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Involvement of Cytochrome P450 in (-)-(4*R*)-Isopiperitenone Oxidation by Cell Suspension Cultures of *Mentha piperita*

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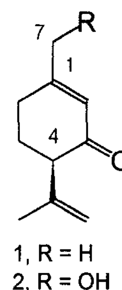
Abstract Biotransformation of exogenous (-)-(4*R*)-isopiperitenone in cell suspension cultures of *Mentha piperita* resulted in oxidized products, with (-)-7-hydroxyisopiperitenone being the major compound. The mass of products obtained under $^{18}O_2$ atmosphere was two units higher than that under normal atmosphere. The biotransformation was inhibited by several cytochrome P450-specific inhibitors as well as by carbon monoxide. Carbon monooxide inhibition was substantially overcome by irradiation of cells with blue light including light at 450 nm wavelength. These results suggested that a cytochrome P450-type monooxygenase was involved in the biotransformation.

Key words: *Mentha piperita*, biotransformation, isopiperitenone, hydroxylation, cytochrome P450

Cytochrome P450-dependent oxidation plays a prominent role in plant terpenoid biosynthesis [1]. It has been observed to catalyze the oxidation of (-)-limonene, the initial step in menthol biosynthesis of *Mentha piperita* L [2]. Biological oxygenation of foreign compounds is one of the important metabolic reactions in plant cells cultured *in vitro* [9]. Although the involvement of cytochrome P450 in biotransformation of exogenous monoterpenes by plant cell cultures has often been implied, rigorous demonstrations have seldom been accomplished until the publication of a report by Tang and Suga [10].

Previous studies demonstrated that *M. piperita* cells cultured as suspension *in vitro* hydroxylate the allylic positions and epoxidize the terminal double bond of (-)-(4*R*)-isopiperitenone [6]. In the oxygenation process of terpenoids by cells, 7-hydroxylation and subsequent glucosylation account for more than 50% of the conversion [5, 7]. The nature of the enzyme(s) which catalyzes the oxidation remains unknown. This paper presents the evidence for the involvement of cytochrome P450-type monooxygenases in the hydroxylation and epoxidation of (-)-(4*R*)-isopiperitenone.



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MATERIALS AND METHODS

General Methods

Electron impact mass spectra (EIMS) were obtained by using an AX505WA mass spectrometer (JEOL, Japan) at 70 eV. GC-EIMS analysis was performed on an AX505WA equipped with HP 5890 series II GC. The GC condition was as described earlier [7]. Light intensity was measured with an IL1700 Photometer (International Light Inc., Newbarryport, U.S.A.).

Reagents

For the suspension culture, plant cell culture-grade reagents were purchased from Sigma (St. Louis, U.S.A.). [^{18}O]-Water (97 atom % ^{18}O) and carbon monooxide gas (99%) were purchased from Aldrich (Milwaukee, U.S.A.). The other reagents were GR grade. (-)-(4*R*)-Isopiperitenone was synthesized from (-)-(4*S*)-limonene as described previously [10].

Culture and Feeding Method

The cell suspension culture of *Mentha piperita* was maintained and cultured as described earlier [5]. Twenty

milligrams of neat (-)-(4*R*)-isopiperitenone per 100 ml culture in a 250-ml Erlenmeyer flask was administered to a 3-week-old culture which contained about 12 g of cells. Cytochrome P450-specific inhibitors were dissolved in 100 μl DMSO before addition. Cells and culture broth were separated after two days of incubation. The broth was extracted with an equal volume of CH_2Cl_2 and concentrated under reduced pressure for direct GC-MS analysis. Isolation of 7-hydroxyisopiperitenone 7-*O*-β-D-glucopyranose was performed as reported earlier [7]. Amount of the (-)-7-hydroxyisopiperitenone was estimated by combining the free compound with the glucoside form, and was used to calculate the hydroxylase activity.

Source of Oxygen for Oxygenation

Labeled oxygen was prepared by electrical decomposition of a mixture of 3 M $Na^{18}OH$ and [^{18}O]-water (1:2, v/v). After electrolysis with 15-20 volts of direct current, $^{18}O_2$ collected at the anode was mixed with N_2 to make an oxygen concentration of 20% (v/v). Before the oxygenation process, the culture flask containing suspension cells supplied with (-)-(4*R*)-isopiperitenone was evacuated and flushed with N_2. The procedure was repeated before the gas mixture of 20% $^{18}O_2$ in N_2 was transferred into the flask. Biotransformation was performed as indicated above.

Recovery of CO Inhibition by Blue Light Irradiation

The atmosphere of the suspension culture was replaced by a mixture of CO and O_2 (4:1, v/v) as mentioned above. Ten microliters of (-)-(4*R*)-isopiperitenone was then administered. The suspension cells were incubated for two days under illumination as follows. Intensity of white light at the surface of the flask was 120 W/m^2, and the intensities of light after passing through blue (350~500 nm) and red filters (>600 nm) were 27 and 16 W/m^2, respectively.

RESULTS AND DISCUSSION

Incubation of (-)-(4*R*)-isopiperitenone with peppermint suspension cells resulted in four hydroxylated and two epoxidized products. Their chemical structures had been determined previously [5, 6]. The experiments here are mainly described in terms of 7-hydroxylation because (-)-7-hydroxyisopiperitenone is the major product of the biotransformation. The other products followed the same pattern as the 7-hydroxy compound.

The source of oxygen for the oxygenation of the monoterpene was examined by $^{18}O_2$ labeling. GC-MS analysis showed that molecular ion peaks of all hydroxylated and epoxidized products were increased by two mass units compared with products obtained under normal atmosphere as shown for (-)-7-hydroxyisopiperitenone (Fig. 1). EIMS of 7-hydroxyisopiperitenone 7-*O*-β-D-glucopyranose [7]

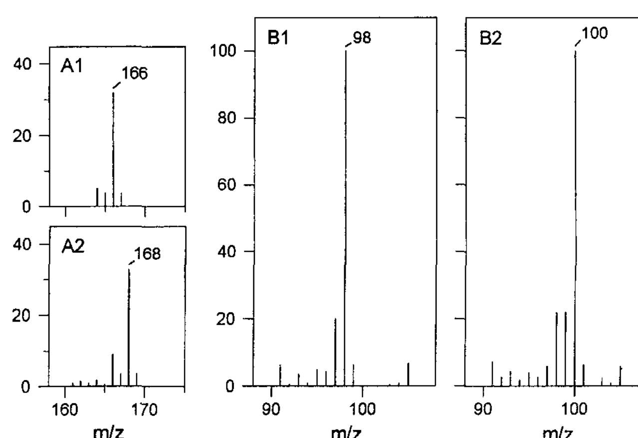

Fig. 1. Regions of interest in GC-EIMS of ^{18}O-labeled 7-hydroxyisopiperitenone (A2 and B2) and the spectra of the control experiment (A1 and B1) in *M. piperita* cell suspension culture.

The peaks at *m/z* 98 and 100 represent the fragments resulting from the loss of isopropenyl side chain and C_4-C_5 moiety, and peaks at *m/z* 166 and 168 represent the molecular ions.

also showed the molecular ion peak at *m/z* 330 instead of at *m/z* 298, suggesting the oxygen atom of aglycon was retained in the glycosylation process. The reaction confirmed the concept that the oxygen source for hydroxylation and epoxidation was molecular oxygen, as is typical for a cytochrome P450-type monooxygenase.

In the biotransformation as described above, the reaction of monooxygenase to several known cytochrome P450-specific inhibitors supported the above concept (Table 1). Isopiperitenone monooxygenase was only moderately sensitive to metyrapone as has been observed for other plant-derived systems [10]. However, binding of metyrapone is known to be selective only for certain forms of the

Table 1. Effect of inhibitors on the 7-hydroxylase activity in *M. piperita* cell suspensions.

Inhibitor	Relative activity (%)	IC_{50} [a] (μM)
Control	100	
KCN (1 mM)	100	
CO + dark	0	
CO + white light	44[b]	
CO + blue light	42[b]	
CO + red light	16[b]	
Metyrapone		> 200
Clotrimazole		22.4
Miconazole		57.9
1-Aminobenzotriazole		14.9
Plumbagin		38.6

[a]Concentration of inhibitor needed to reduce the enzyme activity by 50%.
[b]Corrected for light intensity.

cytochrome [11]. *N*-Substituted imidazoles such as clotrimazole and miconazole had similar inhibitory effects. 1-Aminobenzotriazole with IC_{50} of 14.9 μM was more potent than imidazole inhibitors. Plumbagin, a naphthoquinone known as one of the most potent cytochrome P450 inhibitors [4], completely inhibited the oxygenation of isopiperitenone at 200 μM.

The best-established criterion for cytochrome P450 involvement is the light reversible inhibition by carbon monoxide [10]. Incubation of the suspension cells under an atmosphere of $CO{:}O_2$ (4:1, v/v) in the dark led to a complete inhibition of the hydroxylation, whereas light irradiation induced a partial recovery of hydroxylase activity (Table 1). Irradiation with blue light, including light at 450 nm wavelength, restored the hydroxylase activity of the cells as much as that of the white light. In comparison, when red light was employed, the recovery was only 16% of the control experiment. The difference in recovery of the hydroxylase activity between blue and red light irradiation was consistent with the absorption maximum of cytochrome P450 at 450 nm [10]. In addition, KCN in concentrations of up to 1 mM did not inhibit the isopiperitenone monooxygenase activity, which is typical for cytochrome P450 enzymes. Recently, we found that mRNA of cytochrome P450 in cells of *M. piperita* cultured as suspension was induced by (-)-(4*R*)-isopiperitenone and elicitors as shown by Northern blot analysis [8]. Therefore, the restoration of hydroxylase activity by blue light and the resistance to KCN inhibition were highly suggestive for the involvement of cytochrome P450 in the oxygenation of (-)-(4*R*)-isopiperitenone.

Oxidation of terpenes by plant cells is known to be catalyzed mostly by cytochrome P450-dependent enzymes [2]. 2-Oxoglutarate-dependent cytosolic dioxygenases have also been reported as responsible in kaurene metabolism [3]. All data combined, however, cytochrome P450 must be considered functional in the oxidative biotransformation of monoterpenes in cell cultures of *M. piperita* as has been shown in the case of *Nicotiana* [10].

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REFERENCES

1. Bolwell, G. P., K. Bozak, and A. Zimmerlin. 1994. Plant cytochrome P450. *Phytochemistry* **37:** 1491–1506.
2. Karp, F., C. A. Mihaliak, J. L. Harris, and R. Croteau. 1990. Monoterpene biosynthesis: Specificity of the hydroxylations of (-)-limone by enzyme preparations from peppermint, spearmint, and perilla leaves. *Arch. Biochem. Biophys.* **276:** 219–226.
3. Kwak, S. S., Y. Kamiya, A. Sakurai, N. Takahashi, and J. E. Graebe. 1988. Partial purification and characterization of gibberellin 3β-hydroxylase from immature seeds of *Phaseolus vulgaris* L. *Plant Cell Physiol.* **29:** 935–943.
4. Muto, N., A. Inouye, A. Inada, T. Nakanishi, and L. Tan. 1987. Inhibition of cytochrome P-450-linked monooxygenase systems by naphthoquinones. *Biochem. Biophys. Res. Commun.* **146:** 487–494.
5. Park, S. H., Y. A. Chae, H. J. Lee, Y. H. Lim, and S. U. Kim. 1994. Production of (-)-7-hydroxyisopiperitenone from (-)-isopiperitenone by a suspension cell culture of *Mentha piperita*. *Planta Med.* **60:** 297–390.
6. Park, S. H. and S. U. Kim. 1998. Modified monoterpenes from biotransformation of (-)-isopiperitenone by suspension cell culture of *Mentha piperita*. *J. Nat. Prod.* **61:** 354–357.
7. Park, S. H., K. S. Kim, Y. Suzuki, and S. U. Kim. 1997. Metabolism of isopiperitenones in cell suspension culture of *Mentha piperita*. *Phytochemistry* **44:** 623–626.
8. Son, J. S., Y. J. Chang, Y. D. Choi, and S. U. Kim. 1998. Role of jasmonic acid in biotransformation of (-)-isopiperitenone in suspension cell culture of *Mentha piperita*. *Mol. Cells* **8:** 366–369.
9. Suga, T. and T. Hirata. 1990. Biotransformation of exogenous substrates by plant cell cultures. *Phytochemistry* **29:** 2393–2406.
10. Tang, Y. X. and T. Suga. 1994. Evidence for the involvement of cytochrome P450-dependent monooxygenases in the allylic hydroxylation of terpineols by *Nicotiana tabacum* cell cultures. *Phytochemistry* **37:** 731–735.
11. Testa, B. and P. Jenner. 1981. Inhibitors of cytochrome P450s and their mechanism of action. *Drug Metab. Rev.* **12:** 1–117.