Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

From solution to in-cell study of the chemical reactivity of acid sensitive functional groups: a guideline for rational design of cleavable linker for biospecific endosomal release

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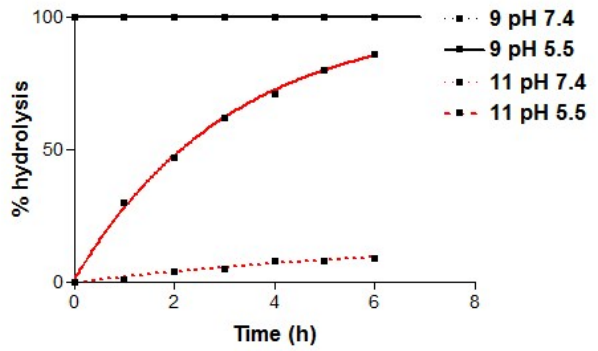


Fig. S1 Hydrolysis kinetics of compounds **9** and **11** at pH 5.5 and 7.4

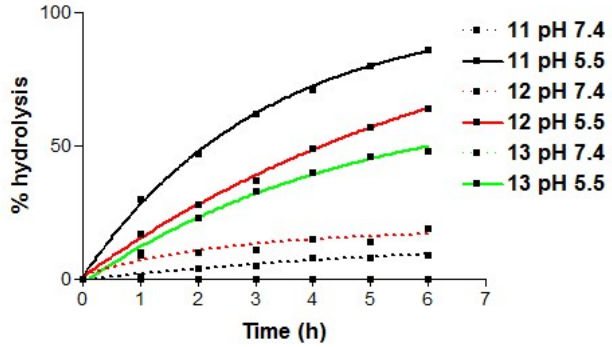


Fig. S2 Hydrolysis kinetics of compounds **11** – **13** at pH 5.5 and 7.4

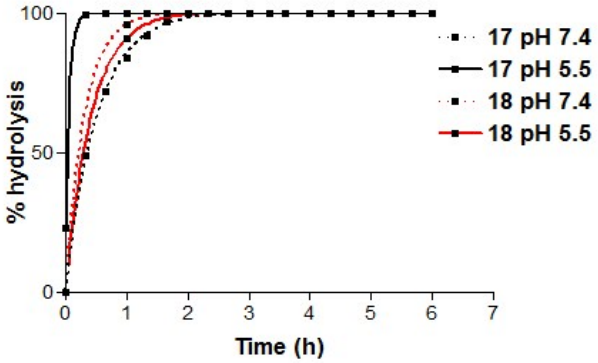


Fig. S3 Hydrolysis kinetics of compounds **17** and **18** at pH 5.5 and 7.4

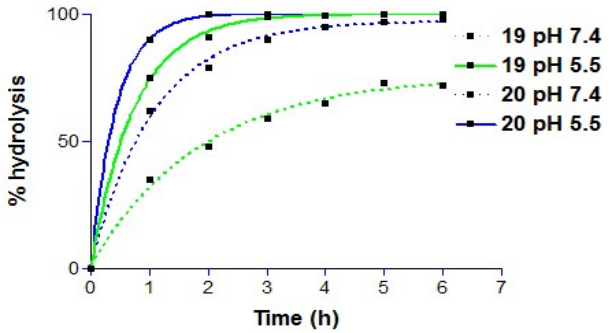


Fig. S4 Hydrolysis kinetics of compounds **19** and **20** at pH 5.5 and 7.4

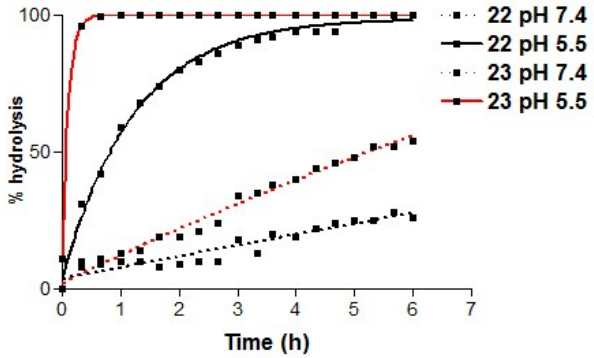


Fig. S5 Hydrolysis kinetics of compounds **22** and **23** at pH 5.5 and 7.4

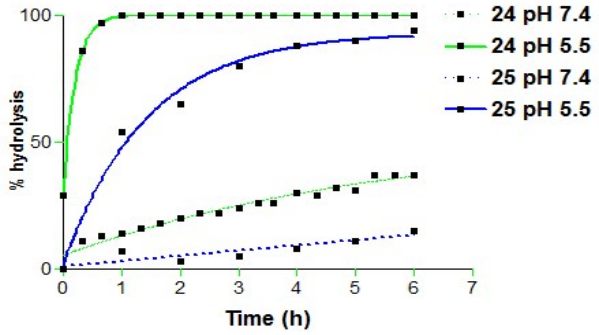


Fig. S6 Hydrolysis kinetics of compounds **24** and **25** at pH 5.5 and 7.4

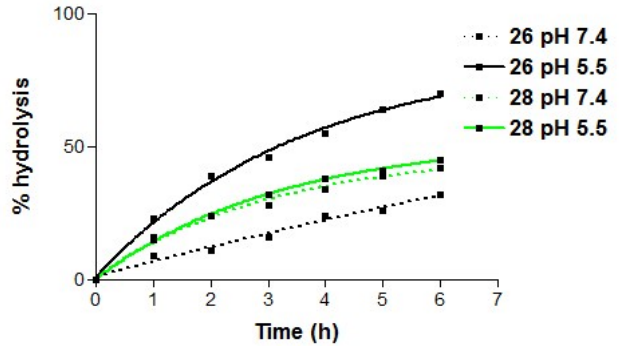


Fig. S7 Hydrolysis kinetics of compounds **26** and **28** at pH 5.5 and 7.4

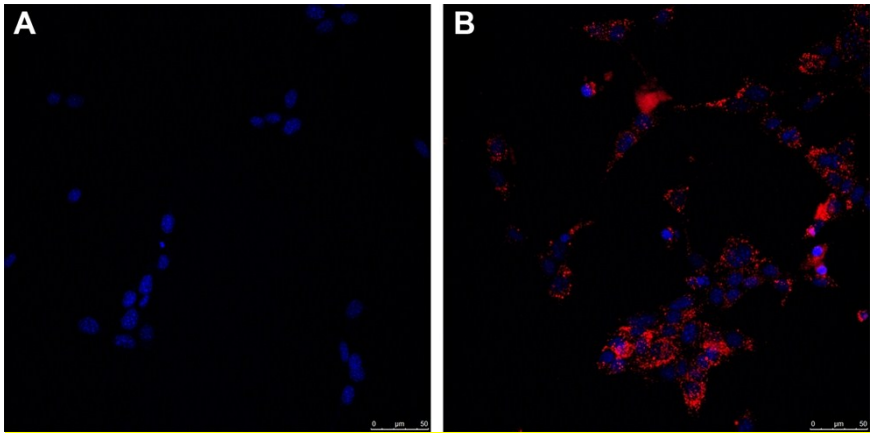


Fig. S8 In-vitro imaging of BNL CL.2 cells loaded with probes **TAMRA-Alkyne** (A) and **P5** (B); Red channel: TAMRA fluorescence (1 µM, 90 min), blue channel: Hoechst nuclei staining (5 µg/mL, 30 min); Scale bar: 50 µM.

General experimental procedures: Unless otherwise indicated, reactions were carried out under argon atmosphere in flame-dried glassware equipped with magnetic stirring. Air or moisture-sensitive liquids were transferred *via* syringe. If required, solutions were degassed by passing a stream of argon through the solutions. Organic solutions were concentrated by rotary evaporation at 25-60 °C at 15-30 torr. Analytical thin layer chromatography (TLC) was performed using plates cut from glass sheets (silica gel 60F-254 from Merck). Visualization was achieved by 254 or 365 nm UV light and by immersion in an ethanolic solution of cerium sulfate, and subsequent treatment with a heat gun. Column chromatography was carried out as "Flash Chromatography" using silica gel G-25 (40-63 µM) from Macherey-Nagel.

Materials: All reagents were obtained from commercial sources and used without further purifications. All dry solvents were obtained from Aldrich or Alfa Aesar.

Instrumentation: ^{1}H and ^{13}C NMR spectra were recorded at 23 °C on a Bruker 400 or 500 spectrometers. Recorded shifts are reported in parts per million (δ) and calibrated using residual undeuterated solvent signals. Data are represented as follows: Chemical shift, mutiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad), coupling constant (*J*, Hz) and integration. High resolution mass spectra (HRMS) were obtained using a Agilent Q-TOF (time of flight) 6520 and low resolution mass spectra using a Agilent MSD 1200 SL (ESI/APCI) with a Agilent HPLC1200 SL. GC-MS analyses were performed by means of Agilent 7890A Gas Chromatograph equipped with DB-5MS 30 m x 0.25 mm column and JEOL AccuTOF-GCv. UV-Vis spectra and kinetics were recorded on a Shimadzu UV-1800 spectrophotometer. Fluorescence kinetics were recorded on a multilabel plate reader (Victor X2, PerkinElmer) or on a Fluorolog (Jobin Yvon, Horiba) spectrofluorometer. Microwave-assisted experiments were carried out in sealed reaction vessels in a Biotage Initiator (Biotage, Sweden). Reaction times listed refer to ´hold time´ at the specified temperature. Melting points were taken on a Stuart Scientific SMP3 apparatus from Bibby and are uncorrected. IR spectra were recorded on a Nicolet 380 FT-IR spectrometer from Thermo Electron Corporation as a CH_2Cl_2 solution or solid on a diamond plate.

Analytical HPLC methods: HPLC experiments were performed on a Shimadzu system (Pump: model LC 20-AD, UV-detector: SPD 20-A, Autosampler: SIL 20-A).

Method 1 (acidic conditions): Column: Sunfire C_{18} (150 mm × 4.5 mm i.d., 5 µm, Waters). Flow: 1 mL/min. Injection volume = 10 µL. Eluant A/B water/CH_3CN, with 0.05% TFA. Gradient: 5% B to 95% B in 20 minutes and 10 minutes of re-equilibration. Detection: 254 nm.

Method 2 (basic conditions): Column: XBridge C_{18} (150 mm × 4.5 mm i.d., 5 µm, Waters). Flow: 1 mL/min. Injection volume = 10 µL. Eluant A/B NH_3HCOOH solution (10 mM, pH 8.5)/CH_3CN. Gradient: 5% B to 95% B in 20 minutes and 10 minutes of re-equilibration. Detection: 254 nm.

Semi-preparative HPLC methods: The semi-preparative HPLC system consisted of a Waters 600 pump, a 2487 detector (Waters) and a 5 mL sample loop.

Method 1 (acidic conditions): Column: Sunfire C_{18} (150 mm × 19 mm i.d., 5 µm, Waters). Flow: 17 mL/min. Injection volume = 1 mL. Eluant A/B water/CH_3CN, with 0.05% TFA. Gradient: 5% B to 95% B in 40 minutes and 10 minutes of re-equilibration. Detection: 254 nm.

Method 2 (basic conditions): Column: XBridge C_{18} (150 mm × 19 mm i.d., 5 µm, Waters). Flow: 17 mL/min. Injection volume = 1 mL. Eluant A/B NH_3HCOOH solution (10 mM, pH 8.5)/CH_3CN. Gradient: 5% B to 95% B in 40 minutes and 10 minutes of re-equilibration. Detection: 254 nm.

Hydrolysis kinetics measured by HPLC

A 10 mM solution of the compound was prepared in DMSO and diluted in corresponding buffer to obtain a 1 mM solution (1 mL). For pH 7.4 and 5.5, phosphate buffers were used (NaH_2PO_4/Na_2HPO_4 pH 7.4 or KH_2PO_4/Na_2HPO_4 pH 5.5, 100 mM). Each solution was immediately analyzed by analytical HPLC (basic conditions as mentioned above). Reaction was performed at 25 °C and crude was injected every 30 minutes for up to 15 hours.

Kinetic fluorescence measurements of TAMRA/BHQ-2 compounds in solution

A 5.0 µM solution of **TAMRA/BHQ-2 compound** was prepared in DMSO and diluted into the corresponding buffer (100 mM citric acid/phosphate buffer systems pH 3-7)[1] to obtain a 0.5 µM dye solution (1 mL). Each solution was immediately distributed into a 96-well Plate (3 × 300 µL/well) and fluorescence was recorded using a multilabel plate reader (Victor X2, PerkinElmer - Excitation and emission used filters were 550/8 nm and 580/10 nm). Fluorescence was measured every 5 minutes for up to 15 hours and the plate was shaken 10 seconds before each measurement.

Synthesis

General procedure A: The aldehyde (0.42 mmol), the β-aminoalcohol or amine compound (0.42 mmol) and molecular sieves 4Å (0.4 g) were added to $CHCl_3$ (5.0 mL) and sealed in a microwave tube and heated to 120 °C under microwave irradiation for 10 minutes (ref. 2). After cooling, the crude mixture was filtered and the solvent was removed under reduced pressure. The crude material was purified by distillation or preparative HPLC (method 2).

General procedure B: A solution of aldehyde (12 mmol), β-aminoalcohol or amine compound (36 mmol) and *p*-toluenesulfonic acid (0.09 mmol) in toluene (27 mL) was refluxed using a Dean-Stark apparatus (ref 3). The resulting mixture was cooled down to room temperature, quenched with a saturated solution $NaHCO_3$ (10 mL) and extracted with Et_2O (3 × 20 mL). The organic layers were washed with water (50 mL), brine (50 mL), dried over Na_2SO_4 and evaporated under reduced pressure. The crude material was purified by distillation or silica gel chromatography.

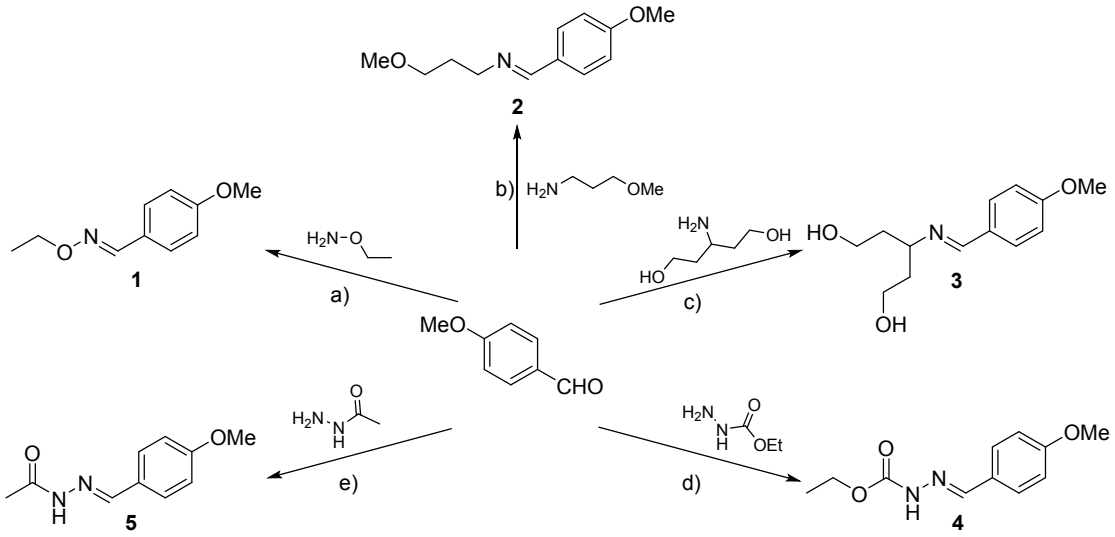
General procedure C: modification of BHQ-2 by peptide coupling

To a solution of BHQ-2 (0.272 mmol) in a mixture of DMF/CH_2Cl_2 (1:2, 9.0 mL) were successively added Et_3N (0.816 mmol) and the amine (0.408 mmol). The mixture was cooled to 0 °C and PyBOP (0.408 mmol) was added. The solution was allowed to warm up to room temperature and stirred for 16 hours. The reaction mixture was diluted with saturated $NaHCO_3$ solution (50 mL) and extracted with EtOAc (3 × 50 mL). The organic layers were combined, washed successively with saturated $NaHCO_3$ solution (50 mL), water (50 mL), brine (50 mL) and dried over Na_2SO_4. The crude product was purified by silica gel column chromatography (CH_2Cl_2/MeOH 100:0 to 95:5) to yield the desired product.

General procedure D: modification of 5-TAMRA by peptide coupling

5-TAMRA (0.167 mmol) was dissolved in DMF (1.20 mL) and Et_3N (0.334 mmol) was added. The mixture was cooled to 0 °C and PyBOP (0.167 mmol) was added. After 10 minutes the amine (0.200 mmol) was added and the resulting mixture was stirred for additional 16 hours at room temperature. The crude mixture was directly purified by preparative HPLC (method 1) and lyophilized to afford the modified **TAMRA**.

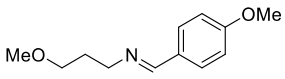
Compounds in Scheme 1 were synthesized by condensation of *p*-anisaldehyde with primary amines following literature procedures.



Scheme 1 – Synthesis of compounds 1 – 5

Reagents and conditions: a) $MgSO_4$, CH_2Cl_2/MeOH (3:1), rt, 16 h, 76%; b) $CHCl_3$, 4Å molecular sieves, reflux, 12 h, 45%; c) toluene, reflux, 16 h, 79%; d) $CHCl_3$, 4Å molecular sieves, 140 °C, microwave, 40 min, 74%; e) EtOH, rt, 16 h, 53%.

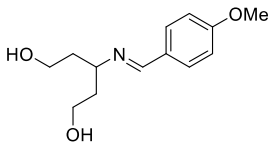
(*E*)-1-(4-Methoxyphenyl)-N-(3-methoxypropyl)methanimine 2



This compound was synthesized following a reported protocol synthesis.[4]

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.24 (s, 1H), 7.67 (d, J = 8.7 Hz, 2H), 6.98 (d, J = 8.7 Hz, 2H), 3,79 (s, 3H), 3.54 (t, J = 6.9 Hz, 2H), 3.37 (t, J = 6.5 Hz, 2H), 3.22 (s, 3H), 1.84-1.78 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.1, 160.0, 131.7, 129.3, 113.9, 69.7, 57.8, 57.1, 55.2, 30.6.

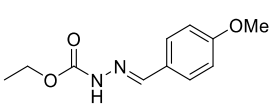
(*E*)-3-((4-Methoxybenzylidene)amino)pentane-1,5-diol 3



This compound was synthesized following a reported protocol synthesis.[5]

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.22 (s, 1H), 7.68 (d, J = 8.5 Hz, 2H), 6.98 (d, J = 8.5 Hz, 2H), 4.51 (t, J = 5.5 Hz, 2H), 3.79 (s, 3H), 3.64 – 3.59 (m, 2H), 3.44 – 3.38 (m, 2H), 3.29 – 3.23 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.0, 160.1, 129.5, 129.1, 113.8, 74.6, 62.4, 55.2.

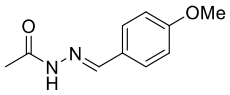
Ethyl (*E*)-2-(4-methoxybenzylidene)hydrazine-1-carboxylate 4[6]



Following general procedure A (140 °C, 40 min), compound **4** was obtained by reaction of *p*-anisaldehyde and ethylcarbazate. Purification was achieved by preparative HPLC (method 2).

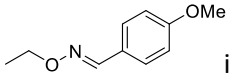
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.19 (brs, 1H), 7.80 (s, 1H), 7.59 (d, *J* = 8.6 Hz, 2H), 6.86 (d, *J* = 8.6 Hz, 2H), 4.27 (dt, *J* = 6.2, 8.0 Hz, 2H), 3.80 (s, 3H), 1.30 (t, *J* = 6.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.4, 144.6, 129.0, 126.6, 114.3, 62.1, 55.5, 14.8.

(*E*)-*N*'-(4-Methoxybenzylidene)acetohydrazide 5[7]



This compound was synthesized following a reported protocol.[8]

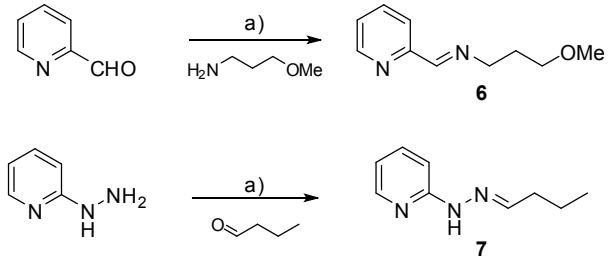
(*E*)-4-Methoxybenzaldehyde *O*-ethyl oxime 1[9]



To a stirred solution of *O*-ethylhydroxylamine hydrochloride (94 mg, 0.97 mmol) in a mixture of CH_2Cl_2/MeOH (3:1, 7 mL) $MgSO_4$ (370 mg), Et_3N (135 µL, 0.97 mmol) and *p*-anisaldehyde (120 mg, 0.88) were added. The reaction mixture was stirred for 16 hours at room temperature and filtered off. The filtrate was evaporated under reduced pressure and the residue was purified by preparative HPLC (method 2) to give compound **1** (120 mg, 76%) as a pinkish oil.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.14 (s, 1H), 7.54 (d, *J* = 8.6 Hz, 2H), 6.96 (d, *J* = 8.6 Hz, 2H), 4.11 (q, *J* = 7.1 Hz, 2H), 3.78 (s, 3H), 1.23 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.5, 147.9, 128.2, 124.6, 114.2, 66.7, 55.1, 14.4.

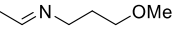
Compounds **6** and **7** were synthesized from picolinaldehyde and 2-hydrazinylpyridine, respectively by condensation with 3-methoxypropan-1-amine and butanal, respectively (Scheme 2).



Scheme 2 – Synthesis of compounds 6 and 7

Reagents and conditions: a) $CHCl_3$, 4Å molecular sieves, 140 °C, microwave, 40 min, 60% for **6** and 98% for **7**.

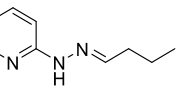
(*E*)-*N*-(3-Methoxypropyl)-1-(pyridin-2-yl)methanimine 6[10]



Following general procedure A (140 °C, 40 min), compound **6** was obtained by reaction of 2-pyridinecarboxaldehyde and 3-methoxypropylamine. The reaction mixture was filtered and then immediately purified on basic alumina gel chromatography (cyclohexane/EtOAc 5:5). Imine **6** (453 mg, 60%) was obtained as orange oil.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.63 (dd, *J* = 1.8, 4.9 Hz, 1H), 8.33 (s, 1H), 7.95 (dt, *J* = 1.0, 7.9 Hz, 1H), 7.86 (dt, *J* = 1.8, 7.9 Hz, 1H), 7.45 (ddd, *J* = 1.8, 4.9, 7.9 Hz, 1H), 3.66 (dt, *J* = 1.5, 6.9 Hz, 2H), 3.39 (t, *J* = 6.8 Hz, 2H), 3.23 (s, 3H), 1.86 (tt, *J* = 6.8, 6.9 Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.9, 154.1, 149.3, 136.7, 125.0, 120.3, 69.6, 57.8, 57.0, 30.2.

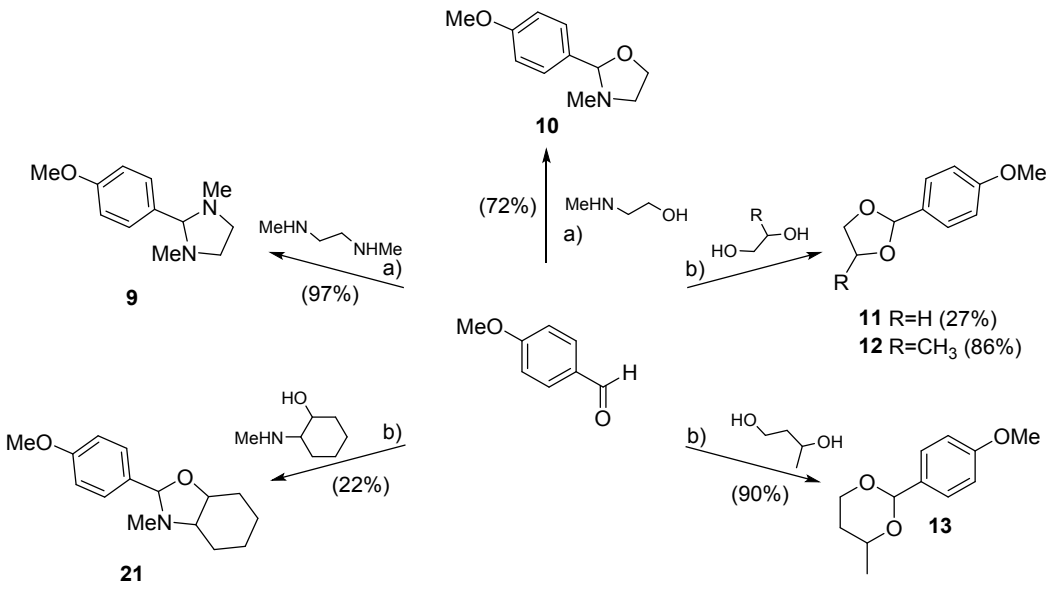
(*E*)-2-(2-Butylidenehydrazinyl)pyridine 7



This compound was synthesized following a reported protocol for acylhydrazone synthesis.[8]

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.62 (brs, 1H), 8.04 (dd, *J* = 1.9, 4.9 Hz, 1H), 7.51 (ddd, J = 2.0, 7.2, 8.5 Hz, 1H), 7.17-7.10 (m, 2H), 6.69-6.65 (m, 1H), 2.26 (dt, *J* = 5.5, 7.4 Hz, 2H), 1.60-1.50 (m, 2H), 0.95 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.5, 147.4, 143.3, 138.2, 116.0, 115.2, 107.4, 34.4, 20.5, 13.9.

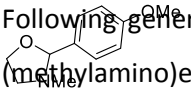
Heterocycles derived from benzaldehyde. Imidazolidine **9** and oxazolidines **10** and **21** were prepared by treatment of *p*-anisaldehyde with the corresponding 1,2-diamine, 1,2- and 1,3-diol in the microwave in the presence of molecular sieves (Scheme 3). Similarly dioxolanes **12 – 13** and dioxane **13** were prepared using classical acetalization methods.



Scheme 3 – Synthesis of compounds 9 – 13 and 21

Reagents and conditions: a) $CHCl_3$, 4Å molecular sieves, 120 °C, microwave, 10 min; b) *p*-TsOH, toluene, 100 °C, 24 h.

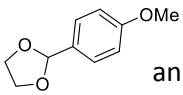
2-(4-Methoxyphenyl)-3-methyloxazolidine 10[11]



Following general procedure A, compound **10** was obtained by reaction of *p*-anisaldehyde and 2-(methylamino)ethanol. Purification was achieved by distillation.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (d, J = 8.5 Hz, 2H), 6.87 (d, J = 8.5 Hz, 2H), 4.55 (s, 1H), 4.09 – 3.96 (m, 2H), 3.77 (s, 3H), 3.31 (ddd, J = 2.8, 6.7, 9.3 Hz, 2H), 2.65 (dt, J = 7.9, 9.3 Hz, 2H), 2.23 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 129.0, 113.9, 98.2, 65.4, 55.4, 54.9, 38.2.

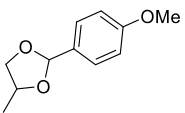
2-(4-Methoxyphenyl)-1,3-dioxolane 11[12]



Following general procedure B, compound **11** was obtained by reaction of *p*-anisaldehyde and ethylene glycol. Purification was achieved by silica gel column chromatography (Cyclohexane/EtOAc/Et_3N, 700:100:1).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 (d, J = 8.5 Hz, 2H), 6.89 (d, J = 8.5 Hz, 2H), 5.74 (s, 1H), 4.12 – 4.09 (m, 2H), 4.00 – 3.97 (m, 2H), 3.79 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 128.0, 113.9, 103.9, 65.4, 55.4.

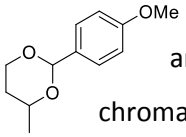
2-(4-Methoxyphenyl)-4-methyl-1,3-dioxolane 12[13]



Following general procedure B, compound **12** was obtained by reaction of *p*-anisaldehyde and 1,2-propanediol. Purification was achieved by silica gel column chromatography (Cyclohexane/EtOAc/Et_3N, 700:100:1). Dioxolane compound **12** was obtained with an enantiomeric ratio of 60/40.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 (dt, J = 2.1, 8.6 Hz, 2H), 6.88 (dd, J = 2.7, 8.6 Hz, 2H), 5.89 (s, 0.4H, isomer A), 5.75 (s, 0.6H, isomer B), 4.39 – 4.29 (m, 1H), 4.25 (dd, J = 6.1, 8.1 Hz, 0.4H, isomer A), 4.08 (dd, J = 6.1, 7.5 Hz, 0.6H, isomer B), 3.79 (s, 3H), 3.59 (t, J = 7.5 Hz, 0.6H, isomer B), 3.52 (t, J = 8.1 Hz, 2H, 0.4H, isomer A), 1.37 (d, J = 6.1 Hz, 2H, isomer B) 1.32 (d, J = 6.1 Hz, 1H, isomer A); ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.4, 128.2, 127.9, 113.9, 104.2, 103.1, 73.5, 72.4, 72.2, 71.6, 55.5, 18.9, 18.6.

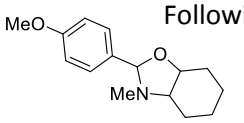
2-(4-Methoxyphenyl)-4-methyl-1,3-dioxane 13[14]



Following general procedure B, compound **13** was obtained by reaction of *p*-anisaldehyde and 1,3-butanediol. Purification was achieved by silica gel column chromatography (Cyclohexane/EtOAc/Et_3N, 700:100:1). Dioxane compound **13** was obtained with an enantiomeric ratio of 91/9.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 (d, J = 8.6 Hz, 2H), 6.88 (d, J = 8.6 Hz, 2H), 5.45 (s, 0.91H, isomer A), 5.23 (s, 0.09H, isomer B), 4.21 (ddd, J = 1.4, 5.0, 11.5 Hz, 1H), 3.97 – 3.87 (m, 2H), 3.76 (s, 3H), 1.77 (dddd, J = 5.0, 11.5, 12.4, 13.3 Hz, 1H), 1.48 (dtd, J = 1.4, 2.5, 13.3 Hz, 1H), 1.30 (d, J = 6.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.9, 131.5, 127.4, 113.5, 101.2, 73.3, 67.0, 55.2, 33.0, 21.8.

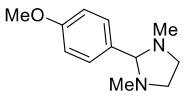
2-(4-Methoxyphenyl)-3-methyloctahydrobenzo[*d*]oxazole 21



Following general procedure B, compound **21** was obtained by reaction of *p*-anisaldehyde and 2-(methylamino)cyclohexanol. Purification was achieved by preparative HPLC (method 2).

^{1}H NMR (400 MHz, CD_3CN) δ 7.37 (d, J = 8.6 Hz, 2H), 6.91 (d, J = 8.6 Hz, 2H), 4.53 (s, 1H), 3.79 (s, 3H), 3.6 (ddd, J = 3.9, 8.9, 11.2 Hz, 2H), 2.11 (s, 3H), 2.00 – 1.93 (m, 3H), 1.82 – 1.78 (m, 2H), 1.46 – 1.20 (m, 4H); ^{13}C NMR (100 MHz, CD_3CN) δ 161.0, 134.4, 130.2, 118.3, 114.5, 99.8, 83.0, 71.5, 56.0, 36.2, 31.1, 28.7, 24.8.

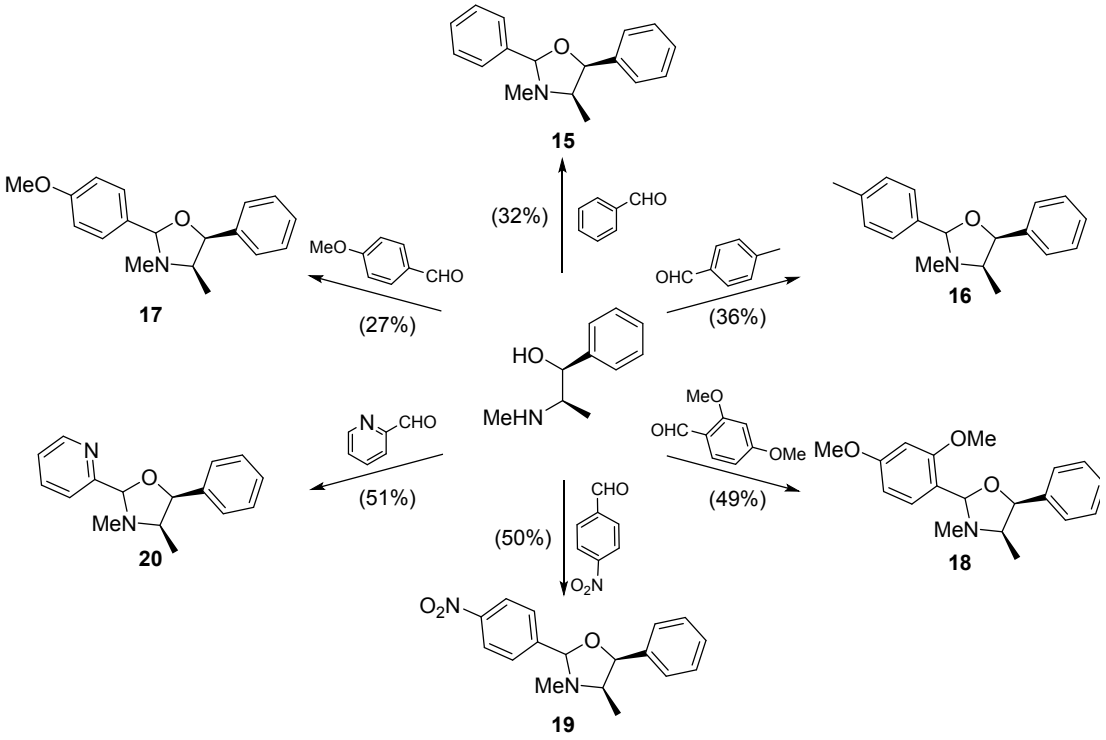
2-(4-Methoxyphenyl)-1,3-dimethylimidazolidine 9[15]



Following general procedure A, compound **9** was obtained by reaction of *p*-anisaldehyde and *N*,*N*'-dimethylethylenediamine. Purification was achieved by distillation.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 (d, J = 8.5 Hz, 2H), 6.86 (d, J = 8.5 Hz, 2H), 3.79 (s, 3H), 3.37 (dt, J = 3.5, 4.5 Hz, 2H), 3.19 (s, 1H), 2.52 (dt, J = 3.5, 4.5 Hz, 2H), 2.14 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 130.1, 113.9, 92.1, 55.4, 53.4, 39.6.

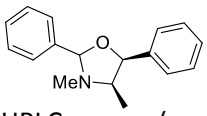
Oxazolidines **15 – 20** were synthesized by condensation of (-)-(1*R*,2*S*)-ephedrine with the corresponding aldehyde (Scheme 4).



Scheme 4 – Synthesis of oxazolidines 15 – 20 starting from (-)-(1R,2S)-ephedrine

Reagents and conditions: $CHCl_3$, 4Å molecular sieves, 120 °C, microwave, 10 min.

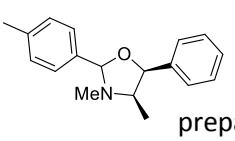
(4*R*,5*S*)-3,4-Dimethyl-2,5-diphenyloxazolidine 15[2]



Following general procedure A, compound **15** was obtained by reaction of benzaldehyde and (-)-(1*R*,2*S*)-ephedrine. Purification was achieved by preparative HPLC (method 2).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 (dd, J = 1.9, 7.7 Hz, 2H), 7.44 – 7.24 (m, 8H), 5.14 (d, J = 8.3 Hz, 2H), 4.69 (s, 1H), 2.98 (qd, J = 6.5, 8.3 Hz, 1H), 2.18 (s, 3H), 0.78 (d, J = 6.5 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$-d_1) δ 140.0, 138.3, 129.4, 128.7, 128.6, 128.2, 128.1, 127.8, 99.0, 82.7, 64.2, 36.0, 15.2.

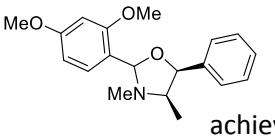
(4*R*,5*S*)-3,4-Dimethyl-5-phenyl-2-(*p*-tolyl)oxazolidine 16[2]



Following general procedure A, compound **16** was obtained by reaction of *p*-tolualdehyde and (-)-(1*R*,2*S*)-ephedrine. Purification was achieved by preparative HPLC (method 2). Compound **16** was obtained in an enantiomeric ratio of 93/7.

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.48 (d, J = 7.6 Hz, 2H), 7.42 – 7.24 (m, 7H), 5.46 (d, J = 5.6 Hz, 0.07H, isomer A), 5.27 (s, 0.07H, isomer A), 5.11 (dd, J = 8.3 Hz, 0.93H, isomer B), 4.60 (s, 0.93H, isomer B), 2.96-2.90 (m, 1H), 2.34 (s, 3H), 2.10 (s, 0.20H, isomer A), 2.06 (s, 2.80H, isomer B), 0.66 (d, J = 6.4 Hz, 2.80H, isomer B), 0.59 (d, J = 6.6 Hz, 0.20H, isomer A); ^{13}C NMR (100 MHz, DMSO-d_6) δ 140.1, 138.3, 135.3, 128.9, 128.1, 127.7, 127.4, 97.7, 81.1, 63.0, 35.2, 20.8, 14.7.

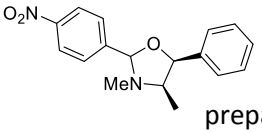
(4*R*,5*S*)-2-(2,4-Dimethoxyphenyl)-3,4-dimethyl-5-phenyloxazolidine 18



Following general procedure A, compound **18** was obtained by reaction of 2,4-dimethoxybenzaldehyde and (-)-(1*R*,2*S*)-ephedrine. Purification was achieved by preparative HPLC (method 2). Compound **18** was obtained with an enantiomeric ratio of 93/7.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (d, J = 8.5 Hz, 1H), 7.29 – 7.13 (m, 6H), 6.52 (dd, J = 2.4, 8.5 Hz, 1H), 6.41 (d, J = 2.4 Hz, 1H), 5.77 (s, 0.07H, isomer A), 5.43 (d, J = 5.3 Hz, 0.07H, isomer A), 5.09 (s, 0.93H, isomer B), 5.06 (d, J = 8.0 Hz, 0.93H, isomer B), 3.76 (s, 3H), 3.75 (s, 3H), 2.92 – 2.85 (m, 1H), 2.18 (s, 0.20H, isomer A), 2.11 (s, 2.80H, isomer B), 0.69 (d, J = 6.4 Hz, 2.80H, isomer B), 0.66 (d, J = 7.1 Hz, 0.2H, isomer A); ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.5, 160.1, 140.4, 129.5, 128.2, 127.9, 127.6, 105.0, 98.7, 91.9, 82.7, 64.0, 55.8, 55.6, 36.2, 15.1.

(4*R*,5*S*)-3,4-Dimethyl-2-(4-nitrophenyl)-5-phenyloxazolidine 19[2]

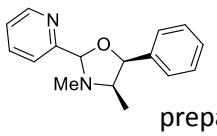


Following general procedure A, compound **19** was obtained by reaction of 4-nitrobenzaldehyde and (-)-(1*R*,2*S*)-ephedrine. Purification was achieved by preparative HPLC (method 2). Compound **19** was obtained in an enantiomeric ratio of 9/1.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.27 (d, J = 8.6 Hz, 1.8H, isomer A), 8.24 (d, J = 8.6 Hz, 0.2H, isomer B), 7.81 (d, J = 8.6 Hz, 1.8H, isomer A), 7.70 (dd, J = 8.6 Hz, 0.2H, isomer B), 7.38 – 7.26 (m, 5H), 5.55 (d, J = 5.3 Hz, 0.1H, isomer B), 2.38, (s, 0.1H, isomer B), 5.17 (d, J = 8.2 Hz, 0.9H, isomer A), 4.79 (s, 0.9H, isomer A), 3.73 – 3.68 (m, 0.1H, isomer B), 3.03 (qd, J = 6.5, 8.2 Hz, 0.9H, isomer A), 2.27 (s, 0.3H, isomer B), 2.21 (s, 2.7H, isomer A), 0.78 (d, J = 6.5 Hz, 2.7H, isomer A), 0.73 (d, J = 6.6 Hz, 0.3H,

isomer B); ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.8, 145.6, 139.3, 129.5, 129.5, 128.3, 128.1, 128.0, 123.9, 97.5, 83.1, 64.2, 36.1, 15.3.

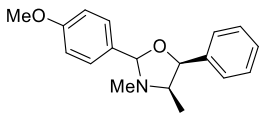
(4*R*,5*S*)-3,4-Dimethyl-5-phenyl-2-(pyridin-2-yl)oxazolidine 20[16]



Following general procedure A, compound **20** was obtained by reaction of 2-pyridinecarboxaldehyde and (-)-(1*R*,2*S*)-ephedrine. Purification was achieved by preparative HPLC (method 2). Compound **20** was obtained in an enantiomeric ratio of 93/7.

^{1}H NMR (400 MHz, $CDCl_3$-d_1) δ 8.76 (d, *J* = 4.8 Hz, 0.93H, isomer A), 8.72 (d, *J* = 4.8 Hz, 0.07H, isomer B), 7.95-7.83 (m, 2H), 7.58 (d, *J* = 8.1 Hz, 2H), 7.48 – 7.38 (m, 4H), 5.73 (d, *J* = 5.3 Hz, 0.07H, isomer B), 5.53 (s, 0.07H, isomer B), 5.40 (s, 0.93H, isomer A), 5.31 (d, *J* = 8.1 Hz, 0.93H, isomer A), 4.96 (s, 1H), 3.83 (dq, *J* = 5.4, 6.7 Hz, 0.07H, isomer B), 3.15 (qd, *J* = 6.4, 8.2 Hz , 0.93H, isomer A), 2.46 (s, 0.21H, isomer B), 2.43 (s, 2.79H, isomer A), 0.90 (d, *J* = 6.4 Hz , 2.79H, isomer A), 0.85 (d, *J* = 6.9 Hz, 0.21H, isomer B); ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.4, 149.2, 139.9, 137.1, 128.2, 128.1, 127.9, 123.9, 122.3, 98.9, 83.0, 64.4, 36.2, 15.1.

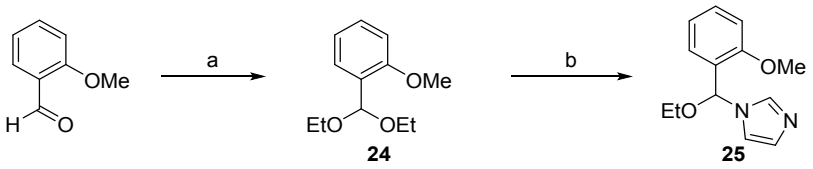
(4*R*,5*S*)-2-(4-Methoxyphenyl)-3,4-dimethyl-5-phenyloxazolidine 15[17]



Following general procedure A, compound **15** was obtained by reaction of *p*-anisaldehyde and (-)-(1*R*,2*S*)-ephedrine. Purification was achieved by preparative HPLC (method 2). Compound **15** was obtained in an enantiomeric ratio of 93/7.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 (d, *J* = 8.6 Hz, 2H), 7.41 (d, *J* = 7.8 Hz, 2H), 7.33 – 7.24 (m, 4H), 6.94 (d, *J* = 8.3 Hz, 2H), 5.55 (d, *J* = 5.3 Hz, 0.07H, isomer A), 5.27 (s, 0.07H, isomer A), 5.11 (d, *J* = 8.3 Hz, 0.93H, isomer B), 4.63 (s, 0.93H, isomer B), 3.83 (s, 3H), 2.97 – 2.90 (m, 1H), 2.15 (s, 3H), 0.76 (d, *J* = 6.4 Hz, 2.91H, isomer B), 0.7 (d, *J* = 6.9 Hz, 0.14H, isomer A); ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.4, 140.0, 130.3, 129.6, 128.0, 127.9, 127.6, 113.9, 98.6, 82.3, 64.0, 55.3, 35.7, 15.1.

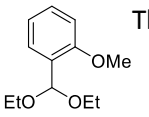
Following a reported procedure,[18] compound **25** was synthesized in two steps by treatment of *o*-anisaldehyde with triethylorthoformate in the presence of HCl to give **24** followed by reaction with imidazole (Scheme 5).



Scheme 5 – Synthesis of compound 25

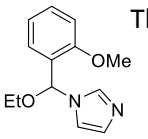
Reagents and conditions: a) Triethylorthoformiate, EtOH, HCl (cat.), reflux, 24 h, 72%; b) imidazole, *p*-TsOH, 110 °C, 72 h, 45%.

1-(Diethoxymethyl)-2-methoxybenzene 24[18]



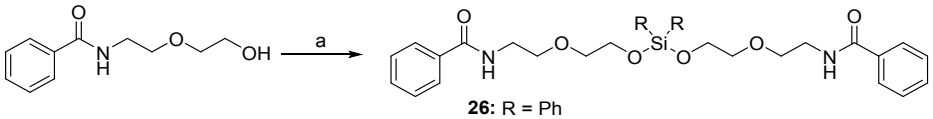
This compound was synthesized following a reported protocol.[18]

1-(Ethoxy(2-methoxyphenyl)methyl)-1*H*-imidazole 25[18]



This compound was synthesized following a reported protocol.[18]

Treatment of *N*-(2-(2-hydroxyethoxy)ethyl)benzamide with dichlorodiphenylsilane successfully gave compound **26** (Scheme 6). It is worth noting that analogues **26a** and **26b** were also prepared but could not be purified due to their high instability.

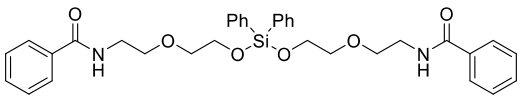


26: R = Ph
26a: R = Me, degradation upon purification
26b: R = *n*-Bu, degradation upon purification

Scheme 6 – Synthesis of siloxane-based compound 26

Reagents and conditions: a) Ph_2SiCl_2, DMF, 0 °C to rt, 16 h, 73%.

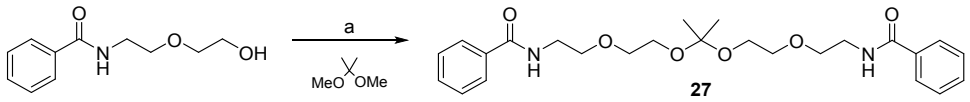
***N*,*N*'-(7,7-Diphenyl-3,6,8,11-tetraoxa-7-silatridecane-1,13-diyl)dibenzamide 26**



This compound was synthesized following a reported protocol for silyl ether synthesis.[19] *N*-(2-(2-hydroxyethoxy)ethyl)benzamide (116 mg, 0.56 mmol) was dissolved in CH_2Cl_2 (1 mL) and Et_3N (78 µL, 0.56 mmol) was added. After stirring for 5 minutes at 0°C, dichlorodiphenyl silane (30 µL, 0.14 mmol) was added dropwise and stirring was continued for 16 hours while the mixture was warmed up to room temperature. CH_2Cl_2 was removed under reduced pressure and the crude material was directly purified by preparative HPLC (method 2) and lyophilized to afford **26** (61 mg, 73%) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69 (d, J = 7.7 Hz, 4H), 7.62 (d, J = 7.7 Hz, 4H), 7.44 – 7.36 (m, 4H), 7.33 – 7.28 (m, 8H), 6.66 (s, 2H), 3.91 (t, J = 5.0 Hz, 4H), 3.58 – 3.57 (m, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.7, 135.1, 134.7, 132.5, 131.5, 130.7, 128.7, 128.1, 127.2, 72.3, 69.9, 62.8, 40.0.

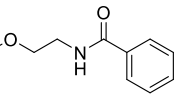
N-(2-(2-hydroxyethoxy)ethyl)benzamide was reacted with 2,2-dimethoypropane in the presence of *p*-TsOH to give **27** (Scheme 7). Here again it is worth noting that the low yield (7%) is due to the hydrolysis of **27** during purification.



Scheme 7 – Synthesis of compound 27

Reagents and conditions: a) *p*-TsOH, $CHCl_3$, 4Å molecular sieves, rt, 16 h, 7%.

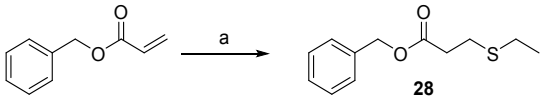
_N_,_N_'-(7,7-dimethyl-3,6,8,11-tetraoxatridecane-1,13-diyl)dibenzamide 27



To a stirred solution of *N*-(2-(2-hydroxyethoxy)-ethyl)benzamide (212 mg, 1.01 mmol) in $CHCl_3$ (3:1) (10 mL) 4Å molecular sieves (200 mg), 2,2-dimethoxypropane (57 µL, 0.46 mmol) and *p*-toluenesulfonic acid (2 mg, 0.01 mmol) were added. The reaction mixture was stirred for 16 hours at room temperature and was filtered off. The filtrate was concentrated under reduced pressure and the residue was purified by preparative HPLC (method 2) to give **27** (15 mg, 7%) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, J = 7.4 Hz, 4H), 7.48 – 7.37 (m, 6H), 6.80 (brs, 2H), 3.73 (t, J = 4.7 Hz, 4H), 3.64 (s, 8H), 3.58 (t, J = 4.7 Hz, 4H), 2.13 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.9, 134.7, 131.6, 128.7, 127.2, 72.5, 70.1, 61.9, 40.0, 31.1, 29.9.

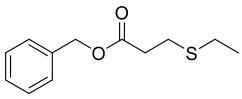
β-thiopropionate derivative **28** was synthesized by Michael addition of ethanethiol on benzyl acrylate (Scheme 8).



Scheme 8 – Synthesis of β-thiopropionate 28

Reagents and conditions: a) Ethanethiol, Et_3N, THF, rt, 16 h, 55%.

Benzyl 3-(ethylthio)propanoate 28

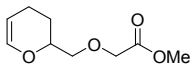


To a stirred solution of benzylacrylate (118 mg, 0.73 mmol) in THF (2 mL) Et_3N (304 µL, 2.18 mmol) and ethanethiol (107 µL, 1.46 mmol) were successively added. The reaction mixture was stirred for 16 hours at room temperature and concentrated under reduced pressure. The crude residue was purified by preparative HPLC (method 2) to yield **28** (88 mg, 55%) as a white solid.

^{1}H NMR (400 MHz, CD_3CN) δ 7.46 – 7.29 (m, 5H), 5.10 (s, 2H), 2.77 (t, *J* = 7.2 Hz, 2H), 2.63 (t, *J* = 7.2 Hz, 2H), 2.52 (q, *J* = 7.4 Hz, 2H), 1.21 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, CD_3CN) δ 172.1, 136.6, 129.0, 128.6, 128.5, 117.4, 66.6, 35.3, 26.8, 26.1, 15.0.

Synthesis of TAMRA-THP-(BHQ-2) probe P1:

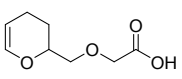
Methyl 2-((3,4-dihydro-2*H*-pyran-2-yl)methoxy)acetate



3,4-Dihydro-2*H*-pyran-2-methanol **31** (0.90 mL, 8.67 mmol) was added dropwise to a suspension of NaH (60% in mineral oil, 0.52 g, 13.00 mmol) in anhydrous THF (100 mL) at 0 °C. 30 minutes after addition was complete, bromomethyl acetate (1.65 mL, 17.34 mmol) was added dropwise. After 3 hours stirring at 0 °C, the reaction was quenched with aqueous saturated NH_4Cl and the organic solvent was removed under reduced pressure. The obtained aqueous solution was extracted twice with CH_2Cl_2 (2 × 100 mL) and the combined organic layers were washed with saturated $NaHCO_3$ solution (100 mL), water (100 mL), brine (100 mL) and dried over Na_2SO_4. The crude material was purified by silica gel column chromatography (Cyclohexane/EtOAc 8:2) to afford the desired product (1.13 g, 70%) as a colorless oil.

R*f* 0.34 (Cyclohexane/EtOAc 8:2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.34 (td, *J* = 6.3, 1.6 Hz, 1H), 4.67 – 4.64 (m, 1H), 4.15 (d, *J* = 5.5 Hz, 2H), 4.04 – 3.98 (m, 1H), 3.72 (s, 3H), 3.63 (d, *J* = 5.05 Hz, 2H), 2.12 – 2.02 (m, 1H), 1.99 – 1.91 (m, 1H), 1.85 – 1.81 (m, 1H), 1.71 – 1.61 (m, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.9, 143.5, 100.6, 74.2, 74.1, 68.8, 51.9, 24.4, 19.4; IR (neat) : 2921, 1754, 1734, 1437, 1143, 1067 cm^{-1}.

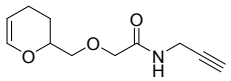
2-((3,4-Dihydro-2*H*-pyran-2-yl)methoxy)acetic acid 33



To a solution of methyl 2-((3,4-dihydro-2*H*-pyran-2-yl)methoxy)acetate (1.13 g, 6.07 mmol) in MeOH/water (1:1, 5 mL) was added LiOH (145 mg, 6.07 mmol) in one portion. The reaction mixture was stirred at room temperature for 5 hours. The crude material was dried by lyophilization to give **33** (1.04 g, quant.) as a white solid. The crude product was used without any further purification.

^{1}H NMR (400 MHz, MeOD-d_4) δ 6.35 (td, J = 6.3, 1.6 Hz, 1H), 4.72 – 4.68 (m, 1H), 4.06 – 4.00 (m, 1H), 3.92 (s, 2H), 3.63 – 3.56 (m, 2H), 2.16 – 2.08 (m, 1H), 2.02 – 1.94 (m, 1H), 1.92 – 1.86 (m, 1H), 1.69 – 1.61 (m, 1H); ^{13}C NMR (101 MHz, MeOD-d_4) δ 178.0, 144.4, 102.0, 75.4, 74.3, 72.1, 25.6, 20.4; IR (neat): 2920, 1605, 1429, 1066, cm^{-1}.

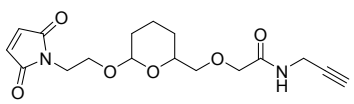
2-((3,4-Dihydro-2*H*-pyran-2-yl)methoxy)-*N*-(prop-2-ynyl)acetamide 34



33 (247 mg, 1.44 mmol) was dissolved in DMF (18.00 mL) and DIEA (0.49 mL, 1.72 mmol) was added. The mixture was cooled to 0 °C and HBTU (6.52 mg, 1.72 mmol) was added. After 10 minutes, propargylamine (0.11 mL, 1.72 mmol) was added dropwise and the reaction mixture was stirred for 16 hours at room temperature. The reaction was quenched with saturated $NaHCO_3$ solution (100 mL) and extracted with EtOAc (3 × 50 mL). The organic layers were combined, washed with saturated $NaHCO_3$ solution (100 mL), water (100 mL), brine (100 mL), dried over Na_2SO_4 and concentrated under reduced pressure. The crude oil was purified by silica gel column chromatography (Cyclohexane/EtOAc 5:5) to afford pure **34** (214 mg, 71%) as yellow oil.

R*f* 0.40 (Cyclohexane/EtOAc 5:5); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.12 (brs, 1H), 6.38 (td, J = 1.6, 6.3 Hz, 1H), 4.72 – 4.68 (m, 1H), 4.05 (dd, J = 2.6, 5.5 Hz, 2H), 4.00 (d, J = 5.0 Hz, 2H), 3.64 – 3.54 (m, 2H), 2.21 (t, J = 2.6 Hz, 1H), 2.14 – 2.05 (m, 1H), 2.00 – 1.93 (m, 1H), 1.80 – 1.75 (m, 1H), 1.72 – 1.68 (m, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.7, 143.3, 101.0, 79.4, 74.6, 74.0, 71.7, 70.7, 28.8, 24.2, 19.5; IR (neat): 3291, 2921, 1667, 1650, 1523, 1239, 1066 cm^{-1}; ESI-MS: 210 $[M+H]^+$. HRMS calcd 209.1052 for $C_{11}H_{15}NO$, found 209.1050.

2-((6-(2-(2,5-Dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethoxy)tetrahydro-2*H*-pyran-2-yl)methoxy)-*N*-(prop-2-ynyl)acetamide 36

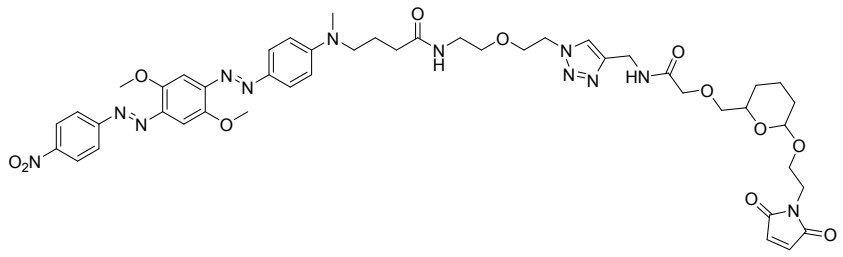


*p*TsOH (6 mg, 0.03 mmol) was added to a cold solution of **34** (300 mg, 1.44 mmol) in anhydrous CH_2Cl_2 (1.2 mL) and the reaction was stirred at 0 °C for 5 minutes. A solution of **35** (244 mg, 1.73 mmol) in CH_2Cl_2 (1.7 mL) was added dropwise and the reaction mixture was stirred for additional 3 hours at room temperature. The reaction was quenched with a 1N NaOH solution (1.0 mL) and extracted with CH_2Cl_2 (2 × 10 mL). The organic layers were combined, washed with water (20 mL) and dried over Na_2SO_4. The crude oil was purified by silica gel column chromatography (Cyclohexane/EtOAc 5:5 to 3:7) to afford **36** (388 mg, 77%) as colorless oil.

R*f* 0.53 (Cyclohexane/EtOAc 3:7); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 (brs, 0.7H), 7.17 (brs, 0.3H), 6.69 (s, 1.5H), 6.67 (s, 0.5H), 4.88 – 4.87 (m, 0.7H), 4.40 (dd, J = 2.2, 9.5 Hz, 0.3H), 4.10 – 3.35 (m, 13H), 2.21 – 2.20 (m, 1H), 1.81 – 2.21 (m, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.7, 169.8, 134.2, 96.7, 79.9,

75.3, 71.6, 70.6, 68.5, 63.5, 37.7, 29.5, 28.8, 26.9, 17.6; IR (neat): 2929, 1708, 1666, 1592, 1518, 1339, 1098 cm^{-1}.

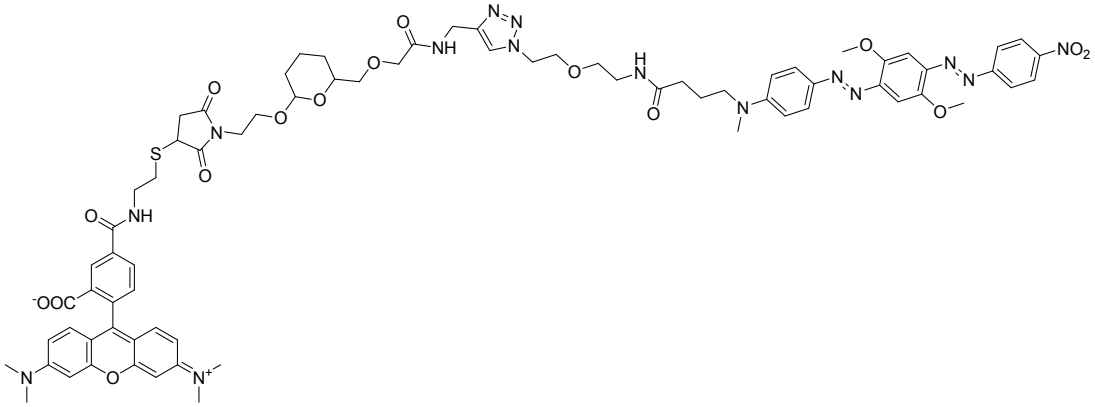
4-((4-((2,5-Dimethoxy-4-((4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)-*N*-(2-(2-(4-((2-((6-(2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethoxy)tetrahydro-2*H*-pyran-2-yl)methoxy)acetamido)methyl)-1*H*-1,2,3-triazol-1-yl)ethoxy)ethyl)butanamide 37



(BHQ-2)-N_3 (131 mg, 0.211 mmol) and **36** (111 mg, 0.317 mmol) were dissolved in a mixture of THF/*t*-BuOH (1:1, 10 mL). Water (5 mL), copper(II) sulfate pentahydrate (16 mg, 0.063 mmol) and sodium ascorbate (25 mg, 0.126 mmol) were successively added to the reaction mixture which was heated under microwave irradation at 50 °C for 1 hour. The solvents were evaporated under reduced pressure and the resulting aqueous solution was extracted with CH_2Cl_2 (50 mL). The organic layer was washed with water (20 mL) and dried over Na_2SO_4. The crude product was purified by silica gel column chromatography (EtOAc then CH_2Cl_2/MeOH 95:5) to yield **37** as a violet solid (151 mg, 74%).

R*f* 0.56 (CH_2Cl_2/MeOH 95:5); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.33 (d, *J* = 9.0 Hz, 2H), 8.00 (d, *J* = 9.0 Hz, 2H), 7.88 (d, *J* = 9.3 Hz, 2H), 7.68 (t, *J* = 5.6 Hz, 1H), 7.64 (s, 1H), 7.46 (s, 1H), 7.42 (s, 1H), 6.75 (d, *J* = 9.3 Hz, 2H), 6.69 – 6.68 (m, 2H), 6.30 (t, *J* = 5.3 Hz, 1H), 4.77 (d, *J* = 2.6 Hz, 1H), 4.58 – 4.48 (m, 2H), 4.45 (t, *J* = 4.8 Hz, 2H), 4.06 (s, 3H), 4.00 (s, 3H), 3.92 (s, 2H), 3.78 – 3.71 (m, 5H), 3.66 – 3.63 (m, 1H), 3.51 – 3.45 (m, 5H), 3.40 – 3.33 (m, 4H), 3.06 (s, 3H), 2.27 (t, *J* = 7.1 Hz, 2H), 1.96 (t, *J* = 7.1 Hz, 2H), 1.80 – 1.30 (m, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.5, 170.9, 170.4, 156.6, 153.8, 152.4, 151.1, 148.5, 147.0, 145.0, 144.6, 142.2, 134.3, 126.4, 124.9, 123.8, 123.7, 111.6, 101.2, 100.2, 96.9, 75.3, 70.8, 70.0, 69.1, 68.4, 63.3, 57.0, 51.9, 50.2, 39.4, 38.6, 37.7, 34.8, 33.11, 29.4, 27.0, 23.0, 17.6; IR (neat) : 2925, 1709, 1659, 1593, 1518, 1098 cm^{-1}; ESI-MS: 991 $[M+Na]^+$. HRMS calcd 968.4141 for $C_{46}H_{56}N_{12}O_{12}$, found 968.4141.

4-(2-(1-(2-(6-((2-((1-(2-(2-(4-((4-((2,5-Dimethoxy-4-((4-nitrophenyl)diazenyl)phenyl)diazenyl) phenyl)(methyl)amino)butanamido)ethoxy)ethyl)-1*H*-1,2,3-triazol-4-yl)methylamino) -2-oxoethoxy)methyl)tetrahydro-2*H*-pyran-2-yloxy)ethyl)-2,5-dioxopyrrolidin-3-ylthio) ethylcarbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3*H*-xanthen-9-yl)benzoate TAMRA-THP-(BHQ-2) or P1

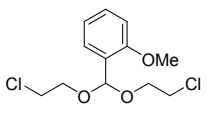


TAMRA-SH[20] (14 mg, 0.028 mmol) was dissolved in degassed anhydrous MeOH (1.8 mL) and Et_3N (9 µL) was added. **37** (21 mg, 0.022 mmol) in CH_2Cl_2 (1.8 mL) was added to the solution and the reaction mixture was stirred for 2 hours at room temperature. The solvents were removed under reduced pressure and the residue was dissolved in DMSO (1.0 mL), purified by preparative HPLC (method 2) and lyophilized to afford the desired **TAMRA-THP-(BHQ-2) P1** (20 mg, 66%) as a dark fluffy violet-pink solid.

^{1}H NMR (400 MHz, $CDCl_3$-d_1) δ 8.89 – 8.88 (m, 1H), 8.72 – 8.68 (m, 1H), 8.35 – 8.32 (m, 2H), 8.02 (d, *J* = 8.9 Hz, 2H), 7.84 – 7.81 (m, 3H), 7.47 (s, 2H), 7.42 (s, 1H), 7.27 (d, *J* = 7.9 Hz, 1H), 7.13 – 7.06 (m, 2H), 6.79 – 6.39 (m, 6H), 4.72 (s, 1H), 4.60 – 4.47 (m, 4H), 4.06 (s, 3H), 4.00 (s, 3H), 3.98 – 3.93 (m, 2H), 3.84 – 3.75 (m, 6H), 3.63 – 3.60 (m, 2H), 3.49 – 3.33 (m, 10H), 3.20 (s, 12H), 3.06 – 3.03 (m, 1H), 2.99 (s, 3H), 2.55 – 2.49 (m, 1H), 2.33 – 2.25 (m, 3H), 2.01 – 1.98 (m, 1H), 1.86 – 1.81 (m, 2H), 1.65 – 1.45 (m, 6H); ESI-MS: 1458 $[M+H]^+$. HRMS calcd 1457.5863 for $C_{73}H_{83}N_{15}O_{16}S$, found 1457.5862.

Synthesis of TAMRA-Acetal-(BHQ-2) probe P2:

1-(Bis(2-chloroethoxy)methyl)-2-methoxybenzene 39

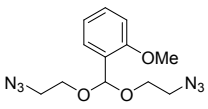


Starting from 2-anisaldehyde **38**, 1-(bis(2-chloroethoxy)methyl)-2-methoxybenzene **39** was synthesized as described for 4-anisaldehyde.[21] 2-Chloroethanol (8.30 mL, 123 mmol), 2-anisaldehyde **38** (6.98 g, 51.3 mmol), benzene (25 mL), and *p*-toluenesulfonic acid (10 mg, 0.05 mmol) were mixed and heated to reflux for 16 hours while the water formed was removed by a Dean-Stark trap. The reaction mixture was cooled to room temperature and quenched with a solution of sodium methoxide (100 mg) in

methanol (2 mL). The solvent was removed under reduced pressure and the crude material was purified using a Kugelrohr distillation apparatus to give **39** (5.54 g, 40%) as an oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57 (dd, *J* = 7.7, 1.8 Hz, 1H), 7.31 (ddd, *J* = 8.3, 7.7, 1.8 Hz, 1H), 6.97 (t, *J* = 7.7 Hz, 1H), 6.88 (d, *J* = 8.3 Hz, 1H), 8.93 (s, 1H), 3.83 (s, 3H), 3.80 (dt, *J* = 5.8, 2.3 Hz, 1H), 3.62 (t, *J* = 5.8 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 130.2, 127.6, 120.5, 110.9, 97.5, 66.2, 55.8, 43.1.

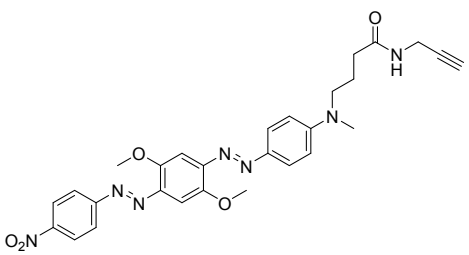
1-(Bis(2-azidoethoxy)methyl)-2-methoxybenzene 40



39 (710 mg, 2.55 mmol) was dissolved in DMF (4 mL) and sodium azide (827 mg, 12.72 mmol) was added to the solution. The reaction mixture was stirred at 80 °C for 16 hours. After cooling, the reaction mixture was extracted with hexane (3 × 5 mL) and the combined organic layers were washed with brine (20 mL), dried over Na_2SO_4 and concentrated under reduced pressure to give **40** (194 mg, 26%) as a yellow oil.

^{1}H NMR (400 MHz, CD_3CN-d_3) δ 7.53 (dd, *J* = 7.7, 1.8 Hz, 1H), 7.35 (dt, *J* = 7.7, 1.8 Hz, 1H), 7.01 – 6.96 (m, 2H), 5.87 (s, 1H), 3.83 (s, 3H), 3.78 – 3.66 (m, 4H), 3.40 – 3.37 (m, 4H); ^{13}C NMR (101 MHz, CD_3CN-d_3) δ 131.1, 128.1, 121.1, 118.3, 112.1, 98.7, 66.3, 56.4, 51.8; HRMS calcd 292.1284 for $C_{12}H_{16}N_6O_3$, found 292.1276.

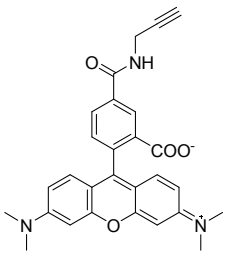
4-((4-((2,5-Dimethoxy-4-((4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)-*N*-(prop-2-ynyl)butanamide (BHQ-2)-Alkyne or (BHQ-2)-Alkyne



(BHQ-2)-Alkyne was synthesized according to the general procedure C. **BHQ-2** (138 mg, 0.272 mmol) reacted with propargylamine (22 mg, 0.408 mmol) to give **(BHQ-2)-Alkyne** as a dark violet solid (111 mg, 75%).

R*f* 0.43 (CH_2Cl_2/MeOH 95:5); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.42 (d, *J* = 9.0 Hz, 2H), 8.31 (t, *J* = 5.5 Hz, 2H), 8.04 (d, *J* = 9.0 Hz, 2H), 7.81 (d, *J* = 9.0 Hz, 2H), 7.42 (s, 1H), 7.36 (s, 1H), 6.87 (d, *J* = 9.0 Hz, 2H), 4.00 (s, 3H), 3.94 (s, 3H), 3.87 (dd, *J* = 5.5, 2.4 Hz, 2H), 3.46 (t, *J* = 7.4 Hz, 2H), 3.09 (t, *J* = 2.4 Hz, 2H), 3.06 (s, 3H), 2.19 (t, *J* = 7.4 Hz, 2H), 1.84 – 1.77 (m, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.0, 155.6, 152.8, 151.9, 150.2, 147.9, 146.3, 143.4, 141.5, 128.5, 125.2, 124.5, 123.0, 111.2, 101.3, 100.8, 80.9, 72.0, 56.7, 50.9, 37.7, 27.5, 22.1; ESI-MS: 544 $[M+H]^+$. HRMS calcd 543.2230 for $C_{28}H_{29}N_7O_5$, found 543.2229.

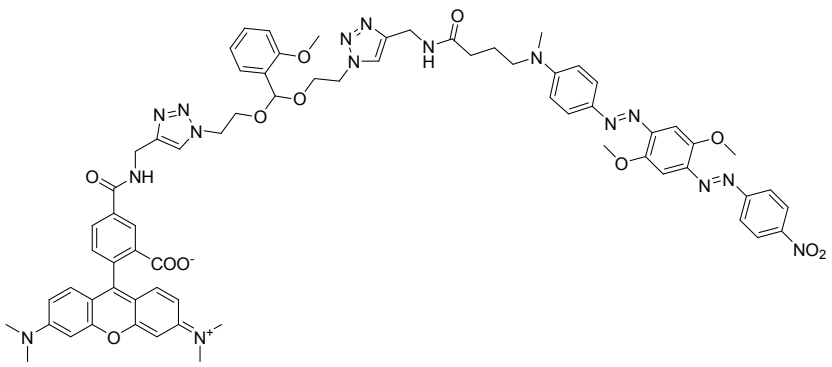
2-(6-(Dimethylamino)-3-(dimethyliminio)-3*H*-xanthen-9-yl)-5-(prop-2-ynylcarbamoyl) benzoate or TAMRA-Alkyne



TAMRA-Alkyne was synthesized according to the general procedure D. 5-TAMRA (246 mg, 0.57 mmol) was reacted with propargylamine (44 µL, 0.69 mmol) to give **TAMRA-Alkyne** as a fluffy pink solid (215 mg, 80%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.37 (t, J = 5.6 Hz, 1H), 8.70 (d, J = 1.8 Hz, 1H), 8.31 (dd, J = 1.8, 7.9 Hz, 1H), 7.59 (d, J = 7.9 Hz, 1H), 7.06 – 7.00 (m, 4H), 6.94 (brs, 2H), 4.15 (dd, J = 2.6, 5.6 Hz, 1H), 3.26 (s, 12H), 3.19 (t, J = 2.6 Hz, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.9, 164.4, 156.6, 135.3, 131.3, 130.5, 114.4, 96.3, 81.0, 73.1, 40.5, 28.7; ESI-MS: 468 $[M+H]^+$. HRMS calcd 467.18451 for $C_{28}H_{25}N_3O_4$, found 467.1847.

5-((1-(2-((2-(4-((4-((4-((2,5-Dimethoxy-4-((4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl) (methyl)amino)butanamido)methyl)-1*H*-1,2,3-triazol-1-yl)ethoxy)(2-methoxyphenyl)methoxy) ethyl)-1*H*-1,2,3-triazol-4-yl)methylcarbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3*H*-xanthen-9-yl)benzoate TAMRA-Acetal-(BHQ-2) or P2



(BHQ-2)-Alkyne (37 mg, 0.068 mmol) and **TAMRA-Alkyne** (32 mg, 0.068 mmol) were dissolved in a mixture of THF/DMSO (1:1, 1 mL). When solution was clear, water (0.5 mL), copper(II) sulfate pentahydrate (7 mg, 0.027 mmol) and sodium ascorbate (11 mg, 0.054 mmol) were added successively to the reaction mixture which was further stirred at room temperature for 3 hours. The crude mixture was diluted with DMSO (0.5 mL), directly purified by preparative HPLC (method 2) and lyophilized to afford the desired **TAMRA-Acetal-(BHQ-2) P2** (8 mg, 10%) as a fluffy violet-pink solid.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.40 (t, J = 5.6 Hz, 1H), 8.48 (s, 1H), 8.44 (d, J = 9.0 Hz, 1H), 8.36 (t, J = 5.6 Hz, 1H), 8.24 (dd, J = 8.0, 1.8 Hz, 1H), 8.06 (d, J = 9.0 Hz, 2H), 7.95 (s, 1H), 7.83 (s, 1H), 7.79 (d, J = 9.0 Hz, 2H), 7.44 (s, 1H), 7.37 (s, 1H), 7.31 (d, J = 8.0 Hz, 1H), 7.23 (d, J = 8.0 Hz, 2H), 6.94 (d, J = 8.6 Hz, 1H), 6.86 – 6.82 (m, 3H), 6.51 – 6.44 (m, 6H), 5.68 (s, 1H), 4.56 (d, J = 5.5 Hz, 2H), 4.48 – 4.39 (m, 4H), 4.29 (d, J = 5.5 Hz, 2H), 3.99 (s, 3H), 3.93 (s, 3H), 3.70 – 3.63 (m, 8H), 3.46 – 3.42 (m, 2H), 3.03 (s, 3H), 2.93 (s, 12H), 2.17 (t, J = 7.1 Hz, 2H), 1.83 – 1.76 (m, 2H); HRMS calcd 1302.5359 for $C_{68}H_{70}N_{16}O_{12}$, found 1302.5358.

Cell culture: Mice embryo NIH/3T3 cells (ATCC CRL-1658™) were grown in Dulbecco's Modified Eagle's Medium containing 1 g/L glucose (Eurobio, Les Ulis, France) supplemented with fetal bovine serum to a final concentration of 10 % (Perbio, Brebieres, France), 2 mM L-Glutamine, 100 U/mL Penicillin and 100 µg/mL Streptomycin (Eurobio). Cells are maintained in a 5 % CO_2 humidified atmosphere at 37 °C.

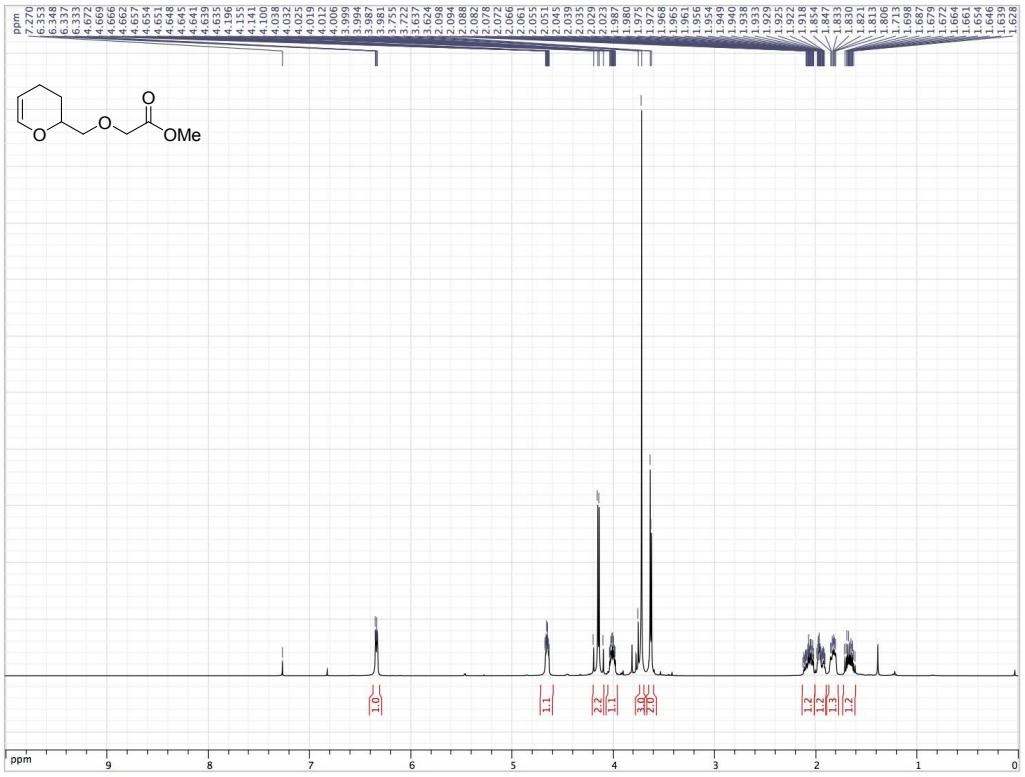
Cell culture: Mice liver BNL CL2 cells (ATCC TIB-73™) were grown in Dulbecco's Modified Eagle's Medium containing 1g/L glucose (Eurobio, Les Ulis, France) supplemented with fetal bovine serum to a final concentration of 10 % (Perbio, Brebieres, France), 2 mM L-Glutamine, 100 U/mL Penicillin and 100 µg/mL Streptomycin (Eurobio). Cells are maintained in a 5 % CO_2 humidified atmosphere at 37 °C.

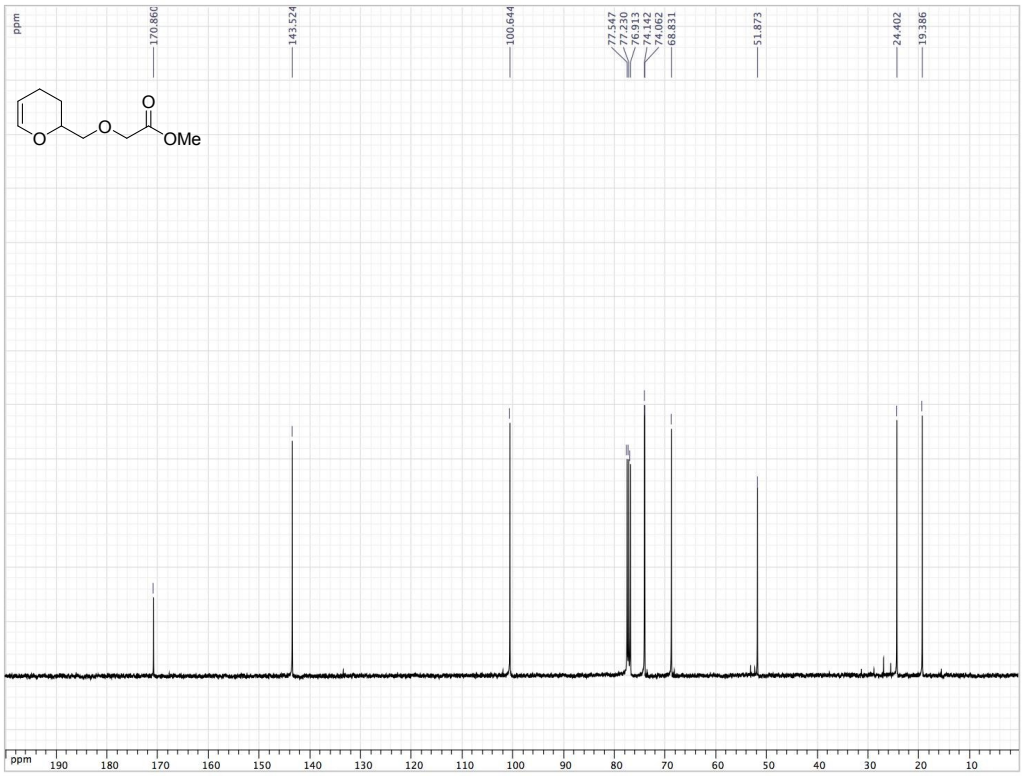
Flow Cytometry: 24 hours prior to the experiment $2.0x10^4$ NIH/3T3 cells were seeded in 96-well plates (Greiner Bio One, Frickenhausen, Germany) in Dulbecco's Modified Eagle's complete Medium. The cells were incubated during 1.5 hours with the different probes prepared at 1 µM in fresh complete medium. After removing the probes cells were incubated 30 minutes more with complete medium, then washed, re-suspended in 40 µL of trypsine and diluted in 160 µL of Phosphate-Buffered Saline with 5 mM of EDTA. A minimum of 2.000 of suspended cells per sample were analyzed using a microcapillary flow cytometer Guava EasyCyte Plus 6C (Guava technologies, Millipore Merck, CA, USA) equipped with a blue (488 nm) and a red (630 nm) laser.

Fluorescence microscopy: 24 hours prior to the experiment $2.5x10^4$ BNL CL2 cells were seeded per well in 8-well Lab-Tek II Chambered coverglass plates (ref. 155409, Nunc, Naperville, IL, USA). The fluorescent probes were diluted up to 300 µL in Dulbecco's Modified Eagles's complete Medium and added onto the cells for 1.5 hours. After washing cells were incubated with 5 µg/mL of Hoechst 33258 (ref. H1399, Invitrogen, Carlsbad, CA, USA) diluted in complete medium during 30 minutes. Cells were then washed and incubated with red phenol-free Dulbecco's Modified Eagle's Medium for microscopic observation. Cells images were acquired on a confocal Leica TSC SPE II microscope (405, 488 and 561 nm) with an image acquisition software (Leica confocal LAS AF, Leica).

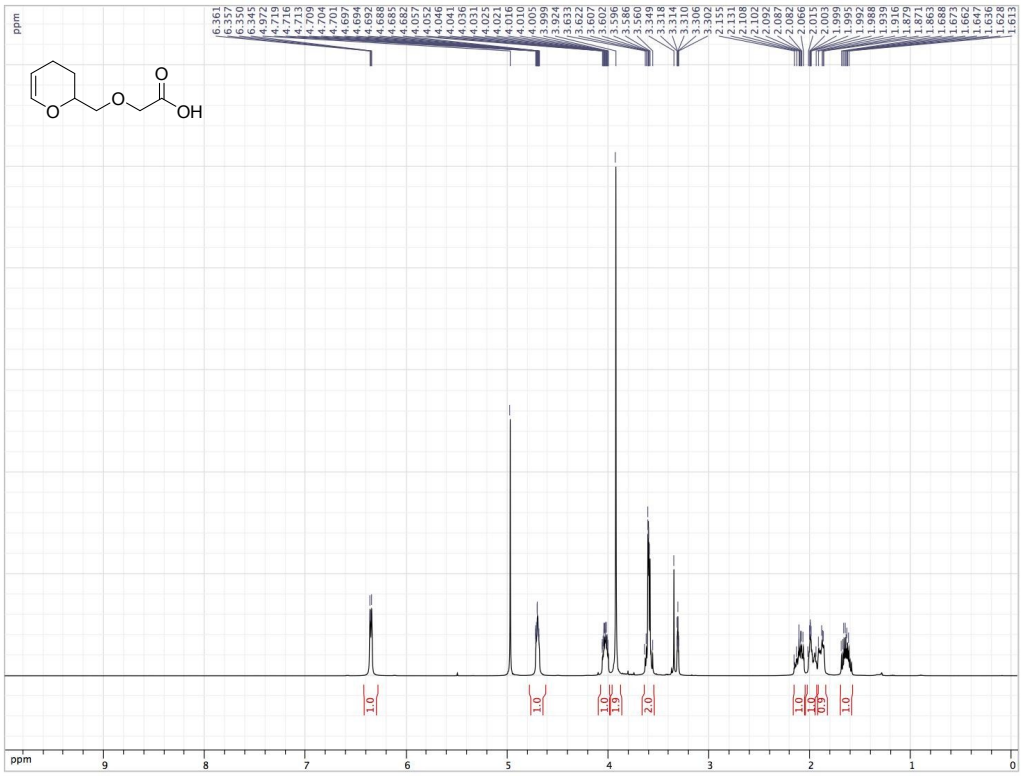
NMR Spectra

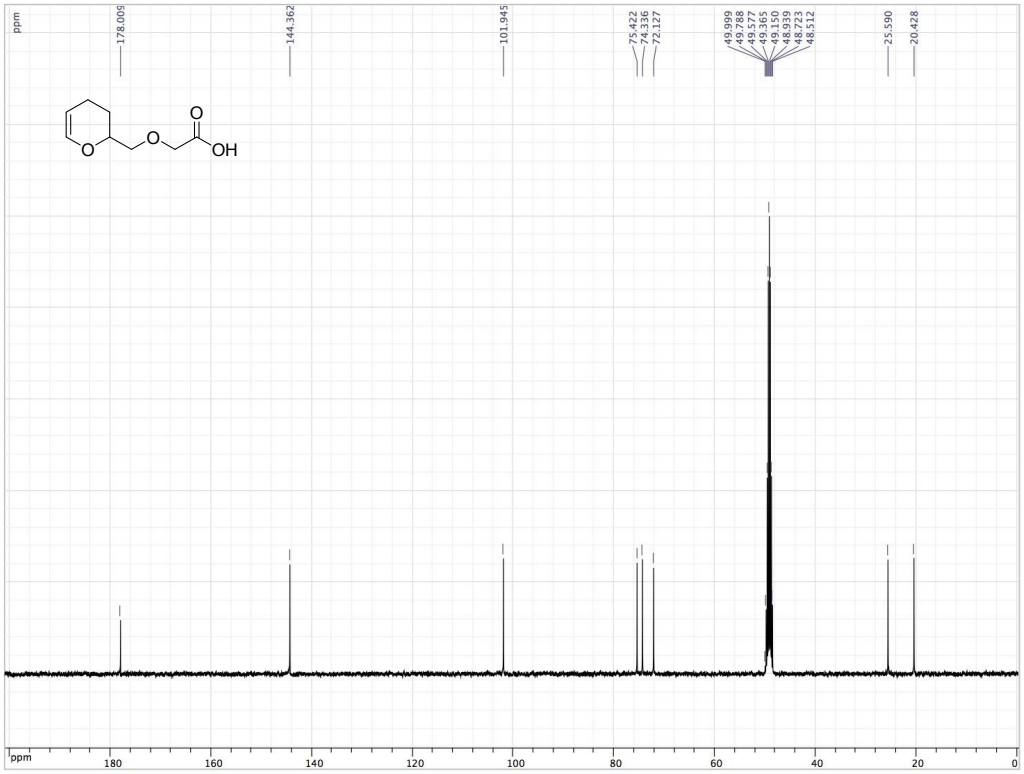
Methyl 2-((3,4-dihydro-2*H*-pyran-2-yl)methoxy)acetate



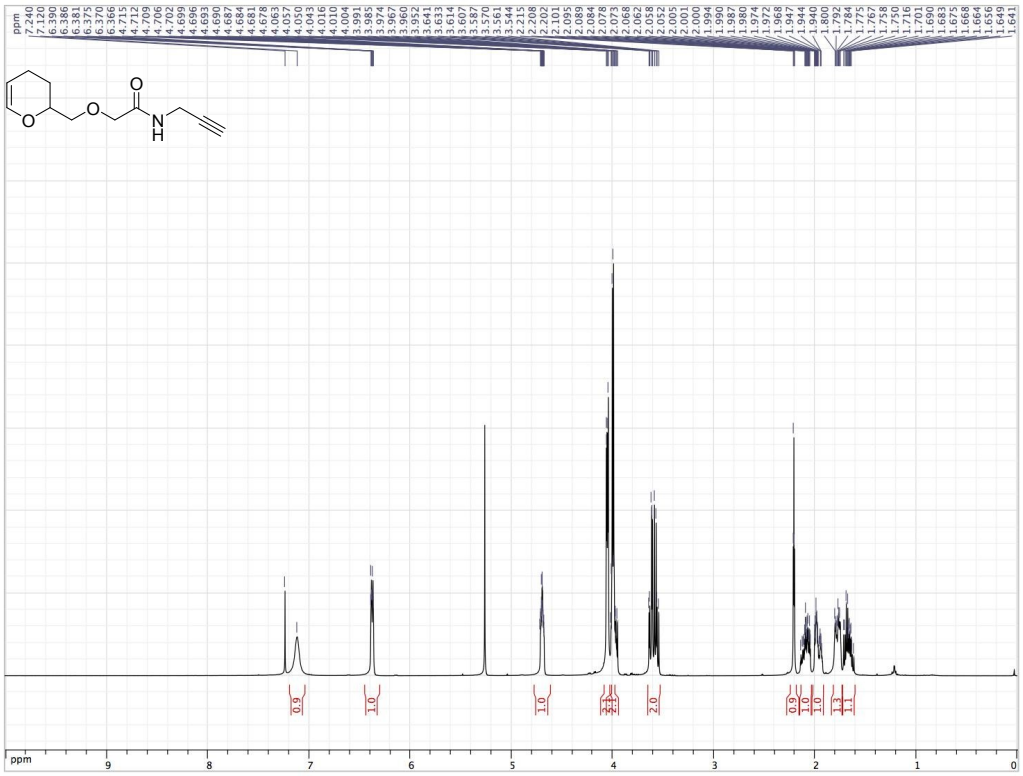


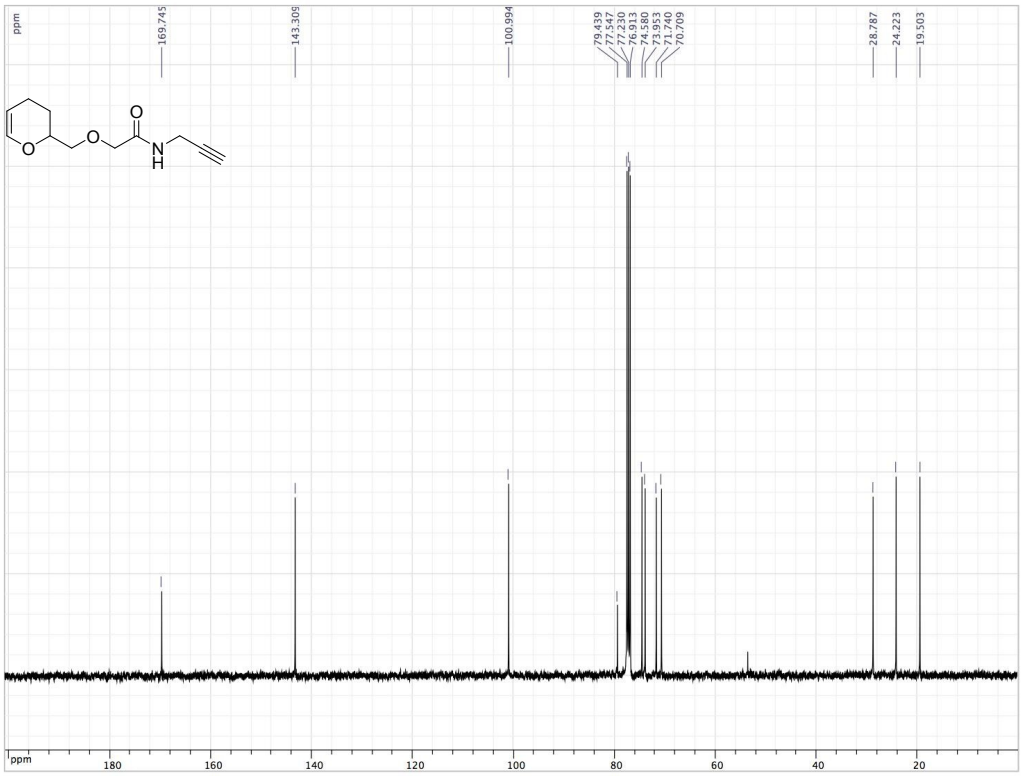
Compound 33



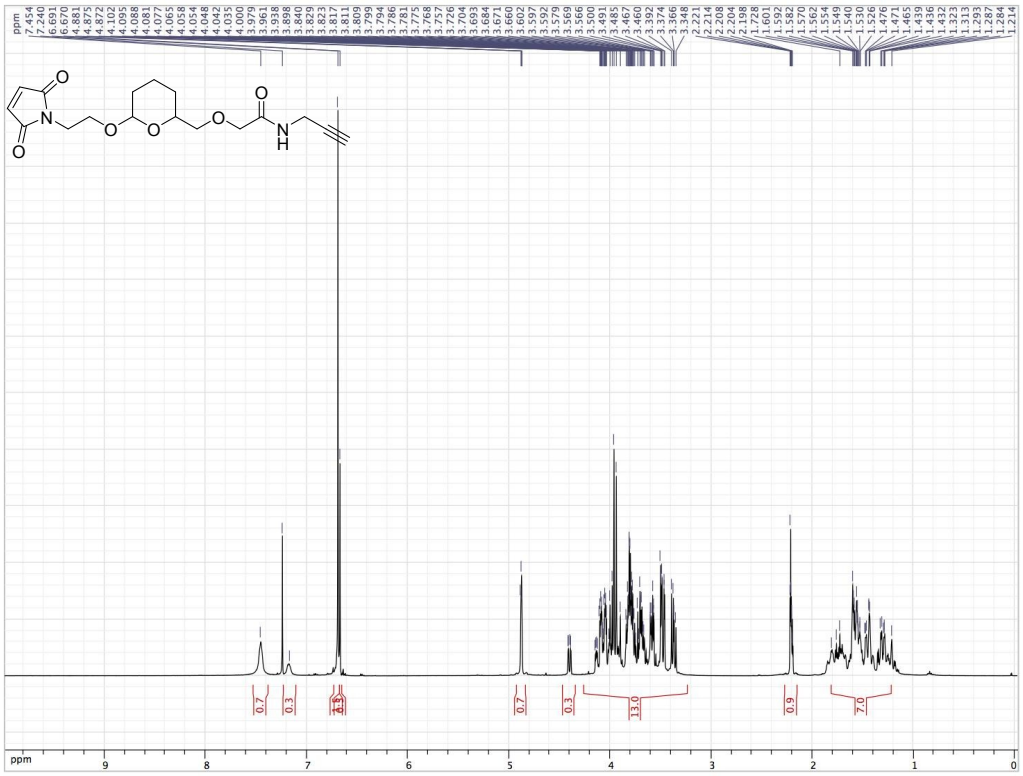


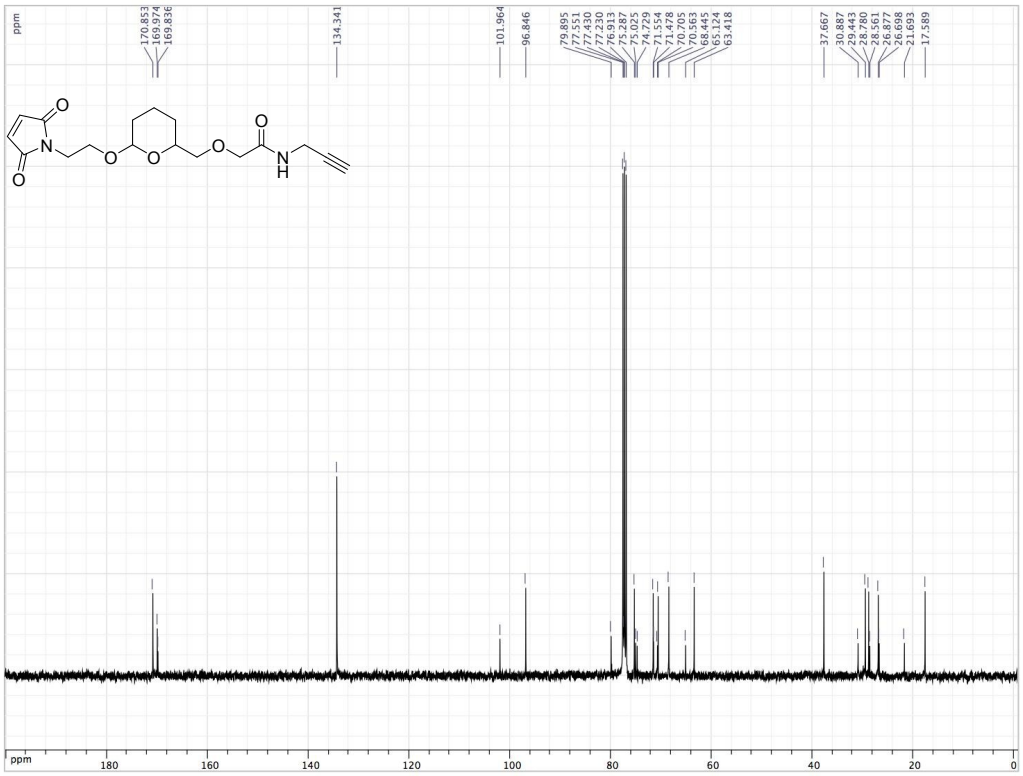
Compound 34



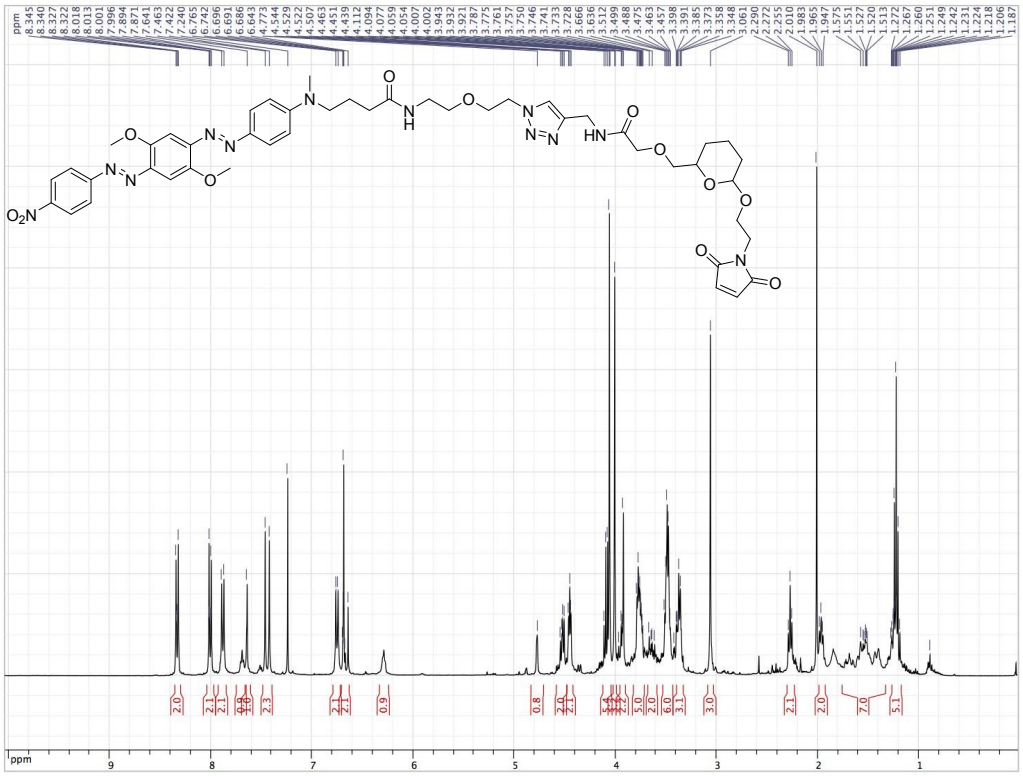


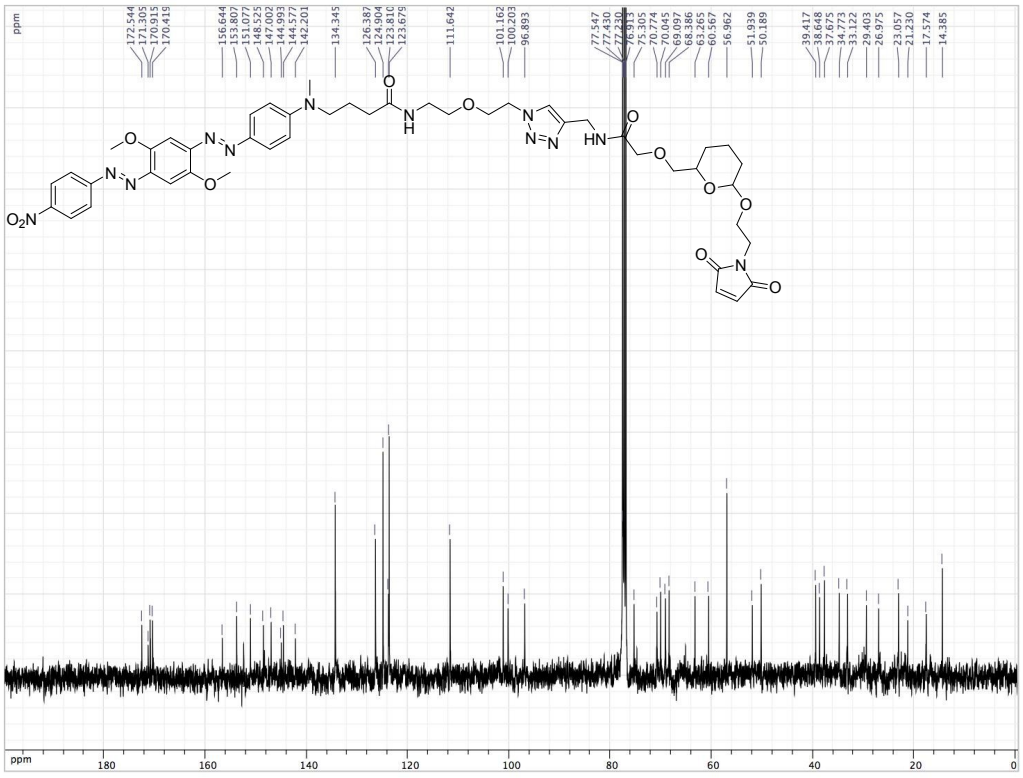
Compound 36



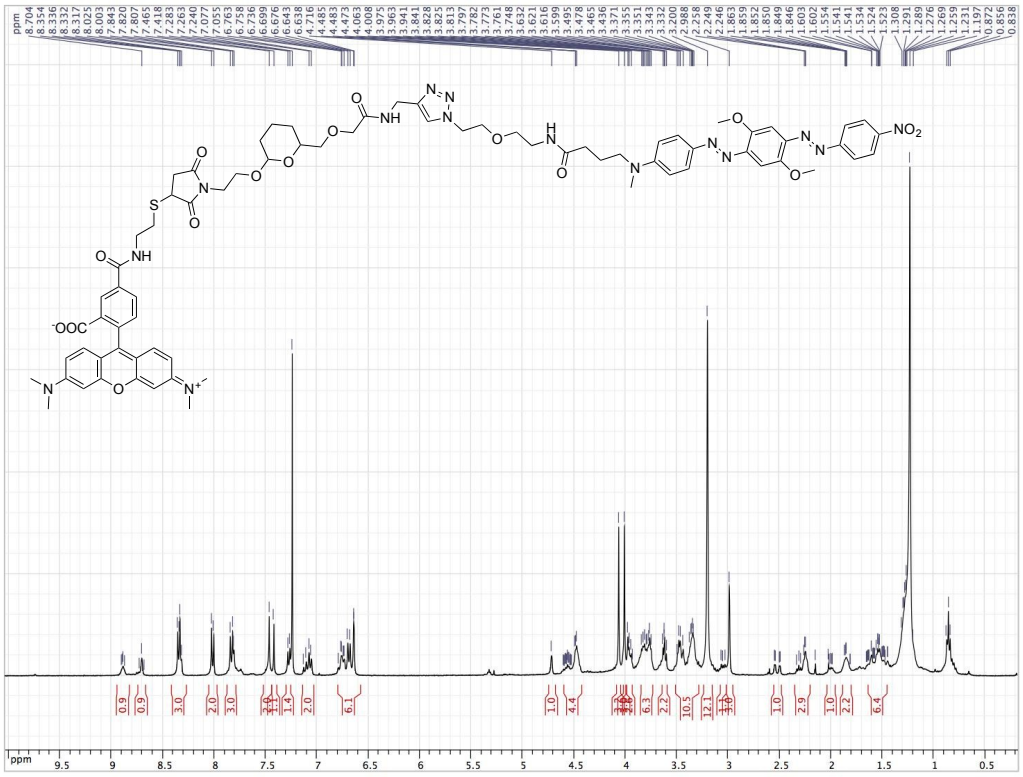


Compound 37

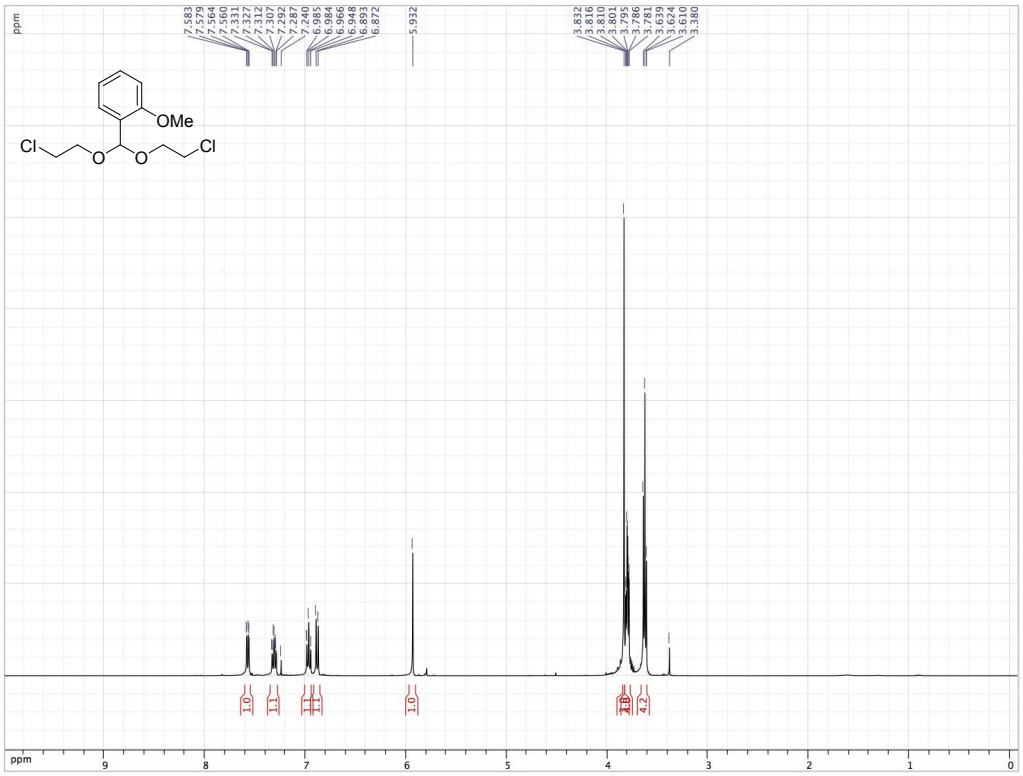




TAMRA-THP-(BHQ-2) P1



Compound 39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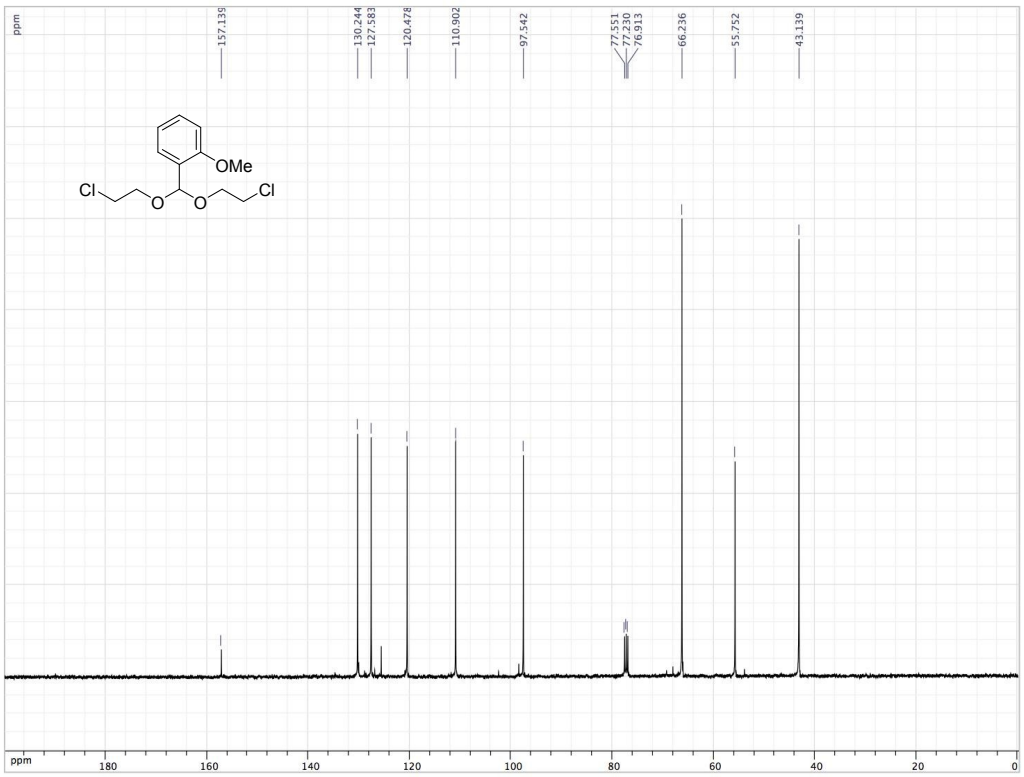
7.579
7.564
7.560
7.331
7.327
7.312
7.307
7.292
7.287
7.240
6.985
6.984
6.966
6.948
6.893
6.872
5.932
3.832
3.816
3.810
3.801
3.795
3.786
3.781
3.639
3.624
3.610
3.380
OMe
Cl
O
O
Cl
1.0
1.1
1.1
1.1
1.0
4.2
ppm
9
8
7
6
5
4
3
2
1
0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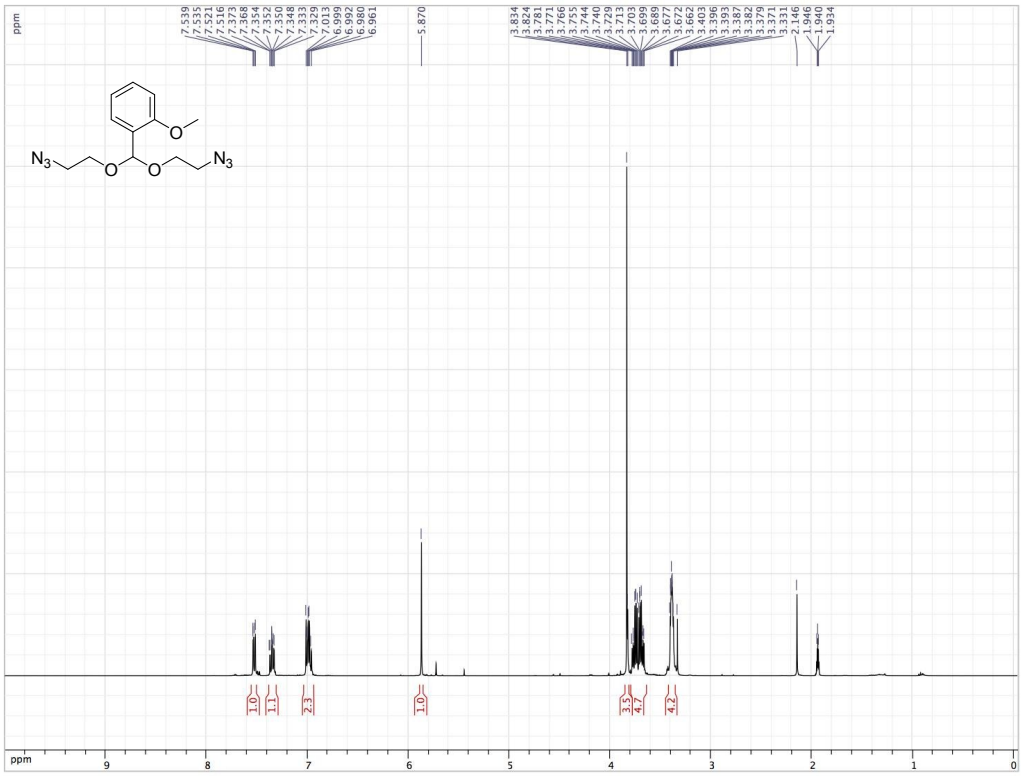
130.244
127.583
120.478
110.902
97.542
77.551
77.230
76.913
66.236
55.752
43.139
OMe
Cl
O
O
Cl
ppm
180
160
140
120
100
80
60
40
20
0

Compound 40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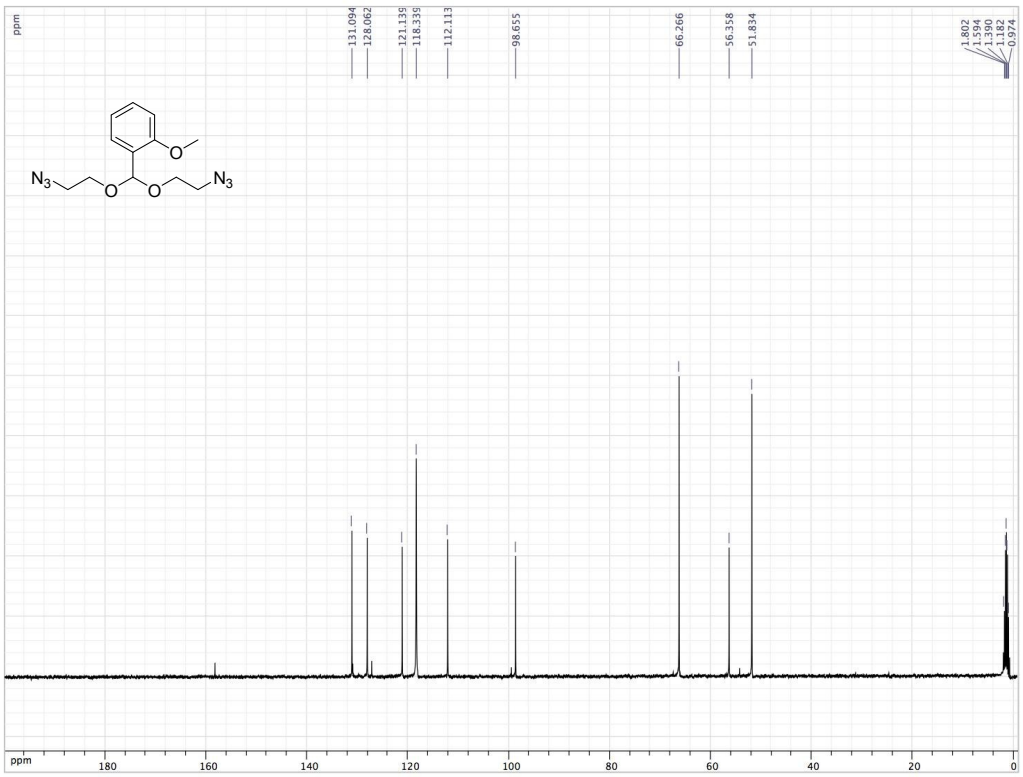
7.535
7.521
7.516
7.373
7.368
7.354
7.352
7.350
7.346
7.333
7.329
7.013
6.999
6.992
6.980
6.961
5.870
3.834
3.824
3.781
3.771
3.766
3.755
3.744
3.740
3.729
3.713
3.703
3.699
3.689
3.677
3.672
3.662
3.403
3.396
3.393
3.387
3.382
3.379
3.371
3.331
3.146
1.946
1.940
1.934
1.0
1.1
2.3
1.0
3.5
4.7
4.2
O
N3
O
O
N3
ppm
9
8
7
6
5
4
3
2
1
0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128.062
121.139
118.339
112.113
98.655
66.266
56.358
51.834
1.802
1.594
1.390
1.182
0.974
O
N3
O
O
N3
ppm
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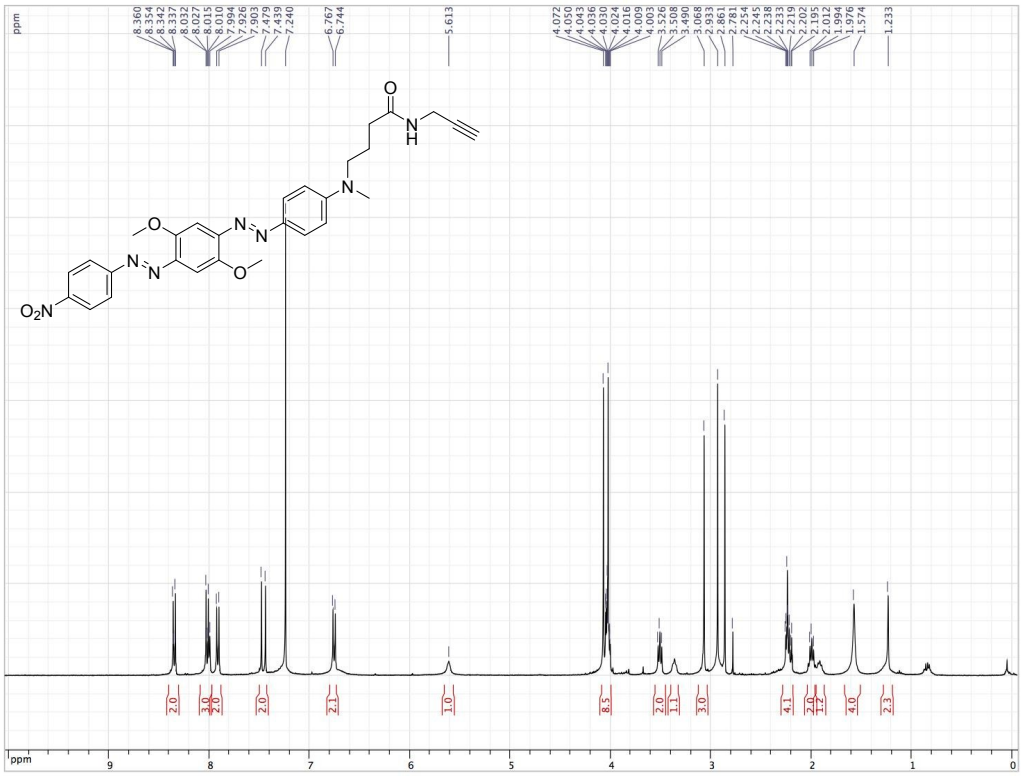
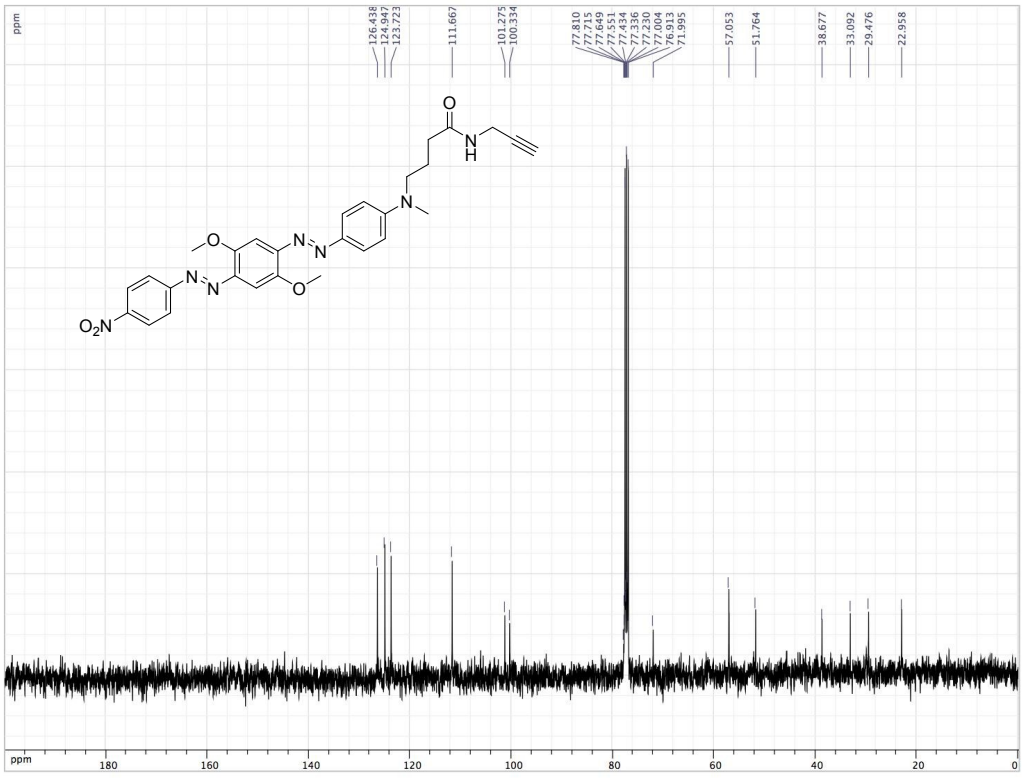
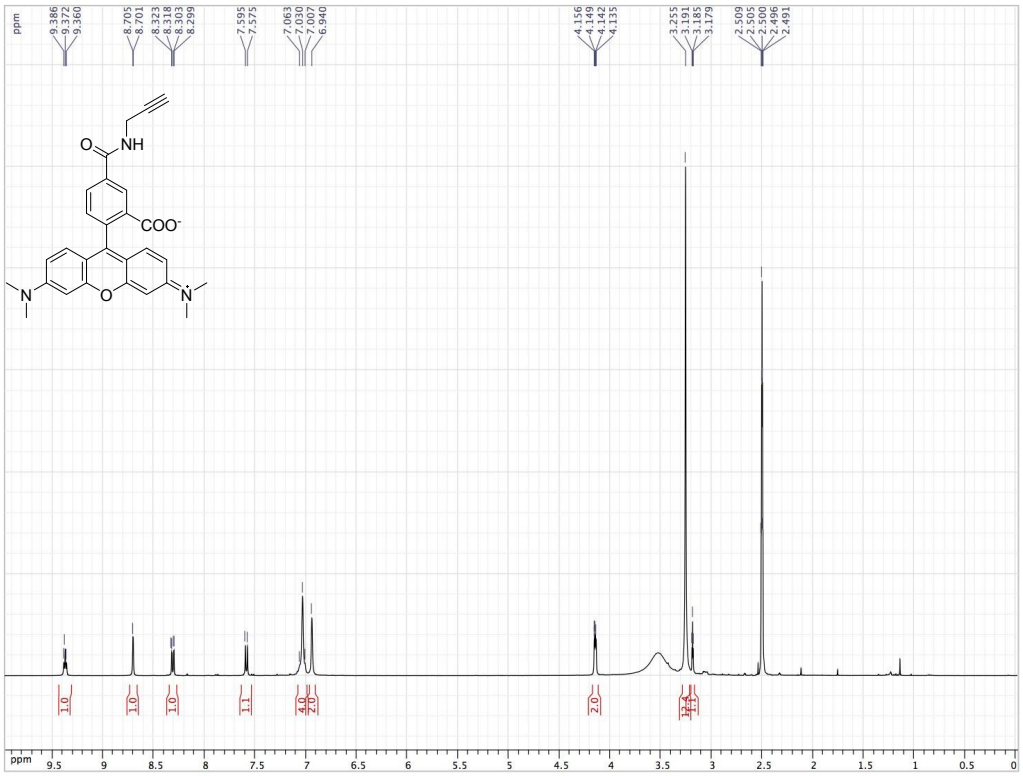
160
140
120
100
80
60
40
20
0

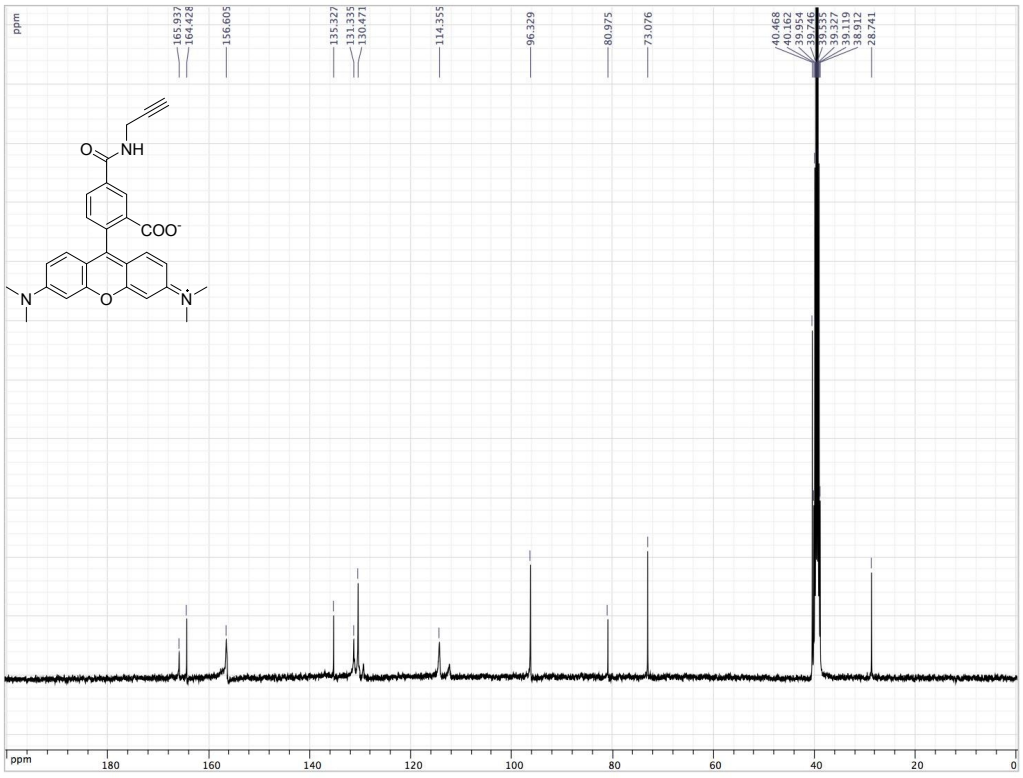
(BHQ-2)-Alkyne



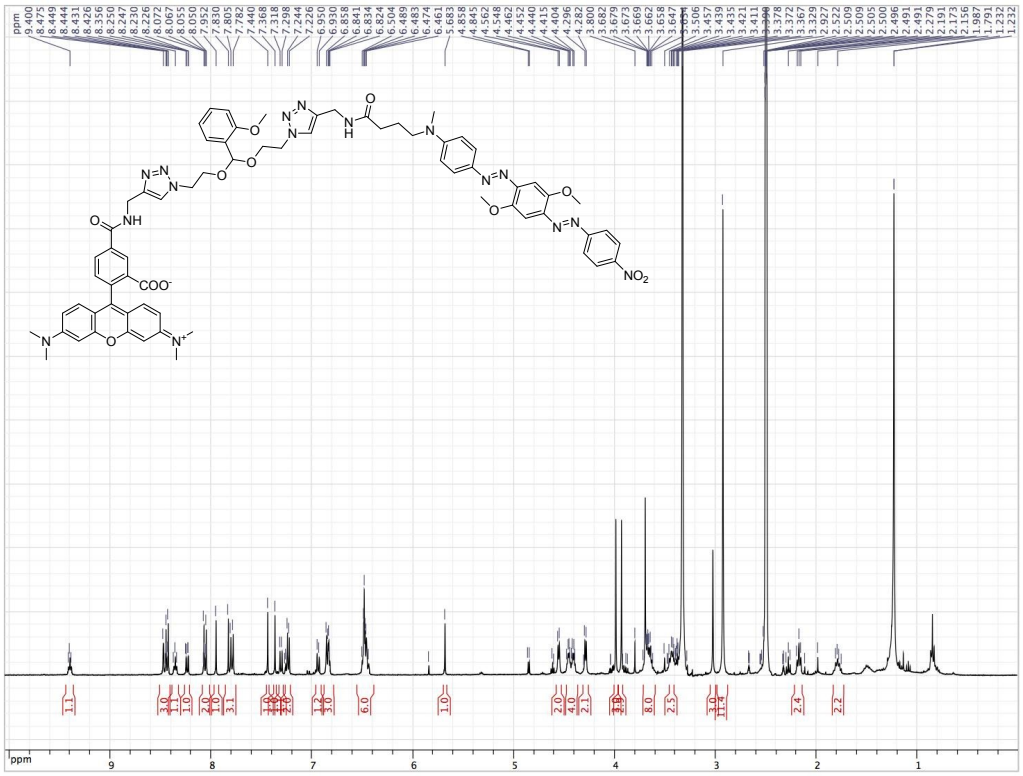


TAMRA-Alkyne





TAMRA-Acetal-(BHQ-2) P2



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