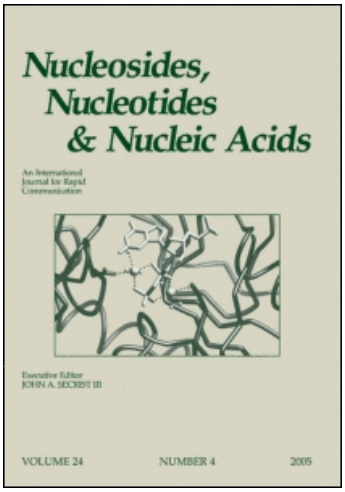

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RANDOM MUTAGENESIS USING 2-AMINO-9-(2-DEOXY-β-D-RIBOFURANOSYL)PURINE-5'-TRIPHOSPHATE AND THE POLYMERASE CHAIN REACTION.

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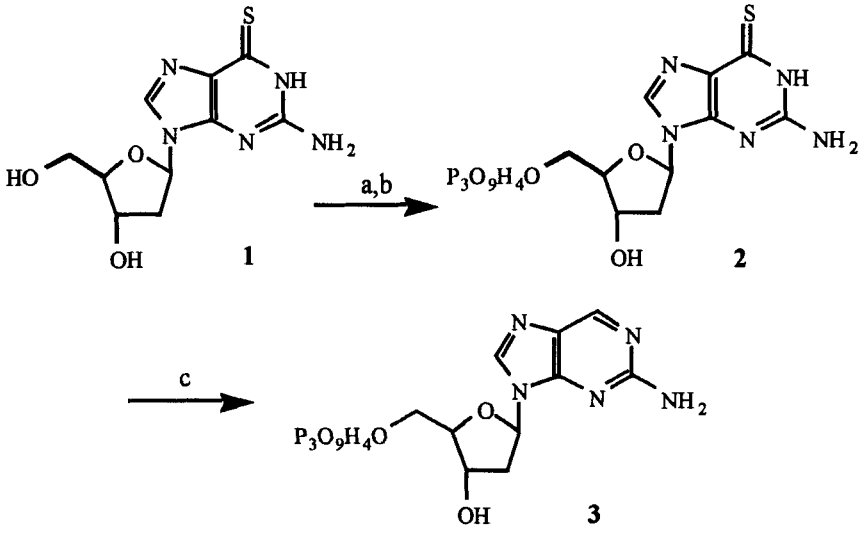
ABSTRACT: Base analogues offer an attractive method for mutagenising DNA in combination with the polymerase chain reaction (PCR). We have synthesised the 5'-triphosphate-2'-deoxyribosyl derivative of 2-aminopurine (dAPTP), one of the first base analogues to be used for mutagenesis, and examined its utility in PCRs. An *E. coli* amber suppressor gene, supF, was used as a template for mutagenesis. The analogue induced exclusively transition mutations, but at a low frequency, consistent with its weak mutagenicity *in vivo*.

Introduction

Analogues of the natural DNA bases have long provided a simple method for generating mutations in bacteria and phages.[1] Since the invention of the polymerase chain reaction (PCR), a number of base analogue 5'-triphosphates have been added to PCR reactions in order to mutagenise the DNA being amplified.[2-4] 2-Aminopurine (2-AP) was one of the earliest base analogues to be used;[5] it generates transition mutations, albeit at a low frequency *in vivo*.[6,7] We have synthesised the 5'-triphosphate of 2-aminopurine-2'-deoxyribonucleoside (dAPTP) (**3**) and examined whether it can produce mutations *in vitro*.



a: $POCl_3$, $P(OMe)_3$, $P(OEt)_3$. b: $[HNBu_3]_2H_2P_2O_7$, NBu_3, 1M TEAB. c: Raney nickel, 50°C.

Scheme 1 Strategy for the synthesis of dAPTP.

Results and Discussion

The strategy for the synthesis of dAPTP is shown in Scheme 1. The nucleoside, 2'-deoxy-6-thioguanosine (1), was phosphorylated8 with phosphoryl chloride followed by addition of bis-(tri-n-butylammonium) pyrophosphate to form 2'-deoxy-6-thioguanosine-5'-triphosphate (2), which upon desulphurization with Raney nickel formed 2-amino-9-(2'-deoxy-β-D-ribofuranosyl)purine-5'-triphosphate, dAPTP, (3).

An amber suppressor gene from *E. coli*, supF, was chosen as a target for mutagenesis because almost all single base substitutions in the region corresponding to the mature tRNA inactivate gene function.[9] In addition, the complete gene is short, allowing the entire nucleotide sequence to be determined in one gel run. Indeed, the 237bp 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 (Figure 1; row 1, lane 3). However, the addition of dAPTP, in amounts from 400μM to 2mM, decreased the yield of the desired full length PCR product (Figure 1; row 1, lanes 4-8). Indeed, no detectable PCR product was obtained when 2mM 2-aminopurine-2'-deoxyribose-5'-triphosphate was added to the reactions (Figure 1, lane 8, rows 1-3).

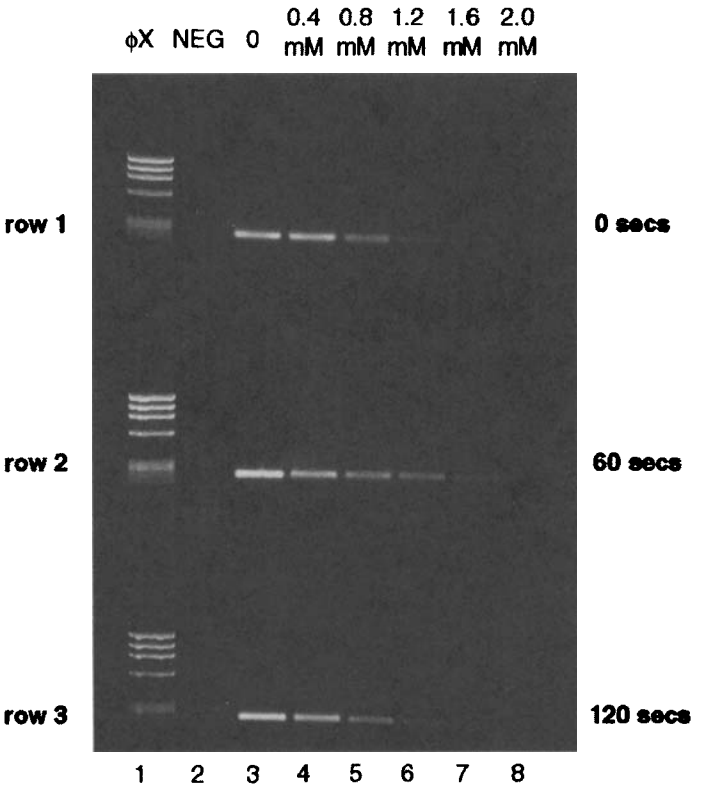


Figure 1. Inhibition of DNA synthesis by 2-aminopurine 2'-deoxyribosyl-5'-triphosphate. Amplification of the supF gene on plasmid pCDM8 (column 3) in the presence of increasing amounts of the analogue, as indicated, results in decreasing yields of product (columns 4-8). Column X shows reactions performed without any template DNA. The size markers (column ΦX) are ΦX174 Hae III fragments.

This inhibitory effect was specific to the analogue; adding excess dATP to the reactions instead of the analogue triphosphate had no effect on the yield of PCR product (result not shown). Two parameters of the reaction were modified in attempt to minimise this inhibition. Firstly, addition of the analogue with an equivalent molar amount of magnesium chloride (to exclude depletion of $MgCl_2$ by the excess triphosphate), in fact, decreased the yield of product (not shown). Secondly, the extension time at 72°C was increased to sixty or one hundred and twenty seconds to allow for delayed extension kinetics following incorporation of the analogue (Figure 1,

```
                          GT
CTTTCTCAAC GTAACACTTT ACAGCGGCGC GTCATTTGAT ATGATGCGCC   50

 T
CCGCTTCCCG ATAAGGGAGC AGGCCAGTAA AAGCATTACC TGTGGTGGGG  100

                                C
TTCCCGAGCG GCCAAAGGGA GCAGACTCTA AATCTGCCGT CATCGACTTC  150

                                          CT
GAAGGTTCGA ATCCTTCCCC CACCACCATC ACTTTCAAAA GTCCGAA     197
```

Figure 2. The nucleotide sequence of the supF gene on the plasmid pCDM8, used as a template for PCR mutagenesis. Transcription initiates at position 53; the DNA sequence corresponding to the mature tRNA is shown in bold. The six mutations, found in separate clones in this study, are shown above the sequence. The transition at position 185 was the only mutation found in the twenty-five control sequences.

rows 2 and 3). This change enhances the yield of PCR product using several other analogue 5'-triphosphates,[10] it made only a slight difference in this case (compare rows 2 and 3 with row 1).

To determine whether the addition of the analogue 5'-triphosphate to the reactions had induced mutations, the PCR products of the supF gene initially amplified in the presence of 1.6mM 2-APTP were reamplified in the presence of only the four normal dNTPs, to remove any analogue bases from the DNA, and then cloned. Twenty-five randomly picked clones were sequenced. In addition, to determine the spontaneous mutation rate, twenty-five clones obtained from DNA amplified in parallel, under identical conditions except for the absence of the analogue, were sequenced. Forty-four of the clones had no mutations; the remaining six clones each had single, different, point mutations. One of the mutant clones was from the control set. All six mutations were transitions (Figure 2). Thus, addition of the analogue increased the mutation rate five-fold in this experiment: from one transition in 4,925 base-pairs of control DNA sequenced, to one transition in every 985 base-pairs of DNA amplified in the presence of the analogue.

All fifty supF clones were assayed for their ability to suppress simultaneously two amber mutations using an *E. coli* strain carrying the p3 plasmid.[11] This plasmid has amber mutations in both its beta-lactamase gene, conferring ampicillin resistance, and in its

tetracycline-resistance gene. DNA from each clone was introduced into this strain, and then equal volumes of the transformation mixture from each clone were spread on plates containing chloramphenicol (resistance to which is carried on the vector) and on plates containing chloramphenicol, ampicillin and tetracycline. All but one of the clones produced several hundred colonies on plates containing ampicillin and tetracycline and were therefore $supF^+$; thus, five of the point mutants in supF did not affect suppression of the amber mutations. The single clone which did not confer resistance to ampicillin and tetracycline had a mutation in the anticodon of the tRNA (position 129 in Figure 2).

A previous report described the incorporation of 2-aminopurine-2'-deoxyribosyl-5'-triphosphate by Epstein-Barr virus DNA polymerase *in vitro*; however, no mutations were reported.[12] This communication is, to our knowledge, the first account of mutagenesis *in vitro* using this compound.

Conclusions

The compound, dAPTP (**3**), was synthesised; its addition to PCR reactions in increasing concentration progressively inhibited the amplification of DNA. There was a five-fold increase in the number of transition mutations in the DNA synthesised in the presence of 1.6mM of the analogue triphosphate.

Experimental

General methods. ^{31}P NMR spectra were recorded on a Varian Gemini 300 instrument. Chemical shifts are referenced from external 1% phosphoric acid. Preparative HPLC was done on a Waters model 600 gradient system and analytical HPLC on a Waters 510 gradient system using a Waters 440 detector at 254 nm. DEAE-Sephadex chromatography was conducted at 5°C using an Amersham Pharmacia Biotech LCC-501 plus system. Triethylammonium bicarbonate buffer (TEAB) was prepared from Millipore Super Q H_2O and distilled triethylamine, bubbling anaerobic grade CO_2 (Praxair) at 5°C to obtain a pH = 7.0 buffer. Tributylammonium pyrophosphate was prepared by passing sodium pyrophosphate through the acid form of Dowex 50WX8, 100-200 mesh (Supelco) ion-exchange resin. The effluent was collected into a vessel containing 80 % ethanol and tributylamine. Repeated evaporation with absolute ethanol followed by anhydrous DMF yielded the final product. Ultraviolet spectra were recorded

on a Perkin Elmer Lambda 2 spectrophotometer. Samples were dissolved in water unless otherwise stated. Tlc was carried out on pre-coated F_{254} silica plates and column chromatography with Merck kieselgel 60.

2'-Deoxy-6-thioguanosine-5'-triphosphate (2). To 1.13 g (4 mmol) of 2'-deoxy-6-thioguanosine[13] under argon was added 30 ml of trimethylphosphate and 30 ml of triethylphosphate. The reaction mixture was cooled in an ice bath and 0.56 µl (6 mmol) of phosphoryl chloride (redistilled) was added. The reaction was stirred with cooling for three hours. Both 1M tri-*n*-butylammonium pyrophosphate in anhydrous DMF (20 ml, 20 mmol) and tri-*n*-butylamine (5 ml, 20 mmol) were then added slowly to the cooled solution simultaneously. This was stirred for 10 mins, then warmed to room temperature, and stirred for another 20 mins. Cooled 1M triethylammonium bicarbonate buffer (TEAB, pH=7.0) was added to the reaction mixture until the solution became neutral as observed by pH paper. The buffer was added to a final volume of 100 ml and the mixture stirred overnight at room temperature. The solution was evaporated under high vacuum, and the crude product applied to a 1 L Sephadex A25 column which was eluted with a gradient of 0.05M to 1M TEAB (pH 7.0) at a flow rate of 1.2 ml min^{-1}. A peak containing the product was collected at approximately 0.9M. After evaporation, the triphosphate was finally purified on a reverse phase Δ PAK C 18 HPLC column (5x30 cm) using a 0% B to 100 % B gradient in 45 mins at 130 ml min^{-1} (A=0.1M TEAB, pH=7.0 and B=25% acetonitrile in 0.1M TEAB, pH=7.0). A total of 1.1 g (38%) of the triphosphate **2** as its bis(triethylammonium) salt was collected. ^{31}P NMR δ -10.52 (d), -11.27 (d), -23.18 (t). HPLC (Δ PAK C 18, 3.9 x 30 mm, 0% B to 100% B in 30 mins at 1 ml min^{-1}) 15.6 mins. UV 346 nm (λmax) and 262 nm.

2-Amino-1-(2'-deoxy-β-D-ribofuranosyl)purine-5'-triphosphate (dAPTP) (3). To 200 mg (0.27 mmol) of the triethylammonium salt of 6-thio-2'-deoxyguanosine-5'-triphosphate (**2**) in 25 ml H_2O was added 1 ml of activated Raney nickel (50% in H_2O). The slurry was stirred overnight at 50°C. After cooling to room temperature, the solution was passed through a small plug of celite, and washed with water, and the combined solution evaporated to dryness. The retention time of the product on reverse phase Δ PAK C 18 was the same as the starting material 2'-deoxy-6-thioguanosine-5'-triphosphate; therefore purification was performed on a Synchropak AX 100 ion exchange column (21.2 x 250 mm) using a 0-50% B gradient in 30 mins at 15 ml min^{-1}

(A= 10% aqueous ethanol and B= 10% ammonium bicarbonate solution (w/w in H_2O) in 10% aqueous ethanol). A total of 60 mg (41%) of tris ammonium salt was collected. ^{31}P NMR δ -6.20 (d), -10.94 (d), -21.72 (t). HPLC (Synchropak AX 100 ion exchange column, 21.2 x 250 mm, 0 to 50% B gradient in 30 mins at 1ml min^{-1}) 12.0 mins (2'-Deoxy-6-thioguanosine-5'-triphosphate: 13.8 mins). UV 304 nm (λmax), and 244 nm.

Polymerase chain reactions. The amber suppressor supF gene was amplified using the plasmid pCDM8 (available from Invitrogen) as a template. The sequences of the oligonucleotide primers used were: 5'-CAGCTGGATTACCGCGGTCT and 5'-CACACACAAGCAGGGAGCAG. Each 50 µl reaction contained 5 ng of the template linearised at its unique HindIII site, 50 pmoles of each primer, 50 µM of each natural dNTP, 5 µl of 10 x PCR buffer and 2.5 units of *Taq* polymerase. A 10 mM stock solution of dAPTP was used to obtain final concentrations of 0.4 mM, 0.8 mM, 1.2 mM, 1.6 mM or 2 mM 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 mins (during which time *Taq* polymerase was added), followed by thirty cycles of: denaturation at 96°C for 5 s, annealing at 50°C for 5 s; extension at 72°C for 0, 60 or 120 s. After the last cycle, a final extension at 72°C for five mins completed the reactions. Reaction products were analysed by electrophoresis on 2% agarose TAE gels. The expected product was 237 base-pairs long. A control reaction, without template, was run with each set of reactions.

Cloning and sequencing of PCR products. PCR products to be cloned were gel purified and reamplified, as above, but for only fifteen cycles, with only normal dNTPs and zero extension time. These PCR products were cloned using a T-vector, prepared, as described,[14] from pBC KS^+ (Stratagene) and the resulting colonies were picked randomly and screened first for the presence of the supF gene in the plasmid and then for supF activity, as described below. Double-stranded plasmid DNA was sequenced both with an Applied Biosystems 373 sequencing machine and a Thermo Sequenase™ dye terminator cycle sequencing pre-mix kit (Nycomed Amersham plc) and manually using a ThermoSequenase™ (Nycomed Amersham plc) radiolabelled terminator cycle sequencing kit and ^{33}P-labelled ddNTPs (Nycomed Amersham plc).

Assaying clones for supF function. The strain KO1 is identical to MC1061[p3][11,15] but carries a deletion within *endA*, eliminating Endonuclease I activity

in plasmid DNA preparations (F.H., unpublished). The p3 plasmid carries ampicillin- and tetracycline-resistance genes, but each of these genes has an amber mutation rendering them inactive in the absence of supF.[11] Each of the fifty clones was transformed into competent KO1 bacteria; equal amounts of the transformation mix was plated on plates containing either chloramphenicol (34 μg/ml) or chloramphenicol, ampicillin (100 μg/ml) and tetracycline (12.5 μg/ml).

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