

Supporting Information

Display selection of exotic macrocyclic peptides expressed under a radically reprogrammed 23 amino acid genetic code

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General Experimental Materials and Methods

Materials. Boc-protected amino acids were purchased from Watanabe Chemical Industries, Ltd. Canonical Fmoc-protected amino acids were purchased from Merck Millipore unless otherwise noted. Recombinant IL-6R was purchased from Sino Biological. Oligonucleotides were purchased from Eurofins Genomics Japan. Other reagents were from Nacalai Tesque, Inc. unless otherwise specified.

Synthesis of aminoacylated tRNAs. Flexizyme aminoacylating ribozymes and tRNAs were prepared by *in vitro* transcription from PCR generated DNA templates (Supplementary Table 1): Sequential PCRs were performed with nesting primers using Taq polymerase (95°C for 40s, 50°C for 40s, 72°C for 40s), and the resulting DNA was used as a template for T7 RNA polymerase mediated transcription at 37°C for 16 hrs prior to polyacrylamide gel purification. Aminoacylation of tRNAs using activated amino acids was performed as follows: 25 μ M each flexizyme (dFx for DBE activated amino acids and eFx for CME activated amino acids) and tRNA in 50 mM HEPES.KOH pH7.5 were heated to 95°C for 2 min and allowed to cool slowly to 25°C. MgCl₂ was added to a final concentration of 600 mM and the reaction was incubated at 25°C for 5 min, before being cooled on ice. Amino acid substrate was added to a final concentration of 5 mM, and the reaction was allowed to proceed on ice for the optimal time determined for each amino acid (Supplementary Figure 1). Aminoacylated tRNA was recovered by ethanol precipitation and stored dry at -80°C until use. For optimisation of reaction duration, a short RNA hairpin oligoribonucleotide corresponding to the tRNA acceptor stem (μ -helix RNA: 5'-GGCUCUGUUCGCAGAGCCGCCA-3') was used as a surrogate for full-length tRNA, and aminoacylation efficiency was assessed by polyacrylamide electrophoresis under acidic conditions with quantification performed using a Typhoon FLA 7000 imager (GE healthcare).

***In vitro* translation.** *In vitro* translation was performed using a purified recombinant expression system as previously described.¹ For testing the translation of individual non-canonical amino acids at the glycine GGG codon, MK3G_(GGG)flag DNA template (amino acid sequence: MKKKGDYKDDDDK) was included at a concentration of 0.1 μ M, and aminoacylated tRNA_{ACC} was included at a concentration of 25 μ M. Translation products were analysed by MALDI-TOF-MS (using a Bruker UltraFlex mass spectrometer) or

autoradiography. For autoradiography, 50 μM [^{14}C]-Asp was included in the reaction, and the translation product was separated on a 15% tricine SDS PAGE gel and imaged using a Typhoon FLA 7000 imager (GE Healthcare).

Synthesis and screening of peptide library. Library construction and screening were performed essentially as previously described.²⁻³ Briefly, DNA templates for construction of mRNA libraries were synthesized by primer extension and PCR using primers shown in Supplementary Table 1. Cognate mRNAs were generated by T7 polymerase-mediated transcription and covalently linked to PEG-puromycin, before translation in reprogrammed *in vitro* translation reactions without Asp, Glu, His, Lys, Met, Asn, Gln, Arg and their cognate AARSs (NNS library). Each reaction contained 1.2 μM mRNA-puro, 6.25 μM initiator tRNA (tRNA^{fMet} aminoacylated with ClAc-Tyr) and 12.5 μM each elongator tRNA (EnGlu_{xxx} aminoacylated with the specified non-canonical tRNA). For the first round of selection, translation was performed at 150 μL scale, to give an initial theoretical library diversity of approximately 1.2×10^{14} molecules at the RNA level. After translation, peptide-mRNA conjugates were isolated from the ribosomes by EDTA treatment and reverse transcribed at using the CGT3an13.R23 primer (NNS library) and RNase H- reverse transcriptase (Promega). Glycosylated IL6R (baculovirus infected Sf 21 cell expressed Leu20-Asp358 fragment, R&D Systems) was immobilized on Dynabead M-270 carboxylic acid magnetic beads (Thermo Fisher) by EDC/NHS chemistry according to the manufacturer's instructions. Peptide-mRNA conjugate libraries were panned against 200 nM immobilized IL6R at 4°C for 30 min, the beads were washed 3 times with cold PBS-T (10 mM phosphate, 130 mM NaCl, 0.05 vol% Tween-20, pH 7.4) and cDNA recovered by PCR using the primers T7g10m.F46 and CGT3an13.R23, prior to T7 transcription to generate the mRNA for the subsequent round. For the second and later rounds of selection, translation was performed at 5 μL scale, and libraries were subjected to 3 rounds of counter selection against mock immobilized beads (EDC/NHS treated) prior to panning against IL6R. Assessment of recovery following each round was performed by quantitative real time PCR using Sybr Green I dye (Lonza Japan Ltd) on a light cycler nano thermal cycler (Roche). DNA from each round was sequenced using a MiSeq high-throughput sequencer (Illumina) and analysed using CLC workbench software (Qiagen). Selection using the 19 canonical amino acid library was performed as above, with the exception that a 6-15 codon NNK library was used and it was translated in an

in vitro reaction including all canonical amino acids (except Met) in addition to 20 μM initiator tRNA aminoacylated with *N*-chloroacetyl-tyrosine.

Solid phase peptide synthesis. Macrocyclic peptides were synthesized at 25 μmol scale using standard solid phase peptide synthesis methodology with an Fmoc-protection strategy using diisopropylcarbodiimide and Oxyma Pure and using a Liberty Blue microwave synthesizer (CEM Japan K.K.). *N*-Fmoc-Sarcosine was purchased from Merck Millipore. *N*-Fmoc protected *O*-methyl-tyrosine, *O*-methyl-homoserine, 2-thienyl-alanine, and *O*-methyl glutamate were purchased from Watanabe Chemical Ltd. For synthesis of *N*-methyl-*O*-methyl-Tyrosine residues, *O*-methyl-tyrosine coupling was followed by Fmoc deprotection, Nosyl protection of the resulting free amine and on-resin methylation using dimethyl sulphate as follows: following Fmoc deprotection, 2-nitrobenzenesulfonyl chloride (22mg, 0.1 mmol) in *N*-methyl-2-pyrrolidone (NMP) and 2,4,6-trimethylpyridine (33 μL , 0.25 mmol) were added to the resin, the reaction was allowed to proceed for 15 min at room temperature and the resin was washed (x5) with NMP. Methylation was performed twice using 1,8-Diazabicyclo(5.4.0)undec-7-ene (11.25 μL , 0.075 mmol) and dimethylsulfate (22.25 μL , 0.25 mmol) in NMP for 5 minutes, after which the resin was washed (x5) with NMP. Nosyl deprotection was performed twice using 1,8-Diazabicyclo(5.4.0)undec-7-ene (18.75 μL , 0.125 mmol) and 2-mercaptoethanol (17.5 μL , 0.25 mmol) in NMP for 5 minutes, after which the resin was washed (x5) with NMP and solid phase peptide synthesis was continued.

Ultra-high performance liquid chromatography. UHPLC was performed using a Nexera X2 system (Shimadzu) fitted with a C18 reversed phase column. Approximately 1 nmol of peptide was separated using a 10 vol% to 70 vol% aqueous acetonitrile gradient supplemented with 0.1 vol% trifluoroacetic acid, and monitored by absorbance at 280 nm.

Surface plasmon resonance. Binding kinetics of each peptide towards human IL6R (see above) or murine IL6R (Mouse myeloma cell line, NS0-derived mouse IL-6R alpha protein Leu20-Glu357 fragment, R&D Systems) were determined using a Biacore T200 machine (GE Healthcare). Running buffer was PBS-T + 0.1% DMSO. 500 nM recombinant IL6R was immobilized on a CM5 chip using EDC/NHS chemistry following the manufacturer's instructions (baseline response 2000-5000 refraction units). Peptides were injected as analyte at a flow rate of 60 $\mu\text{L}\cdot\text{min}^{-1}$ and kinetics were modelled using a 1:1 binding model.

Synthesis of activated amino acid substrates.

Synthesis of amino acid substrates. Norvaline dinitrobenzyl ester (DBE), norleucine-DBE, cyclopentyl alanine-DBE, cyclohexyl alanine-DBE, 4-*O*-methyl-tyrosine cyanomethyl ester (CME), (*S*)-2-amino-3-(1-methyl-1*H*-indol-3-yl) propanoic cyanomethyl ester, 2-Naphthyl alanine-CME, 3-benzothiazyl alanine-CME, *N*-chloroacetyl-tyrosine-CME, sarcosine-DBE, *N*-methyl-leucine-DBE, *D*-alanine-DBE and *N*-methyl-4-*O*-methyl-tyrosine-CME were synthesized previously and this material was used for the present study.³⁻⁵ Other CME and dinitrobenzyl ester DBE activated amino acids were synthesized from commercially available boc-protected precursors as described below. DBE activated *N*-methyl- β -*O*-methyl threonine, *N*-methyl- γ -*O*-methyl-homoserine and *N*-methyl- ϵ -*O*-methyl-glutamate were synthesized by *N*-methylation of the corresponding DBE activated amino acid using Ag₂O and methyl iodide as previously described.⁶ Characterization was performed by ¹H and ¹³C NMR using a Bruker AV300 spectrometer. Chemical shifts are expressed in ppm downfield from internal standard (tetramethylsilane, 0.00 ppm), and coupling constants are reported as hertz. Splitting patterns are indicated as follows: br, broad; s, singlet; d doublet; t, triplet; q, quartet; m, multiplet. Electron spray ionization mass spectrometry was performed using a JEOL AccuTOF LC-Plus 4G or a Bruker micrOTOF focus II ESI mass spectrometer.

General Procedure for the Synthesis of Amino Acids with cyanomethyl ester (CME) derivatization. (Procedure A). (*S*)-cyanomethyl 2-amino-heptanoate trifluoroacetate (Ahp-CME)(for the introduction of 2-amino-heptanoic acid): To a solution of 2-(*tert*-butyloxycarbonyl)amino-heptanoic acid (204 mg, 0.83 mmol) in chloroacetonitrile (0.3 mL), *N,N*-diisopropylethylamine (260 μ l, 1.5 mmol) was added at room temperature. After stirring for 16 h, the reaction mixture was diluted by adding EtOAc and washed with 1M HCl *aq.* (x2), saturated NaHCO₃ (x2) and brine, dried (Na₂SO₄), and concentrated *in vacuo*. The reaction mixture was directly applied to a Biotage SNAP Ultra 25 g column and purified with an appropriate gradient. The solvent was removed *in vacuo* to yield the corresponding Boc-protected cyanomethyl ester as a white solids. Boc deprotection was conducted in neat TFA for 15 min, and the solvent was quickly removed by rotary evaporation at room temperature, followed by overnight drying of the salt under vacuum.

¹H NMR (300 MHz, DMSO-d₆): δ 0.88 (t, 3H, *J* = 7.0 Hz), 1.27-1.43 (m, 6H), 1.79-1.88 (m, 2H), 4.20 (t, 1H, *J* = 6.3 Hz), 5.17 (s, 2H), 8.60 (s, 3H).

^{13}C NMR (75 MHz, DMSO- d_6): δ 13.64, 21.65, 23.58, 28.37, 29.78, 30.53, 50.16, 51.67, 115.31, 168.84.

HRMS (ESI) calculated for $\text{C}_9\text{H}_{16}\text{N}_2\text{O}_2$: ($[\text{M}+\text{H}]^+$) m/z 185.1285, observed: m/z 185.1278.

(S)-cyanomethyl 2-amino-octanoate trifluoroacetate (Aoc-CME)(for the introduction of 2-amino-octanoic acid): The compound was synthesized by procedure A (above).

^1H NMR (300 MHz, D_2O): δ 0.84 (m, 3H), 1.30 (m, 8H), 1.96 (m, 2H), 4.26 (t, $J = 6.3$ Hz, 1H), 5.06 (s, 2H).

^{13}C NMR (75 MHz, D_2O): δ 13.28, 21.78, 23.91, 27.73, 29.53, 30.53, 50.35, 52.68, 114.96, 169.26.

HRMS (ESI) calculated for $\text{C}_{10}\text{H}_{18}\text{N}_2\text{O}_2$: ($[\text{M}+\text{H}]^+$) m/z 199.1441, observed: m/z 199.1441

(S)-cyanomethyl 2-amino-decanoate trifluoroacetate (Adec-CME)(for the introduction of 2-amino-decanoic acid): The compound was synthesized by procedure A (above).

^1H NMR (300 MHz, DMSO- d_6): δ 0.87 (t, 3H, $J = 6.5$ Hz), 1.26 (m, 12H), 4.20 (t, 1H, $J = 6.3$ Hz), 5.17 (s, 2H), 8.59 (s, 3H).

^{13}C NMR (75 MHz, DMSO- d_6): δ 13.88, 22.02, 23.90, 28.37, 28.39, 28.53, 29.83, 30.17, 50.16, 51.67, 115.31, 168.84.

HRMS (ESI) calculated for $\text{C}_{12}\text{H}_{22}\text{N}_2\text{O}_2$: ($[\text{M}+\text{H}]^+$) m/z 227.1754, observed: m/z 227.1761

(S)-cyanomethyl 2-amino-dodecanoate trifluoroacetate (Adod-CME)(for the introduction of 2-amino-dodecanoic acid): The compound was synthesized by procedure A (above).

^1H NMR (300 MHz, DMSO- d_6): δ 0.79 (m, 3H), 1.18 (m, 16H), 1.72 (m, 2H), 5.09 (s, 2H), 4.12 (m, 1H), 8.46 (s, 3H).

^{13}C NMR (75 MHz, DMSO- d_6) δ 13.89, 22.04, 23.92, 28.38, 28.57, 28.63, 28.75, 28.90, 29.86, 31.23, 50.16, 51.70, 115.30, 168.88.

MS (ESI) calculated for $\text{C}_{14}\text{H}_{26}\text{N}_2\text{O}_2$: ($[\text{M}+\text{H}]^+$) m/z 255.2067, observed: m/z 255.2075

(S)-cyanomethyl 2-amino-3-(thiophen-2-yl) propanoate trifluoroacetate (Ala(2-thi)-CME)(for the introduction of 2-thienyl-alanine): The compound was synthesized by procedure A (above).

^1H NMR (300 MHz, D_2O): δ 3.59 (d, $J = 3.0$ Hz, 2H), 4.38, (m, 1H), 5.08 (s, 2H), 7.04 (m, 2H), 7.40 (s, 1H).

^{13}C NMR (75 MHz, D_2O) δ 29.44, 50.58, 53.74, 114.81, 126.73, 127.92, 128.70, 134.27, 167.96.

MS (ESI) calculated for $\text{C}_9\text{H}_{10}\text{N}_2\text{O}_2\text{S}$: ($[\text{M}+\text{H}]^+$) m/z 211.0536, observed: m/z 211.0564

(S)-cyanomethyl 2-amino-3-(thiophen-3-yl) propanoate trifluoroacetate (Ala(3-thi)-CME)(for the introduction of 3-thienyl-alanine): The compound was synthesized by procedure A (above).

^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 3.38 (m, 2H), 7.47 (d, $J = 4.9$ Hz, 1H), 5.06 (s, 2H), 4.55 (m, 1H), 7.04 (s, 1H), 7.31 (s, 1H), 7.49 (s, 1H).

^{13}C NMR (75 MHz, D_2O) δ 29.88, 50.50, 53.32, 114.84, 125.03, 128.01, 133.12, 168.33.

MS (ESI) calculated for $\text{C}_9\text{H}_{10}\text{N}_2\text{O}_2\text{S}$: ($[\text{M}+\text{H}]^+$) m/z 211.0536, observed: m/z 211.0571

(S)-cyanomethyl 2-amino-3-(3,4-dimethoxyphenyl) propanoate trifluoroacetate (Tyr(3,4-OMe)-CME)(for the introduction of 3-methoxy-4-O-methyl-tyrosine): The compound was synthesized by procedure A (above).

^1H NMR (300 MHz, MeOD) δ 3.09-3.14 (m, 2H), 3.73 (s, 3H), 3.76 (s, 3H), 4.36 (t, $J = 6.7$ Hz, 1H), 4.95 (s, 2H), 6.72 – 6.87 (m, 3H).

^{13}C NMR (75 MHz, MeOD) δ 36.88, 50.97, 55.04, 56.54, 113.56, 114.09, 115.32, 123.07, 127.20, 150.50, 151.03, 169.39.

MS (ESI) calculated for $\text{C}_{13}\text{H}_{16}\text{N}_2\text{O}_4$: ($[\text{M}+\text{H}]^+$) m/z 265.1183, observed: m/z 265.1208

General Procedure for the Synthesis of amino Acids with dinitrobenzyl ester (DBE) derivatization. (Procedure B). (S)-3,5-dinitrobenzyl-2-amino-3-methoxypropanoate hydrochloride (Ser(Me)-DBE)(for the introduction of β -O-methyl-serine): To a solution of 2-(*tert*-butyloxycarbonyl)amino-3-methoxypropanoic acid (DHCA salt)(301 mg, 0.75 mmol) in DMF (1 mL), 1-(chloromethyl)-3,5-dinitrobenzene (232 mg, 1.1 mmol) and *N,N,N*-triethylamine (590 μl , 4.2 mmol) was added at room temperature. After stirring for 16 h, the reaction mixture was diluted by adding EtOAc and washed with 1M HCl *aq.* (x2), saturated NaHCO_3 (x2) and brine (x5), dried (Na_2SO_4), and concentrated *in vacuo*. The residue was purified by preparative thinlayer chromatography (PTLC) (hexane:ethylacetate 4:1) to afford the 3,5-dinitrobenzylester (DNB) derivative. To a solution of the DNB derivative in CH_2Cl_2 (0.5 mL), 4 M HCl in EtOAc (4 mL) was added. After stirring for 30 min, the reaction mixture was concentrated *in vacuo*, and the final product precipitated by addition of

cold diethylether (5mL). The Solids were washed with cold diethylether (x5) and dried *in vacuo* to yield the final DBE esterified amino acid.

¹H NMR (300 MHz, DMSO-d₆): δ 3.32 (s, 3H), 3.80-3.90 (dd, *J* = 26.7, 3.0 Hz, 2H), 4.51 (t, 1H, *J* = 4.0 Hz), 5.56 (q, 2H, *J* = 13.7 Hz), 8.72 (m, 5H), 8.83 (s, 1H).

¹³C NMR (75 MHz, DMSO-d₆): δ 52.43, 58.74, 64.89, 69.58, 118.28, 127.99 (2C), 139.97, 148.06 (2C), 167.64.

MS (ESI) calculated for C₁₁H₁₃N₃O₇: ([M+H]⁺) *m/z* 300.0826, observed: *m/z* 300.0678

(S)-3,5-dinitrobenzyl 2-amino-3,3-dimethyl butanoate hydrochloride (Gly(tBu)-DBE)(for the introduction of *tert*-butylglycine): The compound was synthesized by procedure B (above).

¹H NMR (300 MHz, DMSO-d₆) δ 1.02 (s, 9H), 3.90 (m, 1H), 5.52 (s, 2H), 8.74 (s, 1H), 8.83 (s, 1H).

¹³C NMR (75 MHz, DMSO-d₆): δ 26.27, 33.33, 60.83, 64.98, 118.59, 128.73, 139.48, 147.93, 168.20.

MS (ESI) calculated for C₁₃H₁₇N₃O₆: ([M+H]⁺) *m/z* 312.1192, observed: *m/z* 312.1044

(S)-3,5-dinitrobenzyl 2-amino-3-(thiazol-4-yl) propanoate hydrochloride (Ala(4-Thz)-DBE)(for the introduction of 4-thiazolylalanine): The compound was synthesized by procedure B (above).

¹H NMR (300 MHz, DMSO-d₆): δ 3.41 (m, 2H), 4.53 (m, 1H), 5.44 (m, 2H), 7.52 (d, 1H, *J* = 2.1 Hz), 8.65 (m, 2H), 8.76-8.81 (m, 4H), 9.00 (s, 1H).

¹³C NMR (75 MHz, DMSO-d₆): δ 30.95, 51.60, 65.01, 117.64, 118.37, 128.55 (2C), 139.46, 148.01 (2C), 149.77, 154.64, 168.50.

MS (ESI) calculated for C₁₃H₁₂N₄O₆S: ([M+H]⁺) *m/z* 353.0550, observed: *m/z* 353.0404

(S)-3,5-dinitrobenzyl 2-amino-3-methoxybutanoate hydrochloride (Thr(Me)-DBE)(for the introduction of β-*O*-methyl-threonine): The compound was synthesized by procedure B (above).

¹H NMR (300 MHz, DMSO-d₆): δ 1.24 (d, 3H, *J* = 6.5 Hz), 3.26 (s, 3H), 3.91-3.99 (m, 1H), 4.31 (d, 1H, *J* = 3.9 Hz), 5.56 (q, 2H, *J* = 11.4 Hz), 8.60 (s, 3H), 8.74 (s, 2H), 8.84 (s, 1H).

¹³C NMR (75 MHz, DMSO-d₆): δ 15.13, 56.19, 56.41, 65.01, 74.12, 118.40, 128.34 (2C), 139.69, 148.10 (2C), 167.71.

MS (ESI) calculated for C₁₂H₁₅N₃O₇: ([M+H]⁺) *m/z* 314.0983, observed: *m/z* 314.0814

(S)-3,5-dinitrobenzyl 2-amino-4-methoxybutanoate hydrochloride (Hse(Me)-DBE)(for the introduction of γ -O-methyl-homoserine): The compound was synthesized by procedure B (above).

¹H NMR (300 MHz, DMSO-d₆): δ 2.12 (m, 2H), 3.19 (s, 3H), 3.47 (t, 2H, *J* = 5.4 Hz), 4.20 (t, 1H, *J* = 6.0 Hz), 5.48 (s, 2H), 8.60 (s, 3H), 8.75 (s, 2H), 8.82 (s, 1H).

¹³C NMR (75 MHz, DMSO-d₆): 29.97, 49.89, 57.96, 64.88, 66.86, 118.40, 128.44 (2C), 139.64, 148.04 (2C), 169.11.

MS (ESI) calculated for C₁₂H₁₅N₃O₇: ([M+H]⁺) *m/z* 314.0983, observed: *m/z* 314.0814

(S)-1-(3,5-dinitrobenzyl) 5-methyl 2-aminopentanedioate hydrochloride (Glu(Me)-DBE)(for introduction of ϵ -O-methyl-glutamate): The compound was synthesized by procedure B (above).

¹H NMR (300 MHz, DMSO-d₆): δ 2.13 (m, 2H), 2.51 (m, 2H), 4.19 (t, 1H, *J* = 6.9 Hz), 5.49 (m, 2H), 8.71 (d, 2H, *J* = 2.0 Hz), 8.75-8.30 (m, 5H).

¹³C NMR (75 MHz, DMSO-d₆): δ 25.18, 28.98, 51.20, 51.77, 65.13, 118.45, 128.57 (2C), 139.55, 148.13 (2C), 168.81, 172.18.

MS (ESI) calculated for C₁₃H₁₅N₃O₈: ([M+H]⁺) *m/z* 342.0932, observed: *m/z* 342.0776

(S)-3,5-dinitrobenzyl 2-(methylamino)butanoate hydrochloride (MeAbu-DBE)(for introduction of *N*-methyl-2-aminobutyric acid): The compound was synthesized by procedure B (above).

¹H NMR (300 MHz, DMSO-d₆): δ 0.91 (t, 3H, *J* = 7.2 Hz), 1.85-1.99 (m, 2H), 2.61 (s, 3H), 4.18 (t, 1H, *J* = 6.0 Hz), 5.54 (s, 2H), 8.74 (s, 2H), 8.84 (s, 1H), 9.19 (s, 2H).

¹³C NMR (75 MHz, DMSO-d₆): δ 8.90, 21.77, 31.46, 60.86, 118.85, 128.86 (2C), 139.51, 148.25 (2C), 168.43.

MS (ESI) calculated for C₁₂H₁₅N₃O₆: ([M+H]⁺) *m/z* 298.1034, observed: *m/z* 298.0949

(S)-3,5-dinitrobenzyl 2-amino-3-(perfluorophenyl)propanoate hydrochloride (Phe(F5)-DBE)(for introduction of perfluorophenylalanine): The compound was synthesized by procedure B (above).

¹H NMR (300 MHz, DMSO-d₆) δ 3.23-3.31 (m, 2H), 4.39 (t, 1H, *J* = 7.3 Hz), 5.44 (s, 2H), 8.65 (s, 2H), 8.84 (s, 1H), 8.91 (s, 3H).

¹³C NMR (75 MHz, DMSO-d₆): δ 23.06, 50.76, 65.20, 109.09, 109.15, 109.33, 109.39, 109.58, 109.63, 118.48, 128.55 (2C), 139.15, 148.03 (2C), 167.77.

MS (ESI) calculated for C₁₄H₁₇N₃O₈: ([M+H]⁺) *m/z* 436.0563, observed: *m/z* 436.0318

General Procedure for the Synthesis of α-N-methyl amino acids with dinitrobenzyl ester (DBE) derivatization (Procedure C). (S)-3,5-dinitrobenzyl 3-methoxy-2-(methylamino)butanoate hydrochloride (MeThr(Me)-DBE)(for introduction of N-methyl-β-O-methyl-threonine): To a solution of (S)-3,5-dinitrobenzyl 2-(*tert*-butyloxycarbonyl) 2-amino-3-methoxybutanoate (337 mg, 0.79 mmol) in DMF (0.5 mL), Ag₂O (920 mg, 4.0 mmol) and methyl iodide (500 μL, 8.0 mmol) was added at room temperature. The mixture was stirred at room temperature for 8 hours under a nitrogen atmosphere in the dark. The solids were filtered off and the filtrate was partitioned between water and diethylether. The aqueous phase was extracted with diethylether, the combined organic layers were washed with brine, dried (Na₂SO₄), and concentrated *in vacuo*. The residue was purified by preparative thinlayer chromatography (PTLC) (hexane:ethylacetate 4:1) to afford the boc-protected α-N-methyl 3,5-dinitrobenzylester (DNB) derivative. This was dissolved in CH₂Cl₂ (0.5 mL), and 4 M HCl in EtOAc (4 mL) was added. After stirring for 30 min, the reaction mixture was concentrated *in vacuo*, and the final product precipitated by addition of cold diethylether (5mL). The solids were washed with cold diethylether (x5) and dried *in vacuo* to yield the final DBE esterified α-N-methyl amino acid.

¹H NMR (300 MHz, DMSO-d₆): δ 1.25 (d, 3H, *J* = 7.4 Hz), 2.65 (s, 3H), 3.27 (s, 3H), 3.91 (m, 1H), 4.33 (m, 1H), 5.56 (m, 2H), 8.73 (s, 2H), 8.83 (s, 1H), 9.14 (s, 2H).

¹³C NMR (75 MHz, DMSO-d₆): δ 15.20, 32.78, 56.37, 63.81, 64.92, 74.27, 118.45, 128.42 (2C), 139.34, 148.90 (2C), 167.85.

MS (ESI) calculated for C₁₃H₁₇N₃O₇: ([M+H]⁺) *m/z* 328.1139, observed: *m/z* 328.1059

(S)-3,5-dinitrobenzyl 4-methoxy-2-(methylamino)butanoate hydrochloride (MeHse(Me)-DBE)(for introduction of γ-O-methyl-homoserine): This compounds was synthesized following procedure C (above).

¹H NMR (300 MHz, DMSO-d₆): δ 2.16 (m, 2H), 2.61 (s, 3H), 3.17 (s, 3H), 3.46 (m, 2H), 4.22 (m, 1H), 5.50 (s, 2H), 8.76 (s, 2H), 8.84 (s, 1H), 9.41 (s, 2H).

^{13}C NMR (75 MHz, DMSO- d_6): δ 28.60, 31.46, 52.83, 57.50, 58.25, 66.98, 118.45, 128.39 (2C), 139.53, 148.26 (2C), 168.42.

MS (ESI) calculated for $\text{C}_{13}\text{H}_{17}\text{N}_3\text{O}_7$: ($[\text{M}+\text{H}]^+$) m/z 328.1139, observed: m/z 328.1059

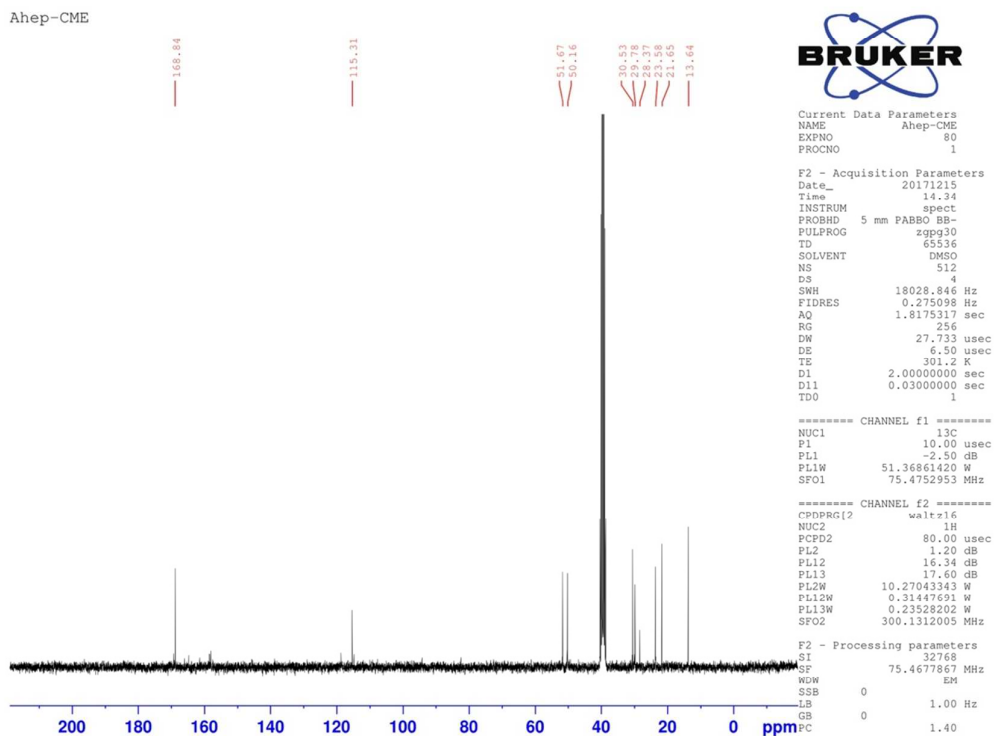
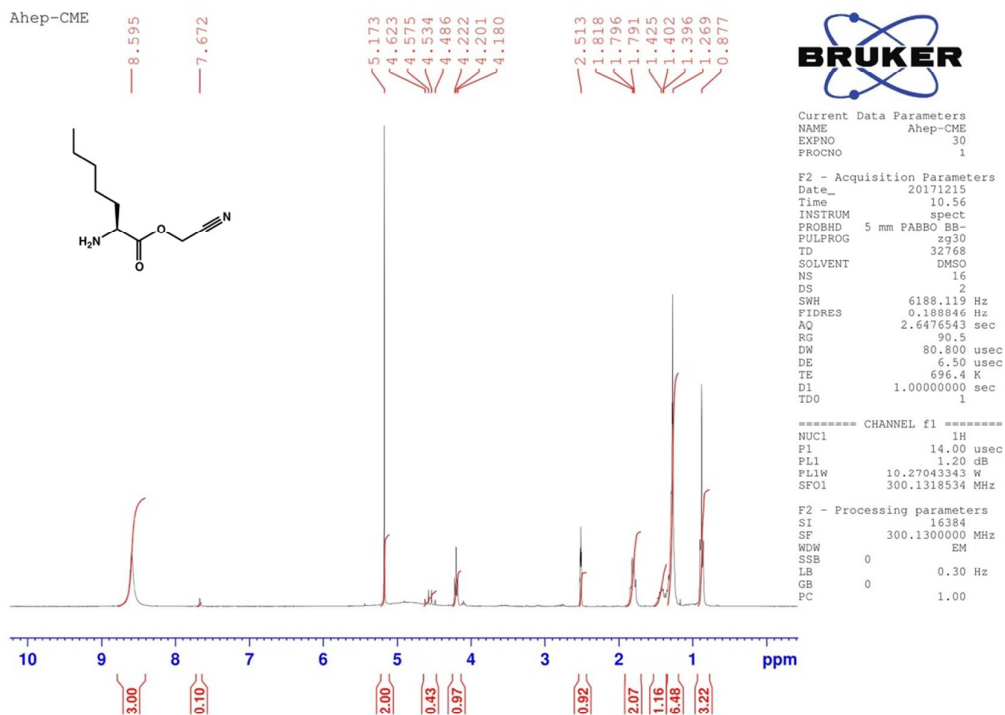
(S)-1-(3,5-dinitrobenzyl) 5-methyl 2-(methylamino) pentanedioate hydrochloride (MeGlu(Me)-DBE)(for introduction of *N*-methyl- ϵ -*O*-methyl-glutamate): This compound was synthesized following procedure C (above).

^1H NMR (300 MHz, DMSO- d_6) δ 2.12 (m, 2H), 2.54 (m, 2H), 2.60 (s, 3H), 3.58 (s, 3H), 4.22 (t, 1H, $J = 6.1$ Hz), 5.50 (m, 2H), 8.76 (s, 2H), 8.84 (s, 1H), 9.61 (s, 2H).

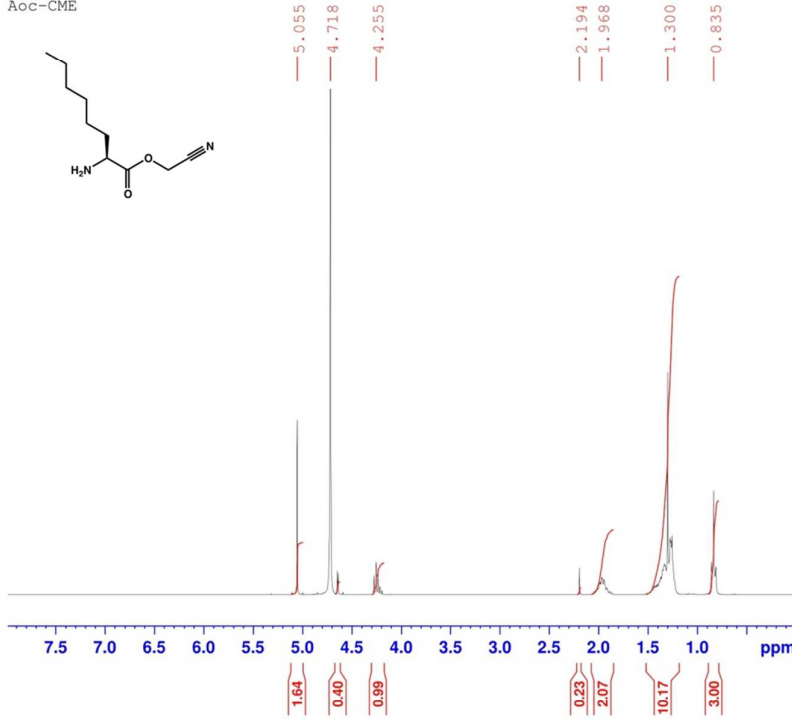
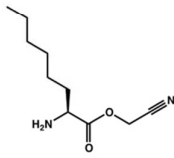
^{13}C NMR (75 MHz, DMSO- d_6) 23.46, 28.69, 30.93, 51.40, 58.57, 65.60, 118.34, 128.80 (2C), 139.11, 148.22 (2C), 167.95, 171.98.

MS (ESI) calculated for $\text{C}_{14}\text{H}_{17}\text{N}_3\text{O}_8$: ($[\text{M}+\text{H}]^+$) m/z 356.1088, observed: m/z 356.0959

NMR Spectra of previously undescribed compounds



Aoc-CME



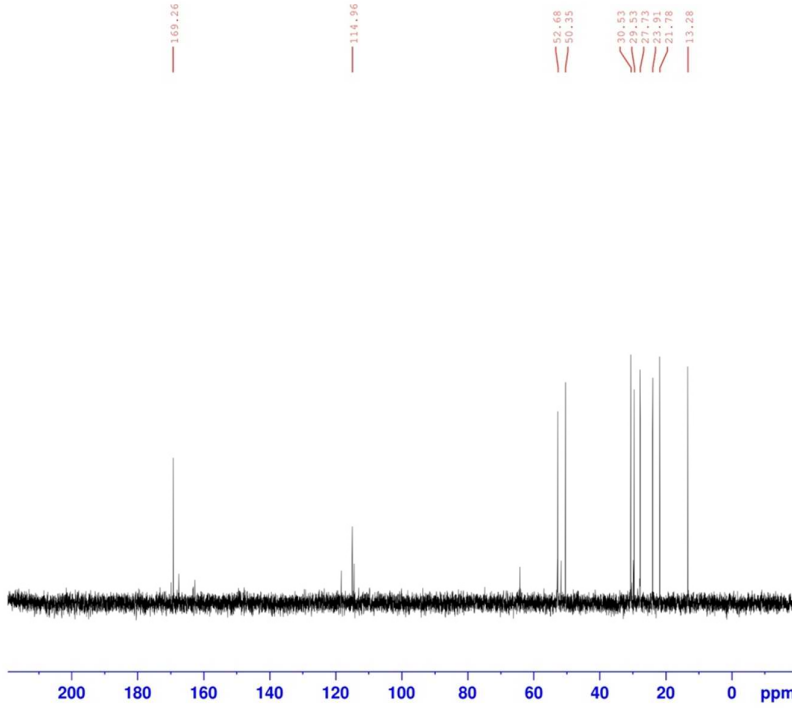
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 PULPROG zg30
 TD 32768
 SOLVENT D2O
 NS 64
 DS 2
 SWH 6188.119 Hz
 FIDRES 0.188846 Hz
 AQ 2.6476543 sec
 RG 80.6
 DW 80.800 usec
 DE 6.50 usec
 TE 301.2 K
 D1 1.00000000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 1H
 P1 14.00 usec
 PL1 1.20 dB
 PL1W 10.27043343 W
 SFO1 300.1318534 MHz

F2 - Processing parameters
 SI 16384
 SF 300.1300000 MHz
 WDW EM
 SSB 0
 LB 0.30 Hz
 GB 0
 PC 1.00

Aoc-CME



Current Data Parameters
 NAME Aoc-CME
 EXPNO 32
 PROCNO 1

F2 - Acquisition Parameters
 Date_ 20170721
 Time 19.21
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zgpg30
 TD 65536
 SOLVENT D2O
 NS 1024
 DS 4
 SWH 18028.846 Hz
 FIDRES 0.275098 Hz
 AQ 1.8175317 sec
 RG 228
 DW 27.733 usec
 DE 6.50 usec
 TE 301.8 K
 D1 2.00000000 sec
 D11 0.03000000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 13C
 P1 10.00 usec
 PL1 -2.50 dB
 PL1W 51.36861420 W
 SFO1 75.4752953 MHz

===== CHANNEL f2 =====
 CPDPRG12 waltz16
 NUC2 1H
 PCPD2 80.00 usec
 PL2 1.20 dB
 PL12 16.34 dB
 PL13 17.60 dB
 PL2W 10.27043343 W
 PL12W 0.31447691 W
 PL13W 0.23528202 W
 SFO2 300.1312005 MHz

F2 - Processing parameters
 SI 32768
 SF 75.4677490 MHz
 WDW EM
 SSB 0
 LB 1.00 Hz
 GB 0
 PC 1.40

Adec-CME

8.593

7.672

5.170
4.623
4.575
4.529
4.481
4.217
4.196
4.175

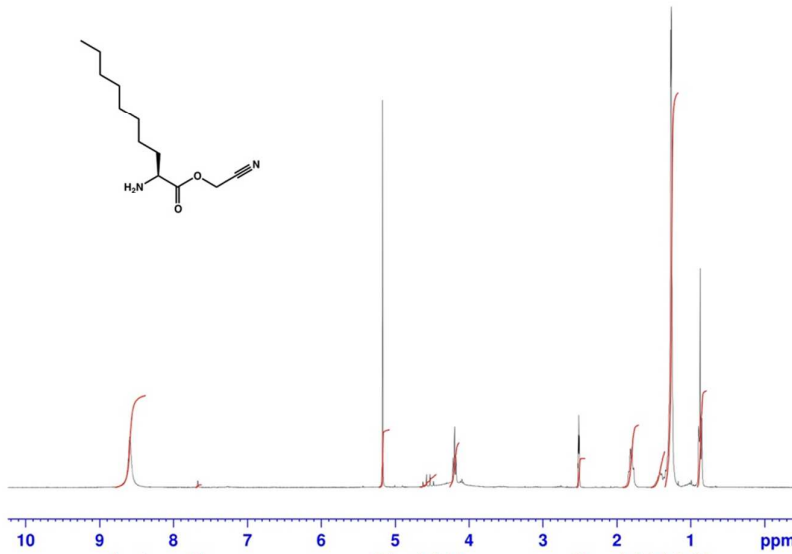
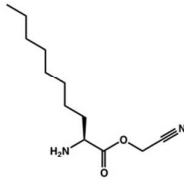
2.513

1.816

1.793

1.261

0.870



Current Data Parameters
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 PROCNO 1

F2 - Acquisition Parameters
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 Time 11.01
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zg30
 TD 32768
 SOLVENT DMSO
 NS 16
 DS 2
 SWH 6188.119 Hz
 FIDRES 0.188846 Hz
 AQ 2.6476543 sec
 RG 80.6
 DW 80.800 usec
 DE 6.50 usec
 TE 303.2 K
 D1 1.0000000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 1H
 P1 14.00 usec
 PL1 1.20 dB
 PL1W 10.27043343 W
 SFO1 300.1318534 MHz

F2 - Processing parameters
 SI 16384
 SF 300.1300000 MHz
 WDW EM
 SSB 0
 LB 0.30 Hz
 GB 0
 FC 1.00

Adec-CME

168.84

115.31

51.67

50.16

31.17

29.83

28.33

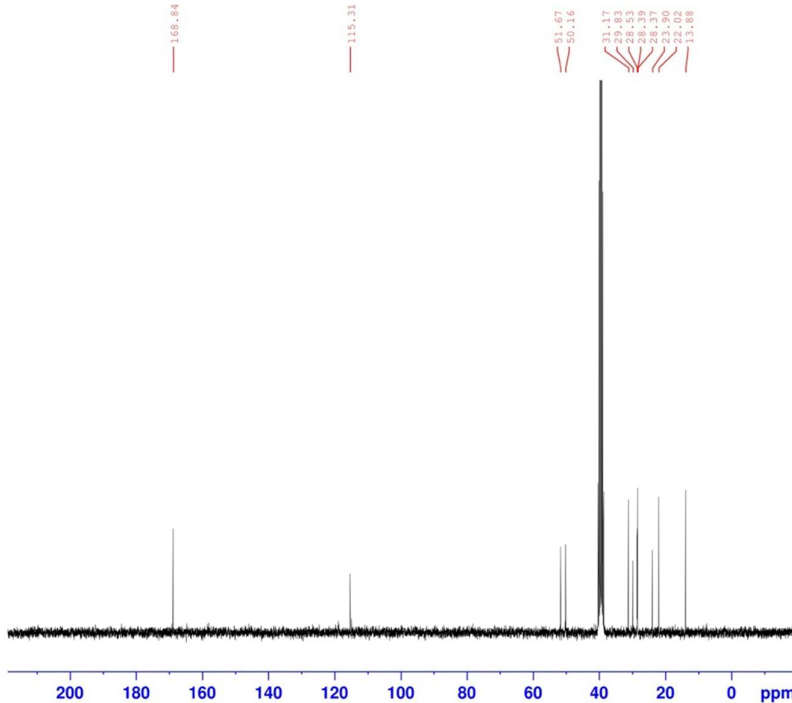
28.37

28.37

23.90

22.02

11.98



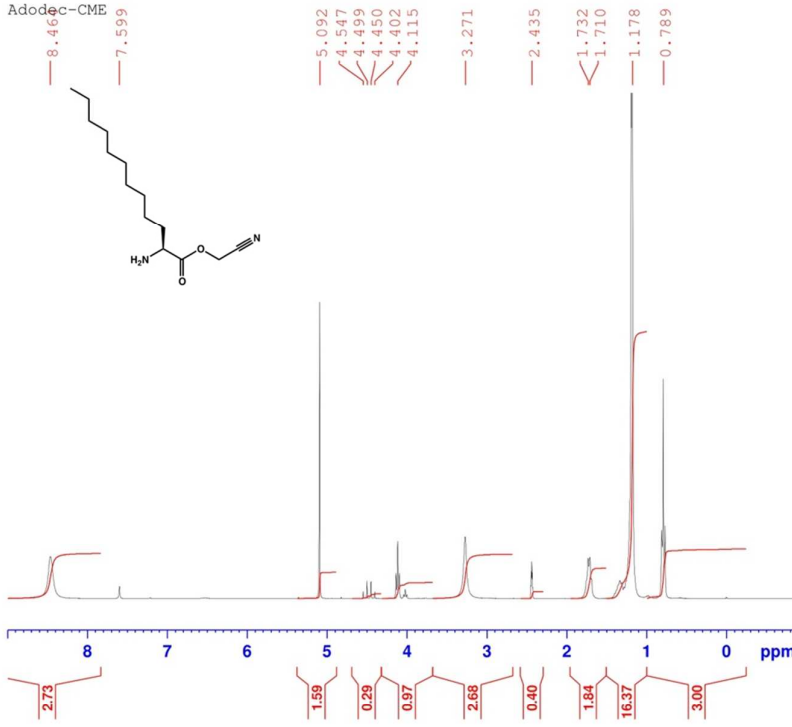
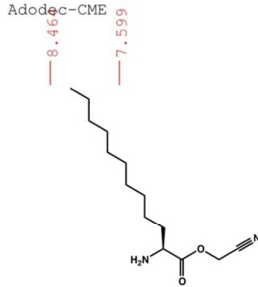
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 PROCNO 1

F2 - Acquisition Parameters
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 PULPROG zgpg30
 TD 65536
 SOLVENT DMSO
 NS 512
 DS 4
 SWH 18028.846 Hz
 FIDRES 0.275098 Hz
 AQ 1.8175317 sec
 RG 228
 DW 27.733 usec
 DE 6.50 usec
 TE 694.6 K
 D1 2.0000000 sec
 D11 0.0300000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 13C
 P1 10.00 usec
 PL1 -2.50 dB
 PL1W 51.36861420 W
 SFO1 75.4752953 MHz

===== CHANNEL f2 =====
 CPDPRG12 waltz16
 NUC2 1H
 PCPD2 80.00 usec
 PL2 1.20 dB
 PL12 16.34 dB
 PL13 17.60 dB
 PL2W 10.27043343 W
 PL12W 0.31447691 W
 PL13W 0.23528202 W
 SFO2 300.1312005 MHz

F2 - Processing parameters
 SI 32768
 SF 75.4677867 MHz
 WDW EM
 SSB 0
 LB 1.00 Hz
 GB 0
 FC 1.40



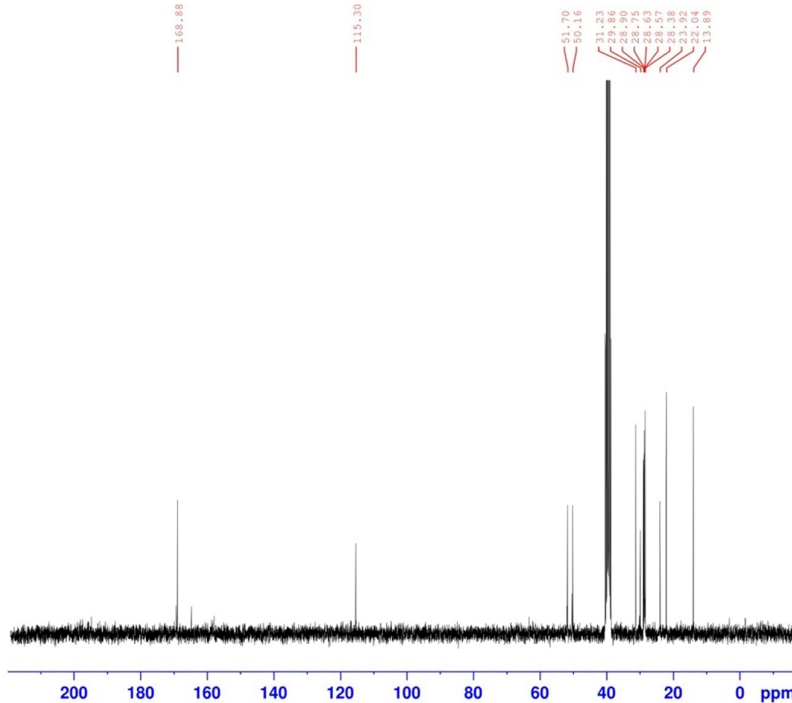
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 PROCNO 1

F2 - Acquisition Parameters
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 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zg30
 TD 32768
 SOLVENT DMSO
 NS 64
 DS 2
 SWH 6188.119 Hz
 FIDRES 0.188846 Hz
 AQ 2.6476543 sec
 RG 90.5
 DW 80.800 usec
 DE 6.50 usec
 TE 301.4 K
 D1 1.00000000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 1H
 P1 14.00 usec
 PL1 1.20 dB
 PL1W 10.27043343 W
 SFO1 300.1318534 MHz

F2 - Processing parameters
 SI 16384
 SF 300.1300245 MHz
 WDW EM
 SSB 0
 LB 0.30 Hz
 GB 0
 PC 1.00

Adodec-CME



Current Data Parameters
 NAME Adod-CME
 EXPNO 21
 PROCNO 1

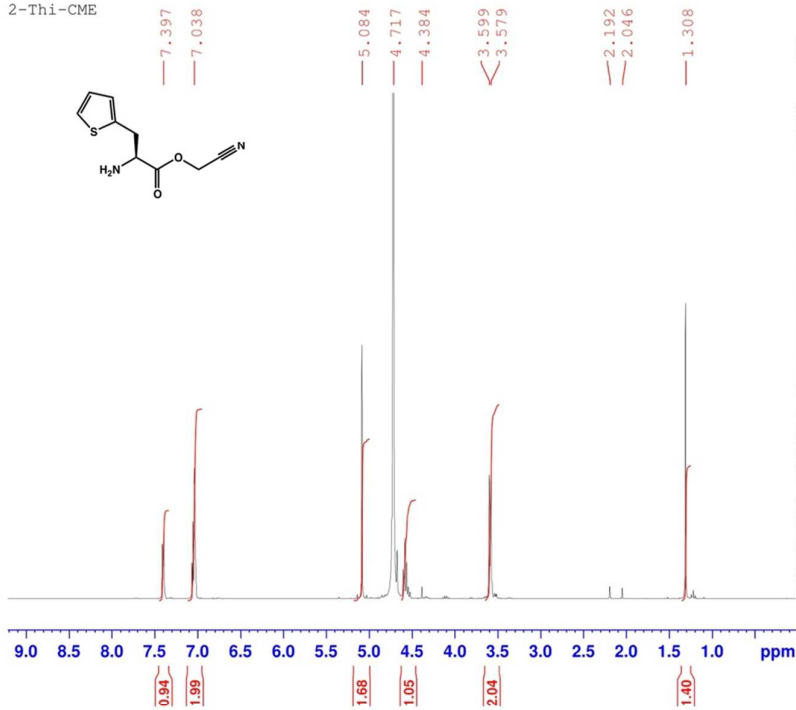
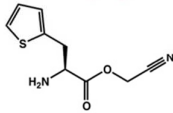
F2 - Acquisition Parameters
 Date_ 20170721
 Time 17.31
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zgpg30
 TD 65536
 SOLVENT DMSO
 NS 800
 DS 4
 SWH 18028.846 Hz
 FIDRES 0.275098 Hz
 AQ 1.8175317 sec
 RG 228
 DW 27.733 usec
 DE 6.50 usec
 TE 302.1 K
 D1 2.00000000 sec
 D11 0.03000000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 13C
 P1 10.00 usec
 PL1 -2.50 dB
 PL1W 51.36861420 W
 SFO1 75.4752953 MHz

===== CHANNEL f2 =====
 CPDPRG12 waltz16
 NUC2 1H
 PCPD2 80.00 usec
 PL2 1.20 dB
 PL12 16.34 dB
 PL13 17.60 dB
 PL2W 10.27043343 W
 PL12W 0.31447691 W
 PL13W 0.23528202 W
 SFO2 300.1312005 MHz

F2 - Processing parameters
 SI 32768
 SF 75.4677867 MHz
 WDW EM
 SSB 0
 LB 1.00 Hz
 GB 0
 PC 1.40

2-Thi-CME



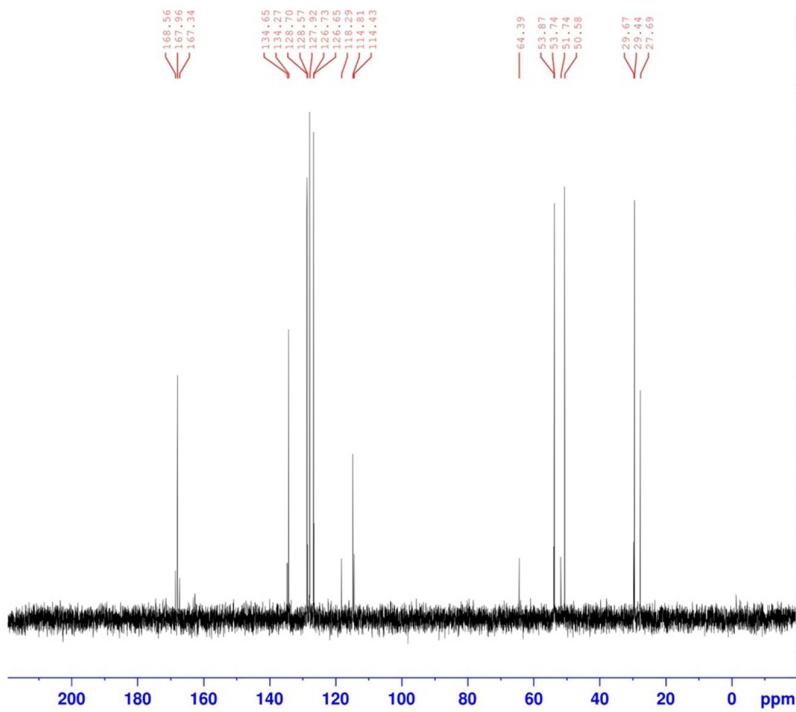
Current Data Parameters
 NAME 2-Thi-CME
 EXPNO 15
 PROCNO 1

F2 - Acquisition Parameters
 Date_ 20170721
 Time 10.30
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zg30
 TD 65536
 SOLVENT D2O
 NS 16
 DS 2
 SWH 6188.119 Hz
 FIDRES 0.094423 Hz
 AQ 5.2953086 sec
 RG 80.6
 DW 80.800 usec
 DE 6.50 usec
 TE 301.3 K
 D1 1.00000000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 1H
 P1 14.00 usec
 PL1 1.20 dB
 PL1W 10.27043343 W
 SFO1 300.1318534 MHz

F2 - Processing parameters
 SI 32768
 SF 300.1300000 MHz
 WDW EM
 SSB 0
 LB 0.30 Hz
 GB 0
 PC 1.00

2-Thi-CME



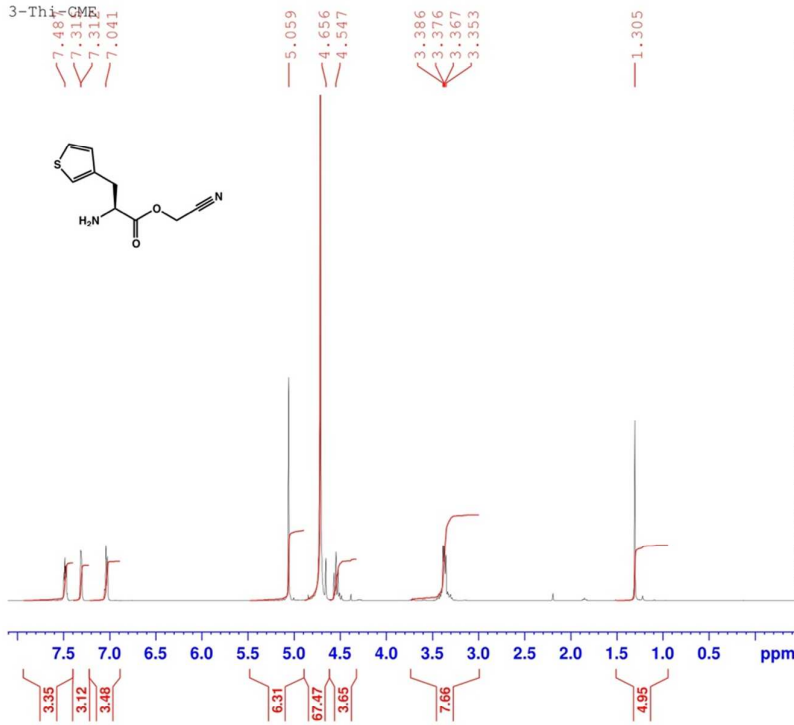
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 NAME 2-Thi-CME
 EXPNO 14
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F2 - Acquisition Parameters
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 Time 10.27
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zgpg30
 TD 65536
 SOLVENT D2O
 NS 1024
 DS 4
 SWH 18028.846 Hz
 FIDRES 0.275098 Hz
 AQ 1.8175317 sec
 RG 256
 DW 27.733 usec
 DE 6.50 usec
 TE 301.6 K
 D1 2.00000000 sec
 D11 0.03000000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 13C
 P1 10.00 usec
 PL1 -2.50 dB
 PL1W 51.36861420 W
 SFO1 75.4752953 MHz

===== CHANNEL f2 =====
 CPDPRG12 waltz16
 NUC2 1H
 PCPD2 80.00 usec
 PL2 1.20 dB
 PL12 16.34 dB
 PL13 17.60 dB
 PL2W 10.27043343 W
 PL12W 0.31447691 W
 PL13W 0.23528202 W
 SFO2 300.1312005 MHz

F2 - Processing parameters
 SI 32768
 SF 75.4677490 MHz
 WDW EM
 SSB 0
 LB 1.00 Hz
 GB 0
 PC 1.40

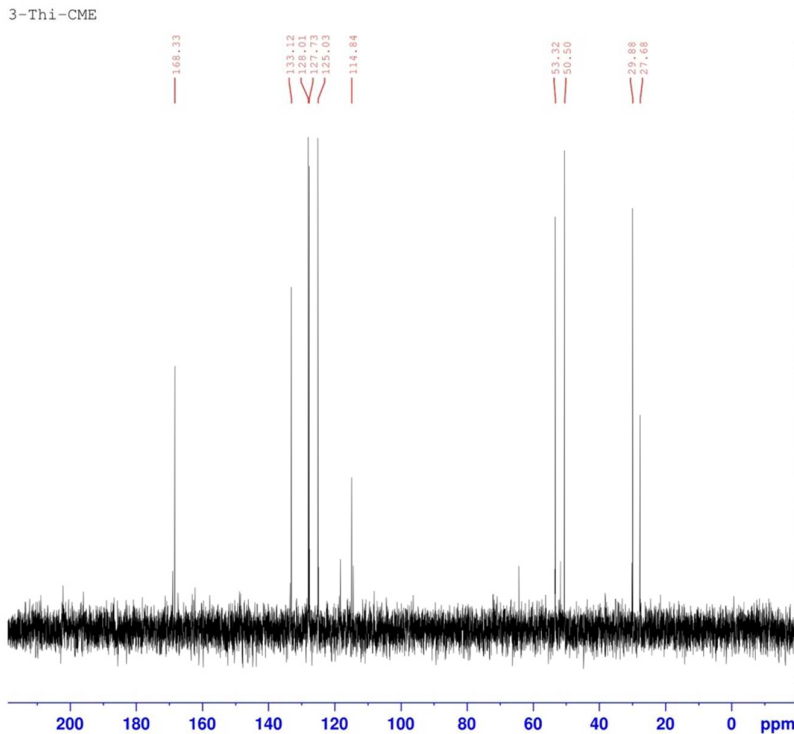


Current Data Parameters
 NAME 3-Thi-CME
 EXPNO 42
 PROCNO 1

F2 - Acquisition Parameters
 Date_ 20170720
 Time 18.42
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zg30
 TD 32768
 SOLVENT D2O
 NS 64
 DS 2
 SWH 6188.119 Hz
 FIDRES 0.188846 Hz
 AQ 2.6476543 sec
 RG 128
 DW 80.800 usec
 DE 6.50 usec
 TE 301.1 K
 D1 1.00000000 sec
 TDO 1

===== CHANNEL f1 =====
 NUC1 1H
 P1 14.00 usec
 PL1 1.20 dB
 PL1W 10.27043343 W
 SFO1 300.1318534 MHz

F2 - Processing parameters
 SI 16384
 SF 300.1300000 MHz
 WDW EM
 SSB 0
 LB 0.30 Hz
 GB 0
 PC 1.00



Current Data Parameters
 NAME 3-Thi-CME
 EXPNO 41
 PROCNO 1

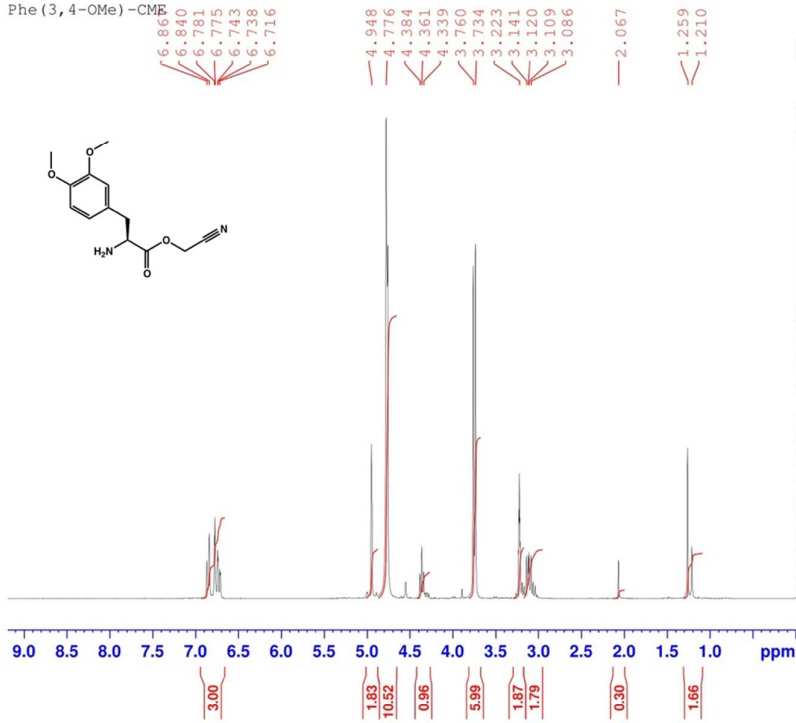
F2 - Acquisition Parameters
 Date_ 20170720
 Time 18.27
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zgpg30
 TD 65536
 SOLVENT D2O
 NS 1024
 DS 4
 SWH 18028.846 Hz
 FIDRES 0.275098 Hz
 AQ 1.8175317 sec
 RG 287
 DW 27.733 usec
 DE 6.50 usec
 TE 301.6 K
 D1 2.00000000 sec
 D11 0.03000000 sec
 TDO 1

===== CHANNEL f1 =====
 NUC1 13C
 P1 10.00 usec
 PL1 -2.50 dB
 PL1W 51.36861420 W
 SFO1 75.4752953 MHz

===== CHANNEL f2 =====
 CPDPRG12 waltz16
 NUC2 1H
 PCPD2 80.00 usec
 PL2 1.20 dB
 PL12 16.34 dB
 PL13 17.60 dB
 PL2W 10.27043343 W
 PL12W 0.31447691 W
 PL13W 0.23528202 W
 SFO2 300.1312005 MHz

F2 - Processing parameters
 SI 32768
 SF 75.4677490 MHz
 WDW EM
 SSB 0
 LB 1.00 Hz
 GB 0
 PC 1.40

Phe(3,4-OMe)-CME



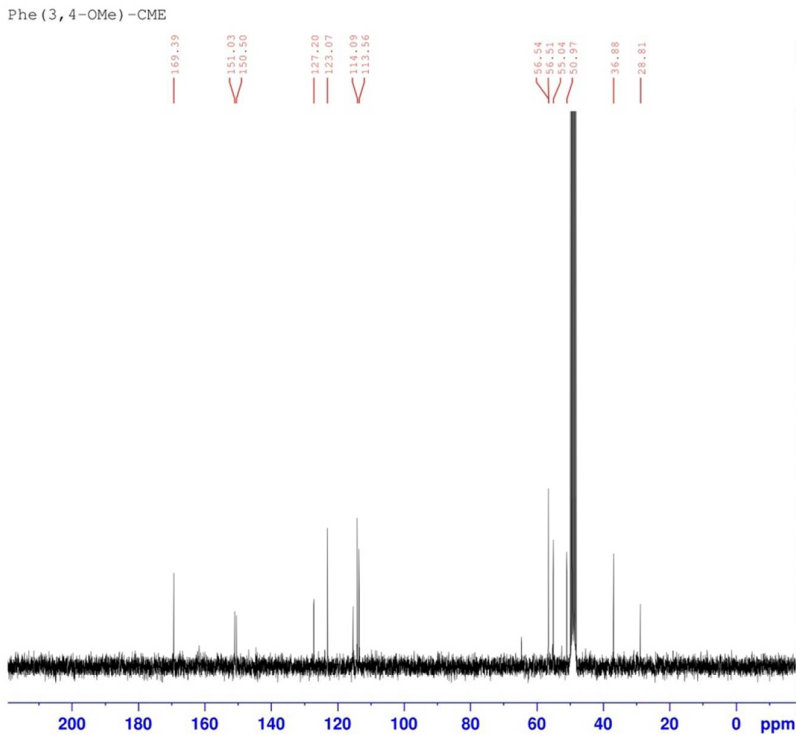
Current Data Parameters
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 PROCNO 1

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 PULPROG zg30
 TD 32768
 SOLVENT MeOD
 NS 64
 DS 2
 SWH 6188.119 Hz
 FIDRES 0.188846 Hz
 AQ 2.6476543 sec
 RG 128
 DW 80.800 usec
 DE 6.50 usec
 TE 301.0 K
 D1 1.0000000 sec
 TDO 1

===== CHANNEL f1 =====
 NUC1 1H
 P1 14.00 usec
 PL1 1.20 dB
 PL1W 10.27043343 W
 SFO1 300.1318534 MHz

F2 - Processing parameters
 SI 16384
 SF 300.1300317 MHz
 WDW EM
 SSB 0
 LB 0.30 Hz
 GB 0
 FC 1.00

Phe(3,4-OMe)-CME



Current Data Parameters
 NAME Phe(3,4-OMe)-CME
 EXPNO 41
 PROCNO 1

F2 - Acquisition Parameters
 Date_ 20170721
 Time 21.52
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zgpg30
 TD 65536
 SOLVENT MeOD
 NS 1024
 DS 4
 SWH 18028.846 Hz
 FIDRES 0.275098 Hz
 AQ 1.8175317 sec
 RG 256
 DW 27.733 usec
 DE 6.50 usec
 TE 301.7 K
 D1 2.0000000 sec
 D11 0.0300000 sec
 TDO 1

===== CHANNEL f1 =====
 NUC1 13C
 P1 10.00 usec
 PL1 -2.50 dB
 PL1W 51.36861420 W
 SFO1 75.4752953 MHz

===== CHANNEL f2 =====
 CPDPRG12 waltz16
 NUC2 1H
 PCPD2 80.00 usec
 PL2 1.20 dB
 PL12 16.34 dB
 PL13 17.60 dB
 PL2W 10.27043343 W
 PL12W 0.31447691 W
 PL13W 0.23528202 W
 SFO2 300.1312005 MHz

F2 - Processing parameters
 SI 32768
 SF 75.4676418 MHz
 WDW EM
 SSB 0
 LB 1.00 Hz
 GB 0
 FC 1.40

4-Thz-DBE

9.00
8.99
8.97
8.76
8.66
8.65

7.53
7.52

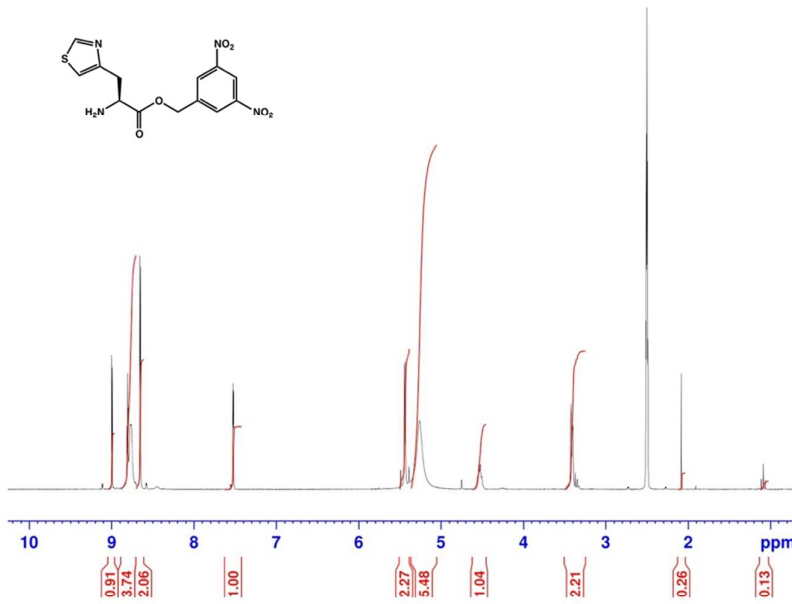
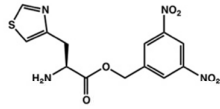
5.45
5.43
5.26

4.54
4.52

3.42
3.40
3.40

2.08

1.09



Current Data Parameters
NAME 4-Thz-DBE
EXPNO 3
PROCNO 1
F2 - Acquisition Parameters
Date_ 20170219
Time 17.12
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zg30
TD 32768
SOLVENT DMSO
NS 50
DS 2
SWH 6188.119 Hz
FIDRES 0.188846 Hz
AQ 2.6476543 sec
RG 228
DW 80.800 usec
DE 6.50 usec
TE 299.6 K
D1 1.00000000 sec
TDO 1
===== CHANNEL f1 =====
NUC1 1H
P1 14.00 usec
PL1 1.20 dB
PL1W 10.27043343 W
SFO1 300.1318534 MHz
F2 - Processing parameters
SI 16384
SF 300.1300036 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

4-Thz-DBE

168.50

154.64

149.77

148.01

139.46

128.55

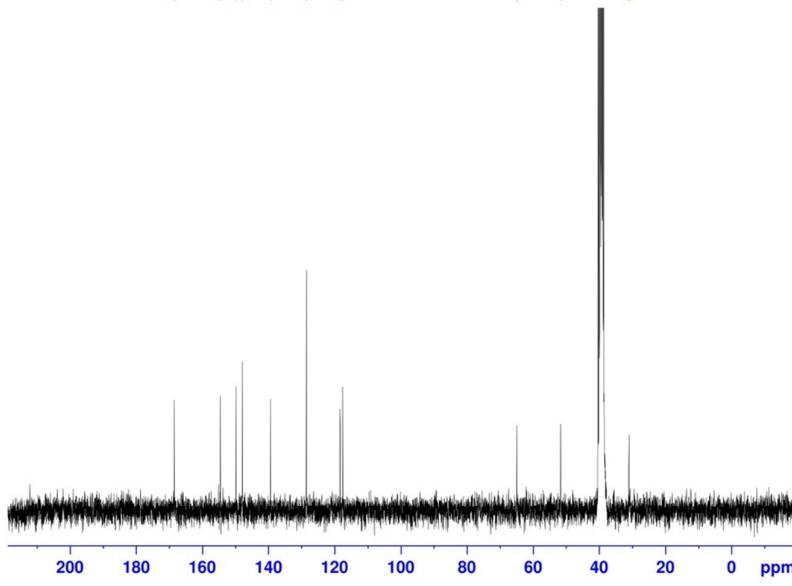
118.37

117.64

65.01

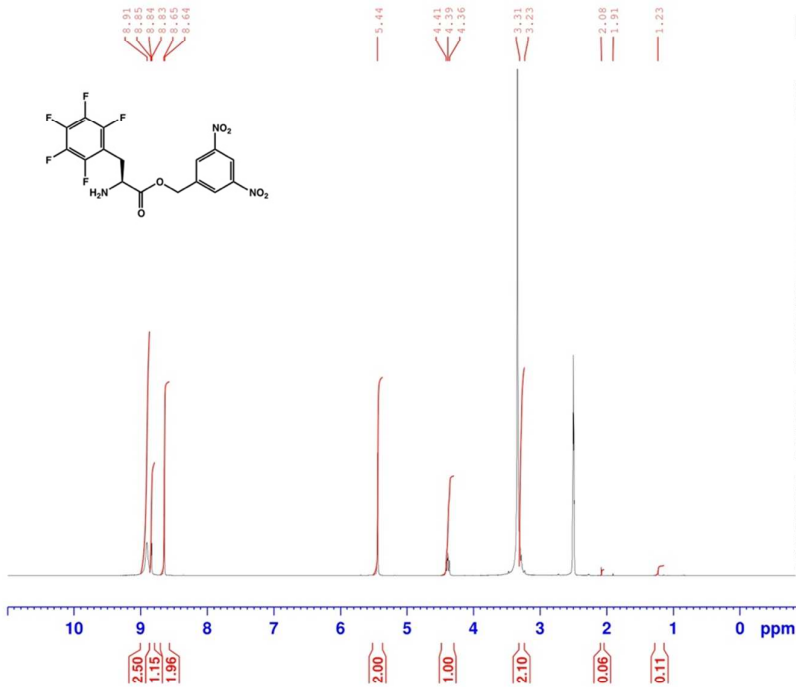
51.60

30.96



Current Data Parameters
NAME 4-Thz-DBE
EXPNO 4
PROCNO 1
F2 - Acquisition Parameters
Date_ 20170219
Time 18.50
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zgpg30
TD 65536
SOLVENT DMSO
NS 1500
DS 4
SWH 18028.846 Hz
FIDRES 0.275098 Hz
AQ 1.8175317 sec
RG 181
DW 27.733 usec
DE 6.50 usec
TE 299.5 K
D1 2.00000000 sec
D11 0.03000000 sec
TDO 1
===== CHANNEL f1 =====
NUC1 13C
P1 10.00 usec
PL1 -2.50 dB
PL1W 51.36861420 W
SFO1 75.4752953 MHz
===== CHANNEL f2 =====
CPDPRG12 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 1.20 dB
PL12 16.34 dB
PL13 17.60 dB
PL2W 10.27043343 W
PL12W 0.31447691 W
PL13W 0.23528202 W
SFO2 300.1312005 MHz
F2 - Processing parameters
SI 32768
SF 75.4677867 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

Phe (F5) -DBE



```

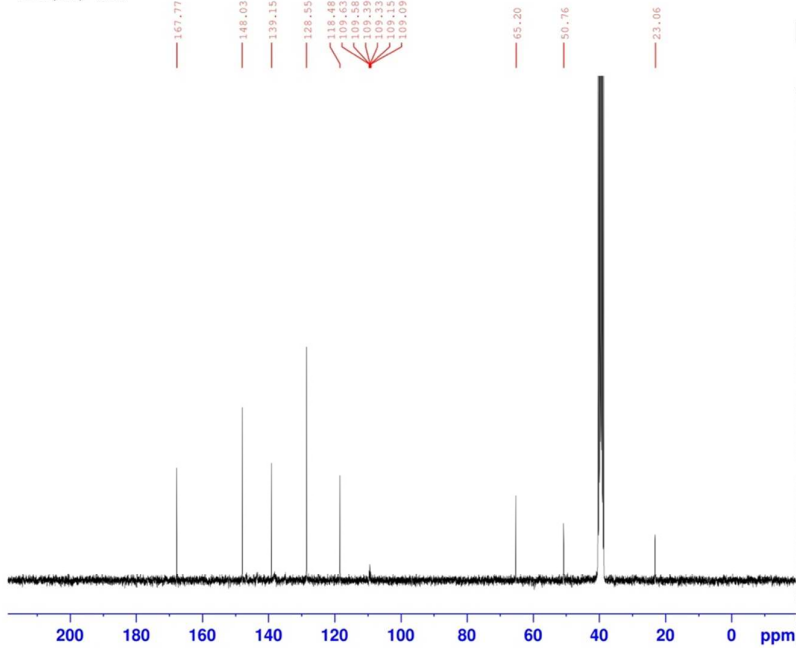
Current Data Parameters
NAME      Phe (F5)-DBE
EXPNO    20
PROCNO   1

F2 - Acquisition Parameters
Date_    20170427
Time     23.46
INSTRUM  spect
PROBHD   5 mm PABBO BB-
PULPROG  zg30
TD       32768
SOLVENT  DMSO
NS       50
DS       2
SWH      6188.119 Hz
FIDRES   0.188846 Hz
AQ       2.6476543 sec
RG       128
DW       80.800 usec
DE       6.50 usec
TE       298.0 K
D1       1.00000000 sec
TDO     1

===== CHANNEL f1 =====
NUC1     1H
P1       14.00 usec
PL1      1.20 dB
PL1W    10.27043343 W
SFO1    300.1318534 MHz

F2 - Processing parameters
SI       16384
SF       300.1300052 MHz
WDW      EM
SSB      0
LB       0.30 Hz
GB       0
PC       1.00
    
```

Phe (F5) -DBE



```

Current Data Parameters
NAME      Phe (F5)-DBE
EXPNO    21
PROCNO   1

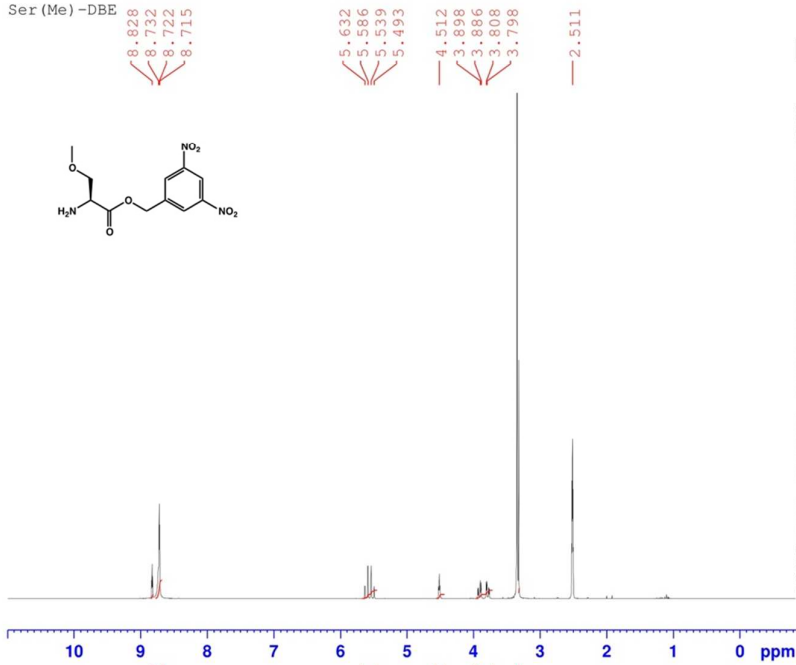
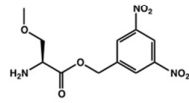
F2 - Acquisition Parameters
Date_    20170428
Time     8.25
INSTRUM  spect
PROBHD   5 mm PABBO BB-
PULPROG  zgpg30
TD       65536
SOLVENT  DMSO
NS       8000
DS       4
SWH      18028.846 Hz
FIDRES   0.275098 Hz
AQ       1.8175317 sec
RG       144
DW       27.733 usec
DE       6.50 usec
TE       297.6 K
D1       2.00000000 sec
D11     0.03000000 sec
TDO     1

===== CHANNEL f1 =====
NUC1     13C
P1       10.00 usec
PL1      -2.50 dB
PL1W    51.36861420 W
SFO1    75.4752953 MHz

===== CHANNEL f2 =====
CPDPRG2  waltz16
NUC2     1H
PCPD2    80.00 usec
PL2      1.20 dB
PL12     16.34 dB
PL13     17.60 dB
PL2W    10.27043343 W
PL12W   0.31447691 W
PL13W   0.23528202 W
SFO2    300.1312005 MHz

F2 - Processing parameters
SI       32768
SF       75.4677867 MHz
WDW      EM
SSB      0
LB       1.00 Hz
GB       0
PC       1.40
    
```

Ser (Me) -DBE



```

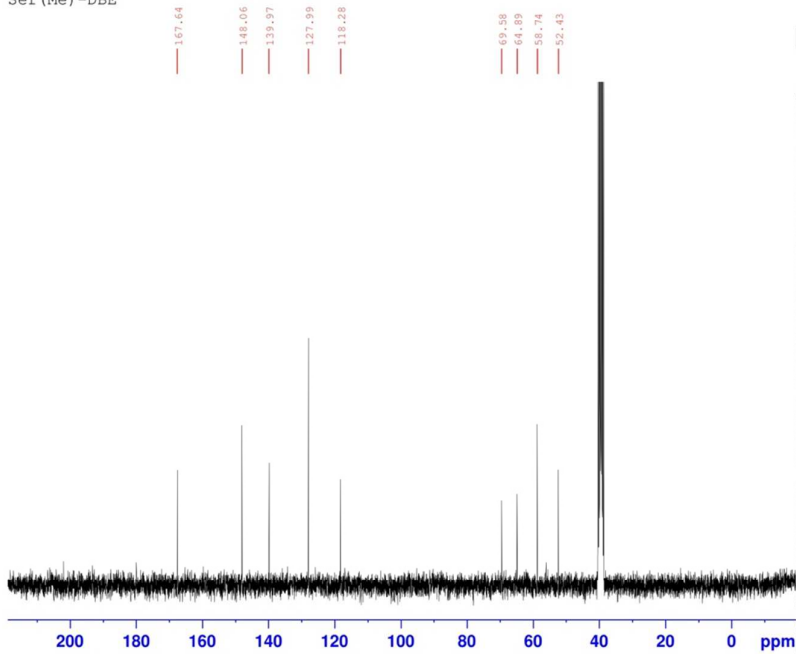
Current Data Parameters
NAME      Ser (Me)-DBE
EXPNO    3
PROCNO   1

F2 - Acquisition Parameters
Date_    20170221
Time     18.33
INSTRUM spect
PROBHD   5 mm PABBO BB-
PULPROG  zg30
TD       32768
SOLVENT  DMSO
NS       20
DS       2
SWH      6188.119 Hz
FIDRES   0.188846 Hz
AQ       2.6476543 sec
RG       203
DW       80.800 usec
DE       6.50 usec
TE       296.4 K
D1       1.0000000 sec
TDO     1

===== CHANNEL f1 =====
NUC1     1H
P1       14.00 usec
PL1      1.20 dB
PL1W    10.27043343 W
SFO1    300.1318534 MHz

F2 - Processing parameters
SI       16384
SF       300.1300000 MHz
WDW      EM
SSB      0
LB       0.30 Hz
GB       0
PC       1.00
  
```

Ser (Me) -DBE



```

Current Data Parameters
NAME      Ser (Me)-DBE
EXPNO    4
PROCNO   1

F2 - Acquisition Parameters
Date_    20170221
Time     19.53
INSTRUM spect
PROBHD   5 mm PABBO BB-
PULPROG  zgpg30
TD       65536
SOLVENT  DMSO
NS       1200
DS       4
SWH      18028.846 Hz
FIDRES   0.275098 Hz
AQ       1.8175317 sec
RG       128
DW       27.733 usec
DE       6.50 usec
TE       296.9 K
D1       2.0000000 sec
D11     0.0300000 sec
TDO     1

===== CHANNEL f1 =====
NUC1     13C
P1       10.00 usec
PL1      -2.50 dB
PL1W    51.36861420 W
SFO1    75.4752953 MHz

===== CHANNEL f2 =====
CPDPRG12 waltz16
NUC2     1H
PCPD2    80.00 usec
PL2      1.20 dB
PL12     16.34 dB
PL13     17.60 dB
PL2W    10.27043343 W
PL12W   0.31447691 W
PL13W   0.23528202 W
SFO2    300.1312005 MHz

F2 - Processing parameters
SI       32768
SF       75.4677867 MHz
WDW      EM
SSB      0
LB       1.00 Hz
GB       0
PC       1.40
  
```

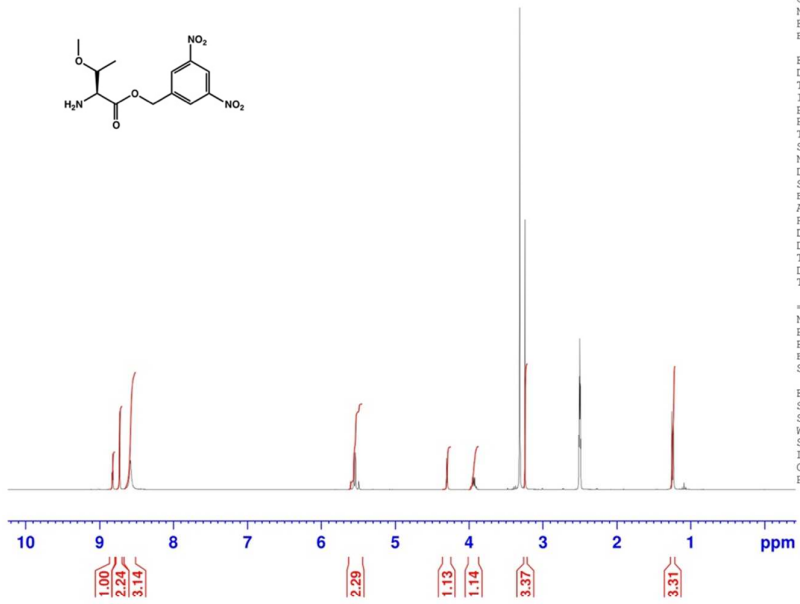
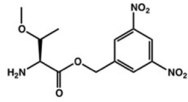
Thr (Me)-DBE

8.85
8.83
8.75
8.74
8.69

5.62
5.57
5.55
5.51

4.32
4.30
3.99
3.96
3.93
3.91

3.26
1.25
1.23



Current Data Parameters
NAME Thr (Me)-DBE
EXPNO 1
PROCNO 1
F2 - Acquisition Parameters
Date_ 20170302
Time 13.26
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zg30
TD 32768
SOLVENT DMSO
NS 50
DS 2
SWH 6188.119 Hz
FIDRES 0.188846 Hz
AQ 2.6476543 sec
RG 161
DW 80.800 usec
DE 6.50 usec
TE 300.1 K
D1 1.00000000 sec
TDO 1
===== CHANNEL f1 =====
NUC1 1H
P1 14.00 usec
PL1 1.20 dB
PL1W 10.27043343 W
SFO1 300.1318534 MHz
F2 - Processing parameters
SI 16384
SF 300.1300038 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

Thr (Me)-DBE

167.71

148.10

139.69

128.34

118.40

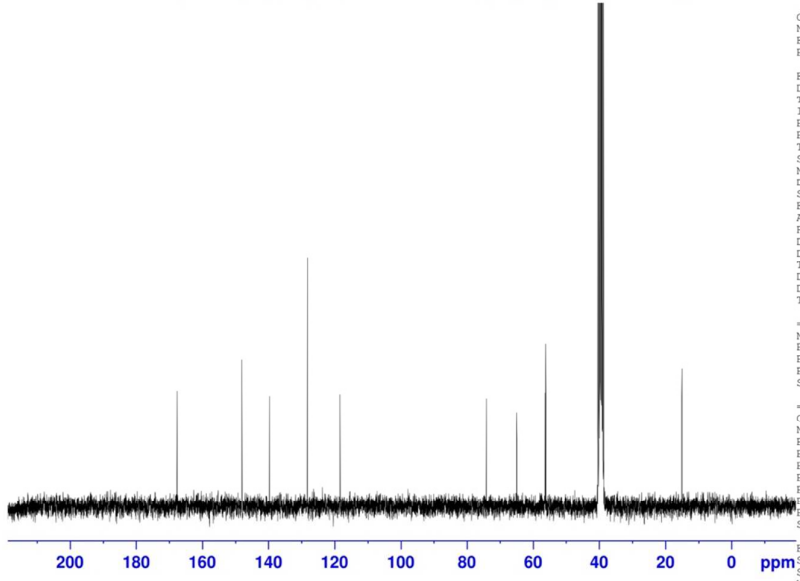
74.12

65.01

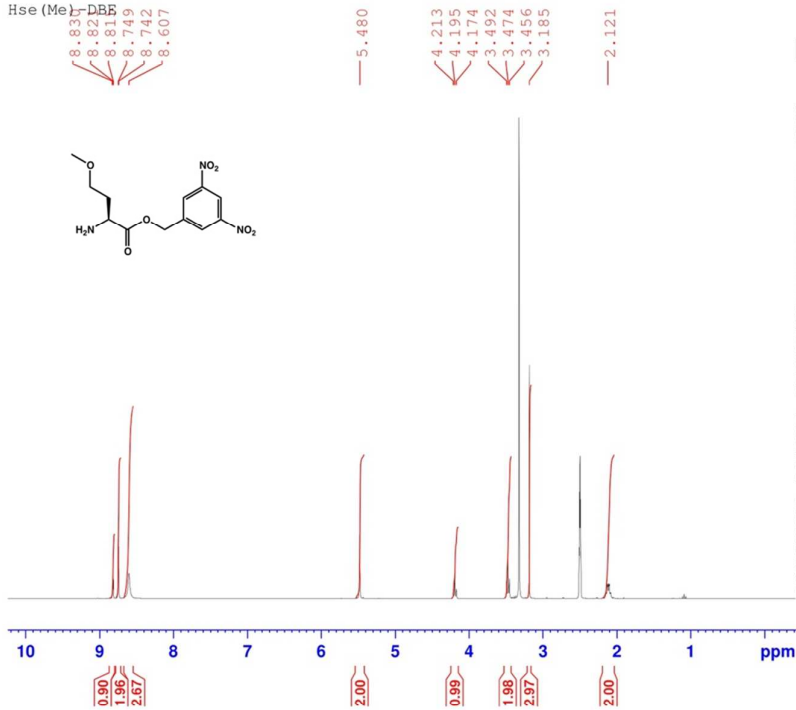
56.41

56.19

15.13



Current Data Parameters
NAME Thr (Me)-DBE
EXPNO 2
PROCNO 1
F2 - Acquisition Parameters
Date_ 20170302
Time 15.05
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zgpg30
TD 65536
SOLVENT DMSO
NS 1500
DS 4
SWH 18028.846 Hz
FIDRES 0.275098 Hz
AQ 1.8175317 sec
RG 161
DW 27.733 usec
DE 6.50 usec
TE 300.2 K
D1 2.00000000 sec
D11 0.03000000 sec
TDO 1
===== CHANNEL f1 =====
NUC1 13C
P1 10.00 usec
PL1 -2.50 dB
PL1W 51.36861420 W
SFO1 75.4752953 MHz
===== CHANNEL f2 =====
CPDPRG12 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 1.20 dB
PL12 16.34 dB
PL13 17.60 dB
PL2W 10.27043343 W
PL12W 0.31447691 W
PL13W 0.23528202 W
SFO2 300.1312005 MHz
F2 - Processing parameters
SI 32768
SF 75.4677867 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

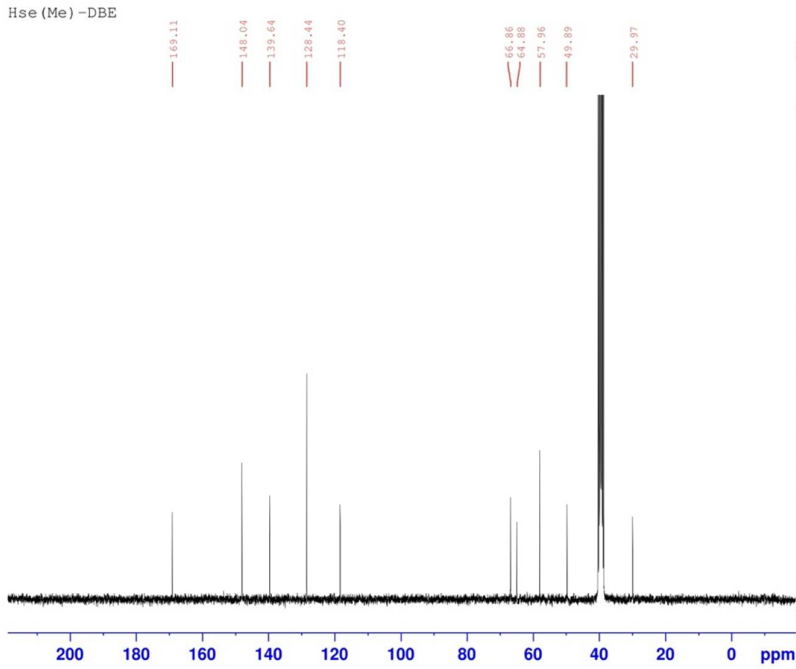


Current Data Parameters
 NAME Hse (Me)-DBE
 EXPNO 3
 PROCNO 1

F2 - Acquisition Parameters
 Date_ 20170302
 Time 21.01
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zg30
 TD 32768
 SOLVENT DMSO
 NS 50
 DS 2
 SWH 6188.119 Hz
 FIDRES 0.188846 Hz
 AQ 2.6476543 sec
 RG 161
 DW 80.800 usec
 DE 6.50 usec
 TE 297.7 K
 D1 1.00000000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 1H
 P1 14.00 usec
 PL1 1.20 dB
 PL1W 10.27043343 W
 SFO1 300.1318534 MHz

F2 - Processing parameters
 SI 16384
 SF 300.1300048 MHz
 WDW EM
 SSB 0
 LB 0.30 Hz
 GB 0
 PC 1.00



Current Data Parameters
 NAME Hse (Me)-DBE
 EXPNO 4
 PROCNO 1

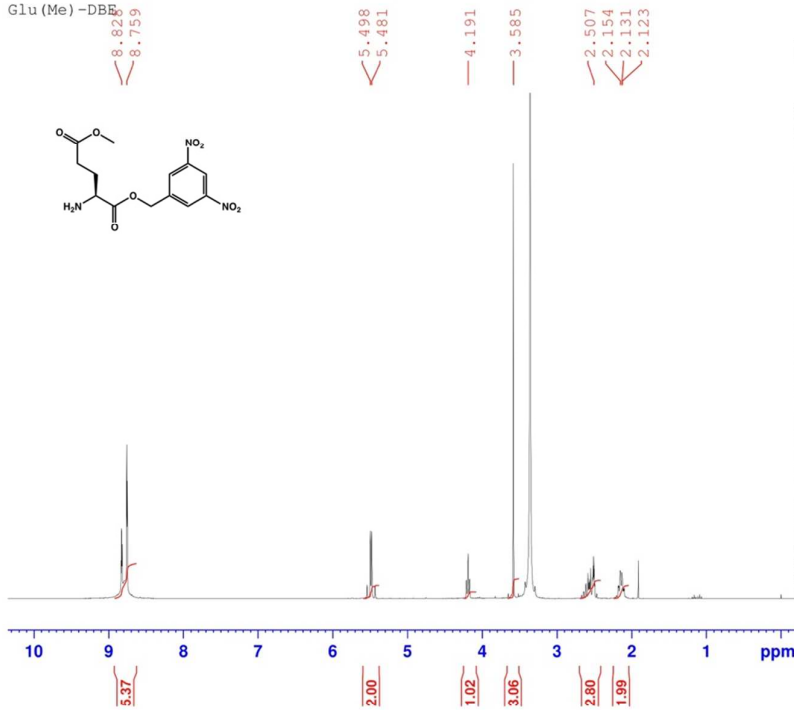
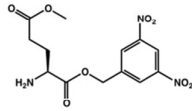
F2 - Acquisition Parameters
 Date_ 20170303
 Time 5.41
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zgpg30
 TD 65536
 SOLVENT DMSO
 NS 8000
 DS 4
 SWH 18028.846 Hz
 FIDRES 0.275098 Hz
 AQ 1.8175317 sec
 RG 128
 DW 27.733 usec
 DE 6.50 usec
 TE 297.1 K
 D1 2.00000000 sec
 D11 0.03000000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 13C
 P1 10.00 usec
 PL1 -2.50 dB
 PL1W 51.36861420 W
 SFO1 75.4752953 MHz

===== CHANNEL f2 =====
 CPDPRG12 waltz16
 NUC2 1H
 PCPD2 80.00 usec
 PL2 1.20 dB
 PL12 16.34 dB
 PL13 17.60 dB
 PL2W 10.27043343 W
 PL12W 0.31447691 W
 PL13W 0.23528202 W
 SFO2 300.1312005 MHz

F2 - Processing parameters
 SI 32768
 SF 75.4677867 MHz
 WDW EM
 SSB 0
 LB 1.00 Hz
 GB 0
 PC 1.40

Glu (Me)-DBE



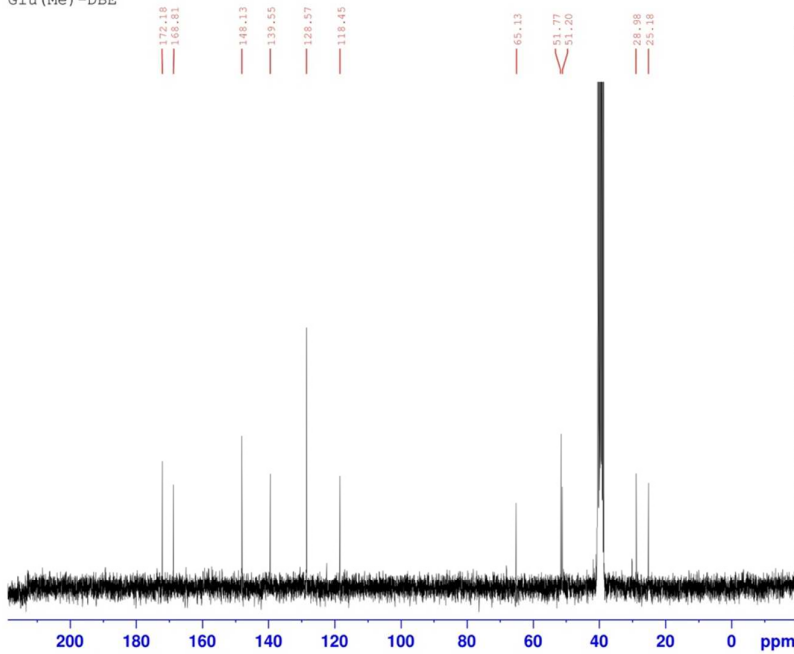
Current Data Parameters
 NAME Glu(Me)-DBE
 EXPNO 190
 PROCNO 1

F2 - Acquisition Parameters
 Date_ 20150806
 Time 15.37
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zg30
 TD 32768
 SOLVENT DMSO
 NS 16
 DS 2
 SWH 6188.119 Hz
 FIDRES 0.188846 Hz
 AQ 2.6476543 sec
 RG 80.6
 DW 80.800 usec
 DE 6.50 usec
 TE 654.7 K
 D1 1.0000000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 1H
 P1 14.00 usec
 PL1 1.80 dB
 PL1W 8.94517326 W
 SFO1 300.1318534 MHz

F2 - Processing parameters
 SI 16384
 SF 300.1300005 MHz
 WDW EM
 SSB 0
 LB 0.30 Hz
 GB 0
 PC 1.00

Glu (Me)-DBE



Current Data Parameters
 NAME Glu(Me)-DBE
 EXPNO 2
 PROCNO 1

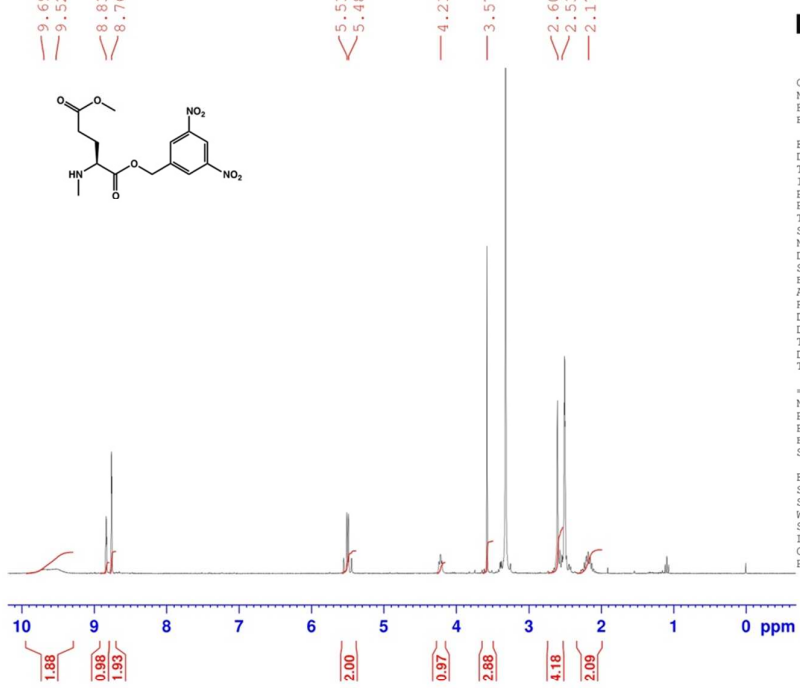
F2 - Acquisition Parameters
 Date_ 20170304
 Time 16.10
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zgpg30
 TD 65536
 SOLVENT DMSO
 NS 1500
 DS 4
 SWH 18028.846 Hz
 FIDRES 0.275098 Hz
 AQ 1.8175317 sec
 RG 161
 DW 27.733 usec
 DE 6.50 usec
 TE 298.7 K
 D1 2.0000000 sec
 D11 0.0300000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 13C
 P1 10.00 usec
 PL1 -2.50 dB
 PL1W 51.36861420 W
 SFO1 75.4752953 MHz

===== CHANNEL f2 =====
 CPDPRG12 waltz16
 NUC2 1H
 PCPD2 80.00 usec
 PL2 1.20 dB
 PL12 16.34 dB
 PL13 17.60 dB
 PL2W 10.27043343 W
 PL12W 0.31447691 W
 PL13W 0.23528202 W
 SFO2 300.1312005 MHz

F2 - Processing parameters
 SI 32768
 SF 75.4677867 MHz
 WDW EM
 SSB 0
 LB 1.00 Hz
 GB 0
 PC 1.40

MeGlu(Me)-DBE



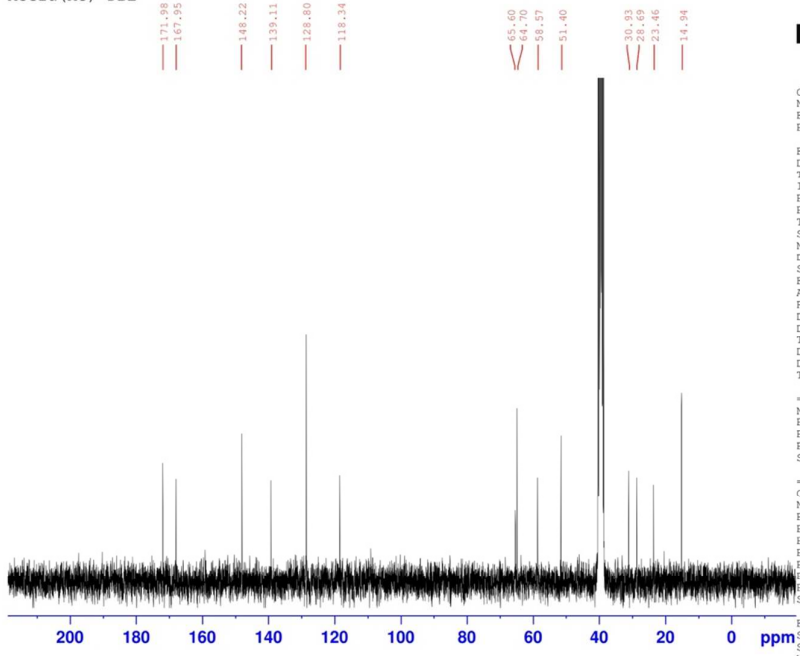
Current Data Parameters
 NAME MeGlu(Me)-DBE
 EXPNO 10
 PROCNO 1

F2 - Acquisition Parameters
 Date_ 20150828
 Time 12.11
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zg30
 TD 32768
 SOLVENT DMSO
 NS 16
 DS 2
 SWH 6188.119 Hz
 FIDRES 0.188846 Hz
 AQ 2.6476543 sec
 RG 144
 DW 80.800 usec
 DE 6.50 usec
 TE 645.3 K
 D1 1.00000000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 1H
 P1 14.00 usec
 PL1 1.80 dB
 PL1W 8.94517326 W
 SFO1 300.1318534 MHz

F2 - Processing parameters
 SI 16384
 SF 300.1300020 MHz
 WDW EM
 SSB 0
 LB 0.30 Hz
 GB 0
 PC 1.00

MeGlu(Me)-DBE



Current Data Parameters
 NAME MeGlu(Me)-DBE
 EXPNO 2
 PROCNO 1

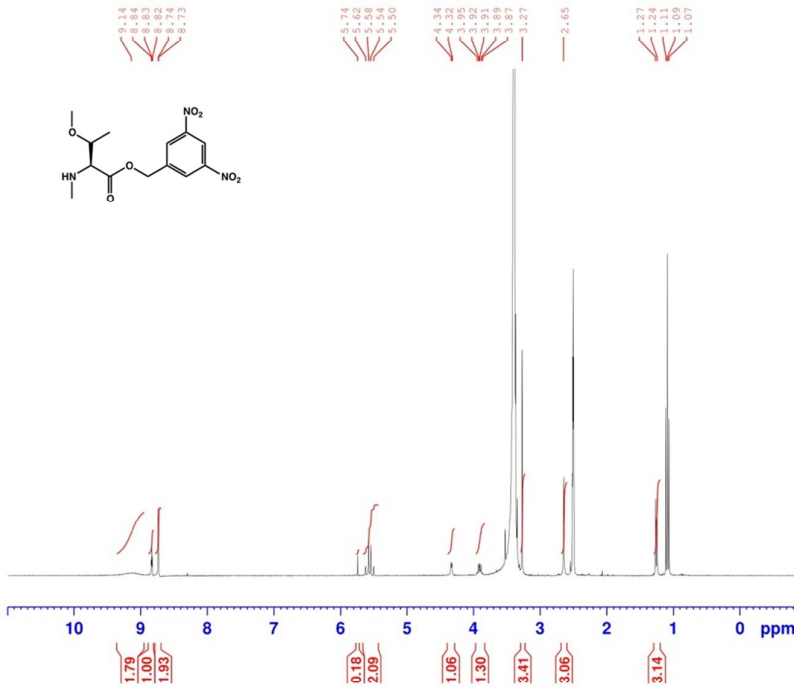
F2 - Acquisition Parameters
 Date_ 20170220
 Time 14.49
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zgpg30
 TD 65536
 SOLVENT DMSO
 NS 1200
 DS 4
 SWH 18028.846 Hz
 FIDRES 0.275098 Hz
 AQ 1.8175317 sec
 RG 144
 DW 27.733 usec
 DE 6.50 usec
 TE 300.6 K
 D1 2.00000000 sec
 D11 0.03000000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 13C
 P1 10.00 usec
 PL1 -2.50 dB
 PL1W 51.36861420 W
 SFO1 75.4752953 MHz

===== CHANNEL f2 =====
 CPDPRG12 waltz16
 NUC2 1H
 PCPD2 80.00 usec
 PL2 1.20 dB
 PL12 16.34 dB
 PL13 17.60 dB
 PL2W 10.27043343 W
 PL12W 0.31447691 W
 PL13W 0.23528202 W
 SFO2 300.1312005 MHz

F2 - Processing parameters
 SI 32768
 SF 75.4677867 MHz
 WDW EM
 SSB 0
 LB 1.00 Hz
 GB 0
 PC 1.40

MeT (Me) -DBE



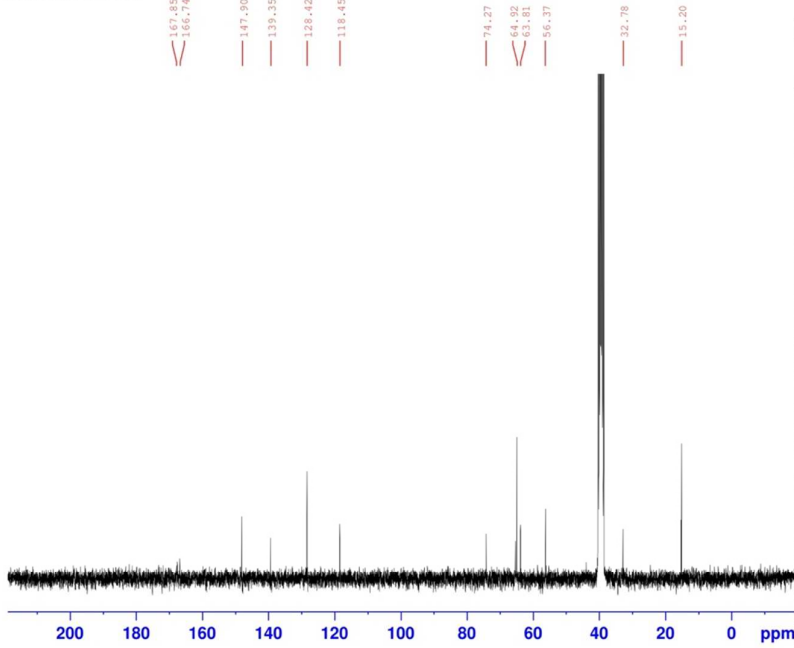
Current Data Parameters
 NAME MeThr (Me)-DBE
 EXPNO 3
 PROCNO 1

F2 - Acquisition Parameters
 Date_ 20170220
 Time 19.55
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zg30
 TD 32768
 SOLVENT DMSO
 NS 50
 DS 2
 SWH 6188.119 Hz
 FIDRES 0.188846 Hz
 AQ 2.6476543 sec
 RG 114
 DW 80.800 usec
 DE 6.50 usec
 TE 298.2 K
 D1 1.0000000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 1H
 P1 14.00 usec
 PL1 1.20 dB
 PL1W 10.27043343 W
 SFO1 300.1318534 MHz

F2 - Processing parameters
 SI 16384
 SF 300.1300035 MHz
 WDW EM
 SSB 0
 LB 0.30 Hz
 GB 0
 PC 1.00

MeThr (Me) -DBE



Current Data Parameters
 NAME MeThr (Me)-DBE
 EXPNO 4
 PROCNO 1

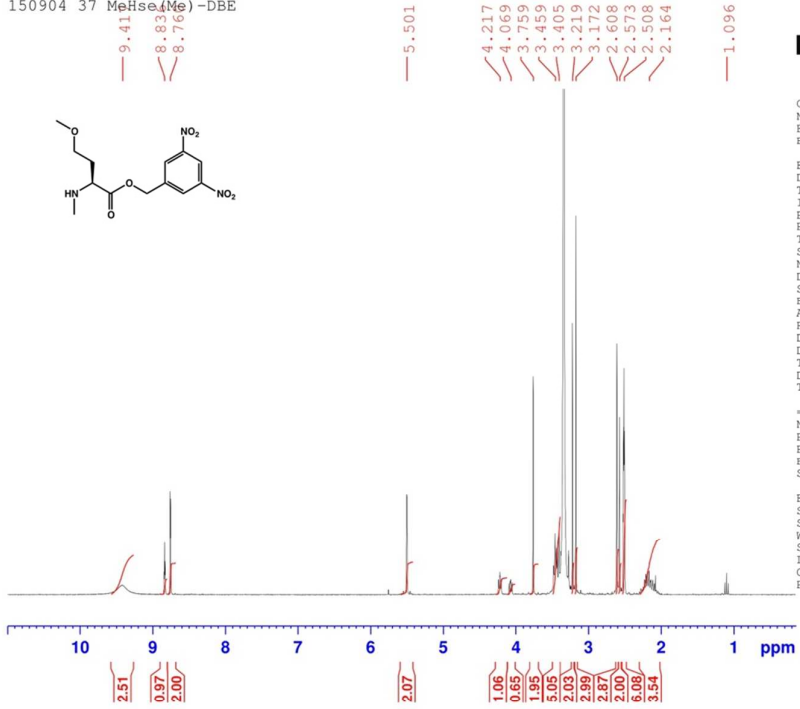
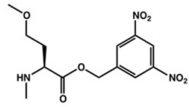
F2 - Acquisition Parameters
 Date_ 20170221
 Time 4.25
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zgpg30
 TD 65536
 SOLVENT DMSO
 NS 8000
 DS 4
 SWH 18028.846 Hz
 FIDRES 0.275098 Hz
 AQ 1.8175317 sec
 RG 144
 DW 27.733 usec
 DE 6.50 usec
 TE 297.4 K
 D1 2.0000000 sec
 D11 0.0300000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 13C
 P1 10.00 usec
 PL1 -2.50 dB
 PL1W 51.36861420 W
 SFO1 75.4752953 MHz

===== CHANNEL f2 =====
 CPDPRG12 waltz16
 NUC2 1H
 PCPD2 80.00 usec
 PL2 1.20 dB
 PL12 16.34 dB
 PL13 17.60 dB
 PL2W 10.27043343 W
 PL12W 0.31447691 W
 PL13W 0.23528202 W
 SFO2 300.1312005 MHz

F2 - Processing parameters
 SI 32768
 SF 75.4677867 MHz
 WDW EM
 SSB 0
 LB 1.00 Hz
 GB 0
 PC 1.40

150904 37 MeHse(Me)-DBE



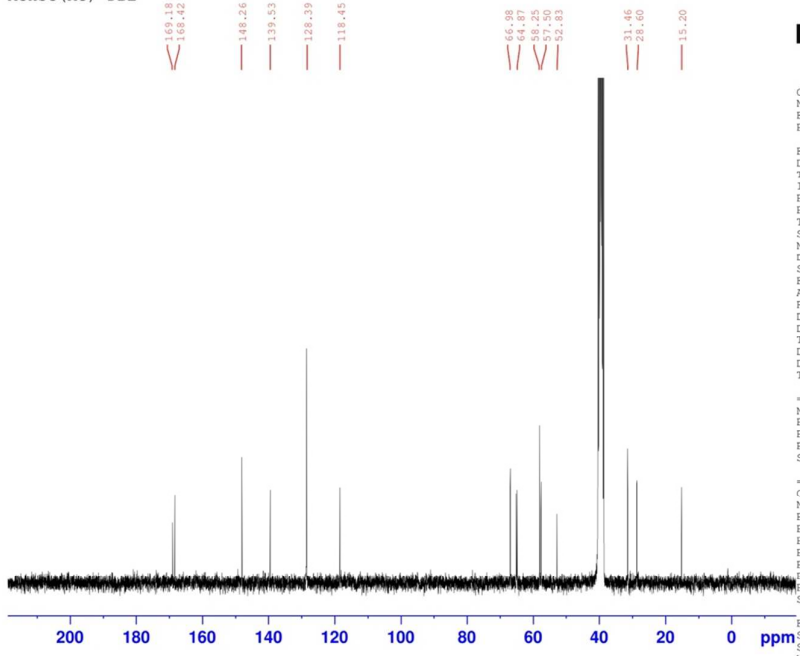
Current Data Parameters
 NAME 150904
 EXPNO 30
 PROCNO 1

F2 - Acquisition Parameters
 Date_ 20150904
 Time 13.43
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zg30
 TD 32768
 SOLVENT DMSO
 NS 16
 DS 2
 SWH 6188.119 Hz
 FIDRES 0.188846 Hz
 AQ 2.6476543 sec
 RG 114
 DW 80.800 usec
 DE 6.50 usec
 TE -2532.9 K
 D1 1.0000000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 1H
 P1 14.00 usec
 PL1 1.80 dB
 PL1W 8.94517326 W
 SFO1 300.1318534 MHz

F2 - Processing parameters
 SI 16384
 SF 300.1300000 MHz
 WDW EM
 SSB 0
 LB 0.30 Hz
 GB 0
 PC 1.00

MeHse (Me)-DBE



Current Data Parameters
 NAME MeHse(Me)-DBE
 EXPNO 1
 PROCNO 1

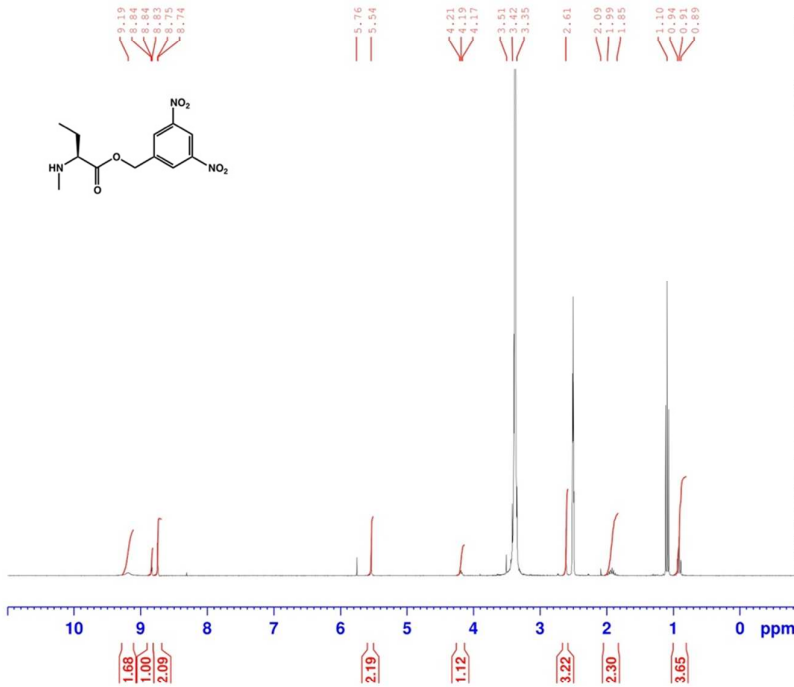
F2 - Acquisition Parameters
 Date_ 20170217
 Time 8.09
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zgpg30
 TD 65536
 SOLVENT DMSO
 NS 8000
 DS 4
 SWH 18028.846 Hz
 FIDRES 0.275098 Hz
 AQ 1.8175317 sec
 RG 161
 DW 27.733 usec
 DE 6.50 usec
 TE 300.6 K
 D1 2.0000000 sec
 D11 0.0300000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 13C
 P1 10.00 usec
 PL1 -2.50 dB
 PL1W 51.36861420 W
 SFO1 75.4752953 MHz

===== CHANNEL f2 =====
 CPDPRG12 waltz16
 NUC2 1H
 PCPD2 80.00 usec
 PL2 1.20 dB
 PL12 16.34 dB
 PL13 17.60 dB
 PL2W 10.27043343 W
 PL12W 0.31447691 W
 PL13W 0.23528202 W
 SFO2 300.1312005 MHz

F2 - Processing parameters
 SI 32768
 SF 75.4677867 MHz
 WDW EM
 SSB 0
 LB 1.00 Hz
 GB 0
 PC 1.40

MeAbu-DBE



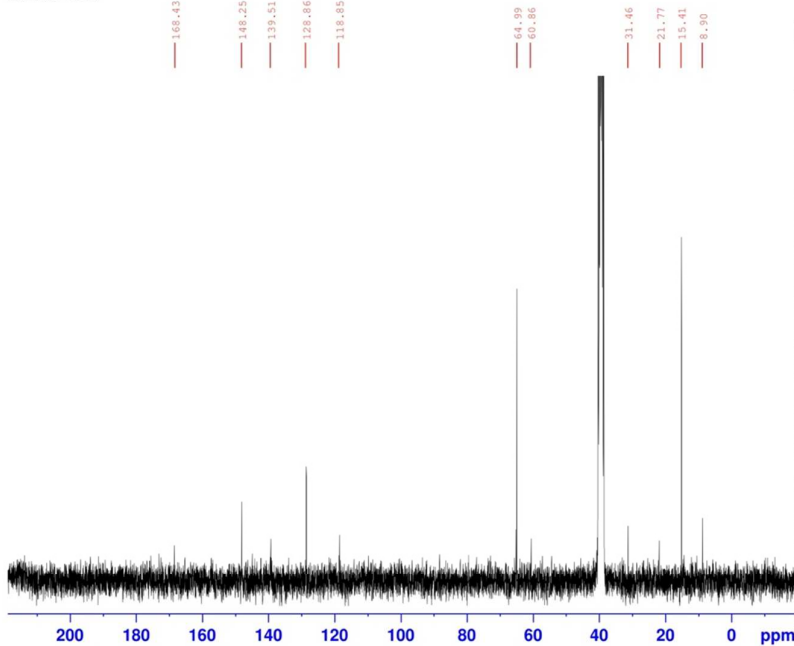
Current Data Parameters
 NAME MeAbu-DBE
 EXPNO 5
 PROCNO 1

F2 - Acquisition Parameters
 Date_ 20170221
 Time 21.42
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zg30
 TD 32768
 SOLVENT DMSO
 NS 50
 DS 2
 SWH 6188.119 Hz
 FIDRES 0.188846 Hz
 AQ 2.6476543 sec
 RG 128
 DW 80.800 usec
 DE 6.50 usec
 TE 295.6 K
 D1 1.00000000 sec
 TD0 1

===== CHANNEL f1 =====
 NUC1 1H
 P1 14.00 usec
 PL1 1.20 dB
 PL1W 10.27043343 W
 SFO1 300.1318534 MHz

F2 - Processing parameters
 SI 16384
 SF 300.1300024 MHz
 WDW EM
 SSB 0
 LB 0.30 Hz
 GB 0
 PC 1.00

MeAbu-DBE



Current Data Parameters
 NAME MeAbu-DBE
 EXPNO 6
 PROCNO 1

F2 - Acquisition Parameters
 Date_ 20170222
 Time 7.25
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zgpg30
 TD 65536
 SOLVENT DMSO
 NS 9000
 DS 4
 SWH 18028.846 Hz
 FIDRES 0.275098 Hz
 AQ 1.8175317 sec
 RG 101
 DW 27.733 usec
 DE 6.50 usec
 TE 295.7 K
 D1 2.00000000 sec
 D11 0.03000000 sec
 TD0 1

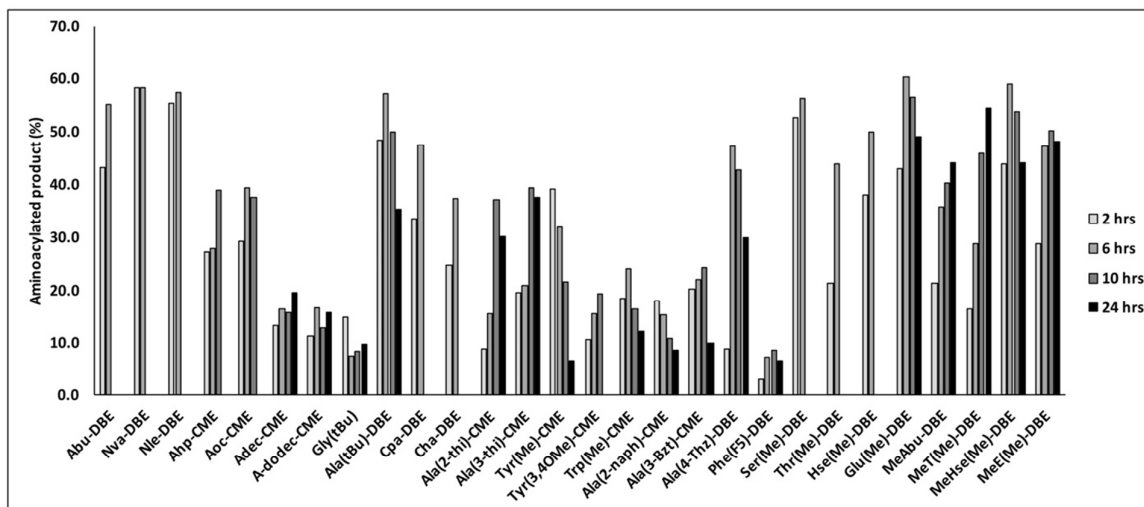
===== CHANNEL f1 =====
 NUC1 13C
 P1 10.00 usec
 PL1 -2.50 dB
 PL1W 51.36861420 W
 SFO1 75.4752953 MHz

===== CHANNEL f2 =====
 CPDPRG12 waltz16
 NUC2 1H
 PCPD2 80.00 usec
 PL2 1.20 dB
 PL12 16.34 dB
 PL13 17.60 dB
 PL2W 10.27043343 W
 PL12W 0.31447691 W
 PL13W 0.23528202 W
 SFO2 300.1312005 MHz

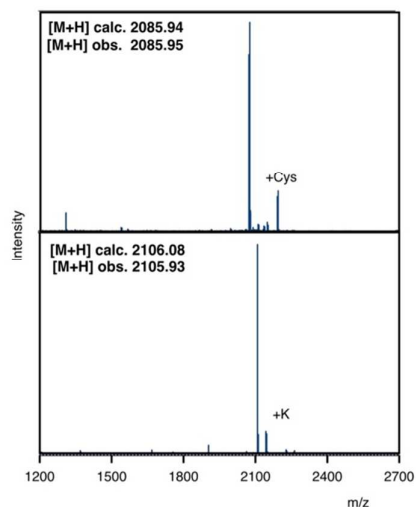
F2 - Processing parameters
 SI 32768
 SF 75.4677867 MHz
 WDW EM
 SSB 0
 LB 1.00 Hz
 GB 0
 PC 1.40

Supplementary References

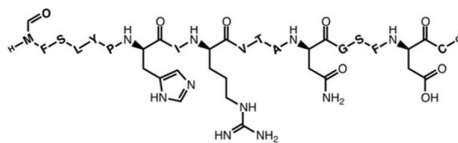
- (1) Goto, Y.; Katoh, T.; Suga, H., *Nat. Protoc.* **2011**, *6*, 779-90.
- (2) Yamagishi, Y.; Shoji, I.; Miyagawa, S.; Kawakami, T.; Katoh, T.; Goto, Y.; Suga, H., *Chem. Biol.* **2011**, *18*, 1562-70.
- (3) Hayashi, Y.; Morimoto, J.; Suga, H., *ACS Chem. Biol.* **2012**, *7*, 607-13.
- (4) Kawakami, T.; Murakami, H.; Suga, H., *Chem. Biol.* **2008**, *15*, 32-42.
- (5) Ojemalm, K.; Higuchi, T.; Jiang, Y.; Langel, U.; Nilsson, I.; White, S. H.; Suga, H.; von Heijne, G., *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, E359-64.
- (6) Danner, P.; Morkunas, M.; Maier, M. E., *Org. Lett.* **2013**, *15*, 2474-7.



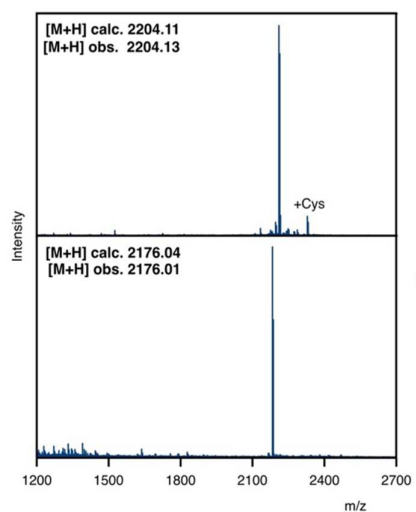
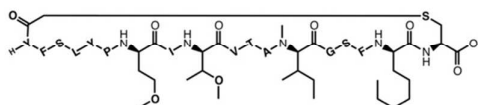
Supplementary Figure S1 – Optimization of flexizyme mediated aminoacylation. μ -helix RNA was aminoacylated with each amino acid substrate for the indicated times on ice, resolved by polyacrylamide gel electrophoresis and quantified by densitometry of the ethidium bromide stained product.



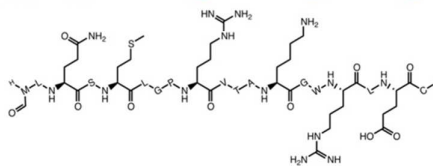
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 fMet His Arg Asn Asp



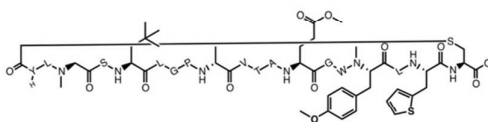
ATG TTC TCC CTC TAC CCC CAC ATC CGC GTC ACC GCC AAC GGC AGC TTC GAC TGC TAA
 ClAc-Tyr Hse(Me) Thr(Me) MeLeu Aoc



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 fMet Gln Met Arg Lys Arg Glu

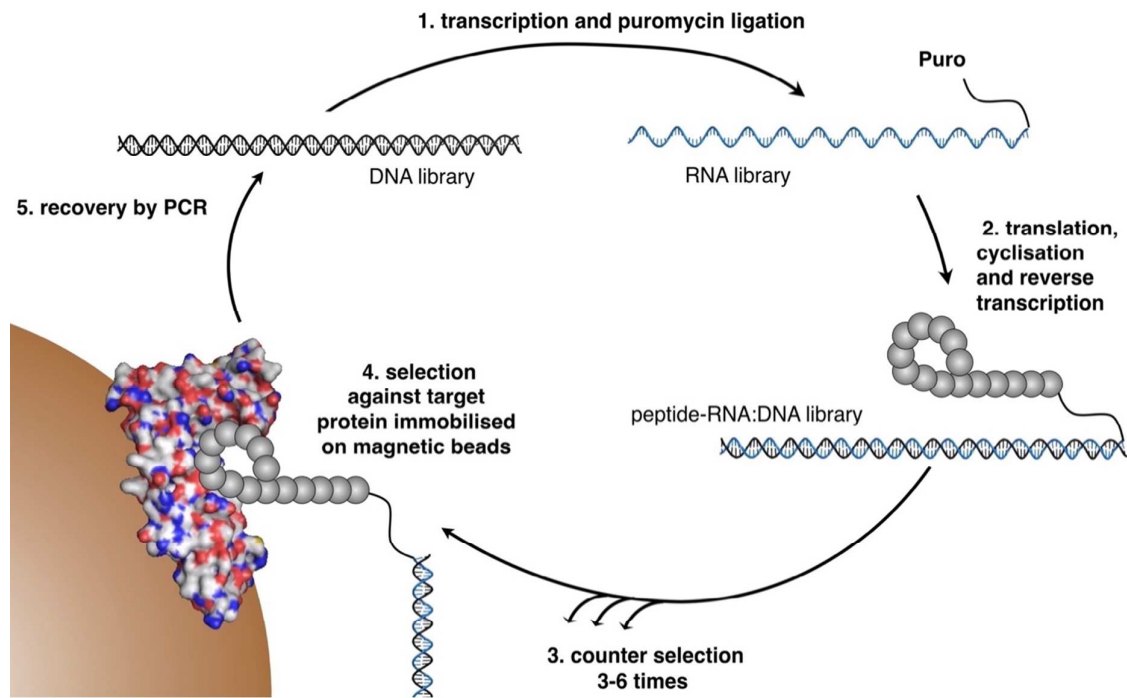


ATG TTG CAG TCG ATG CTG GGG CCG CGG GTG ACG GCG AAG GGG UGG AGG TTG GAG TGC TAA
 ClAc-Tyr MeG Ala(Bu) D-Ala Glu(Me) MeTyr(Me) Ala(2-Thi)



Supplementary Figure S3 – Fidelity of translation under hydrophobic genetic code.

Two model peptides were translated under a standard genetic code (upper panel of each set) or the novel genetic code (lower panel of each set) described in Figure 2, and analyzed by MALDI-TOF mass spectrometry.

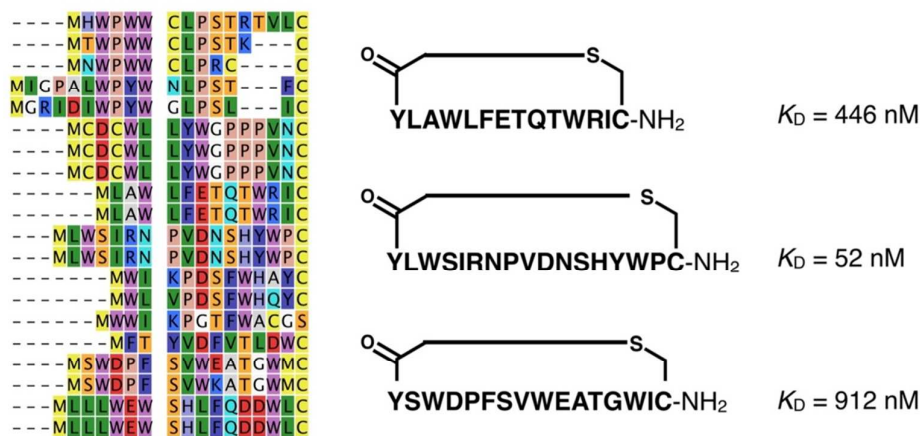


Supplementary Figure S4 – RaPID selection process. A DNA library assembled from synthetic oligonucleotides is transcribed into RNA and ligated to puromycin at the 3' end (1.). Translation of this library in a genetically reprogrammed FIT reaction leads to the formation of a macrocyclic peptide library in which each peptide is covalently linked to its cognate mRNA through the puromycin moiety, and which is then reverse transcribed to generate a combined peptide-RNA:DNA molecule (2.). Counter selections are used to remove peptides that bind non-specifically during the selection process (3.), followed by isolation of target protein binding peptides through panning of the library against target immobilised on magnetic beads (4.). An enriched DNA library is recovered by PCR and the process is repeated until increased rates of target binding are observed. Deconvolution of the library is then achieved through sequencing of the final enriched DNA library.

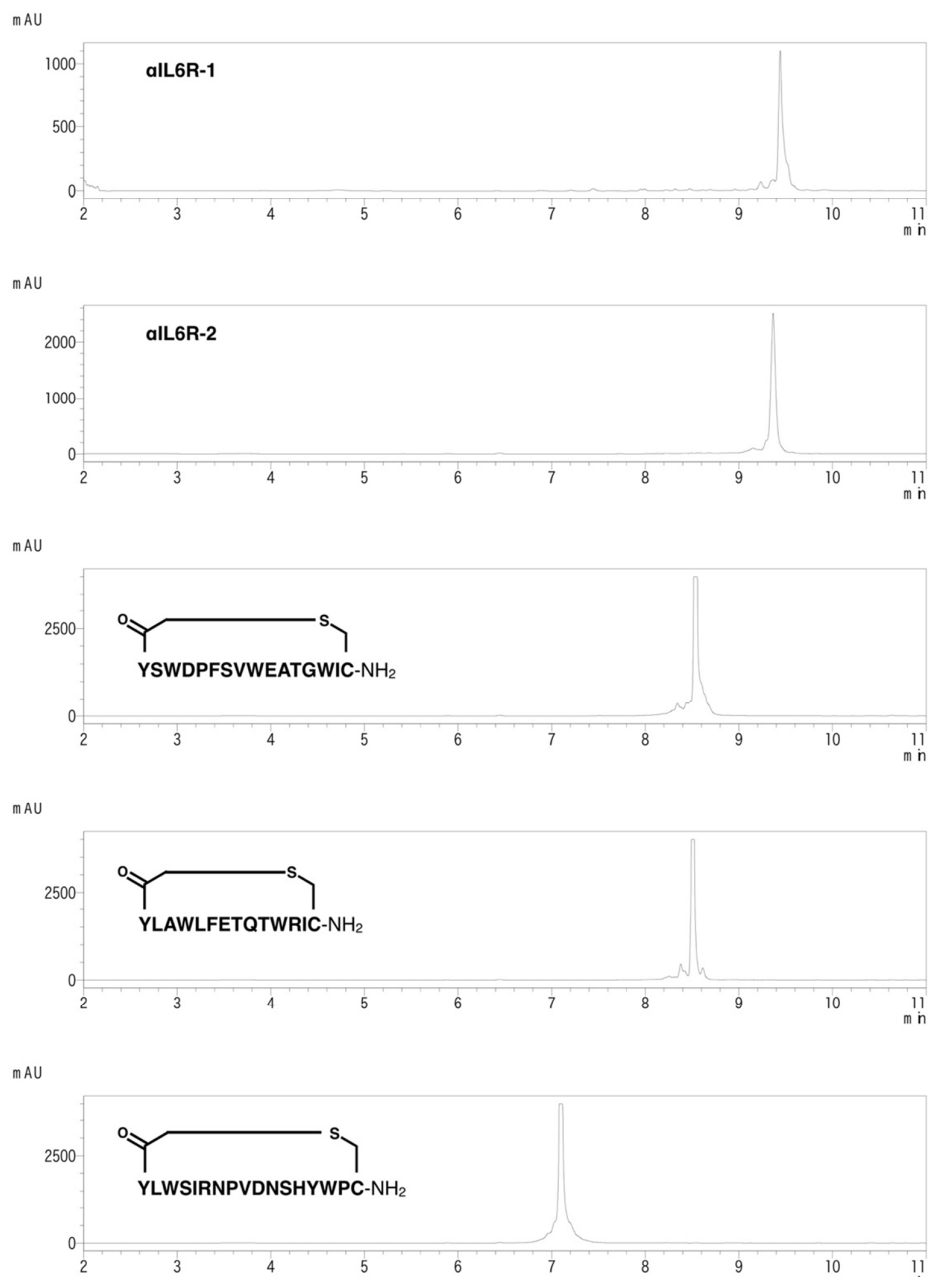
12.30% - YLEZNMHECGTGTGT*
 0.20% - YLEGNMHECGTGTGT*
 0.15% - YLEDNMHECGTGTGT*
 1.82% - YLEZNFHECGTGTGT*
 1.08% - YLEZNMFMCGTGTGT*
 0.88% - YLEZNMHSCGTGTGT*
 0.82% - YLEZNHUHECGTGTGT*
 0.78% - YLEZNFMECGTGTGT*
 0.20% - YLEZNMHECGTGAGT*
 0.18% - YLEZNMHBCTGTGTGT*
 0.18% YTLEZNMHECGTGTGT*
 6.00% YTLEZNMHBCTGTGTGT*
 4.30% - YLEZNFEMCGTGTGT*
 0.22% - YFEZNFEMCGTGTGT*
 1.46% - YLEZNHUFECTGTGTGT*
 0.35% - YLEZNHUFECACTGTGT*
 0.13% - YLEZNYEFECTGTGT*
 1.54% - YFEZNFESHCTGTGT*
 0.50% - YFEZNFEMHCTGTGT*
 1.42% - YFEZNFEMHCTGTGT*
 0.44% - YFEZNFEMHCTGTGT*
 34.09% YTTEZNIHYCGTGTGT*
 0.51% YTTNZNIHYCGTGTGT*
 0.46% YTTHZNIHYCGTGTGT*
 0.16% YTTEZNIHYUGTGTGT*
 3.69% YTTEZNIHYCGTGT* - -
 0.80% YTTEZNIHYCGTGAGT*
 0.35% YTTEZNVHYCGTGTGT*
 0.32% - YLEZNIHYCGTGTGT*
 0.28% YTTEZNIHYCGAGTGT*
 0.27% YTAENZNIHYCGTGTGT*
 0.21% YTTEZNIHYCGTGBGT*
 0.19% YTLEZNIHYCGTGTGT*
 0.17% YATEZNIHYCGTGTGT*
 0.14% YTBENZNIHYCGTGTGT*
 0.14% YTTEZNIHYCGBGTGT*
 0.13% YPTEZNIHYCGTGTGT*
 0.12% YTTEZDHIHYCGTGTGT*
 1.21% YMVEZNXBHYCGTGTGT*
 0.16% YEAEZNIHHCCTGTGT*
 0.25% - YLEZNFIAHACGTGTGT*
 0.18% YTLEZNMHBCTGTGT* - -
 1.02% - YHEZNFEECGTGTGT*
 0.35% - YHEZNFEMCGTGTGT*
 0.12% YMHEZNFESCCTGTGT*
 0.15% - YTEZNFMECGTGTGT*
 0.42% YMVEZNXBHYCGTGZ* - -
 2.06% - YLLNHYANPCGTGTGT*
 1.33% - YBLNHYSNPCGTGTGT*
 0.20% - YEBNHYNILCGTGTGT*

B = Ala(tBu) E = Glu_{Me} D = D-Ala N = MeGly M = Hse_{Me}
H = Ala(2-Thi) X = Aoc U = Thr_{Me} J = MeLeu Z = MeTyr_{Me}

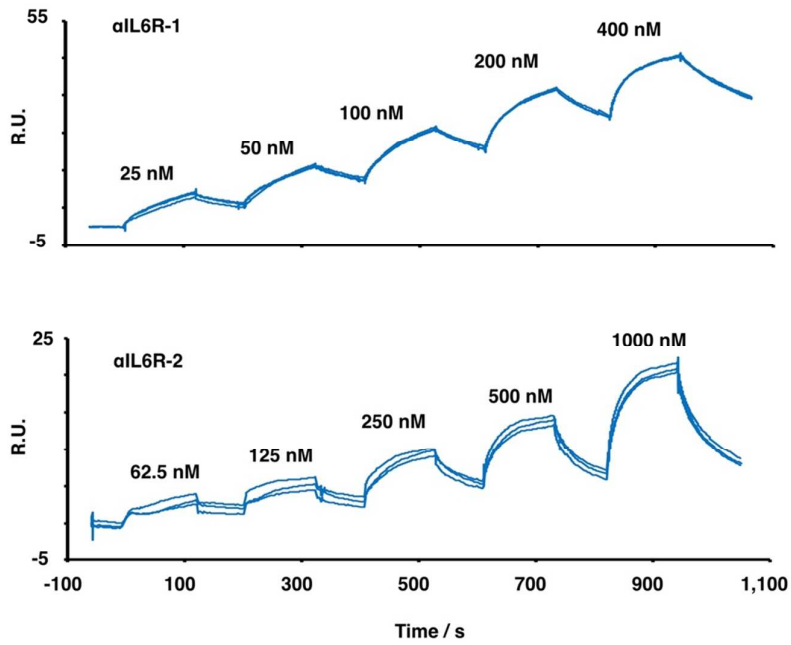
Supplementary Figure S5 – Sequence alignment of 50 most enriched peptides from library screening.



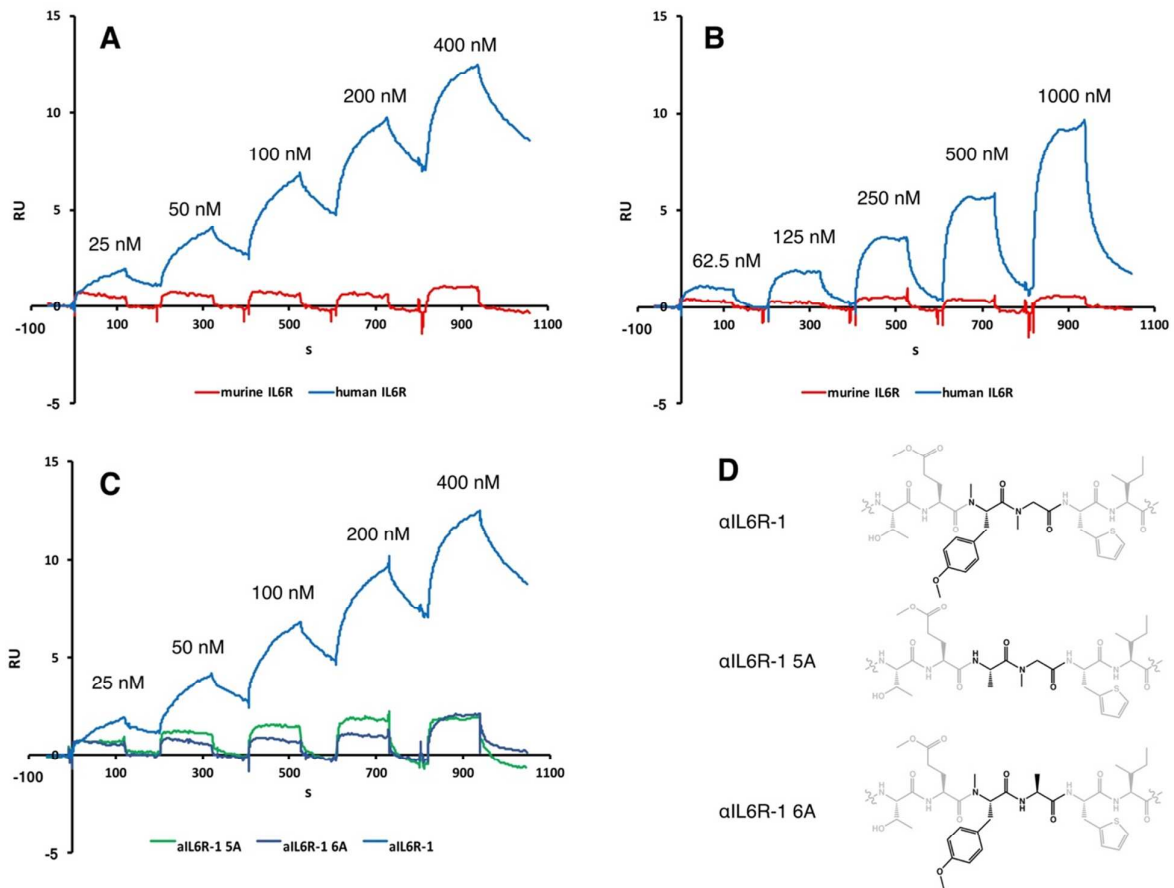
Supplementary Figure S6 – Summary of RaPID selection against IL6R using a canonical amino acid-based macrocyclic peptide library. Selection against IL6R was performed using a 19 canonical amino acid (excluding methionine) library, initiated with *N*-chloroacetyl-tyrosine to produce a cyclizing thioether through reaction with a downstream Cys residue. Selection was performed as described for the heavily reprogrammed library, and the final DNA library was sequenced to determine the sequence of the corresponding peptides. Alignment of the 20 most enriched sequences is shown (left panel – initiating Met residues represent *N*-chloroacetyl-tyrosine). 3 peptides were synthesized by solid phase methodology and binding to IL6R was assessed by surface plasmon resonance, with dissociation constants (K_D) indicated for each.



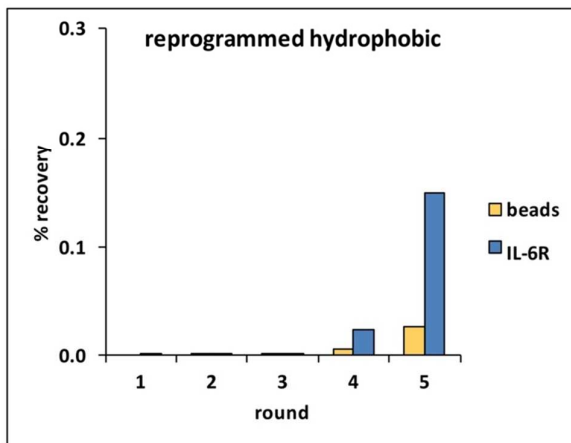
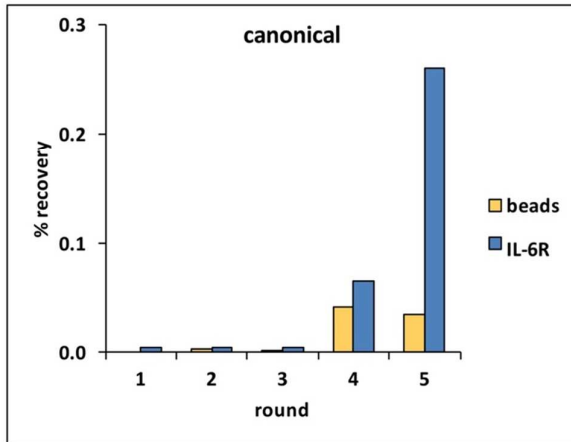
Supplementary Figure S7 – Reversed phase UHPLC analysis. Peptides from hydrophobic (upper two panels) or canonical selections were separated on a C18 reversed phase column under an aqueous acetonitrile gradient.



Supplementary Figure S8 – Surface plasmon resonance studies of α IL6R-1 and α IL6R-2 binding to IL6R. Surface plasmon resonance was performed against immobilised IL6R with each peptide injected in triplicate at 5 concentrations as shown.



Supplementary Figure S9 – Surface plasmon resonance studies of α IL6R-1 5A and 6A mutants and α IL6R-1 and α IL6R-2 binding to human and murine IL6R. Surface plasmon resonance was performed against immobilised human or murine IL6R (A, B) with α IL6R-1 (A) and α IL6R-2 (B) injected at 5 concentrations as shown. (C) Targeted mutants of α IL6R-1 with the conserved MeTyr(Me) or MeGly (positions 5 and 6 respectively) replaced with Ala (D) were assessed for binding to immobilized human IL6R as above.



Supplementary Figure S10 – Library recovery following each round of selection. Library cDNA was quantified before and after selection by real time PCR, and the percentage library recovery (*i.e.* proportion of library bound to the magnetic beads) was calculated. To control for binding to the bead surface cDNA recovered from both the final counter-selection beads (“beads”) and the IL-6R coated beads (“IL-6R”) was quantified. Upper panel shows recovery from the canonical (proteinogenic amino acids not including Met) selection, and lower panel shows recovery from the hydrophobic peptide library selection.

