Pergamon

Bioorganic & Medicinal Chemistry, Vol. 3, No. 1, pp. 55–65, 1995

0968-0896/95 $9.50 + .00

0968-0896(94)00141-3

Application of Chemical Cytochrome P-450 Model Systems to Studies on Drug Metabolism—VIII. Novel Metabolism of Carboxylic Acids via Oxidative Decarboxylation

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Abstract—The oxidative decarboxylation of carboxylic acids by the chemical cytochrome P-450 model and rat liver microsomal systems was investigated. In the chemical system using *meso*-tetrakis(pentafluorophenyl)porphyrin iron chloride [Fe(TPFPP)Cl] with iodosylbenzene (PhIO), α-arylcarboxylic acids and α,α,α-trisubstituted acetic acids are converted to the corresponding one-carbon-reduced alcohol (**I**) and carbonyl derivatives (**II**) via oxidative decarboxylation. These products were then used as standards to identify the metabolites *in vivo* and *in vitro*. Biliary excretion of $\mathbf{I}_a$ and $\mathbf{II}_a$ in bile duct-cannulated rats after oral administration of ketoprofen amounted to 0.22 and 0.03% of the dose, respectively. In the case of indomethacin, $\mathbf{I}_b$ and $\mathbf{II}_b$ were detected as metabolites in the rat liver microsomal system, in yields of 2.8 and 0.29%, respectively. Further, the yields of $\mathbf{I}_b$ and $\mathbf{II}_b$ were decreased in the presence of SKF-525A. Thus, these metabolites were formed by cytochrome P-450-dependent reactions. Metabolites $\mathbf{I}_a$, $\mathbf{I}_b$, $\mathbf{II}_a$ and $\mathbf{II}_b$ had moderate to strong inhibitory activities on arachidonic acid-induced platelet aggregation and cyclooxygenase activity *in vitro*, comparable to those of the parent compounds.

Introduction

Cytochrome P-450 is an important component of the microsomal mixed function oxidase system.[1] This enzyme mediates the oxidative and reductive metabolism of a wide variety of endogenous as well as exogenous compounds.[2] In particular, the metabolism of most xenobiotics involves cytochrome P-450 *in vivo*. It is widely recognized that heme in the enzyme is mainly responsible for its chemistry. Therefore, extensive investigations have been conducted to develop chemical models of cytochrome P-450 using synthetic metalloporphyrins.[3] These studies have contributed not only to the elucidation of the mechanism of cytochrome P-450 reactions, but also to the progress of catalytic oxidation and coordination chemistry. Our interest has been focused on the application of chemical models to study drug metabolism,[4] because it is usually laborious and expensive to obtain sufficient amounts of drug metabolites by using animals, during the development of new medicines. Some oxidation products identical with metabolites are expected to be afforded by the catalytic oxidation of drugs with metalloporphyrin systems since their reactivities are thought to resemble, at least to some extent, that of cytochrome P-450. In our series of studies on the oxidation of multi-functional compounds such as drugs by various chemical models of cytochrome P-450, we have recently found that carboxylic acids with an aryl group or three substituents at the α carbon are readily decarboxylated oxidatively to afford the one-carbon-reduced alcohol and carbonyl products in the iron porphyrin–iodosylbenzene system (Fig. 1).[5] These results encouraged us to examine whether such oxidative decarboxylation of carboxylic acids would occur with cytochrome P-450. Though some enzymes, such as carboxylase, pyruvate decarboxylase (EC 4.1.1.1, PDC) and DOPA decarboxylase (EC 4.1.1.28, PDC) and so on, are known to catalyze decarboxylation of their substrates, the reactions are not oxidative. We report here a novel decarboxylative reaction of carboxylic acids mediated by cytochrome P-450.

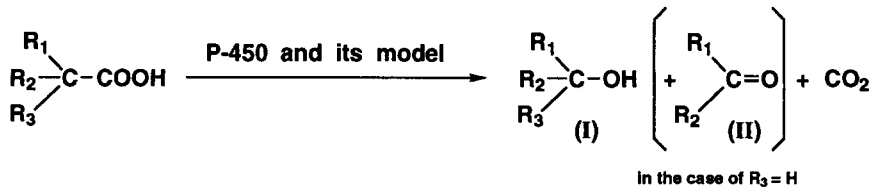


Figure 1.

Results

Reactions with the chemical cytochrome P-450 model system

The oxidative decarboxylation of carboxylic acids by a chemical cytochrome P-450 model was conducted as described in the Experimental section. Ketoprofen, indomethacin and 2,2-dimethyl-3-phenylpropionic acid were efficiently decarboxylated to give the corresponding one-carbon-reduced alcohol (**I**) and carbonyl (**II**) compounds by the PhIO–Fe(TPFPP)Cl system. As we reported in the previous communication, α-aryl or α,α,α-trisubstituted acetic acids generally show high reactivity in this system (Table 1). However, α-mono- or α,α-disubstituted acetic acids with no aryl group at the α position (e.g. lauric acid or 3-heptanoic acid) were inert under the reaction conditions. In the case of clofibric acid, *p*-chlorophenol was afforded, probably by rapid decomposition of an unstable hemiacetal directly formed by the oxidative decarboxylation. Products (**III**) containing a chlorine atom were also formed in the reactions. Compounds **III** have the structure in which the carboxyl group of the original carboxylic acids is substituted with chlorine derived from CH_2Cl_2.

Detection of carbon dioxide by FT-IR in the reaction of ketoprofen with the PhIO–Fe(TPFPP)Cl system

In these decarboxylative reactions, evolution of carbon dioxide was expected. A CO_2 solution in CH_2Cl_2 exhibited strong IR absorption at 2337 cm^{-1} due to CO_2. Therefore, measurement of IR spectra of the reaction mixture (4 h after the addition of ketoprofen) was carried out by using FT-IR instruments. The spectra obtained from the mixture had a clear absorption band at 2337 cm^{-1}, of which the position and shape were identical with those of CO_2 solution in CH_2Cl_2 (Fig. 2).

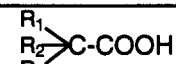
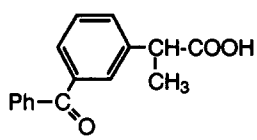
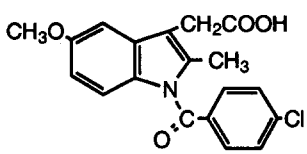
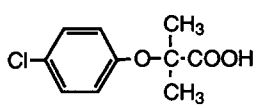
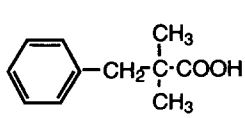
Determination of $^{14}CO_2$ in the reaction of [1-^{14}C]phenylacetic acid with the PhIO–Fe(TPFPP)Cl system

The amount of evolved CO_2 was determined by using [1-^{14}C]phenylacetic acid as a substrate. The formed [^{14}C]CO_2 was released from the liquid phase by argon bubbling, trapped in the usual way, and then the radioactivity was counted. The trapped amount of CO_2 at 3 h after the start of the reaction was 6.1%. The detected amount of CO_2 was rather small, nevertheless, CO_2 was apparently formed.

Detection of carbon radical in the reaction of indomethacin with the PhIO–Fe(TPFPP)Cl system by the spin-trapping ESR method

A spin adduct was obtained by treating the reaction mixture with *N*-phenyl-*N*-tert-butylnitrone (PBN) (Fig. 3). The control incubation mixture (without ketoprofen) exhibited a very weak EPR signal. In contrast, the complete incubation mixture showed a strong signal of a spin adduct. The a_β^H and a^N values of the triplet-doublet signal were 2.0 and 13.8 G, respectively.

Table 1. Yield of the products formed by the reaction of carboxylic acids with the iodosylbenzene–Fe(TPFPP)Cl system

		Products (%)			
		$R_1R_2R_3C$-OH	R_1R_2C=O	$R_1R_2R_3C$-Cl	others
	ketoprofen	**I**$_a$ (48.2)	**II**$_a$ (25.7)	**III**$_a$ (12.5)	
	indomethacin	**I**$_b$ (8.5)	**II**$_b$ (7.6)		**B** (14.2)
	clofibric acid				**C** (29.5)
		**I**$_c$ (29.5)		**III**$_c$ (14.2)	

Reaction conditions: see experimental section. Yields are based on substrate used. **B**: The ester formed from indomethacin with **I**$_b$. **C**: *p*-Chlorophenol.

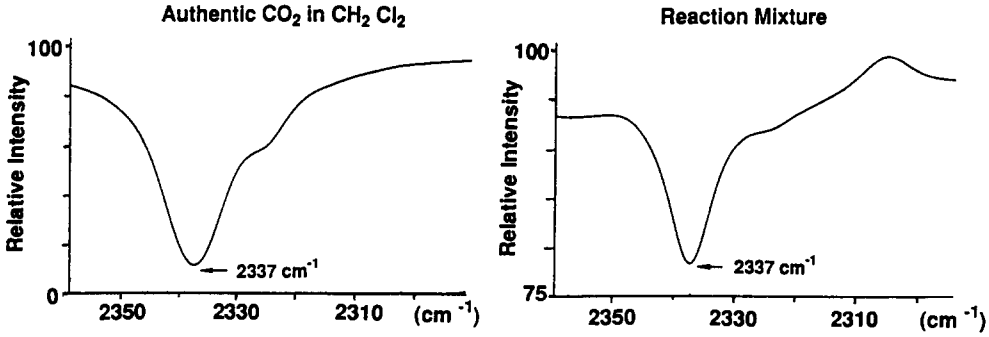


Figure 2. Detection of carbon dioxide in the Fe(III)TPFPPCl–PhIO system by using FT-IR spectroscopy.

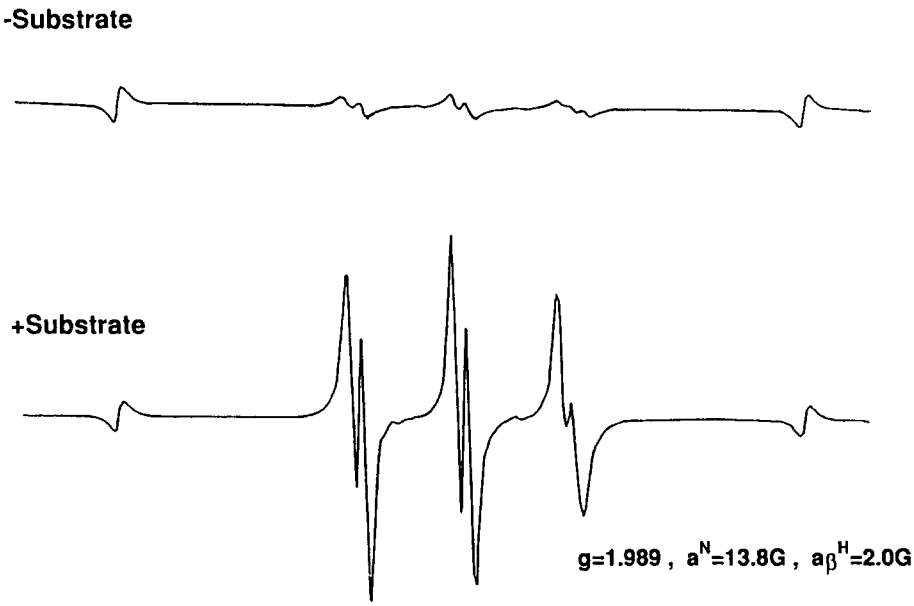


Figure 3. ESR spectra of phenyl-*N*-tert-butylnitrone–ketoprofen-derived radical adduct.

Metabolites of ketoprofen in rats

The metabolites in the bile duct-cannulated and/or intact rats after oral administration of ketoprofen were isolated and purified by TLC and HPLC from the extract. Their structures were identified by using GC–MS. Figure 4 shows the mass spectra and chromatogram of the trimethylsilylated derivatized metabolites in bile, together with authentic compounds $\mathbf{I}_a$ and $\mathbf{II}_a$. The retention times (R_t) in total ion chromatography (TIC) and the mass fragmentation patterns of the two isolated metabolites were similar to those of the authentic compounds, respectively. Figure 5 shows typical HPLC chromatograms exhibiting well-separated peaks of $\mathbf{I}_a$, $\mathbf{II}_a$ and an internal standard ($\mathbf{III}_a$). Those peaks were not interfered with by substances originating from biological specimens. The analytical procedure described in the experimental section gave linear and reproducible calibration curves over a wide range above the quantification limit, *i.e.* 50 ng mL^{-1}, for the two metabolites in bile. Extraction ratios of metabolites and internal standard from bile were greater than 85 %. Biliary recoveries of $\mathbf{I}_a$ and $\mathbf{II}_a$ after oral administration of ketoprofen in bile duct-cannulated rats are presented in Table 2. Biliary excretion during one day of $\mathbf{I}_a$ and $\mathbf{II}_a$ were 0.22 and 0.03% of the dose, respectively.

Table 2. Biliary excretion of metabolites in male rats during 24 h after oral administration of ketoprofen at the dose of 100 mg kg^{-1}

Rat No.	% of Dose	
	Alcohol formed (I_a)	Aldehyde formed (II_a)
1	0.27	0.03
2	0.07	0.03
3	0.32	0.04
Mean ± S.E.	0.22 ± 0.08	0.03 ± 0.00

Metabolites (I_a, II_a) were quantified by using HPLC analysis.

Metabolites in rat liver microsomal systems

The metabolites in rat liver microsomal systems were isolated and purified by TLC from the extract. Their structures were identified by using GC–MS. Figure 6 shows the spectra of the trimethylsilylated derivatized metabolites of indomethacin. The R_t of TIC and mass fragmentation patterns of the isolated metabolites were similar to those of the authentic compounds, respectively. The 2-D TLC of authentic compounds and

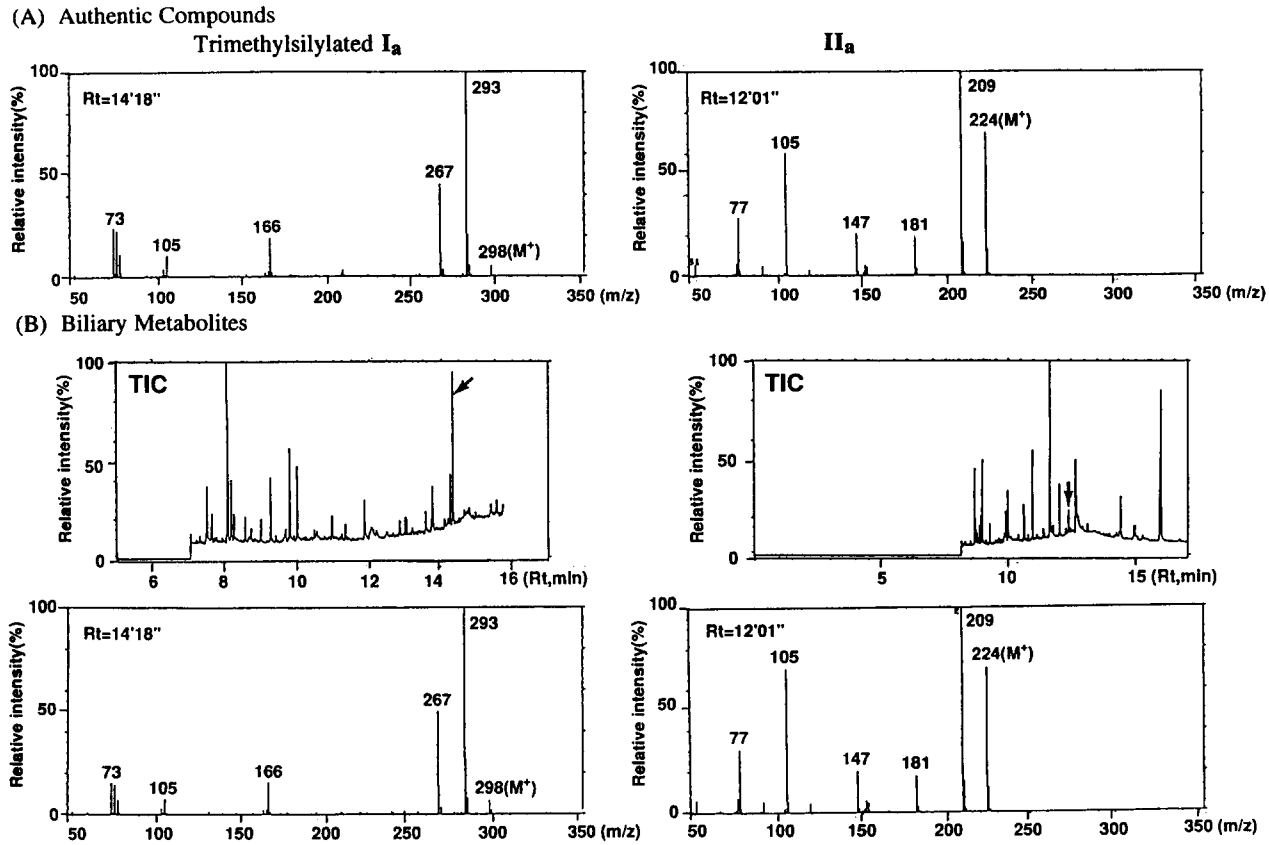


Figure 4. Mass spectra of biliary metabolites of ketoprofen and their authentic compounds.

autoradiograms of the indomethacin metabolites produced by various microsomal systems are shown in Figure 7. The spots of I_b and II_b were identified by comparison with authentic compounds from the position on the TLC plate. The amounts of products are shown in Table 3. When indomethacin metabolism was carried out in the complete microsomal system, the amounts of I_b and II_b formed were 129 ± 5 and 9.3 ± 0.9 nmol mg^{-1} microsomal protein, respectively. In the absence of microsomes or NADPH, I_b and II_b were not detected in both cases. Incubation using atmosphere of an 80:20 ratio of carbon monoxide to oxygen resulted in the 93% inhibition of I_b formation. Furthermore, the formation of I_b and II_b was markedly inhibited by 2 mM SKF-525A, a specific inhibitor of cytochrome P-450. Figure 8 shows the spectra of the metabolites of clofibric acid and 2,2-dimethyl-3-phenylpropionic acid separated from the liver microsomal system. The mass spectral patterns of the trimethylsilylated derivatives were identical with those of the authentic compounds, respectively.

Biological activity of the novel metabolites of indomethacin and ketoprofen

Formation of novel metabolites via oxidative decarboxylation would influence the pharmacological activities and the toxicities of drugs bearing a carboxylic group, especially if the metabolites themselves are biologically active. Therefore, it is necessary to assay the activities of the metabolites, for clarifying their role in in the pharmacological effect of

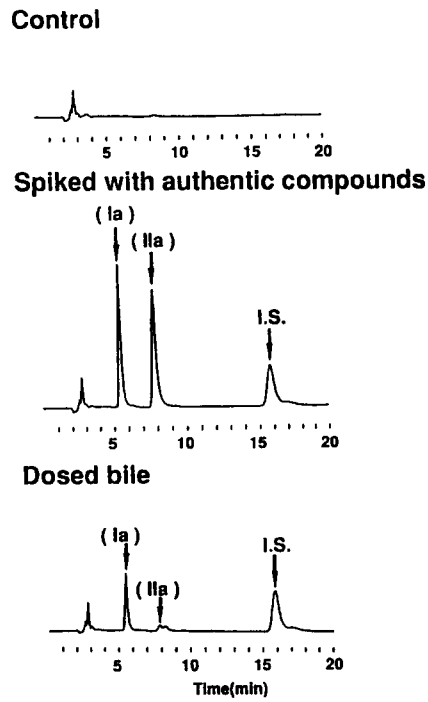


Figure 5. Typical chromatograms of biliary extract.

the parent drugs. Inhibitory activities of decarboxylation products of indomethacin and ketoprofen on arachidonic acid-induced platelet aggregation in rabbits are shown in Table 4. Indomethacin and ketoprofen strongly inhibited the platelet aggregation, with IC_{50} values of

10^{-8} ~ 10^{-7} M. The IC_{50}'s of compounds $\mathbf{I_a}$ and $\mathbf{I_b}$ were 1.6×10^{-7} and 1.0×10^{-6} M, respectively. The activities of compounds $\mathbf{I_a}$ and $\mathbf{I_b}$, though less than those of mother compounds, were still significant. In particular, the IC_{50} value of $\mathbf{I_a}$ was almost equal to that of indomethacin. The aldehyde $\mathbf{II_b}$ had only a slight activity. The chlorinated product $\mathbf{III_a}$, which is not a metabolite, unexpectedly exhibited potent inhibitory activity. Furthermore, these compounds also inhibited cyclooxygenase activity (Table 5).

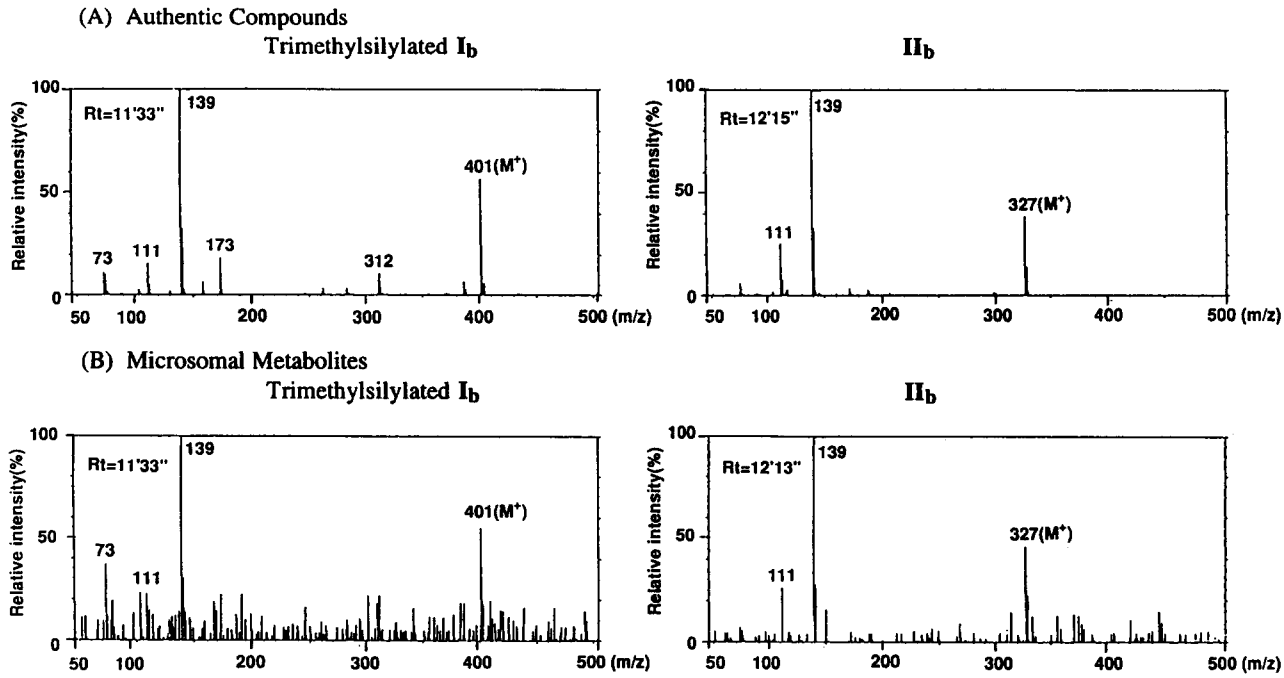


Figure 6. Mass spectra of microsomal metabolites of indomethacin and their authentic compounds.

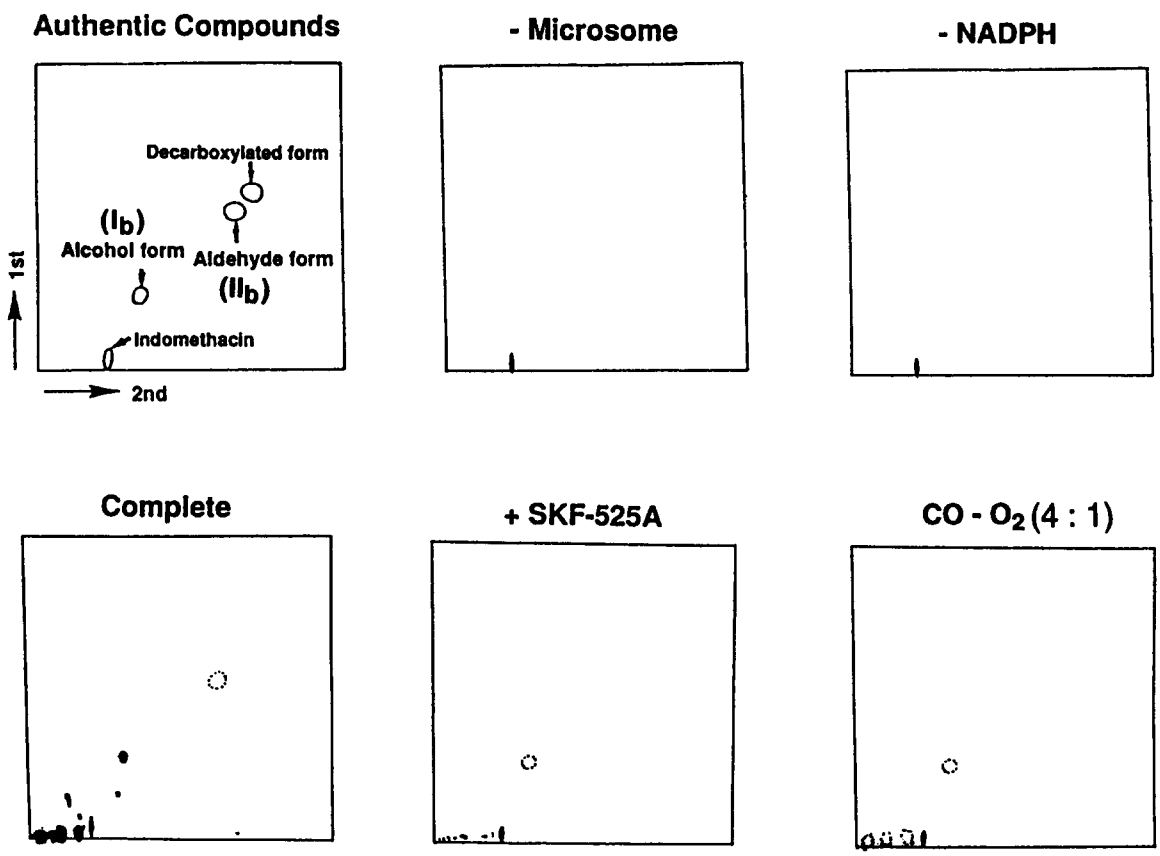


Figure 7. Detection of the metabolites formed by oxidative decarboxylation of indomethacin *in vitro*.

Table 3. Amount of products obtained by rat liver microsome system with indomethacin as a substrate

System	Amount of Products pmol mg^{-1} protein 60 min^{-1})	
	Alcohol formed (I_b)	Aldehyde formed (II_b)
Complete	129 ± 5 (2.87%)	13.1 ± 0.9 (0.29%)
-NADPH	n.d.	n.d.
under $CO:O_2$ (4:1)	8.8 ± 2.1 (0.20%) [1)]	n.d.
+ SKF-525A	9.3 ± 0.9 (0.29%) [1)]	n.d.
No microsomes	n.d.	n.d.

The data represent the mean ± S.E. of three experiments. The data in parentheses are % yields based on dosed indomethacin.
n.d.: Not detected. [1)]This value was significantly different from the value in the complete system ($P < 0.01$).

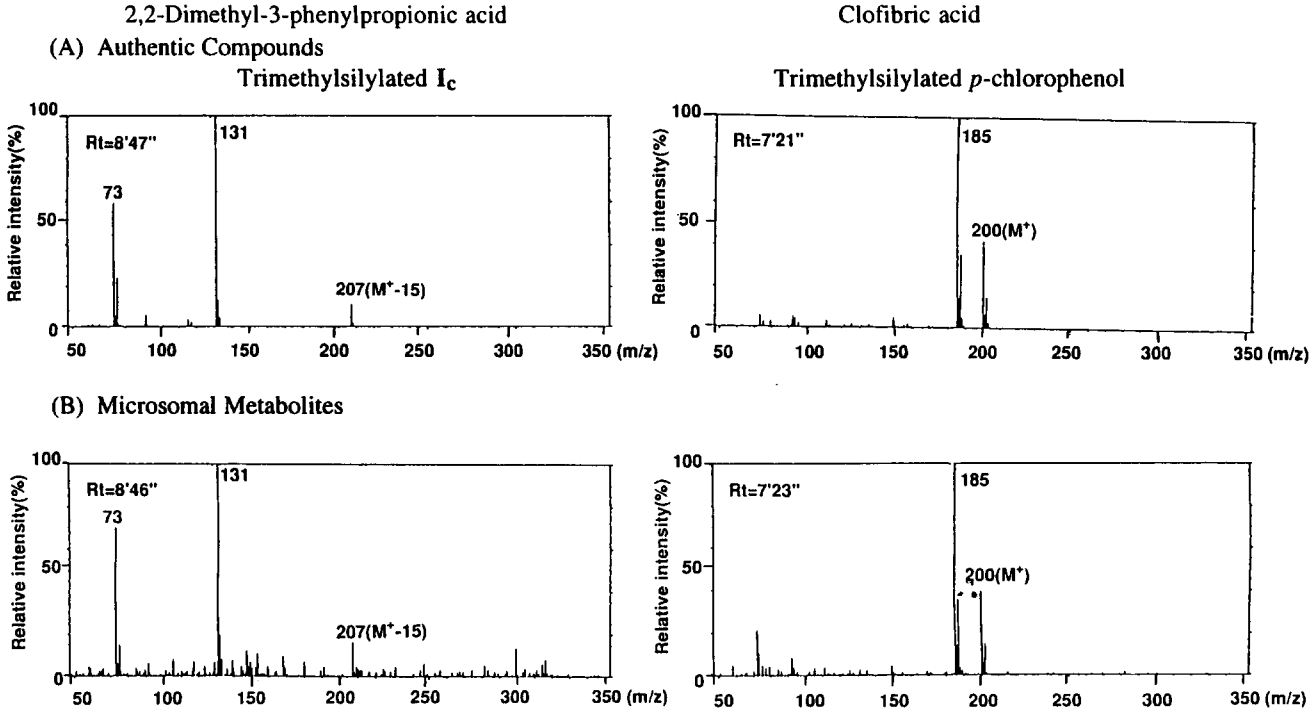


Figure 8. Mass spectra of microsomal metabolites of 2,2-dimethyl-3-phenylpropionic acid and clofibric acid and their authentic compounds.

Table 4. Fifty per cent inhibitory concentration of ketoprofen, indomethacin and their oxidative products effective on platelet aggregation in rabbits

Compound	IC_{50} (M)
Ketoprofen	1.0×10^{-8}
I_a	1.6×10^{-7}
II_a	5.2×10^{-6}
III_a	7.7×10^{-8}
Indomethacin	2.5×10^{-7}
I_b	1.0×10^{-6}
II_b	5.0×10^{-5}

Conditions: see Experimental section.

Table 5. Cyclooxygenase inhibitions of indomethacin and its oxidative products

Compound	IC_{50}(M)
Indomethacin	3.5×10^{-7}
I_b	2.5×10^{-6}
II_b	7.5×10^{-5}

Conditions: see experimental section

Discussion

We have found that several types of compounds having carboxyl groups are efficiently decarboxylated to give the corresponding one-carbon-reduced alcohol (**I**) and carbonyl (**II**) compounds in an artificial cytochrome P-450 model system [Fe(TPFPP)Cl–PhIO]. Carboxylic acids which possess an aromatic substituent(s) or three substituents at the α position undergo facile decarboxylation in this oxidation system. FT-IR spectra of the reaction mixture in the case of I_a exhibited a strong absorption due to carbon dioxide. ESR spectra of the same reaction mixture in the presence of PBN showed a clear doublet–triplet signal at room temperature (Fig. 3). The a_β^H and a^N values of the signal were close to those of the known PBN–cumyl radical adducts in benzene (a_β^H = 2.19, a^N = 14.25).[6] The values of adduct parameters obtained are, however, also comparable to those of alkoxyl radical–PBN adducts.[6] This EPR study provides strong evidence for the formation of a radical (probably a carbon-centered

radical) in the decarboxylation. Under a dioxygen atmosphere, no chlorinated products (**III**) were obtained while considerable amounts of **III** were afforded under argon. This result also supports carbon-centered radical formation, because the radical abstracts a chlorine atom from CH_2Cl_2 under argon, while it is likely to react preferentially with dioxygen, if present, to form an alkylperoxyl radical. Thus, we propose that the decarboxylation reaction proceeds as shown in Figure 9 (path a). The two-electron-oxidized active species ($O=Fe^{IV}(Por)^{+\bullet}$) formed by the reaction of $Fe^{III}(Por)$ with Ph-IO[7] reacts with a carboxylic acid to cause one-electron oxidation. The resulting carboxyl radical readily decomposes to afford carbon dioxide and a carbon radical. Rebound of ·OH equivalent [$HO-Fe^{IV}(Por)$] or dioxygen then gives the alcohol (**I**) and/or carbonyl compound (**II**) (path b). An alternative pathway is possible, as follows. Firstly, carboxylic acid is coupled with Ph-IO to afford a complex **1**. Complex **1** decarboxylates by coordination to the iron atom of $Fe^{III}(Por)$ and subsequent reactions are the same as in path a. Path b seems preferable since mCPBA, which efficiently converts $Fe^{III}(Por)$ into $O=Fe^{IV}(Por)^{+\bullet}$ in CH_2Cl_2, was a less effective oxidant (yield of $\mathbf{I}_b$ = 1.5% in the case of mCPBA) for the decarboxylation than Ph-IO. However, we have no clear evidence to indicate which of the two postulated pathways, actually operates.

Our group's studies on the reactivity of chemical P-450 model systems have revealed good similarity of the reactivity between the model system and microsomes in several cases. Most non-steroidal anti-inflammatory drugs have a carboxyl moiety. Therefore, the findings in the model system led us to investigate the existence of this new metabolic pathway of carboxylic acids via oxidative decarboxylation *in vivo,* since many drugs could be affected. The products derived from drugs with a carboxyl moiety could be used as authentic samples, so-called 'candidate metabolites', to identify novel metabolites in urine and bile of dosed rats or in microsomal reaction mixtures. The two metabolites of ketoprofen isolated from rat bile and urine were consistent with the authentic $\mathbf{I}_a$ and $\mathbf{II}_a$, in terms of the retention times and mass fragmentation in GC–MS experiments. Kitagawa and co-workers have studied in detail the metabolism of [^{14}C]ketoprofen *in vivo* by the 2-D TLC method.[9] Two metabolites which remain unidentified in their report are in fair agreement with authentic $\mathbf{I}_a$ and $\mathbf{II}_a$ in terms of the location of the spots on TLC in our study. Experiments *in vitro* using rat microsomes have revealed that metabolites are formed via oxidative decarboxylation in the incubation mixtures of microsomes plus indomethacin or clofibric acid or 2,2-dimethyl-3-phenylpropionic acid. Therefore, it is highly probable that oxidative decarboxylation of carboxylic acids *generally occurs* as a pathway of hepatic metabolism *in vivo*. In the absence of NADPH, $\mathbf{I}_b$ and $\mathbf{II}_b$ were not formed in microsomal oxidation of indomethacin (Table 3). This result indicates that the decarboxylation is NADPH-dependent. Carbon monoxide strongly inhibited $\mathbf{I}_b$ and $\mathbf{II}_b$ formation (93% inhibition). Flavin-containing monooxygenase is *not* responsible for this decarboxylation, since flavin-catalyzed reactions are unaffected by CO. Formation of $\mathbf{I}_b$ and $\mathbf{II}_b$ by rat microsomes was strongly inhibited by SKF-525A, a selective inhibitor of cytochrome P-450 (*ca* 90%). Each of these results provides evidence for the involvement of cytochrome P-450 in the new metabolism. Cederbaum and Winston reported that benzoate is decarboxylated oxidatively to afford carbon dioxide with rat liver microsomes.[10] However, SKF-525A did not significantly affect this decarboxylation of benzoate, and organic hydroperoxides did not act as oxidants for benzoate decarboxylation. They assumed from these results that the decarboxylation by rat liver microsomes is a result of the interaction of benzoate with •OH or a species with the oxidizing power of •OH that is generated during microsomal electron transport. Thus, the decarboxylative metabolic pathway of the α-substituted acetic acid or propionic acid derivatives should be regarded as different from that of benzoate, of which the carboxyl group is directly attached to an aromatic carbon. The rate of decarboxylation of R-COO$^{\bullet}$ (k_{dec}) is known to change greatly according to the structure of the R- moiety. The k_{dec} of $PhCH_2COO^{\bullet}$ is about 50 times larger than k_{dec} of $PhCOO^{\bullet}$[11]. The Fe(TPFPP)Cl–Ph-IO system did not cause decarboxylation of benzoate. It is highly probable that P-450 decarboxylates carboxylic acid in the same

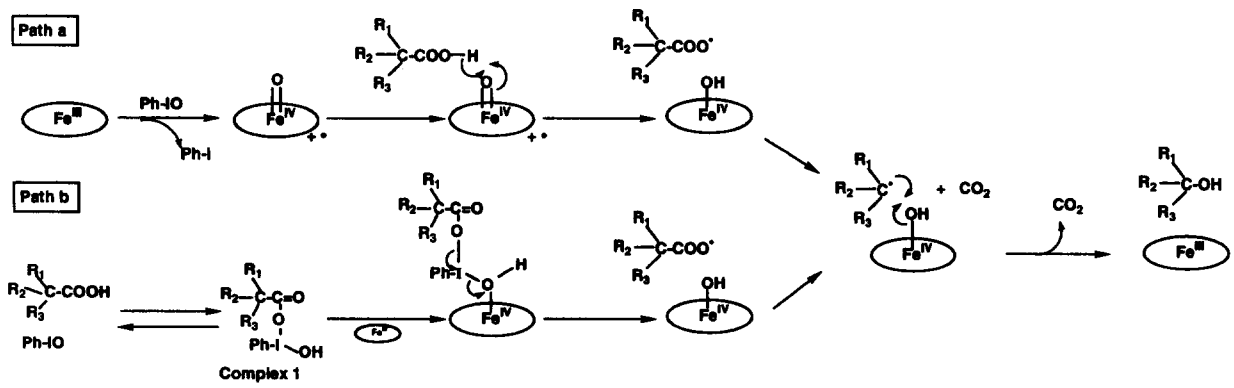


Figure 9. Proposed mechanisms of oxidative decarboxylation in the iron porphyrin–iodosylbenzene system.

manner as the chemical model system, if the mechanism of this model reaction is consistent with path a in Figure 9. Therefore, it would appear that cytochrome P-450 preferentially decarboxylates carboxylic acids of which carboxyl radicals have large k_{dec}. After our findings described above, we found a report claiming that the same mode of metabolism of indole-3-acetic acid occurs in plants[12] and is catalyzed by horseradish peroxidase,[13] though the substrate is restricted to indole-3-acetic acid. Therefore, oxidative decarboxylation by the Fe(TPFPP)Cl–PhIO system can also be regarded as a model reaction of the metabolism of indole-3-acetic acid in plants.

Furthermore, the biological activities of the novel metabolites were assayed (Tables 4 and 5). Compounds $\mathbf{I_a}$ and $\mathbf{I_b}$ exhibited significant inhibitory activities on arachidonic acid-induced platelet aggregation. It is noteworthy that loss of the carboxyl group does not always cause a large decrease in activity of non-steroidal anti-inflammatory drugs. The results suggest that these metabolites do contribute to the biological activity of these drugs, though the decarboxylative pathway is not a metabolic activation in these cases.

The existence of new metabolism via oxidative decarboxylation would imply that carbon radicals are formed *in vivo* following administration of drugs with carboxyl groups. The radical itself or its dioxygen adduct, i.e. alkylperoxyl radical, is likely to damage biopolymers such as proteins and DNA, since these radicals have high reactivity to various functional groups. Such radicals may result in covalent binding of drug metabolites to protein. Furthermore, the formed alcohols (**I**) and ketones (**II**) may have other bioactivities than those of the original compounds. The physiological significance of this metabolism, i.e. in metabolic activation, could be enormous, and we are planning further studies along this line.

Experimental

Materials and methods

[^{14}C]Indomethacin was obtained from NEN Research Products. The specific radioactivity was 3.91 MBq mg^{-1} and radiochemical purity was greater than 98.2% by TLC. This compound was purified prior to use by HPLC. The unlabelled indomethacin and ketoprofen were purchased from Sigma Company. SKF-525A was a generous gift from Smith Kline and Fujisawa Co., Ltd. *p*-Chlorophenol, 2,2-dimethyl-3-phenyl-1-propanol and 1,1-dimethyl-2-phenylethanol were purchased from Wako Pure Chemical Industries Ltd. Clofibric acid was a gift from Kyorin Pharmaceutical Co., Ltd. *meso*-Tetrakis(pentafluorophenyl)porphyrin iron chloride [Fe(TPFPP)Cl] was prepared according to the literature,[14] and the UV-vis spectrum was consistent with the literature values. Iodosylbenzene (PhIO) was purchased from Tokyo Kasei Chemicals. [1-^{14}C]Phenylacetic acid was purchased from American Radiolabeled Chemicals Inc. All other chemicals were of reagent grade. For TLC separation, Kieselgel 60F_{254} (E. Merck Darmstadt, F.R.G.), 0.25 mm thickness was used, and the spots were visualized under UV light (254 nm). The plate was developed with the following solvent systems: ketoprofen metabolites, benzene-methanol (9:1,v/v); indomethacin metabolite, EtOAc:*n*-hexane (7:3,v/v); 2,2-dimethyl-3-phenylpropionic acid and clofibric acid metabolites, chloroform (100%). In the case of [^{14}C]indomethacin metabolism, EtOAc:*n*-hexane (1:1, v/v) was used first, followed by $CHCl_3$:Me_2CO:HOAc (10:2:0.1, v/v). A Jeol DX-303 mass spectrometer equipped with a Hewlett-Packard 5890A gas chromatograph and a JMA-DA5000 data system was used for the measurement of mass spectra. The metabolites were analyzed on fused silica capillaries, 30 m × 0.25 mm DB-5 (J&W Scientific, Rancho Cordova, CA), operated at 60–280 °C or 200–280 °C. The injection and source temperatures were 250 and 260 °C, respectively. Helium was used as the carrier gas, with the column head pressure set at 98 psi. The ionization potential and ionization current were set at 70 eV and 300 μA, respectively. Before the measurement, the metabolites were trimethylsilylated with *N,O*-bis-(trimethylsilyl)acetamide:pyridine:chlorotrimethylsilane (5:4:1, v/v). A Hitachi 655A-11 liquid chromatograph equipped with a Hitachi L-5000 LC controller and a Hitachi 655A UV monitor was used for analysis of urine and bile metabolites of dosed ketoprofen. The column was an Asahi Chemical Industry Asahipak ODP-50 (6 mm diameter × 150 mm). Mobile phase, MeCN:0.1% phosphate (6:4), was passed at a rate of 1.0 mL min^{-1}. The eluate was monitored at 280 nm. In the case of isolation of ketoprofen metabolites, a Jasco Twincle liquid chromatograph equipped with a Jasco UVIDEC-100-V UV monitor was used. The column was Tosoh TSK gel ODS-120T (4.6 mm diameter × 250 mm). Mobile phase, methanol:H_2O (8:2, v/v), was passed at a rate of 1.0 mL min^{-1}. Detection wavelength was 260 nm. For the measurement of radioactivity, a Beckman liquid scintillation counter (model 3801) was employed. Counting efficiency was determined by automatic external standardization. Jeol JNM-EX400 and GSX-400 (400 MHz) FT nuclear magnetic resonance spectrometers were used to measure ^{1}H NMR spectra. The sample was dissolved in $CDCl_3$, and analyzed with TMS as an internal standard. A Perkin-Elmer 204 elemental analyzer was used for elemental analysis. A Perkin-Elmer 1600 series Fourier-Transform infrared spectrometer was used for IR spectroscopy. Melting points were determined on a Yanagimoto micro melting point apparatus, without correction.

Synthesis of 2,2-dimethyl-3-phenylpropionic acid

2,2-Dimethyl-3-phenylpropionic acid was synthesized by oxidation of 2,2-dimethyl-3-phenyl-1-propanol according to Omura and Swern.[15] Analytical data of 2,2-dimethyl-3-phenylpropionic acid: mp 55–57 °C (recryst. from *n*-hexane); ^{1}H NMR (400 MHz), ($CDCl_3$/TMS) δ 1.21 (6H, *s*, CH_3), 2.90 (2H, *s*, CH_2), 7.16–7.28 (5H, *m*,

H_{arom}); IR (KBr, cm^{-1}) 3400 (COOH), 1690 (C=O); MS (*m/z*); 178 $[M]^+$. Anal. calcd for $C_{11}H_{14}O_2$: C, 74.13; H, 7.92. Found: C, 73.85; H, 8.07.

Reactions with the chemical model system

(A) Ketoprofen–PhIO–Fe(TPFPP)Cl. A mixture of Fe(TPFPP)Cl (5.3 mg, 5 μmol), ketoprofen (254 mg, 1 mmol), iodosylbenzene (440 mg, 2 mmol), and 10 mL of CH_2Cl_2 was stirred for 20 h at room temperature under argon atmosphere. Excess PhIO was filtered off and the resulting mixture was isolated by silica gel column chromatography and preparative TLC to give 3-benzoyl-α-phenethyl alcohol ($\mathbf{I_a}$) (oil, yield 48.2%), 1-acetyl-3-benzoylbenzene ($\mathbf{II_a}$) (oil, yield 25.7%) and 3-benzoyl-1-(1-chloroethyl)-benzene ($\mathbf{III_a}$) (oil, yield 12.5%).

Analytical data of $\mathbf{I_a}$: ^1H NMR (400 MHz, $CDCl_3$/TMS) δ 1.53 (3H, *d*, *J* = 6.3 Hz), 1.97 (1H, *br s*), 4.99 (1H, *q*, *J* = 6.3 Hz), 7.47 (1H, *dd*, *J* = 7.6, 7.6 Hz), 7.49 (2H, *dd*, *J* = 7.6, 7.6 Hz), 7.58 (1H, *d*, *J* = 7.6 Hz), 7.63 (1H, *dd*, *J* = 7.6, 7.6 Hz), 7.68 (1H, *d*, *J* = 7.6 Hz), 7.80 (2H, *d*, *J* = 7.6 Hz) 7.82 (1H, *s*); IR (neat, cm^{-1}) 3420 (OH), 1655 (C=O); high-resolution mass spectrum (HRMS); calcd for $C_{15}H_{14}O_2$ 226.0994, found 226.0987.

Analytical data of $\mathbf{II_a}$: ^1H NMR (400 MHz, $CDCl_3$/TMS) δ 2.66 (3H, *s*), 7.51 (2H, *dd*, *J* = 7.6, 7.6 Hz), 7.61 (1H, *dd*, *J* = 7.8, 7.8 Hz), 7.63 (1H, *dd*, *J* = 7.6, 7.6 Hz), 7.81 (2H, *dd*, *J* = 7.6, 1.4 Hz), 8.00 (1H, *ddd*, *J* = 7.8, 1.8, 1.8 Hz), 8.19 (1H, *ddd*, *J* = 7.8, 1.8, 1.8 Hz), 8.37 (1H, *dd*, 1.8, 1.8 Hz); IR (neat, cm^{-1}) 1690 (C=O), 1660 (C=O); HRMS; calcd for $C_{15}H_{12}O_2$ 224.0838, found 224.0861.

Analytical data of $\mathbf{III_a}$: ^1H NMR (400 MHz, $CDCl_3$/TMS) δ 1.88 (3H, *d*, *J* = 6.6 Hz), 5.15 (*q*, *J* = 6.6 Hz), 7.48 (1H, *dd*, *J* = 7.7, 7.7 Hz), 7.50 (2H, *dd*, *J* = 7.7, 7.7 Hz), 7.67 (1H, *d*, *J* = 7.7 Hz), 7.61 (1H, *d*, *J* = 7.7 Hz), 7.72 (1H, *dd*, *J* = 7.7, 7.7 Hz), 7.81 (2H, *dd*, *J* = 1.8, 7.7 Hz), 7.86 (1H, *d*, *J* = 1.8 Hz); IR (neat, cm^{-1}) 1600 (C=O); HRMS; calcd for $C_{15}H_{13}ClO$ 244.0626, found; 244.0635.

(B) Indomethacin–PhIO–Fe(TPFPP)Cl. The reaction mixture with indomethacin was worked-up according to the previously outlined procedure, giving *N*-(4-chlorobenzoyl)-3-hydroxymethyl-5-methoxyindole ($\mathbf{I_b}$) (yield 8.5%), *N*-(4-chlorobenzoyl)-3-formyl-5-methoxy-indole ($\mathbf{II_b}$) (yield 7.6%) and the ester form (**B**) (yield 14.2%).

Analytical data of $\mathbf{I_b}$: mp 140–142 °C (recryst. from CH_2Cl_2:*n*-hexane); ^1H NMR (400 MHz, $CDCl_3$/TMS) δ 1.46 (1H, *br s*), 2.44 (3H, *s*), 3.85 (3H, *s*), 4.83 (2H, *s*), 6.68 (1H, *dd*, *J* = 2.4, 9.2 Hz), 6.83 (1H, *d*, *J* = 9.2 Hz), 7.11 (1H, *d*, *J* = 2.4 Hz), 7.48 (2H, *d*, *J* = 8.4 Hz), 7.66 (2H, *d*, *J* = 8.4 Hz); IR (KBr, cm^{-1}) 3500 (OH), 1665 (C=O); MS (*m/z*); 329 $[M]^+$; Anal.: Calcd for $C_{18}H_{16}ClNO_3$: C, 65.56; H, 4.89; N, 4.25. Found: C, 65.21; H, 4.83; N, 4.29.

Analytical data of $\mathbf{II_b}$: mp 154–155 °C (recryst. from CH_2Cl_2:n-hexane); ^1H NMR (400 MHz, $CDCl_3$/TMS) δ 2.77 (3H, *s*), 3.88 (3H, *s*), 6.74 (2H, *d*, *J* = 1.5 Hz), 7.51 (2H, *d*, *J* = 8.8 Hz), 7.71 (2H, *d*, *J* = 8.8 Hz), 7.82 (1H, *dd*, *J* = 1.5, 1.5 Hz), 10.33 (1H, *s*); IR (KBr, cm^{-1}) 1697 (C=O), 1655 (C=O); MS (*m/z*); 327 $[M]^+$. Anal.: Calcd for $C_{18}H_{14}ClNO_3$: C, 65.96; H, 4.31; N,4.27. Found: C, 65.83; H, 4.26; N, 4.34.

Analytical data of **B**: mp 164–165 °C (recryst. from CH_2Cl_2:*n*-hexane); ^1H NMR (400 MHz, $CDCl_3$) δ 2.34 (3H, *s*), 2.40 (3H, *s*), 3.67 (3H, *s*), 3.68 (2H, *s*), 3.70 (3H, *s*), 5.29 (2H, *s*), 6.63 (1H, *dd*, *J* = 2.6, 8.8 Hz), 6.67 (1H, *dd*, *J* = 2.6, 8.8 Hz), 6.85 (1H, *d*, *J* = 8.8 Hz), 6.87 (1H, *d*, *J* = 2.6 Hz), 6.88 (1H, *d*, *J* = 2.6 Hz), 7.45 (2H, *d*, *J* = 8.4 Hz), 7.47 (2H, *d*, *J* = 8.4 Hz), 7.61 (2H, *d*, *J* = 8.4 Hz), 7.61 (2H, *d*, *J* = 8.4 Hz) 7.64 (2H, *d*, *J* = 8.4 Hz); IR (KBr, cm^{-1}) 1730 (C=O), 1670 (C=O); FAB-MS (*m/z*); 669 $[M + 1]^+$. Anal.: calcd for $C_{37}H_{30}Cl_2N_2O_6$: C, 66.37; H, 4.52; N, 4.18. Found: C, 66.53; H, 4.57; N, 4.11.

(C) Clofibric acid–PhIO–Fe(TPFPP)Cl. The reaction mixture with clofibric acid was worked-up according to the previously outlined procedure, giving *p*-chlorophenol (**C**) (yield 29.5%).

(D) 2,2-Dimethyl-3-phenylpropionic acid–PhIO–Fe(TPFPP)Cl. The reaction of 2,2-dimethyl-3-phenylpropionic acid was worked-up according to the previously outlined procedure, giving a 1,1-dimethyl-3-phenylethanol ($\mathbf{I_d}$) (oil, yield 29.5%) and 2-chloro-2-methyl-1-phenylpropane ($\mathbf{III_d}$) (oil, yield 18.1%).

Analytical data of $\mathbf{III_d}$: ^1H NMR(400 MHz, $CDCl_3$/TMS) δ 1.58 (6H, *s*), 3.08 (2H, *s*), 7.18–7.31 (5H, *m*); IR (neat, cm^{-1}) 3080, 3060, 3030, 2970, 2930 (CH); HRMS; calcd for $C_{10}H_{13}Cl$ 168.0706, found 168.0723.

FT-IR studies

Incubation mixtures for FT-IR studies contained, in a total of 10 mL, Fe(TPFPP)Cl (5 μmol), ketoprofen (1 mmol), and iodosylbenzene (2 mmol) in CH_2Cl_2 under argon. Reaction mixtures were stirred at room temperature for 4 h. Aliquots of the reaction mixture were loaded into a KBr cell for liquid samples and subjected to FT-IR analysis.

Determination of CO_2 by using ^{14}C-labeled substrate

[1-^{14}C]Phenylacetic acid (0.76 μg, 15.0 MBq mg^{-1}) and cold phenylacetic acid (68 mg, 0.50 mmol) were mixed and added to the mixture of iodosylbenzene (220 mg, 1.0 mmol) and Fe(TPFPP)Cl (2.7 mg, 2.5 μmol) in CH_2Cl_2 (5 mL). After 3 h, 1 mL of the reaction mixture was taken from the reaction vessel and placed in a two-necked flask equipped with an argon gas inlet and outlet. Argon was flowed through the flask. Then, the gas from the outlet was once passed through a cold trap

(dry ice:Me_2CO), and finally introduced to a solution for CO_2 trap (ethanolamine:MeOH, 1:1; 100 mL). Radioactivity of the amine solution was determined with a liquid scintillation counter.

EPR studies on the oxidative decarboxylation by the model system

Incubation mixtures for EPR studies contained, in a total of 5 mL, Fe(TPFPP)Cl (1 μmol), ketoprofen (0.2 mmol), and iodosylbenzene (0.4 mmol) in benzene under argon. Reaction mixtures were stirred at room temperature for 10 min. Control incubation was carried out in the absence of ketoprofen. Aliquots (500 μL) of the resulting mixture were mixed with PBN (2 mmol) for radical-trapping and subjected to EPR analysis. EPR parameters are given in the text (Fig. 3).

Bile duct cannulation and drug administration

Male Wistar rats, weighing 200–450 g were purchased from the Nippon Biosupply Center. For bile duct cannulation, rats were anesthetized with ether prior to surgery. After surgery, rats were housed in restraining cages with free access to water and food and placed under a lamp to maintain the body temperature. The animals were orally given 100 mg kg^{-1} of ketoprofen dissolved in 80% propylene glycol aqueous solution after recovery from anesthesia, and bile was collected at intervals for 24 h.

Isolation of metabolites from urine and bile after ketoprofen administration

Aliquots of urine and bile samples of rats were treated with the same volume of β-glucuronidase/arylsulfatase solution at 37 °C for 16 h. The enzyme solution was prepared by diluting enzyme juice (from *Helix pomatia*, β-glucuronidase 5.2 IU mL^{-1}, arylsulfatase 2.6 IU mL^{-1}, Boehringer Mannheim GmbH) to 1/5 with 1 M acetate buffer (pH 5.2). After the hydrolysis, the urine and bile samples were extracted with Et_2O under alkaline conditions. The Et_2O solution was evaporated under reduced pressure and the resulting residue was dissolved in MeOH. The MeOH solution was spotted on a TLC plate and then developed with the appropriate solvent system. The regions with UV absorption were scraped off and extracted with MeOH:$CHCl_3$ (1:9). The products were further purified by HPLC.

Analysis of metabolites in bile after ketoprofen administration

The alcohol and keto form of ketoprofen in bile were simultaneously determined by HPLC. Aliquots of hydrolyzed test sample and 6 N NaOH were added to test tubes containing an internal standard compound (**C**). Bile samples were extracted with 6 mL of *n*-hexane. Five milliliters of the organic layer was evaporated under reduced pressure and then dissolved in 0.1 mL of MeOH. Ten microliters of the methanol solution was injected into the HPLC column.

Preparation of microsomes

Rats (5 weeks, *ca* 160 g) received intraperitoneal injection of 60 mg kg^{-1} sodium phenobarbital (Wako Pure Chemical Industries Co., Ltd) once daily for 3 days, then were fasted overnight and killed by exsanguination from the abdominal aorta. The livers were removed immediately, and perfused with ice-cold phosphate buffer (10 mM phosphate buffer containing 0.15 M KCl, pH 7.4) and homogenized in 3 volumes of the 100 mM sodium phosphate buffer (pH 7.4). Microsomes were prepared according to the method of Sato and Omura.[16] The protein concentration of microsomal preparation was determined by the method of Lowry *et al.* with bovine serum albumin as a standard.[17]

Incubation conditions of microsomal systems and TLC autoradiograms

The standard incubation mixtures contained Cofactor-I (Oriental Yeast Co., Ltd) 8.6 mM $MgCl_2$, 17.0 mM KCl, 2.6 mM G-6-P, 2.0 mM NADPH, 2.2 mM NADH, 42.1 mM Na_2HPO_4, 10.3 mM NaH_2PO_4 (final concentrations), 20 units glucose-6-phosphate dehydrogenase, 6 mg microsomal protein and 100 mM phosphate buffer (pH 7.4). The final volume of each incubation mixture was 2 mL. The reaction was initiated by the addition of 500 nmol of substrate ([^{14}C]indomethacin; 27 nmol) in 50 μL EtOH and run at 37 °C for 60 min. The non-NADPH incubation medium was prepared independently by the use of the solution in which NADPH, NADH and Cofactor I were eliminated from the complete medium. For the inhibition study, SKF-525A was added directly to the system at a final concentration of 2 mM. The incubation mixture was chilled and immediately 3 volumes of EtOH was added twice for deproteinization. The solution was evaporated under reduced pressure and the resulting residue was dissolved in MeOH. The MeOH solution was spotted on a TLC plate and subjected to 2-D development. The plate was sprayed with Enhancer (NEN) and wrapped with Lumirrormembrane (6 μm, Mitsubishi Rayon) before being placed on X-ray film (Fuji Medical RX type) to obtain the autoradiograms. Each region having ^{14}C-activity, visualized as a dark spot on the autoradiograms was scraped from the plate, suspended in 1 mL of MeOH and mixed with 10 mL of scintillator (ACS-II, Amersham), for counting.

Assay of antiplatelet activity in vitro

Blood was collected from rabbits with a plastic syringe containing 1/10 volume of 3.8% sodium citrate solution. Platelet-rich plasma (PRP) was prepared by centrifugation at 4 °C for 10 min at 400 × *g*. Platelet-poor plasma (PPP) was obtained as the supernatant fraction of the residual blood by centrifugation at 2000 × *g* for 10 min.

The antiplatelet effect of ketoprofen and indomethacin and their oxidative products was determined by the

turbidimetric method using an aggregometer (Hematracer: Niko Bioscience Co., Ltd, Japan). A 200 μL sample of PRP and 25 μL of a test compound solution or vehicle were placed in the cuvette of the aggregometer, and incubated at 37 °C for 5 min. After incubation, platelet aggregation was induced by the addition of 25 μL of sodium arachidonate solution (150 μM final concentration, Sigma Chemical Co.).

The extent of aggregation was expressed taking the maximum change in light transmission for PRB against PPP as 100%. The per cent inhibition of aggregation by the test compounds was calculated by dividing the per cent aggregation by that observed in the vehicle run, and then multiplying by 100.

Cyclooxygenase activity of platelets

The cyclooxygenase activity of platelets was measured according to a modified method of Takayama *et al.* A hundred microliters of platelets (1×10^8) suspension and 25 μL of the indomethacin solution or vehicle instead of drug were incubated at 37 °C for 5 min in Hepes–Tyrode buffer (pH 7.4), and shaken for 1 min after the addition of 50 μL of solution of arachidonate (200 μM final concentration, Sigma Chemical Co.). The reaction was terminated by the addition of 0.5 mL thiobarbituric acid–reactive substance (0.8% thiobarbituric acid:7% perchloric acid, 2:1). After the sample was boiled for 10 min, 10% Triton X-100 and 1 N NaOH were added. The sample was measured by a Hitachi double beam spectrophotometer (Model 100-40) in optical absorbance at 548 nm just 1 min after the addition of NaOH. The IC_{50} against cyclooxygenase was calculated by the least square method.

Acknowledgement

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan.

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(*Received in U.S.A.* 12 *July* 1994; *accepted* 5 *October* 1994)