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MECHANISM OF ACTION OF THE NITROSOUREAS—IV

SYNTHESIS OF THE 2-HALOETHYLNITROSOUREA-INDUCED DNA CROSS-LINK 1-(3-CYTOSINYL),2-(1-GUANYL)ETHANE

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Abstract—The 2-haloethylnitrosoureas have been shown to form the cross-linked structure 1-(3-cytosinyl),2-(1-guanyl)ethane in DNA. This cross-link has now been synthesized by the reaction of O^6-(2-fluoroethyl)guanosine with deoxycytidine in dimethyl sulfoxide followed by removal of the sugars by acid hydrolysis. This synthetic route supports the mechanism for cross-link formation in DNA that involves an initial attack on the O^6-position of guanine, followed by a rearrangement and subsequent reaction with cytosine. It also provides a practical route to the synthesis of 1-(3-cytosinyl),2-(1-guanyl)ethane for studies involving formation of this cross-link in DNA.

The 2-haloethylnitrosoureas are antitumor agents whose biological activities apparently result from their ability to modify DNA [1, 2]. Specifically, their cytotoxic effects have been related to DNA cross-linking as determined by the alkaline elution technique [3]. Studies from our laboratory have shown that cross-linking can be attributed, at least in part, to formation of the structure 1-(3-cytosinyl),2-(1-guanyl)ethane [4].

1-(3-Cytosinyl),2-(1-guanyl)ethane is apparently formed in DNA by the 2-haloethylnitrosoureas following an initial attack on the O^6-position of guanine. According to this scheme, an O^6-substituted-guanine, e.g. O^6-(2-fluorethyl)guanine or O^6-(2-chlorethyl)guanine, is formed initially and rearranges to the intermediate, 1,O^6-ethanoguanine. 1,O^6-Ethanoguanine then reacts with cytosine, probably in the opposite DNA strand, to yield $CytCH_2CH_2Gua$§ [4, 5]. This mechanism is supported by the observation that cells lacking in the ability to repair O^6-alkylguanines are more sensitive to the cytotoxic action of the 2-haloethylnitrosoureas than cells which are able to remove these alkyl groups [6]. Also in agreement with this mechanism is the observation that O^6-alkylguanine-DNA alkyltransferase prevents DNA cross-linking *in vitro*, as determined by physical measurements [7, 8]. Recently, we have shown that alkyltransferase specifically prevents formation of $CytCH_2CH_2Gua$ [9].

Here, we describe the synthesis of $CytCH_2CH_2Gua$ at the monomer level by a route which involves initial formation of $dCydCH_2CH_2Guo$ from the reaction of O^6-(2-fluorethyl)guanosine with deoxycytidine, as reported previously in abstract form [10]. This synthetic scheme follows the mechanism proposed above and, therefore, strongly supports it as the route to cross-linking in DNA. The initial synthetic product, $dCydCH_2CH_2Guo$, is suitable for use as a hapten to produce antibodies for the detection of this cross-link in DNA, and as a source of marker material in HPLC separations.

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§ Abbreviations: $CytCH_2CH_2Gua$, 1-(3-cytosinyl),2-(1-guanyl)ethane; $CytCH_2CH_2Guo$, 1-(3-cytosinyl),2-(1-guanosinyl)ethane; $dCydCH_2CH_2Guo$, 1-(3-deoxycytidyl),2-(1-guanosinyl)ethane; $dCydCH_2CH_2dGuo$, 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane; and FABMS, fast atom bombardment mass spectrometry.

MATERIALS AND METHODS

Materials. O^6-(2-Fluoroethyl)guanosine was prepared as described previously from 6-chloro-2-aminopurine-9-riboside (PWA, Mannheim) [5]. Deoxycytidine was obtained from P-L Biochemicals (Milwaukee, WI); both nucleosides were dried by repeated washings with absolute ethanol and evaporations to dryness. Marker amounts of $dCydCH_2CH_2dGuo$ were isolated from DNA which had been reacted with *N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea [4]. Marker 1-(2-hydroxyethyl)guanosine was synthesized as described previously [5]; 3-methyldeoxycytidine was purchased from Calbiochem (San Diego, CA) and 1-methylguanosine from the Sigma Chemical Co. (St Louis, MO).

Reaction of O^6(2-fluoroethyl)guanosine with deoxycytidine. O^6-(2-Fluoroethyl)guanosine (2 mg) was added to 40 μl of dimethyl sulfoxide containing 4 mg deoxycytidine, and the solution was incubated at 55° for 1 week. At the end of this time, it was added to 8 ml of water and the components were separated, 1 ml at a time, on a C_{18} column as described below. Increasing the time of incubation or the temperature, or adding pyridine or other bases did not seem to improve the yield significantly; the most important factor was the elimination of water.

High-performance liquid chromatography. HPLC separations were performed on a Spherisorb 5 μm (4.6 × 250 mm) C_{18} column from Alltech (Deerfield, IL). The column was eluted at 1 ml/min with a gradient of 1 to 10% acetonitrile in 50 mM KH_2PO_4,

pH 6, over 20 min, and then continued with 10% acetonitrile in the same buffer for 20 min. Elution profiles were monitored at 254 nm with a Hewlett–Packard 1040A detector system. This detector is interfaced with a Hewlett–Packard 85B computer and is able to record spectra of derivative peaks as they are separated, thus providing valuable information as to the purity and identity of absorbance peaks. Peak areas were recorded on a Hewlett–Packard 3390A integrating recorder.

Spectrometry. Ultraviolet spectra were obtained on a Beckman model 35 spectrophotometer in 0.1 HCl, in 0.1 M sodium cacodylate buffer, pH 7, and in 0.1 M NaOH. Fast atom bombardment mass spectrometry was performed on a Varian MAT 311A instrument using a sulfolane-glycerol matrix and an 8 keV beam of xenon atoms. The low volatility of the cross-linked structures makes it essential to remove all traces of sodium and potassium buffers; accordingly, derivatives were purified for mass spectrometry by elution from a C_{18} column with dilute triethylammonium formate buffer, then reisolated from a C_{18} column with a water–acetonitrile system, and finally dried over P_2O_5 *in vacuo*.

RESULTS

HPLC analyses of controls and of the reaction mixture of deoxycitidine and O^6-(2-fluoroethyl)guanosine are shown in Fig. 1. Deoxycytidine was essentially stable under these conditions (Fig. 1A), but O^6-(2-fluoroethyl)guanosine (peak 5 in Fig. 1B) gradually decomposed into two products labeled peaks 2 and 3. Peak 2 was shown to be 1-(2-hydroxethyl)guanosine by co-chromatography with known marker material and by its ultraviolet spectra as described previously [5]. The new product, peak 3, had UV spectra in acid, base, and neutral pH identical with those of peak 2, indicating that it was a 1-substituted guanosine. Mass spectrometry of the purified derivative showed that it had a molecular weight of 329, in agreement with a structure of 1-(2-fluorethyl)guanosine for this derivative.

As shown in Fig. 1C, an additional product (peak 4) appeared when deoxycytidine was reacted with O^6-(2-fluoroethyl)guanosine. This peak was collected and shown to consist of a single component by rechromatography on a C_{18} column and by analysis on a strong cation exchange column. Its UV spectra, as shown in Fig. 2, were identical with those of 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane [4]. FABMS revealed (M + 1) = 537 in agreement with the proposed structure 1-(3-deoxycytidyl),2-(1-guanosinyl)ethane for this compound.

Proof that we had synthesized 1-(3-deoxycytidyl),2-(1-guanosinyl)ethane came from an analysis of the acid hydrolysate of this compound and a comparison of the hydrolysis products with those of authentic 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)-ethane isolated from DNA which had been reacted with *N*,*N*′-bis(2-chlorethyl)-*N*-nitrosourea.

Putative 1-(3-deoxycytidyl),2-(1-guanosinyl) ethane, isolated as shown in Fig. 1, was treated with 0.1 N HCl for 1 hr at 100° and then separated by HPLC under the same conditions as those used in

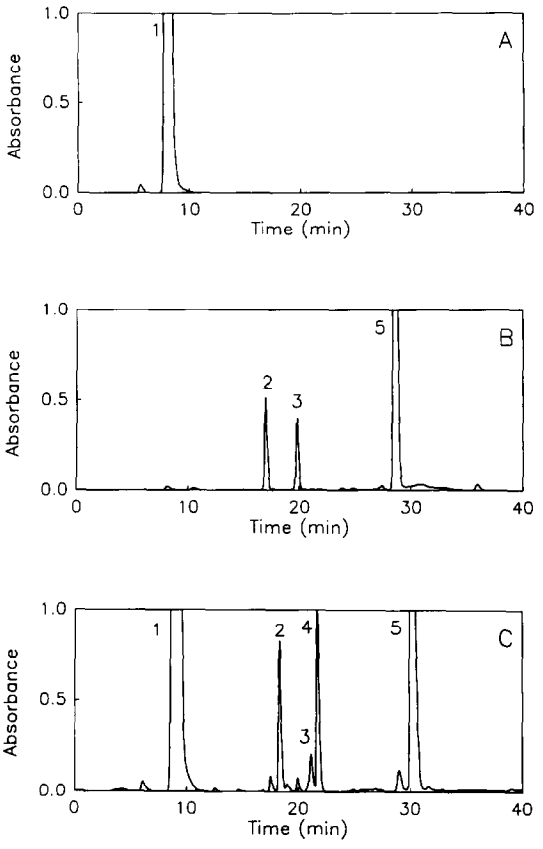

Fig. 1. HPLC profiles after incubation for 7 days at 55°: (A) deoxycytidine alone; (B) O^6-(2-fluoroethyl)guanosine alone; and (C) a mixture of deoxycytidine and O^6-(2-fluoroethyl)guanosine. See text for elution conditions and peak identifications.

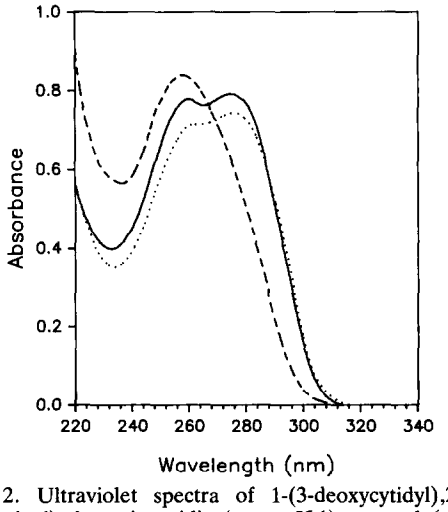

Fig. 2. Ultraviolet spectra of 1-(3-deoxycytidyl),2-(1-guanosinyl)ethane in acidic (· · ·, pH 1), neutral (——, pH 7), and basic (– – –, pH 12) solution.

Fig. 1. As shown in Table 1, three major ultraviolet-absorbing peaks were observed with retention times of 15.9, 19.8, and 21.7 min; the last of these corresponded to unchanged peak 4 material as verified

Table 1. HPLC retention times and molecular weights of cross-linked structures as determined by mass spectrometry

Derivative	Retention time (min)	Mol. wt
$CytCH_2CH_2Gua$	15.9	288
$CytCH_2CH_2Guo$	19.8	420
$dCydCH_2CH_2Guo$	21.7	536
$dCydCH_2CH_2dGuo$	21.9	520

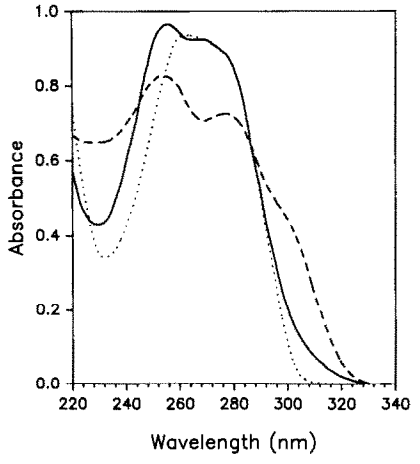

Fig. 3. Ultraviolet spectra of 1-(3-cytosinyl),2-(1-guanosinyl)ethane in acidic (· · ·, pH 1), neutral (——, pH 7), and basic (– – –, pH 12) solution.

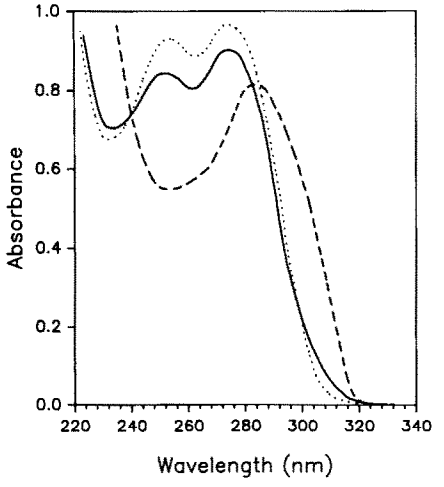

Fig. 4. Ultraviolet spectra of 1-(3-cytosinyl),2-(1-guanyl)ethane in acidic (· · ·, pH 1), neutral (——, pH 7), and basic (– – –, pH 12) solution.

by its characteristic ultraviolet spectrum and its retention time. The two earlier peaks were collected for ultraviolet and mass spectrometry and were assigned the structures 1-(3-cytosinyl),2-(1-guanyl)ethane and 1-(3-cytosinyl),2-(1-guanosinyl)ethane, respectively, as described below.

Material from the 19.8 min peak had the ultraviolet spectra shown in Fig. 3. while material in the 15.9 min peak had the spectra shown in Fig. 4. FABMS revealed (M + 1) = 421 for the 19.8 min peak and (M + 1) = 289 for the 15.9 min peak, both of which are in agreement with the predicted molecular weights for the structures shown in Table 1. The 15.9 min peak had lost both the ribose and the deoxyribose sugars, while the 19.8 min peak had evidently lost the deoxyribose sugar from the substituted deoxycytidine but not the ribose sugar from the substituted guanosine.

Since we thought the purine nucleoside might be less stable than the pyrimidine nucleoside, we compared the stability of 1-methylguanosine with 3-methyldeoxycytidine (data not shown). These studies, performed spectrometrically, showed that 3-methyldeoxycytidine was less stable in an acidic solution than 1-methylguanosine.

Hydrolysis of $dCydCH_2CH_2dGuo$ isolated from DNA under the same conditions used for hydrolysis of synthetic $dCydCH_2CH_2Guo$ yielded a single ultraviolet-absorbing peak with a retention time of 15.9 min and the same ultraviolet spectra (shown in Fig. 4) as that obtained from synthetic 1-(3-deoxycytidyl),2-(1-guanosinyl)ethane. The products were shown to be the same by co-chromatography on a C_{18} column.

Thus, on the basis of the ultraviolet and mass spectrometric data and the fact that it could be acid hydrolyzed to the same product that we obtained by acid hydrolysis of $dCydCH_2CH_2dGuo$ isolated from DNA, we concluded that we had synthesized $dCydCH_2CH_2Guo$ from O^6-(2-fluoroethyl)-guanosine and deoxycytidine. The yield of $dCydCH_2CH_2Guo$ in the reaction whose analysis is shown in Fig. 1 was 13%, based on O^6-(2-fluoroethyl)guanosine. Yields ranged up to 18% depending, apparently, on our success in excluding water from the reaction. The yield was not increased significantly by varying time and temperature, or by the addition of pyridine or other bases. A systematic study of other solvent systems was not undertaken, however.

DISCUSSION

The successful synthesis of $CytCH_2CH_2Gua$ from O^6-(2-fluoroethyl)guanosine and deoxycytidine shows that our proposed route to this cross-link in DNA, which involves an initial attack on the O^6-position of guanine, is probably valid. Furthermore, since the presence of O^6-(2-fluoroethyl)guanine has been demonstrated in DNA that was treated with *N*,*N*′-bis(2-fluoroethyl)-*N*-nitrosourea, at least some of the cross-links presumably arise from O^2-(2-fluoroethyl)guanine, specifically [5]. This would not exclude the possibility that other intermediates involving some other residue of the parent 2-haloethylnitrosourea as a leaving group may also lead to cross-link formation. For example, attack on DNA by a cyclized form of *N*,*N*′-bis(2-fluoroethyl)-*N*-nitrosourea may lead to initial substitution at the O^6-position of guanine by the

$$—CH_2CH_2N(NO)CONHCH_2CH_2F$$

group [1, 11]. Alternatively, initial substitution at the O^6-position of guanine may involve the $—CH_2CH_2NNOH$ group, as suggested by Buckley

and Brent [12]. Either of these O^6-substituted guanines could probably form the intermediate 1,O^6-ethanoguanine and lead to the CytCH_2CH_2Gua cross-link.

In this regard, recent studies of the reaction of *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea with guanosine may be relevant [13]. These studies have shown that *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea reacts with guanosine in the presence of bromide ion to form 7-bromoethylguanosine in addition to 7-chloroethylguanosine. This observation indicates that some of the initial substitution of guanosine involves a highly reactive intermediate.

It is also of interest to compare the slow rate of cross-link formation from O^6-(2-fluoroethyl)-guanosine reported here (1 week at 55°) with the observation that *N,N'*-bis(2-fluoroethyl)-*N*-nitrosourea cross-links DNA in a few hours [14]. Although this difference may indicate that the cross-linking measured by Kohn [14] occurs by a different mechanism than that described here or that the difference in solvent affects the rate, it seems probable that steric factors are influencing the rate in DNA. The local DNA structure may bring the substituted guanine into a favourable position for reaction with cytosine and thereby accelerate the reaction.

In summary, we have described a relatively straightforward synthetic route to the DNA cross-link, CytCH_2CH_2Gua. A similar synthesis could be used to prepare 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane, but this would require the use of the less readily available 6-chloro-2-aminopurine-9-deoxyriboside as a starting material rather than 6-chloro-2-aminopurine-9-riboside. Finally, the synthesis reported here offers further insight into the likely mechanism by which the CytCH_2CH_2Gua cross-link is formed in DNA.

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