Formation of O\textsuperscript{6}-Ethylthioethyldeoxyguanosine from the Reaction of
Chloroethyl Ethyl Sulfide with Deoxyguanosine\textsuperscript{1}

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\textit{ABSTRACT}

O\textsuperscript{6}-Ethylthioethyldeoxyguanosine has been synthesized from 6-chloro-3\textsuperscript{'}5\textsuperscript{'}-di-O-acetyldideoxyguanosine and characterized by UV, fluorescence, and mass spectrometry. High-pressure liquid chromatography studies have shown that this modified nucleoside is formed when the one-armed sulfur mustard, chloroethyl ethyl sulfide, reacts with deoxyguanosine. This result supports the hypothesis that the mutagenic effects of the sulfur mustards are caused in part by substitution of the O\textsuperscript{6}-position of deoxyguanosine.

\textit{INTRODUCTION}

The sulfur mustards are a highly reactive group of compounds which, besides being mutagenic and carcinogenic, have some therapeutic activity as antitumor agents (1). The cytotoxicity of bifunctional sulfur mustards is probably related to DNA cross-linking, while it has been hypothesized that mutagenic effects in bacteria are caused in part by alkylation of the O\textsuperscript{6}-position of deoxyguanosine (4).

The model single-armed mustard CEES\textsuperscript{3} has been studied extensively as a mutagen and has been shown to react with the N-7 and N\textsuperscript{2} positions of deoxyguanosine, but no evidence has been reported previously for reaction at the O\textsuperscript{6}-position (5, 8).

Here, we would like to describe the synthesis and characterization of O\textsuperscript{6}-ethylthioethyldeoxyguanosine and present data which show that this compound is formed from the reaction of CEES with deoxyguanosine.

\textit{MATERIALS AND METHODS}

Chloroethyl ethyl sulfide was obtained from Chemicals Procurement Laboratories, Inc.; sodium hydride was from Aldrich Chemical Company, Inc.; and deoxyguanosine was from P-L Biochemicals.

High-pressure liquid chromatography was performed on a modular apparatus consisting of a Milton-Roy 5000-psi minipump, Rheodyne Model 7125 injector, an LDC Fluoromonitor III detector equipped with a 254-nm excitation filter and a 300 to 400-nm emission filter, and a Hewlett-Packard 1040A diode array detector which recorded UV spectra of chromatographic peaks as they eluted. Separations were performed on a Spherisorb octadecylsilane 5-\mu m (4.6 x 250-mm) column at room temperature.

UV spectra were obtained with a Beckman Model 55 spectrophotometer in 50 mM K\textsubscript{2}HPO\textsubscript{4} buffer, pH 7, and after adjustment of the pH to 12 with KOH and to 2.2 with HCl. Fluorescent spectra were obtained in a Perkin-Elmer 512 fluorescence spectrophotometer with excitation and emission slit widths of 20. Mass spectrometry was performed on a Varian MAT 311A mass spectrometer with the electron impact technique. The compound was introduced directly on the probe, and spectra were obtained with 70-eV electrons with the ion source and probe both at 220\textdegree.

\textit{Synthesis of O\textsuperscript{6}-Ethylthioethyldeoxyguanosine.} This compound was synthesized in a manner analogous to that reported for the synthesis of O\textsuperscript{6}-methyldeoxyguanosine (7), and it involved formation of the intermediate, 6-chloro-3\textsuperscript{'}5\textsuperscript{'}-di-O-acetyldideoxyguanosine. The 6-chloro group in this compound was then displaced by reaction with sodium ethylthio-ethoxide.

Since 6-chloro-3\textsuperscript{'}5\textsuperscript{'}-di-O-acetyldideoxyguanosine is not available commercially, this compound was synthesized by the method of Gerster et al. (3) as described previously (7). The first step in this synthesis is to acetylate deoxyguanosine to protect its deoxyribose sugar from chlorination. Five g of deoxyguanosine were reacted with an excess of acetic anhydride (15 ml) in pyridine (75 ml) for 3 days at room temperature. The product was filtered, washed with warm pyridine (50 ml) and with dry ether (50 ml), and then vacuum dried at 65\textdegree for 2 days before the next step. This procedure completely acetylates the deoxyribose sugar and protects it from chlorination.

Chlorination of the acetylated deoxyguanosine followed the procedure we have described (7), taking great care to exclude moisture which leads to a rapid decomposition of the product. All of the glassware was dried under vacuum at 75\textdegree, and the POCl\textsubscript{3} was freshly distilled immediately before use. Two g of acetylated deoxyguanosine were added with stirring to a solution of POCl\textsubscript{3} (75 ml) and N,N-diethylaniline (2 ml) at room temperature. The suspension was heated until all of the solid was dissolved and a clear yellowish solution was obtained. Excess POCl\textsubscript{3} was removed under a vacuum, and the resulting syrup was stirred slowly into excess ice until hydrolysis was complete. Then, the solution was extracted 3 times with 100 ml of dichloromethane. Organic extracts were combined, washed 3 times with 100 ml of cold 1 N HCl and, finally, washed with cold water until neutral. The dichloromethane solution was dried over anhydrous magnesium sulfate, and the solvent was evaporated under vacuum to leave a thin oil. When this oil was extracted with dry ether, amorphous 6-chloro-3\textsuperscript{'}5\textsuperscript{'}-di-O-acetyldideoxyguanosine was obtained. Its UV spectrum, UV (0.1 n HCl), \lambda\textsubscript{max} 315, 241, \lambda\textsubscript{<max> 272, 234, was in agreement with that reported for this compound previously (7).

This material was used directly for the synthesis of O\textsuperscript{6}-ethylthioethyldeoxyguanosine. Compound 6-chloro-3\textsuperscript{'}5\textsuperscript{'}-di-O-acetyldideoxyguanosine (5 mg) was dissolved in 0.5 ml of dimethylformamide, and an excess (100 mg) of ethyl hydroxyethyl sulfide was added. Sodium hydride (10 mg) was added to convert ethyl hydroxyethyl sulfide to sodium ethylthio-ethoxide, and the reaction was incubated at 37\textdegree for 18 hr. By this time, the alkoxide had displaced the chlorine in position 6 of the acetylated deoxyguanosine, and the acetate groups had been hydrolyzed from the 3\textsuperscript{'}- and 5\textsuperscript{'}-positions of the deoxyribose. The reaction mixture was dried under vacuum, and the product was purified by high-pressure liquid chromatography using System A as described in the footnote to Table 1. The major peak, a late, intensely fluorescent compound eluting at 24.9.

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\textsuperscript{3}The abbreviation used is: CEES, chloroethyl ethyl sulfide.

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min, was verified to be the expected product by UV and mass spectrometry as described below. Material for these studies was collected from repeated chromatographic runs; samples were pooled, and most of the salt was removed by additional purification on a C\textsubscript{18} column with 15% acetonitrile in 25 mM triethylammonium formate, pH 4.5.

Reaction of CEES with Deoxyguanosine. Deoxyguanosine (35 mg) was dissolved in 35 ml of 50 mM cacodylate buffer, pH 7, at 37°. A total of 125 \mu l of CEES (approximately 35 \mu mol/ml) were added to the reaction vessel with stirring. The pH was maintained at approximately 7 with the addition of small amounts of 1 N NaOH. After 1 hr, there was no further change in pH, but the incubation was continued for an additional 6 hr to ensure complete hydrolysis of the CEES. The reaction mixture was filtered through a 0.2-\mu m nitrocellulose filter and separated by high-pressure liquid chromatography as described in the legend to Chart 4.

RESULTS AND DISCUSSION

Synthesis of the marker compound, O\textsuperscript{6}-ethylthioethyldeoxyguanosine, closely followed our synthesis of O\textsuperscript{6}-methyldeoxyguanosine (7). Although some difficulties were encountered in synthesizing the intermediate, presumably because of residual traces of water (3), displacement of the chlorine by the alkoxide proceeded smoothly. The major product was isolated as described above and was shown to be chromatographically pure in the 4 systems described in Table 1.

Although the identity of the product could be predicted from its manner of synthesis, structural confirmation was provided by UV, fluorescence, and mass spectrometry. The position of substitution of deoxyguanosine by a moiety which does not absorb in the UV region can be established by UV spectrometry. The UV spectra of O\textsuperscript{6}-ethylthioethyldeoxyguanosine at different pHs are shown in Chart 1; the 2 absorption maxima shown in this chart are typical of O\textsuperscript{6}-substituted deoxyguanosines (10). The fluorescence spectra shown in Chart 2 are also very similar to those reported by Singer for O\textsuperscript{6}-ethylguanosine (9). The nature of the substituent group in the O\textsuperscript{6}-position was confirmed by mass spectrometry as shown in Chart 3. This chart reveals a molecular ion peak at m/e 355 corresponding to the structure O\textsuperscript{6}-ethylthioethyldeoxyguanosine. The fragmentation pattern shows peaks at m/e 285 due to loss of a thiocetyl group and at 266 due to loss of the ethylthioethyl group. This spectrum also shows a large peak at m/e 151 which corresponds to (guanine + H\textsuperscript{+}). A similar peak appears in the mass spectrum of deoxyguanosine itself, and its presence in the O\textsuperscript{6}-ethylthioethyldeoxy-
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Guanosine spectrum indicates that the O₆-substituent is labile.

Mass spectrometry using both positive and negative fast atom bombardment confirmed the molecular weight of 355. Again, an intense peak was observed which corresponds to the dealkylated base.

Thus, the structure of the synthetic material was confirmed, and it could be used to show that the reaction of CEES with deoxyguanosine produced the same compound. Deoxyguanosine was incubated with CEES as described above, and the mixture of derivatives which was obtained was separated as shown in Chart 4. The peak which appeared at 68 min was intensely fluorescent and had the spectrum of an O₆-substituted deoxyguanosine, as shown in Chart 5. Material from this peak was collected in repeated chromatographic runs, lyophilized to dryness, redissolved, and shown to have the same chromatographic properties as marker O₆-ethylthioethyldeoxyguanosine in the 4 chromatographic systems shown in Table 1. Based on the amount of deoxyguanosine in the reaction mixture, 0.7% was converted to the O₆-derivative, and this derivative amounted to 5.3% of the total derivatives obtained. Structures of the other deoxyguanosine derivatives have not been completely established, but they evidently include the N-7 and N² derivatives reported previously (8).

Thus, we have shown that the sulfur mustards, like the nitrosoureas but evidently unlike the nitrogen mustards (11), react with the O₆-position of deoxyguanosine. Since the significance of substitution in this position was first pointed out by Loveless (6), evidence has gradually accumulated that O₆-substitution of guanine is both mutagenic and carcinogenic. Thus, the formation of O₆-ethylthioethyldeoxyguanosine would help to explain the strongly mutagenic effects of the sulfur mustards as suggested previously (2, 4). Investigations are currently underway to determine the extent and importance of O₆-ethylthioethyldeoxyguanosine formation in DNA.


Chart 4. High-pressure liquid chromatographic separation of a CEES-deoxyguanosine reaction mixture. A sample containing 20 μg of nucleosides was separated on a 5-μm Spherisorb column (4.6 x 250 mm) at a flow rate of 1 ml/min with: 3% acetonitrile in 25 mM KH₂PO₄, pH 4.5 for 25 min; a 3 to 30% gradient of acetonitrile in the same buffer for 50 min; and, finally, 30% acetonitrile in buffer for 25 min. Peak 4, unmodified deoxyguanosine; Peak 10, O₆-ethylthioethyldeoxyguanosine.

Chart 5. UV spectrum of the O₆-ethylthioethyldeoxyguanosine peak (Peak 10) in the chromatographic run shown in Chart 4. The spectrum was obtained by the Hewlett-Packard 1040A detector in an elution buffer of approximately 25% acetonitrile in 25 mM KH₂PO₄, pH 4.5.
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