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A Novel Combined Chemical–Enzymatic Synthesis of Cross-Linked DNA Using a Nucleoside Triphosphate Analogue†

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ABSTRACT: A novel method using combined chemical and enzymatic reactions to allow the preparation of covalently cross-linked DNA duplexes has been described. The method can be used to specifically link two complementary bases of a DNA duplex containing all four natural bases. The modified nucleotide 9-(2-deoxy-5-*O*-triphospho-β-D-ribofuranosyl)-N^6,N^6-ethano-2,6-diaminopurine (6edDTP) was prepared by total chemical synthesis and was found to be incorporated into DNA duplexes in the place of 2′-deoxyguanosine 5′-*O*-triphosphate by the Klenow fragment of *Escherichia coli* DNA polymerase I, T4 and T7 DNA polymerases, avian myeloma virus reverse transcriptase, and rat DNA polymerase β. Once incorporated, the aziridine of the nucleotide is rapidly opened by the N4 of the cytosine on the complementary strand to give cross-linked DNA, where the modified nucleotide is covalently joined to the complementary base by an ethano linkage. The duplexes produced were found to be recognized as substrates by various DNA polymerases. The K_m for the incorporation of the 6edDTP into DNA catalyzed by the Klenow fragment of *E. coli* DNA polymerase I was found to be 29 μM, and the k_{cat} was found to be 0.014 s^{-1}. The modified nucleoside also served as a substrate for terminal deoxynucleotidyltransferase, where it was added to single-stranded DNA and then hybridized to a complementary strand, after which cross-linking of the two strands occurred within 1 min.

The ability to covalently cross-link polynucleotide duplexes is of considerable biochemical and clinical importance. The mechanisms of action of various enzymes (such as integrases, recombinases, restriction enzymes, and exonucleases) on DNA duplexes require local melting or distortion of the duplexes for activity. The study of the interaction of these enzymes with DNA would be aided by the availability of covalently cross-linked DNA duplexes, if such duplexes could be prepared to resemble closely normal DNA, except for the cross-linked bases. The interaction of the exonuclease activity of the Klenow fragment of *Escherichia coli* DNA polymerase I has been studied, for example, by the action of the enzyme on duplexes containing a cross-linked cytosine–cytosine base pair (Cowart et al., 1989). It was found that at least four base pairs of the primer terminus must melt out in order for the enzyme to be able to remove a base pair through exonuclease activity. Other polymerases had differing requirements.

The cytotoxicity of many antitumor compounds is thought to be due to their ability to induce interstand cross-linking of cellular DNA (Mitchell et al., 1989). DNA cross-linked by naturally occurring compounds like psoralens or the clinically important synthetic bifunctional alkylating agents becomes resistant to in vivo repair, and these lesions impair the ability of the target to undergo transcription. This has been implicated as the mechanism of efficacy for some compounds active against malignant cells, and as a result, various cross-linked DNA duplexes have been synthesized for study (Kohn, 1979).

The ability of single-stranded polynucleotides to recognize and bind to their complementary strands forms the basis for various exciting clinical and biological applications (Knorre & Vlassov, 1985). A DNA oligomer containing an alkylating agent could bind to its complementary messenger RNA and, once covalently cross-linked, shut down the synthesis of the corresponding protein. The inhibition of protein biosynthesis with antisense oligonucleotides has been reported (Zamecnik & Stevenson, 1978; Jayaraman et al., 1981), but the ability to form a covalent bond with their complementary oligonucleotides might make them more efficient in this process. This principle has been demonstrated by Vlassov et al. (1984), who showed that antibody production in a mouse myeloma

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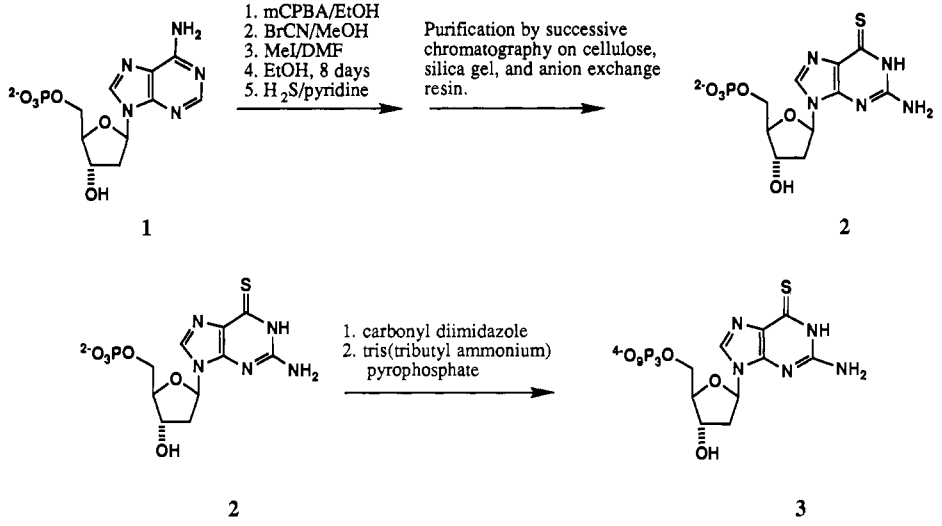
Scheme I: Chemical Synthesis of 6-Thio-2′-deoxyguanosine 5′-*O*-Triphosphate (3)



cell line could be halted by perfusion of the cells with an alkylating oligonucleotide complementary to the mRNA coding for the κ light chain of the IgG.[1]

Various methodologies have been developed to allow the preparation of cross-linked DNA duplexes, each with its own advantages and disadvantages. Certain drugs (nitrogen and sulfur mustards, etc.) and natural agents can cross-link DNA, but these suffer from a lack of complete specificity with regard to which bases become covalently attached. Synthetic ribo- and deoxyribooligonucleotides have been chemically modified so as to place mustard or α-haloketone alkylating groups at the 3′ or 5′ end, or to attach noble metals to oligomers, and thus make these capable of cross-linking to their complementary strands (Summerton & Bartlett, 1978; Iverson & Dervan, 1987; Meyer et al., 1989). These methods suffer from the fact that they produce adducts which grossly perturb the structure of the DNA duplex, and so their utility as substrates for the study of enzymes that act on normal DNA is minimal.

Webb and Matteucci (1986a,b, 1987) prepared phosphoramidite derivatives of deoxynucleotide bases which, after chemical conversion of oligodeoxynucleotides, could be treated with aziridine to give polynucleotides, which bound to and cross-linked their complementary oligonucleotides. The method allows the placement of the covalent linkage at specific places in the duplexes but requires a cytosine–cytosine or cytosine–adenosine mismatch at the site of the cross-link. Also, the method does not allow the preparation of duplexes containing all four natural bases of DNA.

We desired a high-yield method for the preparation of covalently cross-linked DNA duplexes containing all four bases, in which the cross-links might be specifically incorporated into synthetic oligonucleotides or natural substrates like plasmids. It was also desired that the method place the cross-link between two complementary base pairs so that the lesion could be accommodated in the normal B-DNA structure with a minimum of structural perturbation.

We report a methodology for covalently cross-linking DNA duplexes which improves on the methods described previously. An aziridine-containing triphosphate analogue is prepared which can be incorporated into DNA by DNA polymerases opposite a cytosine. Once incorporated, the nucleophilic N4 of cytosine apparently opens the aziridine ring, forming cross-linked DNA duplexes in high yield.

Experimental Procedures

Materials. Deoxyribonucleoside triphosphates were from Pharmacia. Radiolabeled nucleotides were from New England Nuclear. Deoxyadenosine monophosphate was from Aldrich. Klenow fragment, an exonuclease-deficient mutant of Klenow fragment, and the Y766S exonuclease deficient mutant of Klenow fragment of *E. coli* DNA polymerase I were prepared and purified from plasmids supplied by Catherine Joyce (Yale University School of Medicine). T4 DNA polymerase was purified from a cloned overproducer supplied by William Konigsberg (Yale University School of Medicine). T4 polynucleotide kinase, avian myeloma virus reverse transcriptase, terminal deoxynucleotidyltransferase from calf thymus, and Sequenase were from U.S. Biochemical Corp. Mammalian (rat) DNA polymerase β was prepared and purified from *E. coli* strains containing the corresponding plasmid, supplied by Samuel Wilson at the National Institutes of Health. T7 DNA polymerase was prepared by mixing T7 gene 5 protein and *E. coli* thioredoxin (both prepared from cloned overproducers supplied by Arne Holmgren of the Karolinska Institute of Stockholm, Sweden) in a ratio of 1:7. Scintiverse II liquid scintillation cocktail was from Fisher. Poly(ethylenimine) thin-layer chromatography plates, flash chromatography grade silica gel, and fluorescent silica gel TLC plates were from E. Merck Co. (Darmstadt, FRG). Fibrous cellulose was from Whatman. Fluorescent cellulose on polyester TLC plates was from Sigma. Sephadex A-25 was from Sigma. NMR spectra were recorded on a Bruker WM-360 spectrometer. Ultraviolet absorbance spectra were recorded on a Perkin-Elmer Lambda 3840 diode array spectrometer. All other reagents were of the highest quality commercially available.

Electrophoresis. DNA oligomers were analyzed by quenching reaction solutions into 90% formamide buffer (90 mM Tris–borate and 10 mM EDTA, pH 8.3), followed by electrophoresis on polyacrylamide denaturing gels (PAGE).

[1] Abbreviations: 6edDTP, 9-(2-deoxy-5-*O*-triphospho-β-D-ribofuranosyl)-N^6,N^6-ethano-2,6-diaminopurine; IgG, immunoglobulin G; TLC, thin-layer chromatography; PEI, poly(ethylenimine); EDTA, ethylenediaminetetraacetic acid; PAGE, (denaturing, 8 M) polyacrylamide gel electrophoresis; TdGMP, 6-thio-2′-deoxyguanosine 5′-*O*-monophosphate; TdGTP, 6-thio-2′-deoxyguanosine 5′-*O*-triphosphate; TEAB, triethylammonium bicarbonate; dAMP, 2′-deoxyadenosine 5′-*O*-monophosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; exo$^-$, exonuclease deficient; NMR, nuclear magnetic resonance; DTT, dithiothreitol; dNTP, 2′-deoxyribonucleoside 5′-*O*-triphosphate; mCPBA, *m*-chloroperbenzoic acid; DMF, dimethylformamide.

Bands corresponding to oligomers were visualized by autoradiography and quantified by cutting out the bands and counting in Scintiverse II with a Beckman LS 6800 scintillation counter.

Preparation of 6-Thio-2′-deoxyguanosine 5′-O-Monophosphate (**2**). 2′-Deoxyadenosine monophosphate (**1**) was converted to 6-thio-2′-deoxyguanosine 5′-*O*-monophosphate (TdGMP, **2**) in five steps (Scheme I). However, the material produced by the published procedure (Ueda et al., 1978) was found to be contaminated with at least 20 products and was wholly unsuitable for chemical synthesis without further purification. The ammonium salt of the TdGMP (95 mg) was adsorbed onto a 1.9 × 45 cm column of Whatman CF-11 fibrous cellulose and eluted with a 7:3:2 mixture of acetonitrile, 0.1 M NH_4CO_3H, and 12 M ammonium hydroxide. The product eluting at R_f 0.14 was collected and concentrated in vacuo. This was the only product collected that was found to be hydrolyzed by 5′-nucleotidase from *Crotalus adamanteus*. Products were assayed in 10-μL reactions containing 0.3 unit of nucleotidase and 0.1 μmol of the suspected monophosphate in 50 mM glycine and 10 mM $MgCl_2$ at pH 8.9 for 2 h. Samples were spotted on TLC plates, and those exhibiting a change in R_f were assumed to be 5′-monophosphates with the base in the β configuration. The crude TdGMP was further purified by chromatography on a 1.9 × 30 cm column of silica gel, eluting with 0.3 M ammonium acetate in 65% ethanol. The material with R_f 0.49 was found to have a λ_{max} of 343 nm and was a substrate for nucleotidase. Fractions containing this product were combined and concentrated in vacuo. This material was further purified by ion-exchange chromatography on a 1.0 × 10 cm column of Sephadex A-25, eluting with 0–0.6 M TEAB (pH 7.6). The desired product eluted at 0.5 M TEAB and has a λ_{max} of 343 nm at pH 7.0. Fractions containing the TdGMP were combined and concentrated in vacuo to a clear glass, for an overall yield of 1.5% from dAMP.

Preparation of 6-Thio-2′-deoxyguanosine 5′-O-Triphosphate (**3**). 6-Thio-2′-deoxyguanosine 5′-*O*-monophosphate (**2**, 59 μmol) was converted to 6-thio-2′-deoxyguanosine 5′-*O*-triphosphate (Yoshida et al., 1979) by the method of Hoard and Ott (1965) and purified on a 2.5 × 25 cm column of Sephadex A-25, eluting with 0–0.7 M TEAB (pH 7.6). Fractions eluting at 0.65 M TEAB were concentrated in vacuo to a glass. The product, λ_{max} 343 (pH 7.0), had R_f 0.15 on PEI–cellulose TLC plates developed with 0.3 M sodium phosphate (pH 7.0) and was obtained in 48% yield after purification (Scheme I). The TdGMP and TdGTP desulfurize in aqueous solution with a $t_{1/2}$ of 4 days at room temperature and were kept at −70 °C.

Preparation of 9-(2-Deoxy-5-O-triphospho-β-D-ribofuranosyl)-N^6,N^6-ethano-2,6-diaminopurine (6edDTP, **5**). To a stirred solution of 2.8 μmol of TdGTP (**3**) in 1.9 mL of 20 mM sodium phosphate at pH 7.0 was added 0.4 mL of a 0.1 M solution of sodium periodate (Scheme II). After 10 min at room temperature, 64 μL of 1 M rhamnose was added to quench the excess periodate. After 10 min, 160 μL of aziridine (Allen et al., 1963) was added, and the mixture was kept at room temperature for 24 h. The reaction was worked up by addition of 1 mL of 50 mM HEPES–KOH (pH 7.9), followed by lyophilization until all but about 0.1 mL of ice remained. The sample was immediately diluted to 1 mL with water and purified on a Whatman Partisil 10-SAX anion-exchange column, eluting with a 10-mL gradient of 50 mM HEPES–KOH (pH 7.9) to 0.75 M KCl–50 mM HEPES–KOH (pH 7.9), and then with a 60-mL gradient of 0.75 M KCl–50 mM HEPES–KOH (pH 7.9) to 1 M KCl–50 mM HEPES–KOH (pH 7.9), at a flow rate of 2 mL/min. The 6edDTP (35% yield) eluted at 0.8 M KCl as the major product and was immediately frozen and stored under liquid nitrogen. Thin-layer chromatography on PEI TLC plates revealed R_f 0.43 (0.3 M sodium phosphate, pH 7.0). The ultraviolet absorbance spectrum showed λ_{max} 297 nm and ϵ 10 500 M^{-1} cm^{-1} at pH 7.5. ^{31}P NMR (pH 7.9, 30% D_2O) showed resonances at δ 10.6 (doublet), δ 14.2 (doublet), and δ 25.5 (triplet) with trimethyl phosphate in water as an external reference.

Scheme II: Chemical Synthesis of the Cross-Linking Agent 9-(2-Deoxy-5-*O*-triphospho-β-D-ribofuranosyl)-N^6,N^6-ethano-2,6-diaminopurine (6edDTP, **5**)

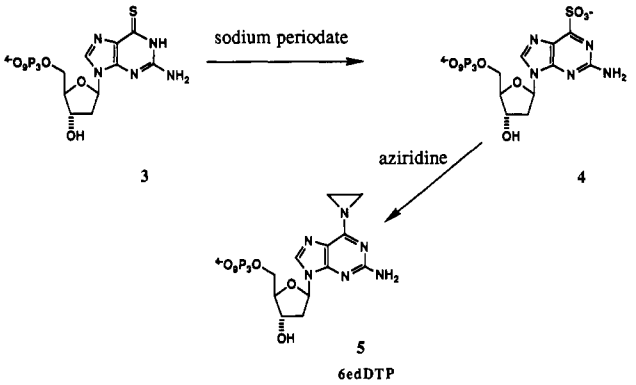


Chart I: Oligonucleotides Used as Substrates for Cross-Linking by 6edDTP (**5**)

5′	TCGCAGCCGTCCT	**6**
5′	AAACCACAGGACGGCTGCGA	**7**
5′	TCAGATCCAAGTCAGGACGGCTGCGA	**8**
5′	TCGCAGCCGTCCTD	**9**
5′ 3′	TCGCAGCCGTCCTD AGCGTCGGCAGGACACCAAA	**10**
5′ 3′	TCGCAGCCGTCCTD AGCGTCGGCAGGACTGAACCTAGACT	**11**
5′ 3′	TCGCAGCCGTCCA AGCGTCGGCAGGTTCCCAAA	**12**
5′ 3′	TCGCAGCCG AGCGTCGGCAGGTTCCCAAA	**13**
5′ 3′	TCGCAGCCGT AGCGTCGGCAGGTTCCCAAA	**14**
5′ 3′	TCGCAGCCGTCCT AGCGTCGGCAGGACACCAAA	**15**

Enzymatic Reactions of 6edDTP. The incorporation of 6edDTP into 13/26-mer (**6**/**8**; see Chart I) was catalyzed by DNA polymerases. A reaction solution (10.3 μL) containing 56 μM 13-mer (**6**), 3.3 pmol of [γ-^{32}P]ATP, and 6 units of T4 polynucleotide kinase in buffer (50 mM HEPES–KOH, 10 mM $MgCl_2$, at pH 7.6) was incubated at 37 °C for 30 min before adding 4.1 μL of 170 μM [5′-^{32}P]-26-mer (**8**). The reaction was then heated at 70 °C for 5 min and cooled to room temperature to give a solution of 40 μM 13/26-mer, with 5′-^{32}P labels on the 13/26-mer. Aliquots of a solution of the labeled 13/26-mer (8 μM) and 190 μM 6edDTP in the above buffer were quenched into gel loading buffer after 0, 1, 4, and 20 h (lanes 1–4, Figure 1). Aliquots of a solution of 8 μM 13/20-mer (**6**/**7**), 8 μM 6edDTP, and 11 μM Klenow fragment (exo$^-$) in buffer were quenched into gel loading buffer after 2 and 10 min and 1, 4, and 20 h (lanes 5–9). Aliquots of a solution of 8 μM 13/26-mer, 8 μM 6edDTP, and 6 μM of the Y766S mutant of Klenow fragment (exo$^-$) in buffer were

quenched into gel loading buffer after 2 and 10 min and 1, 4, and 20 h (lanes 10–14). The samples were analyzed after electrophoresis on 16% PAGE, with subsequent autoradiography of the gel (Figure 1).

The apparent K_m and k_{cat} for the incorporation of 6edDTP into the 13/20-mer (**15**) were determined by quenching aliquots of a solution containing 1.0 μM labeled 13/20-mer (prepared as above), 2.0 μM of the Y766S mutant of Klenow fragment (exo$^-$), and varying amounts of 6edDTP in polymerase buffer into gel loading buffer at time points ranging from 10 s to 30 min. The samples were analyzed after electrophoresis on a 16% PAGE, with subsequent autoradiography of the gel. Gel slices containing the products were cut out and quantitated by counting in scintillation cocktail. In this way, elongation and cross-linking of the 13/20-mer could be followed over time (Figure 2). Since the enzyme is present in excess over the 13/20-mer, and both are at much higher concentration than the known K_d of the enzyme for DNA (personal communication from Dr. Steve Carroll, The Pennsylvania State University), all the DNA should be complexed with the enzyme, and the reaction should be pseudo-first-order. The pseudo-first-order progress curves were plotted and rate constants of the reactions fit to $f(t) = A - Ae^{-kt}$, where $f(t)$ is the amount of elongated product formed at time t, A is the mole fraction of 13/20-mer ultimately elongated, and k is the pseudo-first-order rate constant. The reaction of 13/20-mer with 6edDTP showed substrate inhibition above 80 μM, but an apparent K_m and k_{cat} were obtained by plotting $[6edDTP]^{-1}$ versus k^{-1} for the data below 80 μM (Figure 2).

The addition of 6edDTP onto 13-mer (**6**), catalyzed by terminal deoxynucleotidyltransferase, was studied by quenching aliquots of a 10-μL reaction containing 1.6 μM 13-mer (5′-^{32}P labeled, prepared as above), 42 μM 6edDTP, and 17 units of terminal transferase in buffer (40 mM sodium cacodylate, 8 mM $MgCl_2$, and 0.5 mM DTT, pH 7.5) into gel loading buffer after 0, 1, 2, 5, and 30 min and 3, 7.5, and 21 h. The samples were analyzed after electrophoresis on a 16% PAGE, with subsequent autoradiography of the gel. Analysis of the autoradiogram of the gel showed that, after 30 min, much of the 13-mer was elongated to a 14-mer (**9**) but that after 21 h most of the 13-mer had been elongated to much longer products.

The reaction of the 14-mer (**9**, formed by elongation of the 13-mer with 6edDTP using terminal transferase) with a complementary 26-mer (**8**) to give the cross-linked duplex (**11**) was examined. A 35-μL reaction containing 1.6 μM 13-mer (**6**) (5′-^{32}P labeled), 42 μM 6edDTP and 60 units of terminal transferase in buffer was incubated at 22 °C for 30 min and quenched by addition of 0.5 M EDTA to 30 mM. To the 14-mer reaction was added excess 26-mer (1 μL of 170 μM), after 0.5, 2, 5, 10, 15, 30, and 60 min and 3, 16, and 18 h. Aliquots were quenched into gel loading buffer for electrophoresis on a 16% PAGE. Gel slices containing the products were cut out and quantitated by counting in scintillation cocktail.

Elongation of the cross-linked 14/20-mer product (**10**) with various DNA polymerases was examined. A reaction containing 1.6 μM labeled 13/20-mer (the 13-mer was 5′-^{32}P end labeled as described above), 4.9 μM 6edDTP, and 1.6 μM Klenow fragment (exo$^-$) was incubated at 22 °C for 24 h, quenched with 0.5 M EDTA to 40 mM, and run on a 14% denaturing polyacrylamide gel. The band corresponding to the cross-linked 14/20-mer product (**10**) was cut out and purified as previously described (Cowart et al., 1989). A 10-μL reaction containing the 14/20-mer cross-linked duplex (9 nM, 4000 cpm), 4.5 μM Klenow fragment, and 80 μM TTP (lanes 1–3, Figure 3), or all four dNTPs (80 μM, lanes 4–6) in polymerase buffer, was incubated at 22 °C, and aliquots were quenched into gel loading buffer after 0, 1, and 5 min. A 10-μL reaction containing the 14/20-mer cross-linked duplex (9 nM, 4000 cpm), 14 units of avian myeloma virus reverse transcriptase, and all four dNTPs (80 μM) in polymerase buffer was incubated at 22 °C, and aliquots were quenched into gel loading buffer after 0, 1, 5, and 15 min (lanes 7–10). A 10-μL reaction containing the 14/20-mer cross-linked duplex (9 nM, 4000 cpm), 3.7 μM T4 DNA polymerase, and all four dNTPs (80 μM) in polymerase buffer was incubated at 22 °C, and aliquots were quenched into gel loading buffer after 0, 1, and 5 min (lanes 11–13). A 10-μL reaction containing the 14/20-mer cross-linked duplex (9 nM, 4000 cpm), 1.5 μM T7 DNA polymerase, and all four dNTPs (80 μM) in polymerase buffer was incubated at 22 °C, and aliquots were quenched into gel loading buffer after 0, 1, 5, and 15 min (lanes 14–17). Samples were analyzed by autoradiography following electrophoresis on a 16% PAGE.

Cross-linked 14/26-mer (**11**) was prepared and purified in the same manner as the labeled cross-linked 14/20-mer above. A 15-μL reaction containing 14/26-mer duplex (75 nM), 10 pmol of [γ-^{32}P]ATP, and 6 units of T4 polynucleotide kinase in polynucleotide kinase buffer was incubated at 37 °C for 30 min. The reaction was then heated to 70 °C for 5 min and cooled to room temperature to give radiolabeled cross-linked 14/26-mer. A reaction containing cross-linked duplex (**11**) and 1 μM Klenow fragment in polymerase buffer was incubated and was quenched 1 min after the following additions: no addition (lane 1, Figure 4); addition of dATP to 20 μM (lane 2); addition of dATP and dCTP to 20 μM (lane 3); addition of dATP, dCTP, and dTTP to 20 μM (lane 4); addition of all four dNTPs to 20 μM (lane 5). Samples were analyzed by autoradiography following electrophoresis on 16% PAGE.

The incorporation of 6edDTP into DNA duplexes by polymerases opposite any four of the nucleic acid bases followed by elongation was examined. The primer strand of duplex was labeled as described above and annealed to the corresponding template strand. Aliquots of a reaction containing the 13/20-mer (**12**) (1 μM), 5 μM 6edDTP, and Klenow fragment (exo$^-$, 4 μM) in polymerase buffer were quenched into gel loading buffer after 0, 5, and 30 min and 10 and 26 h (lanes 1–5, Figure 5). All four dNTPs were added to the reaction to 160 μM, and after 15 min, an aliquot was quenched into gel loading buffer to give lane 6. Aliquots of a reaction containing the 9/20-mer (**13**) (1 μM), 5 μM 6edDTP, and Klenow fragment (exo$^-$, 4 μM) in polymerase buffer were quenched into gel loading buffer after 0, 5, and 30 min and 10 and 26 h (lanes 7–11). All four dNTPs were added to the reaction to 160 μM, and after 15 min, an aliquot was quenched into gel loading buffer to give lane 12. Aliquots of a reaction containing the 9/20-mer (**13**) (1 μM), 5 μM 6edDTP, 7 μM dTTP (to convert **13** into **14**), and Klenow fragment (exo$^-$, 40 μM) in polymerase buffer were quenched into gel loading buffer after 0, 5, and 30 min and 10 and 26 h (lanes 13–17). All four dNTPs were added to 160 μM, and after 15 min, an aliquot was quenched into gel loading buffer to give lane 18. Aliquots of a reaction containing the 13/20-mer (**15**) (1 μM), 5 μM 6edDTP, and Klenow fragment (exo$^-$, 4 μM) in polymerase buffer were quenched into gel loading buffer after 0, 5, and 30 min and 10 and 26 h (lanes 19–23). All four dNTPs were added to 160 μM, and after 15 min, an aliquot was quenched into gel loading buffer to give lane 24. Samples

were analyzed by autoradiography following electrophoresis on 16% PAGE.

Results

A deoxynucleoside triphosphate analogue (6edDTP, **5**) has been prepared by chemical synthesis. When incorporated into DNA duplexes by DNA polymerases, a covalent cross-link is formed between this base analogue and the complementary cytosine on the opposite strand. The cross-linked duplexes have been isolated and can support polymerization by all DNA polymerases tested.

The 2′-deoxyadenosine monophosphate (**1**) was convertd to 6-thio-2′-deoxyguanosine monophosphate (TdGMP, **2**) in five steps by the method of Ueda et al. (1978) as shown in Scheme I. Although this method was found to give some of the desired monophosphate, the product was found to be contaminated with at least 20 products and was thus unsuitable for chemical synthesis without further purification. No one chromatographic procedure was found to give the TdGMP in pure form, but a combination of chromatographic steps was required. The crude TdGMP was purified successively on cellulose, silica gel, and anion-exchange chromatography. The desired product was found to possess λ_{max} 343 nm at pH 7.0 and to be susceptible to dephosphorylation by 5′-nucleotidase from *C. adamanteus* to give a nucleoside of different chromatographic mobility. After every chromatographic step, products were checked, and only the fractions meeting these two criteria were saved. In this way, TdGMP was prepared and purified in an overall 1.5% yield from dAMP. The compound was found to undergo desulfurization to dGMP with a half-life of 4 days in water at pH 7.0 at room temperature, so the product was stored at −70 °C for subsequent use.

The 6-thio-2′-deoxyguanosine monophosphate (TdGMP, **2**) was converted to the phosphorimidazolidate and subsequently to 6-thio-2′-deoxyguanosine triphosphate (TdGTP, **3**) (Yoshida et al., 1979) by the method of Hoard and Ott (1965). This compound was obtained in 48% yield after purification by anion-exchange chromatography and stored at −70 °C for subsequent use.

The 6-thioinosine and 6-thioguanosine nucleosides have been oxidized to the electrophilic 6-sulfonate derivatives in a 1 M solution of sulfite/bisulfite ion (Rackwitz & Scheit, 1974). The 6-sulfonates were readily displaced by amine nucleophiles to give the corresponding 6-aminopurine and 2,6-diaminopurine derivatives. Although 1 M sulfite/bisulfite was found to oxidize TdGTP (**3**) to the corresponding sulfonate derivative (**4**), the presence of the large amount of sulfite ion would probably interfere with the reaction of the 6-sulfonated nucleosides with amines, so an alternate reagent for the oxidation of the 6-thio moiety was desired (Scheme II). Sodium periodate has been used for the oxidation of naturally occurring 4-thiouridylate residues in transfer RNAs (Ziff & Fresco, 1969). It was found that sodium periodate at 20 mM quantitatively oxidized TdGTP to the 6-sulfonate derivative in less then 10 min. The reaction was followed by the disappearance of the 343 nm λ_{max} absorbance of the TdGTP and the appearance of the 320 nm λ_{max} absorbance of the sulfonate. Excess periodate was destroyed by the addition of rhamnose to 30 mM.

The 6-sulfonate nucleotide (**4**) was found to be readily displaced with methylamine within 15 min. The desired compound, 9-(2-deoxy-5-*O*-triphospho-β-D-ribofuranosyl)-N^6,N^6-ethano-2,6-diaminopurine (6edDTP, **5**), was produced by oxidation of TdGTP with sodium periodate, followed by reaction of the 6-sulfonate with 2 M aziridine for 24 h. 6edDTP was purified as the major product of the reaction by anion-exchange chromatography.

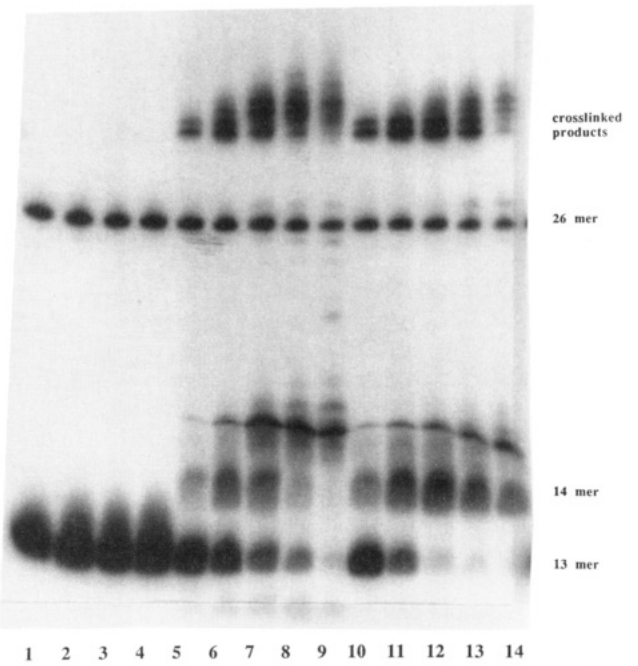


FIGURE 1: Lanes 1–4 are the control reaction of 190 μM 6edDTP and 8 μM 13/26-mer (**6**/**8**, 5′-^{32}P end labeled on both strands) after 0, 1, 4, and 20 h and show no cross-linked product formation. Lanes 5–9 show the incorporation of 6edDTP (8 μM) into 13/26-mer by excess Klenow fragment (exo$^-$) and subsequent cross-linking after 2, 10, and 60 min and 4 and 20 h. Lanes 10–14 show the incorporation of 6edDTP (8 μM) into 13/26-mer by excess Y766S mutant of Klenow fragment (exo$^-$) and subsequent cross-linking after 2, 10, and 60 min and 4 and 20 h.

The aziridine-containing nucleotide 6edDTP was found to be incorporated into test duplexes containing a 13-mer as primer and a 20- or 26-mer as template. Once incorporated, the analogue induced the two strands to become covalently cross-linked, forming a product of much lower electrophoretic mobility. As seen in Figure 1, with labeled 13-mer (**6**) as primer and 26-mer (**8**) as template, some of the 13-mer is elongated to the 14-mer (**9**), and a certain proportion reacts to give a cross-linked 14/26-mer duplex (**11**). Since the products were analyzed by electrophoresis under extreme denaturing conditions (>60 °C, 8 M urea), it can be assumed that the low-mobility products are covalently cross-linked. The reduced mobility of the cross-linked products, relative to the single-stranded primer and template from which they are produced, is due to the larger size of the cross-linked duplex. However, these cross-linked products are always more mobile than single-stranded oligonucleotides containing the same number of bases (data not shown). The reaction of the test 13/26-mer duplex with 6edDTP was found to be catalyzed by exonuclease-deficient mutants of Klenow fragment, avian myeloma virus reverse transcriptase, T7 DNA polymerase (exo$^-$, Sequenase), and rat DNA polymerase β and gave identical cross-linked duplexes with all exonuclease-deficient polymerases tested (data not shown). The concentrations and times required for 6edDTP incorporation/cross-linking were different for Klenow fragment, Sequenase, reverse transcriptase, and rat DNA polymerase β, indicating different K_m's and/or k_{cat}'s for incorporation.

A control reaction with 13/26-mer duplex in the presence of 190 μM 6edDTP was found to give no new products in the absence of a polymerase even after 20 h, showing the requirement for incorporation of the analogue into the duplex for cross-linking to occur. It should be noted that the distribution of cross-linked products seen was identical when the

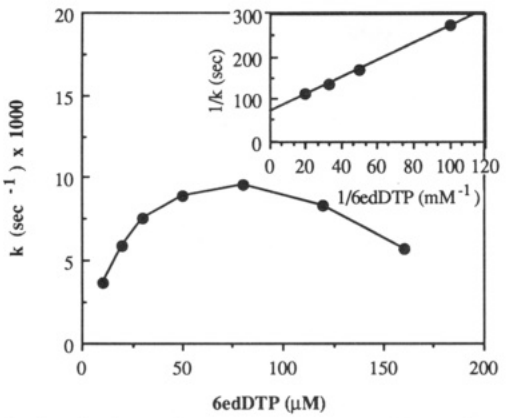


FIGURE 2: Pseudo-first-order rate constant at various 6edDTP concentrations shows that at high 6edDTP concentrations there is substantial substrate inhibition. However, from the data at 6edDTP concentrations up to 50 μM (inset), an apparent K_m of 29 μM and k_{cat} of 0.014 s^{-1} could be obtained.

13/26-mer duplex was used as a substrate, with either the 13-mer or the 26-mer 5′-end labeled with ^{32}P, indicating that the products contain both the 13-mer and the 26-mer, as a cross-linked duplex should (data not shown).

The requirement that the level of exonuclease activity be low or negligible in the polymerase used to incorporate the 6edDTP can be understood when the kinetic parameters for incorporation of the 6edDTP are examined. Under conditions of excess Klenow over DNA, all the DNA is complexed with the enzyme, and the incorporation of 6edDTP is a pseudo-first-order process. The time course of the reaction can be used to extract rate constants for incorporation. When the concentration of the 6edDTP is varied, a series of rate constants is obtained (Figure 2). It was found that the rate of incorporation of 6edDTP increased as the concentration of the triphosphate increased up to 80 μM, after which the rate actually decreased, due to substrate inhibition. An apparent K_m and k_{cat} may be calculated, however, from the rate constants up to 50 μM to give K_m (apparent) = 29 μM, and k_{cat} = 0.014 s^{-1}. This K_m is seen to be about 5-fold higher than that for the natural deoxynucleoside triphosphate substrates, but the k_{cat} is about 3000 times slower than with the natural dNTPs. Because of this reduction in k_{cat}, the incorporation of the 6edDTP proceeds with a maximal rate comparable to the exonuclease rate of wild-type Klenow (exo$^+$) fragment (k_{exo} = 0.005 s^{-1}), so that a significant amount of the primer strand of the substrate duplex is exonucleolytically degraded by this enzyme during 6edDTP incorporation. Consequently, use of exonuclease-deficient Klenow fragment produces more of the desired product. The low rate of 6edDTP incorporation precludes use of polymerases with extremely active exonuclease activities, like T4 and T7 DNA polymerases.

Terminal deoxynucleotidyltransferase was found to accept 6edDTP as a substrate, catalyzing the elongation of radiolabeled 13-mer in a time-dependent fashion. Conditions were found which allowed the elongation of the 13-mer (**6**) to mostly the 14-mer (**9**), with a smaller amount of longer products and some 13-mer left over. It was found that the terminal transferase reaction could be quenched with EDTA and that, following quenching, addition of the complementary 26-mer (**8**) to the reaction quickly generated the same cross-linked DNA products seen when 6edDTP is incorporated by Klenow fragment. Thus the cross-linking reaction of 6edDTP-terminated 13-mer with the complementary 26-mer was found to be complete within 30 s.

The ability of the cross-linked 14/20-mer duplex (**10**) to serve as a substrate for elongation by various polymerases was examined (Figure 3). The duplex could be elongated by all polymerases tested (Klenow fragment, reverse transcriptase, T4 DNA polymerase, and T7 DNA polymerase). This may indicate that the structure of the cross-linked products closely resembles normal primer–template duplexes. As seen in Figure 3, Klenow fragment and T7 DNA polymerase are sluggish in catalyzing the addition of the last base to a duplex to give a blunt-ended duplex. This has been previously noted with normal duplexes and is a feature of these polymerases, and not due to the cross-linked base pair in the duplex (Cowart et al., 1989).

If the cross-linked 14/26-mer product indeed has the structure shown in Scheme III, then successive addition of the appropriate deoxynucleoside triphosphates should give con-

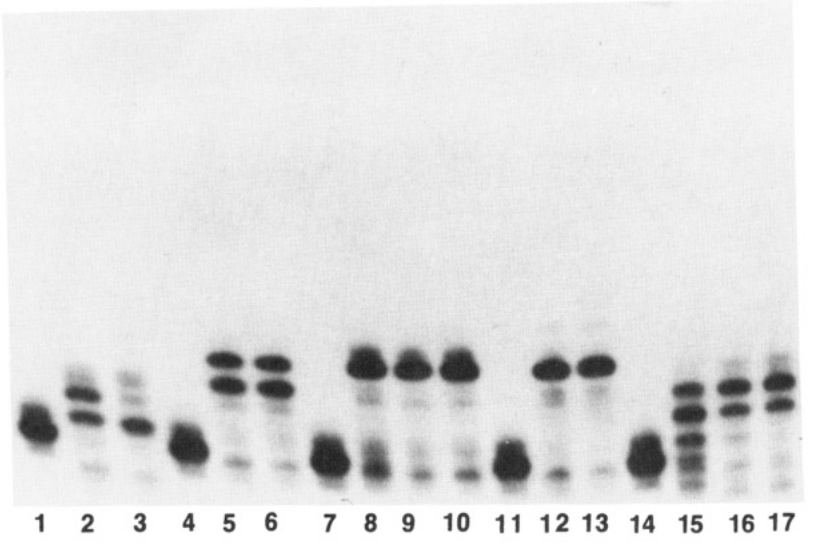
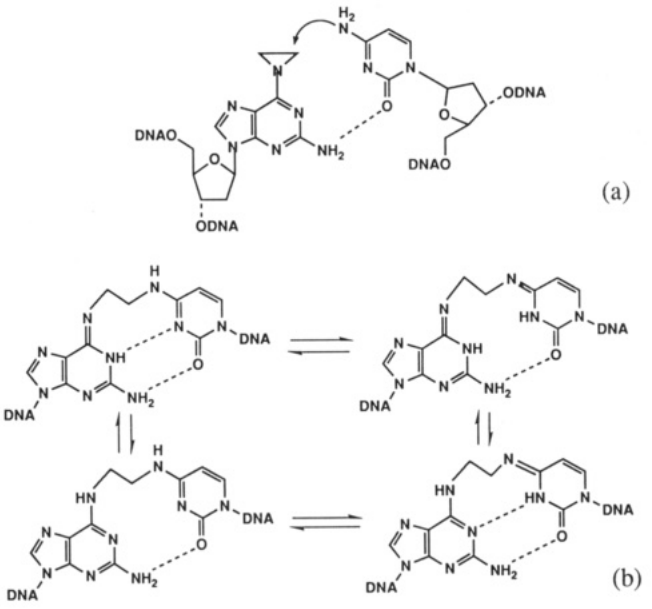


FIGURE 3: In the presence of 80 μM TTP, the cross-linked duplex (**10**, 9 nM) was elongated by excess Klenow fragment after 0, 1, and 5 min (lanes 1–3). The cross-linked 14/20-mer duplex (**10**, 9 nM) was incubated with various polymerases in the presence of all four deoxynucleotide triphosphates (80 μM). The elongation of **10** with excess Klenow fragment after 0, 1, and 5 min (lanes 4–6) is seen. The elongation of **10** with excess reverse transcriptase after 0, 1, 5, and 15 min is shown in lanes 7–10. The elongation of **10** with excess T4 DNA polymerase after 0, 1, and 5 min is seen in lanes 11–13. The extension of **10** with excess T7 DNA polymerase after 0, 1, 5, and 15 min is shown in lanes 14–17. The reluctance of some polymerases to extend duplexes to a blunt end has been previously noted with un-cross-linked control duplexes (Cowart et al., 1988). At much longer time points, more of the fully extended blunt-ended duplexes are formed.

Scheme III: Proposed Mechanism of Cross-Link Formation between 6edDTP Incorporated into the Primer Strand and a Template Strand Cytosine (a) and Putative Structure of the Cross-Linked Base Pair (b)



trolled elongation of this primer–template system. The radiolabeled cross-linked 14/26-mer product (**11**) was prepared and purified by denaturing gel electrophoresis. In the presence of Klenow fragment, the only single nucleotide which could be added to extend the 14/26-mer by one base was dATP (not dTTP, dGTP, or dCTP). It was found that the only two nucleotides that allowed extension of the 14/26-mer by two bases were dATP and dCTP (not dATP and dTTP, or dATP and dGTP). It was found that only three nucleotides which could be added to further extend the 14/26-mer cross-linked duplex were dATP, dCTP, and dTTP. Addition of all four deoxynucleoside triphosphates allowed complete extension of the 14/26-mer cross-linked duplex (Figure 4). These results are consistent with the structure (**11**) assigned to the cross-linked duplex. The controlled extension of purified 14/20-mer duplex (**10**) with Klenow fragment also gave results consistent with the structure proposed. An attempt was made to sequence the single-stranded region of the template strand of the cross-linked duplexes according to the dideoxynucleoside triphosphate technique. Under conditions where it was easy to read the sequence in *non-cross-linked* duplexes, the corresponding cross-linked duplexes gave uninterpretable results, with only the last three or four bases clearly determined. This difficulty is likely caused by the presence of the cross-linked base pair, which prevents complete melting of the elongated duplexes even under extremely denaturing conditions. Thus the electrophoretic mobility of the cross-link does not have the strict dependence of the number of bases that is seen for single-stranded DNA.

The ability of 6edDTP to be incorporated into primer–template duplexes opposite each of the four natural nucleotides was examined. Figure 5 shows the results of the incorporation of 6edDTP into model duplexes where only the primer strand was radiolabeled. Klenow fragment (exo$^-$) rapidly incorporated 6edDTP opposite a thymidine in duplex **12**, but almost no cross-linked products were formed. The triphosphate analogue was found to be incorporated opposite an adenosine in duplex **13**, and significant amounts of cross-linked duplexes were formed. When all four triphosphates were added to the reaction mixture, only one of the two duplexes was found to

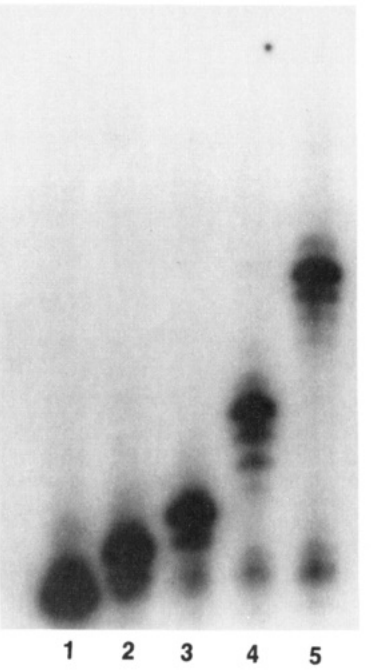


FIGURE 4: The 14/26-mer cross-linked duplex (**11**) undergoes controlled extension with excess Klenow fragment and the successive addition of nothing (lane 1), dATP (lane 2), dCTP (lane 3), dTTP (lane 4), and dGTP (lane 5).

undergo elongation by the polymerase. The 6edDTP was found to be incorporated opposite a guanosine in duplex **14**, but almost no cross-linked products were formed. When 6edDTP was incorporated opposite a cytosine in duplex **15**, significant amounts of cross-linked duplexes were formed, and only one of these was elongated by the polymerase when all four dNTPs were added to the reaction mixture. The identity of these contaminating cross-linked duplexes is not known but may be the duplexes formed by attack of template strand bases other than the complementary base expected.

Although the triphosphate analogue 6edDTP was designed to resemble dGTP, so that it might be selectively incorporated into primer–template systems opposite a template cytosine, it was found that Klenow fragment incorporated the analogue into duplexes opposite any of the four deoxynucleotides with little selectivity. However, once incorporated into duplexes, significant amounts of cross-linked duplexes were formed only when the 6edDTP was opposite the more nucleophilic bases cytosine or adenosine. Webb and Matteucci (1986a,b) obtained a similar result when their aziridine-containing bases were incorporated into oligonucleotides, and these oligonucleotides cross-linked with their complementary oligonucleotides. This is most likely because cytosine (pK_a of protonated N4 is 4.3) and adenosine (pK_a of protonated N6 is 3.7) are the most nucleophilic bases and are most capable of opening the aziridine ring of the 6edDTP. Contaminating cross-linked duplexes may arise from partial melting or slipping, allowing alkylation of a noncomplementary base to occur.

Discussion

A deoxynucleoside triphosphate analoge, 6edDTP (**5**), has been prepared by chemical synthesis, from TdGTP (**3**), which, when incorporated into DNA duplexes by DNA polymerases, causes a covalent cross-link to form between the analogue and the complementary cytosine on the opposite strand. It should be noted that the 6-sulfonate triphosphate (**4**) is electrophilic and can be displaced by aziridine to form 6edDTP (**5**). It was originally thought that this compound (**4**) could be used to incorporate cross-links specifically into DNA duplexes.

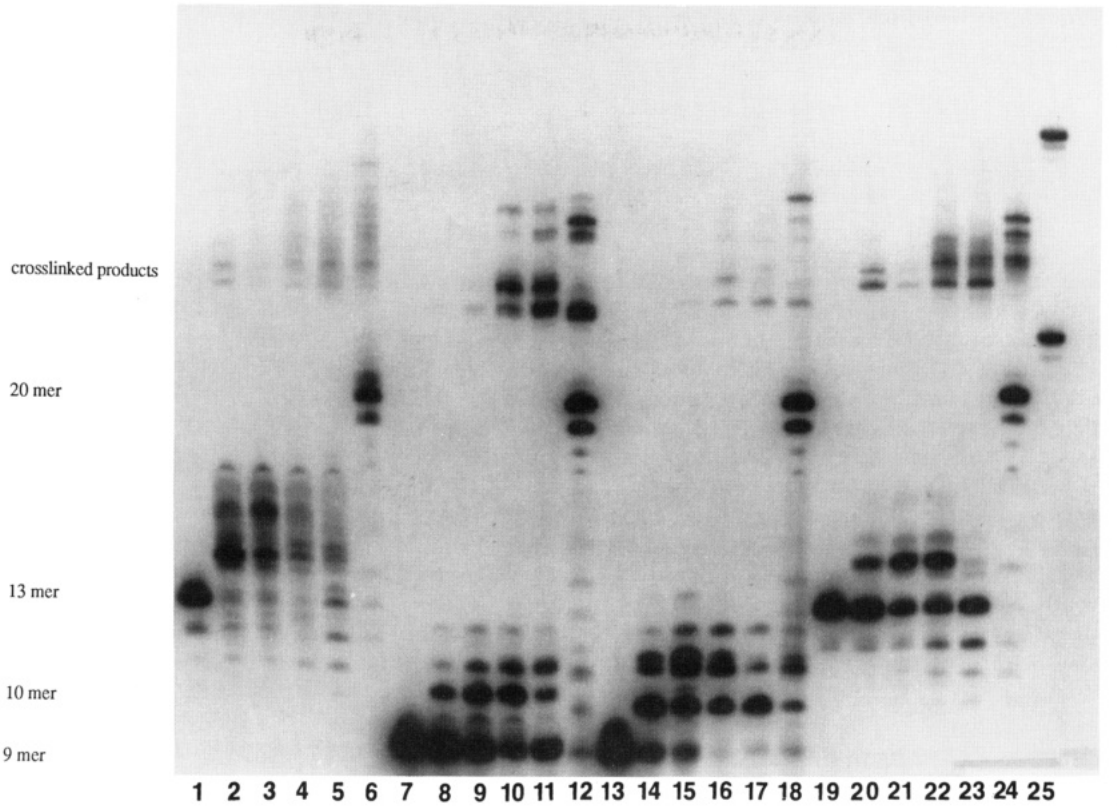


FIGURE 5: The incorporation of 6edDTP (**5**) opposite the four nucleic acid bases was examined. Only the primer strand of each duplex was labeled. Reactions contained duplex (1 μM), 6edDTP (5 μM), and excess Klenow fragment. Lanes 1–5 show that 6edDTP is incorporated opposite thymidine in the 13/20-mer (**12**) after 0, 5, and 30 min and 10 and 26 h, but that little cross-linking occurs. Addition of all four natural deoxynucleoside triphosphates allows extension of the primer to a 20-mer (lane 6). Lanes 7–11 show that 6edDTP is incorporated opposite an adenosine in the 9/20-mer (**13**) after 0, 5, and 30 min and 10 and 26 h, with the formation of a substantial amount of cross-linked products at the longer time points. Addition of all four dNTPs allows extension of the 13-mer to a 20-mer and extension of only one of the cross-linked duplexes formed (lane 12). Lanes 13–17 show that 6edDTP is incorporated opposite guanosine in the 10/20-mer (**14**) after 0, 5, and 30 min and 10 and 26 h, but that little cross-linking occurs. Following addition of all four dNTPs, the primer is extended to a 20-mer (lane 18). Lanes 19–23 demonstrate that incorporation of 6edDTP opposite cytosine in the 13/20-mer duplex (**15**) occurs after 0, 5, and 30 min and after 10 and 26 h, with the formation of substantial amounts of cross-linked products. Following addition of all four dNTPs, the primer is extended to a 20-mer, but only one of the cross-linked products is elongated (lane 24). Lane 25 consists of authentic labeled single-stranded 24-mer and 36-mer as size standards.

However, it was found that the 6-sulfonate (**4**) could not serve as a substrate for incorporation into duplexes when either Klenow fragment (exo$^-$) or avian myeloma virus reverse transcriptase was used. 6-Thio-2′-deoxyguanosine triphosphate (**3**) could however, be incorporated into duplexes and subsequently oxidized to the 6-sulfonate after incorporation into the primer strand of the duplex. However, this was not found to give any of the cross-linked duplex.

The 6edDTP was found to be quite unstable, losing its ability to cross-link DNA within 10 h of storage at room temperature, and within 3 weeks at −70 °C (data not shown). Because of this instability, the compound was placed under liquid nitrogen (−197 °C) for long-term storage. The compound was found to be more stable in slightly basic solution. This may be due to a lack of protonation at N6, which would make the aziridine much more susceptible to nucleophilic opening by water (the pK_a of the structurally related base 2,6-diaminopurine is 5.1). Nucleophilic attack of water on the aziridine ring of 6edDTP should give the 2-hydroxyethyl derivative, which should be capable of incorporation into DNA by polymerases, but devoid of the ability to cross-link DNA. This was found, as solutions containing 6edDTP rapidly lost their ability to form cross-linked DNA rapidly but retained their ability to be support polymerization (data not shown). Because of the instability of the 6edDTP, the yield of cross-linked DNA products is variable, ranging up to 45% for freshly prepared 6edDTP. This maximal value may reflect partitioning between reaction of the aziridine of the incorporated 6edDTP with the complementary base and reaction of the aziridine ring with water.

When the reaction of 6edDTP with DNA duplexes is examined, the distribution of cross-linked products produced in the reaction is seen to change over time, with usually a single product formed early in the reaction, this being replaced by products of lower electrophoretic mobility after some time. This was assumed to be due to the initial formation of a single cross-linked duplex, which undergoes further elongation, since there is an excess of 6edDTP over duplex. When it is desired to isolate only the initially formed cross-linked product, this problem can be circumvented by using conditions of excess duplex DNA over the triphosphate analogue, so that only a single cross-linked product is formed (data not shown). The use of the Y776S mutant of Klenow fragment (exo$^-$) for incorporation of 6edDTP into duplexes also improves the purity of the product. We have found that the tyrosine-766 to serine-766 mutant of Klenow fragment has unusual properties in that it misincorporates mismatched dNTPs more readily than wild-type Klenow fragment but is reluctant to elongate these products, compared to Klenow fragment (personal communication from Stephen Carroll). This suggested that the exonuclease-deficient Y766S variant of Klenow fragment might be more suitable for the preparation of pure cross-linked product, because the enzyme should incorporate the nucleotide analogue 6edDTP more readily than wild-type Klenow (exo$^-$) fragment, but should be less likely to elongate such a product.

As seen in Figure 1, this mutant enzyme gave the desired highest mobility cross-linked product in somewhat greater purity than the wild-type Klenow fragment.

Webb and Matteucii (1986a,b) found that the half-life of cross-linking with duplexes containing 4-ethenocytosine and 6-ethenoadenosine was about 30 h (rate constant = 6×10^{-5} s^{-1}). It was speculated that this slow rate of cross-linking was due to the lack of steric complementarity between the aziridine-containing bases and the opposing cytosine on the other strand. We have found that the ratio of cross-linked duplex DNA to the total amount of 6edDTP incorporated into duplex DNA was approximately constant throughout the course of cross-linking reactions, indicating that the rate of cross-linking is as fast as or faster than the rate of incorporation of the 6edDTP (data not shown). As mentioned previously, the maximum rate of cross-linking (and of 6edDTP incorporation) is 0.014 s^{-1} (a half-life of 50 s), which represents a 2200-fold increase in the rate of cross-linking of duplexes containing 6edDTP/cytosine over duplexes containing 4-ethenocytosine/cytosine and 6-ethenoadenosine/cytosine base pairs. In order to determine whether this increase in rate was due to direct catalysis of the cross-linking by Klenow fragment or due to a very fast Klenow-independent cross-linking reaction occurring *after* polymerization, terminal transferase was used to add 6edDTP onto the end of labeled 13-mer, and this was subsequently mixed with the complementary 26-mer and the rate of the cross-linking measured in the absence of Klenow fragment. The results indicated that the cross-linking reaction was complete within 30 s in the absence of Klenow fragment. The steric resemblance of 6edDTP to dGTP may allow the 6edDTP to assume a conformation more suitable for aziridine ring opening, thus the enhancement in cross-linking rate. The results indicate that the role of the polymerase is to incorporate the 6edDTP, with subsequent cross-linking occurring at a rapid rate not necessarily dependent on the presence of the polymerase.

The structure of the covalent cross-link in the duplex DNA is not known, but it may closely resemble a C·G base pair (Scheme IIIa). It was assumed that the atom on the template cytosine which attacks the aziridine is N4, since this is the most nucleophilic atom in this base, but this must remain as only reasonable speculation, as the method does not allow the preparation of the large quantities of duplex required for analysis of spectroscopic (NMR or X-ray crystallographic) means, due to the instability of the 6edDTP, the low yields in the steps leading to the preparation of the 6edDTP, and the high cost of synthetic oligonucleotides. As seen in Scheme IIIb, the cross-linked base pairs may tautomerize to structures allowing two hydrogen bonds between the base pairs. In normal duplex DNA, base pair tautomers are present at about 5–15% of the "normal" base pairing and interconvert at about 100–300 s^{-1}, and so are in rapid equilibrium. The structures shown represent the four possible tautomers of the cross-linked base pair and probably also interconvert rapidly. Since this base pair resembles a normal C·G base pair so closely, that DNA-utilizing enzymes may not easily be able to distinguish this structural unit from normal duplex DNA, except for the fact that the DNA is prevented from local melting near the cross-linked bases. The normal guanosine-O6 to cytosine-N4 distance in DNA varies from 2.7 to 3.1 Å (Saenger, 1984). Calculations of exocyclic nitrogen to exocyclic nitrogen bond distance in the modified base pairs indicate that the distance may vary from 3.2 Å in the eclipsed/syn conformation of the ethano linkage between the base pairs to 3.5 Å in the trans/anti conformation, indicating a close but not identical distance to that in normal duplex DNA.

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