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Metabolism of Magnolol from Magnoliae Cortex. I.[1] Application of Liquid Chromatography-Mass Spectrometry to the Analysis of Metabolites of Magnolol in Rats

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As a part of our studies on the metabolism of active components from traditional Chinese medicines, magnolol was orally administered to rats. The urinary and fecal metabolites were analyzed by high-performance liquid chromatography and liquid chromatography-mass spectrometry, and their structures were determined to be tetrahydromagnolol(M1), 5-(1-propen-1(*E*)-yl)-5′-propyl-2,2′-dihydroxybiphenyl(M2), 5-allyl-5′-propyl-2,2′-dihydroxybiphenyl(M3), isomagnolol (5,5′-di(1-propen-1(*E*)-yl)-2,2′-dihydroxybiphenyl)(M4), 5-allyl-5′-(1-propen-1(*E*)-yl)-2,2′-dihydroxybiphenyl(M5). On the other hand, magnolol was transformed to M4 and M5 together with small amounts of M2 and M3 by anaerobic incubation with rat feces. This suggests that the isomerization of magnolol in the rat could be carried out by the action of intestinal bacteria.

Keywords——*Magnolia obovata*; *Magnolia officinalis*; LC-MS; magnolol; isomagnolol; dihydromagnolol; tetrahydromagnolol; 5-allyl-5′-(1-propen-1(*E*)-yl)-2,2′-dihydroxybiphenyl; metabolism

Magnolol[2] and honokiol[3,4] are major phenolic components of Magnoliae Cortex which is the most important constituent of prescriptions for the therapy of anxiety, neuronal disturbance, or gastrointestinal disorder in traditional Chinese medicine. These compounds are relatively simple, naturally occurring phenylpropanoids, but are distributed only in limited Magnoliaceous plants,[5] *Magnolia officinalis* REHD *et* WILS., *M. obovata* THUNB., *M. tripetala*, *M. grandiflora* L.[6] and a Laureaceous plant, *Sassafras randaiense* (HAYATA) REHD.[7,8]

Pharmacological studies on magnolol and honokiol as well as the extracts of Magnoliae Cortex have been extensively conducted by Watanabe *et al.*[9,10] They showed that these compounds and the extracts have distinct central depressant and centrally acting muscle relaxant effects which could in part account for the efficiency of the prescription containing Magnoliae Cortex for treating anxiety, neuronal disturbance and Parkinson's disease. On the other hand, we recently reported that magnolol and honokiol have potent antibacterial action against a primary cariogenic bacterium, *Streptococcus mutans*, as well as other Gram-positive bacteria.[11-14]

In the present paper, we report the metabolism of magnolol administered orally to rats and the *in vitro* transformation of the compound by intestinal bacteria.

Materials and Methods

Instruments——All melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Proton magnetic resonance (^{1}H-NMR) spectra were measured with an FX-90Q spectrometer (JEOL

Ltd., Tokyo) with tetramethylsilane as an internal standard. Ultraviolet (UV) spectra were measured with a Shimadzu UV-210A digital double beam spectrophotometer and infrared (IR) spectra with a Hitachi 260-01 infrared spectrometer. Electron impact mass spectra (EI-MS) were measured with a JMS-DX 300 mass spectrometer (JEOL) at an ionization voltage of 70 eV. High-performance liquid chromatography (HPLC) was carried out on a Tri Rotar SR-1 (JASCO Ltd., Tokyo) equipped with a UVIDEC-100V detector (JASCO) using a column (50 cm × 2.1 mm i.d. for analysis or 25 cm × 7.2 mm i.d. for preparatory purposes) of TSK-ODS-120A (10 μ, Toyo Soda Kogyo Co., Tokyo). Liquid chromatography-mass spectrometry (LC-MS) was carried out on a JMS-D 300 (JEOL) equipped with a direct inlet LC-MS interface and a FAMILIC-300 HPLC system (JASCO).

Chemicals——Magnolol was isolated from Magnoliae Cortex.[12] Isomagnolol[15,16] and tetrahydromagnolol were synthesized as reported previously.[13] GAM broth was purchased from Nissui Seiyaku Co. (Tokyo). The dilution medium consisted of 0.78% K_2HPO_4 (37.5 ml), 0.47% KH_2PO_4–1.18% NaCl–1.2% $(NH_4)_2SO_4 \cdot H_2O$–0.12% $CaCl_2$–2.5% $MgSO_4 \cdot H_2O$ (37.5 ml), 0.1% resazurin (1 ml), L-cysteine·HCl·H_2O (0.5 g), 25% L-ascorbic acid (2 ml), 8% Na_2CO_3 (50 ml), agar (0.5 g) and distilled water (860 ml), and was prepared immediately before use.

Administration of Magnolol to Rats——A suspension of magnolol in arabic gum (2 ml, 25 mg/ml) was orally administered daily (at 4:30 p.m.) to the rats (10 male, Wistar rats, 4 weeks of age, weighing 218—252 g) for 6 d. During the experiments, food and drinking water were allowed *ad libitum*. The feces and urine excreted were collected daily (at 4:30 p.m.).

Analysis of the Urinary Metabolites——Sodium chloride was added to the collected urine (pooled at 24-h intervals) to give a final concentration of 10% (w/v). The resulting urine was then extracted three times with 2 volumes of benzene. After the filtration, the benzene solution was concentrated to a small volume and diluted with MeOH to give a volume of exactly 10 ml. A 4 μl aliquot of this solution was injected into a column of TSK-ODS-120A (50 cm × 2.1 mm i.d.).

Analysis of the Fecal Metabolites——The feces (collected and combined at 24-h intervals) were extracted with MeOH (200 ml × 3). After evaporation of the MeOH, the residue was suspended in water (200 ml) and extracted with benzene (200 ml × 3). The combined benzene solution was evaporated *in vacuo* to give an oily residue. The residue was dissolved in $CHCl_3$ and the solution was adjusted to a volume of exactly 100 ml. A 2 μl aliquot was applied to a semi-micro column of μs-Finepak SIL C_{18} (25 cm × 1.5 mm i.d.) or a column of TSK-ODS-120A (50 cm × 2.1 mm i.d.) for analysis. Quantitative analysis of magnolol and tetrahydromagnolol was carried out by using standard lines of authentic samples.

LC-MS Measurement——The sample (10 μg) was injected into a semi-micro column (25 cm × 1.5 mm i.d.) of μs-Finepak SIL C_{18} (JASCO) attached to the HPLC system. The effluent (100 μl/ml) from the column was introduced into a mass spectrometer through a direct inlet LC-MS interface. Mass detection was carried out by repetitive scanning (3 s/scan) in the CI mode from *m/z* 50 to *m/z* 500 under the following conditions: ionization voltage of 200 eV; ionization current of 300 μA. The pressure in the ion source was maintained at *ca.* 1×10^{-4} Torr during the operation.

Isolation of the Fecal Metabolites——The feces (24—50 g), which had been collected at 24-h intervals after the administration of magnolol, were extracted three times with MeOH (200 ml) and the solution was concentrated *in vacuo*. The residue was suspended in water (200 ml) and extracted three times with benzene (200 ml), which was then evaporated off *in vacuo*. The residue was dissolved in $CHCl_3$ (10 ml) and applied to a column of alumina (Merck, Art. 1077, 100 g). The column was washed with MeOH (100 ml) and eluted with *n*-BuOH–AcOH–H_2O (3:1:1). The eluate was evaporated to dryness *in vacuo*, dissolved in ether (200 ml) and washed three times with 5% Na_2CO_3 (100 ml), then dried over Na_2SO_4. The solution was concentrated, and the residue was dissolved in $CHCl_3$–MeOH (1:1) and adsorbed on C-18 SEP-PAK (Waters Associates, Milford, Mass.), which was eluted with CH_3CN–H_2O (1:1). The eluate was evaporated to dryness *in vacuo*, and the residue was dissolved in MeOH (1.0 ml). The solution was repeatedly chromatographed on TSK-ODS-120A (25 cm × 7.2 mm i.d.) at a pressure of 110 kg/cm^2. The metabolites, M1, M2, M3, M4, M5 and M6, were isolated and the structures were determined on the basis of the physical properties and direct comparison with authentic samples.

M1: colorless prisms from benzene–hexane, mp 142—143 °C, EI-MS *m/z*: 270 (M^+, base peak), UV λ_{max}^{MeOH}: 291 nm. M1 was identical with an authentic sample of tetrahydromagnolol on the basis of comparisons of mp, IR, H^1-NMR (see Table I) and retention time of HPLC.

M2: EI-MS *m/z*: 268 (M^+, base peak), UV λ_{max}^{MeOH}: 277 nm.

M4: EI-MS *m/z*: 266 (M^+, base peak), UV λ_{max}^{MeOH}: 243 nm.

M5: EI-MS *m/z*: 266 (M^+, base peak), UV λ_{max}^{MeOH}: 245, 291 nm.

M6: EI-MS *m/z*: 266 (M^+, base peak), UV λ_{max}^{MeOH}: 291 nm.

IR (KBr): 3150 (OH), 1230 (OH), 1638 (–CH=CH_2), 1610 (aromatic C=C) cm^{-1}.

Incubation of Magnolol with Rat Feces——Fresh feces (1 g) of rats were suspended in 10^4 volumes of dilution medium. An aliquot (0.3 ml) of the suspension was added to GAM broth (10 ml) containing 0.13 mg of magnolol and anaerobically incubated at 37 °C for 48 h. The culture was then extracted three times with benzene (10 ml). The benzene layer was evaporated to dryness *in vacuo* and the residue was dissolved in MeOH (0.3 ml). A 2 μl aliquot of the methanolic solution was then analyzed by HPLC and LC-MS.

Results

Analysis of the Fecal Metabolites by HPLC and LC-MS

Figure 1 shows a high-performance liquid chromatogram of the metabolites excreted in the feces of rats to which magnolol had been repeatedly administered. The metabolites corresponding to these peaks were tentatively designated as M1, M2, M3, M4, M5 and M6 from the last peak. M6 was identical with authentic magnolol in terms of the retention time on HPLC and the ultraviolet absorption spectrum. To determine the structures of the other metabolites, the effluent from a semi-micro LC column was introduced into the mass spectrometer through a direct inlet LC-MS interface. Figure 2 shows a reconstructed ion current chromatogram (RIC) and mass chromatograms. M1 and M6 were detected as major peaks and M2, M3 as minor peaks. Contrary to the result obtained by UV monitoring (Fig. 1), M4 and M5 gave small and unresolved peaks. Based on the quasi-molecular ions (QM^+, Fig. 3) corresponding to the peaks labelled M1—M6 on the RIC and the mass chromatograms (Fig. 2B), the molecular weights of the metabolites were estimated to be 270 for M1, 268 for M2 and M3, and 266 for M4—M5 and M6, suggesting that M1 is a tetrahydro derivative and M2 and M3 are dihydro derivatives. The structures of M4 and M5 were not clarified in this experiment but these compounds may be isomers of magnolol (M6).

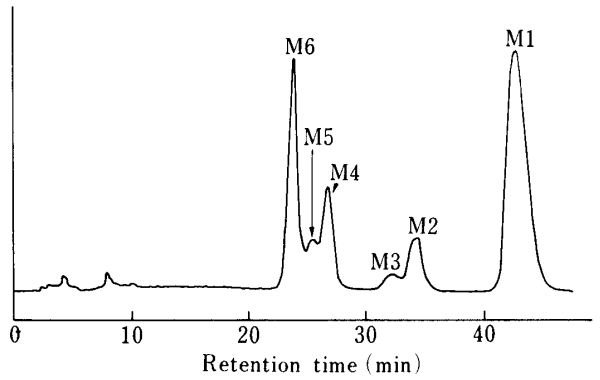

Fig. 1. A Chromatogram of the Fecal Metabolites Following Oral Administration of Magnolol

HPLC was carried out by using a semi-micro column (25 cm × 1.5 mm i.d.) of μs-Finepak SIL C_{18}; solvent, CH_3CN–H_2O (50 : 50); flow rate, 100 μl/min; ultraviolet trace at 250 nm; 3 μl of the sample was injected.

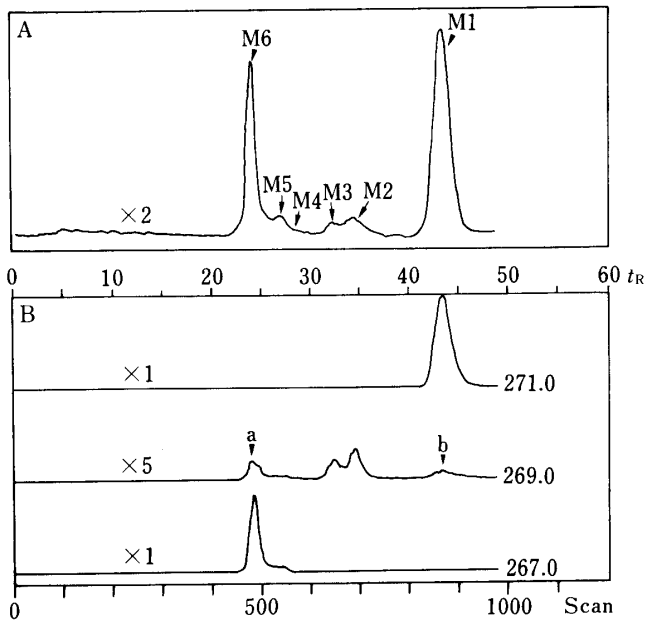

Fig. 2. A Reconstructed Ion Current Chromatogram (RIC, A) and Mass Chromatograms (B) of Metabolites Excreted in the Feces

A sample was chromatographed on the FAMILIC-300 system. The column was 25 cm × 1.5 mm i.d. μs-Finepak SIL C_{18} and the solvent was CH_3CN–H_2O (50 : 50), flow rate 100 μl/min. The mass spectrometer was a JMS-D 300 equipped with a direct inlet LC-MS interface, operated in the positive chemical ionization mode. a, attributable to a weak $(M+3)^+$ ion of magnolol (4.3% of the QM^+ ion); b, attributable to a weak $(M-2)^+$ ion of tetrahydromagnolol (1.4% of the QM^+ ion).

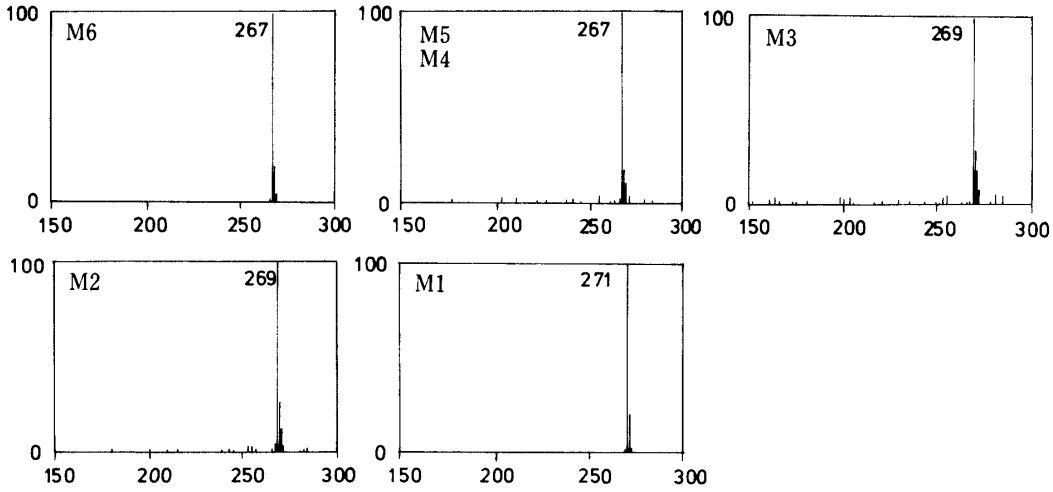

Fig. 3. Chemical Ionization Mass Spectra Corresponding to the Peaks on the RIC

Though the mass range was scanned from *m/z* 50 to *m/z* 500 in the LC-MS measurement, mass spectra are represented here only in the quasi-molecular ion region (*m/z* 150—300). Intensity is represented as a percentage of the base peak.

TABLE I. ^{1}H-NMR Spectra Data for the Metabolites in $CDCl_3$

Metabolites	CH_3–CH_2–CH_2–	CH_3–CH_A=CH_B–	CH_2=CH–CH_2–	Aromatic-H
M1	0.96 (6H, t, J=7 Hz) 1.41—1.82 (4H, m) 2.57 (4H, t, J=8 Hz)			6.36—7.32 (6H, m)
M2	0.93 (3H, t, J=7 Hz) 1.50—1.74 (2H, m) 2.56 (2H, t, J=7 Hz)	1.85 (3H, d, J=6 Hz) 5.91—6.29 (1H, m) 6.35 (1H, d, J_{AB}=14 Hz)		6.80—7.30 (6H, m)
M4		1.85 (6H, d, J=5 Hz) 5.90—6.47 (2H, m) 6.40 (2H, d, J_{AB}=16 Hz)		6.90—7.33 (6H, m)
M5		1.86 (3H, d, J=7 Hz) 5.74—6.30 (1H, m) 6.38 (1H, d, J_{AB}=14 Hz)	3.37 (2H, d, J=7 Hz) 4.97—5.16 (2H, m) 5.7—6.2 (1H, m)	6.90—7.30 (6H, m)
M6 (magnolol)			3.37 (4H, d, J=7 Hz) 4.99—5.16 (4H, m) 5.75—6.18 (2H, m)	6.89—7.20 (6H, m)

The values are represented as δ (ppm); m, multiplet; d, doublet; t, triplet.

Isolation of the Fecal Metabolites and Identification of Their Structures

Each metabolite was isolated by preparative HPLC and the structure was determined as below. In agreement with the result obtained by LC-MS, M1 gave a molecular ion peak (M^+) 4 mass units higher than that of magnolol (m.w. 266) on electron impact ionization mass spectrometry (EI-MS). It showed characteristic signals at δ 0.96 (6H, t, J=7 Hz), 1.41—1.82 (4H, m) and 2.57 ppm (4H, t, J=8 Hz) due to two propyl groups and at 6.36—7.32 ppm due to six aromatic protons in the ^{1}H-NMR spectra (Table I). M1 was finally identified as tetrahydromagnolol (5,5′-dipropyl-2,2′-dihydroxybiphenyl) by direct comparisons of the mp, ^{1}H-NMR, IR and retention time on HPLC with those of an authentic sample synthesized from magnolol by catalytic hydrogenation.[4,13] M2 gave a molecular ion peak two mass units higher than that of magnolol and showed characteristic ^{1}H-NMR signals[17] at δ 1.85 (3H, d, J=6 Hz), 5.91—6.29 (1H, m) and 6.35 (1H, d, J=14 Hz) ppm due to a propenyl group as well

as at 0.93 (3H, t, $J=7$ Hz), 1.50—1.74 (2H, m) and 2.56 ppm (2H, t, $J=7$ Hz) due to a propyl group. The structure was determined to be 5-(1-propen-1(*E*)-yl)-5′-propyl-2,2′-dihydroxybiphenyl. M3 was not obtained in an appreciable amount, but it seemed to be an isomer of M2, namely 5-allyl-5′-propyl-2,2′-dihydroxybiphenyl, based on the mass chromatogram at *m/z* 269.0 corresponding to the quasi-molecular ion $(M+1)^+$ (Fig. 2B) and the CI-MS fragment pattern in the LC-MS (Fig. 3). M4 showed characteristic ^{1}H-NMR signals at δ 1.85 (6H, d, $J=5$ Hz), 5.90—6.47 (2H, m) and 6.40 ppm (2H, d, $J=16$ Hz) due to two *trans*-propenyl groups, indicating that two allyl side chains of magnolol were isomerized to *trans*-propenyl groups. It was then identified as isomagnolol by comparing it directly with an authentic sample synthesized from magnolol in the presence of $RhCl_2$.[13] M5 showed a molecular ion peak of *m/z* 266 in the EI-MS, identical with those of M4 and M6 (magnolol), and one allyl group (δ 3.37 (2H, d, $J=7$ Hz), 4.97—5.16 (2H, m), 5.7—6.2 ppm (1H, m)) and one propenyl group (δ 1.86 (3H, d, $J=7$ Hz), 5.74—6.30 (1H, m), 6.38 ppm (1H, d, $J=14$ Hz)) in the ^{1}H-NMR. The structure was determined to be 5-allyl-5′-(1-propen-1(*E*)-yl)-2,2′-dihydroxybiphenyl. M6 was identical with magnolol on the basis of direct comparisons of mp and spectroscopic properties.

Change of the Fecal Metabolites

Figure 4 shows the time course of the metabolites excreted in the feces following repeated administration of magnolol. In the feces excreted in 24 h after the first administration, only small amounts of metabolites were detected, but magnolol (M6) was recovered as a major constituent (22% of the administered dose). Isomagnolol (M4) and tetrahydromagnolol (M1), however, increased in amount almost linearly during 48—72 h accompanied by a significant decrease of magnolol (M1). The former metabolite became constant in amount after 96 h but the latter increased further and finally reached a maximum after 120 h.

Analysis of the Urinary Metabolites by HPLC

Figure 5 shows chromatograms of the urinary metabolites measured by HPLC at the 2nd day and 4th day under the same conditions. The elution patterns were essentially similar to that observed in the case of the fecal metabolites except for several peaks eluted earlier. The metabolites (M1—M6) were detected on the basis of the retention times and CI-MS. We tried to isolate the constituents of two peaks (M7 and M8) that were absent in the control experiment by repeated preparative HPLC, but sufficient amounts for structure determination could not be obtained. HPLC analysis of urinary metabolites collected at 24-h intervals revealed that the composition of these metabolites (M1—M8) also varied significantly with the collecting period, as observed in the case of the feces (data not given).

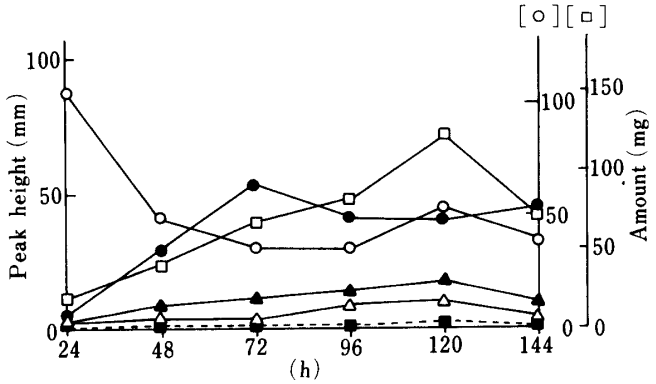

Fig. 4. Changes of the Fecal Metabolites Following the Repeated Oral Administrations of Magnolol to Rats

A dose of 50 mg was administered daily to each of ten rats and the feces were collected and combined at 24-h intervals. The benzene-soluble metabolites were analyzed by HPLC. Excreted amounts of M1 and M6 per 10 rats were calculated at 24-h intervals after the administration (the right Y-axis). Variations in the amounts of other metabolites as well as M1 and M6 are also represented as relative changes in peak height (the left Y-axis).

□—□, M1 (tetrahydromagnolol); △—△, M2 (5-(1-propen-1(*E*)-yl)-5′-propyl-2,2′-dihydroxybiphenyl); ■---■, M3 (5-allyl-5′-propyl-2,2′-dihydroxybiphenyl); ●—●, M4(5,5′-di(1-propen-1(*E*)-yl)-2,2′-dihydroxybiphenyl); ▲—▲, M5 (5-allyl-5′-(1-propen-1(*E*)-yl)-2,2′-dihydroxybiphenyl); ○—○, M6 (magnolol).

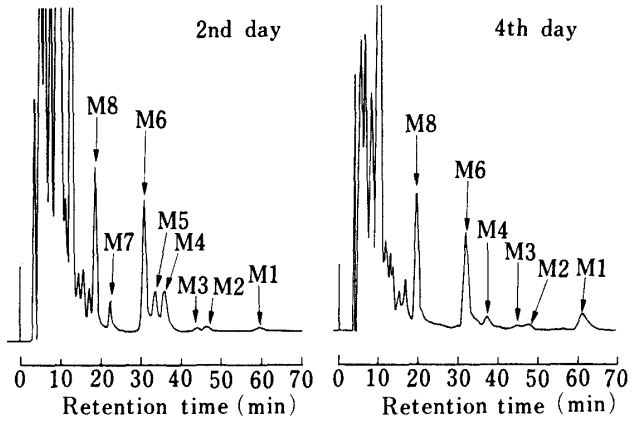

Fig. 5. HPLC Chromatograms of the Urinary Metabolites Following Repeated Oral Administrations of Magnolol

HPLC was carried out using a column (50 cm × 2.1 mm i.d.) of TSK-ODS-120A; mobile phase, CH_3CN–H_2O–AcOH (50 : 50 : 0.5); flow rate, 0.4 ml/min; ultraviolet trace at 250 nm. The relative ratio of the metabolites varied depending on the period after the repeated administrations of magnolol.

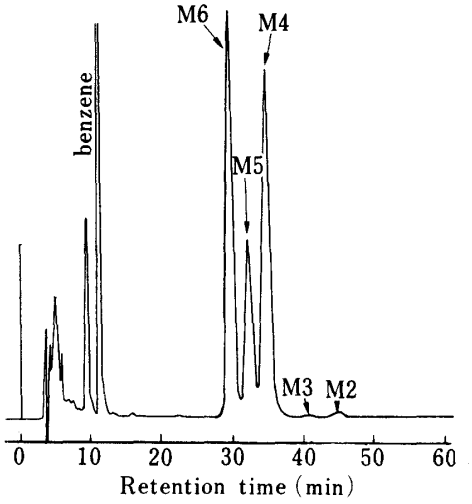

Fig. 6. A Chromatogram of the Metabolites Obtained by the *in Vitro* Incubation with Rat Feces

HPLC was carried out under the same conditions as described in the legend to Fig. 5.

In Vitro Transformation of Magnolol by Rat Intestinal Flora

To investigate the role of the intestinal bacteria in the metabolism of magnolol, the compound was anaerobically incubated with fresh rat feces. Since magnolol has antibacterial action against various bacteria including anaerobes in the intestinal tract,[14] the cultivation was carried out at an extremely low concentration of 10 μg/ml. In contrast to the case of the fecal and urinary metabolites in rats, the HPLC profile showed three major peaks corresponding to M4 (isomagnolol), M5 and M6 (magnolol) and two minor peaks corresponding to M2 and M3 (Fig. 6). No peak for tetrahydromagnolol (M1) was observed even after prolonged incubation. This result indicates that the intestinal bacteria mostly take part in the isomerization of magnolol but not in the reduction under the conditions used.

Discussion

By using HPLC and LC-MS techniques, various metabolites were identified in the benzene soluble fractions from the urine and feces following repeated administrations of magnolol to rats. The on-line LC-MS system provided useful information for structure elucidation without requiring isolation and prior derivatization, though it failed in the present experiment to yield satisfactory separation between isomagnolol (M4) and 5-allyl-5′-(1-propen-1(*E*)-yl)-2,2′-dihydroxybiphenyl (M5) in the RIC. However, these compounds appeared to decompose or polymerize easily prior to the ionization process. In fact, no satisfactory RIC was obtained when isomagnolol was directly injected into an ion source through an LC-MS interface without an LC column in-line.

Figure 7 shows a possible metabolic process deduced from the results of experiment. Magnolol is isomerized to isomagnolol (M4) *via* an intermediate, 5-allyl-5′-(1-propen-1(*E*)-yl)-2,2′-dihydroxybiphenyl (M5), and these isomers as well as magnolol are further reduced in part or in all of the unsaturated side chains to give dihydro and tetrahydro derivatives. Although the reduction of double bonds by intestinal flora occurs in a wide variety of

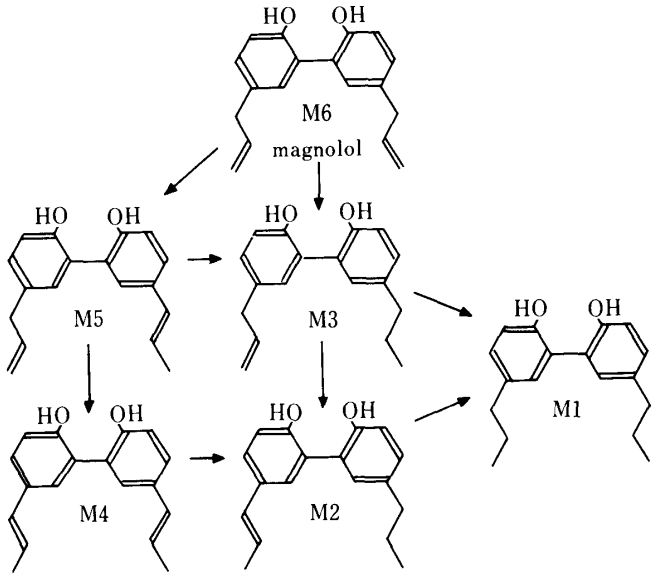

Fig. 7. Possible Metabolic Processes of Magnolol in Rats

compounds including hydroxycinnamic acids (*e.g. o*-coumaric, *p*-coumaric, caffeic, ferulic and sinapic acids) and 4-vinylcatechol,[18] as has been demonstrated in both *in vivo* and *in vitro* investigations using intestinal bacteria, it is uncertain whether the reduction of magnolol and its metabolic intermediates occurs in tissue or is carried out by intestinal bacteria. In our experiments, only small amounts of partly reduced metabolites (M2 and M3) were detected after anaerobic incubation with the intestinal bacterial mixture, although a major component of the metabolites in the feces was a fully reduced derivative, tetrahydromagnolol (M1). This suggests that the reduction of the unsaturated side chains of magnolol could be carried out by tissue enzymes. If this is so, some tissue enzymes responsible for the reduction seem to be inducible because the reduced products were scarcely detected after a single administration of magnolol but increased significantly in amount after repeated administrations. On the other hand, the isomerization of magnolol was due to intestinal bacterial enzymes, as demonstrated by the *in vitro* experiment. This is the first report to show that an allyl substituent is easily converted to a propenyl one by intestinal bacteria. Since isomagnolol has a weaker antibacterial action than magnolol,[13] the isomerization is likely to be one of the detoxication processes by intestinal bacteria.

References and Notes

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15) The name isomagnolol was first adopted by Sugii (ref. 2) and then by Erdtman *et al.* (ref. 16) for a 5,5′-di(1-propen-1(*E*)-yl)-2,2′-dihydroxybiphenyl compound obtained from magnolol by treatment with alkali, but

recently it was inappropriately applied by El-Feraly *et al.* (ref. 8) to a 4,4′-diallyl-2-hydroxybiphenyl ether compound isolated from *Sassafras randaiense* root. Here, we use this name for the former compound.
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