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Supporting Information

Revealing the Indispensable Role of the RFamide Functionality using a Novel Acid Labile Benzofuranone based Amine (ALBA) Linker

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Biological Assays

Inositol-(1, 4, 5)-triphosphates accumulation assay

COS-7 cells electroporated with GPR54 were seeded in 12 well plates (100 000 cells/1ml) and incubated for 24 hours in a 37 °C 5% CO_2 incubator. Thereafter, the medium was aspirated and cells were rinsed with 0.5 mL of Med199 medium containing 2% FCS for 15 minutes. Cells were radiolabelled with 0.5 mL 2uCi/ mL myo [2-3H (N)]-inositol (Perken-Elmer) in Med199 / 2 %FCS and incubated for 20 hours. After which, cells were incubated with Buffer-I (140mM NaCl, 4 mM KCl, 20 mM Hepes, 8 mM glucose, 0.1 % bovine serum albumin (BSA), 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM LiCl) for 15 minutes at 37 ˚C. Peptides were diluted (0-1 µM) in Buffer- I and cells were stimulated for 90 minutes at 37 ˚C. The media was aspirated and 1 mL 10 mM formic acid was added to each well and the cells were extracted by incubation at 4 ˚C for 30 minutes.

Columns packed with Dowex 1x 8-200 ion-exchange resin (Sigma) were washed sequentially with 3 mL 3M ammonium formate / 0.1 M formic acid and 10 mL distilled water. The cell extracts were added to the columns, followed by 10 mL distilled water and then 5 mL 0.1 M ammonium formate / 0.1 M formic acid. Samples were then eluted in 1 mL 1M ammonium formate / 0.1M formic acid. Scintillation vials were prepared with 2.6 mL scintillation fluid and 1 mL of elute was added to each vial and mixed well. Samples were counted in a Tri-carb 2100TR liquid scintillation analyser (Packard).

Chemical Syntheses

All chemicals were obtained from Sigma Aldrich unless stated otherwise.

Analytical Tools

HPLC:

HPLC analysis was performed on an Agilent 1100 series HPLC system, consisting of a quaternary pump (G1311A), a degasser (G1322A), an FLD detector (G1321A) and a DAD detector; column: Vydac peptide C8, 4.6 mm x 150 mm, 5 µm particle diameter size. Analyses were performed using a linear gradient of A: H_2O containing 0.1 % TFA and B: MeCN (HPLC grade) containing 0.1 % TFA with a flow rate of 0.8 mL/min, Retention times (t_R) are denoted in minutes. All analyses use standard gradient unless stated otherwise.

Standard gradient: 5 min 0 % B, 25 min 0 – 100 % B, 27.5 min 100 – 0 % B, 30 min 0 % B.

LCMS:

Samples were analyzed on an HPLC-ESI-MS system consisting of a Finnigan Deka XP Plus ESI-MS detector connected to an Agilent 1100 capillary LC with a G1376A capillary pump, a G1377A Micro-autosampler and a G1325B DAD detector with micro flow cell. The system was run with a spray voltage of 5 kV, capillary temperature of 275 °C, a capillary voltage of 15 V in single MS mode. The LC-system contained a Zorbax-SB-C18 0.5 x 35 mm 3.5 µm particle size column and was run with a gradient of solvent A: H_2O containing 0.1% TFA and B: MeCN (LCMS grade) containing 0.1 % TFA. All analyses use standard gradient unless stated otherwise.

Standard Gradient: 2.5 min 0 % B, 22.5 min 0 – 100 % B, 25 min 100 – 0 % B, 28 min 0 % B at a flow rate of 200 µL/min.

HPLC-ESI-MS data were processed using the Xcalibur software package (version 2.0, Thermo Electron Corporation, MA, USA).

Preparative HPLC:

Purification of mg quantities of peptides and dyes was carried out using a preparative HPLC system (Agilent 1100 prep-HPLC system), equipped with a preparative autosampler (G2260A), preparative scale pumps (G1361A), a fraction collector (G1364B-prep) and a multiwavelength UV detector (G13658 MWD with preparative flow cell). Material was separated at a flow rate of 20 mL/min on an Agilent RP-C-18 column (21.2 x 150 mm, 10 µm particle size), using a H_2O/acetonitrile gradient and a detection wavelength of 210 nm.

Solvent A: H_2O), 0.1% TFA; solvent B: Acetonitrile, 0.1% TFA.

Method A: 15% B for 5 minutes, the increase to 55% B over 20 minutes, then 100% B over 10 minutes.

Method B: 5% to 40% B in 20 minutes, 40% B to 95 % B in 5 minutes.

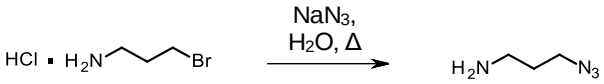
NMR Instrumentation:

^{1}H NMR spectra were recorded on a Bruker AVA500 (500 MHz) or a Bruker AVA400 (400 MHz) spectrometer. Chemical shifts (δ) are quoted in parts per million (ppm) downfield of tetramethylsilane, using residual protonated solvent as internal standard (e.g. CDCl3 at 7.27 ppm). Abbreviations used in the description of resonances are: s (singlet), d (doublet), t (triplet), q, (quartet), app (apparent), br (broad), m (multiplet). Coupling constants (*J*) are quoted to the nearest 0.1 Hz.

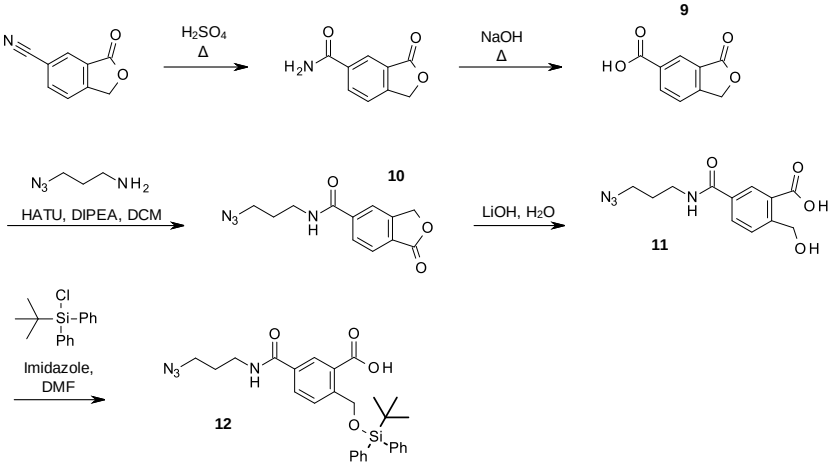
Proton-decoupled ^{13}C NMR spectra were recorded on a Bruker AVA500 (125.8 MHz) Spectrometer or a Bruker AVA400 (100.6 MHz) spectrometer. Chemical shifts (δ) are quoted in parts per million (ppm) downfield of tetramethylsilane, using deuterated solvent as internal standard (e.g. $CDCl_3$ at 77.0 ppm). Assignments were made using the DEPT sequence with secondary pulses at 90° and 135°.

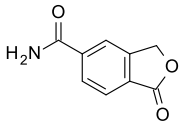
Synthesis of ALBA Linker

3-Azidopropylamine:

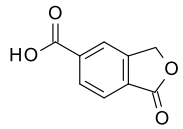


1-Bromo-3-aminopropane hydrochloride (1.0 g, 5.73 mmol) was stirred in water (5 mL) followed by the addition of NaN_3 (1.1 g, 17.0 mmol) in water (5 mL). The mixture was heated to reflux overnight then concentrated to a third of the volume under partial pressure. The remaining mixture was cooled to 0ºC and Et_2O (10 mL) and KOH pellets (1.2 g) added. The organic layer was separated and the aqueous washed twice more with Et_2O and the organics pooled and dried over $MgSO_4$ and concentrated to dryness. This gave a yellow oil (380 mg, 67 %) which was used without further purification. Rf = 0.17 (10:1:0.1 DCM/MeOH/7M NH_3 in MeOH); ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.38 (2H, t, *J* = 6.8 Hz, $N_3C\mathbf{H}_2$), 2.82 (2H, t, *J* = 6.8 Hz, $NH_2C\mathbf{H}_2$), 1.74 (2H, quint, *J* = 6.8 Hz, $CH_2C\mathbf{H}_2CH_2$); ^{13}C NMR (125.8 MHz, $CDCl_3$) δ 49.3 (CH_2), 39.4 (CH_2), 32.4 (CH_2). MS (ESI) Exact mass calcd for $C_3H_9N_4$ $[M+H]^+$ 101.08 found: 100.95. Data is in agreement with the literature[1].

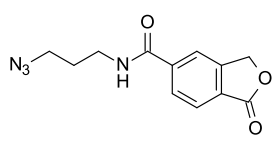




5-Carbamoylphthalide: 5-Cyanophthalide (4.10 g, 25.8 mmol) was stirred in 75 % sulphuric acid (40 mL) and the mixture heated to 80 °C for 3 hours. The solution was allowed to cool to rt then poured over ice, causing a white solid to precipitate which was filtered under vacuum, washed with water and dried overnight at 120 °C to give 5-Carbamoylphthalide (4.25 g, 93 %). R_f = 0.52 (10 % MeOH/DCM); ^{1}H NMR (500 MHz, $(CD_3)_2SO$) δ 8.22 (1H, s, N$\mathbf{H}_2$), 8.11 (1H, s, N$\mathbf{H}_2$), 8.03 (1H, t, *J* = 8.0 Hz, Ar**H**), 7.92 (1H, d, *J* = 8.0 Hz, Ar**H**), 7.66 (1H, s, Ar**H**), 5.47 (2H, s, C$\mathbf{H}_2$); ^{13}C NMR (125.8 MHz, $(CD_3)_2SO$) δ 170.1 (C), 167.0 (C), 147.4 (C), 139.6 (C), 128.1 (C), 127.1 (CH), 124.9 (CH), 122.1 (CH), 70.0 (CH_2); MS (ESI) Exact mass calcd for $C_9H_8NO_3$ $[M+H]^+$ 178.05, found: 178.16. Data is in agreement with the literature.[2]

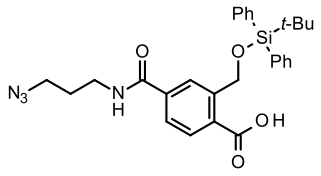


5-Carboxyphthalide (9): 5-Carbamoylphthalide (4.10 g, 23.02 mmol) was stirred in NaOH 2M solution and heated to reflux overnight. The solution was cooled to rt and acidified to pH1 using HCl which caused a white solid to precipitate. This was filtered under vacuum, washed with water and dried at 120 °C overnight to give 5-Carboxyphthalide **10** (4.01 g, 98 %). R_f = 0.33 (10 % MeOH/DCM); ^{1}H NMR (500 MHz, $(CD_3)_2SO$) δ 13.51 (1H, br s, COO**H**), 8.22 (1H, s, Ar**H**), 8.10 (1H, dd, *J* = 8.0, 0.6 Hz, Ar**H**), 7.95, (1H, d, *J* = 8.0 Hz, Ar**H**), 5.47 (2H, s, C$\mathbf{H}_2$); ^{13}C NMR (125.8 MHz, $(CD_3)_2SO$) δ 170.4 (C), 169.9 (C), 166.5 (C), 147.6 (C), 135.9 (C), 129.8 (CH), 125.1 (CH), 124.1 (CH), 70.1 (CH_2); MS (ESI) Exact mass calcd for $C_9H_7O_4$ $[M+H]^+$ 179.03, found: 179.24. Data is in agreement with the literature.[2]

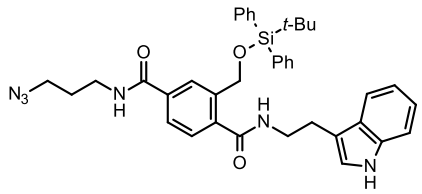


***N*-(3-azidopropyl)-1-oxo-3H-*iso*benzofuran-5-carboxamide (10):** 3-Amino-1-propylazide (380 mg, 3.80 mmol), 5-carboxyphthalide **9** (676 mg, 3.80 mmol) and HBTU (1.73 g, 4.56 mmol) were stirred in dry DCM (40 mL) at rt and DIPEA (1580 mL, 9.12 mmol) was added. The solution was stirred overnight then washed with sat. $NaHCO_3$ x 2. The organics were dried over $MgSO_4$ then concentrated to dryness. The residue was purified by flash chromatography (20 % EtOAc/Cyclohexane) to give compound **10** as a white solid (602 mg, 61 %). R_f = 0.28 (20 % EtOAc/Cyclohexane); ^{1}H NMR (500 MHz, $(CD_3)_2SO$) δ 8.79 (1H, t, *J* = 5.3 Hz, N**H**), 8.08 (1H, s, Ar**H**), 8.00 (1H, d, *J* = 8.0 Hz, Ar**H**), 7.93 (1H, d, *J* = 8.0 Hz, Ar**H**), 5.47 (2H,

s, C=OOC**H**$_2$), 3.43 (2H, t, *J* = 6.7 Hz, N_3C**H**$_2$), 3.38-3.34 (2H, m, NHC**H**$_2$), 1.80 (2H, quin, *J* = 6.7 Hz, CH_2C**H**$_2CH_2$); ^{13}C NMR (125.8 MHz, $(CD_3)_2SO$) δ 170.4 (C), 165.8 (C), 147.8 (C), 140.2 (C), 128.2 (C), 127.3 (CH), 125.2 (CH), 122.3 (CH), 70.3 (CH_2), 48.8 (CH_2), 37.2 (CH_2), 28.6 (CH_2); MS (ESI) Exact mass calcd for $C_{12}H_{13}N_4O_3$ $[M+H]^+$ 261.26, found: 260.94.

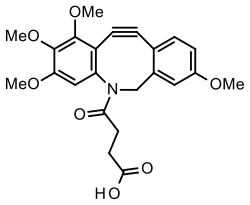


ALBA linker (12): Phthalide **10** (560 mg, 2.15 mmol) was stirred in MeOH (85 % in H_2O, 5 mL) and to this was added $LiOH.H_2O$ (90 mg, 2.15 mmol). The mixture was heated to 50 °C overnight. Solvents were removed under reduced pressure then the residue dissolved in pyridine (5 mL) and TBDPSiCl (7.1 mmol, 1.8 mL). The mixture was stirred overnight then saturated $NaHCO_3$ was added and extracted with DCM. The organics were dried ($MgSO_4$) then concentrated to dryness. The resulting oil was dissolved in MeOH (20 mL) and THF (7 mL) and treated with an aqueous solution of K_2CO_3 (700 mg, 7 mL) and stirred for 30 minutes. The solution was then concentrated to around one quarter. The mixture was cooled on ice and adjusted to pH 1 with $KHSO_4$ 1M, extracted with diethyl ether and dried over $MgSO_4$. The residue was purified using flash chromatography (9:1 Cyclohexane/EtOAc → EtOAC) to give the title compound **12** as a white solid (548 mg, 49 %). R_f = 0.33 (EtOAc); ^{1}H NMR (500 MHz, $(CD_3)_2SO$) δ 8.68 (1H, t, *J* = 5.6 Hz, N**H**), 8.40 (1H, d, *J* = 1.7 Hz, Ar**H**), 7.93 (1H, d, *J* = 8.0 Hz, Ar**H**), 7.80 (1H, dd, *J* = 8.0, 1.7 Hz, Ar**H**), 7.68 - 7.63 (4H, m, Ar**H**), 7.48 - 7.42 (6H, m, Ar**H**), 5.13 (2H, s, CC**H**$_2$O), 3.44 (2H, t, *J* = 6.8 Hz, N_3C**H**$_2$), 3.39 - 3.35 (2H, m, NHC**H**$_2$), 1.80 (2H, quin, *J* = 6.8 Hz, CH_2C**H**$_2CH_2$), 1.06 (9H, s, C(C**H**$_3$)$_3$); ^{13}C NMR (125.8 MHz, $(CD_3)_2SO$) δ 167.5 (C), 166.0 (C), 142.4 (C), 137.9 (C), 135.0 (CH), 132.7 (C), 130.2 (CH), 130.1 (C), 130.0 (CH), 128.0 (CH), 125.6 (CH), 125.1 (CH), 63.4 (CH_2), 48.5 (CH_2), 36.7 (CH_2), 28.3 (CH_2), 26.6 (CH), 18.9 (C), 14.1 (CH_3); MS (ESI) Exact mass calcd for $C_{28}H_{33}N_4O_4Si$ $[M+H]^+$ 517.23, found: 517.12.



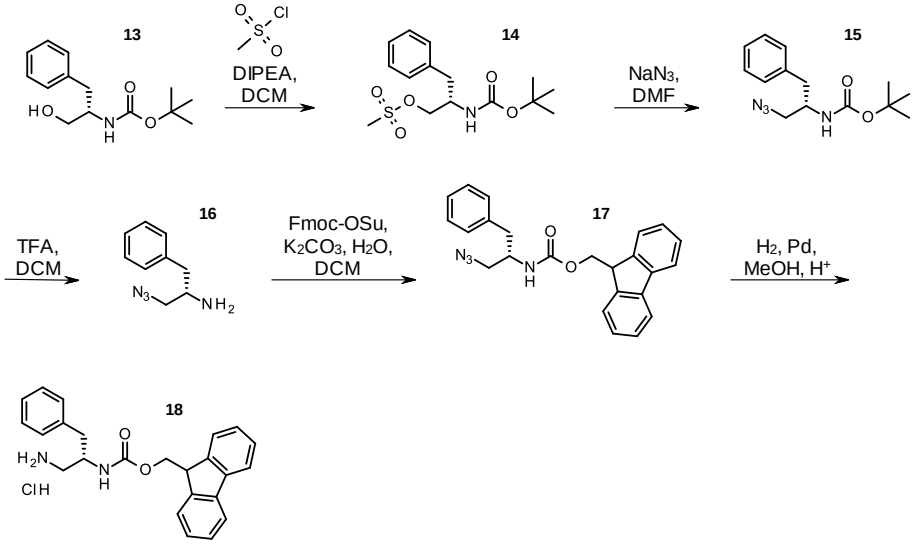
Tryptamine loaded ALBA linker: ALBA linker **12** (50.0 mg, 0.097 mmol) was stirred with tryptamine (19 mg, 0.116 mmol) in DCM 5 mL. To this was added HATU (44 mg, 0.116 mmol) and DIPEA (34 µL, 0.194 mmol) at room temperature with stirring. After 3 hours, the mixture was washed with water and the organics separated and dried over $MgSO_4$. Solvents were removed *in vacuo* and the residue purified using silica gel chromatography (8:2 EtOAc / Cyclohexane) to give the amide as a white solid (52 mg, 82 %). R_f = 0.49 (EtOAc); ^{1}H NMR (500 MHz, $(CD_3)_2SO$) δ 7.96 (1H, br s, N**H**), 7.70 (1H, dd, *J* = 8.0, 1.8 Hz, Ar**H**), 7.65 - 7.55 (6H, m, Ar**H**), 7.46 - 7.42 (2H, m, Ar**H**), 7.39 - 7.32 (5H, m, Ar**H**), 7.23 – 7.08 (3H, m, Ar**H**), 6.96 (1H, d, *J* = 2.3 Hz, Ar**H**), 6.20 (1H, br t, *J* = 5.8 Hz, N**H**), 4.84 (2H, s, C**H**$_2$Si), 3.79 - 3.72 (2H, m, Tryptamine C**H**$_2CH_2NH$), 3.51 (2H, q, *J* = 6.6 Hz, N_3C**H**$_2CH_2CH_2$), 3.42 (2H, t, *J* = 6.6 Hz, $N_3CH_2CH_2C$**H**$_2$), 3.06 (2H, t, *J* = 7.0 Hz, Tryptamine CH_2C**H**$_2$NH), 1.89 (2H, quin, *J* = 6.6 Hz, N_3CH_2C**H**$_2CH_2$), 1.03 (9H, s, C(C**H**$_3$)$_3$); ^{13}C NMR (125.8 MHz, $(CD_3)_2SO$) δ 167.8 (C), , 166.7 (C), 138.1 (C), 137.9 (C), 136.4 (C), 135.8 (C), 135.7 (CH), 132.8 (C), 130.0 (CH), 128.9 (CH), 127.9 (CH), 127.3 (C), 127.2 (CH), 126.6 (CH), 122.2 (CH), 121.9 (CH), 119.5 (CH), 118.7 (CH), 112.9 (C), 111.2 (CH), 64.3 (CH_2), 49.4 (CH_2), 40.4 (CH_2), 37.7 (CH_2), 28.8 (CH_2), 26.8 (CH_3), 25.4 (CH_2), 19.2 (C); MS (ESI) Exact mass calcd for $C_{38}H_{42}N_5O_4Si$ $[M+H]^+$ 659.31, found: 659.22.

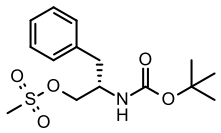
Synthesis of Copper-Free Click Reagent



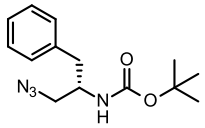
Oxo(1,2,3,8-tetramethoxy-11,12-didehydrodibenzo[*b*,*f*]- azocin-5(6*H*)-yl)butanoic acid: This was prepared according to the literature.[3]

Synthesis of Phe-Amine Building Block

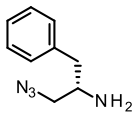




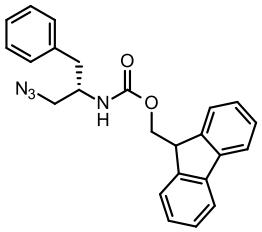
(2S)-2-({[(1,1-dimethylethyl)oxy]carbonyl}amino)-3-phenylpropyl methanesulfonate (14): *N*-Boc-L-Phenylalaninol **13** (2.00 g, 7.96 mmol) was added to DCM (100 mL) and to this was added DIPEA (1.70 mL, 9.55 mmol). The mixture was cooled to 0 °C and methanesulfonyl chloride (0.7 mL, 9.15 mmol) was added slowly and the mixture allowed to warm to room temperature over 2 hours. The organics were washed with H_2O then dried and concentrated to dryness to give a white solid, which was purified using flash chromatography, eluting with DCM to give the mesylate **14** (1.74 g, 66 %) as a white solid. R_f = 0.47 (50 % Cyclohexane/EtOAc); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.34 – 7.22 (5H, m, Ar**H**), 4.79 – 4.69 (1H, br s, N**H**), 4.30 – 4.21 (1H, m, NHC**H**), 4.15 – 4.06 (2H, m, CHC**H**$_2$O), 3.02 (3H, s, SC**H**$_3$), 2.96 – 2.84 (2H, m, CHC**H**$_2$Ar), 1.43 (9H, s, C(C**H**$_3$)$_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 155.1 (C), 136.6 (C), 129.2 (CH), 128.8 (CH), 128.6 (CH), 127.0 (CH), 126.6 (CH), 80.0 (C), 69.8 (CH_2), 52.5 (CH), 50.8 (CH_2), 37.2 (CH_3), 28.3 (CH_3); MS (ESI) Exact mass calcd for $C_{15}H_{23}NO_5SNa$ $[M+Na]^+$ 352.12, found: 352.05. Spectroscopic data is consistent with that reported previously.[4]



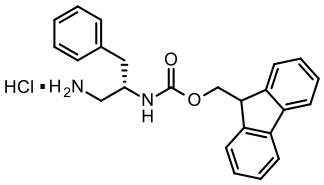
Tert-butyl-*N*-[(1S)-1-(azidomethyl)-2-phenyl-ethyl] carbamate (15): Methane sulfonate **14** (1.7 g, 5.15 mmol) was stirred in DMF (55 mL) and to this was added sodium azide (768 mg, 5.66 mmol) and the mixture stirred at 55°C overnight. Solvents were removed under partial pressure and the residue dissolved in DCM and washed with water. The organics were dried over $MgSO_4$ then concentrated to dryness. The residue was purified using flash chromatography, eluting with 50 % Cyclohexane in EtOAc to give compound **15** (738 mg, 52 %) as a white solid. R_f = 0.80 (50 % Cyclohexane/EtOAc); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38 – 7.19 (5H, m, Ar**H**), 4.68 (1H, br s, N**H**), 4.05 – 3.93 (1H, br m, NHC**H**), 3.45 (1H, dd, *J* = 12.1, 3.9 Hz, C**H**$_2$N$_3$), 3.34 (1H, dd, *J* = 12.3, 4.4 Hz, C**H**$_2$N$_3$), 2.94 – 2.86 (1H, m, CHC**H**$_2$Ar), 2.84 – 2.79 (1H, m, CHC**H**$_2$Ar), 1.45 (9H, s, C(C**H**$_3$)$_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 155.1 (C), 137.1 (C), 129.3 (CH), 128.7 (CH), 126.7 (CH), 79.8 (CH), 53.1 (CH_2), 51.3 (CH), 38.1 (CH_2), 28.3 (CH_3) MS (ESI) Exact mass calcd for $C_{14}H_{21}N_4O_2$ $[M+H]^+$ 277.17, found: 276.68. The data is in agreement with the literature.[5]



1-Azido-3-phenyl-propan-2-amine (16): Azide **15** (731 mg, 2.65 mmol) was stirred in 50 % TFA in DCM for 5 hours. Solvents were removed *in vacuo* and the residue taken up in DCM and washed with saturated $NaHCO_3$ three times. The organics were dried and concentrated to dryness and the residue purified by flash chromatography, eluting with 5 % MeOH in DCM to give amine **16** (387 mg, 83 %) as a colourless oil. R_f = 0.33 (5 % MeOH/DCM,); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40 – 7.20 (5H, m, Ar**H**), 3.45 – 3.40 (1H, m, NH$_2$C**H**), 3.29 – 3.20 (2H, m, C**H**$_2$N$_3$), 2.82 (1H, dd, *J* = 13.4, 5.5 Hz, C**H**$_2$Ar), 2.65 (1H, dd, *J* = 13.4, 5.8 Hz, C**H**$_2$Ar), 1.70 (2H, s, N**H**$_2$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.0 (C), 129.2 (CH), 128.7 (CH), 126.7 (CH), 57.2 (CH_2), 52.4 (CH), 41.3 (CH_2). MS (ESI) Exact mass calcd for $C_9H_{13}N_4$ $[M+H]^+$ 177.11, found: 177.00. The data is in agreement with the literature.[6]



9H-Fluoren-9-ylmethyl *N*-[(1S)-1-(azidomethyl)-2 phenyl-ethyl]carbamate (17): Amine **16** (350 mg, 1.99 mmol) was stirred in water (15 mL) and to this was added sodium carbonate (421 mg, 3.98 mmol). The mixture was cooled in an ice bath and Fmoc succinimidyl ester (737 mg, 2.19 mmol) in DCM 15 mL was added with stirring. This was left to warm to room temperature overnight. The organics were separated and washed with H_2O (3x), dried over $MgSO_4$ and concentrated to dryness. The residue was purified using flash chromatography, eluting with 20 % EtOAc in Cyclohexane to give fmoc protected amine **17** (753 mg, 95 %) as a white solid. R_f = 0.27 (20 % EtOAc in Cyclohexane); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.80 (2H, d, *J* = 7.6 Hz, Ar**H**), 7.58 (2H, t, *J* = 6.5 Hz, Ar**H**), 7.43 (2H, t, *J* = 7.5 Hz , Ar**H**), 7.34 (4H, t, *J* = 7.3 Hz, Ar**H**), 7.30 – 7.25 (1H, m, Ar**H**), 7.22 (2H, d, *J* = 6.8 Hz, Ar**H**), 7.20 (1H, br d, *J* = 7.7 Hz, N**H**), 4.44 - 4.39 (2H, m, CHC**H**$_2$O), 4.20 (1H, t, *J* = 6.6 Hz, C**H**CH$_2$O), 4.06 - 4.03 (1H, m, NHC**H**), 3.42 - 3.40 (2H, m, C**H**$_2$N$_3$), 2.89 - 2.85 (2H, m, C**H**$_2$Ar); ^{13}C NMR (125 MHz, $CDCl_3$) δ 155.6 (C), 143.8 (C), 141.4 (C), 136.8 (C), 129.3 (CH), 128.8 (CH), 127.4 (CH), 127.1 (CH), 126.9 (CH), 125.1 (CH), 120.0 (CH), 66.8 (CH$_2$), 53.2 (CH$_2$), 51.8 (CH), 47.3 (CH), 38.1 (CH$_2$); MS (ESI) Exact mass calcd for $C_{24}H_{23}N_4O_2$ [M+H]$^+$ 399.18, found: 398.96. The data is in agreement with the literature.[7]



9H-Fluoren-9-ylmethyl-*N*-[(1S)-1-(aminomethyl)-2-phenyl-ethyl]carbamate Hydrochloride (18): Compound **17** (350 mg, 0.88 mmol) was stirred in MeOH 25 mL and to this was added HCl in dioxane (2M, 1.36 mL, 2.72 mmol). The reaction was put under an argon atmosphere and palladium on carbon 5 % loading (25 mg) was added. The flask was then evacuated and filled with a hydrogen atmosphere and stirred overnight. The mixture was filtered through celite and concentrated to dryness. The resulting gum was triturated with diethyl ether to give amine hydrochloride salt **18** (275 mg, 74 %) as an off white solid. R_f = 0.68 (10 % MeOH/DCM); ^{1}H NMR (400 MHz, DMF-d_7) δ 8.93 (2H, br s, N**H**$_2$), 7.94 (2H, d, *J* = 7.6 Hz, Ar**H**), 7.75 (2H, t, *J* = 8.4 Hz, Ar**H**), 7.45 (2H, t, *J* = 7.5 Hz, Ar**H**), 7.4 - 7.26 (6H, m, Ar**H**), 7.25 - 7.18 (1H, m, Ar**H**), 4.29 – 4.13 (4H, m, CHC**H**$_2$O, C**H**CH$_2$O, NHC**H**), 3.28 – 3.17 (2H, m, C**H**$_2$NH$_2$), 3.09 – 3.01 (2H, m, C**H**$_2$Ar); ^{13}C NMR (200 MHz, DMF-d_7) δ 157.3 (C), 145.3 (C), 142.2 (C), 139.4 (C), 130.8 (C), 130.5 (C), 129.8 (CH), 129.4 (CH), 128.8 (CH), 128.2 (CH), 128.2 (CH), 127.5 (CH), 126.8 (CH), 126.6 (CH), 121.1 (CH), 67.3 (CH), 52.5 (CH), 48.1(CH$_2$), 39.1 (CH$_2$), 37.7 (CH$_2$); MS (ESI) Exact mass calcd for $C_{24}H_{25}N_2O_2$ [M+H]$^+$ 373.19, found: 373.17.

Solid Phase Synthesis Methods

TentaGel S NH$_2$ (RAPP-Polymers) resins were used for all solid phase work, unless otherwise stated.

General coupling conditions

Beads were swelled in DCM then drained. Couplings were carried out using 5 equiv. acid (or amine in the case of Phe-amine) (relative to resin), 5 equiv. HATU and 10 equiv. DIPEA in DMF (10 ml/g resin). Beads were shaken in this mixture for 1 hour then washed extensively with DMF and DCM.

Removal of Fmoc protecting group

Beads were swollen in DCM then drained and 20% Piperidine in DMF was added (10 ml/g resin) and the mixture shaken for 30 minutes, then washed with DMF and DCM.

Loading ALBA Linker onto Resin

ALBA linker (10eq) was dissolved in Acetonitrile/H_2O (1:1) then the solution applied to resin loaded with Oxo(1,2,3,8-tetramethoxy-11,12-didehydrodibenzo[b,f]- azocin-5(6H)-yl)butanoic acid. The mixture was shaken overnight at room temperature. The resin was drained and the excess linker retained for future use. Beads were washed extensively with DMF, then DCM.

TFA Cleavage of Linker

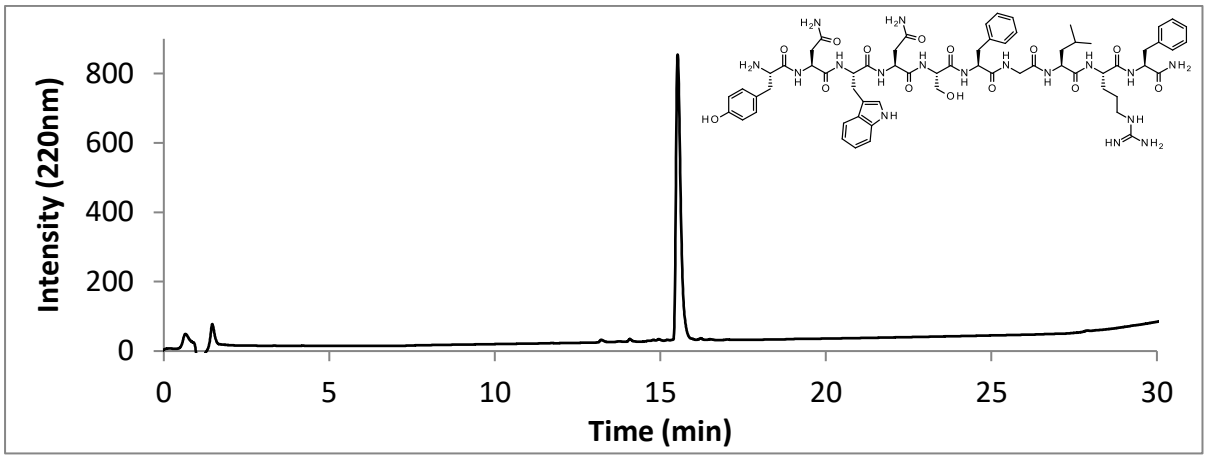
Resin was subjected to a solution of 2.5 % TIS, 2.5 % H_2O in TFA for 4 hours. Following this, the resin was drained and the liquid collected. The resin was rinsed with TFA again and the filtrate concentrated to dryness. The residue was taken up in cold diethyl ether to precipitate the side chain deprotected peptide, which was filtered off and dried.

Fluoride / Dilute Acid Cleavage of Linker

Resin was subjected to a solution of 0.1 M TBAF in THF for 30 minutes. The solution was then drained from the resin & the resin washed with DMF 3 times. A solution of 5 % TFA, 5 % water in DCM and this was added to the resin. The mixture was shaken for 4 hours. This solution was collected and concentrated to dryness to yield the side chain protected peptide.

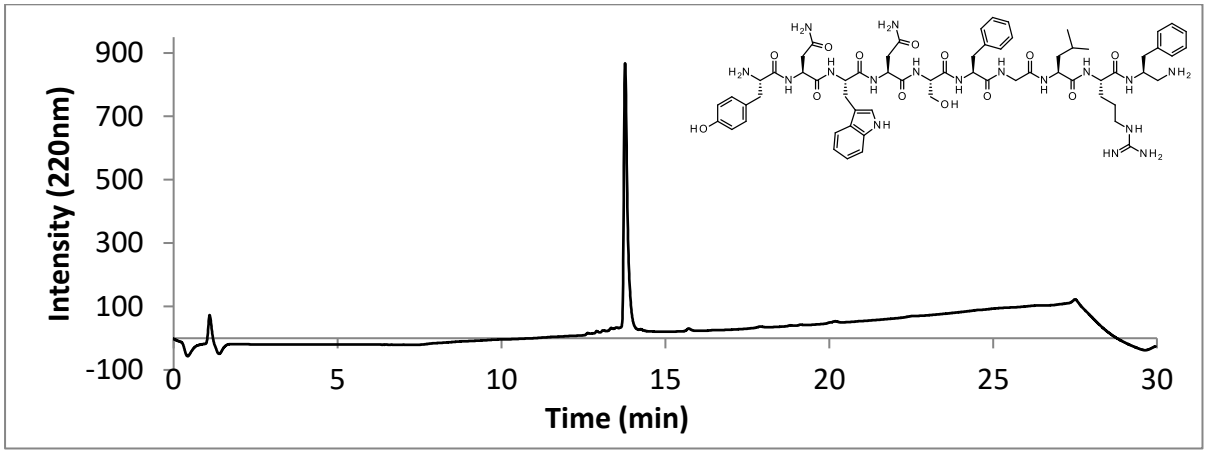
Kisspeptin10

Kisspeptin was synthesised on tentagel S RAM resin using procedures outlined above. MS (ESI) Exact mass calcd for $C_{63}H_{84}N_{17}O_{14}$ $[M+H]^+$ 1302.64, found: 1303.02; Crude purity (RP-HPLC): 92%. The peptide was purified using semi preparative RP-HPLC method A. >95% purity.



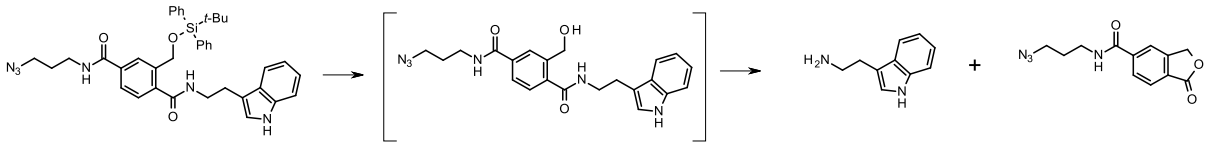
Kisspeptin10-amine (24)

The peptide was synthesised using the ALBA linker according to procedures outlined above. MS (ESI) Exact mass calcd for $C_{63}H_{86}N_{17}O_{13}$ $[M+H]^+$ 1288.66, found: 1288.66; Crude purity (RP-HPLC): 68%. The peptide was purified using semi preparative RP-HPLC method B. >95% purity.



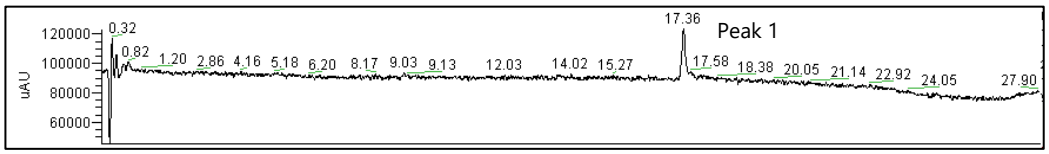
Solution Based Linker Cleavage Tests

In order to test the stability of the ALBA linker to certain conditions, it was loaded with Tryptamine (see above for experimental procedure) in solution. This compound was then weighed into a vial (1 mg/mL) and stirred in different solutions for a given amount of time. LCMS analysis of the product showed how the linker behaved in each. LCMS photodiode array traces shown.



A Mass : 658.31 | B Mass : 420.19 | C Mass : 160.10 | D Mass : 260.09

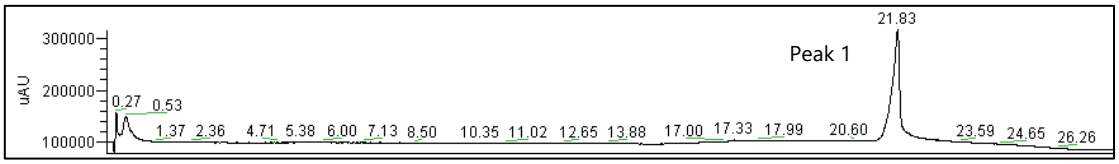
1) Reference - Tryptamine loaded ALBA linker (A)



Peak	t_R	m/z observed	Species Observed
1	17.36	658.8	Tryptamine Loaded ALBA Linker (A)

2) 1 hour 20% Piperidine in DMF

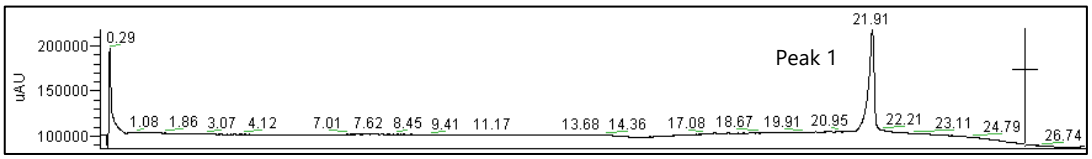
Linker stable to these conditions



Peak	t_R	m/z observed	Species Observed
1	21.83	659.0	Tryptamine Loaded ALBA Linker (A)

3) 1 hour 10% DIPEA in DMF

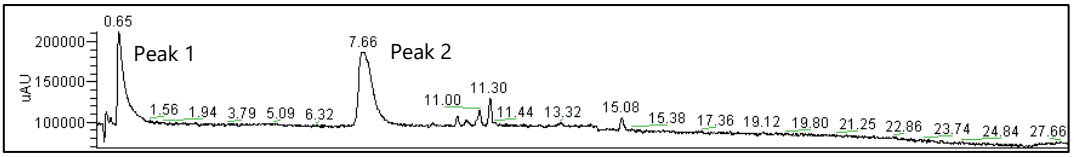
Linker stable to these conditions



Peak	t_R	m/z observed	Species Observed
1	21.91	659.2	Tryptamine Loaded ALBA Linker (A)

4) 4 hours TFA

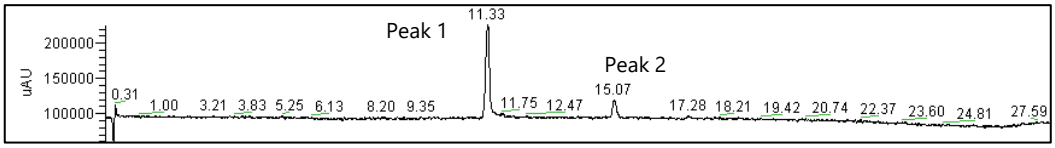
Linker cleavage appears complete after 4 hours due to disappearance of starting material and formation of benzofuranone compound D (peak 2) and release of tryptamine C (peak 1).



Peak	t_R	m/z observed	Species Observed
1	0.65	161.0	Tryptamine (C)
2	7.66	260.9	Benzofuranone (D)

5) 30 min 1mM TBAF in THF

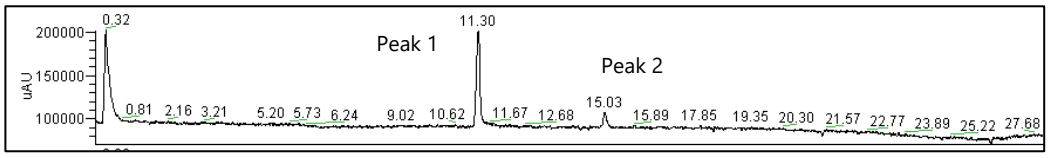
TBDPS group removed after 30 minutes due to disappearance of starting material and appearance of deprotected linker compound B.



Peak	t_R	m/z observed	Species Observed
1	11.33	421.1	TBDPS deprotected linker (B)
2	15.07	none	TBDPS group

6) 30 min 1mM TBAF in THF then overnight pyridine/H_2O

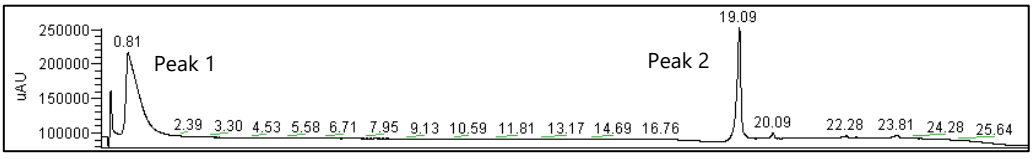
Linker cleavage with pyridine appears unsuccessful, even overnight with no evidence of benzofuranone, only deprotected linker compound B.



Peak	t_R	m/z observed	Species Observed
1	11.30	421.1	TBDPS deprotected linker (B)
2	15.03	none	TBDPS group

7) 4 hours 0.1 M TBAF, 5% TFA, 5 % H_2O in DCM

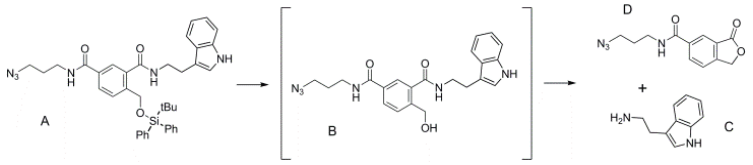
Linker cleavage appears complete after 4 hours due to disappearance of starting material and formation of benzofuranone compound D (peak 2) and release of tryptamine C (peak 1).



Peak	t_R	m/z observed	Species Observed
1	0.81	160.97	Tryptamine (C)
2	19.09	260.92	Benzofuranone (D)

Table S1 - Species present during ALBA linker cleavage tests as determined by LC-MS analysis.

Entry	Conditions	Species present
1	10% DIPEA / DMF	A
2	20% Piperidine / DMF	A
3	1 mM TBAF	B
4	TFA	C + D
5	1 mM TBAF then Pyridine / H2O	B
6	1 mM TBAF then 5% TFA in DCM	C + D



GPR54-Kisspeptin 10 model building

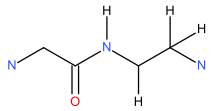
A model of GPR54 was created using the sequence with Uniprot accession Q969F8 and AlphaFold2 via the ColabFold server[8]. An approximate model of the peptide was built manually, and docked into the internal cavity of the protein model using the HDOCK protein-protein docking server[9]. Further refinement of the top-scoring predicted HDOCK binding mode was performed using Vina via its "--local_only"-switch invoked energy minimisation mode[10]. All program parameters remained as defaults. Binding mode analysis was performed visually with PyMOL[11] (version 2.5.4).

Cheminformatics analysis of C-terminal amines

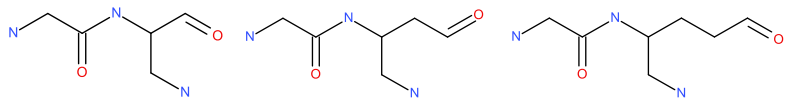
The ChEMBL[12] bioactivity database was queried for peptides containing C-terminal amines. However, online web-based searching was insufficient for specification of primary and secondary amines likely due to the automated removal of explicit hydrogens. The ChEMBL version 33 database was downloaded in SQLite format and Python code in a Jupyter notebook used to interrogate the dataset. The Jupyter notebook documenting all steps, code and output of processing is available on GitHub in the following gist: https://gist.github.com/stevenshave/c98b8075ab01b689e9cbee551c2aadfe. Using Python 3.10.11, the SQLite version of ChEMBL version 33 was queried using functionality of the Pandas (version 1.5.3) package using the query below to extract SMILES and ChEMBL IDs to a file.

```
"SELECT s.canonical_smiles, m.chembl_id AS compound_chembl_id FROM compound_structures s RIGHT JOIN molecule_dictionary m on s.molregno = m.molregno;"
```

Substructure searching using OpenBabel (version 3.0.0) was then applied to find molecules containing the following substructure



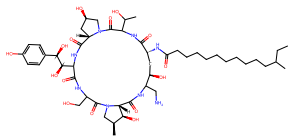
And exclude the three substructures below to remove molecules containing β^3 glutamic, homo-glutamic and longer amino acids.



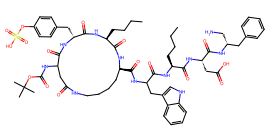
Substructure searching was invoked using the following command from within the notebook:

```
!obabel -ismi chembl33.smi --filter "s='NCC(=O)[NH1][CH1][CH2][NH2]' s!='NCC(=O)[NH1][CH1](C(=O))[CH2][NH2]' s!='NCC(=O)[NH1][CH1](CC(=O))[CH2][NH2]' s!='NCC(=O)[NH1][CH1](CCC(=O))[CH2][NH2]'" -O alba_cleaved_substructure_matches.smi
```

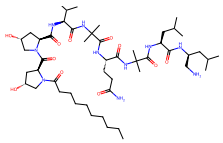
This resulted in the following molecules being identified as substructure matches: CHEMBL438649, CHEMBL216677, CHEMBL448005, CHEMBL449446, CHEMBL554386, CHEMBL3219497, CHEMBL4756686, CHEMBL4778987, CHEMBL4856898, CHEMBL4861854, CHEMBL4867275, CHEMBL4872372, CHEMBL4878945, CHEMBL4879341, CHEMBL5028624, CHEMBL5029024, CHEMBL5084213



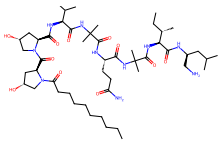
CHEMBL438649



CHEMBL216677



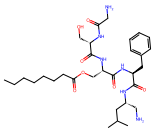
CHEMBL448005



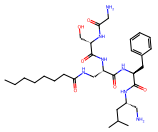
CHEMBL449446

CHEMBL554386

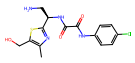
CHEMBL3219497



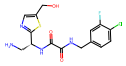
CHEMBL4756686



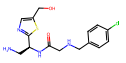
CHEMBL4778987



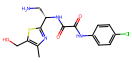
CHEMBL4856898



CHEMBL4861854

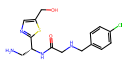


CHEMBL4867275

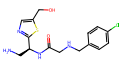


CHEMBL4872372

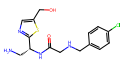
CHEMBL4878945



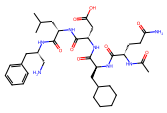
CHEMBL4879341



CHEMBL5028624



CHEMBL5029024



CHEMBL5084213

The cyclic peptides CHEMBL438649 and CHEMBL216677 may be excluded from analysis as they match the substructure query by virtue of having an ethanamine sidechain. CHEMBL554386 and CHEMBL3219497 were discounted as substructure matching was only successful due to sidechain cyclization turning them into small cyclic molecules with the C-terminal amine motif.

CHEMBL448005 and CHEMBL449446 are cicadapeptin I, and cicadapeptin II respectively, natural product extracts from the parasitic fungus Cordycepsheteropoda with literature noted antibacterial activity[13].

CHEMBL4756686, CHEMBL4778987 are covered in patent number US 2020/0102365 A1, and are recorded in ChEMBL with IC_{50}s of 45 and 1 μM respectively. No C-terminal amide derivative was available for comparison.

The CHEMBL IDs CHEMBL4856898, CHEMBL4861854, CHEMBL4867275, CHEMBL4872372, CHEMBL4878945, CHEMBL4879341, CHEMBL5028624, and CHEMBL5029024 are part of a series of compounds produced during studies of HIV gp120. Whilst many of these inhibitors contain the C-terminal amine functionalities, no amide is available for comparison[14].

CHEMBL5084213 is noted in literature to bind to the Escherichia coli sliding clamp [15] with an K_D of 877 ± 45 nM. A matching amide derivative is also reported with a K_D of 268 ± 68 nM.

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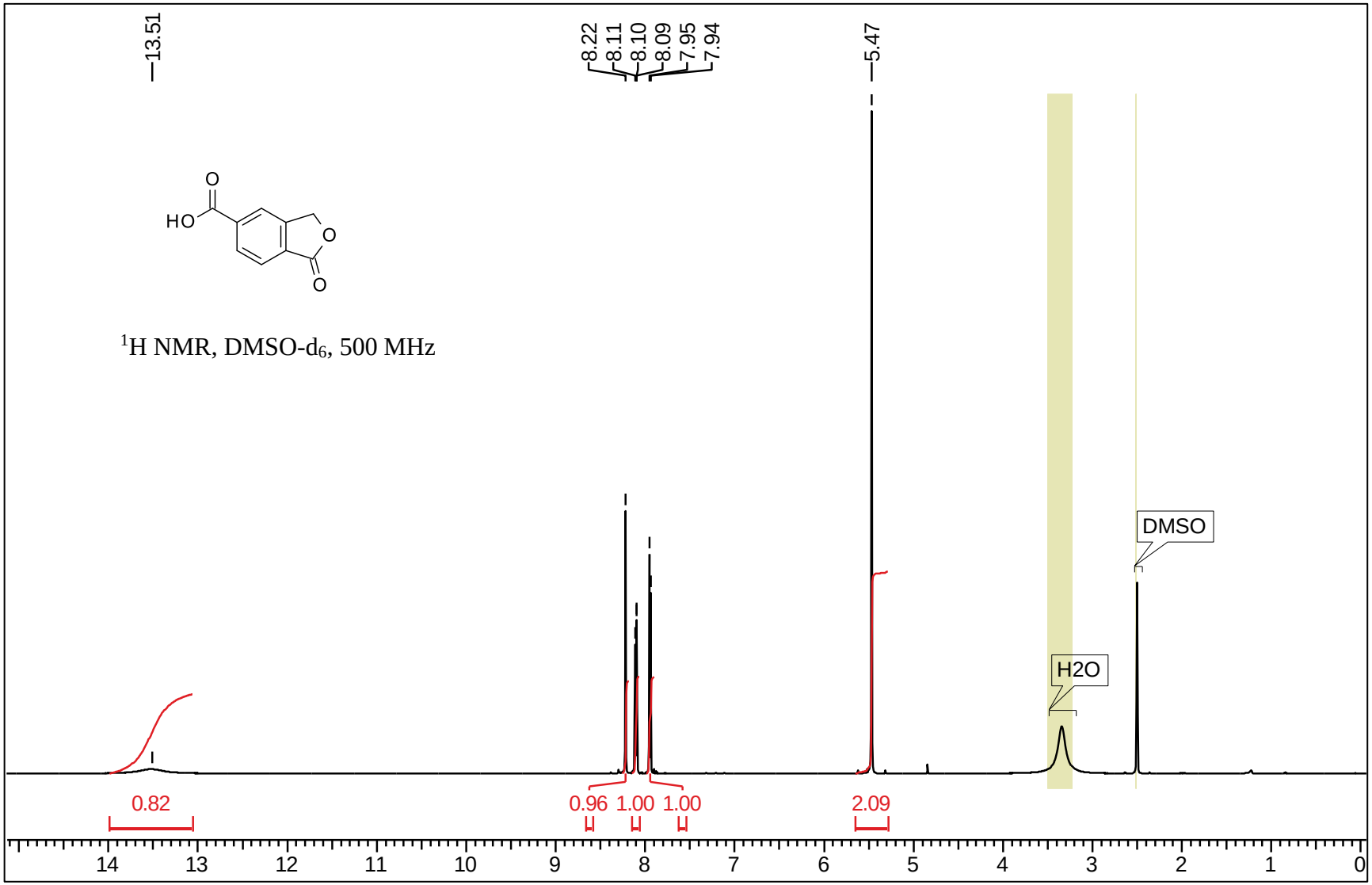
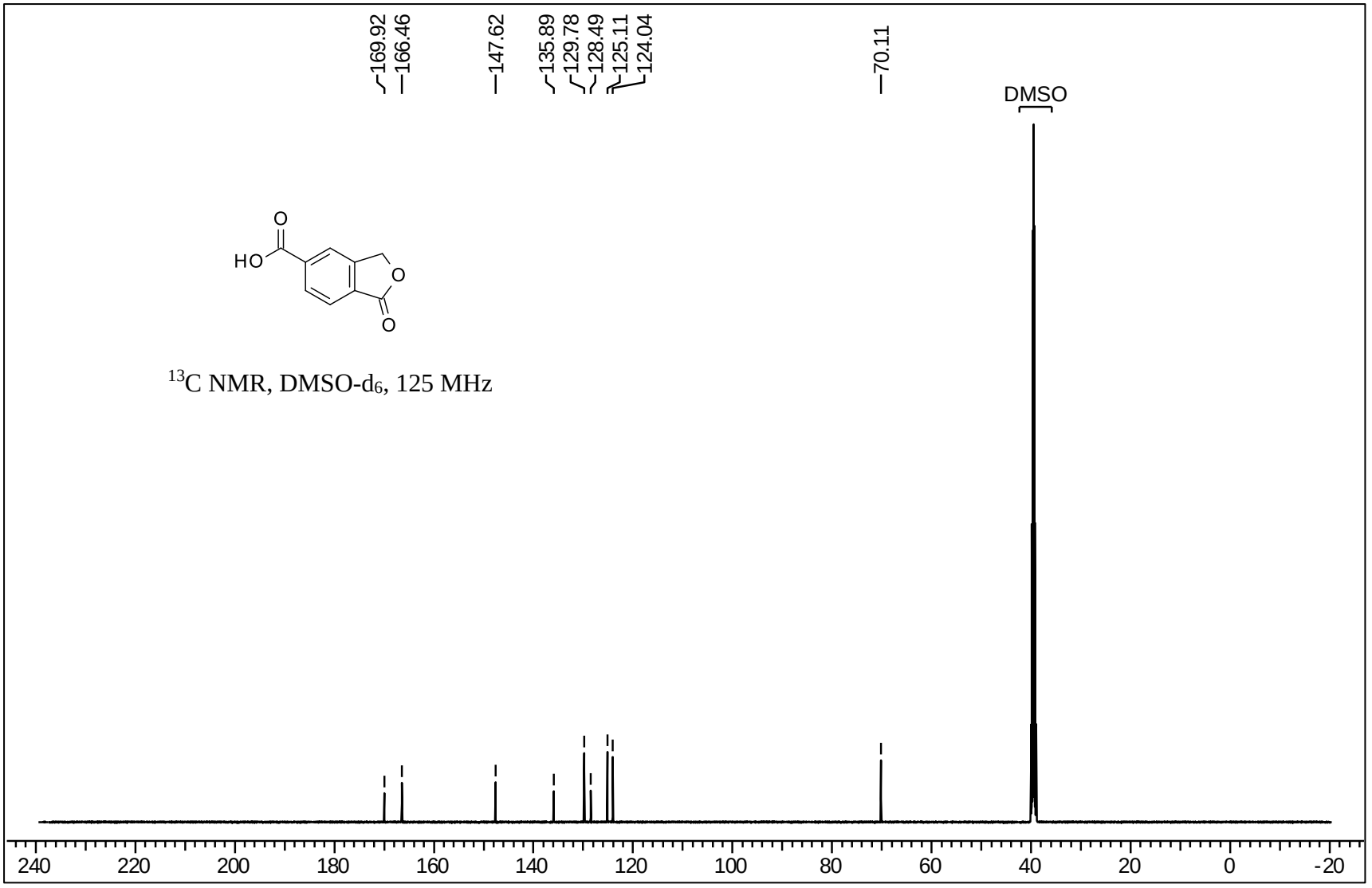
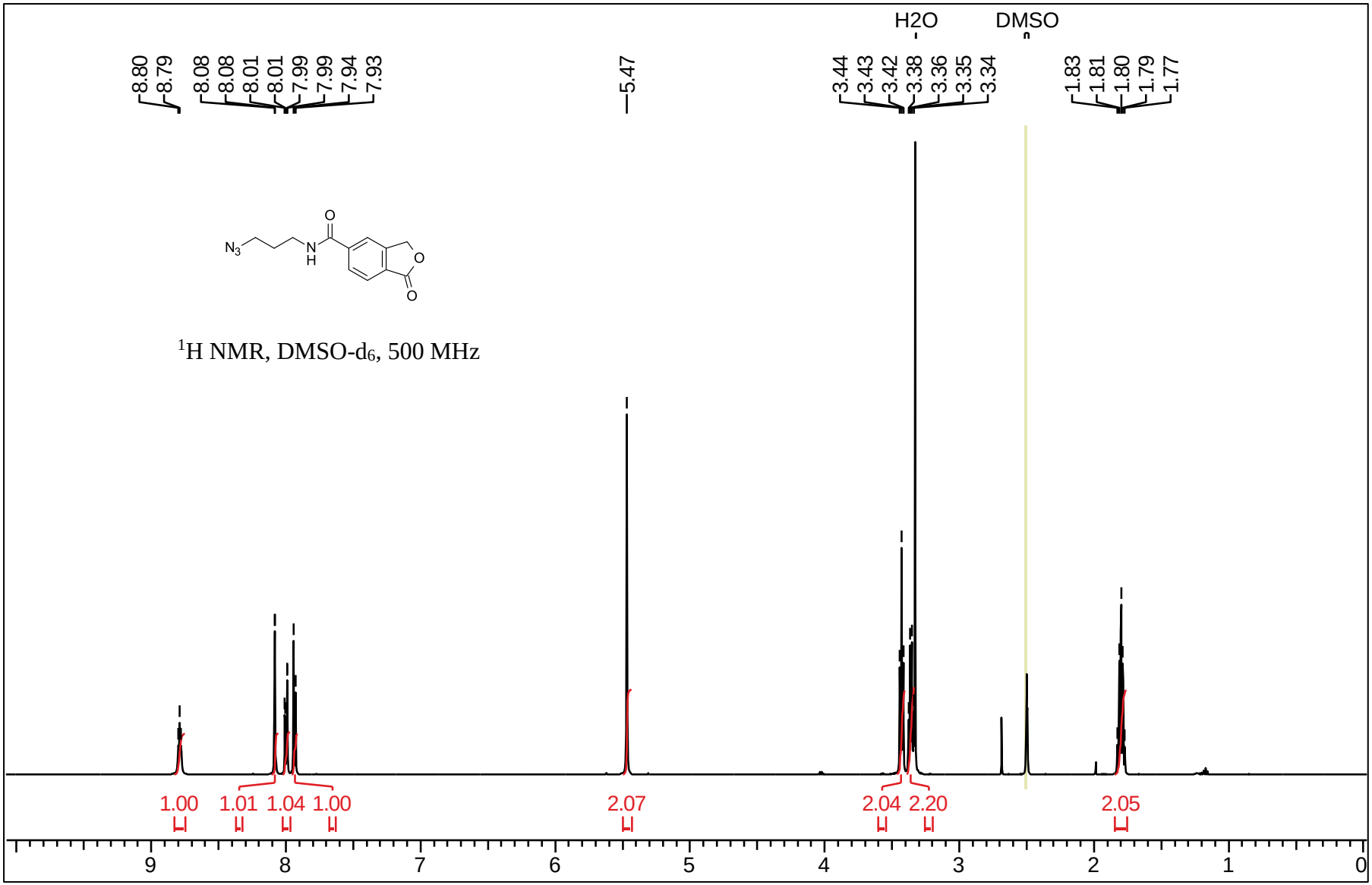
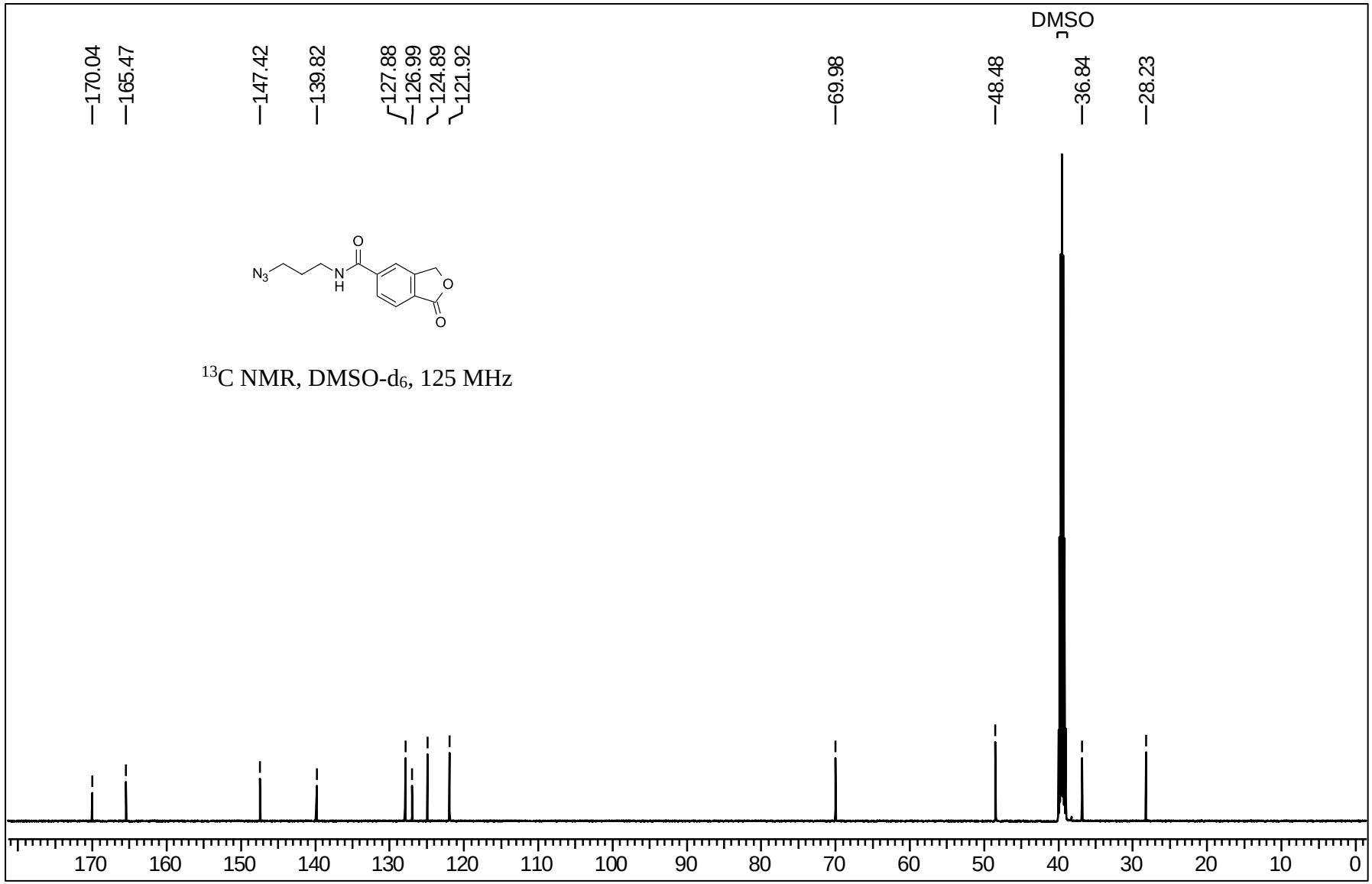
NMR Spectra follow


13.51
8.22
8.11
8.10
8.09
7.95
7.94
5.47
HO
O
O
O
^{1}H NMR, DMSO-d$_6$, 500 MHz
DMSO
H2O
0.82
0.96
1.00
1.00
2.09
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0

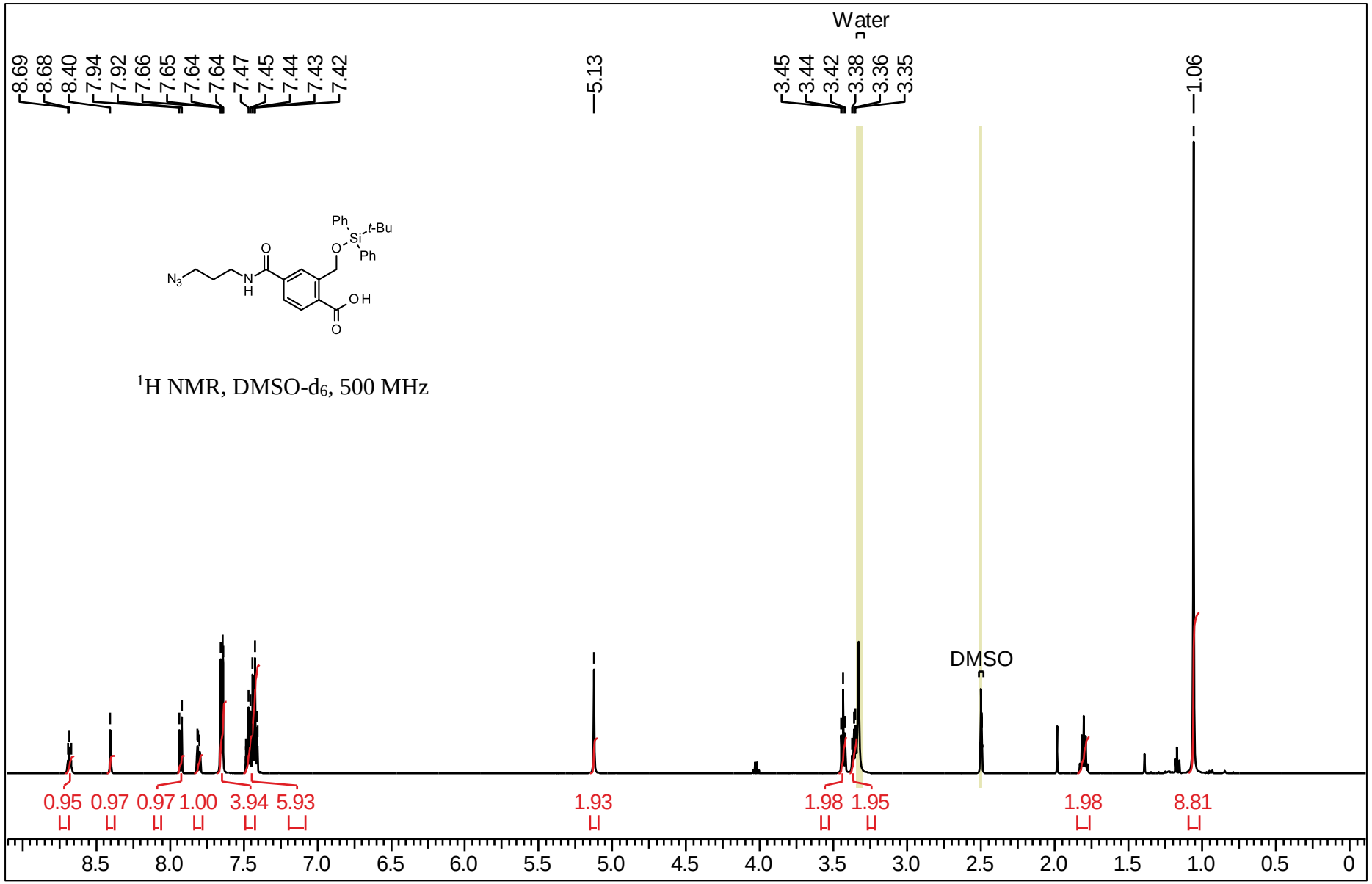


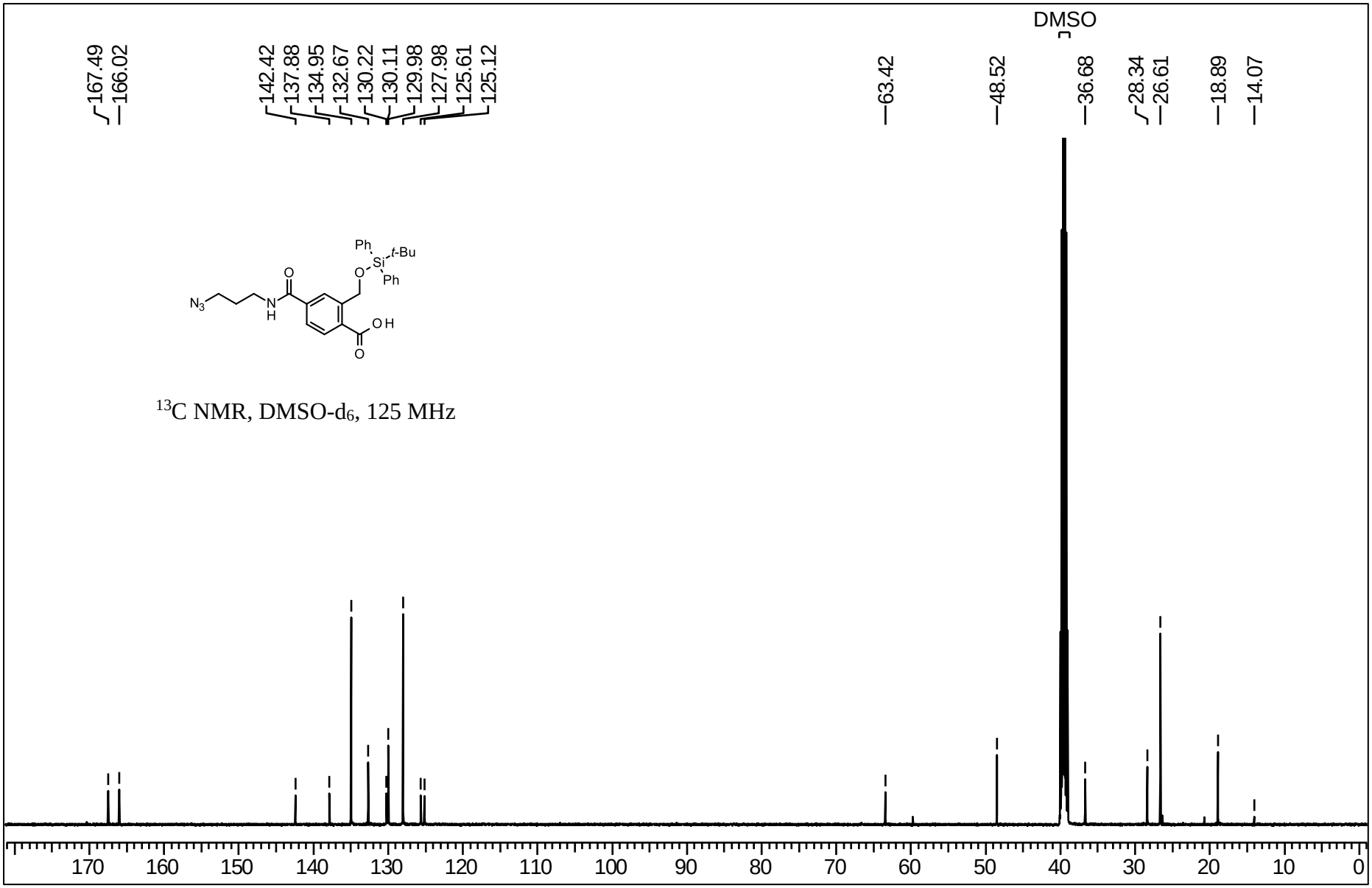
^{13}C NMR, DMSO-d_6, 125 MHz


H2O
DMSO
8.80
8.79
8.08
8.08
8.01
8.01
7.99
7.99
7.94
7.93
5.47
3.44
3.43
3.42
3.38
3.36
3.35
3.34
1.83
1.81
1.80
1.79
1.77
N3
H
N
O
O
O
O
[1]H NMR, DMSO-d6, 500 MHz
1.00
1.01
1.04
1.00
2.07
2.04
2.20
2.05
9
8
7
6
5
4
3
2
1
0

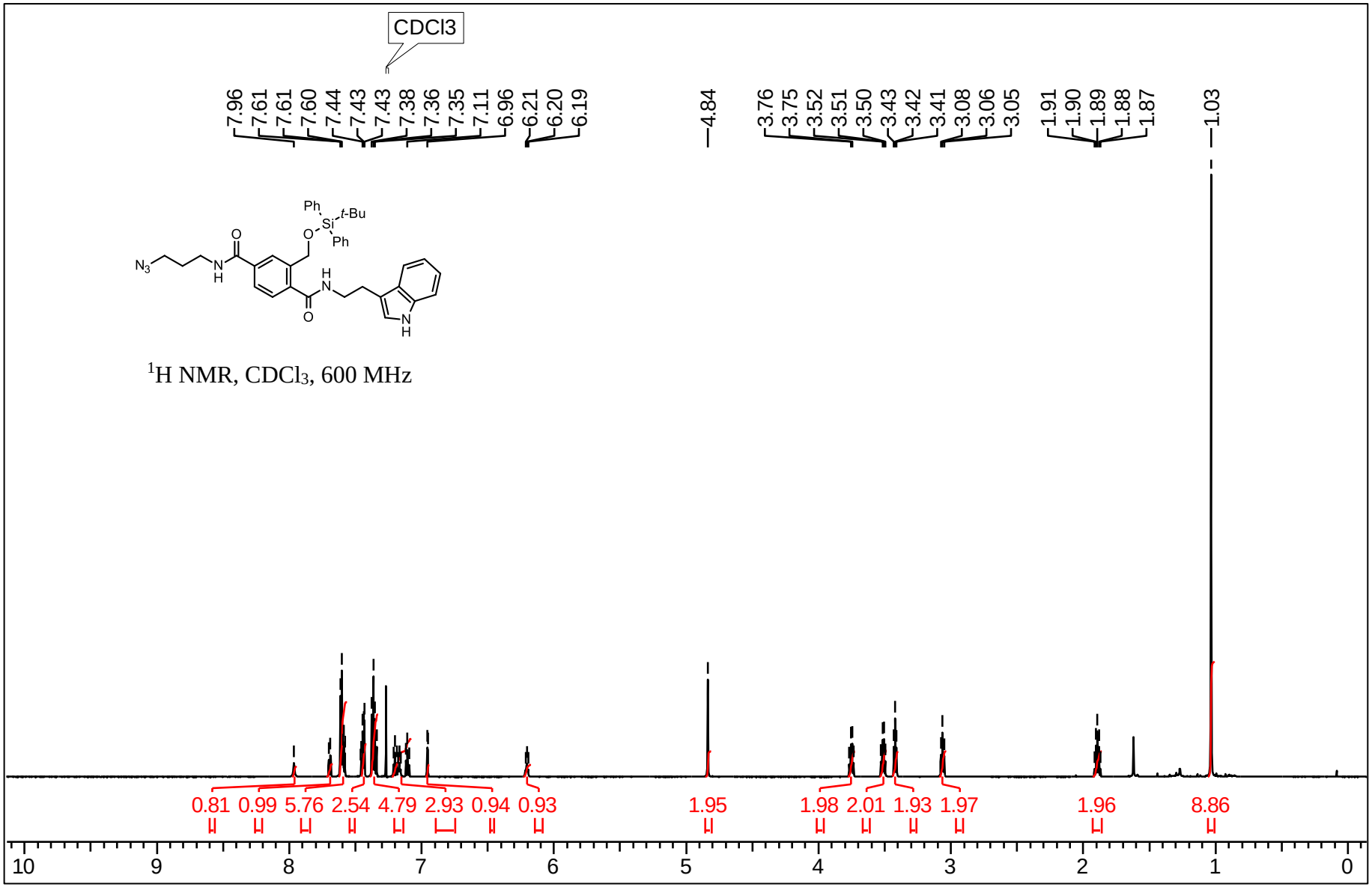


^{13}C NMR, DMSO-d_6, 125 MHz

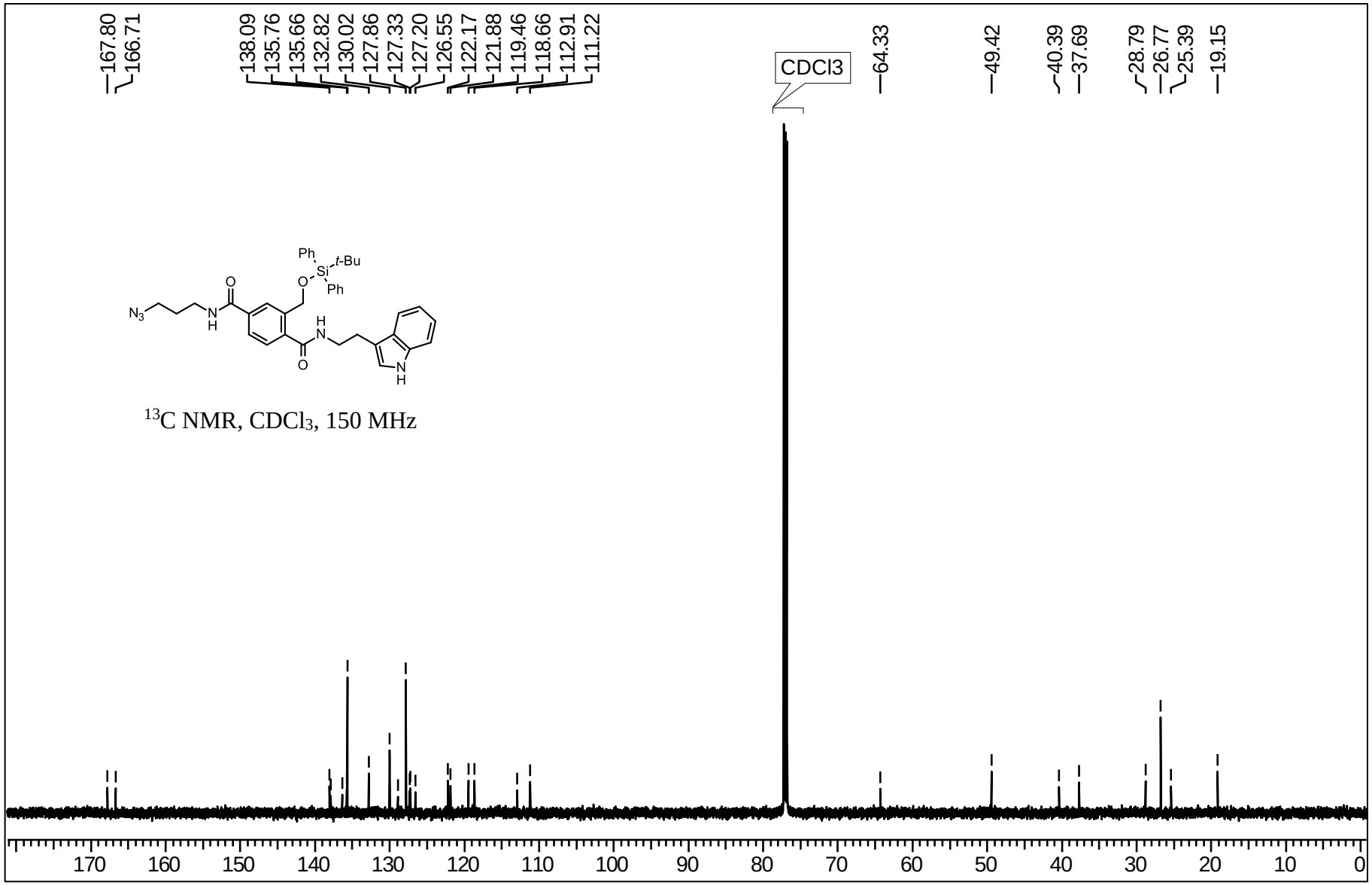

Water
DMSO
8.69
8.68
8.40
7.94
7.92
7.66
7.65
7.64
7.64
7.47
7.45
7.44
7.43
7.42
5.13
3.45
3.44
3.42
3.38
3.36
3.35
1.06
Ph
Si
t-Bu
Ph
O
O
N3
N
H
OH
O
1H NMR, DMSO-d6, 500 MHz
0.95
0.97
0.97
1.00
3.94
5.93
1.93
1.98
1.95
1.98
8.81
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0



^{13}C NMR, DMSO-d_6, 125 MHz



^{1}H NMR, $CDCl_3$, 600 MHz



^{13}C NMR, $CDCl_3$, 150 MHz