JPP Journal of Pharmacy and Pharmacology

JPP 2003, 55: 1583–1591

Received March 19, 2003
Accepted July 22, 2003
DOI 10.1211/0022357022188
ISSN 0022-3573

Metabolites of orally administered *Magnolia officinalis* extract in rats and man and its antidepressant-like effects in mice

Takahiro Nakazawa, Takaaki Yasuda and Keisuke Ohsawa

Abstract

As a part of our search for the active metabolite from the bark of *Magnolia officinalis* (Magnoliaceae), the aqueous extract was orally administered to rats, and metabolites in the urine were analysed by a high-performance liquid chromatograph equipped with a photodiode array detector. When the extract was given to rats, five metabolites (sinapic acid-4-*O*-sulfate (**1**), sinapic acid-4-*O*-β-glucuronide (**2**), sinapic acid (**3**), 3-[2′,6-dihydroxy-5′-(2-propenyl)[1,1′-biphenyl]-3-yl]-(*E*)-2-propenoic acid (**4**), and an unchanged form, magnolol (**5**)) were detected in the urine. It was revealed that metabolites **1**–**3** and **4** were respectively derived from syringin and magnolol contained in the extract. In a human urine sample, metabolites **3**–**5** and dihydroxydihydromagnolol (**6**) were detected. These structures were identified by a combination of spectral methods and/or by comparison with authentic compounds obtained by synthesis. Among these free form metabolites (**3**–**6**), acute treatments with magnolol and dihydroxydihydromagnolol (50–100 mg kg^{-1}, i.p.) attenuated the forced swim-induced experimental depression in mice. The results indicated that magnolol and dihydroxydihydromagnolol were the antidepressant constituents of *Magnolia officinalis*.

Introduction

The bark of *Magnolia officinalis* is a useful drug prescribed in Hangekouboku-to, Saiboku-to, and other traditional Chinese medicines, used primarily to treat clinical depression and anxiety-related disorders such as anxiety neurosis and anxiety hysteria (Narita 1990; Ozaki & Shimomura 1993; Yamagiwa 1998). One of the goals in our laboratory is to characterize the antidepressant compounds of *M. officinalis*. Many components such as essential oils (Fujita et al 1973a; Baek et al 1992), neolignans (Fujita et al 1973b; Yahara et al 1991), alkaloids (Tomita et al 1951; Ito & Yoshida 1966) and phenylpropanoids (Hasegawa et al 1988a, b) have been identified in *M. officinalis*. Recent findings demonstrated that Hangekouboku-to, consisting of *M. officinalis*, *Pinellia ternata*, *Perilla frutescens*, *Zingiber officinale*, and *Poria cocos*, showed an antidepressant-like effect in the mouse tail suspension and the forced swimming tests (Luo et al 2000), widely accepted pharmacological models for detecting antidepressant activity (Porsolt et al 1977, 1978). However, the principal active components were still obscure. In most cases, traditional herbal medicines are prepared by extraction with hot water and are orally administered, so that the components present in the aqueous extract may be metabolized by the gut flora before being absorbed into the body. Accordingly, to evaluate the activity of the herbal medicines, investigation of the compounds actually absorbed into the body is necessary. It is well known that some metabolites e.g. paeonimetabolin-I, the metabolite of paeoniflorin (Abdel-Hafez et al 1998), are related to the pharmacological effects of herbal medicines (Yamauchi et al 1993; Nose et al 1994; Hasegawa & Uchiyama 1998; Kim et al 2000). We therefore speculated that the activity of *M. officinalis* might be induced by the active metabolites.

In this study, we have described the structure determination of the metabolites after oral administration of the extracts of *M. officinalis* in rats and man, and have

Department of Phytochemistry, Tohoku Pharmaceutical University, 4-1 Komatsushima 4-chome, Aoba-ku, Sendai, Miyagi 981-8558, Japan

Takahiro Nakazawa, Takaaki Yasuda, Keisuke Ohsawa

Correspondence: K. Ohsawa, Department of Phytochemistry, Tohoku Pharmaceutical University, 4-1 Komatsushima 4-chome, Aoba-ku, Sendai, Miyagi 981-8558, Japan. E-mail: ohsawa@tohoku-pharm.ac.jp

assessed the antidepressant-like activity of the metabolites in the mouse forced swimming test.

Materials and Methods

Apparatus

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. IR spectra were measured with a Perkin Elmer FT-IR1725X spectrometer. Optical rotations were in methanol using a Jasco DIP-360 digital polarimeter (cell length 50 mm). NMR spectra were recorded on a JEOL JNM-EX 400 (^{1}H 400, ^{13}C 100 MHz) spectrometer. Chemical shifts were given in δ value (ppm) downfield relative to tetramethylsilane. Electron impact (EI) and FABMS were measured with a JEOL JMS-DX 303 mass spectrometer. The HPLC system comprised a CCPM pump, CO-8010 column oven (Tosoh, Tokyo, Japan) and model MCPD-3600 photodiode array detector (Otsuka, Osaka, Japan).

Reagents

The bark of *M. officinalis* was commercially obtained from the Japanese market, Matuura Kampo Co., Ltd (Nagoya, Japan). Sinapic acid and β-glucuronidase Type H-2 were purchased from Sigma (St Louis, MO). For column chromatography, Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden) and Wakogel C-200 (Wako Pure Chemical Industries Ltd, Osaka, Japan) were used. Magnolol and honokiol were isolated from *M. officinalis* as reported by Yahara et al (1991). Syringin, magnoloside A, and magnoloside B were isolated from aqueous extract of *M. officinalis* by a combination of column chromatography with Sephadex LH-20 (H_2O/MeOH) and prep. HPLC. Racemic dihydroxydihydromagnolol ($[a]_D^{20}$ 0.40 (c = 1.0, MeOH)) was synthesized by oxidation of magnolol in acetone–H_2O (1:1) with osmium tetroxide and N-methylmorpholine-N-oxid e $2H_2O$ (VanRheenen et al 1976). The identity of the compounds were confirmed by MS, ^{1}H, and ^{13}C NMR spectroscopies before use (Gewali et al 1988; Hasegawa et al 1988a, b; Yahara et al 1991). The purity of each compound was verified by HPLC (> 98%).

HPLC conditions

For qualitative and quantitative analysis, the HPLC conditions were as follows: column, TSK gel ODS-120T (Tosoh, Tokyo, 250 × 4.6 mm i.d.); column temperature, 40 °C; flow rate, 1 mL min^{-1}; detection, by UV at 250 nm (magnolol and dihydroxydihydromag nolol), 270 nm (syringin, magnoloside A and B), 290 nm (honokiol), 295 nm (randainic acid), 340 nm (sinapic acid, and its sulfate and glucuronide). The mobile phase was a gradient system with 0.1% trifluoroacetic acid in H_2O (A) and CH_3CN (B). The gradient systems of qualitative and quantitative analysis were A/B = 100/0 (0 min) 80/20 (40 min) → 40/60 (50 min) → 80/20 (80 min) or A/B = 100/0 (0 min) → 0/100 (60 min).

Animals

Male Sprague-Dawley rats (200–250 g) and ddY mice (26–28 g) were purchased from Japan SLC, Inc. These animals were housed in a temperature- (22 ± 2 °C), humidity- (55 ± 10%), and light- (0800–2000 h) controlled room with free access to distilled water and commercial rodent chow (CE-2, Clea Japan Inc., Tokyo, Japan).

Drug administration

Rats

After seven days of feeding, food was withheld for 18 h, and thereafter a suspension of the aqueous extract and syringin in H_2O (1 g kg^{-1}, 17 mg kg^{-1}) and of magnolol (8 mg kg^{-1}) in propylene glycol were orally administered by direct stomach intubation at a constant volume of 5 mL kg^{-1} body weight. The animals were allowed free access to water and sugar during the experiments.

Mice

Sinapic and randainic acids, magnolol, and dihydroxydihydromagnolol were uniformly dispersed in Tween 80 and then dissolved in distilled water. All drugs were administered in an injection volume of 4 mL kg^{-1} (i.p.); 1% Tween 80 alone was administered as a vehicle. All behavioural experiments were carried out between 1300 h and 1500 h. Experimental procedures were conducted according to protocols approved by the Committee of Animal Experiments of Tohoku Pharmaceutical University and in accordance with the ethical guidelines of the Japanese Pharmacological Society.

Preparation of aqueous extract

The bark of *M. officinalis* (100 g) was added to 2 L distilled water, and the entire mixture was boiled until the volume decreased to half of the original volume. The extract was then filtered through five layers of gauze to the filtrate, which was freeze-dried. Two hundred milligrams of the freeze-dried extract corresponded to 1.0 g of the herbal medicine. This freeze-dried extract (9.0 g) was stored at −20 °C until used.

Major metabolites of urine sample

The urine samples were collected for 48 h at 12 h intervals after the oral administration of the aqueous extract (1.0 g kg^{-1}), syringin (17 mg kg^{-1}) and magnolol (8 mg kg^{-1}) to rats, using a metabolic cage. Each urine sample was filtered through a 0.45-μm membrane filter, and then 20 μL of the sample was injected into the HPLC.

Minor metabolites of urine sample

The urine samples collected as described above were extracted three times with equal volumes of ethyl acetate. The organic layer was combined and then evaporated to dryness at 40 °C. The residue was dissolved in 1 mL methanol. A 20-μL sample was injected into the HPLC.

Enzymatic hydrolysis of urine sample

The urine sample was transferred to a test tube to which was added 5.0 mL 0.2 M sodium acetate buffer (pH 5.5) and 50 μL β-glucuronidase solution followed by incubation at 37 °C for 24 h. The incubated solution was extracted three times with ethyl acetate (20 mL). The organic layer was then evaporated to dryness at 40 °C. The residue was dissolved in 1 mL methanol. A 20-μL sample was injected into the HPLC.

Human urine sample

A healthy male volunteer from this college took part in the testing program. The volunteer (29-years old, 63 kg) was given the aqueous extract (2 g) after obtaining informed consent to participate. Urine samples (1.2 L) were collected for 24 h after administration. The samples were treated in a manner similar to that described above. Informed consent was obtained from the volunteer according to the ethical guidelines of the Helsinki Declaration.

Isolation of urinary metabolites

For the isolation of urinary metabolites, rat urine (approximately 400 mL) was collected as described above after the aqueous extract administration. The urine sample was successively subjected to Sephadex LH-20 with H_2O and MeOH as eluents, the H_2O eluate fraction containing sinapic acid-4-*O*-sulfate (**1**) and sinapic acid-4-*O*-β-glucuronide (**2**) was lyophilized and the MeOH fractions containing sinapic acid (**3**) were evaporated to dryness under reduced pressure at 40 °C. Each fraction was further purified by a combination of column chromatography with Sephadex LH-20 (H_2O) and prep. HPLC, giving **1** (6 mg), **2** (4 mg), and **3** (2 mg). For isolation of metabolites 3-[2′,6-dihydroxy-5′-(2-propenyl)[1,1′-biphenyl]-3-yl]-(*E*)-2-propenoic acid (**4**) and magnolol (**5**), a urine sample (640 mL) incubated with β-glucuronidase was extracted with ethyl acetate (1000 mL) three times. The organic layer was evaporated to dryness at 40 °C. The residue was dissolved in a small amount of methanol and chromatographed on Sephadex LH-20 with 50% methanol as the eluant. The fractions containing metabolites were subjected to prep. HPLC under the above described conditions. Each metabolite fraction was evaporated to dryness at 40 °C in-vacuo to afford **4** (3 mg) and **5** (5 mg).

1 *(sinapic acid-4-*O*-sulfate)*
Colourless amorphous powder; mp 180 °C; IR (KBr) ν max 1673, 1639, 1500, 1048, 994 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ3.73 (6H, s, 3,5-OC$\underline{H}_3$), 6.46 (1H, d, J = 15.1 Hz, H-8), 6.84 (2H, s, H-2,6), 7.31 (1H, d, J = 15.1 Hz, H-7); ^{13}C NMR(DMSO-d_6) δ 56.1 (-O$\underline{C}$H$_3$), 105.4 (C-2, 6), 123.8(C-8), 131.4 (C-1), 132.8 (C-4), 140.6 (C-7), 153.9 (C-3, 5), 170.4 (C-9); negative-ion FABMS m/z 325[M–H + Na]$^-$, 303[M–H]$^-$, 223[M–H–SO$_3$]$^-$; negative-ion HRFABMS m/z 303.0115, calcd for $C_{11}H_{11}O_8S$, 303.0174.

2 *(sinapic acid-4-*O*-β-glucuronide)*
Colourless amorphous powder; mp 208–210 °C(dec.); IR (KBr) ν max 1690, 1620, 1508, 979, 834 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.21–3.41 (4H, m, H-2′–5′), 3.85 (6H, s, 3,5-OC$\underline{H}_3$), 4.94 (1H, d, J = 7.1 Hz, H-1′), 6.53 (1H, d, J = 15.6 Hz, H-8), 7.23(2H, s, H-2,6), 7.37(1H, d, J = 15.6 Hz, H-7); ^{13}C NMR (DMSO-d_6) δ56.9 (-O$\underline{C}$H$_3$), 71.8 (C-4′), 73.0 (C-2′), 73.6 (C-5′), 76.6 (C-3′), 102.7 (C-1′), 106.5 (C-2, 6), 126.4 (C-8), 128.3 (C-1), 135.7 (C-4), 141.7 (C-7), 152.6 (C-3,5), 169.7 (C-9), 172.9 (C-6′); negative-ion FABMS m/z 421[M–H+Na]$^-$, 399[M–H]$^-$, 223[M–H–GlcUA]$^-$; negative-ion HRFABMS m/z 399.1801, calcd for $C_{17}H_{19}O_{11}$, 399.0927.

3 *(sinapic acid)*
The mp and ^{13}C NMR spectra were identical to those reported (Kung & Huang 1949; Fujita et al 1984).

4 *(3-[2′,6-dihydroxy-5′-(2-propenyl)[1,1′-biphenyl]-3-yl]-(*E*)-2-propenoic acid; randainic acid)*
Peel yellow powder; mp 130–131 °C; IR (KBr) ν max 3500–3330, 1671, 1601, 1499, 825 cm^{-1}; ^{1}H NMR (CD_3OD) δ 3.34 (2H, d, J = 6.8 Hz, H-7′), 5.02 (1H, brd, J = 10.0 Hz, H-9′), 5.08 (1H, brd, J = 18.5 Hz, H-9′), 5.98 (1H, ddt, J = 18.5, 10.0, 6.8 Hz, H-8′), 6.33 (1H, d, J = 15.9 Hz, H-8), 6.83 (1H, d, J = 8.1 Hz, H-5′), 6.93 (1H, d, J = 8.3 Hz, H-5), 7.04 (1H, d, J = 8.1 Hz, H-6′), 7.05 (1H, s, H-2′), 7.42 (1H, d, J = 2.2 Hz, H-2), 7.46 (1H, dd, J = 8.3, 2.2 Hz, H-6), 7.58 (1H, d, J = 15.9 Hz, H-7); ^{13}C NMR (CD_3OD) δ 40.4 (C-7′), 115.6 (C-9′), 117.0 (C-5′), 117.3 (C-5), 117.9 (C-8), 126.8 (C-3), 127.3 (C-3′), 128.3 (C-1), 129.5 (C-6), 130.1(C-6′), 132.7 (C-2′), 133.0 (C-2), 133.1 (C-1′) 139.5 (C-8′), 145.3 (C-7), 153.6 (C-4′), 157.8 (C-4), 172.5 (C-9); HREIMS m/z 296.1068, calcd for $C_{18}H_{16}O_4$, 296.1049.

5 *(magnolol)*
The mp, ^{1}H and ^{13}C NMR spectra were identical to those reported (Fujita et al 1973b; Yahara et al 1991).

Synthesis of randainic acid

3-Bromo-4-methoxymethylcinnamic acid methoxy methyl ether
A mixture of 3-bromo-4-hydroxybenzaldehyde (2.5 g, 12.5 mmol), malonic acid (2.6 g, 25 mmol), pyridine (5.0 mL), and piperidine (0.05 mL) was irradiated for 1 min in a microwave oven. The mixture was cooled. The excess pyridine was neutralized by the addition of 3 M HCl. The precipitated acid was filtered and washed with cold water. The product (2.5 g) was dissolved in dimethylformamide (40 mL) and to this was added 21 g K_2CO_3. The mixture was stirred at room temperature for 15 min, and chloromethylmethyl ether (MOMCl; 11 mL, 129 mmol) was added dropwise over 5 min. The mixture was stirred and refluxed for 2 h. After removal of the solvent, the crude product was purified by column chromatography (n-hexane/ethyl acetate 9:1) to afford 3-bromo-4-methoxymethylcinnamic acid methoxy methyl ether (white solid, 3.02 g, 74%).

3-Allyl-6-methoxymethylphenylboronic acid

K_2CO_3 (13.7 g) was added to a stirred solution of 2,4-dibromophenol (25.0 g, 99.2 mmol) in 270 mL acetone. The mixture was stirred at room temperature for 15 min, and MOMCl (10.7 mL, 141 mmol) was added dropwise over 5 min. The mixture was stirred and refluxed for 2 h. The solvent was evaporated in-vacuo. Crude products were dissolved in water and then extracted with ether. The extract was dried (Na_2SO_4), the ether was distilled off, the residue was purified by column chromatography (n-hexane/ethyl acetate 17:1) to afford 2,4-dibromophenol methoxy methyl ether (colourless oil, 24.3 g, 82.3%). A stirred Grignard reagent of 2,4-dibromophenol methoxy methyl ether was prepared with 2,4-dibromophenol methoxy methyl ether (24.3 g, 82.1 mmol) and magnesium turnings (3.00 g) in dry tetrahydrofuran (THF; 450 mL), and allyl bromide (14.6 g, 121 mmol) was added dropwise at room temperature over 30 min. The reaction mixture was refluxed for 3 h. The THF was distilled off, and the residue was flash chromatographed using n-hexane and ethyl acetate (100:2, v/v) as eluent. The colourless oil, 2-bromo-4-allylphenol methoxy methyl ether (13.68 g), was obtained in 53% yield. To a solution of the oil (3.10 g, 12.1 mmol) and tetramethylethylenediamine (1.82 g, 6.56 mmol) in anhydrous ether (23.9 mL), BuLi (1.6 M, 7.56 mL) was added dropwise at −78 °C under nitrogen. The organic solution was stirred at −78 °C under nitrogen for 1 h. Trimethyl borate (1.89 g, 181 mmol) was added to the lithio reagent. The mixture was stirred at −78 °C for 1 h, followed by stirring at room temperature for 6 h. Dilute HCl was added to the reaction mixture until pH < 2. The mixture was stirred for 30 min and then extracted with ethyl acetate. The solution was dried over Na_2SO_4, and a yellow solid was obtained after removal of the solvent. The crude product was purified by column chromatography (n-hexane/ethyl acetate 15:1) to afford a white solid as 3-allyl-6-methoxymethyl phenylboronic acid (1.18 g, 44.0%).

Randainic acid

3-Bromo-4-methoxymethylcinnamic acid methoxy methyl ether (0.4 g, 1.21 mmol) and $Pd(PPh_3)_4$ (233 mg, 0.20 mmol) were dissolved in dioxane (5.0 mL) under nitrogen. K_2CO_3 (253 mg) and 3-allyl-6-methoxymethylphenylboronic acid (0.51 g, 2.29 mmol) in dioxane (5.0 mL) were added to the solution. The mixture was degassed and refluxed under nitrogen for 12 h. After cooling, the mixture was extracted with ethyl acetate. After removal of the solvent, the product was dissolved in THF (7 mL). HCl 3 M (9 mL) was then added to the solution and refluxed for 3 h. The reaction mixture was extracted with ethyl acetate, and the solution was dried. After removal of the solvent, the crude product was purified by column chromatography (n-hexane/ethyl acetate 7:3) to afford randainic acid as a white solid (222 mg, 62%).

Forced swimming test

The procedure was a modification of the method described by Porsolt et al (1977, 1978). Mice were placed in a glass aquarium (15 cm × 12 cm diameter) containing 9-cm deep cold water (23 ± 1 °C) for 15 min followed by a 5-min retest (test session) 24 h later. The immobility time was recorded during the test session: the mouse was considered immobile whenever it stopped swimming and remained floating in the water, with its head just above the water level. Following the test, the animals were dried and returned to their home cage. The vehicle or test drugs were administered 1 h before a test session.

Statistics

The data were analysed using the one-way analysis of variance. If any statistically significant change was found, post-hoc comparisons were performed using the Student–Newman–Keuls test. Data were deemed significant when $P < 0.05$. Results are expressed as mean ± s.e.m.

Results

Identification of the metabolites

Using an HPLC equipped with a photodiode array detector, main metabolites **1**–**3** were detected in urine samples after the oral administration of *M. officinalis* aqueous extract as shown in Figure 1. Analysis of an ethyl acetate extract of the urine sample showed the presence of two minor metabolites **4** and **5** (Figure 2). Metabolite **1** was obtained as an amorphous powder, and the molecular formula was determined to be $C_{11}H_{11}O_8S$ based on a

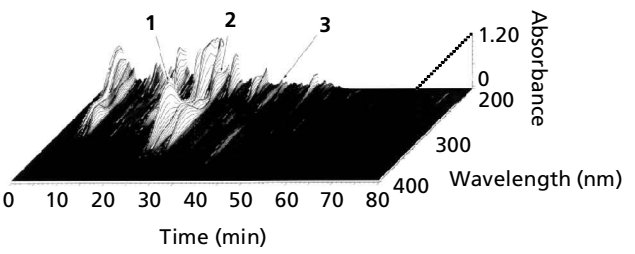

Figure 1 HPLC chromatogram of a urine sample following the oral administration of an aqueous extract of *Magnolia officinalis* to rats.

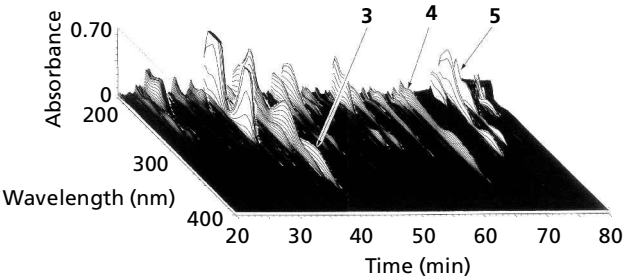

Figure 2 HPLC chromatogram of ethyl acetate extract of a urine sample following the oral administration of an aqueous extract of *Magnolia officinalis* to rats.

negative HRFABMS. The IR spectrum of **1** showed absorption bands at 1673 and 1639 cm^{-1} due to the α, β-unsaturated carboxylic function, and the intense absorption at 1048 cm^{-1} suggested a sulfate-conjugated structure for **1**. The ^{1}H and ^{13}C NMR data of **1** were similar to those of sinapic acid. Furthermore, hydrolysis of **2** using 2 M HCl gave a product that was tentatively identified as sinapic acid by comparing its retention time and UV spectrum during HPLC with those of an authentic sample. Comparison of the ^{13}C NMR spectrum of **1** with that of sinapic acid indicated that the C-4 signal of **2** shifted upfield by 5.2 ppm, accompanied by downfield shifts of C-3 and -5 (6.0 ppm), indicating that the sulfate group was situated at C-4. Thus, the structure of **1** was concluded to be sinapic acid-4-*O*-sulfate. Metabolite **2** was obtained as an amorphous powder, and the molecular formula was determined to be $C_{17}H_{19}O_{11}$ based on a negative HRFABMS (*m/z*: 399.91801$(M-H)^{-}$, calcd for $C_{17}H_{19}O_{11}$, 399.0927). The IR spectra of **2** exhibited absorption due to the α,β-unsaturated carboxylic function (1690, 1620 cm^{-1}). The ^{1}H NMR spectra of **2** showed signals due to two methoxy and *trans* double bond signals (δ 3.85 (6H, s, 3,5-OC$\underline{H}_3$), 6.53 (1H, d, $J = 15.6$ Hz), 7.37(1H, d, $J = 15.6$ Hz)), a 2H-singlet at δ 7.23 in the aromatic proton region, and a β-glucuronic acid moiety (3.21–3.41 (4H, m, H-2′–5′), 4.94 (1H, d, $J = 7.1$ Hz, H-1′)). Enzymatic hydrolysis of **2** with β-glucuronidase gave the aglycone, which was assumed to be sinapic acid from the behaviour of the HPLC. Furthermore, the ^{13}C NMR data of **2** was superimposable on that of sinapic acid, except for the signals due to a β-glucuronic acid. The location of the glucuronic acid moiety was found by HMBC between the anomeric proton at δ 4.94 and C-4 at δ 135.7 for the sinapic acid moiety. Thus, **2** was identified as sinapic acid-4-*O*-β-glucuronide. Metabolite **4** was obtained as a peel yellow powder. The HREIMS of **4** showed a molecular ion peak at *m/z* 296.1068, indicating the molecular formula

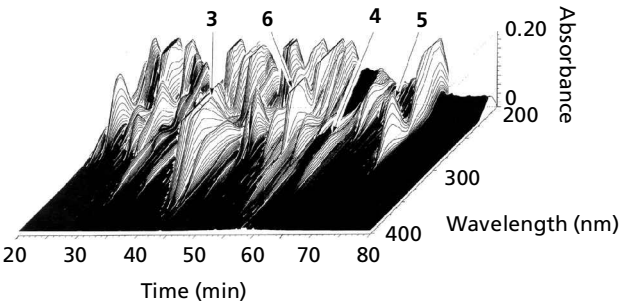

Figure 3 HPLC chromatogram of ethyl acetate extract of a urine sample after enzymatic hydrolysis following the oral administration of an aqueous extract of *Magnolia officinalis* to a volunteer.

to be $C_{18}H_{16}O_4$. The IR spectrum showed the presence of hydroxyl (3500–3330), α,β-unsaturated carboxylic (1671, 1625) and aromatic groups (1601). The ^{13}C NMR spectra of **4** was very similar to that of randainal (2, 2′-dihydroxy-5-allylbiphenyl-5′- propenal) (Chen et al 1983). However, the ^{1}H and ^{13}C NMR spectra suggested the signals were due to a *trans*-disubstituted alkene (δ 6.33 (1H, d, $J = 15.9$ Hz, H-8), and 7.58 (1H, d, $J = 15.9$ Hz, H-7)) and a carboxylic acid (δ 172.5), respectively. Furthermore, the molecular ion peak at *m/z* 296 was 16 mass units greater than that of randainal. Thus, metabolite **4** was determined to be 3-[2′, 6-dihydroxy-5′-(2-propenyl)[1,1′-biphenyl]-3-yl]-(*E*)-2-propenoic acid and was designated as randainic acid. Metabolites **3** and **5** were identified to be sinapic acid and magnolol by direct comparison with authentic samples. For the human urine sample, two metabolites were detected as shown in Figure 3. These metabolites were identified to be dihydroxydihydromagnolol (**6**) and magnolol by comparing their retention times and UV spectra during HPLC with those of authentic and synthesized samples. The chemical structures of these metabolites are shown in Figure 4.

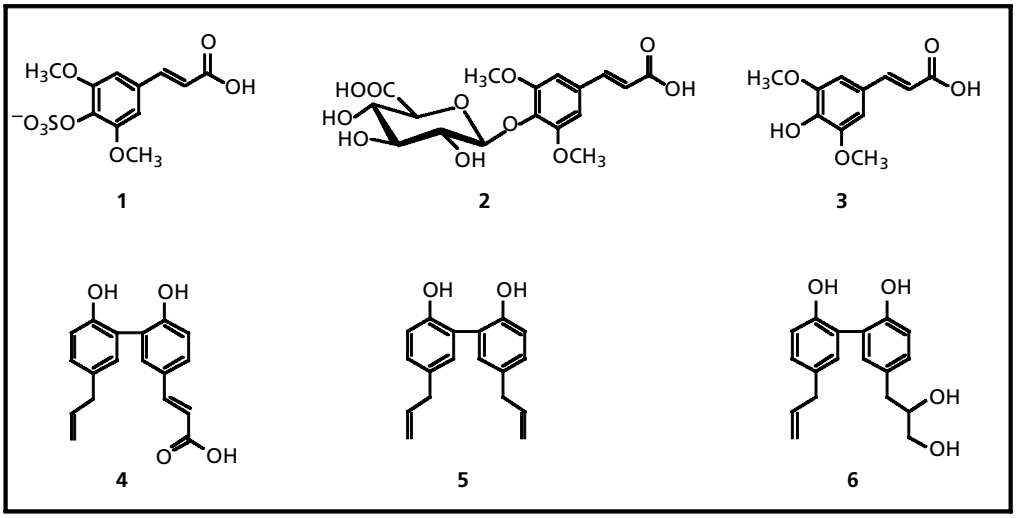

Figure 4 Chemical structures of sinapic acid-4-*O*-sulfate (**1**), sinapic acid-4-*O*-β-glucuronide (**2**), sinapic acid (**3**), 3-[2′,6-dihydroxy-5′-(2-propenyl)[1,1′-biphenyl]-3-yl]-(*E*)-2-propenoic acid (**4**), magnolol (**5**) and dihydroxydihydromagnolol (**6**).

Identification and quantification of the major constituents in the aqueous extract

HPLC analysis detected five major peaks in the aqueous extract of *M. officinalis* (Figure 5). These constituents were identified as syringin, magnoloside A and B, honokiol and magnolol by direct comparison with authentic samples. Table 1 shows the contents of each constituent in the aqueous extract (1 g) and the relative standard deviations in this analysis, respectively.

The metabolites after the administration of syringin and magnolol

When syringin (17 mg kg^{-1}), corresponding to the extract 1 g kg^{-1}, was orally administered to rats, three metabolites were detected in the urine. These metabolites were identified as **1**, **2** and **3** by comparing retention times and UV spectra during HPLC with those of the samples isolated from the rat urine. The total cumulative amounts of **1**–**3** in the urine excreted during 48 h are shown in Table 2. When magnolol 8 mg kg^{-1}, corresponding to the extract 1 g kg^{-1}, was orally administered to rats, two peaks were detected in the urine sample (chromatogram not shown). These constituents were identified to be **4** and **5** by comparing their HPLC behaviour with those of authentic and synthesized samples. The total cumulative amounts of **4** and **5** in the urine and the β-glucuronidase-treated urine sample are shown in Table 3.

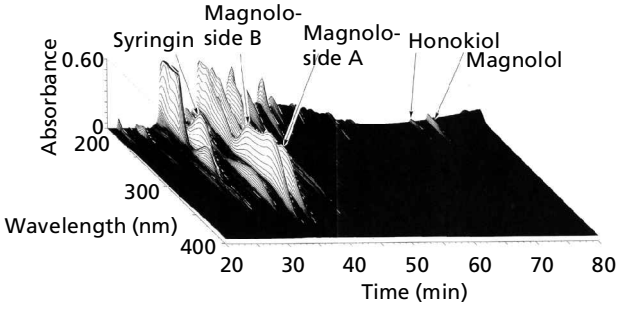

Figure 5 HPLC chromatogram of an aqueous extract of *Magnolia officinalis*.

Table 1 Contents of major components in the aqueous extract of *Magnolia officinalis*.

Component	Content in the aqueous extract	
	mg g^{-1}	r.s.d.%
Syringin	16.98 ± 0.07	0.92
Magnoloside B	36.14 ± 0.20	1.25
Magnoloside A	22.01 ± 0.20	2.03
Honokiol	4.34 ± 0.03	1.37
Magnolol	7.94 ± 0.04	1.16

Data expressed as mean ± s.e.m. (n = 5). r.s.d., relative standard deviation.

Table 2 Urinary excretion of the metabolites in rats after the oral administration of syringin (17 mg kg^{-1}).

Metabolites	% of dose		
	0–12 h	12–48 h	Total
1	12.22 ± 2.68	N.D.	12.22 ± 2.68
2	20.10 ± 2.42	N.D.	20.10 ± 2.42
3	0.72 ± 0.13	N.D.	0.72 ± 0.13
Total	33.04 ± 5.23	0	33.04 ± 5.23

Data expressed as mean ± s.e.m. (n = 5). N.D., not determined.

Antidepressant-like effect of the metabolites

Table 4 shows the effects of desipramine (positive control) or the free-form metabolites **3**–**6**, in acute treatment (12.5–100 mg kg^{-1}, i.p.), on the immobility time in the mouse forced swimming test. There was a significant effect of magnolol (**5**) and dihydroxydihydromagnolol (**6**) treatment on immobility time in the forced swimming test ($F(4, 70) = 8.40$, $P < 0.0001$; $F(4, 70) = 3.68$, $P < 0.01$, respectively). Post-hoc analysis revealed that **5** produced a significant reduction in the immobility time at doses of 50 and 100 mg kg^{-1} ($P < 0.01$) and **6** at doses of 100 mg kg^{-1} ($P < 0.01$). The effects of sinapic acid (**3**) and randainic acid (**4**) treatment at doses from 12.5 to 100 mg kg^{-1} did not reach statistical significance ($F(4, 70) = 0.83$, $P > 0.05$; $F(4, 70) = 1.80$, $P > 0.05$, respectively) under this condition.

Discussion

The bark of *Magnolia officinalis* is one of the most important traditional herbal medicines in China and Japan and is used to treat clinical depression and anxiety-related disorders. Generally, traditional herbal medicines are orally administered as the decoctions (aqueous extracts), and the components in the extracts may be metabolized by the gut flora before being absorbed into the body. Accordingly, to evaluate the bioactive compounds in these drugs, an investigation of the compounds actually absorbed into body is necessary, and knowledge of the metabolism of the compounds may help in understanding the mechanism of action and therapeutic effects of the traditional herbal medicines. We isolated sinapic acid-4-*O*-sulfate (**1**), sinapic acid-4-*O*-β-glucuronide (**2**), sinapic acid (**3**), 3-[2′,6-dihydroxy-5′-(2-propenyl)[1,1′-biphenyl]-3-yl]-(*E*)-2-propenoic acid (**4**), and the unchanged form magnolol (**5**) in the rat urine after the oral administration of an aqueous extract of *M. officinalis*. To identify the origins of the metabolites, we conducted a qualitative and quantitative analysis of the major constituents in the aqueous extract administered to rats. According

Table 3 Urinary excretion of the metabolites in rats after the oral administration of magnolol (8 mg kg^{-1}).

Metabolites		% of dose				
		0–12 h	12–24 h	24–36 h	36–48 h	Total
Free form	**4**	0.17 ± 0.04	0.17 ± 0.01	0.17 ± 0.06	0.15 ± 0.09	0.66 ± 0.19
Conjugated form	**4**	0.19 ± 0.11	0.36 ± 0.13	0.31 ± 0.09	0.24 ± 0.03	1.09 ± 0.36
Total	**4**	0.36 ± 0.15	0.52 ± 0.14	0.49 ± 0.14	0.39 ± 0.12	1.75 ± 0.55
Free form	**5**	1.96 ± 0.42	1.16 ± 0.13	0.81 ± 0.05	0.15 ± 0.02	4.08 ± 0.61
Conjugated form	**5**	0.08 ± 0.02	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.31 ± 0.06
Total	**5**	2.04 ± 0.44	1.23 ± 0.14	0.90 ± 0.06	0.22 ± 0.03	4.39 ± 0.67

Data expressed as mean ± s.e.m. (n = 5).

Table 4 The effects of acute metabolite (**3**–**6**) treatment on immobility time in the mouse forced swimming test.

Compounds	Dose (mg kg^{-1})	Immobility time (s)	*f* value	*P* value	df
Vehicle		217.0 ± 8.2			
Desipramine	25	163.9 ± 11.7**	13.70	0.0009	1, 28
Sinapic acid (**3**)	12.5	200.8 ± 13.0	0.83	0.5090	4, 70
	25	208.3 ± 8.3			
	50	195.2 ± 10.3			
	100	195.2 ± 9.7			
Randainic acid (**4**)	12.5	187.0 ± 9.0	1.80	0.1394	4, 70
	25	193.3 ± 10.7			
	50	188.9 ± 8.6			
	100	179.1 ± 8.9			
Magnolol (**5**)	12.5	209.1 ± 11.0	8.40	0.0001	4, 70
	25	213.3 ± 11.1			
	50	162.1 ± 16.2**			
	100	139.7 ± 12.1**			
Dihydroxydihydromagnolol (**6**)	12.5	185.4 ± 15.0	3.68	0.0088	4, 70
	25	179.7 ± 12.1			
	50	177.7 ± 9.3*			
	100	170.3 ± 11.2**			

Data expressed as means ± s.e.m. (n = 15). *$P < 0.05$, **$P < 0.01$ compared with vehicle (Student–Newman–Keuls test).

to our analysis, the aqueous extract contained substantial amounts of syringin, magnoloside A and B, honokiol and magnolol (Table 1). Yahara et al (1991) isolated sinapic aldehyde and randainal from *M. officinalis*, yielding 0.001% and 0.003%, respectively. However, neither of these compounds nor **3** and **4** were contained in the aqueous extract (detection limited 0.2–0.5 μg mL^{-1}, signal/noise = 5), which suggested that **3** and **4** were the metabolites of the constituents in the extract. When syringin (17 mg kg^{-1}), corresponding to 1-g extract, was given to rats, metabolites **1**–**3** were detected in the urine. Therefore, we concluded that syringin was the most possible precursor of metabolites **1**–**3**. The total cumulative amounts of **1**–**3** in the urine excreted during 12 h corresponded to 33.04 ± 5.23% (mean ± s.e.m., n = 5) of the dose for syringin administration (Table 2). In general, orally administered glycosides are usually absorbed after being hydrolysed by bacterial enzymes to the corresponding aglycones. Kim et al (1999) reported that syringin was converted to its aglycone, synapyl alcohol, by incubation with intestinal bacteria. However, in this study, we could not detect any syringin or synapyl alcohol in the urine after syringin administration (detection limited 0.5 μg mL^{-1}, signal/noise = 5). Thus, in-vivo orally administered syringin might be completely hydrolysed and then oxidized to the corresponding acid, sinapic acid. In contrast, when magnolol 8 mg kg^{-1}, corresponding to 1-g extract, was orally administered to rats, magnolol was detected in the urine sample together with metabolite **4**. According to Hattori et al (1986), after single oral administration of [ring-^{14}C]magnolol to rats, most of the radioactivity was eliminated into the faeces and urine within the first 12 h. Moreover, the single-dosed magnolol was recovered to a greater extent from the faeces (72%) than from the urine (7.4%) in 144 h, while in the case of repeated oral administration the faecal constituents significantly changed, and the five hydrogenated metabolites were detected.

HPLC analysis showed that 4.39 ± 0.67% of orally administered magnolol was excreted in the urine as the free and conjugated form within 48 h. Furthermore, we observed that **4** (1.75 ± 0.55%) was derived from magnolol orally administered in spite of the single dose (Table 3). On the other hand, in the case of man, Homma et al (1992) reported that magnolol was metabolized to dihydroxydihydromagnolol. Our preliminary experiment detected the presence of dihydroxydihydromag nolol (**6**) in addition to **3**–**5** in human urine following administration of a *M. officinalis* extract.

Recent findings demonstrated that when Hangekouboku-to, the aqueous extract consisting of *M. officinalis*, *Pinellia ternata*, *Perilla frutescens*, *Zingiber officinale*, and *Poria cocos* was orally administered to mice for 15 days, they showed an antidepressant-like effect in the forced swimming and tail suspension tests (Luo et al 2000). However, the principal active components were not identified. As described above, we observed that **3**–**6** were detected in the urine following oral administration of *M. officinalis* extract to rats and/or man. Further, **4** and **5**, but not **3** were detected in the urine at least up to 48 h after a single administration of each origin (magnolol and syringin) to rats. We therefore hypothesized that repeated administration of the extracts may be leading to metabolite accumulation and that the antidepressant-like effect observed after chronic administration of the herbal medicine may be induced by the active metabolites. Among four free-form metabolites, **5** (unchanged form), and **6** in acute treatments significantly decreased the duration of immobility in the forced swimming test. Ai et al (2001) showed that magnolol selectively interacted with γ-aminobutyric acid$_A$ (GABA$_A$) receptor subtypes in-vitro. It is known that GABAergic systems were involved in antidepressant action in the forced swimming test (Nakagawa et al 1996). Moreover, clinical and biochemical findings suggested that GABA may play a major role in affective disorders such as depression (Lloyd et al 1983; Suzdak & Gianutsos 1985). We speculate therefore that magnolol (**5**) and its metabolite, dihydroxydihydromagnolol (**6**), are the possible major active principles responsible for the antidepressant effect of *M. officinalis* and that the effect may be related to GABAergic systems. Further studies are necessary to confirm this suggestion.

A traditional phytomedicine "*Magnolia officinalis*" has been used empirically for a long time in the psychiatric field for treatments of depressant disorders. This study, in which experimentally-caused depression in mice was inhibited by acute treatment with magnolol and its metabolite, dihydroxydihydromagnolol, would support clinical application of *M. officinalis* as an antidepressant.

Conclusion

In this study, after the oral administration of an aqueous extract of *M. officinalis* to rats or man, we identified six metabolites in the urine, sinapic acid and its sulfate, glucuronide, randainic acid, magnolol (unchanged form), and dihydroxydihydromag nolol. In addition, among four free-form metabolites, magnolol and dihydroxydihydromagnolol in acute treatments showed antidepressant-like effects in the mouse forced swimming test. These results suggested that magnolol and dihydroxydihydromag nolol were the antidepressant active constituents of *M. officinalis*.

References

Abdel-Hafez, A. A., Meselhy, M. R., Nakamura, N., Hattori, M., Watanabe, H., Mohamed, T. A., Mahfouz, N. M., el-Gendy, M. A. (1998) Potent anticonvulsant paeonimetabolin-I derivatives obtained by incubation of paeoniflorin and thiol compounds with *Lactobacillus brevis*. *Chem. Pharm. Bull.* **46**: 1486–1487

Ai, J., Wang, X., Nielsen, M. (2001) Honokiol and Magnolol selectively interact with GABA$_A$ receptor subtypes in vitro. *Pharmacology* **63**: 34–41

Baek, N. I., Kim, H., Lee, Y. H., Park, J. D., Kang, K. S., Kim, S. I. (1992) A new dehydrodieugenol from *Magnolia officinalis*. *Planta Med.* **58**: 566–568

Chen, F. C., Lee, J. S., Lin, Y. M. (1983) Biphenyls from the heartwood of Taiwan *sassafras*. *Phytochemistry* **22**: 616–617

Fujita, M., Itokawa, H., Sashida, Y. (1973a) Studies on the components of *Magnolia obovata* Thunb. I. On the components of the essential oil of the bark. *Yakugaku Zasshi* **93**: 415–421

Fujita, M., Itokawa, H., Sashida, Y. (1973b) Studies on the components of *Magnolia obovata* Thunb. II. On the components of the methanol extract of the bark. *Yakugaku Zasshi* **93**: 422–428

Fujita, M., Yamada, M., Nakajima, S., Kawai, K., Nagai, M. (1984) *O*-Methylation effect on the carbon-13 nuclear magnetic resonance signals of ortho-disubstituted phenols and its application to structure determination of new phthalides from *Aspergillus silvaticus*. *Chem. Pharm. Bull.* **32**: 2622–2627

Gewali, M. B., Hattori, M., Namba, T. (1988) Constituents of the stem of *Eucommia ulmoides* Oliv. *Shoyakugaku Zasshi* **42**: 247–248

Hasegawa, H., Uchiyama, M. (1998) Antimetastatic efficacy of orally administered ginsenoside Rb1 in dependence on intestinal bacterial hydrolyzing potential and significance of treatment with an active bacterial metabolite. *Planta Med.* **64**: 696–700

Hasegawa, T., Fukuyama, Y., Ymada, T. (1988a) Isolation and structure of Magnoloside A, a new phenylpropanoid glycoside from *Magnolia obovata* Thunb. *Chem. Lett.* 163–166

Hasegawa, T., Fukuyama, Y., Ymada, T., Nakagawa, K. (1988b) Structures of Magnoloside B and C. Novel phenylpropanoid glycoside with allopyranose as the core sugar unit. *Chem. Pharm. Bull.* **36**: 1245–1248

Hattori, M., Endo, Y., Takebe, S., Kobashi, K., Fukasaku, N., Namba, T. (1986) Metabolism of magnolol from Magnoliae cortex. II. Absorption, metabolism and excretion of [ring-^{14}C] magnolol in rats. *Chem. Pharm. Bull.* **34**: 158–167

Homma, M., Oka, K., Yamada, T., Niitsuma. T., Ito, H., Takahashi, N. (1992) A strategy for discovering biologically active compounds with high probability in traditional Chinese herb remedies: an application of saiboku-to in bronchial asthma. *Anal. Biochem.* **202**: 179–187

Ito, K., Yoshida, A. (1966) Studies on the alkaloids of Magnoliaceous plants. XXXVII. Alkaloids of *Magnolia obovata* Thunb. (2). *Yakugaku Zasshi* **86**: 124–126

Kim, D. H., Lee, K. T., Bae, E. A., Han, M. J., Park, H. (1999) Metabolism of liriodendrin and syringin by human intestinal bacteria and their relation to in vitro cytotoxicity. *J. Arch. Pharm. Res.* **22**: 30–34

Kim, D. H., Hong, S. W., Kim, B. T., Bae, E. A., Park, H. Y., Han, M. (2000) Biotransformation of glycyrrhizin by human

intestinal bacteria and its relation to biological activities. *J. Arch. Pharm. Res.* **23**: 172–177

Kung, H. P., Huang, W. Y. (1949) Chemical investigation of Draba nemorosa, L.; the isolation of sinapine iodide. *J. Am. Chem. Soc.* **71**: 1836–1837

Lloyd, K. G., Morselli, P. L., Depoortere, H., Fournier, V., Zivkovic, B., Scatton, B., Broekkamp, C., Worms, P., Bartholini, G. (1983) The potential use of GABA agonists in psychiatric disorders: evidence from studies with progabide in animal models and clinical trials. *Pharm. Biochem. Behav.* **18**: 957–966

Luo, L., Nong, W. J., Kong, L. D., Jiang, Q. G., Tan, R. X. (2000) Antidepressant effects of Banxia Houpu decoction, a traditional Chinese medicinal empirical formula. *J. Ethnopharmacol.* **73**: 277–281

Nakagawa, Y., Ishima, T., Ishibashi, Y., Yoshii, T., Takashima, T. (1996) Involvement of GABA(B) receptor systems in action of antidepressants: baclofen but not bicuculline attenuates the effects of antidepressants on the forced swim test in rats. *Brain Res.* **709**: 215–220

Narita, H. (1990) Kanmpo-therapy for psychiatric region. *Jpn. J. Neuropsychopharmacol.* **12**: 165–172

Nose, M., Ito, M., Kamimura, K., Shimizu, M., Ogihara, Y. (1994) A comparison of the antihepatotoxic activity between glycyrrhizin and glycyrrhetinic acid. *Planta Med.* **60**: 136–139

Ozaki, T., Shimomura, T. (1993) Hangekouboku-to no kouutsu-koukanituite. *Komposinryo* **12**: 21–25

Porsolt, R. D., Brochet, D., Simon, P. (1977) Behavioral despair in mice: a primary screening test for antidepressants. *Arch. Int. Pharm. Ther.* **229**: 327–336

Porsolt, R. D., Anton, G., Blavet, N., Jalfre, M. (1978) Behavioural despair in rats: a new model sensitive to antidepressant treatments. *Eur. J. Pharmacol.* **47**: 379–391

Suzdak, P. D., Gianutsos, G. (1985) Parallel changes in the sensitivity of gamma-aminobutyric acid and noradrenergic receptors following chronic administration of antidepressant and GABAergic drugs. A possible role in affective disorders. *Neuropharmacology* **24**: 217–222

Tomita, M., Inubushi, Y., Yamagata, M. (1951) Alkaloids of *Magnolia obovata* Thunb. *Yakugaku Zasshi* **71**: 1069–1075

VanRheenen, V., Kelly, R. C., Cha, D. Y. (1976) An improved catalytic OsO_4 oxidation of olefins to *cis*-1,2-glycols using tertiary amine oxides as the oxidant. *Tetra. Lett.* **17**: 1973–1976

Yahara, S., Nishiyori, T., Kohda, A., Nohara, T., Nishioka, I. (1991) Isolation and characterization of phenolic compounds from Magnolia Cortex produced in China. *Chem. Pharm. Bull.* **39**: 2024–2036

Yamagiwa, M. (1998) Kouutsuteki Inkoutouijoukansyoukanjya nitaisuru Saiboku-to nokouka. *Kampoigaku* **22**: 19–22

Yamauchi, K., Yagi, T., Kuwano, S. (1993) Suppression of the purgative action of rhein anthrone, the active metabolite of sennosides A and B, by calcium channel blockers, calmodulin antagonists and indomethacin. *Pharmacology* **47**: 22–31