6x), the corresponding Fmoc-AA-glycolic acid (2 equiv.) was coupled to the resin using HBTU (4 equiv.), Oxyma Pure (4 equiv.) and DIPEA (10 equiv.) in DMF (45 min). Fmoc-AAx-glycolic acid was prepared according to Nuijens *et al.*[8] After washing with DMF and Fmoc deprotection the next amino acid Fmoc-AAx-OH was coupled using DIC (4 equiv.) and Oxyma Pure (4 equiv.) in DMF (45 min). After the final Fmoc-deprotection, the resin was dried in a stream of nitrogen gas.

3.2 Synthesis of peptide amides

Peptide amides were synthesized using a Rink amide resin. After washing the resin with DMF (3x) and Fmoc deprotection using piperidine/DMF (20%(v/v), 2x 8 min)., the amino acids Fmoc-AAx-OH were coupled using DIC (4 equiv.) and Oxyma Pure (4 equiv.) in DMF (45 min). After final Fmoc-deprotection, the resin was dried

3.3 Cleavage of peptides containing unnatural amino acids

Peptides containing unnatural amino acids were cleaved using the following protocols:

1) Aminooxy peptides with **hS**(ONH_2), a cocktail of TFA/MiliQ/thioanisole/DODT/TIS (90/5/2/5/5/2.5, v/v/v/v/v) for 2 hours at room temperature.
2) Ketone-peptides with either **F**(C=O) or **D**(C=O), a cocktail of TFA/MilliQ/thioanisole/TIS/Phenol (80/5/5/5/2.5/7.5, v/v/v/v/v) for 2 hours at room temperature.

Precipitation of the peptide with diethylether/ pentane (1:1, v/v) followed by lyophilization of the precipitated peptide afforded the crude peptide. Purification of the crude peptide was performed by reversed-phase HPLC (mobile phase consists of gradient mixture of eluent-A (milliQ containing 0.05% (v/v) TFA) and eluent-B (CH_3CN containing 0.05% (v/v) TFA).

4 Tolerance of unnatural amino acids hS(ONH_2), F(C=O), and D(C=O) in the omniligase-1 binding pockets: model studies

Reaction procedure

Peptides bearing an **hS**(ONH_2), **F**(C=O) or **D**(C=O) residue in either of the positions P4-P2' were synthesized according to the procedures described in section 2. For a list of sequences including their respective molecular weights see Table 1.

For testing the acceptance of each respective substrate by omniligase-1 stock solutions of acyl donor (ester) fragment (10 mM) and acyl acceptor fragment (amine) (15 mM) were prepared in deionized water. 25 µL of both ester and amine fragment stock solution were added. The mixture was diluted with 100 µL 1 M potassium phosphate buffer pH 8.5. Final concentration of the ester fragment was 1.66 mM and of the amine fragment 2.5 mM (5 eq.). In order to start the reaction 2 µg of Omniligase-1 (final concentration 0.5 µM) was added. After 0 and 30 min 25 µL of reaction mixture were quenched in 475 µL of quenching solution (0.5% (v/v) methanesulfonic acid in water). Samples were analyzed using HPLC-MS. HPLC yields were calculated based on integration of the product peak and the remaining peak area of the acyl donor fragment. Analytical HPLC was performed on an Agilent 1260 liquid chromatography system using a reversed-phase column (Waters XSelect CSH C18, 2.5 µm particle size, 150 × 3.0 mm) at 40 °C, coupled with an Agilent 6130 quadrupole LC/MS system. UV detection was performed at λ= 220 nm using a UV-VIS 204 linear spectrometer and peptides were identified by their mass using LC-MS. As eluents A (water+ 0.05% (v/v) MSA) and B (MeCN+ 0.05% (v/v) MSA) were used.

All reactions performed are listed in Figure 1a. In the HPLC trace the peaks of product (''synthesis''), hydrolyzed ester (''hydrolysis'') as well as potentially remaining educt (''ester'') were integrated. The results are displayed in Figure 1b,c,d. The reaction was deliberately performed under sub-optimal reaction conditions and stopped after 30 min in order to highlight differences between the substrates. After 30 min the control ''benchmark'' reaction (Ac-DFSKL-Cam-L-OH + H-ALKKF-NH_2) is usually complete with exclusive formation of the desired ligation product.

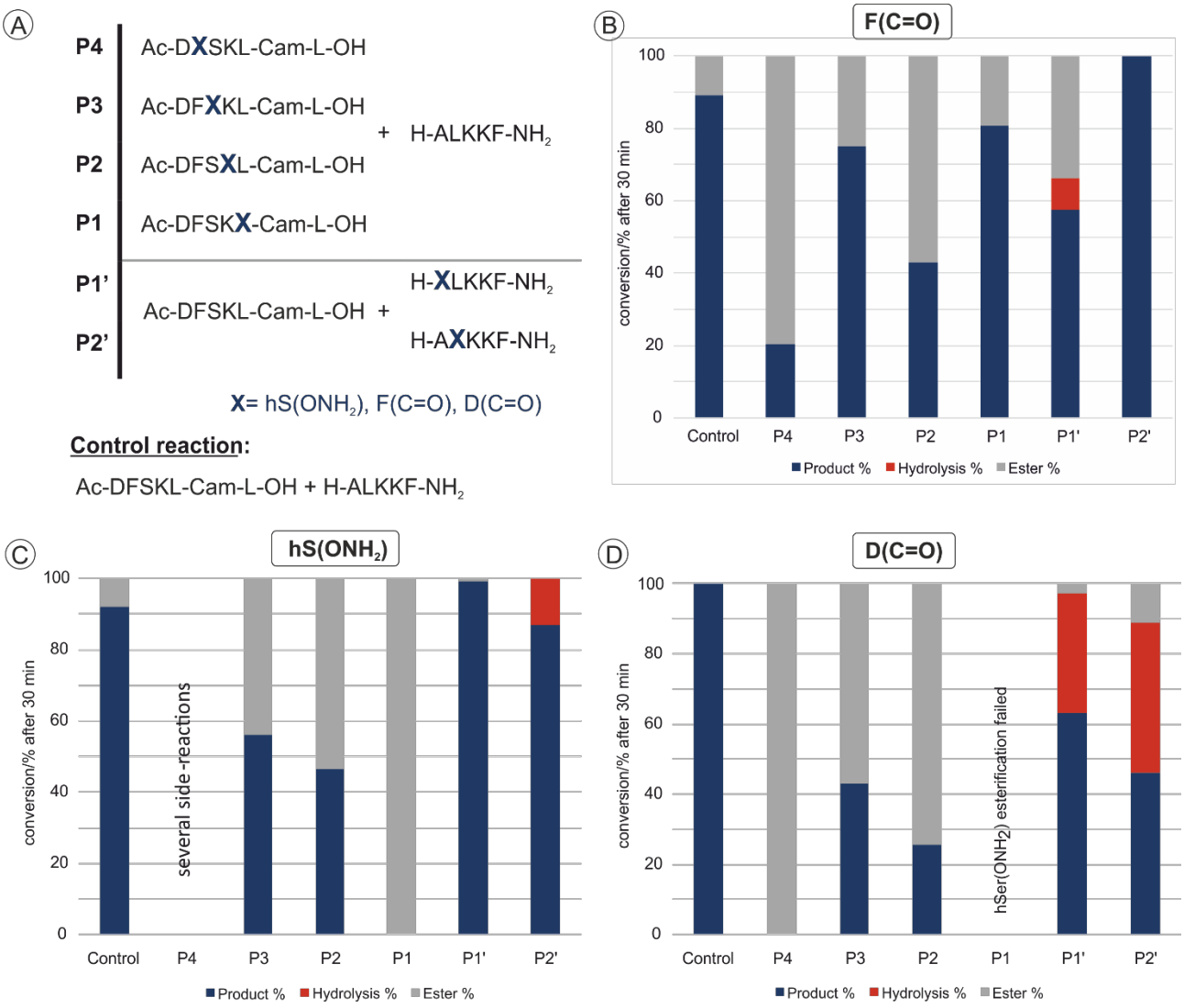


Figure 1. a) Model reactions performed for testing the acceptance of [Aha] in each respective peptide binding pocket of omniligase-1. X represent either **hS**(ONH_2), **F**$(C{=}O)$ or **D**$(C{=}O)$. The reaction Ac-DSFKL-Cam-L-OH + H-ALKKF-NH_2 served as a control ligation. b) Reaction yields after 30 min (blue bars) of **F**$(C{=}O)$ containing model peptides, corresponding hydrolysis of the Cam-ester (red bars) Fnd remaining Cam-ester peptide (grey bars). c) Reaction yields after 30 min (blue bars) of **1**(ONH_2) containing model peptides. c) hSeaction yields after 30 min (blue bars) of **D**$(C{=}O)$ containing model peptides.

Table 1. Codes, sequences and exact masses (calculated and experimental) of synthesized Cam esters.

Peptide	Sequence	MW_{calc}	MW_{exp}
hS(ONH_2)**-S4**	Ac-D-**hS**(ONH_2)-SKL-Cam-L-OH	790.4	790.3
hS(ONH_2)**-S3**	Ac-DF-**hS**(ONH_2)-KL-Cam-L-OH	850.4	850.3
hS(ONH_2)**-S2**	Ac-DFS-**hS**(ONH_2)-L-Cam-L-OH	809.4	809.3
hS(ONH_2)**-S1**	Ac-DFSK-**hS**(ONH_2)-Cam-L-OH	843.4	*
hS(ONH_2)**-S1'**	H-**hS**(ONH_2)-LKKF-NH_2	649.4	649.3
hS(ONH_2)**-S2'**	H-A-**hS**(ONH_2)-KKF-NH_2	607.4	607.3
F(C=O)**-S4**	Ac-D-**F**(C=O)-SKL-Cam-L-OH	863.4	863.3
F(C=O)**-S3**	Ac-DF-**F**(C=O)-KL-Cam-L-OH	923.5	923.4
F(C=O)**-S2**	Ac-DFS-**F**(C=O)-L-Cam-L-OH	882.4	882.3
F(C=O)**-S1**	Ac-DFSK-**F**(C=O)-Cam-L-OH	897.4	897.3
F(C=O)**-S1'**	H-**F**(C=O)-LKKF-NH_2	722.5	722.3
F(C=O)**-S2'**	H-A-**F**(C=O)-KKF-NH_2	680.4	680.3
D(C=O)**-S4**	Ac-D-**D**(C=O)-SKL-Cam-L-OH	886.5	886.3
D(C=O)**-S3**	Ac-DF-**D**(C=O)-KL-Cam-L-OH	946.5	946.3
D(C=O)**-S2**	Ac-DFS-**D**(C=O)-L-Cam-L-OH	905.4	905.3
D(C=O)**-S1**	Ac-DFSK-**D**(C=O)-Cam-L-OH	920.5	*
D(C=O)**-S1'**	H-**D**(C=O)-LKKF-NH_2	745.5	745.3
D(C=O)**-S2'**	H-A-**D**(C=O)-KKF-NH_2	703.4	703.3
Control ester	Ac-DFSKL-Cam-L-OH	821.4	821.3
Control amine	H-ALKKF-NH_2	604.4	604.3

*synthesis failed.

5 Enzymatic cyclizations

CEPS:

Linear Cam-ester peptides were dissolved in potassium phosphate buffer solution (500 mM, pH = 8.5) to a concentration of approx. 0.5 mg/mL (0.15- 0.25 mM), followed by addition of omniligase-1 (0.15- 0.5 µM). The reaction was followed by HPLC-MS using an individualized gradient. After completion of the reaction the reaction mixture was purified *via* preparative RP-HPLC. If the linear precursor peptides exhibited low solubility, guanidinium hydrochloride was added to a concentration of 1 M.

Example:

Linear Cam-ester **2**$_{3333}$-**F**(C=O) (H-CYKQ**F**(C=O)SIK**F**(C=O)AKGCSKL-CamL-OH, 20 mg, 7.5 µmol) was dissolved in 500 mM phosphate buffer (30 mL, pH = 8), followed by addition of omniligase-1 to a concentration of 0.4 µM. After a reaction time of 60 min the reaction mixture was purified using RP-HPLC. The monocyclic peptide c**2**$_{3333}$-**F**(C=O) was isolated using preparative RP-HPLC.

The following linear peptide Cam-esters were cyclized into their head-to-tail cyclic counterpart:

Table 2: The sequences of linear peptide-Cam-esters that were synthesized and subjected to cyclization.

Name	Sequence
1$_{3333}$-**hS**(ONH_2)	H-CYKQ**hS**(ONH_2)SIK**hS**(ONH_2)AKGCSKL-O-Cam-L-OH
2$_{3333}$-**F**(C=O)	H-CYKQ**F**(C=O)SIK**F**(C=O)AKGCSKL-O-Cam-L-OH
3$_{3333}$-**D**(C=O)	H-CYKQ**D**(C=O)SIK**D**(C=O)AKGCSKL-O-Cam-L-OH
4$_{4444}$-**hS**(ONH_2)	H-R**hS**(ONH_2)FRLPCRQLRCFRLP**hS**(ONH_2)RQL-O-Cam-L-OH
5$_{4444}$-**F**(C=O)	H-R**F**(C=O)FRLPCRQLRCFRLP**F**(C=O)RQL-O-Cam-L-OH
6$_{5555}$-**hS**(ONH_2)	H-CYKGKQ**hS**(ONH_2)SIKAS**hS**(ONH_2)AKVRGCKFSKL-O-Cam-L-OH
7$_{5555}$-**F**(C=O)	H-CYKGKQ**F**(C=O)SIKAS**F**(C=O)AKVRGCKFSLK-O-Cam-L-OH
8$_{5555}$-**D**(C=O)	H-CYKGKQ**D**(C=O)SIKAS**D**(C=O)AKVRGCKFSKL-O-Cam-L-OH

Table 3: The synthesized cyclic peptides with the corresponding retention time on UPLC and masses.

Name	Sequence	t_R	m/z found	m/z calc	Species
c**1**$_{3333}$-**hS**($SONH_2$)	*cyc*CYKQ**hS**(ONH_2)SIK**hS**(ONH_2)AKGCSKL	0.59	1771.91	1771.46	$[M+H]^+$
c**2**$_{3333}$-**F**(C=O)	*cyc*CYKQ**F**(C=O)SIK**F**(C=O)AKGCSKL	0.97	1918.32	1918.64	$[M+H]^+$
c**3**$_{3333}$-**D**(C=O)	*cyc*CYKQ**D**(C=O)SIK**D**(C=O)AKGCSKL	1.01	983.02	983.85	$[M+2H]^{2+}$
c**4**$_{4444}$-**hS**(ONH_2)	*cyc*R**hS**(ONH_2)FRLPCRQLRCFRLP**hS**(ONH_2)RQL	1.04	859.04	859.18	$[M+3H]^{3+}$
c**5**$_{4444}$-**F**(C=O)	*cyc*R**F**(C=O)FRLPCRQLRCFRLP**F**(C=O)RQL	1.29	907.80	907.90	$[M+3H]^{3+}$
c**6**$_{5555}$-**hS**(ONH_2)	*cyc*CYKGKQ**hS**(ONH_2)SIKAS**hS**(ONH_2)AKVRGCKFSKL	0.69	1325.27	1325.31	$[M+2H]^{2+}$
c**7**$_{5555}$-**F**(C=O)	*cyc*CYKGKQ**F**(C=O)SIKAS**F**(C=O)AKVRGCKFSLK	0.90	1397.57	1397.40	$[M+2H]^{2+}$
c**8**$_{5555}$-**D**(C=O)	*cyc*CYKGKQ**D**(C=O)SIKAS**D**(C=O)AKVRGCKFSKL	0.96	1420.30	1420.43	$[M+2H]^{2+}$

5.1 c1$_{3333}$-hS(ONH$_2$)

linear starting sequence (**1**$_{3333}$-**hS**(ONH$_2$)): H-CYKQ[**HS**(ONH$_2$)]SIK[**hS**(ONH$_2$)]AKGCSKL-CamL-OH

cyclic product (c**1**$_{3333}$-**hS**(ONH$_2$)): c[CYKQ[**hS**(ONH$_2$)]SIK[**hS**(ONH$_2$)]AKGCSKL]

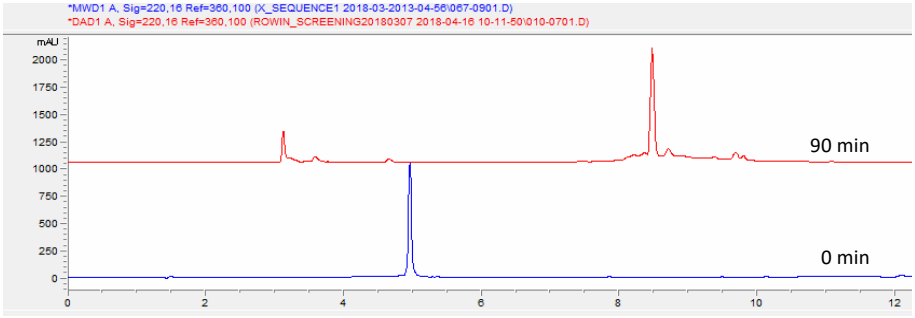


Figure 3. Omniligase-1 mediated cyclization of **1**$_{3333}$-**hS**(ONH$_2$). HPLC trace after 0 min (blue, **1**$_{3333}$-**hS**(ONH$_2$)) and 90 min (red,c**1**$_{3333}$-**hS**(ONH$_2$)) are shown. The cyclization yield is approx. 90% (a/a) based on the HPLC trace.

5.2 c2$_{3333}$-F(C=O)

linear starting sequence (**2**$_{3333}$-**F**(C=O)): H-CYKQ[**F**(C=O)]SIK[**F**(C=O)]AKGCSKL-CamL-OH

cyclic product (c**F**$_{3333}$-**2**(C=O)): c[CYKQ[**F**(C=O)]SIK[**F**(C=O)]AKGCSKL]

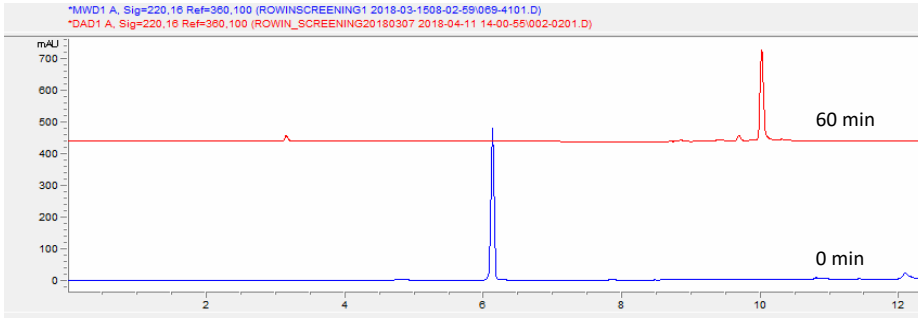


Figure 2. Omniligase-1 mediated cyclization of **2**$_{3333}$-**F**(C=O). HPLC trace after 0 min (blue, **2**$_{3333}$-**F**(C=O)) and 60 min (red, c**2**$_{3333}$-**F**(C=O)) are shown. The cyclization yield is approx. 95% (a/a) based on the HPLC trace.

5.3 c3_{3333}-D(C=O)

linear starting sequence (**3**$_{3333}$-**D**(C=O)): H-CYKQ[**D**(C=O)]SIK[**D**(C=O)]AKGCSKL-Cam-L-OH

cyclic product (c**3**$_{3333}$-**D**(C=O)): c[CYKQ[**D**(C=O)]SIK[**D**(C=O)]AKGCSKL]

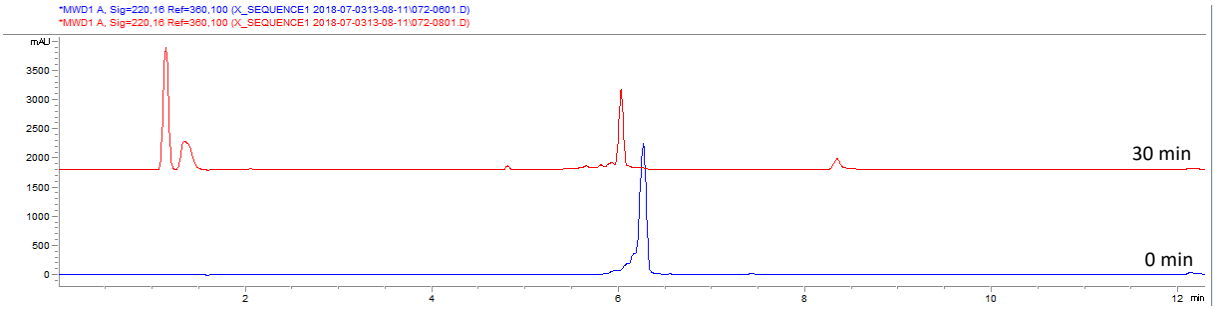


Figure 4. Omniligase-1 mediated cyclization of **3**$_{3333}$-**D**(C=O). HPLC trace after 0 min (blue, **3**$_{3333}$-**D**(C=O)) and 30 min (red, c**3**$_{3333}$-**D**(C=O)) are shown. The cyclization yield is approx. 95% (a/a) based on the HPLC trace.

5.4 c4_{4444}-hS(ONH_2)

linear starting sequence (**4**$_{4444}$-**hS**(ONH_2)): H-R[**hS**(ONH_2)]FRLPCRQLRCFRLP[**hS**(ONH_2)]RQL-Cam-L-OH

cyclic product (c**4**$_{4444}$-**hS**(ONH_2)): c[R[**hS**(ONH_2)]FRLPCRQLRCFRLP[**hS**(ONH_2)]RQL]

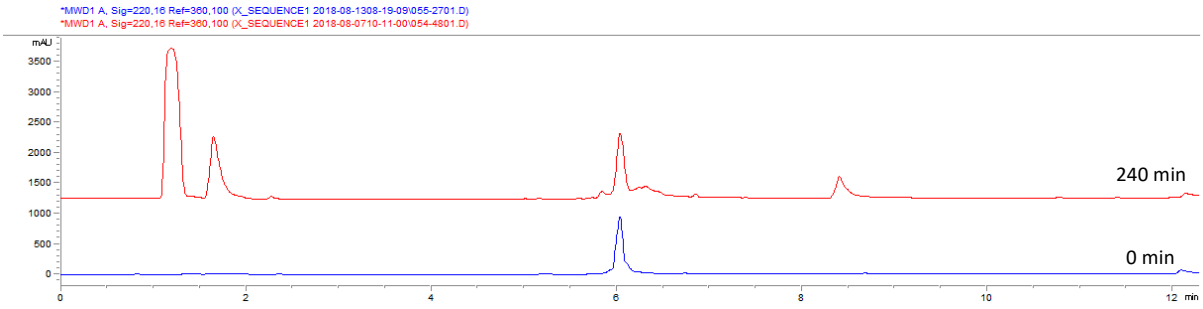


Figure 6. Omniligase-1 mediated cyclization of **4**$_{4444}$-**hS**(ONH_2). HPLC trace after 0 min (blue, **4**$_{4444}$-**hS**(ONH_2)) and 240 min (red, c**4**$_{4444}$-**hS**(ONH_2)) are shown. The cyclization yield is approx. 75% (a/a) based on the HPLC trace.

5.5 c5_{4444}-F(C=O)

linear starting sequence (**5**$_{4444}$-**F**(C=O)): H-R[**F**(C=O)]FRLPCRQLRCFRLP[**F**(C=O)]RQL-Cam-L-OH

cyclic product (c**5**$_{4444}$-**F**(C=O)): c[R[**F**(C=O)]FRLPCRQLRCFRLP[**F**(C=O)]RQL]

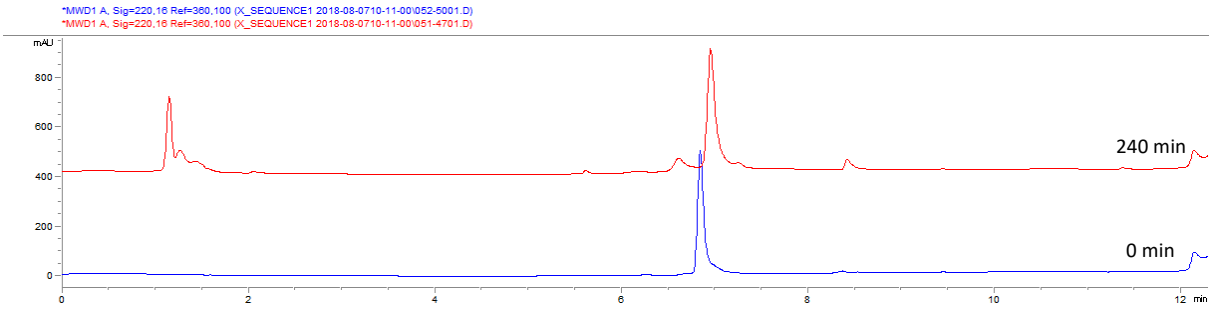


Figure 5. Omniligase-1 mediated cyclization of **5**$_{4444}$-**F**(C=O). HPLC trace after 0 min (blue, **5**$_{4444}$-**F**(C=O)) and 240 min (red, c**5**$_{4444}$-**F**(C=O)) are shown. The cyclization yield is approx. 85% (a/a) based on the HPLC trace.

5.6 c6_{5555}-hS(ONH_2)

linear starting sequence (**6**$_{5555}$-**hS**(ONH_2)): H-CYKGKQ[**hS**(ONH_2)]SIKAS[**hS**(ONH_2)]AKVRGCKFSKL-Cam-L-OH

cyclic product (c**6**$_{5555}$-**hS**(ONH_2)): c[CYKGKQ[**hS**(ONH_2)]SIKAS[**hS**(ONH_2)]AKVRGCKFSKL]

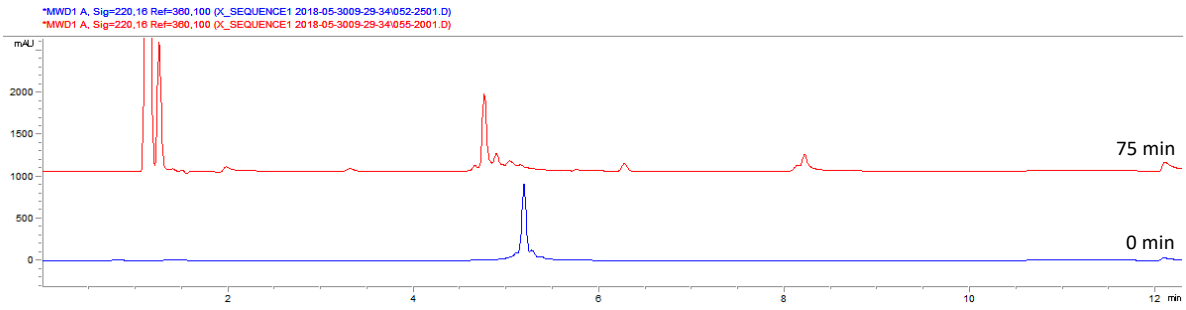


Figure 8. Omniligase-1 mediated cyclization of **6**$_{5555}$-**hS**(ONH_2). HPLC trace after 0 min (blue, **6**$_{5555}$-**hS**(ONH_2)) and 75 min (red, c**6**$_{5555}$-**hS**(ONH_2)) are shown. The cyclization yield is approx. 70% (a/a) based on the HPLC trace.

5.7 c7$_{5555}$-F(C=O)

linear starting sequence (**7**$_{5555}$-**F**(C=O)): H-CYKGKQ[**F**(C=O)]SIKAS[**F**(C=O)]AKVRGCKFSKL-Cam-L-OH

cyclic product (c**7**$_{5555}$-**F**(C=O)): c[CYKGKQ[**F**(C=O)]SIKAS[**F**(C=O)]AKVRGCKFSKL]

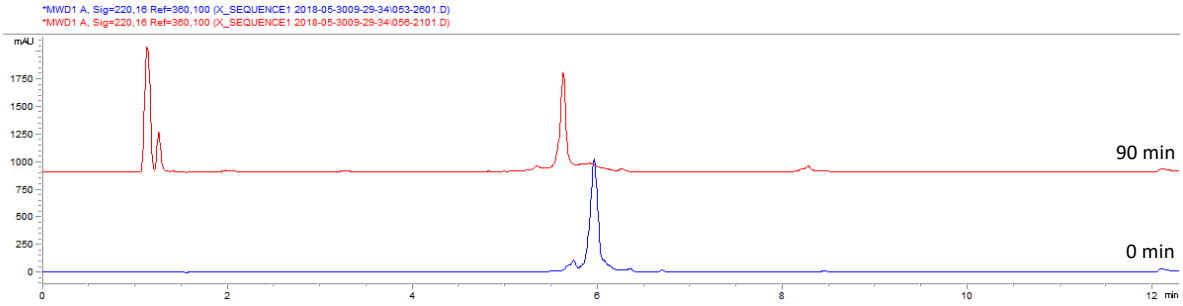


Figure 7. Omniligase-1 mediated cyclization of **7**$_{5555}$-**F**(C=O). HPLC trace after 0 min (blue, **7**$_{5555}$-**F**(C=O)) and 90 min (red, c**7**$_{5555}$-**F**(C=O)) are shown. The cyclization yield is approx. 95% (a/a) based on the HPLC trace.

5.8 c8$_{5555}$-D(C=O)

linear starting sequence (**8**$_{5555}$-**D**(C=O)): H-CYKGKQ[**D**(C=O)]SIKAS[**D**(C=O)]AKVRGCKFSKL-Cam-L-OH

cyclic product (c**8**$_{5555}$-**D**(C=O)): c[CYKGKQ[**D**(C=O)]SIKAS[**D**(C=O)]AKVRGCKFSKL]

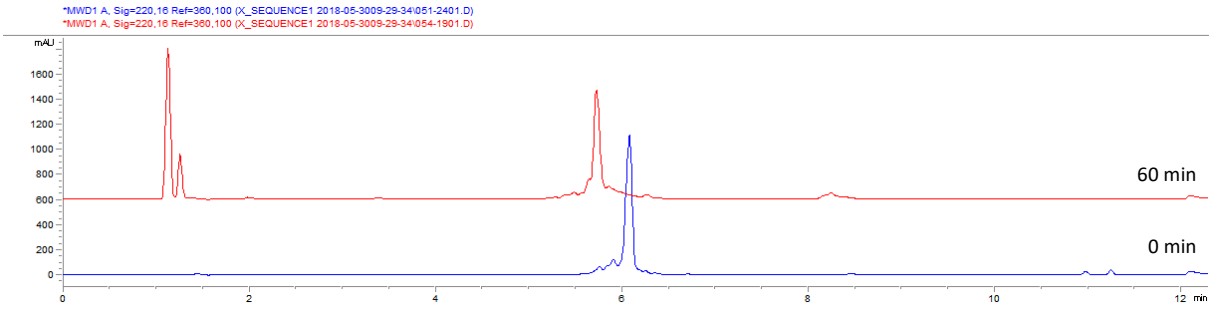
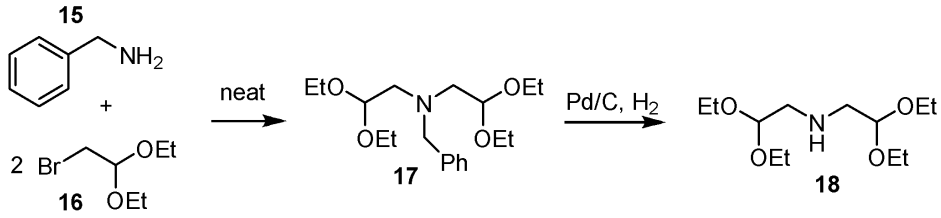


Figure 9. Omniligase-1 mediated cyclization of **8**$_{5555}$-**D**(C=O). HPLC trace after 0 min (blue;c**8**$_{5555}$-**D**(C=O)) and 60 min (red, c**8**$_{5555}$-**D**(C=O)) are shown. The cyclization yield is approx. 95% (a/a) based on the HPLC trace.

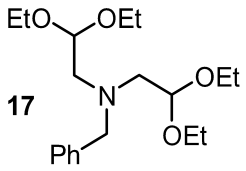
6 Scaffold Synthesis

6.1 Strategy C, 'C' scaffolds

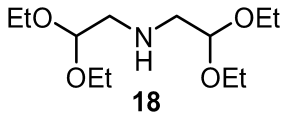
6.1.1 T4-C=O amine 18.



Scheme 4: The synthesis of the T4C amine, bearing the acetal protected aldehyde. This amine is used for both T4C scaffolds.

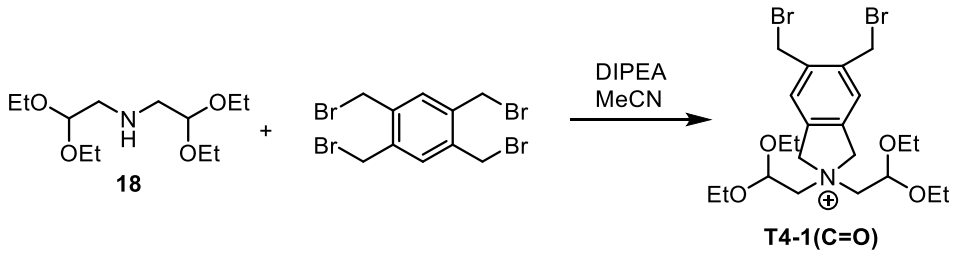


To a flame dried flask, under N_2 flow, benzylamine (**15**) (5 ml, 45.77 mmol, 1 equiv) was added, followed by bromoacetaldehyde dietethyl acetal (**16**) (16 ml, 106.36 mmol, 2.3 equiv) and triethylamine (18 ml, 129.05 mmol, 2.8 equiv). The yellowish mixture was stirred at 100 °C for 18h, resulting is a thick slurry. The solids were filtered off and washed with EtOAc, which was subsequently washed with water, a saturated solution of $NaHCO_3$ and brine, and dried over Na_2SO_4. The volatiles were removed under reduced pressure to yield an orange oil. The oil was immobilized on silica, and the desired compound was purified *via* flash column chromatography (20:1 – Petroleum ether:EtOAc) yielding the benzyl-protected amine **17** as a pale yellow oil in 37% yield (5.715 g, 16.83 mmol). **^{1}H NMR** (300 MHz, Chloroform-*d*) δ 7.30 (ddt, *J* = 21.7, 13.9, 7.0 Hz, 5H), 4.58 (t, *J* = 5.2 Hz, 2H), 3.81 (s, 2H), 3.65 (dq, *J* = 9.2, 7.1 Hz, 4H), 3.51 (dq, *J* = 9.3, 7.0 Hz, 4H), 2.76 (d, *J* = 5.2 Hz, 4H), 1.20 (t, *J* = 7.1 Hz, 12H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 139.75, 128.68, 127.86, 126.59, 102.25, 61.75, 59.97, 57.21, 15.17. **IR** (cm^{-1}) 3027, 2973, 2928, 2876, 1602, 1494, 1452, 1372, 1345, 1267, 1114, 1056, 1023, 916, 849, 816, 739, 698. **HR-MS** (FD) 339.23924, (calc. 339.23828).

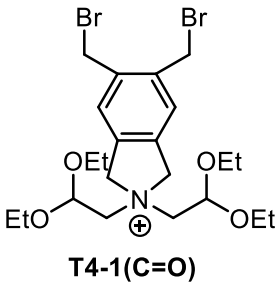


The benzyl-protected amine **17** (5.711 g, 16.82 mmol, 1 equiv) was dissolved in 160ml EtOH, and the solution was degassed and flushed with N_2. Pd/C (10% loading, 267 mg) was added and hydrogen pressure was applied (H_2 filled balloon) after evacuation/saturation (3x).The mixture was stirred for 4h at rt, after TLC indicated the reaction had finished. The solution was filtered over a Na_2SO_4/Celite pad and eluted with EtOH. The volatiles were removed under reduced pressure, yielding the product amine **18** as a pale-yellow oil (4.050 g, 16.24 mmol, 97%). **^{1}H NMR** (400 MHz, Chloroform-*d*) δ 4.44 (t, *J* = 5.6 Hz, 2H), 3.60 – 3.50 (m, 4H), 3.44 – 3.34 (m, 4H), 2.59 (d, *J* = 5.6 Hz, 4H), 1.06 (t, *J* = 7.1 Hz, 12H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 101.70, 61.98, 51.62, 14.99. **IR** (cm^{-1}) 2974, 2876, 1455, 1372, 1346, 1282, 1223, 1118, 1056, 1021, 944, 854, 813, 603, 504. **HR-MS (FD)** 249.19077 (calc. 249.19401).

6.1.2 T4-1(C=O)

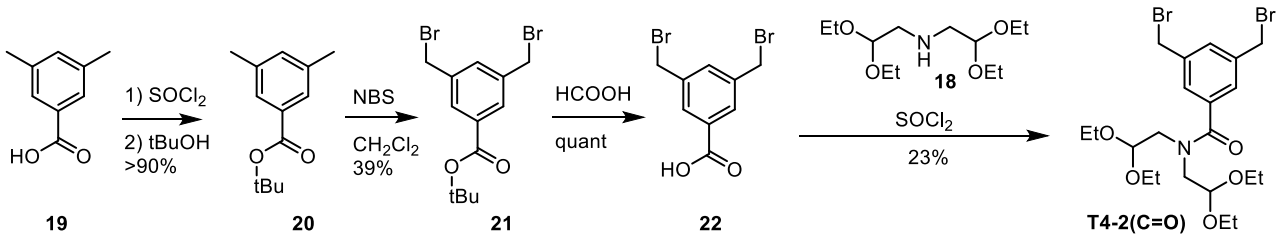


Scheme 5: The synthesis of **T4**-**1**(C=O).

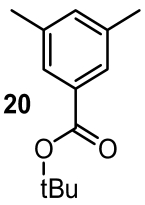


In a flame-dried flask, under N2 flow, 1,2,4,5- tetrakis (bromomethyl)benzene (1.50 g, 3 mmol, 3 equiv) was dissolved in 110 ml of freshly distilled MeCN and DIPEA (209 µL, 1.2 mmol, 1.2 equiv) was added. The secondary amine product (250 mg, 1 mmol, 1 equiv) was dissolved in 2 ml MeCN, and added dropwise to the durene solution. After stirring for one hour, the reaction mixture was concentrated, and immobilized on silica, after which the scaffold was obtained *via* flash column chromatography (EtOAc to EtOAc:EtOH - 4:1), as a slight orange foam (510 mg, 0.94 mmol, 94%). **^{1}H NMR** (500 MHz, Acetonitrile-d_3) δ 7.50 (s, 2H), 5.05 (s, 4H), 4.86 (t, *J* = 5.0 Hz, 3H), 4.76 (s, 4H), 3.72 (d, *J* = 4.9 Hz, 4H), 3.71 – 3.63 (m, 4H), 3.46 (dq, *J* = 9.3, 7.0 Hz, 4H), 1.17 (t, *J* = 7.1 Hz, 12H). **^{13}C NMR (**125 MHz, CD_3CN) δ 138.63, 134.90, 126.08, 97.95, 70.33, 65.05, 64.27, 30.13, 15.09. **IR** (cm^{-1}) 2973, 2929, 2886, 1443, 1374, 1349, 1219, 1121, 1046, 998, 958, 893, 832, 789. **HR-MS** (LC-ESI) for $C_{22}H_{36}Br_2NO_4^+$ calc. 536.1011, found 536.1033. **mp** 32 °C. Even though the scaffold is reactive during CLIPS, it cannot be used in oxime ligation. Due to the quaternary ammonium ion, the acetal deprotection does not proceed. It was therefore omitted from this study.

6.1.3 T4-2(C=O)

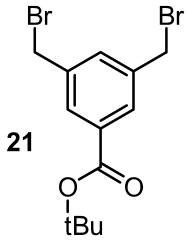


Scheme 6: The synthesis of scaffold **T4**-**2**(C=O). Procedures are detailed below.

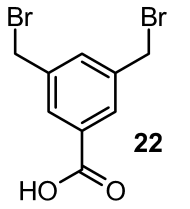


To a flame-dried flask, under N_2 flow, 3,5-dimethyl benzoic acid (**19**) (2.00g, 13.31 mmol, 1 equiv) was suspended in 1.6 ml of toluene. Thionyl chloride (2 ml, 27.6 mmol, 2.06 equiv) was added and the mixture was warmed to a gentle reflux and stirred for 4 hours. The volatiles were removed under reduced pressure, after which the oily residue is diluted with 4 ml of freshly distilled CH_2Cl_2. *t*-BuOH (2.05 ml, 21.31mmol, 1.6 equiv) is added followed by pyridine (1.13 ml, 13.98 mmol, 1.05 equiv). The mixture was stirred for 12 hours, after which the solids are removed by filtration and washed with CH_2Cl_2. The organic phase is washed with 4M HCl, water, 2M NaOH and water. After drying over K_2CO_3, the volatiles are removed, yielding

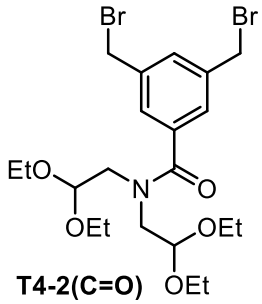
20 as a colorless oil (2.48 g, 12.03 mmol, 90%). **^{1}H NMR** (400 MHz, Chloroform-*d*) δ 7.63 (s, 2H), 7.17 (s, 1H), 2.38 (s, 6H), 1.62 (s, 9H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 166.12, 137.75, 133.99, 127.09, 80.70, 28.18, 21.13. Analytical data are in concurrence to those found in literature.[9]



To a flame-dried flask, under N_2 flow, OtBu-ester **20** (13.95 g, 67.64 mol, 1 equiv) was dissolved in freshly distilled CH_2Cl_2 (250ml). NBS (25.28 g, 142.04 mmol, 2.1 equiv) was added and the mixture was degassed and flushed with N_2. The flask was irradiated with a lamp (500W), heating the mixture to a gentle reflux. The mixture was stirred for 1.5 hours, after which ^{1}H-NMR showed the reaction completed*. The mixture was diluted with CH_2Cl_2 and washed with water. After drying over Na_2SO_4, the volatiles were removed under reduced pressure, yielding a colorless oil. The mixture was crystallized from hexane, providing the desired product **21** as a white solid (7.56 g, 20.77 mmol, 31%). A second crystallization yielded another 1.71 g (4.70 mmol, 7%). ***** *The mixture contains both doubly-brominated product, as well as some incomplete bromination of the starting material.* **^{1}H NMR** (400 MHz, Chloroform-*d*) δ 7.94 (s, 2H), 7.60 (s, 1H), 4.51 (s, 4H), 1.61 (s, 9H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 164.31, 138.54, 133.28, 133.10, 129.69, 81.56, 31.94, 28.01. **IR** (cm^{-1}) 3013, 2982, 2969, 2932, 1790, 1714, 1604, 1472, 1449, 1390, 1369, 1319, 1236, 1213, 1154, 1110, 1060, 999, 973, 953, 918, 893, 846, 794, 771, 753, 734, 692. **mp** 52 °C. **HR-MS** FD m/z [M+] calcd for $C_{13}H_{16}Br_2O_2$: 361.951, found 361.950.



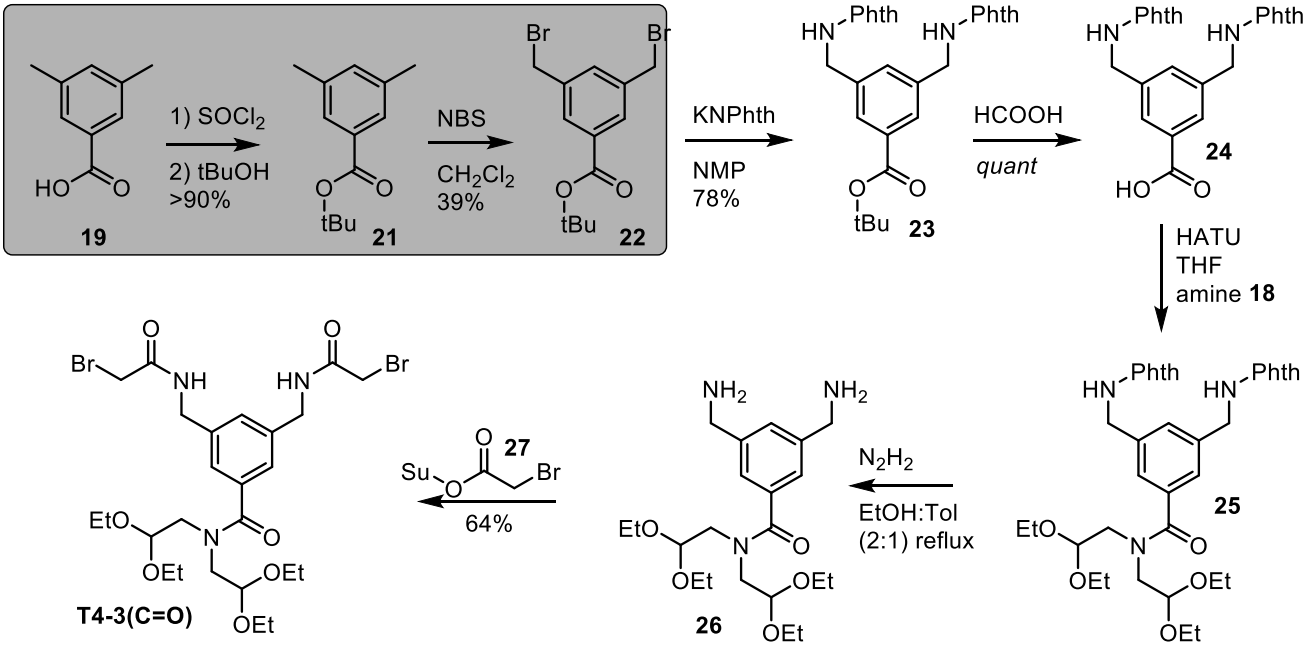
Bromide compound **21** (5.00 g, 13.73 mmol, 1 equiv) was dissolved in freshly distilled CH_2Cl_2 (50 ml). HCOOH was added and the solution was stirred overnight at rt, after which ^{1}H-NMR showed completion of the reaction. The volatiles were removed under reduced pressure and co-evaporation with CH_2Cl_2 (3x) yielded **22** as a white solid (3.98 g, 12.92 mmol, 94%) which was used without further purification.**^{1}H NMR** (400 MHz, Chloroform-*d*) δ 8.10 (s, 2H), 7.71 (s, 1H), 4.55 (s, 4H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 170.03, 139.08, 134.64, 130.45,130.31, 31.53. **IR** (cm^{-1}) 2971, 2821, 2710, 2604, 2539, 1686, 1603, 1460, 1437, 1420, 1308, 1278, 1248, 1211, 1162, 1111, 1056, 998, 938, 927, 904, 855, 771, 728, 691, 662. **mp** 123 °C (sublimates), 142 °C (melts). **HRMS** (FD^+) for $C_9H_8Br_2O_2$ calc. 307.8886, found 307.8891.



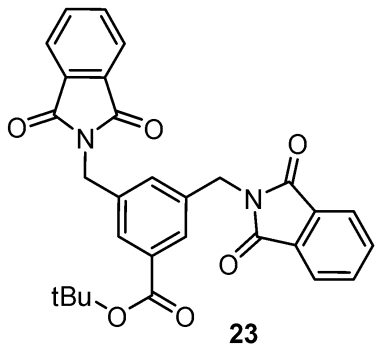
In a flame-dried flask, under N_2 flow, the acid **22** (1.047 g, 3.39 mmol) was dissolved in 5 ml of CH_2Cl_2. $SOCl_2$ (5 ml, excess) was added and the mixture was heated to 75 °C, and stirred for 5 hours. The mixture was cooled to rt and the volatiles were removed under reduced pressure. The remaining oil was dissolved in 18 ml of anhydrous CH_2Cl_2. $NaHCO_3$ (s, 2.60 g, 30.94 mmol 9.1 equiv), NEt_3 (1 ml, 7.17 mmol, 2.1 equiv) and DMAP (25 mg, 0.20 mmol, 6 mol%) were added. The amine **18** (676 mg, 2.71 mmol, 0.8 equiv) was added dropwise, and the mixture was stirred overnight. After extraction with CH_2Cl_2, the organic phase was washed with water (2x) and brine, and subsequently dried over Na_2SO_4. Filtration and evaporation yielded the crude product, which was purified *via* column chromatography (3:1 to 2:1 P.E:EtOAc) and **T4-2**(C=O) was obtained as an off-white waxy solid (414 mg, 0.77 mmol, 23%). **^{1}H NMR** (500 MHz, Chloroform-d) δ 7.43 (s, 1H), 7.40 (s, 2H), 4.84 (s, 1H), 4.49 (t, *J* = 4.6 Hz, 1H), 4.46 (s, 4H), 3.81 (p, *J* = 7.1 Hz, 2H) 3.71 (d, *J* = 5.0 Hz, 1H), 3.61 (dt, *J* = 16.3, 8.4 Hz, 4H), 3.50 (d, *J* = 4.8 Hz, 2H), 3.40 (p, *J* = 7.0 Hz, 2H), 1.29 (t, *J* = 6.9 Hz, 6H), 1.17 (t, *J* = 4.6 Hz, 6H). **^{13}C**

NMR (125 MHz, CDCl3) δ *171.41*, 171.33, 138.66, *138.60*, 137.79, *137.75*, 130.23, *129.75*, 127.71, *127.19*, 101.20, 100.90, 63.39, 52.73, 49.01, 45.22, 32.13, 32.08, 15.45, 15.33. (italic are rotamers). **IR** (cm^{-1}) 2974, 2929, 2877, 2349, 1735, 1634, 1600, 1468, 1441, 1417, 1374, 1345, 1306, 1236, 1215, 1162, 1116, 1055, 932, 896, 837, 757, 704. **HR-MS** FD m/z [M^+] calcd for $C_{21}H_{33}Br_2NO_5$: 537.0725, found 537.0702 **mp** 27 °C. *It was estimated, based on ^{1}H-NMR, that the scaffold has a mixture of Br and Cl substituents. About 38% Cl is incorporated. Determined via LC-MS, there is 9.9% Cl-Cl present, 44.4% of the Cl-Br, and 45.7% of the Br-Br.*

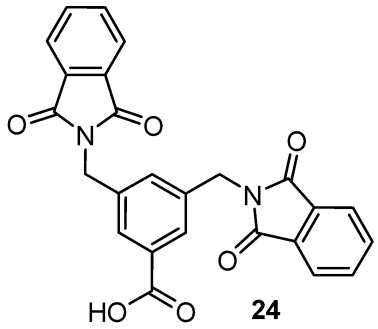
6.1.4 T4-3(C=O) scaffold



Scheme 7: The synthesis of **T4**-**3**(C=O) scaffold. Highlighted in grey is treated in the section of T4-2(C=O). Phth = phthalimide.

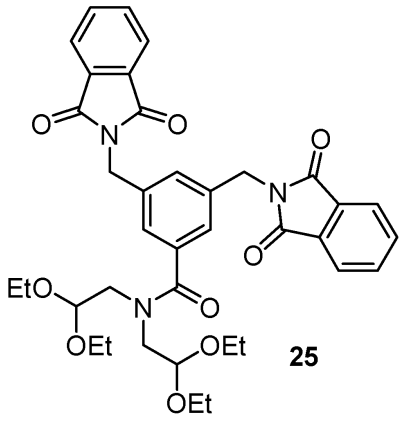


To a flame-dried flask, under N_2 flow, bromide **21** (500 mg, 1.37 mmol, 1 equiv) was dissolved in 27 ml anhydrous DMF. KPhth (1015 mg, 5,48 mmol, 4 equiv) was added next, and the mixture was heated to 125 ° and stirred overnight. The suspension was cooled to rt and the mixture was evaporated to dryness. The mixture was dissolved in CH_2Cl_2 and washed with water, 1M $KHSO_4$, saturated aqueous $NaHCO_3$ and water. After drying over Na_2SO_4 the volatiles were removed under reduced pressure. The Phth remnants were removed *via* flash column chromatography (3:1 - P.E:EtOAc to remove first spot, then increased to 4:1 - EtOAc: Petroleum ether), yielding **23** as a white solid (533 mg, 1.07 mmol, 78%). **^{1}H NMR** (300 MHz, Chloroform-*d*) δ 7.90 (d, *J* = 1.4 Hz, 2H), 7.85 (dd, *J* = 5.4, 3.1 Hz, 4H), 7.72 (dd, *J* = 5.4, 3.1 Hz, 4H), 7.64 (s, 1H), 4.87 (s, 4H), 1.56 (s, 9H). **^{13}C NMR** (75 MHz, $CDCl_3$) δ 167.88, 164.97, 137.03, 134.06, 133.01, 132.31, 132.02, 128.57, 123.46, 81.35, 41.10, 28.11. **IR** (cm^{-1}) 2975, 2938, 1769, 1706, 1607, 1554, 1536, 1466, 1427, 1391, 1367, 1342, 1310, 1257, 1231, 1155, 1122, 1099, 1086, 973, 957, 918, 896, 845, 794, 774, 726, 710, 695. **HR-MS** FD m/z [M^+] calcd for $C_{29}H_{24}N_2O_6$: 496.1634, found 496.1634. **mp** 212 °C.



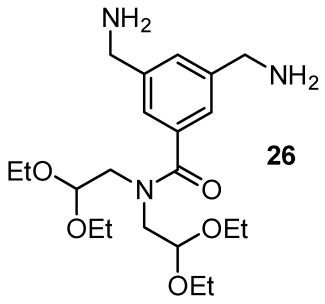
24

To a flask, the phthalimide ester **23** (468 mg, 0.94 mmol) was dissolved in CH_2Cl_2 (5 ml), after which HCOOH (10 ml) was added. A precipitate starts to form, and the mixture was stirred overnight. The solids were filtered off, and washed with CH_2Cl_2. The product was confirmed by ^{1}H-NMR. The off-white solid **24** was dried under high vacuum, yielding 303 mg (0.69 mmol, 73%). No further purification is necessary for subsequent reactions. **^{1}H NMR** (400 MHz, DMSO-d_6) δ 13.10 (s,1H), 7.88 (q, *J* = 4.4 Hz, 8H), 7.76 (s, 2H), 7.53 (s, 1H), 4.82 (s, 4H).**^{13}C NMR** (125 MHz, $CDCl_3$) δ 167.61, 166.70, 137.65, 134.61, 131.43, 130.93, 127.27, 123.26, 40.44. **IR** (cm^{-1})3064, 1771, 1704, 1604, 1466, 1428, 1393, 1359, 1343, 1311, 1261, 1240, 1190, 1170, 1112, 1102, 1086, 1071, 980, 960, 914, 885, 834, 792, 773, 746, 725, 710. **mp** 228 °C (sublimates), 351 °C melts. **HRMS FD** m/z [M^+] calcd for $C_{25}H_{16}N_2O_6$: 440.1008, found 440.1004.



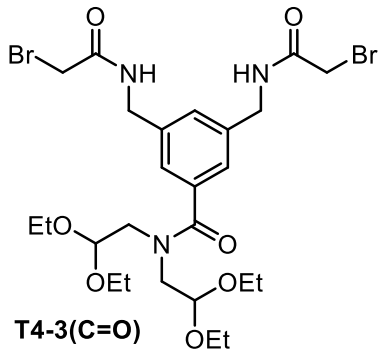
25

In a flame-dried flask, under N_2 flow, the Phth-acid **24** (1000 mg, 2.27 mmol), was suspended in 15 ml of anhydrous DMF. HATU (949 mg, 2.497 mmol, 1.1 equiv) and DIPEA (1 ml, 5.74 mmol, 2.5 equiv) were added, yielding a clear solution. The mixture was stirred for 30 min, after which the amine **18** (594 mg, 2.38 mmol, 1.05 equiv) was added. The mixture was stirred overnight, after which it was diluted with EtOAc and washed with H_2O and brine. After drying over Na_2SO_4, filtration and evaporation of the volatiles, **25** is obtained as a pale brown solid, which is used without further purification (1540 mg, 2,27 mmol, quant). **^{1}H NMR** (500 MHz, Chloroform-*d*) δ 7.82 (dd, *J* = 5.3, 3.1 Hz, 4H), 7.70 (dd,*J* = 5.4, 3.0 Hz, 4H), 7.54 (s, 1H), 7.33 (s, 2H), 4.82 (s, 4H), 4.77 (t, *J* = 4.8 Hz, 1H), 4.33 (t, *J* = 4.7 Hz, 1H), 3.80 – 3.68 (m, 2H), 3.63 (d, *J* = 4.9 Hz, 2H), 3.57 (dt, *J* = 15.5, 6.7 Hz, 3H), 3.44 (dt, *J* = 14.8, 7.0 Hz, 3H), 3.38 (d, *J* = 4.8 Hz, 2H), 3.25 (p, *J* = 7.1 Hz, 2H), 1.20 (t, *J* = 6.6 Hz, 8H), 1.02 (t, *J* = 6.8 Hz, 6H). **^{13}C NMR** (125 MHz, $CDCl_3$) δ 171.76, 167.76, 137.71, 137.21, 134.03, 132.05, 129.72, 126.25, 123.39, 101.49, 100.98, 63.56, 63.26, 52.70, 49.26, 41.12, 15.42, 15.20. **IR** (cm^{-1}) 2974, 2929, 2877, 1765, 1702, 1635, 1605, 1468, 1445, 1421, 1394, 1348, 1329, 1313, 1260, 1233, 1173, 1119, 1103, 1054, 1016, 958, 960, 938, 914, 886, 846, 799, 733, 712. **HR-MS** FD m/z [M^+] calcd for $C_{37}H_{41}N_3O_9$: 671.2843, found 671.2842 **mp** 161-164 °C.



26

The Phthalimide compound **25** (1530 mg, 2.27 mmol) was suspended in 20 ml of Toluene/EtOH (1:2). Hydrazine hydrate (51% solution in water, 1.42 ml, 22.77 mmol, 10 equiv) was added and the mixture was stirred at reflux for 2 hours, during which a thick precipitate has formed. The mixture was cooled to rt after which the solids were filtered off, and washed with CH_2Cl_2. The volatiles were removed under reduced pressure, yielding the crude diamine **26** in quantitative yield, which was used without further purification. **^{1}H NMR** (400 MHz, DMSO-d6) δ 7.33 (s, 1H), 7.14 (s, 2H), 5.76 (s, 2H), 4.73 (s, 1H), 4.54 (s, 1H), 3.73 (s, 4H), 3.71 – 3.63 (m, 2H), 3.62 – 3.55 (m, 3H), 3.55 – 3.40 (m, 5H), 3.35 (d, J = 12.5 Hz, 4H), 1.16 (t, J = 6.3 Hz, 8H), 1.08 – 0.95 (m, 6H).

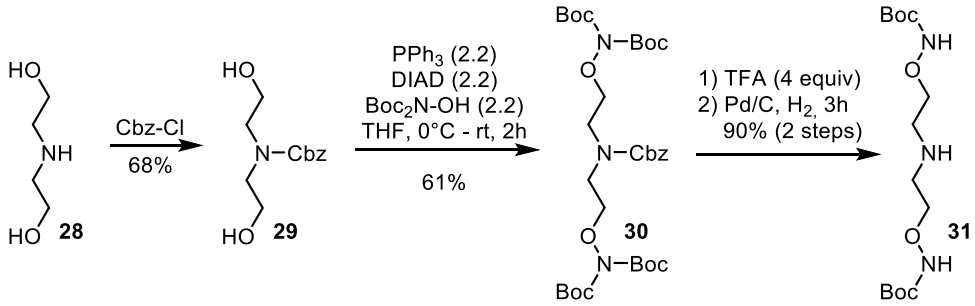


T4-3(C=O)

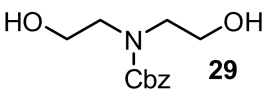
The crude diamine **26** (contains water, 1190 mg, est. 2.27 mmol) was dissolved in 55 ml CH_2Cl_2 with 5 ml EtOH added. Then $NaHCO_3$ (1334 mg, 15.89 mmol, 7 equiv) and Bromoacetic acid *N*-hydroxysuccinimide ester (**27**) (1768 mg, 7.49 mmol, 3.3 equiv) were added and the mixture was stirred for 1 hour, after which TLC-analysis showed full conversion. The reaction mixture was diluted with water and extracted with CH_2Cl_2. The organic phase was subsequently washed with water, brine and a sat. solution of $NaHCO_3$. After drying over Na_2SO_4, filtration and evaporation of the volatiles under reduced pressure, the crude product was obtained, which was purified by column chromatography (7:1 – EtOAc:P.E.),yielding scaffold **T4-3**(C=O) as a fluffy white solid (950 mg, 1.54 mmol, 64% overall). **^{1}H NMR** (500 MHz, Chloroform-*d*) δ 7.20 (t, *J* = 5.9 Hz, 2H), 7.18 (s, 2H), 7.13 (s, 1H), 4.79 (t, *J* = 4.9 Hz, 1H), 4.46 (t, *J* = 4.8 Hz, 1H), 4.36 (d, *J* = 5.9 Hz, 4H), 3.88 (s, 4H), 3.75 (p, *J* = 7.2 Hz, 2H), 3.66 (d, *J* = 4.9 Hz, 2H), 3.58 (dq, *J* = 14.1, 6.9 Hz, 5H), 3.43 (d, *J* = 4.8 Hz, 2H), 3.37 (dt, *J* = 15.5, 7.2 Hz, 2H), 1.22 (t, *J* = 6.9 Hz, 6H), 1.13 (t, *J* = 6.9 Hz, 6H). **^{13}C NMR** (125 MHz, $CDCl_3$) δ 172.11, 165.89, 138.56, 137.35, 127.32, 125.09, 101.14, 100.80, 63.33, 52.68, 48.78, 43.40, 28.86, 15.28. **IR** (cm^{-1}) 3262, 3067, 2971, 2873, 1678, 1665, 1599, 1480, 1424, 1373, 1341, 1297, 1266, 1248, 1227, 1209, 1177, 1120, 1063, 1027, 933, 905, 858, 830, 799, 763, 723, 708. **HR-MS** FD m/z [M^+] calcd for $C_{25}H_{39}Br_2N_3O_7$: 651.1155, found 651.1136. **mp** 74°C.

6.2 Strategy for the alkoxy amine centered scaffolds

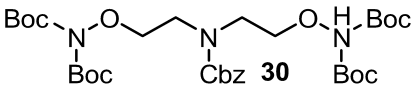
6.2.1 Amine for aminooxy centered scaffolds



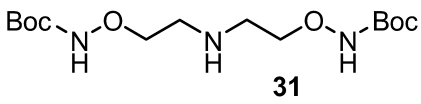
Scheme 8: Synthesis of the aminooxy type scaffold amine. This amine is used for all three scaffolds in this strategy.



To a flame-dried flask, under N_2 flow, diethanolamine (**28**) (3.4 ml, 25 mmol, 1 equiv) was dissolved in 35 ml of freshly distilled MeCN. The solution was cooled to 0 °C, after which solid K_2CO_3 (6.931 g, 50 mmol, 2 equiv) was added. Then, Cbz-Cl (3.7 ml, 26 mmol, 1.01 equiv) was added to the mixture in a dropwise fashion. The suspension was warmed to rt. and stirred overnight. The volatiles were removed *via* rotary evaporation. The resulting slurry was redissolved in EtOAc and washed with water and brine. After drying over Na_2SO_4, the volatiles were removed under reduced pressure, yielding a colorless oil. Flash column purification (5% EtOH in EtOAc) provided **29** as a colorless oil (4.097 g, 17.12 mmol, 68%).**^{1}H NMR** (400 MHz, Chloroform-*d*) δ 7.38-7.30 (m, 5H), 5.13 (s, 2H), 3.84 (s, 2H), 3.77 (s, 2H), 3.55 – 3.44 (m, 4H), 3.44 – 3.33 (s, 2H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 156.58, 136.17, 128.33, 127.90, 127.63, 67.16, 61.36, 60.99, 52.33, 51.76. **IR** (cm^{-1}) 3368, 3064, 3032, 3942, 2879, 1671, 1496, 1473, 1455, 1415, 1363, 1262, 1217, 1130, 1046, 989, 906, 858, 768, 734, 696. **HR-MS** FD m/z [M^+] calcd for $C_{12}H_{17}NO_4$: 239.1158, found 239.1157.



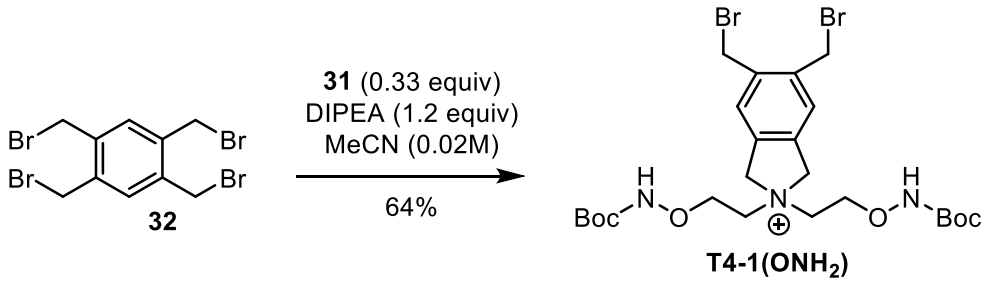
To a flame-dried flask, under N_2 flow, Cbz-protected diethanolamine **29** (10.0 g, 41.79 mmol, 1 equiv) was dissolved in 230 ml of freshly distilled THF. PPh_3 (23.02 g, 87.76 mmol, 2.1 equiv) and Boc_2*N*-OH (20.47 g, 87.76 mmol, 2.1 equiv) were added, and the solution was cooled to 0 °C. DIAD (17.3 ml, 87.76 mmol, 2.1 equiv) was added in dropwise fashion *via* a syringe pump (5ml/h). The mixture was warmed to rt and stirred overnight. The volatiles were removed under reduced pressure, providing a yellow oil. Flash column chromatography (3:2:0.5 – Petroleum ether:CH_2Cl_2:EtOAc) yielded **30** as a colorless oil (21.39 g, 31.94 mmol, 76%). **^{1}H NMR** (400 MHz, Chloroform-d) δ 7.39 – 7.30 (m, 5H), 5.14 (s, 2H), 4.10 (t, J = 5.0 Hz, 2H), 4.04 (t, J = 5.5 Hz, 2H), 3.67 (q, J = 5.2 Hz, 4H), 1.53 (s, 18H), 1.51 (s, 18H). **^{13}C NMR** (100 MHz, CDCl3) δ 155.73, 149.88, 136.43, 128.49, 128.03, 127.93, 83.72, 83.68, 75.00, 74.90, 67.21, 47.26, 46.68, 28.01. **IR** (cm^{-1}) 2979, 2936, 1792, 1751, 1703, 1475, 1457, 1412, 1393, 1368, 1344, 1271, 1247, 1140, 1109, 1092, 1038, 1004, 912, 890, 848, 794, 768, 751, 735, 697. **HR-MS** FD m/z [M+H]$^+$ calcd for $C_{32}H_{52}N_3O_{12}$: 670.3551, found 670.6468.



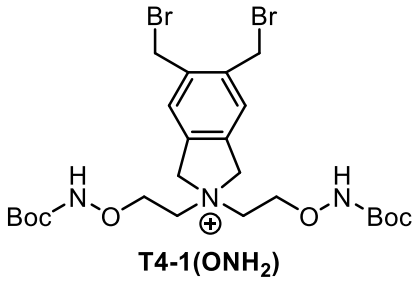
31

The protected amino-oxy compound **30** (840 mg, 1.2 mmol, 1 equiv) was dissolved in 20 ml of CH_2Cl_2. TFA (368 µL, 4.8 mmol, 4 equiv) was added in dropwise fashion, after which the mixture was stirred for 16h at rt. TLC and NMR analysis showed the *mono*-deprotection. The volatiles were removed under reduced pressure. To remove the Cbz-group, the oily residue was redissolved in 25 ml of EtOH. The solution was evacuated, and purged with N_2. Pd/C (10 wt%, 75 mg) was added. H_2 pressure was applied *via* a balloon. The solution was thrice evacuated and saturated with H_2. The solution was stirred for 3h at rt, after which it was filtered over a Celite pad. After elution with EtOH the volatiles were removed under reduced pressure, yielding an opaque oil. Further purification *via* flash column chromatography (EtOAc:EtOH – 5:1) yielded the deprotected amine **31** as a very sticky foam (363 mg, 1.08 mmol, 90%) (can also be a solid, as the oil solidifies). **^{1}H NMR** (400 MHz, Chloroform-*d*) δ 4.26 – 4.13 (m, 4H), 3.40 – 3.18 (m, 4H), 1.50 (s, 18H). **^{13}C NMR** (125 MHz, $CDCl_3$) δ 158.30, 82.98, 71.54, 45.93, 28.17. **IR** (cm^{-1}) 3198, 2981, 2938, 1672, 1446, 1395, 1369, 1287, 1253, 1201, 1161, 1112, 1051, 1011, 925, 837, 799, 774, 721. **mp** 61 to 70 °C. the melting is very slow, yielding a gloopier substance. **HR-MS** FD m/z $[M]^+$ calcd for $C_{14}H_{29}N_3O_6$: 336.2134, found 336.2192.

6.2.2 T4-N1

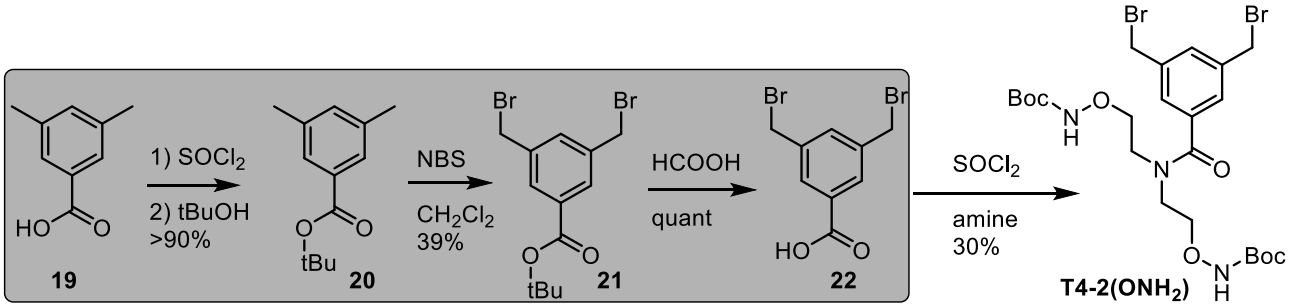


Scheme 9: The synthesis of **T4-1**(ONH_2).

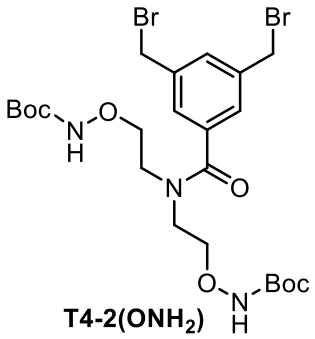


To a flame-dried flask, under N_2-flow, 1,2,4,5-tetrakis (bromo-methyl)benzene **32** (1.350 g, 3 mmol, 3 equiv) was added and dissolved in 175 ml of freshly distilled MeCN. DIPEA (348 µL, 2 mmol, 2 equiv) was added and the mixture was stirred until all solids had dissolved. A solution of the deprotected amine **31** (335 mg, 1 mmol, 1 equiv) in 20 ml of MeCN was added to the durene solution in dropwise fashion over the course of an hour, and the mixture was stirred overnight. Full consumption of the starting material was shown *via* LC-MS analysis. (EtOAc, then 5:1 up to 2:1 EtOAc:EtOH) yielded scaffold **T4-1**(ONH_2) as an off-white foam (400 mg, 0.64 mmol, 64%). **^{1}H NMR** (400 MHz, CH_3CN+D_2O) δ 7.47 (s, 2H), 5.05 (s, 4H), 4.74 (s, 4H), 4.18 (s, 4H), 3.95 – 3.88 (m, 4H), 1.43 (s, 18H). **^{13}C NMR** (100MHz, CH_3CN+D_2O) δ 157.78, 138.90, 134.88, 126.62, 82.51, 70.58, 69.38, 60.80, 30.16, 28.10. **IR** (cm^{-1}) 3255, 3236, 3134, 2976, 2934, 2177, 2164, 2157, 2033, 1712, 1453, 1393, 1367, 1273, 1250, 1213, 1159, 1107, 1007, 946, 926, 841, 799, 772. **HR-MS** (LC-ESI) for $C_{24}H_{38}Br_2N_3O_6^+$ calc 622.1127 found 622.1148. **mp** 101 °C.

6.2.3 T4-2(ONH_2)

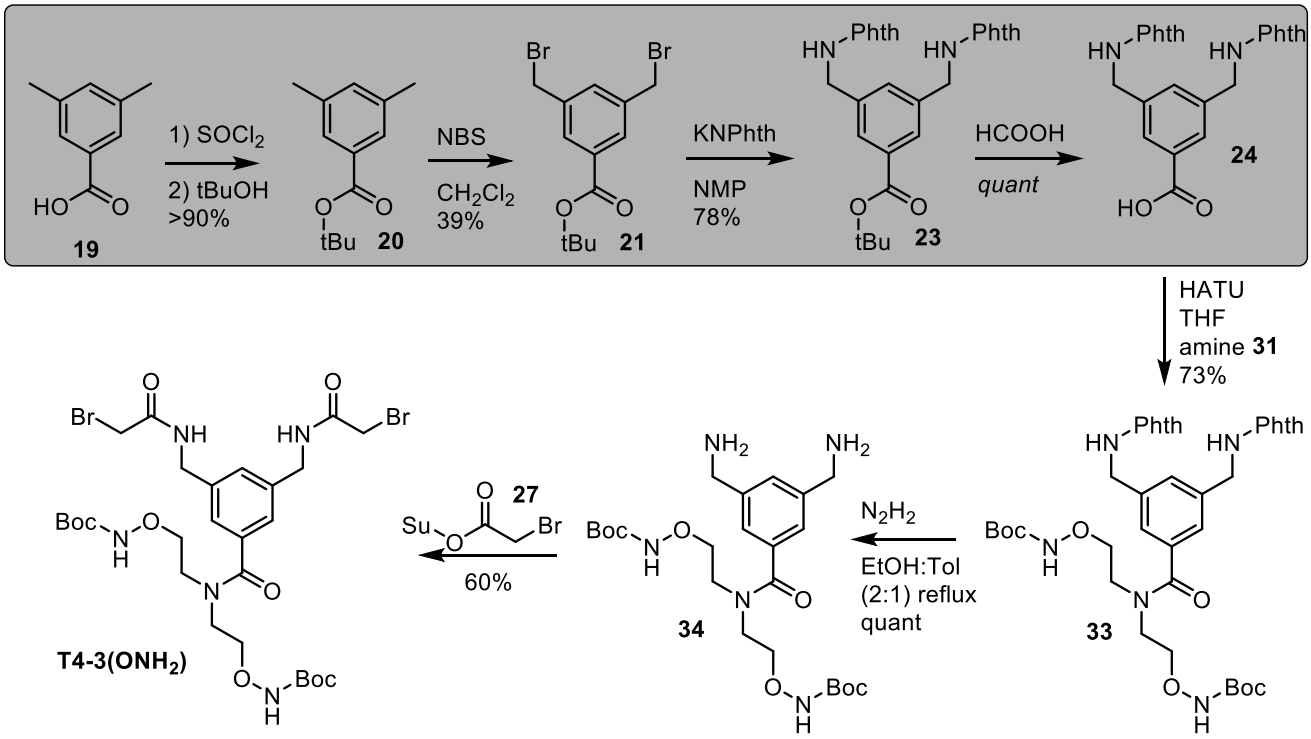


Scheme 10: The synthesis of **T4-2**(ONH_2), where the steps highlighted in grey are treated under strategy 1 for **T4-2**(C=O)

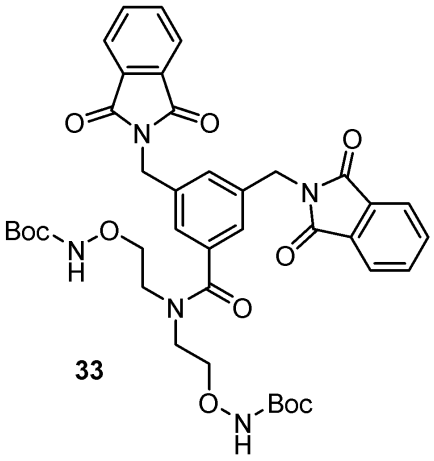


To a flame-dried Schlenk flask, under N_2 atmosphere, acid **22** (2.00 g, 6.49 mmol) is added, followed by $SOCl_2$ (9 ml, excess). The mixture is heated to reflux, for 1.5 hours, after which the remaining $SOCl_2$ is removed *in vacuo*. After co-evaporation with toluene, the acid-chloride was obtained as a pale-yellow solid. CH_2Cl_2 (50 ml) was added, followed by DMAP (50 mg, 6mol%), The amine **31** (1.94 g, 5.78 mmol, 0.9 equiv) was dissolved with NEt_3 (1.13 ml, 6.49 mmol, 1 equiv) in CH_2Cl_2 (5 ml). The mixture was stirred for 2 hours at rt. After extraction with CH_2Cl_2, the organic phase was washed with 1M $KHSO_4$ (1x) and water (2x), and subsequently dried over Na_2SO_4. Filtration and evaporation yielded the crude product, which was purified *via* column chromatography (3:1 to 2:1 P.E:EtOAc) and **T4-2**(ONH_2) was isolated as a colorless oil (763 mg, 1.22 mmol, 21%). *Theproduct is isolated as a single spot on TLC. In LC-MS, the presence of the desired product was shown. Additionally, the presence of partially chlorinated product was shown by mass, and confirmed via the isotope pattern. As the peaks are too close to integrate, the ratio of Cl-substitution was determined via NMR, as in ^{1}H-NMR and ^{13}C-NMR the -CH_2-Cl and the -CH_2-Br signals can be distinguished. Using a long delay time, the ratio can be determined by integration of the signals. In ^{1}H-NMR, with a delay of 10s, the ratio Br:Cl was determined at 1 : 0.30. In ^{13}C-NMR, with a delay of 15s, the ratio of Br:Cl was determined at 1 : 0.29.* **^{1}H NMR** (300 MHz, Chloroform-*d*) δ 8.37 (s, 1H), 7.96 (s, 1H), 7.38 (s, 1H), 7.33 (s, 1H), 4.50 (s, 1H, of CH_2-Cl, 4.39 (s, 3H, of CH_2-Br), 4.01 (s, 2H), 3.87 (s, 2H), 3.79 (s, 2H), 3.49 (s, 2H), 1.47 – 1.33 (m, 18H, overlapping singlet of Boc-groups). **^{13}C NMR** (75 MHz, $CDCl_3$) δ 171.36, 171.30, 156.68, 156.42, 138.61, 138.28, 136.93, 130.16, 129.68, 127.12, 126.60, 81.39, 81.09, 74.01, 73.23, 47.79, 44.80, 43.66, 31.76, 27.93. **IR** (cm^{-1}) 3247, 2977, 2933, 1715, 1620, 1598, 1475, 1444, 1392, 1366, 1270, 1247, 1159, 1109, 1049, 1010, 910, 836, 771, 757, 729, 704. **mp** 38 °C **HR-MS** FD, for $[C_{23}H_{35}Br_2N_3O_7]^+$, calc 624.0920, found, 624.0880. The Boc-group is easily lost during ionization. The mono-Chloride mass is also found.

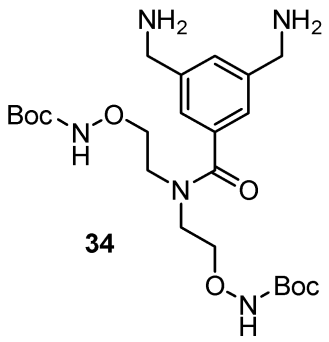
6.2.4 T4-3(ONH_2)



Scheme 11: The synthesis of scaffold **T4-3**(ONH_2). Highlighted in grey are compounds whereof the synthesis was detailed under strategy I.

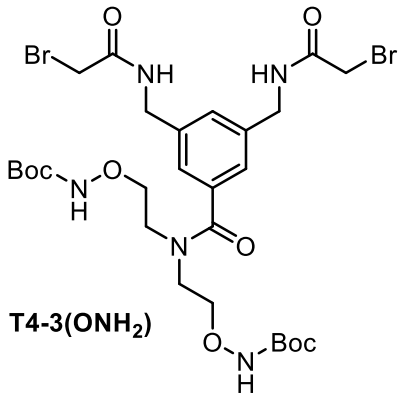


Phthalimide *tert*-butyl ester **23** (1.82 mg, 3.67 mmol) was dissolved in 1:2 CH_2Cl_2:HCOOH (60 ml). The reaction mixture was stirred for 2 days at rt yielding a white suspension. The solids were filtered off, and washed with CH_2Cl_2, yielding **24** as a very fine white powder (1.8 g, contains water, *quant*). The free acid **24** was suspended in DMF (25 ml). HATU (1.53 g, 4.04 mmol, 1.1 equiv) was added followed by DIPEA (1.28 ml, 7.34 mmol, 2 equiv). This mixture is left for 15 minutes, during which a yellow solution forms, which later forms a precipitate. The aminooxy amine **31** (1.32 g, 3.92 mmol, 1.07 equiv) was dissolved in 17 ml DMF, and added dropwise to the HATU solution. The reaction mixture remains yellow but becomes quite opaque with more precipitate. The reaction is finished after 2h. The reaction mixture was evaporated to yield a very viscous yellow oil. EtOAc was added and the organic phase was washed with H_2O, sat. $NaHCO_3$ solution, H_2O, 1M $KHSO_4$, H_2O, brine and was subsequently dried over Na_2SO_4. The product was purified by column chromatography (1:1 to 1:2 P.E:EtOAc). **33** is isolated as a white powder (1.87 g, 2.36 mmol, 67%).**^{1}H NMR** (300 MHz, Chloroform-*d*) δ 8.42 (s, 1H), 8.08 (s, 1H), 7.68 (dd, *J* = 5.3, 3.1 Hz, 4H), 7.58 (dd, *J* = 5.4, 3.1 Hz, 4H), 7.43 (s, 1H), 7.28 (s, 2H), 4.71 (s, 4H), 3.97 (s, 2H), 3.85 (s, 2H), 3.75 (s, 2H), 3.42 (s, 2H), 1.33 (s, 18H). **^{13}C NMR** (75 MHz, $CDCl_3$) δ 171.87, 167.75, 156.88, 156.63, 137.24, 137.06, 134.10, 131.77, 129.64, 126.22, 123.33, 81.39, 81.07, 74.27, 73.41, 47.96, 43.87, 40.98, 28.17. **IR (cm^{-1})** 3266, 2976, 2934, 1770, 1707, 1624, 1468, 1426, 1391, 1366, 1344, 1248, 1161, 1108, 1050, 1012, 955, 728, 711. **HR-MS** FD m/z [M+H$^+$] calcd for $C_{39}H_{44}N_5O_{11}^+$: 758.3032 found 758.3005. **mp** 78 °C.



34

Phthalidmide compound **33** (1866 mg, 2.36 mmol, 1 equiv) was added to a flask, and dissolved in EtOH:Toluene – 2:1 (31 ml). Hydrazine hydrate (50% solution in water, 1.6 ml, 23.6 mml, 10 equiv) was added and the mixture was heated to reflux, where a solid started to precipitate after 30 min. The mixture was refluxed overnight, after which the yellow suspension was cooled to rt. The solids were filtered off and washed with CH_2Cl_2 (3x). The volatiles were removed under reduced pressure, yielding the desired product **34** as a white solid (1084 mg, 2.18 mmol, 92%), which was used in the next reaction step without further purification. **^{1}H NMR** (500 MHz, Chloroform-d) δ 8.51 (s, 1H), 8.21 (s, 1H), 7.33 (s, 2H), 7.31 (s, 1H), 4.11 (s, 2H), 4.00 (s, 2H), 3.92 (s, 4H), 3.87 (s, 2H), 3.59 (s, 2H), 1.49 (s, 18H). **^{13}C NMR** (125 MHz, CDCl3) δ 173.28, 157.34, 156.60, 143.71, 136.76, 127.11, 124.05, 81.65, 81.48, 74.01, 73.35, 48.03, 45.97, 43.39, 38.62, 28.26.



T4-3(ONH_2)

The free amine **34** (688 mg, 1.38 mmol) was dissolved in 36 ml CH_2Cl_2. To fully dissolve the compound, 7 ml of EtOH is added. Solid $NaHCO_3$ (381 mg, 4.53 mmol, 3.3 equiv) is added, followed by Bromoacetyl-O-succinimide ester **27** (1.07 g, 4.53 mmol, 3.3 equiv) which is added in one portion. The reaction mixture is stirred at rt for 30 min, after which LC-MS shows the reaction had completed. The volatiles were removed under reduced pressure and the remainder was dissolved in CH_2Cl_2 and washed with 1M $KHSO_4$ (3x) and brine (2x) and dried over Na_2SO_4. The volatiles were removed under reduced pressure. The product was further purified by column chromatography (7:1 EtOAc: Petroleum ether), yielding the finished scaffold **T4-3**(ONH_2) as a white foam (635 mg, 0.89 mmol, 62%).**^{1}H NMR** (500 MHz, Chloroform-*d*) δ 8.30 (d, *J* = 15.0 Hz, 2H), 7.62 (t, *J* = 5.9 Hz, 2H), 7.21 (s, 2H), 7.16 (s, 1H), 4.32 (d, *J* = 5.9 Hz, 4H), 4.02 (s, 2H), 3.92 (s, 2H), 3.84 (s, 4H), 3.77 (s, 2H), 3.46 (s, 2H), 1.44 (s, 18H). **^{1}H NMR** *(500 MHz, DMSO-d_6, 25 °C)* δ 9.99 (s, 2H), 8.82 (t, *J* = 5.8 Hz, 2H), 7.23 (s, 1H), 7.17 (s, 2H), 4.32 (d, *J* = 5.8 Hz, 4H), 3.94 (s, 2H), 3.92 (s, 4H), 3.79 (s, 2H), 3.70 (s, 2H), 3.46 (s, 2H), 1.42 (d, *J* = 13.8 Hz, 18H). **^{1}H NMR** *(500 MHz, DMSO-d_6, 75 °C)* δ 9.75 (s, 2H), 8.61 (s, 2H), 7.25 (s, 1H), 7.19 (s, 2H), 4.34 (d, *J* = 5.8 Hz, 4H), 3.92 (s, 4H), 3.89 (s, 4H), 3.60 (s, 4H), 1.44 (s, 18H). **^{13}C NMR** *(125 MHz, $CDCl_3$, rt)* δ 172.47, 166.58, 157.14, 156.76, 138.92, 136.62, 128.38, 127.39, 125.56, 124.58, 81.80, 81.66, 73.71, 73.61, 73.20, 72.99, 48.22, 44.09, 43.72, 43.35, 42.97, 29.64, 29.50, 28.86, 28.74, 28.23, 27.85, 26.96. (note: many double peaks due to rotameric effect). **IR (cm^{-1})** 3257, 3076, 2975, 2928, 1713, 1657, 1619, 1598, 1536, 1476, 1424, 1392, 1366, 1274, 1249, 1160, 1106, 1048, 1026, 899, 836, 792, 761, 688. **HR-MS** FD m/z [M-Boc+H$^+$] calcd for $C_{22}H_{34}Br_2N_5O_7^+$: 638.0820 found 638.0867. **mp** 60-63 °C.

7 Preparation of tetracyclic peptides

7.1 General information

Sample preparation

Peptides

Approximately 0.2 mg of peptide was dissolved in 100µL of 1:1 (v/v) MeCN:MilliQ. 10 µL of this solution is diluted with 50 µL of MilliQ. For analysis, 6 µL was injected onto the UPLC column. Reaction samples were measured on a UPLC-ESMS system (3 min, 5-55%B, where B=MeCN, column temperature of 50°C), Acquity UPLC Peptide BEH C18 Column, 130Å, 1.7 µm, 2.1x50 mm with UV detection (λ = 215 nm) and positive ion current for MS analysis.

Reaction mixtures in MeCN:MilliQ solvent mixtures

20 µL of sample was diluted with 40 µL MilliQ, and filtered over a pipet-tip frit filter. For analysis, 6 µL was injected onto the UPLC column. Reaction samples were measured on a UPLC-ESMS system (3 min, 5-55%B, where B=MeCN, column temperature of 50°C), Acquity UPLC Peptide BEH C18 Column, 130Å, 1.7 µm, 2.1x50 mm with UV detection (λ = 215 nm) and positive ion current for MS analysis.

Reaction mixtures in DMSO:MilliQ solvent mixtures

30 µL of sample was diluted with 30 µL MilliQ and filtered over a pipet-tip frit filter. For analysis, 8 was is injected onto the UPLC column. Reaction samples were measured on a UPLC-ESMS system (3 min, 5-55%B, where B=MeCN, column temperature of 50°C), Acquity UPLC Peptide BEH C18 Column, 130Å, 1.7 µm, 2.1x50 mm with UV detection (λ = 215 nm) and positive ion current for MS analysis.

7.2 General CLIPS/oxime procedure

7.2.1 Strategy I

In a glass vial (5 ml), the peptide (0.2 mg) was dissolved in DMSO: MilliQ (1:1, v/v) at a concentration of 0.50 mM. The scaffold (1 to 2 mg/100 µL) was added with a molar equivalent relative to the peptide whereby the peptide weight was taken uncorrected for any TFA-salts present in the dry material (**T4-2**(C=O) at 0.95 equiv, **T4-3**(C=O) at 1.05 equiv). A solution of 1M NH_4HCO_3 was added to reach pH > 8 (30 µL). The reaction mixture is analyzed after 20 min. Upon completion, the reaction mixture was acidified with a 15% TFA solution (volume of base+ 20%, generally 40 µL), to remove the acetal protecting groups of the aldehydes. Oxime ligation occurs simultaneously. The reaction mixture was analyzed at certain time intervals, until the reaction had completed.

7.2.2 Strategy II

CLIPS

In a glass vial (5 ml), the peptide (0.2 mg) was dissolved in MeCN: MilliQ (1:1) at a concentration of 0.50 mM. The scaffold (1 to 2 mg/100 µL) was added with a molar equivalent relative to the peptide, whereby the peptide weight was taken uncorrected for any TFA-salts present in the dry material (**T4**-**1**(ONH_2) at 0.95 equiv, **T4**-**2**(ONH_2) at 1.05 equiv and **T4-3**(ONH_2) at 0.85 equiv). A solution of 1M NH_4HCO_3 is added to reach pH>8 (30 µL). The reaction mixture is analyzed after 20 min. Upon completion, the reaction mixture is lyophilized to dryness. The reaction can also be performed in DMSO:MilliQ mixtures, but DMSO is less suitable for lyophilizing. This has, however, no noticeable effect on the further reactions.

<u>Scaffold deprotection</u>

To remove the Boc-groups on the scaffold aminooxy, the lyophilized product was treated with excess 2:1 TFA: CH_2Cl_2 (300 µL total). The solution was left for 2 hours at rt, after which the volatiles were evaporated under a flow of N_2. For larger scale reactions, the material was again dissolved in CH_2Cl_2, and evaporated to dryness, to remove all TFA remnants.

Oxime Ligation

The free aminooxy peptide was dissolved in DMSO:MilliQ to reach a peptide concentration of 0.50 mM (same as the CLiPS reaction). The pH does not necessarily need adjustment, and was usually omitted. If the pH needs adjustment, the optimum is considered to be pH 4.5, reached by adding a 1M acetate buffer of pH 4.5. The reaction can be carried out at rt, but some systems show the best results at 40 °C. The glass reaction vessel is placed on a heating plate with a temperature set of 40°C.

7.2.3 Isolated tetracycle c5$_{4444}$-F(C=O)•T4-2(ONH$_2$)$^{C/O}$

Peptide c**5**$_{4444}$-**F**(C=O) (5.35 mg, 1.96 µmol) was suspended in 1.95 ml of MeCN, followed by the addition of 1.95 ml of MilliQ, to fully dissolve the peptide (0.50 mM). 87.75 µL (1.05 equiv, uncorrected) of scaffold **T4-2**(ONH$_2$) solution (1.44 mg in 100µL) was added to the peptide. 100 µL of a of 1M NH_4HCO_3 solution was added, with the resulting pH of 8.5. Analysis of the CLIPS mixture showed full conversion to the desired product c**5**$_{4444}$-**F**(C=O)•**T4-2**(ONH$_2$)^C, with some excess scaffold present (Figure 10). The solution was lyophilized, yielding a white powder. To remove the Boc-groups of the scaffold, 4 ml of TFA was added, followed by 2 ml of CH_2Cl_2. The solution was stirred for 2h at rt, resulting in a slightly yellowish solution. Under a stream of nitrogen, the volatiles were evaporated, yielding a sticky residue. This residue was dissolved in CH_2Cl_2, and the volatiles were removed once more, yielding a sticky white residue film on the vial. This residue was dissolved in 1ml DMSO, followed by the addition of 3 ml water. The reaction mixture was stirred at 40 °C over the weekend. The mixture was analyzed, showing 2 products with identical mass, at 1.30 and 1.34 min.

Preparative HPLC purification of the peptides, on a Supelco column (C5-C10) flow 8 ml/min, with phase A: water+ 0.05% (v/v) TFA, and phase B: water MeCN+ 0.05% (v/v) TFA. Purification is performed at rt (no column heater).
The reaction mixture was diluted with water to a final volume of 80 ml (2.5% DMSO). The peptide was loaded onto the column *via* a dilution method, where the reaction solution is taken as the mobile phase (4 ml/min). Once the loading peak of DMSO is completely off the column, the gradient is started (8 ml/min, 0 to 38% MeCN, over 35 min). Products start to elute after approximately 27 min. The fractions (~3 ml) were collected in 10 ml glass vials, analyzed on UPLC and lyophilized.

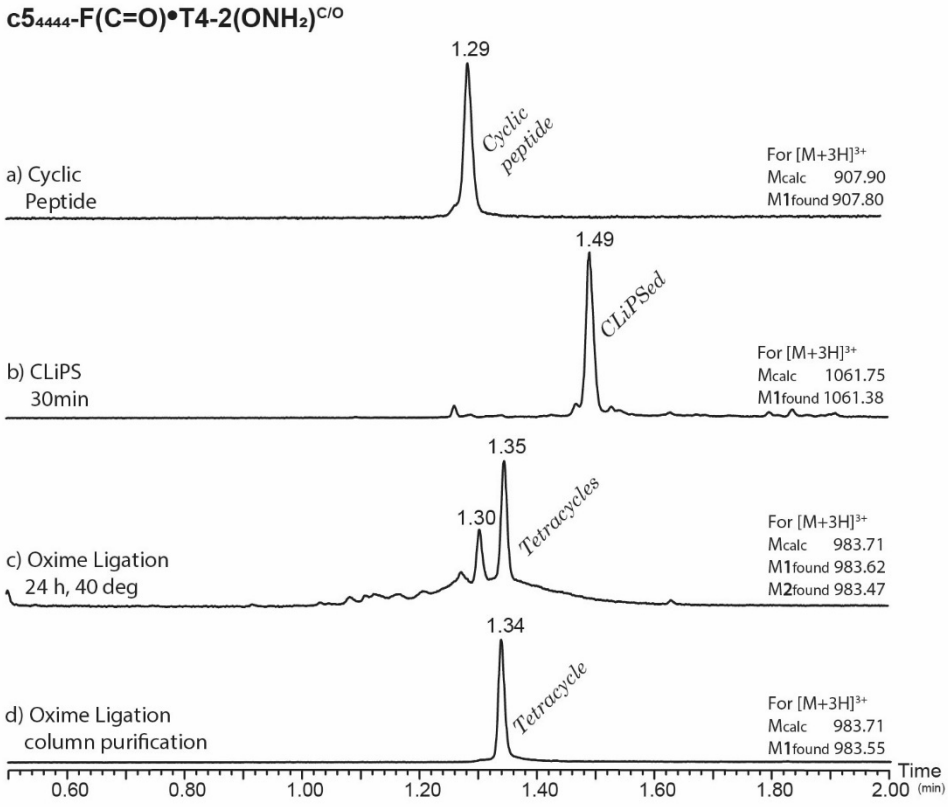


Figure 10. The chromatograms of the cyclization and isolation of c**5**$_{4444}$-**F**(C=O)•**T4-2**(ONH$_2$)$^{C/O}$, where a) the cyclic peptide c**5**$_{4444}$-**F**(C=O), b) the CLIPSed product c**5**$_{4444}$-**F**(C=O)•T4-N2^C, c) the two products obtained for c**5**$_{4444}$-**F**(C=O)•**T4-2**(ONH$_2$)$^{C/O}$ d) isolated tricycle of c**5**$_{4444}$-**F**(C=O)•**T4-2**(ONH$_2$)$^{C/O}$

Product 1 (tr 1.30 min) was present in fr. 10 and 11, but not pure.

HR-MS (ESI+), for $C_{139}H_{209}N_{43}O_{25}S_2$, mw_{calc} 2945.5924,

$[M+2H]^{2+}$ calc 1473.8015, found 1473.8158.

$[M+3H]^{3+}$ calc 982.8703, found 982.8719.

$[M+4H]^{4+}$ calc 737.4047, found 737.4042.

Product 2 (tr 1.34 min) was present in fr. 12 to 15, in pure form (1.85 mg total).

HR-MS: ESI-QTOF for $[C_{139}H_{209}N_{43}O_{25}S_2]^+$ $[M+H]^+$ calc 2945.5919, found 2945.8657. HR-MS (ESI+), for $C_{139}H_{209}N_{43}O_{25}S_2$, mw_{calc} 2945.5924

HR-MS (ESI+), for $C_{139}H_{209}N_{43}O_{25}S_2$, mw_{calc} 2945.5924,

$[M+2H]^{2+}$ calc 1473.8015, found 1473.8117.

$[M+3H]^{3+}$ calc 982.8703, found 982.8701.

$[M+4H]^{4+}$ calc 737.4047, found 737.4029.

Product 2 was used for ^{1}H-NMR analysis.

7.3 NMR analysis of c54444-F(C=O)•T4-2(ONH2)C/O

7.3.1 Materials and methods

The NMR sample of c**5**$_{4444}$-**F**(C=O)•**T4-2**$(ONH_2)^{C/O}$ was prepared as a 1.07 mM solution in 540 µl total volume (5 mm NMR tube) containing 25 mM NaAc-d^3 buffer (pH 4.45), 0.1 mM EDTA, 0.2 mM sodium azide, 2 µM DSS-d^6 as chemical shift reference and 2% (v/v) D_2O for deuterium lock. NMR spectra (1D ^{1}H, DIPSI 80 ms mixing time, NOESY 350 ms mixing time, ROESY 150 ms mixing time, natural abundance ^{13}C-^{1}H HSQC, ^{13}C-^{1}H HMBC, and ^{15}N-^{1}H HSQC[10] of 1.07 mM c**5**$_{4444}$-**F**(C=O)•T4-N2$^{C/O}$ were recorded on a Bruker Avance III HD 700 MHz spectrometer, equipped with a TCI cryoprobe. Spectra were recorded at various temperatures varying between 25 °C and 37 °C, Temperature-dependent exchange processes of c**5**$_{4444}$-**F**(C=O)•**T4-2**$(ONH_2)^{C/O}$ were studied in both NOESY and ROESY spectra. Processing and analysis was carried out by Topspin 3.2 (Bruker, Rheinstetten, Germany).

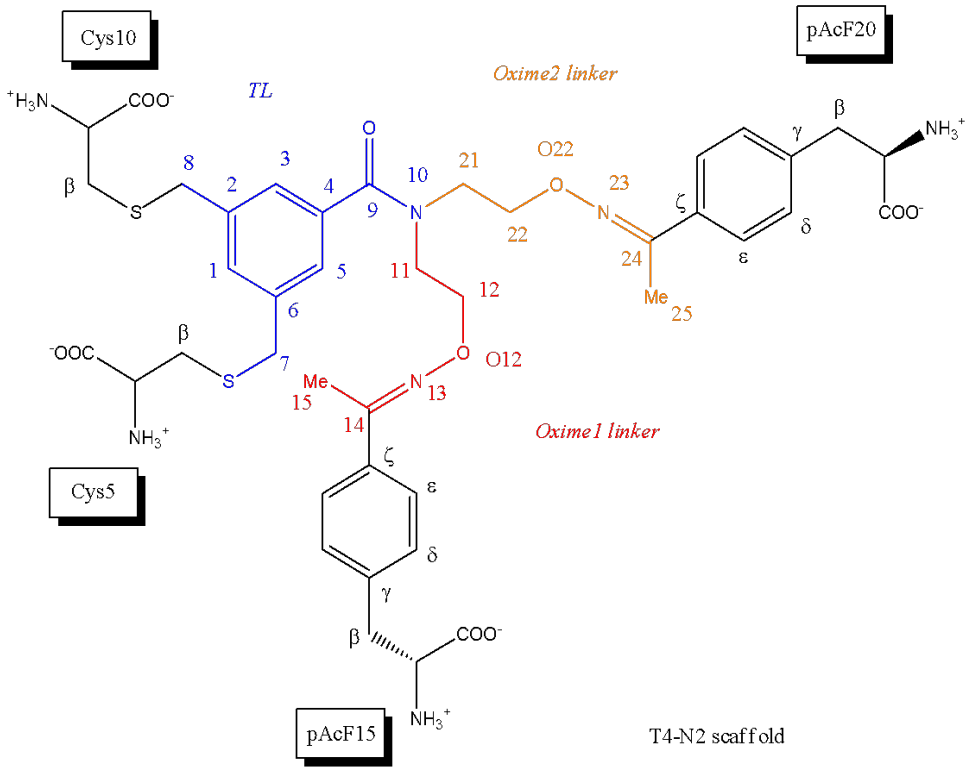


Figure 11. Topology and atom numbering in the NMR analysis of **c5$_{4444}$-F**(C=O)•**T4-2**(ONH$_2$)$^{C/O}$.

7.3.2 Results

Molecule c**5**$_{4444}$-**F**(C=O)•**T4-2**(ONH$_2$)$^{C/O}$ has been studied by NMR spectroscopy to determine the conformational characteristics and dynamics of the peptide part and scaffold system, the latter schematically shown in Figure 11. A systematic study of concentration and temperature shows that the system adopts a complex ^{1}H NMR spectrum that corresponds to multiple conformational ensemble in slow exchange. Figure 12 shows the 1D proton spectrum recorded at 25, 30 and 37 ^{o}C. At all three temperatures a broad envelop at 8.1 ppm is observed that contains most amino acid amide resonances. In contrast, aromatic protons from F(C=O)15, F(C=O)20 and the ring protons of Linker group TL (grouped at 7.25 ppm) adopt sharp resonances, but these overlapping signals are rather divided up in numerous peaks. In the end, the conformational set of structures considered is too complex to structurally elucidate by NMR. In order to estimate the number of distinct conformers present in the conformational mixture, different types of additional 2D NMR spectra were recorded. Figure 13 displays the region in the ^{13}C-^{1}H HSQC spectrum in which part the oxime methyl resonances, denoted Me-15 and Me-25, are observed. At least twelve individual large methyl cross peaks can be separated in this 2D region, while one expects only a maximum of four peaks that would correspond to the *syn* and *anti* orientation of Methyl C15 and C25 with respect to the C=N double bond in both linkers Oxime1 and Oxime2 (Fig S1). ROESY spectra on the scaffold clearly demonstrate the existence of various equilibrium states in this conformational ensemble, with many off-diagonal exchange peaks visible for methyl resonances Me-15 and Me-25 (Figure 14). In addition, other regions of the 13C-1H HSQC spectrum are consistent with a global equilibrium ensemble affecting the entire scaffold and the protein segments. The large number of individual Leu methyl resonances present (Figure 15), many more than the expected eight (4 x Hδ1/Hδ2 Leu) peaks, shows that the protein loops do not adopt a well-defined,

single low-energy state in solution. Based on these NMR results we did not attempt to assign individual resonances. The small ^{1}H chemical shift dispersion observed for amino acid backbone resonances together with the similarity of group-based resonances in the linker region of c**5**$_{4444}$-**F**(C=O)•**T4-2**$(ONH_2)^{C/O}$ however agree with a lack of structural rigidity.

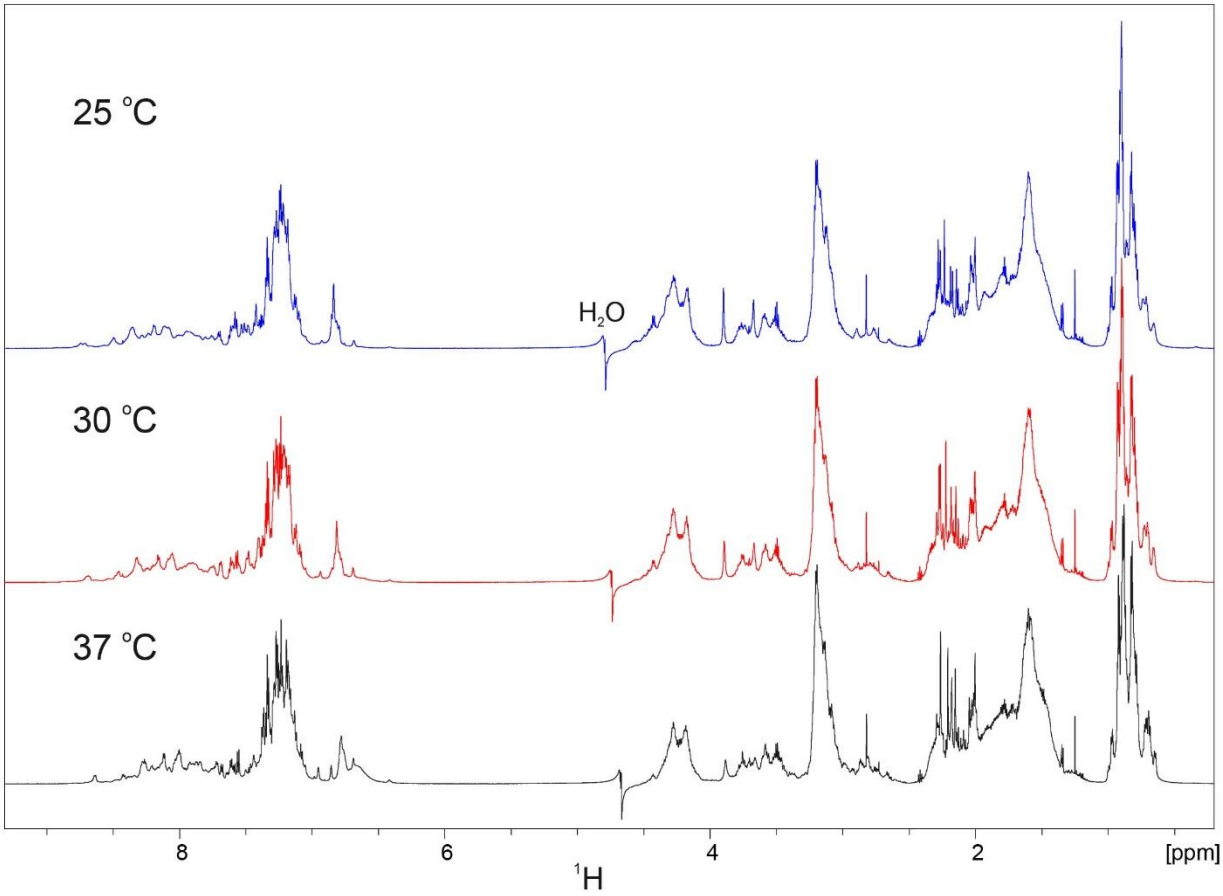


Figure 12. 700 MHz ^{1}H spectrum of c**5**$_{4444}$-**F**(C=O)•**T4-2**$(ONH_2)^{C/O}$ recorded at three different temperatures, using excitation sculpting to selectively suppress the water resonance positioned in the spectrum at the pointer annotated *H2O*.

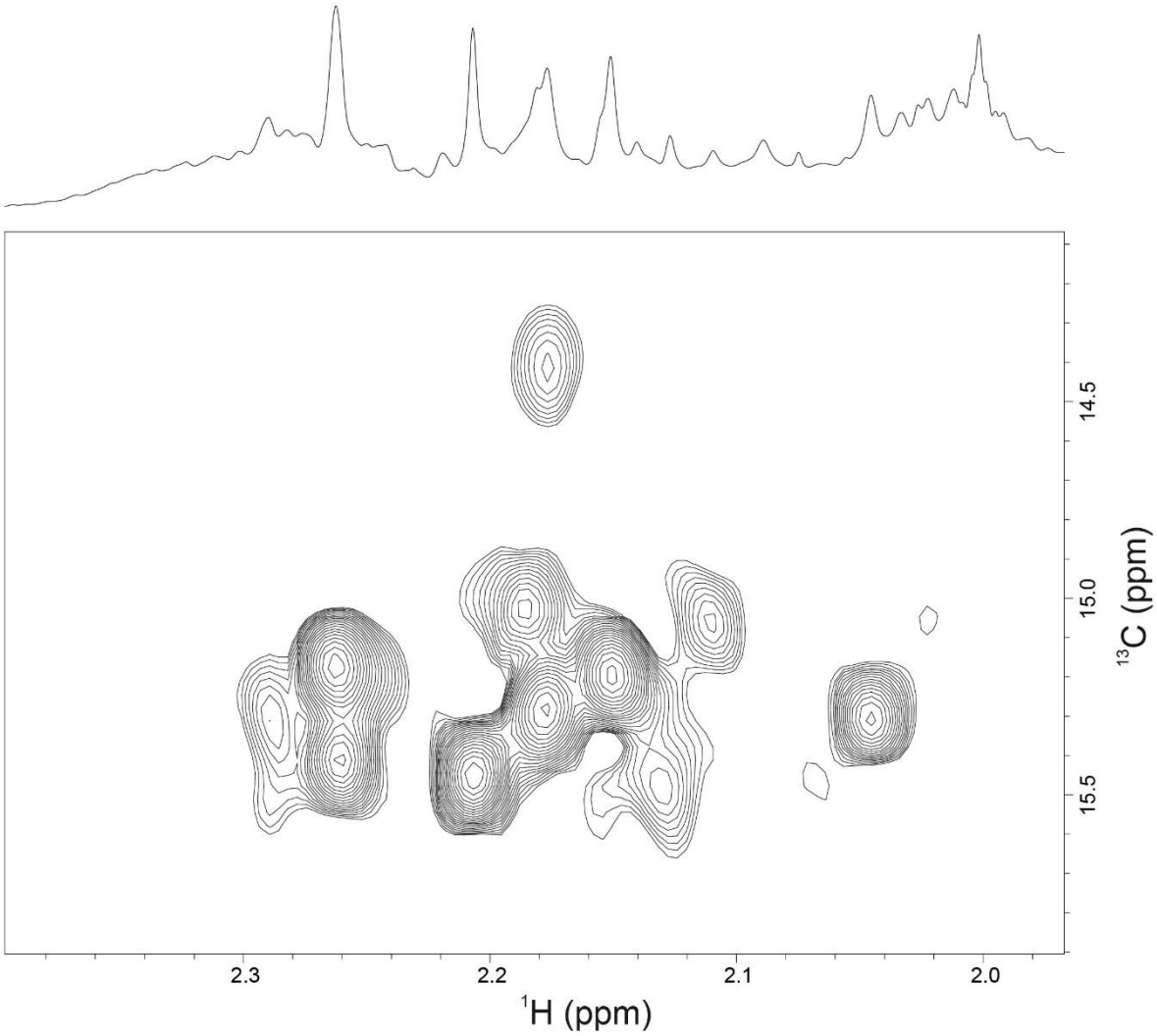


Figure 13. Part of the alifatic methyl region of the HSQC ^{13}C-^{1}H spectrum of c**5**$_{4444}$-**F**(C=O)•**T4-2**(ONH$_2$)$^{C/O}$ at 37 °C. This region contains the cross peaks corresponding to oxime methyl peaks Me-15 and Me-25 positioned in the two oxime linkers (Scheme Fig. 11). Multiple peaks are observed in slow to medium exchange, the complexity of the spectrum prevents unambiguous assignment of these multiple peaks.

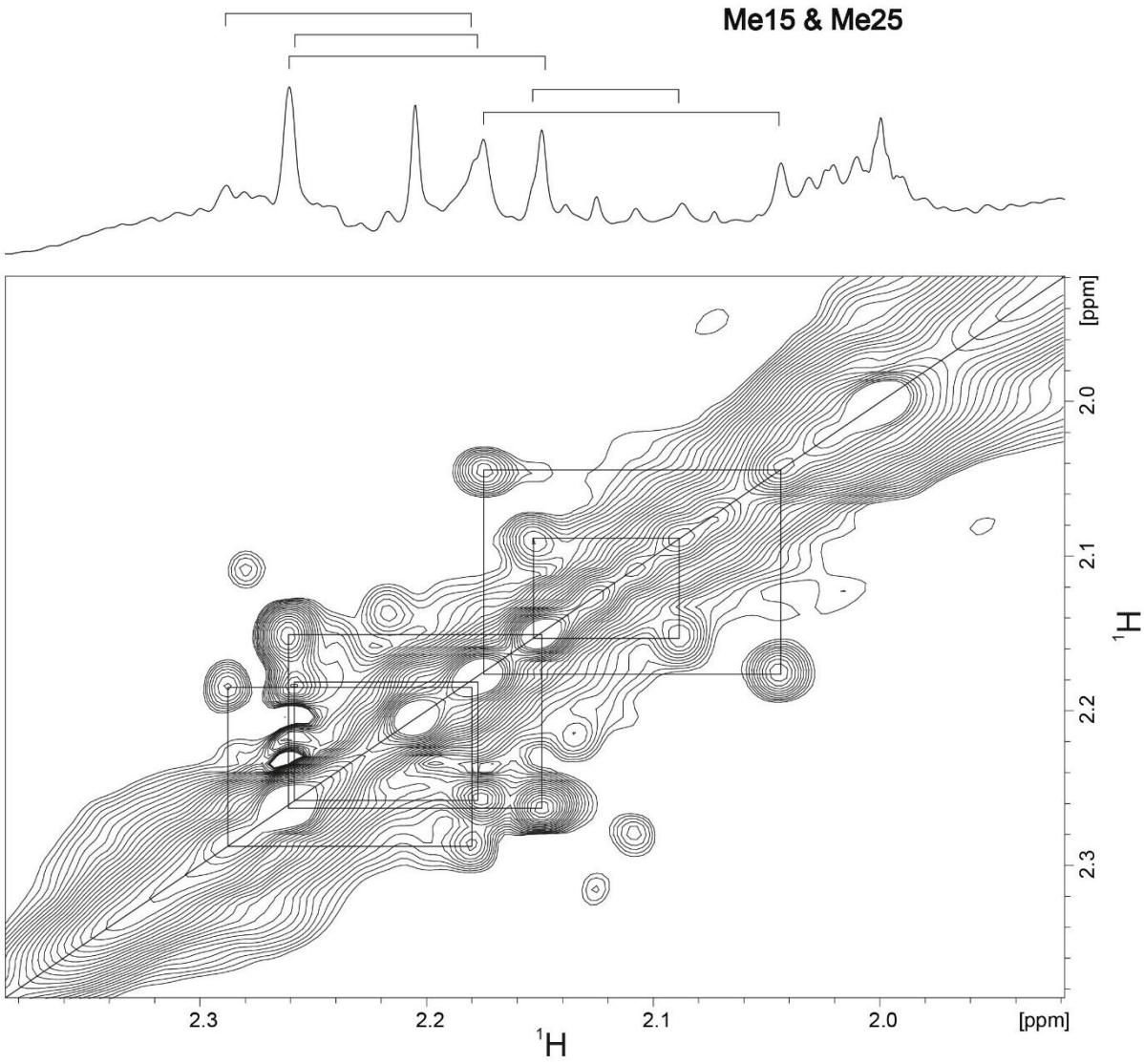


Figure 14. Part of the ROESY spectrum (150 ms mixing time) of c**5**$_{4444}$-**F**(C=O)•**T4-2**$(ONH_2)^{C/O}$ at 37 °C, containing the methyl peaks Me-15 and Me-25 that are positioned in the two oxime linkers of the scaffold (Scheme Fig 11). Discrimination between NOE and exchange follows from the sign of off-diagonal cross peaks relative to the diagonal intensity, negative for NOE peaks and positive for ROE exchange peaks. In this region only positive exchange peaks are observed, and strongest pair-wise ROE correlations are indicated by rectangles and with horizontal bars in the corresponding 1D ^{1}H reference spectrum.

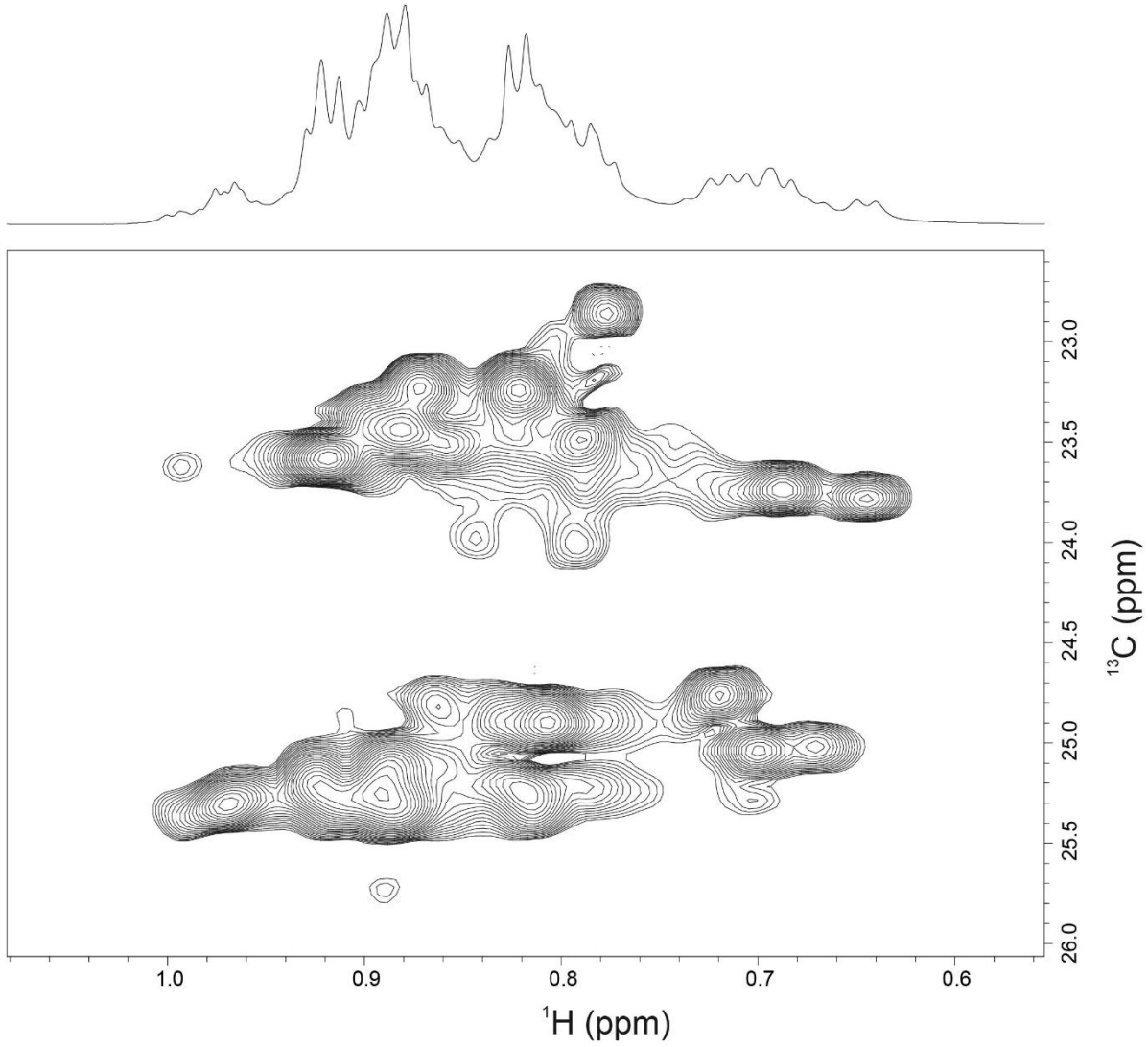


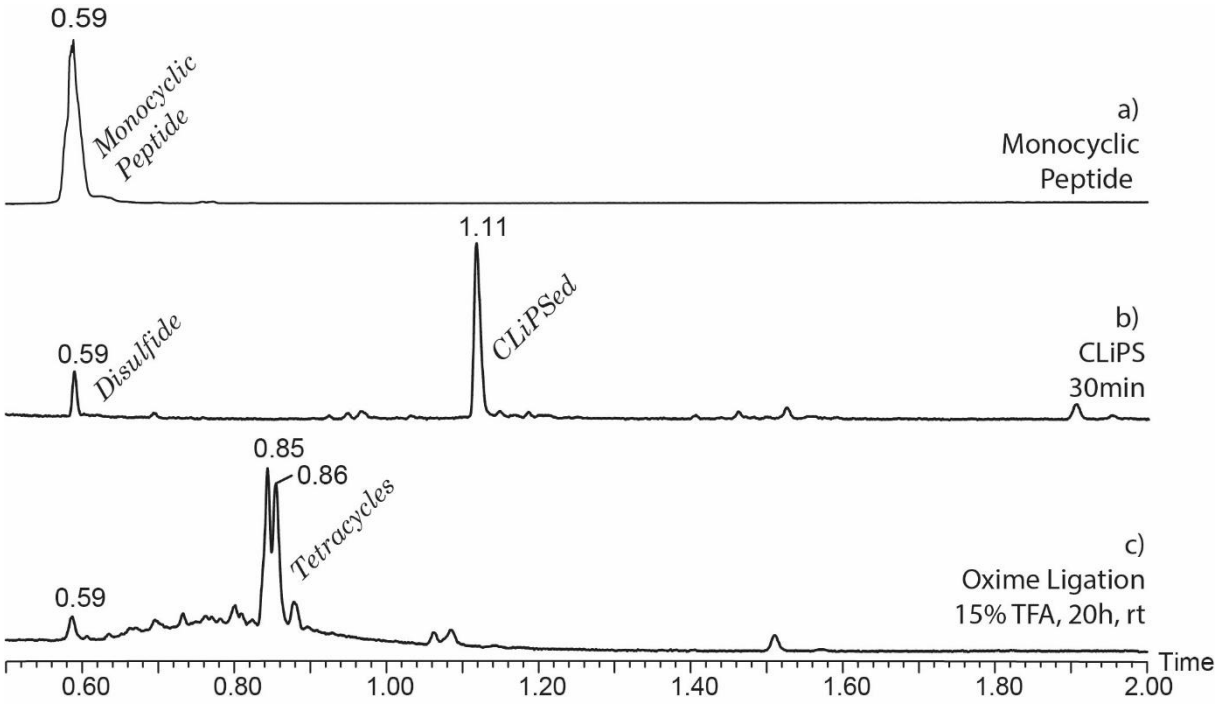
Figure 15. Part of the aliphatic methyl region of the HSQC ^{13}C-^{1}H spectrum of c$\mathbf{5}_{4444}$-**F**(C=O)•**T4-2**$(ONH_2)^{C/O}$ at 37 °C. This region contains the cross peaks corresponding to Leu methyl pairs Hδ1#-CD1 and Hδ2#-CD2 in amino acid residues Leu3, Leu8, Leu13 and Leu18 of the peptide loops. The complexity of the spectrum prevents unambiguous assignment of these methyl peaks.

8 UPLC graphs of the synthesized tetracycles

8.1 hS(ONH_2) peptides

8.1.1 $c1_{3333}$-hS(ONH_2)•T4-2(C=O)$^{c/o}$

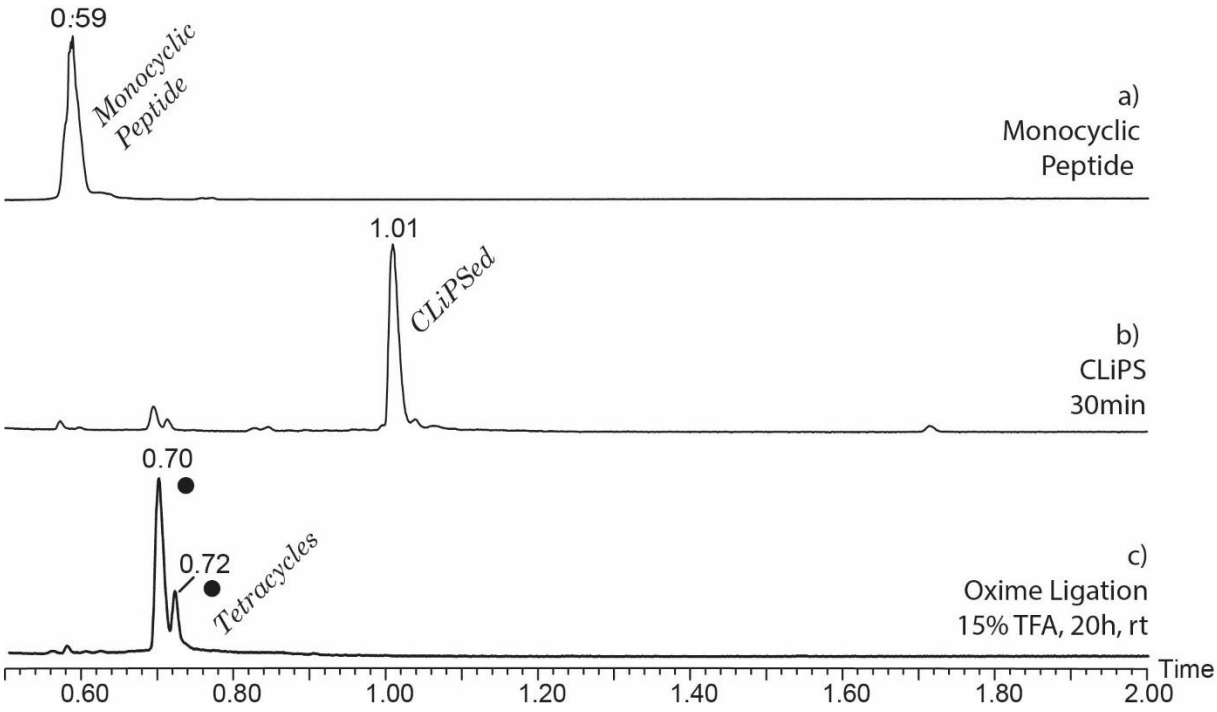
cyc-CYKQ**hS**(ONH_2)SIK**hS**(ONH_2)AKGCSKL with the **T4-2**(C=O) scaffold



Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocylic peptide	0.59	1771.91	1771.46	$[M+H]^{+}$
				886.64	886.23	$[M+2H]^{2+}$
				591.58	591.15	$[M+3H]^{3+}$
b	CLIPS reaction	Disulfide	0.59	885.97	886.23	$[M+2H]^{2+}$
				591.13	591.16	$[M+3H]^{3+}$
		CLIPSed	1.11	1075.58	1075.49	$[M+2H]^{2+}$
				702.29	701.98	$[M-OEt+3H]^{3+}$
c	Oxime ligation	Disulfide	0.59	885.97	886.23	$[M+2H]^{2+}$
				591.13	591.16	$[M+3H]^{3+}$
		Tetracycle	0.85	984.00	983.86	$[M+2H]^{2+}$
				656.38	656.24	$[M+3H]^{3+}$
		Tetracycle	0.86	983.70	983.86	$[M+2H]^{2+}$
				656.16	656.24	$[M+3H]^{3+}$

8.1.2 $c1_{3333}$-1hS(ONH_2)•T4-3(C=O)$^{c/o}$

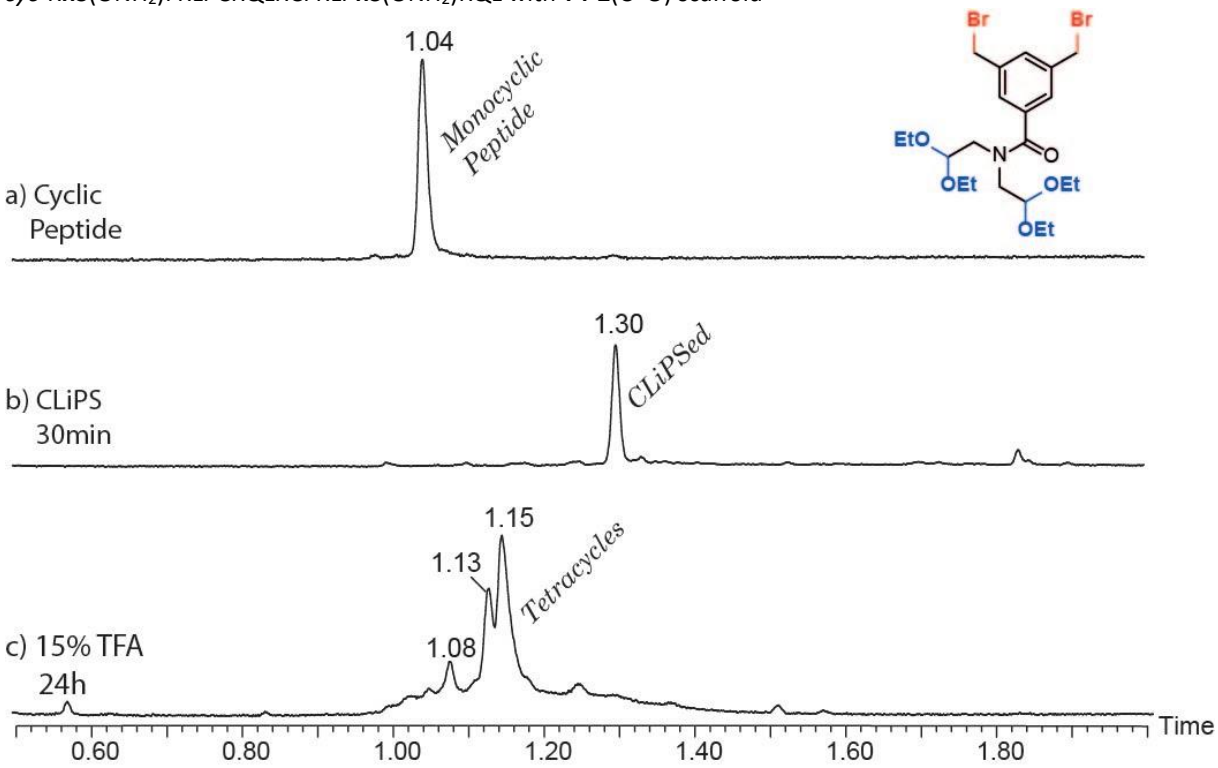
cyc-CYKQ**hS**(ONH_2)SIK**hS**(ONH_2)AKGCSKL with he **T4-3**(C=O) scaffold



Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocylic peptide	0.59	1771.91	1771.46	$[M+H]^+$
				886.64	886.23	$[M+2H]^{2+}$
				591.58	591.15	$[M+3H]^{3+}$
b	CLIPS reaction	CLIPSed	1.01	1132.73	1132.53	$[M+2H]^{2+}$
				740.24	740.33	$[M-OEt+3H]^{3+}$
c	Oxime ligation	Tetracycle	0.70	1040.40	1040.40	$[M+2H]^{2+}$
				694.11	693.93	$[M+3H]^{3+}$
		Tetracycle	0.72	1040.48	1040.40	$[M+2H]^{2+}$
				694.11	693.93	$[M+3H]^{3+}$

8.1.3 $c4_{4444}$-hS(ONH_2)•T4-2(C=O)$^{c/o}$

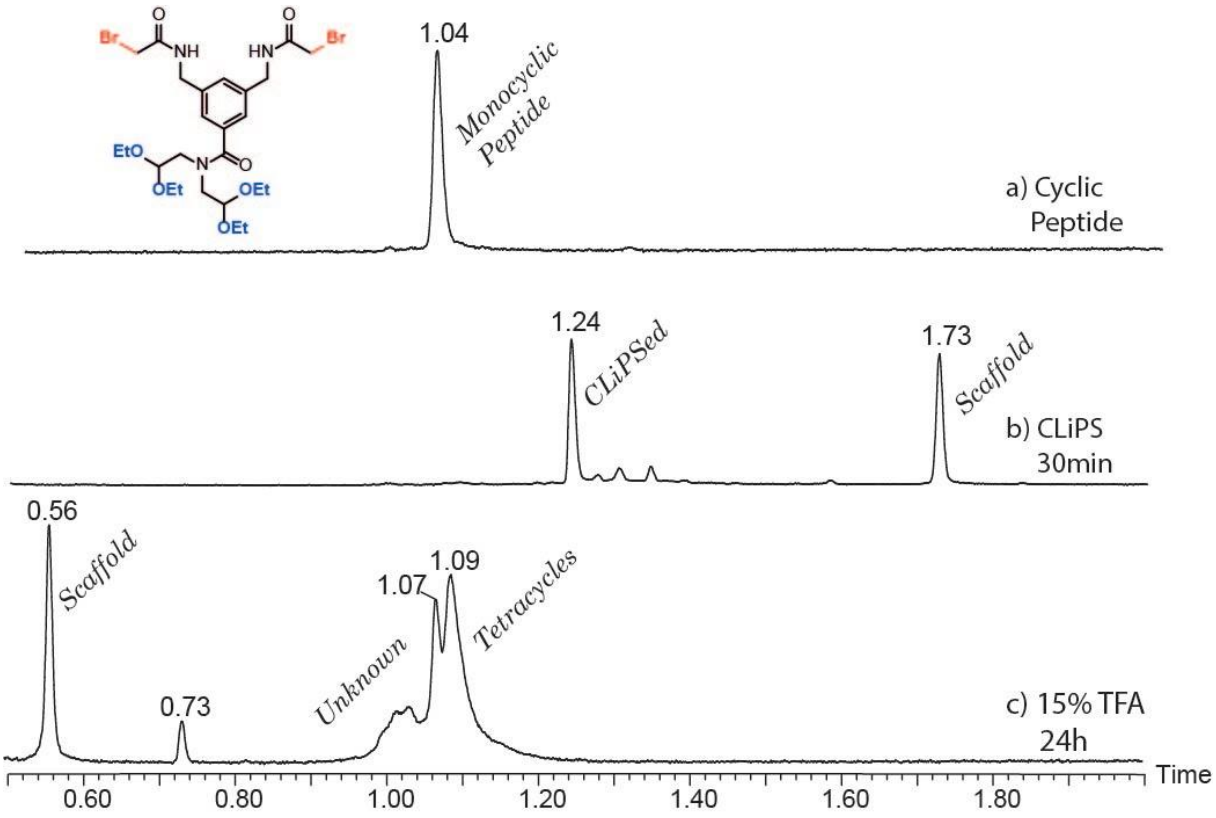
cyc-R**hS**(ONH_2)FRLPCRQLRCFRLP**hS**(ONH_2)RQL with **T4-2**(C=O) scaffold



Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocylic peptide	1.04	859.04	859.18	$[M+3H]^{3+}$
				644.76	644.63	$[M+4H]^{4+}$
				516.13	515.91	$[M+5H]^{5+}$
b	CLIPS reaction	CLIPSed	1.30	984.82	985.01	$[M+3H]^{3+}$
				738.96	739.01	$[M+4H]^{4+}$
				583.60	582.40	$[M-OEt+5H]^{5+}$
c	Oxime ligation	Unknown	1.08			
		Tetracycle	1.13	923.55	923.59	$[M+3H]^{3+}$
				692.91	692.95	$[M+4H]^{4+}$
				554.68	554.56	$[M+5H]^{5+}$
		Tetracycle	1.15	923.47	923.59	$[M+3H]^{3+}$
				692.92	692.95	$[M+4H]^{4+}$
				554.53	554.56	$[M+5H]^{5+}$

8.1.4 $c4_{4444}$-hS(ONH_2)•T4-3(C=O)$^{c/o}$

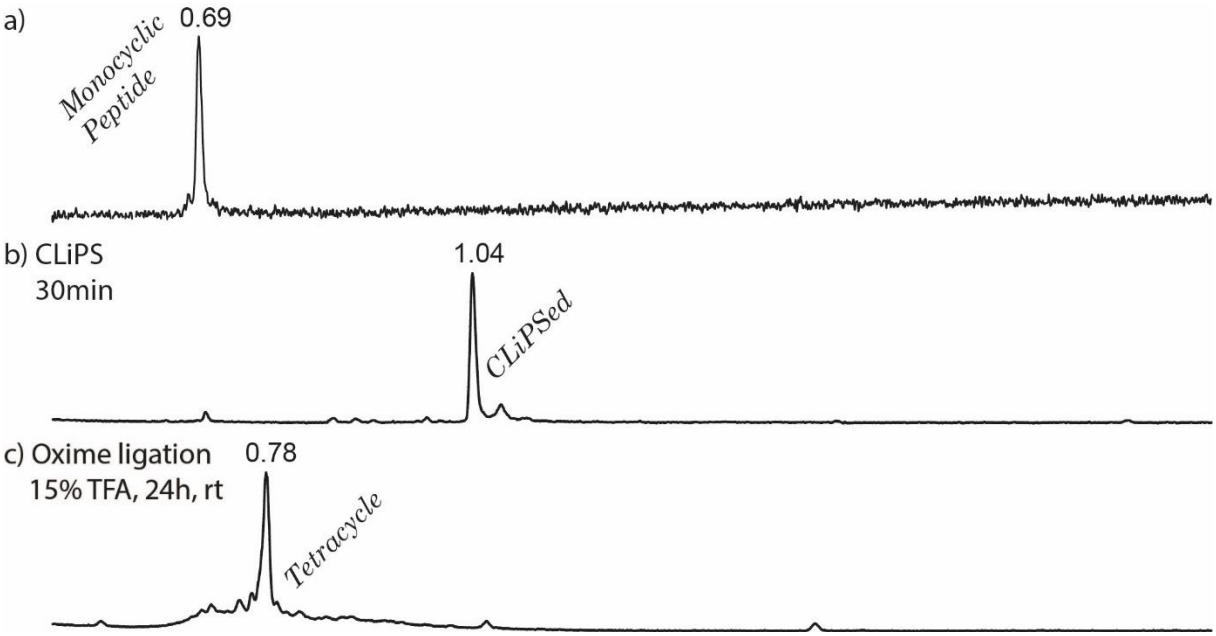
cyc-R**hS**(ONH_2)FRLPCRQLRCFRLP**hS**(ONH_2)RQL with **T4-3**(C=O) scaffold



Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocylic peptide	1.04	859.04	859.18	$[M+3H]^{3+}$
				644.76	644.63	$[M+4H]^{4+}$
				516.13	515.91	$[M+5H]^{5+}$
b	CLIPS reaction	CLIPSed	1.24	1533.48	1533.56	$[M+2H]^{2+}$
				1023.00	1023.04	$[M+3H]^{3+}$
				767.61	767.54	$[M+4H]^{4+}$
		scaffold	1.73	608.53	608.10	$[M-OEt+H]^{+}$
c	Oxime ligation	Scaffold	0.56			
		Unknown	0.73			
		Tetracycle	1.07	961.65	961.62	$[M+3H]^{3+}$
				721.41	721.47	$[M+4H]^{4+}$
				578.00	577.57	$[M+5H]^{5+}$
		Tetracycle	1.09	961.57	961.62	$[M+3H]^{3+}$
				721.64	721.47	$[M+4H]^{4+}$
				576.88	577.57	$[M+5H]^{5+}$

8.1.5 c6$_{5555}$-hS(ONH$_2$)•T4-2(C=O)$^{c/o}$

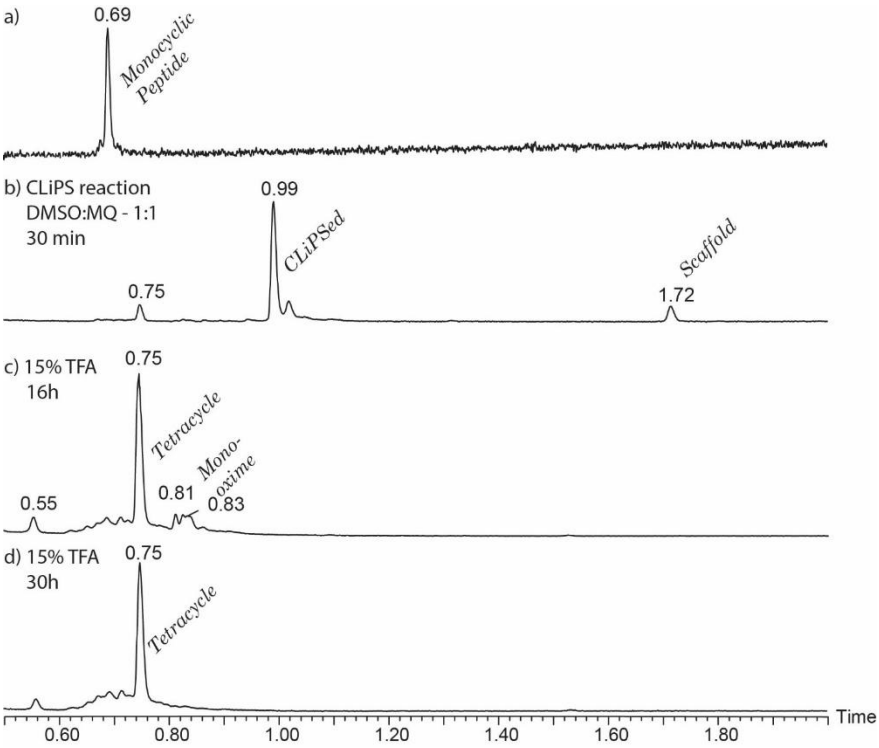
cyc-CYKGKQ**hS**(ONH$_2$)SIKAS**hS**(ONH$_2$)AKVRGCKFSKL with **T4-2**(C=O) scaffold



Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocylic peptide	0.69		2645.62	$[M+H]^{+}$
				1325.27	1325.31	$[M+2H]^{2+}$
				883.49	883.54	$[M+3H]^{3+}$
				662.91	662.66	$[M+4H]^{4+}$
				530.38	530.12	$[M+5H]^{5+}$
b	CLIPS reaction	CLIPSed	1.04	1512.63	1512.56	$[M+2H]^{2+}$
				1008.98	1008.71	$[M+3H]^{3+}$
				722.46	722.49	$[M-2OEt-H_2O+3H]^{3+}$
				578.45	578.19	$[M-2OEt-H_2O+4H]^{4+}$
c	Oxime ligation	Tetracycle	0.78	1420.45	1420.43	$[M+2H]^{2+}$
				947.62	947.29	$[M+3H]^{3+}$
				710.99	710.72	$[M+4H]^{4+}$

8.1.6 c6$_{5555}$-hS(ONH$_2$)•T4-3(C=O)$^{c/o}$

cyc-CYKGKQ**hS**(ONH$_2$)SIKAS**hS**(ONH$_2$)AKVRGCKFSKL with **T4-3**(C=O) scaffold

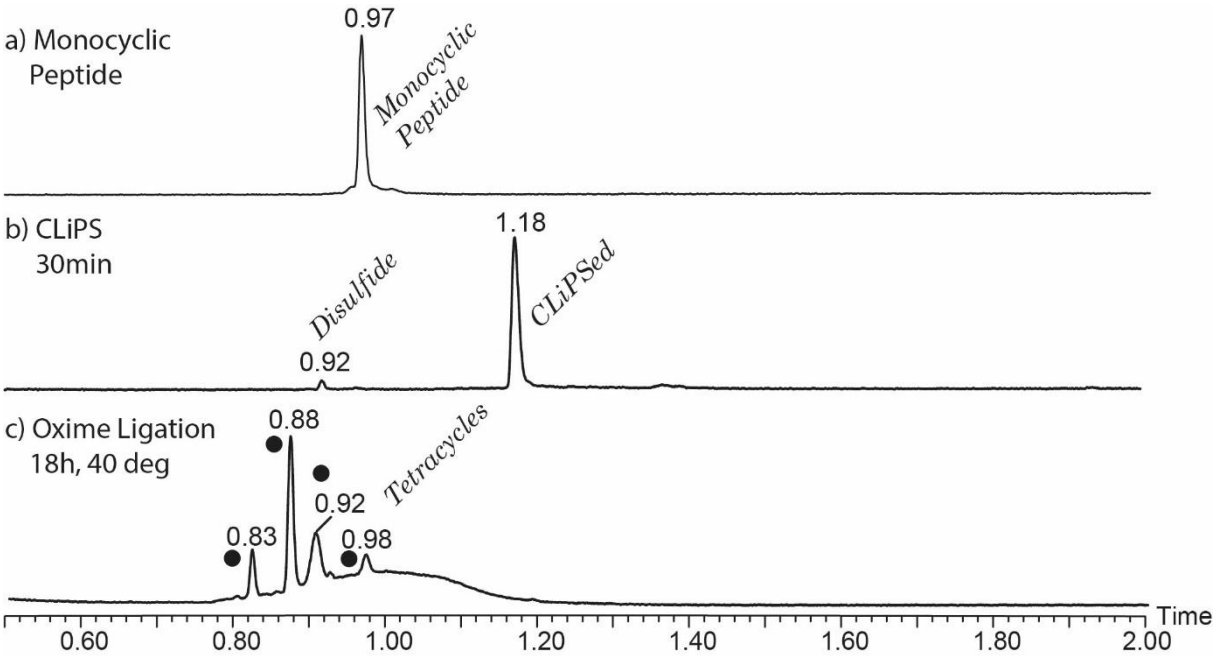


Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocylic peptide	0.69		2645.62	$[M+H]^{+}$
				1325.27	1325.31	$[M+2H]^{2+}$
				883.49	883.54	$[M+3H]^{3+}$
				662.91	662.66	$[M+4H]^{4+}$
				530.38	530.12	$[M+5H]^{5+}$
b	CLIPS reaction	CLIPSed	0.99	1569.85	1569.61	$[M+3H]^{3+}$
				1047.00	1046.74	$[M+4H]^{4+}$
				1016.06	1016.03	$[M-2OEt-H_2O+3H]^{3+}$
			1.72	606.58	608.10	$[M-OEt+H]^{+}$
c	Oxime ligation	Scaffold	0.55	506.30	506.17	
		Tetracycle	0.75	1477.67	1477.48	$[M+2H]^{2+}$
				985.43	985.31	$[M+3H]^{3+}$
				739.49	739.24	$[M+4H]^{4+}$
				591.88	591.60	$[M+5H]^{5+}$
		Mono-oxime	0.83	1523.95	1523.54	$[M+2H]^{2+}$
				1016.25	1016.03	$[M+3H]^{3+}$
				762.44	762.27	$[M+4H]^{4+}$
				739.56	739.24	$[M-2OEt-H_2O+4H]^{4+}$
d	Oxime ligation	Tetracycle	0.75	1477.30	1477.48	$[M+2H]^{2+}$
				985.57	985.31	$[M+3H]^{3+}$
				739.41	739.24	$[M+4H]^{4+}$
				591.35	591.60	$[M+5H]^{5+}$

8.2 Tetracycles of F(C=O) Peptides

8.2.1 $c2_{3333}$-F(C=O)•T4-1$(ONH_2)^{C/O}$

cyc-CYKQ**F**(C=O)SIK**F**(C=O)AKGCSKL with **T4-1**(ONH_2) scaffold

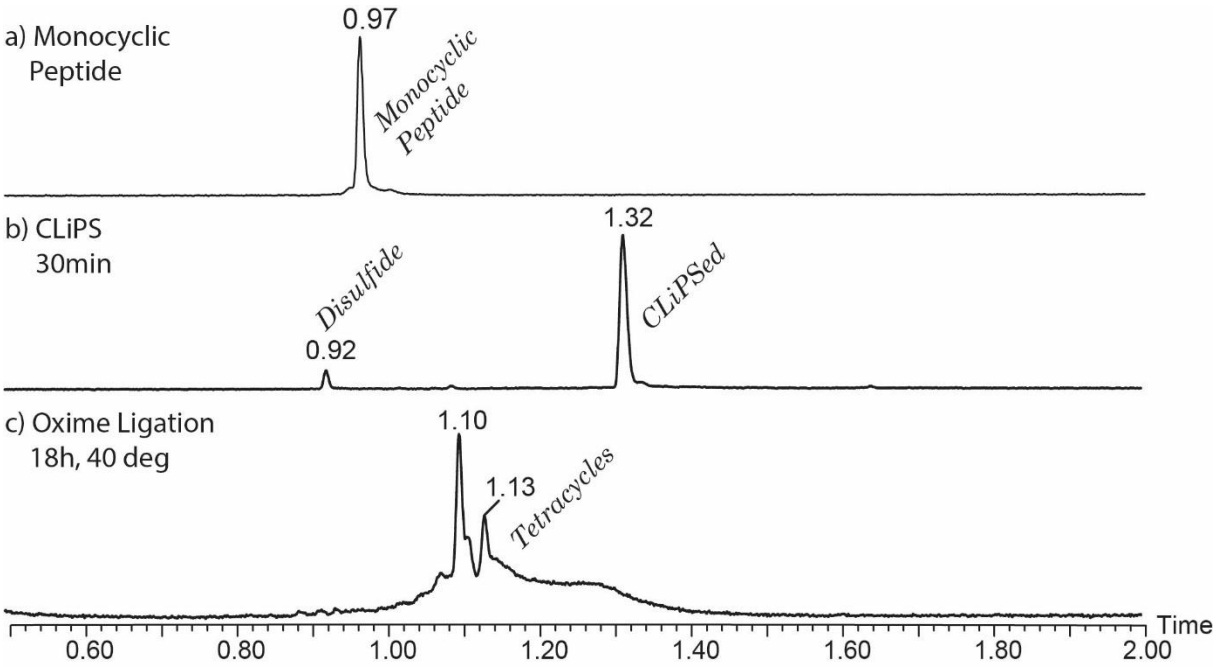


• = isomers

Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocyclic peptide	0.97	1918.32	1918.64	$[M+H]^+$
				959.70	959.32	$[M+2H]^{2+}$
				640.26	639.88	$[M+3H]^{3+}$
b	CLIPS reaction	CLIPSed	1.18	1191.02	1191.11	$[M+2H]^{2+}$
				794.39	794.07	$[M+3H]^{3+}$
				760.94	760.72	$[M-Boc+2H]^{3+}$
				727.49	727.37	$[M-2Boc+3H]^{3+}$
c	Oxime ligation	Tetracycle	0.83	1072.88	1073.04	$[M+2H]^{2+}$
				715.49	715.70	$[M+3H]^{3+}$
		Tetracycle	0.88	1072.58	1073.04	$[M+2H]^{2+}$
				715.49	715.70	$[M+3H]^{3+}$
		Tetracycle	0.92	1072.50	1073.04	$[M+2H]^{2+}$
				715.64	715.70	$[M+3H]^{3+}$
		Tetracycle	0.98	1072.73	1073.04	$[M+2H]^{2+}$
				715.41	715.70	$[M+3H]^{3+}$

8.2.2 $c2_{3333}$-F(C=O)•T4-2(ONH_2)$^{C/O}$

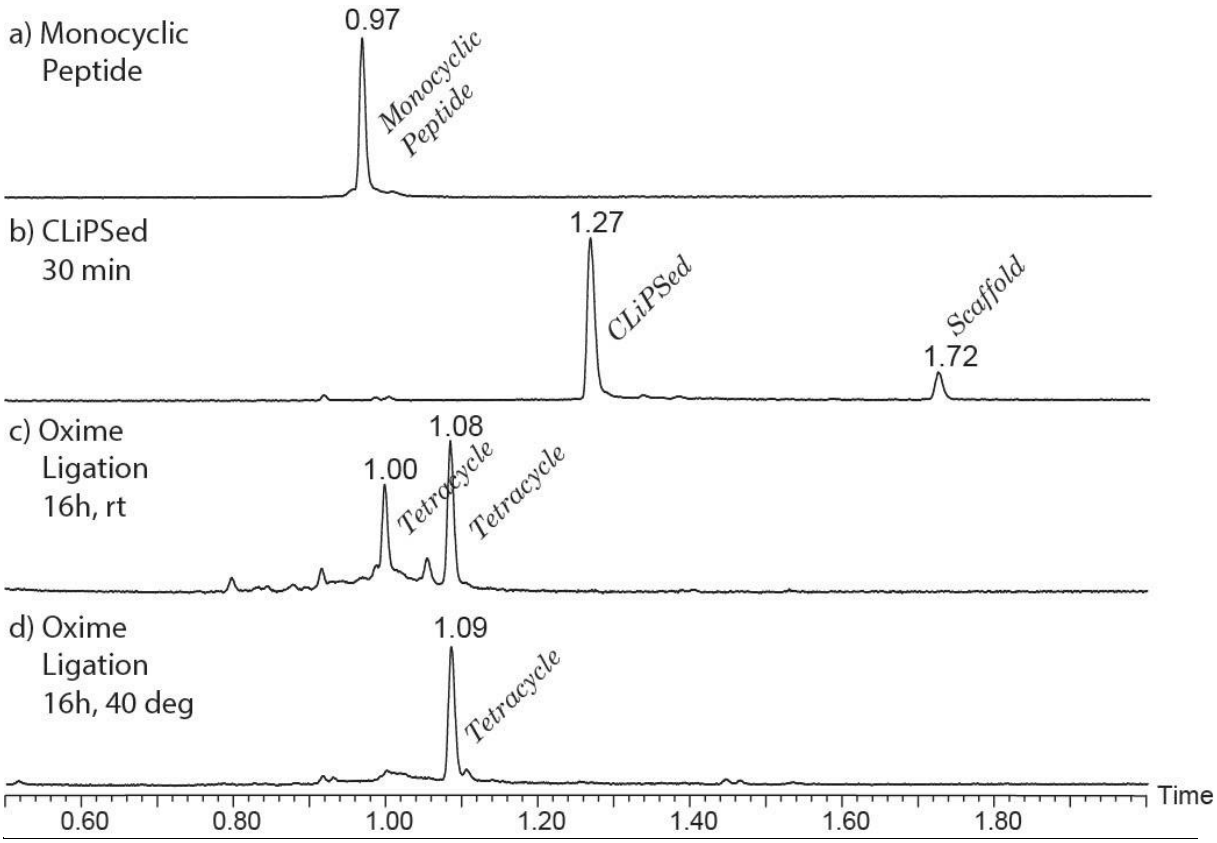
cyc-CYKQ**F**(C=O)SIK**F**(C=O)AKGCSKL with **T4-2**(ONH_2) scaffold



Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocyclic peptide	0.97	1918.32	1918.64	$[M+H]^{+}$
				959.70	959.32	$[M+2H]^{2+}$
				640.26	639.88	$[M+3H]^{3+}$
b	CLIPS reaction	Disulfide	0.92	959.10	959.82	$[M+2H]^{2+}$
				640.11	640.22	$[M+3H]^{3+}$
		CLIPSed	1.32	1191.83	1191.59	$[M+2H]^{2+}$
				794.76	794.40	$[M+3H]^{3+}$
				761.54	761.05	$[M-Boc+2H]^{3+}$
				728.24	727.70	$[M-2Boc+3H]^{3+}$
c	Oxime ligation	Tetracycle	1.10	1073.85	1073.52	$[M+2H]^{2+}$
		Tetracycle	1.11	716.09	716.02	$[M+3H]^{3+}$
				1074.00	1073.52	$[M+2H]^{2+}$
		Tetracycle	1.12	716.09	716.02	$[M+3H]^{3+}$
				1073.25	1073.52	$[M+2H]^{2+}$
				716.16	716.02	$[M+3H]^{3+}$

8.2.3 $c2_{3333}$-F(C=O)•T4-3$(ONH_2)^{C/O}$

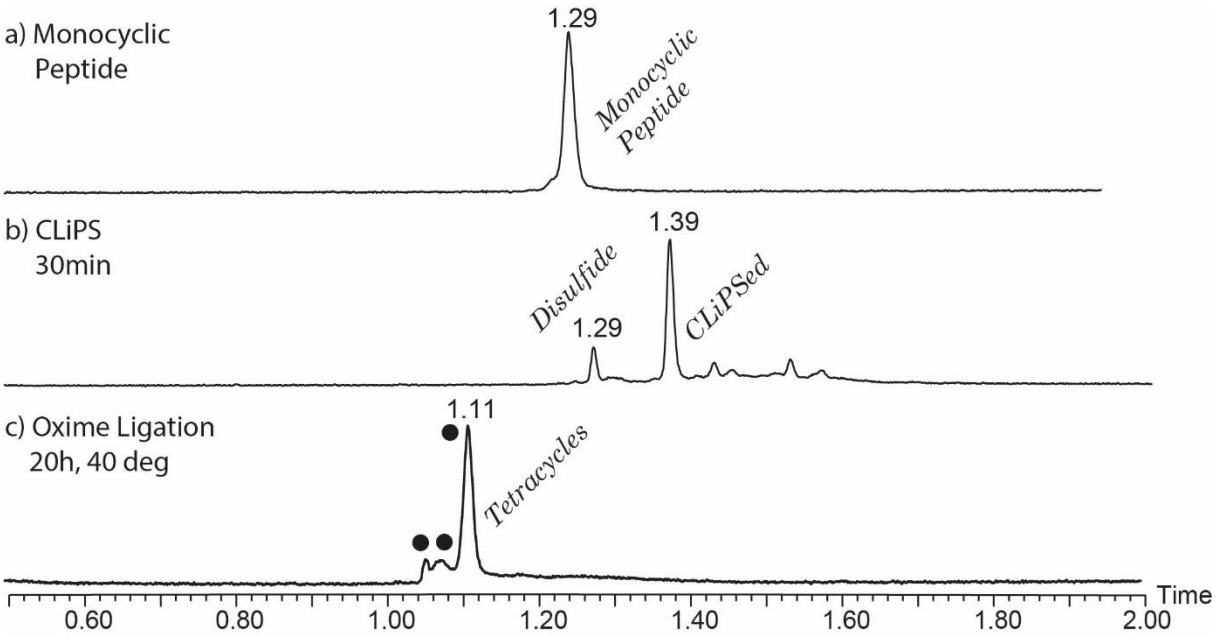
cyc-CYKQ**F**(C=O)SIK**F**(C=O)AKGCSKL with **T4**-**3**(ONH_2) scaffold



Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocyclic peptide	0.97	1918.32	1918.64	$[M+H]^+$
				959.70	959.32	$[M+2H]^{2+}$
				640.26	639.88	$[M+3H]^{3+}$
b	CLIPS reaction	CLIPSed	1.27	1248.91	1248.64	$[M+2H]^{2+}$
				833.02	832.43	$[M+3H]^{3+}$
				799.64	799.08	$[M-Boc+2H]^{3+}$
				766.26	765.73	$[M-2Boc+3H]^{3+}$
		Scaffold	1.72	540.43	540.23	$[M-2Boc+2H]^{2+}$
c	Oxime ligation	Tetracycle	1.00	1130.48	1130.58	$[M+2H]^{2+}$
				754.34	753.72	$[M+3H]^{3+}$
		Tetracycle	1.06	1130.56	1130.58	$[M+2H]^{2+}$
				754.19	753.72	$[M+3H]^{3+}$
		Tetracycle	1.09	1130.56	1130.58	$[M+2H]^{2+}$
				754.19	753.72	$[M+3H]^{3+}$
d	Oxime ligation	Tetracycle	1.09	1130.78	1130.58	$[M+2H]^{2+}$
				754.19	753.72	$[M+3H]^{3+}$

8.2.4 $c5_{4444}$-F(C=O)•T4-1$(ONH_2)^{C/O}$

cyc-R**F**(C=O)FRLPCRQLRCFRLP**F**(C=O)RQL with **T4-1**(ONH_2) scaffold

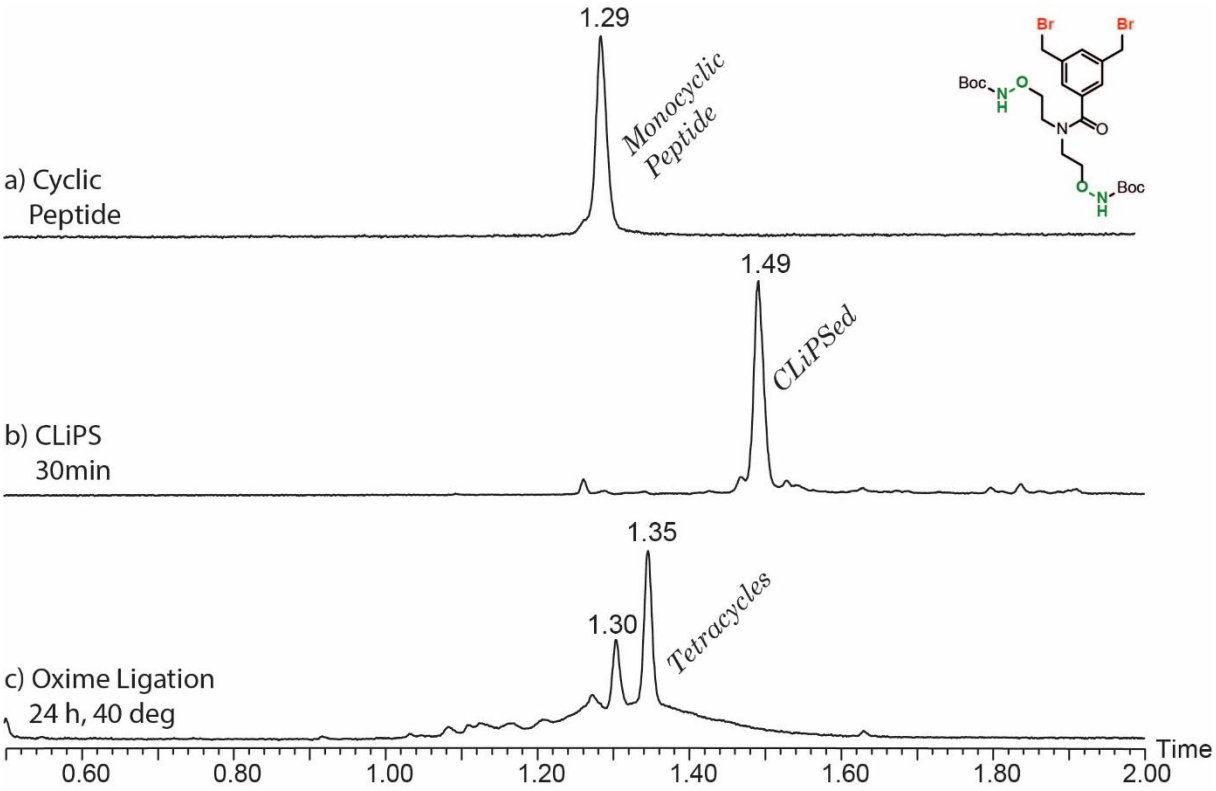


• = isomers

Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocyclic peptide	1.29	907.80	907.90	$[M+3H]^{3+}$
				681.21	681.18	$[M+4H]4^{+}$
b	CLIPS reaction	Disulfide	1.29	906.97	907.90	$[M+3H]^{3+}$
				681.06	681.18	$[M+4H]^{4+}$
		CLIPSed	1.39	1061.78	1061.43	$[M+3H]^{3+}$
				796.64	796.88	$[M+4H]^{4+}$
				597.58	597.84	$[M-2Boc+5H]^{5+}$
c	Oxime ligation	Tetracycle	1.05	983.09	983.05	$[M+3H]^{3+}$
				737.58	737.85	$[M+4H]^{4+}$
				590.47	590.24	$[M+5H]^{5+}$
		Tetracycle	1.06	982.70	983.05	$[M+3H]^{3+}$
				737.51	737.85	$[M+4H]^{4+}$
				589.76	590.24	$[M+5H]^{5+}$
		Tetracycle	1.07	982.77	983.05	$[M+3H]^{3+}$
				737.58	737.85	$[M+4H]^{4+}$
				590.54	590.24	$[M+5H]^{5+}$
		Tetracycle	1.11	982.90	983.05	$[M+3H]^{3+}$
				737.71	737.85	$[M+4H]^{4+}$
				589.82	590.24	$[M+5H]^{5+}$

8.2.5 $c5_{4444}$-F(C=O)•T4-2$(ONH_2)^{C/O}$

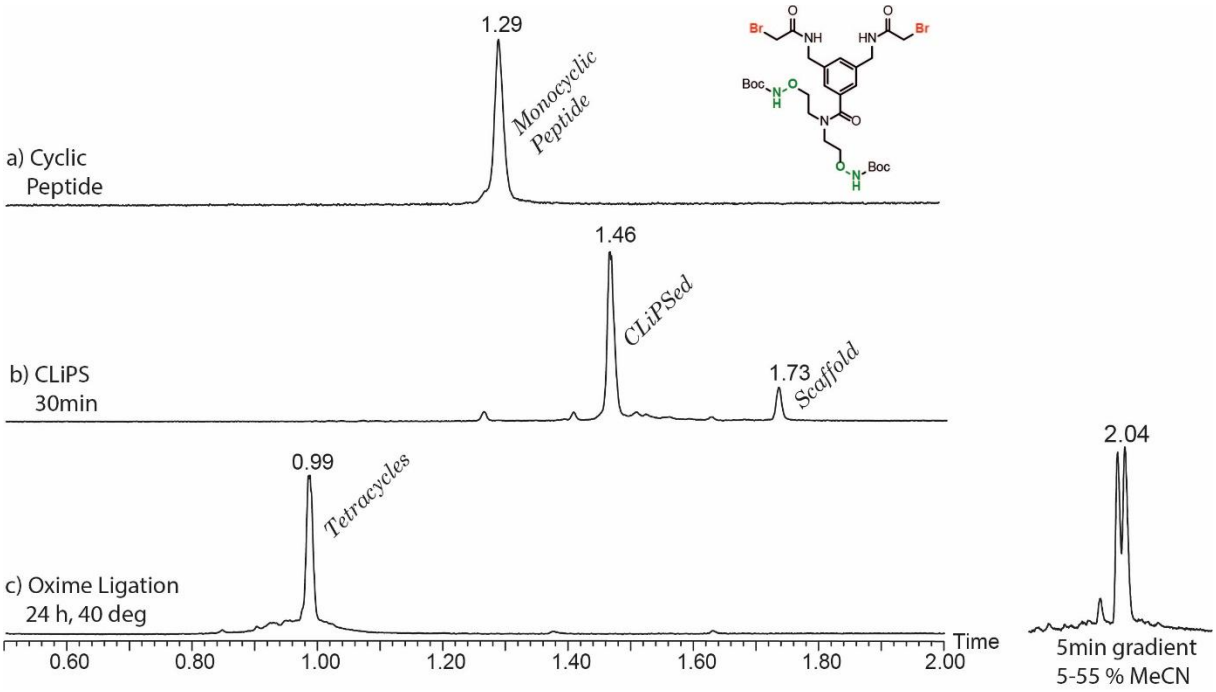
cyc-R**F**(C=O)FRLPCRQLRCFRLP**F**(C=O)RQL with **T4-2**(ONH_2) scaffold



Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocyclic peptide	1.29	907.80	907.90	$[M+3H]^{3+}$
				681.21	681.18	$[M+4H]4^{+}$
b	CLIPS reaction	CLIPSed	1.49	1061.38	1061.75	$[M+3H]^{3+}$
				797.01	797.12	$[M+4H]^{4+}$
				771.96	772.11	$[M-Boc+3H]^{4+}$
				747.06	747.10	$[M-2Boc+5H]^{5+}$
				597.80	598.04	$[M-2Boc+2H]^{2+}$
c	Oxime ligation	Tetracycle	1.30	983.62	983.71	$[M+3H]^{3+}$
				738.66	738.34	$[M+4H]^{4+}$
				591.05	590.63	$[M+5H]^{5+}$
		Tetracycle	1.35	983.47	983.71	$[M+3H]^{3+}$
				738.06	738.34	$[M+4H]^{4+}$
				590.68	590.63	$[M+5H]^{5+}$

8.2.6 $c5_{4444}$-F(C=O)•T4-3$(ONH_2)^{C/O}$

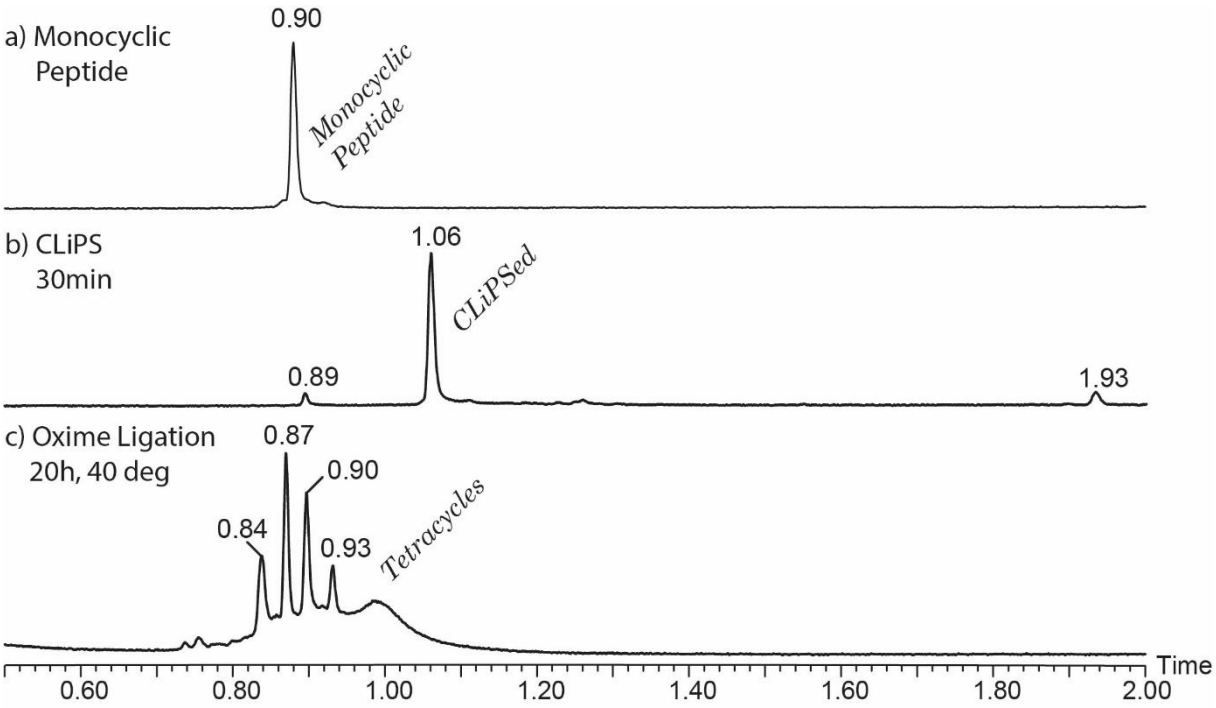
cyc-R**F**(C=O)FRLPCRQLRCFRLP**F**(C=O)RQL with **T4-3**(ONH_2) scaffold



Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocyclic peptide	1.29	907.80	907.90	$[M+3H]^{3+}$
				681.21	681.18	$[M+4H]^{4+}$
b	CLIPS reaction	CLIPSed	1.46	1100.33	1100.12	$[M+3H]^{3+}$
				825.59	825.65	$[M+4H]^{4+}$
				800.39	800.63	$[M-Boc+3H]^{4+}$
				620.83	620.86	$[M-2Boc+5H]^{5+}$
		Scaffold	1.73	540.43	540.23	$[M-2Boc+2H]^{2+}$
c	Oxime ligation	Tetracycle	0.99	1022.49	1022.08	$[M+3H]^{3+}$
				767.09	766.86	$[M+4H]^{4+}$
				613.42	613.45	$[M+5H]^{5+}$
		Tetracycle	0.99	1022.29	1022.08	$[M+3H]^{3+}$
				767.02	766.86	$[M+4H]^{4+}$
				613.88	613.45	$[M+5H]^{5+}$

8.2.7 $c7_{5555}$-F(C=O)•T4-1$(ONH_2)^{C/O}$

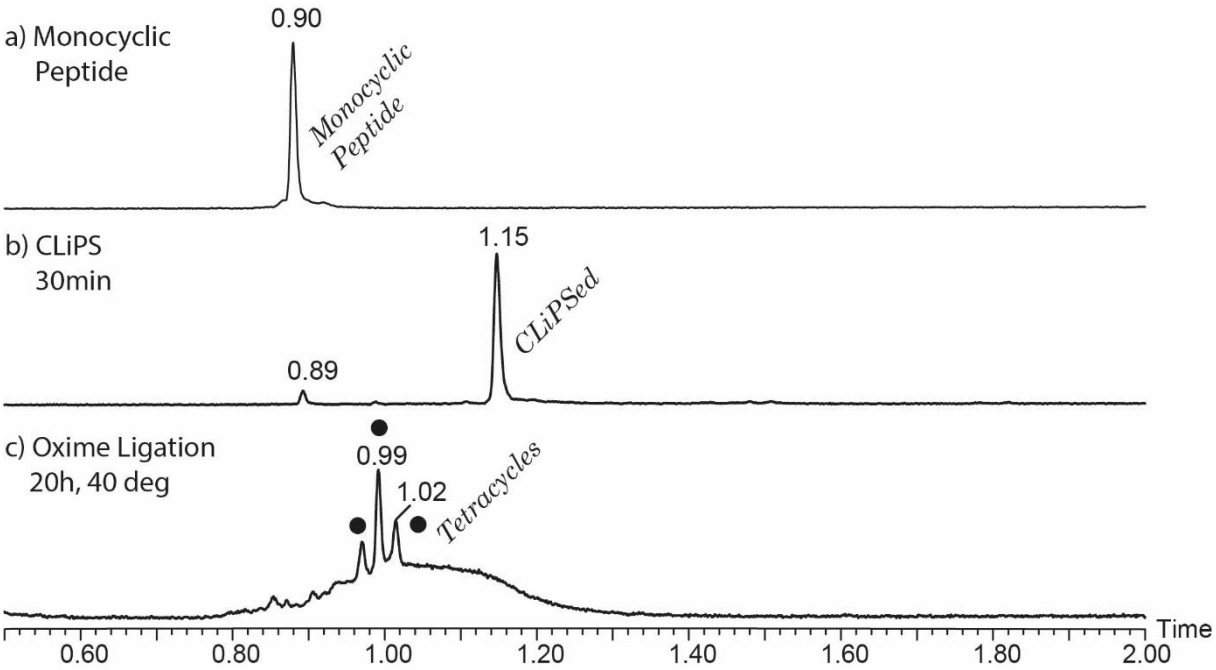
cyc-CYKGKQ**F**(C=O)SIKAS**F**(C=O)AKVRGCKFSKL with **T4-1**(ONH_2) scaffold



Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocyclic peptide	0.90		2791.80	$[M+H]^{+}$
				1397.57	1397.40	$[M+2H]^{2+}$
				931.95	931.60	$[M+3H]^{3+}$
				699.21	699.20	$[M+4H]^{4+}$
b	CLIPS reaction	CLIPSed	1.06	1628.28	1628.19	$[M+2H]^{2+}$
				1085.70	1085.46	$[M+3H]^{3+}$
				814.57	814.65	$[M+4H]^{4+}$
				790.04	789.89	$[M-2Boc+4H]^{4+}$
				612.43	612.06	$[M-2Boc+5H]^{5+}$
		Scaffold	1.93	624.66	624.39	$[M+H]^{+}$
c	Oxime ligation	Tetracycle	0.84	1007.10	1007.08	$[M+3H]^{3+}$
				755.39	755.62	$[M+4H]^{4+}$
		Tetracycle	0.87	1006.73	1007.08	$[M+3H]^{3+}$
				755.24	755.62	$[M+4H]^{4+}$
		Tetracycle	0.90	1006.88	1007.08	$[M+3H]^{3+}$
				755.46	755.62	$[M+4H]^{4+}$
		Tetracycle	0.93	1007.03	1007.08	$[M+3H]^{3+}$
				755.69	755.62	$[M+4H]^{4+}$

8.2.8 $c7_{5555}$-F(C=O•T4-2(ONH_2)$^{c/o}$

cyc-CYKGKQ**F**(C=O)SIKAS**F**(C=O)AKVRGCKFSKL with **T4-2**(ONH_2) scaffold

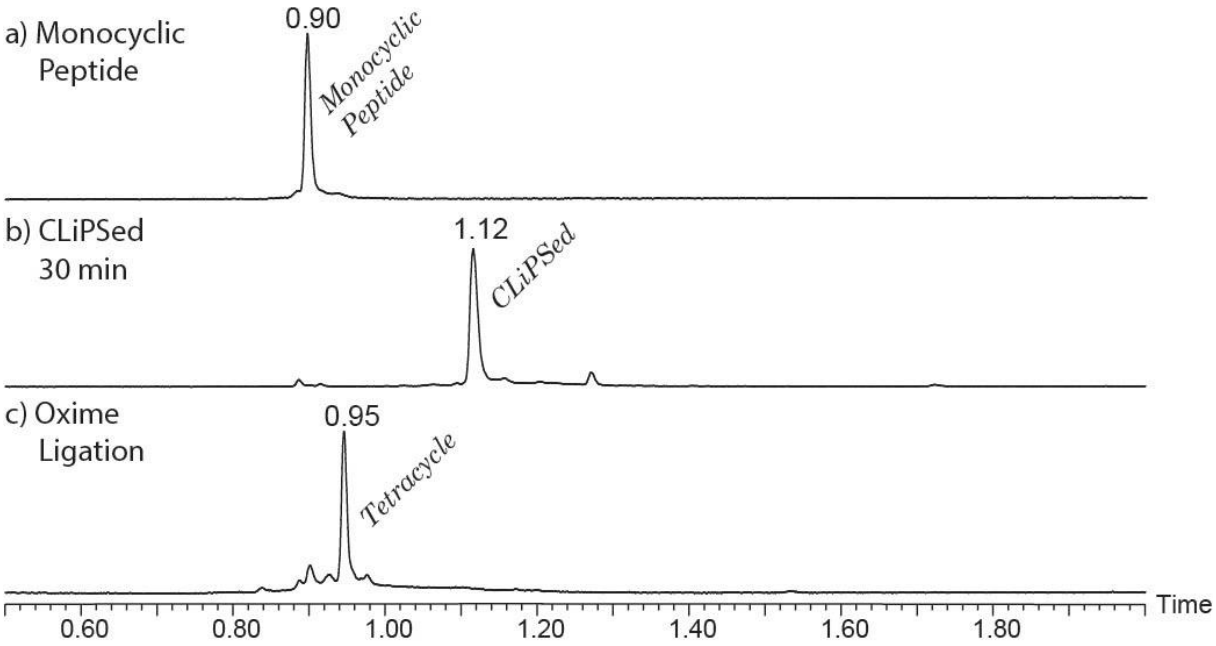


• = isomers

Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocyclic peptide	0.90		2791.80	$[M+H]^{+}$
				1397.57	1397.40	$[M+2H]^{2+}$
				931.95	931.60	$[M+3H]^{3+}$
				699.21	699.20	$[M+4H]^{4+}$
b	CLIPS reaction	CLIPSed	1.15	1628.66	1628.67	$[M+2H]^{2+}$
				1086.30	1086.12	$[M+3H]^{3+}$
				815.02	814.89	$[M+4H]^{4+}$
				765.06	764.87	$[M-2Boc+4H]^{4+}$
				611.91	612.26	$[M-2Boc+5H]^{5+}$
c	Oxime ligation	Tetracycle	0.96	1007.63	1007.41	$[M+3H]^{3+}$
				756.51	756.11	$[M+4H]^{4+}$
		Tetracycle	0.99	1007.70	1007.41	$[M+3H]^{3+}$
				756.44	756.11	$[M+4H]^{4+}$
		Tetracycle	1.02	1007.55	1007.41	$[M+3H]^{3+}$
				756.06	756.11	$[M+4H]^{4+}$

8.2.9 c7$_{5555}$-F(C=O)•T4-3(ONH$_2$)$^{C/O}$

cyc-CYKGKQ**F**(C=O)SIKAS**F**(C=O)AKVRGCKFSKL with **T4-3**(ONH$_2$) scaffold

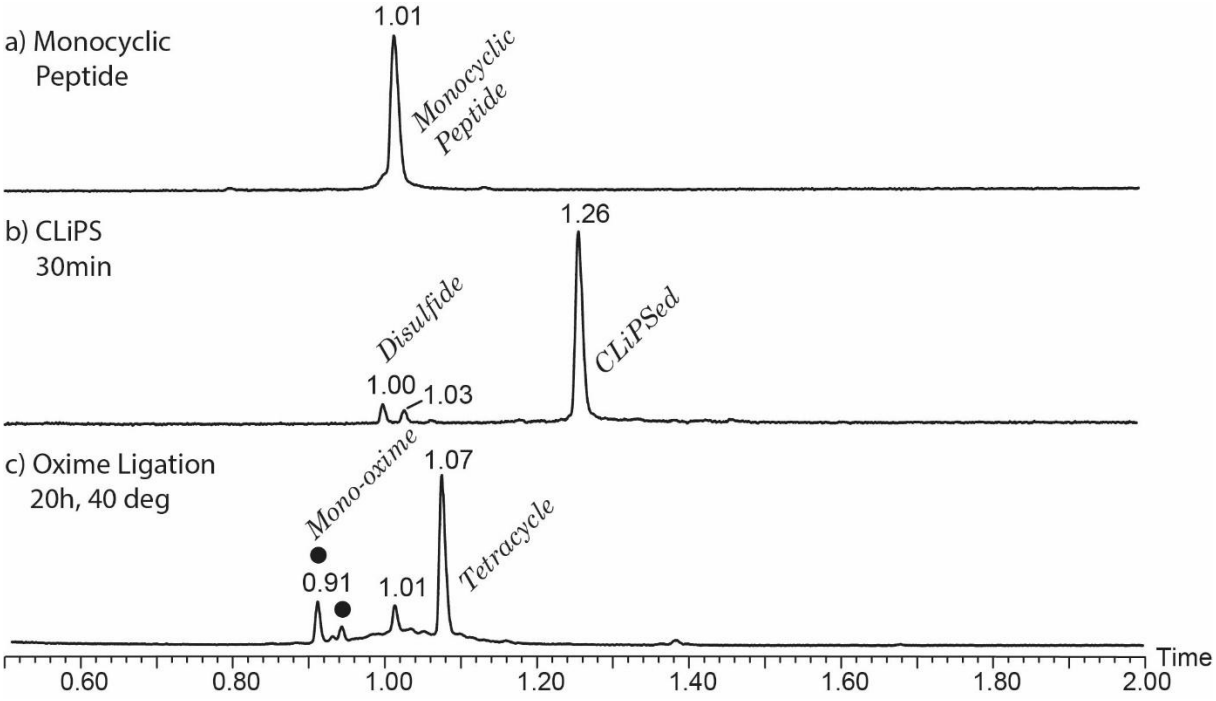


Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a	sm	Monocyclic peptide	0.90		2791.80	$[M+H]^{+}$
				1397.57	1397.40	$[M+2H]^{2+}$
				931.95	931.60	$[M+3H]^{3+}$
				699.21	699.20	$[M+4H]^{4+}$
b	CLIPS reaction	CLIPSed	1.12	1686.18	1686.22	$[M+3H]^{3+}$
				1124.41	1124.48	$[M+4H]^{4+}$
				818.69	818.41	$[M-Boc+4H]^{4+}$
				793.71	793.39	$[M-2Boc+4H]^{4+}$
c	Oxime ligation	Tetracycle	0.95	1567.23	1567.65	$[M+2H]^{2+}$
				1045.58	1045.44	$[M+3H]^{3+}$
				784.41	784.39	$[M+4H]^{4+}$

8.3 Cyclization results for D(C=O) peptides

8.3.1 $c3_{3333}$- D (C=O)•T4-1$(ONH_2)^{c/o}$

cyc-CYKQ**D**(C=O)SIK**D**(C=O)AKGCSKL with **T4-1**(ONH_2) scaffold

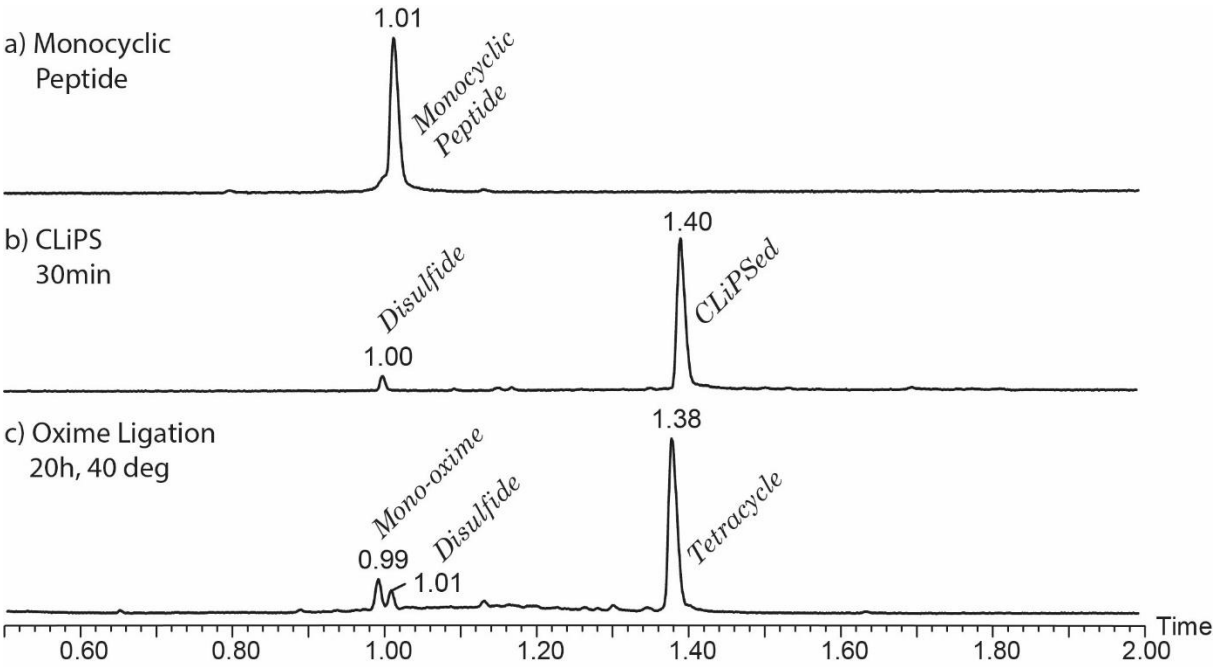


• = mono-oxime

Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocyclic peptide	1.01		1964.71	$[M+H]^{+}$
				983.02	983.85	$[M+2H]^{2+}$
				655.86	655.90	$[M+3H]^{3+}$
b	CLIPS reaction	Disulfide	1.00	982.65	982.36	$[M+2H]^{2+}$
				655.56	655.24	$[M+3H]^{3+}$
		CLIPSed	1.26	1214.04	1214.64	$[M+2H]^{2+}$
				809.69	809.76	$[M+3H]^{3+}$
				776.31	776.41	$[M-Boc+3H]^{3+}$
				557.60	557.85	$[M-2Boc+4H]^{4+}$
c	Oxime ligation	Mono-Oxime	0.91	1104.61	1104.58	$[M+2H]^{2+}$
				736.94	736.39	$[M+3H]^{3+}$
		Mono-Oxime	0.94	1104.23	1104.58	$[M+2H]^{2+}$
				736.86	736.39	$[M+3H]^{3+}$
		Monocycle	1.01	981.90	982.36	$[M+2H]^{2+}$
				655.18	655.24	$[M+3H]^{3+}$
		Tetracycle	1.07	1095.53	1095.57	$[M+2H]^{2+}$
				730.94	730.72	$[M+3H]^{3+}$
				548.45	548.60	$[M+4H]^{4+}$

8.3.2 $c3_{3333}$-D(C=O)•T4-2$(ONH_2)^{c/o}$

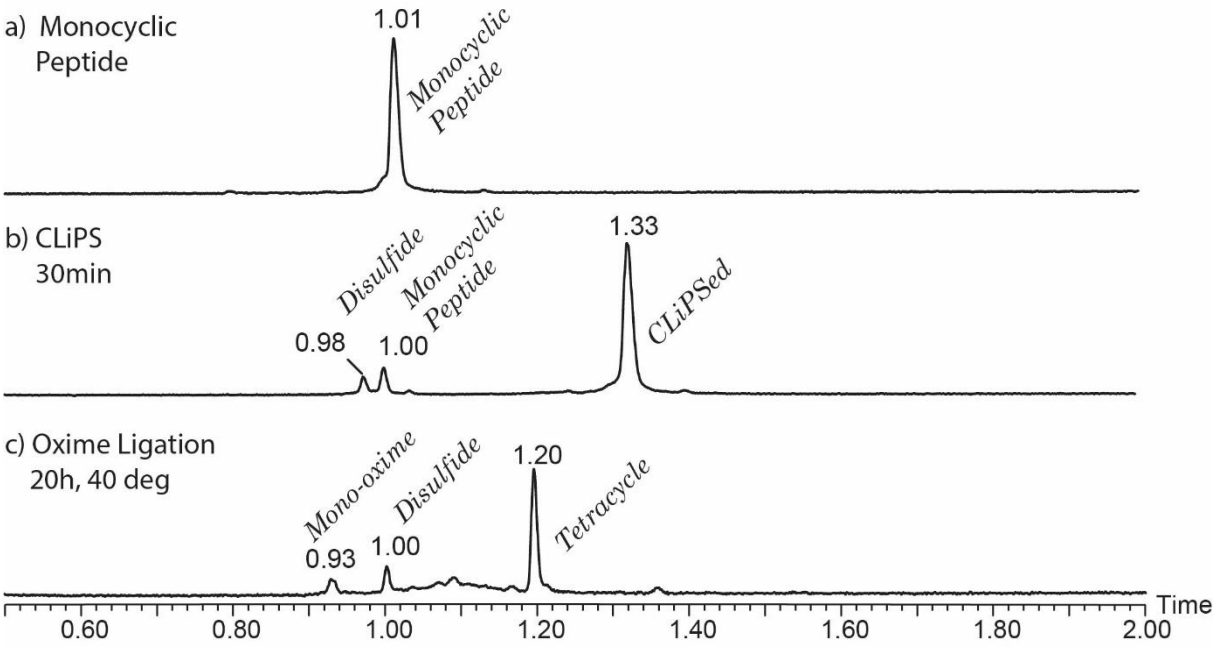
cyc-CYKQ**D**(C=O)SIK**D**(C=O)AKGCSKL with **T4-2**(ONH_2) scaffold



Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocyclic peptide	1.01		1964.71	$[M+H]^{+}$
				983.02	983.85	$[M+2H]^{2+}$
				655.86	655.90	$[M+3H]^{3+}$
b	CLIPS reaction	Disulfide	1.00	982.20	982.36	$[M+2H]^{2+}$
				655.26	655.24	$[M+3H]^{3+}$
		CLIPSed	1.40	1214.71	1214.63	$[M+2H]^{2+}$
				810.29	810.09	$[M+3H]^{3+}$
				743.61	743.39	$[M-2Boc+3H]^{3+}$
				558.20	558.10	$[M-2Boc+4H]^{4+}$
c	Oxime ligation	Mono-Oxime	0.99	1106.63	1106.07	$[M+2H]^{2+}$
				737.54	737.38	$[M+3H]^{3+}$
				553.33	553.59	$[M+4H]^{4+}$
		Tetracycle	1.38	1096.58	1096.56	$[M+2H]^{2+}$
				731.54	731.71	$[M+3H]^{3+}$

8.3.3 $c3_{3333}$-D(C=O•T4-3$(ONH_2)^{c/o}$

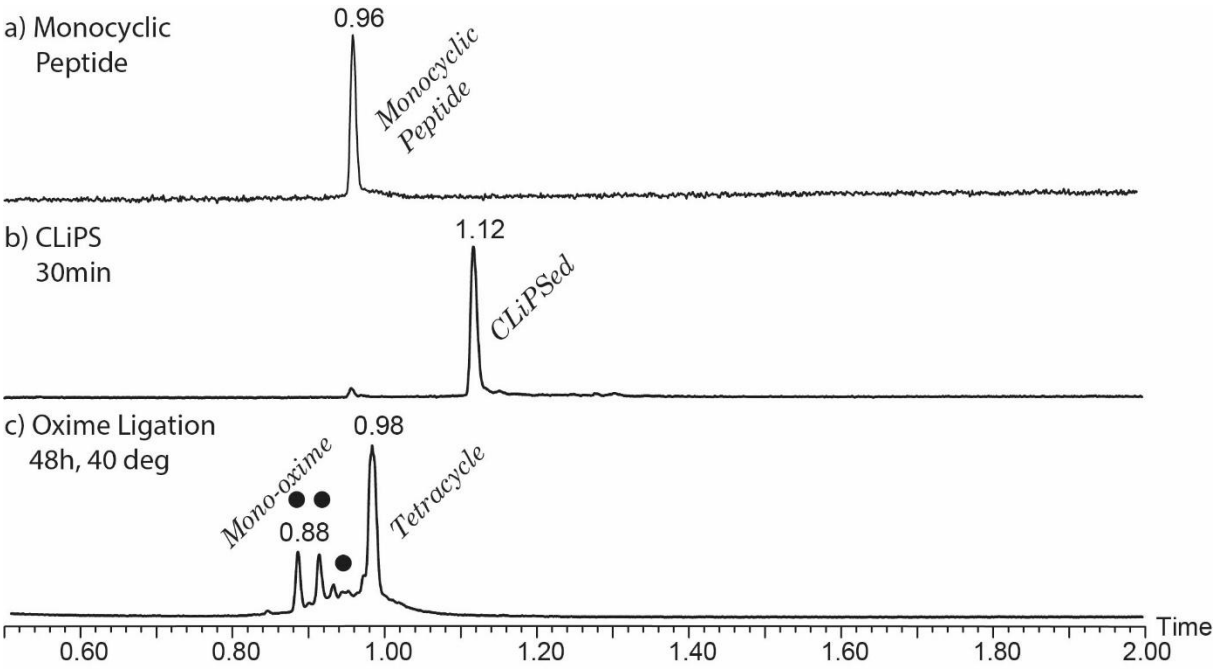
cyc-CYKQ**D**(C=O)SIK**D**(C=O)AKGCSKL with **T4-3**(ONH_2) scaffold



Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocyclic peptide	1.01		1964.71	$[M+H]^+$
				983.02	983.85	$[M+2H]^{2+}$
				655.86	655.90	$[M+3H]^{3+}$
b	CLIPS reaction	Disulfide	0.98	982.42	982.36	$[M+2H]^{2+}$
				655.33	655.24	$[M+3H]^{3+}$
		Monocycle	1.00	982.95	982.86	$[M+2H]^{2+}$
				655.71	655.91	$[M+3H]^{3+}$
		CLIPSed	1.33	1271.79	1271.68	$[M+2H]^{2+}$
				848.32	848.12	$[M+3H]^{3+}$
				814.87	814.77	$[M-Boc+3H]^{3+}$
				781.56	781.42	$[M-2Boc+3H]^{3+}$
c	Oxime ligation	Mono-Oxime	0.93	1162.58	1163.12	$[M+2H]^{2+}$
				775.56	775.42	$[M+3H]^{3+}$
		Monocycle	1.00	981.90	982.36	$[M+2H]^{2+}$
				655.11	655.24	$[M+3H]^{3+}$
		Tetracycle	1.20	1153.51	1153.61	$[M+2H]^{2+}$
				769.56	769.74	$[M+3H]^{3+}$

8.3.4 $c8_{5555}$-D(C=O)•T4-1(ONH_2)$^{c/o}$

cyc-CYKGKQ**D**(C=O)SIKAS**D**(C=O)AKVRGCKFSKL with **T4-1**(ONH_2) scaffold

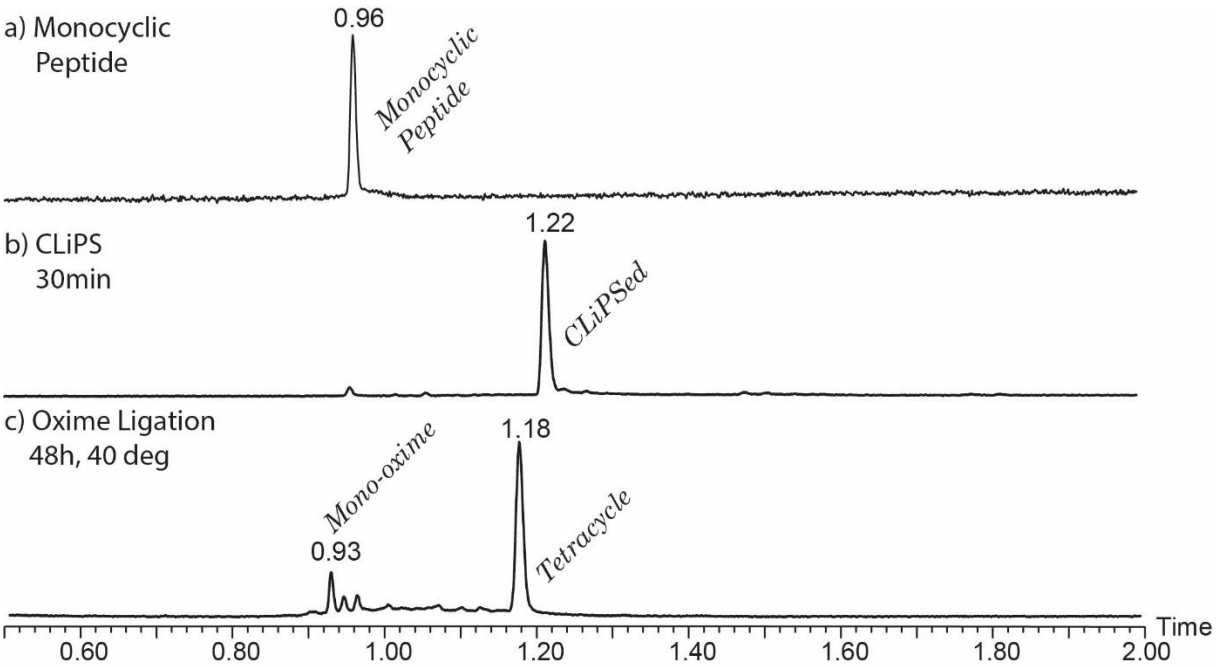


• = mono-oxime

Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocyclic peptide	0.96		2837.86	$[M+H]^{+}$
				1420.30	1420.43	$[M+2H]^{2+}$
				947.25	947.29	$[M+3H]^{3+}$
				710.69	710.72	$[M+4H]^{4+}$
				567.95	568.17	$[M+5H]^{5+}$
b	CLiPS reaction	CLiPSed	1.12	1650.86	1650.72	$[M+2H]^{2+}$
				1101.16	1101.15	$[M+3H]^{3+}$
				826.19	826.17	$[M+4H]^{4+}$
				801.07	801.15	$[M-Boc+4H]^{4+}$
				775.79	775.89	$[M-2Boc+4H]^{4+}$
c	Oxime ligation	Mono-Oxime	0.88	1541.35	1541.66	$[M+2H]^{2+}$
				1028.25	1028.11	$[M+3H]^{3+}$
				771.51	771.64	$[M+4H]^{4+}$
				618.06	617.67	$[M+5H]^{5+}$
		Mono-Oxime	0.92	1541.95	1541.66	$[M+2H]^{2+}$
				1028.18	1028.11	$[M+3H]^{3+}$
				771.89	771.64	$[M+4H]^{4+}$
		Mono-Oxime	0.93	1542.63	1541.66	$[M+2H]^{2+}$
				1028.25	1028.11	$[M+3H]^{3+}$
				771.89	771.64	$[M+4H]^{4+}$
		Tetracycle	0.98	1533.33	1533.15	$[M+2H]^{2+}$
				1022.15	1022.44	$[M+3H]^{3+}$
				767.01	767.13	$[M+4H]^{4+}$
				613.63	613.87	$[M+5H]^{5+}$

8.3.5 $c8_{5555}$-D(C=O)•T4-2(ONH_2)$^{c/o}$

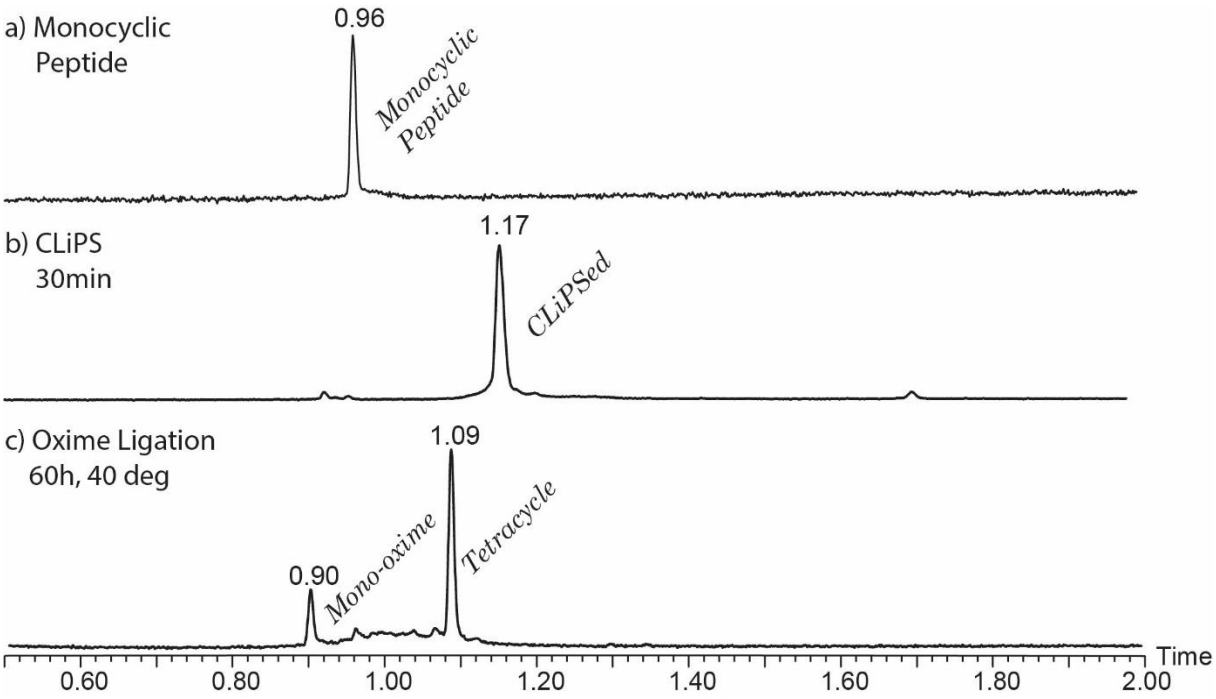
cyc-CYKGKQ**D**(C=O)SIKAS**D**(C=O)AKVRGCKFSKL with **T4-2**(ONH_2) scaffold



Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocyclic peptide	0.96		2837.86	$[M+H]^{+}$
				1420.30	1420.43	$[M+2H]^{2+}$
				947.25	947.29	$[M+3H]^{3+}$
				710.69	710.72	$[M+4H]^{4+}$
				567.95	568.17	$[M+5H]^{5+}$
b	CLIPS reaction	CLIPSed	1.22	1651.69	1651.70	$[M+2H]^{2+}$
				1101.76	1101.14	$[M+3H]^{3+}$
				826.64	826.41	$[M+4H]^{4+}$
				801.59	801.40	$[M-Boc+4H]^{4+}$
				776.61	776.38	$[M-2Boc+4H]^{4+}$
c	Oxime ligation	Mono-Oxime	0.93	1543.30	1028.77	$[M+2H]^{2+}$
				1028.93	771.88	$[M+3H]^{3+}$
				771.96	617.87	$[M+4H]^{4+}$
		Tetracycle	1.18	1533.78	1533.63	$[M+2H]^{2+}$
				1022.93	1022.76	$[M+3H]^{3+}$
				767.54	767.38	$[M+4H]^{4+}$
				614.01	614.26	$[M+5H]^{5+}$

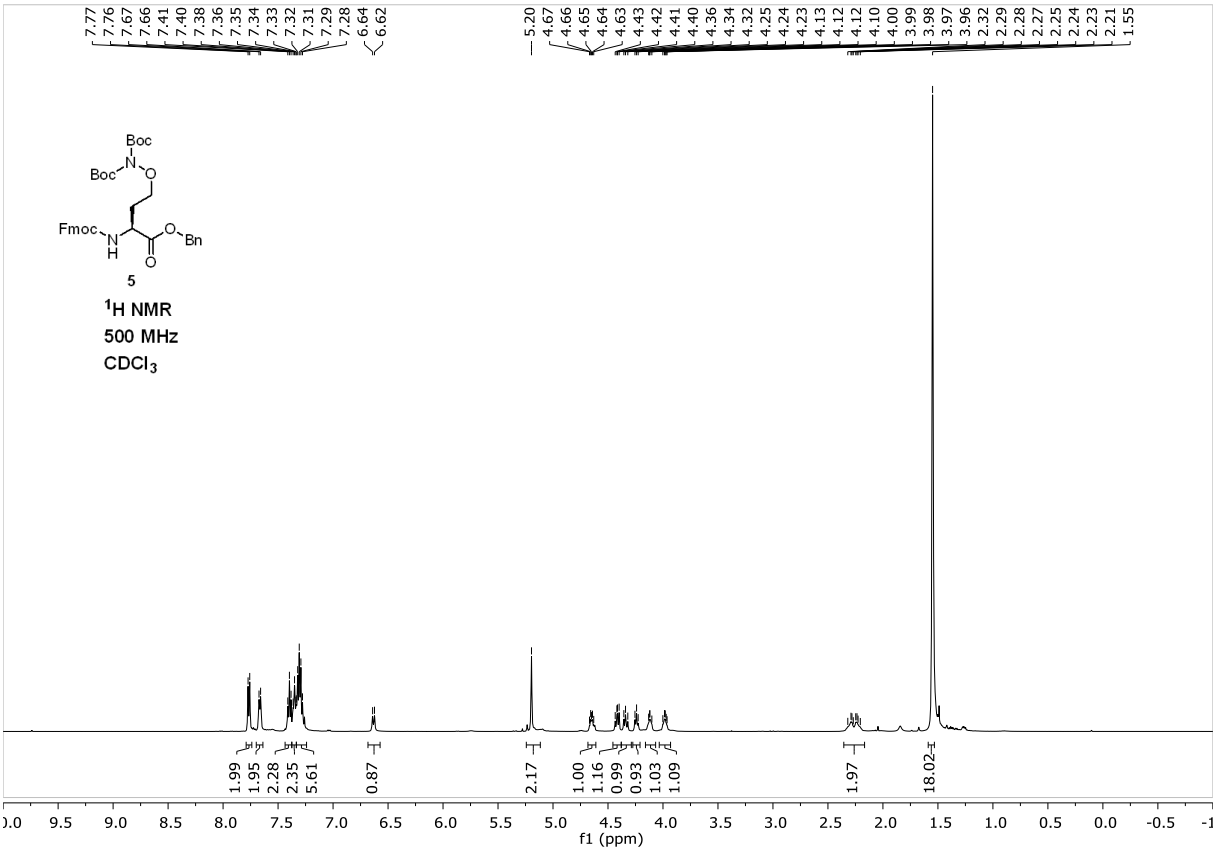
8.3.6 $c8_{5555}$-D(C=O)•T4-3$(ONH_2)^{c/o}$

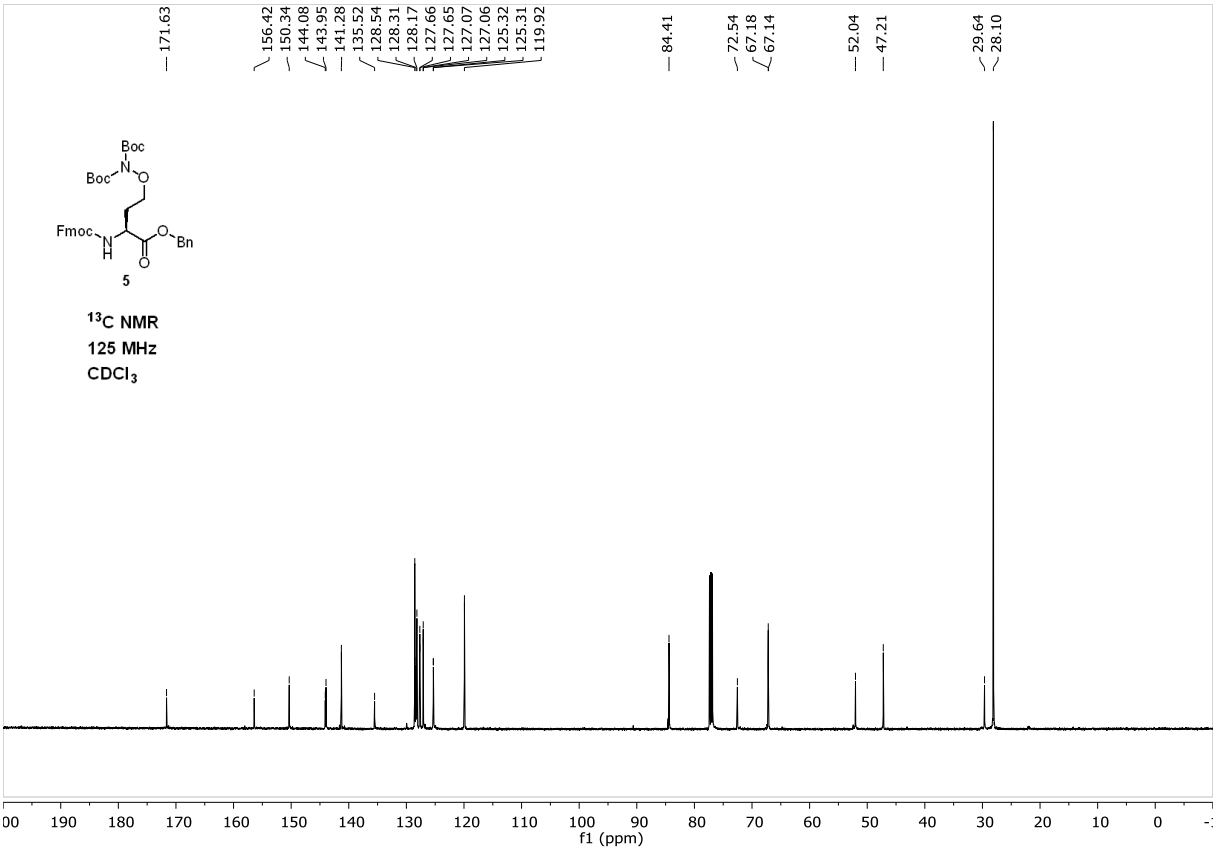
cyc-CYKGKQ**D**(C=O)SIKAS**D**(C=O)AKVRGCKFSKL with **T4-3**(ONH_2) scaffold

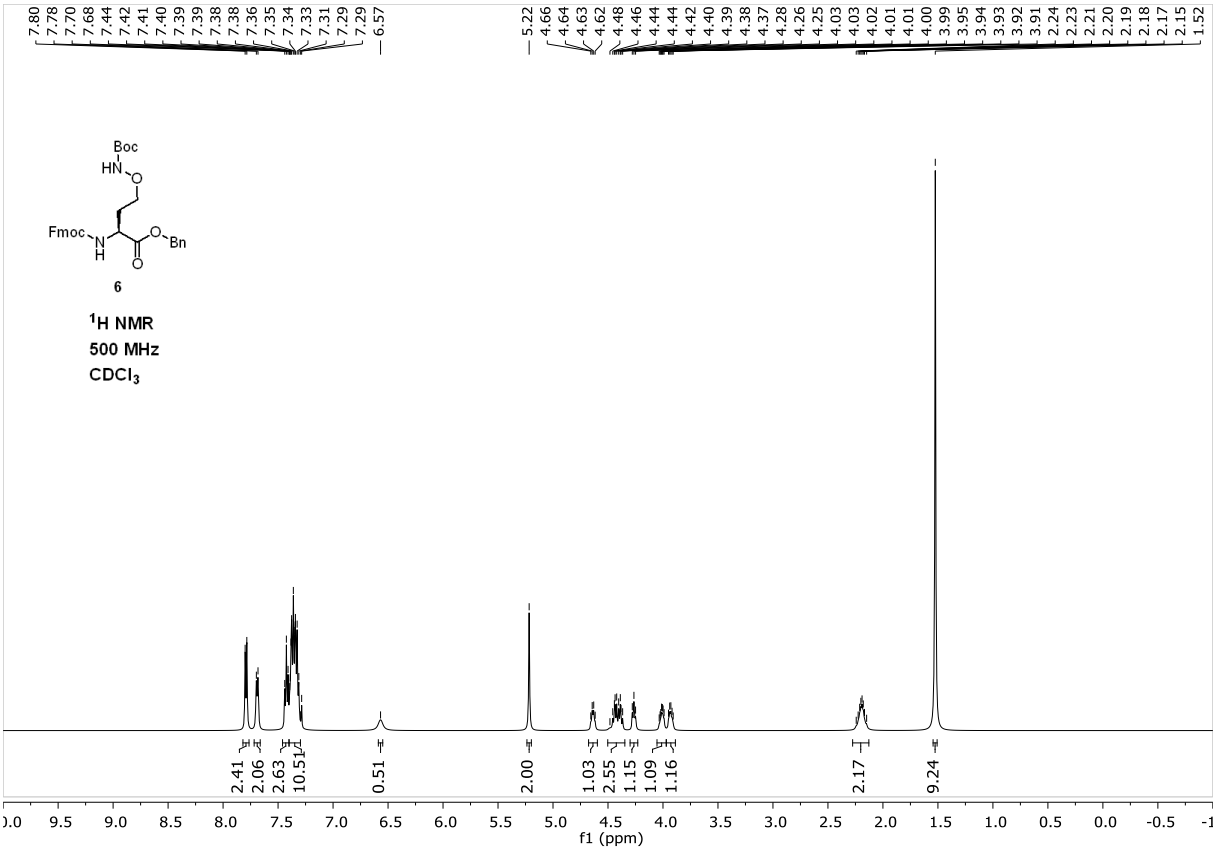


Entry	Reaction	Compound	tr	m/z found	calc	Ionized species
a		Monocyclic peptide	0.96		2837.86	$[M+H]^{+}$
				1420.30	1420.43	$[M+2H]^{2+}$
				947.25	947.29	$[M+3H]^{3+}$
				710.69	710.72	$[M+4H]^{4+}$
				567.95	568.17	$[M+5H]^{5+}$
b	CLIPS reaction	CLIPSed	1.17	1708.99	1708.75	$[M+2H]^{2+}$
				1139.78	1139.17	$[M+3H]^{3+}$
				855.14	854.93	$[M+4H]^{4+}$
				804.97	804.91	$[M-2Boc+4H]^{4+}$
c	Oxime ligation	Mono-Oxime	0.90	1066.80	1066.47	$[M+3H]^{3+}$
				801.07	800.66	$[M+4H]^{4+}$
		Tetracycle	1.09	1591.08	1591.18	$[M+2H]^{2+}$
				1061.10	1061.13	$[M+3H]^{3+}$
				796.04	796.15	$[M+4H]^{4+}$
				636.66	636.88	$[M+5H]^{5+}$

9 NMR spectra



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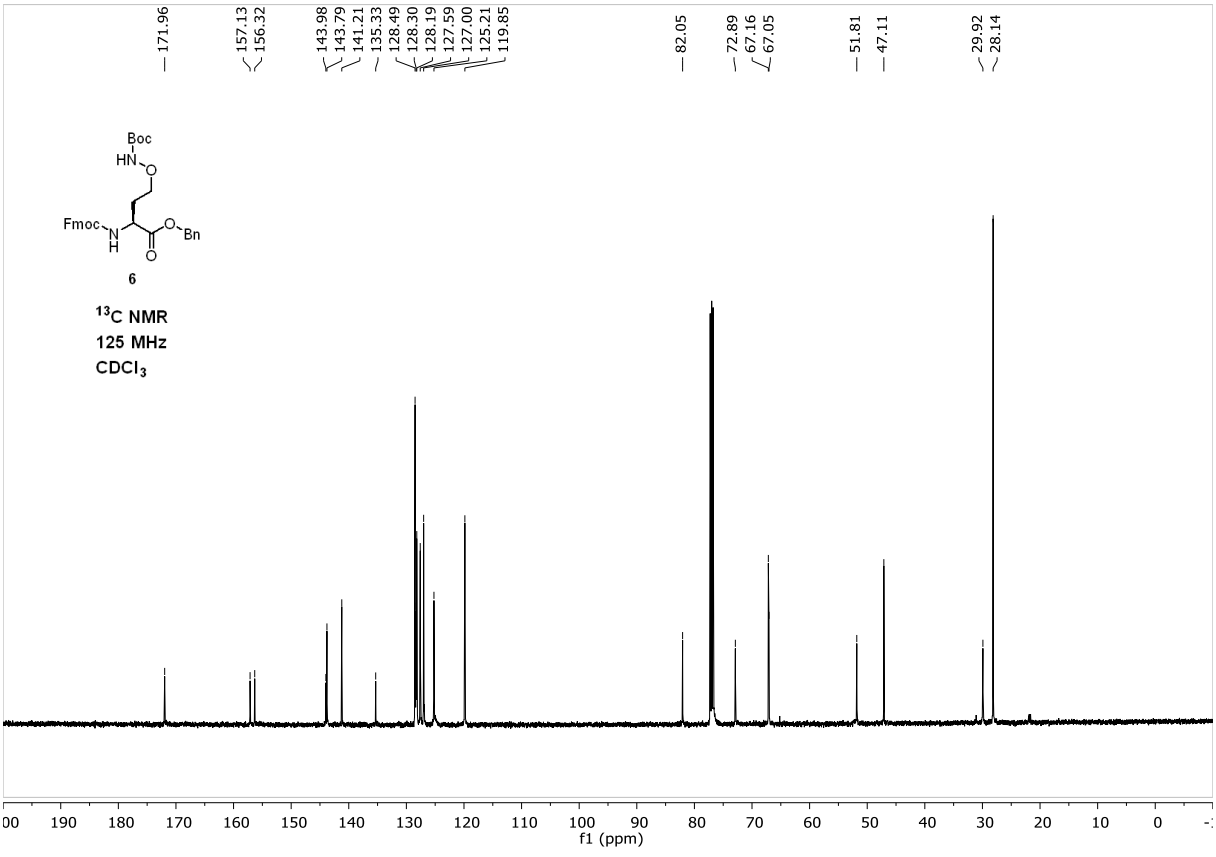
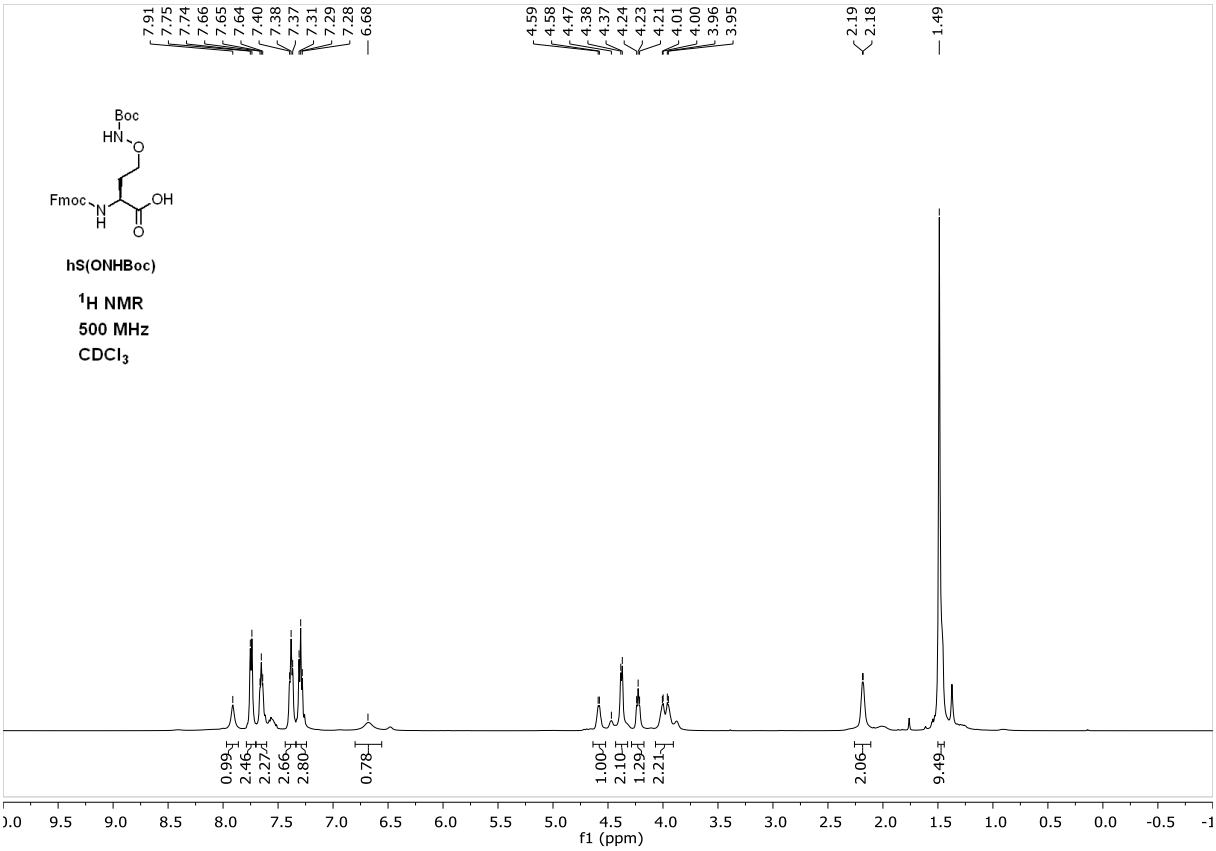
HN
O
Fmoc
N
H
O
O
Bn
O
6
1H NMR
500 MHz
CDCl3
f1 (ppm)


Boc
HN
O
Fmoc
N
H
O
O
Bn
O
6
13C NMR
125 MHz
CDCl3
f1 (ppm)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7.65
7.64
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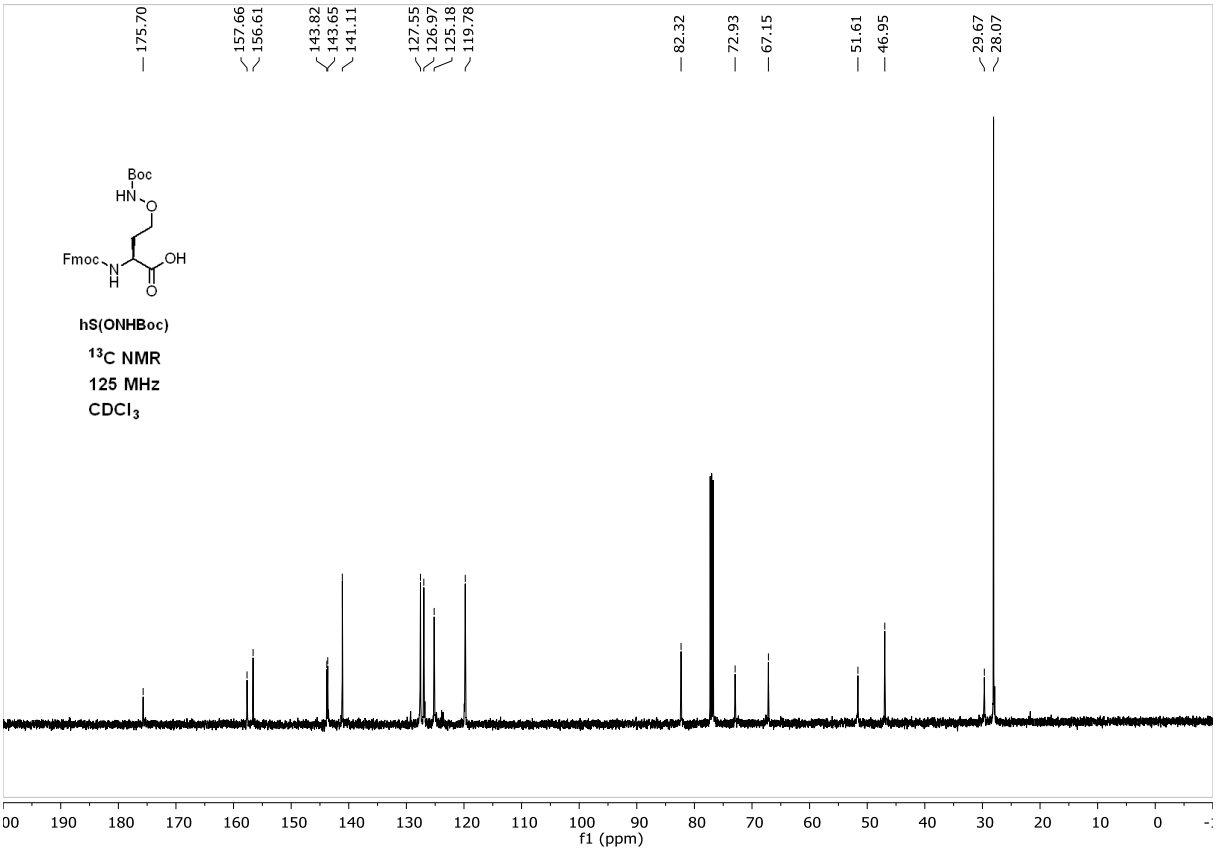
7.38
7.37
7.31
7.29
7.28
6.68
4.59
4.58
4.47
4.38
4.37
4.24
4.23
4.21
4.01
4.00
3.96
3.95
2.19
2.18
1.49
Boc
HN
O
Fmoc
N
H
OH
O
hS(ONHBoc)
1H NMR
500 MHz
CDCl3
0.99
2.46
2.27
2.66
2.80
0.78
1.00
2.10
1.29
2.21
2.06
9.49
f1 (ppm)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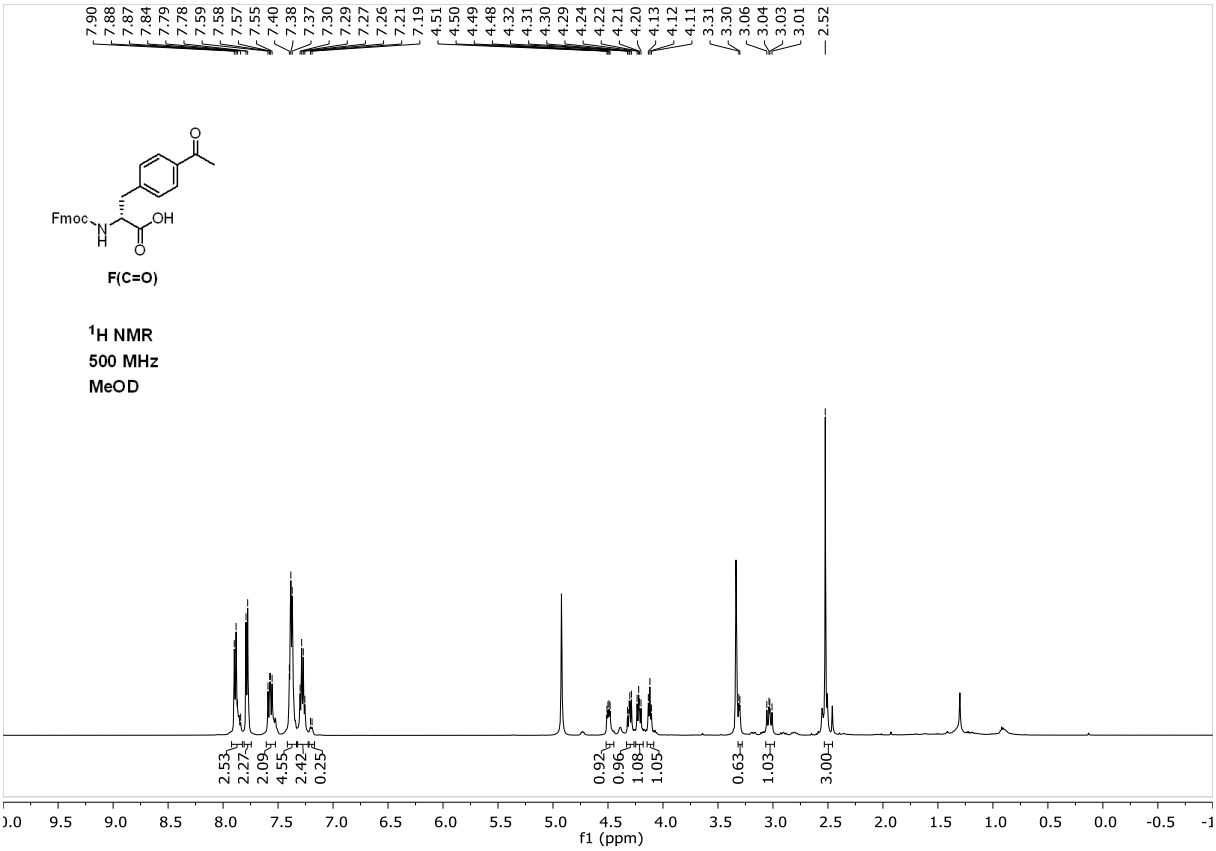
156.61
143.82
143.65
141.11
127.55
126.97
125.18
119.78
82.32
72.93
67.15
51.61
46.95
29.67
28.07
Boc
HN
O
Fmoc
N
H
OH
O
hS(ONHBoc)
13C NMR
125 MHz
CDCl3
f1 (ppm)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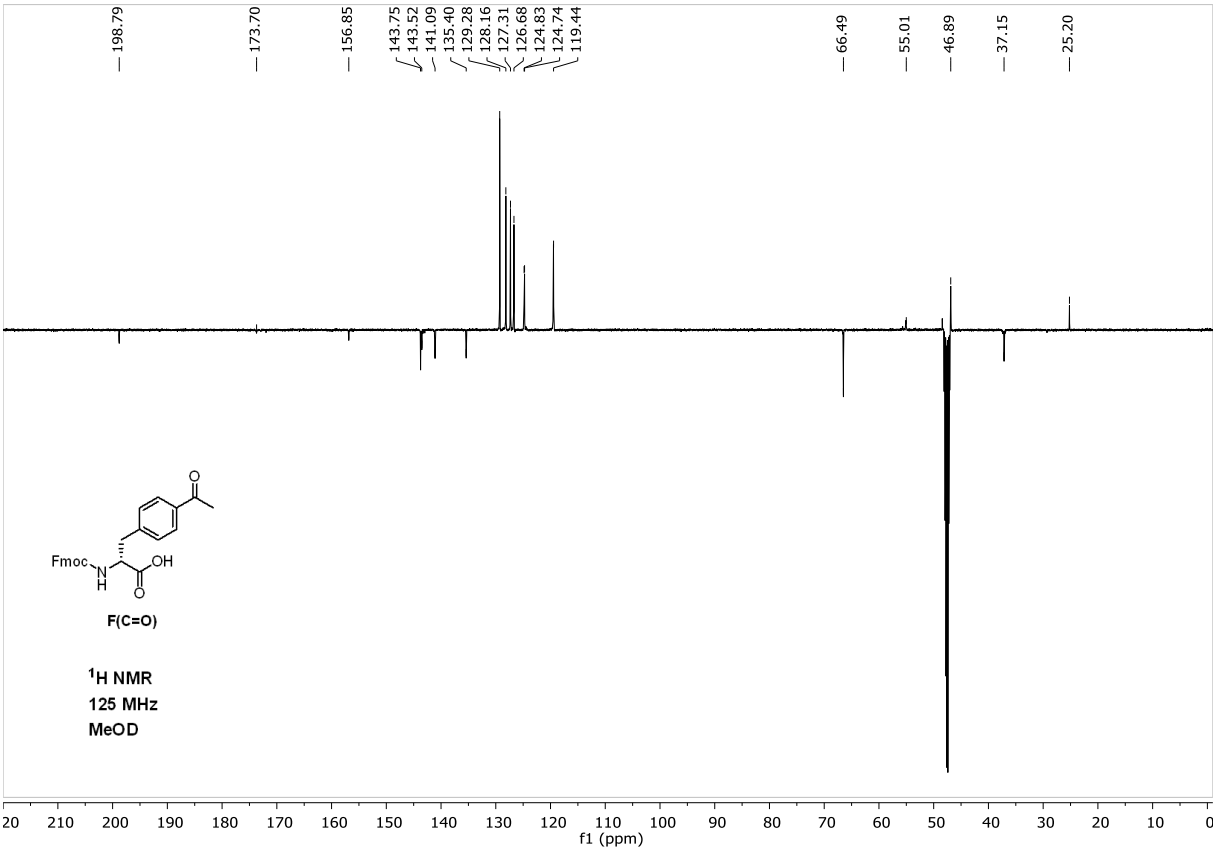
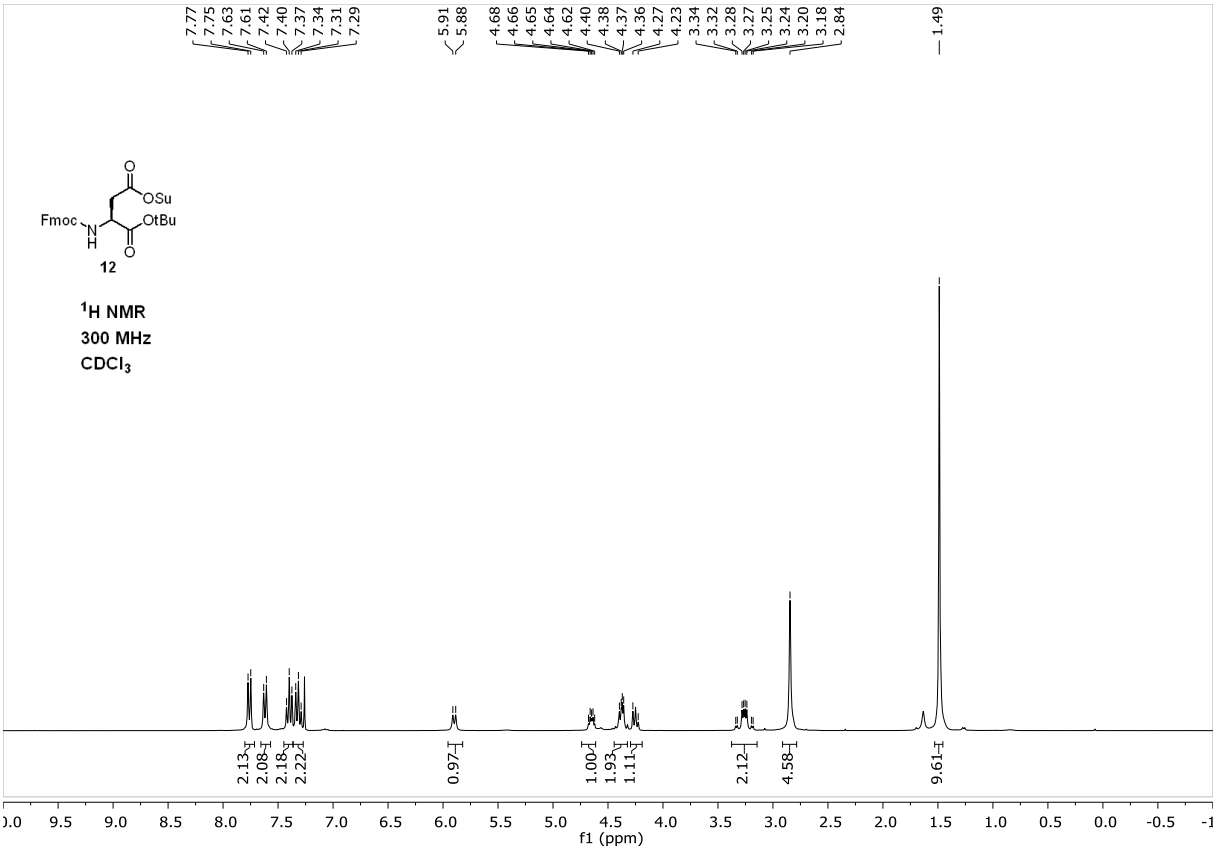
7.79
7.78
7.59
7.58
7.57
7.55
7.40
7.38
7.37
7.30
7.29
7.27
7.26
7.21
7.19
4.51
4.50
4.49
4.48
4.32
4.31
4.30
4.29
4.24
4.22
4.21
4.20
4.13
4.12
4.11
3.31
3.30
3.06
3.04
3.03
3.01
2.52
Fmoc
OH
F(C=O)
1H NMR
500 MHz
MeOD
2.53
2.27
2.09
4.55
2.42
0.25
0.92
0.96
1.08
1.05
0.63
1.03
3.00
f1 (ppm)


198.79
173.70
156.85
143.75
143.52
141.09
135.40
129.28
128.16
127.31
126.68
124.83
124.74
119.44
66.49
55.01
46.89
37.15
25.20
Fmoc
OH
F(C=O)
1H NMR
125 MHz
MeOD
f1 (ppm)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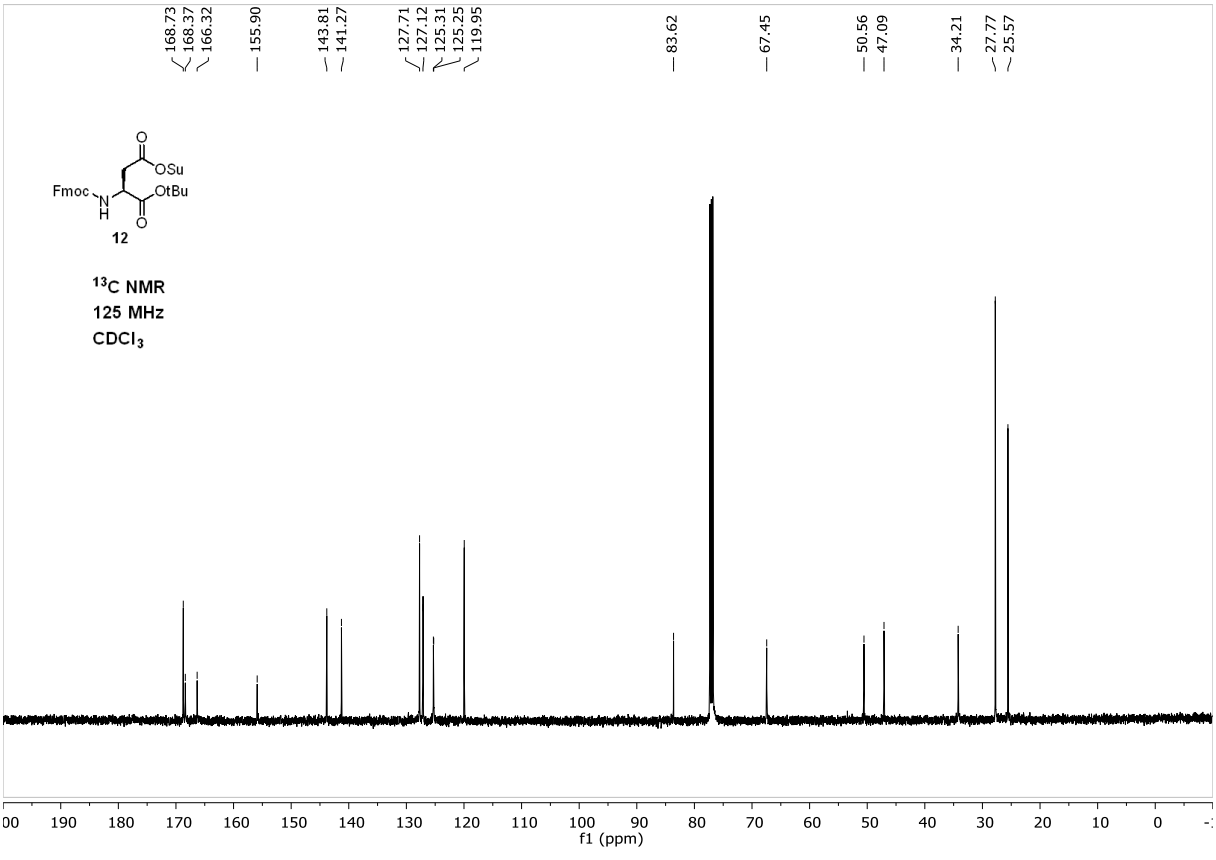
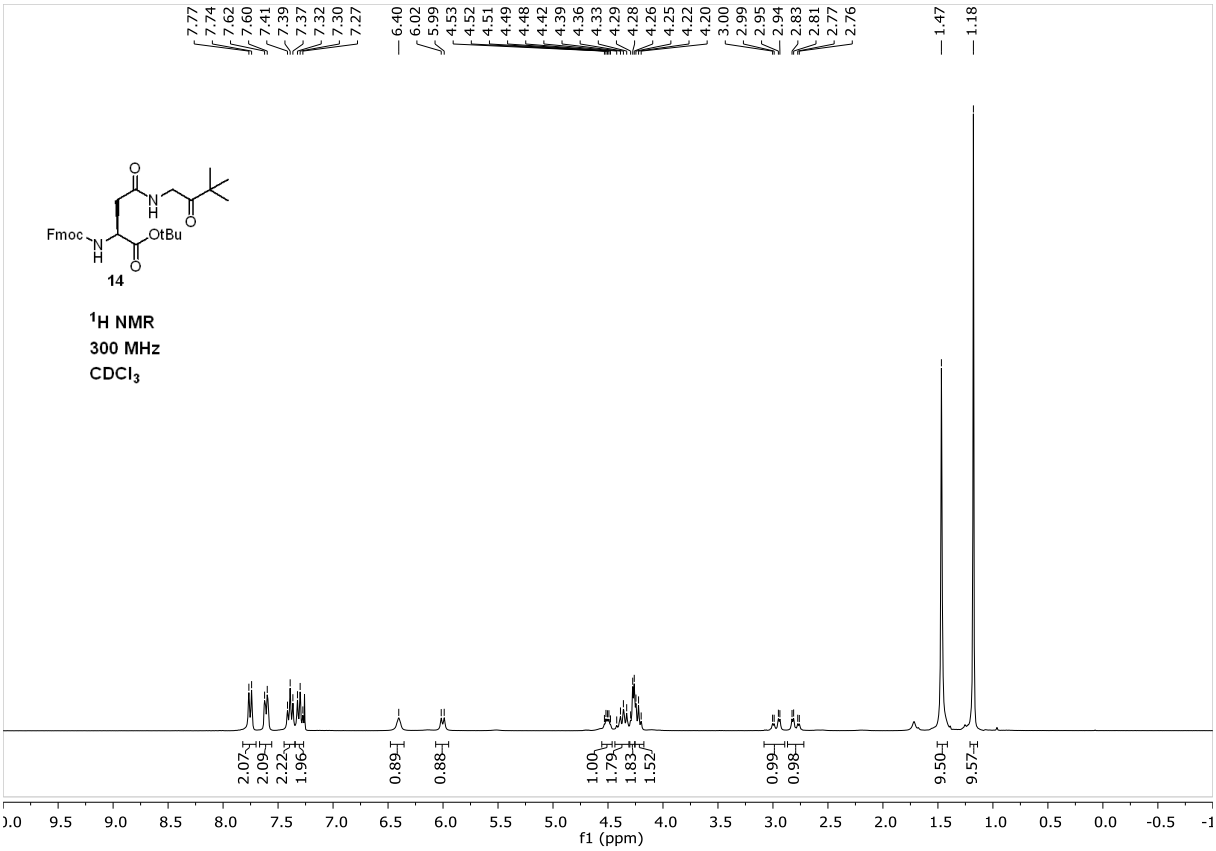
7.42
7.40
7.37
7.34
7.31
7.29
5.91
5.88
4.68
4.66
4.65
4.64
4.62
4.40
4.38
4.37
4.36
4.27
4.23
3.34
3.32
3.28
3.27
3.25
3.24
3.20
3.18
2.84
1.49
Fmoc
OSu
OtBu
12
1H NMR
300 MHz
CDCl3
2.13
2.08
2.18
2.22
0.97
1.00
1.93
1.11
2.12
4.58
9.61
f1 (ppm)


168.73
168.37
166.32
155.90
143.81
141.27
127.71
127.12
125.31
125.25
119.95
83.62
67.45
50.56
47.09
34.21
27.77
25.57
Fmoc
OSu
OtBu
12
13C NMR
125 MHz
CDCl3
f1 (ppm)


7.77
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7.39
7.37
7.32
7.30
7.27
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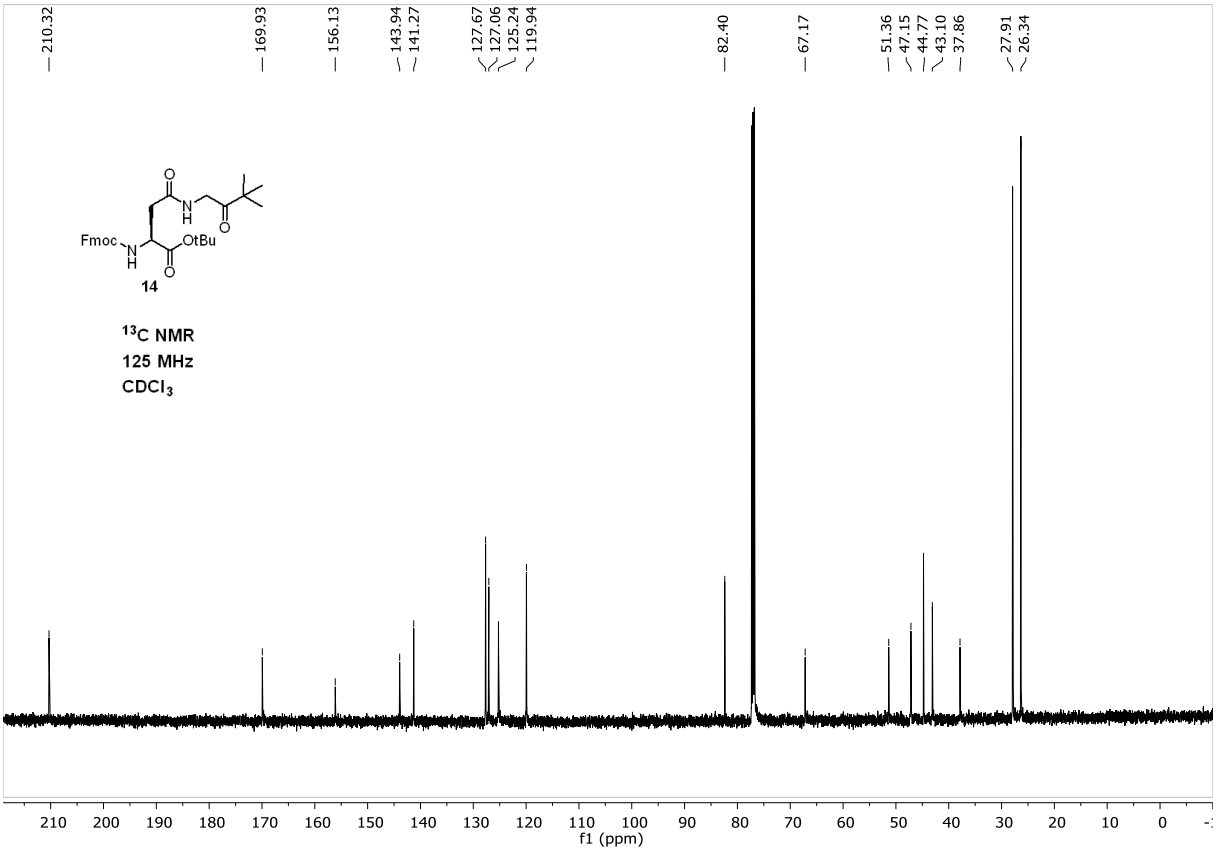
6.02
5.99
4.53
4.52
4.51
4.49
4.48
4.42
4.39
4.36
4.33
4.29
4.28
4.26
4.25
4.22
4.20
3.00
2.99
2.95
2.94
2.83
2.81
2.77
2.76
1.47
1.18
Fmoc
OtBu
14
1H NMR
300 MHz
CDCl3
2.07
2.09
2.22
1.96
0.89
0.88
1.00
1.79
1.83
1.52
0.99
0.98
9.50
9.57
f1 (ppm)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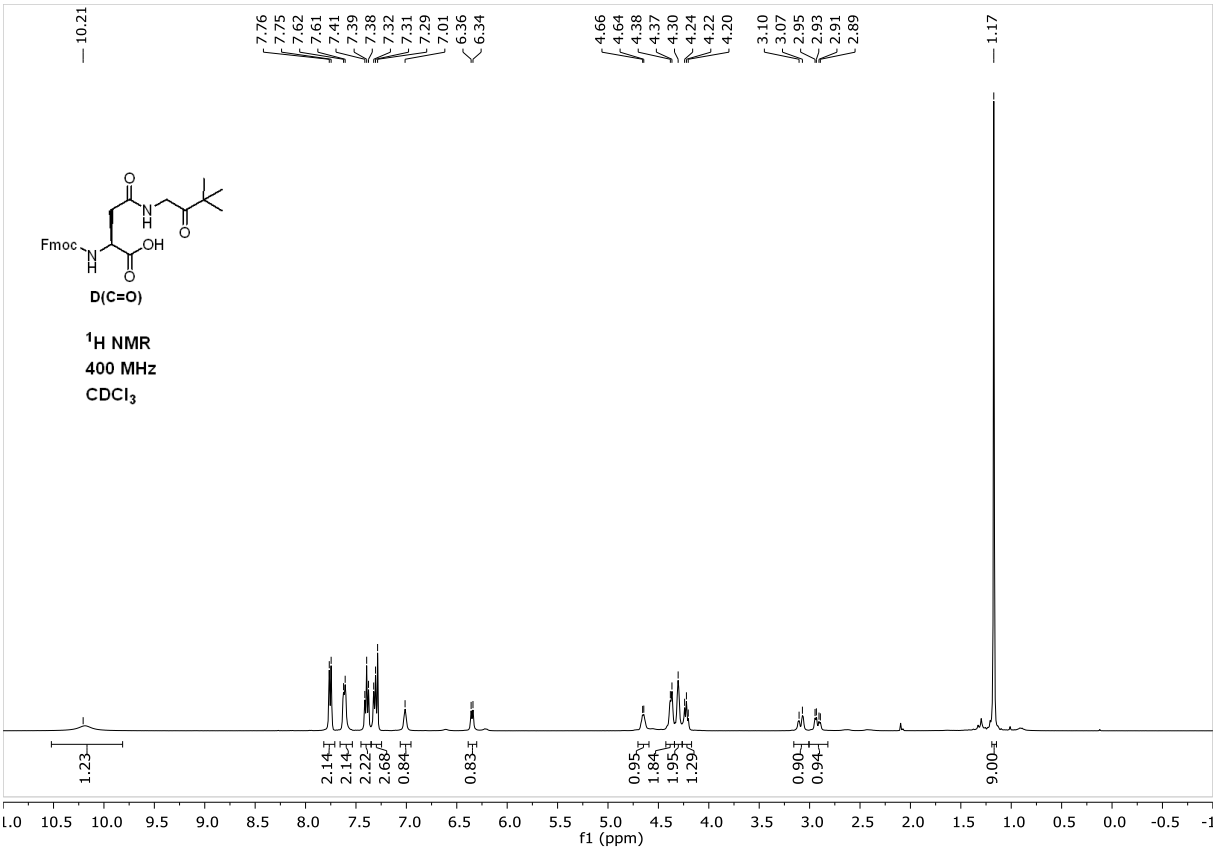
143.94
141.27
127.67
127.06
125.24
119.94
82.40
67.17
51.36
47.15
44.77
43.10
37.86
27.91
26.34
Fmoc
OtBu
14
13C NMR
125 MHz
CDCl3
f1 (ppm)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7.62
7.61
7.41
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7.32
7.31
7.29
7.01
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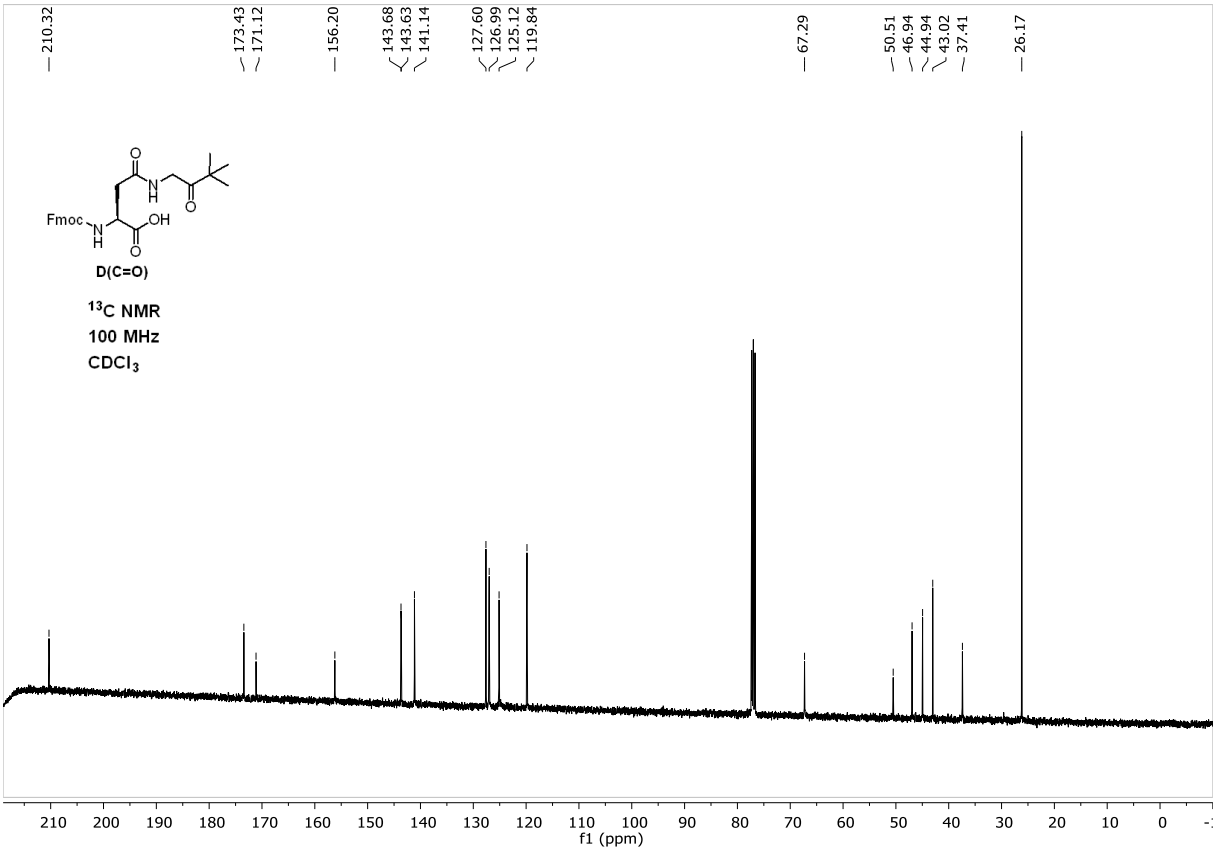
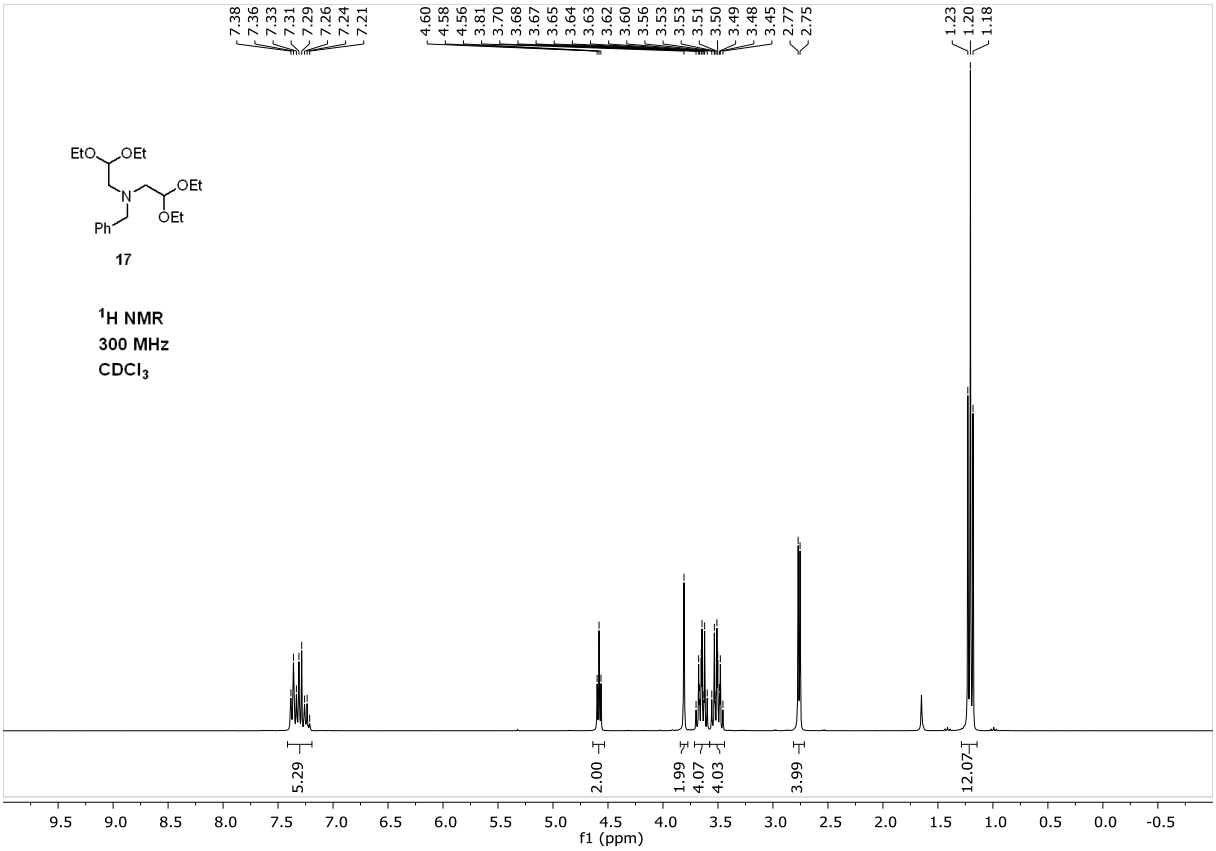
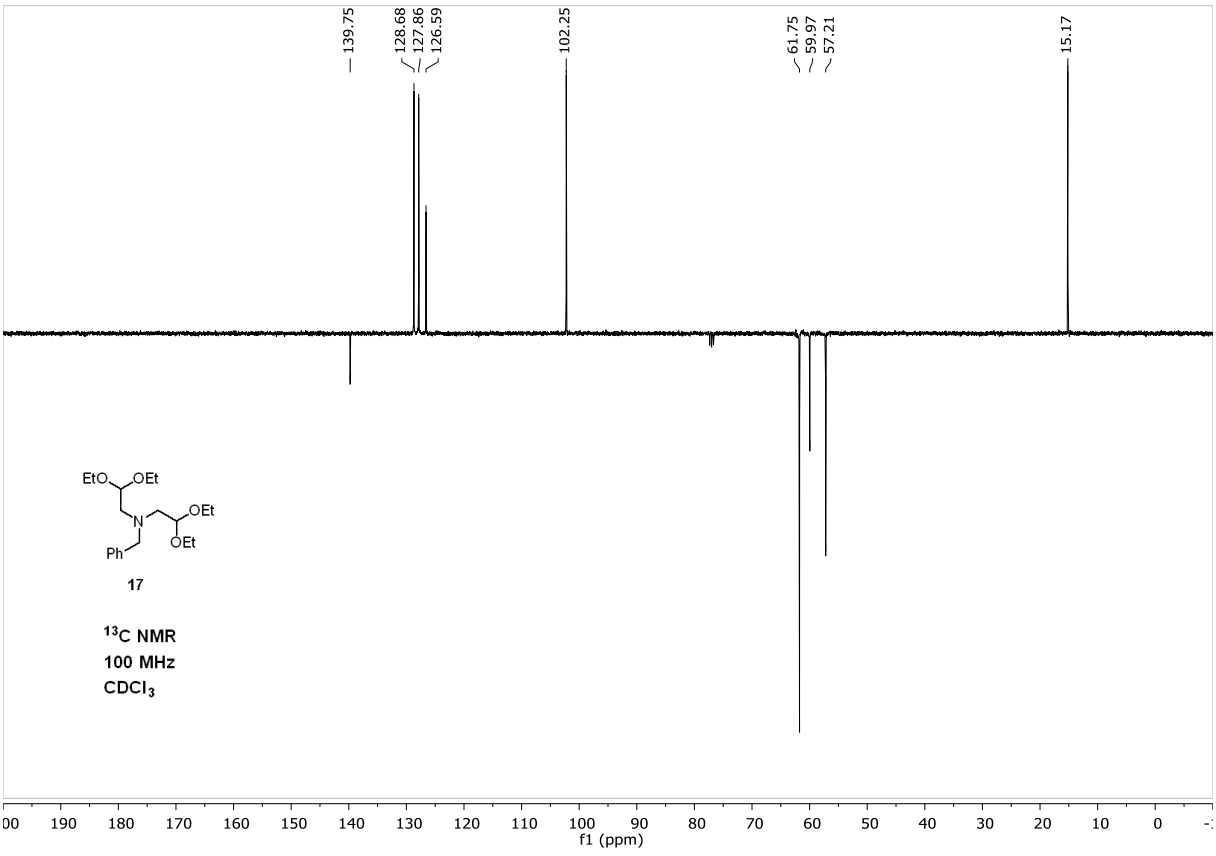
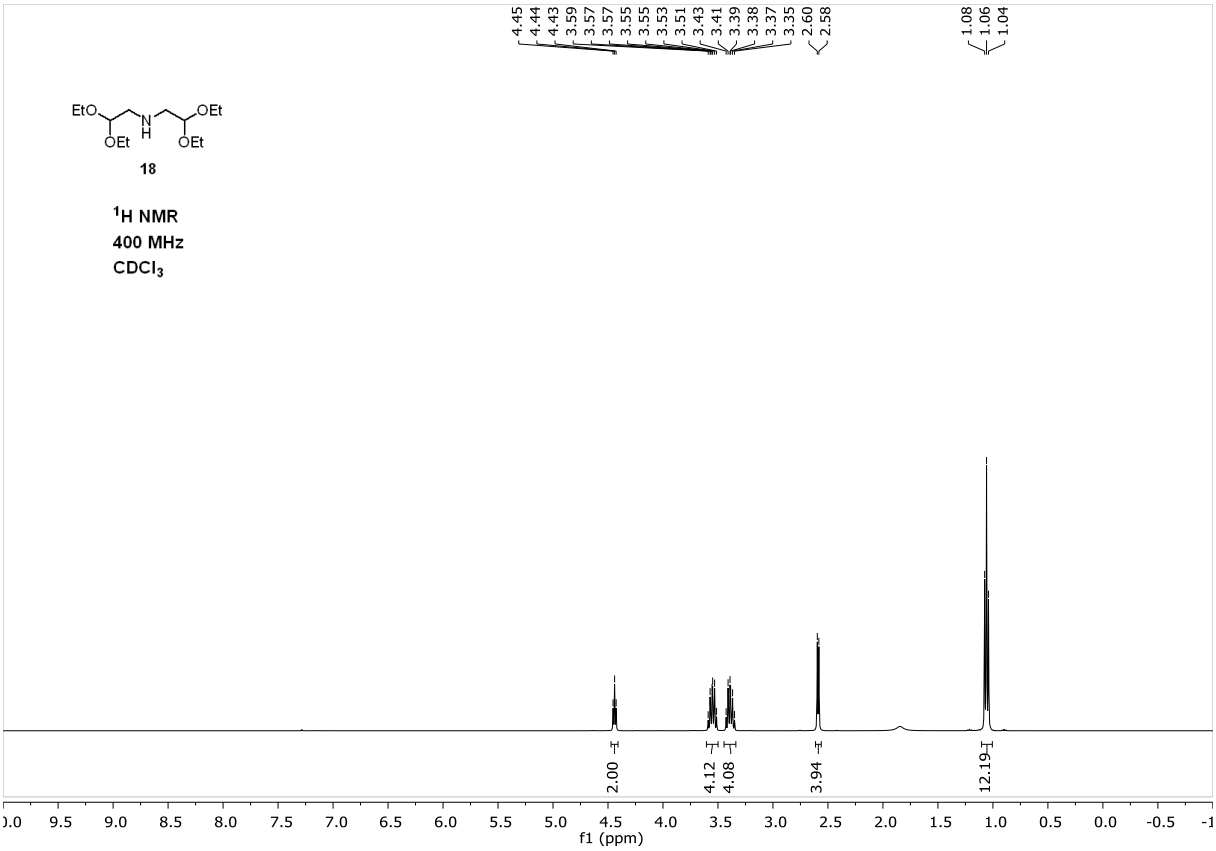
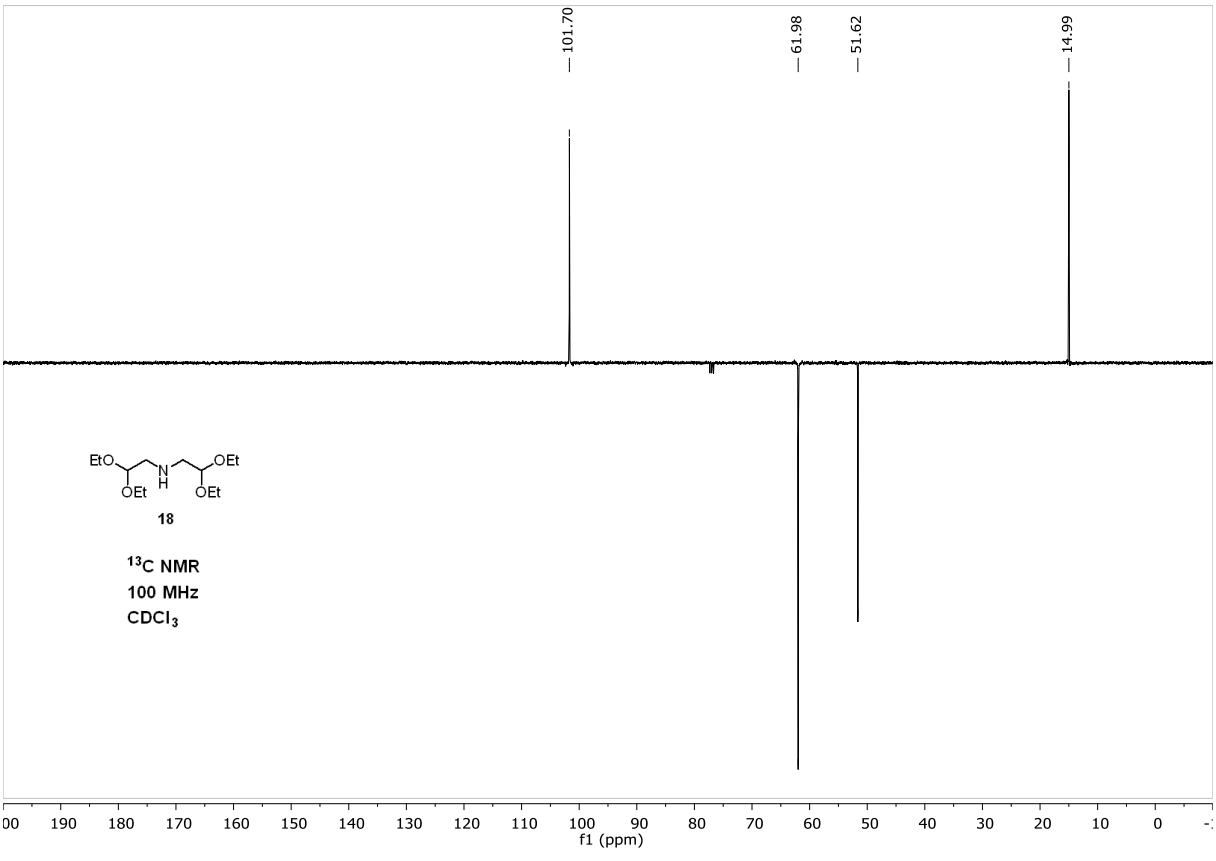
4.64
4.38
4.37
4.30
4.24
4.22
4.20
3.10
3.07
2.95
2.93
2.91
2.89
1.17
Fmoc
OH
D(C=O)
1H NMR
400 MHz
CDCl3
1.23
2.14
2.14
2.22
2.68
0.84
0.83
0.95
1.84
1.95
1.29
0.90
0.94
9.00
f1 (ppm)

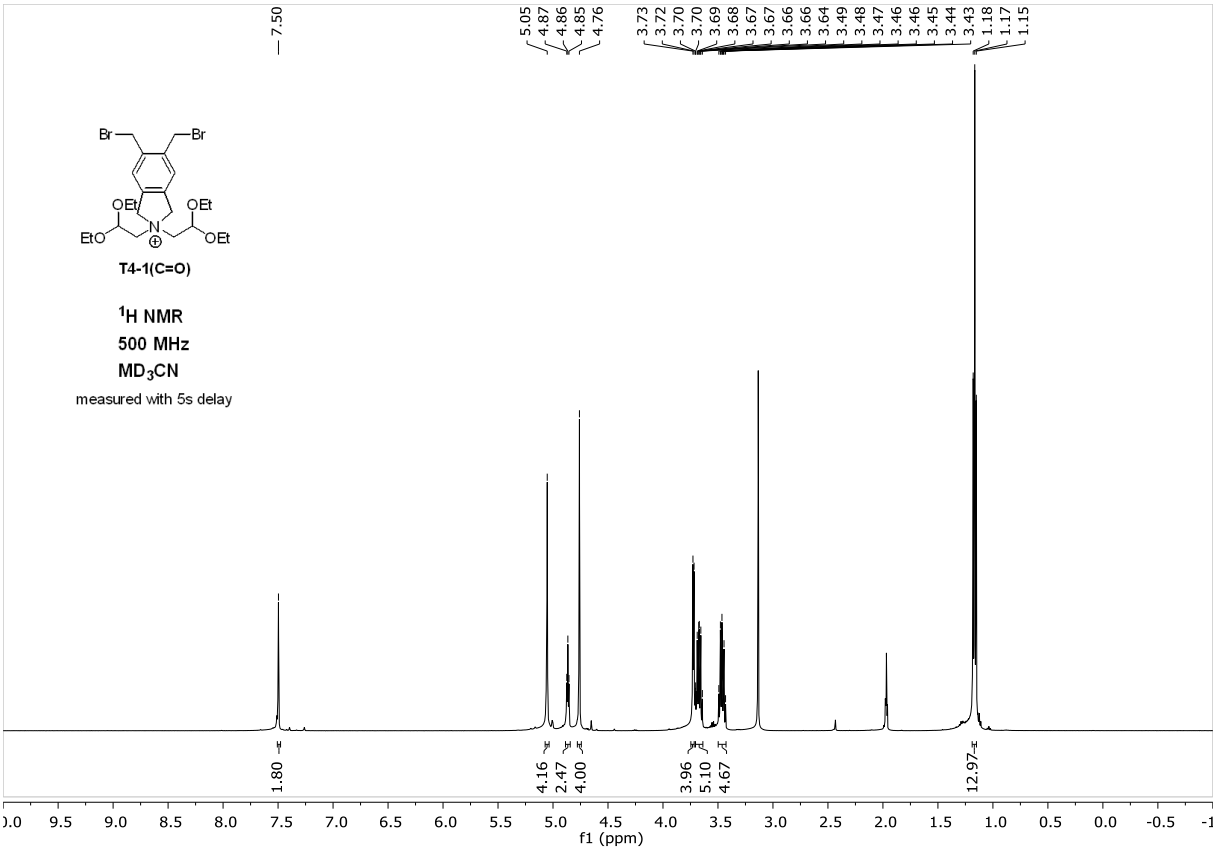

210.32
173.43
171.12
156.20
143.68
143.63
141.14
127.60
126.99
125.12
119.84
67.29
50.51
46.94
44.94
43.02
37.41
26.17
Fmoc
OH
D(C=O)
13C NMR
100 MHz
CDCl3
f1 (ppm)

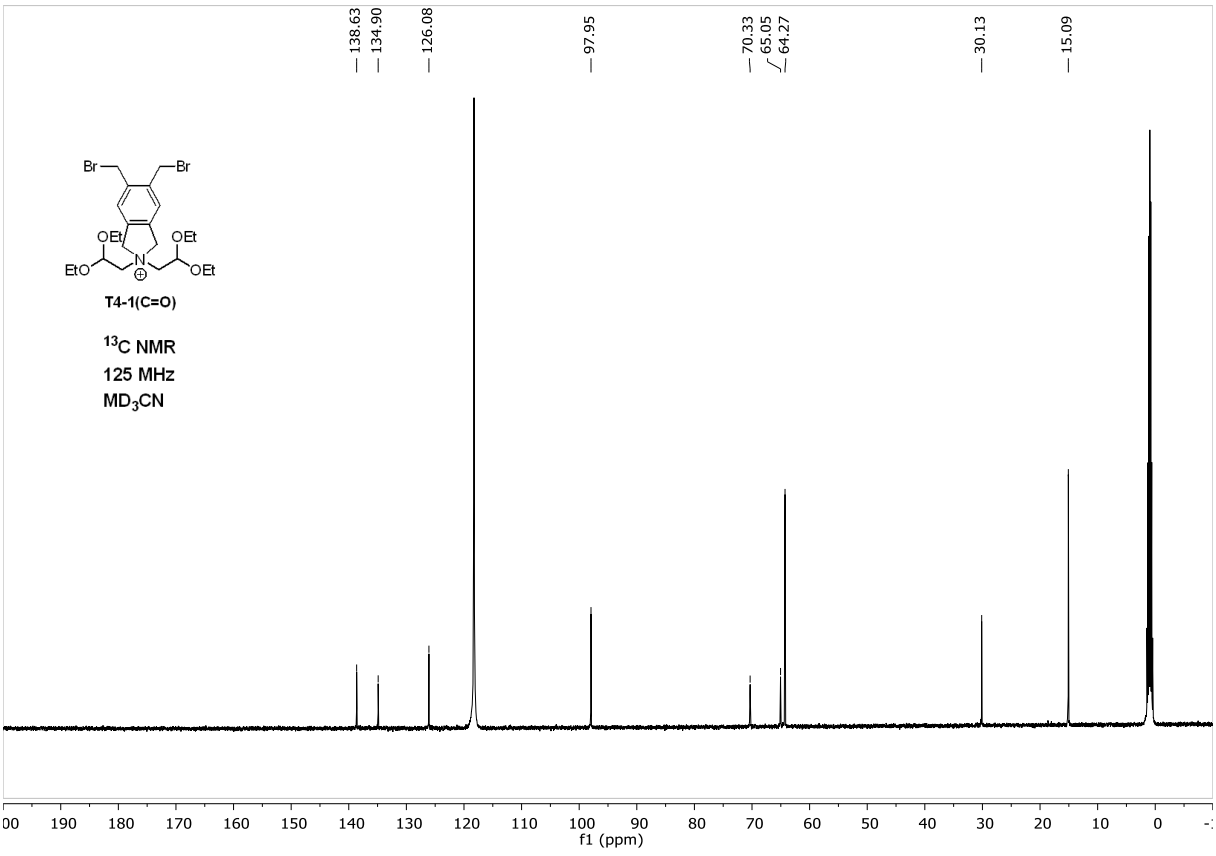

7.38
7.36
7.33
7.31
7.29
7.26
7.24
7.21
4.60
4.58
4.56
3.81
3.70
3.68
3.67
3.65
3.64
3.63
3.62
3.60
3.56
3.53
3.53
3.51
3.50
3.49
3.48
3.45
2.77
2.75
1.23
1.20
1.18
EtO
OEt
N
OEt
OEt
Ph
17
1H NMR
300 MHz
CDCl3
5.29
2.00
1.99
4.07
4.03
3.99
12.07
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)

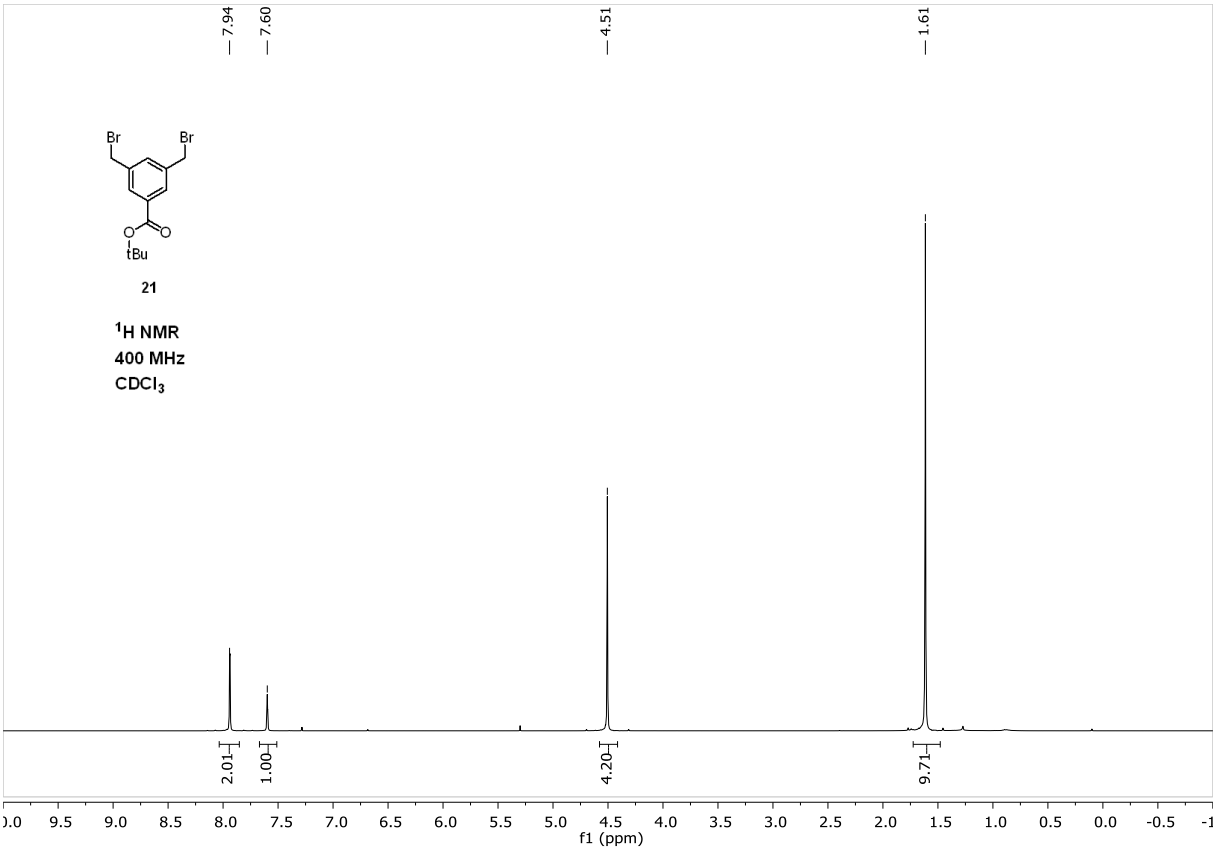

139.75
128.68
127.86
126.59
102.25
61.75
59.97
57.21
15.17
EtO
OEt
N
OEt
OEt
Ph
17
13C NMR
100 MHz
CDCl3
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

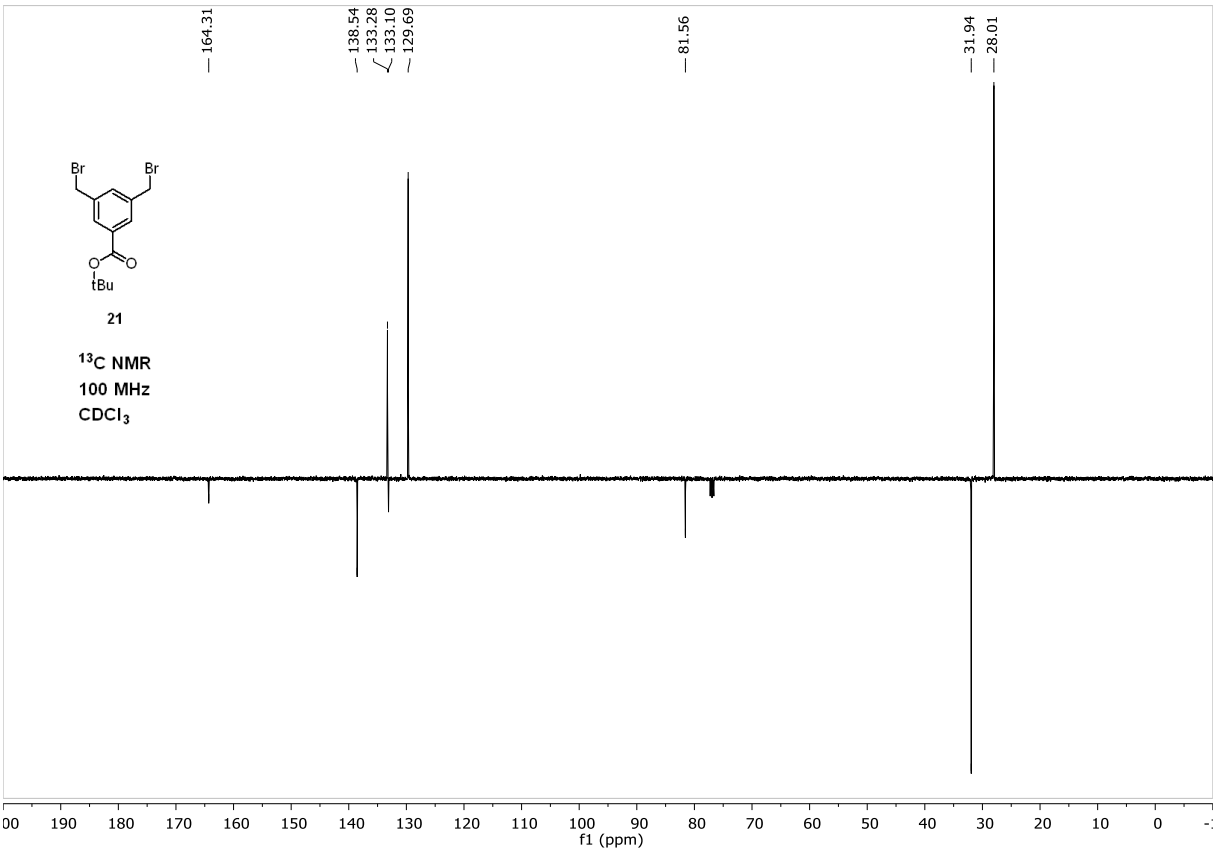

4.45
4.44
4.43
3.59
3.57
3.57
3.55
3.55
3.53
3.51
3.43
3.41
3.39
3.38
3.37
3.35
2.60
2.58
1.08
1.06
1.04
EtO
OEt
N
H
OEt
OEt
18
1H NMR
400 MHz
CDCl3
2.00
4.12
4.08
3.94
12.19
f1 (ppm)

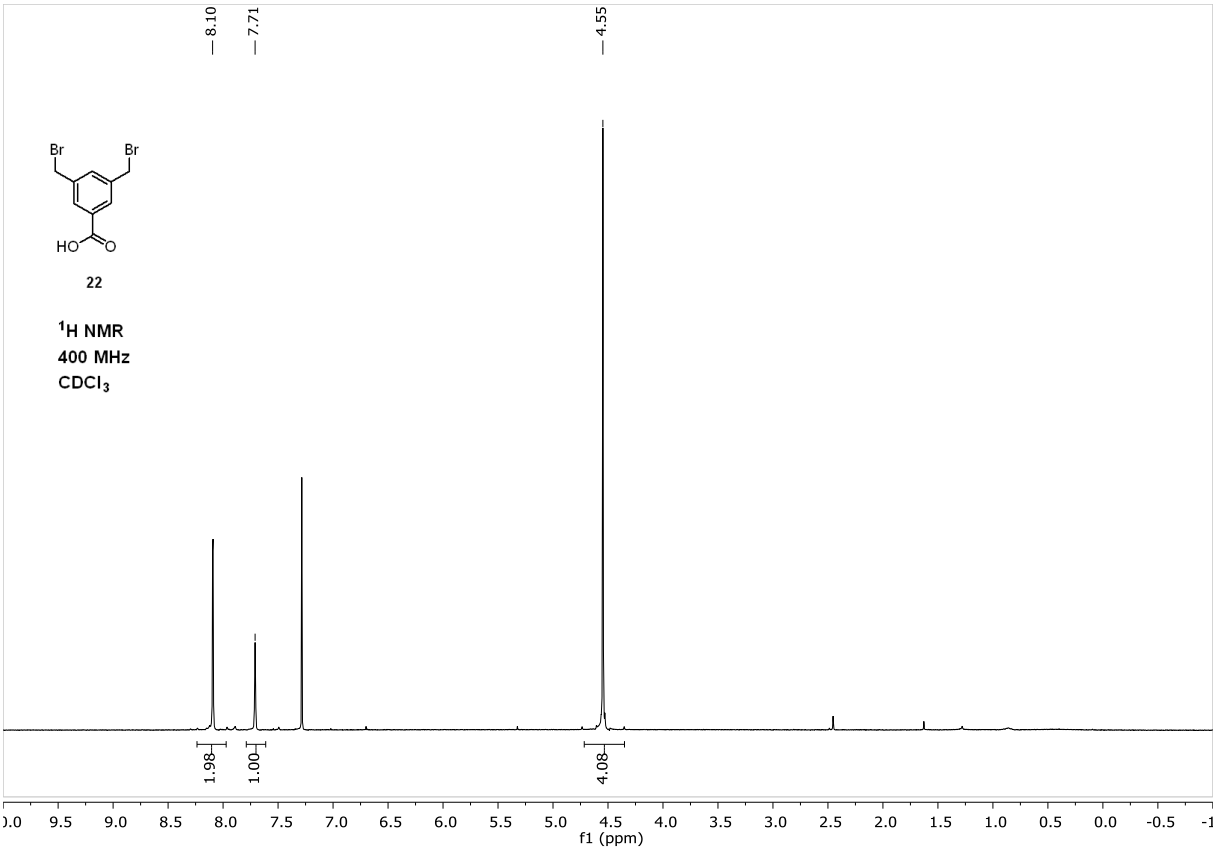

101.70
61.98
51.62
14.99
EtO
OEt
N
H
OEt
OEt
18
13C NMR
100 MHz
CDCl3
f1 (ppm)

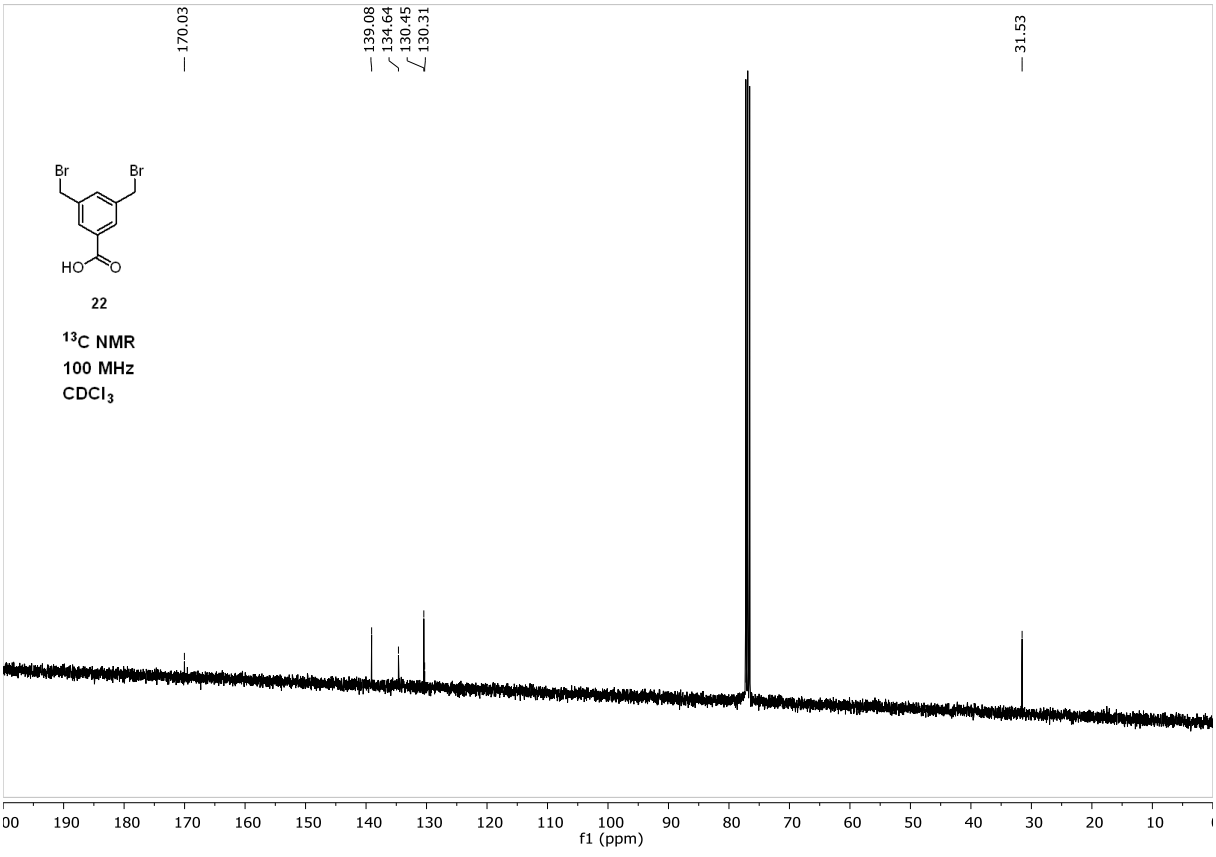

7.50
5.05
4.87
4.86
4.85
4.76
3.73
3.72
3.70
3.70
3.69
3.68
3.67
3.67
3.66
3.66
3.64
3.49
3.48
3.47
3.46
3.46
3.45
3.44
3.43
1.18
1.17
1.15
Br
Br
OEt
OEt
EtO
OEt
N
T4-1(C=O)
1H NMR
500 MHz
MD3CN
measured with 5s delay
1.80
4.16
2.47
4.00
3.96
5.10
4.67
12.97
f1 (ppm)

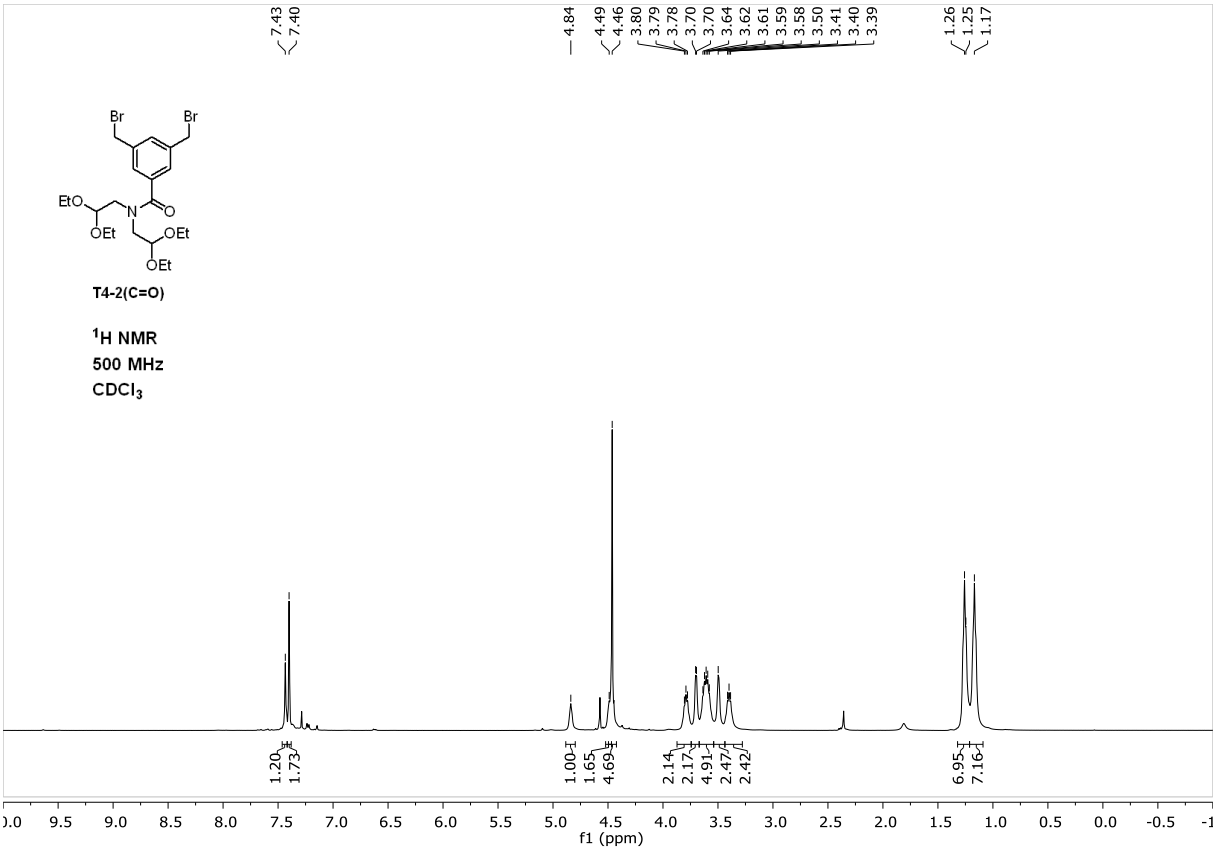

138.63
134.90
126.08
97.95
70.33
65.05
64.27
30.13
15.09
Br
Br
OEt
OEt
EtO
OEt
N
T4-1(C=O)
13C NMR
125 MHz
MD3CN
f1 (ppm)

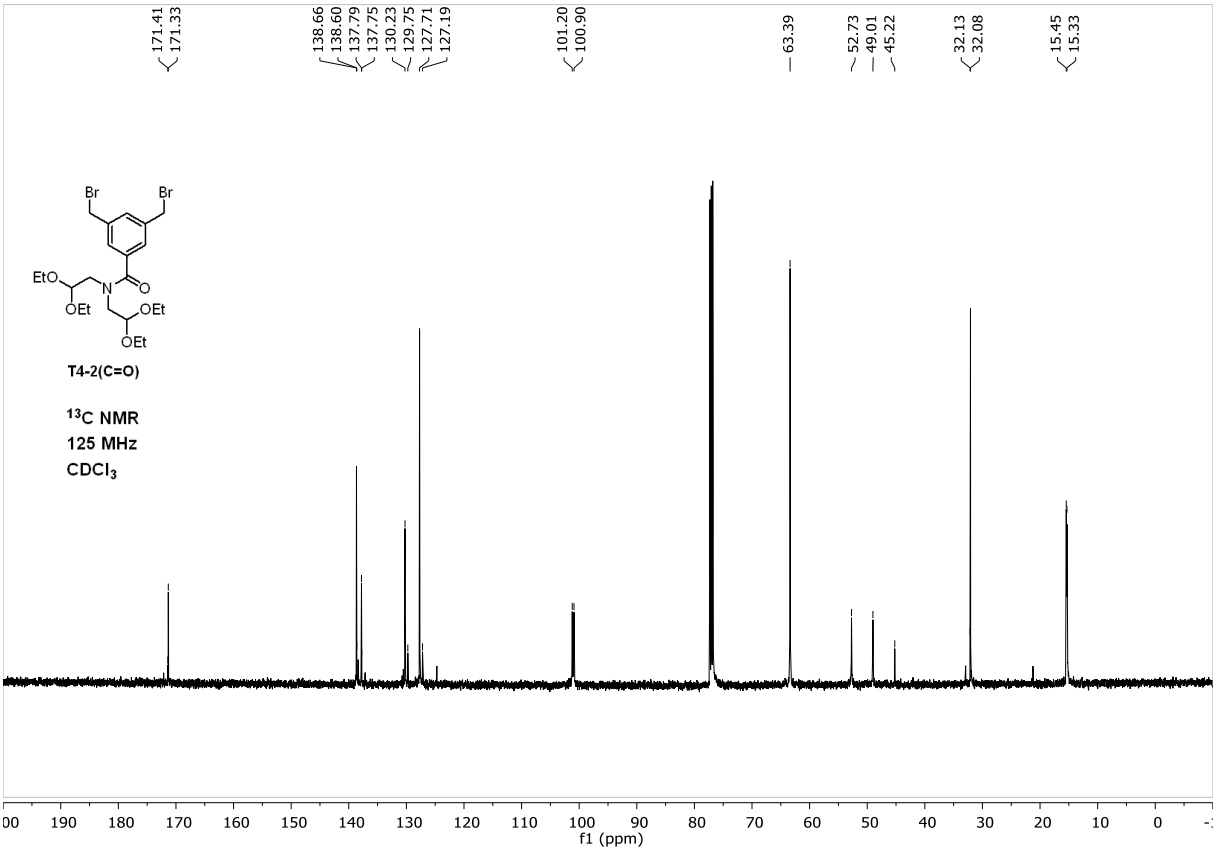

7.94
7.60
4.51
1.61
Br
Br
O
O
tBu
21
1H NMR
400 MHz
CDCl3
2.01
1.00
4.20
9.71
f1 (ppm)

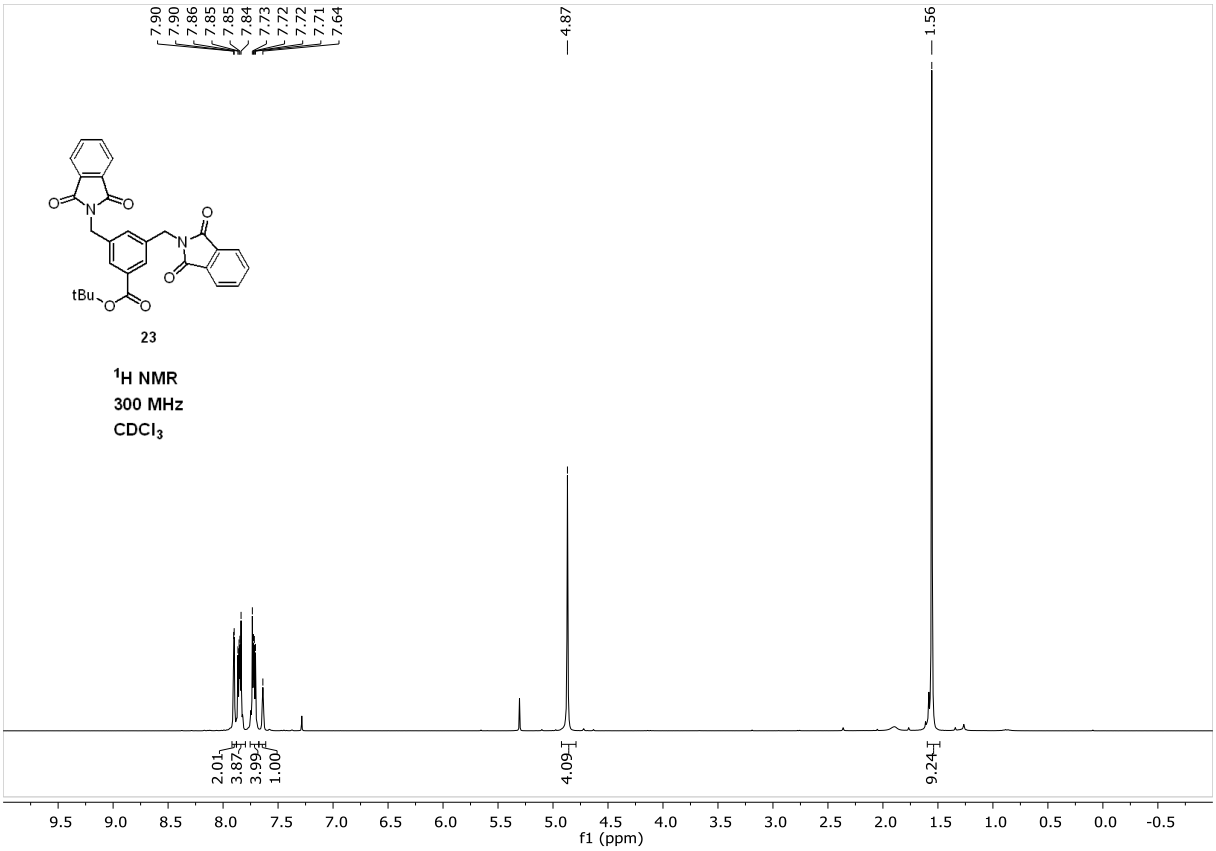

164.31
138.54
133.28
133.10
129.69
81.56
31.94
28.01
Br
Br
O
O
tBu
21
13C NMR
100 MHz
CDCl3
f1 (ppm)

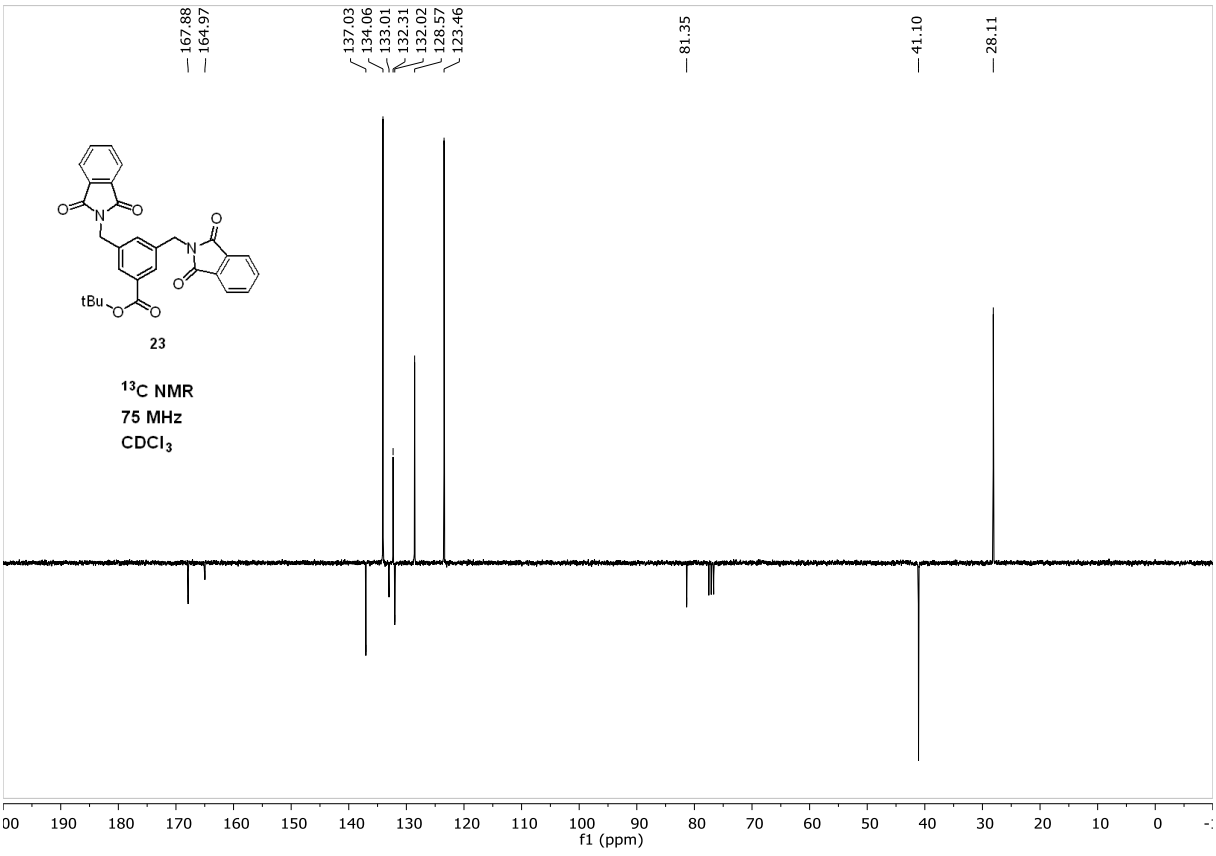

8.10
7.71
4.55
Br
Br
HO
O
22
1H NMR
400 MHz
CDCl3
1.98
1.00
4.08
f1 (ppm)

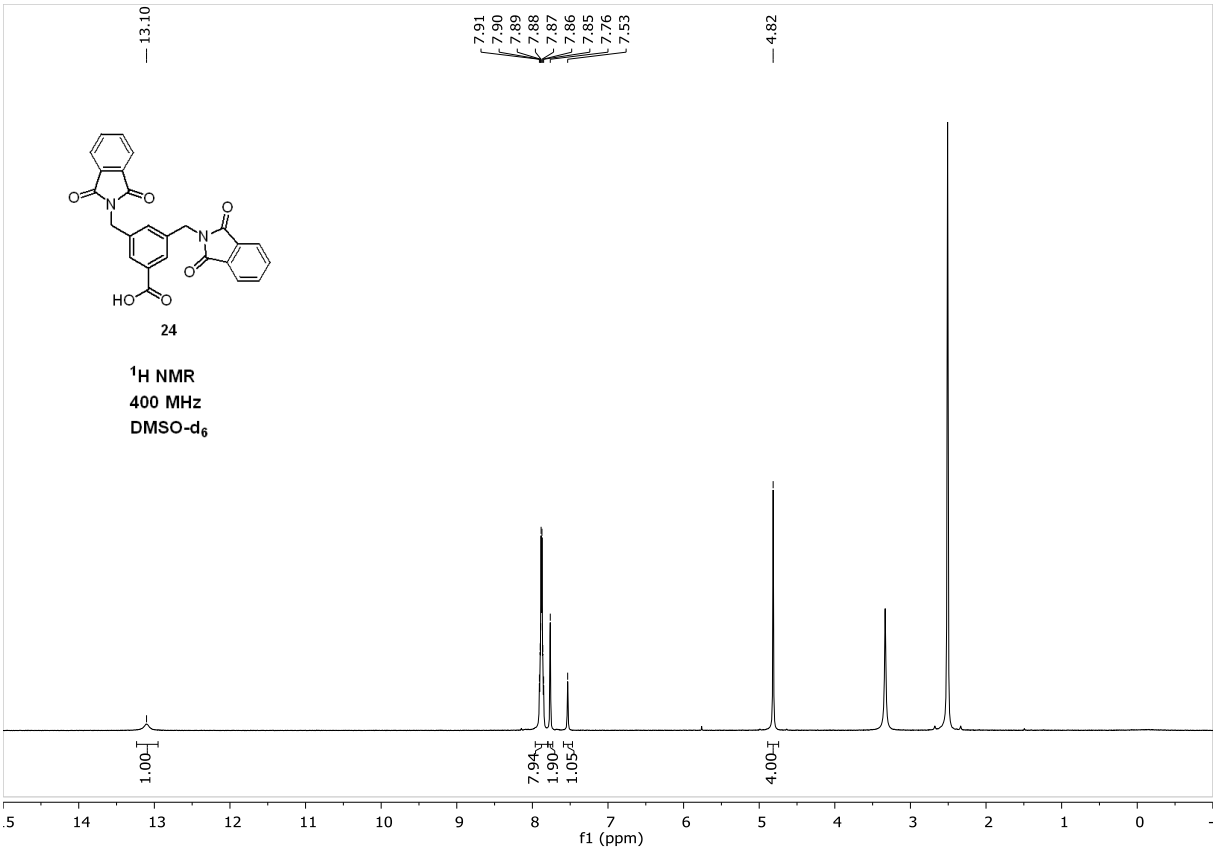

170.03
139.08
134.64
130.45
130.31
31.53
Br
Br
HO
O
22
13C NMR
100 MHz
CDCl3
f1 (ppm)

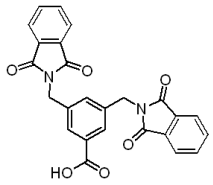

T4-2(C=O)
1H NMR
500 MHz
CDCl3
f1 (ppm)


T4-2(C=O)
13C NMR
125 MHz
CDCl3
f1 (ppm)


7.90
7.90
7.86
7.85
7.85
7.84
7.73
7.72
7.72
7.71
7.64
4.87
1.56
tBu
23
1H NMR
300 MHz
CDCl3
2.01
3.87
3.99
1.00
4.09
9.24
f1 (ppm)


167.88
164.97
137.03
134.06
133.01
132.31
132.02
128.57
123.46
81.35
41.10
28.11
tBu
23
13C NMR
75 MHz
CDCl3
f1 (ppm)



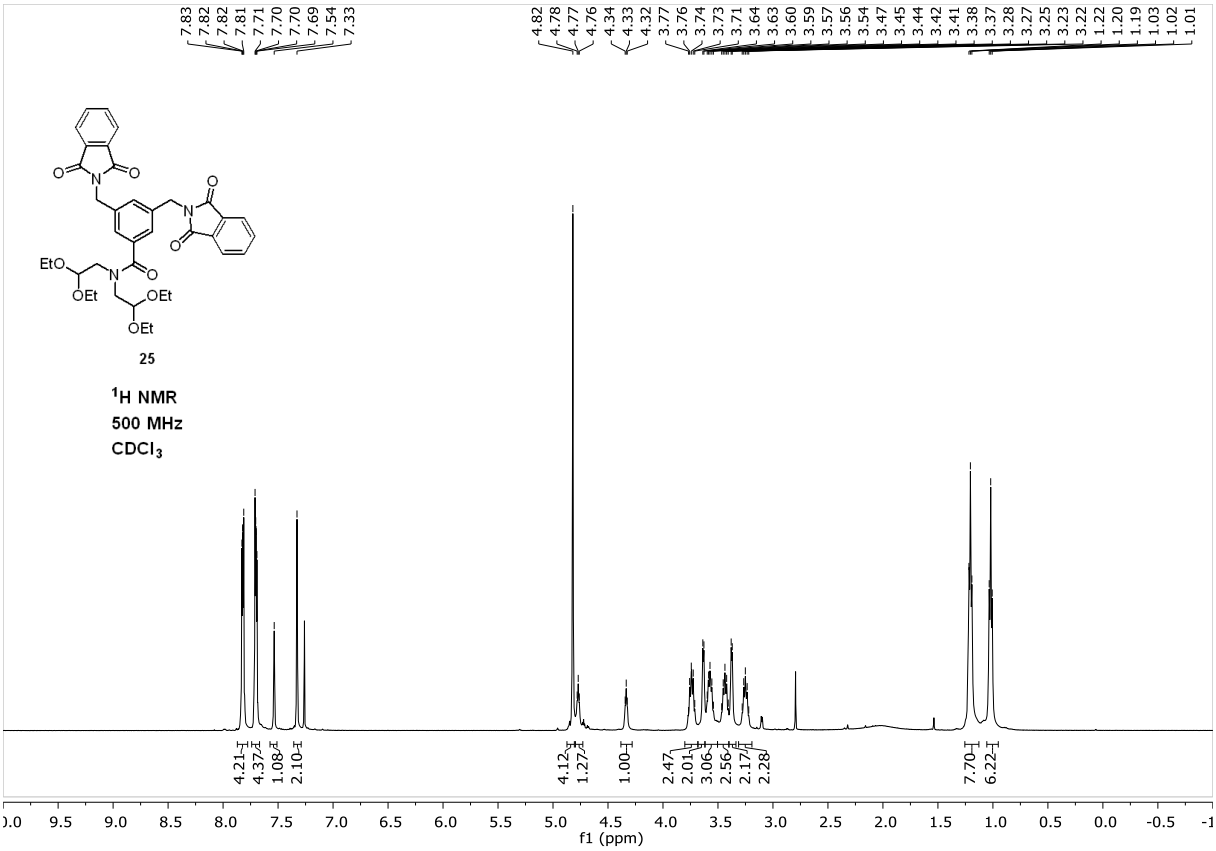


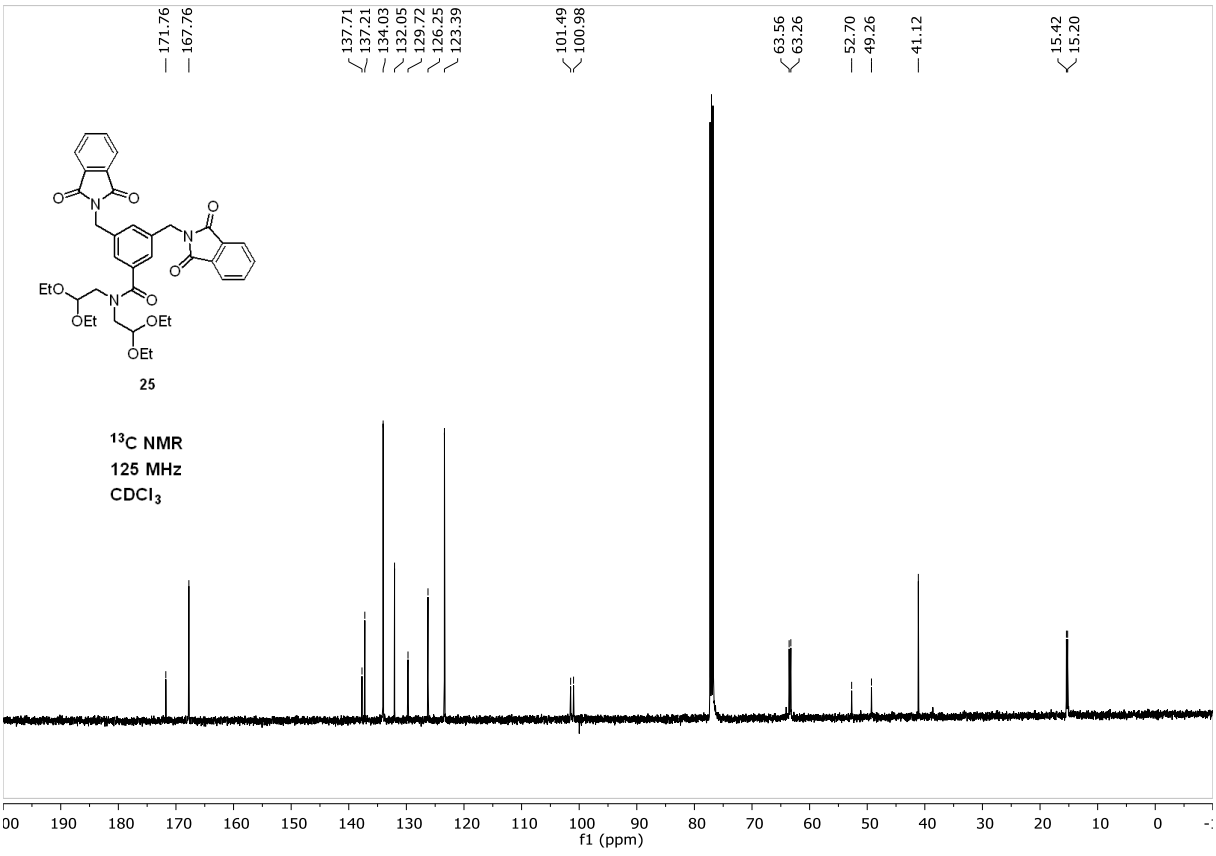
24

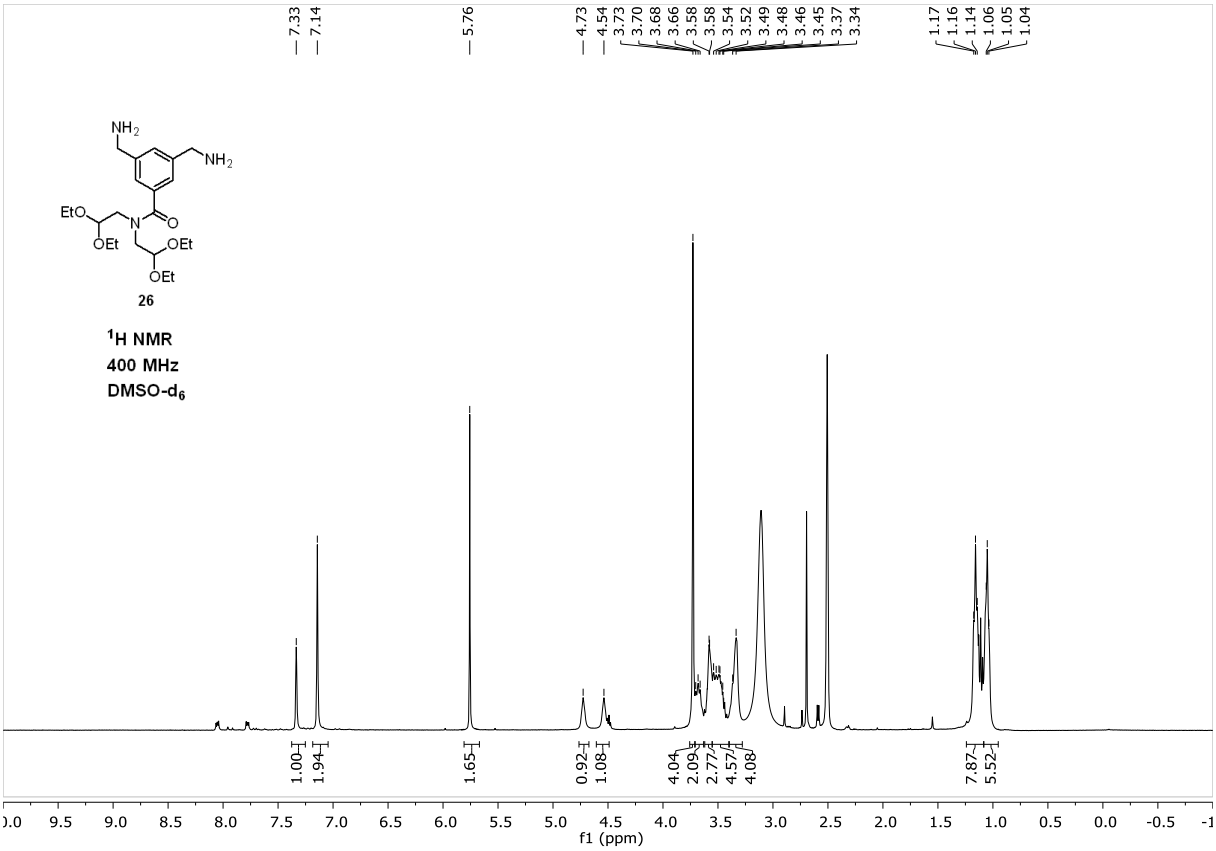
^{1}H NMR
400 MHz
DMSO-d_6

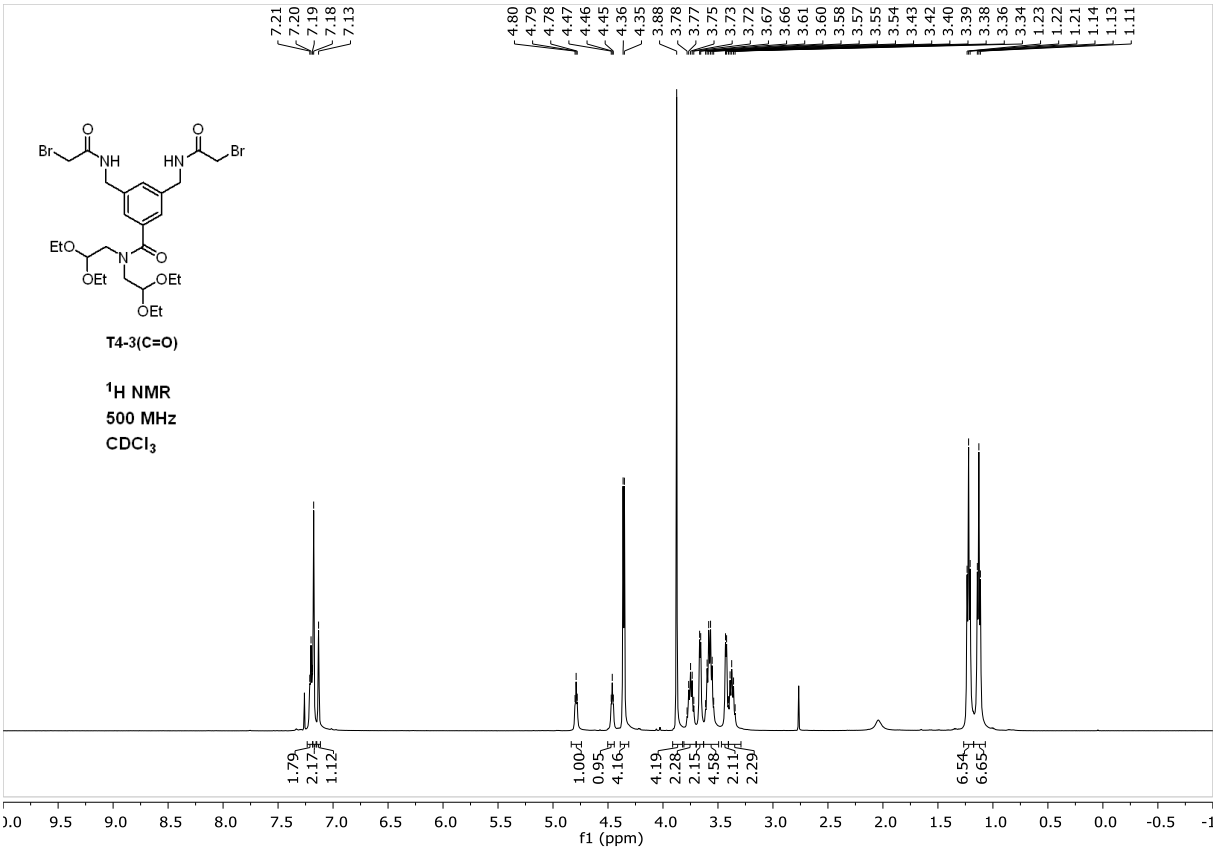
24

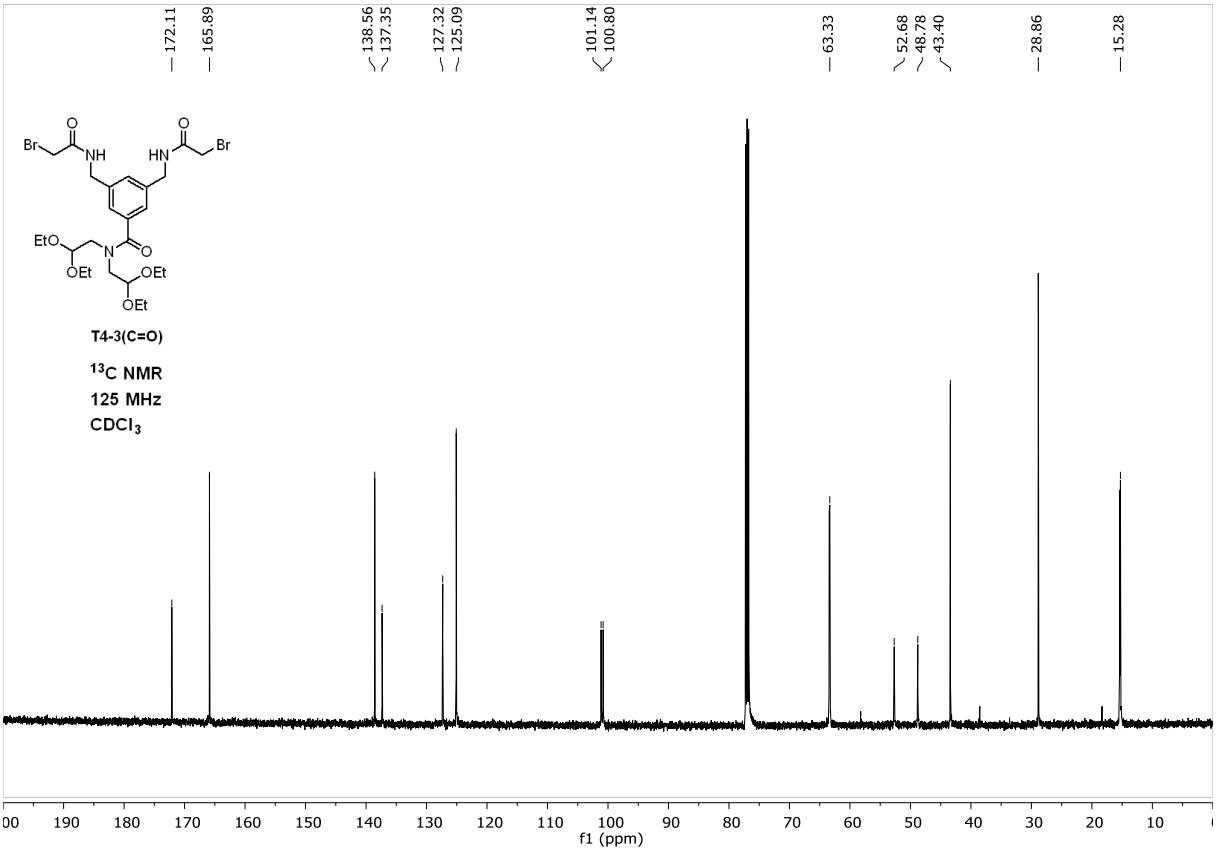
^{13}C NMR
125 MHz
DMSO

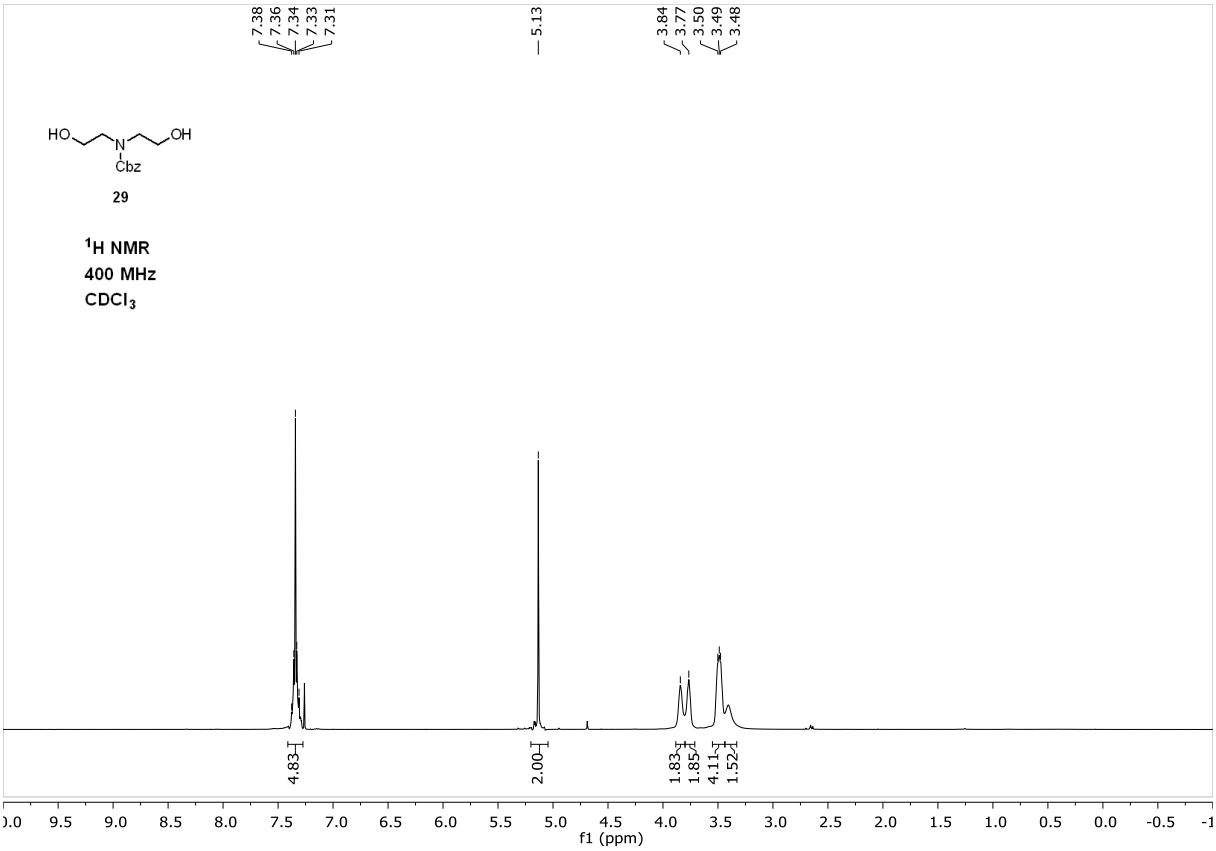

7.83
7.82
7.82
7.81
7.71
7.70
7.70
7.69
7.54
7.33
4.82
4.78
4.77
4.76
4.34
4.33
4.32
3.77
3.76
3.74
3.73
3.71
3.64
3.63
3.60
3.59
3.57
3.56
3.54
3.47
3.45
3.44
3.42
3.41
3.38
3.37
3.28
3.27
3.25
3.23
3.22
1.22
1.20
1.19
1.03
1.02
1.01
EtO
OEt
OEt
OEt
25
1H NMR
500 MHz
CDCl3
4.21
4.37
1.08
2.10
4.12
1.27
1.00
2.47
2.01
3.06
2.56
2.17
2.28
7.70
6.22
f1 (ppm)

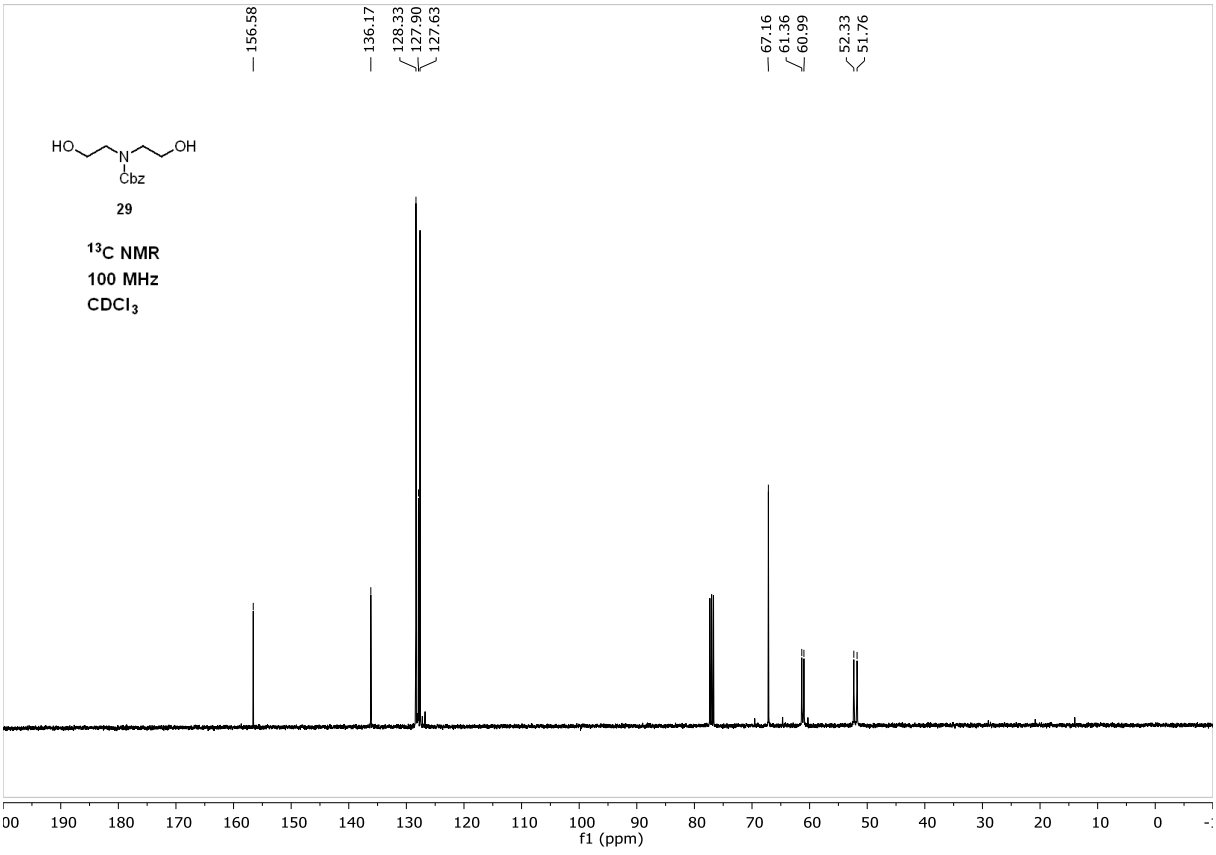

171.76
167.76
137.71
137.21
134.03
132.05
129.72
126.25
123.39
101.49
100.98
63.56
63.26
52.70
49.26
41.12
15.42
15.20
EtO
OEt
OEt
OEt
25
13C NMR
125 MHz
CDCl3
f1 (ppm)

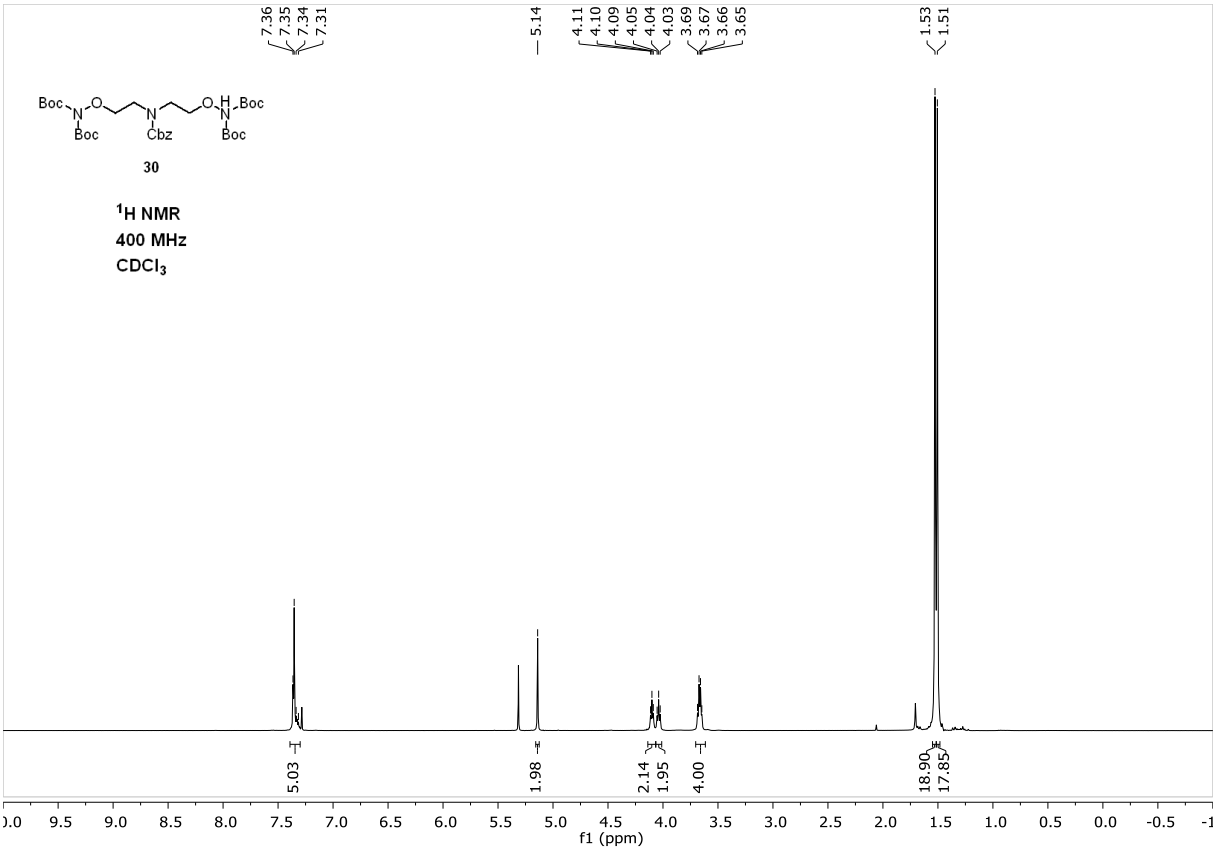

7.33
7.14
5.76
4.73
4.54
3.73
3.70
3.68
3.66
3.58
3.58
3.54
3.52
3.49
3.48
3.46
3.45
3.37
3.34
1.17
1.16
1.14
1.06
1.05
1.04
NH2
NH2
EtO
OEt
N
O
OEt
OEt
26
1H NMR
400 MHz
DMSO-d6
1.00
1.94
1.65
0.92
1.08
4.04
2.09
2.77
4.57
4.08
7.87
5.52
f1 (ppm)

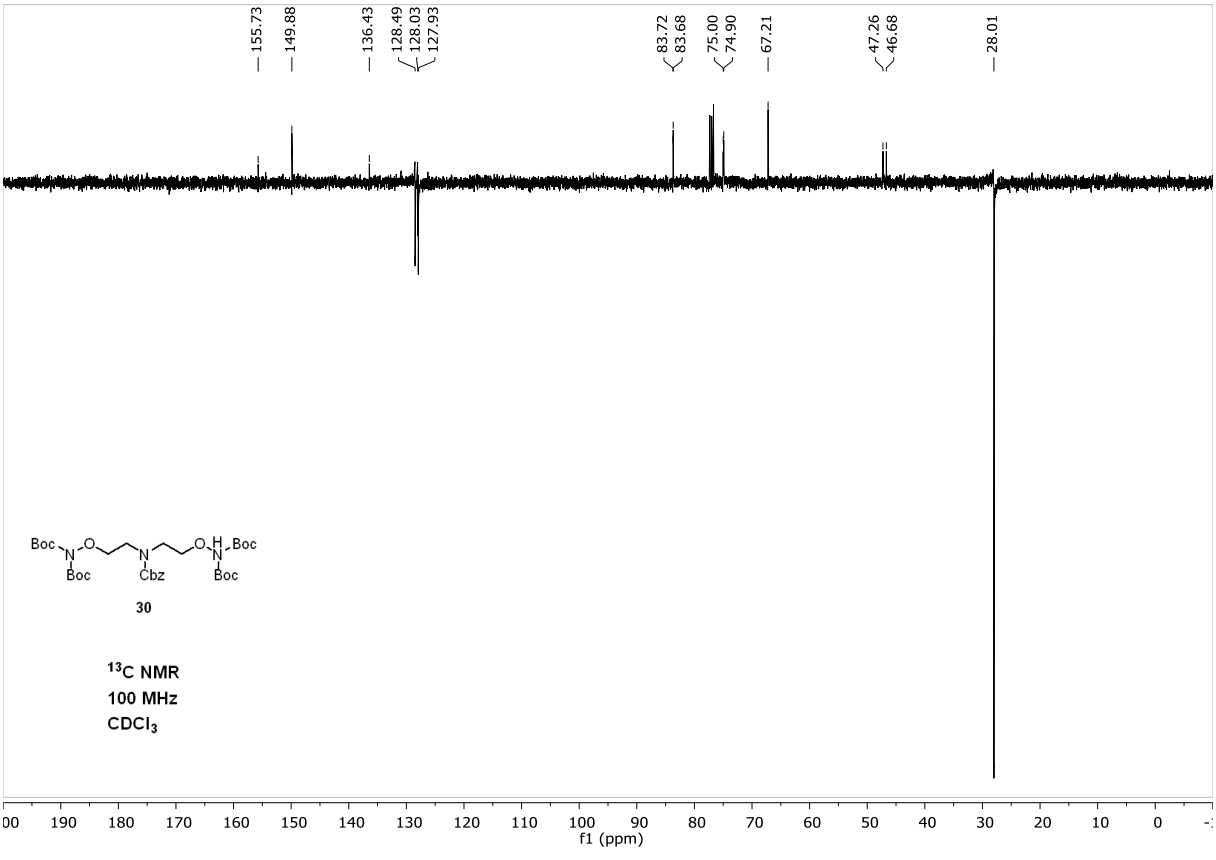

T4-3(C=O)
^{1}H NMR
500 MHz
CDCl$_3$
f1 (ppm)

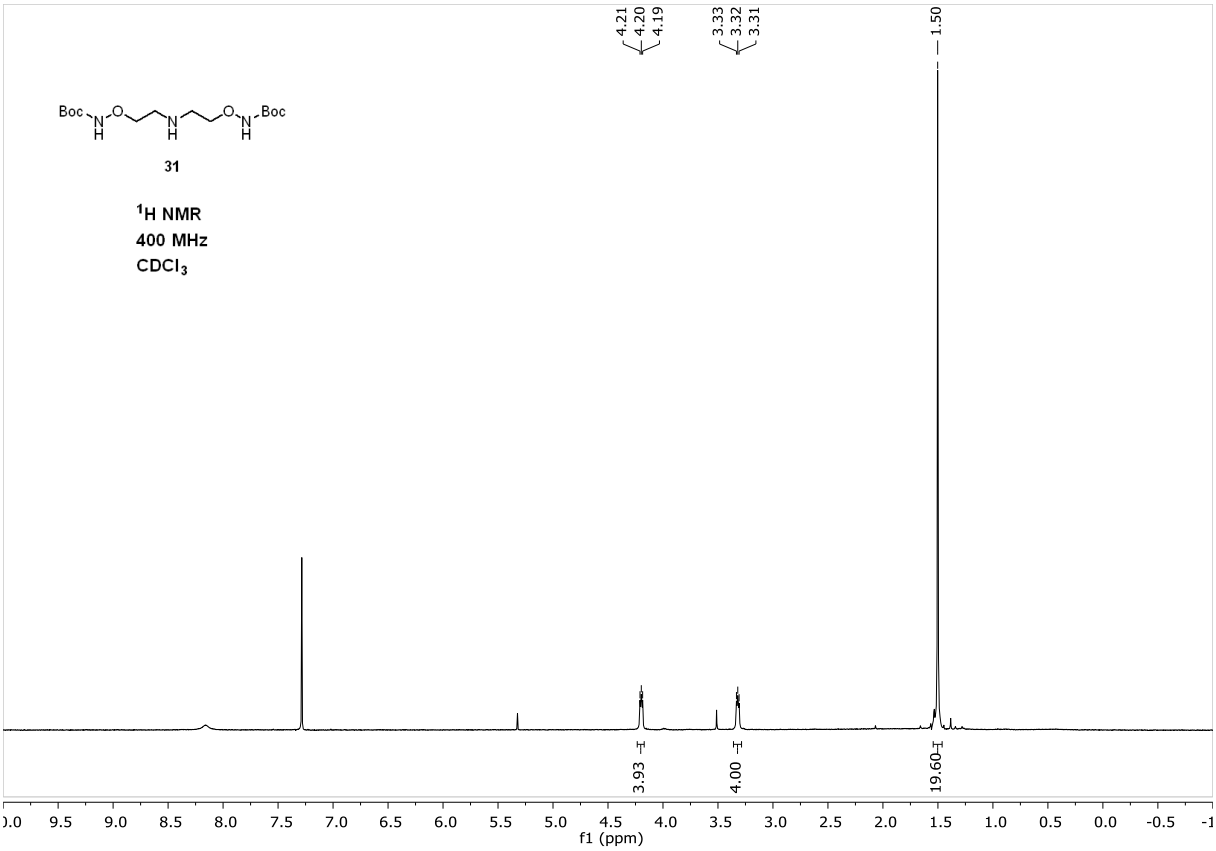

T4-3(C=O)
^{13}C NMR
125 MHz
CDCl$_3$
f1 (ppm)

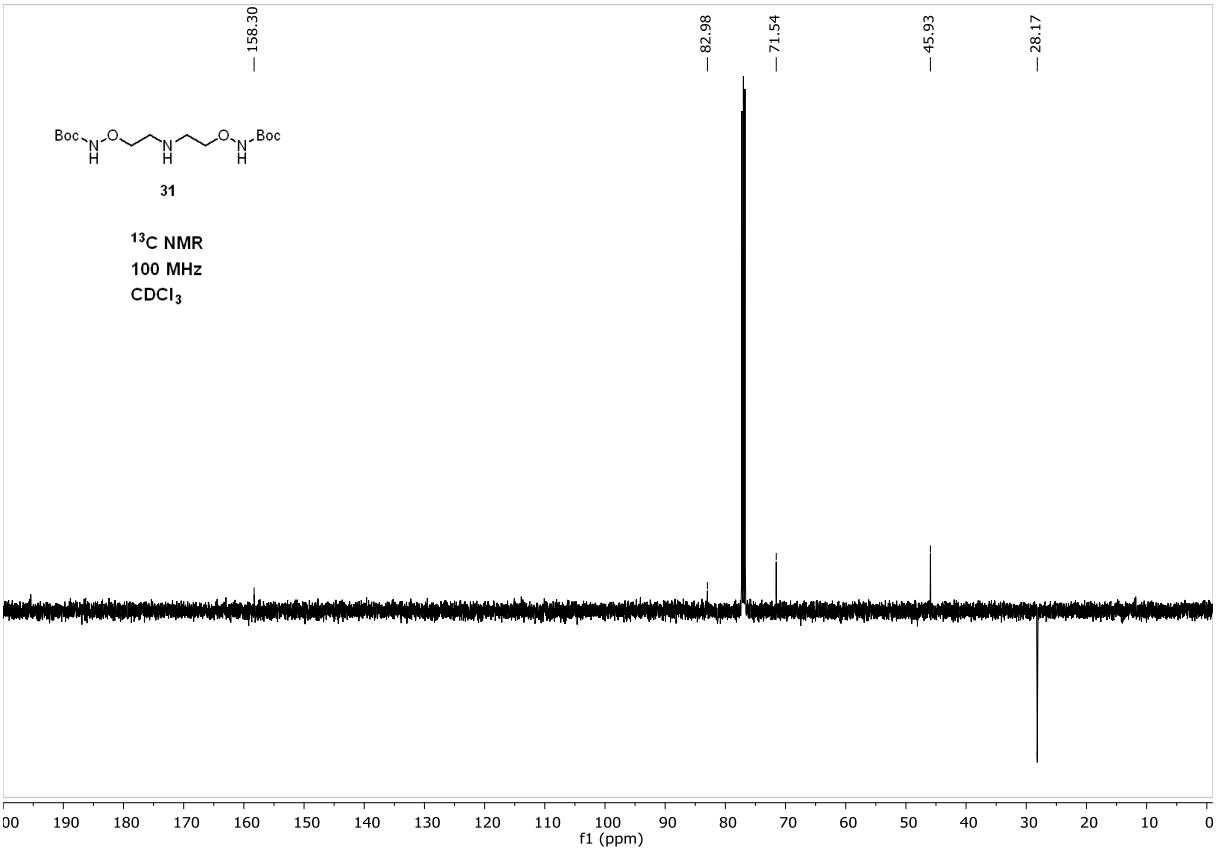

7.38
7.36
7.34
7.33
7.31
5.13
3.84
3.77
3.50
3.49
3.48
HO
N
OH
Cbz
29
1H NMR
400 MHz
CDCl3
4.83
2.00
1.83
1.85
4.11
1.52
f1 (ppm)

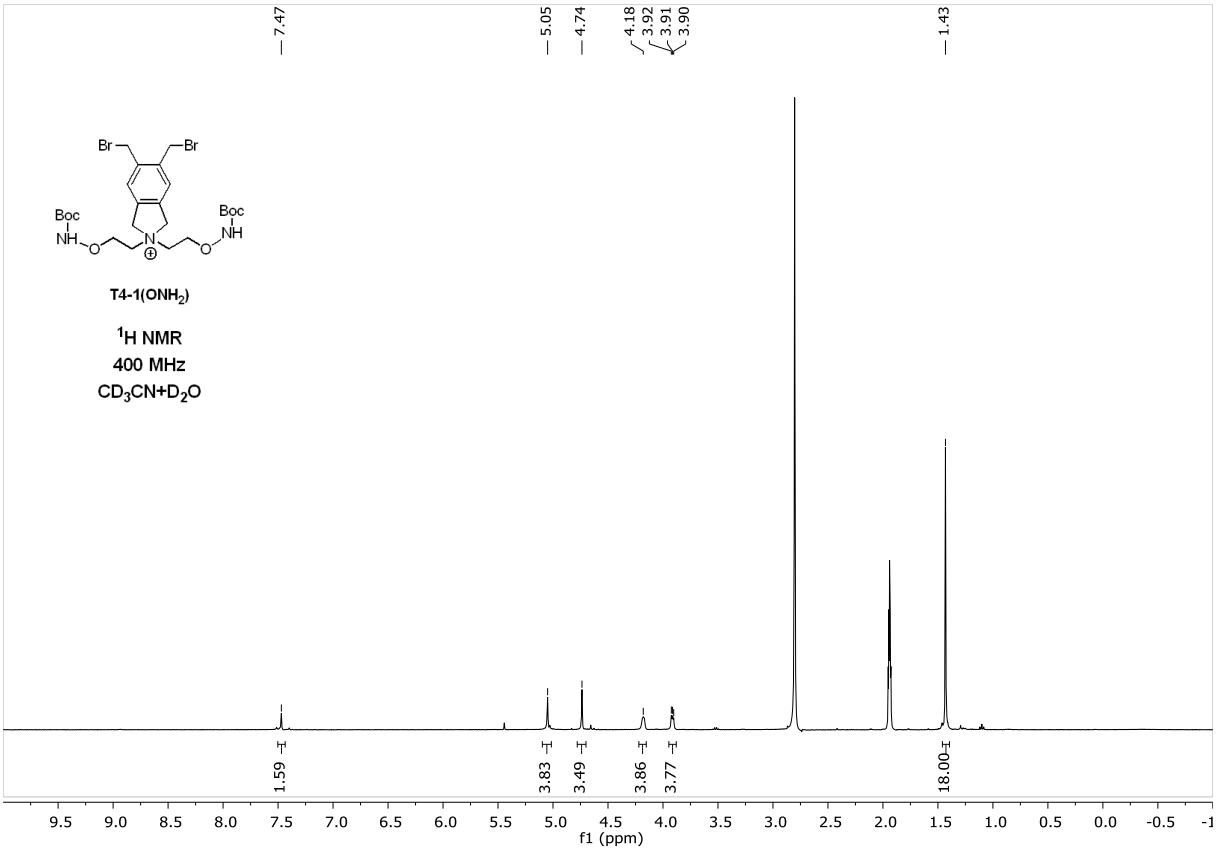

156.58
136.17
128.33
127.90
127.63
67.16
61.36
60.99
52.33
51.76
HO
N
OH
Cbz
29
13C NMR
100 MHz
CDCl3
f1 (ppm)

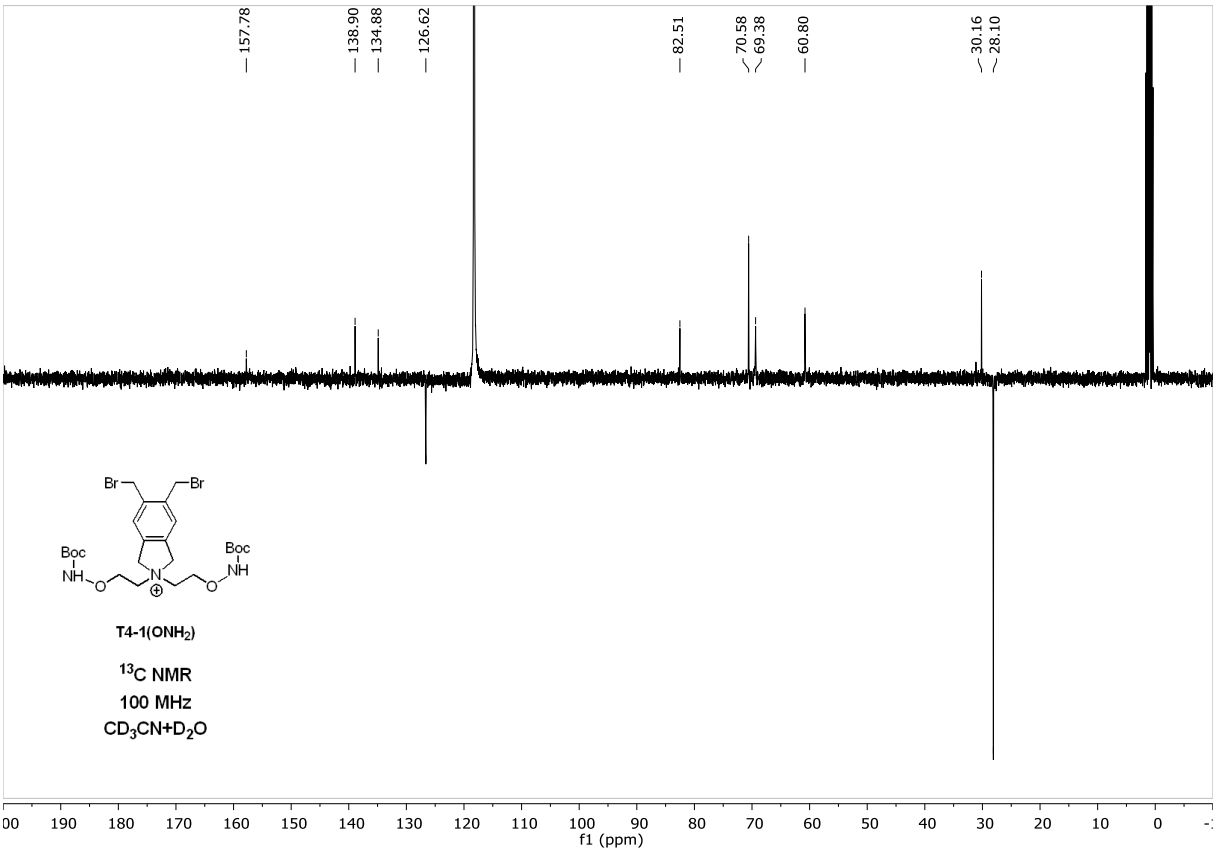

7.36
7.35
7.34
7.31
5.14
4.11
4.10
4.09
4.05
4.04
4.03
3.69
3.67
3.66
3.65
1.53
1.51
Boc
N
O
Boc
N
Cbz
O
H
N
Boc
Boc
30
1H NMR
400 MHz
CDCl3
5.03
1.98
2.14
1.95
4.00
18.90
17.85
f1 (ppm)

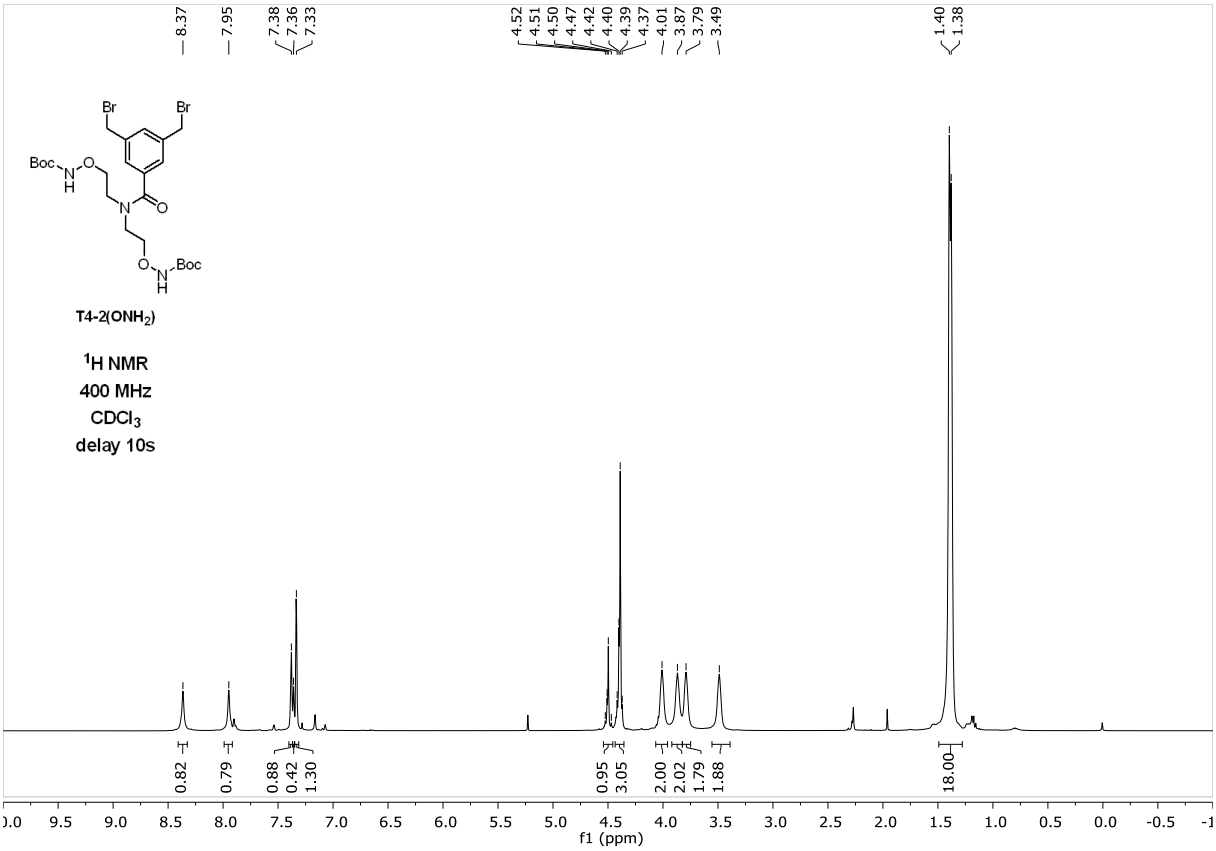

155.73
149.88
136.43
128.49
128.03
127.93
83.72
83.68
75.00
74.90
67.21
47.26
46.68
28.01
Boc
N
O
Boc
N
Cbz
O
H
N
Boc
Boc
30
13C NMR
100 MHz
CDCl3
f1 (ppm)

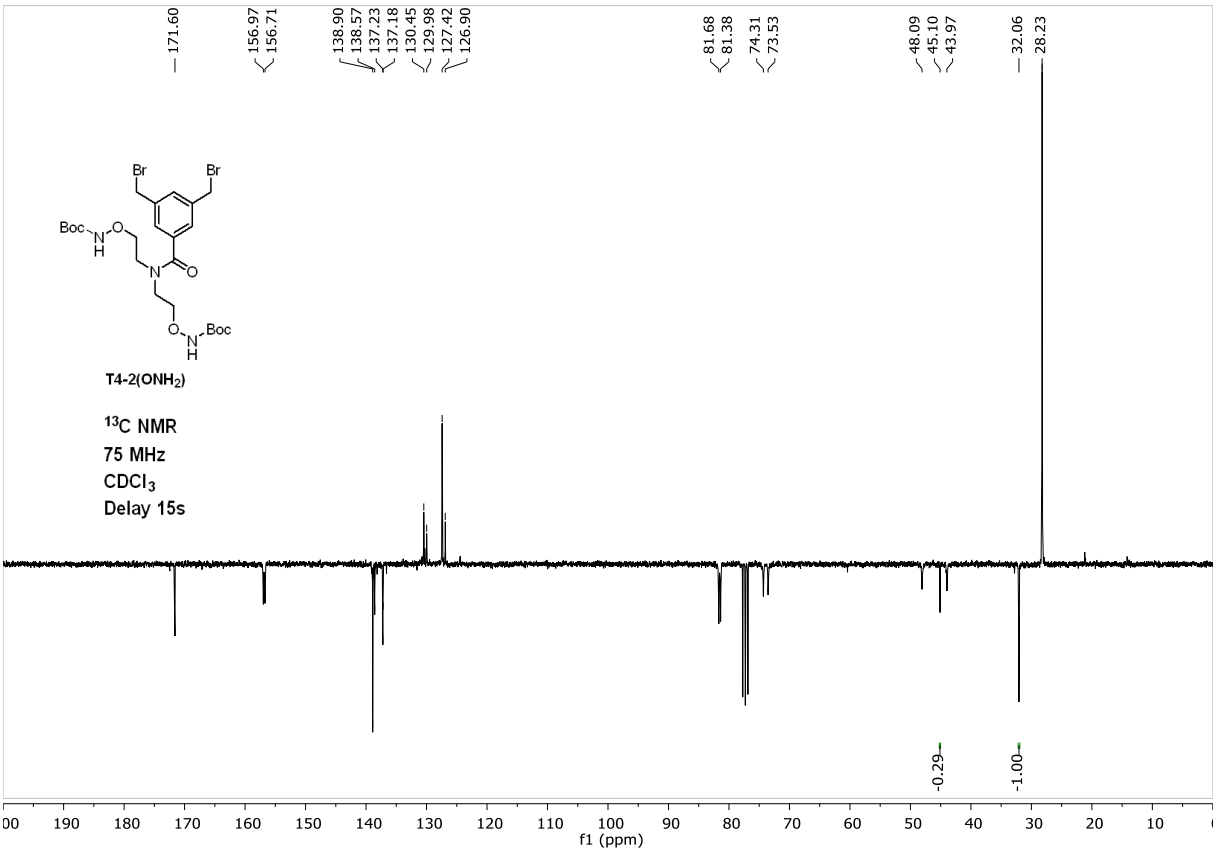

4.21
4.20
4.19
3.33
3.32
3.31
1.50
Boc
O
N
H
Boc
31
1H NMR
400 MHz
CDCl3
3.93
4.00
19.60
f1 (ppm)

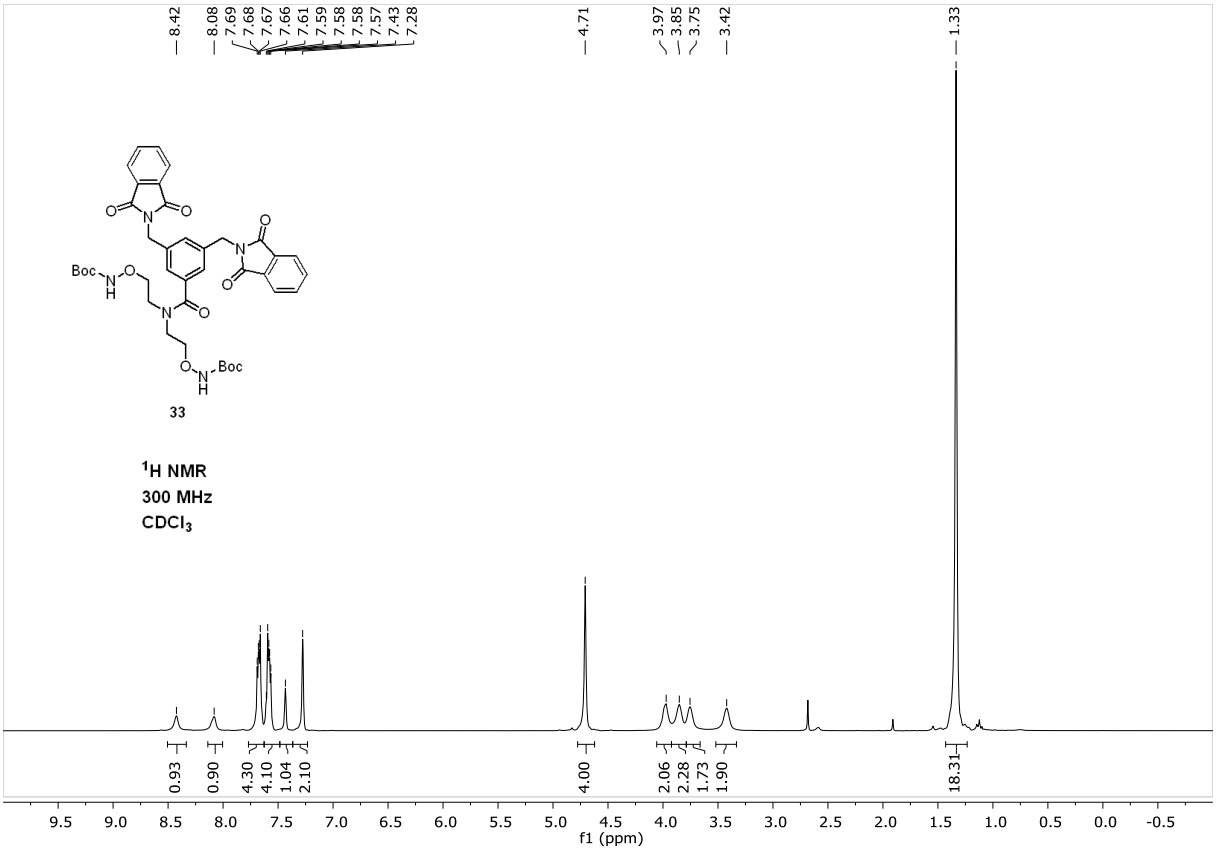

158.30
82.98
71.54
45.93
28.17
Boc
O
N
H
Boc
31
13C NMR
100 MHz
CDCl3
f1 (ppm)

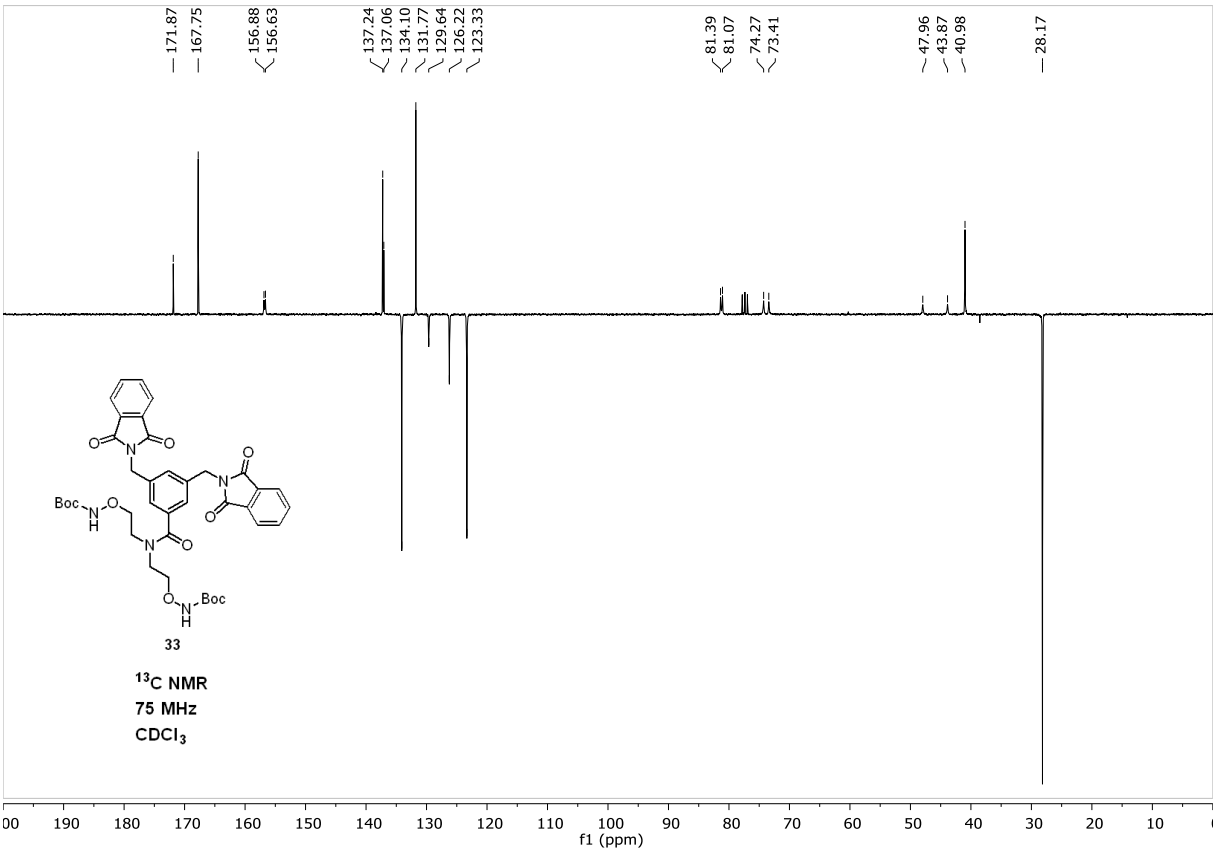

7.47
5.05
4.74
4.18
3.92
3.91
3.90
1.43
Br
Br
Boc
NH
Boc
NH
O
O
N
T4-1(ONH2)
1H NMR
400 MHz
CD3CN+D2O
1.59
3.83
3.49
3.86
3.77
18.00
f1 (ppm)

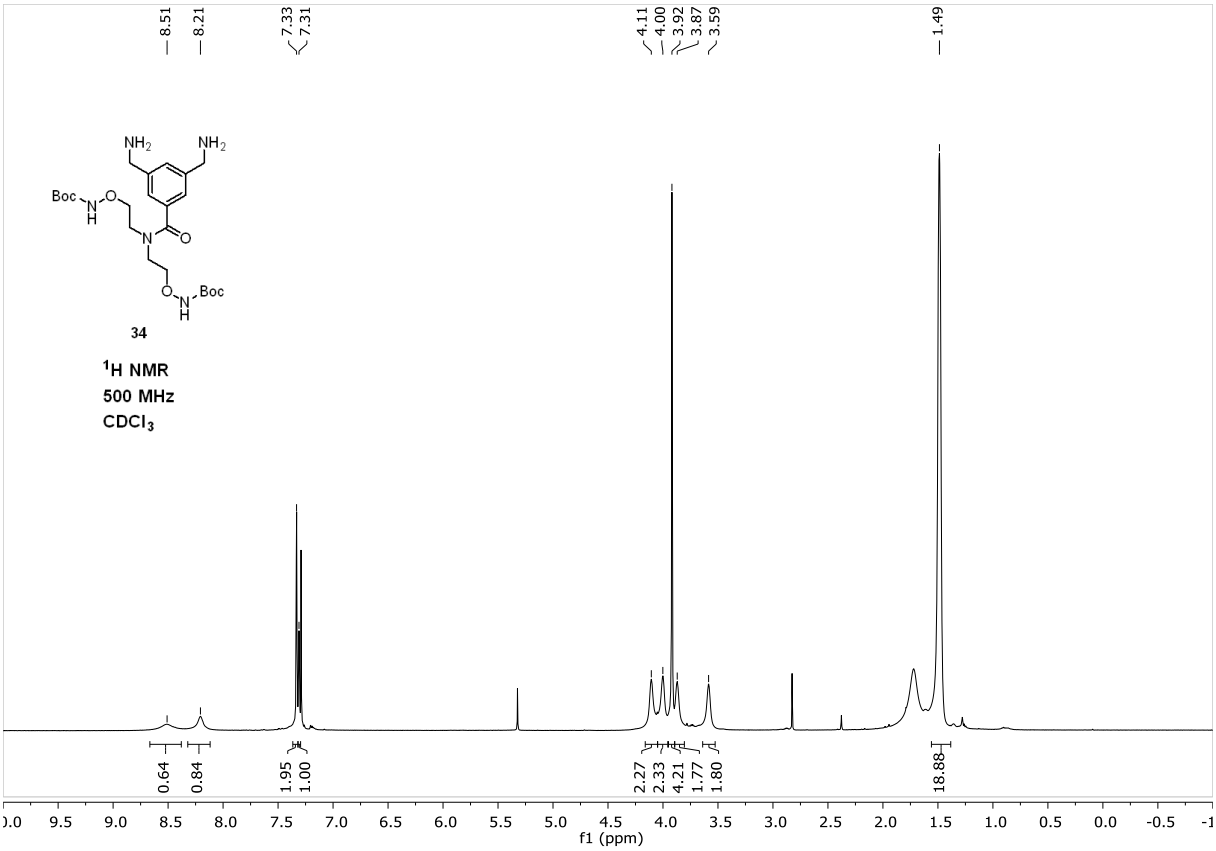

157.78
138.90
134.88
126.62
82.51
70.58
69.38
60.80
30.16
28.10
Br
Br
Boc
NH
Boc
NH
O
O
N
T4-1(ONH2)
13C NMR
100 MHz
CD3CN+D2O
f1 (ppm)

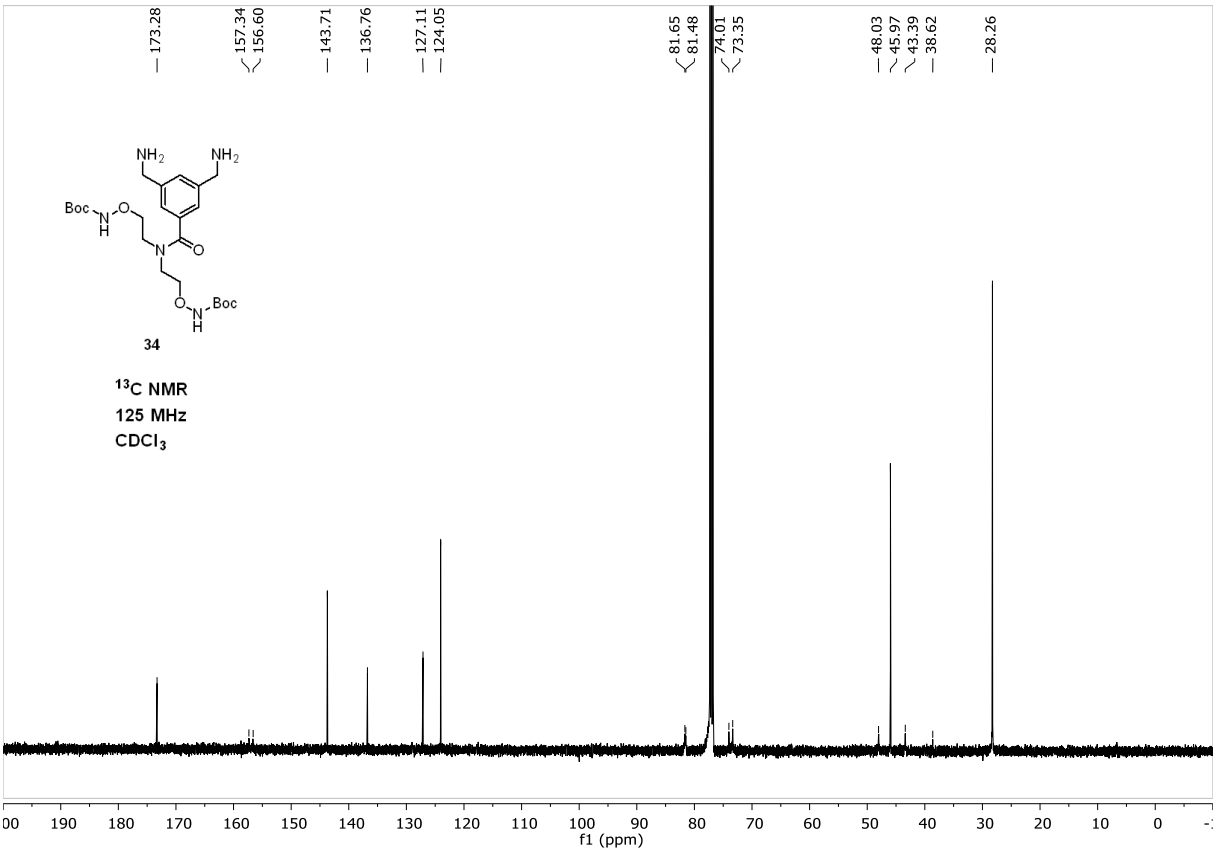

8.37
7.95
7.38
7.36
7.33
4.52
4.51
4.50
4.47
4.42
4.40
4.39
4.37
4.01
3.87
3.79
3.49
1.40
1.38
Br
Br
Boc
N
H
O
N
O
O
N
H
Boc
T4-2(ONH2)
1H NMR
400 MHz
CDCl3
delay 10s
0.82
0.79
0.88
0.42
1.30
0.95
3.05
2.00
2.02
1.79
1.88
18.00
f1 (ppm)

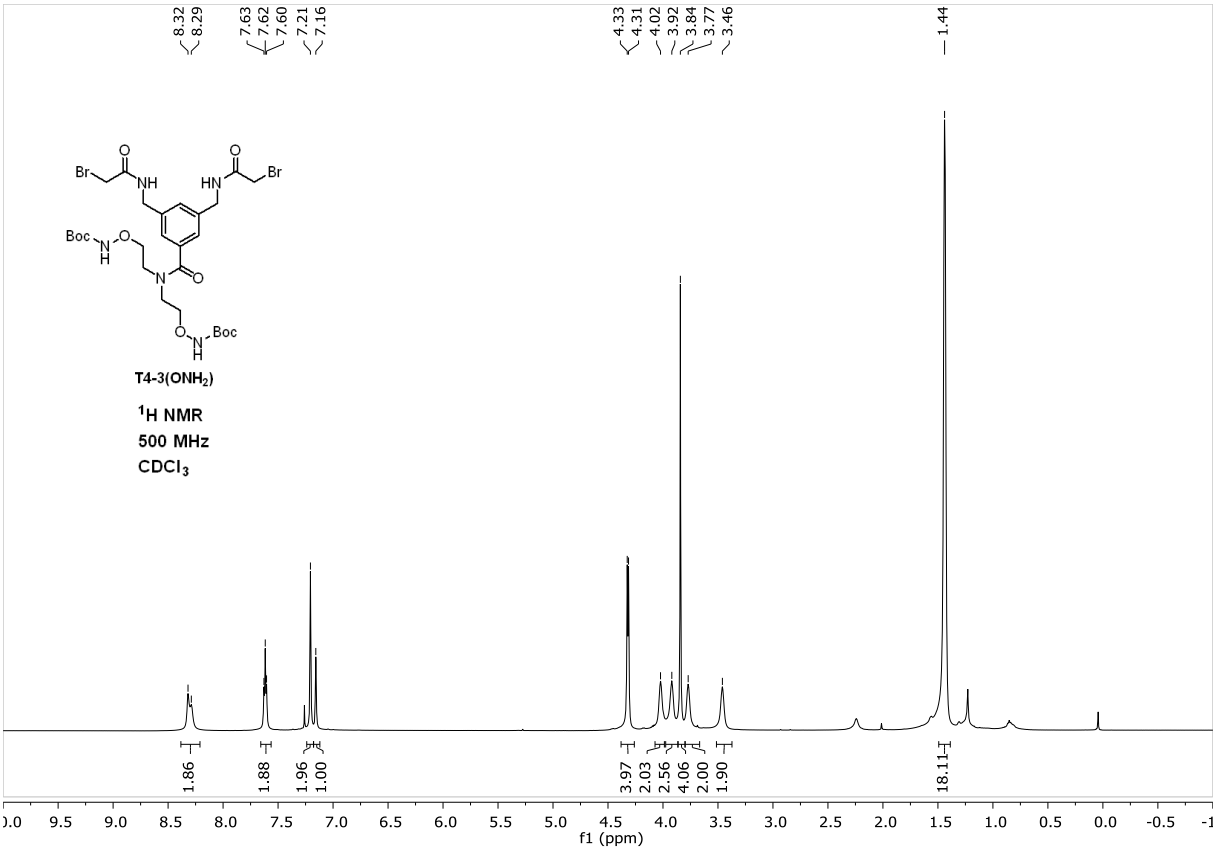

171.60
156.97
156.71
138.90
138.57
137.23
137.18
130.45
129.98
127.42
126.90
81.68
81.38
74.31
73.53
48.09
45.10
43.97
32.06
28.23
Br
Br
Boc
N
H
O
N
O
O
N
H
Boc
T4-2(ONH2)
13C NMR
75 MHz
CDCl3
Delay 15s
-0.29
-1.00
f1 (ppm)

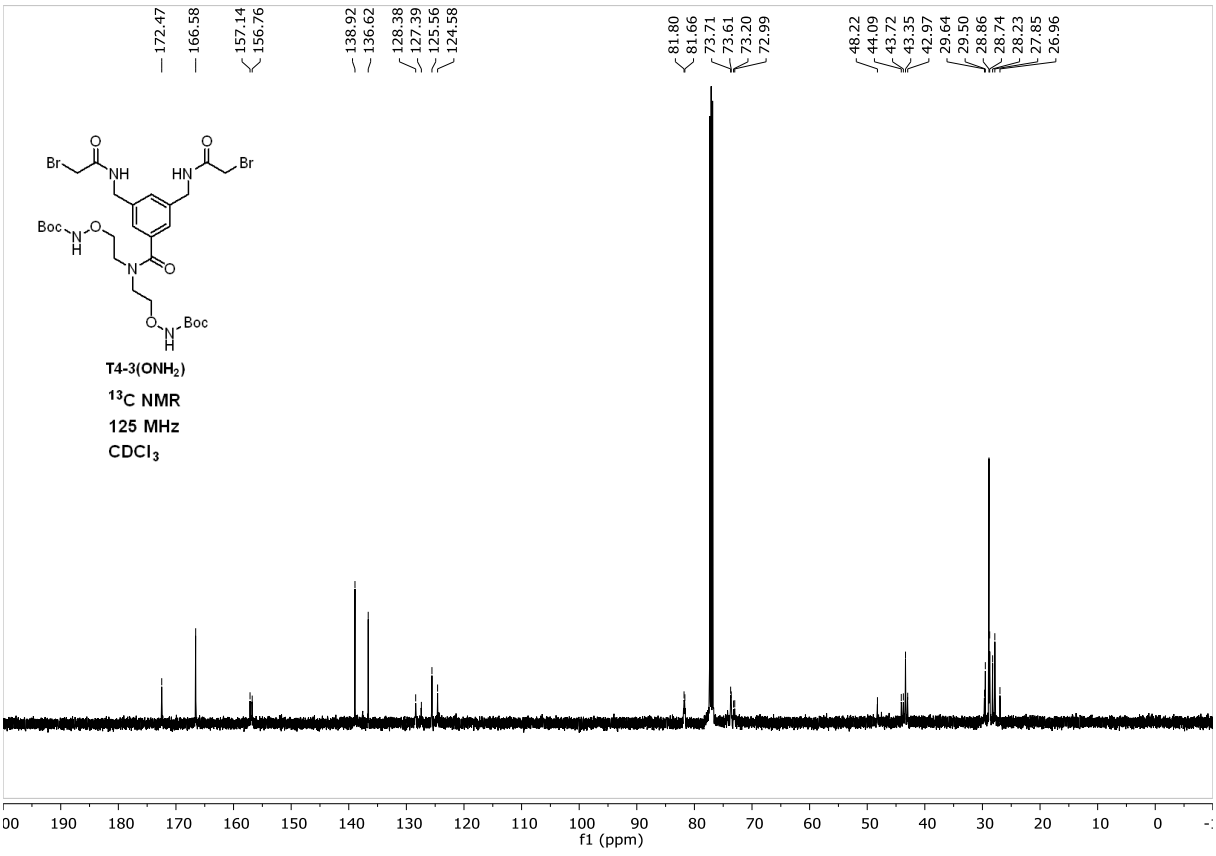

8.42
8.08
7.69
7.68
7.67
7.66
7.61
7.59
7.58
7.58
7.57
7.43
7.28
4.71
3.97
3.85
3.75
3.42
1.33
Boc
Boc
33
1H NMR
300 MHz
CDCl3
0.93
0.90
4.30
4.10
1.04
2.10
4.00
2.06
2.28
1.73
1.90
18.31
f1 (ppm)


171.87
167.75
156.88
156.63
137.24
137.06
134.10
131.77
129.64
126.22
123.33
81.39
81.07
74.27
73.41
47.96
43.87
40.98
28.17
Boc
Boc
33
13C NMR
75 MHz
CDCl3
f1 (ppm)


8.51
8.21
7.33
7.31
4.11
4.00
3.92
3.87
3.59
1.49
34
1H NMR
500 MHz
CDCl3
0.64
0.84
1.95
1.00
2.27
2.33
4.21
1.77
1.80
18.88
f1 (ppm)


173.28
157.34
156.60
143.71
136.76
127.11
124.05
81.65
81.48
74.01
73.35
48.03
45.97
43.39
38.62
28.26
34
13C NMR
125 MHz
CDCl3
f1 (ppm)


8.32
8.29
7.63
7.62
7.60
7.21
7.16
4.33
4.31
4.02
3.92
3.84
3.77
3.46
1.44
T4-3(ONH2)
1H NMR
500 MHz
CDCl3
1.86
1.88
1.96
1.00
3.97
2.03
2.56
4.06
2.00
1.90
18.11
f1 (ppm)


172.47
166.58
157.14
156.76
138.92
136.62
128.38
127.39
125.56
124.58
81.80
81.66
73.71
73.61
73.20
72.99
48.22
44.09
43.72
43.35
42.97
29.64
29.50
28.86
28.74
28.23
27.85
26.96
T4-3(ONH2)
13C NMR
125 MHz
CDCl3
f1 (ppm)

10 References

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