Characterization of Nucleoside and DNA Adducts Formed by *S*-(1-Acetoxymethyl)glutathione and Implications for Dihalomethane−Glutathione Conjugates

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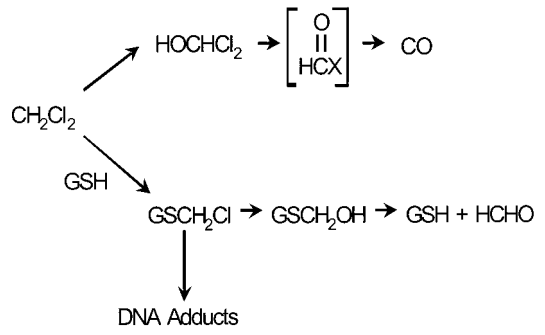
S-(1-Acetoxymethyl)glutathione ($GSCH_2OAc$) was synthesized and used as a model for the reaction of glutathione (GSH)-dihaloalkane conjugates with nucleosides and DNA. Previously, *S*-[1-(N^2-deoxyguanosinyl)methyl]GSH had been identified as the major adduct formed in the reaction of $GSCH_2OAc$ with deoxyguanosine. $GSCH_2OAc$ was incubated with the three remaining deoxyribonucleosides to identify other possible adducts. Adducts to all three nucleosides were found using electrospray ionization mass spectrometry (ESI MS). The adduct of $GSCH_2OAc$ and deoxyadenosine was formed in yield of up to 0.05% and was identified as *S*-[1-(N^7-deoxyadenosinyl)methyl]GSH. The pyrimidine deoxyribonucleoside adducts were formed more efficiently, resulting in yields of 1 and 2% for the $GSCH_2OAc$ adducts derived from thymidine and deoxycytidine, respectively, but their lability prevented their structural identification by ^{1}H NMR. On the basis of the available UV spectra, we propose the structures *S*-[1-(N^3-thymidinyl)methyl]GSH and *S*-[1-(N^4-deoxycytidinyl)methyl]GSH. Because adduct degradation occurred most rapidly at alkaline and neutral pH values, an enzymatic DNA digestion procedure was developed for the rapid hydrolysis of DNA to deoxyribonucleosides at acidic pH. DNA digests were completed in less than 2 h with a two-step method, which consisted of a 15 min incubation of DNA with high concentrations of deoxyribonuclease II and phosphodiesterase II at pH 4.5, followed by incubation of resulting nucleotides with acid phosphatase. Analysis of the hydrolysis products by HPLC-ESI-MS indicated the presence of the thymidine adduct.

Introduction

CH_2Cl_2 is a solvent used in the United States at the level of 3×10^8 kg/year (*1*, *2*). Although CH_2Cl_2 is not extremely toxic to humans, its biological effects are of concern because of the risk of widespread exposure to the compound in the chemical and other industries (*3*, *4*). A major concern is the possible carcinogenicity (*2*, *4*); CH_2Cl_2 is known to be tumorigenic in mice but not in rats (*5*, *6*). Due to the potential danger of CH_2Cl_2 to workers in the chemical industry and others exposed to the compound in large quantities, the Environmental Protection Agency and the Occupational Safety and Health Administration have regulated CH_2Cl_2 exposure in the workplace (*4*).

The detoxication of CH_2Cl_2 occurs through two competing pathways, both of which convert this hydrophobic molecule to water-soluble products that can be easily excreted (Scheme 1). The first of these pathways is an oxidative P450 pathway that yields carbon monoxide (*7*).

Scheme 1. Dual Pathway for CH_2Cl_2 Metabolism



The second pathway is through GSH conjugation by GSH S-transferase (GST)[1] enzymes and results in formation of HCHO (*7*). It is likely that the genotoxicity of CH_2Cl_2 results from DNA binding by the electrophilic intermediate $GSCH_2Cl$ (*7*) rather than DNA binding by HCHO. While this is an efficient detoxication method, DNA adducts are probably formed as a side reaction of this

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[1] Abbreviations: CID, collision-induced dissociation; ESI, electrospray ionization; DNase, deoxyribonuclease; DTNB, 5, 5′-dithio-bis(2-nitrobenzoic acid); ESI, electrospray ionization; $GSCH_2OAc$, *S*-(1-acetoxymethyl)GSH; GST, GSH *S*-transferase; PDE, phosphodiesterase; SIM, selected ion monitoring. See Instructions to Authors for the standard abbreviations of nucleosides and bases [*Chem. Res. Toxicol.* (1998) **11**, 5A−10A].

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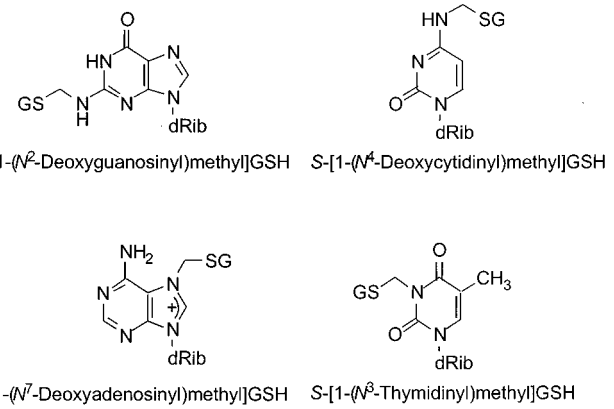
Scheme 2. Adduct Structures of $GSCH_2OAc$−Nucleoside Products



pathway (Scheme 1). The tumors that have been observed in mice are proposed to occur by way of the GST pathway and not the P450 pathway (*8*).

S-(1-Acetoxymethyl)GSH ($GSCH_2OAc$) was developed as a model compound for the potentially toxic *S*-(1-halomethyl)GSH intermediates in the GST detoxication pathway (*9*). The rationale for using $GSCH_2OAc$ is that the acetate models the halogen leaving group of the putative conjugate generated enzymatically. In contrast to the halides, $GSCH_2OAc$ can be prepared, stored as a dry powder, and used to generate adducts in aqueous solutions. Use of this compound in the formation of DNA adducts eliminates the requirement for GST enzymes. Previous research identified a single adduct of the $GSCH_2OAc$ to dGuo at the exocyclic N2 position, *S*-[1-(N^2-deoxyguanosinyl)methyl]GSH (Scheme 2) (*9*). Evidence for adduct formation with dCyd had been observed (*9*), but the product could not be characterized.

The goals of the present study were to synthesize, purify, and characterize the structures of any other nucleoside adducts using UV, MS, and NMR methodologies. Adduction of $GSCH_2OAc$ to double-stranded DNA in vitro was also investigated. A rapid protocol was developed for an enzymatic digestion of duplex DNA to nucleosides under mild acidic conditions (Scheme 3) because of the lability of the dGuo and other DNA adducts (*9*).

Experimental Procedures

Warning! *$GSCH_2OAc$ is a potential mutagen and carcinogen (9) and should be handled carefully according to appropriate environmental safety and health protocols.*

Chemicals. GSH, nucleosides, and DNA base analogues were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. $GSCH_2OAc$ was prepared by dissolving GSH (9.8 g, 32 mmol) in 40 mL of dry CH_3OH and then adding dropwise a solution of Na° (2.3 g, 0.10 mol, 3 equiv) dissolved in 80 mL dry CH_3OH (*9*). The resulting solution of tri-anionic GSH was added dropwise, at room temperature, to a solution of bromomethyl acetate (Aldrich Chemical Co., Milwaukee, WI, 3.3 mL, 34 mmol) dissolved in 40 mL of CH_3OH, and the product was allowed to precipitate at 0 °C for 20 min (*9*). The product was collected by centrifugation ($3 \times 10^3 g$, 10 min), the supernatant was decanted, and the product was dried in vacuo. The recovered $GSCH_2OAc$ (15.4 g) was stored desiccated at −80 °C and used without further purification.

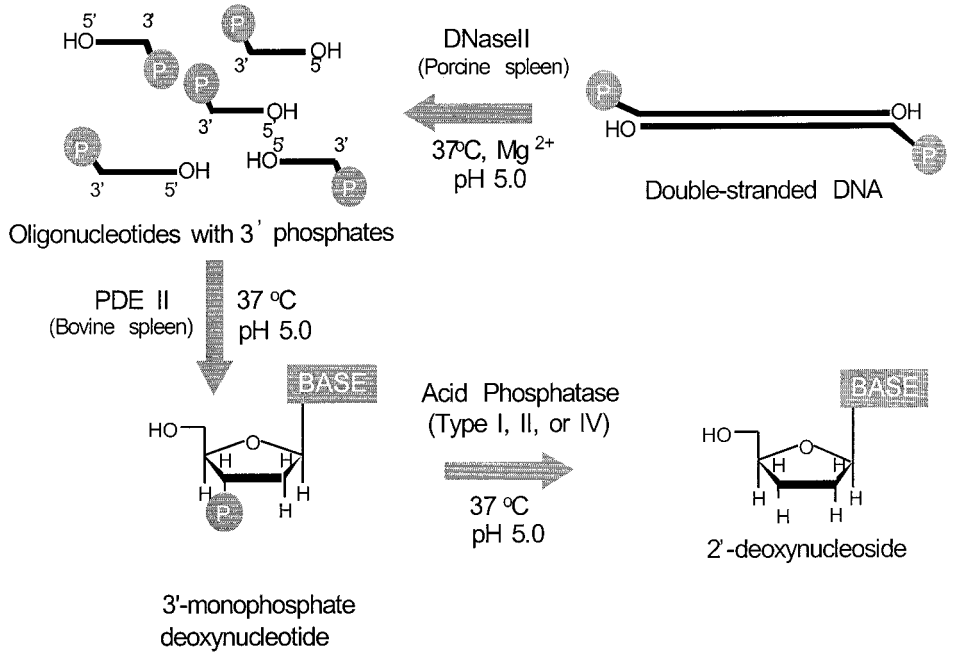
The dAdo adduct was synthesized by reacting dAdo (250 mg, 0.93 mmol) with $GSCH_2OAc$ (1.0 g, 2.6 mmol) in 50 mL of H_2O. The reaction was incubated at room temperature for 2.5 h, and the reaction was stopped by adding 12.5 mL of 3.0 M $NH_4CH_3CO_2$.

The dThd adduct was synthesized by reacting dThd (300 mg, 1.2 mmol) with $GSCH_2OAc$ (850 mg, 2.2 mmol) in 50 mL of H_2O. The reaction was incubated at room temperature for 10 min and was stopped by the addition of 12.5 mL of 3.0 M $NH_4CH_3CO_2$.

The dCyd adduct was synthesized by reacting dCyd (64 mg, 0.28 mmol) with $GSCH_2OAc$ (100 mg, 0.26 mmol) in 12.5 mL of H_2O. The reaction was incubated at room temperature for 30 min, and another 100 mg of $GSCH_2OAc$ was added to the solution, followed by a 30 min incubation. The reaction was stopped by the addition of 2.5 mL of 3.0 M $NH_4CH_3CO_2$.

Kinetic Studies of $GSCH_2OAc$ and $dGuoCH_2SG$ Stabilities. UV spectra were acquired at room temperature with a OLIS/Cary 14 spectrophotometer (On-Line Instrument Systems, Bogart, GA).

Scheme 3. Rapid Digestion of DNA to Nucleosides



For the estimation of the $t_{1/2}$ of $GSCH_2OAc$, a cuvette (1.0 mL, 1 cm path length) was filled with 0.98 mL of a solution of 0.10 M Tris-HCl (pH 8.0) and 20 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) (at 23 °C), in a Cary 14/OLIS spectrophotometer. A 10 μL aliquot of 10 mM GSH was added, and the final A_{412} value (1.15) was reached within 0.1 min (allowing for 5 s deadtime after manual mixing). The experiment was done again but by quickly dissolving 14 mg of $GSCH_2OAc$ in 5.0 mL of H_2O and adding 30 μL of this solution to the cuvette to start the assay). An exponential increase in A_{412} was recorded and fit to a pseudo-first-order plot, assuming that the fit is for decay of $GSCH_2OAc$ to GSH and without correcting for the reaction of GSH with DTNB.

The stability of *S*-[1-(N^2-deoxyguanosinyl)methyl]GSH (*9*) as a function of pH was studied by MS methods. Standard adduct was dissolved in 50 mM $NH_4CH_3CO_2$ and adjusted with either NH_4OH or CH_3CO_2H to pH 4.0, 5.0, 6.0, 7.0, 8.0, or 9.0. For adduct incubated at a given pH value, aliquots were extracted at specified times in a time course that extended to 4.2 h. Samples were immediately lyophilized and resuspended in 1.0 mL of HPLC buffer A [2% CH_3OH (v/v) in 10 mM $NH_4CH_3CO_2$, pH 4.5]. Samples were immediately placed in an Alliance 2690 Separations Module (Waters, Milford, MA), and the autosampler injected 5 μL aliquots onto a C18 guard cartridge at 5 min intervals. Adduct was eluted from the guard column with 95% CH_3OH (v/v) and was introduced on-line into a TSQ-7000 triple stage quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) outfitted with an API-1 ESI source (vide infra for MS parameters). Absorption at 260 nm was used to monitor total Gua chromophore levels of eluted analyte from each injection. Any sample-to-sample injection differences could be corrected by normalizing the 260 nm UV peaks to unity. Intact adduct levels in each injected sample were measured by selected ion monitoring (SIM) at *m*/*z* 587 and 294 (doubly charged adduct). Correction factors resulting from the normalized UV traces were then applied to the ion level detected by the mass spectrometer, with the zero time sample given an adduct level of unity (or 100%). While the absolute concentration of intact adduct cannot be quantified by this procedure, the relative amount of adduct remaining during the time course can.

Separation and Purification of Nucleoside Adducts. Nucleoside adducts were separated using HPLC. Buffer A contained CH_3OH and 10 mM $NH_4CH_3CO_2$ (pH 4.5) (2−98, v/v). Buffer B contained CH_3OH and 10 mM $NH_4CH_3CO_2$ (pH 4.5) (95−5, v/v). The flow rate was 3.0 mL min^{-1}, and the octadecylsilane (C18) column used was a Beckman ODS Ultrasphere (5 μm, 10 × 250 mm) (Beckman, San Ramon, CA). Initial small-scale separations were performed to determine the t_Rs of the unreacted deoxynucleosides, possible N-acetylated nucleosides, and the nucleoside adducts. Subsequent large-scale HPLC separations were performed with 2.0 mL injection volumes of nucleoside/$GSCH_2OAc$ reaction mixtures.

Following removal of CH_3OH in vacuo, pooled adduct fractions were concentrated using Bakerbond C18 cartridges (J. T. Baker, Milford, MA); nucleoside adducts were eluted with 2.0 mL of a CH_3OH:H_2O mixture (60/40, v/v). CH_3OH was quickly removed using a Labconco vacuum centrifuge (Labconco, Kansas City, MO). UV spectra of purified adducts were acquired to verify identities of eluted materials.

Concentrated nucleoside adduct samples were diluted to 4.0 mL with H_2O and injected into the HPLC in two separate 2.0 mL repurification runs. The nucleoside fraction was collected from each of the runs, and CH_3OH was removed in vacuo. Adduct fractions were concentrated using Bakerbond cartridges (vide supra) and the eluted material was concentrated to dryness in vacuo.

MS of Adducts. MS was performed using a Finnigan TSQ-7000 triple stage quadrupole mass spectrometer with a standard API-1 electrospray ionization source outfitted with a 150 μm inner diameter deactivated fused silica capillary. The mass spectrometer was operated in the positive ion mode. MS detection parameters were optimized using dGuo for a standard and depended on the type of experiment conducted and the flow rate of introduced sample. Typical parameters for a flow rate of 20 μL min^{-1} from a syringe pump were N_2 sheath gas, 54 psi; N_2 auxiliary gas, 0 psi; spray voltage, 3.6 kV; capillary temperature, 200 °C.

Data acquisition and spectral analysis were conducted using Finnigan ICIS software, version 8.3.2, on a Digital Equipment Co. Alpha workstation. When samples were introduced by direct infusion, a Harvard Apparatus (Holliston, MA) model 22 syringe pump was used at a flow of 10−20 μL min^{-1}.

For qualitative analysis, different scan modes were applied. Simple full scans covered the *m*/*z* range from 100 to 2000 with scan times up to 2 ms/amu (the range was extended to check condensation products). Tandem MS experiments were conducted with Ar at 2.5 mTorr as the collision gas and a collision voltage of −22 V. Daughter scans were performed to screen the fragmentation pattern of the parent ions of interest, and parent scans were performed to identify the source molecules of characteristic daughter ions. A Waters on-line UV monitor was used (in parallel) to record analytes absorbing at 260 nm in order to correlate ions with molecules bearing DNA base chromophores.

HPLC-MS of Adducts. Prior to purification, crude $GSCH_2OAc$-nucleoside adduct samples were analyzed using HPLC-MS. The autosampler and HPLC system consisted of an Alliance 2690 Separations Module from Waters. The buffers used for this separation were identical to those used in preparative HPLC purifications of nucleoside adducts. The flow rate was 200 μL min^{-1}. Analytical 250 mm C18 columns (5 μm particle diameter) from several manufacturers were employed in the separation of adducts in the overall course of this work. The gradient was as follows: 0 to 6 min, B = 0%; 6 to 8 min, B = 0 to 5%; 8 to 36 min, B = 5 to 25%; 36 to 54 min, B = 25 to 100%; 46 to 54 min, B = 100%. From 0 to 6 min, the valve was switched to waste in order to desalt samples on-line; at 6 min the valve switched back to the mass spectrometer ESI source.

NMR of $dAdoCH_2SG$. ^1H NMR spectra were acquired with a Bruker DRX400 Avance instrument operating at 400.13 MHz (Bruker, Billerica, MA). Nucleoside adducts, nucleosides, and GSH were all dissolved in ^2H_2O. Proton scans were then performed on dAdo, GSH, and the $dAdoCH_2SG$ adduct, also in ^2H_2O. The data were processed using a Silicon Graphics O2 computer with XWINN NMR software.

Formation of $GSCH_2OAc$-Modified Duplex DNA. Double-stranded calf thymus DNA (Sigma) was dissolved in H_2O and sonicated for 1 h in a Fisher FS9H sonicator bath (Fisher Scientific, Pittsburgh, PA). The DNA was repurified by a phenol:$CHCl_3$:isoamyl alcohol (25:24:1, v/v/v) extraction followed by three C_2H_5OH precipitations. Ethanolic DNA pellets were resuspended in H_2O and the purity and double-stranded character of the DNA were monitored by UV spectroscopy.

$GSCH_2OAc$ (67 mg, 180 μmol) was added to DNA (2.2 mg, 7.0 μmol DNA bases) in 2.0 mL of 83 mM sodium acetate buffer (pH 5.0). The reaction progressed for 1 h at 37 °C. The DNA was precipitated by C_2H_5OH precipitation, washed twice with 70% C_2H_5OH (v/v), and dried in vacuo. A mixture of porcine spleen deoxyribonuclease (DNase) II (EC 3.1.22.1, Sigma) and bovine spleen phosphodiesterase (PDE) II (EC 3.1.16.1, Sigma) was used to degrade duplex DNA to 3′-phosphate nucleotides in 15 min at 37 °C. The concentrations used were the following: DNA, 1 mM (bases); sodium acetate buffer (pH 5.0), 83 mM; $MgSO_4$, 8.3 mM; NaCl, 20 mM; DNase II, 1 unit (μg DNA)$^{-1}$; PDE II, 0.74 units (mg DNA)$^{-1}$.

Three nonspecific acid phosphatases proved useful in hydrolyzing $GSCH_2OAc$-modified DNA to nucleosides (all from Sigma): Type I wheat germ, 15−20 units (mg DNA)$^{-1}$; Type II white potato, 3.00 units (mg DNA)$^{-1}$; and Type IV white potato, 11.5 units (mg DNA)$^{-1}$. Enzymes and any nondigested DNA were removed with Amicon Centricon-30 membranes (Amicon, Lexington, MA, M_r cutoff 30 kDa) and the filtrate was stored at −80 °C until further analysis. The lability of the $GSCH_2OAc$-

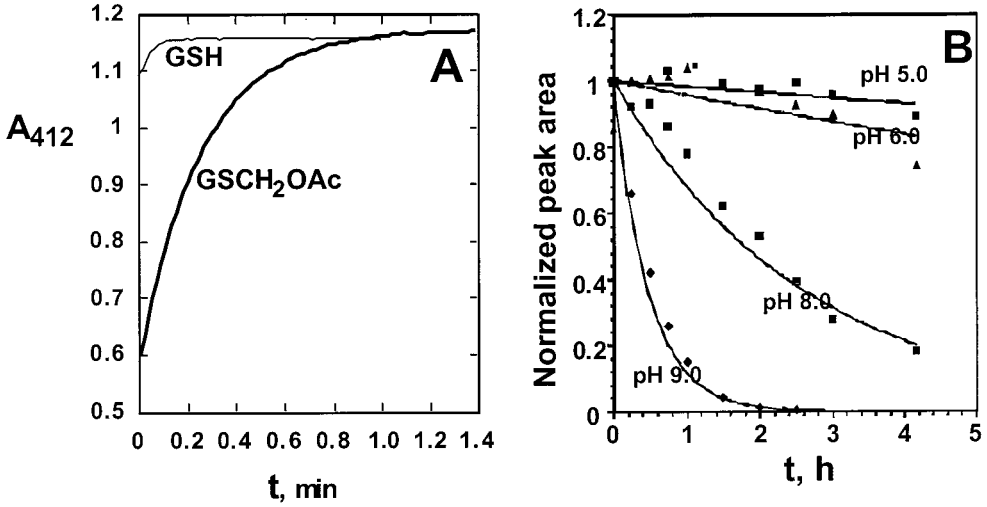


Figure 1. Stability of reagent and a nucleoside adduct. (A) Decomposition of $GSCH_2OAc$. (B) Stability of *S*-[1-(N^2-deoxyguanosinyl)-methyl]GSH at varying pH values. See Experimental Procedures for details.

derived adducts necessitated a rapid, efficient digest with all aspects of the experiment performed in 1 day.

Results

Stabilities of $GSCH_2OAc$ and $dGuoCH_2SG$ Adduct. In pH 8.0 Tris-HCl buffer, the half-life of $GSCH_2OAc$ was 11 s (Figure 1A). The reaction kinetics were best fit to an exponential decay curve, $C_{(t)} = C_o e^{-kt}$, suggesting a pseudo-first-order reaction in which $GSCH_2OAc$ is hydrolyzed to GSH (partially GS^- at pH 8.0), which in turn reacts with DTNB.

HPLC-purified $dGuoCH_2SG$ was observed to be alkaline labile (Figure 1B). Using an exponential decay as a best fit, the half-lives of dGuo adduct at pH 5.0, 6.0, 8.0, and 9.0 were estimated to be 38, 14, and 1.8 h and 19 min, respectively, in qualitative agreement with previous work in this laboratory.[2,3] The instability of dGuo adduct under alkaline conditions led to the development of a rapid DNA digest protocol at low pH, affording the opportunity to study adducts in double-stranded DNA (vide infra).

HPLC Purification of Adducts. Initial reaction conditions were designed using studies with the dGuo adduct, because this had been most extensively characterized (*9*). The adduct *S*-[1-(N^2-deoxyguanosinyl)methyl]-GSH was the only one detected in the reaction of dGuo with $GSCH_2OAc$, using HPLC monitored by UV or MS.

Following the reaction of each deoxyribonucleoside with $GSCH_2OAc$, components of the reaction mixtures were analyzed and subsequently purified by HPLC.[4] The initial analytical HPLC was followed by a series of preparative injections (Figure 2). The peak eluting at 11.4 min in part A was shown by UV and MS spectra to be a

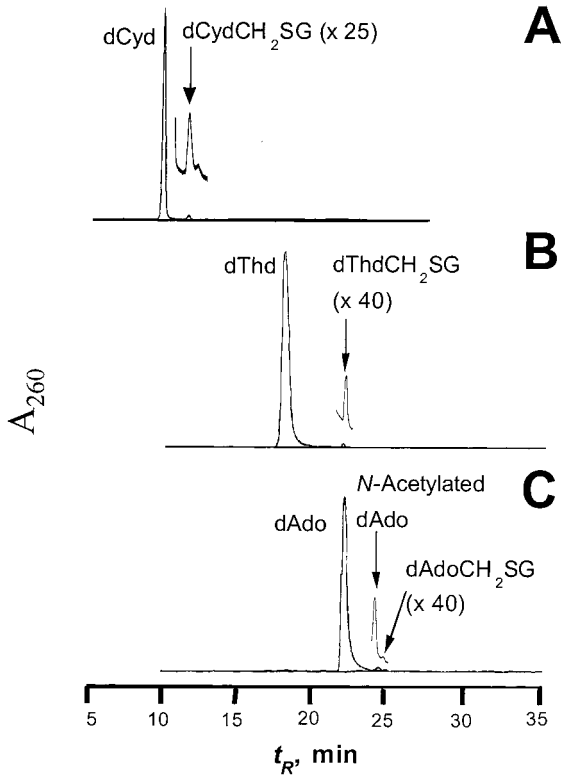


Figure 2. HPLC chromatograms of reactions of $GSCH_2OAc$ with deoxynucleosides, followed by separation with a semi-preparative C18 (octadecylsilane) column. Absorbance was monitored at 260 nm (HPLC buffers at pH 4.5). (A) Injection of 50 μL of $GSCH_2OAc$ plus dCyd reaction mixture. The inset shows injection of 1.0 mL of the same reaction mixture. (B) Injection of 50 μL of $GSCH_2OAc$ plus dThd reaction mixture. The inset shows an injection of 2.0 mL of the reaction mixture. (C) Injection of 50 μL of $GSCH_2OAc$ plus dAdo reaction mixture. The inset shows an injection of 2.0 mL of the reaction mixture.

dCyd adduct (vide infra). In the case of the dAdo, the peaks at t_R 25 and 26 min were both collected; preliminary MS identified the t_R 25 min peak as an acetylated dAdo derivative[3] and the t_R 26 min peak as the putative adduct of interest. The peak at 22 min in the dThd chromatogram was tentatively identified as the dThd adduct (vide infra). For the larger-scale reactions, the efficiencies of dCyd, dThd, and dAdo adduct formation

[2] Müller, M., Doerschuk, B., and Guengerich, F. P., unpublished results. The results were similar to those described here but were compromised by the apparent presence of an acetyl group in the GSH moiety that was inadvertently formed due to too extensive treatment with $GSCH_2OAc$ in the synthetic reaction.[3]

[3] With concentrations of $GSCH_2OAc$ higher than used here, the products were sometimes complicated by the presence of acetylated products, presumably resulting from attack of a deoxyribose alcohol or the GSH amine by the acetyl group of $GSCH_2OAc$.

[4] With each of the nucleosides, efforts were made to improve the reaction yields with the addition of *N*,*N*-dimethylformamide to improve the stability of the reagent $GSCH_2OAc$. However, yields of each nucleoside adduct were lowered when any of this cosolvent (or Me_2SO) was used, possibly reflecting the need for S_N1 reactions.

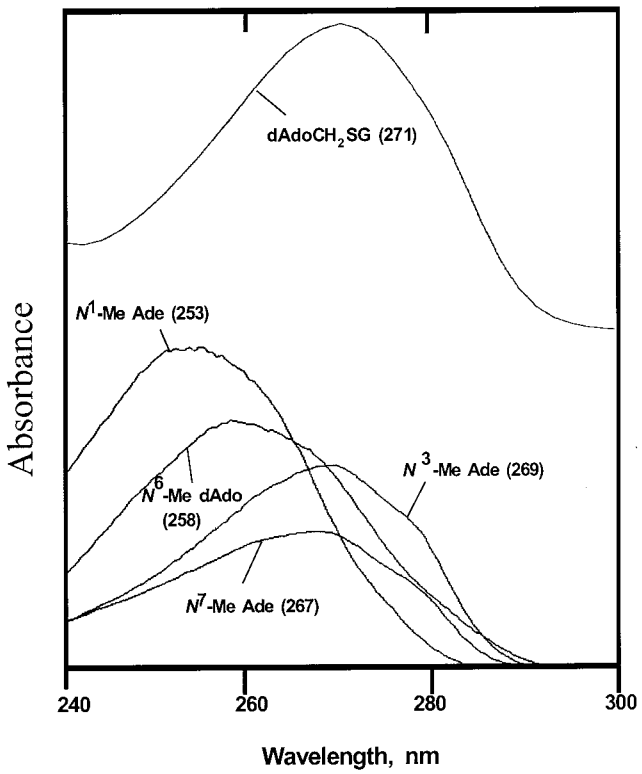


Figure 3. UV-vis spectra of $dAdoCH_2SG$ and methylated dAdo analogues in H_2O at pH 4.5. λ_{max} values are in parentheses.

were approximately 2, 2, and 0.05% of the starting nucleoside, respectively, but some smaller-scale preparations showed pyrimidine adduct yields of 11%.

Characterization of dAdo Adduct. The on-line UV spectrum of the dAdo adduct indicated a λ_{max} at 271 nm (Figure 3). This is significantly red-shifted from the dAdo standard, λ_{max} 256 nm. Spectra of methylated base analogues were obtained for comparison. The UV spectra of N^1-CH_3 Ade, N^3-CH_3 Ade, N^6-CH_3 dAdo, and N^7-CH_3 Ade had λ_{max} values at 253, 269, 258, and 267 nm, respectively (Figure 3). The literature indicates λ_{max} values (at neutral to acidic pH) of 259 (*10*, *11*), 270 (*12*), and 271 (*11*, *13*) for N^1-, N^3-, and N^7-alkyl (d)Ado, respectively, indicating a lack of effect of the ribosyl moiety on the Ade spectra. The $dAdoCH_2SG$ adduct was characterized by a UV spectrum similar to those of N^3-CH_3 dAdo and N^7-CH_3 dAdo.

MS of the purified dAdo adduct (Figure 4, panel A) showed a parent ion (MH^+) at *m/z* 571. The fragment at *m/z* 455 represents a loss of deoxyribose, and the fragment at *m/z* 308 indicates the presence of GSH in the sample. CID of the *m/z* 571 peak (Figure 4, panel B) and analysis by MS/MS showed the loss of deoxyribose (−116 Da) and the presence of GSH. In addition, the fragment at *m/z* 320 represents a $GSCH_2^+$ carbocation fragment and the *m/z* 252 fragment confirmed the presence of dAdo in the parent compound.

The ^1H NMR spectrum of the dAdo adduct (^2H_2O) appeared to indicate a mixture of dAdo and GSH spectra, except for the downfield, aromatic region (Figure 5). The only significant difference between the two spectra was the absence of the C8 proton resonance in the adduct spectrum, indicating that the adduct was formed at either the N7 or C8 position on the purine ring. Tomasz first showed that alkylation at the N7 position of Guo induces C8 proton exchange with the solvent, and thus the H8

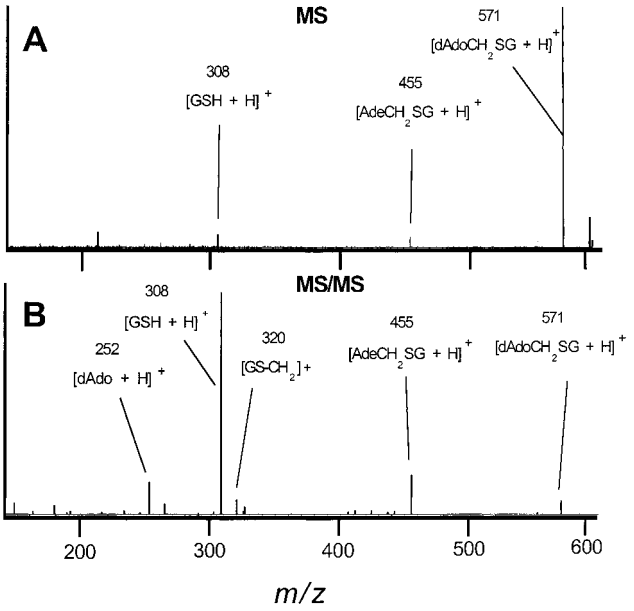


Figure 4. ESI-MS of HPLC-purified $dAdoCH_2SG$ Adduct. (A) Direct infusion at 10 μL min^{-1} with a full ion scan in positive chemical ionization mode. The trace of the adduct ion is shown at *m/z* 571. Ion fragmentation of $dAdoCH_2SG$, after loss of deoxyribose, is shown at *m/z* 455. (B) ESI-MS/MS of purified $dAdoCH_2SG$, showing fragmentation of the *m/z* 571 parent ion. Introduction of the sample was also by direct infusion at 10 μL min^{-1}.

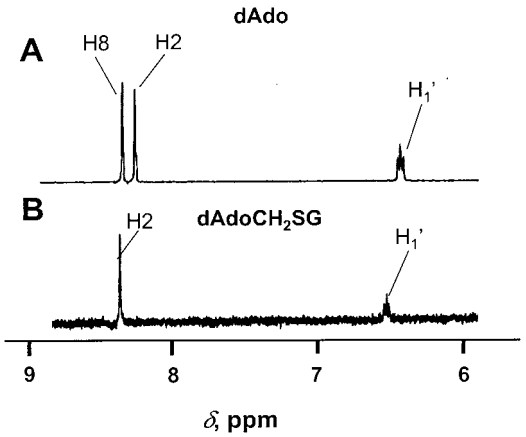


Figure 5. ^1H NMR downfield aromatic regions of dAdo and $dAdoCH_2SG$ (^2H_2O). (A) dAdo. (B) $dAdoCH_2SG$.

proton disappears from a ^1H NMR spectrum (*14*). Such facile exchange has also been documented for N^7-alkyl dAdo adducts (*15*).

On the basis of all of this information, the dAdo adduct was determined to form through the N7 position and assigned the structure *S*-[1-(N^7-deoxyadenosinyl)methyl]-GSH (Scheme 2).

Characterization of dThd Adduct. The online UV spectrum of the dThd adduct indicated a λ_{max} at 266 nm (Figure 6), corresponding to a red shift of 2 nm with respect to standard dThd (λ_{max} 264 nm). Spectra of methylated base analogues were obtained for comparison (Figure 6). The UV spectra for O^2-CH_3 dThd, N^3-CH_3 dThd, and O^4-CH_3 dThd exhibited lowest-energy λ_{max} values of 250, 260, and 274 nm, respectively. The adduct spectrum is most similar to the N^3-CH_3 dThd and O^4-CH_3 dThd spectra.

The crude dThd plus $GSCH_2OAc$ reaction mixture was analyzed by HPLC-ESI-MS, and an eluate at 36 min was

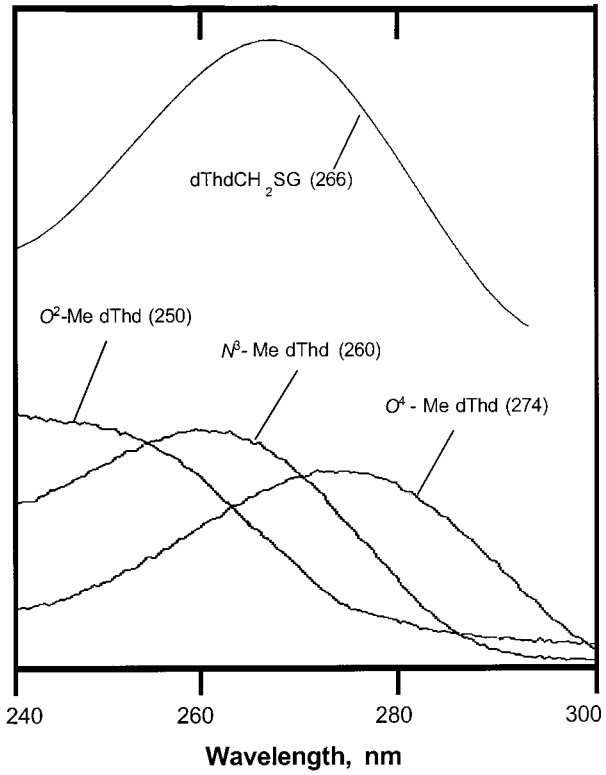


Figure 6. UV spectra of methylated dThd analogues. λ_{max} values are in parentheses. All spectra were recorded at pH 4.5 in 18% aqueous CH_3OH (v/v).

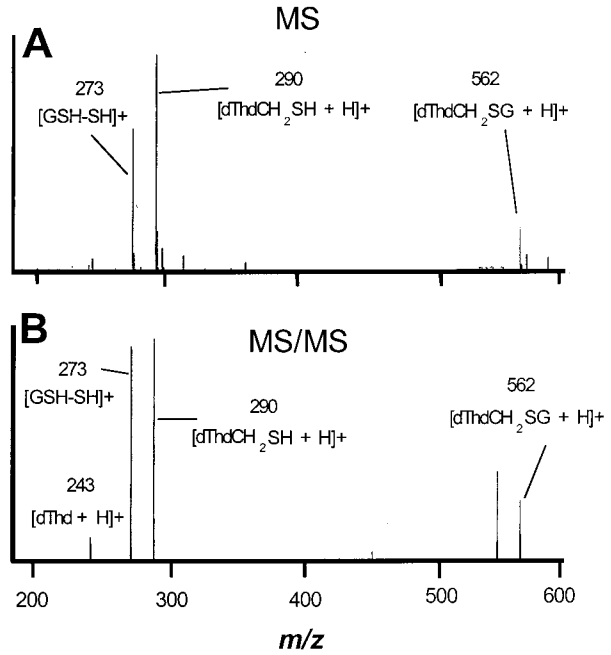


Figure 7. HPLC-ESI-MS of $dThdCH_2SG$ adduct. The pH of HPLC buffers was maintained at pH 4.5. (A) Full scan of dThd adduct (*m*/*z* 562) acquired on-line after elution from analytical reversed-phase C18 column. (B) Fragmentation of the *m*/*z* 562 dThd adduct ion by HPLC-ESI-MS/MS.

identified as the $dThdCH_2SG$ adduct (*m*/*z* 562). Full-scan ESI-MS of the analyte showed strong ion peaks at *m*/*z* 273 and *m*/*z* 290 (Figure 7). These fragments were verified to be adduct-related by CID and analysis by MS/MS (Figure 7). The *m*/*z* 273 and 290 fragments corresponded to GSH minus the sulfhydryl and $dThdCH_2SH$, respectively. In addition, fragmentation of adduct released dThd (*m*/*z* 243). Several attempts to obtain ^1H NMR spectra of the labile dThd adduct were unsuccessful.

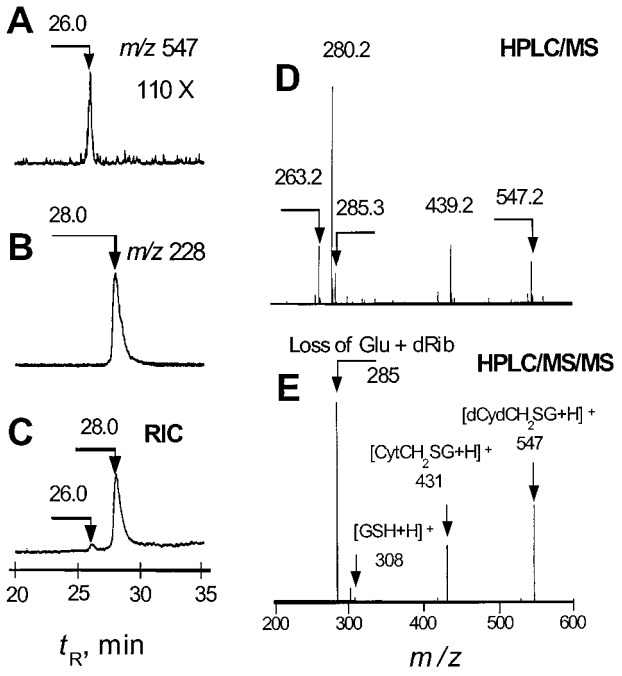


Figure 8. HPLC-ESI-MS of the $dCydCH_2SG$ adduct. The pH of the HPLC buffers was maintained at 4.5. Left: Mass chromatograms of crude dCyd plus $GSCH_2OAc$ reaction mixture, monitoring elution of dCyd adduct (A), dCyd (B), and the total ion current (C). (D) HPLC/MS of 26 min eluate. (E) HPLC-ESI-MS/MS of the dCyd ion.

Characterization of dCyd Adduct. The λ_{max} of the dCyd adduct was 271 nm, in agreement with previous work in this laboratory (*9*). This λ_{max} is similar to that reported for N^4-ethyl Cyd (272 nm) at neutral or alkaline pH but not N^3-ethyl Cyd (279 nm) (*16*). HPLC-ESI-MS of crude nucleoside adduct mixtures showed the dCyd adduct eluting 2 min before dCyd (Figure 8, panels A−C). The full-scan mass spectrum showed several prominent ion peaks (Figure 8D). The mass chromatogram of the HPLC run suggested that the analyte corresponding to *m*/*z* 439 did not precisely coelute with the adduct. Upon collision with Ar gas (Figure 8E), the adduct parent ion yielded fragments with *m*/*z* 431, 308 (weak), and 285, corresponding to adduct minus deoxyribose, GSH, and adduct minus deoxyribose *and* the glutamyl residue of GSH, respectively. The dCyd adduct decomposed quickly at room temperature and proved refractory to NMR structure determination.

DNA Digests. The lability of the $dGuoCH_2SG$ adduct at alkaline and neutral pH necessitated the development of a relatively rapid DNA digest to nucleosides under acid conditions (Figure 1B). Although we did not analyze the kinetics of hydrolysis of the other adducts, the similarity of the assigned structures and initial work on separation of products suggested similar behavior. Porcine spleen DNase II and bovine spleen PDE II efficiently digested DNA to nucleotides in 15 min. Four different nonspecific acid phosphatases, Type I from wheat germ and Types II, IV, and VII from white potato, were then used to digest the nucleotides to nucleosides in as little as 1.7 h. In this examination, Types I, II, and IV acid phosphatases worked best and yielded all four nucleosides in approximately equimolar concentrations (data not shown).

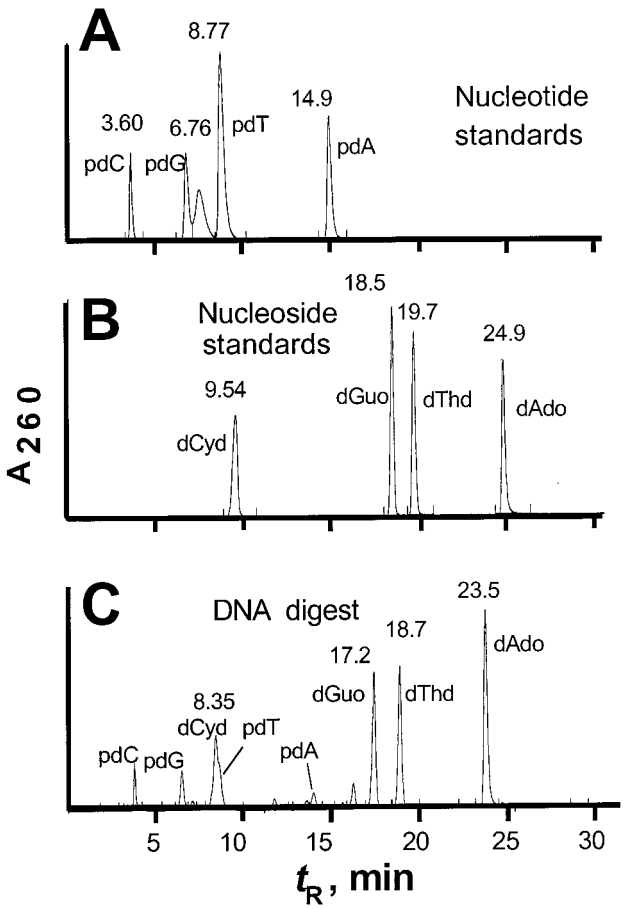


Figure 9. Acid DNA digests of calf thymus DNA. The upper two panels (A, B) show the HPLC chromatograms of nucleotide and nucleoside standards. The lower panel (C) shows the chromatogram of DNA digested to nucleosides by DNase II, PDE II, and wheat germ acid phosphatase Type I (Beckman Ultrasphere ODS analytical column).

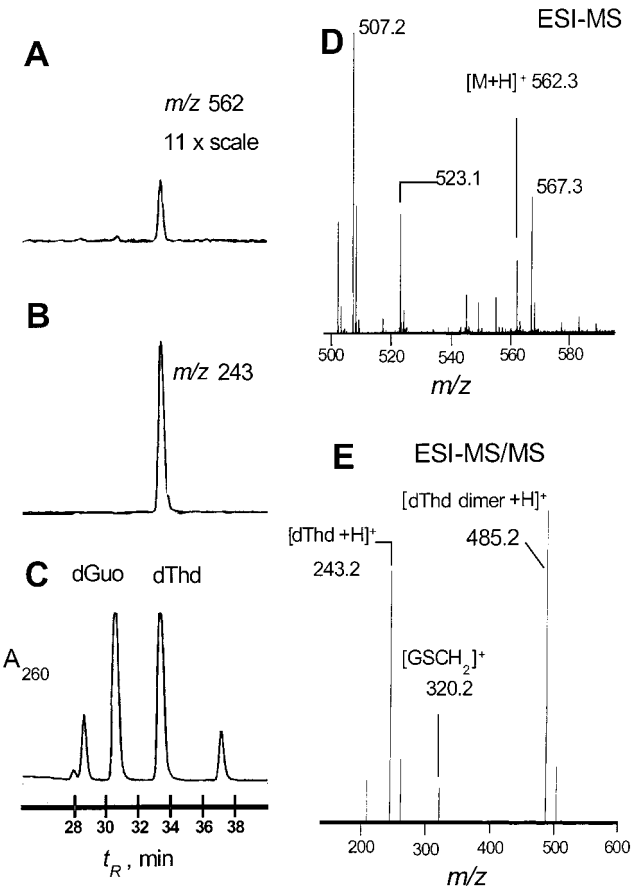


Figure 10. HPLC-ESI-MS and HPLC-ESI-MS/MS of $GSCH_2$-OAc-modified calf thymus DNA digested to nucleosides with DNase II, PDE II, and acid phosphatase Type IV. (A–C) Mass chromatograms monitored at *m/z* 243 and 562, corresponding to dThd and $dThdCH_2SG$. (D, E) Full scans of the eluted adduct (t_R 33.5 min). The adduct ion *m/z* 562 was selected for the CID tandem MS experiment (E).

DNA digests in citrate, acetate, and succinate buffers were performed at pH values of 5.0, 5.5, or 6.0 to optimize reaction conditions. Acetate buffer at pH 5.0 was found to work well for rapid reaction with few reaction byproducts and a high ratio of nucleosides to nucleotides. A 2-h digest in sodium acetate buffer (pH 5.0) is shown in Figure 9, with an 8:1 ratio of nucleosides to nucleotides (right-hand portion of the chromatogram shows a level baseline where $GSCH_2OAc$ adducts should elute).

HPLC-ESI-MS of DNA modified with $GSCH_2OAc$ and digested to nucleosides indicated the presence of dThd lesions. The mass chromatogram (Figure 10) showed a *m/z* 562 peak, corresponding to a dThd adduct. The dThd adduct coeluted with the unreacted dThd at 33.5 min with a 0.25 mL min^{-1} flow rate. CID of the *m/z* 562 parent ion efficiently fragmented the dThd adduct into dThd nucleoside (*m/z* 243) and $GSCH_2^+$ (*m/z* 320). Thus, the compound in the peak eluted at 33.5 min contained both GSH and nucleoside, verifying its identity as a dThd DNA lesion.

Discussion

One approach to better understanding the mutagenicity of GSH conjugates of CH_2Cl_2 and other dihalomethanes is the characterization of deoxyribonucleoside adducts and ultimately DNA adducts. A major technical obstacle is the preparation of the relevant electrophilic product, presumably $GSCH_2X$ (X = halide). Bonse et al. (*17*) prepared model compunds in the series $RSCH_2X$ (where R is a simple alkyl or aryl group) and measured the stabilities in 1,4-dioxane solutions containing low concentrations of H_2O, where the half-lives were on the order of hours. Dekant (*18*) prepared *S*-(1-chloromethyl)-Cys and estimated that the $t_{1/2}$ in aqueous buffer was <4 s at 0 °C. Stourman and Armstrong have recently estimated the $t_{1/2}$ of CH_3SCH_2Cl in 90% H_2O to be ~11 ms.[5] We continued to utilize a model alkylating agent we developed, $GSCH_2OAc$ (*9*). This compound can be readily prepared and stored. Its $t_{1/2}$ was estimated to be 11 s at pH 8.0 (Figure 1), which allows this reagent to be practical in model reactions with nucleosides because of the apparent stability of the acetate relative to the chloride.

We consider $GSCH_2OAc$ to be a reasonable model and employed this reagent in our studies. A liablity is the deomonstrated tendency to acetylate when very high concentrations are used.[3]

dAdo Adduct. Using the extinction coefficient for Ado (ϵ_{260} = 13 400 M^{-1} cm^{-1}) as a basis, we estimate that the yield for $dAdoCH_2SG$ formation was only 0.05%, yet the 100 μg obtained were sufficient for structure determination. By comparing the UV spectrum of the dAdo adduct

[5] Stourman, N. V., and Armstrong, R. N., unpublished results (estimated by appearance of thiolate absorbance at 240 nm).

to that of the methylated base analogues, we were able to determine that the N1 and N6 positions were unlikely sites of adduction, whereas the absorbance spectra of the N^3- and N^7-alkyl analogues were similar to the spectrum of the dAdo adduct derived from $GSCH_2OAc$.

Definitive evidence for the structure of the dAdo adduct was provided by the absence of the C8 proton resonance in the NMR spectrum recorded in ^2H_2O, consistent with the known exchange of an N^7alkyl dAdo adduct (*15*). The dAdo adduct, determined to form at the N7 position, was therefore characterized as *S*-[1-N^7-deoxyadenosinyl)-methyl]GSH (Scheme 2). Although the N7 atom of dAdo is not as nucleophilic as the N7 atom of dGuo, it is a major site of alkylation on Ado by several electrophiles (*15*, *19*).

dThd Adduct. The dThd adduct was produced in a yield of 2% (based on the absorbance and $\epsilon_{260} = 9500\ M^{-1}\ cm^{-1}$) but the nucleoside adduct proved to be rather labile. MS showed that the adduct was formed, and a characteristic pattern of fragmentation occurred between the GSH and the sulfhydryl group of its Cys residue. This fragmentation produced the ion at *m*/*z* 290, representing dThd with the addition of the methylene bridge and the sulfhydryl. The other resulting ion at *m*/*z* 273 represents loss of the sulfhydryl group (−32) from GSH.

Due to the instability of the $dThdCH_2SG$ adduct, attempts at ^1H NMR were unsuccessful. When the UV spectrum of the adduct was compared to that of standard dThd, the adduct showed a 2 nm red shift, indicating a small change in the aromatic character of the chromophore. Among the methylated base-adduct analogues, the most likely site of adduction is at the N3 position of the pyrimidine ring, which would result in an unchanged resonance structure. The UV spectrum of O^2-CH_3 Thd is very dissimilar to the dThd adduct, and the lowest energy λ_{max} of the dThd adduct is blue-shifted 8 nm with respect to O^4-CH_3 Thd. It is highly unlikely that adduction at any position of an intact pyrimidine would induce a large increase in energy of $\pi-\pi^*$ transitions, and we therefore propose that the dThd adduct is *S*-[1-(N^3-thymidinyl)-methyl]GSH (Scheme 2).

dCyd Adduct. The dCyd adduct was also highly unstable at room temperature and under the pH conditions tested, from pH 5.0 to 8.0, but its UV spectrum could be acquired on-line during HPLC purification. The adduct spectrum was very similar to the UV spectrum of unmodified dCyd nucleoside, with the adduct λ_{max} blue-shifted by just 1 nm with respect to the nucleoside λ_{max} (271 vs 272 nm for dCyd). The similarity of the spectra suggest little perturbation of the Cyt π electrons, a condition that would result from alkylation at the exocyclic N4 position of the cytosine moiety (*16*). Thus, we propose that the dCyd adduct is *S*-[1-(N^4-deoxycytidinyl)-methyl]GSH (Scheme 2).

Detection of Adducts in Double-Stranded DNA. The dThd adduct was detected in calf thymus DNA treated with $GSCH_2OAc$, as shown by the MS evidence (Figure 10). The concentration is difficult to estimate, but a rough comparison of ion currents suggests that ~1 dThd in 10^3 was modified.[6] A particularly pertinent question is the in vivo applicability of the $GSCH_2OAc$-DNA adducts, which were formed at pH 5.0 and have short lifetimes at neutral to alkaline pH. However, it is important to note that the stability in the nucleosides may be much less than in double-stranded DNA, as is the case with some other DNA adducts (*20*). We have not yet attempted to estimate stabilities within DNA.

Conclusions. In conclusion, the adducts generated with $GSCH_2OAc$ are considerably different from those we have observed with half-mustards of the form $GSCH_2CH_2X$ (*21*−*25*). With the ethylene series, the predominant reactions are S_N2-like, with an episulfonium ion intermediate (*26*) and the N7 atom of dGuo the most reactive site in DNA (*21*−*25*). We hypothesize that the reactions with $GSCH_2OAc$ may have more S_N1 character. Future efforts will involve the extension of strategies for the rapid hydrolysis of DNA, the characterization of GSH-containing DNA adducts, and more sensitive analysis by HPLC-MS or other methods.

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[6] Surprisingly, $dGuoCH_2SG$ adducts in double-stranded DNA were never detected, although the nucleoside formed adduct complexes in yields higher than 5% in earlier (*9*) and subsequent work. Steric hindrance may have blocked binding by $GSCH_2OAc$, but another possibility is that the PDE II or acid phosphatase may not have been able to catalyze the hydrolysis of DNA with bulky $dGuoCH_2SG$ lesions. In our hands, the $dAdoCH_2SG$ adduct was moderately stable (Figure 1), although the very low nucleoside yield might have precluded its detection in a less-sensitive duplex DNA reaction.

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