Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

Ribosome-mediated incorporation of fluorescent amino acids into peptides *in vitro*

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1. General Methods

1.1 Materials

All reagents were purchased from commercial suppliers and used as received unless otherwise stated. Dry dichloromethane (DCM) and dimethylformamide (DMF) were obtained from a Solvent Delivery System (SDS) equipped with activated neutral alumina columns under argon. Column chromatography were performed on Biotage Isolera System using Silicycle Siliasep HP flash cartridges.

1.2 Characterisation

NMR. ^{1}H and (500 MHz) were recorded at room temperature (298 K). Chemical shifts are reported in δ (ppm) referenced on residual solvent peaks. Coupling constants (J) are expressed in Hertz (Hz). Splitting patterns are designated as: s (singlet), d (doublet), t (triplet), q (quartet), p (pentet or quintet), m (multiplet).

1.3 General Synthetic Procedures

A. Formation of cyanomethyl ester

Carboxylic acid (1 equiv.), triethylamine (1.5 equiv.), chloroacetonitrile (excess), and CH_2Cl_2 were combined in a round bottom flask with a stir bar. After stirring overnight at 25 °C, the reaction mixture was diluted with CH_2Cl_2 and washed with 1N HCl, saturated $NaHCO_3$ solution, water and brine. The organic phase was dried over $MgSO_4$ and concentrated to provide the crude product. The product was purified by flash column chromatography.

B. Formation of dinitrobenzyl ester

Carboxylic acid (1 equiv.), 3,5-dinitrobenzyl alcohol (1 equiv.), EDCI (1 equiv.), DMAP (0.2 equiv.), triethylamine (2 equiv.), and CH_2Cl_2 were combined in a round bottom flask with a stir bar was added. After stirring overnight at 25 °C, the reaction mixture was diluted with CH_2Cl_2 and washed with 1N HCl, water, and brine. The organic phase was dried over $MgSO_4$ and concentrated to provide the crude product. The product was purified by flash column chromatography.

C. Formation of ABT ester

According to standard procedure[1], tert-butyl (2-(4-(mercaptomethyl)benzamido)ethyl) carbamate (ABT) (1 equiv.), carboxylic acid (1.4 equiv.), CH_2Cl_2 (0.3 M), DMAP (2.8 equiv.), and EDC•HCl (2.8 equiv.) were combined in a flask with a stir bar. After stirring for 3 h at 25 °C, the reaction was evaporated under reduced pressure, diluted with EtOAc, and washed with 1M HCl, saturated

$NaHCO_3$ and brine. The organic phase was dried and concentrated to provide the crude product. The product was purified by flash column chromatography.

D. Boc deprotection

Purified product was dissolved in 4M HCl•dioxane (dinitrobenzyl ester and ABT ester) or 10% TFA in DCM (dinitrobenzyl ester) and stirred for 1 h or overnight, respectively. Concentration under reduced pressure provided the product in sufficient purity.

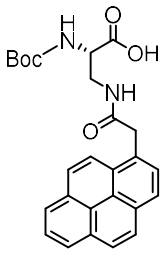
E. Fmoc deprotection

Fmoc-protected ester (1 equiv.) and CH_2Cl_2 were combined in a flask with a stir bar. Then, DBU (0.1 equiv.) was added dropwise. The reaction mixture was stirred at 25 °C for 10 min. The resulting mixture was concentrated to provide the crude product. The product was purified by flash column chromatography.

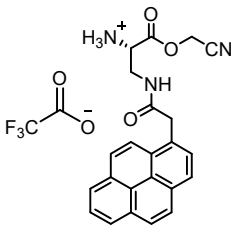
2. Synthesis of FAAs

2.1 Dap-based FAAs

General reaction scheme shown in Supplemental Figures below.

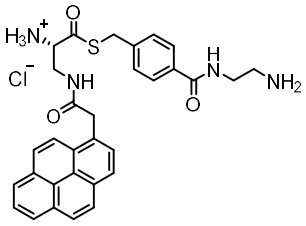


(*S*)-2-((tert-butoxycarbonyl)amino)-3-(2-(pyren-1-yl)acetamido)propanoic acid (Boc-Dap-1). 1-pyreneacetic acid (260 mg, 1.0 mmol) and dry CH_2Cl_2 (25 mL) under N_2 were combined in a flask with a stir bar. Then, oxalyl chloride (0.093 mL, 1.1 mmol) was added followed by a single drop of DMF. The resulting solution was stirred until the gas formation ceased. Then, the reaction mixture was condensed to remove residual oxalyl chloride and redissolved in dry CH_2Cl_2. In a separate vial, Boc-Dap-OH (204 mg, 1.0 mmol) and pyridine (0.161 mL, 2.0 mmol) was dissolved in dry CH_2Cl_2. The solution of acid chloride was then added dropwise to the solution of Boc-Dap-OH and pyridine. The reaction mixture was stirred for 1 h, then diluted with CH_2Cl_2 and washed with water, saturated $CuSO_4$ solution and brine. The organic phase was dried over $MgSO_4$ and concentrated.

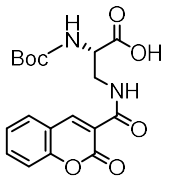


Cyanomethyl (*S*)-2-amino-3-(2-(pyren-1-yl)acetamido)propanoate (Dap-1-CME). Prepared according to the procedure for the formation of cyanomethyl ester using **Boc-Dap-1** (89 mg, 0.20 mmol), triethylamine (0.042 mL, 0.30 mmol), and chloroacetonitrile (5 mL) and deprotected using general procedure D. ^{1}H NMR (500 MHz, DMSO-*d*6) δ 8.49 (br, 4H), 8.29-7.97 (m,

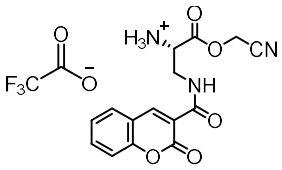
9H), 4.23 (s, 2H), 4.10 (br, 3H), 3.48 (s, 2H), 3.45 (s, 2H) ppm. ^{13}C NMR (125 MHz, DMSO-*d*6) δ 172.1, 167.9, 131.2, 130.8, 130.5, 130.3, 129.5, 129.2, 127.8, 127.7, 127.4, 126.7, 125.6, 125.4, 125.2, 124.5, 124.4, 124.3, 115.8, 52.2, 51.0, 50.9, 28.8. Exact mass for $C_{23}H_{19}N_3O_3$: $[M+H]^+$ = 386.21 (obs) 386.14 (calc)



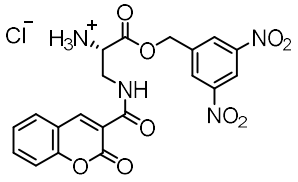
S-(4-((2-aminoethyl)carbamoyl)benzyl) (*S*)-2-amino-3-(2-(pyren-1-yl)acetamido)propanethioate (Dap-1-ABT). Prepared according to the procedure for the formation of ABT ester using **Boc-Dap-1** (89 mg, 0.20 mmol), tert-butyl (2-(4-(mercaptomethyl)benzamido)ethyl) carbamate (ABT) (68 mg, 0.20 mmol), EDCI (76 mg, 0.40 mmol), DMAP (49 mg, 0.40 mmol), and CH_2Cl_2 (5 mL). The final product was purified by flash chromatography and deprotected using general procedure D. ^{1}H NMR (500 MHz, MeOD) δ 8.19 – 8.02 (m, 1H), 7.99 – 7.84 (m, 1H), 7.77 (d, *J* = 8.0 Hz, 1H), 7.39 (d, *J* = 7.9 Hz, 1H), 4.55 (s, 1H), 3.62 – 3.43 (m, 2H), 3.25 (s, 2H), 3.07 (dd, J = 12.2, 6.1 Hz, 2H) ppm. ^{13}C NMR (125 MHz, MeOD) δ 169.53, 142.52, 132.75, 130.83, 127.41, 127.33, 127.24, 127.22, 127.06, 126.76, 125.74, 124.92, 124.87, 124.77, 124.73, 124.50, 122.98, 71.41, 42.96, 39.61, 37.77, 37.38, 36.83, 34.74, 29.35, 25.91, 25.38. Exact mass for $C_{31}H_{30}N_4O_3S$: $[M+H]^+$ theoretical mass not observed, 539.20 (calc)



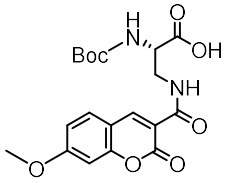
(*S*)-2-((*tert*-butoxycarbonyl)amino)-3-(2-oxo-2*H*-chromene-3-carboxamido)propanoic acid (Boc-Dap-2). Coumarin-3-carboxylic acid (930 mg, 4.9 mmol), CDI (795 mg, 4.9 mmol), and DMF (10 mL) were combined in a flask with a stir bar. After stirring 2 h at 25 °C, Boc-Dap-OH (1.0 g, 4.9 mmol) was added. The reaction mixture was stirred overnight, then diluted with EtOAc and washed with water and brine. The organic phase was dried over $MgSO_4$ and concentrated. Flash column chromatography yielded the product as white solid.



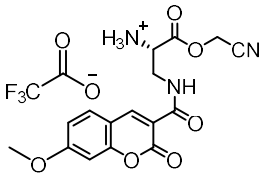
Cyanomethyl (*S*)-2-amino-3-(2-oxo-2H-chromene-3-carboxamido)propanoate (Dap-2-CME) Prepared according to the procedure for the formation of cyanomethyl ester using **Boc-Dap-2** (800 mg, 2.1 mmol), chloroacetonitrile (0.14 mL, 2.2 mmol), triethylamine (0.44 mL, 3.2 mmol), and CH_2Cl_2 (10 mL). ^{1}H NMR (500 MHz, MeOD) δ 8.92 (s, 1H), 7.87 (d, *J* = 7.7 Hz, 1H), 7.78 (t, *J* = 7.8 Hz, 1H), 7.47 (dt, *J* = 7.4, 3.2 Hz, 2H), 4.84 – 4.76 (m, 2H), 4.47 (t, *J* = 5.1 Hz, 1H), 4.12 – 4.05 (m, 2H) ppm. ^{13}C NMR (125 MHz, MeOD) δ 167.11, 164.02, 160.83, 154.62, 148.58, 134.43, 130.00, 125.18, 118.46, 117.74, 116.12, 68.29, 63.14, 53.74, 52.89, 39.14. Exact mass calcd for C15H14N3O5 $[M+H]^+$ 316.09, found 316.09.



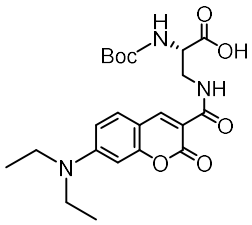
3,5-dinitrobenzyl (*S*)-2-amino-3-(2-oxo-2H-chromene-3-carboxamido)propanoate (Dap-2-DNB). Prepared according to the procedure for formation of dinitrobenzyl ester using **Boc-Dap-2** (800 mg, 2.1 mmol), 3,5-dinitrobenzyl alcohol (420 mg, 2.1 mmol), DDC (440 mg, 2.1 mmol), DMAP (52 mg, 0.42 mmol), and CH_2Cl_2 (10 mL). The product was purified by flash chromatography and deprotected using general procedure D. The final product was obtained as white solid. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.90 (d, *J* = 1.65, 1H), 8.83 (s, 1H), 8.73 (s, 2H), 7.83 (td, *J* = 20.0, 7.6 Hz, 2H), 7.47 (td, *J* = 10.0, 8.4 Hz, 2H), 5.59 (q, *J* = 32.5, 12.9, 2H), 4.47 (t, *J* =4.9 Hz ,1H), 3.76 (t, *J* = 4.8 Hz, 1H) ppm. ^{13}C NMR (125 MHz, DMSO-*d*6) δ 168.3, 162.6, 160.2, 154.2 (2C), 148.3, 139.9, 134.9, 130.7, 129.0 (2C), 125.7, 118.5, 116.6, 72.6, 70.9, 65.7, 60.6, 51.6, 44.0 Exact mass for $C_{20}H_{16}N_4O_9$: $[M+H]^+$ = 457.16 (obs) 457.09 (calc)



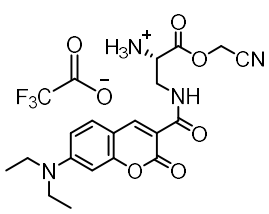
(*S*)-2-((tert-butoxycarbonyl)amino)-3-(7-methoxy-2-oxo-2H-chromene-3-carboxamido)propanoic acid (Boc-Dap-3). 7-methoxycoumarin-3-carboxylic acid (540 mg, 2.45 mmol), CDI (437 mg, 2.69 mmol), and DMF (40 mL) were combined in a round bottom flask with a stir bar. After stirring 2 h at 25 °C, Boc-DAP-OH (500 mg, 2.45 mmol) was added. The reaction mixture was stirred overnight, then diluted with EtOAc and washed with water, and brine. The organic phase was dried over $MgSO_4$ and concentrated. Flash column chromatography yielded the product as white solid.



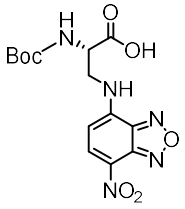
Cyanomethyl (*S*)-2-amino-3-(2-oxo-2H-chromene-3-carboxamido)propanoate (Dap-3-CME). Prepared according to the procedure for the formation of cyanomethyl ester using **Boc-Dap-3** (100 mg, 0.25 mmol), triethylamine (0.15 mL, 0.38 mmol), and chloroacetonitrile (5 mL). The product was purified by flash chromatography and deprotected using general procedure D. The final product was obtained as yellow powder. ^{1}H NMR (500 MHz, MeOD) δ 9.19 (dt, *J* = 17.7, 6.4 Hz, 1H), 8.70 (d, *J* = 6.4 Hz, 2H), 7.63 (d, *J* = 8.7 Hz, 2H), 6.93 (d, *J* = 11.1 Hz, 2H), 6.89 (s, 2H), 5.05 – 4.93 (m, 2H), 4.62 (d, *J* = 14.4 Hz, 1H), 4.55 (d, *J* = 14.5 Hz, 1H), 4.37 (t, *J* = 5.2 Hz, 1H), 4.33 (t, *J* = 5.2 Hz, 1H), 3.94 – 3.87 (m, 4H) ppm. ^{13}C NMR (125 MHz, MeOD) δ 167.17, 166.91, 166.25, 165.73, 165.67, 164.62, 164.56, 164.53, 161.38, 161.21, 156.98, 156.96, 148.80, 148.58, 131.29, 131.24, 114.00, 113.85, 113.80, 113.77, 113.75, 113.50, 112.12, 112.11, 99.96, 63.56, 55.42, 53.75, 52.94, 52.59, 51.09, 51.05, 50.04, 42.56, 39.29, 39.18, 39.06, 27.42, 27.21. δ Exact mass for $C_{16}H_{16}N_3O_6$: $[M+H]^+$ = 346.10 (obs), 346.10 (calc)



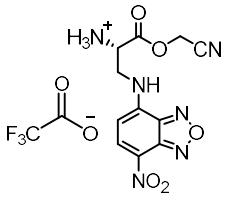
(*S*)-2-((*tert*-butoxycarbonyl)amino)-3-(7-(diethylamino)-2-oxo-2*H*-chromene-3-carboxamido)propanoic acid (Boc-Dap-4). 7-(diethylamino)coumarin-3-carboxylic acid (1.0 g, 3.83 mmol), CDI (651 mg, 4.02 mmol), and DMF (10 mL) were combined in a round bottom flask with a stir bar. After stirring 2 h at 25 °C, Boc-DAP-OH (782 mg, 4.9 mmol) was added. The reaction mixture was stirred overnight, then diluted with EtOAc and washed with water and brine. The organic phase was dried over $MgSO_4$ and concentrated. Flash column chromatography yielded the product as yellow solid.



Cyanomethyl (*S*)-2-amino-3-(7-(diethylamino)-2-oxo-2H-chromene-3-carboxamido)propanoate (Dap-4-CME). Prepared according to the procedure for the formation of cyanomethyl ester using **Boc-Dap-4** (100 mg, 0.22 mmol), triethylamine (0.1 mL, 0.33 mmol), and chloroacetonitrile (3 mL). The product was purified by flash chromatography and deprotected using general procedure D. The final product was obtained as yellow powder. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.94 (t, *J* = 6.0 Hz, 1H), 8.66 (s, 1H), 8.54 (s, 3H), 7.70 (d, *J* = 9.0 Hz, 1H), 6.82 (dd, *J* = 8.2, 2.1 Hz, 1H), 6.63 (d, *J* = 1.6 Hz), 5.11 (s, 2H), 4.37 (t, *J* = 5.1 Hz, 1H), 3.81-3.77 (m, 2H), 3.48 (q, *J* = 7.0 and 7.1 Hz, 4H), (t, *J* = 7.1 Hz, 6H) ppm. ^{13}C NMR (125 MHz, DMSO-*d*6) δ 168.0, 164.1, 161.8, 157.8, 153.1, 148.5, 132.2, 115.8, 110.7, 109.1, 108.0, 96.3, 65.3, 51.9, 51.0, 44.8 (2C), 12.7 (2C). Exact mass for $C_{19}H_{22}N_4O_5$: $[M+H]^+$ = 387.24 (obs), 387.41 (calc)

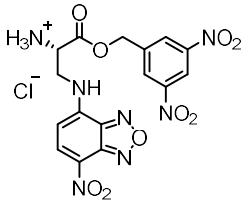


(*S*)-2-((tert-butoxycarbonyl)amino)-3-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)propanoic acid (Boc-Dap-5). Boc-Dap-OH (1.0 g, 4.89 mmol), $NaHCO_3$ (1.56 g, 14.7 mmol), and H_2O (1.25 mL) were combined in a round bottom flask with a stir bar and heated to 55 °C. In a separate vial, 4-chloro-7-nitrobenzofurazan (1.0 g, 5.13 mmol) was dissolved in MeOH (8 mL) and then added to the reaction mixture dropwise over 10 minutes. The reaction mixture was refluxed for 1 h. Then, MeOH was removed rotatory evaporation. The remaining mixture was acidified to pH 3-4 and rinsed with CH_2Cl_2 three times. The organic phase was dried over $MgSO_4$ and concentrated.

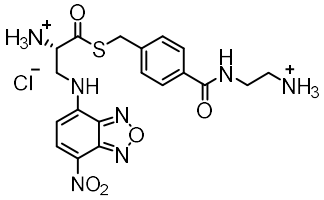


Cyanomethyl (*S*)-2-amino-3-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)propanoate (Dap-5-CME). Prepared according to the procedure for the formation of cyanomethyl ester using **Boc-Dap-5** (273 mg, 0.74 mmol), triethylamine (0.16 mL, 1.11 mmol), and chloroacetonitrile (3 mL). The product

was purified by flash chromatography and deprotected using general procedure D. The final product was obtained as orange powder. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.64 (s, 3H), 8.52 (d, *J* = 8.75 Hz, 1H), 6.46 (d, *J* = 13.85 Hz, 1H), 5.46 (s, 2H), 4.565 (t, *J* = 7.0 Hz, 1H), 4.05 (br, 2H) ppm. ^{13}C NMR (125 MHz, DMSO-*d*6) δ 167.7, 148.2, 139.6, 129.1, 126.7, 118.7, 117.2, 67.7, 65.9, 65.3, 61.6. Exact mass for $C_{11}H_{10}N_6O_5$: [M+DMSO-d_6 (84)]$^+$ = 390.35 (obs), 390.07 (calc)



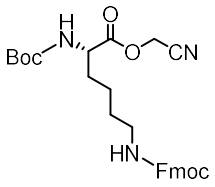
3,5-dinitrobenzyl (*S*) 2-amino-3-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)propanoate (Dap-5-DNB). Prepared according to the procedure for the formation of dinitrobenzyl ester using **Boc-Dap-5** (273 mg, 0.74 mmol), 3,5-dinitrobenzyl alcohol (147 mg, 0.74 mmol), EDCI (143 mg, 0.744 mmol), DMAP (18 mg, 0.15 mmol), triethylamine (0.21 mL, 1.49 mmol), and CH_2Cl_2. The product was purified by flash chromatography and deprotected using general procedure D. The final product was obtained as orange powder. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.23 (s, 1H), 8.87 (s, 3H), 8.57 (d, *J* = 7.2 Hz, 1H), 6.63 (m, 1H), 4.58 (s, 2H), 4.21-4.12 (m, 2H), 3.8-3.67 (br, 1H) ppm. ^{13}C NMR (125 MHz, DMSO-*d*6) δ 168.3, 167.4 (2C), 145.0, 144.4, 138.1, 122.6, 115.6, 70.8, 68.2, 64.7, 64.0, 62.5, 51.6, 51.1, 43.9. Exact mass for $C_{16}H_{13}N_7O_9$: [M+H]$^+$ = 447.27 (obs), 447.32 (calc)



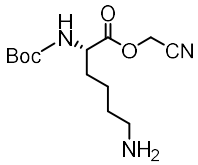
S-(4-((2-aminoethyl)carbamoyl)benzyl) (*S*)-2-amino-3-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)propanethioate (Dap-5-ABT). Prepared according to the procedure for formation of ABT ester using **Boc-Dap-5** (158 mg, 0.43 mmol), tert-butyl (2-(4-(mercaptomethyl)benzamido)ethyl) carbamate (ABT) (145 mg, 0.43 mmol), EDCI (165 mg, 0.86 mmol), DMAP (105 mg, 0.86 mmol), and CH_2Cl_2 (5 mL). The product was purified by flash chromatography and deprotected using general procedure D. The final product was obtained as orange powder. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.89 (s, 3H), 8.71 (t, *J* = 5.2 Hz, 1H), 8.51 (d, *J* = 8.8 Hz, 1H), 8.06 (s, 3H), 7.75 (d, *J* = 8.0 Hz) 7.31 (d, *J* = 8.0 Hz, 2H), 6.53 (d, *J* = 8.8 Hz, 1H), 4.62 (t, *J* = 6.2 Hz, 1H), 4.26 (s, 2H), 4.15-4.00 (m, 2H), 3.51 (q, J = 5.65, 5.60Hz, 2H), 2.98 (q, *J* = 5.35, 5.30 Hz, 2H) ppm. ^{13}C NMR (125 MHz, DMSO-*d*6) δ 194.5, 166.5, 140.6, 133.2 (2C), 129.0 (4C), 127.9 (4C), 57.3, 39.0, 37.5, 34.6, 32.7, 31.7. Exact mass for $C_{19}H_{21}N_7O_5S$: [M+H]$^+$ = 460.22 (obs), 460.48 (calc)

2.2 Lys-based FAAs

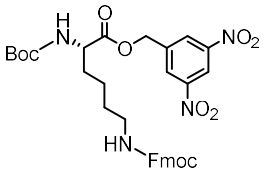
General reaction scheme shown in Supplemental Figures below.



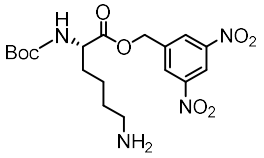
Cyanomethyl N6-(((9H-fluoren-9-yl)methoxy)carbonyl)-N2-(tert-butoxycarbonyl)-L-lysinate (Boc-Lys(Fmoc)-CME). Boc-Lys(Fmoc)-OH (937 mg, 2.0 mmol), chloroacetonitrile (1.26 mL, 20.0 mmol), K_2CO_3 (414 mg, 3.0 mmol), and DMF (20 mL) were combined in a round bottom flask with a stir bar. The reaction mixture was stirred at 25 °C for 72 h. Then, the reaction was quenched by $NaHSO_4$ and rinsed with EtOAc three times. The organic phase was washed with water and brine, dried over $MgSO_4$, and concentrated.



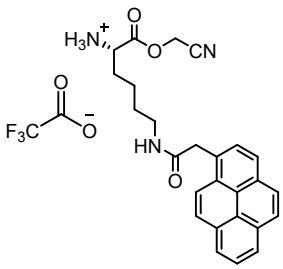
Cyanomethyl (*tert*-butoxycarbonyl)-*L*-lysinate (Boc-Lys-CME). Boc-Lys(Fmoc)-CME (690 mg, 1.21 mmol) and CH_2Cl_2 (10 mL) were combined in a round bottom flask with a stir bar. Then, DBU (0.15 mL. 0.121 mmol) was added dropwise. The reaction mixture was stirred at 25 °C for 10 min. Then, the solvent was removed by rotatory evaporation. Flash column chromatography yielded the product as white powder.



3,5-dinitrobenzyl N^6-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-N^2-(*tert*-butoxycarbonyl)-*L*-lysinate (Boc-Lys-(Fmoc)-DNB). Boc-Lys(Fmoc)-OH (937 mg, 2.0 mmol), 3,5-dinitrobenzyl chloride (866 mg, 4.0 mmol), K_2CO_3 (828 mg, 6.0 mmol), and DMF (10 mL) were combined in a round bottom flask with a stir bar. The reaction mixture was stirred at 25 °C for 72 h. Then, the reaction was quenched by $NaHSO_4$ and rinsed with EtOAc three times. The organic phase was washed with water and brine, dried over $MgSO_4$, and concentrated. Flash column chromatography yielded the product as white solid.

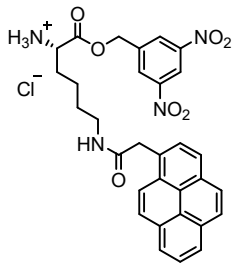


3,5-dinitrobenzyl (tert-butoxycarbonyl)-L-lysinate (Boc-Lys-DNB). Prepared according to general procedure E for Fmoc deprotection using **Boc-Lys-(Fmoc)-DNB**. The product was purified by flash chromatography.

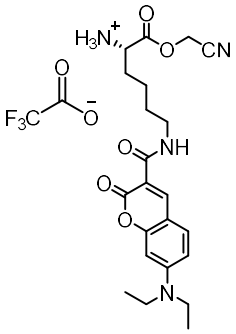


Cyanomethyl N6-(2-(pyren-1-yl)acetyl)-*L*-lysinate (Lys-1-CME). Need a preparation method. **Boc-Lys-CME** (29 mg, 0.10 mmol), 1-pyreneacetic acid (26 mg, 0.10 mmol), BOP (54 mg, 0.12 mmol), DMAP (15 mg, 0.12 mmol) and CH_2Cl_2 (1 mL) were combined in a round bottom flask with a stir bar under N_2. After stirring 4 h at 25 °C, the reaction mixture was diluted with CH_2Cl_2 and neutralized with saturated $NaHSO_4$ solution. The resulting mixture was washed with saturated $NaHCO_3$ solution, water, and brine. The organic phase was dried over $MgSO_4$ and concentrated. Flash column chromatography yielded the product as yellow solid. ^1H

NMR (500 MHz, DMSO-*d6*) δ 8.54 (s, 4H), 8.23-7.97 (m, 8H), 5.23 (td, *J* = 61.0, 13.2 Hz, 2H), 4.17 (d, *J* = 11.9 Hz), 4.10 (br, 1H), 3.07 (br, 2H), 1.84 (br, 2H), 1.43 (br, 2H), 1.23 (t, *J* = 10.8 Hz) ppm. ^{13}C NMR (125 MHz, DMSO-*d6*) δ $C_{26}H_{27}N_3O_3$ $[M+H]^+$ = 430.21 (obs), 430.52 (calc)

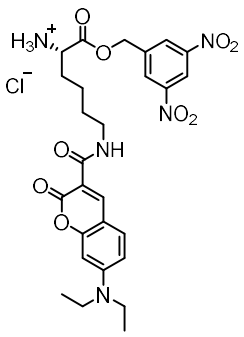


3,5-dinitrobenzyl N6-(2-(pyren-1-yl)acetyl)- *L*-lysinate (Lys-1-DNB). Boc-Lys-DNB (28 mg, 0.067 mmol), 1-pyreneacetic acid (21 mg, 0.086 mmol), BOP (59 mg, 0.134 mmol), DMAP (16 mg, 0.134 mmol) and CH_2Cl_2 (1 mL) were combined in a round bottom flask with a stir bar under N_2. After stirring 4 h at 25 °C, the reaction mixture was diluted with CH_2Cl_2 and neutralized with saturated $NaHSO_4$ solution. The resulting mixture was washed with saturated $NaHCO_3$ solution, water, and brine. The organic phase was dried over $MgSO_4$ and concentrated. Flash column chromatography yielded the product as yellow solid. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.60 (t, *J* = 2.0 Hz, 1H), 8.54 (br, 2H), 8.51 (d, *J* = 1.9 Hz, 2H), 8.30-8.26 (m, 2H), 8.18-8.04 (m, 6H), 7.91 (t, *J* = 8.2 Hz, 1H), 7.92 (d, *J* = 7.8 Hz, 1H), 5.20 (q, *J* = 38.7, 13.3 Hz, 2H), 4.11 (s, 2H), 4.03 (q, *J* = 5.5, 5.0 Hz, 1H), 3.07-2.97 (m, 2H), 1.84-1.77 (m, 2H), 1.38 (s, 3H), 1.29-1.10 (m, 1H) ppm. ^{13}C NMR (125 MHz, DMSO-*d6*) δ 170.4, 169.5, 148.2 (2C), 131.6, 131.2, 130.7, 130.0, 129.4, 129.0, 128.7, 127.8 (4C), 127.5, 127.2, 126.6, 125.4, 125.3, 125.1, 124.6, 124.4, 124.2, 118.5, 65.2, 52.2, 38.4, 29.9, 29.0, 21.9 Exact mass for $C_{31}H_{28}N_4O_7$: $[M+H]^+$ = 569.20 (obs), 569.28 (calc)

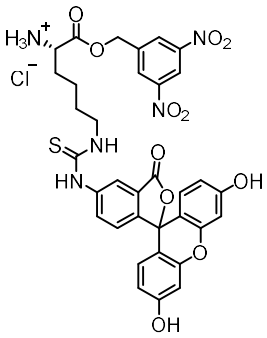


Cyanomethyl N6-(7-(diethylamino)-2-oxo-2H-chromene-3-carbonyl)-*L*-lysinate (Lys-4-CME)

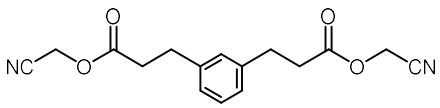
Lys-CME (29 mg, 0.102 mmol), 7-(diethylamino)coumarin-3-carboxylic acid (22 mg, 0.102 mmol), BOP (54 mg, 0.123 mmol), DMAP (10 mg, 0.082 mmol), triethylamine (0.050 mL, 0.356 mmol) and CH_2Cl_2 (5 mL) were combined in a round bottom flask with a stir bar under N_2. After stirring overnight at 25 °C, the reaction mixture was diluted with CH_2Cl_2 and neutralized with saturated $NaHSO_4$ solution. The resulting mixture was washed with saturated $NaHCO_3$ solution, water, and brine. The organic phase was dried over $MgSO_4$ and concentrated. Flash column chromatography yielded the product as bright yellow powder. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.72 (s, 1H), 7.72 (d, 1H, *J* = 9.0 Hz), 7.28 (t, *J* = 51 Hz), 6.86 (dd, *J* = 9.05, 2.3 Hz), 6.62 (d, *J* = 1.9 Hz), 4.63 (s, 2H), 4.31-4.29 (m, 1H), 3.79-3.71 (m, 2H) 3.55 (q, *J* = 7.0 , 7.0 Hz, 4H) 1.20 (t, *J* = 12.0 Hz, 9H) ppm. ^{13}C NMR (125 MHz, DMSO-*d6*) δ 169.3, 163.0, 158.6, 157.7, 153.5, 150.4, 132.3, 110.4, 107.5, 106.8, 96.3, 72.6, 70.9, 68.4, 64.5, 62.7, 52.4, 49.6, 44.8 (2C), 12.8 (2C). Exact mass for $C_{22}H_{28}N_4O_5$: $[M+H]^+$ = 429.38 (obs), 429.50 (calc).



3,5-dinitrobenzyl N6-(7-(diethylamino)-2-oxo-2H-chromene-3-carbonyl)-*L*-lysinate (Lys-4-DNB) Boc-Lys-DNB (28 mg, 0.067 mmol), 7-(diethylamino)coumarin-3-carboxylic acid (21 mg, 0.080 mmol), BOP (59 mg, 0.134 mmol), DMAP (16 mg, 0.134 mmol) and CH_2Cl_2 (1 mL) were combined in a round bottom flask with a stir bar under N_2. After stirring 4 h at 25 °C, the reaction mixture was diluted with CH_2Cl_2 and neutralized with saturated $NaHSO_4$ solution. The resulting mixture was washed with saturated $NaHCO_3$ solution, water, and brine. The organic phase was dried over $MgSO_4$ and concentrated. carbonyl)lysinate (Lys-4-DNB). ^{1}H NMR (500 MHz, MeOD) δ 8.70 (t, *J* = 2.1 Hz, 1H), 8.51 (dd, *J* = 6.7, 3.3 Hz, 3H), 8.41 (s, 1H), 7.45 (d, *J* = 9.0 Hz, 1H), 7.40 (d, *J* = 9.0 Hz, 1H), 6.72 (td, *J* = 8.9, 2.4 Hz, 2H), 6.39 (d, *J* = 2.4 Hz, 1H), 5.37 – 5.25 (m, 2H), 3.62 (t, *J* = 6.3 Hz, 1H), 3.56 (dd, *J* = 7.8, 5.6 Hz, 1H), 3.43 (q, *J* = 7.1 Hz, 5H), 1.20 – 1.12 (m, 15H) ppm. ^{13}C NMR (125 MHz, MeOD) δ 147.67, 131.18, 127.16, 125.90, 117.61, 110.32, 107.92, 95.77, 64.12, 53.46, 44.58, 38.56, 29.38, 28.62, 22.35, 11.27. Exact mass for $C_{27}H_{31}N_5O_9$: theoretical mass not observed, 570.57 (calc)

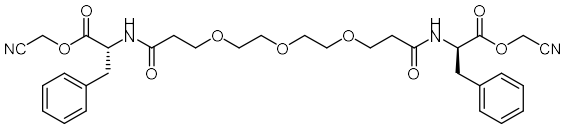


3,5-dinitrobenzyl N6-((3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)carbamothioyl)-*L*-lysinate (Lys-6-DNB) Boc-Lys-DNB (28 mg, 0.067 mmol), fluorescein isothiocyanate (29 mg, 0.073 mmol), and DMF (1 mL) were combined in a round bottom flask with a stir bar under N_2. After stirring overnight at 25 °C, the reaction mixture was diluted with EtOAc and washed water, and brine. The organic phase was dried over $MgSO_4$ and concentrated. Flash column chromatography yielded the product as yellow solid. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 10.60 (s, 1H), 10.20 (br, 2H), 8.80 (s, 1H), 8.75 (s, 2H), 8.57 (br, 3H), 8.05 (s, 1H), 7.81 (d, *J* = 8.2 Hz, 1H), 7.35 (d, *J* = 7.25 Hz, 1H), 7.16 (d, *J* = 8.15 Hz, 1H), 6.61-6.56 (m, 6H), 5.54 (s, 2H), 4.21 (s, 1H), 3.47 (s, 2H), 1.89 (br, 2H), 1.58-1.42 (m, 4H) ppm. ^{13}C NMR (125 MHz, DMSO-*d*6) δ 169.6, 167.7, 160.3, 152.4, 152.3, 148.5 (2C), 140.0, 136.1, 133.8, 132.5, 129.6, 128.9 (3C), 128.3, 126.2, 122.2, 118.8, 113.2 (2C), 110.3, 109.3, 102.7 (2C), 66.8 (3C), 65.5, 52.3, 43.4, 30.1, 28.1, 22.2 Exact mass for $C_{34}H_{29}N_5O_{11}S$: $[M+H]^+$ = 716.17 (obs), 716.16 (calc)



Bis(cyanomethyl) 3,3'-(1,3-phenylene)dipropionate (7) 3,3'-(1,3-phenylene)dipropionic acid (111 mg, 0.05 mmol), triethylamine (104 µL, 1.5 eq), chloroacetonitrile (38 µL, 1.2

eq), and CH_2Cl_2 (1 mL) were combined in a round bottom flask with a stir bar under N_2. After stirring overnight at 25 °C, the reaction mixture was diluted with EtOAc and washed with 4 % $NaHCO_3$, and brine. The organic phase was dried over $MgSO_4$ and concentrated. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 7.20 (t, *J* = 7.4 Hz,1H), 7.10-7.06 m, 3H), 4.96 (s, 4H), 2.84 (t, *J* = 7.5, 4H), 2.74 (t, *J* = 7.5, 4H) ppm. ^{13}C NMR (125 MHz, DMSO-*d*6) δ 171.8 (2C), 140.5 (2C), 128.8, 128.7, 126.6 (2C), 116.4 (2C), 49.3 (2C), 34.7 (2C), 30.3 (2C). Exact mass for $C_{16}H_{16}N_2O_4$: $[M+H]^+$ = 301.34 (obs) 301.31 (calc)



Bis(cyanomethyl) (2R,18R)-2,18-dibenzyl-4,16-dioxo-7,10,13-trioxa-3,17-diazanonadecanedioate (8). 1-(cyanomethoxy)-1-oxo-3-phenylpropan-2-aminium[2] (150 mg, 0.62 mmol), 3,3'-((oxybis(ethane-2,1-diyl))bis(oxy))dipropionic acid (0.31 mmol), HBTU (0.62 mmol), DIPEA (1.55 mmol), and 8 mL of acetonitrile were combined in a round bottom flask with a stir bar. After stirring overnight at 25 °C, the reaction mixture was diluted with EtOAc and washed with 4 % $NaHCO_3$, and brine. The organic phase was dried over $MgSO_4$ and concentrated. Flash column chromatography yielded the product as oil. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.46 (d, *J* = 7.4 Hz, 2H), 7.28 (t, *J* = 6.7 Hz, 4H), 7.23 (d, *J* = 7.1 Hz, 6H), 4.97 (s, 4H), 4.51 (q, *J* = 6.3, 7.4 Hz, 2H), 3.52 (t, *J* = 6.5 Hz, 4H), 3.44-3.42 (m, 8H), 3.06-2.92 (m, 4H), 2.33 (t, J = 6.5 Hz, 4H) ppm. ^{13}C NMR (125 MHz, DMSO-*d*6) δ 171.1 (2C), 170.8 (2C), 137.2 (2C), 129.5 (4C), 128.7 (4C), 127.7 (2C), 116.1 (2C), 70.1 (2C), 66.9 (2C), 53.7 (2C), 49.8 (2C), 38.7 (2C), 36.7 (2C), 36.1 (2C). Exact mass for $C_{32}H_{38}N_4O_9$: $[M+H]^+$ = 623.68 (obs) 623.68 (calc)

3. Extended Methods

3.1 Preparation of DNA templates

The DNA templates for flexizyme (Fx) and tRNA preparations were synthesised by using the following DNA sequences as previously described[2-4].

- Sequences of the final DNA templates used for *in vitro* transcription by T7 RNA polymerase

eFx	GGCGTAATACGACTCACTATAGGATCGAAAGATTTCCGCGGCCCCGAAAGGGGATTAGC GTTAGGT
dFx	GGCGTAATACGACTCACTATAGGATCGAAAGATTTCCGCATCCCCGAAAGGGTACATGGC GTTAGGT

aFx	GGCGTAATACGACTCACTATAGGATCGAAAGATTTCCGCACCCCCGAAAGGGGTAAGTGGCGTTAGGT
fMet (CAU)	GTAATACGACTCACTATAGGCGGGGTGGAGCAGCCTGGTAGCTCGTCGGGCTCATAACCCGAAGATCGTCGGTTCAAATCCGGCCCCCGCAACCA
Pro1E2(GGU)	GTAATACGACTCACTATAGGGTGATTGGCGCAGCCTGGTAGCGCACTTCGTTGGTAACGAAGGGGTCAGGGGTTCGAATCCCCTATCACCCGCCA
GluE2(GAU)	GTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTGATAAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGCCA
GluE2(GGC)	GTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTGGCAGGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGCCA

*Note that the underlined sequences are the T7 promoter sequence.

3.2 Preparation of Fx and tRNAs

Flexizymes and tRNAs were prepared using the HiScribe™ T7 High yield RNA synthesis kit (NEB, E2040S) and purified by previously reported methods[2].

3.3 General Fx-mediated acylation reaction

A. Microhelix

1 µL of 0.5 M HEPES (pH 7.5) or bicine (pH 8.8), 1 µL of 10 µM microhelix, and 3 µL of nuclease-free water were mixed in a PCR tube with 1 µL of 10 µM eFx, dFx, and aFx, respectively. The mixture was heated for 2 min at 95 °C and cooled down to room temperature over 5 min. 2 µL of 300 mM $MgCl_2$ was added to the cooled mixture and incubated for 5 min at room temperature. Followed by the incubation of the reaction mixture on ice for 2 min, 2 µL of 25 mM activated ester substrate in DMSO was added to the reaction mixture. The reaction mixture was further incubated for 12 h on ice in cold room. Microhelix was purchased from IDT. (Sequence: 5'-GGCUCUGUUCGCAGAGCCGCCA-5'

B. tRNA

2 µL of 0.5 M HEPES (pH 7.5) or 0.5 M bicine (pH 8.8), 2 µL of 250 µM tRNA, 2 µL of 250 µM of a Fx selected on the microhelix experiment and 6 µL of nuclease-free water were mixed in a PCR tube. The mixture was heated for 2 min at 95 °C and cooled down to room temperature over 5 min. 4 µL of 300 mM $MgCl_2$ was added to the cooled mixture and incubated for 5 min at room temperature. Followed by the incubation of the reaction mixture on ice for 2 min, 4 µL of 25 mM activated ester substrate in DMSO was added to the reaction mixture. The reaction mixture was

further incubated for the optimal time determined on the microhelix experiment on ice in cold room. The tRNAs were ethanol-precipitated before further usage.

3.4 *In vitro* synthesis of peptides with FAAs

A. N-terminal incorporation

As reporter peptide, a T7 promoter-controlled DNA template (pJL1_StrepII_1, see plasmid map) was designed to encode a streptavidin (Strep) tag and additional Ser and Thr codons (**X**WHSPQFEKST (strep-tag), where **X** indicates the position of the long-carbon chain substrate). The translation initiation codon AUG was used for N-terminal incorporation of the long-carbon chain substrates, **X**. Peptide synthesis was performed using only the 9 amino acids that decode the initiation codon AUG and the purification tag in the absence of the other 11 amino acids to prevent corresponding endogenous tRNAs from being aminoacylated and used in translation. The PURExpress® Δ (aa, tRNA) kit (NEB, E6840S) was used for polyamide synthesis reaction and the reaction mixtures were incubated at 37 °C for 3 h. The synthesised peptides were then purified using Strep-Tactin®-coated magnetic beads (IBA), denatured with 0.1 % SDS, and characterised by MALDI-TOF mass spectroscopy.

B. C-terminal incorporation

Three plasmids (pJL1-StrepII_1, pJL1-StrepII_2, pJL1-StrepII_3, see plasmid map) encoding (MWHSPQFEKS**X**, MWHSPQFEKS**XY**, MWHSPQFEKS**XYZ**, where **X**, **Y**, and **Z** indicate the position of the FAA substrate) was used for C-terminal incorporation. The FAAs were incorporated at Thr (ACC), Ile (AUC), and Ala (GCC) codons using three tRNAs (Pro1E2 (GGU), GluE2(GAU), GluE2 (GGC)).

3.5 Protein expression in the presence of EF-P

EF-P was prepared as described in the previous study.[4] Dap-**2**, Dap-**4**, and Dap-**5** were charged to Pro1E2 (GGU) for C-terminal incorporation. The tRNA charging reaction was carried out under the same reaction condition optimized in the microhelix experiment shown in **Fig. S3**. The FAA-charged tRNAs were treated as described in 3.3.B and added into the PURExpress reaction in the presence of 10 μM (in final) EF-P. The reaction was carried out as described in section 3.4.B.

3.6 Purification of peptides with FAAs

The polypeptides containing FAAs were purified using affinity tag purification as described previously[2].

3.7 Characterisation of peptides

A. Mass spectrometry

1.5 µL of the peptides purified by the strep affinity tag was mixed on a MALDI plate with 1 µL of saturated α-cyano-4-hydroxycinnamic acid (CHCA) in THF containing 0.1 % TFA. The samples were dried at room temperature for 30 min. MALDI-TOF mass spectra of the peptides were obtained on a Bruker rapifleX using the positive reflectron mode.

B. Fluorescence

The fluorescent characteristics of peptides containing a single or multiple FAAs were visualized on a Bio-Rad Gel-doc™ by exposure of UV light filtered at 560/50 nm for 13 s. The obtained images were not further processed by other image software.

C. UV-Vis

The FAA substrates (Dap-**3** and Dap-**5**) were diluted in several different concentration 0.1% SDS solution in water to keep the condition consistent with the peptides eluted from the purification procedure using Strep-Tactin®-coated magnetic beads. The UV-Vis absoprion spectra for substrates and peptides containing FAA were obtained using the light at 200-850 nm on a Nanodrop 2000C (ThremoScience).

4. Supplemental Figures

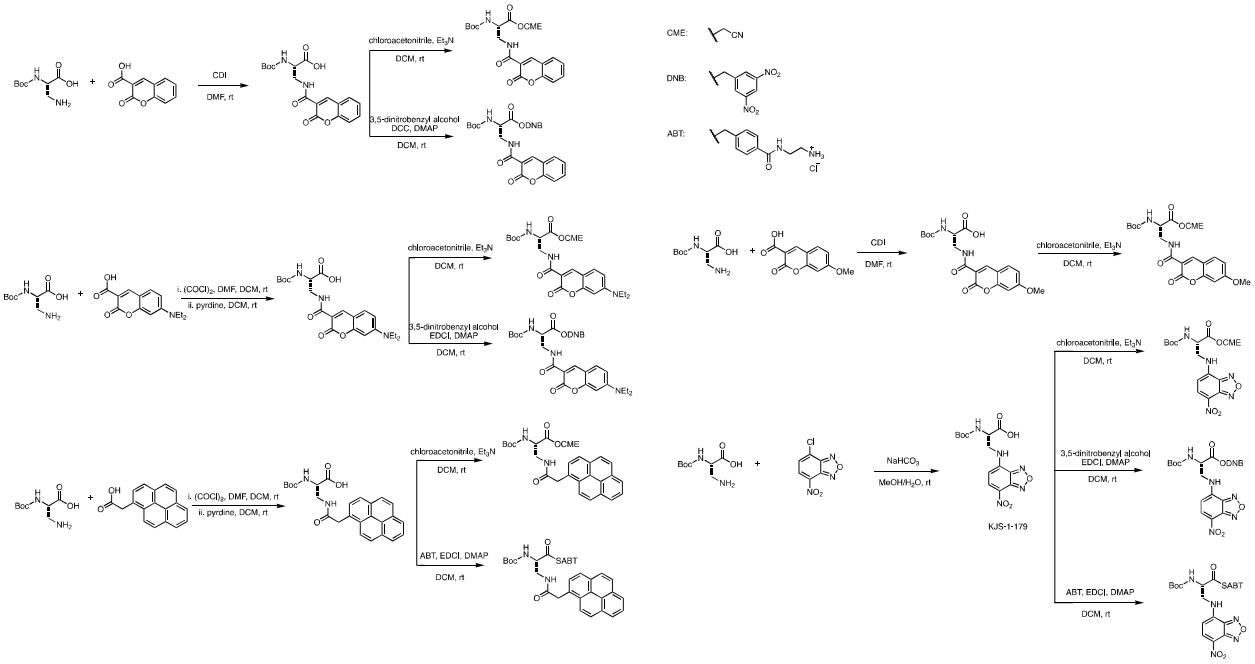


Figure S1: Synthetic scheme of Dap-based derivatives with activating groups.

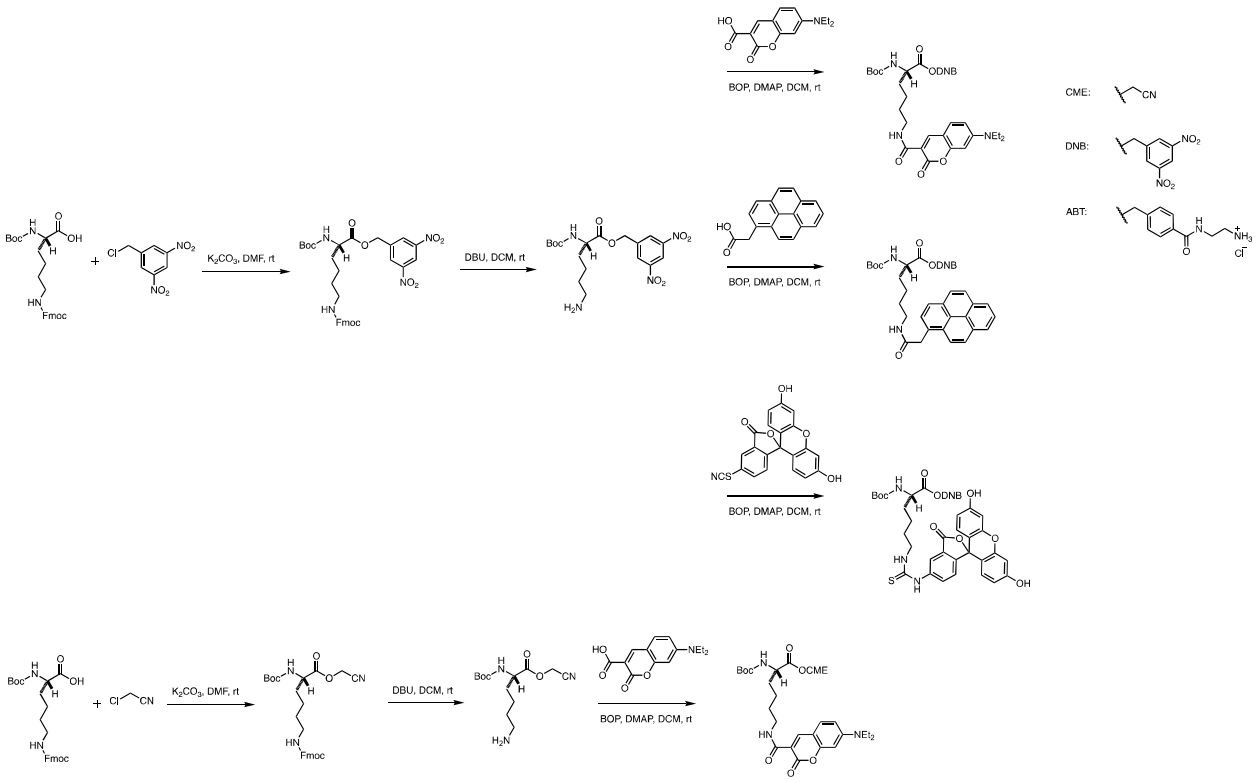


Figure S2: Synthesis of Lys-based FAA derivatives with activating group.

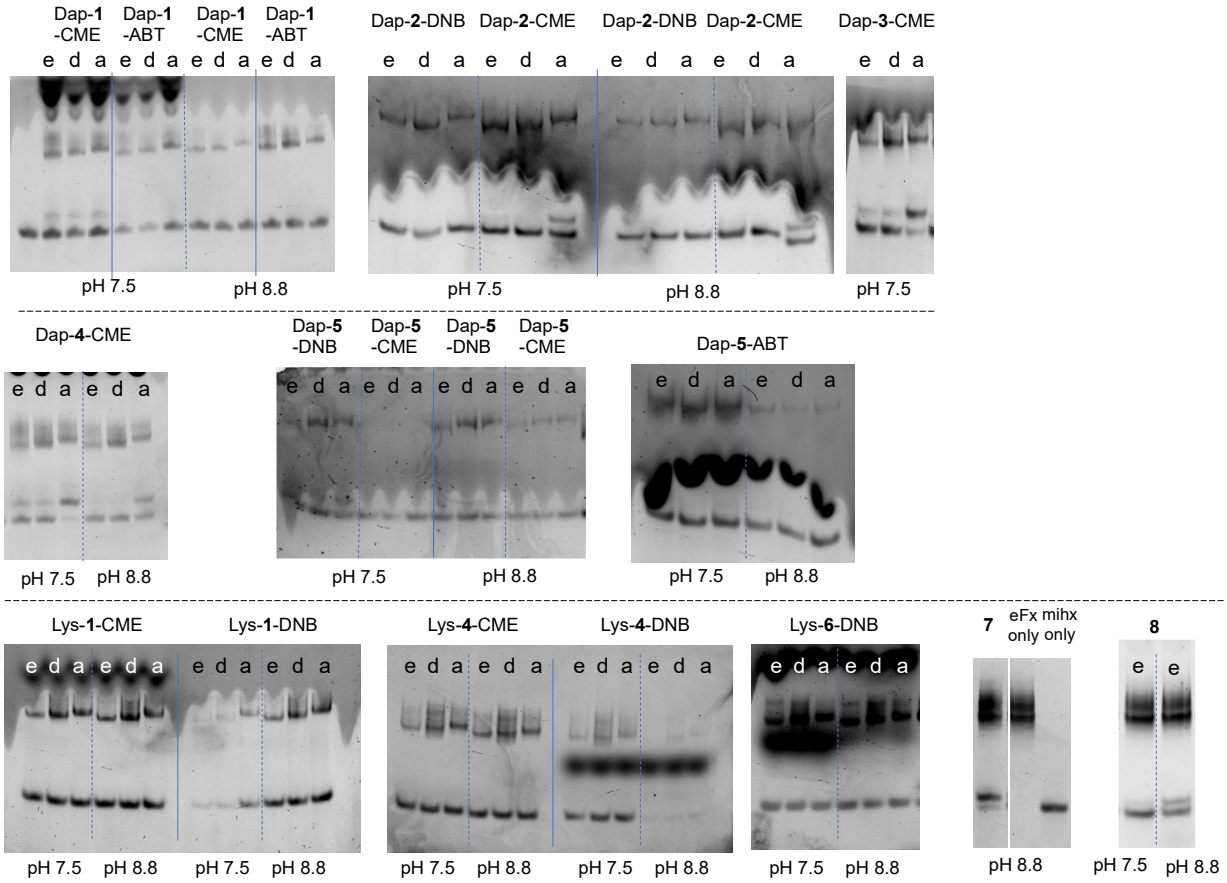


Figure S3. Fx-charging of FAAs. The Fx-catalyzed acylation reaction of FAAs (Dap-**1**-CME;ABT, Dap-**2**-CME;DNB, Dap-**3**-CME, Dap-**4**-CME, Dap-**5**-CME;DNB;ABT, and Lys-**1**-CME:DNB, Lys-**4**-CME:DNB, Lys-**6**-DNB) were carried out using microhelix or tRNA and monitored at two different pHs (7.5 and 8.8). Gels are representative of three independent experiments. The acylation yields were analyzed by electrophoresis on 15 % polyacrylamide gel containing 50 mM NaOAc (pH 5.2). The crude products containing the chemical substrates were loaded on the gel and separated by the electrophoretic mobility at 135 mV in cold room over 1.5 h. The acylation reactions were monitored over 16 h and the yields were quantified using densitometric analysis using the ImageJ software. The gel was stained using GelRed (Biotium); abs. 300 nm, em. 600 nm., while the FAAs absorb and emit the light in the range of abs. 310-460 nm, em. 350-515 nm.

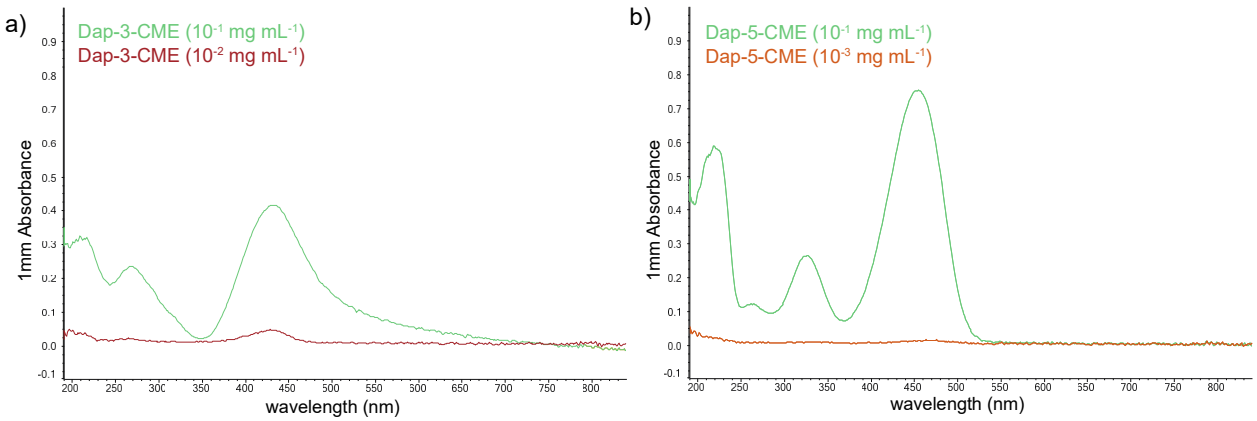


Figure S4. UV-Vis characterization of activated FAAs. The signature absorption of Dap-**3**-CME and Dap-**5**-CME was observed at 300-550 nm. At lower concentrations, the signature trends are not observed (0.1 % SDS in water).

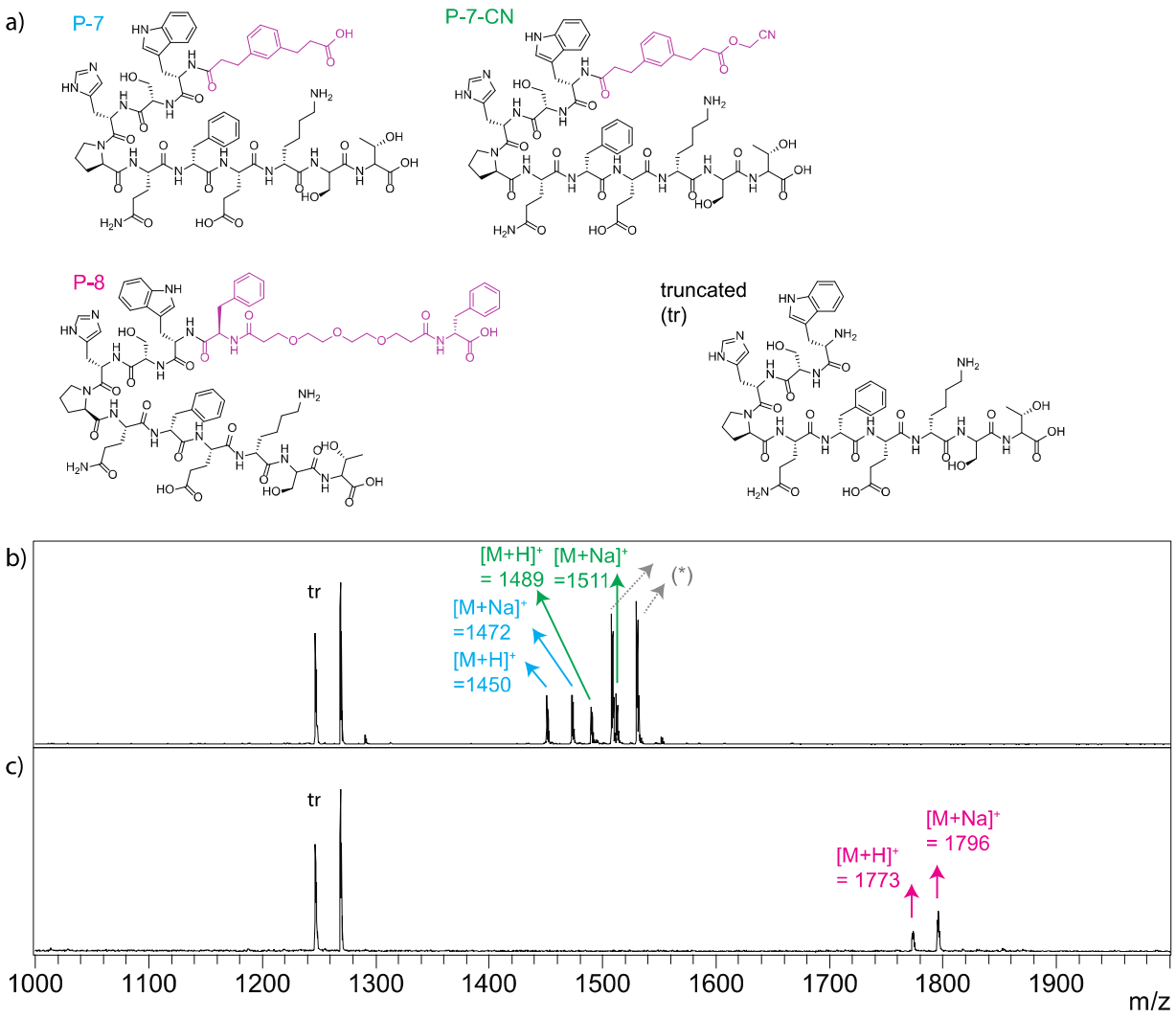
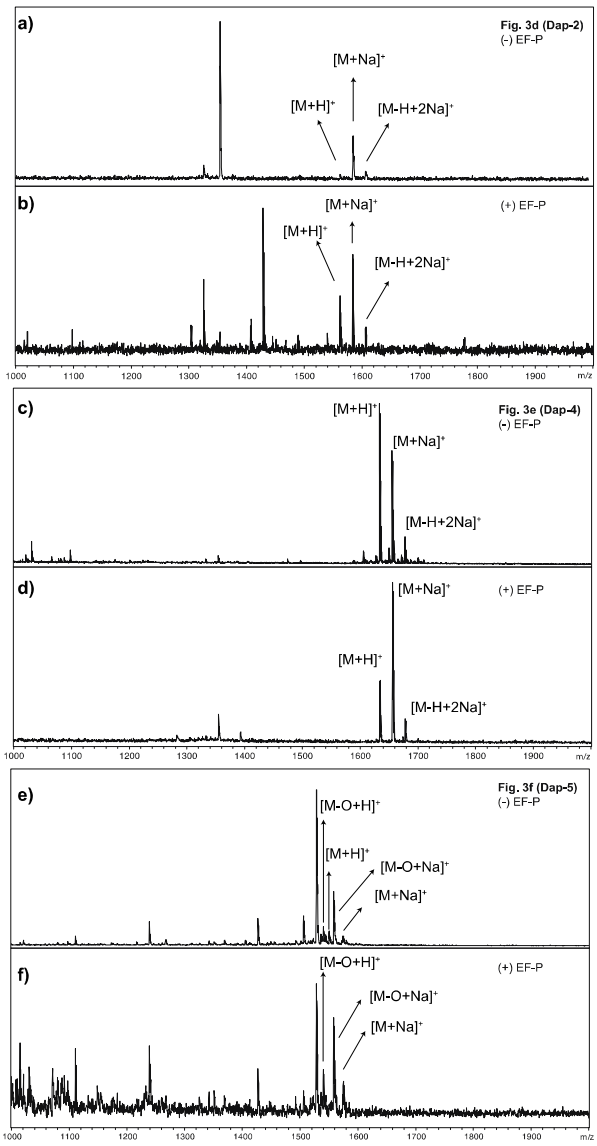


Figure S5. Characterization of the N-terminus functionalized peptides with 7 and 8. (a) Structure and molecular weight of full-length and truncated byproduct peptides produced in the PURE reaction. (b) MALDI-TOF mass spectrometry data for incorporation of 7 into the N-terminus of a peptide. Partial hydrolysis of the cyanomethyl group of 7 is observed. The theoretical masses of produced peptides are $[M+H]^+$ = 1450, $[M+Na]^+$ = 1472 for partially hydrolyzed peptides (P-7, blue arrows) and $[M+H]^+$ = 1489, $[M+Na]^+$ = 1511 for unhydrolyzed peptides (P-7-CN, green arrows). (c) Produced peptides containing 8 at the N-terminus. Theoretical mass of the target peptide: $[M+H]^+$ = 1772, $[M+Na]^+$ = 1794, pink arrows. Theoretical mass of the truncated peptides (tr) is $[M+H]^+$ = 1246, $[M+Na]^+$ = 1268. The marked peaks by an asterisk ($[M+H]^+$ = 1507; $[M+Na]^+$ = 1529, gray arrows) were unidentified. Data are representative of three independent experiments.

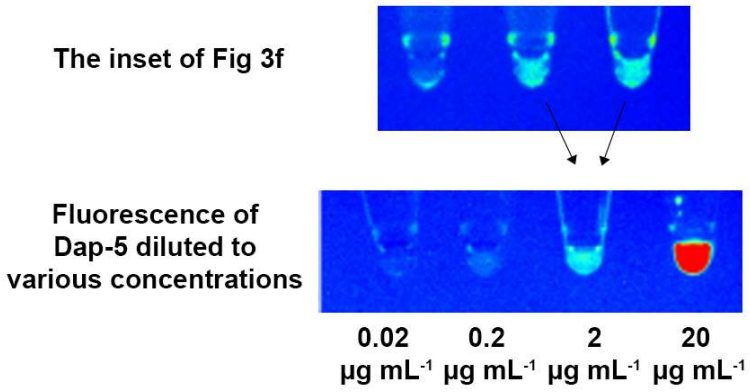


g)

	Dap-**2**	Dap-**4**	Dap-**5**
Relative yield (%) of peptides			
(-) EF-P	34 (a)	81 (c)	29 (e)
(+) EF-P	32 (b)	88 (d)	28 (f)

Figure S6. Comparison of peptide expression level in the absence and presence of EF-P. The incorporation of FAAs was carried out in the presence of 10 μM of EF-P to address the low incorporation efficiency of FAAs (e.g., Dap-**2** and Dap-**5**) in the PURExpress reaction. However, a significant increase was not observed. The arrows indicated the peaks corresponding to the theoretical masses of the peptides: (a) fMWSHPQFEKS-(Dap-**2**) (-) EF-P, (b) fMWSHPQFEKS-(Dap-**2**) (+) EF-P, (c) fMWSHPQFEKS-(Dap-**4**) (-) EF-P, (d) fMWSHPQFEKS-(Dap-**4**) (+) EF-P (e) fMWSHPQFEKS-(Dap-**5**) (-) EF-P, (f) fMWSHPQFEKS-(Dap-**5**) (+) EF-P ($[M+H]^+$ = 1562; $[M+Na]^+$ = 1584, $[M-H+2Na]^+$ = 1606 for a and b, $[M+H]^+$ = 1633; $[M+Na]^+$ = 1655; $[M-H+2Na]^+$ = 1677 for c and d, $[M+H]^+$ = 1553; $[M+Na]^+$ = 1575; $[M-O+H]^+$ = 1537, $[M-O+Na]^+$ = 1559 for e and f, respectively). (g) Relative yield of produced peptides containing an FAA at the C-terminus. Data are representative of n=3 independent experiments.

a)



b)

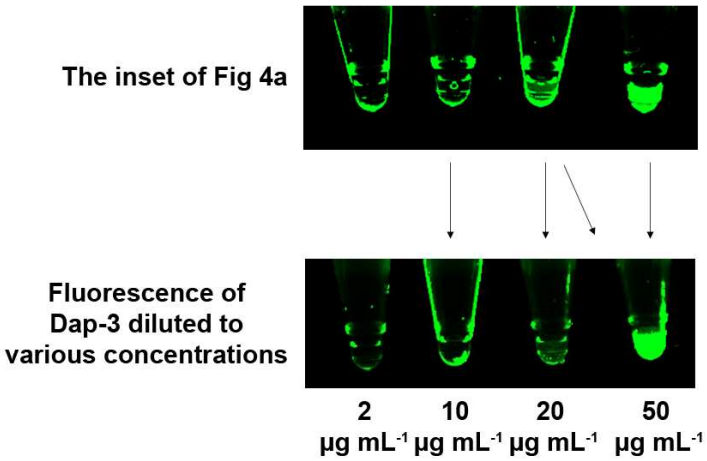


Figure S7. Determination of the yield of peptides based on the fluorescence. (a) Based on the similar intensity observed of the pure FAA from the same exposure, the intensity of the produced peptide containing Dap-**5** either at the N- and C-terminus is comparable to the intensity of 2 μg mL^{-1} of Dap-**5**-CME in 10 μL of water (0.1% SDS), which gives ~116 ng (75 nmol) of peptide with Dap-5 when calculated using the molar mass of the produced peptide. (b) Compared to the intensity obtained from several known concentration of Dap-**3**-CME, the yield of peptides containing one, two, or three Dap-**3** is determined approximately as ~520 ng (330 nmol), ~670-1200 ng (340-570 nmol), or ~1200 ng (570 nmol), respectively.

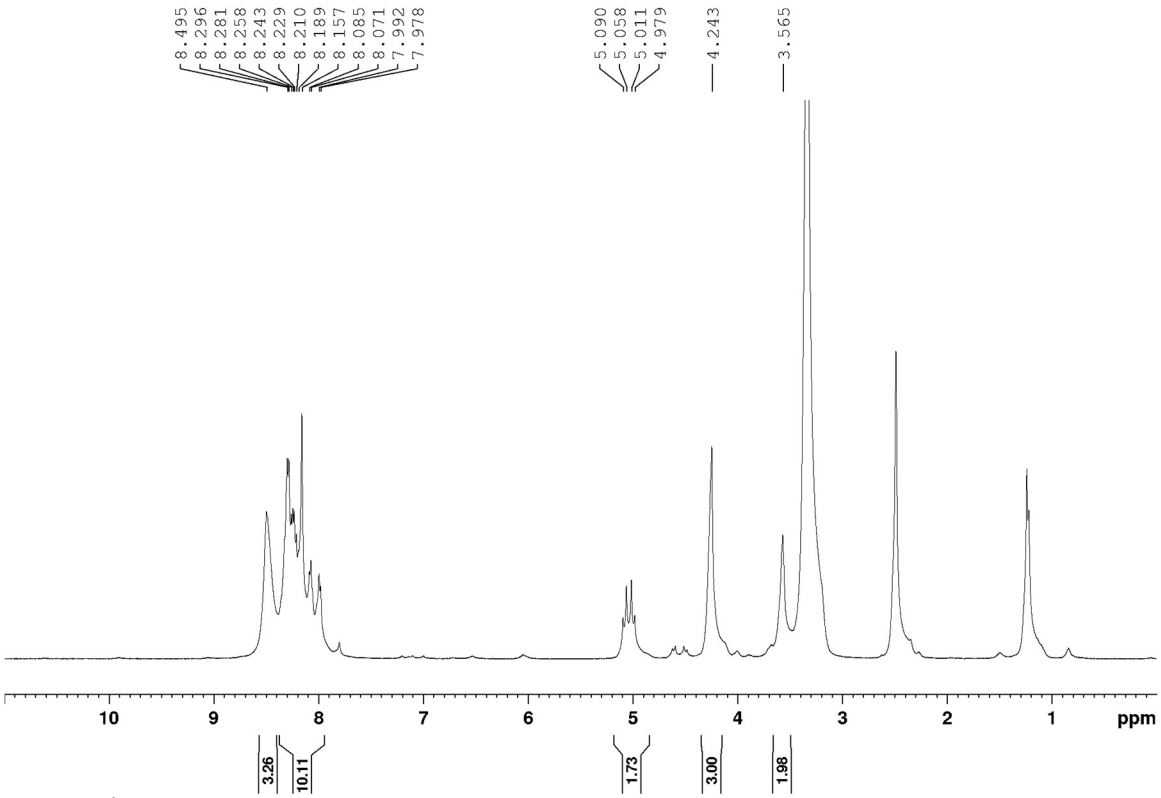


Figure S8. ^{1}H NMR spectrum of Dap-1-CME (500 MHz, DMSO-d_6)

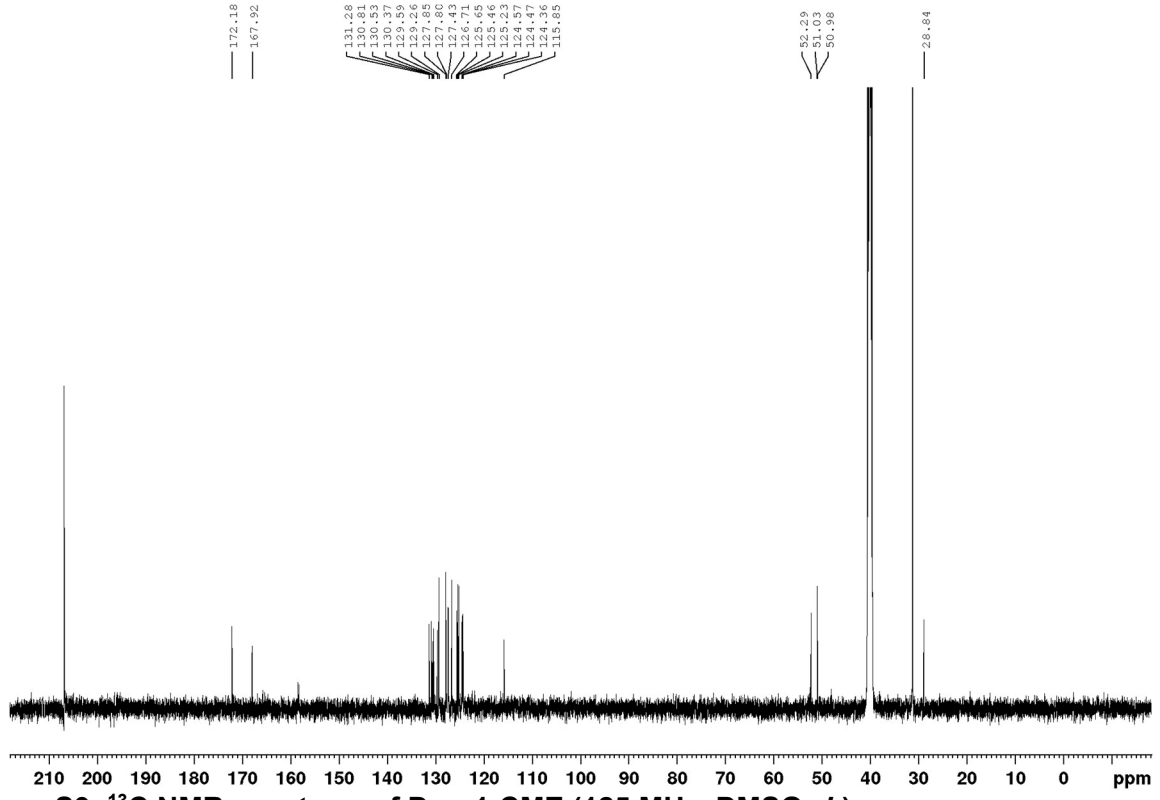


Figure S9. ^{13}C NMR spectrum of Dap-1-CME (125 MHz, DMSO-d_6)

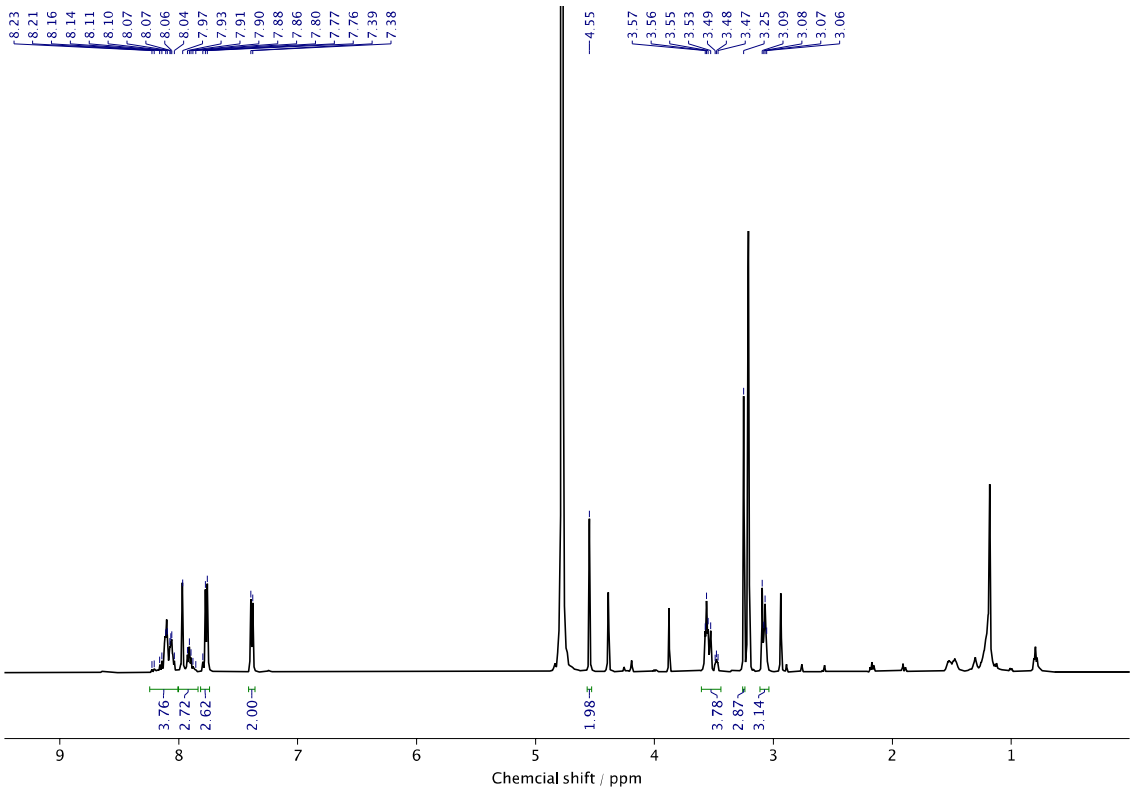


Figure S10. ^{1}H NMR spectrum of Dap-1-ABT (500 MHz, MeOD-d_4)

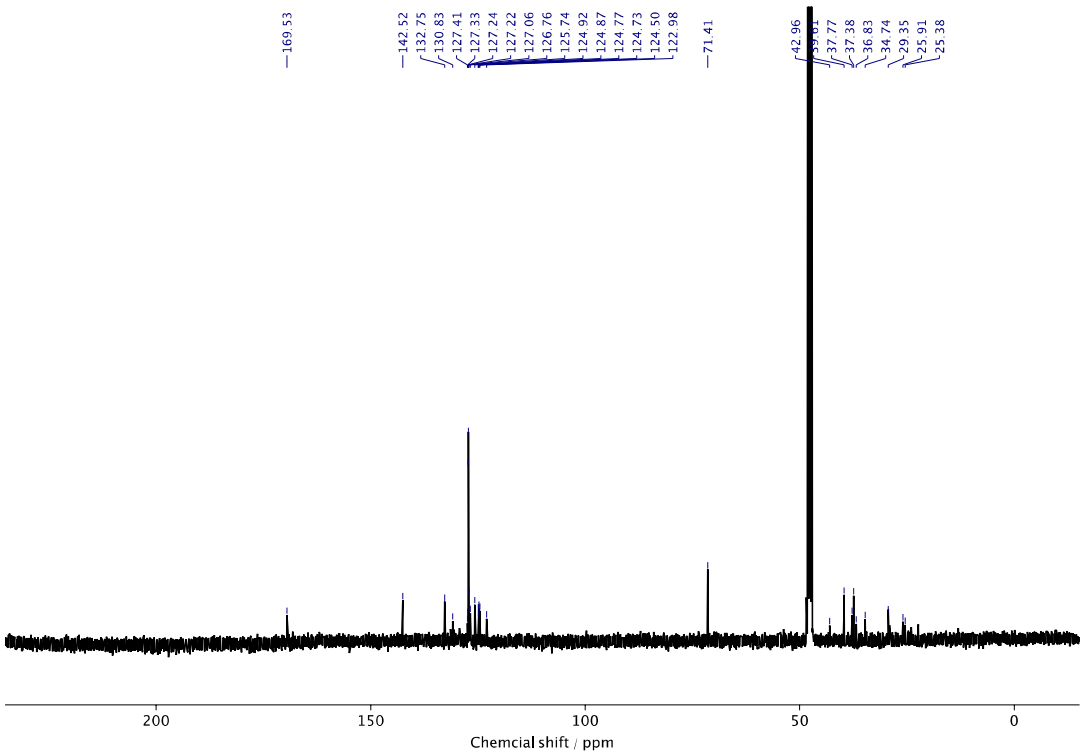


Figure S11. ^{13}C NMR spectrum of Dap-1-ABT (125 MHz, MeOD-d_4)

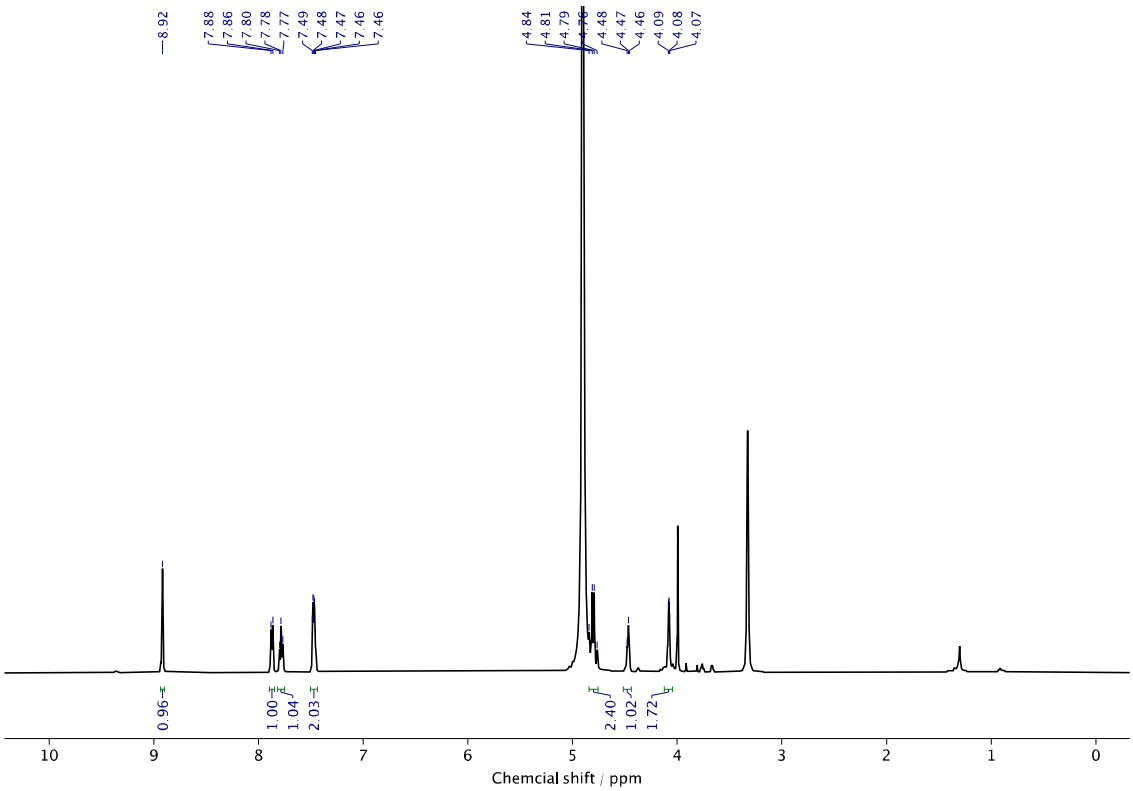


Figure S12. ^{1}H NMR spectrum of Dap-2-CME (500 MHz, MeOD-d_4)

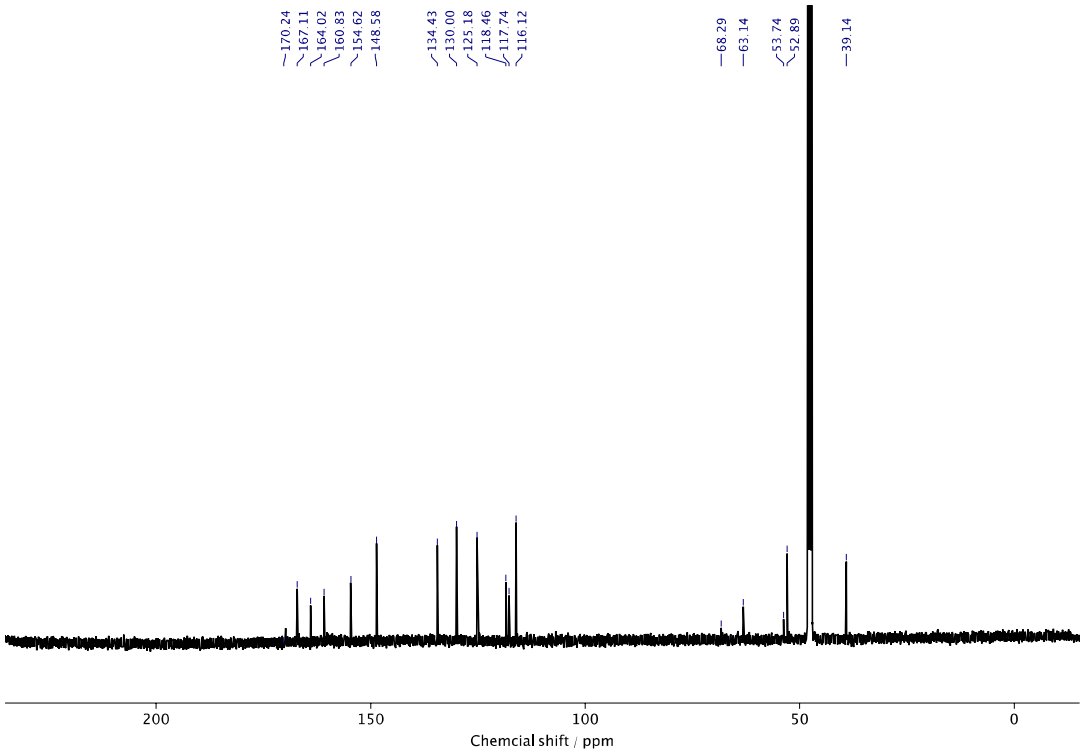


Figure S13. ^{13}C NMR spectrum of Dap-2-CME (125 MHz, MeOD-d_4)

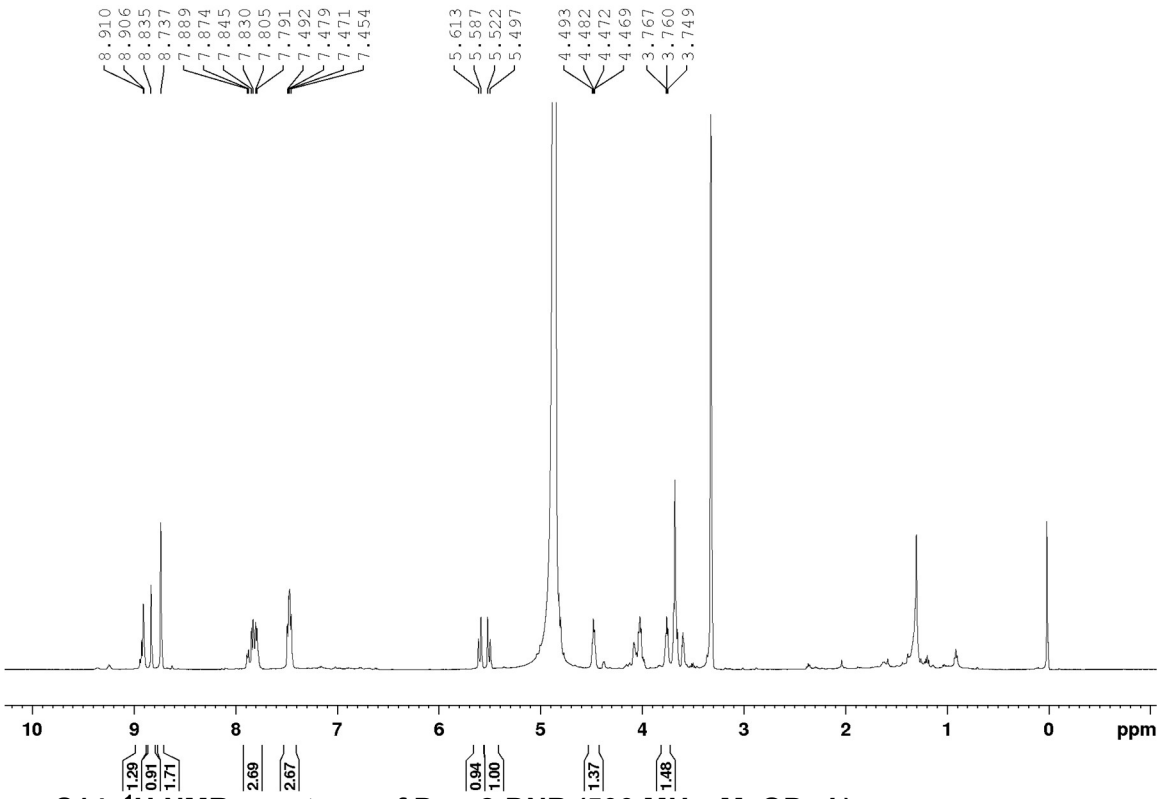


Figure S14. ^{1}H NMR spectrum of Dap-2-DNB (500 MHz, MeOD-d_4)

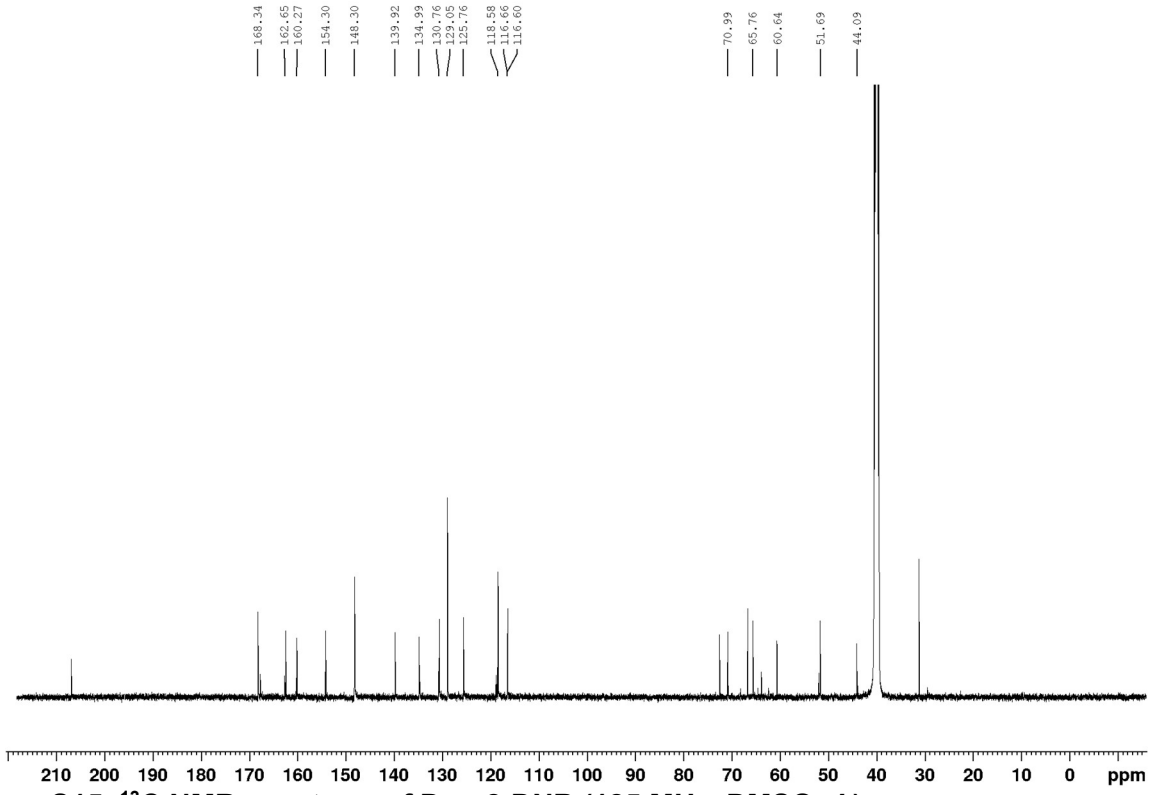


Figure S15. ^{13}C NMR spectrum of Dap-2-DNB (125 MHz, DMSO-d_6)

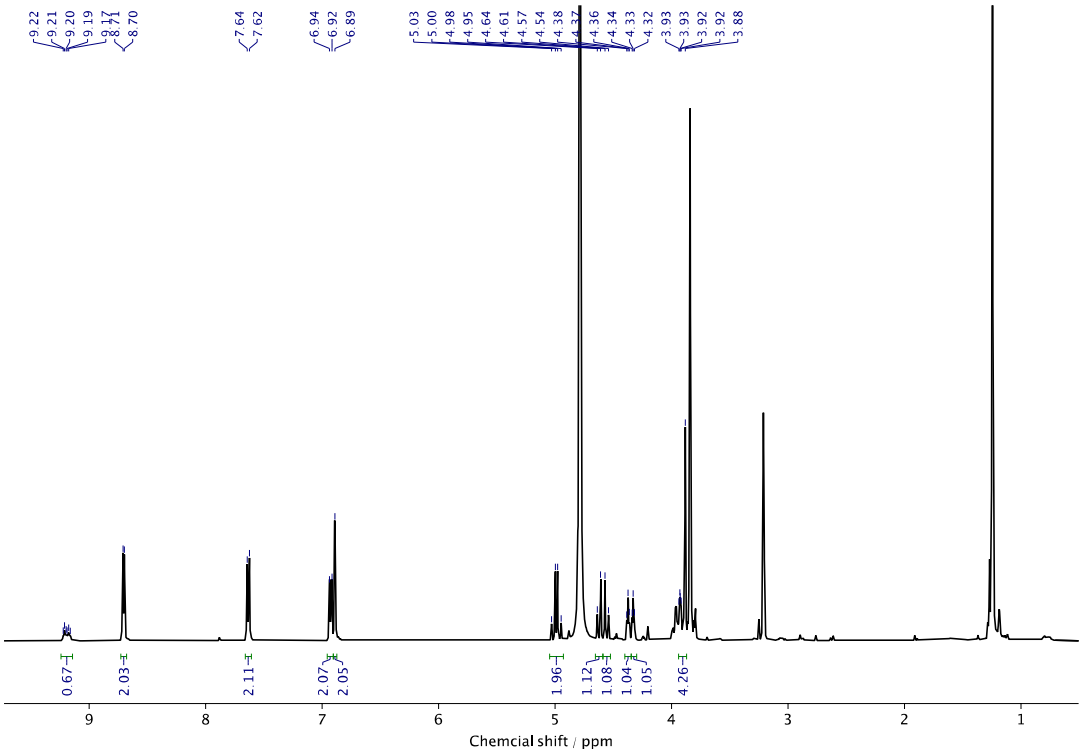


Figure S16. ^{1}H NMR spectrum of Dap-3-CME (500 MHz, MeOD-d_4)

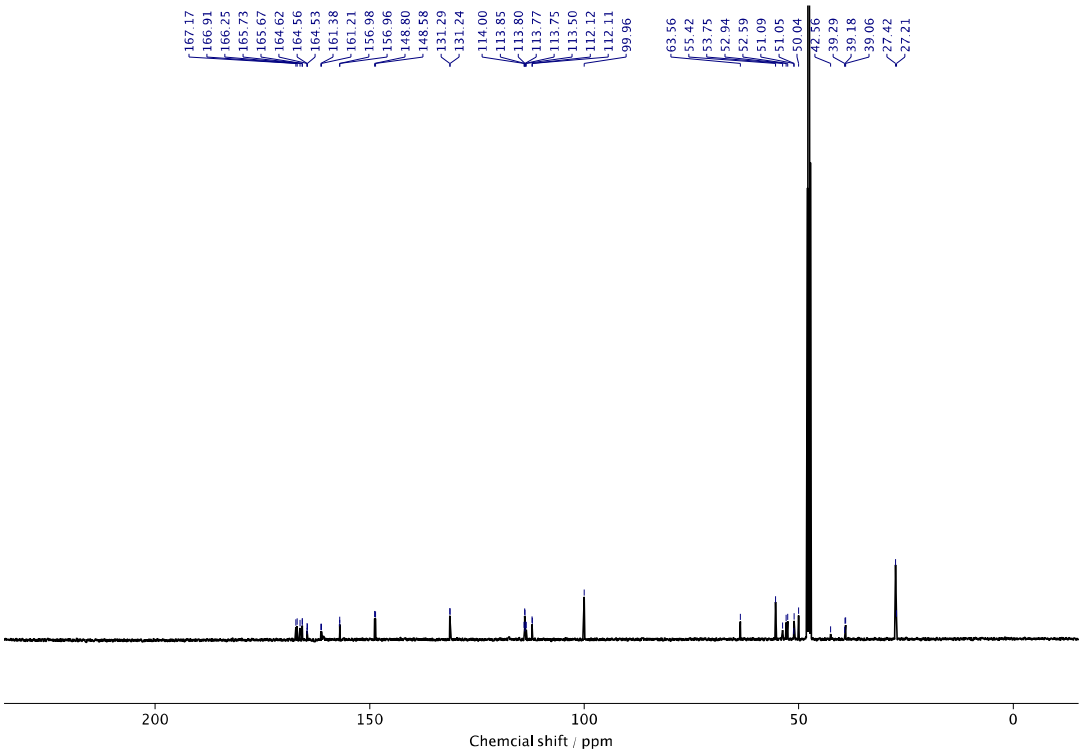


Figure S17. ^{13}C NMR spectrum of Dap-3-CME (125 MHz, MeOD-d_4)

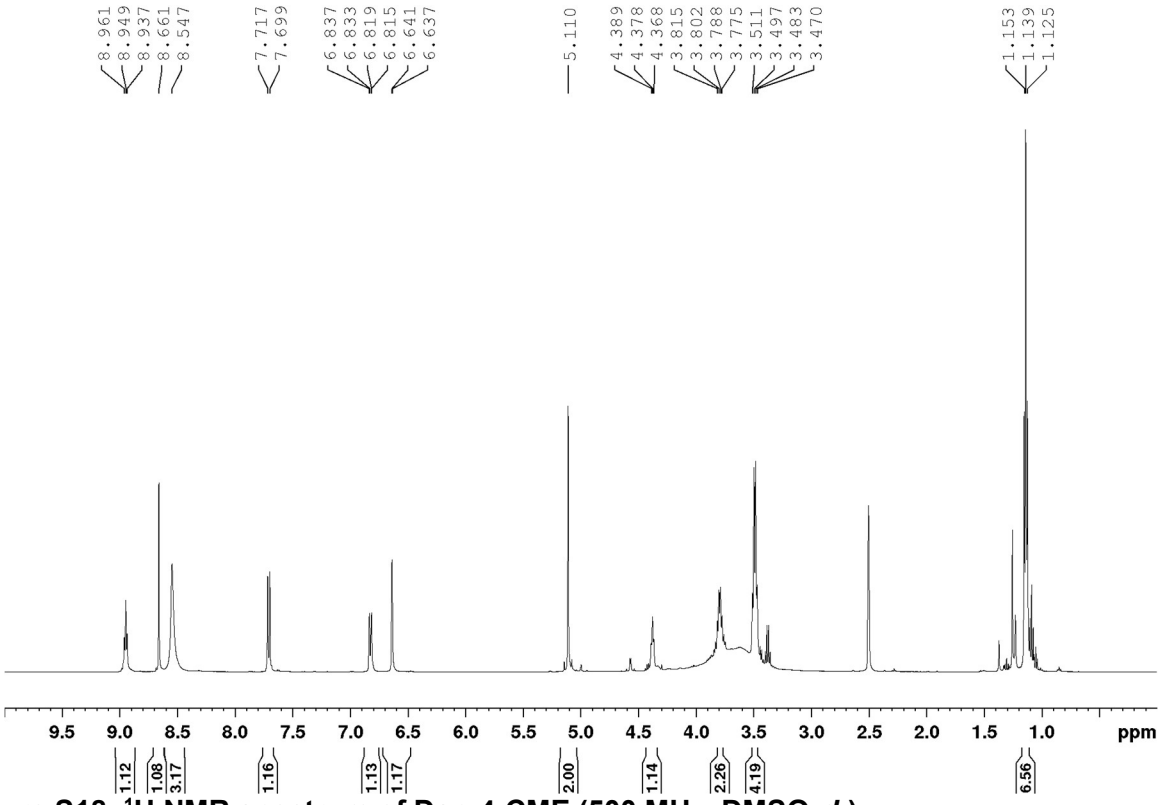


Figure S18. ^{1}H NMR spectrum of Dap-4-CME (500 MHz, DMSO-d_6)

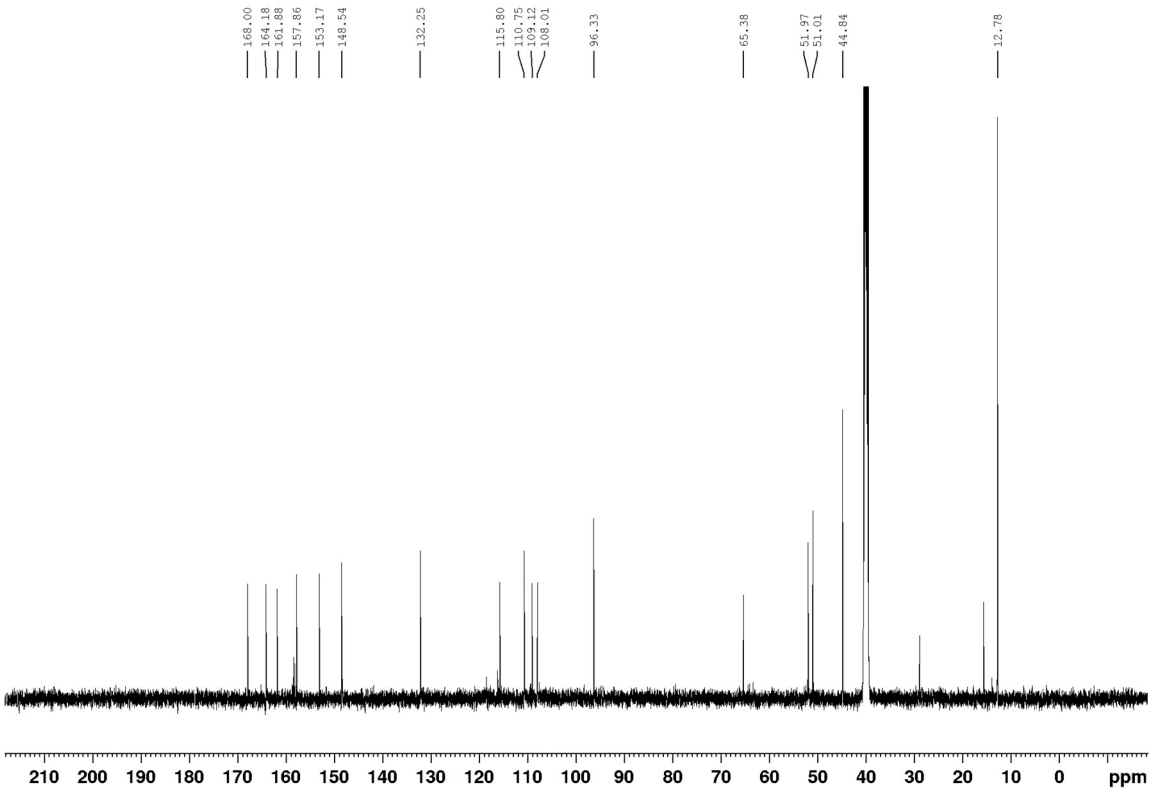


Figure S19. ^{13}C NMR spectrum of Dap-4-CME (125 MHz, DMSO-d_6)

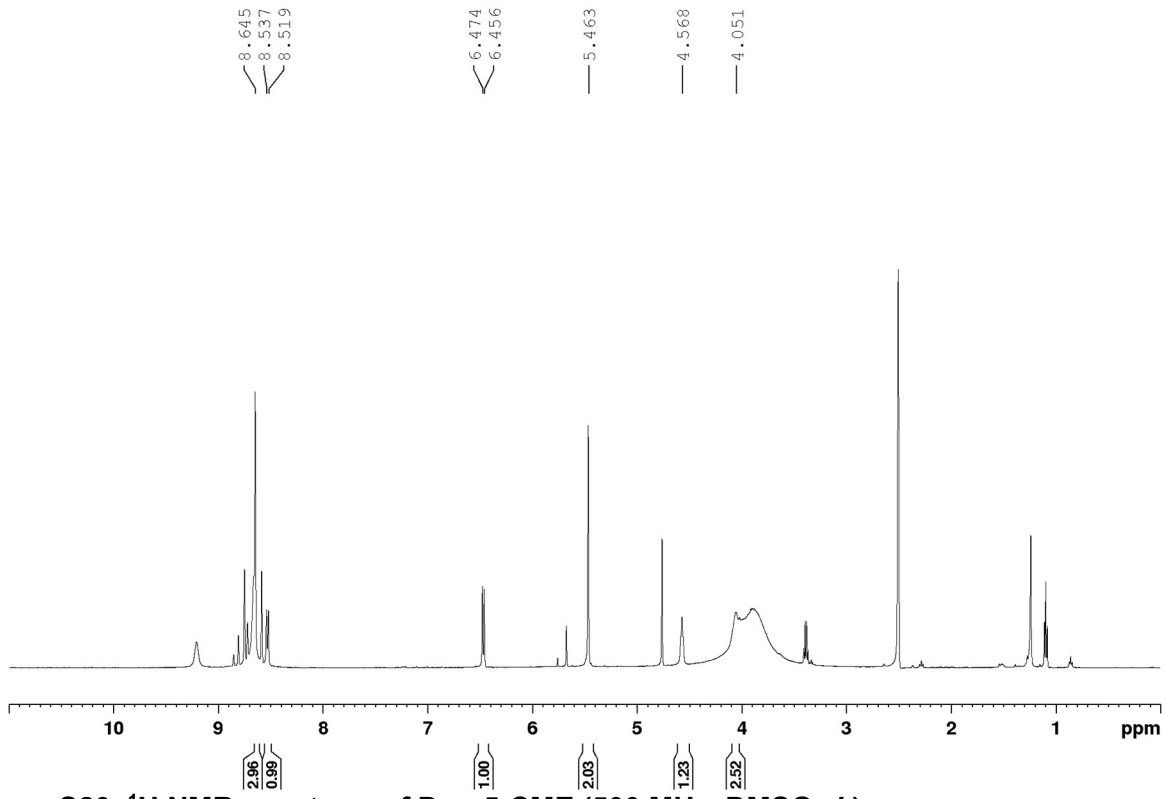


Figure S20. ^{1}H NMR spectrum of Dap-5-CME (500 MHz, DMSO-d_6)

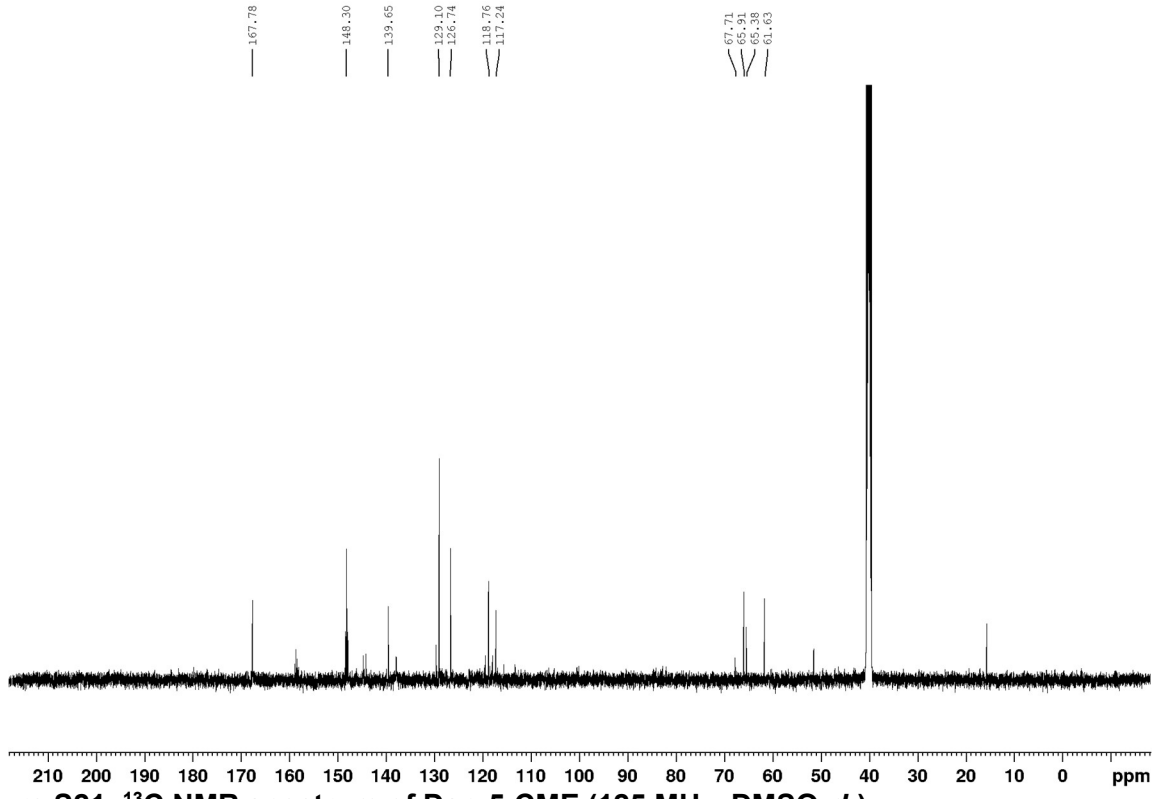


Figure S21. ^{13}C NMR spectrum of Dap-5-CME (125 MHz, DMSO-d_6)

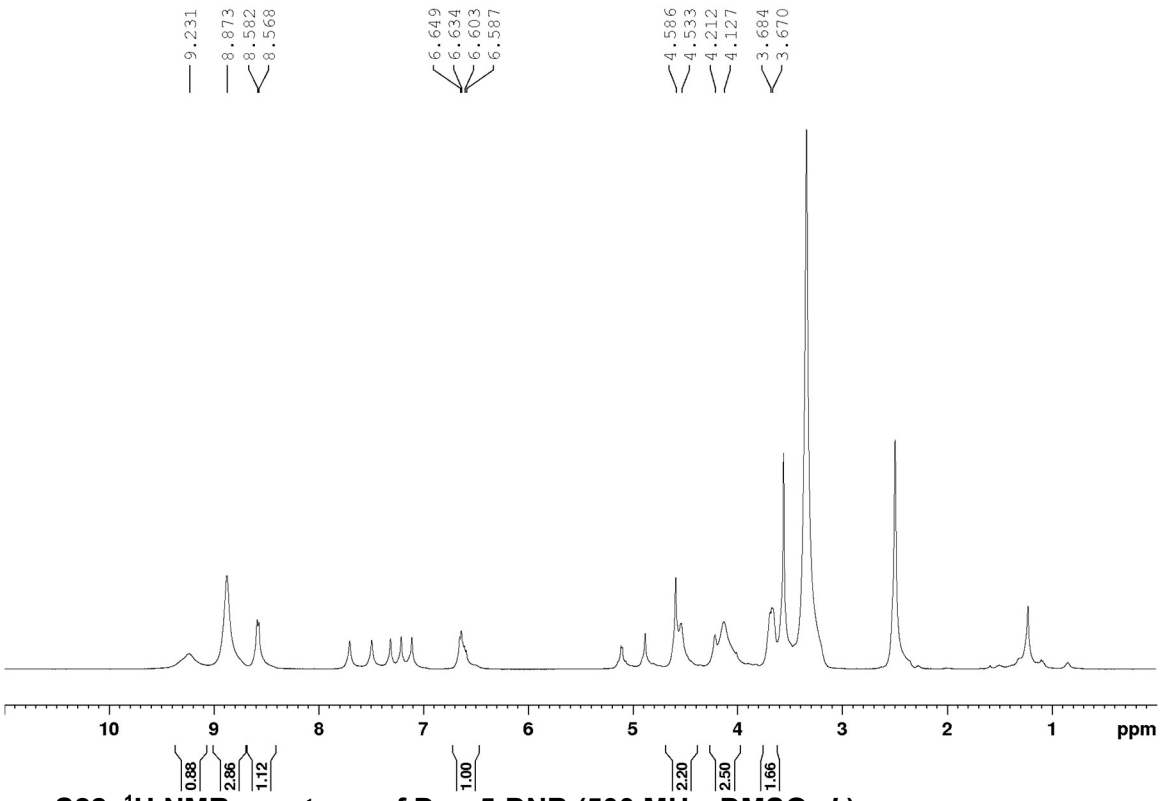


Figure S22. ^{1}H NMR spectrum of Dap-5-DNB (500 MHz, DMSO-d_6)

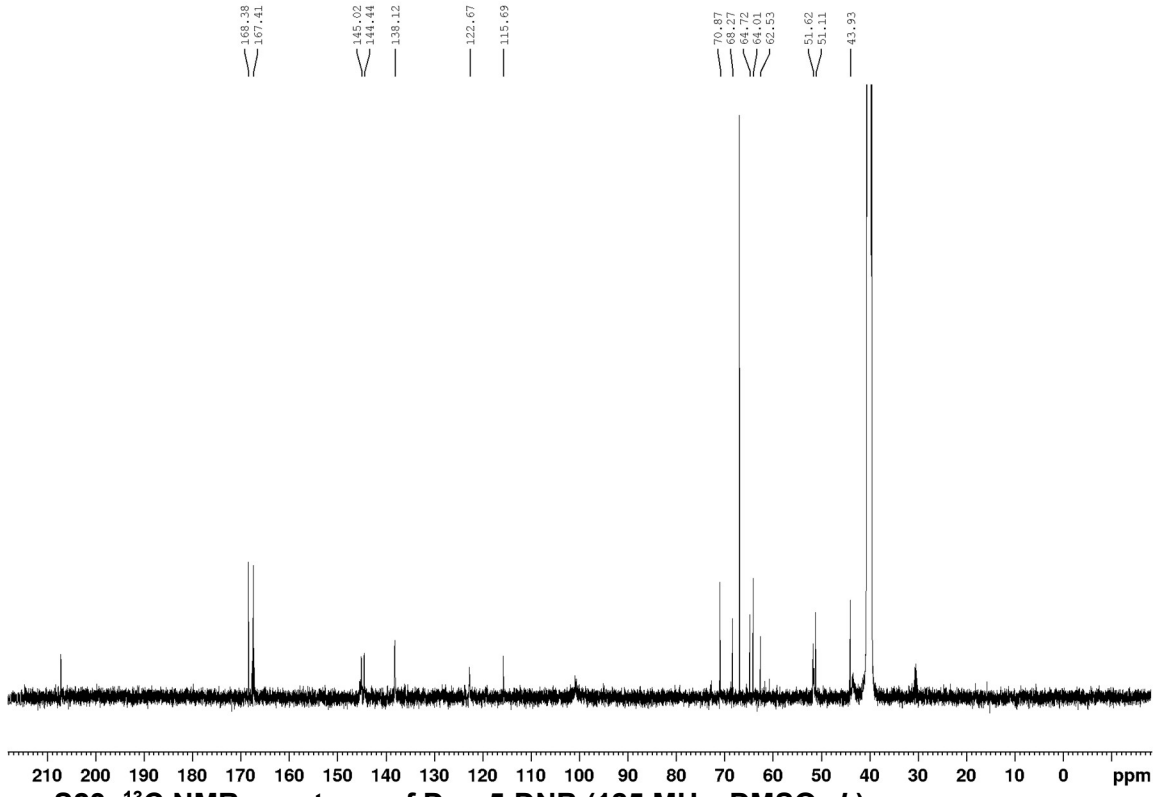


Figure S23. ^{13}C NMR spectrum of Dap-5-DNB (125 MHz, DMSO-d_6)

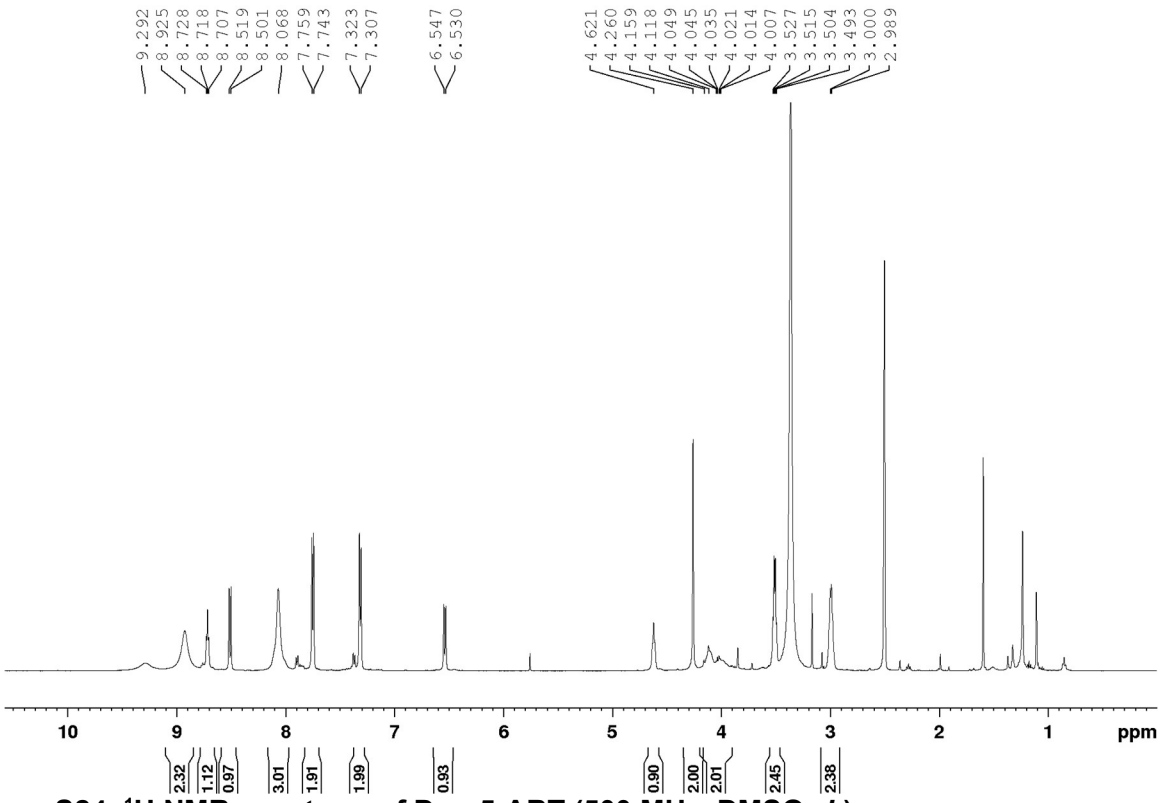


Figure S24. ^{1}H NMR spectrum of Dap-5-ABT (500 MHz, DMSO-d_6)

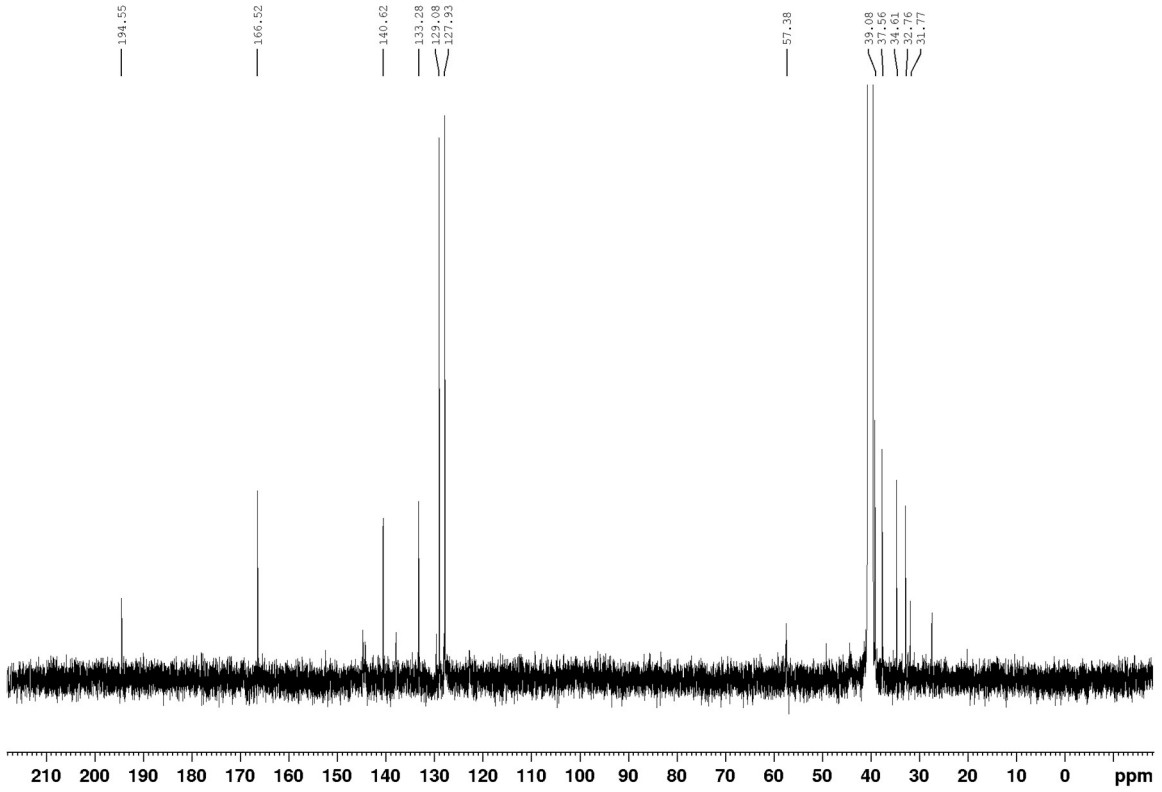


Figure S25. ^{13}C NMR spectrum of Dap-5-ABT (125 MHz, DMSO-d_6)

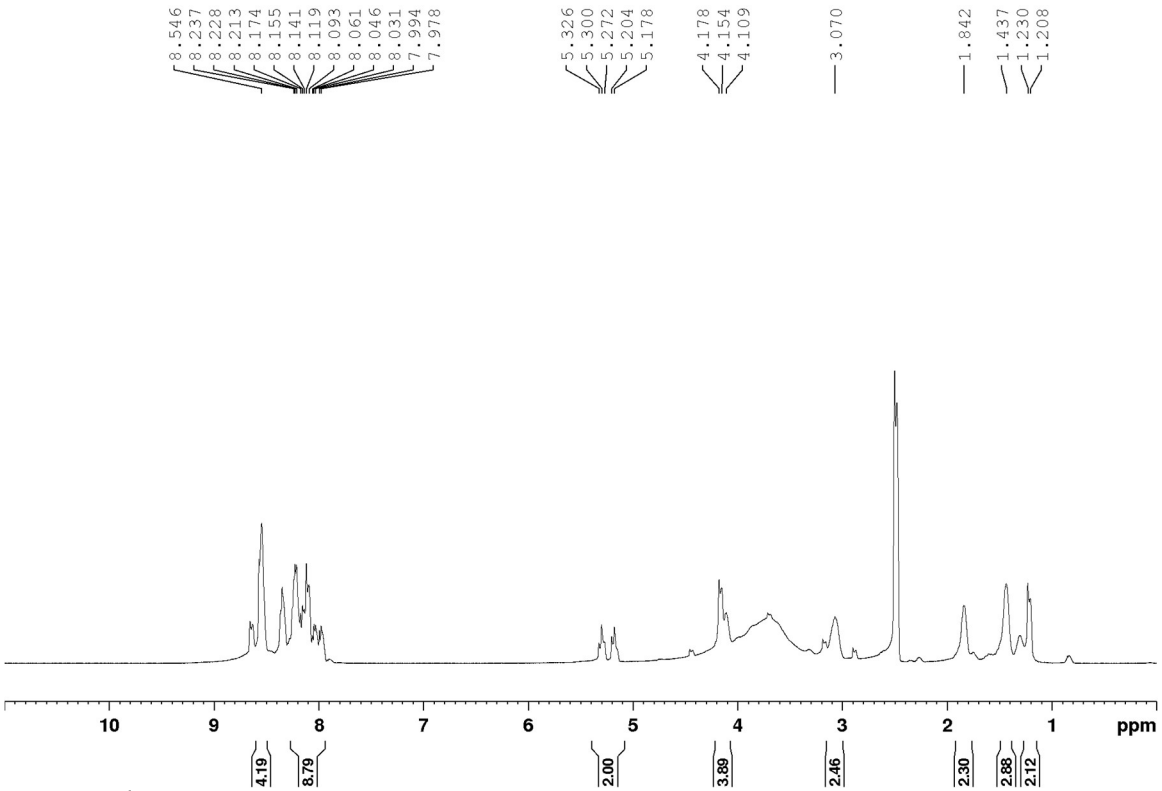


Figure S26. ^{1}H NMR spectrum of Lys-1-CME (500 MHz, DMSO-d_6)

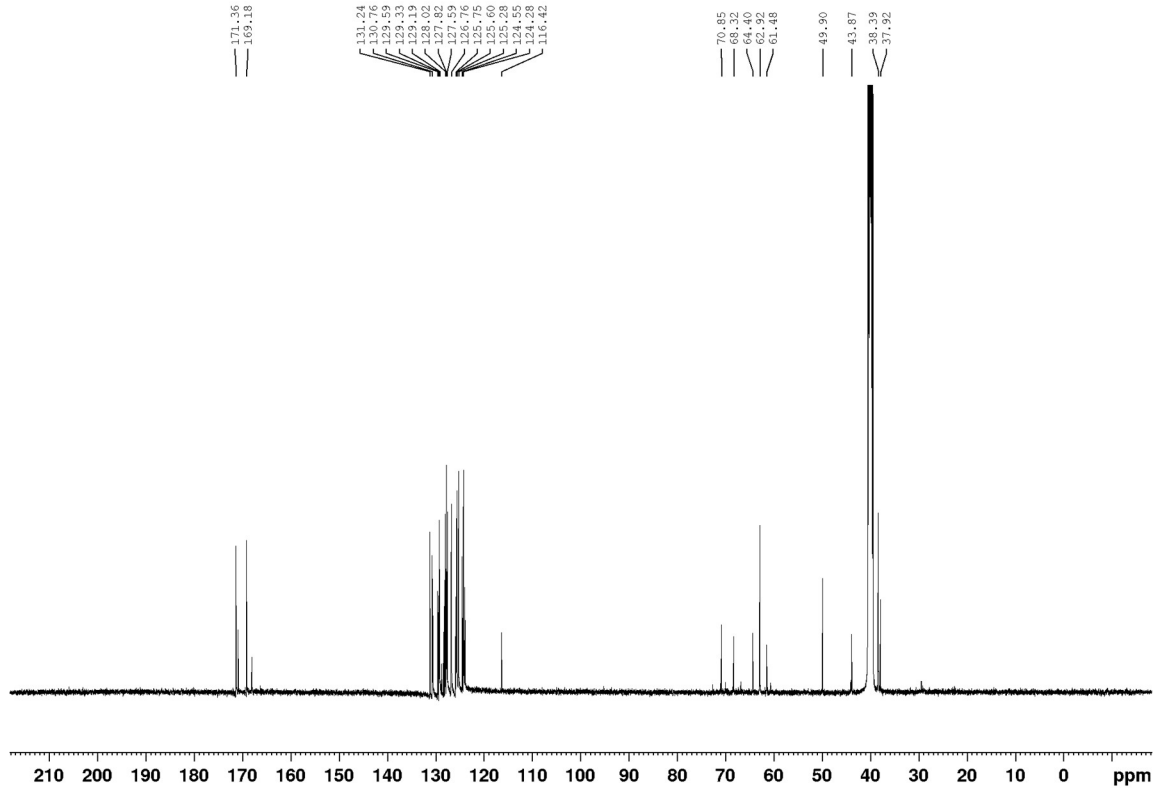


Figure S27. ^{13}C NMR spectrum of Lys-1-CME (125 MHz, DMSO-d_6)

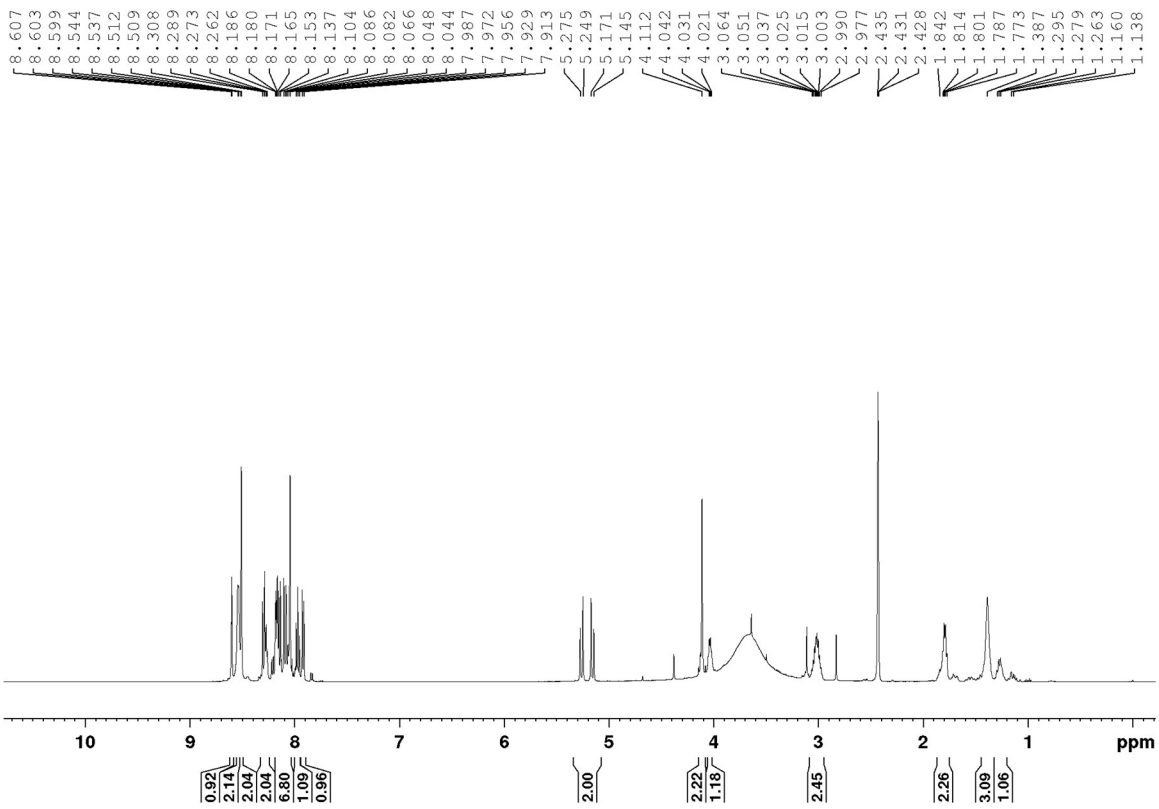


Figure S28. ^{1}H NMR spectrum of Lys-1-DNB (500 MHz, DMSO-d_6)

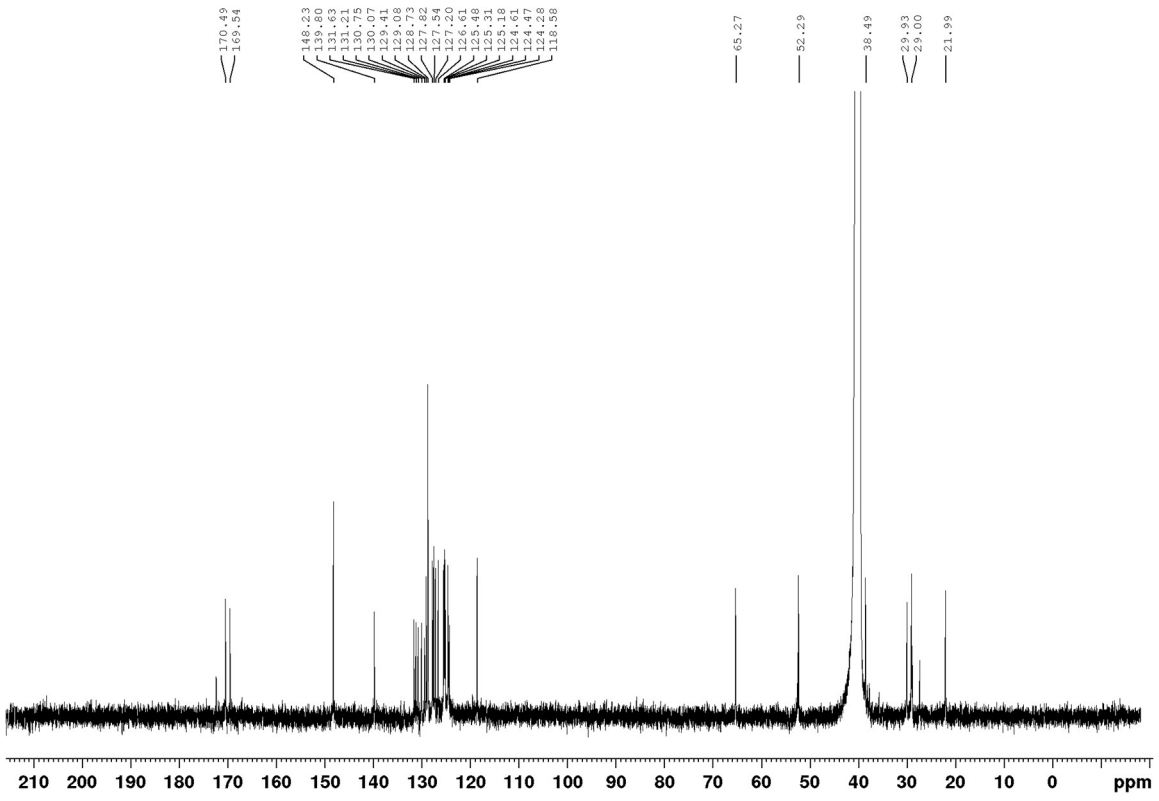


Figure S29. ^{13}C NMR spectrum of Lys-1-DNB (125 MHz, DMSO-d_6)

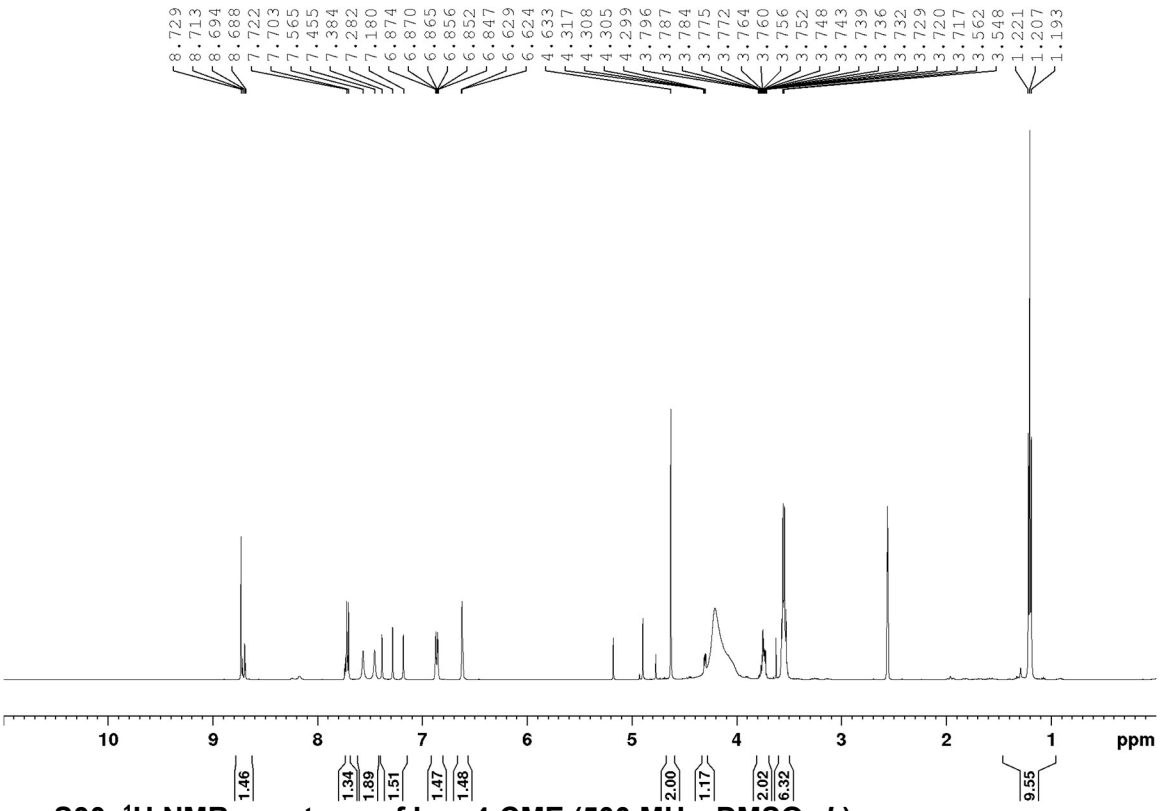


Figure S30. ^{1}H NMR spectrum of Lys-4-CME (500 MHz, DMSO-d_6)

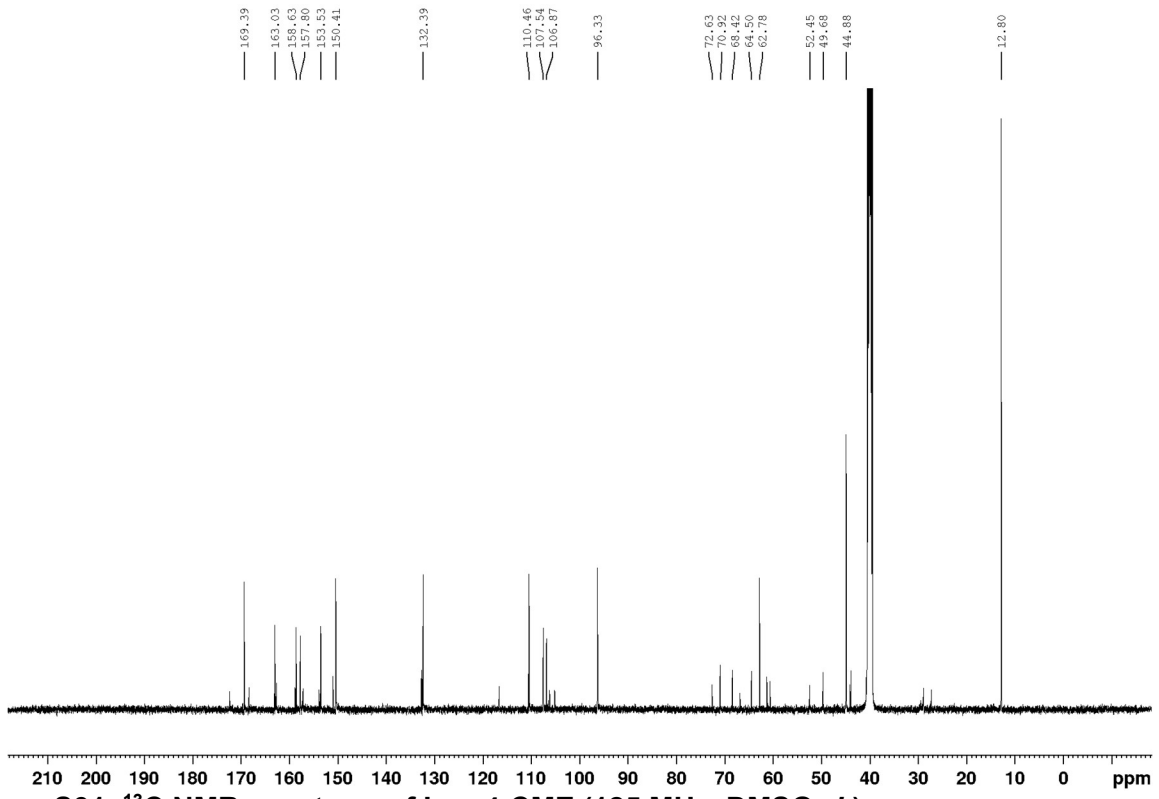


Figure S31. ^{13}C NMR spectrum of Lys-4-CME (125 MHz, DMSO-d_6)

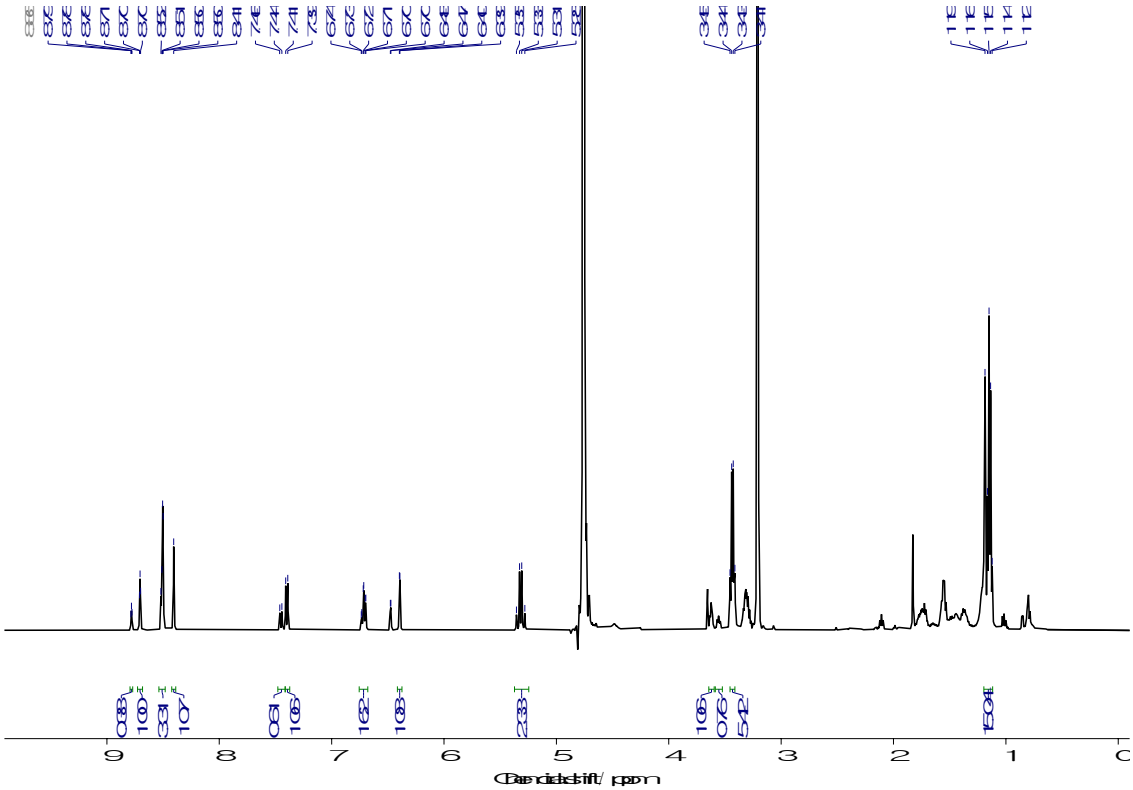


Figure S32. ^{1}H NMR spectrum of Lys-4-DNB (500 MHz, MeOD-d_4)

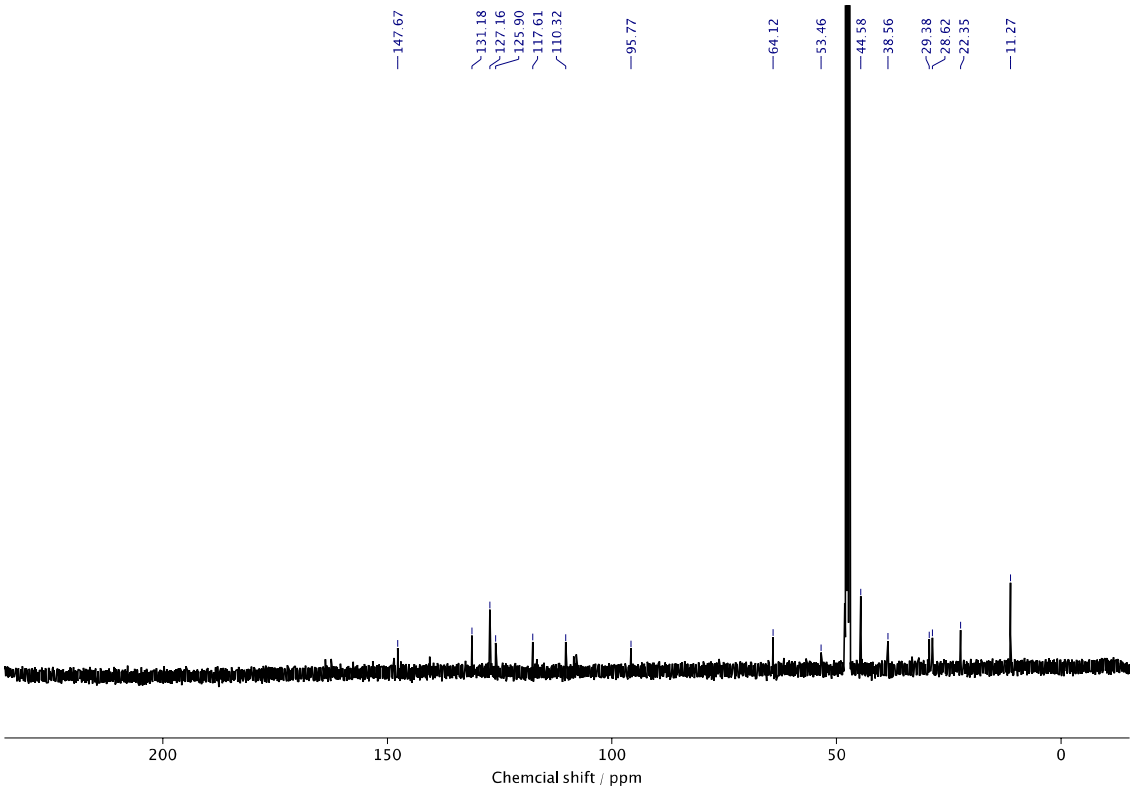


Figure S33. ^{13}C NMR spectrum of Lys-4-DNB (125 MHz, MeOD-d_4)

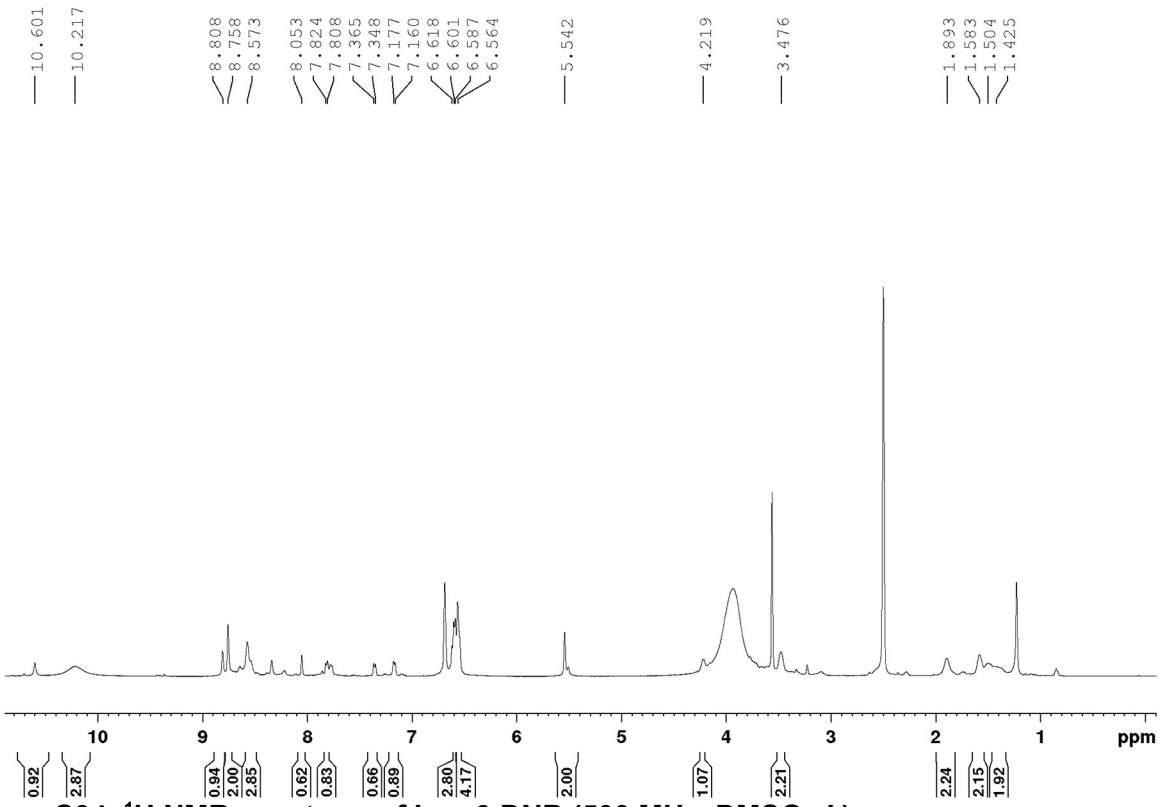


Figure S34. ^{1}H NMR spectrum of Lys-6-DNB (500 MHz, DMSO-d_6)

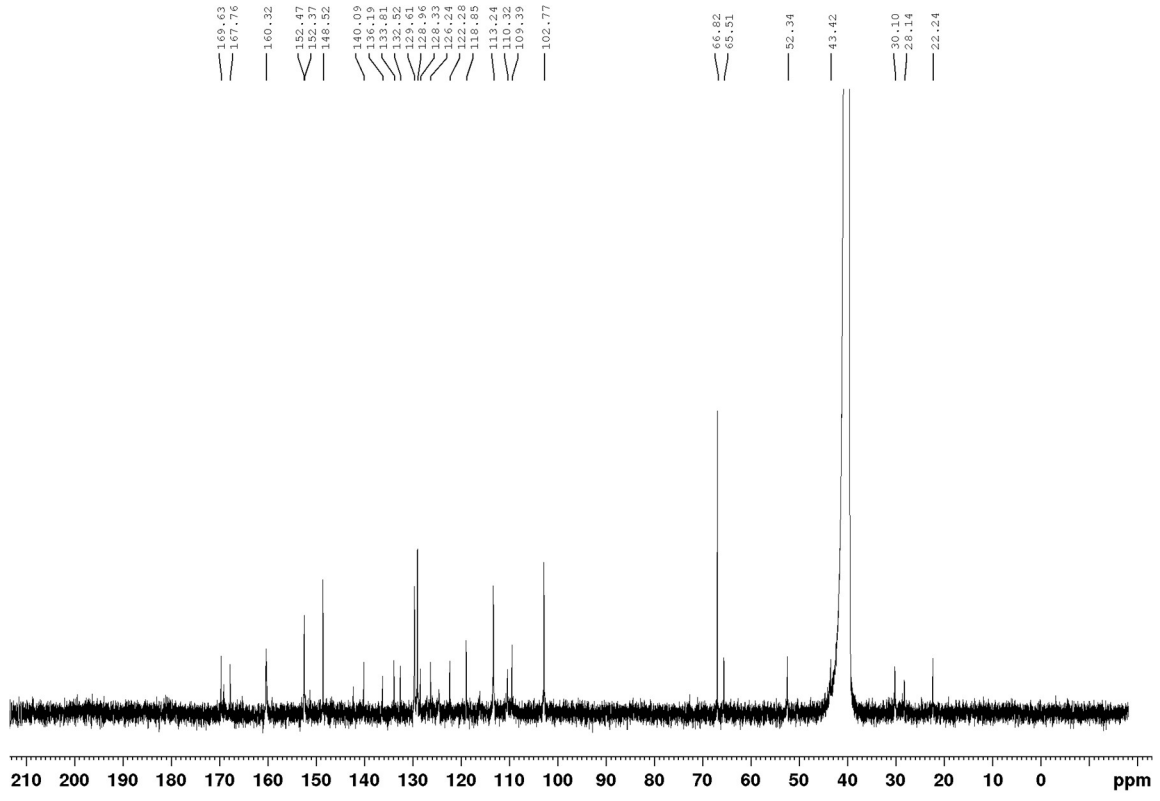


Figure S35. ^{13}C NMR spectrum of Lys-6-DNB (125 MHz, DMSO-d_6)

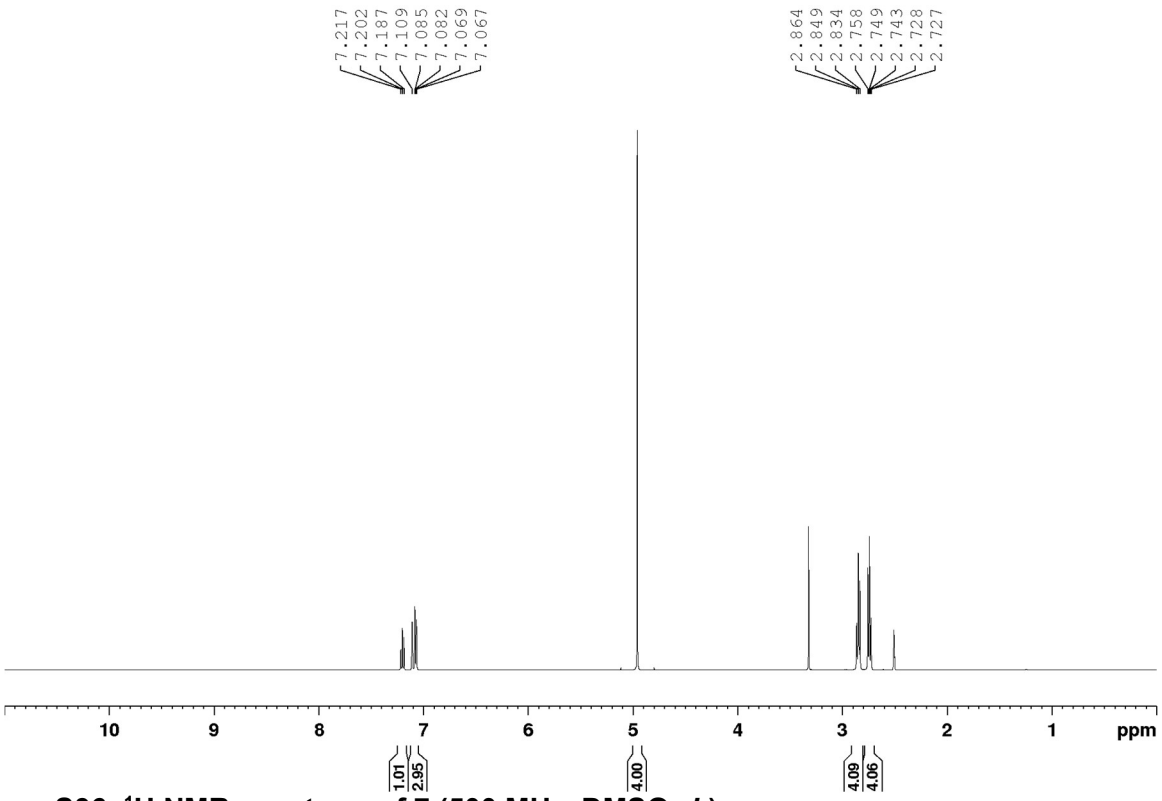


Figure S36. ^{1}H NMR spectrum of 7 (500 MHz, DMSO-d_6)

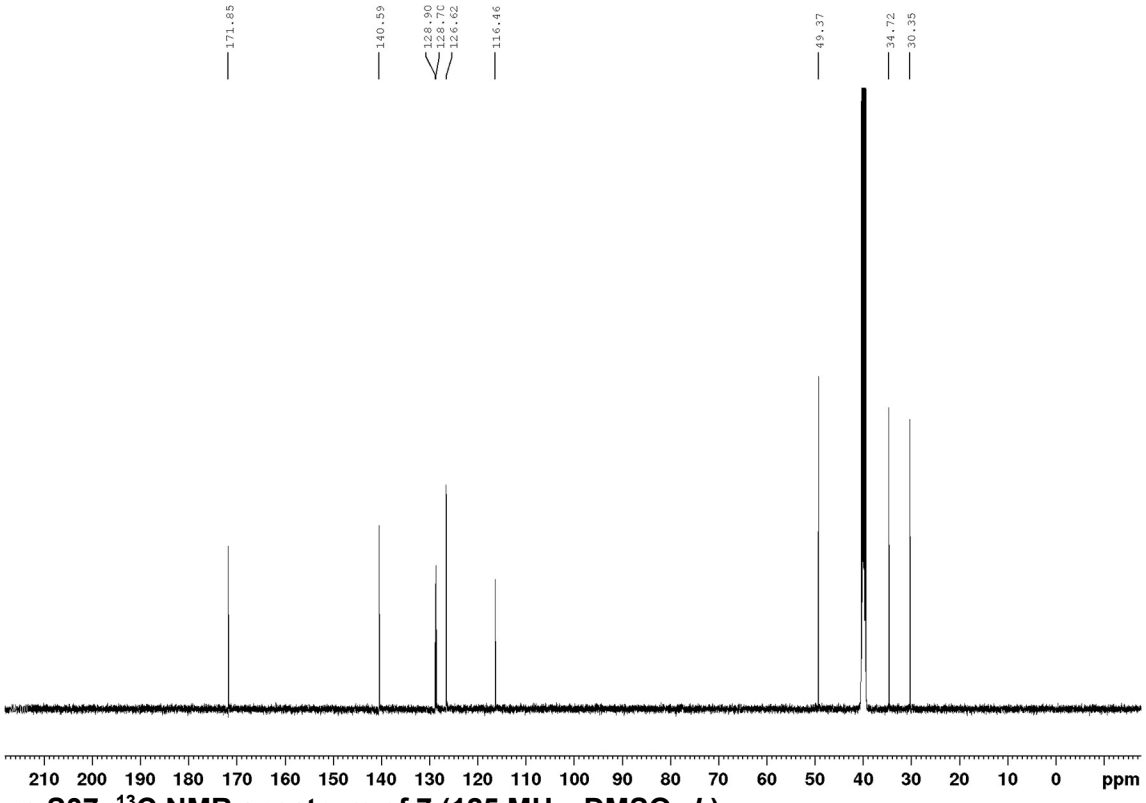


Figure S37. ^{13}C NMR spectrum of 7 (125 MHz, DMSO-d_6)

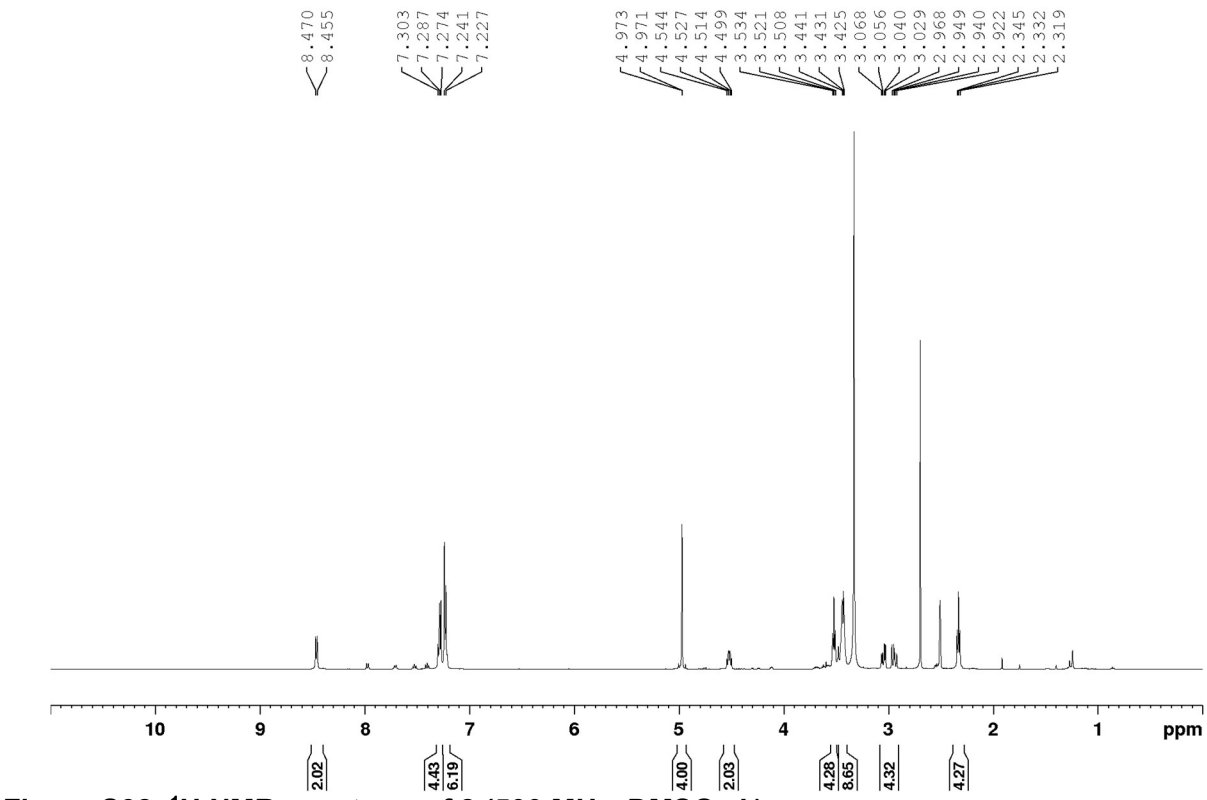


Figure S38. ^{1}H NMR spectrum of 8 (500 MHz, DMSO-d_6)

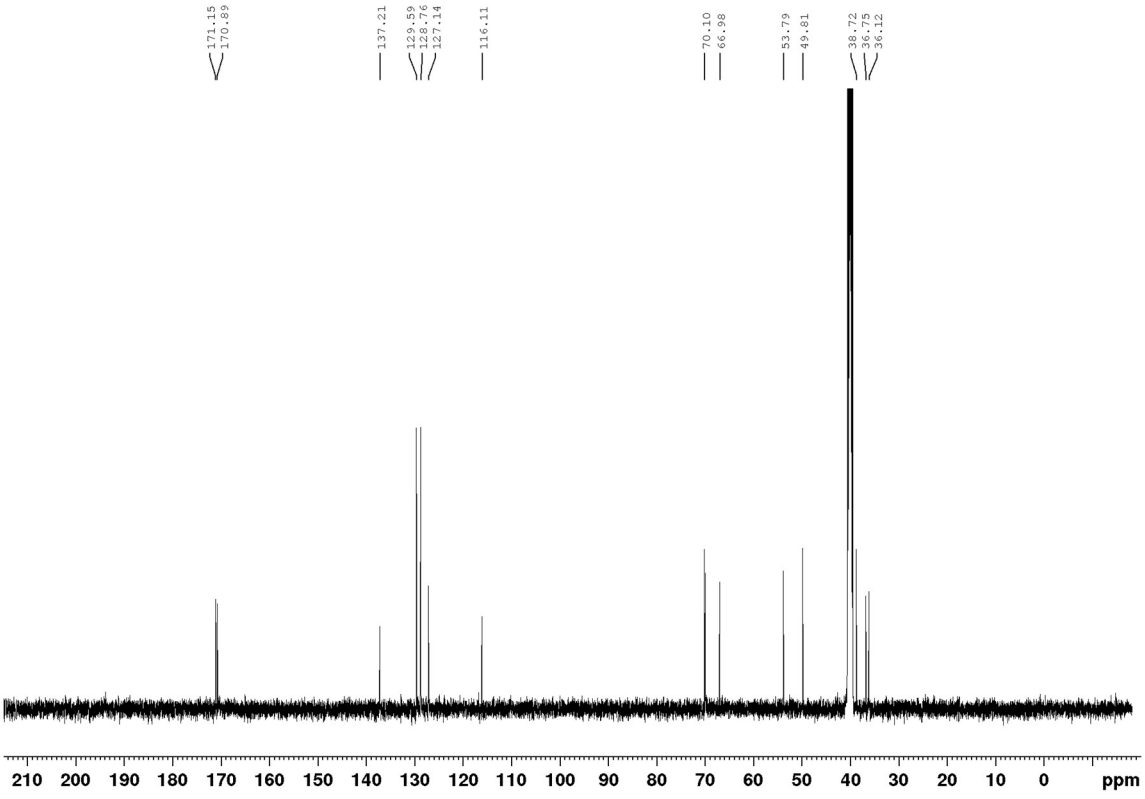


Figure S39. ^{13}C NMR spectrum of 8 (125 MHz, DMSO-d_6)

5. Plasmids map

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CATATG: NdeI
GTCGAC: SalI
TGGTCTCATCCGCAGTTCGAAAAA: strep tag
TAGTAA: Stop

>pJL1_StrepII_1
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>pJL1_StrepII_3
CATATGTGGTCTCATCCGCAGTTCGAAAAATCC**ACCATCGCC**TAGTAAGTCGAC]
fMetTrpSerHisProGlnPheGluLysSerThrIleAla

6. References

1. N. Niwa, Y. Yamagishi, H. Murakami and H. Suga, *Bioorg Med Chem Lett*, 2009, **19**, 3892-3894.
2. J. Lee, K. E. Schwieter, A. M. Watkins, D. S. Kim, H. Yu, K. J. Schwarz, J. Lim, J. Coronado, M. Byrom, E. V. Anslyn, A. D. Ellington, J. S. Moore and M. C. Jewett, *Nat Commun*, 2019, **10**, 5097.
3. J. Lee, K. J. Schwarz, D. S. Kim, J. S. Moore and M. C. Jewett, *Nat Commun*, 2020, **11**, 4304.
4. J. Lee, R. Torres, M. Byrom, A. D. Ellington and M. C. Jewett, *Chem Comm*, 2020, **56**, 5597-5600.
5. C. Tuckey, H. Asahara, Y. Zhou and S. Chong, *Curr Protoc Mol Biol*, 2014, **108**, 16 31 11-22.