In Vitro Regio- and Stereoselective Oxidation of β-Ionone by Human Liver Microsomes

Authors Shinsuke Marumoto[1], Ryoyu Shimizu[2], Genzoh Tanabe[3], Yoshiharu Okuno[4], Mitsuo Miyazawa[2,5]

Affiliations The affiliations are listed at the end of the article

Key words
- β-ionone
- human liver microsome
- cytochrome P450
- regio- and stereoselective oxidation
- 4-hydroxy-β-ionone

Abstract

The metabolism of the norisoprenoid β-ionone was investigated *in vitro* using human liver microsomes and 11 different recombinant cytochrome P450 enzymes expressed in *Trichoplusia ni* cells. β-Ionone was found to be oxidized via 4*S*-hydroxylation by CYP2B6 in human liver microsomes. CYP1A2 also regioselectively catalyzed the hydroxylation of β-ionone to yield 4-hydroxylation; this conversion was not stereoselective. Further kinetic analysis revealed that CYP2B6 exhibited the highest activity for β-ionone 4-hydroxylation. Kinetic analysis showed that K_m and V_{max} for oxidation of β-ionone by CYP1A2 and CYP2B6 was 107.9 ± 36.0 µM and 3200.3 ± 323.0 nmol/min/nmol P450 and 5.6 ± 1.2 µM and 572.8 ± 29.8 nmol/min/nmol P450, respectively. The reaction rates observed using human liver microsomes and recombinant CYP2B6 were very high compared with those of other CYP2B6 substrates reported thus far. These results suggest that β-ionone, a norisoprenoid present in nature, is one of the effective substrates for CYP2B enzymes in human liver microsomes. To the best of our knowledge, this is the first time that 4-hydroxy β-ionone has been described as a human metabolite of β-ionone.

Introduction

The ionone derivatives, α-, β- and γ-, mainly occur in plants containing β-carotene. β-Ionone is found in a variety of foods including carrots, raspberries, pumpkin, and many other fruits and herbs [1–3]. These plants generally share the woody-floral, violet scent characteristics of natural compounds and also have odors. Humans are also exposed to β-ionone through the daily dietary intake of β-ionone-rich food or the passive transfer of pasture species alfalfa (lucerne) through the consumption of dairy products [4].

Human metabolism of naturally occurring volatile compounds is interesting because their metabolites are important determinants of food safety and biological activity. A variety of components of numerous plant species are oxidized by cytochrome P450 (CYP) in laboratory animals and humans [5–7]. The CYP enzymes detoxify and/or toxify these compounds to more polar and sometimes more reactive metabolites [7–11]. In mammals, the liver plays a major role in the metabolism and systemic elimination of xenobiotics after exposure. For this reason, investigations of β-ionone metabolic pathways, patterns of metabolite and intermediate formation, and kinetics are often studied using CYP human liver microsomes (HLMs). Studies of HLM metabolism indicate that CYP is a major enzyme in the metabolism of monoterpenes 1,8-cineole, (+)- and (−)-limonenes, (+)-fenchone, and (−)-camphor [12–15]. The major pathways used for the metabolism of these compounds involve CYP-dependent hydroxylation or oxidation.

Although β-ionone is contained in foods consumed daily and is widely used in cosmetic products, no studies describe its metabolism by HLMs. Investigation of β-ionone metabolism by HLMs will provide a better understanding of the metabolism and toxicity of related naturally occurring volatile compounds in the human body.

The present study uses HLMs to investigate the metabolism of β-ionone, including identification of metabolites, elucidation of CYP-catalyzed metabolism by 11 types of recombinant human CYP enzymes, and determination of enzyme kinetics.

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Correspondence
Mitsuo Miyazawa
Department of Applied Chemistry
Faculty of Science and Engineering
Kinki (Kindai) University
Kowakae, Higashiosaka-shi
Osaka 577–8502
Japan
Phone: + 81 6 67 21 23 32
Fax: + 81 6 67 27 20 24
miyazawa@apch.kindai.ac.jp

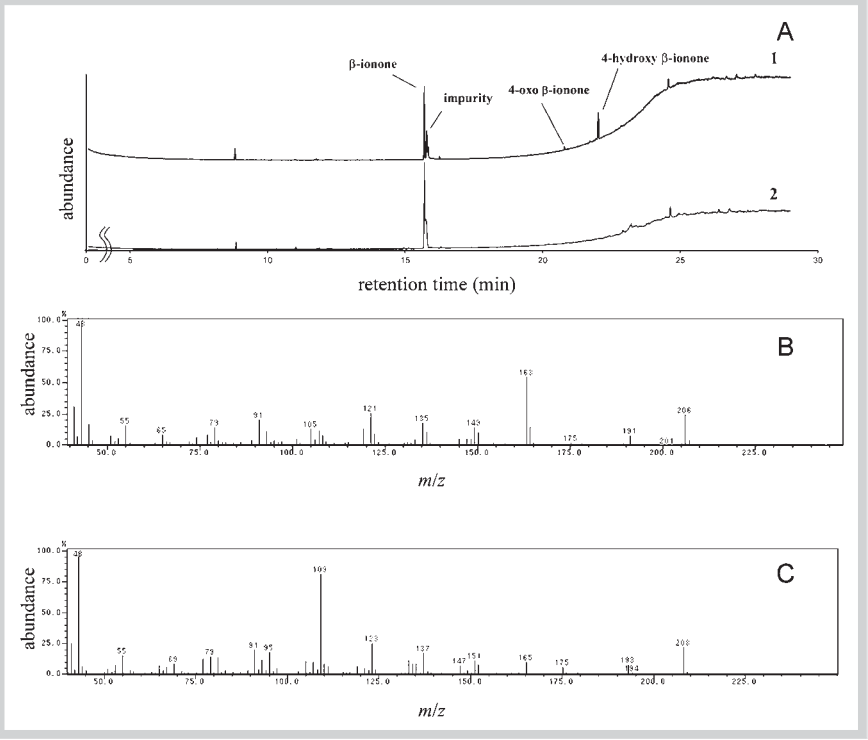

Fig. 1 GC-MS analysis of *β*-ionone and its transformation products, 4-oxo *β*-ionone and 4-hydroxy *β*-ionone. Panel **A** shows the GC-MS chromatogram of *β*-ionone with HLMs in the presence of an NADPH-generating system (1) and without HLMs (2). Panel **B** shows the full scan mass spectrum of 4-oxo *β*-ionone. Panel **C** shows the full scan mass spectrum of 4-hydroxy *β*-ionone.

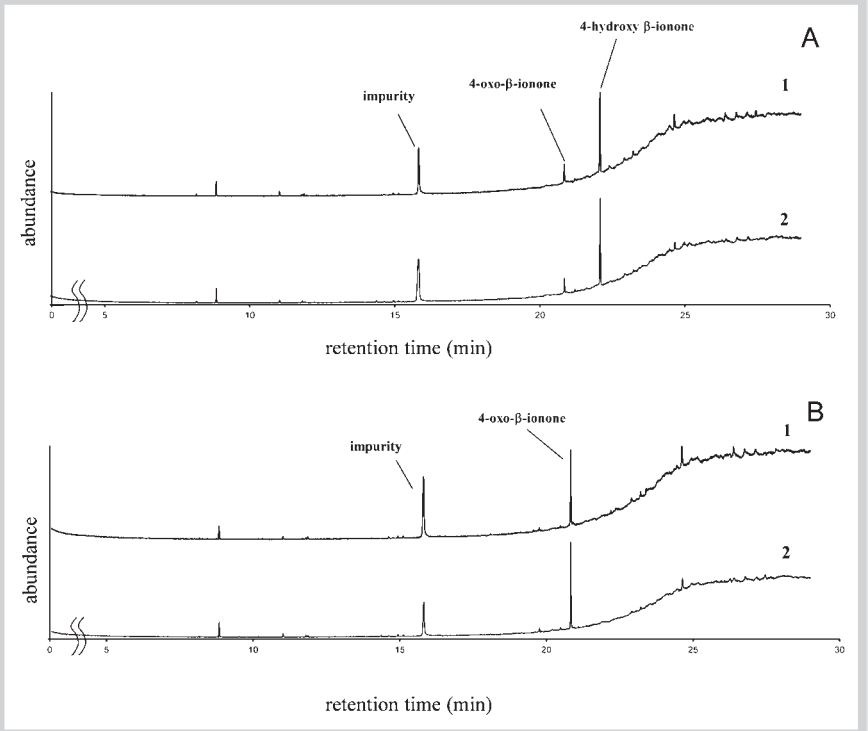

Fig. 2 Panel **A** shows the GC-MS chromatogram of 4-hydroxy *β*-ionone with HLMs in the presence of an NADPH-generating system (1) and without HLMs (2). Panel **B** shows the GC-MS chromatogram of 4-oxo *β*-ionone with HLMs in the presence of an NADPH-generating system (1) and without HLMs (2).

Results

The metabolism of *β*-ionone was examined using HLMs (HF207) in the presence of an NADPH-generating system. Using GC-MS analysis, we identified the two metabolites as 4-hydroxy-*β*-ionone and 4-oxo-*β*-ionone (**Fig. 1**). Component identification was made based on mass spectral fragmentation and comparison of retention time to an authentic sample obtained by organic synthesis. In addition to chromatographic behavior, panel C (**Fig. 1**) shows spectral data indicating the presence of a 4-hydroxylated metabolite. The molecular mass of the metabolite increased by from 192 to 208 upon the introduction of an oxygen atom. Although the 4-oxo metabolite was generated by HLMs from 4-hydroxy-*β*-ionone, the production of two metabolites via HLMs indicated that the 4-oxo metabolite was formed by the auto-oxidation of 4-hydroxy-*β*-ionone (**Fig. 2**). The chirality of

the metabolite was determined using chiral HPLC. The metabolism of β-ionone by HLM (HFC207) produced isomers of (*S*)- and (*R*)-4-hydroxylated metabolites at a ratio of isomers 2 : 1 (**Figs. 3** and **4**). Recombinant human CYPs expressed in *T. ni* cells were tested for their ability to catalyze β-ionone oxidation. CYP1A2 catalyzed the oxidation of (+)-(*S*)-4-hydroxy- and (−)-(*R*)-4-hydroxy-β-ionones. In contrast, CYP2B6 catalyzed the enantioselective oxidation of (+)-(*S*)-4-hydroxy-β-ionone (**Fig. 5**). All other CYP enzymes had very low activity or activity below the limit of detection.

To further assess whether the β-ionone metabolic pathways involved catalysis by CYP1A2 and CYP2B6, we examined the effects of chemical inhibitors of CYP1A2 (α-naphthoflavone) and CYP2B6 (ticlopidine) on β-ionone metabolism by the HLMs HFC205 and recombinant CYP enzymes. HLMs contained relatively high CYP1A2 and CYP2B6 activity. The concentrations of α-naphthoflavone and ticlopidine needed to effectively inhibit oxidation of β-ionone were determined using recombinant CYP1A2 (**Fig. 6A**) and CYP2B6 (**Fig. 6C**). In assays of the recombinants, α-naphthoflavone and ticlopidine strongly inhibited the oxidation of β-ionone by CYP1A2 and CYP2B6, respectively. The inhibition by various concentrations of chemical inhibitors on the metabolism of β-ionone by HLMs is shown in **Fig. 6B, D**. The formation of (*S*)- and (*R*)-4-hydroxy β-ionones in the presence of 50-donor pool HLMs, recombinant CYP1A2 and CYP2B6 with an NADPH-generating system varied depending on incubation time, CYP concentration, and substrate concentration (**Fig. 7A–I**, respectively). Using different incubation times, we found a linearity of the β-ionone 4-hydroxylation activities catalyzed by the enzymes up to 40 min. Increasing of the CYP enzyme concentration or the β-ionone concentration led to increasing 4-hydroxy β-ionone formation. In the absence of the CYP enzymes, we detected no β-ionone hydroxylation at all. Kinetic analysis of β-ionone oxidation activity was carried out using recombinant CYP1A2, CYP2B6, and 50-donor pool HLMs of human samples (**Table 1**). Kinetic analysis showed that the V_{max}/K_m values for (+)-(*S*)- and (−)-(*R*)-4-hydroxy-β-ionone, catalyzed by HLMs, were 0.3 and 0.1 µL/min/mg protein, respectively. Recombinant CYP1A2 and CYP2B6 catalyzed the production of (+)-(*S*)-4-hydroxy-β-ionone. The V_{max}/K_m values were 29.4 and 102.1 µM/min, respectively. The V_{max}/K_m value for (−)-(*R*)-4-hydroxy-β-ionone, catalyzed by CYP1A2, was 11.8 µM/min.

Our results suggest that CYP1A2 and CYP2B6 are important enzymes in β-ionone oxidation by HLMs. **Fig. 8** depicts the relationship between the oxidation activity of β-ionone and specific activities of CYP1A2 and CYP2B6 in eight different HLMs, indicating a good correlation between (+)-(*S*)-4-hydroxy-β-ionone and CYP2B6 activity ($r = 0.854$). The formation rates of (+)-(*S*)- and (−)-(*R*)-4-hydroxy-β-ionone correlated slightly with the activity of CYP1A2. There was no correlation between CYP2B6 activity and the formation of (−)-(*R*)-4-hydroxy-β-ionone.

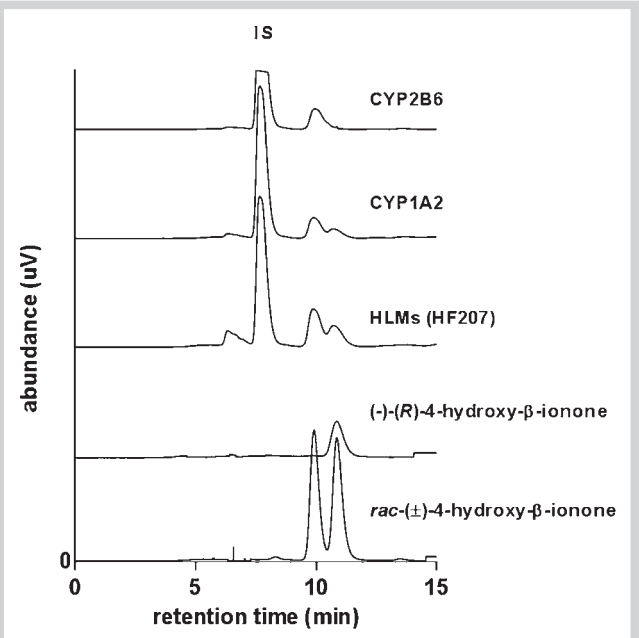

Fig. 3 Representative chromatographic traces for 4-hydroxylation of β-ionone in HLMs, CYP1A2, and CYP2B6 incubates.

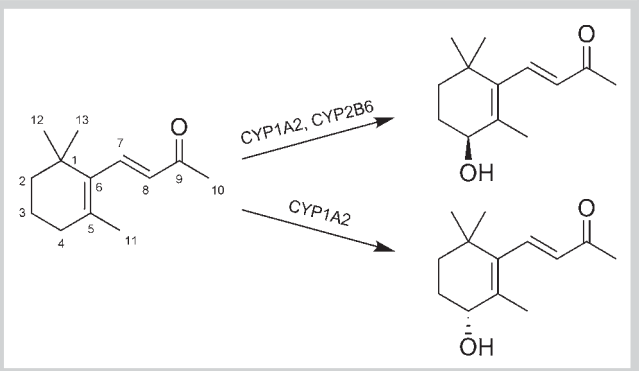

Fig. 4 Metabolism of β-ionone by human CYP1A2/CYP2B6 and *in vitro*.

Discussion

In this study, we found that β-ionone was selectively oxidized to its respective 4-hydroxylation metabolites by CYP1A2 and CYP2B6 in HLMs. This activity preceded stereoselective oxidation (*S* form) by CYP2B6 (**Fig. 4**). Although the enzyme efficiency (V_{max}/K_m ratio) of CYP2B6 was much higher than that of CYP1A2 for the formation of (*S*)-4-hydroxy β-ionone from the substrate by recombinant CYP, CYP2B6 was found to be more active than CYP1A2 in catalyzing β-ionone using HLMs. Ticlopidine, a specific CYP2B6 inhibitor, significantly suppressed the activity of (*S*)-4-hydroxylation β-ionone catalyzed by HLMs. β-Ionone (*S*)-4 oxidation activity was found to correlate with CYP2B6, but not CYP1A2, present in eight types of HLMs. The reason that CYP2B6 is more active than CYP1A2 in the stereoselective oxidation of β-ionone by HLMs may be related to the higher content of this CYP species in the human liver. Our data suggested that the mean levels of CYP2B6 in HLMs of eight human samples were approximately fivefold higher than those of CYP1A2, probably resulting in a more important role of CYP2B6 in the catalysis of β-ionone oxidation by HLMs.

Species differences have been reported in the metabolism of β-ionone by CYPs, and CYP101B1 has been shown to produce two oxidized metabolites: 3-hydroxy-β-ionone and 4-hydroxy-β-ionone [16]. Similarly, CYP102A1 and P450 SU1, SU2, and SOY have been shown to metabolize 4-hydroxy-β-ionone [17]. In HLMs, β-ionone is converted to 4-hydroxy-β-ionone. Therefore, our re-

sults, together with those of previous studies, show that the metabolism of *β*-ionone by CYPs proceeds via preferential oxidation at the 4-position (allylic position). Allylic hydroxylation of *β*-ionone at position 4, which contains conjugated *π*-bonded carbon atoms, producing allylic alcohols, has been reported as a common CYP-mediated reaction [18]. Accordingly, allylic alcohols have been identified as enzymatic products of other monoterpenes, such as limonene, 1,8-cineole, and Δ^3-carene [19,20]. Δ^3-Carene is also hydroxylated by CYP2B6. Allylic hydroxylation of monoterpenes is catalyzed by other CYPs, such as CYP2C9, 2C19 2D6, and CYP3A4.

We have recently reported the metabolism of monoterpenes by HLMs. Of these, (+)-fenchone, was most efficiency catalyzed by human CYP2B6 (10-hydroxylation) and CYP2A6 (6-*endo* and -*exo* hydroxylations), with metabolic rates (V_{max}/K_m) of 175, 167, and 150 /nM/min, respectively. The V_{max}/K_m of *β*-ionone 4-hydroxylation for CYP2B6 (*S* form) and CYP1A2 (*S* and *R* forms) was determined as 102.1, 29.4, and 11.8 /μM/min, respectively. Compared with the V_{max} and V_{max}/K_m values of other monoterpenes metabolized by CYP enzymes, the values described here, particularly for CYP2B6, are high. CYP2B6 is expressed mainly in human liver, although this enzyme has also been detected in various extra-hepatic tissues [21]. Considerable variability exists not only in hepatic expression of CYP2B6 mRNA (280-fold) and protein (> 288-fold) but also in CYP2B6 enzyme activity (80-fold) [22–24]. The magnitude of CYP2B6 catalytic activity may be altered as a result of enzyme inhibition by various synthetic drugs [25,26] and naturally occurring compounds such as curcuminoid extract [27], phenethyl isothiocyanate [28], and citral [29]. Important CYP2B6 drug substrates include the alkylating anticancer prodrug cyclophosphamide [30] and the tobacco use cessation agent bupropion [31]. Bupropion and efavirenz are often administered in combination with several other drugs. For example, tenofovir increases the plasma concentration of efavirenz, a substrate of CYP2B6, under conditions of limited efavirenz metabolism [32]. Since the rate of *β*-ionone metabolism by CYP2B6 is high, it's possible that high amounts of these agents might alter the bioavailability of drugs that are metabolized by CYP2B6; however, *in vitro* inhibition of CYP2B6 activity does not necessarily translate into *in vivo* drug interactions.

In summary, our results show that *β*-ionone is regioselectively oxidized to its respective 4-hydroxy derivatives by CYP1A2 and CYP2B6 in HLMs. CYP2B6 regio- and stereoselectively catalyzes *β*-ionone metabolism. Our results also show that CYP2B6 plays a more important role than CYP1A2 in catalyzing *β*-ionone oxidation. The precise role of the CYP2B6 enzyme in *β*-ionone metabolism in response to human exposure to these chemicals is still unknown.

Materials and Methods

Chemicals and reagents

β-Ionone (purity > 99%) was purchased from Tokyo Chemical Industry Co., Ltd. *α*-Naphthoflavone (purity ≥ 98%) and ticlopidine (purity ≥ 99%), were purchased from Sigma-Aldrich. $NADP^+$, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast, Ltd. The other regents and chemicals used in this study were of the highest quality commercially available and obtained from sources as described previously [12–15].

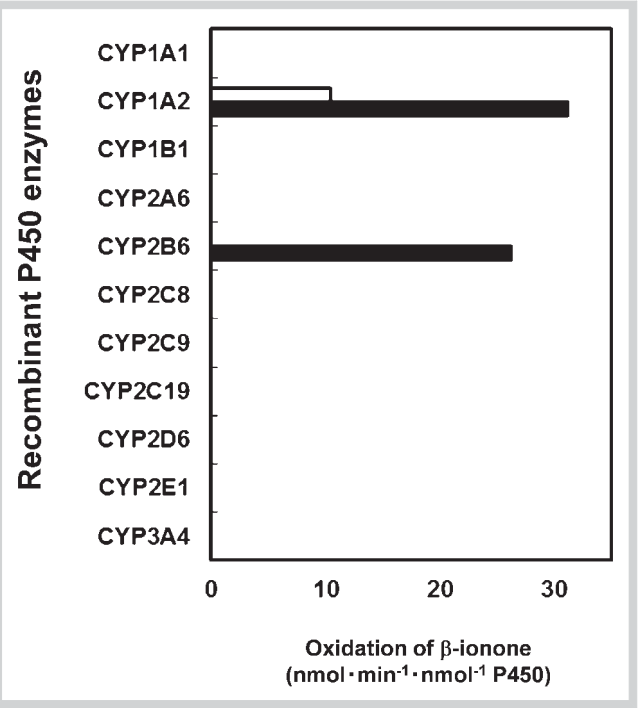

Fig. 5 Oxidation of *β*-ionone by 11 types of recombinant human P450 expressed in *T. ni* cells.

Synthesis of (±)-4-hydroxy-*β*-ionone

(±)-4-Hydroxy-*β*-ionone was synthesized as described previously [33]. The epoxidation of *α*-ionone (1 g, 5.3 mmol) with *m*-CPBA (1.4 equivalents) at 5 °C for 1 h in 20 mL of CH_2Cl_2 yielded 4,5-epoxide. The 4,5-epoxide was subjected to a base catalyzed rearrangement by refluxing with K_2CO_3 (3.0 equivalent) in methanol for 4 h to yield the final compound. Most of the methanol was then evaporated, and the residue was dissolved in ethyl acetate. The organic phase was washed successively with water and brine. The solution was evaporated and the residue was purified by silica gel column chromatography (ethyl acetate-hexane 65 : 35) to give the (±)-4-hydroxy-*β*-ionone white powder (420 mg, 48%) with ^{1}H NMR ($CDCl_3$, 800 MHz) *δ* 7.18 (1H, dq, *J* = 16.4, 1.3 Hz, H-8), 6.11 (1H, d, *J* = 16.4 Hz, H-7), 4.01 (1H, t, *J* = 4.9 Hz), 2.55–2.53 (1H, m, H-3), 2.35 (3H, s, H-11) 1.90–1.89 (1H, m, H-2), 1.80 (3H, d, *J* = 1.0 Hz, H-10), 1.19 (6H, s, H-12, 13); ^{13}C NMR ($CDCl_3$, 200 MHz) *δ* 198.5 (C, C-9), 142.7 (CH, C-7), 139.4 (C, C-6), 133.9 (C, C-5), 133.0 (C, C-8), 69.9 (CH, C-4), 35.5 (C, C-1), 34.6 (CH_2, C-2), 28.8 (CH_3, C-12 or − 13), 28.3 (CH_2, C-3), 27.5 (CH_3, C-12 or − 13), 27.3 (CH_3, C-10), 18.4 (CH_3, C-11); EIMS *m*/*z* 208 $[M]^+$ (21), 137 (17), 123 (25), 109 (79), 95 (18), 91 (20), 43 (100), and 41 (24).

Preparation of (−)-(*R*)-4-hydroxy-*β*-ionone

(−)-(*R*)-4-Hydroxy-*β*-ionone enantiomer was prepared as described previously [33]. A mixture of (±)-4-hydroxy-*β*-ionone (200 mg, 1.0 mmol), dry lipase (100 mg), activated molecular sieves 4 Å (150 mg), and vinyl acetate (0.5 equivalent) in 10 mL of dry *n*-hexane-THF (9 : 1 v/v) was stirred at room temperature for 18 h. The reaction was monitored by chiral HPLC and terminated at ~ 50% conversion. The reaction mixture was filtered and the filtrate was concentrated *in vacuo*. The residue was purified further by silica gel column chromatography. Elution with a

mixture of ethyl acetate and hexane (65 : 35) yielded (−)-(*R*)-4-hydroxy-*β*-ionone (103 mg; *ee* 95% as analyzed by chiral HPLC; $[\alpha]_D^{20.4}$ −8.01° [$CHCl_3$, *c* 1.0]). The spectral data of enantiomer were identical to those of racemate.

Synthesis of 4-oxo-*β*-ionone

Dess-Martin periodinane (1.0 equivalents) was added to a solution of (±)-4-hydroxy-*β*-ionone (200 mg, 1.0 mmol) in 10 mL of CH_2Cl_2. The reaction mixture was stirred until the starting alcohol was consumed, as determined by TLC analysis. Once the reaction was judged complete, the mixture was diluted with aqueous $NaHCO_3$ and CH_2Cl_2. The precipitated solids were filtered through a bed of diatomaceous earth. The filter cake was rinsed with CH_2Cl_2, and the biphasic filtrate was separated. The organic layer was extracted and evaporated. The resulting residue was eluted through a silica gel column with a ratio of ethyl acetate-hexane (65 : 35) to obtain the corresponding 4-oxo-*β*-ionone (143 mg, 75%), with a pale yellow solid; ^1H NMR ($CDCl_3$, 800 MHz) *δ* 7.24 (1H, dq, *J* = 16.5, 1.0 Hz, H-8), 6.19 (1H, d, *J* = 16.5 Hz, H-7), 2.55–2.53 (1H, m, H-3), 2.35 (3H, s, H-11) 1.90–1.89 (1H, m, H-2), 1.80 (3H, d, *J* = 1.0 Hz, H-10), 1.19 (6H, s, H-12, 13); ^{13}C NMR ($CDCl_3$, 200 MHz) *δ* 198.6 (C, C-4), 197.4 (C, C-9), 157.7 (C, C-6), 140.3 (CH, C-7), 133.5 (CH, C-8), 131.4 (C, C-5), 37.3 (CH_2, C-2), 35.5 (C, C-1), 34.2 (CH_2, C-3), 27.9 (CH_3, C-11), 27.3 (CH_3, C-12, 13), 13.4 (CH_3, C-10); EIMS *m/z* 206 $[M]^+$ (26), 164 (16), 163 (59), 135 (17), 122 (18), 121 (35), 43 (100), and 41 (26).

Enzymes

HLMs HG18, HG64, HH37, HH519, HH581, HH715, HFC205, HFH705, and 50-donor pool were obtained from Gentest Co., Inc. and were stored at −80 °C. HLMs were available for complete catalytic assays of the major CYPs: phenacetin-*O*-deethylase (CYP1A2), coumarin 7-hydroxylase (CYP2A6), (*S*)-mephenytoin *N*-demethylase (CYP2B6), paclitaxel 6*α*-hydroxylase (CYP2C8), 4′-hydroxylase (CYP2C9), (*S*)-mephenytoin 4′-demethylase (CYP2C19), bufuralol 1′-hydroxyase (CYP2D6), chlorzoxazone 6-hydroxylase (CYP2E1), testosterone 6*β*-hydroxylase (CYP3A4), and lauric acid 12-hydroxylase (CYP4A). Recombinant human CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 expressed in *T. ni* cells infected with a baculovirus containing CYP and NADPH-P450 reductase *c*DNA inserts were obtained from Gentest. The CYP content was determined using these materials as described in the manufacturers protocols.

β-Ionone oxidation assays

β-Ionone oxidation by CYP enzymes was determined as follows: Standard reaction mixtures contained HLMs (0.2 mg protein/ml) or recombinant CYP (11 different recombinants; 20 pmol/ml) with 200 µM *β*-ionone in a final volume of 0.50 mL 100 mM potassium phosphate buffer (pH 7.4) containing an NADPH-generating system (0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, and 0.5 units glucose-6-phosphate dehydrogenase/ml) [12–15]. Incubations were carried out at 37 °C for 30 min and terminated by the addition of 1.0 mL CH_2Cl_2 followed by vigorous mixing. The extracts (organic layer) were collected by centrifugation at 3000 rpm for 10 min and transferred to an insert for analysis by GC-MS.

GC-MS analysis was performed using an Agilent 6890 N gas chromatograph (Agilent Technologies) equipped with an Agilent 5973 N quadrupole mass selective detector. The metabolites were separated using an HP-5MS nonpolar capillary column (30 m × 0.25 mm i.d; film thickness 0.25 µm) and a DB-WAX polar capillary column (15 m × 0.25 mm i.d; film thickness 0.25 µm) using helium (at 1.5 mL/min) as the carrier gas. The column temperature was programmed as isothermal at 80 °C for 5 min, then raised to 260 °C at a rate of 8 °C/min and held at 260 °C for 5 min. The injector temperature was maintained at 270 °C. The effluent of the GC column was introduced directly into the source via a transfer line (280 °C). The ion source temperature was set at 230 °C. The electron impact (EI) ionization voltage was set to 70 eV and positively charged ions were analyzed in full scan mode applying a scan of *m/z* 40–500 amu.

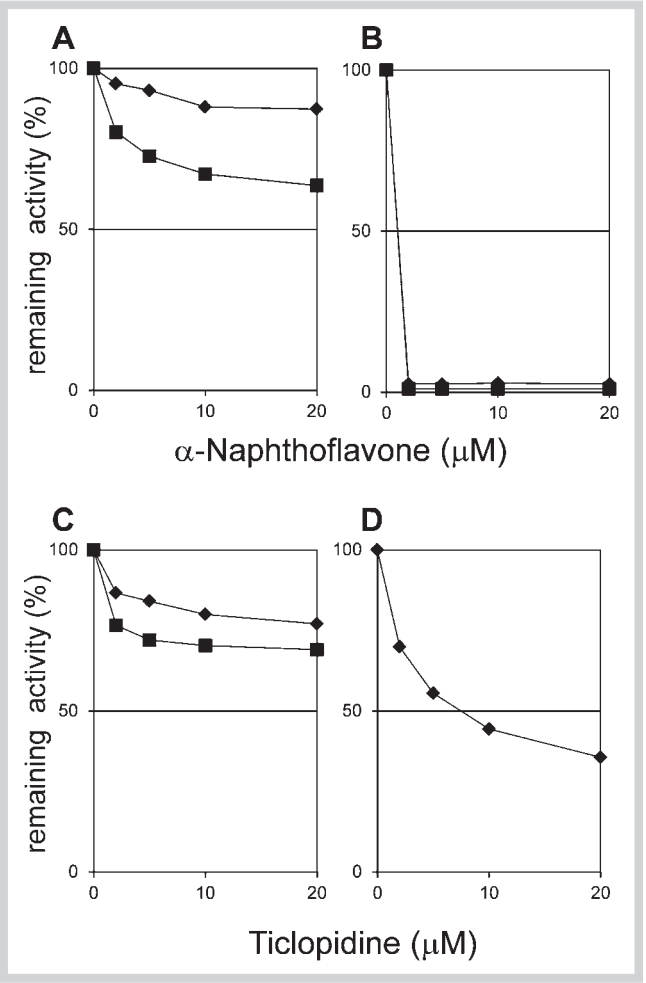

Fig. 6 Effects of *α*-naphthoflavone (**A** and **B**) as an inhibitor of CYP1A2 and ticlopidine (**C** and **D**) as an inhibitor of CYP2B6 on the oxidation of *β*-ionone to (+)-(*S*)-4-hydroxy-*β*-ionone (■) and (−)-(*R*)-4-hydroxy-*β*-ionone (•) by HLMs.

Inhibition experiments

The inhibitory effects of known selective inhibitors of CYP on the metabolism of *β*-ionone by HLMs and recombinant CYP were evaluated to determine the specific CYP enzymes involved in each metabolic pathway. The inhibitors included *α*-naphthoflavone (1–20 µM), a selective CYP1A2 inhibitor [34], ticlopidine (1–20 µM), a selective CYP2B6 inhibitor [35]. The reaction mixture contained 20 nM recombinant CYP, inhibitor, 100 mM phosphate buffer (pH 7.4), and an NADPH-generating system (0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, and 0.5 units of glucose

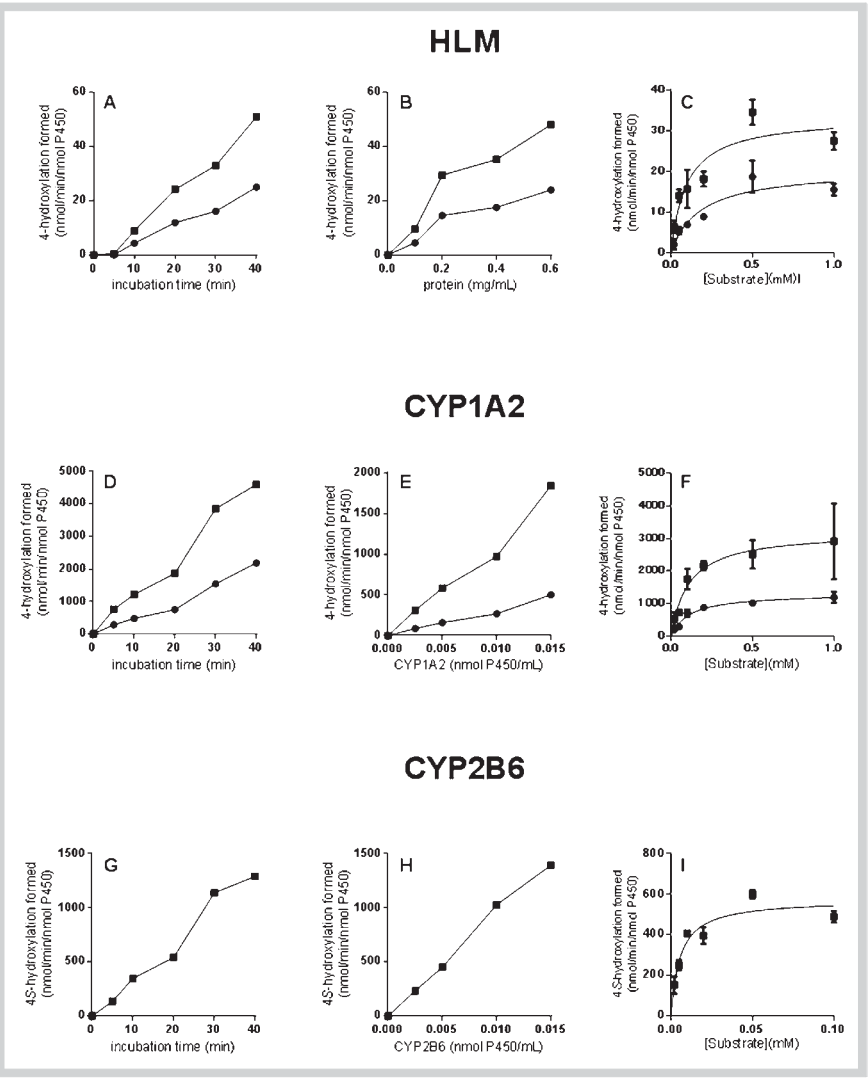

Fig. 7 *β*-Ionone was incubated with HLMs (**A–C**), CYP1A2 (**D–F**), and CYP2B6 (**G–I**) in the presence of an NADPH-generating system, and the (*S*)-4-hydroxy-*β*-ionone (■) and (*R*)-4-hydroxy-*β*-ionone (•) formed were analyzed on a chiral HPLC system. In **A, D**, and **G**, concentrations of *β*-ionone, HLMs, CYP1A2, and CYP2B6 were 0.2 mM, 0.2 mg/ml, 20 pmol/P450, and 20 pmol/P450, respectively. In **B, E**, and **H**, the incubation time and substrate concentration was 30 min and 0.2 mM, respectively. In **C, F**, and **I**, the incubation time and concentrations of HLMs, CYP1A2, and CYP2B6 were 30 min, 0.2 mg/ml, 20 pmol/P450, and 20 pmol/P450, respectively.

Table 1 Kinetic analysis of the (*S*)- and (*R*)-4-hydroxylation of *β*-ionone by CYPs.

Enzyme source	Oxidation of *β*-ionone					
	(+)-(*S*)-4-hydroxy-*β*-ionone			(−)-(*R*)-4-hydroxy-*β*-ionone		
	K_m(μM)	V_{max}	V_{max}/K_m	K_m(μM)	V_{max}	V_{max}/K_m
HLM[a,c]	96.5 ± 27.9	33.5 ± 2.8	0.3	179.1 ± 55.6	20.5 ± 2.2	0.1
CYP1A2[b,d]	107.9 ± 36.0	3200.3 ± 323.0	29.4	111.7 ± 19.8	1312.1 ± 71.1	11.8
CYP2B6[b,d]	5.6 ± 1.2	572.8 ± 29.8	102.1			

[a] V_{max} expressed in nmol/min/mg protein for HLMs; [b] V_{max} expressed in nmol/min/nmol P450 for recombinant P450; [c] Intrinsic clearance (V_{max}/K_m) expressed in μL/min/mg protein for HLMs; [d] Intrinsic clearance (V_{max}/K_m) expressed in/μM/min

6-phosphate dehydrogenase/ml). To the reaction mixture, 100 μM of substrate was added. The incubation was carried out for 30 min at 37 °C.

Kinetic analysis

The kinetic parameters (V_{max} and K_m) for *β*-ionone metabolite formation by recombinant human CYP were estimated by using a computer program designed (GraphPad Prism) for nonlinear regression analysis.

Blank tests

Incubation was carried out at 37 °C for 30 min with 200 μM of substrate or metabolites; 4-hydroxy-*β*-ionone and 4-oxo-*β*-ionone in a final volume of 0.5 mL of 100 mM potassium phosphate buffer (pH 7.4) in combination with the NADPH-generating system, but without the addition of microsomes.

Chiral HPLC method

A simple reversed-phase chiral HPLC method was developed and validated for the direct separation of the (±)-4-hydroxy-*β*-ionone enantiomers. The enzyme reaction mixtures were then applied

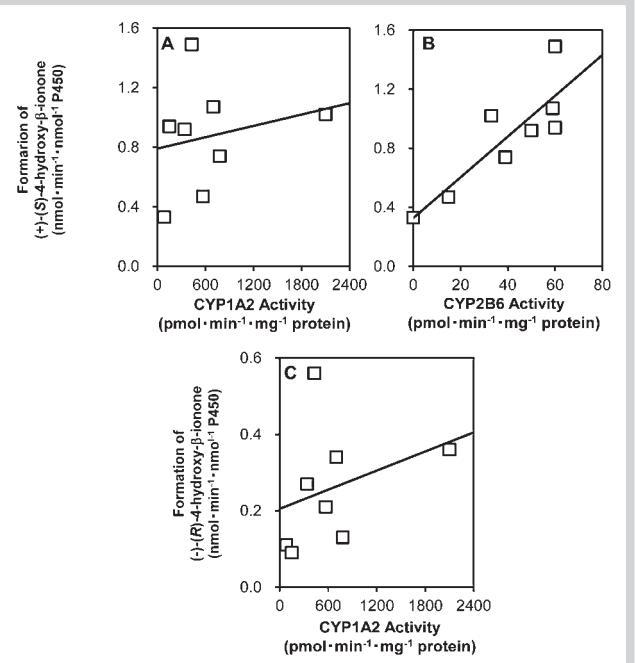

Fig. 8 Correlation between CYP1A2 (**A** and **C**) or CYP2B6 (**B**) content and the catalytic activity of eight samples of HLMs toward oxidation of *β*-ionone. In **A** and **B**, CYP1A2 and CYP2B6 were compared with respect to the formation of (+)-(*S*)-4-hydroxy-*β*-ionone in these human samples; in **C**, CYP1A2 content of the eight human samples was compared with respect to the formation of (−)-(*R*)-4-hydroxy-*β*-ionone.

to a Smart-SPE C18–30 cartridge (AiSTI SCIENCE CO., Ltd.) that had been previously conditioned with 2 mL of acetonitrile and 2 mL of acetonitrile-distilled water (50 : 50 v/v) and then washed with 5 mL of distilled water. The reaction mixture retained in the cartridge was eluted with acetonitrile/distilled water (300 µL; 80 : 20 v/v) containing the internal standard into a disposable sample tube, and a 10-µL aliquot was analyzed using chiral HPLC. The synthetic standards of 4-hydroxy-*β*-ionones were prepared similarly. A standard curve was constructed by plotting the peak area (PA) versus the concentration of (−)-(*R*)- and (+)-(*S*)-4-hydroxy-*β*-ionone using the internal standard method with chiral HPLC. Chromone was added as an internal standard to this solution at a final concentration of 1 µM. Within-day precision of the assay was determined by analysis of a replicate sample (n = 3) of four different concentrations on the same day. Standard curves for (−)-(*R*)- and (+)-(*S*)-4-hydroxy-*β*-ionone were plotted for the concentration range of 0.4–100 µg/ml. UV intensities increased linearly between 0.4 and 100 µg/ml (−)-(*R*)- and (+)-(*S*)-4-hydroxy-*β*-ionone with R^2 values > 0.99. Aliquots (usually 10 µL) of the extracts described above were analyzed by HPLC using an LC-CCPS system (Tosoh) with a spectrometer UV-8020 (Tosoh). Samples were separated on a Chiralcel OD-H analytical column (250 × 4.6 mm, Daicel Corp.) equipped with a Chiralcel OD-H guard column (10 × 4 mm, Daicel Corp.). The eluent was a mixture of 25% acetonitrile (v/v) containing 20 mM potassium phosphate buffer (pH 2.6). The flow rate was 0.5 ml/min and the UV was measured at 260 nm. Peak areas thus obtained were integrated using Chromato-PRO (Run Time Corp.).

Conflict of Interest

The authors declare no conflict of interest.

Affiliations

[1] Joint Research Center, Kinki (Kindai) University, Kowakae, Higashiosaka-shi, Osaka, Japan
[2] Department of Applied Chemistry, Faculty of Science and Engineering, Kinki (Kindai) University, Kowakae, Higashiosaka-shi, Osaka, Japan
[3] Pharmaceutical Research and Technology Institute, Kinki (Kindai) University, Higashi-osaka, Osaka, Japan
[4] Department of Materials Science, Wakayama National College of Technology, Gobo, Wakayama Japan
[5] Graduate School of Materials Science Nara Institute of Science and Technology, Ikoma, Nara, Japan

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