

Design and synthesis of fluorescence-labeled nucleotide with cleavable azo linker for DNA sequencing

Lianjiang Tan[†], Yazhi Liu^{†,‡}, Qinglai Yang[†], Xin-Yan Wu[‡], Xiaowei Li[§], Bing Gong^{¶,‡} Yu-Mei Shen^{†,*}
and Zhifeng Shao[§]

[†]Shanghai Center for Systems Biomedicine, Key Laboratory of Systems Biomedicine (Ministry of Education), Collaborative Innovation Center of Systems Biomedicine, and [§]Bio-ID Center, School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai 200240, China

[‡]Key Laboratory for Advanced Materials and Institute of Fine Chemicals, East China University of Science and Technology, Shanghai 200237, China

[¶]College of Chemistry, Beijing Normal University, Beijing 100875, China

[‡]Department of Chemistry, University at Buffalo, State University of New York, Buffalo, NY 14260, USA

Supporting Information

Contents

General Methods	S2
Experimental Section	S2-S5
Supplementary Data	S6-S7
DNA Extension/Cleavage Cycles	S7-S11
Copies of ¹ NMR, ¹³ C NMR and ³¹ P NMR spectra	S12-S20

General Methods

Chemicals needed in this work were purchased from commercial sources (Alfa and Shanghai Chemical Reagent Company) and directly used without further purification. All the chemicals were stored in the presence of CaCl₂ in a desiccator under vacuum at room temperature and protected from light. All reactions were followed by thin-layer chromatography (precoated 0.25 mm silica gel plates from Aldrich) and silica gel column chromatography was carried out with silica gel 60 (mesh 200-400). The NMR spectra were recorded on a Varian MERCURY plus-400 (400 MHz for ¹H-NMR; 100 MHz for ¹³C-NMR; 162 MHz for ³¹P-NMR) spectrometer with chemical shifts reported in ppm relative to the residual deuterated solvents or the internal standard tetramethylsilane. High-resolution mass spectrometry analysis was carried out using a Quadrupole-Time-of-Flight Premier Mass Spectrometer (Waters, US) equipped with an electrospray interface. Data were collected in centroid mode from mass-to-charge ratio (*m/z*) 50 to 1000 at scan time of 0.25 s with an interval of 30 s. High-performance liquid chromatography (HPLC) was carried out on a Prominence LC-20A HPLC (Shimadzu, Japan) at ambient temperature. An SB-C18 analytical column (4.6 × 250 mm, 5 μm, Agilent) was used and the traces were recorded with a UV detector at 254 nm. Conditions for compound **1**: 1 mL/min, methanol in 8.6 mM Et₃N and 100 mM HFIP aqueous solution with 15 % methanol in 5 min, followed by 15 %~40 % methanol in 5 min and 40 %~70 % methanol in 30 min. Conditions for compound **5** and its cleavage products: 1 mL/min, methanol in water with 2 % methanol in 0.01 min, followed by 5 % methanol in 5 min, 20 % methanol in 15 min, 40 % methanol in 50 min, and 80 % methanol in 50.01 min. The measurements were conducted in triplicate. The fluorescence spectra of **dUTP-azo linker-5(6)TAMRA (1)** and TAMRA in water were recorded on a LS 55 luminescence spectrometer (PerkinElmer, US), applying an excitation wavelength of 560 nm.

Experimental Section

4-ethyl-2-((4-(2-hydroxyethyl)phenyl)diazenyl)phenol (3). 274 mg (2 mmol) of 2-(4-aminophenyl)ethanol dissolved in mixture of ethanol (5 mL) and water (5 mL), followed by sodium nitrite (145mg, 2.1mmol) dissolved in water (10 mL) and then added dropwise, the solution was cooled to 0 °C. The mixture was stirred for 40 min, sodium hydroxide (80 mg, 2 mmol) was added, followed by 244 mg (2 mmol) of 4-ethylphenol dissolved in mixture of ethanol (45 mL) and water (15 mL) in an ice bath. Then the new synthesized diazonium salt was added dropwise to 4-ethylphenol solution. The mixture was stirred for 5 hrs. The precipitate was filtered and washed with water (3 x 10 mL). Then the precipitate was dried under vacuum overnight to give crude product 225 mg (yield 42%). ¹H NMR (400 MHz, CDCl₃): δ ppm 12.72 (s, 1H), 7.80 (d, 2H, J = 8.4 Hz), 7.75 (d, 1H, J = 2.4 Hz), 7.37 (d, 2H, J = 8.4 Hz), 7.18 (d, 1H, J = 2.4 Hz, 8.4 Hz), 6.95 (d, 2H, J = 8.4 Hz), 3.90 (t, 2H, J = 6.4 Hz), 2.94 (t, 2H, J = 6.4 Hz), 2.69 (q, 2H, J = 7.6 Hz), 1.75 (s, 1H), 1.29 (t, 3H, J = 7.6 Hz). ¹³C NMR (100 MHz, CDCl₃): δ ppm 150.8, 149.5, 142.3, 137.2, 135.8, 133.2, 131.8, 130.1, 122.4, 118.0, 63.4, 39.1, 27.9, 15.7.

Tert-butyl(2-(2-(2-(4-ethyl-2-((4-(2-hydroxyethyl)phenyl)diazenyl)phenoxy)ethoxy)ethoxy)ethyl)carbamate (4). 50 mg (0.124 mmol) of 2, 2-dimethyl -4-oxo -3, 8, 11-trioxa -5-azatriodecan-13-yl 4-methylbenzenesulfonate **2** and 19 mg (0.136 mmol) of K₂CO₃ was added to a 10 mL flask, followed by 101 mg (0.372 mmol) of 4-ethyl-2-((4-(2-hydroxyethyl)phenyl)diazenyl)phenol **3**. The mixture was heated to 120°C for 2.5 hrs under a nitrogen atmosphere and then cooled to room temperature. Ethyl acetate (20 mL) was added and the organic layer was washed with water (3 x 20 mL) and concentrated. The crude mixture was purified by column chromatography (2:1 PE/EA) to give 56 mg of product **4** (yield 90 %). ¹H NMR (400 MHz, CDCl₃): δ ppm 7.85 (d, 2H, J = 8.4 Hz), 7.47 (d, 1H, J = 2.0 Hz), 7.36 (d, 2H, J = 8.4 Hz), 7.25 (dd, 1H, J = 2.0 Hz, 8.4 Hz), 7.03 (d, 1H, J = 8.4 Hz), 5.05 (s, 1H), 4.34 (t, 2H, J = 4.8 Hz), 3.95~3.91 (m, 4H), 3.78 (t, 2H, J = 4.8 Hz), 3.60 (t, 2H, J = 4.8 Hz), 3.49 (t, 2H, J = 5.2 Hz), 3.26~3.23 (m, 2H), 2.94 (t, 2H, J = 6.4 Hz), 2.65 (q, 2H, J = 7.6 Hz), 1.40 (s, 9H), 1.24 (t, 3H, J = 7.6 Hz). ¹³C NMR (100 MHz, CDCl₃): δ ppm

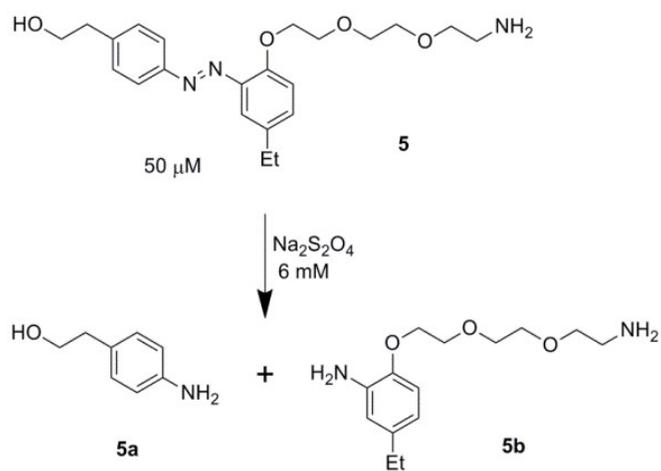
154.6, 152.0, 142.7, 142.1, 137.6, 131.7, 129.8, 123.2, 116.2, 115.5, 79.3, 71.0, 70.3, 70.3, 69.9, 63.4, 40.4, 39.2, 28.5, 28.1, 15.7.

2-(4-((2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-5-ethylphenyl)diazenyl)phenyl)ethanol (5). To a 10 mL flask containing 60 mg (0.12 mmol) of tert-butyl (2-(2-(2-(4-ethyl-2-((4-(2-hydroxyethyl) phenyl) diazenyl)phenoxy)ethoxy)ethoxy)ethyl)carbamate **4** in an ice bath, followed by 1 mL of TFA. The mixture was stirred for 1 hr and then quenched with the addition of saturated sodium bicarbonate. The solution was then concentrated. The crude mixture was spotted onto a silica gel plate to give 20 mg of product **5** (yield 42%). ¹H NMR (400 MHz, CDCl₃): δ ppm 7.63 (d, 2H, J = 8.0 Hz), 7.46 (d, 1H, J = 2.0 Hz), 7.40 (d, 2H, J = 8.4 Hz), 7.29 (dd, 1H, J = 2.0 Hz, 8.8 Hz), 6.97 (d, 1H, J = 8.4 Hz), 4.32~4.30 (m, 2H), 3.92 (m, 4H), 3.76~3.68 (m, 4H), 3.62~3.58 (m, 4H), 2.91 (s, 2H), 2.75 (s, 2H), 2.64 (q, 2H, J = 7.6 Hz), 1.24 (t, 3H, J = 7.6 Hz); ¹³C NMR (100 MHz, CDCl₃): δ ppm 154.1, 253.3, 143.3, 141.8, 137.4, 132.3, 129.9, 122.6, 116.7, 113.3, 70.8, 69.7, 69.5, 68.2, 67.1, 62.7, 40.1, 38.6, 28.1, 15.7.

2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)-4-((2-(2-(2-(4-ethyl-2-((4-(2-hydroxyethyl)phenyl)diazenyl)phenoxy)ethoxy)ethoxy)ethyl)carbamoyl)benzoate (6). To a 10 mL flask 19 mg (0.136 mmol) of 2-(4-((2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-5-ethylphenyl) diazenyl) phenyl) ethanol **5** was added, followed by 10 mg (0.019 mmol) of 5(6)-TAMRA, SE(II) dissolved in anhydrous DMF and triethylamine. The mixture was stirred for 3 hrs and then concentrated. The crude material was spotted onto a silica gel plate to give 13 mg (yield 84%) of product **6**. ¹H NMR (400 MHz, CD₃OD): δ ppm 8.51 (d, 1H, J = 2.0Hz), 8.01~7.98 (m, 1H), 7.76 (d, 2H, J = 8.4Hz), 7.38~7.34 (m, 3H), 7.25~7.20 (m, 2H), 7.16 (d, 2H, J = 9.2Hz), 7.10 (d, 1H, J = 8.4Hz), 6.94~6.88 (m, 4H), 4.30 (t, 2H, J = 4.8Hz) 3.96~3.92 (m, 2H) 3.83~3.77 (m, 4H), 3.72~3.68 (m, 4H), 3.62~3.58 (m, 2H), 3.27 (s, 3H), 3.26 (s, 6H), 3.25 (s, 3H), 2.87 (t, 2H, J = 6.8Hz), 2.56 (q, 2H, J = 7.6Hz), 1.17 (t, 3H, J = 7.6Hz). HRMS: calcd for C₄₇H₅₀N₅O₈ [M-H]⁺ 812.3659, found 812.3657.

dUTP-AZO-5(6)TAMRA (1). To a 10 mL flask containing 13 mg (0.016 mmol) of 2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)-4-((2-(2-(2-(4-ethyl-2-((4-(2-hydroxyethyl)phenyl)diazenyl)phenoxy)ethoxy)ethoxy)ethyl)carbamoyle)benzoate **7**, 2 mL of dry acetonitrile was added, followed by 24.6 mg (0.096 mmol) of DSC, 3.9 mg (0.032 mmol) of DMAP and 5.2 mg (0.04 mmol) DIPEA. The mixture was stirred for 6 hrs. Then the crude product was dissolved in 0.8 mL of acetonitrile. The prepared **dUTP(AP₃)** (20 mg, 38.4 mmol) dissolved in 1.5 mL of Na₂CO₃/NaHCO₃ buffer was added, followed by 6 μ L triethylamine. The mixture was stirred for 3 hrs and then concentrated. The crude product was purified by preparative HPLC with C-18 reverse phase column (250 mm x 4.6 mm) to give the terminal product **1** (3.5 mg, yield 16%). ¹H NMR (400 MHz, D₂O): δ ppm 8.25 (s, 1H), 7.83 (s, 1H), 7.41 (d, 2H, J = 7.2 Hz), 7.16 (s, 2H), 6.92~6.66 (m, 7H), 6.55~6.49 (m, 2H), 6.40 (s, 2H), 5.93 (s, 1H), 4.49 (s, 1H), 4.24~3.99 (m, 8H), 3.87~3.68 (m, 9H), 3.10 (s, 3H), 3.08 (s, 3H), 3.06 (s, 3H), 3.03 (s, 3H), 2.86-2.88 (m, 2H), 2.19~2.07 (m, 3H), 1.97~1.92 (m, 1H), 0.64-0.66 (m, 3H); ³¹P NMR (D₂O, 162 MHz): δ -6.76, -10.82, -20.07; HRMS: calcd for C₆₀H₆₆N₈O₂₃P₃ [M+3H]⁺ 1359.3454, found 1359.3459.

A disulfide linker-based reversible terminator dUTP-S-S-TAMRA. The detailed synthetic procedure and characterization for the reversible terminator dUTP-S-S-TAMRA were reported in Ref. (1). ¹H-NMR (400 MHz, D₂O): δ 8.29 (s, 1H), 8.18 (d, J = 7.6 Hz, 1H), 7.80 (s, 1H), 7.60 (d, J = 8.0 Hz, 1H), 7.30 (d, J = 9.6 Hz, 1H), 7.18 (d, J = 9.6 Hz, 1H), 7.00~7.05 (m, 1H), 6.91 (d, J = 9.6 Hz, 1H), 6.70~6.76 (m, 2H), 5.84~5.88 (m, 1H), 5.47 (s, 1H), 4.49 (s, 1H), 4.10~4.16 (m, 2H), 3.96 (s, 2H), 3.85~3.90 (m, 3H), 3.72~3.77 (m, 2H), 3.25 (d, J = 9.6 Hz, 8H), 3.17 (t, J = 4.8 Hz, 2H), 2.94~2.99 (m, 2H), 2.65~2.75 (m, 2H), 2.26~2.32 (m, 1H), 2.04~2.15 (m, 1H), 1.24~1.31 (m, 1H). HRMS: calc for C₄₂H₄₆N₆O₁₉S₂P₃ [M-H]⁻ 1095.1459, found 1095.1483.



Scheme S1. Schematic cleavage of the azo linker **5**.

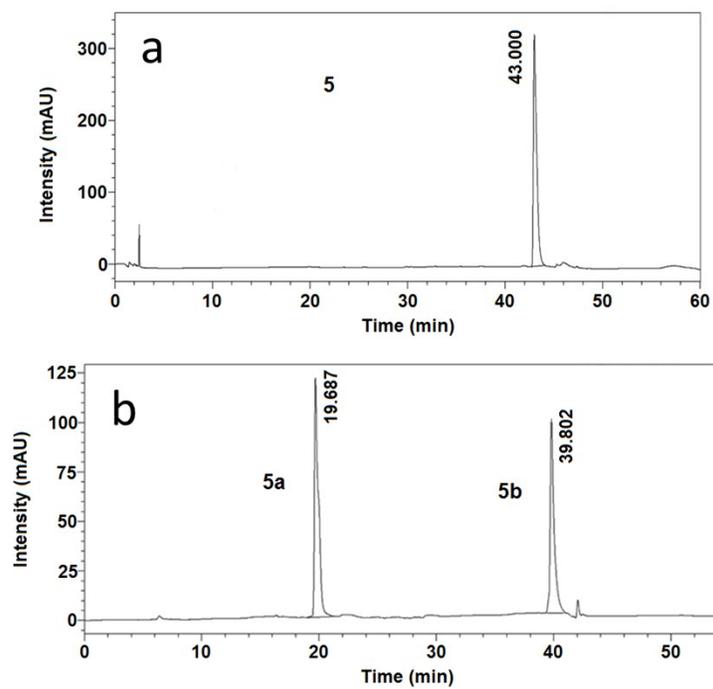


Fig. S1. HPLC spectra of the azo linker **5** (a) and the cleavage products **5a** and **5b** (b).

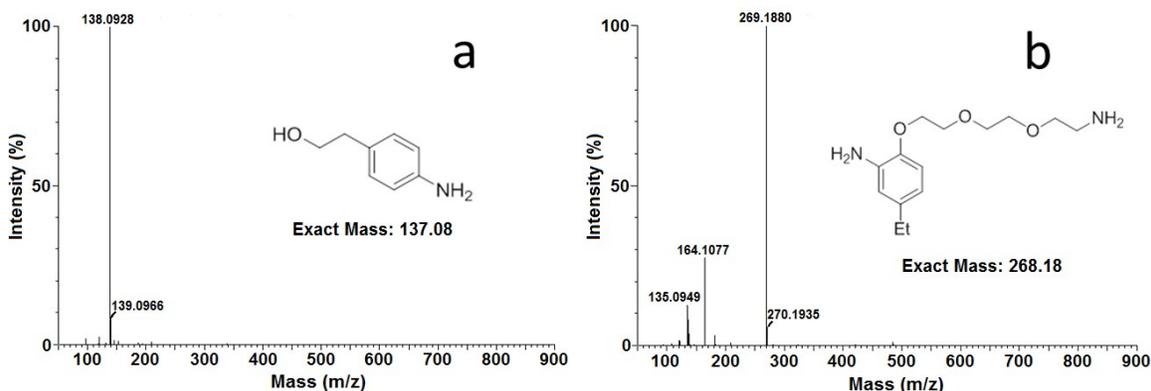


Fig. S2. HRMS spectra of the cleavage products **5a** and **5b** from the azo linker **5**.

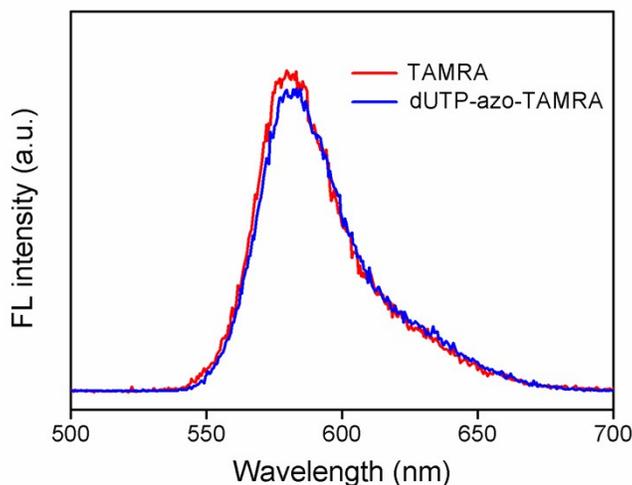


Fig. S3. Fluorescence spectra of TAMRA and the nucleotide analogue **dUTP-azo linker-5(6)TAMRA (1)** in water at the concentration of 10 μ M.

DNA Extension/Cleavage Cycles

Primer extension and cleavage of the extension product. A 24-mer oligonucleotide, 5'-GAGGAAAGGGAAGGGAAAGGAAGG-3' (molecular weight of 7663), and a fluorescence-labeled 24-mer oligonucleotide with the same base sequence (Dylight 800-5'-GAGGAAAGGGAAGGGAAAGGAAGG-3'), were used as a primer for MALDI-TOF MS and denaturing gel electrophoresis, respectively. Two oligonucleotides with slightly different base sequences

were used as templates, and another three oligonucleotides were for comparison purpose, as listed in Table S1. The molecular weight of the template 2 was 11998. The primer and template were annealed in a Tris-EDTA buffer (TE, pH 7.5) according to the following protocol: 95 °C for 3 min, followed by a decrease to 4 °C at a ramp rate of 0.1 °C /sec and holding at 4 °C. The annealed primer and template mixture was used to perform extension reaction. The components were mixed in the order in a microfuge tube on ice: 2 µL of 10× Klenow reaction buffer, 1 µL of 1 M NaCl, 2 µL of annealed template/primer (1 µg/µL), 5 µL of 1 mM **dUTP-azo linker-5(6)TAMRA (1)**, 0.4 µL (2 U) of Klenow Fragment exo⁻ DNA polymerase and Type I water to a final volume of 20 µL. After gentle vortex mix, the mixture was incubated at 37 °C for 12 min and then heated at 72 °C for 10 min, followed by cooling to 16 °C. The extension products were obtained by phenol chloroform extraction, ethanol precipitation, and vacuum drying. The products were treated with 30 mM sodium dithionite in NaAc/HAc buffer (pH 5.2) for 4.5 min at 37 °C to cleave the fluorophore. The cleavage process was stopped by adding an appropriate amount of Tris-HCl buffer (pH 8.5), yielding cleavage products that could be used for the next cycle of extension and cleavage. At each step, a portion of the extension or cleavage products was dissolved in water for denaturing gel electrophoresis and MALDI-TOF MS.

Denaturing Polyacrylamide Gel Electrophoresis and MALDI-TOF MS. DNA sequencing gel electrophoresis was performed on 12 % denaturing polyacrylamide gels containing 7 M of urea, using a Sequi-Gen GT Nucleic Acid Electrophoresis Cell (Bio-Rad, US). Before electrophoresis, the prepared gels were run at a voltage of 2000 V and a constant power of 40 W for 30 min at 55 °C. In the meantime, the sequencing reaction samples were mixed with a small amount of 0.1 M sodium hydroxide and were denatured into single strands by heating to 95 °C for 3 min and then cooling to room temperature rapidly. Then 1 µL of the samples (~ 15 ng/µL DNA) were mixed with 1.5 µL of 6×

loading buffer containing marker dyes, and the mixtures were then loaded on each well of the gels. Maintained at 55 °C, the gels were run under the above-mentioned conditions for 70 min. The resultant gels were observed using an Odyssey Infrared Imaging System (LI-COR Biosciences, US) under excitation of 785 nm laser. The samples at each step were resolved in water, purified by HPLC, desalted by using the ZipTip protocol and then analyzed on an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics, US). 3-Hydroxypropionaldehyde (3-HPA) was used as matrix. The samples were spotted on the layer of the matrix and measured in negative ion mode.

Superparamagnetic Beads Experiments. Dynabeads® magnetic beads (10 mg/mL, Thermo Fisher, US) were used as substrates for cycles of extension and cleavage reactions. A 60-mer 5'-dual biotin modified oligonucleotide (dual biotin-5'-CAGTCGGTGATAGAGTGGTGCGCGTGCGCGTCAA AAAAGAGAATGAGGAACCCGGGGCAG-3') was used as the template. A 23-mer 5'-AMCA modified oligonucleotide (AMCA-5'-CTGCCCCGGGTTCTCATTCTCT-3') was used as the primer. The beads were washed with 1× B&W Buffer for three times before use. The biotinylated template was added to the washed beads, which were incubated for 15 min at room temperature under gentle rotation to immobilize the template. The template-coated beads were separated with a magnet for 2 min and washed with 1× B&W Buffer twice. The number of template molecules bound to one bead was about 10⁶. Then the primer was added to the beads and an annealing process as mentioned above was performed. The annealed primer and template-coated beads were separated with the magnet, washed with 1× B&W Buffer twice, and used for cycles of extension and cleavage. The **dUTP-azo linker-5(6)TAMRA (1)** was incorporated into the primer on the beads using the same extension protocol as described above. The resultant beads were separated, washed, and immersed in the 30 mM sodium dithionite in NaAc/HAc buffer (pH 5.2) for 4.5 min at 37 °C to cleave the fluorophore. The beads were

then separated and washed for the next cycle of extension and cleavage. The beads at each step were observed using a Ti-U inverted phase contrast microscope (Nikon, Japan). The fluorescence emission in the TAMRA channel under excitation of 560 nm laser was detected for both the DNA-coated beads and blank beads. For each image, 20 beads were analyzed and the emission intensity was given as average \pm standard deviation. The emission in the AMCA channel under excitation of 350 nm was also detected to ensure that the amount of primer on the beads kept approximately constant during the cycles of extension and cleavage.

Table S1. Sequence of Templates.

No.	Sequence
1	3'-CTCCTTTCCTTCCCTTTCCTTCCATCGATCGCCATGTGC-5'
2	3'-CTCCTTTCCTTCCCTTTCCTTCCAAAAGTCGCCATGTGC-5'
3	3'-CTCCTTTCCTTCCCTTTCCTTCCGTCGATCGCCATGTGC-5'
4	3'-CTCCTTTCCTTCCCTTTCCTTCCCTACGATCGCCATGTGC-5'
5	3'-CTCCTTTCCTTCCCTTTCCTTCCCTCGATCGCCATGTGC-5'



Fig. S4. Fluorescent image of a denaturing polyacrylamide gel after electrophoresis. The primer was extended using

dUTP-azo linker-5(6)TAMRA (1) and different templates. Lane 1: the primer (Dylight 800-5'-GAGGAAAGGGAAGGGAAGGAAGG-3'). Lane 2: the 25-mer oligonucleotide(Dylight 800-5'-

GAGGAAAGGGAAGGGAAGGAAGGT-3'). Lane 3: extended primer using template 1 (cleaved). Lane 4: primer extension product using template 3. Lane 5: primer extension product using template 4. Lane 6: primer extension product using template 5. In lanes 4-6, the primer was not extended as the signals were similar to that of the primer.

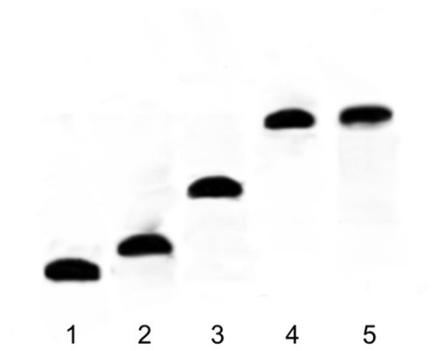
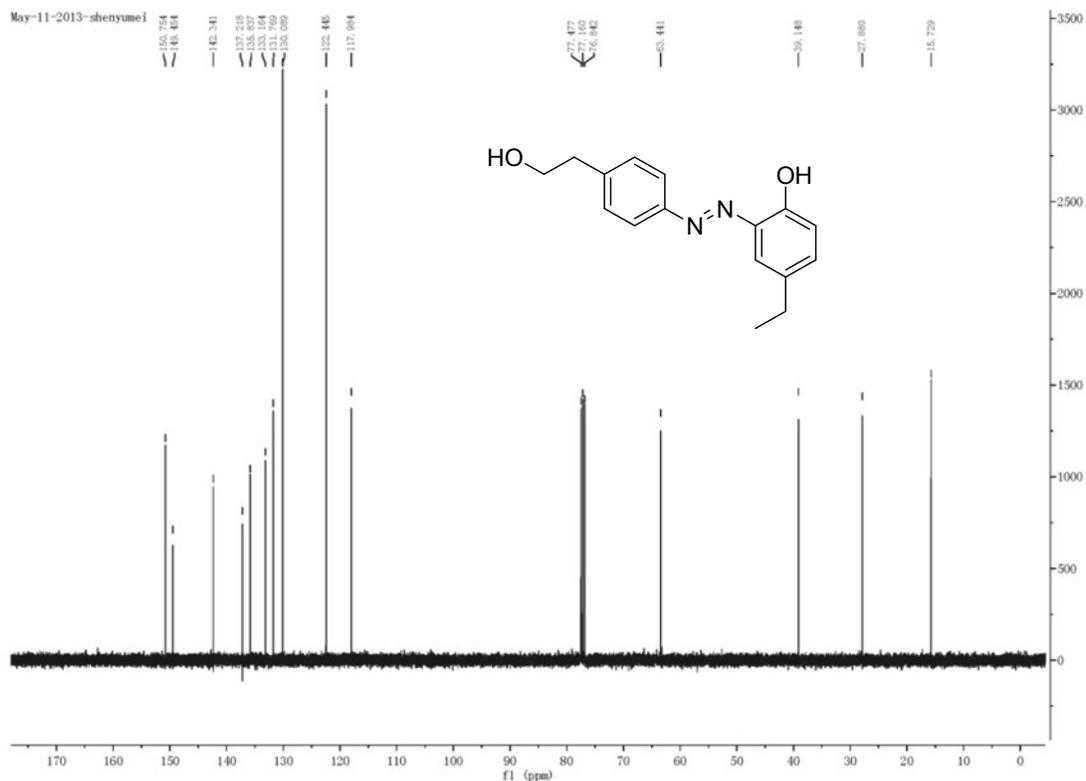


Fig. S5. Denaturing polyacrylamide gel electrophoresis results of the primer extended by **dUTP-S-S-TAMRA**. Lane 1: the primer (Dylight 800-5'-GAGGAAAGGGAAGGGAAGGAAGG-3'). Lane 2: the 25-mer oligonucleotide (Dylight 800-5'-GAGGAAAGGGAAGGGAAGGAAGGT-3'). Lane 3: extended primer using template 1. Lane 4: extended primer using template 2. Lane 5: extended primer using template 3. The protocol of extension reaction was the same as that for the **dUTP-DMKL-5(6)TAMRA (7)**. These results suggest that the primer was extended by two **dUTP-S-S-TAMRAs** at a time under the same conditions.

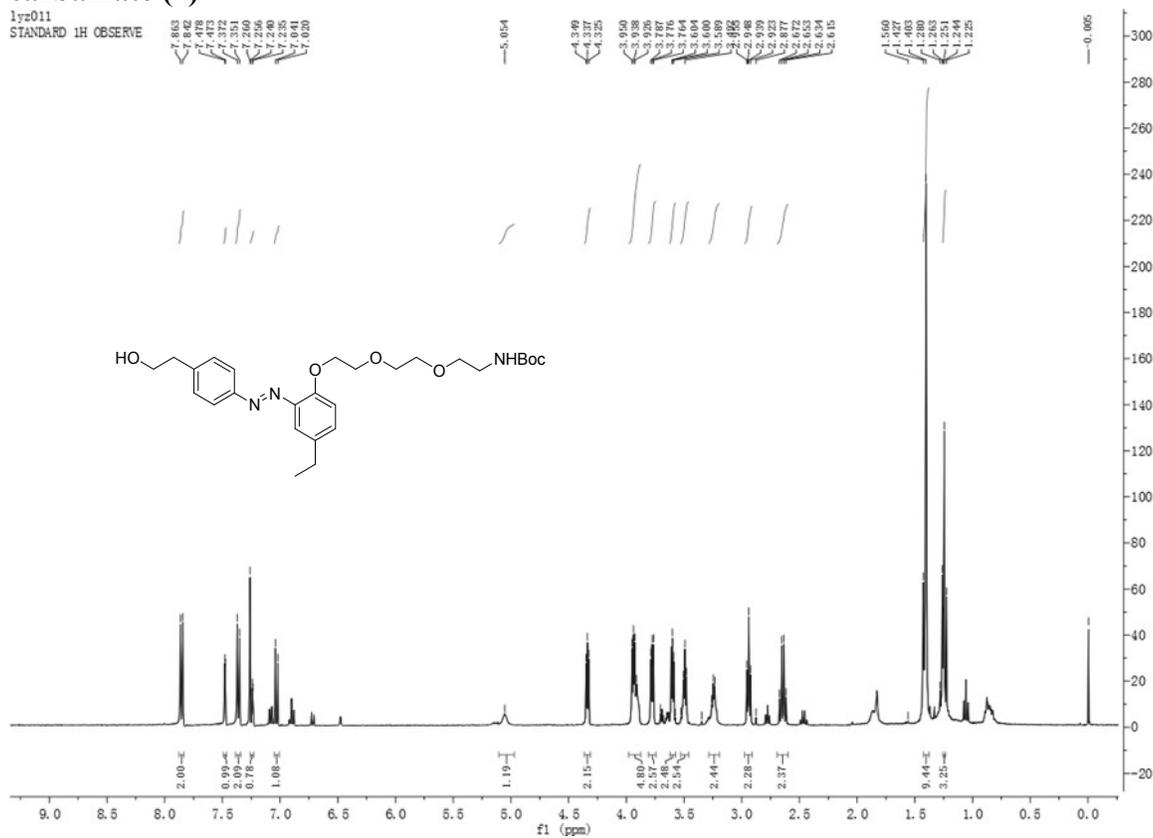
Reference

(1) Tang, D.; Jiang, M.; Li, X.; Kang, Y.; Wu, X.; Gong, B.; Shao, Z.; Zhao, X.; Shen, Y. *Chem. J. Chinese U.* **2014**, *35*, 2346-2352.

4-ethyl-2-((4-(2-hydroxyethyl)phenyl)diazenyl)phenol (3)

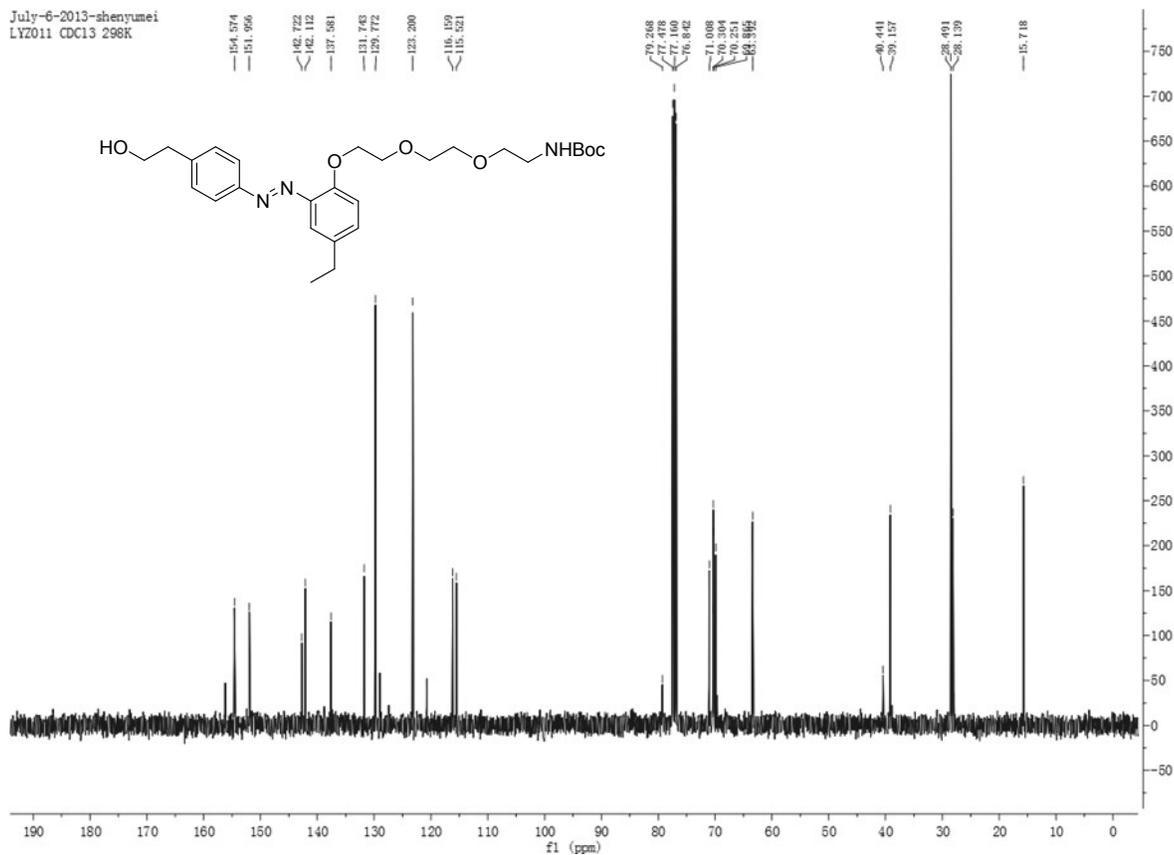


tert-butyl(2-(2-(2-(4-ethyl-2-((4-(2-hydroxyethyl)phenyl) diazenyl) phenoxy) ethoxy) ethoxy) ethyl) carbamate (4)



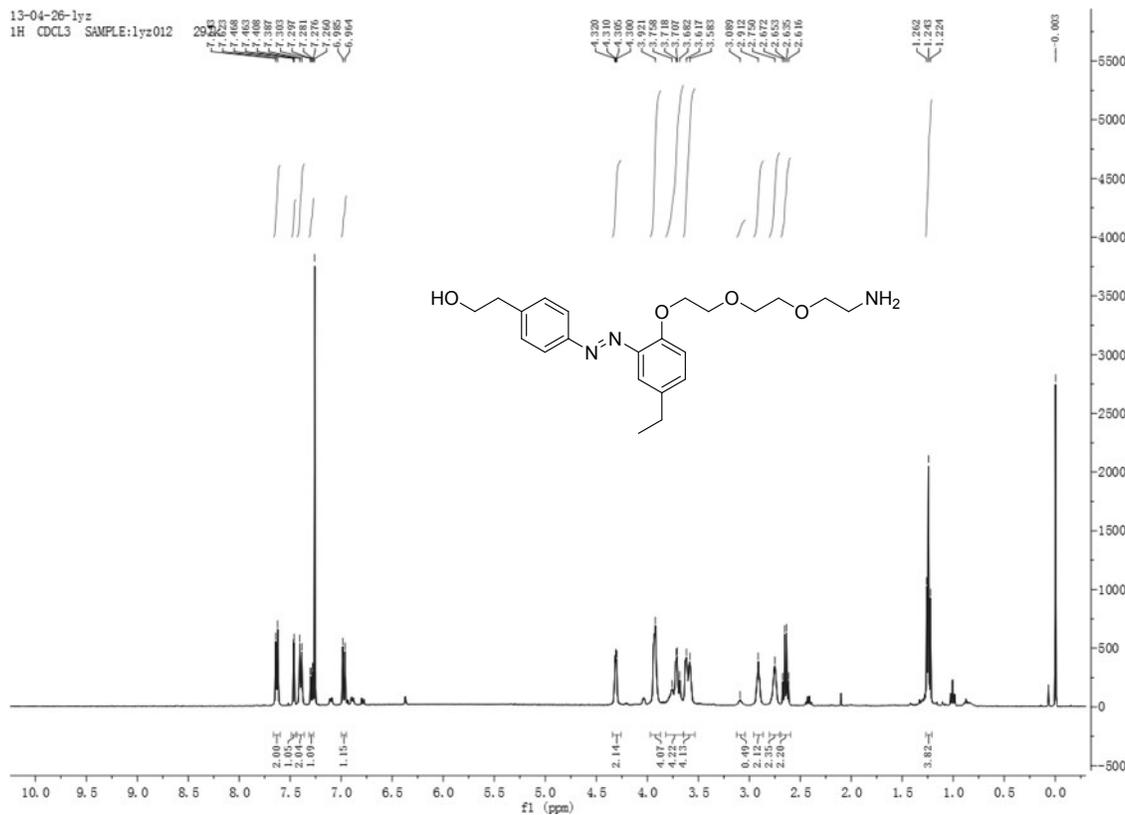
tert-butyl(2-(2-(2-(4-ethyl-2-((4-(2-hydroxyethyl)phenyl)diazenyl) phenoxy)ethoxy) ethoxy) ethyl) carbamate (4)

July-6-2013-shenyumei
LYZ011 CDCl3 296K



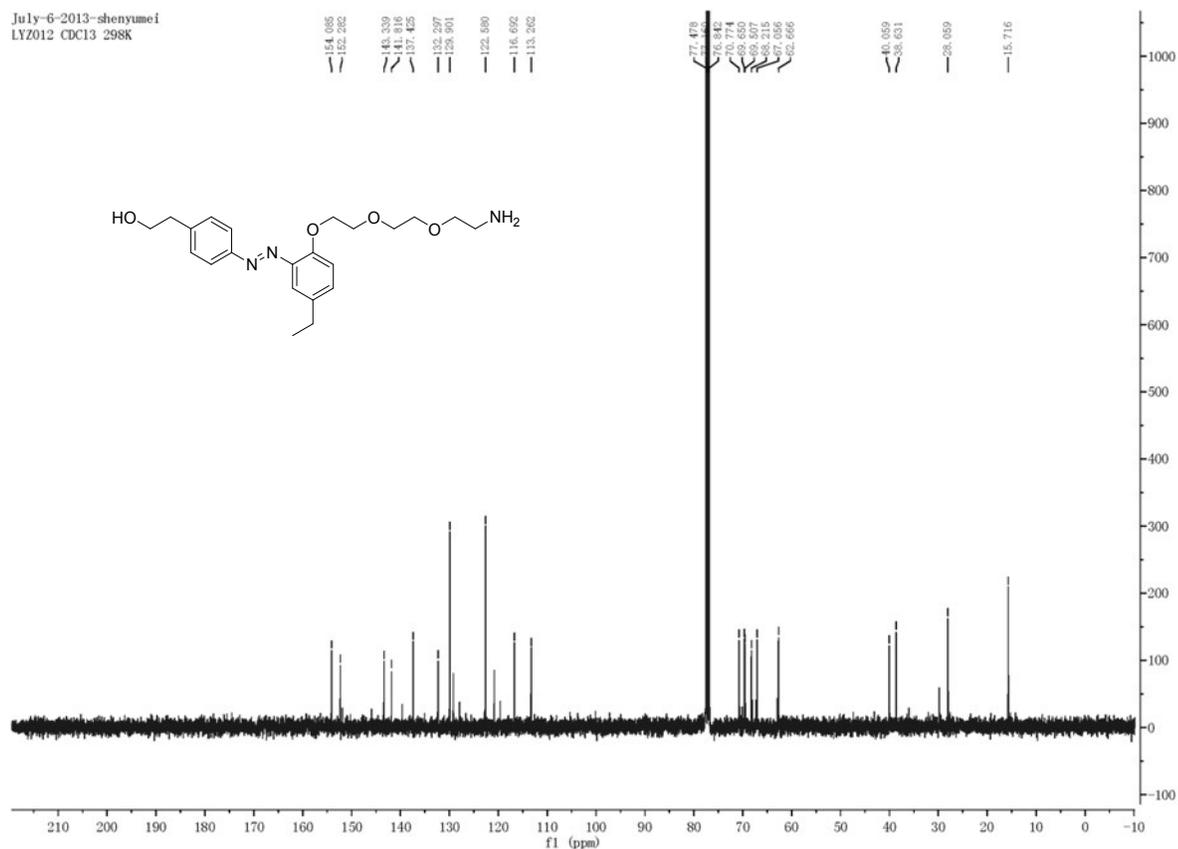
2-(4-((2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-5-ethylphenyl)diazenyl)phenyl)ethanol (5)

13-04-26-lyz
1H CDCl3 SAMPLE:lyz012

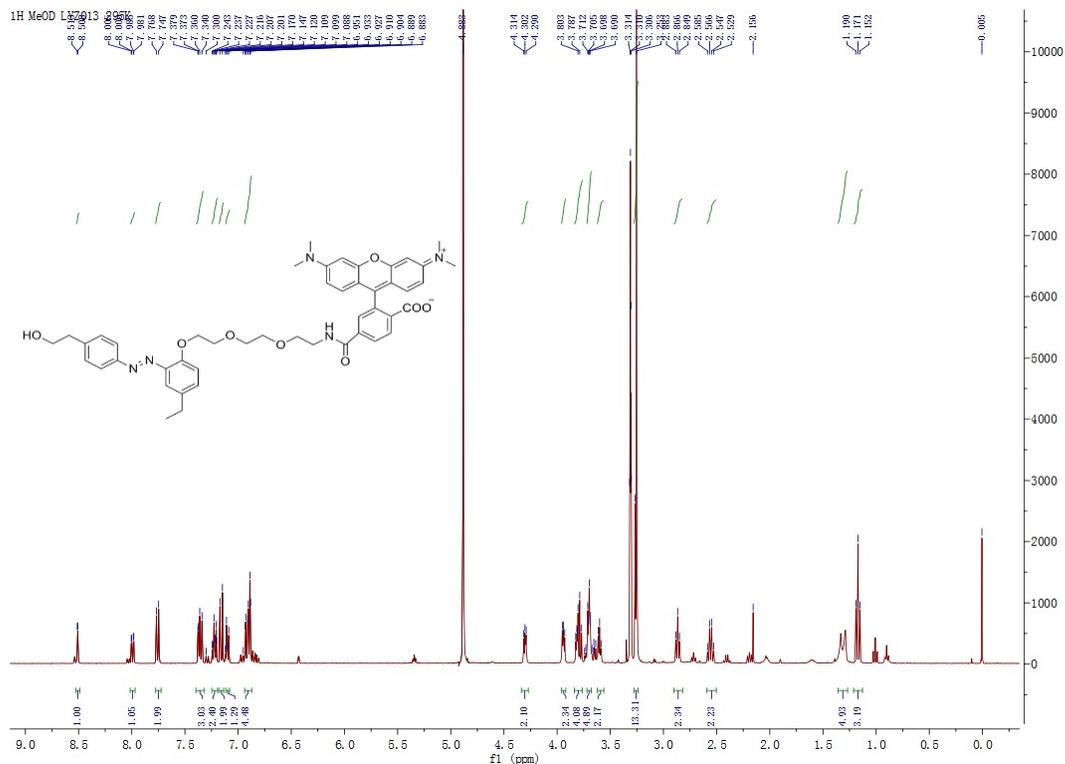


2-(4-((2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethoxy)-5-ethylphenyl)diazenyl)phenyl)ethanol (5)

July-6-2013-shenyumei
LYZ012 CDCl3 298K



2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)-4-((2-(2-(2-(4-ethyl-2-((4-(2-hydroxyethyl)phenyl)diazenyl)phenoxy)ethoxy)ethoxy)ethyl)carbamoyl)benzoate (6)

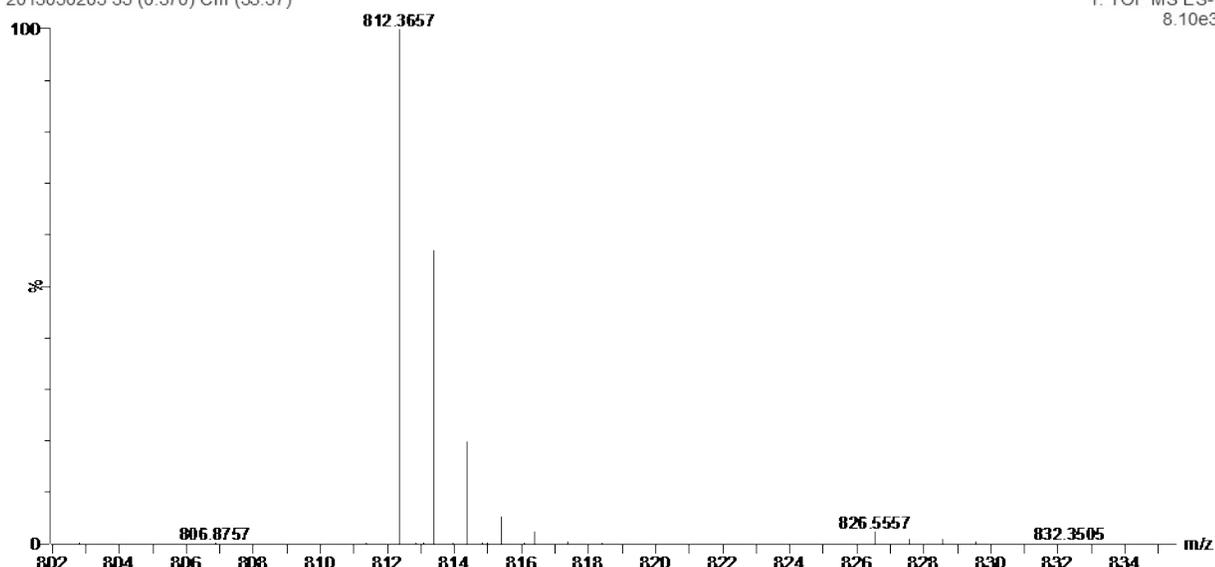


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LYZ013

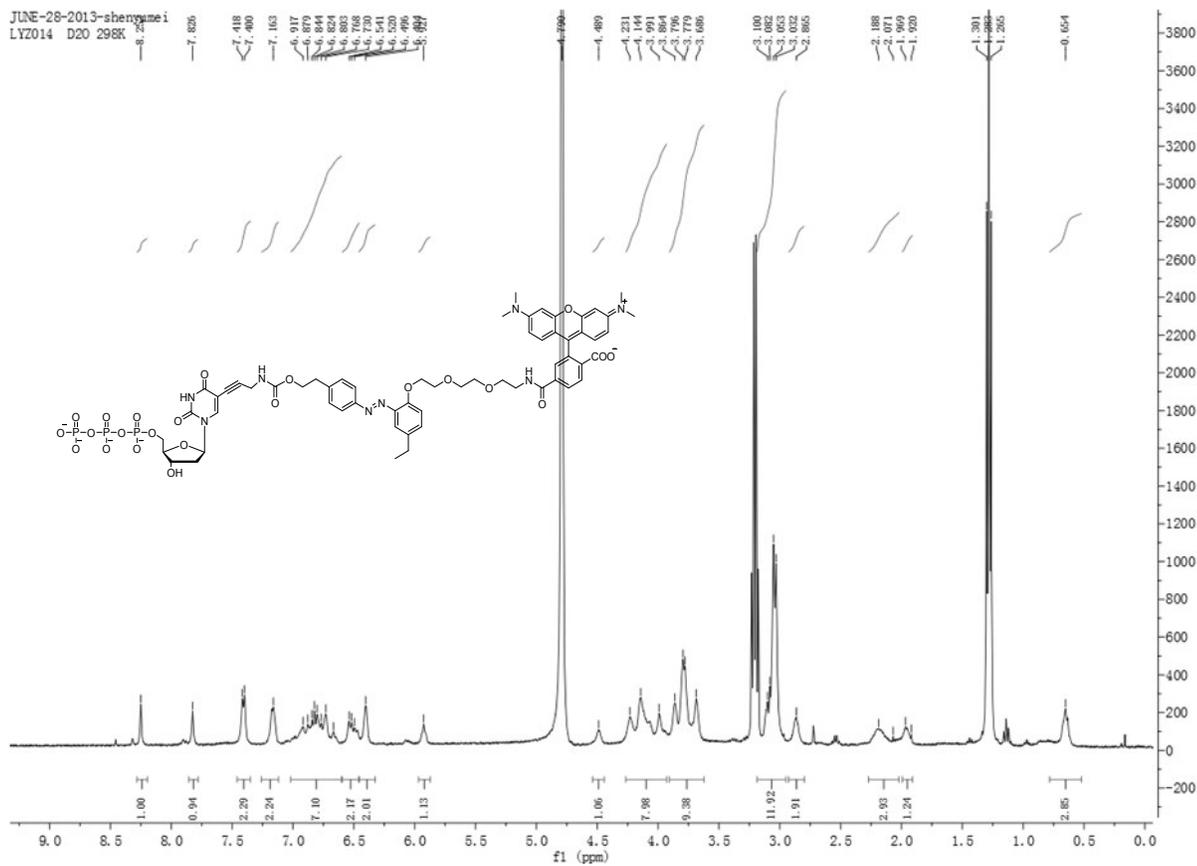
2013050203 35 (0.370) Cm (33:37)

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8.10e3



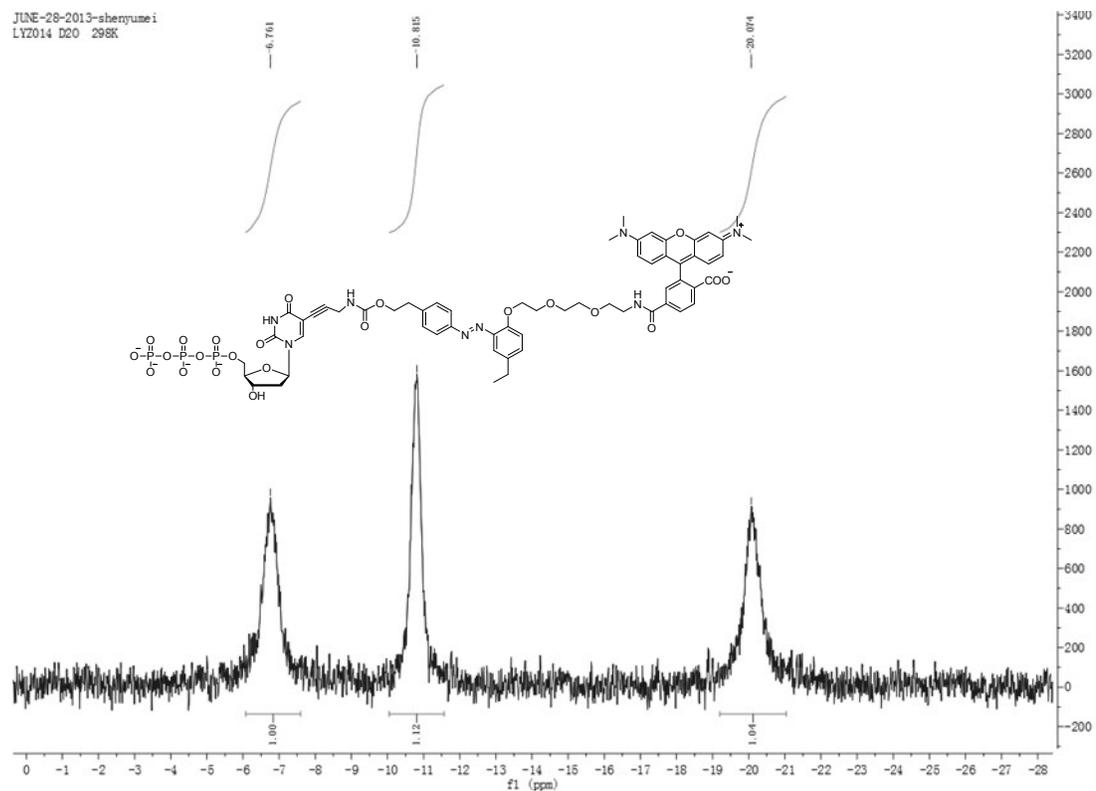
¹H-NMR for dUTP-azo-5(6)TAMRA (1)

JUNE-26-2013-shenyamei
LYZ014 D20 298K



³¹P-NMR for dUTP-azo-5(6)TAMRA (1)

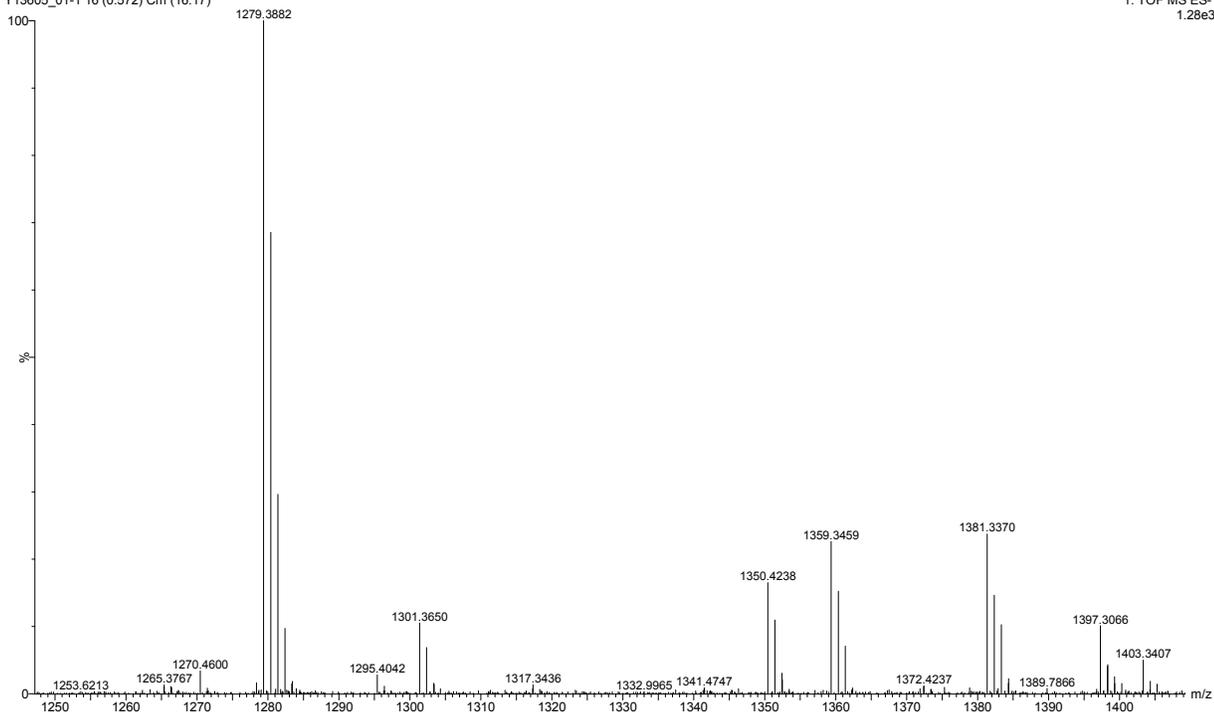
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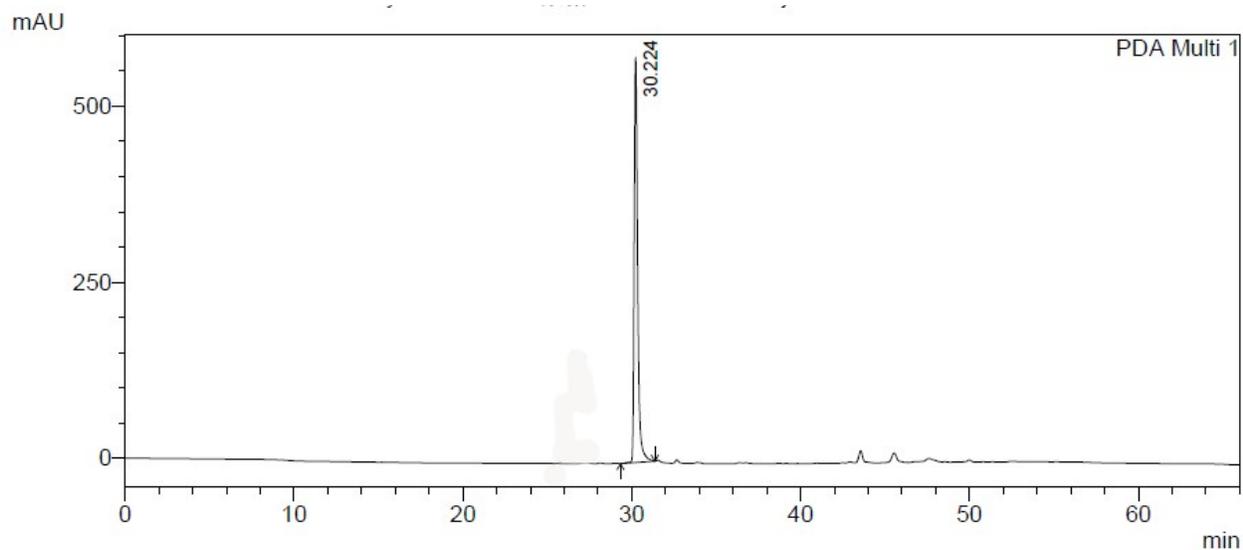
HRMS for dUTP-azo-5(6)TAMRA (1)

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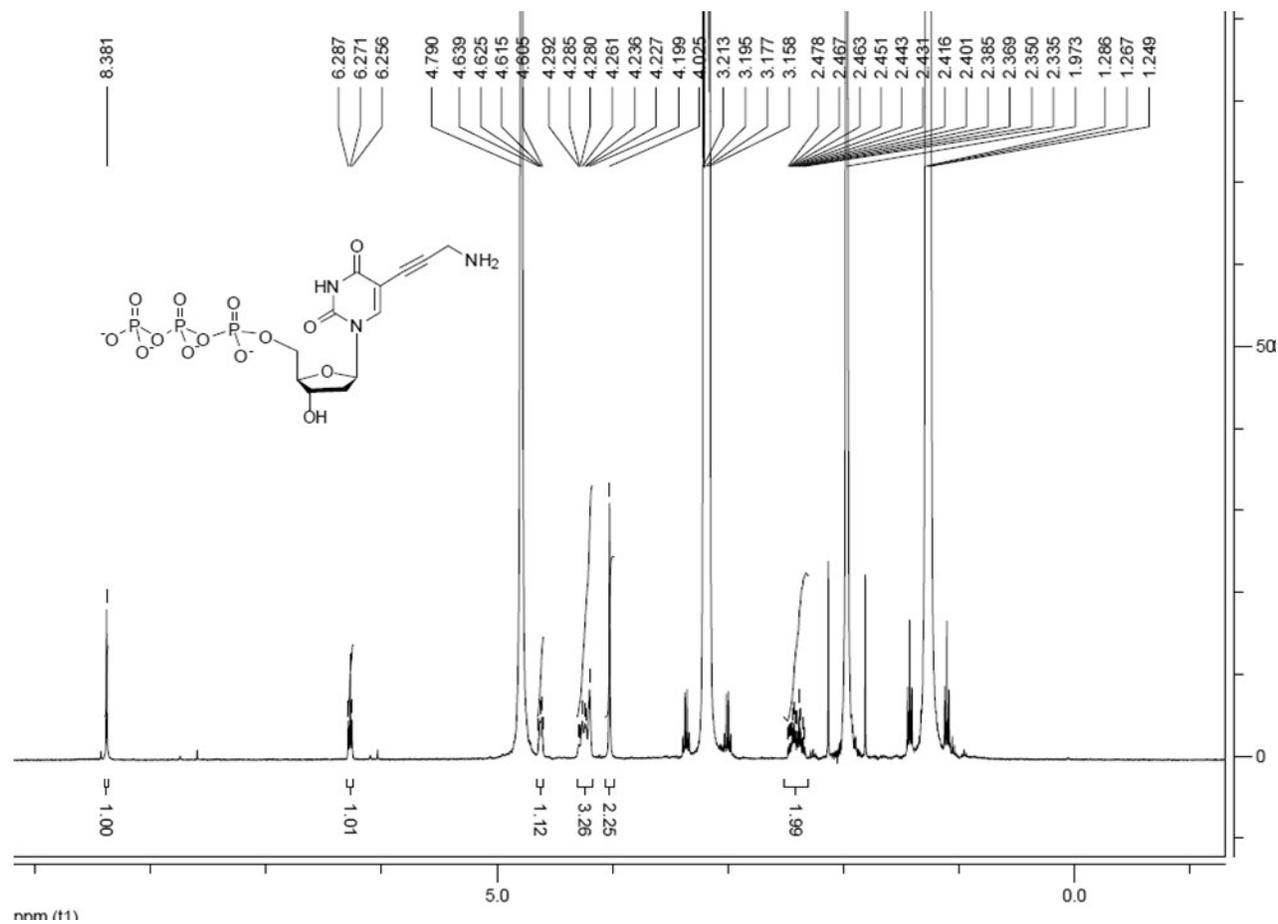


HPLC purity for dUTP-azo-5(6)TAMRA (1)

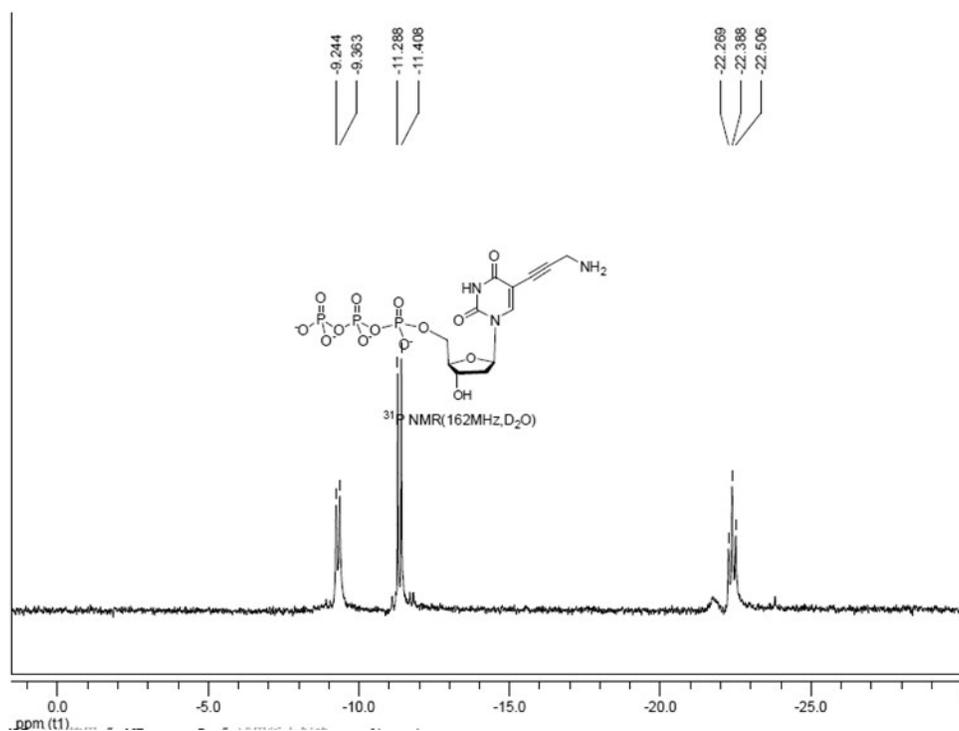


1 PDA Multi 1/546nm 4nm

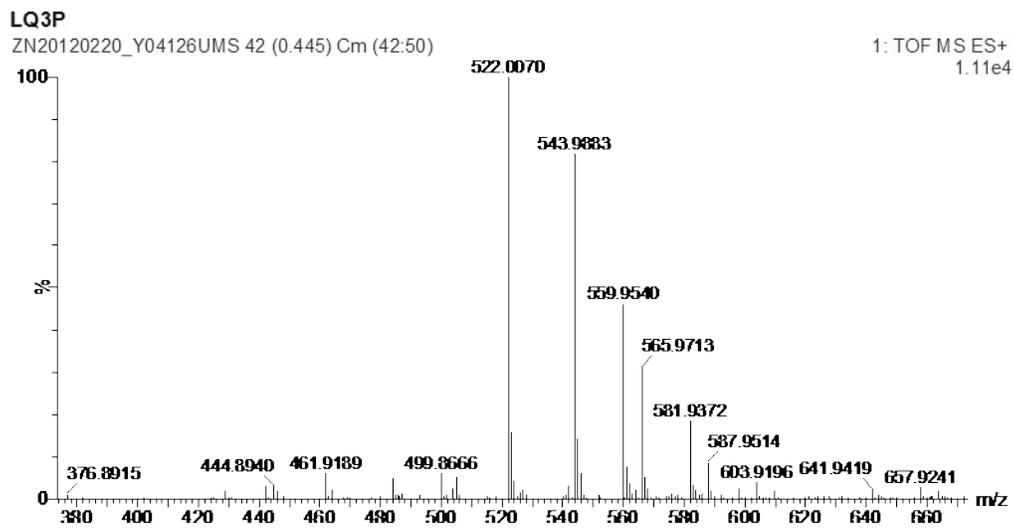
¹H-NMR for dUTP(AP₃)



³¹P-NMR for dUTP(AP₃)



HRMS for dUTP(AP₃)



HPLC for dUTP(AP₃)

