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DITHIAZEPANE FORMATION FROM REACTIVE BIFUNCTIONAL ALKYLATING AGENTS

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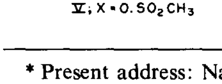
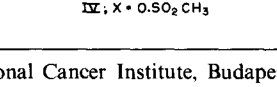
Abstract—The technique of trapping a reactive alkylating metabolite with sodium sulphide-^{35}S, previously applied to the azo-mustard, 2′-carboxy-4-di-(2-chloroethyl)amino-2-methylazobenzene (CB 1414) has been extended to the related di-(2-methanesulphonyloxyethyl)amino analogue (CB 10221). In addition to the previously reported 4-(4-amino-3-methylphenyl)-1,4-thiazane, mass spectrometry afforded evidence for the additional formation of 5-(4-amino-3-methylphenyl)-1,2-dithia-5-azepane when CB 10221 was incubated with a rat liver homogenate in the presence of sodium sulphide. Dithiazepane formation was further exemplified by the production of 4-(4-hydroxyphenyl)-1,4-thiazane and 5-(4-hydroxyphenyl)-1,2-dithia-5-azepane from *N*,*N*-di-(2-chloroethyl)-4-hydroxyaniline (*p*-hydroxyaniline mustard) and sodium sulphide. The preparation of thiazanes of some additional compounds of interest in relation to metabolic studies is also reported. The implications of dithiazepane formation for the identification and quantitative determination of alkylating metabolites by this trapping procedure are discussed.

In a previous report from this laboratory, the use of radioactively labelled sodium sulphide to trap a reactive metabolite of an antitumour alkylating agent was exemplified [1]. Thus, 4-di-(2-chloroethyl)amino-2-methylaniline (I), produced when the azo mustard, 2′-carboxy-4-di-(2-chloroethyl)amino-2-methylazobenzene (CB 1414, II) was incubated with a 9000*g* supernatant from rat liver, reacted rapidly *in situ* with sodium sulphide-^{35}S to yield a radioactively labelled thiazane (III). This work formed part of an investigation into the use of azo-mustards as selective agents in the treatment of hepatomas. Reduction of these compounds in the liver to reactive amines such as (I) would liberate the cytotoxic agent in proximity to its intended site of action. Hopefully, the shorter the half-life for hydrolysis of such an amine [for (I), $t_{\frac{1}{2}} = 8{\cdot}5$ min at 37°] the less will be the damage to rapidly proliferating normal tissues at sites remote from the liver. Azo-mustards which by reduction might yield amines with an even shorter half-life than (I), have been prepared and their suitability as selective agents has been evaluated [2] by assessing toxicity and the rate of reduction by the 9000*g* supernatant obtained from liver homogenates. The latter data was obtained by determining the released anthranilic acid by the Bratton–Marshall reaction [3]. We now report on the trapping of the reactive alkylating product from the metabolism of one such compound, 2′-carboxy-4-di-(2-methanesulphonyloxyethyl)-amino-2-methylazobenzene (CB 10221, IV), a potential precursor of the amine, 4-di-(2-methanesulphonyloxyethyl)amino-2-methylaniline (V) with a half-life at 37° of 4·7 min.



I; X = Cl
V; X = O.SO$_2$CH$_3$



II; X = Cl
IV; X = O.SO$_2$CH$_3$

MATERIALS AND METHODS

Quantities and conditions for the metabolism of CB 10221 and for the extraction, chromatography and mass spectrometry of products were closely similar to those reported for the metabolism of CB 1414 (see "Preparation of 9000*g* supernatant" and "Isolation of metabolites" in Materials and Methods section of ref. 1) except that, additionally, one mass spectrum was obtained at an ionizing voltage of 12 eV (see Fig. 1a).

The progress of the chemical reactions reported below, and the homogeneity of the products was also monitored by the previously reported [1] TLC procedure.

Nomenclature of compounds

The nomenclature "thiazane" is now preferred to the previously used "thiazan" [1] and in this paper is taken to be synonymous with "1,4-thiazane".

Synthesis of thiazanes

4-*Hydroxyphenylthiazane.* *N*,*N*-Di-(2-chloroethyl)-4-hydroxyaniline hydrochloride [4] (0·5 g) was converted into the free base by shaking with saturated

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aqueous sodium bicarbonate (50 ml) and ether (50 ml). The dried ($MgSO_4$) organic phase was concentrated and the residue was treated with a boiling solution of sodium sulphide (micro-analytical grade, BDH Chemicals Ltd., 2·5 g in methanol (30 ml) for 1 hr. Silicic acid (Merck, Kieselgel G, 5 g) was then added and the mixture was concentrated to dryness. The residue was applied to the top of a dry column of silicic acid (25 g) which was eluted with chloroform (10 ml fractions). The concentrate from fractions 20–40 was rechromatographed on a larger column (35 × 2 cm) of silicic acid (60 g). Fractions 31–50 (eluted with chloroform) contained two components, R_f (TLC, chloroform–methanol, 97:3) 0·30 and 0·40, each of which gave a positive reaction with the potassium dichromate–silver nitrate spray test for bivalent sulphur compounds [5]. After separation by TLC these components were extracted from the adsorbent with methanol, and their mass spectra were determined (Fig. 2). The more mobile product gave a spectrum appropriate for a dithiazepane (VII, Fig. 2a) and the less mobile product, for a thiazane (VIII, Fig. 2b) (see Results and Discussion). The latter compound was obtained as colourless rods (0·11 g, 30%), m.p. 151–152° when the residue from fractions 31–50 was crystallized from toluene (~5 ml). (Found: C, 61·4; H, 6·85; N, 7·1; S, 16·65% $C_{10}H_{13}NOS$ requires C, 61·5; H, 6·7; N, 7·15; S, 16·45%).

A lower m.p. (132°) is reported for the product obtained [6] from 4-hydroxyaniline and *bis*(2-chloroethyl)sulphide.

4-*Phenylthiazane*. This compound was prepared from *N*,*N*-di-(2-chloroethyl)aniline (aniline mustard) by a method similar to that of Ross [7] except that methanol replaced aqueous acetone as a reaction medium. The product had m.p. 34° (ref. 7,31°). The mass spectrum showed the appropriate molecular ion at *m/e* 179 (base peak). There was no corresponding signal for the dithiazepane (mol. wt 211).

4-(*Thiazan*-4-*yl*)*phenyl sulphate*. A solution of the lutidine salt of 4-di-(2-chloroethyl)aminophenyl sulphate [8] (0·1 g) and sodium sulphide (0·2 g) in water (3 ml) was kept at room temperature for 15 hr then concentrated to dryness. A solution of the residue in methanol (5 ml) was treated with silicic acid (1 g), the mixture was concentrated to dryness, and the residue was applied to the top of a dry column (25 × 1 cm) of silicic acid (10 g) which was eluted with butan-1-ol–water (86:14, 5 ml fractions). The first 50 ml of eluate contained the product with R_f (TLC, butan-1-ol–water, 86:14) 0·35 (starting material has R_f 0·44). Dissolution of the concentrated eluate in methanol–propan-2-ol and concentration to 1 ml afforded the sodium salt of the title compound as colourless needles (16 mg, 21%) of the dihydrate. (Found: N, 4·4; S, 19·2%. $C_{10}H_{12}NaNO_4S_2 . 2H_2O$ requires N, 4·2; S, 19·25%).

4-(*Thiazan*-4-*yl*)*phenyl β*-D-*glucopyranosiduronic acid*. The isolation procedure described for the preceding compound was applied to the reaction of the tetra-*n*-butylammonium salt of [*N*,*N*-di-(2-chloroethyl)amino]phenyl-*β*-D-glucopyranosiduronic acid [8] (0·22 g) with sodium sulphide (0·44 *g*) in water (6 ml). Fractions 15–27 (10 ml fractions, silicic acid 30 g, column 18 × 2 cm) afforded the product, R_f (TLC, butanol–water, 86:14) 0·10 (starting material has R_f 0·20), which was crystallized as described above to yield the sodium salt of the title compound (30 mg, 19%). (Found: N, 3·55; S, 8·25%. $C_{16}H_{20}NaNO_7S$ requires N, 3·55; S, 8·15%).

4-[4-(*Thiazan*-4-*yl*)*phenyl*]*butyric acid*. A solution of 4-[4-di-(2-chloroethyl)aminophenyl]butyric acid (chlorambucil [9], 0·5 g) and sodium sulphide (0·5 g) in methanol (5 ml) was heated under reflux for 16 hr. The supernatant was concentrated to dryness after the addition of silicic acid (3 g) and the residue was applied to the top of a column (10 × 2 cm) of silicic acid (15 g) which was eluted with chloroform–methanol (19:1, 10 ml fractions). Fractions 7–15 contained the title product (0·3 g) which gave colourless plates (0·12 g, 28%), m.p. 67·5–69·5° from ether–petroleum ether (b.p. 30–40°) (1:1). (Found: C, 63·25; H, 7·4; N, 5·4; S, 12·05%. $C_{14}H_{19}NO_2S$ requires C, 63·4; H, 7·15; N, 5·35; S, 12·1%).

The mass spectrum showed the appropriate molecular ion (*m/e* 265, base peak) and no signal for a dithiazepane (*m/e* 297).

3-[4-(*Thiazan*-4-*yl*)*phenyl*]-L-*alanine*. The preparative conditions for the preceding compound were applied to 3-[4-di-(2-chloroethyl)aminophenyl]-L-alanine (melphalan [10], 0·5 g) except that the product (0·32 g, 73%) was eluted from the column with chloroform–methanol (3:1, 400 ml) as an amorphous white solid (m.p. 208–210°). (Found: C, 58·6; H, 6·5; N, 10·7; S, 12·1%. $C_{13}H_{18}N_2O_2S$ requires C, 58·6; H, 6·8; N, 10·5; S, 12·0%).

The mass spectrum showed a molecular ion at *m/e* 266 (3% of base peak at *m/e* 192). The signal for the dithiazepane (*m/e* 298) was absent.

Hydrolysis of 4-(*thiazan*-4-*yl*)*phenyl sulphate and* 4-(*thiazan*-4-*yl*)*phenyl β*-D-*glucopyranosiduronic acid*. A solution of each thiazane (1 mg) in N HCl (0·1 ml) sealed in a capillary, was immersed in boiling water for 16 hr. The hydrolysates were neutralized with Amberlite IR45 (HO^-) resin then extracted with chloroform. The extracts each contained a single u.v.-absorbing component identical (TLC, mass spectrometry) with synthetic 4-hydroxyphenylthiazane.

Reaction of 4-*di*-(2-*bromopropyl*)*amino*-2-*methylaniline with sodium sulphide*. The hydrochloride of the title compound [2] (0·1 g) was added to a solution of sodium sulphide (0·5 g) in water (5 ml) and acetone (5 ml). After 2 hr, the solution was extracted with chloroform and the dried ($MgSO_4$) extract was subjected to TLC in chloroform. The mass spectrum of the product (R_f 0·20) contained signals appropriate for the molecular ions of 5-(4-amino-3-methylphenyl)-3,7-dimethyl-1,2-dithia-5-azepane (*m/e* 268, base peak) and 4-(4-amino-3-methylphenyl)-2,5-dimethylthiazane (*m/e* 236, 12%).

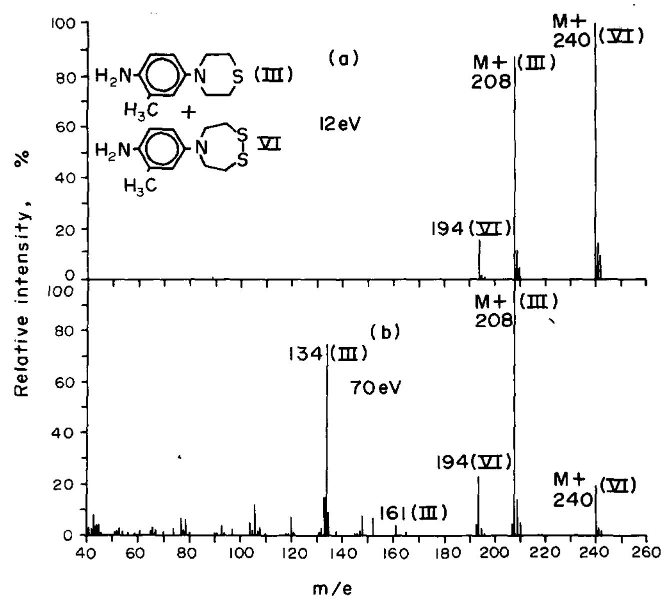

Fig. 1. Mass spectra of the mixture of products from the incubation of CB 10221 with 9000 *g* liver supernatant and radioactively labelled sodium sulphide: (a) 12 eV spectrum; (b) 70 eV spectrum. Figures in brackets after the *m/e* values refer to the component to which the signal is ascribed: 4-(4-amino-3-methylphenyl)-1,4-thiazane (III) or 5-(4-amino-3-methylphenyl)-1,2-dithia-5-azepane (VI).

RESULTS AND DISCUSSION

When the azo mustard CB 10221 (IV) was incubated with a 9000*g* liver supernatant and radioactively labelled sodium sulphide under the conditions previously applied to CB 1414 (II), a sulphur-containing product additional to the expected, and previously observed [1], thiazane (III) was detected by mass spectrometry. Thus, the mass spectra at 70 and 12 eV (Fig. 1) of the radiochromatographically homogeneous product contained major peaks at *m/e* 240 and 194, in addition to the signals previously reported for thiazane (III) of which the principal were the molecular ion (*m/e* 208) and the fragment ion at *m/e* 134. The additional signals were more prominent in the spectrum measured at reduced ionizing voltage (12 eV, Fig. 1a) than at the conventional ionizing potential of 70 eV (Fig. 1b). The intensity ratio of 10: 1 for the peaks at *m/e* 240 and 242* and comparison of the molecular weight with that of the thiazane both indicated that the new component was the dithiazepane, 5-(4-amino-3-methylphenyl-1,2-dithia-5-azepane (VI). The ion of *m/e* 194 which contains one sulphur atom (see footnote) could be ascribed to the loss of the fragment CH_2S from the molecular ion of (VI) which may be compared with the loss of CH_3S from the molecular ion of the thiazane (III) which was reported previously [1].

Since the supposed dithiazepane (VI) could not be separated from the thiazane (III) by chromatography, other evidence was sought for the formation of dithiazepanes as products of the reaction between bifunctional alkylating agents and sodium sulphide. Such evidence was obtained during an evaluation of sodium sulphide as a trapping agent for metabolites [12] of aniline mustard [*N*,*N*-di-(2-chloroethyl)aniline]. Sodium sulphide reacted readily with aniline mustard and the ethereal sulphate and glucuronide conjugates of *p*-hydroxyaniline mustard [N,N-di-(2-chloroethyl)-4-hydroxyaniline] to give thiazanes apparently as the sole, sulphur-containing products. However, two such products were formed when *p*-hydroxyaniline mustard reacted with sodium sulphide. These were separable by TLC and gave mass spectra appropriate for a dithiazepane (VII, Fig. 2a) and a thiazane (VIII, Fig. 2b), respectively. The presence of appropriate metastable peaks at *m/e* 144·3 (*m/e* 227→181) and at *m/e* 100·7 (*m/e* 181→135) was evidence for the successive loss of the two sulphur atoms from the molecular ion of (VII) as parts of two CH_2S fragments. The intensities of $(X + 2)^+$ ions corresponding to *m/e* 227, 181, and 135 were also consistent with this scheme*. The major fragmentation pathways for the molecular ion of the thiazane (VIII) were those previously recorded for the thiazane (III) [1]. Attempts to isolate (VII) in bulk by column chromatography were unsuccessful; mixtures with the thiazane (VIII) resulted. Günther and Mautner, who first isolated the parent dithiazepane,

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* The number of sulphur atoms present in an ion may be deduced from the contribution of the sulphur isotope ^{34}S. Thus, an ion X^+ containing only ^{32}S is accompanied by an ion $(X + 2)^+$ of intensity, relative to X^+, of 4·4% if X^+ contains one sulphur atom, and 8·9 per cent if two sulphur atoms are present [11]. The slightly higher intensities observed in practice for $(X + 2)^+$ are due to small additional isotopic contributions, principally from ions containing two ^{13}C atoms.

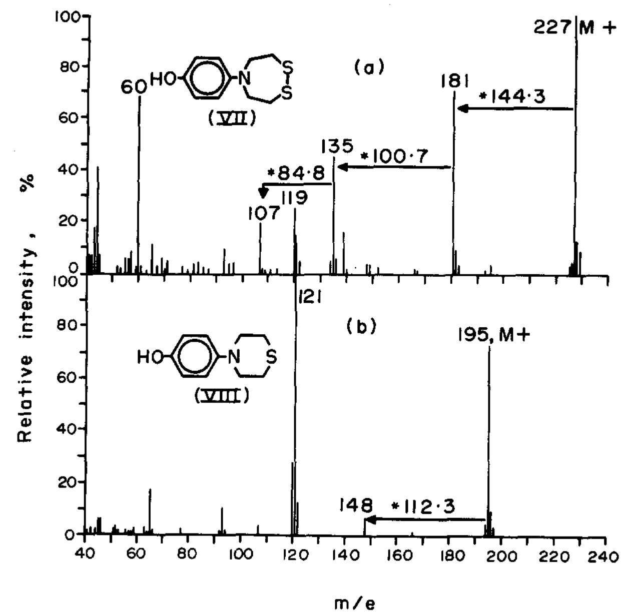
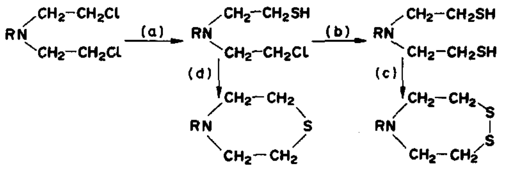


Fig. 2 Mass spectra at 70 eV of products from the reaction of *p*-hydroxyaniline mustard with sodium sulphide: (a) 5-(4-hydroxyphenyl)-1,2-dithia-5-azepane (VII); (b) 4-(4-hydroxyphenyl)-1,4-thiazane (VIII).

1,2-dithia-5-azepane, as its hydrochloride in 1960, reported the free base to be unstable [13].

The mechanism of dithiazepane formation in the reactions reported here is unclear. It is unlikely to be caused by a sodium disulphide impurity, despite the fact that the above-mentioned workers [13] prepared the parent compound by reacting *N*,*N*-di-(2-chloroethyl)amine with sodium disulphide. Thus, in contrast to p-hydroxyaniline mustard, aniline mustard and the above-mentioned conjugates do not appear to afford dithiazepanes on reaction with sodium sulphide. The thiazanes formed from the conjugates were too involatile to afford mass spectra, but supporting evidence for their structures was their acidic hydrolysis to 4-hydroxyphenylthiazane. Two other important antineoplastic alkylating agents, chlorambucil and melphalan, when condensed with sodium sulphide, also afforded only thiazanes. In contrast, 4-di-(2-bromopropyl)amino-2-methylaniline, which hydrolyses even more rapidly ($t_{\frac{1}{2}}$ = 41 sec at 37°) [2] than (V), abundantly formed a dithiazepane.

An alternative mechanism for dithiazepane formation is shown in Scheme 1 (a–c). Intermolecular displacement of each chlorine atom (the argument also applies to other leaving groups) by a sulphide ion is followed by atmospheric oxidation of the intermediate bis(2-thioethyl) derivative to the dithiazepane. The extent of dithiazepane formation would then depend upon the relative rates of the intermolecular displacement (b) and the intramolecular displacement (d) leading to the thiazan. Whereas this limited study suggests that dithiazepanes are formed from compounds which are relatively reactive to hydrolysis, and hence to attack by other nucleophiles, it is not clear why such a property would favour the intermolecular reaction (b) over the intramolecular reaction (d).



Scheme 1. Mechanisms for the formation of thiazanes and dithiazepanes from *N*,*N*-*bis*(2-chloroethyl)amines

From a qualitative viewpoint, the observation that dithiazepanes can be formed from certain reactive bifunctional alkylating agents does not detract from the potential utility of sodium sulphide as a trapping agent, since the dithiazepanes give characteristic mass spectra from which the structure of the original alkylating species can be readily deduced. In quantitative determinations, however, allowance must be made for the two-fold incorporation of sulphur in dithiazepane formation, and if mass spectrometry affords evidence that both a thiazane and a dithiazepane are formed, these must be separately determined.

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REFERENCES

1. T. A. Connors, A. B. Foster, A. M. Gilsenan, M. Jarman, and M. J. Tisdale, *Biochem. Pharmac.* **21**, 1309 (1972).
2. M. A. Bukhari, T. A. Connors, A. M. Gilsenan, W. C. J. Ross, M. J. Tisdale, G. P. Warwick and D. E. V. Wilman, *J. natn. Cancer Inst.* **50**, 243 (1973).
3. A. C. Bratton and E. K. Marshall, *J. biol. Chem.* **128**, 537 (1939).
4. M. H. Benn, A. M. Creighton, L. N. Owen and G. R. White, *J. chem. Soc.* 2365 (1961).
5. R. H. Knight and L. Young, *Biochem. J.* **70**, 111 (1958).
6. N. Tcherkerzoff, *Rev. Inst. Franc. Petrole Ann. Combust. Liquides* **18**, 438 (1963).
7. W. C. J. Ross, *J. chem. Soc.* 815 (1950).
8. M. A. Bukhari, J. L. Everett and W. C. J. Ross, *Biochem. Pharmac.* **21**, 963 (1972).
9. J. L. Everett, J. J. Roberts and W. C. J. Ross, *J. chem. Soc.* 2386 (1953).
10. F. Bergel and J. A. Stock, *J. chem. Soc.* 2409 (1954).
11. J. H. Beynon, R. A. Saunders, and A. E. Williams, *The Mass Spectra of Organic Molecules*, pp. 352. Elsevier, Amsterdam (1968).
12. T. A. Connors, P. B. Farmer, A. B. Foster, A. M. Gilsenan, M. Jarman and M. J. Tisdale, *Biochem. Pharmac.* **22**, 1971 (1973).
13. W. H. H. Günther and H. G. Mautner, *J. Med. Chem.* **82**, 2762 (1960).