Synthesis of fluorescent derivatives of 3′-*O*-(6-aminohexanoyl)-pyrimidine nucleosides 5′-triphosphates that act as DNA polymerase substrates reversibly tagged at C-3′

Robert S. Sarfati,[*,a] **Thomas Berthod,**[a] **Catherine Guerreiro**[a] **and Bruno Canard**[b]
[a] *Unité de Chimie Organique, UA-CNRS 487, 28, Rue du Dr. Roux, 75724 Paris cedex 15, France*
[b] *Faculté de Médecine, URA-CNRS 1462, Av. de Valombrose, 06107 Nice cedex 2, France*

Treatment, of 5′-*O*-dimethoxytritylthymidine **1** and of 4-*N*-benzyloxycarbonyl-2′-deoxy-5′-*O*-dimethoxytrityl-cytidine **12** with a mixture of 6-(benzyloxycarbonylamino)hexanoic acid, dicyclohexylcarbodiimide and 4-(dimethylamino)pyridine yielded 3′-*O*-acylated derivatives **2** and **13**.These were further converted into 3′-*O*-(6-aminohexanoyl)thymidine **7** and 3′-*O*-(6-aminohexanoyl)-2′-deoxycytidine **16** monophosphates. The latter compounds were labelled at the aliphatic amino group with fluorescent probes and transformed into their triphosphates **9** and **18**.

DNA sequencing[1,2] has revolutionized the speed and depth of our understanding of complex molecular processes. Introduced about 15 years ago, dideoxy sequencing[2] has gained a wide acceptance, and is still now the method of choice to determine a nucleotide sequence from a single-stranded DNA template. The method relies on gel electrophoresis to resolve DNA fragments produced from larger fragments according to their size. Great efforts have been made to automate the electrophoresis[3,4] step as well as the subsequent detection of the separate DNA fragments. The need for alternative methods for sequencing is great and several strategies to overcome the drawbacks of electrophoresis have been described recently.[5–8] In particular, techniques enabling rapid detection of a single or few bases would be a great help in genetic analysis applied to medicine, since certain cancers and other genetic diseases are related to minor mutations.

We have recently described a sequencing scheme[9] to determine the nucleotide sequence in a DNA molecule which avoids the use of both electrophoresis and harmful radiolabels. The method uses 3′-substituted-2′-deoxynucleoside 5′-triphosphates corresponding to the four nucleobases A, C, G and T. The 3′-position is esterified by a separate anthranilic derivative (3′-tag)[9,10] to give specific fluorescent properties to each nucleotide. These nucleotides act as substrates for several DNA polymerases, leading to chain termination. However, chemical removal of a tag by alkali required for saponification of ester functionality might melt the primer–template duplex. Since esterases, ubiquitous enzymes have broad substrate specificity, we anticipated that it would be possible to remove the tag by using a much milder approach such as enzymic hydrolysis.

For these purposes, in furthering our approach towards a new non-gel- and non-radioactive-based method devoted to nucleotide sequencing, we describe in this paper the synthesis of pyrimidine nucleoside 5′-triphosphates bearing, at the 3′-position, a six-bond aminoacyloxy group with fluorescent probes attached to the amino function.

Results and discussion

The reaction sequences utilized in the preparation of 3′-*O*-(6-aminohexanoyl)-thymidine and -cytosine 5′-triphosphates, respectively labelled with fluorescein and *N*-methylisatoic acid, are depicted in Schemes 1 and 2. The key steps are discussed below.

Protection of the amino function of 6-aminohexanoic acid

To block the amino function of 6-aminohexanoic acid, we chose the *N*-benzyloxycarbonyl group (Cbz). This blocking group is stable to a variety of hydrophilic conditions and most notably is removable under neutral hydrogenolysis conditions. Treatment of 6-aminohexanoic acid with 1-(benzyloxycarbonyl)-3-methylimidazolium chloride[11] in aq. sodium hydrogen carbonate for 16 h at room temperature, and subsequent purification, gave the desired 6-(benzyloxycarbonylamino)hexanoic acid in ~65% yield.

Synthesis of labelled 3′-*O*-(aminohexanoyl)thymidine 5′-triphosphate 7

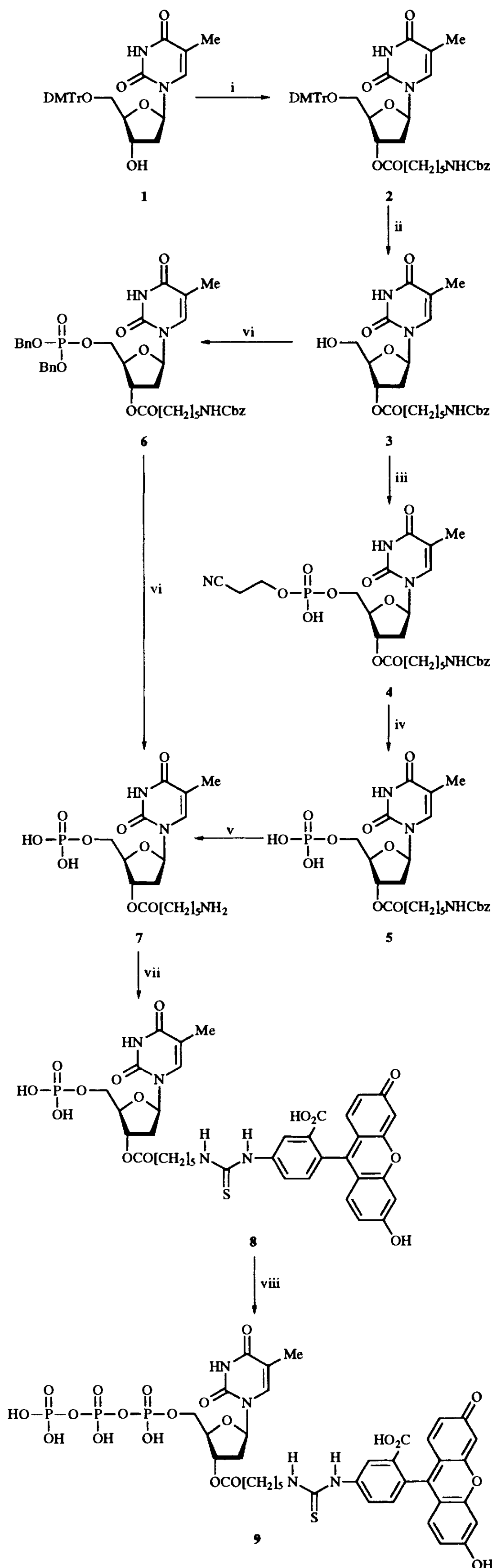
The selective protection of thymidine with dimethoxytrityl chloride (DMTrCl) in pyridine gave compound **1**.[12]

Acylation of 3′-OH with 6-(benzyloxycarbonylamino)hexanoic acid and dicyclohexylcarbodiimide (DCC) in the presence of a catalytic amount of 4-(dimethylamino)pyridine (DMAP) in acetonitrile at room temperature led to compound **2** in 87% yield. Removal of the DMTr group by treatment with 2% benzenesulfonic acid gave compound **3**.

In a first approach to phosphorylation, the free 5′-OH group of compound **3** was phosphorylated by condensation with β-cyanoethyl dihydrogen phosphate[13] in the presence of DCC in anhydrous pyridine to give compound **4**. (i) Treatment of compound **4** with a pyridine–water mixture, at 65 °C led to simultaneous decyanoethylation and deacylation. By replacement of water by 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), a less nucleophilic base, the phosphodiester was converted into the phosphomonoester **5** without hydrolysis of the 3′-ester. (ii) Incubation of compound **4** with phosphodiesterase I of *Crotalus* venom at pH 8.3 for 5 days led to compound **5**. Compound **5** was purified by HPLC using a preparative reversed-phase RP-18 column.

These two reactions of selective removal of the β-cyanoethyl group were successfully applied to small-scale preparations with yields of 58 and 52%, respectively. However, they were not readily adaptable to larger scale preparations, since the former led to some thymidine monophosphate and the latter might be too expensive.

For these reasons, we used a different approach. This involved phosphorylation of the free 5′-OH group of compound **3** with freshly prepared dibenzylphosphorochloridate[14] in pyridine at −20 °C. After work-up and purification on silica gel we obtained a fully protected nucleotide **6** in 48% yield.



Scheme 1 *Reagents and conditions:* i, $HO_2C[CH_2]_5NHCbz$, DCC, DMAP, MeCN; ii, 2% $PhSO_3H$; iii, $NC[CH_2]_2PO_3^{2-}$, DCC, pyridine; iv, (a) DBU, pyridine (b) phosphodiesterase; v, H_2, Pd–$BaSO_4$, 90% EtOH; vi, $ClPO(OBn)_2$, pyridine, −20 °C; vii, FITC, aq. pyridine; viii, CDI, $[Bu_3NH]_4^+[P_2O_7]^{4-}$–DMF–MeCN

Nucleosides blocked by benzyl and benzyloxycarbonyl groups were introduced by Rapoport and co-workers[15] for oligonucleotide synthesis. These Cbz and benzyl protecting groups could be deblocked by using hydrogen over palladium on carbon or palladium on barium sulfate. However, these conditions led to significant reduction of the 5,6-double bond of thymidine and similar observations were made[16] when benzyl groups were removed from uridine-containing oligonucleotides. To avoid this over-reduction of pyrimidine bases, several authors turned to transfer hydrogenolysis[17,18] using cyclohexadiene as the hydrogen source and 10% palladium on carbon as the catalyst.

In a first experiment we tried to remove the *N*-Cbz and *O*-benzyl groups of compound **6** by using hydrogen over palladium on barium sulfate as catalyst and 90% aq. ethyl alcohol as solvent. In our hands, under these conditions, there was no reduction of the thymidine moiety even after 2 days, and compound **6** was almost quantitatively converted into thymidine 5′-monophosphate acylated at the 3′-position by 6-aminohexanoic acid, compound **7**. The latter product was confirmed by analysis of its ^1H and ^{13}C NMR spectra in which neither proton nor carbon peaks of 3′-*O*-(6-aminohexanoyl)-dihydrothymidine 5′-phosphate **10** were detectable.

Generally, primary amino groups were labelled by fluorescein isothiocyanate (FITC) in dimethylformamide (DMF) at pH 10. To avoid any hydrolysis of the ester function at position 3′, compound **7** was labelled by FITC in a mixture of pyridine–water (5:3) at room temperature in the dark for 2 days. The labelled thymidine monophosphate derivative **8** was isolated at 70% yield after purification by HPLC on a reversed-phase RP-18 Lichoprep (25–40 mm) column.

The method of preparation of nucleotide triphosphates which has been most widely employed,[19] involving condensation of an activated nucleoside, *e.g.*, a nucleoside 5′-phosphoromorpholidate[20] with inorganic pyrophosphate,[21] is not readily applicable to the synthesis of 3′-*O*-acylated nucleotide triphosphates for our purpose. This is because of a requirement for high temperatures and basic conditions, under which esters are highly saponified.

Thus, we turned to the preparation of compound **9** *via* the phosphoroimidazolate,[22] prepared under very mild conditions from phosphomonoester **8** and 1,1′-carbonyldiimidazole (CDI).

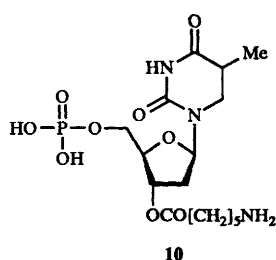
Synthesis of labelled 3′-*O*-(6-aminohexanoyl)-2′-deoxycytidine 5′-triphosphate 16

The 4-amino group of 2′-deoxycytidine was blocked in two steps as the benzyl carbamate **11** as described by Rapoport and co-workers.[15]

Selective protection of the 5′-OH group of *N*-Cbz-2′-deoxycytidine **11** with DMTrCl in pyridine gave compound **12**. Acylation of the 3′-OH group of compound **12** with 6-(benzyloxycarbonylamino)hexanoic acid and DCC in the presence of a catalytic amount of DMAP at room temperature led to compound **13**. Removal of the DMTr group by treatment with 2% benzenesulfonic acid gave compound **14**.

Phosphorylation of the free 5′-OH group of compound **14** with freshly prepared dibenzyl phosphorochloridate in pyridine at −20 °C after work-up, and purification on silica gel, gave the fully protected nucleotide **15** in 48% yield.

The protected phosphotriester **15** was also deblocked by using hydrogen over palladium on barium sulfate and 90% aq. ethyl alcohol as solvent and thus converted into compound **16** in 78%. Again, no reduction of pyrimidine base was observed, as demonstrated by analysis of ^1H and ^{13}C NMR spectra in which neither proton nor carbon peaks of 3′-*O*-(6-aminohexanoyl)-2′-*O*-deoxy-5,6-dihydrouridine 5′-monophosphate **20** were detectable. The latter would be the deaminated product[23] if 3′-



O-(6-aminohexanoyl)-2'-O-deoxy-3,4,5,6-tetrahydrocytidine 5'-monophosphate **19** were to be formed.

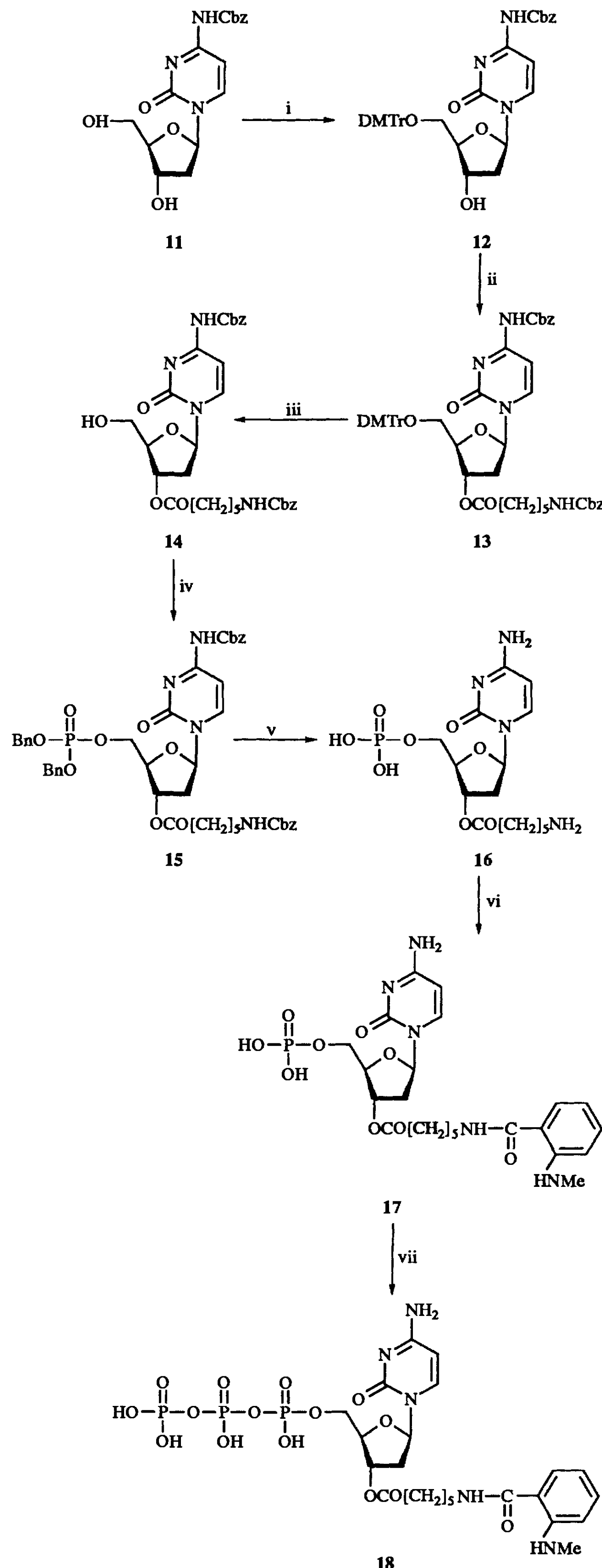
The 6-amino function of compound **16** was labelled by *N*-methylisatoic anhydride in a mixture of pyridine–water (5 : 3) at room temperature in the dark for 2 days. The fluorescent cytosine 5'-monophosphate derivative **17** was isolated after purification by preparative chromatography on a reversed-phase column 47% yield and was then converted into the triphosphate **18** by the same method described above.

Incorporation of triphosphates 9 and 18 into DNA

When incubated in the presence of a DNA polymerase and a synthetic DNA substrate, these 3'-substituted-2'-deoxynucleotides acted as substrates and were thus incorporated into DNA. Fig. 1 shows complete incorporation of 3'-substituted-dCMP† from compound **18** in less than 1 min at 37 °C when using a modified T7 DNA polymerase, and to a lesser extent, incorporation of 3'-substituted TMP† from compound **9** when using *Taq* DNA polymerase. As reported by others[24] for 2',3'-dideoxynucleotide triphosphates, incorporation was greatly enhanced when Mg^{2+} was replaced by Mn^{2+}, leading to an almost complete incorporation of substrate **9** under the reaction conditions used here. Two band-products were observed when compound **18** was used as a substrate (Fig. 1, bands 1 and 2). This may indicate that part of the fluorescent reporter group had been cleaved. Indeed, fluorescence analysis of the products immobilized on magnetic beads as described[10] indicated that some quenching might have occurred, but precise characterization of the products awaits their individual purification (unpublished data). However, since several other DNA polymerases were able to produce these two addition products when the modified nucleotide carried a cytosine moiety only, an alternative explanation may be that the added 3'-substituted-dCMP conferred a special conformation to a fraction of the 22-mer product that was responsible for the two bands observed upon autoradiography.

Experimental

All solvents were of analytical grade and were distilled and dried before used. Adsorption column chromatography was performed on column of silica gel 60 (Merck). TLC was conducted on silica-60 F_{254} plates (Merck). All compounds were located by spraying with 10% sulfuric acid and heating, or with anisaldehyde–H_2SO_4–EtOH (1 : 1 : 18, v/v/v) and charring at 110 °C for a few minutes. Phosphorus-containing compounds were revealed by spraying with Dittmer–Lester reagent.[25] All pH values were measured using a 632 pH Meter, Metrohm. NMR spectra were recorded on a Bruker MSL-300 instrument operating at 300, 75 and 121 MHz for ^{1}H, ^{13}C and ^{31}P, respectively. Coupling constants (J) are given in Hz. Tetramethylsilane was used as internal reference (^{1}H, ^{13}C) and H_3PO_4 as external reference (^{31}P). ^{13}C NMR data of the compounds prepared are listed in Tables 1 and 2. Assignments of ^{1}H NMR spectral peaks were achieved by using ^{1}H–^{1}H-shift-correlated 2D-NMR spectroscopy (COSY). Mass spectra were recorded on a VG 70-250 double-focussing instrument (VG



Scheme 2 *Reagents and conditions:* i, DMTrCl, pyridine; ii, $CbzNH[CH_2]_5CO_2H$, DCC, DMAP, MeCN; iii, 2% $PhSO_3H$; iv, $ClPO(OBn)_2$, pyridine, −20 °C; v, H_2, Pd–$BaSO_4$, 90% EtOH; vi, *N*-Methylisatoic anhydride, aq. pyridine; vii, CDI, $[Bu_3NH]^+$-$[P_2O_7]^{4-}$–DMF–MeCN

† dCMP = 2-deoxycytidine 5'-monophosphate; TMP = thymidine 5'-monophosphate.

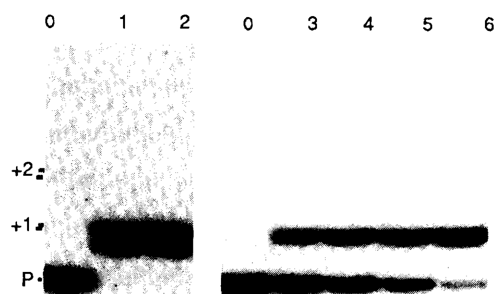
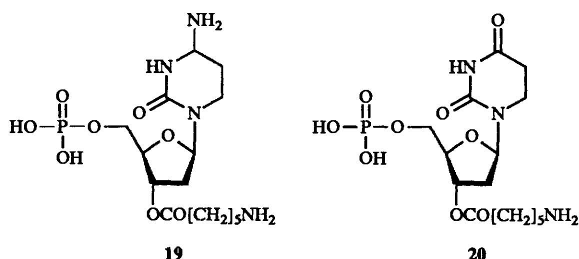


Fig. 1 Incorporation of 3'-substituted-TMP and 3'-substituted-dCMP into DNA. Compound **18** (1 mmol dm^{-3}) was incubated with modified T7 DNA polymerase (6 units) and 1 μmol dm^{-3} ^{32}P-labelled DNA substrate at pH 8.0, 37 °C, in a 2 × 10^{-2} cm^3 volume, for 0 min (band 0), 1 min (band 1) or 5 min (band 2).

Compound **9** (2 mmol dm^{-3}) was incubated with *Taq* polymerase at pH 8.0, 50 °C, in a buffer containing 2.5 mmol dm^{-3} Mg^{2+} (bands 3 and 4) or 1 mmol dm^{-3} Mn^{2+}, 5 mmol dm^{-3} sodium citrate (bands 5 and 6) in a 2 × 10^{-2} cm^3 volume for 15 min (bands 3 and 5) or 30 min (bands 4 and 6). P: primer (21-mer), + 1: 22-mer. + 2: 23-mer.



instruments, Le Chesnay, France) equipped with a fast-atom bombardment gun (Ion Tech., UK) for fast bombardment (FAB). The gun was operated with xenon at 8 kV and 1 mA. Caesium iodide and glycerol were used for calibration, glycerol as the matrix. Purification by HPLC was accomplished on a Nucleosil 5-C18 column, using a gradient of acetonitrile (A) in 0.05 mol dm^{-3} triethylammonium acetate buffer (pH 7.5) (B). Purity of each nucleoside was checked by HPLC on a Nucleosil 5-C18 column by using a gradient of 5–50% of A in B during 20 min at a flow rate of 0.5 cm^3 min^{-1} (retention times t_R are given in min) on a Perkin-Elmer Series 4 HPLC instrument coupled to a Hewlett Packard 1040 A diode-array detector. Thymidine and cytosine were purchased from Sigma, and FITC and *N*-methylisatoic anhydride from Aldrich. Phosphodiesterase I was purchased from Boerhinger Mannheim. Elementary analyses were performed by the Laboratoire de Microanalyse de l'Université Paris VI, France.

6-(Benzyloxycarbonylamino)hexanoic acid

6-Aminohexanoic acid (1.0 g, 7.6 mmol) was dissolved in water (20 cm^3) and $NaHCO_3$ (1.3 g, 2 mol equiv.) was added to the solution. After 10 min, 1-(benzyloxycarbonyl)-3-methylimidazolium chloride [11] (4.8 g, 19.1 mmol) was added to the mixture, which was then stirred at room temperature overnight. The pH of the solution was brought to 2 with HCl (2.0 mol dm^{-3}), after which the mixture was extracted with diethyl ether (2 × 50 cm^3). The organic layer was washed with water (3 × 30 cm^3), dried over Na_2SO_4, and then concentrated. The residue was purified on a silica gel column with CH_2Cl_2 containing 0–5% MeOH as eluent. The *title compound* was obtained as a powder (1.3 g, 65%), R_f 0.61 [silica gel; CH_2Cl_2–MeOH (97.5:7.5, v/v)]; δ_H[$(CD_3)_2SO$] 12.5 (1 H, s, CO_2H), 8.4–8.2 (5 H, m, ArH), 5.0 (2 H, s, CH_2Ph), 3.38 (1 H, s, NH), 2.95 (2 H, m, 6-H_2), 2.18 (2 H, t, $J_{2',3'}$ 8.33, 2-H_2), 1.5 (2 H, m, 3-H_2), 1.42 (2 H, m, 5-H_2) and 1.28 (2 H, m, 4-H_2); δ_C[$(CD_3)_2SO$] 174.56 (C-1), 156.20 (NHCO), 137.42, 128.45, 127.83 (arom.), 65.19 (CH_2Ph), 40.07 (C-2), 33.71 (C-6), 29.26 (C-3), 25.91 (C-5) and 24.31 (C-4) (Found: C, 63.3; H, 7.05; N, 5.35. $C_{14}H_{19}NO_4$ requires C, 63.37; H, 7.22; N, 5.28%).

3'-*O*-[6-(Benzyloxycarbonylamino)hexanoyl]-5'-*O*-dimethoxytritylthymidine 2

Compound **1** (2.18 g, 4 mmol) was dissolved in dry acetonitrile (80 cm^3) and 6-(benzyloxycarbonylamino)hexanoic acid (1.167 g, 4.4 mmol), DMAP (0.1 g), pyridine (0.785 cm^3) and DCC (0.45 g, 2.2 mmol) were added. The mixture was stirred at room temperature until no starting material could be detected by TLC. The reaction was quenched by addition of methyl alcohol (2 cm^3), and the mixture was filtered. The filtrate was evaporated to dryness and the residue was purified on a silica gel column with CH_2Cl_2 containing 0–1% MeOH as eluent. The *title compound* **2** was obtained as a powder (2.75 g, 87%), R_f 0.37 [silica gel; CH_2Cl_2–MeOH 95:5 (v/v)]; δ_H[$(CD_3)_2SO$] 7.5 (1 H, s, 6-H), 7.4–6.83 (18 H, ArH), 6.21 (1 H, t, $J_{1',2'} = J_{1',2''} =$ 8.33, 1'-H), 5.3 (1 H, m, 3'-H), 5.0 (2 H, s, CH_2Ph), 4.05 (1 H, m, 4'-H), 3.75 (6 H, s, 2 × OMe), 3.2 (2 H, m, 5'-H_2), 2.95 (2 H, m, CH_2NH), 2.35 (2 H, m, 2'-H_2), 2.2 (2 H, m, $COCH_2$), 1.5 (2 H, m, CH_2), 1.45 (3 H, s, 5-Me), 1.38 (2 H, m, CH_2) and 1.1 (2 H, m, CH_2) (Found: C, 67.8; H, 6.5; N, 5.5. $C_{45}H_{49}N_3O_{10}$ requires C, 68.25; H, 6.23; N, 5.30%).

3'-*O*-[6-(Benzyloxycarbonylamino)hexanoyl]thymidine 3

Compound **2** (2.64 g, 3.33 mmol) was dissolved in dichloromethane–methanol (7.3 v/v; 10 cm^3). The mixture was cooled in an ice-bath and a solution of 2% benzenesulfonic acid in dichloromethane–methanol (7:3 v/v; 70 cm^3) at 0 °C was added. The mixture was stirred at 0 °C until all starting material had disappeared, and was then washed respectively with aq. $NaHCO_3$ (3 × 30 cm^3) and water (2 × 30 cm^3). The organic layer was dried (Na_2SO_4), filtered, and evaporated to dryness. The resulting solid was purified on a silica gel column, with CH_2Cl_2 containing 0–2% MeOH as eluent. Pure fractions were combined and concentrated to give the *title compound* **3** as powder (1.3 g, 80%), R_f 0.23 [silica gel; CH_2Cl_2–MeOH (92.5:7.5 v/v)]; δ_H[$(CD_3)_2SO$] 7.75 (1 H, s, 6-H), 7.4–7.19 (5 H, m, ArH), 6.2 (1 H, t, $J_{1',2'} = J_{1',2''} =$ 8.33, 1'-H), 5.21 (2 H, m, 3'-H and 5'-OH), 5.0 (2 H, s, CH_2Ph), 3.95 (1 H, m, 4'-H), 3.61 (2 H, m, 5'-H_2), 2.98 (2 H, m, CH_2NH), 2.35 (2 H, m, 2'-H_2), 2.25 (2 H, m, $COCH_2$), 1.79 (3 H, s, 5-Me), 1.55 (2 H, m, CH_2), 1.5 (2 H, m, CH_2) and 1.25 (2 H, m, CH_2) (Found: C, 59.2; H, 6.3; N, 8.6. $C_{24}H_{31}N_3O_8$ requires C, 58.88; H, 6.38; N, 8.58%).

3'-*O*-[6-(Benzyloxycarbonylamino)hexanoyl]thymidin-5'-yl β-cyanoethyl hydrogen phosphate 4

An anhydrous solution of 3'-*O*-[6-(benzyloxycarbonylamino)-hexanoyl]thymidine **3** (1.04 g, 2.13 mmol), pyridinium β-cyanoethyl phosphate (4.31 mmol), and DCC (1.66 g, 12.75 mmol) in dry pyridine (20 cm^3) was kept at room temp. for 2 days. Water (7 cm^3) was then added and the reaction mixture was kept at room temp. for 1 h. Dicyclohexylurea was removed by filtration and was washed thoroughly with 1:1 aq. pyridine. The total aq. pyridine solution was evaporated at a temperature below 20 °C. The residue was dissolved in water (5 cm^3), and the solution was passed through a short column of Dowex 50 (H^+) resin. The pH of the total effluent including washings was adjusted carefully with barium hydroxide to 7.5. The solution was filtered from the precipitate which had deposited out, and was concentrated at reduced temperature. The remaining syrup was purified by preparative HPLC using a reversed-phase RP-18 (25–40 μm) column. Elution with water containing 0–1% acetonitrile gave the *title compound* **4** (0.97 g, 65%), R_f 0.49 [silica gel; Pr^iOH–conc. ammonia–water (7:1:2 v/v/v)]; $\delta_H(D_2O)$ 7,65 (1 H, s,

Table 1 ^{13}C NMR chemical shifts of the thymidine analogues prepared

	Chemical shift											
Compound	C-2	C-4	C-5	C-6	5-Me	C-1′	C-2′	C-3′	C-4′	C-5′	CH_2 aliphatics	Others
2[a]	150.55	163.44	109.86	135.03	11.58	83.01	40.42	74.34	83.76	63.74	36.46, 33.26, 28.95, 25.53, 23.90	172.40 and 155.99 (C=O), 158.12–113.20 (arom), 86.00 (C of DMTr), 54.98 (MeO), 64.99 (CH_2 of Cbz)
3[a]	150.70	163.88	109.95	136.03	12.51	83.86	40.51	74.82	84.83	61.54	36.73, 33.59, 29.27, 25.85, 24.24	172.74 and 156.30 (C=O), 137.52–127.93 (arom), 65.30 (CH_2 of Cbz)
4[b]	152.24	167.03	112.52	137.08	12.40	83.86	40.84	76.05	85.54	61.31	37.21, 34.39, 29.20, 25.92, 24.62	176.65 and 159.18 (C=O), 137.66–128.85 (arom), 120.00 (CN), 67.35 and 61.25 (CH_2 of cyanoethyl), 65.30 (CH_2 of Cbz)
6[a]	150.68	163.85	110.36	135.75	12.30	82.74	40.23	74.75	83.92	65.28	36.35, 33.51, 29.24, 25.82, 24.17	172.95 and 156.45 (C=O), 136.63–128.01 (arom), 69.69 (CH_2 of Bn), 67.04 (CH_2 of Cbz)
7[c]	150.71	163.83	111.80	134.87	12.34	82.75	40.75	74.54	84.36	66.50	37.09, 33.83, 29.60, 26.10, 24.34	176.39 (C=O)
8[b]	152.40	167.15	112.61	137.80	12.25	84.16	39.94	76.24	85.61	65.70	37.12, 34.12, 27.05, 25.72, 24.21	132.26–103.95 (CH of fluorescein)
9[b]	152.40	167.15	112.61	137.70	12.32	86.84	45.55	76.56	84.34	65.39	37.22, 34.47, 28.43, 26.18, 24.63	132.26–103.95 (CH of fluorescein)

[a] In $(CD_3)_2SO$. [b] In D_2O. [c] In $CDCl_3$.

Table 2 ^{13}C NMR chemical shifts of the 2′-deoxycytidine analogues prepared

	C-2	C-4	C-5	C-6	C-1′	C-2′	C-3′	C-4′	C-5′	CH_2 aliphatics	Others
11[a]	153.35	162.91	94.38	144.89	86.25	40.75	70.13	88.05	61.15		154.40 (C=O), 136.14–128.13 (arom), 66.69 (CH_2 of Cbz)
12[a]	153.30	162.76	94.20	144.55	85.91	40.80	69.30	88.05	62.88		154.14 (C=O), 158.17–113.28 (arom), 85.7 (C of DMTr), 66.53 (CH_2 of Cbz), 55.04 (MeO)
13[a]	153.21	162.97	94.51	144.50	83.56	40.77	73.57	86.16	63.12	37.73, 33.42, 29.09, 25.67, 24.07	172.47, 156.67 and 156.14 (C=O), 158.22–113.32 (arom), 86.16 (C of DMTr), 66.62 and 65.14 (CH_2 of Cbz), 55.07 (MeO)
14[a]	144.80	163.09	94.69	144.48	85.69	40.19	74.76	86.36	61.34	38.03, 33.58, 29.25, 25.82, 24.22	172.72 and 156.28 (C=O), 136.10–127.91 (arom), 66.74 and 65.28 (CH_2 of Cbz)
15[b]	152.01	162.20	95.04	143.68	83.40	40.78	74.44	86.88	68.00	38.73, 33.86, 29.64, 26.12, 24.40	172.94, 156.45 and 154.83 (C=O), 136.63–128.03 (arom), 69.83 and 69.75 (CH_2 of Bn), 66.86 and 66.56 (CH_2 of Cbz)
16[c]	158.29	166.79	97.53	142.50	84.68	39.89	76.78	86.46	64.52	37.84, 34.17, 27.01, 25.67, 24.20	176.45 (C=O)
17[c]	157.97	166.58	97.42	142.22	84.17	39.81	76.47	86.22	64.90	37.65, 34.37, 28.71, 26.09, 24.56	176.62 and 172.20 (C=O), 149.10–119.01 (arom), 30.24 (Me)
18[c]	157.97	166.58	97.42	142.20	84.17	39.81	76.47	86.22	64.90		

[a] In $(CD_3)_2SO$. [b] In $CDCl_3$. [c] In D_2O.

6-H), 7.35 (5 H, m, ArH), 6.28 (1 H, t, $J_{1',2'} = J_{1',2''} = 8.33$, 1'-H), 5.39 (1 H, m, 3'-H'), 5.08 (2 H, s, CH_2Ph), 4.29 (1 H, m, 4'-H), 4.2–4.0 (4 H, m, 5'-H_2 and CH_2CN), 3.15 [2 H, t, J(CH_2, CH_2NH) 7.55, CH_2NH], 2.82 (2 H, t, J_{H^a,H^b} 6.66, CH_2CH_2CN), 2.4 (4 H, m, 2'-H_2 and CH_2CO), 1.8 (3 H, s, 5-Me), 1.65 (2 H, m, CH_2), 1.5 (2 H, m, CH_2) and 1.3 (2 H, m, CH_2); $\delta_P(D_2O)$ 0.1 (m, 1 P) (Found: C, 46.3; H, 5.1; N, 8.0. $C_{27}H_{35}N_4O_{12}P\cdot0.5\,Ba\cdot0.5\,H_2O$ requires C, 45.85; H, 4.98; N, 7.92%).

3'-*O*-[6-(Benzyloxycarbonylamino)hexanoyl]thymidin-5'-yl dihydrogen phosphate 5

(a) By action of phosphodiesterase I from snake venom. Compound **4** (0.2 g, 0.3 mmol) was dissolved in water (4 cm^3) and hydrolysed by phosphodiesterase I from snake venom (0.6 units) at pH 8.3 and room temp. for 5 days. The mixture was passed through a short column of Dowex 50 (pyridinium) resin and the eluate was evaporated to small bulk. Purification was performed by HPLC using a reversed-phase RP-18 (25–40 μm) column. Elution with water containing 0–15% acetonitrile gave the *title compound* **5** (102 mg, 58%), R_f 0.42 [silica gel; Pr^iOH–conc. ammonia–water (7:1:2 v/v/v)]; $\delta_H(D_2O)$ 7.75 (1 H, s, 6-H), 7.32 (5 H, m, ArH), 6.3 (1 H, t, $J_{1',2'} = J_{1',2''} = 8.33$, 1'-H), 5.36 (2 H, m, 3'-H), 5.05 (2 H, s, CH_2Ph), 4.3 (1 H, m, 4'-H), 3.99 (2 H, m, 5'-H_2), 3.15 (2 H, m, CH_2NH), 2.4–2.23 (4 H, m, 2'-H_2, CH_2CO), 1.85 (3 H, s, 5-Me), 1.62 (2 H, m, CH_2), 1.48 (2 H, m, CH_2) and 1.3 (2 H, m, CH_2); $\delta_P(D_2O)$ 0.1 (m, 1 P) (Found: C, 47.3; H, 5.55; N, 6.9. $C_{24}H_{30}N_3O_{11}P\cdot Li_2\cdot1.5\,H_2O$ requires C, 47.38; H, 5.46; N, 6.90%).

(b) By action of DBU. Compound **4** (0.14 g, 0.19 mmol) was dissolved in dry pyridine (10 cm^3) and the mixture was evaporated to dryness under reduced pressure. The residue was dissolved again in pyridine (16.5 cm^3) and DBU (0.284 cm^3, 1.9 mmol) was added. The solution was kept at 60 °C for a day. After cooling in an ice–water-bath, the solution was diluted with water (6 cm^3) and passed through a short column of Dowex 50 (pyridinium) resin. The eluate was evaporated to small volume and purified as described above to give compound **5** (60 mg, 52%).

Dibenzyl-3'-*O*-[6-(benzyloxycarbonylamino)hexanoyl]thymidin-5'-yl phosphate 6

Dibenzyl phosphorochloridate was prepared by reaction of dibenzyl phosphite (6.36 cm^3, 28.8 mmol) with *N*-chlorosuccinimide (3.9 g, 89 mmol) in anhydrous toluene (60 cm^3) for 2 h at room temperature. The solution was decanted from the succinimide precipitate, concentrated to small bulk at low temperature, and the concentrate was added to a frozen solution of compound **3** (4 g, 8.2 mmol) in dried pyridine (30 cm^3). The shaken mixture was allowed to warm up gradually to form a clear, homogeneous melt and was then kept at −20 °C for 16 h. The solution was then mixed with sodium hydrogen carbonate (6 g) and a small volume of water. The mixture was evaporated under reduced pressure at 20 °C and the residual syrup was dissolved in a mixture of chloroform (35 cm^3) and water (35 cm^3). The organic layer was successively washed with water, HCl (1.0 mol dm^{-3}) and 5% aq. $NaHCO_3$ and was then dried over sodium sulfate. Evaporation gave an oil, which was purified on a silica gel column by elution with CH_2Cl_2 containing 0–1% MeOH. The *title compound* **6** obtained was a syrup (4.26 g, 48.7%), R_f 0.51 [silica gel; CH_2Cl_2–MeOH (92.5:7.5 v/v)]; $\delta_H(CDCl_3)$ 9.75 (1 H, s, 3-H), 7.41 (1 H, s, 6-H), 7.35 (6 H, m, ArH, exchangeable proton), 6.35 (1 H, m, 1'-H), 5.1 (7 H, m, 3'-H, CH_2Ph, exchangeable proton), 4.24–4.1 (2 H, m, 5'–H_2), 4.05 (1 H, m, 4'-H), 3.19 (2 H, m, CH_2NH), 2.3 (2 H, m, $COCH_2$), 2.21 (2 H, m, 2'-H_2), 1.8 (3 H, s, 5-Me), 1.62 (2 H, m, CH_2), 1.48 (2 H, m, CH_2) and 1.3 (2 H, m, CH_2); $\delta_P(CDCl_3)$ −2.54 (1 P, m) (Found: C, 60.8; H, 5.9; N, 5.4. $C_{38}H_{44}N_3O_{11}P$ requires C, 60.87; H. 5.91; N, 5.60%).

3'-*O*-(6-Aminohexanoyl)thymidin-5'-yl dihydrogen phosphate 7

Compound **6** (2 g, 2.67 mmol) was dissolved in 90% aq. alcohol (50 cm^3) and hydrogenated in the presence of palladium oxide–barium sulfate catalyst (1 g) at room temp. and atmospheric pressure for 2 days. The reaction mixture was diluted with water (6 cm^3) and the catalyst was removed by centrifugation. The precipitate was washed several times with 50% aq. ethyl alcohol (10 cm^3) until the tests for primary amine and sugar were negative. Supernatants were pooled and freeze-dried. The resulting material was purified by HPLC on a reversed-phase RP18 (25–40 μm) column eluted with water containing 0–1% MeCN. Freeze-drying of the eluate gave compound **7** as a powder (1.25 g, 98%), R_f 0.25 [silica gel; Pr^iOH–conc. ammonia–water (7:1:2 v/v/v)]; an aliquot was repurified by reversed-phase HPLC [Nucleosil 5C18, 5 μm, 250 mm × 10 mm; A = 0.01 mol dm^{-3} triethylammonium acetate (TEAA), B = MeCN from 5–50% B in 20 min, flow rate 6 cm^3 min^{-1}, t_R **7** = 6.49 min]; $\delta_H[(CD_3)_2SO]$ 7.79 (1 H, s, 6-H), 6.35 (1 H, t, $J_{1',2'} = J_{1',2''} = 8.08$, 1'-H), 5.38 (1 H, m, 3'-H), 4.31 (1 H, m, 4'-H), 4.05 (2 H, m, 5'-H_2), 2.95 [2 H, t, J(CH_2, CH_2NH_2) 7.56, CH_2NH_2], 2.40 (4 H, m, $CONH_2$, 2'-H_2), 1.84 (3 H, s, 5-Me), 1.61 (4 H, m, 2 × CH_2) and 1.38 (2 H, m, CH_2); $\delta_P(D_2O)$ 0.65 (1 P, m) (Found: C, 40.3; H, 6.1; N, 8.7. $C_{16}H_{24}Li_2N_3O_9\cdot1.5\,H_2O$ requires C, 40.52; H, 5.73; N, 8.86%).

Labelling of compound 7 with FITC

Compound **7** pyridinium salt (0.58 g, 1.13 mmol) was dissolved in a mixture of pyridine–water (5:3 v/v; 16 cm^3) and FITC (0.66 g, 1.7 mmol) was added. The mixture was stirred at room temp. in the dark for 2 days. The solution was evaporated to dryness under reduced pressure at 20 °C and the residual material was washed successively with acetone and diethyl ether. The crude product was purified by HPLC on a preparative reversed-phase RP-18 column with water containing 0–20% acetonitrile as eluent. Pure fractions of compound **8** were pooled, concentrated and freeze-dried (76%), R_f 0.27 [silica gel; Pr^iOH-conc. ammonia–water (7:1:2 v/v/v)]; an aliquot was repurified by reversed-phase HPLC [Nucleosil 5C18, 5 μm, 250 mm × 10 mm; A = 0.01 mol dm^{-3} TEAA, B = MeCN from 5–50% B in 20 min, flow rate 6 cm^3 min^{-1}, t_R **8** = 10.3 min]; $\delta_H(D_2O)$ 7.75 (1 H, s, 6-H), 7.62–6.75 (9 H, m, fluorescein), 6.1 (1 H, m, 1'-H), 5.32 (1 H, m, 3'-H), 4.15 (1 H, m, 4'-H), 4.02 (2 H, m, 5'-H_2), 3.65 (2 H, m, CH_2NH), 2.45 (2 H, m, CH_2CO), 2.23 (2 H, m, 2'-H_2), 1.81 (3 H, s, 5-Me), 1.71 (4 H, m, 2 × CH_2) and 1.41 (2 H, m, CH_2); $\delta_P(D_2O)$ 0.76 (m, 1 P); *m/z* (FAB^+) 843 (M + $3Li^+$ + $2H^+$).

3'-*O*-(6-Aminohexanoyl)thymidin-5'-yl tetrahydrogen triphosphate labelled with FITC

Compound **8** pyridinium salt (83 mg, 0.1 mmol) was dissolved in a mixture of water (5 cm^3), and tributylamine (0.025 cm^3, 0.1 mmol) was added. The reaction mixture was evaporated under reduced pressure and the residue was dissolved in water (5 cm^3) and then freeze-dried. Dry acetonitrile (3 cm^3) was added to the lyophilized material. After evaporation under high vacuum, dry acetonitrile (2 cm^3), DMF (0.5 cm^3) and CDI (0.065 g, 0.4 mmol were added. The mixture was shaken for 6 h at room temp. in a well sealed container. The reaction was quenched by addition of methyl alcohol (0.012 cm^3, 0.3 mmol). After 45 min, tetrakis(tributylammonium) pyrophosphate (0.4 cm^3, 0.4 mmol) of a 1.0 dm^{-3} stock solution in dry acetonitrile was added. The stoppered mixture was vigorously mixed and held at room temp. for 3 days. The reaction mixture was concentrated under reduced pressure. Water (2 cm^3) was added twice, and the solution was freeze-dried to remove completely all traces of DMF. The crude product was purified on a preparative RP-18 (25–40 μm) column eluted with water containing 0–4% acetonitrile to give

the title compound **9**, R_f 0.11 [silica gel; Pr^iOH–conc. ammonia–water (7:1:2 v/v/v)]; an aliquot was repurified by reversed-phase HPLC [Nucleosil 5C18, 5 μm (250 mm × 10 mm); A = 0.01 mol dm^{-3} TEAA, B = MeCN 5–50% B in 20 min, flow rate 6 cm^3 min^{-1}, t_R **9** = 11.92 or 9.32 min]; $\delta_H(D_2O)$ 7.75 (1 H, s, 6-H), 7.62–6.75 (9 H, m, fluorescein), 6.1 (1 H, m, 1′-H), 5.32 (1 H, m, 3-H), 4.15 (1 H, m, 4′-H), 4.02 (2 H, m, 5′-H_2), 3.65 (2 H, m, CH_2NH), 2.45 (2 H, m, CH_2CO), 2.23 (2 H, m, 2′-H_2), 1.81 (3 H, s, 5-Me), 1.71 (4 H, m, 2 × CH_2) and 1.41 (2 H, m, CH_2); $\delta_P(D_2O)$ −22.63 [1 P, t, $J(P_\gamma, P_\alpha) = J(P_\gamma, P_\beta)$ = 20.36, P_γ], −11.19 (1 P, d, $J(P_\alpha, P_\gamma)$ 20.36, P_α) and −10.24 (1 P, d, $J(P_\beta, P_\gamma)$ 20.36, P_β); *m/z* (FAB^+) 1009 (M + 4Li^+ + 2H^+).

4-*N*-Benzyloxycarbonyl-2′-deoxy-5′-*O*-dimethoxytritylcytidine 12

Di-*p*-methoxytrityl chloride (9.07 g, 26.8 mmol) was added to a solution of 4-*N*-benzyloxycarbonyl-2′-deoxycytidine **11** (8.8 g, 24.37 mmol) in dry pyridine (20 cm^3), and the mixture was stirred for 2 h at room temperature. Methyl alcohol (5 cm^3) was then added, the solution was evaporated, and the residue was dissolved in CH_2Cl_2 (100 cm^3) and washed successively with 5% aq. $NaHCO_3$ and water. The organic layer was dried over Na_2SO_4, filtered, and then concentrated. The resulting syrup was purified by silica gel column chromatography. Elution with 0–2% MeOH in CH_2Cl_2 gave the title compound **12** (13.36 g, 83%), R_f 0.63 [silica gel; CH_2Cl_2–MeOH (92.5:7.5 v/v)]; $\delta_H[(CD_3)_2SO]$ 10.85 (1 H, s, 4-NH), 7.95 (1 H, d, $J_{6,5}$ 7.55, 6-H), 7.22–6.66 (19 H, m, 5-H and ArH), 5.91 (1 H, t, $J_{1',2'} = J_{1',2''}$ = 6.03, 1′-H), 5.18 1 H, d, $J_{HO,3'}$ 4.73, 3′-OH), 4.99 (2 H, s, CH_2Ph), 4.1 (1 H, m, 3′-H), 3.76 (1 H, m, 4′-H), 3.35 (6 H, s, 2 × OMe), 3.25 (2 H, m, 5′-H_2) and 2.36–2.05 (2 H, m, 2′-H_2).

4-*N*-Benzyloxycarbonyl-3′-*O*-[6-(benzyloxycarbonylamino)-hexanoyl]-2′-deoxy-5′-*O*-dimethoxytritylcytidine 13

From compound **12** (5 g, 7.55 mmol), and following the procedure described previously for compound **2**, the title compound **13** was obtained (5.66 g, 82.5%) as a powder, R_f 0.71 [silica gel; CH_2Cl_2–MeOH (92.5:7.5 v/v)]; $\delta_H[(CD_3)_2SO]$ 10.85 (1 H, s, 4-NH), 8.05 (1 H, d, $J_{6,5}$ 7.55, 6-H), 7.6 (1 H, s, CH_2NH), 7.4–6.8 (19 H, m, 5-H and ArH), 6.1 (1 H, t, $J_{1',2'} = J_{1',2''}$ = 6.03, 1′-H), 5.19 (1 H, m, 3′-H), 5.15 and 4.96 (4 H, 2 s, 2 × CH_2Ph), 4.13 (1 H, m, 4′-H), 3.7 (6 H, s, OMe), 3.3 (2 H, m, 5′-H_2), 2.95 (2 H, m, CH_2NH), 2.25 (4 H, m, 2′-H_2, $COCH_2$), 1.5 (2 H, m, CH_2), 1.45 (2 H, m, CH_2) and 1.25 (2 H, m, CH_2).

4-*N*-Benzyloxycarbonyl-3′-*O*-[6-(benzyloxycarbonylamino)-hexanoyl]-2′-deoxycytidine 14

From compound **13** (3.35 g. 3.37 mmol), and following the procedure described previously for compound **3**, the *title compound* **14** was prepared as a powder (3.05 g, 91%), R_f 0.59 [silica gel; CH_2Cl_2–MeOH (92.5:7.5 v/v)]; $\delta_H[(CD_3)_2SO]$ 10.9 (1 H, s, 4-NH), 8.35 (1 H, d, $J_{6,5}$ 7.57, 6-H), 7.5–7.25 (11 H, m, 5-H and ArH), 7.1 (1 H, d, $J_{6,5}$ 7.57, 5-H), 6.1 (1 H, dd, $J_{1',2'}$ 7.75, $J_{1',2''}$ 5.95, 1′-H), 5.25 (2 H, m, 3′-H, 5′-OH), 5.21 and 5.02 (4 H, 2 s, 2 × CH_2Ph), 4.13 (1 H, m, 4′-H), 3.68 (2 H, m, 5′-H_2), 3.02 (2 H, m, CH_2NH), 2.41–2.16 (4 H, m, 2′-H_2, $COCH_2$), 1.57 (2 H, m, CH_2), 1.45 (2 H, m, CH_2) and 1.29 (2 H, m, CH_2) (Found: C, 60.8; H, 6.0; N, 9.4. $C_{31}H_{36}N_4O_9$ requires C, 61.27; H, 5.80; N, 9.22%).

Dibenzyl-4-*N*-benzyloxycarbonyl-3′-*O*-[6-(benzyloxycarbonyl-amino)hexanoyl]-2′-deoxycytidin-5′-yl phosphate 15

From compound **14** (2 g, 3.28 mmol), and following the procedure described previously for compound **6**, the title compound **15** was obtained as a syrup (1.56 g, 48%), R_f 0.45 [silica gel; CH_2Cl_2–MeOH (92.5:7.5 v/v)]; $\delta_H(CDCl_3)$ 8.0 (1 H, d, $J_{6,5}$ 7.55, 6-H), 7.5–7.25 (22 H, m, ArH, 4-NH, $NHCH_2$), 7.15 (1 H, d, $J_{6,5}$ 7.55, 5-H), 6.21 (1 H, dd, $J_{1',2'}$ 5.24, $J_{1',2''}$ 8.55, 1′-H), 5.21–4.98 (5 H, m, 2 × CH_2Ph, 3′-H), 4.13 (3 H, m, 4′-H, 5′-H_2), 3.2 (2 H, m, CH_2NH), 2.6 (1 H, m, 2′-H), 2.32 (2 H, m, CH_2CO), 1.75 (1 H, m, 2′-H), 1.61 (2 H, m, CH_2), 1.5 (2 H, m, CH_2) and 1.38 (2 H, m, CH_2); $\delta_P(CDCl_3)$ −2.33 (m, 1 P) (Found: C, 60.1; H, 5.7; N, 6.4. $C_{45}H_{49}N_4O_{12}P{\cdot}0.5\ CH_2Cl_2$ requires C, 59.96; H, 5.53; N, 6.15%).

3′-*O*-(6-Aminohexanoyl)-2′-deoxycytidin-5′-yl dihydrogen phosphate 16

From phosphate **15** (2.03 g, 2.33 mmol), and following the procedure described previously for compound **7**, the title compound **16** was obtained as a powder (0.90 g, 78%), R_f 0.11 [silica gel; Pr^iOH–conc. ammonia–water (7:1:2, v/v/v)]; an aliquot was repurified by reversed-phase HPLC [Nucleosil 5C18, 5 μm, 250 mm × 10 mm; A = 0.01 mol dm^{-3} TEAA, B = MeCN 5–50% B in 20 min, flow rate 6 cm^3 min^{-1}, t_R **16** = 3.88 min]; $\delta_H(D_2O)$ 8.02 (1 H, d, $J_{6,5}$ 7.61, 6-H), 6.32 (1 H, m, 1′-H), 6.09 (1 H, d, $J_{5,6}$ 7.61, 5-H), 5.35 (1 H, m, 3′-H), 4.30 (1 H, m, 4′-H), 3.9 (2 H, m, 5′-H_2), 2.92 (2 H, t, *J* 7.55, CH_2NH_2), 2.39 (4 H, m, $COCH_2$, 2′-H_2), 1.61 (4 H, m, 2 × CH_2) and 1.34 (2 H, m, CH_2); $\delta_P(D_2O)$ 4.29 (1 P, t); *m/z* (FAB^+) 433 (M + 2Li^+ + 2H^+).

Labelling of compound 16 with *N*-methylisatoic anhydride. 2′-deoxy-3′-*O*-[6-*N*-methylanthranilamido)hexanoyl]cytidin-5′-yl dihydrogen phosphate 17

3′-*O*-(6-Aminohexanoyl)-2′-deoxycytidin-5′-yl phosphate pyridinium salt **16** (0.251 g, 0.5 mmol) was dissolved in a mixture of pyridine (10 cm^3) and water (5 cm^3), and *N*-methylisatoic anhydride (0.28 g, 1.75 mmol) was added. The mixture was stirred for 2 days at room temp. and was then evaporated under reduced pressure repeatedly after addition of water. The residue was washed twice with diethyl ether and then was further purified on a preparative RP-18 (25–40 μm) column containing 0–25% acetonitrile to give the title compound **17** (0.149 g, 47%), R_f 0.56 [silica gel; Pr^iOH–conc. ammonia–water (7:1:2, v/v/v)]; an aliquot was repurified by reversed-phase HPLC [Nucleosil 5C18, 5 μm, 250 mm × 10 mm; A = 0.01 mol dm^{-3} TEAA, B = MeCN 5–50% B in 20 min, flow rate 6 cm^3 min^{-1}, t_R **17** = 11.29 min]; $\delta_H(D_2O)$ 7.90 (1 H, d, $J_{6,5}$ 7.61, 6-H), 7.40–6.60 (4 H, m, anthranilyl), 6.22 (1 H, m, 1′-H), 6.05 (1 H, d, $J_{5,6}$ 7.61, 5-H), 5.32 (1 H, m, 3′-H), 4.29 (1 H, m, 4′-H), 3.95 (2 H, m, 5′-H_2), 3.32 (2 H, m, CH_2NH_2), 2.75 (1 H, s, *NH*Me), 2.39 (2 H, m, $COCH_2$), 2.25 (2 H, m, 2′-H_2), 1.65 (2 H, m, CH_2), 1.57 (2 H, m, CH_2) and 1.43 (2 H, m, CH_2); $\delta_P(D_2O)$ 2.66 (1 P, m); *m/z* (FAB^+) 567 (M + 2Li^+ + 2H^+).

2′-Deoxy-3′-*O*-[6-*N*-methylanthranilamido)hexanoyl]cytidin-5′-yl tetrahydrogen triphosphate 18

From compound **17** (64 mg, 0.1 mmol), and following the procedure described for compound **9**, the title compound **18** was prepared; R_f 0.12 [silica gel; Pr^iOH–conc. ammonia–water (7:1:2 v/v/v)]; an aliquot was repurified by reversed-phase HPLC [Nucleosil 5C18, 5 μm, 250 mm × 10 mm; A = 0.01 mol dm^{-3} TEAA, B = MeCN 5–50% B in 20 min, flow rate 6 cm^3 min^{-1}, t_R **18** = 9.82 min]; $\delta_H(D_2O)$ 7.90 (1 H, d, $J_{6,5}$ 7.61, 6-H), 7.40–6.60 (4 H, m, anthranilyl), 6.22 (1 H, m, 1′-H), 6.05 (1 H, d, $J_{5,6}$ 7.61, 5-H), 5.32 (1 H, m, 3′-H), 4.29 (1 H, m, 4′-H), 3.95 (2 H, m, 5′-H_2), 3.32 (2 H, m, CH_2NH_2), 2.75 (1 H, s, *NH*Me), 2.39 (2 H, m, $COCH_2$) 2.25 (2 H, m, 2′-H_2), 1.65 (2 H, m, CH_2), 1.57 (2 H, m, CH_2) and 1.43 (2 H, m, CH_2); $\delta_P(D_2O)$ −22.30 (1 P, d, P_γ), −10.78 (1 P, d, P_α) and −9.66 (1 P, t, P_β); *m/z* (FAB^+) 714 (M + H^+).

Incorporation into DNA

Single-base addition was performed exactly as described previously.[10] Briefly, a synthetic DNA, single-stranded 31-mer (5′-TTTTTTTTT(G or A)GGATACATATCCTTAAAGTAT-3′)

was annealed to a ^{32}P-labelled 21-mer primer (5′-^{32}P-ATACTTTAAGGATATGTATCC-3′) at pH 8.0 in a water-bath at 65 °C slowly cooling to room temperature over a period of time up to 1 h. The labelling reaction mixture contained 1.6 nmol of primer and 20 pmol of γ-^{32}P-ATP in order to achieve an incorporation of the label greater than 90% in a final volume of 20 mm^3. T4 polynucleotide kinase (10 units) was heat-inactivated for 10 min at 68 °C. Primer-extension reactions were performed by using 5 units of DNA polymerase in the buffer recommended by the manufacturer, the 3′-modified 2′-deoxy-nucleotide triphosphate, and the DNA substrate (80 pmol) at pH 7.5 in a final volume of 20 mm^3. Aliquots were withdrawn and heated to 70 °C in formamide buffer before being loaded on a 15% polyacrylamide denaturing gel, which was subsequently autoradiographed.

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