# Metabolism of Isorhynchophylline in Rats Detected by LC-MS

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**ABSTRACT – Purpose.** This paper investigates the metabolic fate of isorhynchophylline (ISOR) as a main bioactive oxindole alkaloid in the traditional Chinese medicine, *Uncaria* species. **Methods.** After oral administration of ISOR to rats, plasma, bile, urine and feces were analyzed by LC-MS. Hydroxylation of ISOR and successive glucuronidation proceeded in vitro by incubation with rat liver microsomes. **Results.** ISOR was identified in plasma, bile, urine and feces. 11-Hydroxyisorhynchophylline 11-*O*-$\beta$-D-glucuronide (MI1) and 10-hydroxyisorhynchophylline 10-*O*-$\beta$-D-glucuronide (MI2) were found in bile, and free 11-hydroxyisorhynchophylline (MI3) and 10-hydroxyisorhynchophylline (MI4) were found in urine and feces. Within 24 h, 71.6% of ISOR was found in the feces and 13.8% into the urine of rats after oral administration of 37.5 mg/kg. Monitoring by LC-MS showed that 8.5% of ISOR was metabolized to MI3 and MI4 in a ratio of ca. 1:1. Specific inhibition of CYP isozymes indicated that CYP2D, CYP1A1/2 and CYP2C participate in ISOR hydroxylation. **Conclusions.** ISOR was found in the circulatory system after oral administration. Cytochrome P450 in rat liver microsomes played a key role in ISOR hydroxylation.

## INTRODUCTION

The tetracyclic oxindole alkaloids isorhynchophylline (ISOR) and its isomeric rhynchophylline were separated from the root bark of *Uncaria* (*U.*) *tomentosa* (Wild.) DC. by HPLC (1). These alkaloids confer protective effects on ischemia-induced neuronal damage in the rat hippocampus (2), suppressive effects on 5-$HT_{2A}$ receptor function in the mouse brain (3) and vasodilator action (4). Recently, ISOR was reported to be effective against angiotensin II induced proliferation in rat vascular smooth muscle cells (5).

The typical indole alkaloid, yohimbine, is metabolized to 10-hydroxyyohimbine and 11-hydroxyyohimbine in humans (6). The biopharmaceutics of yohimbine and the pharmacokinetics of these metabolites in healthy men have also been described (7). However, despite significant pharmacological effects, the metabolism and pharmacokinetics of ISOR have not been reported. Here, we describe the absorption, distribution, metabolism and excretion of ISOR in rats using LC-MS.

## METHODS

### Apparatus

Both $^{1}$H-NMR (400 MHz) and $^{13}$C-NMR (100 MHz) spectra were recorded using a Jeol ECX-400P spectrometer with $CD_3OD$ as the solvent and tetramethylsilane (TMS) as the internal standard. Chemical shifts are shown as $\delta$ values in ppm downfield to TMS. Coupling constants ($J$) are described in Hertz (Hz). Singlet, doublet, triplet, multiplet and broad types of multiplicity are shown as s, d, t, m, and br, respectively. High-resolution fast atom bombardment mass spectrometry (HRFAB-MS) proceeded using a Jeol JMS-AX505HAD mass spectrometer. Circular dichroic (CD) spectra ($c$ = 0.2 mg/ml, MeOH, cell length of 1 cm, volume of 2 ml, 25 °C) were recorded on a Jasco J-805 spectropolarimeter. Optical rotation was measured using a Jasco DIP-140 digital polarimeter. Melting points were measured on a Yanaco micro melting point apparatus without correction.

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**Enzymes and Chemicals**

β-Glucuronidase (Type B-1), pooled microsomes from male rat liver (product number M9066, protein ≥ 20 mg/ml), β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (β-NADPH), alamethicin, SKF-525A (SKF), quinine (QUI), α-naphthoflavone (NAP), cimetidine (CIM), erythromycin (ERY), uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA) and other chemicals were purchased from Sigma (St. Louis MO, USA). Elution solvents for LC-MS were of HPLC grade. We isolated ISOR (1.9 g) from stems and hooks of a mixture (3 kg) of *U*. species including *U. rhynchophylla* Miquel, *U. sinensis* Haviland, and *U. macrophylla* Wallich (*Rubiaceae*) (Tochimoto Tenkaido Co., Osaka, Japan) by refluxing with $CHCl_3$, and purification by repeated silica gel column chromatography (1), followed by preparative HPLC. The isolated ISOR was identified by comparing its retention time on HPLC, and NMR, LC-MS, and CD spectra with those of an authentic sample (provided by Tsumura Co., Tokyo, Japan). We used ISOR at > 99.5% purity confirmed by HPLC for all experiments.

**Animals and ISOR Administration**

Male Wistar rats aged 8 weeks and weighing 240-250 g were used (Sankyo Labo Service Corporation, Inc., Tokyo, Japan). ISOR was dissolved in DMSO at a concentration of 10.7 mg/ml. The solution was administered orally to rats at a dosage of 37.5 mg/kg for ISOR. All animal care and experiments proceeded at the animal experimental center of the University of Toyama in accordance with the guidelines provided by the Life Science Research Center of the same institution. No appreciable side effects were evident in the rats throughout the study. Unless otherwise indicated, the experiments were repeated in 3 rats and the mean values were presented.

**Preparation of Plasma Samples after Oral Administration**

The rats were acclimated for 3 days in metabolic cages with 12-h light intervals. They were fasted overnight with free access to drinking water before oral ISOR administration by gavage and the fast was continued for 3 h. The rats were anesthetized with an intraperitoneal (i.p.) injection of 12 mg (64.8 mg/ml) of pentobarbital sodium (Kyoritsu Seiyaku Co., Tokyo, Japan) before collecting blood samples using heparinized (heparin sodium salt solution, 40 units) containers, syringes and needles. Blood (6 - 8 ml) samples were collected from the inferior vena cava of the rats with a syringe at 30 min, 1, 2, 3, 4, 6, 8, 12, 24, 36, 48, 72 and 96 h after oral administration. After centrifugation at 2220 × *g* for 15 min, the plasma was analyzed immediately or stored at -22 °C. One sample was obtained per rat and 3 rats were sampled at each sample time.

Plasma samples (2.0 ml) were lyophilized to dryness. Half of the residue was digested with β-glucuronidase (32,000 Sigma units) at 37 °C in a buffer (2.0 ml, pH 5.0) for 12 h with a Taitec Personal-11 water bath shaker at 100 strokes/min. A 3-ml Waters Sep-Pak® Vac cartridge was washed with methanol (3.0 ml) followed by water (6.0 ml). The incubation mixture was then passed through the cartridge with 3.0 ml of methanol. The eluate was concentrated under vacuum at room temperature to yield a light pink residue that was dissolved in methanol (4.0 ml) and analyzed by LC-MS. The other half of the residue was directly dissolved in methanol (4.0 ml) without enzymatic digestion for LC-MS analysis.

**Preparation of Bile Samples after Oral Administration**

Isorhynchophylline was administered orally after the common bile ducts of rats were cannulated (i.d. 0.58 mm, o.d. 0.96 mm; PE-50). Bile (0.6 ml/h) was collected at 1-h intervals for 12 h and lyophilized.

Half of the residue was digested with β-glucuronidase (60,000 Sigma units) in a buffer (2.0 ml, pH 5.0) by agitation at 100 strokes/min for 12 h at 37 °C. The mixture was processed as described above for plasma samples after oral administration. The other half of the residue was dissolved in methanol (4.0 ml) without enzymatic digestion for LC-MS analysis.

**Preparation of Urinary and Fecal Samples after Oral Administration**

Rat urinary (15 - 21 ml over a period of 24 h), and fecal (15 - 20 g over 24 h) samples were collected at 24-h intervals for 4 days after oral administration of ISOR and stored at -22 °C until analysis.

Half of the lyophilized residue of urinary

samples (10.0 ml) was digested with β-glucuronidase (60,000 Sigma units) in a buffer (5.0 ml, pH 5.0) at 37 °C for 12 h. The incubation mixture was processed as described above for the plasma samples, except that a 6-ml Waters Sep-Pak® Vac cartridge was used to treat the incubation mixture with 6.0 ml of methanol. The other half of the residue was dissolved in methanol and analyzed by LC-MS without enzymatic digestion.

All fecal samples collected within 24 h were ground and extracted with ethyl acetate (3 × 200 ml). The extracts were concentrated under vacuum at room temperature to yield a yellow residue that was dissolved in methanol and analyzed by LC-MS.

**Controls**

Control rat blood, bile, urine and feces were collected after the administration of DMSO vehicle and processed as described above.

**Isolation of Urinary and Fecal Metabolites**

ISOR (60 mg/kg every 8 h) was administered orally to four rats for one week. The feces were ground and extracted with methanol. The Methanol extract was combined with urine samples and applied to a Diaion HP-20 column (6 × 40 cm). The column was eluted with 5 l methanol and the methanol eluate was concentrated. The metabolites were separated by a Sephadex LH-20 column (2.5 × 30 cm) eluated with methanol and purified with preparative HPLC under the following conditions: Cosmosil column, 5C18-AR-II, Waters type, 20 i.d. × 250 mm; flow rate, 3.0 ml/min; temperature, 30 °C; detection, UV at 254 nm; elution, stepwise gradient of increasing solvent B (0.01% v/v acetic acid in acetonitrile) in solvent A (0.01% v/v acetic acid) from 10 to 30% in 60 min, 30 to 100% in 40 min and 100% for another 20 min.

**Isolation of Biliary Metabolites**

Rat bile was collected from 10 rats after oral ISOR administration at a dose of 60 mg/kg every 8 h for 1 day. The bile was lyophilized and then applied to an LH-20 column (2.5 × 30 cm). The methanol eluate was subjected to preparative HPLC under the same conditions described above.

**Incubation of ISOR with Rat Liver Microsomes**

The reaction mixture (1.0 ml) comprised 100.0 μM ISOR, pooled rat liver microsomes (50.0 μl), and 5.0 mM $MgCl_2$ in 0.1 M PBS buffer (pH 7.4). The reaction was triggered by 2.0 mM β-NADPH after heating for 3 min, and continued at 37 °C under agitation by 100 strokes per minute for 1 h. The reaction was terminated by adding ice-cold ethyl acetate (1 ml) and then incubation mixture was extracted with ethyl acetate (3 × 1 ml, including the terminator). The extracts were pooled and evaporated *in vacuo* to yield a residue that was dissolved in methanol for analysis by LC-MS.

The CYP-dependent hydroxylation of ISOR was confirmed by adjusting ISOR concentrations from 50.0 to 200.0 μM, rat liver microsomes from 12.5 to 100.0 μl, incubation from 10 to 120 min, adding respective inhibitors of CYP isozymes and incubation without β-NADPH. The inhibitors were 400.0 μM SKF (general inhibitor of CYP) (8-9), 25.0 μM NAP (inhibitor of CYP1A1/2) (10-11), 200.0 μM CIM (inhibitor of CYP2C) (9), 25.0 μM QUI (inhibitor of CYP2D) (10), and 200.0 μM ERY (inhibitor of CYP3A) (11). Both ISOR and the inhibitors were dissolved in DMSO and diluted in the reaction mixture so that the final DMSO concentrations were < 1% (v/v). The reaction was started by adding ISOR, and terminated to prepare analytical samples as described above.

**Glucuronidation of Hydroxylated ISOR**

Rat liver microsomes (50.0 μl) were activated with alamethicin (50.0 μg) in 0.1 M PBS buffer (pH 7.4, 0.5 ml) on ice for 15 min (12-13). A mixture of rat liver microsomes, β-NADPH (2.0 mM), ISOR, alamethicin, and $MgCl_2$ (5.0 mM) was incubated at 37 °C for 3 min in the same buffer (total 1.0 ml) and then UDPGA (5.0 mM) was added to start the reaction. The same volume of ice-cold methanol was added to stop the reaction at 2 h. The mixture was separated by centrifugation at 5368 × *g* for 5 min, passed through a 0.45 μm filter and then the filtrate was analyzed by LC-MS. A portion of the filtrate after solvent evaporation was agitated at 100 strokes per min at 37 °C for 6 h with β-glucuronidase (5,000 Sigma units) in 1.0 ml of buffer (pH 5.0). The reaction mixture was separated by centrifugation at 5368 × *g* for 5 min to yield a supernatant that was analyzed by LC-MS.

### LC-ESI-Ion Trap $MS^n$ Measurements

Both *in vivo* and *in vitro* samples were analyzed using an Agilent 1100 series HPLC system coupled with a Bruker Daltonics® Esquire $3000^{plus}$ mass spectrometer. Integrated LC-MS control and data were processed using the Agilent ChemStation for the LC-MS system and Bruker Daltonics Esquire 5.1 (Esquire Control Version 5.1, and DataAnalysis Version 3.1).

The samples were applied to a Cosmosil packed column (5C18-MS-II, 4.6 i.d. × 150 mm) at 30 °C, and UV detection was set at 245, 254, 230, 208 and 280 nm. The elution system comprised an increase of solvent B (0.01% v/v acetic acid in $CH_3CN$) from 10 to 25% in solvent A (0.01% v/v acetic acid) within 40 min, 25 to 65% within 10 min, then to 100% within 10 min at a flow rate of 1.0 ml/min unless indicated otherwise. All samples were passed through a 0.45 μm filter before analysis. The injection volume was 2.0 μl for LC-MS. Through a splitting device, 20% of the eluate from a diode array detector was introduced to atmospheric pressure interface-electrospray ionization (API-ESI) for total ion monitoring (TIM) or selective ion monitoring (SIM). The fragmentation cut-off was set at 27% of the precursor mass. The scan range of the ion trap was from *m/z* 50 to 1,000 in the positive ion mode, the nebulizer was set at 50 psi, dry gas at 10.0 l/min and the dry temperature was 360 °C.

Both ISOR and its metabolites were quantified by LC-MS. Calibration curves of ISOR and its metabolites were constructed based on areas in the extracted ion chromatogram (EIC) against known concentrations of each in methanol. The correlation coefficient $r^2$ was > 0.999 in a concentration range from $4.0 \times 10^{-10}$ to $4.0 \times 10^{-7}$ mol/l.

### Recovery Experiments

The recovery of ISOR and its metabolites was examined by adding precise amounts of the compounds ($4.0 \times 10^{-10}$, $1.2 \times 10^{-8}$, and $4.0 \times 10^{-7}$ mol/l, respectively) to body fluids (6.0 ml blood from inferior vena cava, and 0.6 ml bile) and excretions (20.0 ml urine, and 20.0 g feces), and then measuring the respective concentrations by LC-MS. The recoveries from five analyses varied from 95.2 to 102.5%.

### Precision Experiments

The precision of the analysis of ISOR and its metabolites was examined by comparing the results of intra- and inter-day assays with relative standard deviation (RSD) values. The RSD values for intra-day (5 measurements on the same day for each sample) and inter-day (5 measurements per day for each sample for 5 continuous days) assay were both < 5.0%.

All of the data in Figs. 2 and 5, are shown as means ± SD (n = 3), unless otherwise indicated.

## RESULTS AND DISCUSSION

### ISOR in Plasma

After oral administration of ISOR at a dose of 37.5 mg/kg to rats, the free form of ISOR was detected in plasma (ESI-MS *m/z*: 385 $[M+H]^+$) at a retention time of 20.0 min in the EIC at *m/z* 385 (Figure 1).

The compound was identified by comparing the retention time and MS spectra including MS/MS and $MS^3$ with those of an authentic sample. The time course of ISOR in plasma is shown in Figure 2.

### ISOR and Its Metabolites in Bile

ISOR and its metabolites MI1 and MI2 were detected in rat bile by LC-MS after the oral administration of 37.5 mg/kg of ISOR. Both MI1 and MI2 had the same quasi-molecular ion peak at *m/z* 577 ($[M+H]^+$) in the ESI-MS spectrum (Figure 3; Table 1), but different retention times of 8.9 and 9.7 min, respectively, in the EIC at *m/z* 577 (Figure 3a). MI1 and MI2 were quantitatively converted to MI3 and MI4, respectively, by *β*-glucuronidase, and their MS spectra both showed *m/z* 401 $[M+H]^+$ (Figure 4; Table 1).

About 10.0% of the administered dose of ISOR was excreted into bile within 12 h, during which 65.5% of ISOR were transformed to MI1 and MI2 in a ratio of 45:55.

### ISOR and Its Metabolites in Urine and Feces

ISOR, MI3 and MI4 were detected in rat urine and feces by LC-MS, which were identical to those of metabolites excreted into bile.

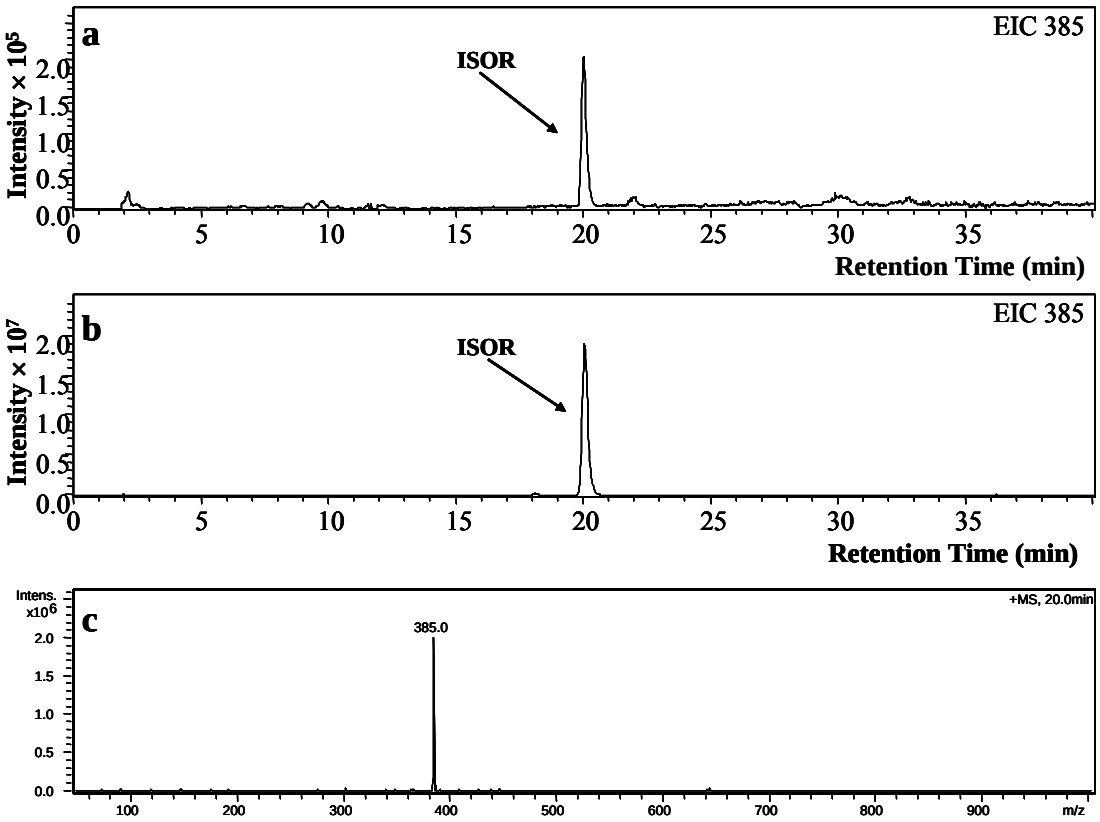


**Figure 1.** LC-MS (EIC) elution profile monitored at *m/z* 385 ± 0.5 (ISOR) in plasma sample collected 3 h after oral ISOR administration, a: ISOR in plasma, b: ISOR standard, c: MS of ISOR.

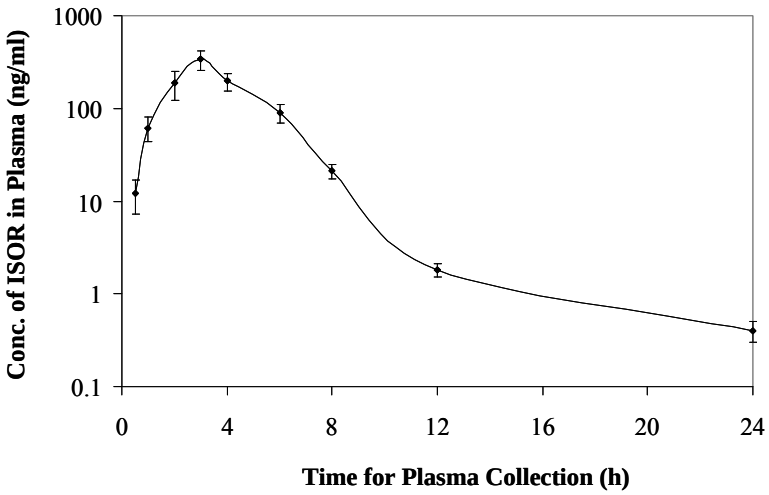


**Figure 2.** Time course of ISOR in rat plasma after oral administration. After oral administration of 37.5 mg/kg, ISOR concentrations in plasma were quantified by LC-MS (EIC) monitored at *m/z* 385 ± 0.5 in the positive ion mode.

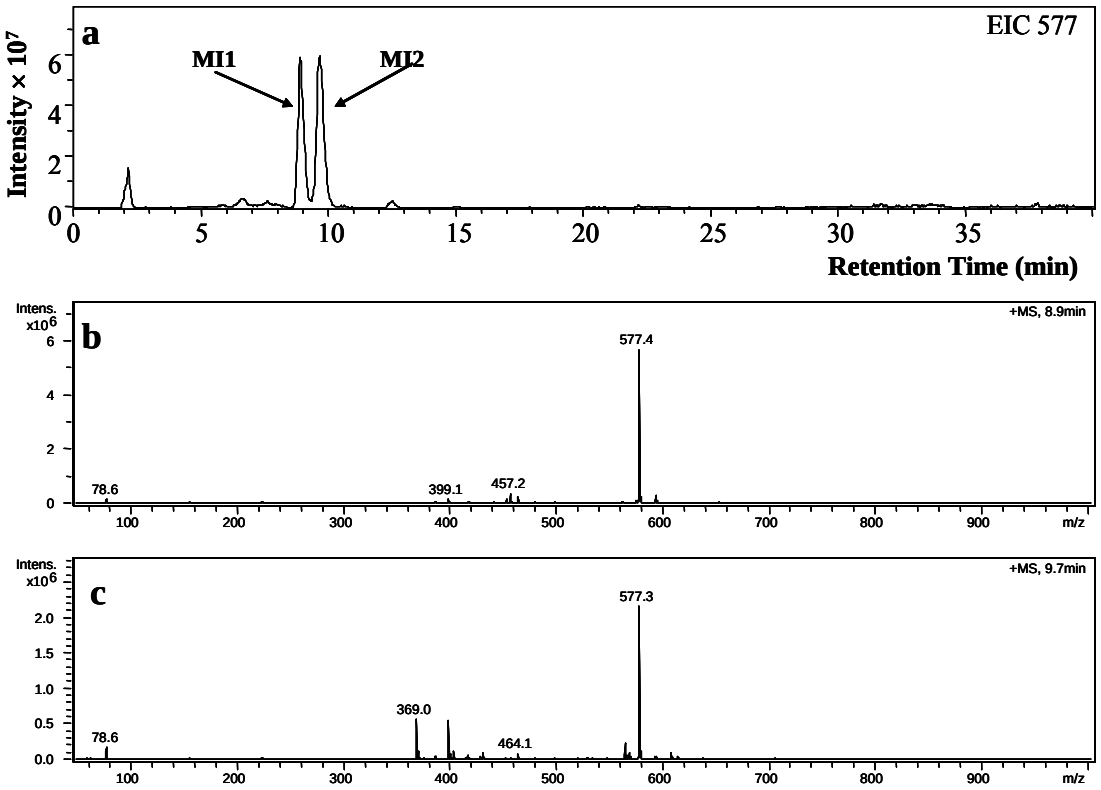


**Figure 3.** LC-MS (EIC) elution profile monitored at *m/z* 577 ± 0.5 (a, MI1 and MI2) in bile sample collected 2 to 3 h after oral ISOR administration and MS of MI1 (b) and MI2 (c).

Table 1. Retention times and major mass spectral data in positive ion mode ($[M+H]^+$).

| Compound | Retention time (min) | Mass spectral data (*m/z*) | | |
|---|---|---|---|---|
| | | MS | MS/MS | $MS^3$ |
| MI1 | 8.9 | 577 | 401 | 369, 337 |
| MI2 | 9.7 | 577 | 401 | 369, 337 |
| MI3 | 12.1 | 401 | 369, 337 | 337, 281 |
| MI4 | 14.6 | 401 | 369, 337 | 337, 281 |
| ISOR | 20.0 | 385 | 353 | 321, 269 |

The amount of ISOR excreted into feces (20.0 g, in 24 h) was 5-fold higher than that excreted into urine (20.0 ml, within 24 h) with total 85.5% of ISOR in excretion unchanged.

The results indicated that ISOR is absorbed orally since unchanged ISOR was identified in urine, as were metabolites MI3 and MI4, and MI1 and MI2 were identified in bile. However, the large fraction of the oral dose of ISOR found in the feces may be due to the fact that only about 20% of ISOR is absorbed following oral administration to rats.

Metabolites MI1 and MI2 were identified in bile but not in urine or feces. It is possible that when these metabolites are excreted back into the intestine, the glucuronidases in the gut hydrolyse MI1 and MI2 back to MI3 and MI4, respectively.

**Hydroxylation of ISOR by Rat Liver Microsomes**

Isorhynchophylline was hydroxylated at the aromatic ring to yield MI3 and MI4. Independent experiments showed that MI3 and MI4 were converted to MI1 and MI2, respectively, in yields of about 9.8% by rat liver microsomes in the presence of UDPGA. Furthermore, digestion with $\beta$-glucuronidase returned these products to the original MI3 and MI4, respectively. All of which are identical with the metabolites of ISOR *in vivo*. These findings confirmed that hepatic enzymes metabolized ISOR in rats. The hydroxylation of ISOR by rat liver microsomes was significantly inhibited by SKF-525A (general inhibitor of CYP) (Figure 5).

Neither of the hydroxylated metabolites of ISOR was detected in the absence of $\beta$-NADPH. The amounts of MI3 and MI4 were appreciably increased by an increase in rat liver microsomes concentrations from 12.5 to 100.0 μl or incubation periods from 10 to 120 min (data not shown). All of this evidence indicates that CYP in rat liver microsomes was responsible for the ISOR hydroxylation. The hydroxylation of ISOR by rat liver microsomes was decreased by 50-80% in the presence of specific inhibitors such as QUI, NAP and CIM against CYP2D, CYP1A1/2, and CYP2C isozymes, respectively, but not altered in the presence ERY (Figure 5). Thus, CYP2D, CYP1A1/2, and CYP2C, but not CYP3A, might participate in ISOR hydroxylation.

**Structures of Metabolites**

Urine, bile and feces of rats were collected after repeated oral administration of ISOR to determine the structures of metabolites MI3 and MI4. Repeated column chromatography yielded two pure compounds with a quasi-molecular ion peak at 401 $[M+H]^+$, which was 16 mass units higher than that of ISOR, suggesting that they were monohydroxylated. MI3 was assigned the molecular formula $C_{22}H_{28}N_2O_5$ by HRFAB-MS at *m/z* 401.4825 ($[M+H]^+$), Calcd for $[C_{22}H_{28}N_2O_5+H]$: 401.4827. The $^1$H-NMR spectrum of MI3 was similar to that of ISOR, except for an aromatic region, in which an ABX-type signal appeared at $\delta$ 6.85 (1H, d, $J_{12,10}$ = 1.2 Hz, H-12), 7.06 (1H, dd, $J_{10,9}$ = 8.0 Hz, $J_{10,12}$ = 1.2 Hz, H-10), and 7.45 (1H, d, $J_{9,10}$ = 8.0 Hz, H-9). Since a proton signal at $\delta$ 7.45 (H-9) neighboring a signal at $\delta$ 7.06 (H-10) correlated with a carbon signal at $\delta$ 55.7 (C-7) in the HMBC experiment (Figure 6), we concluded that MI3 was 11-hydroxyisorhynchophylline. Similarly, MI4 was assigned the molecular formula $C_{22}H_{28}N_2O_5$ by HRFAB-MS at *m/z* 401.4826 ($[M+H]^+$); Calcd for $[C_{22}H_{28}N_2O_5+H]$: 401.4827. The $^1$H-NMR spectrum of MI4 was similar to that of MI3 with an ABX-type signal at $\delta$ 6.82 (1H, d, $J_{12,11}$ = 7.6 Hz, H-12), 7.08 (1H, dd, $J_{11,9}$ = 1.2 Hz, $J_{11,12}$ = 7.6 Hz, H-11), and 7.40 (1H, d, $J_{9,11}$ = 1.2 Hz, H-9). The HMBC spectrum revealed a significant correlation between a proton signal at $\delta$ 7.40 (H-9) and a carbon signal at $\delta$ 149.6 (C-10), or at $\delta$ 55.8 (C-7), indicating a hydroxy group attached at C-10 in the aromatic ring. We consequently determined MI4 as 10-hydroxyisorhynchophylline (Figure 6).

Since MI1 and MI2 were converted to MI3 and MI4, respectively, by $\beta$-glucuronidase digestion and the difference in molecular units between the latter metabolites corresponded to the liberation of the glucuronic acid residue $C_6H_8O_6$, we speculated that MI1 and MI2 are glucuronide conjugates of MI3 and MI4. MI1 was assigned the molecular formula $C_{28}H_{36}N_2O_{11}$ by HRFAB-MS *m/z*: 577.6083 ($[M+H]^+$); Calcd for $[C_{28}H_{36}N_2O_{11}+H]$: 577.6087. The $^1$H-NMR and $^{13}$C-NMR spectra of MI1 and MI2 resembled that of the basic 11-hydroxyisorhynchophylline and 10 -hydroxyisorhynchophylline structures, respectively, except for signals due to the glucuronide moiety. The appreciable correlation between a proton signal at $\delta$ 4.92 (1H, d, $J$ = 7.6 Hz, H-1′) and a carbon signal at $\delta$ 151.3 (C-11) in the HMBC experiment of MI1 indicated that a glucuronic acid residue is attached to C-11 of 11-hydroxyisorhynchophylline. From the coupling constant $J_{1',2'}$ = 7.6 Hz of an anomeric proton of the sugar, we determined the structure of MI1 as 11-hydroxyisorhynchophylline11-*O*-$\beta$-D-glucuronide (Figure 6). Similarly, MI2 was assigned the molecular formula $C_{28}H_{36}N_2O_{11}$ by HRFAB-MS *m/z*: 577.6084 ($[M+H]^+$); Calcd for $[C_{28}H_{36}N_2O_{11}+H]$: 577.6087. The appreciable correlation of a proton signal at $\delta$ 4.89 (1H, d, $J$ = 7.6 Hz, H-1′) and a carbon signal at $\delta$ 149.9 (C-10) in the HMBC experiment of MI2 indicated that a glucuronic acid residue is attached to C-10 of 10-hydroxyisorhynchophylline.

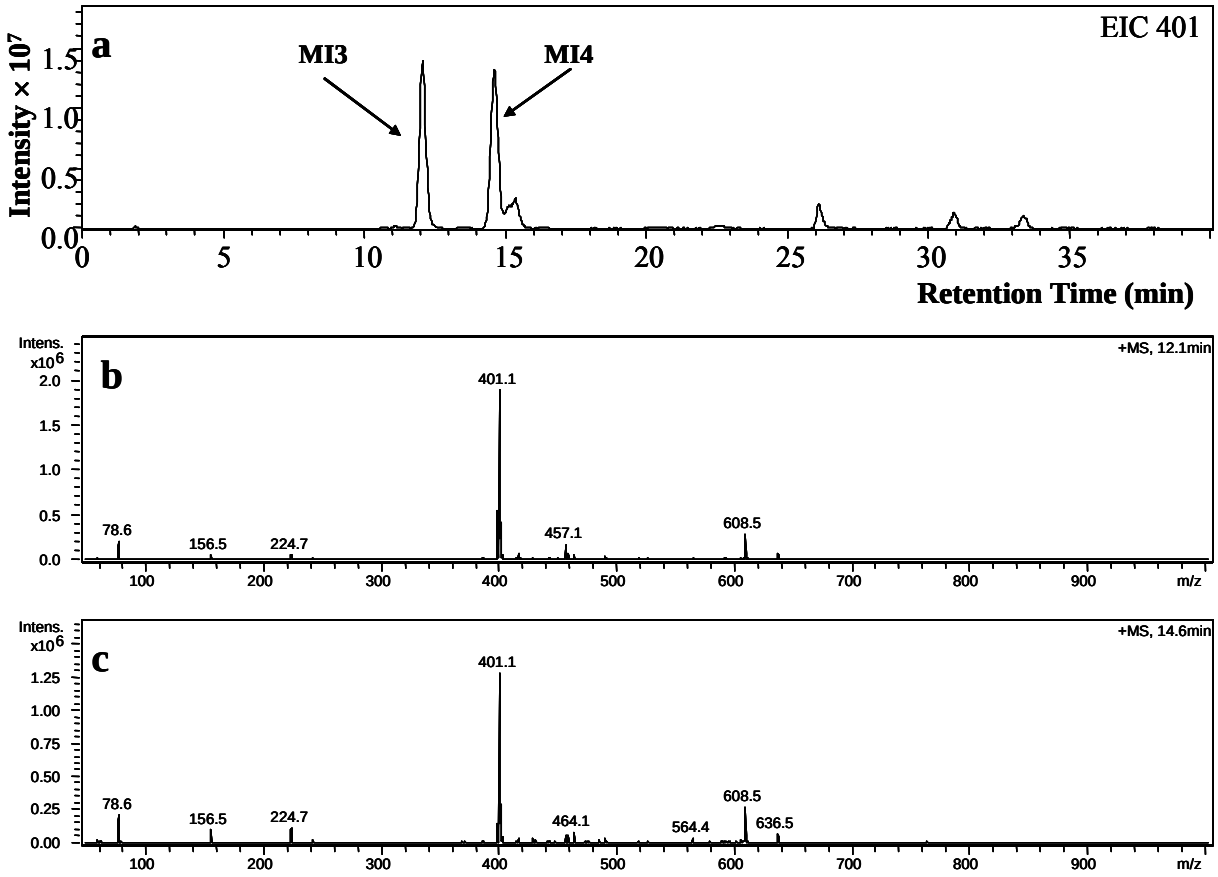


**Figure 4.** LC-MS (EIC) elution profile monitored at *m/z* 401 ± 0.5 (a, MI3 and MI4) in enzyme-treated bile sample collected 2 to 3 h after oral ISOR administration and MS of MI3 (b) and MI4 (c).

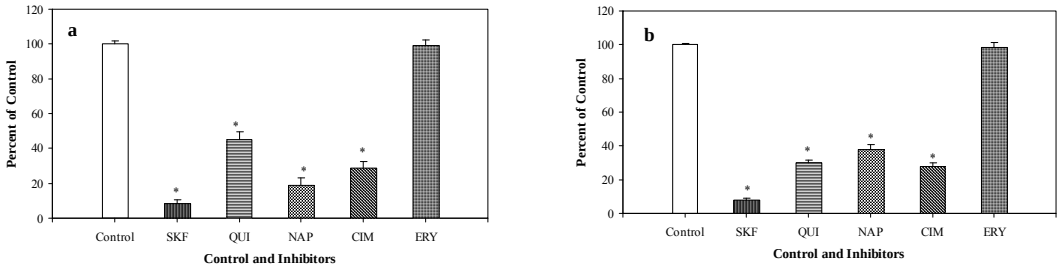


**Figure 5.** Effects of isozyme inhibitors on hydroxylation of ISOR to MI3 (a) and MI4 (b). *Significant value at $p < 0.01$ (Student's *t*-test). SKF (general CYP inhibitor), QUI (CYP2D inhibitor), NAP (CYP1A1/2 inhibitor), CIM (CYP2C inhibitor) and ERY (CYP3A inhibitor).

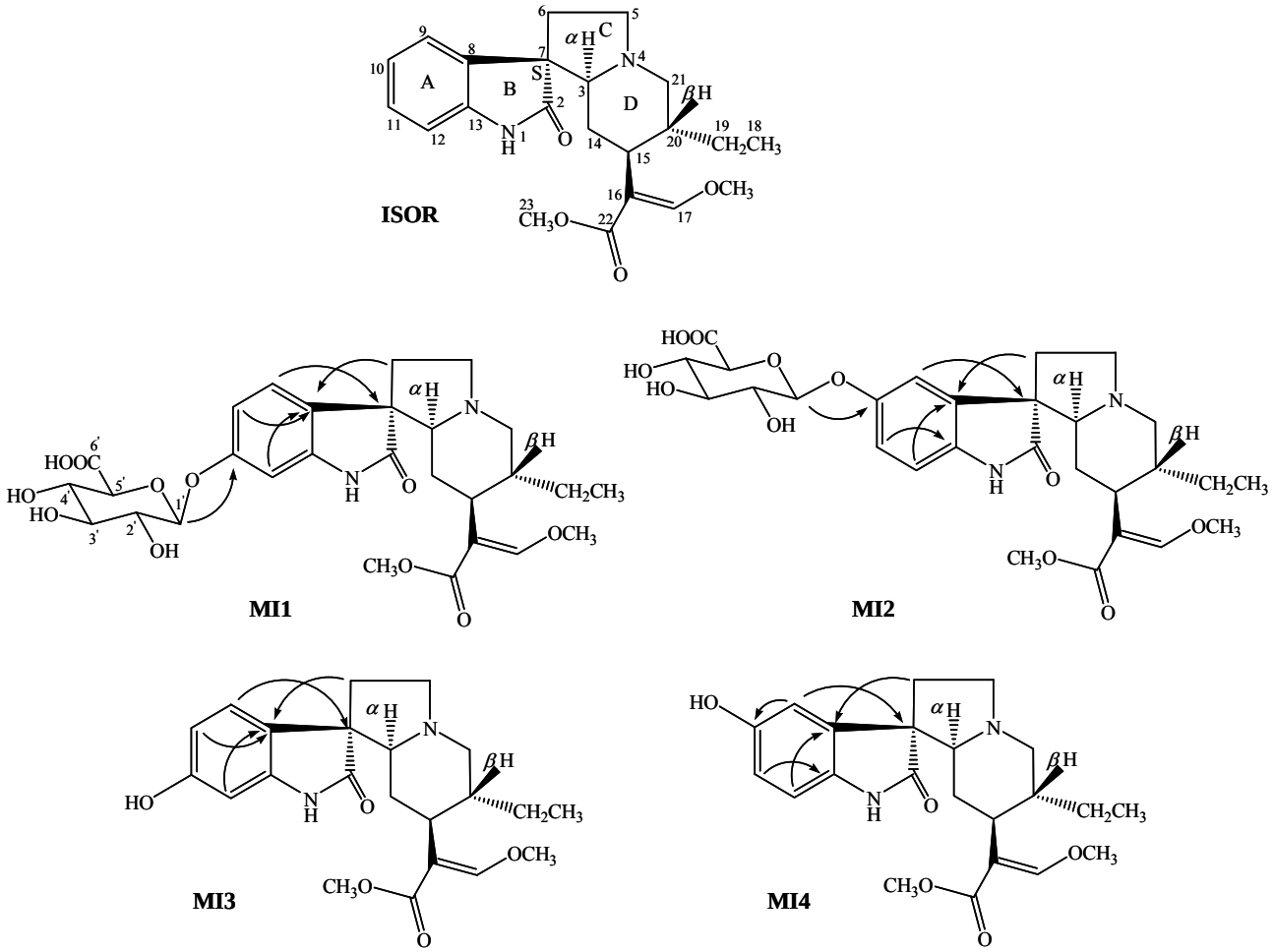


**Figure 6.** Structures of ISOR, MI1, MI2, MI3, and MI4 with key correlations in HMBC (H to C).

Based on the coupling constant $J_{1',2'}$ = 7.6 Hz of an anomeric proton of the sugar, we concluded that MI2 was 10-hydroxyisorhynchophylline 10 -*O*-*β*-D-glucuronide (Figure 6). The absolute configuration of the asymmetric centers at C-3 and C-7 for MI1 to MI4 were assigned to *S* and *S*, respectively, due to a negative Cotton effect (CE) at 283-285 nm, a negative CE at 255-260 nm, and a positive CE at 228-233 nm, which resemble the CD spectrum of ISOR (14). Also the apparent characteristics in NMR of MI1-4 appeared identically with *normal*-type oxindole alkaloids at spiro C-7 (15). The absolute configuration of C-15 of MI1-4 was fixed in *S* form as that of ISOR. From the coupling constant (11.2 Hz) of the proton on C-20 and H-15$\alpha$, C-20 was fixed as *R* form, which is identical to that of ISOR (15).

## CONCLUSIONS

ISOR was detected in rat plasma, bile, urine, and feces after oral administration without being conjugated. No metabolites were detected in rat plasma by LC-MS, whereas 10- and 11-hydroxy isorhynchophyllines were detected in urine and feces. Two metabolites conjugated with *β*-D-glucuronic acid were detected in rat bile. The incubation of ISOR with rat liver microsomes *in vitro* verified that CYP1A1/2, CYP2C, and CYP2D are involved in ISOR hydroxylation in the rat liver.

## ACKNOWLEDGMENTS

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## REFERENCES


1. Laus, G., Keplinger, D. Separation of stereoisomeric oxindole alkaloids from *Uncaria tomentosa* by high performance liquid chromatography. *J. Chromatogr. A*, 662: 243-249, 1994.
2. Kang, T.-H., Murakami, Y., Takayama, H., Kitajima, M., Aimi, N., Watanabe, H., Matsumoto, K. Protective effect of rhynchophylline and isorhynchophylline on in vitro ischemia-induced neuronal damage in the hippocampus: putative neurotransmitter receptors involved in their action. *Life Sci*, 76: 331-343, 2004.
3. Matsumoto, K., Morishige, R., Murakami, Y., Tohda, M., Takayama, H., Sakakibara, I., Watanabe, H. Suppressive effects of isorhynchophylline on 5-$HT_{2A}$ receptor function in the brain: behavioural and electrophysiological studies. *Eur. J. Pharmacol*, 517: 191-199, 2005.
4. Zhang, W.-B., Chen, C.-X., Sim, S.-M., Kwan, C.-Y. In vitro vasodilator mechanisms of the indole alkaloids rhynchophylline and isorhynchophylline, isolated from the hook of *Uncaria rhynchophylla* (Miquel). *Naunyn-Schmiedeberg's Arch. Pharmacol*, 369: 232–238, 2004.
5. Zhang, F., Sun, A.-S., Yu, L.-M., Wu, Q., Gong, Q.-H. Effects of isorhynchophylline on angiotensin II-induced proliferation in rat vascular smooth muscle cells. *J. Pharm. Pharmacol*, 60: 1673-1678, 2008.
6. Le Verge, R., Le Corre, P., Chevanne, F., Döe De Maindreville, M., Royer, D., Levy, J. Determination of yohimbine and its two hydroxylated metabolites in humans by high-performance liquid chromatography and mass spectral analysis. *J. Chromatogr*, 574: 283-292, 1992.
7. Le Corre, P., Dollo, G., Chevanne, F., Le Verge, R. Biopharmaceutics and metabolism of yohimbine in humans. *Eur. J. Pharm. Sci*, 9: 79-84, 1999.
8. Lee, S. K., Kim, G. H., Kim, D. H., Kim, D. H., Jahng, Y., Jeong, T. C. Identification of a tryptanthrin metabolite in rat liver microsomes by liquid chromatography/electrospray ionization-tandem mass spectrometry. *Biol. Pharm. Bull*, 30: 1991-1995, 2007.
9. Nakazawa, T., Banba, K.-i., Hata, K., Nihei, Y., Hoshikawa, A., Ohsawa, K. Metabolites of hirsuteine and hirsutine, the major indole alkaloids of *Uncaria rhynchophylla*, in rats. *Biol. Pharm. Bull*, 29: 1671-1677, 2006.
10. Wang, Y., Wang, S., Liu, Y., Yan, L., Dou, G., Gao, Y. Characterization of metabolites and cytochrome P450 isoforms involved in the microsomal metabolism of aconitine. *J. Chromatogr. B*, 844: 292-300, 2006.
11. Daniel, W. A., Syrek, M., Haduch, A. The contribution of cytochrome P-450 isoenzymes to the metabolism of phenothiazine neuroleptics. *Eur. Neuropsychopharmacol*, 12: 371-377, 2002.
12. Ghosal, A., Yuan, Y., Hapangama, N., Su, A. D. (I.), Alvarez, N., Chowdhury, S. K., Alton, K. B., Patrick, J. E., Zbaida, S. Identification of human UDP-glucuronosyltransferase enzyme(s) responsible for the glucuronidation of 3-hydroxydesloratadine. *Biopharm. Drug Dispos*, 25: 243-252, 2004.
13. Yu, L., Lu, S., Lin, Y., Zeng, S. Carboxyl-glucuronidation of mitiglinide by human UDP-glucuronosyltransferases. *Biochem. Pharmacol*, 73: 1842-1851, 2007.
14. Yuan, D., Ma, B., Wu, C., Yang, J., Zhang, L., Liu, S., Wu, L., Kano, Y. Alkaloids from the leaves of *Uncaria rhynchophylla* and their inhibitory activity on NO production in lipopolysaccharide-activated microglia. *J. Nat. Prod*, 71: 1271-1274, 2008.
15. Seki, H., Takayama, H., Aimi, N., Sakai, S.-i., Ponglux, D. A nuclear magnetic resonance study on the eleven stereoisomers of heteryohimbine-type oxindole alkaloids. *Chem. Pharm. Bull*, 41: 2077-2086, 1993.


## APPENDIX

The NMR data for the metabolites are provided here.

### Metabolite MI1 (11-Hydroxyisorhynchophylline 11-*O*-$\beta$-D-Glucuronide)

White amorphous solid, mp 276-280 °C. $[\alpha]_D^{22}$ +12.3° (*c* = 0.42, MeOH). CD $\Delta\varepsilon$ (nm): -1.2 (285), -9.3 (255), +10.2 (233). HRFAB-MS *m/z*: 577.6083 ($[M+H]^+$, Calcd for $[C_{28}H_{36}N_2O_{11}+H]$: 577.6087). ESI-MS *m/z*: 577 ($[M+H]^+$). $^1$H-NMR ($CD_3OD$) $\delta$: 0.62 (1H, br m, H-14$\beta$), 0.90 (3H, t, H-18), 1.82 (2H, m, H-19), 2.10 (1H, br m, H-21$\alpha$), 2.12 (1H, m, H-6$\alpha$), 2.15 (1H, br m, H-14$\alpha$), 2.22 (1H, br m, $J_{20,15}$ = 11.2 Hz, $J_{20,21\alpha}$ = 11.2 Hz, $J_{20,21\beta}$ = 3.2 Hz, H-20), 2.36 (1H, br m, $J_{15,14\beta}$ = 11.2 Hz, $J_{15,20}$ = 11.2 Hz, $J_{15,14\alpha}$ = 3.2 Hz, H-15), 2.46 (1H, m, H-6$\beta$), 2.57 (1H, m, H-5$\alpha$), 2.65 (1H, m, H-3), 3.16 (1H, m, H-21$\beta$), 3.18 (1H, m, H-5$\beta$), 3.28 (1H, m, H-2′), 3.41 (1H, m, H-3′), 3.42 (1H, m, H-4′), 3.69 (1H, d, $J_{5',4'}$ = 7.6 Hz, H-5′), 3.70 (3H, s, H-23), 3.75 (3H, s, O$\underline{CH}_3$), 4.92 (1H, d, $J_{1',2'}$ = 7.6 Hz, H-1′), 6.89 (1H, d, $J_{12,10}$ = 1.2 Hz, H-12), 7.09 (1H, dd, $J_{10,9}$ = 8.0 Hz, $J_{10,12}$ = 1.2 Hz, H-10), 7.35 (1H, s, H-17), 7.43 (1H, d, $J_{9,10}$ = 8.0 Hz, H-9).

$^{13}$C-NMR ($CD_3OD$) $\delta$: 11.8 (C-18), 24.3 (C-19), 28.8 (C-14), 35.7 (C-6), 37.3 (C-20), 39.3 (C-15), 50.4 (C-23), 55.6 (C-7), 56.5 (C-5), 57.5 (C-21), 63.3 ($O\underline{C}H_3$), 71.8 (C-3), 72.3 (C-4′), 75.5 (C-2′), 78.3 (C-5′), 79.0 (C-3′), 99.8 (C-12), 100.6 (C-1′), 111.3 (C-10), 113.8 (C-16), 126.7 (C-9), 130.1 (C-8), 140.6 (C-13), 151.3 (C-11), 159.6 (C-17), 169.3 (C-22), 177.0 (C-6′), 182.6 (C-2).

## Metabolite MI2 (10-Hydroxyisorhynchophylline 10-*O*-$\beta$-D-Glucuronide)

White amorphous solid, mp 273-278 °C. $[\alpha]_D^{22}$ +9.6° (*c* = 0.68, MeOH). CD $\Delta\varepsilon$ (nm): -2.1 (283), -7.2 (260), +9.1 (230). HRFAB-MS *m/z*: 577.6084 ($[M+H]^+$, Calcd for $[C_{28}H_{36}N_2O_{11}+H]$: 577.6087). ESI-MS *m/z*: 577 ($[M+H]^+$). $^1$H-NMR ($CD_3OD$) $\delta$: 0.51 (1H, br m, H-14$\beta$), 1.02 (3H, t, H-18), 1.79 (2H, m, H-19), 1.95 (1H, br m, H-21$\alpha$), 2.02 (1H, m, H-6$\alpha$), 2.19 (1H, br m, H-14$\alpha$), 2.29 (1H, br m, $J_{20,15}$ = 11.2 Hz, $J_{20,21\alpha}$ = 11.2 Hz, $J_{20,21\beta}$ = 3.2 Hz, H-20), 2.35 (1H, br m, $J_{15,14\beta}$ = 11.2 Hz, $J_{15,20}$ = 11.2 Hz, $J_{15,14\alpha}$ = 3.2 Hz, H-15), 2.41 (1H, m, H-6$\beta$), 2.46 (1H, m, H-5$\alpha$), 2.58 (1H, m, H-3), 3.11 (1H, m, H-21$\beta$), 3.20 (1H, m, H-5$\beta$), 3.27 (1H, m, H-2′), 3.40 (1H, m, H-3′), 3.46 (1H, m, H-4′), 3.65 (1H, d, $J_{5',4'}$ = 7.6 Hz, H-5′), 3.66 (3H, s, H-23), 3.70 (3H, s, $OC\underline{H}_3$), 4.89 (1H, d, $J_{1',2'}$ = 7.6 Hz, H-1′), 6.72 (1H, d, $J_{12,11}$ = 7.6 Hz, H-12), 7.10 (1H, dd, $J_{11,9}$ = 1.2 Hz, $J_{11,12}$ = 7.6 Hz, H-11), 7.36 (1H, s, H-17), 7.46 (1H, d, $J_{9,11}$ = 1.2 Hz, H-9). $^{13}$C-NMR ($CD_3OD$) $\delta$: 11.6 (C-18), 24.1 (C-19), 28.9 (C-14), 35.6 (C-6), 37.5 (C-20), 39.1 (C-15), 50.3 (C-23), 55.7 (C-7), 56.0 (C-5), 57.3 (C-21), 63.3 ($O\underline{C}H_3$), 71.3 (C-3), 71.8 (C-4′), 75.2 (C-2′), 78.5 (C-5′), 79.1 (C-3′), 100.4 (C-1′), 109.7 (C-12), 112.7 (C-16), 116.3 (C-9), 119.4 (C-11), 134.1 (C-13), 134.4 (C-8), 149.9 (C-10), 159.1 (C-17), 169.4 (C-22), 176.3 (C-6′), 182.2 (C-2).

## Metabolite MI3 (11-Hydroxyisorhynchophylline)

Obtained as a white amorphous solid, mp 231-234 °C. $[\alpha]_D^{20}$ +112.6° (*c* = 0.62, MeOH). CD $\Delta\varepsilon$ (nm): -1.7 (285), -6.3 (258), +7.6 (228). HRFAB-MS *m/z*: 401.4825 ($[M+H]^+$, Calcd for $[C_{22}H_{28}N_2O_5+H]$: 401.4827). ESI-MS *m/z*: 401 ($[M+H]^+$). $^1$H-NMR ($CD_3OD$) $\delta$: 0.61 (1H, br m, H-14$\beta$), 0.87 (3H, t, H-18), 1.80 (2H, m, H-19), 2.08 (1H, br m, H-21$\alpha$), 2.10 (1H, br m, H-6$\alpha$), 2.11 (1H, m, H-14$\alpha$), 2.12 (1H, br m, $J_{20,15}$ = 11.2 Hz, $J_{20,21\alpha}$ = 11.2 Hz, $J_{20,21\beta}$ = 3.2 Hz, H-20), 2.32 (1H, br m, $J_{15,14\beta}$ = 11.2 Hz, $J_{15,20}$ = 11.2 Hz, $J_{15,14\alpha}$ = 3.2 Hz, H-15), 2.43 (1H, m, H-6$\beta$), 2.55 (1H, m, H-5$\alpha$), 2.61 (1H, m, H-3), 3.11 (1H, m, H-21$\beta$), 3.17 (1H, m, H-5$\beta$), 3.68 (3H, s, H-23), 3.78 (3H, s, $OC\underline{H}_3$), 6.85 (1H, d, $J_{12,10}$ = 1.2 Hz, H-12), 7.06 (1H, dd, $J_{10,9}$ = 8.0 Hz, $J_{10,12}$ = 1.2 Hz, H-10), 7.33 (1H, s, H-17), 7.45 (1H, d, $J_{9,10}$ = 8.0 Hz, H-9). $^{13}$C-NMR ($CD_3OD$) $\delta$: 11.7 (C-18), 24.2 (C-19), 28.9 (C-14), 35.5 (C-6), 37.3 (C-20), 39.2 (C-15), 50.3 (C-23), 55.7 (C-7), 56.3 (C-5), 57.3 (C-21), 63.5 ($O\underline{C}H_3$), 72.0 (C-3), 99.5 (C-12), 111.1 (C-10), 113.6 (C-16), 126.8 (C-9), 130.0 (C-8), 140.7 (C-13), 151.1 (C-11), 159.2 (C-17), 169.2 (C-22), 182.3 (C-2).

## Metabolite MI4 (10-Hydroxyisorhynchophylline)

Obtained as a white amorphous solid, mp 227-231 °C. $[\alpha]_D^{20}$ +103.8° (*c* = 0.76, MeOH). CD $\Delta\varepsilon$ (nm): -1.4 (284), -5.9 (257), +8.1 (228). HRFAB-MS *m/z*: 401.4826 ($[M+H]^+$, Calcd for $[C_{22}H_{28}N_2O_5+H]$: 401.4827. ESI-MS *m/z*: 401 ($[M+H]^+$). $^1$H-NMR ($CD_3OD$) $\delta$: 0.53 (1H, br m, H-14$\beta$), 0.97 (3H, t, H-18), 1.73 (2H, m, H-19), 1.91 (1H, br m, H-21$\alpha$), 2.01 (1H, m, H-6$\alpha$), 2.11 (1H, br m, H-14$\alpha$), 2.19 (1H, br m, $J_{20,15}$ = 11.2 Hz, $J_{20,21\alpha}$ = 11.2 Hz, $J_{20,21\beta}$ = 3.2 Hz, H-20), 2.32 (1H, br m, $J_{15,14\beta}$ = 11.2 Hz, $J_{15,20}$ = 11.2 Hz, $J_{15,14\alpha}$ = 3.2 Hz, H-15), 2.37 (1H, m, H-6$\beta$), 2.50 (1H, m, H-5$\alpha$), 2.56 (1H, m, H-3), 3.16 (1H, m, H-21$\beta$), 3.20 (1H, m, H-5$\beta$), 3.62 (3H, s, H-23), 3.75 (3H, s, $OC\underline{H}_3$), 6.82 (1H, d, $J_{12,11}$ = 7.6 Hz, H-12), 7.08 (1H, dd, $J_{11,9}$ = 1.2 Hz, $J_{11,12}$ = 7.6 Hz, H-11), 7.37 (1H, s, Hz, H-17), 7.40 (1H, d, $J_{9,11}$ = 1.2 Hz, H-9). $^{13}$C-NMR ($CD_3OD$) $\delta$: 11.7 (C-18), 24.2 (C-19), 28.7 (C-14), 35.6 (C-6), 37.9 (C-20), 39.1 (C-15), 50.1 (C-23), 55.8 (C-7), 56.6 (C-5), 57.1 (C-21), 63.3 ($O\underline{C}H_3$), 71.2 (C-3), 110.1 (C-12), 113.0 (C-16), 116.2 (C-9), 119.0 (C-11), 134.0 (C-13), 134.5 (C-8), 149.6 (C-10), 161.1 (C-17), 169.4 (C-22), 182.3 (C-2).