Inhibition of DNA polymerase reactions by pyrimidine nucleotide analogues lacking the 2-keto group

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ABSTRACT

To investigate the influence of the pyrimidine 2-keto group on selection of nucleotides for incorporation into DNA by polymerases, we have prepared two C nucleoside triphosphates that are analogues of dCTP and dTTP, namely 2-amino-5-(2′-deoxy-β-D-ribofuranosyl)pyridine-5′-triphosphate (d*CTP) and 5-(2′-deoxy-β-D-ribofuranosyl)-3-methyl-2-pyridone-5′-triphosphate (d*TTP) respectively. Both proved strongly inhibitory to PCR catalysed by Taq polymerase; d*TTP rather more so than d*CTP. In primer extension experiments conducted with either Taq polymerase or the Klenow fragment of *Escherichia coli* DNA polymerase I, both nucleotides failed to substitute for their natural pyrimidine counterparts. Neither derivative was incorporated as a chain terminator. Their capacity to inhibit DNA polymerase activity may well result from incompatibility with the correctly folded form of the polymerase enzyme needed to stabilize the transition state and catalyse phosphodiester bond formation.

INTRODUCTION

DNA replication is dependent on faithful copying of DNA by polymerases (1). Structural studies have indicated that the majority of polymerases belong to an enzyme superfamily having closely related active sites similarly positioned within a polymerase cleft (2–3). Steitz (4) has pointed out that specific recognition and positioning of the O2 atoms of pyrimidines and the N3 atoms of purines by protein side chains may be important to the mechanism by which polymerases enhance the fidelity of Watson–Crick base pairing and promote correct nucleotide incorporation at the active site. A similar conclusion was reached by Georgiadis *et al.* (5), arising from a detailed modelling study with reverse transcriptase.

DNA polymerases can sometimes tolerate chemical changes in their nucleotide substrates and in previous work we have used PCR with Taq DNA polymerase to incorporate nucleotide analogues with added/deleted functional groups into DNA (6–8). Analogue triphosphates, such as d(2,6-diaminopurine)triphosphate instead of dATP (addition of the 2-amino group) and dITP in place of dGTP (deletion of the 2-amino group), as well as a wide variety of pyrimidine nucleotides modified at the 5 position, have been enzymatically incorporated into DNA sequences (Guo,M.J.and Waring,M.J. unpublished results). An opportunity therefore arises to determine whether pyrimidine nucleotide analogues modified at the O2 carbonyl can serve as substrates for DNA polymerases or not and thereby to put the specific predictions of the crystallographic and modelling studies (4,5) to the test.

In order to probe functional group interactions involving the O2 carbonyl of thymidine and deoxycytidine we first set out to prepare pyrimidine-like nucleoside triphosphate derivatives of dT and dC from which the O2 carbonyls had been selectively deleted without otherwise altering hydrogen bonding characteristics. Such derivatives should permit normal dA-dT (or dG-dC)-like Watson–Crick hydrogen bonding between the incoming triphosphate analogue and the corresponding template purine residue. We initially prepared a 4-pyrimidinone derivative to serve as a dT analogue (9), but elimination of the O2 carbonyl from the pyrimidine nucleobase resulted in a tautomeric change at the N3 nitrogen such that a hydrogen bond donor was converted to a hydrogen bond acceptor. Incorporation of this derivative into DNA sequences (paired with dA) resulted in destabilized duplexes (Lan,T. and McLaughlin,L.W., unpublished results), presumably due to apposition of the electron lone pairs of the N1 of dA and the N3 of the 4-pyrimidinone derivative. Similar concerns arise with the pyrimidine analogue of dC, in which the exocyclic amino group most likely exists in the imino tautomer. To eliminate these undesirable tautomers from the analogue triphosphates we designed C nucleosides based upon 2-pyridone and 2-aminopyridine ring systems (10). 2-Hydroxypyridine heterocycles generally prefer the keto or 2-pyridone tautomer and as such a 2-pyridone nucleoside should function as a bidentate

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hydrogen bond donor/acceptor and base pair with a complementary dA in much the same manner as does the native thymine derivative. Similarly, the 2-aminopyridine nucleoside should prefer the amino tautomer and engage in two complementary hydrogen bonds to a template dG residue. According to this reasoning the best analogues to test for acceptability to polymerases would be 5-(2′-deoxy-β-D-ribofuranosyl)-3-methyl-2-pyridone-5′-triphosphate (d*TTP) (**3**) and 2-amino-5-(2′-deoxy-β-D-ribofuranosyl)pyridine-5′-triphosphate (d*CTP) (**8**). Here we report our results on synthesis of these compounds and their ability to function with DNA polymerases.

A secondary goal of the present work was to construct by PCR a series of *tyr*T DNA molecules containing pyrimidines lacking the O2 substituent in order to investigate the role of the third (minor groove) hydrogen bond in G·C and diaminopurine·T base pairs as a potential determinant of sequence-specific recognition by ligands. Previous studies (11–14) have furnished abundant evidence of the importance of the purine 2-amino group as regards ligand binding, but have left open the question whether the influence of that group depends upon its forming a minor groove Watson–Crick hydrogen bond to the O2 of the complementary pyrimidine or not. Studies involving incorporation of the two novel bases from d*TTP and d*CTP, together with dA or dDAP and dG or dI in the other strand (8), should provide a definitive answer to the question.

MATERIALS AND METHODS

Chemicals and biochemicals

Ammonium persulphate, Tris base, acrylamide, bis-acrylamide, ultrapure urea, boric acid and tetramethylethylenediamine were from BDH. Photographic requisites were from Fuji. Bromophenol blue and xylene cyanol were from Sigma. [γ-^{32}P]ATP (6000 Ci/mmol) was obtained from Amersham International. The natural nucleotides dGTP, dATP, dCTP and dTTP were purchased from Pharmacia. Restriction endonuclease *Eco*RI, Taq polymerase (Promega) and T4 polynucleotide kinase (Pharmacia) were used according to the supplier's recommended protocols in the activity buffer provided. Synthetic oligonucleotides were obtained from the Protein and Nucleic Acid Chemistry Facility Laboratory in the Department of Biochemistry, Cambridge University. All other chemicals were analytical grade reagents and all solutions were prepared using doubly deionized Millipore filtered water. ^{1}H and ^{31}P NMR spectra were obtained on a Bruker DRX 300 spectrometer. ^{31}P NMR spectra were referenced to phosphoric acid.

Synthesis of nucleotide analogues

Analytical HPLC was performed on a 50 × 7.8 mm BioRad HRLC® MA7Q anion exchange column, running a linear gradient of 100% A (0% B) to 50% A (50% B) over 8 min at a flow rate of 4 ml/min, which provides an increase in ammonium bicarbonate concentration from 50 to 375 mM. Semi-preparative HPLC was performed on a Hichrom Partisil 10 SAX anion exchange column running a gradient of 100% C (0% D) to 10% C (90% D) over 18 min at a flow rate of 7 ml/min, providing a linear increase in KH$_2$PO$_4$ concentration from 50 to 635 mM. (Buffers: A, 50 mM ammonium bicarbonate; B, 0.7 M ammonium bicarbonate; C, 50 mM potassium dihydrogen phosphate, pH 7.0; D, 0.7 M potassium dihydrogen phosphate, pH 7.0.)

The buffer phosphate was removed by running the solution through a DEAE-Sephadex A-25 anion exchange column, washing with 100 mM triethylammonium bicarbonate (TEAB) and then eluting with 400 mM TEAB. The fractions containing product were pooled together, evaporated to dryness and converted into the sodium salt by dissolving in water and passing through a column of sodium Dowex 50 X 2 ion exchange resin.

Tetra-n-butylammonium pyrophosphate (15,16). Tetrasodium pyrophosphate decahydrate (13.4 g, 30 mmol) was converted to its pyridinium salt by dissolving it in water and passing through a column of pyridinium Dowex 50 X 2 ion exchange resin (100g, 200–400 mesh). The salt was eluted with five times the column volume of deionized water. The eluate was evaporated to dryness under reduced pressure. After dissolving the residue in dry pyridine, 26.4 ml (120 mmol) tri-*n*-butylamine were added and the resulting liquid was shaken until a clear solution resulted. Solvent was then removed under reduced pressure. The residue was co-evaporated with dry pyridine three times, dissolved in dry DMF and adjusted to a final volume of 60 ml of a 0.5 M solution. This solution is stable for at least two months when stored at 4°C.

General procedure for phosphorylation (17–19). The requisite nucleoside (0.5 mmol) and 1,8-bis(dimethylamino)naphthalene (Proton Sponge) (0.75 mmol, 1.5 equiv.) were dissolved in 3 ml trimethylphosphate (dried with 4A molecular sieves) and cooled to –20°C before phosphorus oxychloride (1.5–2 equiv.) was pipetted into the solution (suspension). The mixture was stirred with cooling for 3 h and the temperature was kept between –10 and –20°C. Aliquots of 5 ml 0.5 M pyrophosphate solution (in DMF, 5 equiv.) were added, followed by 0.5 ml tri-*n*-butylamine. The mixture was again stirred with cooling for 3 min. TEAB (30 ml, 0.4 M) was added and stirring continued at room temperature for 10 min. Volatiles were removed under reduced pressure and to the residue was added 30 ml diethyl ether. Liquid was removed by desiccation and the solid residue was washed with 30 ml diethyl ether. It was then dissolved in 20 ml water and loaded onto a DEAE-Sephadex A-25 anion exchange column (3 × 25 cm) for separation. Elution was conducted from 2L 0.1 M to 2L 0.7 M TEAB; the product eluted from the column near 0.35 M TEAB. Appropriate fractions were collected and volatiles removed under reduced pressure. The product was converted into the sodium salt by passing through a Dowex 50 X 2 ion exchange column (in the sodium form).

*5-(2′-Deoxy-β-D-ribofuranosyl-3-methyl-2-pyridone-5′-triphosphate (3) (d*TTP)* (Fig. 1). Phosphorylation of 57.2 mg (0.19 mmol) 2-benzyloxy-5-(2′-deoxy-β-D-ribofuranosyl)-3-methylpyridine (**1**) (10) with 60 mg Proton Sponge, 2 ml trimethyl phosphate, 35 µl phosphorus oxychloride, 2 ml 0.5 M tetra-*n*-butylammonium pyrophosphate and 0.2 ml tri-*n*-butylamine yielded 70 mg 2-benzyloxy-5-(2′-deoxy-β-D-ribofuranosyl)-3-methyl-pyridine-5′-triphosphate (**2**) as its sodium salt after column separation. This product was then dissolved in 10 ml water/ethanol (1:1 v/v), 5 mg 10% Pd/C was added and the mixture was stirred under a hydrogen atmosphere for 7.5 h. After removing catalyst and solvent the residue was purified by HPLC [retention time (RT) 9.8 min], resulting in 28.3 mg 5-(2′-deoxy-β-D-ribofuranosyl)-3-methyl-2-pyridone-5′-triphosphate (**3**). ^{1}H NMR in D$_2$O δ (p.p.m.): 7.56 (s, 1H, H6), 7.42 (s, 1H, H4), 4.92 (t, 1H, H1′), 4.48 (bs, 1H, H3′), 4.08 (bs, 1H, H4′), 4.02 (m, 2H, H5′), 2.08 (m, 2H, H2′), 2.0 (s, 3H, CH$_3$). ^{31}P NMR in D$_2$O δ (p.p.m.): –6.5 (m), –11.1 (m), –21.9 (m).
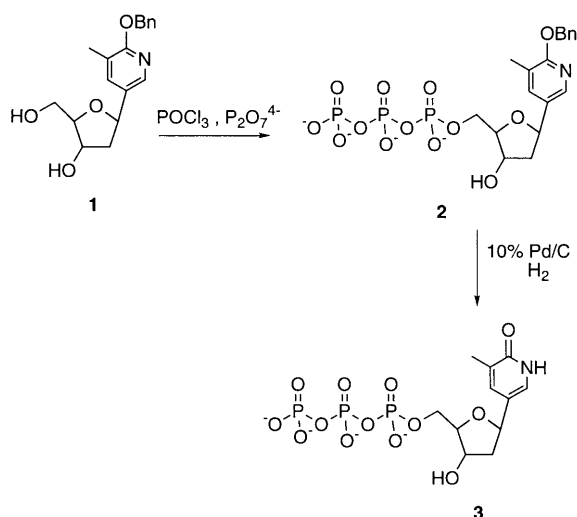
Figure 1. Reaction scheme for synthesis of d*TTP (**3**).

*2-Amino-5-(2′-deoxy-β-D-ribofuranosyl)pyridine-5′-triphosphate (8) (d*CTP)* (Fig. 2). A solution of 2-(benzoylamino)-5-(2′-deoxy-β-D-ribofuranosyl)pyridine (**4**) (130 mg, 0.41 mmol) (20) in 15 ml 40% methylamine was heated at 70°C for 26 h. The mixture was concentrated *in vacuo* and the residue dissolved in 30 ml dry methanol, 3 ml *N,N*-dimethylformamide dimethylacetal were added and the solution was refluxed overnight. After removing solvent the residue was purified on a silica gel column with 5–10% methanol in chloroform as eluent. This gave 77 mg (0.29 mmol) 2-(*N,N*-dimethylamidinyl)-5-(2′-deoxy-β-D-ribofuranosyl)pyridine (**6**). The product was phosphorylated with 90 mg Proton Sponge, 3 ml trimethyl phosphate, 53 µl phosphorus oxychloride, 3 ml 0.5 M tetra-*n*-butylammonium pyrophosphate and 0.3 ml tri-*n*-butylamine. The reaction mixture was separated on a DEAE-Sephadex column using the conditions

described before, appropriate fractions were collected and volatiles were removed under reduced pressure. The residue [2-(*N,N*-dimethylamidinyl)-5-(2′-deoxy-β-D-ribofuranosyl)-pyridine-5′-triphosphate (**7**)] was suspended in 20 ml concentrated ammonia and heated at 50°C for 4 h. Insoluble material was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was further purified by semi-preparative anion exchange HPLC (RT 9.8 min), resulting in 17.5 mg 2-amino-5-(2′-deoxy-β-D-ribofuranosyl)pyridine-5′-triphosphate (**8**) as its sodium salt. ^{1}H NMR in D_2O δ (p.p.m.): 8.99 (s, 0.5 H, not fully exchanged NH_2), 8.28 (bs, 1H, H6), 7.91 (dd, 1H, H4), 7.81 (d, 0.5H, H3, $J = 8.6$ Hz), 7.03 (d, 0.5H, H3, $J = 8.3$ Hz), 5.22 (dd, 1H, H1′, $J = 5.1$ Hz, $J = 11$ Hz), 4.87 (dd, 1H, H3′, $J = 6.7$ Hz, $J = 6.9$ Hz), 4.44 (bs, 1H, H4′), 3.81 (m, 2H, H5′), 2.51 (dd, 1H, $J = 5.3$ Hz, $J = 14$ Hz, H2a′), 2.15 (m, 1H, H2b′). ^{31}P NMR in D_2O δ (p.p.m.): –6.4 (d), –12.8 (d), –21.9 (m)

Primer 5′-labelling and purification

A Watson primer representing the first 20 nt of the top strand of *tyr*T DNA (21) having the sequence 5′-GTT ACC TTT AAT CCG TTA CG-3′ was 5′-end-labelled with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase according to a standard procedure (22). Thus, a 200 µl solution containing 4 µl 5.3 mM Watson primer, 167 µl TN buffer, 20 µl 10× T4 polynucleotide kinase buffer, 5 µl [γ-^{32}P]ATP (6000 Ci/mmol) and 4 µl T4 polynucleotide kinase was incubated for 40 min at 37°C and then heated at 70°C for 10 min. Loading dye (20 µl) was added and the solution was loaded onto a 10% polyacrylamide gel running at 270 V for 1.5 h. The labelled primer band was cut out and electroeluted into 10 M ammonium acetate solution at 140 V for 40 min. The labelled primer was precipitated from the ammonium acetate solution by adding 3 volumes of absolute ethanol and was further desalted by repeated ethanol precipitation. The primer was finally dissolved in 1 ml TN buffer to give a 5.8 µM solution, according to UV measurements.
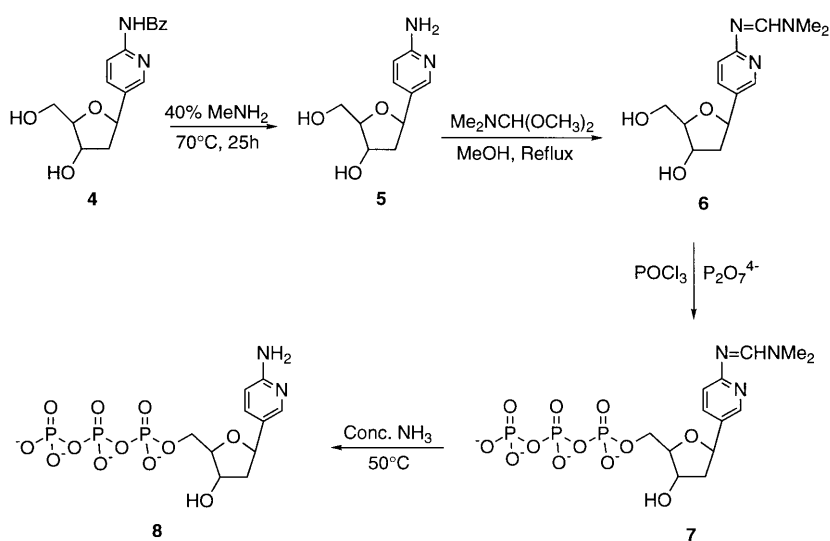

Figure 2. Reaction scheme for synthesis of d*CTP (**8**).

PCR protocol

In general the procedure used for PCR experiments was adapted from the standard protocol described previously (23,24). Reaction mixtures (50 µl) contained 10 ng *tyr*T(A93) template (the *Eco*RI–*Ava*I fragment) (5), 1 µM each primer [top (Watson) strand primer 5′-end labelled by prior incubation with [γ-^{32}P]ATP and polynucleotide kinase; bottom (Crick) strand primer 5′-GGG CTC GGG AAC CCC CAC CA-3′], 250 µM each natural nucleoside triphosphate, varying concentrations of either d*CTP or d*TTP as stated and 1 U Taq DNA polymerase. The PCR reactions were buffered with the commercially supplied solutions to final conditions of 50 mM KCl, 10 mM Tris–HCl, pH 9.0, 1.5 mM MgCl$_2$ and 0.1% Triton X-100. In order to prevent non-specific primer annealing the reaction mixtures were heated to 60°C prior to addition of the appropriate DNA polymerase. To each tube ~50 µl mineral oil was then added to prevent evaporation. The samples were heated to 94°C for 3 min. Following this initial denaturation step 20 amplification cycles were performed consisting of 94°C denaturation for 1 min, a 37°C primer annealing step for 2 min and a 72°C polymerization step for 10 min. After completion of the last amplification cycle the samples were heated to 72°C for another 10 min to complete unfinished chains, following which the reactions were cooled slowly to allow efficient annealing of complementary DNA strands. Two additional 5 min steps were included, one at 55 and one at 37°C. After the entire process was complete the mineral oil was removed by extracting with chloroform, 5 µl of loading dye were added to each sample and 8 µl were then loaded onto a 6% non-denaturing polyacrylamide gel to analyse the products.

Primer extension experiments

To investigate whether d*CTP and d*TTP would serve as substrates for particular DNA polymerases or not two templates, a 25mer having the sequence 5′-T CAG TCG TAA CGG ATT AAA GGT AAC-3′ and a 24mer with the sequence 5′-CGA CCG TAA CGG ATT AAA GGT AAC-3′ were used. The difference between these two templates lies only in the bases extending beyond the region complementary to the primer (the 25mer contains all four bases, while the 24mer lacks T and therefore will not direct incorporation of dA into the product). The complementary 20 nt Watson primer was labelled with ^{32}P at its 5′-end as described above.

Aliquots of 0.5 µM each appropriate template, primer, 100 µM each natural nucleoside triphosphate and 1 U DNA polymerase were used in a 50 µl reaction mixture buffered with the commercially supplied solutions. In order to prevent non-specific primer annealing all reactions were heated to 80°C for 5 min and then cooled to ambient temperature prior to addition of the appropriate DNA polymerase. Mixtures were incubated at 37°C overnight, then 5 µl loading dye were added to each sample and 8 µl were loaded onto a 20% denaturing polyacrylamide gel to analyse the results.

Gel electrophoresis and autoradiography

For primer extension reactions the products were analysed by PAGE under denaturing conditions (0.3 mm, 20% acrylamide gels containing 7 M urea and TBE). Electrophoresis was carried out at 60 W in 1× TBE buffer for ~4 h. After electrophoresis was complete the gel was fixed in 10% (v/v) acetic acid containing 10% methanol (v/v) for 15 min. For PCR reactions the products were analysed on polyacrylamide gels under non-denaturing conditions (0.3 mm, 6% acrylamide). Electrophoresis was carried out at 900 V in 1× TBE buffer for ~2 h. The gel was then transferred to Whatman 3MM paper, dried on a gel drier under vacuum at 80°C and exposed to a PhosphorImager screen and/or an X-ray film (Fuji R-X) for ~48 h.

Quantification by storage phosphorimaging

A Molecular Dynamics 425E PhosphorImager was used to collect data from the storage screens exposed to dried gels overnight at room temperature (25). Baseline-corrected scans were analysed by integrating all the densities between two selected boundaries using ImageQuant v.3.3 software.

RESULTS

Chemical syntheses

In the initial synthesis of the 2′-deoxycytidine analogue **4** a benzoyl group was used to protect the 2-amino substituent, which could be removed with either 40% methylamine at 70°C for 25 h (20) or concentrated ammonia at 60°C for 3 days (10). Unfortunately, under these conditions the triphosphate will be destroyed. Therefore, a new protecting group for the exocyclic amino substituent had to be sought. We used anion exchange HPLC to examine the stability of dNTPs under varying reaction conditions. In 40% methylamine at 70°C 40% of the dNTP decomposed after 1 h, 70% after 5 h and >95% had been lost after 17 h, but at ambient temperature with potassium carbonate in methanol/water (5:1), 40% methylamine, and 0.5 M TEAB solutions dNTPs were stable for >24 h. In concentrated ammonia at ambient temperature dNTPs are stable for long periods of time, but at 50°C 25% decomposition was observed after 17 h. The dNTPs treated for 1 h in 2 M TEAB were completely hydrolysed and 50% was lost after 4 h in 1 M TEAB. With 10% Pd/C in ethanol and 8 h at ambient temperature about one third decomposed. We tried trifluoroacetyl as an amine protecting group but were unable to remove it from the 3′,5′ positions selectively. Eventually we chose an amidine (Me$_2$NCH=) to protect the exocyclic amino group (26), as it can be incorporated easily under mild conditions and readily removed by concentrated ammonia at 50°C. After deprotection and separation of the dNTP product by DEAE-Sephadex column chromatography the phosphorylated product contained a small amount of an unidentified by-product which was removed by HPLC purification using an anion exchange column.

We note that the pK_a of d*C is 6.26, compared with 4.3 for natural 2′-deoxycytidine (20), so that at pH 7 d*CTP exists in two forms (protonated and unprotonated) in approximately equal proportions, as revealed by NMR analyses of the protons on the base, which appear as two sets of signals at different positions in the ^{1}H NMR spectrum.

PCR

Initial experiments quickly revealed that substituting d*CTP for dCTP or d*TTP for dTTP in the PCR amplification procedure under standard conditions using Taq polymerase failed to yield detectable amounts of full-length product. Other conditions were explored and the concentration of each analogue was varied over a wide range in separate experiments, but little or no PCR product
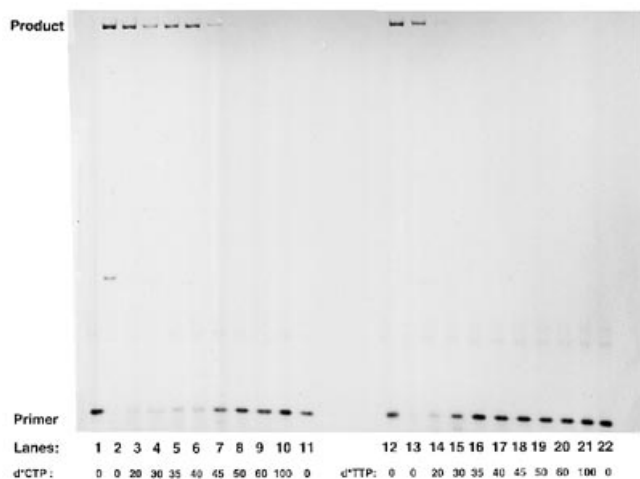
Figure 3. Formation of PCR products in the presence of d*CTP (lanes 3–10) or d*TTP (lanes 14–21). Lanes 1, 11, 12 and 22 contained the primer alone. Lanes 2 and 13 show the results of the control PCR without any nucleotide analogue added.

Figure 4. Inhibition of the PCR reaction by d*CTP (○) and d*TTP (△). The ordinate shows the fractional conversion of added primer to full-length PCR product.

Figure 5. Failure of d*TTP to substitute for dTTP in the primer extension reaction with Taq polymerase. The position of the primer band is shown in the lane at the extreme left; the direction of migration was from the top to the bottom. The 25mer template was used. Each set of three lanes shows the products resulting from incubation with the natural nucleotides indicated in the box below them, with d*TTP added as indicated above. The bands corresponding to products with 1–5 nt added are identified at the left.

was obtained. Other heat-stable enzymes were also tested, but to no avail. Suspecting that the compounds might be inhibitory to polymerase activity, they were then added to standard reaction mixtures (containing all four natural nucleoside triphosphates at 250 µM each) and both analogue dNTPs were found to cause progressive concentration-dependent suppression of full-length product synthesis without accumulation of any discrete bands corresponding to truncated chains (Fig. 3). The d*TTP derivative proved to be a more powerful inhibitor than d*CTP, causing 50% inhibition at ~20 µM, while the same extent of inhibition by the cytidine derivative required a slightly higher concentration of 40 µM (Fig. 4). Given the pK_a of the deoxycytidine analogue (6.26), this factor of two difference in inhibitory concentrations may simply reflect the available concentration of the unprotonated form of d*CTP.

Primer extension reactions

The observed inhibition of polymerase activity might result from simple competition between analogues and natural substrates for the active site, from analogue incorporation in the fashion of a chain terminator, or from some non-specific inhibitory action on the polymerase–template–primer complex with or without template-directed false nucleotide incorporation. To distinguish between these various possibilities we designed two template sequences, each complementary to the same primer used in the PCR experiments but carrying a 4 or 5 nt 5′-overhang, so as to permit extension of the primer by polymerase action. The results of an experiment with d*TTP and Taq polymerase are shown in Figure 5. The first thing to note is that the product in each control lane (no d*TTP added) consists of two bands, of which the stronger is 1 nt longer than the predicted product strictly complementary to the template overhang. This addition of an 'extra' nucleotide is a peculiarity observed with several polymerases under artificial conditions involving long incubation times, often exacerbated when dATP is present (27). Interestingly, the presence of d*TTP tends to suppress addition of this 'extra' nucleotide such that the predominant product corresponds to
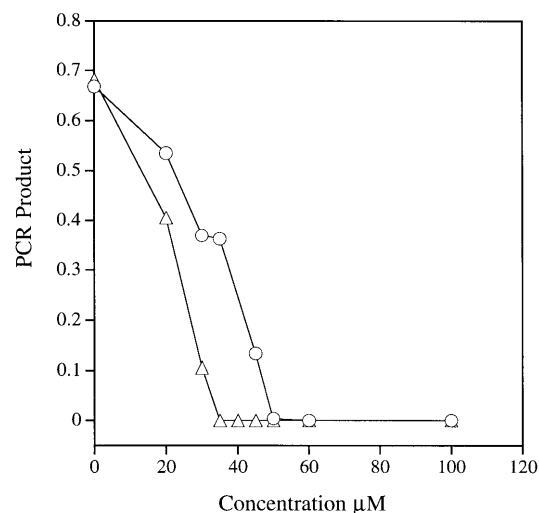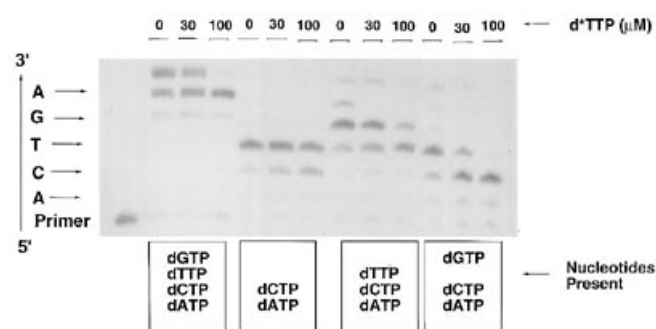
correct template-directed incorporation of the natural nucleotides present. Evidently there is no significant inhibition of the normal extension reaction under these conditions, though the rate of nucleotide addition may be slowed considerably. Equally evidently, d*TTP cannot substitute for dTTP, because whenever d*TTP is added and dTTP is omitted the length of the product corresponds to one terminated immediately before the T incorporation step, i.e. with A and C incorporated plus, sometimes, an 'extra' nucleotide. There is absolutely no sign of an increase in the length of any product when reactions containing 30 or 100 µM d*TTP are compared with control reactions lacking the analogue, as would be the case if d*TMP had been incorporated and then served as a chain terminator. Similar results were obtained in parallel experiments with d*CTP (data not shown).

Taq polymerase is characterized by low stringency as regards incorporation of unnatural nucleotides, so the experiments were
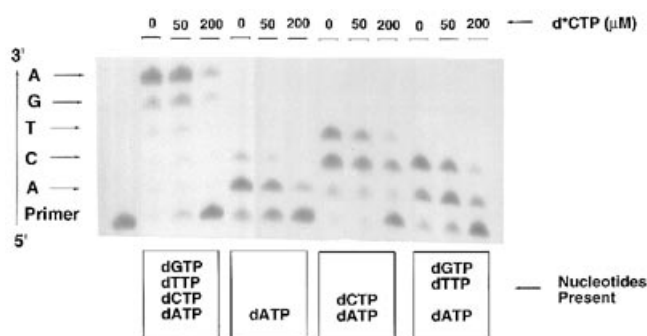
Figure 6. Failure of d*CTP to substitute for dCTP in the primer extension reaction with the Klenow fragment of DNA polymerase. Details of the experiment as described in the legend to Figure 5.

Figure 7. Failure of nucleoside triphosphate analogues to serve as substrates for Taq polymerase in the primer extension reaction with a 24mer template. Details of the experiment as for Figure 5.

repeated with the Klenow fragment of *E.coli* DNA polymerase I (Fig. 6). Again, in the control reactions (no d*CTP) some incorporation of an 'extra' nucleotide at the ends of truncated products is evident, though generally not nearly so marked as was observed with Taq polymerase, and the magnitude of the effect is more or less proportional to the number of substrate nucleotides present. This suggests that the 'extra' nucleotide may arise at least partially from trace contamination of one nucleoside triphosphate with another, though it remains entirely possible that it is simply nucleotide misincorporation, which might occur over prolonged periods, particularly at a lower than optimum temperature that might alter fidelity. Nevertheless, the phenomena noted with Taq polymerase are largely confirmed: there is now substantial inhibition of normal chain extension by 200 µM d*CTP, but in no case is there evidence of the analogue substituting for dCTP or leading to formation of a product longer than that seen in the corresponding reaction performed without d*CTP. Similar results were obtained in parallel experiments with d*TTP (data not shown).

In an effort to alleviate any ambiguity arising from 'extra' nucleotide incorporation by Taq polymerase the primer extension experiment was redesigned with a slightly shorter template having a 4 nt overhang devoid of thymine residues, so that dATP could be omitted from the reaction mixture (Fig. 7). In this experiment a much weaker 'extra' band or bands appeared in control reactions, fully consistent with slight contamination between nucleotides, and the effects of d*CTP and d*TTP were unchanged: there was little or no inhibition of formation of the expected product (though consistent suppression of 'extra' bands) and each analogue completely failed to substitute for the natural nucleotide in the primer extension reaction.

DISCUSSION

In comparing the d*CTP and d*TTP analogues with the natural dCTP and dTTP nucleotides there are two major differences. First, the normal C–N glycosidic bond has been changed to a C–C bond, which, being slightly longer, may alter the conformation around the ribose ring due to different steric and electronic interactions between segments of the nucleotides and polymerases. Benner and colleagues (28) have demonstrated that this kind of modification is accepted by T7 RNA polymerase using a C–C ribonucleotide analogue, and a variety of C nucleoside com-
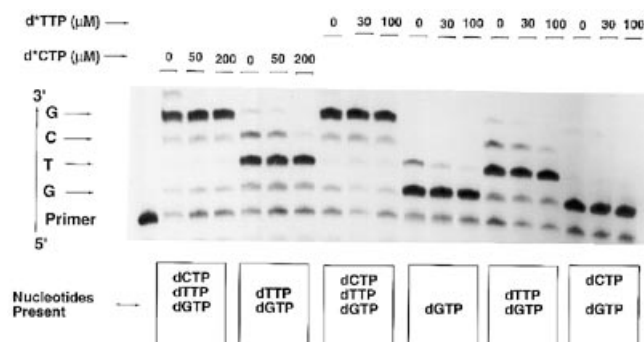
pounds (including several antibiotics) have long been known whose biological properties may include facile phosphorylation to the triphosphate level leading to extensive incorporation into nucleic acids *in vivo* (29). So the altered glycosidic bond seems unlikely to be responsible for the observed inhibitory effects. The second modification in the analogues is deletion of the 2-keto group from the pyrimidine ring. It is known that both hydrogen bond acceptors, the O2 atoms of pyrimidines and the N3 atoms of purines, lie in overlapping positions in the minor groove for all four base pairs (4) and these functional groups are invariant within normal sequences but can be differently positioned for non-Watson–Crick base pairs. Recently Steitz and colleagues have published a crystal structure of Taq polymerase containing DNA at the active site (4). They compared the structure of the duplex DNA in the complex with those of A- and B-form DNA and showed that it displays characteristics of both A and B forms, but is not identical to either. The minor groove is wider than that of B-form DNA and is accessible to protein side chains that can hydrogen bond to the O2 atoms of pyrimidines and N3 atoms of purines. They note that specific recognition and positioning of these base hydrogen bond acceptors by protein side chains may be an important mechanism by which polymerases enhance the fidelity of Watson–Crick base pairing and ensure correct nucleotide incorporation at the polymerase active site.

Our results show that pyrimidine nucleotides lacking the 2-keto group are not substrates for the polymerases tested and, indeed, inhibit activity of the enzymes. This observation is entirely consistent with the view of Eom *et al.* (4) and Georgiadis *et al.* (5) that the 2-keto group is vital for polymerase recognition. The crystallographic and modelling studies of these authors led in each case to the observation of a close contact between the O2 carbonyl and an amino acid side chain which strongly suggests a critical contact, but in the absence of studies of function cannot confirm it. The present work provides the necessary functional study, indicating that such a contact is indeed important for polymerase activity. It is possible that the 2-keto group may assist the polymerase to adopt a properly folded form, by forming a hydrogen bond(s) with the protein side chain(s) and bringing appropriate functional groups into the correct positions in the active site to catalyse formation of a new internucleotide bond and facilitate release of inorganic pyrophosphate from the complex. The inhibitory activity of these analogues would then be the result of the absence of a necessary conformational change and/or

improper folding at the active site caused by the presence of nucleotides lacking the 2-keto group. The unnatural triphosphate will bind in what amounts to the 'right' place but block formation of the activated state and prevent elongation of the chain.

Recently Moran *et al.* (30) have incorporated a difluorotoluene nucleoside into DNA by solid phase synthesis and used it as a template for DNA polymerase extension. They noted that this analogue can be copied as if it were thymidine. On this evidence they suggested that the shape of the DNA base may be the chief property by which DNA replicating enzymes select the correct nucleosides to insert into a growing strand, and shape might be more important than hydrogen bonding in explaining the extreme accuracy by which DNA polymerases copy DNA. In another paper (31) Kool and co-workers have demonstrated that the difluorotoluene nucleoside triphosphate (dFTP) is a substrate for Klenow fragment polymerase. dFMP was incorporated into newly synthesized DNA opposite A in the template. Again, it was argued that shape complementarity is likely to be the most important criterion and hydrogen bonds might even be eliminated if the steric requirements are met. However, a more recent paper by Evans and Seddon (32) provides evidence that 2,4-difluoro-toluene can act as a hydrogen bond donor and acceptor, which contradicts the hypothesis of Kool *et al.* and would tend to support the conclusions of the present paper in terms of the relevance of hydrogen bonding to fidelity. Calculations (33) and measurement (34) reveal that although the hydrogen bonding ability of difluorotoluene in chloroform is small, it remains difficult to predict that ability in a protein environment such as the active site of a polymerase. Based on our results presented here, together with a wider examination of pyrimidine analogues as polymerase substrates, it appears that the right hydrogen bonding properties are important both for a compound to serve as a substrate for DNA polymerase and the speed with which the enzyme can use it to extend the growing chain. Even if the canonical Watson–Crick hydrogen bonds are not absolutely required for high fidelity of replication, the ability to engage in some kind of hydrogen bonding between the polymerase and the groups at position 2 (for pyrimidine) or 3 (for purine) appears vital. It remains to be determined whether other pyrimidine analogues with altered hydrogen bonding capability at position 2 are acceptable as polymerase substrates, such as variants of d*CTP and d*TTP with fluorine at the 2 position. The present analogues might also be useful in crystallographic studies of the polymerase active site if crystals of complexes can be grown. Sadly, the goal of investigating the importance of the third (minor groove) hydrogen bond in a G-C or DAP-T base pair for ligand binding interactions must be addressed in some other way. There are indications that if the 2-keto function of cytosine is lost the 2-amino group of guanine may also need to be eliminated in order to form a stable base pair of the Watson–Crick type (35).

REFERENCES

1 Kornberg,A. and Baker,T.A. (eds) (1992) *DNA Replication*, 2nd Edn. Freeman, San Francisco, CA.
2 Joyce,C.M. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 1619–1622.
3 Joyce,C.M. and Steitz,T.A. (1994) *Annu. Rev. Biochem.*, **63**, 777–822.
4 Eom,S.H., Wang,J.M. and Steitz,T.A. (1996) *Nature*, **382**, 278–281.
5 Georgiadis,M.M., Jessen,S.M., Ogata,C.M., Telesnitsky,A., Goff,S.P. and Hendrickson,W.A. (1995) *Structure*, **3**, 879–892.
6 Bailly,C. and Waring,M.J. (1995) *Nucleic Acids Res.*, **23**, 885–892.
7 Bailly,C., Payet,D., Travers,A.A. and Waring,M.J. (1996) *Proc. Natl. Acad. Sci. USA*, **93,** 13623–13628.
8 Jennewein,S. and Waring,M.J. (1997) *Nucleic Acids Res.*, **25,** 1502–1509.
9 Rajur,S.B. and McLaughlin,L.W. (1992) *Tetrahedron Lett.*, **33**, 6081–6084.
10 Hsieh,H.-P. and McLaughlin,L.W. (1995) *J. Org. Chem.*, **60**. 5356–5359.
11 Bailly,C., Møllegaard,N.E., Nielsen,P.E. and Waring,M.J. (1995) *EMBO J.*, **14,** 2121–2131.
12 Bailly,C. and Waring,M.J. (1995) *J. Am. Chem. Soc.*, **117,** 7311–7316.
13 Bailly,C., Waring,M.J. and Travers,A.A. (1995) *J. Mol. Biol.*, **253,** 1–7.
14 Bailly,C., Braña,M. and Waring,M.J. (1996) *Eur. J. Biochem.*, **240,** 195–208.
15 Moffatt,J.G. (1964) *Can. J. Chem.*, **42**, 599–603.
16 Chen,J.T. and Benkovic,S.J. (1983) *Nucleic Acid Res.*, **11**, 3737–3751.
17 Ludwig,J. (1981) *Acta Biochem. Biophys. Acad. Sci. Hung.*, **16**, 131–133.
18 Ruth,J.L. and Cheng,Y.C. (1981) *Mol. Pharmacol.* **20**, 415–422.
19 Seela,F., Muth,H.-P. and Roling,A. (1991) *Helv. Chim. Acta*, **74**, 554–556.
20 Hildbrand,S. and Leumann,C. (1996) *Angew. Chem. Int. Edn English*, **35**, 1968–1970.
21 Drew,H.R., Weeks,J.R. and Travers,A.A. (1985) *EMBO J.*, **4**, 1025–1032.
22 Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
23 Sayers,E.W. and Waring,M.J. (1993) *Biochemistry*, **32**, 9094–9017.
24 Marchand,C., Bailly,C., McLean,M.J., Moroney,S.E. and Waring,M.J. (1992) *Nucleic Acid Res.*, **20**, 5601–5606.
25 Johnston,R.F., Pickett,S.C. and Barker,D.L. (1990) *Electrophoresis*, **11**, 355–360.
26 McBride,L.J., Kierzek,R., Beaucage,S.L. and Caruthers,M.H. (1986) *J. Am. Chem. Soc.*, **108**, 2040–2048.
27 Newton,C.R. and Graham,A. (eds) (1994) *PCR, Introduction to Biotechniques Series*. Bios Scientific Publications, Oxford, UK, p. 13.
28 Piccirilli,J.A., Moroney,S.E. and Benner,S.A. (1991) *Biochemistry*, **30**, 10350–10356.
29 Suhadolnik,R.J. (1970) *Nucleoside Antibiotics*. Wiley, New York, NY.
30 Moran,S., Ren,R.X.F., Rumney, S. and Kool,E.T. (1997) *J. Am. Chem. Soc.*, **119**, 2056–2057.
31 Moran,S., Ren,R.X.-F. and Kool,E.T. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 10506–10511.
32 Evans,T.A. and Seddon,K.R. (1997) *J. Chem. Soc. Chem. Commun.*, 2023–2024.
33 Howard,J.A.K., Hoy,V.J., O'Hagan,D. and Smith,G.T. (1996) *Tetrahedron*, **52**, 12613–12622.
34 Schweitzer,B.A. and Kool,E.T. (1995) *J. Am. Chem. Soc.*, **117**, 1863–1872.
35 Hildbrand,S., Blaser,A., Parel,S.P. and Leumann,C.J. (1997) *J. Am. Chem. Soc.*, **119**, 5499–5511.