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Microbial metabolism of monoterpenes – recent developments

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Abstract

Monoterpenes are important renewable resources for the perfume and flavour industry but the pathways and enzymology of their degradation by microorganisms are not well documented. Until recently the acyclic monoterpene alcohols, (+)-camphor and the isomers of limonene were the only compounds for which significant sections of catabolic pathways and associated enzymology had been reported. In this paper recent developments in our understanding of the enzymology of ring cleavage by microorganisms capable of growth with 1,8-cineole and α-pinene are described. 1,8-Cineole has the carbocyclic skeleton of a monocyclic monoterpene with the added complication of an internal ether linkage. Ring hydroxylation strategy and biological Baeyer-Villiger oxygenation lead to an efficient method for cleaving the ether linkage. α-Pinene is an unsaturated bicyclic monoterpene hydrocarbon. At least two catabolic pathways exist. Information concerning one of them, in which α-pinene may be initially converted into limonene, is rudimentary. The other involves attack at the double bond resulting in formation of α-pinene epoxide. Ring cleavage is then catalysed by a novel lyase that requires no additional components and breaks both carbocyclic rings in a concerted manner.

Introduction

The monoterpenoids (C_{10}) are major components of plant oils and are synthesized from two isoprene units. Parent structures are acyclic, monocyclic or bicyclic and the latter consist of fused C6/C5, C6/C4 and C6/C3 ring systems. In addition to the parent hydrocarbons a number of oxygenated derivatives are also formed. Some representative structures are shown in Fig. 1.

Some of the earliest reported studies of monoterpene metabolism, by organisms capable of growth on them as sole carbon sources, were those of Seubert (1960), Seubert & Remberger (1963), Seubert et al. (1963), and Seubert & Fass (1964) using *Pseudomonas cintronellolis*. They are of particular interest since they firmly established a novel catabolic route for the degradation of cintronellol, geraniol and nerol in which oxidation of the primary alcohol to carboxyl and formation of a common CoA ester is followed by biotin-dependent carboxylation of a methyl group. Double bond hydration forms a 3-hydroxyacid CoA ester from which the two carbon unit side chain is eliminated as acetate by the action of a lyase. Two unimpeded cycles of β-oxydation are followed by a repetition of the methyl group elimination reactions which completes the degradation of these compounds to central metabolites (Fig. 2). Although this work was published over twenty five years ago the proposed pathway was supported by subcellular studies, enzyme isolation and ^{14}C experiments and, more recently, it has been extended to cover *P. putida* and *P. mendocia* strains by Cantwell et al. (1978). At the same time a group at the University of Illinois was investigating the degradation of the C6/C5 bicyclic monoterpene (+)-camphor by strains of *Pseudomonas putida* (Bradshaw et al.

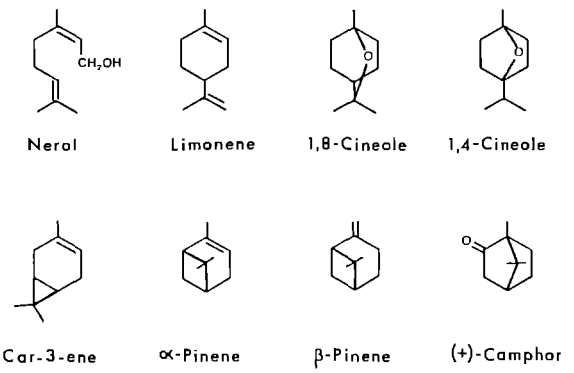

Fig. 1. Some naturally occurring monoterpenes.

1959; Conrad et al. 1961, 1965; Trudgill et al. 1966a, b) and *Mycobacterium rhodochrous* (Chapman et al. 1966). Although only limited success was achieved in understanding the catabolic pathways, key roles for methylene group hydroxylation and biological Baeyer-Villiger monooxygenases in ring cleavage strategies were established (Figs 3 and 4). No further experimental studies with *M. rhodochrous* T1 have been published but the (+)-camphor 5-hydoxylase from *P. putida* ATCC 17453 has been the subject of extensive and detailed research (see Gunsalus & Marshall 1971; Gunsalus & Lipscomb 1973; Gunsalus et al. 1974 for reviews). More recently, the oxygenating component of 2,5-diketocamphane 1,2-monooxygenase has been purified to homogeneity from *P. putida* (Taylor & Trudgill 1986) and the enzymology of the second ring cleavage step which involves CoA ester formation prior to biological Baeyer-Villiger oxygenation has been elucidated (Ougham et al. 1983; Trudgill 1986).

These particular areas of research probably represent the most detailed metabolic studies to date of monoterpene catabolism by organisms capable of growth with them as sole carbon sources although Bhattacharyya and his research group investigated the transformation of a number of monterpenes including geraniol, linalool, limonene and α-pinene and β-pinene, primarily by *Pseudomonas* strains. Very thorough and detailed analyses of metabolites accumulated in culture media were made. However, primarily because of the large number of metabolites accumulated, it was often difficult to identify a clear cut route leading to

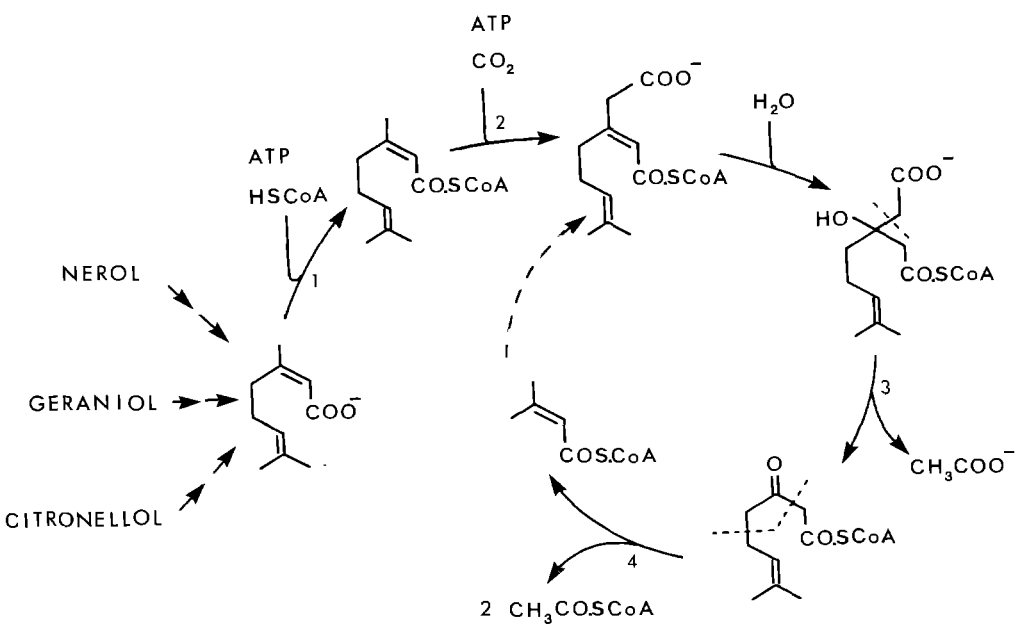

Fig. 2. Degradation of acyclic monoterpene alcohols by *Pseudomonas citronellolis*. (*1*) CoA ester synthetase; (*2*) biotin-dependent carboxylase; (*3*) lyase; (*4*) two cycles of β-oxidation. From the work of Seubert (1960); Seubert & Remberger (1963); Seubert, Fass & Remberger (1963).

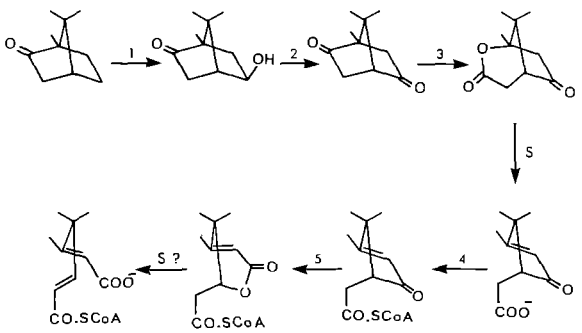

Fig. 3. Established steps of (+)-camphor degradation by *Pseudomonas putida* ATCC 17453. Enzymes: (*1*) camphor 5-hydroxylase; (*2*) 5-*exo*-hydroxy-camphor dehydrogenase; (*3*) 2,5-diketocamphane 1,2-monooxygenase (a Baeyer-Villiger monooxygenase); (*4*) 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA synthetase; (*5*) 2-oxo-Δ^3-trimethylcyclopentenylacetyl-CoA monooxygenase (a Baeyer-Villiger monooxygenase). S = spontaneous reaction.

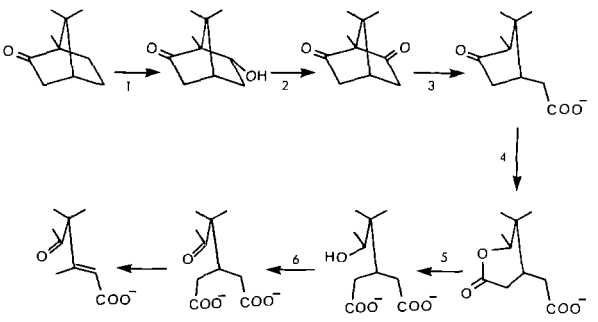

Fig. 4. Proposed steps of (+)-camphor degradation by *Mycobacterium rhodochorus* T1. Enzymes: (*1*) (+)-camphor 6-*endo*-hydroxylase; (*2*) 6-*end*-hydroxycamphor dehydrogenase; (*3*) 2,6-diketocamphane lyase; (*4*) 3-oxo-4,5,5-trimethylcyclopentanylacetic acid monooxygenase (a Baeyer-Villiger monooxygenase); (*5*) a lactone hydrolase; (*6*) a dehydrogenase. Based on results of Chapman, Meerman, Gunsalus, Srinivasan & Rinehart (1966).

central metabolites. Exceptions to this were the catabolism of limonene through perillic acid and a β-oxidative ring cleavage cycle (Dhavalikar & Bhattacharyya 1966; Dhavalikar et al. 1966) and evidence for the degradation of α-pinene through limonene or a closely related compound (Shukla & Bhattacharyya 1968; Shukla et al. 1968).

Current studies

More recent studies have been concentrated on two compounds where knowledge is limited and microorganisms are presented with particular structural complications in the growth substrate. The first of these is 1,8-cineole where the monocyclic monoterpene ring system has an ether linkage superimposed upon it and the second is α-pinene where work by Best et al. (1987) and ourselves (see below) has revealed a unique and quite novel ring cleavage enzyme.

1,8-Cineole

1,8-Cineole [1,3,3-trimethyl-2-oxabicyclo(2,2,2) octane] is isomeric with α-terpineol but contains neither a hydroxyl group nor a double bond. The trivial name eucalyptol is therefore rather misleading. It is found in considerable quantities in eucalyptus oil from the leaves of *Eucalyptus radiata* var Australiana (Nishimura et al. 1982). The molecule has the carbon skeleton of the monocyclic monoterpenes but, because of the ether linkage, is a bicyclic compound and it presents organisms capable of growing with it as sole carbon source with the additional problem of cleaving the ether linkage.

Metabolism of 1,8-cineole by Pseudomonas flava

Metabolite accumulation

Until recently, reports of microbial 1,8-cineole metabolism were confined to the isolation of metabolites from culture media and, following investigations of the dynamics of their production and disappearance, suggestions of a metabolic progression (MacRae et al. 1979; Carman et al. 1986). *Pseudomonas flava*, isolated by enrichment culture on 1,8-cineole accumulated the metabolites shown in Fig. 5. It is reasonable to suggest that initial attack occurs by hydroxylation at carbon 2, followed by dehydrogenation to form 2-oxocineole. Further metabolic steps are obscure, although a biological Baeyer-Villiger oxygenation can be envisaged. The

source of the lactone (R)-5,5-dimethyl-4-(3′-oxobutyl)-4,5-dihydrofuran-2(3H)-one was not immediately evident.

Metabolism of 1,8-cineole by Rhodococcus C1

Metabolite accumulation

Rhodococcus strain C1 was isolated by enrichment culture on 1,8-cineole (Wiliams et al. 1989) and grew in shake flasks with a doubling time of about 8 h. Gas Chromatographic (GC) analysis of diethyl ether extracts of neutral culture media showed the short-term accumulation of two compounds during the 15–18 h period of a 24 h growth regime. No acidic metabolites were detected. Sufficient quantities of the compounds for unequivocal identification and for metabolic studies were obtained from a 40 litre culture. One of these compounds gave a mass fragmentation pattern almost identical with that reported for the two isomers of 2-hydroxycineole and an ^{1}H-NMR spectrum indetical with that reported for 2-*endo*-hydrocycineole* (MacRae et al. 1979). These authors have reported that 2-*endo*-hydroxycineole is laevorotatory, $[\alpha]_D$-26° (c = 0.2 in ethanol) the alcohol accumulated by *Rhodococcus* C1 was dextrorotatory $[\alpha]_D$ + 27.5° in the same solvent. These result are only compatible with the compound being the optical isomer 6-*endo*-hydroxycineole. The second metabolite reacted with acidic 2,4-dinitrophenylhydrazine (Friedemann and Haugen, 1943) to give an orange insoluble 2,4-dinitrophenylhydrazone and a mass spectrum almost identical with that reported for 2-oxocineole (MacRae et al. 1979). The ^{1}H-NMR spectrum displayed all the diagnostic features attributable to 2-oxocineole. However, while 2-oxocineole is dextrorotatory the ketone accumulated by *Rhodococcus* C1 was laevorotatory, compatible with the compound being 6-oxocineole.

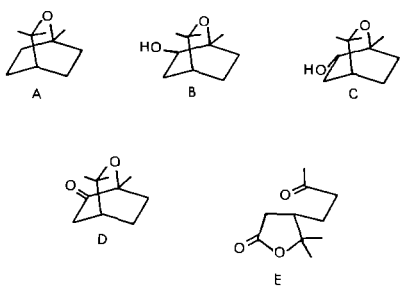

Fig. 5. Metabolites accumulated by *Pseudomonas flava* during growth on 1,8-cineole. Compounds are: (*A*) 1,8-cineole (growth substrate); (*B*) 2-*endo*-hydroxycineole; (*C*) 2-*exo*-hydroxycineole; (*D*) 2-oxocineole; (*E*) (R)-5,5-dimethyl-4-(3′-oxobutyl)-4,5-dihydrofuran-2(3H)-one. Data from MacRae, Alberts, Carman & Shaw (1979).

Rhodococcus C1 was also capable of growth with 6-endo-hydroxycineole and 6-oxocineole as sole carbon sources and it is logical to conclude that, by attacking the compound at carbon 6, it degrades 1,8-cineole in a manner that is analogous but enantiomeric to that utilized by *P. flava*. Further investigations of the route of 1,8-cineole degradation were made with subcellular systems.

1,8-Cineole hydroxylation

Although it is logical to assume that initial attack upon the molecule is by a monooxygenase that inserts an oxygen atom at the 6-*endo* position to form 6-*endo*-hydroxycineole we failed to identify any such hydroxylating system in subcellular preparations from induced cells. The use of a variety of different cell disruption procedures, inclusion of potential stabilizing agents and a range of electron donors and acceptors (in case the oxygen atom was derived from water) were all unsuccessful.

6-endo-hydroxycineole dehydrogenase

In contrast, an induced NAD+ linked 6-*endo*-hydroxycineole dehydrogenase was readily detected (specific activity 0.62 μmol/min.mg protein, pH optimum 10.5) and GC analysis showed 6-oxocineole to be the only product. The reverse reaction (specific activity 1.1 μmol/min.mg protein, pH optimum 7.5) yielded only 6-*endo*-hydroxycineole.

* Strict application of chemical nomenclature does not allow use of *endo* and *exo* in the (2,2,2)bicyclo system in relation to 1,8-cineole derivatives. To simplify presentation of results we have (a) designated as *endo* those alcohols in which the hydroxyl substitution is on the opposite side of the reference plane to the lowest priority bridge (see Fig. 5) and (b) made use of the numbering system consistent with the trivial name 1,8-cineole.

Further metabolism of 6-oxocineole

Not infrequently the catabolic transformation of cyclic hydrocarbons and alcohols into cyclic ketones is a preparation for ring oxygen insertion by a biological Baeyer-Villiger reaction (see Trudgill 1984, 1986) for reviews. These enzymes are flavoproteins and display typical monooxygenase characteristics, requiring an electron donor and incorporating one atom of oxygen into the organic product. When crude extract of 1,8-cineole-grown *Rhodococcus* C1 was incubated with 6-oxocineole and NADPH an oxygen-dependent oxidation of the NADPH was observed. Polarographic assays in the presence of limited amounts of either NADPH or 6-oxocineole allowed an approximate (1 : 1 : 1) stoichiometry of the reaction to be established. GC analysis of diethyl ether extracts of acidified reaction mixtures showed that the 6-oxocineole had been converted into a single more polar metabolite. GC-mass spectral analysis of the product gave a spectrum identical with that reported for the lactone (R)-5,5-dimethyl-4-(3′-oxobutyl)-4,5-dihydrofuran-2(3H)-one isolated by MacRae et al. (1979) (Fig. 5). Although the initial products of biological Baeyer-Villiger oxygenation of cyclic ketones are lactones there is no way in which this particular lactone can be formed directly from 6-oxocineole and the identity of the initial product of oxygenation was therefore uncertain.

Reaction with partially purified 6-oxocineole oxygenase

Purification of the NADPH-linked 6-oxocineole oxygenase from *Rhodococcus* C1 was a valid approach to determining the identity of the immediate oxygenation product. Unfortunately the enzyme was not very stable and althought addition of ethanol (5% v/v) did have a stabilizing influence we were unable, using either conventional procedures or by taking advantage of the short processing times of fast protein liquid chromatography (FPLC), to purify the enzyme more than 5–7 fold without rapid loss of activity.

When 15 mg of partially purified enzyme were incubated with 60 μmol of 6-oxocineole and excess NADPH the consumption of 50 μmol of oxygen was observed. Direct ferric hydroxamate assay for lactones (Cain 1961) showed them to be absent. When the aqueous reaction mixture was acidified (pH $\simeq$ 1) and extracted with diethyl ether the ether phase contained 'ferric hydroxamate positive' material which co-chromatographed with the 5,5-dimethyl-4-(3′-oxobutyl)-4,5-dihydrofuran-2(3H)-one obtained with crude cell extracts. This reinforced the view that this lactone is an artifact of isolation and not the immediate product of oxygenation.

5,5-Dimethyl-4-(3′-oxobutyl)-4,5-dihydrofuran-2(3H)-one is the lactone of 3-(1-hydroxy-1-methylethyl)-6-heptanoic acid. 4-Hydroxy acids readily lactonize in acidic solution and organic solvents. Wallach (1895) made use of precisely this spontaneous ring closure of 3-(1-hydroxy-1-methylethyl)-6-heptanoic acid in his investigations into the structure of α-terpineol. The working hypothesis that this acid is a catabolic intermediate from which the lactone is formed during extraction procedures was supported by the observation that 3-(1-hydroxy-1-methyl-ethyl)-6-heptanoic acid, prepared by alkalie hydrolysis of an aqueous solution of the racemic lactone [synthesized chemically from α-terpineol essentially as described by MacRae et al. 1979)], would support growth of *Rhodococcus* C1 and was oxidized rapidly by 1,8-cineole-grown cells.

Chemical Baeyer-Villiger oxygenation of 6-oxocineole

Magnesium monoperoxyphthalate is an efficient chemical Baeyer-Villiger reagent that gives clean reaction products in good yield. When the 6-oxocineole, isolated from culture medium, was incubated with this reagent in dimethylformamide, followed by addition of water, acidification and extraction into diethyl ether, the reaction product had the same GC retention time as 5,5-dimethyl-4-(3′-oxobutyl)-4,5-dihydrofuran-2(3H)-one.

These observations clearly suggested that the initial step in 6-oxocineole degradation is indeed a biological Baeyer-Villiger oxygenation. Chemical Baeyer-Villiger reagents insert the ring oxygen in such a manner that, when there are two different alkyl groups either side of the carbonyl the more highly substituted carbon atom becomes bonded to the new oxygen. From this it is possible to predict

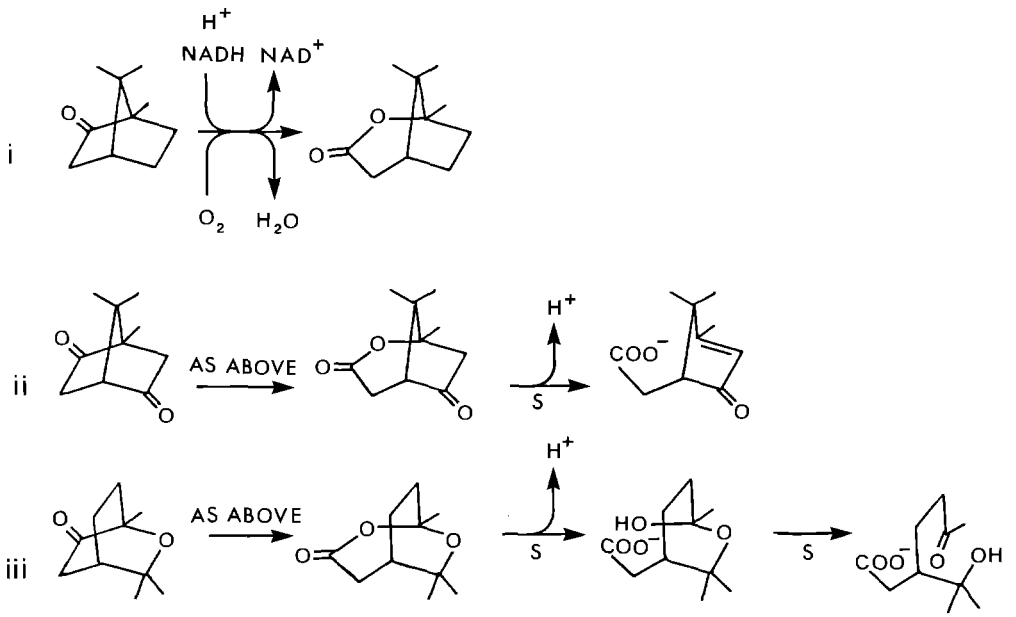

Fig. 6. Reactions catalysed by 2,5-diketocamphane 1,2-monooxygenase from *P. putida* ATCC 17453 with (*i*) (+)-camphor and (*ii*) 2,5-diketocamphane as substrates. The proposed oxygenation reaction with 6-oxocineole (*iii*) is followed by spontaneous cleavage to form the acyclic compound 3-(1-hydroxy-1-methylethyl)-6-oxoheptanoic acid. An alternative postulated spontaneous ring cleavage sequence leading to the same product is shown in Fig. 8. Adapted from Williams, Trudgill & Taylor (1989).

that the ring oxygenation product is 1,6,6-trimethyl-2,7-dioxabicyclo(3,2,2)-nonan-3-one. However, the steps between this lactone and 3-(1-hydroxy-1-methyethyl)-6-oxoheptanoic acid and any enzymes involved were not immediately clear.

Studies with 2,5-diketocamphane 1,2-monooxygenase and 6-oxocineole

2,5-Diketocamphane 1,2-monooxygenase of *P. putida* ATCC 17453 is a well described NADH-linked biological Baeyer-Villiger monooxygenase that is known to insert an oxygen atom into camphor between the carbonyl group and the bridgehead. The enzyme consists of NADH oxidase and oxygenating flavoprotein components that reversibly dissociate and which have been purified to homogeneity from (+)-camphor-grown cells (Taylor & Trudgill 1986). Although the enzyme is know to have a broad ketone substrate specificity it might be expected that 2-oxocineole would be preferred to 6-oxocineole. Preliminary experiments established that 6-oxocineole is a good substrate. This is presumably a result of the spatial symmetry of the (2,2,2) bicyclo skeleton (Fig. 6). Reactions in which 0.65 unit of enzyme was incubated with 10 μmol of 6-oxocineole and excess NADPH were used for product accumulation and analysis. Direct assay for lactones at the end of the reaction showed none to be present and, as occurred with the oxygenase preparations from *Rhodococcus* C1, acidification and diethyl ether extraction yielded, once again, the lactone 5,5-dimethyl-4-(3'oxobutyl)-4,5-dihydrofuran-2(3H)-one. This observation is of particular interest since camphor-grown *P. putida* ATCC 17453 is not known to have a lactone hydrolase. The clear implication is that the initial product of Baeyer-Villiger oxygenation is unstable. It was possible to confirm this fact in further investigations with a pH-stat. When the 2,5-diketocamphane 1,2-monooxygenase complex is incubated with (+)-camphor oxygenation takes place, a proton is consumed and the lactone product accumulates. In contrast the lactone formed from 2,5-diketocamphane is unstable and spontaneously ring opens to give an unsaturated acid so that overall proton balance is maintained. The experimental consequences of this are shown in Fig. 6. When 6-oxocineole is used as substrate proton balance is

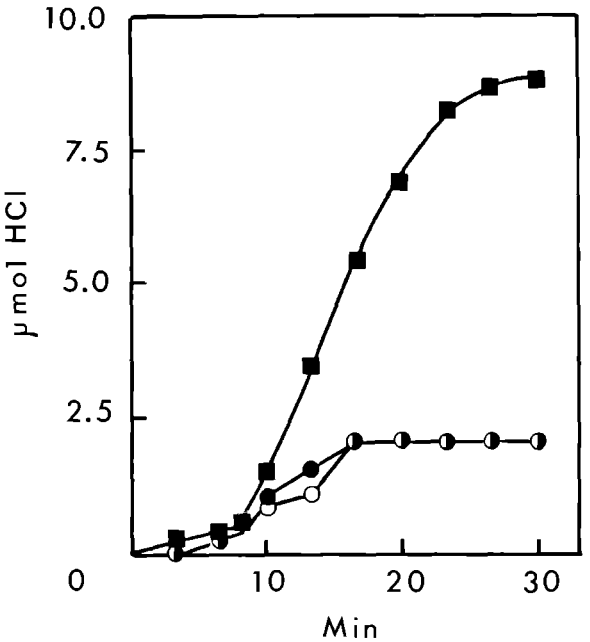

Fig. 7. A comparison of proton balance in the oxygenation reactions depicted in Fig. 6. The reaction vessel of a pH-stat contained 0.5 unit of 2,5-diketocamphane monooxygenase complex from *P. putida* ATCC 17453 and 10 μmol of NADH in 11 ml of distilled water adjusted to pH 6.7 with a minimum of phosphate buffer. After establishment of endogenous rates 10 μmol of (+)-camphor (■), 2,5-diketocamphane (●) or 6-oxocineole (○) were added and proton release monitored by controlled addition of 2 mM-HCl to maintain constant pH.
Adapted from Williams, Trudgill & Taylor (1989).

maintained confirming that, subsequent to the initial oxygenation step, further non-enzymic reaction(s) occur in which a proton is regenerated (Fig. 7).

Reaction sequence and ring cleavage

A reaction sequence for the cleavage of 6-oxocineole that is compatible with the experimental observations is shown in Fig. 8. The presumed lactone, 1,6,6-trimethyl-2,7-dioxabicyclo(3,2,2)nonan-3-one, is a strained molecule. We suggested originally that spontaneous cleavage is hydrolytic (Williams et al. 1989). This would form a hemiacetal with consequent further spontaneous cleavage of the ether linkage and formation of the anticipated acyclic acid. A possible alternative involving internal electron shifts would result in vinyl ether formation. Vinyl ethers are relatively unstable, especially in

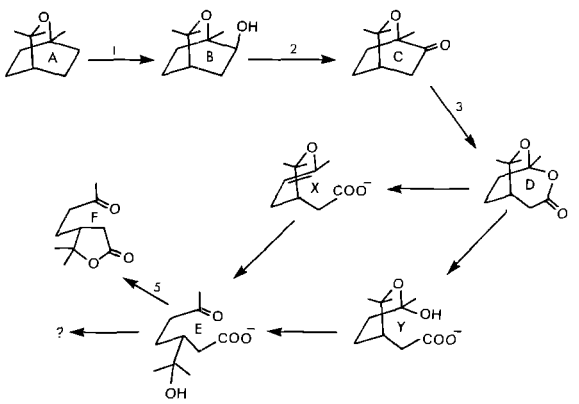

Fig. 8. Proposed ring cleavage reactions in the metabolism of 1,8-cineole by *Rhodococcus* strain C1. Compounds are: (*A*) 1,8-cineole; (*B*) 6-*endo*-hydroxycineole; (*C*) 6-oxocineole; (*D*) 1,6,6-trimethyl-2,7-dioxabicyclo(3,2,2)nonan-3-one; (*E*) 3-(1-hydrox-1-methylethyl)-6-oxoheptanoic acid; (*F*) 5,5-dimethyl-4-(3′-oxobutyl)-4,5-dihydrofuran-2(3H)-one. Compounds X, 2,6,6-trimethyl-5-acetyl-4,5-dihydropyran-2-ol and Y, 2,6,6-trimethyl-5-acetyltetrahydropyran-2-ol are proposed alternative transient intermediates of spontaneous ring cleavage. Enzymes are: (*1*) putative 1,8-cineole 6-*endo*-hydroxylase; (*2*) NAD-linked dehydrogenase; (*3*) NADPH-linked monooxygenase (a Baeyer-Villiger oxygenase). Reaction 5 is a spontaneous hydroxyacid ring closure (with loss of water) that occurs upon acidification of reactions and extraction with diethyl ether. Adapted from Williams, Trudgill & Taylor (1989).

dilute acidic solution, and spontaneous cleavage would again yield the anticipated acid. It is not known which of these alternative routes is followed.

The further metabolism of 3-(1-hydroxy-1-methylethyl)-6-oxoheptanoic acid has not been investigated but in this context it is of interest that 1,8-cineole-grown *Rhodococcus* C1 also oxidizes levulinic acid and acetone which can be derived theoretically from the open chain acid (Fig. 8) by fairly conventional metabolic steps.

MacRae et al. (1979) established the absolute configuration of the lactone (Fig. 5) accumulated in culture media by *P. flava*. Although we have not established the absolute configuration of the lactone formed by *Rhodococcus* C1 and 3-(1-hydroxy-1-methylethyl)-6-oxoheptanoic acid from which it is formed, logic would dictate that thay will have the (S) configuration.

Metabolism of 1,4-cineole

In principle the sequence of ring cleavage steps is also theoretically applicable to the less widely distributed isomer 1,4-cineole which, through a sequence of more highly strained intermediates, would yield 3-hydroxy-3-(1-methylethyl)-6-oxoheptanoic acid. In this context it is of interest that Rosazza et al. (1987) have reported 2-*endo*-hydroxy and 2-oxo derivatives as products of 1,4-cineole fermentation by a variety of bacteria and fungi grown in rich media.

Bacterial metabolism of α-pinene

Naturally occurring bicyclic monoterpenes are homocyclic and are fused 6/5 (borneol, camphor), 6/4 (α-pinene, β-pinene) or 6/3 (car-3-ene) carbon rings. The degradation of camphor has been studied in most detail and preparation for ring cleavage is dominated by hydroxylation and Baeyer-Villiger oxygenation reactions which form lactones although, in the case of *P. putida* ATCC 17453 lactone hydrolase activity has not been detected. (+)-Camphor-grown *P. putida* ATCC 17453 hydroxylates the compound at C5 and oxidises this to 2,5-diketocamphane. This appears to be a two-fold strategy; on the one hand it produces a molecule which is unstable and spontaneously ring opens once a biological Baeyer-Villiger oxygenase has inserted an oxygen between the keto group at C2 and the bridgehead, on the other it places a second keto group in such a position that the molecule can be manipulated, by formation of a CoA ester and a second ring oxygenation, so that a second spontaneous ring cleavage occurs (Fig. 3). Further metabolic studies on the degradation of the acyclic metabolite have not been reported.

Unlike camphor, in which the keto group might be expected to have a directing influence on catabolism, car-3-ene and α-pinene are unsaturated hydrocarbons. There is no significant published information on the microbial degradation of car-3-ene but more progress has been made in understanding the degradation of α-pinene.

Early studies of α-pinene metabolism by Pseudomonas strains

Shukla & Bhattacharyya (1968) and Shukla et al. (1968) isolated a variety of metabolites from culture medium when *Pseudomonas* strain PL was grown with α-pinene as sole carbon source. Accumulated acidic metabolites, primarily associated with oxidation of the C10 methyl group included perillic acid, which had previously been identified as a metabolite produced from limonene by this organism (Dhavaliker & Bhattacharyya 1966) and the acyclic acids 3-isopropylpimelic acid and 3-isopropenylpimelic acid. Although Shukla & Bhattacharyya (1968) presented, on the basis of accumulation experiments, a rather complex pattern of metabolic pathways leading from α-pinene to other compounds their evidence, including limited subcellular studies, is consistent with a primary degradative route that involves initial cleavage of the cyclobutane ring to form limonene or 1-*p*-menthene, oxidation of the C7 methyl group (C10 of α-pinene) to carboxyl and β-oxidative ring cleavage (Fig. 9).

In contrast to these observations Gibbon & Pirt (1971), Gibbon et al. (1972) and Tudroszen et al. (1977), again on the basis of metabolites accumulated by parent Pseudomonas strains and mutants, proposed a quite different catabolic route in which cleavage of the C6 ring occurred between carbon atoms 3 and 4 leading to the formation of 2-methyl-5-isopropylhexa-2,5-dienoic acid (Fig. 10). Although a biological Baeyer-Villiger oxygenation step was proposed no supporting subcellular evidence was reported.

α-Pinene metabolism by P. fluorescens NCIMB 11671

Recently Best et al. (1987) reported that extracts of α-pinene-grown *Pseudomonas fluorescens* NCIMB 11671 catalysed an NADH-linked consumption of oxygen in the presence of α-pinene. Rates of NADH and oxygen consumption suggested a 1 : 1 stoichiometry and the activity was located

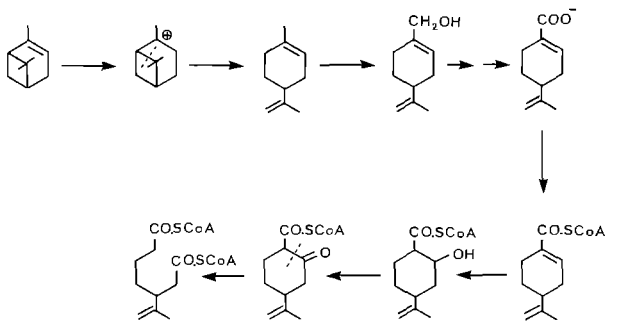

Fig. 9. Proposed pathway for cleavage of α-pinene by *Pseudomonas* PL. A prototrophic rearrangement of α-pinene to form limonene has been suggested, followed by methyl group oxidation and ring cleavage mediated by a β-oxidation cycle. Based on the results of Shukla & Bhattacharyya (1968) and Shukla, Moholay & Bhattacharyya (1968).

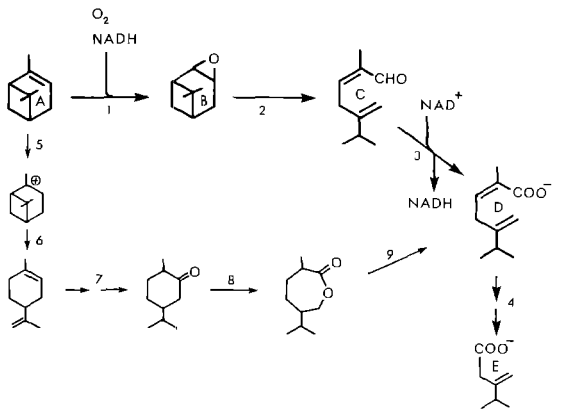

Fig. 10. Partial catabolism of α-pinene by *Nocardia* sp. strain P18.3, *P. fluorescens* NCIMB 11761 and *P. putida* NCIMB 10684. Compounds are: (*A*) α-pinene; (*B*) α-pinene epoxide; (*C*) *cis*-2-methyl-5-isopropylhexa-2,5-dienal; (*D*) *cis*-2-methyl-5-isopropylhexa-2,5-dienoic acid; (*E*) 3-isopropylbut-3-enoic acid. Reactions are: (*1*) NADH-dependent α-pinene monooxygenase (*P. putida* NCIMB 11671); (*2*) α-pinene epoxide lyase (all three organisms); (*3*) NAD-linked 2-methyl-5-isopropylhexa-2,5-dienal dehydrogenase (*Nocardia* P18.3); (*4*) proposed β-oxidation cycle to give the acid identified by Tudroszen, Kelly & Millis (1977). Reactions 5–9 were originally proposed for *P. putida* NCIMB 10684 by Gibbon & Pirt (1971).

in the soluble protein fraction. The neutral reaction product was extracted into diethyl ether and mass spectral, ^{1}H and ^{13}C NMR analyses identified the compound as 2-methyl-5-isopropylhexa-2,5-dien-1-al, although the isomeric configuration was not reported. It is of particular interest that, on oxidation, this aldehyde would yield 2-methyl-5-isopropylhexa-2,5-dienoic acid, both isomers of which were reported (Gibbon & Pirt 1971; Gibbon et al. 1972; Tudroszen et al. 1977) as being accumulated from α-pinene by *Pseudomonas* PX1 (NCIMB 10684), *P. putida* PIN11 and mutants thereof.

A particularly remarkable feature was the rapid cleavage of both carbocyclic rings of α-pinene that was catalysed by very small amounts of protein. It was provisionally assumed that an initial hydroxylation of α-pinene occurred. However, the inclusion of atebrin in assays, as a supposed inhibitor of enzymes with flavin prosthetic groups and thus of a flavoprotein hydroxylase, resulted in an inhibition of ring cleavage and the accumulation of α-pinene epoxide. Subsequent experiments showed that

- the initial oxygenation step formed α-pinene epoxide,
- β-pinene and (+)-limonene were also substrates for the enzyme,
- the enzyme was sensitive to inhibitors that react with thiol groups and that complex Fe^{2+}.

Further preliminary studies with α-pinene epoxide showed that the soluble protein extract of α-pinene-grown *P. fluorescens* NCIMB 11671 catalysed a very rapid cleavage of α-pinene epoxide with the formation of the acyclic aldehyde 2-methyl-5-isopropylhexa-2,5-dienal.

α-Pinene metabolism by Nocardia sp. strain P18.3

We have isolated a Gram-positive organism *Nocardia* sp. strain P18.3 by elective culture with α-pinene. Cells grown on the terpene oxidize the growth substrate and α-pinene epoxide at comparable rates. Although we have been unable to demonstrate the putative epoxide-forming monooxygenase in this organism, incubation of extracts of α-pinene-grown cells with α-pinene epoxide resulted in its very rapid conversion into the *cis* isomer of 2-methyl-5-isopropylhexa-2,5-dienal [specific activity 16 μmol/min · mg protein] (Griffiths et al. 1987). Quite rapid transition to the *trans* isomer took place non-enzymically in the glycine NaOH

buffer (pH 9) which was used to suppress non-enzymic breakdown of the α-pinene epoxide. An induced NAD-linked dehydrogenase oxidized either isomer of the aldehyde to 2-methyl-5-isopropylhexa-2,5-dienoic acid.

Bacteria investigated and distribution of catablic pathways

The enzymic cleavage of α-pinene epoxide by extracts of α-pinene-grown bacteria would appear to be a useful diagnostic test. An examination (Table 1) of the distribution of the enzyme in available *Pseudomonas* strains provides additional support for there being two distinct degradative pathways. The reports by Gibbon & Pirt (1971) and Tudroszen et al. (1977) are substantially correct with regard to the identities of metabolites accumulated by *Pseudomonas* PX1 (NCIMB 10684), although it is likely that (+)-*trans*-carveol arises non-biologically from α-pinene epoxide, since carveol isomers are formed spontaneously, especially in slightly acidic solution (Griffiths et al. 1987). They are, however, incorrect as regards the mechanism of ring cleavage and this illustrates the dangers inherent in formulating catabolic pathways on this type of evidence alone. The induction of the epoxide cleaving enzyme firmly places *Pseudomonas* PX1 in the same group as *P. fluorescens* NCIMB 11671 and *Nocardia* P18.3 (Fig. 10). In contrast *Pseudomonas* strains PL and PIN 18 (NCIMB 10687) degraded α-pinene by the alternative route (Fig. 9); a dichotomy first appreciated by Gibbon & Pirt (1971) and Gibbon et al. (1972).

Purification and properties of α-pinene epoxide lyase

This well documented cleavage of α-pinene epoxide, in which a carbon-oxygen bond and two carbon-carbon bonds are cleaved rapidly without any apparent requirement for additional co-factors was worthy of more detailed investigation.

From Nocardia P18.3

The enzyme was purified from *Nocardia* P18.3 (Griffiths et al. 1987) and shown to constitute about 6% of the soluble cell protein. Sephacryl S-200 chromatography gave an M_r of 40000 and ultracentrifugal determination (Yphantis 1964) a value of 50000. SDS-polyacrylamide gel electrophoresis yielded two electrophoretically dissimilar bands (M_r values 17000 and 22000).

The enzyme was devoid of detectable prosthetic groups, had no cofactor requirements and there was no significant variation in activity over a pH range from 7 to 10. The K_m for *a*-pinene epoxide was approx. 9 μM and the turnover number 15000. Production of *cis*-2-methyl-5-isopropylhexa-2,5-dienal in pyrophosphate buffer was stoichiometric. Structurally related terpenes such as β-pinene, pinan-3-ol, pinan-3-one, and carvone were inhibitors and, of a range of group-specific inhibitors, sensitivity was shown only towards sulphydryl active agents such as *p*-hydroxymercuribenzoate and 5,5′-dithiobis-2-nitrobenzoate and to atebrin.

A particularly interesting feature of the enzyme was that, although it was stable to dialysis and prolonged storage at 4° C, it exhibited suicidal catalysis under a range of assay conditions and in different buffers. Product inhibition was not a factor and each molecule of enzyme turned over about 12000 molecules of substrate before being inactivated.

We have proposed a mechanism for the decyclization of *a*-pinene epoxide which is compatible with the observed properties of the enzyme (Griffieths et al. 1987). Donation of a proton from a site

Table 1. Distribution of α-pinene epoxide lyase in extracts of α-pinene and succinate-grown bacteria.

Strain	α-Pinene epoxide lyase (μmol cleaved/min · mg protein) from cells grown on:	
	α-pinene	succinate
Nocardia sp. strain P18.3	16	0.2
Pseudomonas sp. strains		
NCIMB 10684	5.3	0.3
NCIMB 11671	3.9	0.09
NCIMB 10687	0.01	0.001
PL	0.01	0.001

Adapted from Griffiths, Bociek, Harries, Jeffcoat, Sissons & Trudgill (1987).

in the catalytic centre initiates a series of concerted rearrangements, leading to formation of the *cis* isomer of the product (Fig. 11). If this mechanism is substantially correct then the enzyme should be classified as a lyase (EC 4.99.-.-). The proposed mechanism is compatible with the terpene inhibitor profile and the high turnover number. It may explain the origin of the small amounts of carvone accumulated by *P. fluorescens* NCIMB 11671 (European patent application EP 82 30 5540) since the elimination of a proton at any intermediate stage in catalysis would lead to the production of compounds such as carveol, *a*-terpineol or trans-sobrerol. It is also possible that inactivation of the enzyme during catalysis may be associated with a low frequency side reaction of one of the unstable carbocation intermediates with an essential reactive site in the catalytic centre.

From P. putida PX1

We have also purified the *a*-pinene epoxide lyase from *P. putida* PX1 (NCIMB 10684). It also constitutes about 6% of the soluble cell protein and has an M_r of 42000 by ultracentrifugal analysis. It differs from the *Nocardia* enzyme in being formed from two electrophoretically identical subunits and in having a much higher K_m for *a*-pinene epoxide (210 μM). This, in conjunction with a less rapid inactivation during catalysis, made the enzyme a more suitable subject for kinetic and inhibitor studies.

Inhibition studies with atebrin and related compounds

As had previously been reported for the lyase from *P. fluorescens* NCIMB 11671 (Best et al. 1987) the enzymes from *Nocardia* P18.3 and *P. putida* NCIMB 10684 were very sensitive to inhibition by atebrin (quinacrine, 6-chloro-9-[(4-diethylamino)-1-methylbutyl]amino-2-methoxyacridine).

The *a*-pinene epoxide lyase form *P. putida* NCIMB 10684 was selected for detailed study. Kinetic analyses showed that the inhibition was non-competitive with a K_i of about 0.6 μM. A dissociation constant for atebrin was measured fluorimetrically (excitation 424 nm; fluorescence 497 nm in pyrophosphate buffer, pH 9.0) by following the increase in fluorescence that occurred upon adding enzyme to atebrin. Values of between 0.3 and 0.9 μM obtained correlate closely with the K_i obtained from kinetic studies. Inhibiton by atebrin was reversible, dialysis of inhibited enzyme against buffer resulted in good recovery of activity.

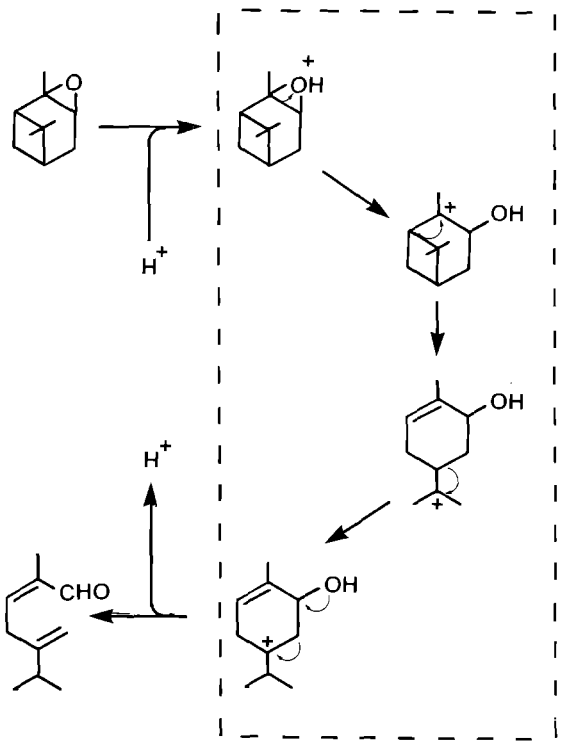

Fig. 11. Proposed reaction scheme for the decyclization of α-pinene epoxide by α-pinene epoxide lyase. Compounds within the box are suggested enzyme-bound carbocations.
Adapted from Griffiths, Harries, Jeffcoat & Trudgill (1987).

In addition to atebrin, chlorpromazine (2-chloro-10-(3-dimethylaminopropyl)phenothiazine) and promethazine (10-(2-dimethylaminopropyl)phenothiazine) were also potent inhibitors (Fig. 12) while Methylene Blue, Pyronin Y and diphenyliodonuim chloride all gave 50% inhibition at 10 μM or less (*a*-pinene epoxide at 2 mM, enzyme at 54 nM, pH 9).

Non-competitive inhibition by compounds that exhibit K_i values two to three orders of magnitude lower than the K_m for the substrate is typical of compounds that act as transition state inhibitors,

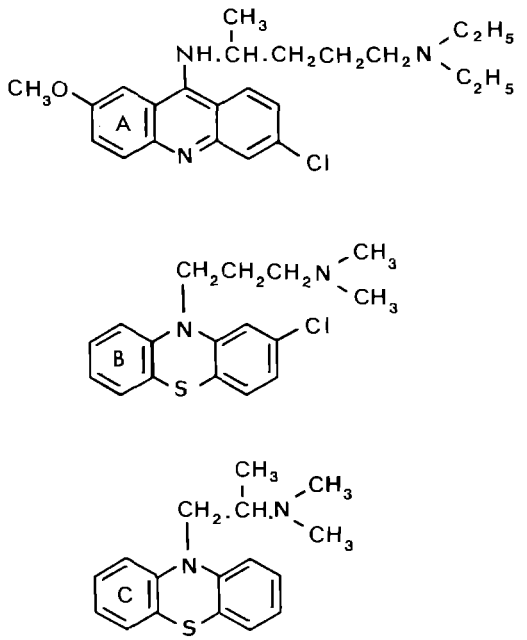

Fig. 12. Compounds that act as transition state inhibitors of α-pinene epoxide lyase. (*A*) atebrin (6-chloro-9-[(4-diethylamino)-1-methylbutyl]-amino-2-methoxyacridine); (*B*) chlorpromazine (2-chloro-10-(3-dimethylaminopropyl)-phenothiazine; (*C*) promethazine (10-(2-dimethylaminopropyl)-phenothiazine).

taking advantage of additional binding interactions of a transition state by incorporating key structural elements of it in the stable structure of the inhibitor. One result is that transition state analogue inhibitors may have a much higher affinity for the enzyme active site than traditional ground state inhibitors. This is exemplified, for example, by the inhibition of 2,3-oxidosqualene cyclases by 2-*aza*-2,3-dihydrosqualene, 2-*aza*-2,3-dihydrosqualene-*N*-oxide and several derivatives thereof that show obvious structural analogy with carbocation intermediates of the cyclization process (see Benveniste 1986 for review).

In the case of α-pinene epoxide lyase the precise structural analogy between the potent inhibitors and proposed carbocation intermediates formed in catalysis (Fig. 11) is not obvious. Structural variations exist but the most potent compounds have both a ring nitrogen and a dimethyl or diethyl substituted amine group. The situation is further complicated by the fact that the two ionizable nitrogen atoms of atebrin have pK_a values of 7.5 (aromatic) and 10.1 (diethylamine) but the compound is a less effective inhibitor at pH7 than at pH9. In conclusion, although we are uncertain as to exactly how these inhibitors mimic transition state carbocations were are encouraged in our proposed catalytic mechanism (Fig. 11) by the proven requirement for both a positive charge and a cyclic component if the inhibitor is to be optimally effective.

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