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Synthesis of Methylketone Containing Nucleoside Triphosphates for RNA Labelling

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Abstract—The three nucleoside triphosphates **1**, **2** and **3** bearing a linker with a terminal methylketone group were prepared for incorporation into RNA fragments and post-labelling by the fluorescein derivative **4** containing an aminooxy group. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

High density oligonucleotide probe arrays (DNA chips) represent powerful new tools for the analysis of DNA and RNA sequences.[1] This technology can be applied to nucleic acid sequence analysis such as pathogen identification, polymorphism, mutations detection, or mapping.[1] The principle of the technology is based on the hybridisation of the labelled nucleic acid sample to be analysed, i.e. the target, with a large set of oligonucleotides immobilised on the chip at precise locations. The hybridisation pattern of the labelled nucleic acid target gives primary structure informations.[2] Quite generally, prior to analysis on the chip, the nucleic acid target is amplified and labelled. This is most frequently achieved in one step by incubating a modified fluorescent nucleotide triphosphate, in addition to the natural, during the PCR-type amplification.[3]

In a recent paper,[4] we proposed an alternative procedure for labelling RNA targets, called 'post-amplification labelling'. The approach first involves enzymatic incorporation of a nucleotide triphosphate previously modified so as to carry a reactive functional group X. The resulting amplified RNA sequences are then labelled by reaction with a fluorophore functionalised by a Y group complementary to X. Severe limitations exist as to the nature of X and Y. The conjugation reaction must be selective, quantitative and rapid to allow for specific labelling and possible automation. The X group must remain inert to the enzymatic conditions for efficient incorporation. The aminooxy-carbonyl coupling reaction meets these criteria of selectivity and efficiency. We used it in the past to prepare fluorescent oligodeoxyribonucleotides.[5] It has been recently reported for conjugation of peptides with oligosaccharides[6] and to immobilise oligonucleotides on solid supports.[7] In our previous post-amplification labelling approach,[4] the modified nucleotide, uridine or adenosine triphosphate, carried the aminooxy function and the fluorescent probe contained the aldehydic function. Enzymatic incorporation of such modified triphosphates into an RNA fragment was found effective and a remarkable efficiency of the labelling by aldehydic fluorescein was reached. However, some drawbacks appeared: the synthesis of the modified triphosphates required protection of the aminooxy group and at the final stage the acidic deprotection had to be carried out using large excess of methoxyamine requiring therefore tedious purification. We thus considered the 'reverse strategy' in which the aminooxy moiety is supported by the fluorescent label and the carbonyl by the triphosphate. The methylketone function was preferred to the aldehyde, on the hypothesis that the aldehyde could be more susceptible to interfere with the enzymatic process. In the present paper, we report on the preparation of the uridine, adenosine and cytidine triphosphate derivatives **1**, **2** and **3**, all of which incorporating a linker with a terminal methylketone group.[8] We describe the preparation of the aminooxy containing fluorescein derivative **4** and subsequent coupling with the methylketone derivatives (see Fig. 1).

Results and Discussion

Synthesis of the uridine triphosphate derivative 1 (Scheme 1)

To introduce the substituent at the C-5 position of uridine, we selected the method described by Hobbs[10] which we previously used for preparing the aminooxy containing triphosphate derivatives.[4] Preparation of the protected

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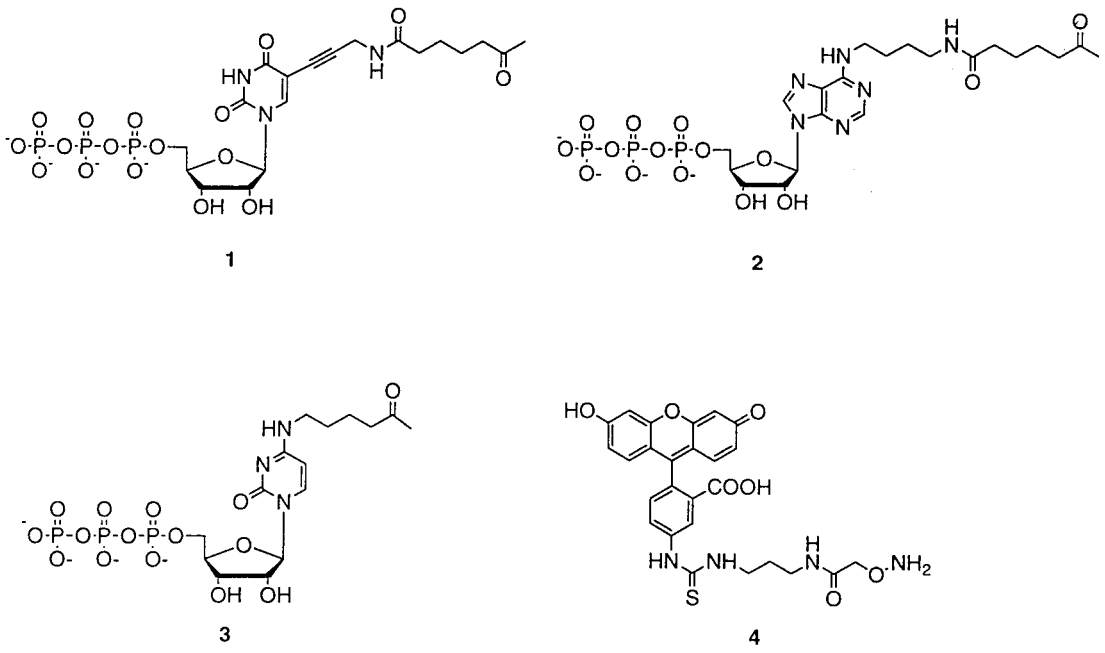
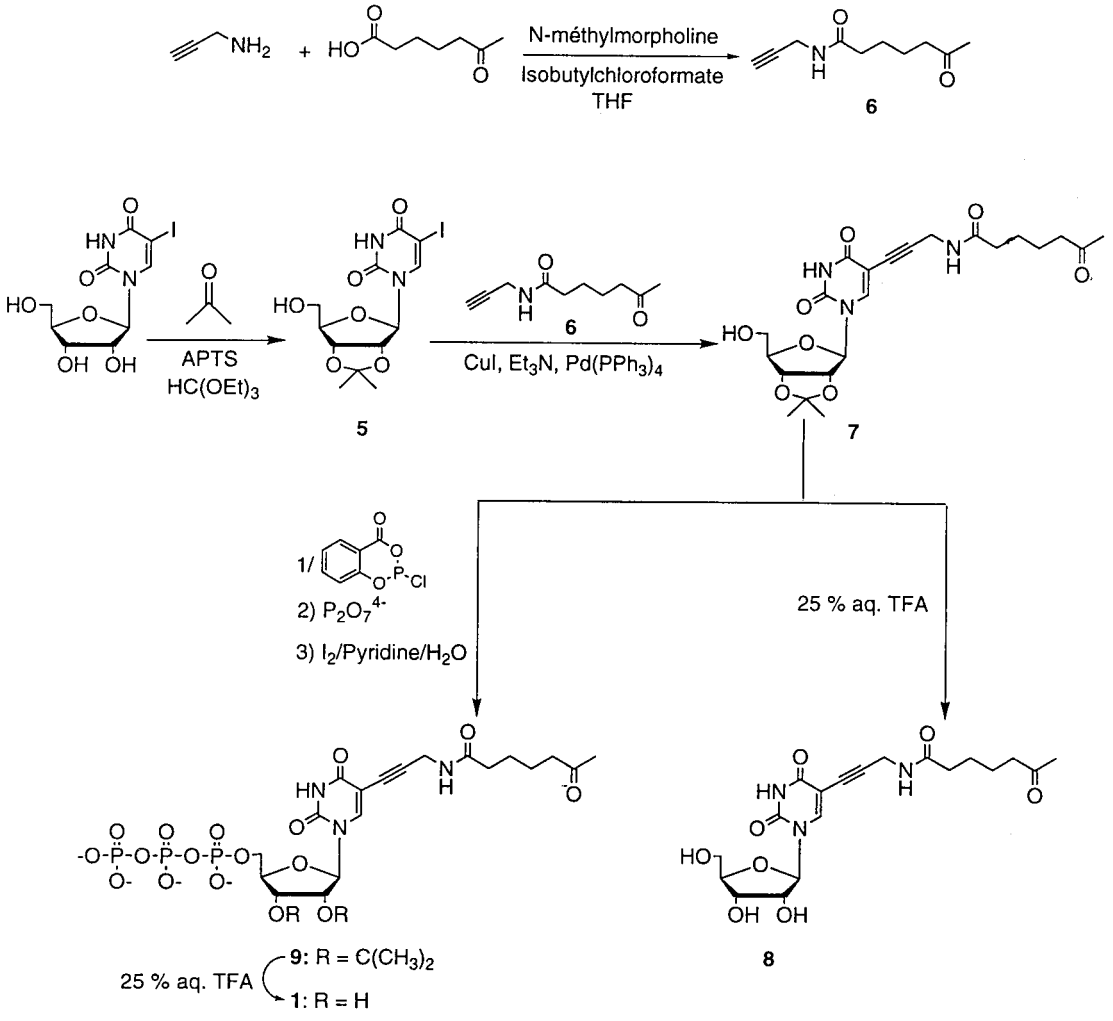
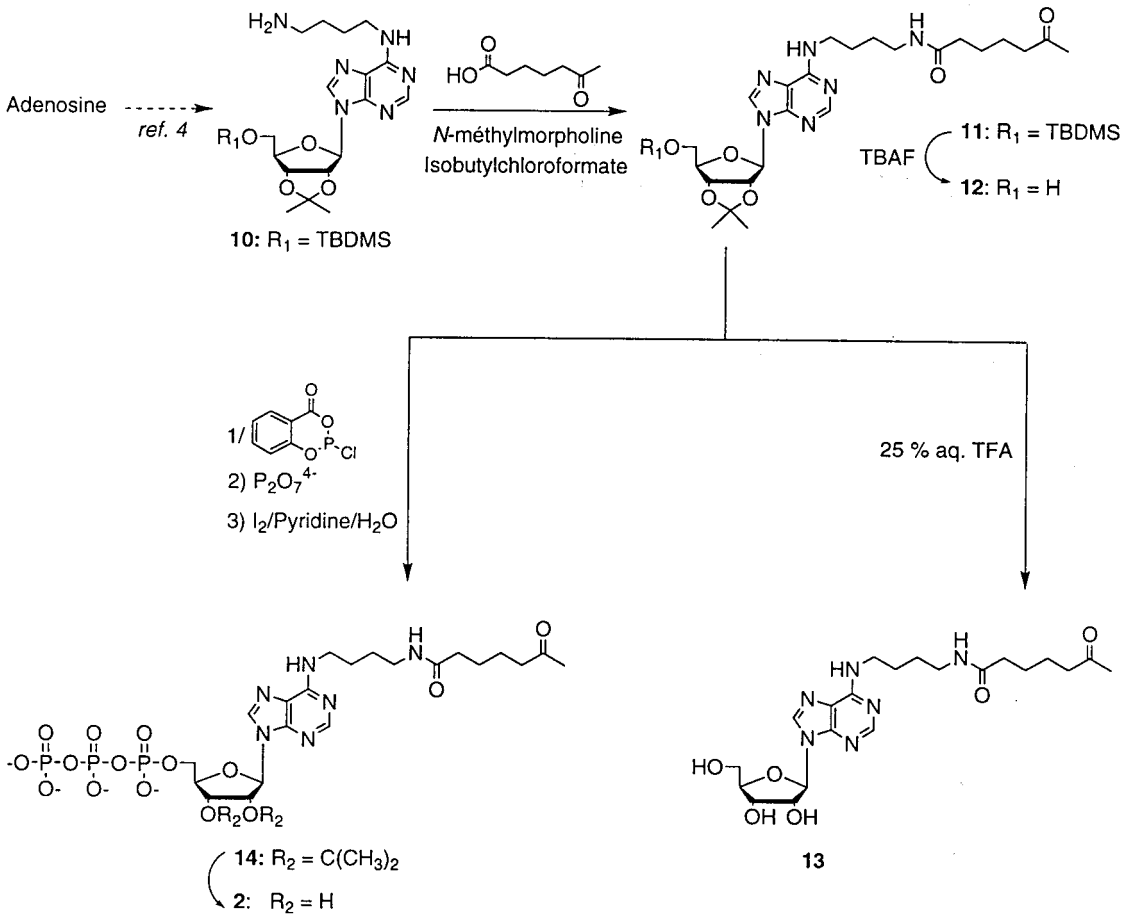


Figure 1. Structure of the modified nucleosides triphosphates **1**, **2**, **3** and of fluorescein derivative **4**.



Scheme 1.



Scheme 2.

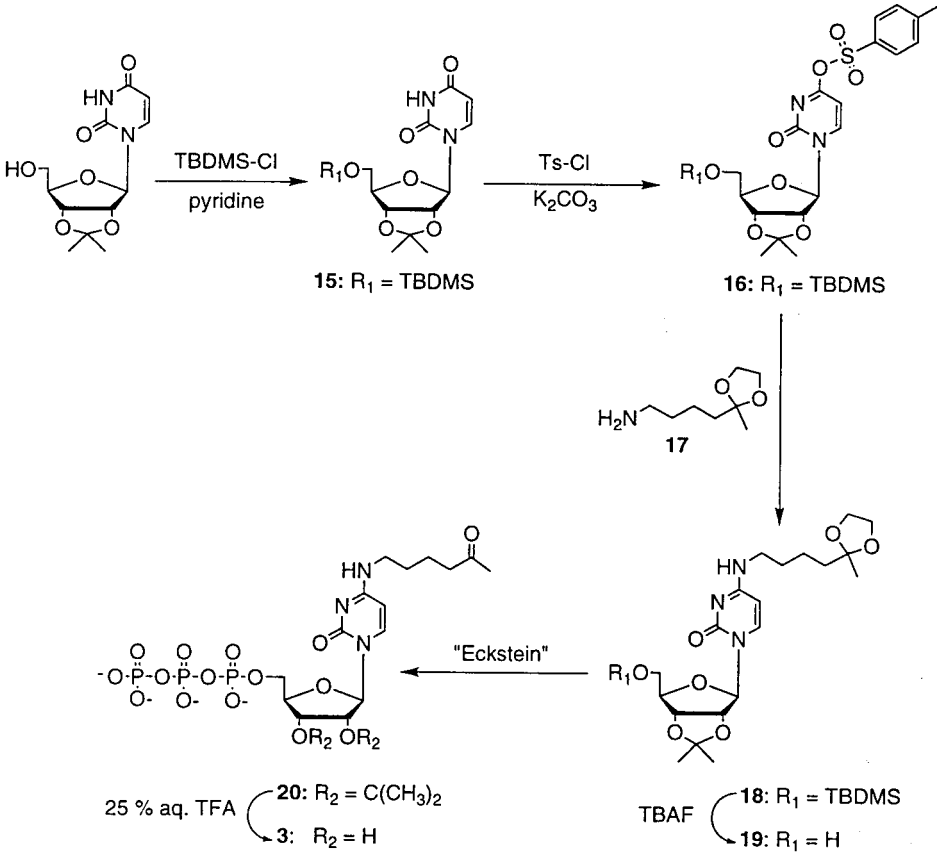
nucleoside **7** was accomplished as depicted in Scheme 1. The alkynyl chain **6** was prepared in 80% yield by coupling 6-oxoheptanoic acid with propargylamine in the presence of isobutylchloroformate. The alkynyl chain **6** was then introduced on to the uridine ring by reaction of **5** with copper iodide in the presence of triethylamine and tetrakis(triphenylphosphine)palladium to afford the protected nucleoside **7** in 60% yield. Protection of the 2′- and 3′-hydroxyl groups of the ribose moiety as an acetonide before introduction of the alkynyl chain facilitated the purification step. We then determined the mildest acidic conditions required for removal of the acetonide protecting group to be applied in the final stage of the syntheses. Treatment of **7** in 25% aqueous trifluoroacetic acid at room temperature for 30 min led to total removal of the acetonide. We thus prepared the nucleoside **1** using Eckstein's procedure[11] and acidic hydrolysis. Reaction of the protected nucleoside **7** with salicyl phosphorochloridite, subsequent reaction with pyrophosphate and final oxidation by iodine furnished the intermediate protected nucleotide **9**. The presence of the acetonide group, of the chain at the C-5 position and of the tributylammonium counterions rendered the nucleotide lipophilic enough to allow for rapid and efficient purification by C18 reverse phase flash chromatography. Deprotection of the 2′- and 3′-hydroxyl groups was then achieved using 25% aqueous trifluoroacetic acid leading to the desired nucleotide **1** as the sodium salt in 20% overall yield. The structure of **1** was confirmed by ^{1}H and ^{31}P NMR spectroscopy and ESMS. The ^{31}P NMR spectra showed in particular three signals at nearly −5.5, −10.5 and −21.3 ppm corresponding, respectively, to the γ, α and β phosphorus atoms.

Synthesis of the adenosine triphosphate derivative 2 (Scheme 2)

To incorporate a chain containing the methyl ketone group on the adenosine ring at the C-6 position, we used the key intermediate **10** prepared by conventional route.[4] Coupling nucleoside **10** with 6-oxoheptanoic acid was performed using isobutylchloroformate in strictly anhydrous conditions leading to the protected nucleoside **11**. Subsequent treatment with tetrabutylammonium fluoride (TBAF) removed the TBDMS protection and afforded the 2′,3′ protected nucleoside **12** in 85% overall yield from the key intermediate **10**. Triphosphorylation was then performed using the same conditions as described above for the uridine derivative **1**. Nucleotide **2** was obtained as the sodium salt in 33% overall yield and characterised by ^{1}H and ^{31}P NMR spectroscopy and ESMS.

Synthesis of the cytidine triphosphate derivative 3 (Scheme 3)

The methyl ketone chain at the C-4 position of cytidine was introduced using the procedure developed by Czernecki and



Scheme 3.

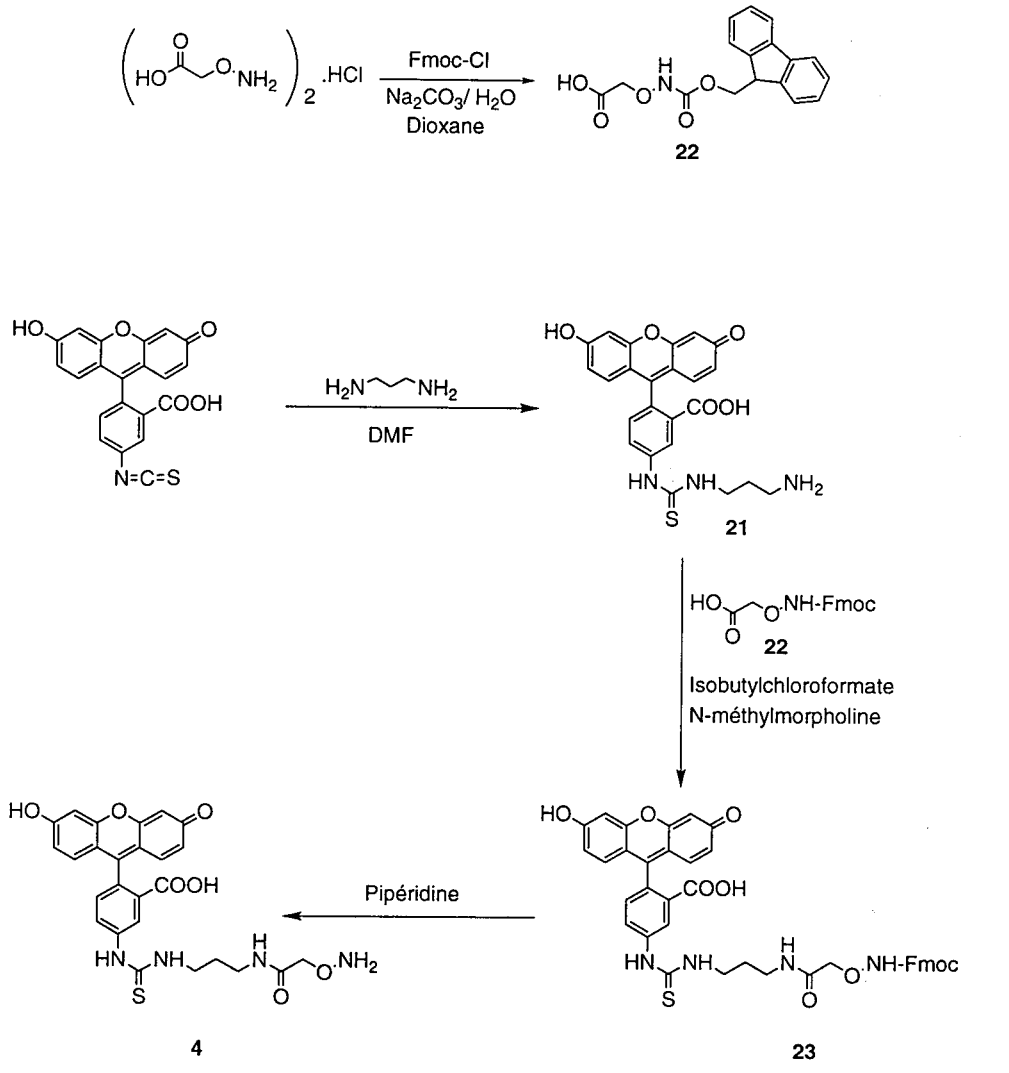
Coll.[12] This approach involves conversion of the hydroxyl group at the C-4 position of uridine into a good leaving group, i.e. a tosylate, followed by displacement with an amino compound. Preparation of nucleotide **3** was accomplished by the route depicted in Scheme 3. The 5′-hydroxyl group was first protected as the *tert*-butyldimethylsilyl ether **15**. The C-4 position was then activated by treatment of **15** with tosyl chloride in basic conditions. The 'activated' nucleoside **16** was obtained in 76% overall yield from commercial 2′,3′-isopropylidene uridine. The amine **17** was prepared in two steps from 5-oxohexanitrile as described.[13] Displacement of the tosylate in **16** was then performed using 2 equiv. of the amine **17** to afford the protected nucleoside **18** within 10 min at room temperature. Finally, treatment with TBAF gave the desilylated compound **19** in 40% overall yield from 2′,3′-isopropylidene uridine. Nucleoside **19** was then transformed into the triphosphate **3** using Eckstein's procedure and acidic hydrolysis of the two protecting groups as described above for the uridine derivative **1**. The structure of nucleoside **3** was established by ^{1}H and ^{31}P NMR spectroscopy and confirmed by ESMS.

Preparation of the aminooxy fluorophore 4 (Scheme 4)

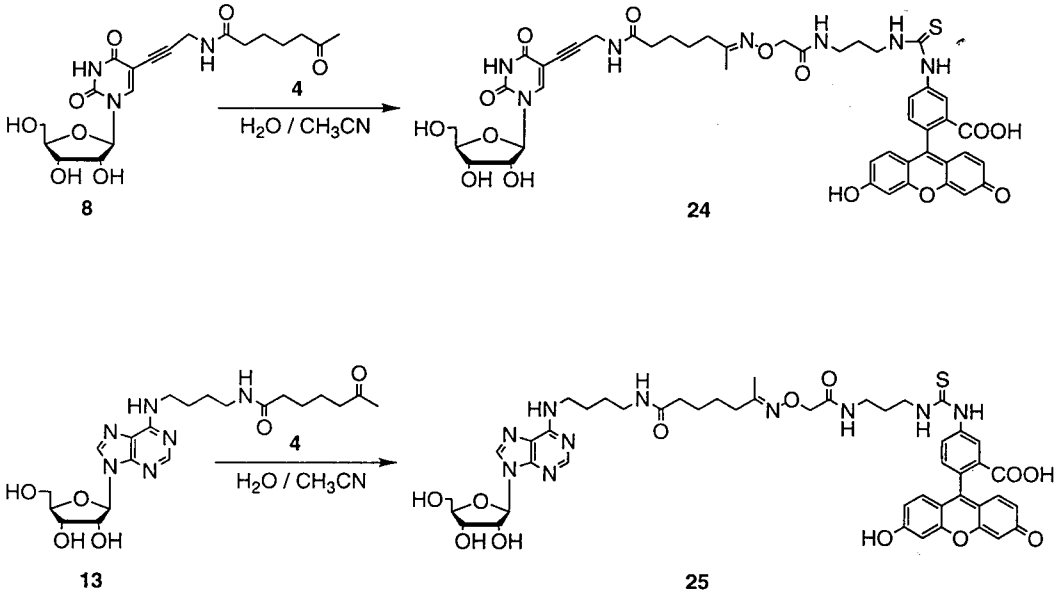
Compound **4** was prepared in a three step reaction from the commercially available fluorescein isothiocyanate (FITC). Reaction of FITC with an excess of 1,3-diaminopropane gave compound **21** in 80% yield. Purification had to be performed using reverse phase chromatography due to the high polar character of **21**. The fluorescein derivative **21** was used directly without protection for coupling with the protected *O*-(methoxycarbonyl)hydroxylamine **22** in the presence of isobutylchloroformate to afford the protected fluorophore **23**. No side reaction was observed in these conditions. The Fmoc group was selected for protection of the aminooxy function as it could be removed in mild alkaline conditions, which did not affect the luminescent probe. Compound **22** was prepared in quantitative yield by treatment of commercial *O*-(carboxymethyl)hydroxylamine hemihydrochloride with 9-fluorenylmethylchloroformate. Finally, the Fmoc protection of **23** was removed by basic hydrolysis using piperidine in DMF to afford the fluorophore **4** in 30% overall yield from FITC.

Aminooxy-methylketone coupling reaction (Scheme 5)

The efficiency of the coupling reaction was verified by reacting the fluorophore **4** with the unprotected nucleosides **8** and **13**. The reactions were carried out at room temperature in water/acetonitrile solution using 2 equiv. of fluorophore **4**. The corresponding oxime ethers **24** and **25** were thus obtained in 80–85% yield in isolated products. The structure of each compound was determined by ^{1}H NMR using DQFcosy sequences and confirmed by HRMS. The ^{1}H NMR spectra showed in particular two signals for the oximic protons which clearly demonstrated that a diastereoisomeric mixture of *Z*/*E* compounds was obtained. The *Z*/*E* ratios, determined by ^{1}H NMR for each compound **24** and **25** were, respectively, 60/40 and 70/30.



Scheme 4.



Scheme 5.

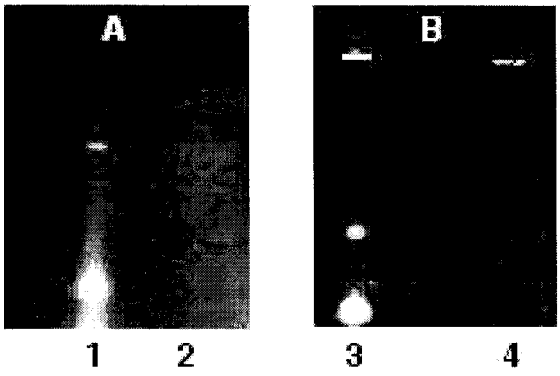


Figure 2. Gel electrophoresis analysis of the transcription products resulting from incorporation of the modified triphosphate **1** (100% **1**; 0% UTP) compared to incorporation of UTP. Visualisation was performed by UV before (A) and after (B) ethidium bromide staining. Lines 1 and 3: modified transcripts resulting from incorporation of **1** and subsequent incubation with fluorophore **4**. Lines 2 and 4: natural transcripts resulting from incorporation of UTP and subsequent incubation with **4**.

The incorporation of the modified triphosphates **1**, **2** and **3** by T7 RNA polymerase into a fragment of 16S ribosomal RNA from *Mycobacterium tuberculosis* or into a fragment of the reverse transcriptase gene of HIV was evaluated. Fig. 2 shows a typical gel electrophoresis analysis of the transcription products resulting from incorporation of nucleoside triphosphate **1** into a fragment of 16S RNA from *M. tuberculosis*. The presence of a fluorescent band (lines 1 and 3) having the same electrophoretic mobility as the transcripts resulting from incubation with the unmodified triphosphate (line 4) indicates that the modified triphosphate **1** has been incorporated. It also shows the efficiency of the subsequent labelling with fluorophore **4**. The selectivity of the labelling of the transcription products by fluorophore **4** was confirmed by the absence of fluorescent transcripts using the unmodified triphosphate (line 2).

Conclusion

We have prepared a new series of nucleotide triphosphates which have been shown to be enzymatically incorporated successfully into RNA fragments. The three nucleotides **1**, **2** and **3** could be prepared in satisfactory yields.

Labelling of the modified RNA transcripts by the fluorescein derivative **4** showed remarkable selectivity and efficiency. These results show that the new activated nucleotides and fluorescent probe can be useful in molecular diagnostic based on nucleic acid targets amplification and DNA chip technology.

Experimental

General

All commercially available chemical reagents were used without further purification. The nucleoside **10** was prepared as previously described.[4] Analytical TLC was performed on 0.2 mm silica 60 coated aluminium foils with F-254 indicator (Merck). Prep. column chromatographies were done using silica gel (Merck 60, 200–63 μm) or reverse phase silica gel (Merck-Lichroprep RP18, 40–63 μm). Analytical HPLC were performed on a Millipore-Waters equipment (two M-510 pumps, solvent gradient M680) with a UV detector (M490 or diode array 990). Reverse phase analyses were performed using μ-bondapak C-18 column (Waters) with a methanol–water pH 2.5 gradient, flow 2 mL/min for 10 min. Anion Exchange Chromatography used for the triphosphates analysis was carried out using a Protein Pak DEAE 8HR column (Waters, 8 μm, 10×100 mm) with Tris–HCl 20 mM, pH 7.6 for eluent A and Tris–HCl 20 mM, NaCl 0.5 M, pH 7.6 for eluent B with a flow rate of 2 mL/min. The gradient used was: 0–35% B in 40 min. Melting points were measured on an Electrothermal Series IA9100 apparatus. UV spectra were performed on a Perkin-Lambda 15UV/VIS and Fourier Transform Infrared spectra on a Perkin–Elmer Impact 400 spectrophotometer. NMR spectra were recorded on Bruker AC200, Avance 300 and Varian Unity plus 500 spectrometers. Spectra were referenced to the residual proton solvent peaks. The DQFCOSY spectra were run with 2048 points in t_2, 400 points in t_1 and 128 scans for each t_1 values. The mass spectra were recorded on a Delsi-Nermag R10-10 spectrometer. ES-MS analyses were performed on a VG Platform II (Micromass) in the negative ion mode. 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′,3′-Isopropylidene-5-iodouridine 5. To a mixture of 5-iodouridine (3 g, 8.1 mmol) and APTS (0.15 g, 0.8 mmol) in acetone (50 mL) was added dropwise under argon, ethylorthoformate (2.7 mL, 16.2 mmol). The mixture was stirred overnight at rt, then sodium hydrogenocarbonate (0.680 g, 8.1 mmol) was added. The solution was stirred for 30 min, filtered and evaporated to afford crude compound **5**. Recrystallisation in EtOH/hexane solution (1/1, v/v) gave compound **5** as a white powder (2.5 g, 75%). Mp 220–221°C. ^{1}H NMR (DMSO d_6, 200 MHz): δ ppm=1.27 and 1.47 (6H, 2s, 2 CH_3 isoprop.), 3.57 (2H, m, $C_{5',5''}$–H), 4.08 (1H, m, $C_{4'}$–H), 4.73 (1H, dd, $C_{3'}$–H), 4.90 (1H, dd, $C_{2'}$–H), 5.14 (1H, t, $C_{5'}$–OH), 5.81 (1H, d, $C_{1'}$–H), 8.31 (1H, s, C_6–H), 11.72 (1H, br s, NH). ^{13}C NMR (DMSO d_6, 50 MHz): δ ppm=24.9 (CH_3), 26.8 (CH_3), 60.9 (*C*H), 69.3 (quat), 80.1 (*C*H), 83.7 (*C*H), 86.7 (*C*H), 91.1 (*C*H), 112.7 (quat), 145.9 (*C*H), 149.8 (quat), 160.4 (quat). MS (FAB (+), glycerol matrix): *m/e*=411 $[M+H]^+$.

***N*-(2-Propynyl)-6-oxoheptanamide 6.** To a solution of 6-oxoheptanoic acid (2.75 g, 18 mmol) in dry THF (40 mL) cooled at 0°C were added successively under argon *N*-methylmorpholine (2 mL, 18 mmol), isobutylchloroformate (2.3 mL, 18 mmol) then after 15 min propargylamine (1.25 mL, 18 mmol) and the reaction was stirred for 30 min at rt. The solvent was evaporated and the residue obtained was dissolved in CH_2Cl_2. The organic layer was washed successively with aqueous NaOH (0.1N), aqueous HCl (0.1 N), brine and dried (Na_2SO_4). The crude product was purified by silica gel column chromatography (EtOAc) to give compound **6** as a white powder (2.6 g,

80%). Mp 88–90°C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ ppm=1.57 (4H, m, CH_2–CH_2), 2.10 (3H, s, $COCH_3$), 2.17–2.20 (3H, m, C C–*H* and $NHCOCH_2$), 2.43 (2H, t, $COCH_2$), 4.00 (2H, dd, –CH_2), 6.09 (1H, br s, N*H*). ^{13}C NMR ($CDCl_3$, 50 MHz): δ ppm=23.0 (CH_2), 24.7 (CH_2), 28.8 (CH_2), 29.8 (CH_3), 35.7 (CH_2), 43.1 (CH_2), 71.0 (*C*H), 79.7 (quat), 172.5 (quat), 209.0 (quat). MS (CI): *m/e*=182 $[M+H]^+$. IR (KBr): 3273, 3077, 2930, 2873, 2114, 1706, 1640, 1550 cm^{-1}. Anal. Calcd for $C_{10}H_{15}NO_2$, 0.25 H_2O: C 64.67 H 8.41 N 7.54, found C 64.51 H 8.30 N 7.69.

Protected nucleoside 7. To a mixture of compound **5** (0.5 g, 1.22 mmol) and copper iodide (44 mg, 0.232 mmol) in degassed DMF (5 mL) were added, under argon, triethylamine (0.33 mL, 2.32 mmol) and alkyne **6** (0.6 g, 3.48 mmol). The reaction was stirred in the dark for 10 min then tetrakis(triphenylphosphine)palladium (0.134 g, 0.116 mmol) was added. After stirring under argon for 3 h, the solvent was evaporated and the crude product was purified by silica gel column chromatography (EtOAc/MeOH: 95/5 then 90/10, v/v) to give nucleoside **7** as a white powder (0.34 g, 60%). Mp 138–140°C. ^{1}H NMR (DMSO d_6, 200 MHz): δ ppm=1.27 and 1.47 (6H, 2s, 2 CH_3 isoprop.), 1.42–1.44 (4H, m, CH_2–CH_2–CH_2–CH_2), 2.05 (3H, s, $COCH_3$), 2.06–2.10 (2H, m, $NHCOCH_2$), 2.40 (2H, t, $COCH_2$), 3.56 (2H, m, $C_{5',5''}$–*H*), 4.04 (3H, m, $C_{4'}$–*H* and CH_2NH), 4.74 (1H, dd, $C_{3'}$–*H*), 4.90 (1H, dd, $C_{2'}$–*H*), 5.13 (1H, t, $C_{5'}$–O*H*), 5.81 (1H, d, $C_{1'}$–*H*), 8.11 (1H, s, C_6–*H*), 8.30 (1H, t, CH_2NH), 11.68 (1H, br s, N*H*). ^{13}C NMR (DMSO d_6, 50 MHz): δ ppm=22.7 (CH_2), 24.4 (CH_2), 24.9 (CH_3), 26.8 (CH_3), 28.3 (CH_2), 29.5 (CH_3), 34.7 (CH_2), 42.2 (CH_2), 60.9 (CH_2), 73.7 (*C* C), 80.1 (*C*H), 83.6 (*C*H), 86.6 (*C*H), 89.7 (C≡*C*), 91.1 (*C*H), 98.0 (quat), 112.7 (quat), 144.7 (*C*H), 149.2 (*C*=O), 161.4 (*C*=O), 171.5 (*C*=O), 208.2 ($COCH_3$). MS (CI): *m/e*=464 $[M+H]^+$. UV (MeOH): $\lambda_{max}(\epsilon)$=288 (13000). IR (KBr): 3494, 3330, 3060, 2930, 1722, 1640, 1560, 1453 cm^{-1}. HRMS (FAB (+)) Calcd for $C_{22}H_{30}N_3O_8$: 464.2033, found 464.2027.

Deprotected nucleoside 8. The protected nucleoside **7** (0.2 g, 0.43 mmol) was dissolved in a 25% aqueous TFA solution (10 mL) and the solution was stirred at rt for 30 min. The solvent was then evaporated and the residue obtained was purified by C18 reverse phase column chromatography (H_2O/MeOH: 50/50, v/v) to give compound **8** as a white powder (0.143 g, 79%). Mp 138–140°C. ^{1}H NMR (DMSO d_6, 300 MHz): δ ppm=1.44 (4H, m, CH_2–CH_2–CH_2–CH_2), 2.04 (3H, s, $COCH_3$), 2.07 (2H, m, $NHCOCH_2$), 2.40 (2H, t, $COCH_2$), 3.50–3.70 (2H, m, $C_{5',5''}$–*H*), 3.85 (1H, m, $C_{4'}$–*H*), 3.96 (1H, m, $C_{3'}$–*H*), 4.00–4.10 (3H, m, CH_2NH and $C_{2'}$–*H*), 5.00 (1H, d, $C_{3'}$–O*H*), 5.12 (1H, t, $C_{5'}$–O*H*), 5.33 (1H, d, $C_{2'}$–O*H*), 5.74 (1H, d, *J*=5 Hz, $C_{1'}$–*H*), 8.19 (1H, s, C_6–*H*), 8.24 (1H, t, N*H*CO). ^{13}C NMR (DMSO d_6, 50 MHz): δ ppm=24.0 (CH_2), 26.1 (CH_2), 30.5 (CH_2), 30.8 (CH_3), 36.7 (CH_2), 44.1 (CH_2), 61.7 (CH_2), 70.5 (*C*H), 74.8 (C *C*), 75.4 (*C*H), 85.7 (*C*H), 90.9 (*C*H), 91.4 (*C* C), 100.2 (quat), 146.1 (*C*H), 151.6 (quat), 165.3 (quat), 177.3 (quat), 212 (quat). MS (FAB (−), glycerol matrix): *m/e*=422 $[M-H]^-$. Anal. Calcd for $C_{19}H_{25}N_3O_8$, 0.5 H_2O: C 52.77 H 6.06 N 9.72, found C 53.01 H 6.06 N 9.88.

Nucleoside triphosphate 1. The protected nucleoside **7** (0.232 g, 0.5 mmol) was dissolved in anhydrous pyridine and coevaporated twice to dryness in vacuo. To the residue obtained, pyridine (0.5 mL), dioxan (1.5 mL) and a solution of 2-chloro-4*H*-1,3,2-dioxaphosphorin-4-one in dioxan (1 M, 0.65 mL, 0.65 mmol) were added under argon. After 20 min, a solution of tributylammonium pyrophosphate (0.5 M) in anhydrous DMF (1.60 mL, 0.80 mmol) and tri-*n*-butylamine (0.65 mL) were added. The reaction was stirred for 30 min, then a solution of 1% iodine (10 mL, 0.39 mmol) in pyridine/water (98/2, v/v) was added. After 20 min, excess iodine was destroyed by addition of a 5% aqueous $NaHSO_3$ solution and after 10 min the solution was evaporated to dryness. The residue was dissolved in water (60 mL) and the aqueous layer was washed with CH_2Cl_2 (20 mL). The crude product was purified by C18 reverse phase column chromatography (H_2O then H_2O/MeOH: 50/50, v/v) giving the intermediate protected triphosphate **9** (0.16 mmol, 30%). t_R=32 min. ^{31}P NMR (D_2O, 80 MHz): δ ppm=−22.40 (t, Pβ), −11.20 (d, *J*=18 Hz, Pα), −9.80 (d, *J*=20 Hz, Pγ). ^{1}H NMR (D_2O, 200 MHz): δ ppm=1.22 and 1.42 (6H, 2s, 2 CH_3 isoprop.), 1.36–1.45 (4H, m, CH_2–CH_2–CH_2–CH_2), 1.99 (3H, s, $COCH_3$), 2.10 (2H, t, $COCH_2$), 2.38 (2H, t, $COCH_2$), 3.98 (2H, s, –CH_2), 4.01–4.09 (2H, m, $C_{5',5''}$–*H*), 4.45 (1H, m, $C_{4'}$–*H*), 4.88 (2H, m, $C_{2'}$–*H* and $C_{3'}$–*H*), 5.67 (1H, m, $C_{1'}$–*H*), 7.89 (1H, s, C_6–*H*). The protected nucleotide **9** (0.045 mmol) was then dissolved in water (15 mL) and a 50% aqueous solution of TFA (15 mL) was added. The mixture was stirred for 15 min then the solvent was evaporated. The residue obtained was dissolved in water (10 mL) and the pH was adjusted at 8.5 by addition of aqueous NaOH (0.1N). The crude product was purified using C18 reverse phase column chromatography by eluting with H_2O. After evaporation, the nucleotide **1** was obtained as a white powder (0.03 mmol, 70%). ^{31}P NMR (D_2O, 80 MHz): δ ppm=−21.28 (t, Pβ), −10.45 (d, Pα), −5.55 (d, Pγ). ^{1}H NMR (D_2O, 200 MHz): δ ppm=1.39 (4H, m, CH_2–CH_2–CH_2–CH_2), 1.99 (3H, s, $COCH_3$), 2.11 (2H, t, $COCH_2$), 2.38 (2H, t, $COCH_2$), 3.98 (2H, s, –CH_2), 4.00–4.10 (3H, m, $C_{4'}$–*H* and $C_{5',5''}$–*H*), 4.18 (1H, m, $C_{3'}$–*H*), 4.27 (1H, m, $C_{2'}$–*H*), 5.76 (1H, d, *J*=4.6 Hz, $C_{1'}$–*H*), 7.97 (1H, s, C_6–*H*). ES-MS (negative mode): *m/e*=662 $[M-H]^-$, 684 $[M-H+Na]^-$, 706 $[M-2H+2Na]^-$.

Protected nucleoside 12. To a solution of 6-oxoheptanoic acid (0.288 g, 2 mmol) in dry THF (5 mL) cooled at 0°C were added successively under argon *N*-methylmorpholine (0.223 mL, 2 mmol), isobutylchloroformate (0.258 mL, 2 mmol) then after 15 min the nucleoside **10** (0.980 g, 2 mmol) and the reaction was stirred at rt for 2 h. The solvent was then evaporated and the oily residue obtained was dissolved in Et_2O. The organic layer was washed with aqueous NaOH (0.1N), brine, then dried (Na_2SO_4). Evaporation of the solvent afforded the intermediate nucleoside **11** as an oily residue which was used without further purification in the next reaction. The crude product **11** was dissolved in THF (10 mL) and a 1 M TBAF solution in THF (3.35 mL, 3.35 mmol) was added and the reaction was stirred for 1 h. The solvent was then evaporated and the residue obtained was dissolved in CH_2Cl_2. The organic layer was washed with brine, dried (Na_2SO_4) and evaporated. The crude product was purified by silica gel column

chromatography (CH_2Cl_2 then CH_2Cl_2/MeOH: 95/5, v/v) to give compound **12** as a white resin (0.860 g, 85%). ^{1}H NMR ($CDCl_3$, 200 MHz): δ ppm=1.34 and 1.61 (6H, 2s, 2 CH_3 isoprop.), 1.50–1.70 (8H, m, 4 CH_2), 2.10 (3H, s, COCH_3), 2.15 (2H, m, COCH_2), 2.43 (2H, t, COCH_2), 3.27 (2H, m, CH_2NH), 3.68 (2H, m, CH_2NH), 3.92 (2H, m, $C_{5',5''}$–*H*), 4.50 (1H, m, $C_{4'}$–*H*), 5.07 (1H, m, $C_{3'}$–*H*), 5.17 (1H, m, $C_{2'}$–*H*), 5.81 (1H, d, *J*=4.8 Hz, $C_{1'}$–*H*), 6.09 (1H, t, $C_{5'}$–O*H*), 6.67 (1H, br m, N*H*), 7.76 (1H, s, C_8–*H*), 8.27 (1H, s, C_2–*H*). ^{13}C NMR ($CDCl_3$, 50 MHz): δ ppm=25.1 (*C*H_2), 27.1 (*C*H_2), 27.3 (*C*H_3), 28.8 (*C*H_2), 29.1 (*C*H_2), 29.7 (*C*H_3), 32.0 (*C*H_3), 38.5 (*C*H_2), 41.1 (*C*H_2), 42.3 (*C*H_2), 45.2 (*C*H_2), 65.4 (*C*H_2), 83.6 (*C*H), 84.9 (*C*H), 88.0 (*C*H), 96.3 (*C*H), 115.8 (quat), 141.4 (*C*H), 154.5 (*C*H), 157.2 (quat), 172 (NH*C*O), 209 (*C*OCH$_3$). MS (CI): *m*/*e*=505 $[M+H]^+$. UV (MeOH): $\lambda_{max}(\epsilon)$=267 (17000). IR (KBr): 3333, 2934, 2863, 1716, 1618 cm^{-1}. HRMS (CI) Calcd for $C_{24}H_{37}N_6O_6$: 505.2775, found 505.2761.

Deprotected nucleoside 13. As described for **8**, compound **13** was prepared by acidic treatment of **12** (yield: 65%). Mp 74–75°C. ^{1}H NMR (DMSO d_6, 300 MHz): δ ppm=1.39–1.46 (6H, m, 3 CH_2), 1.57 (2H, m, CH_2), 2.00 (2H, m, COCH_2), 2.03 (3H, s, COCH_3), 2.38 (2H, t, COCH_2), 3.04 (2H, m, CH_2NH), 3.46 (2H, m, CH_2NH), 3.60 (2H, m, $C_{5',5''}$–*H*), 3.95 (1H, m, $C_{4'}$–*H*), 4.13 (1H, m, $C_{3'}$–*H*), 4.58 (1H, m, $C_{2'}$–*H*), 5.87 (1H, d, *J*=6 Hz, $C_{1'}$–*H*), 7.73 (1H, t, N*H*), 8.05 (1H, t, N*H*), 8.21 (1H, s, C_8–*H*), 8.36 (1H, s, C_2–*H*). ^{13}C NMR (DMSO d_6, 75 MHz): δ ppm=23.0 (*C*H_2), 25.0 (*C*H_2), 26.8 (*C*H_2), 29.8 (*C*H_3), 35.4 (*C*H_2), 38.4 (*C*H_2), 42.6 (*C*H_2), 61.8 (*C*H_2), 70.8 (*C*H), 73.7 (*C*H), 86.1 (*C*H), 88.1 (*C*H), 140.0 (*C*H), 154.3 (*C*H), 158.1 (quat), 158.5 (quat), 171.9 (NH*C*O), 208.5 (*C*OCH$_3$). MS (FAB (+), glycerol matrix): *m*/*e*=465 $[M+H]^+$. HRMS (FAB (+)) Calcd for $C_{21}H_{33}N_6O_6$: 465.2462, found 465.2436.

Nucleoside triphosphate 2. Using the same procedure described for **1**, the nucleotide **2** was obtained from protected nucleoside **12** as a white powder with a 33% overall yield. Protected nucleotide intermediate **14**: t_R=30 min. ^{31}P NMR (D_2O, 80 MHz): δ ppm=−22.22 (t, Pβ), −10.50 (d, Pα), −9.85 (d, Pγ). ^{1}H NMR (D_2O, 200 MHz): δ ppm=1.26 and 1.48 (6H, 2s, 2 CH_3 isoprop.), 1.10–1.30 (4H, m, CH_2–CH_2), 1.40–1.60 (4H, m, CH_2–CH_2), 1.91 (3H, s, COCH_3), 1.97 (2H, t, COCH_2), 2.25 (3H, t, COCH_2), 3.02 (2H, t, CH_2NH), 3.37 (2H, m, CH_2NH), 3.92–4.14 (2H, m, $C_{5',5''}$–*H*), 4.49 (1H, m, $C_{4'}$–*H*), 5.05 (1H, dd, $C_{3'}$–*H*), 5.19 (1H, m, $C_{2'}$–*H*), 6.05 (1H, d, *J*=4 Hz, $C_{1'}$–*H*), 8.01 (1H, s, C_2–*H*), 8.22 (1H, s, C_8–*H*). Nucleotide triphosphate **2**: t_R=30 min. ^{31}P NMR (D_2O, 80 MHz): δ ppm=−21.37 (t, Pβ), −10.38 (d, Pα), −5.40 (d, Pγ). ^{1}H NMR (D_2O, 200 MHz): δ ppm=1.28 (4H, m, CH_2–CH_2), 1.49 (4H, m, CH_2–CH_2), 1.92 (3H, s, COCH_3), 1.98 (2H, t, COCH_2), 2.27 (2H, t, COCH_2), 3.04 (2H, t, CH_2NH), 3.40 (2H, m, CH_2NH), 3.92–4.14 (2H, m, $C_{5',5''}$–*H*), 4.19 (1H, m, $C_{4'}$–*H*), 4.40–4.50 (2H, m, $C_{3'}$–*H* and $C_{2'}$–*H*), 5.92 (1H, d, *J*=5.9 Hz, $C_{1'}$–*H*), 8.05 (1H, s, C_2–*H*), 8.32 (1H, s, C_8–*H*). ES-MS (negative mode): *m*/*e*=703 $[M-H]^-$, 725 $[M-2H+Na]^-$, 747 $[M-3H+2Na]^-$.

5′-*tert*-Butyldimethylsilyl-2′,3′-*O*-isopropylidene-uridine 15. To a solution of commercial 2′,3′-*O*-isopropylidene-uridine (5 g, 17.6 mmol) in anhydrous pyridine (30 mL) was added under argon *tert*-butyldimethylsilyl chloride (3 g, 19.4 mmol). The solution was stirred for 6 h and the solvent was then evaporated. The oily residue obtained was dissolved in CH_2Cl_2 and the organic layer was washed with brine and dried (Na_2SO_4). Evaporation of the solvent gave compound **15** as a white powder (5.9 g, 85%). ^{1}H NMR ($CDCl_3$, 200 MHz): δ ppm=0.04 (6H, s, Si(CH_3)$_2$), 0.85 (9H, s, Si–C(CH_3)$_3$), 1.30 and 1.54 (6H, 2s, 2 CH_3 isoprop.), 3.85 (2H, m, $C_{5',5''}$–*H*), 4.25 (1H, m, $C_{4'}$–*H*), 4.66 (1H, dd, $C_{3'}$–*H*), 4.71 (1H, dd, $C_{2'}$–*H*), 5.64 (1H, d, *J*=7.2 Hz, C_5–*H*), 5.94 (1H, d, $C_{1'}$–*H*), 7.63 (1H, d, *J*=7.2 Hz, C_6–*H*), 9.65 (1H, s, N*H*). ^{13}C NMR ($CDCl_3$, 50 MHz): δ ppm=−5.6 and −5.5 (*C*H_3), 18.2 (quat), 25.3 (*C*H_3), 25.7 (*C*H_3), 27.2 (*C*H_3), 63.2 (*C*H_2), 80.2 (*C*H), 85.2 (*C*H), 86.6 (*C*H), 91.8 (*C*H), 102.1 (*C*H), 113.9 (quat), 140.6 (*C*H), 150.2 (quat), 163.5 (quat). MS (FAB (−), glycerol matrix): *m*/*e*=397 $[M-H]^-$.

5′-*tert*-Butyldimethylsilyl-2′,3′-*O*-isopropylidene-4-*O*-tosyl-uridine 16. To a solution of the protected nucleoside **15** (2.4 g, 6 mmol) in acetonitrile (50 mL) were added potassium carbonate (1 g, 7.2 mmol) and tosyl chloride (1.26 g, 6.6 mmol). The solution was refluxed for 4 h then filtered and evaporated. The oily residue obtained was dissolved in EtOAc and the organic layer was washed with water, dried (Na_2SO_4) and evaporated to afford compound **16** as a white powder (3 g, 90%). ^{1}H NMR ($CDCl_3$, 200 MHz): δ ppm=0.00 (6H, s, Si(CH_3)$_2$), 0.80 (9H, s, Si–C(CH_3)$_3$), 1.30 and 1.53 (6H, 2s, 2 CH_3 isoprop.), 2.40 (3H, s, Tos–CH_3), 3.80 (2H, m, $C_{5',5''}$–*H*), 4.37 (1H, m, $C_{4'}$–*H*), 4.64 (2H, m, $C_{3'}$–*H* and $C_{2'}$–*H*), 5.84 (1H, s, $C_{1'}$–*H*), 5.98 (1H, d, *J*=7.2 Hz, C_5–*H*), 7.32 (2H, d, Ar*H* Tos), 8.02 (2H, d, Ar*H* Tos), 8.15 (1H, d, *J*=7.2 Hz, C_6–*H*). ^{13}C NMR ($CDCl_3$, 50 MHz): δ ppm=−5.7 and −5.6 (*C*H_3), 18.1 (quat), 21.7 (*C*H_3), 25.1 (*C*H_3), 25.7 (*C*H_3), 27.1 (*C*H_3), 63.2 (*C*H_2), 79.9 (*C*H), 86.1 (*C*H), 88.1 (*C*H), 94.2 (*C*H), 94.6 (*C*H), 113.6 (quat), 129.5 (*C*H), 129.8 (*C*H), 132.9 (quat), 145.9 (quat), 146.3 (*C*H), 153.6 (quat), 166.5 (quat).

Protected nucleoside 18. To a solution of nucleoside **16** (3 g, 5.4 mmol) in CH_2Cl_2 (100 mL) was added the amine **17** (1.94 g, 12.2 mmol) and the reaction was stirred under argon for 10 min at rt. The solution was then washed successively with water, with a 5% aqueous HCl solution, with brine then dried (Na_2SO_4) and evaporated. Compound **18** was purified by silica gel column chromatography (EtOAc then EtOAc/MeOH: 95/5, v/v) and was obtained as a white powder (1.8 g, 62%). Mp 51–54°C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ ppm=0.04 (6H, s, Si(CH_3)$_2$), 0.83 (9H, s, Si–C(CH_3)$_3$), 1.22 (3H, s, CH_3), 1.26 and 1.54 (6H, 2s, 2 CH_3 isoprop.), 1.30–1.68 (6H, m, 3 CH_2), 3.42 (2H, m, CH_2NH), 3.70–3.90 (2H, m, $C_{5',5''}$–*H*), 3.84 (4H, m, O–CH_2CH_2–O), 4.15 (1H, m, $C_{4'}$–*H*), 4.75 (2H, m, $C_{3'}$–*H* and $C_{2'}$–*H*), 5.57 (1H, d, *J*=7.5 Hz, C_5–*H*), 5.80 (1H, m, $C_{1'}$–*H*), 7.42 (1H, d, *J*=7.5 Hz, C_6–*H*). ^{13}C NMR ($CDCl_3$, 50 MHz): δ ppm=−5.6 and −5.5 (*C*H_3), 18.2 (quat), 21.4 (*C*H_2), 23.6 (*C*H_3), 25.3 (*C*H_3), 25.8 (*C*H_3), 27.2 (*C*H_3), 29.1 (*C*H_2), 38.6 (*C*H_2), 40.7 (*C*H_2), 63.3 (*C*H_2), 64.5 (*C*H_2), 80.1 (*C*H), 85.5 (*C*H), 87.5 (*C*H), 93.4 (*C*H), 95.1 (*C*H), 109.8 (quat), 113.4 (quat), 140.7 (*C*H), 155.9 (quat), 163.7 (quat). MS (FAB (-), glycerol matrix): *m*/*e*=538 $[M-H]^-$.

Nucleoside 19. The 5′-protected nucleoside **18** (1.1 g, 2.04 mmol) was dissolved in THF (30 mL) and a 1 M TBAF solution in THF (2.24 mL, 2.24 mmol) was added. The reaction was stirred for 30 min and the solvent was evaporated. The oily residue obtained was dissolved in CH_2Cl_2 and the organic layer was washed with brine, dried (Na_2SO_4) and evaporated. Compound **19** was obtained after purification by silica gel column chromatography (CH_2Cl_2 then CH_2Cl_2/MeOH: 95/5, v/v) as a white powder (0.7 g, 80%). Mp 64–65°C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ ppm=1.23 (3H, s, CH_3), 1.28 and 1.49 (6H, 2s, 2 CH_3 isoprop.), 1.36–1.42 (2H, m, CH_2), 1.50–1.56 (4H, m, 2 CH_2), 3.36 (2H, m, CH_2NH), 3.75 (2H, m, $C_{5',5''}$–H), 3.85 (4H, m, O–CH_2CH_2–O), 4.23 (1H, m, $C_{4'}$–H), 5.05 (1H, m, $C_{3'}$–H), 5.17 (1H, m, $C_{2'}$–H), 5.27 (1H, dd, $C_{1'}$–H), 5.69 (1H, d, J=7.2 Hz, C_5–H), 6.22 (1H, t, $C_{5'}$–OH), 7.16 (1H, d, J=7.2 Hz, C_6–H). ^{13}C NMR ($CDCl_3$, 50 MHz): δ ppm= 21.3 (*C*H_2), 23.6 (*C*H_3), 25.1 (*C*H_3), 27.2 (*C*H_3), 28.9 (*C*H_2), 38.5 (*C*H_2), 40.8 (*C*H_2), 62.7 (*C*H_2), 64.5 (*C*H_2), 80.4 (*C*H), 83.5 (*C*H), 87.5 (*C*H), 95.9 (*C*H), 98.8 (*C*H), 109.8 (quat), 113.6 (quat), 143.2 (*C*H), 156.4 (quat), 164.1 (quat). MS (FAB (−), glycerol matrix): *m*/*e*=424 $[M-H]^-$. Anal. Calcd for $C_{20}H_{31}N_3O_7$: C 56.46 H 7.34 N 9.88, found: C 56.00 H 7.61 N 9.79.

Nucleotide triphosphate 3. The nucleotide **3** was obtained as a white powder with an 30% overall yield from protected nucleoside **19** using the same procedure as for obtention of **1**. Protected nucleotide intermediate **20**: t_R=32 min. ^{31}P NMR (D_2O, 80 MHz): δ ppm=−21.20 (t, Pβ), −10.21 (d, Pα), −6.56 (m, Pγ). ^{1}H NMR (D_2O, 200 MHz): δ ppm=1.15 (3H, s, CH_3), 1.22 and 1.43 (6H, 2s, 2 CH_3 isoprop.), 1.23–1.60 (6H, m, 3 CH_2), 3.18 (2H, t, CH_2NH), 3.80 (4H, m, O–CH_2CH_2–O), 4.03 (2H, m, $C_{5',5''}$–H), 4.39 (1H, m, $C_{4'}$–H), 4.76 (1H, m, $C_{3'}$–H), 4.88 (1H, m, $C_{2'}$–H), 5.83–5.87 (2H, m, $C_{1'}$–H and C_5–H), 7.53 (1H, d, J=7.6 Hz, C_6–H). Nucleotide **3**: t_R=30 min. ^{31}P NMR (D_2O, 80 MHz): δ ppm=−22.55 (t, Pβ), −10.75 (d, Pα), −10.01 (d, Pγ). ^{1}H NMR (D_2O, 200 MHz): δ ppm=1.41 (4H, m, 2 CH_2), 2.00 (3H, s, COCH_3), 2.42 (2H, m, COCH_2), 3.17 (2H, m, CH_2NH), 4.04–4.07 (3H, m, $C_{5',5''}$–H and $C_{4'}$–H), 4.14–4.22 (2H, m, $C_{3'}$–H and $C_{2'}$–H), 5.82–5.90 (2H, m, $C_{1'}$–H and C_5–H), 7.64 (1H, d, J=7.6 Hz, C_6–H). ES-MS (negative mode): *m*/*e*=580 $[M-H]^-$.

5-[3-(3-Aminopropyl)thioureido]-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl) benzoic acid 21. To a solution of 1,3-diaminopropane (0.5 mL, 6.9 mmol) in anhydrous DMF (20 mL), was added dropwise under argon a solution of FITC (0.5 g, 1.16 mmol) in dry DMF (7 mL). The reaction mixture was stirred for 30 min and the solvent was then evaporated. The oily residue obtained was purified by C18 reverse phase column chromatography (H_2O then H_2O/MeOH: 50/50, v/v) to give compound **21** as an orange powder (0.42 g, 80%). Mp 205–207°C (dec.). ^{1}H NMR (DMSO d_6–AcOD, 200 MHz): δ ppm=1.91 (2H, m, CH_2), 2.94 (2H, m, CH_2N), 3.58 (2H, m, CH_2N), 6.45–6.57 (6H, m, 6 Ar*H*), 7.01 (1H, d, J=8.2 Hz, Ar*H*), 7.61 (1H, d, J=8.2 Hz, Ar*H*), 8.02 (1H, s, Ar*H*). MS (FAB (+), glycerol matrix): *m*/*e*=464 $[M+H]^+$. HRMS (FAB (+)) Calcd for $C_{24}H_{22}N_3O_5S$: 464.1280, found: 464.1279.

2-[(*N*-Fluorenemethyloxycarbonyl)-aminooxy]-acetic acid 22. To a 10% $NaHCO_3$ aqueous solution (25 mL) of commercially available *O*-(carboxymethyl)hydroxylamine hemihydrochloride (0.847 g, 7.75 mmol) cooled at 0°C, was added dropwise under argon a solution of 9-fluorenylmethylchloroformate (2 g, 7.75 mmol) in dioxan (20 mL). The reaction mixture was stirred overnight at rt and then evaporated. To the residue obtained was added H_2O (250 mL) and the aqueous layer was washed with Et_2O (200 mL). The pH of the aqueous layer was then adjusted pH=3 by addition of aqueous HCl (1N) and extracted with CH_2Cl_2 (3×250 mL). The organic layers were then dried (Na_2SO_4) and evaporated to afford the acid **22** as a white powder (2.4 g, 98%). Mp 125–126°C. ^{1}H NMR (DMSO d_6, 200 MHz): δ ppm=4.23 (1H, m, CH_2–CH), 4.27 (2H, s, OCH_2CO), 4.36 (2H, d, OCH_2–CH), 7.31 (2H, t, 2 Ar*H*), 7.40 (2H, t, 2 Ar*H*), 7.70 (2H, d, J= 6.9 Hz, 2 Ar*H*), 7.86 (2H, d, J=6.9 Hz, 2 Ar*H*). ^{13}C NMR (DMSO d_6, 50 MHz): δ ppm=46.3 (*C*H), 65.9 (*C*H_2), 71.9 (*C*H_2), 119.9 (*C*H), 125.0, 126.9 and 127.5 (*C*H), 140.5 (quat), 143.4 (quat), 156.8 (quat), 169.9 (quat). MS (DCI): *m*/*e*=314 $[M+H]^+$. Anal. Calcd for $C_{17}H_{15}NO_5$: C 65.17 H 4.83 N 4.47, found: C 65.54 H 4.96 N 4.50.

Protected fluorescein probe 23. To a solution of acid **22** (0.473 g, 1.5 mmol) in anhydrous DMF (10 mL) cooled at 0°C were added successively under argon *N*-methylmorpholine (0.166 mL, 1.5 mmol), isobutylchloroformate (0.193 mL, 1.5 mmol) then after 15 min the compound **21** (0.350 g, 0.75 mmol) and the reaction mixture was stirred for 1 h at rt. The solvent was then evaporated and the residue obtained was purified by silica gel column chromatography (CH_2Cl_2/MeOH: 50/50, v/v) to afford compound **23** as an orange powder (0.227 g, 40%). Mp 162–165°C. ^{1}H NMR (DMSO d_6, 200 MHz): δ ppm=1.71 (2H, m, CH_2), 3.17 (2H, m, CH_2NH), 3.49 (2H, m, CH_2NH), 4.13 (2H, s, CH_2–ONH), 4.22 (1H, t, CH–CH_2), 4.42 (2H, m, OCH_2–CH), 6.55–6.68 (6H, m, Ar*H* Fluo), 7.10 (1H, d, Ar*H* Fluo), 7.28 (2H, t, Ar*H* Fmoc), 7.33 (1H, m, Ar*H* Fluo), 7.37 (2H, t, Ar*H* Fmoc), 7.61 (2H, dd, J=6.8 Hz, Ar*H* Fmoc), 7.82 (2H, dd, J=6.8 Hz, Ar*H* Fmoc), 8.01 (2H, m, 2 N*H*), 8.21 (1H, s, Ar*H* Fluo), 9.95, 10.10 and 10.72 (4H, s, NH and OH). ^{13}C NMR (DMSO d_6, 50 MHz): δ ppm=26.08 (*C*H_2), 28.1 (*C*H_2), 35.7 (*C*H_2), 41.0 (*C*H_2), 46.2 (*C*H), 66.1 (*C*H_2), 74.5 (*C*H_2), 83.0 (quat), 102.0 (quat), 109.5 (*C*H), 112.3 (quat), 119.9 (*C*H), 124.0 (quat), 124.8 (*C*H), 126.3 (quat), 126.9 (*C*H), 127.6 (*C*H), 128.8 (*C*H), 140.5 (quat), 143.1 (quat), 147 (quat), 151.6 (quat), 157.2 (quat), 159.0 (quat), 167.9 (quat), 168.5 (quat), 180.0 (quat). MS (FAB (+), glycerol matrix): *m*/*e*=759 $[M+H]^+$. HRMS (FAB (+)) Calcd for $C_{41}H_{34}N_4O_9S$: 759.2125, found 759.2128.

Fluoresceine probe 4. The protected probe **23** (0.116 g, 0.16 mmol) was dissolved in anhydrous DMF (3 mL) and piperidine (0.025 mL, 0.24 mmol) was added. The solution was stirred for 15 min at rt and the solvent was evaporated. The residue obtained was purified by C18 reverse phase column chromatography to give compound **4** as an orange powder (0.078 g, 93%). Mp 188–190°C. ^{1}H NMR (DMSO d_6, 500 MHz): δ ppm=1.72 (2H, m, CH_2), 3.17 (2H, m, CH_2NH), 3.39–3.48 (2H, m, CH_2NH), 3.52 (2H, s, ONH_2), 3.94 (2H, s, CH_2–ONH_2), 6.53–6.63 (6H, m, Ar*H*), 7.16 (1H, d, Ar*H*), 7.72 (1H, m, Ar*H*), 7.86 (1H, t, N*H*), 8.19 (1H, s, Ar*H*). MS (FAB (−), glycerol matrix):

m/e=535 $[M-H]^-$. HRMS (FAB (+)) Calcd for $C_{26}H_{24}N_4O_7S$: 537.1444, found: 537.1445.

Oxime ether 24. To a solution of deprotected nucleoside **8** (5 mg, 0.01 mmol) in a 50% aqueous acetonitrile solution (1 mL) was added a 50% aqueous acetonitrile solution (2.5 mL) of fluorophore **4** (12 mg, 0.02 mmol). The solution was stirred for 2 h at rt and X-treme resin was then added. The mixture was stirred for 30 min and filtered. The resin was washed several times with a 50% aqueous acetonitrile solution and the solvent was then evaporated. The crude product was purified by C18 reverse phase column chromatography (H_2O/CH_3CN: 80/20, v/v) to give compound **24** as an orange powder (9 mg, 81%). ^{1}H NMR (DMSO d_6, 500 MHz, 2 diastereoisomers): δ ppm=1.35–1.52 (4H, m, CH_2CH_2–CH_2–$C(CH_3)$=N–O), 1.67 (2H, m, CH_2–CH_2–NH–C=S), 1.76 and 1.84 (3H, 2s, CH_2–$C(CH_3)$=N–O), 2.09 (2H, m, $NHCOCH_2$), 2.34 and 2.39 (2H, 2t, CH_2–$C(CH_3)$=N–O), 3.16 (2H, m, NH–$CH_2CH_2CH_2$–NH–C=S), 3.51 (2H, m, CH_2–NH–C=S), 3.54–3.65 (2H, m, $C_{5',5''}$–*H*), 3.83 (1H, m, $C_{4'}$–*H*), 3.94 (1H, m, $C_{3'}$–*H*), 4.02 (1H, m, $C_{2'}$–*H*), 4.05 (2H, d, $^-CH_2$), 4.31 and 4.33 (2H, 2s, =N–O–CH_2CO), 5.74 (1H, d, $C_{1'}$–*H*), 6.54–6.66 (6H, m, 6 Ar*H*), 7.16 (1H, d, Ar*H*), 7.66–7.74 (2H, m, Ar*H*), 8.20 (2H, s, Ar*H* and C_6–*H*), 8.29 (1H, t, N*H*CO), 8.29 (1H, t, N*H*CO). MS (FAB (+), glycerol matrix): *m/e*=942 $[M+H]^+$, 965 $[M+H+Na]^+$. HRMS (FAB(+)) Calcd for $C_{45}H_{48}N_7O_{14}S$: 942.2980, found 942.3016.

Oxime ether 25. The oxime ether **25** was obtained in the same manner as compound **24** in a 82% yield. ^{1}H NMR (DMSO d_6, 500 MHz, 2 diastereoisomers): δ ppm=1.36–1.50 (6H, m, NH–CH_2CH_2–CH_2 and $NHCOCH_2CH_2$–CH_2–) 1.67 (2H, quint., CH_2–CH_2–NH–C=S), 1.75 and 1.83 (3H, 2s, CH_2–$C(CH_3)$=N–O), 2.09 (2H, m, NHCO–CH_2), 2.32 and 2.37 (2H, 2t, CH_2–$C(CH_3)$=N–O), 3.02 (2H, m, HN^6–CH_2CH_2–CH_2CH_2NHCO), 3.16 (2H, m, NH–$CH_2CH_2CH_2$–NH–C=S), 3.45 (2H, m, HN^6CH_2), 3.50 (2H, m, CH_2–NH–C=S), 3.53–3.65 (2H, m, $C_{5',5''}$–*H*) 3.94 (1H, m, $C_{4'}$–*H*), 4.13 (1H, m, $C_{3'}$–*H*), 4.31 and 4.32 (2H, 2s, =N–O–CH_2CO), 4.60 (1H, t, $C_{2'}$–*H*), 5.86 (1H, d, $C_{1'}$–*H*), 6.53–6.66 (6H, m, Ar*H*), 7.14 (1H, d, Ar*H*), 7.66–7.74 (2H, m, Ar*H*), 8.32 (1H, s, C_2–*H*), 8.41 (1H, s, C_8–*H*). MS (FAB (+), glycerol matrix): *m/e*=983 $[M+H]^+$, 1005 $[M+Na]^+$. HRMS (FAB(+)) Calcd for $C_{47}H_{54}N_{10}O_{12}S$: 983.3722, found 983.3713.

Incorporation and labelling assay. The PCR target containing the T7 promoter was prepared as described.[14] This PCR target was used for generating the modified single-stranded RNA target by in vitro transcription. Transcriptions were carried out as described previously[14] by incubating the PCR target, T7 RNA polymerase and equimolar concentrations of the nucleotides at 37°C for 1 h. The ratios of modified versus natural nucleotides were varied in the range 0:100, 30:70, 70:30, 100:0. Labelling was then achieved by adding an excess of a solution of the fluorescein derivative **4** (10 equiv.) in DMF to the crude transcription mixture. The reaction was performed at room temperature for 30 min. The labelled transcription products were analysed by electrophoresis on a 6% urea/polyacrylamide gel in Tris–borate buffer (pH 8.5) at 150 V for 1 h.

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