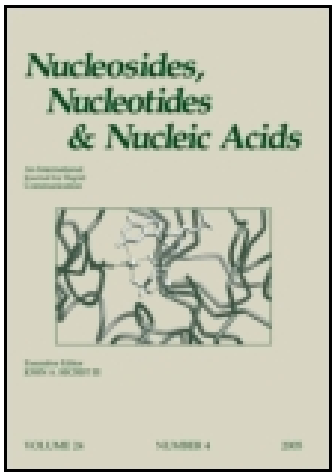

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Design and Synthesis of New Acid Cleavable Linkers for DNA Sequencing by Synthesis

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DESIGN AND SYNTHESIS OF NEW ACID CLEAVABLE LINKERS FOR DNA SEQUENCING BY SYNTHESIS

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□ *A new kind of acid sensitive tetrahydrofuranyl (THF) linker was synthesized and then reacted with 5-(6)-carboxytetramethylrhodaminesuccinimidyl ester (5(6)-TAMRA, SE), followed by di(N-succinimidyl) carbonate (DSC) and modified 2′-deoxyuridine triphosphate (dUTP); the final product, as a reversible terminator for DNA sequencing by synthesis (DNA SBS), was given obtained and confirmed by ^{1}H-NMR, ^{31}P-NMR, and HRMS with purity of up to 99%. The synthesized dye-labeled terminator incorporated into DNA strand successfully, and the fluorophore was cleaved completely under acidic conditions. The preliminary results encourage us to explore more acid-sensitive linkers for DNA SBS to increase the cleavage efficiency under weakly acidic conditions.*

Keywords Triphosphate analogues; fluorescent base analogues; synthetic methodology

INTRODUCTION

Haemophilus influenza was the first free-living organism to have its entire genome sequenced in the late 1990s.[1] Since then, the Human Genome Project (HGP) has cost more than $2 billion and has taken about a decade to

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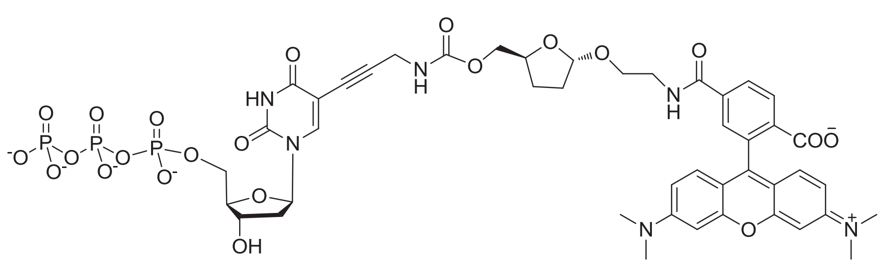
be completed. The workhorse technology behind this project was the Sanger sequencing method.[2] Developed by Frederick Sanger and colleagues in 1977, it has been the most widely used sequencing method for almost 30 years.[3] This method is based on synthesizing DNA on a single-stranded template while incorporating chain terminators randomly.[4] Despite the fact that improvements to this method were made in the 1990s,[5,6] the difficulty in achieving high-throughput and the complexity involved in the automation are still the associated challenges.[7]

To address the limitations of current DNA-sequencing techniques, a series of accurate and high-throughput technologies have been explored and, in particular, Sequencing by Synthesis (SBS) approaches have been investigated and implemented.[8] The cleavable linker used to attach a fluorophore to 2'-deoxynucleotides plays a determinant role in the success of SBS. In 2008, G.Turcatti reported a new class of reduction-cleavable reversible terminator in which the disulfide linker was explored as a chemically cleavable moiety.[8] S. Balasubramanian developed an excellent method of sequencing by synthesis based on fluorescently tagged NTPs for which the fluorescent dye and the azidomethyl protection were from the 3′-group by Staudinger reactions.[8] In addition, photocleavable (PC),[9–13] metal-assisted cleavable,[14–16] and some other kinds of cleavable linkers[17,18] were synthesized and served as reversible terminators successfully in DNA SBS. To our knowledge, acid-cleavable linkers in DNA SBS have never been reported so far.

2-Tetrahydrofuranyl (THF) ethers and 2-tetrahydropyranyl (THP) ethers are widely used in organic synthesis as protecting groups because of their easy installation and stability with most nonacidic reagents.[19,20] In addition, THF ethers have been shown to be more labile than THP ethers under acidic conditions.[21] In this study, we present the synthesis of a novel acid-cleavable reversible terminator dUTP-THF-5(6)TAMRA based on the THF linker (Figure 1) and its preliminary application in DNA SBS. The



12
dUTP-THF-5(6)TAMRA

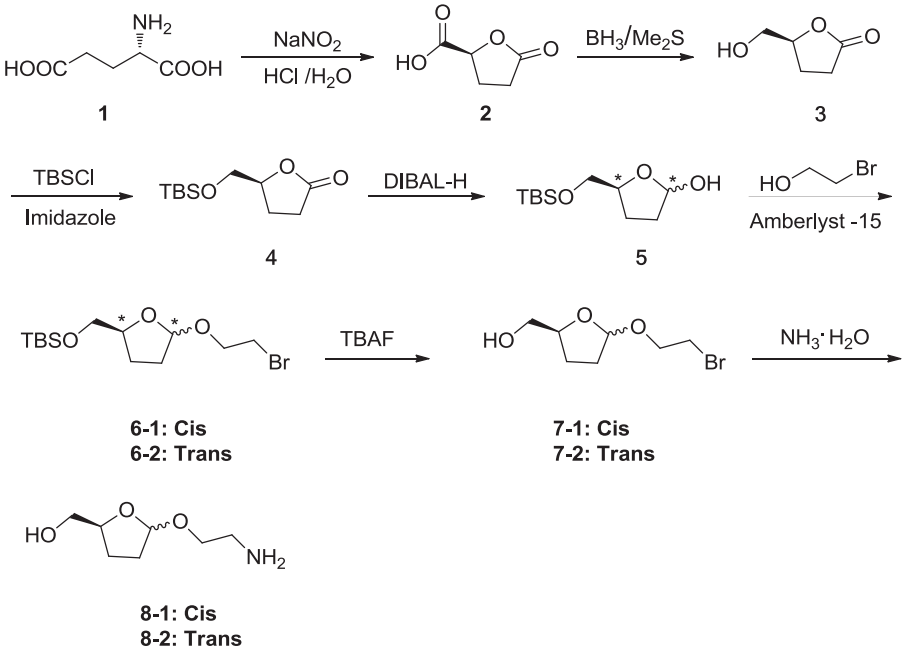
FIGURE 1 Structure of dUTP-THF-5(6)TAMRA.

reversible terminator can be incorporated into a DNA strand faithfully by DNA polymerase, and the fluorescent dye cleaved completely within 25 minutes under acidic conditions.

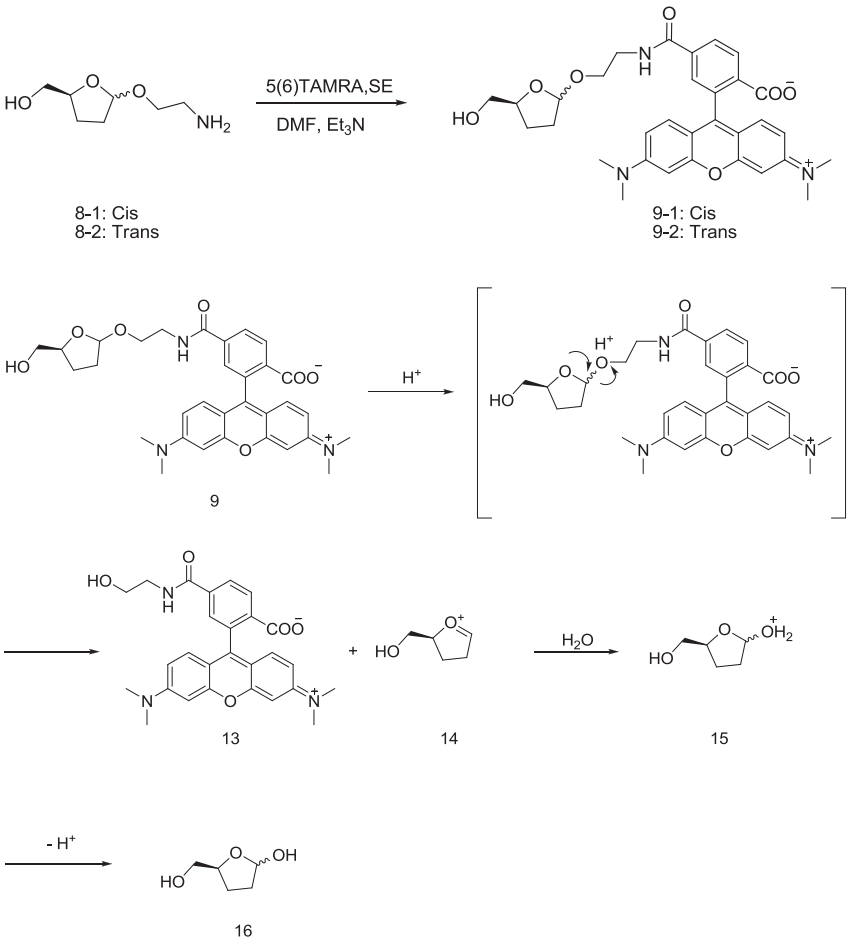
RESULTS AND DISCUSSION

The synthesis of (5S)-5-(((tert-butyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-ol (5) was carried out in a four-step reaction procedure starting from L-glutamic acid, according to published procedures.[22–26] The known compound **5** was made in 22% overall yield as a mixture of diastereoisomers, and was used for the next step directly. Then, treatment of compound **5** with excess 2-bromoethanol and catalytic Amberlyst A-15 in dichloromethane under reflux delivered the desired ethers **6** in good yield. The two isomers for compound **6** were separated by silica gel chromatography column and subjected to ^{1}H-^{1}H COSY and NOE study. De-protection of the silyl group, individually, by using tetra-butylammoniumfluoride (TBAF) in THF furnished **7**, followed by nucleophilic substitution to generate the new cleavable linker **8** (Scheme 1).

To evaluate the cleavability under acidic conditions, compound **9** was synthesized from commercial 5(6)-TAMRA, SE(□), and the linker **8** (Scheme 2).[27]



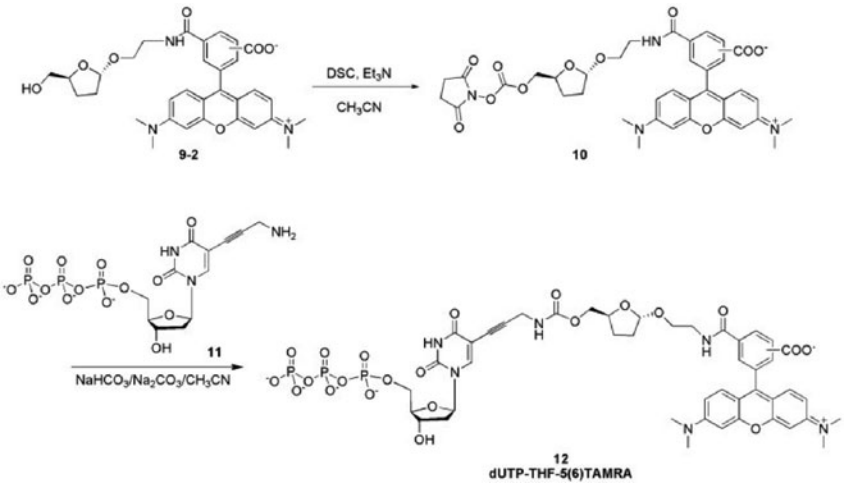
SCHEME 1 Synthesis of the acid cleavable linker.



SCHEME 2 Synthesis of model compound 9 and expected mechanism of cleavage.

With the compound **9** (both of *trans* and *cis* isomers) in hand, cleavage tests were performed on a small scale (3 mg) and analyzed by HPLC, where the UV-visible compounds were detected (Figure 2). After treatment with citric acid/Na_2HPO_4 buffer (pH = 2.25) at 45°C for 20 minutes, both *trans* and *cis* isomers for compounds **9** were cleaved completely into the compound **13**, and this was confirmed by HR-MS. By comparison, the configuration nature of the compound **9** has little effect on the efficiency of cleavage (Table 1). Accordingly, we chose trans-((2S,5S)-5-(2-aminoethoxy)tetrahydrofuran-2-yl)methanol (**9-2**) as the starting material to evaluate the cleavage of the dye-labeled THF linker under acidic condition.

Conditions: yiliteSinoChrom ODS-BP C18 column 5 μm, 4.6 mm×250 mm, flow rate 0.6 mL/minute, gradient 0 minute 100% A, 0% B → 5 minutes 90% A, 10% B→30 minutes 50%A, 50% B→50 minute 0%A, 100% B (A: H_2O, B: Methanol).



SCHEME 3 Synthesis of dUTP-THF-5(6)TAMRA.

Trans-((2S,5S)-5-(2-aminoethoxy)tetrahydrofuran-2-yl)methanol (**9-2**) was treated with DSC in acetonitrile to furnish the mixed succinimide carbonate (**10**) and used in the next step without further purification. The 5-(3-amino-1-propynyl)-2′-deoxyuridine 5′-triphosphate [**dUTP(AP$_3$)**] (**11**) was prepared from the commercially available 5-iodo-2'-deoxyuridine according to the Huang method.[28] The last step was conducted in Na_2CO_3/$NaHCO_3$ buffer (0.1 M, pH 8.7).[29] The **dUTP-THF-5(6)TAMRA(12)** was purified with RP-HPLC on a 250 × 9.4 mm C18 column to obtain the pure product (retention time = 32 minutes) in yield of 33%. The fluorescent nucleotide (**12**) was characterized by ^{1}H NMR, ESI-HRMS, and ^{31}P NMR spectrum.

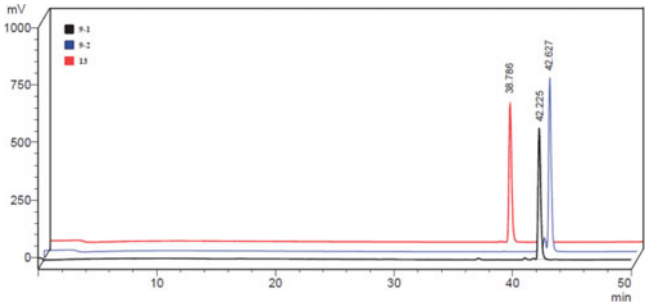
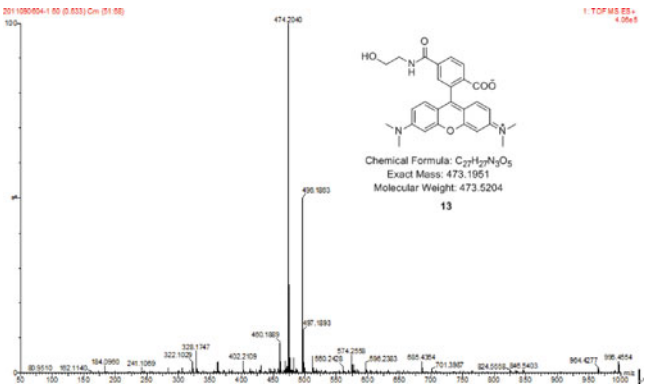


FIGURE 2 HPLC chromatogram of cleavage experiment and HR-MS of cleavage product. (Color figure available online).

TABLE 1 The pyrolysis results at pH 2.25 (45°C)

Time (min)	Comp **9-1**/Cleavage conversion (%)	Comp **9-2**/Cleavage conversion (%)
1	17.87	13.09
3	58.10	55.91
5	80.84	84.02
7	90.98	94.65
9	95.63	98.02
11	97.75	99.18
13	98.63	99.55
15	99.27	99.78
20	99.82	100



To use the **dUTP-THF-5(6)TAMRA(12)** for DNA sequencing, it is critical that the nucleotide analogue be incorporated faithfully into a DNA strand during the strand-extension reaction and be cleaved efficiently under optimized conditions. To this end, we performed a DNA extension assay by using **12** as the substrate in solution, which was further subjected to cleavage reaction. The reaction products were analyzed by polyacrylamide gel electrophoresis (Figure 3). As shown in Figure 3, autoradiogram results illustrated that dUTP-THF-5(6)TAMRA(**12**) is can be incorporated into the DNA strand (Lane 1). The fluorophore group could be partially cleaved under pH 1.6 within 5 minutes and completely removed within 25 minutes.

In conclusion, we have successfully developed a novel acid-cleavable linker for DNA SBS. The dUTP-THF-5(6)TAMRA (**12**) was synthesized successfully based on the novel linker of THF in, overall, 10 steps. In our preliminary experimental effort for DNA sequencing by synthesis, this reversible terminator was shown to be an excellent substrate for DNA polymerase and can be incorporated faithfully into DNA strand. Moreover, the DNA extension product can be completely cleaved at pH 1.6 within 25 minutes whereas DNA duplexes were harmed seriously in acidic conditions. The results of this preliminary research promotes the exploration of more acid-sensitive

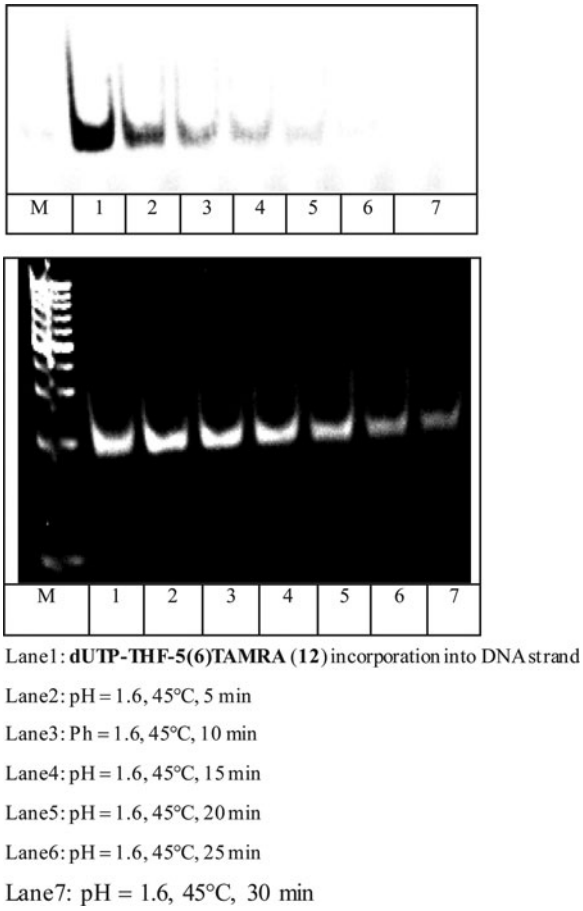


FIGURE 3 Autoradiogram of DNA incorporation and cleavage (up), GR fluorescent staining of DNA incorporation and cleavage (down).

linkers for DNA sequencing by synthesis, and further results will be reported in the future.

EXPERIMENTAL SECTION

(((2S,5R)-5-(2-Bromoethoxy)tetrahydrofuran-2yl)methoxy) (tert-butyl)dimethylsilane (6-1); (((2S,5S)-5-(2-bromoethoxy) tetrahydrofuran-2yl)methoxy)(tert-butyl)dimethylsilane (6-2)

To a solution of **5** (232 mg, 1 mmol) in DCM (10 mL) was added bromoethanol (250 mg, 2 mmol) and Amberlyst-15 (50 mg). The mixture was stirred at reflux for 2 hours; then, the Amberlyst-15 was filtered and the solvent was removed under vacuum. The residue was purified by column

chromatography (eluted with 5% ethyl acetate in petroleum ether) to give **6-1** and **6-2** as a yellow oil.

6-1 (66 mg, 20% yield)**:** ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.13 (d, J = 4.0 Hz, 1H), 4.11–4.14 (m, 1H), 3.92–3.97 (m, 1H), 3.68–3.75 (m, 2H), 3.57–3.61 (m, 1H), 3.44–3.50 (m, 2H), 1.93–2.01 (m, 3H),1.78–1.80 (m, 1H), 0.90 (s, 9H), 0.07 (s, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 104.36, 81.26, 67.21, 67.19, 32.82, 30.97, 26.24, 25.93, 18.36, −5.25, −5.28; HRMS: calc for $C_{13}H_{27}O_3SiBrNa$ $[M+Na]^+$361.0811, found 361.0836.

6-2(85 mg, 25% yield)**:** ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.18 (d, J = 4.8 Hz, 1H), 4.16–4.19 (m, 1H), 3.92–3.96 (m, 1H), 3.73–3.76 (m, 1H), 3.61 (d, J = 4.4 Hz, 2H), 3.46–3.50 (m, 2H), 1.89–2.08 (m, 3H), 1.69–1.73 (m, 1H), 0.89 (s, 9H), 0.06 (d, J = 2.0 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 104.67, 79.02, 67.29, 65.41, 32.08, 31.10, 25.93, 25.31, 18.36, −5.26, −5.31; HRMS: calc for $C_{13}H_{27}O_3SiBrNa$ $[M+Na]^+$ 361.0811, found 361.0835.

(2S,5R)-5-(2-bromoethoxy)tetrahydrofuran-2-yl)methanol (7-1)

To a solutionof **6-1** (100 mg, 0.3 mmol) in 5 mL of THF was added TBAF (0.6 mL, 0.6 mmol).The mixture was stirred at room temperature for 4 hours. Then, the solvent was removed under vacuum and the residue was purified by column chromatography (eluted with 20% ethyl acetate in petroleum ether) to give **7-1**as a colorless oil (60 mg, 88% yield): ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.15 (d, J = 4.4 Hz, 1H), 4.26–4.32 (m, 1H), 3.99–4.02 (m, 1H), 3.74–3.81 (m, 2H), 3.55 (dd, J = 5.2, 12.0 Hz, 1H), 3.49 (t, J = 6.0 Hz, 2H), 1.92–2.07 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 104.74, 81.55, 67.93, 65.61, 33.25, 30.79, 24.33; HRMS: calc for $C_7H_{13}BrO_3Na$ $[M+Na]^+$ 246.9946, found 246.9938.

(2S,5S)-5-(2-bromoethoxy)tetrahydrofuran-2-yl)methanol (7-2)

The procedure is same as **7-1** to yield **7-2** as a colorless oil (60 mg, 83% yield): ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.19 (d, J = 4.8 Hz, 1H), 4.20–4.26 (m, 1H), 3.93–3.96 (m, 1H), 3.69–3.76 (m, 2H), 3.44–3.52 (m, 3H), 1.95–2.06 (m, 3H), 1.64–1.68 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 104.59, 78.62, 67.34, 64.78, 32.41, 30.94, 24.87; HRMS: calc for $C_7H_{13}BrO_3Na$$[M+Na]^+$ 246.9946, found 246.9929.

(2S,5R)-5-(2-aminoethoxy)tetrahydrofuran-2-yl)methanol (8-1)

To a solution of **7-1** (136 mg, 0.6 mmol) was added ammonium hydroxide (2 mL). The mixture was stirred at 40°C for 6 hours, then the solvent was removed under vacuum to give **8-1** as a colorless oil (90 mg, 93%). The product was used for the next step without further purification: ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.15 (d, J = 4.0 Hz, 1H), 4.22–4.23 (m, 1H), 3.97–4.02 (m, 2H), 3.89 (dd, J = 2.4, 12.0 Hz, 1H), 3.67 (dd, J = 4.8, 12.0 Hz, 1H),

3.38–3.44 (m, 1H), 3.19–3.25 (m, 1H), 1.97–2.05 (m, 3H), 1.81–1.87 (m, 1H); ^{13}C NMR (CD_3OD, 100 MHz): δ 105.02, 81.38, 64.01, 63.49, 39.78, 32.68, 24.20; HRMS: calc for $C_7H_{16}NO_3$ $[M+H]^+$ 162.1130, found 162.1128.

(2S,5S)-5-(2-aminoethoxy)tetrahydrofuran-2-yl)methanol (8-2)

The procedure is as same as that of **8-1,** and finally yielded compound **8-2** as a colorless oil (94 mg, 97% yield): ^{1}H NMR (CD_3OD, 400 MHz): δ 5.20–5.22 (m, 1H), 4.17–4.23 (m, 1H), 3.87–3.95 (m, 1H), 3.64–3.78 (m, 1H), 3.56–3.61 (m, 1H), 3.47–3.53 (m, 1H), 3.15 (t, $J = 4.8$ Hz, 1H), 1.91–2.10 (m, 3H), 1.64–1.70 (m, 1H); ^{13}C NMR (CD_3OD, 100 MHz): δ 104.72, 79.11, 63.87, 62.95, 39.53, 31.54, 24.86; HRMS: calc for $C_7H_{16}NO_3$ $[M+H]^+$ 162.1130, found 162.1135.

2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)-4-((2-(((2R,5S)-5-(hydroxymethyl)tetrahydrofuran-2-yl)oxy)ethyl) carbamoyl)benzoate (9-1)

To a solution of **8-1** (15 mg, 0.019 mmol) in DMF (0.5 mL) was added 5(6)-TAMRA, SE (10 mg, 0.019 mmol) in DMF (0.5 mL) and Et_3N (40μL, 0.285 mmol). The mixture was protected from light and stirred at room temperature for 3 hours. Then, the solvent was removed under vacuum and the residue was purified by TLC (eluted with 10% dichloromethane in methanol) to give **9-1** as a red solid (10 mg, 96%): ^{1}H NMR (CD_3OD, 400 MHz): δ 8.13 (d, $J = 8.0$ Hz, 1H), 8.08 (dd, $J = 1.6$,8.0 Hz, 1H), 7.73 (d, $J = 1.6$ Hz, 1H), 7.25 (dd, $J = 1.6$, 9.6 Hz, 2H), 6.99 (dd, $J = 2.0$, 9.2 Hz, 2H), 6.89 (d, $J = 2.4$ Hz, 2H), 5.10 (d, $J = 1.6$ Hz, 1H), 4.07–4.11 (m, 1H), 3.78–3.85 (m, 1H), 3.46–3.57 (m, 5H), 3.26 (s, 12H), 1.87–1.95 (m, 3H), 1.68–1.76 (m, 1H); HRMS: calc for $C_{32}H_{36}N_3O_7$ $[M+H]^+$ 574.2553, found 574.2531; calc for $C_{32}H_{35}N_3O_7Na[M+Na]^+$ 596.2373, found 596.2340.

2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)-4-((2-(((2S,5S)-5-(hydroxymethyl)tetrahydrofuran-2-yl)oxy)ethyl) carbamoyl)benzoate (9-2)

The procedure is the as same as that of **9-1** to yield **9-2** as a red solid (8 mg, 73% yield):^{1}H NMR (CD_3OD, 300 MHz): δ 8.06–8.16 (m, 2H), 7.70 (s, 1H), 7.26 (d, $J = 9.6$ Hz, 2H), 7.00 (dd, $J = 2.1$,9.6 Hz, 2H), 6.92 (d, $J = 2.1$ Hz, 2H), 5.16–5.17 (m, 1H), 4.07–4.11 (m, 1H), 3.76–3.84 (m, 1H), 3.40–3.65 (m, 5H), 3.28 (s, 12H), 1.83–2.02 (m, 3H), 1.60–1.61(m, 1H); HRMS: calc for $C_{32}H_{36}N_3O_7$ $[M+H]^+$ 574.2553, found 574.2534; calc for $C_{32}H_{35}N_3O_7$ Na $[M+Na]^+$ 596.2373, found 596.2363.

2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)-4-((2-(((2S,5S)-5-(((((2,5-dioxopyrrolidin-1-yl)oxy)carbonyl)oxy)methyl) tetrahydrofuran-2-yl)oxy)ethyl)carbamoyl)benzoate (10)

9-2 (6.6 mg, 0.012 mmol) and Et_3N (15 μL, 0.096 mmol) were dissolved in CH_3CN (anhy, 0.5 mL), and N,N′-disuccinimidyl carbonate (12 mg, 0.048 mmol) was added. After the reaction mixture was stirred for 4 hours at room temperature, the solution was used directly for the next step without purification.

dUTP-THF-5(6)TAMRA (12)

A solution of **dUTP(AP3) (11)** (11 mg, 0.012 mmol) in 0.1 M sodium bicarbonate buffer (pH 9.16, 0.5 mL) was added to the solution of crude **10**. The resulting reaction mixture was protected from light and periodically vortexed for 2 hours. After lyophilization, the resulting residue was dissolved in TEAA buffer (20 mM, pH 7.0, 1.0 mL) and purified with RP-HPLC on a 250×9.4 mm C18 column to obtain the product (retention time = 32 minutes) as a triethylammonium salt. The product was then precipitated from NaCl/ethanol to remove significant amounts of TEAA salts to give sodium salt of **12** as a red solid (5 mg, 33%). Mobile phase: A, 20 mm TEAA in water; B, methanol. Elution was performed with 100% A isocratic over 10 minutes, followed by a linear gradient of 0–50% Bfor 20 minutes and then 50% B isocratic over another 20 minutes at a flow rate of 4 mL/minute: ^{1}HNMR (D_2O, 400 MHz): δ8.32 (s, 1H), 7.71 (s, 1H), 7.62 (d, J = 8.0 Hz, 1 H), 7.25 (d, J = 9.6 Hz,2H), 6.93–7.05 (m, 3 H), 6.71 (s, 2H), 5.68–5.72 (m, 1 H),5.28 (d, J = 8.8 Hz,1H),4.47 (s, 1 H), 3.88–4.19 (m, 7 H), 3.68–3.85 (m, 4 H), 3.55–3.63 (m, 1 H), 3.25 (d, J = 8.8 Hz, 12 H), 2.23–2.34 (m, 1 H), 2.02–2.18 (m, 3 H), 1.89–1.96 (m, 1 H), 1.51–1.61 (m, 1H); ^{31}P NMR (D_2O, 162 MHz): −18.95, −10.37, −5.20; HRMS: calc for $C_{45}H_{50}N_6O_{22}P_3$ $[M\text{-}H]^-$ 1119.2191, found 1119.2238.

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SUPPORTING INFORMATION AVAILABLE

Spectral data for **6-12**(^{1}H, ^{13}C, and ^{31}P NMR) and general experimental procedures are available, free of charge, online.

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