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BIOTRANSFORMATION OF LIMONENE AND RELATED COMPOUNDS BY *ASPERGILLUS CELLULOSAE*

YOSHIAKI NOMA, SUMIKA YAMASAKI and YOSHINORI ASAKAWA*

Faculty of Domestic Sciences and *Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770, Japan

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Key Word Index—*Aspergillus cellulosae*; biotransformation; (+)-, (−)- and (±)-limonenes; isopiperitenone; limonene-1,2-*trans*-diol; *cis*-carveol; α-terpineol; 1-methylcyclohexene; cyclohexene; 3-methyl-2-cyclohexenone; 2-cyclohexenone.

Abstract—The biotransformation of (+)-, (−)- and (±)-limonenes by *Aspergillus cellulosae* M-77 has been investigated. (+)-Limonene was transformed mainly to (+)-isopiperitenone, (+)-limonene-1,2-*trans*-diol, (+)-*cis*-carveol and (+)-perillyl alcohol, along with the minor formation of isopiperitenol and α-terpineol, whereas (−)-limonene was transformed to (−)-perillyl alcohol, (−)-limonene-1,2-*trans*-diol and (+)-neodihydrocarveol as the major products, along with the minor products such as (−)-isopiperitenone. In the case of the DL-form, perillyl alcohol, limonene-*trans*-1,2-diol, isopiperitenone and α-terpineol were also formed. 1-Methylcyclohexene and cyclohexene were also transformed to 3-methyl-2-cyclohexenone and 2-cyclohexenone via the corresponding alcohols, respectively.

INTRODUCTION

It is considered that the microbiological introduction of an oxygen functional group is important for the formation of biologically active substances because many biologically active compounds possess hydroxyl, carbonyl, carboxylic and epoxide groups, etc. Previously we reported the introduction of a hydroxyl group into terpenoids such as menthol, fenchone, 1,8- and 1,4-cineoles and carvotanacetone by *Aspergillus niger* [1–5] and *Streptomyces bottropensis* [6]. In our continuing biotechnological investigation of the terpenoid biotransformation, we chose (+)-limonene (**1**), (−)-limonene (**1′**) and (±)-limonene (a mixture of **1** and **1′**) as the substrates from a viewpoint of the production of useful compounds from *Citrus* peel oil as biomass, because 7000 ton/year residue of *C. sudachi*, which contains 95% of **1** in the essential oil, has been obtained in *Tokushima prefecture*. The biotransformation of **1**, **1′** and the racemates have been carried out by using many microorganisms [7–15] except for *A. cellulosae*. We now report the biotransformation of limonenes (**1**, **1′** and **1** and **1′**) and related compounds by *A. cellulosae* M-77.

RESULTS AND DISCUSSION

When **1** (*ca* 400 mg) was added to the cultures of 10 kinds of *Aspergillus* sp., all fungi formed (+)-limonene-*trans*-1,2-diol (**5**) as one of the major products. However, two strains of *A. cellulosae*, M-77 and IFO 4040, formed mainly (+)-isopiperitenone (**2**, 19% as peak area in GC), (+)-perillyl alcohol (**3**, 12%) and (+)-*cis*-carveol (**4**, 5%) together with **5** (21%) and other minor products such as isopiperitenol (**6**), *n*-piperitenone and α-terpineol (**7**) after five days. Compound **1** added to the medium as control was not transformed, whereas **1′** was mainly converted to (−)-perillyl alcohol (**3′**, 20% as peak area in GC), (−)-limonene-*trans*-1,2-diol (**5′**, 10%) and (+)-neodihydrocarveol (**8**, 10%) along with the minor amounts of by-products such as isopiperitenol (**6′**), (−)-isopiperitenone (**2′**), (−)-*trans*- and *cis*-carveols (**9** and **4′**), (−)-carvone (**10**) and (+)-dihydrocarvone (**11**) after five days. It is considered that compound **8** was formed via **9**–**11** on the basis of the biotransformation of the intermediates (**4′**, **9**–**11**). In the case of the biotransformation of the racemates (**1** and **1′**), not only perillyl alcohol, isopiperitenone and limonene-*trans*-1,2-diol but also compound **7** were formed as major products. Of limonene biotransformation by the fungus, we were interested in the allylic hydroxylation and dehydrogenation at the C-3 position of **1** to give **2**. Consequently, the analogous biotransformations for 1-methylcyclohexene (**12**) and cyclohexene (**13**) were carried out. Compounds **12** and **13** were transformed via 3-methyl-2-cyclohexenol (**14a** and **b**, insect pheromones [16]) and 2-cyclohexenol (**16**) to 3-methyl-2-cyclohexenone (**15**) and 2-cyclohexenone (**17**), respectively. The biotransformation of **1**, **1′**, **12** and **13** by *A. cellulosae* is shown in Fig. 1.

We consider that the microbiological introduction of an oxygen functional group at the C-3 position of **1**, **1′**, **12** and **13** shown in this study is a very interesting reaction from the viewpoint of both useful utilization of *Citrus* peel oil as biomass and the possibility of the formation of biologically active substances such as *p*-menthane-3,8-diols as mosquito repellent and allelochemicals [1, 17, 18].

EXPERIMENTAL

Microorganisms and cultivation. Aspergillus cellulosae M-77 was cultivated rotatory (120 rpm min^{-1}) at 30° for 1–7 days in

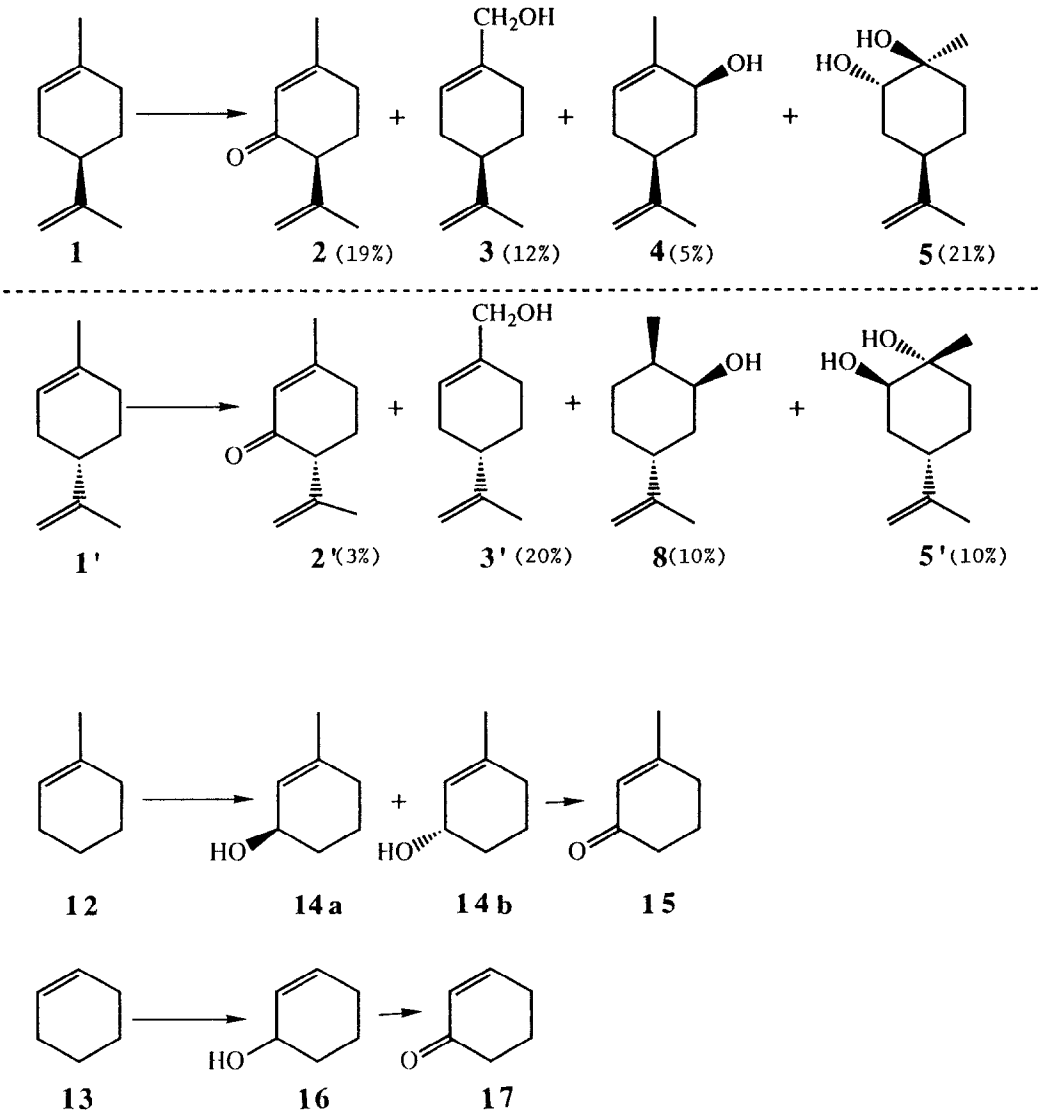

Fig. 1. Biotransformation of (+)- and (−)-limonenes (**1** and **1'**), 1-methylcyclohexene (**12**) and cyclohexene (**13**) by *Aspergillus cellulosae*.

200 ml medium containing sucrose (15 g), glucose (15 g), polypepton (5 g), K_2HPO_4(1 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), KCl (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.01 g) in distilled water (1 l, pH 7.0).

Biotransformation. (+)-, (−)- and (±)-Limonene (**1**, **1'** and the mixture of **1** and **1'**; *ca* 400 mg/200 ml medium) were added to the full growth culture and cultivated under the same conditions as described above. For time course changes an aliquot (*ca* 10 ml) of cultured broth was taken and extd with Et_2O and the extract was examined by GC-MS. For the acquisition of the metabolites, a large scale culture was carried out repeatedly. Recovery yields as the Et_2O extracts of metabolites towards **1**, **1'**, **12** and **13** added were *ca* 7–10%, 7–10%, 1.6% and 1.3%, respectively.

Isolation and identification of the metabolites. The metabolites were sepd and purified by a combination of CC on silica gel and prep. GC. The products were identified by comparison of the GC, R_f, ^{1}H NMR and mass spectra with those of authentic specimens.

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