Supporting Information

Rapidly photocleavable triazene-functionalized nucleotides for DNA sequencing

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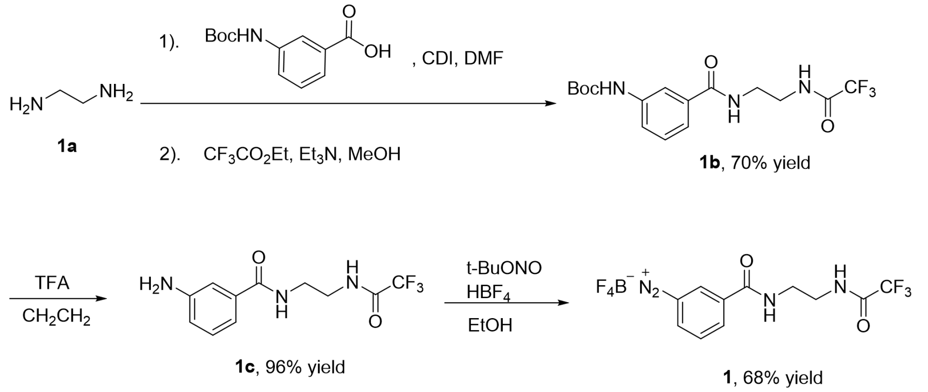
Contents

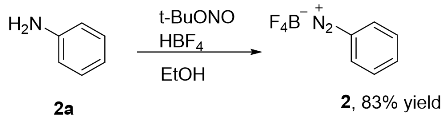
1. General Methods

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 $CaCl_2$ in a desiccator under vacuum at room temperature and protected from light. For the synthesis experimental section that required heating, experiments were conducted on an oil bath. For the DNA extension/cleavage experimental section that required heating, experiments were conducted on a metal bath. 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-700/600/400 spectrometer with chemical shifts reported in ppm relative to the residual deuterated solvents or the internal standard tetramethylsilane. ESI-HRMS was carried out using an electrospray spectrometer Waters Micro mass Q-TOF Premier Mass Spectrometer. 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 (10 × 250 mm or 21.2 × 250 mm) was used and the traces were recorded with a UV detector at 254 nm. Oligonucleotides used as primers or template were designed by ourselves and synthesized by Sangon Biotech. DNA polymerase was provided by New England Biolabs.

2. Synthesis Experimental Section

2.1 Synthesis of diazonium salts





Compound 1b: Boc-3-aminobenzoic acid (10 mmol, 2.37 g) and N,N'-carbonyldiimidazole (12 mmol, 1.95 g) were dissolved in N,N-dimethylformamide (50 mL) and stirred for 30 min at 0 °C. After ethylenediamine (50 mmol, 3.00 g) were added, the solution was stirred at room temperature for 12 h. Then, the reaction solution was diluted with dichloromethane, and the organic phase was washed with water and brine, dried over sodium sulfate. The solvent was removed under vacuum. Desired product was obtained as yellow oil. The product is directly used for the next reaction without additional purification.

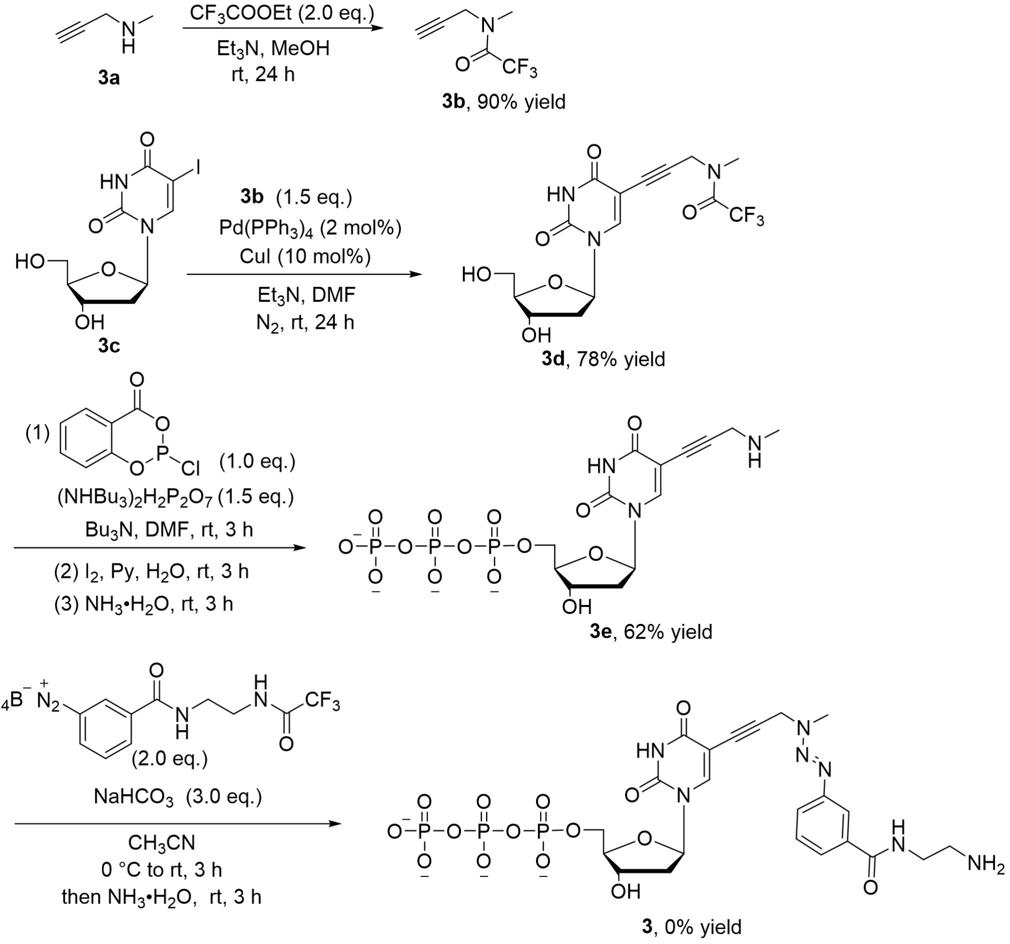
Ethyl trifluoroacetate (20 mmol, 2.84 g) and triethylamine (30 mmol, 3.04 g) were added to a solution of ethylenediamine derivatives in methanol (50 mL) at room temperature for 12 h. After the removal of solvent under reduced pressure, the crude product was purified by silica gel column chromatography (eluent = PE / EA). Desired product was obtained as white solid (2.60 g, 70 % yield, two steps). ^{1}H NMR (700 MHz, CD_3OD) δ 7.85 (s, 1H), 7.54 (d, *J* = 7.6 Hz, 1H), 7.41 (dt, *J* = 7.8, 1.4 Hz, 1H), 7.34 (t, *J* = 7.9 Hz, 1H), 3.54 (t, *J* = 6.3 Hz, 2H), 3.51 (t, *J* = 5.8 Hz, 2H), 1.52 (s, 9H). ^{13}C NMR (176 MHz, CD_3OD) δ 169.3, 158.0 (q, *J* = 36.9 Hz), 153.8, 139.6, 134.9, 128.6, 121.4, 120.9, 117.2, 116.1 (q, *J* = 286.6 Hz), 79.7, 39.1, 38.5, 27.3. HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{16}H_{21}N_3O_4F_3$ 376.1484, found 376.1483.

Compound 1c: Amide condensation products **1b** (5 mmol, 1.88 g) was dissolved in trifluoroacetic acid / dichloromethane (1 : 1, 10 mL) and stirred for 3 h at room temperature. After the removal of solvent under reduced pressure, the crude product was purified by silica gel column chromatography (eluent = DCM / MeOH). Desired product was obtained as light yellow solid (1.33 g, 96 % yield). ^{1}H NMR (700 MHz, CD_3OD) δ 7.15 (t, *J* = 7.8 Hz, 1H), 7.11 (t, *J* = 2.0 Hz, 1H), 7.06 (dt, *J* = 7.7, 1.3 Hz, 1H), 6.85 (ddd, *J* = 8.0, 2.4, 1.0 Hz, 1H), 3.54 – 3.47 (m, 4H). ^{13}C NMR (176 MHz, CD_3OD) δ 170.0, 158.0 (q, *J* = 36.9 Hz), 147.9, 135.0, 128.8, 118.0, 116.1 (q, *J* = 286.6 Hz), 116.0, 113.5, 39.2, 38.5. HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{11}H_{13}N_3O_2F_3$ 276.0960, found 276.0963.

Compound 1: Aniline derivatives **1c** (0.3 mmol, 82.6 mg) and fluoboric acid (50 wt. ％ in H_2O, 0.6 mmol, 105.4 mg) were dissolved in ethanol (2 mL). Tert-butyl nitrite (0.6 mmol, 61.9 mg) was slowly added to the solution. The resulting mixture was stirred at 0 °C for 2 h and then warm up to room temperature for 1 h. An appropriate amount of ether was added to the solution and maintained at 4 °C for 1 h to precipitate solid. After filtering and vacuum drying, desired product was obtained as white solid (76.0 mg, 68 ％ yield). The product is directly used for the next reaction without additional purification. The freshly preparation of diazonium salts is important for improving the yield of synthesizing triazenes.

Compound 2: Aniline **2a** (3 mmol, 0.28 g) and fluoboric acid (50 wt. ％ in H_2O, 6 mmol, 1.05 g) were dissolved in ethanol (10 mL). Tert-butyl nitrite (6 mmol, 1.05 g) was slowly added to the solution. The resulting mixture was stirred at 0 °C for 2 h and then warm up to room temperature for 1 h. An appropriate amount of ether was added to the solution and maintained at 4 °C for 1 h to precipitate solid. After filtering and vacuum drying, desired product was obtained as white solid (0.48 mg, 83 ％ yield). The product is directly used for the next reaction without additional purification. The freshly preparation of diazonium salts is important for improving the yield of synthesizing triazenes.

2.2 Synthesis of triazene compounds



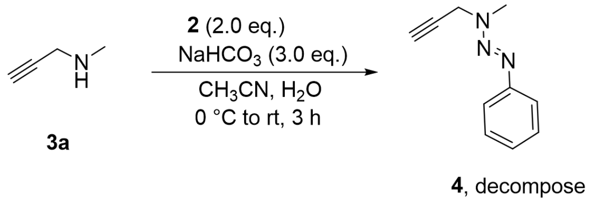
Compound 3b: Dissolve N-methylpropargylamine (5.00 mmol, 0.35 g), triethylamine (10.00 mmol, 1.01 g) and ethyl trifluoroacetate (10.00 mmol, 1.42 g) in methanol (10 mL), and stir the mixture at room temperature for 24 h. After concentration under reduced pressure, the crude product is purified by silica gel column chromatography using petroleum ether and ethyl acetate as the eluent. The product is obtained as a pale yellow liquid (0.74 g, 90% yield). ^{1}H NMR (700 MHz, CDCl$_3$) δ 4.31 – 4.14 (m, 2H), 3.25 – 3.09 (m, 3H), 2.39 – 2.28 (m, 1H). ^{13}C NMR (176 MHz, CDCl$_3$) δ 157.2 – 156.1 (m), 118.9 – 113.6 (m), 76.5, 76.3, 73.7, 73.5, 39.1 (q, *J* = 4.5 Hz), 38.0, 34.2, 34.0 (q, *J* = 3.8 Hz). HRMS (ESI) m/z: [M + H]$^+$ Calcd for C$_6$H$_7$F$_3$NO 166.0474, found: 166.0475.

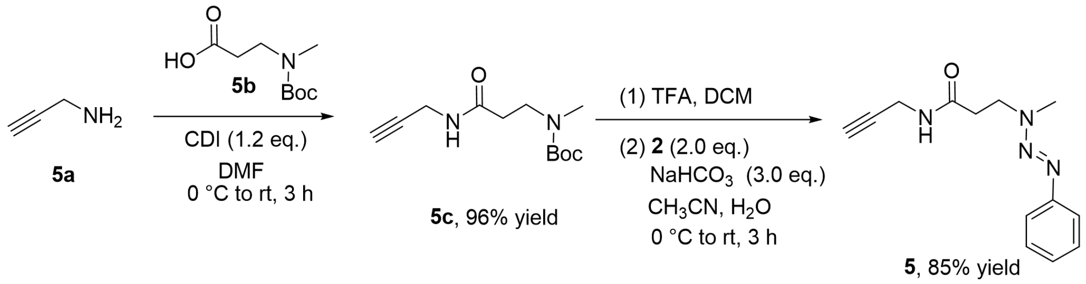
Compound 3d: Dissolve 5-iododeoxyuridine **3c** (2.00 mmol, 0.71 g), copper(I) iodide (0.20 mmol, 38.0 mg), tetrakis(triphenylphosphine)palladium(0) (0.04 mmol, 46.0 mg) and triethylamine (1.6 mL) in *N,N*-dimethylformamide (14 mL) under a nitrogen atmosphere, then add *N*-methyl-*N*-propargyltrifluoroacetamide **3b** (3.00 mmol, 0.50 g). Stir the solution at room temperature for 24 h. After concentration under reduced pressure, purify the crude product by silica gel column chromatography using dichloromethane and methanol as the eluent. The product is obtained as a brown solid (0.61 g, yield 78%).^{1}H NMR (700 MHz,

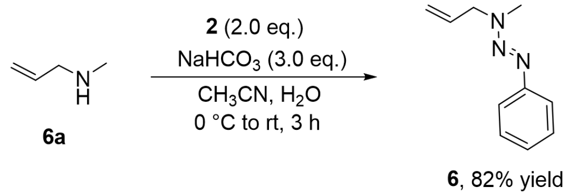
CD_3OD) δ 8.37 (s, 1H), 6.22 (t, *J* = 6.5 Hz, 1H), 4.50 – 4.47 (m, 2H), 4.40 (dt, *J* = 6.9, 3.7 Hz, 1H), 3.93 (q, *J* = 3.4 Hz, 1H), 3.78 (ddd, *J* = 55.3, 12.0, 3.3 Hz, 2H), 3.33 – 3.24 (m, 3H), 2.37 – 2.18 (m, 2H). ^{13}C NMR (176 MHz, CD_3OD) δ 163.0, 162.9, 156.8-156.1 (m), 149.8, 149.7, 144.7, 144.6, 119.0 – 113.5 (m), 98.0, 97.8, 87.7, 87.7, 85.8, 85.7, 85.7, 85.6, 76.4, 76.0, 70.5, 70.4, 61.1, 61.0, 40.4, 40.3, 39.3 (q, *J* = 4.5 Hz), 38.2, 33.4 (q, *J* = 3.8 Hz), 33.2. HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{15}H_{17}F_3N_3O_6$ 392.1064, found: 392.1069.

Compound 3e: Dissolve tributylammonium pyrophosphate (0.30 mmol, 164.4 mg) in *N,N*-dimethylformamide (0.5 mL) and tributylamine (0.5 mL). Dissolve 2-chloro-1,3,2-benzodioxaphosphorin-4-one (0.20 mmol, 40.4 mg) in *N,N*-dimethylformamide (0.5 mL). Mix the above two solutions and allow the reaction to proceed for 30 min. Dissolve the thoroughly dried nucleoside **3d** (0.20 mmol, 78.3 mg) in *N,N*-dimethylformamide (1.5 mL) and add it to the reaction mixture. Stir the resulting solution at room temperature for 3 h. Then, add 3% iodine solution (pyridine/water = 9:1; 3 mL) to the mixture and maintain the brown color without fading for 15 min. Add deionized water (3 mL) to the mixture and continue the reaction for 3 h. Transfer the mixture to a centrifuge tube, followed by the addition of saturated sodium chloride solution (1 mL) and anhydrous ethanol (80 mL), and store the mixture at -20 °C overnight. Collect the solid precipitate by centrifugation of the mixture, then dissolve the precipitate in concentrated ammonia water (5 mL) and react for 3 h. After the removal of solvent under reduced pressure, the crude product was purified by preparative HPLC (C-18, 0.1 M TEAB in water / methanol = 0/100 to 15/85 , flow rate = 4.0 mL/min, I = 254 nm) t_R = 10.6 min. The target product is obtained as a white solid (66.4 mg, yield 62%). ^1H NMR (400 MHz, D_2O) δ 8.28 (s, 1H), 6.17 (t, *J* = 6.4 Hz, 1H), 4.51 (s, 1H), 4.21 – 4.05 (m, 3H), 3.96 (s, 2H), 2.69 (s, 3H), 2.40 – 2.21 (m, 2H). ^{31}P NMR (162 MHz, D_2O) δ -6.09, -11.08, -21.37. HRMS (ESI) m/z: $[M + 3H]^-$ Calcd for $C_{13}H_{19}N_3O_{14}P_3$ 534.0085, found 534.0086.

Compound 3: Dissolve compound **3e** (56.1 μmol, 30.0 mg) and sodium bicarbonate (168.1 μmol, 14.1 mg) in deionized water (4 mL). Dissolve diazonium salt (112.2 μmol, 42.0 mg) in acetonitrile (4 mL). Mix the above two solutions, react at 0 °C for 1 h, and then continue the reaction at room temperature for another 2 h. After the reaction, add concentrated ammonia water (6 mL) and stir the solution at room temperature for 3 h. After concentration under reduced pressure, the target product is not isolated.





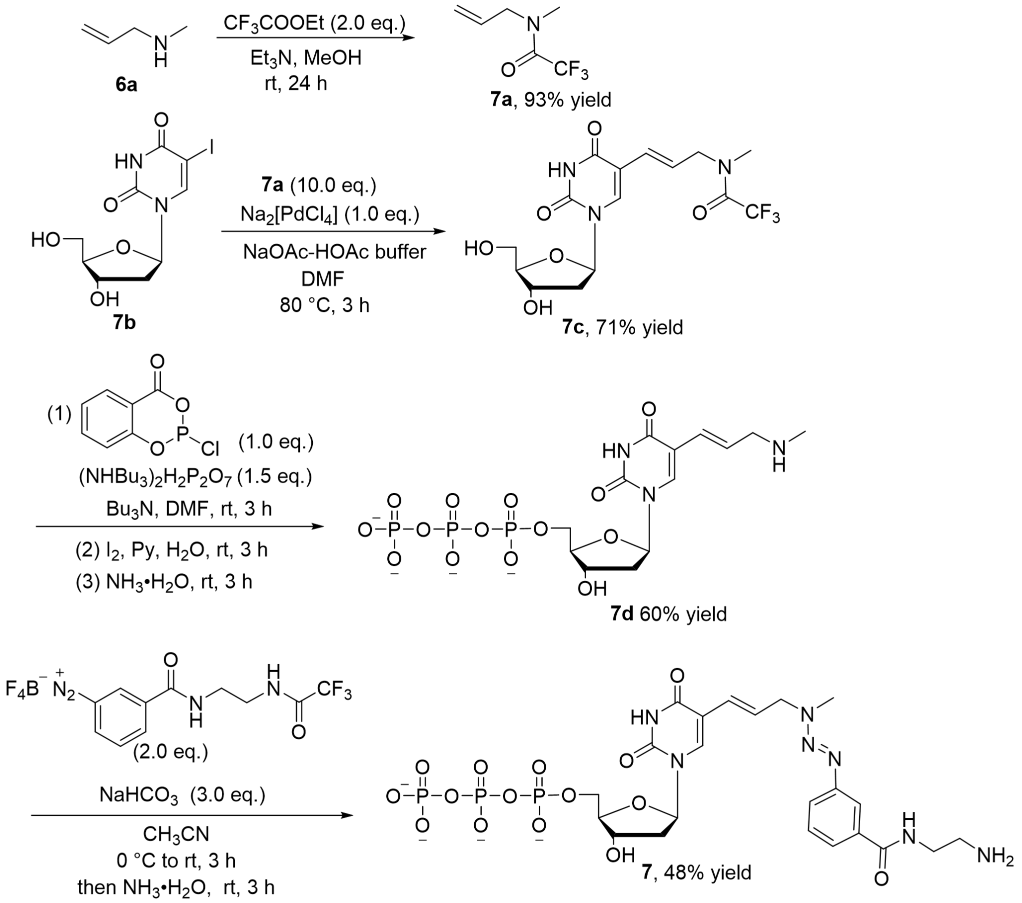


Compound 4: Dissolve N-methylpropargylamine **3a** (0.20 mmol, 13.8 mg) and sodium bicarbonate (0.60 mmol, 50.4 mg) in deionized water (2 mL). Dissolve diazonium salt (0.40 mmol, 76.8 mg) in acetonitrile (2 mL). Mix the above two solutions, react at 0 °C for 1 h, and then continue the reaction at room temperature for another 2 h. The product can be detected by TLC. Subsequently, dilute the reaction mixture with ethyl acetate and wash the organic layer with saturated brine. Collect the organic layer, dry it over anhydrous sodium sulfate, and concentrate it under reduced pressure. Decomposition of the product was detected by TLC during the concentration process.

Compound 5c: Dissolve *N*-Boc-3-(methylamino)propionic acid (1.00 mmol, 0.20 g) and *N,N'*-carbonyldiimidazole (1.20 mmol, 0.19 g) in *N,N*-dimethylformamide (10 mL), and stir the mixture at 0 °C for 30 min. Add propargylamine (5.00 mmol, 0.28 g), then stir the solution at room temperature for 3 h. After the reaction, dilute the reaction mixture with dichloromethane, wash the organic phase with water and saturated brine successively, and dry it over anhydrous sodium sulfate. Concentrate the organic layer and purify the residue by silica gel column chromatography using ethyl acetate/petroleum ether as the eluent. The target product is obtained as a white solid (0.23 g, yield 96%). ^{1}H NMR (400 MHz, CD_3OD) δ 3.97 (s, 2H), 3.53 (s, 2H), 2.88 (s, 3H), 2.61 (t, *J* = 2.5 Hz, 1H), 2.44 (t, *J* = 6.9 Hz, 2H), 1.49 (s, 9H). ^{13}C NMR (101 MHz, CD_3OD) δ 172.1, 156.0, 79.8, 79.1, 70.9, 45.1, 34.4, 34.1, 28.1, 27.3. HRMS (ESI) m/z: $[M + Na]^+$ Calcd for $C_{12}H_{20}N_2NaO_3$ 263.1366, found 263.1383.

Compound 5: Dissolve compound **5c** (0.50 mmol, 120.2 mg) in dichloromethane (2 mL) and trifluoroacetic acid (2 mL), and allow the mixture to react for 1 h. After the reaction is complete, remove the solvents under reduced pressure. Then add deionized water (5 mL) to dissolve the product, followed by an appropriate amount of sodium bicarbonate to adjust the solution to alkaline. Dissolve diazonium salt (1.0 mmol, 192.0 mg) in acetonitrile (5 mL), add this solution to the above reaction mixture, and then add sodium bicarbonate (1.50 mmol, 126.0 mg). Stir the reaction mixture at 0 °C for 1 h, and then continue the reaction at room temperature for another 1 h. Concentrate the mixture under reduced pressure, and purify the crude product by silica gel column chromatography using ethyl acetate and petroleum ether as the eluent. The target product is obtained as a pale yellow oil (103.5mg, yield 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 (d, *J* = 8.2 Hz, 2H), 7.33 (t, *J* = 7.7 Hz, 2H), 7.15 (t, *J* = 7.2 Hz, 1H), 6.11 (s, 1H), 4.12 – 3.96 (m, 4H), 3.29 (s, 3H), 2.60 (t, *J* = 6.6 Hz, 2H), 2.19 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.4, 150.6, 128.9, 125.8, 120.6, 79.3, 71.8, 50.2, 34.9, 29.8, 29.3. HRMS (ESI) m/z: $[M + Na]^+$ Calcd for $C_{13}H_{16}N_4NaO$ 267.1216, found 267.1226.

Compound 6: Dissolve N-methylallylamine **6a** (0.20 mmol, 14.2 mg) and sodium bicarbonate (0.60 mmol, 50.4 mg) in deionized water (2 mL). Dissolve Dissolve diazonium salt (0.40 mmol, 76.8 mg) in acetonitrile (2 mL). Mix the above two solutions, react at 0 °C for 1 h, and then continue the reaction at room temperature for another 2 h. Subsequently, dilute the reaction mixture with ethyl acetate and wash the organic layer with saturated brine. Collect the organic layer, dry it over anhydrous sodium sulfate, and concentrate it under reduced pressure. Purify the crude product by silica gel column chromatography using petroleum ether/ethyl acetate as the eluent. The product is obtained as a pale yellow liquid (28.8 mg, yield 82%). ^{1}H NMR (700 MHz, $CDCl_3$) δ 7.49 (d, *J* = 8.5 Hz, 2H), 7.38 (t, *J* = 7.3 Hz, 2H), 7.19 (t, *J* = 7.3 Hz, 1H), 5.93 (ddt, *J* = 16.5, 10.4, 5.9 Hz, 1H), 5.29 – 5.25 (m, 2H), 4.39 (dt, *J* = 6.0, 1.6 Hz, 2H), 3.22 (s, 3H). ^{13}C NMR (176 MHz, $CDCl_3$) δ 150.8, 133.3, 128.8, 125.5, 120.5, 118.1, 58.7, 33.9. HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{10}H_{14}N_3$ 176.1182, found: 176.1194.



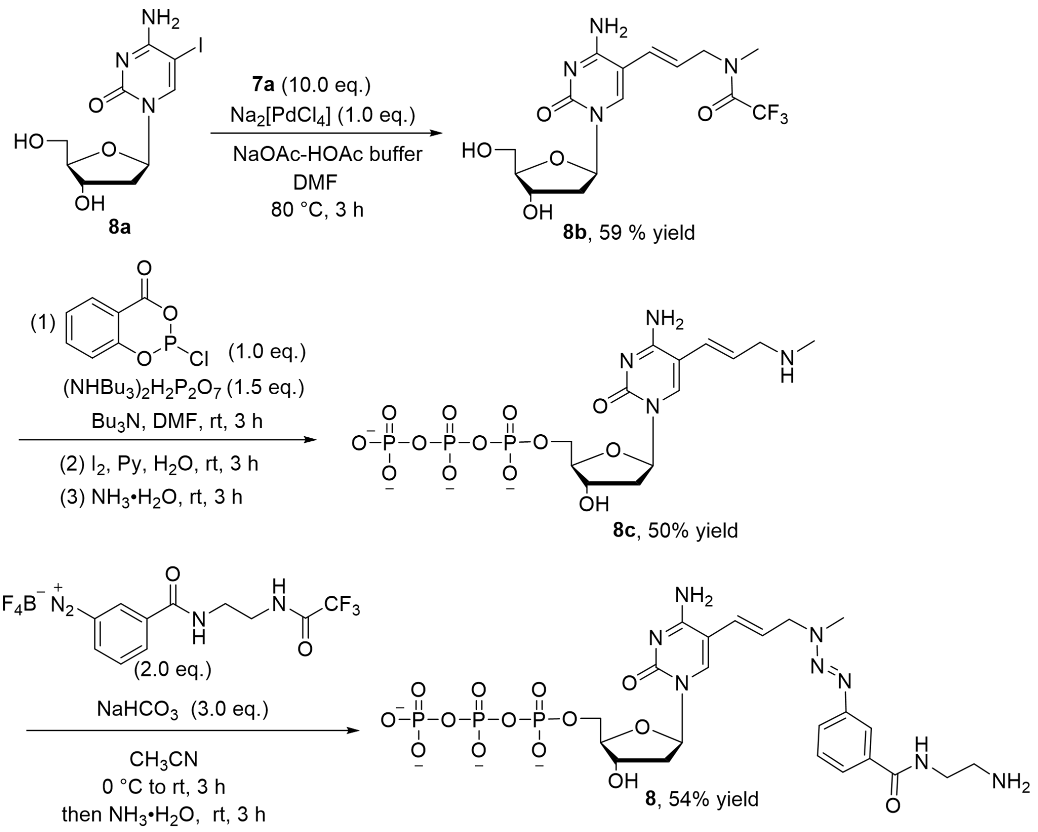
Compound 7a: Dissolve *N*-methylallylamine (5.00 mmol, 0.36 g), triethylamine (10.00 mmol, 1.01 g) and ethyl trifluoroacetate (10.00 mmol, 1.42 g) in methanol (10 mL), and stir the mixture at room temperature for 24 h. After concentration under reduced pressure, the crude product is purified by silica gel column chromatography using petroleum ether and ethyl acetate as the eluent. The product is obtained as a pale yellow liquid (0.78 g, 93% yield). ^{1}H NMR (700 MHz, CDCl$_3$) δ 5.81 – 5.70 (m, 1H), 5.34 – 5.21 (m, 2H), 4.07 – 4.00 (m, 2H), 3.10 (s, 1.75H), 3.00 (s, 1.25H). ^{13}C NMR (176 MHz, CDCl$_3$) δ 157.0 (q, *J* = 35.6 Hz), 156.9 (q, *J* = 35.9 Hz), 131.6, 130.7, 119.3, 119.0, 116.6 (q, *J* = 287.7 Hz), 116.5 (q, *J* = 287.7 Hz), 52.0 (q, *J* = 3.2 Hz), 51.5, 34.3 (q, *J* = 3.8 Hz), 34.1. HRMS (ESI) m/z: [M + H]$^+$ Calcd for $C_6H_9F_3NO$ 168.0631, found 168.0632.

Compound 7c: Under a nitrogen atmosphere, dissolve *N*-methyl-*N*-allyltrifluoroacetamide **7a** (10.00 mmol, 1.67 g) and 5-iododeoxyuridine **7b** (1.00 mmol, 0.35 g) in *N,N*-dimethylformamide (10 mL). After the reactants are completely dissolved, add sodium tetrachloropalladate (1.00 mmol, 0.29 g) and sodium acetate buffer (0.3 M, pH = 5.2, 5 mL). Stir the mixture at 80 °C for 3 h under a nitrogen atmosphere. Upon completion of the reaction, filter the solution through Celite, concentrate the filtrate, and purify the crude product by silica gel column chromatography using dichloromethane and methanol as the eluent. The product is obtained as a pale yellow solid (0.28 g, 71% yield). ^{1}H NMR (700 MHz,

CD_3OD) δ 8.20 (s, 1H), 6.48 (ddt, *J* = 35.3, 15.9, 6.4 Hz, 1H), 6.33 (dd, *J* = 15.8, 4.7 Hz, 1H), 6.28 (t, *J* = 6.5 Hz, 1H), 4.42 (dt, *J* = 6.6, 3.6 Hz, 1H), 4.18 – 4.09 (m, 2H), 3.93 (q, *J* = 3.3 Hz, 1H), 3.79 (ddd, *J* = 53.4, 12.1, 3.2 Hz, 2H), 3.17 – 2.97 (m, 3H), 2.35 – 2.22 (m, 2H). ^{13}C NMR (176 MHz, CD_3OD) δ 162.9, 162.9, 157.0 – 156.2 (m), 150.0, 150.0, 138.8, 138.5, 125.7, 125.6, 123.3, 122.6, 116.7 (q, *J* = 287.1 Hz), 116.6 (q, *J* = 287.4 Hz), 110.6, 110.4, 87.6, 87.6, 85.3, 85.3, 70.5, 70.5, 61.2, 61.1, 51.6 (q, *J* = 3.3 Hz), 51.1, 40.3, 40.2, 33.5 (q, *J* = 3.8 Hz), 33.1. HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{15}H_{19}F_3N_3O_6$ 394.1220, found 394.1226.

Compound 7d: Dissolve tributylammonium pyrophosphate (0.30 mmol, 164.4 mg) in *N,N*-dimethylformamide (0.5 mL) and tributylamine (0.5 mL). Dissolve 2-chloro-1,3,2-benzodioxaphosphorin-4-one (0.20 mmol, 40.4 mg) in *N,N*-dimethylformamide (0.5 mL). Mix the above two solutions and allow the reaction to proceed for 30 min. Dissolve the thoroughly dried nucleoside **7c** (0.20 mmol, 78.7 mg) in *N,N*-dimethylformamide (1.5 mL) and add it to the reaction mixture. Stir the resulting solution at room temperature for 3 h. Then, add 3% iodine solution (pyridine/water = 9:1; 3 mL) to the mixture and maintain the brown color without fading for 15 min. Add deionized water (3 mL) to the mixture and continue the reaction for another 3 h. Transfer the mixture to a centrifuge tube, followed by the addition of saturated sodium chloride solution (1 mL) and anhydrous ethanol (80 mL), and store the mixture at -20 °C overnight. Collect the solid precipitate by centrifugation, then dissolve the precipitate in concentrated ammonia water (5 mL) and react for 3 h. After the removal of solvent under reduced pressure, the crude product was purified by preparative HPLC (C-18, 0.1 M TEAB in water / methanol = 0/100 to 15/85 , flow rate = 4.0 mL/min, l = 254 nm) t_R = 13.7 min. The target product is obtained as a white solid (64.4 mg, yield 60%). ^{1}H NMR (700 MHz, D_2O) δ 7.98 (s, 1H), 6.50 (d, *J* = 15.9 Hz, 1H), 6.24 (t, *J* = 6.5 Hz, 1H), 6.17 (dt, *J* = 15.8, 7.2 Hz, 1H), 4.55 (s, 1H), 4.14 (s, 2H), 4.06 (s, 1H), 3.67-3.54 (m, 2H), 2.56 (s, 3H), 2.33 – 2.16 (m, 2H). ^{31}P NMR (283 MHz, D_2O) δ -5.93 (d, *J* = 19.1 Hz), -10.94 (d, *J* = 19.1 Hz), -21.05. HRMS (ESI) m/z: $[M + 3H]^-$ Calcd for $C_{13}H_{21}N_3O_{14}P_3$ 536.0242, found 536.0229.

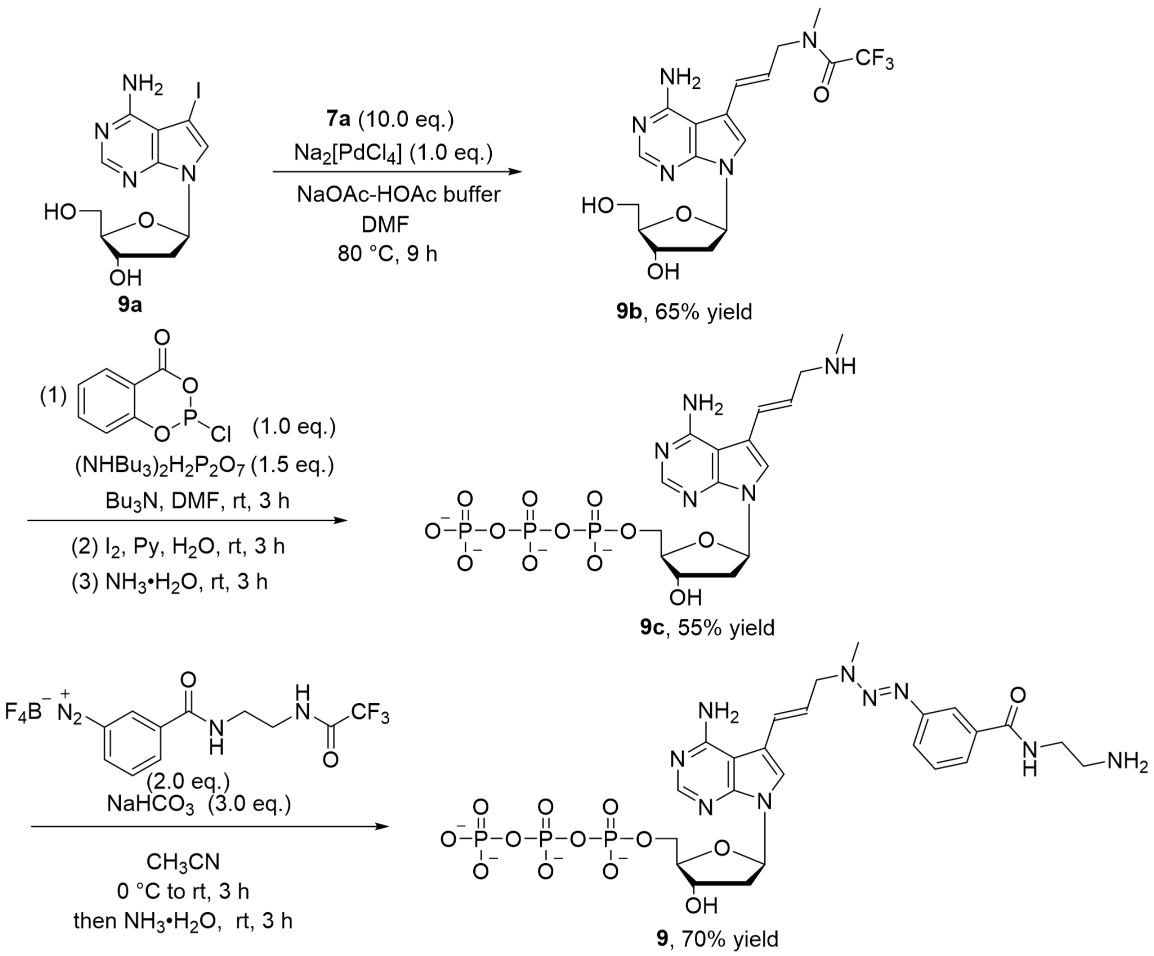
Compound 7: Dissolve compound **7d** (55.8 µmol, 30.0 mg) and sodium bicarbonate (167.6 µmol, 14.1 mg) in deionized water (4 mL). Dissolve diazonium salt (111.6 µmol, 41.7 mg) in acetonitrile (4 mL). Mix the above two solutions, react at 0 °C for 1 h, and then continue the reaction at room temperature for another 2 h. After the reaction, add concentrated ammonia water (6 mL) and stir the solution at room temperature for 3 h. After the removal of solvent under reduced pressure, the crude product was purified by preparative HPLC (C-18, 0.1 M TEAB in water / methanol = 10/90 to 50/50 , flow rate = 4.0 mL/min, l = 254 nm) t_R = 13.4 min. The target product is obtained as a pale yellow solid (18.0 mg, yield 48%). ^{1}H NMR (700 MHz, D_2O) δ 7.78 (s, 1H), 7.74 (s, 1H), 7.50 (d, *J* = 7.0 Hz, 1H), 7.42 (d, *J* = 8.1 Hz, 1H), 7.38 (t, *J* = 7.8 Hz, 1H), 6.43 (dt, *J* = 15.8, 6.0 Hz, 1H), 6.22 (d, *J* = 15.7 Hz, 1H), 6.17 (t, *J* = 6.8 Hz, 1H), 4.52 (s, 1H), 4.43 (s, 2H), 4.16 – 4.03 (m, 3H), 3.63 (t, *J* = 5.9 Hz, 2H), 3.20 – 3.12 (m, 5H), 2.28 – 2.19 (m, 2H). ^{31}P NMR (283 MHz, D_2O) δ -6.06, -11.18, -21.33. HRMS (ESI) m/z: $[M + 3H]^-$ Calcd for $C_{22}H_{31}N_7O_{15}P_3$ 726.1096, found 726.1103.



The synthetic procedure of **Compound 8** is the same as that of **Compound 7**.

Compound 8c: The crude product was purified by preparative HPLC (C-18, 0.1 M TEAB in water / methanol = 0/100 to 15/85 , flow rate = 4.0 mL/min, l = 254 nm) t_R = 9.6 min. White solid (53.6 mg, 50% yield). ^{1}H NMR (700 MHz, D$_2$O) δ 8.02 (s, 1H), 6.41 (d, *J* = 15.6 Hz, 1H), 6.17 (t, *J* = 6.3 Hz, 1H), 6.14 – 6.05 (m, 1H), 4.52 – 4.47 (m, 1H), 4.19 – 4.07 (m, 2H), 4.05 (s, 1H), 3.68 – 3.47 (m, 2H), 2.56 (s, 3H), 2.38 – 2.12 (m, 2H). ^{31}P NMR (283 MHz, D$_2$O) δ -7.01, -11.33 (d, *J* = 15.6 Hz), -20.61. HRMS (ESI) m/z: [M + 3H]$^-$ Calcd for $C_{13}H_{22}N_4O_{13}P_3$ 535.0402, found 535.0408.

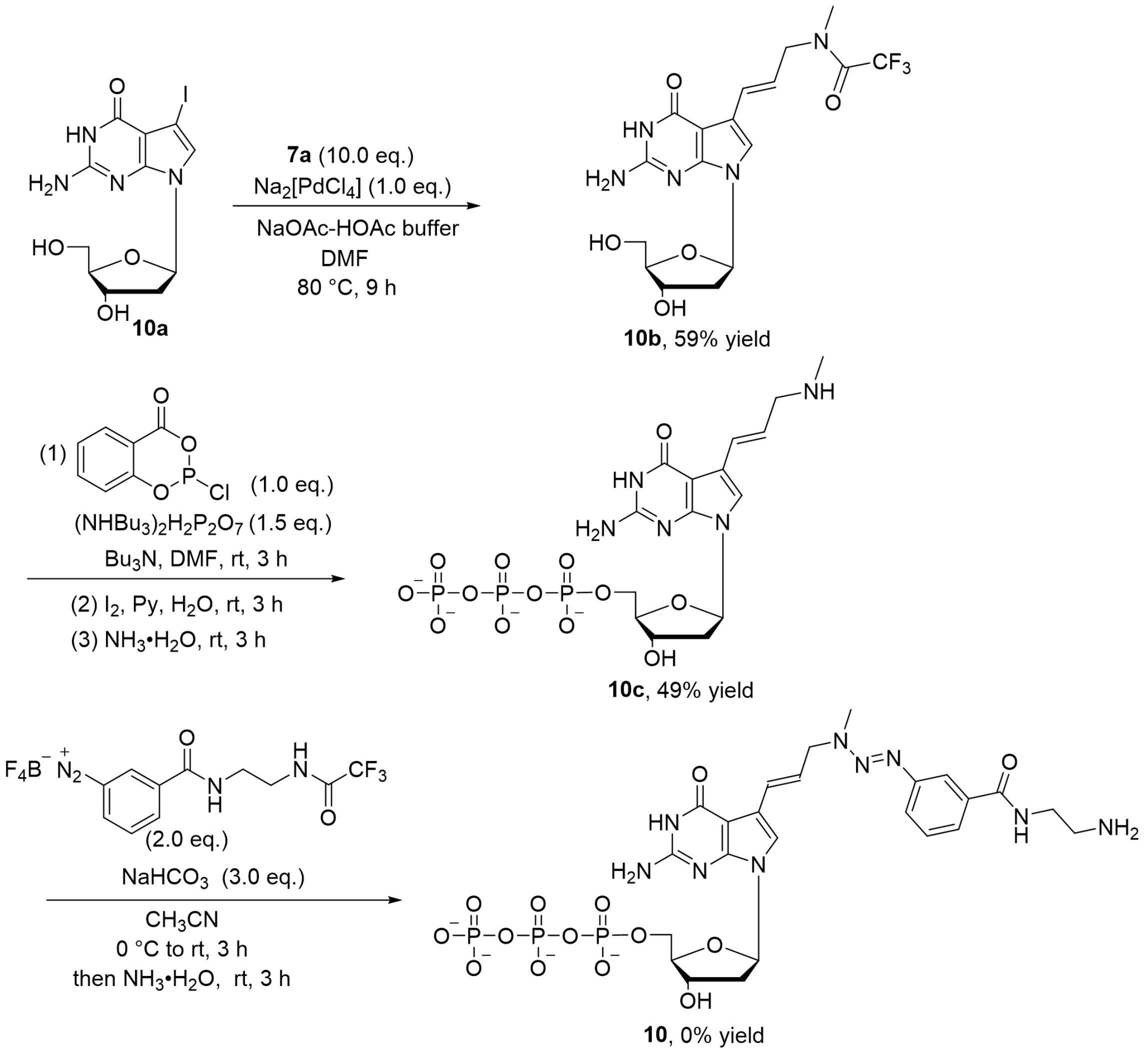
Compound 8: The crude product was purified by preparative HPLC (C-18, 0.1 M TEAB in water / methanol = 0/100 to 50/50 , flow rate = 4.0 mL/min, l = 254 nm) t_R = 15.4 min. Pale yellow solid (7.3 mg, 54% yield). ^{1}H NMR (700 MHz, D$_2$O) δ 7.86 (s, 1H), 7.75 (s, 1H), 7.54 (d, *J* = 7.9 Hz, 1H), 7.52 (d, *J* = 7.9 Hz, 1H), 7.45 (t, *J* = 7.8 Hz, 1H), 6.27 (d, *J* = 16.0 Hz, 1H), 6.22 (t, *J* = 6.7 Hz, 1H), 6.15 (d, *J* = 14.9 Hz, 1H), 4.57 – 4.52 (m, 2H), 4.16 (s, 3H), 3.66 – 3.52 (m, 2H), 3.20 – 3.07 (m, 5H), 2.42 – 2.18 (m, 2H). ^{31}P NMR (283 MHz, D$_2$O) δ -5.36 (d, *J* = 17.2 Hz), -10.67 (d, *J* = 16.5 Hz), -19.35. HRMS (ESI) m/z: [M + 3H]$^-$ Calcd for $C_{22}H_{32}N_8O_{14}P_3$ 725.1256, found 725.1270.



The synthetic procedure of **Compound 9** is the same as that of **Compound 7**.

Compound 9c: The crude product was purified by preparative HPLC (C-18, 0.1 M TEAB in water / methanol = 0/100 to 15/85 , flow rate = 4.0 mL/min, l = 254 nm) t_R = 17.6 min. White solid (61.5 mg, 55% yield). ^{1}H NMR (700 MHz, D_2O) δ 7.93 (s, 1H), 7.71 (s, 1H), 6.80 (d, *J* = 15.5 Hz, 1H), 6.52 (t, *J* = 7.0 Hz, 1H), 6.17 (dt, *J* = 15.2, 7.3 Hz, 1H), 4.25 – 3.98 (m, 3H), 3.83 – 3.56 (m, 2H), 2.64 (s, 3H), 2.61 – 2.31 (m, 2H). ^{31}P NMR (283 MHz, D_2O) δ -6.11 (d, *J* = 19.3 Hz), -10.98 (d, *J* = 17.7 Hz), -21.55. HRMS (ESI) m/z: [M + 3H]$^-$ Calcd for $C_{15}H_{23}N_5O_{12}P_3$ 558.0562, found 558.0566.

Compound 9: The crude product was purified by preparative HPLC (C-18, 0.1 M TEAB in water / methanol = 0/100 to 60/40 , flow rate = 4.0 mL/min, l = 254 nm) t_R = 15.0 min. Pale yellow solid (9.4 mg, 70% yield). ^{1}H NMR (700 MHz, D_2O) δ 7.92 (s, 1H), 7.59 (s, 1H), 7.43 (d, *J* = 7.6 Hz, 1H), 7.41 (d, *J* = 6.8 Hz, 1H), 7.35 (t, *J* = 7.8 Hz, 1H), 7.30 (s, 1H), 6.59 (d, *J* = 15.4 Hz, 1H), 6.39 (t, *J* = 7.1 Hz, 1H), 6.01 (dt, *J* = 15.2, 6.2 Hz, 1H), 4.53 (s, 1H), 4.44 (s, 2H), 4.11 (s, 1H), 4.02 (s, 2H), 3.45 – 3.35 (m, 2H), 3.25 - 3.10 (m, 5H), 2.54 – 2.22 (m, 2H). ^{31}P NMR (283 MHz, D_2O) δ -5.46 (d, *J* = 15.6 Hz), -10.58 (d, *J* = 15.6 Hz), -19.34. HRMS (ESI) m/z: [M + 3H]$^-$ Calcd for $C_{24}H_{34}N_9O_{13}P_3$ 748.1416, found 748.1404.



10a

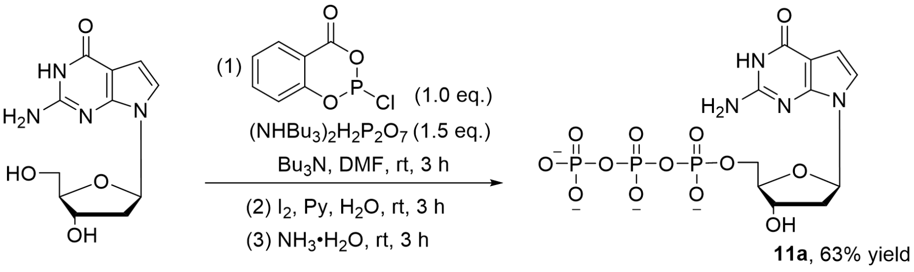
10b, 59% yield

10c, 49% yield

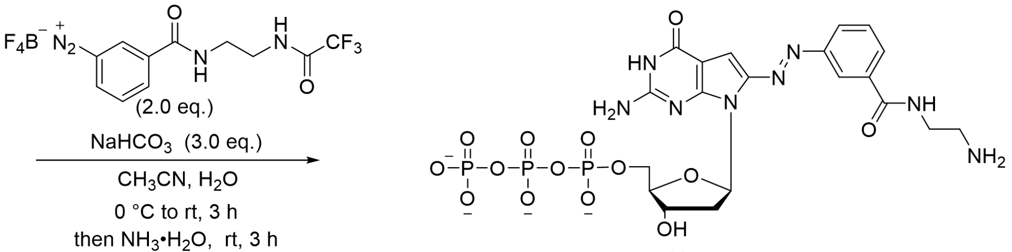
10, 0% yield

The synthetic procedure of **Compound 10** is the same as that of **Compound 7**.

Compound 10c: The crude product was purified by preparative HPLC (C-18, 0.1 M TEAB in water / methanol = 0/100 to 15/85 , flow rate = 4.0 mL/min, l = 254 nm) t_R = 16.3 min. White solid (56.3 mg, 49% yield). ^{1}H NMR (700 MHz, D$_2$O) δ 7.36 (s, 1H), 6.85 (d, *J* = 16.1 Hz, 1H), 6.44 – 6.37 (m, 1H), 6.36 (t, *J* = 7.0 Hz, 1H), 4.15 – 4.01 (m, 3H), 3.72 – 3.60 (m, 2H), 2.62 (s, 3H), 2.57 – 2.27 (m, 2H). ^{31}P NMR (283 MHz, D$_2$O) δ -5.85 (d, *J* = 20.5 Hz), -10.61 (d, *J* = 18.9 Hz), -21.91 (t, *J* = 20.0 Hz). HRMS (ESI) m/z: [M + 3H]$^-$ Calcd for $C_{15}H_{23}N_5O_{13}P_3$ 574.0511, found 574.0526.



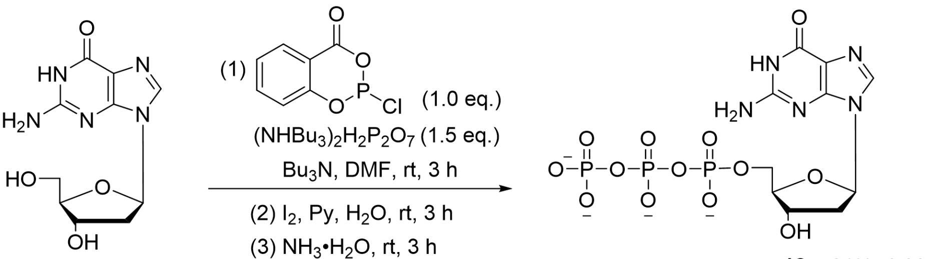
11a, 63% yield



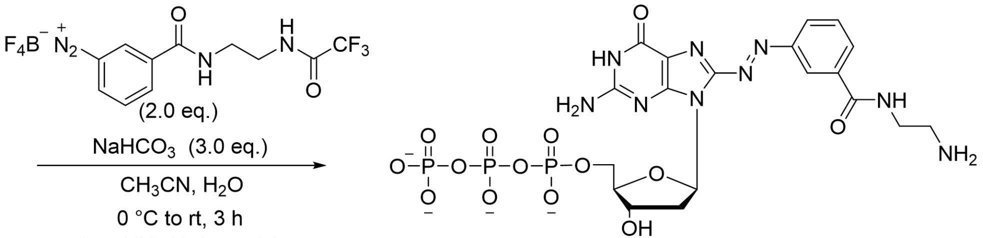
11, 71% yield

Compound 11a: Dissolve tributylammonium pyrophosphate (0.30 mmol, 164.4 mg) in a mixture of *N*,*N*-dimethylformamide (0.5 mL) and tributylamine (0.5 mL). Dissolve 2-chloro-1,3,2-benzodioxaphosphorinane-4-one (0.20 mmol, 40.4 mg) in *N*,*N*-dimethylformamide (0.5 mL). Combine the above two solutions and allow the reaction to proceed for 30 min. Dissolve commercially available deoxyguanosine (0.20 mmol, 53.3 mg) in *N*,*N*-dimethylformamide (1.5 mL) and add the resulting solution to the above reaction mixture. Stir the solution at room temperature for 3 h. Then, add 3% iodine solution (pyridine/water = 9:1; 3 mL) to the mixture and maintain the mixture until the brown color remains unchanged within 15 min. Add deionized water (3 mL) to the mixture and continue the reaction for another 3 h. Transfer the mixture to a centrifuge tube, then add saturated sodium chloride solution (1 mL) and anhydrous ethanol (80 mL). Store the resulting mixture at -20 °C overnight. Collect the solid precipitate by centrifugation of the mixture and subsequently dissolve the precipitate in deionized water. The crude product was purified by preparative HPLC (C-18, 0.1 M TEAB in water / methanol = 5/95 to 10/90 , flow rate = 4.0 mL/min, l = 254 nm) t_R = 10.6 min. The target product is obtained as a white solid (63.7 mg, yield 63%). ^{1}H NMR (700 MHz, Deuterium Oxide) δ 7.06 (d, *J* = 3.8 Hz, 1H), 6.45 (d, *J* = 3.7 Hz, 1H), 6.37 (t, *J* = 6.3 Hz, 1H), 4.22 – 3.96 (m, 3H), 2.79 – 2.15 (m, 2H). ^{31}P NMR (283 MHz, Deuterium Oxide) δ -6.11 (d, *J* = 20.8 Hz), -11.02 (d, *J* = 17.3 Hz), -21.79. HRMS (ESI) m/z: $[M + 3H]^-$ Calcd for $C_{11}H_{16}N_4O_{13}P_3$ 504.9932, found 504.9943.

Compound 11: Dissolve Compound **11a** (19.8 μmol, 10.0 mg) and sodium bicarbonate solution (59.4 μmol, 5.0 mg) in deionized water (2 mL). Dissolve aryl diazonium salt (39.6 μmol, 14.8 mg) in acetonitrile (2 mL). Combine the above two solutions at 0 °C and allow the reaction to proceed for 1 h, followed by stirring at room temperature for another 2 h. After the reaction, add concentrated ammonia solution (6 mL) and stir the mixture at room temperature for 3 h. The crude product was purified by preparative HPLC (C-18, 0.1 M TEAB in water / methanol = 10/90 to 60/40 , flow rate = 4.0 mL/min, l = 254 nm) t_R = 14.5 min. The target product **6** is obtained as an orange-yellow solid (9.8 mg, yield 71%). ^{1}H NMR (700 MHz, D_2O) δ 7.77 (s, 1H), 7.68 (d, *J* = 7.2 Hz, 1H), 7.65 (d, *J* = 7.1 Hz, 1H), 7.45 (t, *J* = 7.5 Hz, 1H), 6.78 (d, *J* = 6.4 Hz, 1H), 6.61 (s, 1H), 4.23 (s, 1H), 4.16 (s, 1H), 4.04 (s, 1H), 3.64 (s, 2H), 3.18 (t, *J* = 7.8 Hz, 2H), 2.36 (s, 2H). ^{31}P NMR (283 MHz, D_2O) δ -5.93 (d, *J* = 20.5 Hz), -10.78 (d, *J* = 19.6 Hz), -22.02 (t, *J* = 20.1 Hz). HRMS (ESI) m/z: $[M + 3H]^-$ Calcd for $C_{20}H_{26}N_8O_{14}P_3$ 695.0787, found 695.0792.



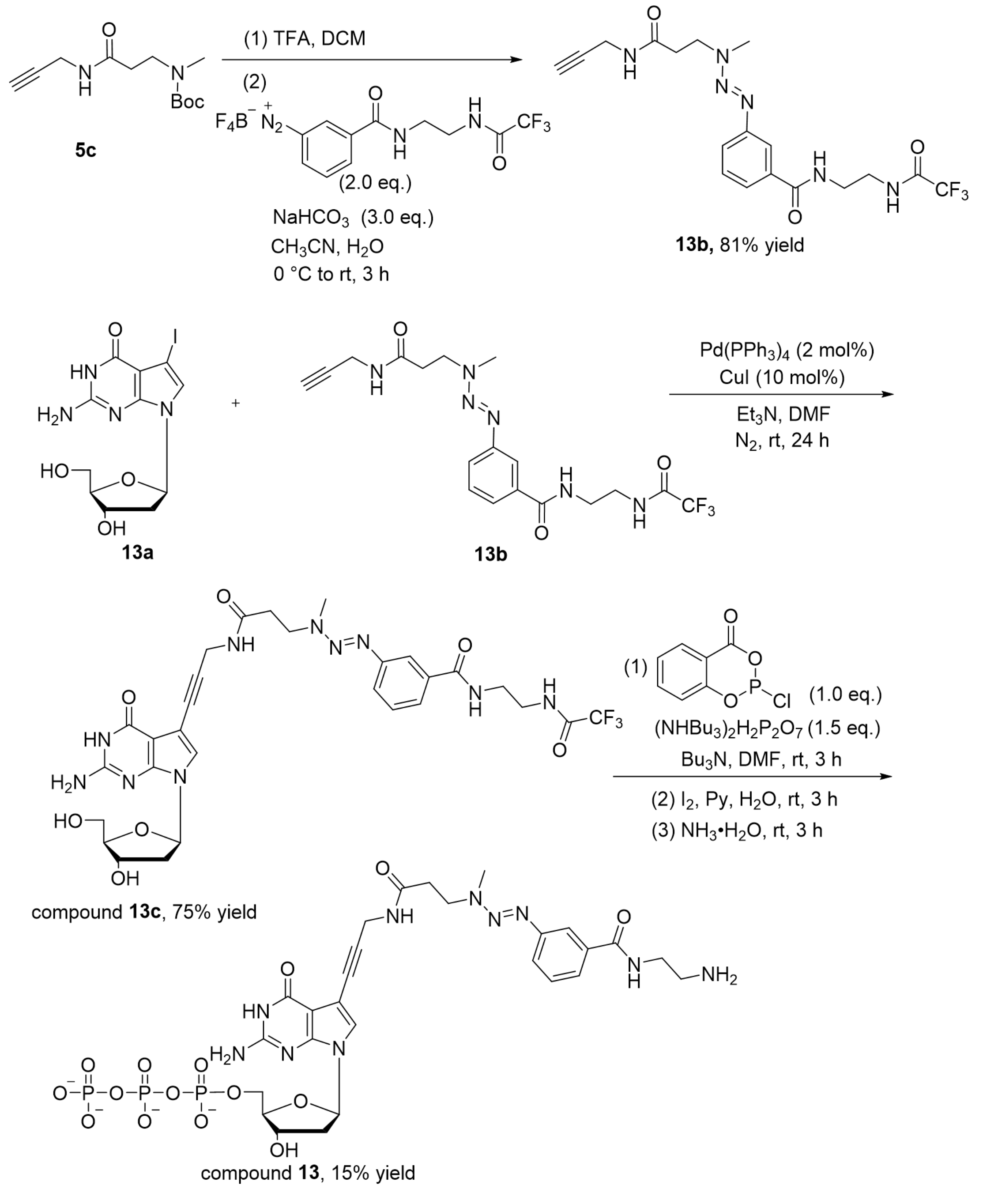
12a, 61% yield



12, 0% yield

The synthetic procedure of **Compound 12** is the same as that of **Compound 11**.

Compound 12a: The crude product was purified by preparative HPLC (C-18, 0.1 M TEAB in water / methanol = 5/95 to 10/90 , flow rate = 4.0 mL/min, l = 254 nm) t_R = 9.0 min. White solid (61.8 mg, 61% yield). ^{1}H NMR (400 MHz, D_2O) δ 7.99 (s, 1H), 6.22 (t, *J* = 6.9 Hz, 1H), 4.31 – 3.95 (m, 3H), 2.79 – 2.32 (m, 2H). ^{31}P NMR (162 MHz, D_2O) δ -5.86 (d, *J* = 20.5 Hz), -10.95 (d, *J* = 19.6 Hz), -21.67 (t, *J* = 19.6 Hz). HRMS (ESI) m/z: [M + 3H]$^-$ Calcd for $C_{10}H_{15}N_5O_{13}P_3$ 505.9885, found 505.9891.

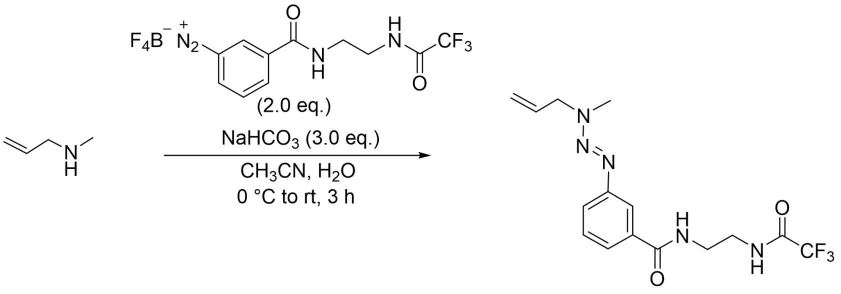


Compound 13b: Dissolve Compound **5c** (0.50 mmol, 120.2 mg) in a mixture of dichloromethane (2 mL) and trifluoroacetic acid (2 mL), and allow the reaction to proceed for 1 h. After the reaction is complete, remove the solvents under reduced pressure. Then, dissolve the resulting product in deionized water (5 mL), and adjust the solution to alkaline pH by adding an appropriate amount of sodium bicarbonate. Dissolve diazonium salt (0.60 mmol, 224.4 mg) in acetonitrile (5 mL), add this solution to the above reaction mixture, followed by the addition of sodium bicarbonate (1.50 mmol, 126.0 mg). Stir the reaction mixture at 0 °C for 1 h, and then continue the reaction at room temperature for another 2 h. After concentration under reduced pressure, purify the crude product by silica gel column

chromatography using ethyl acetate and petroleum ether as the eluent. The target product is obtained as a pale yellow solid (172.7 mg, yield 81%). ^{1}H NMR (700 MHz, CD_3OD) δ 7.85 (t, *J* = 1.9 Hz, 1H), 7.63 – 7.51 (m, 2H), 7.41 (t, *J* = 7.8 Hz, 1H), 4.10 (t, *J* = 6.8 Hz, 2H), 3.95 (d, *J* = 2.5 Hz, 2H), 3.62 – 3.50 (m, 4H), 3.26 (s, 3H), 2.64 (t, *J* = 6.7 Hz, 2H), 2.55 (s, 1H). ^{13}C NMR (176 MHz, CD_3OD) δ 171.7, 169.4, 158.0 (q, *J* = 36.9 Hz), 151.1, 134.8, 128.6, 123.7, 123.4, 119.0, 116.1 (q, *J* = 286.7 Hz), 79.0, 70.8, 51.9, 39.1, 38.6, 34.9, 34.0, 28.1. HRMS (ESI) m/z: $[M - H]^-$ Calcd for $C_{18}H_{20}F_3N_6O_3$ 425.1554, found 425.1556.

Compound 13c: Under a nitrogen atmosphere, dissolve commercially available 5-iododeoxyguanosine **13a** (0.05 mmol, 19.6 mg), copper(I) iodide (5.0 μmol, 19.0 mg), tetrakis(triphenylphosphine)palladium (1.0 μmol, 23.0 mg) and triethylamine (0.15 mmol, 15.2 mg) in *N*,*N*-dimethylformamide (1 mL). Add Compound **13b** (0.15 mmol, 64.0 mg) to the reaction solution, and stir the mixture at room temperature for 24 h under nitrogen protection. After confirming the completion of the reaction by thin-layer chromatography (TLC), concentrate the mixture under reduced pressure. Purify the crude product by silica gel column chromatography using dichloromethane and methanol as the eluent. The product is obtained as a pale yellow solid (25.9 mg, yield 75%). ^{1}H NMR (700 MHz, CD_3OD) δ 7.82 (s, 1H), 7.54 (d, *J* = 7.7 Hz, 1H), 7.51 (d, *J* = 6.7 Hz, 1H), 7.34 (t, *J* = 7.8 Hz, 1H), 7.12 (s, 1H), 6.34 (t, *J* = 7.0 Hz, 1H), 4.46 (dt, *J* = 6.0, 3.1 Hz, 1H), 4.15 (s, 2H), 4.08 (s, 2H), 3.95 (q, *J* = 4.1 Hz, 1H), 3.72 (ddd, *J* = 39.6, 12.0, 4.2 Hz, 2H), 3.60 – 3.49 (m, 4H), 2.66 (t, *J* = 6.8 Hz, 2H), 2.47 (ddd, *J* = 13.7, 8.0, 5.9 Hz, 1H), 2.26 (ddd, *J* = 13.5, 6.1, 3.0 Hz, 1H). ^{13}C NMR (176 MHz, CD_3OD) δ 171.9, 169.4, 159.8, 158.0 (q, *J* = 36.9 Hz), 153.0, 151.1, 150.7, 134.6, 128.7, 123.7, 123.4, 123.1, 119.1, 116.1 (q, *J* = 286.7 Hz), 100.1, 98.8, 87.3, 85.2, 83.9, 76.0, 71.5, 62.3, 51.9, 39.8, 39.2, 38.6, 35.1, 34.2, 29.7. HRMS (ESI) m/z: $[M - H]^-$ Calcd for $C_{29}H_{32}F_3N_{10}O_7$ 689.2413, found 689.2424.

Compound 13: Dissolve tributylammonium pyrophosphate (63.0 μmol, 34.6 mg) in a mixture of *N*,*N*-dimethylformamide (0.5 mL) and tributylamine (0.5 mL). Dissolve 2-chloro-1,3,2-benzodioxaphosphorinane-4-one (42.0 μmol, 8.5 mg) in *N*,*N*-dimethylformamide (0.5 mL). Combine the above two solutions and allow the reaction to proceed for 30 min. Dissolve thoroughly dried compound **13c** (42.0 μmol, 25.0 mg) in *N*,*N*-dimethylformamide (1.5 mL), and add the resulting solution to the above reaction mixture. Stir the solution at room temperature for 3 h. Then, add 3% iodine solution (pyridine/water = 9:1; 1 mL) to the mixture and maintain it until the brown color remains unchanged within 15 min. Add deionized water (2 mL) to the mixture and continue the reaction for another 3 h. Transfer the mixture to a centrifuge tube, then add saturated sodium chloride solution (1 mL) and anhydrous ethanol (80 mL). Store the mixture at -20 °C overnight. Collect the solid precipitate by centrifugation of the mixture, then dissolve the precipitate in concentrated ammonia solution (5 mL) and react for 3 h. After the removal of solvent under reduced pressure, the crude product was purified by preparative HPLC (C-18, 0.1 M TEAB in water / methanol = 10/90 to 60/40 , flow rate = 4.0 mL/min, l = 254 nm) t_R = 16.1 min. The product is obtained as a pale yellow solid (5.2 mg, yield 15%). ^{1}H NMR (700 MHz, D_2O) δ 7.42 (s, 1H), 7.25 – 7.22 (m, 1H), 7.18 (d, *J* = 8.1 Hz, 1H), 7.05 (t, *J* = 7.9 Hz, 1H), 6.79 (s, 1H), 6.10 (t, *J* = 7.2 Hz, 1H), 4.52 (s, 2H), 4.21 – 3.80 (m, 7H), 3.51 (q, *J* = 7.1 Hz, 2H), 3.44 – 3.32 (m, 2H), 3.14 (s, 3H), 2.65 – 2.54 (m, 2H), 2.44 – 2.36 (m, 1H), 2.25 – 2.17 (m, 1H). ^{31}P NMR (283 MHz, D_2O) δ -6.01 (d, *J* = 19.1 Hz), -10.98 (d, *J* = 19.1 Hz), -21.34. HRMS (ESI) m/z: [M + 3H]$^-$ Calcd for $C_{27}H_{36}N_{10}O_{15}P_3$ 833.1580, found 833.1589.



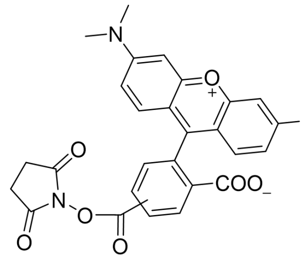
14, 90% yield

Compound 14: Dissolve *N*-methylallylamine (0.20 mmol, 14.2 mg) and sodium bicarbonate (0.60 mmol, 50.4 mg) in deionized water (4 mL). Dissolve diazonium salt (0.40 mmol, 150.0 mg) in acetonitrile (4 mL). Mix the above two solutions, react at 0 °C for 1 h, and then continue the reaction at room temperature for another 2 h. Subsequently, dilute the reaction mixture with ethyl acetate and wash the organic layer with saturated brine. Collect the organic layer, dry it over anhydrous sodium sulfate, and concentrate it under reduced pressure. Purify the crude product by silica gel column chromatography using petroleum ether and ethyl acetate as the eluent. The product is obtained as a pale yellow solid (64.0 mg, yield 90%). ^{1}H NMR (700 MHz, CD_3OD) δ 7.85 (s, 1H), 7.55 (t, *J* = 8.5 Hz, 2H), 7.40 (t, *J* = 7.8 Hz, 1H), 6.01 – 5.85 (m, 1H), 5.36 – 5.19 (m, 2H), 4.41 (d, *J* = 5.9 Hz, 2H), 3.62 – 3.46 (m, 4H), 3.19 (s, 3H). ^{13}C NMR (176 MHz, CD_3OD) δ 169.4, 158.0 (q, *J* = 36.9 Hz), 151.1, 134.8, 133.2, 128.6, 123.7, 123.4, 118.9, 117.3, 116.1 (q, *J* = 286.6 Hz), 58.1, 39.1, 38.5, 33.0. HRMS (ESI) m/z: [M + H]$^+$ Calcd for $C_{15}H_{19}F_3N_5O_2$ 358.1485, found 358.1492.

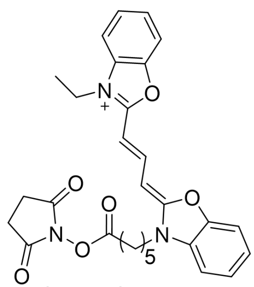
Method for UV-Vis Absorption Spectroscopy Detection of Compound 14: Take four 200 μL centrifuge tubes, and add 50 μL of 20 mM compound **14** methanol solution to each tube, followed by 50 μL 30 mM of aqueous $Na_2S_2O_4$ solution. Irradiate the samples with an UV lamp for 0 s, 3 s, 6 s and 9 s, respectively. Transfer each reaction mixture into a cuvette, add an appropriate amount of 20% aqueous methanol solution and mix well, then measure the samples by UV-vis absorption spectroscopy. Prepare a blank control by adding 50 μL methanol, 50 μL 30 mM of aqueous $Na_2S_2O_4$ solution and an appropriate amount of 20% aqueous methanol solution into another cuvette.

2.3 General procedure for fluorescent labeling

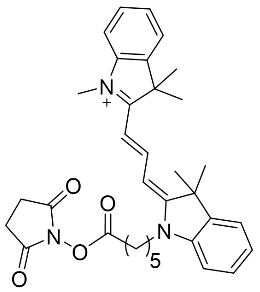
(**a**) Fluorescein is a commercially available product with the following structure.



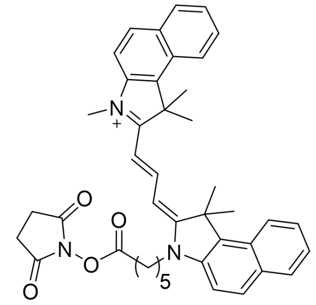
TAMRA NHS ester



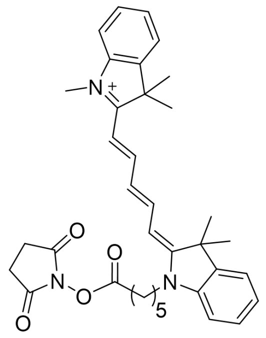
Cy2 NHS ester



Cy3 NHS ester



Cy3.5 NHS ester



Cy5 NHS ester

TAMRA NHS ester: Absorption maximum 541 nm; Emission maximum 567 nm.

Cy2 NHS ester: Absorption maximum 492 nm; Emission maximum 510 nm.

Cy3 NHS ester: Absorption maximum 555 nm; Emission maximum 570 nm.

Cy3.5 NHS ester: Absorption maximum 591 nm; Emission maximum 604 nm.

Cy5 NHS ester: Absorption maximum 646 nm; Emission maximum 662 nm.

(**b**) Fluorescein Labeling Method

Dissolve modified nucleotides (3.0 mg) in a 0.1 M sodium carbonate solution (1 mL). Dissolve fluorescein Dye NHS ester (1.0 mg) and triethylamine (5.0 mg) in N,N-dimethylformamide (1 mL). Mix the above two solutions and react at room temperature for 3 h. After concentration under reduced pressure, purify the product via C-18 high-performance liquid chromatography (HPLC). The resulting fluorescently labeled nucleotides are shown below.

Compound 7-TAMRA: The crude product was purified by preparative HPLC (C-18, 0.1 M TEAB in water / methanol = 10/90 to 50/50 , flow rate = 4.0 mL/min, I = 546 nm) t_R = 16.8 min. White solid (61.5 mg, 55% yield). Red solid (0.9 mg, yield 42%). HRMS (ESI) m/z: [M + 3H]$^-$ Calcd for $C_{47}H_{51}N_9O_{19}P_3$ 1138.2520, found 1138.2532.

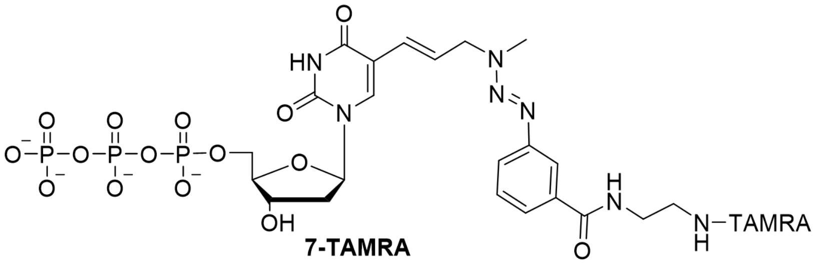
Compound 11-TAMRA: The crude product was purified by preparative HPLC (C-18, 0.1 M TEAB in water / methanol = 10/90 to 40/60 , flow rate = 4.0 mL/min, I = 546 nm) t_R = 15.2 min. Red solid (1.1 mg, yield 52%). HRMS (ESI) m/z: [M + 3H]$^-$ Calcd for $C_{45}H_{46}N_{10}O_{18}P_3$ 1107.2210, found 1107.2214.

Compound 7-Cy3: The crude product was purified by preparative HPLC (C-18, 0.1 M TEAB in water / acetonitrile = 30/70 to 80/20 , flow rate = 4.0 mL/min, I = 555 nm) t_R = 10.8 min. Red solid (1.5 mg, yield 71%). HRMS (ESI) m/z: [M + 2H]$^-$ Calcd for $C_{52}H_{65}N_9O_{16}P_3$ 1164.3768, found 1164.3771.

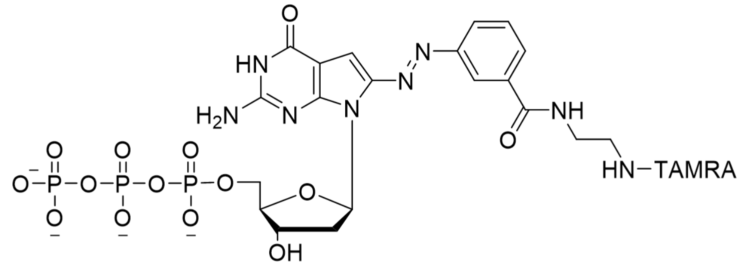
Compound 8-Cy2: The crude product was purified by preparative HPLC (C-18, 0.1 M TEAB in water / acetonitrile = 10/90 to 50/50 , flow rate = 4.0 mL/min, l = 492 nm) t_R = 18.6 min. Orange solid (1.4 mg, yield 64%). HRMS (ESI) m/z: $[M + 2H]^-$ Calcd for $C_{47}H_{56}N_{10}O_{17}P_3$ 1125.3043, found 1125.3062.

Compound 9-Cy5: The crude product was purified by preparative HPLC (C-18, 0.1 M TEAB in water / acetonitrile = 30/70 to 80/20 , flow rate = 4.0 mL/min, l = 646 nm) t_R = 12.4 min. Blue solid (1.5 mg, yield 72%). HRMS (ESI) m/z: $[M + 2H]^-$ Calcd for $C_{56}H_{69}N_{11}O_{14}P_3$ 1212.4244, found 1212.4264.

Compound 13-Cy3.5: The crude product was purified by preparative HPLC (C-18, 0.1 M TEAB in water / acetonitrile = 30/70 to 80/20 , flow rate = 4.0 mL/min, l = 591 nm) t_R = 16.7 min. Purple solid (1.4 mg, yield 67%). HRMS (ESI) m/z: $[M + 2H]^-$ Calcd for $C_{65}H_{74}N_{12}O_{16}P_3$ 1371.4564, found 1371.4555.



7-TAMRA



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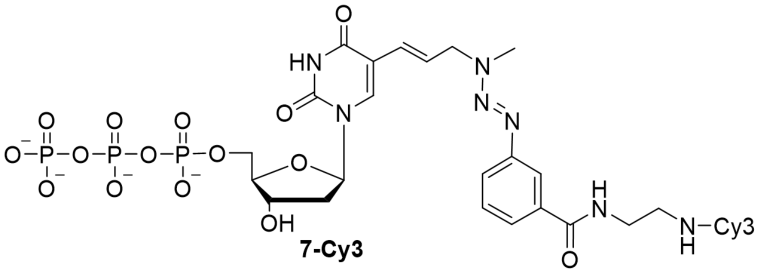
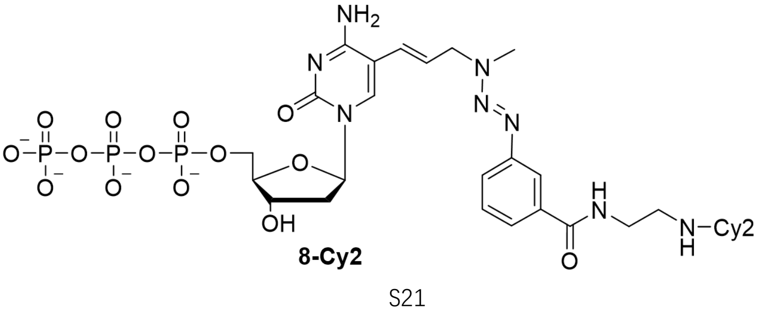


7-Cy3



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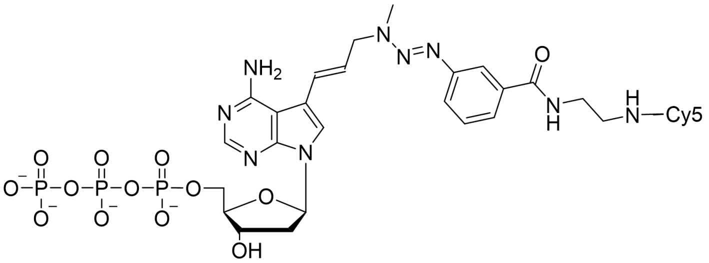
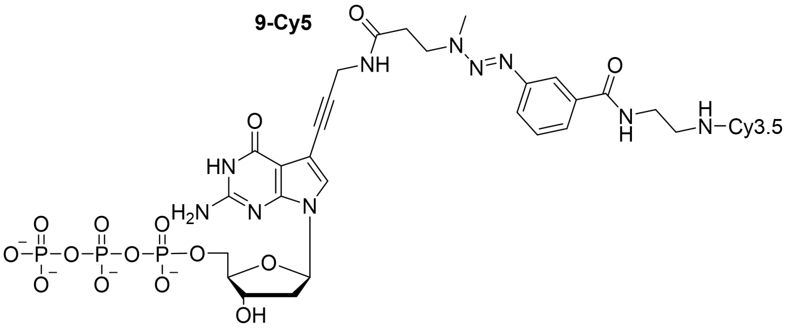


9-Cy5



13-Cy3.5

3. DNA Extension / Cleavage Experimental Section

The oligonucleotides used in the experiment are as follows
The 24 nt primer: DyLight 800-5'-GAGGAAAGGGAAGGGAAAGGAAGG-3'
The 25 nt primer as contrast: DyLight 800-5'-GAGGAAAGGGAAGGGAAAGGAAGGT-3'
Template Ⅰ: 3'-CTCCTTTCCCTTCCCTTTCCTTCC**AAAA**GTCGCCATGTCG -5'
Template Ⅱ: 3'-CTCCTTTCCCTTCCCTTTCCTTCC**A**TCGATCGCCATGTCG -5'
Template Ⅲ: 3'-CTCCTTTCCCTTCCCTTTCCTTCC**G**TCGATCGCCATGTCG -5'
Template Ⅳ: 3'-CTCCTTTCCCTTCCCTTTCCTTCC**T**ACGATCGCCATGTCG -5'
Template Ⅴ: 3'-CTCCTTTCCCTTCCCTTTCCTTCC**C**TCGATCGCCATGTCG -5'

3.1 General procedure for the DNA extension experiment

The primer and template were annealed in a Tris-EDTA buffer (1 X, 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: reaction buffer (10 X, 2 μL), annealed template / primer (50 μM, 1 μL), the triazene reversible terminator (1 mM, 2 μL), Bst DNA polymerase (5U) and ultrapure water to a final volume of 20 μL. After gentle vortex mix, the mixture was incubated at the optimal enzyme temperature 65 °C for 5 min, followed by cooling to 16 °C. The extension products were obtained by phenol chloroform extraction, ethanol precipitation, and G-50 column purification. At each step, a portion of the extension products was dissolved in water for denaturing gel electrophoresis.

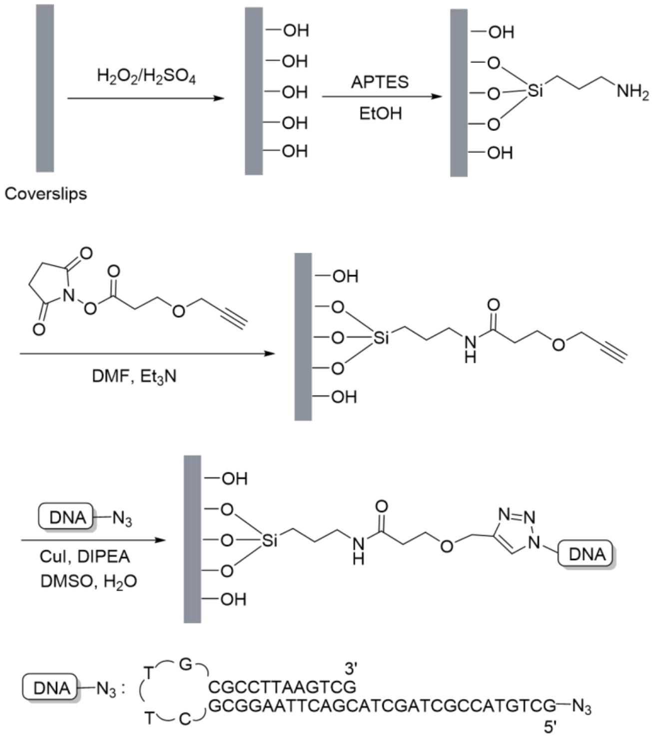
3.2 General procedure for the DNA cleavage experiment

The extension products were irradiated with 365 nm LED UV light for 5 s in a TE buffer solution (0.1 M Tris-EDTA buffer, pH = 8) containing 30 mM $Na_2S_2O_4$ to generate cleavage products. The cleavage products were obtained by ethanol precipitation, and G-50 column purification. Cleavage products could be used for the next extension. At each step, a portion of the cleavage products was dissolved in water for denaturing gel electrophoresis.

3.3 DNA denaturing polyacrylamide gel electrophoresis

DNA electrophoresis was performed on 12 % denaturing polyacrylamide gels containing 7 M of urea, using a S4S aluminum backed sequencer system (Thermo Scientific). Before electrophoresis, the prepared gels were run at a voltage of 2000 V and a constant power of 40 W for 30 min. 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. The gels were run under the above-mentioned conditions for 120 min. The resultant gels were observed using an Odyssey Infrared Imaging System (LI-COR Biosciences, US) under excitation of 785 nm laser.

4. Single-Molecule DNA Sequencing by Synthesis Experiment

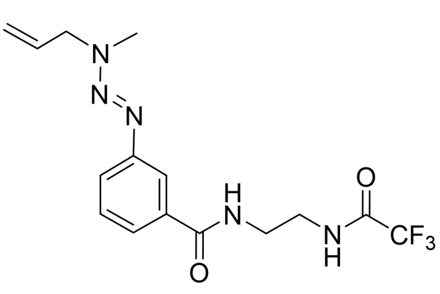


The coverslips were immersed in piranha solution (a mixture of 30% hydrogen peroxide and concentrated sulfuric acid at a volume ratio of 3:7) and heated at 80 °C for 1 h. After removal, the coverslips were rinsed sequentially with ultrapure water and ethanol. The pretreated coverslips were then placed in an ethanol solution of 3-aminopropyltriethoxysilane (APTES), where the mass ratio of APTES to absolute ethanol was 1:49, followed by heating at 60 °C for 2 h. After cooling to room temperature, the coverslips were taken out and rinsed successively with ultrapure water and ethanol to obtain aminated coverslips.

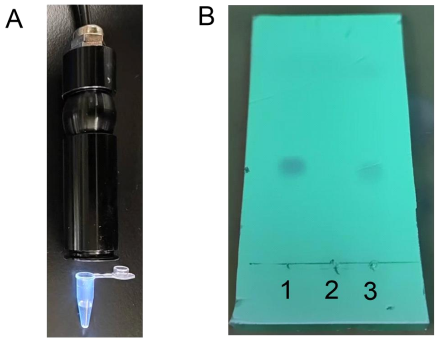
The aminated coverslips were immersed in a 0.1 M N,N-dimethylformamide (DMF) solution of propargyl-PEG1-NHS ester, with the addition of 1% (v/v) triethylamine. The mixture was sonicated at room temperature for 5 min and then soaked for 5 h. Upon completion of the reaction, the coverslips were washed sequentially with DMF, ultrapure water and ethanol to yield alkynylated coverslips. Azide-modified DNA was dissolved in a mixed solvent of dimethyl sulfoxide (DMSO) and water (v/v = 1:2) to prepare a 100 pM DNA solution. Meanwhile, the catalyst system (cuprous iodide and diisopropylethylamine) was also dissolved in DMSO/water mixture (v/v = 1:2) to form a 1 nM catalyst solution. Aliquots of 10 µL DNA solution and 10 µL catalyst solution were mixed thoroughly, then dropped onto the surface of alkynylated coverslips and incubated at room temperature for 1 h. After the reaction, the coverslips were rinsed sequentially with DMSO, ethanol and TE buffer (0.1 M Tris-EDTA buffer, pH = 8) to obtain DNA-immobilized coverslips.

The DNA-immobilized coverslip, a silica spacer with a hollowed-out center, and a glass slide were assembled in a stack to form a microfluidic channel. Two holes were drilled on the top glass slide to serve as the inlet and outlet for reagent delivery, and a heating plate was used to regulate the temperature of the glass assembly. The following components were mixed sequentially in a centrifuge tube placed on ice: reaction buffer (10X, 10 µL), triazene reversible terminator nucleotides (10 µM, 10 µL), Bst DNA polymerase (0.25 U), and ultrapure water, with a final volume adjusted to 100 µL. After gentle vortexing, the mixture was injected into the sandwiched channel and incubated at 65 °C (the optimal enzymatic temperature) for 5 min, followed by cooling to room temperature. The microfluidic channel was rinsed sequentially with TE buffer and ultrapure water, and then subjected to total internal reflection fluorescence (TIRF) microscopy for single-molecule DNA fluorescence imaging. Subsequently, the extension products were irradiated with 365 nm LED UV light for 5 s in TE buffer containing 30 mM $Na_2S_2O_4$ (0.1 M Tris-EDTA buffer, pH = 8) to generate cleavage products. The channel was rinsed again with TE buffer and ultrapure water, and single-molecule DNA fluorescence was visualized via TIRF microscopy once more. The same procedure was repeated for each sequencing cycle.

5. Photocleavage mechanism Section

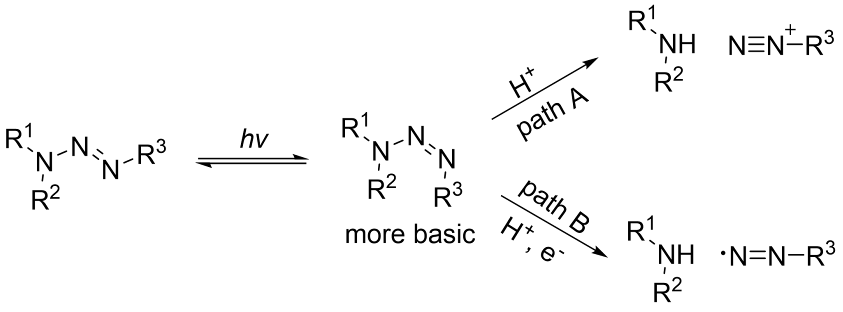


compound **14**



eluent = PE/EA (1:1)

Photocleavage of compound **14** was conducted, and the minor products were characterized by TLC analysis. Figure **A**: Experimental device diagram. Figure **B**: TLC analysis; line 1: compound **14**, line 2: photocleavage of 10 mM compound **14** in MeOH/H_2O (1:1) with 30 mM $Na_2S_2O_4$ within 10 s, line 3: photocleavage of 10 mM compound 14 in MeOH/H_2O (1:1) with 30 mM $Na_2S_2O_4$ and TEMPO within 10 s. It can be seen from the TLC analysis that its fluorescent spot disappeared after UV irradiation (line 2), which confirmed the occurrence of the photocleavage reaction. However, when TEMPO was added to the reaction system, UV irradiation could not completely cleave compound **14** (line 3), suggesting that this process may follow a radical mechanism.

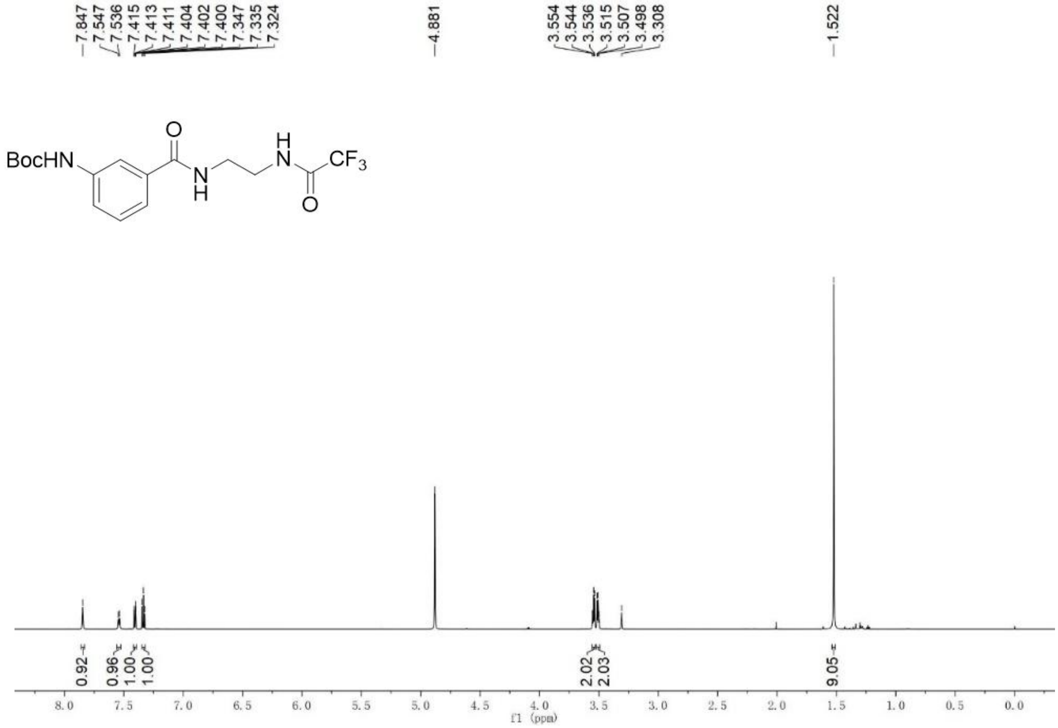


Based on Professor Jewett's work[1] and our research, we propose the following photocleavage mechanism. Firstly, triazene forms a trans-triazene structure under UV irradiation, which makes the nitrogen atoms more basic. Under the action of hydrogen cations (path A), diazonium cations can be released at a relatively high pH value. When an appropriate reducing agent exists in the reaction system (path B), triazene releases diazonium radicals via single electron transfer. Diazonium radicals may form hydrazine under reducing conditions or undergo other quenching reactions.

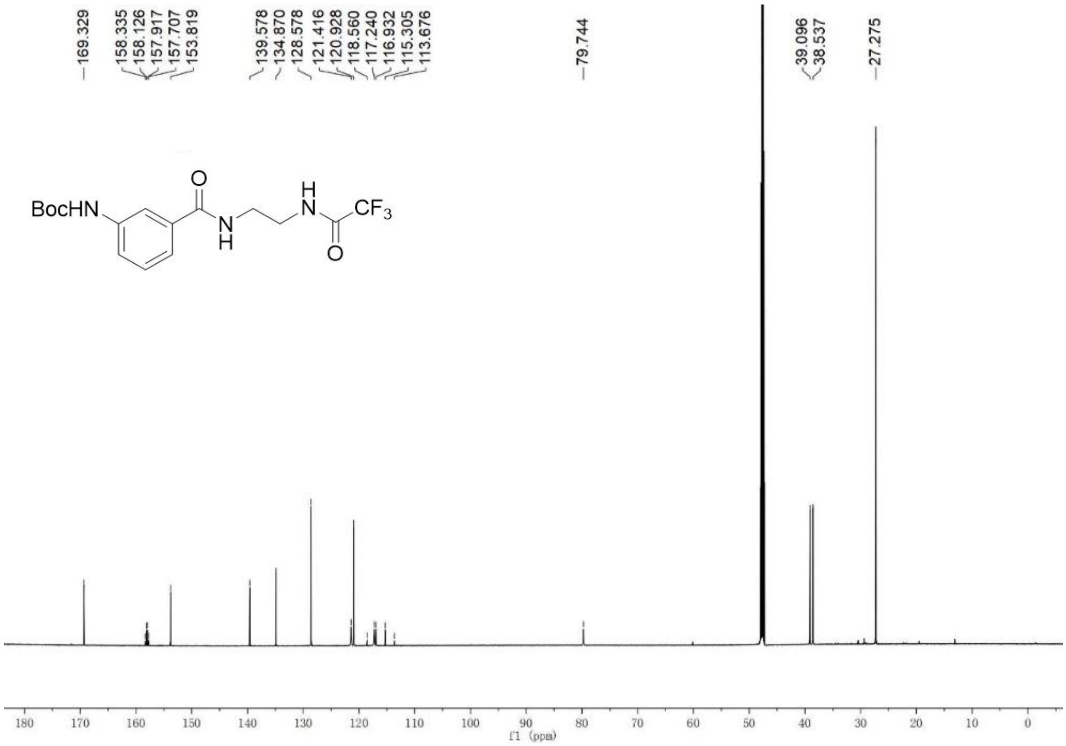
6. References

1. G. J. Davis, J. A. Townsend, M. G. Morrow, M. Hamie, A. J. Shepard, C.-C. Hsieh, Mi. T. Marty and J. C. Jewett, *Bioconjugate Chem.*, 2021, **32**, 2432.

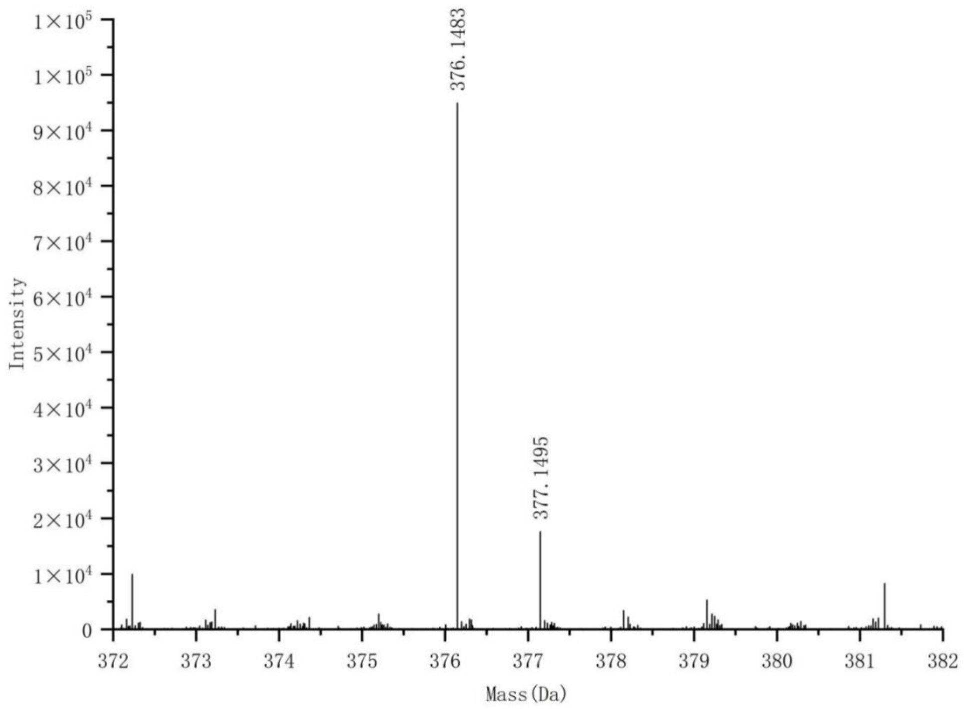
7. Copies of Spectra of Compounds



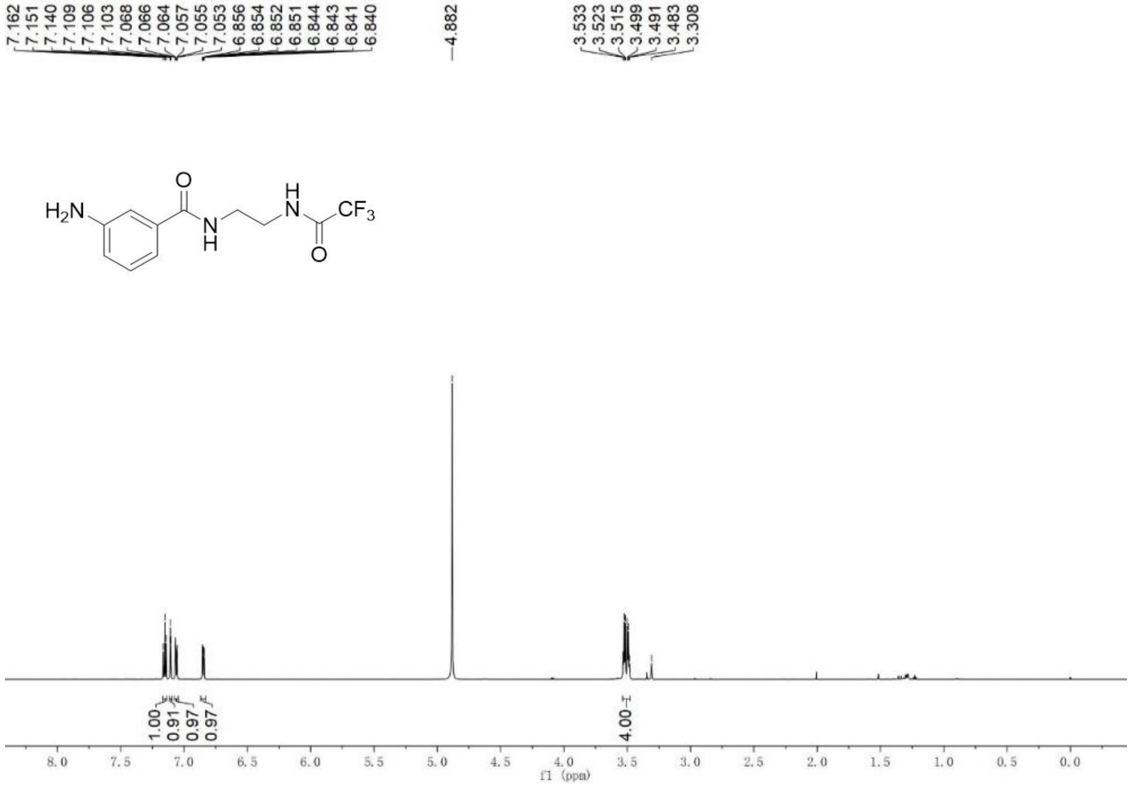
^{1}H NMR spectrum of the compound 1b in CD_3OD



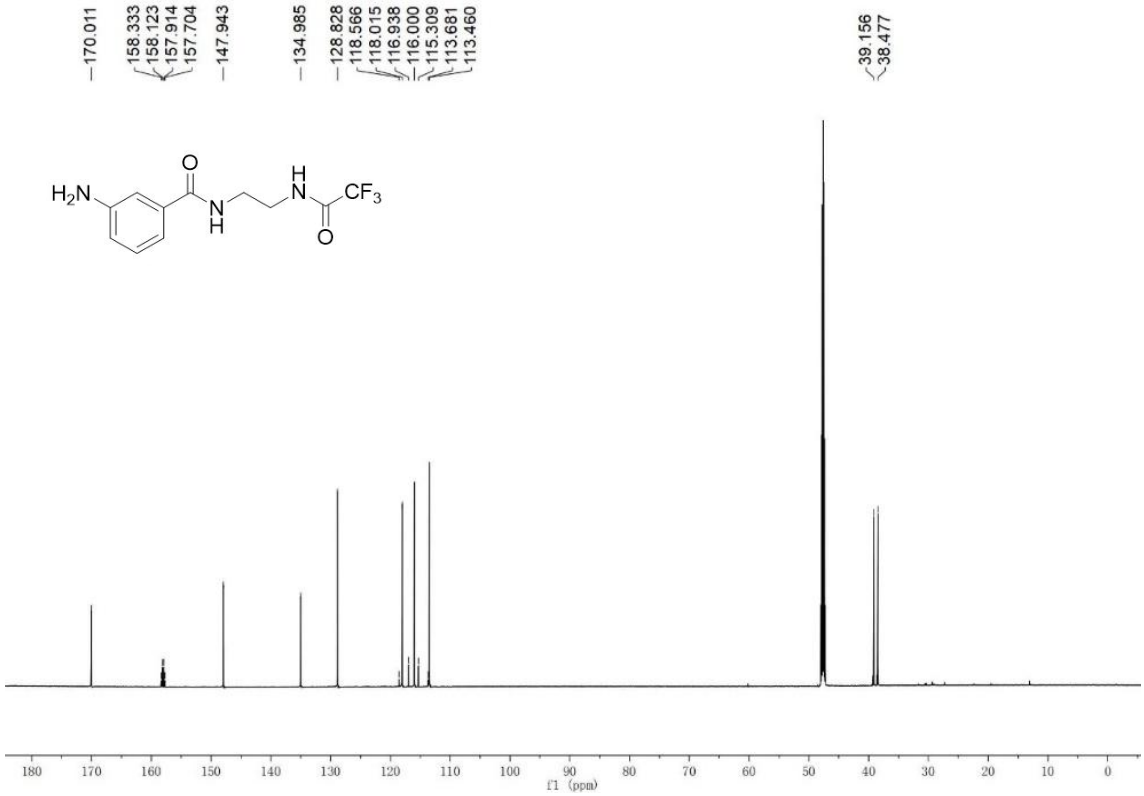
^{13}C NMR spectrum of the compound 1b in CD_3OD



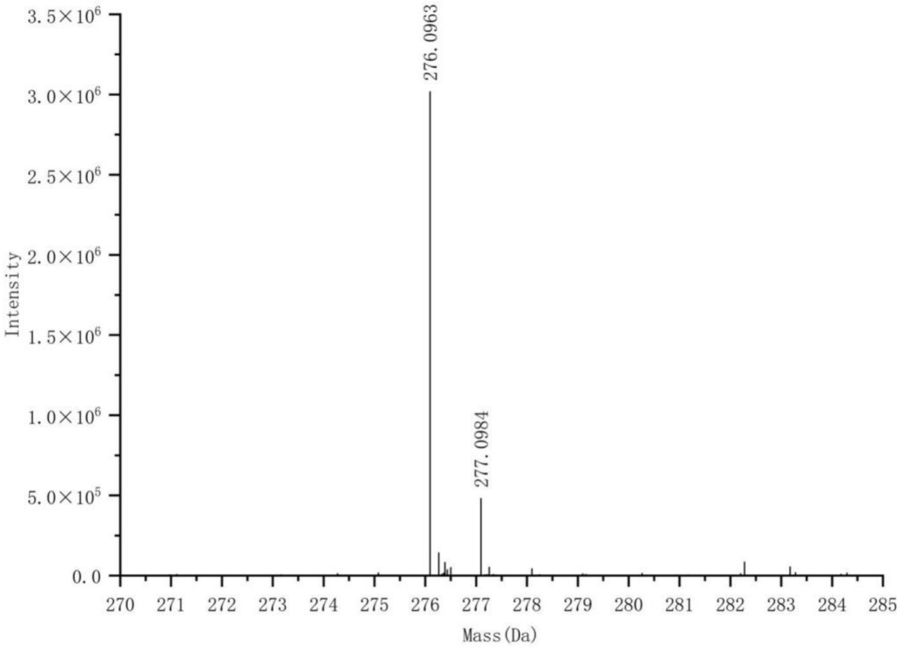
ESI-HRMS spectrum of the compound 1b



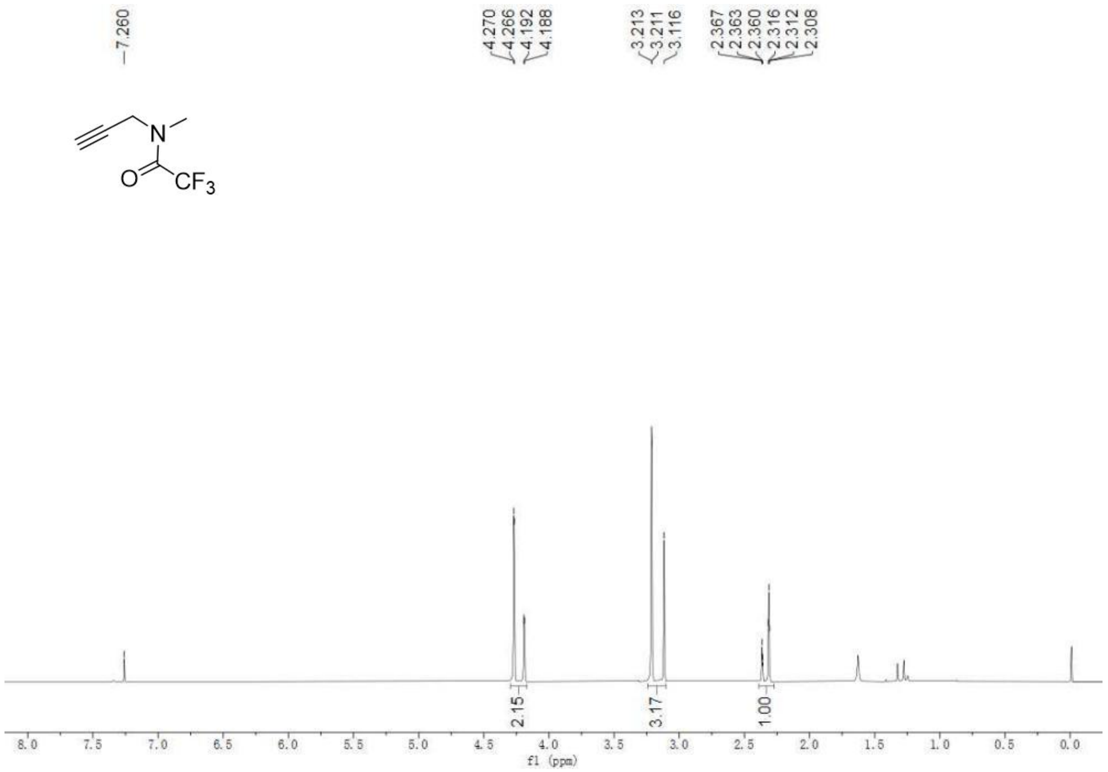
^{1}H NMR spectrum of the compound 1c in CD$_3$OD



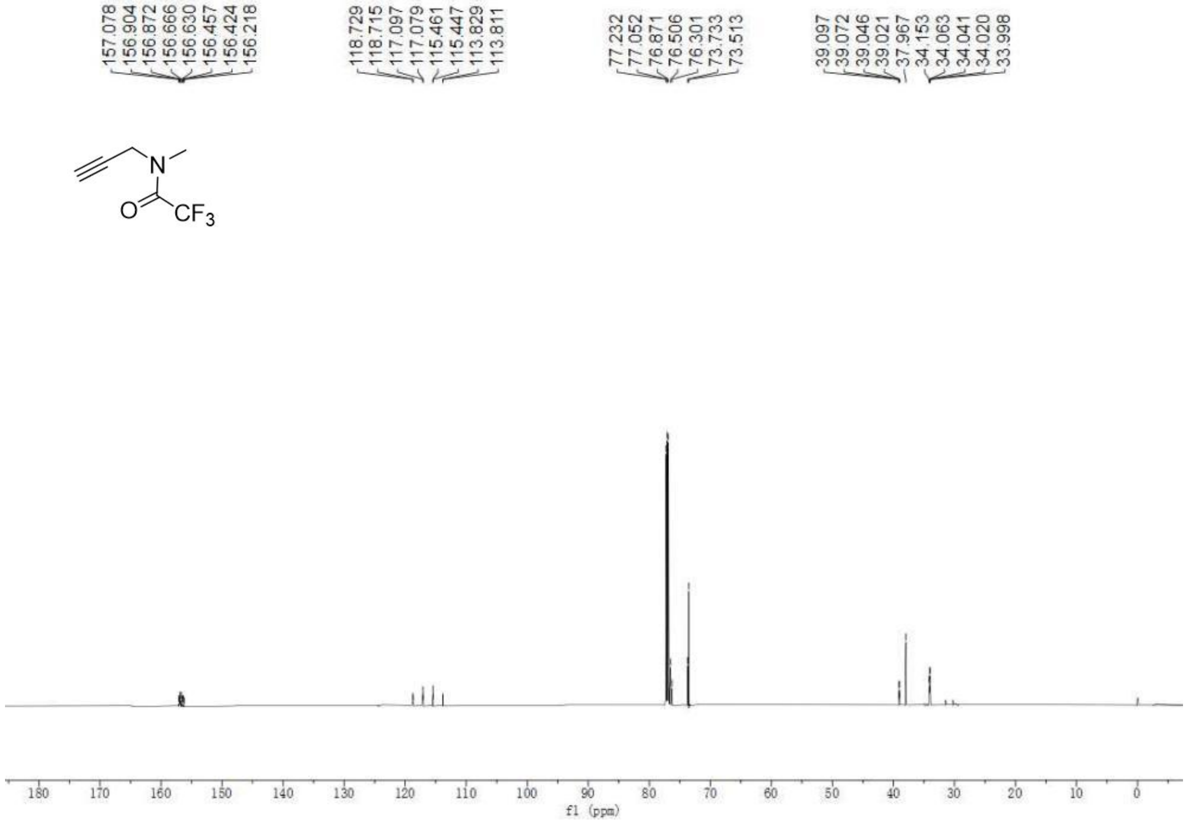
^{13}C NMR spectrum of the compound 1c in CD_3OD



ESI-HRMS spectrum of the compound 1c



^{1}H NMR spectrum of the compound 3b in $CDCl_3$



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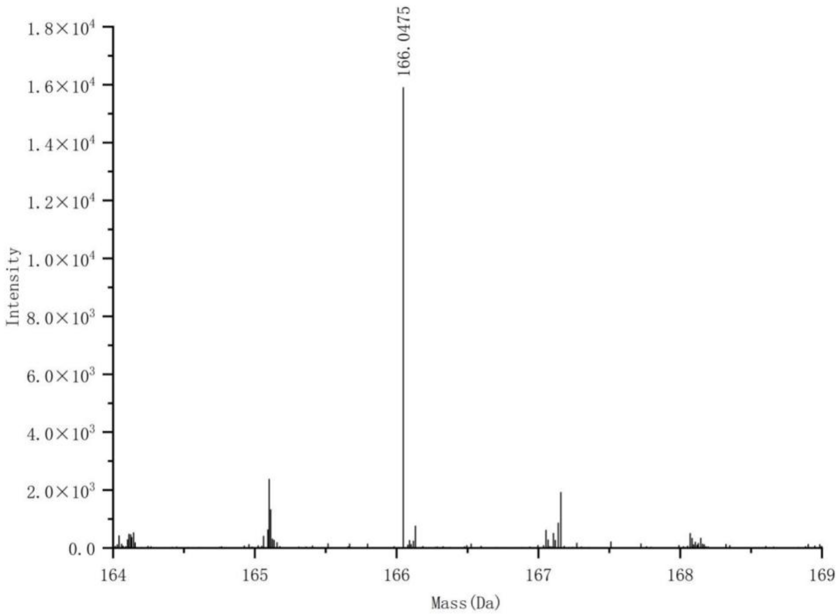
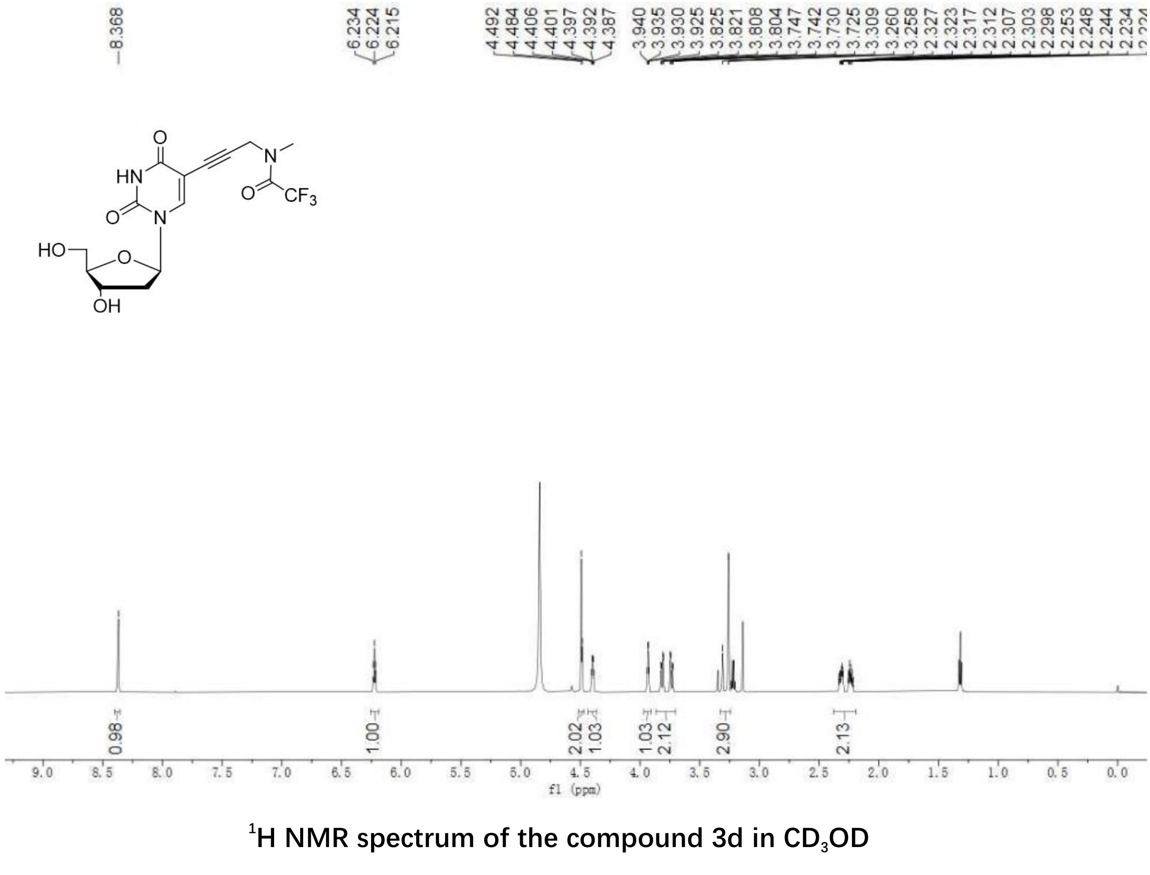
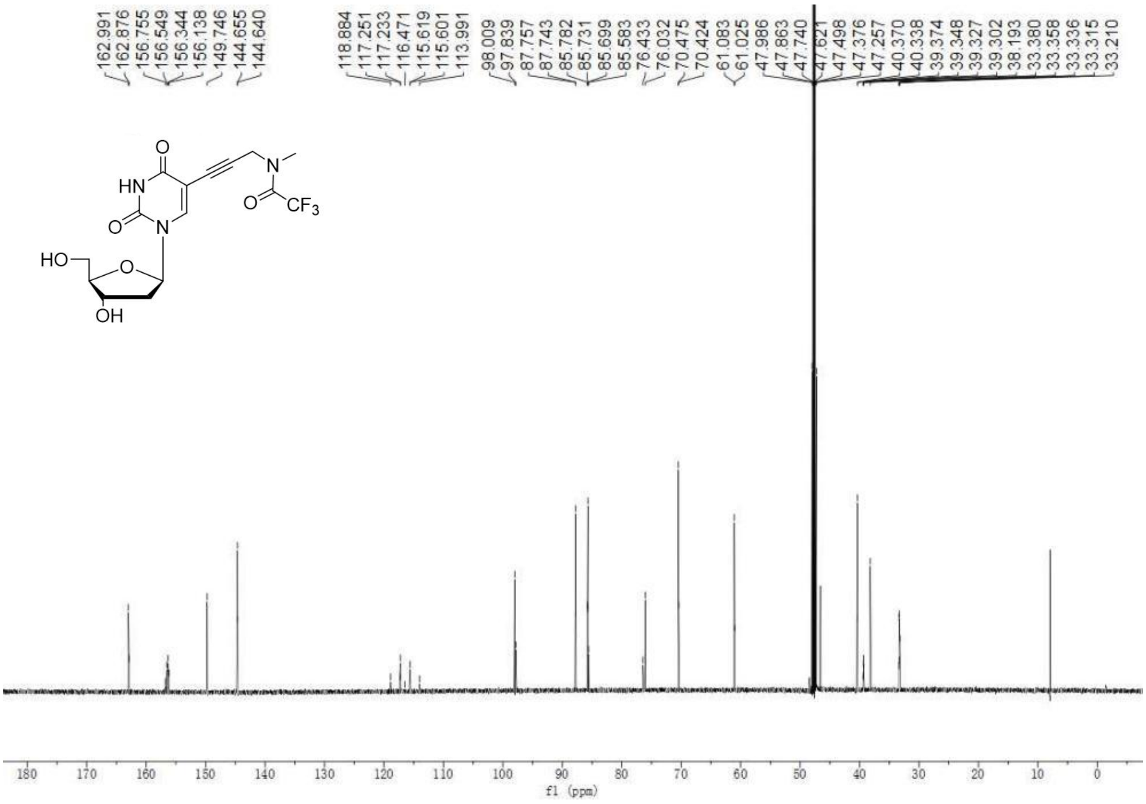


ESI-HRMS spectrum of the compound 3b



^{1}H NMR spectrum of the compound 3d in CD_3OD



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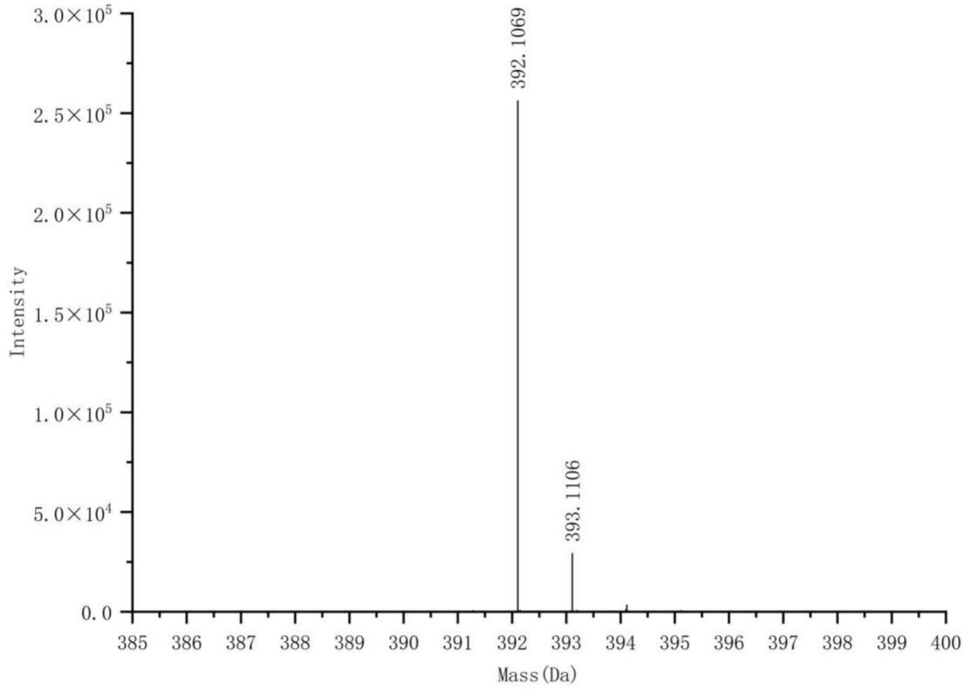

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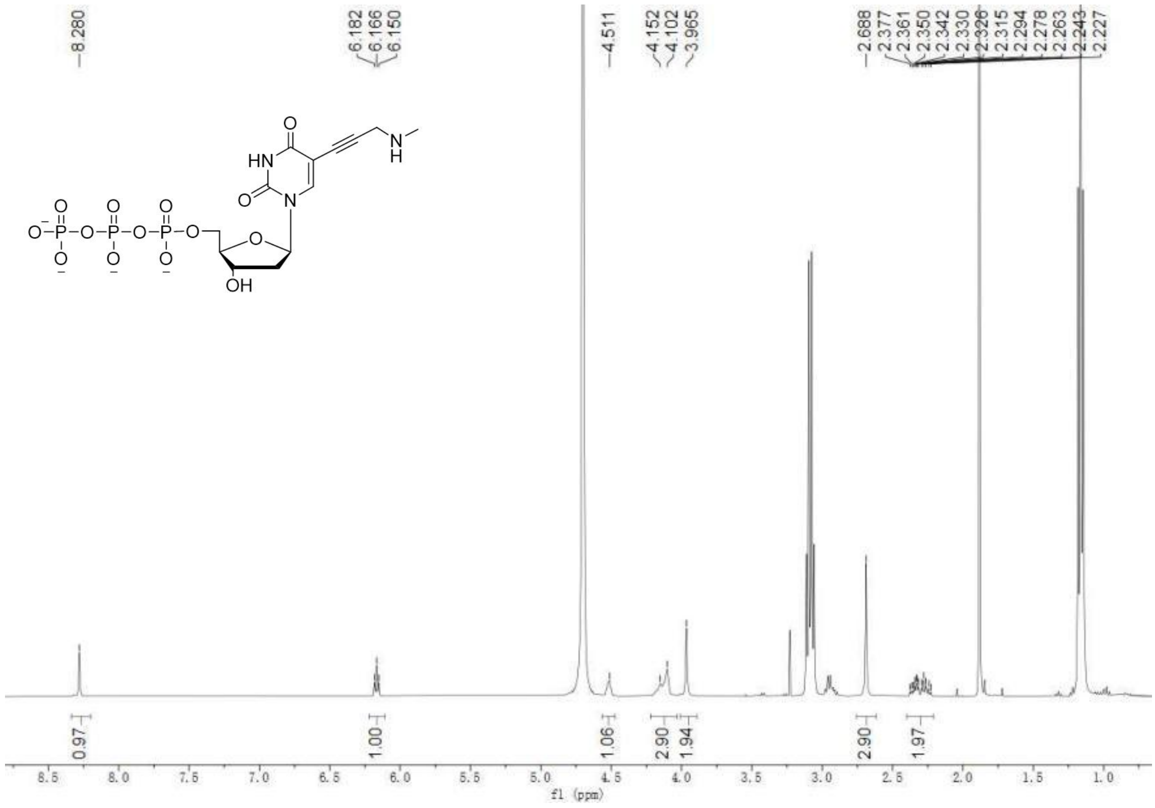
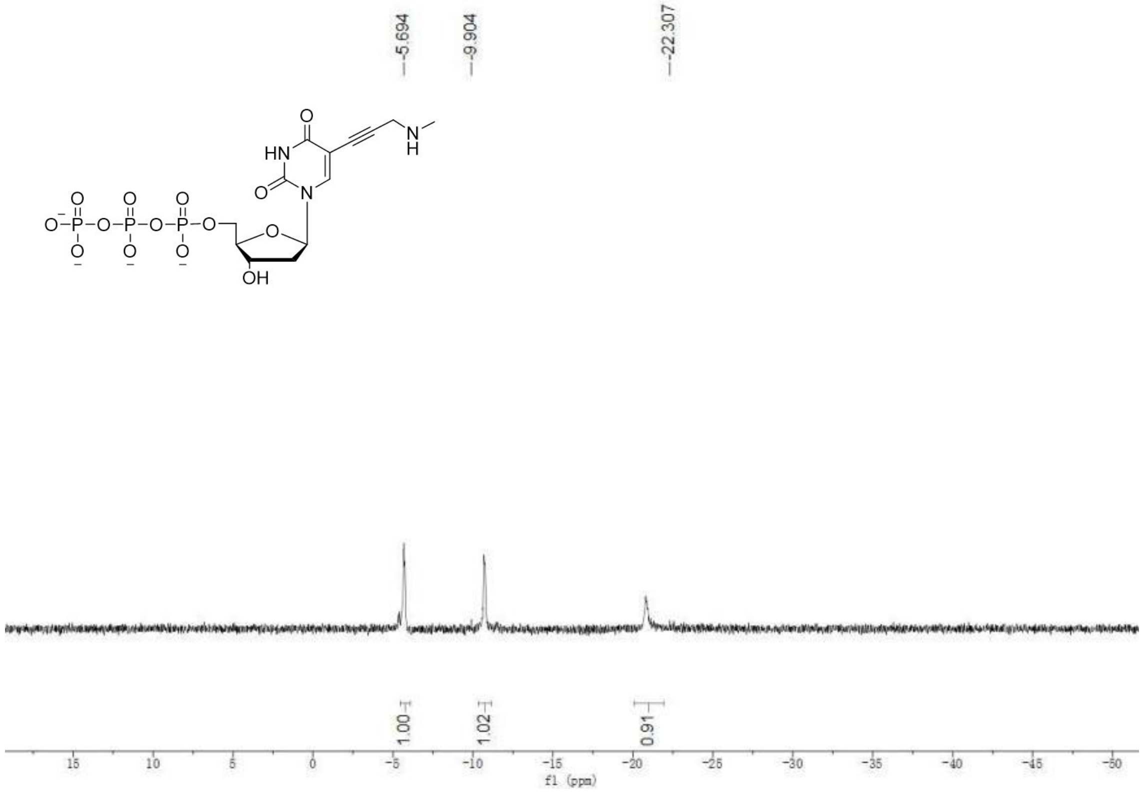
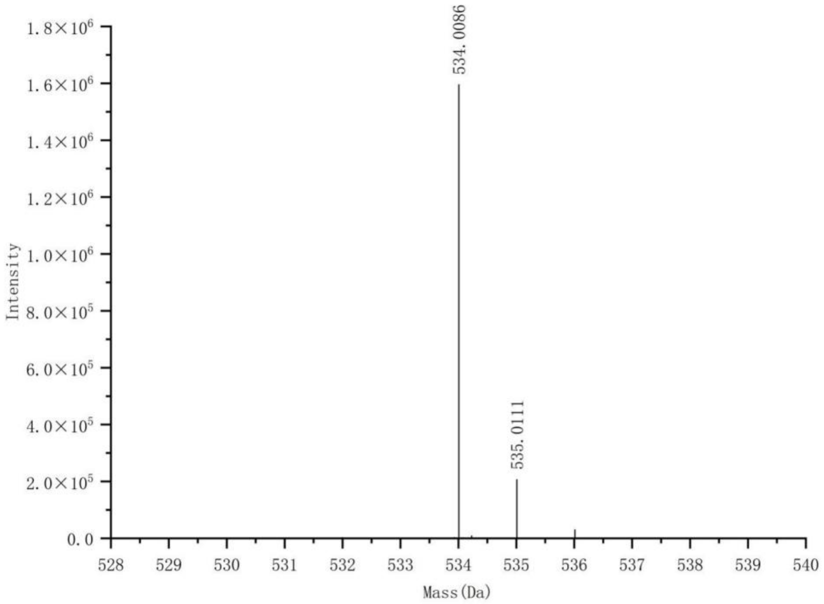


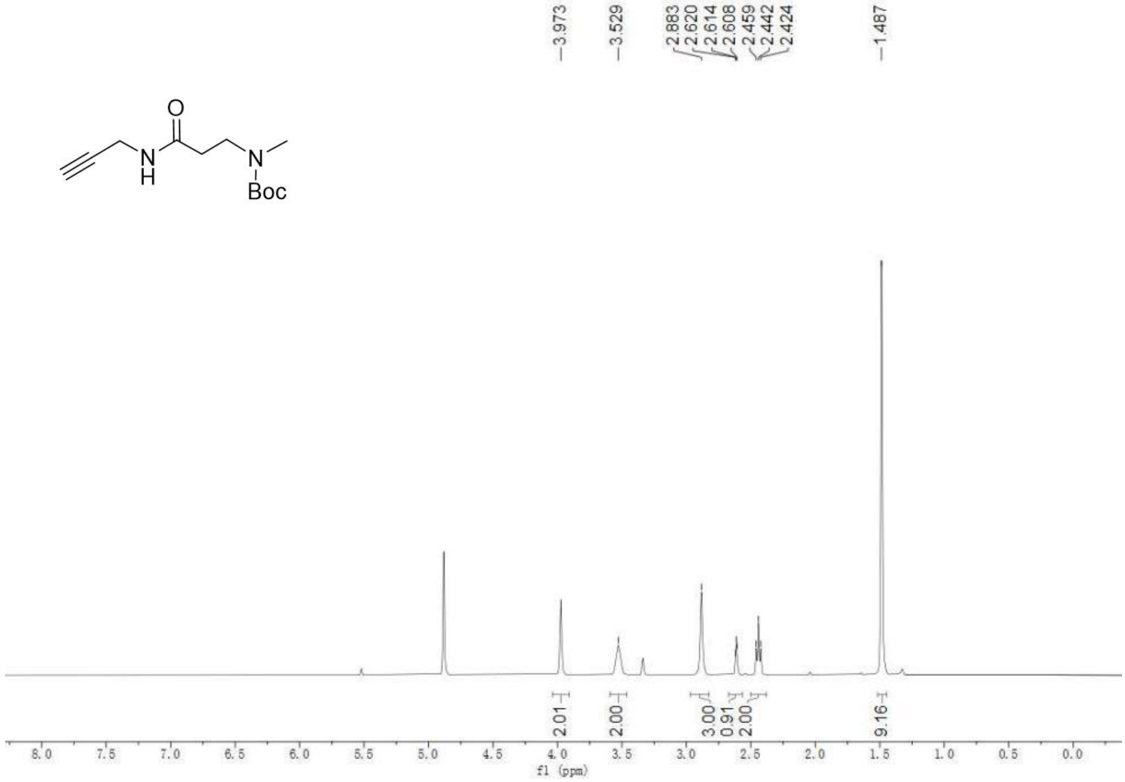
^{1}H NMR spectrum of the compound 3e in D_2O



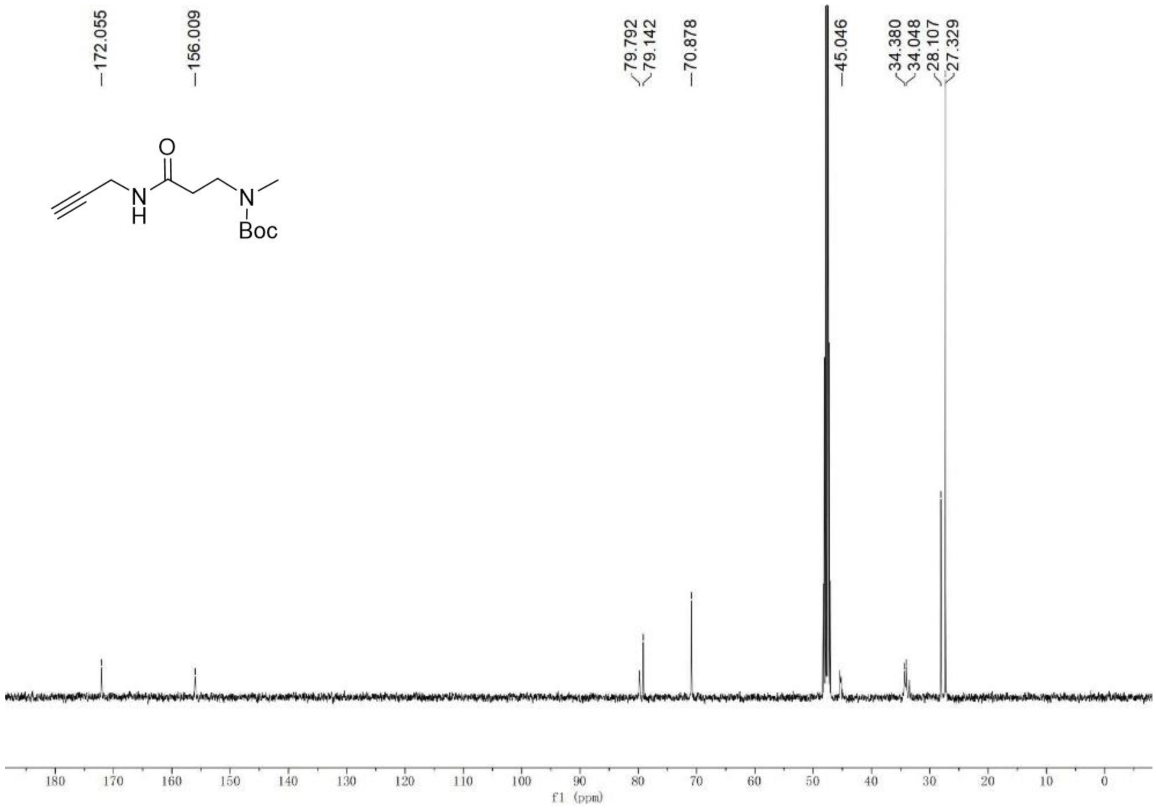
^{31}P NMR spectrum of the compound 3e in D_2O



ESI-HRMS spectrum of the compound 3e



^{1}H NMR spectrum of the compound 5c in CD_3OD



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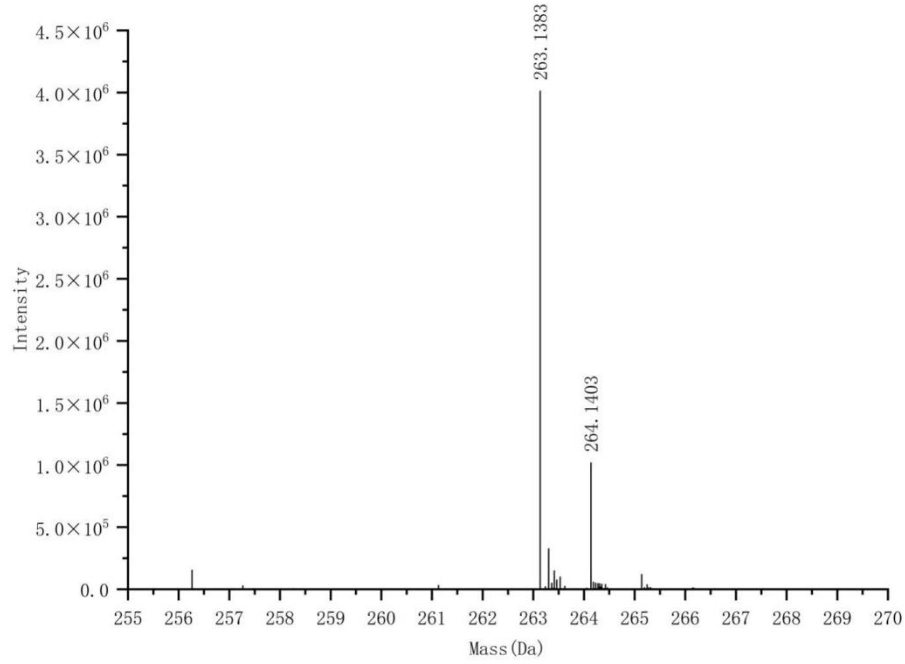
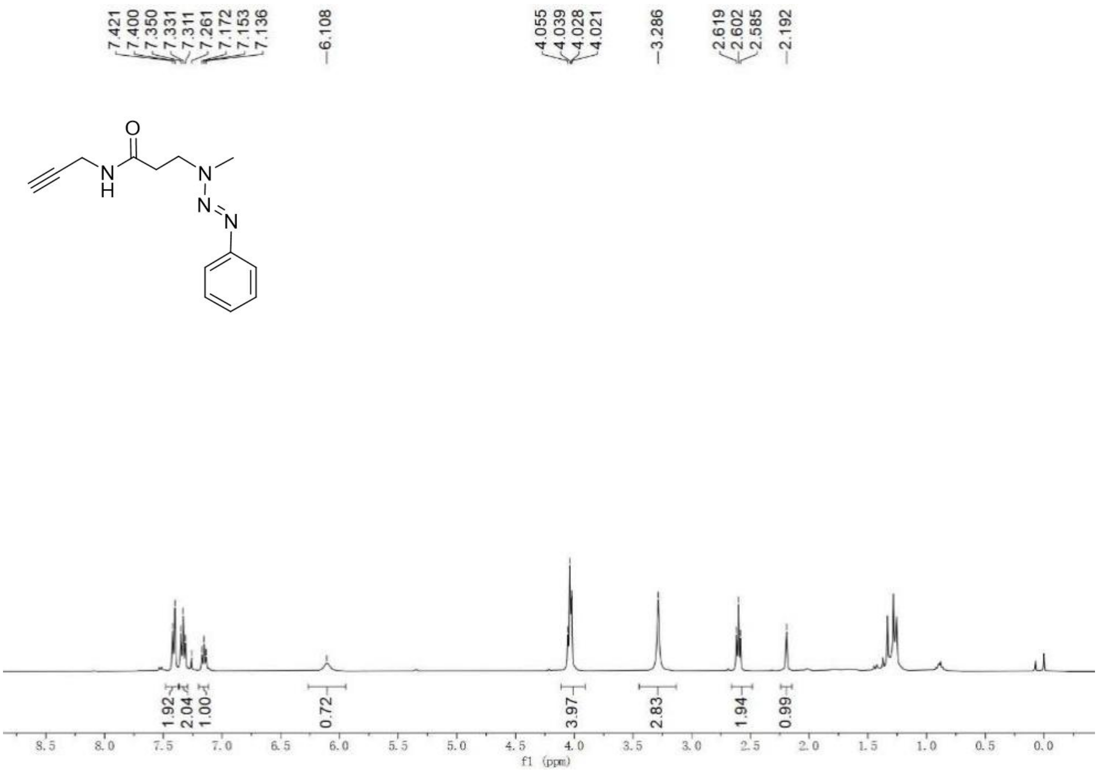
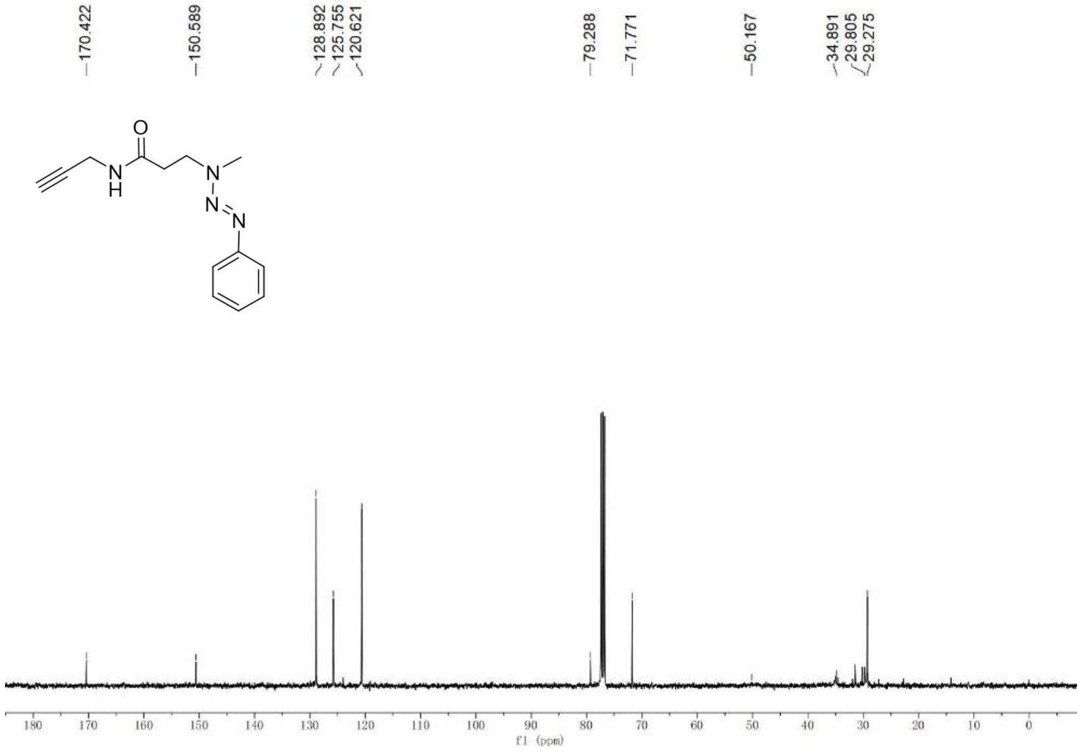


ESI-HRMS spectrum of the compound 5c



^{1}H NMR spectrum of the compound 5 in $CDCl_3$



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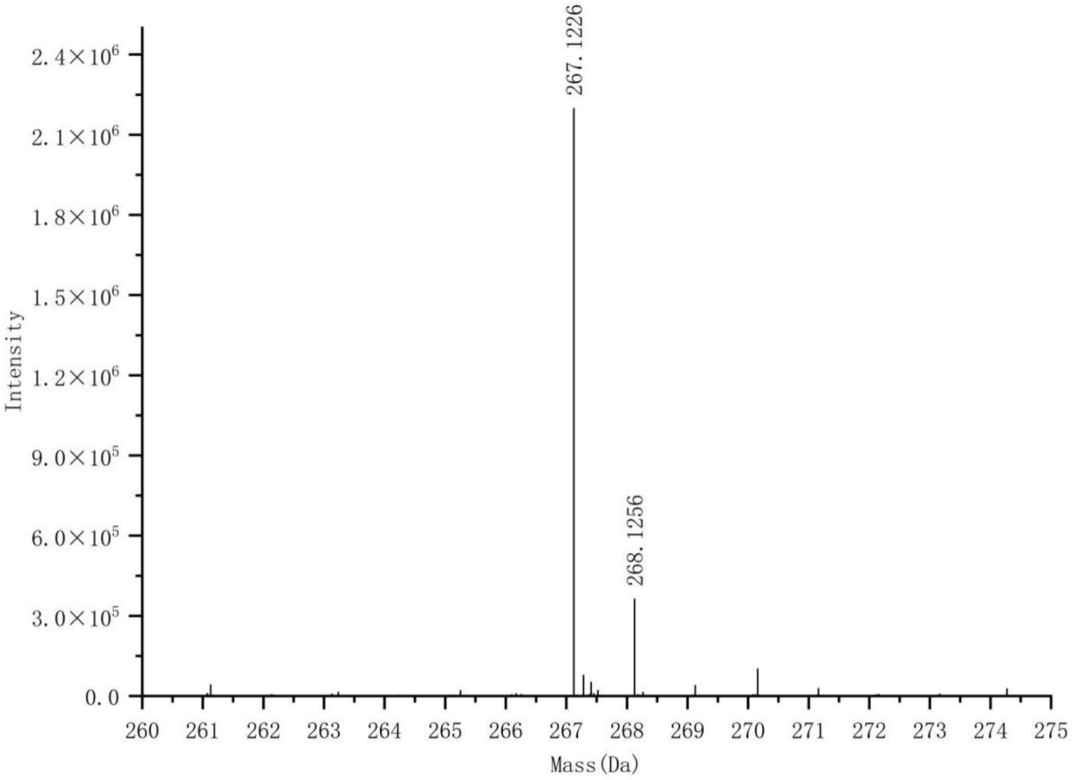
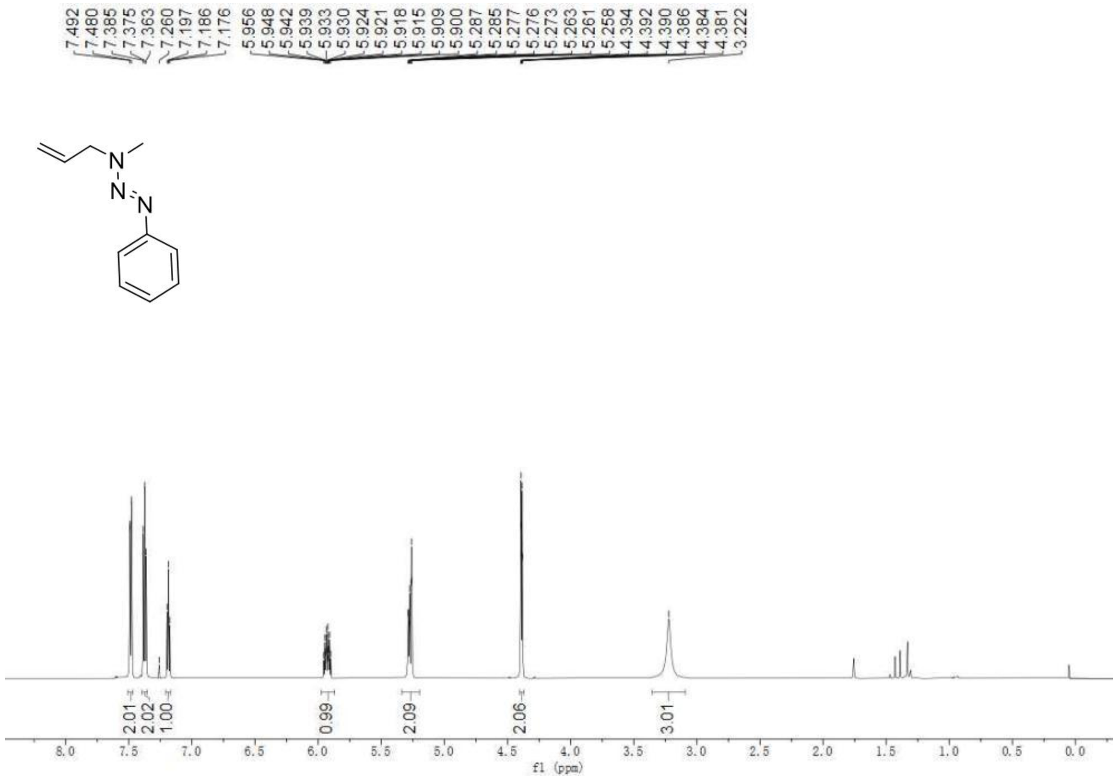
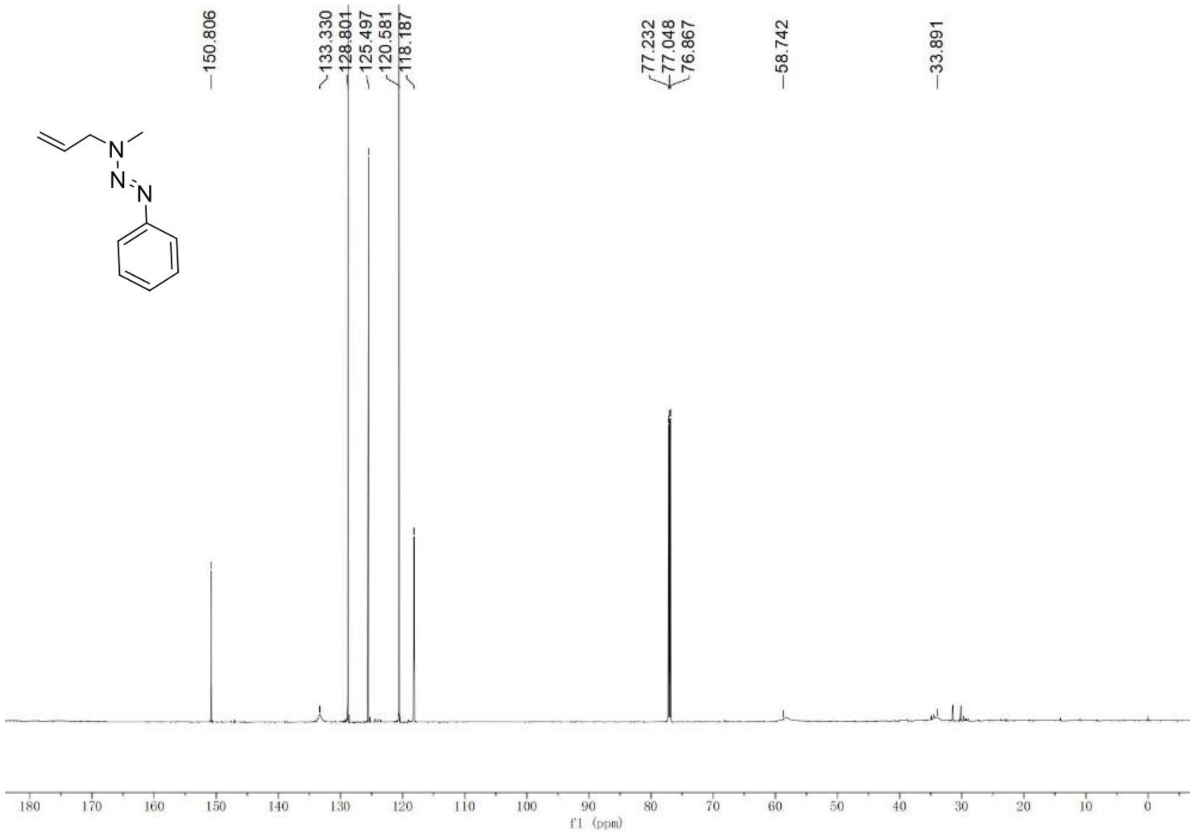


ESI-HRMS spectrum of the compound 5



^{1}H NMR spectrum of the compound 6 in $CDCl_3$



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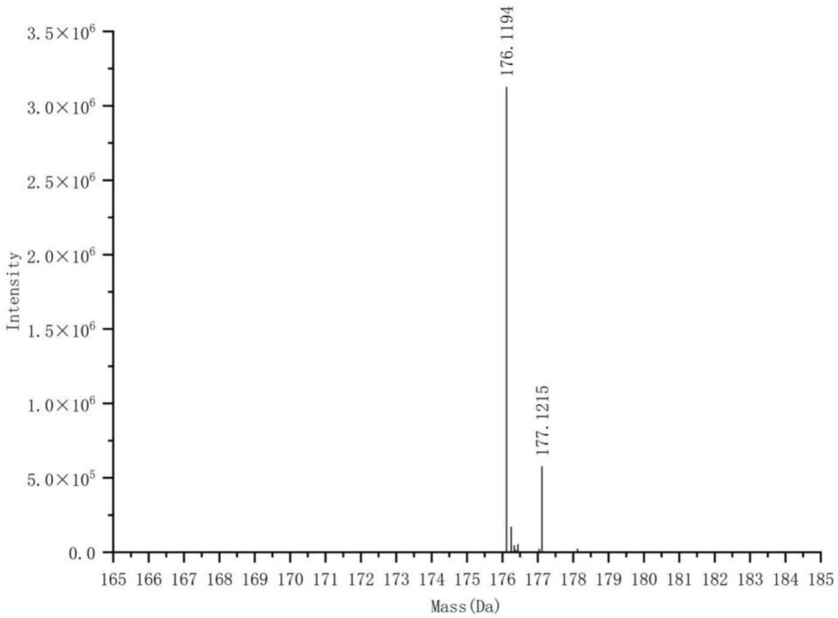
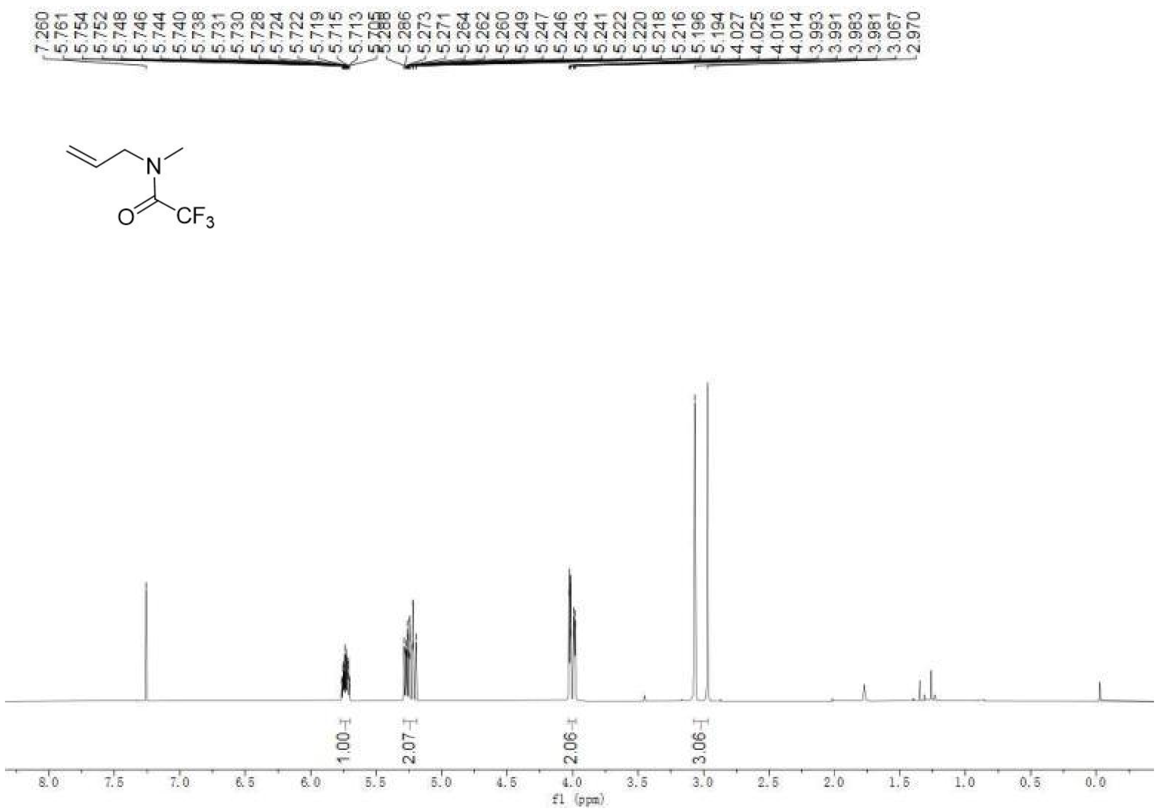
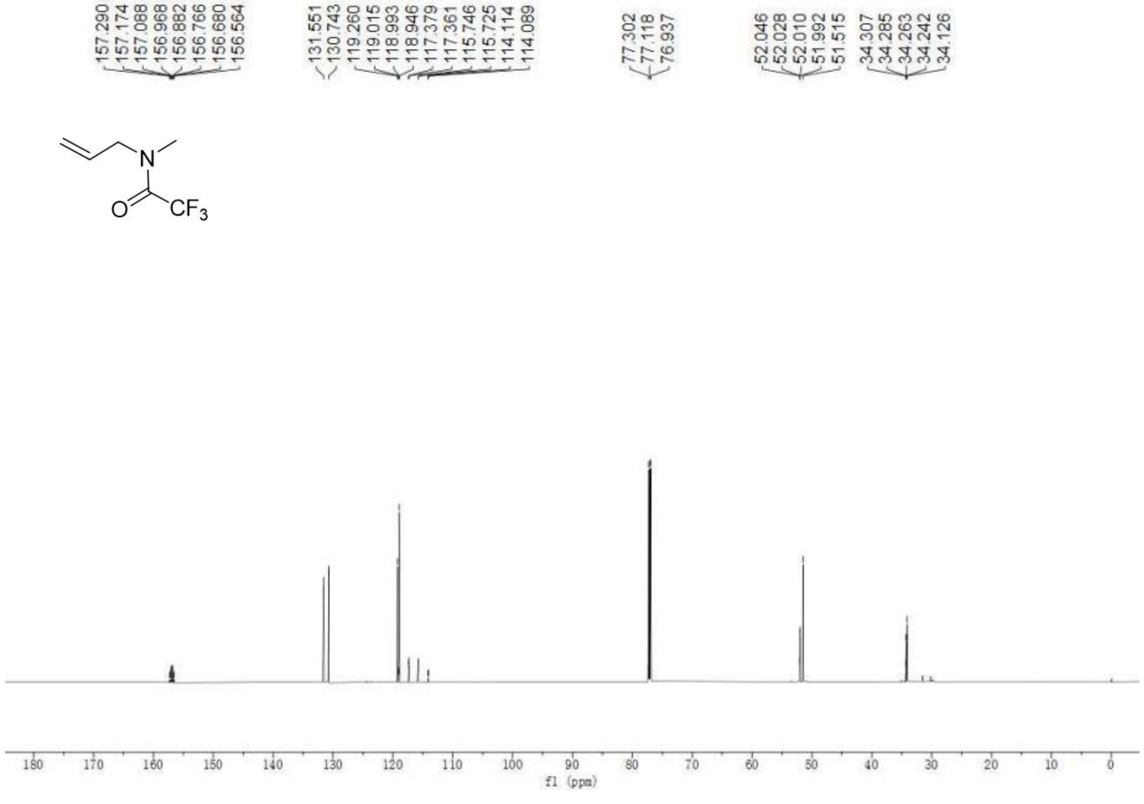


ESI-HRMS spectrum of the compound 6



^{1}H NMR spectrum of the compound 7a in CDCl$_3$



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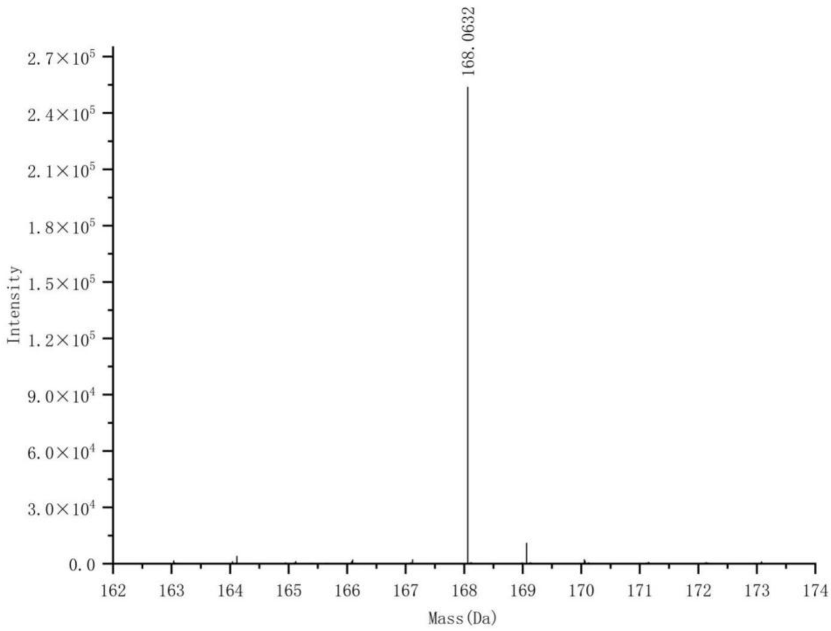
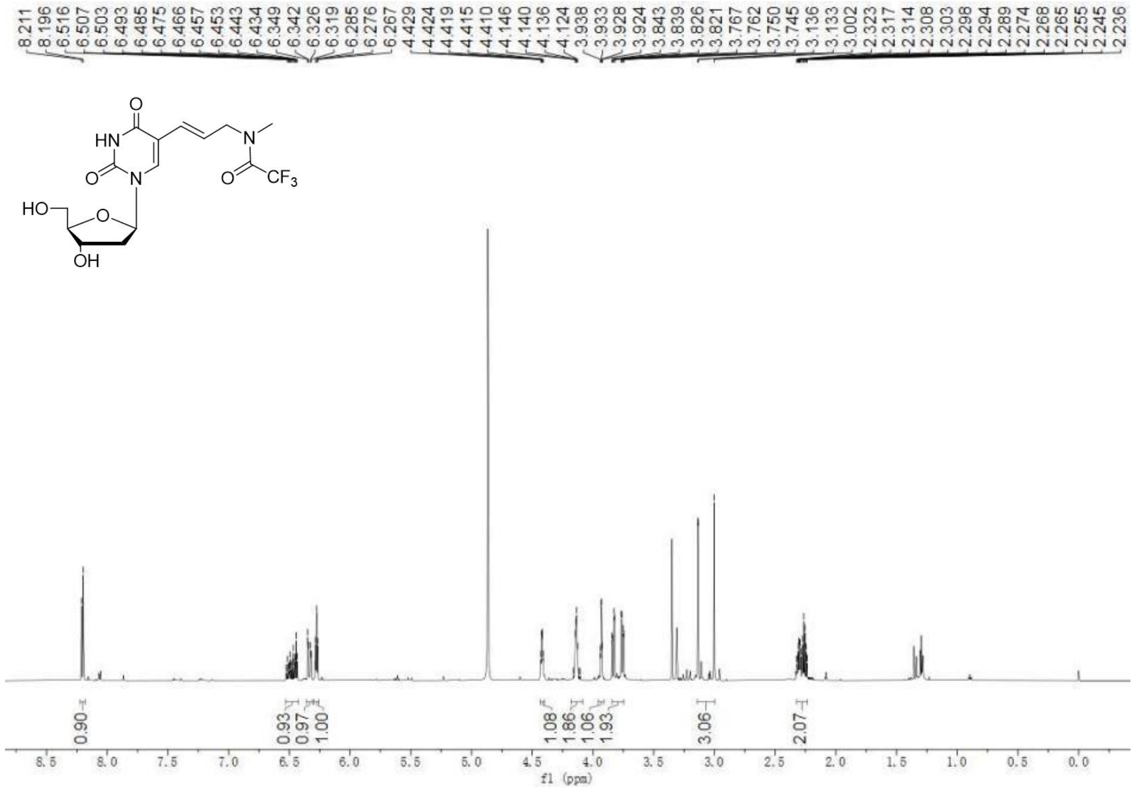
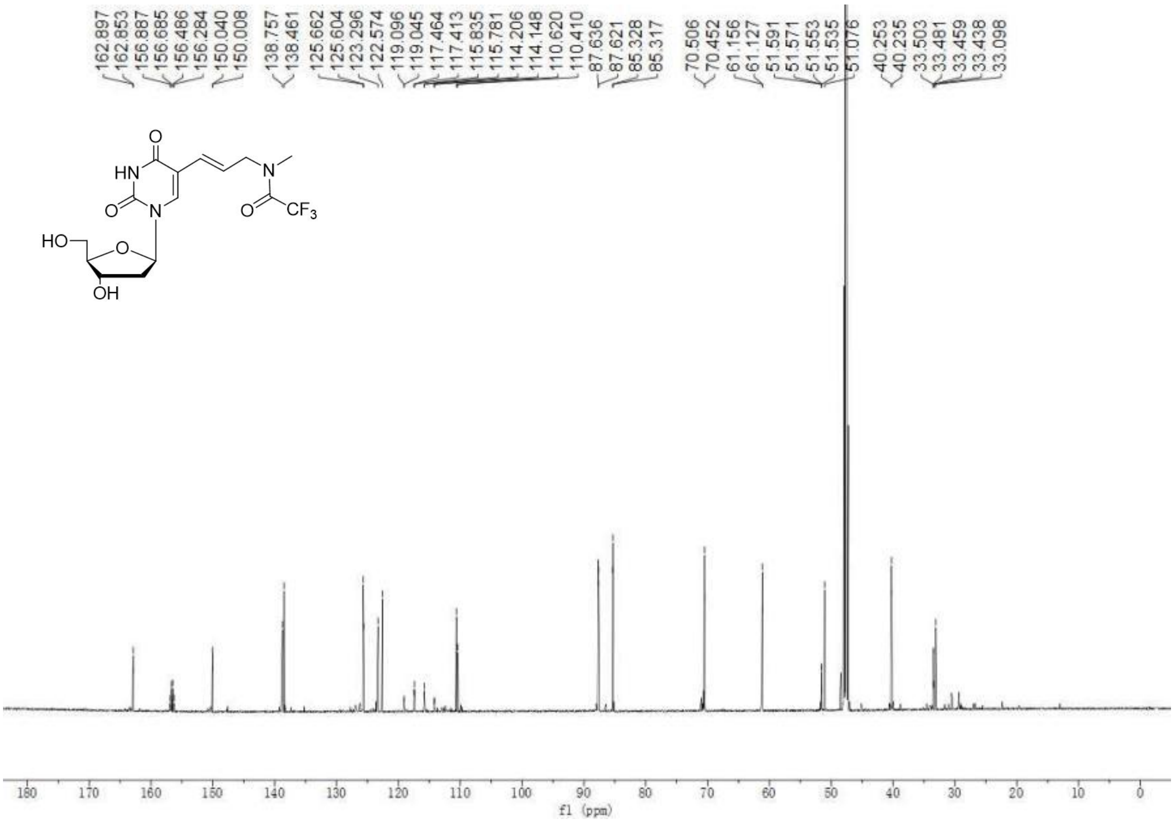


ESI-HRMS spectrum of the compound 7*a*



^{1}H NMR spectrum of the compound 7c in CD_3OD



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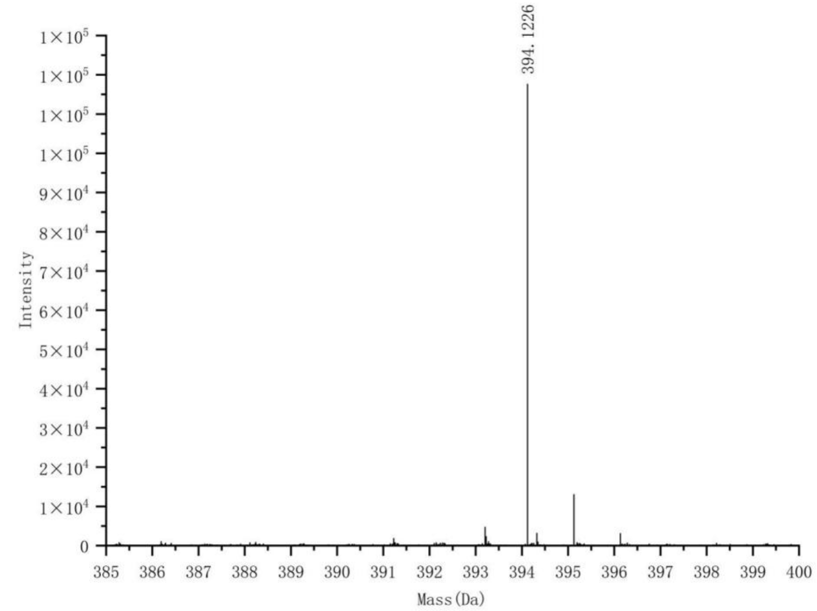
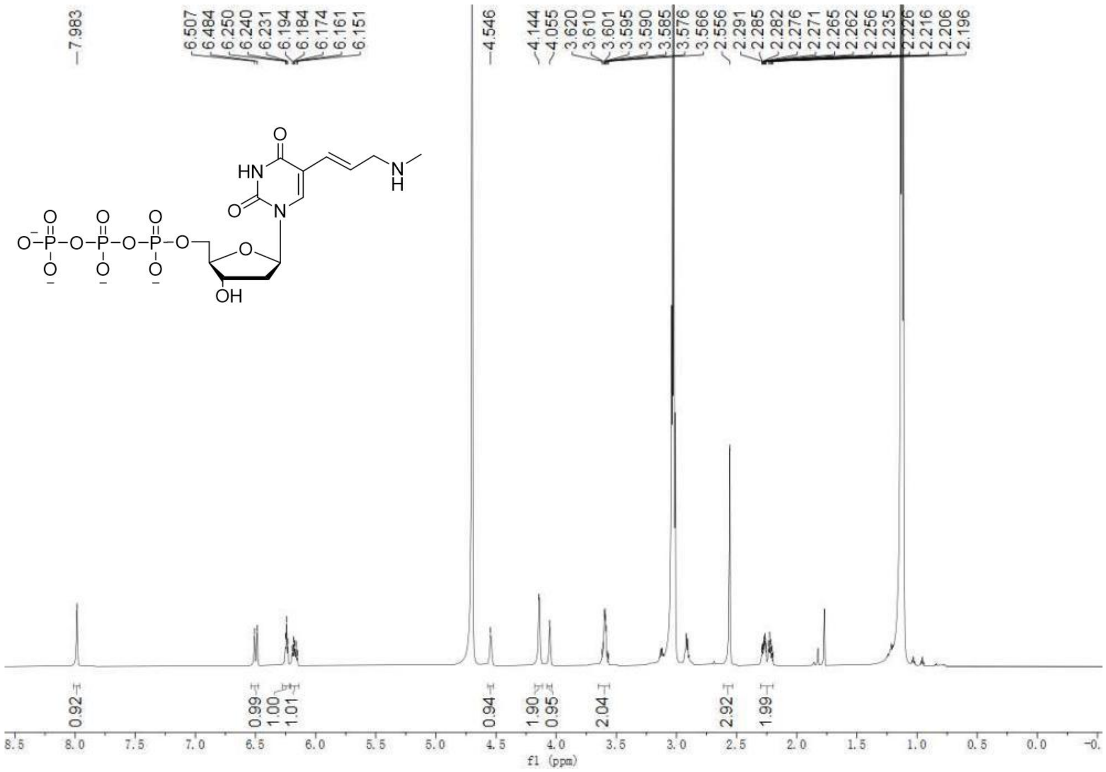
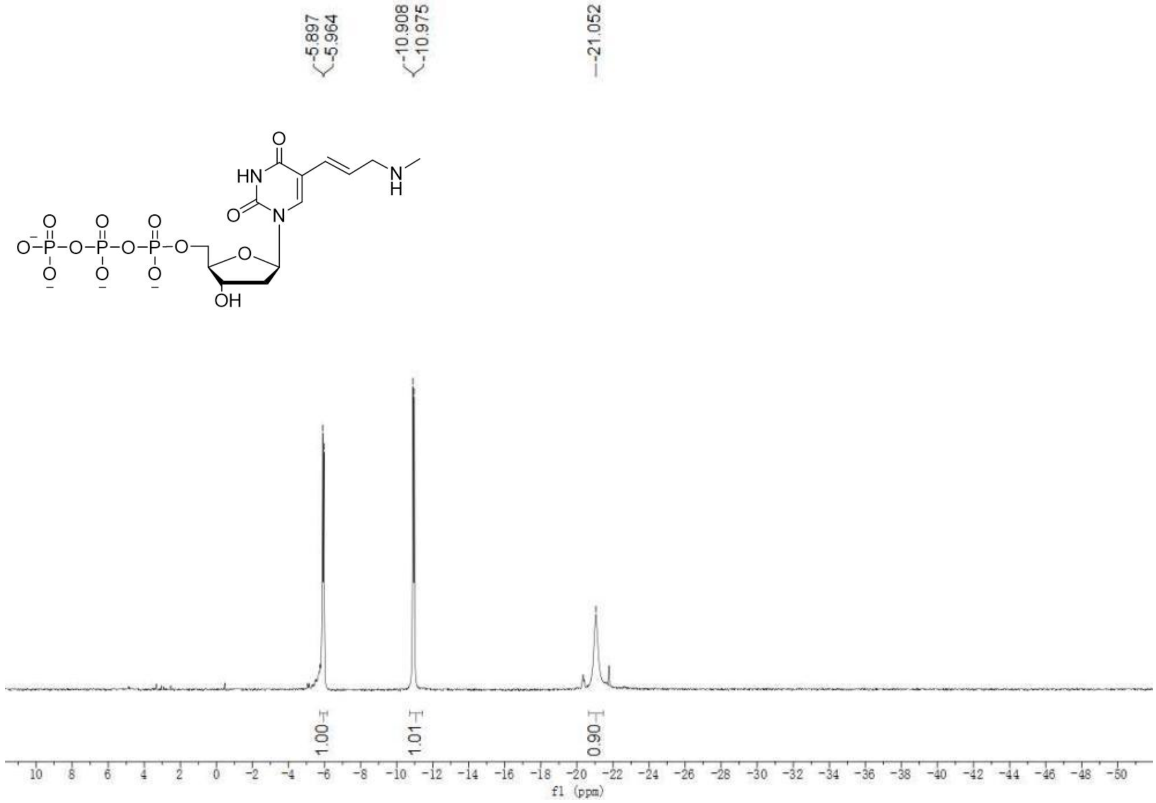


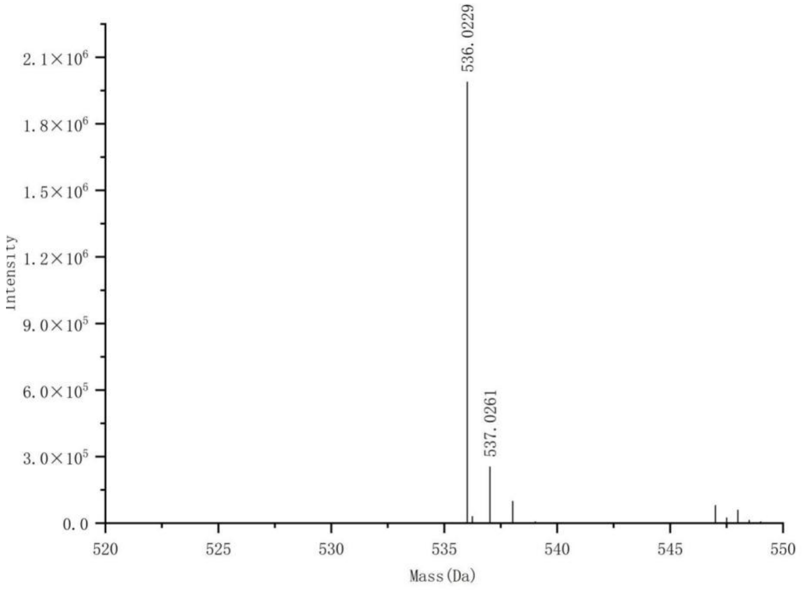
ESI-HRMS spectrum of the compound 7c



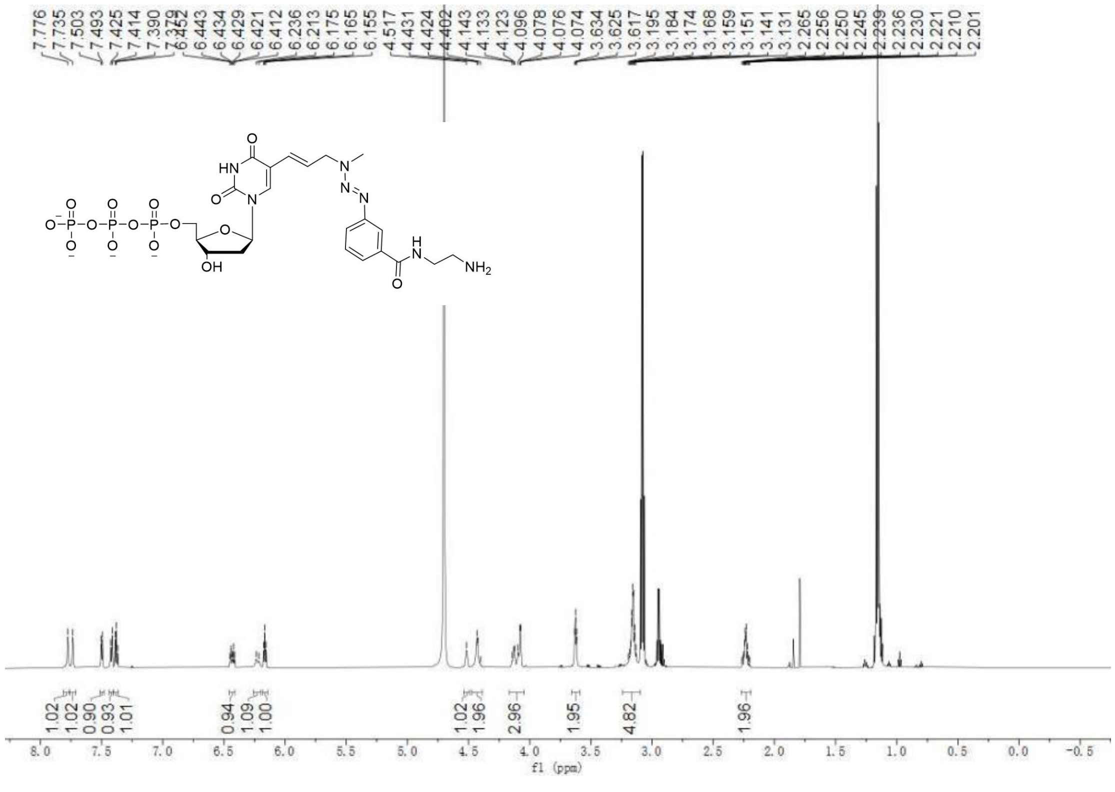
^{1}H NMR spectrum of the compound 7d in D_2O



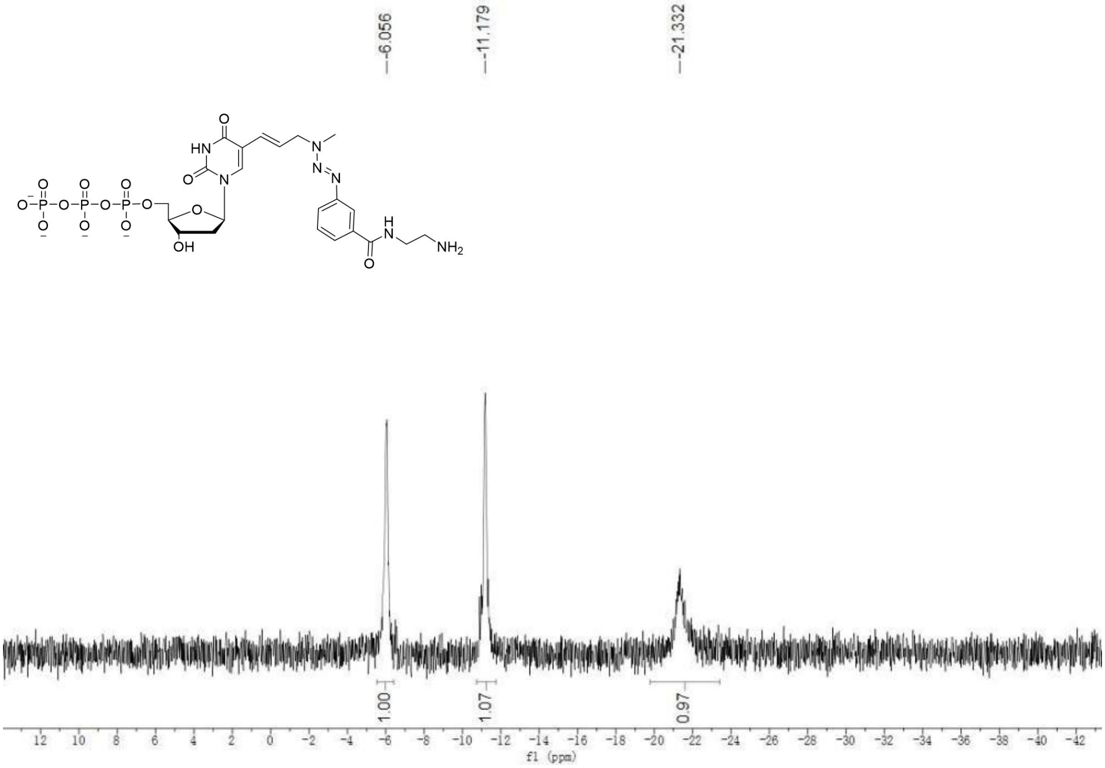
^{31}P NMR spectrum of the compound 7d in D_2O



ESI-HRMS spectrum of the compound 7d



^{1}H NMR spectrum of the compound 7 in D_2O



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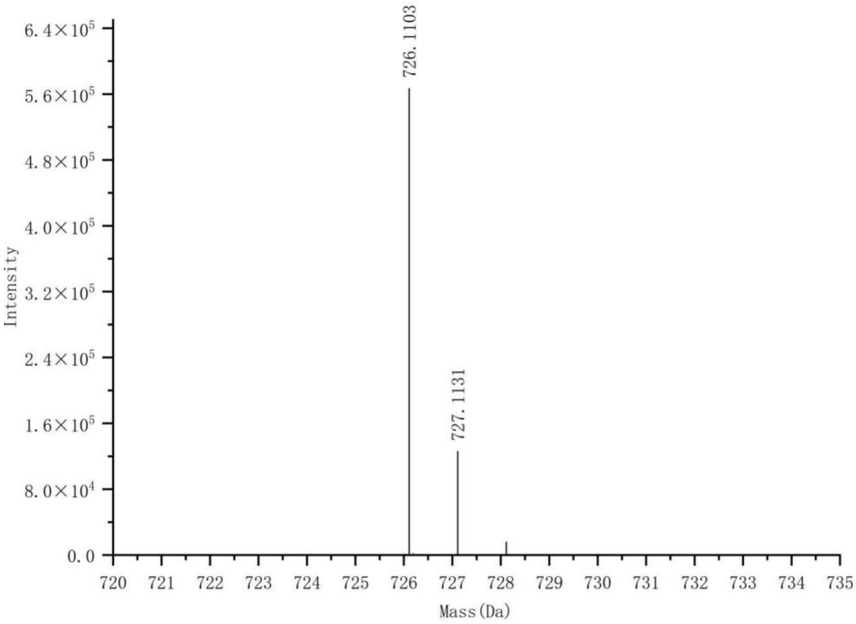
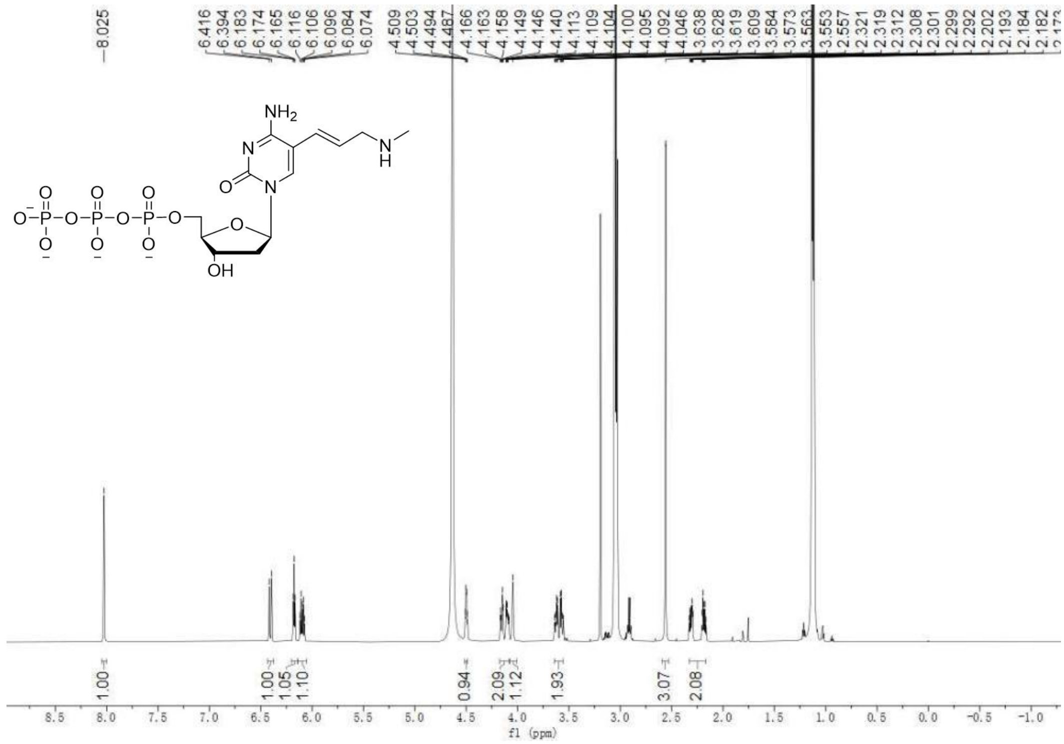


ESI-HRMS spectrum of the compound 7



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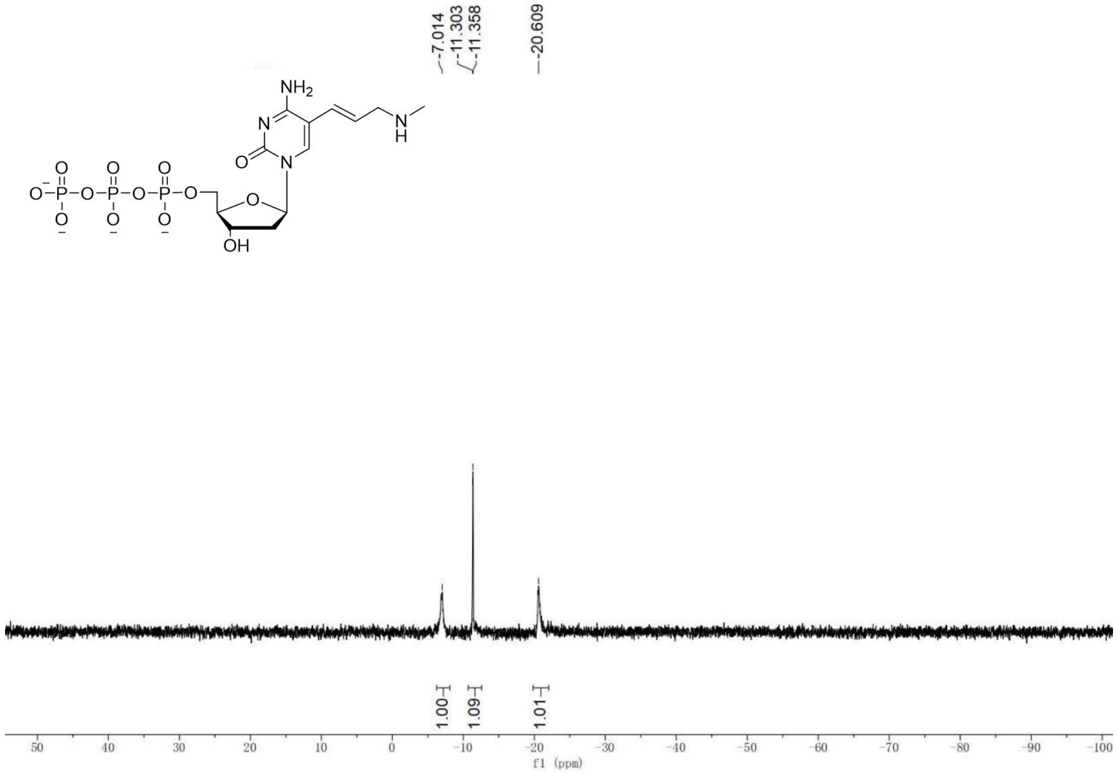
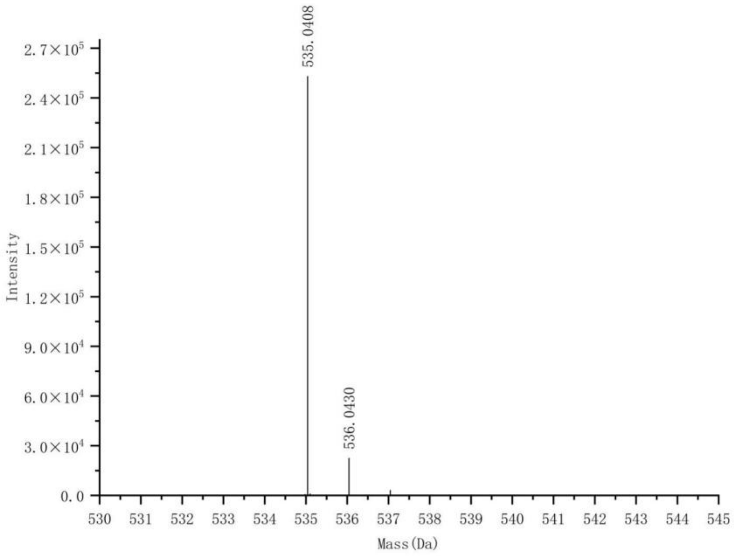
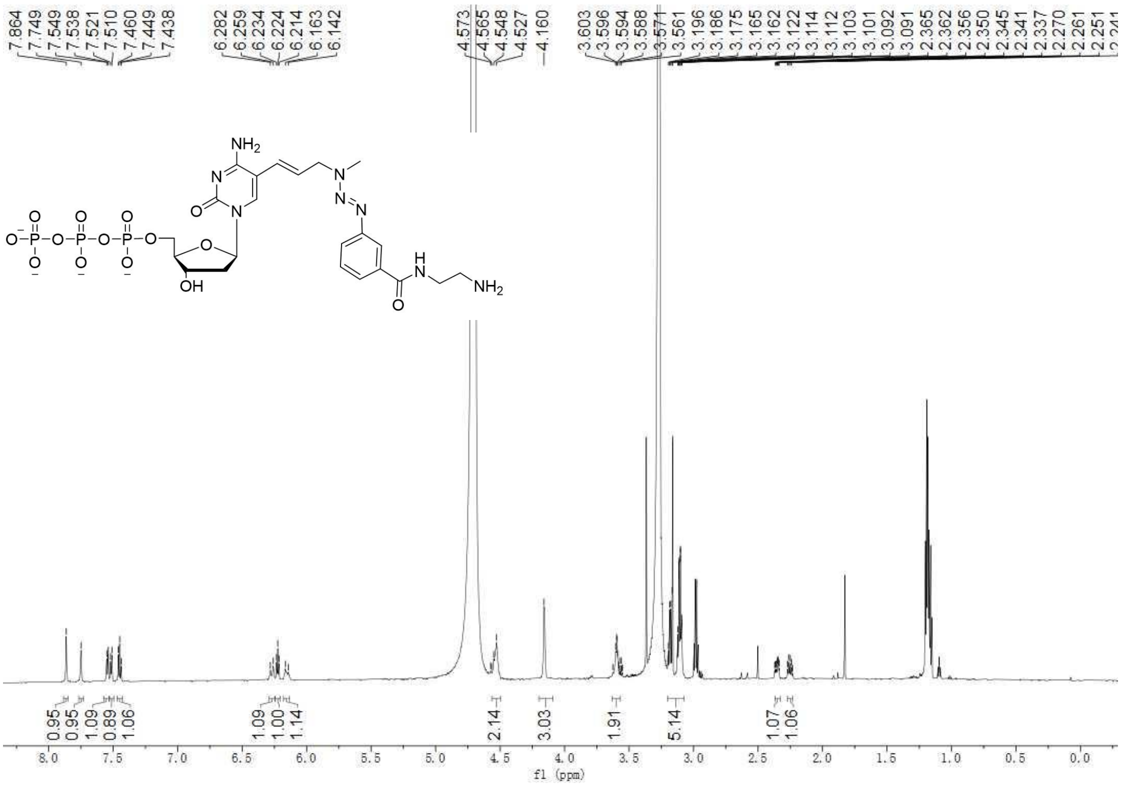


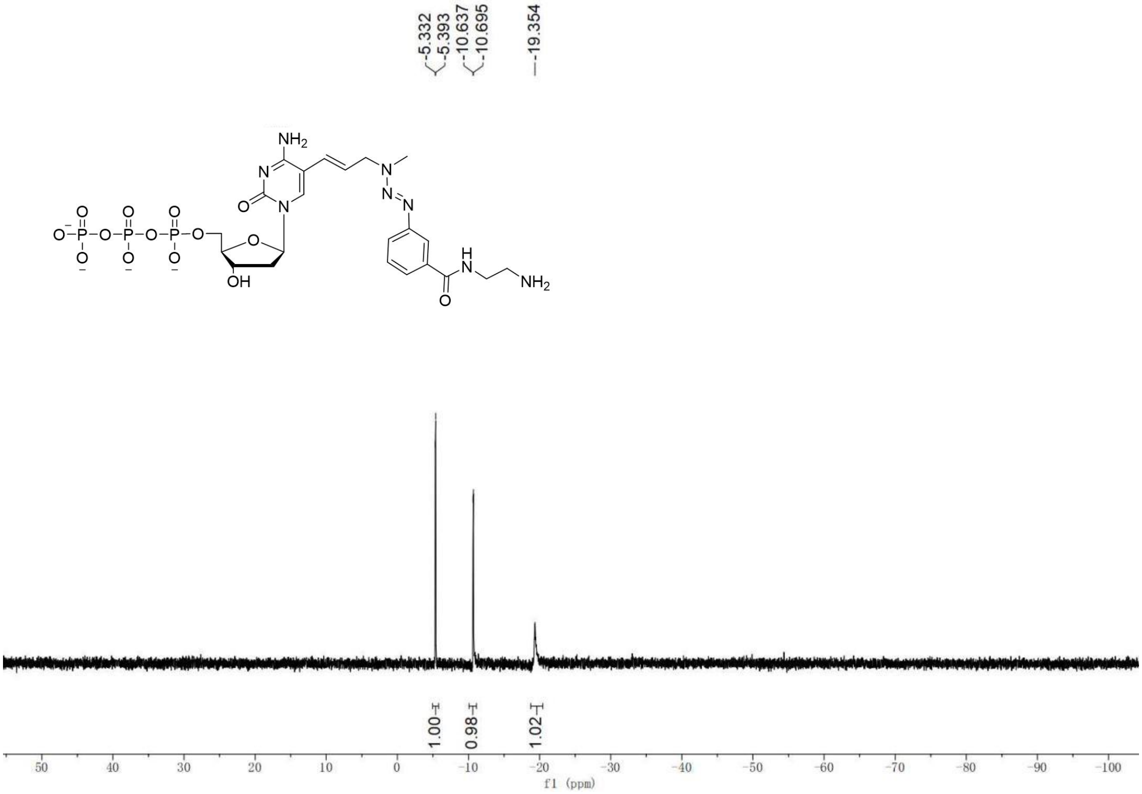
^{31}P NMR spectrum of the compound 8c in D_2O



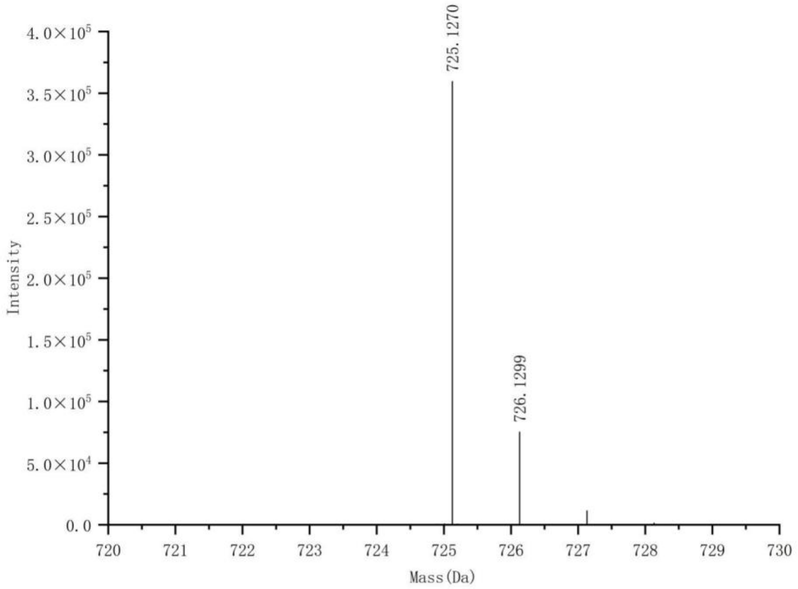
ESI-HRMS spectrum of the compound 8c



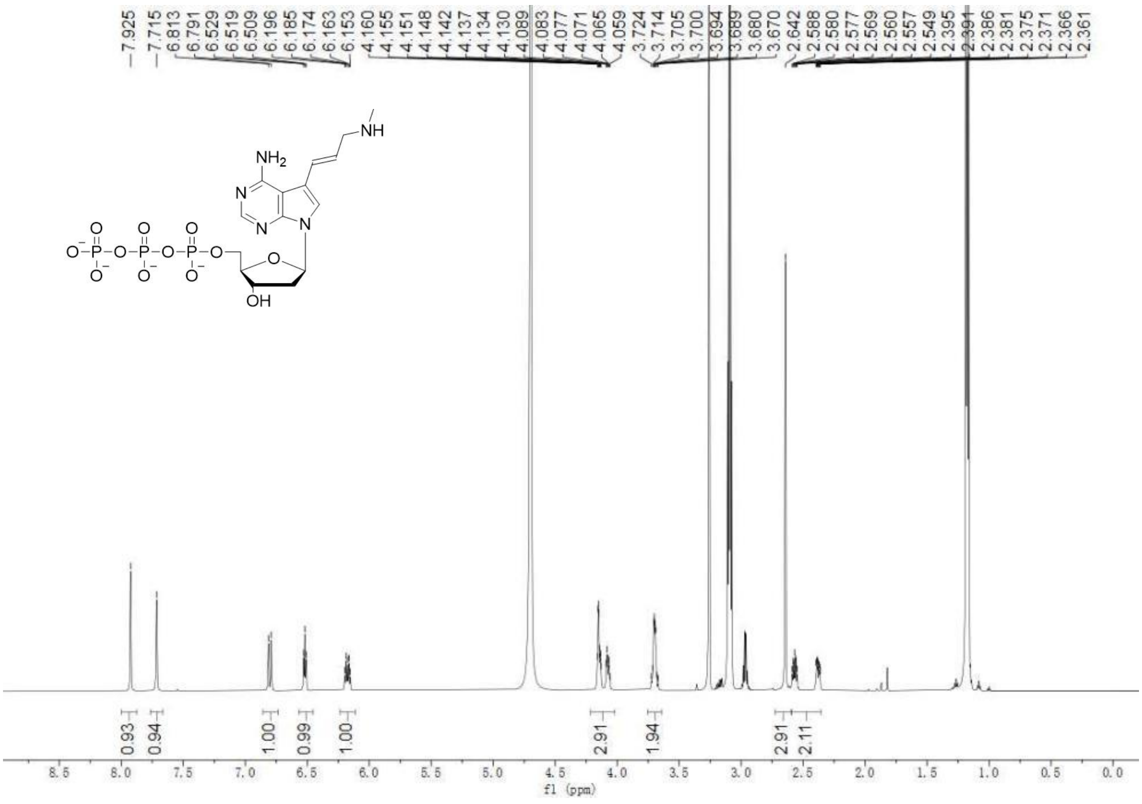
^{1}H NMR spectrum of the compound 8 in D_2O



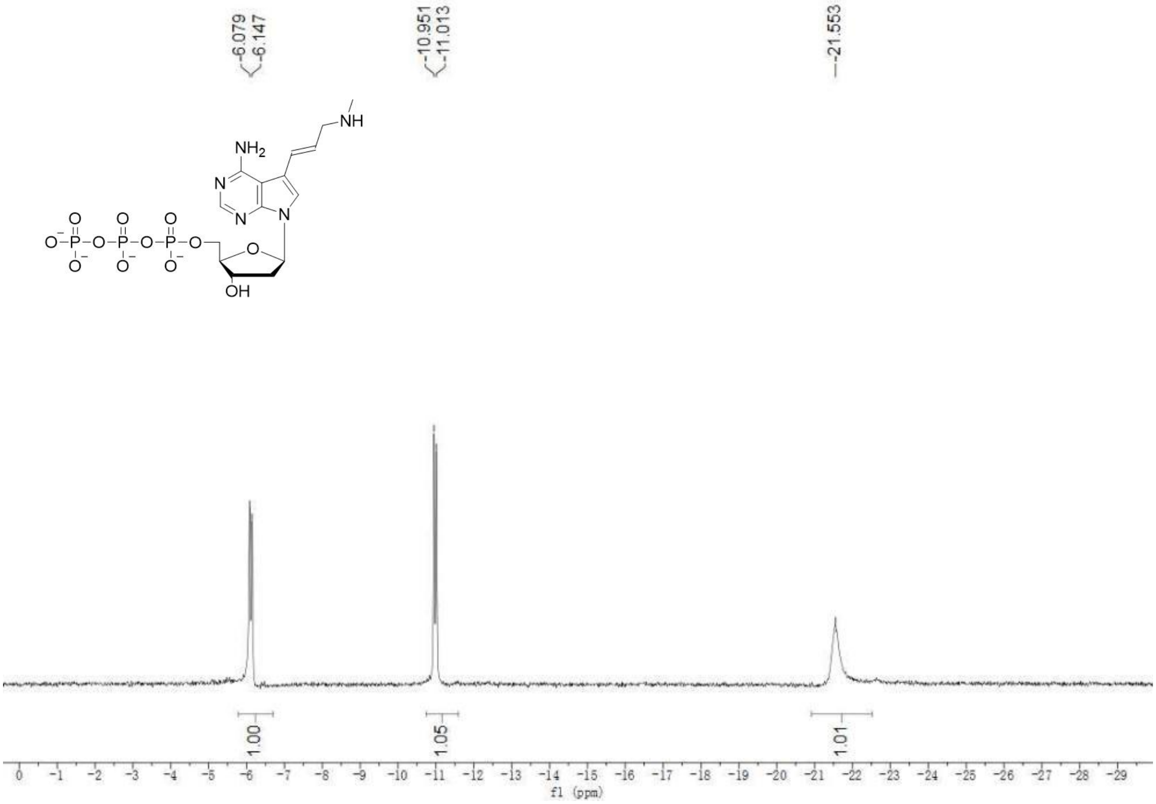
^{31}P NMR spectrum of the compound 8 in D_2O



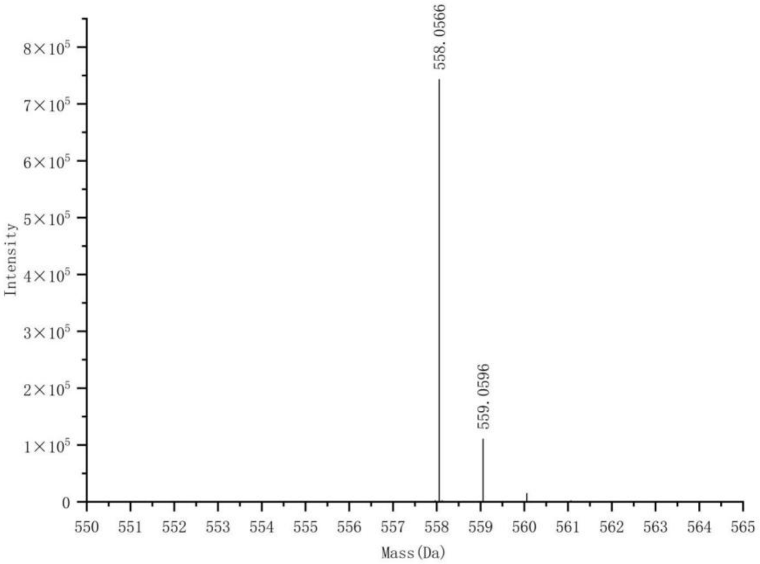
ESI-HRMS spectrum of the compound 8



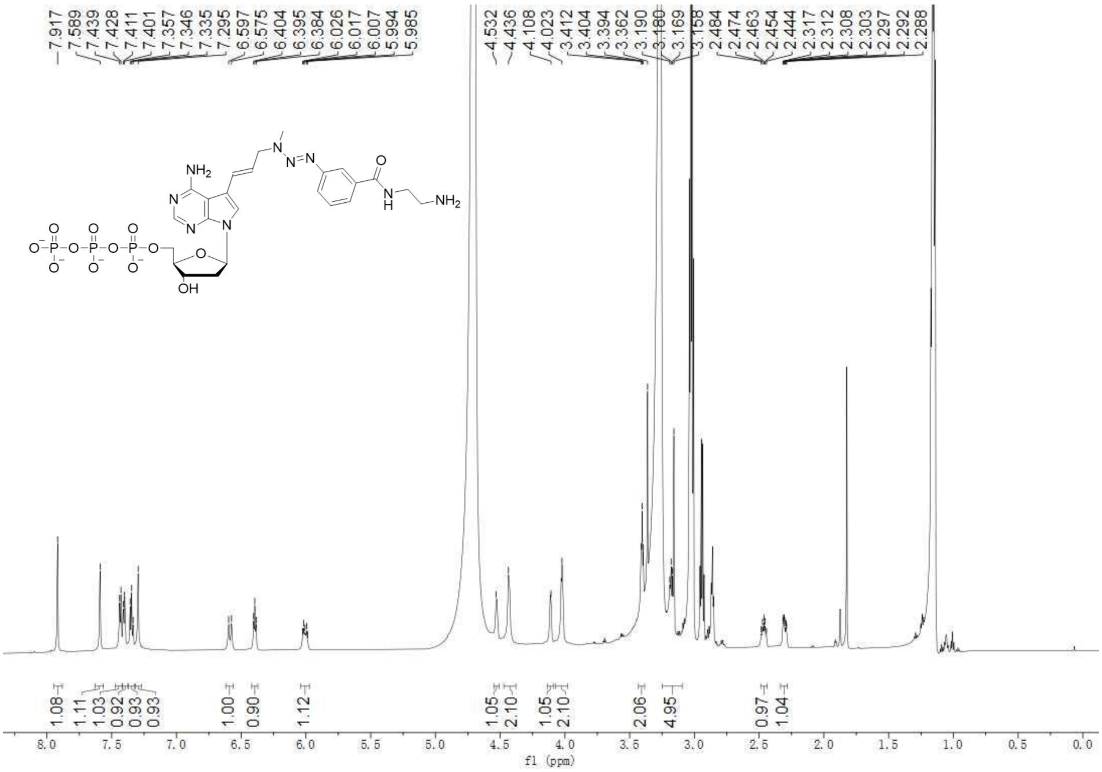
^{1}H NMR spectrum of the compound 9c in D_2O



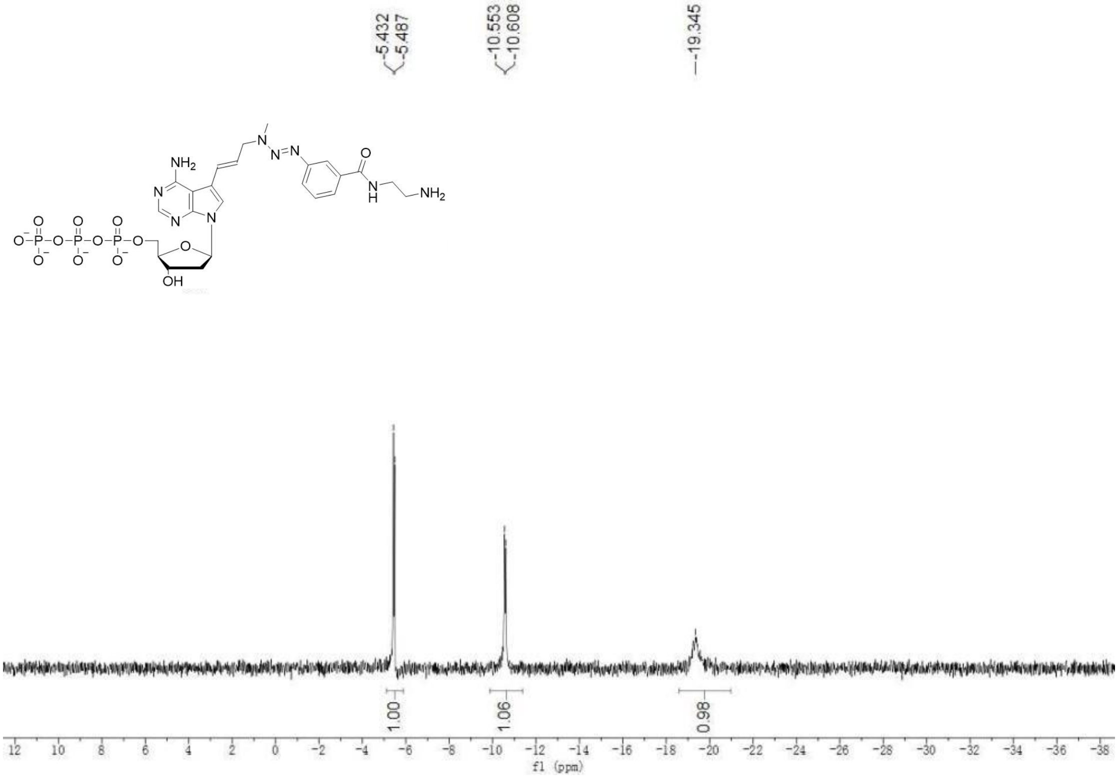
^{31}P NMR spectrum of the compound 9c in D_2O



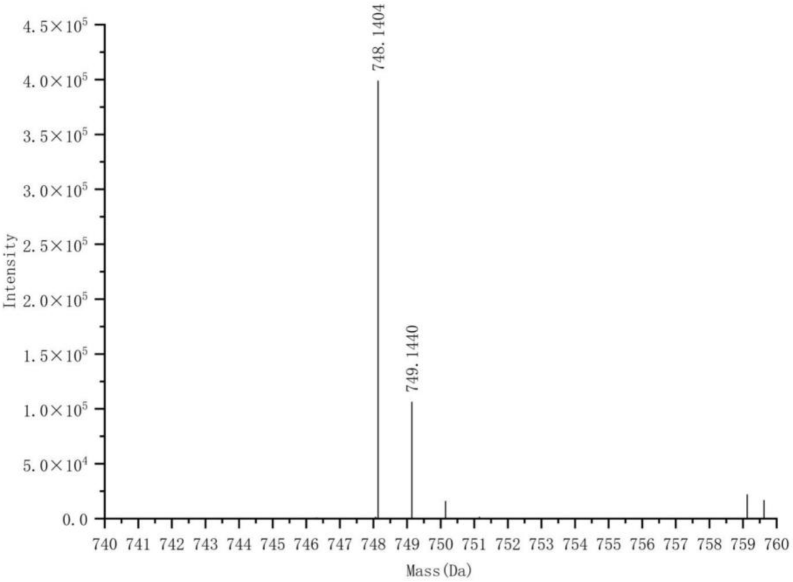
ESI-HRMS spectrum of the compound 9c



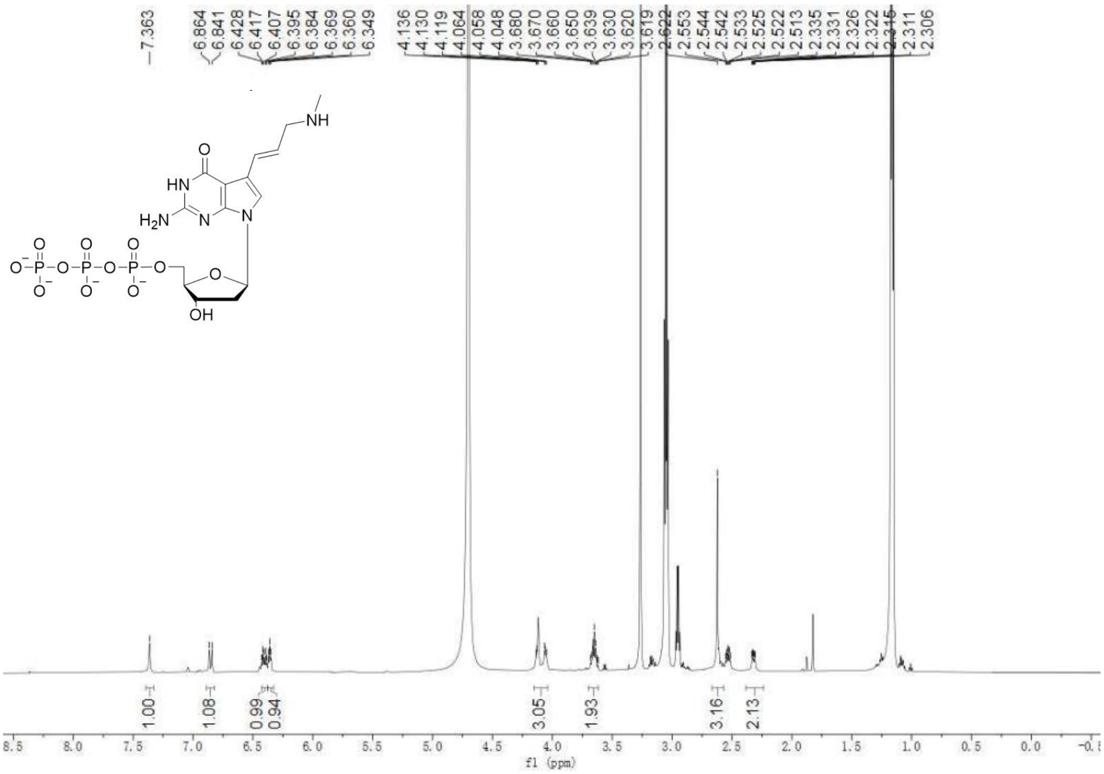
^{1}H NMR spectrum of the compound 9 in D_2O



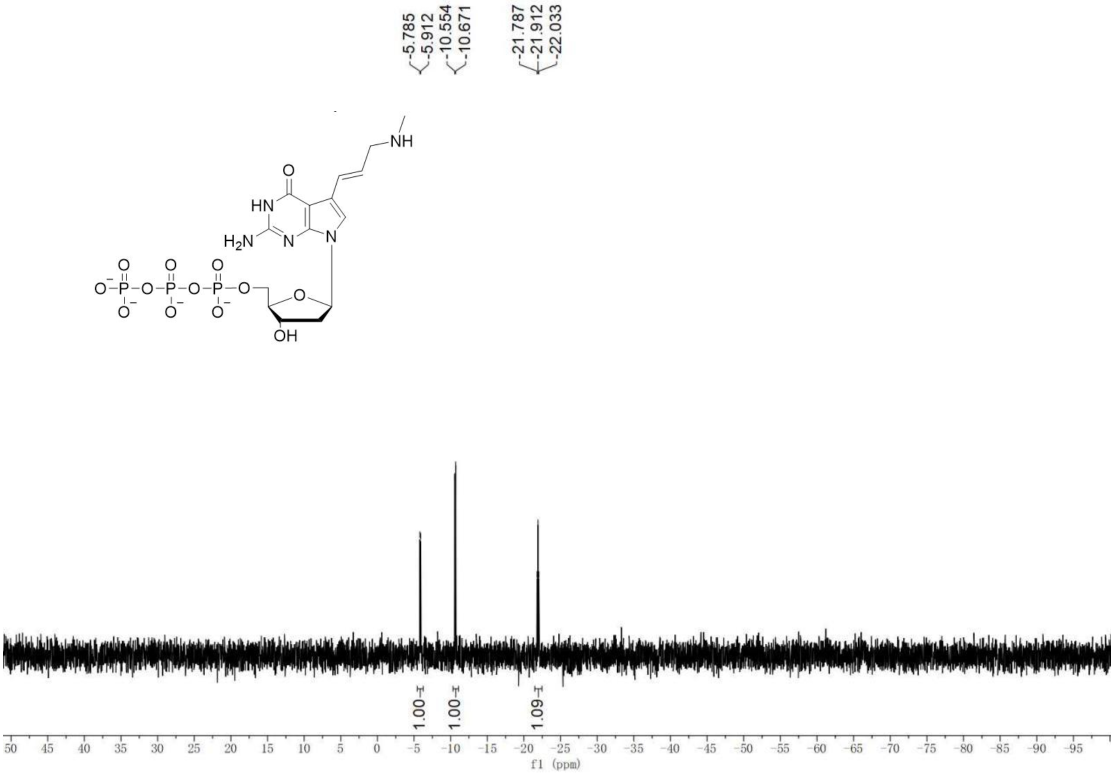
^{31}P NMR spectrum of the compound 9 in D_2O



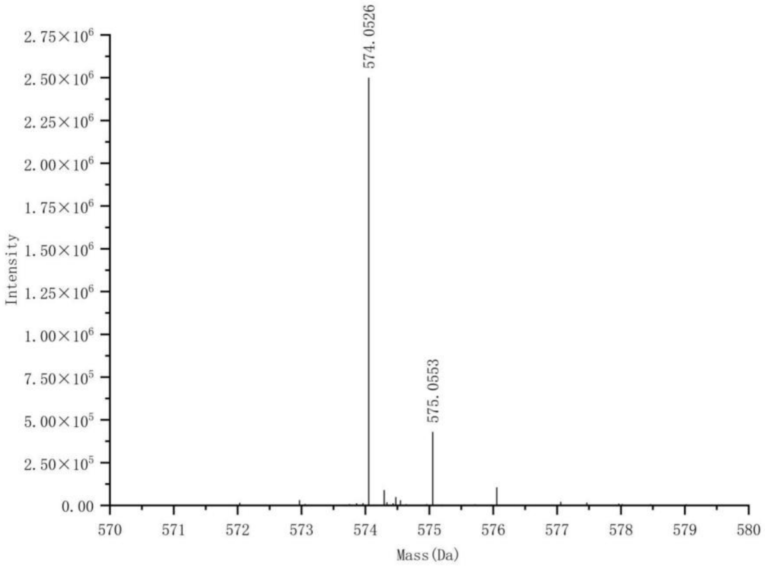
ESI-HRMS spectrum of the compound 9



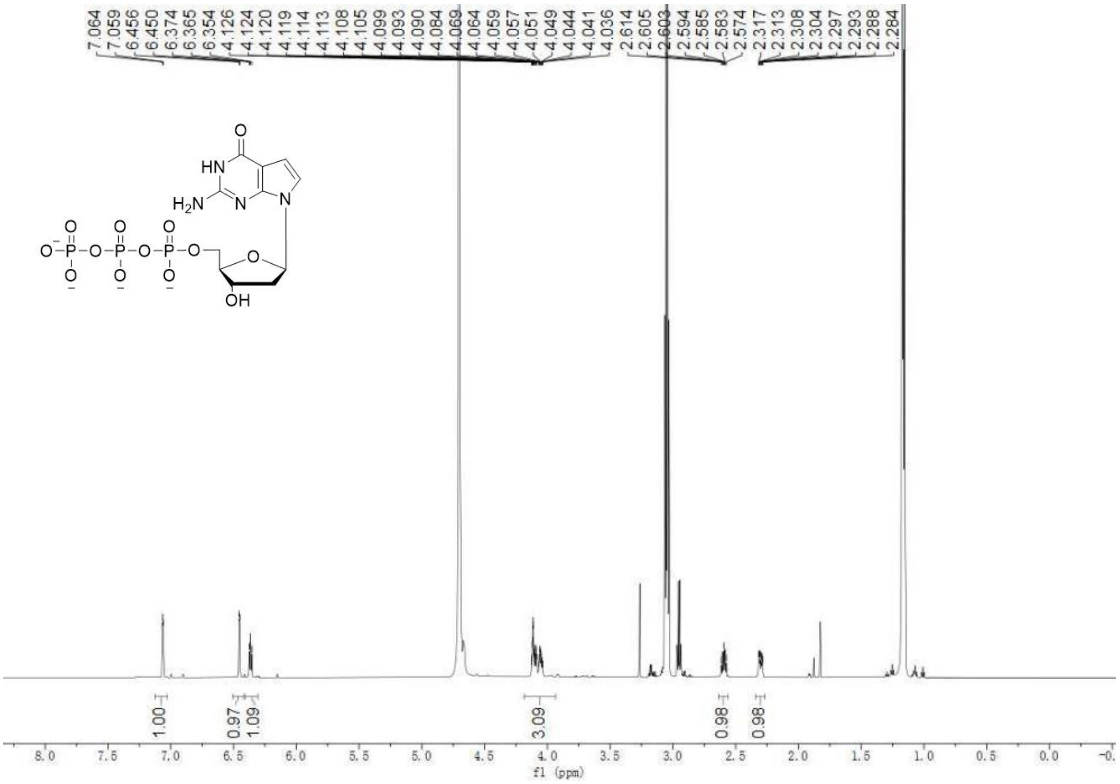
^{1}H NMR spectrum of the compound 10c in D_2O



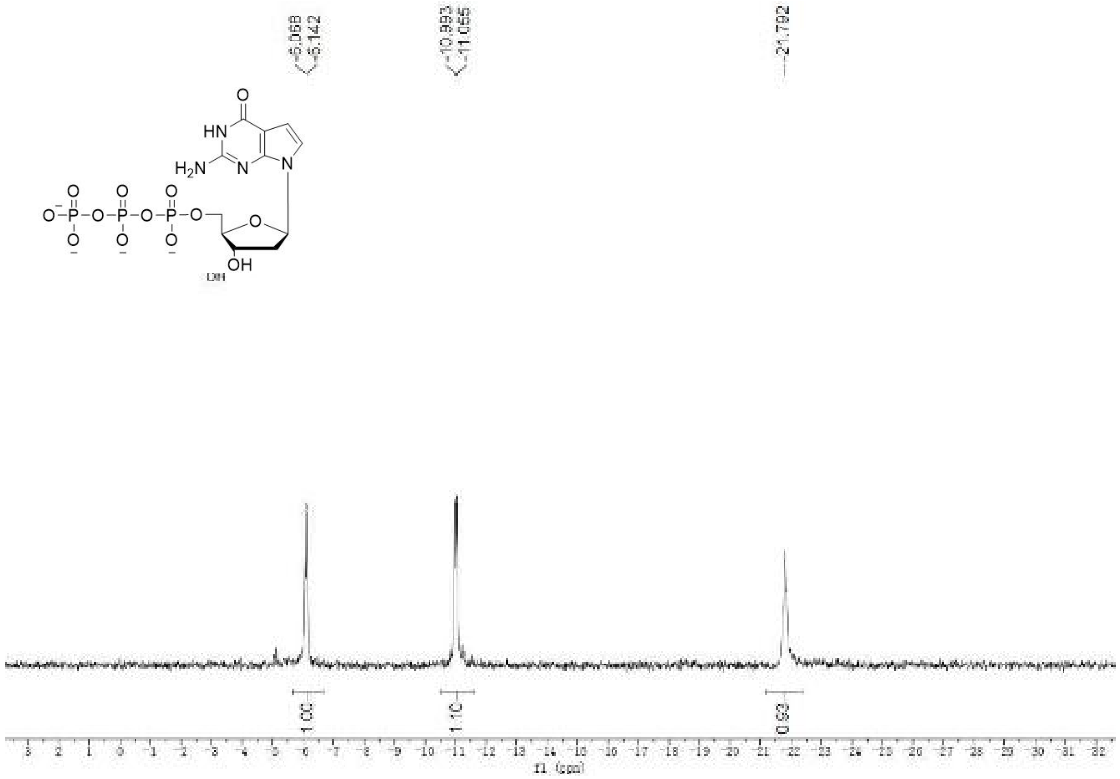
^{31}P NMR spectrum of the compound 10c in D_2O



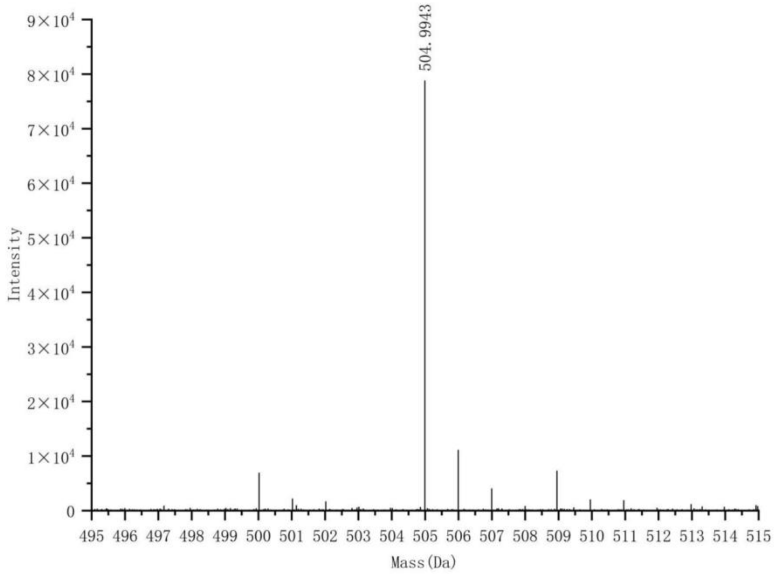
ESI-HRMS spectrum of the compound 10c



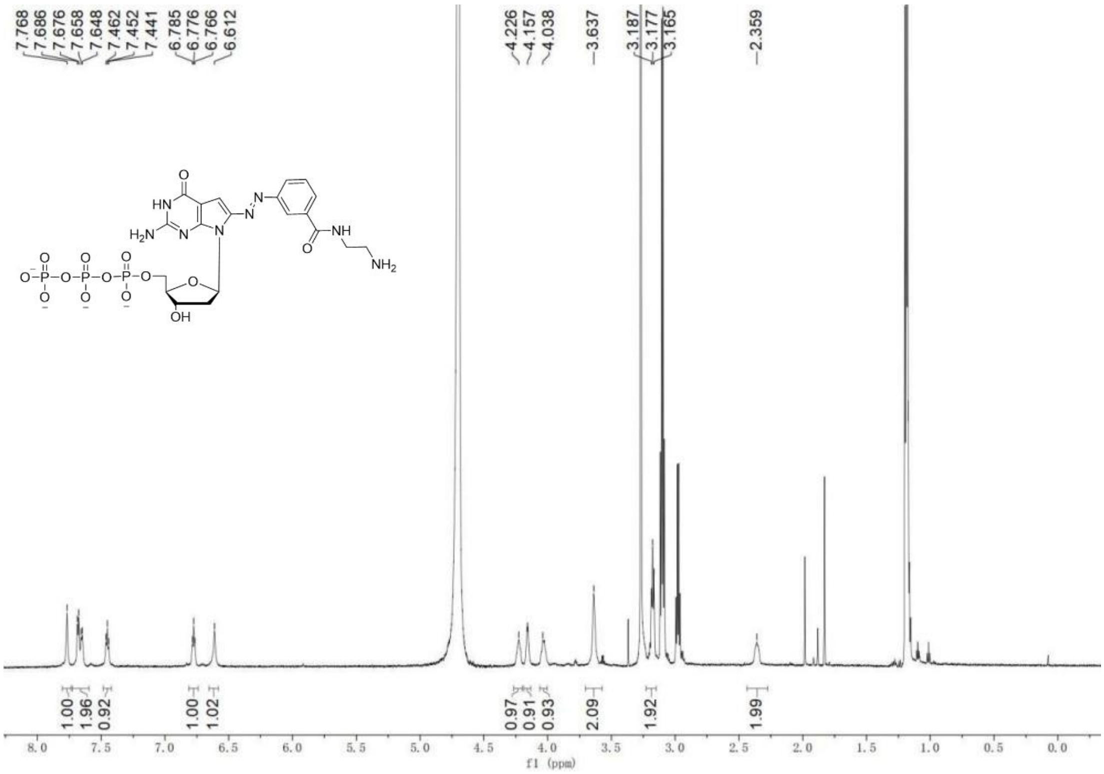
^{1}H NMR spectrum of the compound 11a in D_2O



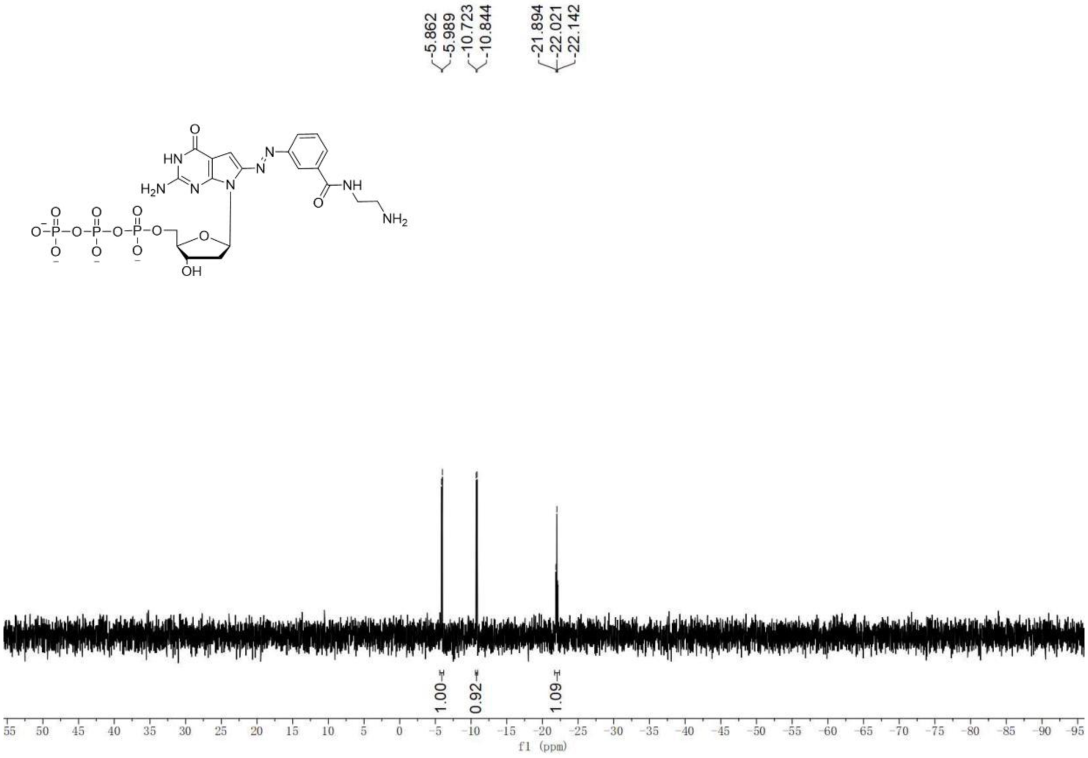
^{31}P NMR spectrum of the compound 11a in D_2O



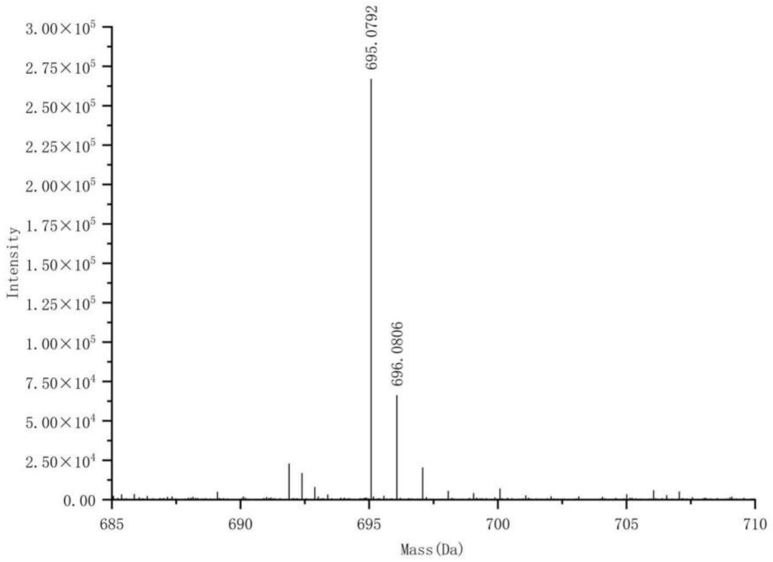
ESI-HRMS spectrum of the compound 11a



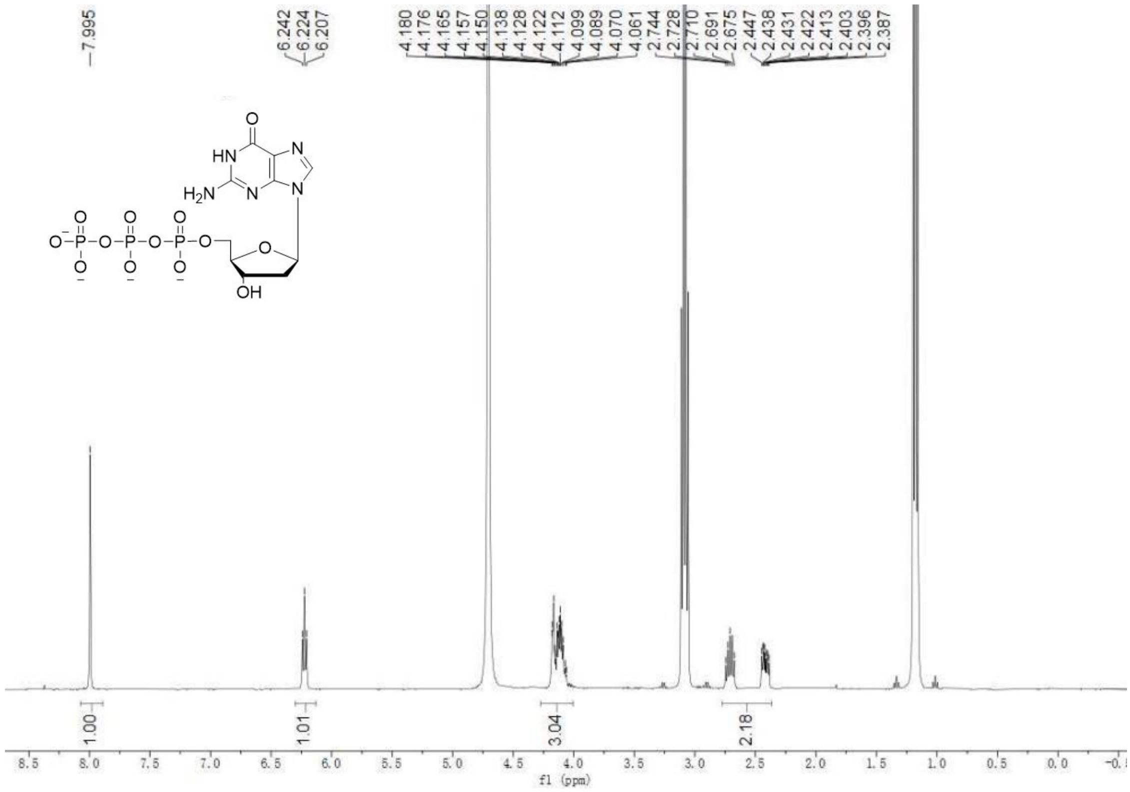
^{1}H NMR spectrum of the compound 11 in D_2O



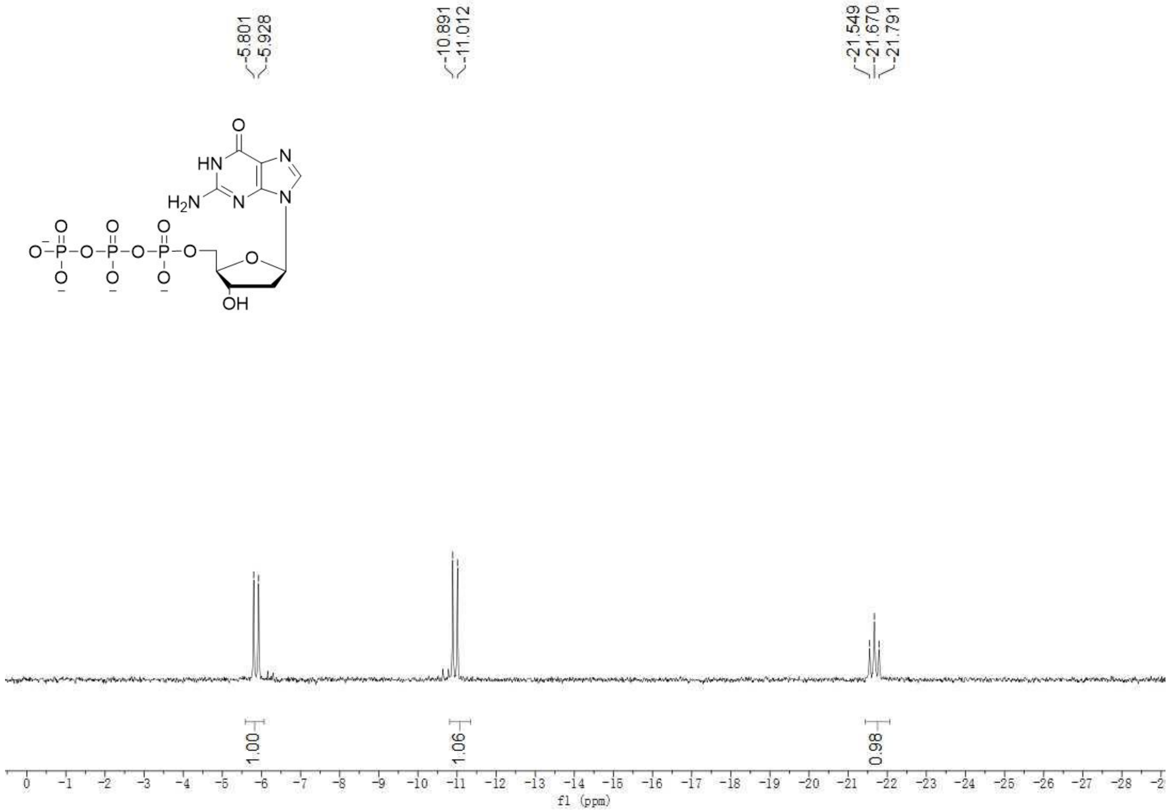
^{31}P NMR spectrum of the compound 11 in D_2O



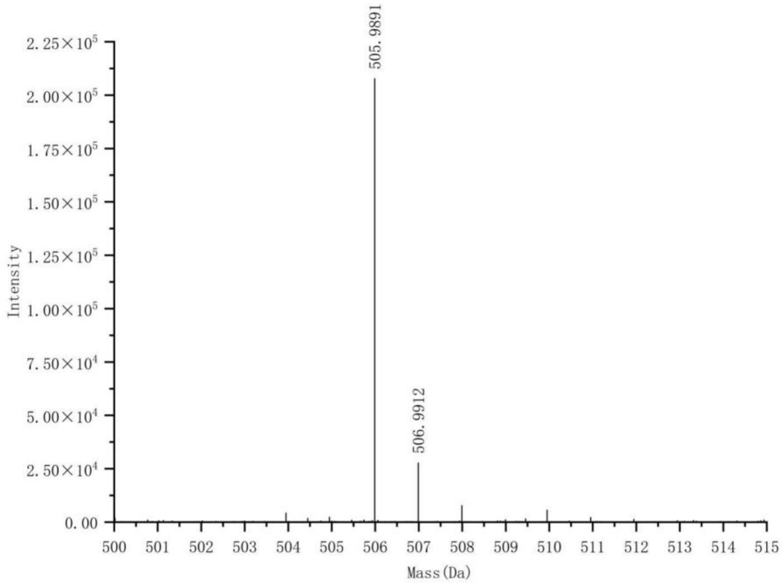
ESI-HRMS spectrum of the compound 11



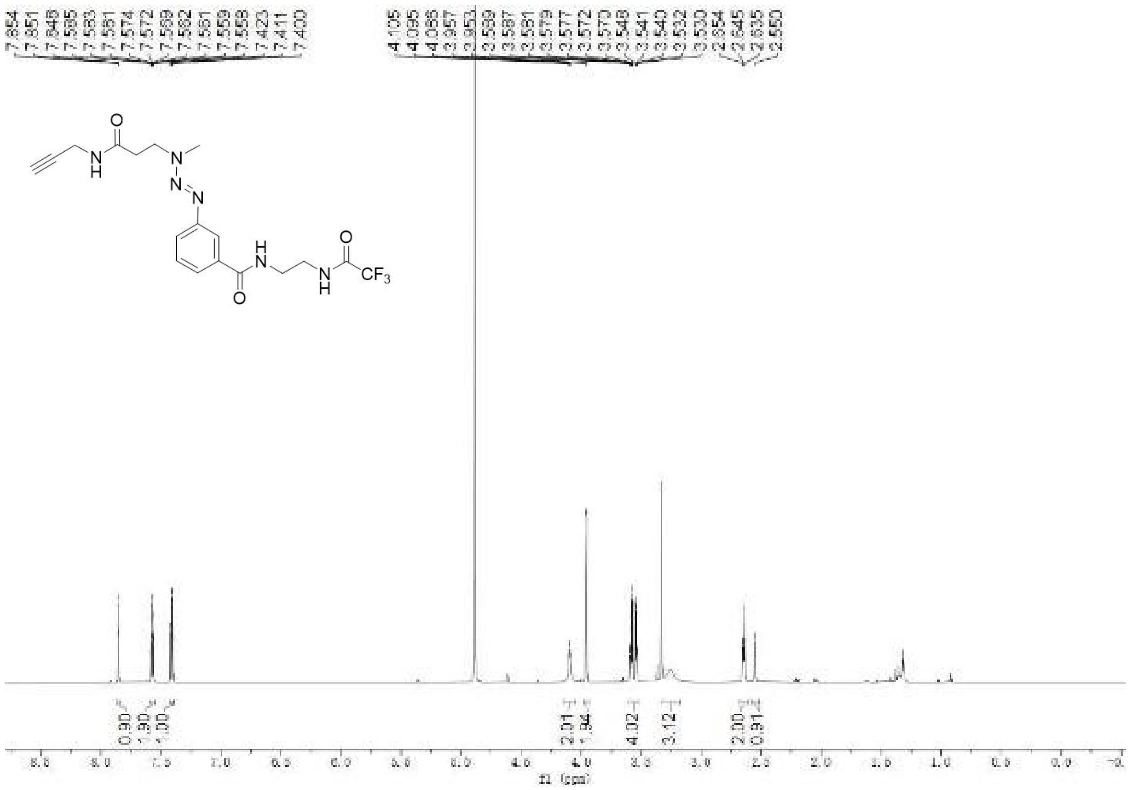
^{1}H NMR spectrum of the compound 12a in D_2O



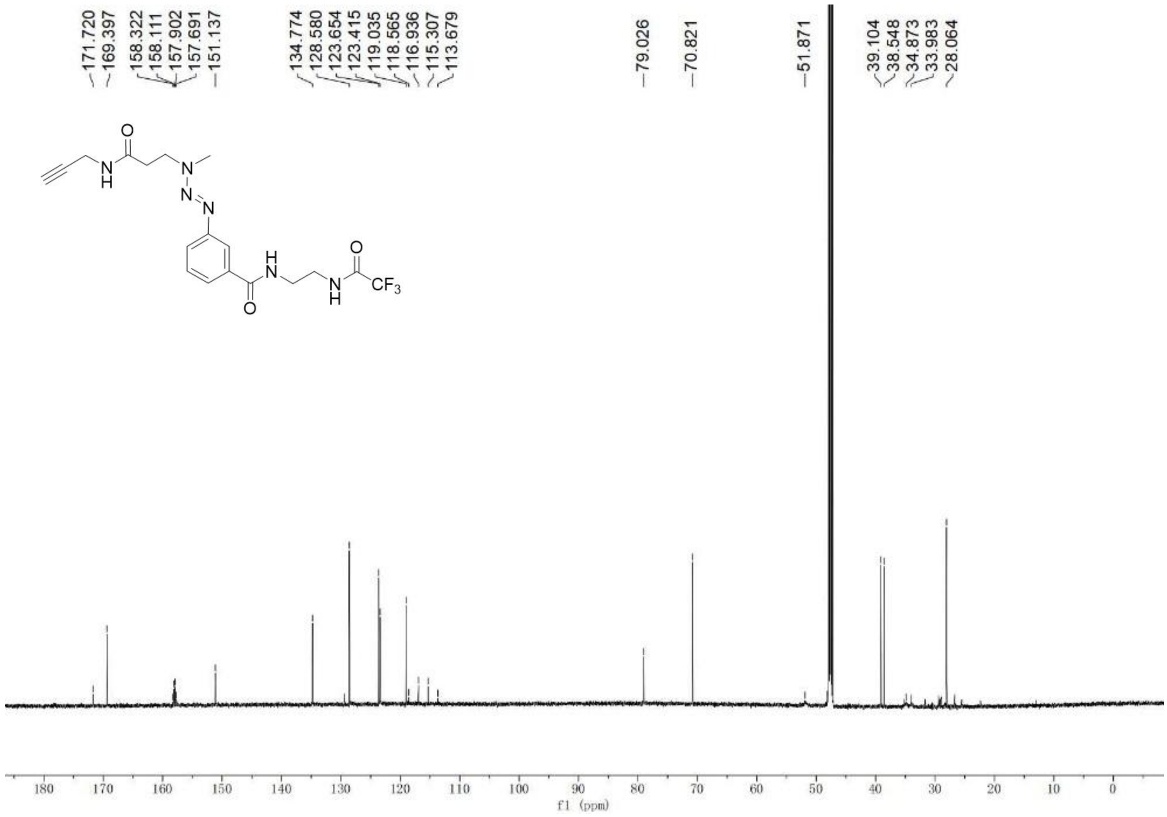
^{31}P NMR spectrum of the compound 12a in D_2O



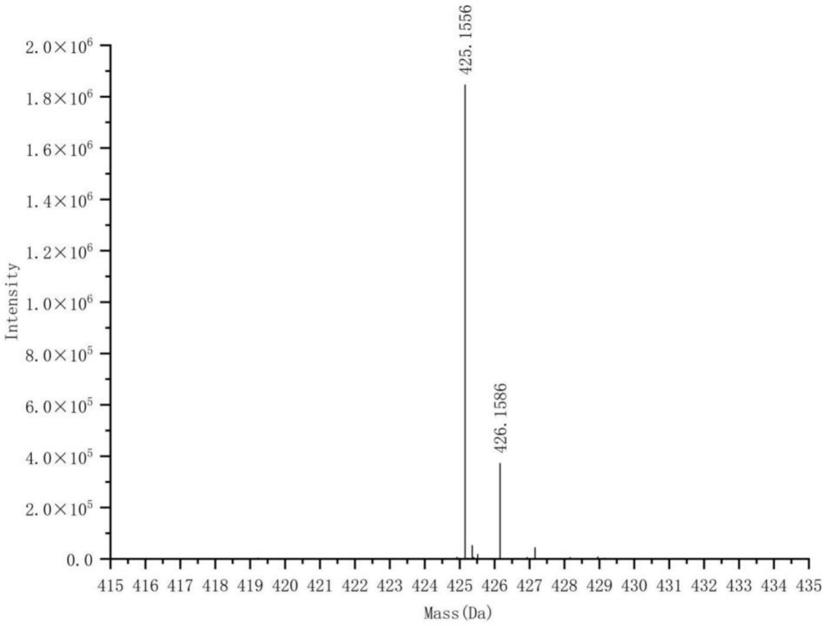
ESI-HRMS spectrum of the compound 12a



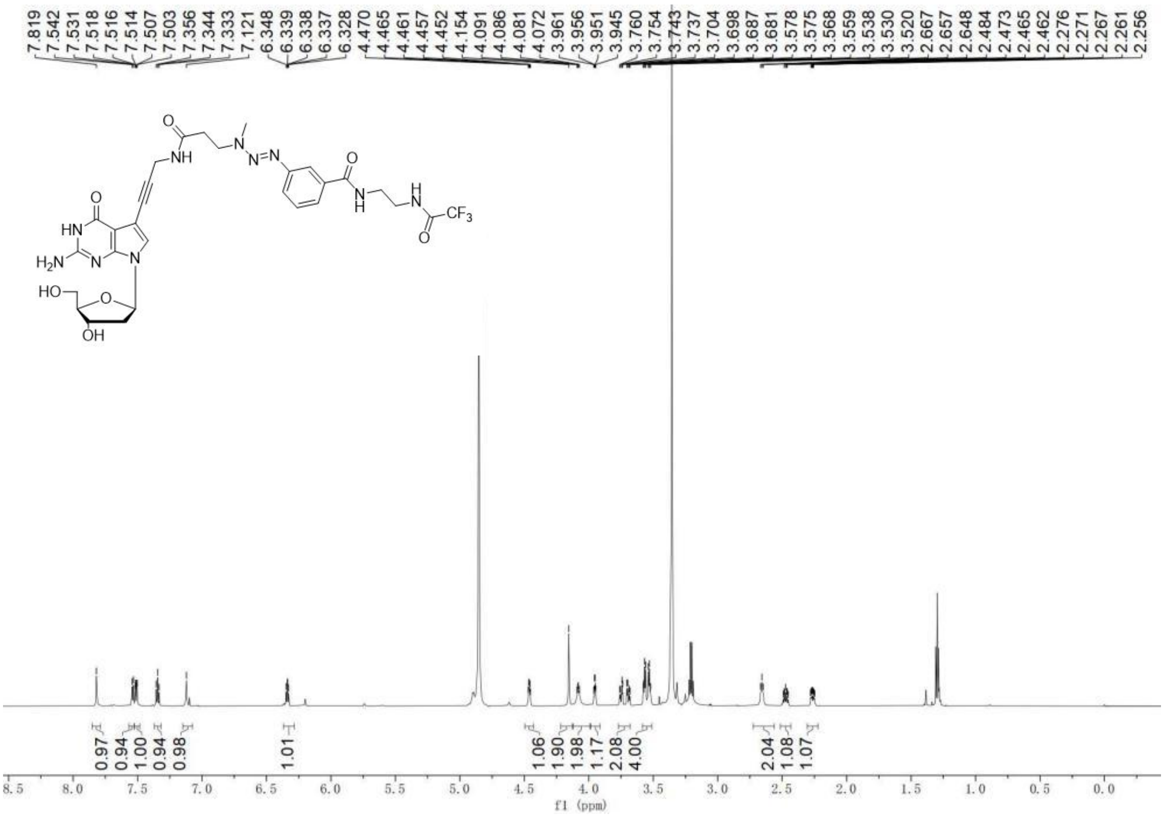
^{1}H NMR spectrum of the compound 13b in CD_3OD



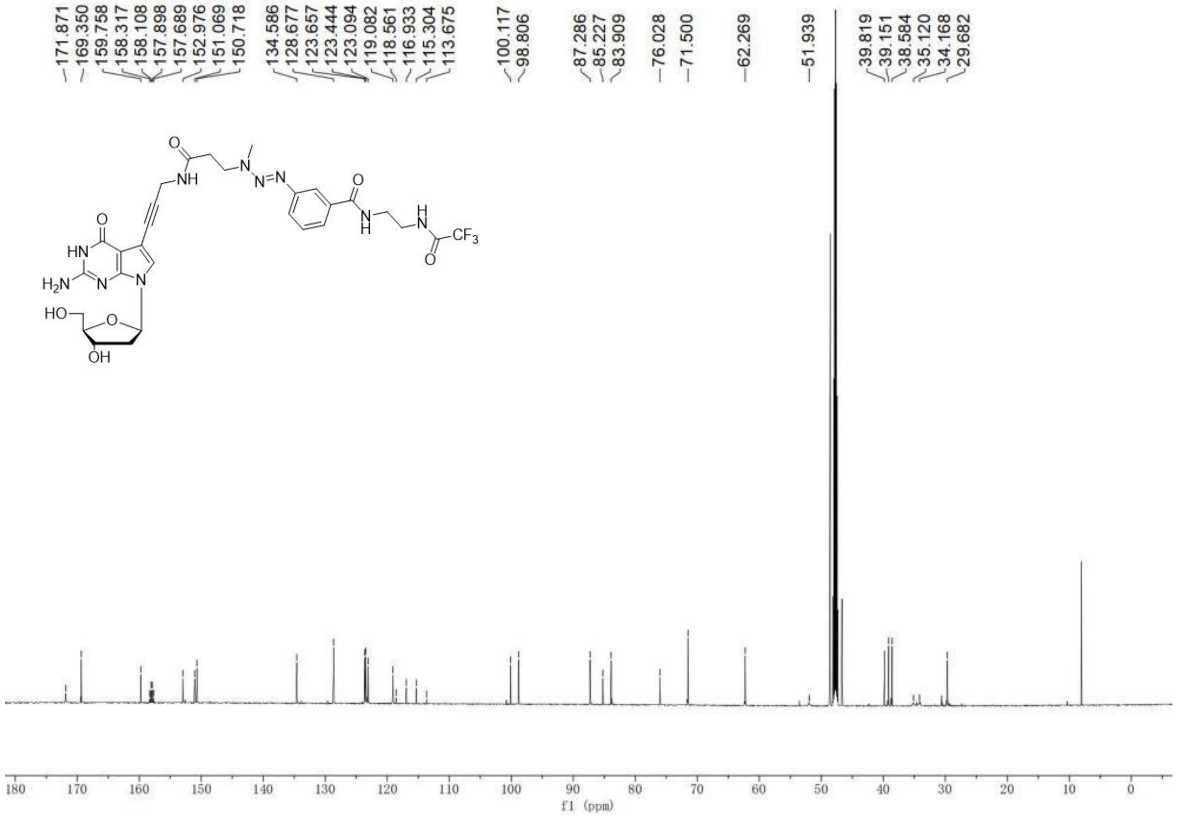
^{13}C NMR spectrum of the compound 13b in CD_3OD



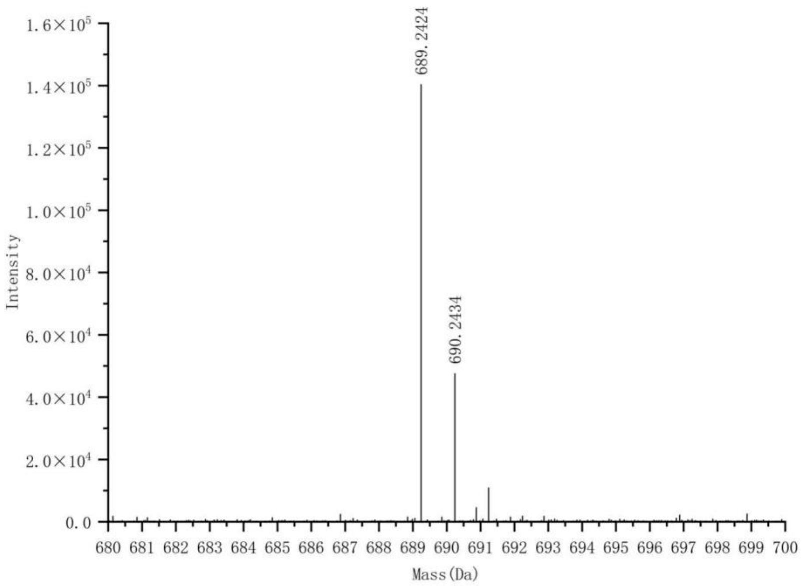
ESI-HRMS spectrum of the compound 13b



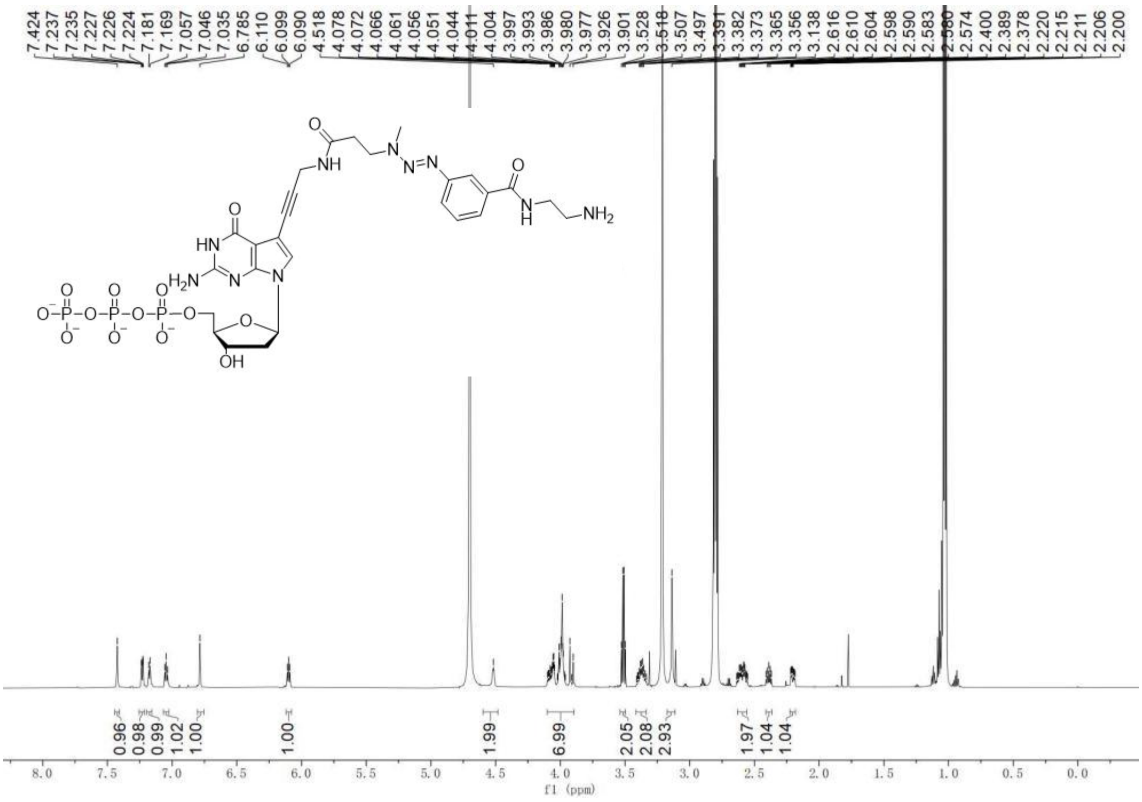
^{1}H NMR spectrum of the compound 13c in CD_3OD



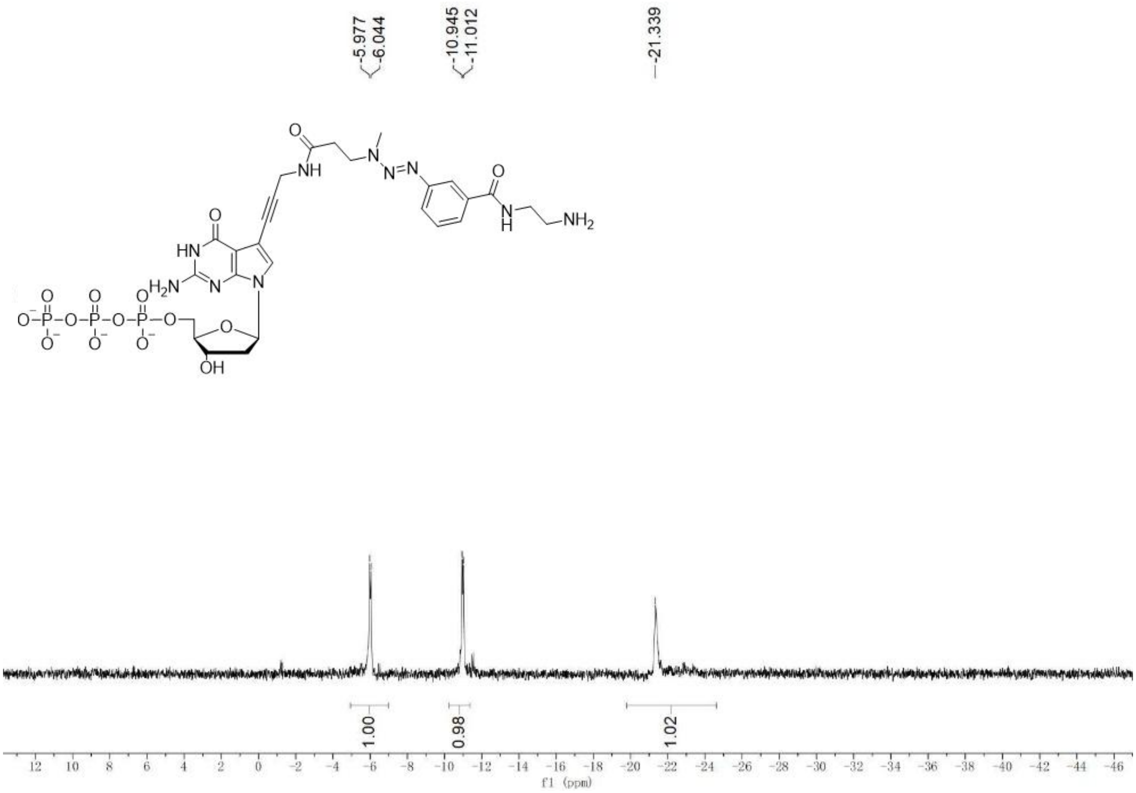
^{13}C NMR spectrum of the compound 13c in CD_3OD



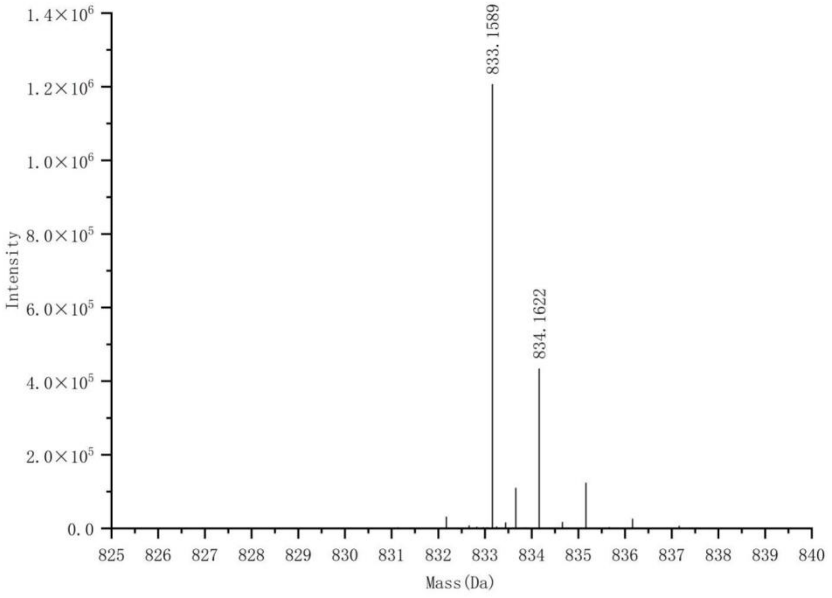
ESI-HRMS spectrum of the compound 13c



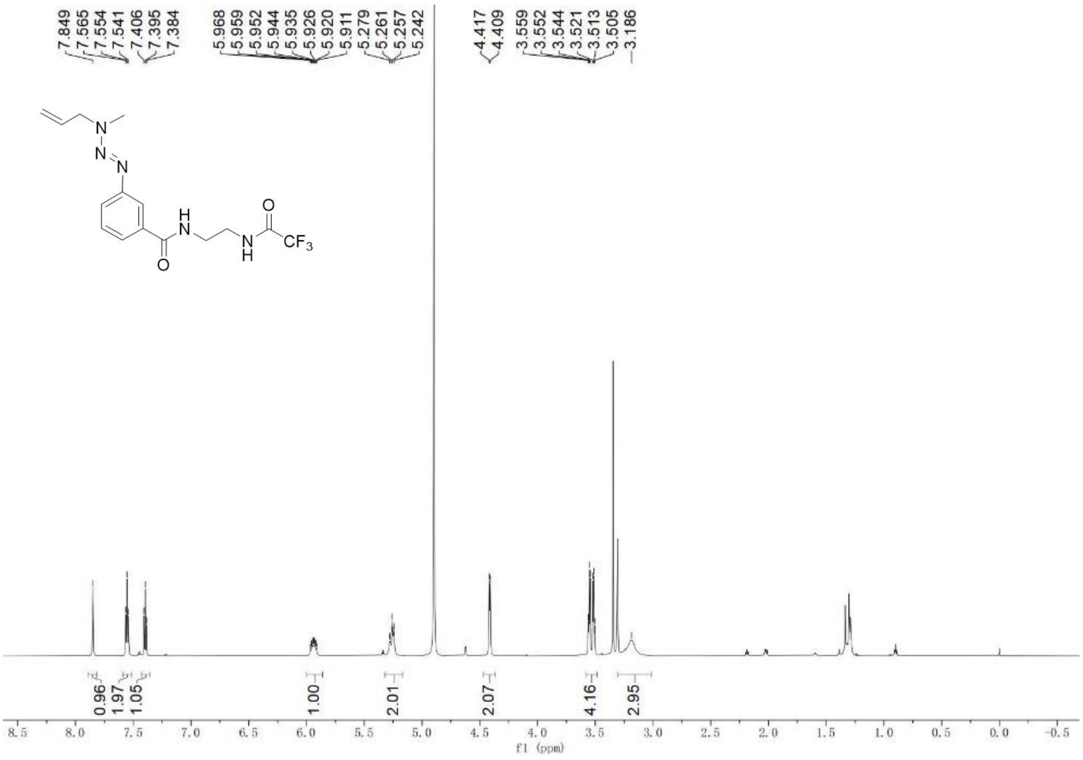
^{1}H NMR spectrum of the compound 13 in D_2O



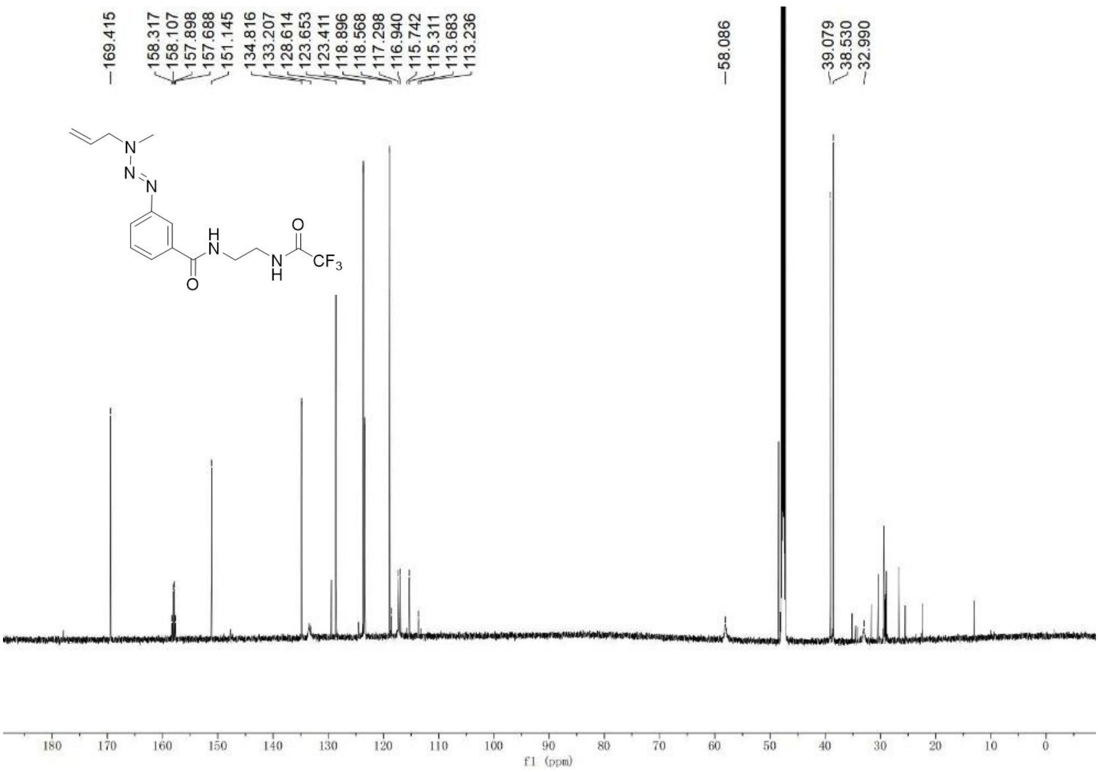
^{31}P NMR spectrum of the compound 13 in D_2O



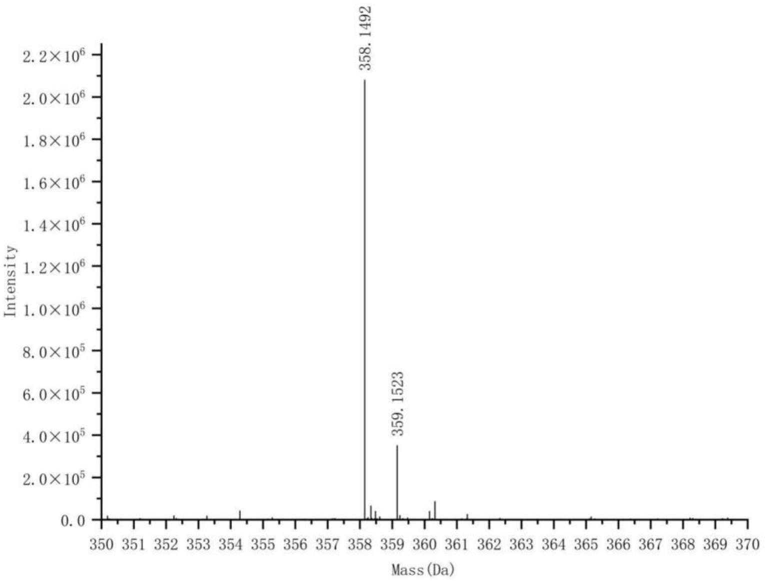
ESI-HRMS spectrum of the compound 13



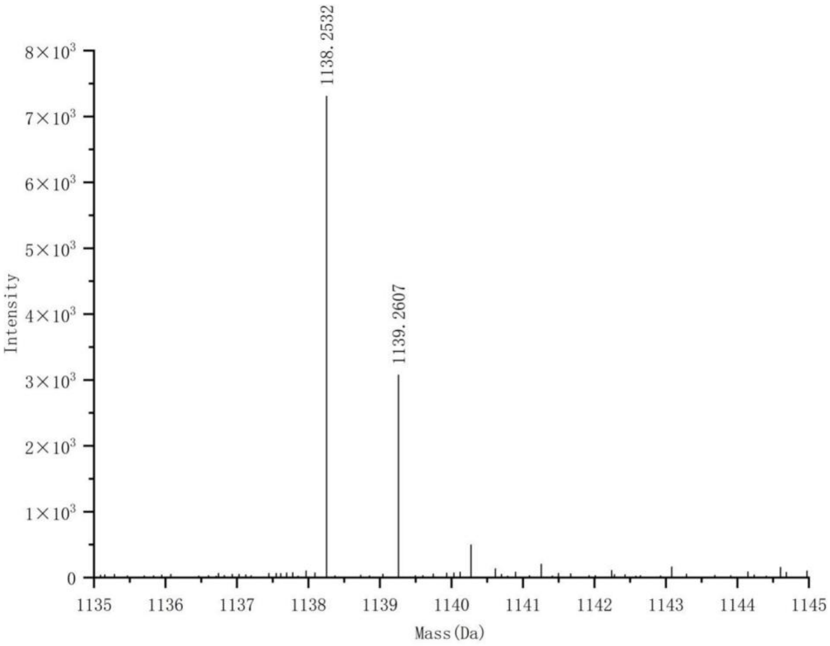
^{1}H NMR spectrum of the compound 14 in CD_3OD



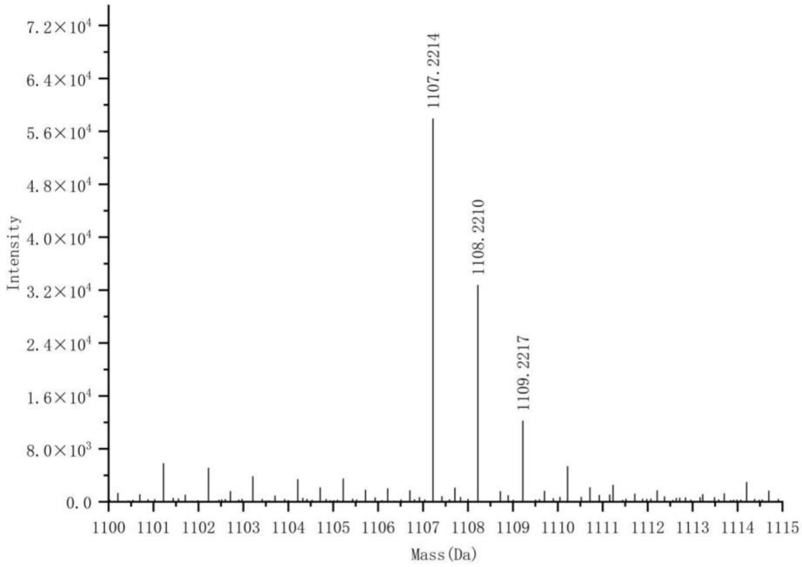
^{13}C NMR spectrum of the compound 14 in CD$_3$OD



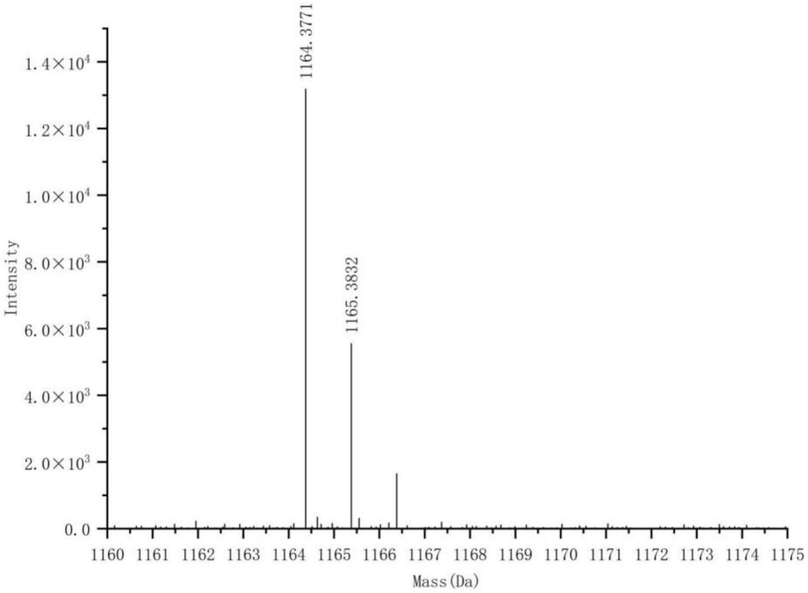
ESI-HRMS spectrum of the compound 14



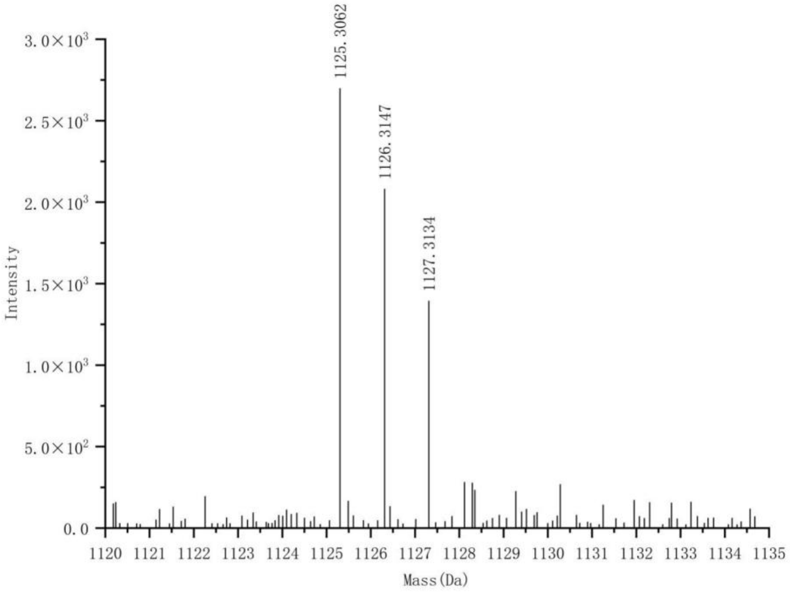
ESI-HRMS spectrum of the compound 7-TAMRA



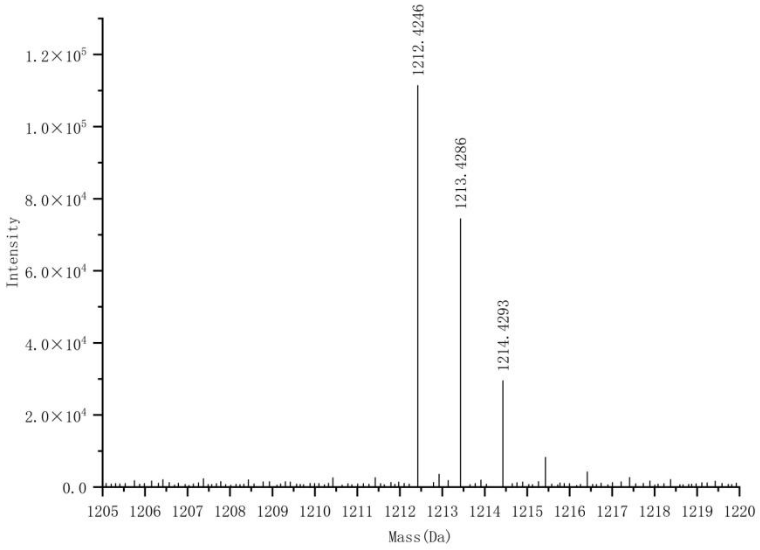
ESI-HRMS spectrum of the compound 11-TAMRA



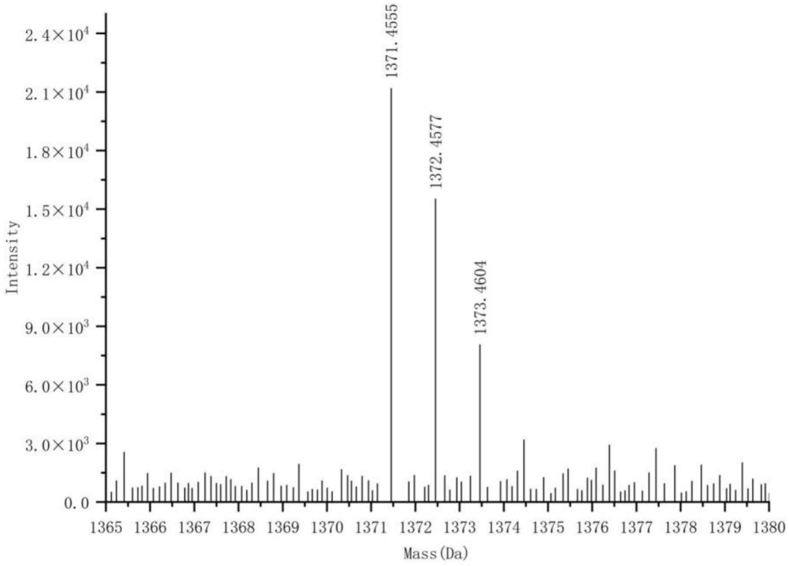
ESI-HRMS spectrum of the compound 7-CY3



ESI-HRMS spectrum of the compound 8-CY2



ESI-HRMS spectrum of the compound 9-CY5



ESI-HRMS spectrum of the compound 13-CY3.5