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EPOXIDE–DIOL PATHWAY IN THE METABOLISM OF 5*H*-DIBENZO[*b,f*]-AZEPINE (IMINOSTILBENE)

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SUMMARY

5*H*-Dibenzo[*b,f*]azepine-10,11-epoxide and 10,11-dihydro-10,11-dihydroxy-5*H*-dibenzo[*b,f*]azepine were identified by gas chromatography–mass spectrometry in rat urine as the main biotransformation products in the metabolism of 5*H*-dibenzo-[*b,f*]azepine (iminostilbene). The presence of these metabolites was confirmed *in vitro* by incubating iminostilbene with rat liver microsomal enzymes.

INTRODUCTION

Iminostilbene (I) represents a heterocyclic structure that is common to several compounds that are widely used both in laboratory investigations and as therapeutic agents in clinical practice for the treatment of neurogical or mental disorders[1–3]. Recently, I has also been identified as a minor metabolite in the biotransformation of carbamazepine, a drug with marked anticonvulsant activity[4].

As a part of a systematic investigation of the relationship between the structure and metabolism of tricyclic compounds[5], we report here a study of the identification of 5*H*-dibenzo[*b,f*]azepine-10,11-epoxide (II) and 10,11-dihydro-10,11-dihydroxy-5*H*-dibenzo[*b,f*]azepine (III) both *in vivo* in rat urine after administration of I and *in vitro* after incubation of I with rat liver microsomes.

EXPERIMENTAL

Standards and reagents

I was purchased from Fluka, Buchs, Switzerland.

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.); and magnesium chloride (Merck, Darmstadt, G.F.R.). The solvents used were of analytical-reagent grade.

Animal experiments

Male Sprague Dawley rats (180–200 g) were injected intraperitoneally with I (80 mg/kg) dissolved in groundnut oil. The animals were housed in individual

metabolic cages and were provided only with water. Urine samples were collected 25 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 by using a procedure that involved centrifugation at a low speed (17,000 *g*) of the liver homogenate in a sucrose–EDTA mixture[6].

The final concentrations of cofactors in 0.2 *M* phosphate buffer (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 m*M* and nicotinamide 10 m*M*. 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

Samples of rat urine (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 under reduced pressure and the residue re-dissolved in acetone, then subjected to gas chromatography–mass spectrometry (GC–MS).

Gas chromatography–mass spectrometry

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.

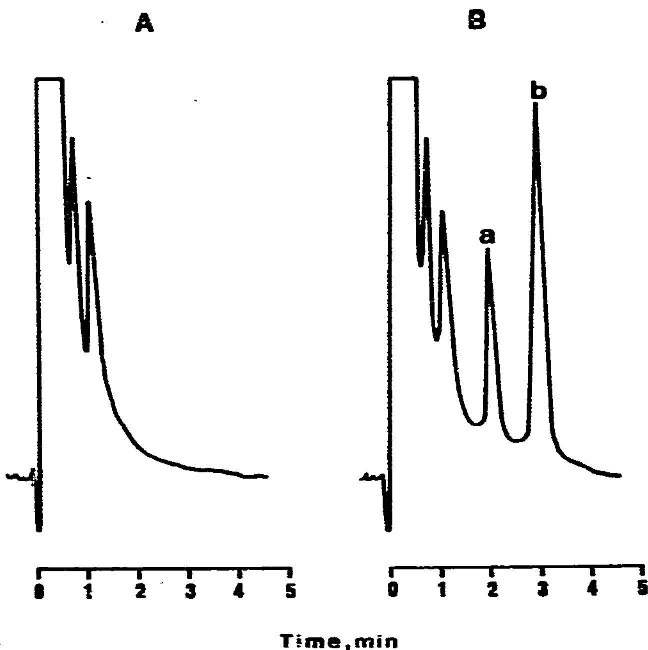


Fig. 1. Gas chromatograms of (A) urine control and (B) urine of rats treated with iminostilbene (80 mg/kg i.p.). Peaks: a, iminostilbene (I); b, iminostilbene-10,11-epoxide (II).

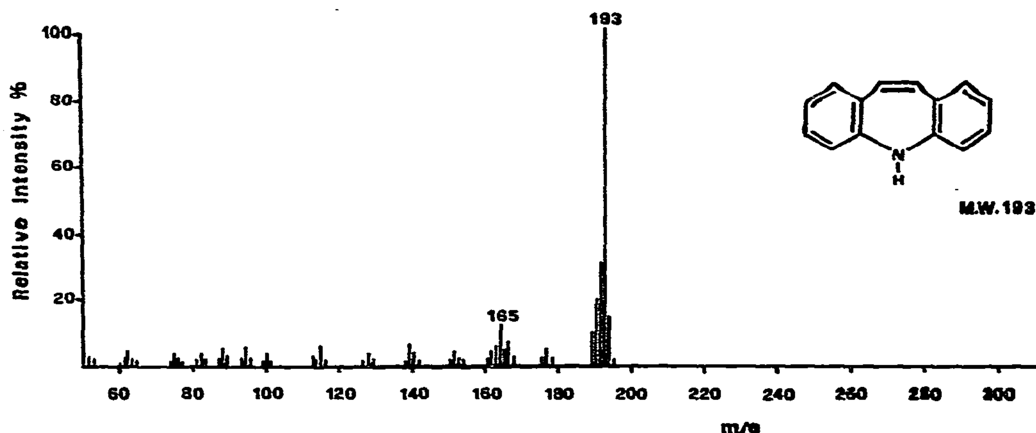


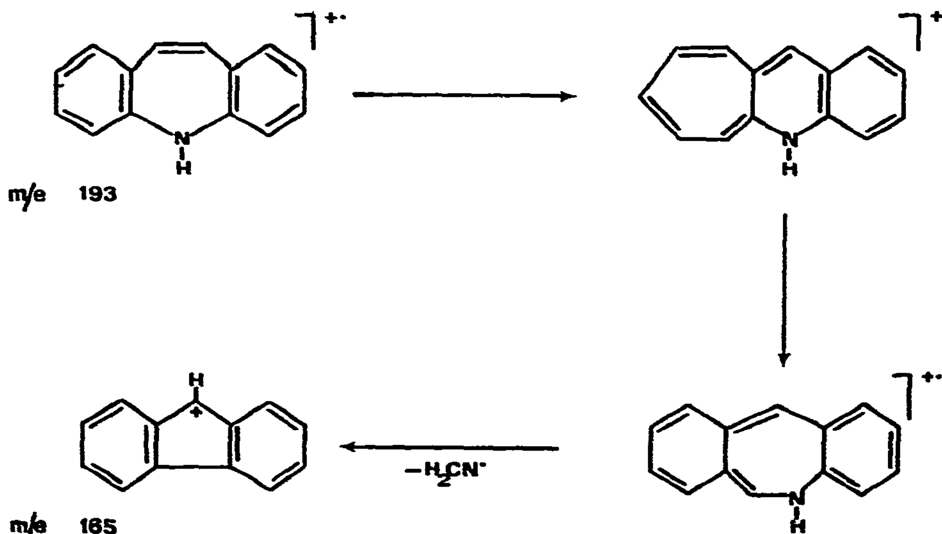
Fig. 2. Mass spectrum of iminostilbene (I).

The chromatographic 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 helium flow-rate of 30 ml/min, for 4 h at 310° with no flow of helium and for 24 h at 275° with a helium flow-rate of 30 ml/min. The injection port temperature was 290° and the oven temperature 270° and 300°, and the flow-rate of carrier gas (helium) was 30 ml/min.

The mass spectrometer was operated under the following conditions: molecular separator temperature, 250°; ion source temperature, 100°; energy of the ionization beam, 70 eV; ionization current, 200 μA.

RESULTS AND DISCUSSION

The gas chromatograms obtained from the urine extract of rats that had been treated with I 24 h earlier are shown in Fig. 1. Peak a (Fig. 1B), when analyzed by



Scheme 1. Proposed fragmentation mechanism for iminostilbene (I).

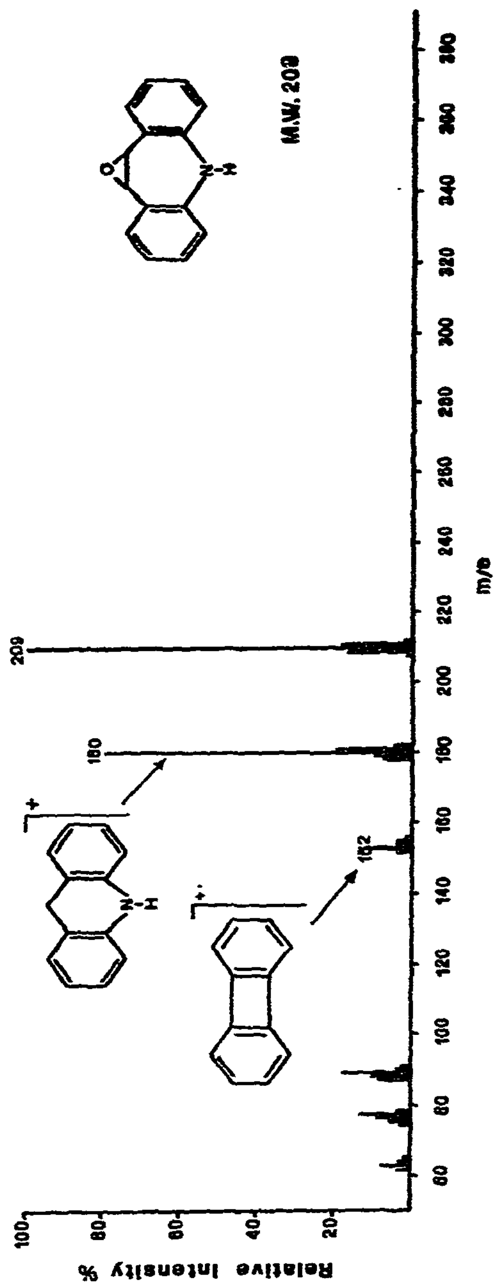


Fig. 3. Mass spectrum of iminostilbene-10,11-epoxide (II).

GC–MS, gave rise to the mass spectrum shown in Fig. 2. The molecular ion at *m/e* 193 and the subsequent loss of $H_2\dot{C}N$ (28 a.m.u.) are consistent with the structure of unchanged I as shown in Scheme I[7].

The mass spectrum (Fig. 3) of peak b (Fig. 1B), obtained at a column temperature of 270°, was similar to that of II, previously found as a metabolite of carbamazepine-10,11-epoxide[8].

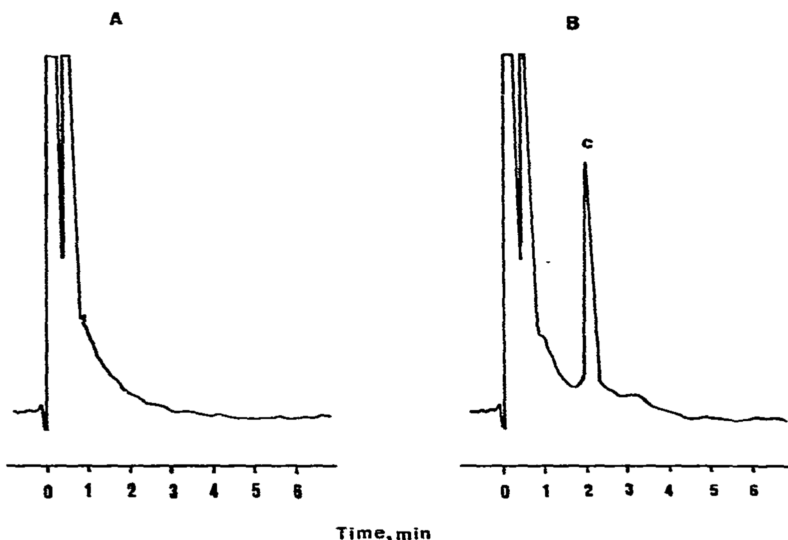


Fig. 4. Gas chromatograms of (A) urine control and (B) urine of rats treated with iminostilbene (80 mg/kg i.p.). Peak c: *n*-butylboronate of iminostilbene-10,11-dihydrodiol.

When the column temperature was increased to 300° and an on-column reaction was carried out by co-injecting 1 μl of an N,N-dimethylformamide solution of *n*-butylboronic acid (10 mg/ml) and 1 μl of an acetone solution of the urine extract, the chromatogram shown in Fig. 4B was obtained. The analysis of this peak (c) by GC–MS gave the mass spectrum shown in Fig. 5.

The molecular ion at *m/e* 293 and occurrence of the reaction with *n*-butylboronic acid, which is a specific reagent for compounds that contain vicinal hydroxy groups[9], is in agreement with the structure reported in Fig. 5. This metabolite has also been detected after incubation of urine with β-glucuronidase, indicating the presence of a glucuronide. By incubating 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 I occurring in the urine.

In order to confirm the enzymatic epoxide–diol metabolic pathway, *in vitro* experiments were performed by incubating I with rat liver microsomes. Metabolites II and III were identified in the incubation medium by means of multiple-ion detection MF. This technique was used as III was present in only trace amounts.

Figs. 6 and 7 show the mass fragmentograms recorded in two successive analyses performed by focusing the instrument on the ions at *m/e* 193 which are specific for I, at *m/e* 180 and 209 which are specific for II, and at *m/e* 180, 208, 236

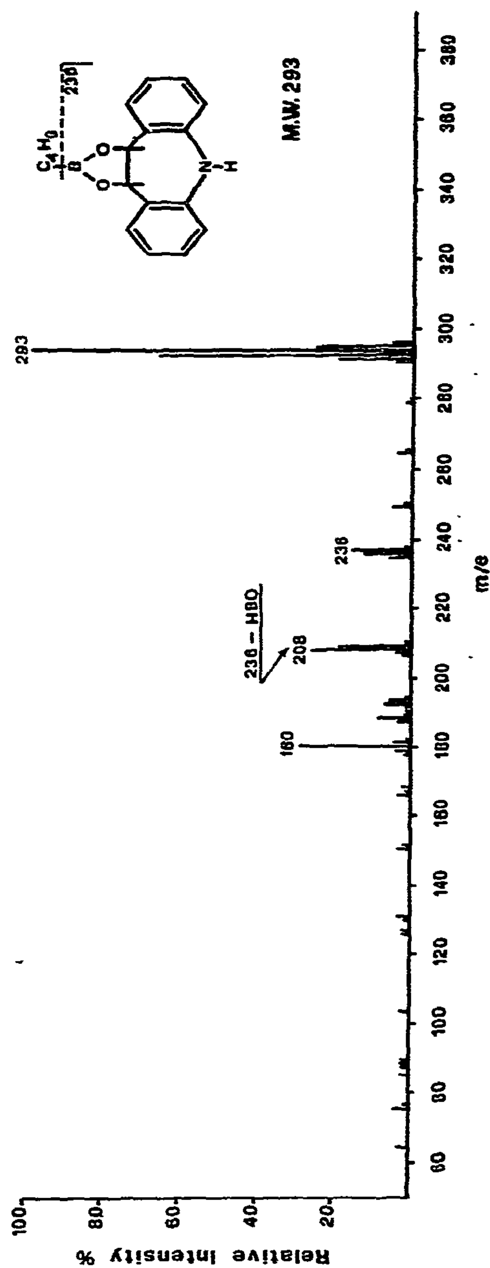


Fig. 5. Mass spectrum of the n-butylboronate of iminostilbene-10,11-dihydrodiol.

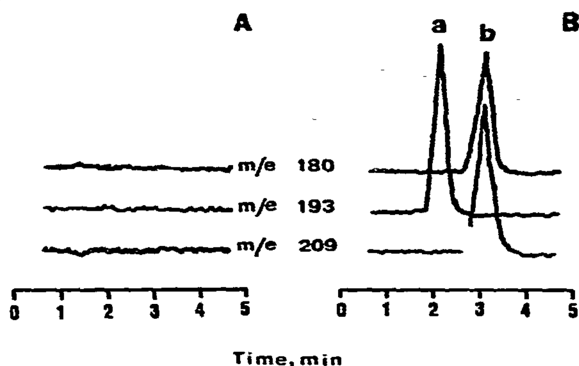


Fig. 6. Mass fragmentograms of (A) control sample of microsomes and (B) microsomes incubated with iminostilbene. Peaks: a, iminostilbene; b, iminostilbene-10,11-epoxide.

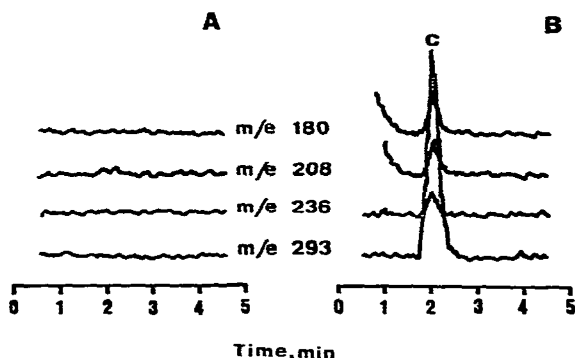


Fig. 7. Mass fragmentograms of (A) control sample of microsomes and (B) microsomes incubated with iminostilbene. Peak c: iminostilbene-10,11-dihydrodiol (*n*-butylboronate).

and 293 which are characteristic of III. In order to ascertain that the formation of these metabolites was due to an enzymatic process, I was incubated under various experimental conditions (Table I).

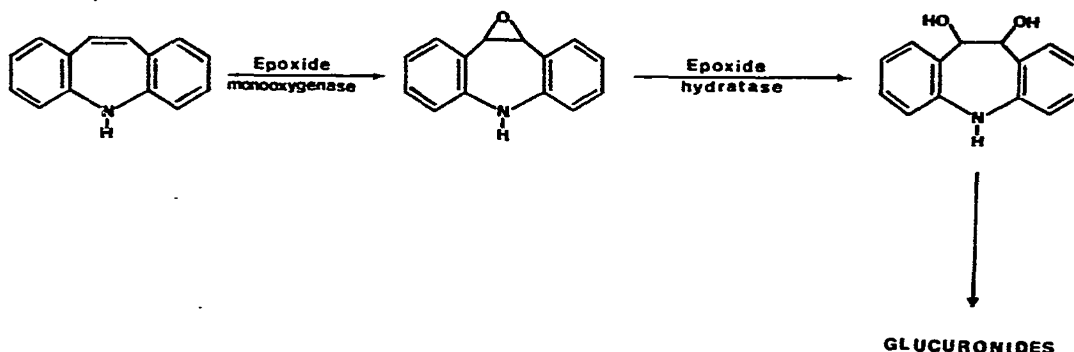
With inactivated microsomes, after boiling or in the absence of NADP, epoxidation and successive formation of the diol were not observed.

TABLE I

INCUBATION OF IMINOSTILBENE WITH RAT LIVER MICROSOMES UNDER VARIOUS EXPERIMENTAL CONDITIONS, 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 suspended in phosphate buffer (0.2 *M*) were heated at 100° for 10 min.



Scheme 2. Possible epoxide–diol metabolic pathway for iminostilbene. Glucuronides are formed only *in vivo*.

In conclusion, I is biotransformed both *in vivo* and *in vitro* following the epoxide–diol metabolic pathway (Scheme 2).

The relatively poor affinity of I for epoxide hydratase makes it a suitable compound for the characterisation of the enzymatic system involved in the formation of epoxides[10]. Further studies are in progress in order to establish whether this epoxide shares the properties of other epoxides that are responsible for several toxic effects, including mutagenesis[11,12].

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