Unraveling tetrazine-triggered bioorthogonal elimination enables chemical tools for ultrafast release and universal cleavage

Jonathan C.T. Carlson[†,#], Hannes Mikula[†,‡,#], and Ralph Weissleder[†,§,*]

† Center for Systems Biology, Massachusetts General Hospital, Boston, MA 02114 (USA).
‡ Institute of Applied Synthetic Chemistry, Vienna University of Technology (TU Wien), Austria.
§ Department of Systems Biology, Harvard Medical School, Boston, MA 02115 (USA).

Supporting Information - Table of Contents

1. General Methods

Unless otherwise noted, reactions were carried out under an atmosphere of nitrogen or argon in air-dried glassware with magnetic stirring. Air- and/or moisture-sensitive liquids were transferred via syringe. All reagents were obtained from commercial sources and used without further purification. BHQ®-10 Succinimidyl ester (BHQ-10-NHS) was purchased from LGC Biosearch Technologies. 3-Cyanopropanoic acid and N-Boc-3-cyano-L-alanine were purchased from Ark Pharm Inc. and Chem-Impex, respectively. m-dPEG®$_4$-NHS was obtained from Quanta BioDesign. Dry solvents were obtained from Sigma Aldrich. Analytical thin layer chromatography (TLC) was performed using plates cut from glass sheets (silica gel 60 F-254, Silicycle). Visualization was achieved under a 254 nm or 365 nm UV light and by immersion in a solution of cerium sulfate in ethanol followed by heating with a heat gun. Column chromatography was carried out using silica gel G-25 (40-63 μM) or C18 flash cartridges (SNAP C18 and SNAP C18 Ultra, Biotage).

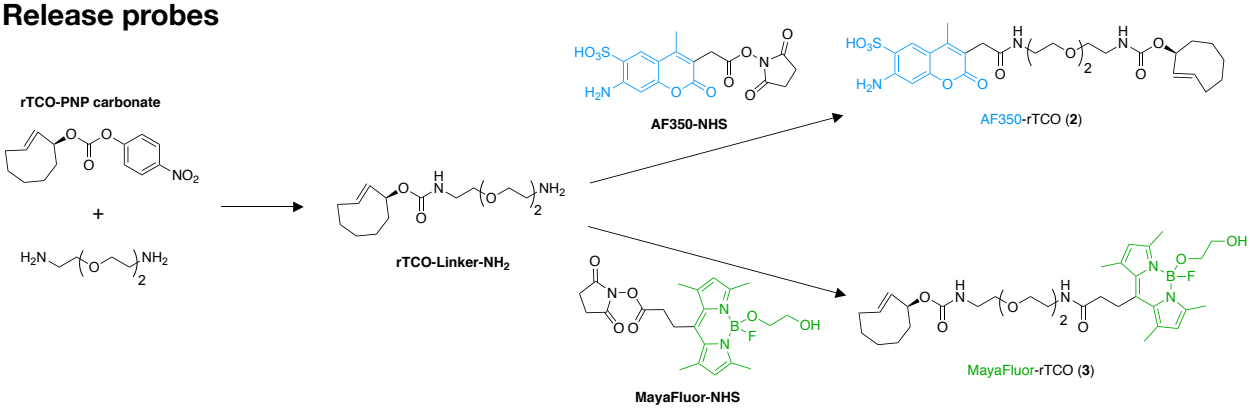
NMR spectra were recorded on a Bruker Avance IIIHD 600 MHz spectrometer equipped with a Prodigy BBO cryo probe, or on a Bruker Avance UltraShield 400 MHz spectrometer. Chemical shifts are reported in parts per million (δ) and calibrated using residual undeuterated solvent. Data are represented as follows: Chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, b = broad), coupling constant (J, Hz) and integration.

High performance liquid chromatography-mass spectrometry analysis (HPLC-MS, LCMS) was performed with on a Waters instrument equipped with a Waters 2424 ELS Detector, Waters 2998 UV-Vis Diode array Detector, Waters 2475 Multi-wavelength Fluorescence Detector, and a Waters 3100 Mass Detector. Separations employed an HPLC-grade water/acetonitrile solvent gradient with one of three columns: XTerra MS C18 Column, 125Å, 5 μm, 4.6 mm X 50 mm column; Waters XBridge BEH C18 Column, 130Å, 3.5 μm, 4.6 mm X 50 mm; Waters XBridge Protein BEH C4 Column, 300Å, 3.5 μm, 2.1 mm X 50 mm. Routine analysis were conducted with 0.1 % formic acid added added to both solvents; buffered analyses were run with ammonium formate buffer (2.5mM)/HPLC-grade acetonitrile. See section 4 below for full details of LCMS buffer preparation.

Fluorescence measurements were conducted with a PTI QuantaMaster 400 fluorimeter (Photon Technologies Incorporated, NJ, USA), and UV-VIS absorption spectra on a Horiba DualFL spectrophotometer (Horiba Instruments). Stopped flow measurements employed an SX20-LED (Applied Photophysics) equipped with a 535nm LED (full width half-maximum 34nm).

2. Synthesis

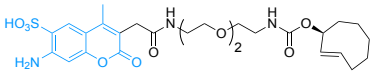
Release probes



rTCO-Linker-NH_2

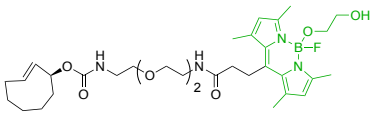
rTCO-PNP carbonate[1] (axial, 58.3 mg, 0.2 mmol) and ethylene glycol bis(2-aminoethyl) ether (296 mg, 2 mmol) were dissolved in DMF (2 mL). DIPEA (103.4 mg, 139 µL, 0.8 mmol) was added and the mixture was stirred at room temperature (rt) overnight. The clear solution was loaded onto a Biotage SNAP Ultra C18 column and the product obtained after reversed phase chromatography (H_2O/MeCN gradient elution, 0.1% formic acid). The product (52 mg, 87%) was used without further characterization. MS $[M+H]^+$ calcd. 301.2 for $C_{15}H_{29}N_2O_4^+$, found 301.3.

*AF350-rTCO (**2**)*



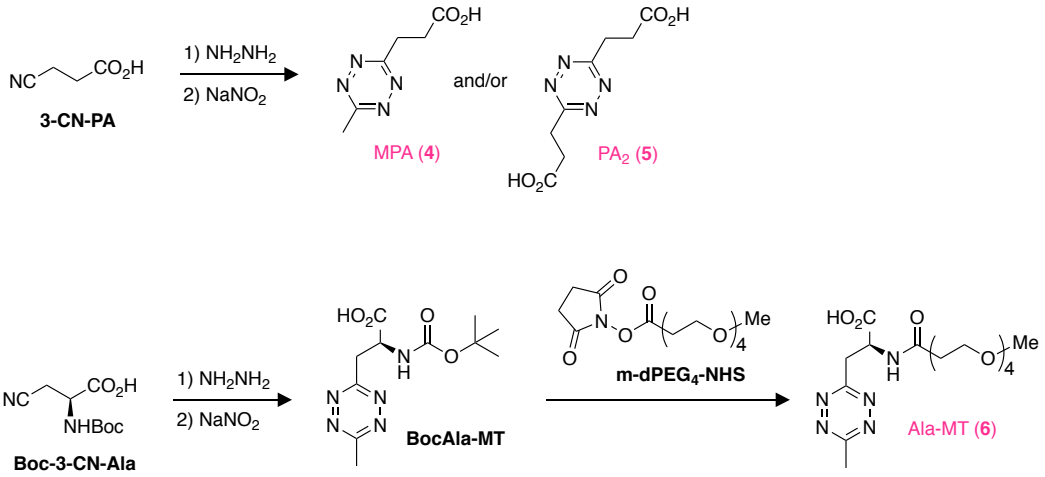
DIPEA (5.2 µL, 0.03 mmol) was added to a solution of rTCO-Linker-NH_2 (4 mg, 0.013 mmol) and AF350-NHS (5 mg, 0.015 mmol) in DMSO (0.5 mL) and the mixture was stirred at rt for 1h. Purification by reversed phase chromatography (H_2O/MeCN gradient elution, 0.1% formic acid) gave the desired product as a pale yellow solid (5.1 mg, 64%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.52 (s, 1H), 8.11 (s, 1H), 6.62 (s, 1H), 5.90-5.79 (m, 1H), 5.61-5.50 (m, 1H), 5.30-5.22 (m, 1H), 3.65-3.58 (m, 6H), 3.58-3.52 (m, 4H), 3.39 (t, J = 5.3 Hz, 2H), 3.29 (t, J = 5.3 Hz, 2H), 3.48-3.39 (m, 4H), 2.08-1.93 (m, 3H), 1.92-1.81 (m, 1H), 1.77-1.58 (m, 2H), 1.56-1.40 (m, 1H), 1.21-1.10 (m, 1H), 0.92-0.81 (m, 1H); ^{13}C NMR (150 MHz, CD_3OD) δ 171.5, 162.9, 157.0, 155.0, 151.2, 148.9, 131.4, 131.2, 125.4, 125.3, 114.1, 109.4, 100.6, 73.8, 69.93, 69.87, 69.6, 69.1, 40.2, 40.1, 39.1, 35.6, 35.4, 33.8, 28.7, 23.8, 13.9; HRMS $[M-H]^-$ calcd. 594.2127 for $C_{27}H_{36}N_3O_{10}S^-$, found 594.2138.

*MayaFluor-rTCO (**3**)*

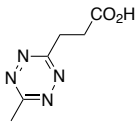


DIPEA (34.8 µL, 0.2 mmol) was added to a solution of rTCO-Linker-NH_2 (16.5 mg, 0.055 mmol) and MayaFluor-NHS[2] (27.6 mg, 0.060 mmol) in DMSO (0.5 mL) and the mixture was stirred at rt for 1h. Purification by reversed phase chromatography (H_2O/MeCN gradient elution, 0.1% formic acid) gave the desired product as an orange solid (28.3 mg, 80%). ^{1}H NMR (400 MHz, DMSO-d_6) δ8.14-8.05 (m, 1H), 7.13 (t, J = 6.0 Hz, 1H), 6.20 (s, 2H), 5.78-5.68 (m, 1H), 5.58-5.48 (m, 1H), 5.24-5.14 (m, 1H), 4.34 (t, J = 5.6 Hz, 1H), 3.51 (s, 4H), 3.45-3.38 (m, 4H), 3.28-3.16 (m, 6H), 3.15-3.08 (m, 2H), 2.75 (t, J = 6.1 Hz, 2H), 2.45 (s, 6H), 2.42 (s, 6H), 2.45-2.35 (m, 3H), 2.02-1.87 (m, 3H), 1.83-1.74 (m, 1H), 1.70-1.33 (m, 4H), 1.08-0.95 (m, 1H), 0.86-0.73 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.3, 155.6, 153.5, 145.2, 139.8, 132.3, 131.3, 130.7, 121.6, 72.7, 69.6, 69.5, 69.14, 69.08, 62.9, 62.85, 62.81, 61.7, 38.7, 36.5, 35.6, 35.2, 28.4, 23.9, 23.8, 16.1, 14.2; HRMS $[M-OC_2H_4OH]^+$ calcd. 583.3462 for $C_{31}H_{45}BFN_4O_5^+$, found 583.3449.

Tz acids

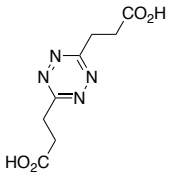


*MPA (**4**)*



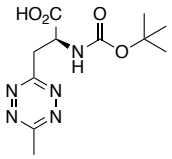
To a mixture of 3-CN-PA (1 g, 10.1 mmol), $Zn(OTf)_2$ (1.82 g, 5 mmol) and MeCN (2.9 g, 70.7 mmol) in a microwave reaction tube cooled to 0°C was added anhydrous hydrazine (11.4 mL, 363.6 mmol). The vessel was sealed and stirred at 60°C for 20h. After cooling to rt the vessel was carefully and slowly vented. The mixture was poured into a solution of $NaNO_2$ (6.27 g, 90.9 mmol) in water (70 mL). After cooling to 5°C 1N HCl was slowly added to acidify to pH 2. The mixture was extracted with EtOAc (5x) and the combined organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography (CH_2Cl_2/EtOAc gradient elution, 0-100% EtOAc, +1% HOAc) to obtain MPA (**4**) as a pink solid (170 mg, 10%). PA_2 (**5**) was obtained as a byproduct (18 mg). ^1H NMR (400 MHz, DMSO-d_6) δ 12.31 (bs, 1H), 3.46 (t, J = 7.0 Hz, 2H), 2.94 (s, 3H), 2.91 (t, J = 7.0 Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.3, 168.3, 167.1, 30.4, 29.2, 20.7; HRMS $[M-H]^-$ calcd. 167.0574 for $C_6H_7N_4O_2^-$, found 167.0565.

*PA_2 (**5**)*



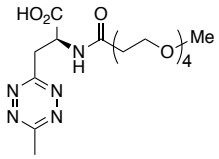
To a mixture of 3-CN-PA (1 g, 10.1 mmol) and $Zn(OTf)_2$ (1.82 g, 5 mmol) in a microwave reaction tube cooled to 0°C was slowly added cold anhydrous hydrazine (4.8 mL, 151.5 mmol). The vessel was sealed and stirred at 60°C for 20h. After cooling to rt the vessel was carefully and slowly vented. The mixture was poured into a solution of $NaNO_2$ (2.09 g, 30.3 mmol) in water (50 mL). After cooling to 5°C 1N HCl was slowly added to acidify to pH 2. The mixture was extracted with EtOAc (5x) and the combined organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography (CH_2Cl_2/EtOAc gradient elution, 0-100% EtOAc, +1% HOAc) to obtain the desired product as a pink solid (185 mg, 16%). ^1H NMR (400 MHz, DMSO-d_6) δ 12.27 (bs, 2H), 3.48 (t, J = 7.0 Hz, 4H), 2.92 (t, J = 7.0 Hz, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.3, 168.7, 30.3, 29.3; HRMS $[M-H]^-$ calcd. 225.0629 for $C_8H_9N_4O_4^-$, found 225.0617.

BocAla-MT



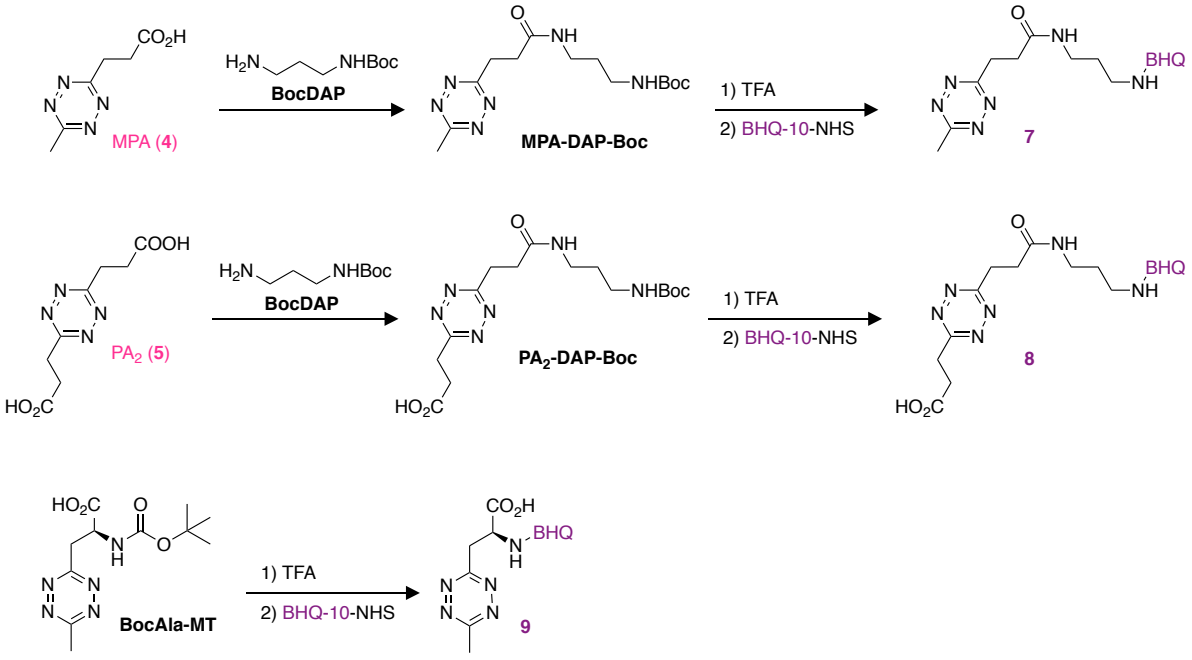
To a mixture of Boc-3-CN-Ala (0.86 g, 4 mmol), $Zn(OTf)_2$ (0.73 g, 2 mmol) and MeCN (1.64 g, 40 mmol) in a microwave reaction tube cooled to 0°C was added anhydrous hydrazine (4.5 mL, 144 mmol). The vessel was sealed and stirred at 60°C for 20h. After cooling to rt the vessel was carefully and slowly vented. The mixture was poured into a solution of $NaNO_2$ (4.14 g, 60 mmol) in water (50 mL). After cooling to 5°C 1N HCl was slowly added to acidify to pH 2. The mixture was extracted with EtOAc (5x) and the combined organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography (CH_2Cl_2/EtOAc gradient elution, 0-100% EtOAc, +1% HOAc) to obtain the product as a pink solid (85 mg, 8%). ^{1}H NMR (400 MHz, CD_2Cl_2) δ 6.45 (bs, 1H), 5.59-5.47 (m, 1H), 4.84-4.74 (m, 1H), 3.80 (dd, J = 15.3, 5.1 Hz, 1H), 3.66 (dd, J = 15.3, 7.0 Hz, 1H), 2.94 (s, 3H), 1.28 (s, 9H); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 174.0, 168.3, 167.0, 155.8, 80.8, 40.2, 37.5, 28.3, 21.3; HRMS $[M-H]^-$ calcd. 282.1208 for $C_{11}H_{16}N_5O_4^-$, found 282.1198.

*Ala-MT (**6**)*

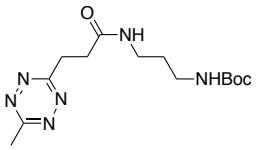


BocAla-MT (12 mg, 42.3 µmol) were dissolved in dry CH_2Cl_2 (1.5 mL) and trifluoroacetic acid (TFA, 0.5 mL) was added. After 30 min the mixture was concentrated and TFA was completely removed by co-evaporation with MeCN (3x). The residue was dried in vacuo (rt, 0.5 mbar) and dissolved in DMSO (0.5 mL). To the solution was added NEt_3 (31 µL, 0.22 mmol) followed by addition of a solution of m-dPEG4-NHS (14.3 mg, 43 µmol) in DMSO (430 µL). The reaction mixture was stirred at rt for 2h and then loaded onto a Biotage SNAP Ultra C18 column. The product was obtained after reversed phase chromatography (H_2O/MeCN gradient elution, 0.1% formic acid) as a pink glassy solid (13.1 mg, 77%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.28 (d, J = 8.2 Hz, 1H), 4.86 (td, J = 8.0, 6.1 Hz, 1H), 3.69 (dd, J = 15.5, 6.1 Hz, 1H), 3.55-3.40 (m, 15H), 3.23 (s, 3H), 2.95 (s, 3H), 2.33-2.23 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.9, 170.1, 167.1, 166.7, 71.3, 69.77, 69.75, 69.6, 69.55, 69.4, 66.5, 58.0, 50.7, 36.6, 35.7, 20.7; HRMS $[M-H]^-$ calcd. 400.1838 for $C_{16}H_{26}N_5O_7^-$, found 400.1850.

BHQ-Tz

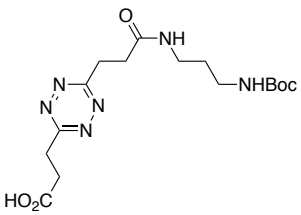


MPA-DAP-Boc



MPA (**4**, 33.6 mg, 0.2 mmol) and DIPEA (174 µL, 1 mmol) were dissolved in dry DMF (2 mL). TSTU (75.3 mg, 0.25 mmol) was added and the solution was stirred at rt for 10 min. After addition of BocDAP (69.7 mg, 0.4 mmol) stirring was continued for 30 min. The mixture was loaded onto a Biotage SNAP Ultra C18 column and the product was obtained after reversed phase chromatography (H_2O/MeCN gradient elution, 0.1% formic acid) as a pink solid (56 mg, 90%). ^{1}H NMR (400 MHz, CD_2Cl_2) δ 6.33 (bs, 1H), 4.98 (bs, 1H), 3.60 (t, J = 7.1 Hz, 2H), 3.24 (q, J = 6.3 Hz, 2H), 3.11 (q, J = 6.2 Hz, 2H), 2.99 (s, 3H), 2.88 (t, J = 7.1 Hz, 2H), 1.56 (quint., J = 6.2 Hz, 2H), 1.41 (s, 9H); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 171.5, 169.4, 168.0, 156.9, 79.4, 37.4, 36.4, 33.2, 30.6, 30.5, 28.5, 21.3; HRMS $[M+H]^+$ calcd. 325.1983 for $C_{14}H_{25}N_6O_3^+$, found 325.1985.

PA_2-DAP-Boc



To a solution of PA_2 (113 mg, 0.5 mmol), BocDAP (43.6 mg, 0.25 mmol) and DIPEA (174.2 µL, 1 mmol) in dry DMF (7 mL) was slowly added a solution of HBTU (94.8 mg, 0.25 mmol) in dry DMF (1 mL). After stirring at rt for 30 min additional BocDAP (17.4 mg, 0.1 mmol) and HBTU (37.9 mg, 0.1 mmol) were added consecutively. After stirring at rt for 2h the mixture was concentrated and the residue purified by reversed phase column chromatography (H_2O/MeCN gradient elution, 0.1% formic acid) to obtain the product as a pink solid (48 mg, 36%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.47 (bs, 1H), 5.04 (bs, 1H), 3.56-3.46 (m, 4H), 3.20-3.07 (m, 2H), 3.03-2.90 (m, 4H), 2.80 (t, J = 6.8 Hz, 2H), 1.51-1.40 (m, 2H), 1.33 (s, 9H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 175.1, 172.0, 169.5, 169.2, 157.2, 79.8, 37.5, 36.6, 33.1, 30.8, 30.5, 30.3, 29.9, 28.5; HRMS $[M-H]^-$ calcd. 381.1892 for $C_{16}H_{25}N_6O_5^-$, found 381.1907.

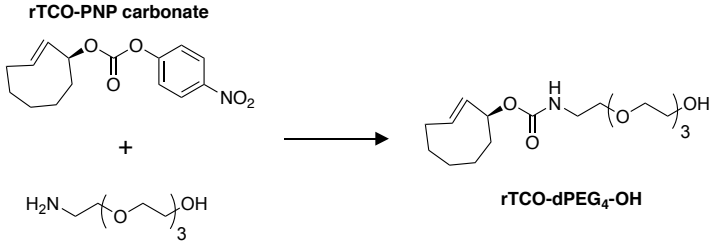
*BHQ-tetrazines **7**, **8** and **9***

MPA-DAP-Boc, PA_2-DAP-Boc and BocAla-MT were deprotected by dissolving in a mixture of CH_2Cl_2 and TFA (3/1). After stirring at rt for 30-60 min the solutions were concentrated and TFA was removed by co-evaporation with acetonitrile (3x) followed by drying in vacuo (rt, 0.5 mbar). BHQ-10 NHS was dissolved in DMSO at a concentration of 10 mg/mL (14.2 mM). To this solution were added successively 1-2 equivalents of the freshly Boc-deprotected tetrazine amine and 3 equivalents of NEt_3. Reaction progress was checked by LCMS with the Waters C4 peptide column; BHQ compounds exhibit anomalous loading and retention characteristics on Waters C18 media, failing to elute on routine gradients and/or exhibiting extremely broad bands. In the event that slow reaction progress was observed, additional equivalents of NEt_3 were added to neutralize residual TFA from the Boc-deprotection. On reaction completion, the reaction mix was loaded directly onto a Biotage SNAP Ultra C18 column for purification with a shallow ammonium-formate buffer (pH 8):acetonitrile gradient (4-15%). This commercial reverse phase media (SNAP Ultra C18) displays artifactual retention of primary amines (potentially due to incomplete capping of the silica) under buffered conditions, allowing separation of the aminotetrazine (which remains in a tight band at the top of the column) and BHQ-Tz product. Purity of the resulting BHQ-Tz conjugates **7**, **8** and **9** was verified by LCMS.

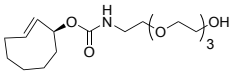
BHQ-tetrazine **7**: ^{1}H NMR (600 MHz, D_2O) δ 7.64-7.57 (m, 3H), 7.47 (d, J = 8.0 Hz, 2H), 7.37-7.30 (m, 1H), 7.03 (d, J = 8.0 Hz, 1H), 6.86 (bs, 2H), 6.50 (bs, 2H), 3.37 (t, J = 7.2 Hz, 2H), 3.09-3.03 (m, 2H), 3.303-2.96 (m, 4H), 2.79 (s, 3H), 2.73 (s, 3H), 2.71 (t, J = 7.2 Hz, 2H), 2.10-2.04 (m, 2H), 1.60-1.53 (m, 2H), 1.53-1.47 (m, 2H); ^{13}C NMR (150 MHz, D_2O) δ 174.4, 174.3, 174.2, 173.9, 173.8, 173.0, 168.4, 167.4, 158.6, 152.0, 147.6, 145.5, 137.8, 137.0, 131.5, 126.7, 123.2, 116.8, 53.6, 40.0, 36.8, 32.5, 31.9, 29.7, 27.8, 22.8, 20.1; HRMS $[M-H]^-$ calcd. 766.2195 for $C_{32}H_{36}N_{11}O_8S_2^-$, found 766.2210.

BHQ-tetrazine **8**: ^{1}H NMR (600 MHz, D_2O) δ 7.80-7.64 (m, 6H), 7.45-7.39 (m, 1H), 7.35-7.24 (m, 2H), 6.74-6.62 (m, 2H), 3.73 (dd, J = 14.8, 4.9 Hz, 1H), 3.51 (dd, J = 14.8, 8.6 Hz, 1H), 3.27-3.20 (m, 2H), 2.86 (bs, 3H), 2.84 (s, 3H), 2.19-2.11 (m, 2H), 1.66-1.60 (m, 2H); HRMS $[M-2H+Na]^-$ calcd. 747.1385 for $C_{29}H_{28}N_{10}NaO_9S_2^-$, found 747.1372.
BHQ-tetrazine **9**: ^{1}H NMR (600 MHz, D_2O) δ 7.70-7.59 (m, 3H), 7.58-7.48 (m, 2H), 7.42-7.33 (m, 1H), 7.15-7.05 (m, 1H), 6.93 (bs, 2H), 6.55 (bs), 3.41-3.34 (m, 3H), 3.15-3.05 (m, 2H), 3.02-2.95 (m, 3H), 2.91-2.84 (m, 2H), 2.82-2.73 (m, 3H), 2.72-2.65 (m, 2H), 2.13-2.03 (m, 2H), 1.67-1.52 (m, 2H), 1.50-1.43 (m, 2H); ^{13}C NMR (150 MHz, D_2O) δ 177.1, 176.7, 176.3, 174.4, 174.2, 173.9, 168.7, 168.5, 166.9, 158.5, 152.1, 147.9, 145.4, 138.6, 137.4, 132.1, 126.7, 123.2, 116.9, 53.5, 39.9, 36.7, 32.5, 31.9, 30.3, 29.8, 28.9, 27.8, 22.8; HRMS $[M-2H+Na]^-$ calcd. 846.2069 for $C_{34}H_{37}N_{11}NaO_{10}S_2^-$, found 846.2059.

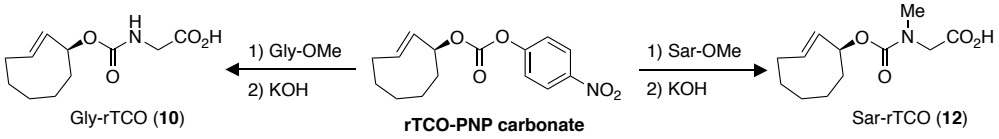
Non-labeled probes for click kinetics and release studies



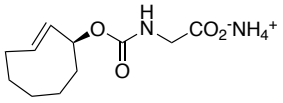
rTCO-dPEG$_4$-OH



To a solution of rTCO-PNP carbonate (29.1 mg, 0.1 mmol) in anhydrous DMF (1 mL) was added 2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethanol (29.0 mg, 0.15 mmol) and DIPEA (52.3 µL, 0.3 mmol). After stirring at rt for 3h the reaction mixture was loaded onto a Biotage SNAP Ultra C18 column and the product was obtained after reversed phase chromatography (H_2O/MeCN gradient elution) as a colorless oil (24 mg, 70%).
^{1}H NMR (400 MHz, D_2O) δ 5.85 (ddd, J = 14.4, 10.5, 3.4 Hz, 1H), 5.60 (dd, J = 16.5, 2.0 Hz, 1H), 5.21 (s, 1H), 3.80-3.51 (m, 14H), 3.48-3.21 (m, 2H) , 2.55-2.27 (m, 1H), 2.16-1.29 (m, 7H), 1.26-0.95 (m, 1H), 0.82 (td, J = 13.7, 13.1, 5.6 Hz, 1H); ^{13}C NMR (100 MHz, D_2O) δ 157.8, 132.0, 131.3, 74.8, 71.6, 69.7, 60.3, 40.0, 35.5, 35.4, 28.5, 23.7; HRMS $[M+H]^+$ calcd. 346.2224 for $C_{17}H_{32}NO_6^+$, found 346.2233



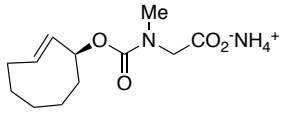
*Gly-rTCO (**10**) ammonium salt*



To a solution of rTCO-PNP carbonate (50 mg, 0.17 mmol) and glycine methyl ester (Gly-OMe) hydrochloride (44 mg, 0.35 mmol) in dry DMF (1 mL) was added DIPEA (122 µL, 0.7 mmol). After stirring at rt overnight an aqueous solution of KOH (4M, 0.35 mL, 1.4 mmol) was added and stirring was continued for 4 h. The solution was loaded directly onto a Biotage SNAP Ultra C18 column, preconditioned with ammonium formate buffer (2.5 mM, pH 9.2). The product was obtained as ammonium salt after reversed phase chromatography (ammonium formate buffer (2.5 mM, pH 9.2)/MeCN gradient

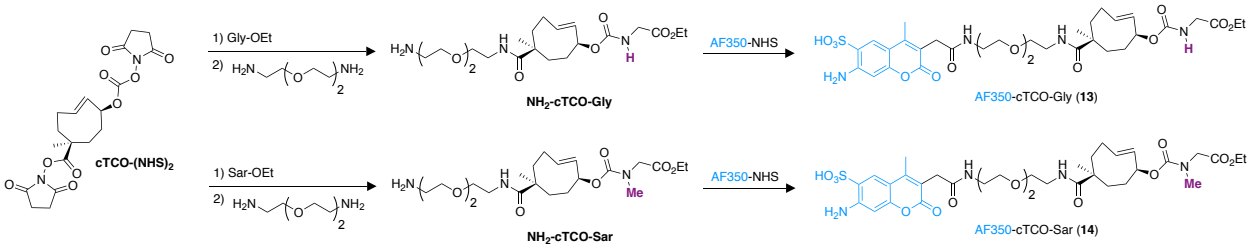
elution) as a colorless solid (28 mg, 72%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 6.76 (t, J = 5.2 Hz, 1H), 5.80-5.72 (m, 1H), 5.57-5.49 (m, 1H), 5.20-5.15 (m, 1H), 3.47-3.39 (m, 2H), 2.41-2.36 (m, 1H), 2.00-1.87 (m, 3H), 1.83-1.75 (m, 1H), 1.69-1.52 (m, 2H), 1.48-1.37 (m, 1H), 1.08-0.98 (m, 1H), 0.83-0.75 (m, 1H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 171.7, 155.3, 132.3, 130.9, 72.7, 43.8, 40.2, 35.6, 35.3, 28.5, 23.7; HRMS [M-H]$^-$ calcd. 226.1085 for $C_{11}H_{16}NO_4^-$, found 226.1077.

*Sar-rTCO (**12**) ammonium salt*

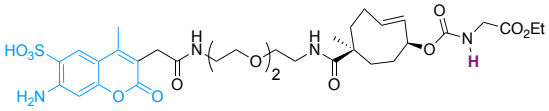


To a solution of rTCO-PNP carbonate (50 mg, 0.17 mmol) and sarcosine methyl ester (Sar-OMe) hydrochloride (49 mg, 0.35 mmol) in dry DMF (1 mL) was added DIPEA (122 µL, 0.7 mmol). After stirring at rt overnight an aqueous solution of KOH (4M, 0.35 mL, 1.4 mmol) was added and stirring was continued for 8 h. The solution was loaded directly onto a Biotage SNAP Ultra C18 column, preconditioned with ammonium formate buffer (2.5 mM, pH 9.2). The product was obtained after reversed phase chromatography (ammonium formate buffer (2.5 mM, pH 9.2)/MeCN gradient elution) as a colorless solid (22 mg, 54%). ^{1}H NMR (600 MHz, DMSO-d_6, mix of two rotamers) δ 5.78-5.68 (m, 1H), 5.58-5.47 (m, 1H), 5.22-5.13 (m, 1H), 3.57-3.45 (m, 2H), 2.87 (s, 1.4H, N-CH_3 of rotamer 1), 2.77 (s, 1.6H, N-CH_3 of rotamer 2), 2.43-2.33 (m, 1H), 2.02-1.85 (m, 3H), 1.84-1.73 (m, 1H), 1.69-1.50 (m, 2H), 1.49-1.36 (m, 1H), 1.08-0.93 (m, 1H), 0.85-0.73 (m, 1H); ^{13}C NMR (150 MHz, DMSO-d_6, two rotamers) δ 171.2, 171.0, 155.2, 155.0, 132.2, 132.1, 130.93, 130.92, 73.18, 73.11, 52.9, 52.5, 40.3, 40.2, 35.6, 35.5, 35.4, 35.2, 34.6, 28.54, 28.51, 23.75, 23.71; HRMS [M-H]$^-$ calcd. 240.1241 for $C_{12}H_{18}NO_4^-$, found 240.1229.

cTCO probes



*AF350-cTCO-Gly (**13**)*

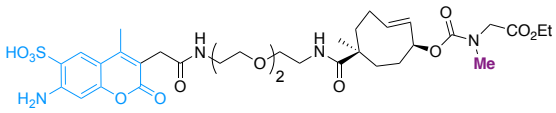


To a solution of cTCO-$(NHS)_2$ (prepared as described by Rossin *et al.*[3]) (10 mg, 0.024 mmol) in DMSO (0.5 mL) were added NEt_3 (8 µL, 6 mg, 0.059 mmol) and glycine ethyl ester hydrochloride (3.3 mg, 0.024 mmol). The mixture was gently rocked for 20 minutes at rt; LCMS indicated complete conversion to the desired monoadduct. An excess of 2,2'-(ethylenedioxy)-bis(ethylamine) (17 µL, 0.118 mmol) was then added and allowed to react at rt with gentle agitation for 90 minutes. LCMS verified complete conversion to the desired double adduct. The product was isolated by direct loading of the reaction mixture onto a Biotage C18 column and purified by reverse phase gradient (ammonium formate buffer (2.5 mM, pH 8)/MeCN, gradient elution, 4-80% MeCN). The intermediate NH_2-cTCO-Gly was briefly dried and then immediately used for the subsequent step.

An estimated 10 mg of NH_2-cTCO-Gly were dissolved in DMSO (0.5 mL) and added to a vial containing 5mg of AlexaFluor 350 succinimidyl ester (AF350-NHS, ThermoFisher Scientific).

The reaction was allowed to proceed overnight at 4°C. LCMS revealed complete consumption of the AF350-NHS and the product was purified by reverse phase chromatography. The reaction mixture was injected directly onto a Biotage C18 Ultra column and the product was eluted with a gradient of ammonium formate buffer (2.5 mM, pH 8)/MeCN (4-50%). The fractions containing AF350-cTCO-Gly were concentrated to obtain **13** (7.6 mg, 83%).
^{1}H NMR (600 MHz, DMSO-d_6, two rotamers, ~5/1) δ 8.11 (s, 1H), 7.44-7.38 (m, 0.35H), 6.63 (s, 1H), 5.99 (ddd, J = 16.4, 10.1, 4.7 Hz, 0.83H), 5.88 (ddd, J = 16.4, 10.1, 4.7 Hz, 0.17H), 5.68 (dd, J = 16.5, 2.4 Hz, 1H), 5.15-5.08 (m, 1H), 4.24 (q, J = 7.1 Hz, 0.35H), 4.18 (q, J = 7.1 Hz, 1.65H), 3.94 (d, J = 17.8 Hz, 0.16H), 3.90 (d, J = 17.8 Hz, 0.16H), 3.85 (s, 1.6H), 3.60 (s, 2H), 3.60-3.57 (m, 4H), 3.55 (t, J = 5.8 Hz, 1H), 3.50 (t, J = 5.8 Hz, 1H), 3.40-3.37 (m, 2H), 3.34-3.30 (m, 2H), 2.43 (s, 3H), 2.32-2.23 (m, 2H), 2.23-2.13 (m, 1H), 2.09-1.97 (m, 2H), 1.95-1.88 (m, 1H), 1.79-1.73 (m, 1H), 1.54 (dd, J = 15.8, 6.6 Hz, 1H), 1.30 (t, J = 7.1 Hz, 0.5H), 1.27 (t, J = 7.1 Hz, 2.5H), 1.13 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 183.5, 173.0, 172.0, 164.3, 158.5, 156.4, 152.6, 150.3, 132.5, 126.71, 126.66, 115.5, 110.9, 102.0, 74.0, 71.3, 71.2, 70.5, 70.46, 68.1, 62.2, 46.5, 45.6, 43.3, 40.5, 40.3, 36.8, 35.2, 32.3, 31.9, 18.3, 15.4, 14.5; HRMS $[M+H]^+$ calcd. 739.2855 for $C_{33}H_{47}N_4O_{13}S^+$, found 739.2844.

*AF350-cTCO-Sar (**14**)*



To a solution of cTCO-$(NHS)_2$ (prepared as described by Rossin *et al.*[3]) (7.4 mg, 0.0175 mmol) in DMSO (0.75 mL) were added NEt_3 (6 µL, 4.4 mg, 0.044 mmol) and sarcosine ethyl ester hydrochloride (2.7 mg, 0.0175 mmol). The mixture was gently rocked for 25 minutes at rt; LCMS indicated complete conversion to the desired monoadduct. An excess of 2,2'-(ethylenedioxy)bis(ethylamine) (26 µL, 0.175 mmol) was then added and allowed to react at rt with gentle agitation for 90 minutes. LCMS verified complete conversion to the desired double adduct. The product was isolated by direct loading of the reaction mixture onto a Biotage C18 column and purified by reverse phase gradient (ammonium formate buffer (2.5 mM, pH 8)/ MeCN, gradient elution, 0-80% MeCN). The intermediate NH_2-cTCO-Sar was briefly dried and then immediately used for the subsequent step.
An estimated 8 mg of NH_2-cTCO-Sar were dissolved in DMSO (0.5 mL) and added to a vial containing 5 mg of AlexaFluor 350 succinimidyl ester (AF350-NHS, ThermoFisher Scientific). The reaction was allowed to proceed overnight at 4°C. LCMS revealed complete consumption of the AF350-NHS. The reaction mixture was injected directly onto a Biotage C18 Ultra column and the product was eluted with a gradient of ammonium formate (2.5 mM, pH 8)/MeCN (4-50%). The fractions containing AF350-cTCO-Sar were concentrated to obtain **14** (6.4 mg, 73%). ^{1}H NMR (600 MHz, DMSO-d_6, two rotamers, ~1/1) δ 8.12 (s, 1H), 7.50-7.46 (m, 0.25H), 7.41-7.37 (m, 0.25), 6.62 (1H), 5.96 (ddd, J = 16.6, 10.0, 5.1 Hz, 0.5H), 5.84 (ddd, J = 16.6, 10.0, 5.1 Hz, 0.5H), 5.71 (dd, J = 16.6, 2.5 Hz, 0.5H), 5.68 (dd, J = 16.6, 2.5 Hz, 0.5H), 5.15-5.14 (m, 0.5H), 5.13-5.12 (m, 0.5H), 4.25 (q, J = 7.2 Hz, 1H), 4.20 (q, J = 7.2 Hz, 1H), 4.17 (d, J = 17.6 Hz, 0.5H), 4.11 (d, J = 17.6 Hz, 0.5H), 4.04 (s, 1H), 3.61-3.59 (m, 2H), 3.59-3.57 (m, 4H), 3.54 (t, J = 5.4 Hz, 2H), 3.50 (t, J = 5.4 Hz, 2H), 3.40-3.36 (m, 2H), 3.10 (s, 1.5H), 2.99 (s, 1.5H), 2.68 (s, 3H), 2.43 (s, 3H), 2.33-2.25 (m, 2H), 2.24-2.13 (m, 1H), 2.11-2.02 (m, 1H), 2.00-1.86 (m, 2H), 1.80-1.74 (m, 1H), 1.61-1.50 (m, 1H), 1.31 (t, J = 7.2 Hz, 1.5 H), 1.28 (t, J = 7.2 Hz, 1.5H), 1.15 (s, 1.5H), 1.13 (s, 1.5H); ^{13}C NMR (150 MHz, DMSO-d_6, mix of two rotamers) δ 183.5, 183.4, 172.9, 171.5, 171.2, 164.3, 157.8, 157.2, 156.4, 152.7, 150.4, 132.7, 132.6, 132.3, 132.2, 126.8, 126.7, 115.5, 110.8, 101.9, 74.94, 74.89, 71.3, 71.2, 70.5, 64.5, 62.2, 51.6, 51.5, 49.8, 46.6, 46.5, 45.6, 45.5, 40.5, 40.4, 40.3, 36.83, 36.79, 36.3, 35.9, 35.2, 32.4, 32.3, 32.0, 31.9, 18.3, 15.3, 14.6, 14.5; HRMS $[M+H]^+$ calcd. 753.3011 for $C_{34}H_{49}N_4O_{13}S^+$, found 753.2991.

3. Click kinetics in PBS by stopped-flow

A. Sample preparation

Tetrazine (**4**-**6**) stock solutions were prepared in DMSO at concentrations ranging from 85-170 mM; concentrations were verified by absorbance measurements. Extinction coefficients for the individual tetrazines **1**, **4**, and **5** at 520nm were determined by titration against a standardized TCO reference solution (data not shown): **1**, 510 $M^{-1}cm^{-1}$; **4**, 484 $M^{-1}cm^{-1}$; **5**, 470 $M^{-1}cm^{-1}$; the stock concentration of Ala-MT (**6**) was determined by aliquoting a measured mass of the compound. Serial dilution into PBS was used to prepare solutions for stopped flow analysis at a Tz concentration of 100μM.

In parallel, rTCO-dPEG$_4$-OH (see Synthesis, section 2) was dissolved in DMSO to target a concentration >150 mM. The exact concentration was determined by absorbance titration with methyltetrazine-PEG4-acid (Click Chemistry Tools, extinction coefficient 438.3 $M^{-1}cm^{-1}$ at 520 nm, data not shown), quantifying the decrease in Tz absorbance upon reaction with rTCO. The initial DMSO stock (162.0 mM) was diluted into PBS (pH 7.4) to prepare solutions for stopped flow analysis at final rTCO concentrations of 1.50 mM and 2.00 mM, respectively.

B. Stopped flow acquisition

The stopped flow spectrophotometer was equipped with a 535 nm LED (FWHM 34 nm) to target the characteristic tetrazine visible light absorbance (peak ~520 nm). The reagent syringes were loaded with tetrazine and rTCO analyte solutions and the instrument primed. Subsequent data were collected in triplicate or quadruplicate for each rTCO concentration and each tetrazine. All Tz samples were benchmarked against the same rTCO solutions to maximize accuracy in relative rate determinations. Reactions were conducted at ambient temperature, recorded automatically at the time of acquisition (T = 22°C).

C. Kinetic fitting

Data were analyzed in GraphPad Prism 6.0/7.0 (Graphpad Software). Second order rate constants were calculated directly from nonlinear fitting of the absorbance vs time curves to the second order rate equation, as derived in *Kinetics and Mechanism*, John W. Moore and Ralph G. Pearson, (John Wiley and Sons, 1961). Tz_o and TCO_o were fixed to the experimental concentrations and to fit the extinction coefficient (ε), rate constant (k) and instrument baseline.

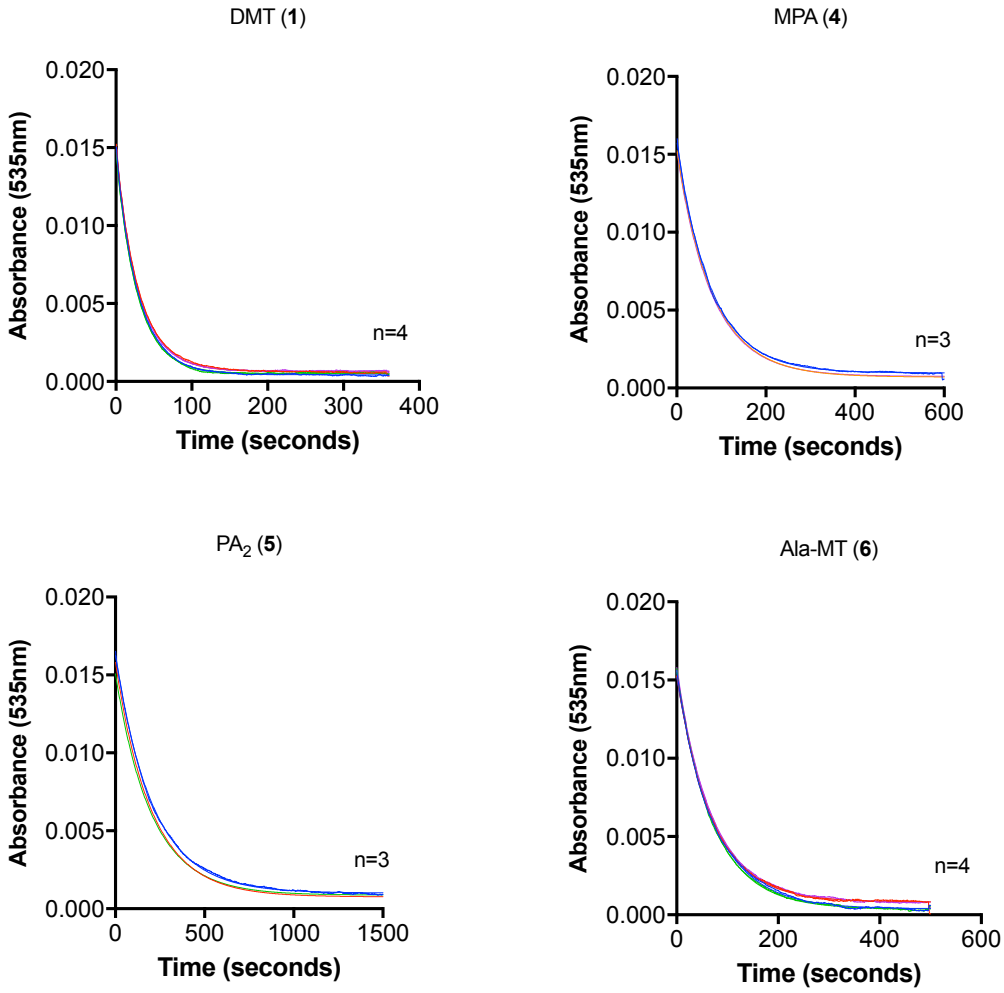
$$[Abs] = \frac{\varepsilon\,(Tz_o TCO_o - {Tz_o}^2)}{TCO_o e^{((TCO_o - Tz_o)kt)} - Tz_o} + baseline$$

D. Click reaction rates with rTCO-dPEG$_4$-OH in PBS, pH 7.4

Reported error estimates represent the standard deviation of the fitted rate constant across N = 3 or 4 runs (indicated on the following page in part E).

Tetrazine	Rate constant [$M^{-1}s^{-1}$] (T= 22°C)
DMT (**1**)	48.7 ± 1.1
MPA (**4**)	17.5 ± 0.3
PA$_2$ (**5**)	6.7 ± 0.2
Ala-MT (**6**)	19.4 ± 0.5

E. Kinetic data with superimposed exponential fits
[rTCO-dPEG$_4$-OH] = 750 μM



4. Release Experiments

A. LCMS

i. Instrument solvents and column conditioning

Solvents for routine HPLC conditions were prepared by addition of 4 mL of neat formic acid to 4 L of HPLC-grade acetonitrile or water to yield a final concentration of 0.1% formic acid.

For buffered LCMS conditions, the aqueous buffer was prepared by dilution of 1mL of 10M ammonium formate (BioUltra, Sigma-Aldrich) into 4 L of HPLC-grade H_2O and the pH adjusted to 8.4 with ~1 M ammonium hydroxide. HPLC grade acetonitrile (without additives) was used as the organic mobile phase. The pH of this volatile buffer declines over time and should be rechecked regularly, particularly if any drift in retention times is observed.

Column conditioning with formic acid and formate buffers is notably slow (many hundreds of column volumes), and was monitored/verified by serial checks of the eluate pH until it matched that of the input solvent.

ii. Analytical stock solutions and buffers

Stock solutions of the release probes AF350-rTCO (**2**), MayaFluor-rTCO (**3**), Gly-rTCO (**10**), Sar-rTCO (**12**), AF350-cTCO-Gly (**13**) and AF350-cTCO-Sar (**14**) were prepared in DMSO at concentrations ≥80-fold higher than the final 100 μM target concentration for analytical LCMS assays, so that the final concentration of DMSO in buffer was never higher than 1.25%. Exact concentrations of the stock solutions were determined from absorbance measurements of samples made from serial dilutions of this stock into water, with the known extinction coefficient of AF350 (19000 $M^{-1}cm^{-1}$, ThermoFisher) and MayaFluor (66,300 $M^{-1}cm^{-1}$)[2].

Tetrazine (**1**[1], **4**-**6**) stock solutions were prepared as described for kinetic experiments (vide supra, Section 3) in DMSO at concentrations ranging from 85-170 mM; Tz concentration in the analytical samples was 200 μM for all endpoint release experiments. For release kinetics, the concentration of **1** was kept constant at 200 μM, while the concentration of **4**-**6** was increased to 400 μM to ensure that the click reaction was complete within 10-20 minutes to allow monitoring of early time points. Comparison of release with **2**+**6** at 200 μM and 400 μM revealed no difference in the rate or magnitude of release observed (data not shown).

Citrate-Phosphate buffers[4] were prepared by ratiometric dilution from standard stocks of 0.1 M Citric Acid and 0.2 M Na_2HPO_4 and their pH verified and adjusted as needed to within ±0.05 pH units by digital pH metering. PBS was used directly from fresh standard solutions (CORNING cellgro, 21-040-CV). Reference buffers at pH 5, 7, 9, and 10 were prepared from Hydrion™ buffer packets (Sigma-Aldrich) and used fresh.

iii. LCMS sample preparation

Analytical samples for LCMS were prepared in a standard sequence: rTCO/cTCO probes were diluted by micropipette directly into the relevant aqueous buffer in the LCMS sample vial and mixed, followed by addition of the tetrazine to initiate the cleavage/release reaction (t=0 for kinetic experiments). Endpoint measurements were made at t ≥ 24 hours.

iv. Analytical LCMS analysis:

Diode array data were collected for all samples and used to discriminate all peaks containing the fluorophore tag from release byproducts. Quantitative product distributions were determined from UV-Vis chromatograms, which were integrated at 350 nm for all probes bearing AF350-rTCO (**2**, **13**, **14**), and at 500 nm for the MayaFluor-rTCO probe **3.**

B. NMR

i. Deuterated phosphate buffer

A 100 mM deuterated phosphate buffer was prepared by dissolving potassium dideuterium phosphate KD_2PO_4 [98% D] (Cambridge Isotope Laboratories) in D_2O [99.9% D] (Sigma Aldrich) and adjusting the pH* to 7.51 by addition of sodium deuteroxide (40 wt.% in D_2O [99% D]). This pH* equates to a pH of 7.4 according to the following equation as reported by Krężel and Bal.[5]

$$pH = 0.929\ pH^* + 0.42$$

ii. Click-to-release reaction and sample preparation

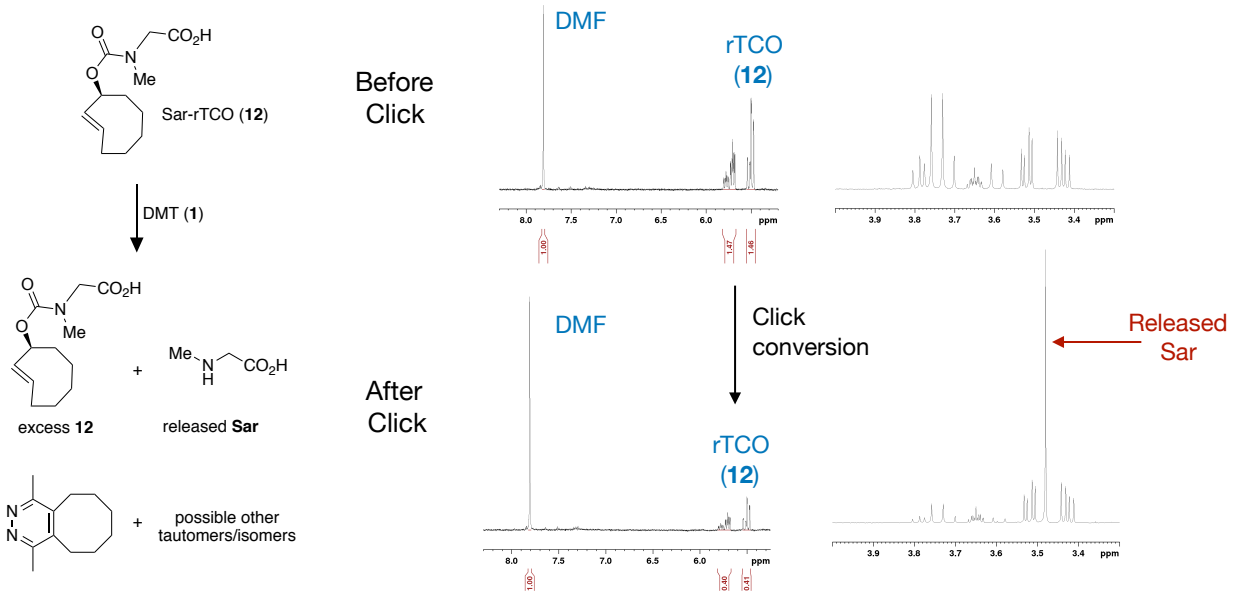
Stock solutions of Gly-rTCO (**10**) and Sar-rTCO (**12**) were prepared in DMSO-d_6 [99.9% D] at a concentration of 150 mM. 100 mM deuterated phosphate buffer was diluted to 10 mM with D_2O. 10 µL of 100 mM Gly-rTCO (**10**) and 100 mM Sar-rTCO (**12**) were added to 990 µL 10mM deuterated phosphate buffer containing 1 mM DMF (as internal standard), respectively, to afford a concentration of 1.5 mM of the rTCO probe for the click-to-release experiment. pH* was re-adjusted to pH* 7.51 = pH 7.4 by addition of sodium deuteroxide (see 4.B.i.) ^{1}H NMR spectra were recorded (128 scans) and the ratio of rTCO probe to DMF was calculated based on the CH-signal of DMF at 7.92 ppm and the CH-signals of rTCO at ~5.75 ppm and ~5.50 ppm.

DMT (**1**) was dissolved in DMSO-d_6 at a concentration of 1 M. 1 µL of this solution was added to the rTCO samples to afford a concentration of 1 mM DMT (**1**). Immediate click reaction was observed (disappearance of pink color). Samples were stored in the dark at room temperature for 36 hours before recording ^{1}H NMR spectra (256 scans) to obtain endpoint values.

iii. Data analysis

NMR spectra were analyzed using TopSpin (Bruker). Click conversion was calculated based on ratio of the CH-signal of DMF at 7.92 ppm and the CH-signals of rTCO at ~5.75 ppm and ~5.50 ppm (see following figure). Released Gly or Sar, respectively, was quantified using the CH_2 signal at ~3.5 ppm, which appears as a distinct singlet as these protons are no longer diastereotopic (see following figure).

To correct for changes of the baseline, all CH_2 signals in the range 4.2-3.2 ppm were set as constant (to include possible other tautomers/isomers/forms) to normalize the CH_2 signal of released Gly/Sar.



5. FRET assays of release rate

A. Analytical stock solutions and buffers

BHQ-Tz probes **7**-**9** were dissolved in water to prepare concentrated stock stock solutions. Concentrations were established by absorbance measurement of serially diluted samples in phosphate buffer pH 7.0 (50 mM); the absorbance of BHQ compounds is pH sensitive, with an absorbance maximum at 516 nM and an extinction coefficient of 28,700 $M^{-1}cm^{-1}$ at that pH (Biosearch Technologies).

MayaFluor-rTCO (**3**) was diluted from the parent DMSO stock into acetonitrile at 100 μM for more rapid mixing with the aqueous samples upon addition to the fluorimeter cuvette.

B. Instrument configuration

The sample chamber of the PTI-Quantamaster 400 fluorimeter was equipped with highly efficient dichroic bandpass filters (OD > 5.0 for rejected wavelengths; Thorlabs) to eliminate passage of reflected/scattered light off the face of the triangular cuvette. A shortpass filter at the entry site of the beam (model FES0500) was paired with a long pass filter (model FELH0500) at the exit from the sample chamber to the emission monochromator.

C. BHQ-Tz - MayaFluor-rTCO click/release fluorescence dynamics

Into a triangular cuvette (model 24-SB-Q-10, Starna Cells) with a magnetic stir bar were added first the buffer solution and then the BHQ-Tz (**7**-**9**) to a final concentration of 1, 2, or 4 mM in 600 μL total volume. For BHQ-Tz-acids **8** and **9**, the pH of the solution was checked by pH micro electrode to verify adequate buffering capacity. Data acquisition was started; after collecting a stable baseline, 6 μL of the MeCN stock solution of **3** were added via the instrument's sample addition port (for a final concentration of MayaFluor-rTCO at 1 μM, and final MeCN concentration ≤1%), with continuous stirring and ongoing data collection.

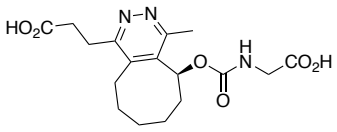
Higher concentrations of BHQ-Tz (2 mM and 4 mM) were used at lower pH values to accelerate the pseudo-first order click rate for kinetic partitioning of click and release. Because these experiments consume a significant quantity of scarce BHQ-Tz probes (~2 mg/cuvette), after allowing the release reaction to go to completion, serial additions of **3** to the same cuvette were employed.

D. Data analysis

The fluorescence traces of intensity vs. time were exported to Graphpad Prism 6.0/7.0 (Graphpad Software) for double exponential nonlinear fitting of fluorescence intensity vs time (falling with click reaction of BHQ-Tz and **3**, then rising after release of the free fluorophore amine).

6. Isolation and characterization of slow releasing isomers and tricyclic dead end

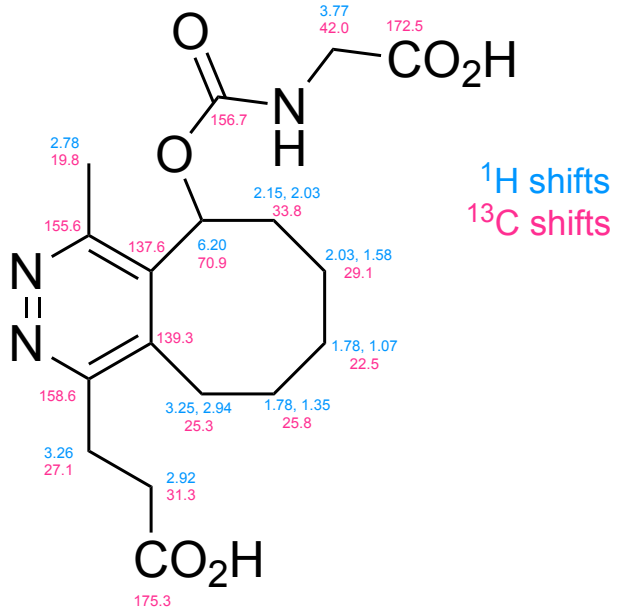
***A**. Isolation of the oxidized slow-releasing MPA-Gly-rTCO tautomer, MPA (**5**) + Gly-rTCO (**10**)*



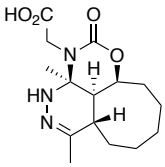
To a solution of 17 mg (0.075 mmol) of Gly-rTCO (**10**) in 25 mL of 10 mM Cit-Phos buffer at pH 6.02 were added 1.25 equivalents (0.093 mmol, 15.7 mg) of MPA (**5**) in 400 µL of DMSO. The solution was mixed thoroughly and allowed to react at room temperature for 15 minutes, whereupon the pH was rapidly uptitrated to pH 7.6 by serial addition of aliquots of 50 mM carbonate/bicarbonate buffer at pH 10, under continuous stirring and continuous pH metering. The reaction mixture was then transferred to a 1 L round bottom flask, placed under an oxygen atmosphere, and kept at 4°C for 72 hours. LCMS at 72 hours indicated substantial conversion to the aromatized product, with minimal residual dihydropyridazine observed.

The reaction mixture was then loaded onto a Biotage C18 Ultra column, washed with water to remove buffer salts, and eluted with an ammonium formate (pH 8.4)/MeCN gradient (4-45% MeCN). Fractions containing the desired product were concentrated by rotary evaporation and dried, and then redissolved in 250 µL of DMSO for loading onto a second Biotage C18 Ultra column equilibrated with H_2O/MeCN with 0.1% Formic acid. Elution with a gradient (4-50% MeCN) and rotary evaporation of the fraction containing the clean compound yielded 2.5 mg of the oxidized slow-releasing MPA-Gly-rTCO click product for NMR analysis. ESI (M-H): expected 364.16; observed 364.19.

^{1}H NMR (600 MHz, CD_3OD) δ 6.20 (dd, J = 10.6, 4.6 Hz, 1H), 3.77 (s, 2H), 3.30-3.20 (m, 3H), 2.98-2.86 (m, 3H), 2.78 (s, 3H), 2.19-2.10 (m, 1H), 2.08-1.98 (m, 2H), 1.82-1.74 (m, 2H), 1.63-1.53 (m, 1H), 1.43-1.28 (m, 1H), 1.18-0.95 (m, 1H); ^{13}C NMR (150 MHz, CD_3OD) δ 175.3, 172.5, 164.8, 158.6, 156.7, 155.6, 139.3, 137.6, 70.9, 42.0, 33.8, 31.3, 29.1, 27.1, 25.8, 25.3, 22.5, 19.8.



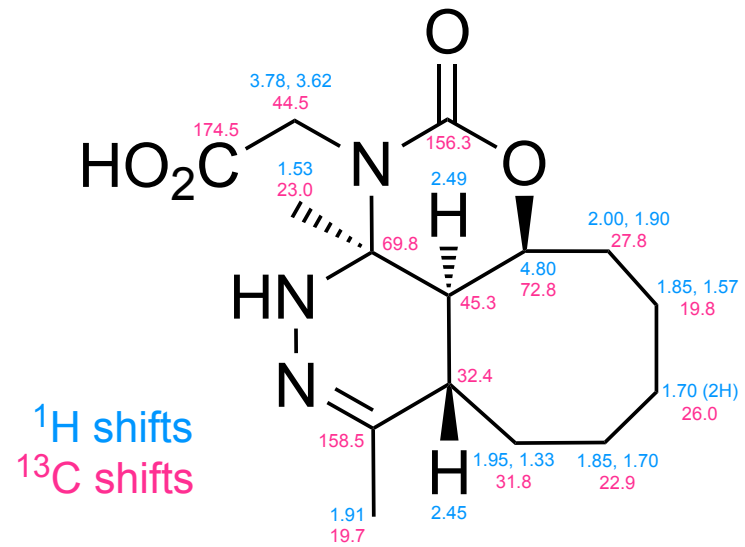
***B**. Isolation of **11**, tricyclic dead end isomer, Gly-rTCO (**10**) + DMT (**1**)*



To a solution of 6mg (0.026 mmol) of Gly-rTCO (**10**) dissolved in 50 mL of PBS were added 3.63 mg (1.25 equivalents, 0.033 mmol) of DMT (**1**) dissolved in 143 µL of DMSO. This solution was allowed to react at room temperature for 24 hours. LCMS after 24 hours and 30 hours were unchanged, and the reaction mixture was then loaded onto a Biotage C18 Ultra column for solid phase extraction and washed with water to remove buffer salts. The loaded organic material was then eluted with and the fractions containing the desired material pooled and concentrated by rotary evaporation. This material was then redissolved in DMSO (300 µL) and loaded onto a Biotage C18 Ultra column for chromatography with H_2O/MeCN, both containing 0.1% formic acid (4-50% MeCN). The fractions containing the desired product were concentrated and dried, yielding 3.5 mg of the dead end isomer for NMR analysis in two fractions of >98% purity. ESI (M-H): expected for a DMT-Gly-rTCO click product (dihydropyridazine oxidation state) 308.17; observed 308.22.

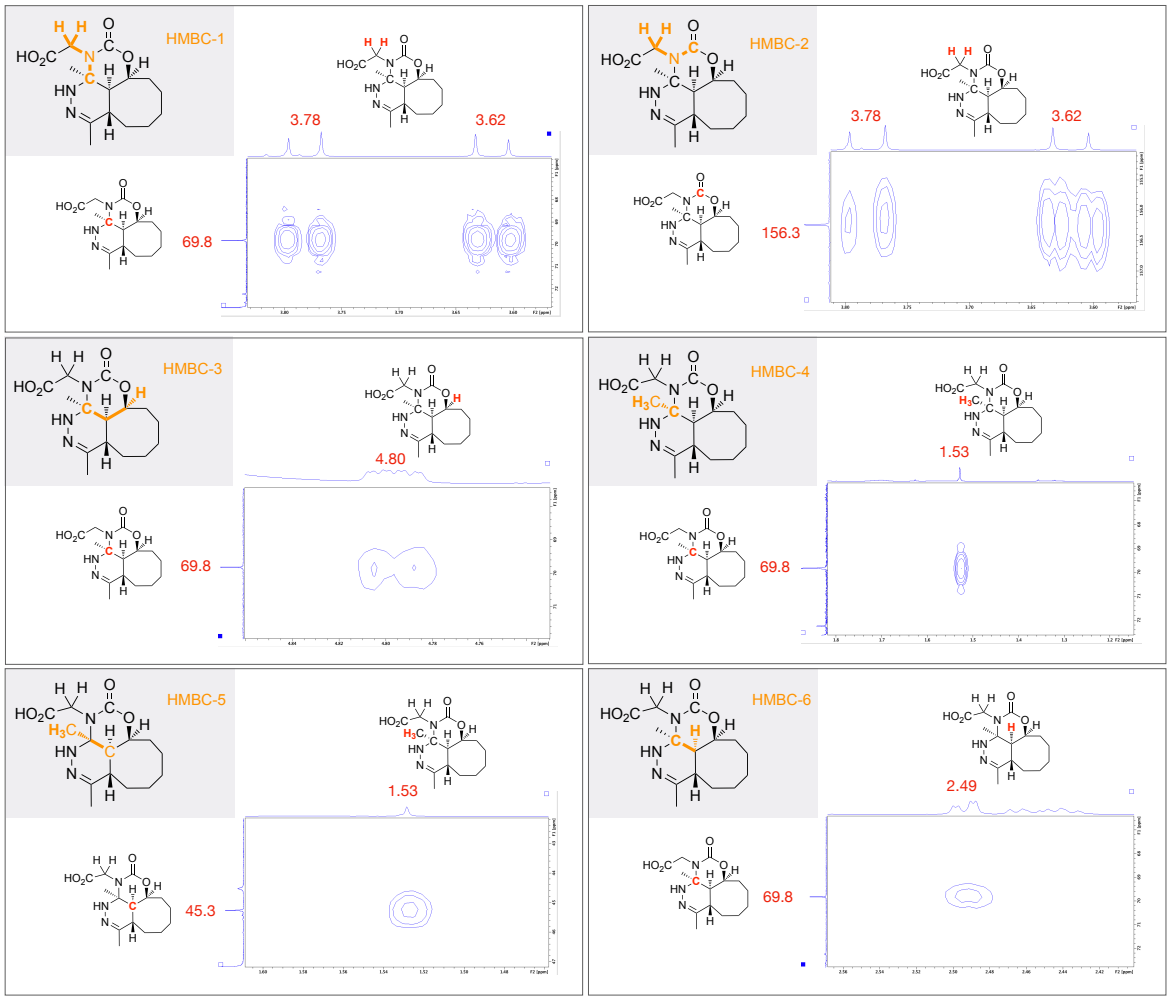
1D NMR (1H, 13C) and 2D NMR (COSY, HSQC, HMBC, NOESY) spectra were recorded to elucidate the chemical structure of compound **11**. All signals were considered and analyzed to assign all carbons and hydrogens of the tricyclic structure (see below).

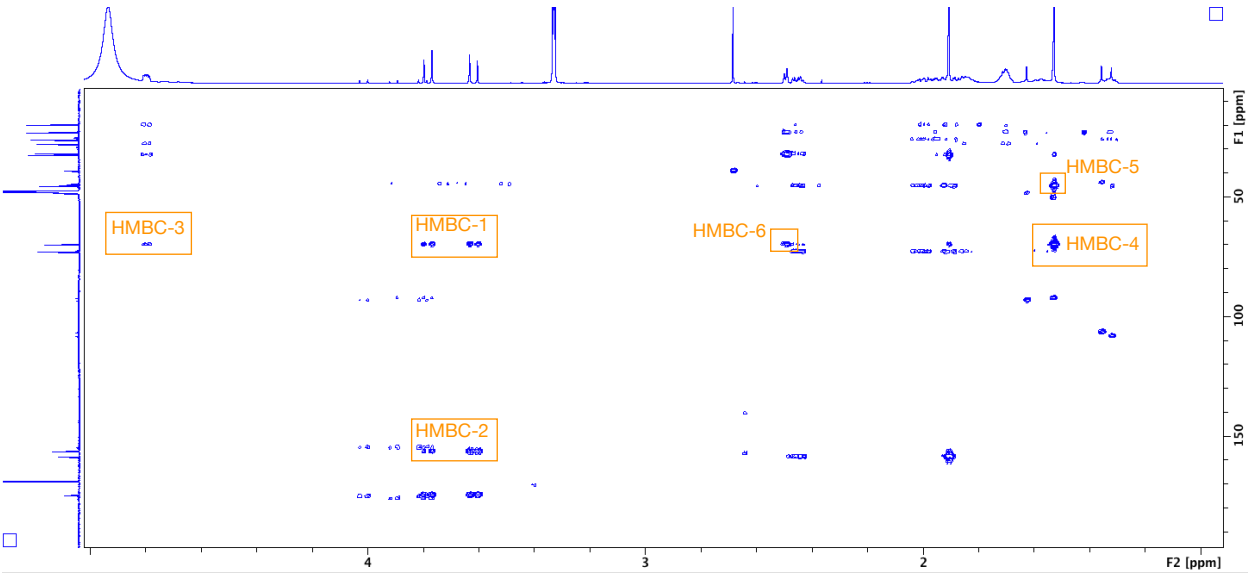
^{1}H NMR (600 MHz, CD_3OD) δ 4.80 (ddd, J = 7.9, 4.0, 1.5 Hz, 1H), 3.78 (d, J = 17.3 Hz, 1H), 3.62 (d, J = 17.3 Hz, 1H), 2.49 (dd, J = 5.7, 1.6 Hz, 1H), 2.47-2.42 (m, 1H), 2.04-1.88 (m, 3H), 1.91 (s, 3H), 1.87-1.80 (m, 2H), 1.73-1.67 (m, 3H), 1.60-1.55 (m, 1H), 1.53 (s, 3H), 1.34-1.30 (m, 1H); ^{13}C NMR (150 MHz, CD_3OD) δ 174.5, 158.5, 156.3, 72.8, 69.8, 45.3, 44.5, 32.4, 31.8, 27.8, 26.0, 23.0, 22.9, 19.8, 19.78.



HMBC map of connectivity:

The tricyclic structure was elucidated based on 1D and 2D NMR data. Key correlations in the HMBC spectrum that establish connectivity are shown below. HMBC-1 and HMBC-2 show the correlation of the (diastereotopic) glycine CH_2 protons at 3.78 / 3.62 ppm with the carbon at 69.8 ppm (formed by cyclization) and the carbamate carbon at 156.3 ppm. The correlations HMBC-3, HMBC-4, HMBC-5 and HMBC-6 support the elucidation of the key fragment.

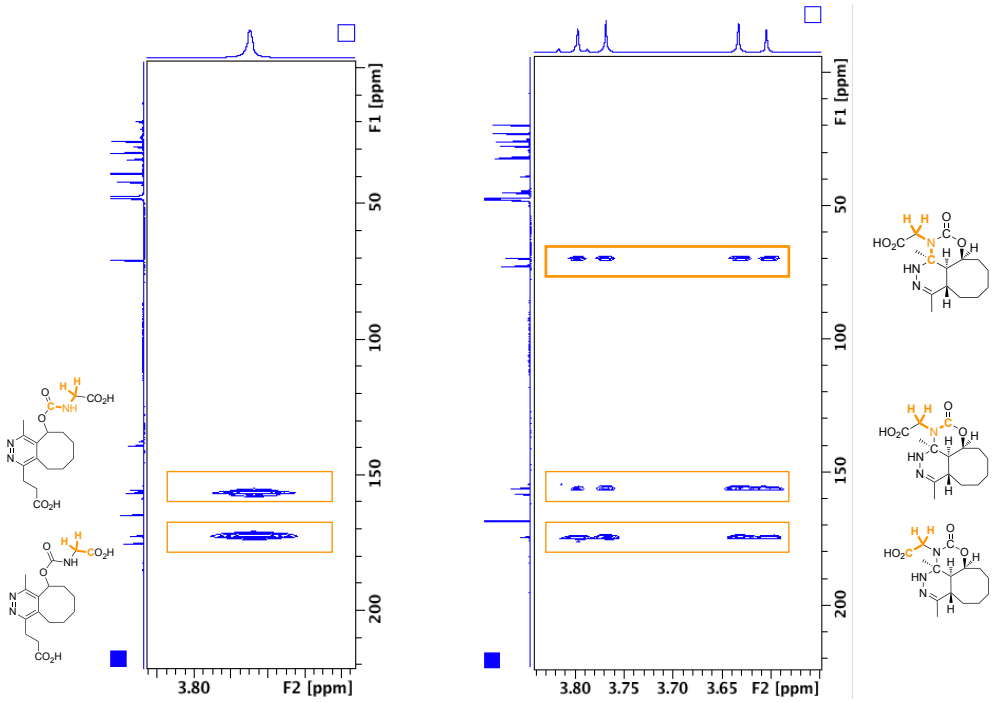




HMBC spectra compared: aromatized slowly releasing tautomer vs. tricyclic dead end (11)
Comparison of the HMBC connectivity for the glycine CH_2 protons between the aromatized slowly releasing tautomer (Fig. 4 in the main text) and the tricyclic dead end **11**.

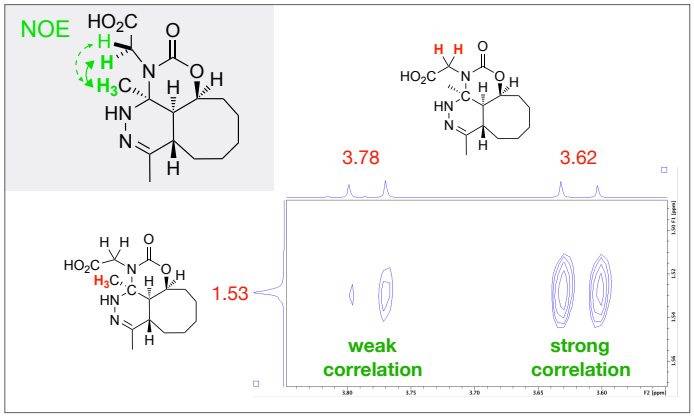
In the absence of cyclization (at left) only two cross peaks are observed, corresponding to the known connectivity to the carbon atoms of the adjacent carboxylic acid and carbamate. This is consistent with the known sensitivity of HMBC, which is capable of detecting heteronuclear correlation at separations ranging from two to a maximum of four bonds.

In contrast, the glycine CH_2 protons in the HMBC spectrum of **11** exhibit three distinct cross peaks, indicating nucleophilic addition of the N-H to the dihydropyridazine ring.

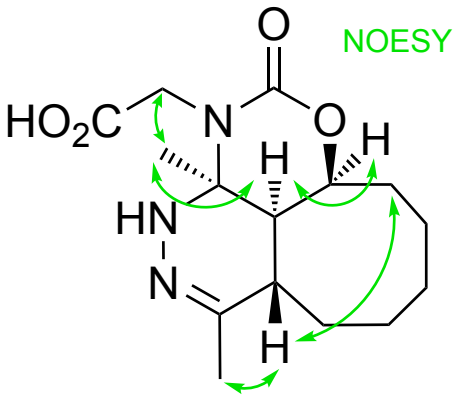


NOESY spectra corroborate connectivity

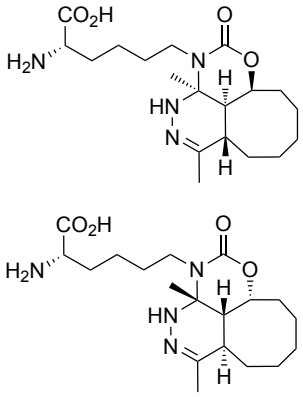
In addition, the structure of the tricyclic dead end is supported by the key NOE between the glycine CH_2 protons at 3.78 / 3.62 ppm and the CH_3 protons at 1.53 ppm. For a visualization of the proximity of those two groups based on the calculated structure see the copy of the NOESY spectrum at page S58. Due to the diastereotopic non-equivalence of the two glycine protons a weak and a strong correlation was observed (see below).



The elucidated structure was further confirmed by additional NOEs (see copy of the NOESY spectrum on page S59).

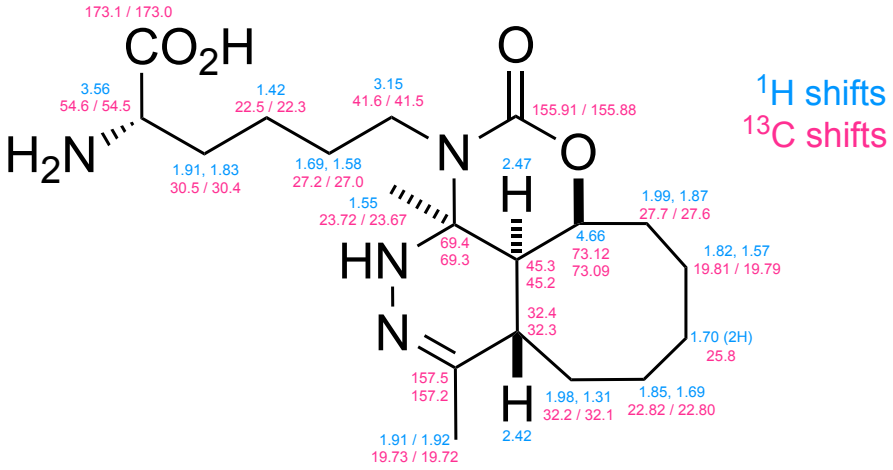


***C**. Isolation of tricyclic dead end isomer, N-ε-rTCO-Lys + DMT (**1**)*

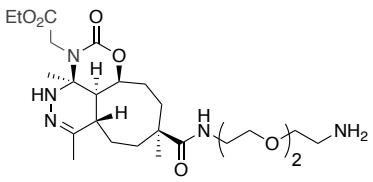


To a solution of 5.5 mg (0.0182 mmol) of N-ε-rTCO-Lysine dissolved directly in 4 mL of PBS were added 1.0 equivalents of DMT (0.018 mmol, 2.0 mg, dissolved in 78 µL of DMSO), with complete disappearance of the pink color and evolution of gas bubbles. The reaction was allowed to proceed for 24hours and then analyzed by LCMS, confirming a major product with a characteristic absorbance maximum at 232 nm. The reaction mixture in PBS was loaded onto a Biotage C18 Ultra column for solid phase extraction and washed with water, then eluted with an ammonium formate (pH 8.4)/MeCN gradient (4-30% MeCN). The fraction containing the desired product was dried by rotary evaporation and evaluated by LCMS, yielding 1.8 mg of the dead end isomer for NMR analysis. ESI (M-H): expected for a DMT-N-ε-rTCO-Lys click product (dihydropyridazine oxidation state) 379.24; observed 379.23.

^{1}H NMR (600 MHz, CD_3OD, two diastereomers) δ 4.67-4.64 (m, 1H), 3.56 (dd, J = 7.1, 5.2 Hz, 1H), 3.22-3.07 (m, 2H), 2.48-2.45 (m, 1H), 2.44-2.39 (m, 1H), 2.03-1.94 (m, 2H), 1.92 (s, 1.5H), 1.91 (s, 1.5H), 1.90-1.77 (m, 5H), 1.74-1.65 (m, 4H), 1.62-1.56 (m, 2H), 1.55 (s, 3H), ;1.46-1.38 (m, 2H), 1.34-1.28 (m, 1H); ^{13}C NMR (150 MHz, CD_3OD, two diastereomers) δ 173.1, 173.0, 157.5, 157.2, 155.91, 155.88, 73.12, 73.09, 69.4, 69.3, 54.6, 54.5, 45.3, 45.2, 41.6, 41.5, 32.4, 32.3, 32.2, 32.1, 30.5, 30.4, 27.7, 27.6, 27.2, 27.0, 25.8, 23.72, 23.67, 22.82, 22.80, 22.5, 22.3, 19.81, 19.79, 19.73, 19.72.

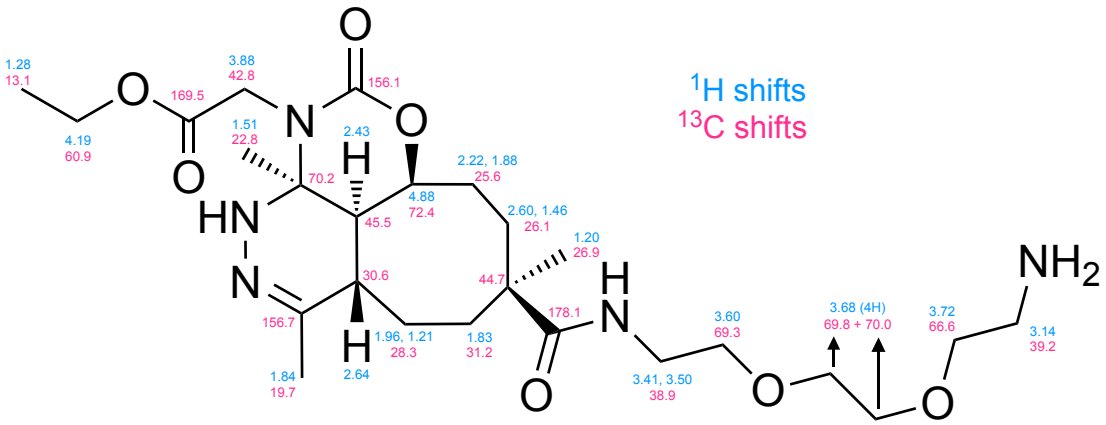


***D**. Isolation of **15**, tricyclic dead end isomer, NH_2-cTCO-Gly + DMT (**1**)*

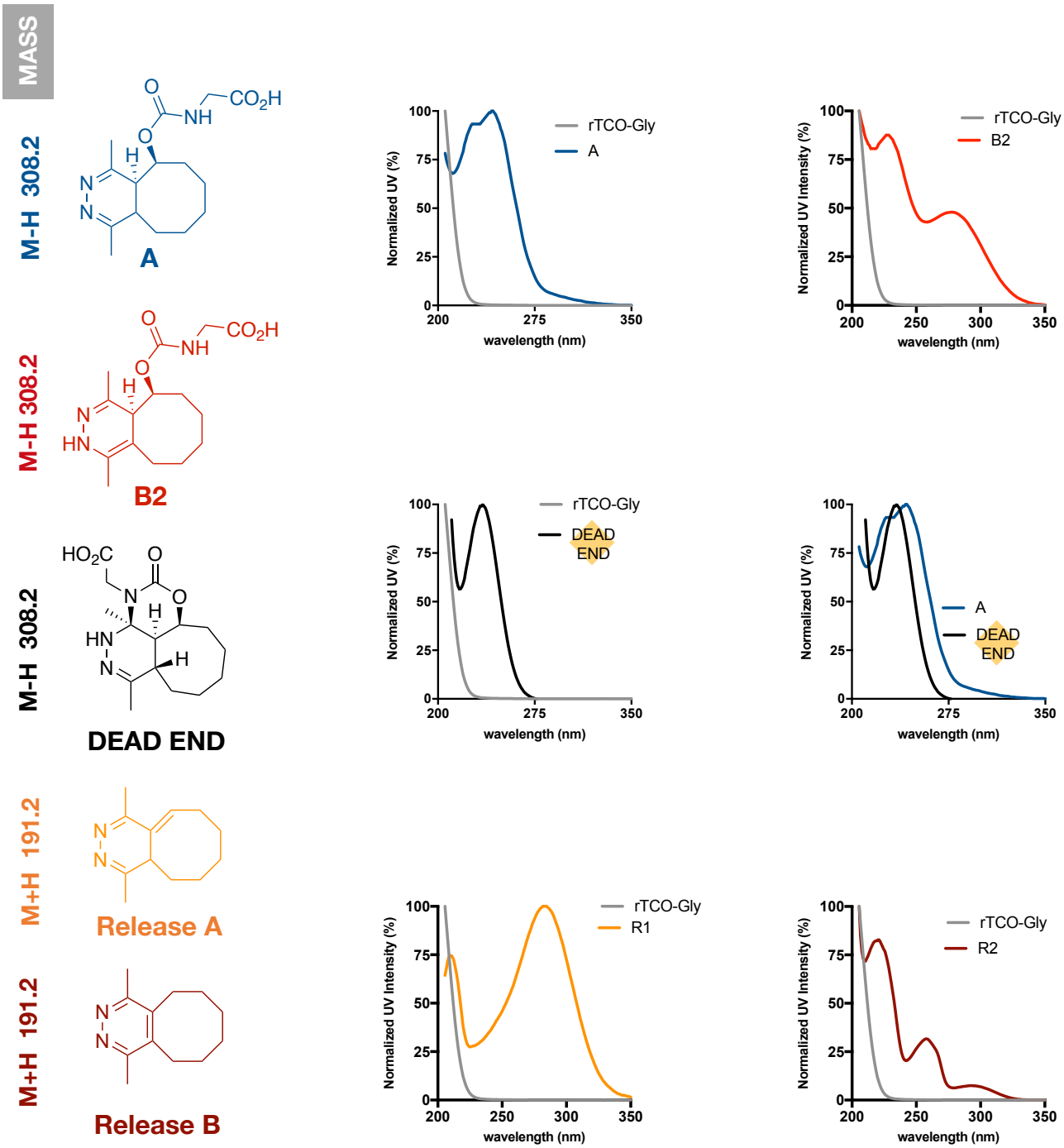


To a solution of 2 mg (0.005 mmol) of NH_2-cTCO-Gly dissolved in 5mL of bicarbonate-carbonate buffer (10 mM, pH 10.2) were added 1.5 equivalents of DMT (0.0075 mmol, 0.82 mg, dissolved in 32.6 µL of DMSO). The reaction was allowed to proceed for 6 hours at room temperature with LCMS analysis indicating complete conversion to a non-released (dead-end) isomer. The reaction mixture was loaded onto a Biotage C18 column for solid phase extraction and rinsed with water to remove salts. (The C18 Ultra column should not be used in this case as the desired product will be anomalously retained due to the primary amine-silica interaction). The column was then eluted with an ammonium formate (pH 8.4)/MeCN gradient (4-30% MeCN) and the desired fractions were pooled and dried by rotary evaporation, yielding 1.8 mg of the dead end isomer for NMR analysis. ESI (M+H): expected, 526.32; observed 526.57. ESI (M-H): expected 524.32; observed 524.44.

^{1}H NMR (600 MHz, CD_3OD) δ 4.90-4.86 (m, 1H), 4.24-4.15 (m, 2H), 3.92 (d, J = 17.6 Hz, 1H), 3.85 (d, J = 17.6 Hz, 1H), 3.72 (t, J = 5.6 Hz, 2H), 3.68 (s, 4H), 3.60 (t, J = 5.6 Hz, 2H), 3.55-3.47 (m, 1H), 3.44-3.38 (m, 1H), 3.15-3.12 (m, 2H), 2.67-2.55 (m, 2H), 2.43 (d, J = 7.0 Hz, 1H), 2.26-2.16 (m, 1H), 2.00-1.92 (m, 1H), 1.91-1.86 (m, 1H), 1.86-1.78 (m, 2H), 1.84 (s, 3H), 1.51 (s, 3H), 1.48-1.42 (m, 1H), 1.28 (t, J = 7.0 Hz, 3H), 1.25-1.19 (m, 1H), 1.20 (s, 3H), ; ^{13}C NMR (150 MHz, CD_3OD) δ 178.1, 169.5, 156.7, 156.1, 72.4, 70.2, 70.0, 69.8, 69.3, 66.6, 60.9, 45.5, 44.7, 42.8, 39.2, 38.9, 31.2, 30.6, 28.3, 26.9, 26.1, 25.6, 22.8, 19.7, 13.1.

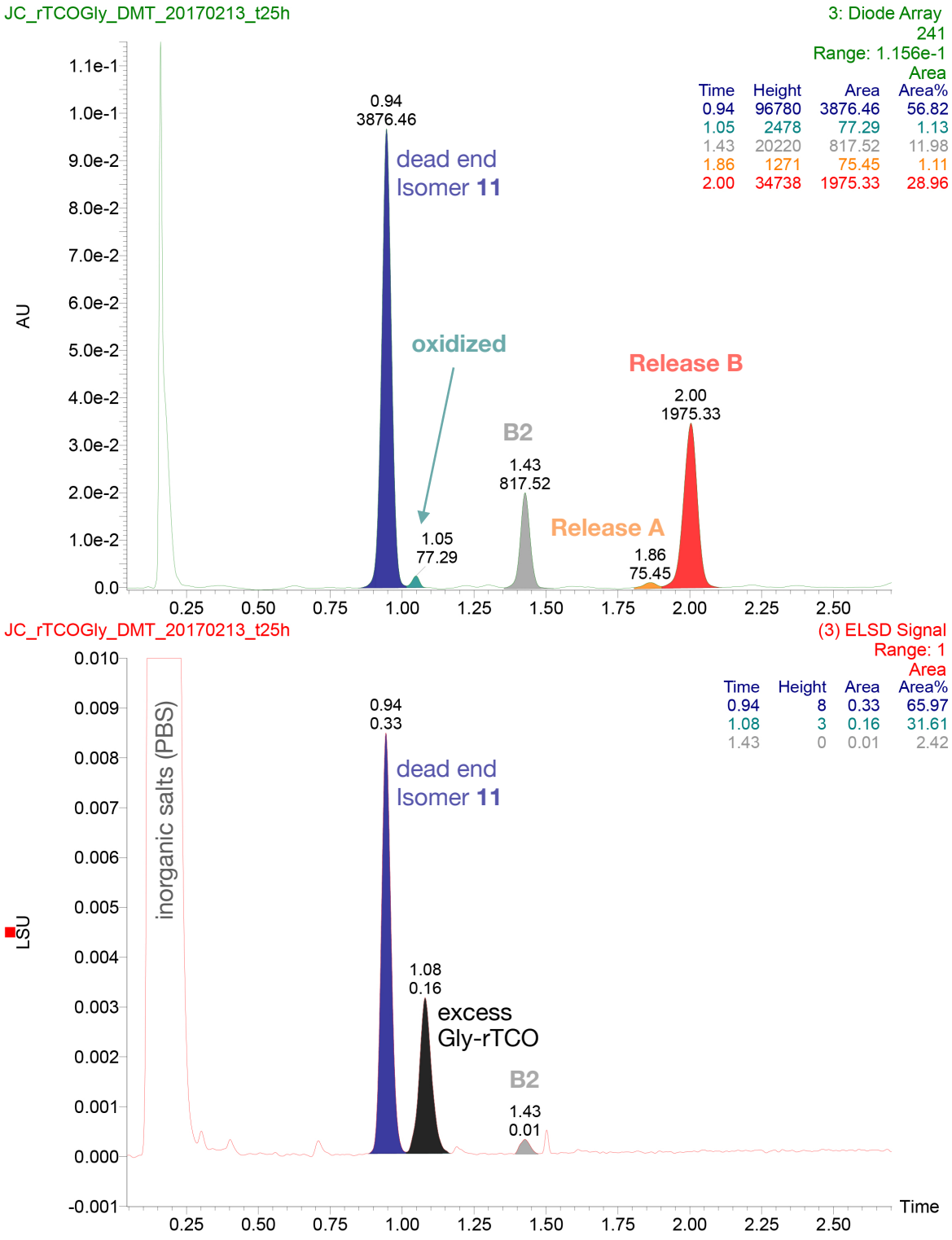


7. Normalized UV Spectra of rTCO-Gly post-click tautomers; ELSD data



LCMS Data - UV and ELSD

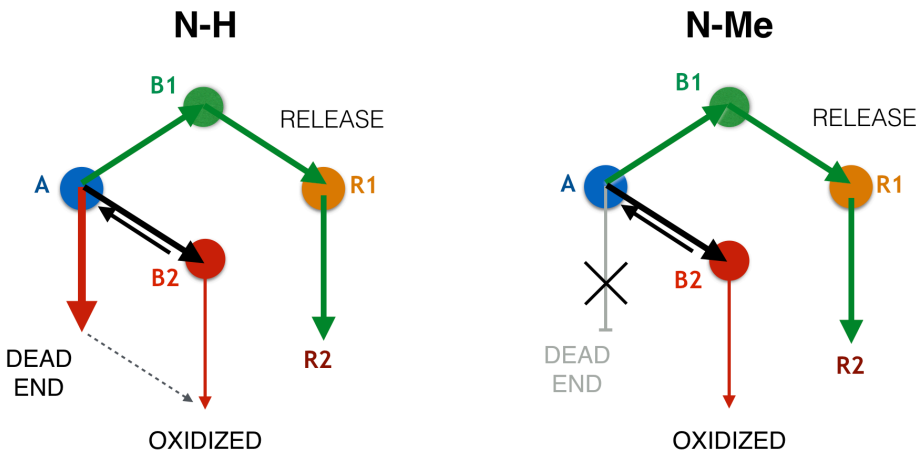
DMT **(1)** + Gly-rTCO **(10)** in PBS**;** Figure 4b(i) of the manuscript.



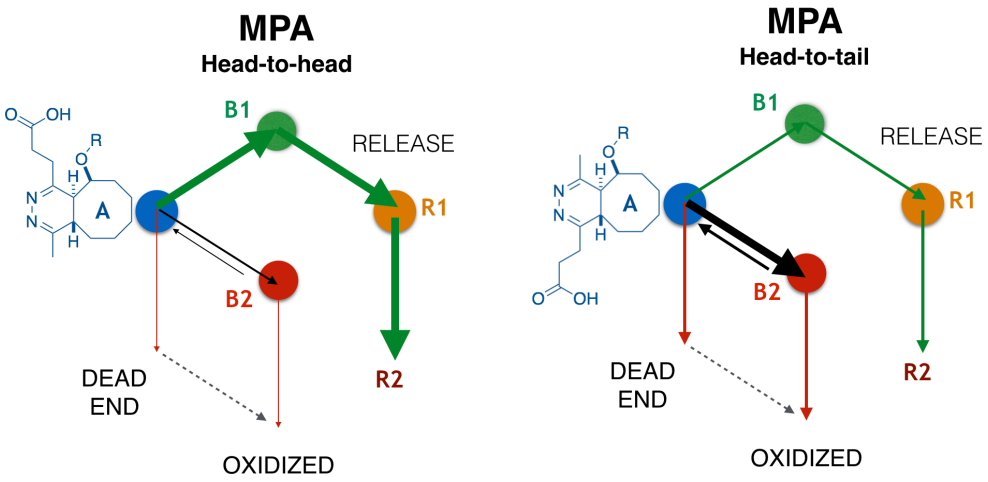
8. Release cube schematic notation

This condensed form of the post-click reaction network, presented in a cube as in the full form of Figure 6 in the main text, provides a visual shorthand for the impact/comparison of Tz/TCO performance in click-to-release.

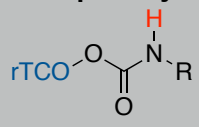
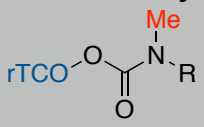
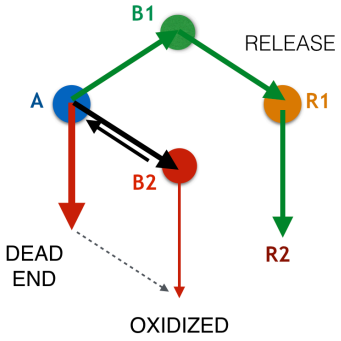
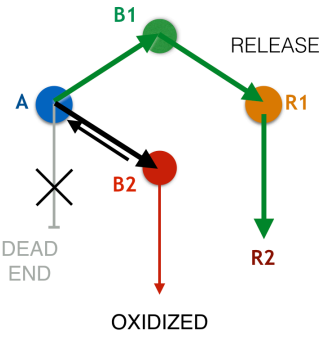
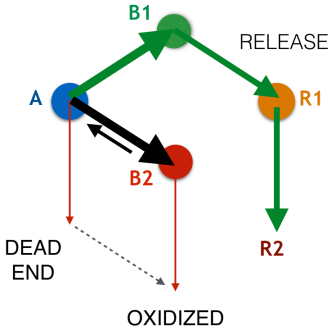
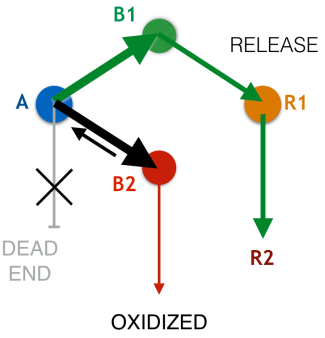
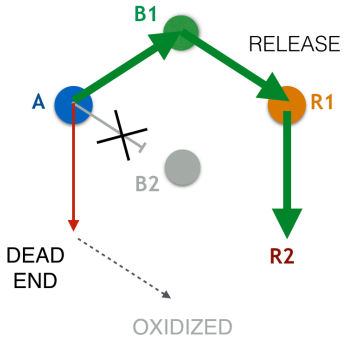
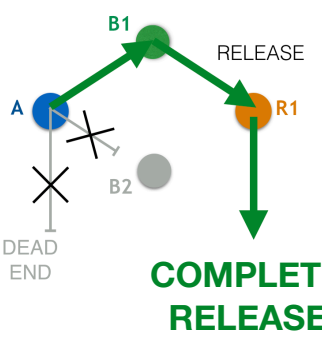
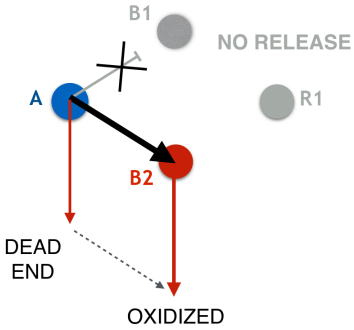
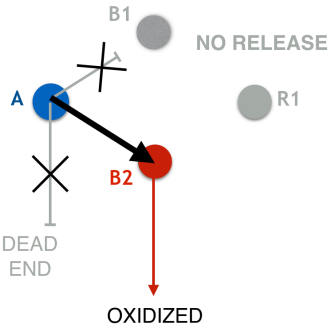
A. Comparison between N-H and N-Me (or release of primary vs. secondary amines in general) in condensed form:



B. Accelerated tautomerization favoring either B1 or B2 formation (e.g. using MPA):

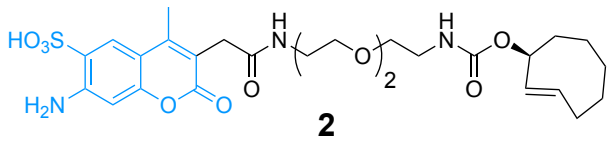


C. In general, tautomerization plays a pivotal role to achieve or even prevent release (see following table). In case of release of secondary amines, controlled tautomerization toward exclusive B1 formation represents the optimal scenario to achieve complete release.

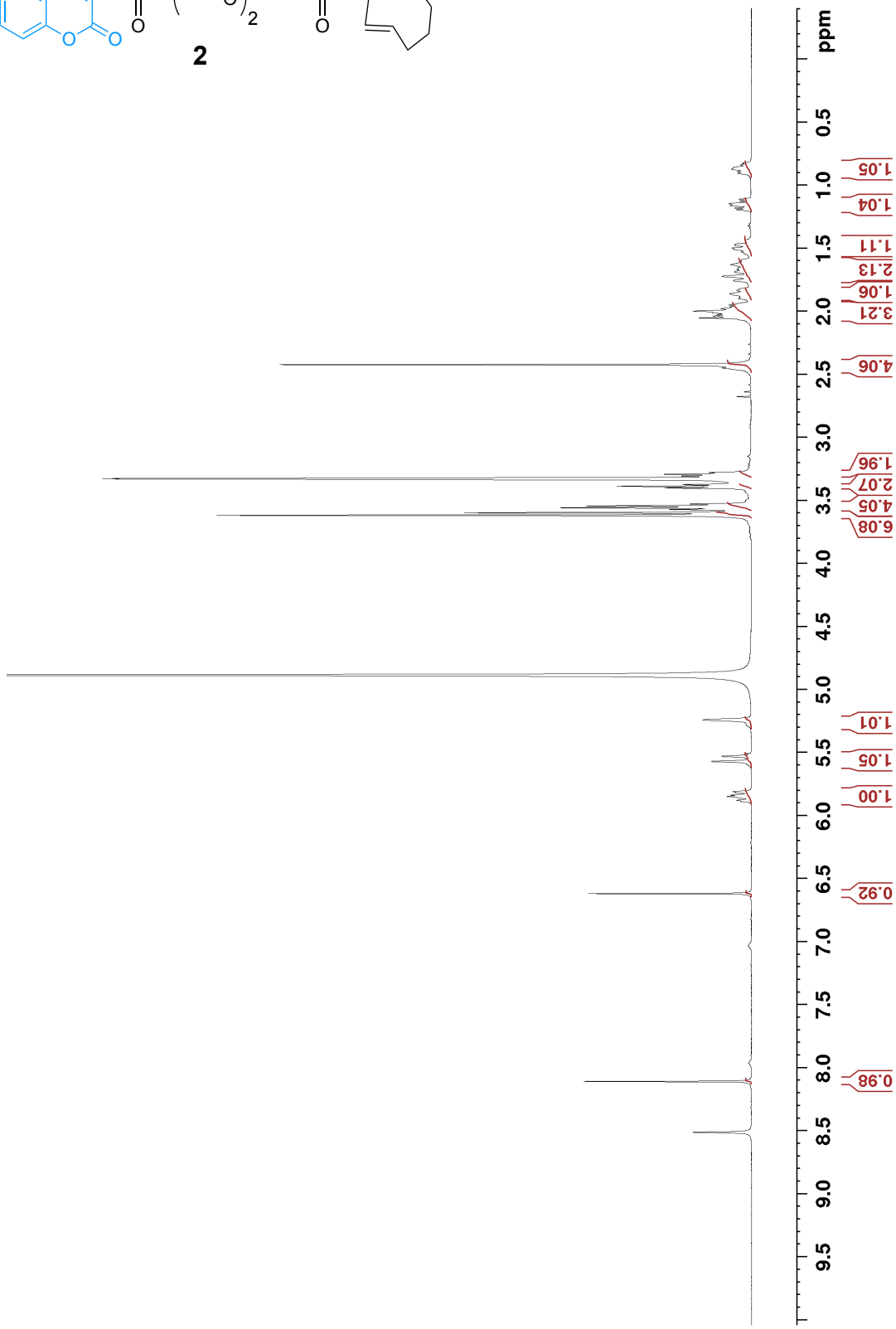
Tautomerization	Release of primary amines 	Release of secondary amines 
No control		
Accelerated		
Controlled B1 formation		
Exclusive B2 formation **CLICK ONLY**		

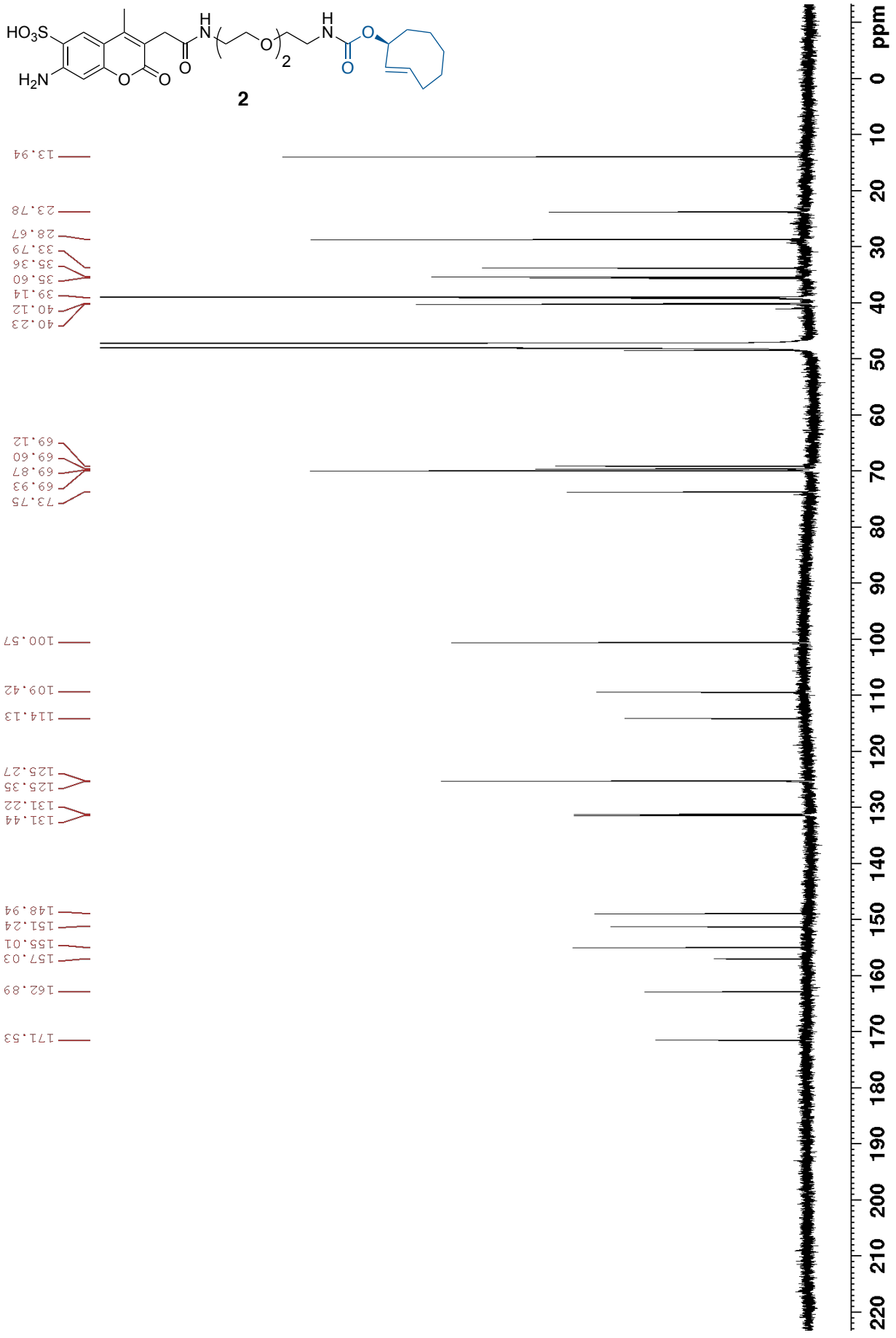
OPTIMAL SCENARIO

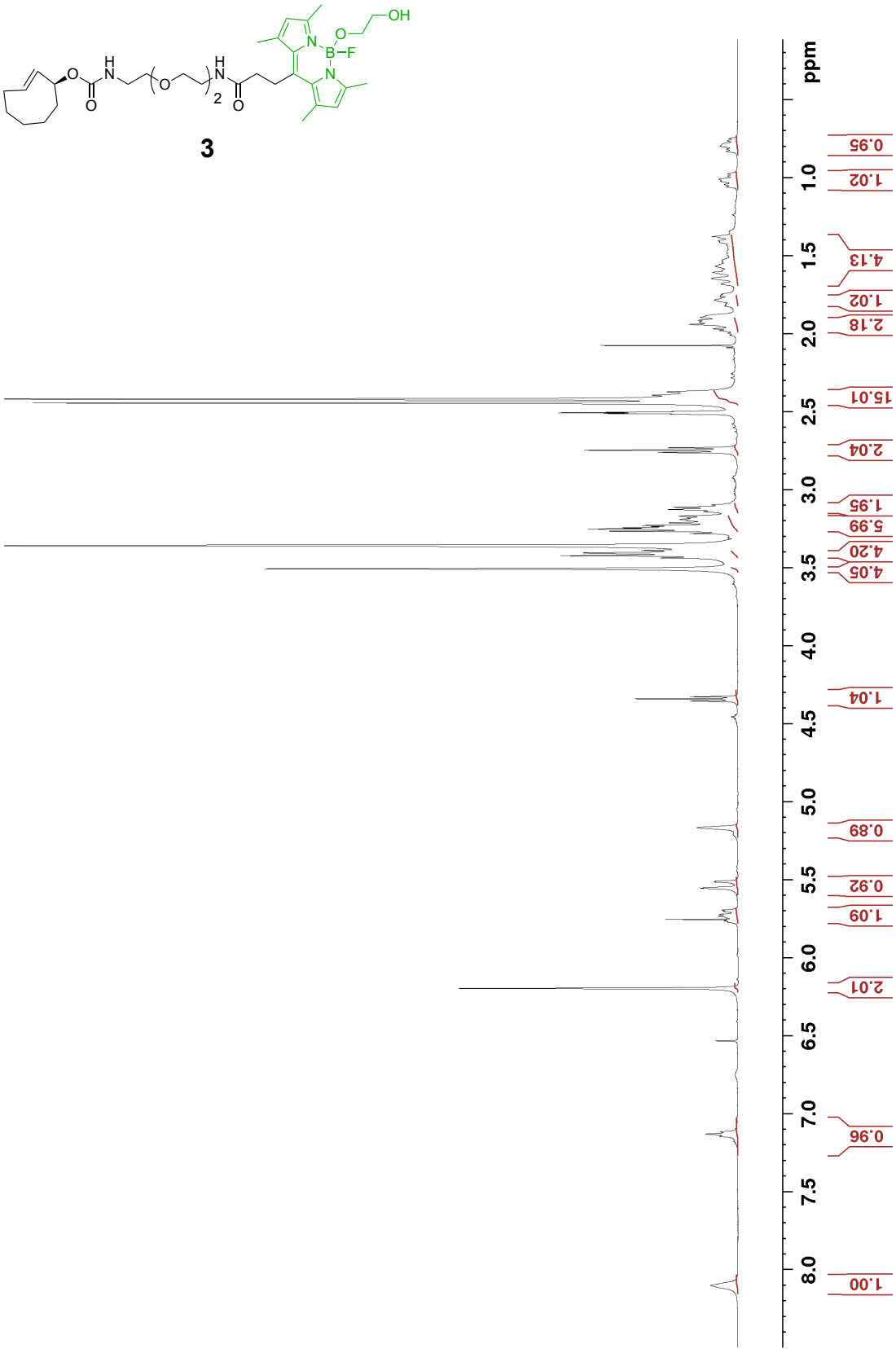
9. NMR Spectra

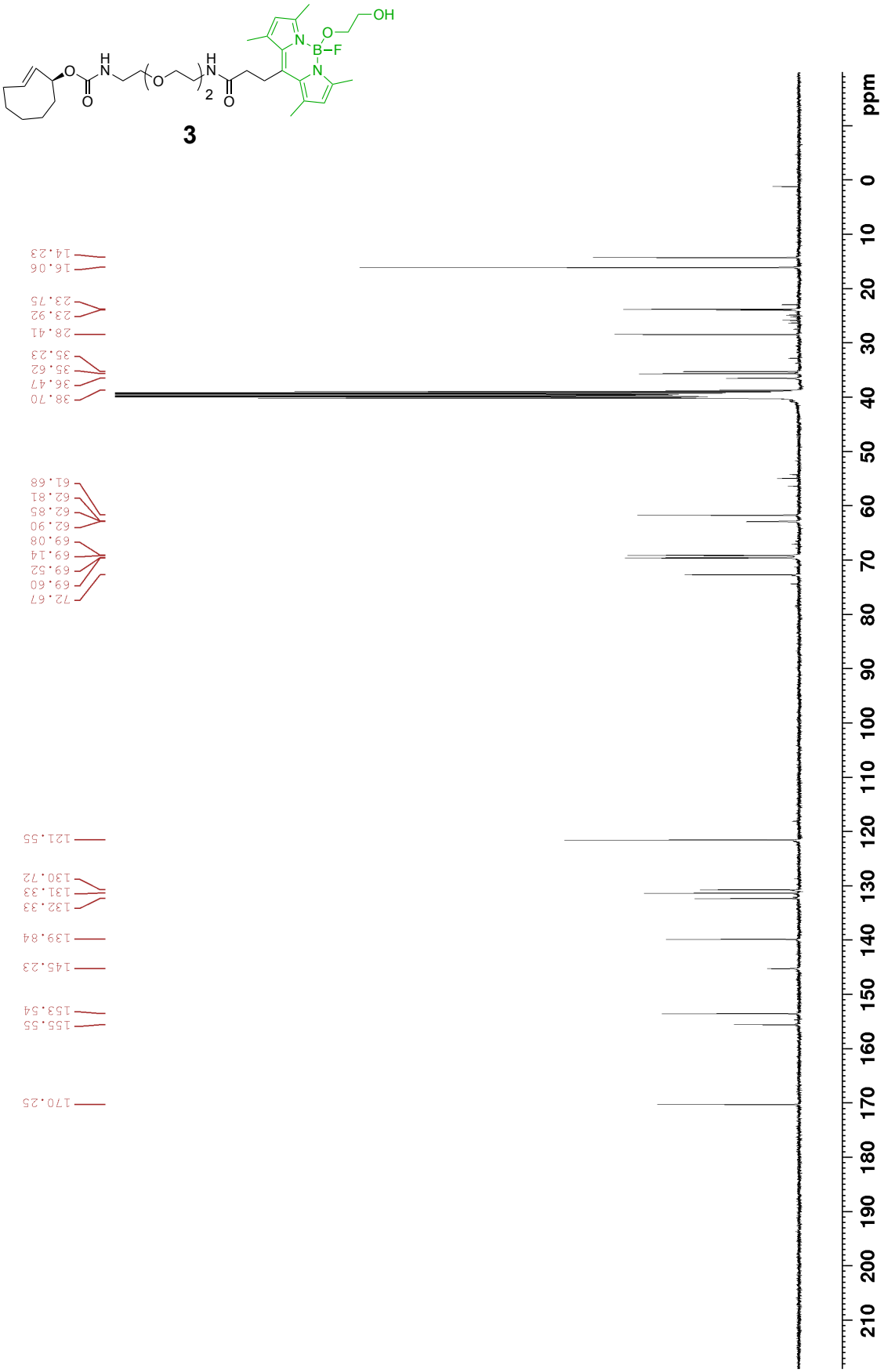


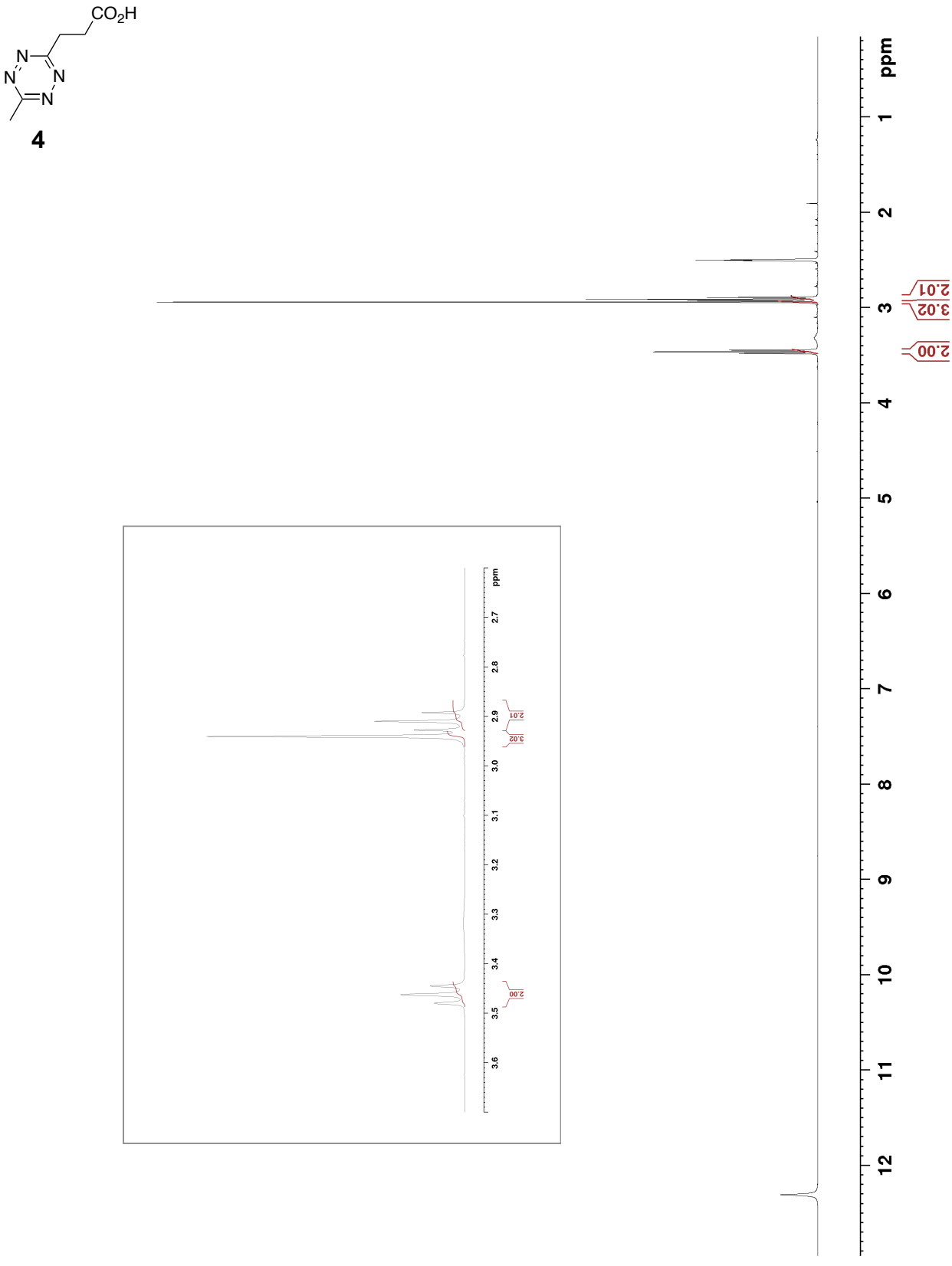
2

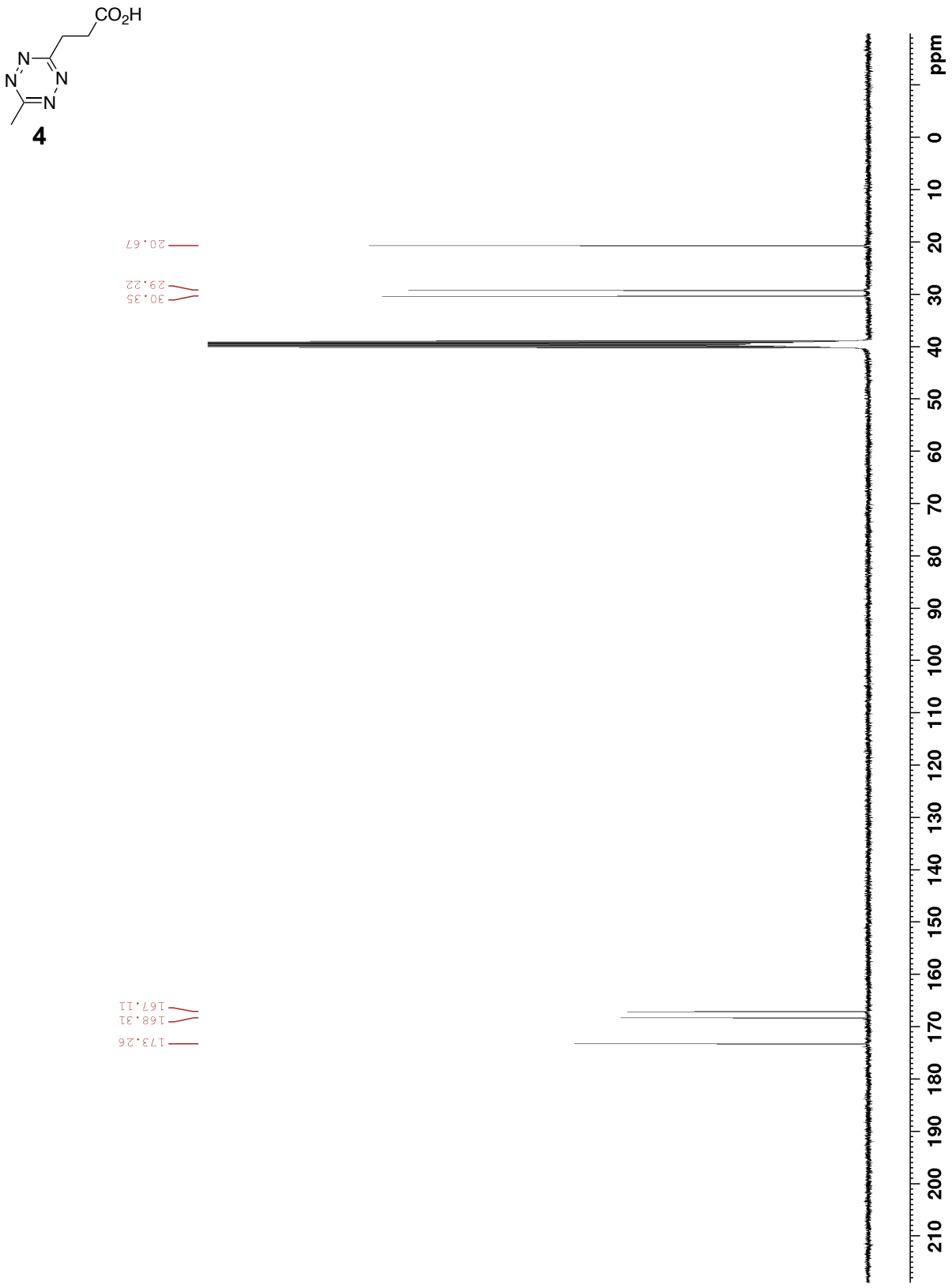


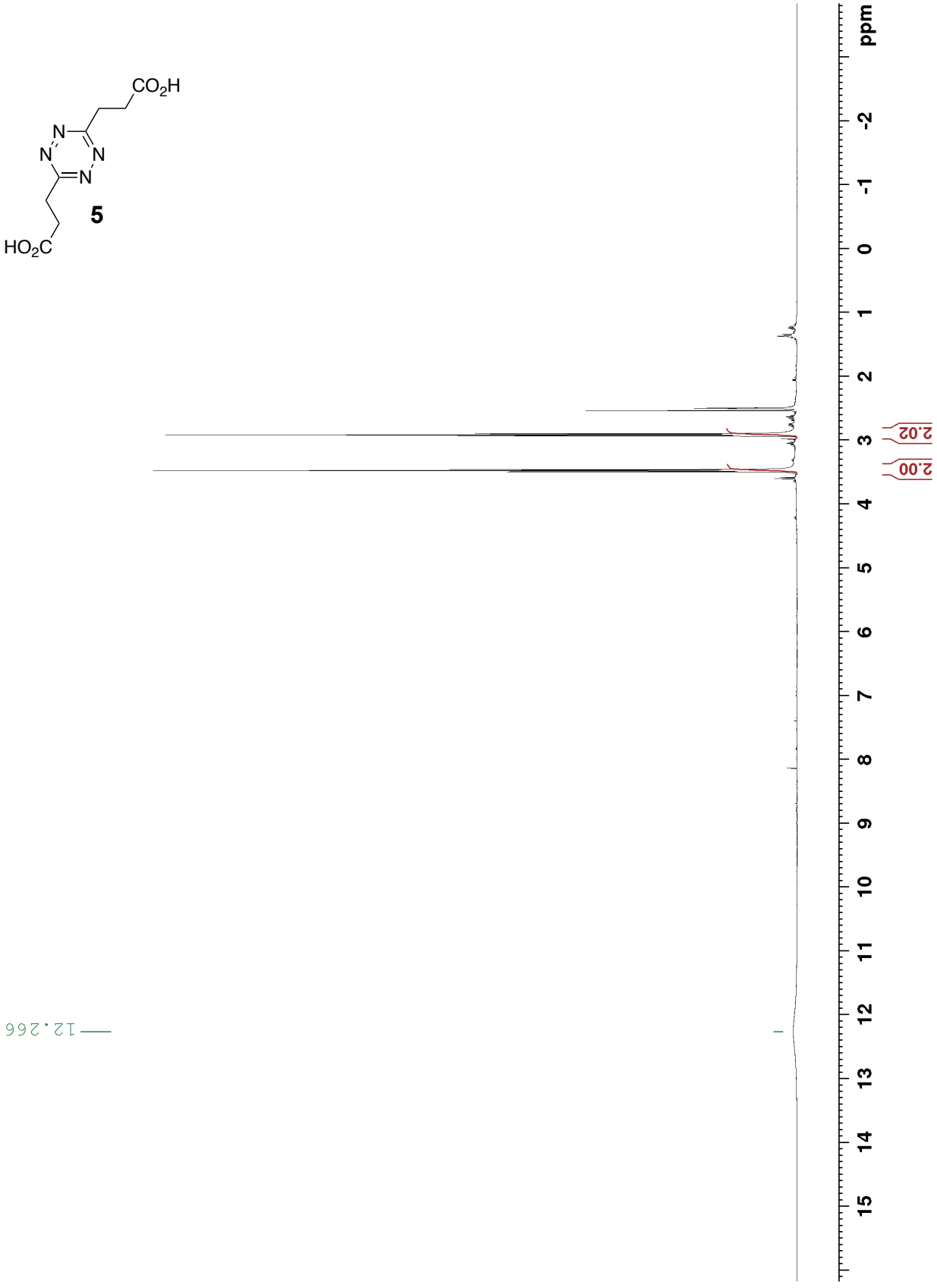

HO3S
H2N
O
O
O
H
N
O
2
O
H
N
O
13.94
23.78
28.67
33.79
35.36
35.60
39.14
40.12
40.23
69.12
69.60
69.87
69.93
73.75
100.57
109.42
114.13
125.27
125.35
131.22
131.44
148.94
151.24
155.01
157.03
162.89
171.53
ppm
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220

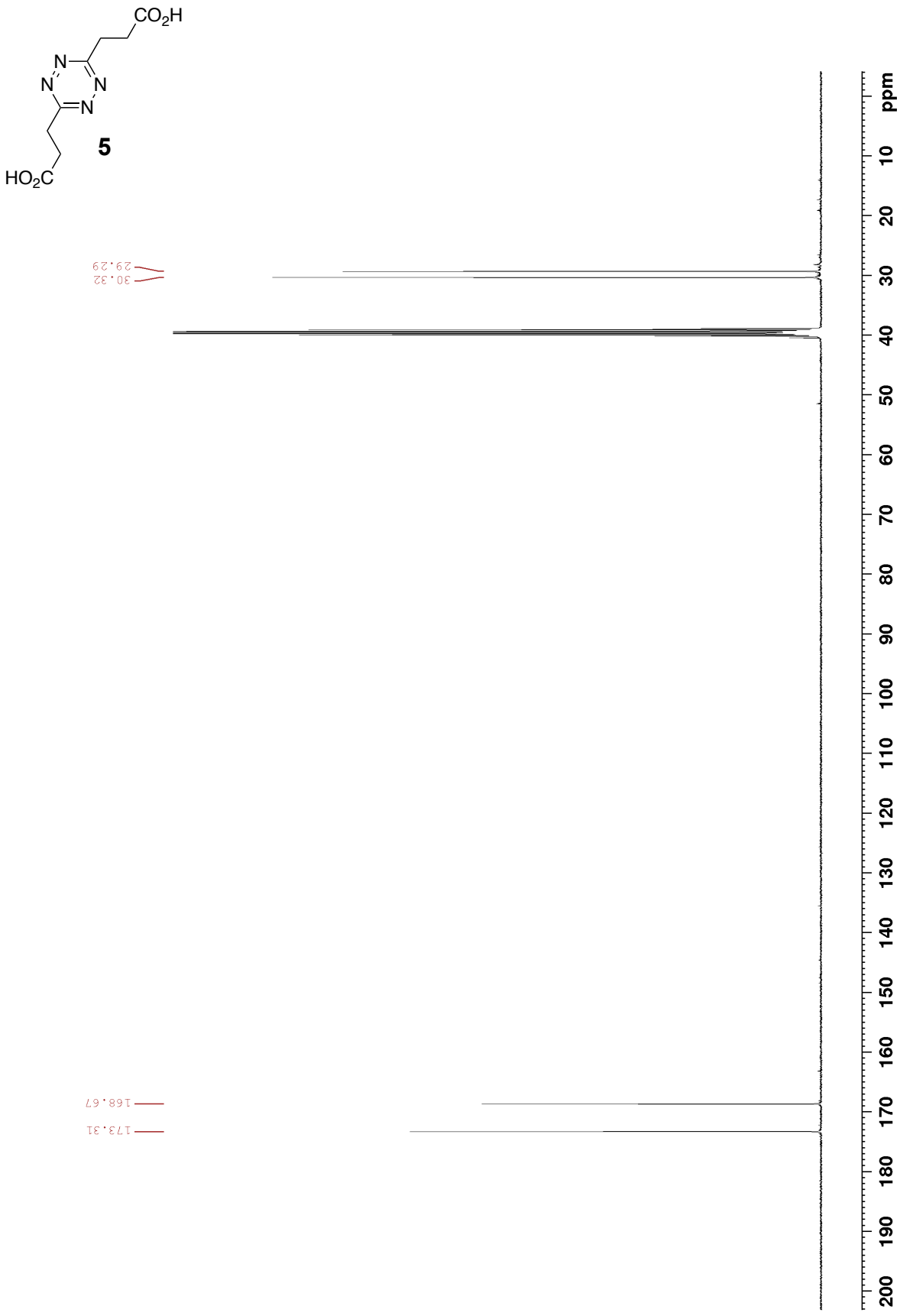

OH
O
B–F
N
N
O
H
N
O
O
O
H
N
2
O
3
ppm
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
0.95
1.02
4.13
1.02
2.18
15.01
2.04
1.95
5.99
4.20
4.05
1.04
0.89
0.92
1.09
2.01
0.96
1.00

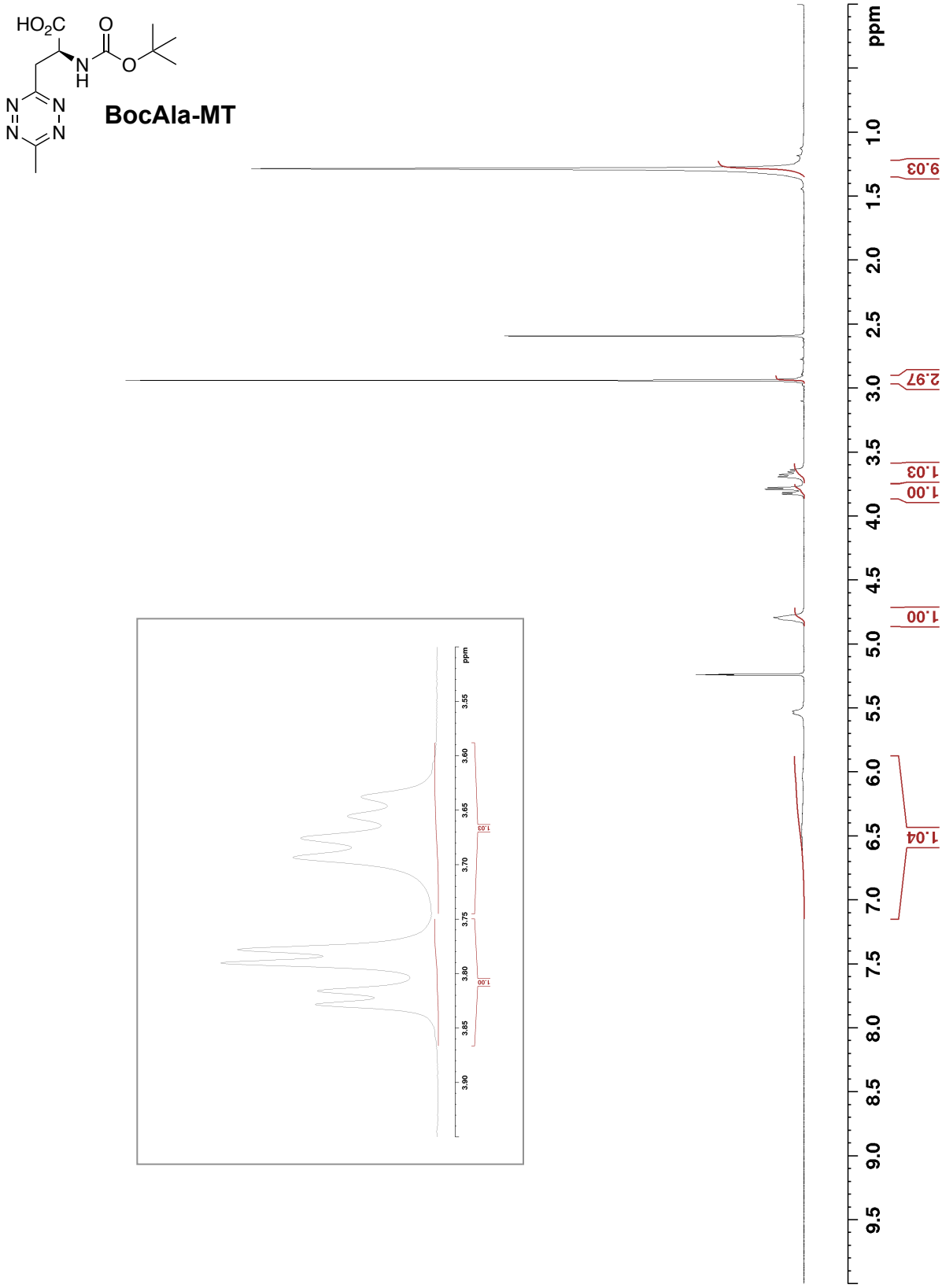

3
14.23
16.06
23.75
23.92
28.41
35.23
35.62
36.47
38.70
61.68
62.81
62.85
62.90
69.08
69.14
69.52
69.60
72.67
121.55
130.72
131.33
132.33
139.84
145.23
153.54
155.55
170.25
ppm

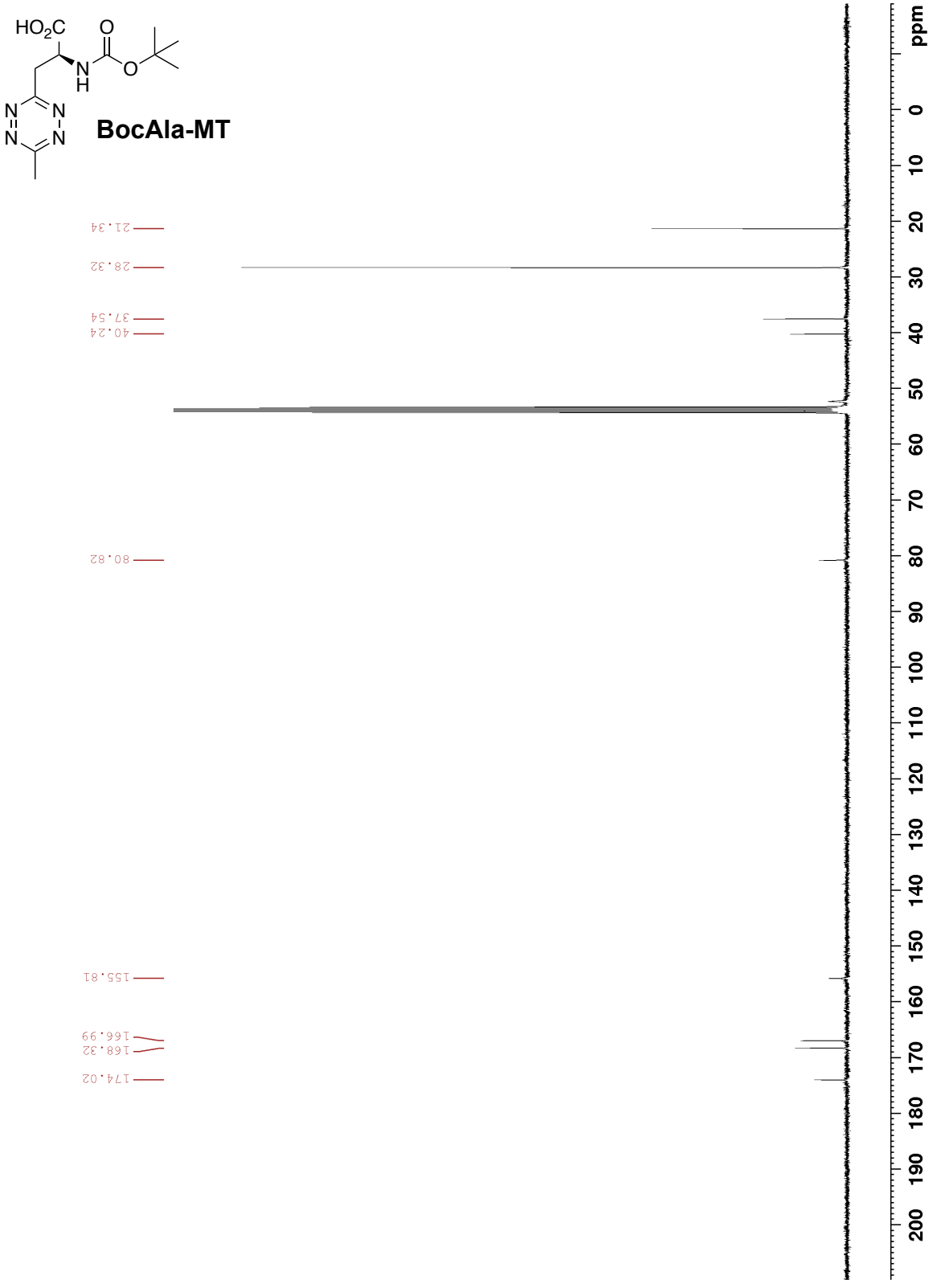

CO2H
N
N
N
N
4
ppm
1
2
3
4
5
6
7
8
9
10
11
12
2.01
3.02
2.00
2.7
2.8
2.9
3.0
3.1
3.2
3.3
3.4
3.5
3.6

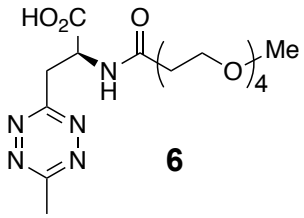

CO2H
N
N
N
N
4
20.67
29.22
30.35
167.11
168.31
173.26
ppm
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210


CO2H
N
N
N
N
5
HO2C
12.266
2.02
2.00
ppm
-2
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15


CO2H
N
N
N
N
5
HO2C
29.29
30.32
168.67
173.31
ppm
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200


HO2C
O
N
H
O
N
N
N
N
BocAla-MT
ppm
9.03
2.97
1.03
1.00
1.00
1.04

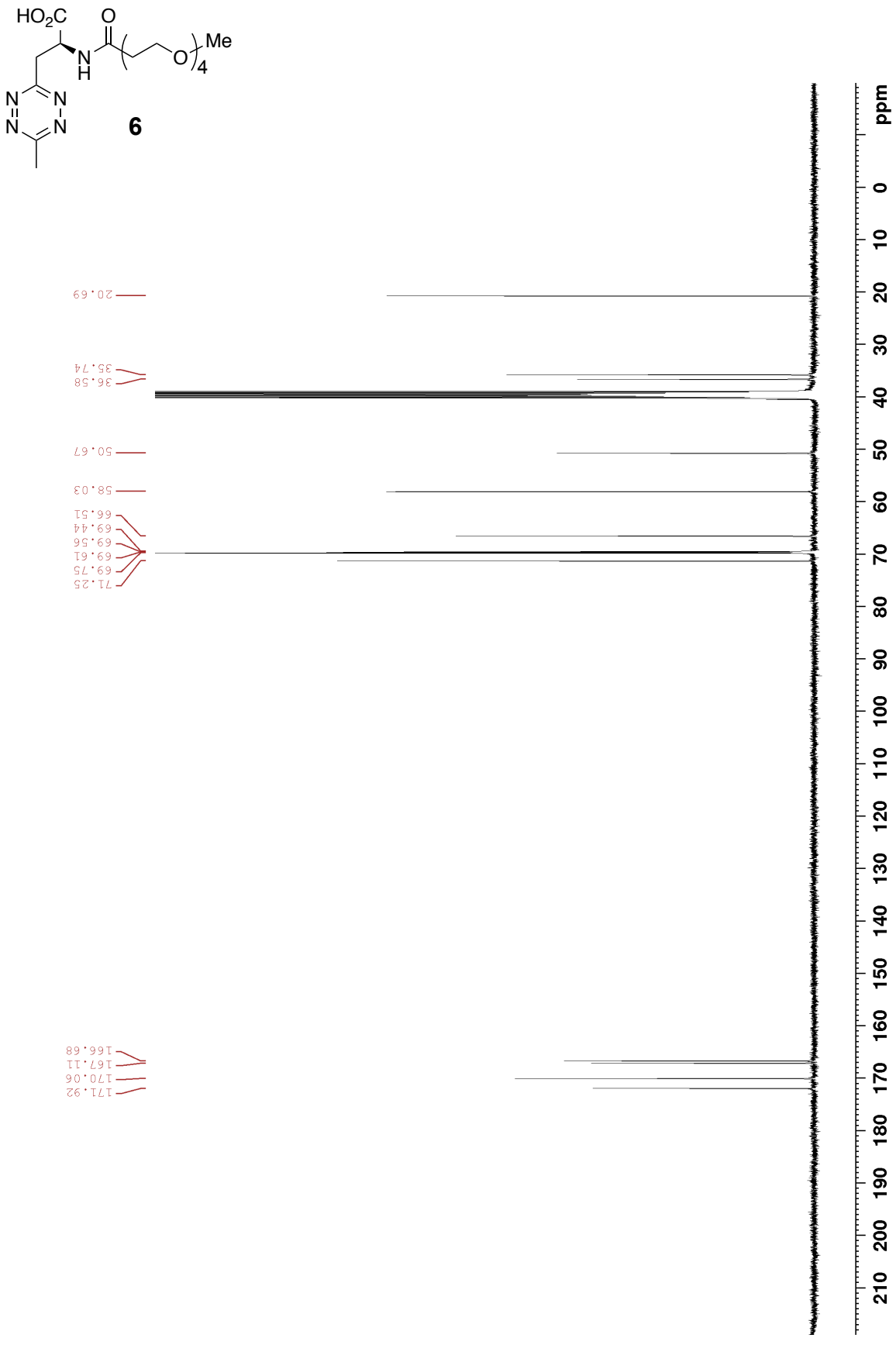

HO2C
O
N
H
O
N
N
N
N
BocAla-MT
21.34
28.32
37.54
40.24
80.82
155.81
166.99
168.32
174.02
ppm
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200

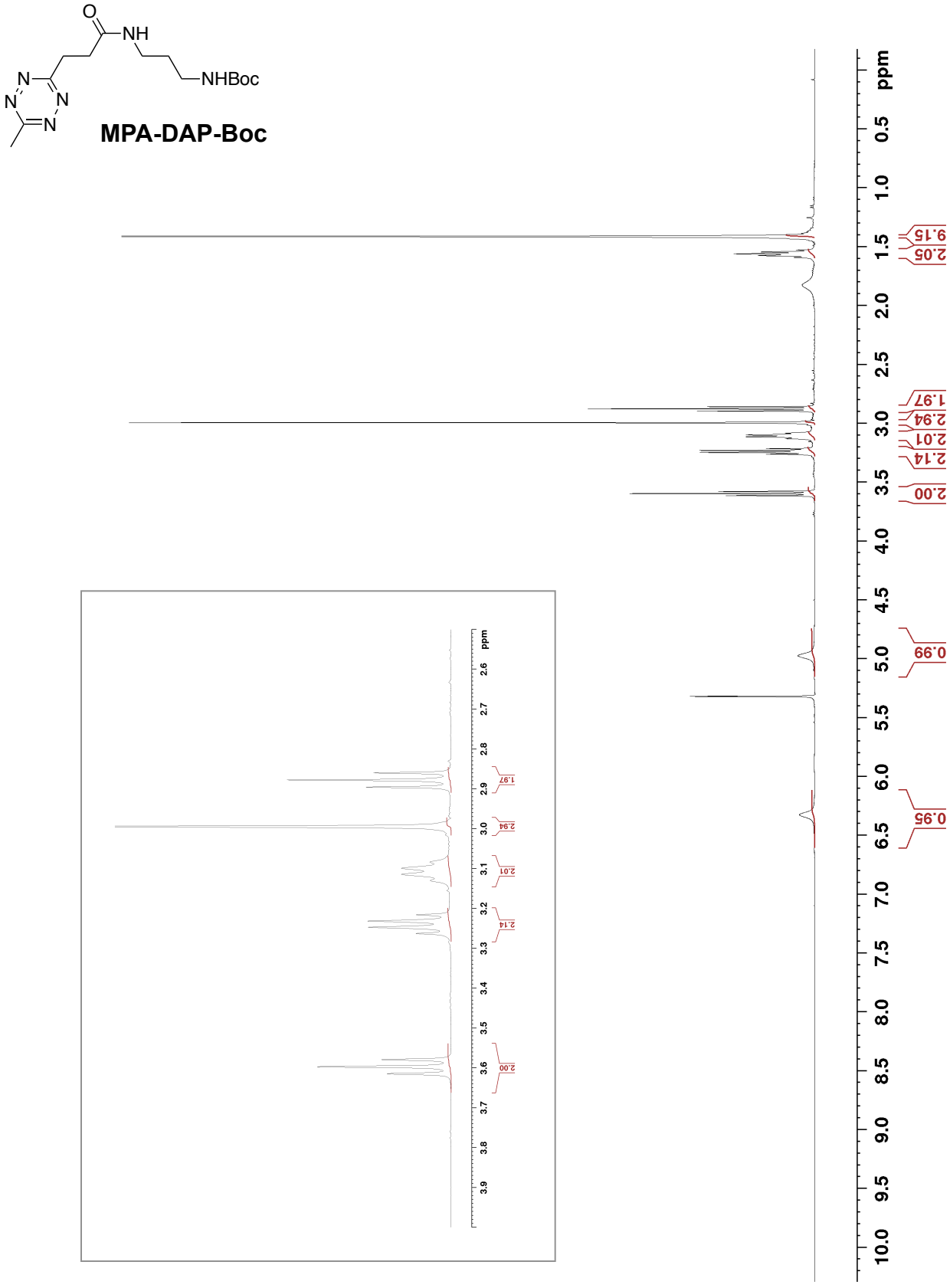

HO2C
O
N
H
Me
O
4
N
N
N
N

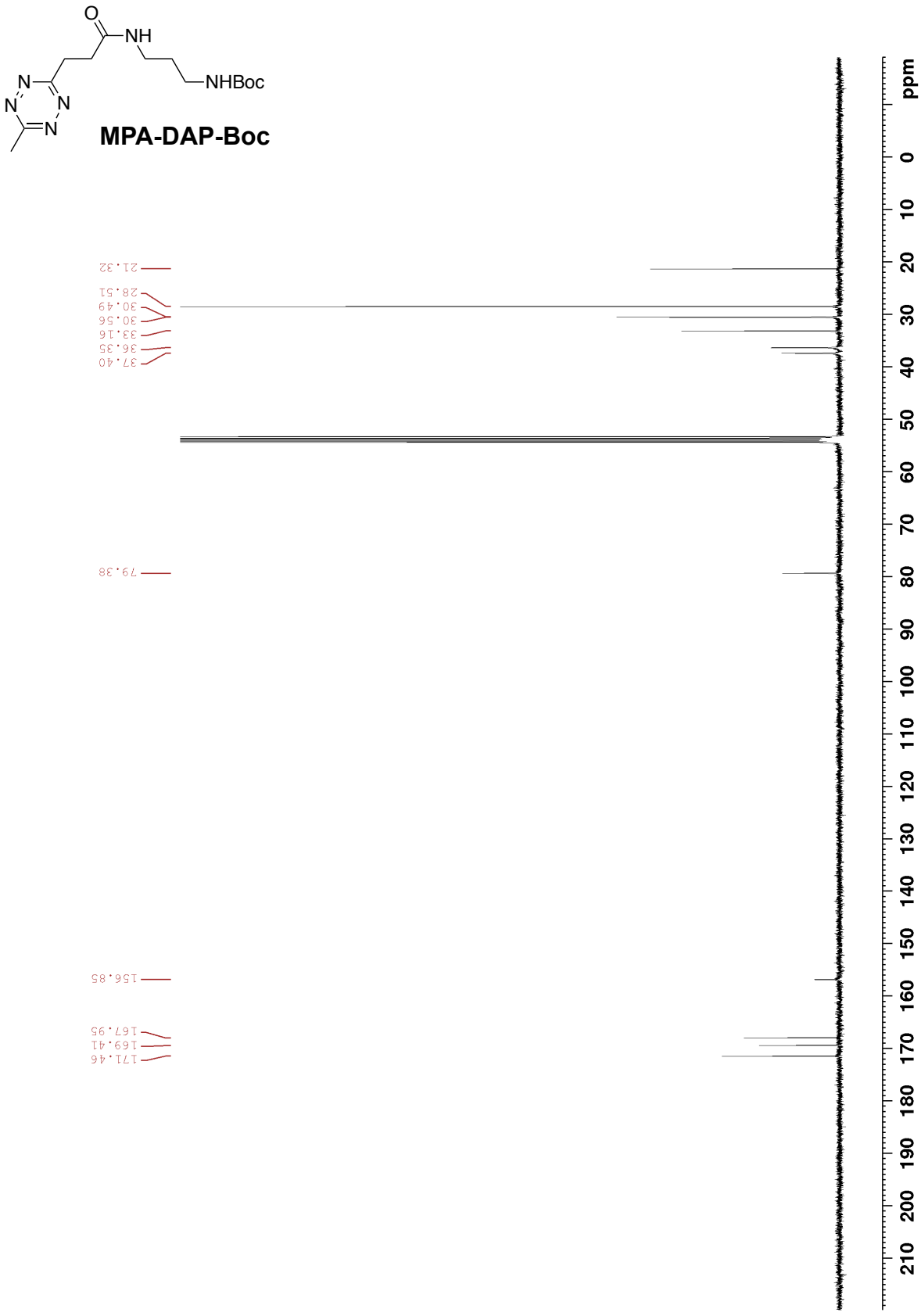
6

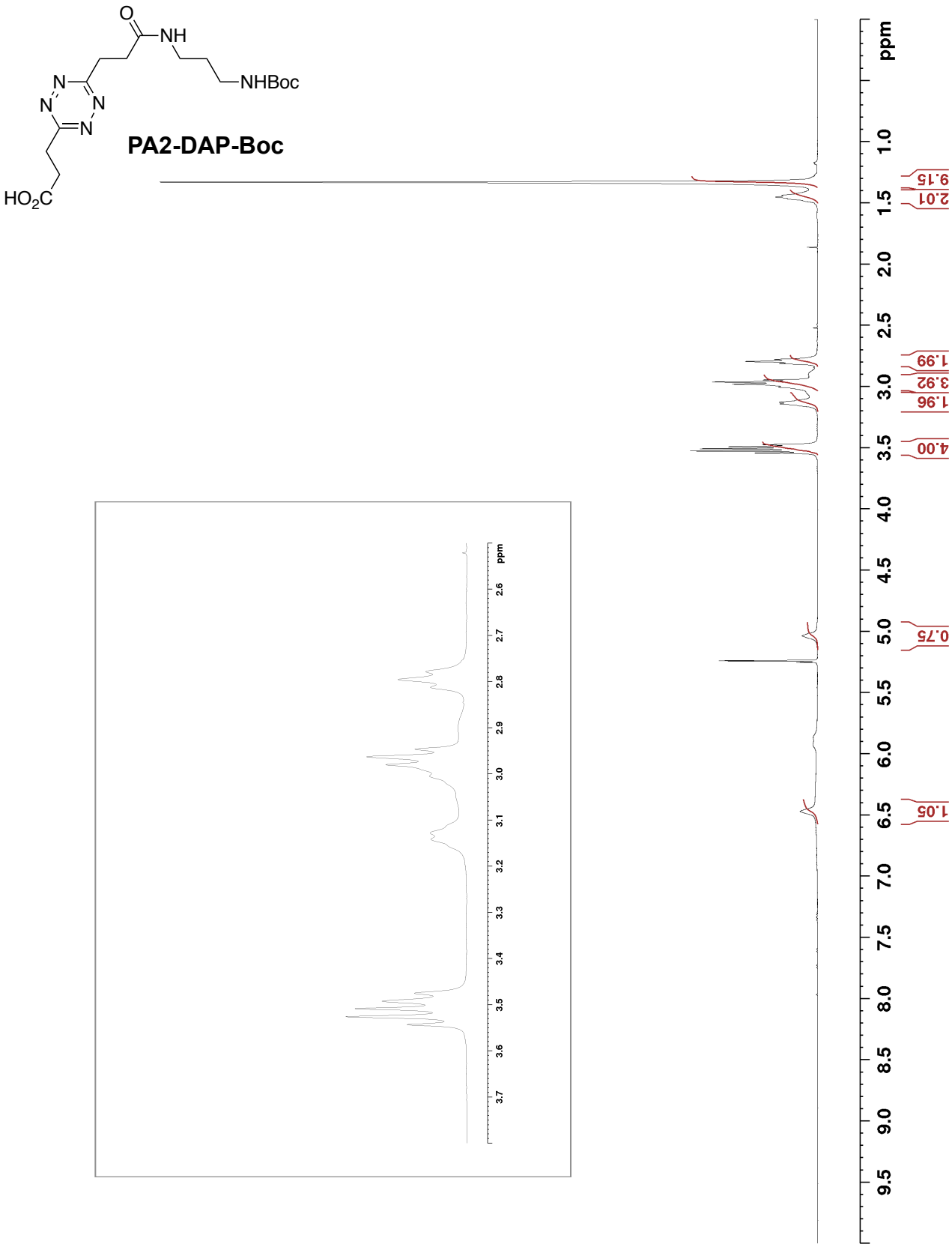
ppm
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
2.06
3.00
3.01
15.01
1.03
1.04
1.00

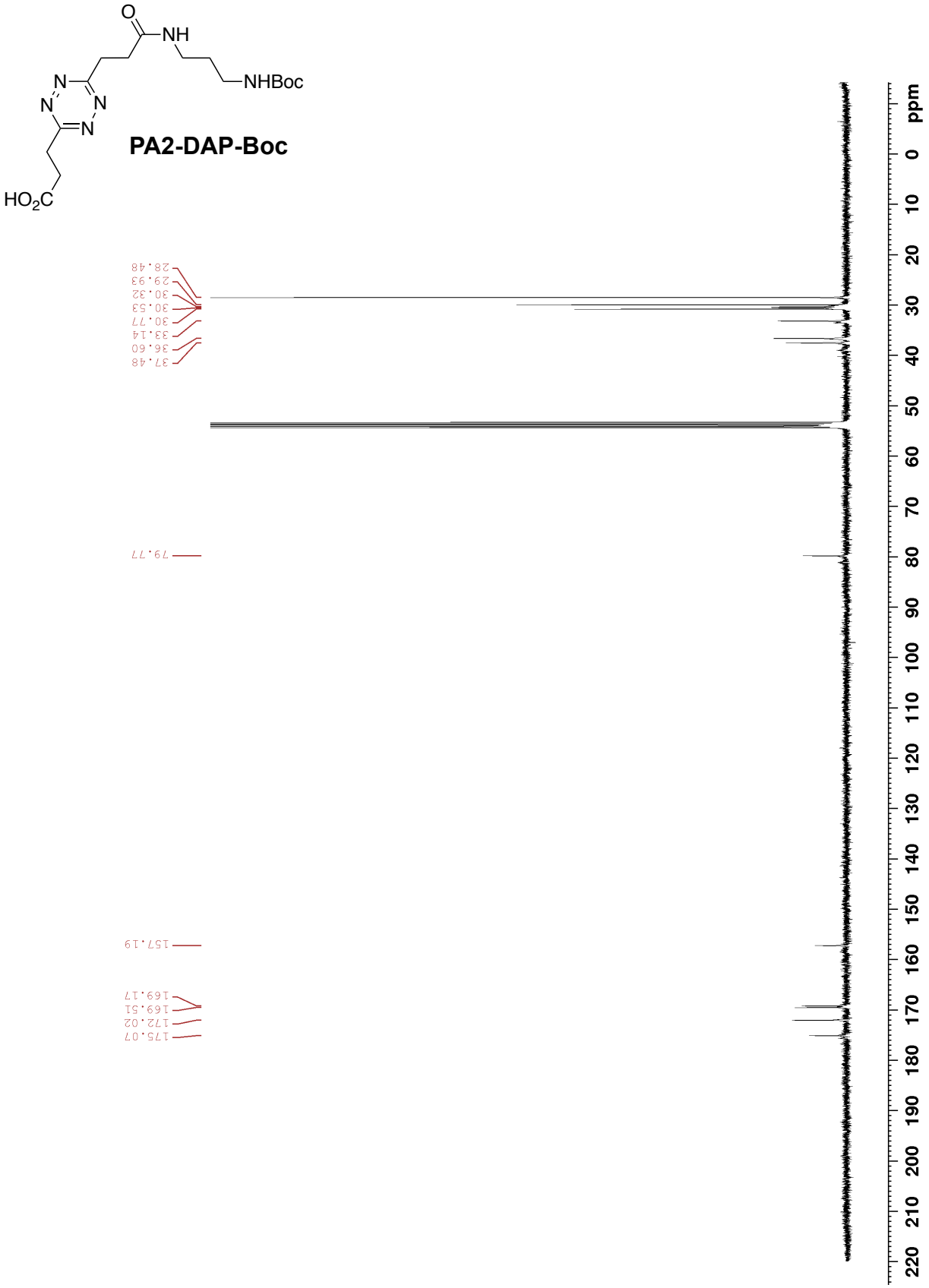
ppm
3.15
3.20
3.25
3.30
3.35
3.40
3.45
3.50
3.55
3.60
3.65
3.70
3.75
3.01
15.01
1.03

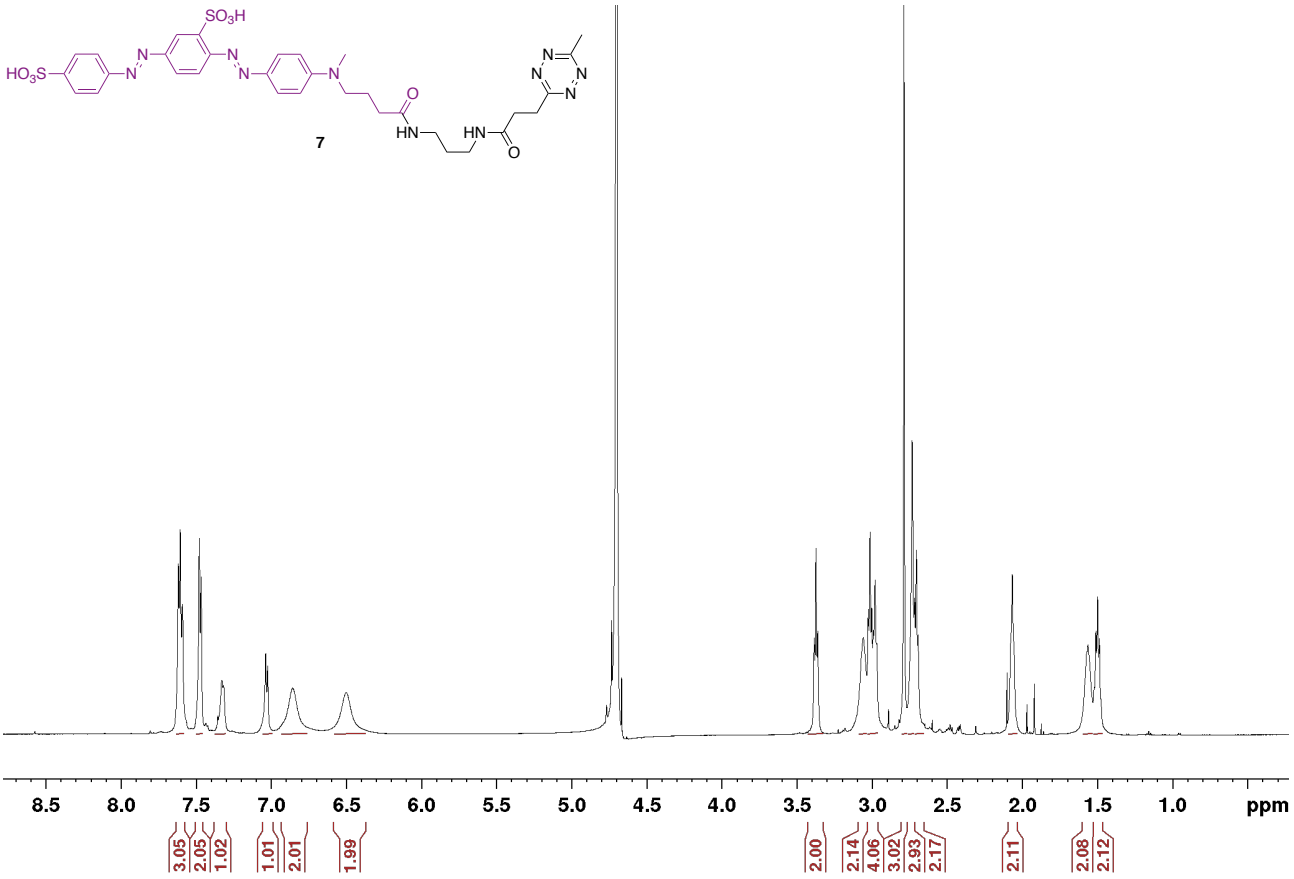

HO2C
O
N
H
O
4
Me
N
N
N
N
6
20.69
35.74
36.58
50.67
58.03
66.51
69.44
69.56
69.61
69.75
71.25
166.68
167.11
170.06
171.92
ppm
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210

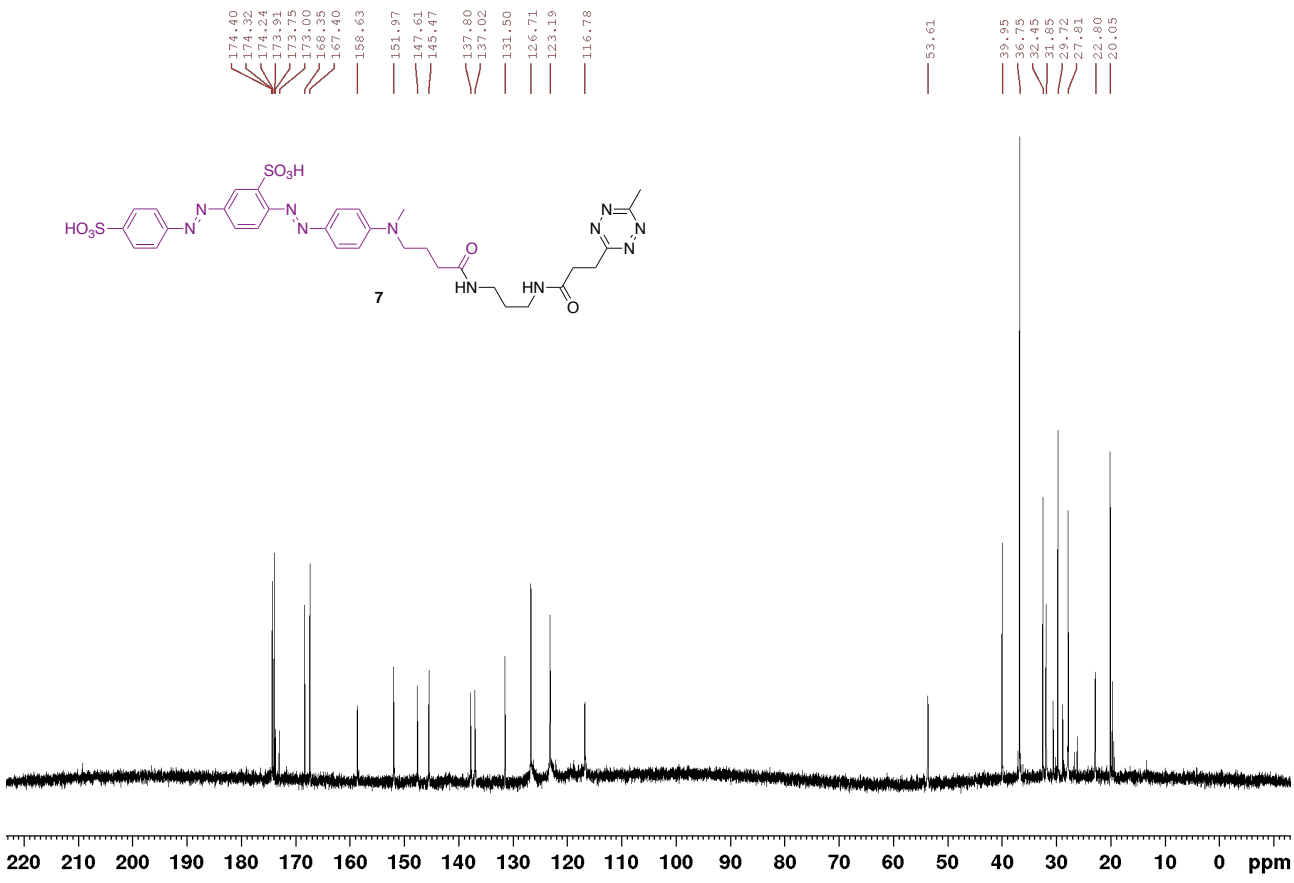

O
NH
NHBoc
N
N
N
N
MPA-DAP-Boc
ppm
9.15
2.05
1.97
2.94
2.01
2.14
2.00
0.99
0.95

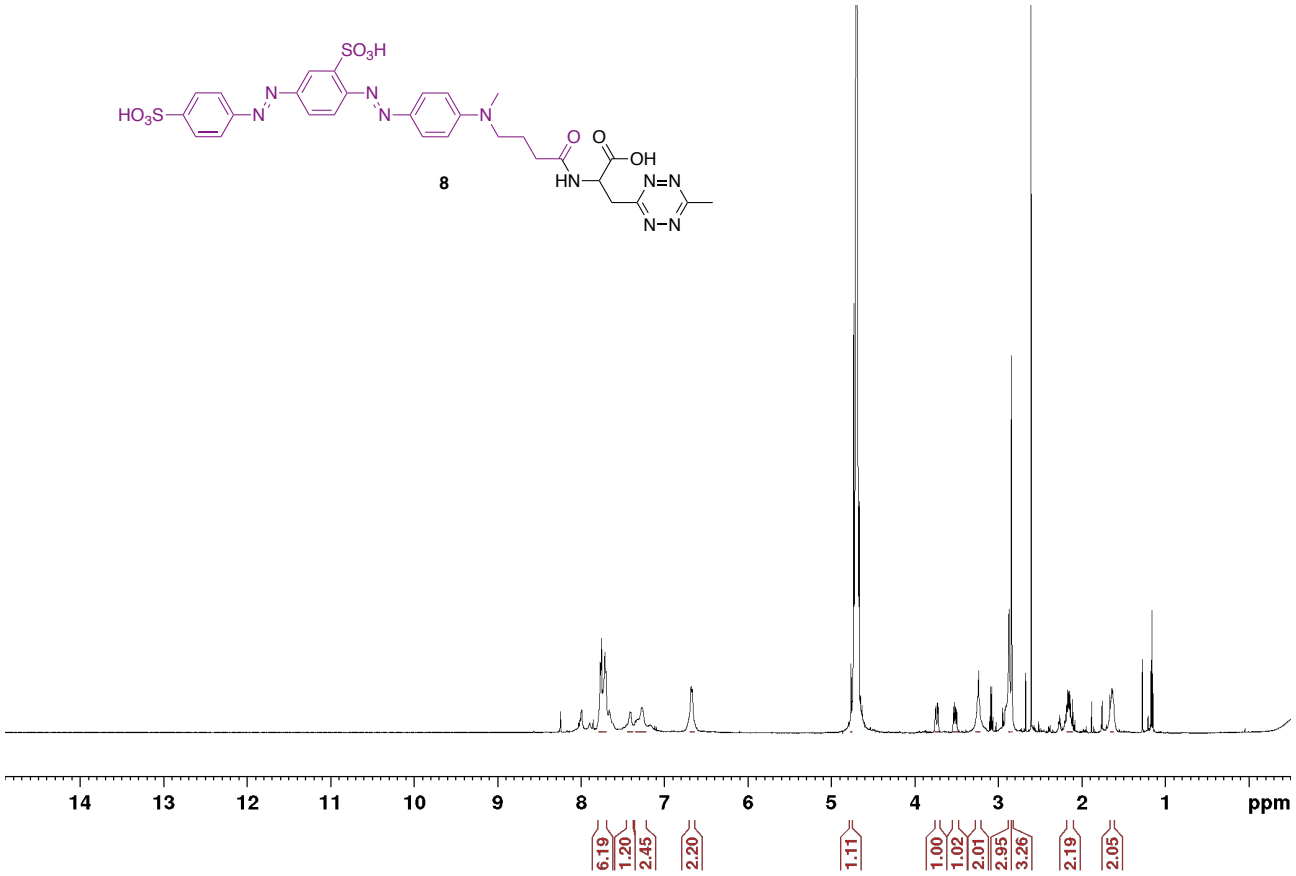

MPA-DAP-Boc
21.32
28.51
30.49
30.56
33.16
36.35
37.40
79.38
156.85
167.95
169.41
171.46
ppm

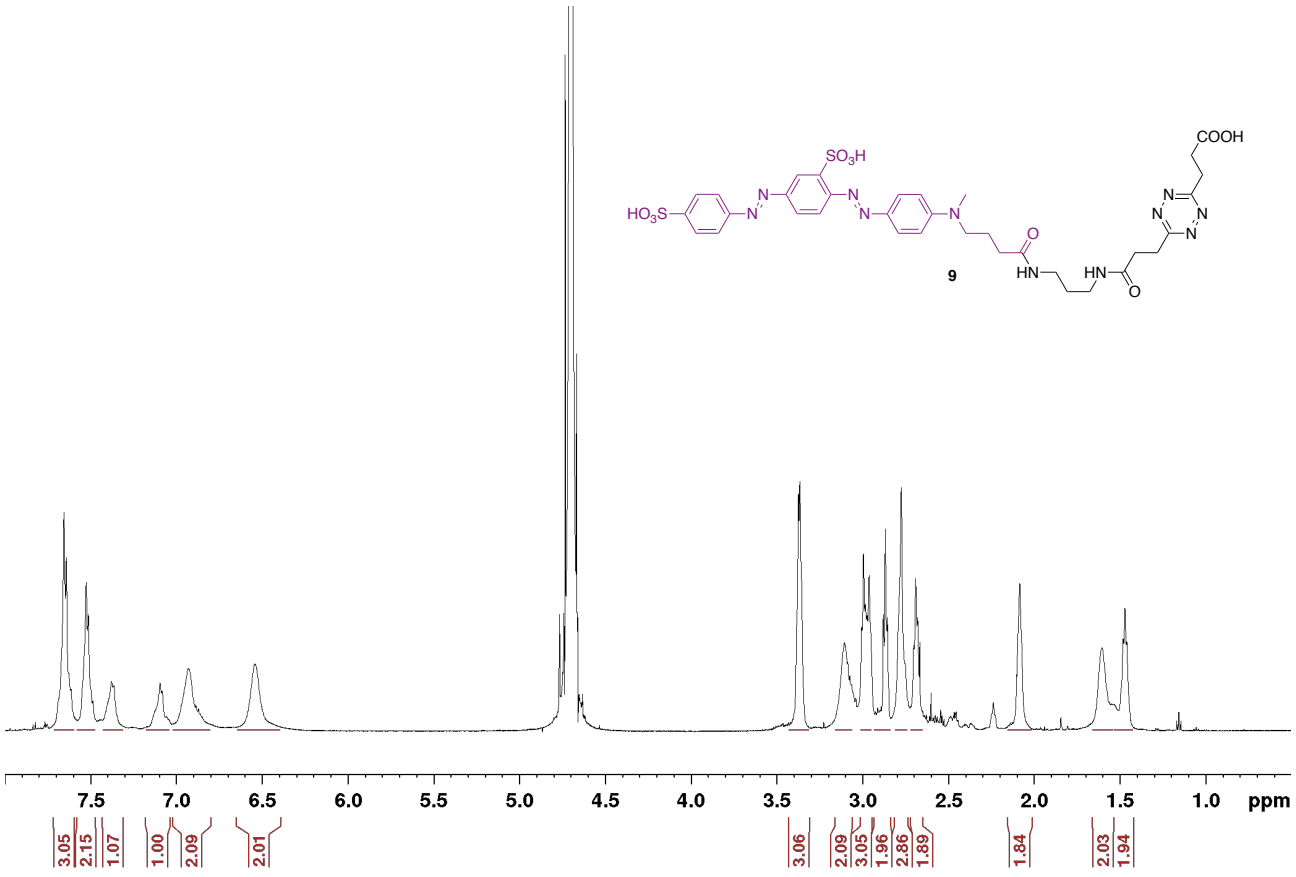

O
NH
NHBoc
N
N
N
N
HO2C
PA2-DAP-Boc
ppm
9.15
2.01
1.99
3.92
1.96
4.00
0.75
1.05

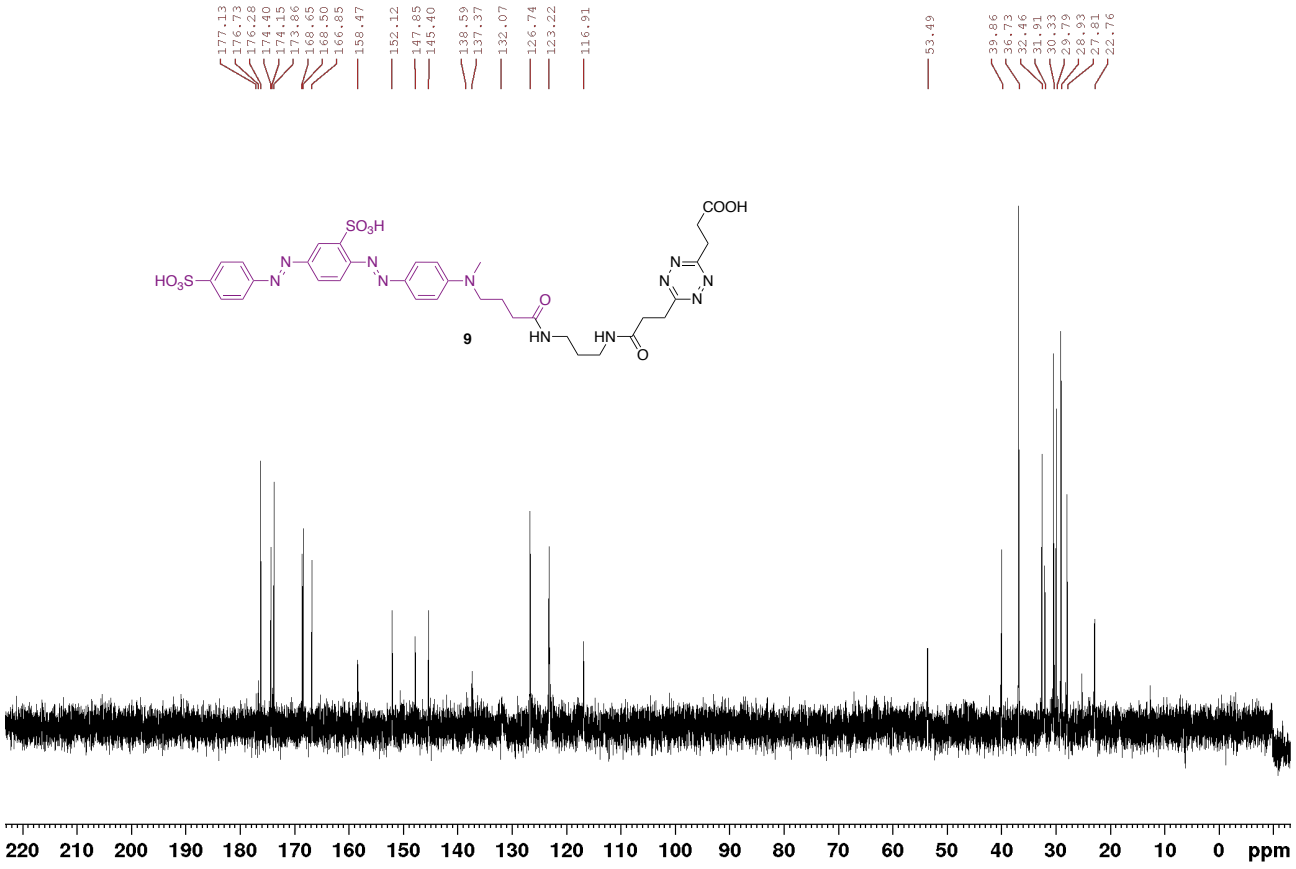

O
NH
NHBoc
N
N
N
N
HO2C
PA2-DAP-Boc
28.48
29.93
30.32
30.53
30.77
33.14
36.60
37.48
79.77
157.19
169.17
169.51
172.02
175.07
ppm
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220

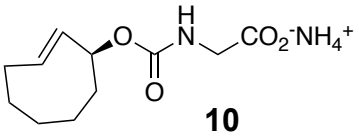

SO3H
HO3S
N
N
N
N
N
O
HN
HN
O
N
N
N
N
7
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm
3.05
2.05
1.02
1.01
2.01
1.99
2.00
2.14
4.06
3.02
2.93
2.17
2.11
2.08
2.12


174.40
174.32
174.24
173.91
173.75
173.00
168.35
167.40
158.63
151.97
147.61
145.47
137.80
137.02
131.50
126.71
123.19
116.78
53.61
39.95
36.75
32.45
31.85
29.72
27.81
22.80
20.05
SO3H
HO3S
N
N
N
N
N
O
HN
HN
O
N
N
N
N
7
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm

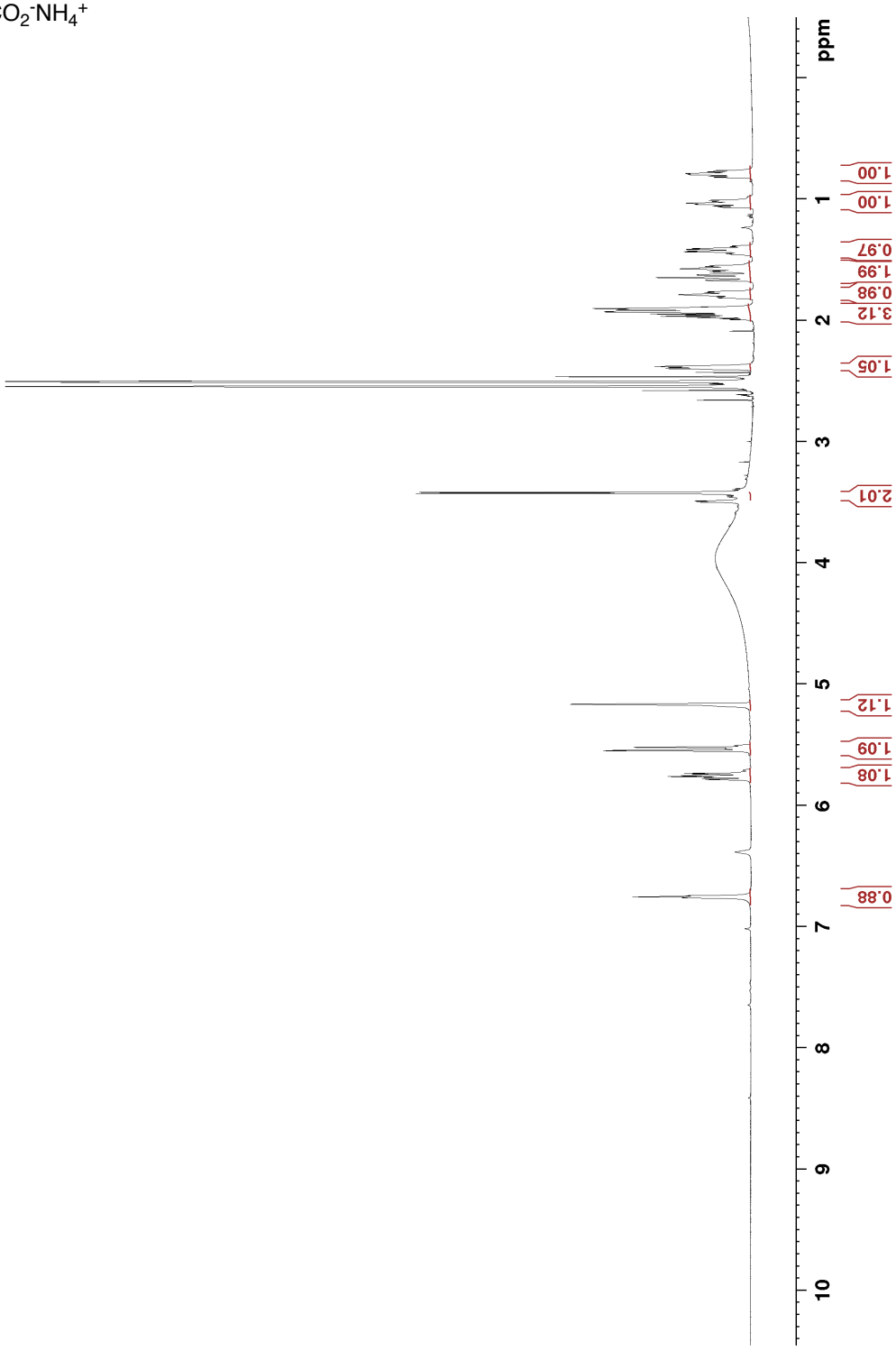

SO3H
HO3S
N
N
N
N
N
O
O
OH
HN
N=N
N–N
8
14
13
12
11
10
9
8
7
6
5
4
3
2
1
ppm
6.19
1.20
2.45
2.20
1.11
1.00
1.02
2.01
2.95
3.26
2.19
2.05

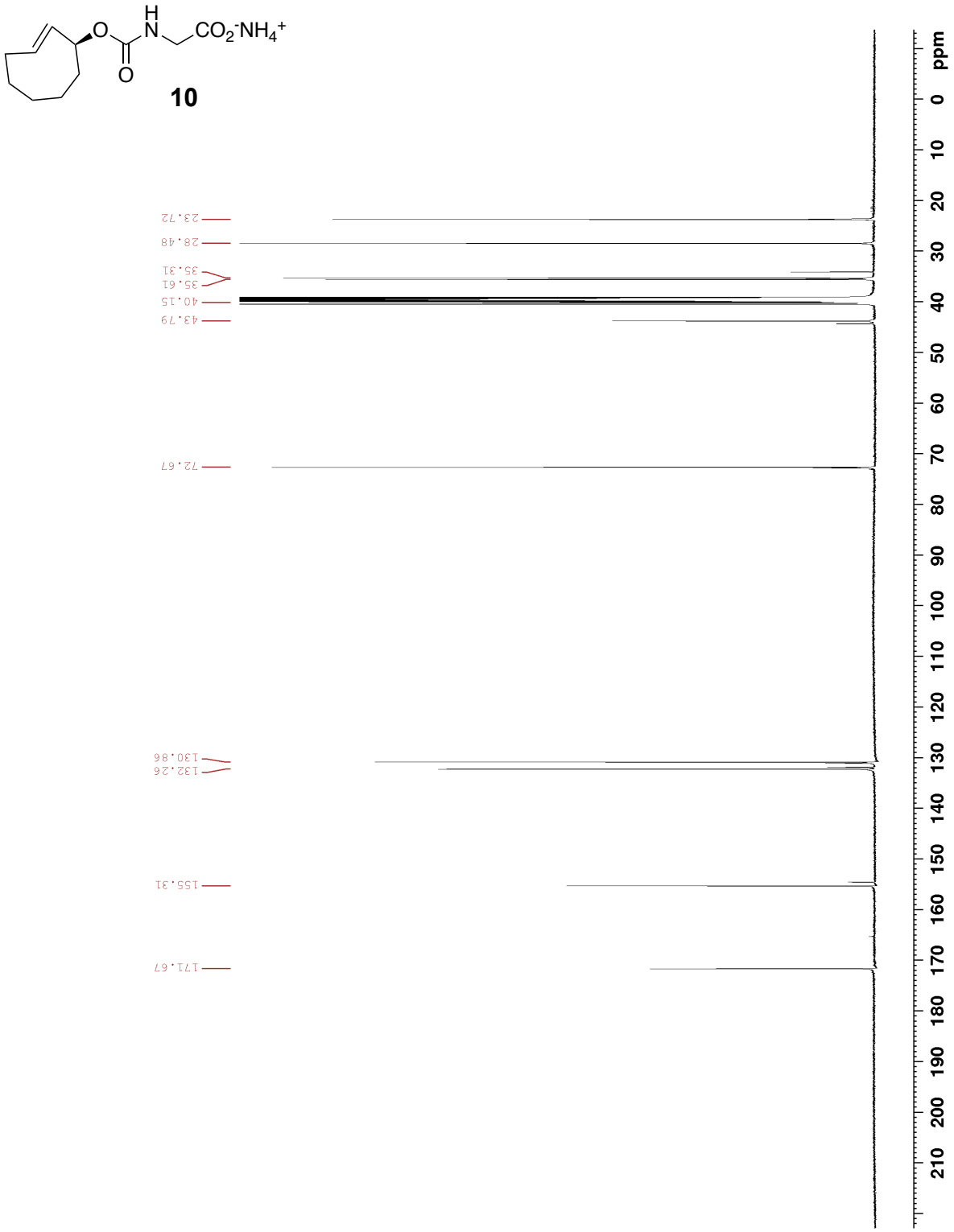

SO3H
HO3S
COOH
9
HN
HN
O
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm
3.05
2.15
1.07
1.00
2.09
2.01
3.06
2.09
3.05
1.96
2.86
1.89
1.84
2.03
1.94

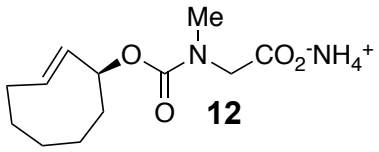

177.13
176.73
176.28
174.40
174.15
173.86
168.65
168.50
166.85
158.47
152.12
147.85
145.40
138.59
137.37
132.07
126.74
123.22
116.91
53.49
39.86
36.73
32.46
31.91
30.33
29.79
28.93
27.81
22.76
SO3H
HO3S
COOH
9
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm


O
H
N
O
$CO_2^-NH_4^+$

10

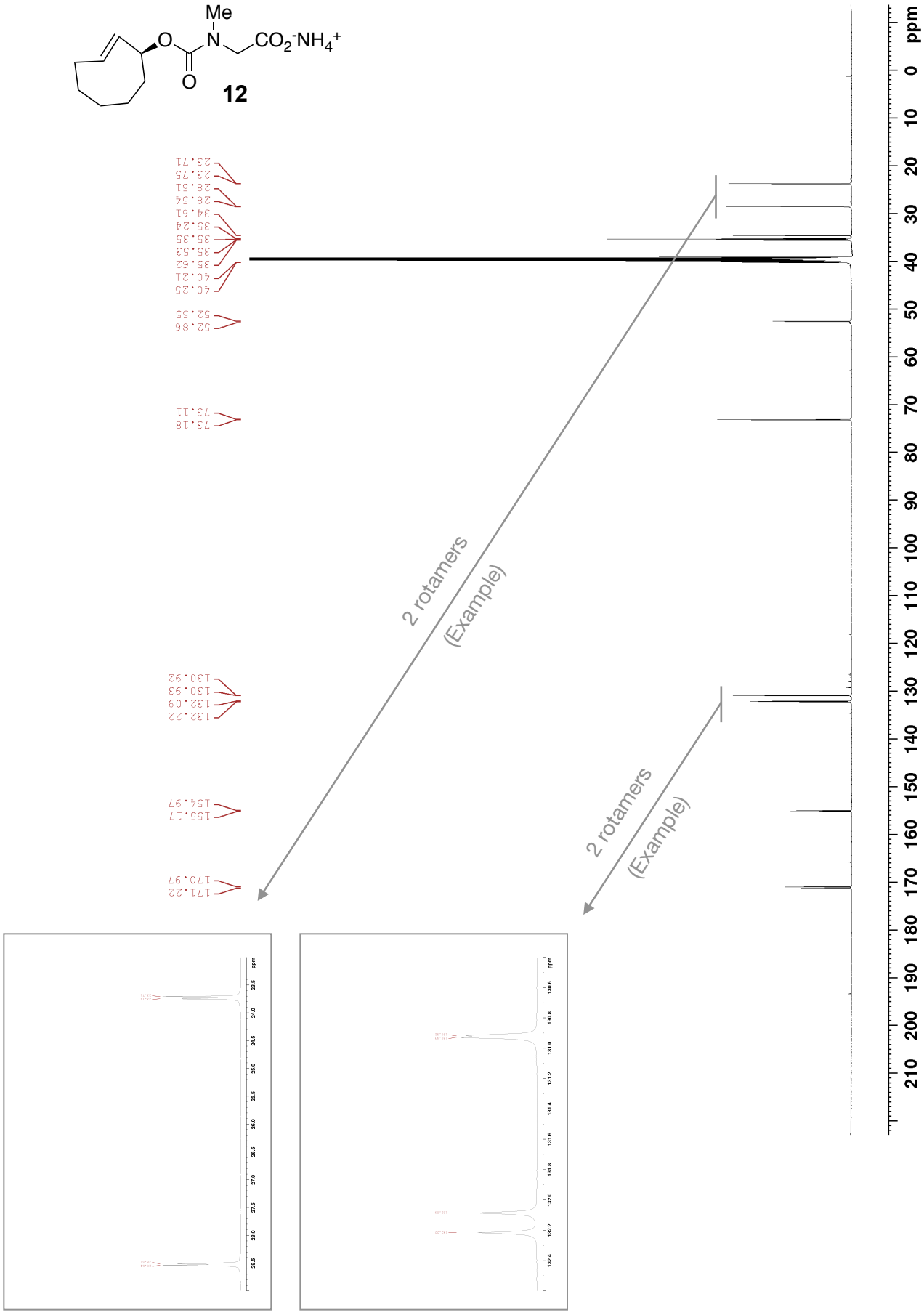

ppm
1
2
3
4
5
6
7
8
9
10
1.00
1.00
0.97
1.99
0.98
3.12
1.05
2.01
1.12
1.09
1.08
0.88

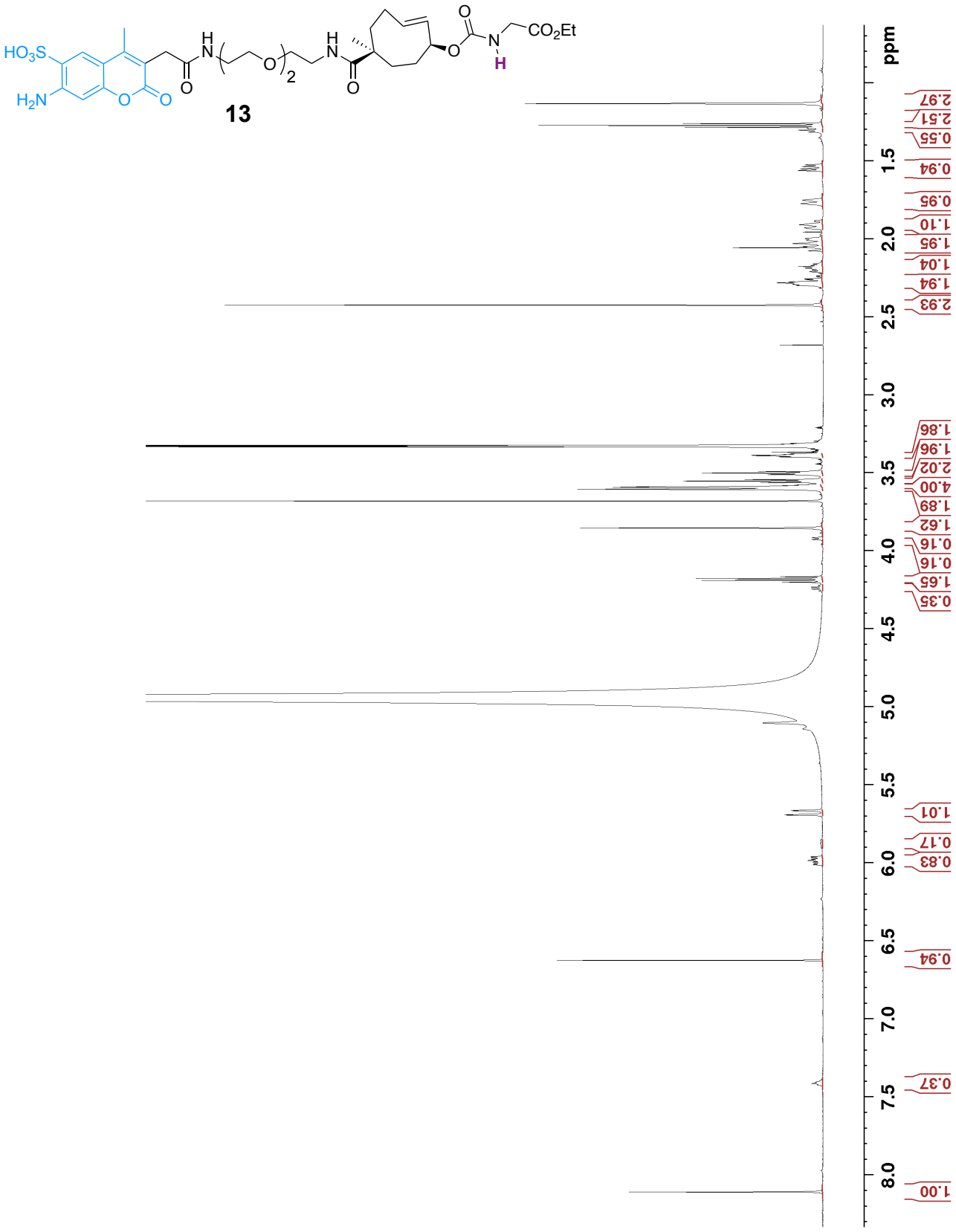

O
H
N
CO2-NH4+
O
10
23.72
28.48
35.31
35.61
40.15
43.79
72.67
130.86
132.26
155.31
171.67
ppm
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210

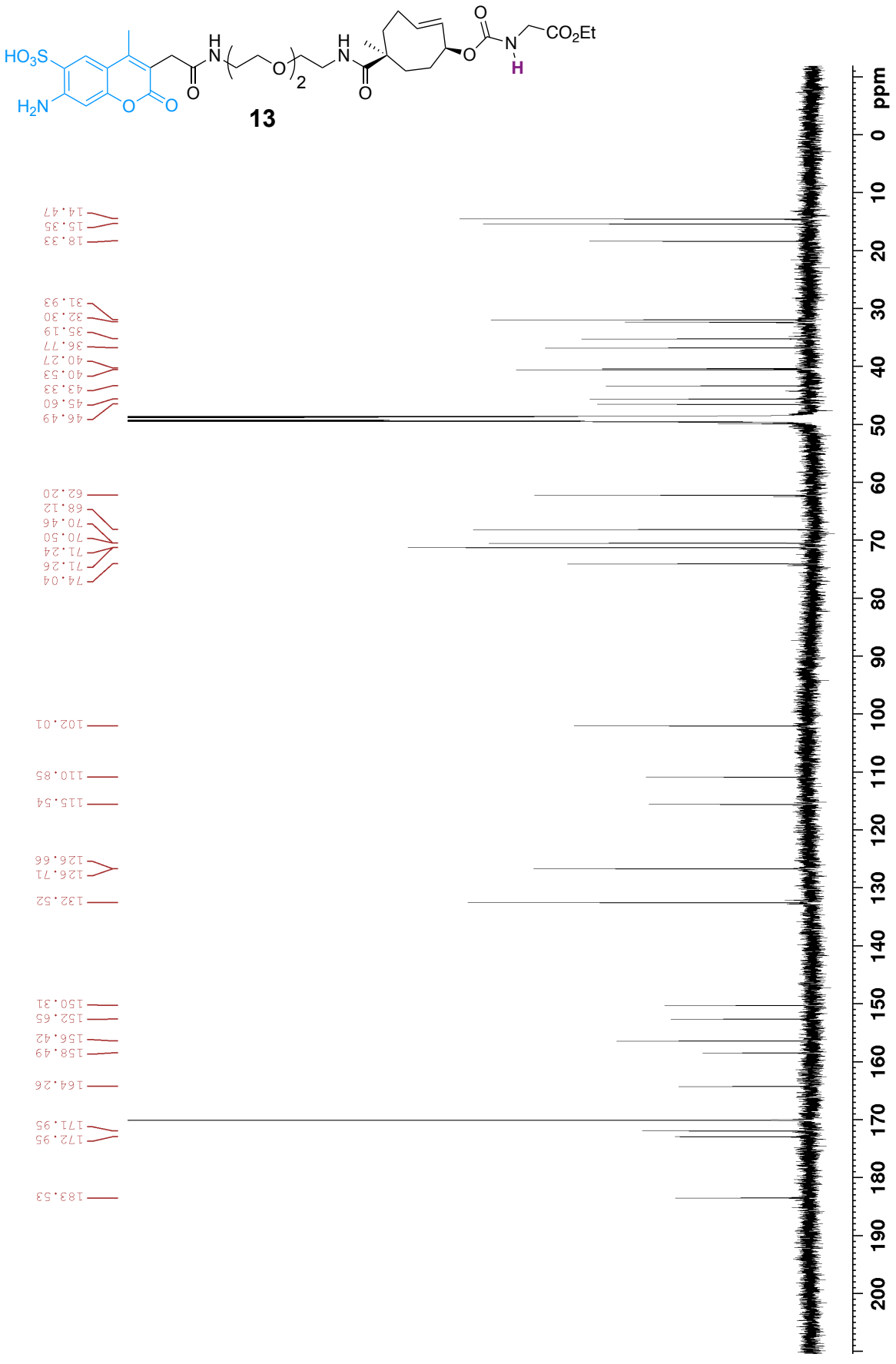

Me
O
N
$CO_2^-NH_4^+$
O

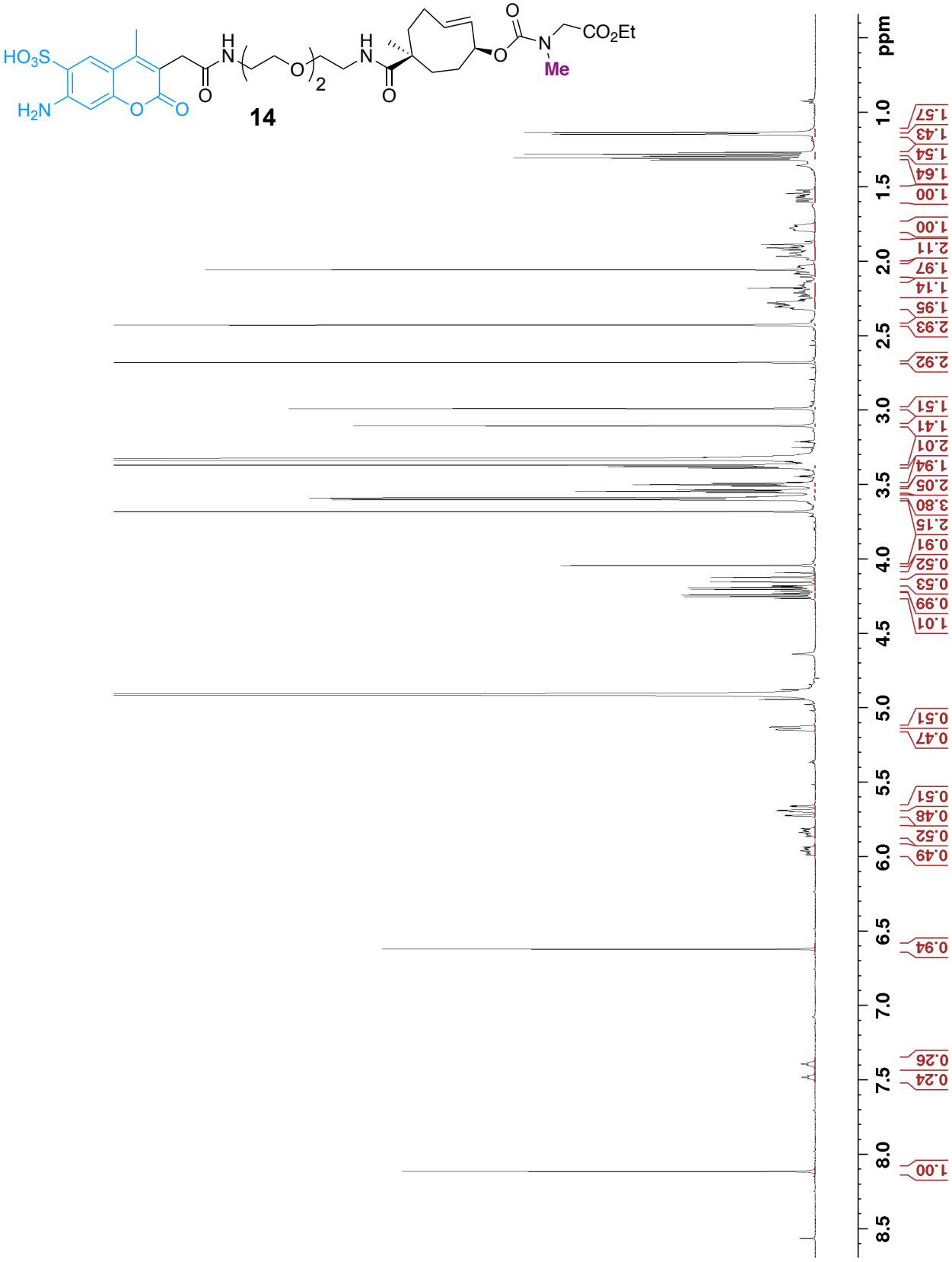
12

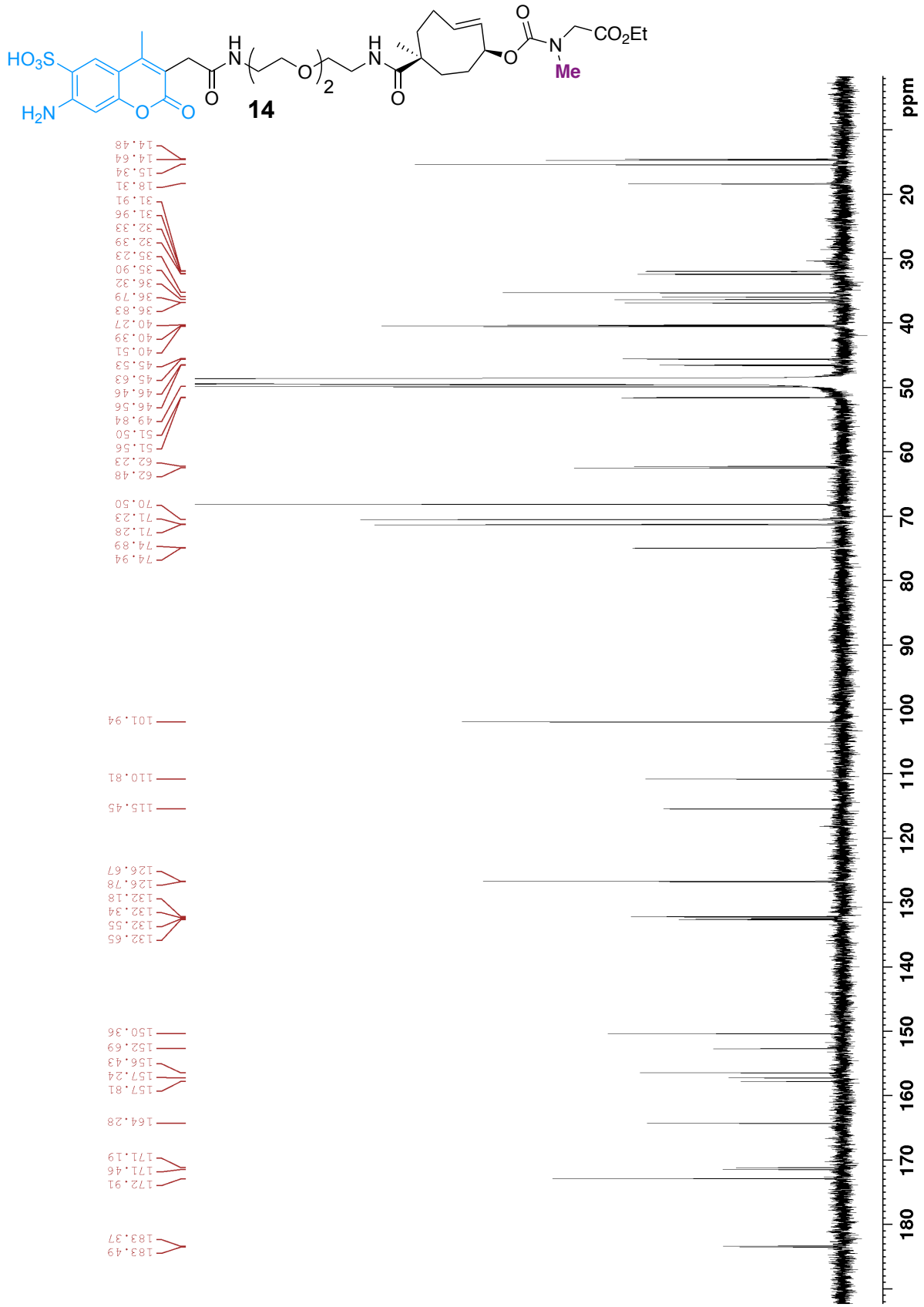
ppm
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
1.07
1.06
1.18
2.18
1.15
3.03
1.06
1.58
1.36
1.30
1.10
1.00
1.02
1.04
5.10
5.15
5.20
5.25
5.30
5.35
5.40
5.45
5.50
5.55
5.60
5.65
5.70
5.75
5.80
5.85
5.90
5.95


Me
O
N
CO2-NH4+
O
12
23.71
23.75
28.51
28.54
34.61
35.24
35.35
35.53
35.62
40.21
40.25
52.55
52.86
73.11
73.18
132.22
132.09
130.93
130.92
155.17
154.97
171.22
170.97
2 rotamers
(Example)
2 rotamers
(Example)
ppm

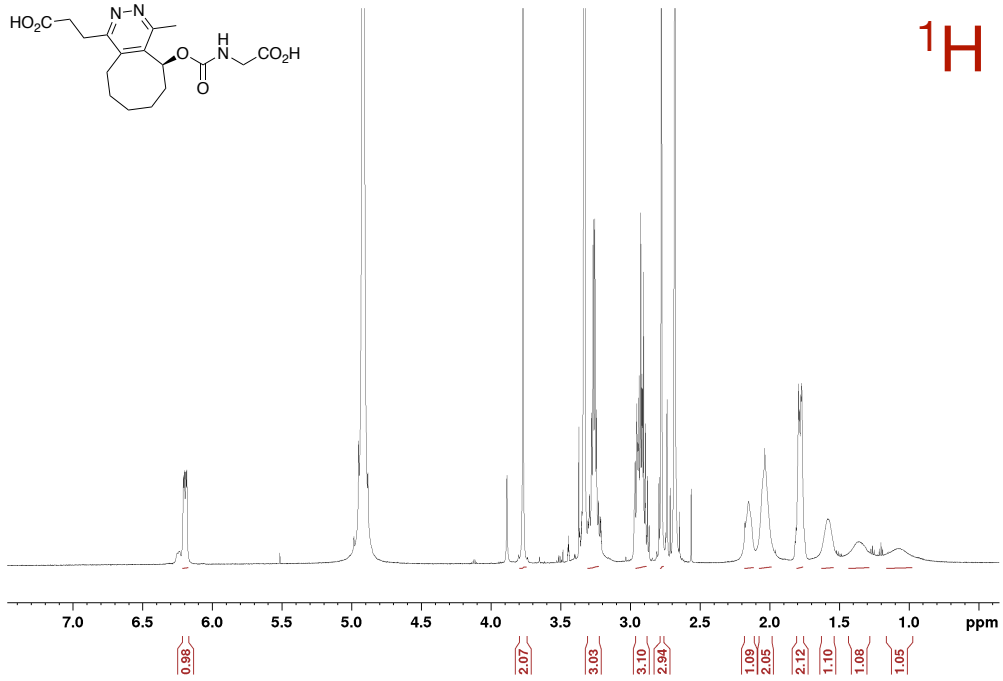

HO3S
H2N
O
O
H
N
O
2
H
N
O
O
O
N
H
CO2Et
13
ppm
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
2.97
2.51
0.55
0.94
0.95
1.10
1.95
1.04
1.94
2.93
1.86
1.96
2.02
4.00
1.89
1.62
0.16
0.16
1.65
0.35
1.01
0.17
0.83
0.94
0.37
1.00

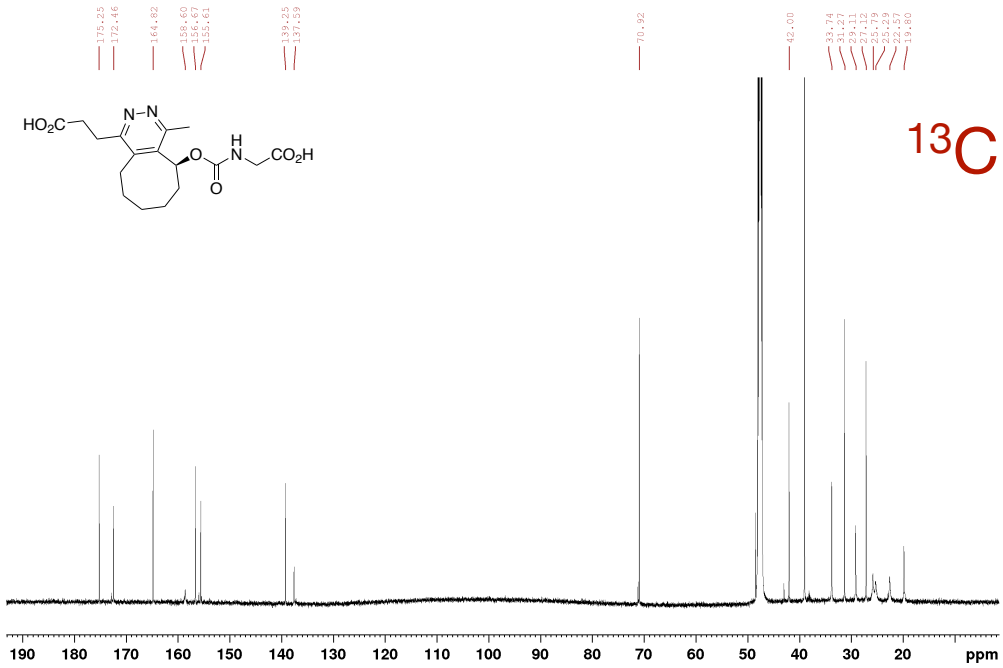

HO3S
H2N
O
O
H
N
O
2
H
N
O
O
O
N
H
CO2Et
13
14.47
15.35
18.33
31.93
32.30
35.19
36.77
40.27
40.53
43.33
45.60
46.49
62.20
68.12
70.46
70.50
71.24
71.26
74.04
102.01
110.85
115.54
126.66
126.71
132.52
150.31
152.65
156.42
158.49
164.26
171.95
172.95
183.53
ppm
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200


HO3S
H2N
O
H
N
O
O
2
H
N
O
O
O
N
CO2Et
Me
14
ppm
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
1.57
1.43
1.54
1.64
1.00
1.00
2.11
1.97
1.14
1.95
2.93
2.92
1.51
1.41
2.01
1.94
2.05
3.80
2.15
0.91
0.52
0.53
0.99
1.01
0.51
0.47
0.51
0.48
0.52
0.49
0.94
0.26
0.24
1.00

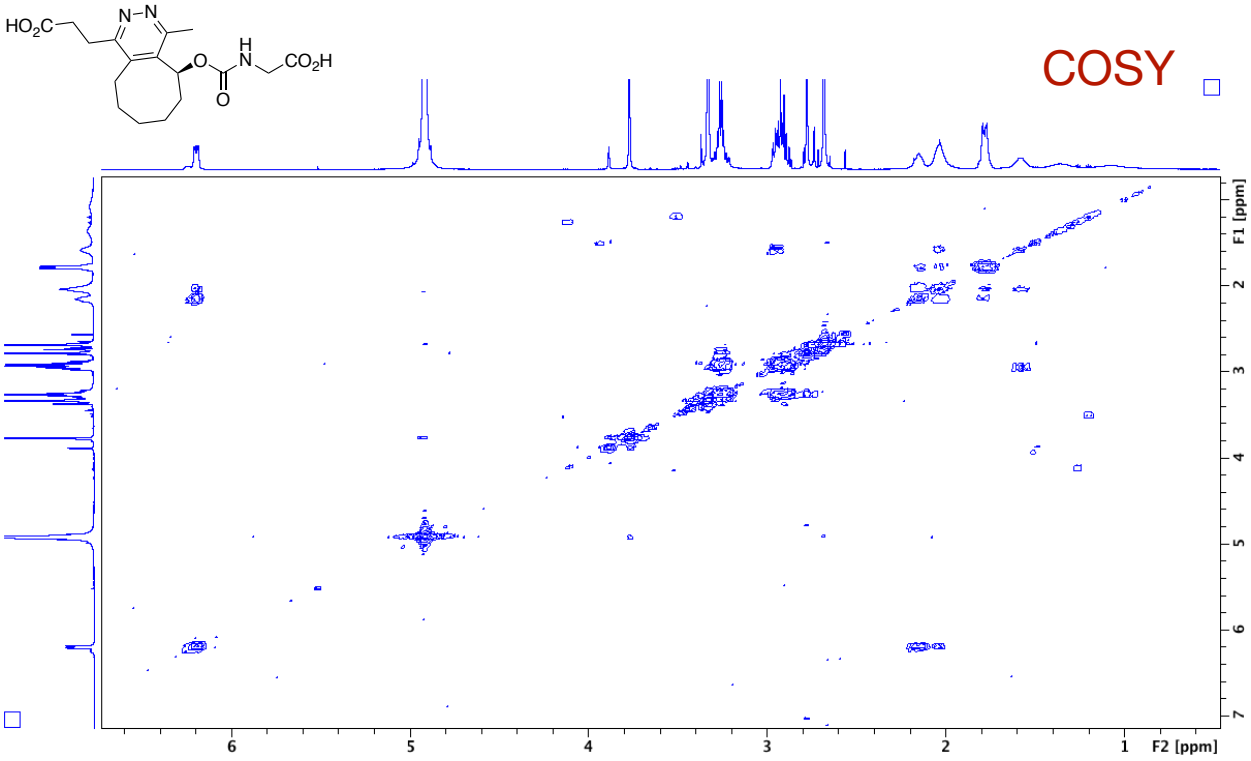

HO3S
H2N
O
O
H
N
O
2
H
N
O
O
O
N
CO2Et
Me
14
14.48
14.64
15.34
18.31
31.91
31.96
32.33
32.39
35.23
35.90
36.32
36.79
36.83
40.27
40.39
40.51
45.53
45.63
46.46
46.56
49.84
51.50
51.56
62.23
62.48
70.50
71.23
71.28
74.89
74.94
101.94
110.81
115.45
126.67
126.78
132.18
132.34
132.55
132.65
150.36
152.69
156.43
157.24
157.81
164.28
171.19
171.46
172.91
183.37
183.49
ppm
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180

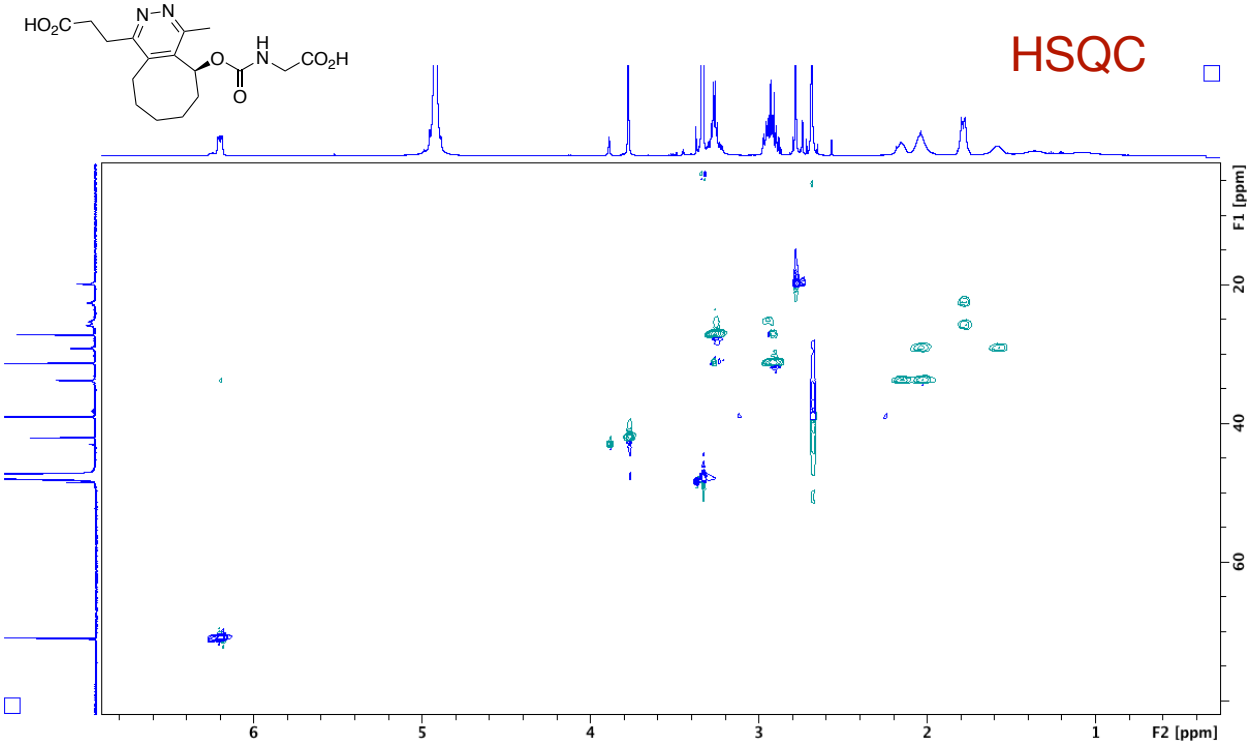
slow-releasing MPA-Gly-rTCO



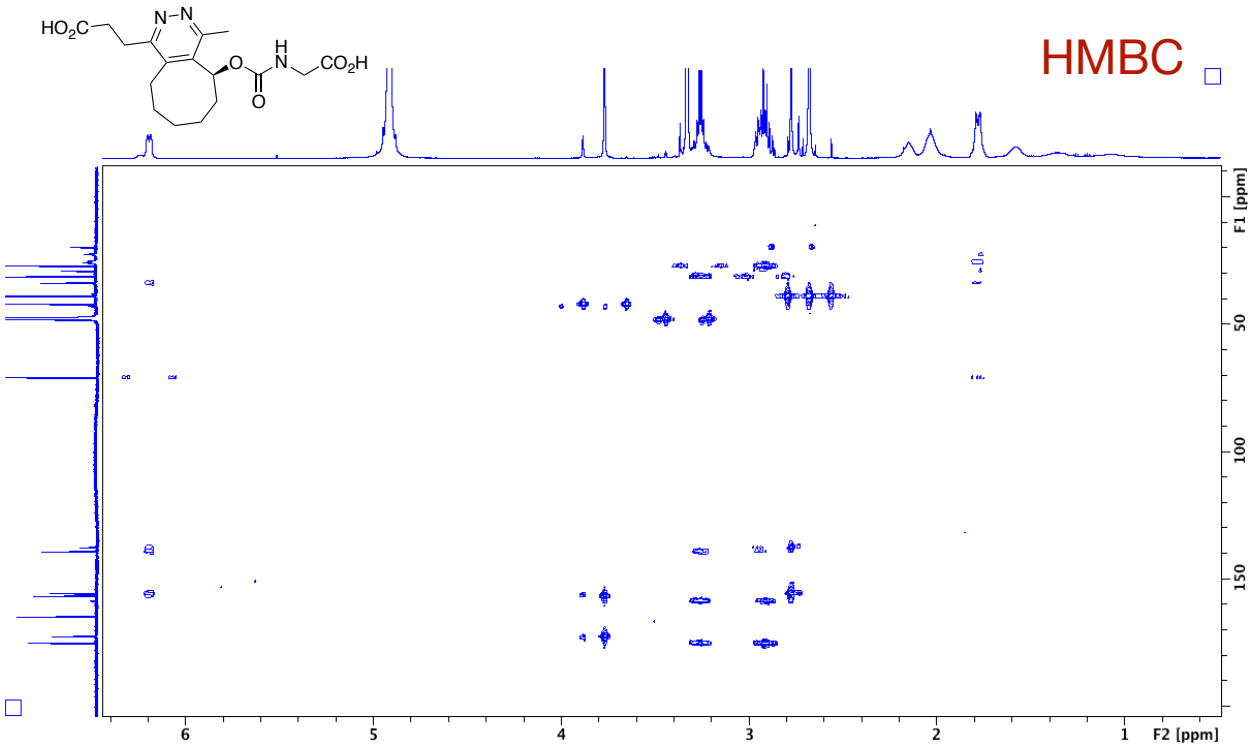


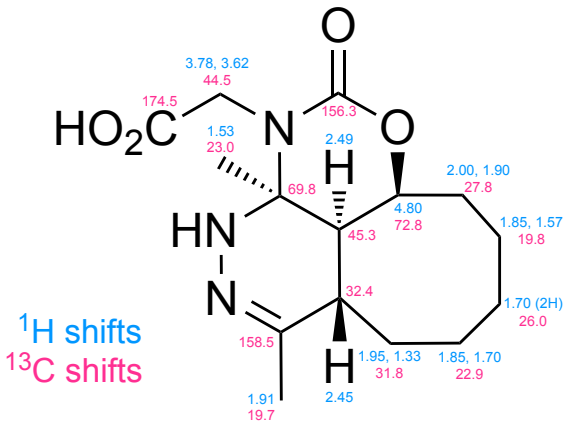
slow-releasing MPA-Gly-rTCO

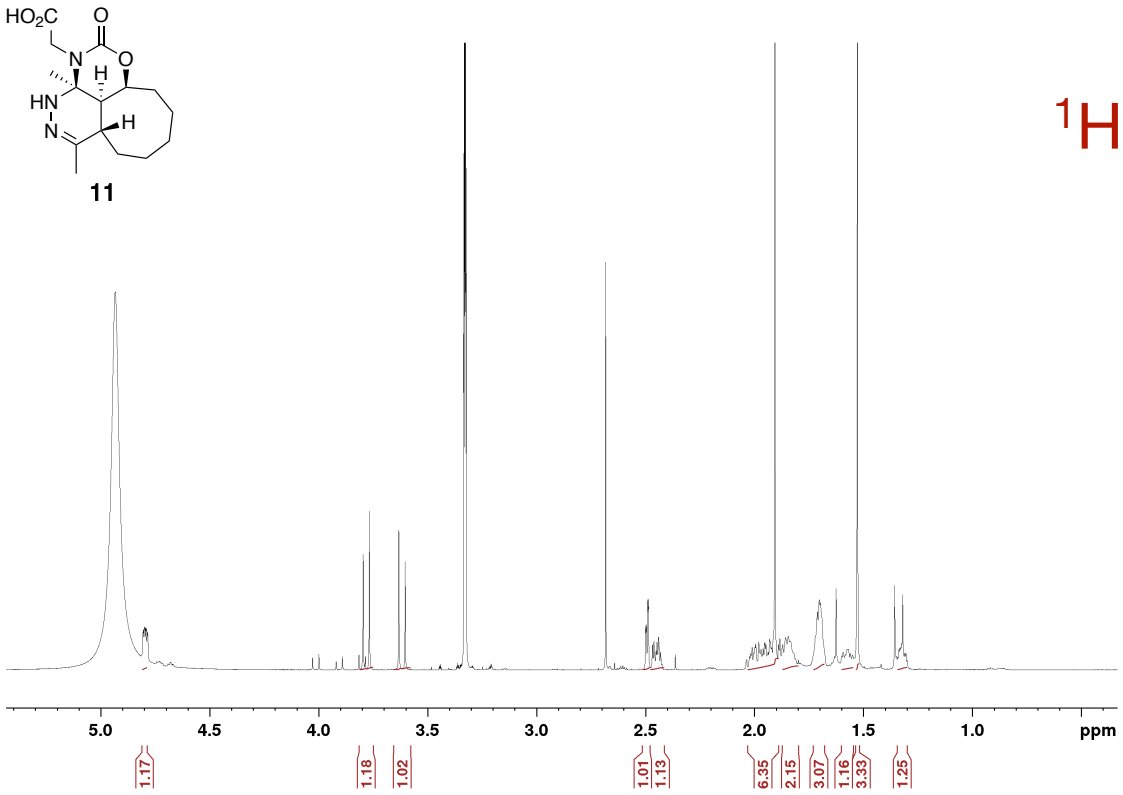


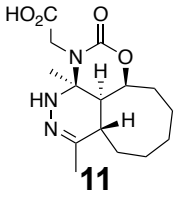


slow-releasing MPA-Gly-rTCO



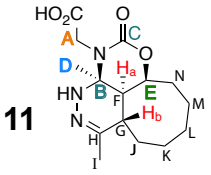

O
3.78, 3.62
44.5
174.5
HO2C
N
156.3
O
1.53
23.0
2.49
H
2.00, 1.90
27.8
69.8
4.80
72.8
1.85, 1.57
19.8
HN
45.3
N
32.4
1.70 (2H)
26.0
158.5
H
1.95, 1.33
31.8
1.85, 1.70
22.9
1.91
19.7
2.45
1H shifts
13C shifts


HO2C
O
N
H
O
HN
N
H
11
1H
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm
1.17
1.18
1.02
1.01
1.13
6.35
2.15
3.07
1.16
3.33
1.25



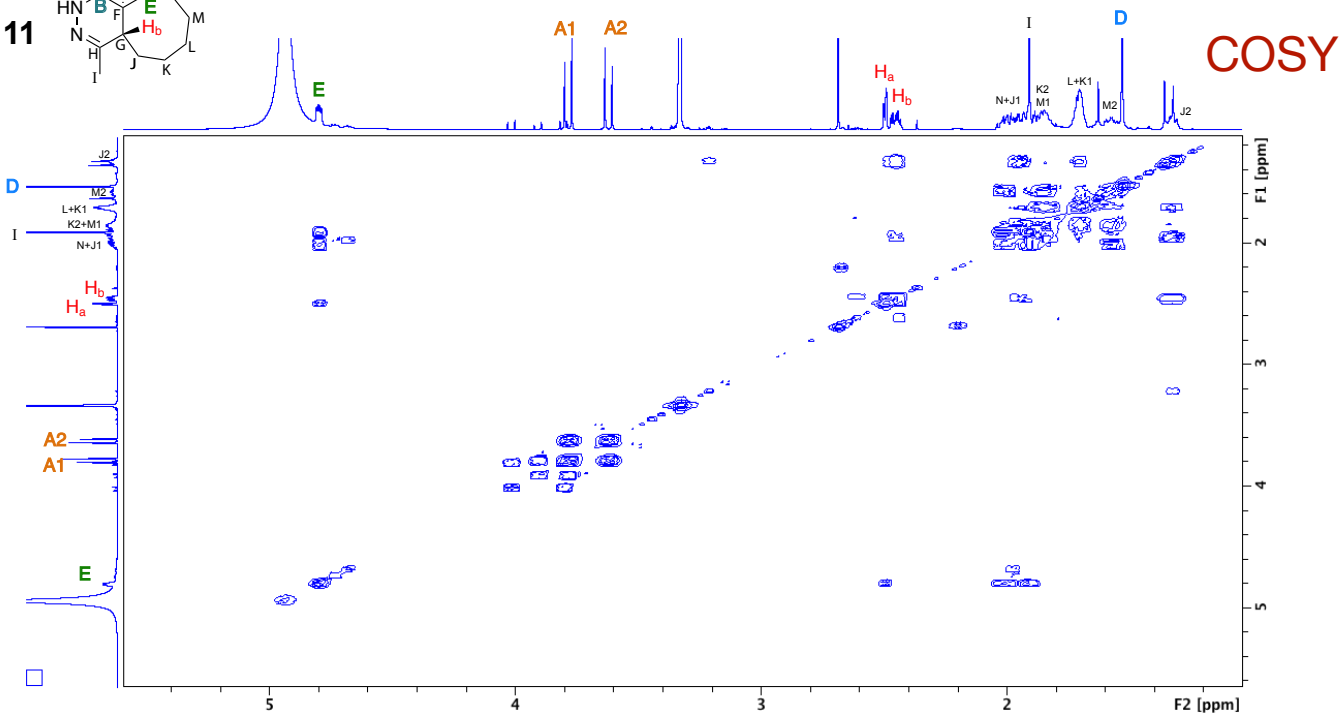
11

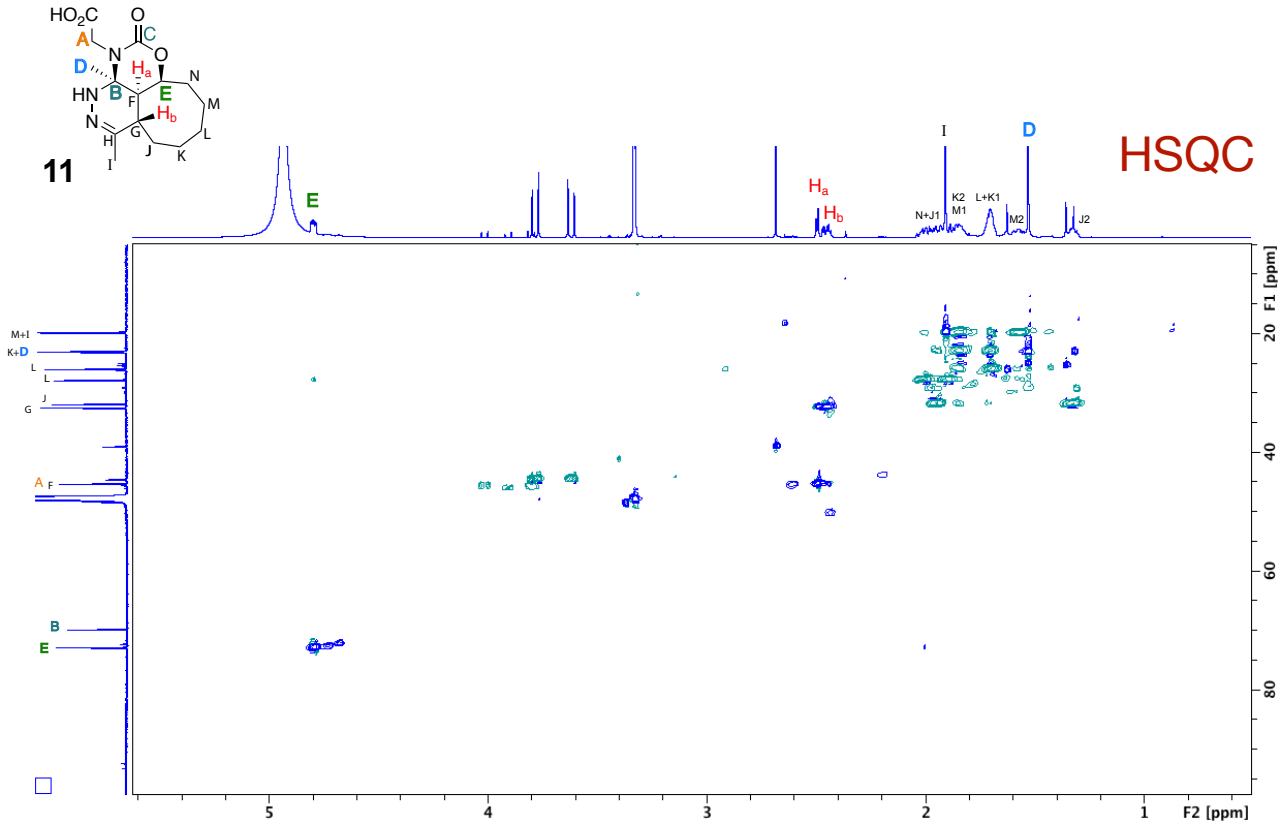
^{13}C

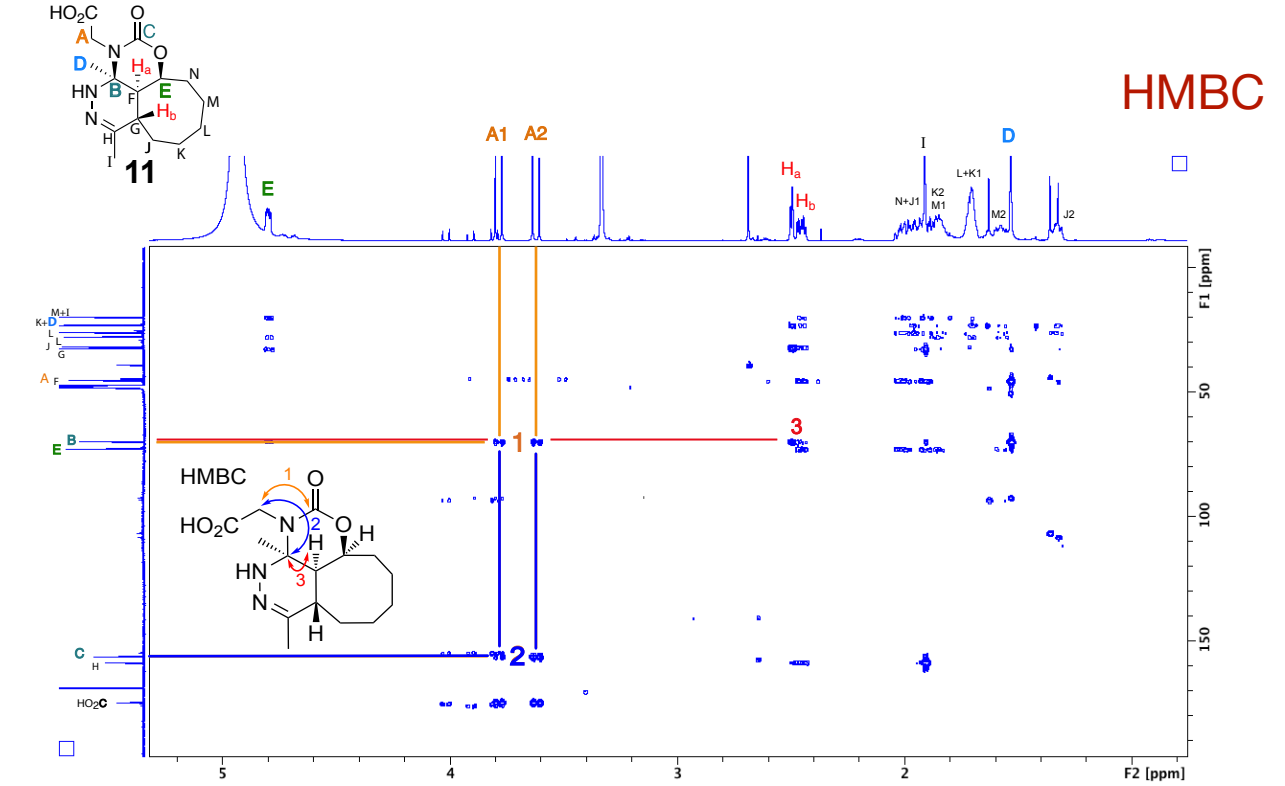


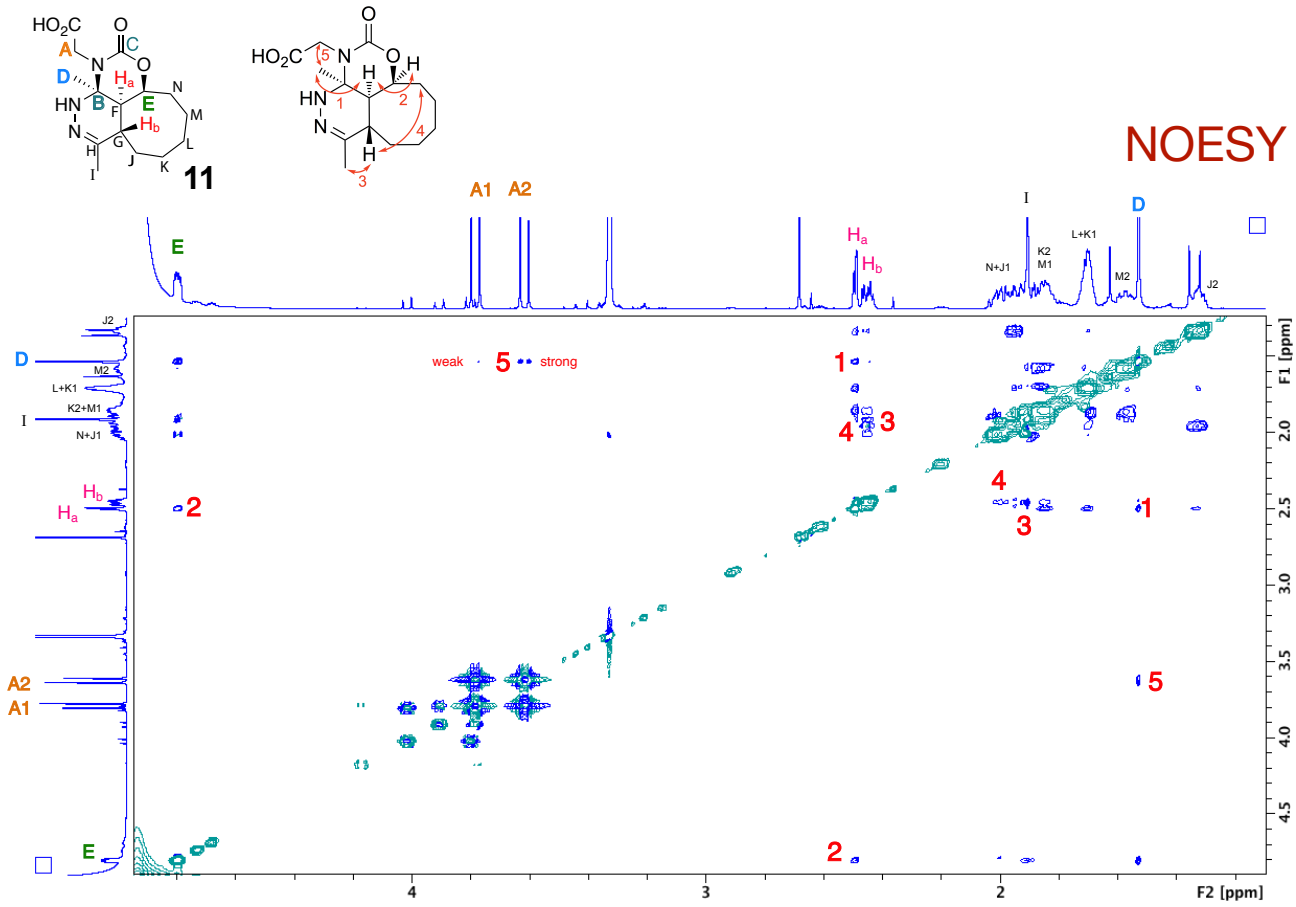
11

COSY

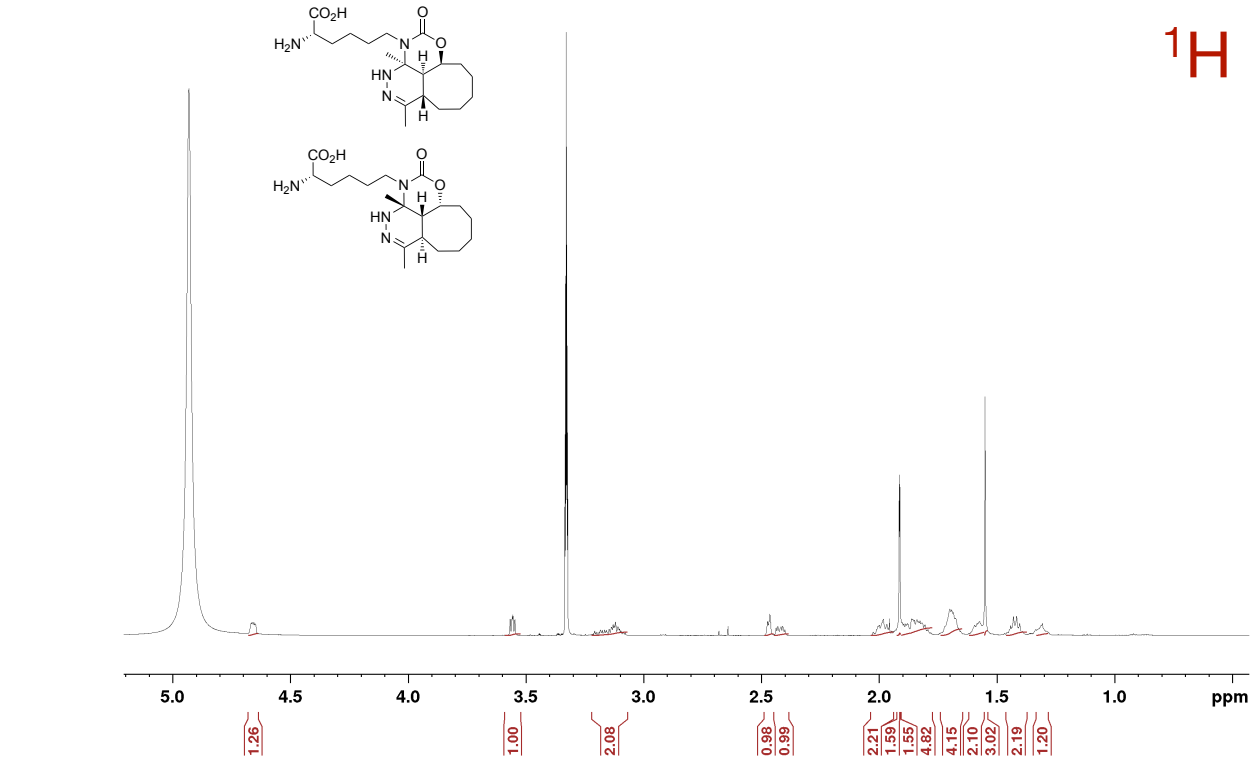



HSQC
11
E
Ha
Hb
I
D
N+J1
K2
M1
L+K1
M2
J2
F1 [ppm]
F2 [ppm]

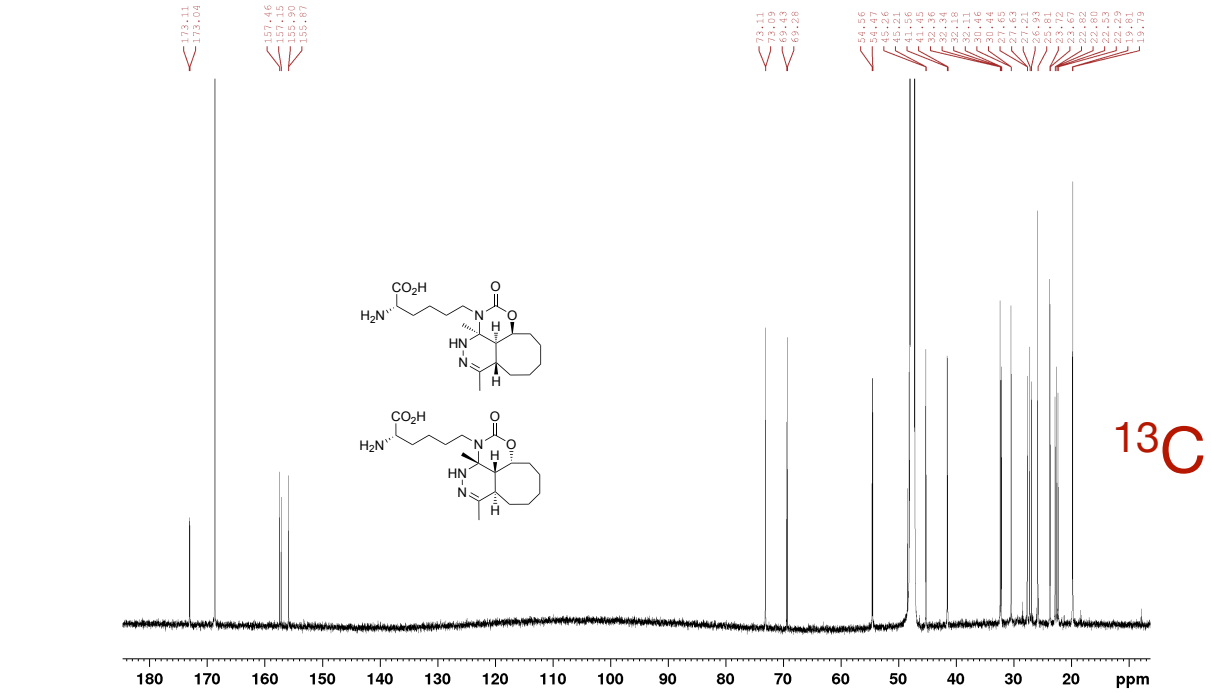

HMBC
11
A1
A2
E
Ha
Hb
I
D
N+J1
K2
M1
L+K1
M2
J2
HMBC
F1 [ppm]
F2 [ppm]

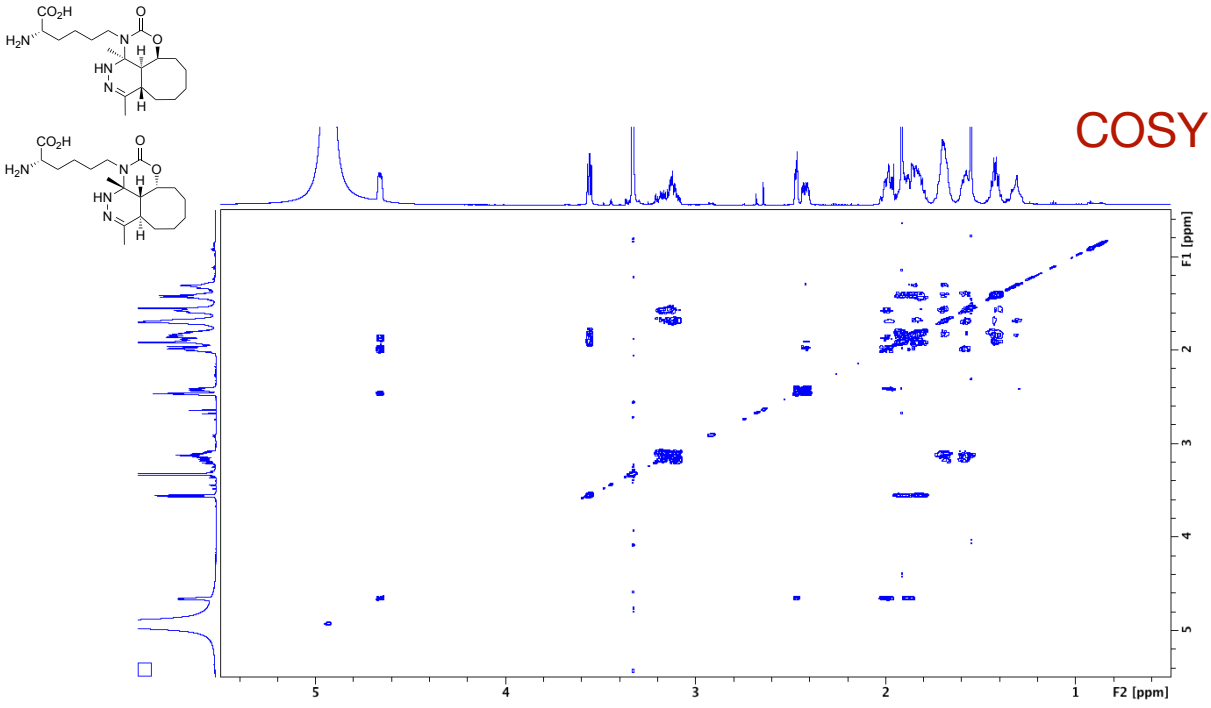


N-ε-rTCO-Lys + DMT dead end isomer



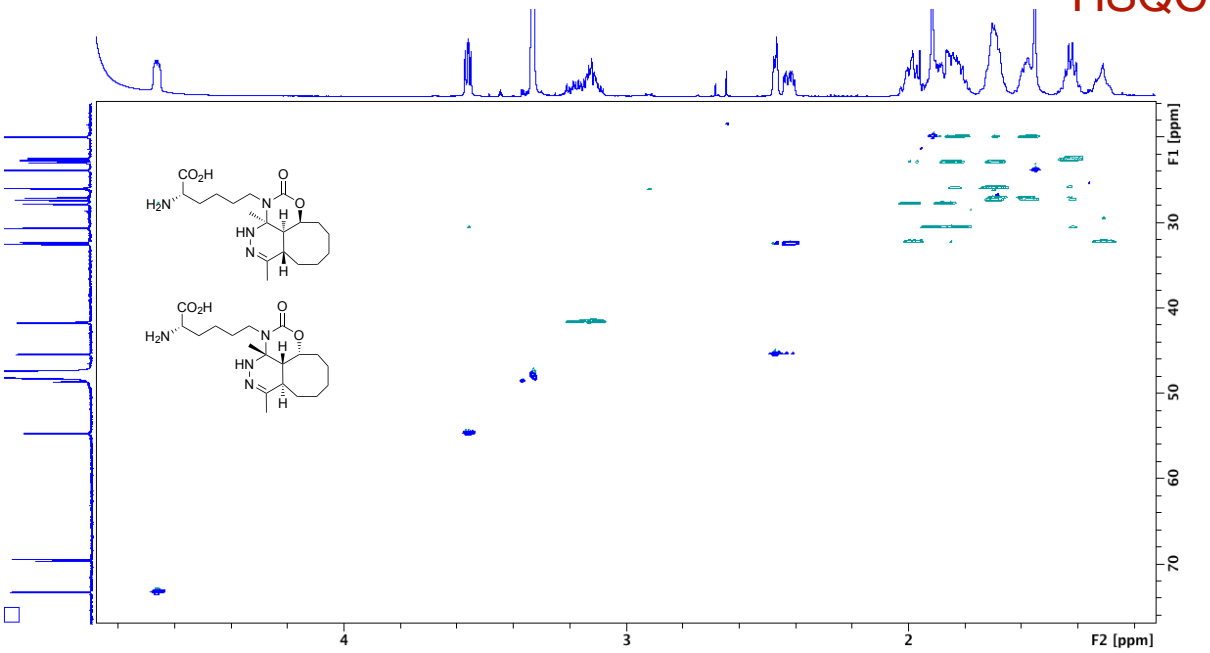
N-ε-rTCO-Lys + DMT dead end isomer



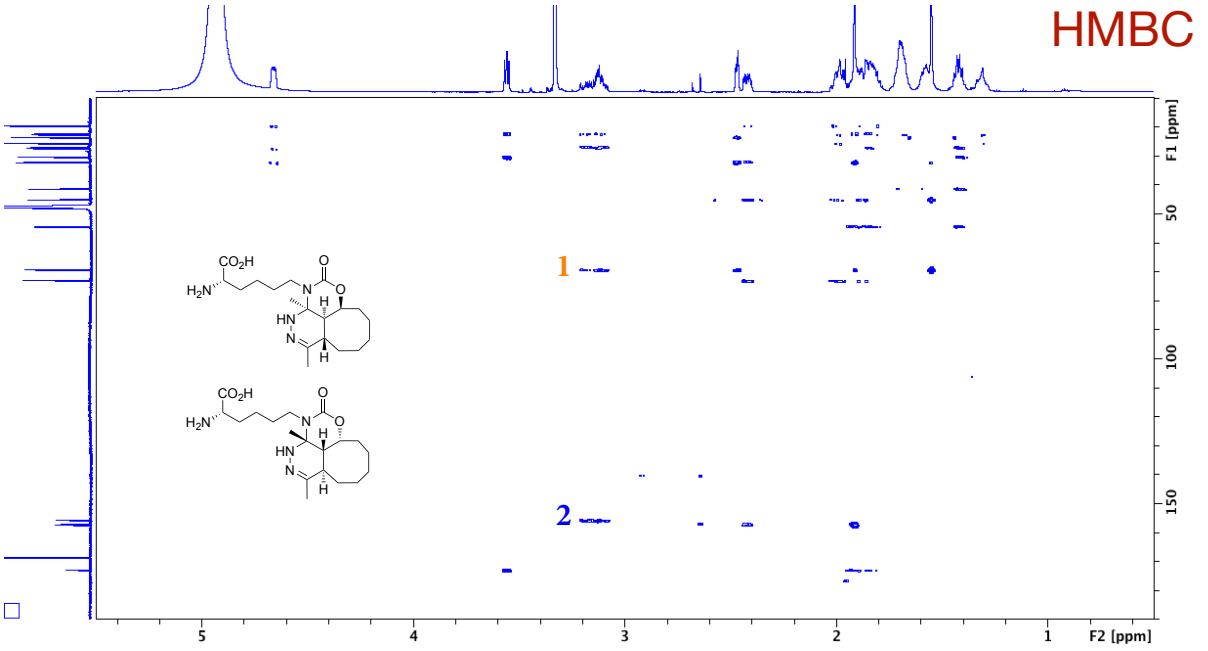


N-ε-rTCO-Lys + DMT dead end isomer

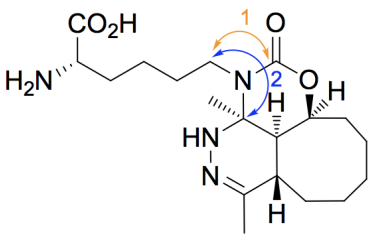
HSQC



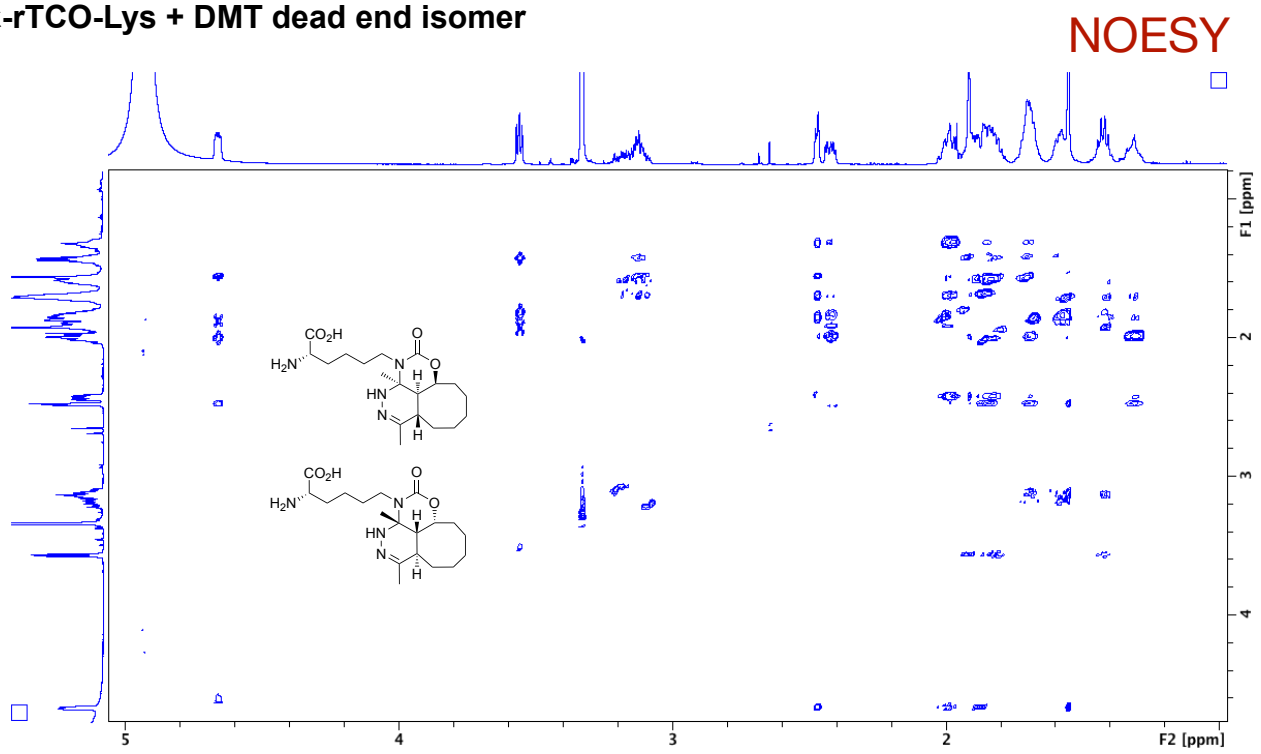
HMBC

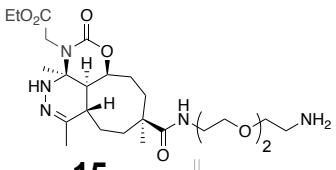


HMBC



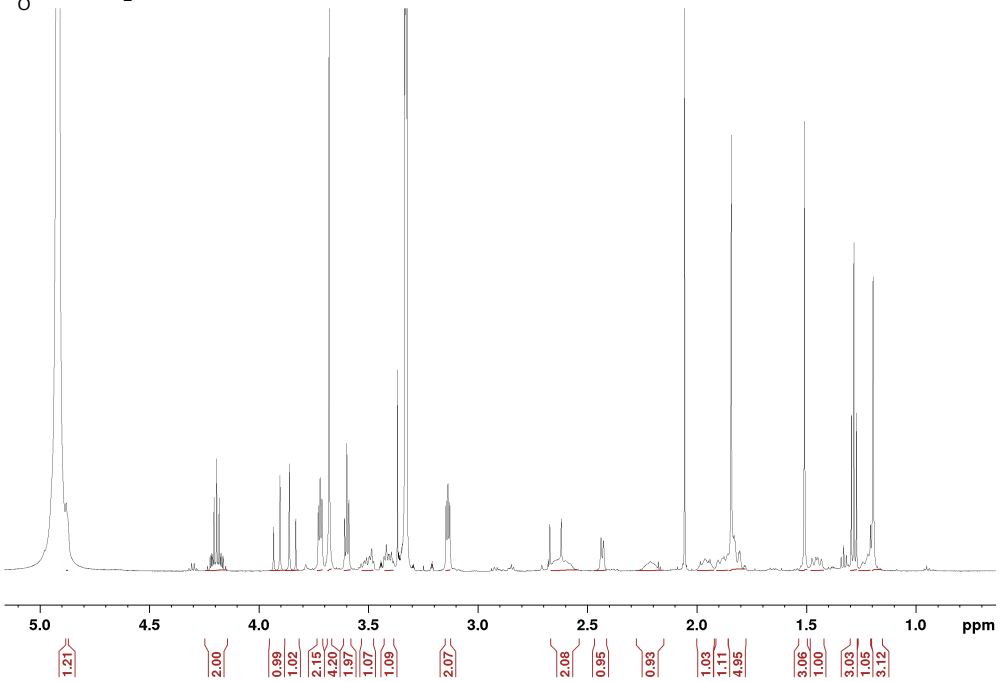
N-ε-rTCO-Lys + DMT dead end isomer

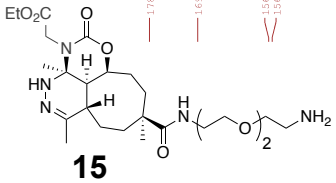




15

^{1}H


5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm
1.21
2.00
0.99
1.02
2.15
4.20
1.97
1.07
1.09
2.07
2.08
0.95
0.93
1.03
1.11
4.95
3.06
1.00
3.03
1.05
3.12

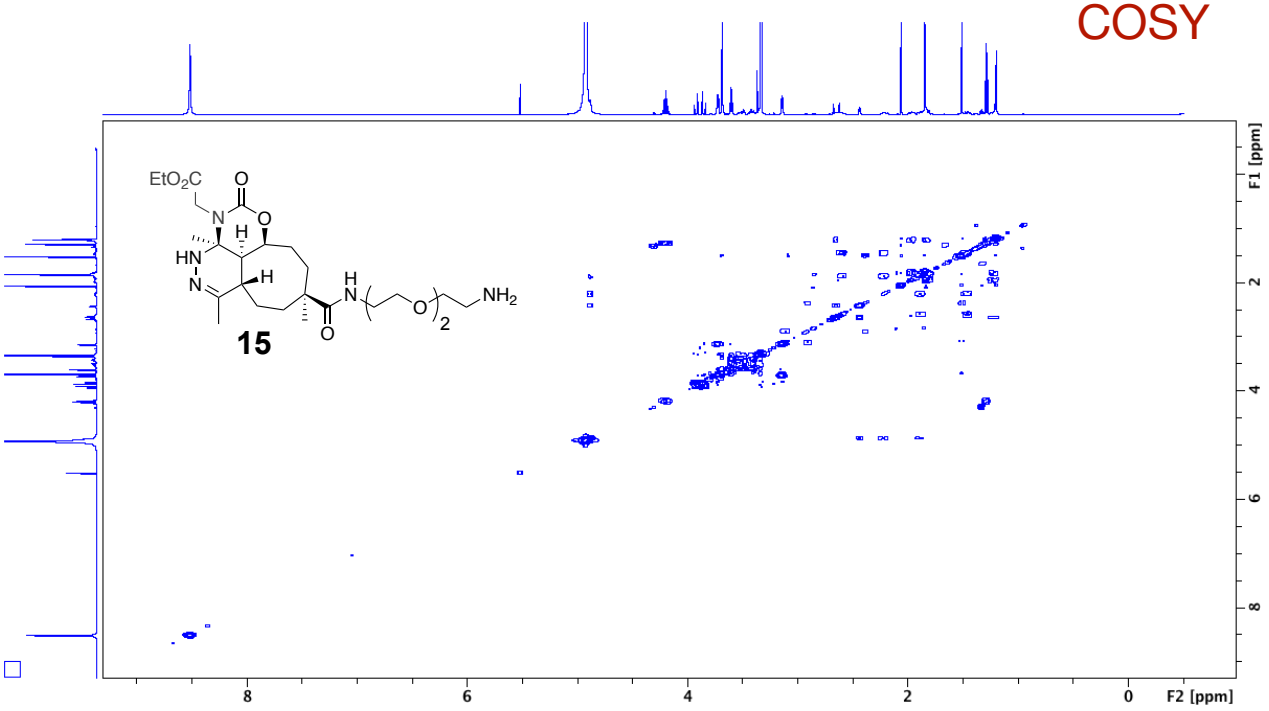


15

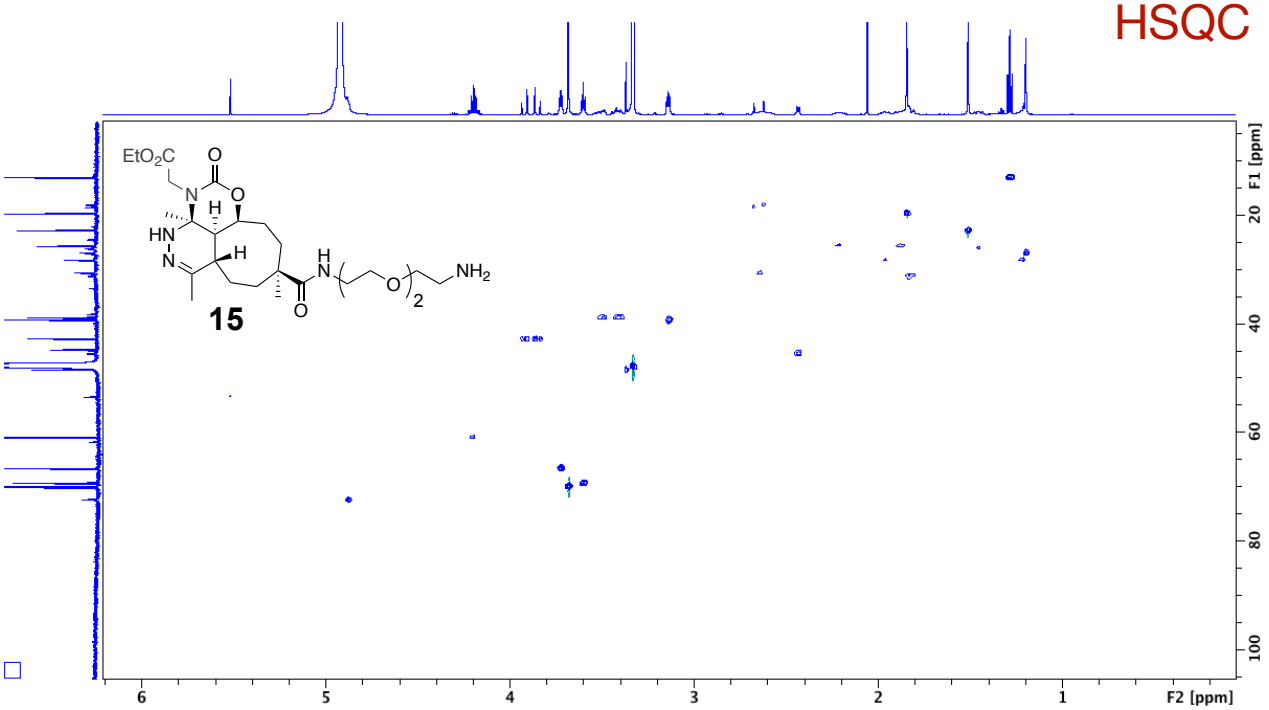
^{13}C

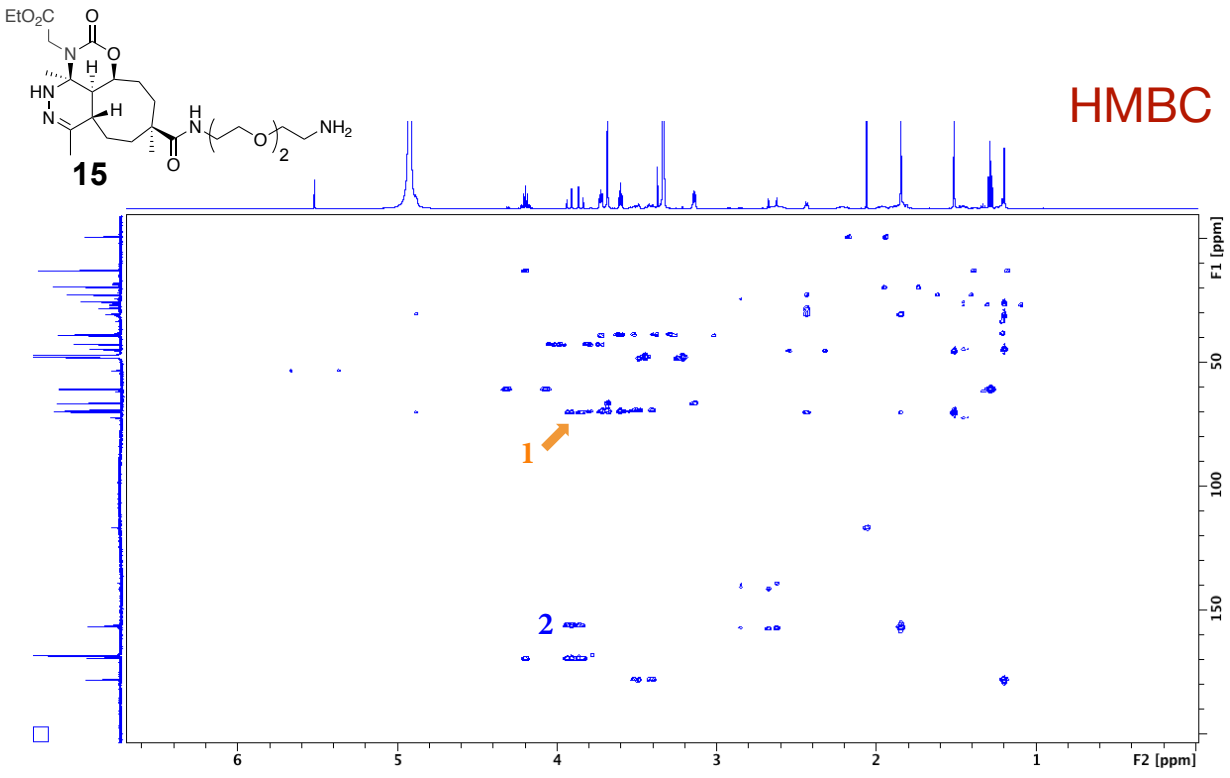
178.07
169.45
156.74
156.08
72.36
70.19
69.98
69.84
69.32
66.59
60.89
45.47
44.72
42.76
39.24
38.86
31.23
30.61
28.26
26.91
26.14
25.64
23.78
19.68
13.06
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm

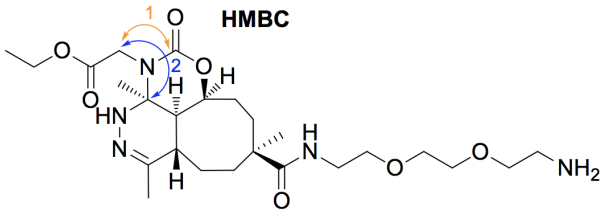
COSY


EtO2C
O
N
H
O
HN
N
H
H
N
O
O
2
NH2
15
F1 [ppm]
2
4
6
8
8
6
4
2
0
F2 [ppm]

HSQC

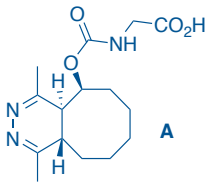

EtO2C
O
N
H
O
HN
N
H
H
N
O
O
2
NH2
15
F1 [ppm]
20
40
60
80
100
6
5
4
3
2
1
F2 [ppm]


15
HMBC
1
2
F1 [ppm]
F2 [ppm]
6
5
4
3
2
1
50
100
150


HMBC
1
2

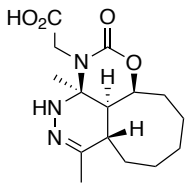
10. XYZ coordinates of optimized geometries calculated using GAUSSIAN 09[6]

Method: M06-2X/6-311+G(d,p)



A

N	0.2884550	1.3969130	1.6430620
N	0.0116910	2.3289790	0.6116680
C	-1.0293640	2.1891580	-0.1128390
C	-1.8929850	0.9504440	-0.1903190
C	-1.3498450	-0.1918680	0.6821660
C	-0.3137690	0.2751300	1.6827780
C	-3.4018700	1.1663880	-0.0187090
C	-4.2628700	0.0465810	-0.6310600
C	-4.1652690	-1.3504000	0.0161310
C	-3.1586150	-2.3660410	-0.5844570
C	-1.8319770	-1.8770140	-1.1824250
C	-0.8107490	-1.3332880	-0.1930870
C	-0.0108720	-0.6380020	2.8346440
C	-1.3362560	3.3000000	-1.0781000
O	0.2711300	-0.7844700	-0.9780710
C	1.4768030	-0.7320080	-0.3766230
O	1.8523690	-1.5345320	0.4602390
N	2.2227480	0.2956780	-0.8292000
C	3.4053400	0.7194030	-0.0879230
C	4.6467120	-0.1193960	-0.4006110
O	5.6586390	0.3679890	-0.8121600
O	4.5389920	-1.4279600	-0.1598630
H	-1.7466450	0.6717630	-1.2411030
H	-2.1523460	-0.6205480	1.2847510
H	-3.6349520	1.2715920	1.0474480
H	-3.6916560	2.1057820	-0.4970290
H	-5.3031640	0.3746380	-0.5588760
H	-4.0495590	-0.0234770	-1.7043190
H	-4.0067220	-1.2324830	1.0914160
H	-5.1471020	-1.8237390	-0.0655100
H	-3.6708490	-2.8864370	-1.3985270
H	-2.9529260	-3.1339590	0.1693540
H	-2.0242820	-1.1283880	-1.9544050
H	-1.3580240	-2.7154520	-1.6995570
H	-0.4178930	-2.1429230	0.4254130
H	-0.9215260	-0.8236230	3.4122880
H	0.3706680	-1.5978420	2.4793160
H	0.7364240	-0.1765550	3.4772050
H	-0.4855960	3.9769070	-1.1343780
H	-1.5715230	2.9089230	-2.0717840
H	-2.2048400	3.8698150	-0.7364690
H	1.7337530	0.9918670	-1.3713120
H	3.6300720	1.7468100	-0.3602740
H	3.1933240	0.6766470	0.9862610



TRICYCLIC DEAD END

H	3.6595900	-1.6430470	0.202832
N	0.8770840	0.8139520	1.6658250
N	1.1235620	1.7534150	0.6707550
C	0.2637600	1.8301730	-0.2647560
C	-1.0275910	1.0394850	-0.3040310
C	-0.9874120	-0.2452290	0.5591790
C	0.3723750	-0.4740580	1.2222380
C	-2.1953970	1.9542820	0.1227070
C	-3.6140980	1.4368110	-0.1656970
C	-4.0879870	0.2095590	0.6368510
C	-3.9151600	-1.1761650	-0.0292500
C	-2.7102310	-1.4053290	-0.9470970
C	-1.3563760	-1.4697640	-0.2603950
C	0.2645810	-1.3695400	2.4543960
C	0.5443550	2.7776350	-1.3932790
O	-0.3951750	-1.6078820	-1.3239820
C	0.9061940	-1.4415830	-1.0633080
O	1.7176970	-1.6190840	-1.9538910
N	1.2731500	-1.0994230	0.2084430
C	2.7034730	-1.1329220	0.4990140
C	3.4953550	0.1095960	0.0658840
O	4.1450170	0.7421790	0.8472350
O	3.4495080	0.3979960	-1.2332730
H	1.7284670	0.7305030	2.2123080
H	-1.1814700	0.7665650	-1.3537030
H	-1.6937280	-0.1485330	1.3812190
H	-2.0906790	2.1593140	1.1948470
H	-2.0873250	2.9156390	-0.3858830
H	-4.2912160	2.2647860	0.0609070
H	-3.7272810	1.2522290	-1.2398970
H	-3.6238140	0.2295250	1.6264510
H	-5.1573740	0.3236470	0.8315500
H	-4.7993330	-1.3577380	-0.6466280
H	-3.9365380	-1.9487000	0.7478000
H	-2.6718200	-0.6352850	-1.7209250
H	-2.8450000	-2.3517490	-1.4770330
H	-1.3033920	-2.3710290	0.3607590
H	-0.4028320	-0.9059110	3.1813710
H	-0.1137130	-2.3566450	2.1896940
H	1.2374890	-1.5019850	2.9318250
H	1.4419450	3.3520100	-1.1713850
H	0.7094950	2.2113930	-2.3154050
H	-0.2924120	3.4577150	-1.5713710
H	2.8499210	-1.2413640	1.5712690
H	3.1340800	-2.0052910	0.0029940
H	2.9285290	-0.2780740	-1.7093670

11. References

1. Versteegen RM, Rossin R, ten Hoeve W, Janssen HM, Robillard MS. *Angewandte Chemie International Edition* 2013, **52**(52): 14112-14116.

2. Courtis AM, Santos SA, Guan Y, Hendricks JA, Ghosh B, Szantai-Kis DM, *et al.* *Bioconjugate Chemistry* 2014, **25**(6): 1043-1051.

3. Rossin R, van Duijnhoven SMJ, ten Hoeve W, Janssen HM, Kleijn LHJ, Hoeben FJM, *et al.* *Bioconjugate Chemistry* 2016, **27**(7): 1697-1706.

4. Dawson RMC, Elliott DC, Elliott WH, Jones KM. Data for Biochemical Research, 3 edn. Oxford University Press (Clarendon): Oxford, 1989.

5. Krężel A, Bal W. *Journal of Inorganic Biochemistry* 2004, **98**(1): 161-166.

6. Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, *et al.* Gaussian 09. Wallingford, CT, USA: Gaussian, Inc.; 2009.