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EPOXIDE–DIOL METABOLIC PATHWAY OF CYTENAMIDE IN THE RAT

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SUMMARY

Cytenamide administered intraperitoneally to rats is biotransformed to cytenamide-10,11-epoxide and 10,11-dihydro-10,11-dihydroxycytenamide. These metabolites were separated by chromatographic methods and their structures elucidated by mass spectrometry. The structure of the epoxide was confirmed by direct comparison with an authentic sample.

The formation of a chemical artifact, cytenamide-*syn*-11-hydroxylactone, in the urine was observed.

The formation of these metabolites *in vitro* was demonstrated by incubation of cytenamide with rat liver microsomes.

INTRODUCTION

Previous work in our laboratory has established that several tricyclic drugs, namely carbamazepine[1], cyproheptadine[2], protriptyline[3], cyclobenzaprine[4,5] and iminostilbene[6], are biotransformed by liver monooxygenases into relatively stable 10,11-epoxides. It appears, therefore, that epoxidation of the 10,11-double bond position can be considered as a metabolic step common to these structurally related compounds.

As part of an investigation of the metabolism of tricyclic drugs, we have examined the biotransformation of cytenamide (5*H*-dibenzo[*a*,*d*]cycloheptatriene-5-carboxamide, I), an analogue of the anticonvulsant cyheptamide[7], in rats. This paper deals with the identification of cytenamide-10,11-epoxide (II) and 10,11-dihydro-10,11-dihydroxycytenamide (III) both *in vivo*, in the urine of rats following intraperitoneal administration of I, and *in vitro*, after incubation of I with rat liver microsomes. Cytenamide-*syn*-11-hydroxylactone (IV) probably arises by intramolecular cyclization of III.

EXPERIMENTAL

Standards and reagents

I, II and IV were obtained from Ayerst Labs., Montreal, Canada.

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 and sodium hydroxide (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) suspended in a 3% solution of hydroxypropylcellulose.

The animals were housed in individual metabolic cages and were 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 by using a procedure that involved centrifugation (17,000 *g*) of the liver homogenate in a sucrose–EDTA mixture[8].

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 in a water-bath at 60° under nitrogen. The residue, redissolved in methanol was then used for thin-layer chromatography (TLC), gas chromatography (GC) and mass spectrometry (MS).

Thin-layer chromatography

Chromatograms on thin-layer glass plates (5 × 20 cm) coated with a 0.25-mm thickness of silica gel F_{254} (Merck) were developed with toluene–isopropanol (80:20, v/v).

Gas chromatography–mass spectrometry

A Finnigan Model 3100 quadrupole gas chromatograph–mass spectrometer and a Model 6000 computer system programmed for mass fragmentography (MF) were used. The gas chromatograph was equipped with a glass column (1 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.). The injection port and the oven temperature were maintained at 290°; the flow-rate of helium (carrier gas) 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

Analytical TLC of the crude extract from the urine of rats treated with I showed the presence of four compounds not present in that of control animals. Their R_F values are reported in Table I.

TABLE I

R_F VALUES OF CYTENAMIDE AND ITS METABOLITES

Silica gel F_{254} glass plates, 0.25 mm thickness. Solvent system: toluene–isopropanol (80:20, v/v).

Compound	R_F
Cytenamide (I)	0.44
II	0.34
III	0.22
IV	0.50

The mass spectrum of the compound with R_F 0.44 (Fig. 1) was identical with that of cytenamide (I). Significantly, the loss of $\dot{C}ONH_2$ from the molecular ion (*m/e* 235) to give an ion at *m/e* 191 is supported by a metastable peak at *m/e* 155.2.

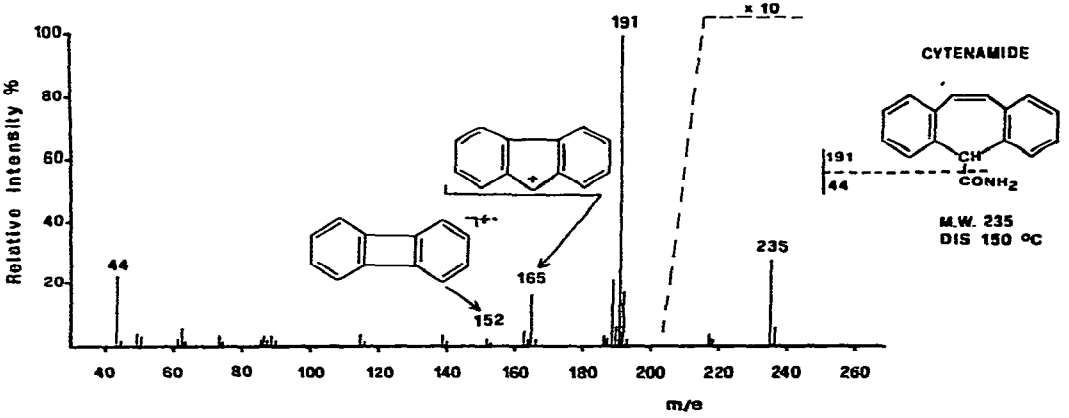


Fig. 1. Mass spectrum of cytenamide extracted from rat urine, identical with that of the authentic compound.

The mass spectrum of the compound with R_F 0.34 (Fig. 2) shows a molecular ion at *m/e* 251, an increase of 16 a.m.u. compared with that of cytenamide. The base peak at *m/e* 178 is believed to arise (Scheme 1) from the ion at *m/e* 207 (loss of $\dot{C}ONH_2$ from the molecular ion) by loss of 29 a.m.u. ($\dot{C}HO$), which is strong evidence in these systems for the presence of a 10,11-epoxide[9]. Confirmation of this evidence was obtained by a direct-inlet MS and TLC comparison with an authentic sample of cytenamide-10,11-epoxide.

The more polar compound (R_F 0.22) showed a molecular ion at *m/e* 269, an increase of 34 a.m.u. with respect to cytenamide. The apparently consecutive losses of 17 and 18 a.m.u. strongly suggest the presence of a 10,11-dihydroxy group (Fig. 3).

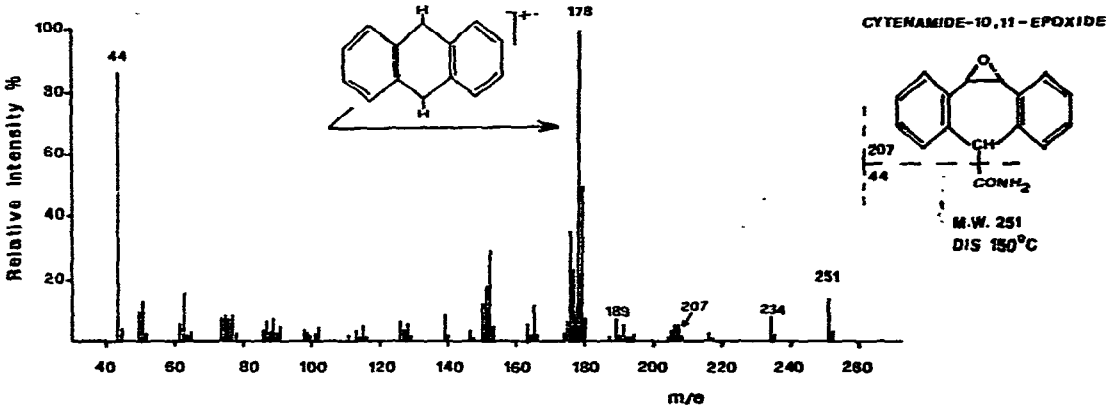
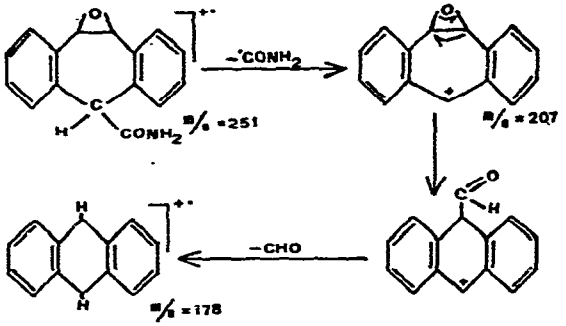


Fig. 2. Mass spectrum of cytenamide-10,11-epoxide extracted from rat urine, identical with that of the authentic compound.



Scheme 1. Possible fragmentation pathway of cytenamide-10,11-epoxide.

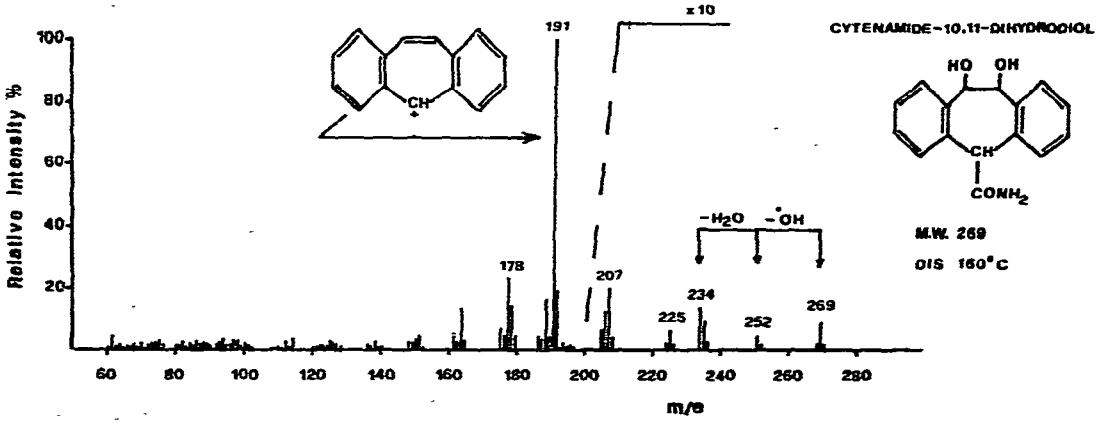


Fig. 3. Mass spectrum of cytenamide-10,11-dihydro-10,11-dihydroxycytenamide extracted from rat urine.

This metabolite was assigned the structure of 10,11-dihydro-10,11-dihydroxycytenamide, a known metabolite of cytenamide[7]. This compound was also obtained after treatment of the urine aqueous fraction with β-glucuronidase.

The mass spectrum of the least polar compound (R_F 0.50) showed a molecular ion at *m/e* 252, corresponding to a structure that does not contain nitrogen. Apparent losses from the molecular ion of 44, 45 and 46 a.m.u. (*m/e* 208, 207 and 206, respectively) followed by loss of 28 and 29 a.m.u., or conversely loss of 29 a.m.u. (*m/e* 223) followed by loss of 44 and 45 a.m.u. to give the ions at *m/e* 179 and 178, strongly suggest the presence of three oxygen atoms in the molecule and the presence of a

–C–O functionality. This evidence and the polarity of this compound indicated the structure shown in Fig. 4, a compound previously found as an artifact in the metabolism of cyheptamide[10]. In fact, incubation of III with rat urine at 37° for 12 h resulted in the formation of IV.

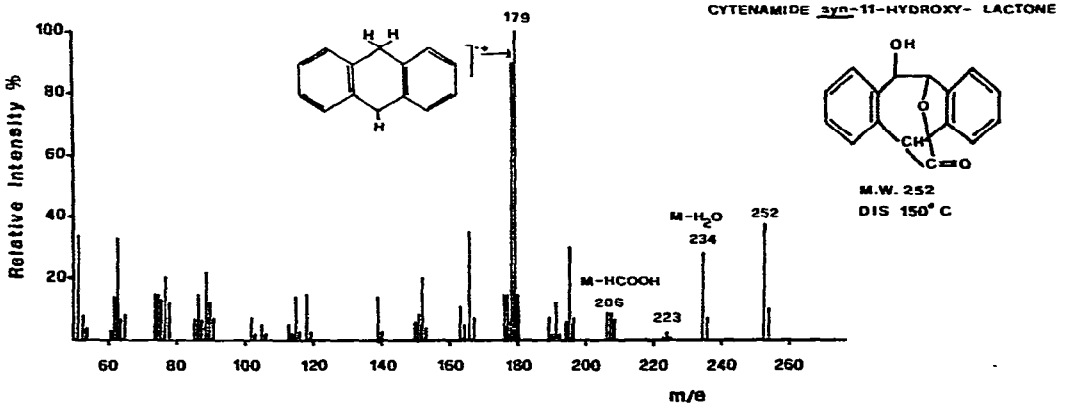


Fig. 4. Mass spectrum of cytenamide-*syn*-11-hydroxylactone extracted from rat urine, identical with that of the authentic compound.

To establish the epoxide–diol pathway *in vitro*, I was incubated with rat liver microsomes.

The formation of II was confirmed by TLC and MS. The diol, on the other hand, was present only in trace amounts and MF of the *n*-butylboronate derivative[11] was used to detect and identify this metabolite. The derivatization reaction with *n*-butylboronic acid was carried out on column in order to analyze III by GLC.

The molecular ion at *m/e* 335 (6%) and the base peak at *m/e* 291 ($M^+ - \dot{C}ONH_2$) in the spectrum of the *n*-butylboronate of III were found highly specific for the MF identification of III. Fig. 5 shows a mass fragmentogram that confirms the presence of minute amounts of III within the microsomal incubation mixture.

In order to ascertain that the formation of these metabolites was due to an enzymatic process, I was incubated under various experimental conditions (Table II).

With inactivated microsomes, after boiling or in the absence of NADP, epoxidation and successive formation of the diol were not observed. IV was never

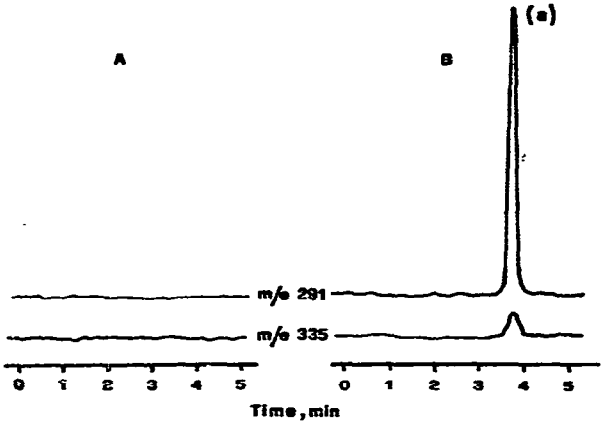


Fig. 5. Mass fragmentogram of: (A) control and (B) microsomes incubated with cytenamide. Peak a: 10,11-dihydro-10,11-dihydroxycytenamide (*n*-butylboronate).

TABLE II

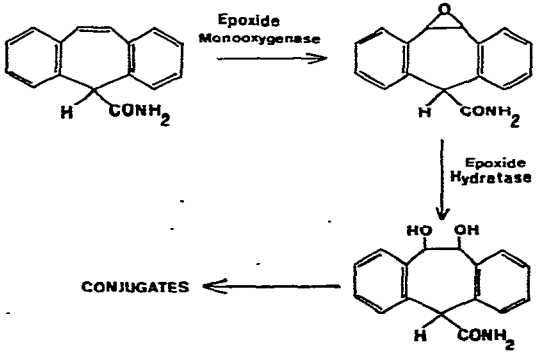
INCUBATION OF CYTENAMIDE, WITH RAT LIVER MICROSOMES UNDER VARIOUS EXPERIMENTAL CONDITIONS, SHOWING THE ENZYMATIC FORMATION OF THE METABOLITES

Experimental conditions	*Compound*		
	II	*III*	*IV*
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.

detected *in vitro* after incubation (pH 7.4) with rat liver microsomes, which is a further confirmation of the fact that this compound may be formed at the slightly acidic pH of the urine.

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



Scheme 2. Suggested metabolic pathway for cytenamide.

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