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Note

"Epoxide–diol" metabolic pathway of 5H-dibenzo[*a*,*d*]cycloheptene in the rat

Studies by gas chromatography and mass spectrometry

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Knowledge of the metabolic fate of drugs has aided the interpretation of their pharmacological action and has provided, in many instances, a more rational basis for therapy. In this field, the correlation between chemical structure and metabolism is currently of considerable interest.

As part of a systematic study on the biotransformation of tricyclic drugs, this paper deals with the metabolism of 5H-dibenzo[*a*,*d*]cycloheptene (I), the basic skeleton of a class of compounds that are widely used both in the laboratory (being important tools for investigations in pharmacological research) and in clinical practice for the treatment of depressive syndromes[1–5].

The hypothesis of an "epoxide–diol" metabolic pathway for this compound, in analogy with other drugs, as previously reported by our group[6–13], has been confirmed. 5H-Dibenzo[*a*,*d*]cycloheptene-10,11-epoxide (II) and 10,11-dihydro-10,11-dihydroxy-5H-dibenzo[*a*,*d*]cycloheptene (III) were identified as the main biotransformation products of I.

EXPERIMENTAL

Standards and reagents

Compound I was kindly donated by Prof. A. Corbella, University of Milan (Milan, Italy). The following reagents were used: glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADP (Boehringer, Mannheim, G.F.R.); nicotinamide and N,N-dimethylformamide (BDH, Poole, Great Britain); *n*-butylboronic acid (Pierce, Rockford, Ill., U.S.A.); magnesium chloride, sodium hydroxide and hydrochloric acid (Merck, Darmstadt, G.F.R.). The solvents used were of analytical-reagent grade.

Animals

Male Sprague Dawley rats (180–200 g body weight) were injected intraperitoneally with compound I (80 mg/kg) dissolved in peanut oil. The animals were housed in individual metabolic cages and provided only with water. Urine samples were collected 24 h after administration of the drug and frozen at −15° until required for analysis.

Incubation system

Microsomes were obtained from the livers of the rats using a procedure involving centrifugation of the liver homogenate in a sucrose–EDTA mixture at low speed (17,000 *g*)[14].

The final concentrations of cofactors in 0.2 *M* phosphate buffer of pH 7.4 were NADP 0.3 m*M*, glucose-6-phosphate 10 m*M*, glucose-6-phosphate dehydrogenase 0.3 U/ml, magnesium chloride 5 mM and nicotinamide 10 m*M*.

Compound I was added as a methanolic solution (0.1 μM) and the mixture was incubated for 2 h at 37° with gentle shaking.

Extraction procedure

Rat urine samples (5 ml) or the incubation mixture (3 ml) were adjusted to pH 9 with 1 *N* sodium hydroxide solution and extracted twice with 5 ml of ethyl acetate. The organic phase was evaporated to dryness in a water-bath at 60° under a gentle stream of nitrogen. The residue, redissolved in methanol, was then used for thin-layer chromatographic, gas chromatographic and mass spectrometric analyses.

Thin-layer chromatography (TLC)

Chromatograms on thin-layer glass plates (20 × 5 cm) coated with silica gel F_{254} (0.25 mm thickness) were developed in chloroform–methanol (95:5).

Gas chromatography (GC)

GC was performed on a Carlo Erba Fractovap Model G-1 gas chromatograph, equipped with a flame-ionization detector. The column was a glass tube (2 m × 4 mm I.D.) packed with 3% OV-17 on Gas-Chrom Q, 100–120 mesh (Applied Science Labs., State College, Pa., U.S.A.), conditioned for 1 h at 250° with a nitrogen flow-rate of 30 ml/min, 4 h at 310° with no nitrogen flow, and 24 h at 250° with a nitrogen flow-rate of 30 ml/min. The column was operated with an injection port temperature of 290° and oven temperatures of 220° and 280° with a flow-rate of nitrogen (carrier gas) of 30 ml/min.

Gas chromatography–mass spectrometry (GC–MS)

A Finnigan Model 3100 quadrupole mass spectrometer, equipped with a gas chromatograph and a Model 6000 computer system programmed for mass fragmentography (MF), was used. The chromatographic conditions were as described above. Helium was used as carrier gas at a flow-rate of 30 ml/min.

RESULTS AND DISCUSSION

TLC of the rat urine extracts gave three spots (R_F values 0.70, 0.58, 0.46) not present in the control urine.

Two peaks (Fig. 1, a and b) were obtained in the GC of the urine extracts at a column temperature of 220°, while another peak (Fig. 2, c) was observed when the temperature was increased to 280° and an on-column reaction with *n*-butylboronic acid, a specific reagent for compounds containing vicinal hydroxy groups[15], was performed.

The eluate of the spot at R_F 0.70 (compound I), when analyzed both by a

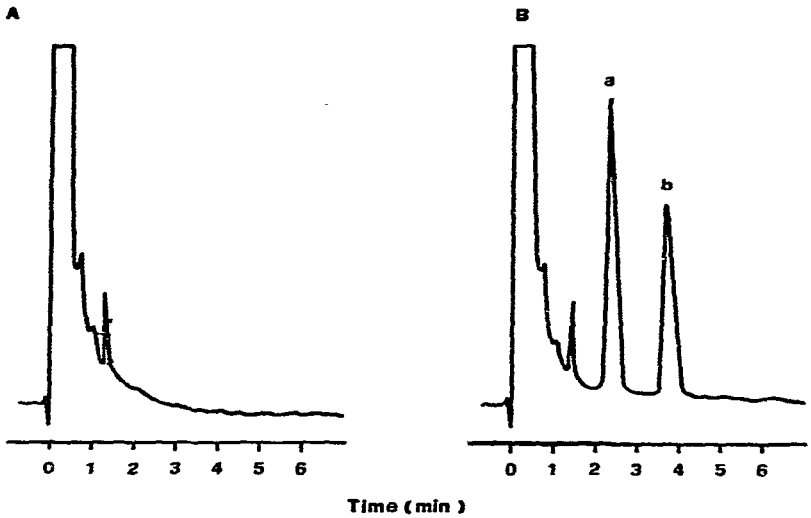

Fig. 1. Gas chromatograms of (A) urine control and (B) urine of rats treated with 5H-dibenzo[a,d]-cycloheptene (80 mg/kg, i.p.). Peaks: a, 5H-dibenzo[a,d]cycloheptene; b, 5H-dibenzo[a,d]cycloheptene-10,11-epoxide.

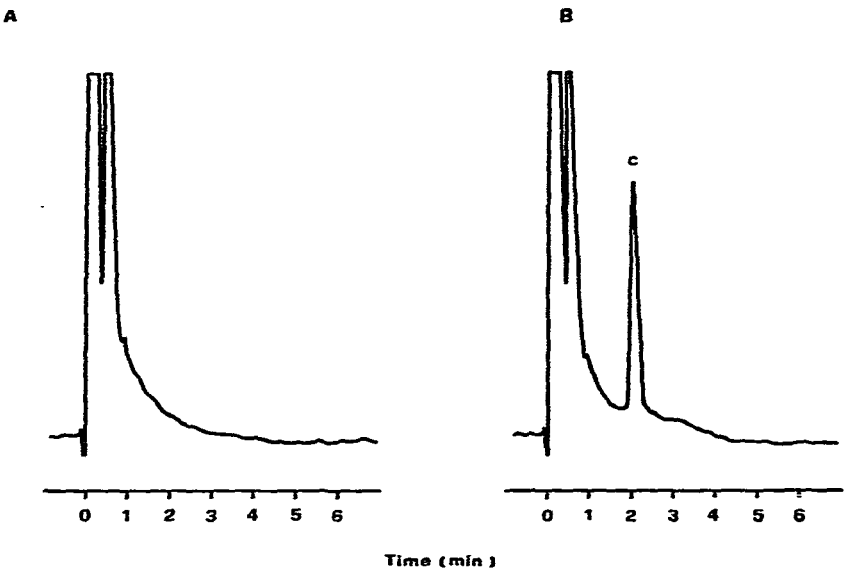

Fig. 2. Gas chromatograms of (A) urine control and (B) urine of rats treated with 5H-dibenzo[*a,d*]-cycloheptene (80 mg/kg, i.p.). Peak c: *n*-butylboronate of 10,11-dihydro-10,11-dihydroxy-5H-dibenzo[*a,d*]cycloheptene.

direct inlet system (DIS) into the ion source of the mass spectrometer, or by GC (peak a), gave the spectrum reported in Fig. 3. The molecular ion (base peak) at *m/e* 192, and the peaks at *m/e* 165 and *m/e* 152, were consistent with the structure of unchanged compound I.

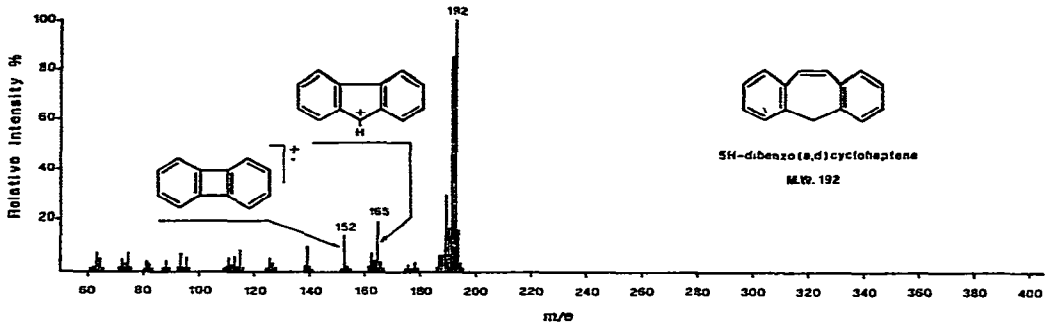

Fig. 3. Mass spectrum of 5H-dibenzo[*a*,*d*]cycloheptene.

The mass spectrum of the spot at R_F 0.58 (compound II), corresponding in GC to peak b, is shown in Fig. 4. The molecular ion at *m/e* 208 (an increase of 16 a.m.u. in comparison with the spectrum of I), and the loss of 29 a.m.u. (·CHO) from the molecular ion to give the base peak at *m/e* 179, suggested[6] epoxidation of the 10,11-double bond (Fig. 5).

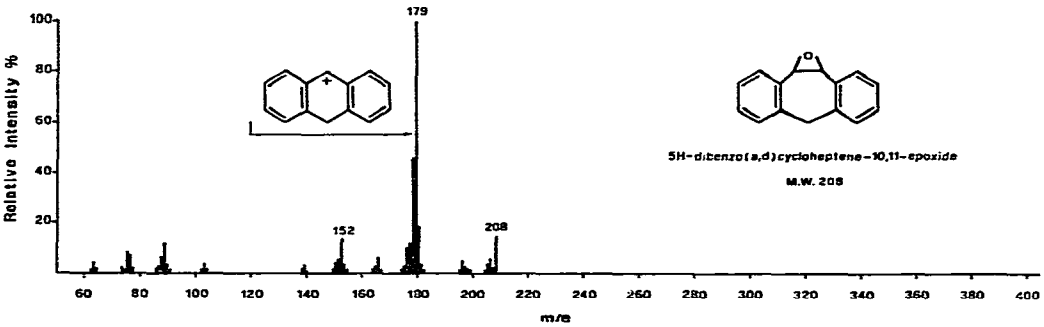

Fig. 4. Mass spectrum of 5H-dibenzo[*a*,*d*]cycloheptene-10,11-epoxide.

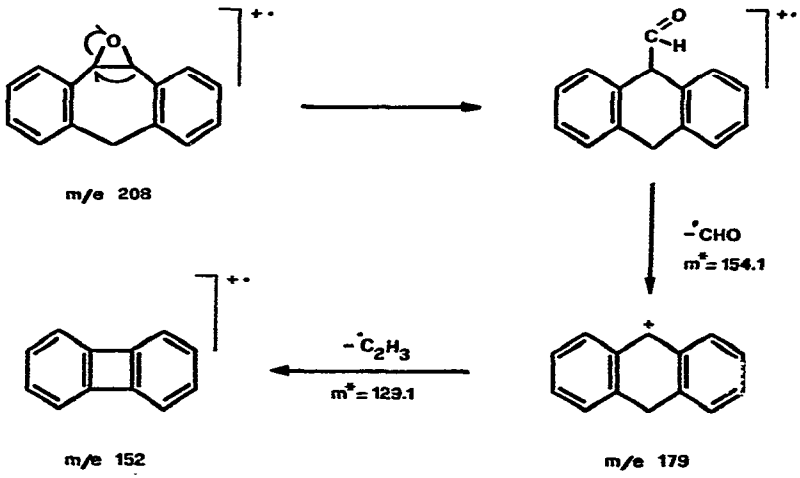

Fig. 5. Proposed fragmentation mechanism for 5H-dibenzo[*a*,*d*]cycloheptene.

In order to confirm this hypothesis, compound II was treated with 1 *N* hydrochloric acid and a compound with a similar TLC mobility (R_F 0.46) to compound III was obtained. GLC analysis of this compound, after on-column reaction with *n*-butylboronic acid, gave a peak at the same retention time as peak c (Fig. 2). The identity of this peak was checked by MS and the spectrum is shown in Fig. 6.

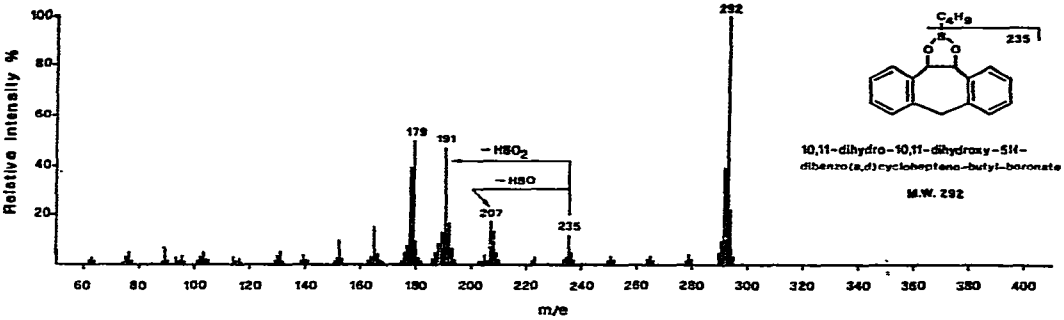

Fig. 6. Mass spectrum of 10,11-dihydro-10,11-dihydroxy-5H-dibenzo[*a*,*d*]cycloheptene butylboronate.

The molecular ion (base peak) at *m/e* 292, and the occurrence of the ions at *m/e* 235, 207, 191 and 179, are consistent with the structure of 10,11-dihydro-10,11-dihydroxy-5H-dibenzo[*a*,*d*]cycloheptene (as the butylboronate). Compound II was therefore demonstrated to be 5H-dibenzo[*a*,*d*]cycloheptene-10,11-epoxide.

Compound III (spot at R_F 0.46) and GC peak c, when analyzed by GC–MS, after on-column reaction with *n*-butylboronic acid, gave a spectrum identical with that of 10,11-dihydro-10,11-dihydroxy-5H-dibenzo[*a*,*d*]cycloheptene. Therefore, the structure of 10,11-dihydro-10,11-dihydroxy-5H-dibenzo[*a*,*d*]cycloheptene can be assigned to compound III. Compound III was also detected in rat urine after incubation with β-glucuronidase, indicating the presence of conjugate compounds.

By incubating compound I with control rat urine at 37° for 12 h, it could be shown that these metabolites were not the result of a chemical reaction of compound I in the urine. To confirm this enzymatic "epoxide–diol" metabolic patway, *in vitro* experiments were performed by incubating compound I with rat liver microsomes. Metabolites II and III were confirmed by means of multiple ion detection mass fragmentography. This technique was applied because the metabolites were present in the incubation medium only in low concentrations.

Figs. 7 and 8 show the mass fragmentograms recorded in two successive analyses performed by focusing the instrument firstly on the ions at *m/e* 192, which are specific for I, at *m/e* 179 and 208, which are specific for II, and secondly upon the ions at *m/e* 191, 179, 235 and 292, which are characteristic of III.

To ascertain that the formation of these metabolites was dûe to an enzymatic process, compound I was incubated under various experimental conditions, as summarized in Table I.

With inactivated microsomes, after boiling, or in the absence of NADP, epoxidation and successive formation of the diol was not observed.

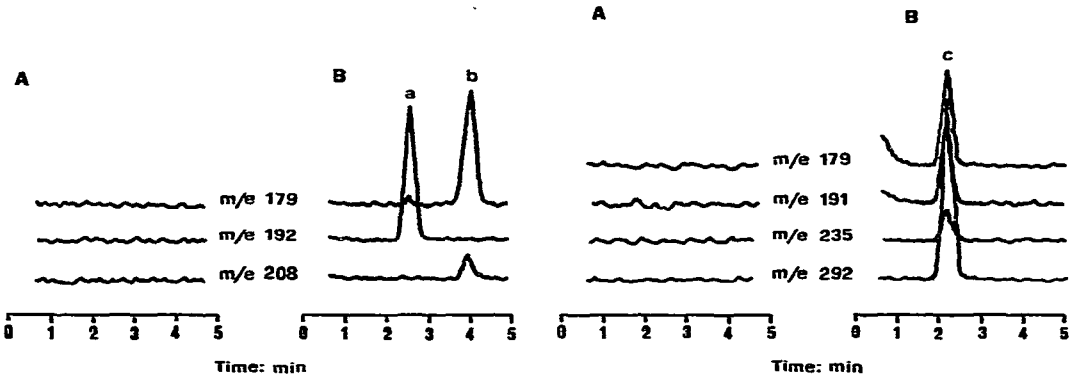

Fig. 7. Mass fragmentograms of (A) control sample of microsomes and (B) microsomes incubated with 5H-dibenzo[*a,d*]cycloheptene. Peaks: a, 5H-dibenzo[*a,d*]cycloheptene; b, 5H-dibenzo[*a,d*]-cycloheptene-10,11-epoxide.

Fig. 8. Mass fragmentograms of (A) control sample of microsomes and (B) microsomes incubated with 5H-dibenzo[*a,d*]cycloheptene. Peak c: *n*-butylboronate of 10,11-dihydro-10,11-dihydroxy-5H-dibenzo[*a,d*]cycloheptene.

TABLE I

INCUBATION OF 5H-DIBENZO[*a,d*]CYCLOHEPTENE UNDER VARIOUS EXPERIMENTAL CONDITIONS WITH RAT LIVER MICROSOMES, SHOWING THE ENZYMATIC FORMATION OF THE METABOLITES

Experimental conditions	*Epoxide*	*Diol*
Microsomes + cofactors	+	+
Denaturated microsomes* + cofactors	—	—
Microsomes + cofactors — NADP	—	—
Cofactors only	—	—

* Microsomes and the sample re-suspended in phosphate buffer (0.2 *M*) were heated at 100° for 10 min.

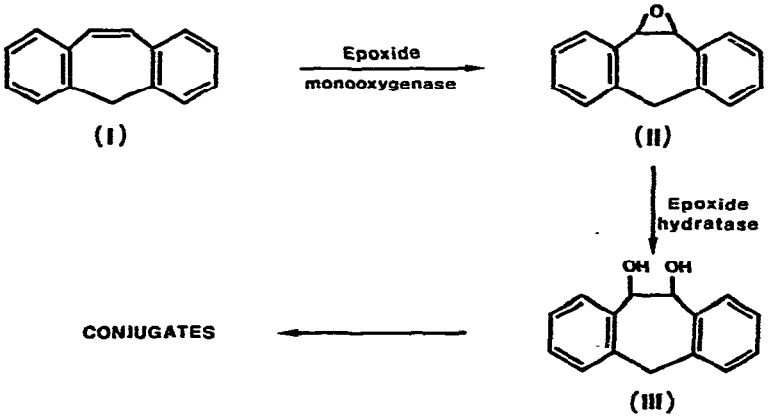

Fig. 9. Possible "epoxide–diol" metabolic pathway for 5H-dibenzo[*a,d*]cycloheptene (I). Conjugates are formed only *in vivo*.

In conclusion, compound I both *in vivo* and *in vitro* is biotransformed following the "epoxide–diol" metabolic pathway (Fig. 9).

The relatively poor affinity of this epoxide as a epoxide hydratase substrate makes it a suitable material for the characterization of the enzymatic systems involved in the formation of epoxide[16]. Further studies are in progress in order to establish whether this epoxide has the same properties as other epoxides that are responsible for several toxic effects[17].

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