7
Microbial Conversion of Terpenoids

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Introduction

It has been well known that terpenoids are basically formed from biological C5-isoprene units, since Ruzicka (1959) formulated the so-called isoprene rule. The biosynthesis of monoterpenes has been recently reviewed by Charlwood and Banthorpe (1978), and that of terpenoids, in a wide sense, by Croteau (1980).

In general, terpenoids or terpenes are classified into monoterpene (C10), sesquiterpene (C15), diterpene (C20), sesterterpene (C25), triterpene (C30), tetraterpene (C40) and polyterpene (more) according to the multiple of biological isoprenoid units. The group of compounds, produced by degradation of terpenes which have lost part of their carbon(s), are called norterpenes. In a narrow sense, terpenes, the major constituents of essential oils, are monoterpenes and sesquiterpenes.

Many of these terpenes, for example abscisin, corriolin, ionone, ipomeamarone, trichochecin, trisporic acid, etc., exhibit considerable physiological activity. Bioconversion of these and related substances deserves further study because they may yield compounds with new or enhanced activity or new and useful properties for antibiotics, growth promotors, flavour and fragrance.

In recent years, microbial conversion of terpenoids has been reviewed by many authors from various viewpoints (Ciegler, 1969; Wood, 1969; Voishvillo, Akhrem and Titov, 1970; Abbott and Glendhill, 1971; Tatsumi, 1974; Kieslich, 1976; Sebek and Kieslich, 1977; Johnson, 1978; Krasnobajew, 1984; Madyastha, 1984). This review focuses on microbial conversion of terpenoids for practical application in the flavour and fragrance industry, and on bioconversion of terpenes with a potential for further biotechnological development.

As early as 1915, Mayer and Neuberg carried out microbial conversion with terpenoids. Since then, most of the studies have involved the microbial breakdown of acyclic as well as cyclic terpenes isolated from essential oils (Wood, 1969). The results were of more theoretical than practical value.

Biotechnology and Genetic Engineering Reviews—Vol. 6, September 1988
0264–8725/88/06/271–320 $20.00 + $0.00

Difficulties encountered in terpenoid bioconversion are due to the fact that most terpenes, especially mono- and sesquiterpenes, have strong antimicrobial activity (Okamura, 1974). Terpenoids are easily decomposed by micro-organisms in nature and it is not difficult to obtain micro-organisms which grow well on a particular terpene in an enrichment culture. However, many micro-organisms selected through screening and adaptation often convert or degrade the terpenoid substrate into an inseparable complex. The substrate and end products are often lost as volatile compounds during aeration under submerged fermentation (Krasnobajew, 1984).

On the basis of these difficulties and earlier studies, industry neglected looking for possibilities for applying bioconversion to terpenes in contrast to the current application of the fermentation technology to steroid chemistry (Imada, Ishikawa and Nishikawa 1981; Martin, 1984). Recently, however, many attempted industrial uses for terpenoid fermentation have been reported in theoretical and patent literature from various research groups. Some of them have been applied practically to the manufacturing of aroma compounds.

Microbial techniques are very useful in organic synthesis. Stereochemistry is a major subject in flavour and fragrance chemistry as well as in bioactive substance chemistry. Micro-organisms can be used as a class of chiral organic reagents (Sih and Rosazza, 1976) and reagents for optical resolution (Prelog, 1964; Moroe *et al.*, 1971; Jones and Beck, 1976; Oritani and Yamashita, 1983). Microbial technique can also be used to advantage to bring about regiospecific changes in terpenes, which is very difficult to accomplish by organic synthesis. Reaction sites on terpene molecules, which are highly susceptible to chemical reagents, are also easily attacked by corresponding microbial enzyme systems. For example, β-ionone has at least five reaction sites in its trimethylcyclohexene ring. Indeed, four biochemical reactions, which are possible by chemical methods, were performed by the fungus, *Aspergillus niger* JTS 191. In addition a non-activated chemically inert carbon atom was functionalized stereospecifically (Mikami *et al.*, 1981a). The type of reaction can be influenced by the fermentation conditions (Mikami *et al.*, 1981c; Mikami, 1984).

It is not possible to formulate a definitive set of reaction rules at present because only a few systematic studies have been made of the action of micro-organisms on various classes of organic compounds. Nevertheless, some useful generalizations are possible. As a rule, yeasts and some other micro-organisms are most suited to catalysing the asymmetric reduction of carbonyl compounds, whereas fungi have the facility of introducing a hydroxyl group into a wide variety of organic compounds. Many bacteria have the ability to oxidize and utilize organic compounds completely to carbon dioxide and water.

Most microbial enzymes possess substrate specificity but microbial cells contain many different types of enzymes which may give rise to undesired side reactions. For this reason, many organic chemists tend to avoid the use of microbial systems.

Side reactions are characteristic of a natural process. Essential oils always contain a series of minor analogous compounds, which may have an important role in the scent (Ohloff, 1978).

In the 1960s, Bhattacharyya and his co-workers (Bhattacharyya *et al.*, 1960) noticed that some essential oils of plant origin such as 'agarwood', *Aquilaria agallocha*, are produced by the micro-organisms infecting these plants (Bose, 1938). In their broadly based studies, they investigated the capability of micro-organisms, particularly fungi, to convert some mono- and sesquiterpens to oxygenated products which might be of potential interest to the perfume industry. At this time, the natural process for the production of agar essential oils was obscure and they did not know the true precursor for fragrant biodegradation products. The results obtained were of more theoretical than practical interest.

In recent years, much attention has been paid to fragrant norterpenes produced by the degradation of higher terpenoids such as carotenoids, diterpenes and polyterpenes. A large number of compounds with the trimethylcyclohexane ring exist throughout nature. They represent important constituents of many essential oils (Naves, 1976) and are presumably generated from carotenoids by complex enzymatic processes (Ohloff, 1978). Ionone is considered to be a key intermediate in this biodegradation process (Stevens, 1970; Enzell, 1976). It would be useful, therefore, to produce compounds with a trimethylcyclohexane ring through microbial conversion of carotenoids or ionones. Following this reasoning, many micro-organisms were investigated to produce fragrant compounds or aroma complexes from ionone substrates (Mikami *et al.*, 1978, 1981a; Krasnobajew and Helminger, 1982), and some of these results are now in practical use (Mikami, 1984).

Although the microbial conversion of terpenes may appear uneconomical compared with organic synthesis, the development of biotechnology brings about the possibility of increasing the efficiency of bioconversion in the future. This is discussed later.

References cited here are not intended to be exhaustive but, rather, illustrative of the research in various areas of microbial conversion of terpenoids.

Monoterpenes

Monoterpenes are widely distributed in nature and are the main constituents of essential oils. They are used as raw materials for perfume and drugs, and various monoterpenes such as citral, citronellal, limonene and pinene are used in large quantities in the chemical and perfume industry for the production of more valuable compounds.

Monoterpenes have strong antibiotic action against diverse microbial flora, however, some soil bacteria such as *Pseudomonas* have the unique ability to degrade and live on them. In the process of utilizing these compounds as a carbon source, the bacteria carry out various interesting conversions in the molecules before finally degrading them to carbon dioxide and water (Hungund, Bhattacharyya and Rangachari, 1970).

Fungi and most other bacteria are normally unable to utilize the terpene molecule all the way to carbon dioxide and water. However, some of them can metabolize or convert terpenes, when grown in energy-rich media. This is

called co-metabolism (Horvath and Alexander, 1970). The main reactions brought about by micro-organisms are oxidation of primary alcohol to carboxylic acid via aldehyde, allylic oxidation, oxygenation on a double bond, and optical resolution or preferential degradation of one of the antipodes.

Although the majority of studies on microbial conversion of monoterpenes are of theoretical value, few of them have been put to practical use.

β-MYRCENE

β-Myrcene (1)* is found in the oil of bay (*Laurus*), verbena (*Verbena*), hop (*Humulus*) etc., and is also obtained by the pyrolysis of β-pinene (Windholz, 1983).

Pseudomonas putida S4–2, newly isolated as a β-myrcene-utilizing bacterium, accumulated (*E*)-2-methyl-6-methylene-2,7-octadienoic acid (2), 4-methylene-5-hexenoic acid (6) and (*E*)-4-methyl-3-hexenoic acid (7) in the culture broth (Narushima, Omori and Minoda, 1982a). Based on the products identified, a metabolic pathway was proposed (*Figure 1*). The degradation of β-myrcene by *Pseudomonas putida* started with the oxidation of the terminal methyl group. The acid (2) produced seems to be degraded via β-oxidation to form 4-methylene-5-hexenoic acid (6), a C3-unit-eliminated compound. Among the monoterpenes of the analogous carbon skeleton, oxidation of the *gem*-methyl terminal has also been found in the microbial conversion of linalool (Mizutani *et al.*, 1971), linalyl acetate (Renganathan and Madyastha, 1983), and 1,2-dihydrolinalyl acetate (Renganathan and Madyastha, 1984), but has not been found in the conversion of geraniol, nerol, citral and citronellal (Devi and Bhattacharyya, 1977; Joglekar and Dhavalikar, 1969).

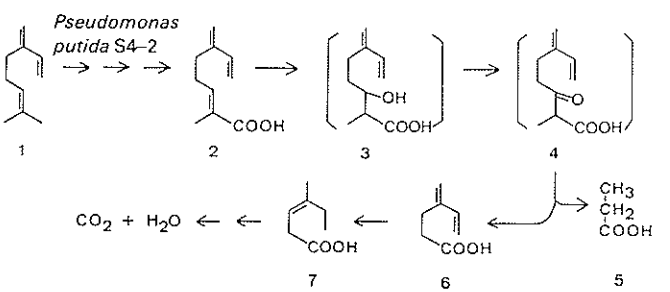


Figure 1. Proposed metabolic pathways of β-myrcene by *Pseudomonas putida*. β-myrcene (1), (*E*)-2-methyl-6-methylene-2,7-octadienoic acid(2), 3-hydroxy-2-methyl-6-methylene-7-octenoic acid(3), 2-methyl-6-methylene-3-oxo-7-octenoic acid(4), propionic acid(5), 4-methylene-5-hexenoic acid(6), (*E*)-4-methyl-3-hexenoic acid(7).

Aspergillus niger JTS 191 has converted β-myrcene by co-metabolism in an energy-rich medium. Major conversion products were 2-methyl-6-methylen-7-

* Throughout this chapter, numbers in parentheses refer to the chemical structures of the compounds, as shown in the relevant Figures.

octene-2,3-diol (8), 6-methyl-2-ethenyl-5-heptene-1,2-diol (9) and 7-methyl-3-methylen-6-octene-1,2-diol (10). The feature of the conversion of β-myrcene by the fungus was the hydroxylation of a double bond resulting in diol (Yamazaki *et al.*, 1988b) (*Figure 2*).

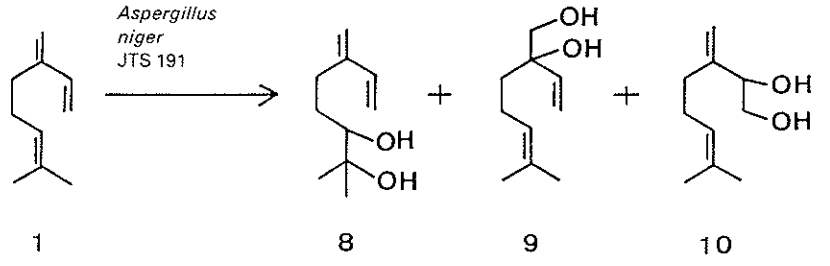


Figure 2. Products resulting from the conversion of β-myrcene by *Aspergillus niger* JTS 191. β-myrcene(1), 2-methyl-6-methylene-7-octene-2,3-diol(8), 6-methyl-2-ethenyl-5-heptene-1,2-diol(9), 7-methyl-3-methylene-6-octene-1,2-diol(10).

LINALOOL

Linalool (11), together with its ester, is the chief constituent of linaloe oil obtained from *Bursera delpechiana* and is also widespread in essential oils.

Mizutani *et al.* (1971) found a cyclization of linalool to camphor (14) using *Pseudomonas pseudomallei* (strain A) isolated from the soil. Other products were 2,6-dimethyl-6-hydroxy-*trans*-2,7-octadienoic acid (12) as well as the degradation products, 2-methyl-2-vinyl-tetrahydrofran-5-one (13) and 4-methyl-*trans*-3-hexenoic acid (15) as shown in *Figure 3*.

In serial studies (Devi and Bhattacharyya, 1977; Madyastha, Bhattacharyya and Vaidyanathan, 1977), *Pseudomonas incognita* (linalool strain), initially isolated by enrichment culture using linalool as the sole source of carbon, produced the following metabolites: 2,6-dimethyl-6-hydroxy-*trans*-2,7-octadienoic acid (12), 2-methyl-2-vinyl-tetrahydrofran-5-one (13), 10-hydroxy-linalool (16), 2-methyl-2-vinyl-5-hydroxyisopropyl-tetrahydrofurane (17), linalool-10-carboxylic acid (18) and oleuropeic acid (19) (*Figure 3*).

Recently, Renganathan and Madyastha (1983) reported the conversion of linalyl acetate by the same *Pseudomonas*. The substrate was metabolized analogously with the acetoxy group intact. From the structure of metabolites, three different pathways for the microbial degradation of linalyl acetate were proposed.

Although these microbial conversions of linalools produced various compounds, a single major metabolite could not be found. However, such metabolite mixtures have the possibility of being used as essential oil-like aroma complexes (*see* page 301).

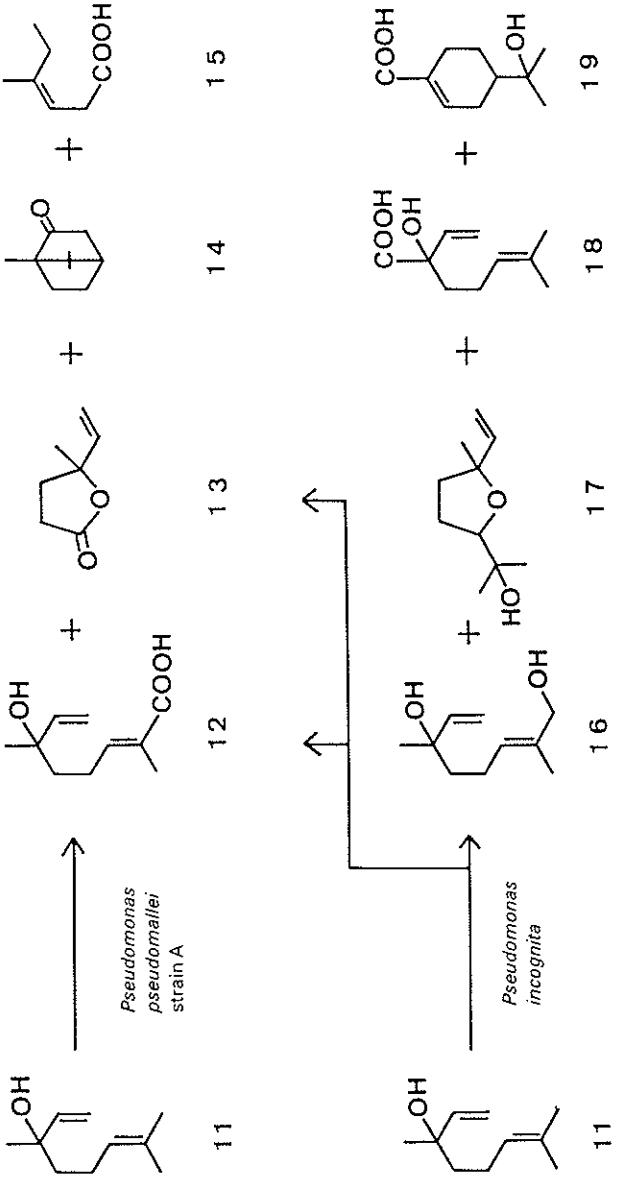


Figure 3. Products resulting from the conversion of linalool by *Pseudomonas*. Linalool(11), 2,6-dimethyl-6-hydroxy-*trans*-2,7-octadienoic acid(12), 2-methyl-2-vinyl-tetrahydrofuran-5-one(13), camphor(14), 4-methyl-*trans*-3-hexenoic acid(15), 10-hydroxylinalool(16), 2-methyl-2-vinyl-5-hydroxyisopropyl-tetrahydrofurane(17), linalool-10-carboxylic acid(18), oleuropeic acid(19).

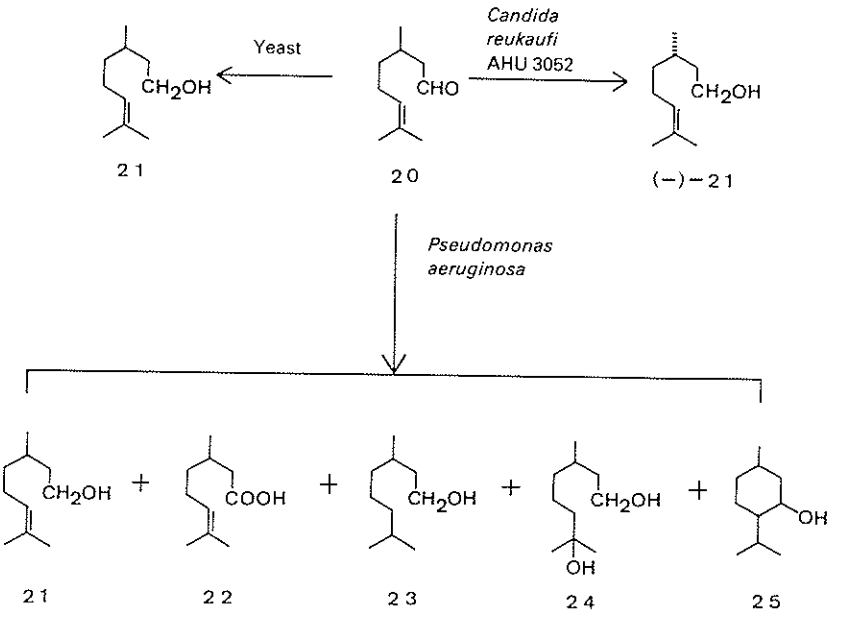


Figure 4. Products resulting from the conversion of citronellal by various micro-organisms. Citronellal(20), citronellol(21), citronellic acid(22), dihydrocitronellol(23), 3,7-dimethyloctane-1,7-diol(24), menthol(25).

CITRONELLAL

In 1915, Mayer and Neuberg reported the reduction of (+)-citronellal (20) to (+)-citronellol (21) by yeast (*Figure 4*). This was the first paper in this research area.

Citronellol exhibits a floral scent and is very important to the fragrance industry. However, (+)-citronellol and (−)-citronellol have different fragrances, the latter being more preferred by perfumers. Yamaguchi *et al.* (1976a) found that various micro-organisms could reduce (±)-citronella asymmetrically to (−)-form-rich citronellol (*Figure 4*). Yeasts such as *Candida reukaufii* AHU-3052 had higher optical selectivities than other micro-organisms. Although the optical purities of (−)-citronellol obtained by microbial reduction of (±)-citronellal were lower than commercial (−)-citronellol ((−)-21), sensory evaluation showed that both had similar scent profiles. This finding was of great interest to the fragrance industry because the key to industrial success of such processes is to find a way of efficient regeneration of cofactors which conjugate with the redox enzyme systems.

Pseudomonas aeruginosa converted citronellal to citronellic acid (22) with a 60–65% yield from concentrations of 0·35–0·10%. (Hayashi *et al.*, 1967a; Joglekar and Dhavalikar, 1969; Tatsumi, 1974). Citronellol (21), dihydrocitronellol (23), 3,7-dimethyloctane-1,7-diol (24) and menthol (25) were identified as minor products (*Figure 4*).

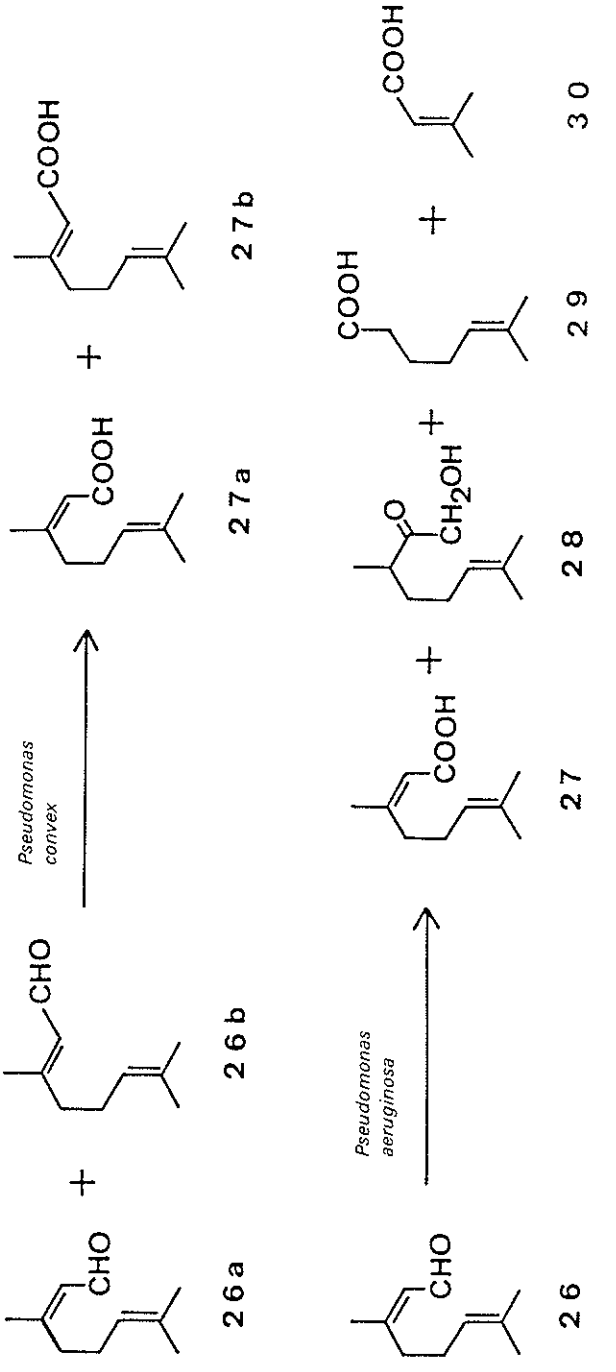


Figure 5. Products resulting from the conversion of citral by *Pseudomonas*. Citral(26), geranial(26b), neral(26a), neroic acid(27 and 27a), geranic acid(27b), 3,7-dimethyl-2-oxo-octanol(28), 6-methyl-5-heptenoic acid(29), 3-methyl-2-butenoic acid(30).

CITRAL

Citral (26), the main constituent of lemon grass, is a mixture of two geometrical isomers, geranial (trans-type, 26b) and neral (cis-type, 26a).

Using *Pseudomonas convex*, isolated from soil, Hayashi et al. (1967b) converted citral to geranic acid (27b) and neroic acid (27a). Neroic acid was found to be converted further to geranic acid (27b).

Pseudomonas aeruginosa also converted citral to geranic acid (27) with a 60% yield. 3,7-Dimethyl-2-oxo-octanol (28), 6-methyl-5-heptenoic acid (29) and 3-methyl-2-butanoic acid (30) were identified as minor products (Joglekar and Dhavalikar, 1969) (*Figure 5*).

LIMONENE

Limonene (31) occurs in various ethereal oils, particularly in oils of lemon, orange, caraway, dill and bergamot.

Dhavalikar and Bhattacharyya (1966) reported detailed studies on the microbial conversion of limonene. A soil pseudomonad, isolated by enrichment culture, was found to grow on limonene as its sole source of carbon. Fermentation of limonene by the bacterium in a mineral salt medium resulted in a large number of neutral and acidic products: carveol (32), carvone (33), 1-*p*-menthene-6,9-diol (34), dihydrocarvone (36), 8-*p*-menthene-1,2-*trans*-diol (37), 8-*p*-menthen-1-ol-2-one (38), perillyl alcohol (39), perillic acid (41), 4,9-dihydroxy-1-*p*-menthen-7-oic acid (42), 2-hydroxy-8-*p*-menthen-7-oic acid (43), 2-oxo-8-*p*-menthen-7-oic acid (44) and β-isopropenylpimelic acid (45). In manometric studies (Dhavalikar, Rangachari and Bhattacharyya, 1966), however, the neutral metabolites such as carveol (32) and dihydrocarvone (36) which accumulated during this fermentation of limonene (31) were found to be incapable of supporting the growth or respiration of the bacterium. Acidic metabolites such as perillic acid (41) were freely metabolized by limonene-grown cells. Based on this data, three pathways in limonene bioconversion were proposed, as shown in *Figure 6*. Pathways involving allylic oxygenation (pathway 1), oxidation via limonene epoxide (35) as an intermediate (pathway 2) and progressive oxidation of the 7-methyl group with ring split which represents the main energy sequence (pathway 3).

The conversion of linonene (1) by moulds was reported by Bowen (1975). *Penicillium italicum* and *Penicillium digitatum* responsible for the most common post-harvest disease of citrus fruits were isolated from decaying oranges. After 9 days of fermentation on a rotary shaker, *Penicillium italicum* produced the following metabolites: *cis*- and *trans*-*p*-mentha-2,8-dien-1-ol (46), *p*-mentha-1,8-dien-4-ol (47), *cis*- and *trans*-carveol (32), carvone (33), perillyl alcohol (39) and *p*-menth-8-ene-1,2-diol (37) (*Figure 7*).

The bioconversion of limonene mentioned above yielded complex mixtures of metabolites. Mukherjee, Kraidman and Hill (1973) obtained a large relative amount of 8-*p*-menthene-*trans*-1,2-diol (37) by microbial conversion of limonene with the *Cladosporium* species. Analogous production of this

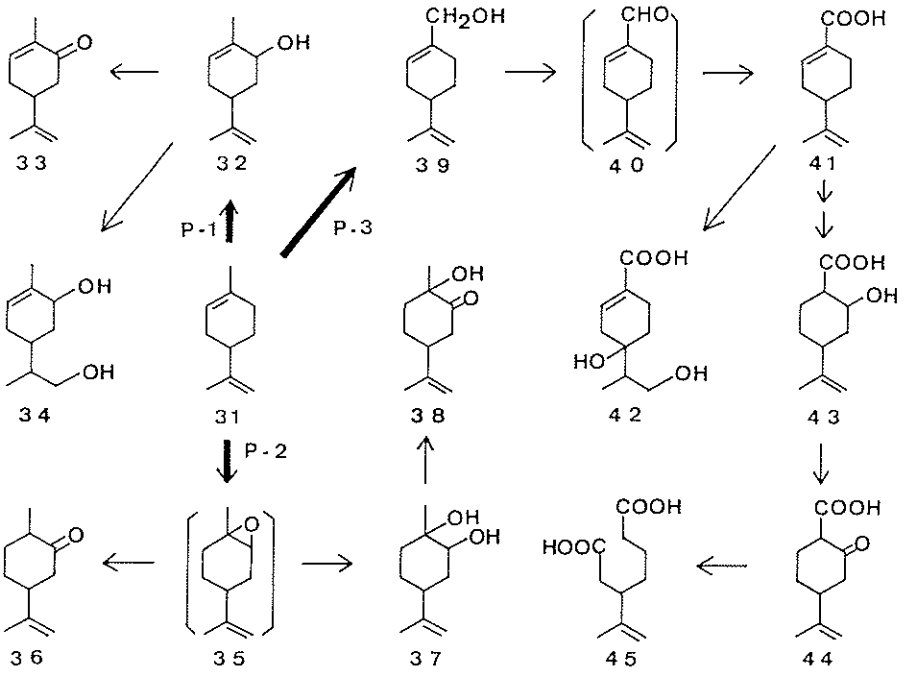


Figure 6. Pathways of the degradation of limonene by a Pseudomonad. Pathway 1(P-1), pathway 2(P-2), pathway 3(P-3), limonene(31), carveol(32), carvone(33), 1-*p*-menthene-6,9-diol(34), limonene epoxide(35), dihydrocarvone(36), 8-*p*-menthene-1,2-diol(37), 8-*p*-menthen-1-o1-2-one(38), perillyl alcohol(39), perillaldehyde(40), perillic acid(41), 4,9-dihydroxy-1-*p*-menthen-7-oic acid(42), 2-hydroxy-8-*p*-menthen-7-oic acid(43), 2-oxo-8-*p*-menthen-7-oic acid(44), β-isopropenylpimelic acid(45).

compound (37) by *Diplodia gossypina* and *Corynespora cassiicola* was reported by Abraham, Stumpf and Kieslich (1986).

In patent literature, Takagi *et al.* (1972) described the preparation of carvone (33) from limonene by co-metabolism of *Arthrobacter* sp.

CARVONE AND CARVEOL

(+)-Carvone is found in caraway seed and dill seed oils and has a caraway-like scent. (−)-Carvone is found in spearmint and kuromoji oils and has a spearmint-like scent.

The microbial conversion of (+)- and (−)-carvones (33) has been studied in detail by Noma and co-workers. (Noma, 1977, 1980, 1984; Noma and Nonomura, 1974; Noma and Tatsumi, 1973). Their stereochemical approach and its results have been discussed by Noma (1984). More recent studies are referred to here.

Using a selected strain of *Aspergillus niger*, Noma and Nonomura (1974) and Noma, Toyota and Asakawa (1985) converted (+)- and (−)-carvones (33)

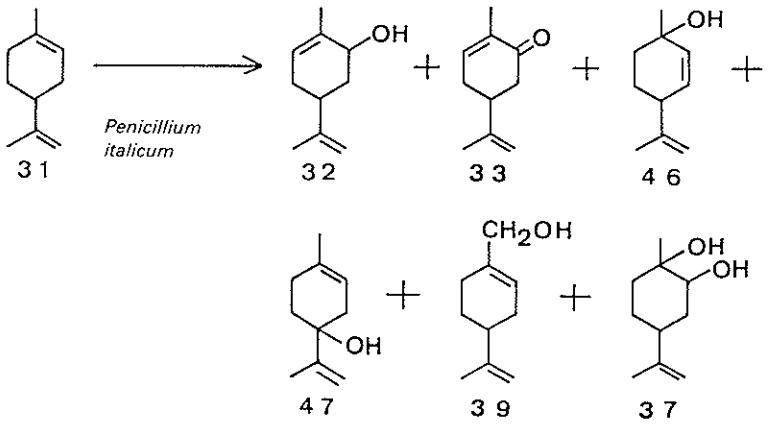


Figure 7. Products resulting from the conversion of limonene by *Penicillium italicum*. Limonene(31), carveol(32), carvone(33), *p*-mentha-2,8-dien-1-ol(46), *p*-mentha-1,8-dien-4-ol(47), perillyl alcohol(39), 8-*p*-menthene-1,2-diol(37).

respectively. (+)-Carvone was transformed to (−)-dihydrocarvone (36), (+)-dihydrocarveol (49), (+)-8-*p*-menthene-1,2-*trans*-diol (37), (+)-isodihydroisobottrospicatol (48) and (+)-9-hydroxyisodihydrocarvone (52). On the other hand, (−)-carvone ((−)-33) was converted to (+)-dihydrocarvone (36), (+)-dihydrocarveol (49), (+)-8-*p*-menthane-2,8-diol (51), (+)-8-*p*-menthene-2,9-diol (53) and (+)-*p*-menthene-2,8,9-triol (54).

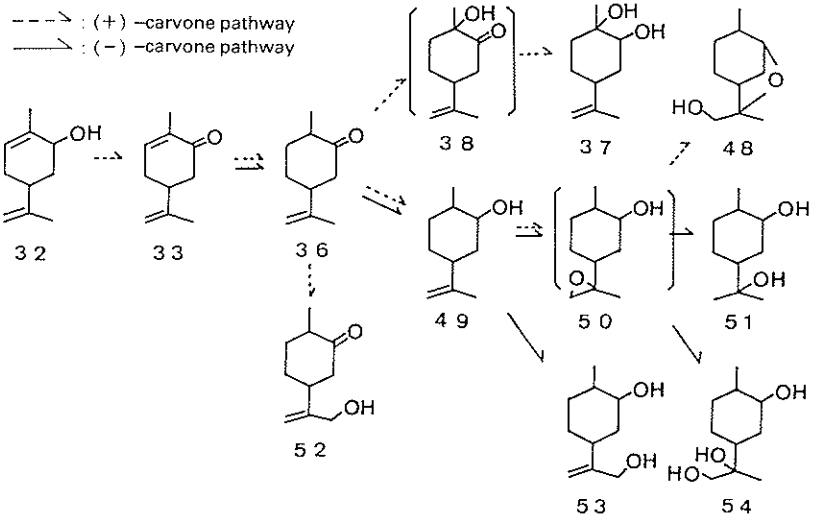


Figure 8. Metabolic pathways of (+)- and (−)-carvone conversion by *Aspergillus niger*. Carveol(32), carvone(33), dihydrocarvone(36), 8-*p*-menthen-1-ol-2-one(38), 8-*p*-menthene-1,2-diol(37), isodihydroisobottrospicatol(48), dihydrocarveol(49), 8-*p*-menthane-2-hydroxy-8,9-epoxide(50), 8-*p*-menthane-2,8-diol(51), 9-hydroxyisodihydrocarvone(52), 8-*p*-menthene-2,9-diol(53), 8-*p*-menthene-2,8,9-triol(54).

To clarify the conversion pathways of (+)-carvone (33), (+)-*cis*-carveol (32) and (−)-dihydrocarvone (36) were incubated with *Aspergillus niger*. Both compounds (32 and 36) resulted in 8-*p*-menthene-1,2-*trans*-diol (37). Dihydrocarveol (49), which was derived from (+)-carvone ((+)-33), did not produce 8-*p*-menthene-1,2-*trans*-diol (37) but isodihydroisobottorospicatol (48) was produced by the fungus.

The conversion pathways for (+)- and (−)-carvone (33) are shown in *Figure 8*. (As space is limited, configurations of stereoisomers are not depicted in this Figure.) Through their studies, it was suggested that micro-organisms such as *Aspergillus niger* distinguished one optical isomer from the other during the conversion of terpenoids.

(−)-MENTHOL

From the stereochemical configuration of menthol, four stereoisomers can exist: menthol (25a), neomenthol (25b) isomenthol (25c), and neoisomenthol (25d). Each of the stereoisomers has a pair of optical isomers: dextrorotatory, (+) and levorotatory, (−) (*Figure 9*). Among the eight stereoisomers, (−)-menthol ((−)-25a) is only obtained from peppermint oil or other mint oils and has the strongest refreshing activity (Arktander, 1969). Thus (−)-menthol ((−)-25a) is widely used in flavouring, perfumes, cosmetics and medicines.

When menthol is synthesized from optically inactive raw materials, one important problem is to separate only optically active (−)-menthol ((−)-25a) from a mixture of (±)-isomers (25a–d), because the simultaneous formation of all of the (±)-isomers (25a–d) is unavoidable. For example, one of the main industrial processes for (±)-menthol synthesis consists of the hydrogenation of

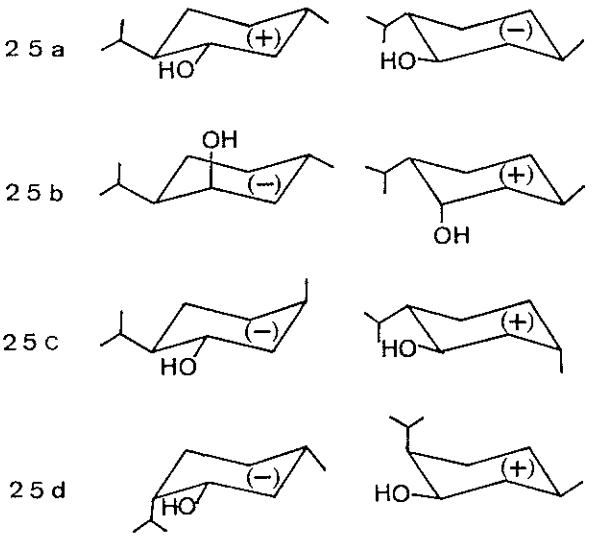


Figure 9. Stereoisomers of menthol. Menthol (25a), neomenthol(25b), isomenthol(25c), neoisomenthol(25d).

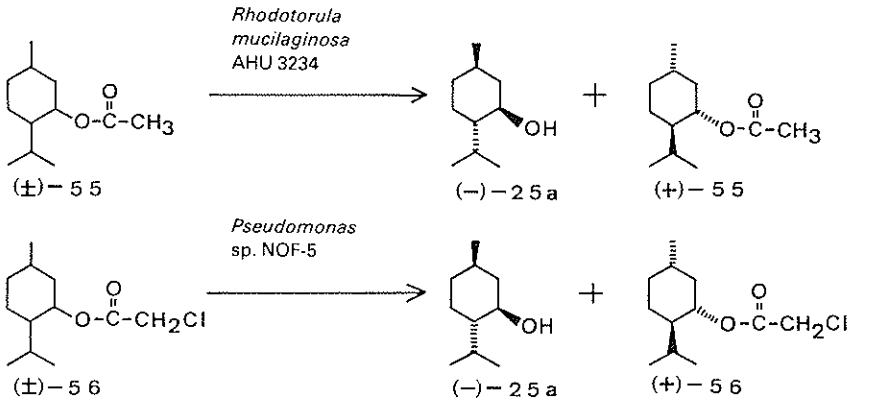


Figure 10. Optical resolution of menthyl esters. (±)-Menthyl acetate ((±)-55), (−)-menthol ((−)-25a), (+)-menthyl acetate ((+)-55), (±)-menthyl chloroacetate ((±)-56), (+)-menthyl chloroacetate ((+)-56).

thymol. The heat equilibration of the resultant mixture produces the following composition: (±)-menthol (25a), *c.* 70%; (±)-isomenthol (25c), *c.* 20%; (±)-neomenthol (25b), *c.* 10%; and neoisomenthol (25d), trace (Moroe *et al.*, 1971). By rectification and other methods (±)-menthol (25a) with a small portion of (±)-isomenthol (25c) could be obtained from the synthetic mixture (Inagaki and Ueda, 1986). Therefore, the optical resolution of (±)-menthol from such mixtures has been required for the industrial production of (−)-menthol ((−)-25a).

Moroe *et al.* (1971) first described, in their patent literature, the optical resolution of (±)-menthyl esters such as acetates by *Rhodotorula, Penicillium, Trichoderma, Streptomyces, Bacillus* and many other micro-organisms. In their serial studies, Yamaguchi *et al.* (1976b) reported that among the micro-organisms capable of hydrolysing menthyl acetate, bacteria and fungi hydrolysed isomenthyl acetate, but certain yeasts could not. This has great advantage over the isolation of (−)-menthol ((−)-25a) from the steric mixtures prepared by the aforementioned industrial process. Subsequently, the asymmetric hydrolysis of (±)-menthyl acetate ((±)-55) by *Rhodotorula mucilaginosa* AHU 3243, a selected yeast strain, was studied using a jar fermenter (Yamaguchi, Komatsu and Moroe, 1977). One of the UV irradiated strains showed *c.* 70% higher activity than the original one. This strain liberated 44·4 g of (−)-menthol ((−)-25a) per 24 h from a 30% (±)-menthyl acetate ((±)-55) mixture in a litre of culture medium kept under optimal operating conditions (*Figure 10*).

Biochemical resolution of (±)-terpenic alcohol by microbial hydrolysis of corresponding esters has been reviewed by Oritani and Yamashita (1983) referring to their serial studies (Oritani and Yamashita, 1973a, b, 1974). Using selected micro-organisms (±)-cyclic terpene alcohols such as menthols, carvomenthols, isopregols, carveols and borneols were resolved via asymmetric hydrolysis of the corresponding acetates and chloroacetates in a high

concentration (1–10%) of racemic substrates. Generally, the acetates of (1*R*)-2,5-disubstituted 1-cyclohexanols of which the acetoxyl group had equatorial conformation, were hydrolysed more rapidly than those of their enantiomeric (1*S*)-alcohols.

Inagaki and Ueda (1986) isolated a bacterium from soil which could hydrolyse (±)-menthyl acetate asymmetrically. The bacterium, tentatively designated as *Pseudomonas* sp. NOF-5, hydrolysed not only (±)-menthyl acetate but also (±)-isomenthyl acetate to give (−)-menthol ((−)-25a) and (−)-isomenthol ((−)-25c) in quantitative yields. However, the hydrolysis of (±)-neoisoementhyl acetate was not stereoselective to give (±)-neoisomenthol, and (±)-neomenthyl (25d) acetate was not hydrolysed. These results corroborate those of the aforementioned studies (Oritani and Yamashita, 1983).

The acid group of the menthyl esters is an important factor in the reaction velocity of hydrolysis (Oritani and Yamashita, 1974). By employing the monochloroacetate of (±)-menthol as the substrate, the reaction velocity was moderately increased. Thus, industrial conditions were established and have now been practically applied for manufacturing (−)-menthol (*Figure 10*) (Inagaki and Ueda, 1987a). Details are discussed on pages 307–308.

A reverse approach to separate (−)-menthol from (±)-menthol has also been reported by Inagaki and Ueda (1987c). The microbial esterification of (−)-menthol from (±)-menthol and various fatty acids by *Pseudomonas* sp. NOF-5 was studied. A water-insoluble fatty acid higher than caproic acid was esterified by using a (−)-menthol with high enantioselectivity (optical purity, 99·6%), especially when lauric acid was used as the fatty acid. The same approach with esterase such as lipase had been described in other patent literature (Nakayama *et al.*, 1977).

Optical resolution of (±)-menthol from the mixture of stereoisomers has been realized on an industrial scale by chemical methods (Jürgen, Kurt and Rudolf, 1971) as well as biochemical methods (Inagaki and Ueda, 1987a). However, simpler methods for optical resolution are being studied. The biochemical approach for optical resolution will be studied and improved by many researchers applying the new biotechnology which is now developing.

(−)-Menthol production by various chemical methods, including biochemical ones, has been reviewed by Traas (1982).

ISOPULEGOL

(−)-Isopulegol ((−)-57a) is one of the raw materials used to synthesize (−)-menthol ((−)-25a), and (+)-isopulegol ((+)-57a) to (−)-citronellol ((−)-21) via (−)-citronellal ((−)-20) (Ueda and Shimizu, 1960) (*Figure 11*).

The optical resolution of (±)-isopulegol was investigated by Inagaki and Ueda (1987b). In the biochemical resolution of (±)-isopulegyl acetate diastereomers with *Pseudomonas* sp. NOF-5, (−)-isopulegol (57a) and (−)-iso-isopulegol (57c) were stereospecifically liberated, but their enantiomers were not hydrolysed. (±)-Neoisopulegyl acetate (58b) was not hydrolysed by the bacterium (*Figure 12*). Furthermore, (±)-isopulegyl chloroacetate was

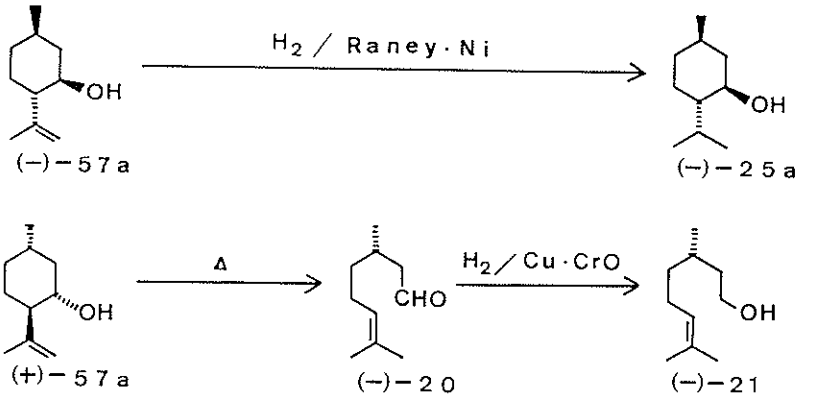


Figure 11. Chemical synthesis of (−)-menthol and (−)-citronellol. (−)-Isopulegol ((−)-57a), (−)-menthol ((−)-25a), (+)-isopulegol ((+)-57a), (−)-citronellal ((−)-20), (−)-citronellol ((−)-21).

hydrolysed faster than its acetate by the bacterium in high substrate concentration (*c.* 50%) to yield (−)-isopulegol and (+)-isopulegyl chloroacetate. The industrial production of (−)-citronellol from (±)-isopulegyl chloroacetate via this asymmetric hydrolysis was also reported (*Figure 11*) (Inagaki and Ueda, 1987b).

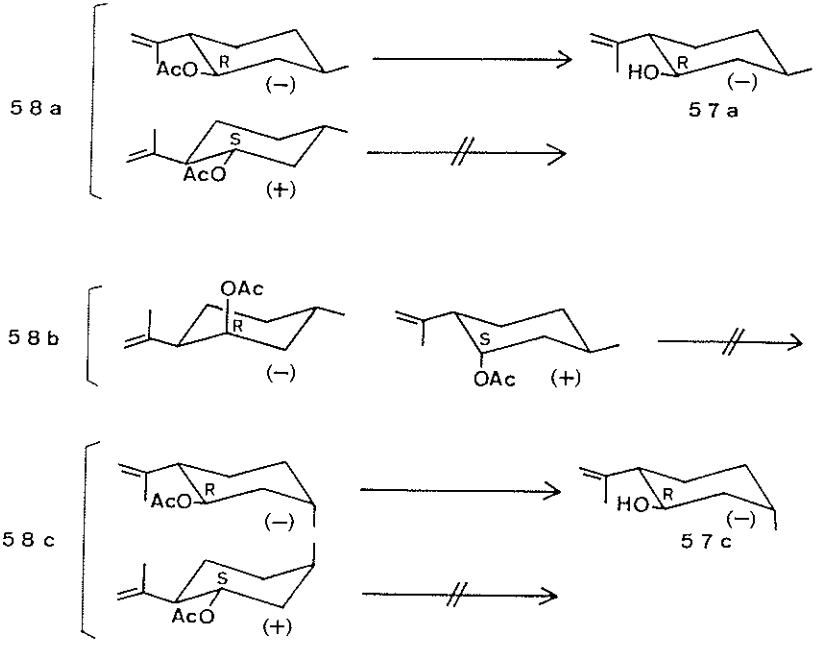


Figure 12. Optical resolution of isopulegyl esters by *Pseudomonas* strain NOF-5. Isopulegyl acetate (58a), neoisopulegyl acetate(58b), iso-isopulegyl acetate(58c), (−)-isopulegol(57a), (−)-iso-isopulegol(57c).

The equatorial acetoxyl group at the *R*-configuration carbon of the cyclohexane ring was preferably hydrolysed as shown in *Figure 12*. This bears out the generalization by Oritani and Yamashita (1973a, 1983).

PINENE

Pinenes (α- and β-pinene), bicyclic monoterpenes, are the principal constituents (58–65%) of turpentine and are the most abundant terpenes in nature (Sully, 1964).

In their broadly based serial studies, Battacharyya *et al.* (1960) first undertook investigation of the capability of micro-organisms, particularly fungi, to convert some easily available terpenes, such as pinene, to oxygenated products. This research was considered potentially interesting to the perfumery industry. *Aspergillus niger* NCIM 612 was selected from a number of fungal strains isolated from infected bark. The conversion of (+)-α-pinene (59) gave (+)-cis-verbenol (60) and (+)-verbenone (61) and (+)-*trans*-sobrerol (62) (*Figure 13*) (Prema and Bhattacharyya, 1962a). Optical antipodes of these products were obtained by incubating (−)-α-pinene with the same fungus. On the other hand, (±)-β-pinene (63) was oxidized to (−)-pinocarveol (64), (−)-pinocarvone (65) and (−)-myrtenol (66) by the same fungus (*Figure 13*) (Bhattacharyya and Ganapathy, 1965).

Recently, Wright *et al.* (1986) reported that a strain of the bacterium *Serratia marcescens*, isolated from sewage sludge, also oxidized α-pinene (59) to produce *trans*-verbenol (60) as the main product, with verbenone (61) and *trans*-sobrenol (62) as minor products. (+)-Verbenone (61) is known as a constituent with the characteristic scent of Spanish verbena oil (Windholz, 1983).

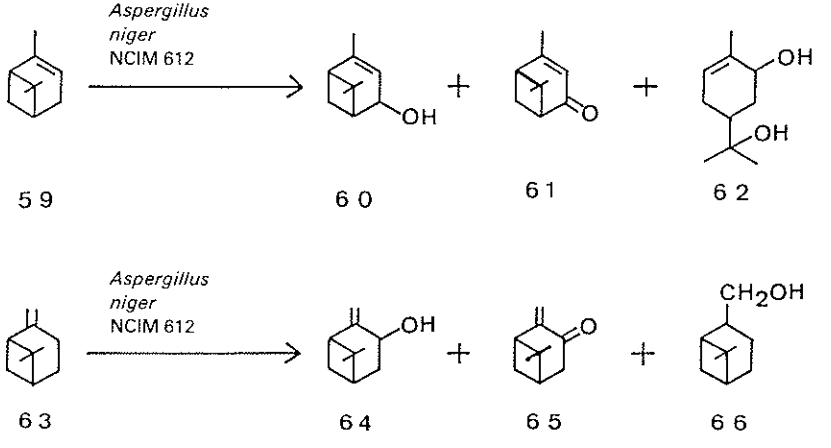


Figure 13. Conversion products of α- and β-pinene by *Aspergillus niger* NCIM 612. α-Pinene(59), verbenol(60), verbenone(61), sobrerol(62), β-pinene(63), pinocarveol(64), pinocarvone(65), myrtenol(66).

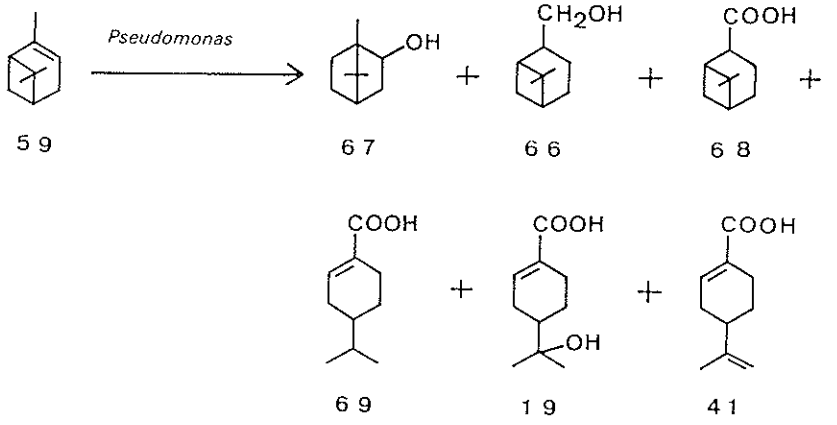


Figure 14. Conversion products of α-pinene by *Pseudomonas*. α-Pinene(59), borneol(67), myrtenol(66), myrtenic acid(68), phellandric acid(69), oleuropeic acid(19), perillic acid(41).

In various studies (Shukla, Moholay and Bhattacharyya, 1968; Shukla and Bhattacharyya, 1968; Gibbon and Pirt, 1971; Gibbon, Mills and Pirt, 1972; Tudrosen, Kelly and Mills, 1977; Narushima, Omari and Minoda, 1982b; Wright *et al.*, 1986) it was shown that *Pseudomonas* sp. metabolized α-pinene to a wide variety of neutral and acidic compounds.

Conversion of α-pinene (59) by a Pseudomonad (PL-strain) resulted in borneol (67), myrtenol (66), myrtenic acid (68), phellandric acid (69), oleuropeic acid (19), perillic acid (41) and various other degradation compounds (*Figure 14*) (Shukla, Moholay and Bhattacharyya, 1968). Subsequently, growth and adaptation studies, using different metabolites obtained by fermentation of pinene with the PL-strain, demonstrated that there may be at least six different metabolic pathways through which the substrate is converted into different transformation products (Shukla and Bhattacharyya, 1968). A later publication by Gibbon and Pirt (1971) described a novel metabolic pathway of α-pinene by *Pseudomonas* sp. PX1 which is not similar to that of the PL-strain.

1,8-CINEOLE

1,8-Cineole or eucalyptol is the chief constituent of *Eucalyptus* oil as well as a component in many essential oils.

MacRae *et al.* (1979) reported the conversion of 1,8-cineole by a bacterium having close affinity to *Pseudomonas flava* which was isolated from eucalyptus leaves. This bacterium utilized 1,8-cineole (70) as a carbon source and produced 2-*exo*- (71a) and 2-*endo*-hydroxycineole (71b), 2-oxocineole (72), and (*R*)-5,5-dimethyl-4-(3′-oxobutyl)-4,5-dihydrofuran-2-(3*H*)-one (73) (*Figure 15*).

Nishimura, Noma and Mizutani (1982) described the formation of 3-*exo*- (74a) and 3-*endo*-hydroxycineole (74b) through conversion of cineole with *Aspergillus niger*.

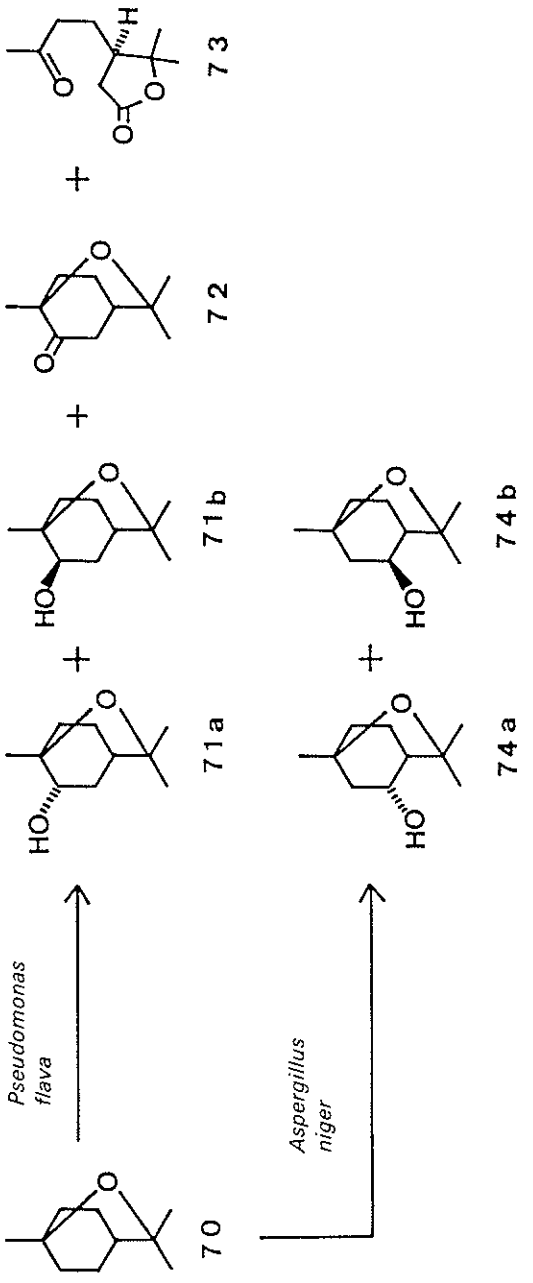


Figure 15. Conversion products of 1,8-cineole by micro-organisms. 1,8-Cineole(70), 2-*exo*-hydroxycineole(71a), 2-*endo*-hydroxycincole(71b), 2-oxocineole(72), (*R*)-5,5-dimethyl-4-(3'-oxobutyl)-4,5-dihydrofuran-2-(3*H*)-one(73), 3-*exo*-hydroxycineole(74a), 3-*endo*-hydroxycineole(74b).

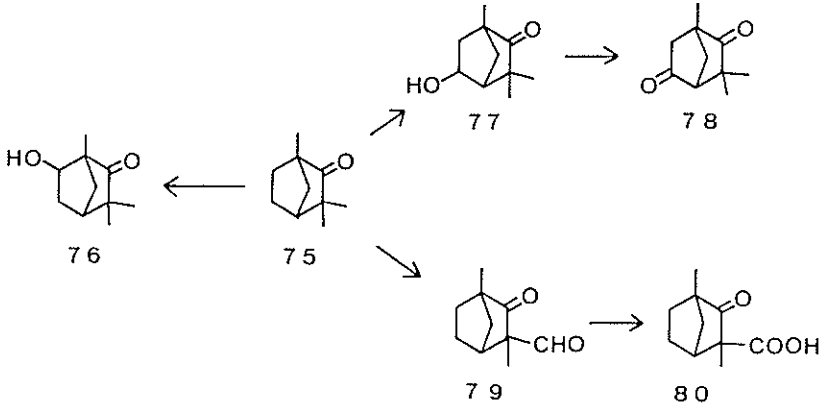


Figure 16. Conversion pathways of fenchone by *Aspergillus niger*. Fenchone(75), 6-hydroxyfenchone(76), 5-hydroxyfenchone(77), 5-oxofenchone(78), 9-formylfenchone(79), 9-carboxyfenchone(80).

FENCHONE

(+)-Fenchone occurs in fennel oil and in the essential oil of *Lavandula stoechas*. This terpene has been used as a counter-irritant (Windholz, 1983).

(+)-Fenchone (75) is hydroxylated by *Absidia orchidis* to 6-*exo*-hydroxyfenchone (76) and 5-*exo*-hydroxyfenchone (77) (Pfrunder and Tamm, 1969). *Aspergillus niger* also converted (+)-fenchone (75) producing 6-hydroxyfenchone (76) as a major product, and 5-hydroxyfenchone (77), 5-oxofenchone (78), 9-formylfenchone (79) and 9-carboxyfenchone (80) as minor products (*Figure 16*) (Yamamoto *et al.*, 1984).

Sesquiterpenes

The number of sesquiterpenes and their derivatives, which are found mainly in essential oils, was estimated to be at least 2000 by the end of 1976 (Ohloff, 1978). Many of them are important flavour and fragrance compounds. Some of them have medicinal uses, such as anthelmintic compounds. However, studies on microbial conversion of sesquiterpenes are meagre compared with monoterpenes, because of the difficulties in obtaining pure compounds.

Microbial conversion of sesquiterpenes has been reported on caryophyllene (85) (Devi, 1979), costunolide (87) (Clark and Hufford, 1979), α-cyperone (82) (Hikino *et al.*, 1975), cyperotundone (90) (Hikino *et al.*, 1968b), farnesolepoxide (89) (Suzuki and Marumo, 1972), germacrone (83) (Hikino *et al.*, 1971), guaioxide (93a) (Ishii, Nakamura and Minato, 1970), kessane (91) (Hikino, Kohama and Takemoto, 1969), α-kessyl alcohol (92) (Hikino *et al.*, 1968a), ligloxide, a diastereomer of guaioxide (93b) (Ishii *et al.*, 1972), patchoulol (94) (Suhara *et al.*, 1981), α-santonin (86) (Hikino, Tokuoka and Takemoto, 1970), trichodiene (84) (Machida and Nozoe, 1972), and valencene (81) (Paknikar and Dhavalikar, 1975) (*Figure 17*). In general, microbial conversion

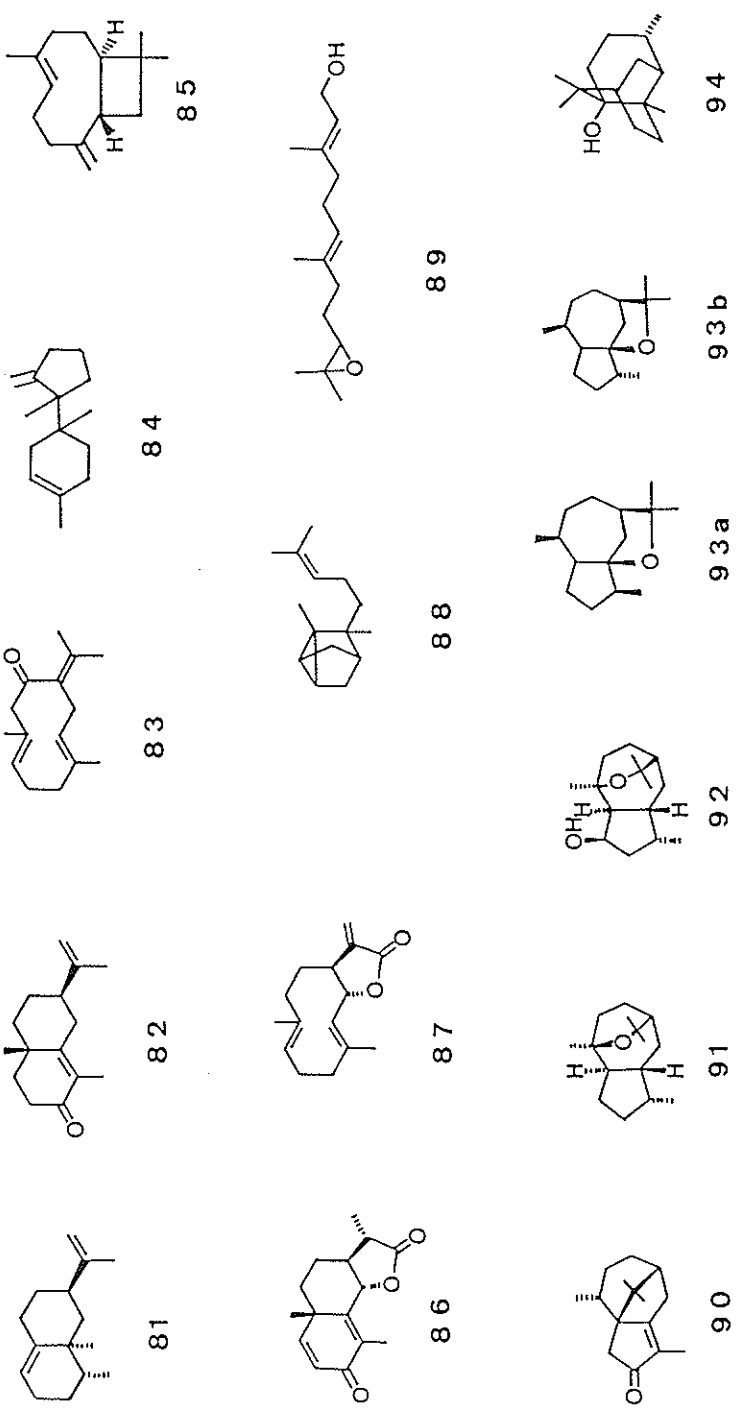


Figure 17. Sesquiterpenes used as substrates for microbial conversion. Valencene(81), α-cyperone(82), germacrone(83), trichodiene(84), caryophyllene(85), α-santonin(86), costunolide(87), α-santalene(88), farnesolepoxide(89), cyperotundone(90), kessane(91), α-kessyl alcohol(92), guaioxide(93a), ligloxide(93b), patchoulol(94).

of sesquiterpenes yields fewer degradation products. The results obtained have helped to elucidate the stereochemistry of these terpenes, as sesquiterpene derivatives are often difficult to prepare by chemical methods.

Kieslich (1976) reviewed the microbial transformation of these compounds. More recent studies on sesquiterpenes are described here.

PATCHOULOL

Patchouli essential oil, which is obtained by steam distillation of the dried leaves of *Pogosterom cablin*, is extensively used in perfumery (Indô, 1975). The major constituent (30–45%) of this oil is patchoulol (94), which has a characteristic odour (Näf *et al.*, 1981). In 1973, however, Teisseire found that the fragrant principle in patchouli oil is norpatchoulol (97), but that its content in the oil amounted to only 0·3% or less. The problematic chemical synthesis of this compound has been achieved recently (Gras, 1977).

Bang, Ourisson and Teisseire (1975) subsequently showed that patchoulol (94) was resio-selectively hydroxylated at the C-10 position by the liver of rabbits and dogs, and that the isolated 10-hydroxypatchoulol (95) could be easily converted to the desired norpatchoulol (97) via 4-carbo-hydroxypatchoulol (96) by chemical methods (*Figure 18*).

Subsequently, Suhara *et al.* (1981) investigated the ability of micro-organisms to convert patchoulol (94) to 10-hydroxypatchoulol (95) which is practically impossible to obtain by purely chemical methods. Of some 350 micro-organisms screened, four strains of *Pithomyces* species were found to carry out regio-selective hydroxylation of patchoulol (94) to 10-hydroxypatchoulol (95). As in the case of other sesquiterpene hydroxylations mentioned above, the strains found positive by the screening were exclusively

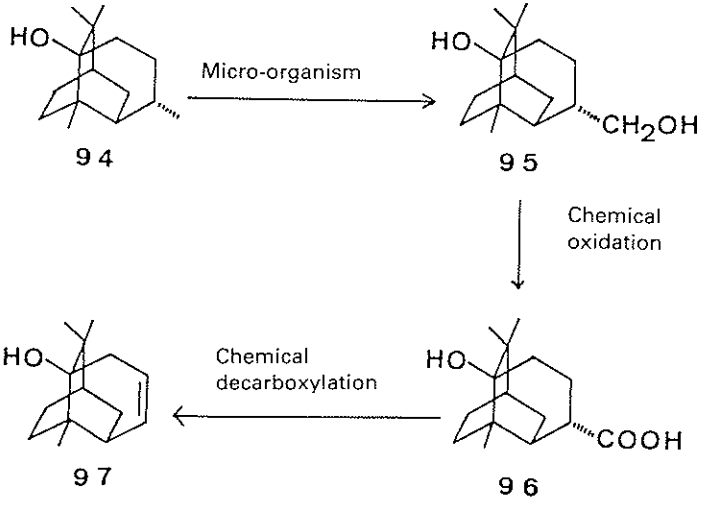


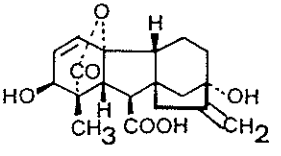
Figure 18. Synthesis of norpatchoulol from patchoulol *via* microbial hydroxylation. Patchoulol(94), 10-hydroxypatchoulol(95), 4-carbohydroxypatchoulol(96), norpatchoulol(97).

fungi, and bacteria were ineffective in this respect. The studies led to the establishment of a microbial process which yields 35–50% of 10-hydroxypatchoulol in 1- and 5-litre fermentation jars.

On the other hand, Fujiwara *et al.* (1980) made screening efforts for the same purpose and found a strain of *Penicillium rubrum* which could produce 10-hydroxypatchoulol from patchoulol (94) with an 80% yield.

Diterpenes

Gibberellin (98) is well known as one of the plant hormones which affects cell growth and division. Various gibberellins have been isolated from higher plants as well as from the culture broth of *Gibberella fujikuroi*, a gibberellin-producing fungus (Takahashi, 1969). To elucidate the biosynthesis of gibberellins, the microbial conversion of several possible intermediates such as kaurene (99) and kaurenoic acid (100) (*Figure 19*) was studied with the fungus (Cross, Galt and Hanson, 1964; Giesman *et al.*, 1966; Ghisalberti *et al.*, 1977).



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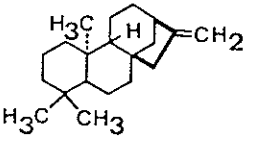

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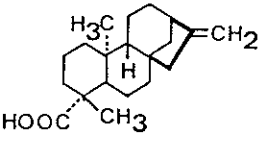

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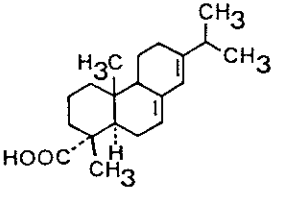

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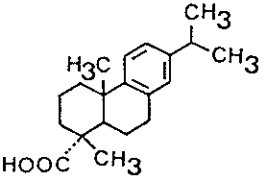

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Figure 19. Diterpenes used as substrates for microbial conversion. Gibberellin A_3(98), kaurene(99), kaurenoic acid(100), abietic acid(101), dehydroabietic acid(102).

The oleoresins of various conifers are major sources of diterpenes. Abietic acid (101) and its related compounds are the main constituents of these oleoresins. Their microbial conversion has been studied mainly by using bacteria isolated from pine forests (Cross and Myers, 1968; Biellmann *et al.*, 1973). Additionally, fungal hydroxylations of dehydroabietic acid (102) have been reported by Kutney *et al.* (1985).

A detailed review on microbial conversion of diterpenes was presented by Kieslich (1976). More recent studies on the bioconversion of diterpenes related to flavour are mentioned here.

LABDANES

The occurrence of labdanes in tobacco was disclosed in the early 1960s by Giles and co-workers (1961, 1962, 1963), who isolated the modified and decomposed labdanes from Greek and Turkish tobaccos. Some of these compounds possess attractive flavour properties and impart a cedar-like aroma to the smoke of turkish tobacco (Enzell, 1976). *cis*-Abienol (103) is known to be a major labdane of Greek and Turkish tobaccos which is present in the cuticular wax of leaves (Reid, 1974). *cis*-Abienol (103) is also abundantly present in the oleoresin of *Abies balsamea*, Canada fir balsam (Gray and Mills, 1964).

Studies were made of the microbial conversion of *cis*-abienol (103) by *Nocardia restricta* JTS-162 and *Rhodococcus erythropolis* JTS-131, bacteria isolated from soil (Hieda *et al.*, 1982a, b, c, d; Hieda, Mikami and Obi, 1983a, b, c). The microbial conversion products were useful for flavouring tobacco.

Five conversion products by *N. restricta* JTS-162 were isolated and identified as follows: (12*Z*)-labda-8(17),12,14-triene (104), (12*Z*)-labda-8(17),12,14,-trien-18-ol (105), (12*Z*)-labda-8(17),12,14-trien-18-al (106), 4,18,19-trinor-3,4-*seco*-5-oxo-(12*Z*)-labda-8(17),12,14-trien-3-oic acid (109) and (12*Z*)-labda-12,14-dien-17α-oic acid (110), while *R. erythropolis* JTS-131 produced four metabolites of *cis*-abienol which were identified as the compounds (105) and (110), and (12*Z*)-labda-12,14-dien-18-oic acid (107) and its methylester (108).

Labd-14-en-13β-ol (sclareol) (111), a constituent of tobacco (Colledge, Reid and Russell, 1975), was analogously converted by *N. restricta* JTS-162 to yield labda-8(17),14-dien-13β-ol (manool) (112), labda-8(17),14-diene-13β,18-diol (113), 13β-hydroxylabda-8(17),14-dien-18-al (114), and 13β-hydroxylabda-8(17)-14-dien-18-oic acid (115). Additional conversion products, 8,13-epoxylabd-14-ene (manoyl oxide) (117), and 14,15-dinorlabd-8(17)-en-13-one (118), were also identified. By *R. erythropolis* JTS-131 the last compound (115) was further converted to its methylester (116).

On the basis of experiments with a degradation sequence, the bioconversion pathways of these labdanes by the bacteria were proposed. An outline of the pathways is illustrated in *Figure 20*.

Subsequently Hieda *et al.* (1982c) and Hieda, Mikami and Obi (1983b) disclosed that *N. restricta* JTS-162 had three plasmids, pCA1, pCA2 and pCA3. Two types of cured strains were obtained by mitomycin C treatment or growth

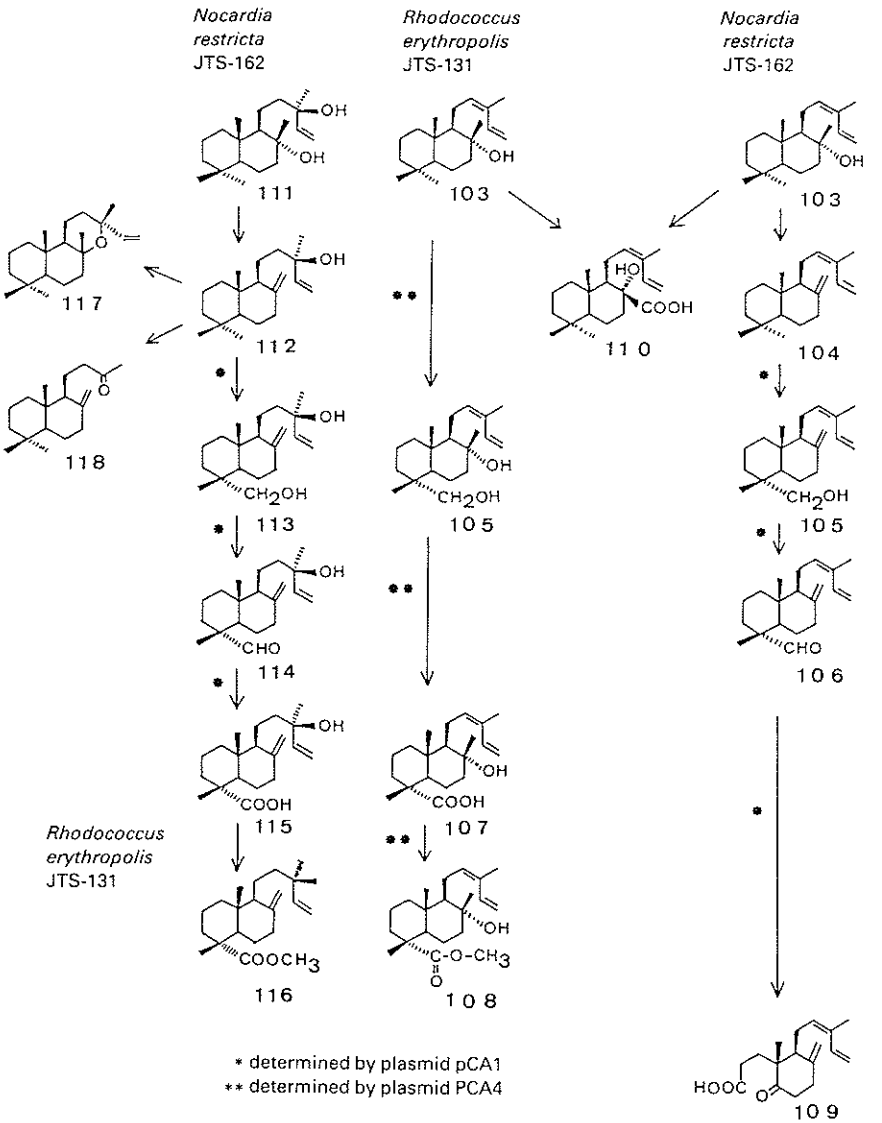

Figure 20. Bioconversion pathways of *cis*-abienol and sclareol by *Nocardia restricta* JTS-162 and *Rhodococcus erythropolis* JTS-131. (12Z)-labda-8(17),12,14-triene(104), (12Z)-labda-8(17),12,14,-trien-18-ol(105), (12Z)-labda-8(17),12,14-trien-18-al(106), (12Z)-labda-12,14-dien-18-oic acid(107), (12Z)-labda-12,14-dien-18-oic acid methyl ester(108), 4,18,19-trinor-3,4-*seco*-5-oxo-(12Z)-labda-8(17),12,14-trien-3-oic acid(109), (12Z)-labda-12,14-dien-17α-oic acid(110), sclareol(111), manool(112), labda-8(17),14-diene-13β,18-diol(113), 13β-hydroxylabda-8(17),14-dien-18-al(114), 13β-hydroxylabda-8(17)-14-dien-18-oic acid(115), manoyl oxide(117), 14,15-dinorlabd-8(17)-en-13-one(118).

at the maximum temperature: one type had two plasmids, pCA2 and pCA3, and the other type had no plasmid. These two types of cured strains lost the ability to oxidize the C-18 methyl group of the labdanes and to split the A-ring of *cis*-abienol. From these results, it was considered that C-18 methyl oxidation and A-ring splitting of these labdanes were determined by plasmid pCA1 in *N. restricta* JTS-162. Analogous studies on *R. erythropolis* JTS-131 exhibited the presence of plasmid pCA4, on which the C-18 methyl oxidation of the labdanes depended. The relationship between plasmids and bioconversion pathways of labdanes is summarized in *Figure 20*.

Triterpenes

Triterpenes from various origins are mainly tri-, tetra- and pentacyclic compounds except squalene, a linear C-30 compound.

Microbial conversion of a few cyclic triterpenes has been reviewed by Kieslich (1976). More recent studies in this field are referred to here.

SQUALENE AND ITS ANALOGUES

Squalene (119) is a naturally abundant linear triterpene and an important biosynthetic precursor of steroids and other triterpenes in many living organisms. An *Arthrobacter* species was found to degrade squalene and to accumulate *trans*-geranylacetone (122) in the medium (Yamada *et al.*, 1975). Subsequently, the authors found that squalene-2,3-epoxide (120) was

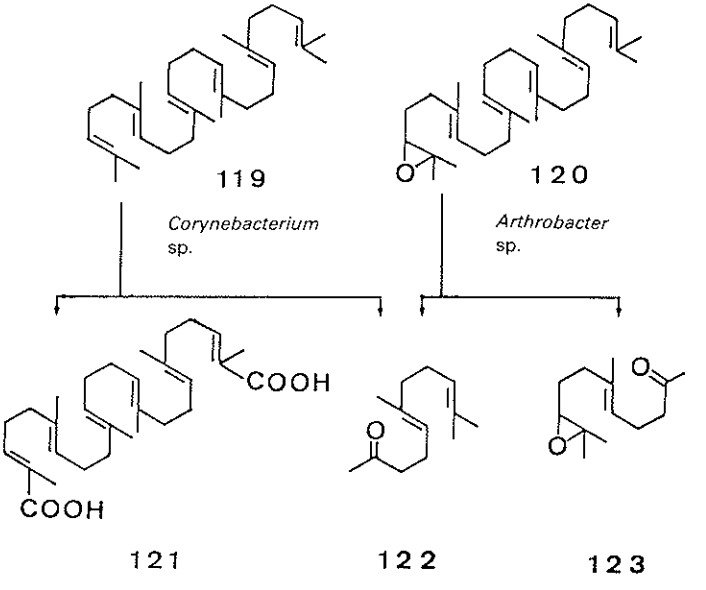

Figure 21. Conversion products of squalenes by bacteria. Squalene(119), squalene-2,3-epoxide(120), squalenedioic acid(121), *trans*-geranylacetone(122), *trans*-9,10-epoxygeranylacetone(123).

degraded by the same bacterium to *trans*-geranylacetone (122) and *trans*-9,10-epoxygeranylacetone (123) (*Figure 21*). As the enzyme system of the *Arthrobacter* species partially recognized the chirality at terminal parts of substrates, the authors proposed the application of such a microbial degradation process to prepare optically active starting materials for the synthesis of bioactive compounds such as the juvenile hormones of insects (Yamada, Kusuhara and Okada, 1977).

(3S)-Squalene-2,3-epoxide (120) has been proved to be the exclusive precursor of steroids in mammalian tissue, yeasts and plants. Yamada, Seo and Okada (1981) reported a direct asymmetric synthesis of this compound (2) starting from squalenedioic acid (121) which was prepared from squalene by microbial conversion with a *Corynebacterium* species (Seo *et al.*, 1983; Yamada, Seo and Okada 1985).

Another strain of *Corynebacterium* was found to convert squalene to mono-, di-, tri-, tetra- and pentahydrated squalene (Seo *et al.*, 1981).

Norterpenes

IONONES AND RELATED COMPOUNDS

A class of unusually potent aroma components comprises substances which are formed by biodegradation of certain odourless and tasteless high-molecular-weight terpenoids occurring in food stuffs, such as carotenoids, diterpenes and polyterpenoids. The breakdown products of nor-isoprenoids are found as a rule in certain vegetable and fruit varieties as well as (in remarkable quantities) in tea and tobacco (Ohloff, 1978). Of the 269 aroma constituents identified in black tea by the end of 1974, 10% of those containing 9–13 carbon atoms can be considered as being derived from carotenoid compounds (Sanderson and Graham, 1973). Of the 200 nor-isoprenoids found in tobacco leaf condensates, 96 are assumed to be carotenoid metabolites (Fujimori, 1984). The explosive development in this field during the last decade is illustrated in some reviews (Enzell, Wahlberg and Aasen, 1977; Ohloff, 1978; Fujimori, 1984).

On the other hand, the need for pure chiral compounds with a trimethylcyclohexane ring as precursors for the chemical synthesis of naturally occurring carotenoids and bioactive terpenoids encouraged the application of microbial conversion.

β-*Ionone*

Microbial conversion has been applied to the preparation of chiral ionone derivatives as precursors for the synthesis of naturally occurring carotenoids. Ito, Masahara and Tsukida (1977) reported the enzymatic reduction of 2-oxo-β-ionone (128a) to (2S)-hydroxy-β-ionone (135a) by yeast for the synthesis of (2S)-hydroxy-β-carotene. (2S)-Hydroxy-β-ionone (135a) was also prepared by the hydroxylation of β-ionone (124a) with *Aspergillus niger* (Mikami *et al.*, 1978).

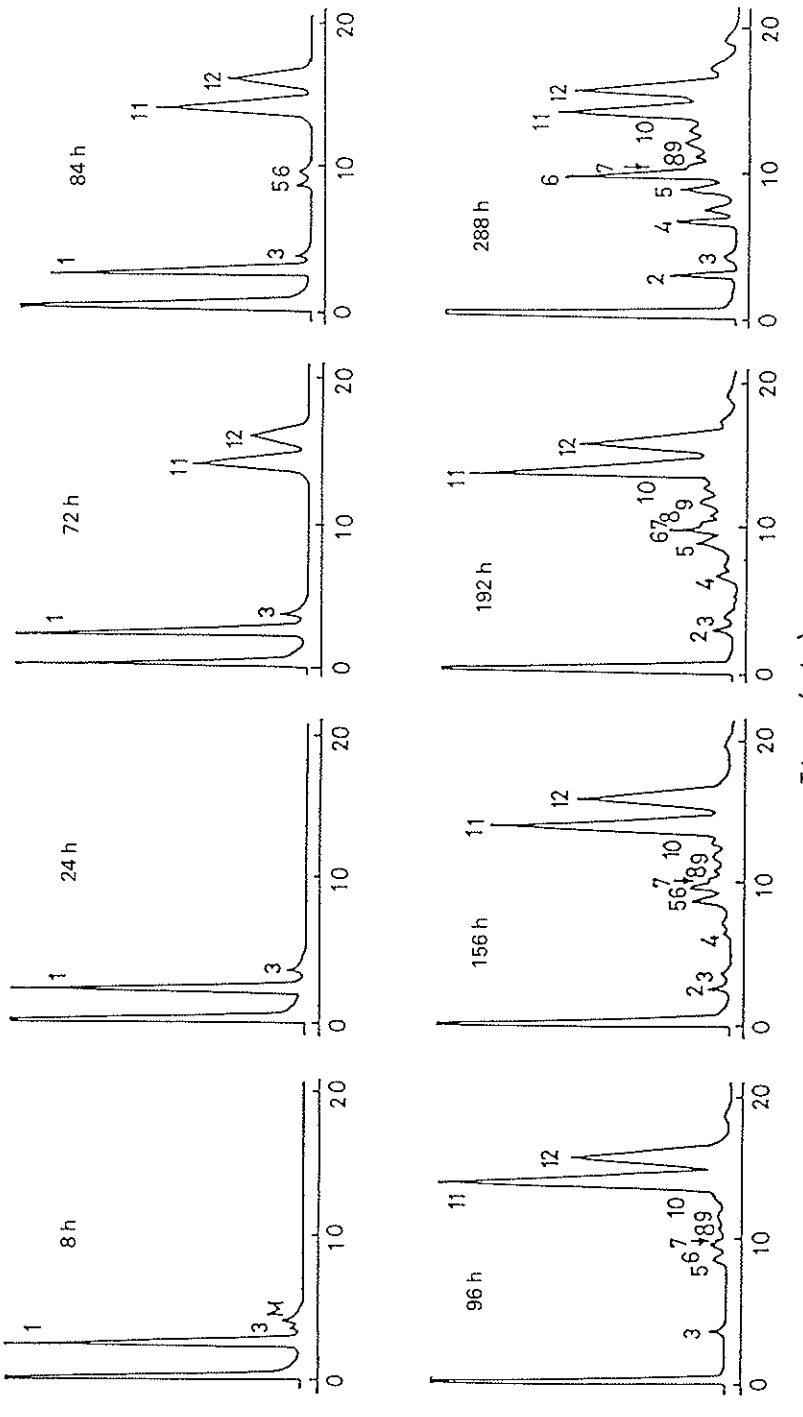

Figure 22. Gas chromatograms in the time course of microbial conversion of β-ionone. Peak(P-1), β-ionone(124a); P-2, 3,4-dehydro-β-ionone(125a); P-3, 5,6-epoxy-β-ionone(126a); P-4, 4-(2,3,6-trimethylphenyl)-but-3-en-2-one(127a); P-5, 2-oxo-β-ionone(128a); P-6, 4-oxo-β-ionone(129a); P-7, 4-acetoxy-β-ionone(130a); P-8, 2-oxo-3,4-dehydro-β-ionone(131a); P-9, 2-acetoxy-β-ionone(132a); P-10, 2,3-dehydro-4-oxo-β-ionone(133a); P-11, (*R*)-4-hydroxy-β-ionone(134a); P-12, (S)-2-hydroxy-β-ionone(135a); M, a metabolite of the fungus. Gas–liquid chromatographic conditions: PEG 20M (5%) on Chromosorb W (AW, DMCS), 3 mm by 2m; temperature: 200°C (isothermal); carrier gas: He 60 ml/min.

About a hundred compounds with a trimethylcyclohexane ring have been isolated from tobacco during the past decade (Fujimori, 1984). The compounds in this group have characteristic and low-threshold odour (Ohloff, 1978). They are thought to be key flavour components in the essential oils of air-cured tobaccos (Enzell, Wahlberg and Aasen, 1977; Fujimori and Kaneko, 1979). These compounds are presumably generated by metabolism or degradation of carotenoids (Enzell, 1976). It would be useful, therefore, to produce these compounds by microbial conversion of carotenoids or ionones. The latter compounds are considered to be key intermediates of carotenoid biodegradation (Stevens, 1970; Enzell, 1976).

Mikami *et al.* (1981a) found that certain strains of *Aspergillus* and *Rhizopus* converted ionones to a complex mixture which resembled an essential oil and had an odour similar to that of tobacco. *Aspergillus niger* JTS 191 was the most suitable for β-ionone conversion, judging from a resistance towards the substrate and flavour of complex products. Fermentation of β-ionones must be carried out with pre-grown mycelial pellets as the substrate inhibits growth (Okamura, 1974).

The typical fermentation procedure was as follows. The spores (4×10^7) of *Aspergillus niger* JTS 191 were inoculated into a 3-litre Erlenmeyer flask containing 1 litre of medium (3% sucrose, 0·2% $NaNO_3$, 0·1% K_2HPO_4, 0·05% KCl, 0·05% $MgSO_4 \cdot 7H_2O$, 0·1% yeast extract and distilled water). Cultivation was at 28°C for 48 h on a rotary shaker at 210 rev/min. To the resulting culture broth, which contained about 4·5 g (dry weight) of mycelial pellets, 1 g of β-ionone was added as a substrate. The broth was incubated at 28°C under continuous shaking, and a 10 ml sample was taken every 12 h and analysed by gas–liquid chromatography (*Figure 22*).

Production on a manufacturing scale is discussed later (page 308).

To determine the organoleptic properties of conversion products of β-ionone, cigarettes made with inferior tobacco were flavoured with the acidic and neutral fraction and evaluated by the olfactory method. The results, together with the odoriferous characteristics of these fractions, are summarized in *Table 1*. It was found that the neutral fraction was very effective at parts-per-million concentrations for improving inferior tobacco. However, the type of aroma varied with the conversion periods. The optimal incubation period was found to be 6–8 days. The acidic fraction was not as effective as the neutral one.

Table 1. Yield and organoleptic properties of acidic and neutral fractions of conversion products of β-ionone

Fraction	Period* (days)	Yield** (%)	Odour characteristics	Smoking aroma
Neutral	4–5	90	Floral, violet-like	Green note, fresh
	6–8	75	Floral, rose-like	Rich aroma, sweet, mild
	9–14	55	Fruity	Poor aroma, harsh, irritative
Acidic	4–5	—	—	
	6–8	15	Acidic	Orient tobacco-like, fruity
	9–14	20	Acidic, disagreeable	Irritative, disagreeable

* The period of incubation; ** Based on the substrate added.

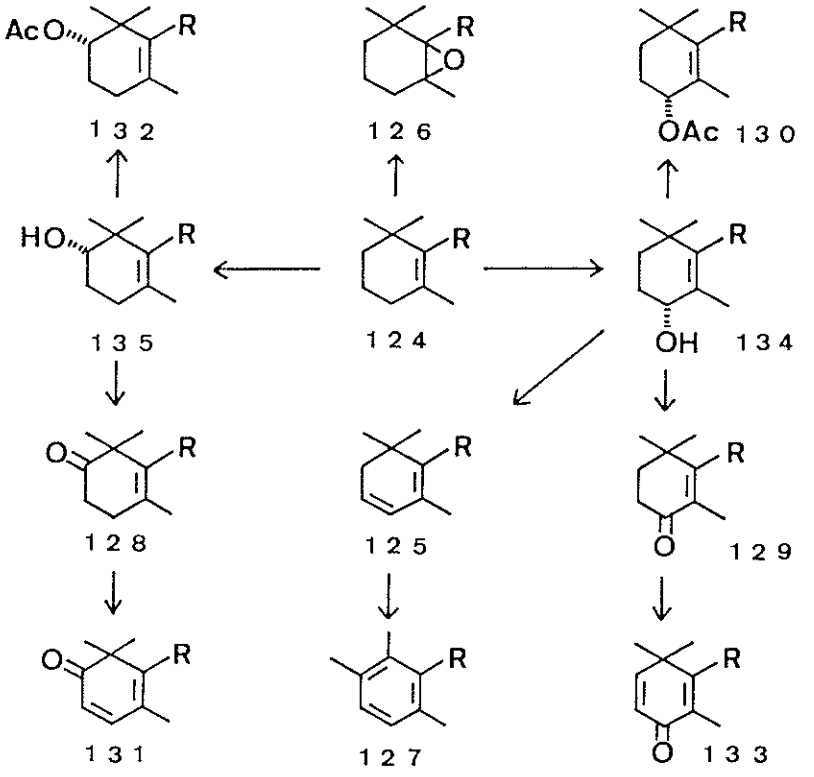

Figure 23. Conversion pathways of β-ionone and β-methylionone by *Aspergillus niger* JTS 191. a, β-Ionone and its conversion products, where R = $-CH{:}CH\text{-}CO\text{-}CH_3$; b, β-methylionone and its conversion products, where R = $-CH{:}CH\text{-}CO\text{-}CH_2\text{-}CH_3$. β-ionone(124a), 3,4-dehydro-β-ionone(125a), 5,6-epoxy-β-ionone(126a), 4-(2,3,6-trimethylphenyl)-but-3-en-2-one(127a), 2-oxo-β-ionone(128a), 4-oxo-β-ionone(129a), 4-acetoxy-β-ionone(130a), 2-oxo-3,4-dehydro-β-ionone(131a), 2-acetoxy-β-ionone(132a), 2,3-dehydro-4-oxo-β-ionone(133a), (R)-4-hydroxy-β-ionone(134a), (S)-2-hydroxy-β-ionone(135a).

Although C14 trimethylcyclohexane compounds have not been isolated from tobacco, the neutral fraction of conversion products from β-methylionone was very effective, in the parts-per-million concentration range, for flavouring tobacco.

The major products from β-ionone (124a) were (*R*)-4-hydroxy-β-ionone (134a) and (*S*)-2-hydroxy-β-ionone (135a). 2-Oxo- (128a), 4-oxo- (129a), 3,4-dehydro- (125a), 2,3-dehydro-4-oxo- (133a), 3,4-dehydro-2-oxo- (131a), (*S*)-2-acetoxy- (132a), (*R*)-4-acetoxy- (130a) and 5,6-epoxy-β-ionone (126a), and 4-(2,3,6-trimethylphenyl)-but-3-en-2-one (127a), and 4-(2,3,6-trimethylphenyl)-but-3-en-2-one (127a) were identified as minor products. Analogous transformation products of β-methylionone also were identified. Based on gas–liquid chromatographic analysis during the fermentation, metabolic pathways of β-ionone and β-methylionone by *A. niger* were proposed (*Figure 23*). There are two main pathways: the enantioselective

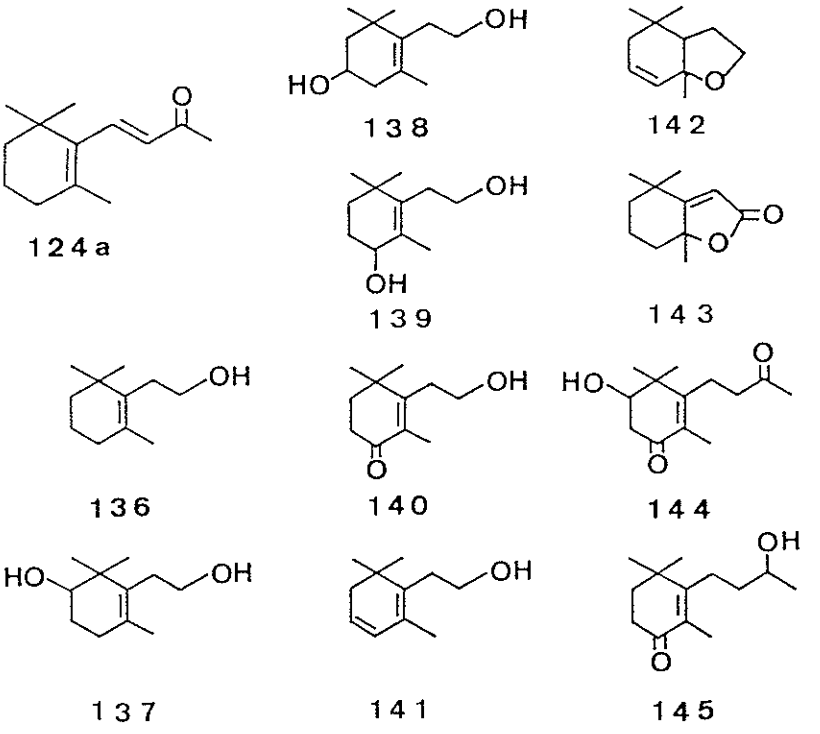

Figure 24. Conversion products of β-ionone by *Lasiodiplodia theobromae* ATCC 28570. β-ionone(124a), β-cyclohomogeraniol(136), 2-hydroxy-β-cyclohomogeraniol(137), 3-hydroxy-β-cyclohomogeraniol(138), 4-hydroxy-β-cyclohomogeraniol(139), 4-oxo-β-cyclohomogeraniol(140), 3,4-dehydro-β-cyclohomogeraniol(141), 4,4,7a-trimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran(142), dihydroactinidiolide(143), 2-hydroxy-4-oxo-7,8-dihydro-β-ionone(144), 4-oxo-7,8-dihydro-β-ionol(145).

hydroxylation at C2 and C4 of β-ionones, followed by further oxidation or dehydration or acetylation.

In general, stereochemistry is a major subject in flavour and fragrance chemistry. In this study, the odour of (*R*)-4-hydroxy-β-ionone (134a) differed from that of chemically synthesized (±)-4-hydroxy-β-ionone. The chiral compounds must play an important part in producing the characteristic odour quality of the conversion products.

Analogous studies were reported by Krasnobajew and Helminger (1982). By employing *Lasiodiplodia*, an essential oil-like product with tobacco flavour could be obtained from β-ionone. In a similar way to the Baeyer–Villiger oxidation, *Lasiodiplodia theobromae* ATCC 28570 efficiently removes a C2-unit from the side-chain of β-ionone giving rise to the main product β-cyclohomogeraniol (136). Further enzymatic action of this fungus such as hydrogenations and hydroxylations led to the production of 2-hydroxy- (137), 3-hydroxy- (138) and 4-hydroxy-β-cyclohomogeraniol (139), 4-oxo-β-cyclohomogeraniol (140), 3,4-dehydro-β-cyclohomogeraniol (141), 4,4,7a-

trimethyl-2-oxo-2,3,3a,4,5,7a-hexahydrobenzofuran (142), dihydroactinidiolide (143), 2-hydroxy-4-oxo-7,8-dihydro-β-ionone (144) and 4-oxo-7,8-dihydro-β-ionol (145) (*Figure 24*).

The studies mentioned above showed that essential oil-like products which are not yet chemically accessible can be obtained through fermentation as a readily available synthetic fragrance. This enzymatic action of the fungi on β-ionones could help to explain the formation of fragrance metabolites from carotenoids in nature.

α-*Ionone*

As the extension of the studies mentioned above on the microbial conversion of the trimethylcyclohexane compounds related carotenoids, the conversion of α-ionones by the same fungus, *Aspergillus niger* JTS 191, has been investigated (Mikami, 1984; Yamazaki *et al.*, 1988a). It was found that fermentation of α-ionones yielded an essential oil-like product with tobacco flavour.

The major products from (±)-α-ionone (146a) were *cis*-3-hydroxy- (*cis*-147a), *trans*-3-hydroxy- (*trans*-147a) and 3-oxo-α-ionone (148a). 2,3-Dehydro-α-ionone (150a), 3,4-dehydro-β-ionone (125a) and 1-(6,6-dimethyl-2-methylene-3-cyclohexenyl)-buten-3-one (3,4-dehydro-γ-ionone) (149a) were also identified as minor products. Analogous bioconversion products from α-methylionone (146b) and α-isomethylionone (146c) were also identified.

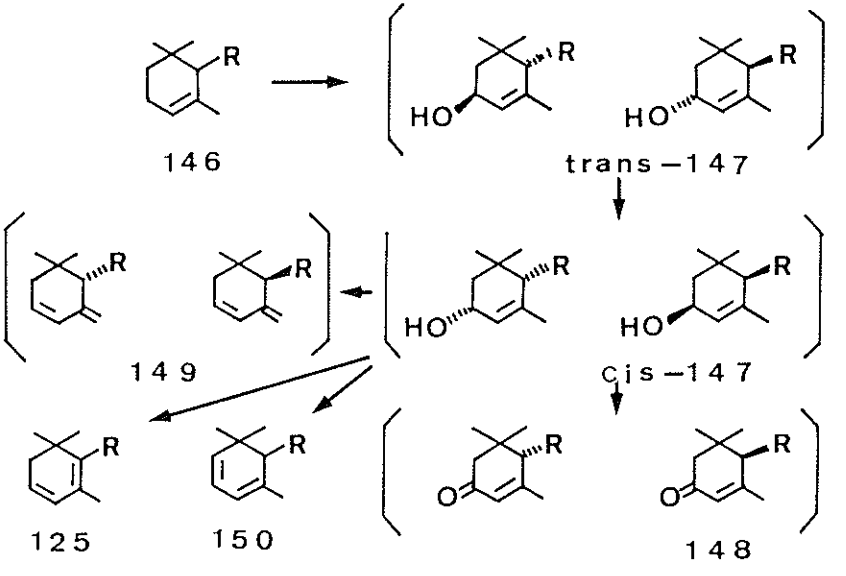

Figure 25. Conversion pathways of α-ionones by *Aspergillus niger*. a, α-Ionone and its conversion products, where R = $-CH{:}CH\text{-}CO\text{-}CH_3$; b, α-methylionone and its conversion products, where R = $-CH{:}CH\text{-}CO\text{-}CH_2\text{-}CH_3$; c, α-isomethylionone and its conversion products, where R = $-CH{:}C(CH_3)\text{-}CO\text{-}CH_3$. α-ionone(146a), *trans*-3-hydroxy-α-ionone(trans-147a), *cis*-3-hydroxy-α-ionone(cis-147a), 3-oxo-α-ionone(148a), 3,4-dehydro-γ-ionone(149a), 2,3-dehydro-α-ionone(150a), 3,4-dehydro-β-ionone(125a).

The same fermentation conditions as elaborated for β-ionone conversion were also optimal for fermentation of α-ionone, and α-ionones were more easily converted than β-ionones. The efficiency of bioconversion, however, depended on the side chain of α-ionones, that is in order of α-isomethyl-, α-methyl- and α-ionone.

Racemic α-ionone was added to the culture broth as the substrate, and the dextrorotatory power of α-ionone in the culture broth rapidly increased. This means that the fungus preferred (6*S*)-(−)-α-ionone to (6*R*)-(+)-α-ionone on incorporation or metabolism. However, with the lapse of time, the substrate was completely incorporated in the fungus cells and the specific rotation of the conversion products became nearly zero. On the basis of experimental results, the metabolic pathway of α-ionones was proposed as shown in *Figure 25*.

On the other hand, *Lasiodiplodia theobromae* also proved to convert α-ionone to an essential oil-like product with black tea or tobacco odour (Krasnobajew and Rytokonen, 1981; Krasnobajew, 1984). As observed for β-ionone, the formation of α-cyclohomogeraniol (151) as well as the occurrence of C11 products suggested that the Bayer–Villiger-type reaction had occurred in the first step of bioconversion. The products identified were as follows: α-cyclohomogeraniol (151), 3-hydroxy-α-cyclohomogeraniol (152), 3-oxo-α-cyclohomogeraniol (153), 4,4,7-trimethyl-2,3,3a,4,5,7a-hexahydro-

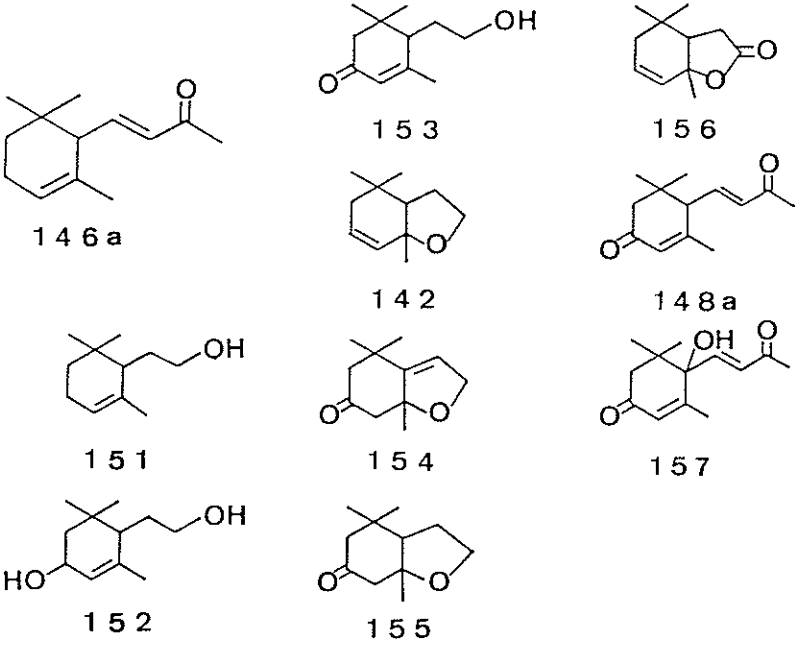

Figure 26. Conversion products of α-ionone by *Lasiodiplodia theobromae*. α-ionone (146a), α-cyclohomogeraniol(151), 3-hydroxy-α-cyclohomogeraniol(152), 3-oxo-α-cyclohomogeraniol(153), 4,4,7a-trimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran(142), 4,4,7a-trimethyl-6-oxo-2,4,5,6,7,7a-hexahydrobenzofuran(154), 4,4,7a-trimethyl-6-oxo-2,3,3a,4,5,6,7,7a-octahydrobenzofuran(155), 4,4,7a-trimethyl-2-oxo-2,3,3a,4,5,7a-hexahydrobenzofuran(156), 3-oxo-α-ionone(148a), dehydrovomifoliol(157).

benzofuran (142), 4,4,7a-trimethyl-6-oxo-2,4,5,6,7,7a-hexahydrobenzofuran (154), 4,4,7a-trimethyl-6-oxo-2,3,3a,4,5,6,7,7a-octahydrobenzofuran (155), 4,4,7a-trimethyl-2-oxo-2,3,3a,4,5,7a-hexahydrobenzofuran (156), 3-oxo-α-ionone (148a) and dehydrovomifoliol (157) (*Figure 26*).

α-*Ionylideneacetic acid*

(+)-Abscisic acid (ABA) (163), a sesquiterpene, is widely known as one of the plant hormones. The analogues of ABA, 2-*cis*, 4-*trans*-xanthoxin (159a) and 2-*trans*, 4-*trans*-xanthoxin (159b), were first produced by photochemical oxidation of violaxanthin (158) (*Figure 27*). These two compounds have been found in many higher plants as plant hormones (Tayler and Burden, 1970a, b). Therefore, as for the origin of ABAs, three possibilities have been proposed: the sesquiterpene biosynthesis from mevalonic acid; biodegradation of carotenoids, or both (Takahashi, Marumo and Ootake, 1973). ABA is also produced by certain fungi such as *Cercospora* and *Botrytis* (Assante, Merlini and Nasini, 1977; Marumo *et al.*, 1982).

An analogue of ABA (163), (±)-(2*Z*, 4*E*)-α-ionylideneacetic acid (160) possesses similar plant growth inhibitory activity to that of ABA. There is the possibility that this strong activity may be caused by its conversion into ABA in plants. Ichimura, Oritani and Yamashita (1983) translated this idea into an experiment.

(±)-(2*Z*, 4*E*)-α-Ionylideneacetic acid (160) was enantioselectively oxidized to (+)-(1′*R*)-(2*Z*,4*E*)-4′-oxo-α-ionylideneacetic acid (161a), (−)-(1′*S*)-(2*Z*,4*E*)-4′-hydroxy-α-ionylideneacetic acid (161b) and (+)-ABA (163) by *Cercospora cruenta* IFO 6164, which can produce (+)-ABA (*Figure 28*). From 40 mg of the (±)-α-acid (160), 1 mg of (+)-ABA (163), 9·6 mg of

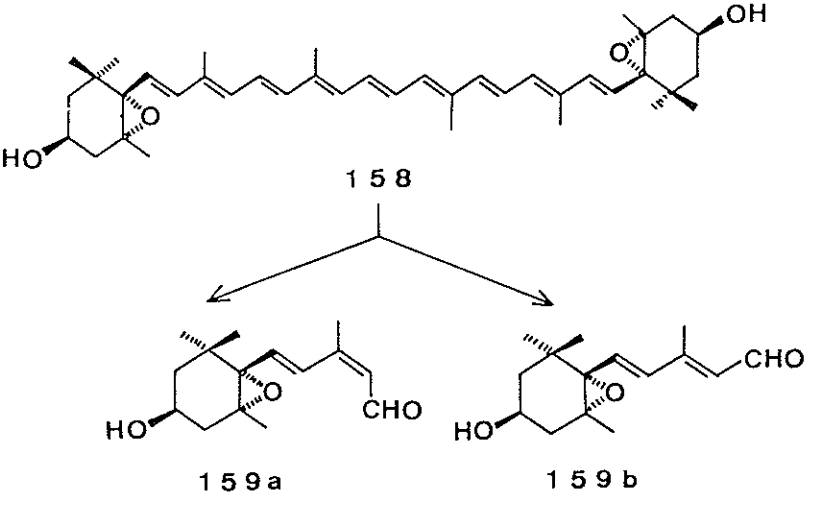

Figure 27. Formation of xanthoxin from violaxanthin. Violaxanthin(158), 2-*cis*,4-*trans*-xanthoxin(159a), 2-*trans*,4-*trans*-xanthoxin(159b).

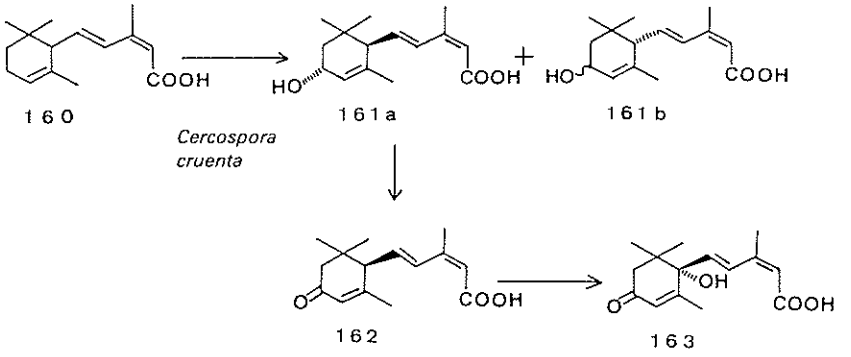

Figure 28a Conversion of α-ionylideneacetic acid by *Cercospora cruenta*. (2*Z*,4*E*)-α-ionylideneacetic acid(160), (+)-(1′*R*,4′*R*)-(2*Z*,4*E*)-4′-hydroxy-α-ionylideneacetic acid(161a), (−)-(1′*S*)-(2*Z*,4*E*)-4′-hydroxy-α-ionylideneacetic acid(161b), (+)-(1′*R*)-(2*Z*,4*E*)-4′-oxo-α-ionylideneacetic acid(162), (+)-abscisic acid(163).

(−)-(2*Z*,4*E*)-4′-hydroxy-α-ionylideneacetic acid (161b) and 8·8 mg of (+)-4′-oxo-α-acid (162) were obtained (*Figure 28*).

Enantioselectivity of the microbial oxidation was re-examined by using optically pure (+)- and (−)-(2*Z*,4E)-α-ionylideneacetic acid as the substrates (Ichimura, Oritani and Yamashita, 1983).

3-Acetoxy-4-oxo-β-ionone

(*S*)-3-Acetoxy-4-oxo-β-ionone (164a) is an important precursor for the synthesis of astaxanthin (166), a carotenoid pigment. Asymmetric hydrolysis of various terpene alcohol acetates with micro-organisms had been reported by Oritani and Yamashita (1973a, b; 1974). Becher *et al.* (1981) examined these reported micro-organisms and found that *Absidia hyalospora* IFO 8022 hydrolysed (±)-3-acetoxy-4-oxo-β-ionone (164) with an optical purity of 60%, in high yield. Furthermore, a strain of *Bacillus* species isolated from soil was found to hydrolyse the (±)-ester (164) to yield the optically active 3-hydroxy-4-oxo-β-ionone (165) leaving the (*S*)-ester (164a) (*Figure 29*). By an optimization of the fermentation conditions using a selected mutant by UV irradiation, the concentration of the racemic substrate (164) could be raised to 30 g/litre from 2·5 g/litre.

4-Oxoisophorone

(4*R*,6*R*)-4-Hydroxy-2,2,6-trimethylcyclohexanone (169a) is a key intermediate in the synthesis of naturally occurring, optically active xanthophylls such as zeaxanthin (170) and cryptoxanthin. These compounds (169a) have also been used for the synthesis of chiral plant growth regulators as well as for various flavouring compounds (Leuenberger *et al.*, 1976; Kienzle *et al.*, 1978).

A synthesis of this compound (169a) starting from the readily available 4-oxoisophorone (167) via biochemical preparation of (6*R*)-2,2,6-

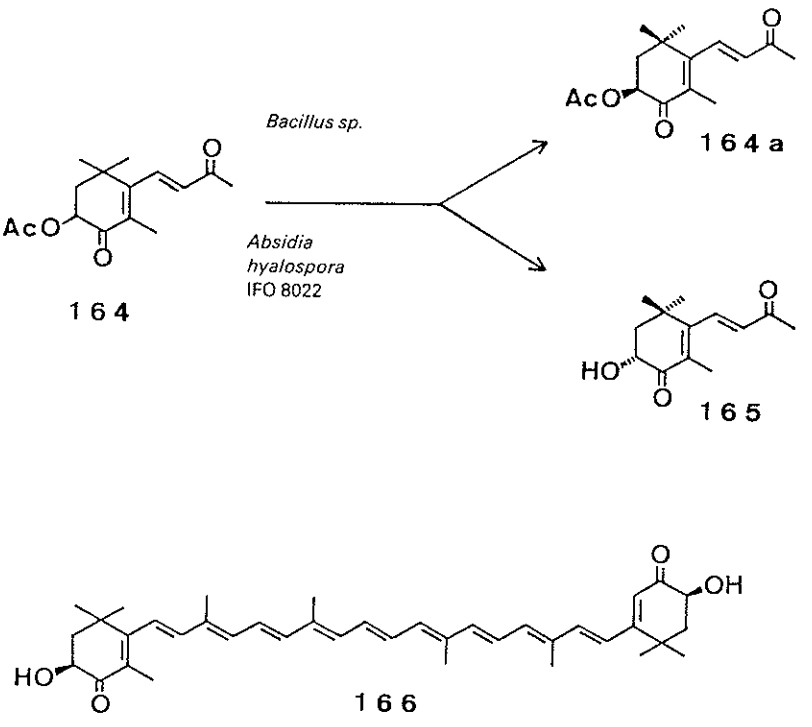

Figure 29. Synthesis of astaxanthin via optical resolution of (±)-3-acetoxy-4-oxo-β-ionone by micro-organisms. (±)-3-Acetoxy-4-oxo-β-ionone(164), (*S*)-3-acetoxy-4-oxo-β-ionone(164a), (*R*)-3-hydroxy-4-oxo-β-ionone(165), astaxanthin(166).

trimethylcyclohexane-1,4-dione (168a) was reported by Leuenberger *et al.* (1976). Chirality was introduced at C-6 of 4-oxoisophorone (167) by a stereoselective hydrogenation of the double bond using baker's yeast as the biocatalyst. Thereafter the carbonyl group at C-4 of (6*R*)-2,2,6-trimethylcyclohexane-1,4-dione (168a) was reduced stereoselectively by chemical methods to the corresponding alcohol (169a) (*Figure 30*). Under the optimized process, the baker's yeast produced about 20 mg of (6*R*)-2,2,6-trimethylcyclohexane-1,4-dione (168a) per 1 g of dry cells per hour, at a 87% yield.

In order to prepare (6*R*)-2,2,6-trimethylcyclohexane-1,4-dione (168a) more efficiently, the application of thermophilic bacteria was attempted. *Thermomonospora curvata* IFO 12384 was selected from many thermophiles tested as a strain capable of efficiently converting 4-oxoisophorone (167) into the dihydro-compound (168a) at 50°C. Under the optimal conditions, the production rate of the compound was 86 mg per gram of dry cells per hour, at a 95% yield (Hori, Hieda and Mikami, 1984).

The continuous asymmetric reduction of 4-oxoisophorone using this immobilized thermophile is discussed on page 311.

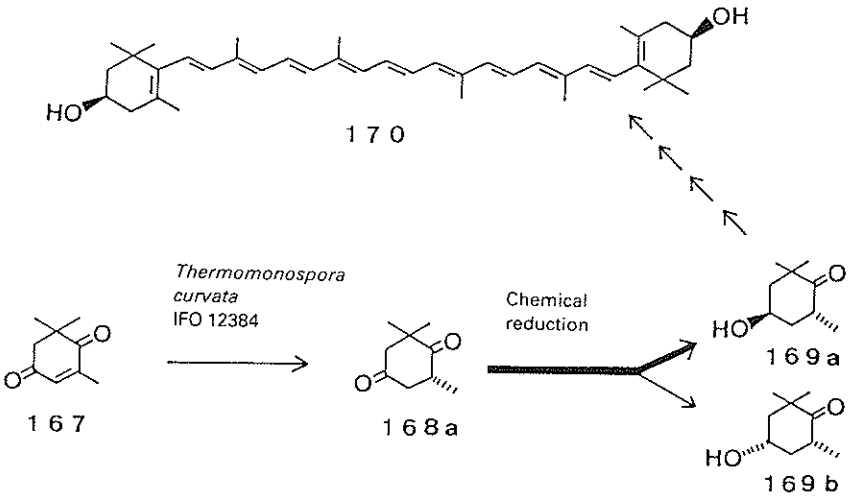

Figure 30. Synthesis of zeaxanthin via microbial conversion of 4-oxoisophorone. 4-Oxoisophorone(167), (6*R*)-2,2,6-trimethylcyclohexane-1,4-dione(168a), (4*R*,6*R*)-4-hydroxy-2,2,6-trimethylcyclohexanone(169a), (4*S*,6*R*)-4-hydroxy-2,2,6-trimethylcyclohexanone(169b), zeaxanthin(170).

The smallest monocyclic aroma compounds derived from carotenoids are C-9 fragments of trimethylcyclohexane ring (Ohloff, 1978). They are important aroma constituents of saffron, tea and tobacco. Tobacco contains 11 of these C9 fragments (Ohloff, 1978; Fujimori, 1984; Zarghami and Heinz 1971).

4-Oxoisophorone (167) was also converted by *Aspergillus niger* JTS 191 to eight or more compounds. The aroma complex produced was effective for flavouring tobacco. Seven conversion products were identified as follows: (6*R*)-2,2,6-trimethylcyclohexane-1,4-dione (168a), *trans*-4-hydroxy-2,2,6-trimethylcyclohexanone (169a), *cis*-4-hydroxy-2,2,6-trimethylcyclohexanone (169b), *cis*-4-hydroxy-3,3,5-trimethylcyclohexanone (174), (4*S*)-4-hydroxy-2,2,6-trimethyl-2-cyclohexen-1-one (171), (4*R*)-4-hydroxy-3,5,5,-trimethyl-2-cyclohexen-1-one (172a), and (2*S*)-2-hydroxy-2,6,6,-trimethylcyclohexane-1,4-dione (173). The metabolic pathway of 4-oxoisophorone using the fungus was proposed as shown in *Figure 31*, and featured an asymmetric reduction of the double bond and carbonyl group (Yamazaki *et al.*, 1988c).

A prolonged incubation of 4-oxoisophorone (167) with *Thermomonospora curvata* brought not only (6*R*)-2,2,6-trimethylcyclohexane-1,4-dione (168a) but also *trans*-4-hydroxy-2,2,6- (169a), *cis*-4-hydroxy-2,2,6- (169b), *cis*-4-hydroxy-3,3,5-trimethylcyclohexanone (174), and 4-hydroxy-2,6,6-trimethyl-2-cyclohexen-1-one (171), all of which, including the substrate, are tobacco constituents (Roberts and Rohde, 1972; Hori, Hieda and Mikami, 1984).

Isophorone

Isophorone (175) is the smallest and the most readily available trimethylcyclohexane compound. *Aspergillus niger* JTS 191 also proved able to convert

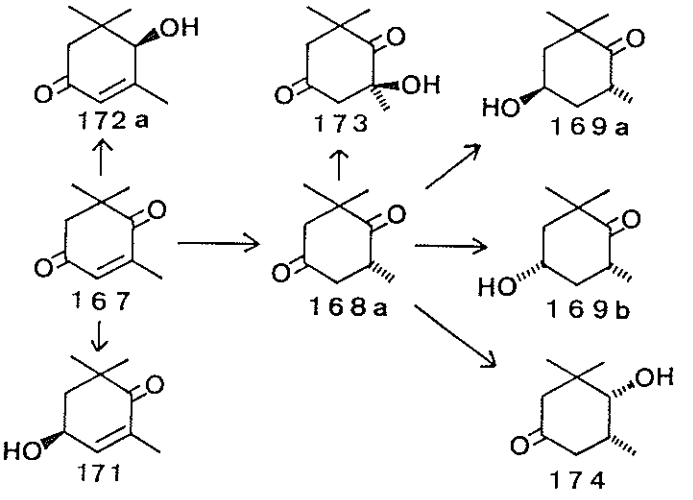

Figure 31. Conversion pathways of 4-oxoisophorone by *Aspergillus niger*. 4-Oxoisophorone(167), (6*R*)-2,2,6-trimethylcyclohexane-1,4-dione(168a), *trans*-4-hydroxy-2,2,6-trimethylcyclohexanone(169a), *cis*-4-hydroxy-2,2,6-trimethylcyclohexanone(169b), *cis*-4-hydroxy-3,3,5-trimethyl-2-cyclohexanone(174), (4*S*)-4-hydroxy-2,2,6-trimethyl-2-cyclohexen-1-one(171), (4*R*)-4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one(172a), (2*S*)-2-hydroxy-2,6,6-trimethylcyclohexane-1,4-dione(173).

isophorone, although the substrate was somewhat less readily converted than 4-oxoisophorone. Only four products were found and identified as follows: (4*S*)-4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one (172b), 3,5,5,-trimethyl-2-cyclohexene-1,4-dione (167), 3,5,5,-trimethylcyclohexane-1,4-dione (168), and 3-hydroxymethyl-5,5-dimethyl-2-cyclohexen-1-one (176). Major products were compounds 172b and 176. From the time course of fermentation followed by gas chromatography, the conversion pathway of isophorone was proposed (*Figure 32*). The main reactions were oxidation although the same fungus reduced 4-oxoisophorone, as mentioned above (Mikami *et al.*, 1981b).

Production process and techniques

MANUFACTURING-SCALE PRODUCTION PROCESS

(−)-Menthol production by asymmetric hydrolysis of (±)-menthyl chloroacetate

Industrial conditions for asymmetric hydrolysis of (±)-menthyl chloroacetate with *Pseudomonas* sp. NOF-5 were investigated and established by Inagaki and Ueda (1987a) as follows. The medium consisted of 6% corn steep liquor containing 0·1% of (−)-menthyl chloroacetate and 0·1% silicone antifoaming agent, initial pH 6·7. A 800 litre jar fermenter containing 400 l medium was

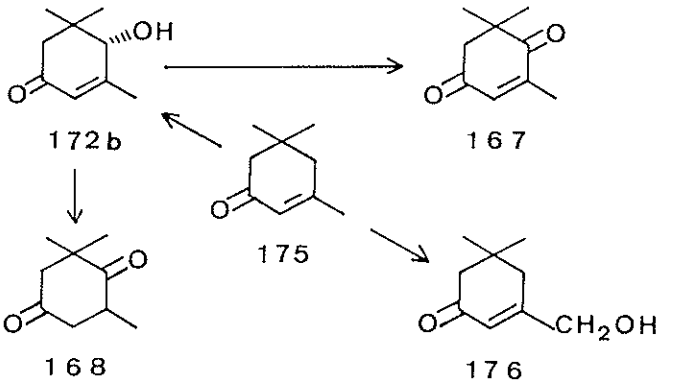

Figure 32. Conversion pathways of isophorone by *Aspergillus niger*. Isophorone(175), (4*S*)-4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one(172b), 3,5,5-trimethylcyclohexene-1,4-dione(167), 3,5,5-trimethylcyclohexane-1,4-dione(168), 3-hydroxymethyl-5,5-dimethyl-2-cyclohexen-1-one(176).

sterilized for an hour at 120°C and inoculated with 14 l of its *Pseudomonas* sp. NOF-5 preculture which was in the middle of logarithmic growth phase. Cultivation was carried out for 30 h at 30°C with an airflow of 200 l/min and stirring at 300 rev/min. To the resulting culture broth, 800 kg of (±)-menthyl chloroacetate was added. Then, asymmetric hydrolysis was performed for 70 h at 30°C, under continuous stirring without aeration, keeping the pH at 7·0 by the addition of ammonia water. After 72 h, the hydrolysis was complete as shown in *Figure 33*. As the reactant mixture was an emulsion, separation into an organic layer and a water layer was carried out by heating it to 70°C and adding ammonium sulphate (20 kg) for salting out. From the organic layer of the reaction mixture, 255 kg of (−)-menthol, $[\alpha]_D$ −49·5° (optical purity 99%) and 388 kg of (+)-menthyl chloroacetate, $[\alpha]_D$ +72° were obtained by fractional distillation at 10 mmHg.

Production of the essential oil-like compounds with tobacco flavour

Manufacturing conditions for the production of essential oil-like compounds with tobacco flavour by microbial conversion of ionones were investigated and established using a 200 l jar fermenter (Mikami, 1984). Under the optimal conditions, 6 g of ionones per litre of culture medium were converted. The manufacturing flow diagram and a typical fermentation process are depicted in *Figure 34* and *Figure 35* respectively.

Aspergillus niger JTS 191 was inoculated on to a potato-dextrose agar medium and incubated for 7 days at 28°C. The resulting spores were suspended in sterilized water at a concentration of 4×10^3/ml. From this spore suspension, 100 ml was inoculated into a 200 l jar fermenter (Suzuki Seiko, Tokyo) equipped with an axial flow-empeller; 100 l growth medium consisting of 3% sucrose, 0·2% $NaNO_3$, 0·1% K_2HPO_4, 0·05% KCl, 0·05% $MgSO_4 \cdot 7H_2O$,

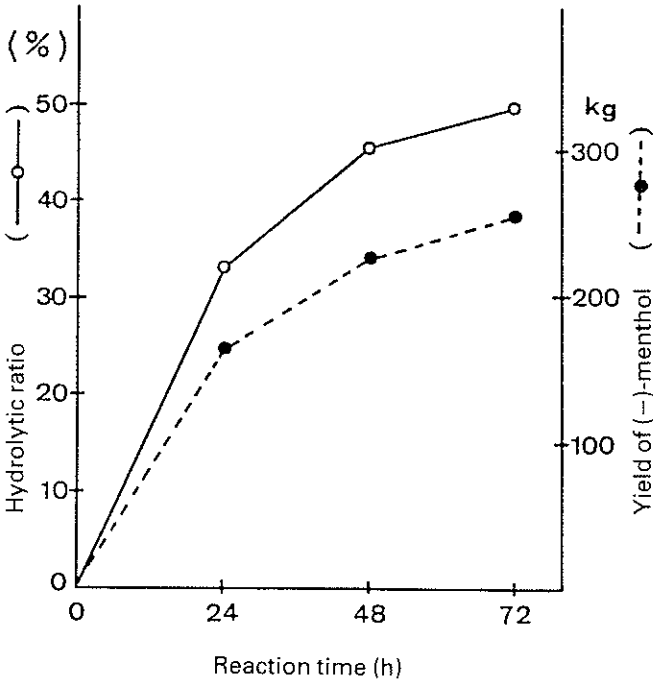

Figure 33. Asymmetric hydrolysis of (±)-menthyl chloroacetate by *Pseudomonas* sp. NOF-5 in 800 litre jar fermenter.

0·1% yeast extract and tap water, at pH 7·0 before sterilization, was also added. Incubation took place at 28°C for 46–48 h with an airflow of 8 l/min during the first 24 h and then 10 l/min, with stirring at 170 rev/min.

Conversion of β-*ionone.* The resulting culture broth contained about 4·5 g (dry weight) of mycelial pellets, pH about 4·5; 0·1–0·15% of the substrate β-ionone was added at the intervals shown in *Figure 35*. The culture broth was stirred and aerated under the same conditions and a 50 ml sample was taken every 12 h and analysed by gas chromatography. The first addition of β-ionone elevated the dissolved oxygen level and decreased mycelial weight because of the antifungal activity of the substrate. After the second addition of the substrate, however, this phenomenon did not occur, suggesting the acquisition of resistance to the toxic substrate. Over the fermentation period of 7 days, 400 g β-ionone were converted while consuming sucrose. Throughout this period, the pH of the culture broth was 4·0–4·5 without adjustment. Judging from gas chromatography and the olganoleptic properties of the conversion products, fermentation was complete.

The fermentation broth was continuously centrifuged and mycelial pellets were removed. The broth was extracted with 20 l ethyl acetate using a

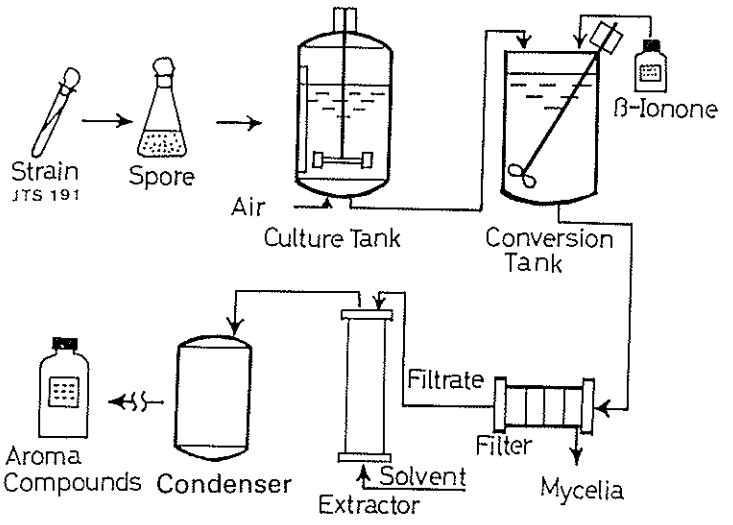

Figure 34. Manufacturing flow diagram of essential oil-like compounds with tobacco flavour by microbial conversion of β-ionone.

countercurrent extracter (Yûki Engineering, Tokyo). After drying over Na_2SO_4, the solvent was evaporated *in vacuo* at 40°C to give about 360 g of conversion products (*c.* 90%) with a tobacco-like odour.

Conversion of methylionone mixture. Methylionone mixture (α-*n*-methylionone, α-isomethylionone and β-n-methylionone) was converted by an analogous procedure using a 2 kl jar fermenter (Mikami, 1984). The resulting aroma complex was better than that from β-ionone for flavouring tobacco, probably because it contained many serial products like natural

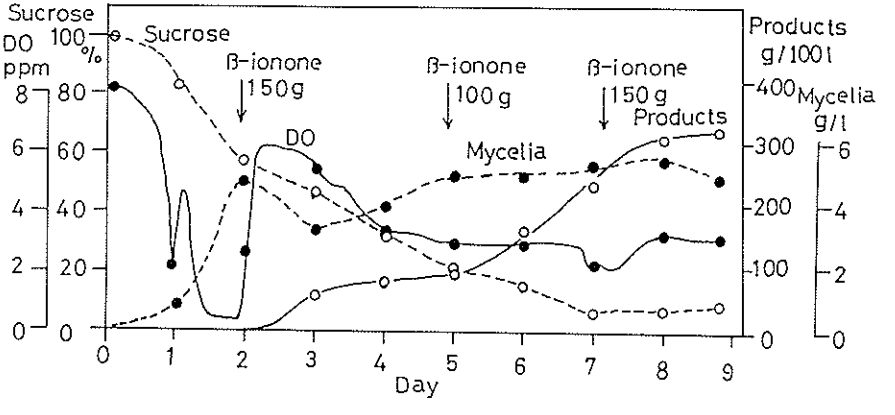

Figure 35. Conversion of β-ionone by *Aspergillus niger* JTS 191 in 200 litre jar fermenter. The substrate was repeatedly added. DO, dissolved oxygen; mycelia, dry weight (g/litre); arrow, addition of β-ionone (g/100 litre medium).

essential oils. As the products contained a number of diastereomers, separation was not complete even when a gas chromatograph equipped with a capillary column was used. In this case, because the substrate was a mixture, the completion of fermentation could not be determined only from the gas chromatogram pattern. Various ratios of functional groups of conversion products were then computed on the basis of gas chromatography. It was found that the progress of fermentation was a function of the ratio of the carbonyl group produced by the introduced hydroxy group. This coincides with the fact that the microbial conversion of ionones by *Aspergillus niger* is an oxidative process.

Among the processes mentioned above, the most important was found to be the pellet formation. The growth of moulds in the form of pellets has been collectively reviewed by Metz and Kossen (1977).

VARIOUS TECHNIQUES

Various techniques have been studied by many researchers hoping to improve the efficiency of microbial conversion.

The application of thermophilic bacteria to the conversion of terpenoids has many advantages because of the high temperature employed. These advantages include a high reaction rate, low interfacial tension which is effective in emulsion formation, freedom from contamination of mesophilic bacteria and the lack of need for a cooling system to remove the heat of fermentation, although a high temperature is incompatible with the volatility and lability of certain terpenoids. As mentioned, *Thermomonospora curvata* converted 4-oxoisophorone to (3*R*)-dihydro-4-oxoisophorone at 50°C more efficiently than mesophiles (Hori, Hieda and Mikami, 1984). This microbial conversion was strongly dependent upon cell growth (Sode *et al.*, 1988c). Subsequently, the thermophile was immobilized in a newly developed air-bubbling hollow-fibre reactor and converted to 4-oxoisophorone with high efficiency and high density cell growth (Sode *et al.*, 1987, 1988b).

Bioconversion by enzymes or spores (Vezina, Sehgal and Sing, 1968) is also feasible in connection with immobilized techniques (Mosbach, 1976; Fukui and Tanaka, 1982).

Micro-organisms such as *Pseudomonas* have diverse abilities to produce various conversion products from a substrate. Treatment with UV or mutational agents are practical methods for obtaining the micro-organisms which affect an enzyme on the conversion pathways, and of accumulating the desired products (Jacobson, 1981). On the other hand, a plasmid-determined transformation of terpenoids has been reported (Hieda *et al.*, 1982c; Hieda, Mikami and Obi, 1983b) suggesting the usefulness of a DNA recombinant to prepare the desired micro-organisms.

In general, terpenoids have antimicrobial activities, and so an excess of substrate inhibits culture growth and brings about cell lysis. Therefore, in order to maintain the conversion ability of micro-organisms, continuous (Krasnobajew and Helminger, 1982) or repeated (Mikami, 1984) addition of small quantities of the substrate have been successfully employed.

Bioconversion in the presence of an organic solvent is of great importance because its application improves the solubility of the water-immiscible substrate and, subsequently, accelerates conversion velocity. In addition, biocatalysts normally exist in the water phase, so the separation of biocatalysts from the reaction mixture is relatively easy. It has been said that the polarity of the organic solvent is the major factor in bioconversions using organic solvents (Omata, Tanaka and Fukui, 1980). Sode *et al.* (1988a) attempted the microbial conversion of β-ionone in the presence of an organic solvent. It was found that the addition of iso-octane (1–2%) accelerated the conversion: it took 3 days to complete the reaction whereas without iso-octane it took more than 6 days. The addition of iso-octane also improved the resistance of *Aspergillus niger* to the antifungal property of β-ionone.

Further improvement of the resistance to the antifungal property of β-ionone was achieved by immobilization. *Aspergillus niger* immobilized by the hydrophobic polymer, PU-3, was repeatedly used for microbial conversion of β-ionone in the presence of iso-octane for more than 480 h. Omata *et al.* (1981) reported the biocatalytic reaction in the organic solvent. (±)-Menthyl succinate was hydrolysed stereoselectively by *Rhodotolura minuta* var. *texensis* cells entrapped within polyurethane resin gels in water-saturated *n*-heptane. The hydrolysed product was pure (−)-menthol. The half-life of the polyurethane-entrapped cells was estimated to be 55–63 days in the organic solvent.

Conclusion

This chapter has attempted to review some of the useful roles of micro-organisms in the preparation of flavour and fragrance compounds as well as bioactive substances. The fact that only a few of them have reached the commercial production stage does not necessarily indicate that the others are unacceptable, but is a reflection of the highly competitive state of the industry. The microbial conversions exhibit characteristics such as substrate specificity, regio-specificity and stereoselectivity. These features make up for the lack of chemical processes. Moreover, an essential oil-like product which contains many constituents was obtained by microbial conversion of one or a few substrates through serial conversion pathways. Although microbial conversion of terpenoids may appear economically discouraging compared with chemical synthesis today, the development of biotechnological processes such as immobilized biocatalysts, bioreactor systems, biochemical reaction in organic solvents and various other techniques mentioned above, will continuously increase the efficiency of manufacturing-scale production.

Acknowledgements

The author is indebted to Drs Y. Yamazaki and N. Hori for corrections of the text, stimulating discussion and valuable suggestions. Mrs Yumiko Hayashi is responsible for the figures and the lay-out of this chapter, which she did with great skill and patience.

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