Pure & Appl. Chem., Vol. 66, No. 4, pp. 729–736.
Printed in Great Britain.

New developments in cytochrome P-450 modeling

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Abstract- Presented here are the results of recent studies which were designed to: (1) evaluate the role of the thiolate ligand, which is characteristic of P-450 structure, by incorporating it into model systems; (2) synthesize polypeptide-bound porphyrin to investigate the effects of heme environment on the activities; (3) apply appropriately designed P-450 mimics to drug metabolism studies and evaluate their utility.

Cytochrome P-450 plays an important role in metabolizing biomolecules and xenobiotics, and the mechanism of its catalytic activities and structural functions have been the subject of extensive investigation in the field of biomimetic chemistry(ref. 1). We have achieved the synthesis of many effective P-450 mimics and clarified their functions with the aim of developing artificial enzymes having high similarity to the native P-450 system for investigating drug metabolism.

Here we present three recent topics in our studies; the evaluation of the effect of the axial ligand and apoprotein by incorporation into model systems, and the application of appropriately designed P-450 mimics to drug metabolism studies.

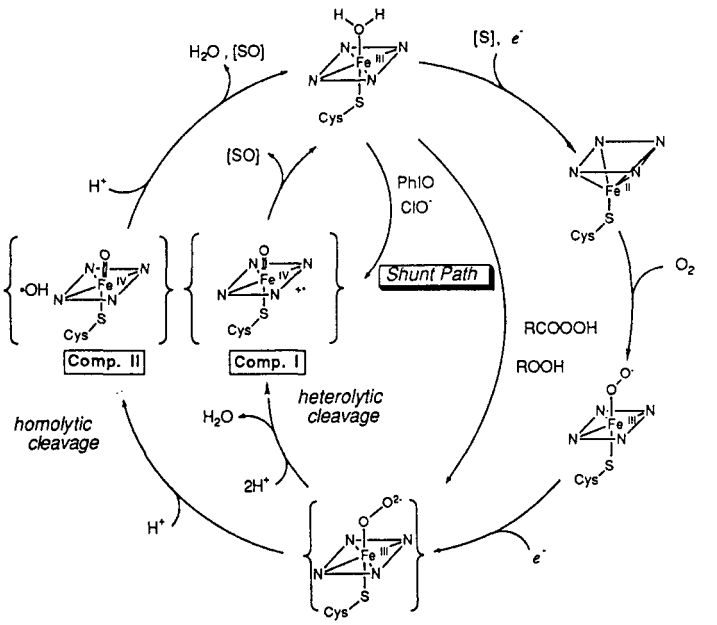
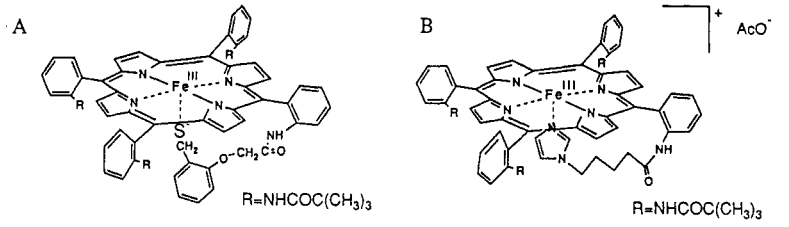


Fig. 1 Catalytic cycle of cytochrome P-450

EVALUATION OF THE ROLE OF THE THIOLATE LIGAND CHARACTERISTIC OF P-450 STRUCTURE

Efficient model systems for the evaluation of thiolate ligation have never been reported, because the thiolate group is labile in oxidation conditions. In order to examine this ligand effect, we synthesized a novel iron porphyrin coordinated by the thiolate anion which is designed to introduce bulky pivaloyl groups on the thiolate coordination face of the porphyrin molecule so that the thiolate ligation could be highly stabilized and protected from oxidation. This P-450 model, termed as the SR-complex, could be stored under air for several months. The characteristic feature of this complex is shown in Figure 2A. It may be the first synthetic example of an isolable low spin ferric porphyrin coordinated by an alkylthiolate with high stability toward dioxygen and thermodynamic stability(ref. 2).



S.R. ("Swan Resting Complex")

FAB·MS. 1160 ($M-CH_3OH$)

EPR. Fe(III) (in DMSO) g_x=1.96, g_y= 2.21, g_z=2.32

EA. Calcd for $C_{69}H_{68}N_8O_6SFe$: C, 69.51; H, 5.66; N, 9.40; S, 2.68. Found: C, 69.23; H, 5.52; N, 9.38; S, 2.60.

UV. Fe(II) (in DMSO) 424, 536, 578 (sh) nm

Fe(II)-CO spectrum. (in DMSO) 459 nm

N.P.

High-resolution FAB·MS. Observed m/e 1131.4668 $(M-AcO)^+$, calcd for $(C_{67}H_{67}N_{10}O_4Fe)^+$; 1134.4696

EPR. (in toluene) g = 6.0, 2.0 (high spin)

EA. Calcd for N.P.$3CH_2Cl_23H_2O$,$(C_{72}H_{81}N_{10}O_9Cl_6Fe)$: C, 57.69; H, 5.45; N, 9.34. Found: C, 57.87; H, 5.00; N, 9.27. Hygroscopic.

UV. Fe(II). (in toluene) 430, 530, 560(sh), 600(sh) nm

Fe(II)-CO spectrum. 423 nm

Fig. 2 Structures of S.R. and N.P.

To clarify the axial ligand effect on the oxygen activation by P-450, which includes a multistep reaction, the effect at each step should be evaluated using the SR-complex. First of all, we examined the catalytic activity of SR on the peroxide shunt reaction of P-450 using alkylperoxides and compared it with that of FeTPPCl to investigate the relative effect of a thiolate ligand on O-O bond cleavage. TBPH and DPPH were chosen as substrates because both are known to trap reactive intermediates with excellent efficiency to almost stoichiometrically

Chart 1 TBP• and DPP• formation catalyzed by heme with alkyl hydroperoxide

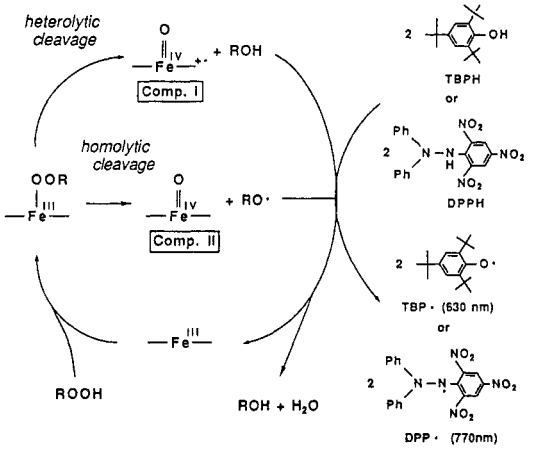


TABLE 1. Observed initial rates of TBP• or DPP• formation on the oxidation of TBPH or DPPH with alkyl hydroperoxides catalyzed by S.R. complex or FeTPPCl.

$$2\ \text{TBPH} + \text{ROOH} \xrightarrow{\text{Catalyst}} 2\ \text{TBP}\cdot + \text{ROH} + H_2O$$

$$2\ \text{DPPH} + \text{ROOH} \xrightarrow{\text{Catalyst}} 2\ \text{DPP}\cdot + \text{ROH} + H_2O$$

Substrate	Oxidant	v (turnover number/ min)[a] S.R.	FeTPPCl	$v_{SR}/v_{FeTPPCl}$
TBPH	$PhC(CH_3)_2OOH$	21	0.35	58
	tert-butyl-OOH	8.5	0.080	110
DPPH	$PhC(CH_3)_2OOH$	20	0.085	235
	tert-butyl-OOH	7.5	0.041	182

Conditions: Solvent = toluene; [TBPH] = 0.2 M; [DPPH] = 0.1 M; [Oxidant] = 5×10^{-2} M; [S.R.] = [FeTPPCl] = 10^{-4} M. These reactions were carried out at 20° under argon atmosphere. a) Observed initial rates of the reactions were based on catalysts (turnover number of catalysts / min).

produce the TBP or DPP radical(chart 1, ref.3). Toluene was used as the solvent, taking into account the highly hydrophobic environment of the P-450 active site. Cumene hydroperoxide or tert-butyl hydroperoxide was used as the oxidant. The observed initial rates of TBP or DPP radical formation were as shown in Table 1. Comparison of the reaction rates derived by monitoring the increase in TBP(630nm) or DPP(700nm) absorbance shows SR to have about 60-240 times higher activity than FeTPPCl(ref.2).

Next, a new iron porphyrin axially and intramolecularly coordinated by imidazole, termed the NP-complex, was prepared in order to compare the effect of imidazole as an axial ligand with that of thiolate. The structure of NP is a modification of the imidazole-ligated heme prepared by Collman(ref. 4) and synthesized according to almost the same procedure used for the SR-complex. All of the UV-Vis. and EPR spectra, elemental analysis and high resolution FAB mass spectra supported the structure shown in Figure 2B. We compared the catalytic activity of NP with that of the SR complex for the same oxidation system as previously mentioned. The oxidation activities were found to be extremely affected by solvent polarity. The catalytic activity of SR was highly inhibited in the polar solvent, while the activity of NP increased with an increase in the dielectric constant of the solvent(ref. 5). These tendencies are of interest in connection with the fact that the environment of P-450's active site is highly hydrophobic and the environments of the hemes active sites having imidazole as the axial ligand, such as peroxidase are generally hydrophilic.

Chart 2. O-O bond cleavage of peroxyphenylacetic acid catalyzed by iron porphyrins

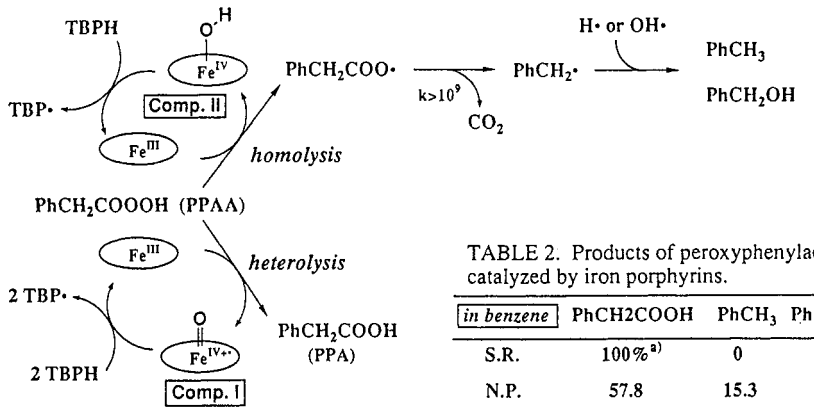


TABLE 2. Products of peroxyphenylaceticacid catalyzed by iron porphyrins.

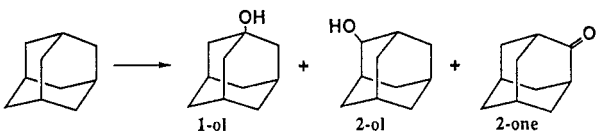
in benzene	PhCH2COOH	PhCH$_3$	PhCH$_2$OH
S.R.	100%[a)]	0	0
N.P.	57.8	15.3	0
FeTPPCl	7.4	61.0	27.6

Reaction conditions ; [Por.]=0.1mM, [PPAA] =1mM,TBPH= 10mM at r.t. under argon. a) Yields were based on PPAA.

The modes of O-O bond cleavage mediated by such iron porphyrins were examined using peroxyphenylacetic acid(PPAA), which has frequently been used as a probe for this purpose(ref. 6). The oxidation of TBPH by PPAA catalyzed by hemes affords phenylacetic acid(PAA) when the O-O bond of PPAA breaks heterolytically, or affords toluene and benzyl alcohol via the benzyl radical formed by rapid decarboxylation from the PAA radical in the case of homolysis as shown in chart 2. The reaction profiles of this catalytic reaction were compared among the three iron porphyrins in benzene, since its hydrophobicity is presumed to be close to that of the environment of P-450's active site. The SR complex quantitatively gave PAA, while FeTPPCl mainly catalyzed the formation of toluene, benzyl alcohol and carbon dioxide. The NP complex showed moderate reactivity intermediate between SR and FeTPPCl(Table 2, ref.7). Therefore, we can conclude from these data that SR heterolytically breaks the O-O bond of peroxyacids heterolytically in benzene. This result indicates that the thiolate

ligand enhances heterolytic cleavage of the peroxyacid-iron porphyrin complex to produce a compound I type intermediate in highly hydrophobic media. On the other hand, FeTPPCl catalyzes the homolysis of peroxyacid to give a compound II type intermediate in hydrophobic media. Thus, it is expected that a more strongly electrodonating axial ligand would more significantly enhance both heterolytic O-O bond scission and its reaction rate. The reactivity of the active species formed by cleavage of the O-O bond of peroxyacid-iron porphyrin complexes was examined by using inactivated alkanes as substrates in benzene. The SR complex catalyzed the hydroxylation of adamantane so efficiently that the yield of adamantanols based on the used mCPBA reached 88%(Table 3, ref. 7). In this reaction, 80% of SR in the reaction mixture was confirmed to remain undecomposed based on EPR and UV-Vis. spectroscopies. On the other hand, only a low or moderate yield of adamantanol was obtained by catalysis with FeTPPCl or the NP-complex, although mCPBA was completely consumed. FeTPPCl and the NP-complex also retained their structures after completion of the reactions. A similar tendency was also observed when cyclohexane was used as a substrate. Thus, the large enhancement in the reactivity of the SR complex in alkane hydroxylation in hydrophobic media provides a rational explanation for the role of thiolate coordination of heme in cytochrome P-450.

TABLE 3. Oxidation of alkanes by iron porphyrin-peracid system



	Oxidant	Adamantanol	1-ol : 2-ol	$PhCH_2COOH$	$PhCH_2OH$ + PhCHO
S.R.	*m*CPBA	88 %	10:1	——	——
N.P.	*m*CPBA	39	7.4:1	——	——
Fe(TPP)Cl	*m*CPBA	11	5:1	——	——
S.R.	PPAA	71	9.5:1	51 %	2 %
Fe(TPP)Cl	PPAA	13	4.8:1	39	13

These reactions were carried out in benzene at r.t. under argon for 10 min. [cat.] = [oxidant] = 1.0 mM; [Adamanntane] = 0.50 M. Yields based on the oxidants used.

SYNTHESIS OF POLYPEPTIDE-BOUND PORPHYRIN AND ITS POLYMER EFFECT ON P-450-LIKE REACTIVITIES

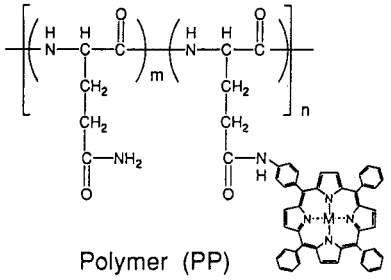
We designed a novel polypeptide-bound porphynato iron complex to investigate the environmental effect of the heme enzyme. This polymer porphyrin(PP), was synthesized from poly-γ-methyl-L-glutamate(MW:100,000) by coupling with FeTPPCl(ref. 8). The ratio of glutamine residues to FeTPPCl was estimated to be about 110 by atomic absorption analysis(chart 3).

We have already reported that this polymer complex exhibited more P-450-like activities than the non-bound FeTPPCl in the olefin oxidation with molecular oxygen in the presence of $NaBH_4$(ref. 8).

It has been well known that cytochrome P-450 catalyzes the demethylation of alkylamines. However, when this polymer was used, unexpected products such as N-N coupling and C-N coupling products were obtained besides the normal demethylation product aniline(chart 4, ref. 9). Table 4 shows the result of this reaction under air in the presence of $NaBH_4$. These coupling compounds were produced in relatively high yield compared with the case of the free FeTPPCl.

Based on this result, we presumed that these products may be unknown metabolites in the P-450 system. So we attempted to detect these compounds in a rat liver microsomal reaction mixture using the GC-SIM method. Two rat liver microsomal systems, the NADPH/O_2 and the cumenehydroperoxide-supported shunt

Chart 3



Polymer (PP)
M = Fe
m = 110

Chart 4

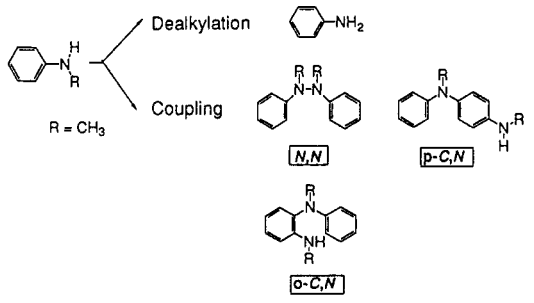


TABLE. 4 Coupling products formation catalyzed by Cyt.P-450 model systems

Catalyst	N,N	o-C,N	p-C,N	aniline
	(μ mol / μ mol cat.)			
Polymer-FeTPPCl	1.84	0.12	1.61	8.92
Free FeTPPCl	0.06	trace	0.12	17.0

Reaction mixture contained catalyst (1μmol), *N*-methylaniline (0.2 mmol), tetramethylammonium hydroxide (0.2 ml of 10 % methanol solution), $NaBH_4$ (0.5 mmol), and methanol (1.3 ml). The reaction mixture was stirred vigorously under air for 3 hr at r.t.

path, were employed. As expected, these coupling compounds were also found as metabolites of N-methylaniline in the microsomal system. Table 5 shows the yields of each coupling product and formaldehyde, which corresponds to the N-demethylation quantified by Nash's method. A significant amount of the coupling product was formed even though at a lower yield than the demethylation product. In addition, P-450 specific inhibitors, SKF-525A and metyrapone, suppressed the formation of these coupling products(data are not shown). These results indicate the coupling reaction is P-450 dependent. Thus, P-450 chemical model systems are available for investigating new metabolic pathways.

TABLE. 5 Coupling products formation of N-methylaniline catalyzed by microsomal systems

	N,N	o-C,N	p-C,N	HCHO
	(n mol / n mol P-450)			
NADPH / O2 Complete	1.38	0.34	0.23	11.35
Cumene hydroperoxide	2.41	0.73	5.32	42.93

Reaction conditions : rat liver microsomes (4.7 mg protein), N-methylaniline (4mM) and NADPH regenerating system (4 mM G-6-P, 5 units G-6-PDHase, 0.4 mM $NADP^+$) or cumene hydroperoxide (1 mM) in 2.5 ml of Na-Pi buffer (0.1 M pH 7.4).

APPLICATION OF P-450 MIMICS TO DRUG METABOLISM STUDIES AND EVALUATION OF THEIR UTILITY

In studies on drug metabolism, it is quite important but difficult to isolate and characterize all the metabolites. We focused on a more complex multi-functional actual drug as a substrate to compare the microsomal oxidation with chemical oxidation systems.

The advantages of using model systems to understand drug metabolism are as follows: 1) metabolite candidates are available in relatively large amounts and can be used to identify the real in vivo metabolites and provide samples for pharmacological testing, 2) the mode of metabolism can be clarified, for example, unstable metabolites can be isolated under selected and controlled

reaction conditions, and 3) the usage of experimental animals can be reduced.

As the first example for this study, phencyclidine(PCP), an anesthetic agent, was chosen as the drug, because, despite being a small molecule, it has an aromatic ring, an aliphatic ring and an N-containing heterocyclic ring moiety, which is expected to exhibit a diversity of reactions in various model systems.

As shown in chart 5, many types of oxidation products were produced by using various chemical model systems; phenolic, alcoholic and ring-opened amino acids(ref. 10).

Chart 5

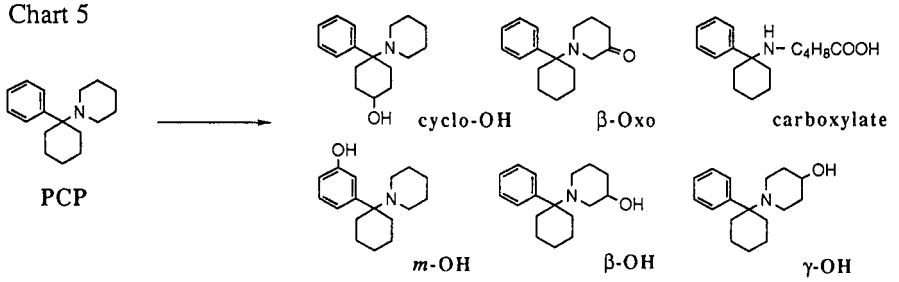


TABLE. 6 Oxidation of PCP by iron porphyrin-mCPBA system.

Catalyst	Yield (based on Oxidant)					
	m-OH	β-OH	γ-OH	cyclo-OH	β-oxo	carboxylate
S.R.	n.d.	1.85%	41%	0.44%	18.5%	n.d.
N.P.	n.d.	2.7	41	0.59	0.41	6.5%
FeTPPCl	n.d.	0.18	22	0.75	1.2	trace

Reaction conditions; [cat.]=1mM, [mCPBA]=1mM, [PCP]=500mM at r.t. under argon.

The SR and NP complexes gave similar products in combination with mCPBA as shown in Table 6. It is noteworthy that the piperidine 3-oxo compound was predominantly obtained in the SR catalyst system.

We then examined the reaction of PCP in the liver microsomal systems. Microsomes-oxidant systems were constituted from three kinds of rat liver microsomes; non-treated, phenobarbital(PB)-treated and 3-methylcholanthrene(3-MC)-treated, and non-treated mouse liver microsomes, and two kind of oxidants; cumene hydroperoxide(A) and iodosylxylene(B).

All products(Table 7) were confirmed by comparison with authentic samples obtained from the chemical model systems. The piperidine-β-oxo compound was generated as in the chemical model systems.

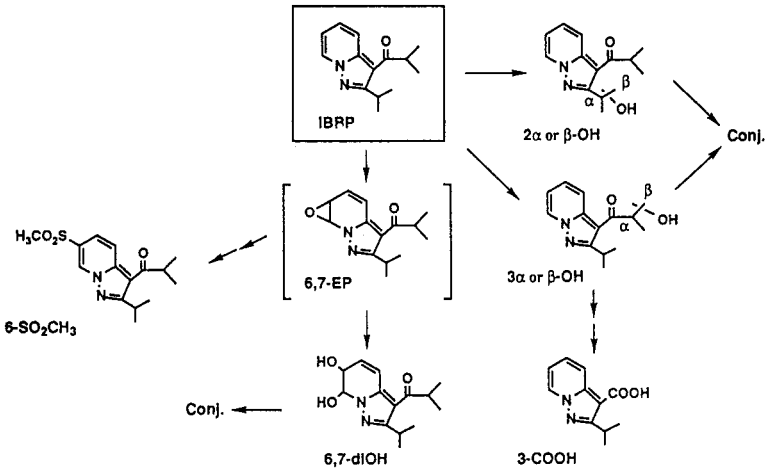
TABLE. 7 Oxidation of PCP by microsomes-oxidant system.

		formation of metabolite (μg / reaction mixture)			
microsomes	oxidant	m-OH	γ-OH	cyclo-OH	β-oxo
Rat non-treated	A	0.40	0.96	4.2	3.7
	B	0.38	3.5	1.7	1.5
Rat PB-treated	A	0.36	2.4	0.37	0.47
	B	0.34	6.0	1.2	2.6
Rat 3-MC-treated	A	0.42	0.89	0.28	0.44
	B	0.38	1.6	8.3	7.3
Mouse	A	0.87	1.3	0.24	0.36
	B	0.34	2.6	3.0	2.7

Oxidant: A) cumene hydroperoxide, B) iodosylxylene

As the next example, we examined the reaction profiles of the antiasthma drug, IBPP, in various P-450 chemical model systems and compared them with those of the rat or human liver microsomal system(ref. 11). The company involved in this

Chart 6 Metabolic Pathways of 3-Isobutyryl-2-isopropylpyrazolo[1,5-a]pyridine (IBPP) in Human and Animals

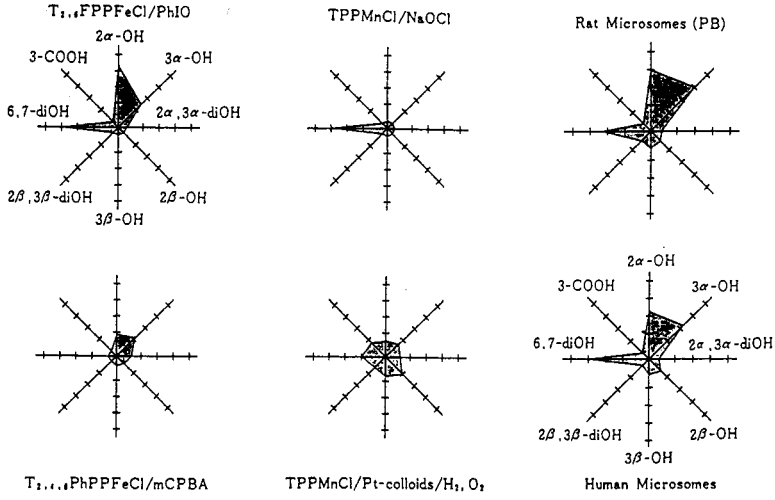


project had difficulty in developing this drug because of the diversity of its metabolic pathways including an unstable metabolite.

The metabolic pathways of IBPP in vivo are as shown in chart 6. The main metabolite is 6,7-diol, but the 6,7-epoxide presumed to be its precursor could not be isolated.

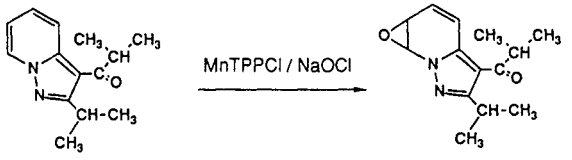
The pattern analysis of IBPP reaction products in some chemical model systems and microsomal systems is shown in Figure 3. The relative yield of the corresponding product is indicated along each axis. These diagrams, formed by linking each plot, show a characteristic pattern of each reaction system. This pattern analysis by a combination of two dimensional TLC is available and would make it possible to select or design a model system most suitable for the metabolism of various drugs(ref. 11). In this method, it is not necessary to confirm each metabolite in advance. After selection of the most suitable model system by comparison with the native P-450 system, the metabolites can be chemically determined by using the model system on a large scale.

Fig. 3 Metabolic patterns of IBPP by P-450 chemical model and liver microsomes



As previously mentioned, an unstable metabolic intermediate of IBPP, the 6,7-epoxide, could not be obtained in vitro and in vivo because of the presence of epoxyhydrase. However, this epoxide could be obtained in good yield by using the Mn-TPPCl/NaOCl system shown in chart 7. Usual epoxidation reagents such as

Chart 7 Synthesis of 6,7-epoxy-IBPP by Cyt.P-450 model system

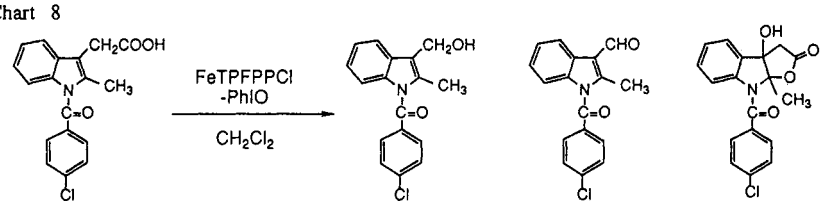


IBPP 6,7-Epoxide Yield 57 %

mCPBA could not give this epoxide. Thus, we could show that many P-450 model systems are useful for identification of minor, new, unstable metabolites.

As the last example, we have found new metabolic pathways of indomethacine, a well-known anti-inflammatory drug, by using the iron porphyrin/PhIO system(ref. 12). As shown in chart 8, indomethacine was converted to an alcohol, aldehyde and hydroxylactone in CH_2Cl_2 at room temperature under argon atmosphere. As far as we know, these types of reactions, decarboxylation and lactonization are novel in oxidation systems catalyzed by a metalloporphyrin. Next we attempted to detect these products as metabolites in rat liver microsomal system as metabolites using ^{14}C-labelled indomethacine. Every compound was confirmed to be formed in the metabolism of indomethacine and suppressed with SKF-525A(Table 8). These results indicate that such types of transformation are new P-450 dependent metabolic pathways of indomethacine.

Chart 8



Indomethacin Alcohol y. 8.5 % Aldehyde y. 7.6 % Lactone y. 17.3 %

TABLE. 8 Metabolism of indomethacine by rat liver microsomes system

System	Amount of Products (pmol / mg protein)		
	Alcohol	Aldehyde	Lactone
Complete	129 ± 5 (2.87 %)	9.3 ± 0.9 (0.29 %)	20.3 ± 3.4 (0.45 %)
+ SKF-525A	9.3 ± 0.9 (0.29 %)	n.d.	0.71 ± 0.13 (0.02 %)
- Microsome	n.d.	n.d.	n.d.

The data represent the mean ± S.E. of three experiments. n.d. : not detectable
The numbers in parenthesis represent % of the dose.

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