Bioactivation of *S*-(2,2-Dihalo-1,1-difluoroethyl)-L-cysteines and *S*-(Trihalovinyl)-L-cysteines by Cysteine S-Conjugate *β*-Lyase: Indications for Formation of both Thionoacylating Species and Thiiranes as Reactive Intermediates

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Received March 22, 1996[⊗]

The covalent binding of reactive intermediates, formed by *β*-elimination of cysteine S-conjugates of halogenated alkenes, to nucleophiles was studied using ^{19}F-NMR and GC-MS analysis. *β*-Elimination reactions were performed using rat renal cytosol and a *β*-lyase model system, consisting of pyridoxal and copper(II) ion. *S*-(1,1,2,2-Tetrafluoroethyl)-L-cysteine (TFE-Cys) was mainly converted to products derived from difluorothionoacetyl fluoride, namely, difluorothionoacetic acid, difluoroacetic acid, and N-difluorothionoacetylated TFE-Cys. In the presence of *o*-phenylenediamine (OPD), as a bifunctional nucleophilic trapping agent, the major product formed was 2-(difluoromethyl)benzimidazole. This product results from initial reaction of difluorothionoacetyl fluoride with one of the amino groups of OPD, followed by a condensation reaction between the thionoacyl group and the adjacent amino group of OPD. In incubations with *S*-(2-chloro-1,1,2-trifluorofluoroethyl)-L-cysteine (CTFE-Cys) and *S*-(2,2-dichloro-1,1-difluorofluoroethyl)-L-cysteine (DCDFE-Cys), formation of thionoacylated cysteine S-conjugates was also observed by GC-MS analysis, indicating formation of the corresponding thionoacyl fluorides. However, according to ^{19}F-NMR analysis, chlorofluorothionoacyl fluoride-derived products accounted for only 10% of the CTFE-Cys converted. In the presence of OPD, next to the corresponding 2-(dihalomethyl)benzimidazoles, 2-mercaptoquinoxaline was identified as the main product in incubations with CTFE-Cys. When chlorofluorothionoacylating species were generated from the unsaturated *S*-(2-chloro-1,2-difluorovinyl)-L-cysteine (CDFV-Cys), 2-(chlorofluoromethyl)benzimidazole and 2-mercaptoquinoxaline were also found as OPD adducts. However, with CDFV-Cys the ratio of 2-(chlorofluoromethyl)benzimidazole to 2-mercaptoquinoxaline was 12-fold higher than in the case of CTFE-Cys. These results suggest an important second mechanism of formation of 2-mercaptoquinoxaline with CTFE-Cys. The formation of 2-mercaptoquinoxaline could also be explained by reaction of OPD with 2,3,3-trifluorothiirane as a second reactive intermediate for CTFE-Cys. Comparable results were obtained when comparing OPD adducts from DCDFE-Cys and TCV-Cys. Both DCDFE-Cys and TCV-Cys form dichlorothionoacylating species. However, DCDFE-Cys forms 21-fold more 2-mercaptoquinoxaline than TCV-Cys, which may be explained by its capacity to form 3-chloro-2,2-difluorothiirane next to dichlorothionoacyl fluoride. In order to explain the apparent differences in the preference of thiols to form different reactive intermediates, free enthalpies of formation ($\Delta_f G$) of thiolate anions and their possible rearrangement products, thionoacyl fluorides and thiiranes, derived from TFE-Cys, CTFE-Cys, and DCDFE-Cys, were calculated by *ab initio* calculations. For TFE-thiolate, formation of difluorothionoacetyl fluoride is energetically favored over formation of the thiirane. In contrast, the thiirane pathway is favored over the thionoacyl fluoride pathway for CTFE- and DCDFE-thiolates. The results of these quantum chemical calculations appear to be consistent with the experimental data.

Introduction

Cysteine S-conjugates are intermediates in the bioactivation mechanisms responsible for the nephrotoxicity of a number of halogenated alkenes, such as hexachloro-1,3-butadiene, tetrafluoroethylene (TFE),[1] chlorotrifluoroethylene (CTFE), 1,1-dichloro-2,2-difluoroethylene (DCDFE), hexafluoropropene, and 1,1,2-trichloro-3,3,3-trifluoropropene, as reviewed recently (*1*−*3*). Formation of the cysteine S-conjugates results from initial enzymic conjugation of the cysteinyl thiol group of glutathione to the parent haloalkenes, followed by subsequent hydrolysis by *γ*-glutamyltranspeptidases and dipeptidases present

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⊗ Abstract published in *Advance ACS Abstracts,* August 15, 1996.

[1] Abbreviations: CDFV-Cys, *S*-(2-chloro-1,2-difluorovinyl)-L-cysteine; CTFE-Cys, *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine; *S*-(2,2-dibromo-1,1-difluoroethyl)-L-cysteine; DCDFE-Cys, *S*-(2,2-dichloro-1,1-difluoroethyl)-L-cysteine; MOP, 3-mercapto-2-oxopropionic acid; OPD, *o*-phenylenediamine; TCV-Cys, *S*-(1,2,2-trichlorovinyl)-L-cysteine; TFE-Cys, *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine; TFE-PMS, *N*-(difluorothionoacetyl)-*S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine.

S0893-228x(96)00049-5 CCC: $12.00

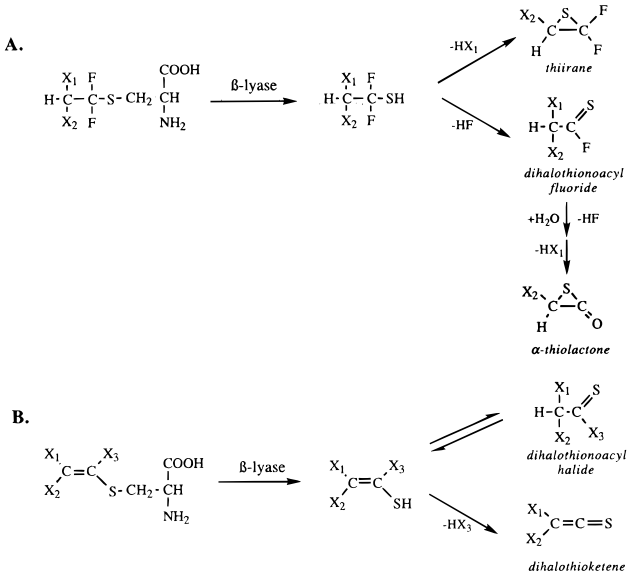

Figure 1. Proposed reactive species formed upon the β-elimination reactions from *S*-(2,2-dihalo-1,1-difluoroethyl)-L-cysteine (A) and *S*-(trihalovinyl)-L-cysteine S-conjugates (B).

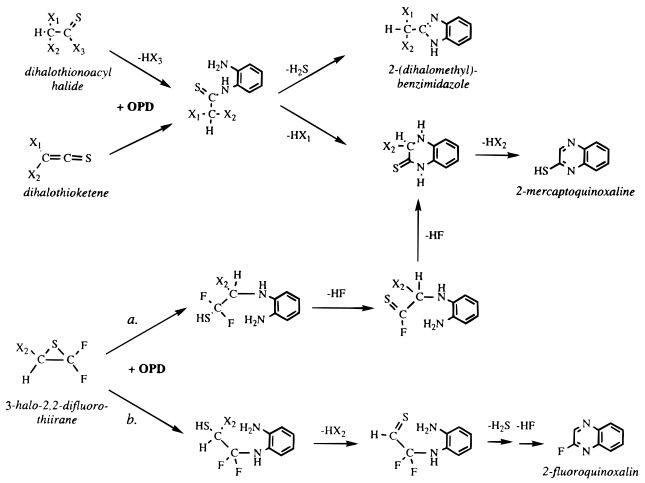

Figure 2. Anticipated products of reactions between *o*-phenylenediamine (OPD) and dihalothionoacylating reactive intermediates (dihalothionoacyl halides and dihalothioketenes) and 3-halo-2,2-difluorothiirane. X_1, X_2, and X_3 represent fluorine and/or chlorine atoms. Anticipated products from α-thiolactones are not shown since these reactive intermediates are only formed from bromine-containing cysteine S-conjugates (*18*).

in several tissues. The toxicity of cysteine S-conjugates of haloalkenes is directed to the proximal tubule of the kidney, due to the presence of active uptake mechanisms and the high activity of the bioactivating enzyme, cysteine S-conjugate β-lyase (β-lyase), in this part of the nephron (*1*, *3*). Cleavage of cysteine S-conjugates of haloalkenes by β-lyase results in the formation of reactive intermediates which may bind covalently to essential biomacromolecules, such as proteins, lipids, DNA, and RNA (*4*−*8*), thus initiating processes leading to cell death, mutagenicity, or carcinogenicity. Reactive intermediates which have been proposed for *S*-(2,2-dihalo-1,1-difluoroethyl)-L-cysteine and *S*-(trihalovinyl)-L-cysteine (*9*−*17*) are shown in Figure 1.

The detection of difluorothionoacylated products from *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFE-Cys, Figure 1A: X_1,X_2 = F, F) is consistent with thionoacylation of target nucleophiles by difluorothionoacetyl fluoride formed by α-elimination of a fluoride anion from the initial thiolate (*11*, *13*, *14*, *16*). Similar evidence has been obtained for the formation of the analogous reactive intermediate, chlorofluorothionoacetyl fluoride, from *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine (CTFE-Cys, Figure 1A: X_1,X_2 = F, Cl) (*10*, *12*, *14*−*16*). However, the relatively low yields of chlorofluorothionoacetylated nucleophiles and the accompanying high yield of fluoride anion observed in ^{19}F-NMR studies may indicate instability of the adduct formed or the formation of another reactive intermediate. Based on the formation of glyoxylate, Finkelstein et al. (*18*) proposed α-thiolactones (Figure 1A) as reactive intermediates formed from bromine-containing cysteine S-conjugates. However, because glyoxylate formation was not observed in the case of TFE-Cys, CTFE-Cys, and *S*-(2,2-dichloro-1,1-difluoroethyl)-L-cysteine (DCDFE-Cys, Figure 1A: X_1,X_2 = F, Cl), this kind of reactive intermediate does not appear to be formed from non-bromine-containing cysteine S-conjugates. Formation of 2,2,3-trifluorothiirane, as another alternative reactive intermediate, has originally been proposed by Dohn et al. (*9*). Very recently, the formation of trihaloalkenes next to glyoxylate in incubations with bromine-containing cysteine S-conjugates was claimed to be the first experimental proof for formation of thiiranes (*19*). However, these authors did not find experimental proof for the formation of thiiranes from CTFE-Cys and DCDFE-Cys.

In the present study, the nature of covalent binding of β-elimination products of three *S*-(2,2-dihalo-1,1-difluoroethyl)-L-cysteine conjugates was investigated using GC-MS and ^{19}F-NMR. Because it is anticipated that the initial products of the reaction of nucleophiles with thionoacyl halides and thiiranes still contain an electrophilic center, *o*-phenylenediamine (OPD) was chosen as a trapping agent. With OPD, the introduced electrophilic center can rapidly react intramolecularly with the adjacent free amino group (Figure 2). Comparable bifunctional nucleophilic trapping agents are DNA bases, such as adenosine, which have been used to trap bifunctional electrophiles, such as 2-bromoacetaldehyde, forming ethenoadenosine (*20*). However, the advantage of OPD over adenosine is that the products formed can easily be analyzed by GC-MS.

It was anticipated that analysis of OPD adducts might discriminate between dihalothionoacylating reactive intermediates (dihalothionoacyl halides and dihalothioketenes) and 3-halo-2,2-difluorothiiranes (Figure 2). The possible contribution of thiiranes was investigated by comparing OPD adducts formed from *S*-(2,2-dihalo-1,1-difluoroethyl)-L-cysteine with those formed from *S*-(trihalovinyl) S-conjugates, which can be bioactivated to similar thionoacylating reactive species (*17*) but cannot form thiirane species (Figure 1B).

The results in the present study support the formation of 3-halo-2,2-difluorothiiranes next to thionoacyl fluorides from *S*-(2,2-dihalo-1,1-difluoroethyl)-L-cysteine conjugates. A theoretical study, using *ab initio* calculations of the free enthalpies of formation ($\Delta_f G$) of reactants and products, was also performed to compare the thermodynamics of the proposed rearrangement reactions.

Materials and Methods

Chemicals and Synthesis. Pyridoxal hydrochloride, *o*-phenylenediamine (OPD), copper(II) chloride hydrate, and 1,2-difluoro-1,1,2,2-tetrachloroethane were obtained from Aldrich (Brussels, Belgium). 1,2-Difluoro-1,2-dichloroethane was prepared by heating 1,2-difluoro-1,1,2,2-tetrachloroethane in pres-

ence of zinc dust in ethanol, by the same procedure as described for the synthesis of 1,1-dichloro-2,2-difluoroethylene (*21*). Difluoroacetic acid (DFA) was obtained from Acros Chimica (Geel, Belgium), and chlorofluoroacetic acid was prepared according to Young and Tarrant (*22*). Difluoroacetic acid anhydride was prepared from difluoroacetic acid according to Sawicki (*23*). TFE-Cys, CTFE-Cys, *S*-(2,2-dichloro-1,1-difluoroethyl)-L-cysteine (DCDFE-Cys), *S*-(2,2-dibromo-1,1-difluoroethyl)-L-cysteine (DBDFE-Cys), *S*-(1,2-dichlorovinyl)-L-cysteine (1,2-DCV-Cys), *S*-(2,2-dichlorovinyl)-L-cysteine (2,2-DCV-Cys), and *S*-(1,2,2-trichlorovinyl)-L-cysteine (TCV-Cys) were synthesized as described previously (*12*, *17*, *24*). Deuterium-labeled CTFE-Cys, [*ethyl*-2-D]-*S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine, was synthesized according to the same method as described for CTFE-Cys, after exchanging all acidic protons of the reagents for deuterium atoms prior to the reaction. Exchange of protons was achieved by repeatedly dissolving the reagents in deuterium oxide and subsequent freeze-drying. ^{1}H-NMR, ^{19}F-NMR, and GC-MS proved the exchange of the proton of the 2-chloro-1,1,2-trifluoroethyl moiety by a deuterium atom.

Synthesis of *N*-Acetyl-*S*-(2-chloro-1,2-difluorovinyl)-L-cysteine. The strategy to synthesize *S*-(2-chloro-1,2-difluorovinyl)-L-cysteine was the preparation of *N*-acetyl-*S*-(2-chloro-1,2-difluorovinyl)-L-cysteine, followed by deacetylation under acidic conditions, as used previously (*12*).

In order to synthesize *N*-acetyl-*S*-(2-chloro-1,2-difluorovinyl)-L-cysteine, the method as described by Gandolfi *et al.* (*25*) was used. In short, 5 g (30 mmol) of *N*-acetyl-L-cysteine was dissolved in 250 mL of liquid ammonia, and 1.4 g (60 mmol) of metallic sodium was added in small pieces. Subsequently, 20 g (150 mmol) of 1,2-dichloro-1,2-difluoroethylene was added slowly to the reaction mixture which was kept at −50 °C. After 4 h, the cooling was removed, and the ammonia and excess 1,2-dichloro-1,2-difluoroethylene were evaporated overnight by a slow nitrogen stream while stirring. The residue was dissolved in 100 mL of 2 N hydrochloric acid and extracted two times with 100 mL of ethyl acetate. The ethyl acetate fractions were combined, treated with active charcoal, and filtered. The solvent was removed by rotaevaporation under vacuum, yielding approximately 6 g of a thick yellow oil. However, according to analysis by ^{1}H-NMR and GC-MS, the sole product formed was *N*-acetyl-*S*-(1,2-dichloro-1,2-difluoroethyl)-L-cysteine, indicating that under these reaction conditions an addition reaction took place instead of the expected substitution reaction. Similar results were obtained when the reaction was performed in methanol instead of liquid ammonia (data not shown).

Characterization of *N*-acetyl-*S*-(1,2-dichloro-1,2-difluoroethyl)-L-cysteine: ^{1}H-NMR (D_2O): δ (intensity, multiplicity) 2.00 (3H, s), 3.20−3.65 (2H, m), 4.45−4.55 (1H, m), 6.62 (1H, d of d, $^2J_{FH} = 48$ Hz, $^3J_{FH} = 6$ Hz). Electron-impact GC-MS of methyl ester: *m*/*z* (rel intensity %, multiplicity, assignment): 309 (0.1, 2Cl, $M^{\bullet+}$), 250 (3.5, 2Cl, $M^{\bullet+} - COOCH_3$ and $M^{\bullet+} - NH_2COCH_3$), 208 (7.6, 2Cl), 176 (18), 144 (8), 134 (14), 117 (19), 88 (57), 43 (100).

We prepared *N*-acetyl-*S*-(2-chloro-1,2-difluorovinyl)-L-cysteine by stirring a 50 mL solution of 50 mM *N*-acetyl-*S*-(1,2-dichloro-1,2-difluoroethyl)-L-cysteine in cold 0.1 N NaOH in the presence of 100 mM silver nitrate. The progress of the reaction was followed by analyzing 100 μL fractions by GC-MS. The 100 μL fractions were added to 2 mL of 2 N hydrochloric acid and extracted with 3 mL of ethyl acetate. The ethyl acetate layer was separated, treated with ethereal diazomethane, and analyzed by electron-impact GC-MS. Using this procedure, two products, with retention times of 11.40 and 11.50 min, in a ratio of approximately 1:2 were obtained. We attribute these two products to the two isomers which were anticipated, having a *cis*- and *trans*-(difluorovinyl) moiety, respectively. The electron-impact mass spectra of both isomers were identical and consistent with that of *N*-acetyl-*S*-(2-chloro-1,2-difluorovinyl)-L-cysteine.

EI-GC-MS of methyl ester: *m*/*z* (rel intensity %, multiplicity, assignment): 273 (0.05, 1Cl, $M^{\bullet+}$), 214 (4, 1Cl, $M^{\bullet+} - COOCH_3$ and $M^{\bullet+} - NH_2COCH_3$), 211 (6, 1Cl, $M^{\bullet+} - C_2F_2$), 172 (10, 1Cl), 144 (21), 88 (30), 84 (9), 43 (100).

After completion of the dehydrohalogenation reaction, the reaction mixture was acidified to pH 2, filtered to remove silver salts, and extracted by ethyl acetate. The ethyl acetate fraction was evaporated to dryness, and the residue was treated with 50 mL of 6 N hydrochloric acid at 80 °C for 16 h, in order to N-deacetylate *N*-acetyl-*S*-(2-chloro-1,2-difluorovinyl)-L-cysteine. After evaporation of solvent by rotavap, the residue was purified by preparative thin layer chromatography using silica gel as stationary phase and 1-propanol/water (70/30 v/v) as eluent. The fraction at R_f 0.6, which was UV-active and ninhydrin-reactive, was collected. ^{1}H-NMR analysis revealed that the final product was free from *N*-acetyl-*S*-(2-chloro-1,2-difluorovinyl)-L-cysteine and *N*-acetyl-*S*-(1,2-dichloro-1,2-difluoroethyl)-L-cysteine: δ (D_2O) 3.30−3.50 (2H, m), 4.20−4.35 (1H, m). GC-MS analysis of the product, after subsequent acetylation by acetic anhydride and methylation by ethereal diazomethane, revealed the two isomers of the methyl esters of *N*-acetyl-*S*-(2-chloro-1,2-difluorovinyl)-L-cysteine as the sole products. This indicates that the final product consists of *S*-(2-chloro-*cis*-1,2-difluorovinyl)-L-cysteine and *S*-(2-chloro-*trans*-1,2-difluorovinyl)-L-cysteine. Because both isomers were anticipated to yield identical products upon the β-elimination reactions, no attempts were undertaken to separate both isomers.

2-Hydroxyquinoxaline and 2-Methoxyquinoxaline. 2-Hydroxyquinoxaline was obtained from Aldrich-Chemie (Steinhein, West Germany). In our GC-MS conditions as described below, 2-hydroxyquinoxaline demonstrated a very poor chromatography yielding a broad peak ranging from 8.7 to 9.1 min and the following electron-impact mass spectrum: *m*/*z* (intensity %) 146 (100, $M^{\bullet+}$), 118 (68), 91 (39), 64 (28), 63 (32).

Methylation of 2-hydroxyquinoxaline by diazomethane yielded a sharp peak with a retention time of 8.53 min and the electron-impact mass spectrum: *m*/*z* (intensity %) 160 (100, $M^{\bullet+}$), 132 (84), 131 (98), 104 (48), 90 (20), 77 (42), 63 (40), 51 (37). A minor product with retention time of 6.95 min also showed a molecular ion at *m*/*z* 160. We propose that this product might be derived from N-methylation of the tautomeric form of 2-hydroxyquinoline: *m*/*z* (rel intensity %, assignment) 160 (100, $M^{\bullet+}$), 159 (47, $M^{\bullet+} - H$), 131 (56), 130 (32), 103 (34), 90 (57), 76 (14), 75 (13), 64 (16), 63 19).

2-Mercaptoquinoxaline and 2-Methylthioquinoxaline. 2-Mercaptoquinoxaline was prepared from 2-hydroxyquinoxaline by the procedure described by Scheeren et al. (*26*). Anhydrous $NaHCO_3$ (4 mmol) was added slowly with stirring to a mixture of 2-hydroxyquinoxaline (1 mmol) and tetraphosphorus decasulfide (0.6 mmol) in diethyl ether, and refluxed for 5 h. The organic phase was evaporated to dryness, the residue was treated with 5 mL of 0.2 N HCl at 40 °C for 2 h and cooled, and the suspension was extracted with ethyl acetate (3 × 5 mL). The combined ethyl acetate fractions were evaporated to dryness to obtain the product. ^{1}H-NMR of the product: ($CDCl_3$, relative to 3-(trimethylsilyl)propionic acid): δ (assignment, intensity, multiplicity) 7.30−7.50 (H^b, 2H, m), 7.55−7.85 (H^c, 2H, m), 8.65 (H^c, s). GC-MS analysis: retention time 8.25 min; electron-impact mass spectrum: *m*/*z* (intensity %, assignment) 162 (100, $M^{\bullet+}$), 134 (10), 129 (24, $M^{\bullet+} - SH$), 118 (75, $M^{\bullet+} - CS$), 108 (8), 102 (21), 90 (10).

Methylation of a solution of 2-mercaptoquinoxaline in ethyl acetate by ethereal diazomethane yielded 2-methylthioquinoxaline: ^{1}H-NMR ($CDCl_3$, relative to 3-(trimethylsilyl)propionic acid): δ (assignment, intensity, multiplicity) 2.62 (H^d, 3H, s), 7.50−7.70 (H^b, 2H, m), 7.82−8.02 (H^a, 2H, m), 8.55 (H^c, 1H, s). GC-MS analysis: retention time 8.45 min; electron-impact mass spectrum: *m*/*z* (rel. intensity %, assignment) 176 (100, $M^{\bullet+}$), 175 (32, $M^{\bullet+} - H$), 161 (13, $M^{\bullet+} - CH_3$), 143, (27, $M^{\bullet+} - SH$), 134 (17), 131 (25, −CHS), 103 (25), 102 (27), 90 (13).

2-(Difluoromethyl)benzimidazole was prepared from difluoroacetic anhydride (3 mmol) and OPD (2 mmol) in dry acetonitrile, according to the method described by Sawicki (*23*). After removal of the solvent, the residue was dissolved in 2 N HCl and extracted with ethyl acetate to remove diacylated OPD; the product was recovered by ethyl acetate extraction of the

aqueous phase adjusted to pH 9 with NaOH. ^{1}H-NMR of the product ($CDCl_3$, relative to 3-(trimethylsilyl)proprionic acid): δ (assignment, intensity, multplicity) 6.92 (H^b, 1H, t, $^2J_{FH} = 53.4$ Hz), 7.32−7.42 (H^a, 4H, m), 11.5 (H^c, 1H, broad peak). ^{19}F-NMR of the product (H_2O, relative to trifluoroacetic acid): δ −41.93 (d, $^2J_{FH} = 51.01$ Hz). GC-MS analysis: retention time 6.88 min; electron-impact mass spectrum: *m/z* (rel. intensity %, assignment) 168 (100, $M^{\bullet+}$), 148 (30, $M^{\bullet+} - HF$), 129 (19, $M^{\bullet+} - HF - F$), 121 (18), 118 (14, $M^{\bullet+} - CF_2$), 103 (18), 90 (37).

Methylation of the ethyl acetate solution of 2-(difluoromethyl)-benzimidazole by ethereal diazomethane yielded *N*-methyl-2-(difluoromethyl)benzimidazole. GC-MS analysis: retention time 6.57 min; electron-impact mass spectrum: *m/z* (rel. intensity %, assignment) 182 (100, $M^{\bullet+}$), 181 (23, $M^{\bullet+} - H$), 167 (5, $M^{\bullet+} - CH_3$), 163 (10, $M^{\bullet+} - F$), 161 (5, $M^{\bullet+} - H - HF$), 147 (3, $M^{\bullet+} - HF - CH_3$), 131 (23, $M^{\bullet+} - CHF_2$), 90 (8).

2-(Dichloromethyl)benzimidazole was prepared from dichloroacetyl chloride (3 mmol) and OPD (2 mmol) in dry acetonitrile, according to the method described by Sawicki (*23*). Because the major product in this reaction mixture was bis-dichloroacetylated OPD, no ^{1}H-NMR spectrum of pure 2-(dichloromethyl)benzimidazole could be obtained. GC-MS analysis, however, showed formation of a product (amounting to 10% of the reaction products) with a mass spectrum consistent with that of 2-(dichloromethyl)benzimidazole. GC-MS analysis: retention time 9.06 min; electron-impact mass spectrum: *m/z* (rel. intensity %, multiplicity, assignment) 200 (18, 2Cl, $M^{\bullet+}$), 165 (88, 1Cl, $M^{\bullet+} - Cl$), 135 (19), 129 (20), 105 (100), 77 (40).

Methylation of reaction mixture by ethereal diazomethane converted this product in a compound with a mass spectrum consistent with that of *N*-methyl-2-(dichloromethyl)benzimidazole. GC-MS analysis: retention time 9.15 min; electron-impact mass spectrum: *m/z* (rel. intensity %, multiplicity, assignment) 214 (19, 2Cl, $M^{\bullet+}$), 179 (100, 1Cl, $M^{\bullet+} - Cl$), 143 (10), 129 (7), 102 (8).

Incubation of Cysteine Conjugate in the Presence of *β*-Lyase Mimetic Model. Cysteine S-conjugates (15 mM) were incubated at 37 °C in 50 mM sodium borate buffer (pH 8.6) in the presence of pyridoxal (0.25 mM) and metal ion (100 μM). In reaction mixtures containing the trapping agent OPD (4 mM), pyridoxal was increased to 2 mM. To identify fluorine-containing reaction products, cysteine S-conjugates were incubated at an initial concentration of 15 mM; after 16 h, trifluoroacetic acid (TFA) was added as internal standard and the incubation mixtures were analyzed with ^{19}F-NMR.

For GC-MS analysis of products, incubations were acidified to pH 2 and subsequently extracted by ethyl acetate. Ethyl acetate fractions were injected on GC-MS directly and after methylation by ethereal diazomethane.

Incubation of Cysteine S-Conjugates in the Presence of Rat Renal Cytosol. Rat renal cytosol was prepared from kidneys of male Wistar rats (180−200 g) as described previously (*12*). Cysteine S-conjugates (4 mM) were incubated in 50 mM sodium borate buffer (pH 8.6) at 37 °C in the presence of 4 mg/mL cytosolic protein and 0.5 mM α-keto-γ-methiolbutyric acid. After 30 min of incubation, reaction mixtures were acidified to pH 2 and extracted two times with ethyl acetate. Combined ethyl acetate fractions were treated with ethereal diazomethane, concentrated, and analyzed by GC-MS.

Instrumental Conditions. All GC-MS analyses were carried out on a Hewlett Packard 5890/MSD system. A CP Sil-SE 30 capillary coloumn (25 m, 0.22 mm i.d.) obtained from Chrompack Ned. BV. was used. The operation temperatures were 280 °C (split injector) and 280 °C (ion source). Electron-impact ionization (electron energy of 70 eV) was used. The carrier gas was helium, at a flow rate of about 3 mL/min, and the column head pressure was 80 kPa. The column temperature was programmed from 60 °C (2.5 min) to 280 °C at 20 °C/min.

^{19}F-NMR spectra of incubations were measured directly in 5 mm NMR tubes using a Bruker MSL 400 system operating at 376.43 Hz. Chemical shifts are with reference to trifluoroacetic acid as internal standard.

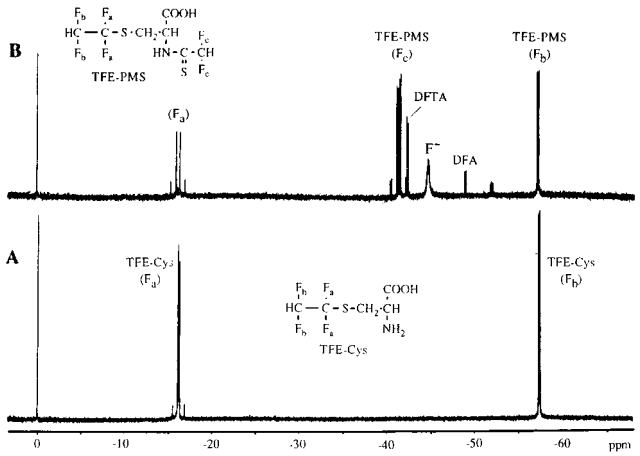

Figure 3. ^{19}F-NMR spectra of 16 h incubations of *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFE-Cys) (15 mM) in a *β*-lyase-mimetic model consisting of pyridoxal (0.25 mM) and Cu(II) (100 μM) in 50 mM potassium borate buffer, pH 8.6. (A) In the absence of Cu(II). (B) In the presence of Cu(II). TFE-PMS, *N*-(difluorothionoacetyl)-*S*-(1,1,2,2,-tetrafluoroethyl)-L-cysteine; DFA, difluoroacetic acid; DFTA, difluorothio(no)acetic acid.

Calculation of Free Enthalpies of Formation of Reactive Intermediates. Free energies of formation of the thiol compounds and possible rearrangement products were calculated by *ab initio* energy calculations using the quantum chemical program package GAMESS (*27*) on the Cyber 205 of the Academic Computer Centre of Amsterdam (SARA). The geometries of the parent compound and reaction products were fully optimized, implying variation of bond distances, bond angles, and torsion angles, using the STO-3G minimal basis set (*28*). After geometry optimization, a SV 6-31G SCF-LCAO-MO energy calculation was performed; d-orbitals were included in calculating sulfur-containing compounds (*29*).

Free enthalpies of hydration of halide ions were obtained from Friedman and Krishnan (*30*).

Results

Incubations of *S*-(2,2-Dihalo-1,1-difluoroethyl)-L-cysteine Conjugates. When the fluoro-containing *S*-(2,2-dihalo-1,1-difluoroethyl)-L-cysteine conjugates (15 mM) were incubated for 16 h in the presence of 0.5 mM pyridoxal and 100 μM Cu(II), the reaction mixtures with CTFE-Cys and DCDFE-Cys developed a dark brown polymeric material. The reaction mixture of TFE-Cys, however, remained a clear pale yellow solution. After 16 h, the parent cysteine S-conjugates were not detected by ^{19}F-NMR anymore, indicating their complete degradation in the *β*-lyase model system (Figures 3B, 4B, and 5B). In the presence of 4 mM OPD, however, significant amounts of cysteine S-conjugates were still present, and might be due to the binding of OPD to pyridoxal. In the absence of Cu(II), the unchanged cysteine S-conjugates were still observed after 16 h (Figures 3A, 4A, and 5A) and there was no evidence for the formation of any other fluorinated products.

The results of ^{19}F-NMR and GC-MS analysis of the reaction products of the *S*-(2,2-dihalo-1,1-difluoroethyl)-L-cysteine conjugates are discussed separately.

(1) TFE-Cys. Upon ^{19}F-NMR analysis of incubations of TFE-Cys with the *β*-lyase-mimetic system, several fluorine-containing products were observed (Figure 3B). These products are similar to those described previously for *β*-cleavage of TFE-Cys by rat renal cytosol (*11*). In addition to fluoride anion, difluoroacetic acid (DFA), and difluorothio(no)acetic acids (DFTA), formation of the self-

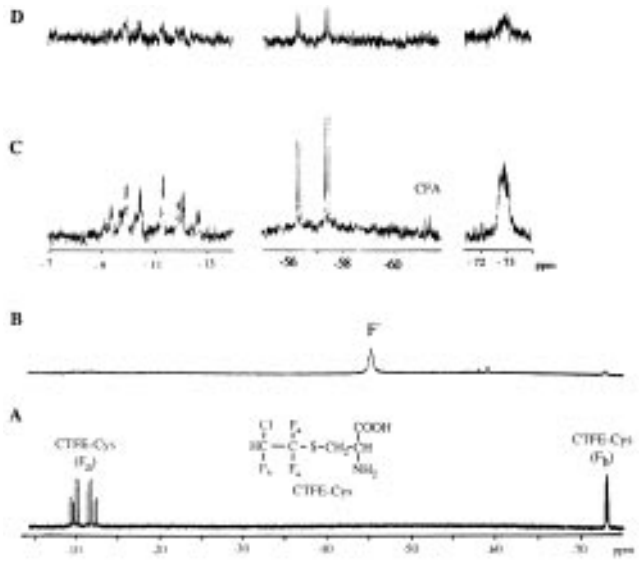

Figure 4. ^{19}F-NMR spectra of 16 h incubations of S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine (CTFE-Cys, 15 mM) in a β-lyase-mimetic model consisting of pyridoxal (0.25 mM) and Cu(II) (100 μM) in 50 mM potassium borate buffer, pH 8.6. (A) Incubation in the absence of Cu(II); (B) incubation in the presence of Cu(II); (C) details of the ^{19}F-NMR spectrum (B); (D) details of the ^{19}F-NMR spectrum of the incubation of 4 mM CTFE-Cys with rat renal cytosol (5 mg/mL). CFA, chlorofluoroacetic acid.

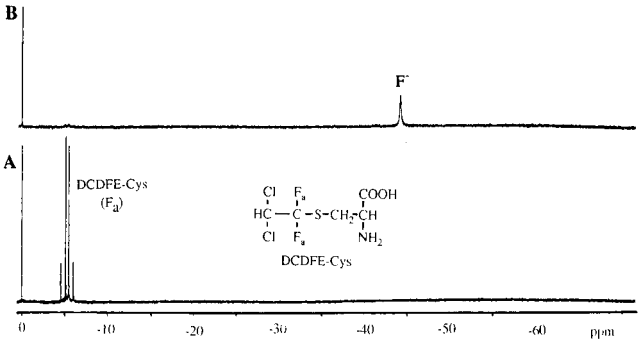

Figure 5. ^{19}F-NMR spectra of 16 h incubations of *S*-(2,2-dichloro-1,1-difluoroethyl)-L-cysteine (DCDFE-Cys, 15 mM) in a β-lyase-mimetic model consisting of pyridoxal (0.25 mM) and Cu(II) (100 μM) in 50 mM potassium borate buffer, pH 8.6. (A) Incubation in the absence of Cu(II). (B) Incubation in presence of Cu(II).

adduct *N*-(difluorothionoacetyl)-*S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFE-PMS; also called pseudomercapturic acid (*11*)) was observed. Based on the ratio of integrals at the end of the incubation, the concentration of the fluoride anion was estimated to be approximately 2.4−2.8 times higher than the sum of the difluoro(thiono)-acetyl-containing compounds. GC-MS analysis of methylated extracts showed formation of TFE-PMS (retention time 7.82 min) as a major product and, in addition, a small amount of *N*-(difluoroacetyl)-*S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFE-PMO (*11*)) (retention time 6.7 min).

When nonenzymic incubations were carried out in the presence of 4 mM OPD after 16 h of incubations, a new fluorine-containing product with δ −41.92 ppm (doublet, J = 51.0 Hz) was detected upon analysis by ^{19}F-NMR (Figure 6, top panel). The chemical shift and coupling constant were identical to those of synthetical 2-(difluoromethyl)benzimidazole. The identity of this product was further supported by GC-MS analysis of an ethyl acetate extract revealing formation of a product with a retention time (6.92 min) and electron-impact mass spectrum

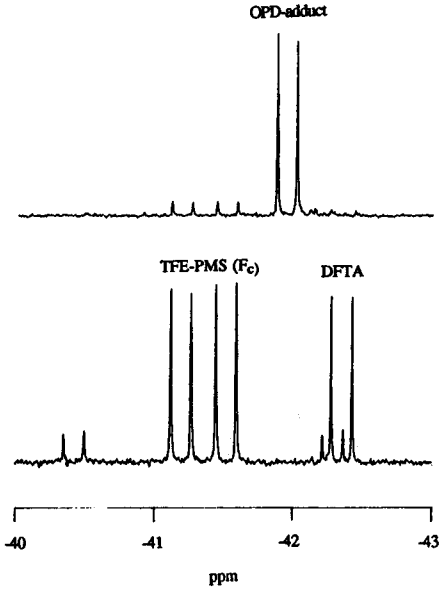

Figure 6. Detail of ^{19}F-NMR spectra of 16 h incubations of *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (15 mM) in the β-lyase-mimetic model in the presence (top) or absence (bottom) of 4 mM *o*-phenylenediamine (OPD). The β-lyase-mimetic model consisted of 2 mM pyridoxal and 0.1 mM Cu(II) in 50 mM potassium borate buffer, pH 8.6.

(Figure 7A) identical to that of 2-(difluoromethyl)benzimidazole. Methylation by diazomethane yielded a peak at 6.57 min with a mass spectrum identical to that of synthetical *N*-methyl-2-(difluoromethyl)benzimidazole (Figure 7D).

GC-MS analysis of cytosolic incubations with TFE-Cys showed formation of the same products as in the β-lyase model system. However, in addition, dimethylated *S*-(1,1,2,2-tetrafluoroethyl)-3-mercapto-2-oxopropionic acid (TFE-MOP) was also observed,[2] pointing to transamination or oxidative deamination of TFE-Cys in cytosol. Addition of (aminooxy)acetic acid (1 mM) to the cytosolic incubation blocked β-elimination reaction; however, formation of TFE-MOP was increased 4-fold, suggesting that amino acid oxidases are the major source of this deamination product in rat renal cytosol (data not shown).

(2) CTFE-Cys. At the end of incubation of CTFE-Cys with the β-lyase-mimetic system, fluoride anion was the major product in the ^{19}F-NMR spectrum (Figure 4B). In addition, the spectrum revealed relatively small amounts of chlorofluoroacetic acid (doublet at δ −61.31 ppm ($^2J_{FH}$ = 52.2 Hz)) and doublets at δ −56.38 ppm (J = 50.3 Hz) and −57.48 ppm (J = 50.8 Hz) (Figure 4C). Also observed were compound(s) with chemical shifts similar to but significantly different from CTFE-Cys at δ −9.25 to −12.75 ppm and at δ −72.9 ppm. When CTFE-Cys was incubated at a concentration of 4 mM with rat renal cytosol (5 mg/mL) for 16 h, similar products were observed (Figure 4D).

GC-MS analysis of methylated extracts revealed the formation of two products with slightly different retention

[2] For identification, references of 3-mercapto-2-oxopropionic acid S-conjugates were prepared by incubating cysteine S-conjugates (4 mM) for 60 min in the presence of 1 mg/mL amino acid oxidase (Sigma, St. Louis, MO) and 40 U/mL catalase (Boehringer Mannheim GmbH, Mannhein, Germany). Incubations were performed in 50 mM potassium phoshate (pH 7.4) at 37 °C. Due to keto−enol tautomerisation of the 3-mercapto-2-oxopropionic acid S-conjugate, methylation by diazomethane also occurs at the enol oxygen. Because the double bond formed can be cis- and trans-substituted, two peaks are observed in GC-MS-analysis.

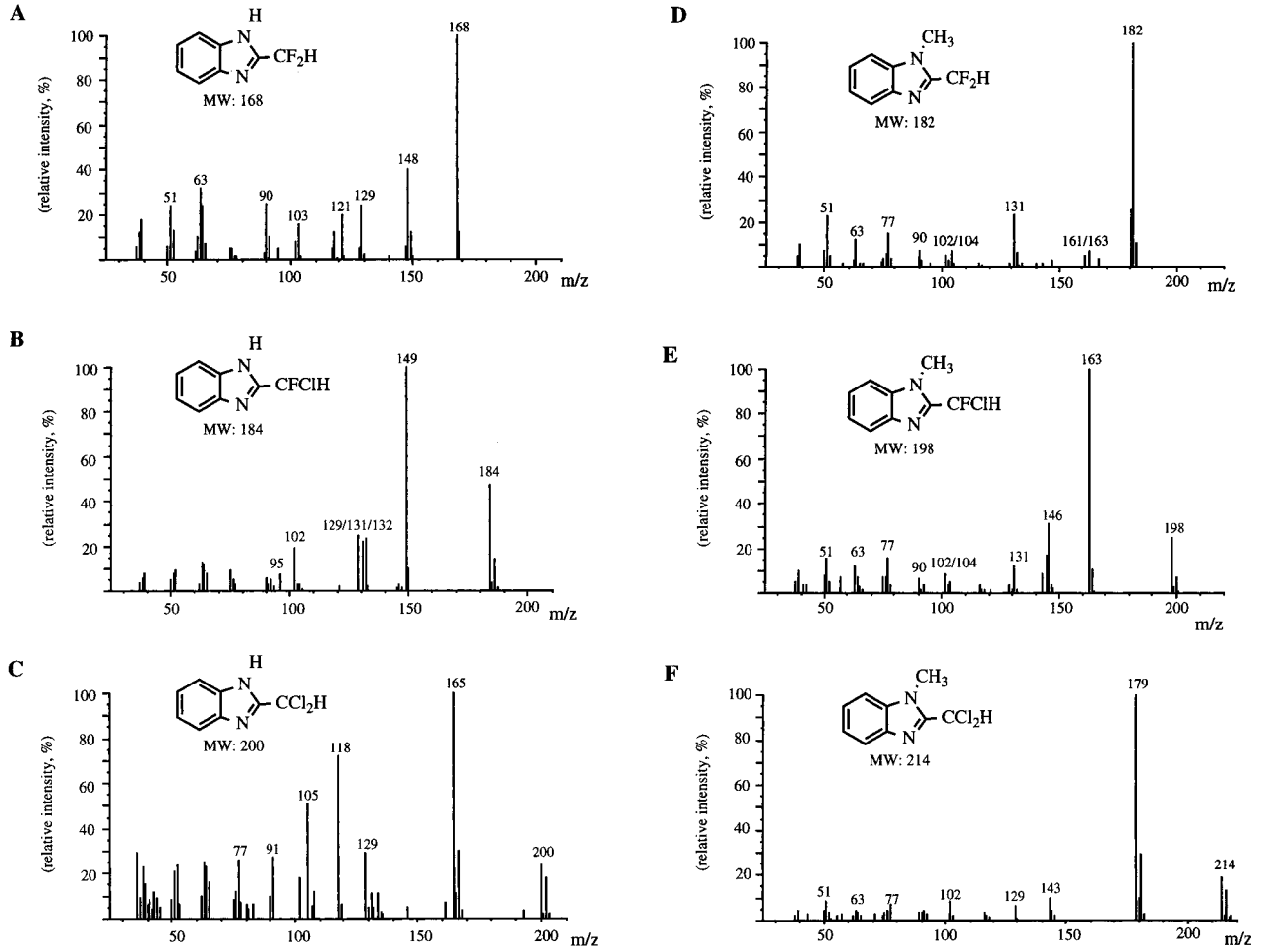

Figure 7. Electron-impact mass spectra of the 2-(dihalomethyl)benzimidazoles formed in incubations of *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFE-Cys, 20 mM) (A), *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine (CTFE-Cys, 20 mM) (B), or S-(2,2-dichloro-1,1-difluoroethyl)-L-cysteine (DCDFE-Cys, 20 mM) (C) in a β-lyase-mimetic model consisting of pyridoxal (2 mM), Cu(II) (100 μM), and OPD (4 mM) in 50 mM potassium borate buffer, pH 8.6. Treatment of the 2-(dihalomethyl)benzimidazoles with ethereal diazomethane converted these compounds into the corresponding *N*-methyl-2-(dihalomethyl)benzimidazoles (D, E, and F).

times, 9.85 vs 9.91 min, but with almost identitical electron-impact mass spectra (Figure 8A). These products were attributed to the pseudomercapturic acid resulting from N-chlorofluorothionoacetylation of CTFE-Cys; the fragmentation pattern was analogous to that of dimethylated TFE-PMS (*11*), except for substitution of fluorine by chlorine atoms in several fragments. Due to the introduction of a racemic *N*-(chlorofluorothionoacetyl) group into L-cysteine, two diastereomeric products were detected by capillary GC. The relative intensity of these products on GC-MS approximately correlated with the relative intensity of the doublets at δ −56.38 ppm and at δ −57.48 ppm observed by ^{19}F-NMR, suggesting that these two doublets represent the *N*-(chlorofluorothionoacetyl) groups of the two diastereomeric pseudomercapturic acids. Based on the ratio of integrals of the ^{19}F-NMR spectrum, at the end of incubation, the concentration of the fluoride anion was more than 10 times higher than the total of chlorofluorothionoacetyl-containing products.

^{19}F-NMR analysis of the reaction mixture of CTFE-Cys in presence of OPD did not result in any additional fluorine-containing products (data not shown) in comparison to that shown in Figure 4C. However, using GC-MS analysis of ethyl acetate extracts showed peaks at 8.01 and 8.25 min as well as a broad peak at 8.70 min, which were not present in incubations without OPD. The EI-mass spectrum of the peak at 8.25 min had a retention time and mass spectrum identical to that of synthetical 2-mercaptoquinoxaline (Figure 9A). Methylation of this extract showed formation of 2-methylthioquinoxaline (Figure 9B), which has a retention time of 8.49 min. When incubations were performed in presence of [*ethyl-2*-D]-*S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine, the molecular ion of 2-methylthioquinoxaline increased one mass unit, proving incorporation of a two-carbon fragment derived from the 2-chloro-1,1,2-trifluoroethyl moiety of CTFE-Cys.

The retention time and mass spectrum of the minor peak at 8.70 min in the nonmethylated extract were identical to those of 2-hydroxyquinoxaline. Methylation of this product resulted in a retention time shift to 8.50 min as was observed with the reference compound. Although 2-methoxyquinoxaline and 2-methylthioquinoxaline show almost identical retention times, these two products can easily be distinguished by selected ion chromatography at *m/z* 160, which is absent in 2-methylthioquinoxaline, and at *m/z* 176, which is absent in 2-methoxyquinoxaline.

The EI-mass spectrum of the peak at 8.01 min revealed a molecular ion of *m/z* 184, having a chlorine isotope pattern (Figure 7B). This mass spectrum is consistent with that of 2-(chlorofluoromethyl)benzimidazole. Although the identity of this adduct was not proved unequivocally by synthesis of the reference compound, its fragmentation pattern and retention time are consis-

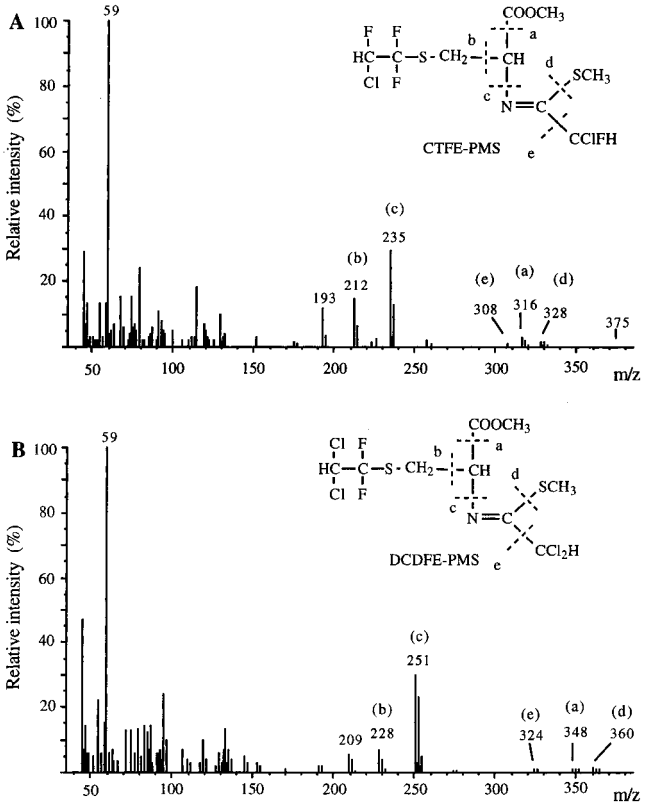

Figure 8. Electron-impact mass spectra of dimethylated pseudomercapturic acids (PMS) formed in incubations of *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine (CTFE-Cys) (A) and *S*-(2,2-dichloro-1,1-difluoroethyl)-L-cysteine (DCDFE-Cys) (B) in a β-lyase-mimetic model consisting of pyridoxal (0.25 mM) and Cu(II) (100 μM) in 50 mM potassium borate buffer, pH 8.6. Fragmentations, as indicated by the dotted lines, are consistent with those obtained from the dimethylated pseudomercapturic acid formed from *S*-(tetrafluoroethyl)-L-cysteine (11).

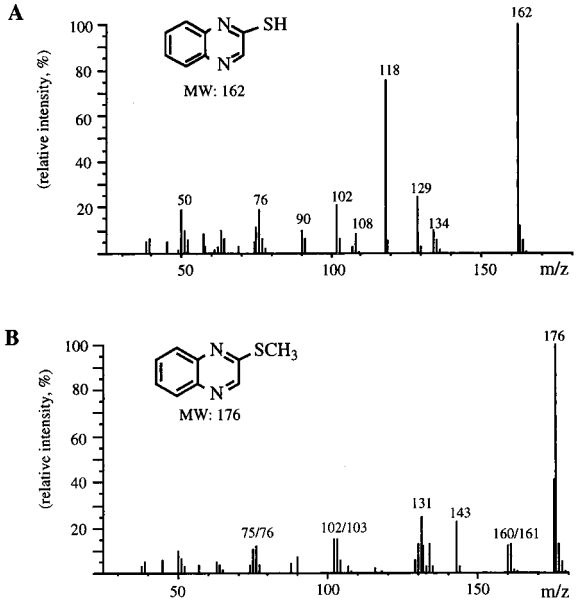

Figure 9. Electron-impact mass spectra of 2-mercaptoquinoxaline (A) formed in incubations of *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine (CTFE-Cys, 20 mM) or *S*-(2,2-dichloro-1,1-difluoroethyl)-L-cysteine (DCDFE-Cys, 20 mM) in a β-lyase-mimetic model consisting of pyridoxal (2 mM), Cu(II) (100 μM), and OPD (4 mM) in 50 mM potassium borate buffer, pH 8.6. Treatment of 2-mercaptoquinoxaline with ethereal diazomethane converted this compound into 2-methylthioquinoxaline (B).

tent with those of 2-(difluoromethyl)benzimidazole and 2-(dichloromethyl)benzimidazole (Figure 7A–C). Methylation of the presumed 2-(chlorofluoromethyl)benzimidazole yielded a new compound with retention time 7.91 min and a mass spectrum consistent with that of *N*-methyl-2-(chlorofluoromethyl)benzimidazole (Figure 7E).

As in the case of TFE-Cys, in incubations of CTFE-Cys with rat renal cytosol the same products could be identified as in the β-lyase model system. However, the yield of products derived from chlorofluorothionoacyl fluoride appeared to be relatively higher in the cytosolic incubations. This may be due to decomposition of the chlorofluorothionoacyl moieties during overnight incubation with the β-lyase-mimetic system (*16*).

In cytosolic incubations of CTFE-Cys, two peaks attributed to cis- and trans-isomers of dimethylated *S*-(2-chloro-1,1,2-trifluoroethyl)-3-mercapto-2-oxopropionic acid (CTFE-MOP) were also observed by GC-MS-analysis,[2] at 6.66 and 6.98 min, pointing to a transamination or oxidative deamination reaction in cytosol.

(3) DCDFE-Cys. As in incubations with CTFE-Cys, fluoride anion was the major product observed in incubations of the β-lyase model with DCDFE-Cys (Figure 5B). The very weak signal observed at approximately −5 ppm also points to a DCDFE-Cys-derived product. Analysis of methylated ethyl acetate extracts of acidified reaction mixtures by GC-MS also pointed to the formation of small amounts of the pseudomercapturic acid, resulting from N-dichlorothionoacetylation of DCDFE-Cys (Figure 8B), with a retention time of 11.66 min.

When DCDFE-Cys incubations were carried out in presence of OPD, different OPD-dependent peaks were observed upon GC-MS analysis of ethyl acetate extracts. GC-MS analysis of ethyl acetate extracts of reaction mixtures revealed the presence of 2-mercaptoquinoxaline (retention time 8.25 min; mass spectrum, Figure 9A) as well as 2-(dichloromethyl)benzimidazole (retention time 9.09 min; mass spectrum, Figure 7C). Treatment of the ethyl acetate extracts by ethereal diazomethane converted these products to 2-(methylthio)quinoxaline (retention time 8.48 min; mass spectrum, Figure 9B) and *N*-methyl-2-(dichloromethyl)benzimidazole (retention time 9.15 min; mass spectrum, Figure 7F). The presence of *m*/*z* 160 in the mass spectrum at 8.48 min also points to the formation of hydroxyquinoxaline in these incubations.

In cytosolic incubations of DCDFE-Cys, two peaks attributed to cis- and trans-isomers of dimethylated *S*-(2,2-dichloro-1,1-difluoroethyl)-3-mercapto-2-oxopropionic acid (DCDFE-MOP) were also observed by GC-MS-analysis,[2] at 7.89 and 8.15 min, again pointing to a transamination or oxidative deamination reaction in cytosol.

Comparison of Products Formed from *S*-(2,2-Dihalo-1,1-difluoroethyl)-L-cysteine Conjugates and *S*-(Trihalovinyl)-L-cysteine Conjugates. It has been reported that adducts of chlorofluorothionoacyl fluoride are labile at basic pH (*16*), so that part of the products after overnight incubations with a β-lyase model already may have been degraded; therefore, it was decided to compare the reaction products of *S*-(trihalovinyl)cysteine S-conjugates and *S*-(2,2-dihalo-1,1-difluoroethyl)-L-cysteine conjugates using enzymic incubations of only 30 min. To avoid decomposition of initially formed products as much as possible, these incubations were extracted immediately and, after methylation, analyzed by GC-MS.

(1) *S*-(2-Chloro-1,2-difluorovinyl)-L-cysteine (CDFV-Cys). GC-MS analysis of extracts of incubations of CDFV-Cys, in the presence of rat renal cytosol and OPD, revealed the formation of similar OPD adducts as ob-

Table 1. Peak Areas of Ion Chromatograms for Quinoxaline and Benzimidazole Compounds

substrate	OPD adduct analyzed	t_{ret}	m/z	ratio[a]
CTFE-Cys	2-methylthioquinoxaline	8.49	176	56.8
	N-methyl-2-(chlorofluoromethyl)benzimidazole	7.90	198/200[b]	
1CDFV-Cys	2-methylthioquinoxaline	8.50	176	4.6
	N-methyl-2-(chlorofluoromethyl)benzimidazole	7.90	198/200[b]	
DCDFE-Cys	2-methylthioquinoxaline	8.49	176	101.1
	N-methyl-2-(dichloromethyl)benzimidazole	9.15	214/216[c]	
TCV-Cys	2-methylthioquinoxaline	8.49	176	4.7
	N-methyl-2-(dichloromethyl)benzimidazole	9.15	214/216[c]	

[a] Ratio of peak area of 2-methylthioquinoxaline relative to that of benzimidazole-products; peak areas were obtained by integration of the ion chromatograms acquired by selected ion monitoring at the indicated m/z values. [b] The ratio of the integrals of m/z 198 to 200 was 3 to 1 due to the chloro isotope pattern. [c] The ratio of the integrals of m/z 198 to 200 was 1.5 to 1 due to the dichloro isotope pattern.

served in incubations of CTFE-Cys. In methylated extracts a peak was observed at 7.90 min with a retention time and mass spectrum identical to the *N*-methyl-2-(chlorofluoromethyl)benzimidazole formed in the incubation of CTFE-Cys. This indeed indicates the formation of the anticipated chlorofluorothionoacylating species as shown in Figure 1B (X_1, X_2, X_3 = Cl, F, F). At 8.50 min, 2-methylthioquinoxaline and minor amounts of 2-methoxyquinoxaline could also be identified. The relative amounts of 2-methylthioquinoxaline and *N*-methyl-2-(chlorofluoromethyl)benzimidazole were determined by integrating the corresponding peaks in ion chromatograms at m/z 176 and 198, respectively. From Table 1 it is clear that the ratio of 2-methylthioquinoxaline to *N*-methyl-2-(chlorofluoromethyl)benzimidazole was 12.3-fold lower when compared to incubations with CTFE-Cys as substrate.

In cytosolic incubations of CDFV-Cys, a cluster of peaks with mass spectra consistent with dimethylated *S*-(2,2-dichloro-1,1-difluoroethyl)-3-mercapto-2-oxopropionic acid (DCDFE-MOP) were also observed by GC-MS analysis, between 6.68 and 6.80 min, pointing to transamination or oxidative deamination of CDFV-Cys in cytosol. The cluster can be explained by the fact that both CDFV-Cys isomers form cis- and trans-isomers of dimethylated MOP S-conjugates.

(2) *S*-(Trichlorovinyl)-L-cysteine (TCV-Cys). GC-MS analysis of extracts of incubations of TCV-Cys, in the presence of rat renal cytosol and OPD, revealed the formation of similar OPD adducts as observed in incubations of DCDFE-Cys. In methylated extracts a peak was observed at 9.15 min with retention time and mass spectrum identical to that of synthetical *N*-methyl-2-(dichloromethyl)benzimidazole. This product is indicative for the formation of the anticipated dichlorothionoacylating species (Figure 1B: X_1, X_2, X_3 = Cl, Cl, Cl). Formation of dichlorothionoacylating species from TCV-Cys has also been demonstrated previously (*17*). As in the case of CDFV-Cys, also in these incubations 2-methylthioquinoxaline and minor amounts of 2-methoxyquinoxaline could be identified (Table 1). The relative amounts of 2-methylthioquinoxaline and *N*-methyl-2-(dichloromethyl)benzimidazole were determined by integrating the corresponding peaks in ion chromatograms at m/z 176 and 214, respectively. In incubations with TCV-Cys as substrate, the ratio of 2-methylthioquinoxaline to *N*-methyl-2-(dichloromethyl)benzimidazole was 21.5-fold lower when compared to that in incubations with DCDFE-Cys as substrate (Table 1).

In cytosolic incubations of TCV-Cys, two peaks with mass spectra consistent with cis- and trans-isomers of dimethylated *S*-(2,2-dichloro-1,1-difluoroethyl)-3-mercapto-2-oxopropionic acid (DCDFE-MOP) were observed by GC-MS analysis, at 9.15 and 9.25 min,[2] pointing to transamination or oxidative deamination of TCV-Cys in cytosol.

Free Enthalpies of Rearrangement Pathways of Fluorinated Ethanethiol Compounds. Free enthalpies of formation ($\Delta_f G$) of presumed reactive intermediates derived from cysteine S-conjugates of TFE, CTFE, and DCDFE were calculated by *ab initio* calculation. The differences between the calculated $\Delta_f G$ values of possible rearrangement products, thiiranes, thionoacyl fluorides, and the corresponding halide anions, are summarized in Table 2. For TFE-thiolate, rearrangement to thionoacyl fluoride is energetically favored by 14 kcal/mol over formation of the corresponding thiirane. In contrast, for both CTFE-thiolate and DCDFE-thiolate, formation of thiirane is energetically favored by 66 and 70 kcal/mol, respectively, over formation of the thionoacyl fluorides.

Because of the relatively high free enthalpies of hydration (ΔG_{hydr}) of fluoride and chloride anions released, −104 and −76 kcal/mol, respectively (*30*), hydration may play an important role in directing the rearrangement reaction in aqueous solution, depending upon which type of halide anion is released by the different rearrangement reactions. In the case of TFE-thiolate, both rearrangements yield a fluoride anion, but formation of thionoacyl fluoride is still energetically favored over formation of thiirane by 14 kcal/mol. However, with both CTFE- and DCDFE-thiolate anions, the nature of released halide anion differs between the thiirane pathway, releasing a chloride anion, and the thionoacyl fluoride pathway, releasing a fluoride anion. Because the free enthalpy of hydration of the fluoride anion is more exothermic than that of the chloride anion, the difference between the thionoacyl fluoride pathway and the thiirane pathway is reduced by 28 kcal/mol (Table 2). However, for both CTFE- and DCDFE-thiolate anions, the thiirane pathway remains energetically favored by 38 and 42 kcal/mol, respectively, over the thionoacyl fluoride pathway. However, the contribution of differences in hydration enthalpies of the thiirane and thionoacyl fluoride products cannot be included because these are not known.

Discussion

In the present study, the nature of the reactive thiol compounds resulting from β-elimination reactions of various cysteine S-conjugates was studied. OPD was used as a trapping agent in order to discriminate between thionoacyl fluorides and thiiranes. The *o*-amino groups of OPD were anticipated to favor ring closure to stable low-molecular-weight products which can easily be detected by GC-MS. Thiiranes were anticipated to form

Table 2. Calculated Free Enthalpies of Formation of the Decomposition Products of Fluorinated Ethanethiolate Compounds

	free enthalpies of formation	
decomposition products (reactive intermediate + halide ion)	$\Sigma(\Delta_f G)$[a]	$\Sigma[(\Delta_f G) + (\Delta G_{hydr}X^-)]$[b]
TFE-thiolate:		
difluorothionoacyl fluoride + F^-	−871.327 a.u.	−871.493 a.u.
2,2,3-trifluorothiirane + F^-	−871.305 a.u.	−871.471 a.u.
	Δ −0.022 a.u. (−13.8 kcal/mol)	Δ −0.022 a.u. (−13.8 kcal/mol)
CTFE-thiolate:		
chlorofluorothionoacyl fluoride + F^-	−1231.372 a.u	−1231.538 a.u.
2,2,3-trifluorothiirane + Cl^-	−1231.477 a.u.	−1231.598 a.u.
	Δ −0.105 a.u. (+65.9 kcal/mol)	Δ +0.060 a.u. (+37.6 kcal/mol)
DCDFE-thiolate:		
difluorothionoacyl fluoride + F^-	−1591.420 a.u.	−1591.586 a.u.
3-chloro-2,2-difluorothiirane + Cl^-	−1591.532 a.u.	−1591.653 a.u.
	Δ +0.112 a.u. (+70.3 kcal/mol)	Δ +0.067 a.u. (+42.0 kcal/mol)

[a] Calculated free enthalpies of formation ($\Delta_f G$): 2,2,3-trifluorothiirane, −771.955 a.u.; 3-chloro-2,2-difluorothiirane, −1132.010 a.u.; difluorothionoacyl fluoride, −771.977 a.u.; chlorofluorothionoacyl fluoride, −1132.022; dichlorothionoacyl fluoride, −1492.070 a.u.; F^-, −99.35 a.u.; Cl^-, −459.522 a.u. [b] Free enthalpies of hydration of halide anions ($\Delta G_{hydr}X^-$): F^-, −104 kcal/mol (−0.166 a.u.); Cl^-, −76 kcal/mol (0.121 a.u.) (*28*).

quinoxaline compounds exclusively, whereas thionoacyl fluorides were anticipated to condense to benzimidazole compounds (Figure 2).

The results of the present investigation demonstrate that with all *S*-(2,2-dihalo-1,1-difluoroethyl)cysteine S-conjugates studied dihalothionoacyl fluorides are formed. This was demonstrated by the formation of self-adducts to the parent cysteine S-conjugates (Figure 6). In the case of TFE-Cys, the so-called pseudomercapturic acid (*11*) resulting from covalent binding of difluorothionoacetyl fluoride to TFE-Cys itself was the major product. Similar results have been obtained by Hayden et al. (*13*) using a nonenzymic model consisting of pyridoxal 5′-phosphate and copper(II). In incubations in the presence of OPD, a high concentration of 2-(difluoromethyl)-benzimidazole was observed, pointing to a intramolecular condensation reaction of the initially difluorothionoacylated OPD (Figure 7).

In incubations with CTFE-Cys and DCDFE-Cys, the corresponding pseudomercapturic acids were also observed, but only in relatively low amounts according to ^{19}F NMR analysis (Figures 4B and 5B). In CTFE-Cys incubations, the ratio of the integrals of the fluoride anion and the products derived from chlorofluorothionoacetyl fluoride was approximately 10 to 1. It was shown by Fisher et al. (*16*) that a lysine adduct of chlorofluorothionoacyl fluoride slowly degrades to non-fluorine-containing products and fluoride anion. At pH 7.4, 50% of the adduct was degraded after 4 days of incubation. When our model system was incubated at pH 7.4, the fluoride anion was still the major product after 16 h of incubation (data not shown). Because instability of chlorofluorothionoacyl amides apparently is not extremely high, we investigated whether alternative reactive intermediates might be formed from CTFE-Cys next to chlorofluorothionoacetyl fluoride.

As proposed previously by Dohn et al. (*9*), a possible alternative reactive intermediate derived from CTFE-Cys is 2,2,3-trifluorothiirane (Figure 1). In the present study, the preference for rearrangement of thiolate anions to either thiiranes or thionoacyl fluorides was investigated theoretically as well by quantum chemical *ab initio* calculations of free enthalpies of formation of reactants and products. Our *ab initio* calculations revealed that the TFE-thiolate anion rearrangement via the thionoacyl fluoride pathway would be energetically favored over the thiirane pathway by 14 kcal/mol (Table 2). Interestingly, in the case of CTFE- and DCDFE-thiolate anions, it was found that formation of the corresponding thiiranes would be thermodynamically favored over that of thionoacyl fluoride even when differences in the hydration energies of the halide anions released were considered (Table 2). When known free energies of hydration of halide anions were included, the thiirane route was still thermodynamically favored over the thionoacyl fluoride route for CTFE- and DCDFE-thiolate anions by 38 and 42 kcal/mol, respectively (Table 2). Because hydration enthalpies of the fluorinated thiiranes and thionoacyl fluorides are not known, their contribution to the thermodynamic control cannot be calculated. However, as these compounds are uncharged and lipophilic, these contributions are expected to be low.

Finkelstein et al. detected glyoxylic acid in incubations of bromine-containing cysteine conjugates, which was attributed to hydrolysis of α-thionolactones as reactive intermediates (*18*). In incubations of TFE-Cys, CTFE-Cys, and DCDFE-Cys, they were not able to detect glyoxylate, indicating that α-thionolactones may not be formed from non-bromine-containing cysteine conjugates. The results in the present study are supportive for their observations. Because α-thionolactones are formed via the thionoacyl fluoride pathway (Figure 1), this route is not energetically favored for CTFE-Cys and DCDFE-Cys. For TFE-Cys, the thio(no)acetic acid formed by hydrolysis of difluorothionoacyl fluoride appears to be relatively stable, because it is detected by ^{19}F-NMR (Figure 3).

In order to find experimental proof for the formation of thiirane products, incubations were performed in the presence of *o*-phenylenediamine (OPD). In incubations with CTFE-Cys the formation of 2-(chlorofluoromethyl)-benzimidazole was indicative for formation of chlorofluorothionoacetyl fluoride. However, in addition, the major OPD adduct in CTFE-Cys incubations was identified as 2-mercaptoquinoxaline. 2-Mercaptoquinoxaline can be explained by a cyclization reaction of the initial chlorofluorothionoacyl adduct of OPD, followed by dehydroha-

logenation reaction (Figure 2) or by a reaction of OPD with 2,2,3-trifluorothiirane (Figure 2, route *a*). In order to distinguish between these two possibilities, additional incubations were performed with *S*-(2-chloro-1,2-difluorovinyl)-L-cysteine (CDFV-Cys). It was anticipated that CDFV-Cys can form a chlorofluorothionoacylating species (chlorofluorothioketene and/or chlorofluorothionoacyl fluoride, Figure 1B) but that it cannot form a thiirane compound. As expected, 2-(chlorofluoromethyl)benzimidazole was indeed identified in incubations with CDFV-Cys. The presence of 2-mercaptoquinoxaline in incubations of CDFV-Cys demonstrates that the initial chlorofluorothionoacyl adduct of OPD indeed reacts intramolecularly, forming a 6-membered ring. This indeed supports the observation of Fisher et al. (*16*) that chlorofluorothionoacetyl moieties are reactive centers prone to hydrolysis or alkylation. Next to 2-mercaptoquinoxaline also small amounts of 2-hydroxyquinoxaline could be identified. This product may result from hydrolysis of 2-mercaptoquinoxaline or 2-fluoroquinoxaline (Figure 2) or can be formed by reacting to glyoxylic acid which can be anticipated as a hydrolysis product formed from both chlorofluoroacetic acid and 2,2,3-trifluorothiirane. Dichloroacetic acid and difluoroacetic acid did not react with OPD under the incubation conditions used, according to GC-MS and ^{19}F-NMR analysis (data not shown), ruling out the contribution of dihaloacetic acids to product formation. Dihalothioacetic acids are also not likely to contribute to formation of 2-mercaptoquinoxaline because reaction of thioacetic acids with amino groups is known to result in release of the sulfur atom as hydrogen sulfide (*31*).

As mentioned above for both CTFE-Cys and CDFV-Cys, formation of 2-(chlorofluoromethyl)benzimidazole and 2-mercaptoquinoxaline was observed. If a chlorofluorothionoacyl adduct of OPD would be the only intermediate leading to these products, a fixed ratio of 2-(chlorofluoromethyl)benzimidazole and 2-mercaptoquinoxaline would be expected in incubations with CTFE-Cys and CDFV-Cys. The fact that for CTFE-Cys the relative amount of 2-mercaptoquinoxaline was 12-fold higher than in the case of CDFV-Cys strongly suggests a second route leading to this product. Based on our quantum chemical calculations, and the fact that formation of 2-mercaptoquinoxaline via thiirane formation can be rationalized (Figure 2), we suggest formation of 2,3,3-trifluorothiirane as a second reactive intermediate for CTFE-Cys.

Comparable results were obtained when comparing products derived from DCDFE-Cys and TCV-Cys. Both compounds are anticipated to form dichlorothionoacylating reactive species (Figure 2: X_1, X_2 = Cl, Cl). Indeed, both 2-mercaptoquinoxaline and 2-(dichloromethyl)benzimidazole were identified as adducts to OPD. In the case of DCDFE-Cys, however, the relative amount of 2-mercaptoquinoxaline was 21-fold higher than in the case of TCV-Cys, which only can form dichlorothionoacylating agents. For the same reasons as discussed for CTFE-Cys, this is indicative for formation of 3-chloro-2,2-difluorothiirane as a second reactive intermediate derived from DCDFE-Cys.

As yet, only limited data are available about the chemical properties of fluorine-containing thiiranes, and their reactivity has only been studied in organic solvents. Tetrafluorothiirane, a relatively stable compound, reacts rapidly with nitrogen compounds and is attacked at its carbon atoms by secondary amines, such as morpholine, ultimately resulting in a diconjugated product which rapidly eliminates all its fluorine atoms on exposure to water (*32*). Thus all the fluorine atoms are lost as fluoride anion, but the sulfur atom is retained. The formation of 2-mercaptoquinoxaline in the reaction of OPD with the presumed 2,2,3-trifluorothiirane can be regarded as an analogous reaction. 2,2-Difluorothiirane is unstable and rapidly extrudes its sulfur atom at 0 °C (*33*). Extrusion of sulfur from several thiiranes is catalyzed effectively by thiolate anions attacking the thiirane sulfur atom. In dimethyl sulfoxide, this process resulted in formation of polysulfides ($n = 2-8$) and 1,1-difluoroethylene; electron-withdrawing substituents on the thiirane ring promoted this reaction strongly (*34*). However, the fact that in our experiments almost all fluorine atoms of CTFE-Cys and DCDFE-Cys were recovered as the fluoride anion indicates that formation of fluorinated ethylenes can only be a minor route. Very recently, glyoxylate and small amounts of trihaloalkenes have been identified as products of bromine-containing cysteine S-conjugates, indicative for thiirane formation (*19*). However, these products could not be identified in incubations with DCDFE-Cys, CTFE-Cys, and TFE-Cys.

In conclusion, the present study demonstrated that dihalothionoacylating species are formed in the case of *S*-(2,2-dihalo-1,1-difluoroethyl)-L-cysteine compounds (TFE-Cys, CTFE-Cys, DCDFE-Cys) and *S*-(trihalovinyl)-L-cysteine compounds (TCV-Cys and CDFV-Cys). For CTFE-Cys and DCDFE-Cys, thiiranes are proposed to be important reactive intermediates next to the dihalothionoacyl fluorides. *Ab initio* calculations showed that rearrangement to thiiranes is thermodynamically favored over formation of thionoacyl fluorides for the thiolate anions formed from CTFE-Cys and DCDFE-Cys. Our results show that, after an initial alkylation reaction step, the products formed from both dihalothionoacylating species and thiiranes still contain an electrophilic center capable of a second alkylation reaction. These electrophiles therefore might lead to intra- and intermolecular cross-links of biomacromolecules.

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