Biochemical Pharmacology, Vol. 28, pp. 859–865.
Pergamon Press Ltd. 1979. Printed in Great Britain.

THE METABOLISM OF A CHLORINATED EPOXIDE (MME): DIOL FORMATION BY PIG LIVER MICROSOMES

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(*Received* 8 *February* 1978; *accepted* 2 *August* 1978)

Abstract—A cyclodiene expoxide MME† with one methyl group attached to the oxirane ring was converted into a single metabolite (M_1) when incubated with pig liver microsomes in the absence of NADPH. Mass spectroscopy of M_1 and its TMS derivative established that it was a diol, indicating that the conversion was mediated by epoxide hydratase. N.m.r. spectra of M_1 with and without the chiral shift reagent, EU $(fod)_3$ suggest that the metabolite is a *cis*-diol. When MME was hydrated in acid solution, a diol was produced which differed from M_1 in its chromatographic behaviour and n.m.r. spectrum. The rate of formation of M_1 from MME by pig liver microsomes was very much slower (less than 0.4 per cent) of that found with the analogous epoxide HEOM‡ which has no methyl group attached to the oxirane ring.

A wide range of epoxides is converted to vicinal diols by one or more forms of epoxide hydratase (EC 4.2.1.63) [1–10]. Substrates for the enzyme include certain chlorinated insecticides [11–13], insect juvenile hormones and expoxides of the carcinogens 3,4-benzopyrene and 1,2-benzanthracene [2, 11, 14–16]. In vertebrates higher epoxide hydratase activity is found in liver microsomes than in kidney microsomes, whilst preparations of lung, muscle and gut have little or no activity [11]. The epoxide hydratase of liver microsomes is inducible by phenobarbitone or 3-methylcholanthrene, is stereoselective [12], and shows greater activity in mammals than in birds and fish when the chlorinated epoxide HEOM is used as a substrate [17, 18]. Many epoxides are hydrated by acid solutions and the final products are often *trans*-diols [13] although dieldrin can also form a *cis*-diol on acid hydrolysis under certain conditions [19, 20]. For some time *trans*-diols were thought to be the sole products of epoxide hydratase action on cyclodiene epoxides, but recently McKinney *et al.* have shown that dieldrin is first converted to its *cis*-diol which is then transformed to the *trans*-diol [21–23].

The present communication is concerned with the hydration of an analogue (MME) of HEOM by vertebrate liver microsomes. MME differs from HEOM in possessing a methyl group in the C_7 position of the unchlorinated ring. This makes the molecule asymmetrical and is likely to sterically hinder enzymic attack on the oxirane ring.

MATERIALS AND METHODS

Chemical reagents Analar grade reagents and solvents were purchased from BDH. Hexachloro [G-^{14}C]cyclopentadiene was purchased from the Radiochemical Centre, Amersham, as a solution in benzene (specific activity 7.41 μCi/mg). Unlabelled hexachlorocyclopentadiene, isoprene, pyridine, hexamethyldisilazane, trimethylchlorosilane, dioxan, vinyl chloride gas and the liquid scintillation fluid KL 354 were purchased from Koch-Light Ltd., tri-sil TBT was bought from Pierce Chemical Company. SE52, Apiezon L, Epikote 1001, OV 225 and Chromosorb W (AW DCMS) were purchased from Perkin Elmer. Deuterochloroform ($CDCl_3$) and Europium 1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedion ($Eu(fod)_3$), were provided by the Chemistry Department, Reading University. Florisil and Kieselgel G were purchased from Anderman Ltd.

Purification procedures (i) Column chromatography was carried out using 3% water/florisil packed into sintered glass columns to a depth of 5 cm. Columns were washed with n-hexane before use and samples were applied as solutions in 30% ether–hexane. The following fractions were collected: (a) 30 ml n-hexane, (b) 64 ml of 40 percent ether–hexane collected as 8 × 8 ml sub-fractions, (c) 30 ml of 100% ether as 3 × 10 ml sub-fractions and (d) 30 ml of 23:1:1 ether–acetone–methanol as 3 × 10 ml sub-fractions.

(ii) Thin layer chromatography (t.l.c.) was carried out using 20 × 20 cm plates coated with 250 microns of Kieselgel G activated before use by heating for 2 hr at 120°. Various mobile phases were used; these are quoted in the text or in the appropriate table.

Radioactivity was located using a Panax t.l.c. radioscanner. Unlabelled material was located by spraying with silver nitrate solution (0.5% w/v $AgNO_3$ in 95% ethanol) and irradiating with ultraviolet light (254 mn). Chlorinated material showed as brown spots. Products were eluted from the silica gel with acetone.

Analytical procedures. (i) Gas–liquid chromatography (g.l.c.) was carried out using a Perkin-Elmer F-11 instrument fitted with a Ni^{63} electron capture detector. Glass columns (1–6 feet long; i.d. 0.5 cm) were used and were packed with DMCS treated Chromosorb W (100–120 mesh) coated with one of the following stationary phases (a) 2.5% Apiezon L and O Epikote

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† MME = 1,2,3,4,9,9-hexachloro-1,4,4a,5,8,8a-hexahydro-6-methyl-6,7-epoxy-1,4-methanonaphthalene.

‡ HEOM = 1,2,3,4,9,9-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-methanonaphthalene.

1001, (b) 2.5% Se52 and 0.5% Epikote 1001, (c) 3% OV225% Epikote 1001. The carrier gas used was oxygen-free nitrogen (OFN) at a flow rate of 70 ml min^{-1}. Operating temperatures were 160° for phase (a), 154° for (b) and 140° for (c).

Samples (3 μl) were injected on to columns as solutions in n-hexane. Relative retention times were calculated with respect to dieldrin which was ascribed a value of 100.

(ii) Liquid scintillation counting for ^{14}C was carried out using a Tracerlab instrument. Quench correction was based on external Standard Channels ratio procedures. Samples were dissolved in 0.5 ml diethyl ether and mixed with 10 ml KL 354 in glass vials for counting.

(iii) Mass spectroscopy (MS) was carried out using electron impact fragmentation (EIMS) and chemical ionisation (CIMS) techniques with respectively, an AEI MS12 and a Finnigan 3200E quad in line to a 6000 data system. Samples were either introduced by direct insertion on a probe heated to 200° or by way of a g.l.c. column. For CIMS, methane was the reactant gas at a source pressure of 700 μm (eV = 100). Multiplier voltage was 1.75 kV.

(iv) Nuclear magnetic resonance (n.m.r.) studies were carried out with a Varian T 60 (60 MHz) attached to a computer of average transients (c.a.t.). Samples (4 mg) were dissolved in 0.5 ml deuterochloroform and 60 MHz n.m.r. spectra were run in the presence and absence of the chiral shift reagent $Eu(fod)_3$. [24]

Synthesis

(i) *Synthesis of 1,2,3,4,9,9-hexachloro [1,2,3,4,9-^{14}C]-1,4,4a-5,8,8a-hexahydro-6-methyl, 1,4-methanonaphthalene ([^{14}C]-MMO) from hexachloro[G-^{14}C]cyclopentadiene.* 612 mg of hexachloro [G-^{14}C]cyclopentadiene (0.765 μCi/mg) were cooled to −70° and reacted with 142 mg of vinyl chloride (initially gas) in a flask. The flask was slowly heated to 180° and kept at this temperature for 20 hr. The reaction products containing 570 mg of 1,2,3,4,5,7,7-heptachloro [1,2,3,4,7-^{14}C]norbornene were refluxed for 8 hr with 10 ml of 8% (v/v) alcoholic potassium hydroxide. The reaction products were added to isoprene in the ratio by weight of 4:1 and were heated in a Carius tube to 130° for 16 hr.

(ii) *Synthesis of a 6,7 epoxide derivative of* [^{14}C]-*MMO.* 20 mg of the major reaction product from the Carius tube ([^{14}C] MMO) were reacted with an excess of perbenzoic acid at room temperature for 48 hr, after which time the reaction mixture was neutralised with a 10% sodium bicarbonate solution. The epoxide, 1,2,3,-4,9,9-hexachloro [1,2,3,4,9-^{14}C]-1,4,4a,5,8,8a-hexahydro-6-methyl, 6,7-epoxy,1,4-methanonaphthalene, hereafter referred to as [^{14}C]-MME, was extracted first into chloroform. This solution of [^{14}C]-MME was purified on a 3% water:Florisil column and by successive t.l.c. separations using three different mobile phases* until a radiochemical purity greater than 99.5 percent and a constant specific activity of 0.36 μCi/mg was obtained.

* Mobile phases: (1) 2:1.5 benzene/ethyl acetate, R_F value of [^{14}C]-MME, 0.70. (2) 1:1 benzene/hexane, R_F value of [^{14}C]-MME, 0.59. (3) 7:2.1 cyclohexane/liquid paraffin/dioxan, R_F value of [^{14}C]-MME, 0.65.

[^{14}C]-MME had the same g.l.c. and t.l.c. and m.s. characteristics as authentic MME and was metabolised in an identical manner to it when incubated with pig, hamster and rabbit 105,000 *g* liver microsomes [30]. With each species, the single product formed from [^{14}C]-MME was identical to that formed from MME, and is referred to as M_1.

(iii) *Synthesis of* S_1. Chemical synthesis of the *trans* diol of MMO was attempted by incubating 0.1 g MMO with 2 ml glacial acetic acid, 0.6 ml formic acid, 1 ml of 30% hydrogen peroxide and 0.04 ml 10M H_2SO_4 at 35° in the procedure described by El Zorgani [25]. The main product S_1 (yield 30 per cent) was purified by t.l.c. and florisil column chromatography and was then re-crystallised from ethyl acetate to give greyish-white flaky crystals (uncorrected m.p. 137–138°). S_1 formed a single TMS derivative after prolonged contact (48 hr) with silylating reagents.

(iv) *Biosynthesis of* M_1 *from* [^{14}C]-*MME using pig liver microsomes.* (*a*) *Enzyme source.* 50 g of liver were removed from a sow 20 min after death and placed on ice. Washed liver microsomes (105,000 *g*) were prepared as described by Walker *et al.* [19] at a concentration of 2 g liver per ml of microsomal suspension.

(*b*) *Incubation procedure.* 25 ml of microsomal suspension were added to 226 ml of buffered reaction mixture (pH 7.4) which contained 0.235 g KCl, 1.898 g $NaH_2PO_4 2H_2O$, 0.398 g NaOH and 0.156 g nicotinamide in an open reaction flask. 2 mg of [^{14}C]-MME (0.036 μCi/mg) dissolved in 1.0 ml of ethanol were added to the reaction flask which was held in a water bath (Mickle Metabolic) maintained at 37°, and shaken at 30 c.p.m. An additional 2 mg of [^{14}C]-MME were added 4 and 11 hr after the start of the incubation.

Samples (0.5 ml) were removed at selected intervals, extracted with ether (2 ml) and these extracts analysed by g.l.c. to monitor the formation of the single metabolite M_1.

After 14 hr the incubation mixture was extracted with 6 × 200 ml of diethyl ether. The combined ether extracts containing M_1 were dried over 10 g anhydrous sodium sulphate and concentrated to 10 ml using oxygen-free nitrogen. M_1 was purified by florisil column chromatography and by t.l.c. to give material sufficiently pure for further analysis. (Radiochemical purity > 99.0 per cent).

(*c*) *Silylation procedures.* A silylating mixture was prepared by adding 0.5 ml trimethylchlorosilane and 1.0 ml hexamethyldisilazane to 5 ml of dry pyridine. A series of tubes containing 2 μg of M_1 or S_1 were incubated at 40° with 0.3 ml of the silylation mixture for periods up to 24 and 48 hr, respectively. Reaction mixtures were then evaporated to dryness with a stream of nitrogen gas and residues were dissolved in 10 ml 40% ether: hexane; 3 μl aliquots were analysed by g.l.c. Blank samples, containing only reagent, showed no interfering peaks.

Alternatively Tri-sil TBT (25 μl) was added to 10 μg quantities of metabolites, heated at 60° for 15 min and the mixture injected directly on column for GC–MS.

RESULTS

Mass spectral examination of M_1 *and* S_1

The EI mass spectrum of M_1 (Fig. 1a) shows a molecular ion of *m/e* 398. This molecular ion possesses

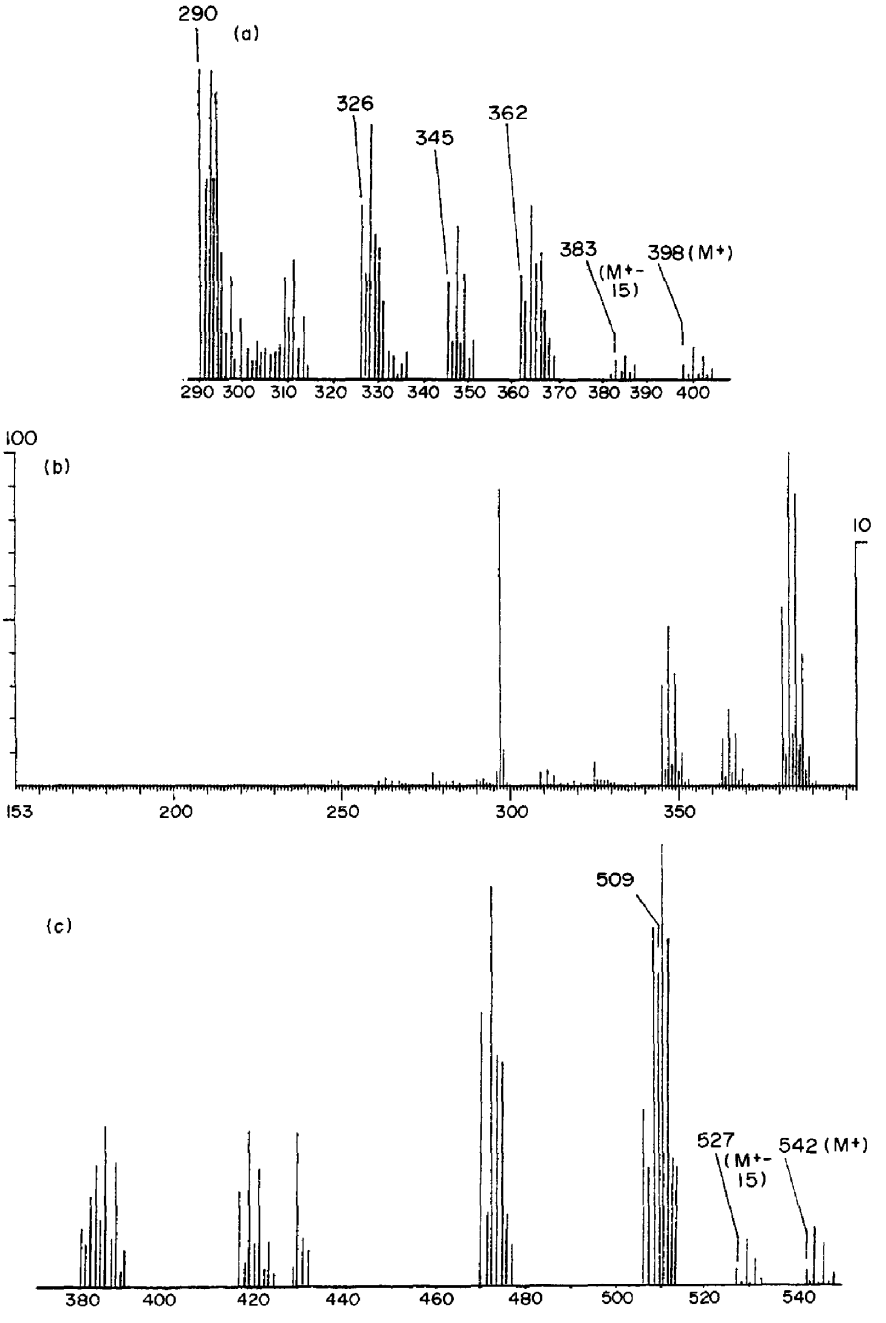

Fig. 1. (a) EI Mass spectrum of metabolite M_1. (b) CI Mass spectrum of metabolite M_1. (c) EI Mass spectrum of the TMS derivative of metabolite M_1.

the distinctive isotope abundance pattern for six chlorine atoms and its mass corresponds to that of a diol formed by hydration of the epoxide ring of MME. Other ions visible in the upper part of the spectrum were identified as $(M\text{-}15)^+$, *m/e* 283; $(M\text{-Cl})^+$, *m/e* 363; $(M\text{-HCl})^+$, *m/e* 362; followed by a series of ions of lower mass arising from successive losses of Cl and HCl (*m/e* 327, 326, 291, 290). It was not possible to distinguish a base peak.

CIMS of M_1 confirms its molecular weight to be 398 (Fig. 1b). Although there is no $(M + 1)^+$ ion seen in the spectrum, two ions (*m/e* 381 and 363) which arise from the successive loss of H_2O from the $(M + 1)^+$ ion are visible. Each contains six chlorine atoms.

Confirmation that the TMS derivative of M_1 is the diTMS ether was made by EIMS (Fig. 1c). The molecular ion is visible (*m/e* 542) and although the base peak is obscured the following ions are visible:- $(M - 15)^+$, *m/e* 527; $(M - Cl)^+$, *m/e* 509; $(M\text{-CL-TMSOH})^+$, *m/e* 417.

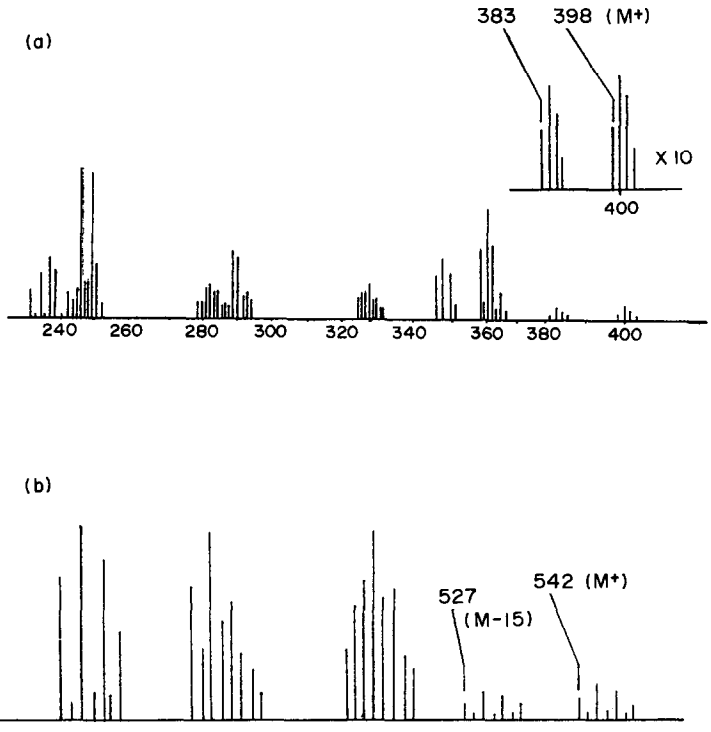

Fig. 2. (a) EI Mass spectrum of S_1. (b) EI Mass spectrum of the TMS derivative of S_1.

The EI mass spectra of S_1 and its diTMS ether (Fig. 2a and b) are similar to those of M_1 and its diTMS ether and confirm S_1 as a diol of MME. However, the chromatographic properties of the two compounds and their TMS derivatives are different, as shown in Table 1.

The silylation characteristics of M_1 and S_1 also differ. M_1 was completely silylated during 24 hr contact with silylating reagent, when a single product thought to be the diTMS ether, was formed (Apiezon RRT = 85). In contrast, the silylation of S_1 was not complete after 24 hr; at this time two products were visible on g.l.c. (Apiezon RRT 79, 110). An additional 24 hr reaction with silylating reagent led to the complete disappearance of the component eluting with RRT 110, and the concomitant increase of that with RRT 79. As stated above, the latter was shown by mass spectrometry to be the diTMS ether of S_1. It is presumed that the component of RRT 110 was the mono TMS ether of S_1.

Table 1. T.l.c. and G.l.c. characteristics of MME, M_1 and S_1

Compound	T.l.c. R_F Values	*T.l.c. Solvent system	G.l.c. Column	G.l.c. relative retention times (HEOD† = 100) Underivatised compound	TMS derivative
MME	0.87	a			
	0.70	b	2.5% Apiezon	83	
	0.59	c	2.5% SE52	92	
	0.65	d			
M_1	0.45	a	3% OV225	720	50
	0.53	e	2.5% Apiezon	380	85
	0.05	f	2.5% SE52	346	—
	0.95	g			
	0.7	h			
S_1	0.53	a	3% OV225	—	44
	0.33	h	2.5% Apiezon	413	79
	0.31	i	2.5% SE52	358	88

* T.l.c. solvent systems—(a) 1:1 benzene:ethyl acetate; (b) 2:1.5 benzene: ethyl acetate; (c) 1:1 benzene:hexane; (d) 7:2:1 cyclohexane/paraffin/dioxan; (e) 35% acetone:hexane; (f) 10% acetone:hexane; (g) 1:1 acetone:hexane; (h) 2:1 benzene:ethyl acetate; (i) 3:1 benzene:ethyl acetate.
—Indicates not measured.

† HEOD is 1, 2, 3, 4, 10, 10-hexachloro-6, 7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-endo-exo-1,4:5,8-dimethanonaphthalene.

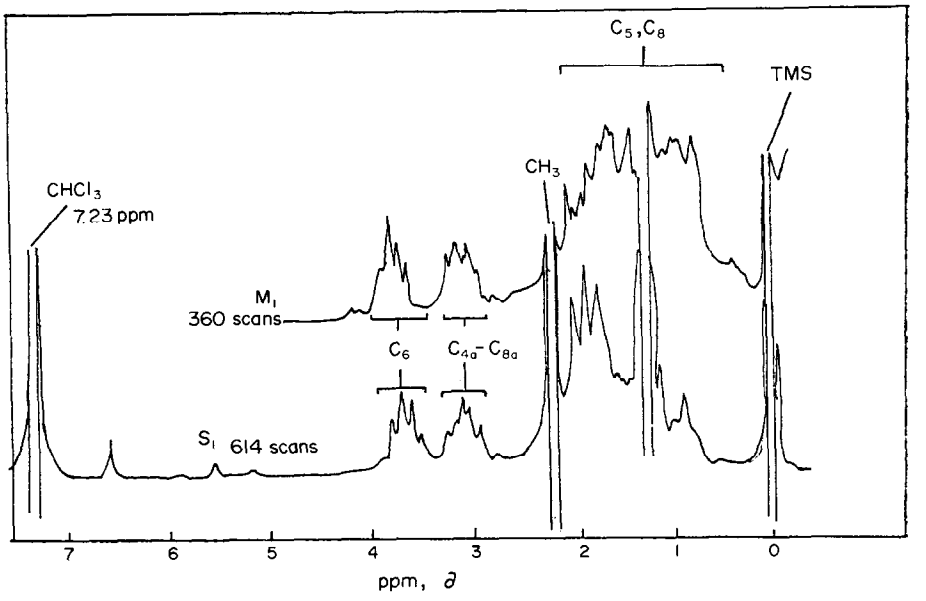

Fig. 3. NMR Spectra of M_1 and S_1.

N.M.R. studies on M_1 and S_1

Comparison of the 60 MHz ^{1}H n.m.r. spectra of M_1 and S_1 (Fig. 3) shows them to be very similar. They both exhibit a resonance frequency for the methyl protons at 1.25 p.p.m., a broad peak which includes the C_{4a} and C_{8a} protons between 2.60 and 3.20 p.p.m. and another broad peak between 3.45 and 3.90 p.p.m. which includes the C_4 proton.

Eu(fod$_3$) was added dropwise to the chloroform solution of M_1 until maximum induced shifts of the proton signals were reached. The small amount of M_1 (4 mg) necessitated use of the c.a.t. attachment (597 scans of 100 sec) to produce a good n.m.r. spectrum (Fig. 4). Protons were identified by measurement of integrated unit areas of their signals and matched with the aid of the solvent peak, which remains constant at 7.23 p.p.m., to the spectra produced in the absence of europium shift reagent. Peak A was ascribed to the C_{4a} and C_{8a} protons (Area = 2) and is seen as a triplet; its slight asymmetry was probably caused by the influence of the methyl group attached to C_7 and seen at 6.23 p.p.m. The C_{4a}–C_{8a} protons had an *induced* shift of 4.75 p.p.m. which is very similar to that reported for the C_2 proton of aldrin *trans*-diol (4.80 p.p.m.) [21]. Since the signals for both protons were shifted by the same amount it is likely that they were equidistant from their respective hydroxyl groups. The C_6 proton of M_1

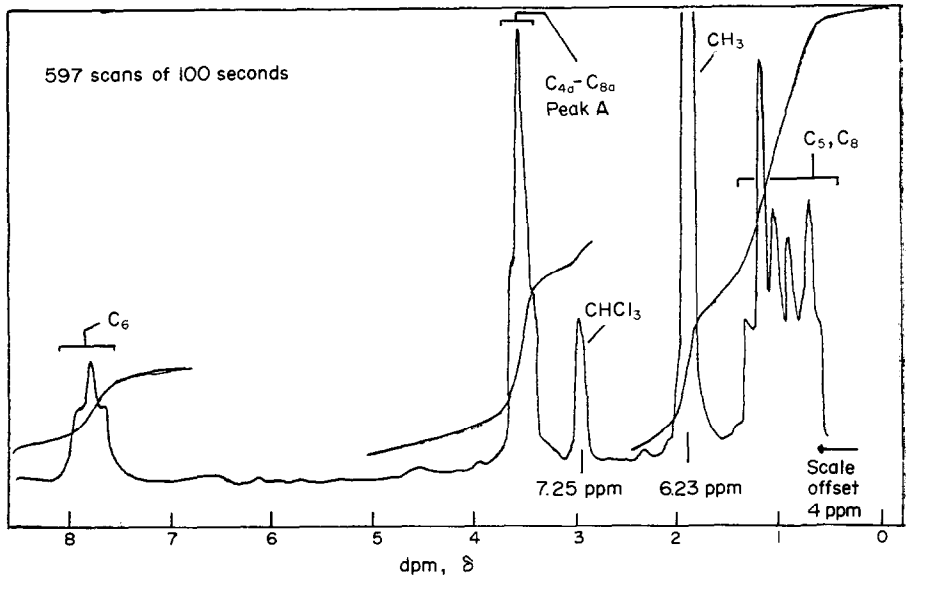

Fig. 4. NMR Spectrum of M_1 in the presence of EU(fod)$_3$.

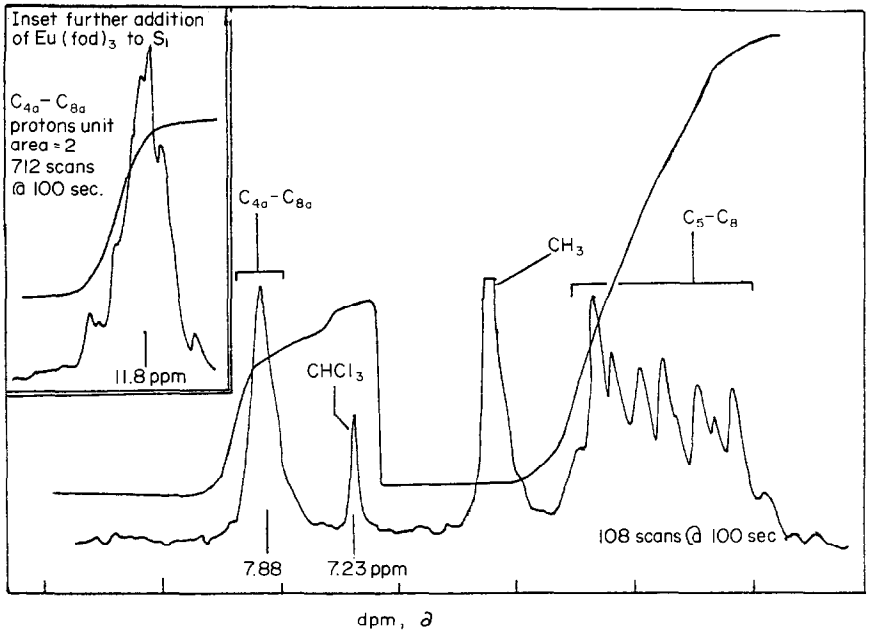

Fig. 5. NMR Spectrum of S_1 in the presence of Eu(fod)$_3$. Inset: Further addition of Eu(fod)$_3$ to S_1.

is a well distinguished triplet with an induced shift of 9.46 p.p.m. and an integrated unit area of 1. These findings indicate that M_1 is a *cis*-diol, and two alternative structures are shown in Fig. 6(a and b). Structure (a) is preferred since this is in accord with the larger induced shift observed with the C_{4a} and C_{8a} proton signals than with the broad peak representing the C_5–C_8 protons. The reverse might be expected if C_6 and C_7 were turned down as in (b) or if the OH groups were in the position occupied by CH_3 and H in either (a) or (b). Structure (a) is in accordance with the proposed structure (c) for the parent epoxide MME.

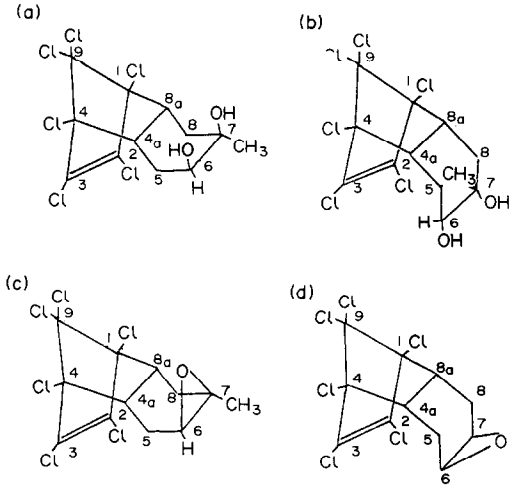

Fig. 6. Two alternative structures, (a) and (b) are shown for M_1. The preferred structure is that of (a). Structure (c) is the proposed structure for the parent epoxide MME. Structure (d) is that of the analogous epoxide HEOM with no methyl group attached to the oxirane ring.

Eu(fod$_3$) was added dropwise to S_1 until it was thought maximum induced shift had been achieved. A multiscan 50 MHz ^{1}H n.m.r. spectrum showed a less intense, broader peak for the C_{4a}–C_{8a} protons with an induced shift of approximately 4.70 p.p.m. (Fig. 5). On the addition of more Eu(fod$_3$) the broad signal containing the C_{4a}–C_{8a} protons was shifted further downfield and was resolved into eight lines. (Fig. 5 inset). This indicates an asymmetrical arrangement of the hydroxyl groups which may be due to a *trans* configuration. The signal for C_4 proton of S_1 differed from that of M_1 and appeared as a singlet. It was likely however to be a triplet which remained unseen because of the peak broadening and the low degree of coupling experienced ($\simeq$ 1Hz).

DISCUSSION

Epoxide hydratase (EC 4.2.1.63) converts several cyclodiene epoxides into their corresponding *trans*-diols. It had been thought that product glycols which are stereochemically fixed by a ring structure invariably have the *trans* configuration [26], however chemical evidence was presented by Chau and Cochrane [20] for the opening of the epoxide ring of dieldrin to form *cis*-dihydroxydihydroaldrin in preference to the *trans*-product, under conditions which favoured intimate ion pair formation. Since then *cis*-aldrin diol has been found to be produced by cockroaches [27] and by rat liver microsomes [22]. McKinney *et al.* using n.m.r. techniques with europium shift reagent (Eu(DPM$_3$)) showed that observation of C_{4a}–C_{8a} protons after an induced shift downfield, provided a useful means for determining the sterochemistry of hydroxyl groups attached to the C_6 and C_7 positions. With aldrin *cis*-diol the induced shift for C_{4a} and C_{8a} protons was the same, indicating that they were equidistant from their respective hydroxyl groups on C_6 and C_7; in contrast, aldrin

trans-diol, where the hydroxyl groups are not equidistant, produced a separated signal for the C_{4a} and C_{8a} protons when shift reagent was present.

In the current study we have compared the structure and formation of two compounds. One, M_1, was formed from the cyclodiene epoxide MME under the same conditions used for the *in vitro* production of HEOM *trans*-diol with liver microsomes from a variety of vertebrate species [28]. The second, S_1, was formed using the same method used to produce HEOM *trans*-diol by purely chemical means [30]. Mass spectral analysis showed that M_1 and S_1 were both dihydroxy derivatives of MME. However, chromatographic evidence suggested a stereochemical difference in the positioning of the hydroxyl groups which subsequent data seems to confirm (Table 1). Firstly, M_1 and S_1 had different silylation characteristics, although both formed a diTMS ether with a molecular weight of 542. Secondly n.m.r. examination of M_1 showed that the signals for protons C_{4a} and C_{8a} were not separated by chiral shift reagent, thus stongly suggesting a *cis*-diol configuration. On the other hand, chiral shift reagent produced a split signal for C_{4a} and C_{8a} protons in the case of S_1, suggesting that these protons are not equidistant from their respective hydroxyl groups. This suggests that S_1 is a *trans*-diol although the separation of the C_{4a} and C_{8a} protons is not complete, thus leaving the matter in some doubt.

The microsomal metabolism of MME differs in certain respects from that found with its analogue HEOM (Fig. 6) which has no methyl groups associated with the oxirane ring. In the first place, HEOM is metabolised much more rapidly by pig liver microsomes than is MME under the same conditions (HEOM = 115 nmoles/mg/min [28]; MME = 0.32 nmoles/mg/min). In the second place HEOM yields a *trans*-diol [28, 29] whereas the evidence reported here strongly suggests that MME forms a *cis*-diol. A possible explanation of this is that the methyl group of MME sterically hinders enzymic attack. Attack from the underside of the oxirane ring should lead to the insertion of a hydroxyl group on this side of the molecule, in a *trans* position with respect to the oxygen released when the ring is subsequently broken. Steric hindrance by the methyl group may restrict attack to the other side of the molecule, where the oxirane ring is situated, with the consequently slow formation of a *cis*-diol.

Acknowledgements—We are grateful to Mr. B. Hatcher, Mr. P. Heath, Dr. N. Isaacs and Mr. C. Ford for performing the n.m.r. and m.s. examinations presented here, and for advice on interpretation of the results.

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