Molecular recognition in the minor groove of the DNA helix. Studies on the synthesis of oligonucleotides and polynucleotides containing 3-deaza-2′-deoxyadenosine. Interaction of the oligonucleotides with the restriction endonuclease *Eco*RV

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ABSTRACT

An improved procedure for the preparation of 3-deaza-2′-deoxyadenosine ($d^{3C}A$) is described which is suitable for the synthesis of gram quantities of this analogue. Using phosphoramidite chemistry $d^{3C}A$ has been incorporated into the *Eco* RV restiction endonuclease recognition sequence (underlined) present in the self-complementary dodecamer d(GACGATATCGTC). The modified oligonucleotides have been thoroughly characterised by nucleoside composition analysis, circular dichroism and thermal melting studies. Studies with *Eco* RV show that incorporation of $d^{3C}A$ into either the central or outer dA-dT base-pair results in a substantial reduction in the rate of cleavage. The two-step conversion of $d^{3C}A$ to 3-deaza-2′-deoxyadenosine-5′-*O*-triphosphate ($d^{3C}ATP$) *via* the 5′-*O*-tosylate is also described. $d^{3C}ATP$ is not a substrate in the poly[d(AT)]·poly[d(AT)] primed polymerisation for either *E.coli* DNA polymerase I or *Micrococcus luteus* DNA polymerase. In a more detailed kinetic analysis $d^{3C}ATP$ was shown to be a competitive inhibitor of *E.coli* DNA polymerase I with respect to dATP.

INTRODUCTION

The manner by which proteins and drugs recognise and bind specific DNA sequences is of fundamental importance to our understanding of basic cellular processes and the design of anticancer and antiviral drugs. It has been established that drugs such as netropsin[1] and Hoechst 33258[2] favour dA-dT sequences and bind to the minor groove of the DNA helix through hydrogen bonds between adenine N-3 and thymine O-2 atoms on the edge of the base-pairs. Water is able to bind in an analogous fashion and gives rise to a 'spine of hydration' in the minor groove that helps to stabilise the B-form of the double helix[3]. Under conditions of low water activity the B-helix can be converted to an A- or Z-helix, but this interconversion can be difficult or even impossible for polymers of high dA-dT composition[4]. In contrast, large protein molecules appear to interact with DNA principally in the major groove of the helix, as exemplified by the helix-turn-helix motif found in prokaryotic activator and repressor proteins[5-9]. Protein binding to the minor groove is rarer but has been observed for DNase I where a flexible loop on the enzyme protrudes into the minor groove[10].

An attractive strategy for studying recognition processes involves the incorporation of modified bases into DNA sequences such that a potential hydrogen bonding site is removed from the DNA. On this basis 3-deaza-2′-deoxyadenosine ($d^{3C}A$ (1)) appears to be a useful probe for this purpose, since it is able to maintain the Watson-Crick hydrogen bonding scheme but removes an essential hydrogen bond acceptor (adenine N-3) from the minor groove.

We report here the synthesis, using phosphoramidite chemistry, of two analogues of d(GACGATATCGTC) in which one of the two central adenine bases is replaced by 3-deazaadenine and studies on their cleavage by the restriction endonuclease *Eco* RV. (During the early stages of this work Ono and Ueda reported the first synthesis of an oligodeoxynucleotide containing (1) using the less efficient phosphotriester chemistry and studied its cleavage with a variety of type II restriction endonucleases[11]). Additionally we describe our attempts to prepare polynucleotides containing 3-deazaadenine through the synthesis of 3-deaza-2′-deoxyadenosine-5′-*O*-triphosphate ($d^{3C}ATP$) (12) and its polymerisation using DNA polymerases. Our results demonstrate that $d^{3C}ATP$ a competitive inhibitor of *E.coli* DNA polymerase with respect to 2′-deoxyadenosine-5′-*O*-triphosphate (dATP).

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RESULTS AND DISCUSSION

Synthesis of 3-deaza-2'-deoxyadenosine (1)

The first and only reported synthesis of (1) utilised 4-chloroimidazo[4,5-c]pyridine as the heterocyclic synthon[12]. However, previous work on the synthesis of the ribose analogue 3-deazaadenosine[13] indicated that synthesis from 4,6-dichloroimidazo[4,5-c]pyridine (9) would provide a more efficient route and in support of this strategy Kazimierczuk *et al.*[14] have demonstrated that the sodium salt of (9) undergoes stereoselective glycosylation to give a high yield of the β-anomer of the corresponding 2'-deoxynucleoside.

The route chosen to (9) (Figure 1) follows closely that originally described by Rousseau and Robins,[15] and therefore only those steps that differ from their procedure will be described. The conversion of 2,6-dichloropyridine-*N*-oxide (2) to 2,6-dichloro-4-nitropyridine-*N*-oxide (3) followed by reduction to 4-amino-2,6-dichloropyridine (5) by hydrogenation in the presence of Raney nickel as previously described[15] proved problematic. Nitration of (2) at temperatures between 100 and 120°C gave crude samples of (3) that were contaminated with significant quantities (15–40%) of 2,6-dichloro-4-nitropyridine (4), whilst the reduction step gave disappointingly low yields (33%) of (5). When (2) was nitrated at temperatures in excess of 150°C (4) was obtained as the sole product in a yield of 56%. Related to this observation Krönke and Schäffer[16] have reported that 4-nitropyridine-*N*-oxide can be converted directly to 4-nitropyridine under similar conditions. It is likely that nitric oxide which is produced from the thermal decomposition of nitrogen dioxide[17] functions as the reductant since it is known to effect the deoxygenation of pyridine-*N*-oxide. Reduction of (4) to 4-amino-2,6-dichloropyridine (5) was accomplished in 82% yield using iron powder in acetic acid. Raney nickel catalysed reduction also gave low yields for the conversion of 4-amino-2,6-dichloro-3-nitropyridine (7) to 3,4-diamino-2,6-dichloropyridine (8) and once again iron in acetic acid was a more effective reagent giving (8) in 64% yield.

Rousseau and Robins[15] had previously used triethyl orthoformate and acetic anhydride to effect ring closure of (8) to 4,6-dichloroimidazo[4,5-c]pyridine (9). Using this procedure we isolated (9) in 70% yield although large scale preparations were contaminated with small amounts of *N*-1-acetyl-5,6-dichloroimidazo[4,5-c]pyridine. Studies conducted by Montgomery and Holum[18] on the cyclisation of 3,6-diaminopyrimidine derivatives suggested that diethoxymethyl acetate should be a superior reagent for this conversion. Thus when (8) was refluxed in diethoxymethyl acetate for 2 hours the product (9) could be obtained in 92% yield. The conversion of (9) to (1) was accomplished in three steps using the glycosylation procedure reported by Kazimierczuk *et al.*[14] followed by ammonolysis and reduction as described by May and Townsend[13] for the synthesis of 3-deazaadenosine. The overall yield for the ten step synthesis of (1) was greater than 6% and the procedure has proved suitable for preparation of gram quantities of (1).

Synthesis of oligodeoxynucleotides containing 3-deaza-2'-deoxyadenosine

The exocyclic amino group of (1)[11] and its ribose analogue[19] have previously been protected as dimethylformamidine derivatives. Formamidine protection appears to have been chosen since it was known that this group could be introduced into adenine nucleosides in excellent yields and is readily removed by ammonolysis[20]. However, more recently it has been reported

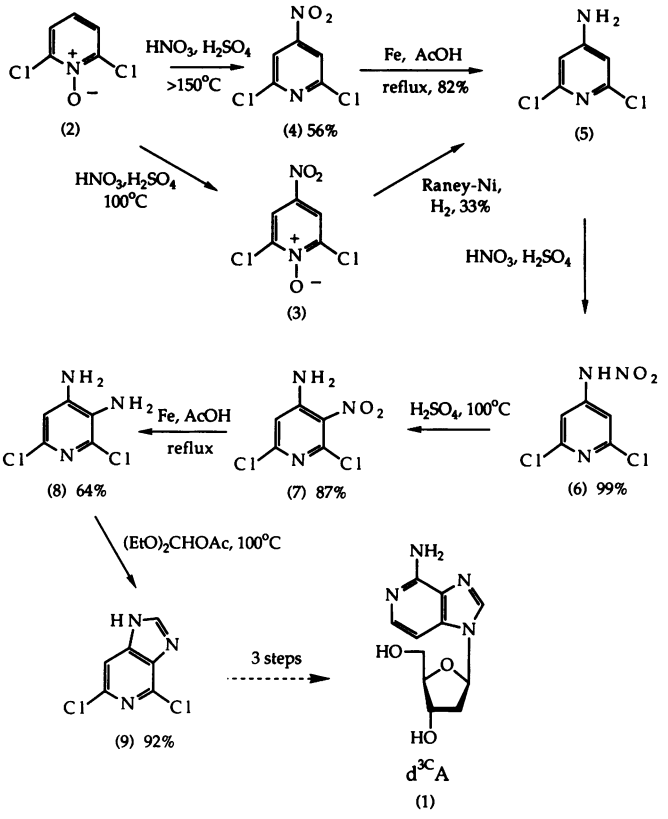

Figure 1. Synthesis of 3-deaza-2'-deoxyadenosine

that dimethylformamidine protection of 2'-deoxyadenosine is incompatible with the acidic and basic conditions used during DNA synthesis[21] and furthermore the instability of this derivative on silica gel precluded its chromatographic purification. Although dimethylformamidine protection has been successfully used for the synthesis of oligodeoxyribonucleotides containing (1)[11] the protected nucleoside was not purified. In order to develop a reliable and efficient procedure for incorporating (1) into oligodeoxyribonucleotides purification of the nucleotide synthons was essential and we therefore decided to use the more stable and widely used benzoyl group. Following the transient protection procedure developed by Ti *et al.*[22] for the synthesis of 6-*N*,*N*-dibenzoyl-2'-deoxyadenosine the 6-*N*,*N*-dibenzoyl derivative of (1) was prepared and the crude product treated with 4,4'-dimethoxytrityl chloride under standard conditions to give 6-*N*,*N*-dibenzoyl-5'-*O*-(4,4'-dimethoxytrityl)-3-deaza-2'-deoxyadenosine (10) in a 56% yield after chromatography.

As expected, on the basis of a previous study in which *N*-benzoyl protection had been used on 7-deaza-2'-deoxyadenosine,[23] debenzoylation of (10) using concentrated aqueous ammonia alone was slow in comparison to 6-*N*,*N*-dibenzoyl-2'-deoxyadenosine. However, in the presence of 10% (w/v) ammonium acetate the reaction was a little more rapid and complete deprotection was achieved in 60 hours at 55°C.

In contrast to the work of Ono and Ueda[11] we chose to synthesise oligodeoxyribonucleotides using phosphoramidite synthons principally because of their compatibility with automated methods of DNA synthesis. Treatment of (10) with 2-cyanoethyl-*N*,*N*-diisopropylaminochlorophosphine under standard conditions gave 6-*N*,*N*-dibenzoyl-5'-*O*-(4,4'-dimethoxytrityl)-3-deaza-2'-

deoxyadenosine-3'-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (11) in 69% yield after purification.

The restriction endonuclease *Eco* RV requires the hexamer sequence d(GATATC) and catalyses the hydrolysis of the central phosphodiester bond[24,25]. Three self-complementary dodecanucleotides containing the recognition sequence were prepared; the unmodified sequence d(GACGATATCGTC) (ODN-1) and two sequences in which one of the central adenine bases was replaced by 3-deazaadenine, d(GACG^{3C}ATATCGTC) (ODN-2) and d(GACGAT^{3C}ATCGTC) (ODN-3). All oligonucleotides were prepared by automated solid phase synthesis as previously described[26] and syntheses were programmed to maintain the dimethoxytrityl group on the terminal dG residue. Removal of the base and phosphate protecting groups and cleavage from the support was accomplished by treatment with 35% aqueous ammonia containing 10% (w/v) ammonium acetate at 50°C for 14 hours for ODN-1 and 55°C for 60 hours for ODN-2 and −3. The resulting DMT-protected oligodeoxyribonucleotides were purified by reverse phase hplc and the DMT group removed with 80% acetic acid. Starting from 2×1 μmole of control pore glass bound deoxycytidine about 30 A_{260} units were generally obtained. Oligonucleotides were greater than 98% pure as determined by hplc.

The composition of each oligonucleotide was determined by digestion with snake venom phosphodiesterase and alkaline phosphatase followed by analysis of the nucleoside mixtures by hplc . Compositions were calculated from the peak area integrals using the nucleoside extinction coefficients and were consistent with the proposed sequence (Table 1). Hyperchromicity values and hence molar extinction coefficients were calculated for all three oligodeoxynucleotides from the increase in uv absorbance obtained after digestion to the constituent nucleosides (Table 2).

An important characteristic of the modified oligonucleotides is the thermal stability of the duplex especially as *Eco* RV requires a double stranded structure as substrate. Melting curves for the three oligonucleotides were determined as previously described[26] (Figure 2). All three oligonucleotides exhibited a double stranded to single coil transition between 50−55°C. The unmodified sequence produced a sharper melting curve indicative of a more co-operative transition. In order to establish that the modified oligonucleotides adopted a B-type helix their circular dichroism spectra were recorded at low salt concentration (Figure 3). As expected, all three oligonucleotides exhibited a positive peak around 280 nm and a trough centred at 250 nm which is characteristic of normal B-DNA.

Cleavage experiments with *Eco* RV

Cleavage experiments with the *Eco* RV endonuclease were performed with the oligonucleotides present at a double strand concentration of 20 μM and at a temperature of 25°C; conditions under which the oligonucleotide substrates are essentially double stranded. The pH and salt conditions were as described in the experimental section and reactions were monitored by hplc. For the studies on ODN-1 *Eco* RV was used at a concentration of 0.1 μM and as expected ODN-1 was cleaved to give d(pATCGTC) and d(GACGAT).

The assay conditions employed for the modified sequences contained 1 μM *Eco* RV but in all other respects the reaction conditions were identical to those described for ODN-1. It was initially assumed that *Eco* RV was cleaving the modified sequences at the correct site to produce the fragments shown in equations (1) and (2). In order to confirm that 'normal'

Table 1. Nucleoside ratios for d(GACGATATCGTC) (ODN-1), d(GACG^{3C}ATATCGTC) (ODN-2) and d(GACGAT^{3C}ATCGTC) (ODN-3). The value for dG was set at 3.0 and other nucleosides ratioed to this number.

	Nucleoside composition (theory)			
	dA	dC	dG	dT	$d^{3C}A$
ODN-1	2.8 (3)	2.8 (3)	3.0 (3)	2.7 (3)	-
ODN-2	1.7 (2)	3.2 (3)	3.0 (3)	3.0 (3)	0.9 (1)
ODN-3	1.6 (2)	3.4 (3)	3.0 (3)	3.2 (3)	1.0 (1)

Table 2. Hyperchromicity, molar extinction coefficients (M^{-1} cm^{-1}per single strand) and rates of oligonucleotide cleavage [nmol min^{-1} mg^{-1} (relative rates)] by *Eco* RV for d(GACGATATCGTC) (ODN-1), d(GACG^{3C}ATATCGTC) (ODN-2) and d(GACGAT^{3C}ATCGTC) (ODN-3).

	Hyperchromicity	Extinction coefficient	Cleavage rate	
ODN-1	1.48	83,000	115	(100)
ODN-2	1.34	88,000	2.9	(2.5)
ODN-3	1.33	89,000	3.5	(3.0)

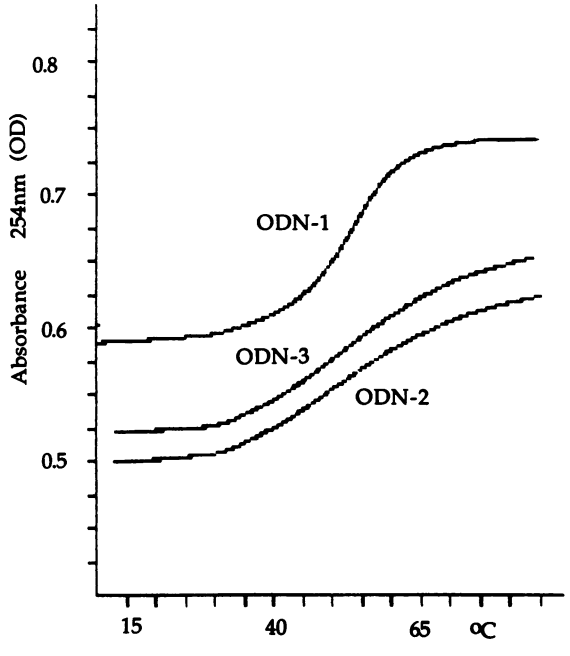

Figure 2. Melting curves for d(GACGATATCGTC) (ODN-1) (53°C), d(GACG^{3C}ATATCGTC) (ODN-2) (52°C) and d(GACGAT^{3C}ATCGTC) (ODN-3) (53°C) recorded in 50 mM Hepes.potassium hydroxide pH 7.5 containing 100 mM sodium chloride and 10 mM magnesium chloride.

$$d(GACG^{3C}ATATCGTC) \xrightarrow{Eco\ RV} d(GACG^{3C}AT) + d(pATCGTC) \quad (1)$$

$$d(GACGAT^{3C}ATCGTC) \xrightarrow{Eco\ RV} d(GACGAT) + d(p^{3C}ATCGTC) \quad (2)$$

cleavage was indeed occurring the products from equations (1) and (2) were isolated and subjected to digestion with snake venom phosphodiesterase and alkaline phosphatase. Analysis of the digestion mixtures by hplc gave nucleoside compositions which

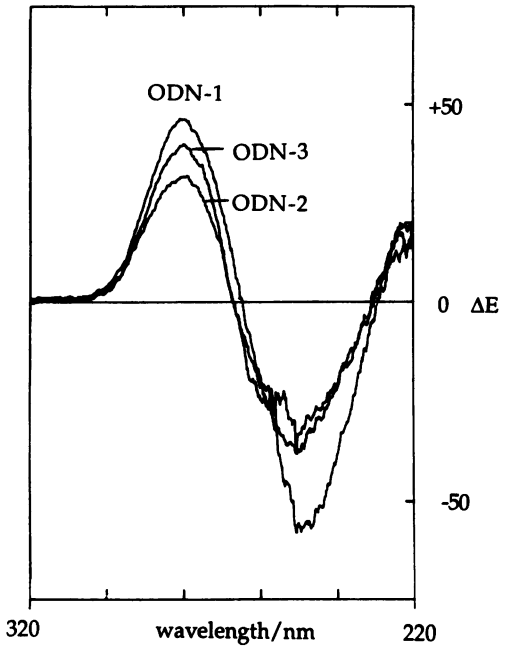

Figure 3 Circular dichroism spectra for d(GACGATATCGTC) (ODN-1), d(GACG^{3C}ATATCGTC) (ODN-2) and d(GACGAT^{3C}ATCGTC) (ODN-3) performed on oligonucleotide solutions (about 1 A_{254} unit/mL) in 25mM KH_2PO_4 pH 7.2 containing 100 mM sodium chloride and 10 mM magnesium chloride.

were correct for the fragments expected from 'normal' cleavage. The cleavage rates for all three oligonucleotides were determined from plots of substrate concentration verses time and are shown in Table 2. Incorporation of 3-deazaadenine into either the outer or inner dA-dT base pairs produced a considerable reduction in the cleavage rate (2.5% and 3% respectively compared to the unmodified sequence).

Recent studies by Mazzarelli *et al.*[27] and those conducted previously by Fliess *et al.*[28] and Nwosu *et al.*[29] have demonstrated that *Eco* RV is particularly sensitive to modifications in the major groove of either the inner or outer dA-dT base-pairs of its substrate DNA. This present study indicates that modification to the minor groove of these base pairs also reduces the cleavage rate. Although it is possible that induced conformational changes could be responsible for the reduced rate of cleavage the Tm studies and CD spectra indicate that structural aberrations, if present, are of a subtle nature.

A more detailed study on the interaction of oligonucleotides containing 3-deazaadenine and other modified dA-dT base-pairs with the *Eco* RV restriction endonuclease and the modification methylase will be reported elsewhere[30, 31].

Synthesis of d^{3C}ATP

3-Deaza-2'-deoxyadenosine was converted to its triphosphate (12) using the two-step procedure developed by Dixit and Poulter[32] (Figure 4). 5'-*O*-*p*-toluenesulphonyl-3-deaza-2'-deoxyadenosine (13) was prepared by treatment of the parent nucleoside with 1.5 equivalents of *p*-toluenesulphonyl chloride in pyridine at 0°C. The product was obtained in 51% yield as a white solid after chromatography on neutral alumina. In model displacement reactions on 5'-*O*-*p*-toluenesulphonyl-2'-deoxyadenosine with tetrakis(tetra-*n*-butylammonium)hydrogen triphosphate (14) it was established that optimum yields of dATP (50–55%) could be

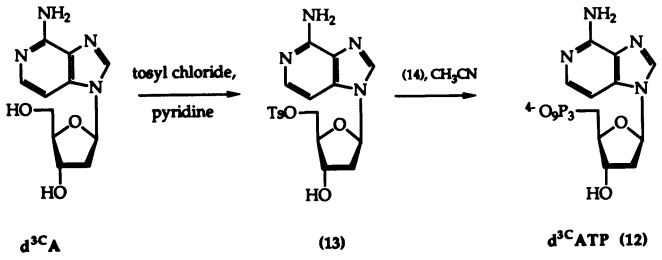

Figure 4. Synthesis of 3-deaza-2'-deoxyadenosine-5'-*O*-triphosphate.

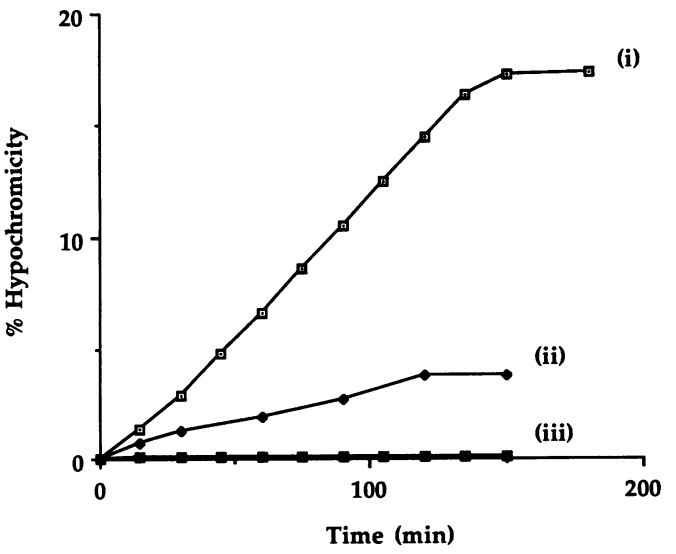

Figure 5. Plots of% hypochromicity against time for poly[d(AT)]·poly[d(AT)] primed polymerisation with 4 units (0.7 μg) *E.coli* DNA polymerase I, 0.25 mM dTTP and the following concentrations of adenine nucleotides: plot (i) 0.25 mM dATP; plot (ii) 0.125 mM dATP and 0.125 mM d^{3C}ATP; plot (iii) 0.25 mM d^{3C}ATP. Buffer and salt concentrations are detailed in the experimental section.

obtained using 1.5 equivalents of (14) in acetonitrile for 48 hours at room temperature. Application of these conditions to (13) were less successful and ion-pair hplc indicated that more than 80% of the tosylate remained after the 48 hour period. However, after an additional 24 hours at 40°C the majority of the starting material had been consumed and following work-up and chromatography on DEAE Sephadex, 3-deaza−2'-deoxyadenosine-5'-O-triphosphate was obtained as its triethylammonium salt in 27% yield. The product was converted to its sodium salt and characterised by FAB mass spectrometry and ^{31}P and ^{1}H nmr spectroscopy.

Polymerisation studies with d^{3C}ATP

In an initial experiment the ability of the synthesised dATP and d^{3C}ATP to act as substrates in the poly[d(AT)]·poly[d(AT)] primed polymerisation with *E.coli* DNA polymerase I (Klenow fragment) was compared (Figure 5). As expected reaction mixtures containing dTTP and dATP (at saturating substrate concentrations) underwent a marked decrease in uv absorption (>17% hypochromicity) over a 3 hour period, demonstrating that a substantial degree of polymerisation had occurred. In an analogous reaction in which d^{3C}ATP was used in place of dATP no hypochromic effect was observed indicating that d^{3C}ATP was

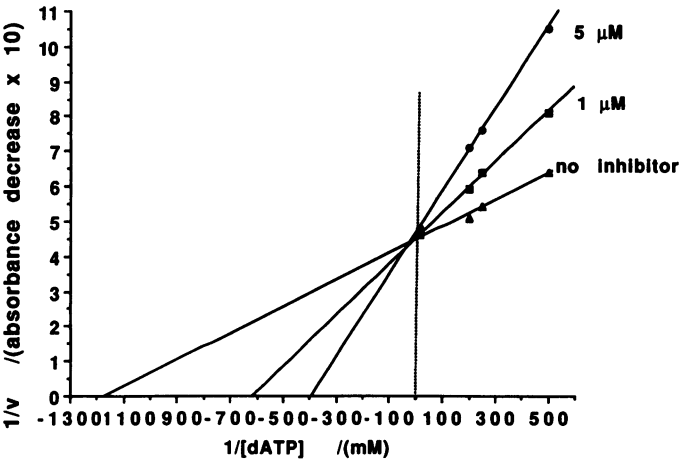

Figure 6. Inhibition of poly[d(AT)]·poly[d(AT)] primed polymerisation of dATP and dTTP by $d^{3C}ATP$. Reaction mixtures contained *E.coli* DNA polymerase I (2 units, 0.35 μg), dTTP (0.1 mM) , dATP (concentrations of 2, 4, 5, and 50 μM) in the presence of $d^{3C}ATP$ (concentrations of 0, 1 and 5 μM). Buffer and salt concentrations are detailed in the experimental section. The decrease in absorbance was recorded after 30 min.

not a substrate for *E.coli* DNA polymerase I. A co-polymerisation experiment which contained equimolar amounts of $d^{3C}ATP$ and dATP developed less than 4% hypochromicity, suggesting that $d^{3C}ATP$ was inhibiting the polymerisation. In a more detailed kinetic analysis Lineweaver-Burk plots for the polymerisation of dATP and dTTP in the presence of varying concentrations of $d^{3C}ATP$ clearly demonstrated that $d^{3C}ATP$ is a competitive inhibitor of *E.coli* DNA polymerase I (Figure 6). From a quadratic Lineweaver-Burk plot (not shown) the K_i value for $d^{3C}ATP$ was calculated to be 4.5 ± 1 μM, this value is greater than Km for dATP which was 0.85 μM (the Km is in good agreement with the previously reported value of 1 μM [33]). $d^{3C}ATP$ was also shown not to be a substrate for *Micrococcus luteus* DNA polymerase, a more detailed kinetic analysis to ascertain whether it was a competitive inhibitor was not performed.

Previous studies have demonstrated that a range of purine analogues[34-37] can be incorporated into DNA using DNA polymerases. Particularly relevant to the present study are the findings that 7-deazapurine-2'-deoxynucleoside triphosphates[35] and derivatives which are labelled at the 7-position[36,37] are substrates for *E.coli* DNA polymerase I. In view of these results it is perhaps surprising that $d^{3C}ATP$, an analogue which does not interfere with Watson-Crick hydrogen bonding or introduce a bulky substituent into the purine ring, is not a substrate for this enzyme. However, the findings presented here are of interest since whilst several 3-deazaadenosine analogues are potent antiviral agents through their ability to inhibit *S*-adenosylhomocysteine hydrolase,[38] this is to our knowledge, the first report of a 3-deazaadenine nucleoside that is potentially (*i.e.* after phosphorylation) able to act as an inhibitor of DNA polymerase.

EXPERIMENTAL

High pressure liquid chromatography was performed on a Varian 5000 liquid chromatograph equipped with a UV50 variable wavelength detector and integration was performed either with a Hewlett Packard integrator or by interfacing to an Apple IIe microcomputer fitted with a standard peak integration programme. Elution gradients for the oligonucleotides were generated by mixing buffer A (0.1 M triethylammonium acetate pH 6.5) and buffer B (0.1 M triethylammonium acetate pH 6.5 containing 65% acetonitrile) as described. Nucleoside triphosphate mixtures were analysed with a linear 10 minute gradient of 5−30% acetonitrile in 50 mM potassium phosphate buffer pH 6.0 containing 25 mM tetra-*n*-butylammonium hydrogen sulphate. Analytical columns (25×0.45 cm) were packed with 5μ reverse phase C18 silica and used with a flow rate of 1 mL/min. Thermal melting point (Tm) measurements on the oligonucleotides and the recording of their circular dichroism spectra were performed as previously described[21]. Proton nmr spectra were recorded on either a Perkin Elmer R34 (220 MHz) or a Brüker WM250 (250 MHz) instrument. The latter machine was also used to obtain ^{31}P nmr spectra at 101.26 MHz. For proton spectra an internal standard of tetramethylsilane was used; phosphorus spectra were referenced to an external standard of 85% phosphoric acid and were recorded with broad band proton decoupling. Uv spectra were recorded on a Perkin Elmer Lambda 5 spectrophotometer equipped with a thermostatic cell holder.

Acetic acid was dried by refluxing with acetic anhydride (5 mL/100 mL) and followed by fractional distillation. Pyridine was refluxed and distilled consecutively from ninhydrin and potassium hydroxide. Iron powder was stirred with dilute hydrochloric acid (4 M) for five minutes then filtered, washed with water and acetone, and dried by suction.

Diethoxymethylacetate was prepared by modification of an existing procedure;[39] in particular, in order to perturb the equilibrium in favour of the product it was found necessary to slowly remove the ethyl acetate as it was formed by distillation. 2-Cyanoethyl *N,N*-diisopropylaminochlorophosphine[40] and tetrakis(tetra-*n*-butylammonium) hydrogen triphosphate[41] were prepared as described previously. The *Eco* RV endonuclease was purified essentially as described previously[24], more detailed information will be reported elsewhere[30]. Polymerase enzymes were purchased from Boehringer (*E.coli* DNA polymerase I Klenow fragment) and Sigma (*Micrococcus luteus* DNA polymerase).

Synthesis of 2,6-dichloro-4-nitropyridine (4)

To 2,6-dichloropyridine-*N*-oxide (50.98g, 0.311 mol) was added 95% fuming nitric acid (82 mL) and 98% concentrated sulphuric acid (200 mL). The mixture was then heated at 148°C for one hour and then at 156°C, until the evolution of nitrogen dioxide had ceased and a dark green/black solution remained. After cooling to room temperature, the mixture was poured onto crushed ice (300 g) and concentrated aqueous ammonia (28%) added with stirring, whilst maintaining the mixture below 40°C (ice bath). After the solution had reached pH 6 the yellow solid which had precipitated was filtered and dried in *vacuo*. The product was obtained as analytically pure yellow flakes after recrystallisation from petroleum ether.

Yield 33.74g, 0.175 mol, 56%; ; Rf (dichloromethane/heptane 95:5): 0.90; ^1H-nmr ($CDCl_3$), 8.02 (2H, s, H3, H5); $n_{max.}$ (KBr disc.), 1553, 1350 cm^{-1} (NO_2); m.p., 99-100°C ; (Found: C, 31.02; H, 1.09; N, 14.50. $C_5H_2Cl_2N_2O_2$ requires C, 31.12; H, 1.04; N, 14.52%).

Preparation of 4-amino-2,6-dichloropyridine (5)

Iron powder was added portionwise to a solution of 2,6-dichloro-4-nitropyridine (**4**) (20.82 g, 108 mmol) in glacial acetic acid (200 mL) and the mixture refluxed for 1 hour. After cooling to room temperature, the reaction mixture was neutralised with 35% (w/v) potassium hydroxide solution. The product was extracted into ethyl acetate (5×200 mL), the combined extracts dried (magnesium sulphate) and evaporated in *vacuo* to produce a white solid. An analytical sample was obtained as colourless needles after recrystallisation from water/methanol.

Yield, 14.3 g, 87.9 mmol, 82%; Rf (dichloromethane/heptane 95:5): 0.53; ^{1}H-nmr ($(CD_3)_2CO$), 6.14, (2H, s, NH_2), 6.54, (2H, s, H3, H5); m.p., 173−175°C; (Found: C, 36.72; H, 2.50; N, 17.50. $C_5H_4Cl_2N_2$ requires C, 36.84; H, 2.47; N, 17.19%).

Preparation of 3,4-diamino-2,6-dichloropyridine (8)

4-Amino-2,6-dichloro-3-nitropyridine (12.9 g, 61.8 mmol) was treated with iron powder (18.3g) in glacial acetic acid (180 mL) and the mixture heated at reflux for 80 minutes. After cooling to room temperature and neutralisation with 35% (w/v) potassium hydroxide solution the mixture was filtered through celite. The celite was then washed with glacial acetic acid (50 mL) and water (50 mL) and the filtrate neutralised once more. The product was extracted into ethyl acetate (4×200 mL) and the combined extracts dried (magnesium sulphate), filtered and then evaporated in *vacuo*. The pure amine was obtained as white needles after recrystallisation from water with decolourising charcoal.

Yield 7.54 g, 39.5 mmol, 64%; Rf (dichloromethane/heptane 95:5): 0.29; ^{1}H-nmr ($(CD_3)_2CO$), 4.44 (2H, s, NH_2), 5.65 (2H, s, NH_2), 6.60 (1H, s, H5); m.p., 191−193°C (Found: C, 33.78; H, 2.84; N, 23.46%. $C_5H_5Cl_2N_3$ requires C, 33.74; H, 2.83; N, 23.60%).

Preparation of 4,6-dichloroimidazo[4,5-c]pyridine

3,4-diamino-2,6-dichloropyridine (5.5 g, 30.3 mmol) was refluxed with diethoxymethylacetate (15 mL, 91 mmol) for 2 hours. After cooling the mixture to room temperature the precipitated product was collected by filtration and washed with a little hot water. The filtrate was evaporated and the residue purified by column chromatography on silica gel eluting with a gradient of methanol (0-15%) in dichloromethane, the appropriate fractions were pooled and the solvent removed in *vacuo* to give an additional portion of the product. An analytical sample was obtained as white flakes after recrystallisation from water.

Yield 5.2 g, 27.9 mmol, 92%; Rf (dichloromethane/methanol 9:1): 0.66; ^{1}H-nmr ($(CD_3)_2SO$), 7.73 (1H, s, H7), 8.54 (1H, s, H2); m.p., 253.5−255°C. (Found: C, 38.30; H, 1.61; N, 22.30%. $C_6H_3Cl_2N_3$ requires C, 38.33; H, 1.61; N, 22.35%).

Preparation of 6-*N*,*N*-dibenzoyl-5′-*O*-(4,4′-dimethoxytrityl)-3-deaza-2′-deoxyadenosine (10)

3-Deaza-2′-deoxyadenosine (0.125 g, 0.50 mmol) was dried by co-evaporation of dry pyridine (3×10 mL) then over phosphorus pentoxide in *vacuo* overnight. It was then suspended in dry pyridine (5 mL) and chlorotrimethylsilane (0.32 mL, 2.5 mmol) added to the stirred solution at room temperature. After 30 minutes, benzoyl chloride (0.29 mL, 2.5 mmol) was added and stirring continued for 3 hours. Cold, saturated aqueous sodium bicarbonate solution (10 mL) was then added and after a further four hours the solution was extracted with ethyl acetate (1×100 mL, 1×50 mL). The combined extracts were dried (sodium sulphate), filtered and then evaporated in *vacuo* to afford an orange-yellow oil. This was then dried by co-evaporation with dry pyridine (2×10 mL) then over phosphorus pentoxide in *vacuo* overnight. The oil was then suspended in dry pyridine (5 mL) and to the solution, was added, 4,4′-dimethoxytrityl chloride (0.237 g, 0.7 mmol, 1.4 equiv.), 4-*N*,*N*-dimethylaminopyridine (3 mg, 0.025 mmol) and dry triethylamine (0.1 mL, 0.72 mmol). The reaction was monitored by silica tlc (developed in 10% methanol/chlorform) and further additions of 4,4′-dimethoxytrityl chloride and triethylamine (in 0.5 equivalent portions) made until all the 5′-hydroxynucleoside (Rf = 0.37) had been consumed. After this time methanol (0.1 mL) was added and after five minutes the mixture was poured onto cold saturated aqueous sodium bicarbonate solution (25 mL), and the product extracted into dichloromethane (3×50 mL). The combined extracts were dried (sodium sulphate), filtered and then evaporated in *vacuo*. The crude product was purified by silica gel column chromatography (60 g) using a gradient of methanol (0−2%) in dichloromethane containing about 0.5% (v/v) pyridine. Appropriate fractions were pooled, evaporated in *vacuo* and the product obtained as a white powder after precipitation from dichloromethane into pentane, followed by centrifugation of the resulting suspension.

Yield, 212.2 mg, 0.28 mmol, 56%; Rf (dichloromethane/methanol 9:1): 0.49; ^{1}H-nmr ($CDCl_3$), 2.40−2.61 (2H, m, H2′ and H2″), 3.34 (2H, m, H5′ and H5″), 3.73 (3H, s, CH_3O), 3.74 (3H, s, CH_3O), 4.09 (1H, m, H4′), 4.61 (1H, m, H3′), 6.19 (1H, t, H1′, J = 6.6 Hz), 6.75 (2H, d, ArH), 6.79 (2H, d, ArH), 7.19−7.39 (16H, m, H3, ArH), 7.82 (4H, d, ArH), 7.95 (1H, d, H2, J 5.7 Hz), 8.05 (1H, s, H8); m/z (FAB$^+$), 761 $(M+H)^+$, 343 $(base(bz)_2+2H)^+$.

Preparation of 6-*N*,*N*-Dibenzoyl-5′-*O*-(4,4′-dimethoxytrityl)-3-deaza-2′-deoxyadenosine-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (11)

6-*N*,*N*-Dibenzoyl-5′-*O*-(4,4′-dimethoxytrityl)-3-deaza-2′-deoxyadenosine (10) (250 mg, 0.329 mmol) was dissolved in dry dichloromethane (5 mL) and stirred under argon whilst dry *N*,*N*-diisopropylethylamine (0.23 mL, 1.32 mmol) and 2-cyanoethyl *N*,*N*-diisopropylaminochloro-phosphoramidite (0.13 mL, 0.660 mmol) were added. After one hour methanol (0.1 mL) was added and the solution was taken up in dry argon-saturated ethyl acetate (100 mL). This was then washed with 5% aqueous sodium bicarbonate solution (2×20 mL) and saturated brine (2×20 mL), dried (sodium sulphate), then evaporated in vacuo. The crude product was purified by silica gel column chromatography (12 g), eluting with dry ethyl acetate/ dichloromethane/triethylamine (5:4:1, v/v/v). Appropriate fractions were pooled, evaporated in *vacuo* then the residue precipitated from dry argon-saturated ethyl acetate into chilled (−78°C), dry pentane in centrifuge tubes. The product was obtained as a white powder after centrifugation and drying over phosphorus pentoxide in *vacuo*.

Yield, 218 mg, 0.227 mmol, 69%; ^{31}P nmr ($CDCl_3$), 148.4, 148.5 ppm; m/z (FAB$^+$), 977 $(M+H+O)^+$, 961 $(M+H)^+$.

Synthesis and characterisation of oligonucleotides

Oligonucleotides were prepared on an Applied Biosystems 381A automatic DNA synthesiser on a 1 μmole scale and were purified by reverse phase hplc as previously described.[20] Base composition analysis was performed by digestion of about 0.5 A_{260} units of the appropriate oligonucleotide with snake venom phosphodiesterase (5 μg) and alkaline phosphatase (special quality

for molecular biology) (5 μg) in 0.5 mL of 10 mM potassium dihydrogen phosphate buffer (pH 7.0) containing 10 mM magnesium chloride. After one hour digestion mixtures were analysed by reverse phase C18 hplc. An elution gradient was employed as follows; buffer A for five minutes, 0−25% B over 10 minutes, and finally 25−75% B over an additional 10 minutes. The deoxynucleosides were generally eluted in the following order dC, $d^{3C}A$, dG, dT and dA. The retention time of $d^{3C}A$ was very sensitive to day-to-day variations on the chromatography conditions and was sometimes eluted after dG. Eluted peaks were identified by coinjection with authentic standards. Nucleosides were detected at 254 nm and quantified by peak integration using the following extinction coefficients (M^{-1} cm^{-1}) dC 6×10^3, dG 13.5×10^3, dT 7×10^3, dA 14.3×10^3 [42] and $d^{3C}A$ 9.3×10^3.

To determine the extinction coefficients of the oligonucleotides the enzymatic digestion was monitored by uv spectrophotometry at 254 nm until the absorbance stopped increasing. The hyperchromicity was calculated by dividing the final absorbance by the initial reading. The extinction coefficient of the oligonucleotide is the sum of the extinction coefficients of the constituent nucleosides divided by hyperchromicity.

Endonuclease assay

The endonuclease reactions were performed at 25°C in 200 μl volumes of 50 mM Hepes.sodium hydroxide pH 7.5 containing 100 mM sodium chloride 10 mM magnesium chloride and 20 μM double stranded oligonucleotide. The enzyme concentration was 0.1 μM (dimeric enzyme) for the natural sequence and 1 μM for the modified sequences. The reactions were monitored by hplc using a gradient of 0−25% buffer B over 20 minutes.

Preparation of 5′-*O*-p-toluenesulphonyl-3-deaza-2′-deoxyadenosine (13)

3-Deaza-2′-deoxyadenosine (0.4 g, 1.6 mmole) was dried by co-evaporation with dry pyridine (4×30 ml) and then dissolved in hot dry pyridine (15 ml). The solution was cooled in an ice bath and a solution of freshly recrystallised (dry 40−60 petroleum ether) *p*-toluenesulphonyl chloride (306 mg, 1.6 mmole) in dry pyridine (6 ml) was added dropwise with stirring. The resulting yellow solution was stirred at 0°C for 2 hours and then at room temperature. After 16 hours tlc indicated that the reaction was not complete and a further portion of *p*-toluenesulphonyl chloride (76 mg, 0.4 mmole) was added. The reaction was stirred for a further 4 hours, and then quenched by the addition of saturated sodium bicarbonate solution (50 ml). The solution was extracted with chloroform (3×20 ml) and the combined organic layers dried and evaporated under reduced pressure. Traces of pyridine were removed by co-evaporation with ethanol and the resulting white foam subjected to purification by chromatography on neutral alumina (35 g). The product was eluted with a gradient of methanol in dichloromethane 0−5%.

Yield, 0.33 g, 0.82 mmol, 51%; 1H nmr (200 MHz, $(CD_3)_2CO$), 2.34 (1H, s, CH_3), 2.46 (1H, m, H2″), 2.68 (1H, m, H2″), 4.16 (1H, m, H4′), 4.25 (2H, m, H5′ and H5″), 4.55 (1H, m, H3′), 6.31 (1H, t, H1′), 6.87 (1H, d, J = 6.0 Hz, H3), 7.30 (2H, d, J = 8.5 Hz, ArH), 7.71 (3H, m, ArH and H2), 8.19 (1H, s, H8); m/z (FAB^+) 405 (M+H) 100%, 135 (base + 2H) 70.5%.

Preparation of 3-deaza-2′-deoxyadenosine-5′-*O*-triphosphate ($d^{3C}ATP$) (1)

3-Deaza-2′-deoxyadenosine (403 mg, 0.62 mmol) dried under *vacuo* over phosphorus pentoxide was dissolved in dry acetonitrile (0.6 ml) and tetrakis(tetra-*n*-butylammonium) hydrogen triphosphate (511 mg, 0.94 mmol) added to the solution. The reaction was stirred under argon at room temperature and 5 μl aliquots withdrawn periodically for hplc analysis. After 48 hours a significant proportion of the starting material remained and the reaction mixture was heated at 40°C for a further 24 hours. Distilled water (5 ml) was then added and the solution purified by ion exchange chromatography on DEAE A-25 Sephadex (5×17 cm) eluting with a linear gradient of triethylammonium bicarbonate 0.05−1.0 M over 2.4 L. Fractions containing the product were pooled, evaporated and rechromatographed on DEAE A-25 Sephadex with a shallower elution gradient 0.05−0.8 M over 3.5 L. The desired fractions were pooled, evaporated, co-evaporated with water and the product converted to its sodium salt by application to a Dowex-50W-X-8 column (100−200 mesh, 30 equivalents sodium form) and elution with three column volumes of distilled water. The product was obtained as a fluffy white powder after lyophilisation.

Yield 1650 A_{260} units, 0.18 mmoles, 27%; hplc retention time 8.8 min; 1H nmr (200 MHz, D_2O), 2.60−2.92 (2H, m, H2′ and H2″), 3.89 (2H, m, H5′ and H5″), 4.24 (1H, m, H4′), 4.73 (1H, m, H3′), 6.47 (1H, t, H1′), 7.20 (1H, d, H3), 7.83 (1H, d, H2), 8.40 (1H, s, H8); ^{31}P nmr (81 MHz, D_2O), −10.96 (d, J = 19 Hz), −11.46 (d, J = 19 Hz), −23.3 (t, J = 19 Hz); m/z FAB^- 489 $(M-H)^-$, 511 $(M-2H + Na)^-$, 533 $(M-3H + 2Na)^-$.

Enzymatic polymerisations

Conditions used for polymerisation are essentially those described previously by Sági *et al.*[43] Reaction mixtures contained 60 mM potassium phosphate pH 7.4, 6 mM magnesium chloride, 0.4 mM EDTA, 5 μM (nucleotide concentration) sonicated poly[d(AT)]·poly[d(AT)] primer and concentrations of nucleotides and polymerase as described in the figure legends, in a total volume of 300 μl. The enzyme was added to the preincubated reaction mixture at 37°C and the polymerisation monitored by the development of hypochromicity at 260 nm.

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