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Bicyclo[6.1.0]nonyne carboxylic acid for the production of stable molecular probes

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Supplementary Information

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Material and methods

Experimental procedures

Unless otherwise indicated, reactions were carried out under an argon atmosphere in flame-dried glassware with magnetic stirring. Air and/or moisture-sensitive liquids were transferred *via* syringe. When required, solutions were degassed by argon bubbling through a needle. Organic solutions were concentrated by rotary evaporation at 25-80 °C and 15- 30 torr. Volume ratios are indicated when referring to mixtures of solvents (e.g. DCM/MeOH 95:5).

Materials

All reagents were obtained from commercial sources and used without prior purifications, except for Jones reagent, which was homemade following reported procedures.[1] Dry solvents were obtained from Merck. Analytical thin layer chromatography (TLC) was performed using plates cut from aluminium sheets (ALUGRAM Xtra SIL G/UV254 from Macherey-Nagel). Visualization was achieved under a 254 or 365 nm UV light and by immersion in an appropriate staining solution. Silica gel for column chromatography was purchased from Merck (Geduran® Si 60, 40-63 µm). Column flash chromatography was carried out using silica gel G-25, G-12 or G-4 (40- 63 µm) from Macherey-Nagel or Büchi. Human plasma was obtained from *Établissement Français du Sang Alsace – Lorraine – Champagne – Ardenne* (*EFS* – French Blood Agency) within the framework of a service contract for the transfer of products derived from blood or its components for non-therapeutic and research purposes, in accordance with French laws and regulations.

Instrumentation and methods associated

^{1}H and ^{13}C NMR spectra were recorded at 23 °C on Bruker Avance III - 400 MHz / 500 MHz spectrometers. Recorded shifts are reported in parts per million (δ) and calibrated using residual nondeuterated solvent. Data are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, app = apparent), coupling constant (*J*, Hz), integration and assignment for ^{1}H NMR data.

Analytical LC-MS analyses were carried out on Waters 2695 separations module equipped with Waters 2487 UV detector, Waters Acquity QDa mass detector and CORTECS, 2.7 µm, C18, 50 x 4.6 mm column. The flow rate was 1 mL/min and the solvent system was composed as follows: solvent A: 0.05% TFA in water; solvent B: acetonitrile. Unless indicated otherwise, the gradient run was 0 to 5 min. – 5% to 95% B; 5 to 6 min. – 95% B; 6 to 7 min. – 5% B. Mass detector was operated in positive MS Scan mode with 600 °C probe temperature, 1.5 kV capillary voltage and 10 V cone voltage.

High-resolution mass spectra (HRMS) were obtained using an Agilent Q-TOF (time of flight) 6520.

IR spectra were recorded in a Thermo-Nicolet FT/IR-380 spectrometer. Spectra were interpreted with OMNIC 9 software and are reporter in cm^{-1}. The abbreviations used are w (weak), m (medium), s (strong).

Melting points were carried out on a melting point apparatus SMP3 from Stuart Scientific.

Concentrations of antibody solutions in DPBS (calcium and magnesium free, Merck, Ref. D8537-6X500ML) were determined by UV absorbance using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Illkirch, France) at 280 nm.

Purification by preparative HPLC were carried out on a Waters 600 controller system (pumps: Waters Delta 600; detector: Waters 2489 UV/Vis) equipped with a SunFire™ Prep C18 OBD 5 µM 19×150 mm column (Waters), using water (0.1% TFA, solvent A) and acetonitrile (solvent B) as a solvent system with a flow rate of 17 $mL.min^{-1}$.

Analytical HPLC analyses for the calculations of second-order rate constants were carried out on a Shimadzu system (pump: LC 20<AD; detector: SPD 20<A; autosampler: SIL 20<A) using a SunFire™ C18 5 µM 4.6 × 150 mm column (Waters). The flow rate was 1 mL/min and the solvent system was composed as follows: solvent A: 0.05% TFA in water; solvent B: acetonitrile. Unless indicated otherwise, the gradient run was 0 to 5 min. – 5% to 95% B; 5 to 6 min. – 95% B; 6 to 7 min. – 5% B.

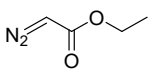
Native mass spectrometry analyses of conjugated antibodies were performed using a LCT mass spectrometer (Waters, Manchester, UK) coupled to an automated chip-based nanoESI infusion source (Triversa Nanomate, Advion, Ithaca, NY) both operating in positive ion mode. Electrospray ionization was conducted at a capillary voltage of 1.75 kV and nitrogen nanoflow of 0.75 psi. The extraction cone value was set to 50 V and the cone voltage was set to 180 V. The pressure in the interface region was

fixed at 6 mbar. Acquisitions were performed in the m/z range 1,000–10,000 with a 4 s scan time. Samples were directly infused after manual desalting step at a concentration around 10 µM. Average DoC values were calculated by using equation 1 (see below). These results were derived from the relative peak intensities measured from deconvoluted mass spectra. External calibration was performed using singly charged ions produced by a 2 g/L solution of cesium iodide in 2-propanol/water (50/50 v/v). MS data interpretations were performed using Mass Lynx V4.1 (Waters, Manchester, UK).

$$DoC = \frac{\left(\sum_{k=0}^{8} k \times intensity\ DoCk\right)}{\sum_{k=0}^{8} intensity\ DoCk} \quad (1)$$

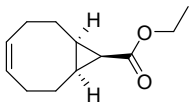
Chemical syntheses and characterizations

Ethyl 2-diazoacetate S1



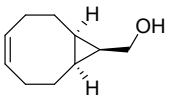
To a solution of glycine ethyl ester hydrochloride (1 equiv., 20.6 g, 147.6 mmol) in water (100 mL) and DCM (150 mL) was added dropwise a solution of $NaNO_2$ (1.3 equiv., 13.2 g, 191.9 mmol) in water (40 mL). The mixture was stirred vigorously at 15 °C for 30 minutes. The organic layer was then isolated, and the aqueous layer was acidified to pH 4.0 with an aqueous solution of $KHSO_4$ (10 %, 20 mL, 0.2 equiv., 4.0 g, 29.5 mmol). DCM (150 mL) was then added to the acidified aqueous solution, and the mixture was stirred at 15 °C for 20 minutes. Organic and aqueous layers were separated, and all organic layers were combined, washed with brine (300 mL), dried over $MgSO_4$, filtered, and concentrated *in vacuo* (water bath at 15 °C) to afford ethyl 2-diazoacetate **S1** (15.9 g, 139.4 mmol, 94%) as a volatile yellow liquid which was used immediately without further purification.

Ethyl (*Z*)-bicyclo[6.1.0]non-4-ene-9-carboxylate 4



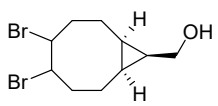
To a solution of cycloocta-1,5-diene (4 equiv., 68.31 mL, 60.11 g, 555.65 mmol) in DCM (250 mL, stabilized with ethanol) containing Rh_2(*S*-BHTL)$_4$ (0.0135 mol%, 24.6 mg, 18.8 µmol) was added a solution of ethyl 2-diazoacetate **S1** (1 equiv., 15.85 g, 138.91 mmol) in DCM (150 mL, stabilized with ethanol) with a rate of 40 mL/h at room temperature. Reaction was monitored by TLC using heptane/diethyl ether 95/5 and stained with a cerium sulfate solution (saturated $Ce(SO_4)_2$ in 15% aqueous sulfuric acid). All volatiles were then removed under vacuum and the crude was purified by silica chromatography (petroleum ether 100% then 97/3 petroleum ether/diethyl ether). Fractions containing the product were combined and solvents were removed to afford the title compound **2** (12.03 g, 61.92 mmol, 45%) as a colorless oil. NMR spectroscopy and MS analysis were found to be in agreement with previously reported characterization data.[2]

(*Z*)-Bicyclo[6.1.0]non-4-en-9-yl)methanol S2



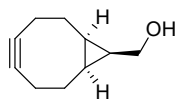
To a solution of **4** (1 equiv., 27.85 g, 133.7 mmol) in diethyl ether (250 mL) at 0 °C and under argon, was added dropwise a solution of $LiAlH_4$ (1.2 equiv., 66.85 mL, 160.4 mmol, 2.4 M in THF). The mixture was stirred 15 minutes at 0 °C, then 30 minutes at room temperature and 1 h at 45 °C until complete disappearance of starting material (reaction was monitored by TLC using heptane/diethyl ether 5/5 revealed with ceric sulfate staining solution – *vide supra*). Upon completion, the mixture was neutralized at 0 °C by the dropwise addition of water. Sodium sulfate was then added to the quenched solution and the mixture was filtered through a pad of Celite and rinced with diethyl ether (100 mL). The resulting solution was concentrated *in vacuo* to afford the title compound as a white solid, which was used in the next step without further purification.

(4,5-Dibromobicyclo[6.1.0]nonan-9-yl)methanol 5



To a solution of **S2** (1 equiv., 10.5 g, 68.97 mmol) in $CHCl_3$ (90 mL) at 0°C was added bromine (1 equiv., 3.54 mL, 11.02 g, 68.97 mmol) dropwise. The reaction mixture was then stirred at 0 °C for 15 min before it was quenched with an aqueous solution of $Na_2S_2O_3$ (10%, 50 mL). The layers were separated and the aqueous layer was extracted twice with chloroform (2 x 50 mL). Organic layers were then combined, washed with an aqueous solution of $Na_2S_2O_3$ (10%, 100 mL) and brine (100 mL), dried over $MgSO_4$, filtered through a pad of Celite, and concentrated *in vacuo* to afford the title compound as a white solid, which was used without further purification.

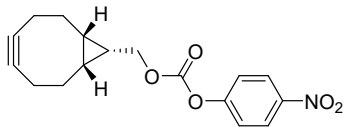
(Bicyclo[6.1.0]non-4-yn-9-yl)methanol 1



Freshly sublimated potassium *tert*-butoxide (6.0 equiv., 32.6 g, 290.3 mmol) was added to degassed 4-methyl-tetrahydropyran (200 mL) and the mixture was stirred for 15 min at room temperature. A solution of **5** (1.0 equiv., 15.1 g, 48.39 mmol) in degassed 4-methyl-tetrahydropyran (100 mL) was then added dropwise and the solution was stirred at 25 °C for 5 h (reaction monitored by TLC, petroleum ether/diethyl ether 50/50, stained with PMA and $KMnO_4$) before being concentrated to dryness. The crude was suspended in an aqueous

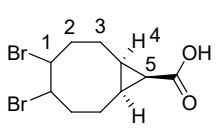
solution of $KHSO_4$ (10%, 20 mL) before diethyl ether (50 mL) was added. The layers were separated and the organic layer washed successively with an aqueous solution of $KHSO_4$ (10%, 3 x 30 mL) and brine (50 mL). The organic layer was then dried over $MgSO_4$, filtered through a pad of Celite, and concentrated *vacuo*. The crude was then purified by flash chromatography (petroleum ether/diethyl ether 50/50) to afford the title compound (5.1 g, 33.87 mmol, 70%) as a white solid. NMR and MS analysis were found to be in agreement with previously reported characterization data.[3]

(Bicyclo[6.1.0]non-4-yn-9-yl)methyl (4-nitrophenyl) carbonate 6



To a solution of BCN **1** (1 equiv., 800.0 mg, 5.33 mmol) in DCM (20 mL) was added *para*-nitrophenol chloroformate (1.2 equiv., 1.29 g, 6.40 mmol) and pyridine (10 equiv ., 4.22 g, 4.31 mL, 53.3 mmol). The reaction was stirred at room temperature for 5 h under a nitrogen atmosphere (reaction monitored by TLC; cHex/EtOAc 1:1). The mixture was quenched with a saturated solution of ammonium chloride (75 mL) and extracted with EtOAc (3 x 75 mL). The organic layers were combined, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The resulting crude was purified by flash chromatography (cHex to cHex/EtOAc 7:3 over 30 min) to afford the title compound (1.58 g, 5.01 mmol, 94%) as a white solid. NMR and MS analysis were found to be in agreement with previously reported characterization data.[4]

4,5-Dibromobicyclo[6.1.0]nonane-9-carboxylic acid 7



To a solution of **5** (1 equiv., 5.0 g, 15.9 mmol) in acetone (200 mL) at 0 °C was added Jones reagent (3.9 equiv., 2.5 M, 24.8 mL, 62.01 mmol) dropwise. The mixture was stirred for 20 min at 0 °C then slowly warmed to room temperature and stirred for 3 h (reaction monitored by TLC; cHex/EtOAc 60:40, stained with ceric sulfate and green bromocresol). Upon completion, the reaction was carefully quenched by the slow addition of isopropanol (20 mL) at 0 °C. Acetone was removed *in vacuo*, and the mixture was extracted with DCM (3 x 30 mL). Organic layers were combined, washed with brine (50 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo* to give the title compound (4.6 g, 14.1 mmol, 89%) as a white solid, which was used without further purification.

R_f = 0.45 (cHex/EtOAc 6:4)

ν_{max} (thin film) /cm^{-1}: 2928 (w), 2876 (w), 2687 (w), 2355 (w), 1683 (s), 1472 (s), 1445 (s), 1385 (s), 1354 (m), 1339 (w), 1318 (w), 1299 (w), 1267 (m), 1216 (s), 1166 (m), 1096 (w)

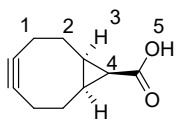
mp: 197 °C

^{1}H NMR (400 MHz, $CDCl_3$): δ 10.89 (s, 1H, OH), 4.84 (m, 1H, H1), 4.77 (m, 1H, H1), 2.81 – 2.63 (m, 2H, H2), 2.37 – 2.14 (m, 4H, H2 + H3), 1.94 – 1.72 (m, 3H, H3 + H4), 1.76 – 1.53 (m, 2H, H4 + H5)

^{13}C NMR (101 MHz, $CDCl_3$): δ 177.5, 55.5, 53.9, 34.7, 34.7, 26.5, 23.9, 22.1, 19.1, 18.0

HR-ESI-MS (M+H$^+$): Calc. 323.9361, found 323.9366

Bicyclo[6.1.0]non-4-yne-9-carboxylic acid 3

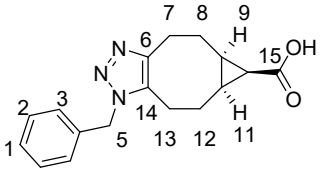


To a solution of freshly sublimated potassium *tert*-butoxide (8.0 equiv., 13.76 g, 122.68 mmol) in dry THF (100 mL) was added a solution of **7** (1.0 equiv., 5.0 g, 15.34 mmol) in dry THF (100 mL) dropwise. The reaction mixture was stirred at 25 °C for 16 h (reaction monitored by TLC; pet. ether/diethyl ether 50:50, stained with ceric sulfate) before it was quenched with glacial acetic acid (16.0 equiv., 14.15 mL, 245.36 mmol). The resulting suspension was filtered and the solid was washed thoroughly with diethyl ether (200 mL). The filtrate was concentrated under reduced pressure and the crude was then purified by flash chromatography (cHex/EtOAc 1:1). Fractions containing the product were combined and concentrated under reduced pressure to afford a yellow solid which was precipitated in heptane (see detailed procedure below) to afford the title compound **3** (1.50 g, 9.13 mmol, 60%) as a white solid, which can be stored at -20 °C under argon indefinitely.

Note – Procedure for precipitation in heptane. The yellow solid obtained after purification by flash chromatography was first dissolved in a minimal amount of DCM, before four volumes of heptane were added. The resulting solution was then concentrated under reduced pressure, with the flask outside of the bath of the rotary evaporator. Upon complete evaporation of DCM, the resulting suspension was filtered and the white solid was rinsed three times with heptane.

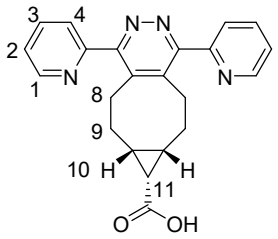
R_f = 0.40 (cHex/EtOAc 6:4)
mp: 197 °C
^{1}H NMR (400 MHz, $CDCl_3$): δ 2.37-2.18 (6H, m, 2 H1 + 4 H2), 2.16-2.04 (2H, m, H1), 1.90 (t, *J* = 8.9 Hz, H4), 1.43-1.37 (2H, m, H3)
^{13}C NMR (101 MHz, $CDCl_3$): δ 179.1, 98.6, 27.7, 26.3, 21.4, 21.1
HR-ESI-MS (M+H$^+$): Calc. 164.0840, Found. 164.0837

12-Benzyl-10,11,12-triazatricyclo[7.3.0.0^{4,6}]dodeca-1(9),10-diene-5-carboxylic acid 8



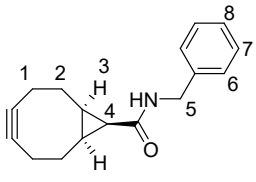
To a solution of **3** (1.0 equiv., 10.0 mg, 60.9 µmol) in DMF (0.5 mL) was added benzyl azide (2.0 equiv., 16.2 mg, 121.8 µmol) and the reaction was stirred at room temperature for 3 hours (reaction monitored by LC-MS). After concentration, the crude was dissolved in MeOH (4.0 mL) and directly purified by preparative HPLC (solvent A/solvent B 95:5 to 5:95 over 20 min). Fractions containing the product were combined and lyophilized to afford the title compound as a white solid (17.0 mg, 57.2 µmol, 94%).
mp: 222 °C
^{1}H NMR (400 MHz, $CDCl_3$): δ 9.72 (1H, s, OH), 7.40-7.33 (3H, m, H1 + H2), 7.17-7.14 (2H, m, H3), 5.57 (1H, d, *J* = 15.6 Hz, H5), 5.51 (1H, d, *J* = 15.6 Hz, H5), 3.23 (1H, ddd, *J* = 11.5, 7.0, 4.6 Hz, H13), 3.04 (1H, ddd, *J* = 14.0, 9.2, 4.6 Hz, H13), 2.91 (1H, ddd, *J* = 10.6, 6.9, 3.7 Hz, H7), 2.66-2.58 (1H, m, H7), 2.27-2.11 (3H, m, 2 H12 + 1 H8), 1.88-1.81 (m, 1H, H8), 1.79 (1H, t, *J* = 8.8 Hz, H10), 1.50-1.42 (1H, m, H11), 1.29-1.20 (1H, m, H9)
^{13}C NMR (101 MHz, $CDCl_3$): δ 141.5,[a] 132.9,[a] 131.9,[a] 129.3, 129.0, 127.2, 53.0,[b] 24.5, 24.0, 22.6, 21.0, 20.2 (2C). C15 could not be detected
[a]: determined by ^{1}H-^{13}C HMBC; [b]: determined by ^{1}H-^{13}C HSQC
HR-ESI-MS (M+H$^+$): Calc. 298.1550, Found. 298.1565

1,4-Di(pyridin-2-yl)-6,6a,7,7a,8,9-hexahydro-5H-cyclopropa[5,6]cycloocta[1,2-d]pyridazine-7-carboxylic acid 9



To a solution of **3** (1.0 equiv., 50.0 mg, 0.30 mmol) in acetonitrile (5.0 mL) was added 3,6-di-2-pyridyl-1,2,4,5-tetrazine (1.2 equiv., 82.3 mg, 0.37 mmol) and the reaction was stirred at room temperature for 30 min. After concentration, the crude solid was triturated in acetone (20.0 mL) and filtered to afford the title compound as a white solid (105.0 mg, 0.28 mmol, 92%).
mp: >250 °C
^{1}H NMR (500 MHz, 373 K, DMSO-d6): δ 11.51 (1H, s, OH), 8.75 (2H, br d, *J* = 4.8 Hz, H1), 8.01 (2H, td, *J* = 7.8, 1.5 Hz, H3), 7.88 (2H, br d, *J* = 7.8 Hz, H4), 7.52 (2H, ddd, *J* = 7.8, 4.8, 0.8 Hz, H2), 3.05 (2H, dt, *J* = 14.4, 7.4 Hz, H8), 2.94 (2H, dt, *J* = 14.4, 6.2 Hz, H8), 2.34-2.22 (2H, br m, H9), 2.14-2.01 (2H, m, H9), 1.61 (1H, t, *J* = 8.6 Hz, H11), 1.42-1.31 (2H, m, H10)
^{13}C NMR (126 MHz, 373 K, DMSO-d6): δ 172.2, 158.5, 156.2, 148.1, 140.6, 136.4, 124.0, 122.9, 26.1, 22.4, 21.5, 20.9
HR-ESI-MS (M+H$^+$): Calc. 373.1659, Found. 373.1670

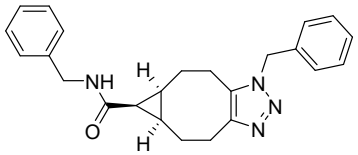
***N*- Benzylbicyclo[6.1.0]non-4-yne-9-carboxamide 10**



To a solution of **3** (1.0 equiv., 50 mg, 0.30 mmol) in DMF (2.0 mL) was added DIPEA (3.0 equiv., 116.5 mg, 151.0 µL, 0.92 mmol) and HATU (1.5 equiv., 173.5 mg, 0.46 mmol) and the solution was stirred at room temperature for 30 min before benzylamine (3.0 equiv., 98.0 mg, 100.0 µL, 0.92 mmol) was added. The mixture was stirred at room temperature for 16 h before it was concentrated to dryness. The resulting crude was dissolved in MeOH (4.0 mL) and purified by preparative HPLC (solvent A/solvent B 95:5 to 5:95 over 20 min). Fractions containing the product were combined and lyophilized to afford the title compound as a yellow solid (63.0 mg, 0.25 mmol, 83%).
mp: 214 °C
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.35-7.27 (5H, m, HAr), 6.07 (1H, br m, NH), 4.43 (2H, d, *J* = 5.8 Hz, H5), 2.47-2.35 (2H, m, H2), 2.32-2.21 (4H, m, H1), 2.14-2.05 (2H, br app dquad, *J* = 13.1, 2.6 Hz, H2), 1.64 (1H, t, *J* = 9.0 Hz, H4), 1.29-1.18 (2H, m, H3)

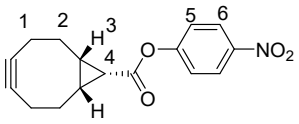
^{13}C NMR (101 MHz, $CDCl_3$): δ 171.2, 138.8, 128.7, 127.7, 127.4, 98.8, 43.5, 27.9, 24.2, 23.2, 21.3
HR-ESI-MS (M+H$^+$): Calc. 254.1539, Found. 254.1550

N,1-Dibenzyl-1,4,5,5a,6,6a,7,8-octahydrocyclopropa[5,6]cycloocta[1,2-d][1,2,3]triazole-6-carboxamide 11



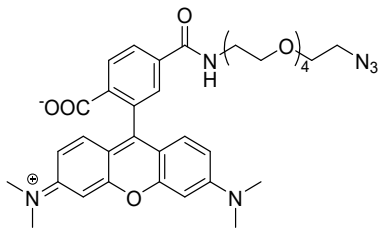
To a solution of **9** (1.0 equiv., 1.3 mg, 5.1 µmol) in DMF (0.5 mL) was added benzyl azide (2.0 equiv., 1.4 mg, 10.2 µmol) and the reaction was stirred at room temperature for 3 hours. After concentration, the crude was dissolved in MeOH (4 mL) and directly purified by preparative HPLC (solvent A/solvent B 95:5 to 5:95 over 20 min). Fractions containing the product were combined and lyophilized to afford the title compound as a white solid (1.7 mg, 4.4 µmol, 86%).
HR-ESI-MS (M+H$^+$): Calc. 387.2179, Found. 387.2192

4-Nitrophenyl bicyclo[6.1.0]non-4-yne-9-carboxylate 12



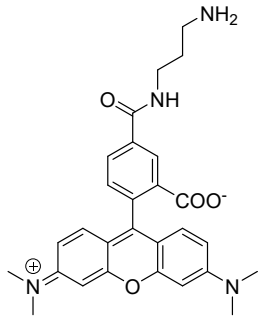
To a solution of **3** (1.0 equiv., 5.0 mg, 30.5 µmol) in DMF (0.5 mL) were added triethylamine (2.0 equiv., 6.2 mg, 8.2 µL, 61.0 µmol) and *bis*(4-nitrophenyl) carbonate (1.0 equiv., 9.3 mg, 30.5 µmol). The reaction was stirred at room temperature for 5 h (reaction monitored by LC-MS) before it was concentrated to dryness. The crude was then dissolved in DCM (5 mL) and washed with a saturated solution of ammonium chloride (5 x 10 mL). The organic layer was finally dried over $MgSO_4$, filtered and concentrated under vacuum to afford the title compound as a yellowish solid, which was used without further purification.

4-((14-Azido-3,6,9,12-tetraoxatetradecyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate (TAMRA-PEG$_4$-N$_3$) 13



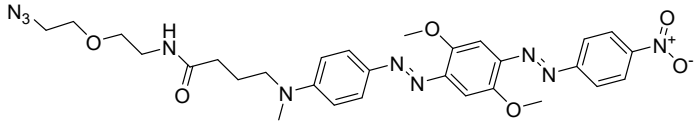
To a solution of TAMRA (1.0 equiv., 5.0 mg, 11.6 µmol) in DMF (0.2 mL) were added DIPEA (3.0 equiv., 4.5 mg, 5.9 µL, 34.8 µmol) and HATU (1.0 equiv., 4.4 mg, 11.6 µmol) and the solution was stirred at room temperature for 15 min before NH_2-PEG_4-N_3 (2.0 equiv., 6.1 mg, 23.2 µmol) was added. The resulting solution was stirred at room temperature for 6 h before it was diluted in methanol (4 mL) and purified directly by preparative HPLC (solvent A/solvent B 95:5 to 5:95 over 20 min). Fractions containing the product were combined and lyophilized to afford the title compound (6.4 mg, 9.5 µmol, 82%) as a pink oil.
HR-ESI-MS (M+2H$^+$): Calc. 338.1532, Found. 338.1526

5-((3-Aminopropyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3*H*-xanthen-9-yl)benzoate (TAMRA-NH$_2$) S3



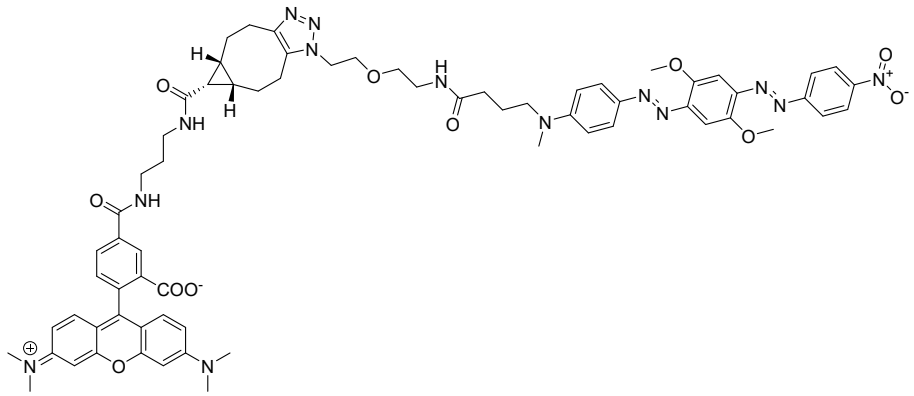
This compound was synthesized according to a previously reported procedure.[4]

_N_-(2-(2-Azidoethoxy)ethyl)-4-((4-((_E_)-(2,5-dimethoxy-4-((_E_)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)butanamide (BHQ-2-N_3) S4



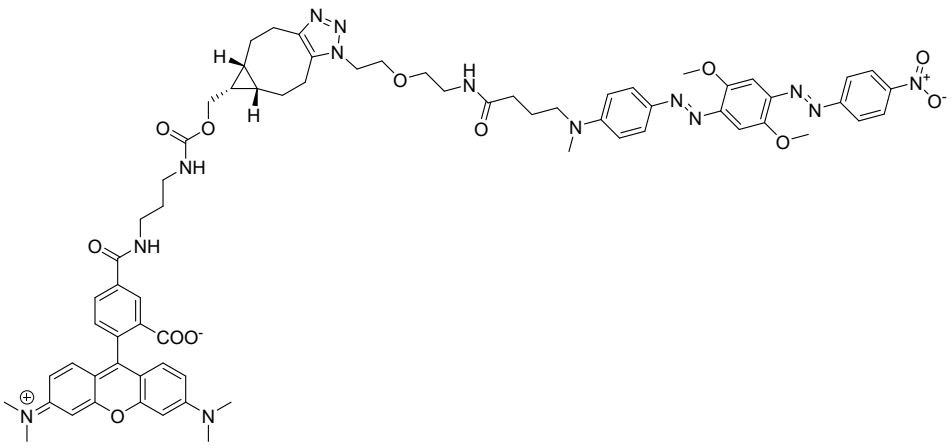
This compound was synthesized according to a previously reported procedure.[4]

5-((3-(-1-(2-(2-(4-((4-((_E_)-(2,5-dimethoxy-4-((_E_)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)butanamido)ethoxy)ethyl)-1,4,5,5a,6,6a,7,8-octahydrocyclopropa[5,6]cycloocta[1,2-d][1,2,3]triazole-6-carboxamido)propyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3_H_-xanthen-9-yl)benzoate 15



To a solution of **3** (1.0 equiv., 0.5 mg, 3.0 µmol) in DMF (0.1 mL) were added DIPEA (3.0 equiv., 1.2 mg, 1.5 µL, 9.0 µmol) and PyBOP (1.5 equiv., 2.3 mg, 4.5 µmol) and the solution was stirred at room temperature for 30 min before TAMRA-NH_2 **S3** (1.5 equiv., 2.2 mg, 4.5 µmol, 1 mM in DMSO) was added. The mixture was then stirred at room temperature for 16 h in the dark. Upon completion, BHQ-2-N_3 **S4** (1.0 equiv., 1.88 mg, 3.0 µmol, 1 mM in DMSO) was added and the solution was stirred at room temperature for 24 h in the dark. After concentration, the crude was dissolved in MeOH (4 mL) and directly purified by preparative HPLC (solvent A/solvent B 95:5 to 5:95 over 20 min). Fractions containing the product were combined and lyophilized to afford the title compound as a purple solid (1.2 mg, 0.96 µmol, 32%).
HR-ESI-MS (M+3H$^+$): Calc. 417.8627, Found. 417.8626

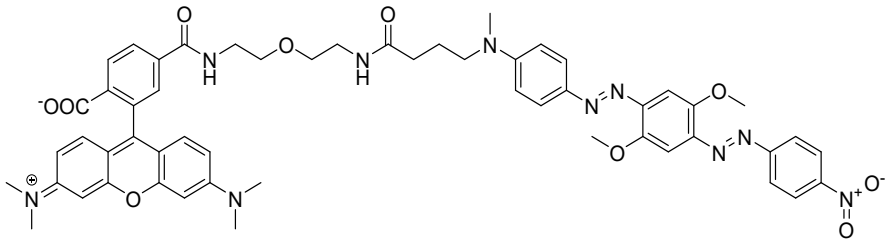
5-((3-((((-1-(2-(2-(4-((4-((_E_)-(2,5-Dimethoxy-4-((_E_)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)butanamido)ethoxy)ethyl)-1,4,5,5a,6,6a,7,8-octahydrocyclopropa[5,6]cycloocta[1,2-d][1,2,3]triazol-6-yl)methoxy)carbonyl)amino)propyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3_H_-xanthen-9-yl)benzoate 16



To a solution of TAMRA-NH_2 **S3** (1.0 equiv., 1.0 mg, 2.1 µmol, 1.0 mM in DMSO) in DMF (0.2 mL) were added triethylamine (2.0 equiv., 0.3 mg, 0.43 µL, 4.2 µmol) and activated BCN **6** (1.0 equiv., 0.7 mg, 2.1 µmol, 1 mM in DMSO) and the solution was stirred

at room temperature for 3 h in the dark. Upon completion, BHQ-2-N_3 **S4** (1.0 equiv., 1.3 mg, 2.1 µmol, 1.0 mM in DMSO) was added and the solution was stirred at room temperature for 24 h in the dark. After concentration, the crude was dissolved in MeOH (4 mL) and directly purified by preparative HPLC (solvent A/solvent B 95:5 to 5:95 over 20 min). Fractions containing the product were combined and lyophilized to afford the title compound as a purple solid (1.2 mg, 0.9 µmol, 43%).
HR-ESI-MS (M+3H$^+$): Calc. 427.8662, Found. 427.8658

4-((2-(2-(4-((4-((*E*)-(2,5-Dimethoxy-4-((*E*)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)butanamido)ethoxy)ethyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3*H*-xanthen-9-yl)benzoate 17



This compound was synthesized according to a previously reported procedure.[4]

Kinetics studies

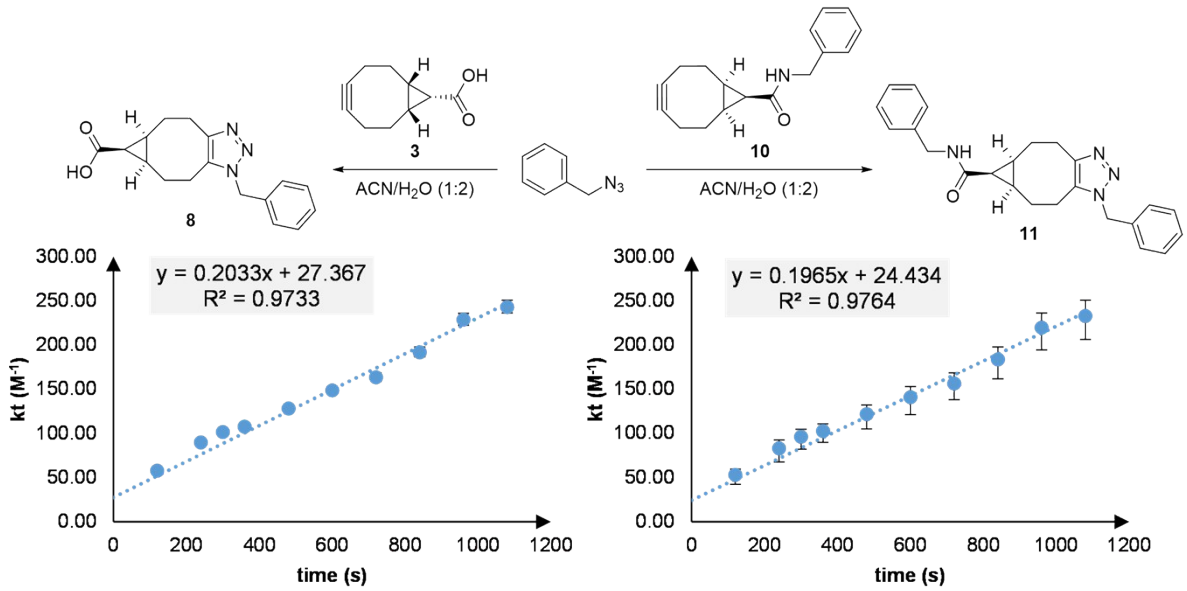


Figure S1. Kinetics studies and determination of second-order rate constants of SPAAC between benzyl azide and BCN **3** and **10**. Experiments were done in triplicates.

Kinetics studies were conducted in triplicate, following the formation of the SPAAC product by HPLC according to the following procedure. 20-mM stock solutions of BCN **3** and **10**, benzyl azide, and benzylamine (internal standard; IS) were prepared in DMSO. In an Eppendorf containing the solvent mixture (ACN/H_2O 1:2; 185 µL), 5 µL of each solution were added sequentially (starting from the IS, then BCN and finally benzyl azide), to reach a final concentration of 500 µM in all compounds. The mixture was agitated at 37 °C and 5-µL aliquots were taken every 2 minutes, diluted with water (195 µL) and analyzed by HPLC at 280 nm (solvent A: water + 0.1% TFA; solvent B: ACN; gradient: 95:5 to 5:95 over 10 min, followed by 5 min equilibration at 5:95).

For each chromatogram obtained, the area under the curve of each peak was divided by that of the IS and the resulting ratios were normalized against the value obtained at t = 0 min.

From the conversion rates thus obtained, the second order rate constants were calculated according to the following equation:

$$kt = \frac{1}{[B]_0 - [A]_0} \times \ln \frac{[A]_0([B]_0 - [P])}{([A]_0 - [P])[B]_0}$$

where k is the second order rate constant expressed in $M^{-1}.s^{-1}$; t, the reaction time in seconds; $[A]_0$, the initial concentration of BCN in $mmol.mL^{-1}$; $[B]_0$, the initial concentration of benzyl azide in $mmol.mL^{-1}$; and [P], the concentration of the triazole product in $mmol.mL^{-1}$.

Stability of FRET probes in aqueous media, human plasma and cell culture medium

Aqueous buffers were prepared as follows:

Calculated pH	Measured pH	Composition
0	<0.7	HCl 1 M
1	1.02	HCl 0.1 M
2	2.00	HCl 0.01M
3	3.03	30 mL KH phthalate standard + 10 mL HCl 0.1 M
4	4.01	KH phthalate (standard solution of pH meter)
5	5.03	0.78 mL NaH_2PO_4 0.1 M + 39.4 mL Na_2HPO_4 0.1 M
7.4	7.40	PBS
9	9.03	15 mL TRIS 4 mM adjusted to pH = 9.0

25 µL of a stock solution of probes **15** and **16** in DMSO (40 µM) were added to 975 µL of aqueous buffered media, human plasma (measured pH 7.35) or Dulbecco's Modified Eagle Medium (DMEM; pH 7.4) to reach a final concentration of 5 µM. These solutions were distributed into 96-well plates and incubated at 23 °C in a SAFAS Xenius XML with excitation/emission wavelengths set to those of TAMRA (550/580 nm). The fluorescence was measured every 3 min for 15 h and normalized against the fluorescence of a solution of TAMRA-NH_2 **S4** (5 µM) and BHQ-2-N_3 **S5** (5 µM) in the same medium. Each measurement was done in triplicate.

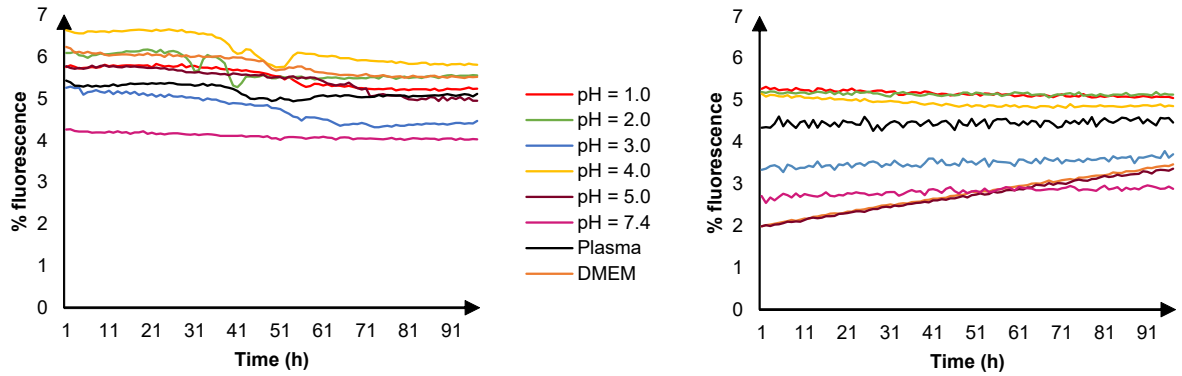


Figure S2. Stability curves of probes **15** (left) and **16** (right), measured at different pH and in different media

Bioconjugation

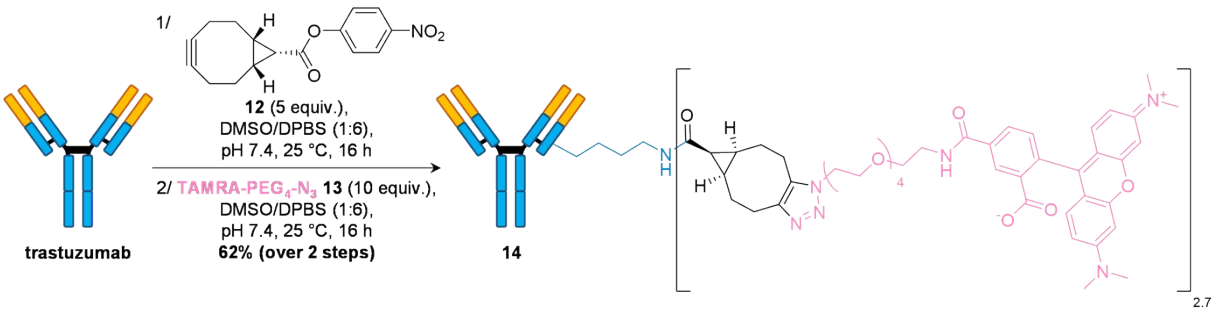


Figure S3. Synthesis of fluorescently labeled trastuzumab **14** via a 'plug-and-play' strategy.

To a solution of trastuzumab (1 equiv., 1 mg, 5 mg/mL in DPBS, pH 7.4) was added activated BCN **12** (5 equiv., 1 mM in DMSO) and the reaction mixture was incubated at 25 °C for 16 h. Excess of reagent and by-products were removed by gel filtration chromatography using Bio-spin P-30 columns (Bio-Rad, Hercules, U.S.A.) pre-equilibrated with DPBS 1X (pH 7.4) to give a solution of BCN-functionalized trastuzumab (0.91 mg, 91%, diluted to 10 mg/mL with DPBS 1X). A solution of TAMRA-PEG_4-N_3 **13** (10 equiv., 10 mM in DMSO) was then added and the mixture was incubated at 25 °C for another 16 h. Excess of reagent was removed by gel filtration chromatography using Bio-spin P-30 columns pre-equilibrated with DPBS 1X (pH 7.4) to give a solution of TAMRA-labeled trastuzumab **14** (0.62 mg, 68%), whose average degree of conjugation was determined to be 2.7 by native mass spectrometry (*vide infra*).

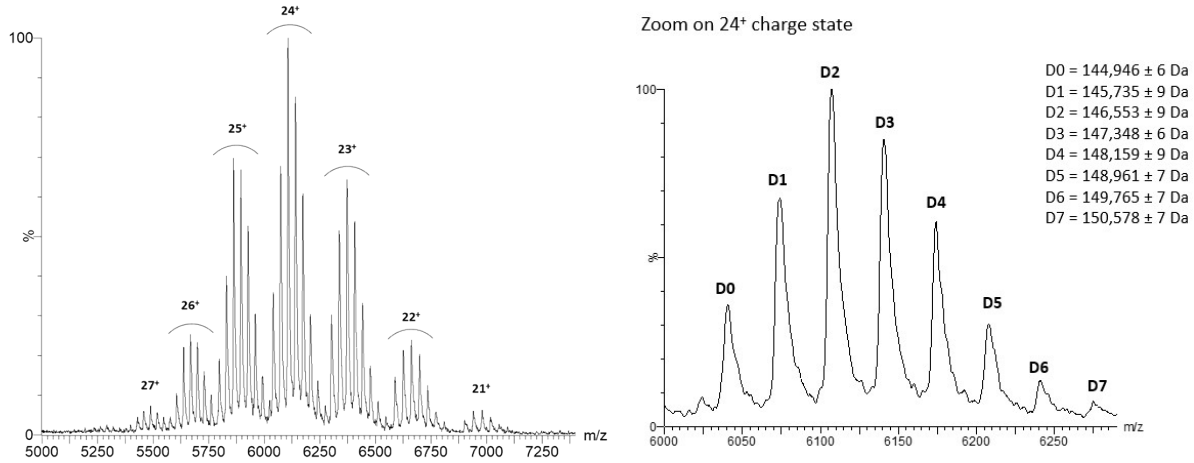


Figure S4. Native mass spectra of TAMRA-labeled trastuzumab **14** (calculated mass of payload: 820.4 Da; found: 804.6 ± 10 Da).

In vitro stability

Cell culture

Human breast adenocarcinoma SKBR3 cells (ATCC HTB-30™) were grown in Dulbecco's Modified Eagle's Medium containing 4.5 g/L glucose (Sigma-Aldrich, St. Louis, MO, USA) supplemented with fetal bovine serum (FBS) to a final concentration of 10% (Perbio, Brebieres, France), 2 mM L-glutamine, 100 U/mL penicillin and 50 µg/mL streptomycin (Merck). Cells were maintained in a 5% CO_2 humidified atmosphere at 37 °C.

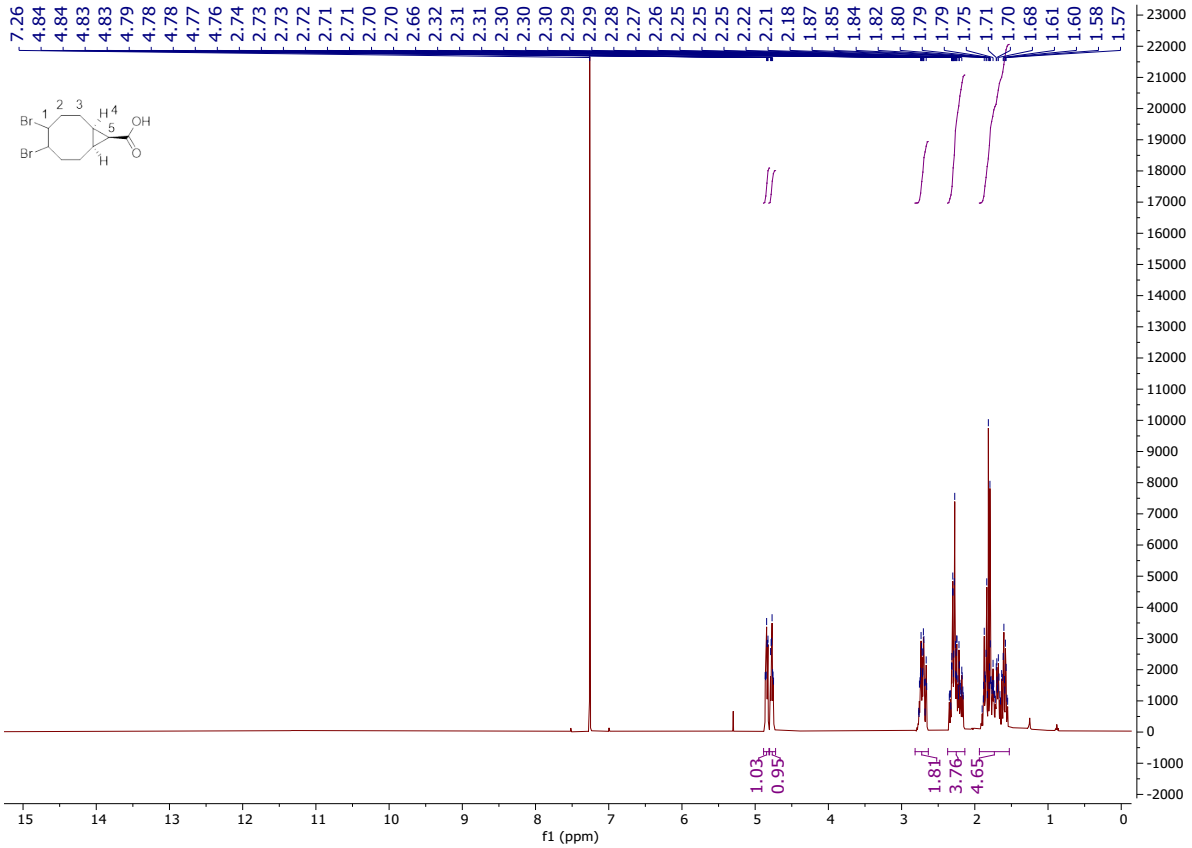
Human breast adenocarcinoma MDA-MB-231 cells (ATCC THB-26™) and Human uterus adenocarcinoma HeLa (ATCC CRM-CCL-2™) were grown in modified Eagle's Medium containing 4.5 g/L glucose (Sigma-Aldrich, St. Louis, MO, USA) supplemented with fetal bovine serum (FBS) to a final concentration of 10%, 2 mM L-glutamine, 100 U/mL penicillin and 50 µg/mL streptomycin. Cells were maintained in a 5% CO_2 humidified atmosphere at 37 °C.

Flow cytometry

Two days prior to the experiment, cells were seeded in 48-well plates (Corning Costar 3548, NY, USA): 3 x 10^4 cells per well in Dulbecco's Modified Eagle's complete Medium for SKBR3, 3 x 10^4 and 1.25 x 10^4 cells per well in Modified Eagle's complete Medium for MDA-MB-231 and HeLa, respectively.

Cells in each well were incubated with 250 µL of a 5-µM solution of fluorescent probes **15**-**17** in complete DMEM for 1, 3, 24 and 48 h at 37 °C. Upon completion, cells in each well were rinsed with 300 µL of PBS (Sigma-Aldrich, St. Louis, MO, USA), incubated with 80 µL of trypsin (0.25%, Merck) for 5 min at 37 °C and resuspended in 500 µL of a PBS solution containing 5% FBS. Cell suspensions were analyzed by flow cytometry on a Fortessa cytometer (BD Biosciences). Each compound was tested in duplicate and each experiment was done twice. Results in Figure 4 of the manuscript are presented as histograms of the mean fluorescence intensity with standard deviations corresponding to four different measurements.

NMR spectra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2.66
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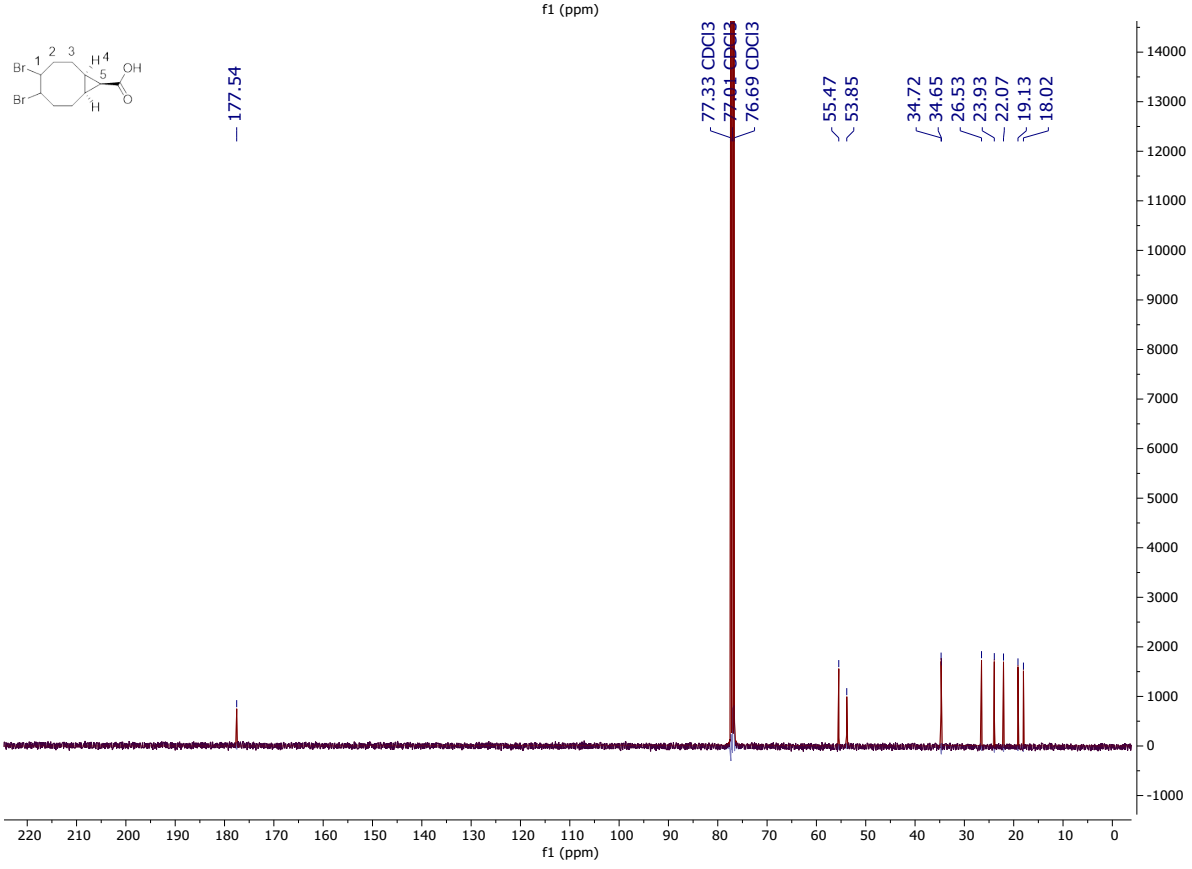
f1 (ppm)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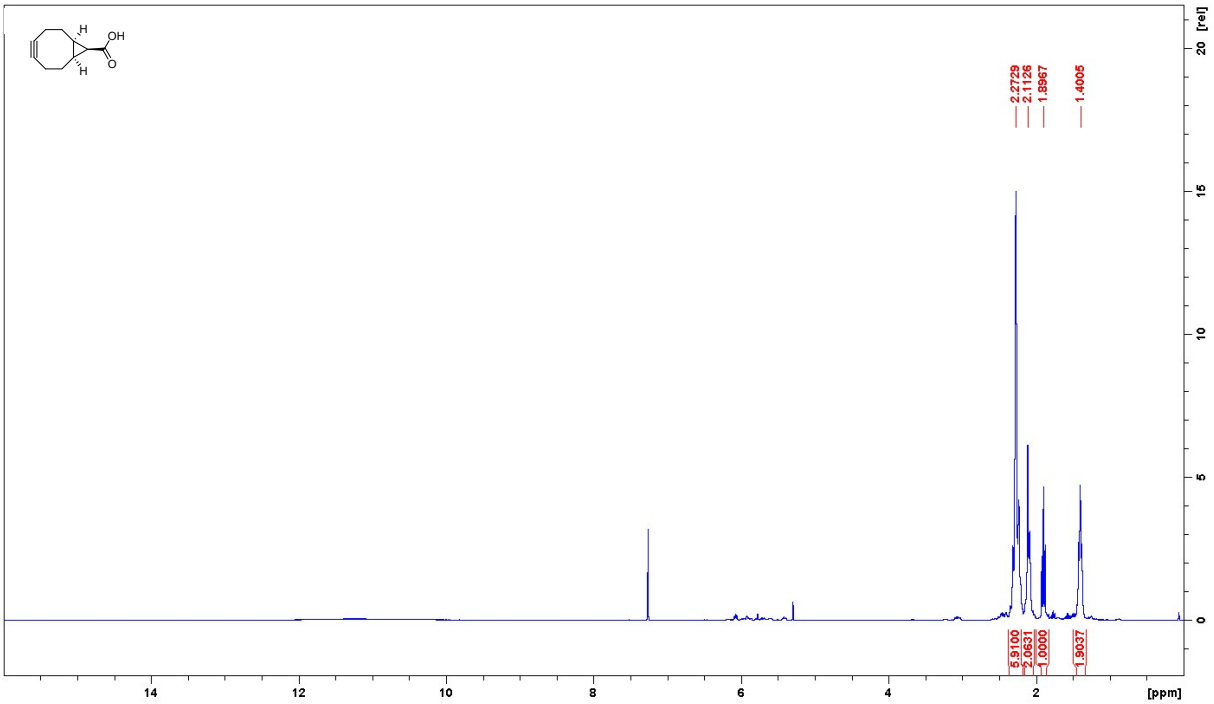
f1 (ppm)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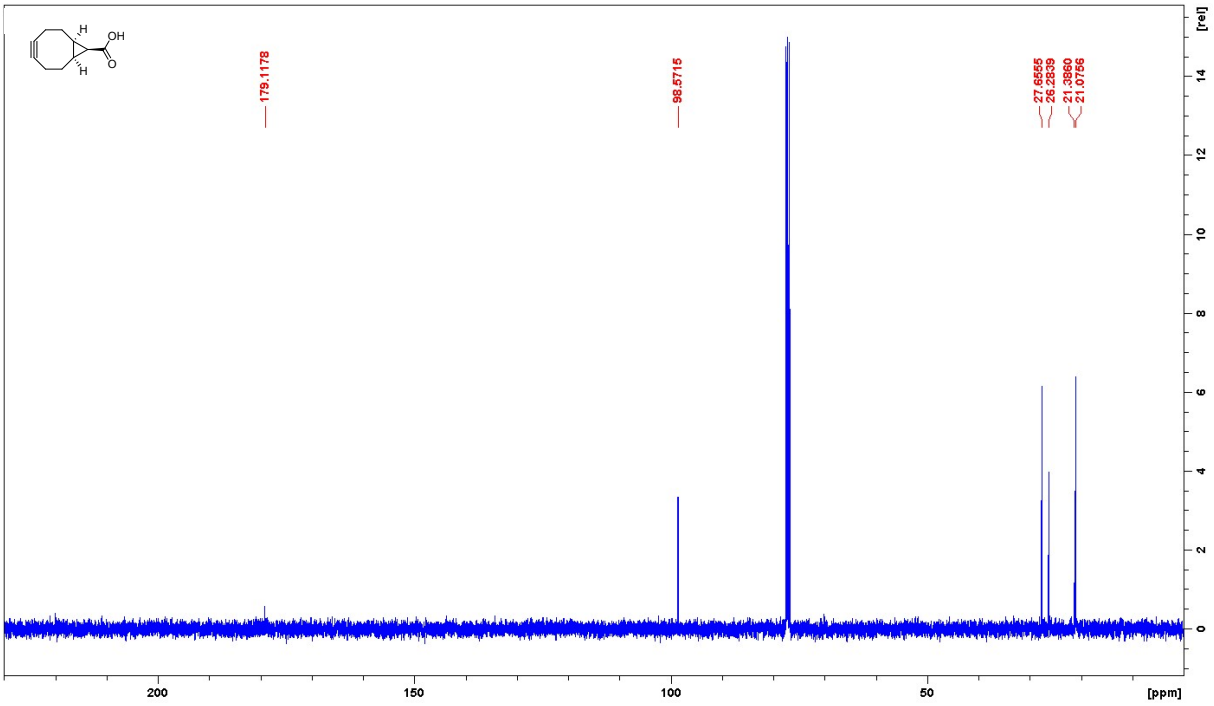
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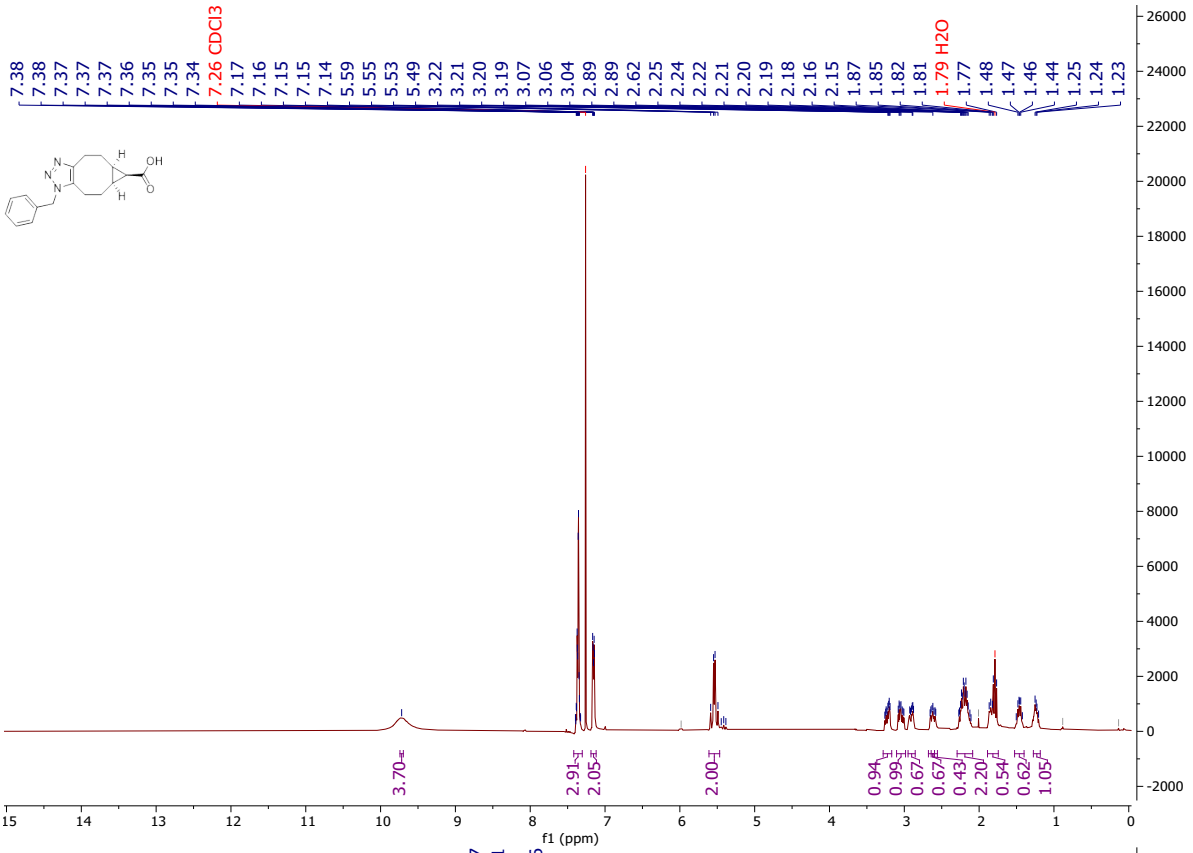
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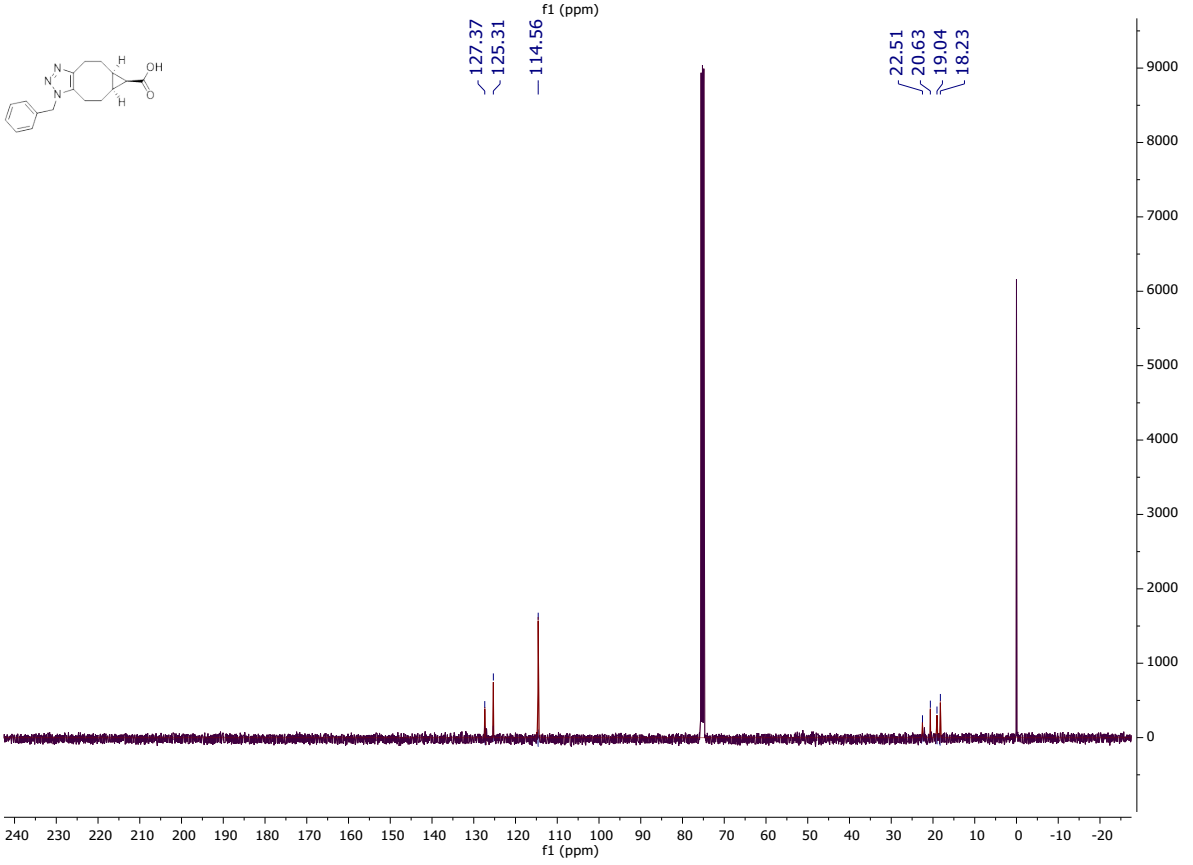
f1 (ppm)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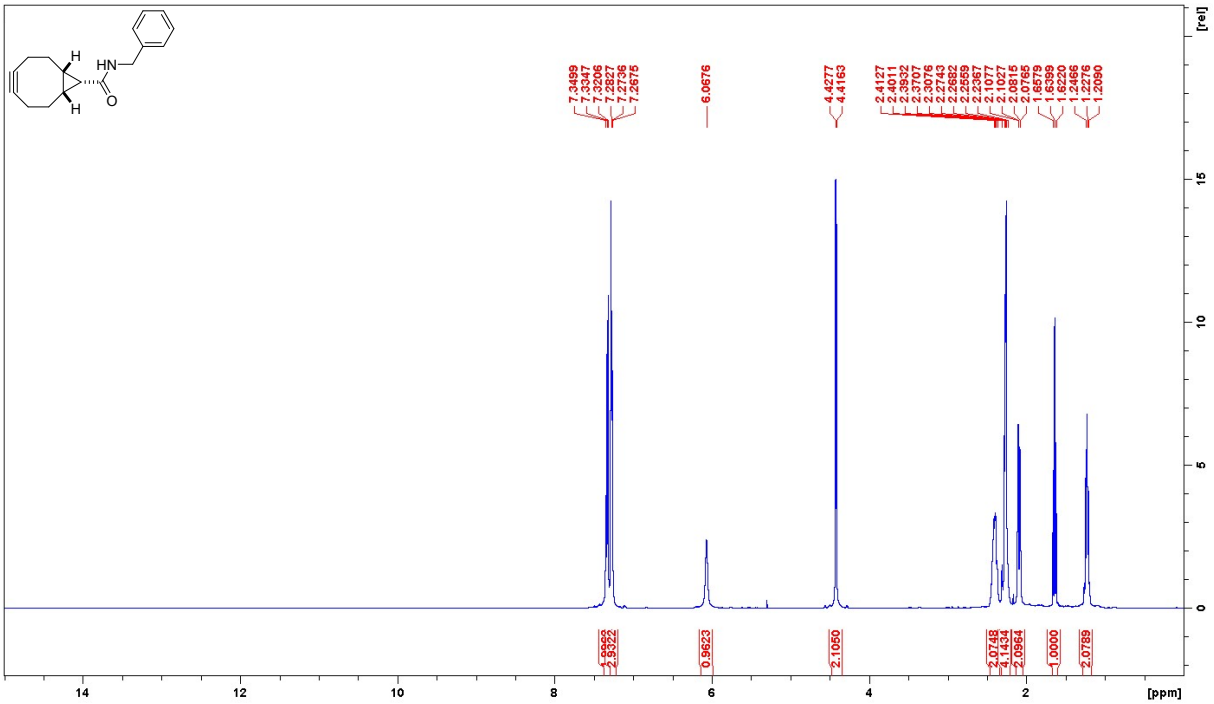
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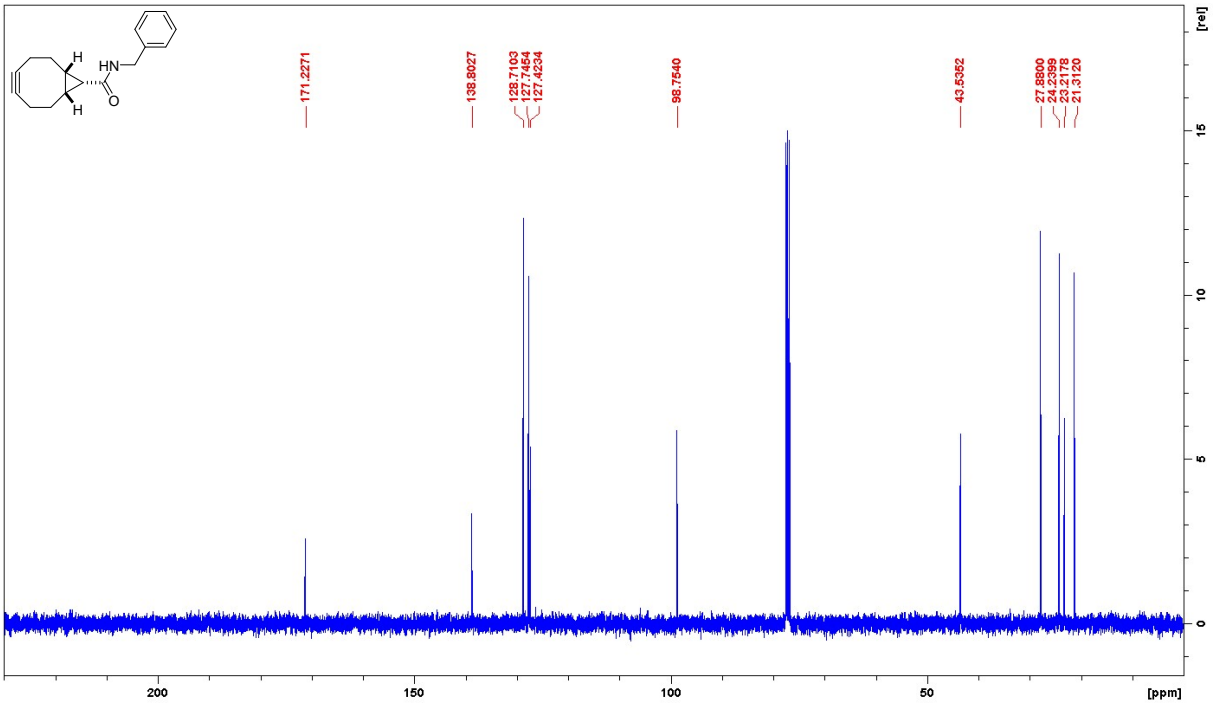
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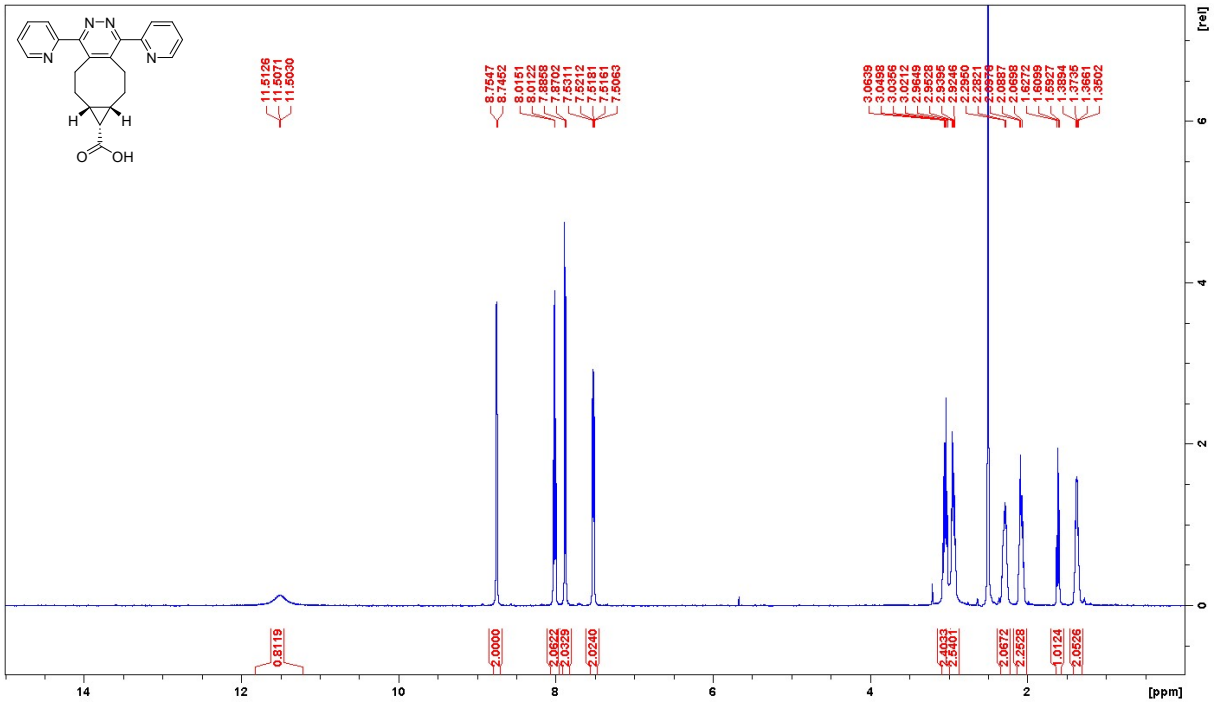
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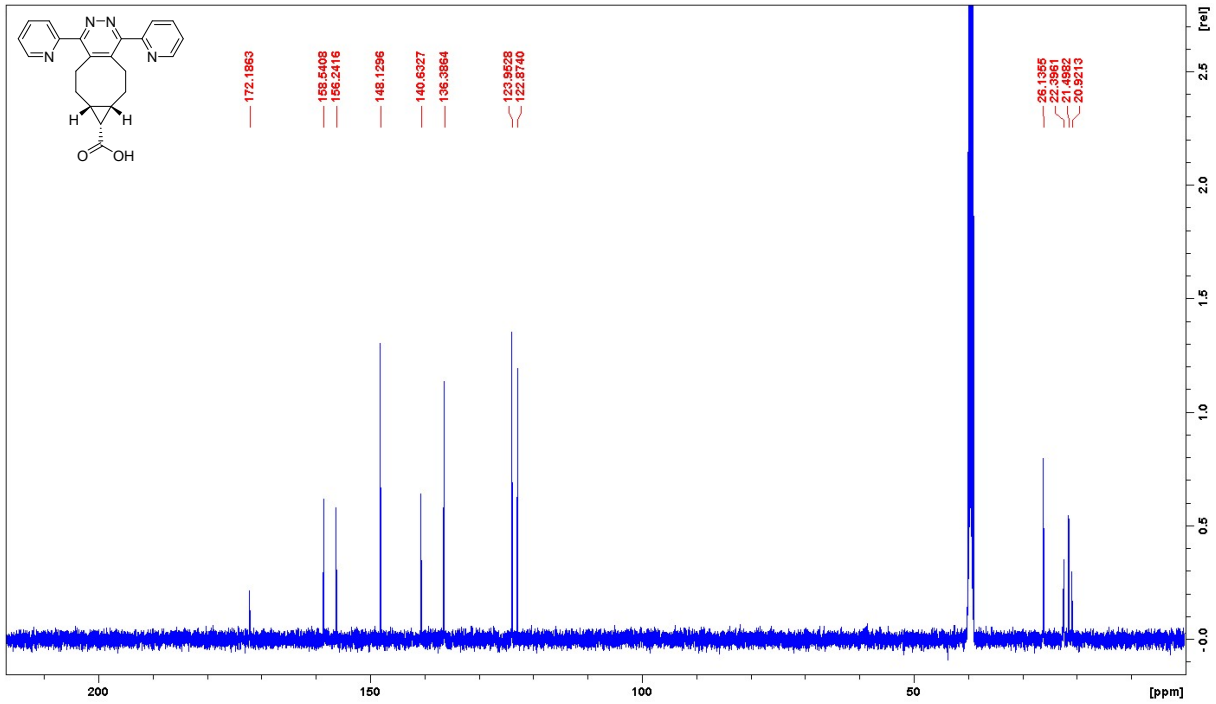
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References

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