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MUTAGENICITY OF BENZYL *S*-HALOALKYL AND *S*-HALOALKENYL SULFIDES IN THE AMES-TEST

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Abstract—The mutagenicity of benzyl 1,2,3,4,4-pentachlorobutadienyl sulfide (BPBS) and benzyl 1,2-dichlorovinyl sulfide (BDVS) was studied in the Ames preincubation assay to investigate the hypothesis that the mutagenic effect of the cysteine *S*-conjugates *S*-(pentachlorobutadienyl)-L-cysteine and *S*-(1,2-dichlorovinyl)-L-cysteine is associated with their metabolism to unstable thiols. Under conditions enabling cytochrome P-450-dependent benzylic hydroxylation of BPBS and BDVS, both benzyl sulfides were mutagenic. These results in combination with the lack of mutagenicity observed with benzaldehyde and with the *tert*-butyl analogues, which cannot be metabolized to a hemimercaptal, indicate that the formation of unstable thiols is responsible for the mutagenic effects of the benzyl sulfides and the corresponding cysteine *S*-conjugates. Benzyl 2-chloro-1,1,2-trifluoroethyl sulfide, which also undergoes benzylic hydroxylation, was negative in the Ames-Test; this is in agreement with the observed lack of mutagenicity of the corresponding *S*-conjugate *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine. Also, benzyl 2-chloroethyl sulfide, which, along with the corresponding *S*-conjugate *S*-(2-chloroethyl)-L-cysteine, does not require bioactivation, was a potent, direct-acting mutagen in the Ames-Test.

Cysteine *S*-conjugates are intermediary metabolites of nephrotoxic haloalkenes. These haloalkenes are conjugated with glutathione (GSH) in the liver; the GSH *S*-conjugates formed are processed to the corresponding cysteine *S*-conjugates and undergo cysteine conjugate β-lyase-dependent bioactivation in the kidney [1–3].

The cysteine *S*-conjugates *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC), *S*-(pentachlorobutadienyl)-L-cysteine (PCBC), and *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine (CTFC) are nephrotoxic in rats and are cytotoxic to renal tubular epithelial cells [4–6]; DCVC and PCBC are also potent mutagens in bacteria [7, 8]. β-Lyase-dependent metabolism of these *S*-conjugates yields pyruvate, ammonia, and unstable thiols. There is considerable evidence that the toxicity and mutagenicity of cysteine *S*-conjugates is associated with the formation of unstable thiols. Both the mutagenicity and cytotoxicity of *S*-conjugates are blocked by inhibitors of β-lyase [4–7); also, *S*-conjugates yielding stable thiols are not mutagenic [9]. Direct evidence for the transformation of unstable thiols to acylating agents has been obtained [10, 11].

The present study was designed to provide an alternative test of the hypothesis that the mutagenicity of cysteine *S*-conjugates is associated with the formation of unstable thiols. Hence, we prepared non-amino acid-based compouds that may undergo metabolism by a different enzymatic reaction to the same unstable thiols as the corresponding cysteine *S*-conjugates. Benzyl 1,2,3,4,4-pentachlorobuta-1,3-dienyl sulfide (BPBS) and benzyl 2-chloro-1,2,2-trifluoroethyl sulfide (BCFS), analogues of PCBC and CTFC, are metabolized by cytochrome $P\text{-}450_{PB\text{-}B}$ to the corresponding hemiacetals, which eliminate unstable thiols and benzaldehyde [12]. On the experiments reported herein, we studied the mutagenicity of several benzyl sulfides and *tert*-butyl sulfides under conditions favoring oxidative biotransformation. *tert*-Butyl sulfides cannot be metabolized by benzylic oxidation [12]. As positive control, we synthesized benzyl 2-chloroethyl sulfide as an analogue of the direct-acting sulfur mustard *S*-(2-chloroethyl)-L-cysteine.

MATERIALS AND METHODS

Syntheses. Benzyl 2-chloro-1,1,2-trifluoroethyl sulfide (BCFS), benzyl 1,2,3,4,4-pentachlorobutadienyl sulfide (BPBS), *tert*-butyl 2-chloro-1,1,2-trifluoroethyl sulfide and *tert*-butyl 1,2,3,4,4-pentachlorobutadienyl sulfide were synthesized and characterized as described previously [12].

Benzyl 1,2-dichlorovinyl sulfide (BDVS). One hundred millimoles of benzyl mercaptan are dissolved in 100 ml of liquid ammonia, and 100 mmol of sodium metal are added followed by 110 mmol of trichloroethene. After 4 hr at −35°, the ammonia is evaporated under reduced pressure, and the dry residue is dissolved in 100 ml of water and extracted twice with 200 ml *n*-hexane. The *n*-hexane layer is separated and the solvent is removed *in vacuo*. The residue was purified by column chromatography on silica gel with *n*-hexane as solvent to give benzyl 1,2-dichlorovinyl sulfide with a purity of 99% (GC) and a yield of 25%.

^{1}H (NMR ($CDCl_3$, int. tetramethylsilane): δ (ppm) = 4.99 (s, 2H); 7.27 (s, 1H); 8.20 (m, 5H). ^{13}C NMR ($CDCl_3$, ^{1}H-decoupled): δ (ppm) = 37.4; 119.9; 127.1; 127.6; 128.6; 128.9; 129.1. Mass spectrum (electron impact, 70eV): m/z (^{35}Cl) = 51

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(5.8%); 57 (5%); 62 (3%); 63 (7.8%); 65 (27%); 77 (2.7%); 83 (3.3%); 89 (6.7%); 91 (100%); 92 (19%); 94 (2.5%); 218 (20%, 2Cl).

tert-Butyl 1,1,2-trichlorovinyl sulfide was prepared analogously to the preparation of *tert*-butyl 1,2,3,4,4-pentachlorobutadienyl sulfide, but with tetrachloroethene as the starting material. ^{1}H NMR ($CDCl_3$, int. tetramethylsilane); δ (ppm) = 1.6 (s) ^{13}C NMR ($CDCl_3$, ^{1}H-decoupled): δ (ppm) = 32.1, 53.3, 125.3, 129.1. Mass spectrum (electron impact, 70eV): m/z (^{35}Cl) = 57 (100%); 58 (4%); 59 (2%); 79 (4%); 91 (4%); 117 (2%); 119 (3%); 126 (4%); 161 (2%, 3Cl); (6%, 3Cl). Purity: 99% (GC), yield 33%).

Benzyl 2-chloroethyl sulfide. To a solution of 10 mmol of sodium metal in 100 ml liquid ammonia, 10 mmol of benzyl mercaptan dissolved in 3 ml of dry dimethylformamide is added followed by the addition of 11 mmol of 1-chloro-2-bromoethane in 5 ml of dimethylformamide. After stirring at −35° for 2 hr the ammonia is evaporated at room temperature, and the dry residue is extracted with *n*-hexane. Purification of the hexane solution by column chromatography on silicagel (hexane:chloroform, 1:1 as the solvent) gives benzyl 2-chloroethyl sulfide in a yield of 45% and a purity of 99%, as determined by GC.

^{1}H NMR ($CDCl_3$, int. tetramethylsilane): δ (ppm) = 2.89 (t, 2H, J = 9Hz); 3.73 (t, 2H, J = 9Hz); 4.65 (s, 2H): 8.13 (m, 5H). ^{13}C NMR ($CDCl_3$, ^{1}H-decoupled): δ (ppm) = 33.5; 37.4; 45.3; 127.8; 128.6; 128.9; 129.1.

Mass spectrum (electron impact, 70eV): m/z (^{35}Cl) = 51 (4%); 63 (5.5%); 65 (15%); 77 (4%); 89 (5%); 91 (100%); 92 (12%); 121 (3.2%, 1Cl); 186 (37%, 1Cl).

Mutagenicity assay. The *Salmonella typhimurium* tester strain TA100, provided by Dr. B. N. Ames, Berkeley, CA, was used for all experiments; UV and crystal violet sensitivity, ampicillin resistance, and mutability by UV were checked regularly. Liver 9000 *g* supernatant ("S9", protein concentration 50 mg/ml) from Aroclor-1254-induced male Wistar rats and "S9"-mix were prepared according to Maron and Ames [13]. The mutagenicity assay employed was a preincubation method similar to that described in the literature [9]. Cultures were grown in Oxoid Nutrient Broth No. 2 for 10 hr and had cell titers of 3–4 × 10^9/ml.

Spontaneous revertant frequencies were comparable with those previously published [13] and with values established in our laboratory [7, 9]. Preincubations were performed in 500 μl 0.1 M phosphate buffer (pH 7.4) with 100 μl of the bacterial culture and 20 μl test compound (dissolved in MeOH) with or without "S9"-mix (0.5–5 mg per plate) at 37° for 2 hr with constant shaking. After preincubation, 2 ml of top agar (45°) containing histidine (0.05 mM) and biotin (0.05 mM) was added, and the mixture was plated on Vogel–Bonner E medium. After 2 days of incubation, colonies of revertants were counted with an automated colony counter; the counts were corrected for overlapping colonies with a computer program. All determinations were made in duplicate, and all experiments were performed at least twice.

RESULTS

tert-Butyl 1,2,3,4,4-pentachlorobuta-1,3-dienyl sulfide, *tert*-butyl 2-chloro-1,1,2-trifluoroethyl sulfide, and *tert*-butyl 1,1,2-trichlorovinyl sulfide failed to induce a mutagenic response at concentrations between 0.1 and 2000 nmol per plate in *S. typhimurium* TA100 either without or with rat liver "S9"-mix (0.5–5 mg protein per plate). Varying the preincubation time (0–120 min) also failed to increase the number of revertants above control values; all *tert*-butyl sulfides were also negative in *S. typhimurium* strains TA2638 and TA98.

The direct-acting sulfur mustard benzyl 2-chloroethyl sulfide was a potent, direct-acting mutagenicity in *S. typhimurium* at concentrations between 0.1 and 2000 nmol per plate and did not cause bacterial toxicity. Addition of rat liver "S9"-mix did not influence the number of revertants obtained (Fig. 1).

The benzyl sulfides BPBS, BCFS and BDVS (0.1–2000 nmol per plate) were neither mutagenic nor bactericidal when mammalian enzymes were not included in the preincubation mixture. Addition of "S9"-mix or rat liver microsomes fortified with a NADPH-generating system resulted in a concentration-dependent increase in the number of revertants in the case of BPBS (Fig. 2) and BDVS (Fig. 3). The maximum response, 5-fold and 6-fold increase over control values with BPBS and BDVS respectively, was obtained when 2.5 mg "S9"-mix per plate were used and preincubations were performed for 120 min (Figs 1 and 2). The capability of "S9"-mix and rat liver microsomes to catalyze oxidative metabolism was tested with isoquinoline oxide in every experiment; these positive controls were consistent with the values previously established in our laboratory. Exclusion of $NADP^+$ from the "S9"-mix or preincubation with rat liver microsomes lacking added NADPH abolished the mutagenic effect.

Hemimercaptals formed by the benzylic hydroxylation of benzyl sulfides yield equimolar amounts of the respective thiol and benzaldehyde. Benzaldehyde (0.1–2000 nmol per plate) was not mutagenic either directly or in the presence of "S9"-mix (data not shown). In contrast to the benzyl sulfides derived from hexachlorobutadiene and trichloroethene, BCFS obtained from chlorotrifluoroethene failed to induce a mutagenic response when tested at concentrations between 0.1 and 2000 nmol per plate under conditions described for BPBS and BDVS. This compound was also not mutagenic in *S. typhimurium* strains TA2638 and TA98.

DISCUSSION

The benzyl sulfides BPBS and BDVS were mutagenic under conditions supporting metabolic oxidation by cytochromes P-450. Cytochrome P-450 hydroxylates benzyl sulfides **1** and **2** at the benzylic carbon atom (Fig. 4) to yield hemimercaptals **3** and **4** [12], which eliminate chlorovinylthiols and benzaldehyde. In contrast, *tert*-butyl sulfides, which cannot undergo benzylic hydroxylation, were not mutagenic. Because benzaldehyde was not mutagenic, the metabolic formation of unstable

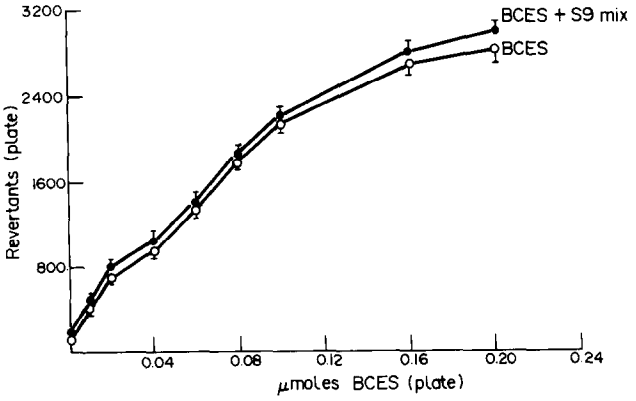

Fig. 1. Mutagenic activity of benzyl 2-chloroethyl sulfide in *S. typhimurium* TA100 with (●) or without (○) the addition of rat liver "S9"-mox (2.5 mg protein/plate). Each data point represents the mean ± SD of 4 determinations from 2 independent experiments.

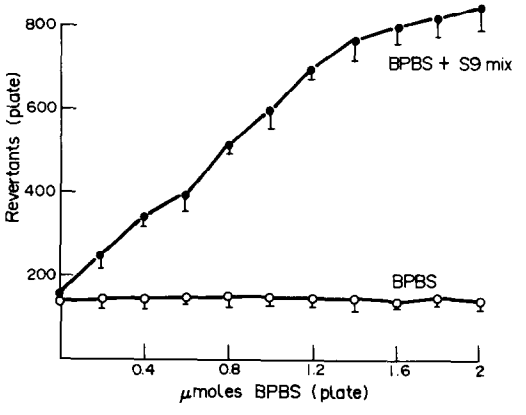

Fig. 2. Mutagenic activity of benzyl 1,2,3,4,4-pentachlorobutadienyl sulfide (BPBS) in *S. typhimurium* TA100 with (●) or without (○) the addition of rat liver "S9"-mix (2.5 mg protein/plate). Each data point represents the mean ± SD of 4 determinations from 2 independent experiments.

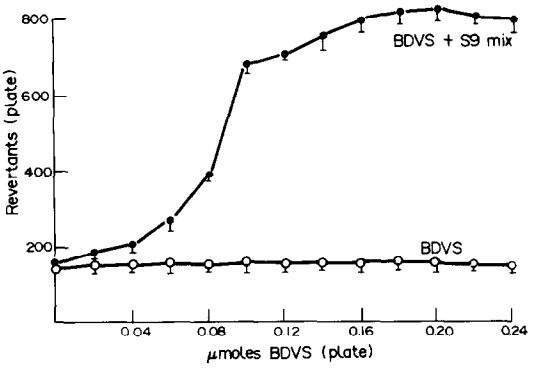

Fig. 3. Mutagenic activity of benzyl 1,2-dichlorovinyl sulfide (BDVS) in *S. typhimurium* TA100 with (●) or without (○) the addition of rat liver "S9"-mix (2.5 mg protein/plate). Each data point represents the mean ± SD of 4 determinations from 2 independent experiments.

chlorovinylthiols represents the crucial reaction responsible for the formation of genotoxic intermediates from chlorovinyl benzyl sulfides (Fig. 4). The unstable thiols formed from BPBS and BDVS, 1,2,3,4,4-pentachloro-1-mercaptobutadiene and 1,2-dichloro-1-mercaptoethane **5**, respectively, are identical to the thiol metabolites formed by cysteine conjugate β-lyase-catalyzed cleavage of the *S*-conjugates PCBC and DCVC [10, 11]. The fate of these enethiols and their transformation to electrophiles has been elucidated: 1,2-dichloro-1-mercaptoethene and 1,2,3,4,4-pentachloro-1-mercaptobutadiene are converted to thioacylating intermediates, either thioketenes **6** or thionoacyl chlorides **7**, or both [11]. These electrophiles are presumably capable of interacting with nucleophilic sites in DNA, as observed for DCVC metabolites [14], and cause mutations.

Similar to the cysteine *S*-conjugate *S*-(2-chloroethyl)-L-cysteine [9], the benzyl sulfide benzyl 2-chloroethyl sulfide **8** is a potent and direct-acting mutagen in bacteria. The mutagenicity of these sulfur mustards is due to the facilitated nucleophilic displacement of the chlorine atom in β-position to the sulphur to yield a highly electrophilic episulfonium ion [15] (Fig. 4).

As found with the cysteine *S*-conjugates CTFC [9], BCFS **2** was not mutagenic under conditions supporting oxidative metabolism. Studies on the biotransformation of CTFC, however, indicate that an electrophile, chlorofluorothionoacetyl fluoride, is an intermediate in the β-lyase catalysed metabolism of CTFC [10]. After benzylic hydroxylation, the unstable thiol 2-chloro-1,2,2-trifluoro-2-mercaptoethane **9** is released from BCFS and eliminates hydrogen fluoride to yield chlorofluorothioacetyl fluoride **10**. This thionacyl fluoride is a strong acylating agent [10] and may be capable of interacting with DNA and causing mutagenicity. Neither the CTFC nor the corresponding benzyl sulfide BCFS were mutagenic; although no satisfactory explanation for this lack of mutagenicity is presently available, our results agree with observations of others on the lack of mutagenicity of fluoroalkyl substituted *S*-conjugates [8]. To explain the differences in mutagenicity between metabolically formed chlorovinyl

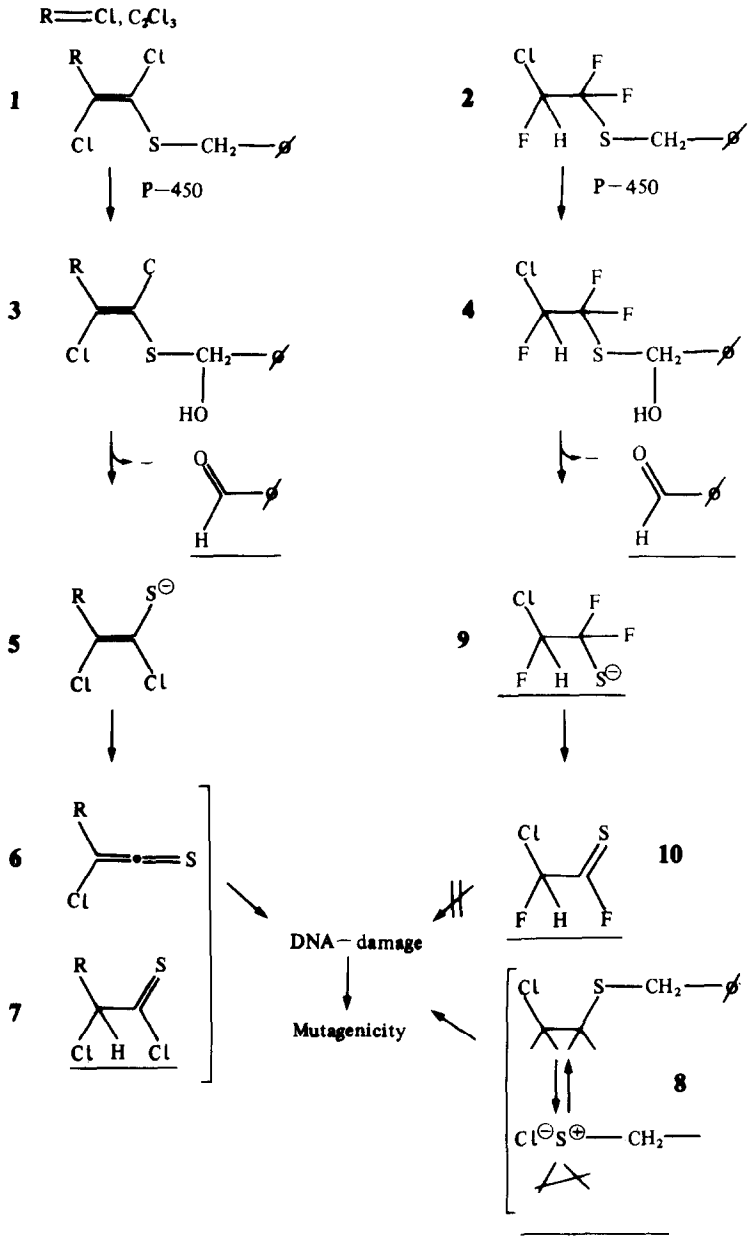

Fig. 4. Metabolic activation of benzyl 1,2-dichlorovinyl sulfide, benzyl 1,2,3,4,4-pentachlorobutadienyl sulfide, benzyl 2-chloro-1,1,2-trifluoroethyl sulfide and benzyl 2-chloroethyl sulfide. Metabolites identified in previous studies [10–12, 15] are underlined.

thiols and fluoroalkyl thiols, a preferential reaction of the fluoroalkyl thiols with protein thiols resulting in disulfide formation has been proposed [8]. Trapping experiments indicate, however, that fluoroalkyl thiols are more stable than chlorovinyl thiols and may be detoxified by disulfide formation [10].

The design and testing of precursors of putative mutagenic metabolites may have broad utility as a strategy for exploring bioactivation mechanisms and merits further exploitation.

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REFERENCES

1. Anders MW, Dohn DR, Elfarra AA, Dekant W and Lash LH, Biosynthesis and metabolism of glutathione conjugates to toxic forms. *CRC Crit Rev Toxicol* **18**: 311–342, 1988.
2. Elfarra AA and Anders MW, Renal processing of glutathione conjugates. Role in nephrotoxicity. *Biochem Pharmacol* **33**: 3729–3732, 1984.

3. Dekant W, Lash LH and Anders MW, Role of glutathione conjugates and bioactivation of cysteine *S*-conjugates by cysteine conjugate β-lyase. In: *Glutathione-Conjugation: Its Mechanism and Biological Significance* (Eds. Ketterer B and Sies H), pp. 415–447. Academic Press, New York 1988.
4. Lash LH and Anders MW, Cytotoxicity of *S*-(1,2-dichlorovinyl)-glutatione and *S*-(1,2-dichlorovinyl)-L-cysteine in isolated rat kidney cells. *J Biol Chem* **261**: 13076–13081, 1986.
5. Dohn DR, Leininger JR, Lash JH, Quebbemann AJ and Anders MW, Nephrotoxicity of *S*-(2-chloro-1,1,2-trifluoroethyl)glutathione and *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine, the glutathione and cysteine conjugates of chlorotrifluoroethene. *J Pharmacol Exp Ther* **234**: 851–857, 1985.
6. Jones TW, Wallin A, Thor H, Gerdes RG, Ormstad K and Orrenius S, The mechanisms of pentachlorobutadienyl–glutathione nephrotoxicity studied with isolated rat renal epithelial cells. *Arch Biochem Biophys* **251**: 504–513, 1986.
7. Dekant W, Vamvakas S, Berthold K, Schmidt S, Wild D and Henschler D, 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, 1986.
8. Green T and Odum J, Structure/activity studies of the nephrotoxic and mutagenic action of cysteine conjugates of chloro and fluoroalkenes. *Chem-Biol Interact* **54**: 15–31, 1985.
9. Vamvakas S, Berthold K, Dekant W and Henschler D, Bacterial cysteine conjugate β-lyase and the metabolism of cysteine *S*-conjugates: structural requirements for the cleavage of *S*-conjugates and the formation of reactive intermediates. *Chem-Biol Interact* **65**: 59–71, 1988.
10. Dekant W, Lash LH and Anders MW, Bioactivation mechanism of the cytotoxic and nephrotoxic *S*-conjugate S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine. *Proc Natl Acad Sci USA* **84**: 7443–7447, 1987.
11. Dekant W, Berthold K, Vamvakas S, Henschler D and Anders MW, 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, 1988.
12. Veltman IC, Dekant W, Guengerich FP and Anders MW, Cytotoxicity and bioactivation mechanism of benzyl 2-chloro-1,1,2-trifluoroethyl sulfide and benzyl 1,2,3,4,4-pentachlorobutadienyl sulfide. *Chem Res Toxicol* **1**: 35–40, 1988.
13. Maron D and Ames B, Revised methods for the *Salmonella* mutagenicity test, *Mutat Res* **113**: 173–215, 1983.
14. Bhattacharya RK and Schultze MO, Properties of DNA-treated with *S*-(1,2-dichlorovinyl)-L-cysteine and a lyase. *Arch Biochem Biophys* **153**: 105–115, 1972.
15. Dohn DR and Casida JE, Thiranium ion intermediates in the formation and reactions of *S*-(2-haloethyl)-L-cysteines. *Bioorg Chem* **15**: 115–124, 1987.