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# Preparation of fluorinated RNA nucleotide analogs potentially stable to enzymatic hydrolysis in RNA and DNA polymerase assays

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ABSTRACT

Analogs of ribonucleotides (RNA) stable to enzymatic hydrolysis were prepared and characterized. Computational investigations revealed that this class of compounds with a modified triphosphate exhibits the correct polarity and minimal steric effects compared to the natural molecule. Non-hydrolysable properties as well as the ability of the modified nucleotide to be recognized by enzymes were probed by performing single-turnover gap filling assays with T7 RNA polymerase and DNA polymerase β.



## 1. Introduction

Nucleotides are central molecules in biology and have high importance in biochemical and medicinal investigations [1]. Hydrolytically stable analogs modified at the α,β-bridging oxygen position in the triphosphate linkage cannot be utilized in enzymatic nucleotidyl transfer reactions. Owing to such hydrolytic stability, such a family of nucleotide analogs is increasingly used for studying active sites of various enzymes, as well as their functions and mechanisms [2–4]. Toward this endeavor, a number of modifications at the triphosphate fragment have been explored, allowing the study of steric and electronic effects with relation to the activity of the resulting analogs [5–8]. However, only a few analogs with a modified triphosphate demonstrate properties similar to natural nucleotides. At the same time, considering the high sensitivity of enzymes toward electronic and steric parameters of the active site binders, these properties of the modified nucleotides are extremely important to effectively mimic of the natural substrates.

Herein, we report the synthesis of novel fluorinated ribonucleotide analogs potentially stable to enzymatic hydrolysis. This has been achieved by substituting bridging oxygen atoms of the triphosphate unit with bioisosteric difluoromethylene groups [9–11]. A computational study demonstrated that such a replacement provides minimal steric perturbation and retains the polarity of the natural triphosphate.

Recently, we reported a simple and efficient synthesis of bis(difluoromethylene)triphosphoric acid (BMF$^4$TPA) **4** [12] (Scheme 1). Correct polarity, low steric demand of the bridging groups, and notable stability of BMF$^4$TPA under a wide range of temperatures and pH prompted us to explore this unique set of properties in biochemical assays via replacement of the triphosphate group in ribonucleotides with the bis(difluoromethylene) analog **4**. Both non-specific and enzyme catalyzed hydrolytic stability of such synthetic analogs were expected to be greater when compared to natural nucleotides.

## 2. Results and discussion

### 2.1. Synthesis

Generally, the BMF$^4$TPA moiety was attached to a nucleoside via nucleophilic displacement of 5′-tosylate in protected nucleosides by the tetra-*n*-butylammonium salt of the acid [13,14]. To prepare the nucleoside tosylates, the A, C and U nucleosides were

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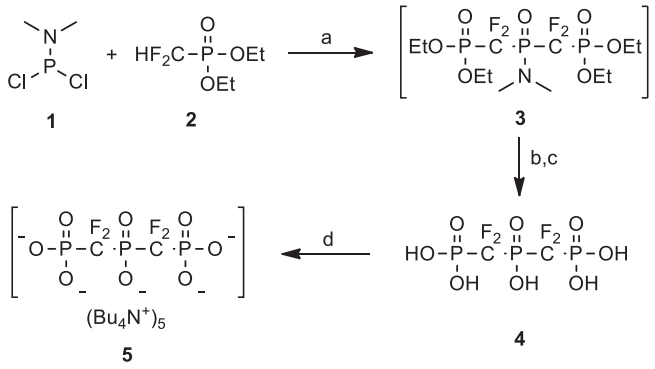


**Scheme 1.** Preparation of BMF[4]TPA [$Bu_4NH^+$]$_5$. Reaction conditions: (a) LTMPA, THF, −78 °C; (b) m-CPBA; (c) TMSBr; (d) $Bu_4NOH$.

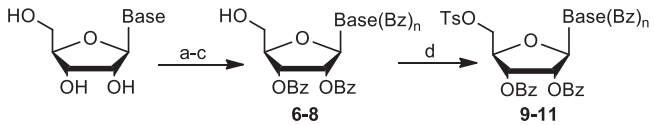
first directly acylated with benzoyl chloride at the *N*-base, 2′- and 3′-positions [15] (Scheme 2). This step was followed by treatment with *p*-toluenesulfonyl chloride in anhydrous pyridine for 7 days at −20 °C to give access to the protected 5′-tosylates **9**–**11** in good yields. However, the described route could not be applied to obtain protected guanosine tosylate directly.

Instead, the solubility of guanosine had to be enhanced by protecting the 5′-hydroxyl with TBDMS and the N2-amino group with a dimethylaminomethylene group [16]. Subsequently, a facile reaction with benzoyl chloride in situ afforded the protection of the 2′- and 3′-hydroxyl groups of **12**. Deprotection of 5′-hydroxyl group was achieved by treating **12** with TBAF/THF/AcOH mixture [17]. The corresponding guanosine tosylate **14** was prepared via the well-established reaction with TsCl in pyridine (Scheme 3).

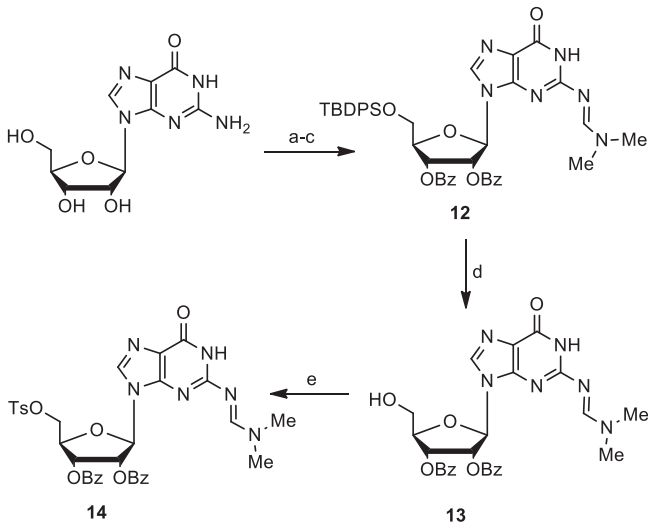
The target nucleotide analogs were prepared (Scheme 4) via the reaction of benzoyl protected 5′-tosyl ribonucleosides **9**–**11** and **14** with the BMF[4]TPA tetra-*n*-butylammonium salt (**5**). It is not surprising that due to the strong electron withdrawing effect of the difluoromethylene bridging groups, BMF[4]TPA acts as a poor nucleophile. However, conversion of BMF[4]TPA into the tetrabutylammonium salt significantly enhanced its solubility and reactivity as a nucleophile.

The substitution reaction was carried out in anhydrous DMF at 110 °C for 1 h, then left stirring at room temperature overnight. The progress of the reaction can be monitored using a weak ion-exchange HPLC using a DEAE-5PW column. The BMF[4]TPA fragment can sustain harsh reaction conditions providing acceptable yields of the products **15**–**18**. Two equivalents of the tosyl nucleoside were required to ensure maximum consumption of BMF[4]TPA [$Bu_4NH^+$]$_5$.

All ribonucleotide analogs exhibited similar retention times and were eluted within 28–35 min using 1 M TEAB and water as eluent. After reactions reached maximum conversions, all nucleotide analogs were subjected to deprotection using water/methanol solution of ammonium hydroxide. In the case of (α,β),(β,γ)-bisCF$_2$ ATP, (α,β),(β,γ)-bisCF$_2$ CTP, and (α,β),(β,γ)-bisCF$_2$ UTP, we were able to achieve conversions close to 90%, although in case of the (α,β),(β,γ)-bisCF$_2$ GTP conversion did not exceed 20%.



**Scheme 2.** Synthesis of benzoyl protected tosylnucleosides. **6**, **9** base = N$^6$-Bz$_2$-adenine, **7**, **10** base = N$^4$-Bz-cytosine, **8**, **11** base = N$^3$-Bz-uracyl. Reaction conditions: (a) TBDMSCl, Py; (b) BzCl, (c) TFA, $H_2O$, THF; (d) TsCl, Py, −24 °C, 7 days.



**Scheme 3.** Preparation of protected 5′-tosylguanosine. Reaction conditions: (a) DMF-DMA, MeOH; (b) TBDPSCl, DMAP, Py; (c) BzCl; (d) TBAF, THF, AcOH; (e) TsCl, Py, −24 °C, 7 days.

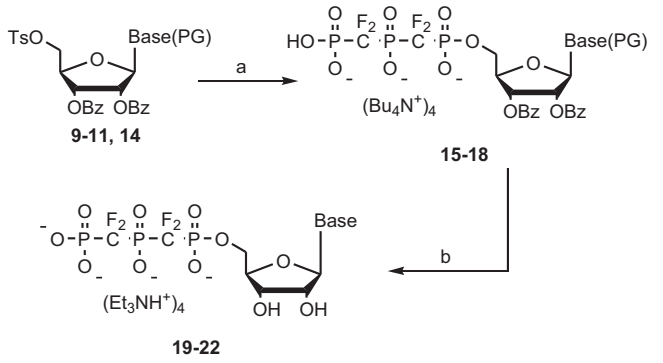
## 2.2. Computational results

A DFT computational study using Gaussian 09 [18] supported the hypothesis that the incorporation of difluoromethylene groups into the (α,β) and (β,γ) positions of triphosphoric acid would be bioisosteric in nature.

Although in the free acid forms these analogs acquire different conformations, the presence of a magnesium dication simulating interactions in enzyme precatalytic state results in similar structures coordinated around the ion for both acids.

The investigation clearly shows that bioisosteric incorporation of difluoromethylene group instead of labile phosphoanhydride oxygen atoms leads to a minimal steric perturbation and retains similar charge distribution compared to the natural triphosphoric acid. Previous studies have revealed that the $pK_a$ values of the BMF[4]TPA ($pK_{a4}$ = 5.33, $pK_{a5}$ = 7.23) are lower than corresponding values of natural triphosphoric acid (5.83–6.50 and 8.73–9.24), [10] but possibly close enough to be considered isoacidic [19].

Natural bond order (NBO) analysis of the BMF[4]TPA analog relative to the natural triphosphoric acid (TPA) showed similar charge distribution. Dipole moments of 4.42 Debye and 4.38 Debye were calculated for the BMF[4]TPA analog and the TPA, respectively, further demonstrating that the overall electrostatic properties of the molecule have not been altered dramatically (Fig. 1B).



**Scheme 4.** Preparation of (α,β),(β,γ)-bisCF$_2$-NTP analogs. Reaction conditions: (a) DMF, 110 °C; (b) $NH_4OH$, MeOH. Yields of bisCF$_2$-NTP analogs: (α,β),(β,γ)-bisCF$_2$-ATP (**19**) 29%, (α,β),(β,γ)-bisCF$_2$-CTP (**20**) 33%, (α,β),(β,γ)-bisCF$_2$-UTP (**21**) 22%, (α,β),(β,γ)-bisCF$_2$-GTP (**22**) 6%.

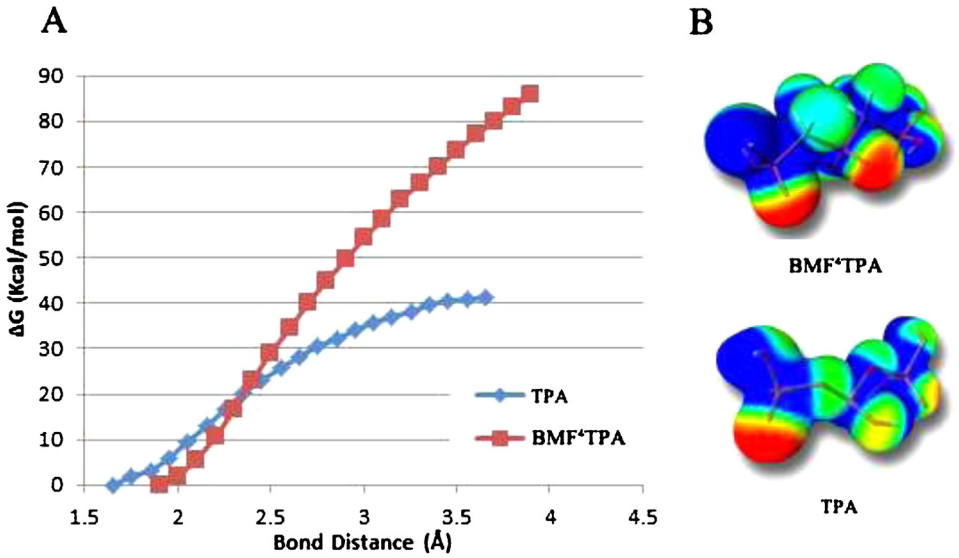



**Fig. 1.** Comparative computational study of natural triphosphoric acid and BM$^{F4}$TPA analog. (A) Bond scan at B3LYP/cc-PVDZ. (B) Charge density maps of the TPA and BMF$^{4}$TPA analog generated by Molden Program [20].

By increasing the bond length of the bridging P–O or P–C bonds of the optimized structure incrementally, bond strength was estimated to be over 80 kcal/mol for the BMF$^{4}$TPA analog compared to approximately 40 kcal/mol for the natural triphosphoric acid (Fig. 1A). The calculated "bond strength" of these analogs also suggests that the isopolar and isosteric replacement makes the molecule significantly more stable and resistant toward potential enzymatic hydrolysis. These computational data, in a preliminary study, encouraged us to evaluate the behavior of prepared nucleotides exposed to biological systems.

### 2.3. Preliminary biological results

The non-hydrolysable nature of prepared ribonucleotide analogs was probed by performing a single-turnover gap-filling assay. Experiments with incorporation of UTP or bis-$CF_2$-UTP (**21**) by T7 RNA pol are shown in Fig. 2A. The position of the first templating dA in a dsDNA substrate following the T7 promoter sequence is labeled on the left side of the gel as U → A as the location of the incorporation of either natural UTP, left lane, or bis-$CF_2$-UTP (**21**), right lane. FL refers to the full length RNA. Each reaction contained CTP, GTP, ATP, and either UTP or bis-$CF_2$-UTP, as labeled above the corresponding lane. The assay was run for 30 min and then analyzed. As predicted, the reaction shown in the left lane, containing the natural substrate, was able to proceed to the full length. The reaction in the right lane, however, stalled due to the inability of the T7 RNA pol to utilize the bis-$CF_2$-UTP analog. However, this study alone does not demonstrate that the nonhydrolyzable derivative is incorporated at the enzymatic site. Studies to obtain crystal structures of such potential ternary complexes are under way.

Subsequently, we decided to test our nucleotide analogs with another class of polymerases. Even though the Tyr271 residue in DNA polymerase β prevents the incorporation of rNTPs to a moderate degree and can discriminate between dNTP and rNTP [21], it is known that pol β does not exhibit high fidelity with respect to base pairing and recognizing ribo- and deoxyribonucleotides. Thus, we tested the hydrolytic stability of our compound with DNA polymerase β. Incorporation of dTTP, UTP, and bis-$CF_2$-UTP (**21**) analog into a single-gapped DNA substrate with dA at the first templating position, using DNA pol β, indeed verified the non-hydrolysable nature of the bis-$CF_2$-UTP (**21**) analog. The position of the unextended primer is labeled P on the left side of the gel, and the incorporation of a single dNTP or NTP base is shown at P + 1.

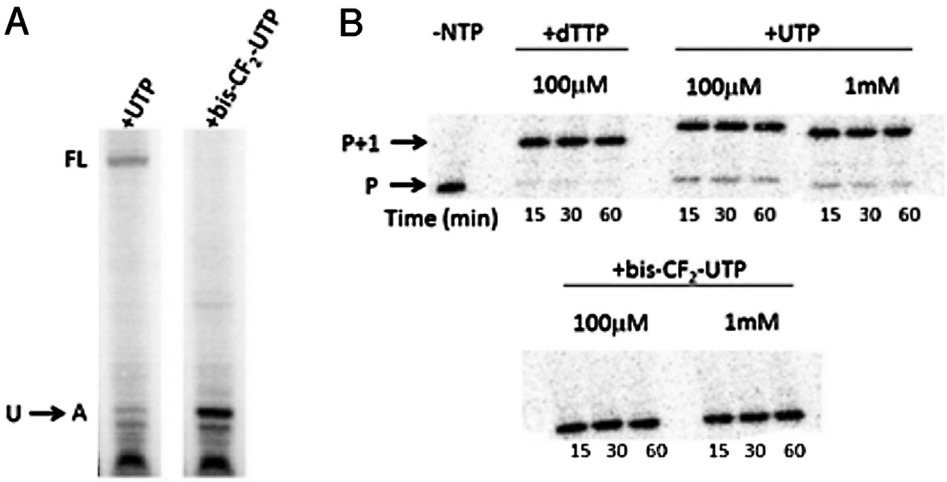



**Fig. 2.** Biochemical assays with bis-$CF_2$-UTP analog. (A) Single turnover assay with T7 RNA Polymerase. (B) Single turnover gap filling assay with DNA polymerase β.

The dNTP or NTP being incorporated, along with the concentration, is labeled across the top of the gel, and the reaction time below each lane. Both experiments indicate that unlike natural UTP and dTTP substrates, the difluoromethylene groups of the bis-$CF_2$-UTP inhibit incorporation into an RNA (Fig. 2A) or DNA (Fig. 2B) oligonucleotide and such nucleotides are not utilized by polymerases. These studies alone do not demonstrate their inhibitory nature in the active site. We believe that such nucleotide analogs stable to enzymatic hydrolysis will help uncover additional details of the mechanism of enzyme catalyzed processes and may be useful to the study of biochemical processes that rely on triphosphate concentration sensing. Such focused studies are underway.

## 3. Conclusion

Analogs of ribonucleotides (RNA) stable to potential enzymatic hydrolysis were synthesized and characterized. Computational investigations revealed that this class of compounds with a modified triphosphate exhibits the correct polarity and minimal steric effects compared to the natural molecule. Preliminary non-hydrolysable properties as well as the ability of the modified nucleotide to be recognized by enzymes were probed by performing single-turnover gap filling assays with T7 RNA polymerase and DNA polymerase β. Further crystallization study of enzyme–substrate complexes and X-ray crystal structure characterization will be required to show incorporation of the non-hydrolyzable RNA analogs in the active sites. Such studies are underway.

## 4. Experimental

Unless otherwise mentioned, all reagents were purchased from commercial sources. All NMR spectra were recorded on a Varian 400 MHz NMR spectrometer. Chemical shifts ($\delta$) are reported in part per million (ppm) relative to internal residual $CHCl_3$ in $CDCl_3$ ($\delta$ 7.25, $^1$H), internal residual HDO in $D_2O$ (pH ~8, $\delta$ 4.8, $^1$H), external chlorotrifluoromethane as the $^{19}$F standard ($\delta$ 0.00, $^{19}$F), and external phosphoric acid ($\delta$ 0.00, $^{31}$P) as a standard for $^{31}$P experiments. High resolution mass spectra were recorded in ESI+ mode on a high resolution mass spectrometer at the Mass Spectrometry facility, University of Arizona. HPLC analysis and purification of the nucleotide analogs **32–35** were performed on a Shimadzu HPLC system (SCL-10A VP, SPD-10A VP, and LC-8A) with Tosoh Bioscience DEAE-5PW 21.5 cm × 15 cm, 13 μm (0–60% 1 M TEAB, pH 8.0) and Shimadzu Premier C18 5 μm 250 mm × 23 mm preparative column.

Anhydrous THF was prepared by distillation over sodium wire. Diethyl difluoromethylphosphonate was obtained from triethylphosphite and chlorodifluoromethane according to a well-established protocol [22,23]. BMF$^4$TPA tetrabutylammonium salt **5** was prepared according to protocol developed by Prakash et al. [12]. For the preparation of protected tosylnucleosides **9**–**11**, we utilized reported procedure [24]. For synthesis of protected guanosine **13** we employed published protocols [16,17], Nucleoside tosylates **9**–**11** and **14** were prepared based on procedure reported by Burton and Flynn [23]

### 4.1. Preparation of $N^2$-[(dimethylamino)methylene] 2′,3′-dibenzoylguanosine **13**

Benzoyl chloride (2.33 g, 1.7 mmol) was added to the stirred solution of 4.4 g (7.58 mmol) of 5′-O-[(*tert*-butyl)diphenylsilyl]-$N^2$-[(dimethylamino)methylene]-guanosine **26** in 80 mL of anhydrous pyridine cooled to 0 °C. The resulting mixture was stirred at room temperature for 8 h. All volatiles were removed under reduced pressure and the residue was distributed between water (300 mL) and dichloromethane (300 mL). The organic fraction was collected and washed three times with water and dried over $MgSO_4$. The solvent was evaporated and without further purification the residue was dissolved in 100 mL of THF and 2.1 mL (36.5 mmol) of acetic acid. Subsequently 14.6 mL (14.6 mmol) of 1 M solution of TBAF in THF was added dropwise to the stirred solution. Reaction mixture was allowed to stir at room temperature overnight. Next all volatiles were evaporated and the residue was dissolved in dichloromethane (200 mL) and washed three times with water and dried over $MgSO_4$. The solvent was evaporated and the reaction mixture was subjected to chromatographic separation on silica gel with $CH_2Cl_2$/MeOH (9/1) as eluent affording 2.05 g of **13** (47%).

$^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 2.86 (s, 3H), 3.07 (s, 3H), 3.8–4.02 (m 2H), 4.5 (m, 1H), 6.1 (m, 1H), 6.17 (m, 1H), 6.4 (m, 1H), 7.2–7.48 (m, 6H), 7.67–7.9 (m, 5H), 8.52 (s, 1H), 10.4 (s, 1H). $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$: 35, 41.3, 61.6, 71.8, 73.6, 84.1, 87.8, 121.1, 128.4, 128.5, 128.9, 129.6, 129.7, 133.5, 133.6, 138.2, 149.8, 157.5, 158.2, 158.7, 165, 165.4.

### 4.2. General procedure for preparation of nucleosides-5′-tosylates **9**–**11** and **14**

Benzoyl protected respective ribonucleosides (500 mg) were dried three times by azeotropic evaporation of pyridine on a rotary evaporator under vacuum. After that, the flask was filled with argon and the ribonucleoside was redissolved in 25 mL of anhydrous pyridine. Subsequently, equimolar solution of *p*-toluenesulfonyl chloride in dry pyridine was added to the stirred solution of ribonucleoside precooled to 0 °C. The reaction mixture was removed from the ice and placed in the freezer (−20 °C) for 5–7 days. Then the flask was warmed to room temperature, pyridine was evaporated by rotatory evaporation and the residue was subjected for chromatographic separation on silica gel using $CH_2Cl_2$/MeOH (1:10) as eluent.

#### 4.2.1. 5′-Tosyl-N,N,2′,3′-tetrabenzoyladenosine **9**

Yield: 61%. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 2.34 (s, 3H), 4.44 (dd, $J$ = 11.2 Hz, 4.8 Hz, 1H), 4.5 (dd, $J$ = 10 Hz, 2.8 Hz, 1H), 4.66 (m, 1H), 5.93 (dd, $J$ = 4.8 Hz, $J$ = 5.6 Hz, 1H), 6.11 (dd, $J$ = 6 Hz, 5.2 Hz, 1H), 6.44 (d, $J$ = 5.6 Hz, 1H), 7.22–7.56 (m,14H), 7.23–7.93 (m, 10H), 8.29 (s, 1H), 8.54 (s, 1H) ppm. $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 21.8, 68.5, 71.5, 74.2, 81, 87.1, 127.9 128.2, 128.4, 128.6, 128.8, 128.8, 129, 129.7, 130, 130.1, 130.3, 132.3, 133.3, 134.1, 143.7, 145.8, 152.3, 152.6, 152.9, 165.2, 165.5, 172.5.

#### 4.2.2. 5′-Tosyl-2′,3′-O,N4-tribenzoylcytidine **10**

Yield: 43%. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 2.34 (s, 3H), 4.38 (dd, $J$ = 11.2 Hz, 3.2 Hz, 1H), 4.48–4.55 (m, 2H), 5.58–5.64 (m, 2H), 6.35–6.36 (m, 1H), 7.18–7.56 (m, 12H), 7.78–7.88 (m, 8H). $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 21.7, 68.2, 70.9, 74.4, 80.7, 97.5, 127.6, 127.9, 128.4, 128.45, 128.5, 129, 129.6, 129.7, 129.8, 129.9, 130.2, 144.7, 145.6, 154.7, 162.7, 165.1, 165.2, 166.5.

#### 4.2.3. 5′-Tosyl-2′,3′-O,N3-tribenzoyluridine **11**

Yield: 55%. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 2.44 (s, 3H), 4.4 (dd, $J$ = 11.2 Hz, 3.2 Hz, 1H), 4.49 (dd, $J$ = 11.2 Hz, 2.4 Hz, 1H), 4.55 (m, 1H), 5.6 (m, 1H), 5.7 (m, 1H), 5.87 (d, $J$ = 8 Hz, 1H), 6.35 (d, $J$ = 6 Hz, 1H), 7.24–7.63 (m, 12H), 7.66 (d, $J$ = 8 Hz, 1H), 7.8–7.93 (m, 7H), ppm. $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 21.7, 68.6, 71.2, 73.5, 80.6, 87.5, 103.4,127.9, 128.1, 128.4, 128.5, 128.6, 129.2, 129.7, 129.8, 130.3, 130.5, 131.1, 132, 133.8, 133.9, 135.2, 139.7, 145.8, 149.4, 161.7, 165.2, 165.3, 168.4.

#### 4.2.4. N-[(dimethylamino)methylene]-,2′,3′-dibenzoylguanosine **14**

Yield: 59%. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 2.2 (s, 3H), 3.03 (s, 3H), 3.18 (s, 3H), 4.27 (dd, $J$ = 11.6 Hz, 4 Hz, 1H), 4.4 (dd, $J$ = 11.6 Hz,

2.4 Hz, 1H), 4.5 (m, 1H), 6 (d, $J$ = 2.4 Hz 1H), 6.2 (m, 1H), 6.3 (m, 1H), 7–7.5 (m, 10H), 7.58 (s, 1H), 7.75–7.8 (m, 4H), 8.7 (s, 1H), 10.16 (s, 1H). $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$: 21.6, 35.3, 41.3, 67.6, 69.8, 73.9, 78.7, 87.9, 121, 127.6, 128.3, 128.4, 128.5, 128.6, 129.6, 129.7, 131.9, 133.7, 133.9, 145.3, 149.7, 157.3, 158.3, 158.8, 165, 165.2.

### 4.3. General procedure for preparation of nucleotide analogs by using reaction of $(Bu_4N^+)_5BMF^4TPA$ salt with benzoyl protected nucleoside 5′-tosylates

A 10 mL flask under argon atmosphere containing 2 mL of dry DMF was charged with 100 mg (0.12 mmol) of 2′-deoxy-5′-tosylnucleoside and 91.5 mg (0.6 mmol) of $(Bu_4N^+)_5BM^{F4}TPA$ salt. The resulting mixture was stirred for 1 h at 100 °C and then left at room temperature for two days. The conversion was checked by $^{19}$F NMR and HPLC using DEAE-5PW weak ion-exchange column with 1 M $Et_3NH^+HCO_3^-/H_2O$ as eluent. After the reaction achieved maximum conversion and most of the starting material was consumed, all volatiles were removed under reduced pressure and the remains were suspended in $H_2O$/MeOH/$NH_4OH$ (1:1:10) mixture and left at room temperature for three days. After deprotection, all undissolved residue was filtered off and the filtrate was concentrated under vacuum, then re-dissolved in water and subjected to HPLC purification on a preparative DEAE-5PW weak ion-exchange column with water/$Et_3NH^+HCO_3^-$ as eluent (0–60% gradient regime). The buffer solution was removed by evaporation and desired nucleotide analogs were dried under high vacuum.

#### 4.3.1. (α,β),(β,γ)-bis($CF_2$) ATP **19**

Yield: 29%. $^1$H NMR (400 MHz, $D_2O$): 1.05 (t, $J$ = 7.6 Hz, 27H, $Et_3NH^+$), 2.97 (q, $J$ = 7.6 Hz, 18H, $Et_3NH^+$) 4.07–4.12 (m, 2H), 4.15–4.19 (m, 1H), 4.25–4.42 (m, 2H), 4.33–4.38 (m, 1H), 4.55–4.59 (m, 1H), 5.92 (d, $J$ = 6.4 Hz, 1H) 8.03 (s, 1H), 8.36 (s, 1H) ppm. $^{19}$F NMR (376 MHz, D2O): −119.2 (t, $J$ = 72.2 Hz), −120.3 (t, $J$ = 83.8 Hz) ppm. $^{31}$P NMR (162 MHz, $D_2O$): 1.8–4.8 (m, 2P), 10.9–13.2 (m, 1P) ppm. HRMS: calculated for [M+$Na^+$] $C_{12}H_{16}F_4N_5O_{11}P_3Na$ 597.9887, found 597.9891.

#### 4.3.2. (α,β),(β,γ)-bis($CF_2$) CTP **20**

Yield: 33%. $^1$H NMR (400 MHz, $D_2O$): 1.1 (t, $J$ = 7.2 Hz, 27H, $Bu_3NH^+$), 2.98 (q, $J$ = 7.2 Hz, 18H, $Et_3NH^+$), 4.07–4.11 (m, 2H), 4.15–4.2 (m, 2H), 4.23–4.27 (m, 1H), 5.86 (d, $J$ = 4.8 Hz, 1H), 5.89 (d, $J$ = 7.6 Hz, 1H), 7.99 (d, $J$ = 7.6 Hz, 1H) ppm. $^{19}$F NMR (376 MHz, $D_2O$): −118.3 (t, $J$ = 75.2 Hz), −118.8 (t, 76.7 Hz) ppm. $^{31}$P NMR (162 MHz, $D_2O$): 2.56–4.42 (m, 2P), 11.87–14.58 (m, 1P) ppm. HRMS: calculated for [M+$Na^+$] $C_{11}H_{15}F_4N_3O_{11}P_3$ 573.9775, found 573.9780.

#### 4.3.3. (α,β),(β,γ)-bis(CF2) UTP **21**

Yield: 22%. $^1$H NMR (400 MHz, $D_2O$): 1.12 (t, $J$ = 7.3 Hz, 27H, $Et_3NH^{+)}$, 3.04 (q, $J$ = 7.3 Hz, 18H, $Et_3NH^+$), 4.07–4.12 (m, 1H), 4.13–4.19 (m, 2H), 4.2–4.25 (m, 2H), 5.78 (d, $J$ = 8 Hz, 1H), 5.81 (d, $J$ = 4.8 Hz, 1H), 7.84 (d, $J$ = 8 Hz, 1H) ppm. $^{19}$F NMR (376 MHz, $D_2O$): −119.36 (t, $J$ = 72.94 Hz), −119.37 (t, $J$ = 76.7 Hz) ppm. $^{31}$P NMR (162 MHz, $D_2O$): 2.32–3.9 (m, 2P), 9.64–12.05 (m, 1P) ppm.

#### 4.3.4. (α,β),(β,γ)-bis($CF_2$) dGTP **22**

Yield: 6%. $^1$H NMR (400 MHz, $D_2O$): 1.1 (t, $J$ = 7.3 Hz, 27H, $Et_3NH^+$), 3.02 (q, $J$ = 7.3 Hz, 18H, $Et_3NH^+$) 4.11–4.22 (m, 4H), 4.36–4.41 (m, 1H), 5.72–5.81 (m, 1H), 8.11 (s, 1H) ppm. $^{19}$F NMR (376 MHz, $D_2O$): −118.9 (t, $J$ = 76.7 Hz) ppm. $^{31}$P NMR (162 MHz, $D_2O$): 2.37–4.25 (m, 2P), 11.7–14.4 (m, 1P) ppm. HRMS: calculated for [M+$H^+$] $C_{12}H_{15}F_4N_5O_{12}P_3$ 589.9872, found 589.9875.

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