1144–1149 *Nucleic Acids Research, 1998, Vol. 26, No. 5*

Comparative mutagenicities of *N*^{6}-methoxy-2,6-diaminopurine and *N*^{6}-methoxyaminopurine 2′-deoxyribonucleosides and their 5′-triphosphates

Fergal Hill, David M. Williams[+], David Loakes and Daniel M. Brown*

Medical Research Council, Laboratory of Molecular Biology and Centre for Protein Engineering, Hills Road, Cambridge CB2 2QH, UK

Received December 16, 1997; Revised and Accepted January 19, 1998

ABSTRACT

The structure of the deoxyribonucleoside derived from *N*^{6}-methoxy-2,6-diaminopurine (dK) was examined by NMR. The methoxyamino residue was found predominantly in the imino rather than the amino tautomer (ratio: 9:1 in DMSO). The nucleoside proved to be a potent transition mutagen in *Escherichia coli*, in contrast to the closely related nucleoside derived from the analogue *N*^{6}-methoxyaminopurine (dZ), which was only weakly mutagenic. The 5′-triphosphate derivatives, dKTP and dZTP, were synthesized; *Taq* polymerase incorporated dKTP opposite both T and, less well, opposite dC in template DNA. Both analogue triphosphates produced transition mutations when added to PCR reactions. In each case, there was a large excess of AT→GC compared to GC→AT mutations (ratios were 15:1 for dKTP and 10:1 for dZTP). Polymerase extension times in each cycle had to be extended, consistent with a decreased rate of DNA synthesis in the presence of the analogues. This and the mutagenic ratios are discussed in terms of *syn-anti* inversion of the methoxyl group.

INTRODUCTION

The bases in nucleic acids are tautomeric systems. A variety of studies indicate that, in the standard bases, the keto and amino tautomers predominate overwhelmingly under normal conditions (reviewed in 1) but the minor tautomers have been implicated in giving rise to transition mutations (2,3). Chemical modification of the bases, however, and in particular the addition of an electronegative atom to the heterocycles, can produce bases with significantly altered tautomeric ratios (4 and references therein). This phenomenon underlies the mutagenicity of a number of chemicals.

Methoxyamine is mutagenic; modification of both cytosine and, to a much lesser extent, adenine residues, occurs. Much work attests to the fact that *N*^{4}-methoxycytosine residues are formed and account for the resulting transition mutations (reviewed in 5). The addition of a methoxyl group to the N^6 position of dA or of 2,6-diaminopurine-2′-deoxyribonucleoside generates a pair of nucleosides, dZ and dK, respectively (Fig. 1), both of which can base pair with either T (as the amino tautomer) or with C (as the imino tautomer). A complication exists however, because the methoxyl group can exist in two configurations, either *syn* or *anti* with respect to N^1 (Fig. 1).

In previous studies, we have examined the duplex stability of oligonucleotides containing dZ and dK (6,7) and the ability of oligonucleotides containing dK to prime DNA synthesis by *Taq* polymerase (8). In this work, we describe NMR studies on dK and the mutagenicity of both nucleosides in *Escherichia coli*. Furthermore, the 5′-triphosphate derivatives, dKTP and dZTP, have been synthesised and used to generate mutations in PCR reactions. A scheme to explain the mutagenesis data obtained is outlined.

MATERIALS AND METHODS

General methods

^{1}H-NMR spectra were recorded in d^6-DMSO on a Bruker DRX 300 spectrometer. Double pulsed field gradient spin echo NOE spectra (DPFGSE NOE) were carried out as described by Stott *et al* (9). Ultraviolet spectra were recorded on a Perkin Elmer Lambda 2 spectrophotometer, and samples were dissolved in water. Tlc was carried out on pre-coated F_{254} silica plates and column chromatography with Merck kieselgel 60. Oligonucleotides were synthesised on an Applied Biosystem ABI 380B synthesiser using the normal synthesis cycle. They were purified by polyacrylamide gel electrophoresis using 20%/7 M urea gels, followed by extraction with 0.5 M ammonium acetate/1 mM EDTA solution and then desalted using a Sephadex G-25 column (NAP-10, Pharmacia). Standard recombinant DNA methods were carried out as described (10).

Synthesis

*9-(2-Deoxy-*β*-D-ribofuranosyl)-N*^{6}*-methoxy-2,6-diaminopurine (dK).* The nucleoside was prepared as previously described (6).

^{1}H-nmr δ (p.p.m.) (DMSO) 2.12–2.17 (1H, m, H2′), 2.42–2.49 (1H, m, H2′), 3.33–3.55 (2H, m, H5′, H5″), 3.72 (3H, s, OCH_3), 3.75–3.78 (1H, m, H4′), 4.28–4.4.31 (1H, H3′), 4.97 (0.9H, t,

*To whom correspondence should be addressed. Tel: +44 1223 248011; Fax: +44 1223 412178; Email: dmb@mrc-lmb.cam.ac.uk

Present address: [+]Department of Chemistry, University of Sheffield, Sheffield S3 7HF, UK

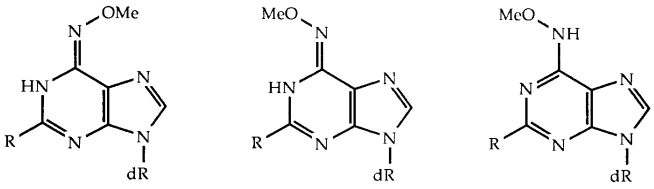

Figure 1. Structure of dK (R = NH_2) and dZ (R = H) nucleosides showing the *syn* and *anti* conformations (left, centre) and the imino/amino (centre, right) tautomers.

5′-OH), 5.08 (0.1H, t, 5′-OH), 5.21 (0.9H, d, 3′-OH), 5.22 (0.1H, d, 3′-OH), 6.03 (0.9H, t, J = 6 Hz, H1′), 6.18 (0.1H, t, H1′), 6.49 (2H, s, NH_2), 7.71 (0.9H, s, H8), 7.98 (0.1H, s, H8), 9.78 (0.9H, s, NH), 10.40 (0.1H, s, NH).

9-(2-Deoxy-β-D-ribofuranosyl)-N^6-methoxy-2,6-diaminopurine-5′-triphosphate, (dKTP). The dK nucleoside (29.6 mg, 0.1 mmol) was dried *in vacuo* over P_2O_5 at 80°C overnight, then suspended in dry trimethylphosphate (0.25 ml) under argon. The solution was then cooled to 0°C and then phosphoryl chloride (12 µl, 0.13 mmol) added and the solution was stirred at 0°C for 70 min. A solution of bis-tributylammonium pyrophosphate (0.5 M in anhydrous DMF, 0.5 ml) was then added to the reaction followed by tributylamine (0.1 ml) and DMF (0.2 ml), and the solution stirred rapidly for 7.5 min. To this was added triethylammonium bicarbonate solution (0.1 M, pH 7.5, 5 ml) and the reaction stirred for a further hour. The reaction was then diluted with water (30 ml) and applied to a column of Sephadex A25 (220×26 mm) in 0.05 M triethylammonium bicarbonate solution (pH 7.5). The column was then eluted with a linear gradient over 30 min of triethylammonium bicarbonate solution (1 l each of 0.05–0.8 M) at 4°C. The triphosphate eluted between 0.5 and 0.68 M buffer. The appropriate fractions were pooled and evaporated, coevaporated with methanol and then dissolved in water (10 ml). The product was further purified by reverse phase HPLC using a Waters 300 × 7.8 mm C18 semi-preparative column and a linear gradient of 0–4.5% acetonitrile in 0.1 M triethylammonium bicarbonate solution (pH 7.5) over 30 min. After evaporation of the buffer the pure triphosphate *tetraki*striethylammonium salt was isolated. Yield 446 A_{260} (pH 7), 0.043 mmol, 43%. The triphosphate was converted into the sodium salt by passage through a Dowex (Na^+) ion exchange column. ^{31}P-NMR δ (p.p.m.) (D_2O) –10.37 (d, γ-P), –10.89 (d, α-P), –23.62 (t, β-P).

9-(2-Deoxy-β-D-ribofuranosyl)-N^6-methoxyaminopurine-5′-triphosphate, (dZTP). N^6-Methoxy-2′-deoxyadenosine (6), (58 mg, 0.2 mmol) was dissolved in dry trimethylphosphate (1.0 ml) by heating under argon. The flask was cooled in an ice-bath and the triphosphate was obtained according to the synthesis of dKTP using phosphoryl chloride (22 µl, 0.24 mmol) and a mixture of bis-tributylammonium pyrophosphate (0.5 M in anhydrous DMF, 1.0 ml), tributylamine (0.2 ml) and anhydrous DMF (0.4 ml). The pure triphosphate as its *tetraki*striethylammonium salt was eluted from DEAE Sephadex A-25 between 0.7 and 0.75 M TEAB as described for dKTP (61 mg, 0.066 mmol, 33%). The triphosphate was converted into its sodium salt by passage through a Dowex (Na^+) ion exchange column. ^{31}P-NMR δ (p.p.m.) (D_2O) –7.12 (d, γ-P), –10.79 (d, α-P), –22.15 (t, β-P).

9-(3,5-O-Tetraisopropyldisiloxane-1,3-diyl-2-deoxy-β-D-ribofuranosyl)-N^6-methoxy-2,6-diaminopurine. To a solution of dK (100 mg, 0.34 mmol) in pyridine (3 ml) was added 1,3-dichlorotetraisopropyldisiloxane (110 mg, 0.35 mmol) and the solution stirred at room temperature overnight. The solvent was removed and the product dissolved in chloroform, extracted (aqueous sodium bicarbonate) and evaporated to a gum which was chromatographed ($CHCl_3$/4% MeOH) to give the product as a foam. Yield 50 mg. ^{1}H-NMR δ (p.p.m.) (DMSO) 0.94–1.06 (28H, m, 4 × ^{i}Pr), 2.42–2.61 (2H, m, H2′, H2″), 3.34 (2H, br, H5′, H5″), 3.72 (3H, s, OCH_3), 3.75–3.89 (1H, m, H4′), 4.640–4.66 (1H, m, H3′), 5.79 (1H, br, H1′), 6.51 (2H, br. s, NH_2), 7.68 (1H, br, H8), 9.84 (1H, br. s, NH).

^{1}H-NMR δ (p.p.m.) ($CDCl_3$) 0.89–1.09 (28H, m, 4 × ^{i}Pr), 2.55–2.69 (2H, m, H2′, H2″), 3.84–3.89 (1H, m, H4′), 3.93 (3H, s, OCH_3), 3.96–4.06 (2H, m, H5′, H5″), 4.80–4.83 (1H, m, H3′), 4.98 (2H, br. s, NH_2), 6.15–6.18 (1H, m, H1′), 7.76 (1H, s, H8).

Oligonucleotide primed incorporation assay for dKTP

A series of 10 µl reactions were performed for each of four DNA polymerases: *Taq*, *E.coli* DNA polymerase I, Klenow fragment and the exonuclease-free version of Klenow fragment (New England Biolabs). Each reaction contained 1 pmol of a 24mer oligonucleotide: 5′-GATCTGGTCATAGCTGTTTCCTGT annealed to 0.33 pmol of a complementary 20mer oligonucleotide 5′-ACAGGAAACAGCTATGACCA which was labelled at the 5′-end with ^{32}P, and 1 µl of 10× buffer [for *Taq* polymerase: 500 mM KCl, 100 mM Tris (pH 9) at room temperature, 1% Triton-X 100, 15 mM $MgCl_2$; for the other three enzymes: 50 mM $MgCl_2$, 100 mM Tris (pH 7.5) at room temperature, 75 mM DTT]. Normal dNTPs in various combinations (see Fig. 3) were added, as required, to 50 µM final concentration; dKTP was used at either 50 or 200 µM final concentration. A single unit of the appropriate polymerase was added to start each of the reactions; these were incubated for 15 min (at 72°C under mineral oil for *Taq*, at 37°C for *Pol*I and its derivatives) before being terminated by the addition of 5 µl of termination mix (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF). The reactions were heated to 70°C for 10 min before electrophoresis on a 20% polyacrylamide/7 M urea gel, which was then exposed to autoradiography film.

Bovine DNA synthesis assay

The incorporation of [α-^{32}P]dCTP into DNA was determined in a series of 50 µl reactions each containing: 5 µl of 10× PCR buffer [500 mM KCl, 100 mM Tris (pH 9) at room temperature, 1% Triton-X 100, 15 mM $MgCl_2$], 12.5 µg of activated bovine thymus DNA, 1 U of *Taq* DNA polymerase (Promega), [α-^{32}P]dCTP (50 µM), and various combinations of dATP, dGTP, TTP and dKTP (each at 50 µM). After 30 min incubation at 72°C, the reactions were stopped by the addition of 10 µl of 100 mM EDTA and placed on ice. Radioactively labelled DNA was collected on DE-81 filters, which were washed five times in 125 mM Na_2HPO_4 (pH 7) and then dried before scintillation counting (11).

Polymerase chain reactions

The *E.coli supF* gene in the plasmid pCDM8 (available from Invitrogen) was used as a template and amplified with the oligonucleotides 5′-CAGCTGGATTACCGCGGTCT and 5′-CACACACAAGCAGGGAGCAG as primers. Each 50 µl reaction contained 5 ng of template DNA linearised at its unique *Hin*dIII site,

50 pmol of each primer, 50 µM of each of the four natural dNTPs, 5 µl of 10× *Taq* buffer and 2.5 U of *Taq* polymerase. Stock solutions of dKTP and dZTP were used to obtain final concentrations of 50, 100, 200, 300, 400 and 500 µM as required. The reactions were overlaid with 35 µl of mineral oil and thermal cycling was carried out on a Techne PHC3 apparatus. Cycling conditions were: denaturation at 96°C for 2 min (during which time *Taq* polymerase was added), followed by 30 cycles of: denaturation at 96°C for 5 s, annealing at 50°C for 5 s; extension at 72°C for variable times. After the last cycle, a final extension at 72°C for 5 min completed the reactions. For dKTP, extension times at 72°C were 0, 2 and 4 min; for dZTP, 0, 1 and 2 min. Reaction products were analysed by electrophoresis on 2% agarose TAE gels. A control reaction, without template, was run with each set of reactions.

Cloning and sequencing of PCR products

The PCR products to be cloned were gel purified and reamplified as above, but for only 15 cycles, using just the four normal dNTPs and without a pause for extension at 72°C. These reamplified PCR products were cloned using a T-vector, prepared as described (12), from pBC KS$^+$ (Stratagene). Double-stranded plasmid DNA was sequenced using a Thermosequenase cycle sequencing kit and ^{33}P-labelled ddNTPs (Amersham International plc).

Bacterial mutation assays

Escherichia coli strains CC101-106 (13) were kindly provided by Dr K. Negishi (Okayama University). Each strain was tested in triplicate. Overnight cultures grown in 2XTY (14) were diluted 1:100 (into 2XTY medium) and grown for 2 h, and the number of colony forming units (cfu) per ml (usually ~10^8) determined by plating dilutions on minimal glucose medium. Approximately 10^7 cfu (100 µl) were mixed with 3 ml of FTOP medium (14) and 100 µg of either nucleoside, dK or dZ and poured onto minimal lactose plates. As controls, the assays were performed without added nucleoside. Colonies were counted after 2 days incubation at 37°C.

RESULTS

N^6-Methoxy-2,6-diaminopurine-2′-deoxyribonucleoside, dK, was observed to exist in two tautomeric forms in d^6-DMSO in its ^{1}H-NMR spectrum, with the two tautomers in a ratio of 9:1. Of particular note are the resonances NH [9.78 (0.9H), 10.40 (0.1H)], H8 [7.71 (0.9H), 7.98 (0.1H)], H1′ [6.02 (0.9H), 6.18 (0.1H)] and 5′-OH [4.97 (0.9H), 5.08 (0.1H)]. We had assumed, from biochemical data (see below), that the major tautomer would be the amino or 'A'-like (N^6-H) one; but the matter was examined further by NMR. Conventional NOE experiments are not sufficiently sensitive for examining minor species, particularly as in this case it involves an exchangeable proton. However, the tautomers were identified using the recently developed double pulsed field gradient spin echo NOE nmr pulse sequence (DPFGSE NOE) (9). Irradiation of the major NH peak at 9.78 p.p.m. gave NOE signals to the NH_2 peak at 6.49 and to the OCH_3 peak at 3.72 p.p.m. (Fig. 2). Irradiation of the minor NH peak at 10.40 p.p.m. gave no NOE signals. Irradiation of the NH_2 signal at 6.49 p.p.m. gave an NOE only to the major NH peak at 9.78 p.p.m. Other (negative) peaks are due to saturation transfer. From these spectra, the major species was deduced to be the imino or 'G'-like (N^1-H) tautomer. It is also clear from these spectra that the *syn* conformer is the major species, because the *anti* form could not have given rise to an NOE signal to the CH_3 group when the major NH signal was irradiated.

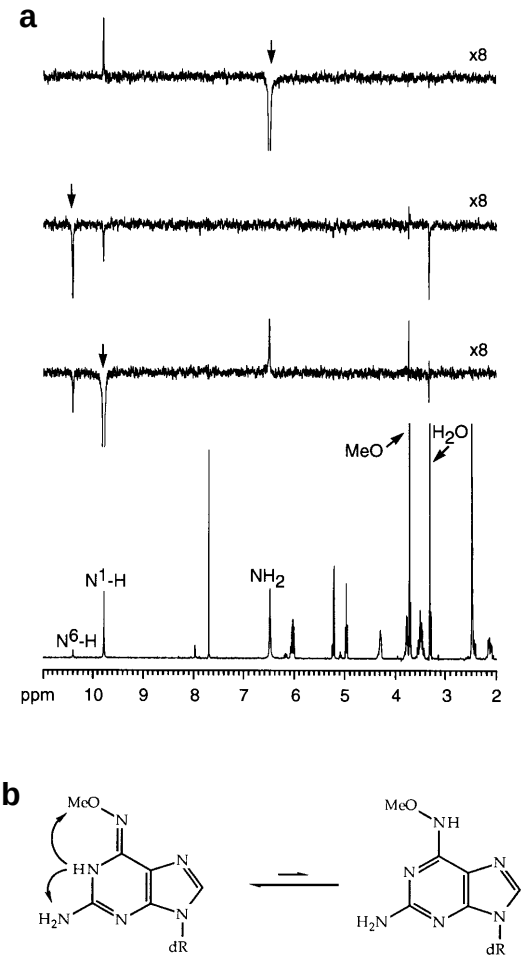

Figure 2. (**a**) ^{1}H-NMR spectrum of dK (bottom) demonstrating the existence of two tautomers in d^6-DMSO, and double pulsed field gradient spin echo NOE spectra. Arrows show signals which have been irradiated. (**b**) Irradiation of the major NH peak at 9.78 p.p.m. gives rise to NOE signals to both the NH_2 and OCH_3 as shown by the arrows and demonstrates that this is the major tautomer.

Table 1. Mutagenesis of *E.coli* strains CC102 annd CC106, which require the transition mutations shown to grow on lactose

	CC102 (GC→AT)	CC106 (AT→GC)
dK	725	220
dZ	9	6
control	8	0

Mean values (from three experiments) of lac$^+$ colonies per 10^7 cfu plated in the presence (100 µg) or absence (control) of dK or dZ.

The mutagenic spectra of dK and dZ in *E.coli* were determined using strains CC101–106, which indicate the occurrence of each of the six possible types of base pair change by reversion of a specific codon in *lacZ* (13). Mutations were produced only in strains reverting to lac$^+$ by transition mutations: CC102 (GC→AT) and CC106 (AT→GC). The mutation rates (lac$^+$ revertants/10^7 bacteria plated/100 µg dK or dZ) are shown in Table 1. While dK was an

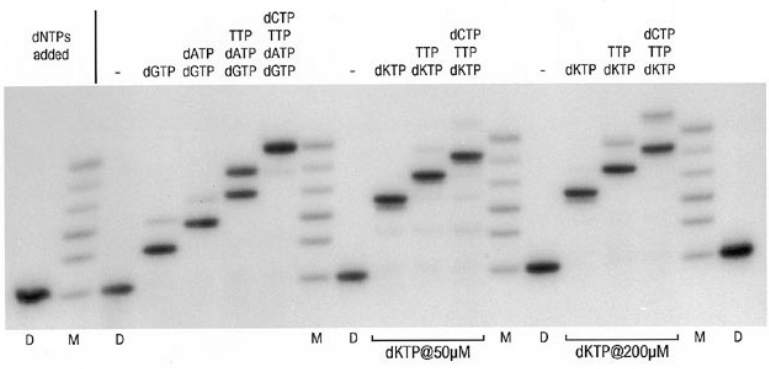

Figure 3. Incorporation of dKTP by *Taq* polymerase opposite dC or T, but not opposite dA or dG, in an oligonucleotide template. Lanes marked D show the labeled primer (20 residues in length), M indicates markers (in increments of one residue). Above each lane are shown the dNTPs added. Note that the extendase activity of *Taq* polymerase generates some product one residue longer than expected.

efficient mutagen in both strains, dZ increased the mutation rate only slightly in strain CC106.

The 5′-triphosphate of dK was synthesised and its incorporation specificity examined in two separate assays using *Taq* polymerase. In the first assay, a radiolabelled primer oligonucleotide, 20 residues in length, was annealed to a 24 residue template strand and primer extension in the presence of various dNTPs was determined by denaturing gel electrophoresis. As Figure 3 shows, *Taq* polymerase can incorporate dKTP as either dGTP or as dATP, that is opposite T or C in a template, but not opposite A or G, because no extension occurs opposite these residues unless TTP or dCTP are present. *Escherichia coli* DNA polymerase I and its derivatives, Klenow fragment and exonuclease-free Klenow showed the same incorporation specificity (results not shown).

In the second assay, incorporation of [α-^{32}P]dCTP into bovine thymus DNA by *Taq* polymerase, in the presence of various other dNTPs at 50 μM concentration was measured. The results, recorded in Table 2, show that DNA synthesis in the presence of all four normal dNTPs is inhibited by the addition of dKTP. As expected, omission of dATP, dGTP or TTP produces a dramatic decrease in the yield of DNA synthesised. Under these circumstances, the addition of dKTP leads to increased synthesis when either dATP or dGTP is omitted but not when TTP is absent. Clearly dKTP substitutes for the absence of dATP much better than it does for the absence of dGTP, although net synthesis is still greatly depressed. Omission of both purine triphosphates is poorly compensated by the addition of the analogue; dKTP cannot substitute for the absence of TTP. The results of both assays are, therefore, consistent.

The 5′-triphosphates of dK and dZ were then used in PCR reactions to determine their mutational spectra *in vitro*. The amber suppressor gene, *supF*, from *E.coli*, was chosen as a target for mutagenesis because almost all single base substitutions in the region corresponding to the mature tRNA inactivate gene function (15), and the complete nucleotide sequence can be determined in one gel run. Indeed, the 237 bp PCR product was efficiently amplified during the time taken for the heating block to change from the annealing temperature of 50°C to the denaturation temperature of 96°C (Fig. 4, first lane). However, the addition of increasing amounts, from 50 to 500 μM of the analogue 5′-triphosphates led to decreasing amounts of the desired PCR product (Fig. 4, row 1, lanes 1–7). Indeed, no detectable PCR product was obtained when >200 μM dKTP or 100 μM dZTP was present in the reactions (Fig. 4).

Table 2. Synthesis of DNA by *Taq* polymerase, using bovine thymus DNA as a template and a variety of dNTPs (each at 50 μM)

dNTPs present	Minus dKTP	Plus dKTP	Change (%)
A G C T	100	80.42 (7.24)	–20
G C T	6.25 (0.58)	16.91 (1.89)	+170
A C T	6.43 (0.61)	9.18 (0.92)	+43
A G C	7.60 (0.53)	7.23 (1.01)	–5
C T	3.85 (0.55)	5.90 (0.82)	+53

Yields of DNA are normalised to that synthesised in the presence of the four normal dNTPs and are the mean values from three experiments. Figures in brackets represent the standard deviations.

This inhibitory effect was specific to the analogues; adding excess dATP to the reactions instead of the analogue 5′-triphosphates had no effect on the yield of PCR product (results not shown). A longer extension time at 72°C was added to each cycle; this change significantly enhanced the yield of PCR products obtained in the presence of both dKTP and of dZTP (compare rows 2 and 3 with row 1). The increased amounts of PCR product synthesised when the time available for extension is prolonged, suggests that incorporation of the analogues creates a 3′-terminus which is more difficult for *Taq* polymerase to extend than one containing only normal nucleotides. This is consistent with the prolonged annealing times required when using oligonucleotide primers containing several dK residues (16).

To characterise the mutations induced by dKTP and dZTP, the PCR products initially amplified in the presence of the analogues were reamplified in the presence of only the four normal dNTPs, to remove any analogue bases from the amplified DNA, and then cloned. Forty-eight clones were sequenced; 16 from DNA amplified in the presence of 200 μM dKTP, 16 amplified in 100 μM dZTP and 16 control clones amplified under identical conditions except for the absence of either analogue. There were no mutations in any of the control clones. The 16 dKTP clones were all mutant, having, in total, 178 mutations in 16 × 197 (3152) bp. All but one of the mutations (a single nucleotide deletion) were transition mutations. The direction of the mutations was highly skewed: A to G mutations [166] were 15 times as frequent as G to A mutations [11]. Four of the 16 dZTP-derived clones were not mutant; the remaining 12 clones contained 22 mutations. Again, the direction of mutation was skewed strongly; A to G mutations [20] were 10 times as common as G to A mutations [2]. The

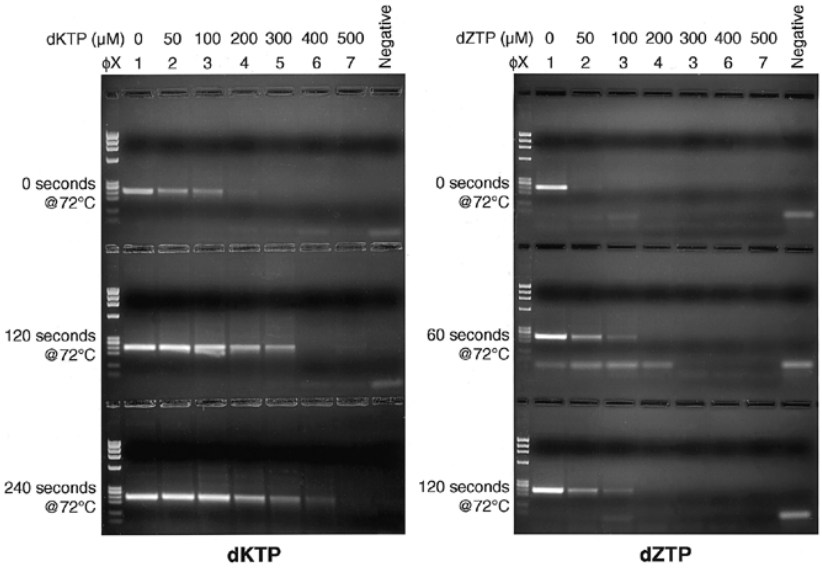

Figure 4. Inhibition of DNA synthesis by dKTP (left) and by dZTP (right) during amplification of the *supF* gene from the plasmid. The presence of increasing amounts of the analogues, as indicated, results in decreasing yields of product (columns 2–7). The last column, labeled negative, shows reactions performed without any template DNA. The size markers are φX174 *Hae*III fragments.

```
 224   41   15  1 111 1         1  2   6  14 32 2  1
CTTTCTCAAC GTAACACTTT ACAGCGGCGC GTCATTTGAT ATGATGCGCC   50
   1              1                    1   1

   122  2  331    21        6 115 13  2511     1    1
CCGCTTCCCG ATAAGGGAGC AGGCCAGTAA AAGCATTACC TGTGGTGGGG  100
     1

14    1        16   3 113 2 2 31 1 1   1  1   1    4
TTCCCGAGCG GCCAAAGGGA GCAGACTCTA AATCTGCCGT CATCGACTTC  150
      1     1 2     1             2   1            11

 44   3  2 12  22       1  1  13    43  1434       12
GAAGGTTCGA ATCCTTCCCC CACCACCATC ACTTTCAAAA GTCCGAA     197
1     1  1                      1    11        1
```

Figure 5. Mutagenic spectra of dKTP and dZTP. The nucleotide sequence of the *supF* gene on the plasmid pCDM8, used as a template for PCR mutagenesis. The transcription start site is position 53; the DNA sequence corresponding to the mature tRNA is shown in bold. The number of transition mutations produced by dKTP at each position are shown above the sequence; those induced by dZTP below.

frequency of mutation at each position in the *supF* sequence is shown in Figure 5.

DISCUSSION

The conversion by methoxyamine of cytosine and, less readily, adenine residues in DNA to the corresponding N^4- and N^6-methoxy-derivatives, has long been held to account for its mutagenic action (17). The electronegative oxygen atom alters the tautomeric equilibrium by as much as 10^4–10^6 and this is undoubtedly the basis for the transition mutagenic changes observed *in vivo* (18–21). However, a more precise description of the process seemed to us to be necessary and to that end we compared the mutational spectra of N^6-methoxydeoxyadenosine, dZ, and its 2-amino derivative, dK, in *E.coli* with the mutations obtained using the deoxyribonucleoside triphosphates as substrates in PCR reactions.

While dZ was, at best, only marginally effective as a mutagen in *E.coli*, dK was very effective in producing both AT→GC mutations and the reverse transition GC→AT. The assay used should be regarded as semi-quantitative, depending as it does to some extent on the leakiness of the *lac* alleles; nevertheless, the relative rates of mutation for the two deoxyribonucleosides are strikingly different. This could arise from poorer utilisation of dZ at any of a number of steps involved in the uptake, phosphorylation, incorporation or repair of the nucleoside. In order to avoid these confounding factors, the 5′-triphosphate derivatives of dZ and dK were synthesized. dKTP was incorporated by *Taq* polymerase as either dGTP or dATP, that is opposite C or T in a template. As expected, it was not incorporated opposite G or A despite prolonged (15 min) incubation. An interesting effect of adding dKTP or dZTP to PCR reactions was the decreasing yield of PCR product as more of the analogue was added. The same phenomenon was not seen with dATP, added in equivalent excess. However, prolonging the time available for chain extension at 72°C, increased the yield. This indicates that both analogues are rather poor substrates for polymerisation. Interestingly, they seem to disrupt further polymerisation after incorporation; this resembles the behaviour of K in primers for PCR (16). Primers containing several K residues required increased annealing times to allow efficient amplification, rather than prolonged extension times. Both observations are consistent with a model in which a DNA chain containing K in proximity to the primer terminus delays chain extension.

The incorporation of dKTP in one cycle requires that it be copied in the following cycle. We have previously shown that K residues in a template are copied by *Taq* polymerase as either G or A with a marked preference for being copied as A rather than as G in a ratio of 7:1 (16). With this information, the bias in the ratio of AT→GC versus GC→AT mutations might be explained as follows: during PCR, dKTP is inserted frequently opposite T, but is then usually (seven times out of eight) copied as A in the next cycle. Only when it is copied as G does a mutation arise. The reverse applies when dKTP replaces dGTP. Although less efficiently inserted opposite C, seven out of eight such incorporations (as

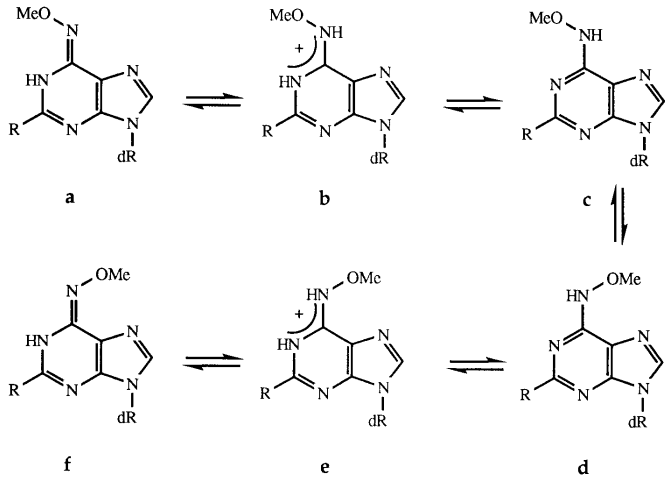

Figure 6. A scheme proposing the acid-base catalysed interconversion of the *syn–anti* and the amino–imino isomers of the N^6-methoxy-2,6-diaminopurine (R = NH_2; dK) and of the N^6-methoxyaminopurine (R = H; dZ) deoxyribosides.

dGTP) result in a mutation. Clearly, dKTP competes with dATP for incorporation very much better than it does with dGTP.

It seems surprising, therefore, that the imino or G-like tautomer of dK is the more abundant, as shown by our NMR data. The tautomeric ratios for dZ in a number of solvents have been measured; decreasing the polarity of the solvent leads to an increase in the amino tautomer, from 25% in DMSO to 70% in chloroform (6). In the present work, the imino tautomer of dK was found to be in excess in d^6-DMSO; however, the exchangeable proton could not be observed in $CDCl_3$, most likely due to its high exchange rate.

In seeking an explanation, we considered a further aspect of alkoxyaminopurines, *viz*, that they exist largely in the *syn* configuration with respect to the hydrogen bonding face (Fig. 1). While N^4-methoxy-dC, with the methoxy group *syn* with respect to N^3, has been demonstrated to form a stable base pair with G in both the wobble and Watson–Crick configurations (22), it is important to note that for dK and dZ inversion of the methoxyl group to the *anti* state is necessary because in their predominant, *syn*, configuration, neither can form a base-pair with either C or T. The scheme shown in Figure 6 outlines how this inversion might occur. As shown by Morozov and co-workers (23), the direct conversion of **a**→**f** is a photochemical process, with a high activation energy for the reverse **f**→**a** dark reaction. A low energy pathway, however, similar to the proton-transfer chemistry involved in DNA breathing can occur (24). Two protonation and deprotonation steps (**a**→**c** and **d**→**f**) would allow the fast interconversion of the tautomeric states and by extension, the *syn*↔*anti* conversion. The rotation about the single bond in the **c**→**d** interconversion must have a lower activation energy, although at equilibrium, the *syn* isomer, **c**, of the oxyamino-tautomer will still be favoured. In the cases of N^6-methyl-dA and N^4-dimethyl C, the activation energies for the C-N bond rotation are ~15 kcal/mol (4).

In summary, although the tautomeric equilibrium may favour the imino (G-like) form, this is incapable of base pairing but may be converted by low energy processes to the (active) A-like species, **d**. At equilibrium, an active G-like species, **f**, will be present but at a very low concentration, in comparison to the active A-like species, **d**. Similarly, the rate retardation might be explained by supposing that the analogue, in reassuming its preferred configuration following incorporation, produces an unacceptable base paired state in the DNA duplex just behind the growth point. The present paper is concerned only with a quantitative comparison of the nucleosides dK and dZ as mutagenic agents *in vivo* and *in vitro*. We have assumed that hydrogen-bonded base pairs are involved in the incorporation of both analogues; this standard assumption has been debated (25). Clearly, both dKTP and dZTP could be of use, in a more detailed kinetic study (26), in investigating the finer questions of polymerase action.

ACKNOWLEDGEMENTS

Dr A.J.H.Smith provided the plasmid pCDM8 and Dr K.Negishi the six Cupples and Miller *E.coli* strains. For assistance and advice with the NOE spectra we thank Drs Ji-Chun Yang and David Neuhaus, and for financial support, the Isaac Newton Trust (to F.H. and D.M.B.) and Amersham International plc (to D.L.).

REFERENCES

1 Saenger, W., (1984) *Principles of Nucleic Acid Structure*, Springer Verlag, New York.
2 Watson, J. D. and Crick, F. H. C., (1953) *Nature*, **171**, 964–967.
3 Topal, M. D. and Fresco, J. R., (1976) *Nature*, **263**, 285–289.
4 Kwiatkowski, J. S. and Pullman, B. (1975) in Katritzky, A. R. and Boulton, A. J. (eds) *Advances in Heterocyclic Chemistry*, Volume 18, pp. 199–335.
5 Singer, B. and Kusmierek, J. T., (1982) *Annu. Rev. Biochem.*, **51**, 655–694.
6 Brown, D. M. and Kong Thoo Lin, P., (1991) *Carbohydrate Res.*, **216**, 129–139.
7 Kong Thoo Lin, P. and Brown, D. M., (1991) *Nucleosides Nucleotides*, **10**, 675–677.
8 Kong Thoo Lin, P. and Brown, D. M., (1992) *Nucleic Acids Res.*, **20**, 5149–5152.
9 Stott, K., Keeler, J., Van, Q. N. and Shaka, A. J., (1997) *J. Magnetic Resonance*, **125**, 302–324.
10 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
11 Brutlag, D. and Kornberg, A., (1972) *J. Biol. Chem.*, **247**, 241–248.
12 Marchuk, D., Drumm, M., Saulino, A. and Collins, F. S., (1991) *Nucleic Acids Res.*, **19**, 1154.
13 Cupples, C. G. and Miller, J. H., (1989) *Proc. Natl. Acad. Sci.*, USA, **86**, 5345–5349.
14 Miller, J. H. (1992) *A Short Course in Bacterial Genetics*, Cold Spring Harbor Laboratory Press, Plainview, NY.
15 Kraemer, K. H. and Seidman, M. M., (1989) *Mutation Res.*, **220**, 61–72.
16 Hill, F., Loakes, D. and Brown, D. M., manuscript submitted.
17 Drake, J. W. (1970) *The Molecular Basis of Mutation*, Holden-Day Inc, San Francisco.
18 Brown, D. M., Hewlins, M. J. E. and Schell, P., (1968) *J. Chem. Soc.* (C), 1925–1929.
19 Kierdaszuk, B., Stolarski, R. and Shugar, D., (1984) *Acta Biochim. Pol.*, **31**, 49–64.
20 Janion, C. and Myszkowska, K., (1981) *Mutation Res.*, **91**, 193–197.
21 Nishio, H., Ono, A., Matsuda, A. and Ueda, T., (1992) *Nucleic Acids Res.*, **20**, 777–782.
22 Gdaniec, Z., Bann, B., Sowers, L.C. and Fazakerley, G.V., (1996) *Eur. J. Biochem.*, **242**, 271–279.
23 Morozov, Y. U., Savin, F. A., Chekhov, V. O., Budowsky, E. I. and Yakovlev, D. Y., (1982) *J. Photochem.*, **20**, 229–252.
24 Teitelbaum, H. and Englander, S. W., (1975) *J. Mol. Biol.*, **92**, 55–78; 79–92.
25 Goodman, M.F., (1995) *Nature*, **378**, 237–238.
26 Frey, M.W., Sowers, L.C., Millar, D.P. and Benkovic, S.J., (1995) *Biochemistry*, **18**, 9185–9192.