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A NEW PATHWAY FOR THE DEGRADATION OF A SESQUITERPENE ALCOHOL, NEROLIDOL BY *Alcaligenes eutrophus*

K.M. Madyastha* and T.L. Gururaja

Department of Organic Chemistry, Bio-organic Section,
Indian Institute of Science, Bangalore 560 012, INDIA

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Summary: An oxidative pathway hitherto unknown for the degradation of a sesquiterpene alcohol, nerolidol (I) by *Alcaligenes eutrophus* is presented. Fermentation of nerolidol (I) by this organism in a mineral salts medium resulted in the formation of geranylacetone (II) and an optically active alcohol (S)-(+)-geranylacetol (III), as major metabolites. Nerolidol (I) induced cells readily transformed 1,2-epoxynerolidol (IV) and 1,2-dihydroxynerolidol (V) into geranylacetone (II). These cells also exhibited their ability to carry out stereospecific reduction of II into (S)-(+)-geranylacetol (III). Oxygen uptake studies clearly indicated that nerolidol induced cells oxidized compounds II, III, IV, V and ethyleneglycol. Based on these observations a new oxidative pathway for the degradation of I is suggested which envisages the epoxidation of the terminal double bond, opening of the epoxide and cleavage between C-2 and C-3 in a manner similar to the periodate oxidation of diol.

Nerolidol (I), a component of many essential oils, is one of the two most important acyclic sesquiterpenes, the other being farnesol. It is used as a base note in many delicate flowery odor complexes (1). Considerable amount of studies have been carried out on the biotransformation of nerolidol (I) and related compounds by various fungi (2-5). However, bacterial degradation of nerolidol (I) has not been reported so far, although an unsuccessful attempt was made by Yamada *et al.* using *Arthrobacter species* (6). Earlier we have studied the bacterial degradation of monoterpene alcohol linalool, linalylacetate as well as various structurally related terpenoids (7-9). Although nerolidol (I) and linalool are acyclic sesquiterpene and monoterpene alcohols respectively, there exists remarkable structural similarities between these two terpenoids. Hence it was of interest to find out whether the bacterial degradation of nerolidol resembles to that of linalool.

We have isolated a bacterial strain, identified as *Alcaligenes eutrophus* capable of utilizing nerolidol (I) as sole source of carbon and energy by enrichment culture technique. Using this organism the mode of degradation of nerolidol was studied. The present paper describes the isolation and identification of metabolites derived from nerolidol. Based on the identification of the metabolites, experiments carried out using nerolidol induced cells and oxygen uptake

*To whom correspondence should be addressed. **FAX: 812-341683.**

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studies, a new oxidative pathway for the degradation of nerolidol is documented. To the best of our knowledge this is the first report on the bacterial degradation of nerolidol.

MATERIALS AND METHODS

Chemicals: Geranylacetol was prepared by reducing geranylacetone using $NaBH_4$. 1,2-epoxynerolidol (**IV**) was prepared by adapting Sharpless epoxidation procedure (10) [**IV**; PMR($CDCl_3$) (90 MHz, 1:1 diastereomers) δ:1.2 & 1.3 (3H,s,H-15), 1.6-1.7 (9H,2s,H-12,H-13, H-14), 1.8 (1H,s,1 OH), 1.9-2.3 (8H,m,H-4,H-5,H-8,H-9), 2.6-3(3H, m,H-1,H-2), 5.0-5.3 (2H,m,H-6,H-10); MS m/z 238(M^+), 220(M^+-H_2O); HRMS $C_{15}H_{26}O_2$ requires 238.1933, found 238.1909]. 1,2-dihydroxy- nerolidol (**V**) was prepared by acid hydrolysis of 1,2-epoxy-nerolidol (**IV**) [**V**; PMR($CDCl_3$) δ:1.23 (3H,s,H-15), 1.6-1.7 (9H,2s, H-12,H-13,H-14), 1.9-2.2 (11H,m,H-4,H-5,H-8,H-9,3 OH), 3.3(1H,dd, J=2.5 & 12.5Hz,H-2), 4.7 (2H,d,H-1),5-5.2 (2H,br s,H-6,H-10); MS m/z 238(M^+-H_2O). All terpenoid substrates were purified by column chromatography on silica gel using 5-10% ethylacetate in hexane as the eluant.

Analytical Methods: Thin Layer Chromatographic (TLC) analyses were performed on silica gel G plates (0.5 mm) developed with ethylacetate-hexane (1:4) as the solvent system. Spectroscopic and Gas Chromatographic (GC) analyses were carried out as reported earlier (11). The column temperature (10% QF_1 on Chromosorb-W) was maintained at 180°C.

Organism: The organism used in this study was identified as a strain of *Alcaligenes eutrophus*. The organism was maintained in the liquid mineral salts medium (12) containing 0.3% nerolidol and incubated aerobically at 28-30°C. Whenever a starter culture was required, an aliquot (5 ml) from the maintenance culture was transferred to 100 ml sterile liquid mineral salts medium containing 0.3% nerolidol and incubated on a rotary shaker (220 rpm) at 28-30°C for 24 h.

Fermentation Conditions: Degradation experiments were conducted in 500 ml Erlenmeyer flasks containing 100 ml sterile salts medium (pH 7.0) to which 5% of 24 h old inoculum (A_{660}= 1.5) and 0.3% nerolidol were added. The flasks were incubated on a rotary shaker (220 rpm) at 28-30°C for 72 h. A control experiment was also run with the substrate but without organism. At the end of the fermentation period, the contents from all the flasks were pooled, acidified to pH 3-4 using 2N HCl, and extracted with distilled diethylether. The organic layer was then separated into acidic and neutral fraction as described earlier (8).

Resting Cell Experiment: *Alcaligenes eutrophus* cells grown on nerolidol for 48 h were harvested by centrifugation (5000 x g, 20 min) and the cells were washed well with Tris-HCl buffer (25 mM, pH 7.3). The cell paste was then suspended in the same buffer to give a final A_{660} of 1.5. Washed cell suspension (100 ml) was incubated at 28-30°C with 100 mg of the substrate for 12 h on a rotary shaker (220 rpm). After the incubation period, the contents of the flasks were acidified to pH 3-4 with 2N HCl and extracted with diethylether as described under "Methods".

Manometric Studies: Manometric studies were performed with a Gilson 5/6 Oxygraph at 30°C. Oxygen consumption was measured using nerolidol induced cells (48 h) prepared as mentioned under Methods. Reaction mixture consists of freshly washed cells (2 mg dry weight), Tris-HCl buffer (25 mM, pH 7.3) and substrate (0.5 μmol in 10 μl acetone) in a total volume of 2 ml.

RESULTS

From 50 flasks, 1.283 g of neutral fraction (excluding the unmetabolized substrate) and 0.293 g of acidic fraction were obtained. Neutral fraction upon TLC examination revealed the presence of two major compounds (R_f : 0.53 and 0.49) and two very minor compounds (R_f : 0.38 and 0.24). The two major compounds were separated and purified on a silica gel column with 5-10% ethylacetate in hexane. Among the two major compounds, the less polar compound (R_f: 0.53; GC R_t: 3.9 min; 57%) was identified as geranylacetone based on the

spectral data (Fig. 1,**II**). The spectral data is in excellent agreement with the earlier report (6) and also by comparing with the authentic geranylacetone. The medium polar compound (R_f: 0.49 ; GC R_t: 2.8 min; 17%) was characterized as (S)-(+)-geranylacetol on the basis of its spectral data (Fig. 1,**III**). Observed $[\alpha]_D$= +3.4 (C=5.0, $CHCl_3$), reported $[\alpha]_D$= +4.0 (C=6.7, EtOH). The spectral characteristics and optical rotation values fully agreed with the earlier report on this compound (13). However, the two very minor compounds could not be obtained in the pure form, hence their characterization was not achieved. Significant portion of the acidic fraction contained cell material and the amount of the acidic metabolites derived from nerolidol was extremely low and hence could not be processed further.

GC profile of the metabolites formed from the incubation of **I**, **II**, **IV** and **V** with the nerolidol induced cells are depicted in Fig. 2. Incubation of **I**/**IV**/**V** with the nerolidol induced cells resulted in the formation of **II**. Similar experiments carried out with geranylacetone (**II**) yielded (S)-(+)-geranylacetol (**III**). Formation of **II** from **IV** and **V** was confirmed by GC analyses where the peak corresponding to **II** was enhanced (R_t: 3.9 min) when mixed with authentic geranylacetone (**II**). The ability of *Alcaligenes eutrophus* whole cells to oxidize proposed nerolidol pathway intermediates (both synthetic and isolated) was determined by measuring the oxygen uptake rates with nerolidol induced cells. These studies indicated that compounds **II**, **III**, **IV** and **V** showed comparable oxygen uptake (Table 1). In fact ethyleneglycol (**VII**), the metabolite possibly derived from compound **V**, showed better oxygen uptake in contrast to other compounds (**II-V**).

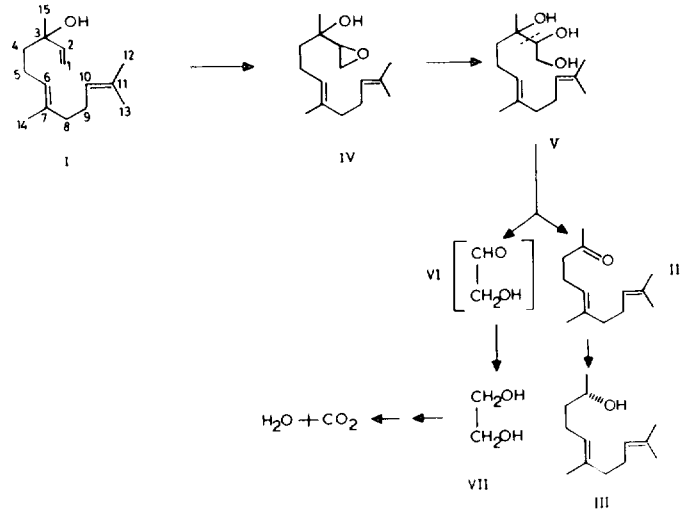

Fig. 1. Proposed pathway for the degradation of nerolidol by *Alcaligenes eutrophus*.

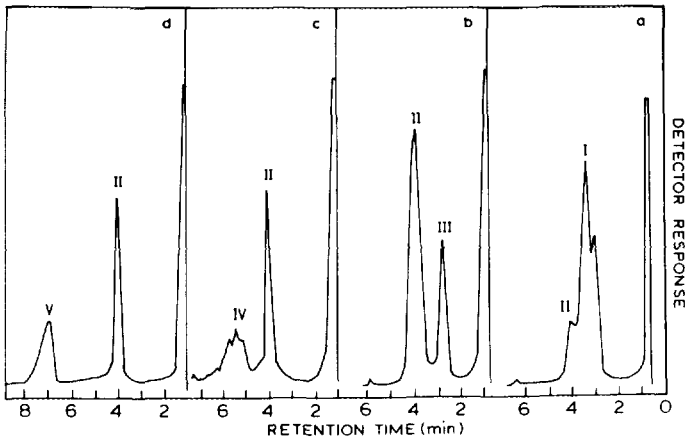

Fig. 2. GC analyses of the reaction products resulted from the incubation of compounds **I/II/IV/V** with nerolidol induced *Alcaligenes eutrophus* cells. (a)-(d) are the product profiles of compounds **I**, **II**, **IV** and **V**, respectively. Control did not show the presence of peak corresponding to R_t: 3.9 min (geranylacetone, **II**).

DISCUSSION

Earlier we have studied the biotransformations of various acyclic monoterpene alcohols in bacterial (7-9), fungal (3), mammalian (14,15) and higher plant (16) systems. One of the striking similarities in all these living systems is their ability to carry out specific ω-methyl hydroxylation. However, the bacterial systems tested so far showed a rigid substrate specificity - while it readily carried out the ω-hydroxylation of tertiary acyclic monoterpene alcohols such as linalool, it failed to accept acyclic primary monoterpene alcohols such as geraniol, citronellol as substrates for the ω-hydroxylation. Contrary to these observations,

TABLE 1. Oxygen uptake by *Alcaligenes eutrophus* cells in presence of various substrates †

Substrates	Oxygen consumed* (nmol/min/mg dry weight)
Nerolidol (**I**)	8.21
Geranylacetone (**II**)	4.11
Geranylacetol (**III**)	3.52
1,2-epoxynerolidol (**IV**)	4.69
1,2-dihydroxynerolidol (**V**)	3.52
Ethyleneglycol (**VII**)	5.86
Glycolic acid	—
Farnesol	—

† Experimental details are described in text.
* All values were corrected for endogeneous respiration.

the fungal systems studied so far showed a broad substrate specificity in their ability to carry out ω-methyl hydroxylation of both primary and tertiary acyclic mono and sesquiterpene alcohols (2,3). Besides, fungal systems are also known to carry out the oxidation of the remote double bond in some of the acyclic sesquiterpene alcohols such as nerolidol and farnesol (5). However, it is interesting to note that *Alcaligenes eutrophus* used in the present investigation failed to carry out the oxidation of either the ω-methyl group or the remote double bond in nerolidol (**I**). Since geranylacetone (**II**) is the major metabolite formed from **I**, it appears that the organism prefers to functionalize the terminal double bond through epoxidation which subsequently opens up to yield the corresponding diol (**V**) (Fig. 1). The resulting triol (**V**) may be cleaved between C-2 and C-3 to generate geranylacetone (**II**) and glycolaldehyde (**VI**). Glycolaldehyde may be converted either to ethyleneglycol (**VII**) or glycolic acid which further gets metabolized to CO_2 and H_2O. However, both growth and oxygen uptake studies clearly revealed that glycolic acid is not further metabolized.

The proposed pathway (Fig. 1) is substantiated based on two important evidences: (i) Cells adapted to nerolidol convert both 1,2-epoxynerolidol (**IV**) and 1,2-dihydroxynerolidol (**V**) into geranylacetone (**II**), (ii) Cells adapted to nerolidol oxidize both compounds **IV** and **V** (Table 1). One can also envisage the formation of geranylacetone (**II**) from farnesol since isomerization of nerolidol (**I**) to farnesol is a feasible process. However, such a process is ruled out since the organism failed to accept farnesol as the substrate. In conclusion, we have demonstrated for the first time that the degradation of nerolidol by *Alcaligenes eutrophus* follows a pathway hitherto unknown.

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