

Expanded ribosomal synthesis of non-standard cyclic backbones *in vitro*

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Methods and Materials

All chemicals were purchased from Sigma-Aldrich, Sejin TCI, and Alpha Aesar, and used as received without further purification. Synthetic DNA oligonucleotides were purchased from IDT Korea, Bioneer (Korea), and CosmoGenetech (Korea). Silica gel flash chromatography was performed using 0.040 - 0.063 mm, 60 Å silica purchased from Supelco®. Thin layer chromatography was performed using glass silica plates coated with fluorescent indicator (F254) purchased from Supelco®. Sand, sodium chloride, sodium bicarbonate, potassium carbonate, concentrated hydrochloric acid, and sodium hydroxide pellets were purchased from Sigma-Aldrich, Biosesang (Korea), Samchun (Korea) and Daejung (Korea). The peptide (**Sa-Hz**-WSHPQFEK-OH) used as internal standards was purchased from Pepton (Korea).

MALDI Mass spectra were recorded on a Bruker Autoflex. High-resolution mass spectrometry (HRMS) analysis was performed at the Department of Chemistry in Pohang University of Science and Technology (POSTECH) using the 6560 Accurate Mass Q-TOF LC/MS system from Agilent Technologies. MALDI mass spectra were processed using Compass DataAnalysis Viewer v6.1 (Bruker) and FlexControl v2.0 (Bruker) software utilizing the smoothing and baseline subtraction. ¹H and ¹³C NMR spectra were collected either using a Bruker AVANCE III™ HD 500 MHz cryoprobe or Bruker AVANCE III™ HD 400 MHz cryoprobe NMR spectrometer and processed with TopSpin (4.3.0). Chemical shifts, denoted in ppm, were assigned relative to the residual NMR solvent peaks. The spectra are representative of at least n = 3 independent experiments.

Synthesis RNAs (tRNA, Fxs, P1c2^{UGGU}, P1c2, P2c^{UGGU}, and Pc2)

DNA template synthesis

Extension: 2.5 µL of 10 µM forward and reverse primer (Ext.) (see **List of primers**) was added to 10 µL KAPA HiFi 2X polymerase, and mixed with 5 µL dH₂O in a PCR tube. The thermocycling conditions were as follows: 5 min at 95 °C followed 37 °C for 10 min and 72 °C for 30 s. PCR amplification: 20 µL of the product was used as an extension template, 20 µL each of 10 µM forward and reverse primer (Amp.) (see **List of primers**) were added to 250 µL KAPA HiFi 2X polymerase, and mixed with 190 µL dH₂O in a PCR tube. The mixture was divided into several PCR tubes, and the DNA was amplified by the following thermocycling conditions: 3 min at 95 °C, followed by 25 cycles of 95 °C for 30 s and T_m (either 55 °C or 65 °C) for 30 s, and 72 °C for 1 min. PCR products were assayed on a 1 % (w/v) agarose gel.

DNA template precipitation

PCR products were extracted using phenol/chloroform/isoamyl alcohol and precipitated with ethanol. Samples were dried at room temperature for 5 min and resuspended in 100 µL nuclease-free water. DNA concentrations were determined from absorbance measured on a Nabi UV/Vis Nano Spectrophotometer. (MicroDigital Co., Ltd)

In vitro transcription

RNAs were prepared using a HiScribe® T7 quick high-yield RNA synthesis kit (NEB, E2050S). For in vitro transcription, 25 µg of DNA template was used with 50 µL of T7 RNA polymerase mix, 250 µL of NTP buffer mix, and nuclease-free water up to 500 µL. The mixture was incubated at 37 °C overnight.

Digestion of DNA templates

The DNA templates were removed by adding 50 µL of DNase I (NEB, E2050S) into the 500 µL of transcription reaction products. The reaction mixture was incubated at 4 °C for 2 h.

Purification of in vitro transcribed RNA

The digested transcription reactions were mixed with 500 µL 2X RNA loading dye and loaded onto a 15 % TBE-Urea gel (24 g of Urea, 18.75 mL of 40 % Acrylamide/Bis solution 19:1 (Bio-Rad, #1610144), and 5 mL of 3 M NaOAc, 10X TBE buffer up to 50 mL using water). The gel was run in 0.5X TBE buffer at 130 V for 3 h at room temperature. The gel was placed on a silica plate and the transcribed RNAs were visualized by irradiation via a UV lamp (260 nm). The RNA products were excised from the gel and added to 5 mL of water. The gels were crushed and then placed in the cold room for 1 h. The gels were transferred to a HiGene™ HiFilter with a collection tube (Biofact) and centrifuged at 4000 × g for 2 min. The flow-through was collected and added to the solution of 0.3 mL of 5 M NaCl and 10 mL of 100 % ethanol. The solution was placed at -20 °C for 16 h and centrifuged at 13,000 × g for 30 min at 4 °C. The supernatant was removed, and the pellet was dried for 5 min at room temperature. The dried RNA pellet was dissolved in nuclease-free water and the concentration was determined using a spectrophotometer.

Dimerization and hybridization assays of PTC analogues

- Dimerization of PTC analogues: 7.3 μL of 137 μM PTC analogue was mixed with 2 μL of 500 mM HEPES (pH 7.5). The mixture was heated to 95 $^{\circ}\text{C}$ for 3 min, then cooled to 25 $^{\circ}\text{C}$ at a rate of 0.1 $^{\circ}\text{C}/\text{sec}$. Once cooled, 4 μL of 0.3 M MgCl_2 was added to reach a final concentration of 60 mM, and the solution was incubated on ice for 10 min to allow self-dimerization.
- Hybridization with tRNAs: To the dimerization solution, 6.7 μL of 149 μM tRNA^{fMet} (CAU) was added (resulting in a final concentration of 50 μM) and the mixture was incubated at 0 $^{\circ}\text{C}$ for additional 24 h.
- Gel assay: 0.5 μL of the prepared sample was combined with 4.5 μL of water and 5 μL of 2X RNA loading dye, (Thermo Scientific, #R0641), and loaded onto a 12% polyacrylamide gel. The gel was run in 0.5X TAM buffer (44.5 mM Tris, 44.5 mM sodium acetate, 15 mM MgCl_2 , pH 7.5) at 100 V for 7 h in a -20°C freezer. Afterward, the gel was stained with 2.5 μL of SYBRTM Gold Nucleic Acid Gel Stain (Invitrogen, #S11494) in 50 mL of water for 10 min, then visualized using a Bio-Rad GelDoc Go system.

Gel electrophoresis

Preparation of acidic gel (pH 5.2)

10.8 g of UREA, 15 mL of 40 % Acrylamide/Bis Solution 19:1 (Bio-Rad, #1610144), and 500 μL of 3 M NaOAc were mixed in a 50 mL tube. Water added to a total volume of 30 mL. After the urea was completely dissolved, 300 μL of 10 % (wt/vol) APS and 24 μL of TEMED were added to the solution. The mixture solution was casted into a gel cassette immediately using a serological pipette and the gel was allowed to polymerize overnight.

Assaying optimal Fx-charging conditions

1 μL of prepared sample was mixed with 5 μL of acidic-loading dye (3 M NaOAc (pH 5.2), 20 μL of 0.5 M EDTA (pH 8.0), 980 μL of 2 % (wt/vol) formamide, and 8 μL of Bromophenol Blue) and loaded onto a prepared 10 % acidic urea gel. The gel was run in 50 mM NaOAc buffer at 135 V for 2 h at 4 $^{\circ}\text{C}$ in a cold room. After electrophoresis, gel was stained by 2.5 μL of SYBRTM Gold Nucleic Acid Gel Stain (Invitrogen, S11494) in 50 mL of water for 10 min. The gel was visualized on a Bio-Rad gel documentation system (GelDoc Go).

Preparation of native gels for electrophoretic mobility shift assay (EMSA)

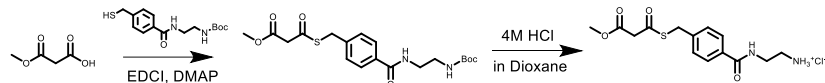
EMSA native gels for RNA dimerization or hybridization were prepared according to a previously published protocol (*Nucleic Acids Research*, 2022, Vol. 50, No. 4 1815–1828). In a 50 mL tube, 12 mL of 40 % Acrylamide/Bis Solution (19:1) (Bio-Rad, #1610144) and 4 mL of 10X TAM buffer (890 mM Tris, 890 mM sodium acetate, and 150 mM MgCl_2 , pH 7.5) were mixed. Water was added to bring the total volume to 40 mL. After thorough mixing, 400 μL of 10 % (wt/vol) APS and 40 μL of TEMED were added to the solution. The mixture was cast into a gel cassette using a serological pipette and allowed to polymerize overnight.

LC-HRMS analysis

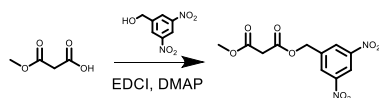
The liquid chromatography high-resolution mass spectrometry (LC-HRMS) analysis was carried out using a 6560 LC/Q-TOF mass spectrometer (Agilent Technologies Inc.). The sample solution was prepared using Amicon Ultra 0.5 mL 3 K centrifugal filters (Tulagreen Carriagtwohill Co.) and automatically loaded with 4 μL into the LC injector. The spray conditions included a voltage offset of 2–3 kV between the spray tip and the inlet capillary, and the sample solution was vaporized and ionized with a flow of heated N_2 gas (~ 3 L/min, 120–200 $^{\circ}\text{C}$) in the source region. The mass-to-charge ratios (m/z) of each ion were measured in the time-of-flight (TOF) region. The mass spectrometric analysis was performed by identifying the retention times of each ion on the LC system using the extracted ion chromatogram (EIC) matched to its m/z . Separation was performed on a Thermo Scientific Acclaim 120 C18 HPLC column with a constant flow rate of 150 $\mu\text{L}/\text{min}$. The mobile phase gradient was initially set at 70/30 vol % (0.1% formic acid in water/acetonitrile) and gradually decreased to 40/60 vol % over a period of 38 min, starting from 2 min.

Synthesis non-canonical monomers (ncMs)

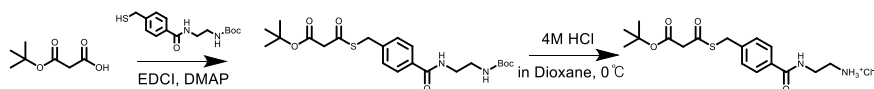
Synthesis of malonate diester



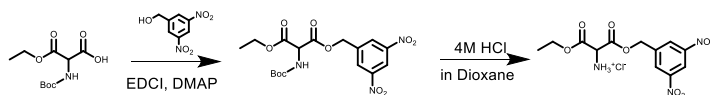
Methyl 3-((4-((2-aminoethyl)carbamoyl)benzyl)thio)-3-oxopropanoate (m) : Prepared according to the general procedure A using methyl hydrogen malonate (341.1 μ L, 3.0 mmol), Boc-ABT (155.2 mg, 0.5 mmol), DMAP (61.1 mg, 0.5 mmol), EDCI·HCl (575.1 mg, 3.0 mmol) and CH_2Cl_2 (10 mL). Purification by flash column chromatography (60 % EtOAc in n-Hex) yielded the Boc-protected product as a white solid (82.9 mg, 40.4 %). The deprotection was achieved upon treatment with 4 M solution of HCl in 1,4-dioxane for 30 min at room temperature, and the resulting product was used without further purification. : ^1H NMR (500 MHz, DMSO-d_6) δ 8.72 (t, J = 5.28 Hz, 1H), 8.01 (br s, 3H), 7.85 (d, J = 8.19 Hz, 2H), 7.41 (d, J = 8.26 Hz, 2H), 4.22 (s, 2H), 3.84 (s, 2H), 3.64 (s, 3H), 3.51 (q, J = 5.90 Hz, 2H), 2.98 (q, J = 5.69 Hz, 2H), ^{13}C NMR (126 MHz, DMSO-d_6) δ 191.4 (1C, O=C-S), 166.9 (1C, O=C-O), 166.9 (1C, O=C-NH), 141.3 (1C, Ph), 133.4 (1C, Ph), 129.1 (2C, Ph), 128.1 (2C, Ph), 52.7 (1C, $\text{H}_3\text{C-O}$), 49.1 (1C, CH_2), 39.1 (1C, CH_2NH_3^+), 37.5 (1C, NHCH_2), 32.8 (1C, SCH_2). HRMS (ESI) : Exact mass calcd. for $\text{C}_{14}\text{H}_{19}\text{N}_2\text{O}_4\text{S}^+ [\text{M}+\text{H}]^+ = 311.1060$; found 311.1089.



3,5-dinitrobenzyl methyl malonate (m-2) : Prepared according to the general procedure B using methyl hydrogen malonate (104.7 μ L, 1.0 mmol), 3,4-dinitrobenzyl alcohol (198.1 mg, 1.0 mmol), DMAP (122.2 mg, 1.0 mmol), EDCI·HCl (191.7 mg, 1.0 mmol) and CH_2Cl_2 (10 mL). Purification by silica gel flash chromatography (30 % EtOAc in n-Hex) yielded a yellow solid (46.9 mg, 15.7 %). : ^1H NMR (500 MHz, DMSO-d_6) δ 8.80 (t, J = 2.20 Hz, 1H), 8.67 (d, J = 1.84 Hz, 2H), 5.43 (s, 2H), 3.69 (s, 2H), 3.68 (s, 3H), ^{13}C NMR (126 MHz, DMSO-d_6) δ 169.3 (1C, O=C), 168.7 (1C, O=C), 150.7 (2C, C-N(=O)=O), 142.7 (1C, Ph), 130.4 (2C, Ph), 120.5 (1C, Ph), 66.6 (1C, O- CH_2), 54.8 (1C, O- CH_3), 43.1 (1C, CH_2 , unidentified due to interference from solvent peaks). HRMS (ESI) : Exact mass calcd. for $\text{C}_{11}\text{H}_{10}\text{N}_2\text{O}_8\text{Na}^+ [\text{M}+\text{Na}]^+ = 321.0329$; found 321.0321.

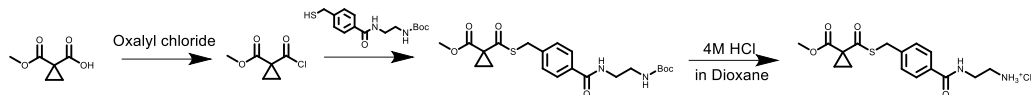


tert-butyl 3-((4-((2-aminoethyl)carbamoyl)benzyl)thio)-3-oxopropanoate (0) : Prepared according to the general procedure A using mono-*tert*-butyl malonate (710.0 mg, 5.0 mmol), Boc-ABT (310.4 mg, 1.0 mmol), DMAP (122.2 mg, 1.0 mmol), EDCI·HCl (383.4 mg, 2.0 mmol) and CH_2Cl_2 (10 mL). Purification by flash column chromatography (30 % EtOAc in n-Hex) yielded Boc-protected product as a white solid. The deprotection was achieved upon treatment with 4 M solution of HCl in 1,4-dioxane at 0 °C for 30 min, and the resulting product was used without further purification. The product was obtained as a white solid (32.8 mg, 8.4 %). : ^1H NMR (500 MHz, DMSO-d_6) δ 8.79 (t, J = 4.94 Hz, 1H), 8.15 (br s, 3H), 7.94 - 7.84 (m, 2H), 7.43 (d, J = 8.03 Hz, 2H), 4.22 (s, 2H), 3.69 (s, 2H), 3.56 - 3.49 (m, 2H), 2.99 - 2.93 (m, 2H), 1.39 (s, 9H) ^{13}C NMR (126 MHz, DMSO-d_6) δ 191.6 (1C, O=C-S), 166.9 (1C, O=C-O), 165.6 (1C, O=C-NH), 141.4 (1C, Ph), 133.3 (1C, Ph), 129.0 (2C, Ph), 128.1 (2C, Ph), 82.0 (1C, C-O), 50.8 (1C, CH_2), 39.0 (1C, CH_2NH_3^+), 37.6 (1C, NHCH_2), 32.7 (1C, SCH_2), 28.0 (3C, $(\text{CH}_3)_3$). HRMS (ESI) : Exact mass calcd. for $\text{C}_{17}\text{H}_{25}\text{N}_2\text{O}_4\text{S}^+ [\text{M}+\text{H}]^+ = 353.1530$; found 353.1555.

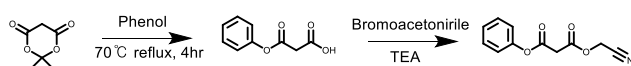


1-((3,5-dinitrobenzyl) 3-ethyl 2-aminomalonate (1) : Prepared according to the general procedure B using 2-((*tert*-butoxycarbonyl)amino)-3-ethoxy-3-oxopropanoic acid (100.0 mg, 0.4 mmol), 3,4-dinitrobenzyl alcohol (198.13 mg, 1.0 mmol), DMAP (122.2 mg, 1.0 mmol), EDCI·HCl (191.7 mg, 1.0 mmol) and CH_2Cl_2 (10 mL). The product was successfully obtained. The deprotection was achieved upon treatment with 4 M solution of HCl in 1,4-dioxane for 30 min at room temperature, and the resulting product was washed with diethyl ether to remove 3,4-dinitrobenzyl alcohol. The solvent was concentrated under vacuum, and the product was obtained as a yellow solid (163.6 mg, 45.0 %). : ^1H NMR (500 MHz, DMSO-d_6) δ 9.23 (br s, 3H), 8.84 (t, J = 2.21 Hz, 1H), 8.74 (d, J

= 2.10 Hz, 2H), 5.58 (q, J = 16.03 Hz, 2H), 5.26 (s, 1H), 3.56 (s, 2H), 1.22 (t, J = 7.08 Hz, 3H), ^{13}C NMR (126 MHz, DMSO- d_6) δ 164.0 (2C, O=C), 148.5 (2C, C-N(=O)=O), 139.7 (1C, Ph), 128.7 (2C, Ph), 119.0 (1C, Ph), 66.8 (1C, O-CH₂-Ph), 66.2 (1C, CH-NH₃⁺), 63.6 (1C, OCH₂), 14.2 (1C, CH₃). HRMS (ESI) : Exact mass calcd. for C₁₂H₁₄N₃O₈⁺ [M+H]⁺ = 328.0775; found 328.0803.

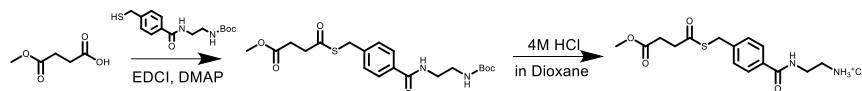


Methyl 1-(((4-((2-aminoethyl)carbamoyl)benzyl)thio)carbonyl)cyclopropane-1-carboxylate (2) : To a stirred solution of 1-(methoxycarbonyl)cyclopropane-1-carboxylic acid (200.0 mg, 1.39 mmol) in CH₂Cl₂ (5 mL) at 0 °C in an inert atmosphere, oxalyl chloride (1.2 mL, 13.9 mmol) was added dropwise and continued stirring for 5 h at room temperature. Removed the solvent and oxalyl chloride under reduced vacuum and redissolved the resulting oily residue in CH₂Cl₂. Added triethylamine (968.0 μL , 6.94 mmol) followed by Boc-ABT (344.5 mg, 1.1 mmol) in CH₂Cl₂ at 0 °C. Allowed the reaction mixture to continue stirring under an inert atmosphere at room temperature for 5 h. Progress of the reaction was monitored using thin-layer chromatography (TLC). Purification of the crude material by flash column chromatography (petroleum ether/ethyl acetate, 95:5 to 85:15) furnished white solid product (92.9 %, 450.0 mg). The deprotection was achieved upon treatment with 4 M solution of HCl in 1,4-dioxane for 30 min at room temperature, and the resulting product was used without further purification. : ^1H NMR (500 MHz, DMSO- d_6) δ 8.79 (t, J = 5.16 Hz 1H), 8.15 (br s, 3H), 7.88 (d, J = 8.16 Hz, 2H), 7.39 (d, J = 8.18 Hz, 2H), 4.18 (s, 2H), 3.66 (s, 3H), 3.53 (q, J = 5.85 Hz, 2H), 2.99 (m, 3H), 1.54 (m, 4H), ^{13}C NMR (126 MHz, DMSO- d_6) δ 194.6 (1C, O=C-S), 170.0 (1C, O=C-O), 166.8 (1C, O=C-NH), 141.4 (1C, Ph), 133.3 (1C, Ph), 129.1 (2C, Ph), 128.1 (2C, Ph), 53.0 (1C, OCH₃), 39.0 (1C, NH₃⁺-CH₂), 37.6 (1C, NH-CH₂), 35.7 (1C, C), 33.0 (1C, SCH₂), 19.1 (2C, CH₂-CH₂). HRMS (ESI) : Exact mass calcd. for C₁₆H₂₁N₂O₄S⁺ [M+H]⁺ = 337.1217; found 337.1247.



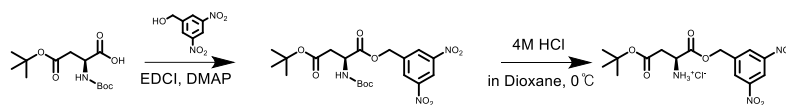
Cyanomethyl phenyl malonate (3) : 2,2-Dimethyl-1,3-dioxane-4,6-dione (721.0 mg, 5.0 mmol) and phenol (615.7 μL , 7.0 mmol) were dissolved in toluene (10 mL). The reaction proceeded under reflux for 4 h. The resulting mixture was then added to saturated NaHCO₃. The layers were separated, and the aqueous layer was washed with toluene. The water layer was acidified with 1 M HCl, and the organic layer was separated using ethyl acetate, dried over MgSO₄, filtered, and concentrated under vacuum. Prepared according to the general procedure C using the resulting product, bromoacetonitrile (5 mL, 718 mol, excess) and triethylamine (139.0 μL , 1.0 mmol). The reaction mixture was stirred at room temperature for 16 h. The resulting solution was washed with 1 M HCl, 4 % NaHCO₃ and brine. The organic layer was dried over MgSO₄, filtered, and concentrated under vacuum. The product was obtained as a colorless liquid (217.0 mg, 19.8%). : ^1H NMR (500 MHz, DMSO- d_6) δ 7.46 (m, 2H), 7.30 (m, 1H), 7.19 (m, 2H), 5.12 (s, 2H), 3.99 (s, 2H), ^{13}C NMR (126 MHz, DMSO- d_6) δ 167.9 (1C, O-O=C), 167.2 (1C, O=C-O), 152.4 (1C, Ph), 132.0 (2C, Ph), 128.6 (1C, Ph), 123.9 (2C, Ph), 117.6 (1C, C $\equiv$ N), 52.1 (1C, OCH₂), 41.8 (1C, CH₂, unidentified due to interference from solvent peaks). HRMS (ESI) : Exact mass calcd. for C₁₁H₉NO₄Na⁺ [M+Na]⁺ = 242.0424; found 242.0428.

Synthesis of succinate diester

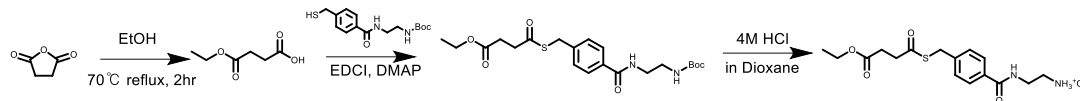


Methyl 4-(((4-((2-aminoethyl)carbamoyl)benzyl)thio)-4-oxobutanoate (5) : Prepared according to the general procedure A using monomethyl succinate (341 μL , 3.0 mmol), Boc-ABT (155.2 mg, 0.5 mmol), DMAP (61.1 mg, 0.5 mmol), EDCI·HCl (575.1 mg, 3 mmol) and CH₂Cl₂ (10 mL). The deprotection was achieved upon treatment with 4 M solution of HCl in 1,4-dioxane for 30 min at room temperature and the product was obtained as a white solid (102.4 mg, 56.8 %). : ^1H NMR (500 MHz, DMSO- d_6) δ 8.78 (t, J = 5.45 Hz, 1H), 8.14 (br s, 3H), 7.85 (d, J = 8.26 Hz, 2H), 7.38 (d, J = 8.25 Hz, 2H), 4.18 (s, 2H), 3.59 (s, 3H), 3.53 (q, J = 5.92 Hz, 2H), 2.98 (m, 2H), 2.90 (t, J = 6.53 Hz, 2H), 2.62 (t, J = 6.53 Hz, 2H), ^{13}C NMR (126 MHz, DMSO- d_6) δ 197.4 (1C, O=C-S), 172.4 (1C, O=C-O), 166.8 (1C, O=C-NH), 141.8 (1C, Ph), 133.3 (1C, Ph), 129.0 (2C, Ph), 129.1 (2C, Ph), 52.0 (1C, CH₃O), 39.03 (1C, CH₂NH₃⁺), 38.3 (1C, CH₂NH), 37.6 (1C, CH₂), 32.3 (1C, SCH₂), 28.9 (1C, CH₂). HRMS (ESI) : Exact

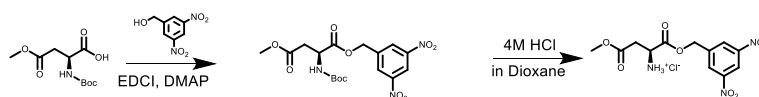
mass calcd. for $C_{15}H_{21}N_2O_4S^+$ $[M+H]^+ = 325.1217$; found 325.1242.



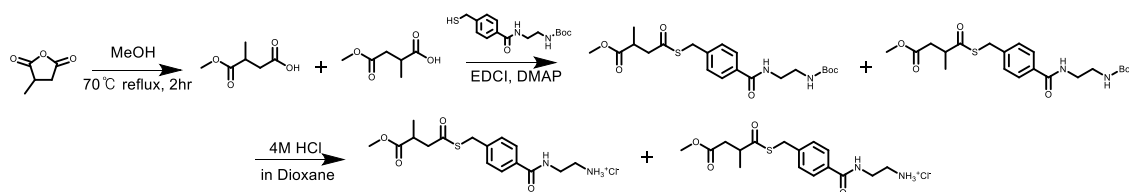
4-(*tert*-butyl 1-(3,5-dinitrobenzyl) L-aspartate (4) : Prepared according to the general procedure B using N-Boc-L-aspartic acid 4-*tert*-butyl ester (289.3 mg, 1.0 mmol), 3,4-dinitrobenzyl alcohol (237.8 mg, 1.2 mmol), DMAP (122.2 mg, 1.0 mmol), EDCI·HCl (230.0 mg, 1.2 mmol) and EtOAc (10 mL). Boc-protected product was successfully obtained. The deprotection was achieved upon treatment with 4 M solution of HCl in 1,4-dioxane at 0 °C for 30 min, and the resulting product was washed with diethyl ether to remove 3,4-dinitrobenzyl alcohol. The solvent was concentrated under vacuum, and the product was obtained as a yellow solid (101.3 mg, 20.5 %). ^1H NMR (500 MHz, DMSO- d_6) δ 8.83 (t, $J = 2.12$ Hz, 1H), 8.82 (br s, 3H, unidentified due to interference from other peaks), 8.76 (d, $J = 2.11$ Hz, 2H), 5.51 (q, $J = 4.57$ Hz, 2H), 4.42 (t, $J = 5.30$ Hz, 1H), 3.00 (m, 2H), 1.36 (s, 9H), ^{13}C NMR (126 MHz, DMSO- d_6) δ 168.9 (1C, O=C), 168.7 (1C, O=C), 148.5 (2C, O=C-NH), 140.0 (1C, Ph), 129.1 (2C, Ph), 118.9 (1C, Ph), 82.1 (1C, OC), 66.8 (1C, OCH $_2$), 48.9 (1C, CHNH $_3^+$), 35.5 (1C, CH $_2$), 28.0 (3C, CH $_3$). HRMS (ESI) : Exact mass calcd. for $C_{15}H_{20}N_3O_8^+$ $[M+H]^+ = 370.1245$; found 370.1245.



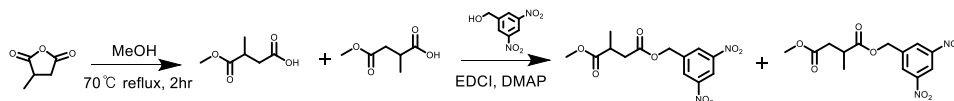
Ethyl 4-((4-((2-aminoethyl)carbamoyl)benzyl)thio)-4-oxobutanoate (5) : Succinic anhydride (1.00 g, 10.0 mmol) was dissolved in ethanol (50 mL). The reaction proceeded under reflux for 3 h. The resulting mixture was evaporated to remove ethanol. The monoethyl succinate was obtained. Then, general procedure A using monomethyl succinate (146.1 mg, 1.0 mmol), Boc-ABT (155.2 mg, 0.5 mmol), DMAP (122 mg, 1.0 mmol), EDCI·HCl (191.7 mg, 1.0 mmol) and EtOAc (10 mL) was carried out. The deprotection was achieved upon treatment with 4 M solution of HCl in 1,4-dioxane at room temperature for 30 min, and the product was obtained as a white solid (163.1 mg, 87.0 %). ^1H NMR (500 MHz, DMSO- d_6) δ 8.78 (t, $J = 5.29$ Hz, 1H), 8.11 (br s, 3H), 7.85 (d, $J = 8.18$ Hz, 2H), 7.37 (d, $J = 8.15$ Hz, 2H), 4.17 (br s, 2H), 4.03 (q, $J = 7.12$ Hz, 2H), 3.54 - 3.52 (m, 2H, unidentified due to interference from solvent peaks), 3.01 - 2.95 (br t, 2H), 2.88 (t, $J = 6.43$ Hz, 2H), 2.63 - 2.57 (br t, 2H), 1.15 (t, $J = 7.10$ Hz, 3H), ^{13}C NMR (126 MHz, DMSO- d_6) δ 197.5 (1C, O=C-S), 172.0 (1C, O=C-O), 166.9 (1C, O=C-NH), 141.8 (1C, Ph), 133.2 (1C, Ph), 129.0 (2C, Ph), 128.1 (2C, Ph), 60.6 (1C, CH $_2$ O), 39.02 (1C, CH $_2$ NH $_3^+$), 38.3 (1C, CH $_2$ NH), 37.6 (1C, CH $_2$), 32.2 (1C, SCH $_2$), 29.1 (1C, CH $_2$), 14.5 (1C, CH $_3$). HRMS (ESI) : Exact mass calcd. for $C_{16}H_{23}N_2O_4S^+$ $[M+H]^+ = 339.1373$; found 339.1393.



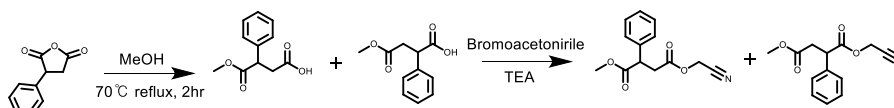
1-(3,5-dinitrobenzyl) 4-methyl L-aspartate (6) : Prepared according to the general procedure B using (*S*)-2-((*tert*-butoxycarbonyl)amino)-4-methoxy-4-oxobutanoic acid (247.2 mg, 1.0 mmol), 3,4-dinitrobenzyl alcohol (198.1 mg, 1.2 mmol), DMAP (122.1 mg, 1.0 mmol), EDCI·HCl (237.8 mg, 1.2 mmol) and CH $_2$ Cl $_2$ (10 mL). Boc-protected product was successfully obtained. The deprotection was achieved upon treatment with 4 M solution of HCl in 1,4-dioxane at 0 °C for 30 min, and the resulting product was washed with diethyl ether to remove 3,4-dinitrobenzyl alcohol. The solvent was concentrated under vacuum, and the product was obtained as a yellow solid (73.7 mg, 20.3%). ^1H NMR (500 MHz, DMSO- d_6) δ 8.90 - 8.51 (br, 3H), 8.82 (t, $J = 1.90$ Hz, 1H), 8.74 (d, $J = 1.90$ Hz, 2H), 5.50 (q, $J = 11.73$ Hz, 2H), 4.49 (t, $J = 5.43$ Hz, 1H), 3.62 (s, 3H), 3.12 (m, $J = 7.59$ Hz, 2H), ^{13}C NMR (126 Hz, DMSO- d_6) δ 170.1 (1C, O=C), 168.5 (1C, O=C), 148.5 (2C, C-N(=O)=O), 140.0 (1C, Ph), 128.9 (2C, Ph), 118.8 (1C, Ph), 65.6 (1C, OCH $_2$), 52.6 (1C, CH $_3$ O), 48.9 (1C, CHNH $_3^+$), 34.4 (1C, CH $_2$). HRMS (ESI) : Exact mass calcd. for $C_{12}H_{14}N_3O_8^+$ $[M+H]^+ = 328.0775$; found 328.0815.



Methyl 4-((4-((2-aminoethyl)carbamoyl)benzyl)thio)-2-methyl-4-oxobutanoate + Methyl 4-((4-((2-aminoethyl)carbamoyl)benzyl)thio)-3-methyl-4-oxobutanoate (7): Methyl succinic anhydride (280.6 μ L, 3.0 mmol) was dissolved in methanol (5 mL). The reaction proceeded under reflux for 3 h. The resulting mixture was evaporated to remove methanol, and the methoxy methyloxobutanoic acid was obtained. Then, general procedure A using methoxy methyloxobutanoic acid (438.4 mg, 3.0 mmol), Boc-ABT (341.4 mg, 1.1 mmol), DMAP (61.1 mg, 0.5 mmol), EDCI·HCl (230.0 mg, 1.2 mmol) and CH_2Cl_2 (10 mL) was carried out. The deprotection was achieved upon treatment with 4 M solution of HCl in 1,4-dioxane at room temperature for 30 min, and the product was obtained as a white solid (406.9 mg, 98.7 %). As a result of the NMR analysis, two products were made. (Methyl 4-((4-((2-aminoethyl)carbamoyl)benzyl)thio)-2-methyl-4-oxobutanoate) (50 %) : ^1H NMR (500 MHz, DMSO-d_6) δ 8.82 (t, $J = 5.25$ Hz, 1H), 8.19 (br s, 3H), 7.89 (d, $J = 8.08$ Hz, 2H), 7.39 (d, $J = 7.85$ Hz, 2H), 4.19 (s, 2H), 3.59 (s, 3H), 3.57 - 3.51 (m, 2H), 3.12 - 2.55 (m, 5H), 1.17 (d, $J = 7.07$, 3H). ^{13}C NMR (126 Hz, DMSO-d_6) δ 197.0 (1C, O=C-S), 175.1 (1C, O=C-O), 166.8 (1C, O=C-NH), 141.8 (1C, Ph), 133.3 (1C, Ph), 129.1 (2C, Ph), 129.0 (2C, Ph), 52.2 (1C, CH_3O), 46.5 (1C, CH_2), 39.0 (1C, CH_2NH_3), 37.6 (1C, CH_2NH), 35.9 (1C, CH), 32.1 (1C, S- CH_2), 16.9 (1C, CH_3). Methyl 4-((4-((2-aminoethyl)carbamoyl)benzyl)thio)-3-methyl-4-oxobutanoate (50 %) : ^1H NMR (500 MHz, DMSO-d_6) δ 8.82 (t, $J = 5.25$ Hz, 1H), 8.19 (br s, 3H), 7.89 (d, $J = 8.08$ Hz, 2H), 7.39 (d, $J = 7.85$ Hz, 2H), 4.19 (s, 2H), 3.60 (s, 3H), 3.57 - 3.51 (m, 2H), 3.12 - 2.55 (m, 5H), 1.12 (d, $J = 6.12$, 3H). ^{13}C NMR (126 Hz, DMSO-d_6) δ 201.5 (1C, O=C-S), 171.8 (1C, O=C-O), 166.8 (1C, O=C-NH), 141.8 (1C, Ph), 133.3 (1C, Ph), 129.1 (2C, Ph), 129.0 (2C, Ph), 52.0 (1C, CH_3O), 44.2 (1C, CH), 39.0 (1C, CH_2NH_3^+), 37.6 (1C, CH_2NH), 37.3 (1C, CH_2), 32.3 (1C, S- CH_2), 18.1 (1C, CH_3). HRMS (ESI) : Exact mass calcd. for $\text{C}_{16}\text{H}_{23}\text{N}_2\text{O}_4\text{S}^+ [\text{M}+\text{H}]^+ = 339.1373$; found 339.1392.

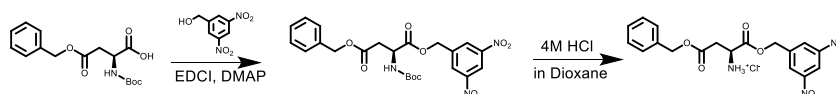


1-(3,5-dinitrobenzyl) 4-methyl 3-methylsuccinate + 1-(3,5-dinitrobenzyl) 4-methyl 2-methylsuccinate (7-2) : Methyl succinic anhydride (280.6 μ L, 3.0 mmol) was dissolved in methanol (5 mL). The reaction proceeded under reflux for 3 h. The resulting solution was washed with 1 M HCl and brine. Prepared according to the general procedure B using resulting product, 3,4-dinitrobenzyl alcohol (594.4 mg, 3.0 mmol), DMAP (122.2 mg, 1.0 mmol), EDCI·HCl (575.1 mg, 3.0 mmol) and CH_2Cl_2 (10 mL). Purification by silica gel flash chromatography (30 % EtOAc in n-Hex) yielded a yellow solid (314.8 mg, 32.2 %). As a result of the NMR analysis, two products were made. : 1-(3,5-dinitrobenzyl) 4-methyl 2-methylsuccinate (50%) : ^1H NMR (500 MHz, DMSO-d_6) δ 8.81 - 8.78 (m, 1H), 8.64 (t, $J = 2.42$ Hz, 2H), 5.36 (s, 2H), 3.57 (s, 3H), 3.01 - 2.80 (m, 1H), 2.74 - 2.58 (m, 2H), 1.21 - 1.12 (m, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 174.2 (1C, O=C), 173.5 (1C, O=C), 150.4 (2C, C-N(=O)=O), 143.0 (1C, Ph), 130.4 (1C, Ph), 130.2 (1C, Ph), 120.5 (1C, Ph), 66.0 (1C, OCH_2), 54.0 (1C, CH_3O), 39.1 (1C, CH_2), 37.5 (1C, CH), 19.0 (1C, CH_3). 1-(3,5-dinitrobenzyl) 4-methyl 3-methylsuccinate (50%) : ^1H NMR (500 MHz, DMSO-d_6) δ 8.81 - 8.78 (m, 1H), 8.64 (t, $J = 2.42$ Hz, 2H), 5.37 (s, 2H), 3.58 (s, 3H), 3.01 - 2.80 (m, 1H), 2.98 - 2.59 (m, 2H), 1.21 - 1.12 (m, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 174.2 (1C, O=C), 173.5 (1C, O=C), 150.4 (2C, 2C, C-N(=O)=O), 142.9 (1C, Ph), 130.4 (1C, Ph), 130.2 (1C, Ph), 120.4 (1C, Ph), 66.0 (1C, OCH_2), 53.8 (1C, CH_3O), 38.9 (1C, CH_2), 37.5 (1C, CH), 18.9 (1C, CH_3). HRMS (ESI) : Exact mass calcd. for $\text{C}_{13}\text{H}_{14}\text{N}_2\text{O}_8\text{Na}^+ [\text{M}+\text{Na}]^+ = 349.0642$; found 349.0641.

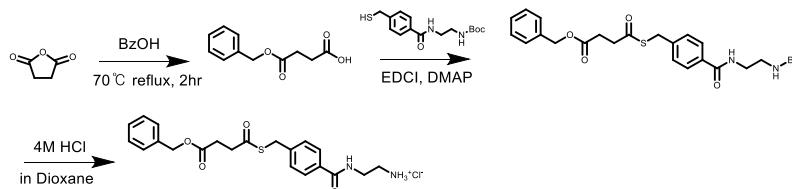


1-(cyanomethyl) 4-methyl 3-phenylsuccinate + 1-(cyanomethyl) 4-methyl 2-phenylsuccinate (8) : Phenylsuccinic anhydride (528.5 mg, 3.0 mmol) was dissolved in methanol (5 mL). The reaction proceeded under reflux for 5 h. The resulting mixture was evaporated to remove methanol. methoxy phenyloxobutanoic acid was obtained. Prepared according to the general procedure C using the resulting product, bromoacetonitrile (348.3 μ L, 5.0 mmol)

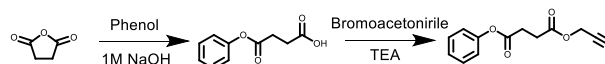
and triethylamine (139.5 μ L, 1.0 mmol). The reaction mixture was stirred at room temperature for 16 h. The resulting solution was washed with 1 M HCl, 4 % NaHCO₃, and brine. The organic layer was dried over MgSO₄, filtered, and concentrated under vacuum. The product was obtained as a colorless liquid (32.8 mg, 4.42 %). As a result of the NMR analysis, two products were made. : 1-(cyanomethyl) 4-methyl 3-phenylsuccinate (33 %) : ¹H NMR (500 MHz, DMSO-d₆) δ 7.39 - 7.25 (m, 5H), 4.95 (s, 2H), 4.11 - 4.06 (m, 1H) 3.57 (s, 3H, unidentified due to interference from solvent peaks), 3.22 - 2.67 (m, 2H), ¹³C NMR (126 MHz, DMSO-d₆) δ 173.4 (1C, O=C), 173.2 (1C, O=C), 137.7 (1C, Ph), 129.3 (2C, Ph), 128.3 (2C, Ph) 128.0 (1C, Ph), 116.2 (1C, C $\equiv$ N), 52.6 (1C, OCH₃), 49.5 (1C, OCH₂), 46.7 (1C, CH), 36.9 (1C, CH₂). 1-(cyanomethyl) 4-methyl 2-phenylsuccinate (67 %) : ¹H NMR (500 MHz, DMSO-d₆) δ 7.39 - 7.25 (m, 5H), 4.97 (s, 2H), 4.19 - 4.13 (m, 1H), 3.59 (s, 3H, unidentified due to interference from solvent peaks), 3.22 - 2.67 (m, 2H), ¹³C NMR (126 MHz, DMSO-d₆) δ 172.1 (1C, O=C), 171.8 (1C, O=C), 137.1 (1C, Ph), 129.4 (2C, Ph), 128.2 (2C, Ph), 128.1 (1C, Ph), 116.0 (1C, C $\equiv$ N), 52.2 (1C, OCH₃), 49.8 (1C, OCH₂), 46.4 (1C, CH), 37.3 (1C, CH₂). HRMS (ESI) : Exact mass calcd. for C₁₃H₁₃NO₄Na⁺ [M+Na]⁺ = 270.0737; found 270.0750.



4-benzyl 1-(3,5-dinitrobenzyl) L-aspartate (9) : Prepared according to the general procedure B using N-Boc-L-aspartic 4-benzyl ester (323.3 mg, 1.0 mmol), 3,4-dinitrobenzyl alcohol (237.8 mg, 1.2 mmol), DMAP (122.2 mg, 1.0 mmol), EDC·HCl (230.0 mg, 1.2 mmol) and EtOAc (10 mL). Boc-protected product was successfully obtained. The deprotection was achieved upon treatment with 4 M solution of HCl in 1,4-dioxane at 0 °C for 30 min, and the resulting product was washed with diethyl ether to remove 3,4-dinitrobenzyl alcohol. The solvent was concentrated under vacuum. The product was obtained as a yellow solid (182.5 mg, 41.5 %). ¹H NMR (500 MHz, DMSO-d₆) δ 8.86 (br, 3H), 8.80 (t, *J* = 2.10 Hz, 1H), 8.71 (d, *J* = 2.10 Hz, 2H), 7.35 - 7.30 (m, 5H), 5.46 (s, 2H), 5.12 (s, 2H), 4.53 (t, *J* = 5.51 Hz, 1H), 3.23 - 3.10 (m, *J* = 5.51 Hz, 2H), ¹³C NMR (126 MHz, DMSO-d₆) δ 169.6 (1C, O=C), 168.5 (1C, O=C), 148.5 (2C, C-N(=O)=O), 140.0 (1C, Ph), 135.9 (1C, Ph), 128.9 (2C, Ph), 128.8 (2C, Ph), 128.6 (1C, Ph), 128.5 (2C, Ph), 118.8 (1C, Ph), 66.8 (1C, OCH₂), 65.6 (1C, OCH₂), 49.0 (1C, CHNH₃⁺), 34.6 (1C, CH₂). HRMS (ESI) : Exact mass calcd. for C₁₈H₁₈N₃O₈⁺ [M+H]⁺ = 404.1088; found 404.1094.

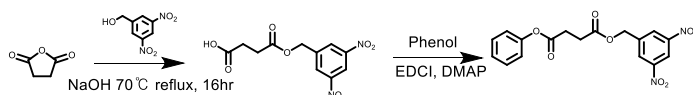


Benzyl 4-((4-((2-aminoethyl)carbamoyl)benzyl)thio)-4-oxobutanoate (10) : Succinic anhydride (1.00 g, 10.0 mmol) was dissolved in benzyl alcohol (50 mL). The reaction proceeded under reflux for 3 h. After the reaction, the resulting mixture was treated with ether and 4 % NaHCO₃ to remove benzyl alcohol. The aqueous layer was washed with diethyl ether and acidified with 1 M HCl. The organic layer was separated using EtOAc, dried over MgSO₄, filtered, and concentrated under vacuum. The monobenzyl succinate was obtained. Prepared according to the general procedure C using monobenzyl succinate (208.2 mg, 1.0 mmol), Boc-ABT (155.2 mg, 0.5 mmol), DMAP (122.2 mg, 1.0 mmol), EDC·HCl (191.7 mg, 1.0 mmol) and EtOAc. (10 mL). The deprotection was achieved upon treatment with 4 M solution of HCl in 1,4-dioxane at 0 °C for 30 min, and the product was obtained as a white solid (171.8 mg, 78.6 %). : ¹H NMR (500 MHz, DMSO-d₆) δ 8.78 (t, *J* = 5.27 Hz, 1H), 8.12 (br, 3H), 7.87 (d, *J* = 8.15 Hz, 2H), 7.41 - 7.31 (m, 7H), 5.09 (s, 2H), 4.18 (s, 2H), 3.52 (q, *J* = 3.52, 2H), 2.98 (m, 2H), 2.93 (t, *J* = 6.47 Hz, 2H), 2.69 (t, *J* = 6.47 Hz, 2H), ¹³C NMR (126 Hz, DMSO-d₆) δ 197.4 (1C, O=C-S), 171.9 (1C, O=C-O), 166.8 (1C, O=C-NH), 141.7 (1C, Ph), 136.5 (1C, Ph), 133.3 (1C, Ph), 129.0 (2C, Ph), 128.9 (2C, Ph), 128.5 (1C, Ph), 128.4 (2C, Ph), 128.1 (2C, Ph), 66.2 (1C, CH₂O), 39.04 (1C, CH₂NH₃⁺), 38.3 (1C, CH₂NH), 37.6 (1C, CH₂), 32.3 (1C, SCH₂), 29.1 (1C, CH₂). HRMS (ESI) : Exact mass calcd. for C₂₁H₂₅N₂O₄S⁺ [M+H]⁺ = 401.1530; found 401.1549.

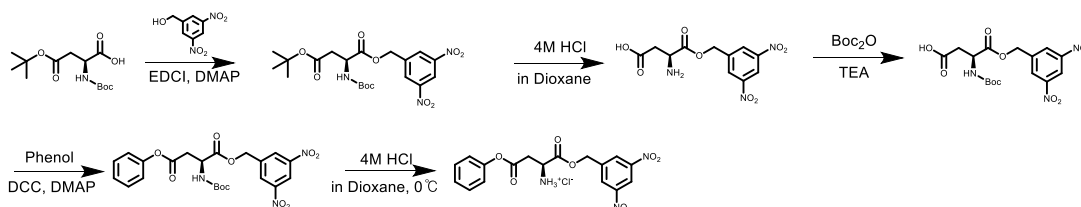


Cyanomethyl phenyl succinate (11) : Succinic anhydride (400.3 mg, 4.0 mmol) and phenol (615.7 μ L, 7.0 mmol)

were dissolved in 1 M NaOH (10 mL). The reaction mixture was stirred at room temperature for 6 h. The resulting mixture was treated with cold 1 M HCl. The product solidified, and the neutralizing solution was removed by filtration. Remaining phenol was removed with an additional wash using cold water. Prepared according to the general procedure C using resulting product, bromoacetonitrile (5 mL, 71.8 mmol, excess) and triethylamine (139.5 μ L, 1.0 mmol). The reaction mixture was stirred at room temperature for 16 h. The resulting solution was washed with 1 M HCl, 4 % NaHCO₃, and brine. The organic layer was dried over MgSO₄, filtered, and concentrated under vacuum. The product was obtained as a colorless liquid (182.0 mg, 19.5 %). : ¹H NMR (500 MHz, DMSO-d₆) δ 7.44 - 7.38 (m, 2H), 7.30 - 7.23 (m, 1H), 7.15 - 7.09 (m, 2H), 7.37 (d, J = 8.15 Hz, 2H), 4.78 (s, 2H), 2.99 - 2.91 (m, 2H), 2.90 - 2.82 (m, 2H), ¹³C NMR (126 MHz, DMSO-d₆) δ 170.7 (1C, O=C), 170.5 (1C, O=C), 150.5 (1C, Ph), 129.5 (2C, Ph), 126.1 (1C, Ph), 121.4 (2C, Ph), 114.2 (1C, C $\equiv$ N), 48.6 (1C, OCH₂), 28.9 (1C, CH₂), 28.5 (1C, CH₂). HRMS (ESI) : Exact mass calcd. for C₁₂H₁₁NO₄Na⁺ [M+Na]⁺ = 256.0580; found 256.0584.

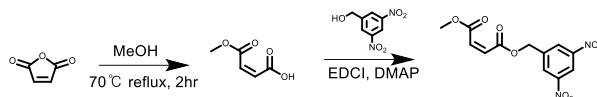


3,5-dinitrobenzyl phenyl succinate (11-2) : Succinic anhydride (500.4 mg, 5 mmol), 3,4-dinitrobenzyl alcohol (990.7 mg, 5 mmol) and NaOH (40.0 mg, 1.0 mmol) were dissolved in EtOAc (10 mL). The reaction proceeded under reflux for 16 h. The resulting solution was washed with 1 M HCl, and brine. The organic layer was dried over MgSO₄, filtered, and concentrated under vacuum. Purification by silica gel flash chromatography (50 % EtOAc in n-Hex) yielded a product. The product was dissolved in EtOAc (10 mL) and treated with phenol (263.9 μ L, 3 mmol), EDCI·HCl (575.1 mg, 3.0 mmol) and DMAP (122.2 mg, 1.0 mmol). The reaction mixture was stirred at room temperature for 16 h. The resulting solution was washed with 1 M HCl, 4 % NaHCO₃, and brine. The organic layer was dried over MgSO₄, filtered, and concentrated under vacuum. Purification by silica gel flash chromatography (50 % EtOAc in n-Hex) yielded a yellow solid (26.3 mg, 2.34 %). : ¹H NMR (500 MHz, DMSO-d₆) δ 8.78 (t, J = 2.10, 1H), 8.67 (d, J = 2.10, 2H), 7.41 - 7.37 (m, 2H), 7.27 - 7.23 (m, 1H), 7.06 - 7.02 (m, 2H), 5.40 (s, 2H), 2.91 - 2.87 (m, 2H), 2.83 - 2.80 (m, 2H), ¹³C NMR (126 MHz, DMSO-d₆) δ 172.2 (1C, O=C), 171.3 (1C, O=C), 150.8 (1C, O-Ph(C)), 148.5 (2C, C-N(=O)=O), 141.1 (1C, Ph), 129.9 (2C, Ph), 128.7 (2C, Ph), 126.3 (1C, Ph), 122.0 (2C, Ph), 118.6 (1C, Ph), 64.3 (1C, OCH₂), 49.0 (1C, CH), 29.1 (1C, CH₂). HRMS (ESI) : Exact mass calcd. for C₁₇H₁₄N₂O₈Na⁺ [M+Na]⁺ = 397.0642; found 397.0633.

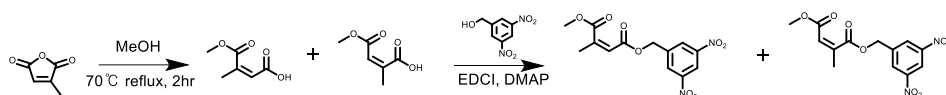


1-(3,5-dinitrobenzyl) 4-phenyl L-aspartate (12) : Prepared according to the general procedure B, using N-Boc-L-aspartic acid 4-*tert*-butyl ester (1.45 g, 5.0 mmol), 3,4-dinitrobenzyl alcohol (1.19 g, 6.0 mmol), DMAP (610.9 mg, 5.0 mmol), EDCI·HCl (1.15 g, 6.0 mmol) and EtOAc (50 mL). Boc and *tert*-butyl protected product was successfully obtained. The deprotection was achieved upon treatment with 4 M solution of HCl in 1,4-dioxane at room temperature for 1 h, and the resulting product was washed with diethyl ether to remove 3,4-dinitrobenzyl alcohol. The resulting product was dissolved in dioxane (20 mL) and water (20 mL). The solution was added to 1 M NaOH (10 mL). The mixture was cooled to 0 °C, Boc₂O (1.09 g, 5 mmol) was slowly added, and the reaction was stirred for 6 h at room temperature. Subsequently, the reaction mixture was diluted with EtOAc (10 mL) and acidified with 1 M HCl. The layers were separated, and the aqueous fraction was extracted with EtOAc. The organic layer was dried over MgSO₄, filtered, and concentrated under vacuum. The resulting product and DCC (1.03 g, 5 mmol) were dissolved in THF (10 mL) and treated with pyridine (402.7 μ L, 5.0 mmol). The reaction was stirred for 18 h at room temperature. The resulting solution was filtered to remove precipitants. The resulting mixture was then added to 4 % NaHCO₃. The layers were separated, and the aqueous layer was washed with EtOAc. The water layer was acidified with 1 M HCl, and the organic layer was separated using EtOAc, dried over MgSO₄, filtered, and concentrated under vacuum. The deprotection was achieved upon treatment with 4 M solution of HCl in 1,4-dioxane at 0 °C for 30 min, and the product was obtained as a white solid (381.7 mg, 17.9 %). ¹H NMR (500 MHz, DMSO-d₆) δ 8.80 (t, J = 2.08 Hz, 1H), 8.76 (d, J = 1.90 Hz, 2H), 8.59 (br, 3H), 7.41 - 7.37 (m, J = 7.80 Hz, 2H), 7.30 - 7.21 (m, 1H), 7.11 - 7.04 (m, 2H), 5.51 (s, 2H), 4.68 (t, J = 5.49 Hz, 1H),

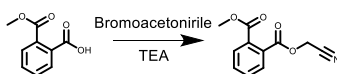
3.41 - 2.85 (m, 2H), ^{13}C NMR (126 MHz, DMSO- d_6) δ 171.3 (1C, O=C), 168.4 (1C, O=C), 150.4 (1C, Ph), 148.5 (2C, C-N(=O)-O), 140.0 (1C, Ph), 129.9 (2C, Ph), 129.2 (2C, Ph), 126.6 (1C, Ph), 121.9 (2C, Ph), 118.9 (1C, Ph), 65.4 (1C, OCH₂), 49.0 (1C, CHNH₃⁺), 34.9 (1C, CH₂). HRMS (ESI) : Exact mass calcd. for C₁₇H₁₆N₃O₈⁺ [M+H]⁺ = 390.0932; found 390.0946.



3,5-dinitrobenzyl methyl maleate (13) : Maleic anhydride (196.1 mg, 2.0 mmol) was dissolved in methanol (4 mL). The reaction proceeded under reflux for 3 h. The resulting solution was washed with 1 M HCl and brine. Then, general procedure B using resulting product, 3,4-dinitrobenzyl alcohol (594.4 mg, 3.0 mmol), DMAP (122.2 mg, 1.0 mmol), EDCI·HCl (575.1 mg, 3.0 mmol) and CH₂Cl₂ (10 mL) was carried out. Purification by silica gel flash chromatography (50% EtOAc in n-Hex) yielded a yellow solid (140.4 mg, 22.6 %). : ^1H NMR (500 MHz, DMSO- d_6) δ 8.81 (t, J = 4.14 Hz, 1H), 8.73 (t, J = 2.07 Hz, 2H), 6.89 (d, J = 6.88 Hz, 2H), 5.49 (s, 2H), 3.76 (s, 3H), ^{13}C NMR (126 MHz, DMSO- d_6) δ 165.2 (1C, O=C-O), 164.5 (1C, O=C-O), 148.6 (2C, C-N(=O)=O), 140.4 (1C, Ph), 134.1 (1C, =CH), 133.2 (1C, =CH), 128.9 (2C, Ph), 118.7 (1C, Ph), 65.0 (1C, CH₂O), 52.8 (1C, CH₃O). HRMS (ESI) : Exact mass calcd. for C₁₂H₁₀N₂O₈Na⁺ [M+Na]⁺ = 333.0329; found 333.0320.

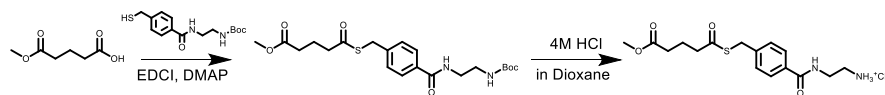


4-(3,5-dinitrobenzyl) 1-methyl 2-methylmaleate + 1-(3,5-dinitrobenzyl) 4-methyl 2-methylmaleate (14) : Citraconic anhydride (179.8 μL , 2.0 mmol) was dissolved in methanol (4 mL). The reaction proceeded under reflux for 3 h. The resulting solution was washed with 1 M HCl and brine. General procedure B using the resulting product, 3,4-dinitrobenzyl alcohol (594.4 mg, 3.0 mmol), DMAP (122.2 mg, 1.0 mmol), EDCI·HCl (512.3 mg, 1.2 mmol) and CH₂Cl₂ (10 mL) was carried out. Purification by silica gel flash chromatography (50 % EtOAc in n-Hex) yielded a yellow solid (60.5 mg, 9.32 %). As a result of the NMR analysis, two products were made. : 4-(3,5-dinitrobenzyl) 1-methyl 2-methylmaleate (25%) ^1H NMR (500 MHz, DMSO- d_6) δ 8.79 (m, 1H), 8.66 (d, J = 3.45 Hz, 2H), 6.22 (s, 1H), 5.41 (s, 2H), 3.66 (s, 3H), 2.04 (s, 3H), ^{13}C NMR (126 MHz, DMSO- d_6) δ 168.3 (1C, O=C), 165.5 (1C, O=C), 148.5 (2C, C-N(=O)=O), 145.2 (1C, C=), 140.4 (1C, Ph), 129.7 (2C, Ph), 120.9 (1C, HC=) 118.9 (1C, Ph), 64.4 (1C, OCH₂), 52.2 (1C, CH₃O), 20.3 (1C, CH₃). 1-(3,5-dinitrobenzyl) 4-methyl 2-methylmaleate (75%) : ^1H NMR (500 MHz, DMSO- d_6) δ 8.81 (m, 1H), 8.68 (t, J = 3.45 Hz, 2H), 6.18 (s, 1H), 5.48 (s, 2H), 3.62 (s, 3H), 2.07 (s, 3H), ^{13}C NMR (126 MHz, DMSO- d_6) δ 168.3 (1C, O=C), 165.5 (1C, O=C), 148.5 (2C, C-N(=O)=O), 145.2 (1C, C=), 140.4 (1C, Ph), 128.8 (2C, Ph), 121.7 (1C, HC=) 118.7 (1C, Ph), 64.8 (1C, OCH₂), 52.6 (1C, CH₃O), 20.2 (1C, CH₃). HRMS (ESI) : Exact mass calcd. for C₁₃H₁₂N₂O₈Na⁺ [M+Na]⁺ = 347.0486; found 347.0485.

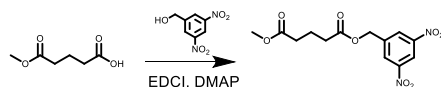


Cyanomethyl methyl phthalate (15) : Prepared according to the general procedure C using monomethyl phthalate (95.5 mg, 0.53 mmol), bromoacetonitrile (1.0 mL, 14.4 mmol) and triethylamine (1.0 mL, 7.17 mmol). The reaction mixture was stirred at room temperature for 2 h. The resulting solution was washed with 1 M HCl, 4 % NaHCO₃ and brine. The organic layer was dried over MgSO₄, filtered, and concentrated under vacuum. The product was obtained as a colorless liquid (109.5 mg, 94.3 %). : ^1H NMR (500 MHz, DMSO- d_6) δ 7.73 - 7.84 (m, 4H), 5.22 (s, 2H), 3.85 (s, 3H), ^{13}C NMR (126 MHz, DMSO- d_6) δ 167.3 (1C, O=C), 166.5 (1C, O=C), 132.8 (1C, Ph), 132.5 (1C, Ph), 131.6 (1C, Ph), 130.3 (1C, Ph), 129.6 (1C, Ph), 129.5 (1C, Ph), 116.1 (1C, C $\equiv$ N), 53.3 (1C, OCH₃), 50.6 (1C, OCH₂). HRMS (ESI) : Exact mass calcd. for C₁₁H₉NO₄Na⁺ [M+Na]⁺ = 242.0424; found 242.0425.

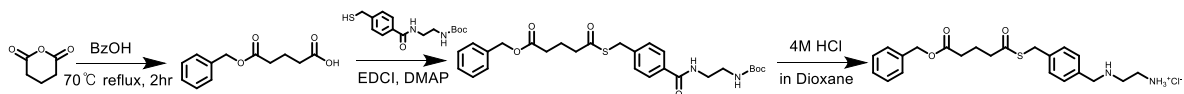
Synthesis of glutarate diester



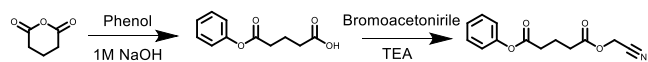
Methyl 5-((4-((2-aminoethyl)carbamoyl)benzyl)thio)-5-oxopentanoate (g) : Prepared according to the general procedure C using monomethyl glutarate (375.0 μ L, 3.0 mmol), Boc-ABT (155.2 mg, 0.5 mmol), DMAP (61.1 mg, 0.5 mmol), EDC·HCl (575.1 mg, 3.0 mmol) and CH₂Cl₂ (10 mL). Purification by flash column chromatography (60 % EtOAc in n-Hex) afforded the corresponding Boc-protected product as a white solid (117.2 mg, 53.5 %). Boc-(g): ¹H NMR (500 MHz, DMSO-d₆) δ 8.42 (t, J = 5.41 Hz, 1H), 7.77 (d, J = 8.18 Hz, 2H), 7.37 (d, J = 8.25 Hz, 2H), 6.91 (t, J = 5.65 Hz, 1H), 4.17 (s, 2H), 3.59 (s, 3H), 3.28 (q, J = 6.19 Hz, 2H), 3.09 (q, J = 6.20 Hz, 2H), 2.66 (t, J = 7.43 Hz, 2H), 2.35 (t, J = 7.39 Hz, 2H), 1.82 (quintet, J = 7.41 Hz, 2H), 1.37 (s, 9H), ¹³C NMR (126 MHz, DMSO-d₆) δ 198.1 (1C, O=C-S), 173.2 (1C, O=C-O), 166.5 (1C, O=C-NH), 156.2 (1C, O=C-NH), 141.5 (1C, Ph), 133.8 (1C, Ph), 129.0 (2C, Ph), 127.9 (2C, Ph), 78.1 (1C, C-O), 51.8 (1C, H₃C-O), 42.5 (1C, CH₂), 40.7 - 39.4 (2C, NH-CH₂-CH₂-NH, unidentified due to interference from solvent peaks), 32.5 (1C, SCH₂), 32.3 (1C, CH₂) 28.7 (3C, CH₃), 20.8 (1C, CH₂). The deprotection was achieved upon treatment with 4 M solution of HCl in 1,4-dioxane, and the resulting product was used without further purification. : HRMS (ESI): Exact mass calcd. for C₁₆H₂₃N₂O₄S⁺ [M+H]⁺ = 339.1370; found 339.1400.



3,5-dinitrobenzyl methyl glutarate (g-2) : Prepared according to the general procedure B using monomethyl glutarate (118.3 mg, 1.0 mmol), 3,4-dinitrobenzyl alcohol (198.13 mg, 1.0 mmol), DMAP (61.1 mg, 0.5 mmol), EDC·HCl (210.9 mg, 1.1 mmol) and CH₂Cl₂ (10 mL). Purification by silica gel flash chromatography (30 % EtOAc in n-Hex) to yield a yellow solid (101.3 mg, 31.1 %). : ¹H NMR (500 MHz, DMSO-d₆) δ 8.79 (t, J = 2.22 Hz, 1H), 8.66 (d, J = 2.12 Hz, 2H), 5.34 (s, 2H), 3.58 (s, 2H), 2.48 (t, J = 7.41 Hz, 2H), 2.37 (t, J = 7.40 Hz, 2H), 1.81 (quint, J = 7.41 Hz, 2H), ¹³C NMR (126 MHz, DMSO-d₆) δ 173.4 (1C, O=C), 172.7 (1C, O=C), 148.5 (2C, C-N(=O)=O), 141.1 (1C, Ph), 128.6 (2C, Ph), 118.6 (1C, Ph), 64.1 (1C, OCH₂), 51.8 (1C, O-CH₃), 32.8 (1C, CH₂), 32.7 (1C, CH₂), 20.2 (1C, CH₂). HRMS (ESI) : Exact mass calcd. for C₁₃H₁₄N₂O₈Na⁺ [M+Na]⁺ = 349.0648; found 349.0642.



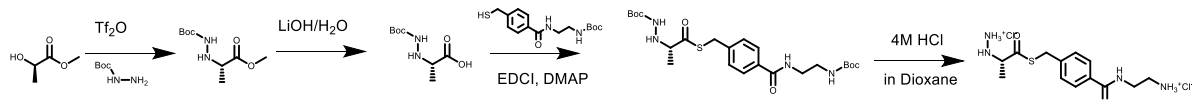
Benzyl 5-((4-(((2-aminoethyl)amino)methyl)benzyl)thio)-5-oxopentanoate (16) : Glutarate anhydride (1.00 g, 8.76 mmol) was dissolved in benzyl alcohol (50 mL). The reaction proceeded under reflux for 3 h. After the reaction, the resulting mixture was treated with diethyl ether and 4 % NaHCO₃ to remove benzyl alcohol. The layers were separated, and the aqueous layer was washed with diethyl ether. The water layer was acidified with 1 M HCl, and the organic layer was separated using EtOAc, dried over MgSO₄, filtered, and concentrated under vacuum. The monobenzyl glutarate was obtained. Prepared according to the general procedure C using monobenzyl glutarate (222.2 mg, 1.0 mmol), Boc-ABT (155.2 mg, 0.5 mmol), DMAP (122.2 mg, 1.0 mmol), EDC·HCl (191.7 mg, 1.0 mmol) and EtOAc. (10 mL). The deprotection was achieved upon treatment with 4 M solution of HCl in 1,4-dioxane at 0 °C for 30 min, and the product was obtained as a white solid (101.3 mg, 44.9 %). : ¹H NMR (500 MHz, DMSO-d₆) δ 8.76 (s, 1H), 8.12 (br s, 3H), 7.87 (d, J = 8.10, 2H), 7.41 - 7.31 (m, 7H), 5.09 (s, 2H), 4.18 (s, 2H), 3.53 (q, J = 5.82, 2H), 2.99 (q, J = 5.72, 2H), 2.66 (q, J = 6.96 Hz, 2H), 2.41 (t, J = 7.33 Hz, 1H), 2.26 (t, J = 7.32 Hz, 1H), ¹³C NMR (126 MHz, DMSO-d₆) δ 198.1 (1C, O=C-S), 174.2 (1C, O=C-O), 166.8 (1C, O=C-NH), 141.9 (1C, Ph), 136.6 (1C, Ph), 133.3 (1C, Ph), 129.0 (2C, Ph), 128.9 (2C, Ph), 128.5 (1C, Ph), 128.4 (2C, Ph), 128.1 (2C, Ph), 66.8 (1C, CH₂O), 42.6 (1C, CH₂), 39.1 (1C, CH₂NH₃⁺), 37.6 (1C, CH₂NH), 32.8 (1C, CH₂), 32.3 (1C, SCH₂), 20.9 (1C, CH₂). HRMS (ESI) : Exact mass calcd. for C₂₂H₂₇N₂O₄S⁺ [M+H]⁺ = 415.1686; found 415.1777.



Cyanomethyl phenyl glutarate (17) : Glutaric anhydride (571.0 mg, 5.0 mmol) and phenol (615.7 mg, 7.0 mmol) were dissolved in 1 M NaOH (10 mL). The reaction mixture was stirred at 0 °C for 6 h. The resulting mixture was then added to 4 % NaHCO₃. The layers were separated, and the aqueous layer was washed with EtOAc. The water layer was acidified with 1 M HCl, and the organic layer was separated using EtOAc, dried over MgSO₄, filtered, and concentrated under vacuum. Crude product was dissolved in bromoacetonitrile (1 mL, 14.4 mol) and treated

with triethylamine (1 mL, 7.17 mmol). The reaction mixture was stirred at room temperature for 16 h. The resulting solution was washed with 1 M HCl, 4 % NaHCO₃, and brine. The organic layer was dried over MgSO₄, filtered, and concentrated under vacuum. The product was obtained as a yellow liquid (27.5 %, 340.2 mg). : ¹H NMR (500 MHz, DMSO-d₆) δ 7.45 - 7.40 (m, 2H), 7.29 - 7.24 (m, 1H), 7.16 - 7.12 (m, 2H), 4.98 (s, 2H), 2.66 (t, *J* = 7.41 Hz, 2H), 2.56 (t, *J* = 7.40 Hz, 2H), 1.91 (quintet, *J* = 7.41 Hz, 2H), ¹³C NMR (126 MHz, DMSO-d₆) δ 172.1 (1C, O=C), 171.7 (1C, O=C), 150.9 (1C, Ph), 129.9 (2C, Ph), 126.3 (1C, Ph), 122.3 (2C, Ph), 115.7 (1C, C≡N), 49.5 (1C, OCH₂), 32.8 (1C, CH₂), 32.2 (1C, CH₂), 19.9 (1C, CH₂). HRMS (ESI) : Exact mass calcd. for C₁₃H₁₃NO₄Na⁺ [M+Na]⁺ = 270.0742; found 270.0737.

Synthesis of hydrazino substrates

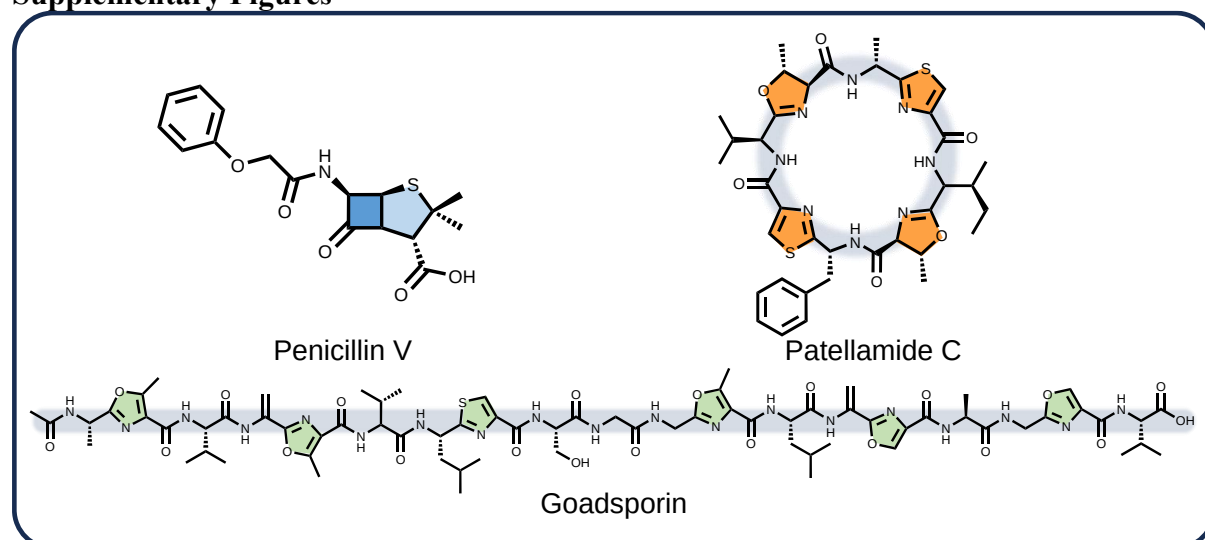


S-(4-((2-aminoethyl)carbamoyl)benzyl) 2-hydrazineylpropanethioate (Hz) : (+)-Methyl D-lactate was obtained from a commercial supplier (Sigma-Aldrich) and used as received: To a solution of (+)-methyl D-lactate (1.43 mL, 15.0 mmol) in CH₂Cl₂ (45 mL) was added trifluoromethanesulfonic anhydride (3.28 mL, 19.5 mmol) and 2,6-lutidine (3.47 mL, 30.0 mmol) at 0 °C, and the reaction was stirred at the same temperature until full consumption of the starting material (confirmed by TLC). To this was then added *tert*-butyl carbazate (3.96 g, 30.0 mmol), and the resulting mixture was further stirred at 0 °C for 4 h, then at room temperature for 16 h. The reaction mixture was diluted with CH₂Cl₂ and washed with H₂O, brine and 1 M HCl. The organic layer was then dried over anhydrous MgSO₄, concentrated under reduced pressure, and purified by flash column chromatography (30 % EtOAc/n-Hex) to furnish [(*tert*-butoxycarbonyl)amino]-L-alanine methyl ester as a yellow oil (2.64 g, 81 %).

The methyl ester (2.44 g, 11.2 mmol) obtained above was then dissolved in 1:1 mixture of THF/H₂O (24 mL) and treated with LiOH·H₂O (940 mg, 22.4 mmol). After stirring at room temperature for 3 h, the mixture was concentrated under reduced pressure and the remaining aqueous layer was washed with EtOAc. The aqueous layer was then acidified to pH ~1 using 1M HCl (aq, extracted with EtOAc, dried over anhydrous MgSO₄, and concentrated under reduced pressure to give [(*tert*-butoxycarbonyl)amino]-L-alanine as a thick colorless oil (2.08 g, 91 %).

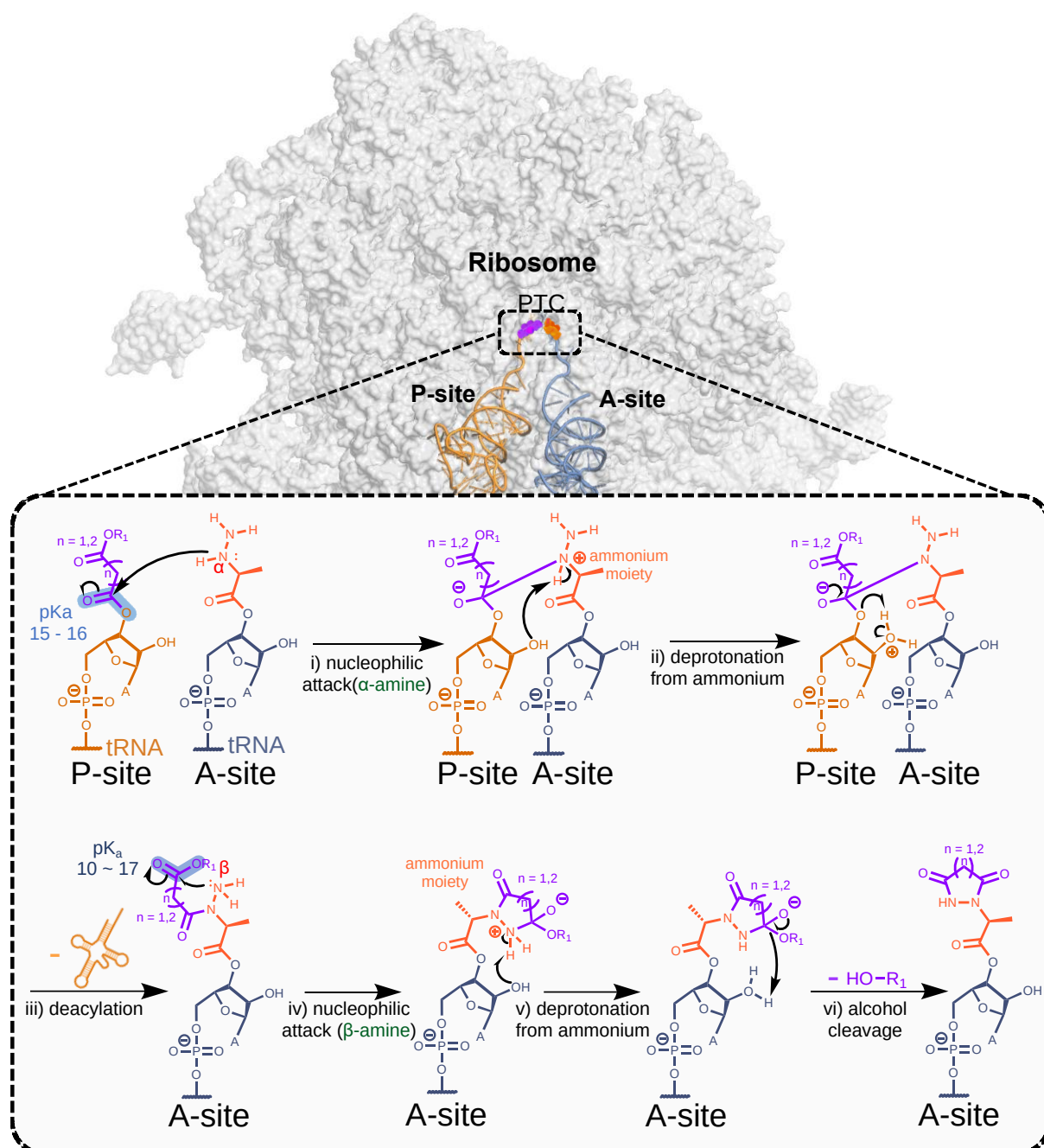
Prepared according to General Procedure C using [(*tert*-butoxycarbonyl)amino]-L-alanine. (428.0 mg, 2.1 mmol), Boc-ABT (465.0 mg, 1.5 mmol), DMAP (512.0 mg, 4.2 mmol), EDC · HCl (803.0 mg, 4.2 mmol) and CH₂Cl₂ (10 mL). Purification by flash column chromatography (60 % EtOAc in n-Hex) afforded the corresponding Boc-protected product as a colorless oil (338.0 mg, 45 %). The deprotection was achieved upon treatment with 4 M solution of HCl in 1,4-dioxane, and the resulting product was used without further purification and characterization. (Boc)₂-(Hz): ¹H NMR (500 MHz, DMSO-d₆) δ 8.40 (t, *J* = 5.11 Hz, 1H), 8.31 (br s, 1H), 7.76 (d, *J* = 7.95 Hz, 2H), 7.37 (d, *J* = 8.15 Hz, 2H), 6.89 (t, *J* = 5.38 Hz, 1H), 4.06 (s, 2H), 3.68 (d, *J* = 5.53 Hz, 1H), 3.28 (q, *J* = 6.04 Hz, 2H), 3.10 (q, *J* = 6.06 Hz, 2H), 1.38 (s, 9H), 1.37 (s, 9H), 1.15 (d, *J* = 6.96 Hz, 3H), ¹³C NMR (126 MHz, DMSO-d₆) δ 204.0 (1C, O=C-S), 166.5 (1C, O=C-NH), 156.2 (2C, O=C-O), 141.8 (1C, Ph), 133.6 (1C, Ph), 129.0 (2C, Ph), 127.8 (2C, Ph), 79.3 (1C, O-C), 78.1 (1C, O-C), 65.5 (1C, NH-CH), 42.2 (2C, NH-CH₂-CH₂-NH, unidentified due to interference from solvent peaks), 31.8 (1C, S-CH₂), 28.7 (3C, (CH₃)₃), 28.6 (3C, (CH₃)₃), 17.3 (1C, CH₃). HRMS (ESI) : Exact mass calcd. for C₂₃H₃₇N₄O₆S⁺ [M+H]⁺ = 497.2428; found 497.2428.

Supplementary Figures



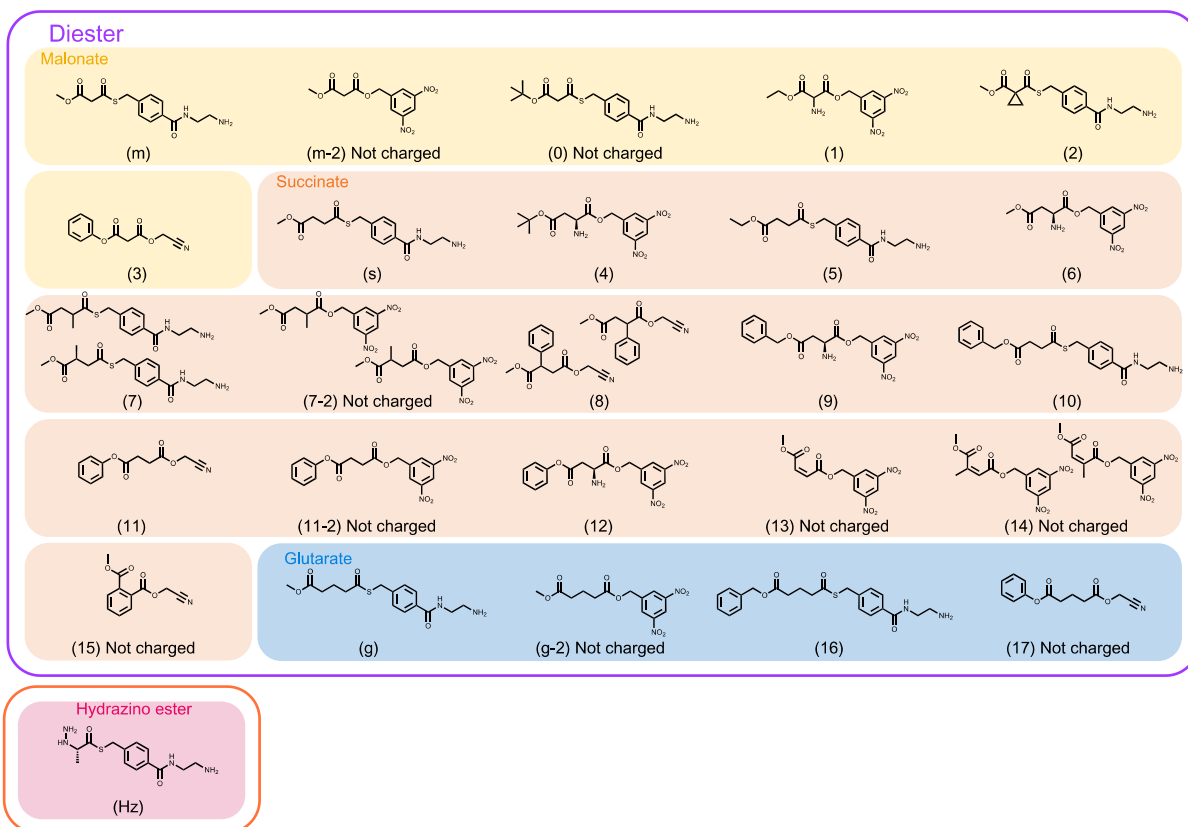
Supplementary Figure 1. Peptide-based natural products with a cyclic motif

Cyclic motifs enhance the drug efficacy of peptides by providing stability against proteases and structural rigidity. Penicillin V, Patellamide C, Goadsporin, peptide with cyclic motif, are representative natural products with known high pharmacological activity.



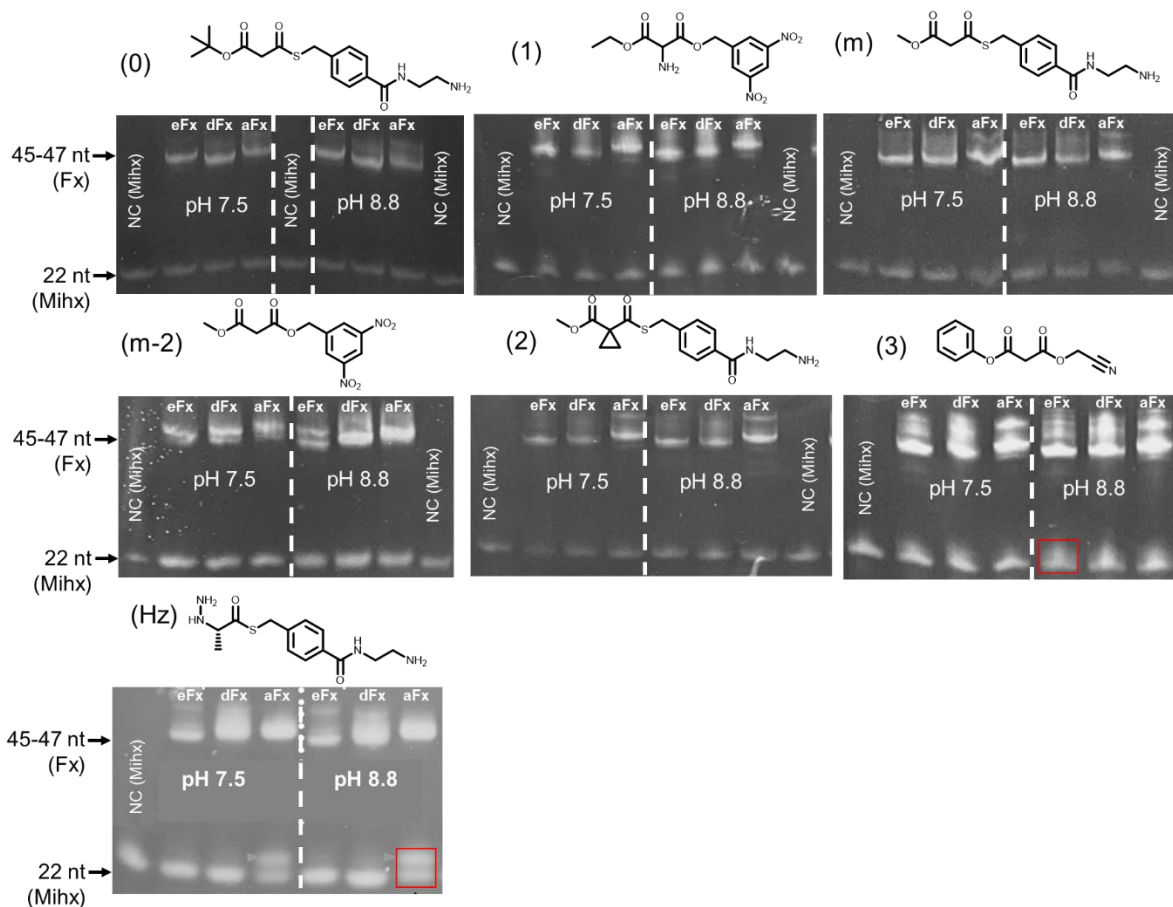
Supplementary Figure 2. Proposed mechanism of cyclic hydrazinedione formation within PTC of ribosome

(i) Nucleophilic attack of the α -amino group on the electrophilic carbonyl carbon in the ester linkage of the P-site tRNA leads to the formation of a zwitterionic tetrahedral intermediate, (ii) the deprotonation from the ammonium moiety results in oxonium formation at the 2'-hydroxy group (OH) of P-site tRNA, (iii) protonation of the 3'-OH group makes the P-site tRNA a good leaving group and transfers the nascent peptide into the A-site, (iv) a subsequent nucleophilic attack of the β -amino group on the electrophilic carbonyl carbon in the ester linkage of the R_1 group leads to the formation of a new zwitterionic tetrahedral intermediate, (v) deprotonation from the ammonium moiety by the 2'-OH group followed by alcohol cleavage and deprotonation from the 2'-OH group (vi) results in cyclic hydrazinedione formation.



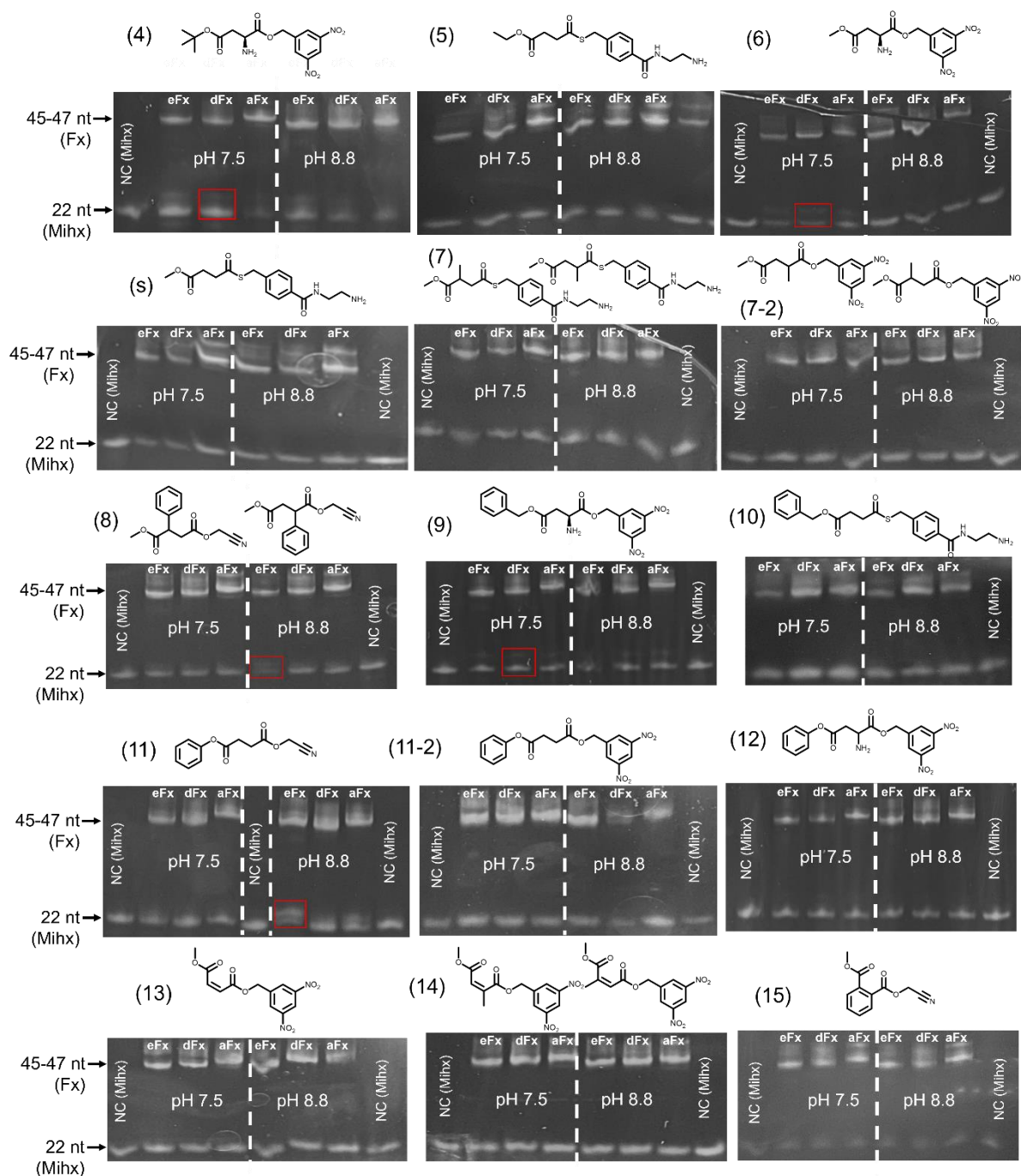
Supplementary Figure 3. Synthetic non-canonical monomers (ncMs) used in this study

We synthesized 25 diesters and one hydrazino ester for this study. We evaluated diverse flexizyme leaving groups (cyanomethylester (CME), dinitrobenzylester (DNB), and 2-(aminoethyl)amidocarboxybenzyl thioester (ABT)) to find optimal conditions for tRNA-charging. For example, substrate **m** was synthesized following the observation that **m-2** was not effectively charged to tRNA. Pairs of substrates such as **m/m-2**, **7/7-2**, **11/11-2** and **g/g-2** share the same structure, but differ in their flexizyme leaving groups (FLGs). Despite our efforts, optimal Fx conditions for charging four of the substrates (**0**, **13**, **14**, **15**, and **17**) were not determined.



Supplementary Figure 4. Acylation of microhelix (mihx) with malonate and hydrazinoesters

Acylation of microhelix with malonate substrates (**m**, **m-2**, **0**, **1**, **2**, **3** and **H_z**) : The Fx-catalyzed acylation reactions for six malonate diester and hydrazinoester substrates were carried by three different flexizymes (eFx, dFx, and aFx) at two different pH levels (7.5 and 8.8) over 16 h. The yield of each acylation reaction was determined by comparing the relative band intensity of unacylated and acylated mihx on the gel. The percent yields are 52.7 % and 50.6 % for substrate **3** and **H_z**. The yields for substrates **0**, **1**, **m**, **m-2**, and **2** could not be determined because the band shifts were unclear. Substrates that did not present clear band shifts were further validated *via* mass spectrometry. The red box was used to indicate bands shifted due to an increase in size after acylation. The same reaction conditions were applied to tRNA acylation with the substrate. Source data are provided as a Source Data file.



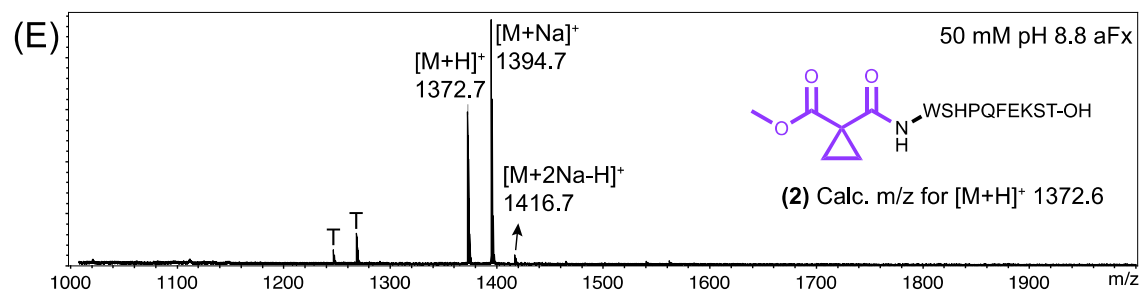
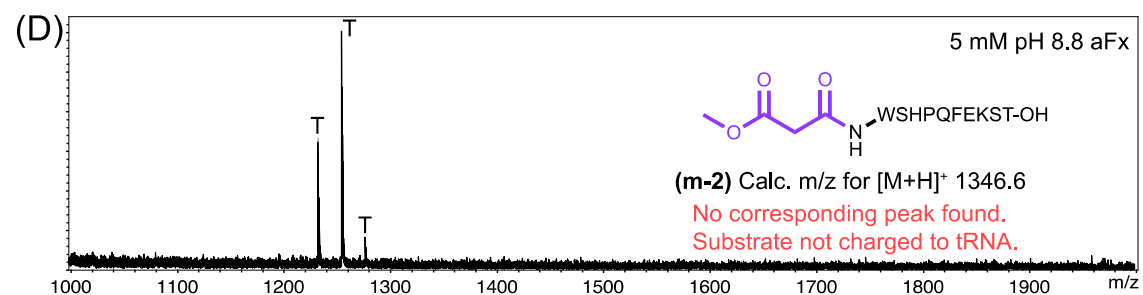
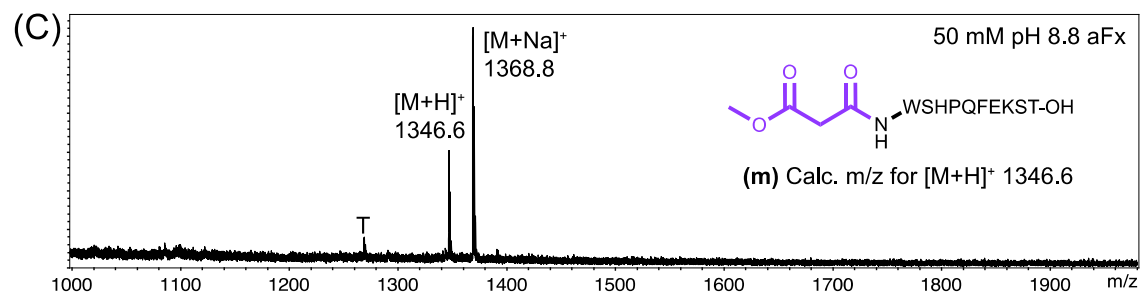
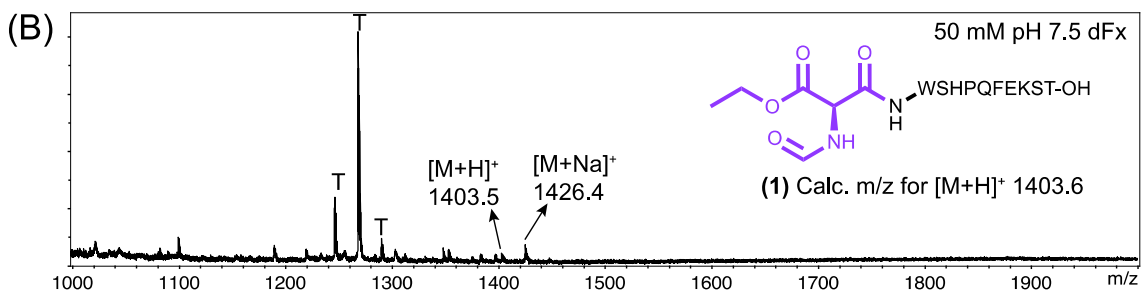
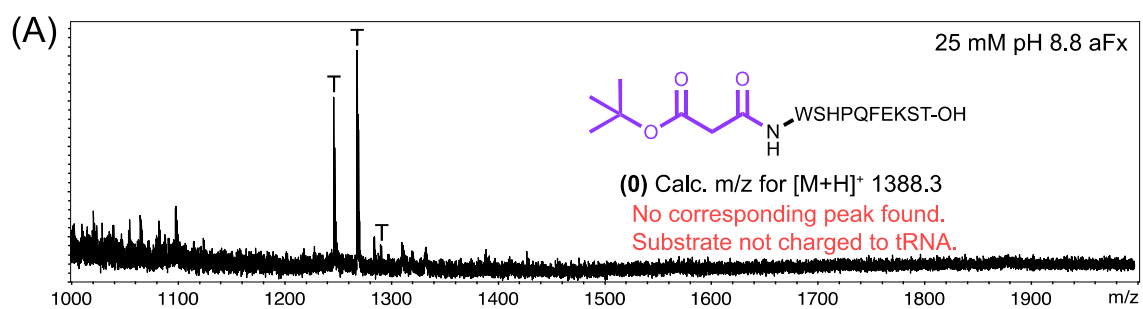
Supplementary Figure 5. Acylation of microhelix with succinate ester

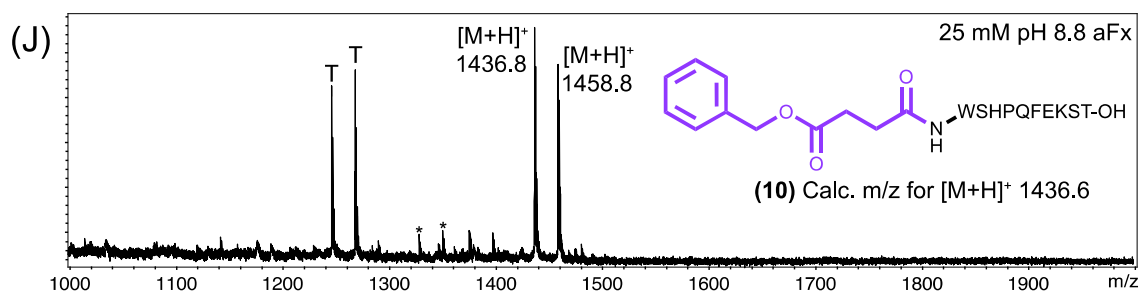
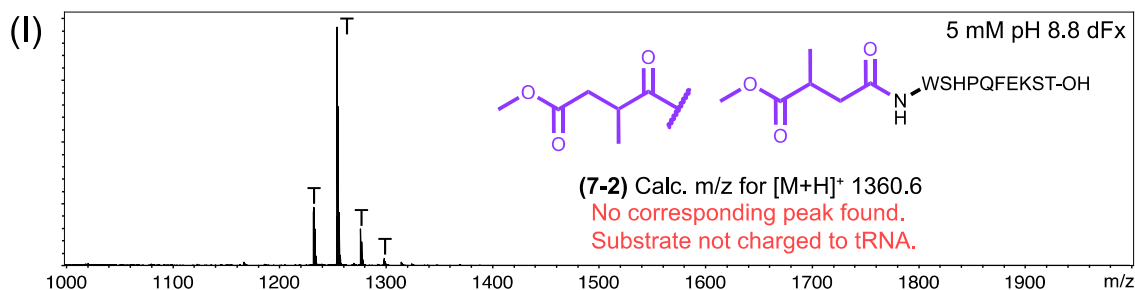
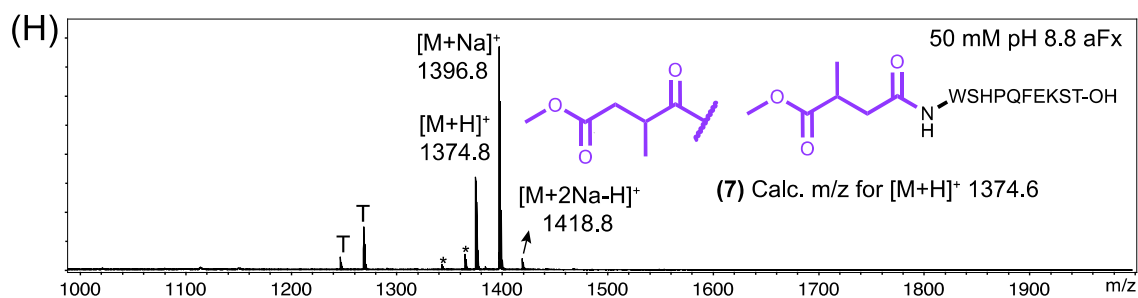
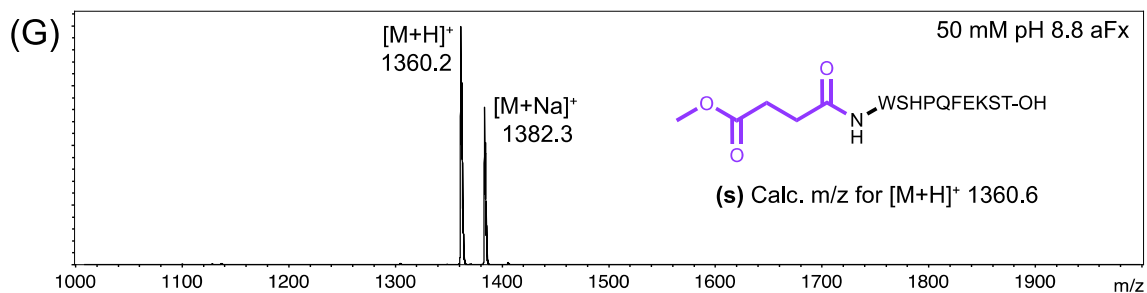
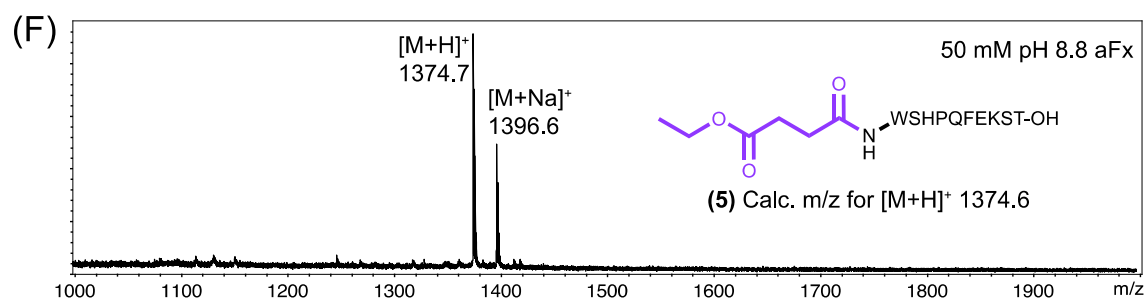
Acylation of microhelix with succinate (**s**, **4-15**): The F_x-catalyzed acylation reactions for 15 succinate diester substrates were carried out with three different flexizymes (eFx, dFx, and aFx) at two different pH levels (7.5 and 8.8) over 16 h. The yield of each acylation reaction was determined by comparing the relative band intensity of unacylated and acylated mihx on the gel. The percent yields are 22.4 %, 34.8 %, 42.0 %, 29.2 %, 57.3 % for substrate **4**, **6**, **8**, **9**, **11**. The yields for substrates **5**, **s**, **7**, **7-2**, **10**, **11-2**, **12**, **13**, **14**, **15** could not be determined because the band shifts were unclear. Substrates that did not present clear band shifts were further validated *via* mass spectrometry. The red box was used to indicate bands shifted due to an increase in size increase after acylation. The same reaction conditions were applied to tRNA acylation with the substrate. Source data are provided as a Source Data file.

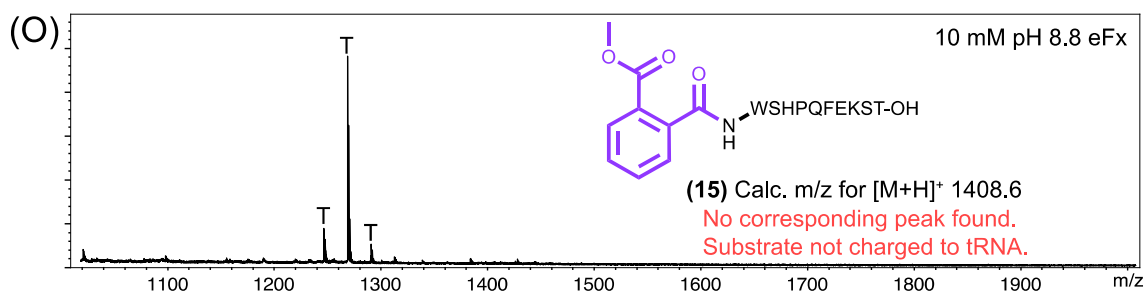
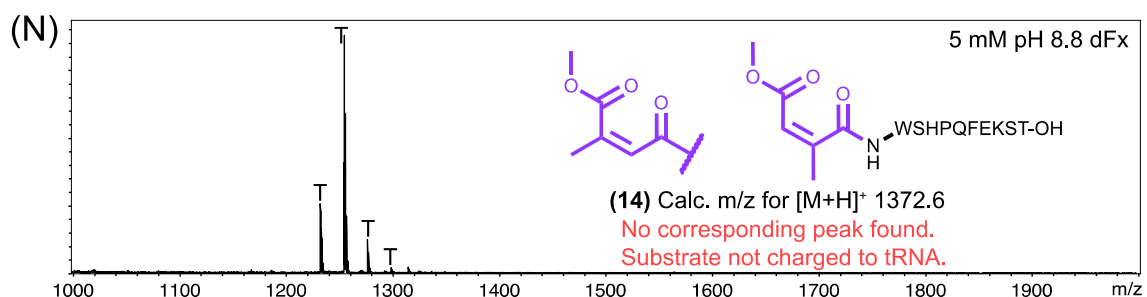
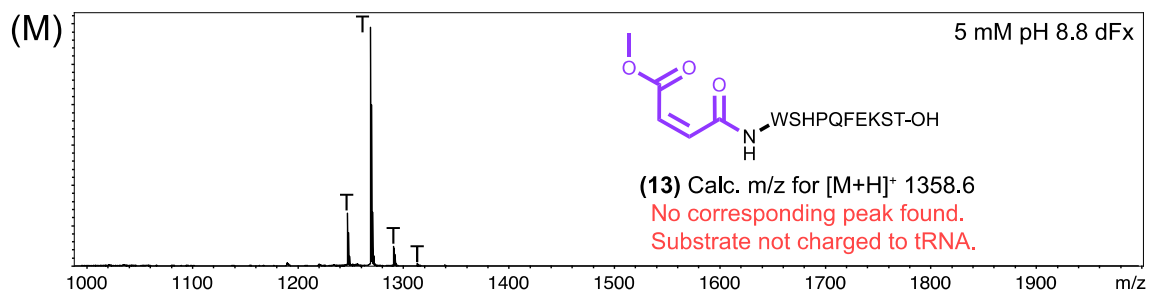
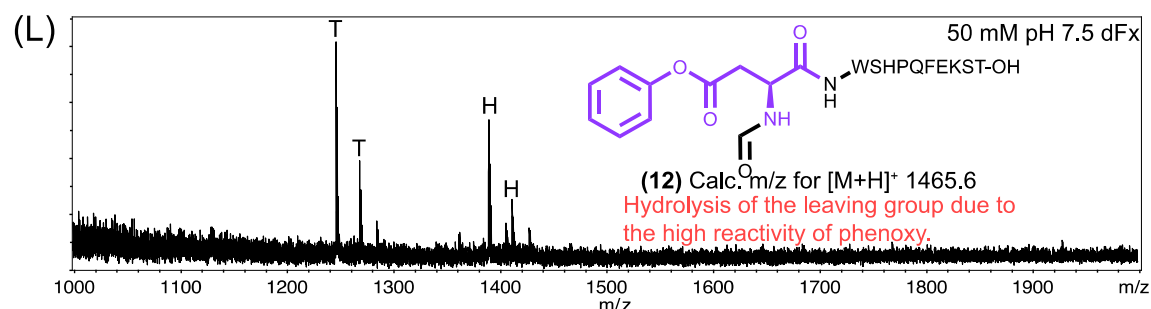
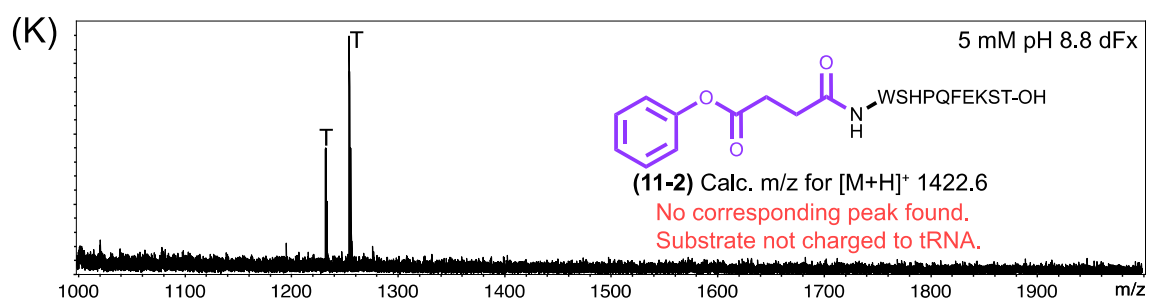


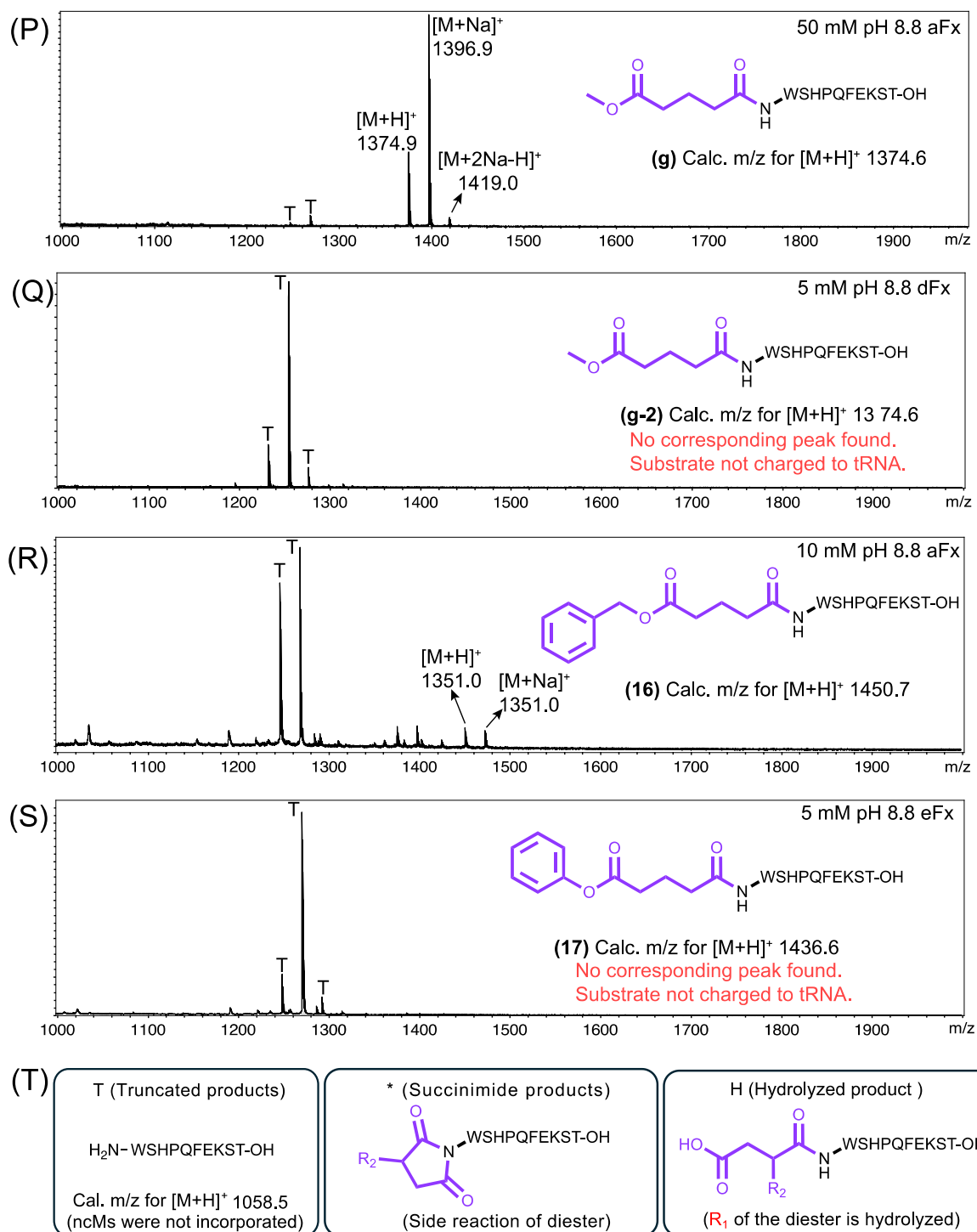
Supplementary Figure 6. Acylation of microhelix with glutarate ester

Acylation of microhelix with glutarate (**g**, **g-2**, **16** and **17**) : The Fx-catalyzed acylation reactions for 4 glutarate diester substrates were carried out with three different flexizymes (eFx, dFx, and aFx) at two different pH levels (7.5 and 8.8) over 16 h. The yield of each acylation reaction was determined by comparing the relative band intensity of unacylated and acylated mihx on the gel. The yields for all substrates could not be determined because the band shifts were unclear. Substrates that did not present clear band shifts were further validated *via* mass spectrometry. The same reaction conditions were applied to tRNA acylation with the substrate. Source data are provided as a Source Data file.





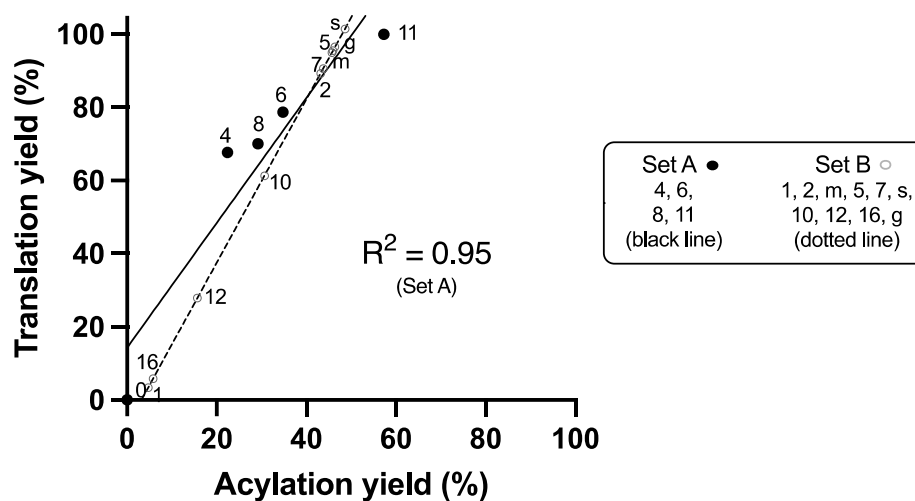




Supplementary Figure 7. Confirmation of tRNA-charging using mass spectrometry analysis

(A-S) For substrates (**m**, **m-2**, **b**, **0**, **1**, **2**, **5**, **7**, **7-2**, **10**, **11-2**, **12**, **13**, **14**, **15**, **g**, **g-2** and **17**) that did not show clear band shift on gel electrophoresis, we further monitored acylation through mass spectrometric analysis. Each substrate was charged to tRNA^{fMet}(CAU) by Fx and supplemented into a PURExpress reaction, where the substrate:tRNA^{fMet}(CAU) conjugate is incorporated at the initiating AUG codon (position X) of a reporter peptide (Strep tag, XWSHPQFEKST). Following the reaction, the peptide was purified using magnetic beads coated with strep-tactin antibody and characterized by MALDI mass spectrometry. For substrates **m-2**, **0**, **7-2**, **12**, **13**, **14**, **15**,

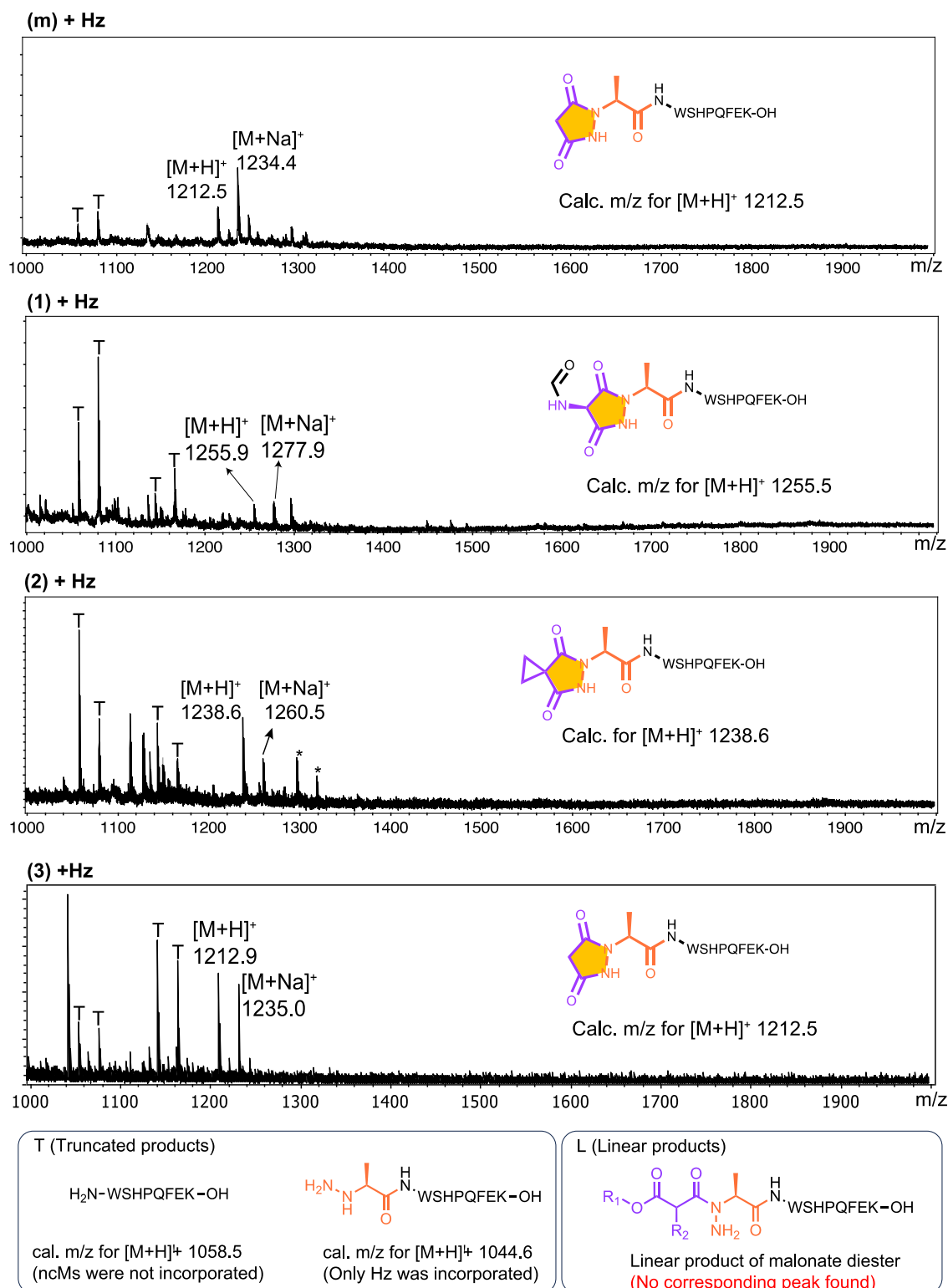
g-2 and **17** that showed no peaks corresponding to the theoretical mass of XWSHPQFEKST, we confirmed that these were not charged to tRNA by Fx. Although band shifts on gel were unclear, substrates **1**, **m**, **2**, **5**, **s**, **7**, **10**, **12** and **g**, **16** were found to be incorporated into a peptide (Supplementary Figures 2 and 3). (T) Peaks marked with **T** indicate a truncated peptide that lacks the first one or two ncMs at the N-terminus. Peaks marked with **H** indicate a peptide in which the substrate was incorporated at the N-terminus, but the ester was found to be hydrolyzed. Peaks marked as an asterisk (*) correspond to succinimide products resulting from side reactions of diesters. Source data are provided as a Source Data file.



Supplementary Figure 8. Acylation yield of ncMs estimated from translation efficiency.

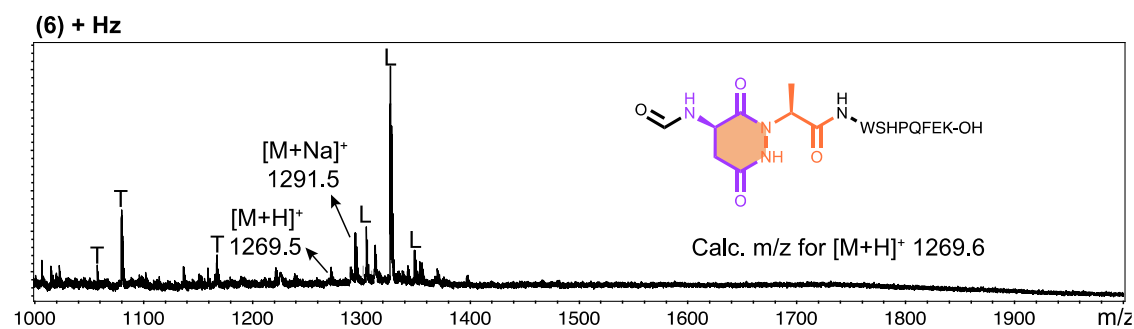
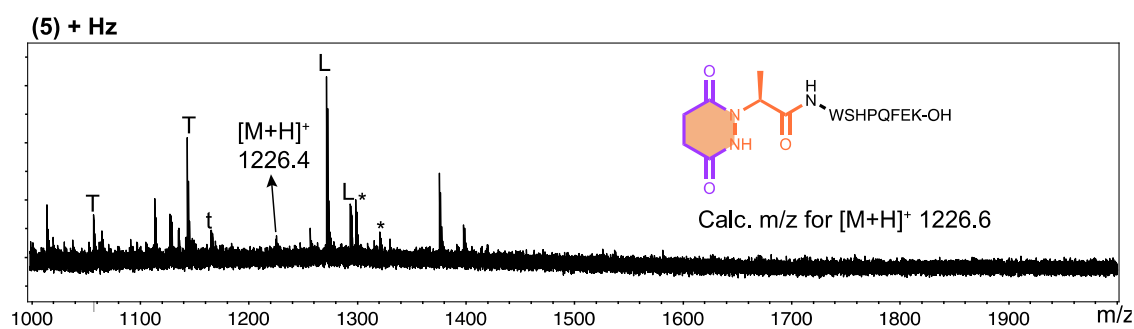
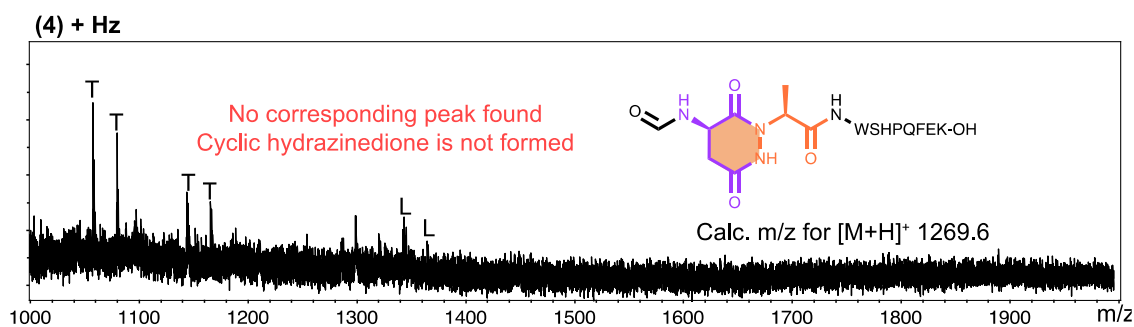
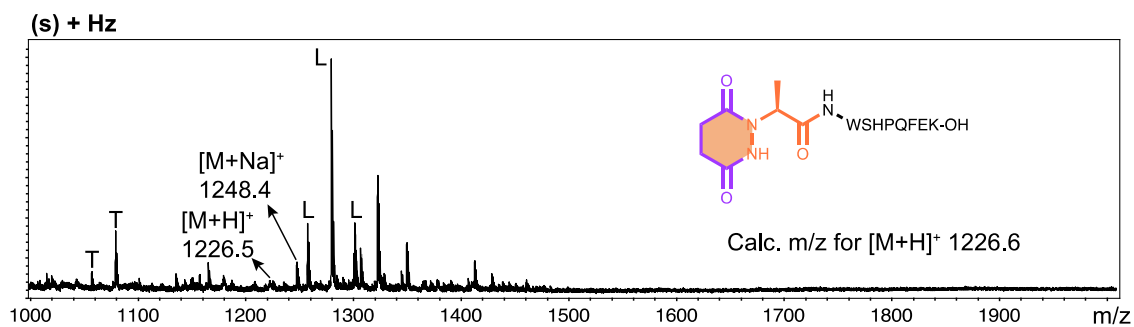
To estimate the acylation yield that did not display a clear band shift on the gel (**Supplementary Figures 4 and 5**), individual value for acylation and N-terminal incorporation efficiency of the four ncMs (**4, 6, 8, and 11**: set A, displayed with black circles) were plotted (black line) and fitted to a linear function. A linear correlation is observed between the acylation against the yield of peptide containing ncM at the N-terminus. The translation yield was determined based on the relative peak intensity. For substrates **1, 2, m, 5, 7, s, 10, 12, 16** and **g** (set B, displayed with white circles), which did not show a clear band shift on the gel, acylation yields were estimated using the plot from previous study (Nat. Commun. 2019, 10, 5097) and plotted alongside the substrates from set A (dotted line). We observed that the two correlations from the different studies were consistent, indicating that using peak intensity to determine acylation yield is a valid approach, at least in our study.

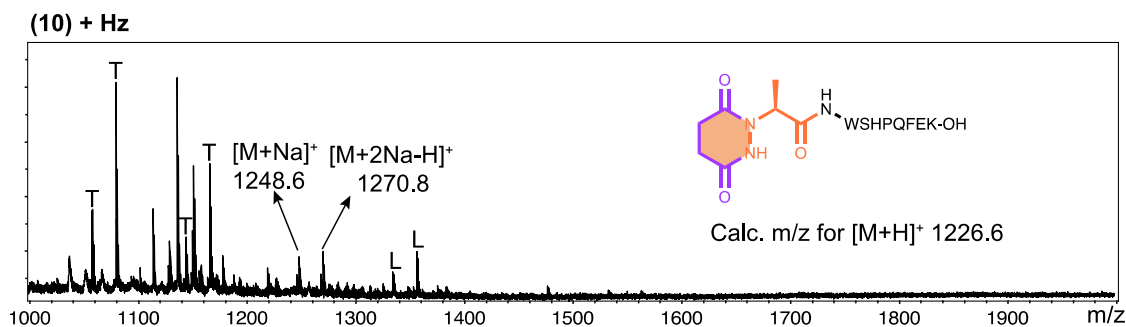
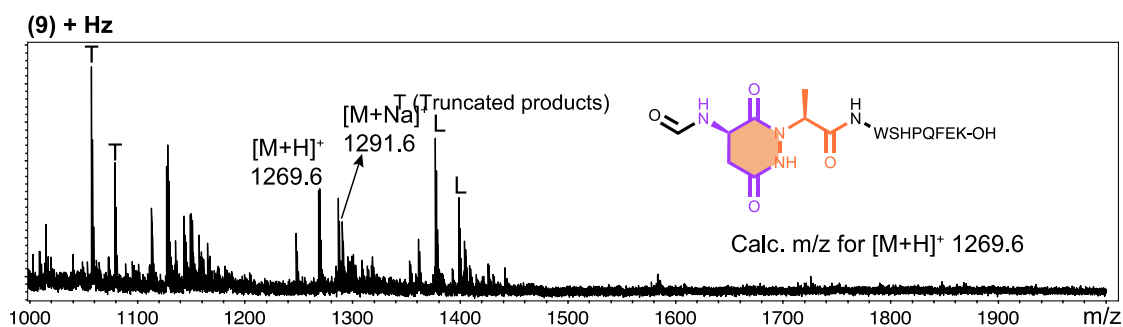
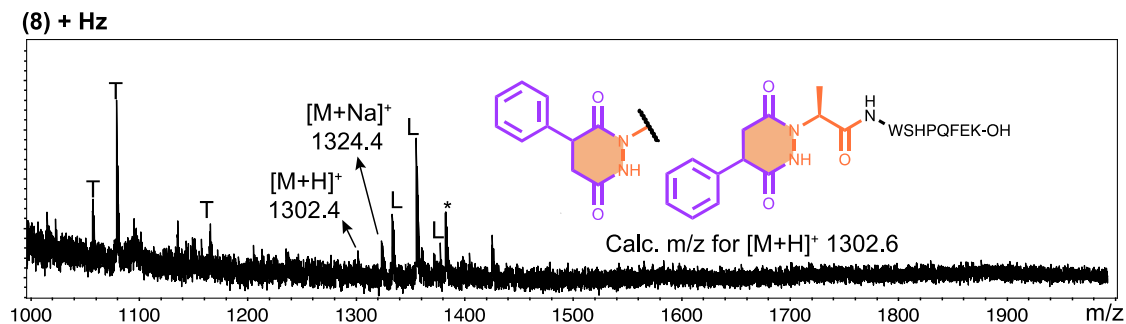
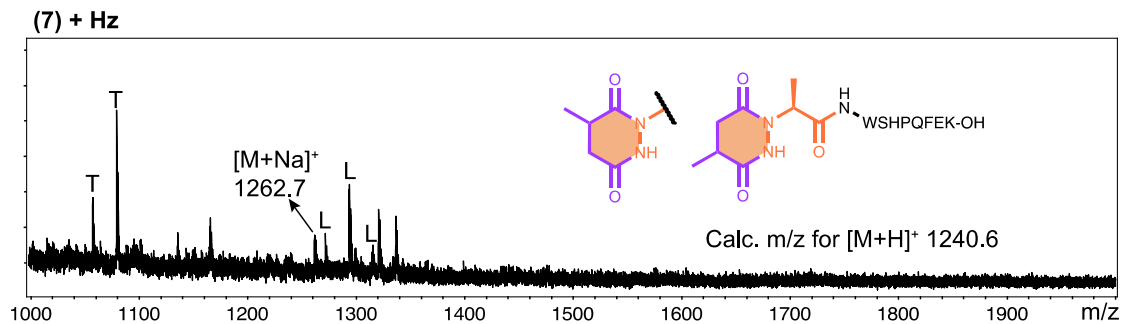
Collectively, low acylation yields tend to reduce the efficiency of N-terminal incorporation, leading to a higher frequency of ribosome's 'skipping (dropping-off)' events, which in turn increases the relative amount of truncated product.

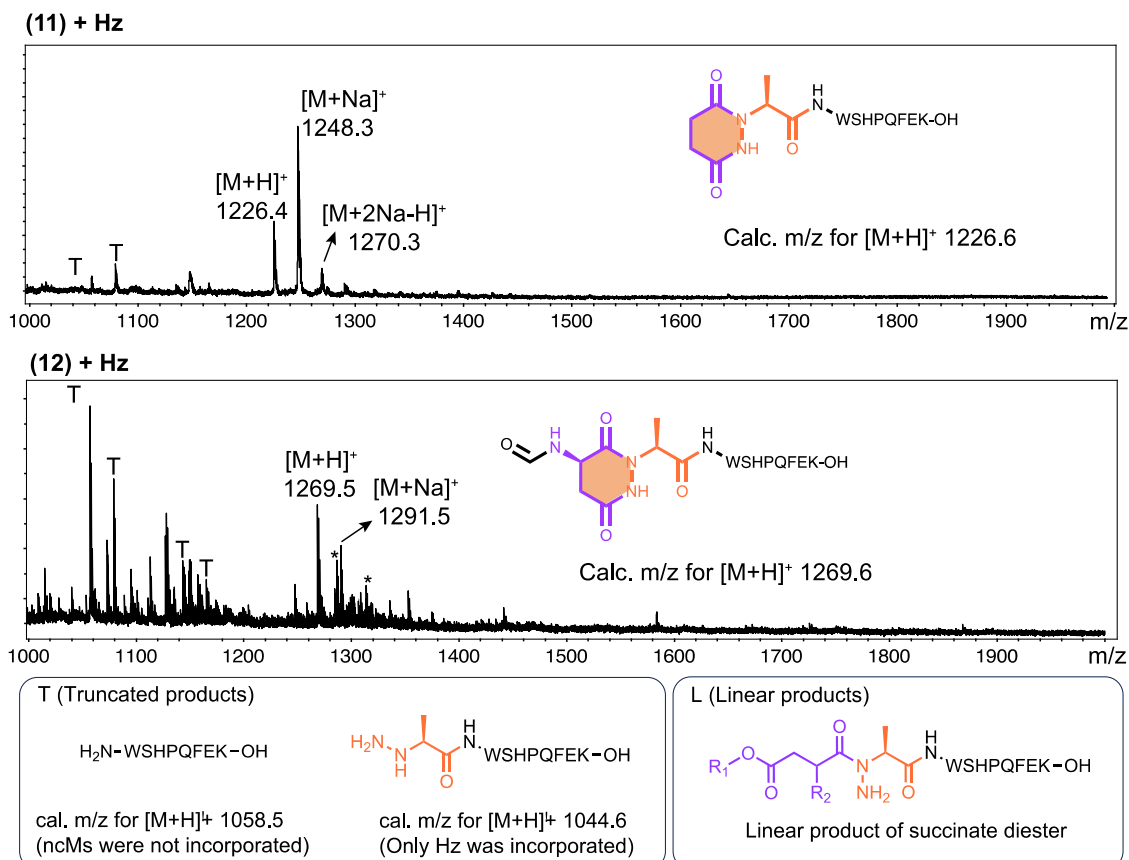


Supplementary Figure 9. Ribosome-mediated synthesis of pyrazolidinedione derivatives

Characterization of the pyrazolidinedione products. Peaks marked with an alphabet (T) indicate truncated peptides that lack the first one or two ncMs at the N-terminus. All malonate diesters, regardless of R_1 and R_2 , formed 5-membered cyclic pyrazolidinedione products. Note that the spectrum for (s) + Hz (upper panel) is reused in Figure 3C of the main text. Source data are provided as a Source Data file.

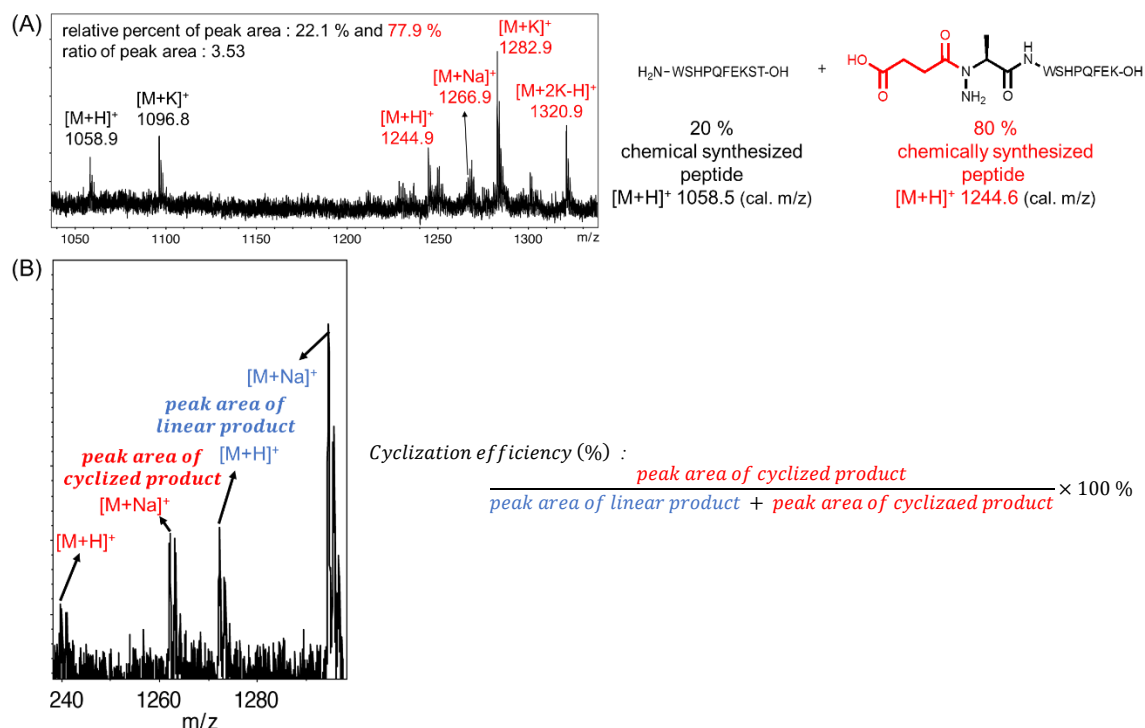






Supplementary Figure 10. Ribosome-mediated synthesis of tetrahydropyridazinedione derivatives

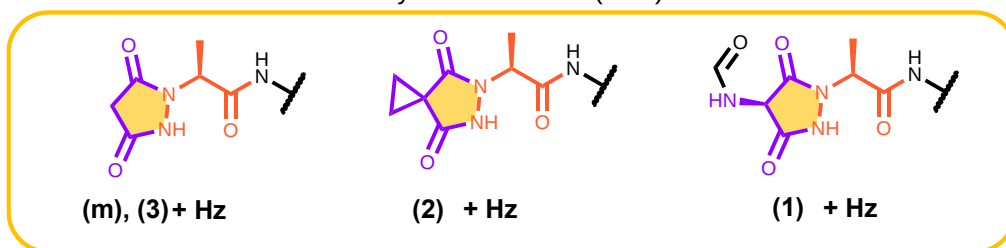
Characterization of tetrahydropyridazinedione products. Peaks marked with T indicate truncated peptides that lack the first one or two ncMs at the N-terminus. Succinate diesters, unlike malonate diesters, formed varying yields of the tetrahydropyridazinedione product depending on R_1 . If R_1 was a good leaving group, a higher yield of tetrahydropyridazinedione products was detected. Peaks marked with L indicate linear products that did not undergo the cyclization reaction to 6-membered ring. Peaks marked with an asterisk (*) correspond to the theoretical mass of the peptide containing Ile at the AUC codon. This product was formed presumably because of contaminations of Ile in the PURExpress™ $\Delta tRNA$, aa kit (NEB). Note that the spectrum for (s) + Hz on page 28 is reused in Figure 4A (lower panel) of the main text. Source data are provided as a Source Data file.



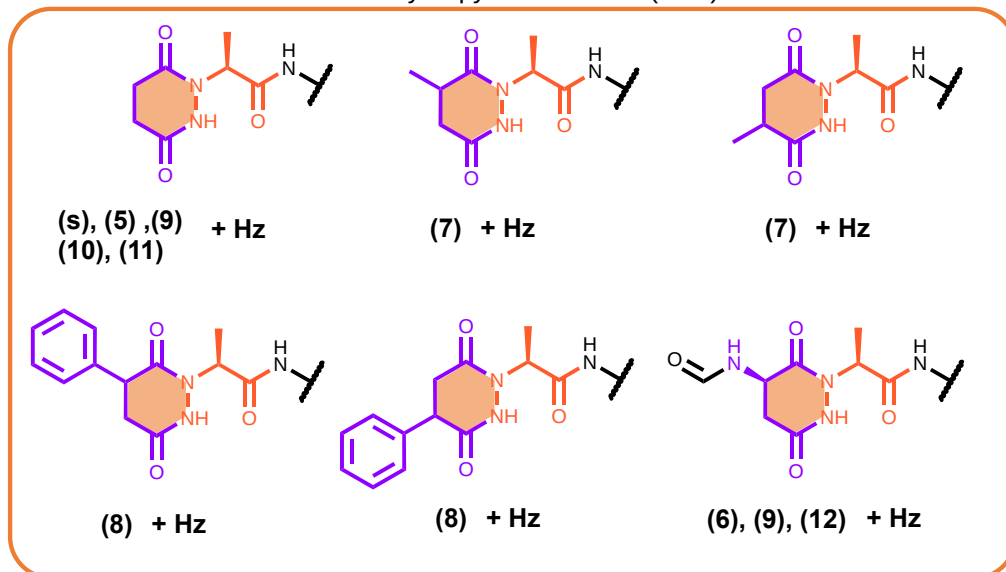
Supplementary Figure 11. Calculation of cyclization efficiency

The cyclization efficiency was determined by calculating the ratio of the sum of peak areas of cyclized products to the sum of peak areas of both linear and cyclized products. This approach is based on a previous study, which demonstrated that peptides with similar amino acid sequences are ionized and detected in proportion to their abundance in the mixture (see main text). (A) We found that the two short chemically synthesized peptides with more than 80 % of compositional similarities, showed comparable intensity on MALDI spectrum. The peptides are $\text{NH}_2\text{-WSHPQFEK-OH}$ (MW 1057.50) vs Sa-Hz-WSHPQFEK-OH (MW 1243.6), where **Sa** and **H_z** refer to succinic acid and hydrazine ester. We mixed the two peptides at a 1:4 ratio of known concentrations and analyzed them using MALDI-TOF mass spectrometry. The integration of peak area on MALDI spectrum yielded values of 22.1 % and 77.9 %, which is very similar to the ratio of sample composition. (B) Based on this analysis, we calculated the cyclization efficiency by determining the ratio of the peak areas corresponding to the mass values of the cyclized product and the linear product. Source data are provided as a Source Data file.

5-membered Pyrazolidinedione (PZD) derivatives

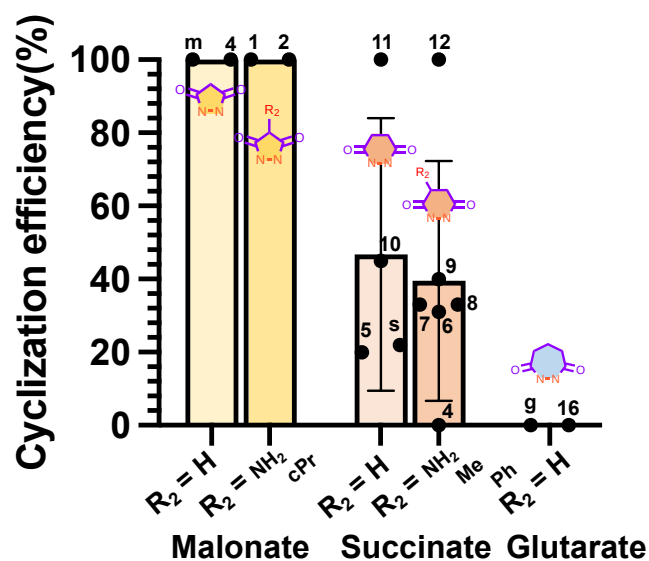


6-membered Tetrahydropyridazinedione (TPD) derivatives



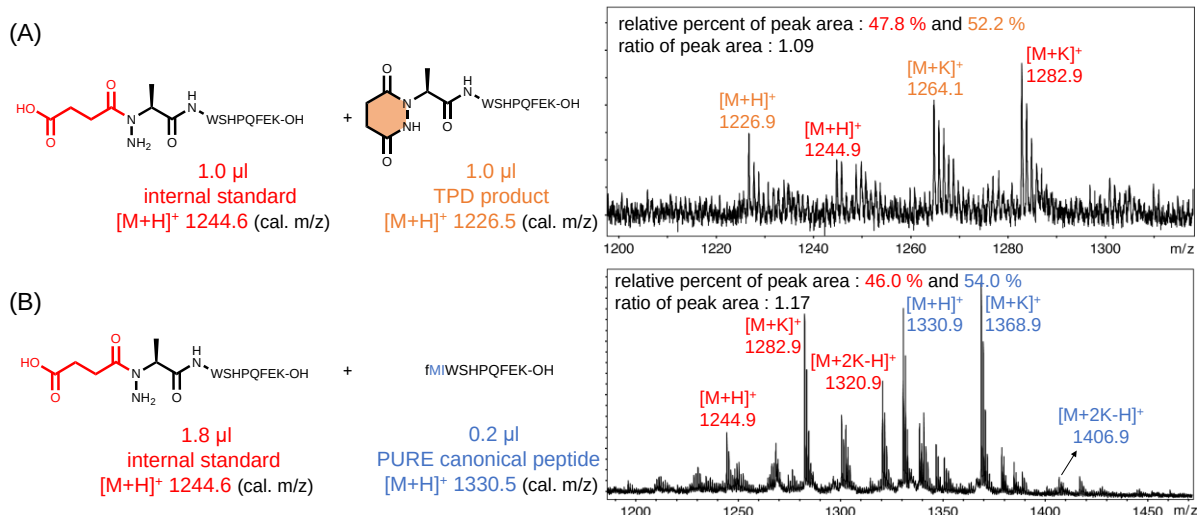
Supplementary Figure 12. Derivatives of cyclic hydrazinedione produced in this work

The genetic code reprogramming approach enabled the formation of misacylated tRNA with synthetic ncMs. By altering the substrates, diverse pyrazolidinedione and tetrahydropyridazinedione derivatives were synthesized by the ribosome in vitro. Importantly, these reactions were carried out under mild conditions in aqueous solution (2 h, 37 °C).



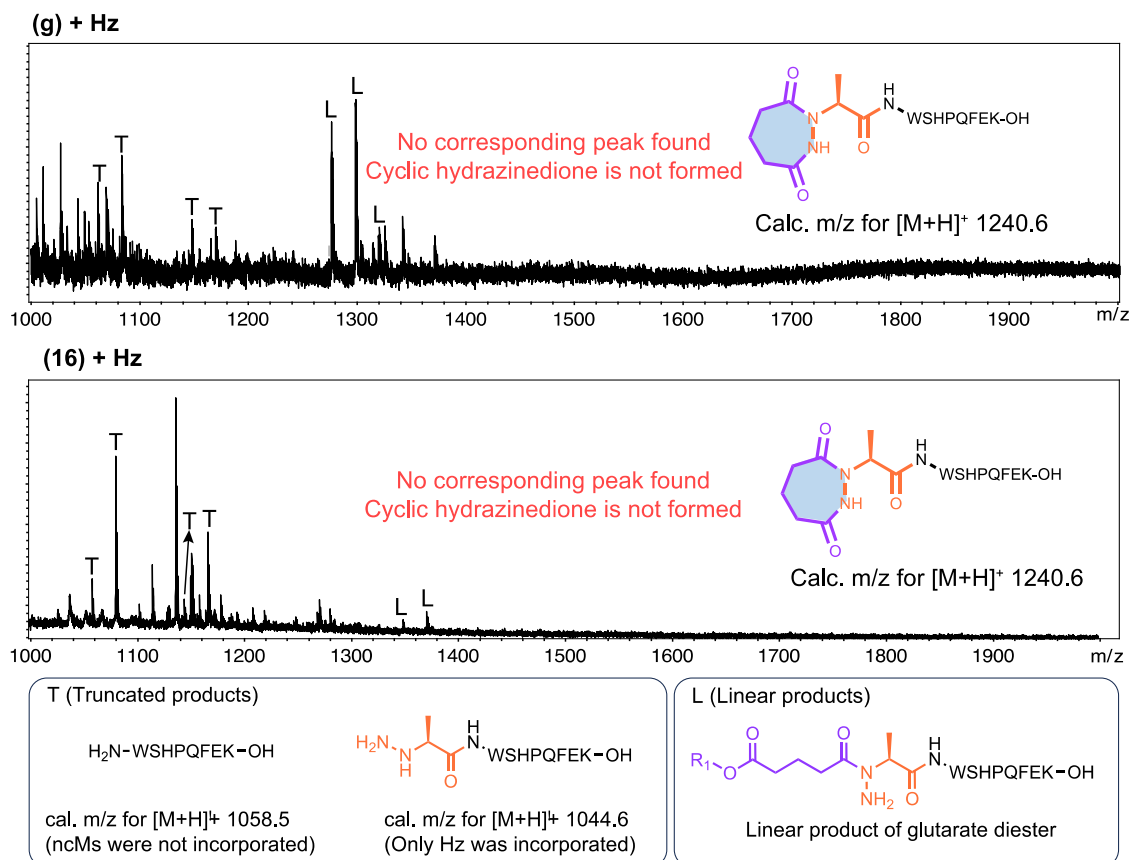
Supplementary Figure 13. Effect of R₂ groups and the length of chain on the cyclization efficiency of ribosome-mediated cyclic hydrazinedione synthesis

Variations in R₂ groups - such as methyl (Me), phenyl (Ph), amino (NH₂), cyclopropyl (cPr) - do not significantly impact cyclization efficiency. Instead, the efficiency is primarily influenced by the kinetics of the ring closure reaction, which is determined by the length of carbon chain. While the R₁ group can partly affect the kinetics of 6-membered ring formation, it has little effect on slower reactions, such as the formation of 7-membered rings. Source data are provided as a Source Data file.



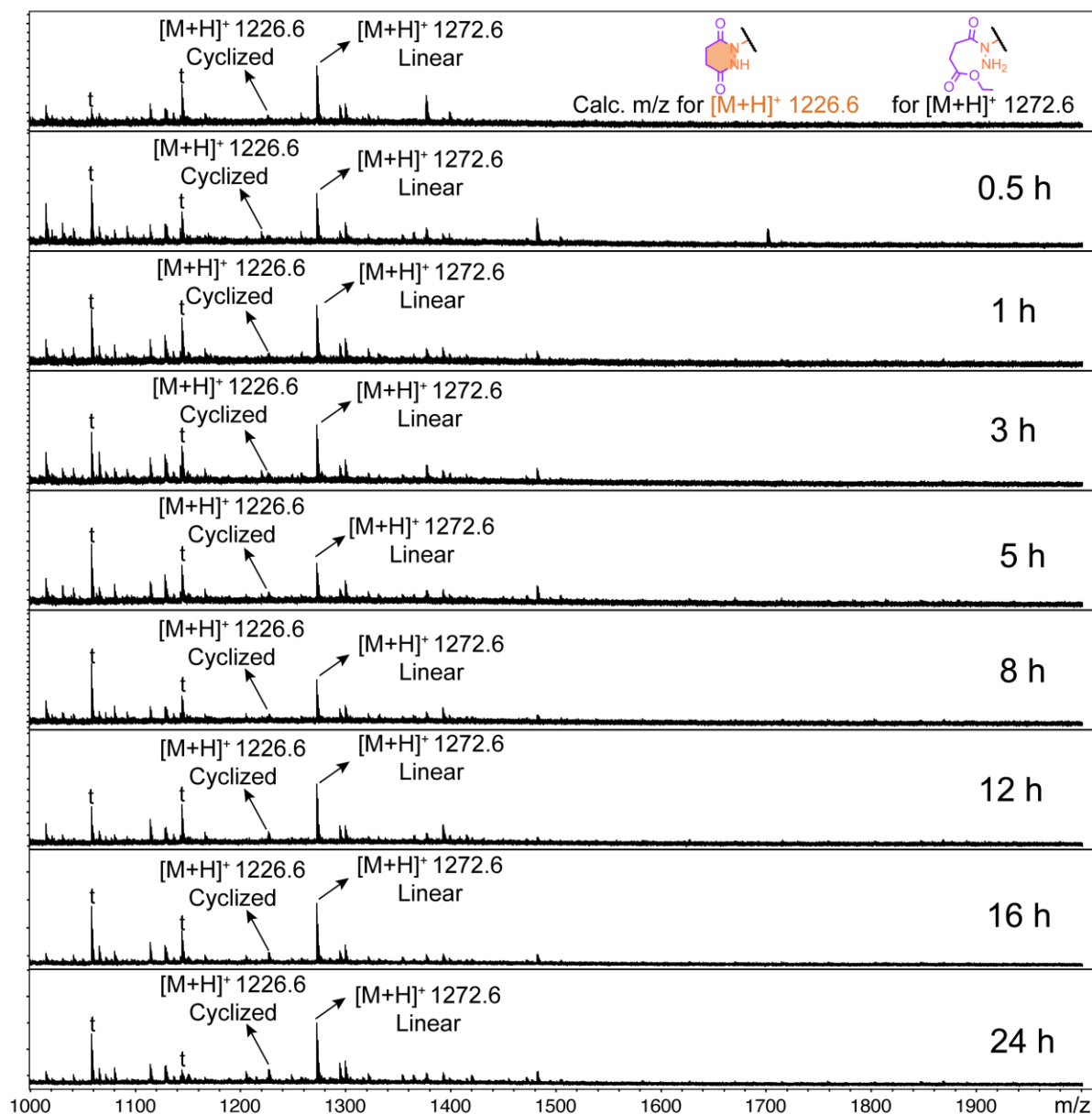
Supplementary Figure 14. Yields of peptides containing the 6-membered cyclic hydrazinedione.

(A) Based on the observations in **Supplementary Figure 10**, we calculated the yield of the TPD (tetrahydropyridazinedione)-WSHPQFEK peptide using an internal standard peptide. A comparable intensity was observed when 1 μL (700 nM) of the internal standard was mixed with an equal volume of target peptide sample. The ratio of peak areas was calculated to be 1.09, indicating that ~ 10 ng of TPD-WSHPQFEK was obtained in 15 μL of the ribosome-mediated polymerization reaction. (B) We also determined the yield of peptides containing only natural amino acids. Comparable intensity was observed when 1.8 μL (700 nM) of the internal standard was mixed with peptides produced in the PURE system sample in the presence of the 20 canonical amino acids. The ratio of peak areas was calculated to be 1.17, indicating that ~ 150 ng ($1.17 \times 700 \text{ nM} \times 1.8 \mu\text{L} \times (1/0.2 \mu\text{L}) \times 15 \mu\text{L} \times 1330 \text{ g/mol}$) of MIWSHPQFEK was obtained in 15 μL of the PURE reaction. This suggests that the ribosome synthesizes peptides containing ncMs at the N-terminus (A) with 15-fold lower efficiency than peptides containing natural amino acids. Source data are provided as a Source Data file.



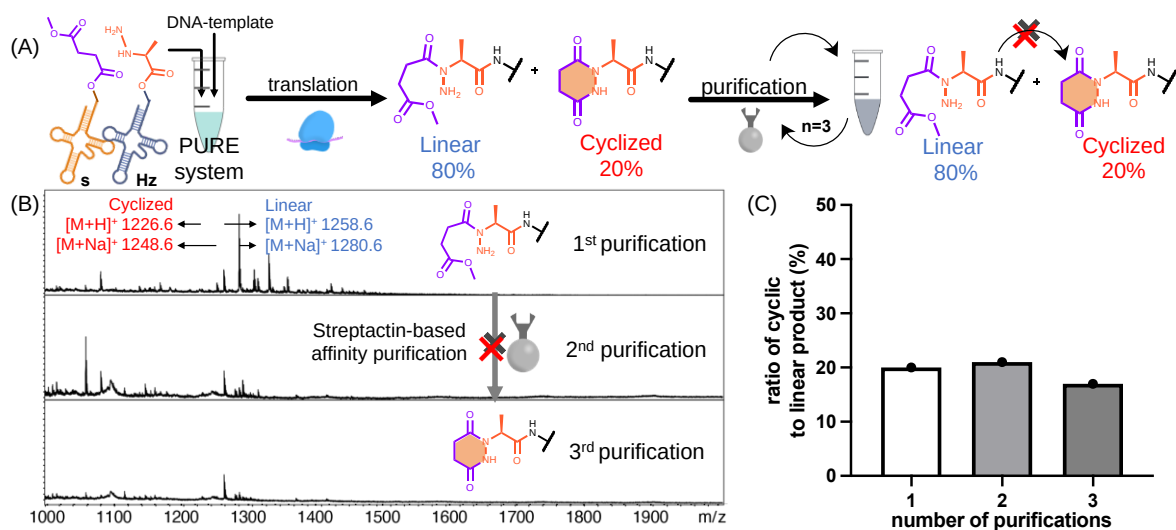
Supplementary Figure 15. Ribosome-mediated synthesis of diazepanedione derivatives

Characterization of diazepanedione products. Peaks marked with T indicate truncated peptides lacking the first one or two ncMs at the N-terminus. Unlike malonate and succinate diesters, glutarate diesters, were only found to form a linear product (L), rather than a 7-membered cyclic diazepanedione product. Source data are provided as a Source Data file.



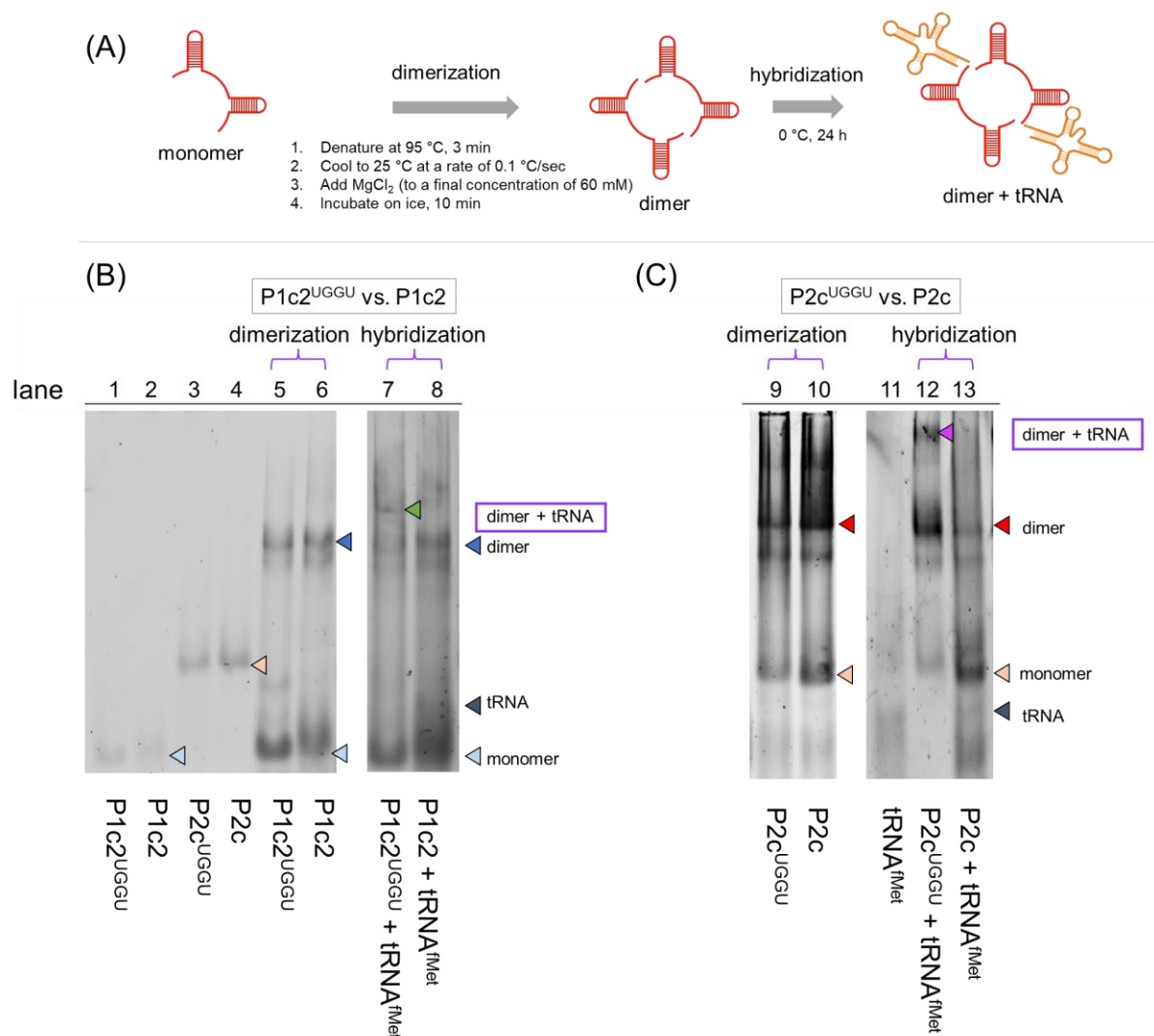
Supplementary Figure 16. Incubation of linear peptide

The full set of MALDI spectra used in Figure 5A in the main text. The linear product bearing ncMs was incubated at 37 °C in Tris-HCl (pH 8.0) buffer and characterized using MALDI-TOF at 0.5, 1, 3, 5, 8, 12, 16 and 24 h. There was no significant variation in cyclization efficiency over time, indicating that self-cyclization did not occur under the experimental conditions in solution. Source data are provided as a Source Data file.



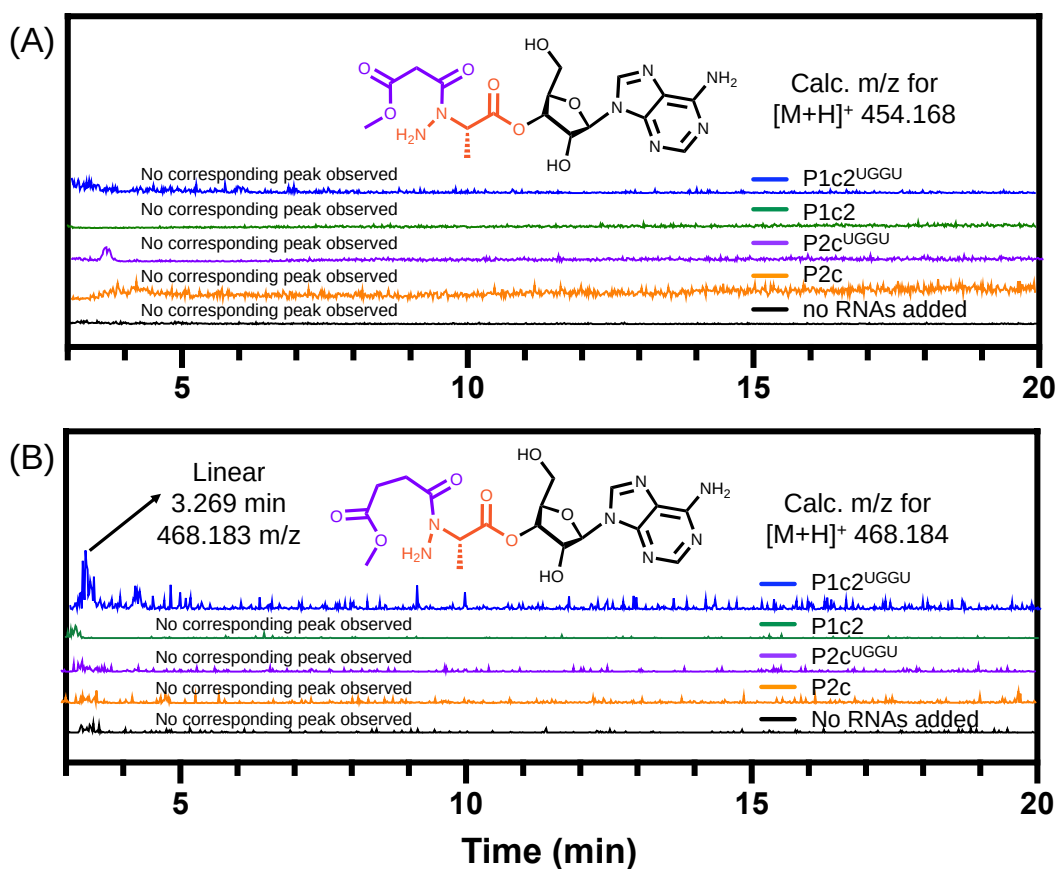
Supplementary Figure 17. Purification of linear peptide

Due to the possibility of self-cyclization during the purification process, the purification steps for the linear peptide containing ncMs were repeated. Then, linear peptide was characterized using MALDI-TOF. There was no significant variation in cyclization efficiency. The iterative (n=3) purification step requiring an antibody and heat does not affect the yield of cyclic product. Source data are provided as a Source Data file.



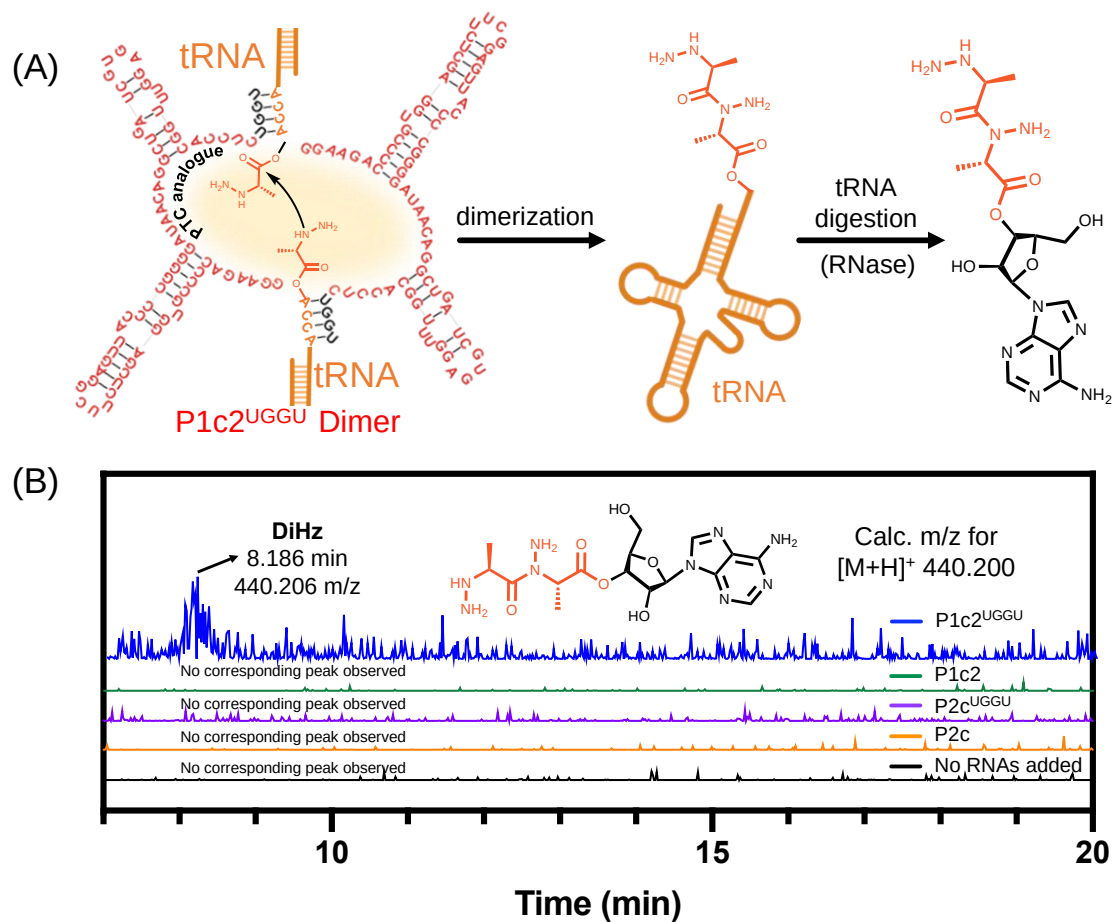
Supplementary Figure 18. Dimerization of PTC analogues (P1c2^{UGGU}, P1c2, P2c^{UGGU}, and Pc2) and their hybridization with tRNAs.

(A) Schematic representation of PTC analogue dimerization and subsequent hybridization with tRNA. We examined the ability of four PTC analogues [P1c2^{UGGU} and P1c2 (marked with a light blue arrow on lanes 1-2) and P2c^{UGGU} and Pc2 (marked with a light orange; lanes 3-4)] to self-dimerize and hybridize with tRNA. (B-C) Consistent with previous findings (*Nucleic Acids Research*, 2022, Vol. 50, No. 4 1815-1828), each monomer produced a new construct likely indicating a dimeric product (lanes 5-6: dark blue; lanes 9-10: red) following the self-dimerization reaction. After subsequent incubation of this dimeric product with tRNA containing the 3'-ACCA sequence (lane 11; dark gray), we observed that P1c2^{UGGU} and P2c^{UGGU} each produced an additional band (lane 7; green, lane 12; pink, respectively), likely indicating a dimer hybridized with tRNA. In contrast, P1c2 and P2c (lanes 8 and 13), which lack the pairing sequence for tRNA hybridization did not produce this band. The faint tRNA band in lanes 7 and 12 suggests that most of the tRNA was utilized for hybridization. Densitometric analysis of the hybridized bands (green and pink) indicates that ~8 and 12 μM of the PTC:tRNA hybridized constructs were used in the reaction for cyclic product production, respectively. Source data are provided as a Source Data file.



Supplementary Figure 19. Monitoring linear product formation through ribosome's catalytic domain analogues (P1c2^{UGGU}, P1c2, P2c^{UGGU}, and P2c)

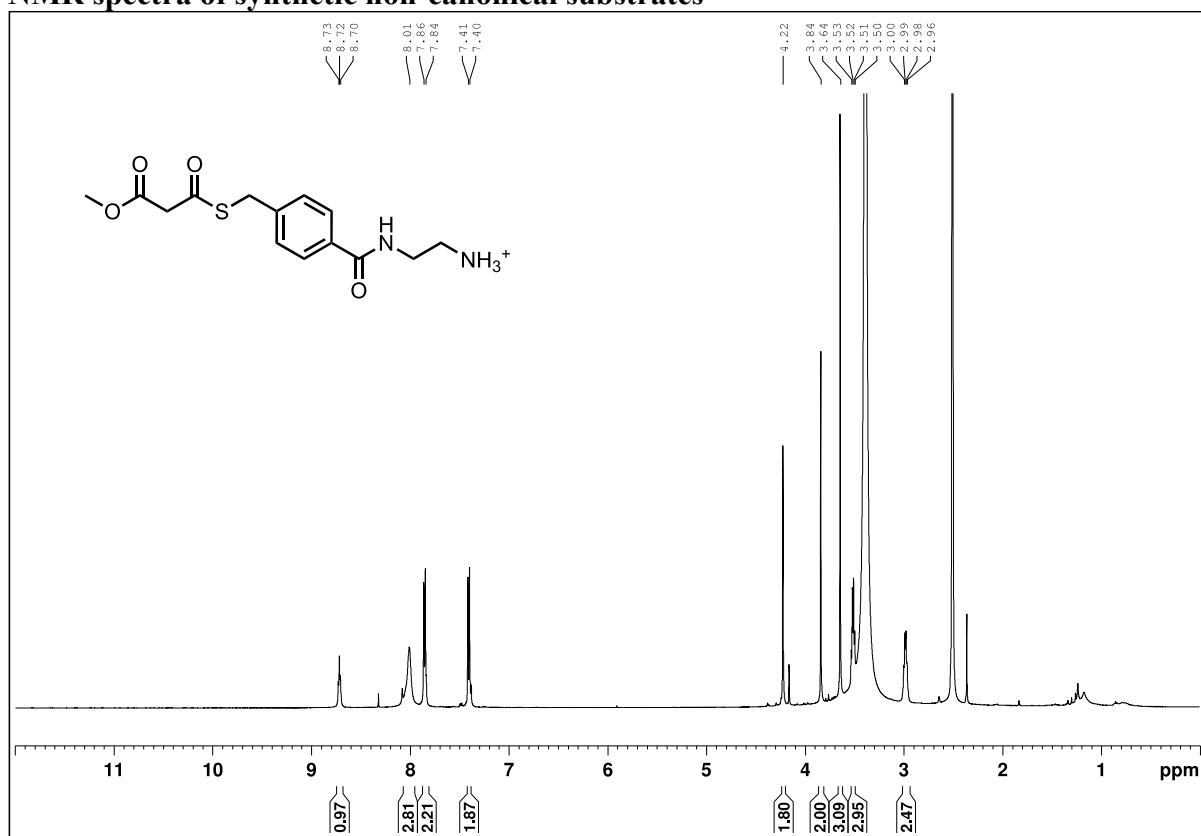
We used extracted ion chromatograms to analyze the formation of linear products within the ribosome's catalytic domain analogues (P1c2^{UGGU}, P1c2, P2c^{UGGU}, and P2c) for the reaction of malonate ester (**m**) and succinate ester (**s**) with hydrazine ester (**H_z**). (A) Consistent with the reactions of **m** and **H_z** with the full ribosome shown in Figure 3C, no linear product linked to the 3'-OH of adenosine was detected (blue). (B) Similarly, consistent with the reactions of **s** and **H_z** with the full ribosome shown in Figure 3D yielding a linear product, a single peak at 3.269 min corresponding to the theoretical mass of a linear product linked to the 3'-OH of adenosine was detected ($[M+H]^+ = 468.184$ (cal), $[M+H]^+ = 468.189$ (obs)). No such peaks were detected in any of the control experiments (green, orange, purple, and black). These findings suggest that P1c2^{UGGU} exhibits biochemical properties comparable to those of the full ribosome, reinforcing its catalytic similarity in facilitating specific reactions. Source data are provided as a Source Data file.



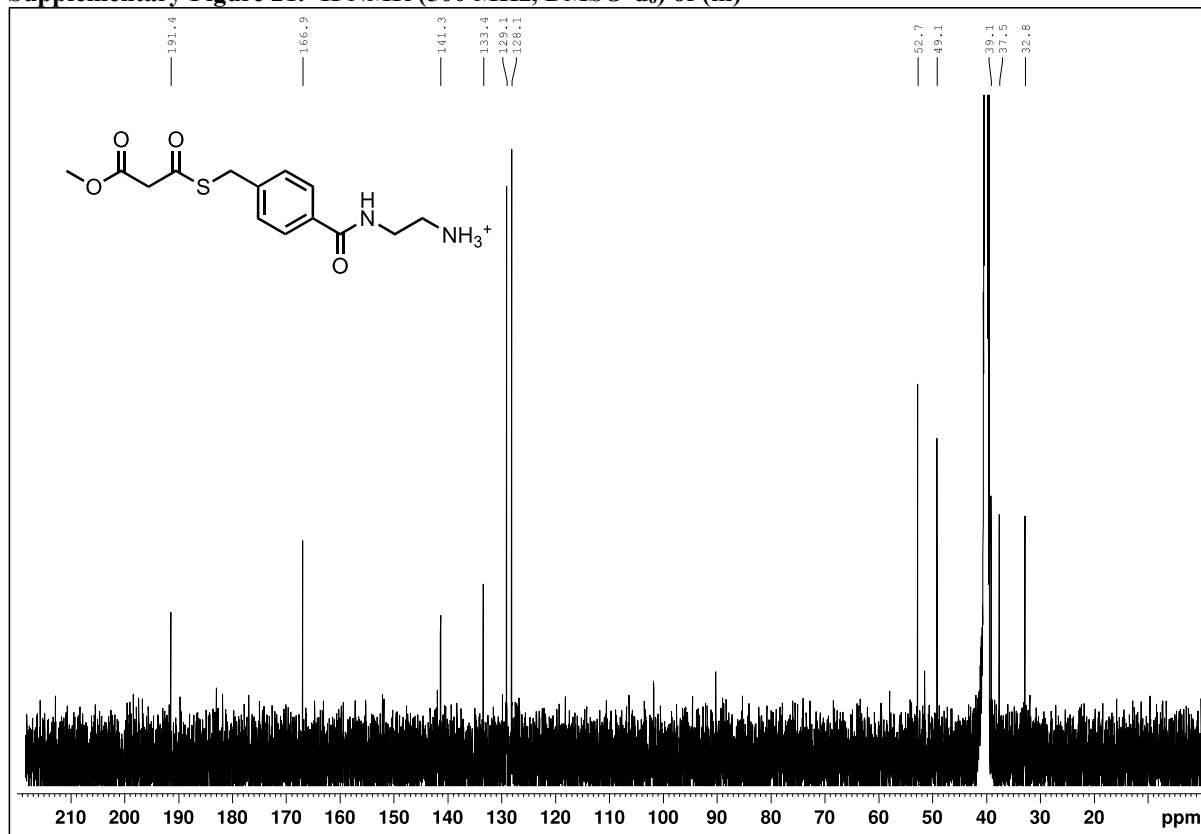
Supplementary Figure 20. Dihydrazinealanine formation through P1c2^{UGGU}

(A) P1c2^{UGGU} facilitates the dimerization of two tRNA-charged substrates. In the absence of a substrate order defined by mRNA, hydrazino alanine (Hz) reacts with itself, resulting in the formation of a dimer (DiHz). (B) The extracted ion chromatograms show a peak at 8.186 min corresponding to the theoretical mass of a DiHz linked to the 3'-OH of adenosine. $[M+H]^+ = 440.200$ (cal), $[M+H]^+ = 440.206$ (obs) exclusively in the presence of P1c2^{UGGU}. No peak corresponding to the theoretical mass was observed in reactions containing non-functional ribosome catalytic domain (P1c2, P2c^{UGGU}, and P2c) or in the absence of catalytic RNA fragments. This finding confirms that P1c2^{UGGU} (blue line) possesses the ability to catalyze the peptide bond formation. Source data are provided as a Source Data file.

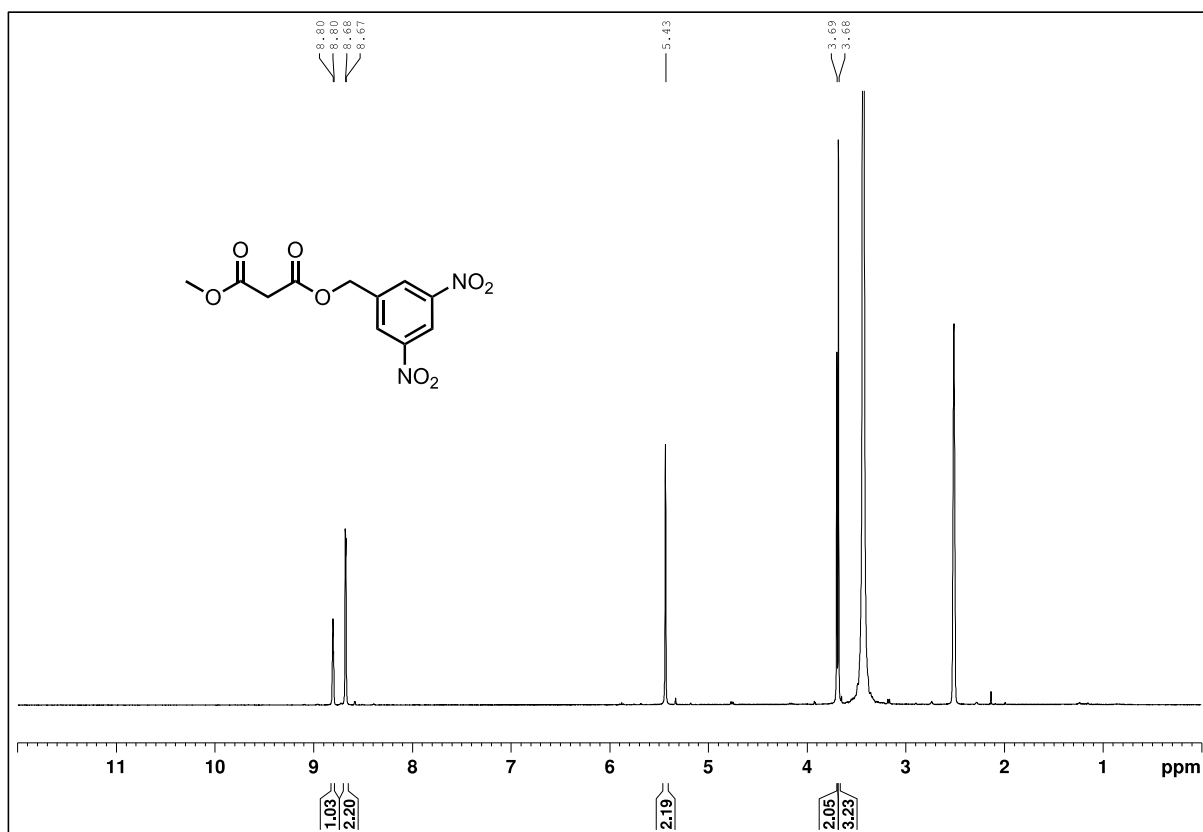
NMR spectra of synthetic non-canonical substrates



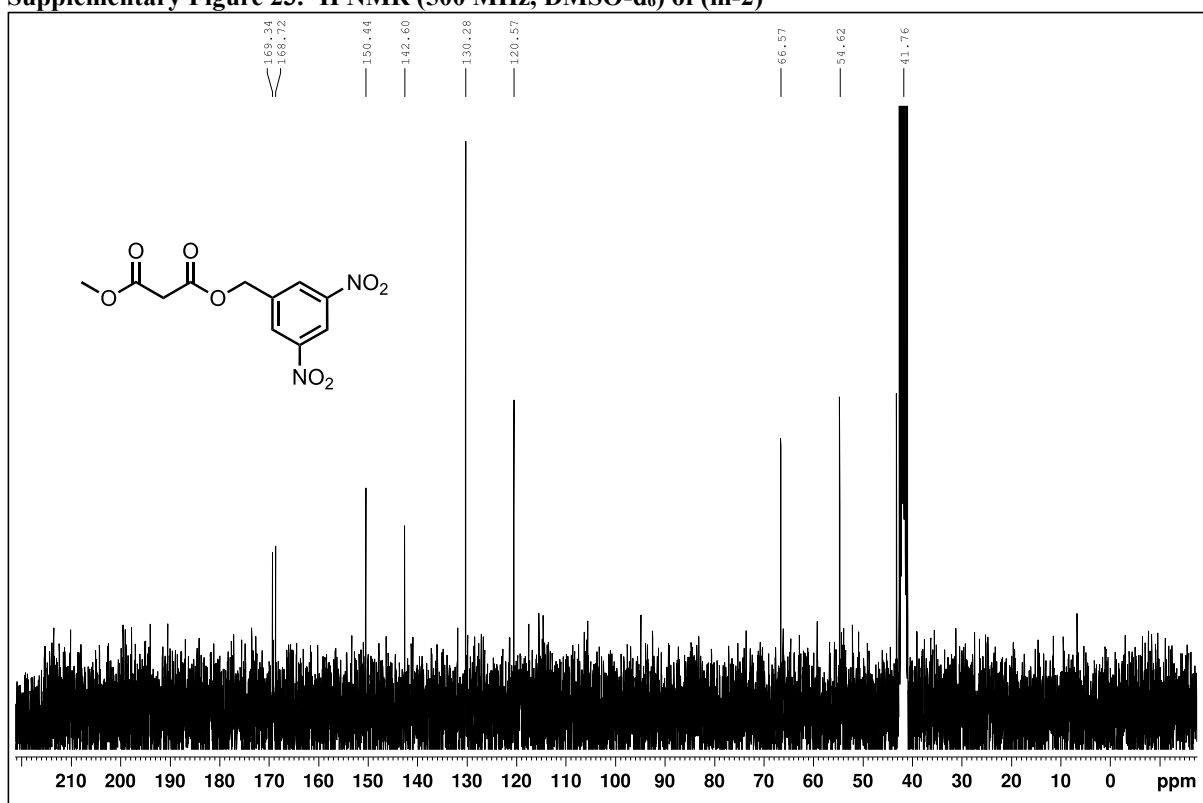
Supplementary Figure 21. ¹H NMR (500 MHz, DMSO-d₆) of (m)



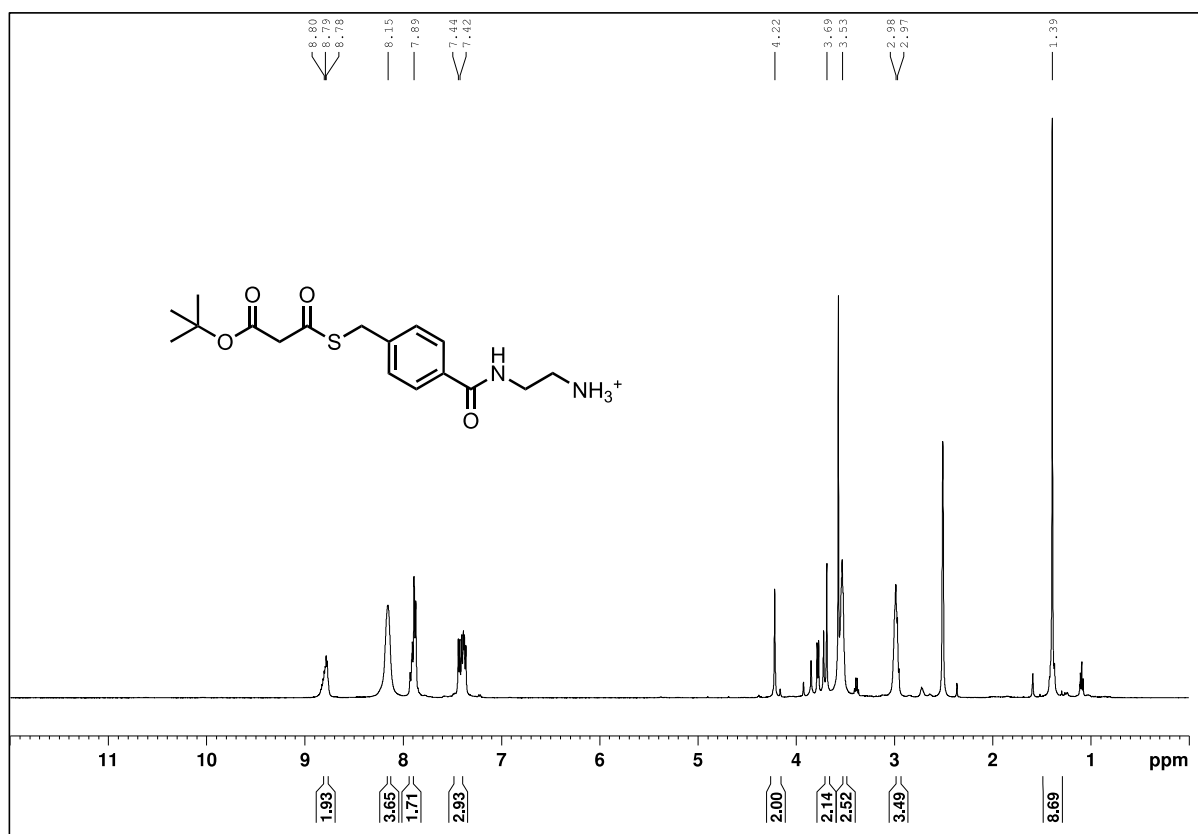
Supplementary Figure 22. ¹³C NMR (126 MHz, DMSO-d₆) of (m)



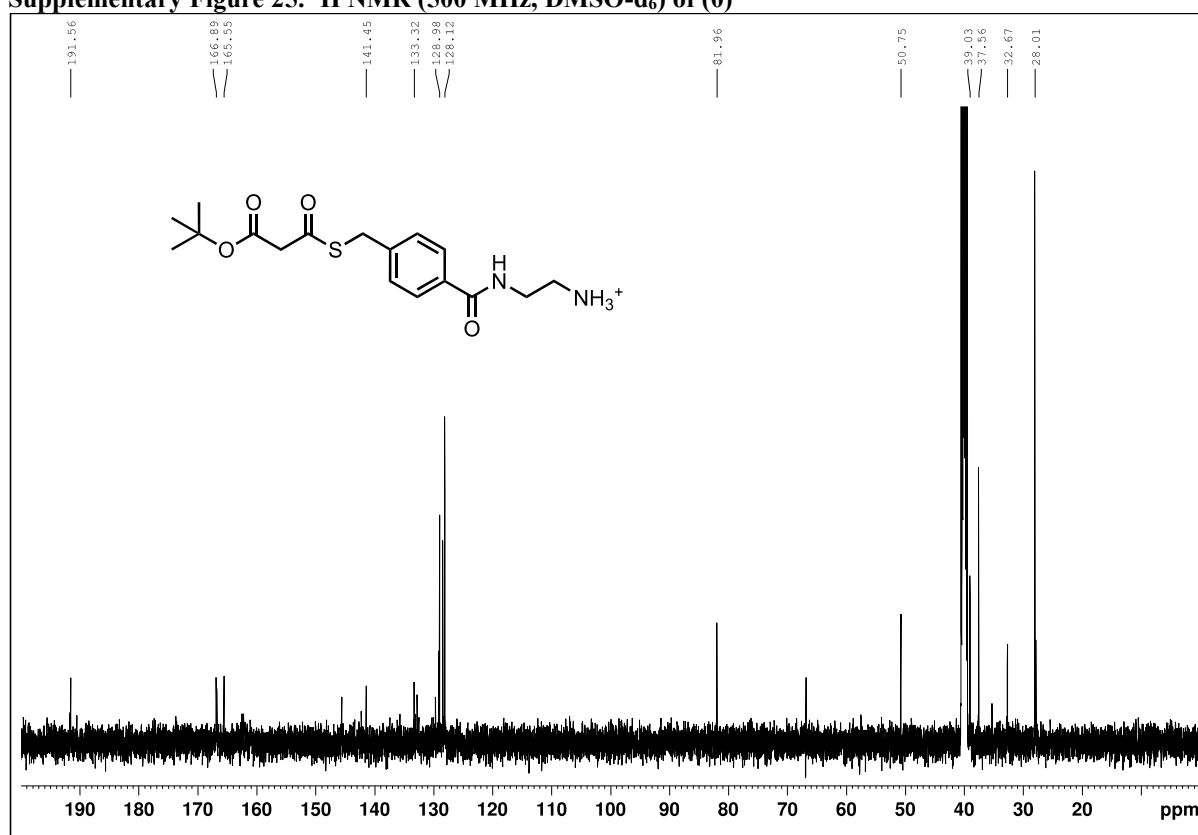
Supplementary Figure 23. ^1H NMR (500 MHz, DMSO-d_6) of (m-2)



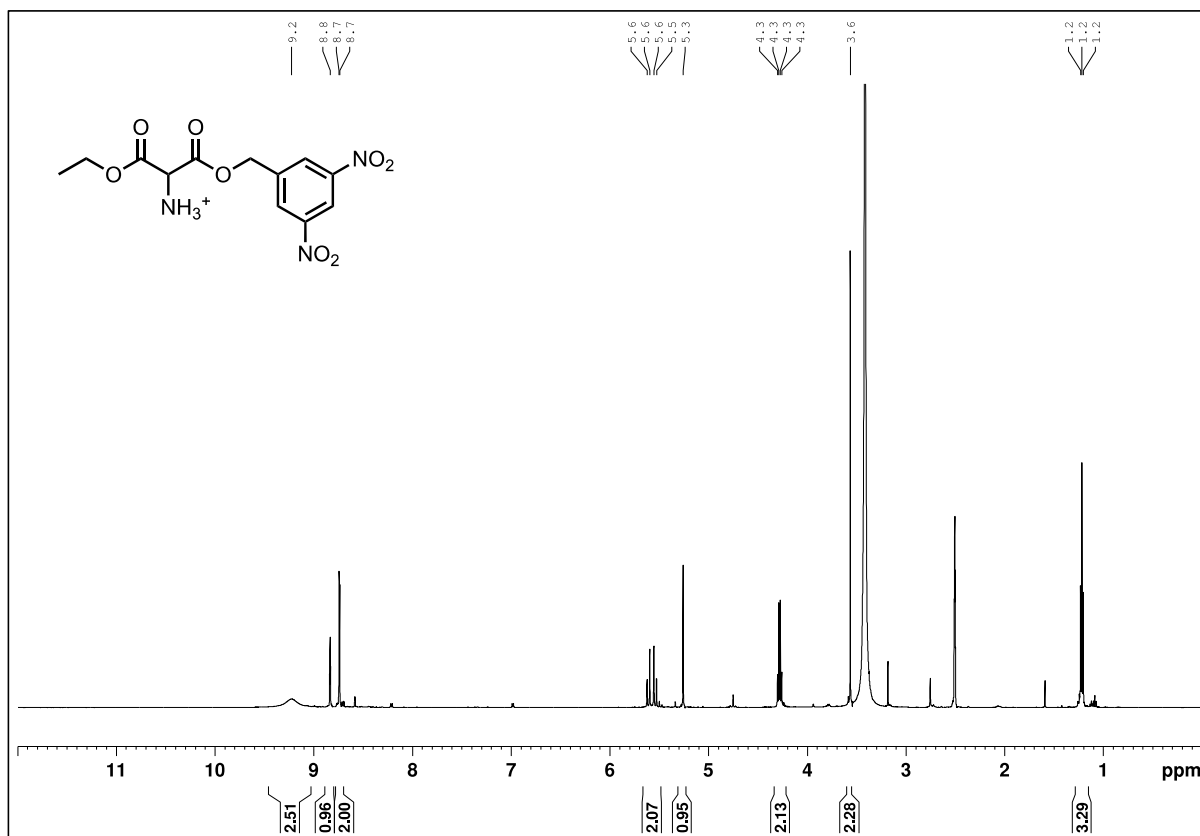
Supplementary Figure 24. ^{13}C NMR (126 MHz, DMSO-d_6) of (m-2)



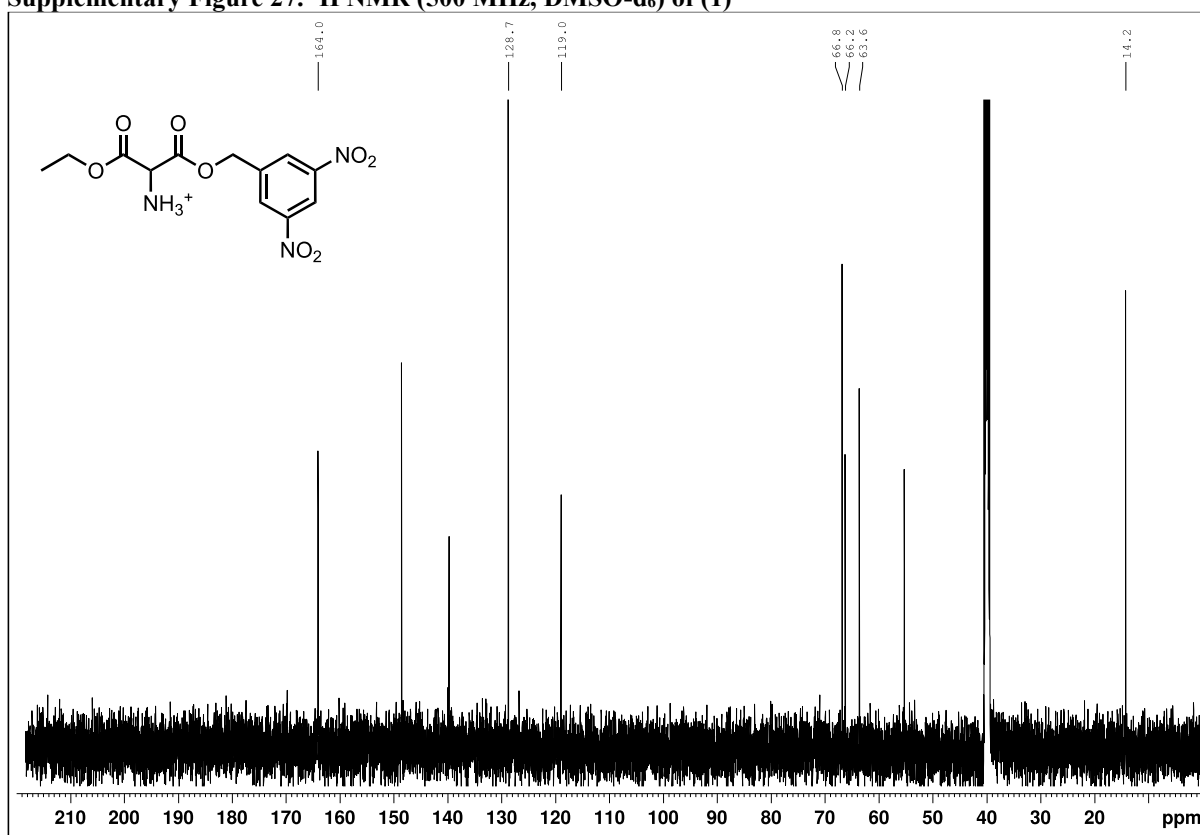
Supplementary Figure 25. ¹H NMR (500 MHz, DMSO-d₆) of (0)



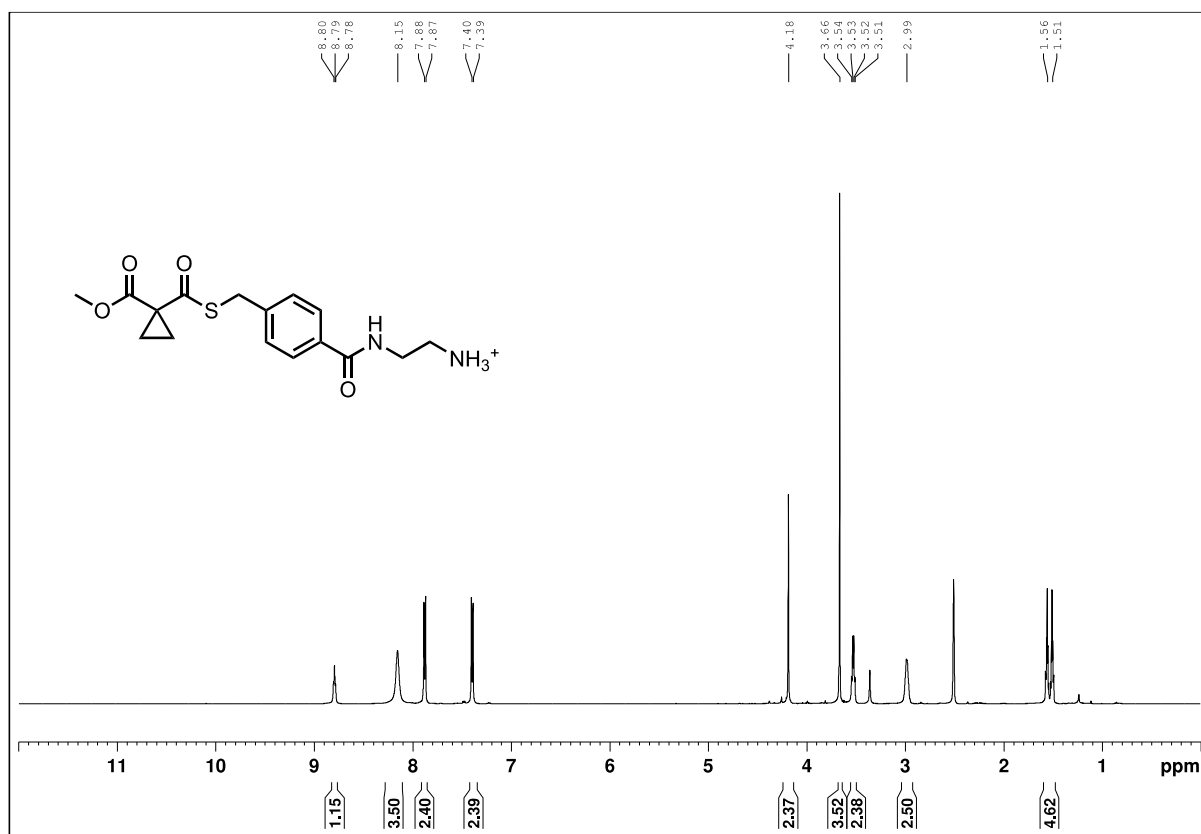
Supplementary Figure 26. ¹³C NMR (126 MHz, DMSO-d₆) of (0)



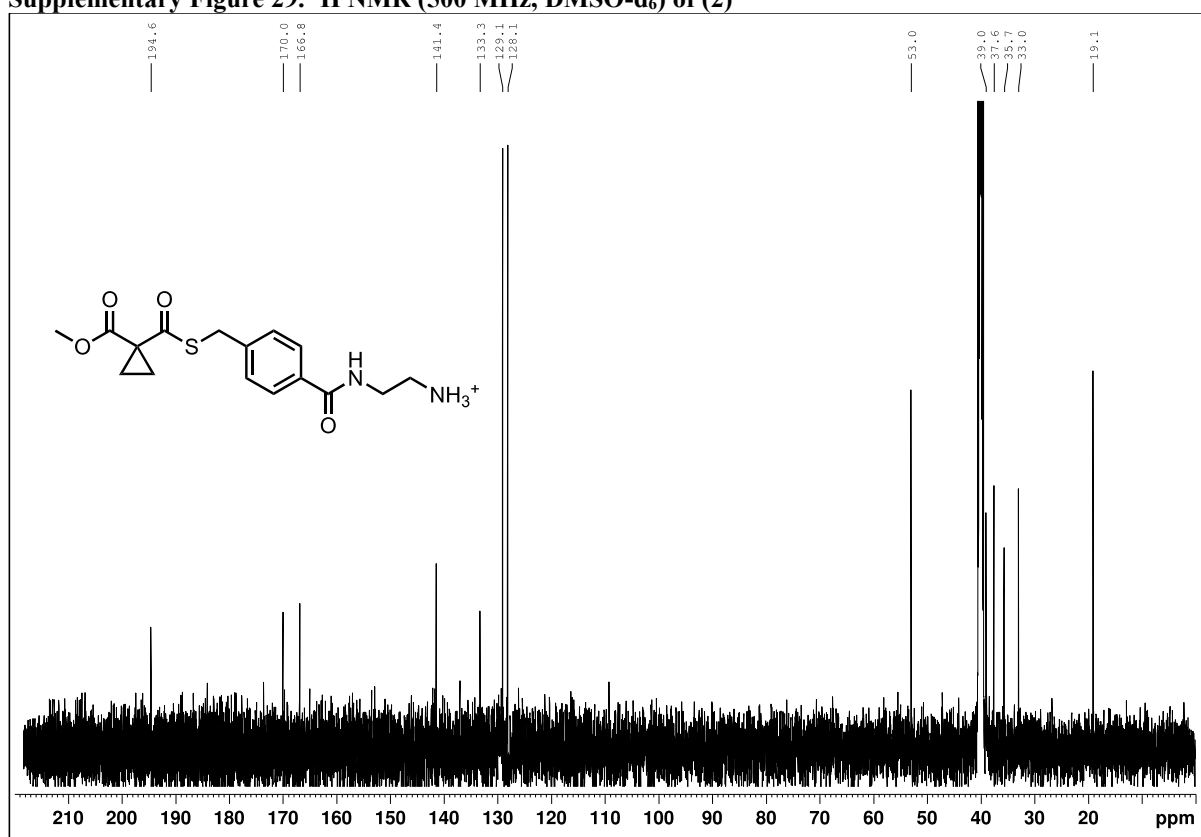
Supplementary Figure 27. ¹H NMR (500 MHz, DMSO-d₆) of (1)



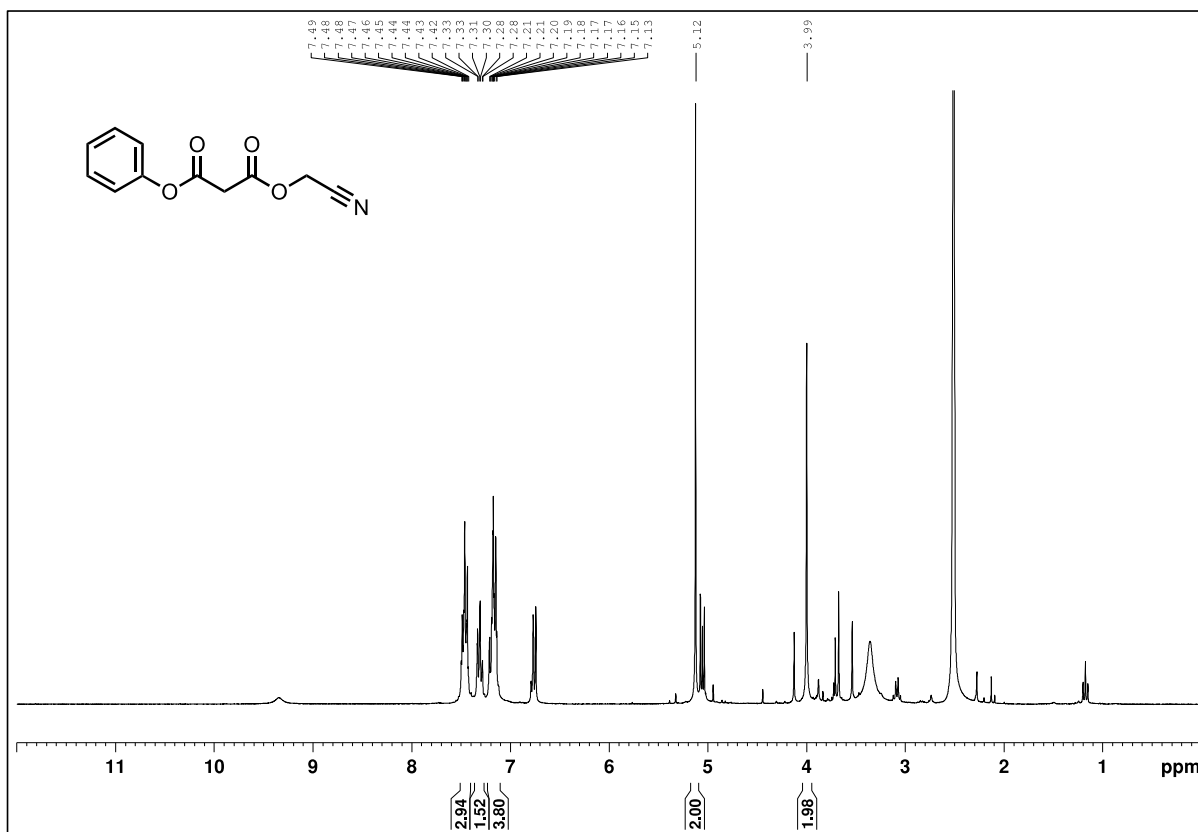
Supplementary Figure 28. ¹³C NMR (126 MHz, DMSO-d₆) of (1)



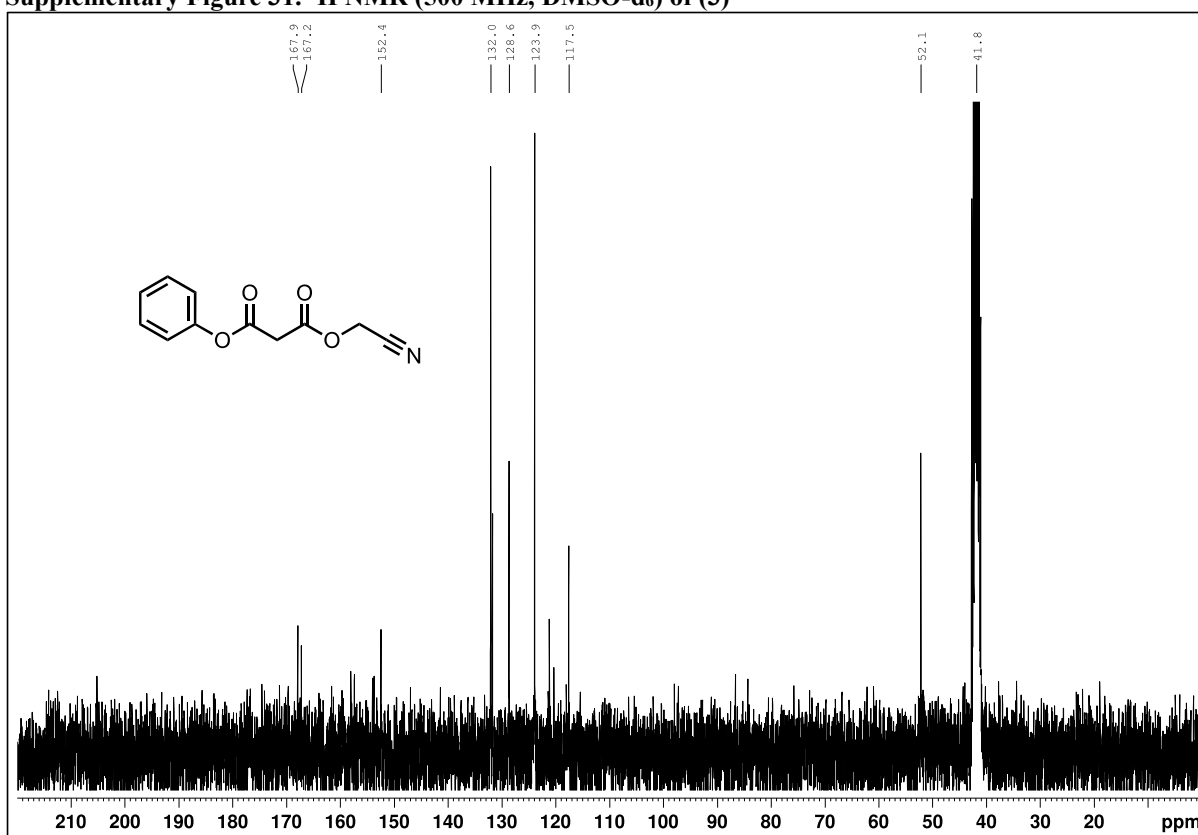
Supplementary Figure 29. ¹H NMR (500 MHz, DMSO-d₆) of (2)



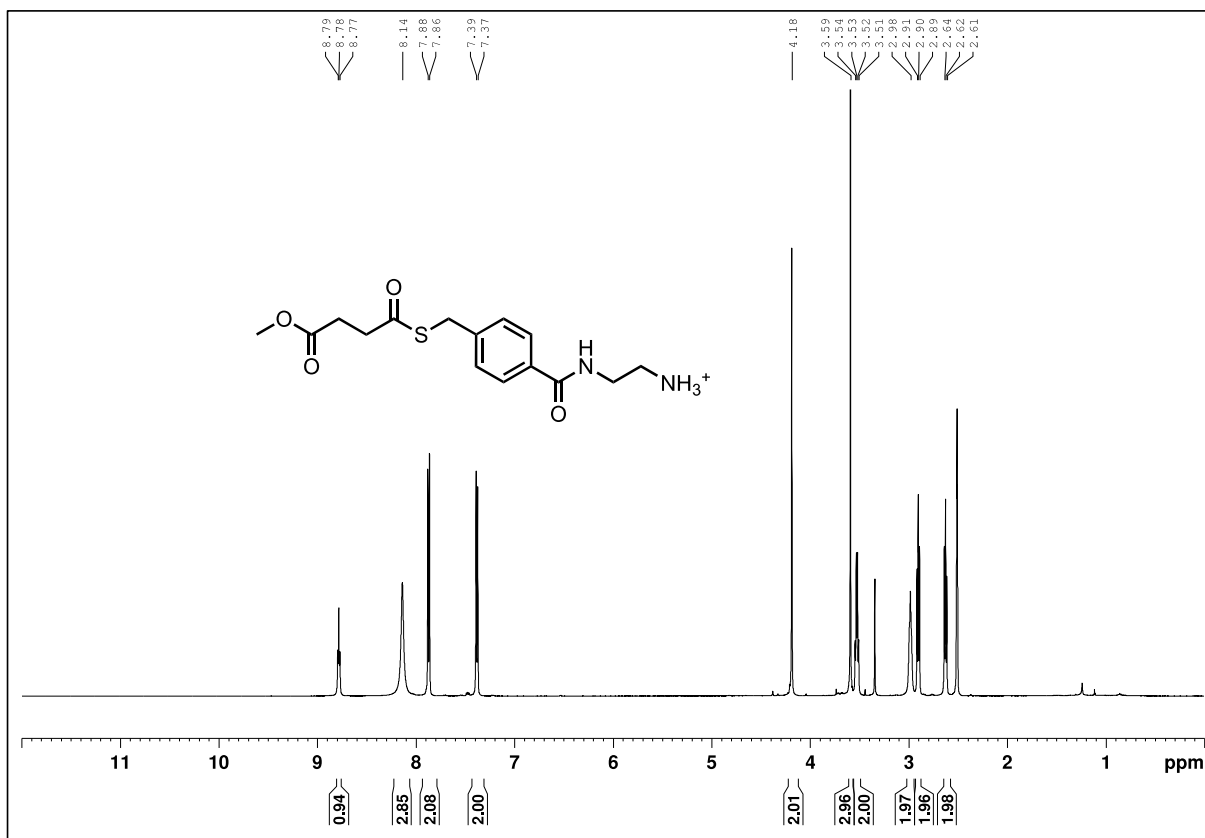
Supplementary Figure 30. ¹³C NMR (126 MHz, DMSO-d₆) of (2)



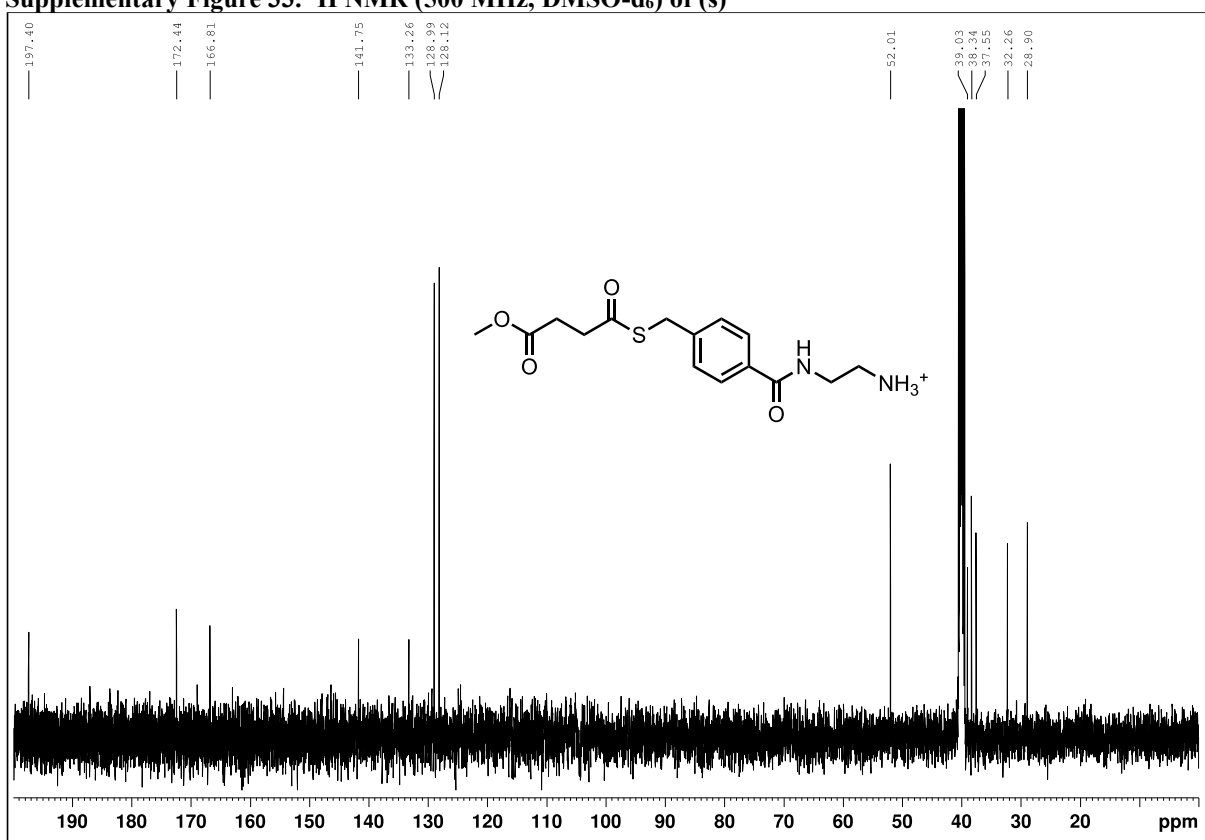
Supplementary Figure 31. ¹H NMR (500 MHz, DMSO-d₆) of (3)



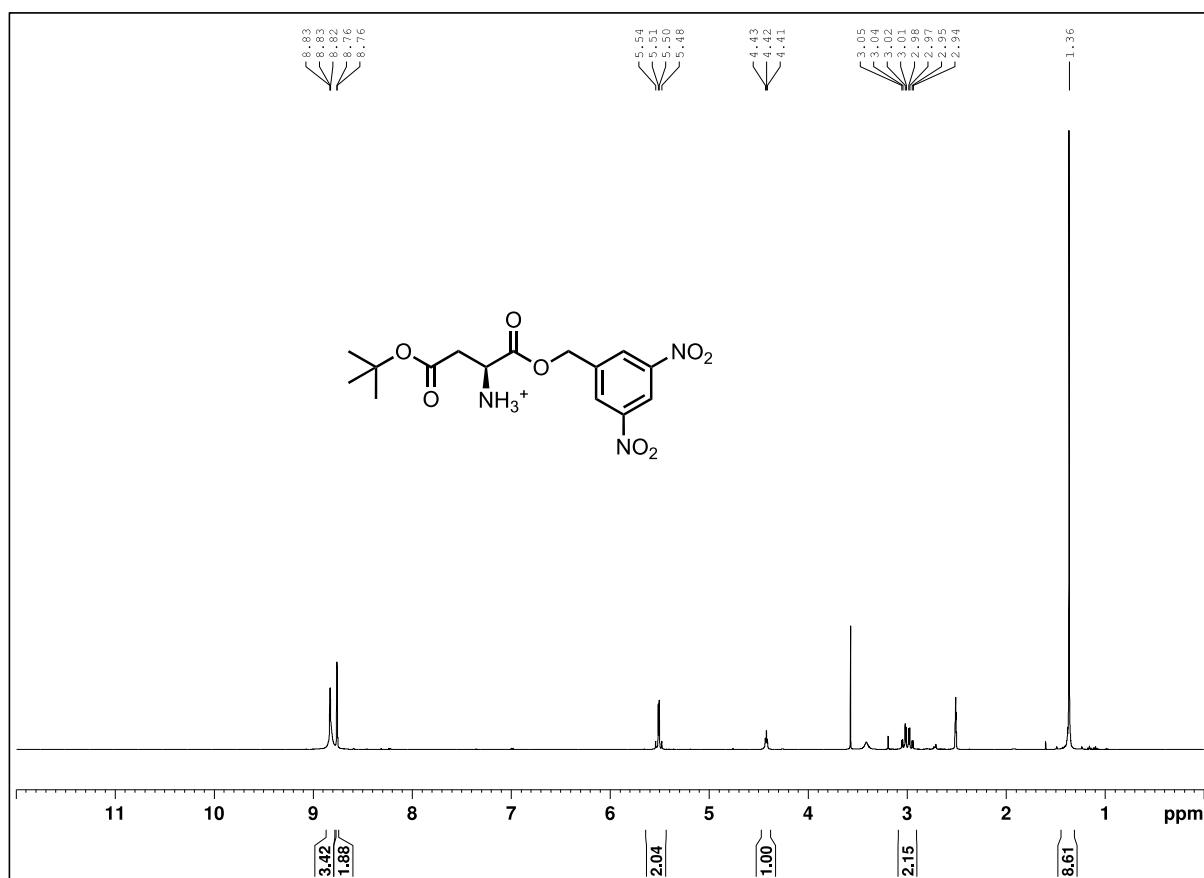
Supplementary Figure 32. ¹³C NMR (126 MHz, DMSO-d₆) of (3)



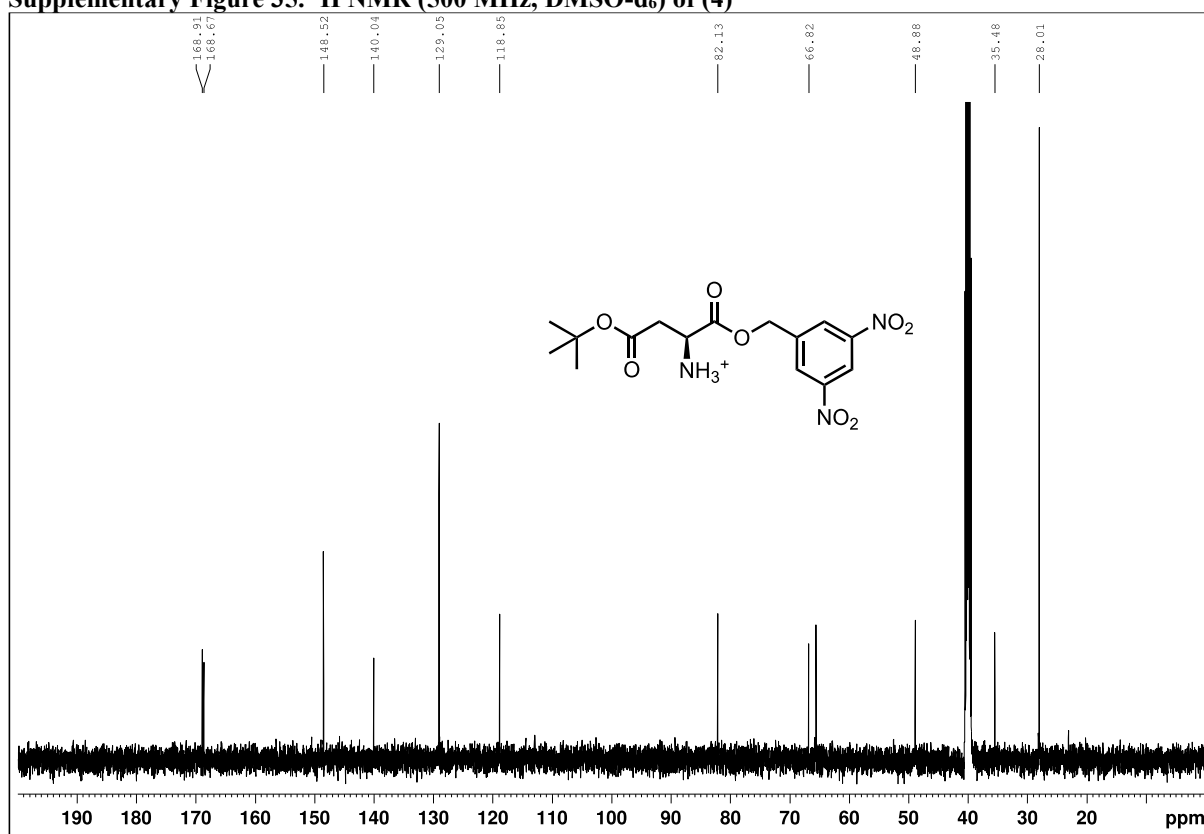
Supplementary Figure 33. ¹H NMR (500 MHz, DMSO-d₆) of (s)



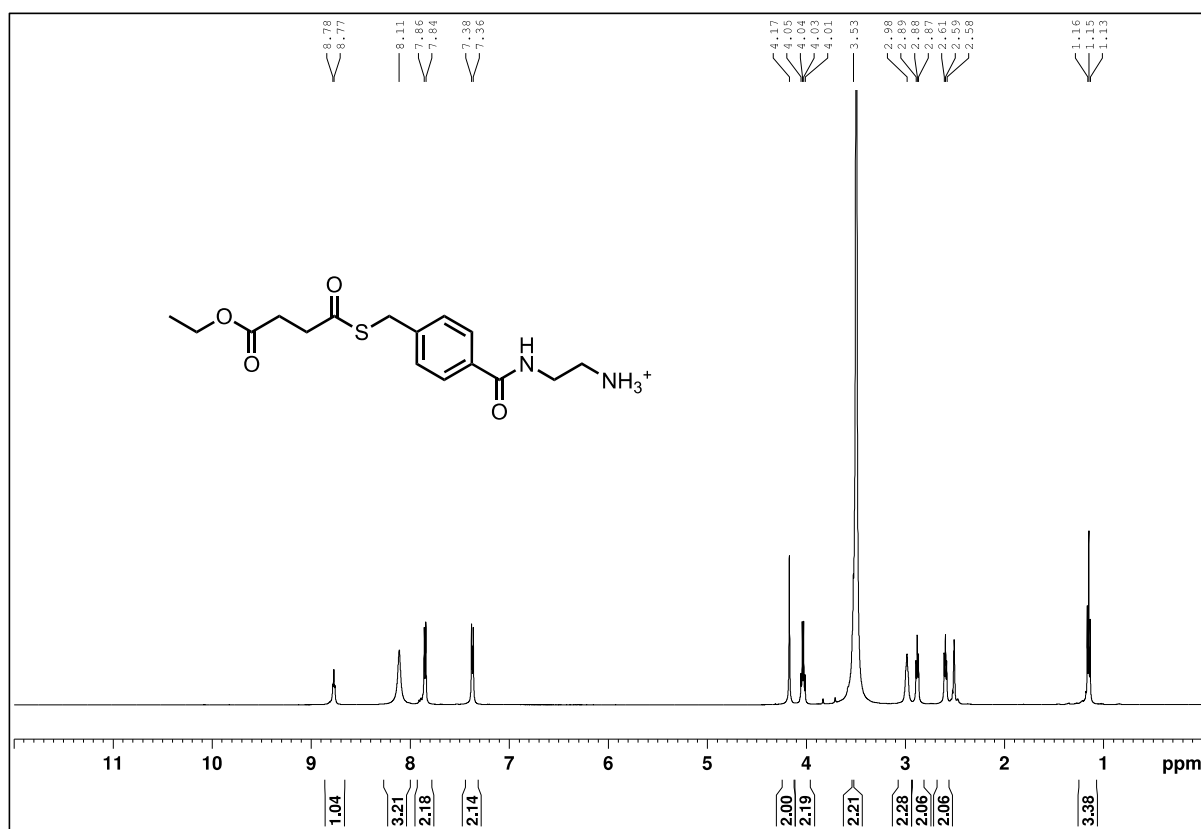
Supplementary Figure 34. ¹³C NMR (126 MHz, DMSO-d₆) of (s)



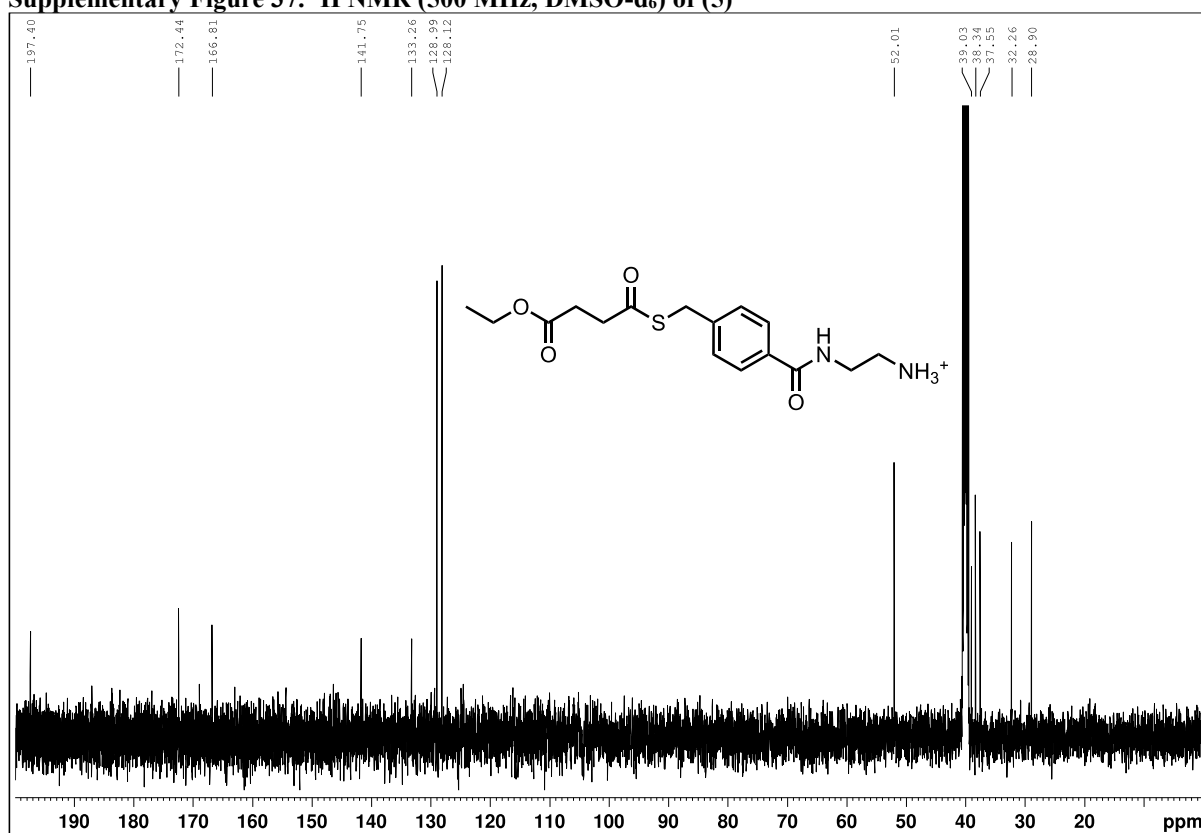
Supplementary Figure 35. ¹H NMR (500 MHz, DMSO-d₆) of (4)



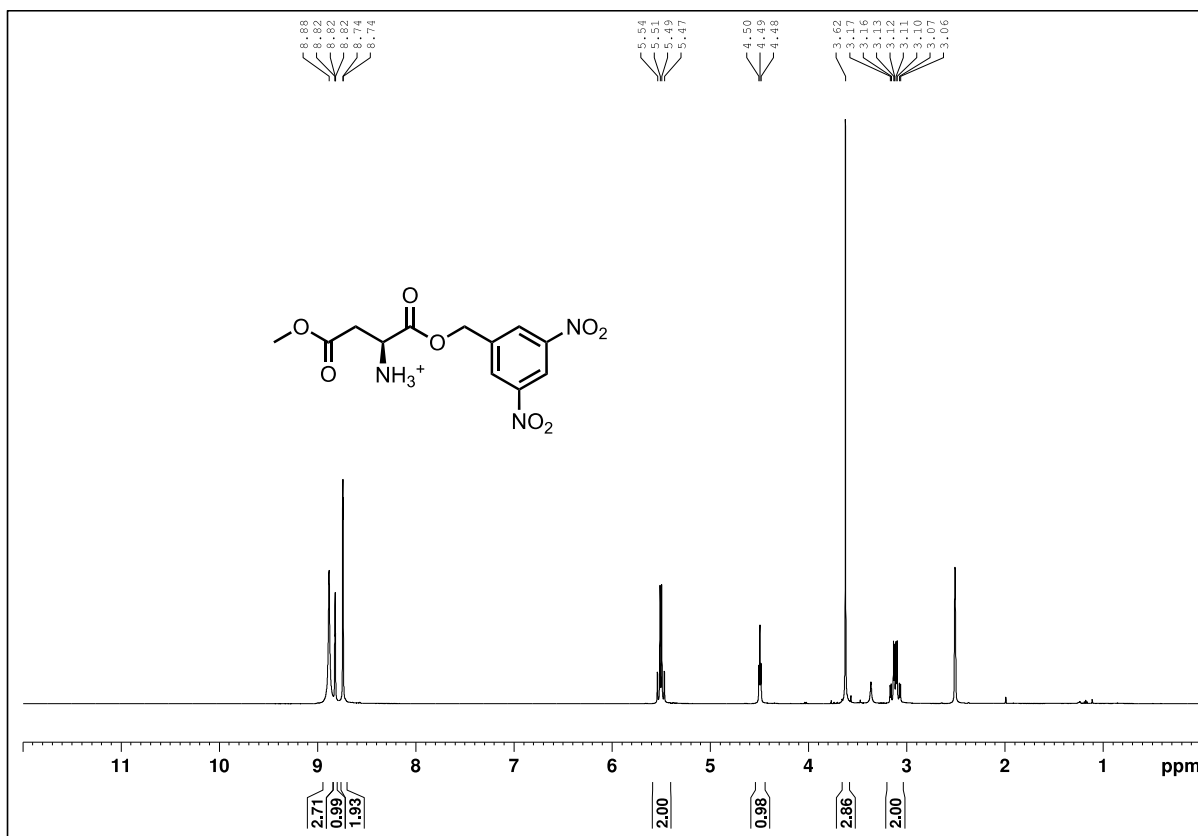
Supplementary Figure 36. ¹³C NMR (126 MHz, DMSO-d₆) of (4)



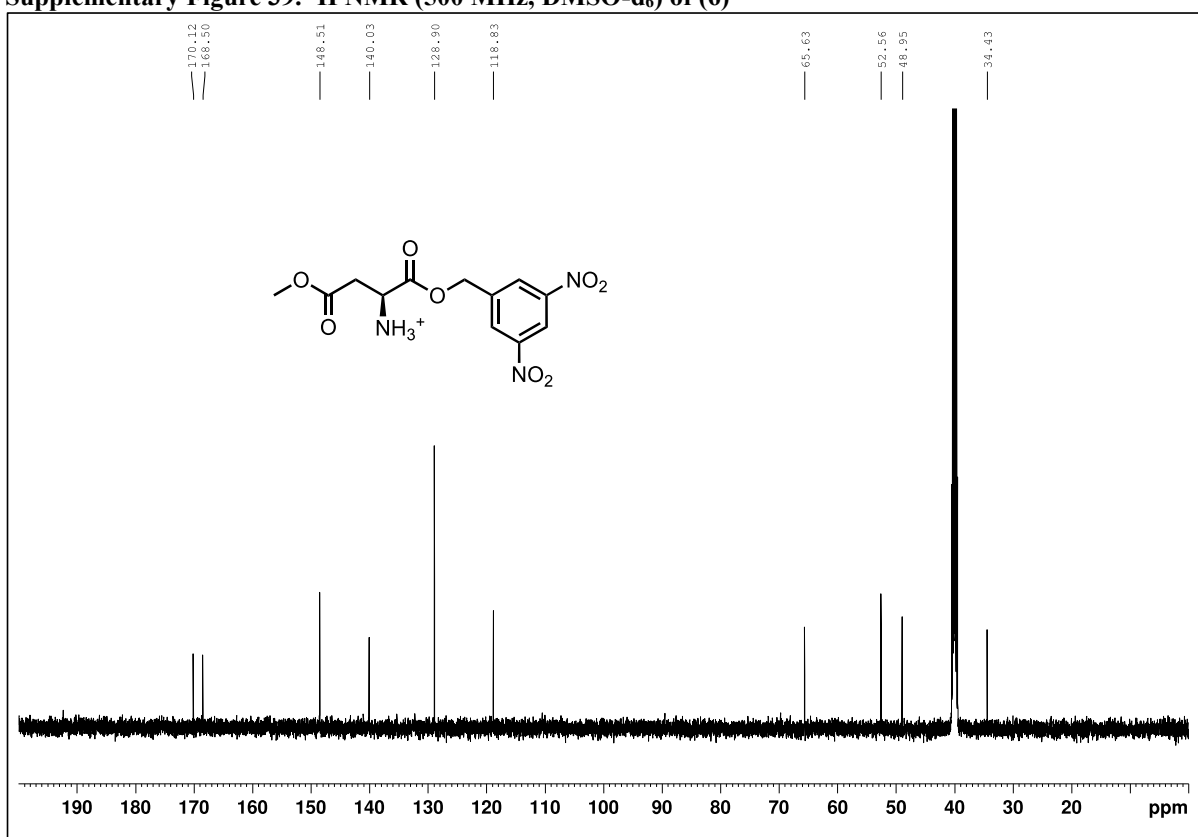
Supplementary Figure 37. ¹H NMR (500 MHz, DMSO-d₆) of (5)



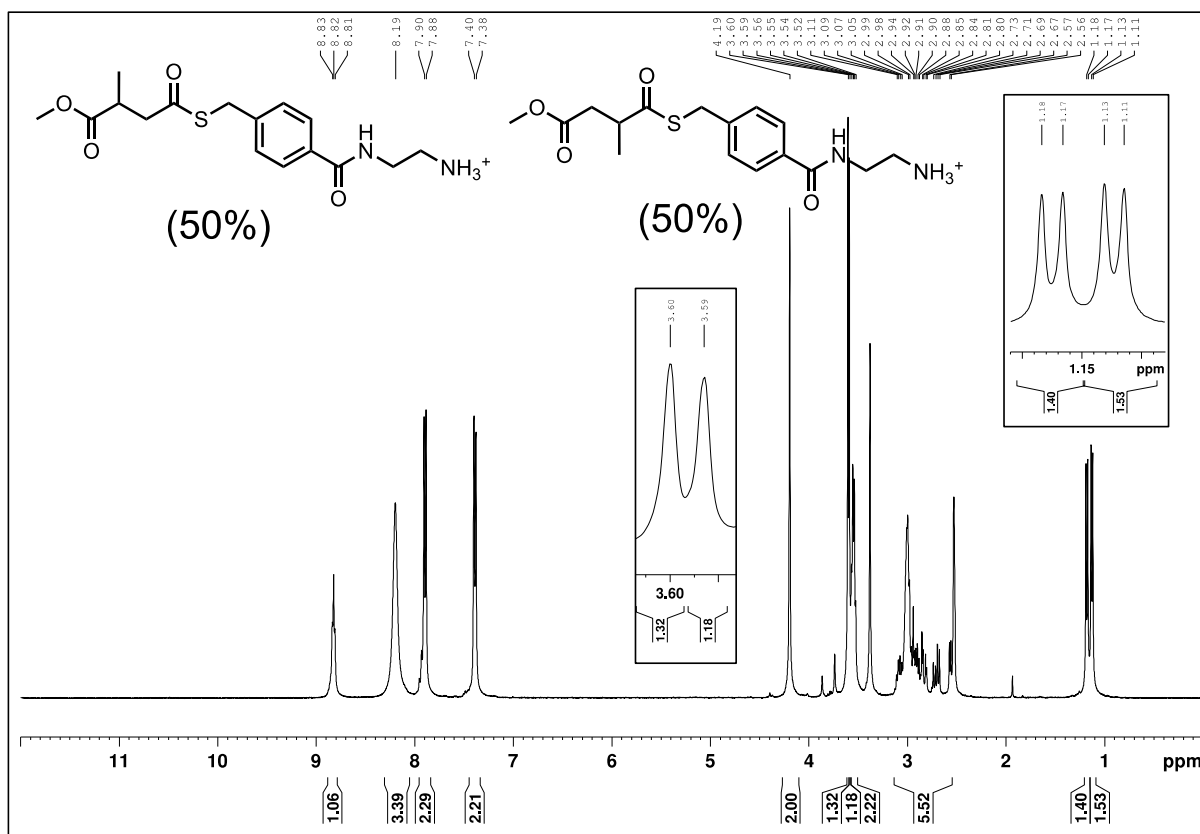
Supplementary Figure 38. ¹³C NMR (126 MHz, DMSO-d₆) of (5)



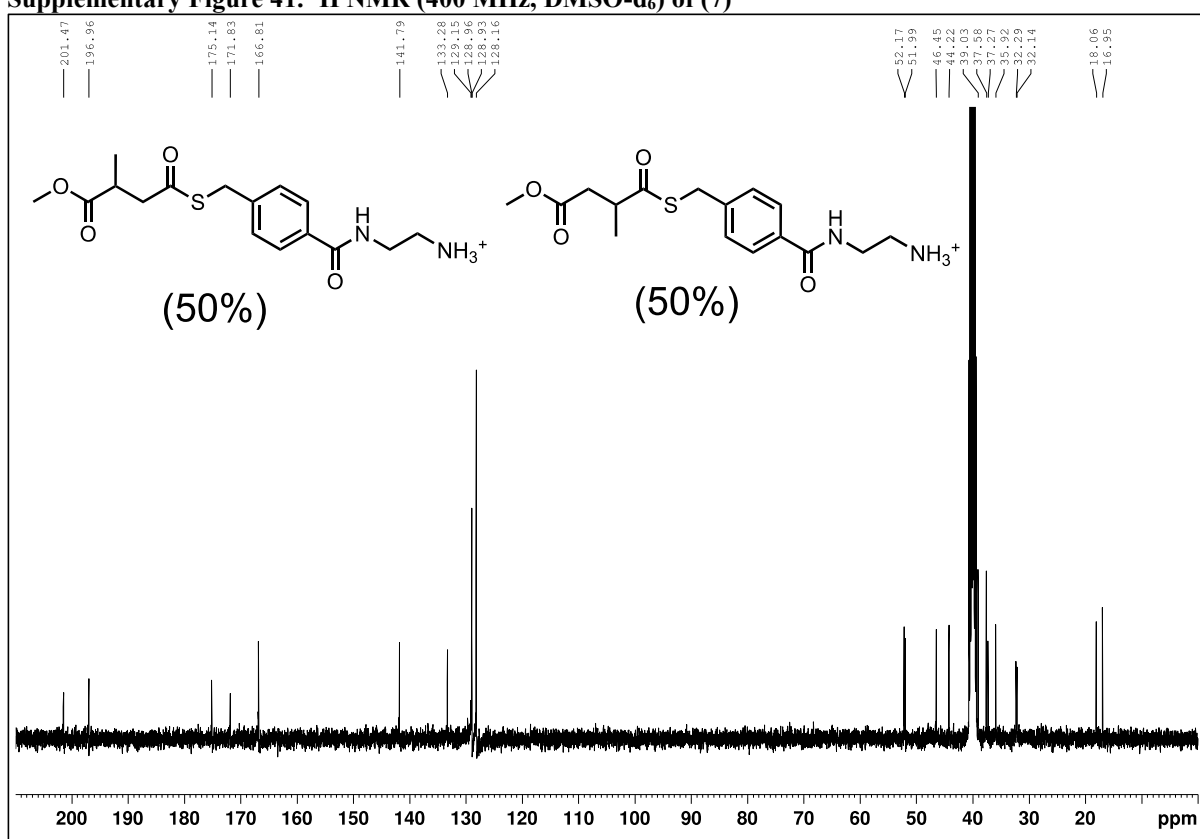
Supplementary Figure 39. ¹H NMR (500 MHz, DMSO-d₆) of (6)



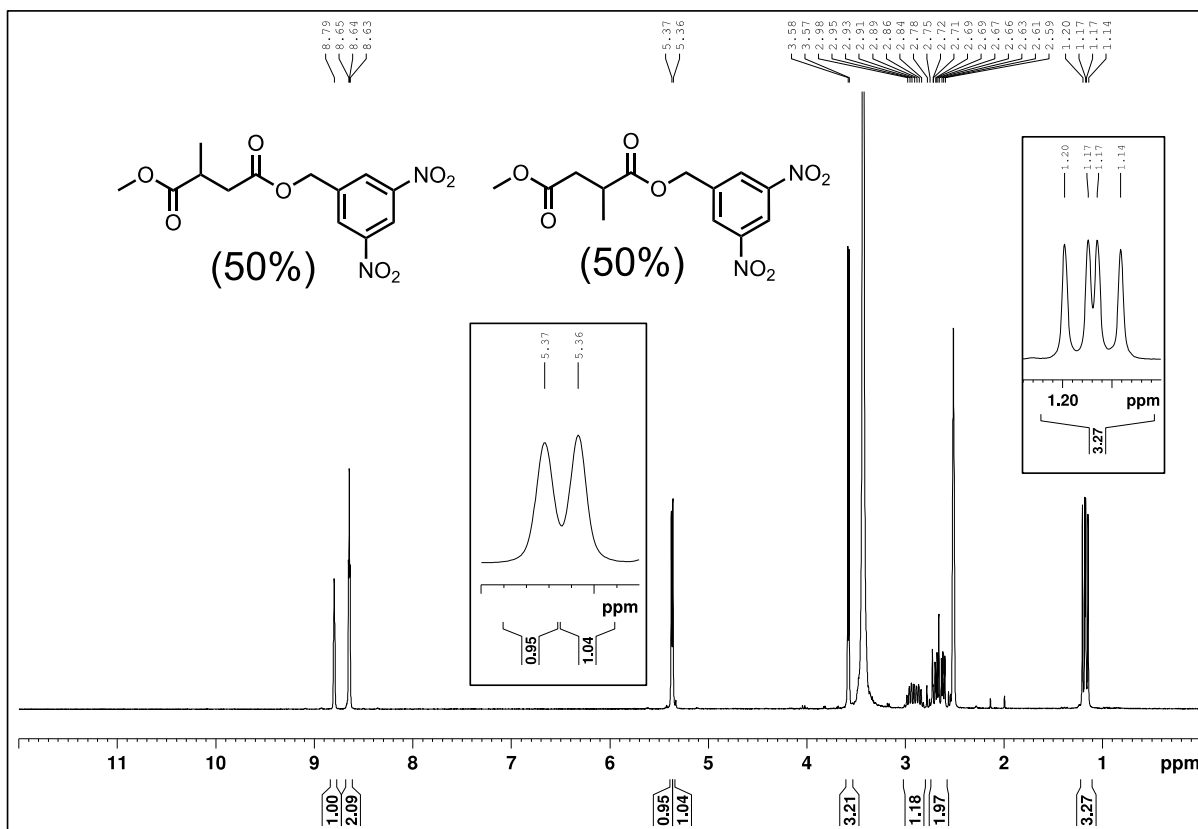
Supplementary Figure 40. ¹³C NMR (126 MHz, DMSO-d₆) of (6)



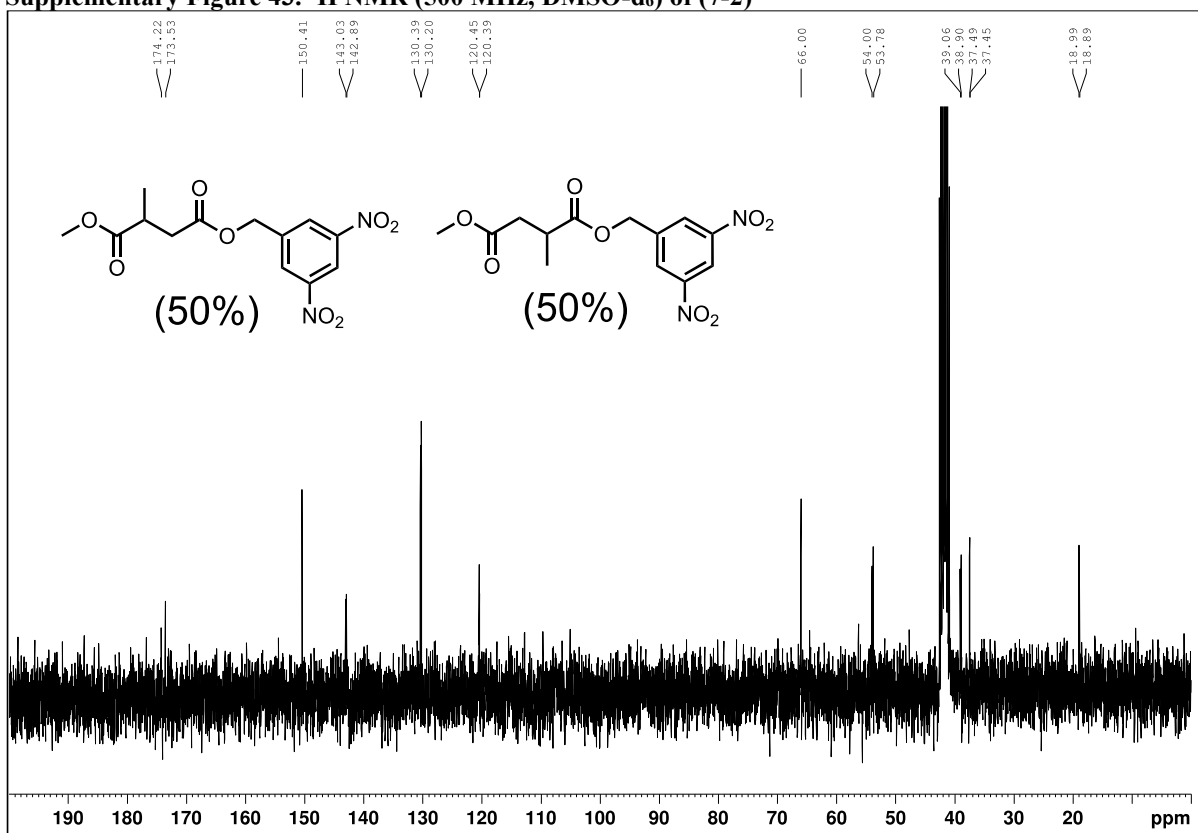
Supplementary Figure 41. ¹H NMR (400 MHz, DMSO-d₆) of (7)



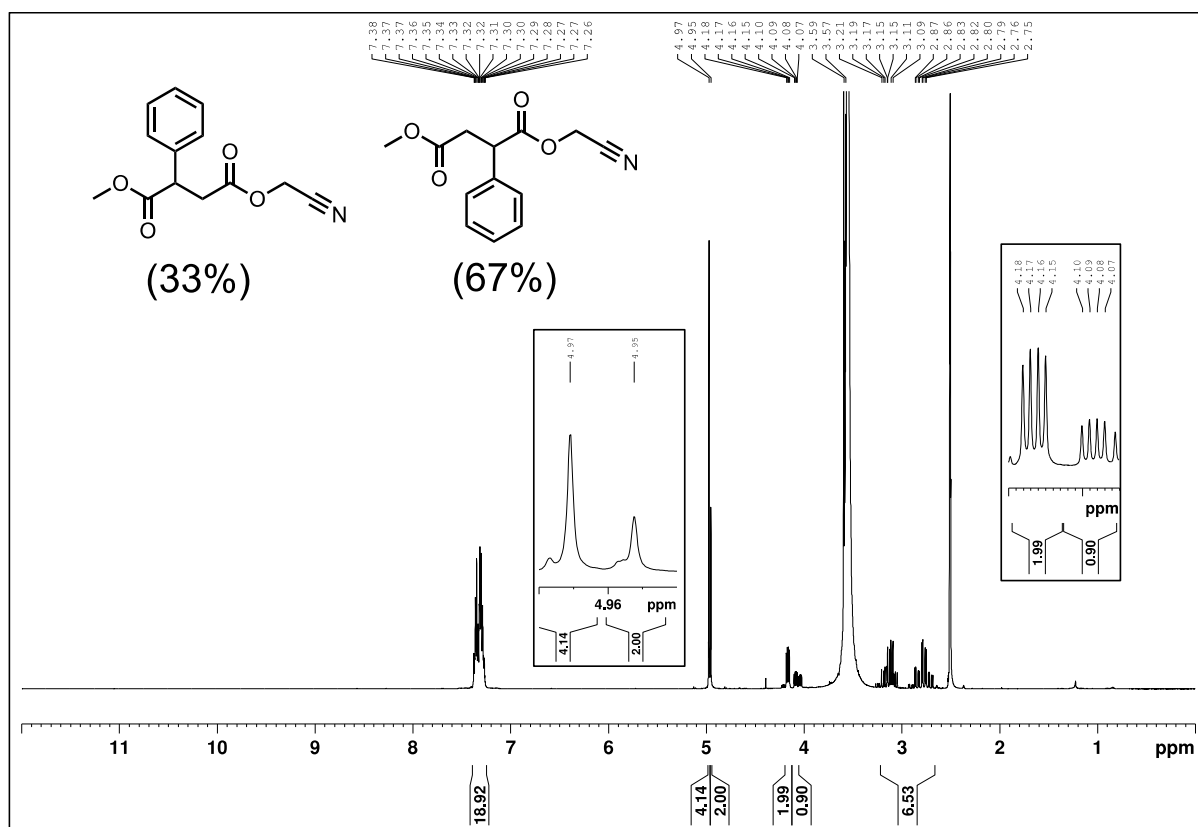
Supplementary Figure 42. ¹³C NMR (101 MHz, DMSO-d₆) of (7)



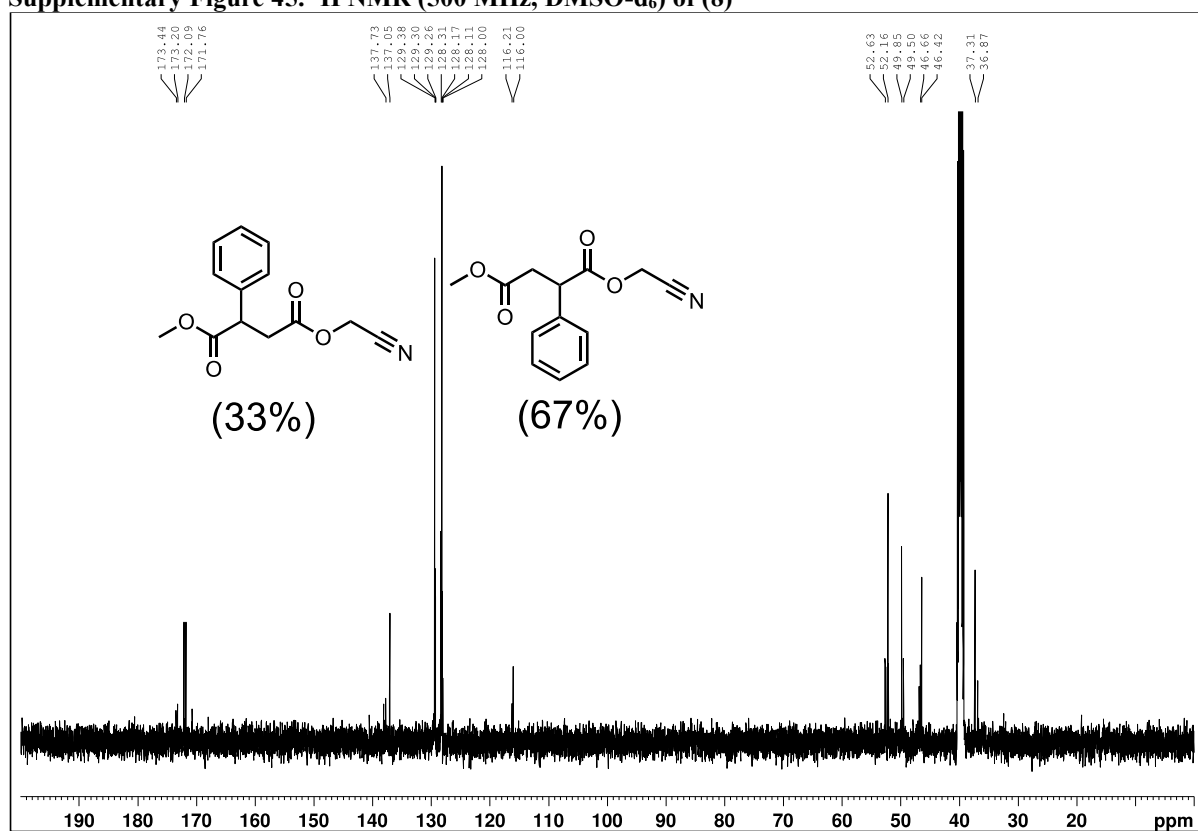
Supplementary Figure 43. ^1H NMR (500 MHz, DMSO-d_6) of (7-2)



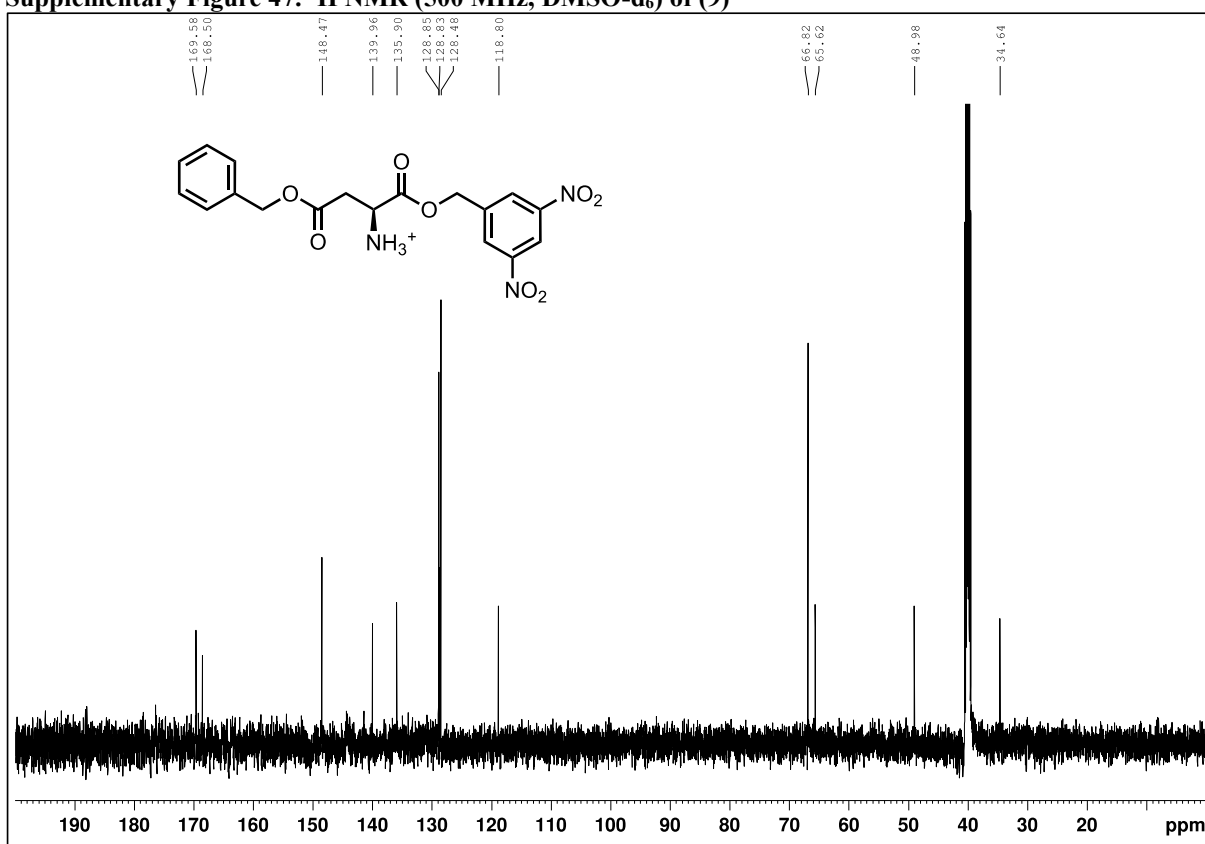
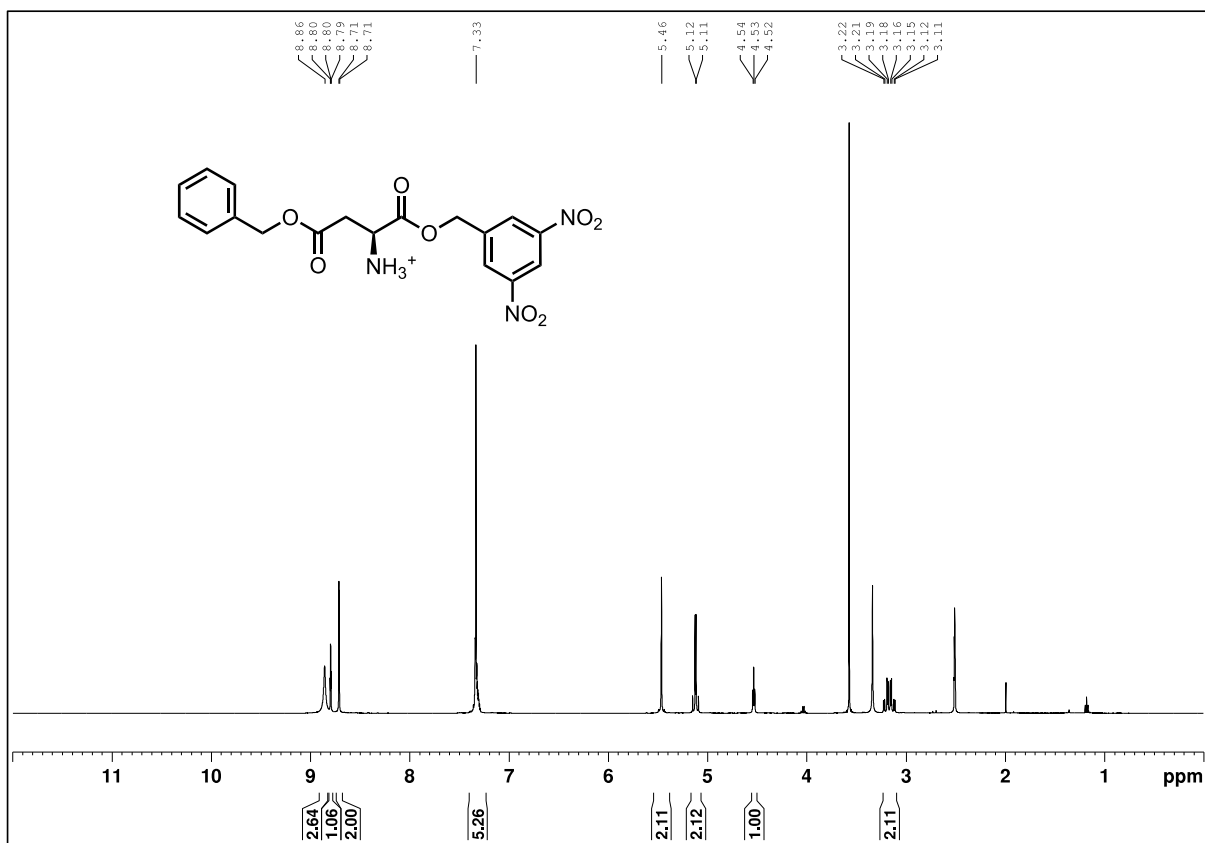
Supplementary Figure 44. ^{13}C NMR (126 MHz, DMSO-d_6) of (7-2)

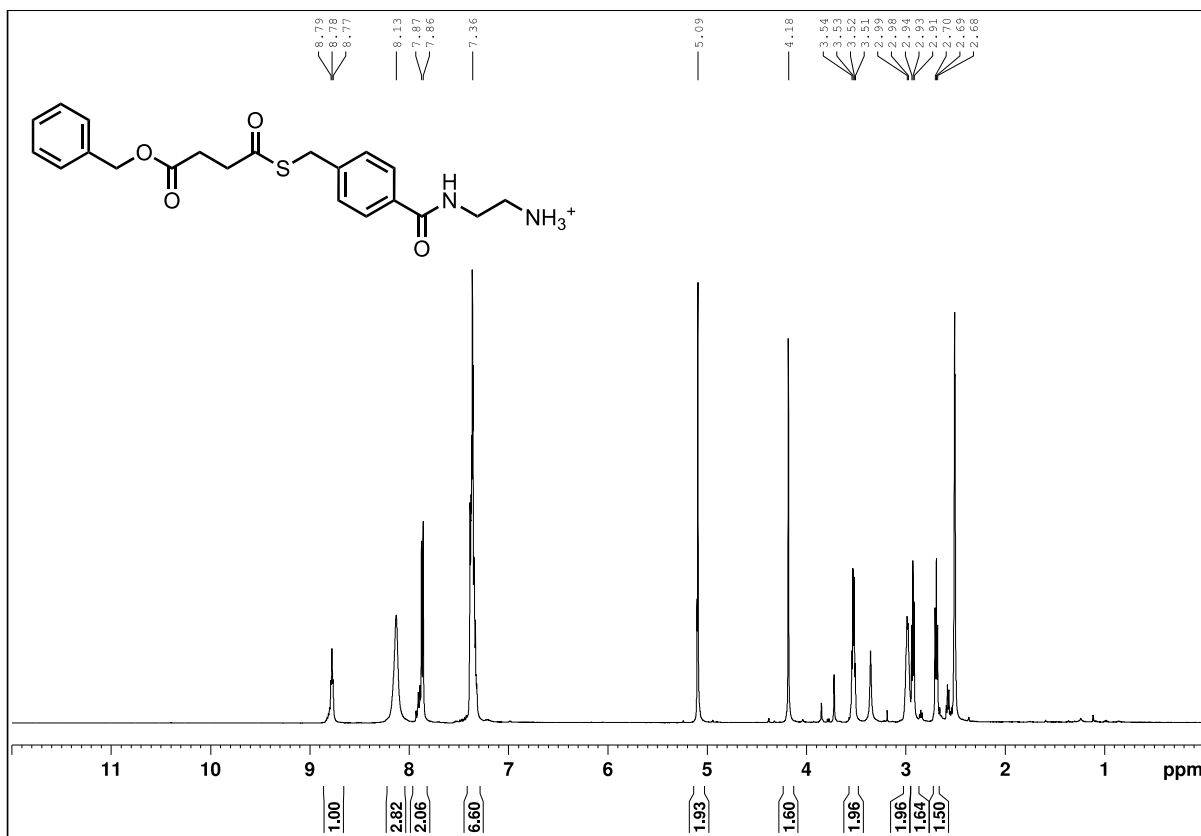


Supplementary Figure 45. ¹H NMR (500 MHz, DMSO-d₆) of (8)

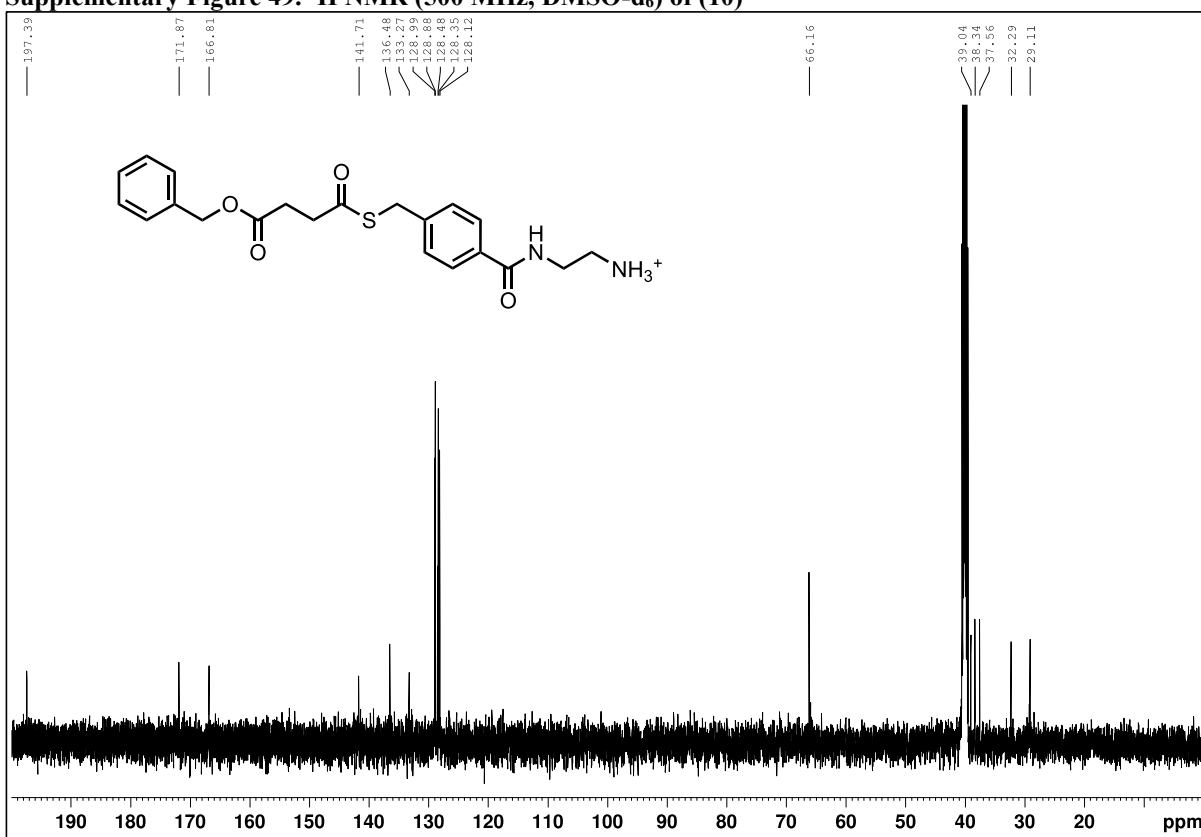


Supplementary Figure 46. ¹³C NMR (126 MHz, DMSO-d₆) of (8)

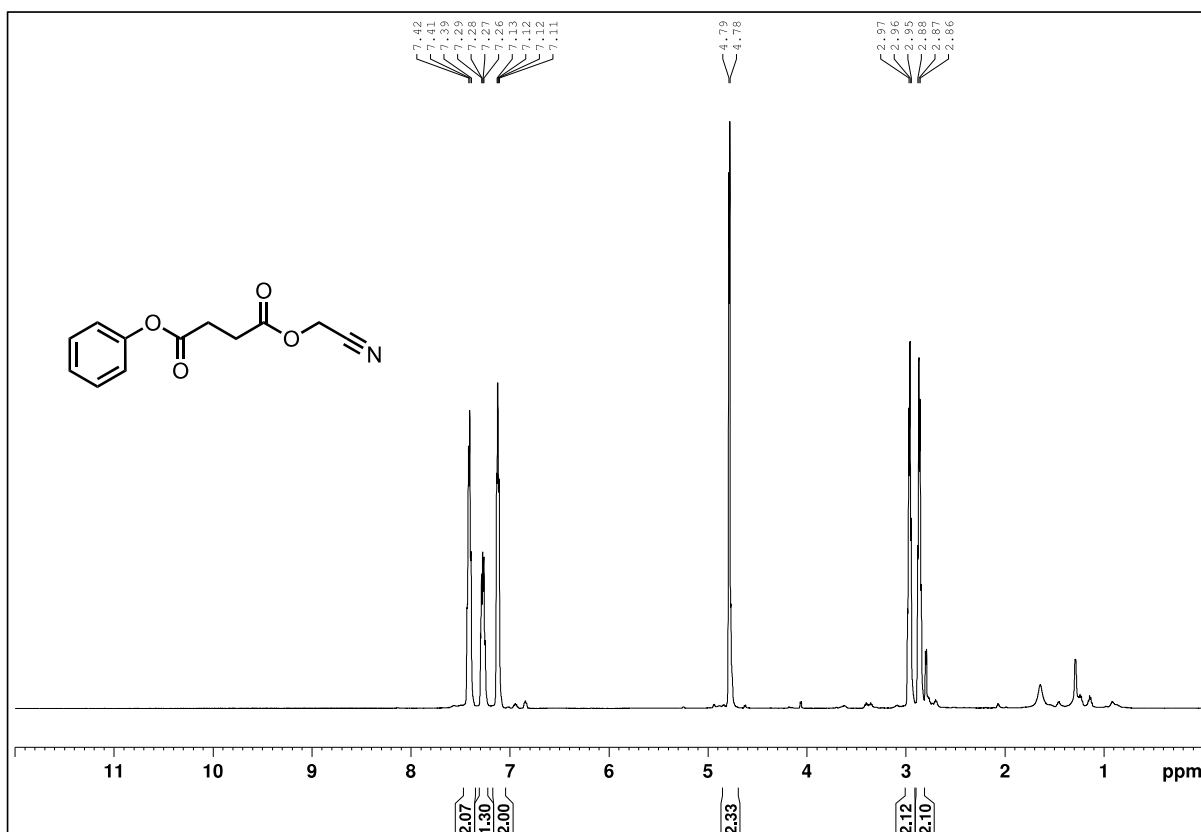




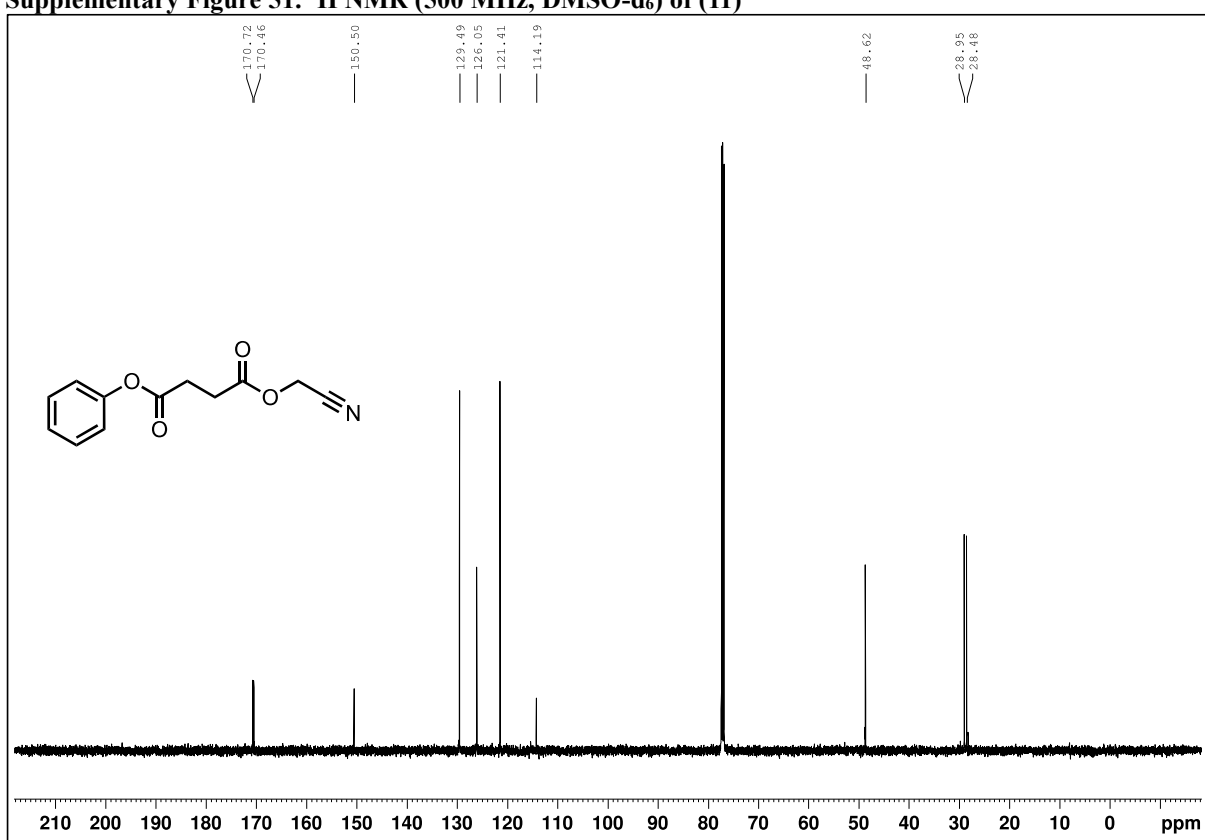
Supplementary Figure 49. ¹H NMR (500 MHz, DMSO-d₆) of (10)



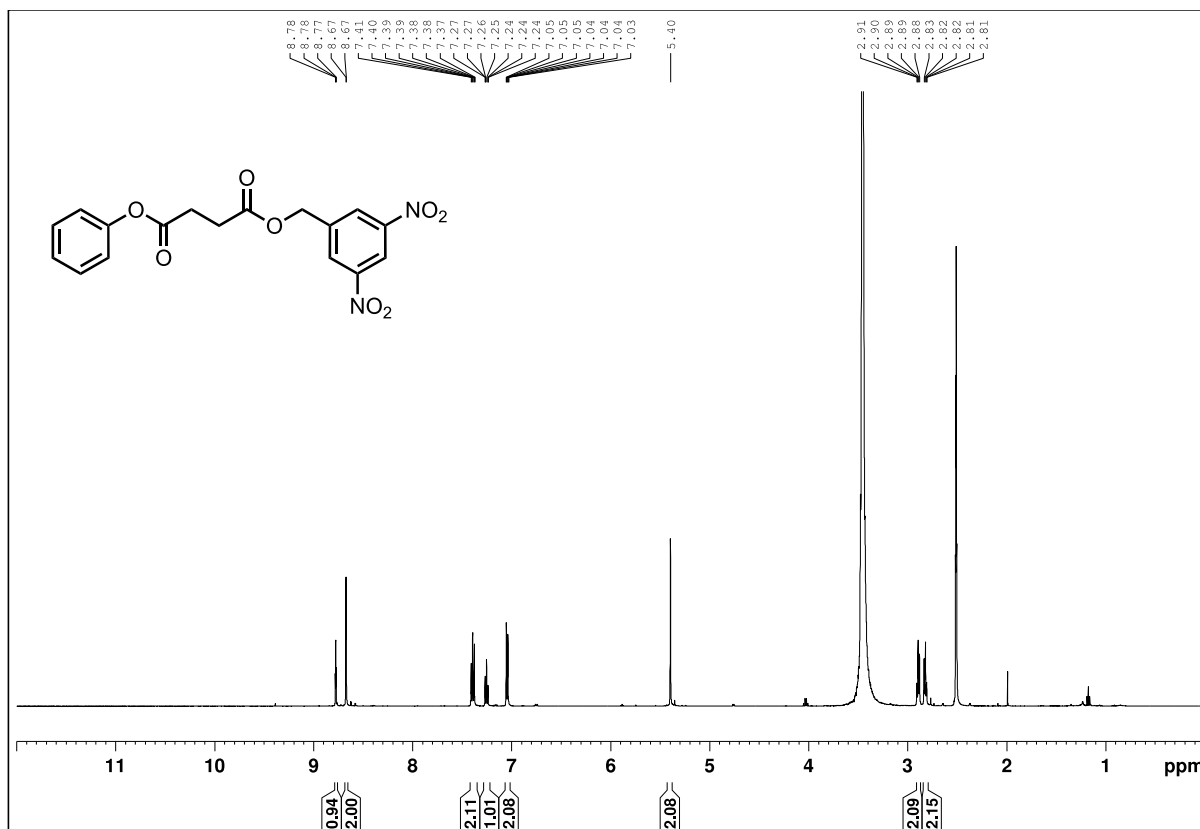
Supplementary Figure 50. ¹³C NMR (126 MHz, DMSO-d₆) of (10)



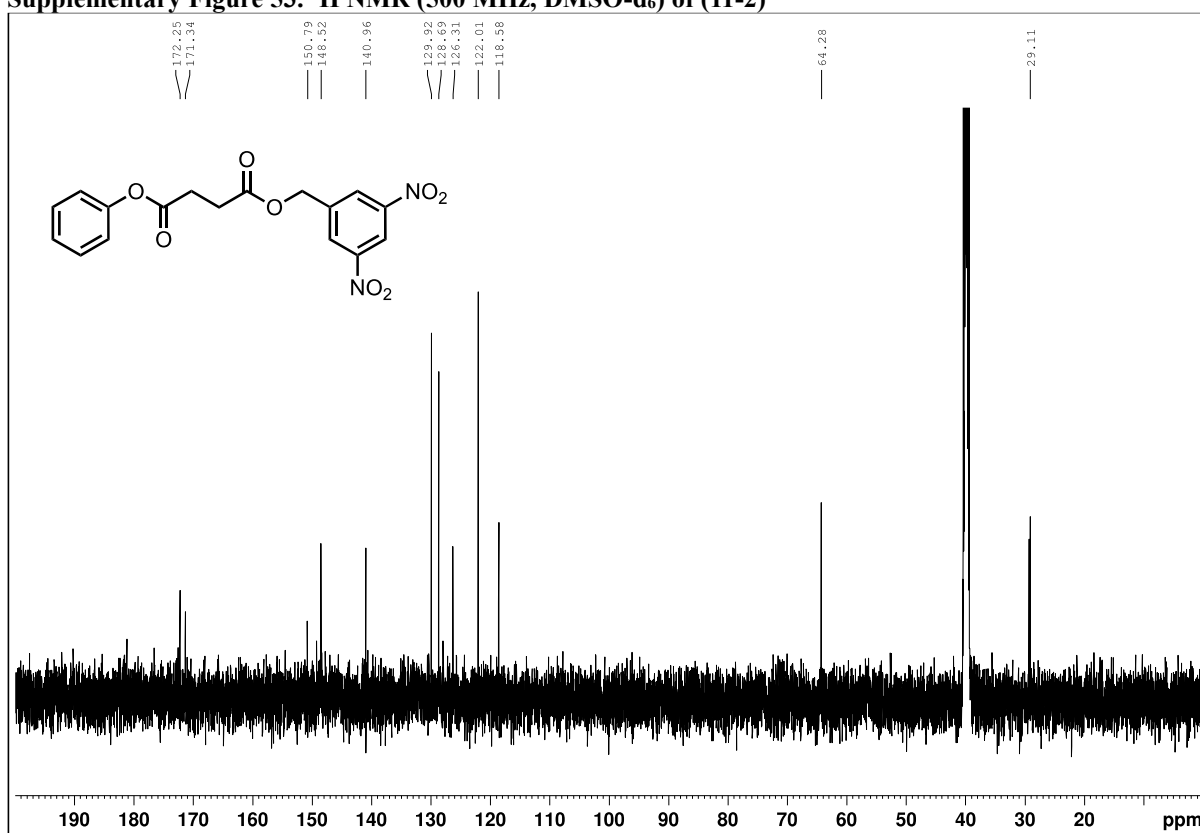
Supplementary Figure 51. ¹H NMR (500 MHz, DMSO-d₆) of (11)



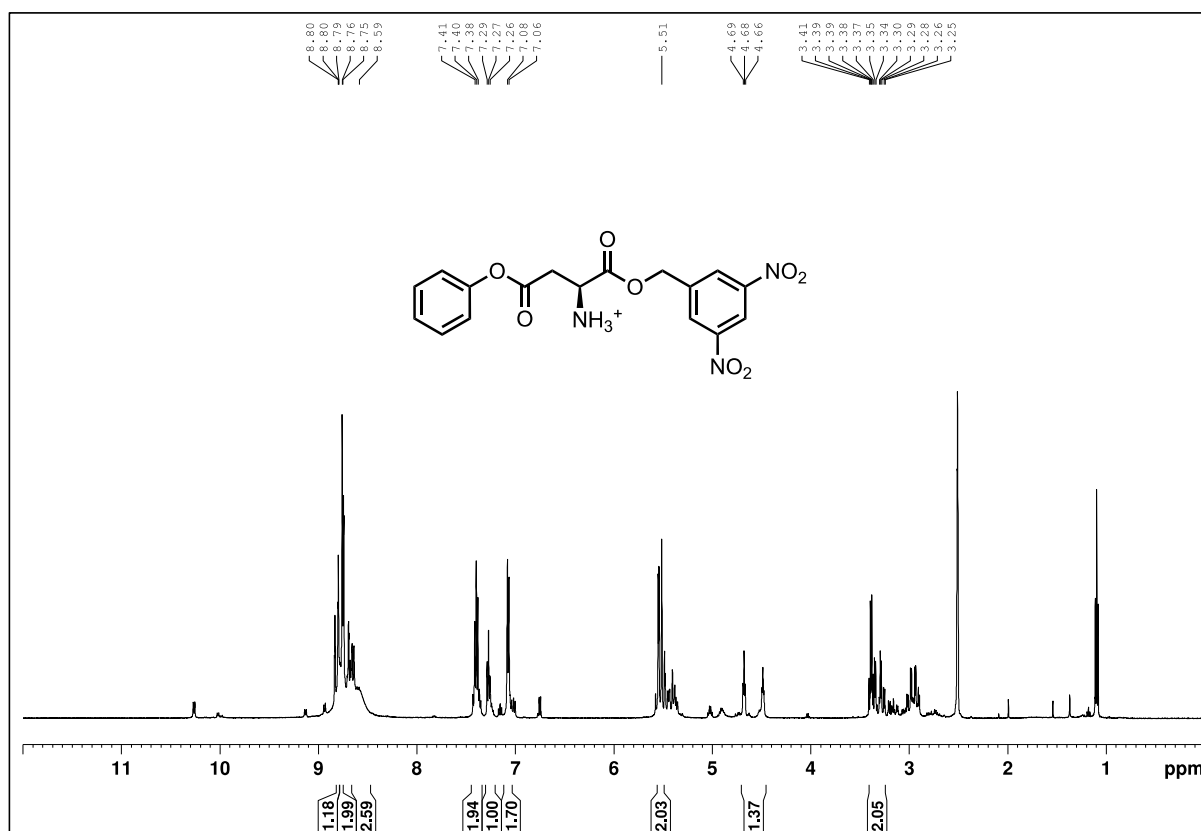
Supplementary Figure 52. ¹³C NMR (126 MHz, DMSO-d₆) of (11)



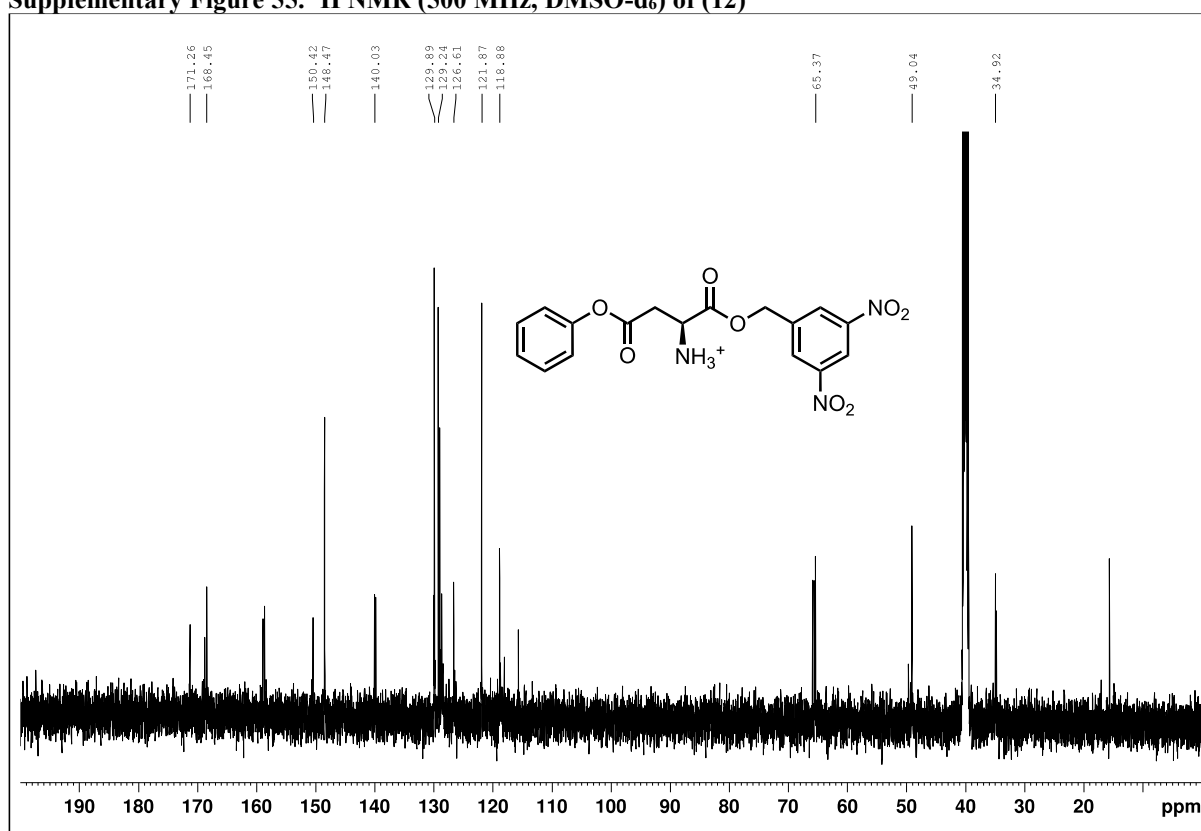
Supplementary Figure 53. ¹H NMR (500 MHz, DMSO-d₆) of (11-2)



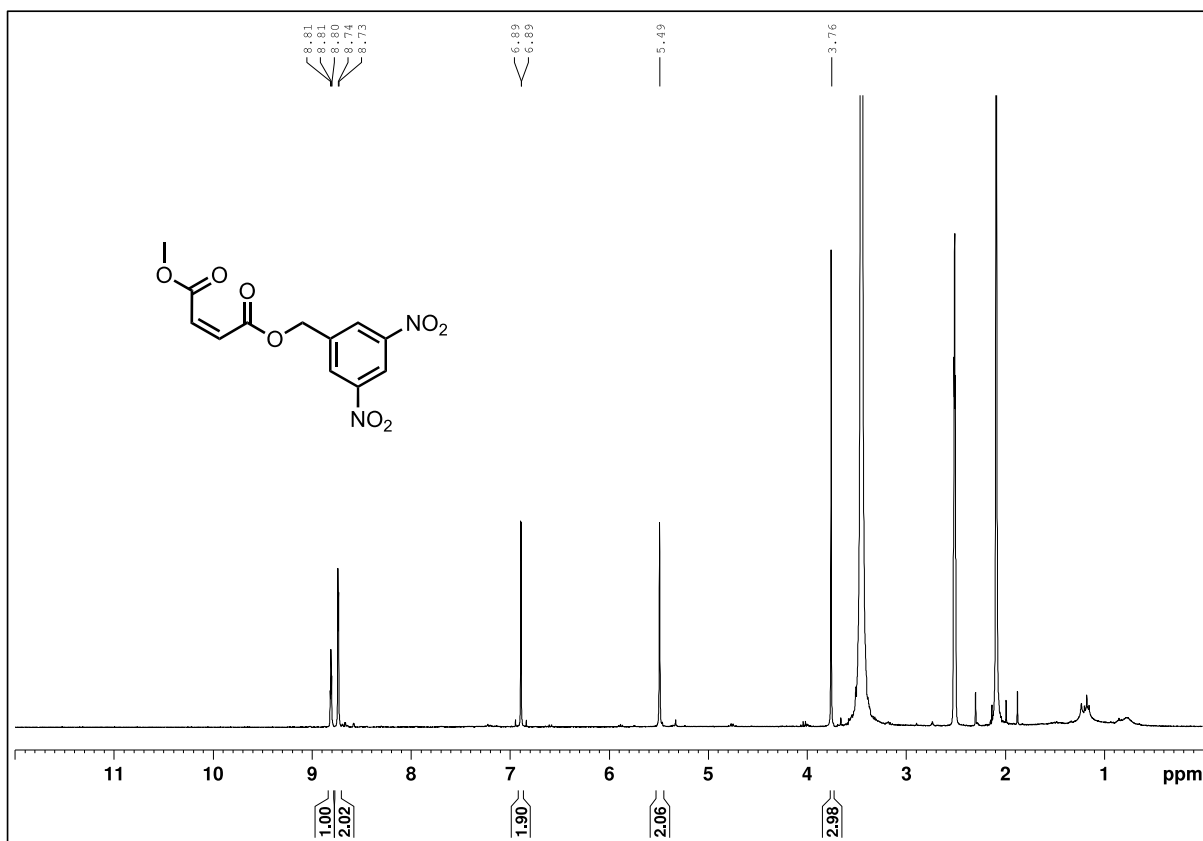
Supplementary Figure 54. ¹³C NMR (126 MHz, DMSO-d₆) of (11-2)



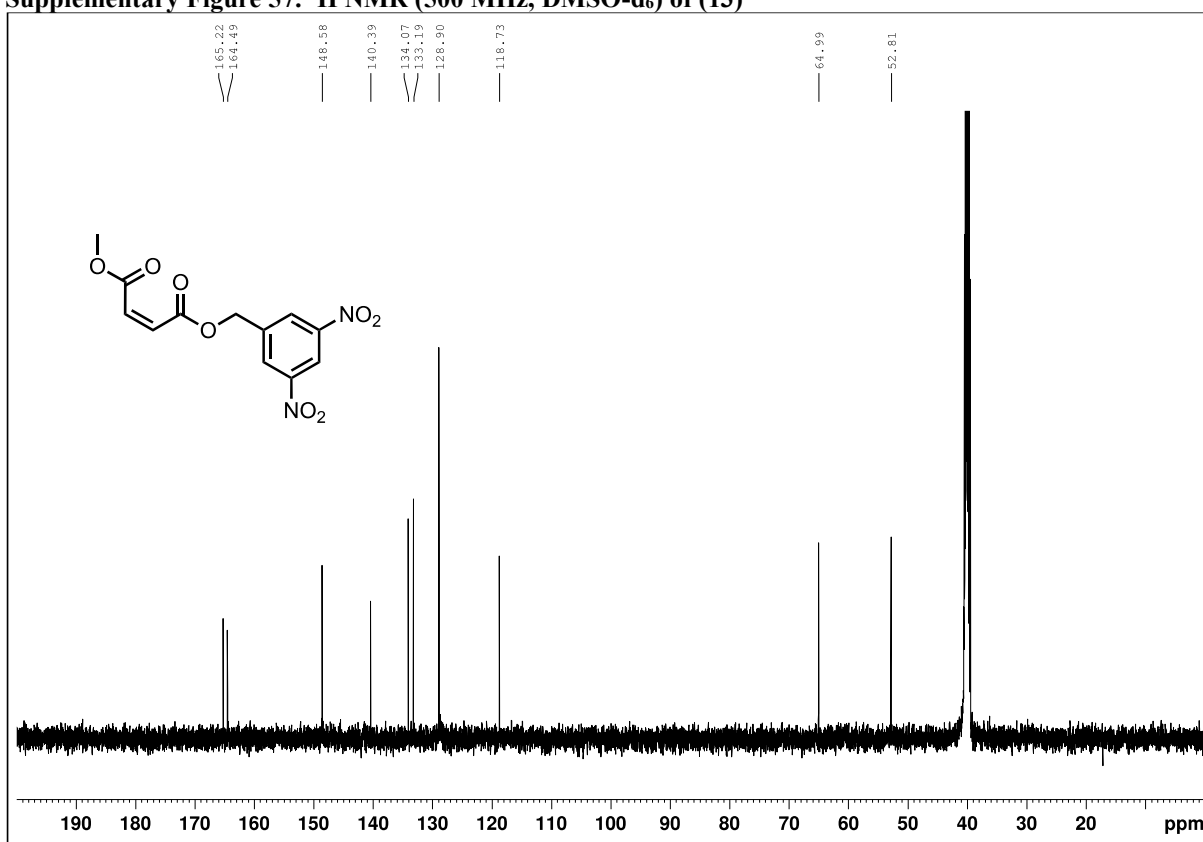
Supplementary Figure 55. ¹H NMR (500 MHz, DMSO-d₆) of (12)



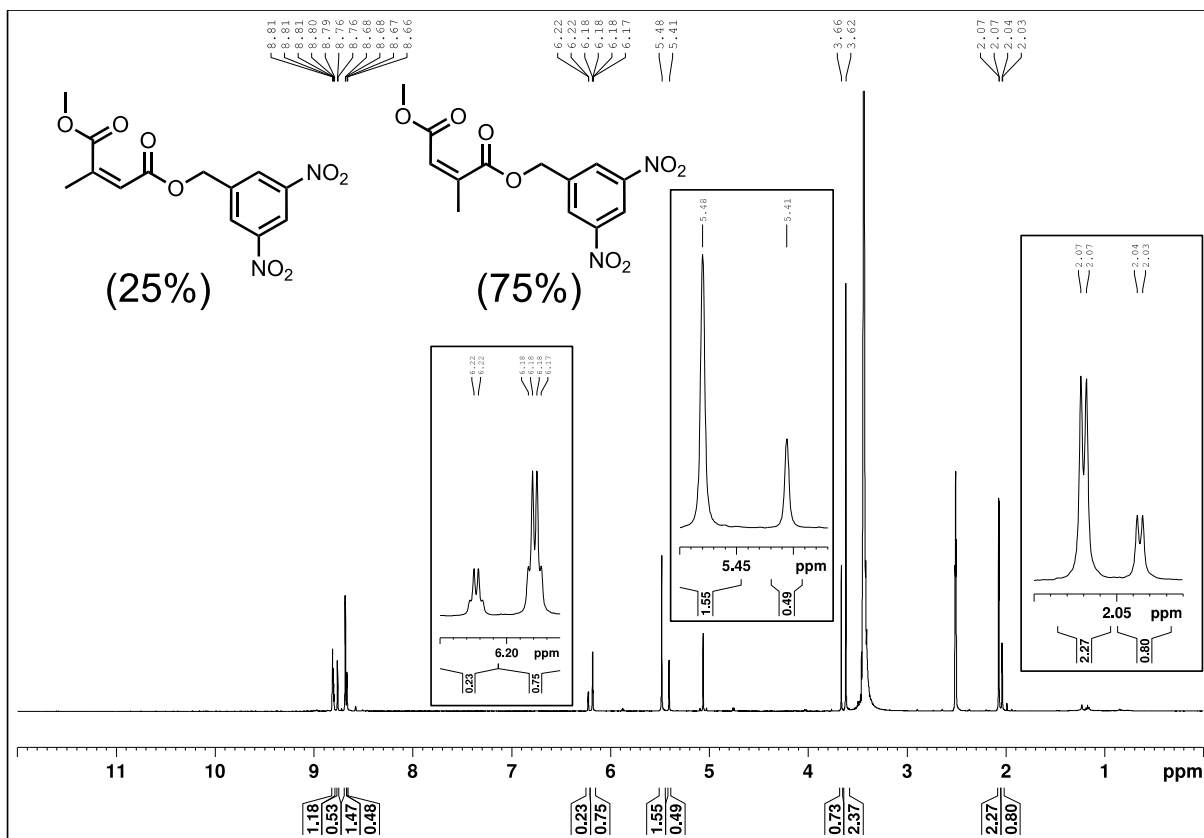
Supplementary Figure 56. ¹³C NMR (126 MHz, DMSO-d₆) of (12)



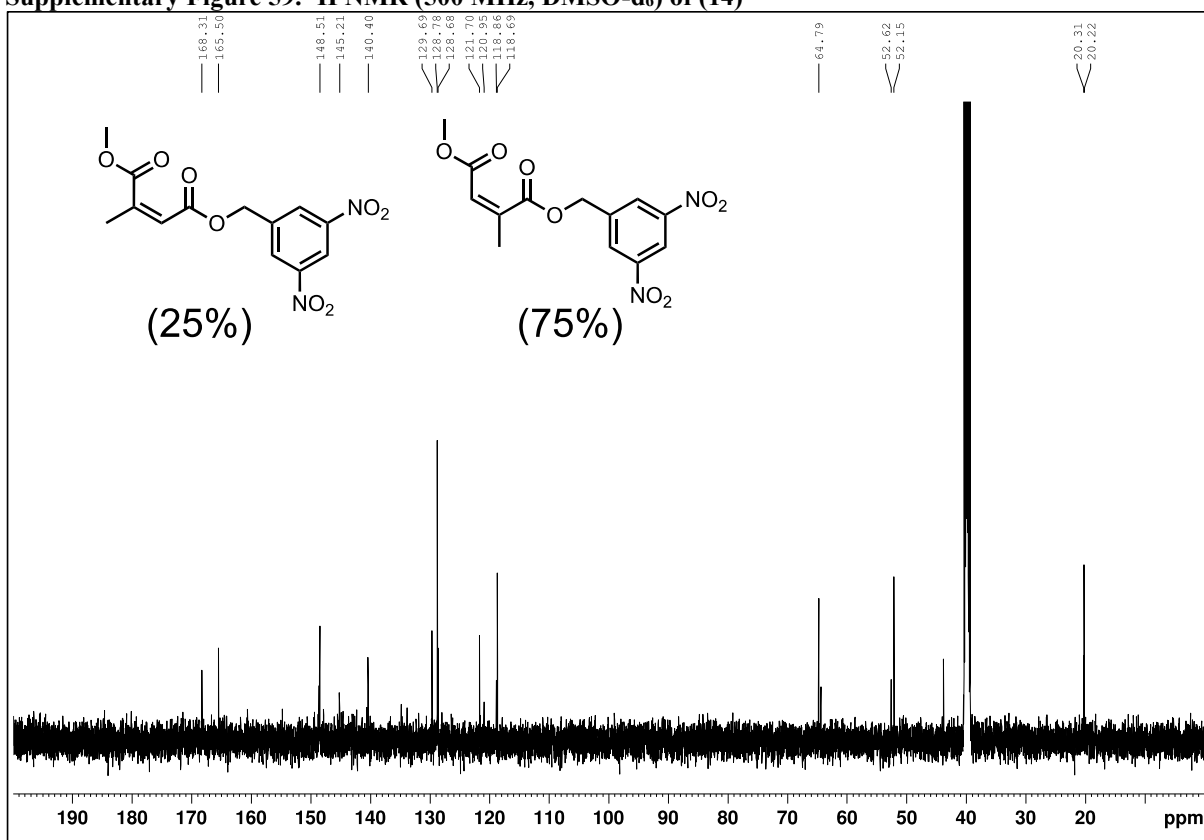
Supplementary Figure 57. ¹H NMR (500 MHz, DMSO-d₆) of (13)



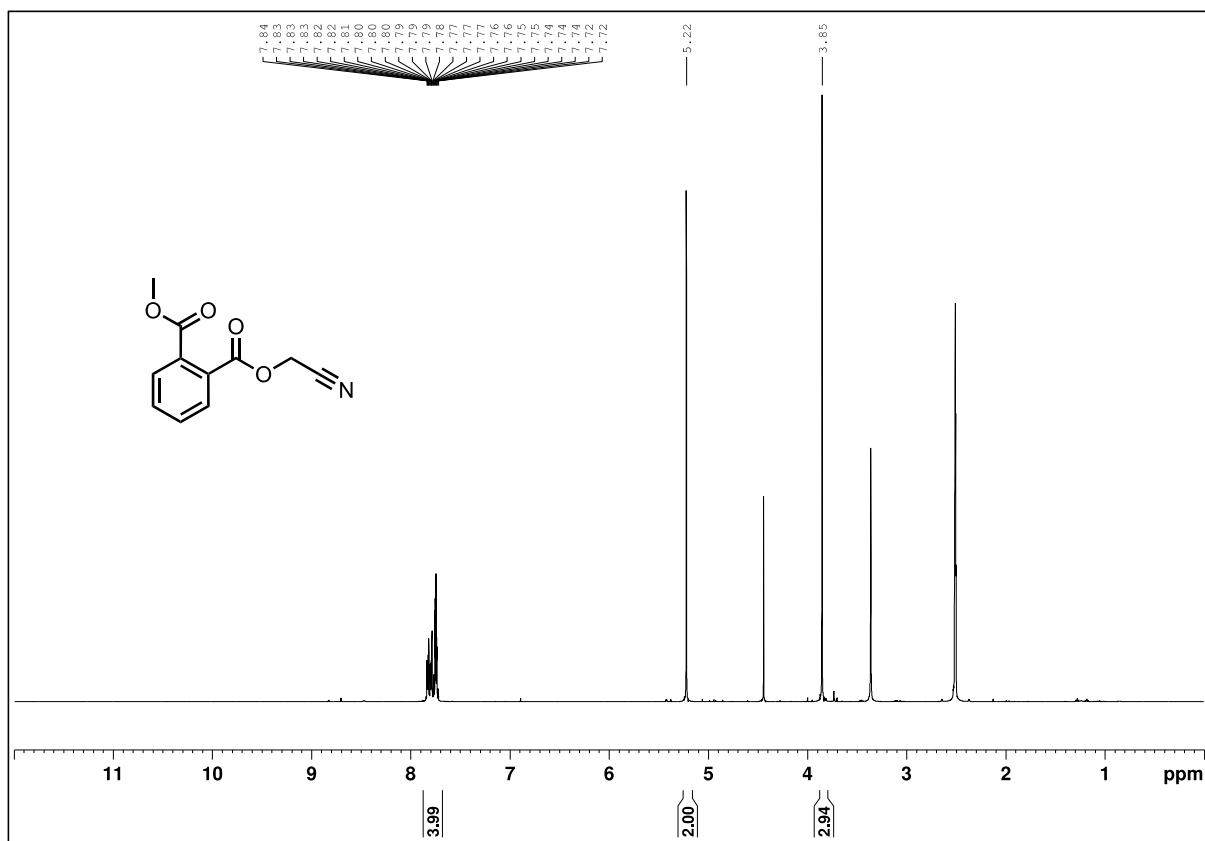
Supplementary Figure 58. ¹³C NMR (126 MHz, DMSO-d₆) of (13)



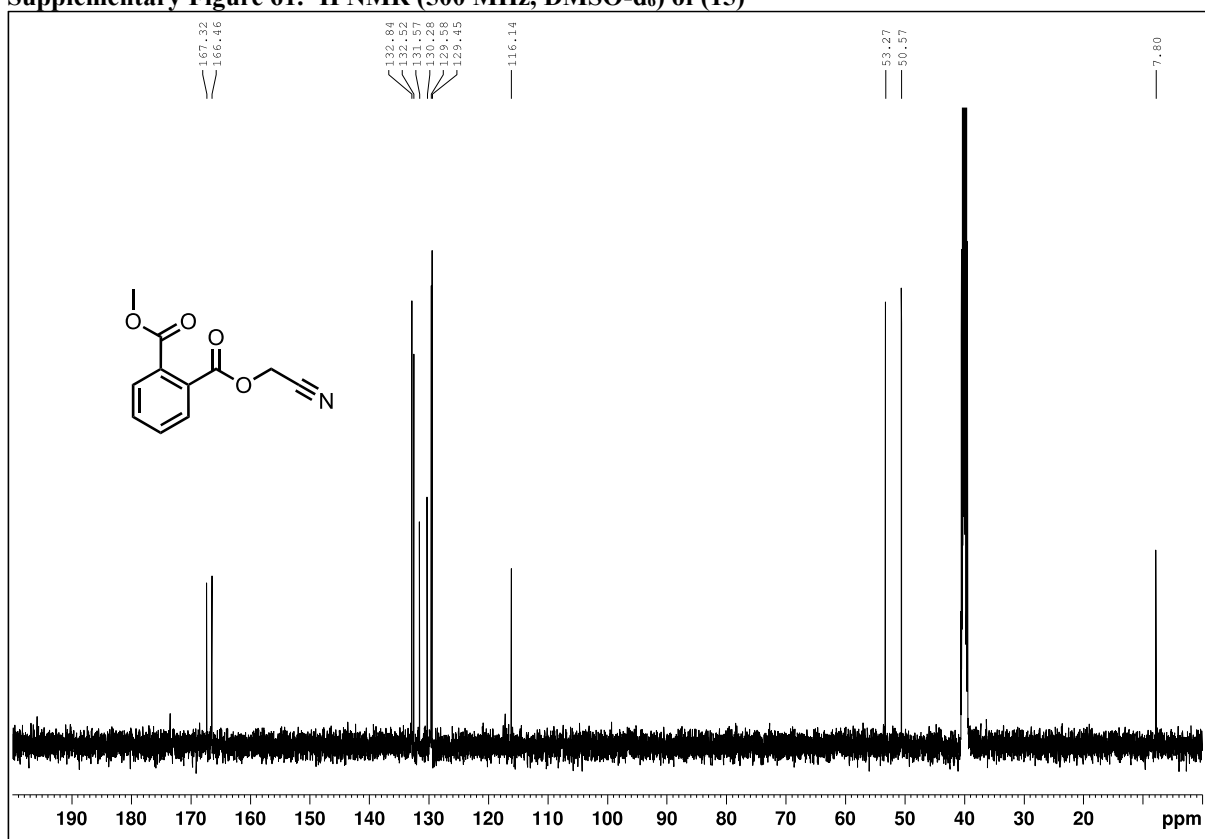
Supplementary Figure 59. ¹H NMR (500 MHz, DMSO-d₆) of (14)



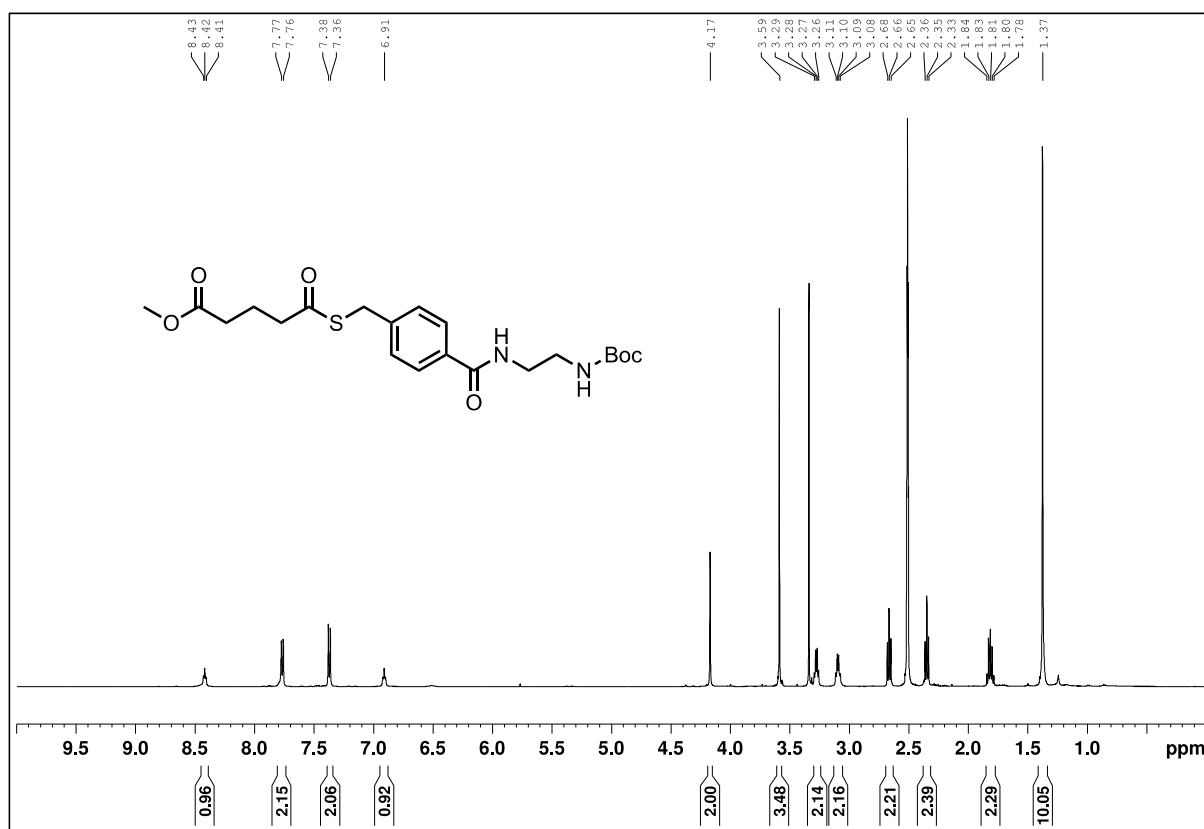
Supplementary Figure 60. ¹³C NMR (126 MHz, DMSO-d₆) of (14)



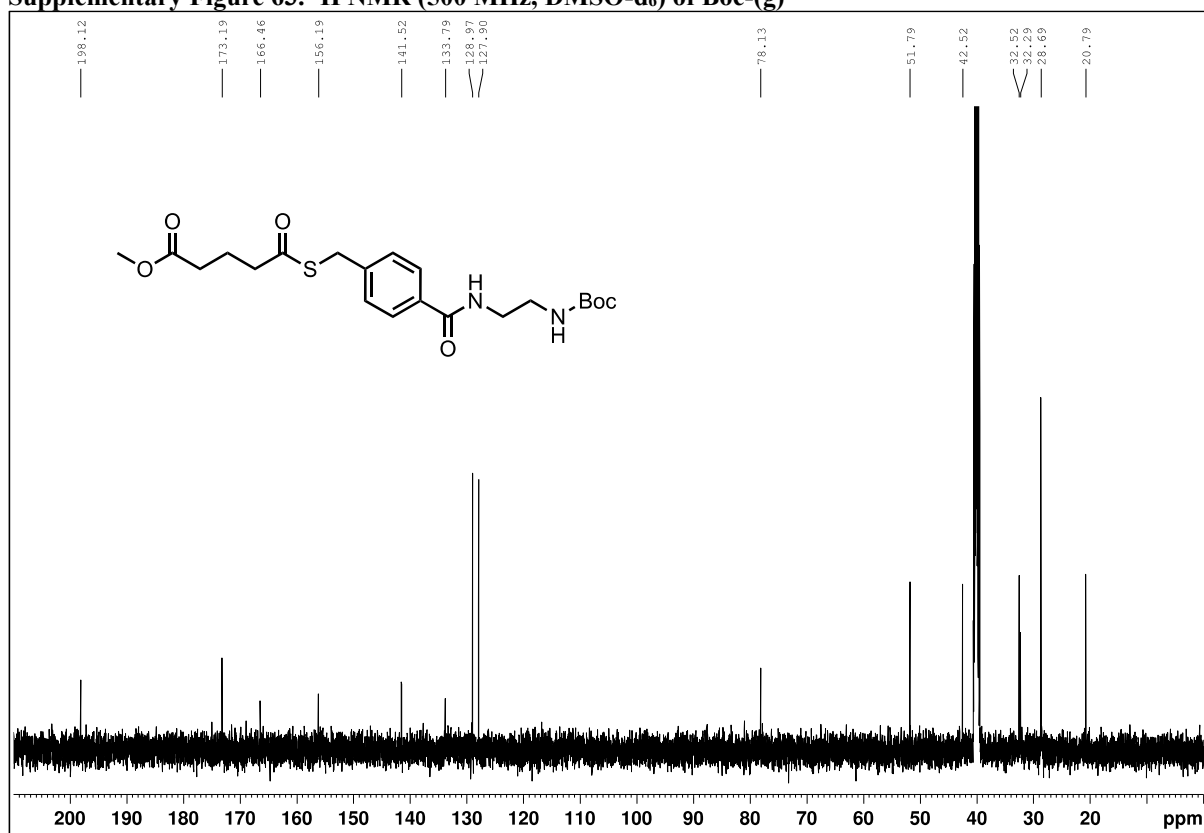
Supplementary Figure 61. ¹H NMR (500 MHz, DMSO-d₆) of (15)



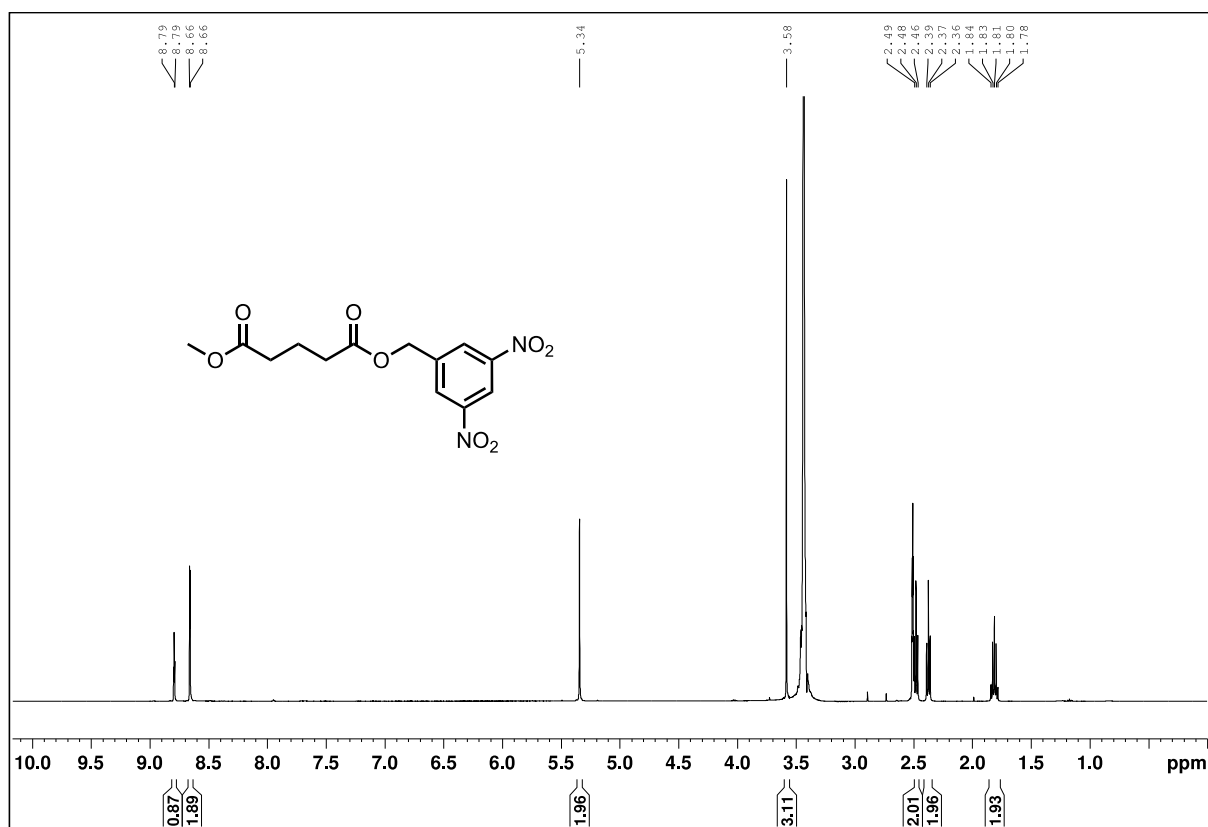
Supplementary Figure 62. ¹³C NMR (126 MHz, DMSO-d₆) of (15)



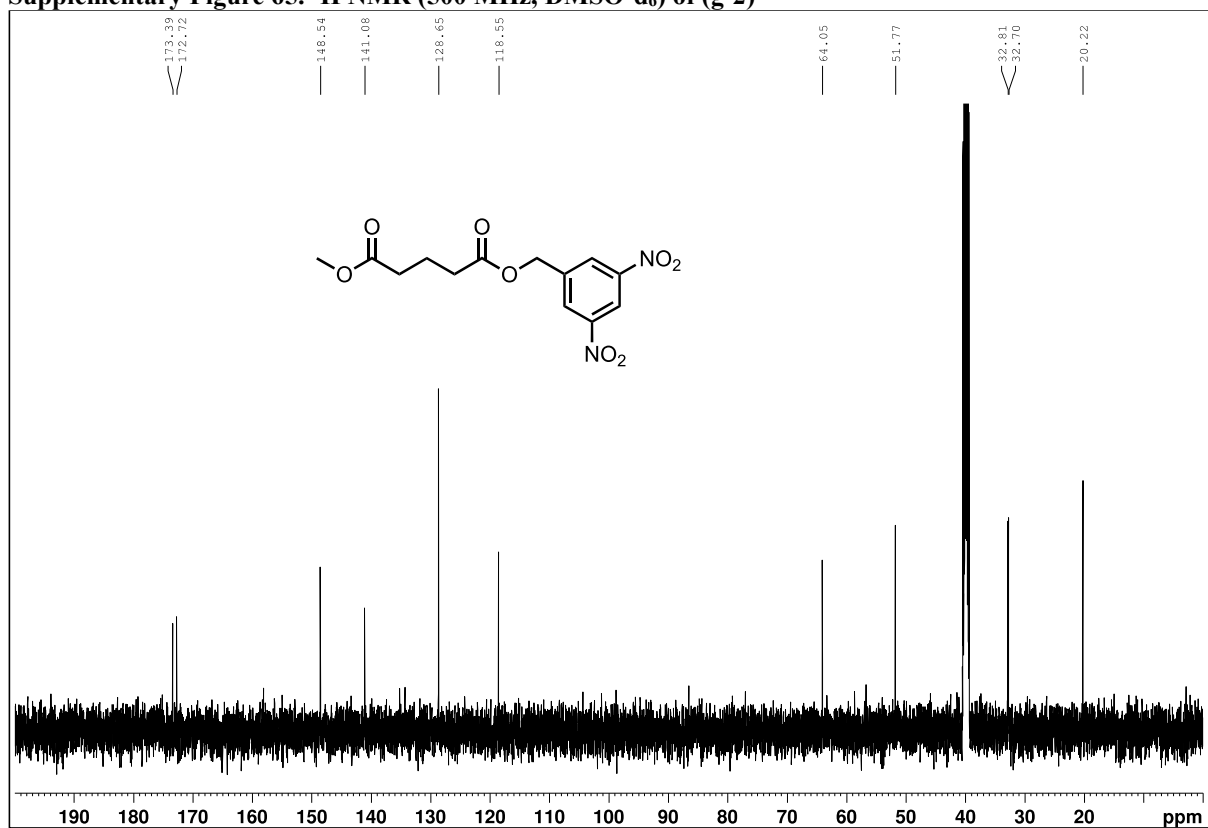
Supplementary Figure 63. ¹H NMR (500 MHz, DMSO-d₆) of Boc-(g)



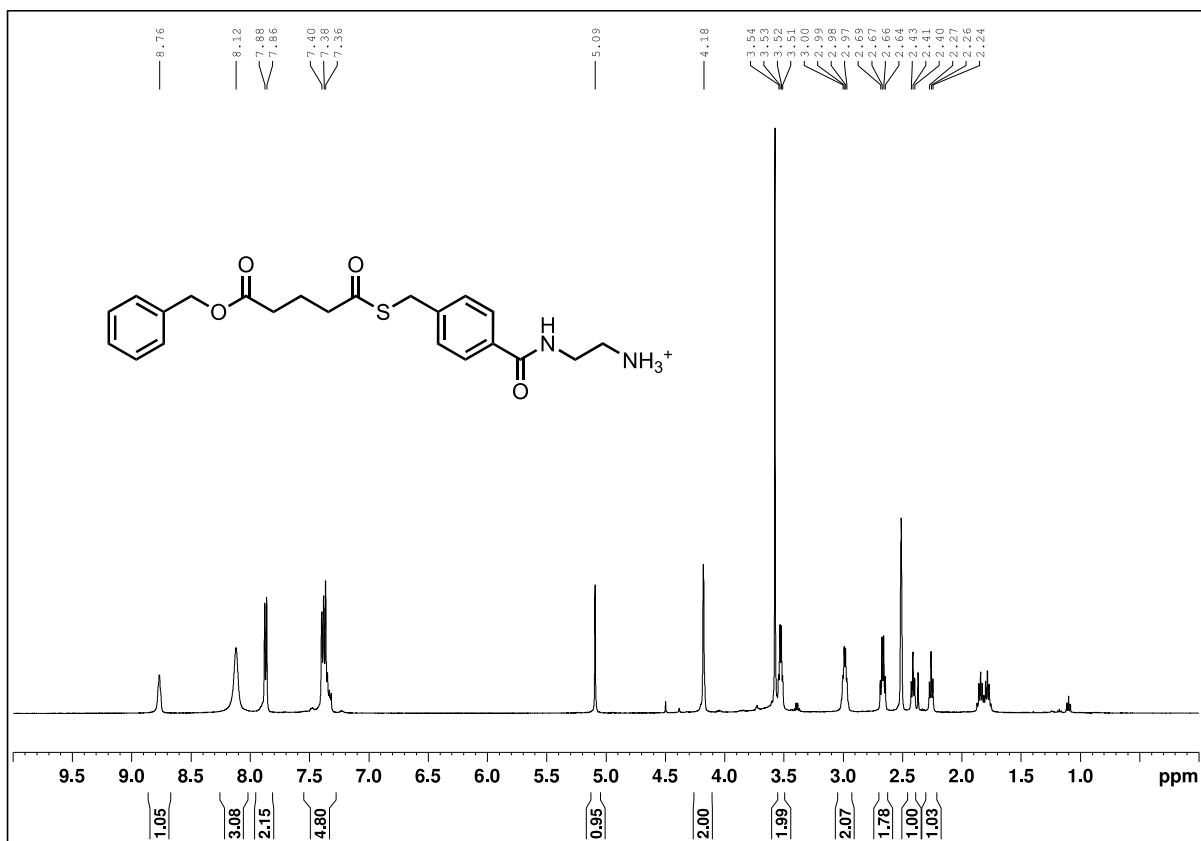
Supplementary Figure 64. ¹³C NMR (126 MHz, DMSO-d₆) of Boc-(g)



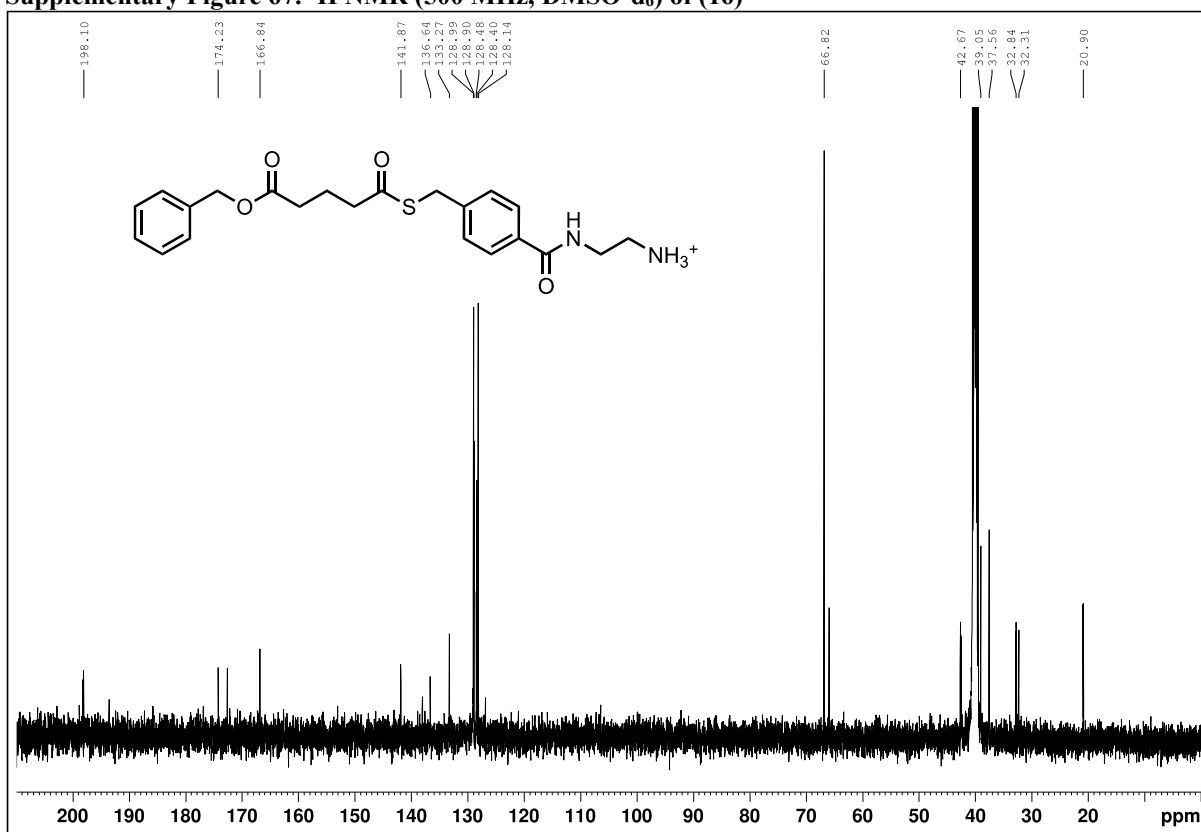
Supplementary Figure 65. ¹H NMR (500 MHz, DMSO-d₆) of (g-2)



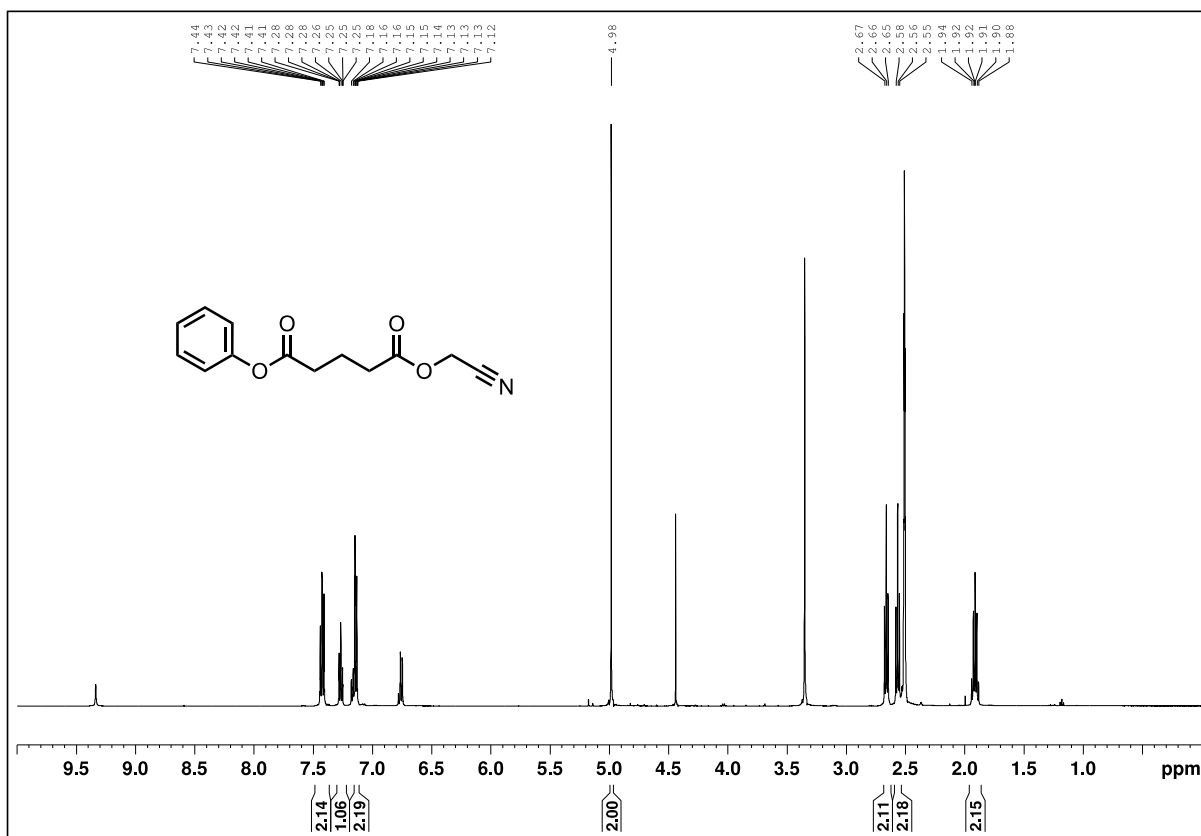
Supplementary Figure 66. ¹³C NMR (126 MHz, DMSO-d₆) of (g-2)



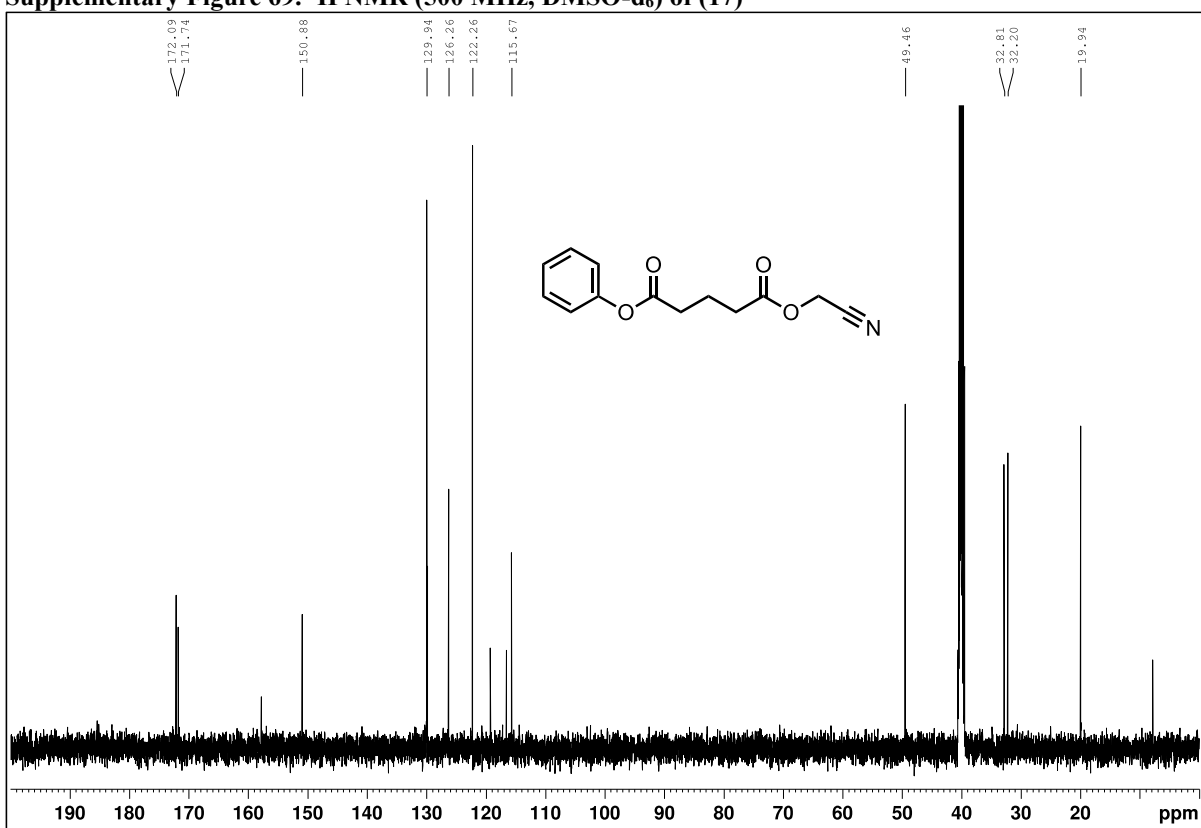
Supplementary Figure 67. ¹H NMR (500 MHz, DMSO-d₆) of (16)



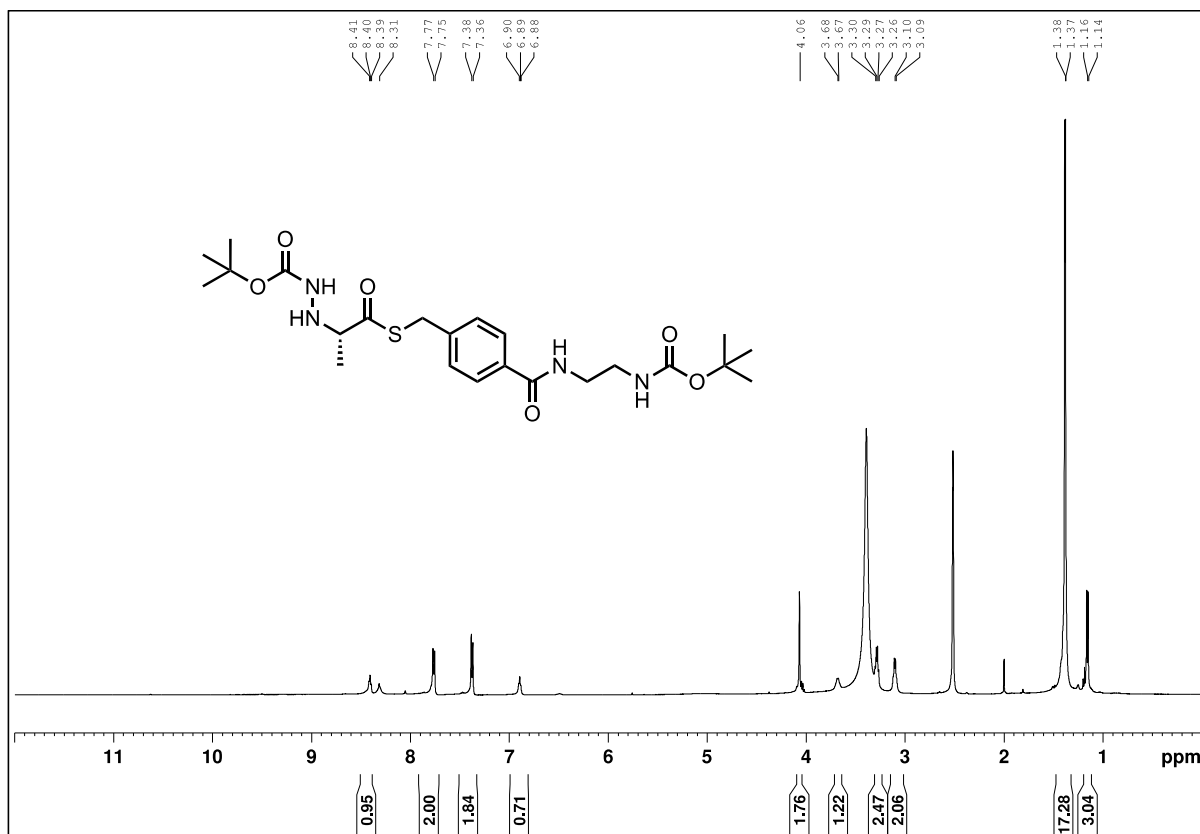
Supplementary Figure 68. ¹³C NMR (126 MHz, DMSO-d₆) of (16)



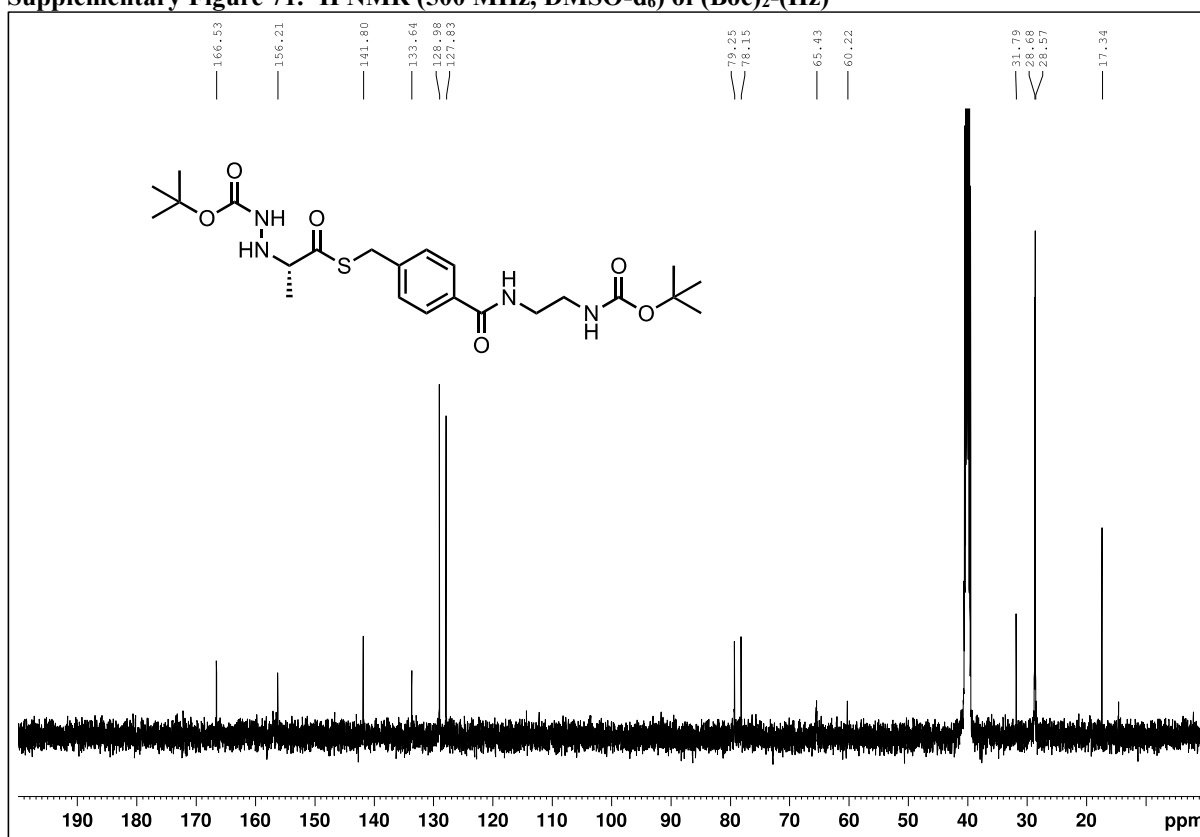
Supplementary Figure 69. ¹H NMR (500 MHz, DMSO-d₆) of (17)



Supplementary Figure 70. ¹³C NMR (126 MHz, DMSO-d₆) of (17)

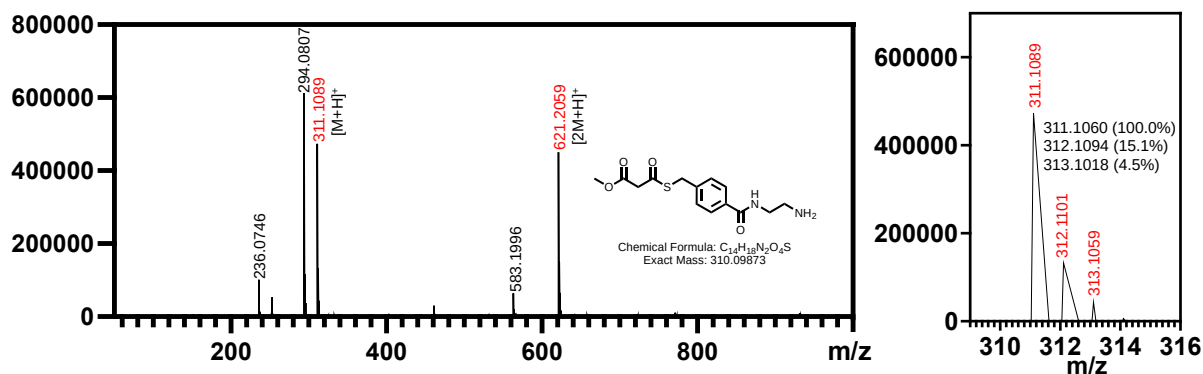


Supplementary Figure 71. ¹H NMR (500 MHz, DMSO-d₆) of (Boc)₂-(Hz)

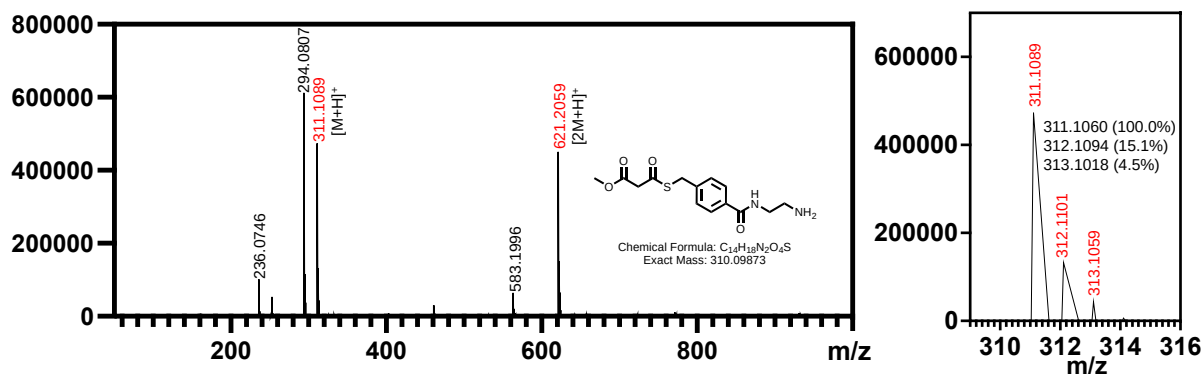


Supplementary Figure 72. ¹³C NMR (126 MHz, DMSO-d₆) of (Boc)₂-(Hz)

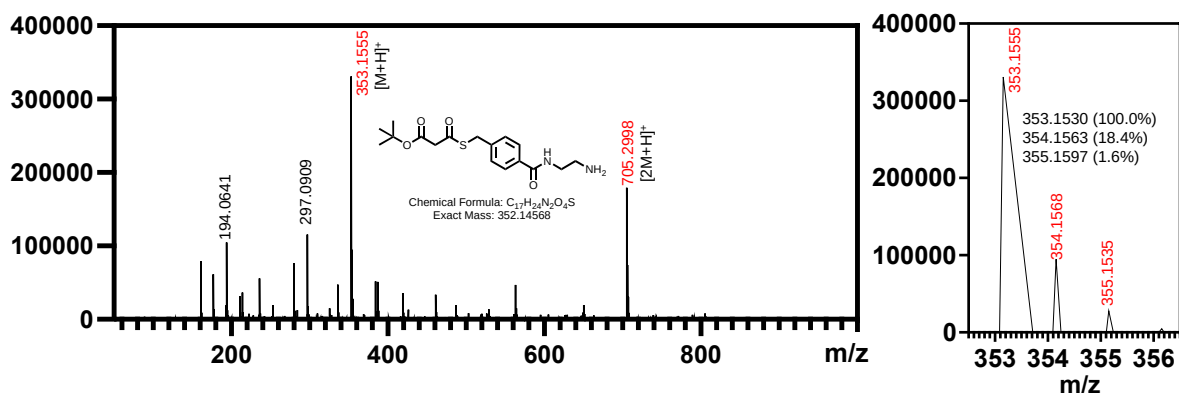
High-Resolution Mass Spectrometry of synthetic non-canonical substrates



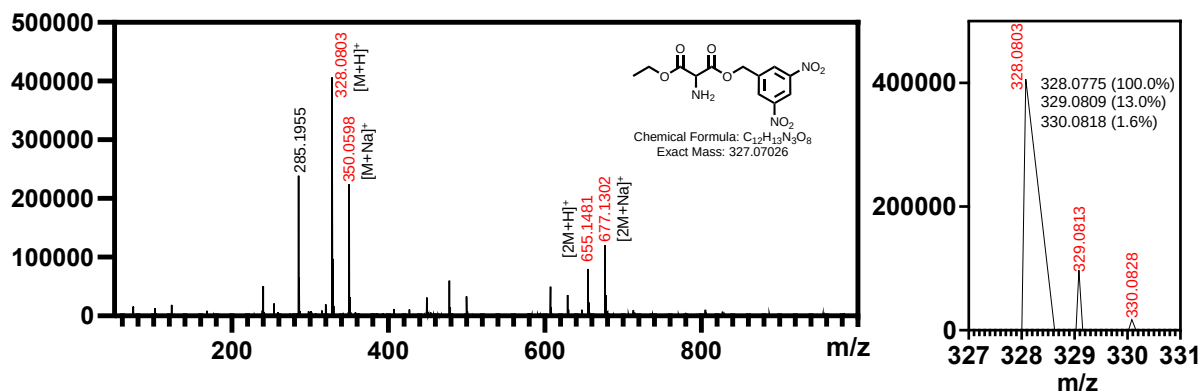
Supplementary Figure 73. HRMS of (m)



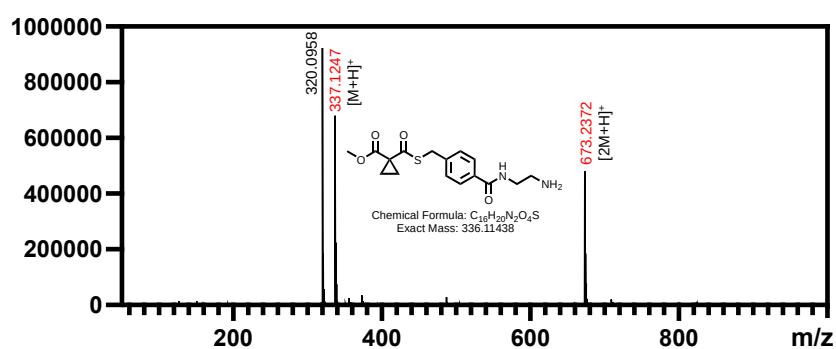
Supplementary Figure 74. HRMS of (m-2)



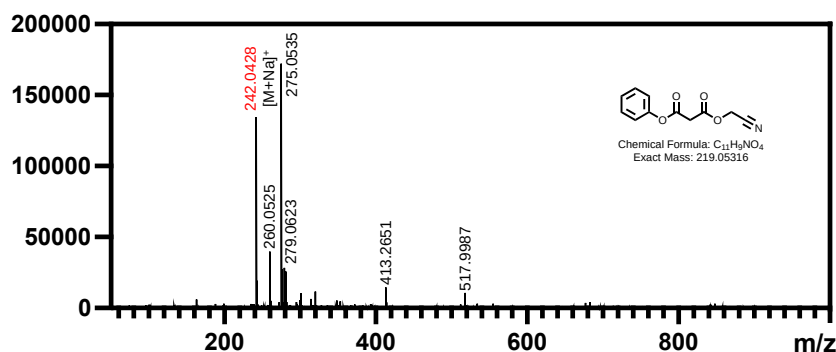
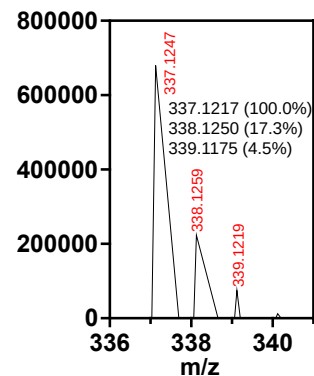
Supplementary Figure 75. HRMS of (0)



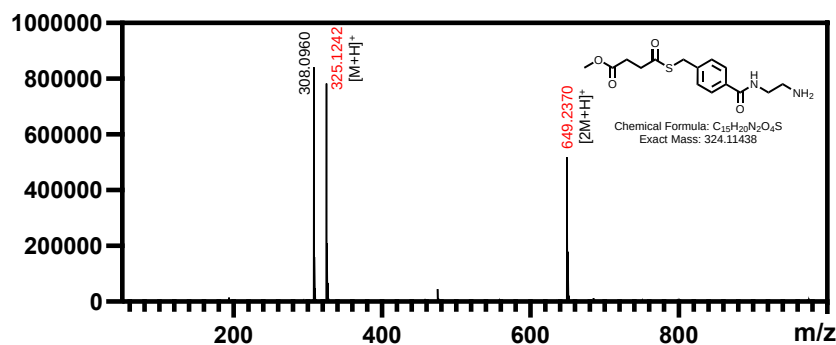
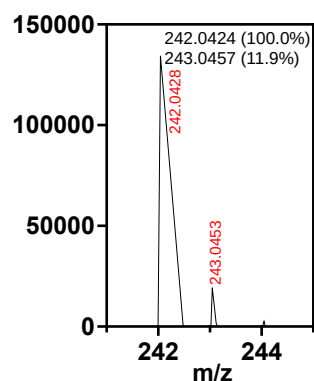
Supplementary Figure 76. HRMS of (1)



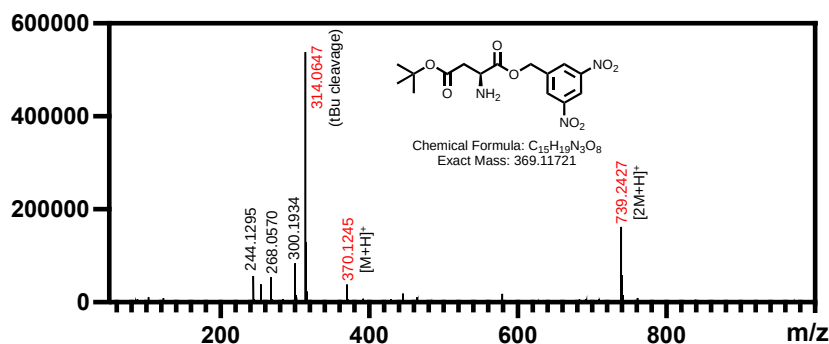
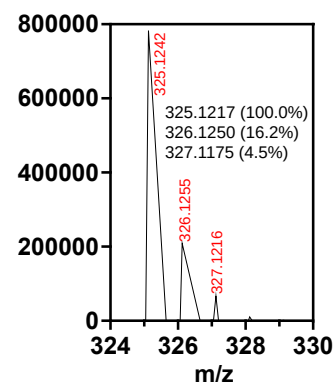
Supplementary Figure 77. HRMS of (2)



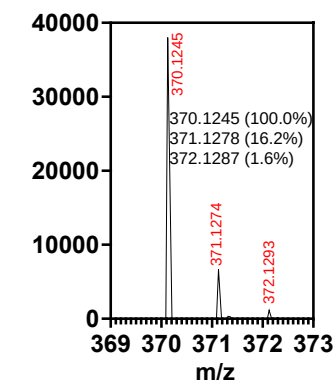
Supplementary Figure 78. HRMS of (3)

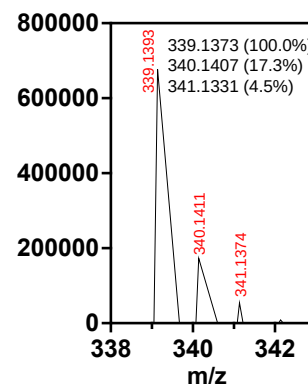
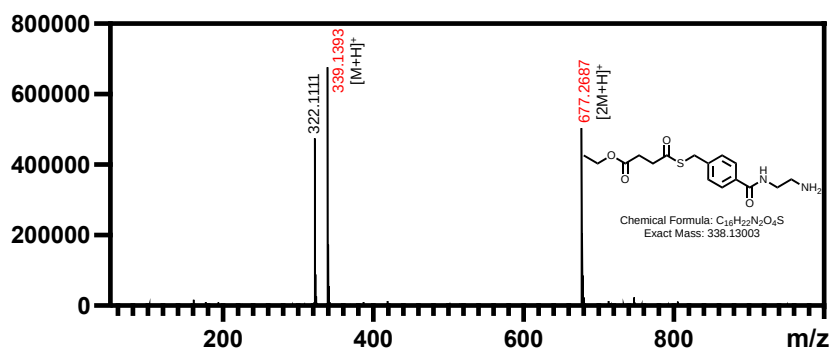


Supplementary Figure 79. HRMS of (s)

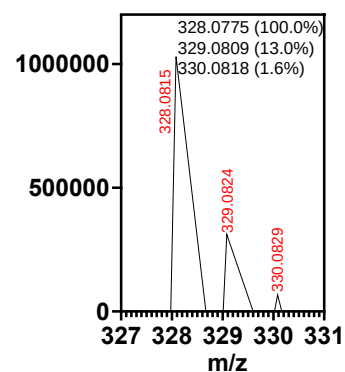
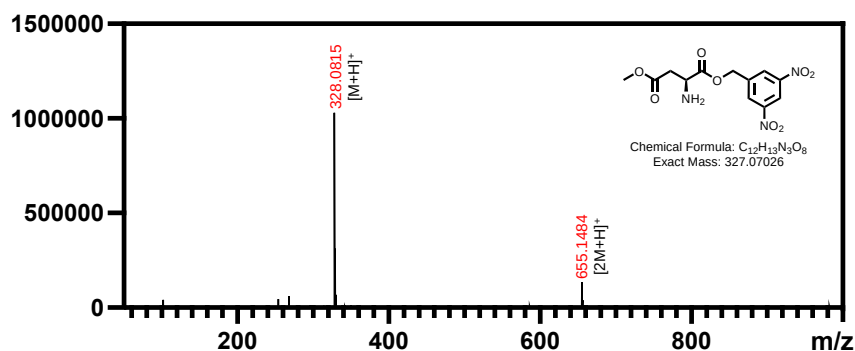


Supplementary Figure 80. HRMS of (4)

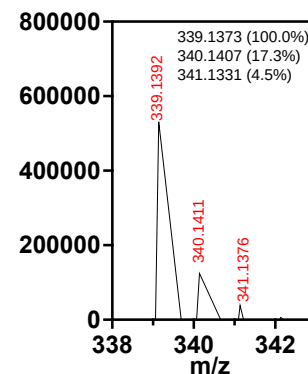
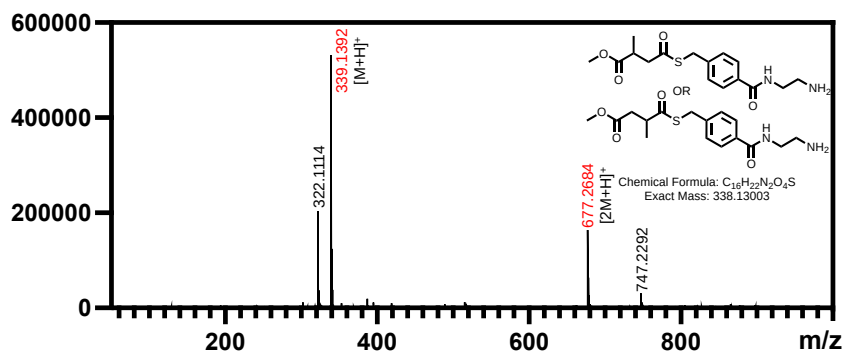




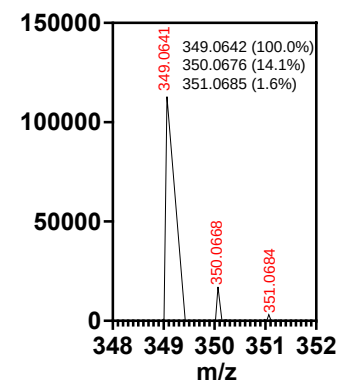
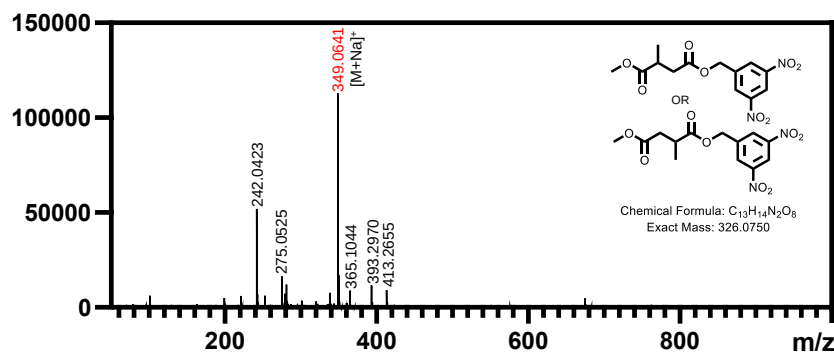
Supplementary Figure 81. HRMS of (5)



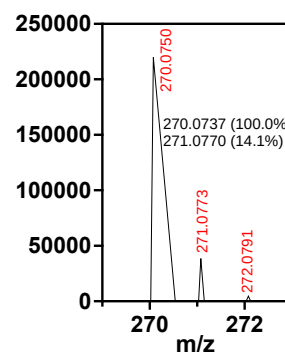
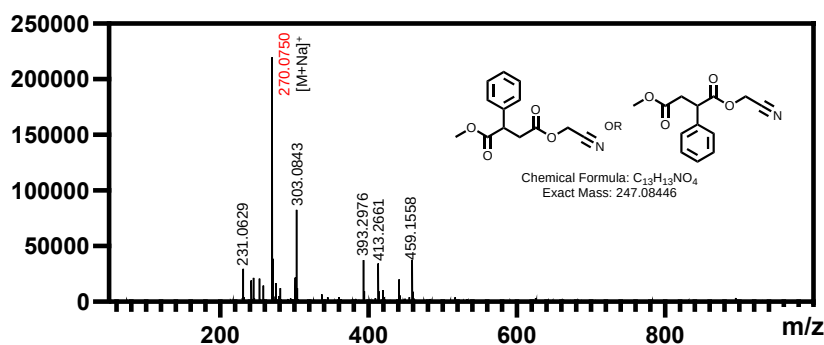
Supplementary Figure 82. HRMS of (6)



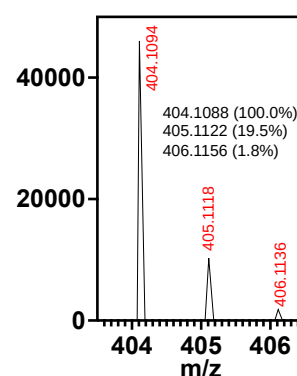
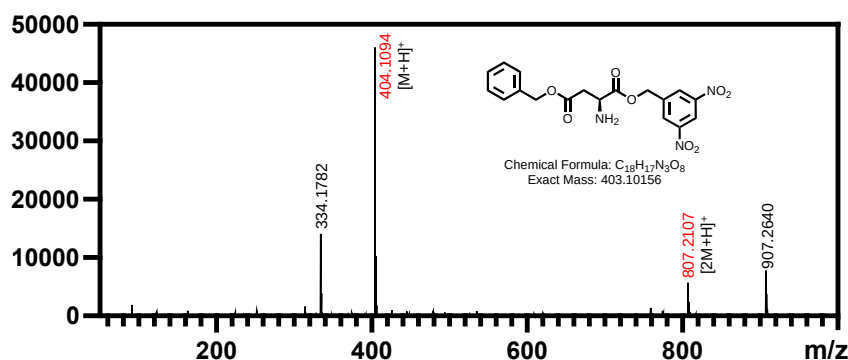
Supplementary Figure 83. HRMS of (7)



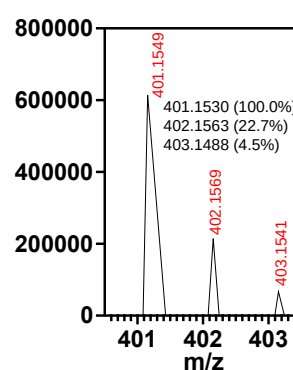
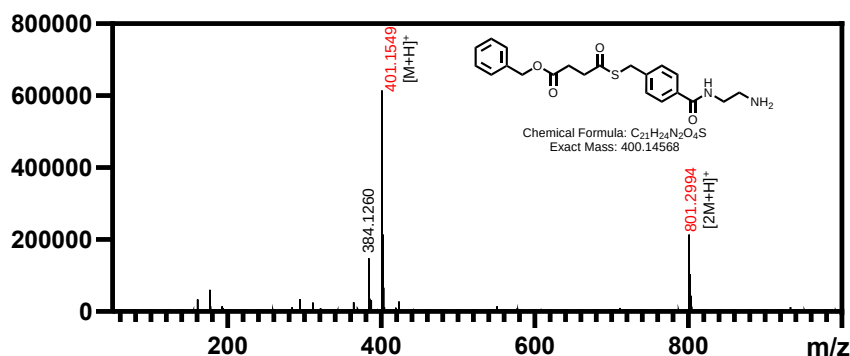
Supplementary Figure 84. HRMS of (7-2)



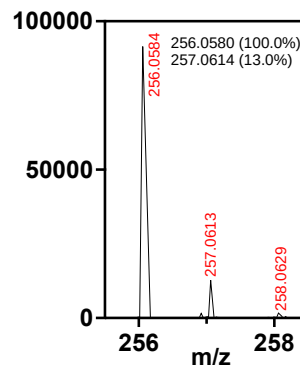
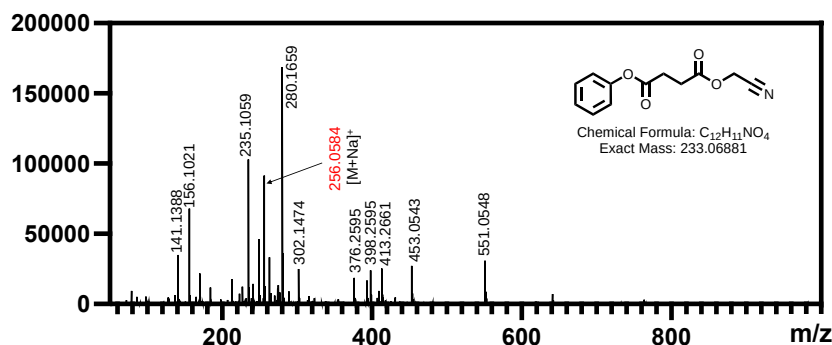
Supplementary Figure 85. HRMS of (8)



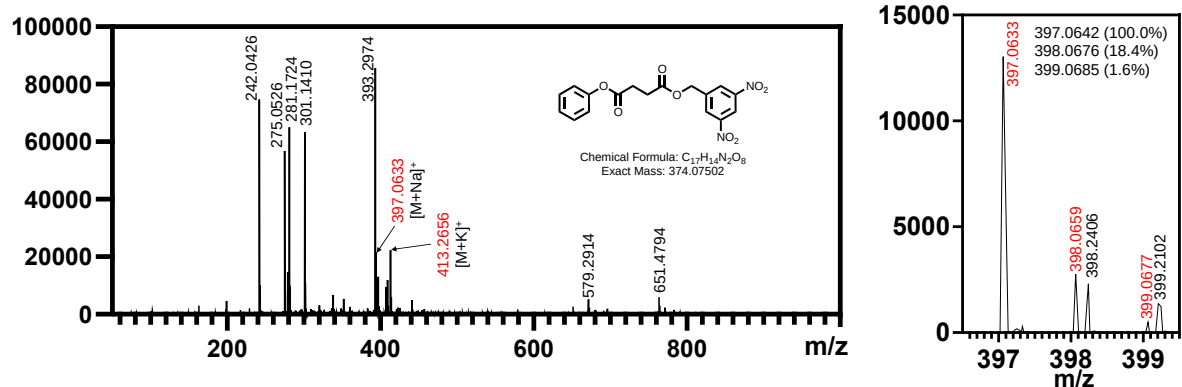
Supplementary Figure 86. HRMS of (9)



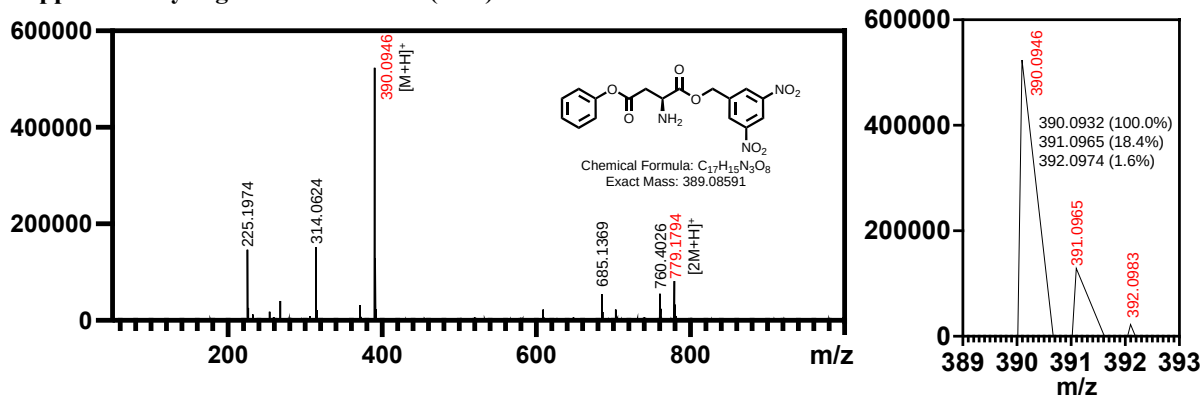
Supplementary Figure 87. HRMS of (10)



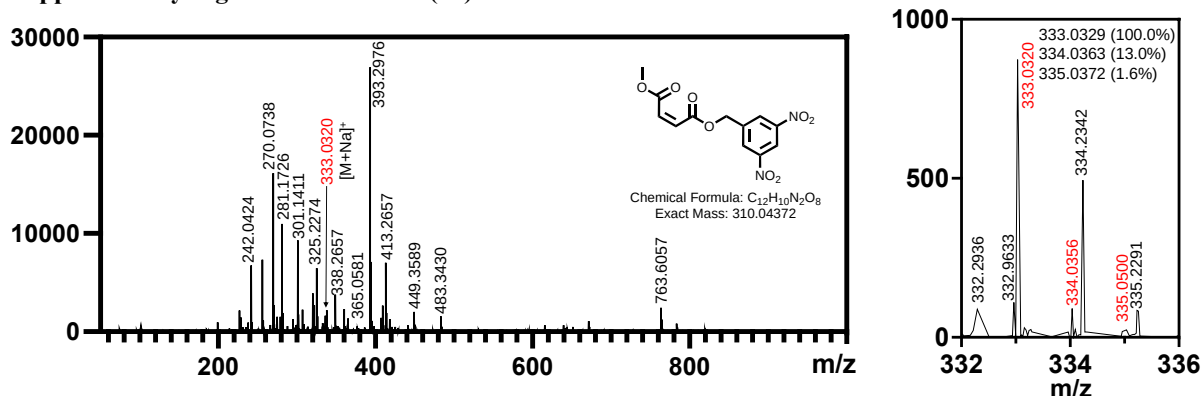
Supplementary Figure 88. HRMS of (11)



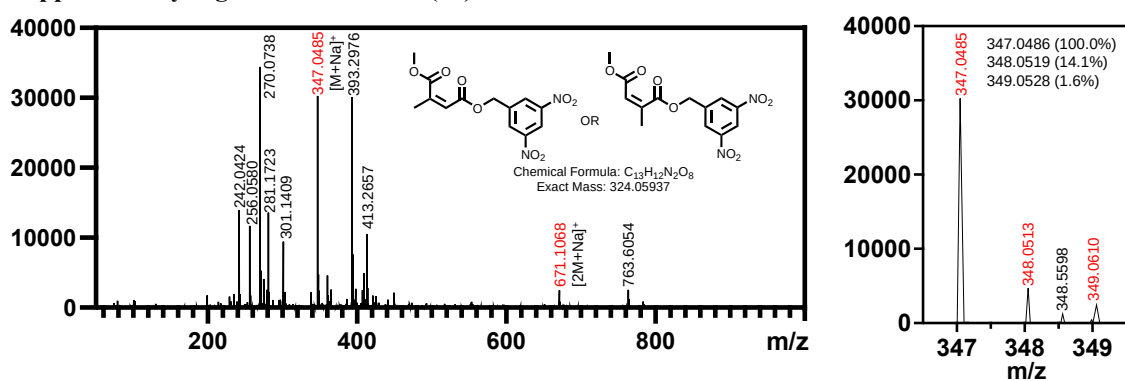
Supplementary Figure 89. HRMS of (11-2)



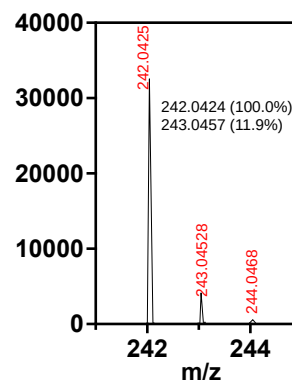
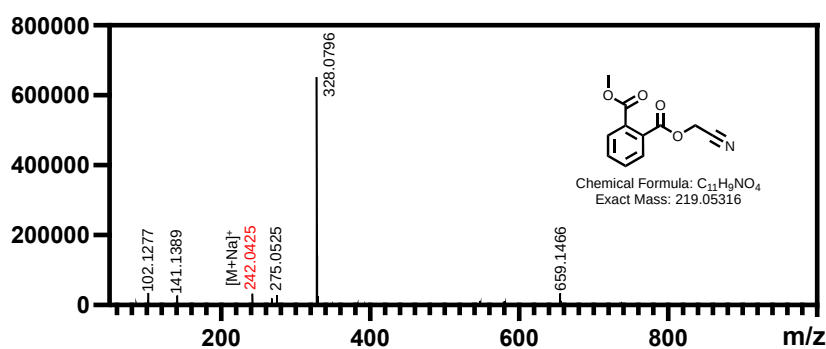
Supplementary Figure 90. HRMS of (12)



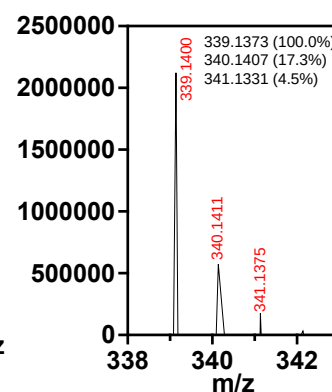
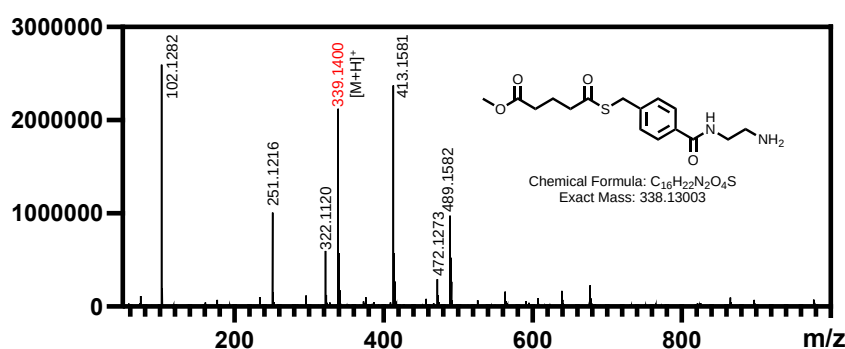
Supplementary Figure 91. HRMS of (13)



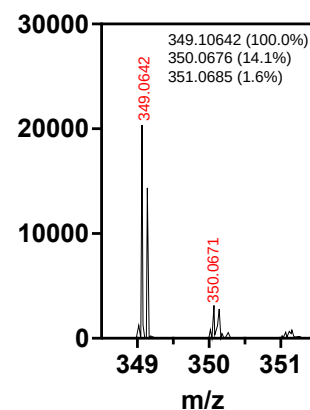
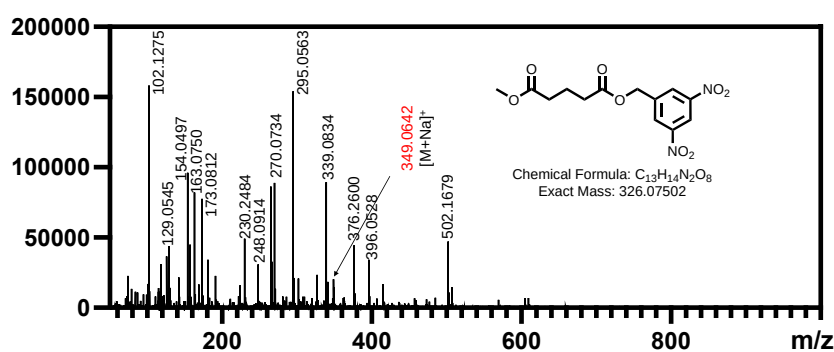
Supplementary Figure 92. HRMS of (14)



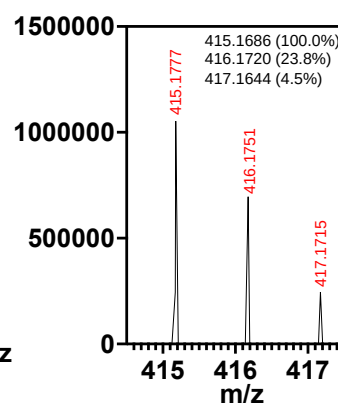
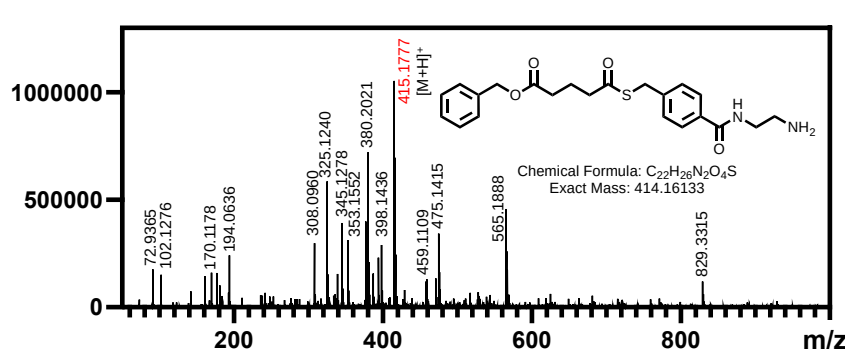
Supplementary Figure 93. HRMS of (15)



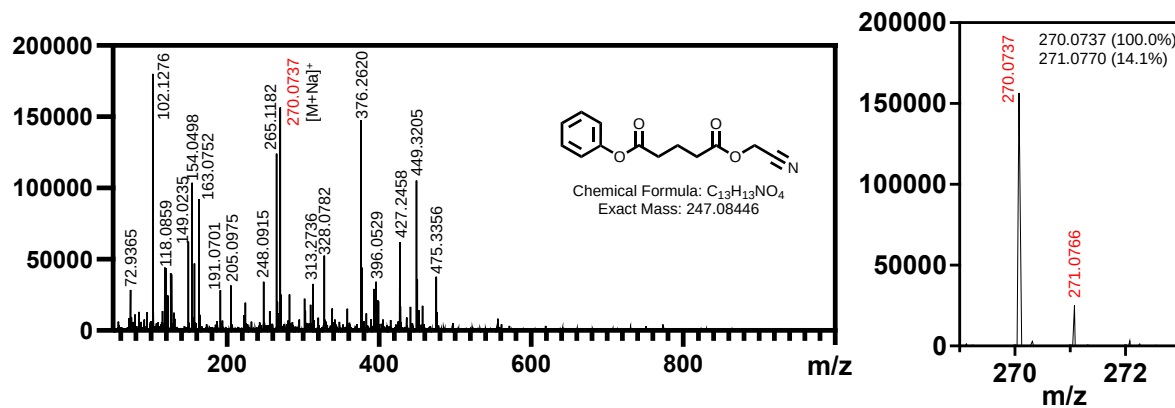
Supplementary Figure 94. HRMS of (g)



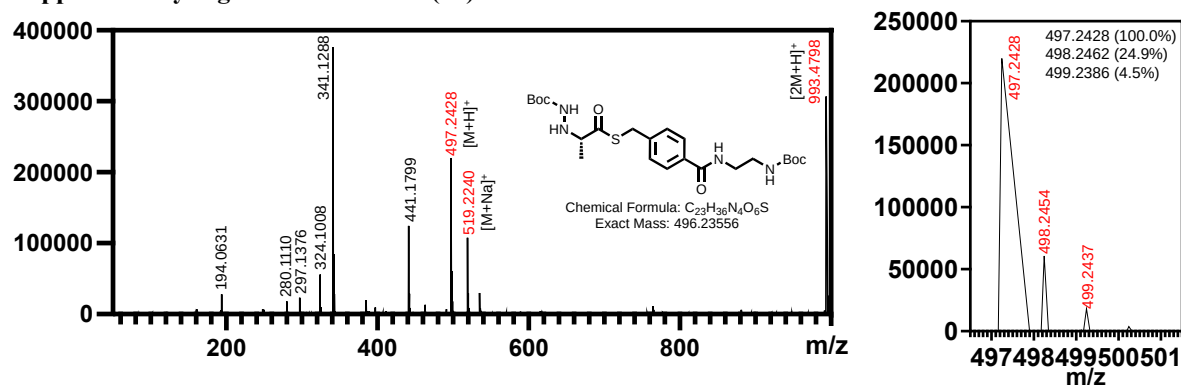
Supplementary Figure 95. HRMS of (g-2)



Supplementary Figure 96. HRMS of (16)

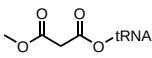
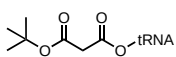
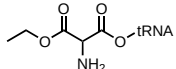
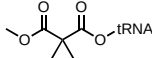
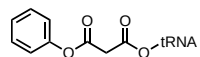
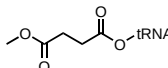
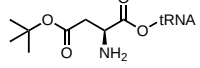
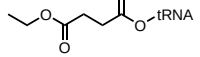
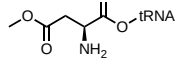
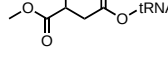
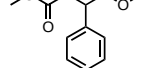
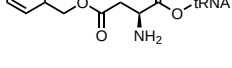
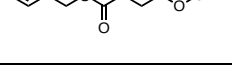


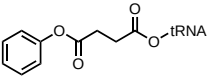
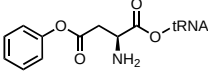
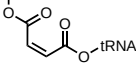
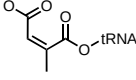
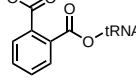
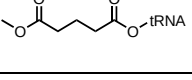
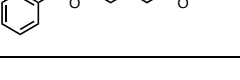
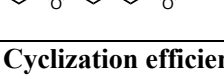
Supplementary Figure 97. HRMS of (17)



Supplementary Figure 98. HRMS of (Boc)₂-(Hz)

Supplementary Tables

	substrates	Structure	LG	Optimal charging condition	Cyclization efficiency
malonate	m m-2		ABT DNB	50 mM pH 8.8 aFx Not charged	100 %
	0		ABT	Not charged	-
	1		DNB	50 mM pH 7.5 dFx	100 %
	2		ABT	50 mM pH 8.8 aFx	100 %
	3		CME	25 mM pH 7.5 eFx	100 %
Succinate	s		ABT	50 mM pH 8.8 aFx	20 %
	4		DNB	50 mM pH 7.5 dFx	0 %
	5		ABT	50mM pH 8.8 aFx	22 %
	6		DNB	50mM pH 7.5 dFx	31 %
	7 7-2		ABT DNB	50 mM pH 8.8 aFx Not charged	33 %
	8		CME	50mM pH 8.8 eFx	33 %
	9		DNB	25 mM pH 8.8 dFx	40 %
	10		ABT	25 mM pH 8.8 aFx	45 %

	11 11-2		CME DNB	50 mM pH 8.8 eFx Not charged	100 %
	12		DNB	10 mM pH 7.5 dFx	100 %
	13		DNB	Not charged	-
	14		DNB	Not charged	-
	15		CME	Not charged	-
Glutarate	g g-2		ABT DNB	50 mM pH 8.8 aFx Not charged	0%
	16		ABT	10 mM pH 8.8 aFx	0 %
	17		CME	Not charged	-

Supplementary Table 1. Cyclization efficiency

Plasmid Map

pJL1_MI-StrepII (MIWSHPQFEK)

GGATCCTGCAGTTGAGATCCTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAACCACCGC
TACCAGCGGTGGTTTGTGGCCGGATCAAGAGCTACCAACTCTTTTCCGAAGGTAAGTGGCTTCAG
CAGAGCGCAGATACCAATACTGTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCT
GTAGCACC GCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGT
CGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTGCGGGCTGAACGG
GGGGTTCGTGCACACAGCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTG
AGCATTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGG
GTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGAAACGCCTGGTATCTTTATAGTCCTGTC
GGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGA
AACGAATTCAGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCC
TCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT[ATGATCTGGTCTCATCCGCAGTTC
GAAAAATAG*]TAAGTCGACCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCAC
CGCTGAGCAATAACTAGCATAACCCCTTGGGGCTCTAAACGGGTCTTGAGGGGTTTTTTTGCTGAAA
GCCAATTCTGATTAGAAAACTCATCGAGCATCAATGAAACTGCAATTTATTCATATCAGGATTATC
AATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGG
ATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCCC
CTCGTCAAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGAGAATGG
CAAAAGCTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCTCGTCATCAAAATCA
CTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCTGAGCGAGACGAAATACGCGATCGCTGT
TAAAAGGACAATTACAAACAGGAATCGAATGCAACCGCGCAGGAACACTGCCAGCGCATCAACA
ATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTCCCGGGGATCGCAGTGGT
GAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTCGGAAGAGGCATAAATCCGTC
AGCCAGTTTAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCTACCTTTGCCATGTTTCAGAAA
CAACTCTGGCGCATCGGGCTTCCCATACAATCGATAGATTGTCGCACCTGATTGCCCCGACATTATCGC
GAGCCCATTTATACCATATAAATCAGCATCCATGTTGGAATTTAATCGCGGCTTCGAGCAAGACGTT
TCCCGTTGAATATGGCTCATAACACCCCTTGTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTTAT
GATGATATATTTTTATCTTGTGCAATGTAACATCAGAGATTTTGAGACACAACGT

pJL1_M-StrepII-ST (MWSHPQFEKST)

[ATGTGGTCTCATCCGCAGTTCGAAAAATCCACCTAG*]

pJL1_MR-StrepII-DR (MRWSHPQFEKDR)

[ATGCGTTGGTCTCATCCGCAGTTCGAAAAATCCACCGACCGTTAG*]

RNA sequences

eFx

GGAUCGAAAGAUUCCGCGGCCCCGAAAGGGGAUUAGCGUUAGGU

dFx

GGAUCGAAAGAUUCCGCAUCCCCGAAAGGGUACAUGGCGUUAGGU

aFx

GGAUCGAAAGAUUCCGCACCCGCGAAAGGGGUAAGUGGCGUUAGGU

microhelix

ACCGCCGAGACGCUUGUCUCGG

tRNA^{fMet} (CAU)

GGCGGGGUGGAGCAGCCUGGUAGCUCGUCGGGCUCAUAACCCGAAGAUCGUCGGUUCAAAUCC
GGCCCCCGCAACCA

tRNA^{Pro1E2} (GAU)

GGGUGAUUGGCGCAGCCUGGUAGCGCACUUCGUUGAUAACGAAGGGGUCAGGGGUUCGAAUCC
CCUAUCACCCGCCA

tRNA^{Pro1E2} (ACG)

GGGUGAUUGGCGCAGCCUGGUAGCGCACUUCGUUACGAACGAAGGGGUCAGGGGUUCGAAUCC
CCUAUCACCCGCCA

tRNA^{Pro1E2} (GUC)

GGGUGAUUGGCGCAGCCUGGUAGCGCACUUCGUUGUCAACGAAGGGGUCAGGGGUUCGAAUCC
CCUAUCACCCGCCA

P1_{c2}^{UGGU}

GUGGAAGACCCCGUGGAGCUCUUCGGAGUUACCCCGGGGAUAACAGGCUGAUCGUGAGGUUUG
GCACCUCUGGU

P2_c^{UGGU}

GAAGACCCCGUGGAGCUUUACUCUUCGGAGUUUGACUGGGGCGGUCCGGAUAAAAGUUACCC
GGGGAUAACAGGCUGAUCGUGAGGUUUGGCACCUUGGU

P1_{c2}

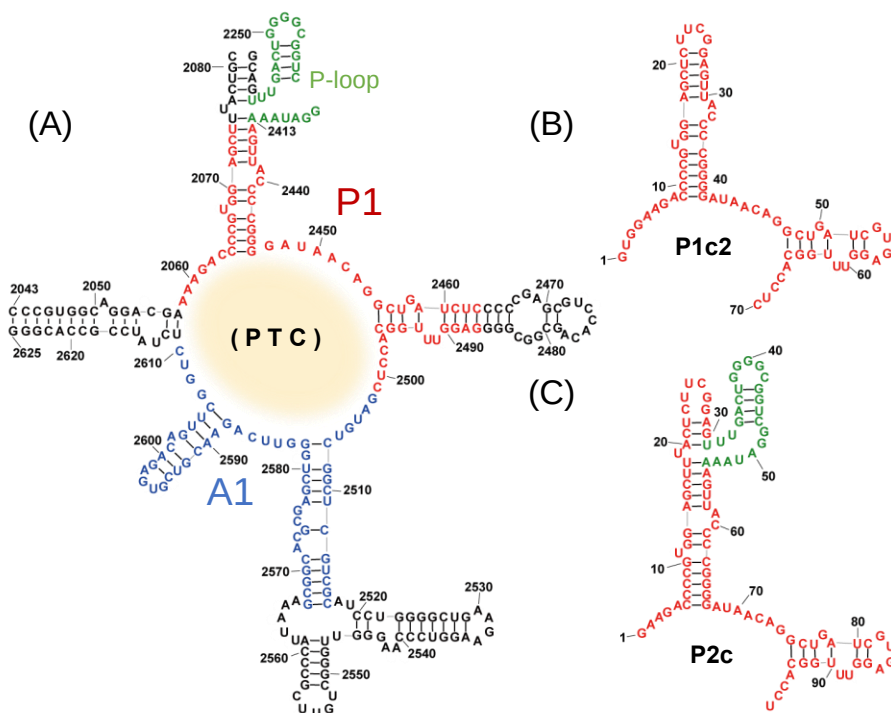
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GCACCUC

P2_c

GAAGACCCCGUGGAGCUUUACUCUUCGGAGUUUGACUGGGGCGGUCCGGAUAAAAGUUACCC
GGGGAUAACAGGCUGAUCGUGAGGUUUGGCACCU

Sequence comparison of two PTC analogues, P1c2 and P2c

P1c2	GUGGAAGACCCCGUGGAGCUCU-----UCG-----GAGU	29
P2c	---GAAGACCCCGUGGAGCUUUACUCUUCGGAGUUUGACUGGGCGGUCCGGAUAAAAGU	57
	***** * * * *	
P1c2	UACCCCGGGGAUAACAGGCUGAUCGUGAGGUUUGGCACCUC	70
P2c	UACCCCGGGGAUAACAGGCUGAUCGUGAGGUUUGGCACCU-	97



(A) The PTC, an aminoacyltransferase ribozyme, resides in the ribosome's large subunit. It facilitates peptide bond formation between adjacent amino acids during translation. The PTC is structurally composed of two major regions: P1 and A1. (B) The P1c2 domain (70-mer) is a functional RNA molecule that mimics the PTC of *T. thermophilus* 23S ribosomal RNA. It is derived from the P1 region within domain V of the ribosome. This domain forms a dimer, creating a symmetrical 3D structure resembling the ribosome's active site and capable of catalyzing peptide bond formation. (C) The P2c domain (97-mer) is also derived from the P1 within domain V of the ribosome, however, it includes an additional P-loop (green), which disrupts the formation of a stable dimeric structure and prevents it from catalyzing peptide bond formation.