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Microbial transformations of acyclic monoterpenes

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Abstract. Microbial degradation of geraniol, citronellol, linalool and their corresponding acetates, structurally modified linalool and linalyl acetate, α-terpineol and β-myrcene are presented. Oxygenative and prototropic rearrangements are normally observed during the microbial metabolism of monoterpenes. Three types of oxygenation reactions are observed, namely, (a) allylic oxygenation (b) oxygenation on a double bond and (c) addition of water across the double bond. The studies indicate commonality in the reaction types or processes occurring during the metabolism of various related monoterpenes and also establish the convergence of degradative pathways at a central catabolic intermediate.

Keywords. Monoterpenes; micro-organisms; metabolism; prototropic-cyclization; oxygenation; fermentation; degradation; aromatization.

1. Introduction

Monoterpenes are widely distributed in nature and they are the usual constituents of essential oils. These natural products, mostly of higher plant origin, are subjected to oxidative metabolism by various types of micro-organisms present in the soil. This process of 'mineralization' is an essential biochemical step in the 'carbon cycle' occurring in nature. Terpenes, particularly the monoterpene alcohols due to their antimicrobial property are generally resistant to microbial degradation. However, during the course of evolution some of the saprophytic micro-organisms, especially the species belonging to *Pseudomonas* have acquired the unique ability to degrade and live on these compounds. In the process of utilizing these compounds as the carbon source, the micro-organisms carry out several interesting rearrangements in the molecule before ultimately degrading them to carbon dioxide and water. On the other hand, fungi is normally unable to degrade a terpene molecule all the way to carbon dioxide and water, although when grown in the presence of energy-rich medium, can metabolize or transform them due to the nonspecific nature of the enzymes present in them.

The unique ability of micro-organisms to carry out different types of reactions has attracted the attention of synthetic organic chemists. Microbes can serve as an important tool in the hands of the organic chemists. The most important aspect of microbes is their ability to catalyze reactions with high degree of stereospecificities. Microbes can introduce stereospecifically oxygen function, bring about the transformations of certain functional groups or carry out asymmetric hydrolysis, which may be either difficult or require a number of intermediate stages in a conventional approach. In fact micro-organisms are extensively used in the steroid transformations. Steroidal intermediates, which are difficult to synthesize are achieved by the use of microbial systems (Vezina and Singh 1976). One such example is the 11α-hydroxylation of progesterone by *A. ochraceus* or *R. nigricans* (Peterson and Murray 1952). Even the

enzyme system isolated from *A. ochraceus* efficiently carries out the 11α-hydroxylation reaction (Jayanthi *et al* 1982; Madyastha *et al* 1984). Microbes are also known to carry out novel transformations of steroids (Madyastha and Valli 1983).

Progress in the microbial degradation of terpenoids in general and monoterpenes in particular, lagged considerably behind as compared to the enormous amount of work carried out with regard to other classes of compounds such as steroids was mainly due to (a) the non-physiological character of terpenoids, and (b) their ability to inhibit the growth of diverse microbial flora. The first detailed work on the microbial degradation of acyclic monoterpenes has been reported in the early part of 1960 (Seubert *et al* 1963; Seubert and Remberger 1963; Seubert and Fass 1964). They have studied the metabolism of citronellal (1), citronellol (2), geraniol (3) and geranic acid (4) by a soil bacterium, *Pseudomonas citronellolis*. It has been observed that the metabolism of these acyclic monoterpenes is initiated by a step-wise oxidation of the primary alcoholic group to the carboxylic group, followed by carboxylation of the C-10 methyl group (β-methyl) by a biotin-dependent carboxylase. The carboxy methyl group gets eliminated at a later stage as acetic acid. Further degradation follows the β-oxidation pattern. On the basis of specificity of the enzyme geranyl carboxylase, it has been suggested that the pathways of degradation of citronellic (5), geranic (4) and neranic (6) acids converge at the neranic acid (Seubert and Fass 1964). The details of the pathway are shown in figure 1.

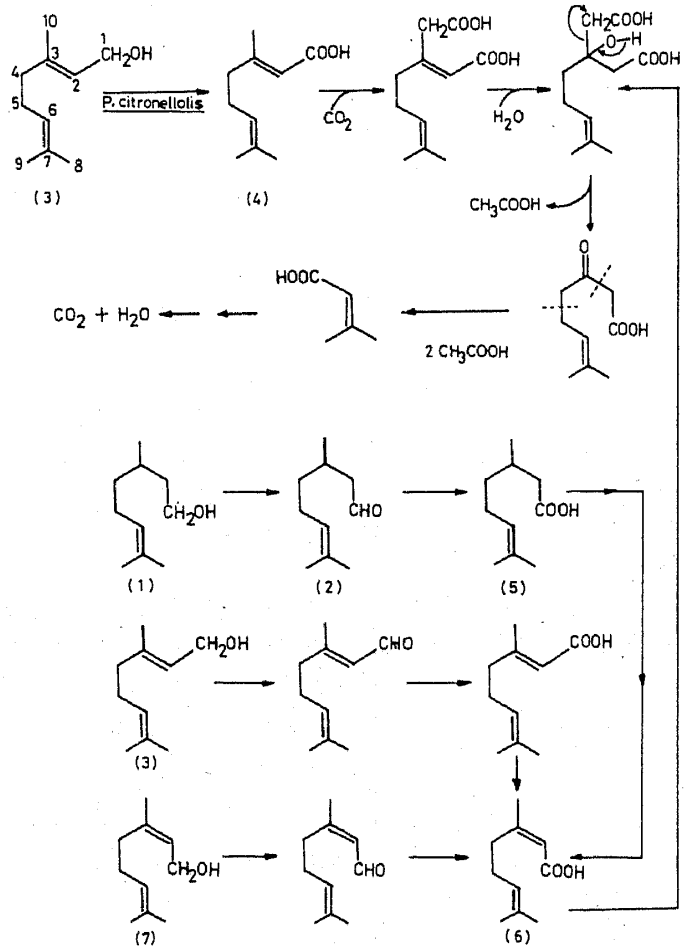

Figure 1. Pathways of degradation of geraniol, nerol and citronellol by *P. citronellolis*.

In contrast to the above findings, higher plants (Madyastha *et al* 1976), fungi (Madyastha and Murthi, Unpublished observation) and mammalian systems (Chadha and Madyastha 1982, 1984) carry out the biotransformation of geraniol (3) and nerol (7) by a totally different pathway. All these living systems specifically carry out the oxidation of the C-8 methyl (ω-methyl) group of acyclic monoterpene alcohols. This observation has prompted us to find a microbial system that has the ability to mimic the mode of metabolism of acyclic monoterpene alcohols observed in fungi, higher plants and mammals. A search for such a microbial system led to the isolation of a very versatile soil pseudomonad, identified as *Pseudomonas incognita*, capable of utilizing geraniol (3), nerol (7), citronellol (1), linalool (8), α-terpineol (9) and their corresponding acetates as the carbon source. Isolation of this versatile micro-organism has given us an opportunity to study the pathways of degradation of acyclic monoterpene alcohols and compare the chemical logic of microbial processes to that seen in the higher plant, fungal and mammalian systems.

2. Metabolism of linalool and linalyl acetate

P. incognita was initially isolated on linalool (8) and hence it was of interest to establish its mode of metabolism in this organism. Isolation and identification of various metabolites from the culture medium have clearly indicated the existence of atleast two different pathways for the biodegradation of linalool (Madyastha *et al* 1977). One of the pathways (figure 2) appears to be initiated by the specific oxygenation of the C-8 methyl group of linalool (8) to 8-hydroxy linalool (10) which further undergoes stepwise oxidation in the presence of NAD-linked dehydrogenases to linalool-8-aldehyde and then to linalool-8-carboxylic acid (11). The second metabolic route consists of prototropic cyclization of linalool to α-terpineol (9) and its further degradation proceeds through the progressive oxidation of the C-10 methyl to yield oleuropeic acid (12). Besides, the presence of linalool oxide (13) and an unsaturated lactone (14) has been noticed. The formation of these compounds probably proceeds through the epoxidation of the 6,7-double bond which on further oxidation could give rise to these

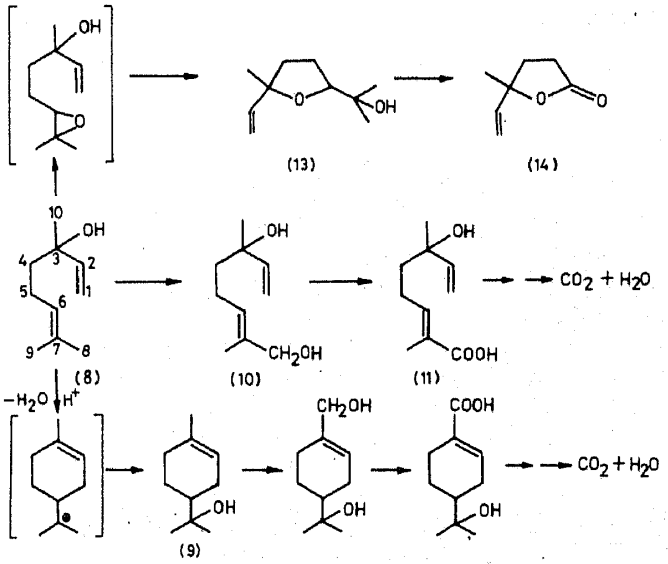

Figure 2. Probable pathways of degradation of linalool by *P. incognita*.

compounds. Chemical analogies of such reactions have been reported earlier (Felix *et al* 1963).

The C-8 methyl oxidation of linalool by a *Pseudomonas* sp. has been reported earlier (Murakami *et al* 1973). In a similar way, the degradation of β-myrcene (15) by a strain of *Pseudomonas putida* (figure 3) commences with the oxidation of the C-8 methyl group (Narushima *et al* 1981). None of the metabolites isolated so far in the linalool fermentation suggests the possible oxidation of the C-1 position (figure 2). In the mammalian metabolism of β-myrcene, the terminal methylene groups are epoxidated and opening of the epoxides lead to the formation of diols (Madyastha and Vatsan, Unpublished observation). However, such a phenomenon is not observed in the microbial degradation of linalool. The interesting aspect of linalool metabolism by *P. incognita* is its ability to initiate one of the major energy-yielding pathways by the specific ω-methyl (C-8 methyl) oxygenation, whereas it fails to carry out similar oxidation with geraniol, nerol and citronellol, although these acyclic monoterpene alcohols are closely related to linalool. The mode of further metabolism of linalool-8-carboxylic acid (11) and oleuropeic acid (12) becomes reasonably clear from the degradation studies carried out with linalyl acetate (16).

P. incognita accepts linalyl acetate (16) better than linalool (8) as the sole source of carbon (Renganathan and Madyastha 1983). Hence it was of interest to find out whether the organism initiates the degradation of linalyl acetate by hydrolyzing the acetate to free alcohol or it has the ability to metabolize the molecule keeping the acetate moiety intact. Cursory examination of various metabolites isolated from the culture medium indicates the existence of atleast three different pathways for the microbial degradation of linalyl acetate (figure 4, Renganathan and Madyastha 1983). Pathways A and C (figure 4) involve hydrolysis of linalyl acetate to linalool which gets further metabolized as discussed earlier (figure 2). The striking aspect of the third pathway (B, figure 4) is the unique ability of the organism to degrade linalyl acetate while keeping the acetate moiety intact as evidenced by the isolation of linalyl acetate-8-carboxylic acid (17) and Δ^5-4-acetoxy-4-methyl hexenoic acid (18) from the culture medium. The formation of linalyl acetate-8-carboxylic acid proceeds in a manner similar to the formation of linalool-8-carboxylic acid (11) from linalool (figure 2). Further, it has been established that NAD-dependent alcohol and aldehyde dehydrogenases catalyze the conversion of 8-hydroxy linalyl acetate (19) to its corresponding acid. The linalyl acetate grown cells readily convert linalyl acetate-8-aldehyde to Δ^5-4-acetoxy-4-methyl hexenoic acid (18) suggesting that the 6,7 double

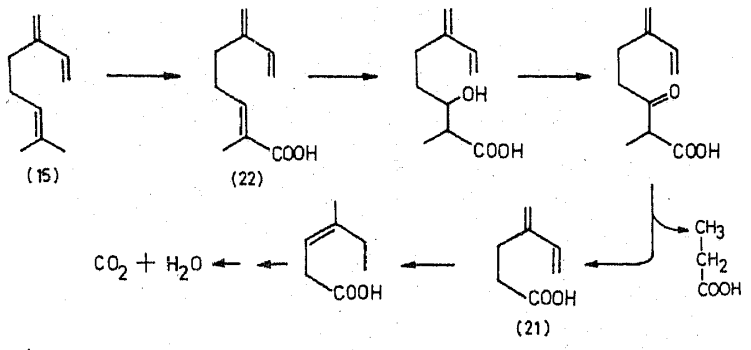

Figure 3. Degradation of β-myrcene by a strain of *P. putida*.

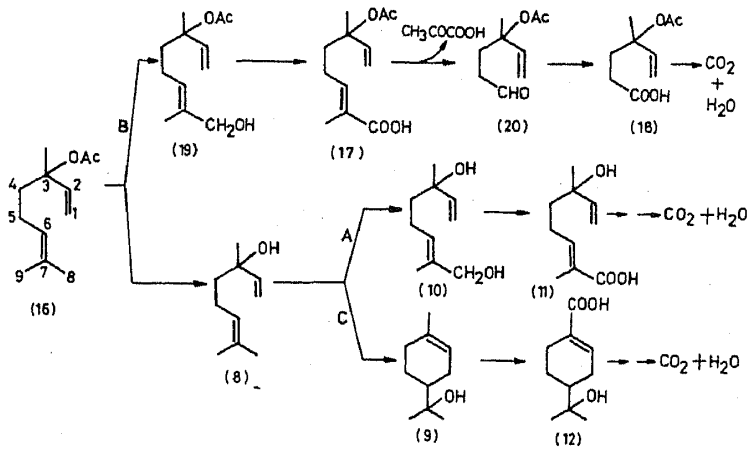

Figure 4. Probable pathways of degradation of linalyl acetate (16) by *P. incognita*.

bond cleavage occurs only after the oxidation of the C-8 methyl group. One of the possible modes for the cleavage of the 6,7 double bond is through epoxidation of the double bond and subsequent opening of the epoxide to the corresponding diol. The resulting diol may be cleaved in a manner similar to the periodate oxidation of diol to Δ^5-4-acetoxy-4-methyl hexenal (20) and pyruvic acid. The hexenal (20) may be dehydrogenated to hexenoic acid (18). The other possibility of formation of hexenoic acid (18) is through the β-oxidation of linalyl acetate-8-carboxylic acid (17). In this mode of cleavage, one would expect Δ^5-4-acetoxy-4-methyl hexenoic acid (18) and propionic acid as the products of degradation. Similar mode of cleavage has been postulated in the microbial degradation of β-myrcene (Narushima *et al* 1981, figure 3) where 4-methylenhex-5-enoic acid (21) is derived from 2-methyl-6-methylen-trans-oct-2,7-dienoic acid (22) through the β-keto oxidation pathway with the elimination of a propionic acid moiety. However, pyruvic acid has been shown to be formed when linalyl acetate-8-aldehyde is incubated with resting cells indicating that the former mode of double bond fission is operative.

The degradation of linalyl acetate (16) by *P. incognita* clearly indicates that part of it gets hydrolyzed to linalool. Several investigators have reported the asymmetric hydrolysis of (±) esters to optically active alcohols by micro-organisms (Fried *et al* 1971; Oritani and Yamashita 1974, 1980). In fact the high enantioselectivity of micro-organisms are often used in the synthesis of chiral organic compounds. Earlier it has been reported that *Bacillus subtilis var niger* hydrolyses racemic linalyl acetate to optically active linalool and acetate of its antipode (Oritani and Yamashita 1973). However, *P. incognita* fails to carry out the enantioselective hydrolysis of racemic linalyl acetate.

3. Metabolism of structurally modified acyclic monoterpenes

In order to ascertain the structural requirements of *P. incognita* to accept acyclic monoterpenes as substrates, its ability to degrade structurally modified linalool and linalyl acetate such as 1,2-dihydrolinalool (23), 1,2-dihydrolinalyl acetate (24), 2,6-dimethyl oct-2-ene, 2,6-dimethyl oct-2,6-diene, tetrahydrolinalyl acetate and 2,6-

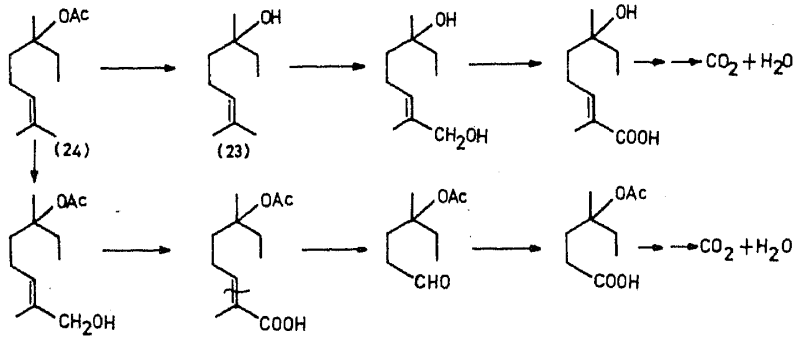

Figure 5. Probable pathways of degradation of 1,2-dihydrolinalyl acetate by *P. incognita.*

dimethyl octane have been studied. Although micro-organisms show great diversity in metabolizing a large number of chemicals, the number of degradative pathways are limited in nature. Often the presence of a single extra enzyme enables the organism to thrive on diverse substrates. However, it has been noticed in the case of 1,2-dihydrolinalool (23) and its corresponding acetate, that the organism fails to convert them to the nearest naturally occurring substrate, linalool and linalyl acetate, before they are further metabolized (Renganathan and Madyastha 1984). The mode of degradation of 1,2-dihydrolinalyl acetate is very similar to that observed for linalyl acetate (figure 5). The organism has shown its ability to grow on 2,6-dimethyl oct-2-ene and 2,6-dimethyl oct-2,6-diene whereas it fails to accept tetrahydrolinalyl acetate and 2,6-dimethyl octane. This observation clearly suggests that 6,7-double bond appears to be an essential structural requirement for these compounds to act as a substrate for this organism.

4. Metabolism of α-terpineol by *P. incognita*

As discussed earlier, one of the pathways for the degradation of linalool and linalyl acetate is initiated by the prototropic cyclization of linalool to α-terpineol (Madyastha *et al* 1977; Renganathan and Madyastha 1983). Besides, α-terpineol has been implicated as one of the intermediates in the microbial degradation of α- and β-pinenes (Shukla and Bhattacharyya 1968). However, earlier studies have failed to establish firmly the degradative pathways of α-terpineol (9). Hence studies were undertaken to get a better insight into the mode of metabolism of α-terpineol by *P. incognita.* On the basis of various metabolites isolated from the culture medium together with the supporting evidence obtained from enzymatic and growth studies, it appears that the organism degrades α-terpineol by atleast three different pathways (Renganathan and Madyastha, Unpublished observation). Among them, pathways A and B seem to be initiated by the oxygenation of the C-7 methyl group of α-terpineol/limonene resulting in the formation of oleuropyl alcohol/perillyl alcohol which further undergoes progressive oxidation to oleuropeic acid (12)/perillic acid (25). 1-Hydroxy-4-isopropenyl-cyclohexane-1-carboxylic acid (26) is presumably formed either from oleuropeic acid through elimination of the tertiary hydroxyl group as water and hydration of the 1,2 double bond or from limonene (27) through the intermediacy of perillic acid (figure 6). In fact limonene has been shown as one of the metabolites derived from α-terpineol (Renganathan and Madyastha, Unpublished observation). The pathways for the

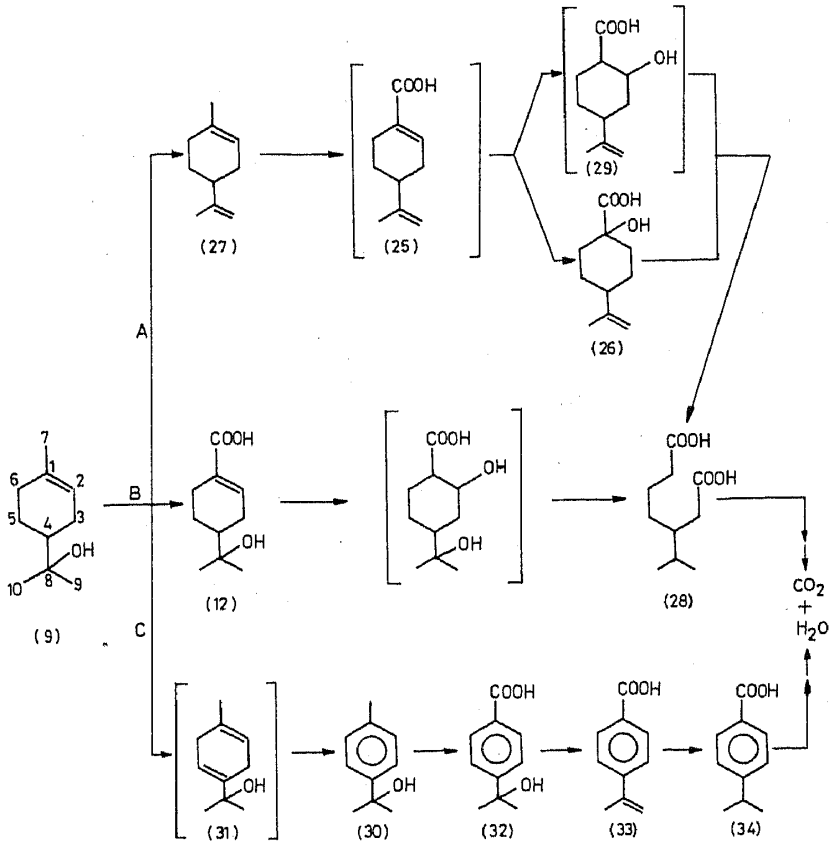

Figure 6. Probable pathways of degradation of α-terpineol (9) by *P. incognita.*

degradation of limonene by PL-strain have been established earlier (Dhavalikar and Bhattacharyya 1966). One can envisage the formation of β-isopropyl pimelic acid (28) either from 1-hydroxy-4-isopropenyl-cyclohexane-1-carboxylic acid (26) or from 2-hydroxy-4-isopropenyl-cyclohexane-1-carboxylic acid (29). The latter acid could arise from perillic acid (25) through the hydration of the 1,2-double bond. In both the cases, the ring fission is expected to proceed between C-1 and C-2 positions, followed by the reduction of the double bond to yield β-isopropyl pimelic acid (28). A similar mode of cleavage has been postulated earlier in the microbial systems (Shukla and Bhattacharyya 1968; Tsukamato *et al* 1977).

The third pathway (figure 6C) involved in the degradation of α-terpineol is the most interesting one. In this pathway, the organism aromatizes α-terpineol to *p*-cymene-8-ol (30) possibly *via* the intermediacy of cyclohexadiene(s). Aromatization of monoterpene by a microbial system has not been documented before, although such a process is believed to take place in higher plants (Poulose and Croteau 1978). The co-occurrence of *p*-cymene-8-ol with structurally related cyclohexadienes such as γ-terpinene suggests their biogenetic relationship. The isolation of *p*-cymene-8-ol from the culture medium implies that α-terpineol undergoes desaturation possibly producing γ-terpinene-8-ol (31) which further gets aromatized to *p*-cymene-8-ol (30). The postulated intermediacy of γ-terpinene-8-ol (31) during the aromatization of α-terpineol is supported by the fact that the organism readily accepts α-terpinene, γ-terpinene and α-phellandrene as growth substrates. Subsequent to the formation of *p*-cymene-8-ol (30), the C-7 methyl

group undergoes progressive oxidation to 8-hydroxycumic acid (32). One could envisage the formation of 4-isopropenyl benzoic acid (33) from 8-hydroxycumic acid through the elimination of the tertiary hydroxyl group. Reduction of the double bond in 4-isopropenyl benzoic acid could give rise to cumic acid (34). The different enzymatic steps involved in the conversion of *p*-cymene-8-ol to cumic acid has also been established. It is quite possible that further metabolism of cumic acid may proceed as proposed earlier (Madyastha and Bhattacharyya 1968a, b; DeFrank and Ribbons 1977a, b).

5. Degradation of geraniol, nerol, citronellol and their corresponding acetates

P. incognita degrades the monoterpene alcohol, geraniol into a number of neutral and acidic metabolites (Rama Devi and Bhattacharyya 1977). Atleast two pathways for its degradation have been proposed (figure 7 A and B). Pathway A involves an oxidative attack of the 2,3-double bond resulting in the formation of an epoxide. Opening of the epoxide yields the triol which upon oxidation forms a ketodiol. The ketodiol is probably converted to 6-methyl-hept-5-en-2-one by a retro-aldol type reaction. Pathway B is initiated by the oxidation of the primary alcoholic group to geranic acid (4) and further metabolism follows the interesting mechanism proposed earlier (Seubert *et al* 1963; Seubert and Remberger 1963). The ability of *P. incognita* to degrade geraniol by atleast two distinct pathways proves its superiority over *P. citronellolis*. In the case of nerol (7) (the *cis*-isomer of geraniol) the degradative pathways analogous to pathways A and B as in geraniol are observed.

It has been observed that *P. incognita* metabolizes acetates of geraniol, nerol and citronellol much faster than their respective alcohols (Madyastha and Renganathan 1983). Blocking the primary alcoholic function by an acetate moiety has not changed the pattern of their degradation. However, the organism does not have the ability to metabolize these compounds keeping the acetate moiety intact as has been noticed in the case of linalyl acetate (Renganathan and Madyastha 1983) and 1,2-dihydrolinalyl acetate (Renganathan and Madyastha 1984). The metabolites isolated from geranyl acetate are geraniol (3), geranic acid (4) and 3-hydroxy citronellic acid (35), and from neryl acetate are nerol (7), neranic acid (6), 2,3-epoxide of nerol (36) and 3-hydroxy citronellic acid (35) (figure 8). The geranic or neranic acid formed possibly gets further metabolized as described earlier. 3-Hydroxy citronellic acid has not been shown to be

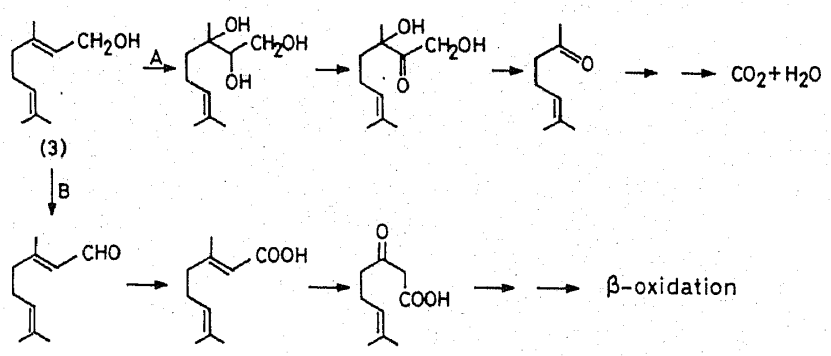

Figure 7. Pathways of degradation of geraniol (3) by *P. incognita*.

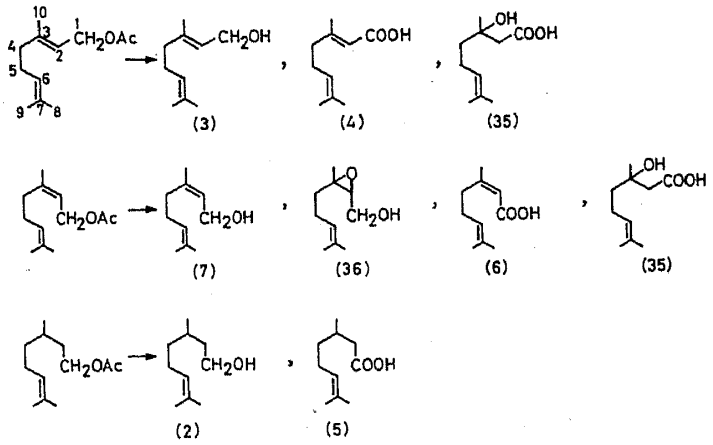

Figure 8. Metabolites of the acetates of geraniol, nerol and citronellol.

formed during the metabolism of geraniol and nerol by *P. citronellolis* (Seubert *et al* 1963). This acid could have been formed by the hydration of the 2,3-double bond of geranic and neranic acid. The significance of this compound in the overall metabolism is not known. It is quite possible that one of the pathways for the degradation of geranyl acetate and neryl acetate converge at 3-hydroxy citronellic acid (35) and from thereon a single pathway may operate for their further metabolism. However, the mode of further degradation of 3-hydroxy citronellic acid has not been established. In contrast to the earlier findings (Seubert and Fass 1964), it appears that *P. incognita* metabolizes geraniol through geranic acid without isomerizing to neranic acid (Madyastha and Renganathan 1983). This has been established by the GC and NMR studies of the methyl esters of the acidic metabolites derived from geranyl acetate.

There has been only limited work carried out so far on the fungal transformations of monoterpenes. Most of these pertain to the transformations of terpene hydrocarbons by fungal system (Prema and Bhattacharyya 1962). Since monoterpene alcohols have considerable antifungal activity, their transformations have not been studied so far. However, a strain of *Aspergillus niger* has been isolated in our laboratory which has the unique ability to transform geraniol, citronellol and linalool to their respective 8-hydroxylated derivatives (Madyastha and Murthi, Unpublished observation). Since free alcohols are toxic to the organism, corresponding acetates have been used in this study.

Examination of the various metabolic pathways of different acyclic monoterpenes by *P. incognita*, reveals that in certain respects, these degradative pathways are similar to their bio-transformation in fungi, higher plants and mammalian systems. One of the striking similarities of all these living systems is their ability to carry out specific ω-methyl hydroxylation of some of the acyclic monoterpene alcohols. It is interesting to note that the higher plant, bacterial and mammalian ω-hydroxylation reaction is mediated by a cytochrome P-450 system. Cytochrome P-450, a haemoprotein, which has the ability to generate a highly reactive hydroxylating species from molecular oxygen. The hydroxylating species is generated from the Fe(II) state of the cytochrome, which is formed by reduction of the Fe(III) oxidation state.

The biochemical pathways involved for the degradation of geraniol, nerol and citronellol seem to be different from what has been observed for linalool, 1,2-

dihydrolinalool and their corresponding acetates. Unlike linalool, the prototropic cyclization of geraniol and nerol has not been observed in *P. incognita*, although conformationally nerol and linalool are very close to each other. Perhaps the logic behind the prototropic rearrangement of linalool to α-terpineol is to converge the catabolic sequence to a specific intermediate which is common for number of structurally related compounds. This is exemplified in the metabolism of α- and β-pinenes by the PL-strain where the rupture of the cyclobutane ring of pinene results in the formation of a *p*-menthenoid cation which can be neutralized through the loss of a proton or by the attack of a hydride or hydroxide ion to yield limonene, *p*-menth-1-ene and α-terpineol (Shukla and Bhattacharyya 1968).

Although micro-organisms have great ability to metabolize a large variety of compounds, the number of biochemical pathways are limited in nature. It is against the cell economy for a microbe with diverse metabolic capacities to have unique degradative pathways for different compounds. Normally microbes have the ability to converge the catabolic sequence of different compounds to a limited number of intermediates and thereon a common pathway operates thus indicating the genetic economy that exists in micro-organisms.

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