Chlorothioketene, the Ultimate Reactive Intermediate Formed by Cysteine Conjugate β-Lyase-Mediated Cleavage of the Trichloroethene Metabolite *S*-(1,2-Dichlorovinyl)-L-cysteine, Forms Cytosine Adducts in Organic Solvents, but Not in Aqueous Solution

Wolfgang Völkel and Wolfgang Dekant*

Department of Toxicology, University of Würzburg, Versbacherstrasse 9, 97078 Würzburg, Germany

Received April 27, 1998

Chlorothioketene has been suggested as a reactive intermediate formed by the cysteine conjugate β-lyase-mediated cleavage of *S*-(1,2-dichlorovinyl)-L-cysteine, a minor metabolite of trichloroethene. Halothioketenes are highly reactive, and their intermediate formation may be confirmed by reactions such as cycloadditions and thioacylations of nucleophiles. A precursor of chlorothioketene, *S*-(1,2-dichlorovinyl)thioacetate, is readly accessible by the reaction of dichloroethyne with thioacetic acid. In presence of base, *S*-(1,2-dichlorovinyl)thioacetate is cleaved to chlorothioketene. Chlorothioketene is not stable at room temperature and was characterized after transformation to stable products by reaction with compounds such as cyclopentadiene, *N*,*N*-diethylamine, and ethanol. In organic solvents, the cleavage of *S*-(1,2-dichlorovinyl)thioacetate in the presence of cytosine results in N^4-acetylcytosine, N^4-(chlorothioacetyl)cytosine, and small amounts of 3-(N^4-thioacetyl)cytosine. No reaction products were seen with guanosine, adenosine, and thymidine under identical conditions. When cytosine was reacted with *S*-(1,2-dichlorovinyl)thioacetate in aqueous solutions, only N^4-acetylcytosine was formed. N^4-(Chlorothioacetyl)cytosine and 3-(N^4-thioacetyl)cytosine were not detected even when using a very sensitive method, derivatization with pentafluorobenzyl bromide and electron capture mass spectrometry with a detection limit of 50 fmol/μL of injection volume. Aqueous solutions of DNA cleave *S*-(1,2-dichlorovinyl)thioacetate to give N^4-acetyldeoxycytidine in DNA, but chlorothioketene adducts of deoxynucleosides were also not detected in these experiments. These results confirm the electrophilic reactivity of chlorothioketene toward nucleophilic groups of DNA constituents in inert solvents but also demonstrate that the formation of DNA adducts under physiological conditions likely is not efficient. Therefore, DNA adducts may not represent useful biomarkers of exposure and biochemical effects for trichloroethene.

Introduction

Thioketenes have been suggested as reactive intermediates formed by the cysteine conjugate β-lyase-mediated cleavage of several halovinyl cysteine S-conjugates such as *S*-(1,2-dichlorovinyl)-L-cysteine (*1*). Halothioketenes are highly reactive electrophiles and are difficult to synthesize and to handle experimentally (*2*); however, several lines of evidence indicate that the α-chloroenethiols formed by cysteine conjugate β-lyase from halovinyl cysteine S conjugates are converted to halothioketenes. For example, dichloroacetylated proteins are formed by the interaction of a reactive intermediate, likely dichlorothioketene, formed by the cysteine conjugate β-lyase-dependent metabolism of the perchloroethene metabolite *S*-(trichlorovinyl)-L-cysteine (*3*). Moreover, conversion products characteristic for thioketenes were observed when α-chloroenethiols were released from precursors in chemical systems (*4*).

An interaction of metabolites formed from halovinyl cysteine S-conjugates with DNA may occur since several compounds from this group such as *S*-(1,2-dichlorovinyl)-L-cysteine and *S*-(trichlorovinyl)-L-cysteine showed β-lyase-dependent mutagenicity in the Ames test (*1*) and did induce some DNA damage in mammalian cells in culture (*5*, *6*). Since nephrotoxic cysteine S-conjugates are minor metabolites of several haloalkenes, which induce a low incidence of renal tumors in rats after long administration of high doses (*7*−*10*).

In this study, we examined the potential of chlorothioketene generated in situ from a novel precursor, *S*-(1,2-dichlorovinyl)thioacetate (**1**), to react with DNA bases and DNA using a highly sensitive GC/MS technique for identification and detection of reaction products under different conditions.

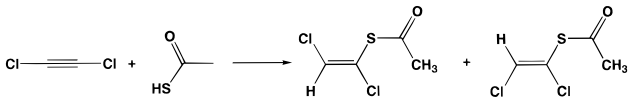
Experimental Procedures

Synthesis of *S*-(1,2-Dichlorovinyl)thioacetate. Dichloroethyne etherate was synthesized as described (**Caution:** *Dichloroethyne is highly flammable, explosive, and highly toxic. Do not attempt to generate pure dichloroethyne. Use an efficient hood!*) (*11*). To generate *S*-(1,2-dichlorovinyl)thioacetate, 45 mmol of thioacetic acid was added dropwise to 45 mmol of dichloroethyne etherate in 10 mL of *n*-hexane (Scheme 1). The reaction mixture was kept at room temperature and stirred for

* To whom correspondence should be addressed: Department of Toxicology, University of Würzburg, Versbacherstrasse 9, 97078 Würzburg, Germany. Telephone: +49(0)931/201-3449. Fax: +49(0)-931/201-3865. E-mail: dekant@toxi.uni-wuerzburg.de.

S0893-228x(98)00084-8 CCC: $15.00
Published on Web 08/25/1998

Scheme 1. Synthesis of *S*-(1,2-Dichlorovinyl)thioacetate



1 h. A mixture of (*E*)- and (*Z*)-*S*-(1,2-dichlorovinyl)thioacetate was isolated by vacuum distillation after removal of the solvent. The purity of the product was >98% as checked by GC/MS. The boiling point was 70 °C at 0.1 Torr.

Characterization of the Two Isomers of *S*-(1,2-Dichlorovinyl)thioacetate. Mass spectra (identical for both isomers) (EI, ^{35}Cl): *m*/*z* (relative intensity) 170 (2Cl, 2%) (M^+), 135 (1Cl, 11%) ($M^+ - Cl$), 127 (2Cl, 6%) ($M^+ - COCH_3$), 92 (1Cl, 100%) ($M^+ - COCH_3 - Cl$). ^{1}H NMR for isomer I (250 MHz, CDCl3): δ 2.30 (s, 3H, CH_3), 6.90 (s, 1H, CHCl). ^{13}C NMR for isomer I (63 MHz, $CDCl_3$): δ 30.2 (s, CH_3), 124.1 (s, CClS), 129.2 (s, CHCl), 188.2 (s, CO). ^{1}H NMR for isomer II (250 MHz, $CDCl_3$): δ 2.40 (s, 3H, CH_3), 6.80 (s, 1H, CHCl). ^{13}C NMR for isomer II (63 MHz, $CDCl_3$): δ 29.2 (s, CH_3), 124.1 (s, CClS), 129.0 (s, CHCl), 188.2 (s, CO).

Synthesis of (Chloroacetylthiono)acetic Acid Ethyl Ester. (Chloroacetylthiono)acetic acid ethyl ester was synthesized by the reaction of 32 mg (0.26 mmol) of chloroacetic acid ethyl ester and 136 mg (0.33 mmol) of Lawessons reagent (*12*) in 1 mL of xylene. After being stirred for 12 h at 140 °C, the mixture was cooled to room temperature and the formed precipitate removed by centrifugation. GC/MS analysis of the supernatant demonstrated the exclusive formation of the thiono ester (stench). The thiono ester was not isolated and characterized by GC/MS. Mass spectrum (EI, ^{35}Cl): *m*/*z* (relative intensity) 138 (1Cl, 100%) (M^+), 110 (1Cl, 75%) ($MH^+ - Et$), 103 (18%) ($M^+ - Cl$), 93 (1Cl, 33%) ($M^+ - OEt$), 77 (1Cl, 68%) ($M^+ - Et - S$).

Synthesis of N^4-Acetylcytosine. An excess of cytosine (36 μmol) was stirred with acetic acid anhydride (23 μmol) in 2 mL of anhydrous dimethylformamide. The obtained solution was analyzed by HPLC with diode array detection and with HPLC/MS. The obtained product had an electronic spectrum identical to that described in the literature (*13*) and an electrospray mass spectrum consistent with the proposed structure. Electronic spectrum (MeOH/H_2O, pH 2): λ_{max} (ϵ in mAU) 217 (100), 246 (90), 302 (60). Electrospray mass spectrum: *m*/*z* 153 (M^+).

Incubation of *S*-(1,2-Dichlorovinyl)thioacetate with DNA Bases. Cytosine (36 μmol) and a 1:2 mixture (based on the GC/MS response) of both isomers of *S*-(1,2-dichlorovinyl)-thioacetate (23 μmol) were stirred in a mixture of dimethylformamide and ethyl acetate (1:3, v/v) at room temperature. After 4 h, ethyl acetate was evaporated in a stream of nitrogen and the residue (5 μL) was analyzed by HPLC or subjected to derivatization with pentafluorobenzyl bromide for GC/MS/NCI (*14*). Reactions with adenine, guanine, and thymine were performed under identical conditions.

Characterization of N^4-(Chlorothioacetyl)cytosine Formed in These Reactions. Electronic spectrum (MeOH/H_2O, pH 2): λ_{max} (ϵ in mAU) 215 (150), 244 (80), 298 (30). Mass spectra after derivatization with PFBnBr (NCI, ^{35}Cl): *m*/*z* (relative intensity) 383 (1Cl, 100%) (M^-), 364 (1Cl, 1%) ($M^- - F$), 203 (1Cl, 8%) ($MH^- - PFBn$). Mass spectra after derivatization with PFBnBr (EI, ^{35}Cl): *m*/*z* (relative intensity) 349 (8%) ($MH^+ - Cl$), 203 (1Cl, 30%) ($MH^+ - PFBn$), 181 (100%) ($PFBn^+$), 92 (1Cl, 18%) ($CHCl - CS$).

^{1}H NMR spectra were identical to those reported for N^4-(chloroacetyl)cytosine (*15*).

Cyclization of N^4-(Chlorothioacetyl)cytosine. N^4-(Chlorothioacetyl)cytosine was stirred at room temperature with catalytic amounts of K_2CO_3 in DMF for 1 h. The obtained solution (10 μL) was directly analyzed by HPLC, and 3-(N^4-thioacetyl)cytosine was characterized by its electronic and by its mass spectrum (*15*). UV (MeOH/H_2O, pH 2): λ_{max} (ϵ in mAU) 202 (600), 302 (1200). Mass spectra after derivatization with PFBnBr (NCI): *m*/*z* (relative intensity) 347 (15%) (M^-), 346 (100%) ($M^- - H$), 326 (50%) ($M^- - 2H - F$). Mass spectra after derivatization with PFBnBr (EI): *m*/*z* (relative intensity) 181 (100%) ($PFBn^+$), 166 (40%) ($M^+ - PFBn$).

Incubation of *S*-(1,2-Dichlorovinyl)thioacetate with DNA Bases, Deoxynucleosides, and DNA in Aqueous Solutions. Incubation of *S*-(1,2-dichlorovinyl)thioacetate with DNA bases, deoxynucleosides, and calf thymus (ct) DNA in buffered aqueous solutions at pH 5−7.5 was performed in the same stoichiometric amounts as described above. Consumption of *S*-(1,2-dichlorovinyl)thioacetate was determined by GC/MS after extraction into *n*-hexane.

DNA Hydrolysis. ctDNA from the reaction mixtures was either precipitated with 2-propanol or enzymatically hydrolyzed if incubations were performed in buffered solutions at pH 5. The precipitated residues were dissolved in buffered solutions at pH 5 and cleaved enzymatically using 1000 units of nuclease S1 (EC 3.1.30.1), 1 unit of acid phosphodiesterase II (EC 3.1.16.1), and 2 units of acid phosphatase (EC 3.1.3.2) per 1 mg of DNA within 12 h to minimize hydrolysis of potential labile adducts (*16*).

Sample Preparation for GC/MS. The method is based on a publication of Fedtke et al. (*14*) with the following modifications. Dry residues of reaction mixtures (≈1 mg) were dissolved in 2 mL of dimethylformamide, 3 mg of K_2CO_3, and 6 μL of pentafluorobenzyl bromide (**Caution:** *Pentafluorbenzyl bromide is a potent lacrymator. Use an efficient hood!*). The mixtures were then stirred for 12 h at room temperature and concentrated in vacuo. The obtained residues were dissolved in 200 μL of dichloromethane, and 2 μL of the obtained solution was injected into the gas chromatograph.

Gas Chromatography/Mass Spectrometry. GC/MS analysis was performed on a MD 800 mass spectrometer equipped with a Carlo Erba 8000 series gas chromatograph (Fisons Instruments, Mainz, FRG). For all separations, a DB-5 (J & W Scientific, Folsom, CA) fused silica capillary GC column (30 m, 0.25 mm i.d., 0.1 μm film thickness) with helium (average linear velocity of 35 mL/min) as the carrier gas was used. Injections were made splitless (valve time of 1.0 min); a temperature gradient starting at an oven temperature of 60 °C and a heating rate of 10 °C/min used to reach 290 °C were used for separation. The transfer line was kept at a temperature of 280 °C. The injector temperature was 250 °C, and the electron source of the mass spectrometer was adjusted to 200 °C both in the electron impact ionization mode and in the chemical ionization mode. Chemical ionization was performed with methane as the reactant gas. The solvent delay was 8 min. Mass spectra (*m*/*z* 100−600) were recorded from 8 to 17 min with a scan time of 0.5 s and an interchannel delay of 0.05 s. The detection limit was measured in SIR mode using characteristic fragments of the relevant compounds (*m*/*z* 347 and 383). Dwell times were 30 ms.

GC/MS data for detection of thioketene trapping products were aquired with a HP 5890 gas chromatograph coupled with a HP 5970 mass-selective detector (Hewlett-Packard, Avondale, PA) with electron-impact ionization (70 eV). For gas chromatographic separations, a DB 1 fused silica capillary column (40 m, 0.18 mm i.d., 0.4 μm film thickness; J&W Scientific) with helium as the carrier gas was used. Injections were made splitless; a temperature gradient starting at an oven temperature of 40 °C and a heating rate of 15 °C/min used to reach 280 °C were used. The injector and transfer line temperatures were kept at 280 °C.

Electrospray Mass Spectrometry (ES/MS). ES/MS was performed on a Trio 2000 mass spectrometer (VG Biotech, Manchester, England) coupled with a HP 1090 HPLC system (Hewlett-Packard) using H_2O/TFA (pH 2)/AcCN (30:70) as a mobile phase with a flow rate of 10 μL/min.

High-Performance Liquid Chromatography (HPLC). HPLC was performed using two Waters M-6000 A pumps (Millipore GmbH, Eschborn, FRG) coupled with a gradient control unit. Steel columns (250 mm × 4 mm i.d.) filled with

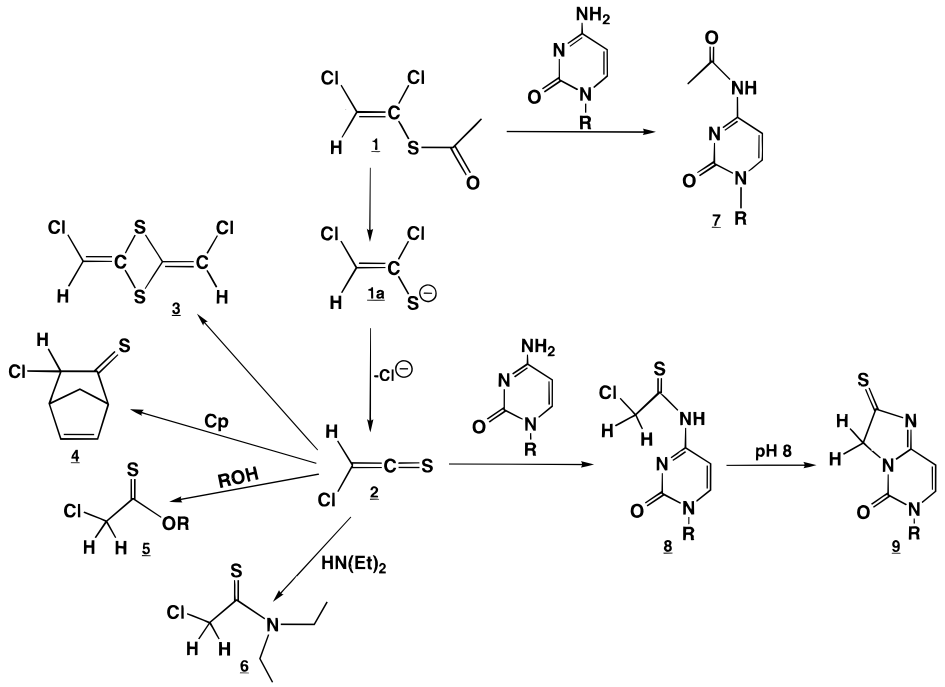


Figure 1. Transformation of the intermediate chlorothioketene to stable reaction products for characterization and reaction of chlorothioketene with cytosine.

Partisil ODS III (5 μm particle size, Bischoff, Leonberg, FRG) were used for separation. Solvents were as follows: (i) solvent A, water with trifluoroacetic acid adjusted to pH 2; solvent B, CH_3OH; 0 to 100% B in 40 min at a flow rate of 1 mL/min. The effluent was passed through a HP 1090 A (Hewlett-Packard) diode array detector.

Results

S-(1,2-Dichlorovinyl)thioacetate (**1**, Figure 1) may be easily obtained by synthesis (Scheme 1), is relatively stable in aqueous solution ($t_{1/2}$ at pH 7 determined to be approximately 12 h), and is cleaved under basic conditions to give *S*-(1,2-dichlorovinyl)thiolate (**1a**, Figure 1) in much higher yields compared to those of previously used precursors for vinyl thiols (*4*).

The formation of chlorothioketene (**2**, Figure 1) from thiol **1a** was confirmed by the detection of characteristic reaction products. Without agents to transform chlorothioketene to stable products, a low concentration of dithiethane **3** was detected using GC/MS (*4*, *17*). In addition, oligomers of the highly reactive thioketene seemed to be formed (*18*). In the presence of cyclopentadiene, 5-thioxo-6-chlorobicyclo[2.2.1]hept-2-ene **4** (*2*, *4*, *18*) was formed as described for structurally similar thioketenes such as di-*tert*-butylthioketene (*19*). In the presence of alcohols, thiono ester **5** [mass spectrum identical to that of synthetic (chloroacetylthiono)acetic acid ethyl ester] was formed, and in the presence of *N*,*N*-diethylamine, thioamide **6** was formed (*20*). An example of the results of these trapping reactions is shown in Figure 2. In the presence of diethylamine, which also functions as a base to catalyze the cleavage of *S*-(1,2-dichlorovinyl)thioacetate to thiol **1a** and chlorothioketene **2**, both *N*,*N*-diethylacetamide (peak at 10.3 min in the chromatogram in Figure 2) and *N*,*N*-diethylchlorothioacetamide **6** (peak at 15.0 min in the chromatogram in Figure 2) were formed. These observations suggest that hydrolytic cleavage of *S*-(1,2-dichlorovinyl)thioacetate represents a useful route for generating high concentrations of chlorothioketene with only one simple byproduct, acetate. The previous method used to generate halovinyl thiols had a much lower yield (as compared in the GC/MS response) and produced a variety of byproducts interfering with further analysis (*4*).

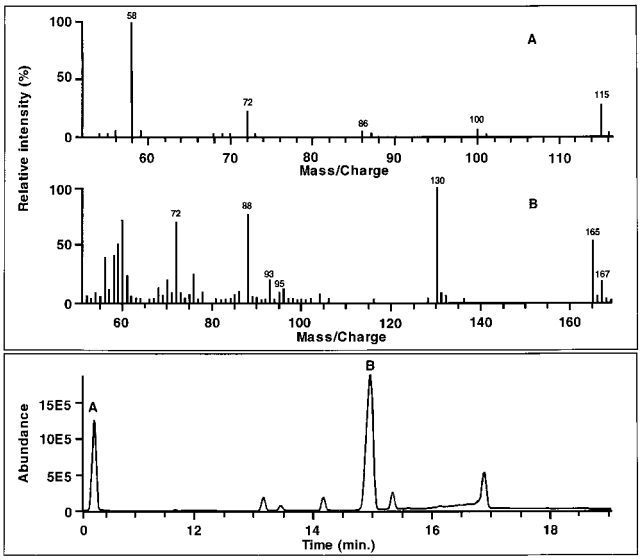


Figure 2. GC separation of an incubation of *S*-(1,2-dichlorovinyl)thioacetate in organic solution in the presence of *N*,*N*-diethylamine. Mass spectrum A represents *N*,*N*-diethylacetamide, and mass spectrum B represents *N*,*N*-diethylchlorothioacetamide (*20*).

When *S*-(1,2-dichlorovinyl)thioacetate was reacted with DNA bases in organic solvents such as dimethylformamide, formation of base modifications was only observed with cytosine by HPLC. Incubations of *S*-(1,2-dichlorovinyl)thioacetate with guanosine, adenosine, and thymidine did not result in the formation of new peaks, even in the presence of additional non-nucleophilic bases such as 1,4-diazabicyclo[2.2.2]octane which promotes hydrolysis of the thioacetate. The formed cytosine derivatives

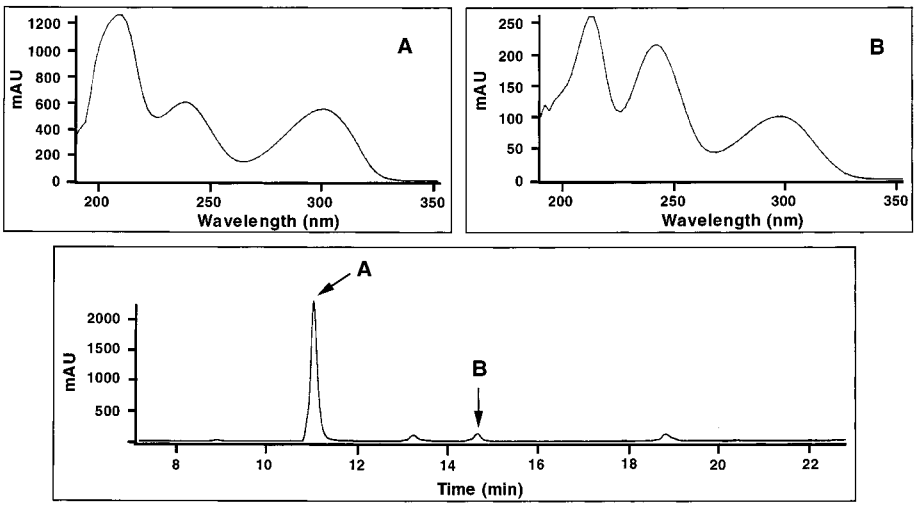


Figure 3. HPLC chromatogram and the electronic spectrum of N^4-acetylcytosine (A) and N^4-(chlorothioacetyl)cytosine (peak B) after incubation of *S*-(1,2-dichlorovinyl)thioacetate in the presence of cytosine in dimethylformamide.

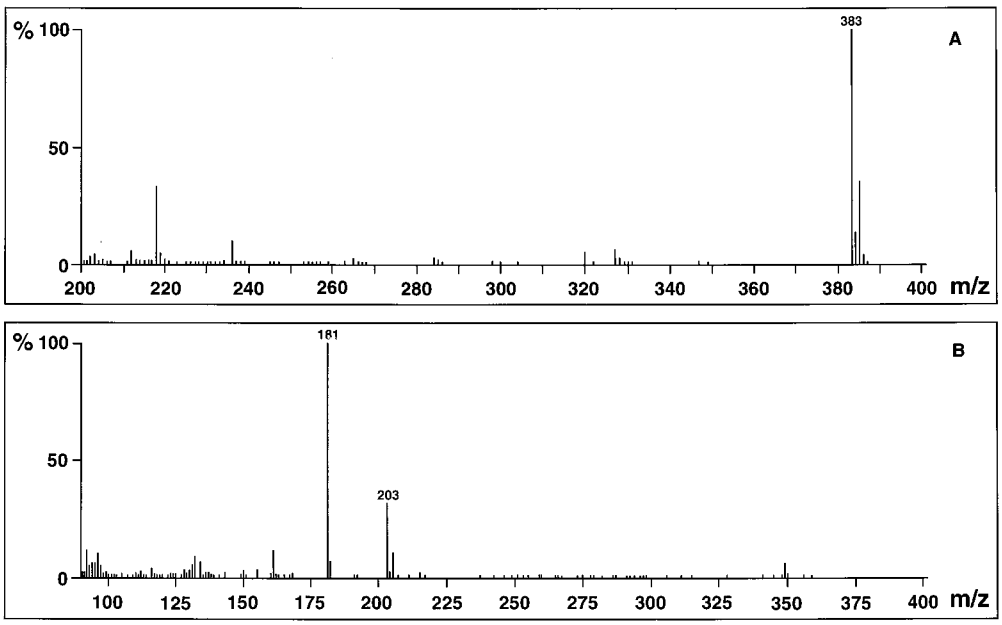


Figure 4. Mass spectra of pentafluorobenzyl derivatives of **8** after chemical ionization (negative ion detection) (A) and electron impact ionization (B).

had characteristic electronic spectra (Figure 3); the compound representing peak A (10.9 min) was identified as N^4-acetylcytosine by comparison of the electronic spectrum with that of a reference compound. Peak B (14.7 min) exhibited an electronic spectrum identical to that of N^4-(chlorothioacetyl)cytosine (*15*). The presence of this compound in the incubations was also confirmed by GC/MS/NCI. Two peaks were obtained by gas chromatographic separation of the pentafluorobenzylated reaction mixtures. One peak contained one chlorine atom (*m*/*z* 383) and represents the pentafluorobenzyl derivative of **8** (Figure 1). Figure 4 shows the mass spectra of **8** (the top shows the electron capture mode and the bottom was recorded in the electron impact mode). The spectra are consistent with the proposed structure of **8**. A value of *m*/*z* 383 (1Cl) represents M^- in the electron capture mode, consistent with the low degree of fragmentation in this ionization process. After electron impact ionization, the molecule fragments intensively and major fragments represent *m*/*z* 181 ($F_5C_6CH_2^+$) and *m*/*z* 203 (1Cl) ($MH - F_5C_6CH_2^+$). The other peak with a single mass fragment (*m*/*z* 347) likely represents the pentafluorobenzyl derivative of **9**.

N^4-(Chlorothioacetyl)cytosine is rapidly hydrolyzed in water at pH 8 back to cytosine and, presumably, chloro(thiono)acetic acid, and ring closure to **9** occurs only in low yield (≈10%) in water (data not shown). The half-life of the hydroslysis of N^4-(chlorothioacetyl)cytosine in water was similar to that of N^4-(chloroacetyl)cytosine (*15*, *21*). In organic solvents in the presence of base, ring closure of **8** to give **9** occurs in high yields. Thus, under the conditions of the derivatization reaction which is performed in dimethylformamide in the presence of base, ring closure may be preferred, thus explaining the higher yields.

On the basis of the results of the reaction of *S*-(1,2-dichlorovinyl)thioacetate in organic solvents, a GC/MS/NCI method was developed to demonstrate a reaction of the intermediate chlorothioketene with DNA bases and DNA under biologically more relevant conditions in

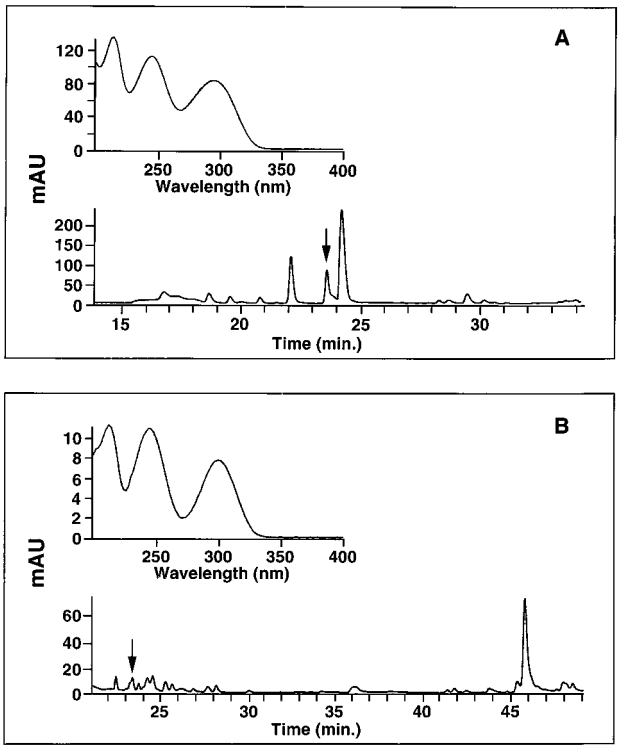


Figure 5. HPLC chromatograms and electronic spectra of N^4-acetyldeoxycytidine formed by incubations of *S*-(1,2-dichlorovinyl)thioacetate **1** with deoxycytidine (A) and DNA after enzymatic hydrolysis (B). The peaks marked with a ↓ have electronic spectra identical to those of N^4-acetyldeoxycytidine and coelute with the reference compound.

aqueous solution. The method based on selected ion monitoring had a limit of detection of 50 pmol/mL of N^4-(chlorothioacetyl)cytosine.

Incubations of *S*-(1,2-dichlorovinyl)thioacetate with deoxycytidine or cytosie in aqueous solutions showed a rapid decrease of the concentration of the thioacetate by GC/MS analysis of hexane extracts and the formation of N^4-acetylcytosine (**7**, Figure 1), respectively, the deoxy derivative in high yield (Figure 5, chart A). The peak at t_R = 23.7 min coeluted with synthetic N^4-acetyldeoxycytidine **7** and had an identical electronic spectrum, suggesting that thiol **1a** is released and that **2** is formed in the reaction mixture. However, peaks indicative of the formation of the deoxy derivatives of **8** or **9** were not observed by HPLC (Figure 5); GC/MS/NCI analysis of the reaction mixtures also was unable to detect the formation of **8** or **9** in these incubations. A GC/MS trace of an incubation of *S*-(1,2-dichlorovinyl)thioacetate with cytosine in water after pentafluorobenzylation by monitoring representative ions of the two products formed from N^4-(chlorothioacetyl)cytosine is shown in Figure 6 (chromatogram B) and compared with the chromatogram (Figure 6A) obtained with approximately 10 pmol/μL of N^4-(chlorthioacetyl)cytosine treated identically. Whereas a clear signal for the synthetic reference compound was obtained, the concentration of N^4-(chlorothioacetyl)cytosine formed by the reaction of chlorothioketene with cytosine in aqueous solution was below the limit of detection. Consistent with these observations, incubations of *S*-(1,2-dichlorovinyl)thioacetate with DNA followed by DNA hydrolysis and HPLC separation also demonstrated the formation of N^4-acetyldeoxycytidine (Figure 5B). The small peak in the chromatogram at t_R = 23.4 min had retention and electronic spectra identical to those of N^4-acetyldeoxycytidine, indicating cleavage of the thioacetate and chlorothioketene formation; attempts to detect N^4-(chlorothioacetyl)deoxycytidine **8** or **9** in these incubations by UV HPLC and/or GC/MS/NCI were also unsuccessful. However, the detection of N^4-acetyldeoxycytidine in DNA treated with the thioacetate demonstrates that chlorothioketene is released in close proximity to the DNA.

Discussion

The results show that chlorothioketenes, most likely representing the toxic metabolites formed from halovinyl cysteine S-conjugates, are highly reactive and short-lived. On the basis of the results with trapping agents, we conclude *S*-(1,2-dichlorovinyl)thioacetate is an easily accessible precursor for the in situ generation of chlorothioketene for studying its reactivity with potential biologically relevant target molecules (*2*). The previous

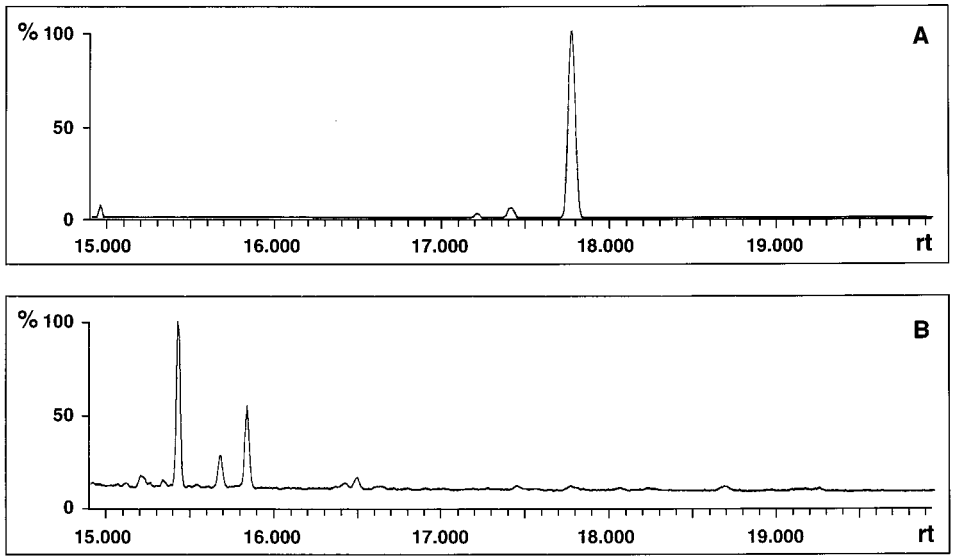


Figure 6. GC/MS traces (*m*/*z* 383) of 10 pmol/μL of N^4-(chlorothioacetyl)cytosine (after pentafluorobenzylation) (A) and an incubation of cytosine with *S*-(1,2-dichlorovinyl)thioacetate in buffered solution at pH 8 treated identically (B). For details of the sample preparation and GC/MS conditions, see Experimental Procedures.

precursors, halovinyl 2-nitrophenyl disulfides, although useful for generating structurally different halothioketenes, require a more complex synthesis and purification procedure. Moreover, their cleavage results in byproducts, which may be chemically reactive and interfere during analytical procedures. The novel chlorothioketene precursor used in this study in contrast is simple to synthesize, relatively stable in water at neutral pH, and cleaved by base to dichlorovinyl thiol. This vinyl thiol is further converted to chlorothioketene. Due to its high reactivity, isolation or direct characterization of this molecule is not possible, but the stable products were detected in the presence of trapping agents often used to demonstrate the intermediate formation of a thioketene (*2*, *17*, *18*). Under favorable conditions, chlorothioketene may interact with DNA bases or deoxynucleosides. In polar organic solvent in the presence of base (cytosine, which catalyzes the release of chlorovinyl thiol as indicated by the formation of N^4-acetylcytosine), relatively high concentrations of chlorothioketene may be present and available for reaction with the DNA base since water is not a completing nucleophile. In presence of methanol, **1** was cleaved at about 50 °C by cytosine to chlorothioketene which immediately reacts with methanol quantitatively to form the corresponding thionoester. Both the thionoester and N^4-acetylcytosine were detected in the methanolic solution either by GC/MS or by UV HPLC (data not shown). Moreover, cytosine shows a higher nucleophilicity in dimethylformamide than in water due to the absence of solvation. Therefore, chlorothioketene reacts in relatively good yields (as compared with the reaction of other electrophiles with DNA constituents). The results presented here show that **1** was also cleaved by cytosine in the presence of water adjusted to pH 6−8 as confirmed by formation of the relatively stable N^4-acetylcytosine (*13*) resulting from the nucleophilic attack of the exocyclic amino group of cytosine on the electrophilic carbonyl function of **1**, but the generation of N^4-(chlorothioacetyl)cytosine by the reaction of cytosine with chlorothioketene did not occur. This may occur for two reasons. (i) The highly reactive chlorothioketene is rapidly hydrolyzed in the presence of water. (ii) Small amounts of N^4-(chlorothioacetyl)cytosine formed may be rapidly hydrolyzed under these conditions like those described for N^4-(chloroacetyl)cytosine (*13*). Our results are confirmed by the observation that chloro(thio)acetylation of cytosine only occurs in anhydrous dimethylformamide (*15*). In the presence of water, chlorothioketenes are very rapidly hydrolyzed and react with DNA constituents, if at all, only in very low yields. On the basis of theoretical considerations (hard and soft electrophiles), a reaction of chlorothioketene, a soft electrophile, with nucleophilic sites in DNA constituents (hard nucleophiles) should be less favored (*22*−*24*). Thus, proteins (softer nucleophilic centers) may be favored targets for interactions of halothioketenes in biological systems. This assumption is supported by the in vivo formation of protein adducts of an intermediate thioketene, formed by β-lyase-mediated cleavage of the perchloroethene metabolite *S*-(trichlorovinyl)-L-cysteine. These protein adducts are formed in relatively good yields. Due to the very low yield of the reactions of halothioketenes with DNA constituents and the low concentrations of halothioketene precursors formed in rodents in vivo after administration of trichloroethene and perchloroethene (*25*, *26*), experimental demonstration of DNA adduct formation in the kidney after administration of tri- or perchloroethene to rodents has to be considered very difficult, if not impossible.

References

(1) Dekant, W., Vamvakas, S., Berthold, K., Schmidt, S., Wild, D., and Henschler, D. (1986) Bacterial β-lyase mediated cleavage and mutagenicity of cysteine conjugates derived from the nephrocarcinogenic alkenes trichloroethylene, tetrachloroethylene and hexachlorobutadiene. *Chem.-Biol. Interact.* **60**, 31−45.

(2) Schaumann, E. (1988) The chemistry of thioketenes: Tetrahedron Report No. 231. *Tetrahedron* **44**, 1827−1871.

(3) Birner, G., Richling, C., Henschler, D., Anders, M. W., and Dekant, W. (1994) Metabolism of tetrachloroethene in rats: identification of N^{ϵ}-(dichloroacetyl)-L-lysine and N^{ϵ}-(trichloroacetyl)-L-lysine as protein adducts. *Chem. Res. Toxicol.* **7**, 724−732.

(4) Dekant, W., Urban, G., Görsman, C., and Anders, M. W. (1991) Thioketene formation from α-haloalkenyl 2-nitrophenyl disulfides: models for biological reactive intermediates of cytotoxic *S*-conjugates. *J. Am. Chem. Soc.* **113**, 5120−5122.

(5) Vamvakas, S., Dekant, W., and Henschler, D. (1989) Genotoxicity of haloalkene and haloalkane glutathione *S*-conjugates in porcine kidney cells. *Toxicol. in Vitro* **3**, 151−156.

(6) Vamvakas, S., Dekant, W., and Henschler, D. (1989) Assessment of unscheduled DNA synthesis in a cultured line of renal epithelial cells exposed to cysteine *S*-conjugates of haloalkenes and haloalkanes. *Mutat. Res.* **222**, 329−335.

(7) National Toxicology Program, National Cancer Institute (1977) Bioassay of tetrachloroethylene for possible carcinogenicity. Technical Report No. 13, U.S. Department of Health and Human Services, Washington, DC.

(8) National Toxicology Program, National Cancer Institute (1986) Carcinogenesis bioassay of tetrachloroethylene. Technical Report No. 232, U.S. Department of Health and Human Services, Washington, DC.

(9) National Toxicology Program, National Cancer Institute (1986) Carcinogenesis bioassay of trichloroethylene. Technical Report No. 311, U.S. Department of Health and Human Services, Washington, DC.

(10) National Toxicology Program, National Cancer Institute (1988) Toxicology and carcinogenesis studies of trichloroethylene in four strains of rats (ACI, August, Marshall, Osborne-Mendel) (gavage studies). Technical Report No. 273, U.S. Department of Health and Human Services, Washington, DC.

(11) Kanhai, W., Dekant, W., and Henschler, D. (1989) Metabolism of the nephrotoxin dichloroacetylene by glutathione conjugation. *Chem. Res. Toxicol.* **2**, 51−56.

(12) Pedersen, B. S., and Lawesson, S.-O. (1979) Studies on Organophosphorus Compounds-XXVIII. *Tetrahedron* **35**, 2433−2437.

(13) Goody, R. S., and Walker, R. T. (1967) Some N^6 acylated cytosines. *Tetrahedron Lett.*, 289−291.

(14) Fedtke, N., Boucheron, J. A., Turner, M. J., and Swenberg, J. A. (1990) Vinyl chloride-induced DNA adducts. I: quantitative determination of N^2,3-ethenoguanine based on electrophore labeling. *Carcinogenesis* **11**, 1279−1285.

(15) Müller, M., Birner, G., and Dekant, W. (1998) Reactivity of haloketenes and halothioketenes with nucleobases: chemical characterization of reaction products. *Chem. Res. Toxicol.* **11**, 454−463.

(16) Müller, M., Birner, G., Sander, M., and Dekant, W. (1998) Reactivity of haloketenes and halothioketenes with nucleobases: reactions in vitro with DNA. *Chem. Res. Toxicol.* **11**, 464−470.

(17) Raasch, M. S. (1970) Bis(trifluoromethyl)thioketene. I. Synthesis and cycloaddition reactions. *J. Org. Chem.* **35**, 3470−3483.

(18) Seybold, G., and Heibl, C. (1975) Blitzhermolyse von 1,2,3-Thiadiazolen: ein einfacher Weg zu Thioketenen (Flash thermolysis of 1,2,3-thiadiazoles. Simple route to thioketenes). *Angew. Chem.* **87**, 171−172.

(19) Elam, E. U., Rash, F. H., Dougherty, J. T., Goodlett, V. W., and Brannock, K. C. (1968) Di-*tert*-butylthioketene. *J. Org. Chem.* **33**, 2738−2741.

(20) Dekant, W., Berthold, K., Vamvakas, S., Henschler, D., and Anders, M. W. (1988) Thioacylating intermediates as metabolites of *S*-(1,2-dichlorovinyl)-L-cysteine and *S*-(1,2,2-trichlorovinyl)-L-cysteine formed by cysteine conjugate β-lyase. *Chem. Res. Toxicol.* **1**, 175−178.

(21) Goody, R. S., and Walker, R. T. (1971) The preparation and properties of some cytosine derivatives. *J. Org. Chem.* **36**, 727−730.

(22) Anders, M. W. (1988) Bioactivation mechanisms and hepatocellular damage. In *The Liver: Biology and Pathology* (Arias, I. M., Jakoby, W. B., Popper, H., Schachter, D., and Shafritz, D. A., Eds.) 2nd ed., pp 389−400, Raven Press, Ltd., New York.

(23) Nelson, S. D., and Pearson, P. G. (1990) Covalent and noncovalent interactions in acute lethal cell injury caused by chemicals. *Annu. Rev. Pharmacol. Toxicol.* **30**, 169−195.

(24) Hinson, J. A., and Roberts, D. W. (1992) Role of covalent and noncovalent interactions in cell toxicity: effects on proteins. *Annu. Rev. Pharmacol. Toxicol.* **32**, 471−510.

(25) Dekant, W., Metzler, M., and Henschler, D. (1986) Identification of *S*-1,2-dichlorovinyl-*N*-acetyl-cysteine as a urinary metabolite of trichloroethylene: A possible explanation for its nephrocarcinogenicity in male rats. *Biochem. Pharmacol.* **35**, 2455−2458.

(26) Dekant, W., Metzler, M., and Henschler, D. (1986) Identification of *S*-1,2,2-trichlorovinyl-*N*-acetylcysteine as a urinary metabolite of tetrachloroethylene: bioactivation through glutathione conjugation as a possible explanation of its nephrocarcinogenicity. *J. Biochem. Toxicol.* **1**, 57−72.

TX980084D