Synthesis of 3'-*O*-(ω-Aminoalkoxymethyl)thymidine 5'-Triphosphates, Terminators of DNA Synthesis that Enable 3'-Labelling

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Treatment of 5'-*O*-benzoylthymidine **1** with a mixture of acetic anhydride, acetic acid and dimethyl sulfoxide yielded 5'-*O*-benzoyl-3'-*O*-methylthiomethylthymidine **2**, which was converted *via* the 3'-*O*-bromomethyl derivative into 3'-*O*-(ω-aminoalkoxymethyl)thymidines **7** bearing a 6, 8 or 10 methylene groups long hydrocarbon chain, and finally to their 5'-triphosphates **10**. The latter compounds were shown to be terminators of DNA synthesis catalysed by thermostable Tet/z DNA-polymerase, and may be labelled at the aliphatic amino group with fluorescent probes.

Quite recently a rapid solid-phase method, called mini-sequencing, has been introduced for the detection of point mutation of DNA.[1,2] The method involves hybridization of immobilized single-stranded DNA with a primer that ends immediately before the site of mutation, and elongation of the chain with a single labelled deoxyribonucleoside 5'-triphosphate. Parallel runs with each of the four possible nucleotides enable identification of the mutated base. Thus far only radiochemically labelled nucleotides have been used. Application of nonradioactive techniques, such as fluorescence detection, is hampered by the fact that the nucleoside triphosphates bearing the fluorescent probe should be reasonably good substrates of DNA polymerase. As a first step in our efforts to find a common labelling strategy for all the nucleotides occurring in DNA, we now report on the preparation of some thymidine 5'-triphosphates bearing a long chain aminoalkoxymethyl side arm at 3'-*O*. Their ability to serve as specific terminators of DNA synthesis catalysed by DNA polymerases were studied before and after attachment of fluorescent probes to their amino function.

Results and Discussion

The reaction sequence utilized in the preparation of 3'-*O*-(ω-aminoalkoxymethyl)thymidine 5'-triphosphates is depicted in Scheme 1. The key steps are discussed below.

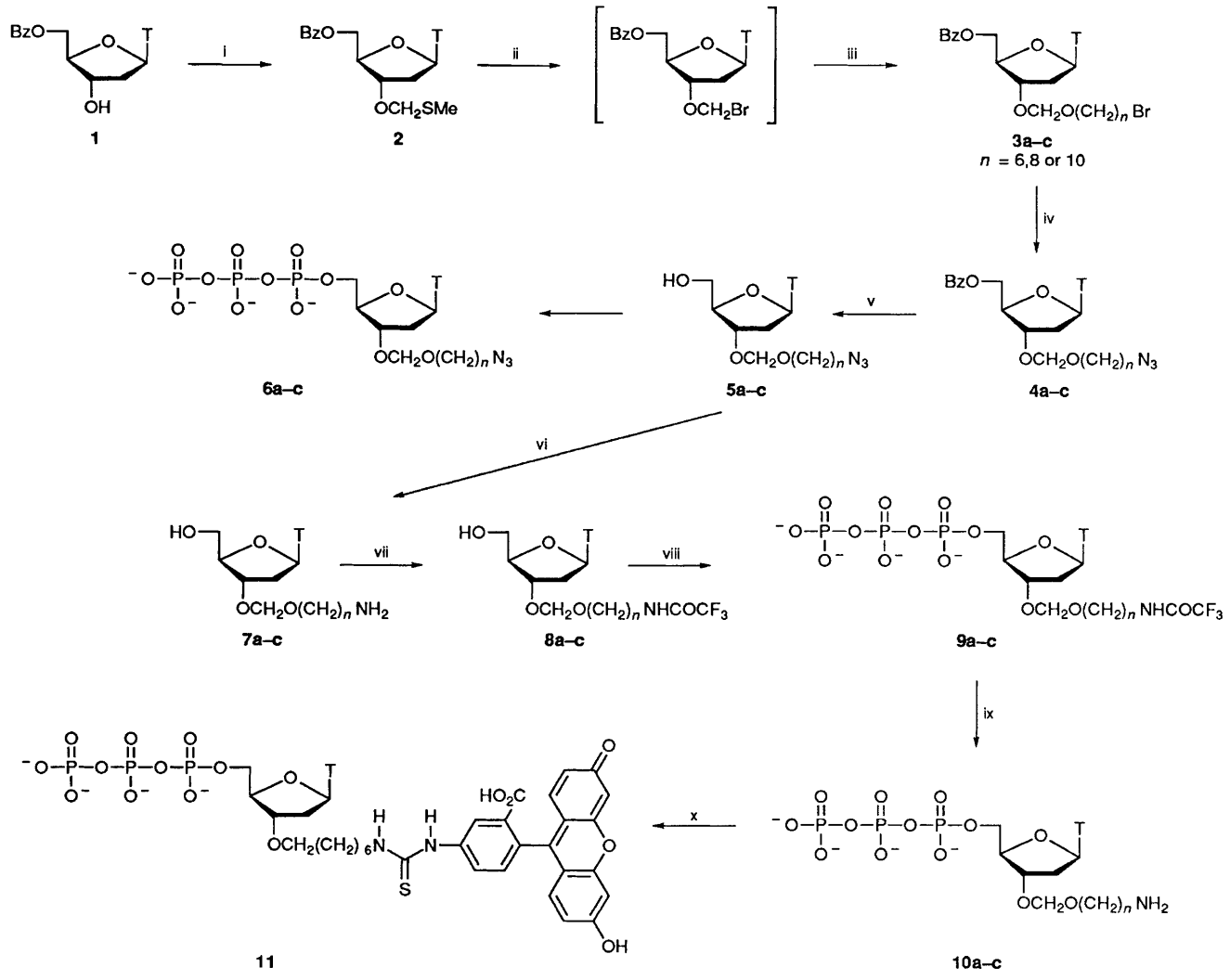
Formation of the Methylthiomethyl Ether **2**.—Methylthiomethyl ethers are well-known by-products of the oxidation of alcohols with a mixture of acetic anhydride and dimethyl sulfoxide (DMSO).[3-8] The amount of these by-products has been shown to vary from traces to more than 70% depending on the nature of the alcohol. Addition of acetic acid to the reaction mixture markedly increases the yield of the methylthiomethyl ether.[9] This observation was exploited in the preparation of 3'-*O*-methylthiomethylthymidine. Treatment of 5'-*O*-benzoylthymidine[10] **1** with a mixture of DMSO, acetic acid and acetic anhydride (54:11:35, v/v) for 2 days at ambient temperature gave, after purification, an almost 70% yield of the desired 3'-*O*-methylthiomethyl derivative **2**.

Attachment of the Aliphatic Arm to O3' **3**.—The methylthiomethyl ether, **2**, was first converted into the more reactive bromomethyl ether,[11-14] by treating **2** with *N*-bromosuccinimide (NBS) or molecular bromine in dry dichloroethane. When the 5'-*O*-benzoyl-3'-*O*-bromomethylthymidine obtained was allowed to react *in situ* with a long-chain ω-bromo alcohol (6 to 10 carbons), in the presence of 2,6-lutidine (2,6-dimethylpyridine), the corresponding 3'-*O*-(ω-bromoalkoxymethyl)thymidines, **3a–c**, were formed. The same methodology has successfully been used also with appropriately protected guanosine, cytidine and adenosine analogues without severe side reactions.[9] It has been shown that when *N*-iodosuccinimide (NIS) is used as the halogen source in a large excess (sixfold), the main product is the corresponding succinimide derivative, **3d**.[12,13] In the present synthesis only traces of this kind of side product were found, and the formation of the side product was completely prevented by using molecular bromide as a thiophilic promoter instead of *N*-halogenosuccinimide. However, a small amount (~ 10%) of another, slow-migrating side product was formed (R_f 0.29 compared to 0.37 of **3a**; MeOH–CH_2Cl_2, 3:97, v/v). The product had an identical UV spectrum with the main product, and its ^{1}H NMR spectrum exhibited double signals for H6 and sugar protons, but only single peaks for the aliphatic arm and a singlet (2 H) at δ 5.47 probably referring to NCH_2O. After deprotection with methanolic ammonia, the ^{13}C NMR showed four signals in the carbonyl carbon region of the thymidine residue. Accordingly, the side product may tentatively be assigned as a dimer **3e** containing a methylene bridge between O3' of one thymidine and N3 of the other.

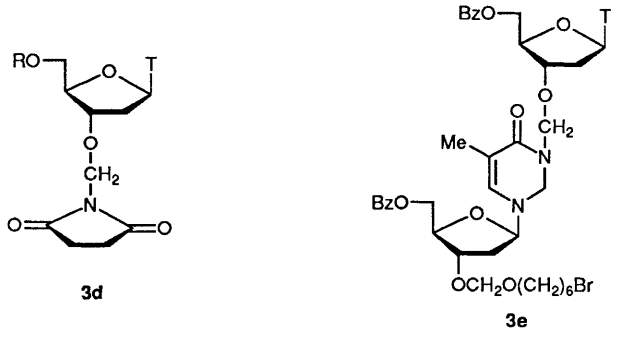
The ω-bromo substituent was displaced by azide ion by heating **3a–c** in a mixture of sodium azide and lithium chloride in dimethylformamide (DMF). The reaction mixture was 'buffered' with ammonium chloride to prevent the cleavage of the base labile 5'-*O*-benzoyl group. The displacement was almost quantitative. After conventional work up, the product, **4a–c**, was purified by silica gel column chromatography. The overall yield starting from **2** was normally about 50%.

The original idea was to debenzoylate **4a–c**, phosphorylate the deblocked 5'-OH (**5a–c**) and then reduce the azido group to an amino function. Unfortunately, the reduction of azido to amino with triphenylphosphine in a mixture of aqueous ammonia and dioxane failed, when applied to triphosphates, although the corresponding azido nucleosides **5a–c** were rapidly reduced with an excess of triphenylphosphine in pyridine. It has also been reported that 3'-azido-3'-deoxythymidine 5'-triphosphate can easily be reduced to the 3'-amino analogue by the method described above.[15]

Since the azido group was reduced at nucleoside level, the resulting amino function has to be protected before phosphorylation of 5'-OH. This was done by treating the aminoalkoxy-



Scheme 1 *Reagents and conditions:* i, $AcOH–Ac_2O$, DMSO; ii, NBS or Br_2, $ClCH_2CH_2Cl$; iii, $Br(CH_2)_nOH$, lutidine; iv, N_3^-, DMF; v, NH_3–MeOH; vi, (*a*) Ph_3P, pyridine (*b*) NH_3 (aq.); vii, CF_3CO_2Me, DMF; viii, (*a*) PO(triazole)$_3$–MeCN (*b*) P_2O_7–DMF (*c*) H_2O; ix, NH_3(aq.); x, FITC, pH 10



methyl nucleosides **7a–c** with an excess of methyl trifluoroacetate in dry DMF. When the ester was dry and freshly distilled, the reaction was quantitative and the product **8a–c** was easily isolated on a short silica gel column.

Synthesis of 5′-Triphosphates.—A modification of the method of Ötvös *et al.*[16] was applied to the preparation of the nucleoside 5′-triphosphates, **9a–c**. According to the original method, the nucleoside is allowed to react with phosphorus oxychloride in dry trimethyl phosphate, and the intermediate formed is treated *in situ* with bis(tributylammonium) pyrophosphate. Unfortunately, phosphorylation of the nucleoside with $POCl_3$ requires a prolonged treatment, and hence the hydrogen chloride liberated may cleave the acetal linkage in **8a–c**. To avoid this, a slightly different approach was applied. The nucleoside was first treated with phosphoryltris(triazole) in dry acetonitrile[17] and the intermediate obtained was allowed to react overnight with bis(tributylammonium) pyrophosphate. The remaining triazole ligand was hydrolysed by addition of water to give **9a–c**. Treatment of **9a–c** with aqueous ammonia gave **10a–c** as the final products.

In order to demonstrate the usefulness of 3′-*O*-(ω-aminoalkoxymethyl)thymidine 5′-triphosphates prepared as substrates of DNA polymerases even when attached to bulky fluorescent groups, **10a** was labelled with fluorescein isothiocyanate. The reaction was quantitative and the product, **11**, was purified by HPLC.

Termination of DNA Synthesis Catalysed by Thermostable Tet/z-DNA Polymerase.—Compounds **10a–c** differ significantly from the well-known terminators of DNA synthesis, such as 2′,3′-dideoxyribonucleoside 5′-triphosphates[18] and 3′-amino-2′,3′-dideoxyribonucleoside 5′-triphosphates.[19] Their ω-aminoalkoxymethyl side arms enable attachment of reporter groups, either before or after enzymatic incorporation into DNA, and hence labelling of the 3′-terminus of DNA. The potential advantages of the usage of a long flexible acetal arm were

expected to be (*i*) the reporter groups may be kept distant from the catalytic centre of the polymerase enzymes, and (*ii*) the flexible arm would not severely restrict the conformational motion that the sugar ring undergoes on binding to enzyme. Indeed, compounds **10a–c**, as well as their analogues bearing a trifluoroacetyl **9a–c** or fluorescein group **11**, at the amino function were observed to be specific terminators of DNA synthesis catalysed by thermostable polymerases.* Fig. 1 shows the results obtained with Tet/z DNA-polymerase, using 2′,3′-dideoxyribonucleoside 5′-triphosphates and compounds **9a**, **10a–c** and **11** as terminators. It is clearly seen that 3′-*O*-(ω-aminoalkoxymethyl)thymidine 5′-triphosphates **10a–c** as well as their analogues, **9a** and **11**, terminate DNA synthesis, giving patterns very similar to that of 2′,3′-dideoxythymidine 5′-triphosphate.

Experimental

All the solvents used were of analytical grade and were distilled and dried before use. Thymidine was purchased from Sigma and ω-bromo alcohols and fluorescein isothiocyanate from Aldrich. Adsorption column chromatography was performed on columns of silica gel 60 (Merck), and triphosphates were purified on Fractogel TSK DEAE-640(M) (Merck). TLC was conducted on silica-60 F_{254} plates (Merck). The melting points reported are uncorrected. NMR spectra were recorded on a JEOL JNM GX-400 spectrometer operating at 399.8, 100.5 and 161.9 MHz for ^1H, ^{13}C and ^{31}P, respectively. Coupling constants are given in Hz. Tetramethylsilane was used as internal (^1H, ^{13}C) and H_3PO_4 as external reference (^{31}P). ^{13}C NMR data of the compounds prepared are listed in Table 1. IR spectra were recorded on Perkin-Elmer 1600 FT-IR spectrophotometer and UV spectra on Perkin-Elmer λ-2 spectrophotometer. Elemental analyses were performed by Analytische Laboratorien, Germany, or at Department of Chemistry, University of Oulu, Finland. Tet/z DNA-polymerase from *Thermus thermophilus* was generously supplied by Dr. V. Kiselev (Research Center of Molecular Diagnostics and Therapy, Moscow, Russia). 2′-Deoxyadenosine 5′-[α-^{35}S]triphosphate (initial specific activity 1300 Ci mmol^{-1}) was obtained from NEN. 2′-Deoxy-and 2′,3′-dideoxy-ribonucleoside 5′-triphosphates were from Pharmacia, and control single stranded M 13 mp 18 DNA from USB.

5′-O-*Benzoyl*-3′-O-*methylthiomethylthymidine* **2**.—Compound **1**[10] (15.0 g, 43.3 mmol) was dissolved in DMSO (145 cm^3) and acetic acid (29 cm^3) and acetic anhydride (93 cm^3) were added. The mixture was stirred at room temperature for 2 days, *i.e.* until no starting material was detected by TLC, and was then evaporated to give an oily residue. The oil was dissolved in methylene chloride (150 cm^3) and washed with sat. aq. $NaHCO_3$ (3 × 75 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, using CH_2Cl_2 containing from 0 to 2% MeOH as eluent. Pure fractions were combined and concentrated, and the residue was crystallized from toluene to give the title compound **2** (12.0 g, 68%) as a white powder; m.p. 135 °C; R_f 0.66 (silica gel, CH_2Cl_2–MeOH, 9:1, v/v); λ_{max}(MeOH)/nm 266; δ_H($CDCl_3$) 8.68 (1 H, br s, H3), 8.04–7.48 (5 H, m, arom), 7.24 (1 H, s, H6), 6.30 (1 H, dd, $J_{1',2'}$ 7.6, $J_{1',2''}$ 6.1, H1′), 4.69 (2H, J_{AB} 11.6, OCH_2S), 4.65 (1 H, dd, $J_{4',5'}$ 3.4, $J_{5',5''}$ 12.2, H5′), 4.58 (1 H, m, H3′), 4.55 (1 H, dd, $J_{4',5''}$ 4.0, H5″), 4.37 (1 H, m, H4′), 2.16 (1 H, ddd, $J_{2',2''}$ 13.8, $J_{2'',3'}$ 3.4, H2″), 2.16 (3 H, s, SCH_3), 2.13 (1 H, m, $J_{2',3'}$ 5.9,

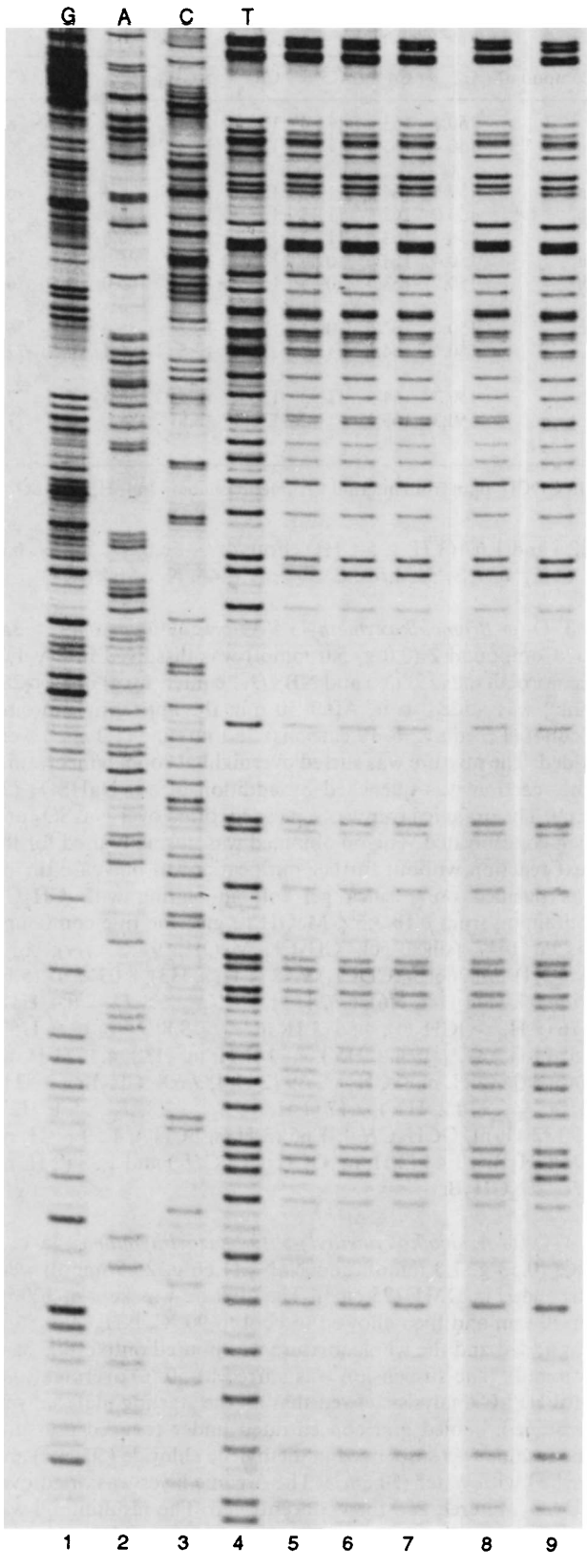


Fig. 1 Gel pattern of the DNA synthesis catalysed by a thermostable DNA-polymerase, Tet/z, and terminated with 2′,3′-dideoxyribonucleoside 5′-triphosphates (ddNTP) and various 3′-*O*-(ω-aminoalkoxymethyl)thymidine 5′-triphosphates (**9a**, **10a–c** and **11**). Tracks 1–4 show termination with ddGTP (7 μmol dm^{-3}), ddATP (147 μmol dm^{-3}), ddCTP (56 μmol dm^{-3}) and ddTTP (86 μmol dm^{-3}), respectively. Tracks 5–9 show termination with **10a** (455 μmol dm^{-3}), **10b** (350 μmol dm^{-3}) and **10c** (210 μmol dm^{-3}), **9a** (455 μmol dm^{-3}) and **11** (260 μmol dm^{-3}), respectively.

* These results will be published elsewhere.

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

Compound	Chemical shift										
	C2	C4	C5	C6	5-CH_3	C1′	C2′	C3′	C4′	C5′	Others
2	150.1	163.4	111.4	134.8	12.3	85.3	37.8	78.0	82.3	64.2	166.2 (C=O), 139.5–134.6 (arom.), 74.1 (OCH_2S), 13.9 (SCH_3)
3a	150.4	163.9	111.3	134.8	12.2	85.0	38.4	76.4	82.5	64.0	166.0 (C=O), 134.8–128.6 (arom), 94.6 (OCH_2O), 68.4, 33.8, 32.6, 29.3, 27.8, 25.3 (6 × CH_2)
5a	150.5	164.0	111.1	136.8	12.6	86.4	38.1	76.6	85.3	62.4	94.8 (OCH_2O), 68.4, 51.4, 36.6, 35.9, 33.7, 32.9 (6 × CH_2)
5b	150.0	162.3	111.3	137.0	12.7	86.7	38.1	76.7	85.2	62.6	94.9 (OCH_2O), 68.6, 51.7, 24.0–20.5 (8 × CH_2)
5c	150.3	163.6	111.2	136.7	12.7	86.6	38.1	76.6	85.3	62.6	94.9 (OCH_2O), 68.7, 51.6, 29.7–26.3 (10 × CH_2)
7a[b]	150.4	164.0	109.4	135.9	12.5	85.0	36.8	76.3	83.5	61.2	93.5 (OCH_2O), 67.3, 41.5, 33.2, 29.1, 26.2, 25.6 (6 × CH_2)
7b[b]	150.7	163.7	109.5	135.9	12.2	85.0	36.9	76.4	83.8	61.3	93.6 (OCH_2O), 67.4, 41.6, 33.2, 29.1, 28.9, 28.8, 26.4, 25.6 (8 × CH_2)
7c[b]	150.4	163.6	109.4	135.9	12.2	85.0	36.9	76.4	83.8	61.2	93.6 (OCH_2O), 67.3, 41.3, 32.9, 28.9–25.6 (10 × CH_2)
8a	150.6	164.2	111.2	136.9	12.5	86.4	38.2	77.2[c]	85.4	62.4	157.6 (C=O), 94.8 (OCH_2O), 68.3, 39.9, 29.4, 28.8, 26.3, 25.7 (6 × CH_2)
8b	150.3	164.2	111.2	136.8	12.6	86.6	38.0	77.2[c]	85.4	62.5	157.6 (C=O), 94.8 (OCH_2O), 68.4, 40.0, 29.5–26.4 (8 × CH_2)
8c	150.3	163.3	111.2	136.8	12.6	86.6	38.0	77.2[c]	85.3	62.5	157.6 (C=O), 94.8 (OCH_2O), 68.6, 40.0, 29.6–26.6 (10 × CH_2)

[a] In $CDCl_3$; ppm from internal tetramethylsilane. [b] In [$^2H_6$]DMSO. [c] Partial overlapping with the solvent signal.

H2′) and 1.67 (3 H, s, 5-CH_3) (Found: C, 56.0; H, 5.4; N, 6.8. $C_{19}H_{22}N_2O_6S_1$ requires: C, 56.2; H, 5.46; N, 6.90%).

3′-O-(ω-*Bromoalkoxymethyl*)-5′-O-*benzoylthymidines* **3a–c**.—Compound **2** (2.0 g, 5.0 mmol) was dissolved in dry 1,2-dichloroethane (25 cm^3) and NBS (1.1 equiv., 1.0 g) or Br_2 (282 mm^3) was added to it. After 10 min the appropriate bromo alcohol (1.5 equiv.; 6–10 carbons) and lutidine (1.2 cm^3) were added. The mixture was stirred overnight at room temperature. The reaction was quenched by addition of aq. $NaHSO_3$ (20 cm^3). The organic layer was separated, dried over Na_2SO_4 and then concentrated. The oil obtained was normally used for the next reaction without further purification. In one case the oil was purified on a silica gel column eluting with CH_2Cl_2 containing from 0 to 3.5% MeOH to give the title compound **3a**; R_f 0.37 (silica gel, CH_2Cl_2–MeOH, 97:3, v/v); λ_{max}(MeOH)/nm 266; δ_H($CDCl_3$) 9.73 (1 H, s, H3), 8.03–7.47 (5 H, arom), 7.24 (1 H, s, H6), 6.32 (1 H, dd, $J_{1',2'}$ 6.8 $J_{1',2''}$ 6.4, H1′), 4.76 (2 H, s, OCH_2O), 4.66 (1 H, m, $J_{4',5'}$ 3.8, $J_{5',5''}$ 12.0, H5′), 4.55 (1 H, m, $J_{4',5''}$ 4.8, H5′), 4.44 (1 H, m, H3′), 4.35 (1 H, m, H4′), 3.56 (2 H, m, OCH_2), 3.39 (2 H, t, *J* 6.8, CH_2Br), 2.52 (1 H, m, $J_{2',2''}$ 14.2, H2″), 2.17 (1 H, m, $J_{2'',3'}$ 2.8, $J_{2',3'}$ 5.1, H2′), 1.83 (2 H, m, OCH_2CH_2), 1.66 (3 H, s, $5CH_3$), 1.59 (2 H, m, CH_2CH_2Br), 1.44 (2 H, m, $OCH_2CH_2CH_2$) and 1.38 (2 H, m, $CH_2CH_2CH_2Br$).

3′-O-(ω-*Azidoalkoxymethyl*)-5′-O-*benzoylthymidines* **4a–c**.—LiCl (0.33 g, 7.8 mmol) and NaN_3 (1.66 g, 25.5 mmol) were suspended in DMF (25 cm^3). The mixture was kept at 120 °C for 30 min and then allowed to cool to 90 °C. NH_4Cl (0.56 g) was added and the whole mixture was poured onto crude **3a–c** (5 mmol). The suspension was stirred at 50 °C overnight, *i.e.* until HPLC analysis showed that all the starting material was consumed, cooled and concentrated under reduced pressure. The residue was suspended in methylene chloride (50 cm^3) and washed with water (50 cm^3). The organic layer was dried over Na_2SO_4, filtered and then concentrated. The resulting oil was dissolved in a small amount of methylene chloride and added dropwise into a mixture of diethyl ether–hexane (1:3, v/v, 250 cm^3) to remove lutidine and unchanged long chain alcohol. The solution was allowed to precipitate and was then decanted. The remaining oil was purified by silica gel column chromatography. Elution with 0 to 4% MeOH in CH_2Cl_2 gave the title compounds **4a–c** as colourless oils. **4a**: (1.60 g, 57% from **2**) R_f 0.37 (silica gel, CH_2Cl_2–MeOH, 97:3, v/v); λ_{max}(MeOH)/nm 268; δ_H($CDCl_3$) 8.20 (1 H, br, H3), 8.04–7.46 (5 H, arom.), 7.23 (1 H, s, H6), 6.30 (1 H, dd, $J_{1',2'}$ 7.2, $J_{1',2''}$ 6.5, H1′), 4.75 (2 H, s, OCH_2O); 4.66 (1 H, dd, $J_{5',5''}$ 12.0, H5′), 4.54 (1 H, dd, H5″), 4.42 (1 H, m, H3′), 4.34 (1 H, m, H4′), 3.54 (2 H, m, OCH_2), 3.25 (2 H, t, *J* 6.8, CH_2N_3), 2.51 (1 H, m, $J_{2',2''}$ 13.8, H2″), 2.15 (1 H, m, H2′), 1.66 (3 H, s, 5-CH_3), 1.60 (4 H, m, 2 × CH_2) and 1.43 (4 H, m, 2 × CH_2). **4b**: (1.20 g, 45% from **2**) R_f 0.39 (silica gel, CH_2Cl_2–MeOH, 97:3, v/v); λ_{max}(MeOH)/nm 269; δ_H($CDCl_3$) 8.23 (1 H, br s, H3), 8.03–7.47 (5 H, arom), 7.23 (1 H, s, H6), 6.30 (1 H, dd, $J_{1',2'}$ 7.3, $J_{1',2''}$ 6.4, H1′), 4.75 (2 H, s, OCH_2O), 4.67 (1 H, dd, $J_{4',5'}$ 2.9, $J_{5',5''}$ 12.2, H5′), 4.54 (1 H, dd, $J_{4',5''}$ 3.9, H5″), 4.43 (1 H, m, H3′), 4.35 (1 H, m, H4′), 3.55 (2 H, m, OCH_2), 3.25 (2 H, t, *J* 6.8, CH_2N_3), 2.52 (1 H, m, $J_{2',2''}$ 13.7, $J_{2'',3'}$ 2.9, H2″), 2.15 (1 H, m, $J_{2',3'}$ 6.8, H2′), 1.66 (3 H, s, 5-CH_3) and 1.60–1.31 (12 H, m, 6 × CH_2). **4c**: (1.28 g, 40% from **2**) R_f 0.40 (silica gel, CH_2Cl_2–MeOH, 97:3, v/v); λ_{max}(MeOH)/nm 269; δ_H($CDCl_3$) 8.20 (1 H, br s, H3), 8.04–7.47 (5 H, arom), 7.20 (1 H, s, H6), 6.30 (1 H, dd, $J_{1',2'}$ 7.3, $J_{1',2''}$ 6.3, H1′), 4.75 (2 H, s, OCH_2O), 4.66 (1 H, dd, $J_{5',5''}$ 12.1, H5′), 4.45 (1 H, dd, H5″), 4.37 (1 H, m, H3′), 4.37 (1 H, m, H4′), 3.57 (2 H, m, OCH_2), 3.25 (2 H, t, *J* 6.8, CH_2N_3), 2.54 (1 H, m, $J_{2',2''}$ 13.6, H2″), 2.15 (1 H, m, H2′), 1.69 (3 H, s, 5-CH_3) and 1.62–1.27 (16 H, m, 8 × CH_2).

3′-O-(ω-*Azidoalkoxymethyl*)*thymidines* **5a–c**.—Compound **4a–c** was dissolved in sat. methanolic ammonia and stirred overnight at room temperature. When the reaction was complete, solvent was evaporated under reduced pressure and the residue was purified on a silica gel column eluting with CH_2Cl_2 containing from 0 to 5% MeOH. The title compounds **5a–c** were obtained as colourless oils (95% yield). **5a**: R_f 0.52 (silica gel, CH_2Cl_2–MeOH, 9:1, v/v); λ_{max}(MeOH)/nm 267; δ_H($CDCl_3$) 9.30 (1 H, br s, H3), 7.43 (1 H, s, H6), 6.18 (1 H, dd, $J_{1',2'}$ 7.0, $J_{1',2''}$ 6.7, H1′), 4.73 (2 H, s, OCH_2O), 4.43 (1 H, m, H3′), 4.07 (1 H, m, H4′), 3.94 (1 H, dd, $J_{4',5''}$ 2.7, $J_{5',5''}$ 11.9, H5′), 3.81 (1 H, dd, $J_{4',5''}$ 3.1, H5″), 3.55 (2 H, t, *J* 6.7, OCH_2), 3.27 (2 H, t, *J* 6.7, CH_2N_3), 2.96 (1 H, br 5′-OH), 2.36 (2 H, m, H-2′, H2″), 1.94 (3 H, s, 5-CH_3), 1.60 (4 H, m, 2 × CH_2) and 1.40 (4 H, m, 2 × CH_2); ν_{max}/cm^{-1} 2096s (N_3). **5b**: R_f 0.54 (silica gel, CH_2Cl_2–MeOH, 9:1, v/v); λ_{max}(MeOH)/nm 267; δ_H($CDCl_3$) 8.30 (1 H, br s, H3), 7.38 (1 H, s, H6), 6.15 (1 H, dd, $J_{1',2''}$ 6.8, H1′), 4.73 (2 H, s, OCH_2O), 4.43 (1 H, m, H3′), 4.08 (1 H, m, H4′), 3.95 (1 H, dd, $J_{5',5''}$ 11.8, H5′), 3.80 (1 H, dd, H5″), 3.54 (2 H, t, *J* 6.8, OCH_2), 3.28 (2 H, t, *J* 6.8, CH_2N_3), 2.96 (1 H, br, 5′-OH), 2.36 (2 H, m, H2′, H2″), 1.92 (3 H, s, 5-CH_3) and 1.61–1.33 (12 H, m, 6 × CH_2); ν_{max}/cm^{-1} 2096s (N_3). **5c**: R_f 0.56 (silica gel, CH_2Cl_2–MeOH, 9:1, v/v); λ_{max}(MeOH)/nm 267; δ_H($CDCl_3$) 8.40 (1 H, br s, H3), 7.38 (1 H, s, H6), 6.16 (1 H, dd, $J_{1',2'}$ 7.0, $J_{1',2''}$ 6.8, H1′), 4.73 (2 H, s, OCH_2O), 4.41 (1 H, m, H3′), 4.07 (1 H, m,

H4'), 3.94 (1 H, dd, $J_{5',5''}$ 11.9, H5'), 3.81 (1 H, dd, H5''), 3.54 (2 H, t, J 6.8, OCH_2), 3.26 (2 H, t, J 6.8, CH_2N_3), 2.90 (1 H, br, 5'-OH), 2.36 (2 H, m, H2', H2''), 1.92 (3 H, s, 5-CH_3) and 1.64–1.29 (16 H, m, 8 × CH_2); ν_{max}/cm^{-1} 2096 (N_3).

3'-O-(ω-*Aminoalkoxymethyl*)*thymidines* **7a–c**.—Compound **5a** (1.12 g, 2.4 mmol) was dissolved in pyridine (30 cm^3) and Ph_3P (2 equiv., 4.0 mmol, 1.05 g) was added. The mixture was stirred at room temperature until no starting material could be detected by TLC. The resulting ylide was hydrolysed with aq. ammonia, evaporated to dryness under reduced pressure, suspended in water (50 cm^3) and then extracted with diethyl ether (3 × 25 cm^3). The aqueous layer was evaporated to dryness to give compound **7a** as a white solid (0.93 g, 93%), R_f 0.0 (silica gel, CH_2Cl_2–MeOH 9:1, v/v). The test for primary amines was positive.* $\lambda_{max}(H_2O)$/nm 267; δ_H([$^2H_6$]-DMSO) 7.70 (1 H, s, H6), 6.12 (1 H, dd, $J_{1',2'}$ 7.8, $J_{1',2''}$ 5.9, H1'), 4.68 (2 H, s, OCH_2O), 4.29 (1 H, m, H3'), 3.91 (1 H, m, H4'), 3.61 (1 H, m, $J_{4',5'}$ 3.9, $J_{5',5''}$ 12.2, H5'), 3.56 (1 H, m, $J_{4',5''}$ 3.9, H5''), 3.47 (2 H, t, J 6.4, OCH_2), 2.50 (2 H, m, CH_2NH_2 partially overlapping with the solvent signal; 2.23 (1 H, m, $J_{2',2''}$ 13.2, $J_{2'',3'}$ 3.6, H2''), 2.14 (1 H, m, $J_{2',3'}$ 6.2, H2'), 1.77 (3 H, s, 5-CH_3) and 1.50–1.29 (8 H, m, 4 × CH_2). With the analogues bearing a longer aliphatic arm (8 and 10 carbons) at O3' the product (**7b** and **c**) was not soluble enough in water to be extracted as described. Accordingly, after hydrolysis and the first evaporation, the remaining triphenylphosphine was removed by stirring the solid material with diethyl ether. The ethereal layer was carefully decanted and the remaining solid material was dried under reduced pressure. HPLC analysis showed that **7b** was homogenous, but **7c** contained 10% of impurities. **7b**: R_f 0.0 (silica gel, CH_2Cl_2–MeOH, 9:1, v/v). The test for primary amines was positive. λ_{max}(MeOH)/nm 266; δ_H([$^2H_6$]DMSO) 7.70 (1 H, s, H6), 6.12 (1 H, dd, $J_{1',2'}$ 7.8, $J_{1',2''}$ 5.9, H1'), 4.68 (2 H, s, OCH_2O), 4.29 (1 H, m, H3'), 3.90 (1 H, m, H4'), 3.61 (1 H, m, $J_{4',5''}$ 3.9, $J_{5',5''}$ 12.2, H5'), 3.56 (1 H, m, $J_{4',5''}$ 3.9, H5''), 3.47 (2 H, t, J 6.8, OCH_2), 2.50 (2 H, m, CH_2NH_2, partially overlapping with the solvent signal), 2.24 (1 H, m, $J_{2',2''}$ 13.7, $J_{2'',3'}$ 2.4, H2''), 2.15 (1 H, m, $J_{2',3'}$ < 1, H2'), 1.77 (3 H, s, 5-CH_3) and 1.50–1.25 (12 H, m, 6 × CH_2). **7c**: R_f 0.0 (silica gel, CH_2Cl_2–MeOH, 9:1, v/v). The test for primary amines was positive. λ_{max}(MeOH)/nm 266; δ_H([$^2H_6$]DMSO) 7.70 (1 H, s, H6), 6.13 (1 H, dd, $J_{1',2'}$ 7.8, $J_{1',2''}$ 6.0, H1'), 4.68 (2 H, s, OCH_2O), 4.29 (1 H, m, H3'), 3.91 (1 H, m, H4'), 3.61 (1 H, m, $J_{5',5''}$ 12.2, H5'), 3.56 (1 H, m, H5''), 3.46 (2 H, t, J 6.8, OCH_2), 2.50 (2 H, m, CH_2NH_2, partially overlapping with the signal of solvent), 2.22 (2 H, m, H2', H2''), 1.77 (3 H, s, 5-CH_3) and 1.50–1.29 (16 H, m, 8 × CH_2).

3'-O-(N-*Trifluoroacetyl*-ω-*aminoalkoxymethyl*)*thymidines* **8a–c**.—Compound **7a–c** (1.0 mmol) was dissolved in dry DMF (2.5 cm^3) freshly distilled methyl trifluoroacetate (1.3 cm^3) was added. The reaction mixture was stirred overnight at room temperature. Solvent and the unchanged ester were evaporated under reduced pressure and the resulting oil was purified on a silica gel column eluting with 3% MeOH in CH_2Cl_2. The title compounds **8a–c** were obtained as colourless oils. **8a**: (0.44 g, 95%); λ_{max}(MeOH)/nm 266; δ_H($CDCl_3$) 9.48 (1 H, br s, H3), 7.51 (1 H, s, H6), 7.09 (1 H, br t, $NHCOCF_3$), 6.18 (1 H, dd, $J_{1',2'}$ 6.8, $J_{1',2''}$ 6.8, H1'), 4.73 (2 H, s, OCH_2O); 4.44 (1 H, m, H3'), 4.09 (1 H, m, H4'), 3.93 (1 H, m, $J_{5',5''}$ 10, H5'), 3.81 (1 H, m, H5''), 3.55 (2 H, t, J 6.8, OCH_2), 3.36 (2 H, q, J 6.8, $CH_2NHCOCF_3$), 3.26 (1 H, br, 5'-OH); 2.39 (1 H, ddd, $J_{2',2''}$ 13.7, $J_{2'',3'}$ 3.7, H2''), 2.31 (1 H, m, $J_{2',3'}$ 6.8, H2'), 1.89 (3 H, s, 5-CH_3), 1.60 (4 H, m, 2 × CH_2) and 1.38 (4 H, m, 2 × CH_2) (Found: C, 49.2; H, 6.2; N, 8.8. $C_{19}H_{28}F_3N_3O_7$ requires C, 48.82; H, 6.04; N, 8.99%). **8b**: (0.44 g, 90%); λ_{max}(MeOH)/nm 266; δ_H($CDCl_3$) 8.94 (1 H, br s, H3), 7.44 (1 H, s, H6), 6.67 (1 H, br t, $NHCOCF_3$), 6.16 (1 H, dd, $J_{1',2'}$ 6.8, $J_{1',2''}$ 6.8, H1'), 4.73 (2 H, s, OCH_2O), 4.42 (1 H, m, H3'), 4.08 (1 H, m, H4'), 3.93 (1 H, m, $J_{5',5''}$ 12.0, H5'), 3.81 (1 H, m, H5''), 3.55 (2 H, t, J 6.8, OCH_2), 3.36 (2 H, q, J 6.8, $CH_2NHCOCF_3$), 2.85 (1 H, br, 5'-OH), 2.36 (2 H, m, H2', H2''), 1.89 (3 H, s, 5-CH_3) and 1.58–1.33 (12 H, m, 6 × CH_2) (Found: C, 50.95; H, 6.4; N, 8.4. $C_{21}H_{32}F_3N_3O_7$ requires C, 50.90; H, 6.51; N, 8.48%). **8c**: (0.46 g, 87%); λ_{max}(MeOH)/nm 268; δ_H($CDCl_3$) 8.63 (1 H, br s, H3), 7.41 (1 H, s, H6), 6.48 (1 H, br t, $NHCOCF_3$), 6.16 (1 H, dd, $J_{1',2'}$ 6.8, $J_{1',2''}$ 6.8, H1'), 4.73 (2 H, s, OCH_2O), 4.42 (1 H, m, H3'), 4.08 (1 H, m, H4'), 3.94 (1 H, m, $J_{5',5''}$ 10.0, H5'), 3.81 (1 H, m, H5''), 3.54 (2 H, t, J 6.8, OCH_2), 3.36 (2 H, q, J 6.8, $CH_2NHCOCF_3$), 2.64 (1 H, br, 5'-OH), 2.37 (2 H, m, H2', H2''), 1.89 (3 H, s, 5-CH_3) and 1.29 (16 H, m, 8 × CH_2) (Found: C, 52.7; H, 6.8; N, 8.0. $C_{23}H_{36}F_3N_3O_7$ requires C, 52.77; H, 6.93; N, 8.03%).

3'-O-(N-*Trifluoroacetyl*-ω-*aminoalkoxymethyl*)*thymidine* 5'-*Triphosphates* **9a–c** *as Triethylammonium Salts*.—Dry, recrystallized triazole (4.5 equiv. to nucleoside, 31 mg, 0.45 mmol) was dissolved in a mixture of dry acetonitrile (0.5 cm^3) and dry triethylamine (4.5 equiv., 63 mm^3). The mixture was cooled on an ice bath and phosphorus oxychloride (1.5 equiv, 14 mm^3) was added. The mixture was allowed to warm to room temperature and stirred for 30 min. The whole mixture was quickly filtered and the filtrate was poured on dried nucleoside **8a–e** (0.1 mmol) and stirred at room temperature until all starting material had disappeared (normally 10 to 20 min). Then bis(butylammonium)pyrophosphate in DMF (0.2 mol dm^{-3}; 1.5 equiv., 2.5 cm^3) was added and the reaction was stirred overnight at room temperature. The remaining triazole ligand was hydrolysed by water (5 cm^3). The reaction mixture was diluted with water (100 cm^3) containing 50% (v/v) acetonitrile. The crude material was applied onto an ion exchange column and eluted with a linear gradient [0.0–0.3 mol dm^{-3} TEAB (triethylammonium hydrogen carbonate) in 50%, v/v, MeCN]. The fraction eluted at 0.2 mol dm^{-3} TEAB was collected and concentrated. The oily residue was further purified on a preparative RP-18 column (Reliasil 300 A, 5 μm, 250 mm × 9 mm) and eluted with water containing 40% (v/v) acetonitrile. **9a**: $\delta_H(D_2O)$ 7.59 (1 H, s, H6), 6.15 (1 H, dd, $J_{1',2'}$ 8.2, $J_{1',2''}$ 6.0, H1'), 4.69 (2 H, s, OCH_2O, partial overlapping with the signal of solvent), 4.40 (1 H, m, H3'), 4.17 (1 H, m, H4'), 4.05 (2 H, m, H5', H5''), 3.49 (2 H, m, OCH_2), 3.14 (2 H, t, J 6.8, $CH_2NHCOCF_3$), 2.31 (1 H, m, $J_{2',2''}$ 14.0, H2''), 2.21 (1 H, m, H2'), 1.76 (3 H, s, 5-CH_3) and 1.43–1.30 (8 H, m, 4 × CH_2). **9b**: $\delta_H(D_2O)$ 7.60 (1 H, s, H6), 6.15 (1 H, d, $J_{1',2'}$ 6.0, H1'), 4.70 (2 H, s, OCH_2O, partial overlapping with the solvent signal), 4.42 (1 H, m, H3'), 4.15 (1 H, m, H4'), 4.07 (2 H, m, H5', H5''), 3.50 (2 H, m, OCH_2), 3.17 (2 H, t, J 6.8, $CH_2NHCOCF_3$), 2.31 (1 H, m, $J_{2',2''}$ 14, H2''), 2.21 (1 H, m, H2'), 1.77 (3 H, s, 5-CH_3) and 1.41–1.29 (12 H, m, 6 × CH_2). **9c**: $\delta_H(D_2O)$ 7.61 (1 H, s, H6), 6.17 (1 H, dd, $J_{1',2''}$ 8.3, H1'), 4.70 (2 H, s, OCH_2O, partial overlapping with the solvent signal), 4.41 (1 H, m, H3'), 4.18 (1 H, m, H4'), 4.06 (2 H, m, H5', H5''), 3.46 (2 H, m, OCH_2), 3.15 (2 H, t, J 6.8, $CH_2NHCOCF_3$), 2.32 (1 H, m, $J_{2',2''}$ 14.3, H2''), 2.22 (1 H, m, H2'), 1.77 (3 H, s, 5-CH_3), 1.44 (4 H, m, 2 × CH_2) and 1.15 (16 H, m, 8 × CH_2). All ^1H NMR spectra also exhibited signals from the triethylammonium group (q 3.03 and t 1.13), which disturbed integration.

3'-O-(ω-*Aminoalkoxymethyl*)*thymidine* 5'-*Triphosphates* **10a–c**.—Compounds **9a–c** were dissolved in aq. ammonia, stirred overnight at room temperature and then evaporated to dryness under reduced pressure to give the title compounds.

* 'Fluram', Roche Spray Reagent for assay of primary amines, F. Hoffman-La Roche Co. Limited, Diacnostica, Basle, Switzerland.

Table 2 Properties of 3'-*O*-modified thymidine 5'-triphosphates prepared

Compound	t_R/min[a]	t_R/min[b]	UV(H_2O)λ/nm λ_{max}	λ_{min}	^{31}P NMR chemical shifts[c] δ[P(α)]	δ[P(β)]	δ[P(γ)]
9a	24.2	23.7	267	235	−11.71	−23.96	−12.34
9b	24.6	26.7	268	234	−11.65	−23.95	−12.32
9c	25.0	32.9	270	235	−11.59	−23.91	−12.17
10a	22.1	14.6	268	235	−8.39	−23.46	−12.23
10b	22.5	17.1	269	233	−8.36	−23.44	−12.19
10c	23.0	22.3	267	234	−8.34	−23.43	−12.18

[a] Ion exchange column (Synchropak AX-300, 6.5 μm, 4.6 × 250 mm), flow rate 1.0 cm^3 min^{-1}, A = 0.03 mol dm^{-3} KH_2PO_4, 50% formamide (pH = 5.2), B = 0.03 mol dm^{-3} KH_2PO_4, 1.0 mol dm^{-3} $(NH_4)_2SO_4$, 50% formamide (pH = 5.6), from A to B in 30 min. [b] Reversed-phase column (Nucleosil 300-5 C4, 5 μm, 250 mm × 9 mm; Macherey-Nagel, A = 0.05 mol dm^{-3} TEAA, pH 5.5; B = A + 50% MeCN, from 40 to 70% B in 45 min, flow rate 0.8 cm^3 min^{-1}). [c] In D_2O, external reference H_3PO_4 (0.00 ppm). Assignments of P(α) and P(γ) are tentative.

Purification was performed on reversed-phase HPLC as described above for **9a–c**. **10a**: $\delta_H(D_2O)$ 7.61 (1 H, s, H6), 6.17 (1 H, dd, $J_{1',2'}$ 6.8, $J_{1',2''}$ 5.4, H1'), 4.70 (2 H, s, OCH_2O, partial overlapping with the solvent signal), 4.40 (1 H, m, H3'), 4.24 (1 H, m, H4'), 4.05 (2 H, m, H5', H5''), 3.45 (2 H, m, OCH_2), 2.88 (2 H, t, CH_2NH_2), 2.32 (1 H, m, H2''), 2.21 (1 H, m, H2'), 1.68 (3 H, s, 5-CH_3) and 1.50–1.25 (8 H, m, 4 × CH_2). **10b**: $\delta_H(D_2O)$ 7.61 (1 H, s, H6), 6.17 (1 H, dd, $J_{1',2'}$ 6.8, $J_{1',2''}$ 5.9, H1'), 4.70 (2 H, s, OCH_2O, partial overlapping with the solvent signal), 4.41 (1 H, m, H3'), 4.18 (1 H, m, H4'), 4.06 (2 H, m, H5', H5''), 3.46 (2 H, m, OCH_2), 2.88 (2 H, t, CH_2NH_2), 2.32 (1 H, m, H2''), 2.23 (1 H, m, H2'), 1.79 (3 H, s, 5-CH_3) and 1.45–1.15 (12 H, m, 6 × CH_2). **10c**: $\delta_H(D_2O)$ 7.61 (1 H, s, H6), 6.19 (1 H, dd, $J_{1',2'}$ 6.7, $J_{1',2''}$ 5.7, H1'), 4.70 (2 H, s, OCH_2O, partial overlapping with the solvent signal), 4.41 (1 H, m, H3'), 4.18 (1 H, m, H4'), 4.06 (2 H, m, H5', H5''), 3.46 (2 H, m, OCH_2), 2.89 (2 H, t, CH_2NH_2), 2.32 (1 H, m, H2''), 2.22 (1 H, m, H2'), 1.77 (3 H, s, 5-CH_3) and 1.45–1.29 (16 H, m, 8 × CH_2); Table 2 records the HPLC retention times, UV absorption maxima and ^{31}P NMR shifts of **9a–c** and **10a–c**.

Labelling of Compound **10a** *with Fluorescein Isothiocyanate.*—Compound **10a** (10 OD) (OD = optical density unit) was dissolved in carbonate buffer (0.1 mol dm^{-3}; 2.5 cm^3, pH = 10.3). Fluorescein isothiocyanate (50 mg) in DMF (2.5 cm^3) was added and the pH was readjusted to 10 with NaOH (0.1 mol dm^{-3}). The mixture was kept overnight in the dark at room temperature. The pH was adjusted to 3 with HCl (1.0 mol dm^{-3}), after which the mixture was extracted with ethyl acetate (4 × 10 cm^3). The organic layer was discarded and the aqueous layer was neutralized with sat. $NaHCO_3$. $NaHCO_3$. The crude product was purified by ion exchange HPLC [Synchropak AX-300, 6.5 μm, 4.6 × 250 mm; A = 0.05 mol dm^{-3} KH_2PO_4, 50% formamide, pH 5.2; B = 0.05 mol dm^{-3} KH_2PO_4, 1 mol dm^{-3} $(NH_4)_2SO_4$, 50% formamide, pH 5.5 from 10 to 100% B in 30 min, then 100% B for 15 min, flow rate 1.0 cm^3 min^{-1}]. The peak eluted at 42 min was collected and repurified by reversed-phase HPLC [Nucleosil 300-5 C4, 5 μm, 250 mm × 9 mm; Macherey-Nagel, A = 0.05 mol dm^{-3} tetraethylammonium acetate (TEAA), pH 5.5; B = A + 50% MeCN, from 40 to 70% B in 45 min, flow rate 0.8 cm^3 min^{-1}, t_R **11** = 17 min] and desalted; $\lambda_{max}(H_2O)$/nm 267, 456 and 477.

* *Abbreviations:* dG, 2'-deoxyguanosine; dA, 2'-deoxyadenosine; dC, 2'-deoxycytidine; T, thymidine; dGTP, 2'-deoxyguanosine 5'-triphosphate; dATP, 2'-deoxyadenosine 5'-triphosphate; dCTP, 2'-deoxycytidine 5'-triphosphate; DTTP, thymidine 5'-triphosphate; ddGTP, 2',3'-dideoxyguanosine 5'-triphosphate; ddATP, 2',3'-dideoxyadenosine 5'-triphosphate; ddCTP, 2',3'-dideoxycytidine-5'-triphosphate; ddTTP, 3'-deoxythymidine 5'-triphosphate.

*Termination of DNA Synthesis.**—Template–primer mixtures contained DNA (1 μg), an equimolar quantity of primer, Tris-HCl (67 mmol dm^{-3}; pH 8.8), $(NH_4)_2SO_4$ (16.6 mmol dm^{-3}) and $MgCl_2$ (1.5 mmol dm^{-3}). Annealing was performed at 70 °C for 2 min with subsequent slow decreasing of temperature to 30 °C. The termination reaction was run in two steps according to USB protocols[20] with some modifications: labelling for 5 min at 45 °C (reaction mixture contained 0.2 μmol dm^{-3} of each of four deoxyribonucleotides, from 5 to 7 μCi [α-^{35}S]dATP and 3 units of Tet/z DNA polymerase), and termination for 10 min at 70 °C after adding terminating compounds and higher concentrations of deoxyribonucleotides. Finally, termination mixtures contained: G-mixture: 3.5 μmol dm^{-3} dGTP; 7 μmol dm^{-3} ddGTP and 35 μmol dm^{-3} of dATP, dCTP and dTTP each; A-mixture: 3.5 mmol dm^{-3} dATP, 50 mmol dm^{-3} ddATP and 35 mmol dm^{-3} of dGTP, dCTP and dTTP each; C-mixture: 3.5 μmol dm^{-3} dCTP; 23 μmol dm^{-3} ddCTP and 35 μmol dm^{-3} of dATP, dCTP and dTTP each; T-mixture: 3.5 μmol dm^{-3} dTTP, 86 μmol dm^{-3} ddTTP and 35 μmol dm^{-3} of dATP, dCTP and dTTP each. Termination mixtures of **9a–c**, **10a–c** and **11** contained 3.5 mmol dm^{-3} dTTP, different concentrations of **9a–c**, **10a–c** and **11**, 35 μmol dm^{-3} of dATP, dCTP and dTTP each. The samples were loaded onto the 8% acrylamide–8 mol dm^{-3} urea gel and electrophoresis was run on Macrophore sequencing system. After electrophoresis the gel was fixed for 30 min in 10% acetic acid–methanol and autoradiographed.

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