Supplementary file

of

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

Contents:

1. General Methods

Chemical reagents used in this work were purchased from Adamas, Sigma-Aldrich and Aladdin, and were used directly without further purification. Oligonucleotides used as primers or templates were designed by ourselves, primers were synthesized by Takara and templates were synthesized by Sangon Biotech. Bst 3.0 DNA polymerase was purchased from New England Biolabs, and ZipTip was purchased from Merk. Magnetic beads (Mag Beads Streptavidin 5 μm, 10 mg/mL) were purchased from TargetMol. The NMR spectra were recorded on 400 MHz Bruker Avance III spectrometer, with chemical shifts reported relative to TMS as an internal standard. The progress of reactions was monitored by thin-layer chromatography (TLC) using silica gel 60 F254 plates. ^{1}H NMR, ^{13}C NMR, ^{31}P NMR and mass spectrometry analyses were performed at the Instrumental Analytical Center of Shanghai Jiao Tong University. Fluorescence spectra were recorded on LS 55 luminescence spectrometer (PerkinElmer, US). Gel electrophoresis was conducted using JY-CX1B Nucleic Acid Electrophoresis Cell (Beijing JUNYI, China), and images were captured using Odyssey Infrared Imaging System (LI-COR Biosciences, US). DNA mass spectrometry analyses were performed by Sangon Biotech using a liquid-mass ion trap mass spectrometer (LTQ XL Thermo, US). Fluorescence images of magnetic beads were observed using Ti-U inverted phase contrast microscope (Nikon, Japan).

2. Chemical Experiment

tert-butyl (2-(2-(2-hydroxyethoxy)ethoxy)ethyl)carbamate (1)

To a solution of 2-(2-(2-aminoethoxy)ethoxy)ethan-1-ol (1.5 g, 10 mmol) in dichloromethane (DCM, 40 mL), triethylamine (2 mL) was added, followed by Boc_2O (3.2 g, 15 mmol) to the mixture. The reaction mixture was stirred at room temperature for 4 h. The resulting mixture was washed successively with 30 mL of water and 30 mL of saturated brine. The organic phase was dried over anhydrous sodium sulfate, and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography (eluted with 50% ethyl acetate in petroleum ether) to yield product **1**

(1.9 g, 76% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 3.74 (t, 2H, *J* = 4.4 Hz), 3.65-3.59 (m, 6H), 3.55 (t, 2H, *J* = 5.2 Hz), 3.32-3.30 (m, 2H), 1.43 (s, 9H).

2,2-dimethyl-4-oxo-3,8,11-trioxa-5-azatridecan-13-yl 4-methylbenzenesulfonate (2)

To a solution of compound **1** (1.9 g, 7.6 mmol) in DCM (20 mL), DMAP (0.1 g, 0.8 mmol) and triethylamine (2 mL) were added. Subsequently, a solution of 4-toluenesulfonyl chloride (1.8 g, 9.5 mmol) in DCM (20 mL) was added dropwise. The reaction mixture was stirred at room temperature overnight. The resulting mixture was concentrated under reduced pressure. The residue was purified by column chromatography (eluted with 30 % ethyl acetate in petroleum ether) to yield product **2** (2.8 g, 91% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.77 (d, 2H, *J* = 8.0 Hz), 7.32 (d, 2H, *J* = 8.0 Hz), 4.96 (s, 1H), 4.14 (t, 2H, *J* = 4.8 Hz), 3.66 (t, 2H, *J* = 4.8 Hz), 3.54-3.45 (m, 6H), 3.27-3.24 (m, 2H), 2.42 (s, 3H), 1.41 (s, 9H).

4-ethyl-2-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)diazenyl)phenol (3)

4-Aminophenylboronic acid pinacol ester (2.2 g, 10 mmol) was dissolved in a mixture of acetone (10 mL) and water (5 mL), and 37% hydrochloric acid (5 mL) was added to the solution. The resulting suspension was cooled to 0-5 ℃ in an ice bath, and solution of sodium nitrite (690 mg, 10 mmol) in water (4 mL) was added slowly, in order to make sure that the temperature was kept below 5 ℃. The mixture was stirred at 0 ℃ for 30 min. Subsequently, the mixture was slowly added to a solution of 4-ethylphenol (1.2 g, 10 mmol) and NaOH (1 g, 25 mmol) in water (20 mL), which was also kept in an ice bath, and the temperature remained below 5 ℃. The reaction mixture was then stirred at room temperature for 5 h. The resulting mixture was concentrated under reduced pressure. The residue was diluted with 30 mL of dichloromethane, washed successively with 20 mL of water and 20 mL of saturated brine, dried over anhydrous sodium sulfate, and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography (eluted with 20% ethyl acetate in petroleum ether) to yield product **3** (1.3 g, 37% yield). ^{1}H NMR (400 MHz, $CDCl_3$) 12.79 (s, 1H), 8.00-7.92 (m, 2H), 7.89 -7.82 (m, 2H), 7.79 (d, *J* = 2.3 Hz, 1H), 7.21 (dd,

J = 8.4, 2.3 Hz, 1H), 6.96 (d, *J* = 8.4 Hz, 1H), 2.69 (q, *J* = 7.6 Hz, 2H), 1.38 (s, 12H), 1.29 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 152.80, 150.71, 147.16, 137.36, 135.91, 133.56, 132.04, 124.30, 121.40, 119.19, 118.05, 117.93, 91.06, 31.71, 30.28, 27.82, 25.16, 15.67. HRMS (ESI): calc. for $C_{20}H_{26}BN_2O_3$ $[M+H]^+$ 353.2036, found 353.2073.

tert-butyl-(2-(2-(2-(4-ethyl-2-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)diazenyl)phenoxy)ethoxy)ethoxy)ethyl)carbamate (4)

A solution of compound **2** (345 mg, 0.85 mmol), compound **3** (603 mg, 1.7 mmol), and K_2CO_3 (130 mg, 1.9 mmol) in anhydrous DMF (5 mL) was stirred at 120 ℃ for 2.5 h. The resulting mixture was concentrated under reduced pressure. The residue was diluted with 15 mL of ethyl acetate, washed with three portions of 10 mL water, dried over anhydrous sodium sulfate, and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography (eluted with 50% ethyl acetate in petroleum ether) to yield product **4** (216 mg, 44% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 7.95-7.91 (m, 2H), 7.87 (d, *J* = 8.4 Hz, 2H), 7.51 (d, *J* = 2.3 Hz, 1H), 7.04 (d, *J* = 8.5 Hz, 1H), 6.92 (dd, *J* = 33.5, 8.2 Hz, 1H), 4.35 (t, *J* = 4.9 Hz, 2H), 3.97-3.93 (m, 2H), 3.82-3.77 (m, 2H), 3.63-3.58 (m, 2H), 3.52 (q, *J* = 5.2, 4.5 Hz, 2H), 3.28 (d, *J* = 4.5 Hz, 2H), 2.65 (q, *J* = 7.6 Hz, 2H), 1.42 (s, 9H), 1.37 (s, 12H), 1.27-1.25 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 156.87, 155.07, 154.89, 142.29, 137.11, 135.42, 132.32, 121.85, 118.72, 115.43, 114.60, 84.05, 77.52, 75.26, 70.68, 70.03, 69.80, 69.53, 60.22, 40.01, 27.64, 24.97, 23.89, 19.79, 15.08, 13.35. HRMS (ESI): calc. for $C_{31}H_{46}BN_3O_7Na$ $[M+Na]^+$ 606.3327, found 606.3333.

tert-butyl(2-(2-(2-(4-ethyl-2-((4-(1-((2R,4R,5R)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)phenyl)diazenyl)phenoxy)ethoxy)ethoxy)ethyl)carbamate (5)

Under nitrogen atmosphere, mixture of H_2O/CH_3CN (2:1, 4 mL) was added to a flask which containing 5- iodo-2′-deoxyuridine (177 mg, 0.5 mmol), compound **4** (300 mg, 0.5 mmol), and Cs_2CO_3 (488 mg, 1.5 mmol). In a separate flask, $Pd(OAc)_2$ (11 mg, 8 μmol) and TPPTS (142 mg, 25 μmmol) were combined, and the flask was evacuated

and purged with argon. Subsequently, H_2O/CH_3CN (2:1, 2 mL) was added to the flask containing the catalyst mixture. The catalyst mixture was then injected into the reaction mixture, then the reaction was stirred at 90 °C for 0.5 h. The resulting mixture was concentrated under reduced pressure to remove acetonitrile. The residual aqueous phase was extracted with ethyl acetate (3 × 5 mL). The organic phase was dried over anhydrous sodium sulfate, and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography (eluted with 20% ethyl acetate in petroleum ether) to yield product **5** (192 mg, 56% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.46-8.35 (m, 1H), 8.15 (s, 1H), 7.98 (d, *J* = 8.0 Hz, 2H), 7.78 (d, *J* = 8.0 Hz, 2H), 7.54 (s, 1H), 7.05 (d, *J* = 8.6 Hz, 1H), 7.01-6.93 (m, 1H), 6.45-6.38 (m, 1H), 5.33 (s, 1H), 4.73-4.64 (m, 1H), 4.40-4.34 (m, 2H), 4.11-4.03 (m, 2H), 4.02-3.96 (m, 2H), 3.95-3.89 (m, 2H), 3.86-3.79 (m, 2H), 3.68-3.64 (m, 2H), 3.56-3.52 (m, 2H), 3.25-3.16 (m, 2H), 2.69 (q, *J* = 7.6 Hz, 2H), 1.48-1.43 (m, 3H), 1.41 (s, 9H); ^{13}C NMR (101 MHz, CD_3OD) δ 162.90, 157.01, 154.81, 152.05, 150.35, 142.33, 139.07, 137.24, 131.20, 124.99, 115.41, 115.32, 113.54, 87.77, 85.67, 78.76, 70.68, 70.62, 69.98, 69.80, 69.72, 69.55, 61.19, 40.56, 39.95, 27.69, 27.50, 14.93; HRMS (ESI): calc. for $C_{34}H_{45}N_5O_{10}Na$ $[M+Na]^+$ 706.3064, found 706.3062.

5-(4-((2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-5-ethylphenyl)diazenyl)phenyl)-1-((2R,4R,5R)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione (6)

Trifluoroacetic acid (1 mL) was added to solution of compound **5** (190 mg, 0.28 mmol) in dichloromethane (DCM, 1 mL), the mixture was stirred in ice bath for 2 h. The reaction was quenched with saturated sodium bicarbonate solution and extracted with DCM (3 × 5 mL). The organic phase was dried over anhydrous sodium sulfate, and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography (eluted with 10% methanol in dichloromethane) to yield product **6** (116 mg, 71% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.54 (s, 1H), 7.94 (d, *J* = 8.6 Hz, 2H), 7.82 (d, *J* = 8.6 Hz, 2H), 7.54 (d, *J* = 2.2 Hz, 1H), 7.39 (dd, *J* = 8.5, 2.3 Hz, 1H), 7.20 (d, *J* = 8.4 Hz, 1H), 6.41 (t, *J* = 6.5 Hz, 1H), 4.42-4.36 (m, 2H), 4.04-

3.97 (m, 3H), 3.92-3.79 (m, 5H), 3.75-3.65 (m, 6H), 3.07-3.00 (m, 2H), 2.43-2.37 (m, 2H), 1.31-1.25 (m, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 163.09, 154.68, 152.13, 150.36, 142.31, 139.16, 137.49, 135.81, 132.10, 128.61, 122.47, 115.46, 115.23, 113.53, 87.81, 85.69, 70.67, 70.57, 69.95, 69.67, 69.54, 66.48, 61.16, 40.50, 39.30, 27.66, 14.85; HRMS(ESI): calc. for $C_{29}H_{37}N_5O_8$ $[M+H]^+$ 584.2720, found 584.2779.

N-(2-(2-(2-(4-ethyl-2-((4-(1-((2R,4R,5R)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)phenyl)diazenyl)phenoxy)ethoxy)ethoxy)ethyl)-2,2,2-trifluoroacetamide (7)

Triethylamine (1 mL) was added to solution of compound **6** (320 mg, 0.55 mmol) in methanol (10 mL), the mixture was stirred in an ice bath, and ethyl trifluoroacetate (93 mg, 0.65 mmol) was subsequently added to the solution. The reaction mixture was then stirred at room temperature for 4 h. The resulting mixture was concentrated under reduced pressure. The residue was diluted with 20 mL of dichloromethane, washed successively with 10 mL of water and 10 mL of saturated brine, dried over anhydrous sodium sulfate, and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography (eluted with 10% methanol in dichloromethane) to yield product **7** (358 mg, 96% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.53 (s, 1H), 7.97-7.90 (m, 2H), 7.86-7.78 (m, 2H), 7.60-7.54 (m, 1H), 7.53 (d, J = 2.3 Hz, 1H), 7.37 (dd, J = 8.5, 2.3 Hz, 1H), 7.19 (d, J = 8.5 Hz, 1H), 6.41 (t, J = 6.5 Hz, 1H), 4.51 (q, J = 4.6 Hz, 1H), 4.40-4.33 (m, 2H), 4.04-3.99 (m, 2H), 4.00-3.95 (m, 2H), 3.83-3.77 (m, 2H), 3.68-3.63 (m, 2H), 3.44 (t, J = 5.4 Hz, 2H), 3.38 (s, 1H), 2.69 (q, J = 7.6 Hz, 2H), 2.40 (dd, J = 6.6, 5.1 Hz, 2H), 1.28 (t, J = 7.6 Hz, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 162.96, 154.75, 152.06, 150.39, 142.33, 137.29, 131.97, 129.34, 128.54, 127.76, 123.27, 122.48, 121.65, 117.55, 115.38, 113.55, 87.76, 85.66, 70.67, 70.59, 69.97, 69.74, 69.54, 68.36, 61.17, 40.47, 39.38, 27.66, 14.85; HRMS (ESI): calc. for $C_{31}H_{35}F_3N_5O_9$ $(M-H)^-$ 678.2387, found 678.2379.

((2R,3R,5R)-5-(5-(4-((2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-5-ethylphenyl)diazenyl)phenyl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphatee (8)

Throughout the entire experiment, syringes were used to transfer solvent reagents from one flask to another under an argon atmosphere. Dried triethylamine (1 mL) was transferred to the septum of a flask containing tributylammonium pyrophosphate (282 mg, 0.51 mmol) dissolved in 1 mL of DMF. The mixture was stirred at room temperature for 0.5 h, then was injected into the septum of flask containing dried 2-chloro-4-H-1,3,2-benzodioxaphosphorin-4-one (104 mg, 0.51 mmol) dissolved in DMF (1 mL) while vigorous stirring. After 30 min, the reaction mixture was added to a flask containing compound **7** (200 mg, 0.29 mmol) dissolved in DMF (1 mL), and continued tirring for 2 h. Iodine solution [3% in 4 mL of 9:1 (pyridine:water)] was injected into the reaction mixture, after 15 min of stirring at room temerature, water (6 mL) was added and the mixture was continued stirring at room temperature for another 2 h. The resulting solution was evenly transferred to two 50 mL centrifuge tubes. NaCl (3 mL, 3 M) solution was added to each tube, followed by the addition of ethanol (30 mL). After placing the tubes at -80 °C for 0.5 h, the crude product was recovered by centrifugation (20 minutes at 3200 rpm). The supernatant was removed, and the residue was transferred to a round-bottom flask, then ammonia solution (6 mL, 25%) was added to the residue solution and stirred at room temperature overnight. Finally the mixture was purified using reverse-phase high-performance liquid chromatography (RP-HPLC) on a 250 × 10 mm C18 column to obtain the product as a triethylammonium salt. After lyophilization, the product **8** was obtained as a yellow solid (64 mg, 27%). Mobile phase: A, 20 mM TEAA in water (pH = 8.5); B, methanol. Elution was performed with a gradient of 5-30% B for 5 minutes, followed by 30-50% B for 20 minutes at a flow rate of 4 mL/min.^{1}H NMR (400 MHz, D_2O) δ 7.98-7.87 (m, 1H), 7.82-7.71 (m, 2H), 7.68-7.57 (m, 2H), 7.42-7.36 (m, 1H), 7.18-7.05 (m, 1H), 7.03-6.87 (m, 1H), 6.30-6.21 (m, 1H), 4.39-4.27 (m, 2H), 4.23-4.08 (m, 3H), 4.06-3.99 (m, 1H), 3.95-3.86 (m, 2H), 3.74-3.63 (m, 4H), 3.61-3.53 (m, 2H), 3.02-2.90 (m, 2H), 2.67-2.50 (m, 2H), 2.43-2.35 (m, 2H), 1.18-1.09 (m, 3H). ^{31}P NMR (162 MHz, D_2O) δ -7.02, -11.24, -21.27. HRMS (ESI): calc. for $C_{29}H_{39}N_5O_{17}P_3$ $(M-H)^-$ 822.1554, found 822.1548.

dUTP-Azo linker-Cy3 (9)

To a solution of compound **8** (11.5 mg, 14 μmol) in 2 mL of 0.5 M Na2CO3-NaHCO3 buffer, a solution of Cy3-NHS (2 mg, 2.7 μmol) in acetonitrile (1 mL) and triethylamine (0.1 mL) were added. The mixture was stirred at room temperature overnight in the dark. The solvent was then removed under vacuum, and the crude product **9** was purified by preparative HPLC using a C-18 reverse phase column (5 μm, 250 mm × 10 mm) to yield the pure final product **9** (retention time = 21 min) as a triethylammonium salt. After lyophilization, the product **9** was obtained as a red solid (2.1 mg, 12% yield). Mobile phase: A, 20 mM TEAA in water (pH = 8.5); B, methanol. Elution was performed with a gradient of 5-60% B for 5 min, followed by 60-66% B for 15 min at a flow rate of 4 mL/min. ^{1}H NMR (400 MHz, D_2O) δ 8.14-8.04 (m, 1H), 7.90-7.90 (m, 2H), 7.86-7.78 (m, 2H), 7.71-7.64 (m, 2H), 7.53-7.42 (m, 4H), 7.41-7.28 (m, 4H), 7.25-7.09 (m, 4H), 6.33-6.19 (m, 1H), 6.12-5.98 (m, 2H), 4.44-4.26 (m, 4H), 4.11-4.04 (m, 2H), 3.98-3.91 (m, 1H), 3.87-3.76 (m, 3H), 3.74-3.67 (m, 2H), 3.51 (s, 3H), 3.47-3.41 (m, 2H), 2.54-2.36 (m, 2H), 2.31-2.19 (m, 4H), 1.73-1.59 (m, 6H), 1.55-1.50 (m, 4H), 1.45 (s, 6H), 1.38 (s, 6H), 0.99-0.93 (m, 3H). ^{31}P NMR (162 MHz, D_2O) δ -10.58, -11.67, -23.11. HRMS (ESI): calc. for $C_{59}H_{74}N_7NaO_{18}P_3^+$ (M-H+Na) $^+$ 1284.4195, found 1284.4192.

3. Biological Evaluations

DNA Extension/Cleavage Cycles

A fluorescence-labeled 24-nt oligonucleotide (Primer 1 in Table S1) was used as primer for denaturing gel electrophoresis. Two oligonucleotides with slightly different base sequences were used as templates (Template 1 and 2 in Table S1), and the other four oligonucleotides were used for comparison (Primer 2, Template 3, 4 and 5 in Table S1). The primers and templates were mixed in a Tris-EDTA buffer (TE, pH 7.5) and annealed 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 components listed in Table S2 were mixed in a microfuge tube to a final volume of 20 μL. After gentle vortex

mixing, the mixture was incubated at 30 °C for 5 min and then heated at 65 °C for 10 min, followed by cooling to 4 °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 5 min at 37 °C to remove the fluorophore. The cleavage process was stopped by adding 4.2 μL of 1 M Tris-HCl buffer (pH 8.5), yielding cleavage product 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.

Table S1. Sequence of primers and templates

No.	Sequence
Primer 1	Dylight 800-5′-GAGGAAAGGGAAGGGAAAGGAAGG-3′
Primer 2	Dylight 800-5′-GAGGAAAGGGAAGGGAAAGGAAGGT-3′
Primer 3	AMCA-5′-CTGCCCCGGGTTCCTCATTCTCT-3′
Template 1	3′-CTCCTTTCCCTTCCCTTTCCTTCC**A**TCGATCGCCATGTCG-5′
Template 2	3′-CTCCTTTCCCTTCCCTTTCCTTCC**AAAA**GTCGCCATGTGC-5′
Template 3	3′-CTCCTTTCCCTTCCCTTTCCTTCC**G**TCGATCGCCATGTCG-5′
Template 4	3′-CTCCTTTCCCTTCCCTTTCCTTCC**T**ACGATCGCCATGTCG-5′
Template 5	3′-CTCCTTTCCCTTCCCTTTCCTTCC**C**TCGATCGCCATGTCG-5′
Template 6	3′-GACGGGGCCCAAGGAGTAAGAG**AAAAAA**CTGCGCGTGCG AGTGGTGAGATAGTGGCTGAC-5′- dual biotin

*The position in the template strands denoting the nucleotide to be incorporated is in bold.

Table S2. Buffer of DNA SBS

Additives	Volume (μL)
10 × isothermol amp buffer II	2
$MgSO_4$ (100 mM)	1.5
Bst 3.0 DNA pol (8 U/μL)	1
9 (5 μM)	4
template/primer (1000 ng/μL)	1.5
ddH_2O	10

Denaturing Polyacrylamide Gel Electrophoresis

DNA sequencing gel electrophoresis was performed on 12% denaturing polyacrylamide gels containing 7 M urea, using a JY-CX1B Nucleic Acid Electrophoresis Cell (Beijing JUNYI, China). Before electrophoresis, the prepared gels were pre-run at a voltage of 2000 V and a constant power of 60 W for 30 min. meanwhile 1 μL of the sequencing reaction samples (~15 ng/μL DNA) was mixed with 1.5 μL of 6 × loading buffer containing marker dyes, and the mixture was then loaded into each well of the gels. The gels were run under the aforementioned conditions for 150 min. The resultant gels were visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences, US) under excitation of a 785 nm laser.

Mass Spectrometry Analysis

The samples at each step were desalted using the ZipTip protocol and then sent to Sangon Biotech for mass spectrometry analyses. The basic experimental parameter settings were as follows:

(a) Ionization spray voltage: 4.5 kV,

(b) Atomization temperature: 350°C,

(c) Ion mode: negative ion mode.

Superparamagnetic Beads Experiments.

Superparamagnetic beads (Mag Beads Streptavidin, 5 μm, 10 mg/mL, TargetMol, US) were used as substrates for extension/cleavage cycles reactions. A 60-mer 5'-dual biotin-modified oligonucleotide (Template 6 in Table S1) was used as the template, and a 23-mer 5'-AMCA-modified oligonucleotide (Primer 3 in Table S1) was used as the primer. The beads were washed three times with 1× B&W Buffer before experiment. The biotinylated template was hybridized with the primer, followed by the annealing procedure. The annealed primer and template were then added to the washed beads, which were incubated for 15 min at room temperature under gentle rotation to immobilize the template. The primer and template-coated beads were separated using a magnetic grate, washed twice with 1× B&W Buffer, and used for extension/cleavage cycles experiment. The dUTP-Azo linker-Cy3 (**9**) was incorporated into the primer on

the beads using the same extension protocol. The resultant beads were separated, washed, and immersed in 30 mM sodium dithionite in NaAc/HAc buffer (pH 5.2) for 5 min at 37 °C to remove the fluorophore. The beads were then separated and washed for the next extension/cleavage cycles. The beads at each step were observed using Ti-U inverted phase contrast microscope (Nikon, Japan). The fluorescence emission in Cy3 channel was detected under excitation of 560 nm laser for both the DNA-coated beads and blank beads. For each image, 110 beads were analyzed, and the emission intensity was reported as average ± standard deviation. The emission in the AMCA channel was also detected under excitation of 350 nm laser to make sure that the amount of primer on the beads remained approximately constant during the cycles of extension and cleavage experiment.

4. Spectra of Compounds

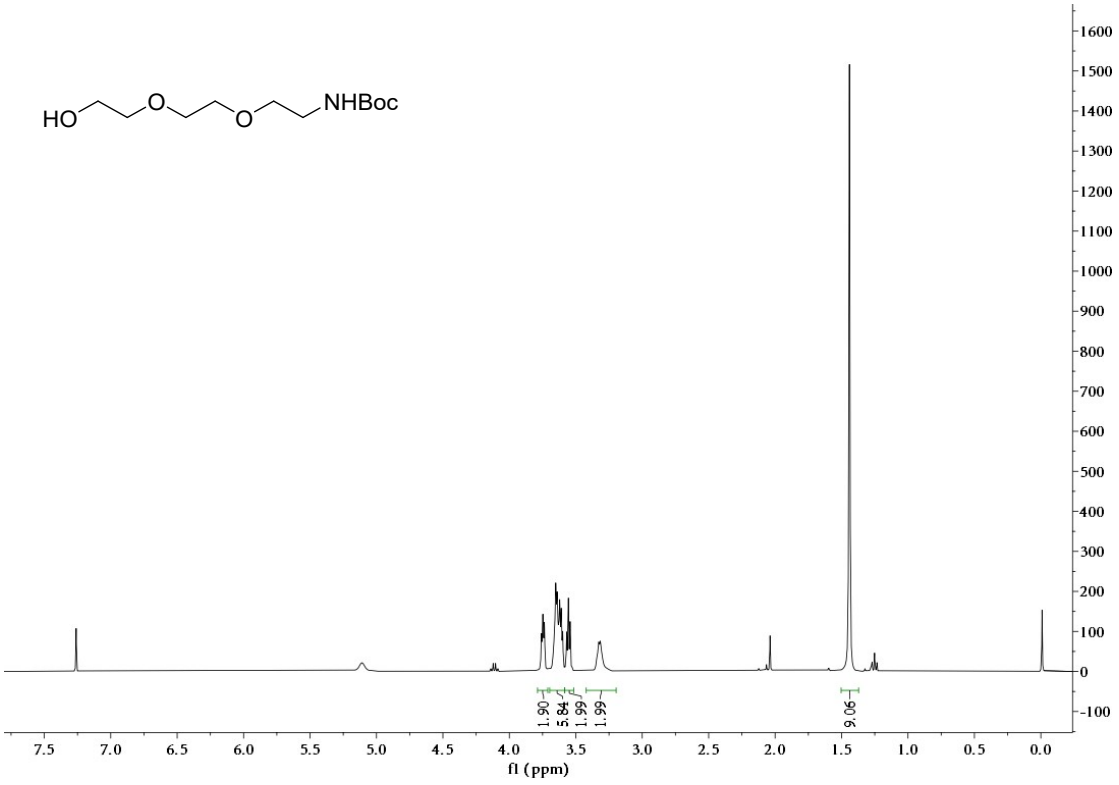

Fig S1: ^{1}H NMR of Compound **1**

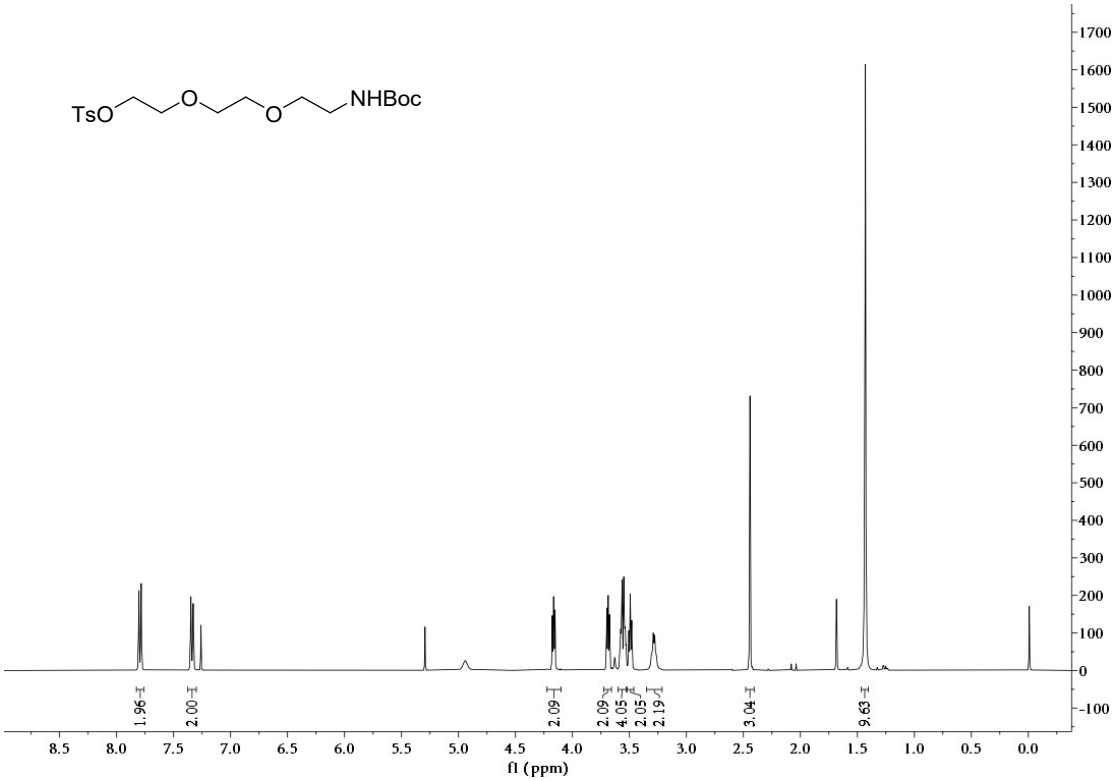

Fig S2: [1]H NMR of Compound **2**

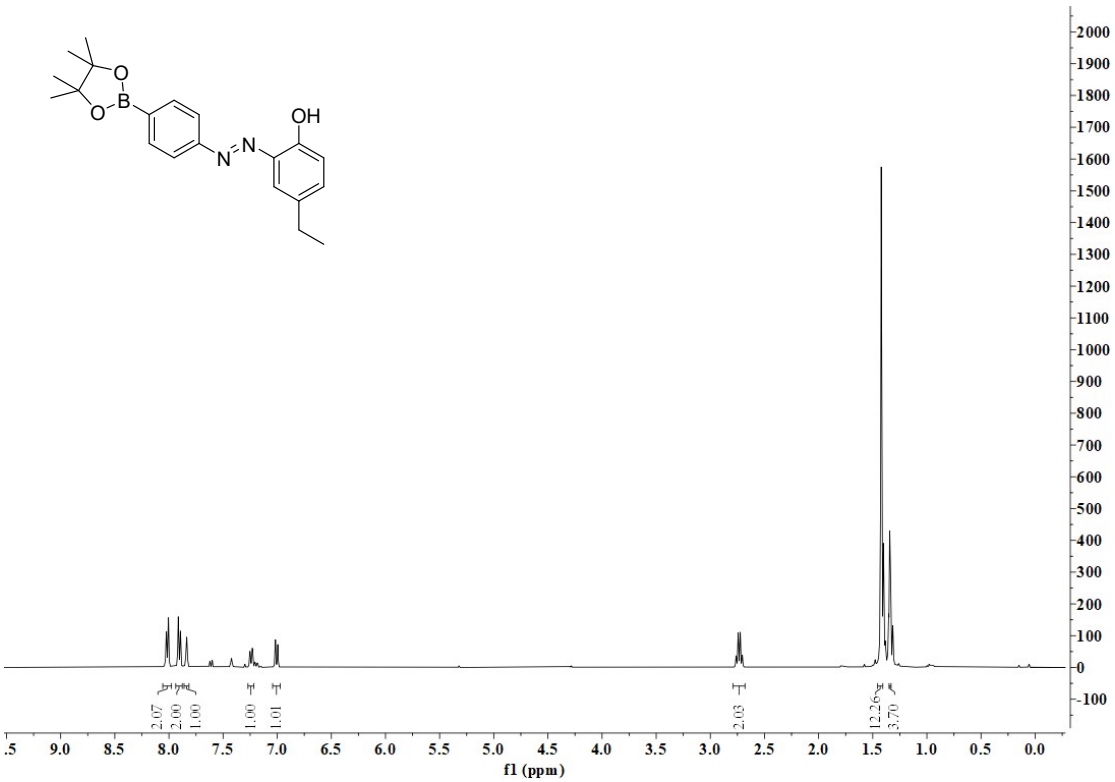

Fig S3: [1]H NMR of Compound **3**

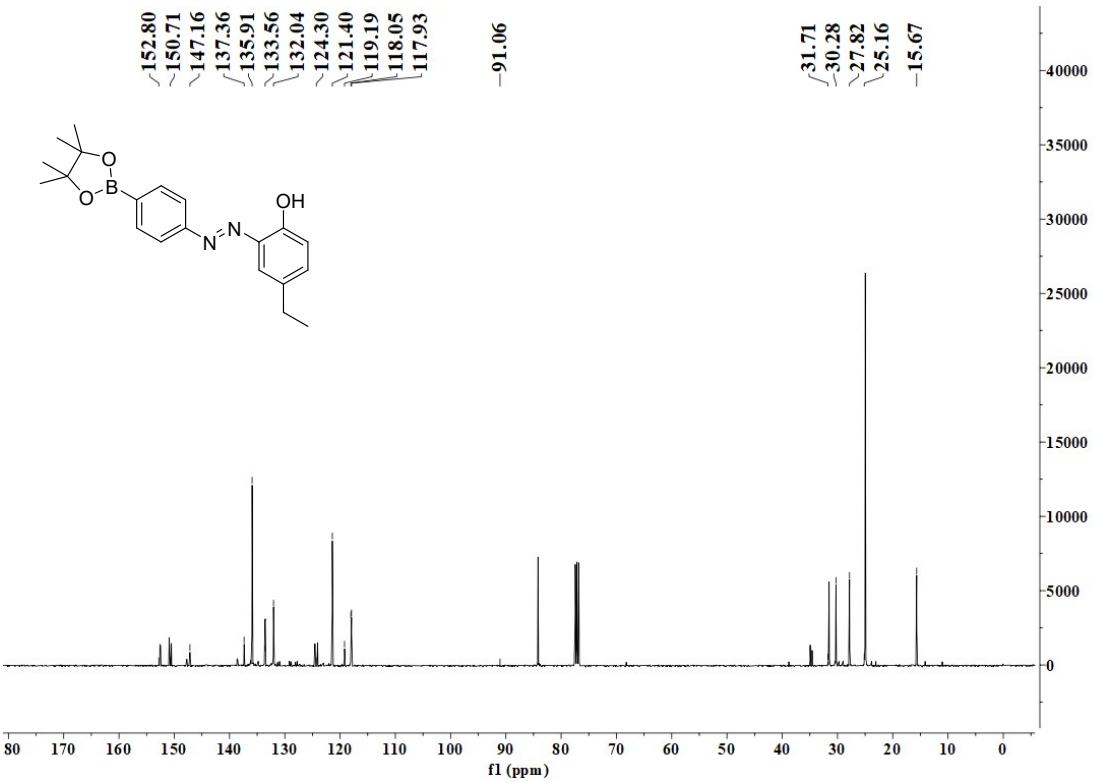

Fig S4: ^{13}C NMR of Compound **3**

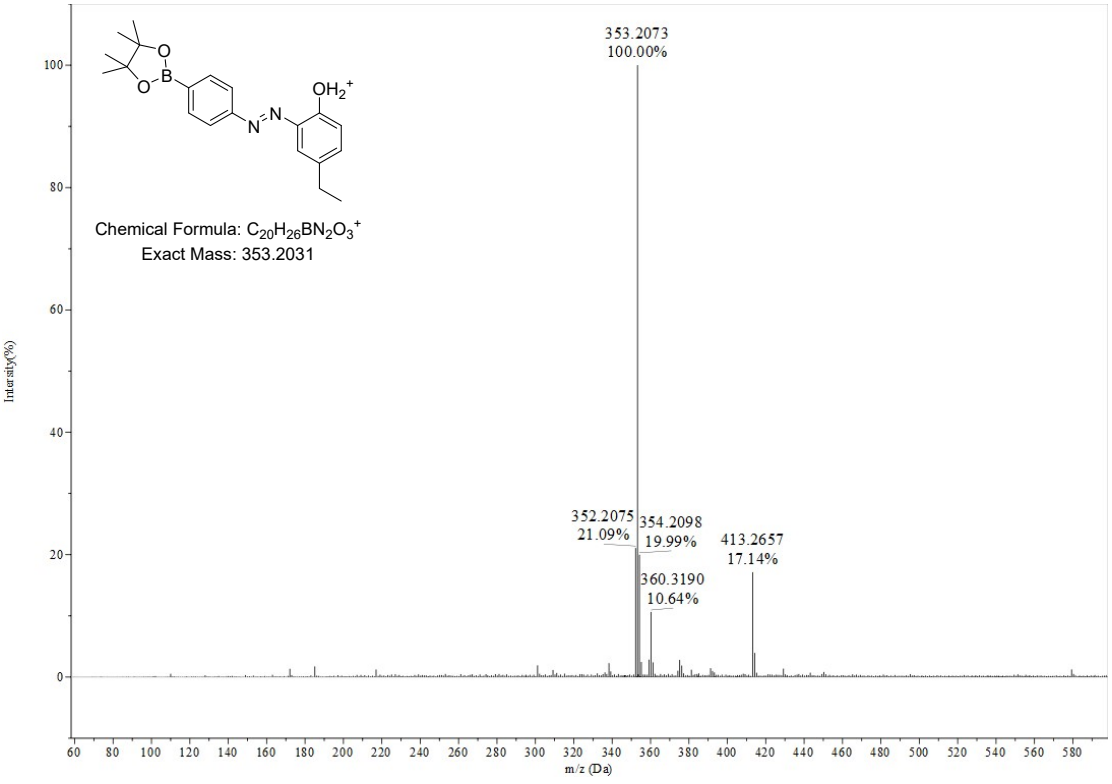

Fig. S5: HR-MS spectrum of Compound **3**

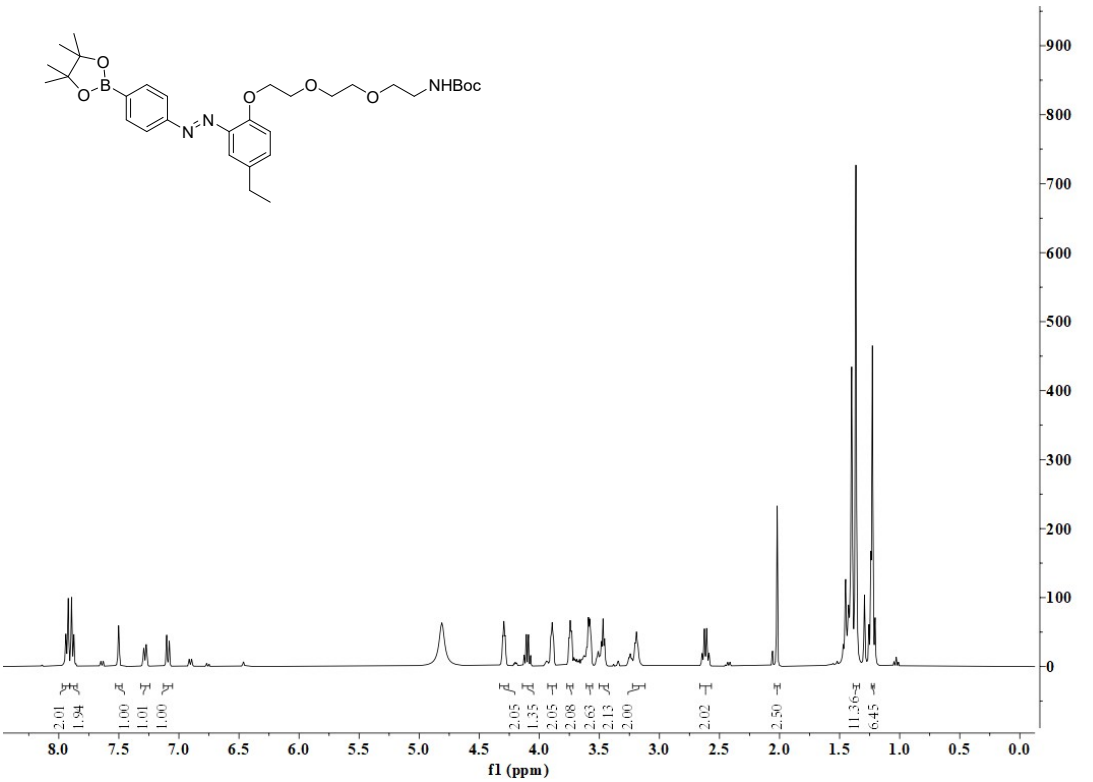

Fig S6: ^{1}H NMR of Compound **4**

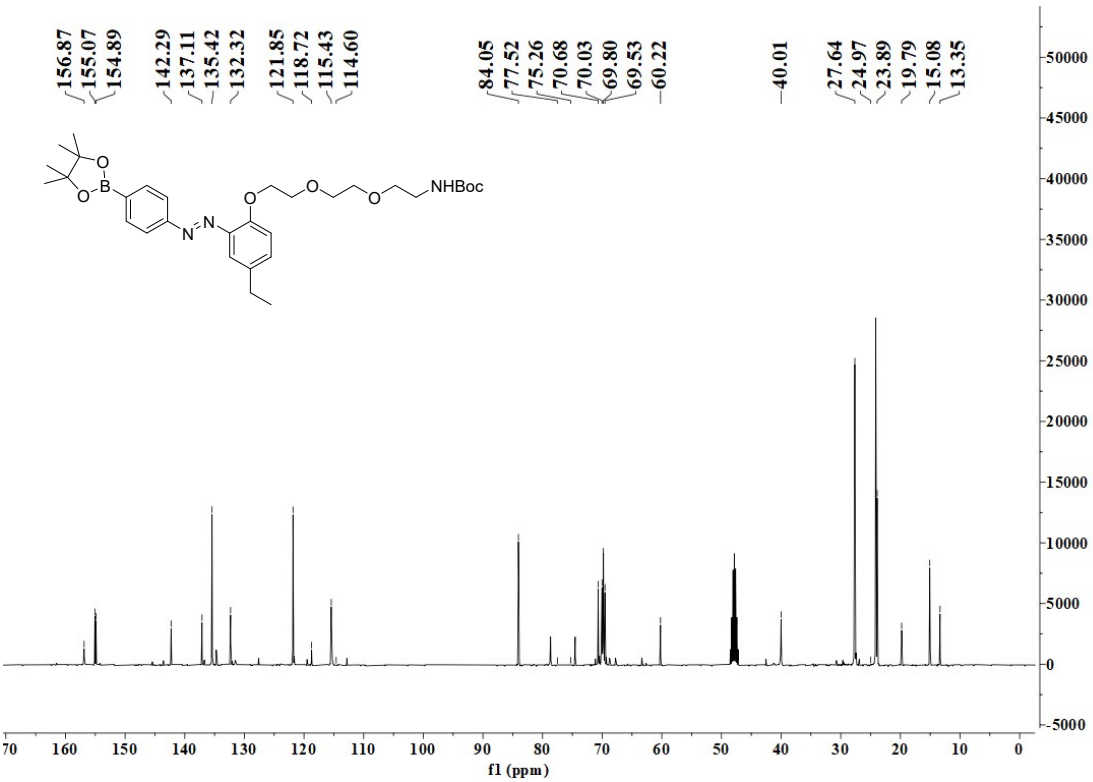

Fig S7: ^{13}C NMR of Compound **4**

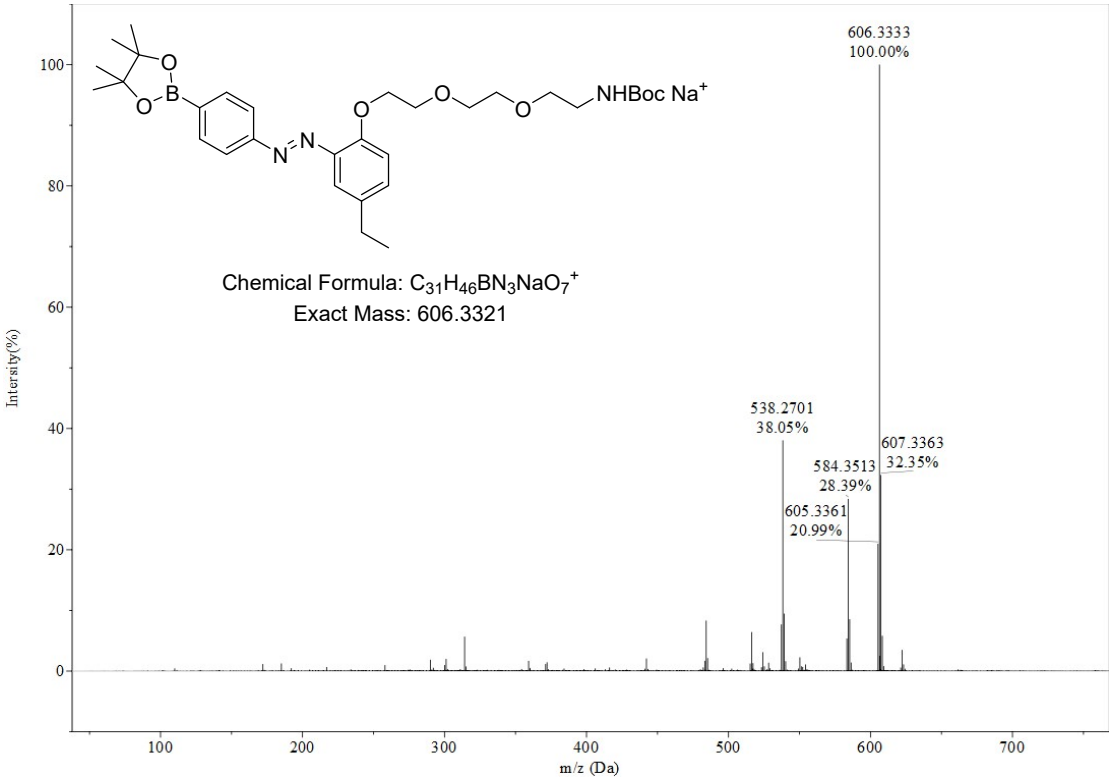

Fig. S8: HR-MS spectrum of Compound **4**

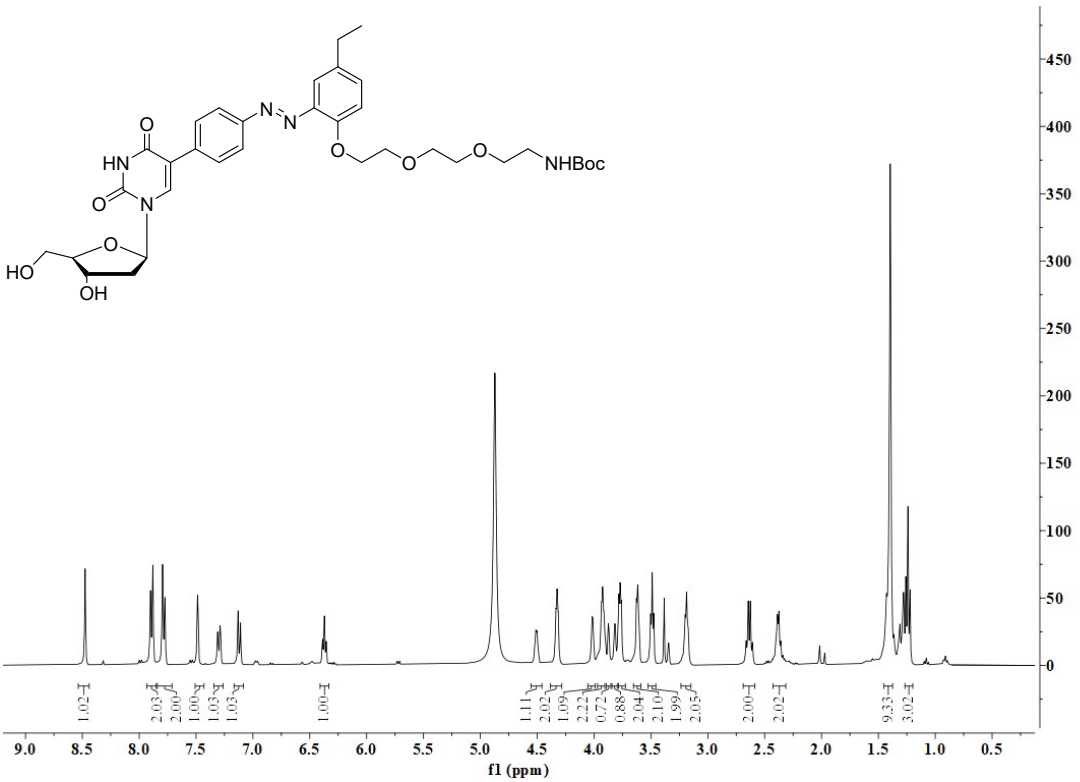

Fig S9: ^{1}H NMR of Compound **5**

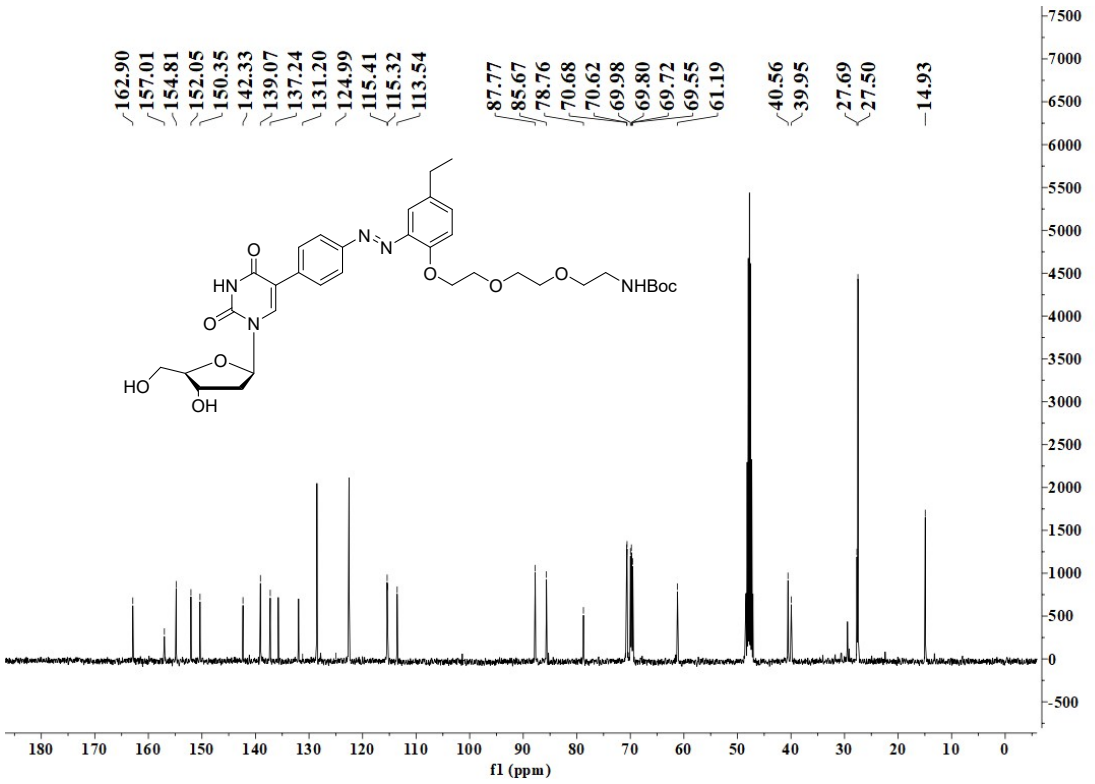

Fig S10: ^{13}C NMR of Compound **5**

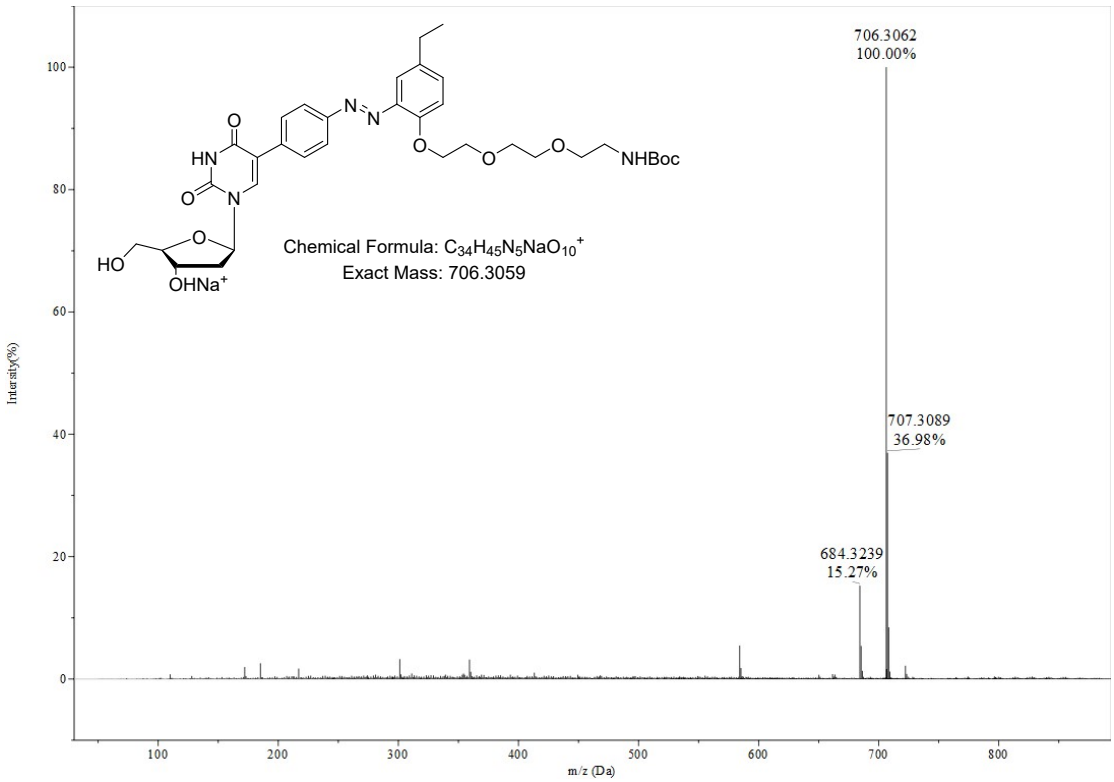

Fig. S11: HR-MS spectrum of Compound **5**

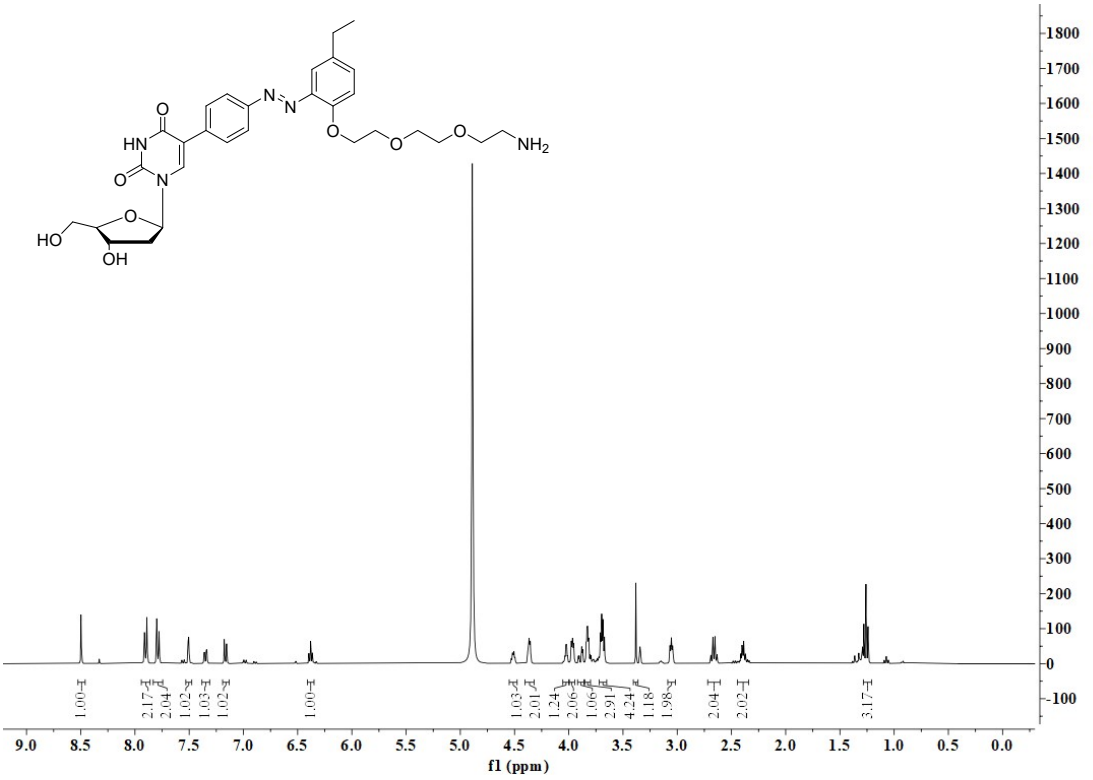

Fig S12: ^{1}H NMR of Compound **6**

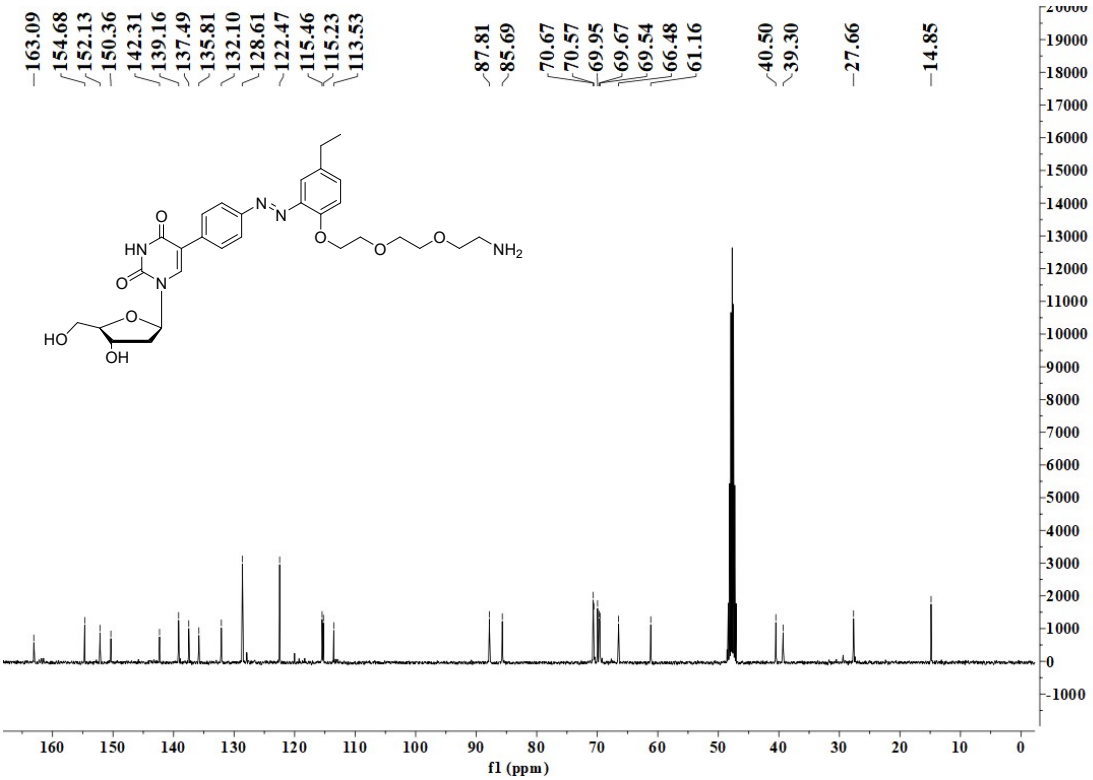

Fig S13: ^{13}C NMR of Compound **6**

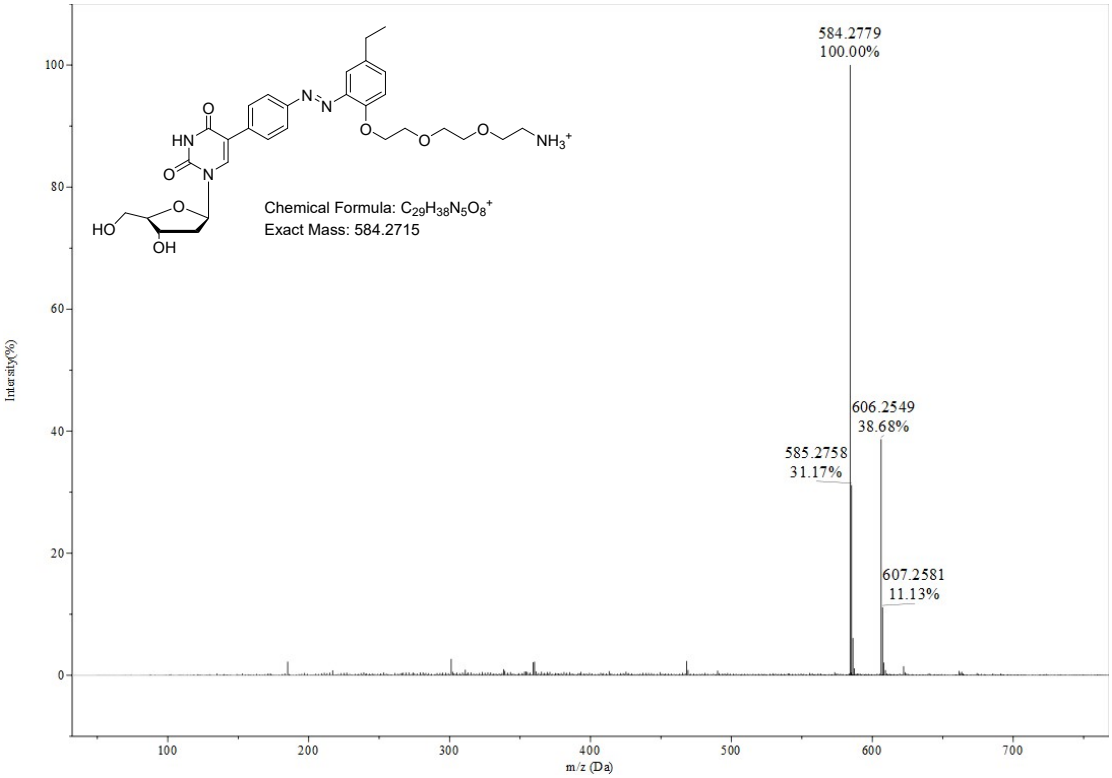

Fig. S14: HR-MS spectrum of Compound **6**

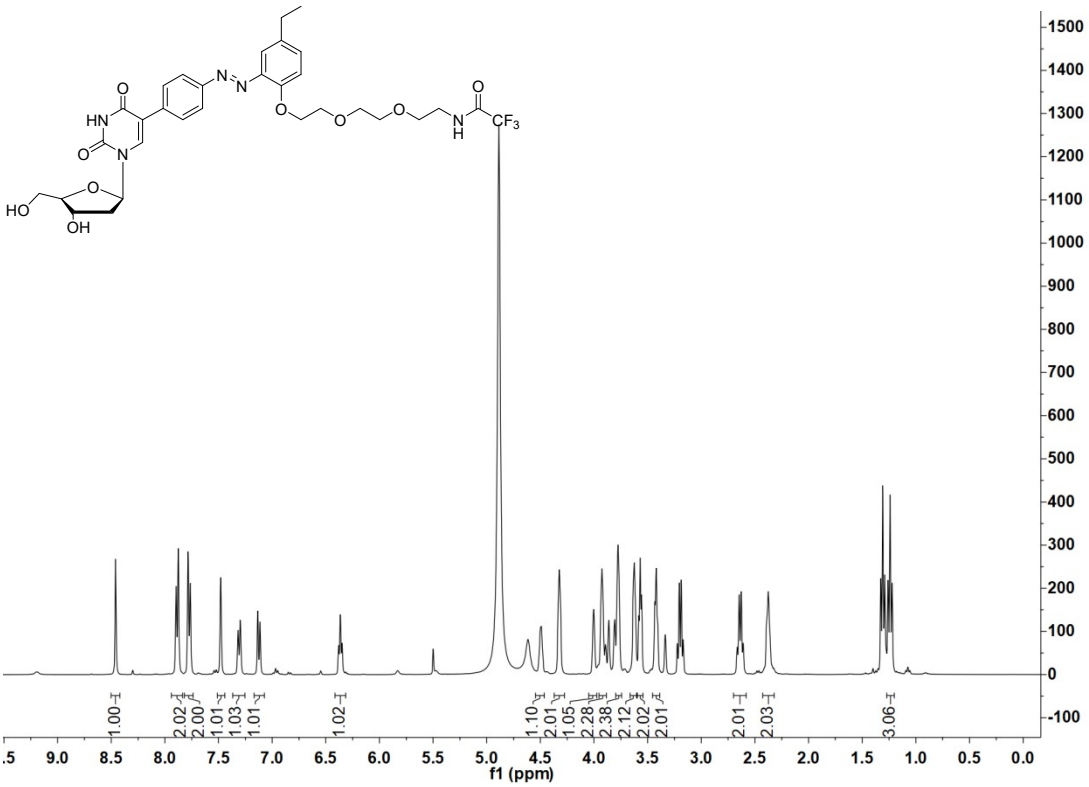

Fig S15: ^{1}H NMR of Compound **7**

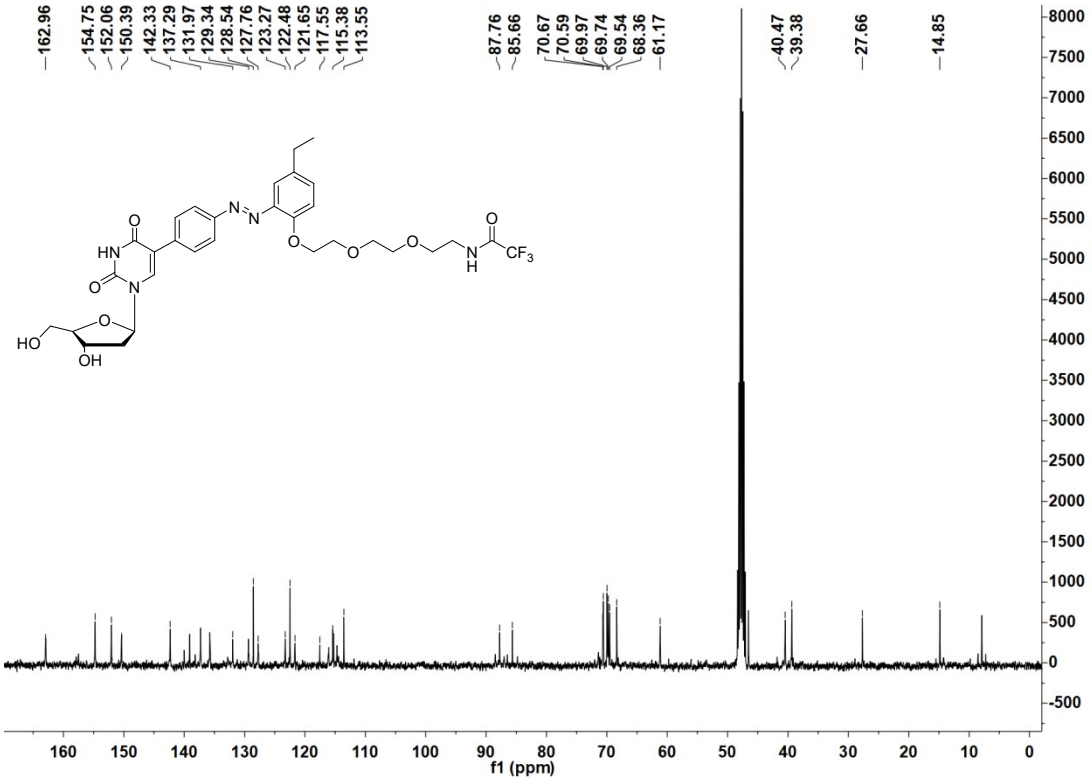

Fig S16: ^{13}C NMR of Compound **7**

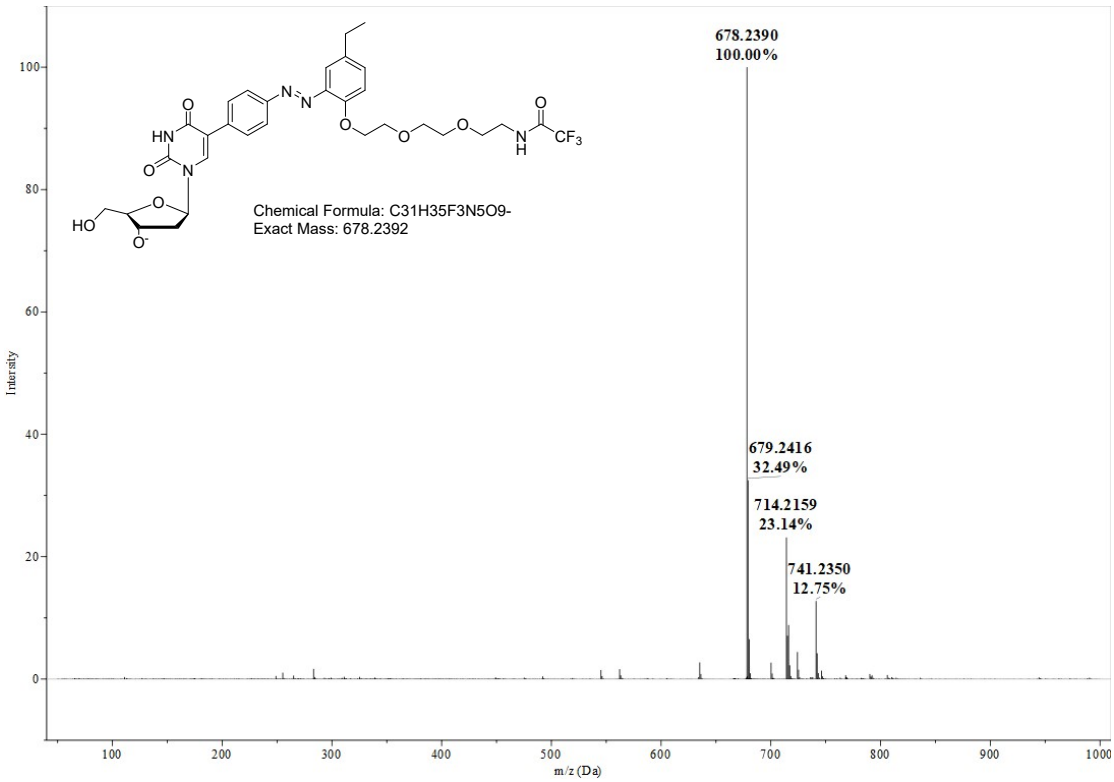

Fig. S17: HR-MS spectrum of Compound **7**

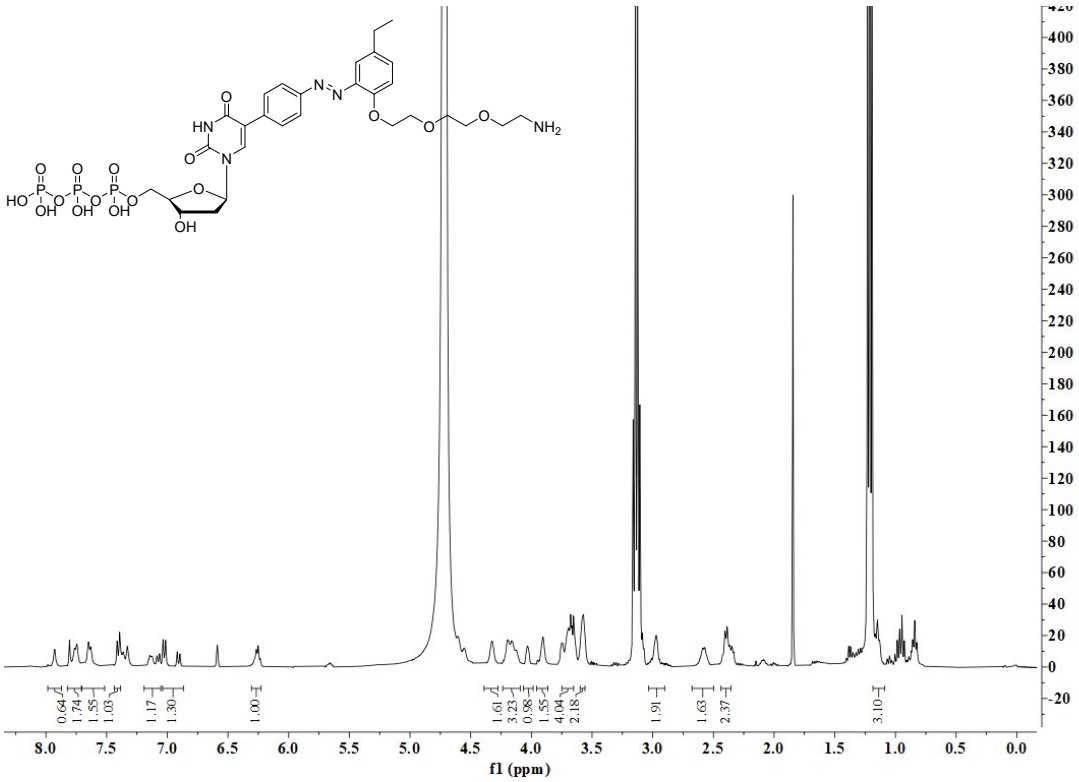

Fig S18: ^{1}H NMR of Compound **8**

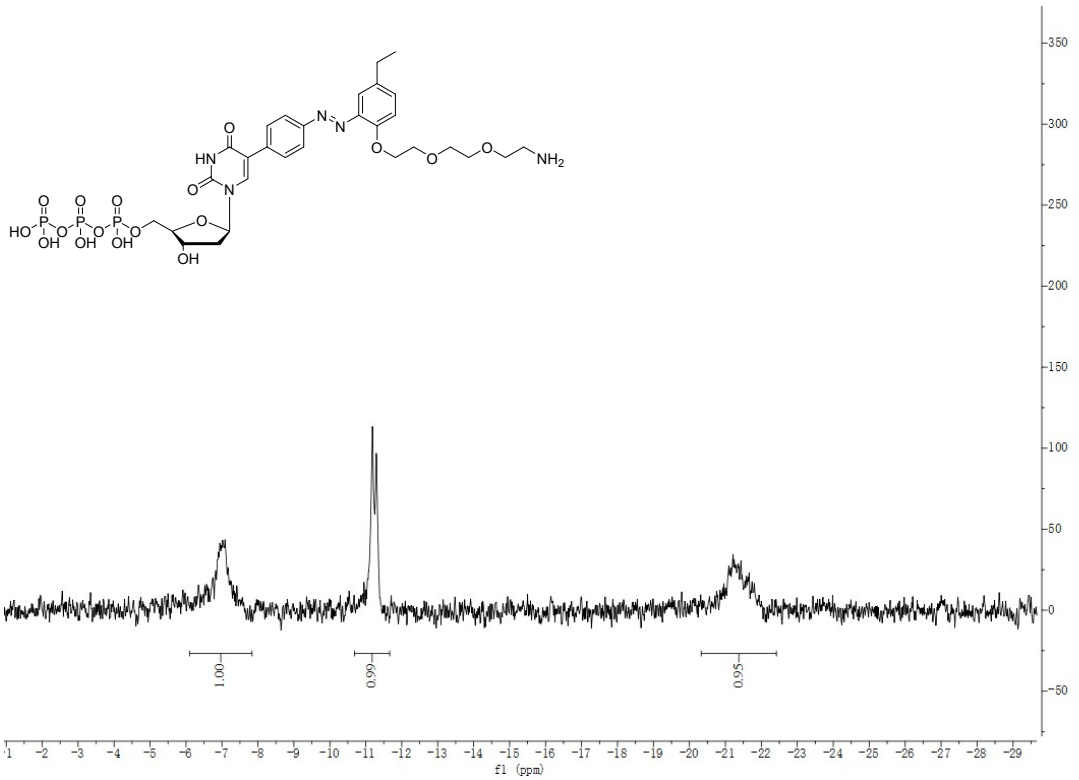

Fig S19: ^{31}P NMR of Compound **8**

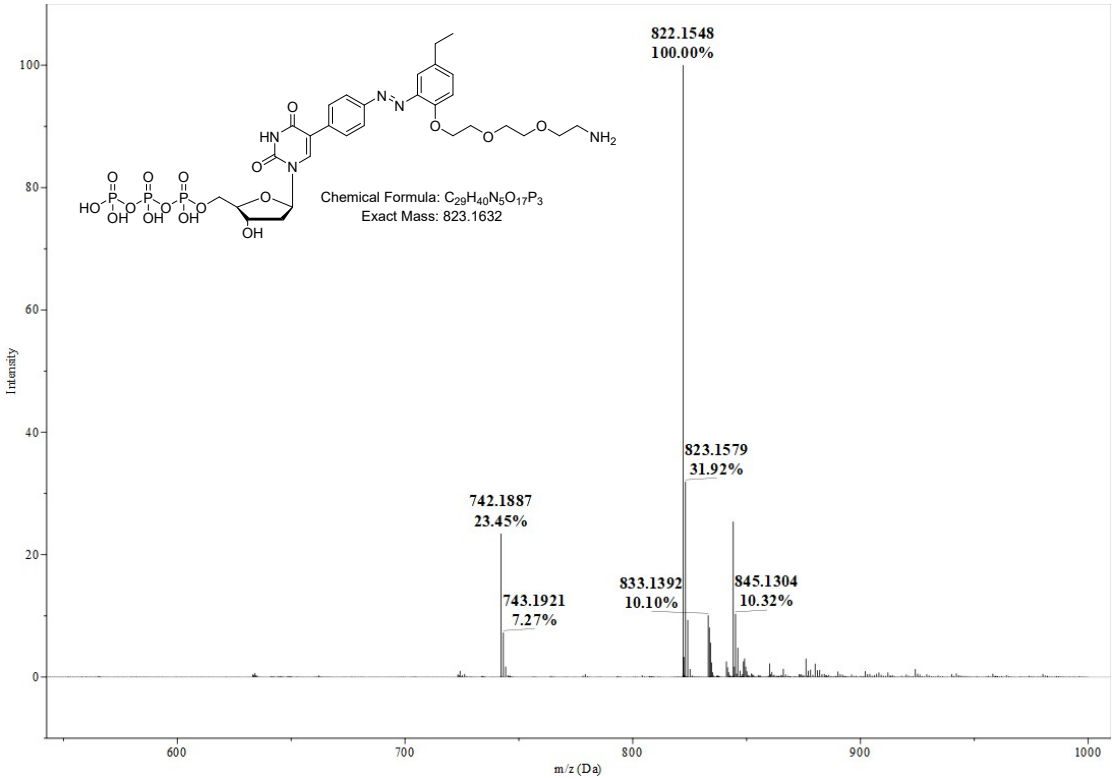

Fig. S20: HR-MS spectrum of Compound **8**

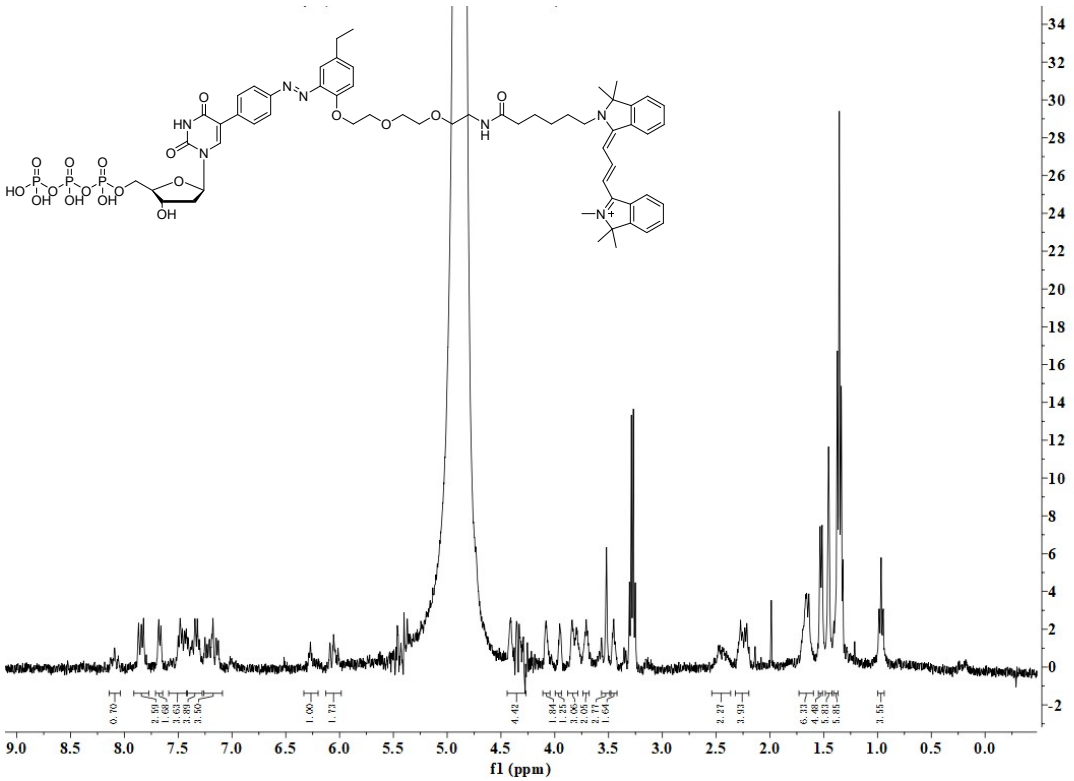

Fig S21: ^{1}H NMR of Compound **9**

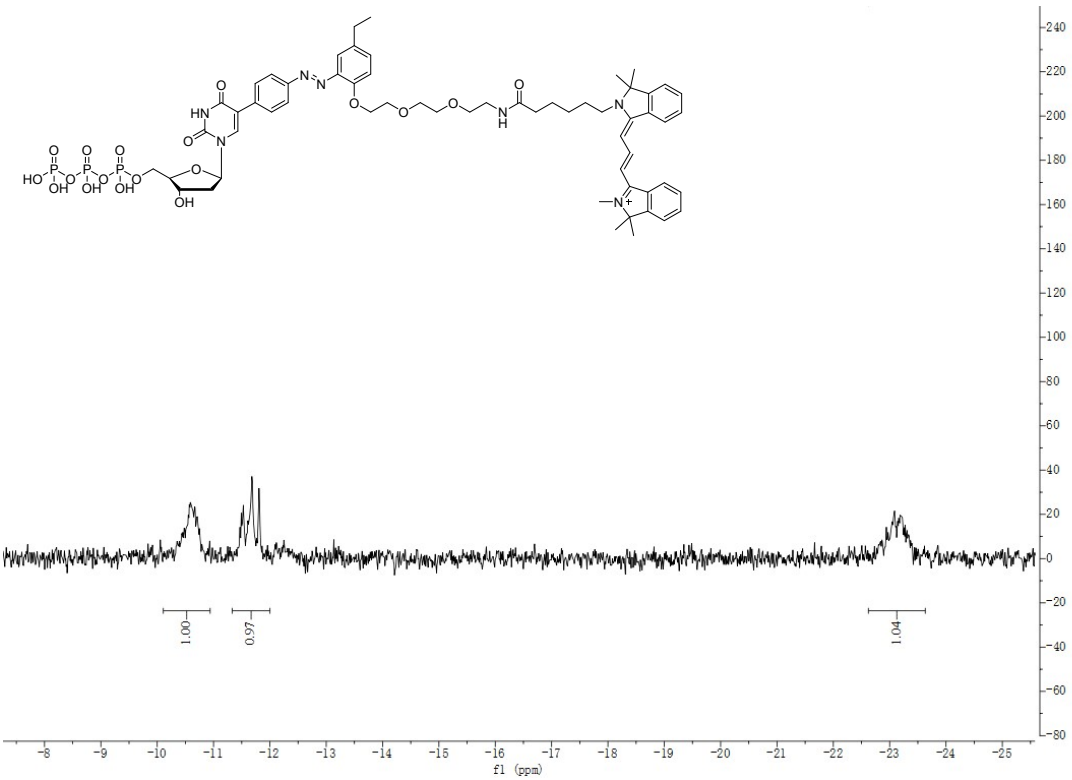

Fig S22: ^{31}P NMR of Compound **9**

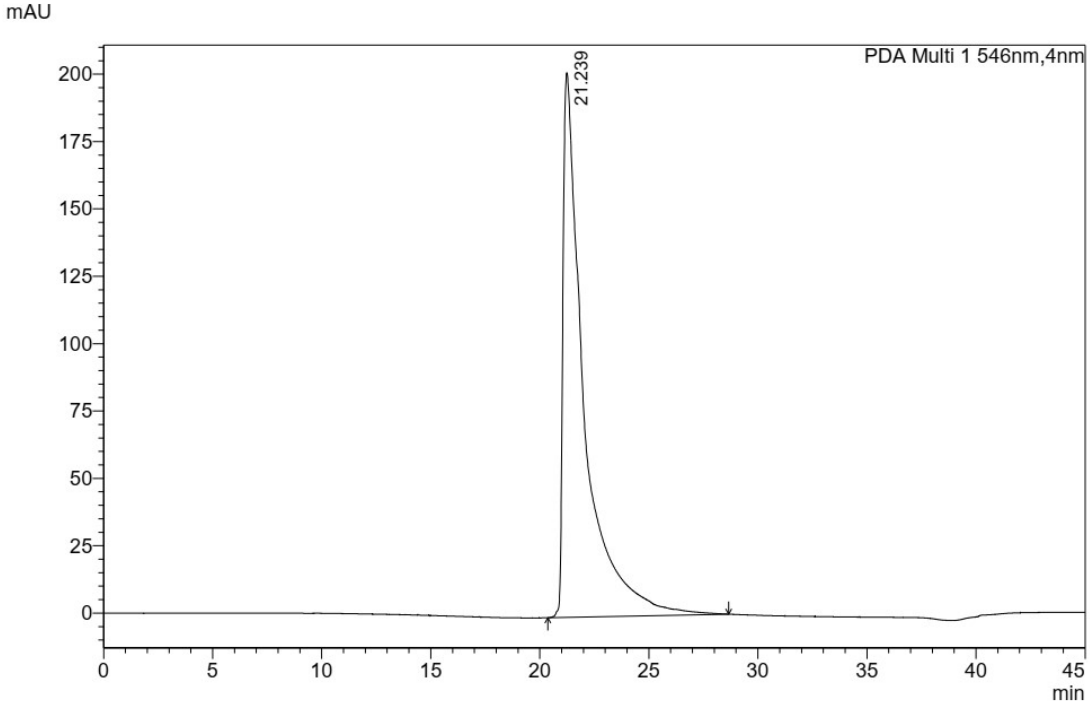

Fig. S23: HPLC of Compound **9**

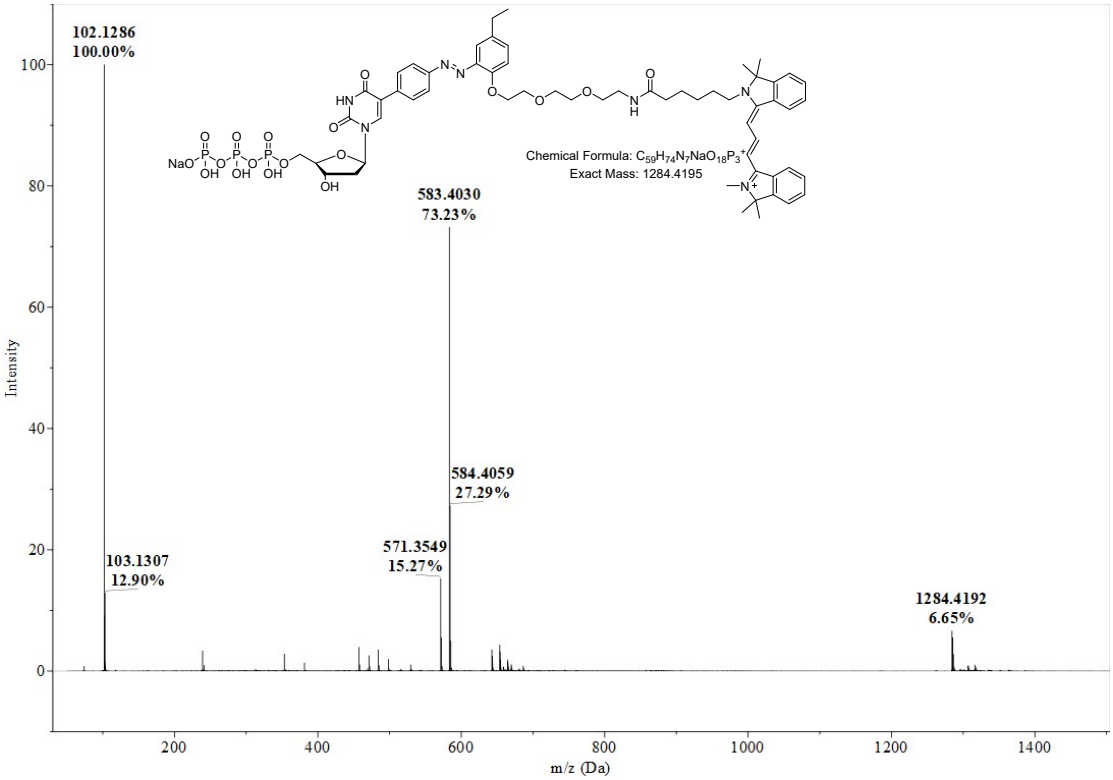

Fig. S24: HR-MS spectrum of Compound **9**