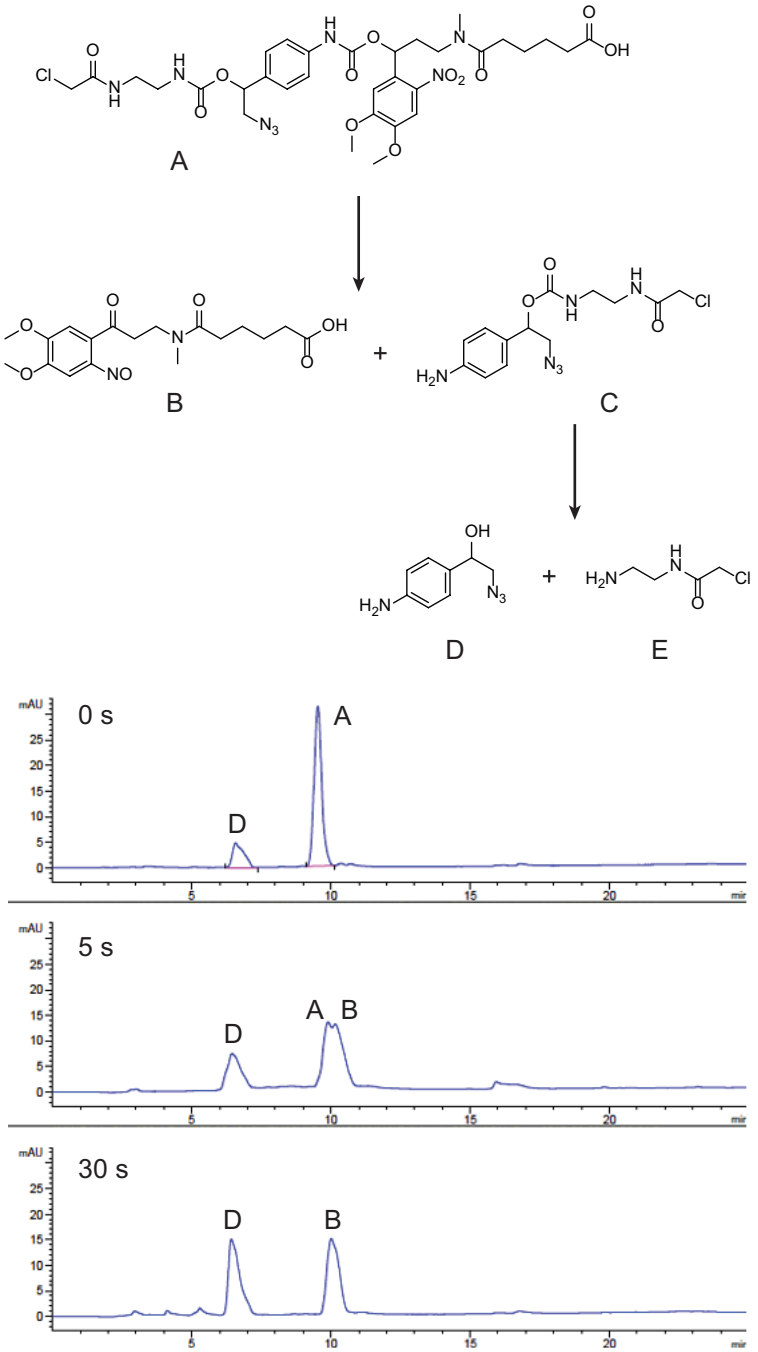


Figure S1. **HPLC analysis of linker photolysis.** The trifunctional self-immolative linker (A; compound **10**) was subjected to UV light for the indicated durations, and the reaction products were analyzed by HPLC 1 hour later. HPLC peaks predicted to concide with individual products are labeled accordingly. Product E is not UV-active and therefore not visible in HPLC chromatogram. The peak for product D at 0 sec is likely due to decomposition during handling.

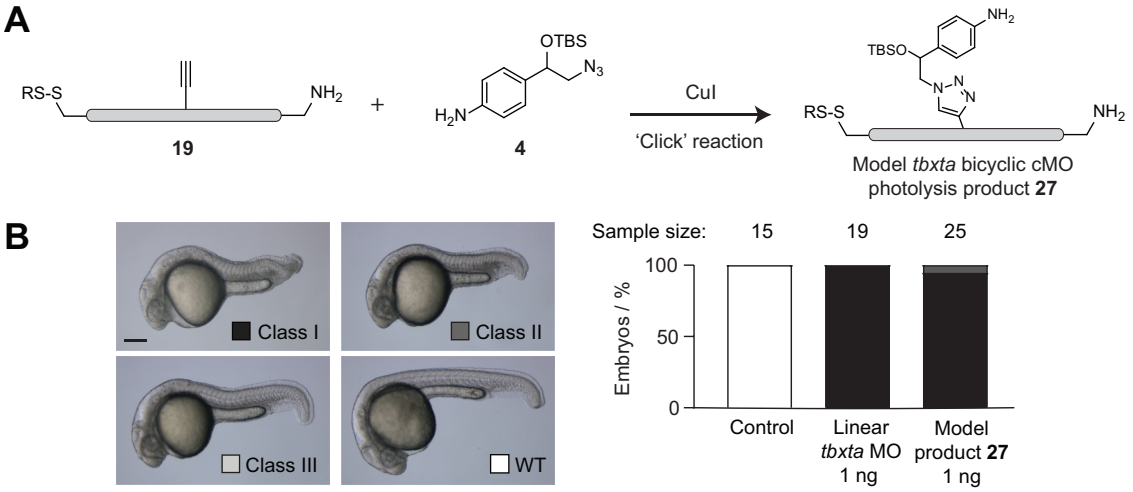


***Figure S2*. Synthesis and phentotypic assessment of a model photorelease product bearing the aminophenyltriazole scar.** (A) 'Click' reaction of the trifunctionalized *tbxta* MO **19** and azidohydroxymethylaniline **4** to yield a model bicyclic cMO photolysis product **27** with an aminophenyltriazole-bearing phosphorodiamidate linkage. The end groups in the MO were not modified. (B) Phenotypic distribution of zebrafish embryos injected with the indicated oligonucleotides (please refer to Figure 3 for a description of the phenotypic classes), demonstrating the functional efficacy of the model photolysis product. Scale bar: 200 µm.

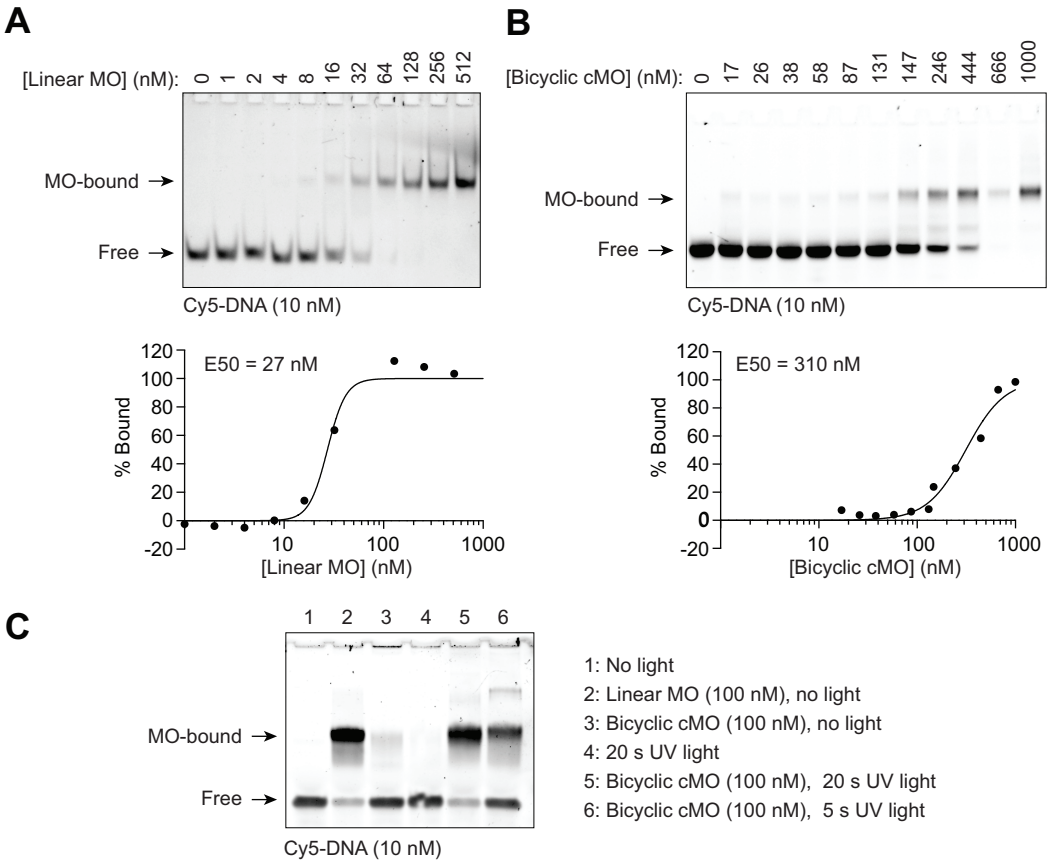


Figure S3. **Comparison of MO-DNA and bicyclic cMO-DNA duplexes.** (A) Gel-shift assay of a *tbxta* MO and Cy5-tagged complementary DNA, and quantification of the MO-bound Cy5-DNA fraction. (B) Corresponding gel-shift assay and quantification for a *tbxta* bicyclic cMO and Cy5-tagged complementary DNA. (C) Gel-shift assay demonstrating UV light-dependent binding of the *tbxta* bicyclic cMO to Cy5-tagged complementary DNA.

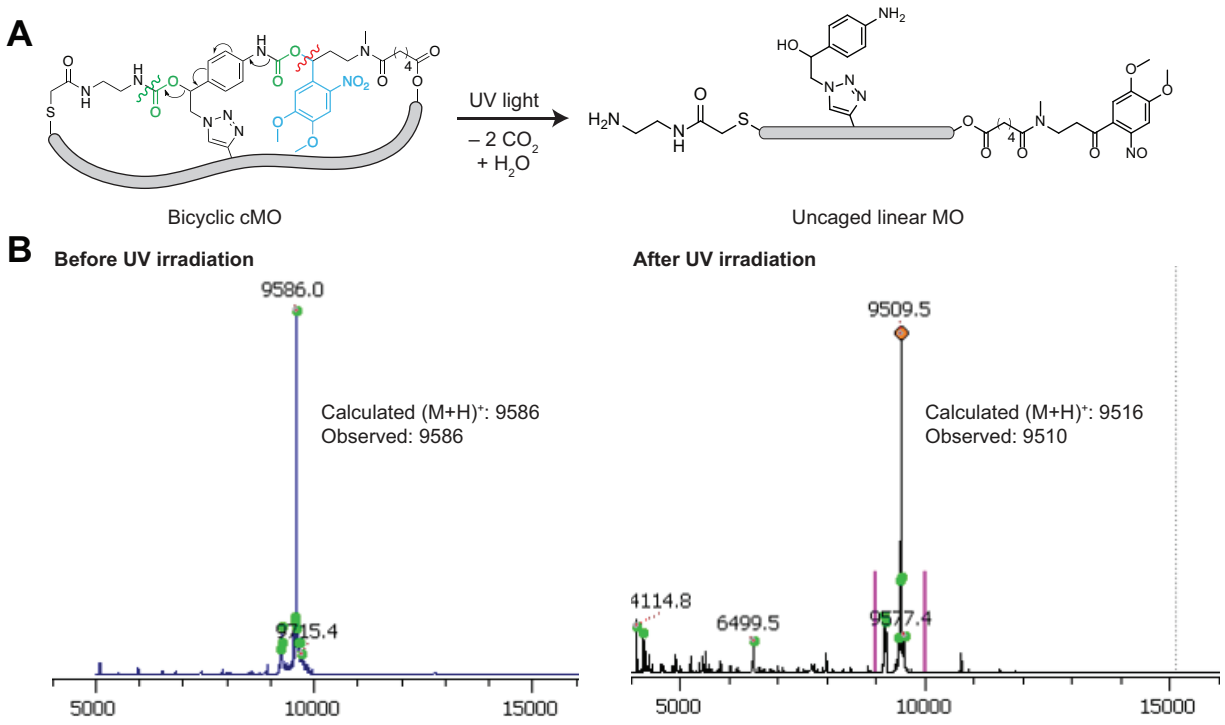


Figure S4. **Mass spectrometry analysis of bicyclic cMO photolysis.** A 10-μM solution of the *tbxta* bicyclic cMO (**25**) in water was irradiated with UV light (27.7 mW/cm^2) for 1 minute using a Leica DM4500B epifluorescence microscope equipped with a 2.5x objective, LED light source, and DAPI filter cube (Ex: 350/50nm), and the reaction was monitored by mass spectroscopy. (A) Schematic representation of the photolysis reaction. (B) Mass spectra of the *tbxta* bicyclic cMO solution before and after UV irradiation. The photolysis reaction yielded a 9510-Da mass that is within 0.1% error of the predicted oligonucleotide product (9516 Da).

MATERIALS AND METHODS

Zebrafish aquaculture and husbandry. Adult zebrafish (wildtype AB strain) were obtained from the Zebrafish International Resource Center. All zebrafish lines were raised according to standard protocols. Embryos used in these studies were obtained by natural matings and cultured at 28.5 °C or 32 °C in E3 embryo medium. All animal procedures were approved by the Administrative Panel on Laboratory Animal Care at Stanford University (Protocol 10511).

MO and cMO microinjections. Linear MO, cyclic cMO, and bicyclic cMO solutions were quantified with a Nanodrop spectrophotometer according to the Gene Tools protocols. The injection solutions were prepared in 100 mM KCl and 0.1% (w/v) phenol red. The solution was heated to 100 °C for 20 sec to dissociate MO aggregates. One-cell stage zebrafish embryos were immobilized on agarose microinjection template with E3 medium and microinjected into the cell with the MO solution (1-3 nL/embryo). All embryos were subsequently cultured in E3 medium, and unfertilized embryos were removed within 3 hours.

Photoactivation of cMOs. Zebrafish embryos were arranged in an 1.5% agarose microinjection template (560-μm x 960-μm wells) with the animal poles facing the light source. The photoactivation of the cMOs was achieved by focusing 365-nm light from a mercury short arc lamp (Osram HBO™ 103W) onto the embryo using a Leica DM4500B upright compound microscope equipped with a 20X water-immersion objective (Leica 506147, HCX APO L 20X /0.5 NA) and a narrow-band DAPI filter cube (Ex: 365 nm, 10-nm bandpass; Chroma). Each embryo was irradiated for 15 seconds. Light intensities for each photoactivation wavelength were measured using a digital energy meter (Thor Labs PM100D) and microscope slide sensor (Thor Labs S170C), yielding the following values: 365-nm mercury lamp, 48 mW/cm^2 (spot diameter = 2.0 mm).

Zebrafish imaging. Live imaging of the zebrafish was performed on manually dechorionated embryos immobilized in E3 medium containing 0.2% (w/v) low-melt agarose and 0.05% (w/v) tricaine mesylate, or in plain E3 medium. Embryos were imaged using a Leica M205FA microscope equipped with a SPOT Flex color camera and images were captured using SPOT software (Molecular Devices).

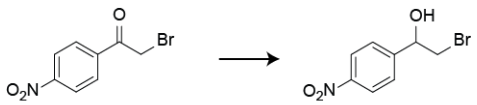
Scoring of *tbxta* mutant phenotypes. *tbxta* cyclic and bicyclic cMO-injected zebrafish embryos were cultured until 24 hours post fertilization (hpf), manually dechorionated and the phenotypes were scored by visual inspection according to the following morphological classes: Class I = a fully penetrant *tbxta* mutant phenotype; Class II = no notochord, U-shaped somites, and some posterior somites; class III = Incompletely vacuolated notochord, V-shaped somites, and shortened anterior-posterior axis; and class IV = wild-type phenotype).

Scoring of *tbx16* mutant phenotypes. Zebrafish injected with *tbx16* cyclic or bicyclic cMOs were cultured until 30 hpf, manually dechorionated and the phenotypes were scored by visual inspection according to the following morphological classes: Class I = loss of trunk somites and spade-like tailbud, Class II = partial loss of trunk mesoderm deficits and enlarged tailbud. WT = wild-type phenotype.

Gel-shift analysis of MO binding with complementary Cy5-tagged DNA. Solutions of Cy5-tagged DNA (10 μM) and varying concentrations of either the *tbxta* linear MO or bicyclic cMO in buffer (Tris 10 mM, EDTA 1 mM, pH 7.4 containing 10 mM $MgCl_2$) were denatured at 80 °C and cooled to room temperature for 20 minutes. The solutions were then maintained in the dark or irradiated with UV light (48 mW/cm^2) using a Leica DM4500B epifluorescence microscope equipped with a DAPI filter cube (Ex: 360/10) and an LED light source. The solution were left for 1 hour to allow completion of the 1,6-elimination reaction. All the samples were then mixed with orange loading dye (6X stock, Thermo Fisher) and resolved on a 15% native polyacrylamide gel at 150 V in 1X TBE buffer for 1.25 hours with an icepack in the gel tank. After electrophoresis, the acrylamide gel was analyzed with a BioRad GelDoc imager.

General synthetic procedures. All reactions were carried out in oven-dried glassware under nitrogen atmosphere. The reagents, chemicals and solvents were purchased from commercial suppliers and used without further purification unless mentioned otherwise. Reactions were stirred magnetically and monitored by thin layer chromatography (TLC), using glass-backed silica gel 60F254 (Merck, 250-μm thickness). Yields of the reactions refer to isolated compounds that are chromatographically and spectroscopically pure unless otherwise indicated. Silica gel flash chromatography was carried out using Teledyne ISCO CombiFlash systems with prepacked SiliCycle columns. The ^{1}H NMR spectra were obtained at Stanford's Nuclear Magnetic Resonance Laboratory (SMRL) using a 500 MHz Bruker Avance Spectrometer and the residual solvent peak was used as the standard. The electrospray mass spectra were obtained at Stanford University Mass Spectrometry (SUMS) facility using a Waters SQD2 LC/MS system. MO and cMO amounts (and yields) were quantified according to their 265-nm absorbances using a Nanodrop spectrophotometer and extinction coefficients and protocols provided by Gene Tools. We assumed that linker structures did not significantly change the extinction coefficients of the MO-based reagents.

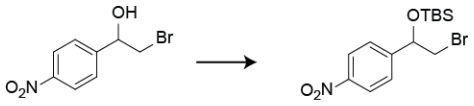
Photocleavable bicyclic linker synthesis



1

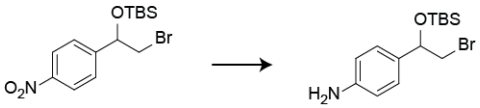
2-bromo-1-(4-nitrophenyl)ethan-1-ol. A solution of 4-nitrophenacyl bromide (3.0 g, 12.29 mmol) in dry methanol (20 mL) was cooled to 0 °C and sodium borohydride (230 mg, 6.08 mmol) in dry methanol (5 mL) was added dropwise to it. After 30 minutes, another batch of sodium borohydride (230 mg, 6.08 mmol) in dry methanol (5 mL) was added and left to stir for 1 hour. When the TLC showed complete conversion, dilute HCl (1M) was added until white precipitation occured. The precipitation was collected by filtration and was washed thoroughly with water and hexane. The filter cake was dried in air and then

under vacuum to obtain the corresponding α-bromoalcohol as an off-white solid (2.10 g, 69%). It was used in the next step without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.26 (d, *J* = 8.4 Hz, 2H), 7.61 (d, *J* = 8.4 Hz, 2H), 5.08 (dd, *J* = 8.4, 3.4 Hz, 1H), 3.71 (dd, *J* = 10.7, 3.4 Hz, 1H), 3.56 (dd, *J* = 10.7, 8.4 Hz, 1H). MS-ESI: m/z calculated for $C_8H_9BrNO_3$ $[M + H]^+$: 246.0; observed: 246.2.



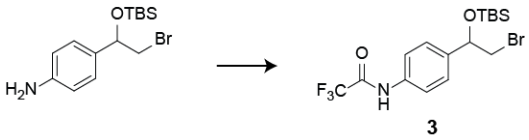
2

(2-bromo-1-(4-nitrophenyl)ethoxy)(*tert*-butyl)dimethylsilane (2). To a stirred solution of the α-bromoalcohol (1.13 g, 4.61 mmol) in dry DMF (10 mL) was added imidazole and *tert*-butyldimethylsilyl chloride at 0 °C. The reaction was left to stir at room temperature for 4 hours and DMF was removed *in vacuo*. The resulting residue was dissolved in ethyl acetate (50 mL) and washed with cold aq. 0.5 N HCl (10 mL) once and with saturated aq. $NaHCO_3$ (20 mL) twice. It was then washed with brine solution (30 mL) and dried over anhydrous Na_2SO_4. The solvents were removed under reduced pressure and the silylated product was purified by silica gel flash chromatography, eluting with hexane/EtOAc (4:1) to afford the TBDMS-protected compound **2** as a yellowish gum (1.03 g, 62%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.28 (d, *J* = 10.0 Hz, 2H), 7.60 (d, *J* = 10.0 Hz, 2H), 5.01 (dd, *J* = 6.8, 5.1 Hz, 1H), 3.58 – 3.45 (m, 2H), 0.96 (s, 9H), 0.19 (s, 3H), 0.07 (s, 3H). MS-ESI: m/z calculated for $C_{14}H_{23}BrNO_3Si$ $[M + H]^+$: 360.1; observed: 360.3.



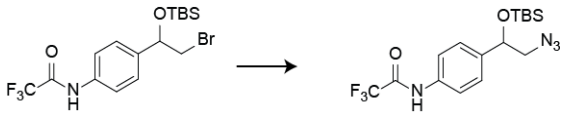
4-(2-bromo-1-((*tert*-butyldimethylsilyl)oxy)ethyl)aniline. The nitro compound (876 mg, 2.43 mmol) was dissolved in THF (30 mL), and the catalyst 5% Pd-C (10 mol%) was added in a round bottom flask. Using a two-way adapter and pump, the flask was evacuated quickly and purged with hydrogen gas from a connected balloon (1 atm). The reaction mixture was then stirred at room temperature for 2 hours.

Debromination was observed if the reaction is done for extended reaction time. The catalyst was filtered through a pad of celite, and the solvent was removed under reduced pressure. The crude material was purified through flash silica gel column, eluting with hexane/EtOAc (4:1) to afford the aryl amine product as a yellow oil (701 mg, 87%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.11 (d, *J* = 10.0 Hz, 2H), 6.64 (d, *J* = 10.0 Hz, 2H), 4.74 (dd, *J* = 8.3, 4.2 Hz, 1H), 3.67 (s, 2H), 3.49 – 3.31 (m, 2H), 0.88 (s, 9H), 0.08 (s, 3H), -0.10 (s, 3H). MS-ESI: m/z calculated for $C_{14}H_{25}BrNOSi$ $[M + H]^+$: 330.1; observed: 330.0.



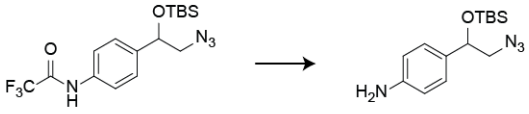
3

***N*-(4-(2-bromo-1-((*tert*-butyldimethylsilyl)oxy)ethyl)phenyl)-2,2,2-trifluoroacetamide (3).** To a stirred solution of the aryl amine (500 mg, 1.51 mmol) and triethylamine (0.231 mL, 1.66 mmol) in dry DCM (25 mL) was added trifluoroacetic anhydride (0.235 mL, 1.66 mmol) dropwise at 0 °C. After 4 hours, the reaction was quenched with water and extracted with DCM. The combined organic fractions were reduced under vacuum and purified by flash chromatography, eluting with hexane/EtOAc (4:1) to afford the product as a yellow oil (457 mg, 71%). 1H NMR (500 MHz, $CDCl_3$) δ 7.78 (s, 1H), 7.45 (d, *J* = 10.0 Hz, 2H), 7.28 (d, *J* = 10.0 Hz, 2H), 4.74 (dd, *J* = 7.6, 4.6 Hz, 1H), 3.37 – 3.25 (m, 2H), 0.78 (s, 9H), 0.00 (s, 3H), -0.18 (s, 3H). MS-ESI: m/z calculated for $C_{16}H_{22}BrF_3NO_2Si$ $[M - H]^-$: 424.1; observed: 424.1.



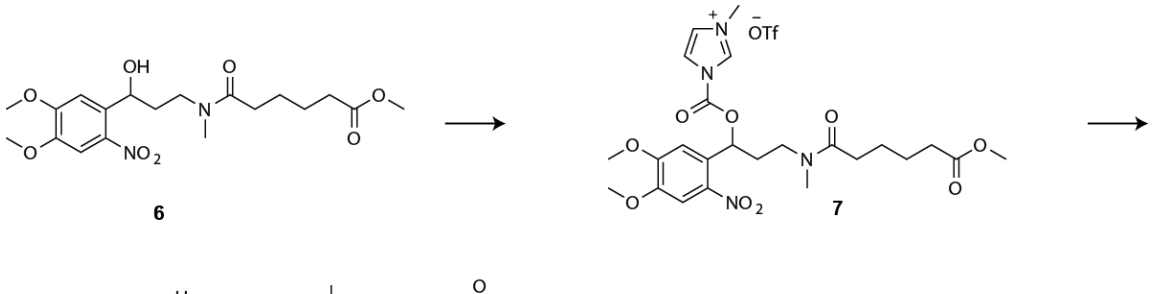
***N*-(4-(2-azido-1-((*tert*-butyldimethylsilyl)oxy)ethyl)phenyl)-2,2,2-trifluoroacetamide**. Sodium azide (300 mg, 4.61 mmol) and tetrabutylammonium iodide (37 mg, 0.01 mmol) were added to a stirred solution of the bromo-compound (368 mg, 0.95 mmol) in dry DMF (10 mL) and heated at 60 °C for overnight. The reaction mixture was concentrated *in vacuo*, diluted in EtOAc (50 mL) and washed with water (3 X 30 mL) and brine (25 mL). The solution was then dried over Na_2SO_4, concentrated under vacuum, and purified

by silica gel flash chromatography, eluting with hexane/EtOAc (4:1) to obtain the azide product as a yellow oil (256 mg, 66%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.46 (d, *J* = 8.6 Hz, 2H), 7.26 (d, *J* = 8.6 Hz, 2H), 4.71 (dd, *J* = 7.6, 3.7 Hz, 1H), 3.25 – 3.05 (m, 2H), 0.79 (s, 9H), -0.01 (s, 3H), -0.20 (s, 3H). MS-ESI: m/z calculated for $C_{16}H_{22}F_3N_4O_2Si$ [M - H]$^-$: 387.1; observed: 387.2.



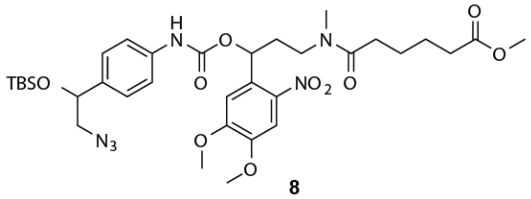
4

4-(2-azido-1-((*tert*-butyldimethylsilyl)oxy)ethyl)aniline (4). The compound (90 mg, 0.23 mmol) from previous step was stirred in 2 mL of 7 M NH_3 in MeOH in an air-tight vial for overnight. The solvent was removed under vacuum, and the residue was quickly purified by silica gel flash chromatography, eluting with DCM/MeOH (25:1) to afford the azidoaniline product as a yellow oil (56 mg, 84%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.02 (d, *J* = 8.3 Hz, 2H), 6.58 (d, *J* = 8.3 Hz, 2H), 4.62 (dd, *J* = 8.2, 3.5 Hz, 1H), 3.22 (dd, *J* = 12.5, 8.1 Hz, 1H), 3.05 (dd, *J* = 12.6, 3.6 Hz, 1H), 0.80 (s, 9H), 0.00 (s, 3H), -0.19 (s, 3H). MS-ESI: m/z calculated for $C_{14}H_{25}N_4OSi$ [M + H]$^+$: 293.1; observed: 293.0.



6

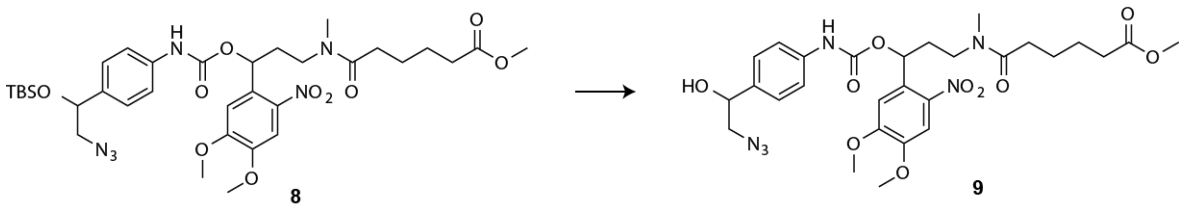
7



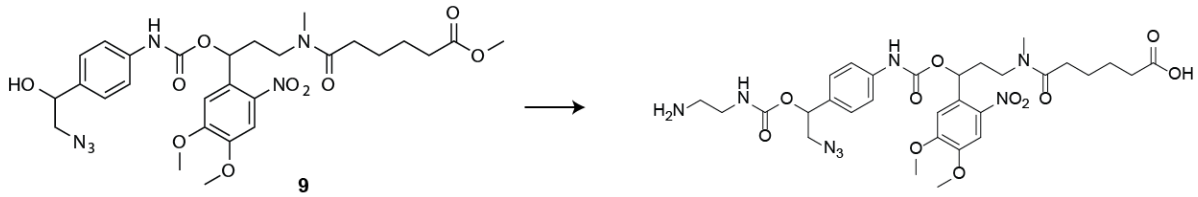
8

Methyl 6-((3-(((4-(2-azido-1-((*tert*-butyldimethylsilyl)oxy)ethyl)phenyl)carbamoyl)oxy)-3-(4,5-dimethoxy-2-nitrophenyl)propyl)(methyl)amino)-6-oxohexanoate (8). Compound **6** (120 mg, 0.29

mmol), synthesized as previously described[1], was dissolved in dry DCM (1.5 mL) and added to a solution of 1,1'-carbonyl diimidazole (118 mg, 0.72 mmol) in anhydrous DCM (1.5 mL). The reaction mixture was stirred at room temperature under N_2 atmosphere for 3 hours, at which point TLC showed complete conversion. The reaction was diluted with additional DCM (5 mL), washed twice with water (2 mL) and dried over Na_2SO_4. Solvent was removed *in vacuo* to yield the crude imidazole carbamate as a yellow gum (136 mg, 93%). The crude material was dissolved in dry DCM (2 mL) and methyl triflate (82 µL, 0.73 mmol) in dry DCM (1 mL) was added dropwise at ~10 °C in an ice-water bath. The reaction was stirred at room temperature for 10 minutes, and the solvent was quickly removed using a rotary evaporator. The vial containing the resulting compound **7** was further connected to a high vacuum pump to remove excess methyl triflate. The crude compound was then dissolved in dry DCM (2 mL) and azidoaniline compound **4** (85 mg, 0.29 mmol) in dry DCM was added directly under N_2 atmosphere. The mixture was stirred for overnight, the solvent was removed under vacuum, and the resulting residue was diluted with ethyl acetate (10 mL). The solution was washed with water and brine, concentrated, and then purified with silica gel flash chromatography, eluting with DCM/MeOH (25:1) to afford the product as a yellow oil (50 mg, 24% over three steps). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.54 (s, 1H), 7.43 (s, 1H), 7.30 – 7.25 (m, 2H), 7.19 – 7.13 (m, 2H), 6.96 (s, 1H), 6.28 – 6.14 (m, 1H), 4.67 (dd, J = 8.0, 3.6 Hz, 1H), 3.86 (s, 3H), 3.84 (s, 3H), 3.74 – 3.63 (m, 1H), 3.58 (s, 3H), 3.52 – 3.41 (m, 1H), 3.23 – 3.16 (m, 1H), 3.12 – 3.04 (m, 1H), 2.98 (s, 3H), 2.34 – 2.18 (m, 4H), 2.18 – 2.04 (m, 1H), 2.04 – 1.88 (m, 1H), 1.68 – 1.50 (m, 4H), 0.80 (s, 9H), -0.06 (s, 3H), -0.20 (s, 3H). MS-ESI: m/z calculated for $C_{34}H_{49}N_6O_{10}Si$ $[M - H]^-$: 729.3; observed: 729.4.

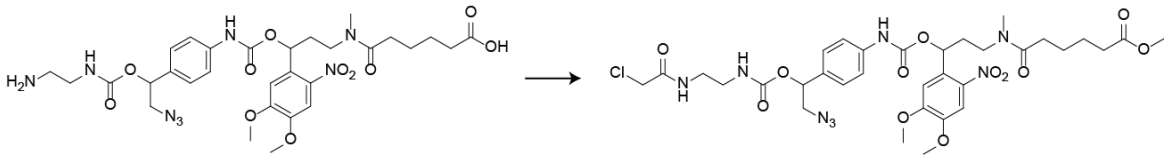


Methyl 6-((3-(((4-(2-azido-1-hydroxyethyl)phenyl)carbamoyl)oxy)-3-(4,5-dimethoxy-2-nitro phenyl)propyl)(methyl)amino)-6-oxohexanoate (9). A solution of tetra-butylammonium fluoride (1 M in THF, 55 µL, 55 µmol) was added dropwise to a stirred solution of the TBS-protected compound **8** (36 mg, 49 µmol) in dry THF (0.5 mL) under N_2 atmosphere. The reaction mixture was left at room temperature for 2 hours, at which point TLC showed complete conversion. The solvent was removed under vacuum, and the residue was purified in a silica gel pipette column, first running with hexane/EtOAc (1:1) and then eluting with DCM/MeOH (25:1) to afford the product as a yellow oil (24 mg, 80%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.63 (s, 1H), 7.46 – 7.38 (m, 1H), 7.35 (d, *J* = 8.5 Hz, 2H), 7.31 – 7.23 (m, 2H), 7.05 (s, 1H), 6.37 – 6.25 (m, 1H), 4.87 – 4.78 (m, 1H), 3.97 – 3.90 (m, 6H), 3.71 – 3.66 (m, 2H), 3.65 (s, 3H), 3.49 – 3.34 (m, 2H), 3.07 (s, 3H), 2.43 – 2.16 (m, 4H), 2.08 – 1.97 (m, 2H), 1.75 – 1.55 (m, 4H). MS-ESI: m/z calculated for $C_{28}H_{37}N_6O_{10}$ $[M + H]^+$: 617.3; observed: 617.4.

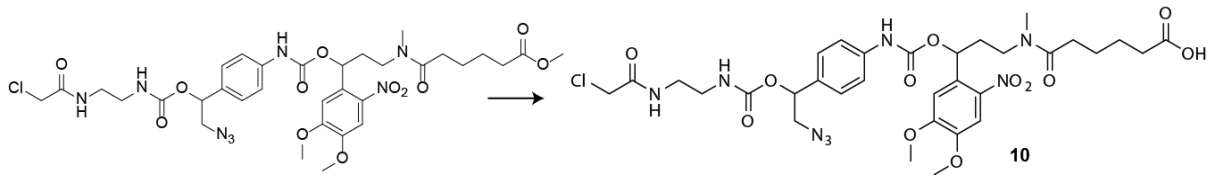


Methyl 6-((3-(((4-(1-(((2-aminoethyl)carbamoyl)oxy)-2-azidoethyl)phenyl)carbamoyl)oxy)-3-(4,5-dimethoxy-2-nitrophenyl)propyl)(methyl)amino)-6-oxohexanoate. The desilylated compound **9** (24 mg, 39 mmol) from the previous step was dissolved in dry DCM (0.5 mL) and was added to a solution of 1,1'-carbonyl diimidazole (24 mg, 150 mmol) in anhydrous DCM (0.5 mL). The reaction mixture was stirred room temperature under N_2 atmosphere for 3 hours, at which point TLC showed complete conversion. The reaction was diluted with DCM (3 mL), washed twice with water (2 mL), and dried over Na_2SO_4. The solvent was removed *in vacuo* to yield the crude imidazole carbamate as a yellow oil (27 mg, quantitative), which was used in the next reaction without further purification. The activated alcohol was dissolved in dry DCM (0.5 mL), and ethylenediamine (32 mL, 0.5 mmol) in dry DCM (0.5 mL) was added to the solution. The resulting mixture was stirred for 2.5 hours at room temperature. The solvent was

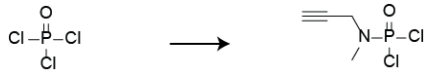
removed under reduced pressure, and the product was purified in a silica gel pipette column, eluting with DCM/MeOH (20:1) to afford the product as a yellow oil (25 mg, 91%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.49 (br s, 1H), 7.69 (s, 1H), 7.40 (d, *J* = 7.8 Hz, 2H), 7.28 – 7.20 (m, 2H), 7.02 (s, 1H), 6.40 – 6.20 (m, 1H), 5.77 (m, 1H), 5.51 (br s, 1H), 3.98 – 3.94 (m, 1H), 3.92 (s, 3H), 3.89 (s, 3H), 3.85 – 3.77 (m, 1H), 3.67 (s, 3H), 3.58 – 3.51 (m, 1H), 3.44 – 3.36 (m, 1H), 3.28 – 3.16 (m, 2H), 3.06 (s, 3H), 2.88 – 2.78 (m, 2H), 2.76 (br s, 2H), 2.45 – 2.27 (m, 4H), 2.23 – 2.14 (m, 1H), 2.07 – 1.97 (m, 1H), 1.72 – 1.55 (m, 4H). MS-ESI: m/z calculated for $C_{31}H_{42}N_8O_{11}$ $[M + H]^+$: 703.3; observed: 703.5.



Methyl 6-((3-(((4-(2-azido-1-(((2-(2-chloroacetamido)ethyl)carbamoyl)oxy)ethyl)phenyl) carbamoyl)oxy)-3-(4,5-dimethoxy-2-nitrophenyl)propyl)(methyl)amino)-6-oxohexanoate. To a stirred solution of the amine from previous step (25 mg, 35 mmol) in dry DCM (1 mL) was added chloroacetyl chloride (6.4 mL, 80 mmol) and triethylamine (25 mL, 175 mmol) at 0 °C. After 2 hours, the reaction mixture was diluted with more DCM (5 mL), washed twice with water (3 mL) and brine (3 mL), and dried over Na_2SO_4. The solvent was removed *in vacuo*, and the residue was purified in a silica gel pipette column, eluting with DCM/MeOH (20:1) to afford the metyl ester product as a yellow oil (13 mg, 48%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.67 – 7.59 (m, 1H), 7.61 (br s, 1H), 7.43 – 7.34 (m, 2H), 7.31 – 7.20 (m, 2H), 7.07 – 6.92 (s, 1H), 6.38 – 6.24 (m, 1H), 5.83 – 5.74 (m, 1H), 5.43 – 5.32 (m, 1H), 4.00 – 3.90 (m, 9H), 3.66 (s, 3H), 3.62 – 3.52 (m, 2H), 3.53 – 3.47 (m, 1H), 3.28 – 3.16 (m, 5H), 3.06 (s, 3H), 2.42 – 2.26 (m, 4H), 2.26 – 2.15 (m, 1H), 2.07 – 1.97 (m, 1H), 1.72 – 1.55 (m, 4H). MS-ESI: m/z calculated for $C_{33}H_{44}ClN_8O_{12}$ $[M + H]^+$: 779.3; observed: 779.4.

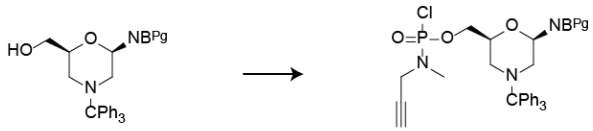


6-((3-(((4-(2-zido-1-(((2-(2-chloroacetamido)ethyl)carbamoyl)oxy)ethyl)phenyl)carbamoyl)oxy)-3-(4,5-dimethoxy-2-nitrophenyl)propyl)(methyl)amino)-6-oxohexanoic acid (10). The purified methyl ester product from the previous step (13 mg, 17 mmol) was dissolved in THF (300 mL), and lithium hydroxide (0.43 mg, 18 mmol) dissolved in water (200 mL) was added to the solution. The reaction mixture was stirred room temperature for overnight and then diluted with 1 mL EtOAc. The resulting solution was washed with 500 mL of 1M HCl, dried over Na_2SO_4 and the solvent was removed under vacuum. The residue was then purified in a silica gel pipette column, eluting with DCM/MeOH (20:1) to afford the final trifunctional linker as a yellow oil (9.5 mg, 75%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.83 (br s, 1H), 7.66 (s, 1H), 7.47 – 7.36 (m, 2H), 7.31 – 7.20 (m, 2H), 7.22 – 7.11 (br s, 1H), 7.07 – 6.92 (s, 1H), 6.40 – 6.20 (m, 1H), 5.85 – 5.69 (m, 1H), 5.68 – 5.53 (m, 1H), 4.00 – 3.84 (m, 9H), 3.63 – 3.25 (m, 7H), 3.11 (s, 3H), 2.54 – 2.14 (m, 5H), 2.08 – 1.94 (m, 1H), 1.72 – 1.55 (m, 4H). HRMS-ESI: m/z calculated for $C_{32}H_{42}ClN_8O_{12}$ $[M + H]^+$: 765.261; observed: 765.434.



Methyl(prop-2-yn-1-yl)phosphoramidic dichloride. Following a previous report of dichlorophosphoramidate reagent synthesis,[2] a solution of phosphorus oxychloride (268 μL, 2.89 mmol) in dry DCM (10 mL) at 0 °C was treated with triethylamine (422 μL, 3.03 mmol) and N-methylpropargylamine (244 μL, 2.89 mmol) under N_2 atmosphere. The reaction was stirred overnight and quenched with 10% aqueous KH_2PO_4 (20 mL). The resulting mixture was extracted thrice with DCM (30 mL), dried over Na_2SO_4, and concentrated under vacuum. The product was then isolated as a colorless oil (420 mg, 78%) by silica gel column chromatography, eluting with DCM/MeOH (20:1). ^{1}H

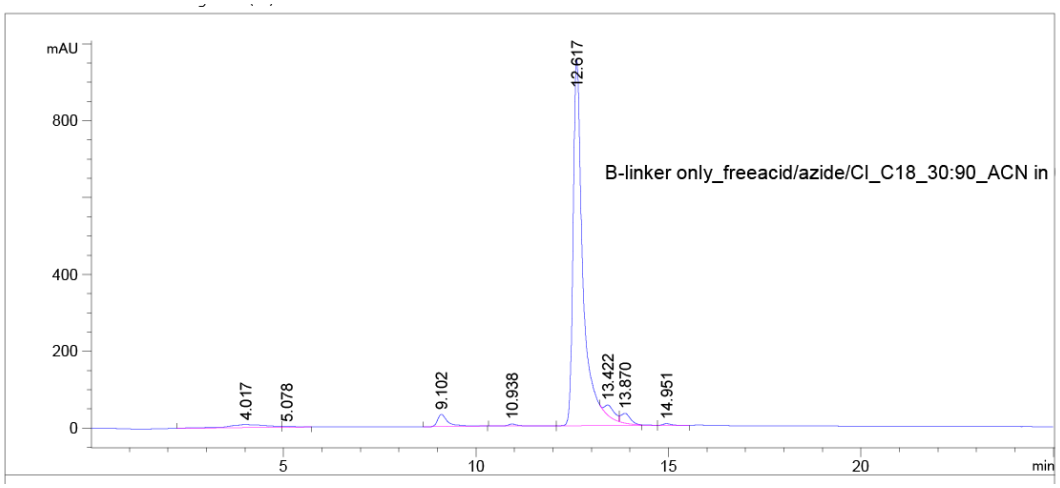
NMR (500 MHz, $CDCl_3$) δ 4.10 (d, *J* = 2.5 Hz, 1H), 4.07 (d, *J* = 2.5 Hz, 1H), 2.99 (s, 1.5H), 2.96 (s, 1.5H), 2.40 (t, *J* = 2.5 Hz, 1H). -ESI: m/z calculated for $C_4H_6Cl_2NaOP$ $[M + Na]^+$: 208.9; observed: 209.0.



13 **17**

(6-(6-benzamido-9H-purin-9-yl)-4-tritylmorpholin-2-yl)methyl methyl(prop-2-yn-1-yl) phosphoramidochloridate (17). 5'-Hydroxyl morpholino monomers were prepared as previously described.[3] LiBr was dried under vacuum using a $CaCl_2$ trap overnight and DBU was passed through a short activated neutral alumina column. A solution of the adenine morpholino monomer **13** (130 mg, 0.22 mmol) in dry DCM and acetonitrile mixture (1:1, 5 mL) was cooled to about 10 °C using an ice-bath under nitrogen atmosphere. Solid dry LiBr (440 mg, 5.0 mmol) was added at once and stirred to dissolve. Dry DBU (76 µL, 5.0 mmol) diluted in 250 µL DCM was then added dropwise, followed by the alkyne chlorophosphororamidate reagent (490 mg, 0.26 mmol) diluted in 750 µL DCM. The reaction was stirred for an hour with continuous TLC monitoring until about 80% of the starting nucleoside was consumed. The reaction mixture was filtered through a cotton plug and concentrated in rotary evaporator without heating. The resulting residue was then rapidly purified by flash chromatography using 2-10% acetone in DCM to yield the activated adenine monomer as a white fluffy solid (45 mg, 46%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.05 (d, *J* = 4.0 Hz, 1H), 8.82 (s, 1H), 8.02 (s, 1H), 7.62 (t, *J* = 7.4 Hz, 1H), 7.53 (t, *J* = 7.6 Hz, 6H), 7.39 – 7.08 (m, 13H), 6.45 (dt, *J* = 10.1, 2.9 Hz, 1H), 4.56 (qd, *J* = 6.2, 2.3 Hz, 1H), 4.28 – 4.08 (m, 2H), 3.94 – 3.67 (m, 2H), 3.57 (ddt, *J* = 10.9, 5.2, 2.4 Hz, 1H), 3.29 (ddt, *J* = 12.0, 4.7, 2.3 Hz, 1H), 2.73 (dd, *J* = 13.1, 6.0 Hz, 3H), 2.28 (t, *J* = 2.5 Hz, 0.5H), 2.22 (t, *J* = 2.5 Hz, 0.5H), 1.87 (ddd, *J* = 12.5, 10.0, 3.0 Hz, 1H), 1.68 (td, *J* = 11.3, 7.7 Hz, 1H). MS-ESI: m/z calculated for $C_{40}H_{37}ClKN_7O_4P$ $[M + K]^+$: 784.2; observed: 784.4.

HPLC analysis of linker 10. HPLC conditions: C18 column, continuous gradient of 30 – 90% acetonitrile (A) in 0.1% TFA in water (B) over 25 minutes at room temperature.



HPLC monitoring of the linker photolysis reaction. A 1.5-mM solution of linker **10** in PBS-acetonitrile (4:1) was irradiated with a 365-nm, 5 mW/cm^2 handheld UV lamp for 0, 5, or 30 seconds. The solution was left at room temperature for 1 hour to allow the 1,6-elimination reaction to proceed and then analyzed by HPLC. We noted partial decomposition of the linker without exposure to the handheld UV lamp, which could be due to light exposure during sample handling. HPLC conditions: analytical C18 column, continuous gradient of 5 – 95% acetonitrile (A) in water (B) over 25 minutes.

Alkyne-functionalized linear morpholinos synthesis. The activated alkyne-chlorophosphoramidate monomers were incorporated during solid-phase synthesis of *tbxta* and *tbx16* MOs by Gene Tools, LLC. The MOs were prepared were 5'-amine, 3'-disulfide modifications and had the following sequences (alkyne-modified monomers are shown in bold font).

tbxta MO: 5′-GACTTGAGGCAG**A**CATATTTCCGAT-3′

tbx16 MO: 5′-CTCTGATAGCC**T**GCATTATTTAGCC-3′

Optimized protocol for bicyclic cMO synthesis

Bicyclic *tbxta* cMO (25). A 25-base *tbxta* MO oligomer with 5'-amine, 3'-disulfide and internal alkyne functionalization **19** (5'-GACTTGAGGCAG**A**CATATTTCCGAT-3') (Gene Tools) was employed for the bicyclic cMO synthesis. The alkyne-modified adenine monomer is shown in bold font. The bicyclic cMO synthesis strategy utilizes three orthogonal reactions: Cu (I)-catalyzed click reaction, amide coupling, and thiol-halogen exchange. In the optimized protocol, the Cu-click reaction was performed first followed by amide coupling and thiol halogen reaction.

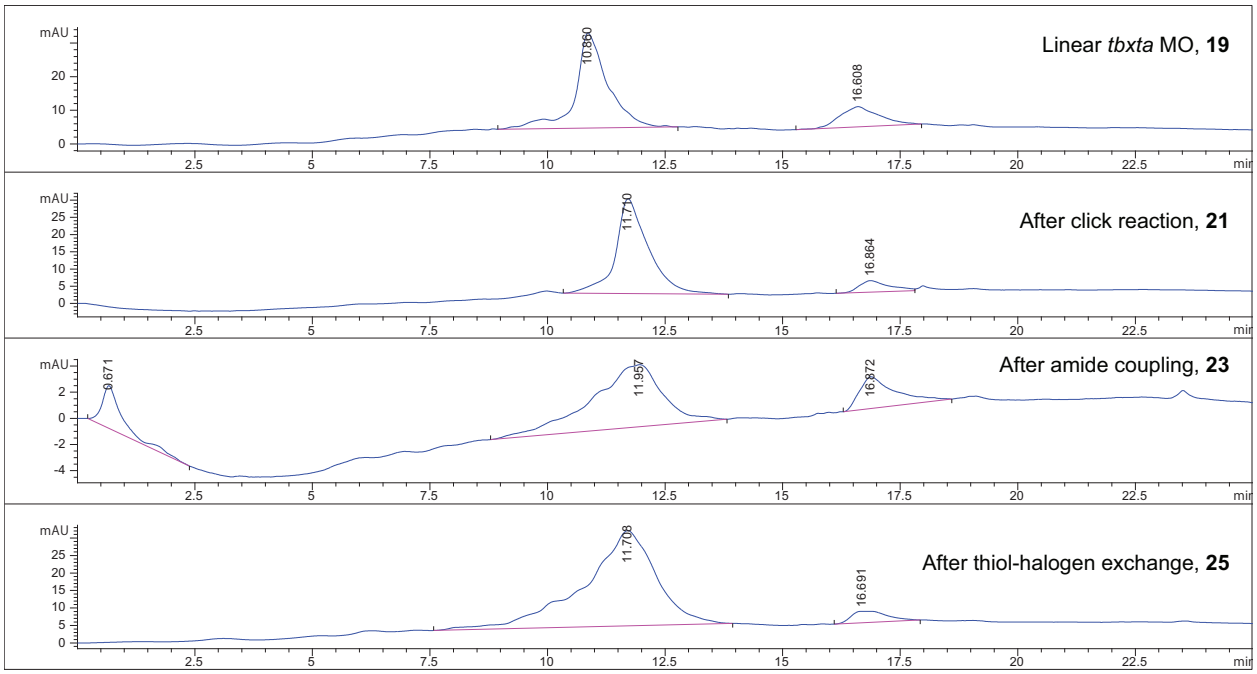
The click reaction was done with 3 equivalent excesses of the linker in a 0.5 M TEAA – DMSO buffer with a CuI – TBTA catalyst. In a typical reaction, 40 nmol of alkyne-morpholino (in 40 µL water) was dissolved in 80 µL of 1 M TEAA buffer and 60 µL DMSO. It was then sonicated to degas and dissolve well. The solution was further purged with N_2 gas to remove dissolve oxygen. 5 µL of CuI (20 mM in DMSO) and 5 µL of TBTA (50 mM in DMSO) was premixed and added to the above reaction. The tube was sealed quickly after purging N_2 and was shaken at 37 °C for 18 hours, at which point HPLC and mass spectroscopy showed complete conversion. It was important to use Cu (I) salts in this reaction, although they may oxidize quickly if the degassing is not optimal and multiple additions were sometimes necessary. Cu (II) and reducing agents like ascorbate were found to react with the disulfide. To remove any unreacted alkyne-morpholino, 100 µL of azide-agarose (~1.5 µmol azide groups), and fresh and same amount of CuI-TBTA added and reacted for 4 hours. The resin was filtered through a spin filter column, washed with water several times, evaporated and purified with Nap-5 column. The solution was lyophilized to dryness, affording the conjugated product **21** (53.3 nmol, 95%) as a white solid. Typical yields for the click reaction yields are 50 – 72%. MS-ESI (m/z): [M+H]+ calculated for **21**: 9758; observed: 9760.

The amide coupling on **21** to generate the first lariat was achieved with an excess of DMTMM (55 mM) in 1 mL MOPS (100 mM)- NaCl (1 M), pH 7.5 buffer at 37 °C for 18 h. Assuming that all the free carboxylic acid was converted to activated ester, the reaction was treated with 200 µL ω-aminohexyl

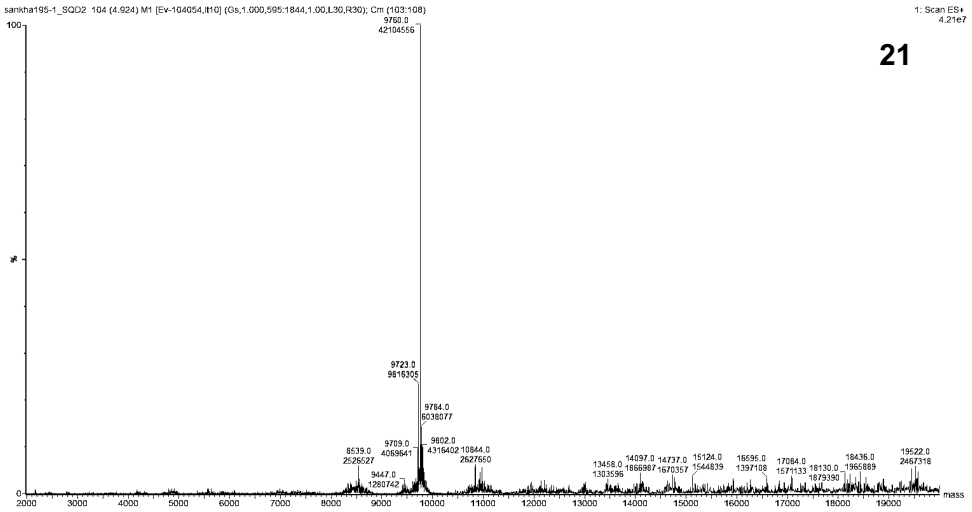
agarose (~ 1 µmol amine groups) to remove unreacted morpholino. The excess reagent and salts were purified with a Nap-25 column. Typical recovered yields are 40 - 55%. MS-ESI (m/z): [M+H]+ calculated for **23**: 9740; observed: 9742.

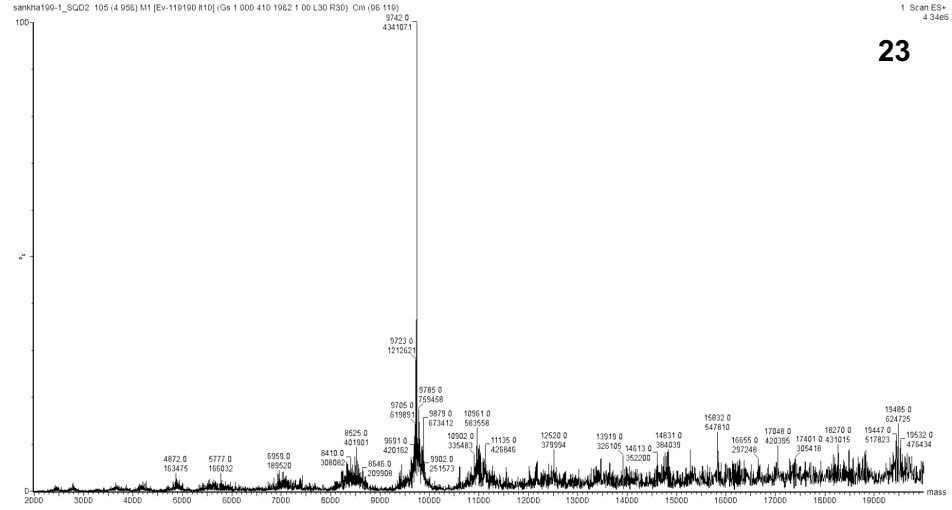
The thiol-halogen exchange reaction was performed as the final step. Typically, the purified morpholino **23** from the previous step was dissolved in 200 µL of 0.1 M Tris-HCl buffer (pH 7.5) and treated with 10 equivalent TCEP-HCl (10 mM solution in water) and mixed gently at room temperature for 15 minutes. It was diluted with another 300 µL of water and dialyzed with Amicon centrifuge tubes (3500-Da MW cutoff) thrice to remove the TCEP. The morpholino was recovered with 0.1 M Tris-HCl buffer (total 1 mL, pH 8.5) and reacted for 18 hours at 37 °C. To remove unreacted thiols, the solution was treated with 100 µL of maleimide agarose beads (~1 µmol groups, Nanocs) and finally purified with Nap-5 columns. Typical isolated yields are 45-60%. MS-ESI (m/z): [M+H]+ calculated for **25**: 9586; observed: 9588.

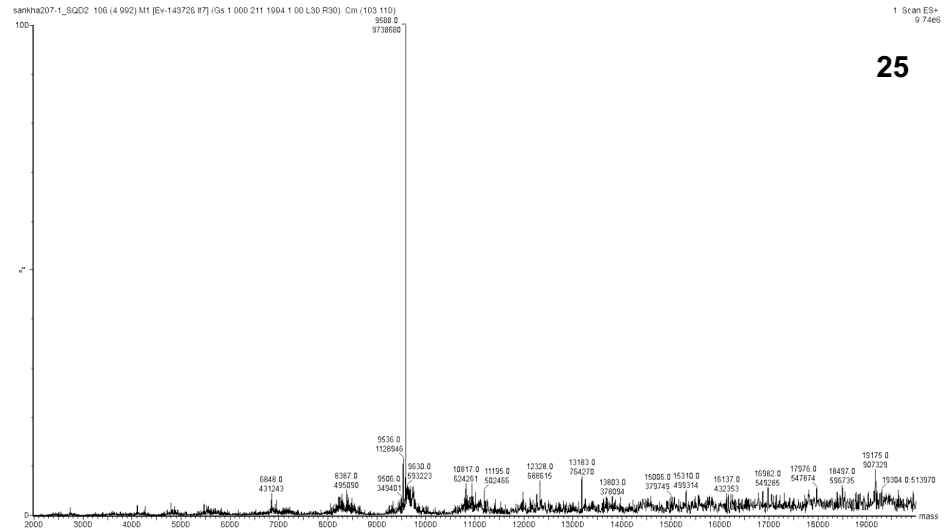
The products in each step did not have efficient separation in the HPLC using all the conditions we tried. However, the click reaction yielded a ~1 minute increase in MO retention time and could be used to monitor its progress. HPLC conditions: analytical C18 column, continuous gradient of 10-95% of acetonitrile (A) in 0.1% TFA in in H_2O (B) over 25 minutes at room temperature.



Representative mass spectra for each purified *tbxta* MO construct are shown below:

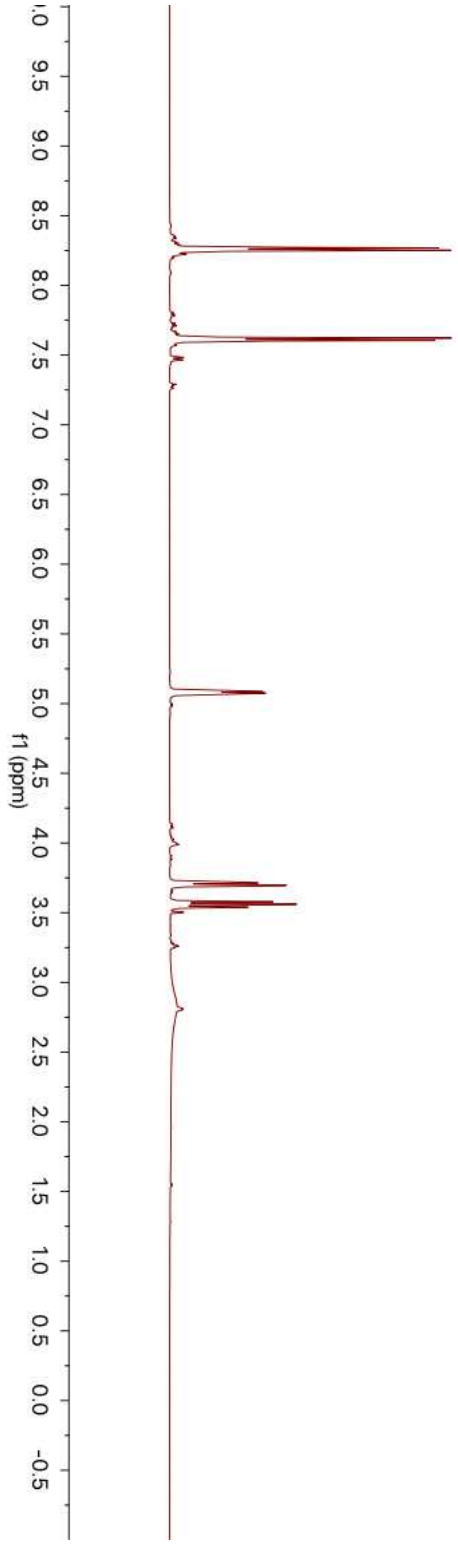


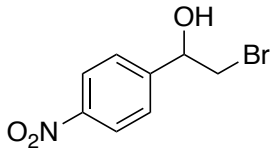




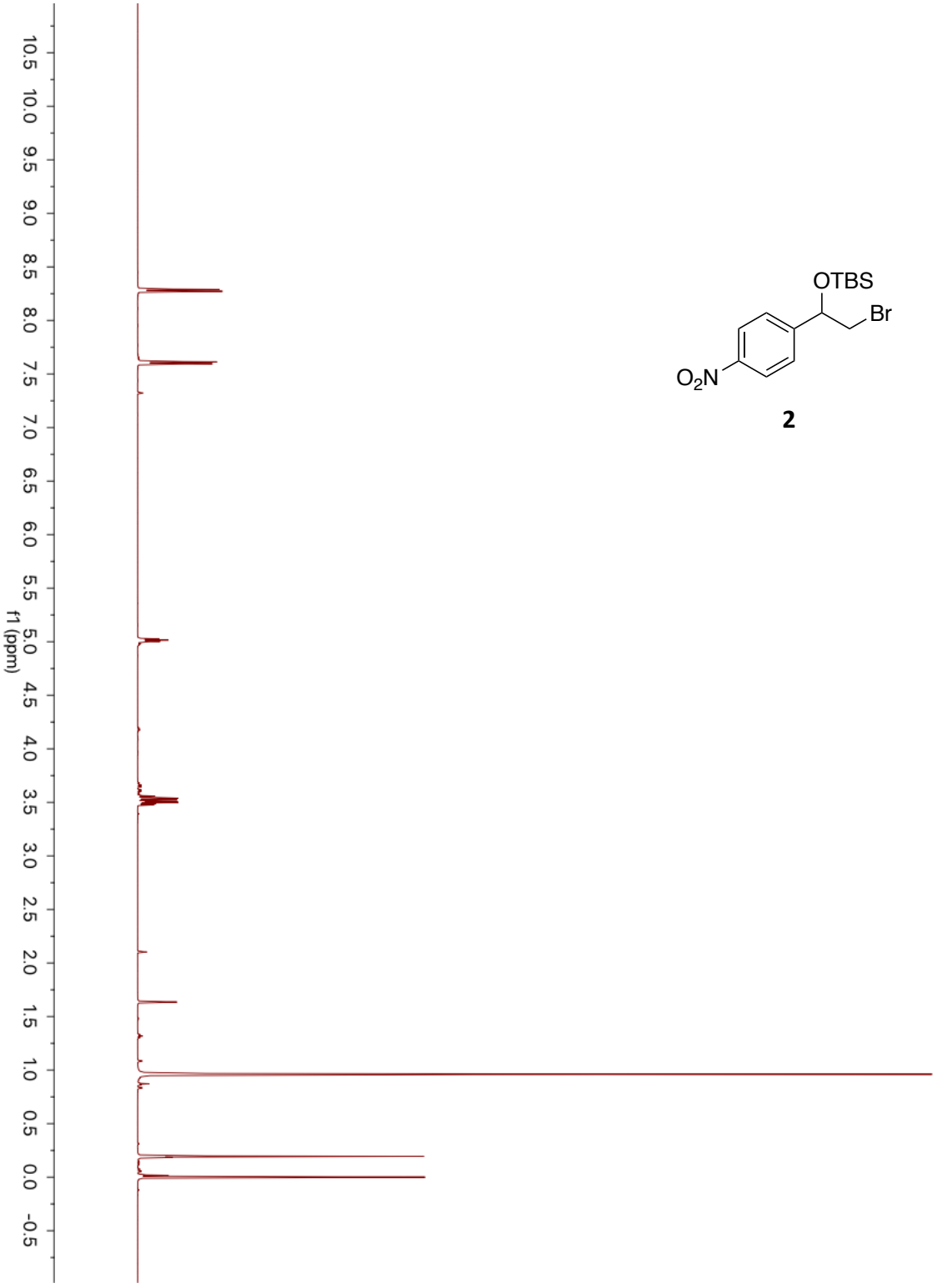
Bicyclic *tbx16* cMO (26). Synthetic procedures identical to that for the bicyclic *tbxta* cMO (**25**) were applied to a *tbx16* MO oligomer with 5'-amine, 3'-disulfide, and internal alkyne functionalization **20** (5′-CTCTGATAGCC**T**GCATTATTTAGCC-3′). Typical isolated yields for the similar to the *tbxta* sequence. For the click reaction, MS-ESI (m/z): $[M+H]^+$ calculated for **22**, 9655: observed: 9661. For the amide coupling reaction, MS-ESI (m/z): $[M+H]^+$ calculated for **24**: 9637; observed: 9642. For the thiol-halogen exchange reaction, MS-ESI (m/z): [M+H]+ calculated for **24**: 9484; observed: 9492.

Synthesis of a model bicyclic cMO photolysis product (27). A 25-base *tbxta* MO with 5'-amine, 3'-disulfide, and internal alkyne functionalization **19** (5'-GACTTGAGGCAG**A**CATATTTCCGAT-3') (Gene Tools) was used to prepare a model photolysis product. The alkyne-modified adenine monomer is shown in bold font. For the click reaction between **19** and **4**, a reaction and purification protocol analogous to that described above for **25** was used. MS-ESI (m/z): $[M+7H]^{+7}$ calculated for **27**: 1324.0; observed 1323.2. $[M+8H]^{+8}$ calculated for **27**: 1158.6; observed 1157.3. $[M+9H]^{+9}$ calculated for **27**: 1030.0; observed: 1029.3. Manual deconvolution of these peaks corresponds to an observed $[M+H]^+$ for **27** of 9261, which conforms to the calculated MW.

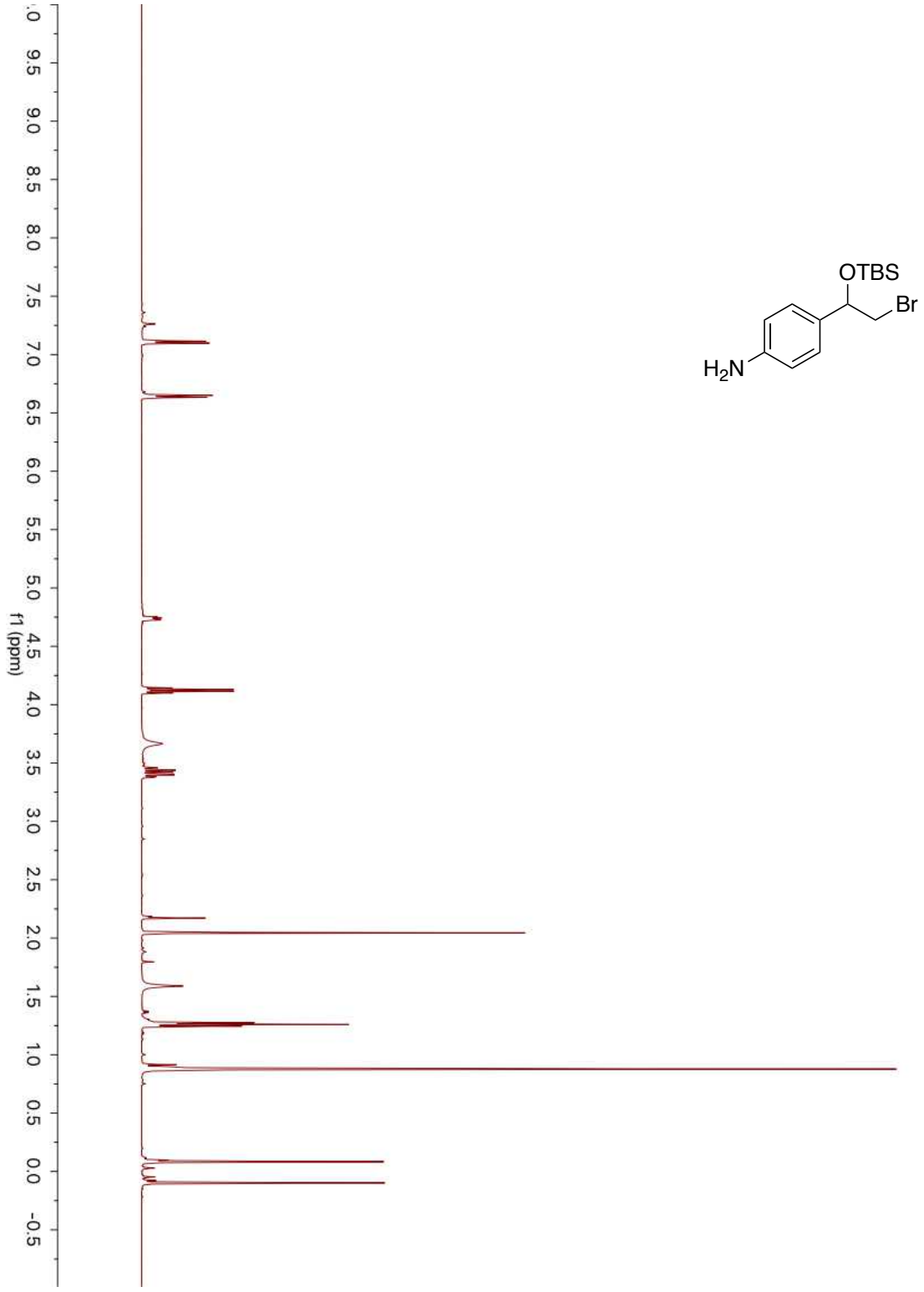

10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f1 (ppm)

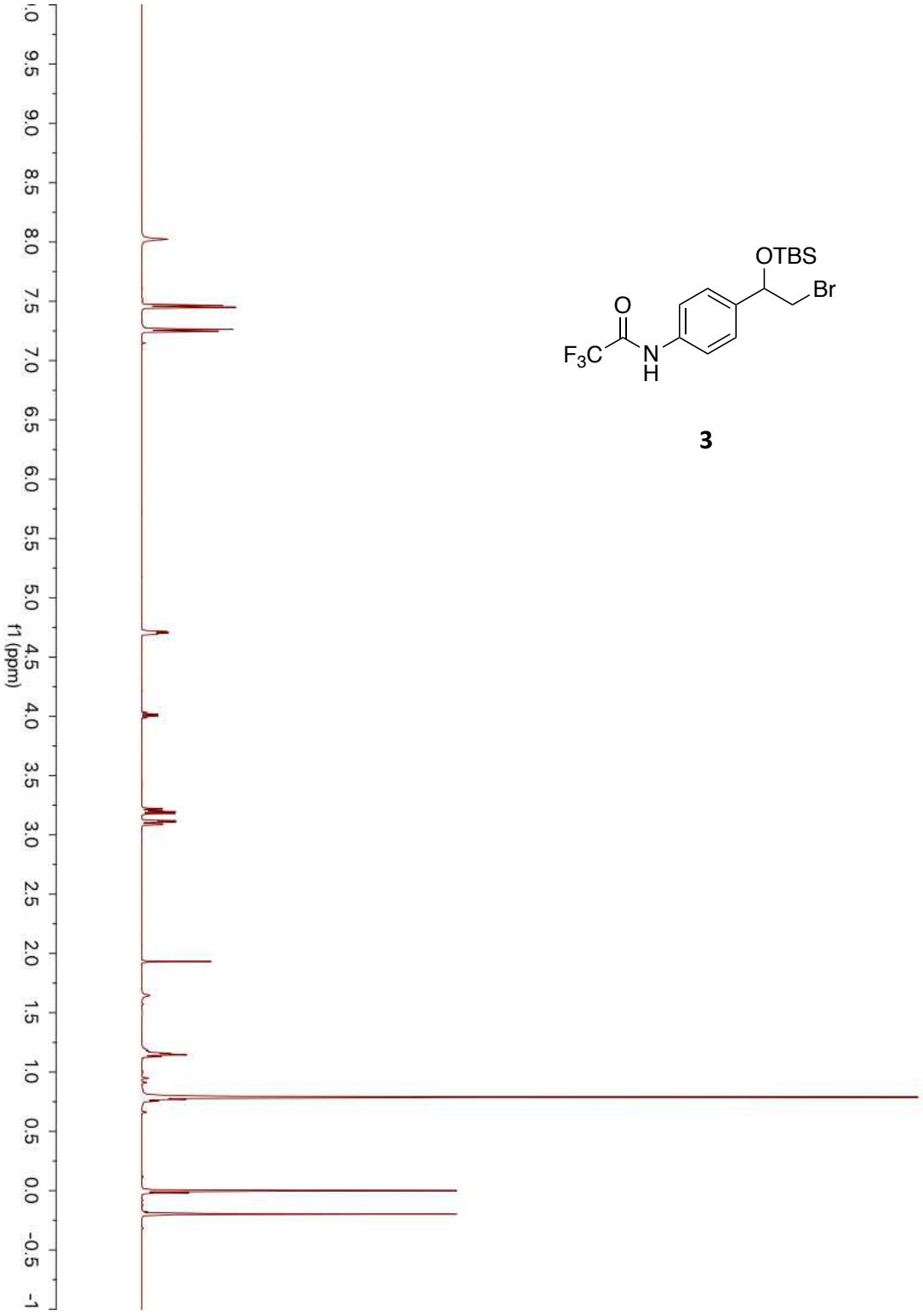

OH
Br
O2N

1


10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f1 (ppm)
OTBS
Br
O2N

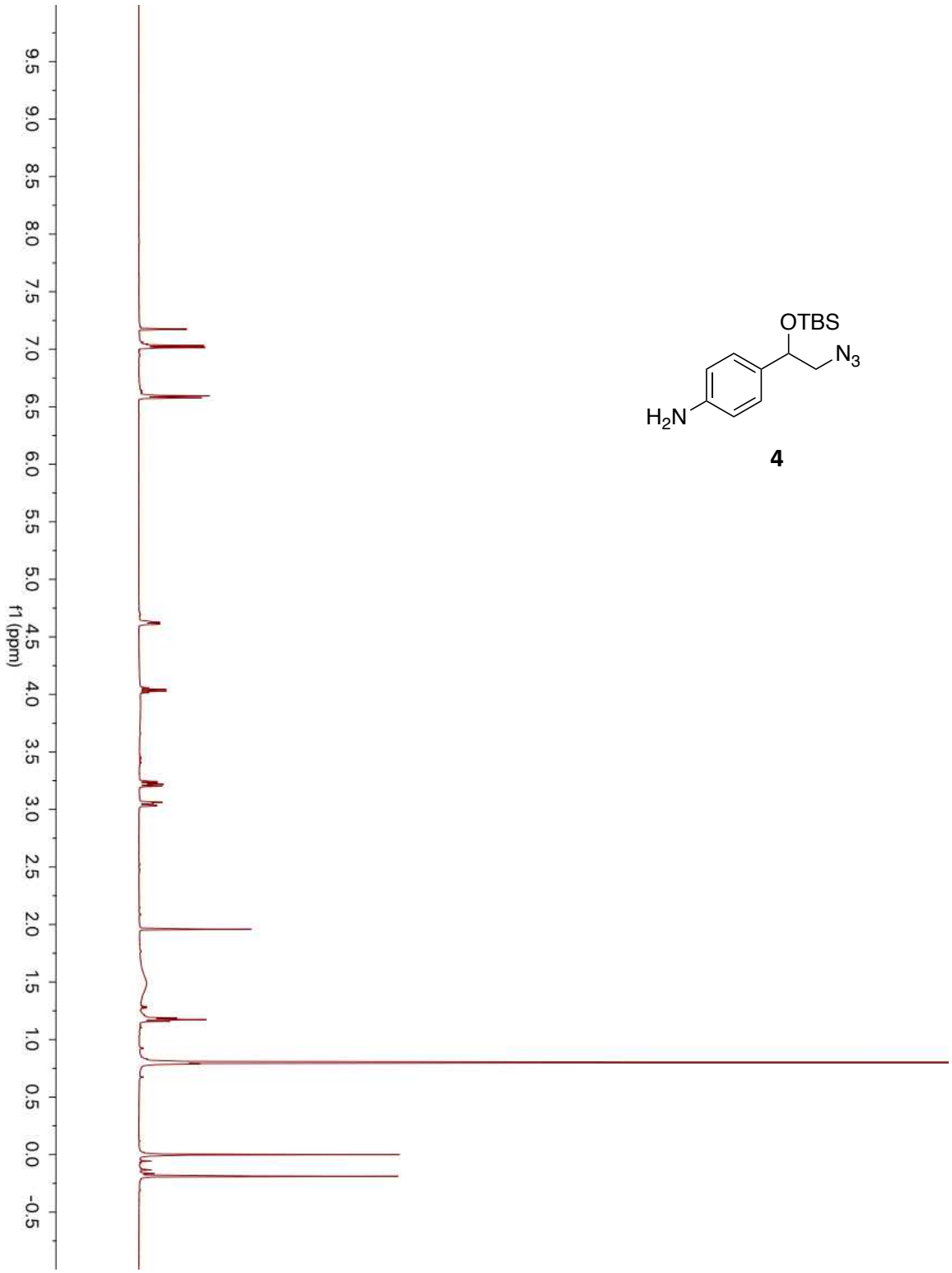
2


OTBS
Br
H2N
f1 (ppm)
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5


f1 (ppm)
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1

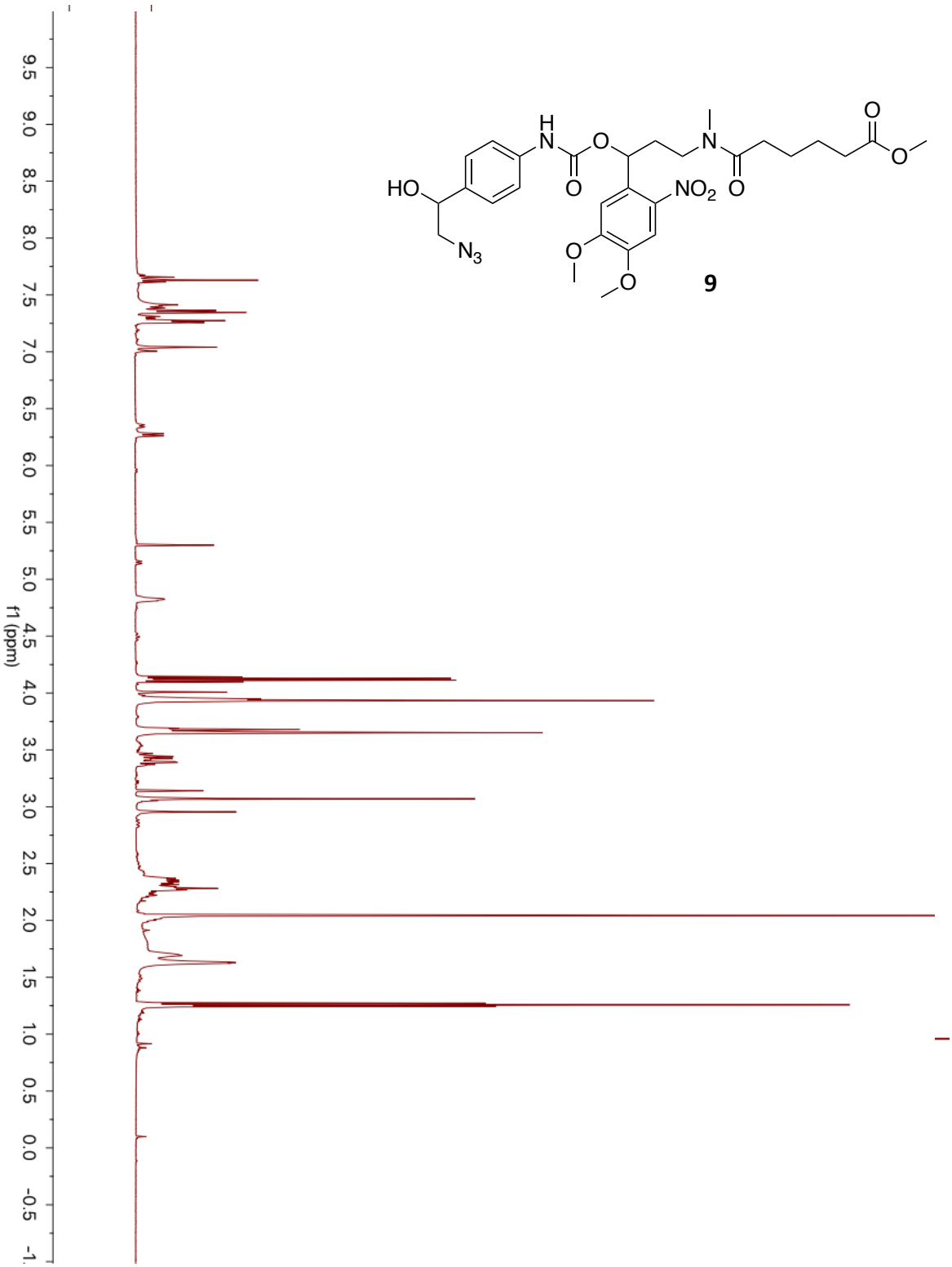
OTBS
Br
O
F3C
N
H

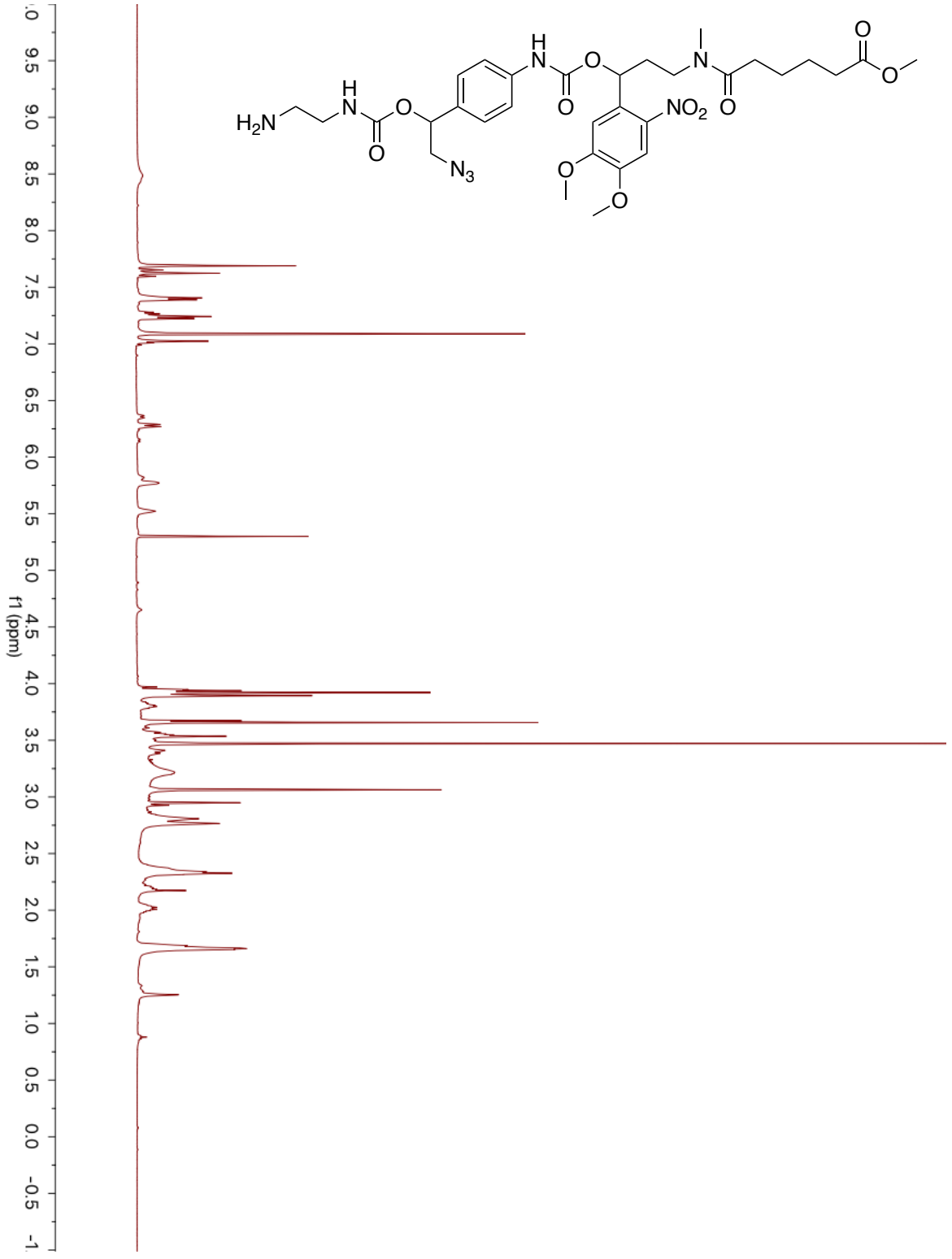
3

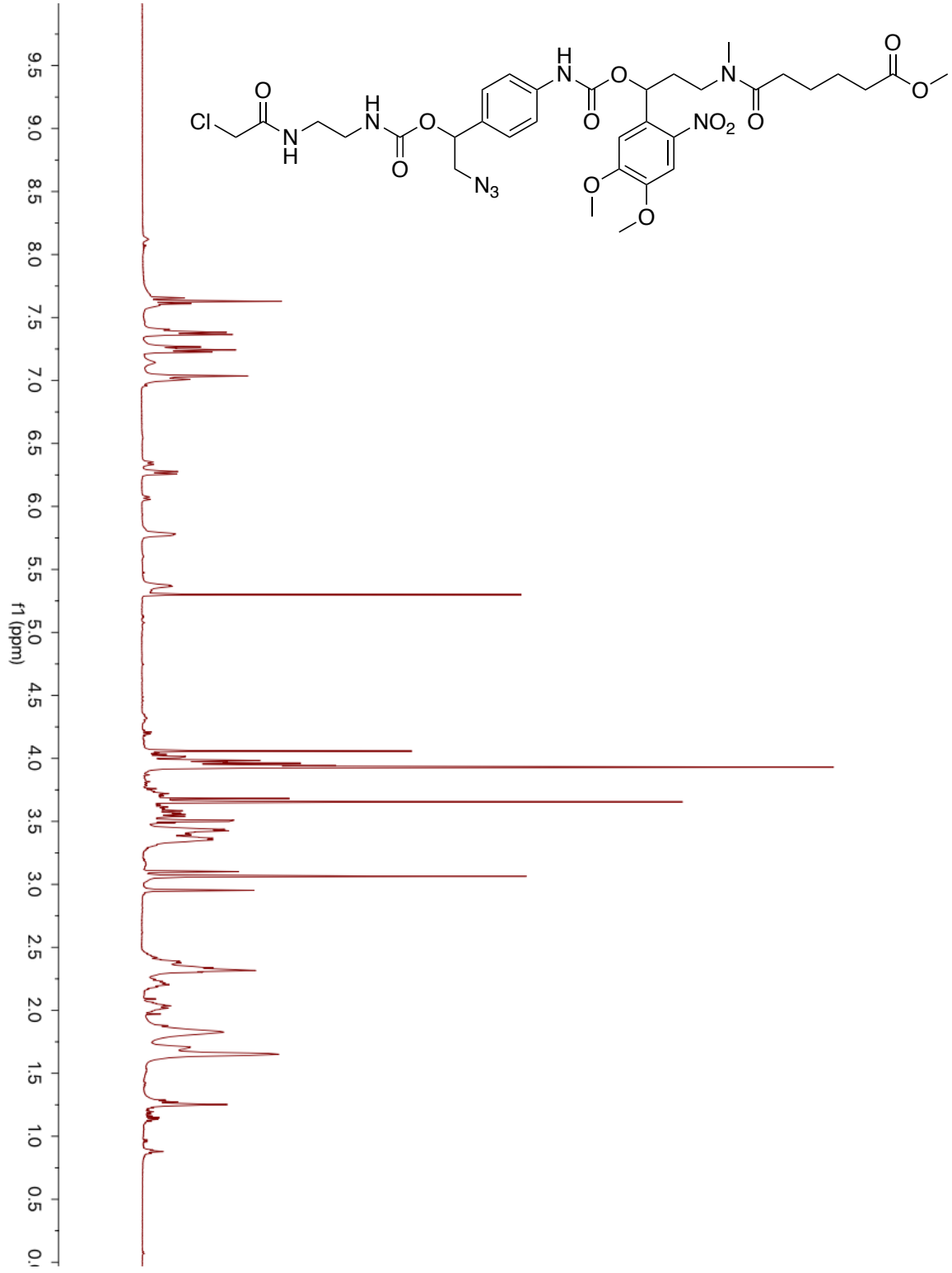

9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f1 (ppm)

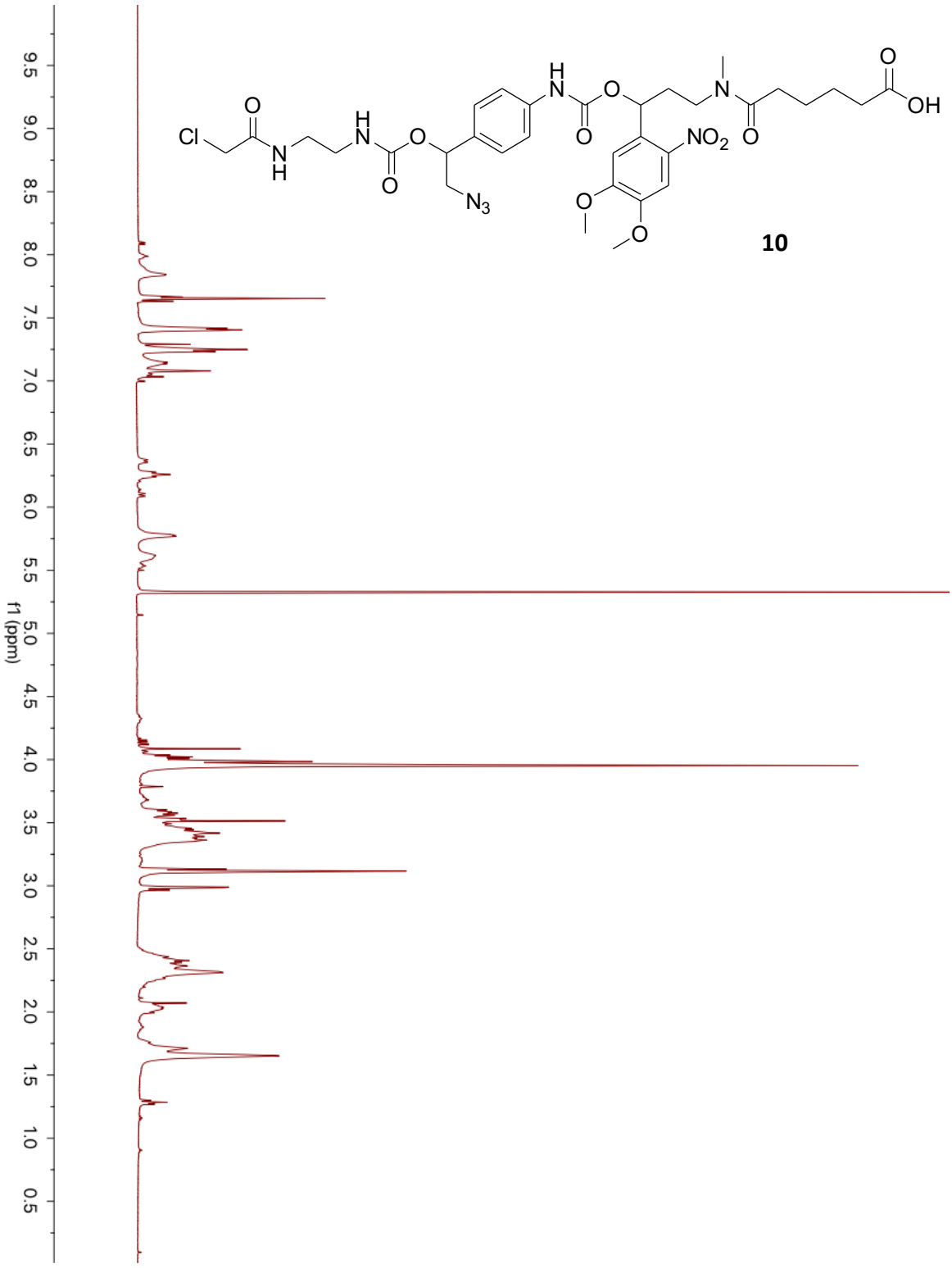
OTBS
N3
H2N

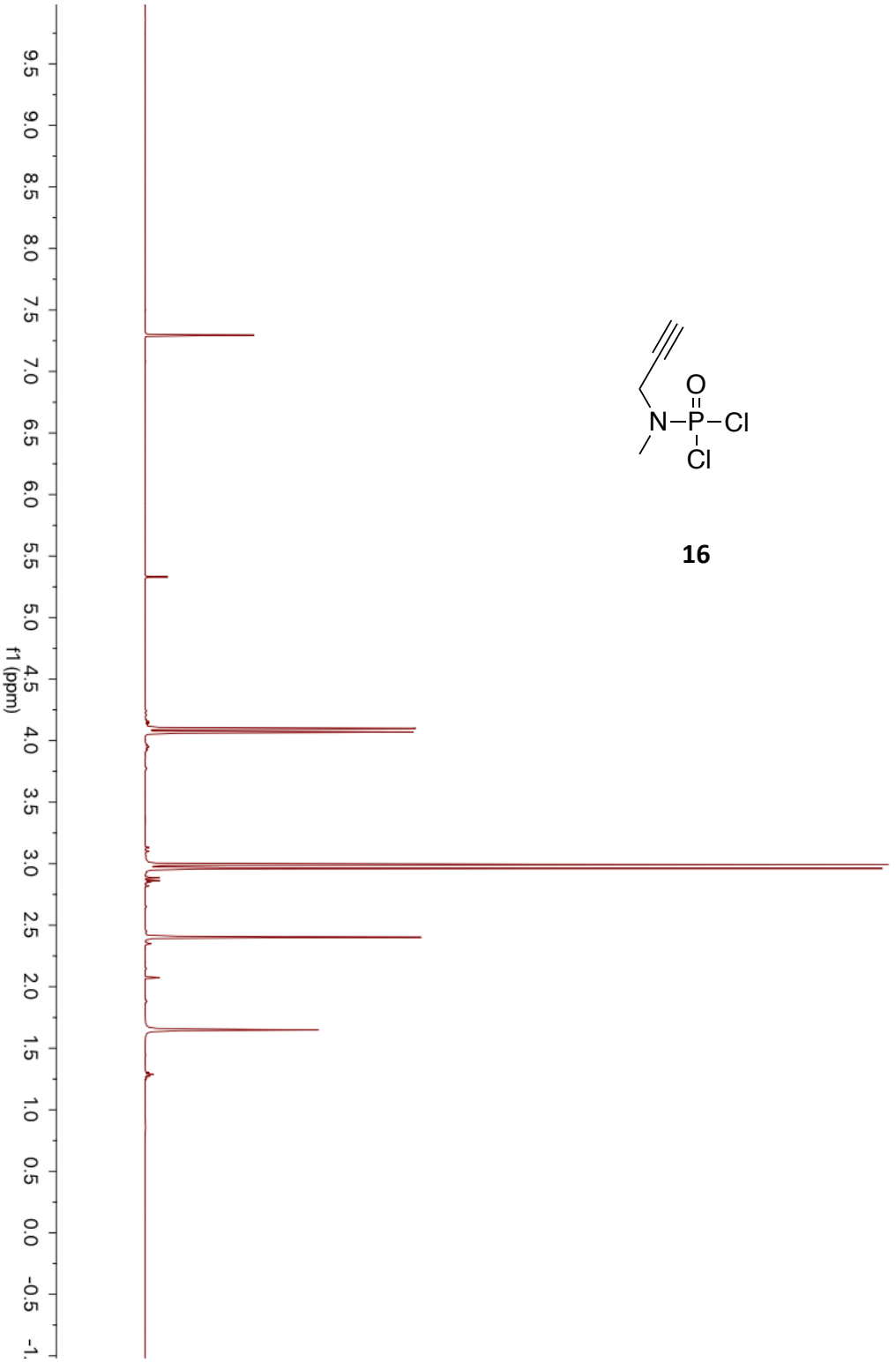
4


HO
H
N
O
O
N
O
O
NO2
O
O
O
N3
O
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.
f1 (ppm)

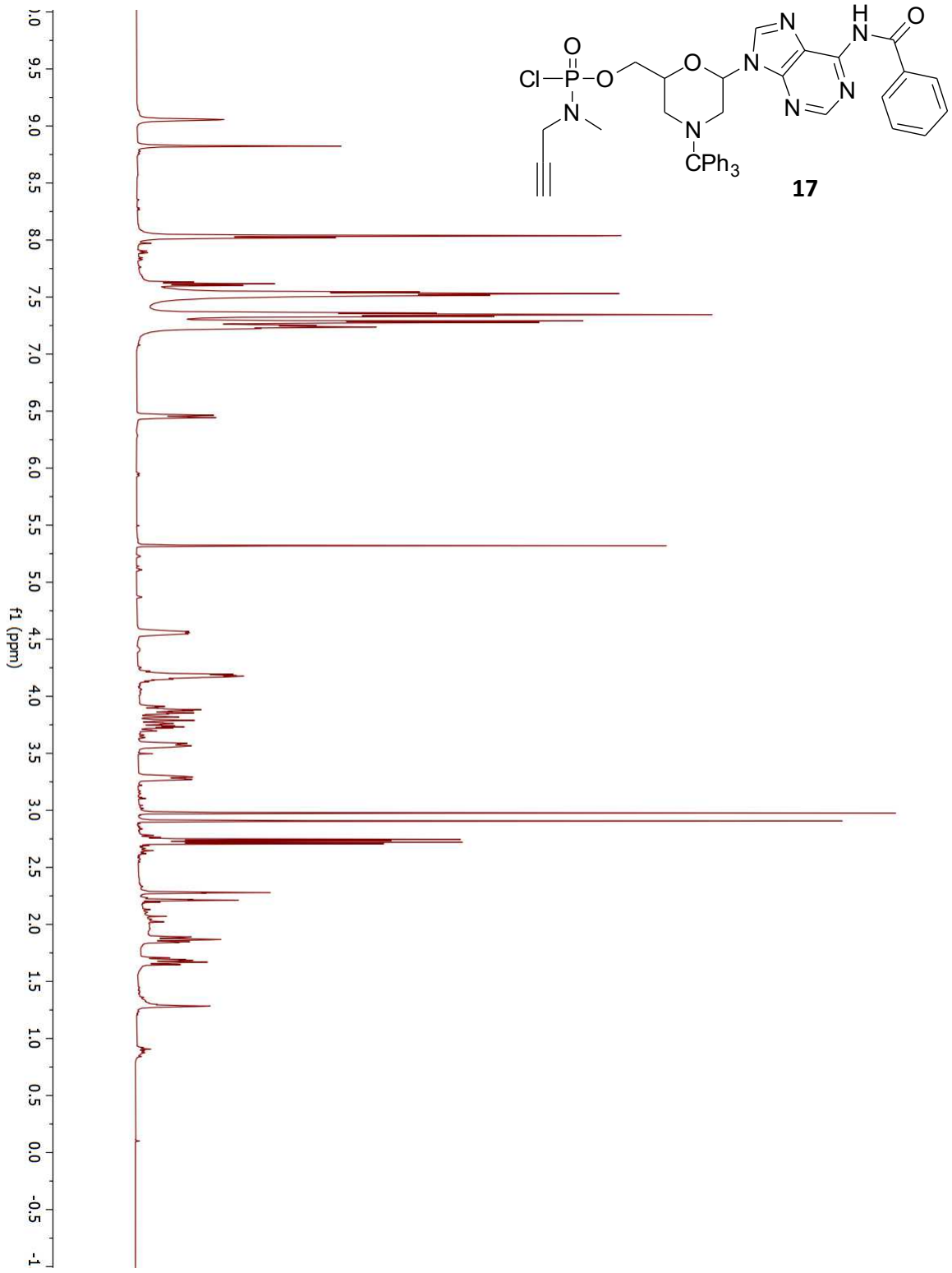

f1 (ppm)
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.
H2N
H
N
O
O
N3
H
N
O
O
NO2
O
N
O
O
O
O
O


f1 (ppm)
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Cl
O
N
H
H
N
O
O
N3
H
N
O
O
O
NO2
O
N
O
O
O
O


Cl
O
N
H
H
N
O
O
N3
H
N
O
O
O
NO2
N
O
OH
O
O
10
f1 (ppm)
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5


9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.
f1 (ppm)
O
N
P
Cl
Cl

16


f1 (ppm)
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1
Cl
P
O
N
CPh3
H
17

REFERENCES

[1] X. Ouyang, I. A. Shestopalov, S. Sinha, G. Zheng, C. L. Pitt, W. H. Li, A. J. Olson, J. K. Chen, *J. Am. Chem. Soc.* **2009**, *131*, 13255-13269.

[2] R. F. Borch, R. R. Valente, *J. Med. Chem.* **1991**, *34*, 3052-3058.

[3] J. Bhadra, S. Pattanayak, S. Sinha, *Curr. Protoc. Nucleic Acid Chem.* **2015**, *62*, 4.65.61-26.