Application of Chemical P-450 Model Systems to Study Drug Metabolism. III.[1] Metabolism of 3-Isobutyryl-2-isopropylpyrazolo[1,5-*a*]pyridine[2]

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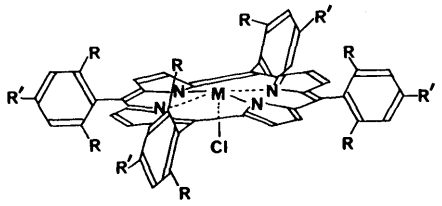
Oxidation of 3-isobutyryl-2-isopropylpyrazolo[1,5-*a*]pyridine (IBPP) was carried out with various chemical model systems for cytochrome P-450 in comparison with the liver microsomal system of rats or humans. α-Hydroxylation of side chains and ring hydroxylation at the 6 and 7 positions were the main reactions in both systems. A pattern analysis of products using two dimensional thin layer chromatography was employed to compare the functions of the chemical model systems with those of microsomal systems. The reaction profile of IBPP by the catalyst/Pt-colloid/H_2, O_2 system was most similar to that of human or rat microsomal system. The utility of these chemical models is discussed from the viewpoint of drug metabolism.

Keywords anti-asthma; cerebral vasodilator; 3-isobutyryl-2-isopropylpyrazolo[1,5-*a*]pyridine; metabolism; liver microsome; oxidation; P-450 chemical model; P-450 mimic; metalloporphyrin

Cytochrome P-450 enzymes metabolize various xenobiotics oxidatively or reductively and play an important role in drug metabolism. To elucidate the functions of these enzymes a wide variety of chemical models for P-450 have been developed. Several models work as efficient oxidation systems for simple substrates such as cyclohexane, cyclohexene, styrene, norbornene and *N*,*N*-dimethylaniline.[3] However, there have been only a few reports on the application of these model systems to the metabolic study of practical drugs having many functional groups.[4] In our previous study,[1] a cytochrome P-450 model system was effectively used to synthesize an unstable metabolic intermediate of 3-isobutyryl-2-isopropylpyrazolo[1,5-*a*]pyridine (IBPP), which has been developed as an antiasthma drug[5] and cerebral vasodilator.[6]

In the present study, we compared the reaction profiles of IBPP in microsomes and various chemical models, to investigate the characteristics of the chemical models as mimics for cytochrome P-450 and their utility for studying drug metabolism. We found that IBPP is oxidized by the following four P-450 chemical models: (i) metalloporphyrin/iodosobenzene (PhIO), (ii) metalloporphyrin/hypochlorite, (iii) metalloporphyrin/*m*-chloroperbenzoic acid (*m*CPBA) and (iv) metalloporphyrin/Pt-colloid/H_2, O_2 systems. The reaction profile in the model system iv was most similar to that of the microsomal system.



	M	R	R′
TPPFeCl :	Fe	H	H
TPPMnCl :	Mn	H	H
$T_{2,6}$FPPFeCl :	Fe	F	H
TMPFeCl :	Fe	CH_3	CH_3
$T_{2,4,6}$OMePPFeCl :	Fe	OCH_3	OCH_3
$T_{2,4,6}$PhPPFeCl :	Fe	C_6H_5	C_6H_5

Chart 1. Structures and Abbreviations of Metalloporphyrins

Experimental

Chemicals IBPP and its metabolites were gifts from the Central Research Laboratories of Kyorin Pharmaceutical Co., Ltd. ^{14}C-IBPP (^{14}C=O at the 3-position) and d_4-IBPP were synthesized after Nagatsu and Takagi.[7] Metalloporphyrins were synthesized by the methods of Adler[8] and Wagner *et al.*[9] Pt-colloid was prepared by the methods of Toshima *et al.*[10] Chart 1 shows the structures and abbreviations of the metalloporphyrins. All other chemicals were of reagent grade and were used without further purification.

Preparation of Microsomes (Ms) The liver was excised from male Wistar rats at 24 h after daily i.p. administration of phenobarbital at a dose of 60 mg/kg. Human liver was obtained 3 h after surgery to treat chronic active hepatitis. The microsomes were prepared by the method of Omura and Sato.[11] Protein concentration was determined by the method of Lowry *et al.*[12] or by using Bio-Rad protein assay kits based on the method of Bradford.[13]

Reactions with the Chemical Models The reactivity of IBPP in the various P-450 chemical model systems was previously examined using 1 mmol of IBPP, 0.01 mmol of metalloporphyrin and 1—5 mmol of oxidant or reductant. Metalloporphyrin was used as a cyclic catalyst.

(i) Catalyst/PhIO System: In accordance with the methods of Cook *et al.*,[14] 10 μmol of ^{14}C-IBPP (0.46 μCi, specific activity was 0.2 μCi/mg for all samples used), 1 μmol of catalyst and 40 μmol of PhIO were dissolved in 1 ml of methylene chloride (CH_2Cl_2), and then the mixture was stirred at room temperature for 16 h under an Ar atmosphere.

(ii) Catalyst/Sodium Hypochlorite System: According to Collman *et al.*[15] and Takagi *et al.*,[16] 5 μmol of ^{14}C-IBPP, 1 μmol of catalyst, 2 μmol of benzalkonium chloride (BKC) and 2.5 μmol of 4′-imidazolylacetophenone (4′-ImAP, used with manganese porphyrin) were dissolved in 1 ml of CH_2Cl_2 at 0 °C (ice bath), then 50 μmol of sodium hypochlorite in 1 ml of H_2O was added under an Ar atmosphere, followed by stirring for 2 h.

(iii) Catalyst/*m*CPBA System: According to Traylor and Miksztal,[17] 10 μmol of ^{14}C-IBPP and 1 μmol of catalyst were dissolved in 1 ml of CH_2Cl_2 with addition of 40 μmol of *m*CPBA, and then the reaction mixture was stirred at room temperature for 4 h.

(iv) Catalyst/Pt-colloid System: According to Tabushi *et al.*,[18] the reaction mixture of 10 μmol of ^{14}C-IBPP, 1 μmol of catalyst and 0.5 μmol of Pt-colloid in EtOH/H_2O (200 μl/200 μl) was stirred under a mixture of 3 l of H_2 and 1 l of O_2 at room temperature for 48 h. In some cases, 10 μl of acetic acid or 10 μl of tetramethylammonium hydroxide was added to the reaction mixture, or 500 μl of acetone was used as a solvent after the removal of EtOH.

Reaction by Microsomes Reaction conditions were optimized on the basis of previous experiments. A mixture of 0.4—0.5 μmol of ^{14}C-IBPP in 50 μl of EtOH, 6.3 mg (rat) or 2.7 mg (human) of microsomes, 25 units of glucose-6-phosphate dehydrogenase (G-6PDH), an reduced nicotinamide adenine dinucleotide phosphate (NADPH)-generating system (final concentrations: 8.6 mM $MgCl_2$, 17 mM KCl, 2.0 mM NADPH, 2.2 mM reduced nicotinamide adenine dinucleotide (NADH), 2.6 mM G-6P, 42.1 mM Na_2HPO_4 and 10.3 mM NaH_2PO_4) and enough 0.1 M phosphate buffer (pH 7.4) to bring the volume to 2 ml, was incubated at 37 °C for 1 h. The protein was removed by adding 5 ml of EtOH twice to the reaction

mixture, then the EtOH layer was concentrated in an evaporator for pattern analysis.

Pattern Analysis of Products Reaction mixtures were concentrated and spotted on a silica gel plate which was then subjected to two-dimensional development. The first developing solvent system was CH_2Cl_2–MeOH (20:1, v/v) and the second was $CHCl_3$–MeOH–C_6H_6–28%NH_4OH (10:4:2:1, v/v). The plate was sprayed with Enhance (NEN) and then wrapped with Lumirror membrane (6 μ, Mitsubishi Rayon) for contact with X-ray film (Fuji Medical RX type) to obtain the autoradiograms. Each fraction having ^{14}C-activity, appearing as a dark spot on the autoradiograms, was removed from the plate together with the silica gel, suspended in 1 ml of MeOH and mixed with 10 ml of scintillator (ACS II, Amersham) for ^{14}C assay. The ^{14}C-activities were determined with a liquid scintillation counter (Packard 2425 or Beckman 3801). An authentic sample of product **1** (3-COOH) was synthesized by the ordinary reaction of 2-isopropylpyrazolo[1,5-*a*]pyridine with BuLi and dry ice in *n*-hexane, 26.0%. mp 85—86 °C (*n*-hexane). *Anal.* Calcd for $C_{11}H_{12}N_2O_2$: C, 64.69; H, 5.92; N, 13.72. Found: C, 64.68; H, 6.11; N, 13.99. The structure of product **2** was estimated by gas chromatography-mass spectrum using the ion cluster method[19] with an equimolar mixture of d_4-IBPP and IBPP.

Results and Discussion

The structures of IBPP and its metabolites are listed in Chart 2. The two-dimensional thin layer chromatograms (TLC) of authentic metabolites and autoradiograms of the reaction products of IBPP produced by various P-450 chemical models and microsomes are shown in Fig. 1. Each spot was identified by comparison with authentic metabolites from the positions on the TLC plate. Unknown products were readily detected. The relative yields of products were determined from the ^{14}C values obtained with a liquid scintillation counter. The conversions and relative yields of the metabolites are listed in Table I.

The last column gives the total amount of unidentified products. The catalyst/PhIO system gave as the main metabolites of IBPP, 2α-OH and/or 3α-OH and 6,7-diOH, which was produced by hydrolysis of the 6,7-epoxide (entries 1a—c). In the catalyst/*m*CPBA system, 2α-OH and 3α-OH were the major products and 6,7-diOH was a minor one (entries 3a—f). In the catalyst/hypochlorite system, 6,7-diOH was the main product (entries 2a, b). The profiles of products in the catalyst/Pt-colloid/H_2, O_2 systems were comparatively similar to those of the microsomal systems. In many cases using a chemical model system, $(CH_3)_2CHCOOH$ was obtained in fairly high yields, while the microsomal systems did not afford it. The 3-position of pyrazolo[1,5-*a*]pyridines is chemically active and can undergo various reactions including C–C bond fission.[20] The production of isobutyric acid from IBPP is considered to be due to this reactivity. When *m*CPBA was used as an oxi-

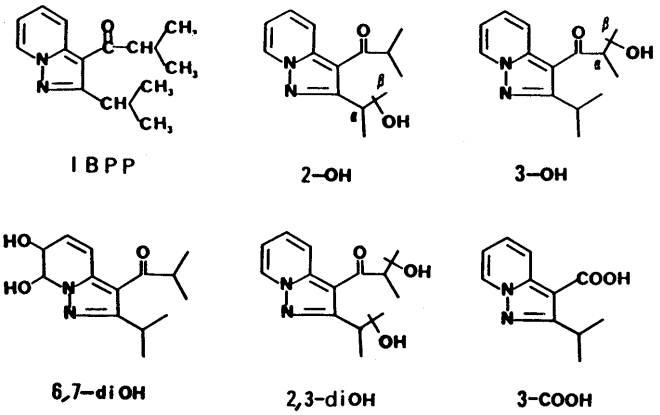


Chart 2. Structures of IBPP and Its Metabolites

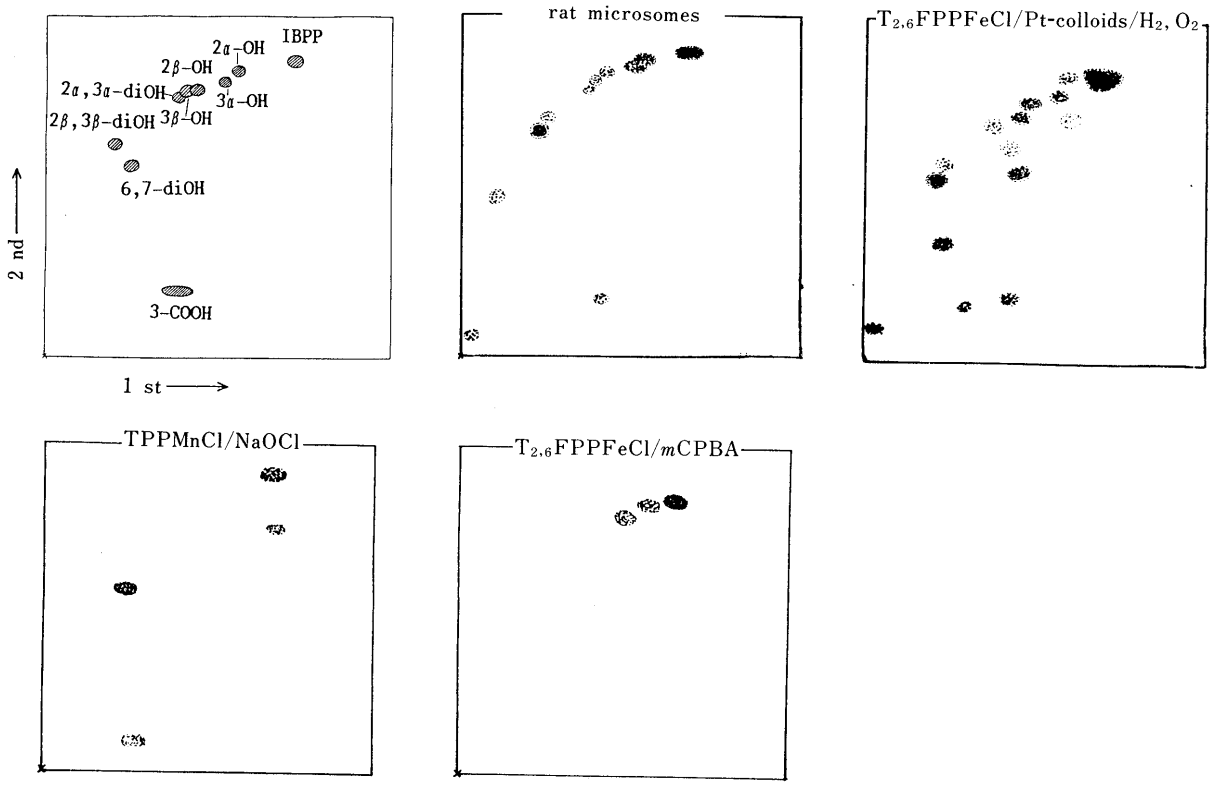


Fig. 1. Two-Dimensional TLC of Authentic Samples and Radiochromatograms of Products of ^{14}C-IBPP Obtained with P-450 Chemical Models and Microsomal System

Solvent: 1st, CH_2Cl_2–MeOH (20:1, v/v); 2nd, $CHCl_3$–MeOH–C_6H_6–28%NH_4OH (10:4:2:1, v/v).

TABLE I. Reaction Products of IBPP Formed by P-450 Chemical Models and Liver Microsomes

Entry No.	System	Relative yield (%)											
		Conversion	2α-OH	3α-OH	2β-OH	3β-OH	2α,3α-diOH	2β,3β-diOH	6,7-diOH	3-COOH[d]	Product 2[e]	$(CH_3)_2$-CHCOOH	Others
1-a	PhIO, TPPFeCl	14.6	5.5	3.2					4.6			70.3	16.4
1-b	PhIO, TPPMnCl	86.8	2.3						2.0			85.7	10.0
1-c	PhIO, $T_{2,6}$FPPFeCl	19.2	34.6	15.2					26.3			23.9	
2-a	NaOCl, TPPFeCl	59.7							33.6			58.7	7.7
2-b	NaOCl, TPPMnCl, 4′-ImAP	65.1							31.2			55.8	13.0
3-a	*m*CPBA, TPPFeCl	21.1	21.3	22.8					2.4			34.5	19.2
3-b	*m*CPBA, TPPMnCl	14.5	27.3	19.5					2.6			28.0	22.6
3-c	*m*CPBA, TMPFeCl	15.7	22.3	21.7	1.9	2.4	2.5		1.1			5.5	42.6
3-d	*m*CPBA, $T_{2,6}$FPPFeCl	23.2	55.5	44.5									
3-e	*m*CPBA, $T_{2,4,6}$OMePPFeCl	16.8	14.6	27.9	5.0	4.7			2.3				45.5
3-f	*m*CPBA, $T_{2,4,6}$PhPPFeCl	67.0	9.1	11.3	1.6	1.2	3.7		1.4				71.7
4-a	Pt-colloid, TPPMnCl	19.1	2.8	4.8	9.6	7.1	2.2	4.3	9.1	5.4	4.7	4.7	50.0
4-b	Pt-colloid, $T_{2,6}$FPPFeCl	16.6	4.9	5.8	10.1	7.7	3.5	4.3	9.8	6.7	14.2	1.8	45.4
4-c	Pt-colloid, $T_{2,6}$FPPFeCl, AcOH	35.7	5.4	9.6				4.0	12.5	5.8	6.9	3.8	58.9
4-d	Pt-colloid, $T_{2,6}$FPPFeCl, Acetone	13.7	6.9	5.1	10.8	6.0		3.0	14.5	9.7			44.0
4-e	Pt-colloid, $T_{2,6}$FPPFeCl, Me_4NOH	1.9	49.0						17.9	15.1			18.0
5 Rat	Ms (PB)[a]	45.4	30.7	29.1	2.9	4.9	1.5	2.8	23.9	0.7			3.5
6 Human	Ms	48.7	44.6[b]		7.8[c]				31.0				16.6

a) Treatment with phenobarbital at doses of 60 mg/kg i.p. daily for 3 d. *b*) Total of 2α-OH and 3α-OH. *c*) Total of 2β-OH and 3β-OH. *d*) Product **1** shown in Chart 2 was identified as 3-COOH. *e*) Product **2** was identified as 2-(2-hydroxy-1-methylethyl)-3-isobutyryl-7-ethoxypyrazolo[1,5-*a*]pyridin-6-one.

dant, large differences were observed in the reaction profile depending on the coupled catalysts. It is noteworthy that the steric effects of ligands in the porphyrin are effective for mimicking the reactivity of microsomes (entries 3c, e, f). In the case of Pt-colloid/catalyst systems, the conversion yields and the product pattern varied with the pH of the solvent. Namely the conversions were higher under acidic (pH 3; entry 4c) and neutral (pH 7; entry 4b) conditions than under basic conditions (pH 10; entry 4e). When the solvent, EtOH–H_2O (1 : 1), was replaced by acetone, the product 2 described later was not obtained (entry 4d). A common reaction product (3-COOH: product **1**) was obtained in the Pt-colloid system and this product was also detected in the rat microsomal system (entries 4a—e, 5). The α oxidation (α-OH) of the side chains of IBPP and the ring hydroxylation (6,7-diOH) were the main pathways in both chemical model systems and microsomes. These findings imply that the present four chemical model systems resemble microsomes in the "metabolic reaction" of IBPP. Among them, the Pt-colloid/catalyst system was closest to microsomes in overall reaction profile. The metabolic patterns in rat and human microsomes differed a little, presumably because of the species difference. However, both microsomal systems were similar in the relative yields of 2α-OH, 3α-OH, 2β-OH, 3β-OH and 6,7-diOH (entries 5, 6). Product **1** and product **2** were obtained only in the Pt-colloid system. Product **1**, which was also detected in the rat microsomal system, was identified as 3-isobutyrylpyrazolo[1,5-*a*]pyridine-3-carboxylic acid by comparison with an authentic sample synthesized by another route (described in Experimental). Product **2** was assumed to be 2-(2-hydroxy-1-methylethyl)-3-isobutyryl-7-ethoxypyrazolo[1,5-*a*]pyridin-6-one on the basis of analysis of the 400 MHz proton nuclear magnetic resonance (^{1}H-NMR) spectrum and of the mass spectrum by use of the ion cluster method.[19] Product **2** was presumed to be derived from the active intermediate 6,7-epoxide by reaction with ethanol used as the solvent in a manner similar to that in the cyclohexene/Pt-colloid/TPPMnCl system.[18]

Conclusion

We attempted to apply some chemical model systems of cytochrome P-450 to the "metabolic oxidation" of the multi-functional drug IBPP, in comparison with the liver microsomal system of mammals (rat or human). Various reaction profiles were shown with various combinations of catalyst and oxidant. The main metabolites of IBPP in microsomes were obtained in most of the chemical model systems though they were accompanied with by-products. The reaction profile of IBPP in the metalloporphyrin catalyst/Pt-colloid/H_2, O_2 system was most similar to that in the rat or human microsomal system. A pattern analysis of products by means of two-dimensional TLC is available and would make it possible to select or design a model system most suitable for the metabolism of each drug. Further applications of chemical model systems for P-450 [P-450 mimics] to metabolism of various drugs are in progress.

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References and Notes

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