Identification of *In Vitro* (Rat Liver Postmitochondrial S9 Fraction) Metabolites of the Antiprotozoal Agent 3a,4,5,6,7,7a-Hexahydro-3-(1-methyl-5-nitro-1*H*-imidazol-2-yl)-1,2-benzisoxazole

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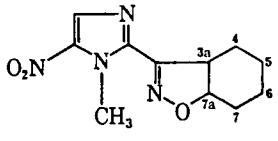
Abstract □ Twelve *in vitro* oxygenated metabolites of 3a,4,5,6,7,7a-hexahydro-3-(1-methyl-5-nitro-1*H*-imidazol-2-yl)-1,2-benzisoxazole (MK-0436) were produced by incubation of this antiprotozoal agent with the postmitochondriai supernatant (S9) fraction isolated from the livers of rats treated with phenobarbital. Metabolite structure elucidation was achieved using NMR and mass spectrometry. Seven monohydroxy and two dihydroxy metabolites were fully characterized; two other metabolites were partially characterized as dihydroxy derivatives of the drug. The major *in vitro* metabolite is the 5 axial hydroxy compound, and a minor metabolite is the corresponding ketone. In all cases metabolite formation involved biotransformation on the hexahydrobenzisoxazole ring.

Keyphrases □ 3a,4,5,6,7,7a - Hexahydro -3- (1-methyl-5-nitro-1*H*-imidazol-2-yl)-1,2-benzisoxazole—synthesis of *in vitro* oxygenated metabolites, characterization by NMR and mass spectrometry, biotransformations on the hexahydrobenzisoxazole ring □ Metabolites—of 3a,4,5,6,7,7a - hexahydro -3- (1-methyl-5-nitro-1*H*-imidazol -2- yl)-1,2-benzisoxazole, characterization by NMR and mass spectrometry □ Antiprotozoal agents—3a,4,5,6,7,7a-hexahydro-3-(1-methyl-5-nitro-1*H*-imidazol-2-yl)-1,2-benzisoxazole and its metabolites, characterization by NMR and mass spectrometry

Seven canine urinary metabolites of the antiprotozoal agent 3a,4,5,6,7,7a-hexahydro-3-(1-methyl-5-nitro-1*H*-imidazol-2-yl)-1,2-benzisoxazole (MK-0436, I) have been identified; all of the compounds (II–VIII) are formed by hydroxylation of the hexahydrobenzisoxazole ring (1, 2). Several dihydroxy analogues of I have been synthesized



I

and at least one, the 6,7-*cis*-dihydroxy compound, exhibits higher antibacterial activity against *Salmonella schottmuelleri* in mice and greater trypanocidal activity *in vivo* against *Trypanosoma cruzi* (Brazil strain) than I (1). Incubation of I with the microsomal cytosol fraction (S9) from the livers of mice produced antibacterial activity not seen with the drug alone, suggesting the *in vitro* formation of antibacterial metabolites (1). The present study was undertaken to identify the metabolites of I generated *in vitro* by the rat liver postmitochondrial supernatant fraction (S9).

EXPERIMENTAL

***In Vitro* Incubations**—The postmitochondrial supernatant fraction was isolated from livers of rats that had been treated with phenobarbital. The 9–12-week-old rats were injected with phenobarbital for four consecutive days at a dose of 80 mg kg/day ip. Twenty-four hours after the last injection the rats were killed and the postmitochondrial S9 supernatant was isolated using the method of Ames (3).

The incubation mixture used was the same as that described by Ames for the S9 mixture (3) and used previously (1). It contained 0.1 *M* sodium phosphate (pH 7.4), 8 m*M* magnesium chloride, 33 m*M* potassium chloride, 5 m*M* glucose-6-phosphate, 4 m*M* nicotinamide adenine dinucleotide phosphate, homogenate equal to 0.78 g wet weight liver, and 4 mg of I (labeled with carbon-14 in the 3a and 7a positions) in 200 μl of dimethylsulfoxide with a total volume of 10.4 ml. The incubation was carried out in a 250-ml Erlenmeyer flask with shaking at room temperature. At the end of the 80-min incubation (determined to be optimum for metabolite production from preliminary experiments) an equal volume of cold methylene chloride was added.

Metabolite Isolation—The metabolites of I were isolated by dilution of the incubation mixture with 10 ml of water followed by three successive extractions with 20-ml portions of methylene chloride. This procedure resulted in the extraction of 80% of the radioactivity present in the incubation mixture. The organic phases were combined and the methylene chloride removed under a stream of nitrogen (water bath, 40°) to yield a tan residue. The latter was dissolved in methanol and subjected to TLC[1] (solvent system A; see below) to yield seven metabolite fractions. Further purification of these fractions, if necessary, was achieved by use of TLC with three other solvent systems (systems B–D; see below). Appropriate zones (visualized by radioscanning[2] or short-wavelength UV[3]) were scraped from the plate and the silica gel washed three times with ethyl acetate–methanol, 4:1. The eluates were combined and, following removal of solvent under a nitrogen stream at 40°, the residue was partitioned between ethyl acetate (2 ml) and water (0.1 ml) to minimize the amount of silica gel present in the isolated metabolite fraction.

TLC and Radioactivity Assay—Isolation of metabolite fractions *via* TLC involved the use of four solvent[4] systems: toluene–ethyl acetate, 3:1 (system A); methylene chloride (system B); methylene chloride–methanol, 9:1 (system C); and trimethylpentane–2-propanol, 4:1 (system D). Metabolite fractions were assayed for radioactivity by use of liquid scintillation counting[5] techniques.

Derivatization—Methoxime formation was carried out by dissolving an aliquot of the metabolite fraction in a pyridine solution of methoxyamine hydrochloride[6] (5 mg/ml) and allowing the reaction to proceed for 2 hr at room temperature. The trimethylsilyl derivative was prepared by dissolving an aliquot of the metabolite fraction in a 1:1 (v/v) mixture of *bis*(trimethylsilyl)trifluoroacetamide[7]–pyridine and allowing the reaction to proceed for 1 hr at room temperature.

NMR and Mass Spectrometry—NMR spectra[8] were obtained from metabolite fractions dissolved in deuterochloroform. Mass spectra[9] of metabolite fractions were obtained by use of the direct probe technique under the following operating conditions: 3.5 kV accelerating potential, 60 μA trap current, 70 eV ionizing energy, and 250° source temperature. Capillary column GLC–mass spectrometry[10] was carried out using a 15 m × 0.25-mm i.d. SE-30 column operated at 220° (helium carrier gas, 1

[1] Quanta/Gram QIF plates, Quantum Industries.
[2] BID System 100 Radioisotope Imaging System.
[3] Chromato-Vue.
[4] Burdick and Jackson, distilled in glass.
[5] Packard Tricarb Liquid Scintillation Spectrometer.
[6] Eastman.
[7] Supelco.
[8] Varian SC-300 MHz spectrometer equipped with a Fourier transform accessory.
[9] LKB 9000 instrument.
[10] Finnigan 3200 instrument.

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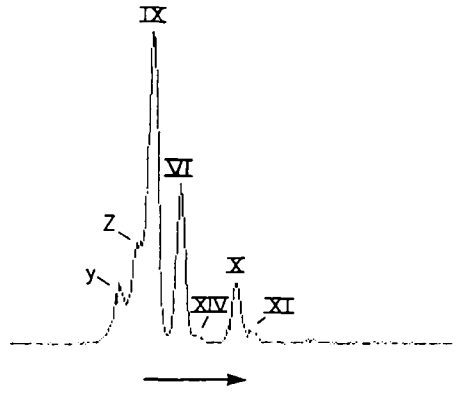

Figure 1—*Radioactivity profile*[11] *arising from TLC (silica gel; toluene–ethyl acetate, 3:1) of the methylene chloride extract from an* in vitro *reaction (rat liver S9 fraction) of [*^{14}*C]I.*

ml/min) interfaced with a spectrometer operated at 70 eV ionizing energy, 0.8 mA emission current, and 1800 V electron multiplier.

RESULTS AND DISCUSSION

The nitroimidazole I and its known hydroxyl group-containing metabolites exhibit strong short-wavelength UV absorption (254 nm) which facilitates their detection on a TLC plate. Further, as the drug used in these studies was ^{14}C-labeled, radioscanning of a developed TLC plate also allowed the detection of metabolites. The electron impact mass spectra of these compounds normally possess intense fragment ions at *m/z* 153 and 195 (1), and these, plus the presence of molecular ions at *m/z* 266 or 282 (16 and 32 AMU greater than the molecular ion of I, respectively), facilitate recognition of the unknown compounds as mono- or dihydroxylated analogues of I. When the metabolites are trimethylsilylated, their molecular ions increase by 72 AMU for each hydroxyl group present to yield signals at *m/z* 338 and 426 (molecular ions, low intensity) and *m/z* 323 and 411 (the corresponding M-15 ions).

Analytical TLC (solvent system A) with UV and/or radioscanning and capillary column GLC–mass spectrometry (as trimethylsilyl derivatives) of a methylene chloride extract of the incubation mixture (~80% of the radioactivity was extractable) demonstrated that it was multicomponent in nature, containing at least seven compounds related to I (Fig. 1, Table I). None of the parent drug was present. Preparative scale TLC (solvent system A) was employed to isolate the seven metabolite fractions in quantities suitable for structure elucidation. These were examined by mass and NMR spectrometry. In all cases the mass spectrometric data showed the metabolites to be mono- or dioxygenated derivatives of I, with the metabolic transformation occurring on the hexahydrobenzisoxazole ring (Table II).

One of the isolated metabolites was shown to be the 7-equatorial hydroxy compound, previously identified as a canine urinary metabolite of I (2) and is thus referred to as metabolite VI (the previous designation). This is the second most abundant of the *in vitro* metabolites.

The major *in vitro* metabolite, IX, is also a monohydroxy derivative of I (molecular ion, *m/z* 266). On the basis of NMR data [4.60 ddd, 7.5, 4.0, 4.0 (H_{7a}); 4.17 br s, $w_{1/2}$: 11 H_3 (H_{5eq}); 3.82 dt, 10.0, 7.5, 7.5 (H_{3a})] the hydroxyl group is assigned to the 5-position with an axial conformation.

Metabolite X is the third most abundant metabolite arising from the S9 incubation. It also possesses a molecular weight of 266, as determined by mass spectrometry. The location of the hydroxyl group was shown by NMR to be at C_{7a}, since the characteristic H_{7a} signal was absent. Other pertinent NMR data included the signal at 3.47 dd, 10.5, 7.5 (H_{3a}). This metabolite was found to dehydrate on prolonged exposure to silica gel to yield an olefin (molecular weight 248) identified as the $\Delta^{3a,7a}$ compound.

Metabolite XI, the fourth of the monohydroxy metabolites, was shown by NMR [key features include signals at 4.66 m (H_{7a}); 3.22 t, 8.3 (H_{3a}); and 3.59 ddd, 11.5, 8.0, 5.0 (H_{4ax})] to possess an equatorial hydroxyl group at C-4. Although hydroxylation at C-4 of I has been recognized previously for an *in vivo* metabolite [4,5-di-equatorial dihydroxy, II (2)], this is the first instance of monohydroxylation at this position.

The minor metabolite fractions Y and Z were found, on the basis of NMR (presence of several N—CH_3 signals) and mass spectrometry (interscan variation of relative ion intensities), to be multicomponent in nature. Preparative scale TLC (solvent system D; Table I) was employed to isolate two metabolites (one major, one minor) from Y and three metabolites (two major, one minor) from Z. The two major components of Z were shown to be the 5- and 6-equatorial monohydroxy metabolites, both previously found as canine urinary metabolites of I (2) and are thus designated (as before) metabolites IV and VII.

The minor components of Y and Z and the major component of Y all possess molecular ions of *m/z* 282 and are thus dihydroxy metabolites of I. As with all of the other known metabolites of this drug, their mass spectrometric behavior indicated that hydroxylation had occurred on the hexahydrobenzisoxazole ring. The exact positions and stereochemistry of the two hydroxyl groups of the major Y metabolite (VIII) were shown by NMR to be 5-axial and 7a. The NMR spectrum of VIII is characterized by the absence of an H_{7a} signal and the presence of a new *H*COH peak of 4.16δ. An equatorial configuration for the 4.16δ methine is inferred based on the broad singlet character and relatively narrow linewidth ($w_{1/2}$10 Hz). Since the coupling patterns of H_{3a} and H_{7a} axial exclude C-4 and C-6 as possible sites, VIII is assigned as the 5-axial,7a-dihydroxy analogue. The minor components of Y (XII) and Z (XIII) were not further characterized.

A very minor metabolite (XIV) was found by mass spectrometry to possess a molecular weight of 264 (*versus* 250 for I). This shift of 14 mass units suggested that the metabolic transformation involved formation of a ketone. Compound XIV was exposed to methoxime-forming conditions (4); mass spectrometry showed a shift in molecular weight from 264 to 293, demonstrating the ketonic nature of the metabolite. The exact site of the keto group on the hexahydrobenzisoxazole ring was shown by NMR [5.19 dt, 11.5, 3.5, 3.5 (H_{7a}); 4.38 ddd, 11.3, 7.3, 3.5 (H_{3a}); 2.96 dd 16.4, 3.5; 2.77 dd 16.4, 7.5 (4-CH_2); 2.44 m, 2.32 m (6,7-CH_2)] to be the 5-position. This metabolite presumably arises from IX *via* oxidation of the 5-axial hydroxyl group.

When the 6,7-epoxide of I is incubated under the conditions that yield the metabolites discussed above, the major metabolite is the 5-axial hydroxy derivative of the 6,7-epoxide. The mass spectrum of this metabolite contains a molecular ion at *m/z* 280 with *m/z* 195 as base peak, the same base peak as found in the mass spectrum of IX (which also possesses an axial hydroxyl group at C-5). The NMR of the hydroxylated epoxide showed peaks at 5.13 d, 11.0 (H_{7a}); 4.10 ddd, 11.0, 5.0, 3.0 (H_{3a}); 3.88 dd, 11.5, 5.0 (H_{5a}); 3.42 br s ($H_{6,7}$); 2.72 ddd, 14.0, 5.0, 4.0 (H_{4eq}); 1.95 ddd, 11.5, 11.5, 5.5 (H_{4ax}).

A preliminary incubation, in which only 10% of the radioactivity was extractable from the aqueous system by methylene chloride, gave six metabolites isolated by TLC (Table I)[12]. Again, no I was found in the extract (as measured by TLC). Four of these compounds were shown by TLC, mass spectrometry, and/or NMR to be metabolites IV, VI, VII, and IX. The two others are newly recognized *in vitro* metabolites of I. One is the 7-axial hydroxy analogue (metabolite V, previously found as a conjugate in dog urine); the second is the 4-equatorial,5-axial dihydroxy analogue (metabolite XV, not found in dog urine). The mass spectrum of this latter metabolite exhibits a molecular ion at *m/z* 282 and fragment

Table I—TLC R_f Values for *In Vitro* Metabolites of I

Compound	Substitution	Solvent System[a] A	B	C	D
I	—	0.72	—	—	—
IV	5-Eq-OH	0.09	—	—	0.28
V	7-Ax-OH	0.14	—	0.51	—
VI	7-Eq-OH	0.22	0.07	—	—
VII	6-Eq-OH	0.09	—	—	0.32
VIII	5-Ax,7a-$(OH)_2$	0.04	—	—	0.39
IX	5-Ax-OH	0.14	—	0.42	—
X	7a-OH	0.38	—	—	—
XI	4-Eq-OH	0.43	—	—	—
XII	$(OH)_2$	0.04	—	—	0.28
XIII	$(OH)_2$	0.09	—	—	0.39
XIV	5-Keto	0.27	—	—	—
XV	4-Eq,5-Ax-$(OH)_2$	0.22	0.11	—	—

[a] (A) Toluene–ethyl acetate, 3:1; (B) methylene chloride; (C) methylene chloride–methanol, 7:1; (D) trimethylpentane-2-propanol, 4:1.

[11] Bioscan BIII System 100/Hewlett-Packard 85.

[12] When incubation of ^{14}C-labeled I in the presence of S9 is carried out with a limited amount of air (without shaking) most of the radioactivity does not extract into methylene chloride following termination of the incubation but is associated with water-soluble species. (H. Skeggs and W. J. A. VandenHeuvel, unpublished data.)

Table II—Major Ions Found in the Mass Spectra of *In Vitro* Metabolites of I

Compound	Substitution	Ion and Relative Intensity											
		m/z 282	*m/z* 266	*m/z* 265	*m/z* 249	*m/z* 247	*m/z* 231	*m/z* 221	*m/z* 195	*m/z* 169	*m/z* 153	*m/z* 149	*m/z* 107
IV	5-Eq-OH	—	77	—	79	—	—	36	54	35	100	—	34
V	7-Ax-OH[a]	—	20	—	9	19	—	2	42	35	100	—	14
VI	7-Eq-OH[b]	—	41	—	54	90	20	16	60	10	100	—	32
VII	6-Eq-OH	—	95	—	80	12	—	—	96	35	100	—	43
VIII	5-Ax,7a-$(OH)_2$[c]	30	—	100	—	26	—	29	28	—	67	—	28
IX	5-Ax-OH[d]	—	29	—	28	22	5	—	100	10	48	—	10
X	7a-OH[e]	—	48	—	100	4	—	—	44	15	36	—	10
XI	4-Eq-OH	—	4	—	2	—	—	—	100	—	3	15	4
XII	$(OH)_2$	28	—	20	—	10	—	—	57	—	100	—	19
XIII	$(OH)_2$	3	—	2	—	—	—	—	6	—	100	—	19
XIV	5-Keto[f]	—	—	—	—	15	—	—	6	25	53	—	—
XV	4-Eq,5-Ax-$(OH)_2$	5	—	1	—	—	—	—	100	4	1	17	7

[a] *m/z* 210, 4%. [b] *m/z* 210, 2%. [c] *m/z* 237, 30%; *m/z* 223, 37%. [d] *m/z* 96, 30%. [e] *m/z* 210, 54%; *m/z* 207, 37%; *m/z* 204, 20%; *m/z* 193, 32%. [f] *m/z* 264, 100%.

ions characteristic of hydroxylation at C-4 and C-7 of the hexahydrobenzisoxazole ring. The NMR of this compound is characterized by signals at 4.68 m (H_{7a}); 3.54 dd, 8.0, 3.5 (H_{4ax}); 4.10 br s, $w_{1/2}$10 Hz (H_{5eq}); and 3.40 t, 8.0 (H_{3a}).

The outcome of this preliminary experiment (which yielded metabolites IV–VII, IX,and XV) might have resulted from a lack of sufficient air in the reaction flask during the incubation or possibly from secondary metabolism resulting from a high enzyme to substrate ratio and extended incubation. In any event, it is clear that routes and extent of *in vitro* metabolism of I are heavily dependent on the incubation conditions. Whether a radically different metabolism (*e.g.*, transformations of the nitro group or the heterocyclic rings) occurs or whether further oxidative metabolism of the hexahydrobenzisoxazole ring leads to highly polar, ring-opened derivatives is not known at present.

Earlier work demonstrated that at least four dihydroxy metabolites (two fully characterized) and at least five monohydroxy metabolites of I are found (free and/or conjugated) in dog urine (1, 2). The current study shows that monohydroxylation is by far the major *in vitro* pathway, but that dihydroxylation is not precluded. A total of 14 *in vivo* and *in vitro* metabolites of I resulting from introduction of one or two oxygen atoms into the hexahydrobenzisoxazole ring have now been recognized.

The extensive oxidative metabolism of I is another example of the ability of the hepatic microsomal mixed-function oxidases to metabolize a compound at several sites (5–7). It is of interest that the alicyclic carbons of the saturated benzisoxazole ring are the targets of metabolism, whereas the N—CH_3 group appears resistent to metabolic attack under the conditions used. Likewise, to date no metabolism has been found to occur at the 3a-position. So although extensive, the metabolism is quite selective. Other nitroimidazoles undergo metabolism on their hydrocarbon moieties. Metronidazole and ipronidazole are hydroxylated on the 2-CH_3 group (8) and the 2-isopropyl group (9), respectively, misonidazole undergoes *O*-demethylation (10), and 5-isopropyl-1-methyl-2-nitro-1*H*-imidazole undergoes extensive metabolism at the isopropyl group (11). Although reduction of the nitro group is considered the mechanism by which nitroimidazoles exert their therapeutic activity, modification of other parts of the molecules can enhance or diminish this therapeutic activity, as shown previously (1).

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