Short Communication

Preparation of Aroma Compounds by Microbial Transformation of Isophorone with *Aspergillus niger*

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Much attention has been paid to the aroma compounds formed by degradation of carotenoids in plants.[1] In the previous paper, the usefulness of the microbial transformation for the preparation of these aroma compounds was demonstrated; β-ionone was converted to more than 13 compounds containing (*S*)-2-hydroxy-β-ionone and (*R*)-4-hydroxy-β-ionone by cultures of a selected strain of *Aspergillus niger*, JTS 191, and the resulting complex was very effective for tobacco flavoring at ppm level.[2,3] As the extension of the studies on the microbial transformation of the trimethylcyclohexane compounds related to carotenoids, the transformation of isophorone (**1**) by the fungus has been investigated. We wish to report here the identification and stereochemistry of four conversion products: 3,5,5-trimethyl-2-cyclohexene-1,4-dione (**2**), 3,5,5-trimethylcyclohexane-1,4-dione (**3**), (*S*)-4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one (**4**), and 3-hydroxymethyl-5,5-dimethyl-2-cyclohexen-1-one (**5**).

The spores of the fungus (4×10^7) were inoculated and cultivated in a liter of a medium consisting of 3% of sucrose, 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4\cdot7H_2O$, 0.1% yeast extract and distilled water, for 48 hr at 28°C under gyratory shaking. To the resulting culture broth which contained mycelial pellets (2～3 mm diameter), 0.1% (wt/vol) of isophorone (**1**) was added as a substrate. The broth was incubated at 28°C under continuous shaking and 10 ml of it was sampled every day and examined by gas chromatography. After 4 days, the conversion products were extracted from the culture broth with ethyl acetate. The organic solution was washed with 5% $NaHCO_3$ *aq.* solution and concentrated *in vacuo*. Four conversion products were observed on a gas chromatogram as shown in Fig. 1. Silicic acid column chromatography and high performance liquid chromatography of the extract gave pure **2**, **3**, **4**, and **5**. The yields of **4** and **5** were about 36% and 35% of the substrate, respectively on the basis of peak areas on the gas chromatogram; dimethyl phthalate was used as an internal standard. Prolonged incubation was needed for complete transformation of the substrate. However, it was always accompanied by partial racemization of **4**.

4: $[\alpha]_D^{23}$ −52.6° ($c=0.9$, methanol), MS *m/z*: 154 (M^+, 5), 112 (37), 98 (100), 70 (37), 69 (30), 43 (20), 41 (23). IR ν_{max}^{film} cm^{-1}: 3400 (OH), 1665 (cyclohexenone). UV λ_{max}^{EtOH} nm: 233

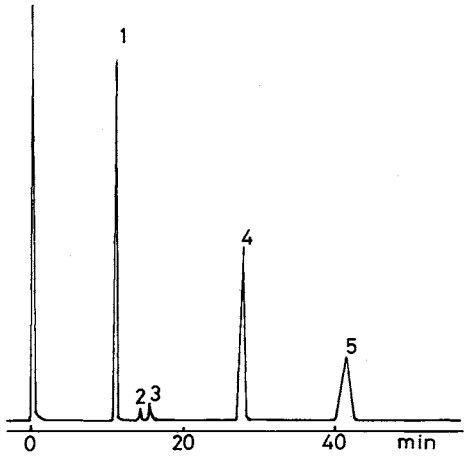

FIG. 1. Gas Chromatogram of Isophorone and Its Microbial Transformation Products.

1: Isophorone, 2: 3,5,5-Trimethyl-2-cyclohexene-1,4-dione, 3: 3,5,5-Trimethylcyclohexane-1,4-dione, 4: 4-Hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one, 5: 3-Hydroxymethyl-5,5-dimethyl-2-cyclohexen-1-one.
Conditions: PEG 20 M (10%) on Chromosorb W (AW·DMCS), 3 mm×2 m; temp., 100°C～5°C/min-210°C; He, 80 ml/min.

(ε=9620). PMR $\delta_{TMS}^{CCl_4}$ ppm: 1.00 and 1.08 (2s, 3H×2, $C(CH_3)_2$), 2.08 (s, 3H, $-C=C-CH_3$), 2.24 (q, 2H, $J_{A,B}$=16, CH_AH_B–CO), 3.99 (broad, 1H, –CH(OH)–), 4.40 (s, 1H, –CH(OH)–, disappeared with the addition of D_2O), 5.82 (broad, 1H, –CO–CH=C–). Oxidation of **4** with chromic acid gave 4-oxoisophorone **2**.

From these physico-chemical data, **4** was identified as chiral 4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one,[4] which was demonstrated to be optically pure by observation of its PMR spectra in the presence of $Eu(tfc)_3$.[5] On application of Horeau's method[6] to **4**, (+)-2-phenylbutanoic acid was liberated showing the *S* configuration at 4-C. The optical yield of the acid was 34.6%.

5: MS *m/z*: 154 (M^+, 47), 139 (M^+-CH_3, 16), 126 (31), 125 (37), 121 (17), 98 (100), 97 (28), 93 (17), 83 (43), 82 (24), 70 (37), 69 (46), 67 (31), 57 (31), 56 (24), 55 (66), 43 (29), 41 (67). IR ν_{max}^{film} cm^{-1}: 3400 (OH), 2950, 2870, 1720 (shoulder), 1660 (CO–C=C), 1120, 1050, 903. UV λ_{max}^{EtOH} nm: 233.5 (ε=13500). PMR $\delta_{TMS}^{CCl_4}$ ppm: 1.07 (s, 6H, $C(CH_3)_2$), 2.16 (2, 2H, $C=C-CH_2-$), 2.22 (s, 2H, $-CO-CH_2-$), 3.77 (very broad, 1H, $-CH_2-OH$), 4.19 (s, 2H, $-CH_2-OH$), 6.08 (broad, 1H, –CH=C–).

The PMR spectrum described above indicated that the olefinic methyl group of isophorone was only converted to hydroxymethyl group. Thus, **5** was deduced to be 3-hydroxymethyl-5,5-dimethyl-2-cyclohexen-1-one, a novel compound. IR, UV, and MS spectra were consistent with this structure.

3 had an IR absorption at 1710 cm^{-1} (non-conjugated carbonyl). The PMR spectrum showed nine protons at 1.09 and 1.19, and other complex signals (5H) between 2.0~3.2 ppm. The MS had a parent peak at *m/z* 154, and other fragments at *m/z* 139 (M^+-15) and 112 (M^+-42). There was no absorption in the UV. On the basis of the data outlined above, **3** was presumed to be 3,5,5-trimethyl-cyclohexane-1,4-dione. The assigned structure was confirmed by its synthesis from the rearrangement of **4**, which was carried out in benzene containing catalitic amounts of *p*-toluene sulfonic acid.[7]

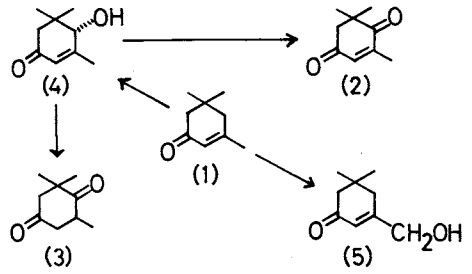

FIG. 2. The Proposed Pathway of Transformation of Isophorone by *Aspergillus niger* JTS 191.

2 had a UV absorption at λ_{max}^{EtOH} 240 nm. The IR spectrum had absorption at 1685 and 1625 cm^{-1}, indicating the presence of a conjugated carbonyl. The MS showed a parent peak at *m/z* 152. The PMR spectrum had signals at 1.22 (s, 6H, $C(CH_3)_2$), 1.98 (d, 3H, *J*=1 Hz, $-C=C-CH_3$), 2.63 (s, 2H, $-CH_2-CO$), and 6.49 (m, 1H, CO–CH=C–). From these data **2** was identified as 3,5,5-trimethyl-2-cyclohexene-1,4-dione, which was confirmed by its synthesis from the oxidation of **4**.

From the time course of fermentation followed by gas chromatography, the conversion pathway of isophorone presumed as Fig. 2. On long standing at room temperature, **4** was spontaneously changed to **2**. **3** had no optical activity, though the fermentation of **2** with baker's yeast gave (*R*)-3.[8] Therefore, it is probable that these minor products were formed from **4** by a chemical process.

In connection with terpene syntheses, **4** is often an important intermediate.[4] Many of C_9-trimethylcyclohexane compounds containing **2**, **3**, and **4** are known as the aroma constituents of saffron,[9] black tea[10] and tobacco.[11,12] These microbial transformation products from isophorone were effective for tobacco flavoring in ppm as a mixture.

The results described above show another useful application of microbial conversion in the preparation of aroma agents.

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