NOTE

Biotransformation of (+)-Fenchone by *Salmonella typhimurium* OY1002/2A6 Expressing Human CYP2A6 and NADPH-P450 Reductase

Hiroshi Nakahashi¹, Nobuo Yagi² and Mitsuo Miyazawa^{1*}

¹ Department of Applied Chemistry, Faculty of Science and Engineering, Kinki University, 3-4-1, Kowakae, Higashiosaka-shi, Osaka 577-8502, Japan

² Picaso Cosmetic Laboratory Limited, 9-20 Ikea-cho, Nishinomiyashi, Hyogo 662-0911, Japan

Abstract: In this study, biotransformation of (+)-fenchone (compound 1) by *Salmonella typhimurium* OY1002/2A6 expressing human CYP2A6 and NADPH-P450 reductase yielded two oxidized metabolites, namely, (+)-(1S,6R)-6-*endo*-hydroxyfenchone (compound 2) and (+)-(1S,6S)-6-*exo*-hydroxyfenchone (compound 3). The conversion rate of compound 1 to compound 2 and 3 was 2.4% and 5.2%, respectively. This is the first study that succeeded in metabolizing compound 1 to obtain large amounts of metabolite 2 and 3 by using *S. typhimurium* OY1002/2A6 expressing human CYP2A6 and NADPH-P450 reductase.

Key words: *S. typhimurium* OY1002/2A6, (+)-fenchone, biotransformation, CYP2A6

1 INTRODUCTION

Study of biotransformation of various materials by using biocatalysts is important for elucidating the metabolic pathways in the human body¹⁻³. Terpenoids are known not only as raw materials for flavor and fragrance, but also as biologically active substances^{4,5}. In our previous studies, we examined the *in vitro* metabolism of monoterpenoids by using human liver microsomes and recombinant enzymes⁶⁻¹⁰. Although terpenoids are essential for life, their metabolism in the human body has not been clarified.

(+)-Fenchone (compound 1), a bicyclic monoterpene, is widely distributed in plants and found in oil extracts from *Foeniculum vulgare* and *Lavandula luisieri*^{11, 12} and has a camphoraceous fragrance¹³. Compound 1 is used as a food flavor and in perfumes, even though compound 1 has been reported to be toxic¹⁴. P450 enzymes have been shown to detoxify and/or toxify cells by converting these compounds to more polar and sometimes to more reactive metabolites¹⁵⁻¹⁷.

We have reported that larvae of the common cutworm (*Spodoptera litura*) convert compound 1 to (+)-(1S,6R)-6-*endo*-hydroxyfenchone (compound 2), (+)-(1S,6S)-6-*exo*-hydroxyfenchone (compound 3), (+)-(1R)-10-hydroxyfenchone, and (+)-(1S,5S)-5-*exo*-hydroxyfenchone,

and that rabbits convert compound 1 to 8-hydroxyfenchone^{18, 19}. Other studies have indicated that cultured cells of *Eucalyptus perriniana* convert compound 1 to several glycosidic metabolites²⁰. In particular, compound 1 was oxidized to compound 2 and compound 3 by the human liver microsomal cytochrome (P450) enzymes cytochrome P450 (CYP2A6) and NADPH-P450 reductase⁷. However, because production of large concentrations of metabolites by using this experimental system was difficult, the metabolites were only identified using gas chromatography (GC) and a combination of gas chromatography-mass spectrometry (GC-MS). We therefore, performed similar reactions using *Salmonella typhimurium* OY1002/2A6 and successfully obtained large amounts of metabolites²¹.

In this study, the biotransformation of compound 1 by *S. typhimurium* OY1002/2A6 expressing human CYP2A6 and NADPH-P450 reductase as a biocatalyst was investigated for the capacity of this microorganism to produce large concentrations of reaction metabolites and to further elucidate the metabolic pathway in humans.

*Correspondence to: Mitsuo Miyazawa, Department of Applied Chemistry, Faculty of Science and Engineering, Kinki University, Kowakae, Higashiosaka-shi, Osaka 577-8502, Japan

E-mail: miyazawa@apch.kindai.ac.jp

Accepted December 17, 2012 (received for review November 12, 2012)

Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online

<http://www.jstage.jst.go.jp/browse/jos/> <http://mc.manuscriptcentral.com/jjocs>

2 MATERIALS AND METHODS

2.1 Materials

(+)-Fenchone (**1**) was purchased from Fluka (Tokyo, Japan). Isopropyl β -D-thiogalactoside was purchased from Wako Pure Chemical (Osaka, Japan), and δ -aminolevulinic acid (δ -ALA) was from Sigma Chemical Co. (St. Louis, MO, USA).

2.2 Strains

S. typhimurium OY1002/2A6 was constructed by introducing plasmid pCW'/2A6: hNPR (carrying cDNA of CYP2A6 and NADPH-P450 reductase), which is an isopropyl β -D-thiogalactopyranoside (IPTG)-inducible bicistronic construct, and *S. typhimurium* TA1535 was constructed by introducing the plasmid pOA101 (carrying the umuC' lacZ fusion gene)^{22–24}.

2.3 Preculture of *S. typhimurium* OY1002/2A6

A single colony of *S. typhimurium* OY1002/2A6 was grown overnight in LB medium containing ampicillin (50 mg/L) at 37°C with shaking (160 rpm).

2.4 Time course in the biotransformation of (+)-fenchone by *S. typhimurium* OY1002/2A6

Precultured *S. typhimurium* OY1002/2A6 was transferred into a 300-mL Erlenmeyer flask containing 100 mL of LB medium supplemented with ampicillin (50 mg/L). The culture conditions were the same as above until the culture reached an optical density of 0.6–0.8 at 600 nm. Thereafter, 0.2 M potassium phosphate buffer (10 mL, pH 7.0), 1 M IPTG (100 μ L), trace elements (25 μ L), 10% glycerol (400 μ L), 1 M δ -ALA (50 μ L), and compound **1** (20 mg, 0.13 mmol) in 1.0 mL of dimethyl sulfoxide (DMSO) were added to the Erlenmeyer flask. The culture was stirred at 37°C for 24 h; thereafter, the medium was salted out with NaCl and extracted with diethyl ether. The extract was analyzed by GC. The ratio of the amount of substrate **1** and to that of metabolic products was determined on basis of the peak areas of GC and GC-MS.

2.5 Isolation of metabolites

Precultured *S. typhimurium* OY1002/2A6 was transferred into a 300-mL Erlenmeyer flask containing 100 mL of LB medium supplemented with ampicillin (50 mg/L) and stirred at 37°C until the optical density of the culture reached 0.6–0.8 at 600 nm. Thereafter, 0.2 M potassium phosphate buffer (10 mL, pH 7.0), 1 M IPTG (100 μ L), trace elements (25 μ L), 10% glycerol (400 μ L), 1 M δ -ALA (50 μ L), and compound **1** (20 mg, 0.13 mmol) in 1.0 mL of dimethyl sulfoxide (DMSO) were added to the Erlenmeyer flask. The medium was stirred at 37°C for 24 h. In order to isolate the metabolites, several similar small-scale cultures were carried out. Subsequently, the medium was salted out with NaCl and extracted with diethyl ether. The diethyl

ether extracts were mixed and dried over Na₂SO₄, and the solvent was evaporated to yield the crude extract (1264 mg). The extract was separated by chromatography using silica-200 columns with diethyl ether-hexane (as explained below). Metabolite **2** (4.8 mg, 2.4%) and **3** (10.4 mg, 5.2%) were isolated.

2.6 Chromatography

For column chromatography (CC), silica gel developed with a hexane-EtOAc gradient was used. For GC, a 5890N gas chromatograph (Agilent Technologies Inc.) equipped with a flame ionization detector, a HP-5 capillary column (30 m length, 0.25 mm inner diameter), and a split injection of 20:1 was used. Helium was used as a carrier gas at a flow rate of 1 mL/min. The oven temperature was programmed from 40°C to 270°C at a rate of 4°C/min. The injector and detector temperatures were 270°C and 280°C, respectively. System software control and data analyses were performed with GC Chemstation software (Agilent Technologies Inc.).

For GC-MS analysis, a 5890A gas chromatograph (Hewlett-Packard) equipped with a split injector HP-5MS capillary column (30 m length, 0.25 mm inner diameter), was combined by direct coupling to a 5972N mass spectrometer (Hewlett-Packard). The same temperature program described above was used. Helium at 1 mL/min was used as a carrier gas. The electron energy was 70 eV, and the ion source temperature was 280°C. Nuclear magnetic resonance (NMR) was performed at 400 MHz (¹H) and 100 MHz (¹³C), in CDCl₃ with tetramethylsilane (TMS) as the internal standard.

3 RESULTS AND DISCUSSION

3.1 Biotransformation of compound **1** by *S. typhimurium* OY1002/2A6

The biotransformation of compound **1** by *S. typhimurium* OY1002/2A6 was investigated. For time-course experiments, a small amount of compound **1** was incubated with *S. typhimurium* OY1002/2A6 for 24 h. Compound **1** was transformed into two metabolites, which were confirmed by GC and GC-MS analyses, and the amount of compound **1** was found to decrease gradually (Fig. 1). Metabolite **2** and **3** were not formed without IPTG, suggesting that compound **1** was transformed to metabolite **2** and **3** by *S. typhimurium* OY1002/2A6 only when human cytochrome P450 2A6 and human NADPH-P450 reductase were expressed. Fig. 2 shows the time course for the appearance of the metabolites.

To isolate the metabolites, several repetitions of the small-scale experiments were carried out, and the culture was extracted as described under Materials and Methods. These metabolites were isolated from the diethyl ether

Fig. 1 GC analysis of (+)-fenchone and its metabolites. (+)-fenchone, 6-*endo*-hydroxyfenchone and 6-*exo*-hydroxyfenchone are shown in 1, 2 and 3 respectively.

Fig. 2 Time course of the biotransformation of compound 1 by *S. typhimurium* OY1002/2A6. 2 and 3 are showed in (▲) and (■).

Fig. 3 Biotransformation of (+)-fenchone by *S. typhimurium* OY1002/2A6.

extract, and the structures were determined to correspond to (+)-*(1S,6R)*-6-*endo*-hydroxyfenchone (compound 2) and (+)-*(1S,6S)*-6-*exo*-hydroxyfenchone (compound 3) by ^1H NMR, ^{13}C NMR, and mass spectra with a comparison to reported data^{7,18)} (Fig. 3).

In previous studies, the metabolites synthesized by human liver microsomes and human CYP2A6 had been identified by GC and GC-MS; however, the amount of metabolites produced was insufficient to be confirmed through ^1H NMR and ^{13}C NMR^{6–10)}. In this study, we succeeded in obtaining large quantities of compound 2 and 3 by using *S. typhimurium* OY1002/2A6. The structures of compound 2 and 3 were determined in detail by ^1H NMR and ^{13}C NMR.

In this experimental system using *S. typhimurium* OY1002/2A6 expressing human CYP2A6 and human NADPH-P450 reductase, 200 mg of compound 1 was converted to 4.8 mg of compound 2 and 10.4 mg of compound 3. In previous kinetic analysis, the V_{max}/K_m values for (+)-fenchone 6-*endo* and 6-*exo* catalyzed by CYP2A6 were 150.2 and 166.8 nmol/(min·nmol) P450 respectively⁷⁾. In this study, compound 3 was preferentially produced by a mechanism similar to human microsomal metabolism.

There have been reports that fenchone shows strong species-specific toxicity²⁵⁾. However, detailed investigations about hydroxyfenchone toxicity have not been reported. Finally, we investigated the biotransformation of bicyclic monoterpenes consisting of a carbonyl group, such as (–)-verbenone, (±)-fenchone, and (–)-camphor, by CYP2A6^{6–10)}. The opposite side of the carbonyl group was oxidized by

CYP2A6 in all substrates. The data suggest that the carbonyl group plays an important role.

Many pharmaceutical medicines and agrochemicals are metabolized by CYPs. In particular, CYP2B6 metabolizes a large number of agrochemicals, and is the most essential P450 isoform in agrochemical metabolism^{26, 27)}. Reproduction of the biokinetics of agrochemical metabolism by using *S. typhimurium* OY1002/2B6 and the methods described in this study may assist in elucidation of the biological mechanisms of human metabolism.

In conclusion, the experimental system consisting of *S. typhimurium* OY1002/2A6 expressing human CYP2A6 and NADPH-P450 reductase produced more than several milligrams of metabolite 2 and 3, while previous experimental systems produced only microgram amounts of the two metabolites^{6–10)}.

REFERENCES

- 1) Xing, Z.; Min, Y.; Rui, L.; Jun, Y.; De, A. G. Microbial transformation of curcumin by *Rhizopus chinensis*. *Biocatal. Biotransform.* **28**(5-6), 380-386 (2003).
- 2) Sakata, K.; Oda, Y.; Miyazawa, M. Suppression of SOS-inducing activity of chemical mutagens by metabolite from microbial transformation of (–)-isolongifolene. *J. Agric. Food Chem.* **58**(4), 2164-2167 (2010).
- 3) Marumoto, S.; Miyazawa, M. Biotransformation of bergapten and xanthotoxin by *Glomerella cingulata*. *J. Agric. Food Chem.* **58**(13), 7777-7781 (2010).

- 4) Gholamreza, K.; Mehrdad, I.; Fatemeh, H.; Bamdad, R.; Amirhossein, S. Screening of Acetylcholinesterase Inhibitory Activity of Terpenoid and Coumarin Derivatives from the Genus *Ferula*. *Pharmacologyonline* **1**, 566-574 (2010).
- 5) Yu, Q.; Bi, J. X.; Yan, Z.; Yun, Z.; Gang, F.; Xiao, L. Y.; Sin, Y. P. Characterization of Aroma Active Compounds in Fruit Juice and Peel Oil of Jincheng Sweet Orange Fruit (*Citrus sinensis* (L.) Osbeck) by GC-MS and GC-O. *Molecules* **13**(6), 1333-1344 (2008).
- 6) Miyazawa, M.; Shindo, M.; Shimada, T. Biotransformation of (–)-verbenone by human liver microsomes. *Biosci. Biotechnol. Biochem.* **66**(11), 2458-2460 (2002).
- 7) Miyazawa, M.; Gyoubu, K. Metabolism of (+)-fenchone by CYP2A6 and CYP2B6 in human liver microsomes. *Biol. Pharm. Bull.* **29**(12), 2354-2358 (2006).
- 8) Miyazawa, M.; Gyoubu, K. Metabolism of (–)-fenchone by CYP2A6 and CYP2B6 in human liver microsomes. *Xenobiotica* **37**(2), 194-204 (2007).
- 9) Gyoubu, K.; Miyazawa, M. In vitro metabolism of (–)-camphor using human liver microsomes and CYP2A6. *Biol. Pharm. Bull.* **30**(2), 230-233 (2007).
- 10) Miyazawa, M.; Marumoto, S.; Takahashi, T.; Nakahashi, H.; Haigou, R.; Nakanishi, K. Metabolism of (+)- and (–)-Menthols by CYP2A6 in Human Liver Microsomes. *J. Oleo Sci.* **60**(3), 127-132 (2011).
- 11) Ozcan, M. M.; Chalchat, J. C. Comparison of chemical composition of essential oil obtained from different parts of *Foeniculum vulgare* ssp. piperitum used as condiment. *J. Food Biochem.* **34**(6), 1268-1274 (2010).
- 12) Azucena, G. C.; Fernanda, D.; Jesus, M. R.; Lucia, S.; Jesus, S.; Jesus B. Chemical and biological profiles of *Lavandula luisieri* essential oil from western Iberia Peninsula populations. *Biochem. Syst. Ecol.* **39**(1), 1-8 (2011).
- 13) Costa, R.; d'Acampora, Z. B.; Crupi, M. L.; De, F.; Maria, R.; Valentino, M. R.; Dugo, P.; Dugo, G.; Mondello, L. GC-MS, GC-O and enantio-GC investigation of the essential oil of *Tarchonanthus camphoratus* L. *Flavour Fragrance J.* **23**(1), 40-48 (2008).
- 14) Opdyke, D. L. J. Monographs on fragrance raw materials. Fenchone. *Food Cosmet. Toxicol.* **14**, 769-771 (1976).
- 15) Guengerich, F. P. Human cytochrome P-450 enzymes. *Life sciences* **50**(20), 1471-1478 (1992).
- 16) David, T.; John, T. S.; Sidney, D. N. Contribution of menthofuran to the hepatotoxicity of pulegone: assessment based on matched area under the curve and on matched time course. *J. Pharmacol. Exp. Ther.* **244**(3), 825-829 (1988).
- 17) Khojasteh-Bakht, S. C.; Chen, W.; Koenigs, L. L.; Pater, R. M. Metabolism of (R)-(+)-pulegone and (R)-(+)-menthofuran by human liver cytochrome P-450s: evidence for formation of a furan epoxide. *Drug Metab. Dispos.* **27**(5), 574-580 (1999).
- 18) Miyazawa, M.; Miyamoto, Y. Biotransformation of (+)-(1R)- and (–)-(1S)-fenchone by the larvae of common cutworm (*Spodoptera litura*). *J. Mol. Catal. B: Enzym.* **32**(4), 123-130 (2005).
- 19) Miyazawa, M.; Kameoka, H. Biotransformation and bioavailability of (+)-fenchone to hydroxyfenchone in rabbits. *Chem. Express* **3**(8), 503-6 (1998).
- 20) Orihara, Y.; Furuya, T. Biotransformation of (+)- and (–)-fenchone by cultured cells of *Eucalyptus per-riniana*. *Phytochemistry* **36**(1), 55-59 (1994).
- 21) Nakahashi, H.; Miyazawa, M. Biotransformation of (–)-camphor by *Salmonella typhimurium* OY1002/2A6 expressing human CYP2A6 and NADPH-P450 reductase. *J. Oleo Sci.* **60**(10), 545-548 (2011).
- 22) Inouye, K.; Shinkyo, R.; Takita, T.; Ohta, M.; Sakaki, T. Metabolism of polychlorinated dibenzo-*p*-dioxins (PCDDs) by human cytochrome P450-dependent monooxygenase systems. *J. Agric. Food Chem.* **50**(19), 5496-5502 (2002).
- 23) Aryal, P.; Yoshikawa, K.; Terashita, T.; Guengerich, F. P.; Shimada, T.; Oda, Y. Development of a new genotoxicity test system with *Salmonella typhimurium* OY1001/1A2 expressing human CYP1A2 and NADPH-P450 reductase. *Mutat. Res.* **442**(2), 113-120 (1999).
- 24) Aryal, P.; Terashita, T.; Shimada, T.; Oda, Y. Use of genetically engineered *Salmonella typhimurium* OY1002/1A2 strain coexpressing human cytochrome P450 1A2 and NADPH-cytochrome P450 reductase and bacterial O-acetyltransferase in SOS/umu assay. *Environ. Mol. Mutagen.* **36**(2), 121-126 (2000).
- 25) Nukenine, E. N.; Adler, C.; Reichmuth, C. Bioactivity of fenchone and *Plectranthus glandulosus* oil against *Prostephanus truncatus* and two strains of *Sitophilus zeamais*. *J. Appl. Entomol.* **134**(2), 132-141 (2010).
- 26) Shimazu, S.; Inui, H.; Ohkawa, H. Phytomonitoring and phytoremediation of agrochemicals and related compounds based on recombinant cytochrome P450s and aryl hydrocarbon receptors (AhRs). *J. Agric. Food Chem.* **59**(7), 2870-2875 (2010).
- 27) Hodgson, E.; Rose, R. L.; The importance of cytochrome P450 2B6 in the human metabolism of environmental chemicals. *Pharmacol. Ther.* **113**(2), 420-428 (2007).