[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY, UNIVERSITY OF MINNESOTA]

The Mechanism of Action of Bisalkylating Agents in Cancer Chemotherapy. II. The Reaction of Myleran with Mercaptans[1]

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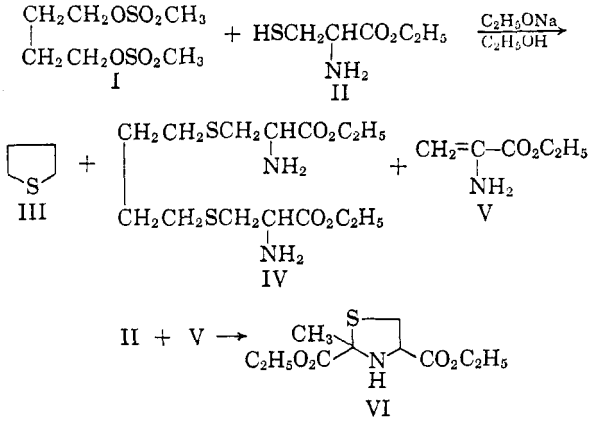
The reaction of myleran with methyl mercaptan, butyl mercaptan, ethyl β-mercaptopropionate, benzyl mercaptan, and glutathione has been investigated. A cyclic sulfonium ion intermediate is proposed in each case, and factors controlling the sulfur-stripping action of myleran, which was previously proposed to explain its physiological activity,[2] are discussed.

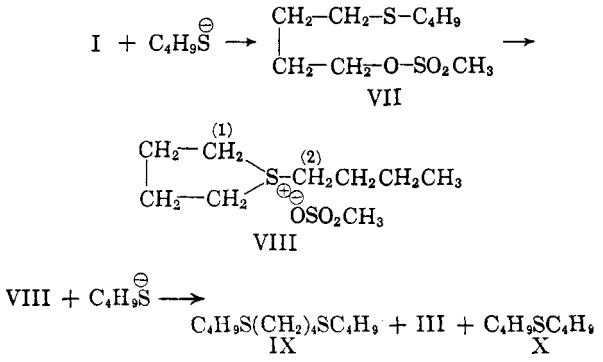
We have recently reported[2] that the reaction of myleran (I) with cysteine ethyl ester (II), in alkaline medium, results in the formation of tetrahydrothiophene (III), the thiazolidine VI, and the expected bisalkylated derivative IV. We have suggested that this "sulfur-stripping" reaction may be responsible for the physiological activity of the



bisalkylating agents in cancer chemotherapy. Roberts and Warwick,[3] in an independent study involving the reaction of myleran and cysteine, observed this sulfur-eliminating reaction, and they have subsequently[4] shown that myleran is metabolized in the mouse and excreted as a derivative of tetrahydrothiophene (3-hydroxytetrahydrothiophene-1,1-dioxide).

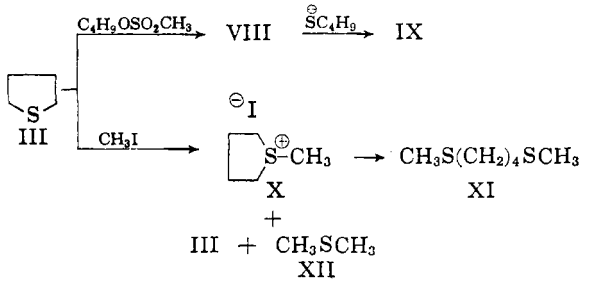
In view of the probable physiological importance of this sulfur-stripping reaction, we have now examined the reaction of myleran with *n*-butyl mercaptan, methyl mercaptan, ethyl β-mercaptopropionate, benzyl mercaptan, and glutathione.

The reaction of myleran (I) with two equivalents of sodium butylmercaptide gave predominantly (68% yield) 1,4-bis(*n*-butylthio)butane (IX). No tetrahydrothiophene or *n*-butyl sulfide (X) was noted, and it was concluded that little, if any, sulfur elimination occurred.



The intermediate VII would be expected to undergo rapid intramolecular sulfonium ion formation (VII → VIII), and the product IX is best explained as resulting from the preferential attack of the butyl mercaptide ion at the methylene carbon atom (1) of the five-membered ring of VIII. Attack of the mercaptide ion at the exocyclic methylene carbon atom (2) would result in the formation of tetrahydrothiophene and *n*-butyl sulfide (X); however, molecular models reveal that carbon atom (2) is significantly more hindered than carbon atom (1).

The sulfide XI was obtained by reaction of myleran and methyl mercaptide. Tetrahydrothiophene (4%, pure) and dimethyl sulfide (1%) were also detected. The slight increase in sulfur elimination (tetrahydrothiophene formation) noted in this reaction, as compared to that involving *n*-butylmercaptide, is consistent with the expected increased shielding of the exocyclic methylene carbon atom (2, in VIII) by the butyl group.



(1) This investigation was supported by the National Cancer Institute, National Institutes of Health, Grant CY 3907.

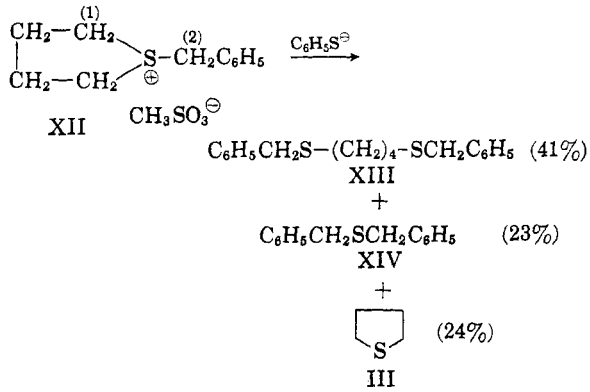
(2) W. E. Parham and J. M. Wilbur, Jr., *J. Am. Chem. Soc.*, **81**, 6071 (1959).

(3) J. J. Roberts and G. P. Warwick, *Nature*, **183**, 1509 (1959).

(4) J. J. Roberts and G. P. Warwick, *Nature*, **184**, 1288 (1959).

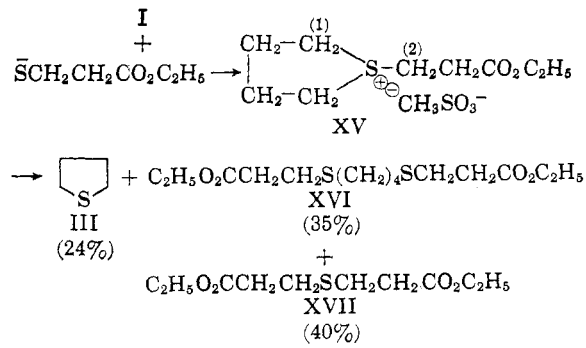
Support for the sulfonium intermediate VIII was obtained by treating the *n*-butyl tetramethylene sulfonium cation VIII, prepared from tetrahydrothiophene and *n*-butyl methane sulfonate, with one equivalent of sodium *n*-butylmercaptide. The sulfide IX was obtained in 74% yield along with a small amount (0.55% yield) of tetrahydrothiophene. Similarly, the reaction of methyl tetramethylene sulfonium iodide (X) with one equivalent of sodium mercaptide gave tetrahydrothiophene (17% yield, pure) and dimethyl sulfide (1.8% yield, yure).

Myleran was found to react with two equivalents of sodium benzyl mercaptide to give tetrahydrothiophene (isolated in 24% yield as the pure mercuric chloride complex), 1,4-bis(benzylthio)butane (XIII, 41% yield), and benzyl sulfide (XIV, 23% yield, pure).

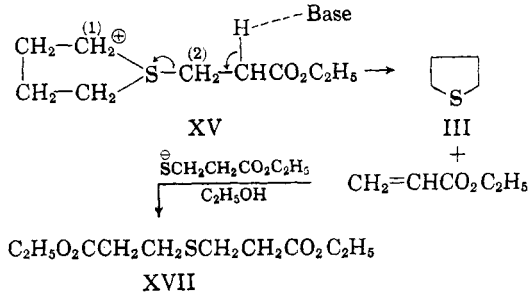


The different course noted for this reaction as compared with that observed with *n*-butyl mercaptan was anticipated, and the preferential reaction of the sulfonium ion XII at carbon (2) with mercaptide ion is probably due to a change from SN-2 to SN-1 type reaction.

The reactions of myleran with two equivalents of the sodium salt of ethyl β-mercaptopropionate was considered next. The esters XVI (35% yield) and XVII (40% yield) were separated from the



reaction mixture by fractional distillation, and characterized as their hydrazides. Tetrahydrothiophene was isolated as the pure mercuric chloride derivative in 23.6% yield. It is proposed that XVI is formed by substitution at carbon 1 in XV by mercaptide ion (SN-2), and that XVII is formed by a *beta*-elimination reaction as shown in the equation below.



It is unreasonable to assume that XVII is formed by either an SN-1 or SN-2 reaction of mercaptide at C_2 (in XV) in view of the results previously obtained with butyl and methyl mercaptan, and incompatible with the previous observation that the reaction of myleran with the sodium salt of cysteine ethyl ester affords VI.

These results suggest that the sulfur-stripping action of myleran is consistent with the *beta*-elimination mechanism proposed,[2] and is to be expected with mercaptans (such as cysteine) bearing a substituent which can further activate the beta carbon atom of the sulfonium intermediate. Sulfur elimination is also observed with mercaptans, such as benzyl mercaptan, which possess a weak carbon-sulfur bond.

The reaction conditions employed in these studies (two equivalents of mercaptide in homogeneous solution) were such that they favored substitution, as opposed to the *beta*-elimination mechanism. This conclusion is consistent with the observation[4] that myleran is converted metabolically almost exclusively to a derivative of tetrahydrothiophene. We have observed that a heterogeneous mixture of myleran and the sodium salt of the tripeptide glutathione, at room temperature for three days, results in the elimination of 60% of the total sulfur of glutathione as tetrahydrothiophene. This observation is in accord with that recently published[4] by Roberts and Warwick who have shown that the reaction of myleran with glutathione, denatured egg albumin and reduced keratin gives tetrahydrothiophene.

EXPERIMENTAL[5]

Reaction of n-butyl mercaptan with myleran. *n*-Butyl mercaptan (18.0 g., 0.20 mole) was added to a solution of sodium ethoxide, prepared by treating sodium (5.1 g., 0.22 mole) with 100 ml. of absolute ethanol. The resulting mixture was added rapidly to a hot solution of myleran (24.6 g., 0.10 mole) in 1600 ml. of absolute ethanol. The stirred reaction mixture was heated under a nitrogen atmosphere at reflux temperature for 6 hr. The mixture was cooled (ice bath) and the sodium methanesulfonate which separated was removed by filtration and washed twice with 100 ml. of ethanol. The washes and filtrate were combined, and the ethanol was re-

(5) All melting points are uncorrected.

moved under reduced pressure on a steam bath. Any further material which separated during the distillation was removed by filtration.

Isolation of tetrahydrothiophene. The ethanol distillate was treated with 275 ml. of saturated alcoholic lead acetate solution to remove unchanged butyl mercaptan. The yellow crystals of lead mercaptide which separated were removed (0.3 g., m.p. 78–80, reported[6] m.p. 80–81°), and the filtrate was distilled under reduced pressure on a steam bath. The ethanol distillate was treated with a solution of mercuric chloride (27.2 g., 0.10 mole) in 200 ml. of ethanol. There was no evidence for the formation of tetrahydrothiophene mercuric chloride after the resulting solution had stood at 0° for 1 week. Only a trace amount (0.0358 g.) of crystalline material, which did not melt up to 280°, was formed.

Isolation of 1,4-bis(n-butylthio)butane (IX). The residual oil remaining after removal of the solvent ethanol was dissolved in 100 ml. of petroleum ether (b.p. 30–60°) and washed five times with 25 ml. of water. The organic layer was collected, dried (magnesium sulfate), and the petroleum ether was removed by distillation. The crude product (18.9 g., 80.7%) was distilled and 16.0 g. (68.5% yield) of product boiling at 137–138°/1.8 mm., $n_D^{25.2°}$ 1.4908 was collected. This material was characterized as 1,4-bis(*n*-butylthio)butane by conversion to the corresponding sulfone.

Preparation of 1,4-bis(butylsulfonyl)butane. To 1,4-bis(*n*-butylthio)butane (1 g., 0.00426 mole) in 5 ml. of glacial acetic acid, was added 25 ml. of 30% hydrogen peroxide, dropwise with stirring during 30 min. An exothermic reaction occurred and cooling was necessary to keep the reaction mixture at room temperature. The mixture was then heated at reflux for 1 hr. The product which separated from the cold mixture was collected and recrystallized from ethanol. There was obtained 1.2 g. (94.5%) of colorless crystals melting at 129–130°.

Anal. Calcd. for $C_{12}H_{26}O_4S_2$: C, 48.29; H, 8.78; S, 21.49. Found: C, 48.31; H, 8.48; S, 21.66.

Preparation of n-butyl tetramethylene sulfonium methanesulfonate (VIII). *Procedure A:* A mixture of methanesulfonoxybutane (15.2 g., 0.10 mole) and tetrahydrothiophene (8.8 g., 0.10 mole) was heated in an oil bath at 100–110° for 17 hr. A dark viscous liquid formed which was purified by treatment with Norite. The filtrate including the ether wash of the filter cake was extracted with 30 ml. of ether, and the ether-insoluble fraction was collected and heated under reduced pressure on a water bath (70–80°) to remove ether and any unchanged tetrahydrothiophene. The residue afforded 15.7 g. (62.5% yield) of a viscous liquid after drying under vacuum (calcium chloride, potassium hydroxide) overnight in a desiccator. This material was hygroscopic but was shown to be *n*-butyl tetramethylene sulfonium methanesulfonate by conversion to the corresponding picrylsulfonate salt.

Preparation of the picryl sulfonate of VIII. A solution of *n*-butyl tetramethylene sulfonium methanesulfonate (1.0 g., 0.00417 mole) in 5 ml. of water was treated with a saturated aqueous solution of sodium picryl sulfonate until no further precipitate formed. The mixture was cooled (0°) for several hours, and the yellow solid was collected and washed with water. The product (1.0 g., 58.9% yield) melted at 155–157°, and when recrystallized from water gave 0.4 g. (23.5%) of pure material; m.p. and mixture m.p. with that prepared by procedure B (m.p. 157–158°) 157–158°.

Procedure B: A mixture of *n*-butyl-4-hydroxybutyl sulfide[7] (4.0 g., 0.025 mole) and 36.5% hydrochloric acid (2.6 g., 0.025 mole) was heated on a steam bath for 1 hr. The mixture was dried by azeotropic distillation with benzene until the benzene distillate (55 ml.) was no longer cloudy. The remaining benzene in the reaction mixture was removed under reduced pressure on a water bath (70–80°). The viscous residue was washed by shaking with ether and decanting the ether. The product was dried in a desiccator (potassium hydroxide, calcium chloride) overnight under vacuum. The viscous oil which remained was treated with an aqueous solution of sodium picrylsulfonate until no further precipitate formed. The crude product melted at 157–158°; the melting point was not changed by additional recrystallizations from water.

Anal. Calcd. for $C_{14}H_{19}N_3S_2O_9$: C, 38.44; H, 4.38; N, 9.60; S, 14.66. Found: C, 38.48; H, 4.41; N, 9.78; S, 14.62.

Reaction of n-butyl mercaptan with n-butyl tetramethylene sulfonium methanesulfonate. Butyl mercaptan (4.5 g., 0.05 mole) was added to a solution of sodium ethoxide, prepared by treating sodium (1.2 g., 0.051 g.-atom) with 25 ml. of absolute ethanol. This mixture was added rapidly to a solution of *n*-butyl tetramethylene sulfonium methanesulfonate (12.0 g., 0.05 mole) in 50 ml. of ethanol. Upon addition of the sodium butanethiolate, a thick white precipitate formed and it was necessary to add 100 ml. of ethanol to facilitate mixing. The reaction was heated at reflux with magnetic stirring for 6 hr. After cooling the mixture in an ice-bath, the insoluble material (sodium methanesulfonate, 5.5 g., 93% yield) was removed and washed with 100 ml. of ethanol. The ethanol washes and filtrate were combined and the ethanol was removed under reduced pressure on a steam bath. Tetrahydrothiophene (0.1 g., 0.55% yield, m.p. crude 125–128°) and 1,4-bis(*n*-butylthio)butane (8.7 g., 74.4% yield, b.p. 127–128°/1.3 mm., n_D^{25} 1.4906–1.4904; disulfone m.p. and mixture m.p. 129–130°) were isolated by procedures essentially identical with those described above.

Reaction of methyl mercaptan with myleran. Methyl mercaptan (10.6 g., 0.22 mole) was added to a solution of sodium ethoxide, prepared by treating sodium (5.5 g., 0.24 g.-atom) with 100 ml. of ethanol. This mixture was added rapidly to a hot solution of myleran (24.6 g., 0.10 mole) in 1700 ml. of ethanol. The reaction was heated at reflux with magnetic stirring for 6 hr. A series of traps cooled with liquid nitrogen and connected at the top of a reflux condenser of the reaction flask served as a means of collecting any dimethyl sulfide formed during the reaction; however, none was noted.

Isolation of XI and the mercuric chloride salt of dimethyl sulfide and tetrahydrothiophene. After the reaction had been concluded, nitrogen was passed into the refluxing mixture for 30 min. The exit gases were passed into a solution of mercuric chloride (27.2 g., 0.10 mole) in ethanol (250 ml.). The solid (4.9 g., m.p. 108–200° dec.) which separated after this mixture had stood for 24 hr. at 0° was collected.

The original reaction mixture was cooled, and the precipitate of sodium methanesulfonate was collected and washed with ethanol (100 ml.). The combined filtrate and washes was concentrated under reduced pressure (60–70°). Additional sodium methanesulfonate precipitated and was collected (combined total 23.0 g., m.p. >230°, 100% yield). The ethanol distillate was treated with a solution of mercuric chloride (81.5 g., 0.30 mole) in ethanol (350 ml.), and the solid (16.0 g., m.p. mostly at 110–155°) was collected and combined with the mercuric chloride complex isolated earlier (combined total, 20.9 g.).

The residue, obtained by removing ethanol, sodium methanesulfonate, and volatile sulfides from the original reaction mixture, was dissolved in ether (50 ml.) and the ether extract was washed with 1*N* hydrochloric acid (25 ml.) and with water (50 ml. total). The ether extract was dried (magnesium sulfate), and distilled. There was obtained 6.8 g. (45% yield) of XI (b.p. 115–119°/25 mm., n_D^{25} 1.5052–1.5108, reported[8] b.p. 115–119°/26 mm.). 1,4-Bis(methylthio)butane (XI) was further characterized by its conversion into 1,4-bis(methylsulfonyl)butane. The procedure used was identical with that described above for preparation of the sulfone of IX. The product was purified by recrystallization from water, and melted at 193.5–194.5°.

(6) E. Wertheim, *J. Am. Chem. Soc.*, **51**, 3661 (1929).

(7) C. D. Hurd and K. Wilkinson, *J. Am. Chem. Soc.*, **71**, 3429 (1949).

(8) D. Jerchel, L. Dippelhofer, and D. Renner, *Chem. Ber.*, **87**, 947–955 (1954).

Anal. Calcd. for $C_6H_{14}S_2O_4$: C, 33.63; H, 6.58; S, 29.93. Found: C, 33.84; H, 6.32; S, 30.27.

A mixture of the combined mercury salts (20.9 g.) and 20% hydrochloric acid (229 ml.) was heated at the reflux temperature for 2 hr. in a flask equipped with a Dean-Stark apparatus and a reflux condenser. The distillate was removed and extracted three times with ether (20 ml.). The ether was dried and distilled and two fractions were collected: (1) b.p. 32–40°, (2) b.p. 117–119° (*ca.* 0.3 g.).

The low-boiling fraction was treated with a solution of mercuric chloride (81.5 g., 0.30 mole) in ethanol. The solid (2.4 g.) which formed after the mixture had stood for 24 hr. at 0° was recrystallized from benzene. There was obtained 1.1 g. (1.2% yield) of the *mercury chloride complex of dimethyl sulfide*[9] (m.p. 153–155°, mixture m.p. with authentic material, m.p. 155–157, was 154–156°).

The higher-boiling fraction was treated with a solution of mercuric chloride (13.6 g.) in ethanol. There was obtained 3.4 g. (9.5% yield) of crude *tetrahydrothiophene mercuric chloride* salt (m.p. 130–145°). This material was recrystallized from ethanol and the product (2.0 g., 5.5% yield, m.p. 127–130°) caused no depression in melting point when admixed with an authentic sample (m.p. 127–130°).

Reaction of methyl mercaptan with methyl-tetramethylene sulfonium iodide. Methyl-tetramethylene sulfonium iodide was prepared according to the procedure of Whitehead, Dean, and Fidler.[10] Methyl mercaptan (4.8 g., 0.10 mole), cooled in Dry Ice–acetone, was added to a solution of sodium ethoxide, prepared by treating sodium (2.3 g., 0.10 mole) with 50 ml. of absolute alcohol. This mixture was added rapidly to a hot solution of methyl-tetramethylene sulfonium iodide (23.0 g., 0.1 mole) in 400 ml. of ethanol. The stirred mixture was heated at reflux for 6 hr. in a round-bottom flask equipped with a reflux condenser and a Dry Ice-acetone condenser. The ethanol solvent was removed from the reaction mixture by distillation into an ice-cooled flask containing 450 ml. of saturated alcoholic lead acetate solution. The ethanol solution was allowed to stand overnight at 0°, and the yellow precipitate of lead methyl mercaptide was removed by filtration.

The residue and the ethanol distillate were processed by procedures similar to that described above for the reaction of myleran with sodium methyl mercaptide.

1,4-Bis(methylthio)butane (4.2 g., 27% yield crude) distilled (2.4 g., 16% yield, b.p. 115–117°/24 mm., n_D^{25} 1.5118; reported[7] b.p. 115/26 mm.) with a colored impurity, and was not processed further.

From combined crude mercury salts (44.5 g.), there was obtained: (1) 2.4 g. (3.8% yield) of *dimethyl sulfide mercuric chloride* (m.p. 151–154°, mixture m.p. with authentic, m.p. 152–154°, 152–154°), and (2) 6.4 g. (17.8% yield) of pure tetrahydrothiophene mercuric chloride (m.p. and mixture m.p. 128–130°).

Reaction of benzyl mercaptan with myleran. Benzyl mercaptan (24.8 g., 0.20 mole) was added to a solution of sodium ethoxide, prepared by treating sodium (4.8 g., 0.21 mole) with 50 ml. of absolute ethanol. The resulting solution was added rapidly to a hot solution of myleran (24.6 g., 0.10 mole) in 1700 ml. of absolute ethanol. The stirred reaction mixture was heated at reflux for 8 hr. Sodium methanesulfonate, which precipitated from the cold (ice bath) reaction mixture was removed by filtration and washed with 100 ml. of ethanol. The washes and filtrate were combined, and the ethanol was removed under reduced pressure on a steam bath. A small amount of additional precipitate formed during this operation, and it was removed from the crude product by filtration.

Isolation of tetrahydrothiophene. The ethanol distillate was treated with 700 ml. of saturated alcoholic lead acetate solution to remove any unchanged benzyl mercaptan. The ethanol solvent was removed from this mixture by distillation under reduced pressure on a steam bath. The distillate was treated with a solution of mercuric chloride (27.1 g., 0.10 mole) in 200 ml. of ethanol. The mixture was allowed to stand at 0° for 1 week. The resulting solid was collected and washed with ethanol which afforded 8.6 g. (24%) of colorless needles melting at 128–130°. A mixture m.p. with authentic mercuric chloride salt, m.p. 127.5–129.5° was 128–130°.

Isolation of benzyl sulfide (XIV). The residual oil (26.3 g.) which remained after removal of the ethanol solvent was dissolved in 50 ml. of ether and washed with 20-ml. portions of water until the water washes were neutral (*p*H paper). The organic layer was dried (magnesium sulfate), and the ether was removed by distillation. Distillation of the yellow oil (25.7 g.) gave 7.8 g. (36.4%) of liquid, b.p. 117–118°/13 mm., which solidified on standing. The crystals were collected and dried on a clay plate to yield 5.0 g. (23.3% yield) of pure benzyl sulfide, m.p. 46–48°; mixture m.p. with authentic material (m.p. 47–49°)[11] was 47–49°.

1,4-Bis(benzylthio)butane (XIII). The residue remaining after removal of the benzyl sulfide weighed 12.5 g. (41.6% yield) and contained the other reaction product, 1,4-bis-(benzylthio)butane. This product was converted to 1,4-bis-(benzylsulfonyl)butane in 77% yield (m.p. 197–198°, from water) by oxidation with hydrogen peroxide by a procedure similar to that described for the oxidation of IX.

Anal. Calcd. for $C_{18}H_{20}S_2O_4$: C, 59.31; H, 5.53; S, 17.60. Found: C, 59.16; H, 6.08; S, 17.70.

Reaction of ethyl β-mercaptopropionate with myleran. The reaction of ethyl β-mercaptopropionate (26.8 g., 0.20 mole) with myleran (24.6 g., 0.10 mole) was carried out as described above for the reaction with benzyl mercaptan. *Tetrahydrothiophene* was isolated from the ethanol distillate as the mercuric chloride salt (8.5 g., 23.6% yield, m.p. and mixture m.p. 128–130°). The esters XVII (9.4 g., 40.2% yield, b.p. 128–130°/1.8 mm., n_D^{25} 1.4642) and XVI (11.1 g., 34.7% yield, b.p. 178–180°/0.14 mm., n_D^{27} 1.4920) were isolated by distillation and characterized by conversion to the corresponding hydrazides.

The hydrazide of XVII was prepared by the usual procedure and melted at 150–152° (85% yield from ethanol).

Anal. Calcd. for $C_6H_{14}N_4SO_2$: C, 34.93; H, 6.84; N, 27.16; S, 15.55. Found: C, 35.20; H, 6.77; N, 26.91; S, 15.37.

The *hydrazide of* XVI was prepared by the usual procedure and melted at 126–127.5° (89.5% yield from ethanol).

Anal. Calcd. for $C_{10}H_{22}S_2N_4O_2$: C, 40.79; H, 7.53; N, 19.03; S, 21.78. Found: C, 40.87; H, 7.59; N, 18.86; S, 22.11.

Reaction of glutathione with myleran. Myleran (12.3 g., 0.05 mole) was added to a basic solution of glutathione, prepared by adding 100 ml. of sodium hydroxide (12.4 g., 0.13 mole) solution to a solution of glutathione (30.7 g., 0.1 mole) in 500 ml. of water. After about 10–15 min., the odor of tetrahydrothiophene was noted. The reaction mixture was stirred under a nitrogen atmosphere in a closed vessel at room temperature for 66 hr. The myleran gradually dissolved and the reaction mixture turned pale yellow. The reaction mixture was extracted three times with 150 ml. of benzene and the organic layer was dried with anhydrous magnesium sulfate. The drying agent was removed and the benzene solution was treated with a solution of mercuric chloride (13.6 g., 0.05 mole) in 100 ml. of ethanol. A white precipitate formed after 1 hr. (0°) which was collected, washed with a little ethanol, and air dried. There was obtained 11.9 g. (66.5% yield) of tetrahydrothiophene mercuric chloride, m.p. and mixture m.p. 127–129°.

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(9) W. F. Faragher, J. C. Morrell, and S. Comay, *J. Am. Chem. Soc.*, **51**, 2781 (1929).

(10) E. V. Whitehead, R. A. Dean, and F. A. Fidler, *J. Am. Chem. Soc.*, **73**, 3634 (1951).

(11) A sample of benzyl sulfide was kindly supplied by R. Koncos, University of Minnesota, prepared as described by J. de Pascual Teresa, *Anales fis. y quim.* (*Madrid*), **40**, 426 (1944).