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Synthesis of a fluorescent derivative of 6-*N*-[*N*-(6-aminohexyl)-carbamoyl)-2′,3′-dideoxyadenosine 5′-triphosphate for detection of nucleic acids

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Treatment of 5′-*O*-TBDMS-2′,3′-dideoxyadenosine **2** with phenoxycarbonyltetrazole followed by *N*-trifluoroacetyl-1,6-diaminohexane yielded 6-*N*-carbamoyl derivative **4**, which was further converted into the deprotected nucleoside **5**. The latter compound was transformed into its triphosphate **6**, which has been dye-labelled at the aliphatic amino group with the fluorescent dye JA242. The conjugate was shown to terminate DNA synthesis catalysed by terminal transferase and different DNA polymerases, allowing DNA detection by time-resolved fluorescence.

The method of choice for the determination of a DNA sequence is Sanger dideoxy DNA sequencing,[1] based on the use of 2′,3′-dideoxynucleoside 5′-triphosphates (ddNTPs) as terminators.

As an alternative for radioactive methods different strategies have been developed in the past, based on the detection of the laser-induced fluorescence of dye-labelled DNA fragments.[2] The strategies used are, preferentially, dye primer sequencing[3] and dye terminator sequencing, with the latter having two main advantages. For the incorporation of the terminator only one extension reaction with four different labelled dideoxynucleosides is needed for each sequence, and DNA fragments formed by false terminations are unlabelled and, therefore, invisible. For dye terminator sequencing there are different approaches to attach a fluorophore *via* a linker arm to the dideoxynucleoside 5′-triphosphate used as sequencing terminator.

The syntheses of base-modified ddNTPs has been described where linker groups with different fluorescent dyes were attached at position C-5 for pyrimidines and at the 7-deaza site for purines as modified nucleobases.[4]

Alternatively the synthesis of different 3′-sugar-modified 2′,3′-dideoxynucleoside 5′-triphosphates has been reported in the literature.[5] 3′-Amino-2′,3′-dideoxynucleoside 5′-triphosphates are well accepted as substrates by different polymerases.[6] The problem of induced cleavage of 3′-ester and -amide linker-modified nucleoside 5′-triphosphates by DNA polymerases[7] and the non-acceptance of different 3′-*C*-modified nucleoside 5′-triphosphates[8] has led to difficulties in applying sugar-modified derivatives as nucleotide terminators in DNA sequencing.

Recently, our group has succeeded in showing that 4-N-dye-labelled 2′,3′-dideoxycytidine 5′-triphosphates serve as efficient terminators in DNA sequencing.[9] These promising results led us to develop a similar synthesis for purine nucleotides. In this paper we describe the synthesis of 2′,3′-dideoxyadenosine 5′-triphosphate bearing a longer chain amino linker at the exocyclic 6-N-position and labelled at the aliphatic amino group with the fluorescent dye JA242. The conjugate **7** acts as terminator in the DNA-primer elongation by terminal deoxynucleotidyl transferase (Tdt) and in the DNA dideoxynucleotide sequencing catalysed by different polymerases.

Results and discussion

The reaction sequence yielding the 6-N-modified 2′,3′-dideoxyadenosine 5′-triphosphate labelled with JA242 is shown in Scheme 1. The key steps are discussed below.

Synthesis of 6-*N*-[*N*-(6-aminohexyl)carbamoyl)-2′,3′-dideoxyadenosine 5′-triphosphate 6

After protection of the 5′-OH-group of 2′,3′-dideoxyadenosine **1** to give the TBDMS ether **2** we used the method of Adamiak and Stawinski[10] to modify the exocyclic C-6-amino group of the nucleobase. This group was activated by phenoxycarbonyltetrazole in 1,4-dioxane for 4 h at 45 °C. 1,6-Diaminohexane was used as linker molecule. It was protected on one side with ethyl trifluoroacetate, according to the method of Agrawal and Tang.[11] Treatment of the intermediate carbamate **3** with the trifluoroacetylated diaminohexane in pyridine for 2 h at 55 °C led to compound **4** (in 60% yield from **2**). The ^{19}F NMR spectrum showed a single peak for the trifluoroacetyl protecting group at −76.08 ppm. The trifluoroacetyl group was completely stable under the reaction conditions. Deprotection of **4** by tetrabutylammonium fluoride (TBAF) in THF yielded the nucleoside **5** in 92% yield after preparative reversed-phase chromatography.

The method of Ludwig and Eckstein[12] was applied for the preparation of the nucleoside 5′-triphosphate **6**. Following the original method, the nucleoside was allowed to react with salicyloyl chlorophosphite (2-chloro-4*H*-1,3,2-benzodioxaphosphinin-4-one) in 1,4-dioxane–pyridine. The intermediate formed was treated *in situ* with bis(tributylammonium) dihydrogen pyrophosphate in DMF. Oxidation of the phosphite was carried out with a freshly prepared solution of iodine in pyridine–water (98:2; v/v). For removal of the trifluoroacetyl

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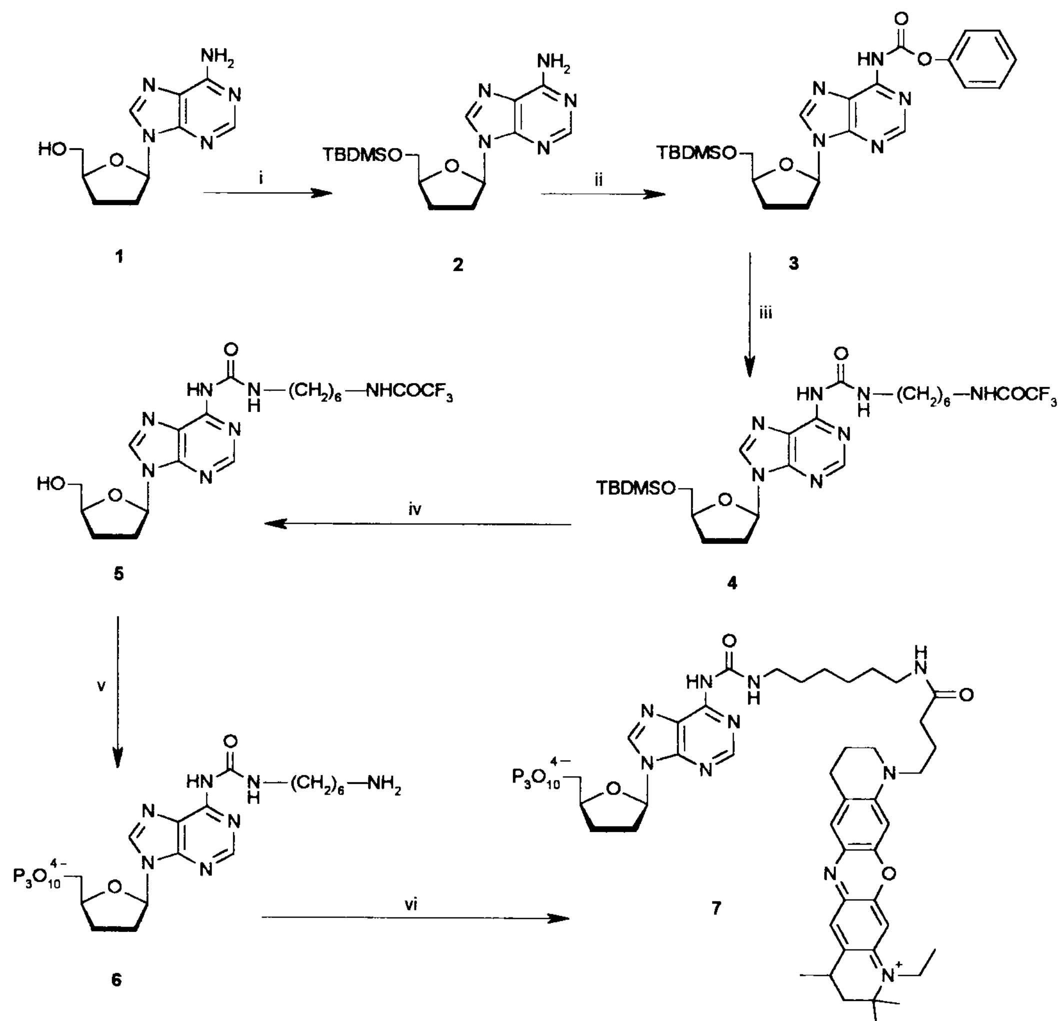

Scheme 1 (i) TBDMSCl, Py, 5 h, rt; (ii) phenoxycarbonyltetrazole, 1,4-dioxane, 4 h, 45 °C; (iii) *N*-trifluoroacetyl-1,6-diaminohexane, Py, 2 h, 55 °C; (iv) TBAF, THF, 1 h, rt; (v) (a) 2-chloro-4*H*-1,3,2-benzodioxaphosphinin-4-one, 1,4-dioxane, pyridine, 15 min, rt; (b) bis(tributylammonium) dihydrogen pyrophosphate, DMF, Bu_3N, 20 min, rt; (c) I_2, pyridine–water (98 : 2 v/v, 20 min, rt; (d) 25% aq. NH_3, 6 h, rt; (vi) JA242, TSTU, DMF–1,4-dioxane, water, DIPEA, rt, 20 min for activation; and then 48 h in the dark, rt.

group, conc. aq. ammonia was used. After purification on DEAE-Sephadex A25 and lyophilisation the product **6** was obtained as the triethylammonium salt in 55% yield. For characterisation the nucleotide was converted into the sodium salt.

Synthesis of dye-labelled 6-*N*-[*N*-(6-aminohexyl)carbamoyl)-2′,3′-dideoxyadenoside 5′-triphosphate 7

The JA242-labelled nucleotide 5′-triphosphate was generated by the reaction of free JA242 dye with the aliphatic amino group of nucleotide **6** according to the method of Bannwarth and Knorr.[13] At first the carboxy group of JA242 was activated with *N*,*N*,*N*′,*N*′-tetramethyl-*O*-succinimidouronium tetrafluoroborate (TSTU) in DMF–1,4-dioxane–DIPEA. The reaction was complete after 20 min as judged by TLC. The reaction was continued by the addition of 0.2 M aq. nucleoside 5′-triphosphate **6**. The free amino group of the nucleotide reacted with the activated carboxy group of the dye, with formation of the corresponding amide within 48 h. Purification of the 6-N^6-dye-labelled nucleoside 5′-triphosphate consisted of three steps. The procedure has proven to be most suitable for purifying dye-labelled triphosphates. In the first step the dye-linker–nucleotide conjugate was subjected to RP-18 silica gel chromatography. To separate the conjugate from the free dye, the formation of the Na^+ salt of compound **7** was required for the next step. The last step, purification on Mono Q-HPLC and desalting, gave the desired product with 6% overall yield and a purity of 95%. The spectroscopic properties corresponded well to the data reported by Lieberwirth *et al.*[14] Determination of the fluorescence quantum yield of the JA242-labelled nucleotide **7** showed a value of 0.82 with respect to the free dye (1.0), which indicates that coupling of the dye to the adenine base moiety resulted in no significant quenching. The value of the fluorescence decay time is 2.4 ns and similar to that of the free dye.[14]

Incorporation of 6-N-dye-modified nucleotide 7 into DNA by terminal deoxynucleotidyl transferase

Tdt from calf thymus catalyses a template-independent addition of deoxyribonucleoside 5′-triphosphates to the 3′-OH ends of double- or single-stranded DNA. When incubated in the presence of Tdt and a single-stranded oligonucleotide substrate (21-mer), the 6-N-substituted 2′,3′-dideoxyadenosine 5′-triphosphate **7** acted as substrate and was thus incorporated into the DNA. The product was observed as a dark band under ethidium bromide-supported UV light (Fig. 1, lane 2). Under visible light the primer could not be observed (Fig. 1, lane 3), but the product was detected as a blue band (Fig. 1, lane 4) caused by the blue fluorescent dye covalently attached to the oligonucleotide substrate.

6-N-JA242-modified nucleotide 7 as terminator in DNA-sequencing reactions

The ability of the nucleotide **7** to serve as a potent chain terminator for DNA polymerases was investigated in standard DNA-sequencing reactions. Their performance was tested by

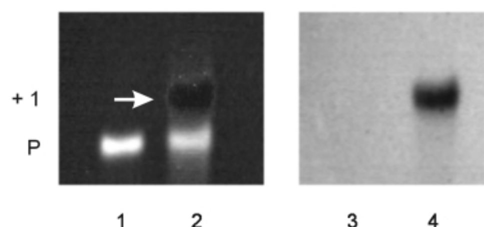

Fig. 1 Primer extension with 6-N-modified ddATP **7** catalysed by Tdt. Ethidium bromide-supported UV light: Lane 1, primer; lane 2, primer + enzyme + **7** (product as dark band); visible light: lane 3, primer; lane 4, primer + enzyme + **7**.

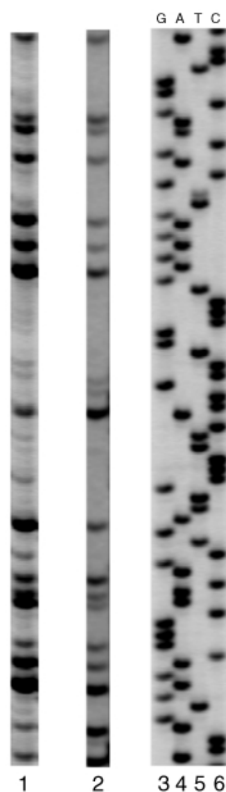

Fig. 2 Sequencing catalysed by a new thermostable DNA polymerase with dye-labelled primers (15 mM of **7**, lane 1) and unlabelled primer (15 mM of **7**, lane 2); lanes 3–6: ddNTP as references.

sequence analysis of the bacteriophage vector M13mp18 with the M13/pUC sequencing primer and two enzymes: ThermoSequenase and a newly developed thermostable DNA polymerase, which had shown improved incorporation of commercially available fluorescent-dye-labelled sequencing terminators in previous experiments.[15] All experiments were performed under cycle-sequencing conditions including denaturing steps at 95 °C, demonstrating that the dye-labelled nucleotide is stable under these conditions in contrast to 6-N-amide-modified nucleotides (data not shown). In two different approaches, the dye-labelled nucleotide **7** was incubated with either a dye-labelled sequencing primer to test only the acceptance of the nucleotide or with an unlabelled primer to detect the multiplex dye JA242 attached to the fragment. The conjugate **7** was well accepted by the enzymes and showed good termination results (Fig. 2). Reading length with the new dideoxynucleotides was up to 600 bases without being optimised.

Similar results were obtained with a 2′,3′-dideoxyadenosine 5′-triphosphate 6-N-labelled with JA242 and CY5[14] *via* a propyl linker as a shorter spacer (data not shown). Together with the 4-N-dye-labelled dideoxycytidine 5′-triphosphates[9] we were able to show that the modification of the exocyclic amino groups does not decrease the acceptance of the nucleotides as potent chain terminators in DNA sequencing.

Experimental

All commercial reagents were used without further purification. Tdt and the new thermostable DNA polymerase were obtained from Roche Diagnostics GmbH, and ThermoSequenase was from Amersham. Column chromatography was performed on Merck silica gel 60 (70–230 mesh), and TLC was performed on silica-60 F254 plates (Merck). Ion-exchange chromatography was performed using DEAE-Sephadex A25 (HCO_3^- form) and Mono Q from Pharmacia. HPLC was performed using pump-system LC 1110, System Organiser LC 1431, UV-detector GAT-PHD 601 and GATZ LCS 502 (Gamma Analysen Technik, Berlin, Germany). UV/VIS spectra were recorded on a Spekord UV 160 A (Shimadzu, Japan). All NMR spectra were obtained on an AM 300 (Bruker Physics, Rheinstetten, Germany). Coupling constants (J) are given in Hz and all chemical shifts are relative to the internal standard TMS (^{1}H and ^{13}C) or to 85% phosphoric acid as external standard (^{31}P). DNA-sequencing reactions were analysed on a LiCor 4000 L. DNA synthesis was performed on a Gene Assembler Plus (Pharmacia LKB, Freiburg, Germany); phosphoramidites were purchased from Pharmacia LKB, FAB mass spectra were obtained on an Autospec, SSQ 7000. MALDI-TOF mass spectra were obtained on a Voyager DE-STR (PerSeptive Biosystems, Framingham, USA).

5′-*O*-*tert*-Butyldimethylsilyl-2′,3′-didexyadenosine 2

2′,3′-Dideoxyadenosine **1** (0.5 g, 2.1 mmol) was dissolved in anhydrous pyridine (10 ml) and the solution was evaporated to dryness *in vacuo*; this process was repeated, and the residue was then suspended in anhydrous pyridine (15 ml). To this solution was added TBDMSCl (0.5 g, 3.3 mmol). The mixture was stirred for 5 h at room temperature and then evaporated to dryness. Purification was achieved by column chromatography on silica gel and elution with chloroform containing from 0 to 10% methanol. Compound **2** (668 mg, 90%) was obtained as a solid white product; [(MALDI-MS) Calc. for $C_{16}H_{28}N_5O_7Si$: m/z (M + H)$^+$, 349.5. Found: m/z 349.2]; δ_H($CDCl_3$) 0.00 [6H, fine d, Si(CH_3)$_2$], 0.82 [9H, s, SiC(CH_3)$_3$], 1.92–2.10 (2H, m, H_2-3′), 2.35–2.45 (2H, m, H_2-2′), 3.67 (1H, dd, $J_{5',4'}$ 3.4, $J_{5',5''}$ −11.3, H-5′), 3.90 (1H, dd, $J_{4',5''}$ 3.4, $J_{5',5''}$ −11.3, H′-5″), 4.14 (1H, m, H-4′), 5.89 (2H, br s, NH_2), 6.23 (1H, dd, $J_{1',2'}$ 6.4, $J_{1',2''}$ 3.0, H-1′), 8.21 (1H, s, H-8), 8.23 (1H, s, H-2); δ_C($CDCl_3$) −5.45, −5.37 [Si(CH_3)$_2$], 18.47 [*C*(CH_3)$_3$], 25.09 (C-3′), 25.95 [C(*C*H_3)$_3$], 33.43 (C-2′), 64.18 (C-5′), 82.10 (C-4′), 85.39 (C-1′), 119.99 (C-5), 130.01 (C-8), 149.17 (C-6), 152.70 (C-2), 155.39 (C-4).

5′-*O*-*tert*-Butyldimethylsilyl-6-2′,3′-dideoxy-*N*-[*N*-(6-trifluoroacetylaminohexyl)carbamoyl]adenosine 4

The nucleoside **2** (0.2 g, 0.57 mmol) and phenoxycarbonyltetrazole (0.13 g, 0.68 mmol) were dissolved in anhydrous 1,4-dioxane and the solution was stirred for 4 h at 45 °C. TLC ($CHCl_3$–MeOH, 95 : 5, v/v) showed the formation of **3**. Then a solution of *N*-trifluoroacetyl-1,6-diaminohexane (362 mg, 1.71 mmol) in anhydrous pyridine (4 ml) was added. The mixture was stirred for 2 h at 55 °C and then evaporated to dryness *in vacuo*. Purification was achieved by column chromatography on silica gel and elution with chloroform containing from 0 to 10% methanol. Compound **4** (200 mg, 60%) was obtained as a solid white product; [(MALDI-MS) Calc. for $C_{25}H_{41}F_3N_7O_4Si$: m/z (M + H)$^+$, 589.71. Found: m/z 589.74]; δ_H($CDCl_3$) 0.00 [6H, s, Si(CH_3)$_2$], 0.82 [9H, s, SiC(CH_3)$_3$], 1.34 and 1.55 (8H, m, 4 × CH_2), 1.96–2.08 (2H, m, H_2-3′), 2.37–2.49 (2H, m, H_2-2′), 3.28 (4H, m, 2 × C*H*$_2$CNHR), 3.70 (1H, dd, $J_{5',4'}$ 3.0 $J_{5',5''}$ −10.9, H-5′), 3.92 (1H, dd, $J_{5'',4'}$ 3.0, $J_{5'',5'}$ −10.9, H′-5′), 4.18 (1H, m,

H-4′), 6.27 (1H, dd, $J_{1',2''}$ 6.2, $J_{1',2''}$ 2.4, H-1′), 8.42 (2H, fine d, H-8, -2), 9.46 (1H, t, J 5.7, NH); δ_C($CDCl_3$) −5.51, −5.44 [$Si(CH_3)_2$], 18.43 [$C(CH_3)_3$], 24.91 (CH_2), 25.89 [$C(CH_3)_3$], 25.92 (C-3′), 26.05, 28.63 and 29.69 (3 × CH_2), 33.43 (C-2′), 39.59 (*C*H_2NHCONH), 42.05 (*C*H_2NHCOCF_3), 63.99 (C-5′), 82.42 (C-4′), 85.62 (C-1′), 115.90 (CF_3), 120.66 (C-5), 139.53 (C-8), 149.52 (C-6), 150.10 (NHCONH), 150.76 (C-2), 154.23 (C-4), 157.06 (*C*OCF_3); δ_F($CDCl_3$) −76.08 (CF_3).

2′,3′-Dideoxy-6-*N*-[*N*-(6-trifluoroacetylaminohexyl)carbamoyl]-adenosine 5

The nucleoside **4** (0.1 g, 0.17 mmol) was dissolved in dry THF (3 ml). A solution of 1 M tetrabutylammonium fluoride in THF (0.5 ml, 0.5 mmol) was added. The reaction was finished after 1 h and the solvent evaporated *in vacuo*. The residue was dissolved in chloroform (50 ml) and extracted twice with water (50 ml). The organic layer was dried ($NaSO_4$), and the solvent removed. The resulting syrup was chromatographed over a reversed phase with water–acetonitrile (1 : 1, v/v). Compound **5** (74 mg, 92%) was obtained as a solid colourless product; [HRMS (EI) Calc. for $C_{19}H_{26}F_3N_7NaO_4$: *m*/*z* $(M + Na)^+$, 496.1896. Found: *m*/*z* 496.1890]; δ_H($CDCl_3$) 1.30 and 1.55 (8H, m, 4 × CH_2), 2.10 (1H, m, H-3′), 2.22 (1H, m, H′-3′), 2.42 (1H, m, H-2′), 2.55 (1H, m, H′-2), 3.29 (4H, m, 2 × C*H*$_2$CNHR), 3.61 (1H, m, H-5′), 3.93 (1H, dd, $J_{4',5''}$ 1.9, $J_{5',5''}$ −12.4, H′-5′), 4.29 (1H, m, H-4′), 6.21 (1H, t, J 6.0, H-1′), 7.51 (1H, br s, NH), 8.39 (1H, s, H-8), 8.56 (1H, s, H-2), 9.19 (1H, s, NH), 9.58 (1H, t, J 5.7, NH); δ_C($CDCl_3$) 25.46 (CH_2), 26.04 (C-3′), 26.18, 28.60 and 29.51 (3 × CH_2), 32.79 (C-2′), 39.67 (*C*H_2NHCONH), 45.92 (*C*H_2NHCOCF_3), 63.99 (C-5′), 82.19 (C-4′), 86.85 (C-1′), 115.90 (CF_3), 121.03 (C-5), 139.83 (C-8), 149.45 (C-6), 150.44 (C-2), 150.47 (NHCONH), 154.56 (C-4), 157.25 (*C*OCF_3); δ_F($CDCl_3$) −75.99 (CF_3).

6-*N*-[*N*-(6-Aminohexyl)carbamoyl]-2′,3′-dideoxyadenosine 5′-triphosphate 6

100 µmol of nucleoside **5** were dissolved in anhydrous pyridine (5 ml) and the solution was evaporated to dryness *in vacuo*. The residue was further dried (P_4O_{10}) overnight. During all the following steps, a high pressure of argon was maintained. Into the vessel containing **5** was injected anhydrous pyridine (100 µl), followed by anhydrous 1,4-dioxane (300 µl), through a septum. A freshly prepared solution of 1 M 2-chloro-4*H*-1,3,2-benzodioxaphosphinin-4-one in anhydrous 1,4-dioxane (110 µl, 110 µmol) was injected into the well stirred solution of nucleoside **5**. After 15 min, a mixture of 0.5 M bis(tributylammonium) dihydrogen pyrophosphate in anhydrous DMF (300 µl) and tributylamine (100 µl) was injected quickly. After 20 min, a solution of 1% iodine in pyridine–water (98 : 2 v/v, 2 ml) was added. After 20 min the excess of I_2 was destroyed by adding 5% aq. $NaHSO_3$ (1 ml). The solution was evaporated and the residue was dissolved in conc. NH_3 (3 ml). The mixture was stirred for 6 h at rt, then evaporated, and the residue was dissolved in water. The aq. triphosphate solution obtained from **5** was purified on a DEAE Sephadex A25 column (2.5 × 25 cm, 4 °C) and eluted with a linear gradient of TEAB (0.05–1 M). After evaporation of the product fractions and repeated co-evaporation with EtOH, the title compound **6** was precipitated from a methanolic triphosphate solution (0.1 mM; 5 ml) by $NaClO_4$ (7.5 mmol) in anhydrous acetone (25 ml) as colourless Na^+ salts. Compound **6** was obtained as a white salt (55% from **5**); λ_{max}(H_2O)/nm 269; *m*/*z* (MALDI-MS) 618.1 $[(M + H)^+$. $C_{17}H_{31}N_7O_{12}P_3$ requires *m*/*z*, 618.4]; δ_H(D_2O) 1.45 and 1.55 (8H, m, 4 × CH_2), 2.04 (1H, m, H-3′), 2.16 (1H, m, H′-3′), 2.41 (1H, m, H-2′), 2.54 (1H, m, H′-2′), 2.88 (2H, t, J 7.5, C*H*$_2$NH$_2$), 3.17 (2H, t, J 6.4, C*H*$_2$NHCO), 4.05 (1H, m, H-5′), 4.17 (1H, m, H′-5′), 4.43 (1H, m, H-4′), 6.24 (1H, dd, $J_{1',2'}$ 3.0, $J_{1',2''}$ 6.7, H-1′), 8.42 (1H, s, H-8), 8.58 (1H, s, H-2); δ_C(D_2O) 25.46 (CH_2), 25.60 (C-3′), 25.95, 26.99 and 28.76 (3 × CH_2), 32.30 (C-2′), 39.66 (CH_2N), 39.99 (*C*H_2NHCO), 67.31 (C-5′), 82.26 (C-4′), 85.55 (C-1′), 120.02 (C-5), 142.01 (C-8), 149.34 (C-6), 149.82 (NHCONH), 151.04 (C-2), 155.63 (C-4); δ_P(D_2O) −9.55 (d, α-P), −20.41 (t, β-P), −5.34 (d, γ-P).

Labelling of 6-*N*-[*N*-(6-aminohexyl)carbamoyl]-2′,3′-dideoxy-adenosine 5′-triphosphate 6 with JA242

To a stirred solution of JA242 (10 mg, 22.4 µmol), DIPEA (23 µl, 134 µmol), and TSTU (6 mg, 22.4 µmol) in 1,4-dioxane (224 µl)–DMF (224 µl) was added a solution of nucleotide **6** (22.4 µmol) in H_2O (112 µl) was added after 20 min. The mixture was stirred in the dark at room temperature for 48 h. After evaporation *in vacuo*, the mixture was applied to an RP-18-column and eluted with 0.1 M triethylammonium acetate with acetonitrile as gradient (0–30%). The first dye-labelled fraction, containing the product **7**, was collected. The product was precipitated as its sodium salt to remove the excess of dye. Compound **7** was purified by preparative anion-exchange-HPLC on Mono Q [solvent A = 20 mM NaOAc, pH 6.5, 20% CH_3CN; solvent B = 20 mM NaOAc, pH 6.5, 20% CH_3CN, 1 M NaCl from 0 to 60% in 35 min (t_R 11.62 min)]. To remove the buffer salts, RP-18 column chromatography was performed. This gave the nucleotide **7** as a blue salt (yield 6%, purity 95% by HPLC), fluorescence quantum yield 0.82: $\lambda_{abs\text{-}max1}$($H_2O$)/nm 669, $\lambda_{abs\text{-}max2}$-($H_2O$)/nm 260; $\lambda_{em\text{-}max}$/nm 685; *m*/*z* (Maldi-MS) 1047.30 $[(M + H)^+$. $C_{44}H_{62}N_{10}O_{14}P_3$ requires *m*/*z*, 1047.37]; δ_P(D_2O) −9.78 (α-P), −20.27 (β-P), −4.76 (γ-P).

3′-End-labelling reaction with terminal deoxynucleotidyl transferase

1.5 nmol of primer 5′-ACACCCAATTCTGAAAATGGA-3′ (11 µl), 125 U of TdT (5 µl) and 15 µl of 1 mM nucleotide **7** were incubated with 10 µl of reaction buffer (5 × conc.), 5 µl of $CoCl_2$ (2.5 mM), and H_2O (4 µl) at 37 °C for 20 h. The reaction was stopped by chilling to −18 °C. The product was electrophoretically separated from unlabelled oligonucleotide on a 20% polyacrylamide 7 M urea gel.

Sequencing reactions with nucleotide 7

Dye–primer and dye–terminator cycle sequencing was performed using two different DNA polymerases (ThermoSequenase and a new thermostable DNA polymerase) on the LiCor-4000L DNA-sequencer. The unlabelled ddNTPs in the termination mixes were replaced by 1.5 µM, 15 µM, 150 µM of **7**. A template–primer–enzyme mix was prepared containing 0.2 µg of M13mp18 DNA (100 fmol), 2 µl of unlabelled or labelled M13/pUC sequencing primer, 2 µl 10 × reaction buffer, 2 µl of one of two thermostable polymerases (4 U μl^{-1}), and deionised H_2O to a total volume of 17.5 µl. Cycle sequencing was performed in 30 cycles: at 95 °C for 30 s (denaturating), 55 °C for 30 s (annealing), 72 °C (elongation). To remove the excess of dye terminator, 92 µl, of H_2O, 10 µl of 3 M NaOAc, and 250 µl of abs. EtOH (rt) were added to the tube. The sample was centrifuged for 15 min, washed with 300 µl of fresh 70% EtOH–H_2O, and the pellet of dye-labelled DNA was dried. The pellet was diluted in 6 µl of stop solution and applied to denaturing polyacrylamide sequencing gel.

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