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Identification of 1-Furan-2-yl-3-pyridin-2-yl-propenone, an Anti-inflammatory Agent, and Its Metabolites in Rat Liver Subcellular Fractions

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1-Furan-2-yl-3-pyridin-2-yl-propenone (FPP-3) has been characterized to have an anti-inflammatory activity through the inhibition of the production of nitric oxide and tumor necrosis factor-α. In the present studies, the phase 1 metabolism of FPP-3 was investigated in rat liver microsomes and cytosols. When FPP-3 was incubated with rat liver microsomes and cytosols in the presence of NADPH, 2 major peaks were detected on a liquid chromatography/electrospray ionization-mass spectrometry. Two metabolites (i.e., M1 and M2) were characterized as reduced forms on propenone: M1 (1-furan-2-yl-3-pyridin-2-yl-propan-1-one) was the initial metabolite and M2 (1-furan-2-yl-3-pyridin-2-yl-propan-1-ol) was a secondary alcohol believed to be formed from M1.

Key words: 1-Furan-2-yl-3-pyridin-2-yl-propenone, LC-ESI/MS, Reduction, Metabolite

INTRODUCTION

1-Furan-2-yl-3-pyridin-2-yl-propenone (FPP-3) is a chemically synthesized novel compound with a propenone moiety. A recent study demonstrated that FPP-3 could inhibit lipopolysaccharide (LPS)-stimulated production of nitric oxide (NO) and tumor necrosis factor-α (TNF-α) in the cultures of RAW 264.7 macrophages (Lee *et al.*, 2004). In addition, FPP-3 could not only inhibit cyclooxygenases (COX) and 5-lipoxygenase (LOX) activity but also inhibit COX-2 by 35-times more selectively than COX-1 (Jahng *et al.*, 2004). FPP-3 (0.5-50 mg/kg, *p.o.*) significantly suppressed the carrageen-induced paw edema in rats and there were no gastric ulcers formed in FPP-3-treated rats (Lee *et al.*, 2006). These results indicated that FPP-3 might have a potent anti-inflammatory activity.

Although the oxidative biotransformation of xenobiotics by cytochrome P450s (CYPs) is the primary metabolic process for a number of xenobiotics, the reduction is also a significant step in the phase 1 biotransformation for a variety of aromatic, alicyclic and aliphatic compounds bearing a carbonyl group (Maser, 1995; Oppermann and Maser, 2000) In mammals, several microsomal and cytosolic carbonyl reducing enzymes have been identified and characterized, including hydroxysteroid dehydrogenases (HSDs), prostaglandin dehydrogenases, dihydrodiol dehydrogenases and cytosolic carbonyl reductase (CBR) (Oppermann *et al.*, 2000).

The objective of our present study was to characterize *in vitro* metabolites of FPP-3 by a liquid chromatography-electrospray ionization tandem mass spectrometry (LC/ESI-MS) and NMR spectroscopy. In this report, two reduced metabolites of FPP-3 which were produced by CYP-independent reduction were identified and characterized in rat liver microsomes and cytosols.

MATERIALS AND METHODS

Chemicals

FPP-3 (purity, >99%) used in this study was chemically synthesized in our group. Metabolites were separated by thin layer chromatography (TLC). Glucose 6-phosphate, β-NADPH, glucose 6-phosphate dehydrogenase and ammonium formate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acetonitrile (ACN) was HPLC-

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grade from Merck Ltd. (Poole, U.K.). All other chemicals were of analytical grade and used as received.

Animals

Specific pathogen-free male Sprague Dawley rats (250-280 g) were obtained from the Orient Co. (Seoul, Korea). The animals received at 5-6 weeks of age were acclimated for at least 1 week. Upon arrival, animals were randomized and housed 3 per cage. The animal quarters were strictly maintained at 23 ± 3°C and 50 ± 10% relative humidity. A 12 h light and dark cycle was used with an intensity of 150-300 Lux. All animal procedures were followed based on a guideline recommended by the Society of Toxicology (U.S.A.) in 1989.

Preparation of subcellular fractions

The livers were perfused with phosphate-buffered saline to remove excess blood and homogenized with four volumes of ice-cold 0.1 M potassium phosphate buffer, pH 7.4. The liver homogenates were centrifuged at 9,000 g for 10 min at 4°C, and the resulting post-mitochondrial supernatants were centrifuged again at 105,000 g for 60 min at 4°C. The microsome pellets and cytosol supernatants were collected and were stored at -70°C until use (Clement *et al.*, 1996). The contents of microsomal and cytosolic proteins were determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Preparation of samples

Metabolism of FPP-3 (25 μM, final concentration) was determined with 1 mg/mL of either microsome or cytosol protein in 0.1 M potassium phosphate buffer, pH 7.4, at 37°C for 1 h in a final incubation volume of 500 μL. The reactions were initiated by the addition of a NADPH-generating system (NGS) containing 0.8 mM β-NADPH, 10 mM glucose 6-phosphate and 1 unit of glucose 6-phosphate dehydrogenase into the reaction mixture. The reaction was stopped after incubation by the addition of 1 mL ethyl acetate. After mixing and centrifugation, 800 μL of the organic layer was separated. The organic layer was dried under a stream of nitrogen gas. A residue was reconstituted in an HPLC grade ACN and injected into an HPLC column.

Isolation and identification of metabolites

According to the method described in the Section of Preparation of Samples, the ethyl acetate extracts were loaded on a TLC aluminum sheet (silica gel 60 F_{254}, Merck). The plate was eluted with hexane:ethyl acetate (2:8). Each spot was separated and extracted by ethyl acetate. The fractions were identified as FPP-3, M1 and M2 by LC/ESI-MS and ^{13}C-, ^{1}H-NMR spectra.

LC/ESI-MS Analysis

The HPLC consisted of surveyor system (Thermo Finnigan, San Jose, CA, U.S.A.) with the LCQ advantage trap mass spectrometer (Thermo Finnigan, San Jose, CA, U.S.A.) equipped with an electrospray ionization (ESI) source. The column used for the separation was an Xterra® (2.1 × 50 mm, 3.5 μm i.d., Waters). The HPLC mobile phases consisted of 20 mM ammonium formate buffer, pH 4.0 (A), and 100% ACN (B). A gradient program was used for the HPLC separation with a flow rate of 210 μL/min. The initial composition was 5% B and programmed linearly to 90% B after 13 min. Nitrogen was used both as the sheath gas at 1.05 L/min and as the auxiliary gas at a 6 L/min with a capillary temperature of 215°C and the spray voltage set to 4 kV. The mass spectrometer was operated in the positive ion mode in *m/z* range 80-450. Helium was used as the collision gas for the tandem mass spectrometric experiments, followed by the isolation of ions over a selected mass window of 1 Da.

NMR Spectroscopy

The NMR spectra were obtained using a Bruker 250 MHz (DMX 250) spectrometer using Bruker's standard pulse program. Samples were dissolved in $CDCl_3$ and

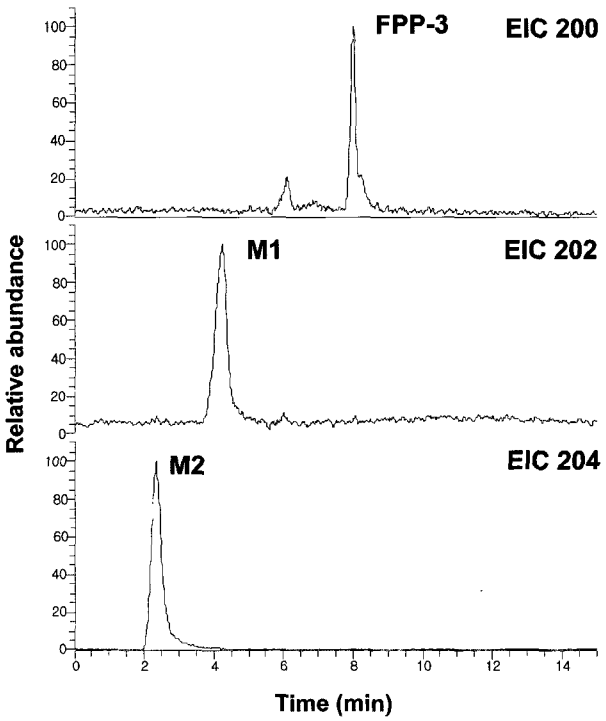

Fig. 1. Extracted ion chromatograms for FPP-3 incubated with rat liver cytosol in the presence of an NADPH-generating system (NGS). Metabolism of FPP-3 (25 μM, final concentration) was determined with 1 mg/mL of cytosol protein in 0.1 M potassium phosphate buffer, pH 7.4, at 37°C for 60 min. The reactions were initiated by the addition of an NGS into the reaction mixture.

chemical shifts were reported in ppm downfield from TMS.

RESULTS

The incubation of FPP-3 with rat liver cytosols in the presence of NGS generated two kinds of metabolites (Fig. 1). No metabolite was generated in the absence of NADPH (data not shown). Rat liver microsomes also produced two metabolites with same retention times (data not shown). M1 and M2 were observed at retention times of 4.3 and 2.3 min, respectively. Their MH^+ ions were observed at *m/z* 202 and 204, respectively. The MH^+ ions of metabolites were identified by full-scan MS detection.

As depicted in Fig. 2A, the MS^2 spectrum of protonated FPP-3 (*m/z* 200) showed product ions at *m/z* 172, 158 and 132. The product ions at *m/z* 172 and 158 might be due to the loss of -CO (28 Da) and $-OC_2H_2$ (42 Da) in the furan ring, but these ions were not important for the determination of metabolite structures. The fragment ion at *m/z* 132 indicated the loss of furan ring (68 Da) to form 3-(pyridin-2-yl)acrylaldehyde. The MS^2 spectra of protonated M1 (*m/z* 202) are shown in Fig. 2B. The MS^2 spectrum of protonated M1 showed a major product ion at *m/z* 134, indicating the loss of furan ring (68 Da) to form 3-(pyridin-2-yl) propanal. The MS^2 spectrum of protonated M2 showed a major product ion at *m/z* 186 (Fig. 2C). The ion at *m/z* 186 was assigned as a form from the ion *m/z* 204 by H_2O loss, indicating the formation of 3-(furan-2-yl)allyl) pyridine. The MS^3 spectrum of the ion at *m/z* 186 showed product ions at *m/z* 158 and 118. The product ion at *m/z* 158 indicated the loss of -CO (28 Da) and the ion at *m/z* 118 correspond to the loss of fuarn ring (68 Da) from the ion at *m/z* 186.

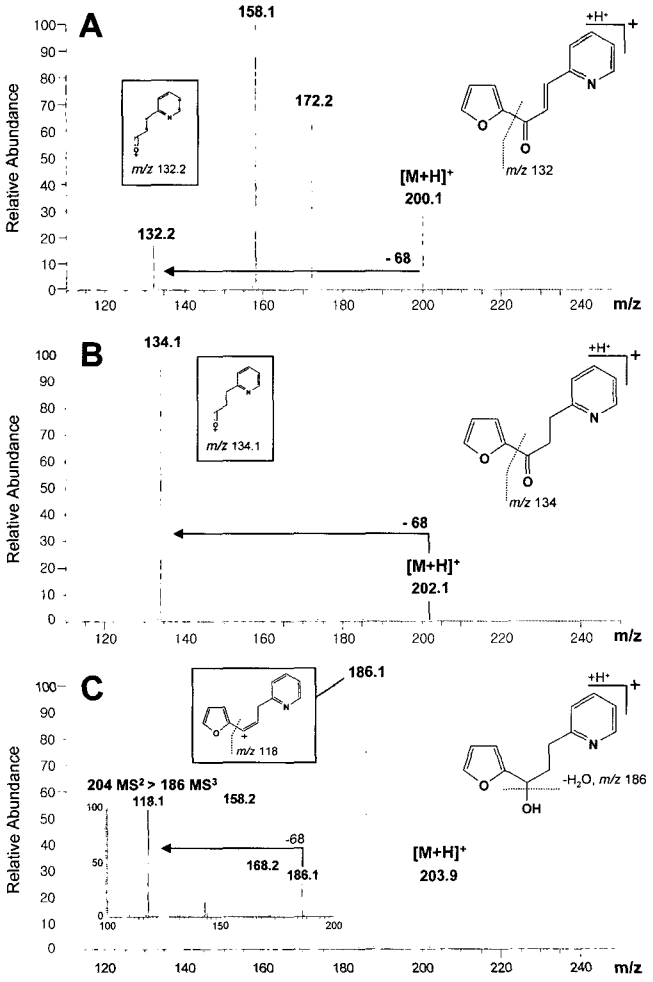

Fig. 2. CID spectra of protonated FPP-3 (A), M1 (B), and M2 (C)

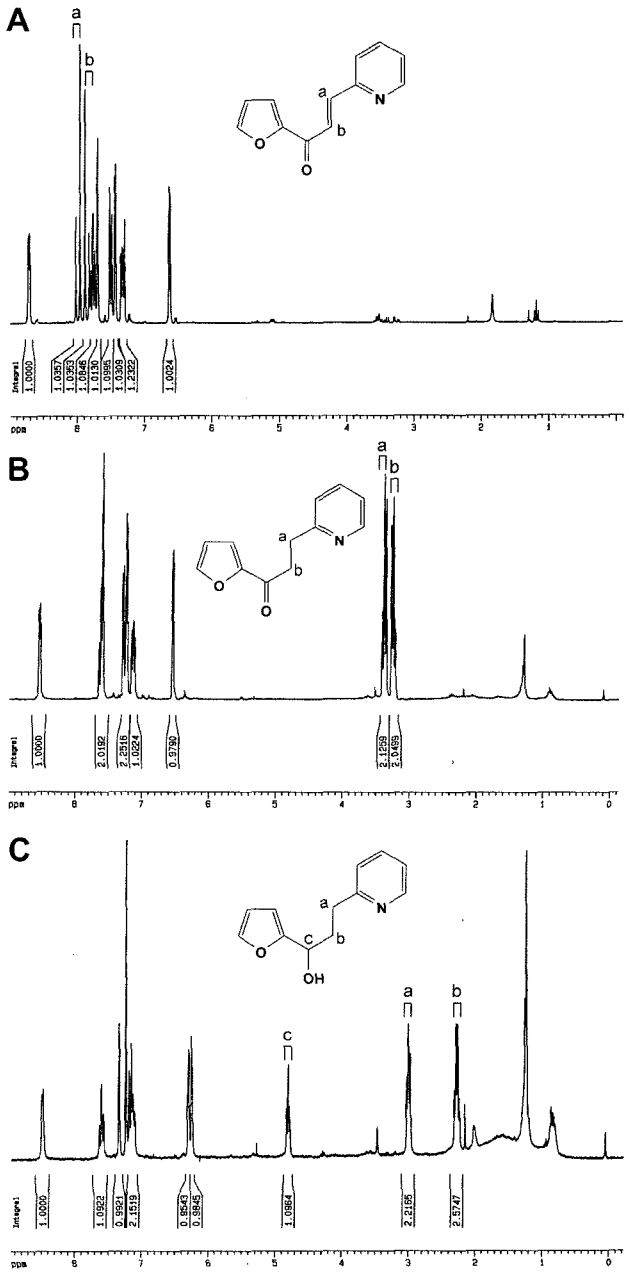

Fig. 3. Proton NMR spectra of FPP-3 (A), M1 (B), and M2 (C)

All metabolites were isolated for the quantification of metabolites and determination of exact structures of individual metabolites by using silica TLC sheets. Each fraction was analyzed by ^{1}H-NMR (Fig. 3) and HPLC (data not shown). ^{1}H-NMR spectrum of FPP-3 showed the proton signals of HC=CH at 7.8 (b) and 7.9 (a) ppm but no peaks at 2 to 5 ppm. ^{1}H-NMR spectrum of M1 showed that novel proton signals of H_2C-CH_2 at 3.2 (b) and 3.4 (a) ppm were detected, and the signals of original HC=CH disappeared as a reduction on C=C bond of acrylaldehyde. Moreover, ^{1}H, ^{1}H-COSY spectra of M1 displayed that novel two CH_2 was connected as one after another (Fig. 4). This structure of M1 was confirmed by ^{13}C-NMR spectrum (data not shown). ^{1}H-NMR spectrum of M2 displayed signals at 2.3 (a), 3.0 (b) and 4.8 (c) ppm. The proton signals at (a) and (b) were believed to be shifted due to the formed novel hydroxyl group by the reduction on carbonyl group of M1. The proton signal at 4.8 ppm was displayed as HO-CH. According to these results, individual metabolites were assigned as 1-furan-2-yl-3-pyridin-2-yl-propan-1-one for M1 and 1-furan-2-yl-3-pyridin-2-yl-propan-1-ol for M2.

Fig. 5 shows the effects of incubation time on the formation of metabolites of FPP-3 in the microsome (Fig. 5A) and the cytosol (Fig. 5B) fractions isolated from male SD rat livers. The results showed similar patterns of metabolites production in both microsomal and cytosol fractions. Following the maximum formation of M1 at 10 min, the formation of M1 was gradually decreased through the incubation period. However, the formation of M2 showed an incubation time-dependent increase. Taken together, FPP-3 metabolism could be divided into two

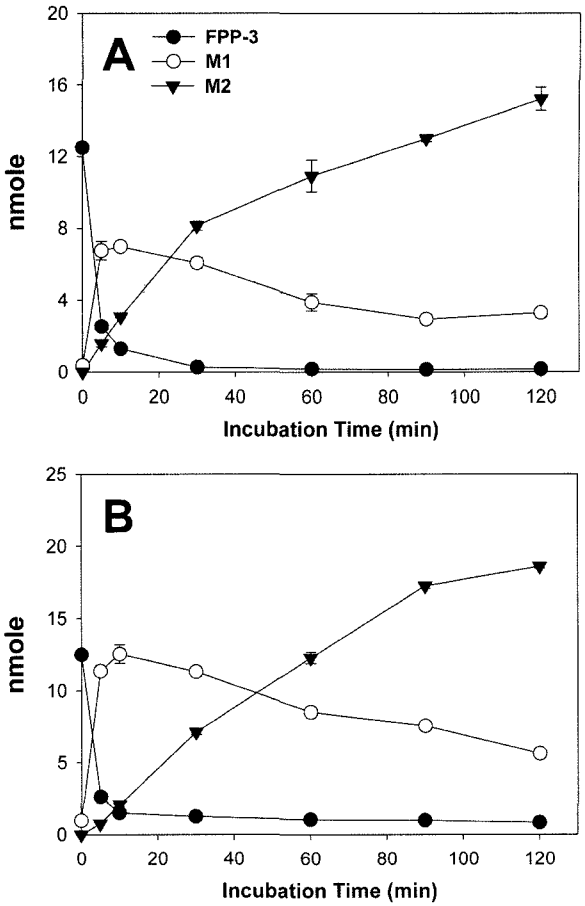

Fig. 5. Time-dependent production of individual metabolites of FPP-3. Metabolism of FPP-3 (25 μM, final concentration) was determined with 1 mg/mL of either microsome (A) or cytosol (B) protein in 0.1 M potassium phosphate buffer, pH 7.4, at 37°C for 120 min. The reactions were initiated by the addition of an NADPH-generating system into the reaction mixture.

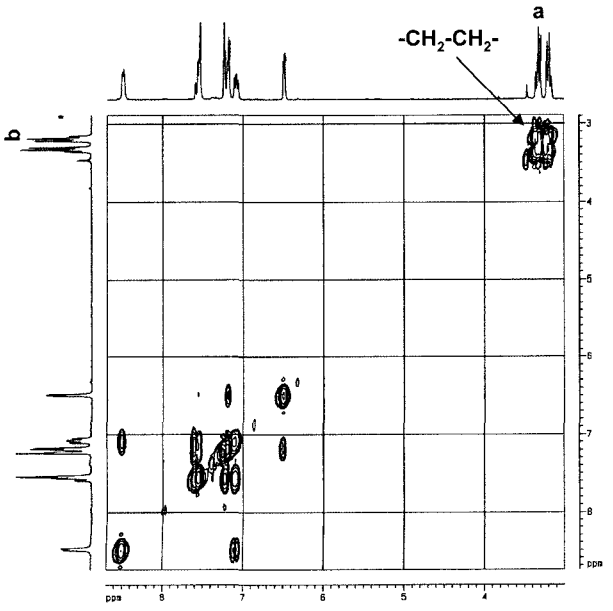

Fig. 4. ^{1}H, ^{1}H -COSY spectra of M1

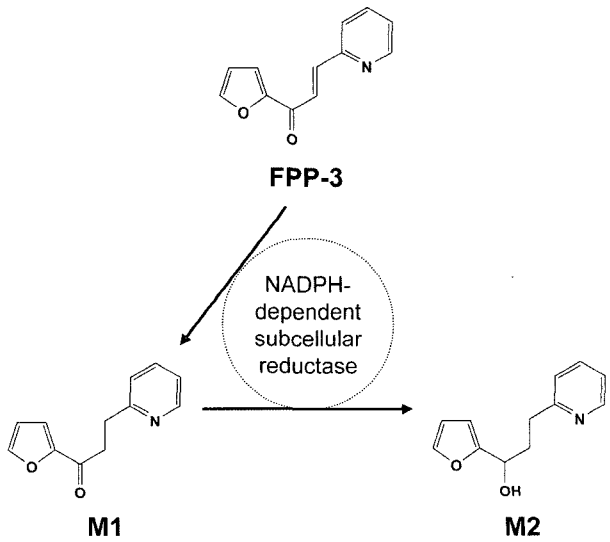

Fig. 6. Proposed metabolic pathway of FPP-3 in rat liver microsomes and cytosols

steps: one is the formation of M1, the initial reduction of FPP-3 by hydrogenation on C=C bond of acrylaldehyde, and the other is the formation of M2 by carbonyl reduction of M1.

DISCUSSION

FPP-3, a synthetic propenone compound, was found to inhibit LPS-stimulated NO and TNF-α production in RAW 264.7 macrophages *in vitro* (Lee *et al.,* 2004) and could not only inhibit COX and 5-LOX activity but also inhibit COX-2 by 35-times more selectively than COX-1 (Jahng *et al.,* 2004). FPP-3 has more potent analgesic and anti-inflammatory effects than conventional NSAIDs such as indomethacin without the gastric ulcer at all in mice and rat (Lee *et al.,* 2006). In this study, we investigated the reductive metabolism of FPP-3 in the rat liver microsome and cytosol.

In these studies, two metabolites of FPP-3 were produced in rat liver microsomes or cytosols in the presence of NGS. Individual metabolites were observed at *m/z* 202 and 204, respectively (Fig. 1). The M1 was 2 Da heavier than the protonated FPP-3 and the M2 was 2 Da heavier than M1. We proposed that the production of M1 was the reduction on C=C bond of acrylaldehyde in FPP-3, and that the production of M2 was by a novel hydroxyl group by carbonyl reduction on M1. The structure of M1 could be confirmed by using ^{1}H-NMR and ^{13}C-NMR, because in case of M1, two kinds of structures could have been possible – i.e., 1-(furan-2-yl)-3-(pyridin-2-yl)-propan-1-one and 1-(furan-2-yl)-3-(pyridin-2-yl) prop-2-en-1-ol. Fortunately, however, we were able to assign only one metabolite (i.e., 1-(furan-2-yl)-3-(pyridin-2-yl)-propan-1-one) by LC/ESI-MS, ^{1}H-NMR and ^{13}C-NMR. According to the results, individual metabolites were determined as 1-furan-2-yl-3-pyridin-2-yl-propan-1-one for M1 and 1-furan-2-yl-3-pyridin-2-yl-propan-1-ol for M2, respectively (Figs. 2 and 3). In addition, the determination of (S)- or (R)-configuration of M2 was important to develop the anti-inflammatory drug. Although the specific configuration of M2 was not characterized in the present study, it would be decided when the M2 with confirmed absolute configurations was chemically synthesized. Studies to characterize stereospecific structure of M2 produced in the present study are currently underway.

Metabolism of FPP-3 might be divided into two steps: the production of M1 from FPP-3 and the subsequent production of M2 from M1 (Figs. 4 and 5). When only M1 was incubated with the subcellular fractions, M2 was produced (data not shown). Because the enzymes involved in the metabolism of FPP-3 were not characterized in the present study, the enzymes could only be speculated at the present time. The hydrogenase (EC 1.12) may contribute to the metabolism of FPP-3 to M1, because the hydrogenases are widespread in prokaryotic and eukaryotic biological systems (Mertens and Liese, 2004). In addition, various enzymes are involved in the carbonyl reduction in microsomes and cytosols. The enzyme(s) for metabolism of M1 to M2 could be suggested to be HSDs in microsomes and CBR in cytosols, because it is generally accepted that HSDs and CBR play important roles in phase 1 biotransformation of pharmacologically active carbonyl compounds (Maser, 1996; Maser *et al.,* 2003). The possible involvement of these enzymes in FPP-3 metabolism is currently under investigation.

In the present study, FPP-3 was found to be metabolized to two metabolites by the reduction. In addition, reasonable structures of two metabolites of FPP-3 could be proposed by using the LC/ESI-MS and ^{1}H-NMR. These proposed structures for the assigned metabolites are summarized with possible metabolic fates in Fig. 6. Knowledge of the proposed structures of the metabolites will be helpful in studies of *in vivo* metabolism of FPP-3 and the specific enzymes related to the reduction of FPP-3.

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