

Synthesis of Nucleoside Triphosphates that Contain an Aminooxy Function for “Post-Amplification Labelling”

Emmanuelle Trévisiol,^[a] Eric Defrancq,^[a] Jean Lhomme,^{*,[a]} Ali Laayoun,^{*,[b]} and Philippe Cros^[b]

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Preparation of the uridine and adenosine triphosphates **1** and **2** bearing a linker with a terminal aminooxy group is described. Both **1** and **2** react readily with the aldehydic

fluorescein derivative **15**. They could each be incorporated into a 330-mer fragment with T7 RNA polymerase.

Introduction

The DNA chip technology field has developed dramatically during the last years, and many applications including gene discovery and expression, detection of mutations or polymorphism, and mapping have been reported.^[1] Most of the conventional oligonucleotide microchip technologies use fluorescent readouts to detect duplex formation, and the fluorescent labels are generally introduced by enzymatic reactions. Organic fluorophores are chemically introduced into primers or into nucleoside triphosphates and are then incorporated by PCR amplification or with DNA or RNA polymerases.^[1] Many modified nucleoside triphosphates bearing a reporter group (e.g., fluorescent or luminescent group) have been described.^[2–5] The label can be attached to various positions of the nucleobase. A number of different fluorophores such as fluorescein derivatives,^[2a] digoxigenin,^[2b] cyanine derivatives...^[2c] were introduced at the C-5 position of pyrimidines with an aminoalkynyl linker. Dansyl and fluorescein derivatives were introduced at the C-8 position of 2'-deoxyadenosine^[3a] or at C-4 of 2'-deoxycytidine.^[4b] A fluorescent label was covalently attached to the 2'- and 3'-hydroxy groups of the ribose ring of ATP.^[4] However, these bulky reporter groups can perturb the amplification efficiency and specificity.

As an alternative procedure for labelling amplified RNA targets without affecting the amplification efficiency and incorporation fidelity, we developed a new strategy called “post-amplification labelling”.^[5] In this approach, activated nucleotides are incorporated before the reaction with the fluorescent label. However, this procedure requires rapid and selective derivatization of the amplified RNA (amplicons) by the fluorescent label. Furthermore, the derivat-

ized amplicons should be stable for the subsequent readout. In our strategy, the coupling reaction is between nucleotides containing a reactive aminooxy group and an aldehydic fluorescent label. Derivatization of oligonucleotides through an aminooxy–aldehyde coupling reaction which forms an oxime ether was previously shown to be highly selective and rapid ($t_{1/2} < 30$ min).^[6] Such oxime ethers are stable under physiological conditions. In the present paper we report on the preparation of nucleoside triphosphates **1** and **2**, both of which incorporate a linker with a terminal aminooxy group. The linker is attached to the C-5 and C-6 positions of uridine triphosphate **1** and adenosine triphosphate **2**, respectively (Figure 1). These two positions were selected to make the aminooxy group accessible to the fluorescent reagent and to reduce the interaction of the fluorophore with the nucleic acid. Preparation of the corresponding fluorescein label **15** which contains an aldehyde group, and its subsequent reaction with the aminooxy derivatives are also reported. Incorporation into a 330-mer RNA fragment by T7 RNA polymerase is described.

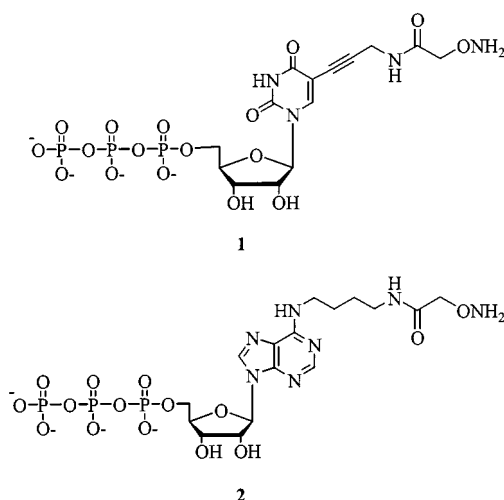


Figure 1. Structure of triphosphates **1** and **2**

^[a] LEDSS, Chimie Bioorganique, UMR CNRS 5616, Université Joseph Fourier, B. P. 53, F-38041 Grenoble Cedex 9, France
Fax: (internat.) + 33-4/76514382
E-mail: Jean.Lhomme@ujf-grenoble.fr

^[b] BioMérieux, Laboratoire Sondes Nucléiques, Chemin de l'Orme, F-69280 Marcy-l'Etoile, France
E-mail: alaayoun@ml.biomerieux.fr

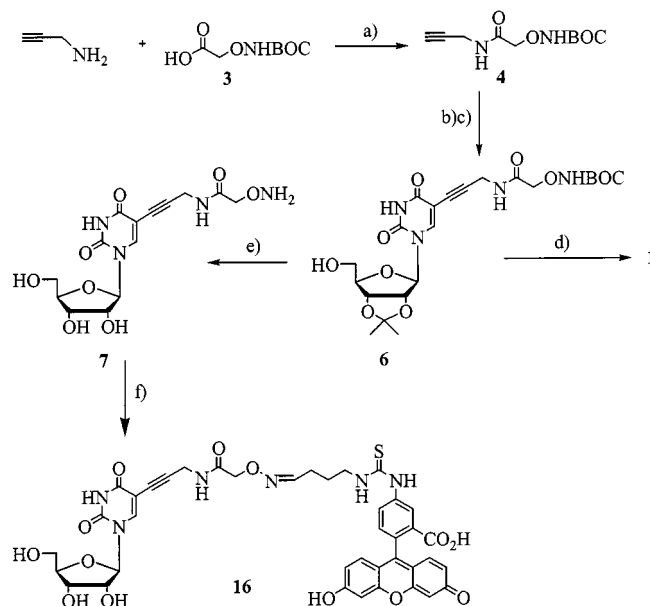
Results and Discussion

Preparation of the Uridine Triphosphate Derivative 1

A number of methods have been developed to phosphorylate ribonucleosides into ribonucleoside 5'-triphosphates.^[7] Most of them involve preparation of the mononucleotide and its conversion into a reactive intermediate such as a morpholidate or an imidazolidate, followed by displacement of the leaving group with pyrophosphate.^[7] Eckstein developed an alternative method by using 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (salicyl phosphorochloridite) which allows direct triphosphorylation of nucleosides.^[8] This procedure requires protection of the 2'- and 3'-hydroxy groups of the sugar, and, if applied to the present target nucleotide **1**, additional protection of the highly reactive aminoxy function. Acetonide and BOC, which can be removed simultaneously under acidic conditions, were selected as protecting groups, allowing the use of basic conditions for the introduction of the functionalized chain onto the uracil ring.

To introduce an alkyl substituent at the C-5 position of uridines, the usual method is to couple 5-iodo or 5-mercuro uridine derivatives with alkenes or alkynes, catalysed by palladium, under basic conditions.^[10] We selected the method described by Hobbs,^[10a] because this coupling reaction was already applied successfully to alkynes containing an amino group.^[10a] Preparation of the protected nucleoside **6** was accomplished as indicated in Scheme 1. The alkynyl chain **4** was obtained in 85% yield from the coupling between the protected *O*-(carboxymethyl)hydroxylamine **3** with propargylamine in the presence of isobutylchloroformate.^[11] The alkynyl chain **4** was then introduced at the C-5 position of uridine by Hobbs' procedure, affording the desired nucleoside **5** in a 60% yield. We then protected the hydroxy groups at positions 2' and 3' with an acetonide, using ethyl orthoformate in acetone with *p*-toluenesulfonic acid as catalyst. The BOC protection of the aminoxy residue remained unchanged under these conditions. The protected nucleoside **6** was thus obtained from commercial 5-iodouridine simply and in 45% overall yield, and the reaction could also be performed on large scale.

At this nucleoside stage we investigated the use of mild experimental conditions, suitable even at the most sensitive triphosphate stage, for the removal of the BOC and acetonide protecting groups. We studied deprotection of **6** in various proportions of trifluoroacetic acid/water mixtures. Treatment of **6** in 25% aqueous trifluoroacetic acid at room temperature for 1 h led to total removal of both the acetal and BOC protecting groups. However, the deprotected nucleoside **7** was not obtained. Instead, the acetone liberated by acetonide hydrolysis was quantitatively trapped by the deprotected oxyamine, with the oxime ether $-O-N=C(CH_3)_2$ forming. Attempts to cleave the two protecting groups selectively and successively were unsuccessful. The solution to the problem was to add ten equivalents of methoxyamine hydrochloride to the deprotection medium to trap the liberated acetone quantitatively. Under these con-



Scheme 1. a) *N*-Methylmorpholine, isobutyl chloroformate; b) 5-iodouridine, CuI, NEt₃, Pd[P(Ph)₃]₄; c) acetone, ethyl orthoformate, *p*-toluenesulfonic acid; d) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one in dioxane, tributylammonium pyrophosphate in DMF then aq. TFA, CH₃ONH₃⁺Cl⁻; e) aq. TFA, CH₃ONH₃⁺Cl⁻; f) **15**, H₂O/DMSO

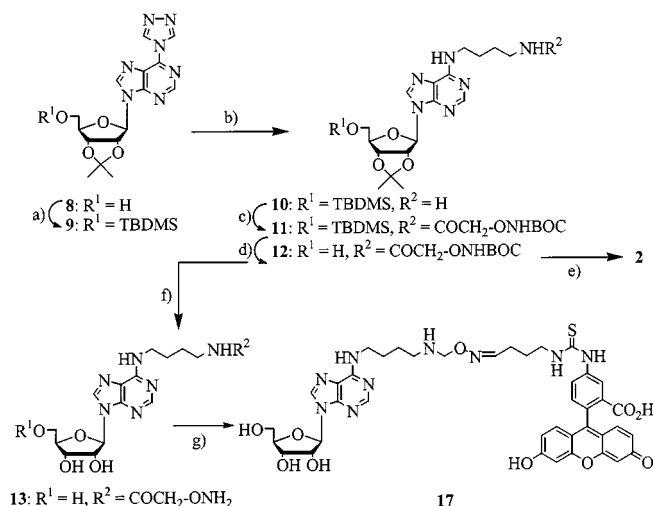
ditions, deprotected nucleoside **7** was obtained in 50% yield from **6** (the yield was low due to loss of product during purification).

We then prepared nucleotide **1** using Eckstein's procedure and acidic hydrolysis. Reaction of the protected nucleoside **6** with salicyl phosphorochloridite was performed under strictly anhydrous conditions to form a cyclic intermediate which on subsequent reaction with pyrophosphate and oxidation by iodine furnished the corresponding protected triphosphate. The intermediate product was isolated by anion-exchange chromatography and was identified by ³¹P- and ¹H-NMR spectroscopy. The protecting groups were then cleaved under the acidic conditions previously found suitable at the nucleoside level, and the corresponding nucleotide **1** was purified by reverse-phase chromatography. Nucleotide **1** was obtained as the sodium salt in 25% overall yield. The structure was established by ¹H- and ³¹P-NMR spectroscopy and confirmed by ES-MS.

Preparation of the Adenosine Nucleotide Derivative 2 (Scheme 2)

To functionalize adenosine at the C-6 position, we chose the procedure developed by Robins and co-workers: The amine at C-6 was converted into the good 1,2,4-triazolo leaving group.^[12] Preparation of the precursor nucleoside **12** was accomplished by the straightforward route depicted in Scheme 2.

The triazolo moiety was introduced by treatment of commercial 2',3'-*O*-isopropylideneadenosine with 1,2-bis[(dimethylamino)methylene]hydrazine dihydrochloride; the 5'-hydroxy group in **8** was protected as the *tert*-butyldimeth-



Scheme 2. a) TBDMS-Cl, pyridine; b) $H_2N-[CH_2]_4-NH_2$, CH_3CN , $50^\circ C$; c) 3, *N*-methylmorpholine, isobutyl chloroformate; d) 1M TBAF in THF; e) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one in dioxane, tributylammonium pyrophosphate in DMF then aq. TFA, $CH_3ONH_3^+Cl^-$; f) aq. TFA, $CH_3ONH_3^+Cl^-$; g) 15, $H_2O/DMSO$

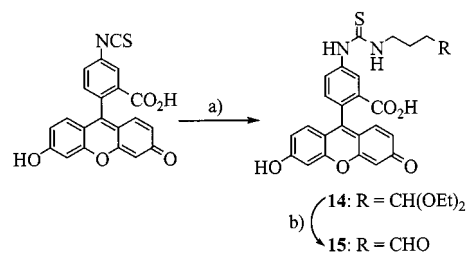
ylsilyl ether **9**. Subsequent treatment of **9** with excess 1,4-diaminobutane gave compound **10** in 80% yield. The presence of the TBDMS protecting group in **10** rendered the molecule relatively lipophilic, therefore elimination of excess 1,4-diaminobutane by silica gel chromatography was facile. The aminooxy moiety was then introduced by coupling nucleoside **10** with the protected *O*-(carboxymethyl)-hydroxylamine **3** in the presence of isobutyl chloroformate under strictly anhydrous conditions. Finally, the TBDMS protection was removed with tetrabutylammonium fluoride (TBAF) to afford nucleoside **12** in 56% overall yield from the triazolo nucleoside **8**. Nucleoside **12** was then transformed into the triphosphate **2** by the same procedure as that described above for the uridine derivative **1**. The structure of the nucleoside triphosphate **2** was confirmed by 1H - and ^{31}P -NMR spectroscopy and by ESMS.

Preparation of the Aldehydic Fluorophore 15

Compound **15** was prepared in a two-step reaction (Scheme 3) from the commercially available fluorescein isothiocyanate (FITC). Reaction of 4-aminobutyraldehyde diethylacetal with FITC in DMF under strictly anhydrous conditions afforded the protected fluorophore **14** quantitatively. Hydrolysis of the acetal was performed under acidic conditions with a 30% aqueous solution of acetic acid to give the aldehydic fluorophore **15**. Compound **15** was characterized by MS and NMR spectra and the purity was confirmed by elemental analysis.

Coupling Reaction Between Nucleosides 7 and 13 and the Aldehydic Fluorophore 15

To check the efficiency of the coupling reaction, the fluorescent label **15** was allowed to react with nucleosides **7**



Scheme 3. a) $H_2N-[CH_2]_3-CH(OEt)_2$, DMF; b) 30% aq. AcOH

and **13**. Reactions were carried out in water containing 10% DMSO, affording the corresponding oxime ethers **16** and **17**. No side reactions could be detected. The structures of **16** and **17** were established by 1H -NMR spectroscopy using DQF-COSY sequences and were confirmed by HR-MS. In particular, the observation of two signals for the oximic protons confirmed that a diastereoisomeric mixture of *Z/E* compounds was obtained. The *Z/E* ratios, determined by 1H NMR, were 65:35 and 80:20 for **16** and **17**, respectively. To determine whether the spectral properties of the fluorescent moiety were modified in adducts **16** and **17**, we measured their absorption and fluorescence in Tris HCl buffer (pH = 9) to make a comparison with **15** possible. The three compounds **15**, **16** and **17** have the same excitation and emission maxima, at 490 and 515 nm, respectively. Furthermore, the fluorescence of the adducts was not quenched.

We studied the incorporation of the modified nucleoside triphosphates **1** and **2** by T7 RNA polymerase into 330 mer fragments of 16S RNA from *Mycobacteria tuberculosis*. Different modified/natural nucleotide ratios were used (from 0 to 100% modified nucleotides). The transcription products were then labelled by reaction with the aldehydic fluorophore **15** (10 equiv.) at room temperature for 30 min, and were subsequently analyzed by gel electrophoresis under denaturing conditions. Typical results for a transcription assay with 100% modified triphosphate **1** are depicted in Figure 2 (lines 3 and 6). The labelled transcripts (lines 3 and 6) result in a fluorescent band with the same electrophilic mobility as the transcripts resulting from incubation of the UTP nucleotide (line 4). Furthermore, the absence of fluorescent transcripts (line 2 and 5) after incubation of the natural transcripts with fluorophore **15** indicate the selectivity of the labelling. These results demonstrate the incorporation of the modified triphosphate **1** into the RNA fragment and the efficiency and the selectivity of the labelling reaction. The purine nucleotide **2** was also incorporated under comparable conditions, but to a smaller extent. Inhibition occurred when 100% nucleotide **2** was used. However, incorporation and selective labelling by fluorophore **15** proceeded efficiently when a 70:30 mixture of the modified triphosphate **2** and ATP was used.

Conclusion

Two new nucleoside triphosphates containing a highly reactive aminooxy moiety were prepared in satisfactory yield. The coupling reaction with an aldehydic fluorophore was

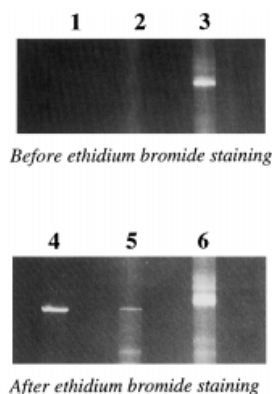


Figure 2. Gel electrophoresis analysis of the transcription products resulting from incorporation of the modified triphosphate **1** (100% **1**; 0% UTP) compared to incorporation of UTP; transcripts are visualized by UV before and after ethidium bromide staining; lines 1 and 4: transcripts resulting from incorporation of UTP; lines 2 and 5: transcripts resulting from incorporation of UTP and subsequent incubation with fluorophore **15**; lines 3 and 6: modified transcripts resulting from incorporation of **1** and subsequent incubation with **15**

investigated at the nucleoside level and we observed the same high reactivity as previously described.^[6] Furthermore, derivatization of the nucleosides by the fluorescent label did not affect the spectral properties of the fluorophore. Incorporation into a 330-mer RNA fragment by T7 RNA polymerase was successful and remarkable efficiency was observed for the modified uridine nucleotide **1**.

Experimental Section

General Methods: All commercially available chemical reagents were used without further purification. 2-Chloro-4*H*-1,2,3-dioxaphosphorin-4-one, dry dioxane, DMF and pyridine (containing less than 0.01% water) were purchased from Aldrich and were used as supplied. 1,2-Bis[(dimethylamino)methylene]hydrazine dihydrochloride was prepared as described.^[12] – TLC: Merck silica gel 60 F₂₅₄ plates. – Preparative column chromatographies: Merck silica gel 60 (200–63 µm) or Merck LiChroprep RP 18 silica gel (40–63 µm). – Analytical HPLC was performed with Millipore-Waters equipment (two M-510 pumps, solvent gradient M680) using a UV detector (M490 or diode array 990). Reverse-phase analysis was performed with a µ-bondapak C-18 column (Waters) using a methanol/water (pH = 2.5) gradient, flow 1 mL/min for 10 min. – Anion-exchange chromatography was carried out with a Protein Pak DEAE 8HR column (Waters, 8 µm, 10 × 100 mm) using Tris-HCl 20 mM (pH = 7.6) as eluent A and Tris-HCl 20 mM, NaCl 0.5 M (pH = 7.6) as B, with a flow rate of 2 mL/min. The gradient used was: 0–35% B in 40 min. – M.p.: Electrothermal Serie IA9100 apparatus, uncorrected values. – UV: Perkin-Lambda 15UV/VIS. – IR: Perkin-Elmer Impact 400 spectrophotometer. – NMR: Bruker AC200, Avance 300 and Varian Unity plus 500 spectrometers. ¹H NMR: CDCl₃ as solvent, δ_H = 7.24, [D₆]DMSO as solvent, δ_H = 2.49; for ¹³C NMR, CDCl₃ as solvent, δ_C = 77.5, [D₆]DMSO as solvent, δ_C = 39.5. The DQF-COSY spectra were run with 2048 points in *t*₂, 400 points in *t*₁ and 128 scans for each *t*₁ values. – MS: Delsi-Nermag R10–10 or VG Platform (Micro-mass) spectrometers. – Elemental analyses were performed by “Service Central de Microanalyse du CNRS” and HRMS by “Centre Régional de Mesures Physiques de l’Ouest”. In several cases

correct elemental analysis could not be obtained due to the polar and hygroscopic character of the compounds.

2-[*N*-(*tert*-Butoxycarbonyl)aminoxy]-*N*-(prop-2-ynyl)acetamide (4**):** To a solution of acid **3** (5 g, 26 mmol) in dry THF (100 mL) cooled at 0°C under argon were added successively *N*-methylmorpholine (2.86 mL, 26 mmol), isobutyl chloroformate (3.3 mL, 26 mmol) and then, after 15 min, propargylamine (1.78 mL, 26 mmol); the reaction mixture was stirred at room temp. for 1 h. After filtration, the solvent was evaporated and the oily residue obtained was dissolved in Et₂O. The organic layer was washed successively with 0.1 N aq. NaOH, 1 N aq. HCl, and brine, then it was dried (Na₂SO₄) and concentrated under reduced pressure. The crude product was purified by silica gel chromatography (AcOEt/cyclohexane, 70:30) to give compound **4** (5 g, 85%) as a white powder, m.p. 69–70°C. – IR (KBr): $\tilde{\nu}$ = 3384 cm⁻¹, 3257, 3165, 2946, 1746, 1659, 1549. – ¹H NMR (200 MHz, CDCl₃): δ = 1.47 [s, 9 H, C(CH₃)₃], 2.17 (t, *J* = 2.4 Hz, 1 H, C–H), 4.07 (dd, 2 H, CH₂–N), 4.31 (s, 2 H, CH₂–ONH), 7.67 (br., 1 H, NH), 8.37 (br., 1 H, NH). – ¹³C NMR (50 MHz, CDCl₃): δ = 28.0 (CH₃), 28.4 (CH₂), 75.9 (CH₂), 79.1 (CH), 82.9 (quat), 157.8 (quat), 168.9 (quat). – MS (DCI); *m/z*: 229 [M + H]⁺. – C₁₀H₁₆N₂O₄ (228.2): calcd. C 52.62, H 7.07, N 12.27; found C 52.59, H 7.09, N 12.33.

5-{2-[*N*-(*tert*-Butoxycarbonyl)aminoxy]-*N*-(prop-2-ynyl)acetamidyl}uridine (5**):** To a mixture of 5-iodouridine (1 g, 2.56 mmol) and copper iodide (97 mg, 0.5 mmol) in degassed DMF (20 mL) was added, under argon, triethylamine (0.72 mL, 5.12 mmol) and alkyne **4** (1.75 g, 7.68 mmol). The reaction was stirred in the dark for 10 min and then tetrakis(triphenylphosphane)palladium (0.30 g, 0.25 mmol) was added. After stirring under argon for 2 h, the solvent was evaporated in vacuum and the crude product obtained was purified by silica gel chromatography (AcOEt/MeOH, 90:10 then 80:20) to give nucleoside **5** (0.75 g, 62%) as a pale yellow powder, m.p. 101–102°C. – IR (KBr): $\tilde{\nu}$ = 3364 cm⁻¹, 2987, 2931, 1721, 1696. – UV (EtOH): λ_{max} (ε) = 288 nm (12000). – ¹H NMR (200 MHz, [D₆]DMSO): δ = 1.40 [s, 9 H, C(CH₃)₃], 3.60 (m, 2 H, 5',5''-H), 3.85 (q, 1 H, 4'-H), 4.05–3.96 (m, 2 H, 2'-H and 3'-H), 4.14 (d, 2 H, CH₂–N), 4.19 (s, 2 H, CH₂–ONH), 5.04 (br. d, 1 H, 3'-OH), 5.16 (br. t, 1 H, 5'-OH), 5.36 (br. d, 1 H, 2'-OH), 5.74 (d, 1 H, 1'-H), 8.21 (s, 1 H, 6-H), 8.42 (br. t, 1 H, NH), 10.24 (br. s, 1 H, NH), 11.63 (br. s, 1 H, NH). – ¹³C NMR (50 MHz, [D₆]DMSO): δ = 27.7 (CH₃), 28.2 (CH₂), 60.3 (CH₂), 69.4 (CH), 73.6 (CH), 74.3 (CH₂), 80.5 (CH), 84.8 (CH), 87.9 (CH), 89.0 (quat), 97.9 (quat), 143.8 (CH), 149.5 (quat), 156.6 (quat), 161.4 (quat), 167.6 (quat). – MS (FAB, glycerol matrix); *m/z*: 469 [M – H]⁻. – C₁₉H₂₆N₄O₁₀·0.5 H₂O: calcd. C 47.58, H 5.68, N 11.69; found C 47.82, H 5.66, N 11.63.

Protected Nucleoside 6: To a suspension of nucleoside **5** (0.5 g, 1.1 mmol) in acetone (3 mL), *p*-toluenesulfonic acid (20 mg, 0.1 mmol) and ethyl orthoformate (0.36 mL, 2.1 mmol) were added. The reaction mixture was stirred under argon at room temp. for 3 h then diluted with CH₂Cl₂. The organic layer was washed with 10% aq. NaHCO₃ followed by brine, dried (Na₂SO₄) and concentrated to afford compound **6** (0.40 g, 73%) as a pale yellow powder, m.p. 110–112°C. – IR (KBr): $\tilde{\nu}$ = 3426 cm⁻¹, 3289, 3073, 2981, 2931, 1715, 1462, 1110. – UV (EtOH): λ_{max} (ε) = 288 nm (12000). – ¹H NMR (200 MHz, [D₆]DMSO): δ = 1.27 (s, 3 H, CH₃), 1.40 [s, 9 H, C(CH₃)₃], 1.47 (s, 3 H, CH₃), 3.56 (m, 2 H, 5',5''-H), 4.06 (q, 1 H, 4'-H), 4.14 (d, 2 H, CH₂–N), 4.19 (s, 2 H, CH₂–ONH), 4.73 (dd, 1 H, 3'-H), 4.91 (dd, 1 H, 2'-H), 5.11 (br. t, 1 H, 5'-OH), 5.82 (d, 1 H, 1'-H), 8.12 (s, 1 H, 6-H), 8.40 (br. t, 1 H, NH), 10.22 (br. s, 1 H, NH), 11.66 (br. s, 1 H, NH). – ¹³C NMR (50 MHz, [D₆]DMSO): δ = 24.9 (CH₃), 26.8 (CH₃), 27.7

(CH₃), 28.1 (CH₂), 60.9 (CH₂), 74.0 (CH), 74.3 (CH), 80.1 (CH), 80.5 (CH₂), 83.6 (CH), 86.6 (CH), 89.1 (CH), 91.1 (quat), 97.9 (quat), 112.8 (quat), 144.8 (CH), 149.2 (quat), 156.6 (quat), 161.4 (quat), 167, 5 (quat). – MS (FAB, *m*NBA matrix); *m/z*: 511 [M + H]⁺. – HRMS (FAB, *m*NBA matrix): calcd. 511.2040; found 511.2040. – C₂₂H₃₀N₄O₁₀·0.5H₂O: calcd. C 50.85, H 6.02, N 10.79; found C 51.08, H 6.03, N 10.44.

Deprotection of Nucleoside 6 To Obtain 7: To a solution of compound **6** (50 mg, 0.1 mmol) in CHCl₃ (3 mL) containing methoxyamine hydrochloride (82 mg, 0.9 mmol) was added 50% aq. TFA solution (3 mL). The reaction mixture was stirred at room temp. for 1 h after which the solvent was evaporated. The crude product was purified by reverse-phase silica gel chromatography to give compound **7** (20 mg, 50%) as a white powder. – IR (KBr): $\tilde{\nu}$ = 3355 cm⁻¹, 3053, 2914, 2816, 1706, 1543, 1461, 1282, 1110. – UV (H₂O): $\lambda_{\max}$ (ϵ) = 288 nm (12000). – ¹H NMR (300 MHz, [D₆]DMSO): δ = 3.59 (m, 2 H, 5',5''-H), 3.84 (m, 1 H, 4'-H), 4.01 (s, 3 H, CH₂-ONH and 3'-H overlapping), 4.05 (m, 1 H, 2'-H), 4.12 (d, 2 H, CH₂-N), 5.04 (d, 1 H, 3'-OH), 5.17 (t, 1 H, 5'-OH), 5.38 (d, 1 H, 2'-OH), 5.73 (d, 1 H, 1'-H), 6.50 (br. s, 2 H, ONH₂), 8.21 (s, 1 H, 6-H), 8.27 (br. t, 1 H, NH), 11.63 (br. s, 1 H, NH). – ¹³C NMR (75 MHz, [D₆]DMSO): δ = 28.5 (CH₂), 60.3 (CH₂), 69.4 (CH), 73.5 (quat), 73.8 (CH), 74.0 (CH₂), 84.7 (CH), 87.9 (CH), 89.5 (quat), 98.0 (quat), 143.6 (CH), 149.5 (quat), 161.4 (quat), 169.4 (quat). – MS (FAB, glycerol matrix); *m/z*: 371 [M + H]⁺.

Nucleoside Triphosphate 1: The protected nucleoside **6** (0.20 g, 0.4 mmol) was dissolved in anhydrous pyridine and coevaporated twice to dryness in vacuum. To the residue obtained, pyridine (0.4 mL), dioxane (1.2 mL) and 2-chloro-4*H*-1,2,3-dioxaphosphorin-4-one solution in dioxane (1 mL, 0.52 mL, 0.52 mmol) was added under argon. After 20 min, a mixture of a 0.5 M solution of tributylammonium pyrophosphate in anhydrous DMF (1.28 mL, 0.64 mmol) and tri-*n*-butylamine (0.52 mL) was added. The reaction mixture was stirred for 30 min, then a solution of 1% iodine (8 mL, 0.31 mmol) in pyridine/water (98:2) was added. After 20 min, excess iodine was destroyed by addition of 5% aq. NaHSO₃ solution; after 10 min, the solution was concentrated to dryness. The residue obtained was dissolved in water and the aq. layer was washed with CH₂Cl₂. The crude product was purified by HPLC on a DEAE column to give the intermediate protected triphosphate which was then desalted by C18 reverse-phase flash chromatography (0.137 mmol, 34%). *t_R* = 35 min. – ³¹P NMR (160 MHz, D₂O): δ = -21.42 (t, P β), -10.87 (d, *J* = 18 Hz, P α), -5.54 (d, *J* = 20 Hz, P γ). – ¹H NMR (400 MHz, D₂O): δ = 1.45 [s, 12 H, CH₃ and C(CH₃)₃], 1.66 (s, 3 H, CH₃), 4.25 (m, 3 H, 4'-H and 5',5''-H), 4.29 (m, 2 H, CH₂-N), 4.38 (s, 2 H, CH₂-O), 4.53 (m, 1 H, 3'-H), 5.07 (m, 1 H, 2'-H), 5.97 (d, 1 H, 1'-H), 7.98 (s, 1 H, 6-H). – The protected nucleotide (0.075 mmol) was then dissolved in water (37.5 mL) and a 50% aq. solution of TFA (37.5 mL) and methoxyamine hydrochloride (63 mg, 0.75 mmol) were added. The mixture was stirred for 30 min, then the pH was adjusted to 8.5 by addition of aq. 0.1 N NaOH, and then the solvent was evaporated to dryness in vacuum. The crude product was purified by reverse-phase chromatography (C18) by eluting with H₂O. The nucleotide **1** was obtained with 25% overall yield. *t_R* = 30 min. – ³¹P NMR (160 MHz, D₂O): δ = -20.93 (t, P β), -10.76 (d, *J* = 19 Hz, P α), -5.51 (d, *J* = 19 Hz, P γ). – ¹H NMR (400 MHz, D₂O): δ = 4.29–4.40 (overlapping m, 3 H, 4'-H and 5',5''-H), 4.29 (m, 2 H, CH₂-N), 4.32 (s, 2 H, CH₂-O), 4.41 (s, 1 H, 3'-H), 4.49 (t, 1 H, 2'-H), 5.99 (d, 1 H, 1'-H), 8.25 (s, 1 H, 6-H). – MS (electrospray, negative mode); *m/z*: 609 [M - H]⁻, 630 [M - 2 H + Na]⁻.

9-(2,3-*O*-Isopropylidene- β -D-ribofuranosyl)-6-(1,2,4-triazol-4-yl)-purine (8): A solution of commercial 2',3'-isopropylideneadenosine

(1 g, 3.2 mmol) and 1,2-bis[(dimethylamino)methylene]hydrazine dihydrochloride (1.4 g, 6.5 mmol) in anhydrous pyridine (20 mL) was heated at 100°C for 48 h. The oily residue obtained after evaporation of pyridine under reduced pressure was dissolved in EtOAc and the organic layer was washed with 1 N aq. HCl, then with brine, dried (Na₂SO₄) and concentrated to give compound **8** (0.70 g, 60%) as a white powder, m.p. 180°C. – IR (KBr): $\tilde{\nu}$ = 3386 cm⁻¹, 3122, 2942, 1611, 1511. – UV (MeOH): $\lambda_{\max}$ (ϵ) = 275 nm (9200), 258 (5700). – ¹H NMR (200 MHz, [D₆]DMSO): δ = 1.33 (s, 3 H, CH₃), 1.55 (s, 3 H, CH₃), 3.55 (m, 2 H, 5',5''-H), 4.33 (1 m, H, 4'-H), 4.60 (br. t, 1 H, 5'-OH), 5.02 (dd, 1 H, 3'-H), 5.41 (dd, 1 H, 2'-H), 6.29 (dd, 1 H, 1'-H), 8.91 (s, 1 H, 2-H), 8.96 (s, 1 H, 8-H), 9.61 (s, 2 H, CH triazolo). – ¹³C NMR (50 MHz, [D₆]DMSO): δ = 25.1 (CH₃), 27.0 (CH₃), 61.2 (CH₂), 81.5 (CH), 83.9 (CH), 87.4 (CH), 90.6 (CH), 113.0 (quat), 122.5 (quat), 140.9 (CH), 142.6 (quat), 145.9 (CH), 152.0 (CH), 153.1 (quat). – MS (DCI); *m/z*: 360 [M + H]⁺, 188 [M - sugar]⁺.

9-[5-*O*-(*tert*-Butyldimethylsilyl)-2,3-*O*-isopropylidene- β -D-ribofuranosyl]-6-(1,2,4-triazol-4-yl)purine (9): A solution of the derivatized triazole **8** (1 g, 2.8 mmol) in anhydrous pyridine (20 mL) was cooled at 0°C and TBDMS-Cl (0.46 g, 3 mmol) was added. The solution was stirred overnight, at room temp. and under argon. The solvent was then evaporated under reduced pressure and the crude mixture obtained was purified by silica gel chromatography (CH₂Cl₂/MeOH, 95:5) to give compound **9** (1.25 g, 94%) as a white powder, m.p. 66–68°C. – IR (KBr): $\tilde{\nu}$ = 3142 cm⁻¹, 2933, 2866, 1607, 1577, 1489. – UV (MeOH): $\lambda_{\max}$ (ϵ) = 275 nm (16000), 258 (10400). – ¹H NMR (200 MHz, CDCl₃): δ = 0.01 (s, 6 H, Si-CH₃), 0.78 [s, 9 H, Si-C(CH₃)₃], 1.41 (s, 3 H, CH₃), 1.65 (s, 3 H, CH₃), 3.86 (m, 2 H, 5',5''-H), 4.54 (q, 1 H, 4'-H), 4.90 (dd, 1 H, 3'-H), 5.19 (dd, 1 H, 2'-H), 6.28 (d, 1 H, 1'-H), 8.47 (s, 1 H, 2-H), 8.86 (s, 1 H, 8-H), 9.61 (s, 2 H, CH triazolo). – ¹³C NMR (50 MHz, CDCl₃): δ = -5.7 (CH₃), 18.0 (quat), 25.1 (CH₃), 25.6 (CH₃), 27.0 (CH₃), 63.5 (CH₂), 81.3 (CH), 85.1 (CH), 87.6 (CH), 92.3 (CH), 113.9 (quat), 122.7 (quat), 140.6 (CH), 142.6 (quat), 144.4 (CH), 152.1 (CH), 153.0 (quat). – MS (DCI); *m/z*: 474 [M + H]⁺.

N⁶-(4-Aminobutyl)-9-[5-*O*-(*tert*-butyldimethylsilyl)-2,3-*O*-isopropylidene- β -D-ribofuranosyl]purine (10): To a solution of compound **9** (1.7 g, 3.6 mmol) in acetonitrile (15 mL) was added 1,4-diaminobutane (3.7 mL, 36 mmol). The reaction mixture was heated at 50°C for 6 h under argon. The solvent was evaporated in vacuum and the oily residue obtained was dissolved in EtOAc. The organic layer was washed twice with brine, then dried (Na₂SO₄) and concentrated under reduced pressure. The crude product was purified by silica gel chromatography (CH₂Cl₂/MeOH/NH₄OH, 80:20:2) to give compound **10** as a pale yellow oil (1.5 g, 80%). – IR (neat): $\tilde{\nu}$ = 3356 cm⁻¹, 3279, 2927, 2858, 1622, 1374. – UV (MeOH): $\lambda_{\max}$ (ϵ) = 266 nm (14100). – ¹H NMR (200 MHz, [D₆]DMSO): δ = -0.07 (s, 6 H, Si-CH₃), 0.78 (s, 9 H, Si-C(CH₃)₃), 1.31 (s, 3 H, CH₃), 1.41 (m, 2 H, CH₂-CH₂), 1.52 (s, 3 H, CH₃), 1.57 (m, 2 H, CH₂-CH₂), 2.48 (br. t, 2 H, NH₂), 2.55 (m, 2 H, N-CH₂), 3.44 (m, 2 H, N-CH₂), 3.69 (m, 2 H, 5',5''-H), 4.20 (m, 1 H, 4'-H), 4.94 (dd, 1 H, 3'-H), 5.39 (dd, 1 H, 2'-H), 6.15 (d, 1 H, 1'-H), 7.80 (br. s, 1 H, NH), 8.20 (s, 1 H, 2-H), 8.24 (s, 1 H, 8-H). – ¹³C NMR (50 MHz, [D₆]DMSO): δ = -5.6 (CH₃), 18.3 (quat), 25.4 (CH₃), 25.8 (CH₃), 27.0 (CH₂), 27.2 (CH₃), 30.7 (CH₂), 40.5 (CH₂), 41.6 (CH₂), 63.5 (CH₂), 81.5 (CH), 84.8 (CH), 87.3 (CH), 91.3 (CH), 113.9 (quat), 120.0 (quat), 138.3 (CH), 148.5 (quat), 153.2 (CH), 154.8 (quat). – MS (FAB, *m*NBA matrix); *m/z*: 493 [M + H]⁺. – HRMS (FAB, *m*NBA matrix): calcd. 493.2959, found 493.2969.

Protected Nucleoside 12: To a solution of acid **3** (0.40 g, 2 mmol) in dry THF (10 mL), cooled at 0°C and under argon, were added, successively, *N*-methylmorpholine (0.23 mL, 2 mmol), isobutyl chloroformate (0.26 mL, 2 mmol), and then, after 15 min, compound **10** (1 g, 2 mmol); the reaction mixture was stirred at room temp. for 2 h. The solvent was evaporated and the oily residue obtained was dissolved in CH₂Cl₂. The organic layer was washed successively with 0.1 N aq. NaOH, 1 N aq. HCl, and brine, then it was dried (Na₂SO₄). Evaporation of the solvent afforded product **11** (1.1 g, 82%) as a white powder which was used without further purification for the next reaction. — ¹H NMR (200 MHz, CDCl₃): δ = −0.01 (s, 6 H, Si—CH₃), 0.82 [s, 9 H, Si—C(CH₃)₃], 1.37 (s, 3 H, CH₃), 1.43 [s, 9 H, C(CH₃)₃], 1.60 (s, 3 H, CH₃), 1.70 (m, 4 H, CH₂—CH₂), 3.37 (q, 2 H, CH₂—N), 3.62 (m, 2 H, CH₂—N), 3.70 (m, 2 H, 5',5''-H), 4.29 (s, 2 H, CH₂—ONH), 4.35 (m, 1 H, 4'-H), 4.95 (dd, 1 H, 3'-H), 5.25 (dd, 1 H, 2'-H), 6.12 (d, 1 H, 1'-H), 7.60 (br. s, 1 H, NH), 7.95 (s, 1 H, 8-H), 8.15 (br. s, 1 H, NH), 8.36 (s, 1 H, 2-H). — MS (DCI); *m/z*: 666 [M + H]⁺. — To the crude product **11** (1 g, 1.5 mmol), dissolved in THF (5 mL), was added a 1 M TBAF (3 mL, 3 mmol) solution in THF and the reaction mixture was stirred for 1 h. The solvent was evaporated and the residue obtained was dissolved in CH₂Cl₂. The organic layer was washed with brine, dried (Na₂SO₄) and concentrated. The crude product was purified by silica gel chromatography (CH₂Cl₂/MeOH, 95:5) to give compound **12** (0.75 g, 91%) as a white powder, m.p. 89–91°C. — IR (KBr): $\tilde{\nu}$ = 3306 cm^{−1}, 2981, 2944, 1737, 1628. — UV (MeOH): λ_{max} (ε) = 267 nm (16600). — ¹H NMR (200 MHz, CDCl₃): δ = 1.35 (s, 3 H, CH₃), 1.43 [s, 9 H, C(CH₃)₃], 1.62 (s, 3 H, CH₃), 1.75 (m, 4 H, CH₂—CH₂), 3.32 (m, 2 H, CH₂—N), 3.68 (m, 2 H, CH₂—N), 3.92 (m, 2 H, 5',5''-H), 4.28 (s, 2 H, CH₂—ONH), 5.09 (m, 1 H, 3'-H), 5.16 (m, 1 H, 2'-H), 5.81 (d, 1 H, 1'-H), 6.00 (br. t, 1 H, 5'-OH), 6.75 (br., 1 H, NH), 7.65 (br., 1 H, NH), 7.74 (s, 1 H, 8-H), 8.15 (br., 1 H, NH), 8.28 (s, 1 H, 2-H). — ¹³C NMR (50 MHz, CDCl₃): δ = 25.1 (CH₃), 26.4 (CH₂), 26.5 (CH₂), 27.4 (CH₃), 27.9 (CH₃), 38.4 (CH₂), 40.0 (CH₂), 63.0 (CH₂), 75.8 (CH₂), 81.5 (CH), 82.6 (quat), 82.9 (CH), 85.9 (CH), 93.8 (CH), 113.8 (quat), 121.1 (quat), 139.3 (CH), 147.5 (quat), 152.6 (CH), 155.0 (quat), 157.9 (quat), 169.1 (quat). — MS (FAB, *m*NBA matrix); *m/z*: 552 [M + H]⁺. — HRMS (FAB, *m*NBA matrix): calcd. 552.2782, found 552.2780. — C₂₄H₃₇N₇O₈ (551.3): calcd. C 52.26, H 6.76, N 17.78; found C 51.87, H 6.61, N 17.18.

Deprotection of Nucleoside 12 To Obtain 13: Deprotected nucleoside **13** was obtained from nucleoside **12** in the same manner as described for compound **7** in 45% yield. — IR (KBr): $\tilde{\nu}$ = 3314 cm^{−1}, 2930, 2865, 1624, 1094. — UV (H₂O): λ_{max} (ε) = 267 nm (16600). — ¹H NMR (300 MHz, [D₆]DMSO): δ = 1.50 (m, 4 H, CH₂—CH₂), 3.10 (q, 2 H, CH₂—N), 3.40 (overlapping m, 4 H, 5',5''-H and CH₂—N), 3.87 (s, 2 H, CH₂—ONH₂), 3.95 (m, 1 H, 4'-H), 4.12 (m, 1 H, 3'-H), 4.58 (m, 1 H, 2'-H), 5.16 (d, 1 H, 5'-OH), 5.42 (m, 2 H, 3'-OH and 2'-OH), 5.86 (d, 1 H, 1'-H), 6.32 (br., 1 H, NH), 7.68 (br., 2 H, ONH₂), 8.18 (br. s, 1 H, 2-H), 8.32 (s, 1 H, 8-H). — ¹³C NMR (75 MHz, [D₆]DMSO) δ = 27.0 (CH₂), 38.1 (CH₂), 60.5 (CH₂), 63.2 (CH₂), 70.9 (CH), 72.5 (CH₂), 73.7 (CH), 74.7 (CH₂), 86.2 (CH), 88.3 (CH), 140.0 (CH), 148.0 (quat), 152.7 (CH), 155.0 (quat), 170.0 (quat). — MS (DCI); *m/z*: 412 [M + H]⁺.

Nucleotide Triphosphate 2: Nucleotide **2** was obtained, as a white powder in 20% overall yield, from protected nucleoside **12** by the same procedure that was used to obtain **1**. Protected nucleotide intermediate: *t*_R = 36 min. — ³¹P NMR (160 MHz, D₂O): δ = −21.62 (t, Pβ), −11.05 (d, *J* = 19 Hz, Pα), −5.66 (d, *J* = 20 Hz, Pγ). — ¹H NMR (400 MHz, D₂O): δ = 1.28 [s, 9 H, C(CH₃)₃], 1.49 (s, 3 H, CH₃), 1.72 (s, 3 H, CH₃), 1.75–1.65 (overlapping m, 4

H, CH₂—CH₂), 3.30 (m, 2 H, CH₂—N), 3.59 (m, 2 H, CH₂—N), 4.30–4.20 (overlapping m, 2 H, 5',5''-H), 4.27 (s, 2 H, CH₂—O), 4.73 (m, 1 H, 4'-H), 5.32 (m, 1 H, 3'-H), 5.42 (t, 1 H, 2'-H), 6.29 (d, 1 H, 1'-H), 8.27 (br. s, 1 H, 2-H), 8.47 (s, 1 H, 8-H). Compound **2**: *t*_R = 30 min. — ³¹P NMR (160 MHz, D₂O): δ = −21.83 (t, Pβ), −10.90 (d, *J* = 19 Hz, Pα), −5.95 (d, *J* = 18 Hz, Pγ). — ¹H NMR (400 MHz, D₂O): δ = 1.74 (m, 4 H, CH₂—CH₂), 3.34 (m, 2 H, CH₂—N), 3.62 (m, 2 H, CH₂—N), 4.17 (s, 2 H, CH₂ONH₂), 4.23–4.34 (m, 2 H, 5',5''-H), 4.43 (m, 1 H, 4'-H), 4.69 (m, 1 H, 3'-H), 4.86 (m, 1 H, 2'-H), 6.16 (d, 1 H, 1'-H), 8.27 (br. s, 1 H, 2-H), 8.55 (s, 1 H, 8-H). — MS (electrospray, negative mode); *m/z*: 650 [M − H][−], 672 [M − 2H + Na][−], 694 [M − 3H + 2Na][−].

Protected Aldehydic Fluorophore 14: To a solution of fluorescein isothiocyanate (0.91 g, 2.35 mmol) in dry DMF (10 mL), 4-amino-butylaldehyde diethylacetal (0.4 mL, 2.35 mmol) was added, and the reaction mixture was stirred at room temp. for 1 h under argon. The solvent was then evaporated under reduced pressure and the crude product was purified by silica gel chromatography (CH₂Cl₂/MeOH, 90:10) to give compound **14** (1.21 g, 94%) as an orange powder, m.p. 200°C (dec.). — IR (KBr): $\tilde{\nu}$ = 3263 cm^{−1}, 2929, 1744, 1610, 1443. — UV (MeOH): λ_{max} (ε) = 480 nm (20800), 454 (20000), 273 (28000), 226 (77400). — ¹H NMR (200 MHz, [D₆]DMSO): δ = 1.09 (t, 6 H, 2 CH₂—CH₃), 1.57 (m, 4 H, CH₂—CH₂), 3.50 (overlapping m, 6 H, CH₂—N and 2 CH₃—CH₂—O), 4.48 (t, 1 H, CH(OEt)₂), 6.61 (m, 6 H, *H*-Ar), 7.15 (d, 1 H, *H*-Ar), 7.70 (d, 1 H, *H*-Ar), 8.10 (br. s, 1 H, NH or OH), 8.20 (s, 1 H, *H*-Ar), 9.85 (br. s, 1 H, NH or OH), 10.11 (br. s, 2 H, NH or OH). — ¹³C NMR (50 MHz, [D₆]DMSO): δ = 15.2 (CH₃), 23.6 (CH₂), 30.6 (CH₂), 35.6 (CH), 43.4 (CH₂), 60.4 (CH₂), 82.8 (quat), 101.8 (CH), 102.0 (CH), 109.5 (quat), 112.3 (CH), 123.8 (CH), 126.3 (quat), 128.8 (CH), 141.2 (quat), 147.0 (quat), 151.7 (quat), 159.3 (quat), 162.1 (CH), 168.3 (quat), 180.2 (quat). — MS (FAB, *m*NBA matrix); *m/z*: 551 [M + H]⁺.

Aldehydic Fluorophore 15: Compound **14** (0.34 g, 0.62 mmol) was dissolved in 30% aq. acetic acid solution (20 mL) and the reaction mixture was stirred at room temp. for 1 h. After the solvent was evaporated under reduced pressure, the crude product was purified by silica gel chromatography (CH₂Cl₂/MeOH, 90:10) to give aldehyde **15** (0.15 g, 50%) as an orange powder, m.p. 200°C (dec.). — IR (KBr): $\tilde{\nu}$ = 3254 cm^{−1}, 2950, 1730, 1586, 1460. — UV (Tris buffer, pH = 9): λ_{max} (ε) = 492 nm (77000), 239 (54000). — ¹H NMR (200 MHz, [D₆]DMSO): δ = 1.90 (m, 2 H, CH₂), 2.06 (m, 2 H, CH₂—CO), 3.63–3.77 (m, 2 H, CH₂—N), 5.72 [m, 1 H, CH(OH)₂], 6.62 (m, 6 H, *H*-Ar), 7.18 (d, 1 H, *H*-Ar), 7.84 (dd, 1 H, *H*-Ar), 8.12 (s, 1 H, *H*-Ar), 9.30 (br., 1 H, NH or OH), 10.14 (br., 2 H, NH or OH). — MS (FAB, *m*NBA matrix); *m/z*: 477 [M + H]⁺, 390 [M − (NH—[CH₂)₃—CHO)]⁺. — HRMS (FAB, *m*NBA matrix): calcd. 477.1120, found 477.1121. — C₂₅H₂₀N₂O₆S·1H₂O (476.1): calcd. C 60.71, H 4.49, N 5.67; found C 60.98, H 4.44, N 5.56.

Oxime Ether 16: To a solution of deprotected nucleoside **7** (5 mg, 0.01 mmol) in water (1 mL) was added fluorophore **15** (6.5 mg, 0.01 mmol) in DMSO (0.1 mL). The reaction mixture was stirred for 1 h, then concentrated under reduced pressure. The crude product was purified by reverse-phase chromatography to give compound **16** as an orange powder (5.7 mg, 70%). — UV (Tris buffer, pH = 9): λ_{max} (ε) = 494 nm (70000), 279 (24000), 239 (50000). — ¹H NMR (500 MHz, [D₆]DMSO, 2 diastereoisomers): δ = 1.75–1.77 (m, 2 H, CH₂—CH₂—NH), 2.21 and 2.41 (2 q, 2 H, N=CH—CH₂), 3.54 (overlapping m, 2 H, CH₂—NH—C=S), 3.56–3.64 (m, 2 H, 5',5''-H), 3.84 (q, 1 H, 4'-H), 3.95 (q, 1 H, 3'-H), 4.03 (q, 1 H, 2'-H), 4.10 (d, 2 H, CH₂—NH—CO), 4.38 and

4.45 (2 s, 2 H, $\text{CH}_2\text{-O-N}$), (s, 2 H, $\text{CH}_2\text{-O-N}$), 5.05 (d, 1 H, 3'-OH), 5.17 (t, 1 H, 5'-OH), 5.38 (d, 1 H, 2'-OH), 5.74 (d, 1 H, 1'-H), 6.54–6.66 (m, 6 H, H-Ar), 6.88 and 7.59 (2 t, 1 H, N=CH), 7.16 (d, 1 H, H-Ar), 7.72 (m, 1 H, H-Ar), 8.11 (br. m, 1 H, NH-C=S), 8.21 (s, 2 H, H-Ar and 6-H), 8.26 (t, 1 H, NH-CO), 8.29 (t, 1 H, NH-CO), 9.86 (br., 1 H, NH or OH), 10.10 (br., 2 H, NH or OH), 11.60 (s, 1 H, NH). – MS (FAB, $m\text{NBA}$ matrix); m/z : 829 $[\text{M} + \text{H}]^+$. – HRMS (FAB, $m\text{NBA}$ matrix): calcd. 829.2139, found 829.2149.

Oxime Ether 17: As described above for oxime ether 16, the coupling product 17 was obtained as an orange powder in 60% yield from nucleoside 13. – UV (Tris buffer, pH = 9): λ_{max} (ϵ) = 494 nm (70000), 268 (24000), 240 (50000). – ^1H NMR (500 MHz, $[\text{D}_6]\text{DMSO}$, 2 diastereoisomers): δ = – 1.43 (m, 2 H, $\text{CH}_2\text{-CH}_2\text{-NH-CO}$), 1.54 (m, 2 H, $\text{N}^6\text{H-CH}_2\text{-CH}_2$), 1.71 (m, 2 H, $\text{CH}_2\text{-CH}_2\text{-NH-C=S}$), 1.93 (br. m, 1 H, NH), 2.13 (br. m, 1 H, NH), 2.17 and 2.36 (2 q, 2 H, N=CH-CH_2), 3.09 (q, 2 H, $\text{CH}_2\text{-NH-CO}$), 3.45 (overlapping m, 2 H, $\text{N}^6\text{H-CH}_2$), 3.51 (overlapping m, 2 H, $\text{CH}_2\text{-NH-C=S}$), 3.52–3.65 (m, 2 H, 5', 5'-H), 3.93 (q, 1 H, 4'-H), 4.11 (q, 1 H, 3'-H), 4.30 and 4.37 (2 s, 2 H, $\text{CH}_2\text{-O-N}$), 4.58 (q, 1 H, 3'-H), 5.14 (d, 1 H, 3'-OH), 5.40 (m, 2 H, 5'-OH and 2'-OH), 5.86 (d, 1 H, 1'-H), 6.54–6.64 (m, 6 H, H-Ar), 6.84 (t, 1 H, N=CH), 7.16 (m, 1 H, H-Ar), 7.56 (t, 1 H, N=CH), 7.70 (m, 1 H, H-Ar), 7.83 (m, 2 H, H-Ar and NH), 8.10 (br. m, 1 H, NH-C=S), 8.21 (s, 1 H, 2-H), 8.32 (s, 1 H, 8-H), 9.30 (br., 1 H, NH or OH), 9.88 (br., 1 H, NH or OH), 10.01 (s, 2 H, NH or OH). – MS (FAB, $m\text{NBA}$ matrix); m/z : 870 $[\text{M} + \text{H}]^+$. – HRMS (FAB, $m\text{NBA}$ matrix): calcd. 870.2881, found 870.2877.

Transcriptions Assay: The PCR target containing the T7 promoter was prepared as previously described.^[13] This PCR target was used to generate the modified single-stranded RNA target by in vitro transcription. Transcriptions were carried out as described previously:^[13] the PCR target, T7 RNA polymerase and equimolar concentrations of the nucleotides were incubated at 37°C for 1 h. The ratios of modified versus natural nucleotides (i.e., 1 vs UTP and 2 vs ATP) were varied, to be 0:100, 30:70, 70:30, and 100:0. For labelling, an excess of a solution of the fluorescein derivative 15 (10 equiv.) in DMF was added to the crude transcription mixture. The reaction was performed at room temperature for 30 min. The labelled transcription products were analyzed by electrophoresis on a 6% urea/polyacrylamide gel in Trisborate buffer (pH = 8.5) at 150 V for 1 h.

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