increase it.[3] The chemistry of the HCN/AlbSAuPEt$_3$ reaction described here suggests, however, that HCN from inhaled smoke may alter auranofin metabolism more subtly by accelerating the formation of Et$_3$PO in vivo. The acetylthioglucose ligand of auranofin is rapidly displaced and metabolized, while the gold-bound phosphine is slowly displaced and oxidized over 24 to 72 h.[7] If, as HCN enters the blood, it reacts with AlbSAuPEt$_3$ (which forms in whole blood, in serum, and in direct reactions of auranofin with albumin), the displacement and subsequent oxidation of the phosphine should occur more rapidly in smokers than in nonsmokers. The extent to which accelerated Et$_3$PO production is observed in vivo will depend on the competition between cyanide binding to gold and its usual metabolic fates: binding to methemoglobin and oxidation to thiocyanate.

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Registry No. HCN, 74-90-8; Et$_3$PAuCN, 90981-41-2; Et$_3$P, 554-70-1; Et$_3$PO, 597-50-2; [(Et$_3$P)$_2$Au]$^+$, 45154-29-8.

Evidence for an Episulfonium Ion Intermediate in the Formation of *S*-[2-(*N*^{7}-Guanyl)ethyl]glutathione in DNA

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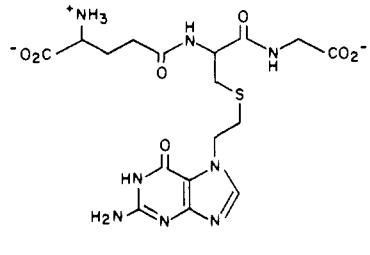
Abstract: The carcinogen ethylene dibromide (EDB) is bioactivated via a pathway involving initial conjugation with the tripeptide glutathione (GSH) in a reaction catalyzed by GSH *S*-transferase. The conjugate then reacts preferentially with DNA guanyl residues to generate *S*-[2-(*N*^{7}-guanyl)ethyl]glutathione. Rates of hydrolysis and alkylation of 4-(*p*-nitrobenzyl)pyridine with several cysteinyl and homocysteinyl analogues of *S*-(2-haloethyl)glutathione at pHs 2.2, 6.4, and 8.5 are consistent with the hypothesis that an episulfonium ion is a common intermediate in both the hydrolysis and alkylation reactions. Consistently, 2-amino-6-chlorohexanoic acid failed to react with 4-(*p*-nitrobenzyl)pyridine. The stereochemical course of the overall reaction was studied with [*threo*-1,2-^{2}H$_2$]EDB and [*erythro*-1,2-^{2}H$_2$]EDB, which were incubated with GSH, rat liver cytosol, and DNA; the resulting DNA *N*^{7}-guanyl adducts were isolated and analyzed by NMR techniques in order to determine the stereochemical course of the reaction. Two-dimensional correlated (COSY) NMR indicated that the reaction had occurred by a single stereochemical course. The magnitude of nuclear Overhauser effects between the ethylene protons suggests that the reaction occurs with net inversion of configuration of the methylene protons. This conclusion was confirmed upon comparison of the COSY NMR spectra of the biologically generated adducts with those that were synthetically prepared from the deuteriated EDB diastereomers via a known stereochemical route. This observation, combined with the kinetic data, supports a reaction mechanism where the EDB–GSH conjugate forms an episulfonium ion prior to reaction with DNA guanyl residues.

Ethylene dibromide (EDB), an extensively used agricultural and industrial chemical, is mutagenic in a variety of test systems[1-4] and is carcinogenic in laboratory animals.[4-8] While epidemiological studies on humans exposed to large EDB doses are inconclusive with regard to carcinogenic risk,[9,10] EDB is acutely toxic and has caused two human deaths.[11] Recent public concern about carcinogenicity has led to the ban of its use as a pesticide.[12]

While EDB is primarily metabolized via an oxidative cytochrome P-450 initiated route,[13,14] the majority of the experimental evidence supports the hypothesis that the pathway responsible for the genotoxicity of this and other *vic*-dihaloethanes involves glutathione *S*-transferase catalyzed conjugation with the tripeptide glutathione (GSH) to yield *S*-(2-bromoethyl)glutathione (route a, Figure 1).[1,14-21] The conjugate reacts preferentially with DNA[16] to form primarily *S*-[2-(*N*^{7}-guanyl)ethyl]glutathione.[15,19,22]



The precise nature of the ultimate DNA alkylating species is unknown. The instability of the putative *S*-(2-bromoethyl)glu-

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[†] Department of Biochemistry.
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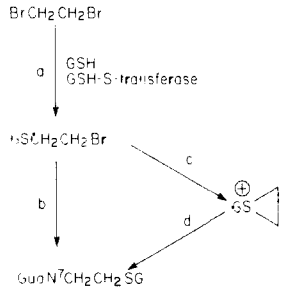


Figure 1. Proposed bioactivation pathway of EDB by GSH *S*-transferase.

tathione conjugate has prevented isolation and characterization; the half-life of the reactive species has been estimated to be less than 10 s.[22] While the conjugate may react directly with DNA guanyl residues (pathway b, Figure 1), it has been proposed that intramolecular displacement of the halogen by the β-sulfur atom to generate a reactive thiiranium (episulfonium) ion occurs prior to interaction with DNA (pathways c and d, Figure 1).[18,19,21,23,24] A third, alternative proposal put forward by Schasteen and Reed[23] suggests, at least in the case of *S*-(2-chloroethyl)glutathione, that the γ-glutamyl bond is cleaved to generate a cysteinyl amine. This species then might react with DNA in a non-episulfonium ion mechanism in which the free amine of cysteine assists the loss of halide prior to reaction with DNA. These authors found support for the pathway in studies in which an enhancement in the rate of hydrolysis of *S*-(2-chloroethyl)cysteine to *S*-(2-hydroxyethyl)cysteine was observed at high pH. The N-acetylated derivative did not show this pH-dependent increase in hydrolysis rate, and the authors postulated that the free amine is responsible for the accelerated rate.

The purpose of our studies was the characterization of the ultimate DNA alkylating species formed following conjugation of EDB with GSH. The first series of experiments presented deals with investigation of the role of the free cysteinyl amine group and the sulfur atom in alkylation reactions by comparing the rates of hydrolysis and alkylation of 4-(*p*-nitrobenzyl)pyridine by *S*-(2-chloroethyl)homocysteine analogues and the desthio compound 2-amino-6-chlorohexanoic acid with those of the cysteine half mustards at various pHs. The second study involved the examination of the stereochemical course of the overall reaction through the use of [*erythro*-1,2-$^2H_2$]EDB and [*threo*-1,2-$^2H_2$]EDB (Scheme I) to determine the number of S_N2 inversions occurring during the overall process.

Results

Kinetic Studies. The kinetic studies were designed to elucidate the role of the free cysteinyl amine group and the sulfur atom in the hydrolysis and alkylation reactions of the cysteine β-halo thioethers. The importance of the sulfur atom in these reactions was examined by comparing the rate of alkylation of 4-(*p*-nitrobenzyl)pyridine by 2-amino-6-chlorohexanoic acid with those of the cysteine and the homocysteine analogues; the finding of similar reaction rates with these compounds would indicate that the sulfur moiety is not involved in the alkylation process. The involvement of the free cysteinyl amine in the hydrolysis of *S*-(2-chloroethyl)-L-cysteine at high pHs has been previously suggested.[23] We explored the ability of the amine moiety to influence the alkylation rates by comparing the rates of hydrolysis and alkylation of 4-(*p*-nitrobenzyl)pyridine by *S*-(2-chloroethyl)-DL-homocysteine analogue with those of cysteine half-mustards at various pHs. The extra methylene group should slow the reaction rates[25] if the cysteinyl amine is important in the reaction, since a seven-membered ring is required for the participation of the amine atom in this analogue. Furthermore, acetylation of the amine group should also slow the reaction rates if such a neighboring group effect occurs.

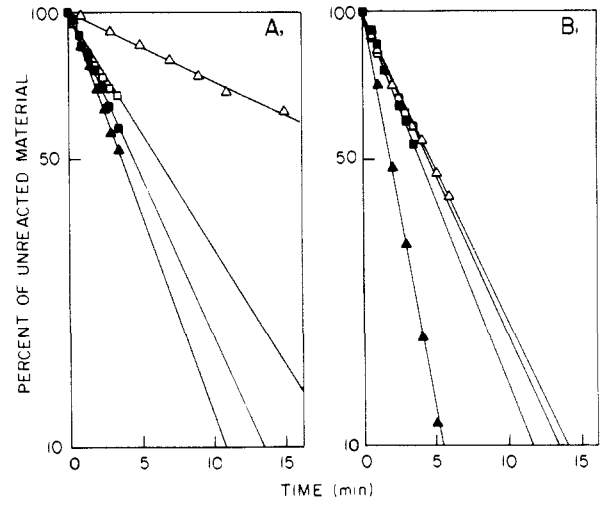
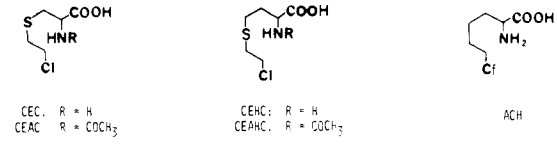


Figure 2. Rates of alkylation of 4-(*p*-nitrobenzyl)pyridine by *S*-(2-chloroethyl)-L-cysteine (Δ) and *S*-(2-chloroethyl)-DL-homocysteine (▲) and their *N*-acetylated derivatives (□ and ■, respectively) at pHs 6.5 (A) and 8.6 (B).

Table I. Effect of pH on the Rates of Hydrolysis and Alkylation of *S*-(2-Chloroethyl)cysteine and *S*-(2-Chloroethyl)homocysteine Analogues



pH	half-life, min				
	CEC	CEHC	CEAC	CEAHC	ACH
	Hydrolysis				
2.2	18.7	2.8	2.3	1.9	nd[a]
6.2–6.4	14.7	2.0	4.3	1.3	nd
8.3–8.6	2.0	1.3	2.6	1.1	nd
	Alkylation				
2.2	43.9	4.0	5.5	4.6	nd
6.3–6.5	18.3	3.5	5.4	4.2	nr[b]
8.5–8.8	4.2	1.3	4.1	3.6	nr

[a] nd = not determined. [b] nr = no reaction.

Table I summarizes half-life data obtained for the hydrolysis and 4-(*p*-nitrobenzyl)pyridine alkylation reactions of the cysteine and homocysteine half-mustards and their *N*-acetylated derivatives at various pH values. 2-Amino-6-chlorohexanoic acid failed to react with 4-(*p*-nitrobenzyl)pyridine at either pH 6.5 or 8.6, indicating that the sulfur moiety is required for alkylation of this nucleophile. With the exception of *S*-(2-chloroethyl)-L-cysteine, all of the β-halo thioesters underwent hydrolysis to the corresponding alcohol or alkylated 4-(*p*-nitrobenzyl)pyridine at rather similar rates independent of pH. The similarity in the rates of

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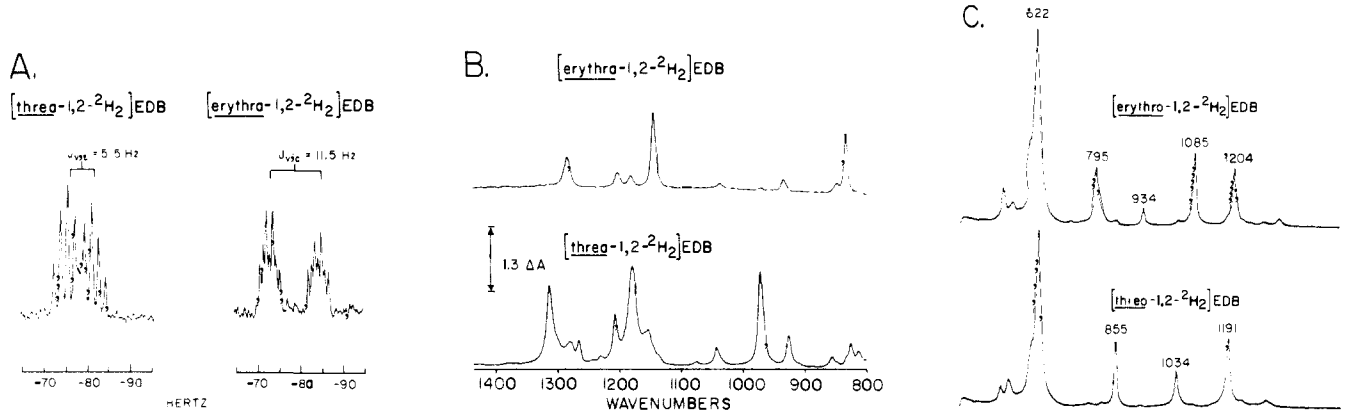

Figure 3. Chemical characterization of [*threo*-1,2-$^2H_2$]EDB and [*erythro*-1,2-$^2H_2$]EDB: (A) ^{13}C satellite signals in the ^1H NMR spectra; (B) IR spectra; (C) Raman spectra.

hydrolysis of *N*-acetyl-*S*-(2-chloroethyl)-L-cysteine and the homocysteine analogues demonstrates that the extra methylene group in the homocysteine derivatives does not affect the reaction rate. This observation supports a common intermediate. The reaction of *S*-(2-chloroethyl)-L-cysteine with water or 4-(*p*-nitrobenzyl)-pyridine is significantly decreased at pH 2.2 or 6.5. However, the half-life of the L-cysteine analogue approaches that of the other sulfur mustards at pH 8.5, suggesting that a protonated amino group retards the reaction of this compound with nucleophiles.

Stereochemical Studies. The pathway by which the EDB–GSH–guanine adduct is formed might (1) involve an episulfonium ion intermediate, causing the overall process to contain three S_N2 reactions, or (2) be a direct alkylation of guanyl residues by the EDB–GSH conjugate, causing the overall process to contain two S_N2 processes. Moreover, the two processes might occur concurrently or the reaction might involve a carbocation; in either case stereospecificity would be lost. These various possibilities, while invisible with EDB itself, become distinguishable when the reaction is carried out with [*threo*-1,2-$^2H_2$]EDB or [*erythro*-1,2-$^2H_2$]EDB. Four different stereoisomers of the EDB–GSH–guanine condensation product can arise from use of [1,2-$^2H_2$]EDB substrates due to the asymmetry of the GSH portion of the adduct. Scheme I shows that the threo form of [1,2-$^2H_2$]EDB, which is actually a racemic mixture of (1*R*,2*R*)- and (1*S*,2*S*)[1,2-$^2H_2$]-EDBs, would produce an equimolar mixture of the two erythro forms of the adduct, if the process occurred via the episulfonium ion (three S_N2 steps) yielding net inversion (path A), whereas it would produce the threo adducts if direct reaction occurred (two S_N2 steps) yielding net retention (path B). By the same token, the erythro form of [1,2-$^2H_2$]EDB, i.e., the meso form, would give threo products by path A and erythro products by path B. With either form of EDB, mixed mechanisms or an S_N1 process would lead to all four of the products. In path A the potential exists for the ring closure to the episulfonium ion to be reversible; however, reversibility will not alter the stereochemical outcome with either of the stereo forms of [1,2-$^2H_2$]EDB.

These mechanistic possibilities can potentially be distinguished by nuclear magnetic resonance (NMR) spectroscopy (Figure 4), because the four EDB protons in the EDB–GSH–guanine adduct are resolved from one another but mutually coupled so that six off-diagonal signals are seen in the two-dimensional correlated (COSY) NMR spectrum; two geminal and four vicinal couplings result in this $A_1A_2X_1X_2$ pattern. Upon deuterium substitution, the ^1H NMR spectra should be simplified. If reaction occurs by a stereospecific pathway, i.e., net retention or net inversion, only minor simplification of the one-dimensional spectrum will occur because a mixture of two products arises from either starting material; however, substantial simplification will occur in the COSY spectrum where the number of off-diagonal signals will reduce to just two vicinal ones, one resulting from each product. The crux of this stereochemical analysis is that, of the four possible vicinal off-diagonal signals in the COSY spectrum, net retention will produce two of them and net inversion the other two. A nonstereospecific mechanism will produce all four.

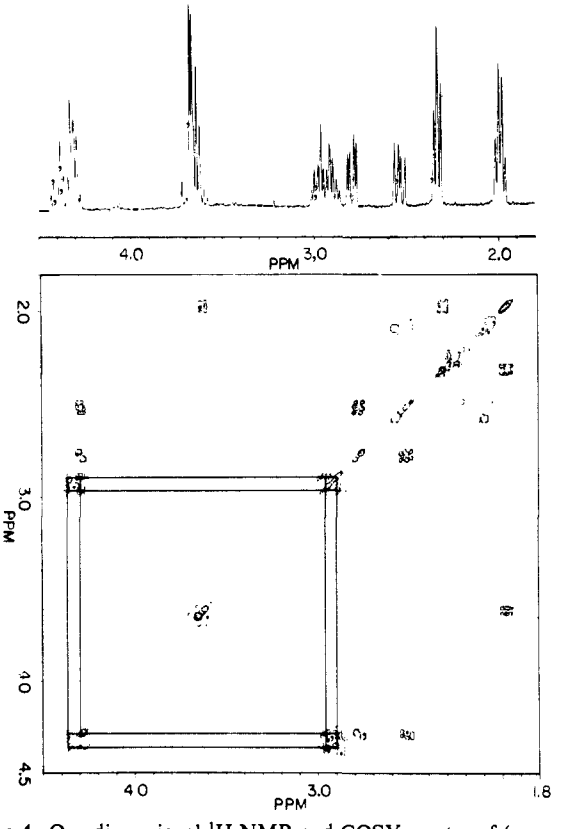

Figure 4. One-dimensional ^1H NMR and COSY spectra of (enzymatically derived) *S*-[2-(*N*^{7}-guanyl)ethyl]glutathione (see ref 15 for original spectrum).

The study required the synthesis of [*threo*-1,2-$^2H_2$]EDB and [*erythro*-1,2-$^2H_2$]EDB. These compounds were prepared according to the methods of Berstein et al.[26] The chemical characterization of the deuteriated material is summarized in Figure 3. The Raman and infrared (IR) spectra of [*erythro*-1,2-$^2H_2$]EDB matched those published by Berstein et al.[26] The signals in our Raman spectra of [*threo*-1,2-$^2H_2$]EDB account for all the signals in that reported for a mixture of [*erythro*-1,2-$^2H_2$]EDB and [*threo*-1,2-$^2H_2$]EDB not pertaining to the erythro deuteriated material.[26] Furthermore, the $^{13}C^1H^2H-^{12}C^1H^2H$ coupling constants, determined from splittings in the ^{13}C satellite signals in the ^1H NMR spectrum of the deuteriated materials, are consistent with the expected spatial orientation of the protons, assuming that EDB exists preferentially in the anti conformation. A large $^1H-^1H$

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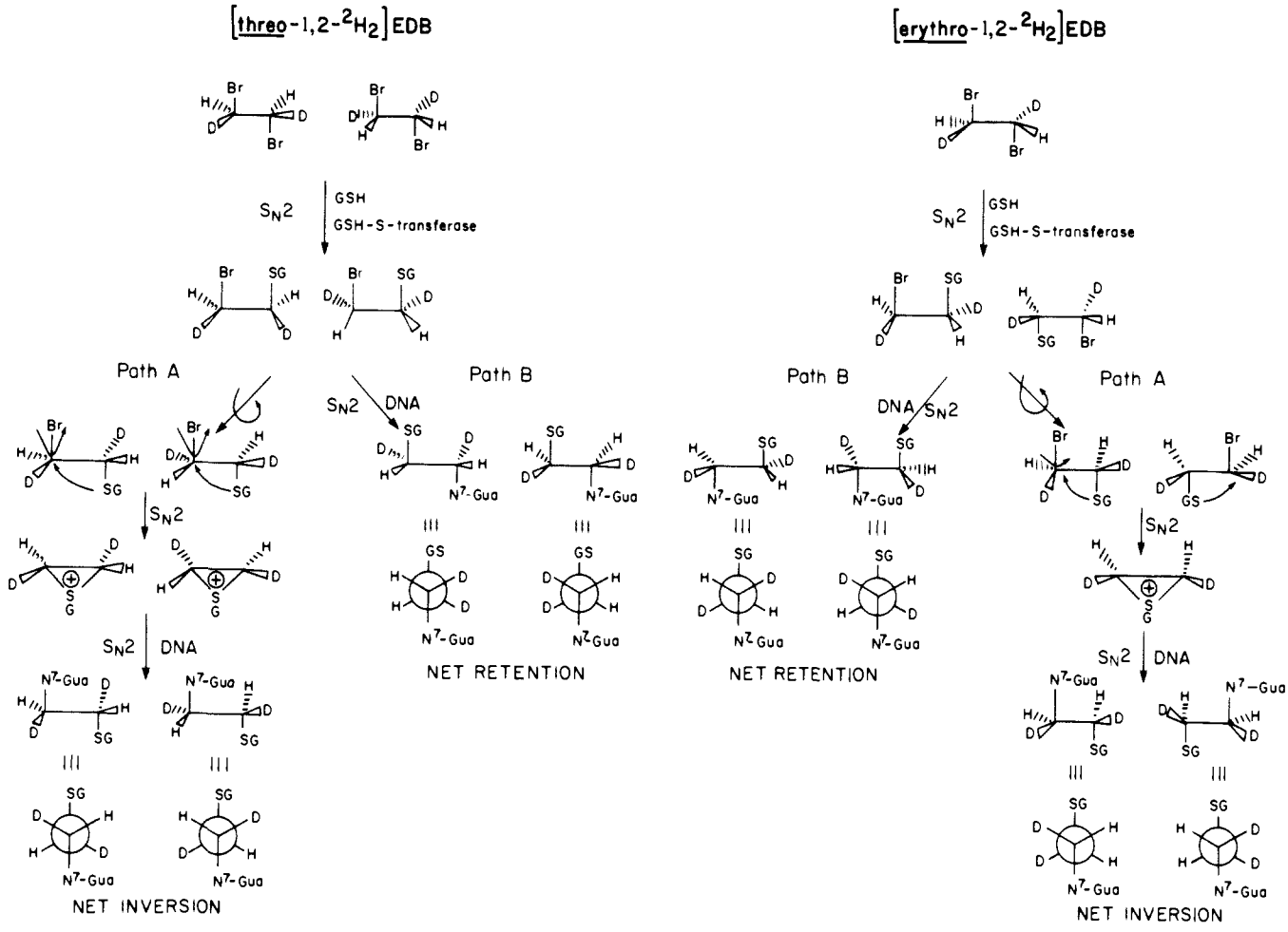
Scheme I. S_N2 Pathways under Consideration for the Reaction of the Stereoselectively Deuteriated EDB Diastereomers with DNA following Conjugation with GSH



vicinal coupling constant (J_{vic} = 11.5 Hz) is observed for protons of [*erythro*-1,2-$^2H_2$]EDB, whereas a small ^1H–^1H vicinal coupling constant (J_{vic} = 5.5 Hz) is measured for the protons of [*threo*-1,2-$^2H_2$]EDB. On the basis of predictions from a Karplus plot of the relationship of torsional angles to vicinal coupling constants, these values suggest that the stereochemical assignments are correct. The spectral analyses demonstrated that both deuteriodiastereomers were greater than 95% stereochemically pure.

[*Erythro*-1,2-$^2H_2$]EDB and [*threo*-1,2-$^2H_2$]EDB were then incubated with chiral GSH, rat liver cytosol (a source of GSH *S*-transferase enzymes), and calf thymus or herring sperm DNA. Tracer amounts of [U-^{14}C]EDB were used to aid in the purification of the adduct. Following isolation and purification by HPLC methods, the *S*-[2-(*N*^{7}-guanyl)ethyl]glutathione adducts were analyzed by one- and two-dimensional NMR techniques. The ^1H NMR and COSY spectra of the adducts generated from [*erythro*-1,2-$^2H_2$]EDB and [*threo*-1,2-$^2H_2$]EDB are shown in Figure 5. A comparison of the ^1H NMR spectra of the adducts derived from the deuteriated EDBs (Figure 5) with that derived from the fully protonated EDB (Figure 4) demonstrates a simplification of the signals of the ethylene bridge protons as a result of loss of the two geminal couplings and two of the vicinal couplings due to deuterium substitution. The observation of only two off-diagonal peaks associated with the ethylene protons in the COSY NMR spectra of the deuteriated adducts demonstrates that the in vitro reaction pathway proceeds by a single bimolecular pathway without involvement of open carbocations or other species that would destroy the stereospecificity of the process. The COSY NMR spectra also show that the coupling patterns of the ethylene protons are complementary in the deuteriated DNA adducts from [*erythro*-1,2-$^2H_2$]EDB and [*threo*-1,2-$^2H_2$]EDB; e.g., in the spectrum of the adduct generated from [*erythro*-1,2-$^2H_2$]EDB, the low-field proton next to the nitrogen atom is coupled to the low-field proton next to the sulfur atom, whereas in the spectrum of the adduct generated from [*threo*-1,2-$^2H_2$]EDB, the low-field proton adjacent to the nitrogen atom is coupled to the high-field proton adjacent to the sulfur atom.

An attempt was made to assign the torsional angle between the two remaining protons in the adducts from the deuteriated EDBs by using Karplus relationships;[27] however, internally consistent values could not be obtained. The failure is possibly due to internal motion in the adduct or due to utilization of an inappropriate mathematical relationship. Attempts to slow rotation by reducing the temperature in $C^2H_3O^2H$–^2H_2O or by conversion of the adduct to the corresponding sulfone with performic acid were unproductive. As an alternative probe of spatial relationships of the ethylene bridge protons, nuclear Overhauser effect (NOE) difference experiments were performed. When the signals for the ethylene protons neighboring the sulfur atom were irradiated in the adduct generated from [*erythro*-1,2-$^2H_2$]EDB, a 4% NOE to their partners adjacent to the nitrogen atom was observed, whereas a NOE of 0.8% was detected in a similar experiment with the adduct from [*threo*-1,2-$^2H_2$]EDB. Assuming that the average conformation of the adducts places guanine and GSH *approximately* anti, the protons are anti to each other in the first case and gauche in the second one (see the Newman projection in Scheme I). On this basis, the in vitro reaction can be assigned as proceeding with net inversion of configuration.

In order to confirm this conclusion, the dideuterio *S*-[2-(*N*^{7}-guanyl)ethyl]glutathiones were prepared stereospecifically by an independent route involving reaction of the deuterio EDBs first with guanosine and then with GSH followed finally by removal of ribose from the adduct (Scheme II). Preliminary studies

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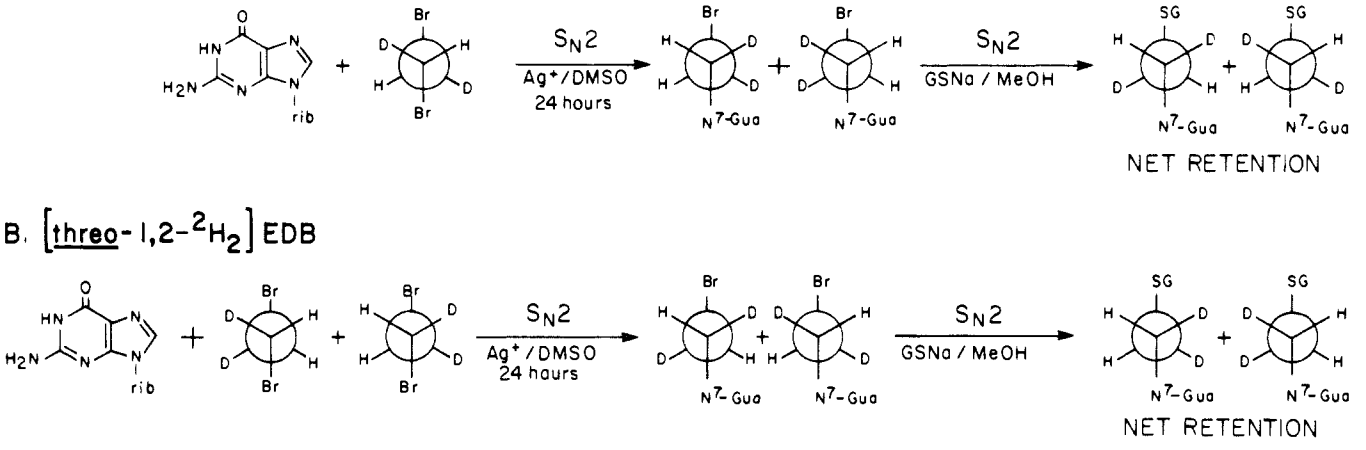
Scheme II. Stereoselective Synthesis of *S*-[2-(N^7-Guanyl)[1,2-^{2}H]ethyl]glutathiones from (A) [*erythro*-1,2-$^2H_2$]EDB and (B) [*threo*-1,2-$^2H_2$]EDB



revealed that the reaction sequence was not stereospecific, yielding adduct in which either form of [1,2-$^2H_2$]EDB gave a mixture of threo and erythro products as indicated by the COSY spectrum of the adducts showing all four off-diagonal signals representing vicinal couplings. Scrambling was demonstrated to occur in the initial condensation where Finkelstein reactions[28] by bromide ion (which is released in the relatively slow reaction of guanosine with EDB) led to loss of stereochemical integrity in the condensation product. The problem was circumvented by carrying out the reaction in the presence of $AgNO_3$ to scavenge the bromide ion. COSY NMR analysis of *N*-(2-bromo[1,2-$^2H_2$]ethyl)guanosine generated from [*erythro*-1,2-$^2H_2$]EDB and [*threo*-1,2-$^2H_2$]EDB in the presence of $AgNO_3$ demonstrates that these compounds were stereochemically pure. The coupling patterns were complementary: the low-field CHN^7 proton was coupled to the high-field CHBr proton in the product derived from [*threo*-1,2-$^2H_2$]EDB, whereas the low-field CHN^7 proton was coupled to the low-field CHS proton in the product formed from [*erythro*-1,2-$^2H_2$]EDB.

A further question concerning the reaction of EDB with guanosine is whether it is a single S_N2 process. A halonium ion is a viable intermediate, particularly in the presence of silver ion,[29] and would result in an additional S_N2 process. To test this possibility, the reaction of 2-bromo-1-chloro[1-^{2}H]ethane with guanosine in the presence of silver nitrate was investigated. No scrambling of label was observed, as the sole product, N^7-(2-chloroethyl)guanosine, contained deuterium only on the carbon atom next to the halogen. Therefore, a halonium ion is probably not an intermediate in this reaction sequence.[30] Consequently, the stereosynthetic route involves two S_N2 reaction steps so there is a net retention of configuration at the ethylene carbon atoms.

S-[2-(N^7-Guanyl)[1,2-$^2H_2$]ethyl]glutathione adducts were then prepared from the stereospecifically synthesized N^7-(2-bromo-[1,2-$^2H_2$]ethyl)guanosine by reaction of purified material with sodium GSH thiolate in CH_3OH [N^7-(2-bromoethyl)guanine did not react with the thiolate anion of glutathione, presumably due

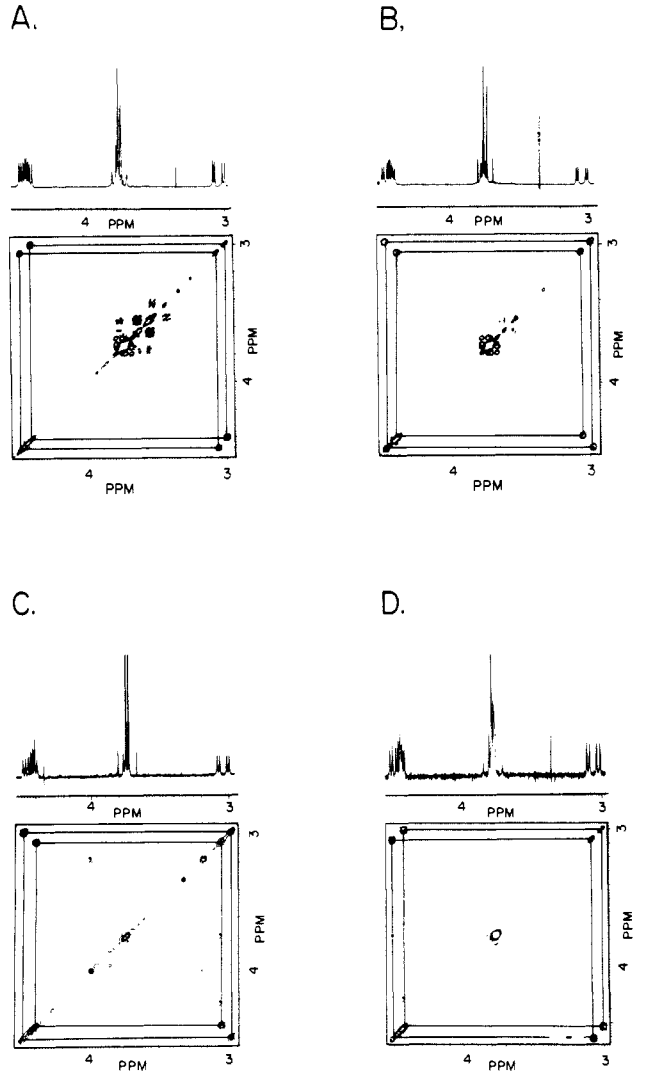


Figure 5. ^{1}H NMR and COSY spectra of *S*-[2-(N^7-guanyl)[1,2-$^2H_2$]-ethyl]glutathiones generated in vitro (A and B) and synthetically (C and D) from [*erythro*-1,2-$^2H_2$]EDB (A and C) and [*threo*-1,2-$^2H_2$]EDB (B and D).

(28) March, J. *Advanced Organic Chemistry: Reactions, Mechanisms, and Structures*; McGraw-Hill: New York, 1977; p 391.

(29) (a) Olah, A. *Halonium Ions*; Wiley-Interscience: New York, 1975; p 8. (b) March, J. *Advanced Organic Chemistry: Reactions, Mechanisms, and Structures*; McGraw-Hill: New York, 1977; p 334.

(30) Further support for a single-displacement reaction mechanism comes from similar studies performed with 2-bromoethyl *p*-toluenesulfonate in which exclusively 2-(N^7-guanosinyl)ethyl *p*-toluenesulfonate was formed. One might have anticipated some formation of N^7-(2-bromoethyl)guanosine if a cyclic bromonium ion were an important intermediate in the reaction. In addition, all three alkylating agents (2-bromo-1-chloro[$^2H_2$]ethane, EDB, and 2-bromoethyl *p*-toluenesulfonate) reacted with guanosine at qualitatively similar rates. This observation argues for a common reaction mechanism for all three compounds. Finally, 2-(phenylthio)[2-$^2H_2$]ethyl *p*-bromobenzenesulfonate reacts directly with methylamine in DMSO without scrambling the label,[31] demonstrating that, under these conditions, episulfonium ion formation does not occur. Since sulfur is more nucleophilic than bromine, it is unlikely that a bromonium ion is formed prior to reaction with the N^7-position of guanosine under our in vitro reaction conditions.

(31) McManus, S. P., personal communication.

to its insolubility in the reaction mixture]. The guanine adduct was released from the ribose by heat treatment of the guanosine adduct at pH 6.5 for 30 min and was purified by the described HPLC technique. COSY NMR analysis (Figure 5C,D) of the products of this sequence indicated that alkylation of GSH had been stereospecific; each adduct showed only two off-diagonal signals due to vicinal coupling of the ethylene protons. The coupling relationships, as revealed in the COSY spectra for these adducts, were reversed from those for the enzymatically generated adducts; i.e., the adduct prepared synthetically from [*erythro*-1,2-$^2H_2$]EDB had the same pattern of couplings as the adduct prepared in vitro from [*threo*-1,2-$^2H_2$]EDB and vice versa. Since the independent synthesis involves two S_N2 steps leading to net retention of configuration at the ethylene carbons, these results demonstrate that the prochiral methylene protons underwent a net inversion of configuration during formation of the in vitro N^7 adducts.

Discussion

In this study we investigated the chemical nature of the DNA alkylating species formed following the GSH *S*-transferase catalyzed conjugation of EDB with GSH. The kinetic studies demonstrated that the sulfur moiety is required for alkylation. The similarity in half-lives of the β-chloro thioethers [with the exception of *S*-(2-chloroethyl)cysteine] is consistent with a common intermediate in the reaction sequence. The pH independence of the reaction rates is suggestive of rate-determining formation of an episulfonium ion. The apparently anomalous pH dependence of the reactions of *S*-(2-chloroethyl)-L-cysteine is also consistent with this proposal. In this case, the hydrolysis and alkylation reactions are hindered at low pH values. However, when the pH of the solution is greater than 8.3, the half-life of the cysteine adduct approaches that of the other analogues. These observations suggest that protonation of the amine slows the reaction. A protonated and unblocked cysteinyl amino group inhibits these reactions, presumably because the positively charged nitrogen atom inductively retards the rate-limiting formation of thiiranium ion. Our results demonstrate that this effect can be reversed by either (1) raising the pH to deprotonate the amine, (2) acetylating the amine, or (3) moving the positively charged amine away from the incipient episulfonium ion by the addition of an extra methylene group (homocysteine analogue). These results collectively suggest that an episulfonium ion is an obligatory intermediate in the model hydrolysis and alkylation reactions. They also suggest that cleavage of the γ-glutamyl bond of the GSH analogue prior to reaction with DNA would serve to lengthen the half-life of the reactive molecule since the resulting free amine would be protonated at physiological pH.

The stereochemical studies demonstrated that the overall process of DNA adduct formation follows a single S_N2 reaction pathway and that the pathway proceeds with net inversion of configuration at the methylene carbon atoms of EDB. Mangold et al.[32,33] demonstrated that GSH *S*-transferase catalyzed conjugation of several alkyl halides with GSH proceeded with inversion of configuration.[34] Consequently, we expected that the initial conjugation reaction in our reaction sequence (Scheme I) also proceeds with inversion of configuration. In order to explain the net inversion of configuration observed in the overall process, two more S_N2 reactions must occur during the reaction of *S*-(2-bromoethyl)glutathione with the N^7-position of DNA guanyl residues. Consequently, we conclude that, following initial conjugation of EDB with GSH, the resulting conjugate undergoes intramolecular displacement of the halogen to generate an episulfonium ion intermediate prior to reaction with the guanyl residues in DNA.[36]

Other studies also support our conclusions that an episulfonium ion is an important intermediate in the bioactivation of dihaloalkanes. For example, 1,3-dibromopropane and 1,4-dibromobutane are unable to alkylate DNA following conjugation with GSH.[21] In addition, van Bladeren et al.[38] found that the sulfur atom had to be positioned trans to the halogen in 1-halo-2-(alkylthio)-cyclohexanes in order to exhibit mutagenicity in a bacterial test system. The importance of an episulfonium ion intermediate in *S*-(2-chloroethyl)cysteine-induced cytotoxicity is suggested by studies in which *S*-(2-chloroethyl)cysteine was quite toxic to isolated rat hepatocytes but *S*-(3-chloropropyl)cysteine was not.[24]

Our work, along with the preexisting data, lends support for a bioactivation sequence in which EDB is conjugated to GSH in a GSH *S*-transferase catalyzed reaction and the resulting conjugate forms an episulfonium ion prior to interaction with the guanyl residues in DNA. It is unclear if the N^7 alkylation reaction is the critical step in EDB-induced genotoxicity. EDB does cause DNA damage in rats in vivo[39] and induces repair in several in vitro systems.[40] *S*-[2-(N^7-Guanyl)ethyl]glutathione accounts for greater than 95% of the radioactivity bound to rat liver DNA following in vivo exposure to radiolabeled EDB.[15] However, we cannot rule out the possibility that an unidentified minor adduct is responsible for the adverse biological effects of EDB, and it is possible that an episulfonium ion is not involved in the formation of such adduct. Research is currently being done to determine the biological relevance of *S*-[2-(N^7-guanyl)ethyl]glutathione.

Experimental Section

GSH, guanosine, and calf thymus and herring sperm DNA were purchased from Sigma Chemical Co. 2-Amino-6-chlorohexanoic acid (mp 174 °C, lit.[41] mp 173–174 °C) was prepared by the method of Ermolaev and Maumind.[41] *S*-(2-Hydroxyethyl)-L-cysteine and its acetylated analogue were synthesized as previously described[42] and converted to the corresponding *S*-(2-chloroethyl)cysteinyl derivatives by published methods.[43] All other chemicals were reagent grade. Male Sprague–Dawley rats (200 g) were purchased from Harlan Industries. NMR spectra were obtained with either a Bruker AM-400-NB or an IBM NR-300 spectrometer. Chemical shifts are reported in ppm; either sodium 2,2-dimethyl-2-silapentane-5-sulfonate or tetramethylsilane was used as an internal or external standard. Dr. Brian Sweetman (Vanderbilt University Mass Spectrometry Laboratory) recorded the fast atom bombardment mass spectrometry (FAB-MS). The spectra were obtained on a VG 70-250 system having extended geometry, a standard VG FAB ion source, a standard Ion-Tech saddle field FAB gun producing xenon atoms of 8-kV energy, and a VG 11/250 data system. Glycerol was used as the matrix for all samples except N^7-(2-bromoethyl)guanosine, where *m*-nitrobenzyl alcohol was the matrix; spectra were recorded over the range m/z 100–900. The electron-impact mass spectrometry (EI-MS) data were recorded by Brian Nobes (Vanderbilt University) on a Nermag R10-10C instrument. Dr. Prasad Polavarapu (Vanderbilt University) recorded the IR and Raman spectra using a Nicolet 6000 Fourier transform IR spectrometer and a self-designed Raman spectrometer.

***S*-(2-Chloroethyl)-DL-homocysteine Hydrochloride.** After sodium (1 g, 43 mmol) had dissolved in dry CH_3OH (25 mL), DL-homocysteine (2.5 g, 18.5 mmol) was added. 2-Bromoethanol (2.6 g, 21.1 mmol) was added 30 min later. After being stirred overnight, the reaction mixture was

(32) Mangold, J. B.; Abdel-Monem, M. M. *Biochem. Biophys. Res. Commun.* **1980**, *96*, 333–340.
(33) Mangold, J. B.; Abdel-Monem, M. M. *J. Med. Chem.* **1983**, *26*, 66–71.
(34) Several different GSH *S*-transferase proteins are found in liver cytosol,[35] and at least four of these isozymes can catalyze the reaction under consideration.[21] Our stereochemical results indicate that the mechanism is the same with all of these involved. For instance, a substantial contribution of an enzymatic double-displacement reaction would lead to scrambling of deuterium substitution.
(35) Mantle, T. J.; Pickett, C. B.; Hayes, J. D., Eds. *Glutathione S-Transferases and Carcinogenesis*; Taylor and Francis: London, 1987.
(36) The preparation of *S*-[(L-cysteinyl)ethyl]thiiranium trifluoromethanesulfonate has been recently reported.[37] Modifications of their procedure might permit synthesis of the corresponding *S*-[(glutathionyl)ethyl]-thiiranium ion, which then could be used for direct study of the reaction of this species with DNA.
(37) Dohn, D. R.; Casida, J. E. *Bioorg. Chem.* **1987**, *15*, 115–124.
(38) Van Bladeren, P. J.; van der Gen, A.; Breimer, D. D.; Mohn, G. R. *Biochem. Pharmacol.* **1979**, *28*, 2521–2524.
(39) Kitchin, K. T.; Brown, J. L. *Biochem. Biophys. Res. Commun.* **1986**, *141*, 723–727.
(40) Working, P. K.; Smith-Oliver, T.; White, R. D.; Butterworth, B. E. *Carcinogenesis (London)* **1986**, *7*, 467–472.
(41) Ermolaev, K. M.; Maumind, V. I. In *Sintez Prirodhnykh Soedinenii, kh Analogov i Fragmentov*; Danilov, S. N., et al., Eds.; Akademiya Nauk SSSR: USSR, **1965**; pp 150–154; *Chem. Abstr.* **1965**, *65*, 9013a.
(42) Van Bladeren, P. J.; Buijs, W.; Breimer, D. D.; van der Gen, A. *Eur. J. Med. Chem. Chim. Ther.* **1980**, *15*, 495–497.
(43) Carson, J. F.; Wong, F. F. *J. Org. Chem.* **1964**, *29*, 2203–2205.

concentrated under reduced pressure and the residue was applied to a Dowex 50 column (H^+ form). The column (1.5 × 25 cm) was washed with 700 mL of H_2O, and the reaction product was eluted with 1 N NH_4OH (200 mL). The aqueous phase was removed in vacuo and the product was recrystallized from 90% ethanol. Following filtration and drying under vacuum overnight, 2.1 g (11.7 mmol) of *S*-(2-hydroxyethyl)-DL-homocysteine was recovered (63% yield): ^1H NMR (^2H_2O) δ 2.15 (m, 2 H β-CH_2), 2.68 (t, 2 H, γ-CH_2) 2.25 (t, 2 H, CH_2S), 3.75 (t, 2 H, $HOCH_2$), 3.85 (dd, 1 H, α-CH); (+)FAB-MS, 180 $(M + H)^+$; (−)FAB-MS, 178 $(M - H)^-$.

S-(2-Hydroxyethyl)-DL-homocysteine (1.5 g, 8.3 mmol) was heated under reflux in concentrated HCl (25 mL) for 6 h. The reaction mixture was concentrated under reduced pressure to yield an oil (1.49 g, 6.4 mmol, 77% yield): ^1H NMR ([$^2H_6$]DMSO) δ 2.05 (m, 2 H, δ-CH_2), 2.7 (m, 2 H, δ-CH_2), 2.9 (t, 2 H, CH_2S), 3.75 (t, 2 H, $ClCH_2$), 3.98 (m, 1 H, α-CH); (+)FAB-MS, 198, 200 $(M + H)^+$.

***N*-Acetyl-*S*-(2-chloroethyl)-DL-homocysteine.** Acetic anhydride (0.6 mL) was added alternately with 1.5 N NaOH (7 mL) to a solution of *S*-(2-hydroxyethyl)-DL-homocysteine hydrochloride (0.5 g, 3 mmol) in 1.5 N NaOH (2.0 mL) at 0 °C. The reaction mixture was applied to a Dowex 50 column (H^+ form, 1.5 × 25 cm, elution with H_2O) to remove any unreacted starting material. Concentration of the void volume yielded a clear oil (0.67 g, 3 mmol, 100% yield): ^1H NMR δ 2.02 (m, 1 H, β-CH), 2.05 (s, 3 H, NCH_3), 2.17 (m, 1 H, β-CH), 2.64 (m, 1 H, δ-CH), 2.72 (m, 1 H, δ-CH), 2.75 (t, 2 H, CH_2S), 3.74 (t, 2 H, CH_2OH), 4.5 (q, 1 H, α-CH).

N-Acetyl-*S*-(2-chloroethyl)homocysteine (0.67 g, 3 mmol) was heated under reflux in concentrated HCl (20 mL) for 3 h. A yellow oil was recovered following concentration under reduced pressure and further drying with a vacuum pump overnight. Attempts to crystallize the oil were unsuccessful: ^1H NMR δ 1.85 (m, 2 H, β-CH_2), 1.85 (s, 3 H, $COCH_3$), 2.65 (m, 2 H, δ-CH_2), 2.85 (td, 2 H, SCH_2), 3.62 (t, 2 H, CHCl), 4.25 (m, 1 H, α-CH), 8.2 (d, 1 H, $NHCOCH_3$); (−)FAB-MS, 238, 240 $(M - H^-)$ 202 $(M^+ - H^+ - HCl^-)$; (+)FAB-MS, 240 and 242 $(M + H^+)$, 204 $(M + H^+ - HCl)$.

Stereochemical Studies. Synthesis. [*threo*-1,2-^{2}H]EDB and [*erythro*-1,2-$^2H_2$]EDB were synthesized from [*cis*-1,2-$^2H_2$]ethylene and [*trans*-1,2-$^2H_2$]ethylene (Merck, Sharp & Dome), respectively, according to the method of Berstein et al.[26] The NMR and mass spectra of these compounds were identical except for the ^{13}C satellite signals in the ^1H NMR spectrum described under Results (Figure 3A): ^1H NMR ($^2HCCl_3$) δ 3.0 (s); EI-MS, 188, 190 (M^+). The Raman and IR spectra are displayed in Figure 3.

***N*^{7}-(2-Bromoethyl)guanosine.** Guanosine (100 mg, 0.35 mmol), $AgNO_3$ (60 mg, 0.35 mmol), and EDB (0.2 mL, 2.3 mmol) were dissolved in DMSO (1.5 mL) in a tightly capped Reacti-vial (Pierce Chemical Co.). After 24 h at 37 °C in a rotating water bath, water (4 volumes) was added, and the mixture was centrifuged (2 × 10^3*g*, 10 min) to separate out the resulting AgBr and unreacted EDB. *N*^{7}-(2-Bromoethyl)guanosine was then purified on an octadecylsilyl (C18) HPLC column (Altex Ultrasphere-ODS, 5 μm, 10 × 250 mm) by a modification of the methods of Ludlum and Tong.[44] The column was eluted with 3% acetonitrile in 25 mM KH_2PO_4 (pH 4.5) for 5 min, followed by a linear gradient to 13% acetonitrile over 10 min. The flow rate was 4.0 mL/min. Under these conditions, *N*^{7}-(2-bromoethyl)guanosine eluted at 17.4 min. The corresponding fraction was collected and rechromatographed on the same column with 20% CH_3OH in 5 mM ammonium acetate (pH 4.0). The corresponding fractions were then collected and lyophilized: ^1H NMR (^2H_2O) δ 3.85 (dd, 1 H, 5′-CH_2OH), 3.93 (m, 2 H, CH_2Br), 4.0 (dd, 1 H, 5′-CH_2OH), 4.33 (quartet, 1 H, C4′), 4.98 (dd, q, 1 H, C3′), 4.68 (dd, 1 H, C3′), 4.86 (m, 1 H, CHN^7), 4.92 (m, 1 H, CHN^7), 6.08 (d, 1 H, C1′); (+)FAB-MS, 428; 430 (M + K), 390, 392 $(M + H^+)$, 258, 260 (M − ribose).

When deoxyguanosine was substituted for guanosine, *N*^{7}-(2-bromoethyl)guanine, not *N*^{7}-(2-bromoethyl)deoxyguanosine, was the major reaction product.

***N*^{7}-(2-Bromo[*threo*-1,2-$^2H_2$]ethyl)guanosine.** The synthesis was repeated with [*erythro*-1,2-$^2H_2$]EDB. The ^1H NMR spectrum (^2H_2O) was identical with the unlabeled compound with the exception of the simplification resulting from deuterium atom substitution: δ 3.89 (d, 0.5 H, *J* = 7.3, BrCH), 3.92 (d, 0.5 H, *J* = 6.3, BrCH), 4.86 (d, 0.5 H, CHN^7). The signal at δ 4.8 (CHN^7) was obscured by ^{2}HHO.

***N*^{7}-(2-Bromo[*erythro*-1,2-$^2H_2$]ethyl)guanosine.** A similar preparation was made with [*threo*-1,2-$^2H_2$]EDB. The ^1H NMR spectrum (^2H_2O) matched that of the unlabeled material with the deuterium atoms, resulting in the following simplifications: δ 3.89 (d, 0.5 H, *J* = 4, CHBr), 3.92 (d, 0.5 H, *J* = 4 Hz, CHBr), 4.86 (d, 0.5 H, *J* = 3.9 Hz, CHN^7). The signal at δ 4.8 was obscured by ^{2}HHO.

***S*-[2-(*N*^{7}-Guanyl)ethyl]glutathione.** Glutathione (25 mg, 0.08 mmol) was reacted with a solution of sodium (7.5 mg, 0.33 mmol) in dry CH_3OH (2.5 mL). When it was dissolved, *N*^{7}-(2-bromoethyl)guanosine was added. The reaction progress was determined by the C18 HPLC assay described below. When all of the *N*^{7}-(2-bromoethyl)guanosine had been consumed (after 1–3 h), the reaction was quenched with 50 mM ammonium acetate (adjusted to pH 6.5) and the CH_3OH was removed under reduced pressure. The resulting solution was heated at 100 °C for 30 min. After being cooled, the adducts were purified by reversed-phase HPLC as previously described.[15] Isolation of the adduct required an initial purification on a C18 HPLC column (Altex Ultrasphere, 5 μm, 10 × 25 mm). A 20-min linear gradient of 5–25% CH_3OH in 5 mM ammonium acetate buffer (pH 4.0) was used to elute the adducts (flow rate 4 mL/min). The pertinent fractions were collected and passed over an aminopropyl column (Altex Ultrasil-NH_2, 10 μm, 0.4 × 25 cm). The sample was loaded onto the column with 8 mM ammonium acetate (in 80% CH_3OH). After 10 min, a linear gradient of 8–760 mM ammonium acetate (in 80% CH_3OH) was used to elute the column (flow rate 1.0 mL/min). The adduct was subjected to one more pass over the initial C18 HPLC system and was collected and lyophilized. Following relyophilization from ^2H_2O, the ^1H and COSY NMR spectra were obtained. The NMR data obtained for the unlabeled material were identical with the adduct previously isolated from in vitro incubations.[15]

***S*-[2-(*N*^{7}-Guanyl)[*erythro*-1,2-$^2H_2$]ethyl]glutathione.** The reaction was repeated with the *N*^{7}-(2-bromo[*threo*-1,2-$^2H_2$]ethyl)guanosine prepared from [*erythro*-1,2-$^2H_2$]EDB. The ^1H NMR spectrum is displayed in Figure 5C.

***S*-[2-(*N*^{7}-Guanyl)[*threo*-1,2-$^2H_2$]ethyl]glutathione.** Similar reaction conditions were used with the *N*^{7}-(2-bromo[*erythro*-1,2-$^2H_2$]ethyl)-guanosine prepared from [*threo*-1,2-$^2H_2$]EDB. The ^1H NMR spectrum is shown in Figure 5D.

***N*^{7}-(2-Chloroethyl)guanosine.** Guanosine (100 mg, 0.35 mmol) was reacted with 1-bromo-2-chloroethane (0.5 g, 3.5 mmol) in the presence of $AgNO_3$ (60 mg, 0.35 mmol) in DMSO (1.5 mL) as described above for the preparation of *N*^{7}-(2-bromoethyl)guanosine. The major reaction product had a retention time similar to that of *N*^{7}-(2-bromoethyl)-guanosine on the C18 HPLC system with the acetonitrile/25 mM KH_2PO_4 (pH 4.5) gradient. NMR and FAB-MS spectral analyses demonstrated that the isolated compound was solely *N*^{7}-(2-chloromethyl)guanosine: ^1H NMR (^2H_2O) δ 3.85 (q, 1 H, C5′), 4.00 (q, 1 H, C5′), 4.17 (t, 2 H, CH_2Cl), 4.3 (m, 1 H, C4′), 4.4 (t, 1 H, C3′), 4.98 (dd, 1 H, C2′), 4.8 (m, obscured by H^2HO, N^7CH_2), 6.05 (d, 1 H, C1′); (+)FAB-MS, 346, 348 (M^+).

***N*^{7}-(2-Chloro[1-$^2H_2$]ethyl)guanosine.** 2-Chloro-1-bromo[1-$^2H_2$]ethane was prepared by the procedure of Kovachic and Leitch.[45] The ^1H NMR spectrum of the product following distillation demonstrated that the material was greater than 99% pure and that the deuterium atom was located on the same carbon atom as the chloride atom: ^1H NMR ($^2HCCl_3$) δ 3.55 (dd, 2 H, CH_2Br), 3.76 (tt, 1 H, CHCl).

The material (0.5 g, 35 mmol) was then reacted with guanosine as described for *N*^{7}-(2-chloroethyl)guanosine. The NMR spectrum of the isolated product upon comparison with the unlabeled compound demonstrated that the deuterium atom was located only on the carbon atom attached to the halogen: ^1H NMR (^2H_2O) δ 3.85 (q, 1 H, C5′), 4.00 (q, 1 H, C-5′), 4.2 (t, 1 H, CHCl), 4.3 (m, 1 H, C4′), 4.4 (t, 1 H, C3′), 4.98 (dd, 1 H, C2′), 4.8 (m, integration prevented by presence of HDO, N^7CH_2), 6.05 (d, 1 H, C1′); (+)FAB-MS, 347, 349 (M^+).

Measurement of Rates of Hydrolysis. A modification of a reported method[46] was used: the compounds under consideration (5 mg) were dissolved in 2.5 mM sodium acetate, 0.1 M *N*-(2-acetamido)-2-iminodiacetic acid (ADA) (pH 6.5), or 0.1 M *N*-ethylmorpholine acetate (pH 9.0) buffer (5.0 mL) and incubated at 37 °C. Aliquots (50 μL) were withdrawn at various times and added to a solution (0.5 mL) of 50 mM 4-(*p*-nitrobenzyl)pyridine (in acetone/0.1 M ADA buffer (pH 6.5)/ethylene glycol (1:2:4, v/v/v)) with mixing. After samples had been heated 20 min at 100 °C, 0.5 mL of an acetone/triethylamine mixture (1:1, v/v) was added with mixing and A_{560} was measured. The percent of unreacted material was determined by

$$[(A_{560})_{t_0} - (A_{560})_t]/(A_{560})_{t_0} \times 100$$

The pH of each reaction solution was measured at the end of the reaction. Similar rates of hydrolysis were obtained for *S*-(2-chloroethyl)-L-cysteine and *S*-(2-chloroethyl)-DL-homocysteine by following the reaction in 2.5 mM sodium [$^2H_3$]acetate in ^2H_2O by integration of the appropriate

(44) Ludlum, D. B.; Tong, W. P. *Nitrosoureas: Current Status and Developments*; Prestayko, A. W., Crooke, S. T., Baker, L. H., Carter, S. K., Schein, P. S., Eds.; Academic: New York, 1981; pp 85–94.

(45) Kovachic, D.; Leitch, L. C. *Can. J. Chem.* **1961**, *39*, 363–374.

(46) Miller, R. E.; Guengerich, F. P. *Biochemistry* **1982**, *21*, 1090–1097.

signals in the ^1H NMR spectrum of the reaction mixture.

Rates of Alkylation of 4-(*p*-Nitrobenzyl)pyridine. A modification of the method of Schasteen and Reed[23] was used: the compounds (5 mg) were dissolved in a solution of 2.5 mM sodium acetate, 0.1 M ADA (pH 6.5), or 0.1 M *N*-ethylmorpholine acetate (pH 9.0) buffer (4.0 mL) and 5% 4-(*p*-nitrobenzyl)pyridine in acetone (1.0 mL). Aliquots (40 μL) were removed at various times and added to a triethylamine/acetone/water mixture (1:1:2, 1.0 mL). A_{600} values were read. At the end of the experiments, the mixture was heated at 100 °C for 20 min and then cooled; the A_{600} value of the sample was treated as the reaction end point. The percent of product formed was determined by

$$[(A_{600})_f - (A_{600})_i]/(A_{600})_f \times 100$$

In Vitro Preparation of *S*-[2-(N^7-Guanyl)ethyl]glutathione. The method used for the preparation of the major EDB adduct has been previously described.[15,19] Briefly, the stereospecifically labeled [1,2-$^2H_2$]-EDBs (10 mM) were incubated individually with liver cytosol prepared from phenobarbital-treated rats (3 mg of protein/mL), herring sperm or calf thymus DNA (3 mg/mL), and GSH (3 mM) in 0.1 M Tris-HCl buffer (pH 7.7) containing 15 mM sodium citrate and 1 mM EDTA (total volume 1000 mL). After the reaction proceeded for 2 h at 37 °C, it was stopped by the addition of a sodium dodecyl sulfate solution to a final concentration of 1% (w/v). The mixture was sequentially washed with equal volumes of a phenol/$CHCl_3$/isoamyl alcohol mixture (25:24:1, v/v/v), ethyl acetate, and finally ether. The DNA was precipitated with cold ethanol (2.5 volumes). Following isolation and resuspension of the DNA in 0.1 M potassium phosphate buffer (pH 7.0, 500 mL), the adducts were released from the DNA by heating for 30 min at 100 °C. After the solution was cooled, DNA was precipitated by the addition of cold ethanol (2.5 volumes) and centrifuged for 30 min at 10^4g. The supernatant was concentrated, and the adducts were purified by the reversed-phase C18 and aminopropyl ion-exchange HPLC systems described for the purification of synthetic compounds (see above). These compounds were then analyzed by one- and two-dimensional NMR techniques to determine the stereochemical orientation of the deuterium atoms. The spectra are displayed in Figure 5A,B.

NMR Spectroscopy. Samples of the purified adducts were prepared in ^2H_2O. The chemical shifts were determined externally by a ^2H_2O solution of DSS or assigning the $^2H^1HO$ signal to 4.8. One-dimensional proton NMR spectra were acquired by using 16 384 data points with a 30° pulse. The spectral resolution was increased by Gaussian multiplication prior to Fourier transformation. The two-dimensional COSY spectra were acquired through the use of the standard COSY software provided by the manufacturer. A 90-τ-45 pulse sequence with 1024 data points in F_2 and 512 in F_1 and magnitude calculations were employed. The spectral window and transmitter frequency were chosen to observe the coupled signals with optimal data point resolution. Prior to the transformations, sine-bell multiplication was used in both dimensions. The data were zero-filled once prior to the F_1 transformation and then symmetrized before it was plotted.

The NOE difference spectra were obtained by collecting 16 scans at low-power on-resonance selective irradiation at all times except during acquisition periods, followed by 16 scans at off-resonance irradiation. After a total of 2560 scans had been recorded, the free induction decay (FID) of the off-resonance data was subtracted from that of the on-resonance data. The resulting FID was Fourier transformed to yield the difference spectrum. The magnitude of the NOE was determined by integration of the signals in the difference spectrum.

Enzymochemical Regioselective Oxidation of Steroids without Oxidoreductases

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Abstract: Two hydrolytic, commercially available enzymes (out of eight tested), *Chromobacterium viscosum* lipase and *Bacillus subtilis* protease (subtilisin), have been found to esterify the model dihydroxy steroid 5α-androstane-3β,17β-diol (**1**) in dry acetone. These enzymes acylate the two hydroxyl groups in **1** with striking—and opposite—regioselectivities: while lipase reacts exclusively with OH in the C-3 position, subtilisin displays a marked preference for the C-17 hydroxyl (chemical reactivities of these two hydroxyl groups in **1** are comparable). The reactivity of various other hydroxy steroids with lipase and subtilisin was examined, and the structural requirements for these enzymatic reactions were elucidated. Several of these enzyme-catalyzed acylations in acetone were scaled up, and 0.7-1.0-g quantities of pure 3β or 17β monobutyryl steroids were obtained with good yields. In the case of the enzymatically prepared 3β-ester of 5α-pregnane-3β,20β-diol, the remaining free hydroxyl group was chemically oxidized, followed by alkaline deacylation at the C-3 position, to afford 5α-pregnan-3β-ol-20-one with a 63% overall isolated yield. Such an enzymochemical process provides an attractive alternative to the currently utilized enzymatic oxidations of steroids catalyzed by hydroxysteroid dehydrogenases.

Site-specific conversion of identical functional groups in steroids, which is of enormous importance for the pharmaceutical industry, has always been a formidable task.[1] In particular, chemical oxidation of OH groups in polyhydroxy steroids seldom affords absolute specificity.[2] Therefore, most practical transformations of this type have been carried out microbiologically.[3] To avoid traditional problems associated with fermentations, a promising recent trend in the preparative oxidation of hydroxyl groups in steroids has been the use of isolated enzymes, hydroxysteroid dehydrogenases, as catalysts.[4] From the standpoint of organic chemists, however, this approach has the drawback that these enzymes require expensive cofactors and are very costly and many are not commercially available.

It occurred to us that perhaps instead of directly exploiting the keen regioselectivity of oxidoreductases at the oxidation step (as

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