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A Study of Side Reactions Occurring during Synthesis of Oligodeoxynucleotides Containing O^6-Alkyldeoxyguanosine Residues at Preselected Sites†

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ABSTRACT: As part of our studies on the molecular mechanisms of mutation by carcinogens we have synthesized 12 oligonucleotides (15-mers) containing an O^6-alkylguanine residue at a preselected position for use as primers in the enzymatic synthesis of biologically active DNA. Ten of these oligonucleotides are derived from a minus strand sequence carrying the modified nucleotide in the third codon of gene G of bacteriophage ΦX174 DNA. Two others are derived from plus strand sequences carrying the modification in the 12th codon of the human Ha-*ras* protooncogene. During this work several potentially serious side reactions, which could complicate interpretation of mutagenesis data, were observed. This paper describes a detailed study of these reactions. Since we were unable to avoid undesirable side products, we developed simple chromatographic methods for detecting and removing them.

O^6-Alkylguanine moieties in DNA have received considerable attention ever since Loveless suggested that they were important premutagenic and precarcinogenic lesions (Loveless, 1969). As part of our studies on the molecular mechanisms of mutation by carcinogens we have synthesized 12 oligonucleotides (15-mers) containing an O^6-alkyl group at a preselected position for use as primers in the enzymatic synthesis of biologically active DNA. Ten of these oligonucleotides are derived from a minus strand sequence carrying the modified nucleotide in the third condon of gene G of bacteriophage ΦX174 DNA. Two others are derived from plus strand sequences carrying the modification in the 12th condon of the

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human Ha-*ras* protooncogene.

Side reactions encountered in these syntheses are potentially serious. A mutation arises from a single change in a single DNA molecule in a single cell, so the mutants one finds are biologically amplified. Since most of our experiments involve biological selection (Bhanot et al., 1979; Chambers et al., 1985), the mutants scored could, in principle, arise from an impurity rather than the desired adduct. This problem is most serious when the mutation frequency is low.

In this paper we will describe our studies of the side reactions that occurred during the synthesis of 12 oligonucleotides containing an O^6-alkylguanine moiety at a preselected position. We will show that in most cases side products cannot be avoided with current methodology. We will describe how we detected and removed these side products before using the modified oligonucleotide for enzymatic synthesis of DNA.

Experimental Procedures

Materials

Unless otherwise noted, all reagents and solvents were from commercial sources and were used without further purification.

Methods

Preparative thin-layer chromatography was carried out on 0.5 mm thick layers of Sil G-25 UV_{254} on glass plates (Brinkman Instruments) with the following solvent systems: (A) *n*-propyl alcohol/NH_4OH/H_2O, 55/10/35 v/v/v; (B) CH_3OH/ethyl acetate, 10/90 v/v; (C) CH_3OH/$CHCl_3$, 10/90 v/v. Analytical thin-layer chromatography was carried out on silica gel 60 F_{254} backed with plastic (Merck). Column chromatography on silica gel was carried out with the following solvent systems: (D) CH_3OH/ethyl acetate, 1/99 v/v; (E) ethyl acetate/benzene, 15/85 v/v. Analytical HPLC[1] and purification of oligonucleotides were performed by reverse-phase chromatography using a 4.6 mm × 75 mm column (Beckman RPSC, 5-μm average particle size) monitored with an Altex Model 322 absorption meter. Vapor-phase chromatography was carried out on a 6 ft × $^1/_8$ in. Poropak Q column at 25 mL/min N_2.[2]

The UV absorption spectra were measured with a Cary 14 spectrophotometer. The NMR spectra were measured on a Nicolet 360-MHz spectrometer at the Atlantic Regional NMR Facility, Department of Chemistry, Dalhousie University.

Nucleoside analysis was carried out on a 0.5-nmol scale by digestion of the oligonucleotide with snake venom phosphodiesterase and *Escherichia coli* alkaline phosphatase as reported previously (Kučan et al., 1971). The resulting nucleosides were fractionated by reverse-phase chromatography on a 4.5 cm × 25 cm column (Lichrosorb RP-18, 10-μm particle size) with either isocratic elution or a linear gradient of CH_3CN in 0.1 M triethylammonium acetate, pH 7.0, as indicated in the appropriate figure legends. Calibration curves relating peak height to nanomoles were prepared from authentic standards. The column calibration was checked before each series of analyses with a standard mixture of nucleosides. When analyses are performed in duplicate, the deviation from the average is approximately 5%.

Alkylation of 5′-O-(Dimethoxytrityl)-N^2-isobutyryl-2′-deoxyguanosine. An ethereal solution of the appropriate diazoalkane (generated from the appropriate *N*-alkyl-*N*′-nitro-*N*-nitrosoguanidine with KOH) was added in two or three portions to a previously cooled (0 °C) solution of the blocked nucleoside **3** (1 mmol) in 50 mL of methanol. After each addition, the reaction mixture was examined by TLC; the main product (depending upon the alkyl group) in solvent B had R_f = 0.45–0.55 and in solvent C had R_f 0.55–0.75. After 30–60 min, when only a small amount of unreacted material remained, the reaction was stopped to avoid prolonged exposure to diazoalkane. The mixture was fractionated by column chromatography on silica gel (2 cm × 30 cm) using solvent system D. The products were precipitated with cold hexane; yields were 45–65%.

For characterization small samples were deprotected first with 80% acetic acid (20 min at 25 °C) to remove the dimethoxytrityl group and then with concentrated ammonia (50 °C, 48 h) to remove the isobutyryl group. After purification by TLC in solvent B, each sample was checked by HPLC and characterized by measuring the ultraviolet spectrum in methanol. The wavelengths corresponding to the maxima and minima for the O^6-alkyl-2′-deoxyguanosines are as follows (in nm): methyl, λ_{max} 247 and 282, λ_{min} 225 and 262; ethyl, λ_{max} 247 and 282, λ_{min} 227 and 262; *n*-propyl, λ_{max} 248 and 282, λ_{min} 228 and 262; *n*-butyl, λ_{max} 248 and 282; λ_{min} 228 and 262. The values for O^6-methyl agree well with those reported in the literature (Friedman, 1963; Farmer et al., 1973).

The ^{1}H NMR spectra of each 5′-*O*-(dimethoxytrityl)-*N*-isobutyryl-O^6-alkyl-2′-deoxyguanosine derivative was measured in Me_2SO-d_6. Our peak assignments are based on (1) comparison with authentic compounds of increasing complexity (2′-deoxyguanosine, 5′-*O*-(dimethoxytrityl)-2′-deoxyguanosine, N^2-isobutyryl-2′-deoxyguanosine, 5′-*O*-(dimethoxytrityl)-N^2-isobutyryl-2′-deoxyguanosine), (2) values reported for 5′-*O*(dimethoxytrityl)-N^2-isobutyryl-O^6-methyl-2′-deoxyguanosine in $CDCl_3$ (Kuzmich et al., 1983), and (3) spectral changes produced by spin decoupling.

5′-(Dimethoxytrityl)-N^2-isobutyryl-O^6-methyl-2′-deoxyguanosine: δ 1.06, 1.08 [2 d, 6, *J* = 6.4 Hz, $(CH_3)_2C$], 2.38 (m, 1, H2″), 2.84–2.90 (m, 2, H2′, $-CHMe_2$), 3.08–3.29 (m, 2, H5′5″), 3.70, 3.71 (2 s, 6, Ar–OCH_3), 3.96 (m, 1, H4′), 4.08 (s, 3, $-OCH_3$), 4.53 (m, 1, H3′), 5.31 (d, 1, *J* = 4.3 Hz, 3′OH), 6.37 (t, 1, *J* = 6.3 Hz, H1′), 6.69–7.30 (m, 13, Ar), 8.32 (s, 1, H8), 10.30 (s, 1, N^2-H).

5′-O-(Dimethoxytrityl)-N^2-isobutyryl-O^6-ethyl-2′-deoxyguanosine: δ 1.06, 1.08 [2 d, 6, *J* = 6.5 Hz, $(CH_3)_2C$], 1.41 (t, 3, *J* = 7.09 Hz, $-CH_3$), 2.37 (m, 1, H2″), 2.83–2.89 (m, 2, H2′, $-CHMe_2$), 3.09–3.29 (m, 2, H5′5″), 3.70, 3.71 (2 s, 6, Ar–OCH_3), 3.96 (m, 1, H4′), 4.53 (m, 1, H3′), 4.57 (q, 2, *J* = 7.03 Hz, $-OCH_2-$), 5.310 (d, 1, *J* = 4.3 Hz, 3′OH), 6.36 (t, 1, *J* = 6.3 Hz, H1′), 6.70–7.30 (m, 13, Ar), 8.31 (s, 1, H8), 10.27 (s, 1, N^2-H).

5′-O-(Dimethoxytrityl)-N^2-isobutyryl-O^6-n-propyl-2′-deoxyguanosine: δ 1.00 (t, 3, *J* = 7.4 Hz, $-CH_3$), 1.06, 1.07 (2 d, 6, *J* = 6.4 Hz, Me_2C), 1.82 (m, 2, $-CH_2-$), 2.36 (m, 1, H2″), 2.82–2.87 (m, 2, H2′, $-CHMe_2$), 3.07–3.26 (m, 2, H5′5″), 3.70, 3.71 (2 s, 6, Ar–OCH_3), 3.96 (m, 1, H4′), 4.48 (t, 2, *J* = 6.7 Hz, $-OCH_2-$), 4.53 (m, 1, H3′), 5.32 (d, 1, *J* = 4.5 Hz, 3′OH), 6.37 (t, 1, *J* = 6.3 Hz, H1′), 6.71–7.30 (m,

[1] Abbreviations: TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; DMT, dimethoxytrityl; iBu, isobutyryl; MsSCl, mesitylenesulfonyl chloride; 2-NH_2-dAdo, 2-amino-2′-deoxyadenosine or 2′-deoxyribosyl-2,6-diaminopurine; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene. ΦX mutants are described by designating the gene, the nature of the mutation (ms = missense, am = amber, etc.), the codon number, the DNA sequence for the codon, the amino acid coded for by the codon, and the phenotype (wt = wild type; ts = temperature sensitive; le = lethal), in that order. For example, ΦXGms3(TCG)^{Ser}(ts) is a temperature-sensitive missense mutant carrying the serine codon, TCG, as the third codon of gene G.

[2] We are indebted to Dr. Carl Breckenridge of this department for the analyses.

13, Ar), 8.30 (s, 1, H8), 10.26 (s, 1, N^2-H).

5′-O-(Dimethoxytrityl)-N^2-isobutyryl-O^6-n-butyl-2′-deoxyguanosine: δ 0.95 (t, 3, J = 7.3 hz, $-CH_3$), 1.05, 1.07 (2 d, 6, J = 6.3 Hz, Me_2C), 1.45 (m, 2, $-CH_2CH_3$), 1.78 (m, 2, $-CH_2-$), 2.35 (m, 1, H2″), 2.83–2.89 (m, 2, H2′, $-CHMe_2$), 3.09–3.29 (m, 2, H5′5″), 3.70, 3.71 (2 s, 6, Ar–OCH_3), 3.96 (m, 1, H4′), 4.53 (t, 2, J = 6.7 Hz, $-OCH_2-$, m, 1, H3′), 5.31 (d, 1, J = 4.3 Hz, 3′OH), 6.36 (t, 1, J = 6.3 Hz, H1′), 6.70–7.29 (m, 13, Ar), 8.30 (s, 1, H8), 10.26 (s, 1, N^2-H).

Preparation of O^6-Alkyl-2′-deoxyguanosines via O^6-Mesitylenesulfonyl Derivatives. 2′-Deoxyguanosine was converted to N^2,3′,5′-triisobutyryl-O^6-(mesitylenesulfonyl)-2′-deoxyguanosine essentially as described by Gaffney and Jones (1982) except that mesitylenesulfonyl chloride instead of triisopropylbenzenesulfonyl chloride was used. After chromatographic purification using solvent system E, the product was obtained as a white foam by evaporation of the solvent (at 70% yield). This was reacted with the appropriate alcohol (*n*-propyl alcohol, isopropyl alcohol, *n*-butyl alcohol, isobutyl alcohol, *sec*-butyl alcohol, *tert*-butyl alcohol, or neopentyl alcohol; 2.5 mmol) at 0 °C (Gaffney & Jones, 1982). Before use each alcohol was examined for purity by vapor-phase chromatography. No isomers or other impurities were detected in the *n*-propyl alcohol or *n*-butyl alcohol at a level that would have detected 0.5%. Small amounts of impurities (<2%) were found in the other alcohols. After the reaction, the products were purified by preparative TLC in solvent B or C. The purity was verified by HPLC.

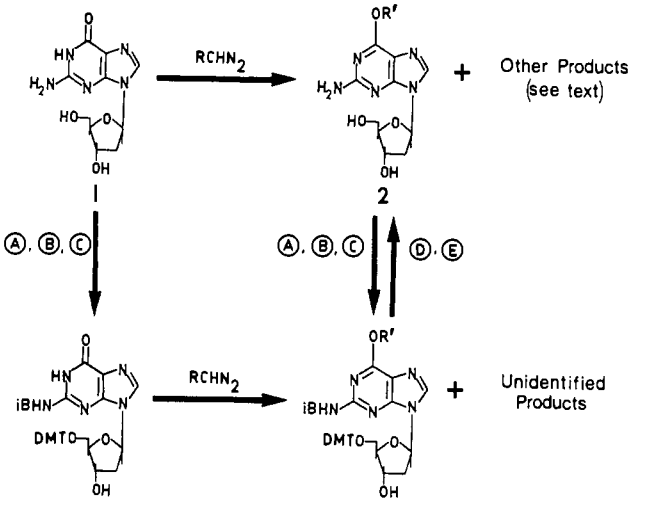
Synthesis of Oligodeoxynucleotides. The pentadecadeoxynucleotides were synthesized on a 5-μmol scale by using the syringe method described by Tanaka and Letsinger (1982). Both the phosphorochloridite derivatives and the methyl *N*,*N*-diisopropylphosphoramidite derivatives activated by benzotriazole (20% in CH_3CN) have been used successfully. In order to minimize depurination, detritylation was carried out with 3% trichloroacetic acid in dry nitromethane containing 1% methanol (Tanaka & Letsinger, 1982). After the last nucleotide had been added, the detritylation step was skipped and the phosphotriesters were deblocked by treatment with thiophenol and triethylamine in dioxane in the usual manner. The oligonucleotide was released from the solid support, and the acyl protecting groups were removed in one of three ways:

(a) Unmodified oligonucleotides were treated with concentrated ammonia at 50 °C for 24 h. The solid support was removed by centrifugation and washed with 1 mL of H_2O. The combined supernatants were lyophilized to dryness. The crude products were dissolved in water and purified by preparative thin-layer chromatography on silica gel in solvent A. The product was eluted from the silica gel with water/methanol (3/1 v/v) and lyophilized to dryness. When the products were taken up in small amounts of water (0.25 mL), spontaneous detritylation took place, usually within 2 h at room temperature. Final purification was achieved by additional thin-layer chromatography or the HPLC method or both.

(b) Alkylated oligonucleotides were treated with concentrated ammonia at 65 °C for 48 h or at 50 °C for 72 h. The workup was as described in (a).

(c) Oligonucleotides were treated in a sealed tube with 10% DBU in methanol at 25 °C for 3 days or, if alkylated, for 5 days. To remove the DBU, the deprotected product was concentrated under reduced pressure, taken up in 1 mL of water, applied to a NACS-52 Prepac Cartridge (Bethesda Research Laboratories, Bethesda, MD), and eluted with 4 M ammonium acetate (0.5 mL). The product was purified by reverse-phase HPLC. The final product was analyzed for its nucleoside content as described above.

Scheme I[a]



[a] R = H, Me, Et, *n*-Pr, or *n*-Bu; R′ = Me, Et, *n*-Pr, or *n*-Bu. (A) Reaction with $(CH_3)_2CHCOCl$. (B) 1 N NaOH, 0 °C. (C) Reaction with dimethoxytrityl chloride (DMT-Cl). (D) 80% HOAc, 25 °C, 20 min. (E) Concentrated NH_3, 50 °C, 16 h.

Phosphorylation of Oligonucleotides. The reaction was carried out with polynucleotide kinase. The product was purified by gel electrophoresis. This material was used for synthesis of form I′ DNA that was part of the mutagenesis experiments reported elsewhere (Chambers et al., 1985).

Results and Discussion

Synthesis and Characterization of 5′-O-(Dimethoxytrityl)-N^2-isobutyryl-O^6-alkyl-2′-deoxyguanosine Derivatives. The classical synthesis of O^6-alkyl-2′-deoxyguanosines (**2**) involves the reaction between the unblocked nucleoside **1** and an active alkyl moiety generated from the appropriate *N*-alkyl-*N*-nitroso compound as shown in Scheme I (Friedman et al., 1963, 1965; Farmer et al., 1973). There are two problems with this approach: formation of positional isomers and rearrangement of the alkyl chain. This reaction appears to occur through an alkyl carbocation (Friedman, 1970). When the reaction is carried out in aqueous solution, rearrangement of the alkyl chain may occur. For example, decomposition of *N*-*n*-propyl-*N*-nitrosourea in Tris buffer at pH 7.4 gave 39% isopropyl alcohol (Park et al., 1977). Treating DNA with *N*-*n*-butyl-*N*-nitrosourea in phosphate buffer at pH 7.4 gave 77% O^6-*n*-butylguanine residues and 23% *sec*-butyl derivatives (Saffhill, 1984). Since the isomers may be repaired at the DNA level by different mechanisms (Todd & Schendel, 1983), it is important either to avoid the rearrangement or to purify the desired nucleoside before incorporating it into DNA for studying mechanisms of mutagenesis.

The extent of the rearrangement is dependent upon the reaction conditions, and polarity of the solvent appears to be important (Friedman et al., 1963; Keating & Skell, 1970). We examined the reaction of ethereal diazopropane or diazobutane with 5′-O-(dimethoxytrityl)-N^2-isobutyryl-2′-deoxyguanosine (**3**) in methanol. The resulting product mixture was examined by HPLC calibrated with authentic standards. Figure 1 shows this calibration. Analyses of the reaction mixtures containing the O^6-propyl and O^6-butyl derivatives indicated each product was >98% normal isomer.[3] Thus, in methanol very little, if

[3] Data supporting this statement were submitted for review but are not shown.

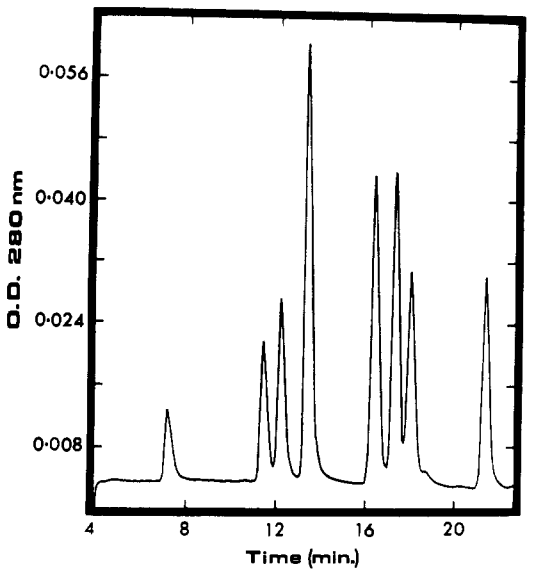


FIGURE 1: Separation of some O^6-alkyl derivatives of 2′-deoxyguanosine by reverse-phase HPLC. The column was a Lichrosorb RP-18 eluted at 1 mL/min with a gradient of 15–40% acetontrile in 0.1 M triethylammonium acetate, pH 7.0, developed over a 25-min period (see Experimental Procedures for details). Peaks 1 and 4 represent compounds **10** and **9**, respectively, of Scheme II. Peaks 2, 3, 5, 6, 7, and 8 represent compound **2** where R = isopropyl, *n*-propyl, *sec*-butyl, isobutyrl, *n*-butyl, and neopentyl, respectively. The retention times were 6.8, 10.9, 11.7, 12.9, 15.9, 16.8, 17.6 and 22.0 min.

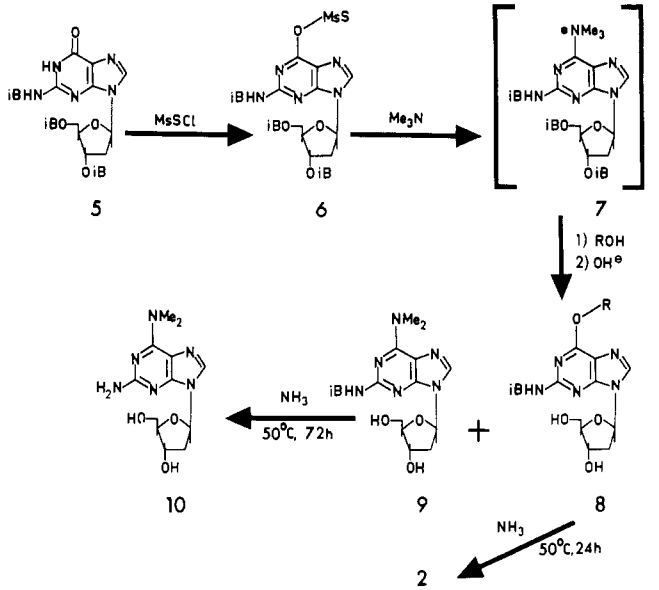
any, rearrangement of the alkylating agent side chain occurred. The small amounts of isomers found (1.5% isopropyl; 0.8% *sec*-butyl)[3] could have been present in the alkylnitrosonitroguanidines used to generate the diazoalkanes, though we did not investigate this directly. It appears that in methanol either the reaction occurs by a mechanism different from that in an aqueous solvent or the alkylation reaction occurs much faster than the rearrangement. In preliminary experiments we found that running the "alkylation reaction" in methanol/tetrahydrofuran *in the absence of the nucleoside* and fractionating the products by vapor-phase chromatography gave a major peak identified by mass spectrometry as α-*n*-butyltetrahydrofuran (data not shown). This result indicates that in this solvent diazabutane may react via a carbene rather than a carbocation.[4]

We also investigated the synthesis of the O^6-alkyldeoxyguanosines by a regiospecific route where isomerization of the alkyl chain cannot occur (Gaffney & Jones, 1982). This synthesis involves displacement of a 6-trimethylammonium ion by the suitable alcohol in the presence of DBU as shown in Scheme II. Although isomerization of the alkyl chain cannot occur in this method, the purity of the alcohol is important. We examined this by vapor-phase chromatography. The *n*-propyl alcohol and *n*-butyl alcohol used were free of isomers and other impurities when analyzed at a level where 0.5% contamination was detectable.

An important side reaction can occur in this synthesis. As shown in Scheme II, attack at the 6-position of the purine ring of the presumed intermediate (**7**) gives the desired product, but attack at one of the exocyclic methyl groups of **7** would give the dimethylamino derivative **9** (Gaffney & Jones, 1982). An earlier report mentions this side reaction, but the purported side product (**9**) was not characterized rigorously (Gaffney & Jones, 1982; Gaffney et al., 1984). It is claimed that

[4] We are indebted to Dr. D. Arnold of the Chemistry Department for suggesting this experiment and to Miles Snow of the Chemistry Department for carrying out the analysis.

Scheme II[a]



[a] R = Me, Et, *n*-Pr, iPr, *n*-Bu, iBu, *s*-Bu, or neopentyl.

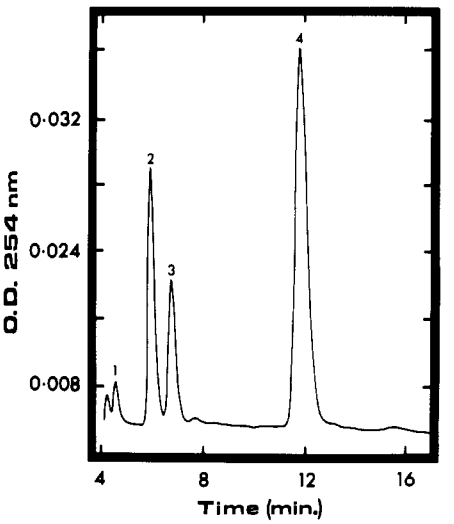


FIGURE 2: Separation of N^2-isobutyryl-O^6-isopropyl-2′-deoxyguanosine (compound **8**) from side products produced in the synthesis shown in Scheme II. Peak 1 = compound **10**; peak 2 = compound **2**, R = isopropyl; peak 3 = compound **9**; peak 4 = the desired product (compound **8**).

reaction of excess alcohol (methyl, ethyl, or *n*-butyl) with **7** in the presence of DBU at 0 °C "gives the desired O^6-alkyl derivative cleanly". In our hands, as the length and/or the bulk of the alkyl chain increased, a side product that appears to be **9** increases. Figure 2 shows its separation of this side product from the O^6-isopropyl derivative. With *tert*-butyl alcohol the side product was the only product. Presumably this is due to steric effects of *tert*-butyl alcohol since we were able to make the neopentyl derivative of **8** without difficulty. However, in the case of neopentyl alcohol the side product was a major product (data not shown).

These results indicate that regardless of the synthetic route it is difficult to avoid side products during preparation of blocked nucleoside required for synthesis of the alkylated oligonucleotide. Fortunately, the desired nucleoside intermediates can be purified fairly easily by HPLC (Figures 1 and 2).

Synthesis and Characterization of Oligonucleotides Carrying an O^6-Alkylguanine Residue at a Preselected Position. The appropriately blocked O^6-alkyl-2′-deoxyguanosine deriv-

atives were converted to either their methoxychlorophosphine derivatives or their methoxy(diisopropylamino)phosphine derivatives (Matteucci & Caruthers, 1981; Beaucage & Caruthers, 1981; Alvarado-Urbina et al., 1981). The activated nucleosides were used for synthesis of the desired oligonucleotides by a manual method (Tanaka & Letsinger, 1982). Twelve oligonucleotides were synthesized: (1) 5′-AAAAGTCTG*AAACAT (G* = O^6-Me or O^6-Bu derivative of G); (2) 5′-AAAAGTCG*AAAACAT (G* = 1-Me, O^6-Me, O^6-Et, O^6-Pr, or O^6-Bu derivative of G); (3) 5′-AAAAGTCGG*AAACAT (G* = O^6-Me, O^6-Et, or O^6-Bu derivative of G); (4) GGCGCCGG*CGGTGTG (G* = O^6-Me or O^6-Bu derivative of G). The choice of oligonucleotide sequence was dictated by biological considerations (Chambers, 1982, 1985). The first three sequences are from the minus (−) strand in gene G of ΦX174. The underlined sequences represent the third codon region and are complementary to either the wild-type codon $(CAG)^{Gln}$ or one of the two mutant codons $(TCG^{Ser}(ts)$ or $(CCG)^{Pro}(le)$ (Chambers et al., 1985). The fourth sequence is from the plus (+) strand of the human Ha-*ras* protooncogene. The underlined sequence corresponds to the 12th codon. Experiments utilizing these oligonucleotides to study the mutagenic effect of these adducts in different DNA repair backgrounds either have been described (Chambers et al., 1985) or are in progress.

The properties of the O^6-alkyl group are such that changes in the deblocking procedure were required. It has been reported that the usual deacylation conditions (concentrated NH_3 at 50 °C for 16 h), which also release the oligonucleotide from the solid support, do not remove the N^2-isobutyryl group quantitatively from the N^2-isobutyryl-O^6-alkylguanine moiety in the oligonucleotide (Gaffney & Jones, 1982). We have confirmed this. Therefore, these conditions are not suitable for deblocking when the oligonucleotide contains an O^6-alkylguanine moiety. We have also confirmed the report that the isobutyryl group can be removed completely from a blocked oligonucleotide at 65 °C in 72 h (Kuzmich et al., 1983). Under these more vigorous conditions, ammonolysis and to a lesser extent hydrolysis occur at the 6-position, displacing the methoxy group and forming either a 2,6-diaminopurine or a guanine moiety (Kuzmich et al., 1983).

Ammonolysis is a particularly serious side reaction because the 2,6-diaminopurine moiety formed is mutagenic (Freese, 1959). Use of an oligonucleotide mixture containing guanine, O^6-methylguanine, and 2,6-diaminopurine rings at the preselected site to synthesize the DNA for studying mutagenesis would make interpretation of the data difficult. Fortunately, the desired oligonucleotide can be separated from the side products by reverse-phase HPLC. A typical elution profile is shown in Figure 3. Peak 1 has the same retention time as an authentic sample of the unmodified oligonucleotide 5′-AAAAGTCG*AAAACAT, with G* = G. By quantitative nucleoside analysis, the products in peaks 2 and 3 were found to contain the 2,6-diaminopurine derivative and the O^6-methylguanine moiety, respectively.[3] Analysis of the oligonucleotide in peak 2 of Figure 3 gave the four unmodified nucleosides in the correct ratio ($T_{2.01}G_{1.04}A_{9.08}C_{1.87}$; theory, $T_{2.00}G_{1.00}A_{9.00}C_{2.00}$) and a peak corresponding to 2′Deoxyribosyl-2,6-diaminopurine, which we could not quantitate accurately.[3] Analysis of the desired oligonucleotide (peak 3 of Figure 3) gave $T_{2.01}G_{1.03}A_{9.08}C_{1.97}m^6G_{0.90}$ (theory, $T_{2.00}G_{1.00}A_{9.00}C_{2.00}m^6G_{1.00}$). There was no evidence of 2′-deoxyribosyl-2,6-diaminopurine. Nor was there any evidence of N^2-isobutyryl-O^6-methyl-2′-deoxyguanosine, indicating that deblocking was complete.[3]

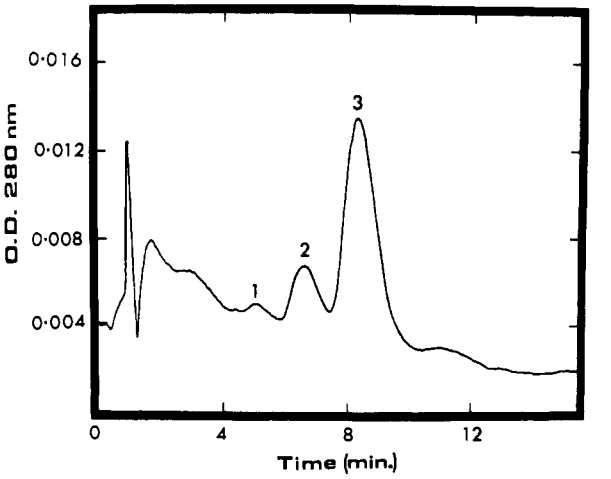
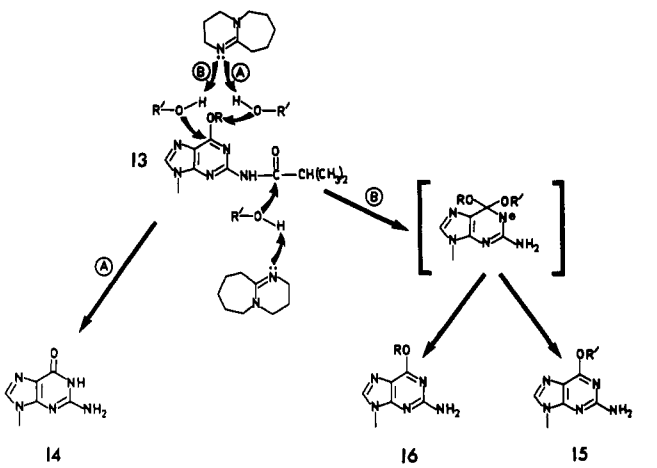


FIGURE 3: Separation of oligonucleotide products produced by deblocking d(AAAGTCG*AAAACAT) with concentrated ammonia at 65 °C for 48 h. In peak 1, G* = guanine; in peak 2, G* = 2,6-diaminopurine; in peak 3, G* = O^6-methylguanine. The HPLC column was RPSC (Beckman) eluted with 7% acetonitrile (see Experimental Procedures for details). The retention times were 5.0, 6.3, and 7.9 min.

Scheme III[a]



[a] R = Me or *n*-Bu; R′ = Me. A and B refer to separate reaction pathways.

Similar results were obtained with the oligonucleotides containing O^6-ethyl-, O^6-*n*-propyl-, and O^6-*n*-butylguanine moieties instead of O^6-methylguanine, but the separations were even easier than with O^6-methylguanine since higher homologues have longer retention times and the side products are unchanged.

These results, taken together, demonstrate it is possible to obtain the desired oligonucleotide containing an O^6-alkylguanine at a preselected site. However, the side reactions caused by NH_3 deblocking are troublesome, so it was desirable to investigate alternative procedures. Kuzmich et al. (1983) have described the use of DBU for deblocking oligonucleotides containing an O^6-methylguanine moiety. DBU does not add to the 6-position of the purine ring, as ammonia does, so one of the side reactions (ammonolysis) is eliminated. We have examined both NH_3 and DBU deblocking with the protooncogene oligonucleotide 5′-GGCGCCGG*CGGTGTG, with G* = m^6G).

Although DBU-catalyzed deblocking gives a simpler mixture of products than does ammonia,[3] two side reactions are still possible if deblocking is carried out in methanol. One of these is removal of the O^6-alkyl group either by hydrolysis if water is present or by direct displacement of the methyl group

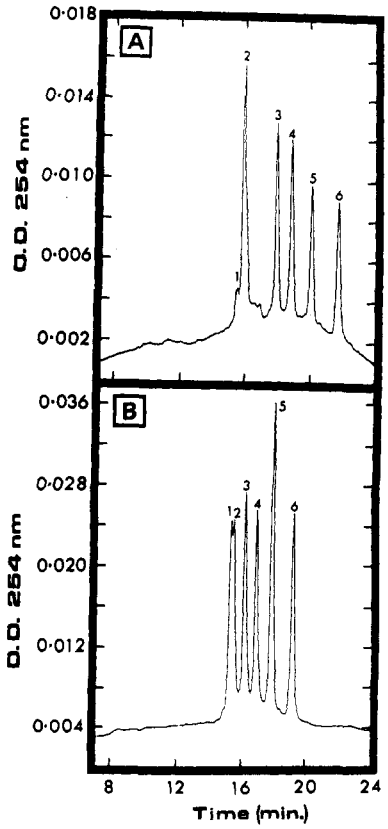


FIGURE 4: Fractionation of oligonucleotides carrying different alkyldeoxyguanosine residues at the G* position in d-(AAAAGTCG*AAAACAT). The mixture was prepared from highly purified synthetic oligonucleotides. The separation was carried out on a reverse-phase column (RPSC) with a 3–15% gradient of acetonitrile in 0.1 M ammonium acetate, pH 7.0, developed over 30 min. In (A) G* corresponds to the following derivatives of guanine: peak 1, unsubstituted; peak 2, 1-methyl; peak 3, N^2-isobutyryl-O^6-methyl; peak 4, N^2-isobutyryl-O^6-ethyl; peak 5, N^2-isobutyryl-O^6-*n*-propyl; peak 6, N^2-isobutyryl-O^6-*n*-butyl. The retention times are 15.2, 15.5, 17.6, 18.6, 19.9, and 21.6 min, respectively. In (B), G* corresponds to the following derivatives of guanine: peak 1, unsubstituted; peak 2, 2,6-diaminopurine; peak 3, O^6-methyl; peak 4, O^6-ethyl; peak 5, O^6-*n*-propyl; peak 6, O^6-*n*-butyl. The retention times are 15.2, 15.4, 16.1, 16.9, 17.9, and 19.2 min, respectively.

by methoxide as shown in Scheme III (**13** → **14**). In the cases we have studied, the desired product (**16**) can be separated from the unmodified oligonucleotide (**14**). In principle, this side reaction can be avoided by carrying out the deblocking in anhydrous methanol. In practice, we always find small amounts of **14**.

For alkyl groups other than methyl, the use of methanol as the solvent produces another side reaction (Scheme III, **13** → **15**). With O^6-butyl-2′-deoxyguanosine, some methanolysis occurs (even at 25 °C), giving a mixture of oligonucleotides containing **14**, **15** (R′ = methyl), and **16** (R = butyl). The obvious way to avoid this alcohol exchange is to carry out the deblocking in the alcohol corresponding to the O^6-alkyl group. In work that is not yet complete, we have found DBU-catalyzed deblocking slows as the chain length of the alcohol increases. The rate is so slow at the oligonucleotide level, where solubility seems to be a problem, that deblocking in the matching alcohol solvent may be impractical when the alkyl group is not methyl. It is important, therefore, to have an HPLC system that separates the different products arising from DBU-catalyzed deblocking when the solvent is methanol and the O^6-alkyl group is not methyl. So far we have studied only derivatives of 5′-AAAAGTCGG*AAACAT (G* = 1-methyl, O^6-methyl, O^6-ethyl, O^6-*n*-propyl, or O^6-*n*-butyl derivative of G). As shown in Figure 4, all of these oligonucleotides are separable from each other and from the unmodified parent compound. Even if some methanolysis occurs to give a mixture of **15** (R′ = methyl) and **16** (R = ethyl, *n*-propyl, or *n*-butyl), the desired product can be separated from the corresponding methyl derivative. Furthermore, the removal of the *N*-isobutyryl blocking group can be monitored at the oligonucleotide level with this HPLC system (compare panels A and B of Figure 4). The ease with which the desired product separates from side products suggests a good prognosis for similar purification of other oligonucleotides containing O^6-alkylguanine moieties.

One final point regarding purity needs emphasis. None of the methods described here or any other chemical or physical method currently available can detect impurities below about 0.1% in the modified nucleoside. The limit may be even higher in the oligonucleotide. Any impurities incorporated into the final DNA that is used for mutagenesis studies represent potential sources of ambiguity. The biological experiments in our prokaryotic system are done by transfection of about 10^8 DNA molecules into an equal number of spheroplasts. If that DNA contains 0.1% of a single impurity at the preselected site, then 10^5 molecules that go into the biological assay will contain the impurity rather than the adduct under study. Since most of the experiments involve a biological selection, the mutants one finds are biologically amplified and could arise from the impurity rather than the desired adduct. It is difficult to decide how serious this is at present, but some of the side products described here (which include O^6-alkylguanine residues containing the N^2-isobutyryl blocking group) are almost certainly present in some of the oligonucleotides containing O^6-methylguanine that have been used in other laboratories to study mutagenesis and repair (Fowler et al., 1982; Topal et al., 1986). One solution to this potential problem is to repeat the synthesis of the oligonucleotide by using a modified nucleoside that has been synthesized by an independent route where the impurities cannot be the same. If these two DNAs give the same results, then it is unlikely that some minor impurity rather than the major modification is responsible for the observed mutation. In this paper, we have described two independent routes to O^6-alkyl-2′-deoxyguanosines. We have examined side reactions that can occur in these syntheses. It appears that the displacement synthesis described by Gaffney and Jones is the method of choice for O^6-alkylguanine residues since both straight- and branched-chain isomers can be prepared without rearrangement and the major side products are easily separated from the desired product at the nucleotide level. Side reactions still occur during oligonucleotide deblocking, so the two syntheses we have examined have some overlapping side products. At present, one must rely on careful purification and analysis of products.

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Influence of Template Strandedness on in Vitro Replication of Mutagen-Damaged DNA†

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ABSTRACT: We analyzed the ability of DNA polymerases to bypass damage on single- and double-stranded templates. In vitro DNA synthesis was studied on UV-irradiated and polyaromatic hydrocarbon reacted (benzo[*a*]pyrenediol epoxide and oxiranylpyrene) double-stranded templates by a protocol involving initiation on a uniquely nicked circular double-stranded template. The template was prepared by treating single-stranded (+)M13mp2 circular strands with mutagen and then hybridizing with restricted M13 RFmp2, followed by isolation of the nicked RFII forms. The protocol permits either (+), (−), or both strands to carry lesions. We found that the rules for termination and bypass of lesions previously observed with single-stranded DNA templates also hold for double-stranded templates. Termination of synthesis occurs primarily one nucleotide 3′ to the lesion in the template strand. Bypass of UV-induced lesions can be followed in a series of three partial reactions in the presence of Mn^{2+} and dGMP, which relax the specificity of nucleotide insertion and 3′ → 5′ exonuclease activity, respectively. There is no evidence for greater permissivity of bypass in double- as opposed to single-stranded templates. As with single-stranded templates, purines and preferentially deoxyadenosine (dA) are inserted opposite lesions. Lesions in the nontemplate strand elicit neither termination nor pausing. The addition of Rec A protein resulted in a measurable increase of bypass in this system.

Termination of DNA synthesis as a result of lesions introduced into the template by mutagenic and/or carcinogenic agents has now been studied in a variety of systems [see Strauss (1985) for review]. For almost all of these cases a set of rules can be formulated, indicating just where the majority of terminations will occur. Most of the lesions previously studied result in termination exactly one nucleotide prior to (3′ to) the lesion on the template strand, especially when polymerases with active 3′ → 5′ exonuclease proofreading functions are utilized. Exceptions do occur but these often are related to the special stereochemical properties of the altered nucleotide [e.g., Moore et al. (1981), Clark and

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