BIOTRANSFORMATION OF (–)-VERBENONE BY *Aspergillus tamarii* AND *Aspergillus terreus*

Kudret YILDIRIM[1,*] and Semra YILMAZER-KESKIN[2]

Chemistry Department, Sakarya University, 54187, Sakarya, Turkey; e-mail: [1] kudrety@sakarya.edu.tr, [2] syilmazer@sakarya.edu.tr

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The biotransformations of (–)-verbenone by *Aspergillus tamarii* and *Aspergillus terreus* were described. The biotransformation of (–)-verbenone with *A. tamarii* and *A. terreus* for 7 days gave (–)-10-hydroxyverbenone. The biotransformation of (–)-verbenone by *A. tamarii* resulted in a higher yield. *A. tamarii* and *A. terreus* were first two microorganisms to hydroxylate (–)-verbenone.

Keywords: (–)-Verbenone; *Aspergillus tamarii*; *Aspergillus terreus*; Biotransformations.

Flavoring compounds are of considerable importance to the food, perfumery and pharmaceutical industries[1]. The investigation of widely and enormously spread cheap and readily available natural terpenoids for biotechnological production of the valuable compounds by biotransformations is driven by special interest in the production of natural flavors and fragrances[2].

Verbenone, a bicyclic ketone, is a natural monoterpene. It is a component of essential oils from *Rosmarinus officinalis* L., *Verbena triphylla* and *Eucalyptus globulus*. (–)-Verbenone (**1**), its levorotatory enantiomer, has been used for a herbal tea, a spice and a perfume due to its spicy odor and camphoraceous fragrance[3]. (–)-Verbenone has also been used as a natural antiattractant pheromone for bark beetle control in pine trees[4], and as a starting material for the preparation of cyclobutyl GABA analogues[5], cyclobutane nucleoside and oligopeptides[6].

In the literature, biotransformations of (–)-verbenone (**1**) by *Nicotiana tabacum* cultured cells[7], human liver microsomes[8], rat liver microsomes[9] and some liver microsomal cytochrome P450 enzymes[10] have been reported. While the first biotransformation afforded *cis*-verbanone by reduction of the double bond, the other biotransformations afforded (–)-10-hydroxyverbenone (**2**).

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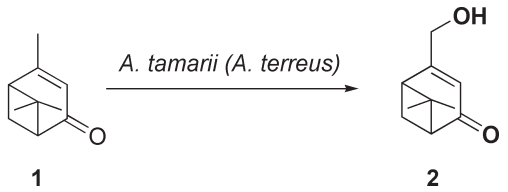
In this paper, we reported the incubations of (–)-verbenone (**1**) with *Aspergillus tamarii* and *Aspergillus terreus*. No previous work on the topic was found in the literature.

RESULTS AND DISCUSSION

The incubation of (–)-verbenone (**1**) with *Aspergillus tamarii* for seven days gave only one metabolite (Scheme 1). IR spectrum of the metabolite showed an absorption at 3430 cm^{-1}. In the ^1H NMR spectrum of the starting material the 10-methyl singlet signal at δ 2.02 was replaced by a new multiplet of methylene group at δ 4.22. In the ^{13}C NMR spectrum of the metabolite, the methyl resonance of starting material at δ 23.30 had been replaced by a secondary carbon resonance at δ 64.04, indicating the presence of a primary alcohol. The metabolite showed an optical rotation of –248.0°. All these results suggested that (–)-verbenone (**1**) is oxidized to (–)-10-hydroxyverbenone (**2**). The exact structure of the metabolite was confirmed by comparison of its spectra (IR, ^1H and ^{13}C NMR) and optical rotation with those in the literature[11]. The optical rotation of the metabolite ($[\alpha]_D^{20}$ –248.0, *c* 0.1, $CHCl_3$) was comparable with that in the literature.



SCHEME 1
Microbial transformations of (–)-verbenone (**1**)

The incubation of **1** with *A. terreus* for seven days also gave only one metabolite. The metabolite was identified as **2**. The structure and stereochemistry of this metabolite were established by comparison of its spectra and optical rotation ($[\alpha]_D^{20}$ –246.0, *c* 0.1, $CHCl_3$) with those of the compound from the previous biotransformation.

CONCLUSION

We have shown that the biotransformations of (–)-verbenone (**1**) by *A. tamarii* and *A. terreus* for seven days afforded (–)-10-hydroxyverbenone (**2**). These two microorganisms converted **1** into **2** as human liver microsomes[8], rat liver microsomes[9] and some liver microsomal cytochrome P450 en-

zymes did[10]. The yield with *A. tamarii* (10.1%) was higher than that with *A. terreus* (8%). *A. tamarii* and *A. terreus* are the first two microorganisms that hydroxylate (–)-verbenone.

EXPERIMENTAL

(–)-Verbenone (94%) was purchased from Sigma–Aldrich and purified by chromatography through a silica gel column eluting with 30% ethyl acetate in hexane. Solvents were of analytical grade and were purchased from Merck. The ingredients for liquid medium were also purchased from Merck. The metabolites were purified by column chromatography on silica gel 60 (Merck 107734) eluting with increasing concentrations of ethyl acetate in hexane. 0.2 mm thick Merck Kieselgel 60 F254 TLC plates were used to check the purity. The spots were visualized with the *p*-anisaldehyde–H_2SO_4 spray reagent. IR spectra (wavenumbers in cm^{-1}) were recorded using a Shimadzu IR Prestige-21. Optical rotation mesurements were carried out on a WXG-4 polarimeter and are given in 10^{-1} deg cm^2 g^{-1} dm^{-1}. ^1H NMR spectra were recorded in deuteriochloroform with tetramethylsilane as an internal standard reference at 300 MHz with a Varian Mercury 300 spectrometer. ^{13}C NMR spectra were recorded in deuteriochloroform at 75 MHz with a Varian Mercury 300 spectrometer. Chemical shifts are given in ppm (δ-scale) and coupling constants (*J*) in Hz. *A. tamarii* Kita MRC 72400 and *A. terreus* Thom MRC 200365 were obtained from TUBITAK Marmara Research Center, Food Science and Technology Research Institute, Culture Collection Unit. Stock cultures were maintained at 4 °C on potato dextrose agar slopes. Gerhardt THO 500 Thermoshake incubator shaker was used for incubations. Biotransformation procedure for each incubation was repeated two times under the same conditions and gave the same results. Biotransformation experiments were monitored by two sets of three different control flasks. The first controls contained the liquid medium and substrate. The second controls contained the liquid medium and microorganism and the third controls contained only liquid medium. After 7 days of incubation, all controls were harvested and analysed by TLC.

Biotransformation of (–)-Verbenone (**1**) by *Aspergillus tamarii*

Glucose (50 g), $NaNO_3$ (2 g), KH_2PO_4 (1 g), KCl (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g) and $FeSO_4 \cdot 7H_2O$ (0.01 g) were mixed in distilled water (1 l) to prepare the liquid medium for *A. tamarii* Kita MRC 72400. The medium was evenly distributed among 10 culture flasks of 250 ml capacity (100 ml in each) and autoclaved at 121 °C for 15 min. Spores were transferred aseptically in a laminar flow hood into one of the flasks containing sterile medium and was incubated at 30 °C and 120 rpm for 3 days. Aliquots (1 ml) from this seed flask were transferred aseptically into the remaining culture flasks and grown for 3 days as above. A clear solution of the substrate (500 mg, 3.33 mmol) in ethanol (10 ml) was then distributed in the culture flasks and fermented for another 7 days. The mycelium was filtered off and washed with ethyl acetate (0.5 l). The broth was then extracted three times with ethyl acetate (3 × 1 l). The collected organic layer was washed with brine and dried over anhydrous sodium sulfate and concentrated in vacuo to afford a brown gum which was chromatographed on silica gel (20 g). Elution with 30% ethyl acetate in hexane gave a colorless oil (90 mg) identified as the unchanged starting material by comparison of its ^1H and ^{13}C NMR spectra with those of an authentic material. Elution with 50% ethyl acetate in hexane gave another colorless oil (56 mg, 10.1%) identified as (–)-10-hydroxyverbenone (**2**) by com-

parison of its spectra and optical rotation ($[\alpha]_D^{20}$ –248.0, *c* 0.1, $CHCl_3$) with those in the literature[11].

Biotransformation of (–)-Verbenone (**1**) by *Aspergillus terreus*

Saccharose (15 g), glucose (15 g), polypeptone (5 g), KH_2PO_4 (1 g), KCl (0.5 g), $MgSO_4$ (0.5 g) and $FeSO_4$ (0.01 g) were mixed in distilled water (1 l) to prepare the liquid medium for *A. terreus* Thom MRC 200365. The biotransformation of (–)-verbenone (**1**) (500 mg, 3.33 mmol) by *A. terreus* at 30 °C and 120 rpm for 7 days was performed as described before and afforded a brown gum which was then chromatographed on silica gel (20 g). Elution with 30% ethyl acetate in hexane afforded a colorless oil (116 mg) idendified as the unreacted starting material by comparison of its ^1H and ^{13}C NMR spectra with those of an authentic material. Elution with 50% ethyl acetate in hexane afforded another colorless oil (44 mg, 8%) idendified as (–)-10-hydroxyverbenone (**2**) by comparison of its spectra and optical rotation ($[\alpha]_D^{20}$ –246.0, *c* 0.1, $CHCl_3$) with those of the metabolite from the previous biotransformation experiment.

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