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METABOLISM OF ISOPIPERITENONES IN CELL SUSPENSION CULTURE OF *MENTHA PIPERITA*

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Key Word Index—*Mentha piperita*; Labiatae; peppermint; metabolism; cell suspension cultures; biotransformation; monoterpene glucoside; isopiperitenone; 7-hydroxyisopiperitenone; 7-hydroxyisopiperitenone 7-*O*-β-D-glucopyranoside.

Abstract—The metabolites of (−)-(4*R*)- and (+)-(4*S*)-isopiperitenone in cell suspension cultures of *Mentha piperita* were isolated from the media and cells. Metabolism proceeded to yield 7-hydroxyisopiperitenones excreted into the medium and ultimately accumulated 7-*O*-β-D-glucopyranosides in the cell. The conversion into the glucopyranosides of the alcohols occurred concomitantly with the formation of the alcohols. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

In the course of the study examining the potential for menthol biosynthesis in *Mentha piperita* suspension cells, we found that they possessed extensive hydroxylation activity towards terpenes. Specifically, (−)-(4*R*)-isopiperitenone (**1a**), a normal biosynthetic precursor fed to the cell suspension cultures, was shown to be converted into (−)-(4*R*)-7-hydroxyisopiperitenone (**1b**) [1]. The same plant cell culture was shown to oxidize artemisinic acid, a biogenetic precursor of the potent antimalarial artemisinin, into 3-oxoartemisinic acid via allylic oxidation [2]. This type of oxidative biotransformation or biodegradation of the nascent endogenous products has been ascribed to be the cause of the lack of essential oil accumulation, for example, in tissue cultures of *Rosa* species [3] and *Salvia officinalis* [4]. The involvement of a cytochrome P-450-type enzyme in this type of allylic oxidation of the exogenous materials has been demonstrated [5].

In the leaves of peppermint, menthol and neomenthol undergo acetylation and glucosylation, respectively, for transport to and degradation in the root [6]. Glucoside formation from exogenous terpenoids, including menthol and neomenthol, as well as aromatic alcohols, has also been shown in cell suspension cultures of peppermint [7]. The reported formation of (−)-(4*R*)-7-hydroxyisopiperitenone from (−)-(4*R*)-isopiperitenone thus strongly suggests further metabolism into its glucoside.

The present paper describes the metabolism of (−)-(4*R*)-7-hydroxyisopiperitenone from (−)-(4*R*)-isopiperitenone by suspension-cultured cells of peppermint into its β-glucoside which accumulate in the cells. Also studied was the analogous metabolism of (+)-(4*S*)-isopiperitenone (**2a**), the enantiomer of the normal metabolite **1a**, in this cell culture.

RESULTS AND DISCUSSION

After the administration of (−)-(4*R*)- or (+)-(4*S*)-isopiperitenone to the peppermint cell suspension cultures, the amounts of 7-hydroxyisopiperitenones (**2a** or **2b**) in the medium were determined. Contents reached a maxima on the third day after the administration. The medium on the third day after feeding (+)-(4*S*)-isopiperitenone was thus extracted with CH_2Cl_2 and the major product purified by silica gel CC and HPLC. The physical properties of the isolated metabolite were identical to those of the previously reported (−)-(4*R*)-7-hydroxyisopiperitenone, except for the sign of optical rotation [1]. The cells were analyzed for the presence of terpene alcohols. Only traces of the alcohols were detected suggesting that the product alcohols were excreted into the medium after oxidation in the cells.

The content of 7-hydroxyisopiperitenones reached maxima on the third day after administration and decreased thereafter in both **1b** and **2b**. This suggested further metabolism of the 7-hydroxy compounds.

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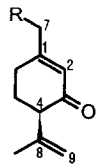
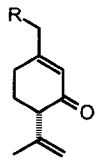
Since glycosylation of the exogenous alcohols administered to the plant cell cultures is known, the polar fraction of the cells and the incubation broth were analysed to demonstrate the formation of glycosides. The culture broth incubated with the isopiperitenones for 7 days was concentrated and subjected to adsorption on XAD-2 followed by elution with MeOH–H_2O (1 : 1) and MeOH. However, no new polar products, presumably monoterpenyl glycosides, were detected in any of the fractions. However, through TLC analysis, the initial MeOH extracts of the cells incubated with the isopiperitenones were shown to contain new polar compounds which had not been found in the control experiment. The polar compounds were purified from the crude MeOH extracts through adsorption on XAD-2 resin, silica gel CC and finally preparative HPLC. The resulting compounds **1c** and **2c** were unequivocally identified as novel β-glucosides of **1b** and **2b** as follows.



1a. R = H
1b. R = OH
1c. R = β-D-glucosyl



2a. R = H
2b. R = OH
2c. R = β-D-glucosyl

Their IR spectra showed absorption bands due to the hydroxyl groups of the sugar (3390 and 1040 cm^{-1}), in addition to the carbonyl and olefinic groups of the monoterpene moiety (1660 and 890 cm^{-1}). Absorption maximum at 234 nm in the UV spectrum indicated one conjugated carbonyl group in the monoterpene moiety. The mass spectra showed a $[M]^+$ at *m/z* 328 and a base peak at *m/z* 98 which resulted from retro-Diels–Alder reaction of the conjugated six-membered carbocyclic moiety. A peak at *m/z* 166 was justified as a fragment due to the cleavage of the glucosidic bond. The ^1H and ^{13}C NMR spectra were assigned based on the 2D NMR spectra (^1H–^1H, ^{13}C–^1H COSY and HMBC). The ^1H NMR spectra revealed the presence of a glucose unit in addition to the 7-hydroxyisopiperitenone moiety. Anomeric proton signals were observed at δ 4.32 (*d*, *J* = 7.7 Hz) for **1c** and at δ 4.31 (*d*, *J* = 7.7 Hz) for **2c**, the coupling constants being typical of β-glucosides. The ^{13}C NMR spectra also showed signals due to the β-glucoside moiety, typical anomeric carbon signals being detected near δ 104 in both **1c** and **2c**.

The identity of the sugar of the glycosides and the configuration at the anomeric position of the sugar moiety were further confirmed by enzymic hydrolysis using β-glucosidase. The released sugar was analyzed by HPLC. The retention time of the sugar from the glycosides was 5.74 min under the given conditions, matching exactly that given by glucose. These results confirmed the presence of a glucose moiety in β-configuration. The analysis of the organic layer by GC also confirmed the presence of either **1b** or **2b** in the glucosides.

Metabolism of terpene alcohols into β-D-glucosides has been well documented. Peppermint cells, especially, are known to possess high glucosyl transferase activity [7]. However, there are conflicting reports on the accumulation site of the glucosides in peppermint cultures. An intracellular presence of the menthyl glucoside [7] or an implied extracellular accumulation [8] has been indicated. Indirect measurement of the glucoside by determining the aglycone which was liberated by the hydrolysis with β-glucosidase treated in the culture, could have resulted in the artefact for the latter case [8]. Our work clearly demonstrates the accumulation of glucosides in the cell. The subcellular accumulation site of the glucoside is not known as yet, though some specialized organelle is highly possible as suggested by the compartmentation of glucosyl transferase in peppermint cells [9].

Fate of the isopiperitenones (**1a** and **2a**) after feeding to the culture was followed in the medium and the cells; representative results obtained showing the general trend are shown in Fig. 1. As reported earlier in the study with open-chain monoterpene oxidation [3] and menthol glucosylation [7], the early onset of the reactions towards the fed substrate was again demonstrated here. The content of the fed substrates decreased rapidly for the first three days with a concomitant increase in the alcohol and the β-D-glucoside content. More than 95% of the isopiperitenones were consumed within one week. After the third day, the content of the alcohols in the medium slowly decreased as the content of the glucoside continued to increase. However, the kinetics of glucoside formation from **1a** was different from that of **2a**. For (−)-(4*R*)-isopiperitenone **1a**, the formation of the glucoside **1c** levelled off after the seventh day of incubation, with a gradual decrease of the intermediate alcohol **1b**. The maximum yield of glucoside **1c** (*ca* 45 μmol) was *ca* 35%. In the case of the (4*S*)-glucoside **2c**, a continuous accumulation until the tenth day after incubation to reach at maximal 60% conversion rate was observed. This difference in the production patterns between the two glucosides might be explained by further metabolism of the (4*R*)-glucoside, as opposed to the (4*S*)-glucoside, which kept accumulating until ten days after the feeding. Glucosylation of menthol in peppermint leaves was indicated as a transport form to be degraded in the rhizome [10]. Preference towards the natural (−)-menthol over (+)-menthol is also reported in peppermint suspension cells [8]. It is known that the glucosyl transferase from peppermint leaves does not discriminate between (−)-menthol and (+)-neomenthol [9]. If one assumes that both of the 7-hydroxyisopiperitenones are equally well glucosylated in the cell culture by the transferase, as is known in peppermint leaves, then discrimination of the hydroxylation enzyme between the substrates **1a** and **2a** could explain the differences in the maximal

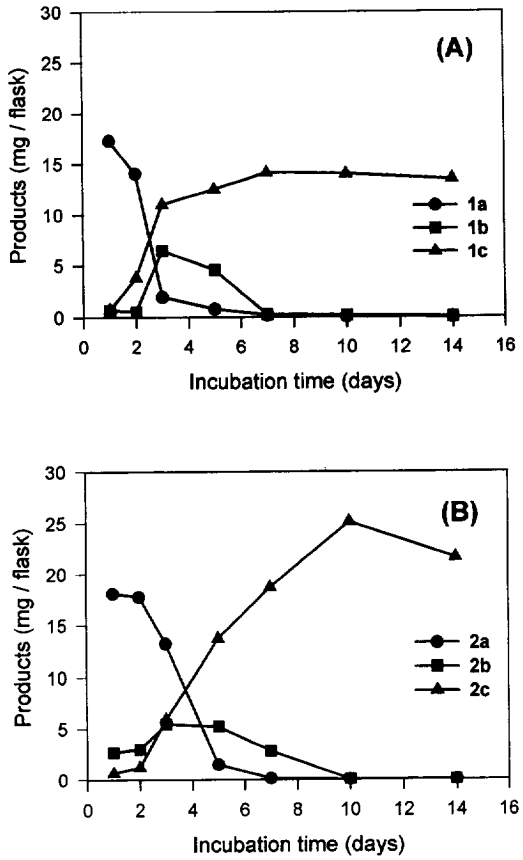


Fig. 1. Changes in contents of isopiperitenones, the 7-hydroxyisopiperitenones and their glucosides in peppermint cell suspension cultures during incubation of (A) (−)-(4*R*)-isopiperitenone and (B) (+)-(4*S*)-isopiperitenone.

amount of glycosides. Further studies on the differences in metabolic patterns should be pursued in order to clarify this observation.

EXPERIMENTAL

Culture and feeding method. Induction and maintenance of peppermint cell suspension cultures was as described earlier [1]. The culture subcultured with an initial inoculum of *ca* 1 g of fr. cell per flask (250 ml) containing the Lin-Staba medium (100 ml) was grown for 2 weeks before the administration of 20 mg of (−)-(4*R*)- and (+)-(4*S*)-isopiperitenone. Cultures were harvested after 7 days of incubation for the isolation of glucosides.

Isolation and identification of (4R)-7-*hydroxyisopiperitenone* 7-O-β-D-*glucopyranoside.* Harvested cells (113 g fr. wt from 10 flasks) were soaked in MeOH (500 ml) and then ground. Cells were separated and extracted a second time with MeOH (500 ml). The MeOH extracts were evapd to dryness and the residue obtained dissolved in 50 ml of H_2O. The residue was chromatographed on XAD-2 column (2.5 cm × 30 cm) with a successive elution of 200 ml of H_2O, 200 ml of 50% aq. MeOH and finally 200 ml of MeOH. The fr. eluted by 50% MeOH was concd and subjected to silica gel CC (100 g, silica gel 60) eluted successively with $CHCl_3$, $CHCl_3$–MeOH (4:1), $CHCl_3$–MeOH (2:1) and $CHCl_3$–MeOH (1:1). The fr. eluted with $CHCl_3$–MeOH (2:1) was finally purified by HPLC. The HPLC conditions were as follows: column, Bondapack C18, 19 mm × 300 mm; solvent, H_2O–MeOH (13:7); detection, UV234 nm; flow rate, 10 ml min^{-1}. The fr. corresponding to the peak at 15.7 min was concd to give a solid. $[\alpha]_D^{22} = 53.9$ (*c* 0.857, MeOH). UV λ_{max}^{MeOH} nm (log ε): 234 (4.10). IR ν_{max}^{KBr} cm^{-1}: 3390 (—OH), 2920 (C—H), 1660 (C=O), 1040 (C—O), 890 (C=C). ^1H NMR (400 MHz, CD_3OD): δ 1.73 (3H, *s*, H-10), 2.04 (1H, *dddd*, *J* = 4.8, 4.9, 4.9, 13.13 Hz, H-5a), 2.15 (1H, *dddd*, *J* = 4.9, 9.0, 10.8, 13.3 Hz, H-5b), 2.41 (2H, *m*, H-6), 3.07 (1H, *dd*, *J* = 4.8, 10.8 Hz, H-4), 3.25 (1H, *m*, H-2′), 3.28 (1H, *m*, H-5′), 3.30 (1H, *m*, H-4′), 3.36 (1H, *m*, H-3′), 3.66 (1H, *dd*, *J* = 5.5, 11.0 Hz, H-6′a), 3.87 (1H, *d*, *J* = 11.0 Hz, H-6′b), 4.29 (1H, *d*, *J* = 16.0 Hz, H-7a), 4.32 (1H, *d*, *J* = 7.7 Hz, H-1′), 4.51 (1H, *d*, *J* = 16.0 Hz, H-7b), 4.75 (1H, *br s*, H-9a), 4.91 (1H, *t*, *J* = 1.6 Hz, H-9b), 6.19 (1H, *t*, *J* = 1.5 Hz, H-2). ^{13}C NMR (100 MHz, CD_3OD): δ 20.9 (C-10), 26.6 (C-6), 28.8 (C-5), 55.8 (C-4), 62.8 (C-6′), 71.4 (C-7), 71.6 (C-4′), 75.0 (C-2′), 78.0 (C-3′), 78.1 (C-5′), 104.0 (C-1′), 114.1 (C-9), 124.9 (C-2), 144.8 (C-8), 164.2 (C-1), 202.2 (C-3). EIMS 70 eV, *m/z* (rel. int): 328[M]$^+$ (11), 195 (35), 166 (76), 148[M − Glucose]$^+$ (74), 98 (100).

Isolation and identification of (4S)-7-*hydroxyisopiperitenone* 7-O-β-D-*glucopyranoside and* (+)-(4S)-7-*hydroxyisopiperitenone.* The procedure for the isolation of (4*S*)-7-hydroxyisopiperitenone 7-*O*-β-D-glucopyranoside was identical to that described above, except that the fr. wt of the harvested cells was 109 g from 10 flasks. Its physical properties were as follows. Solid. $[\alpha]_D^{22} = -14.4$ (*c* 0.457, MeOH). UV λ_{max}^{MeOH} nm (log ε): 234 (4.10). IR ν_{max}^{KBr} cm^{-1}: 3390 (—OH), 2920 (C—H), 1660 (C=O), 1040 (C—O), 890 (C=C). ^1H NMR (400 MHz, CD_3OD): δ 1.73 (3H, *s*, H-10), 2.04 (1H, *dddd*, *J* = 4.8, 4.9, 4.9, 13.4 Hz, H-5a), 2.13 (1H, *dddd*, *J* = 4.9, 9.0, 10.8, 13.4 Hz, H-5b), 2.41 (2H, *m*, H-6), 3.08 (1H, *dd*, *J* = 4.8, 10.8 Hz, H-4), 3.24 (1H, *m*, H-2′), 3.27 (1H, *m*, H-5′), 3.28 (1H, *m*, H-4′), 3.34 (1H, *m*, H-3′), 3.66 (1H, *dd*, *J* = 5.5, 11.9 Hz, H-6′a), 3.87 (1H, *d*, *J* = 11.9 Hz, H-6′b), 4.28 (1H, *d*, *J* = 16.0 Hz, H-7a), 4.31 (1H, *d*, *J* = 7.7 Hz, H-1′), 4.51 (1H, *d*, *J* = 16.0 Hz, H-7b), 4.74 (1H, *br s*, H-9a), 4.91 (1H, *t*, *J* = 1.5 Hz, H-9b), 6.19 (1H, *t*, *J* = 1.5 Hz, H-2). ^{13}C NMR (100 MHz, CD_3OD): δ 20.9 (C-10); 26.7 (C-6), 28.8 (C-5), 55.8 (C-4), 62.8 (C-6′), 71.2 (C-7), 71.6 (C-4′), 75.0 (C-2′), 78.1 (C-3′), 78.1 (C-5′), 103.8 (C-1′), 114.1 (C-9), 125.0 (C-2), 144.8 (C-8), 164.2 (C-1), 202.2 (C-3). EIMS 70 eV, *m/z* (rel. int): 328 [M]$^+$ (14), 195 (31), 166 (77), 148[M—Glucose]$^+$ (79), 98 (100).

(+)-(4*S*)-7-Hydroxyisopiperitenone (**2b**) was isolated from the filtered culture broth fed with **2a** after 3 days of incubation. The isolation method was as

described earlier [1], its physical properties being identical to those of **1b** except for the sign of optical rotation.

Quantitative analysis of metabolites. Cells harvested by filtration after the appropriate days of incubation with the substrate were soaked in 50 ml of MeOH and ground. Cell debris was removed by centrifugation and the MeOH extract concd to 3 ml. Aliquots from the concentrates (100 μl) were adsorbed onto a Sep-Pak Plus C18 cartridge and eluted with 3 ml of MeOH. Then, 20 μl aliquot of the MeOH eluate was subjected to HPLC. The HPLC conditions were as follows: column, Nucleosil 5 C-18, 4.6 mm × 250 mm; solvent, H_2O–MeOH (13 : 7; flow rate, 1 ml min^{-1}; detection at 234 nm. The R_ts of compounds **1c** and **2c** were identical at 7.2 min.

The monoterpene alcohols **1b** and **2b** were determined from the culture filtrate as follows. Cultures were filtered and 2 mg of (−)-(*R*)-carvone was added immediately to the filtrate as int. standard. The filtrates were then extracted × 3 with 50 ml of CH_2Cl_2. The CH_2Cl_2 layer was dried ($MgSO_4$) and concd to 2 ml; 1 μl aliquots of CH_2Cl_2 extracts were subjected to GC. The GC conditions were as follows: column, HP-1, 0.53 mm × 30 m; carrier gas, N_2; flow rate, 15 ml min^{-1}; inj. temp., 250°; det. temp., 300°; oven temp. 120° for 2 min, 120–170° at 5° min^{-1}, 170–250° at 10° min^{-1} and 250° for 5 min; detector, FID. The R_ts of isopiperitenones and 7-hydroxyisopiperitenones were 3.62 and 7.97 min, respectively.

Enzymic hydrolysis of glucosides. A reaction mixt. containing the isolated glucoside (0.5 mg), 1 unit of β-glucosidase from almonds and 100 mM NaOAc buffer (pH 5.0) in a total vol. of 0.3 ml was incubated for 20 hr at 35°. After incubation, the reaction mixt. was extracted with Et_2O for the analysis of the monoterpene alcohols **1b** and **2b**. The Et_2O layer was analysed by GC as described above. Released sugar from enzymic hydrolysis of glucosides was analysed by HPLC. The HPLC conditions were as follows: column, Amide-80, 4.6 mm × 250 mm; solvent, H_2O–MeCN (3 : 7); flow rate, 1 ml min^{-1}; oven temperature, 70°; detection, RI.

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REFERENCES

1. Park, S. H., Chae, Y. A., Lee, H. J., Lim, Y. H. and Kim, S. U., *Planta Medica*, 1994, **60**, 374.
2. Kim, S.-U. and Kwon, H.-J., *Planta Medica*, 1996, **62**, 359.
3. Banthorpe, D. V., Grey, T. J., Poots, I. and Fordham, W. D., *Phytochemistry*, 1986, **25**, 2321.
4. Falk, K. L., Gershenzon, J. and Croteau, R., *Plant Physiology*, 1990, **93**, 1559.
5. Tang, Y. X. and Suga, T., *Phytochemistry*, 1994, **37**, 731.
6. Croteau, R., *Planta Medica*, 1991, **57**, S10.
7. Berger, R. G. and Drawert, F., *Zeitschrift Naturforschung, Teil C*, 1988, **43**, 485.
8. Werrmann, U. and Knorr, D., *Journal of Agricultural Food Chemistry*, 1993, **41**, 517.
9. Martinkus, C. and Croteau, R., *Plant Physiology*, 1981, **68**, 99.
10. Croteau, R., Sood, V. K., Renstrom, B. and Bhushan, R., *Plant Physiology*, 1984, **76**, 647.