Highly Efficient Production of Nootkatone, the Grapefruit Aroma from Valencene, by Biotransformation

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Nootkatone (2), the most important and expensive aromatic of grapefruit, decreases the somatic fat ratio, and thus its demand is increasing in the cosmetic and fiber sectors. A sesquiterpene hydrocarbon, (+)-valencene (1), which is cheaply obtained from Valencia orange, was biotransformed by the green algae *Chlorella* species and fungi such as *Mucor* species, *Botryosphaeria dothidea*, and *Botryodiplodia theobromae* to afford nootkatone (2) in high yield.

Key words nootkatone; valencene; biotransformation; *Chlorella*; *Mucor*

Microorganisms are able to transform a huge variety of organic compounds, such as terpene hydrocarbons, alkaloids, steroids, antibiotics, and amino acids.[1)] We are continuing to study the biotransformation of secondary metabolites such as terpenoids and aromatic compounds from crude drugs and spore-forming green plants by microorganisms[2—8)] and mammals[9,10)] to obtain functional substances such as pheromones and perfumes. Recently, it has been clarified that nootkatone (**2**), the most important grapefruit aromatic, decreases the somatic fat ratio,[11)] and therefore its efficient production has been demanded by the cosmetic and fiber industrial sectors. Previously, valencene (**1**) from the essential oil of Valencia oranges was converted into nootkatone (**2**) by biotransformation using *Enterobacter* sp. in only 12% yield,[12)] *Rodococcus* KSM-5706 in 0.5% yield with a complex mixture,[13)] and using cytochrome P450 in 20% yield with other complex products.[14)] Nootkatone (**2**) was chemically synthesized from valencene (**1**) in three steps with $AcOOCMe_3$ and chromic acid in low yield[15)] and using surface-functionalized silica supported by metal catalysts such as Co^{2+}, Mn^{2+}, *etc.* with *tert*-butyl hydroperoxide in 75% yield.[16)] However, these synthetic methods are not safe because they involve toxic heavy metals. An environment-friendly method for the synthesis of nootkatone which dose not use any heavy metals such as chromium and manganese must be designed. Here we report that the commercially available and inexpensive sesquiterpene hydrocarbon valencene (**1**) from Valencia orange oil can be very efficiently converted into nootkatone (**2**) by biotransformations using *Chlorella*,[17)] *Mucor* species,[18)] *Botryosphaeria dothide*, and *Botryodiplodia theobromae*.

Little attention has been paid to the biotransformation of terpenoids and aromatic compounds using the green algae *Chlorella* species. *Chlorella fusca* var. *vacuolata* IAMC-28 was inoculated and cultivated while stationary under illumination in Noro medium (pH 8.0) at 25 °C for 7 d. (+)-Valencene (**1**) (20 mg/50 ml) was added to the medium and biotransformed by *C. fusca* for a further 18 d to afford nootkatone (**2**) (GC-MS peak area: 89%; isolated yield: 63%). The GC-MS spectrum of the crude metabolites obtained from the culture broth by ether extraction is shown in Fig. 1. The reduction of **2** with $NaBH_4$ and $CeCl_3$ gave 2α-hydroxyvalencene (**3**) in 87% yield, followed by Mitsunobu reaction with *p*-nitrobenzoic acid, triphenylphosphine, and diethyl azodicarboxylate to give nootkatol (2β-hydroxyvalencene) (**4**) in 42% yield, which has calcium-antagonistic activity.[17)] Compounds **3** and **4** were easily biotransformed by *C. fusca* and *Chlorella pyrenoidosa* for only 1 d to give nootkatone (**2**) in good yield (80—90%), respectively. The biotransformation of compound **1** was further performed by *C. pyrenoidosa* and *Chlorella vulgaris* to afford nootkatone in good yield, as shown in Table 1. In the time course (Fig. 2) of the biotransformation of **1** by *C. pyrenoidosa*, the yield of nootkatone (**2**) and nootkatol (**4**) without 2α-hydroxyvalencene (**3**) increased with the decrease in that of **1**, and subsequently the yield of **2** increased with decrease in that of **3**. In the metabolic pathway of valencene (**1**), **1** was slowly converted into nootkatol (**4**), and subsequently **4** was rapidly converted into **2**, as shown in Fig. 3.

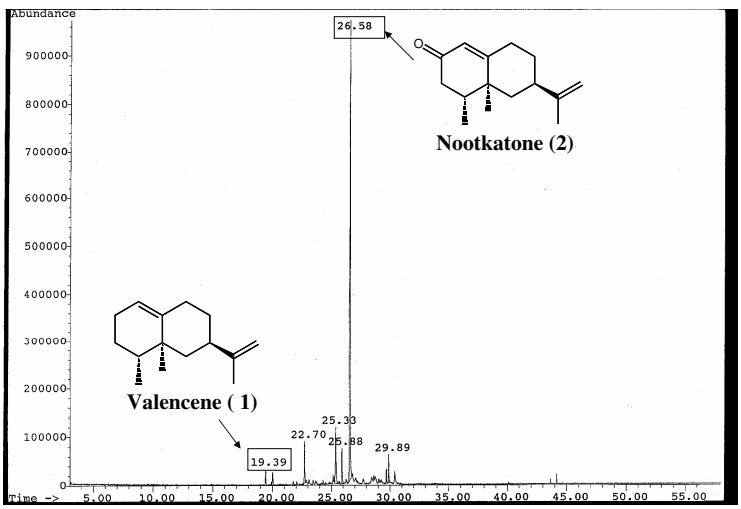

Fig. 1. Total Ion Chromatogram of Metabolites of Valencene (**1**) by *Chlorella fusca* var. *vacuolata*

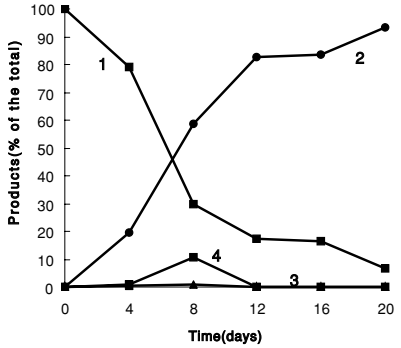

Fig. 2. Time Course Change for the Biotransformation of (+)-Valencene (**1**) by *Chlorella pyrenoidosa* IAM C-101

Compounds: **2**, nootkatone; **3**, 2α-hydroxyvalencene; **4**, nootkatol.

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Table 1. Conversion of Valencene (**1**) to Nootkatone (**2**) by *Chlorella* Species for 14 d

Chlorella sp.	Metabolites (% of the total in GC-MS)				Conversion ratio (%)
	1	**3**	**4**	**2**	
C. fusuca	11	0	0	89	89
C. pyrenoidosa	7	0	0	93	93
C. vulgaris	0	0	0	100	100

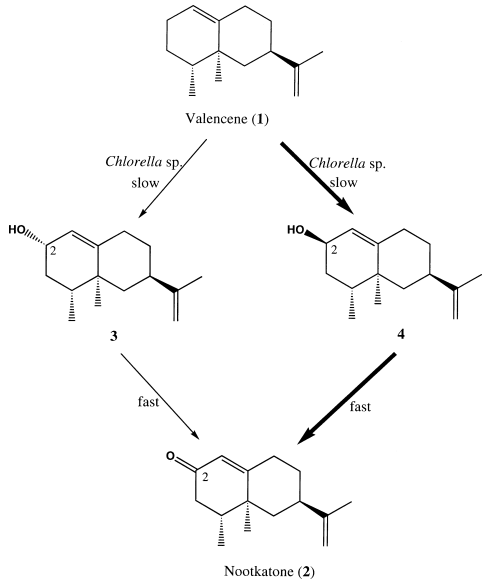

Fig. 3. Possible Metabolic Pathways of Valencene (**1**) by *Chlorella* species

Table 2. Biotransformation of (+)-Valencene (**1**) by *Botryosphaeria dothidea* (in GC-MS)

Botryosphaeria dothidea	Metabolites (% of the total in GC-MS)				Conversion ratio (%)
	1	**3**	**4**	**2**	
Peach PP8402	0	15	21	64	100
Ume BD8398II	8	18	31	43	92
Rose BD8303II	3	8	5	84	97

Compounds: **1**: (+)-valencene; **2**: (+)-nootkatone; **3**: 2α-hydroxyvalencene; **4**: nootkatol.

A fungus strain from the soil adhering to the liverwort *Pallavicinia subcilita* was identified as *Mucor* sp., which was inoculated and cultivated while stationary in Czapek-pepton medium (pH 7.0) at 30 °C for 7 d. Compound **1** (20 mg/50 ml) was added to the medium and incubated for a further 7 d. Nootkatone (**2**) was then obtained in very high yield (82%).

Next, the biotransformation from **1** to **2** was examined using the plant pathogenic fungi *Botryosphaeria dothidea* and *Botryodiplodia theobromae* (a total of 31 strains) separated from fungi infecting various types of fruit, *etc. B. dothidea* and *B. theobromae* were both inoculated and cultivated while stationary in Czapek-pepton medium (pH 7.0) at 30 °C for 7 d. The substrate **1** (20 mg/50 ml) was added to each medium and incubated for a further 7 d to obtain the metabolites. Only the results from five strains characteristic of the 31 strains are shown in Tables 2 and 3.

Table 3. Biotransformation of (+)-Valecene (**1**) by *Botryodiplodia theobromae* (in GC-MS)

Botryodiplodia theobromae	Metabolites (% of the total in GC-MS)				Conversion ratio (%)
	1	**3**	**4**	**2**	
Sudachi BT8603	28	11	20	42	72
Pear BT8002	56	0	9	35	44

Compounds: **1**: (+)-valencene; **2**: (+)-nootkatone; **3**: 2α-hydroxyvalencene; **4**: nootkatol.

In summary, the expensive grapefruit aromatic, nootkatone (**2**) used by cosmetic and fiber manufacturers was obtained in excellent yield by biotransformation of (+)-valencene (**1**), which can be cheaply obtained from Valencia oranges, by *Chlorella* species, fungi such as *Mucor* species, *B. dothidea*, and *B. theobromae*. This is a very inexpensive and clean oxidation reaction that dose not use any heavy metals, and thus this method is expected to find applications in the industrial production of nootkatone.

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