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Microbial transformation of the sweet sesquiterpene (+)-hernandulcin

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Abstract – (+)-Hernandulcin is a sweet bisabolane-type sesquiterpene first isolated from *Lippia dulcis* Trev. (Verbenaceae). This oily compound is 1000-1500 times sweeter than sucrose but with poor solubility in water. Microbial transformation was employed to improve its water solubility, and a variety of microorganisms were screened for their ability to convert (+)-hernandulcin to more polar metabolites. Scale-up fermentation with *Glomerella cingulata*, a fungal strain, has resulted in the isolation of a more polar metabolite (2).

Key words – Microbial transformation, *Glomerella cingulata*, (+)-Hernandulcin, *Lippia dulcis*

Introduction

Hernandulcin was first isolated from a plant as the sweet constituent of the leaves and flowers of *Lippia dulcis Trev.* (Verbenaceae) as a result of literature search in the Mexican ethnobotanical literature for the discovery of sweet-tasting plants (Kinghorn *et al.*, 1985). Its sweetness was rated to be 1000-1500 times as sweet as sucrose on the molar basis (Kinghorn *et al.*, 1985). The naturally occurring (+)-hernandulcin is the only stereoisomer with sweet taste, while the other stereoisomers (6S, 1'*R*; 6*R*, 1'*R*; and 6'*R*, 1'*S*) of this compound prepared by chemical synthesis were bitter and somewhat pungent (Kenji *et al.*, 1986). (+)-Hernandulcin is noncaloric, noncariogenic, nontoxic and nonmutagenic compound (Compadre *et al.*, 1985). Even with these attractive features, (+)-hernandulcin is not yet satisfactory to be considered as an ideal sweetener, partly because of its poor water solubility and stability.

Microbial transformation has been effectively used to obtain more polar metabolites of substrate compounds. In the present investigation, a variety of microorganisms were screened for their ability to transform (+)-hernandulcin to more polar metabolites. Scale-up fermentation with *Glomerella cingulata* has resulted in the isolation of a more polar metabolite (2), however the metabolite was suggested to be non-sweet when considered in the view of Shallenberger and Acree's theory (Compadre *et al.*, 1991).

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Experimental

General procedures – IR spectra were recorded using a Jasco FT-IR 300E spectrophotometer. ^{1}H and ^{13}C NMR spectra were run on a Varian Unity Plus 300 spectrometer in CD_3OD, using TMS as an internal standard. Chemical structure of the metabolite was established on the basis of 1D and 2D NMR experiments including ^{1}H-^{1}H COSY, HMQC, and HMBC. Mass spectra were obtained on a Micromass Platform II Mass Spectrometer (UK).

Chromatographic conditions – TLC was performed using Merck Kiesel gel 60 F_{254} TLC plates with 0.2 mm layer thickness. Developing solvent system used for TLC was $CHCl_3$-MeOH (20:1) solution, and visualization of the TLC plates was performed using anisaldehyde-H_2SO_4 spray reagent.

Microorganisms – A variety of Microorganisms were obtained from Korean Collection for Type Culture. The microorganisms used in this experiment were as follows. *Ambrosiozyma cicatricosa* 7256, *Apiotrichum curvatum* 7225, *Bensingtonia intermedia* 7207, *Circinella simplex* 6008, *Cryptococcus neoformans* 7902, *Debaryomyces hansenii* 7000, *Filobasidium capsuligenum* 7102, *Glomerella cingulata* 6075, *Hanseniaspora valbyensis* 7125, *Humicola grisea* 6010, *Kyeromyces marxianus* var. *marxianus* 7155, *Metschnikowio pulcherrima* 7605, *Microbacterium lacticum* 9230, *Monscus ruber* 6122, *Mucor miehei* 6011, *Pachysolen tannophilus* 7937, *Pichia pastoris* 7190, *Saccharomyces calsbergensis* 7233, *Saccharomyces cerevisiae* 7904, *Schwanniomyces occidentalis* var. *occidentalis* 7194.

Culture Media – All preliminary screening and scale-up experiments were carried out in a medium consisting of dextrose 10 g, yeast extract 3 g, malt extract 3 g, bacto peptone 5 g, in distilled water (1000 ml). Stock cultures of fungi and other microorganisms were stored on slants of 20% agar with medium, respectively, at 4°C.

Fermentation procedures – Microbial transformation studies were carried out by incubating cultures with shaking incubator, operating at 250 rpm, at 25°C. Preliminary screening experiments were carried out in 250 ml Erlenmeyer flasks containing 50 ml of medium. The media were sterilized at 121°C and 18 psi for 15 min. Fermentations were carried out according to a standard two-stage protocol. In stage I, the microorganisms were grown in 50 ml medium for 48 h and transferred to fresh medium (stage II). The substrate was added to the incubation media 24 h after the inoculation of the stage II cultures at a concentration of 0.04 mg/ml of stage II medium. Substrate controls were composed of sterile medium to which the substrate was added and incubated without microorganisms. Culture controls consisted of fermentation blanks in which the microorganisms were grown under identical conditions but without the substrate addition.

Biotransformation studies – A total of 40 mg (+)-hernandulcin was dissolved in 1 ml of EtOH and distributed equally among five 1-liter culture flasks each containing 200 ml of 24 h old stage II culture. After 3 days, the entire incubation mixtures were combined and extracted 3 times with the same amount of EtOAc. The solvent was evaporated under reduced pressure to afford 65 mg of crude residues. The crude extracts were separated on preparative TLC using a mixture of $CHCl_3$ and MeOH as an eluent.

Metabolite 1 (2) – Colorless oil: IR (KBr) ν_{max} 3433, 2972, 1664, 1379, 1008 cm^{-1}, EIMS *m/z* 253 [M+1]. ^1H NMR ($CDCl_3$, 300 MHz) δ5.88 (2H, s), δ3.78 (4'H, t, J = 7.5 Hz), δ2.49 (6H, dd, J = 6 Hz), δ2.25 (4H, m), δ1.83 (5H *ax*, m), δ2.02 (5H eq, m), δ1.94 (7H, s), δ1.74 (2'H, t, J = 6.6 Hz), δ1.36 (6'H, s), δ1.21 (7'H, s), δ1.08 (8'H, s), ^{13}C NMR data, see Table 1.

Table 1. ^{13}C NMR chemical shift assignments of (+)-hernandulcin (**1**) and Metabolite 1 (**2**)

Carbon No.	(+)-Hernandulcin*	Metabolite 1
1	204.0 (0)**	199.9 (0)
2	127.4 (1)	127.7 (1)
3	163.6 (0)	161.7 (0)
4	31.2 (2)	30.1 (2)
5	25.0 (2)	27.7 (2)
6	52.0 (1)	52.6 (1)
7	24.1 (3)	24.5 (3)
1'	73.9 (0)	71.0 (0)
2'	40.1 (2)	35.2 (2)
3'	21.5 (2)	29.7 (2)
4'	124.4 (1)	84.8 (1)
5'	131.4 (0)	84.1 (0)
6'	25.7 (3)	25.5 (3)
7'	17.6 (3)	24.1 (3)
8'	23.6 (3)	23.9 (3)

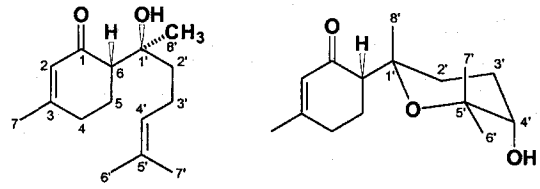
*Nmr data reported in the literature (Compadre, C.M., *et al.*, 1985).

**The numbers in parentheses denote the number of attached protons to the corresponding carbon atoms as determined by APT nmr experiment.

Results and Discussion

The preparative-scale fermentation was performed with 20 species of microorganisms for the screening on the ability of biotransformation of (+)-hernandulcin. The microbial transformation of (+)-hernandulcin using *Glomerella cingulata* led to the isolation of Metabolite 1 (**2**), a more polar biotransformation product.

Metabolite 1 (**2**) had the molecular formula $C_{15}H_{24}O_3$ based on its mass spectrum (M+1, *m/z* 253). The presence of 2-cyclohexen-1-one moiety was indicated clearly in ^1H and ^{13}C nmr spectra. The absence of C-4' methylene signal in the ^1H and ^{13}C nmr spectra together with the appearance of new methine carbon signal corresponding to a hydroxyl group (δC 84.8) suggested that Metabolite 1 (**2**) lacks the double bond between C-4' and C-5' position, and that it was hydroxylated. The position of hydroxyl group at C-4' on the side chain was confirmed by comparison of its nmr data with those of (+)-hernandulcin. Metabolite 1 (**2**) has a cyclized moiety arising from ether linkage formation between C-1' hydroxyl group and C-5'. The



1 **2**

Fig. 1. Chemical structures of (+)-Hernandulcin (**1**) and Metabolite 1 (**2**).

proposed structure was further supported by COSY, HMQC and HMBC experiments by observing the signal connectivities from homonuclear and heteronuclear 2D correlation.

The structural requirement for the sweet taste of hernandulcin, when considered in view proposed by Shallenberger and Acree (Compadre *et al.*, 1988, 1991), includes C-1 keto group, the C-1' tertiary hydroxyl group, and the double bond between C-4' and C-5'. Metabolite 1 (**2**) has a cyclized moiety arising from ether linkage formation between C-1' hydroxyl group and C-5', which caused significant modification in the parent molecular structure, and thus it is suggested that the Metabolite 1 may not be sweet in view of the structure-sweetness relationship model.

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