MICROBIOLOGICAL TRANSFORMATIONS OF TERPENES

A THESIS

SUBMITTED TO THE

UNIVERSITY OF POONA

for the degree of

DOCTOR OF PHILOSOPHY

by

Barandur Prema Raman
National Chemical Laboratory
Poona-8
India.

JANUARY 1962

A C K N O W L E D G E M E N T

The author wishes to express her sincere gratitude to Dr. P. Bhattacharyya, Head of the Biochemistry Division, National Chemical Laboratory, under whose guidance the work was carried out.

She would like to thank Dr. K.Venkataraman, Director, and Dr. V.Jagannathan, Assistant Director, Biochemistry Division, for their keen interest in the work, and Dr. S.C.Bhattacharyya, Assistant Director, Essential Oils Division, for helpful suggestions and for providing the terpene hydrocarbons.

The investigator's thanks are also due to Dr. K.S.Gopala Krishnan, Chief Mycologist, Hindustan Antibiotics Ltd., for identifying the cultures and to Dr. [illegible] Kanekar of the Atomic Energy Establishment, Bombay, for the NMR spectra. Assistance from the Microanalytical and the Infrared Sections of the National Chemical Laboratory is also gratefully acknowledged.

It is a pleasure to acknowledge the helpful discussions and constructive criticisms from her colleagues during the course of this study.

The author is indebted to the Council of Scientific & Industrial Research for the award of a Junior Research Fellowship during the course of the investigation.

******

C O N T E N T S

****

CHAPTER I

INTRODUCTION

INTRODUCTION

Recent developments in the field of "microbiological transformations", particularly of the steroids, have opened up a new line of approach towards the synthesis of many biologically important products.

Microorganisms have been known for a long time to bring about useful chemical conversions which are difficult to achieve with chemical agents. Apart from their use in various types of fermentation utilized to produce alcohol, different organic acids, vitamins, amino acids, antibiotics and other useful industrial and pharmaceutical compounds by normal metabolic processes, they have been used from time to time to effect chemical changes, such as selective and stereospecific oxidation and reduction, in organic substrates. The reduction of furfuraldehyde to furfuryl alcohol,[1] of 4-androstenedione to testosterone by yeast[2] and the oxidation of D-sorbitol to L-sorbose by the acetobacters[3] are a few outstanding examples among many such transformations.

The interest in microbiological transformations, however, received a great stimulus by the discovery of 11 α-hydroxylation of steroids by the mucorale fungi by Peterson and Murray in 1952.[4] Their investigations

paved the way for a totally new approach to the commercial synthesis of steroid hormones such as cortisone, cortisol, corticosterone and aldosterone, all of which possess a vital oxygen function for their biological activity at the position 11, a position not easily accessible by chemical means.

The important discovery of Peterson and Murray[4] in the United States that progesterone can be converted to 11 α-hydroxy progesterone in almost quantitative yields by Rhizopus nigricans caused a wide spread interest in the field of microbiological transformation of steroids in different laboratories of the world. This led to the discovery of several other important microbiological transformations of the steroid nucleus, which have great potentialities in the steroid industry such as 16 α 17α and 21α hydroxylations, degradation of the two carbon C-17 side chain of corticoids and the dehydrogenation of cortisones.

As a result of the intensive studies that followed, it was soon established that the ability to bring about chemical transformations in the steroid nucleus is wide-spread among the microorganisms and the types of reactions encountered are numerous.

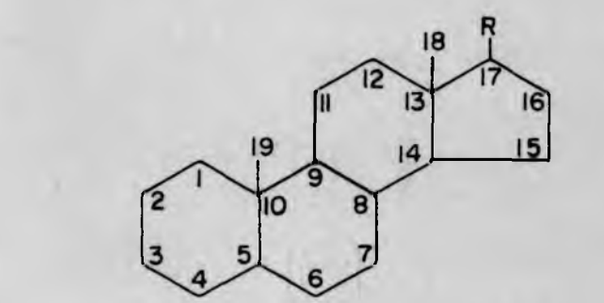
A series of excellent and comprehensive reviews are available on this subject, notably by Peterson,[5]

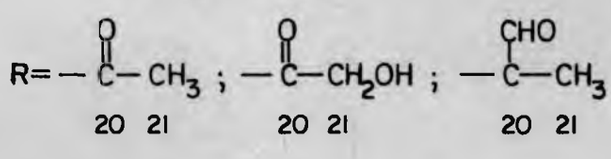
Vischer and Wettstein,[6] Eppstein and coworkers[7] and Fried and collaborators.[8] A few of the important transformations are summarised in Table I.

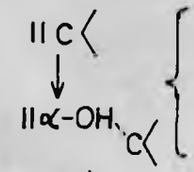
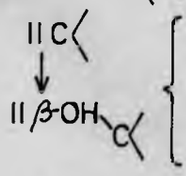
T A B L E I

Microbiological reactions on steroids with typical reactions

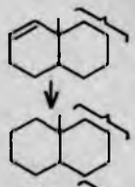
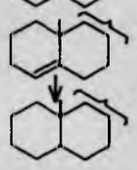
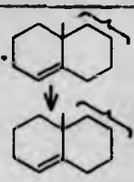
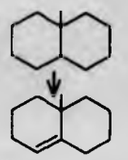
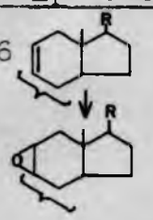
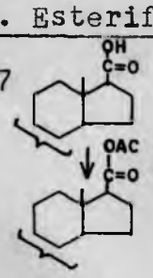
Basic steroid structure





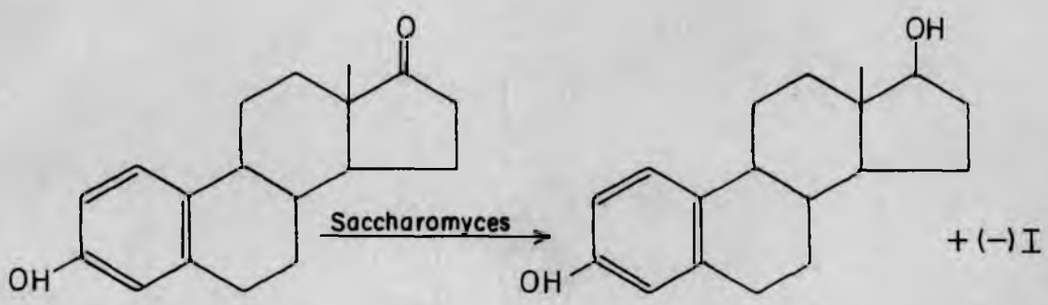
Nos.	Reactions	Substrate	Product	Micro-organisms	Ref
A. Introduction of -OH group					
1		Progesterone	11α-hydroxy-progesterone	Rhizopus-nigricans Neurospora sitophila	6
2		Progesterone	11β-hydroxy progesterone	Curvularia lunata Cunning-hamella. blakesleeana	5

Nos.	Reactions	Substrate	Product	Micro-organisms	Ref.
3	6-C< → 6β-OH-C<	Progesterone 16α-ol	6β-16α dihydroxy-progesterone	Aspergillus nidulans	6
4	6-11-C → 6β-11α-di-OH	Progesterone	6β 11α dihydroxy progesterone	Rhizopus arrhizus	6
	B. Oxidation of -OH group				
5	3-β-OH → 3>C=O	Coprostan-3 β-ol.	Coprostan 3-one	Proactinomyces erythropolis	9
6	Δ5-3 β-OH → Δ4-3>C=O	5-Pregnen-3β-ol-20-one	Progesterone	Corynebacterium mediolanum	9
7	17 β-OH → 17 >C=O	Estradiol-3 17β	Estrone	Proactinomyces erythropolis	9
8	11 β-OH → 11 C>=O	Cortisol	Cortisone	Cunninghamella blakesleena	10
	C. Reduction of C=O group				
9	3 >C=O → 3α-OH + 3β-OH	Androstane-3,17-dione	Androsterone; Epiandrosterone	Mixed putrifactive bacteria	9
10	17 >C=O → 17 β-OH	4-Androstene-3,17-dione	Testosterone	Fermenting-yeast	2
11	20 >C=O → 20 β-OH	Progesterone	4-pregnen-20 β-ol-3-one	Streptomyces lavendulae	9

Nos.	Reactions	Substrate	Product	Micro-organisms	Ref.
D. Hydrogenation of double bonds					
12		Δ^1-androstene-3-17 dione	Androstane-3β 17β diol	Fermenting yeast	9
13		Progesterone	Pregnane 3, 20-dione	Bacillus putrificus	2
E. Introduction of double bonds					
14.		Cortisone	Prednisone	Corynebacterium simplex	6
15		Androstane-3,17 dione	1,4-androstadiene 3,17-dione	Fusarium Sp.	6
F. Epoxidation of steroids					
16		Δ^4-9(11) pregnandiene-17α 21 diol	Δ^4 9β 11β epoxide pregnene 17α 21 diol	Curvularia lunata	9
G. Esterification of steroids					
17		9α-fluoro-11β21-dihydroxy 16α, 17α-isopropylidene-dioxy-pregn-4-ene, 3,20,dione	21-acetyl compound	Trichoderma glaucum	11

Sections B and C in Table I reveal that for nearly every oxidation of a steroid alcohol, a corresponding stereospecific microbiological reduction of a keto-steroid is also known, which clearly indicates that a high degree of specificity exists with respect to the position and steric course of these oxido-reductions.

In many instances the stereo-specificity of pure cultures of microorganisms have been exploited to achieve many highly selective transformations of steroid hormones. Wettstein and colleagues[12] have made ingenious use of this stereo (optical) specificity of the moulds for the resolution of racemic steroids. When synthetic (±)-Oestrone (I) was fermented with yeast, (+) oestradiol-17β(II) and unreacted (-) oestrone were isolated.



(±) I (+) II

Fig. 1

Similarly the Ciba group[13] has shown that only the (+) isomer of the totally synthetic lactone (III) underwent hydroxylation at C_{21} by cultures of Ophiobolus herpotrichus, whereas the enantiomorph remained unaffected.

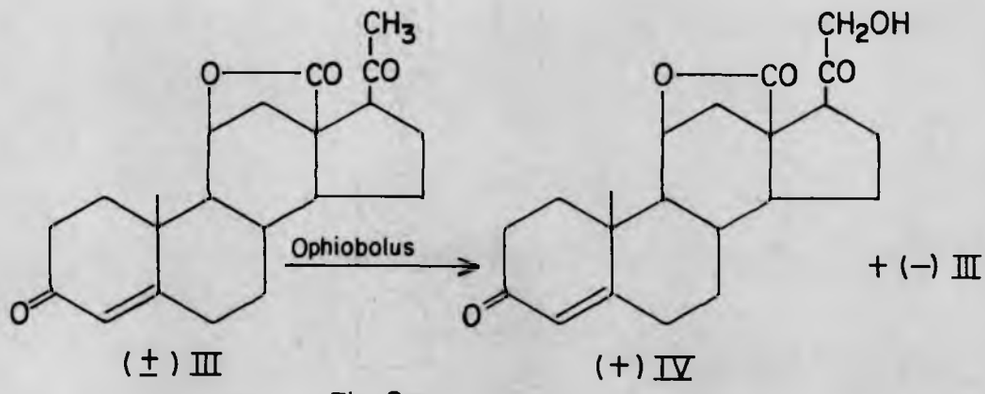


Fig. 2

Utilizing the same principle, the naturally occurring enantiomorph of racemic aldosterone (V) was dehydrogenated at the 1,2-positions by Didymella lycopersici.

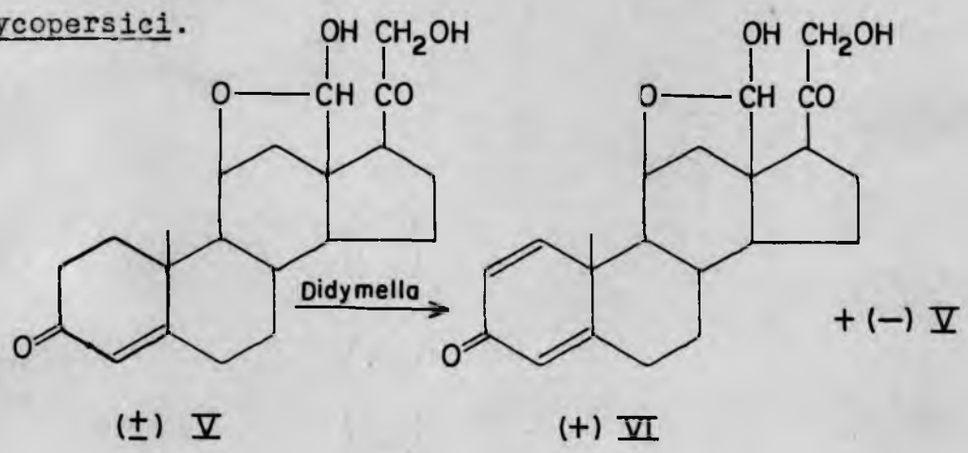


Fig. 3

Another example of such conversion is the transformation of (±) cortisone (VII) to a mixture of (-) cortisone and (+) 1-dehydrocortisone (VIII).

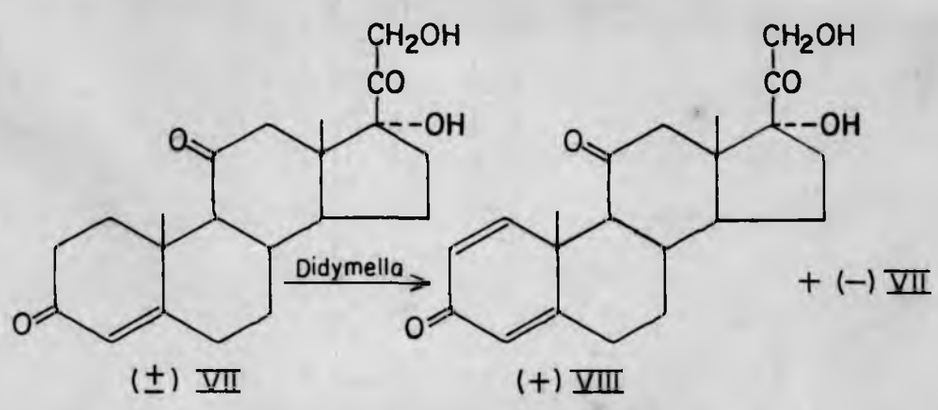


Fig. 4

Microbiological transformations have also been used for proving the structures of new natural or synthetic steroids. For instance, using Streptomyces roseochromogenus testosterone (IX) was converted to 16α-hydroxytestosterone (X).[14] In order to establish the configuration of the hydroxy group at C_{16} position, 16 α-hydroxyprogesterone (XI) was degraded to the known 16 α-hydroxy testosterone (X) by S.lavandulae, an organism known to effect cleavage of the side chain and identity of the compound was thus established.[15]

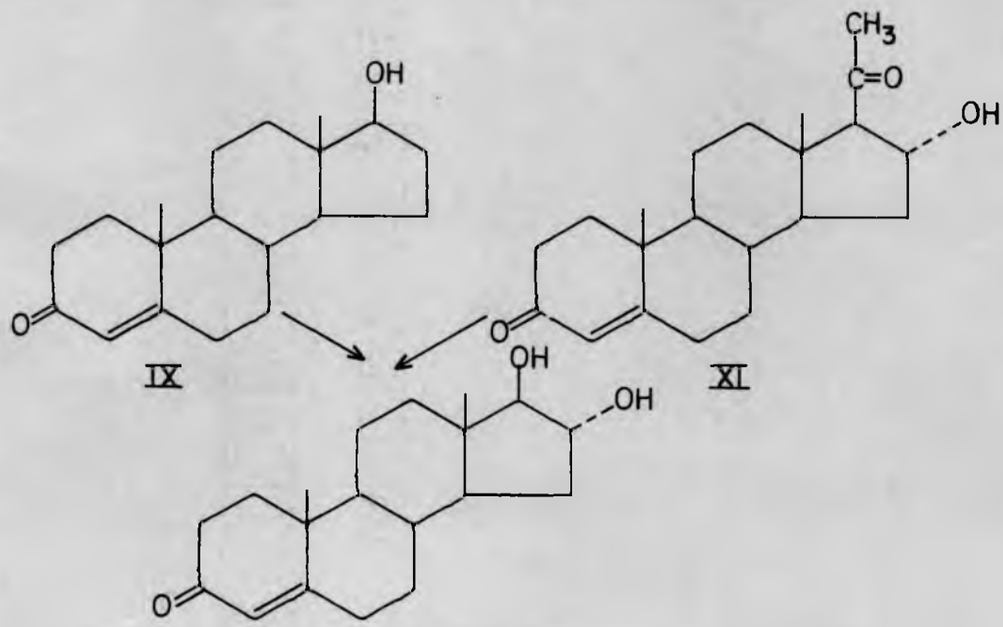


Fig.5

Neher and Wettstein[16] applied microbiological hydroxylation for proving the structure of several new steroids isolated from adrenal glands, such as 6 β,17α-dihydroxy cortexone, 17α-19-dihydroxy-cortexone, 6β-hydroxy corticosterone and 19 -hydroxy corticosterone.

A considerable amount of work has been done on the stereochemical specificity of the microbial enzymes towards substituted decalones by Prelog and co-workers.[17] They have reported that reduction of the carbonyl groups of compounds investigated gave one or more asymmetric carbon atoms, thus forming enantiomeric or diastereomeric products. Based on this stereospecificity of these products, the micro-organisms have been classified into two groups.

The first group is characterised by poor stereospecificity. Aspergillus niger is a representative of this class yielding a mixture of all four of the theoretically possible stereoisomeric Δ^4-8 hydroxy octal-3-ones on reduction of the carbonyl in position 8 of racemic (±) Δ^4-9-methyl-octalin-3-8-dione as shown in Fig.6.

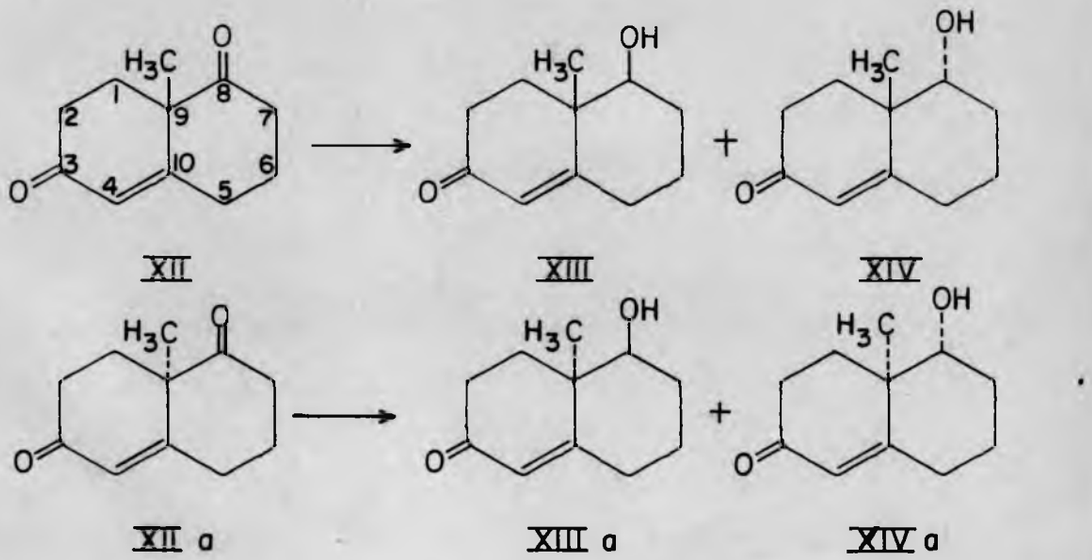


Fig.6

The second group of microorganisms of which Curvularia falcata is typical, has marked stereospecificity. Only two Δ^4-8-hydroxy-octal-3-ones are formed, each enantiomer of the substrate being specifically reduced to the product having 8β-configuration[18] as in Fig.7.

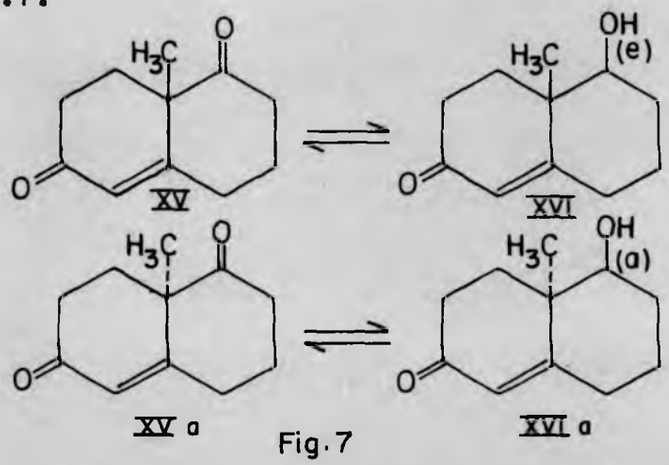


Fig.7

On the basis of these results they have postulated that a clear cut steric course was due to the presence in Curvularia falcata a relatively simple enzyme system as compared to that in Aspergillus-niger.

Acklin, Prelog and Zach[19] have shown that the presence of a double bond in theα-β-position to the carbonyl prevented reduction by C falcata, whereas the non-conjugated carbonyl in the β-position of a decalin derivative was reduced fairly rapidly. Thus, the two stereoisomeric-9-methyl-decalin 1-5 diones yielded the corresponding 5-OH compounds with β-configuration as in Fig. 8.

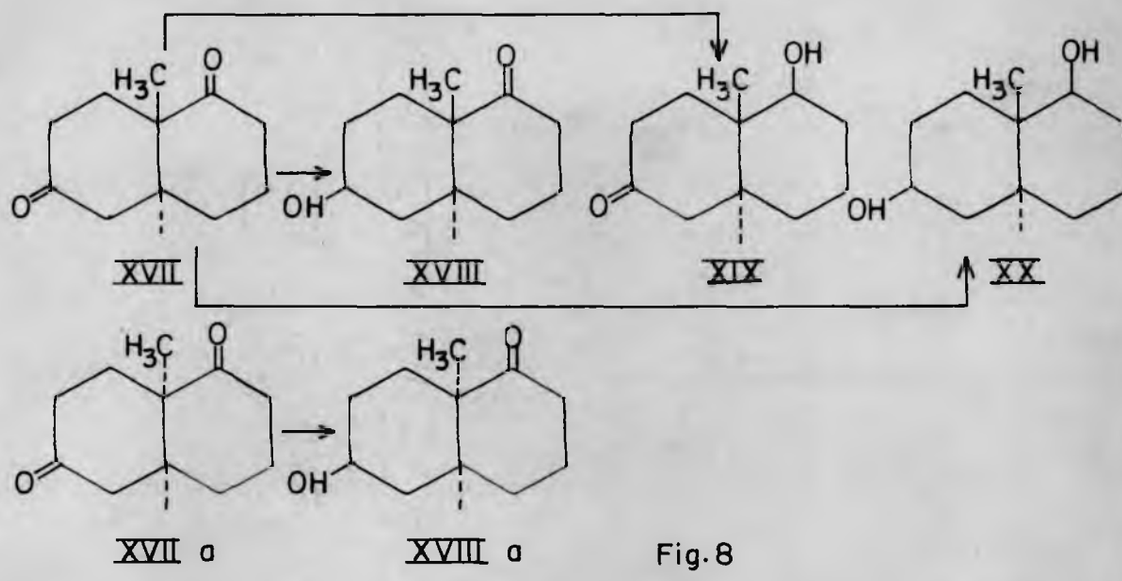
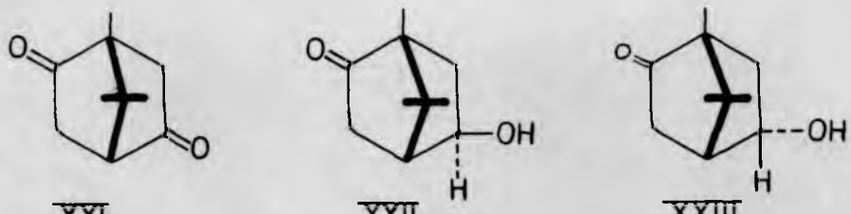


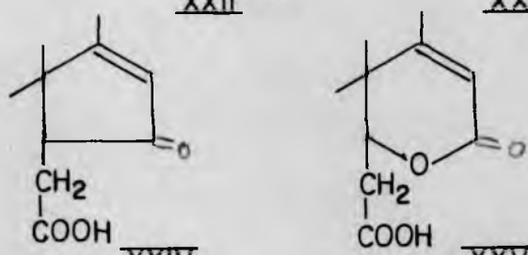
Fig.8

As against the enormous useful data accumulated in the field of steroids, relatively little work has been done on the microbiological transformations of terpenoid compounds. Only during the last four years has some interest been apparent in this field.

In 1958, Corey and collaborators[20] from the University of Illinois first reported on the microbiological degradation of camphor. A Pseudomonad strain (P) which had been isolated from sewage sludge by enrichment techniques using (+) camphor as a carbon source, was grown on a medium containing (+) camphor (0.5%) and minerals. Extraction of the broth at the end of the growth phase followed by chromatography of the neutral fraction yielded three products, viz. a keto camphor (XXI) identified as 2,5-diketocamphane and two hydroxy ketones which proved to be 5-exo (XXII) and 5-endo (XXIII) hydroxycamphors, respectively. (Fig.9).



XXI XXII XXIII



XXIV XXV

Fig.9

The acid fraction afforded a keto acid 3,4,4-trimethyl-5-carboxymethyl cyclopentenone (XXIV). Complete oxidation of this acid by resting bacterial cells was inhibited by addition of 2.2' bipyridine resulting in the accumulation of a new lactonic acid intermediate(XXV). The pathway of degradation of camphor by this organism has been partially formulated as (+) camphor ⟶ 5.hydroxy-camphor ⟶ (XXI) ⟶ (XXIV) ⟶ (XXV).

In continuation of this work Gunsalus and coworkers[21] have reported that Pseudomonads C_1 (formerly P) and C_5 isolated by the enrichment culture technique, oxidise camphor, but differ in their ability to use this compound as a sole carbon and energy source for growth.

Using the same technique, Werner Seubert[22] isolated a new species of the genus Pseudomonas which oxidises citronellal and farnesol. He also found that

the dried cell preparation of these organisms oxidized as well, a number of related compounds which could be intermediates in isoprenoid degradation. Extending the studies to the metabolism of acetate in dried cells and using labelled carbon, Werner Seubert also demonstrated that the substrate citronellal, a likely intermediate in isoprenoid degradation, is partially converted to an ether-soluble fluorescent pigment and a number of amino acids. The nature of the fluorescent pigment has not been established.

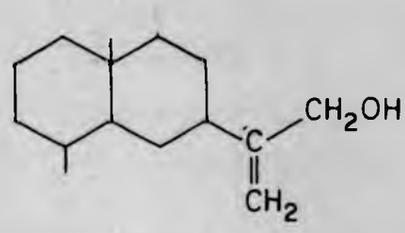
Present Investigation

A review of literature would indicate that although microbiological conversions have been extensively investigated in the case of steroids, such transformations in terpenoid compounds are still relatively unexplored.

A systematic investigation of the microbiological transformations of terpenoid compounds has, in addition to the important fundamental significance in understanding pathways of biosynthesis and biological conversions, a practical bearing on the economics of the perfumery industry.

Among the few isolated reports on the biodegradation of terpenoids is the finding that the yield of essential oils from the plant Aquilaria-agallocha, commonly known as Agarwood, depends on the extent of microbial infestation.[23] Wood from the green healthy tree is odourless, while the wood which is odoriferous is found in comparatively mature trees and has widespread fungal infection associated with irregular patches and dark streaks, highly impregnated with an oleoresin. The organisms associated with the production of this oleoresin, commonly called 'Agar' or 'Agaru' were isolated by earlier workers[24,25] and identified as (i) an Aspergillus sp. which is primarily responsible for the production of 'agar; (ii) a Penicilium sp. and (iii) a Fusarium sp.

One of the main odoriferous principles in 'agar' was identified as a hydroxy compound, agarol,[26] (XXVI); the hydroxylation probably being a direct effect of microbial action on a suitable terpenoid hydrocarbon precursor.



XXVI

With the exception of such indirect evidence for the capacity of microorganisms to transform terpenoid hydrocarbons to oxygenated compounds, no clear-cut experimental evidence in this respect is available in literature. The present studies were, therefore, undertaken to investigate the ability of microorganisms, particularly fungi, to convert mono- and sesqui terpenoid hydrocarbons, auch as α-pinene, Δ^3-carene, carane, camphene, α and β-santalenes and humulene to oxygenated essential oils which could be of some potential value in the perfume industry.

Six isolates from the infected agarwood and six other fungi were screened for their ability to metabolise α-pinene in shake-cultures. Chapter III of this thesis would indicate that all the organisms tested were able to

degrade the test substrate. Among these a strain of A. niger (NCIM 612) was found to be the most efficient, completely metabolising the added α-pinene (0.5%) within 24 hours and was, therefore, used in further studies.

Among the products of microbial action on α-pinene under shake flask conditions three oxygenated derivatives were isolated and identified as (i) cis-verbenol, (ii) verbenone and (iii) trans-sobrerol. The purification and characterisation of these microbial degradation products of α-pinene are recorded in Chapter IV.

Chapter V gives an account of the fermentation of three hydrocarbons, viz., Δ^3-carene, carane and humulene, which were relatively resistant towards microbial action and were recovered almost completely unchanged from the fermentation mixture.

The sesquiterpene hydrocarbon, α-santalene, was degraded by A.niger mainly to tere-santalic acid and to a lesser extent to the corresponding alcohol, tere-santalol. The isolation and identification of these products are detailed in Chapter VI.

In Chapter VII studies on the action of A.niger on two structurally related hydrocarbons, camphene and β-santalene are described. Both of these compounds were oxidised by the mould to an extent of 30 to 35% to the

identical dicarboxylic acid, which could be isolated only as the liquid anhydride, $C_{11}H_{16}O_3$. The anhydride was characterised as 2-nonene-2:3-dicarboxylic acid anhydride from its physicochemical properties, degradation and total synthesis.

Finally, in Chapter VIII some speculations are made on the probable mechanism of action of the mould on the terpenoid substrates with reference to the known oxygen transfer processes in biological systems.

CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS

Spores of *Aspergillus niger* NCIM*(612) were maintained in sterile soil as stock culture and sub-cultures from the stock were used exclusively in these transformations.

Propagation of culture

The cultures were propagated in a potato-dextrose-agar medium[27] of the following composition:

Potato extract	...	20% (W/V)
Dextrose	...	2% (")
Agar	...	1.5%(")
pH adjusted to	...	5.8

For the preparation of slants 5 to 6 ml. of the medium were taken in 15 ml. pyrex tubes and autoclaved at 15 psi pressure (121°) for 20 min. These slants were inoculated from the stock culture and incubated at 28°C for 6 to 7 days.

Preparation of medium and inoculum

Czapek-Dox medium[28] used for growth had the following composition:

Glucose	...	4% (W/V)
Potassium chloride	...	0.05% "
Magnesium sulphate	...	0.025% "
Sodium nitrate	...	0.2% "
Potassium dihydrogen-phosphate	...	0.1% "
Ferrous sulphate	...	0.001% "
Yeast extract	...	0.05% "
Corn steep liquor	...	0.5 % "
pH adjusted to	...	4.8

Aliquots of 100 ml. of the growth medium were distributed in 500 ml. Erlenmeyer flasks, autoclaved at 15 psi pressure for 20 min. and inoculated with a spore suspension in sterile distilled water from potato-dextrose-agar slants (PDA).

Fermentation

The inoculated flasks were incubated at 28° on a rotary shaker at 220 r.p.m. After a good growth was obtained (normally between 24 to 34 hr) the respective substrates (α-pinene, α& β-santalenes, camphene, Δ^3-carene, carane and humulene) were added aseptically in different concentrations depending on the rates of oxidation and the fermentation was allowed to continue for 24 hr with periodic sampling for analysis and examination for the absence of bacterial contamination. Any bacterial contamination was usually detected by estimation of pH and staining broth smears by the standard Gram-staining procedure.

It was observed that when the fermentation proceeded normally, the pH was steady between 4.4 to 4.8 and that bacterial contamination was always associated with a marked increase in pH.

* National collection of industrial microorganisms of the National Chemical Laboratory, Poona, India.

Physical methods

1. The infrared spectra were recorded either in nujol or as pure liquid film on a Grubb-Parson Double beam spectrophotometer or on a Perkin-Elmer Infracord with sodium chloride optics.

2. The ultraviolet absorption spectra were taken in 95% ethanol solution with a Beckmann Quartz Spectrophotometer Model DU.

3. Optical rotations were determined on a Hilger standard polarimeter in ethanol solution unless otherwise stated, using a 1 dm. tube. The concentrations are reported in paranthesis, and are expressed in mg/ml.

4. All melting points and boiling points are uncorrected.

5. The n.m.r. spectra were determined at 60 mcs, with a Varian Associates Model H-R 60 Spectrophotometer in deutero chloroform solution with benzene as an external reference compound. Chemical shifts were estimated using the side bands which develop between two signals in p.p.m.

C H A P T E R III

SCREENING AND SELECTION OF MICROORGANISMS

Screening and Selection of Microorganisms

DISCUSSION

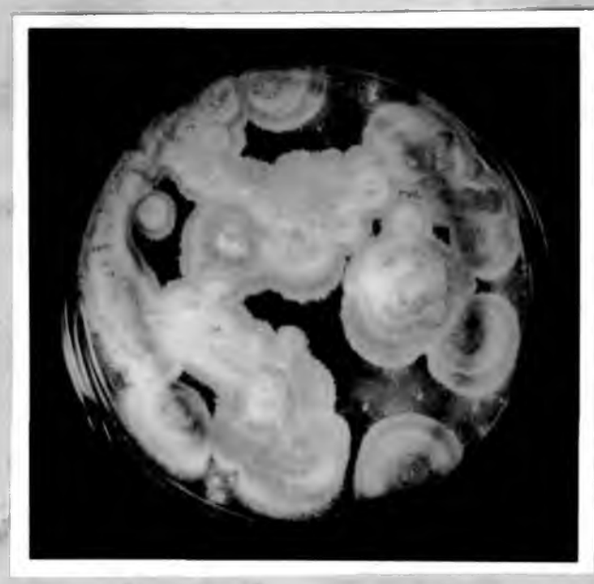
The occurrence of the oleoresinous patches, also known as 'Agar' or 'Agaru', on the plant *Aquilaria agallocha*, which is responsible for the sweet odour is believed to be the result of microbial infestation. This is indicative of the adaptation phenomenon of the microorganisms for the decomposition of the terpenoid hydrocarbon in the Agar plant. On this presumption pure cultures were isolated from the infected bark for studying the microbial conversions of other terpenoid hydrocarbons.

From a sample of infected agarwood, six strains of fungi, identified as (1) *Penicillium steckii*, (2) *Geotrichum sp.*, (3,4,5) *Aspergillus versicolor* and (6) *Cladosporium sp.* were isolated using standard techniques for isolation and identification. Tunstall[24] has isolated from infected agarwood three strains of fungi viz., (1) *Penicillium sp.*, (2) *Aspergillus sp.* and (3) *Fusarium sp.*

A preliminary screening with these microorganisms revealed that one day old shake culture of each was capable of bringing about partial to complete disappearance

of added α-pinene (0.5%) in a period of 24 to 48 hours. In most of the cases extraction of the mycelium and fermented broth with ether or chloroform resulted in the recovery of small amounts of high boiling and sweet smelling oils. This would, therefore, indicate the possibility that a variety of microbial flora may have the ability to metabolise terpenoid hydrocarbons.

A systematic search amoung the common strains of fungi available in the N.C.I.M. collection of National Chemical Laboratory was therefore undertaken to isolate a strain which would rapidly metabolise the added α-pinene. A strain of Aspergillus niger NCIM (612) which was found to be remarkably efficient in this respect and could completely metabolise the added α-pinene (0.5%) in 18 to 24 hours under suitable experimental conditions was, therefore, selected for further studies.



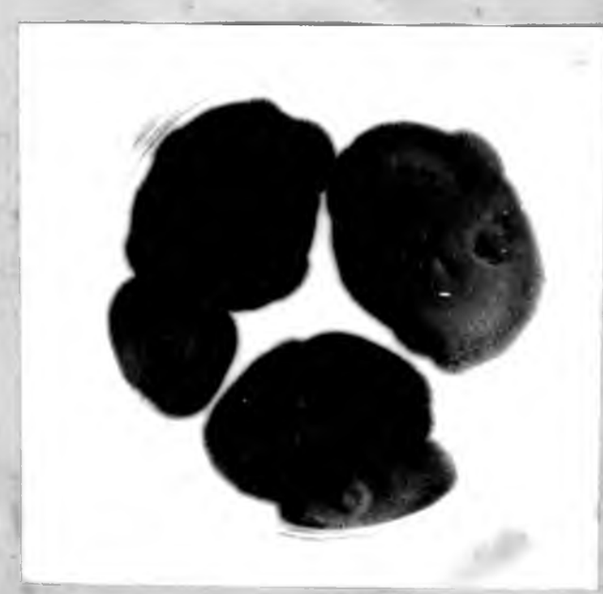


Figure 1

(a) Colonies of Penicillium steckii on Czapek's agar.

(b) Colonies of Cladosporium sp. on Czapek's agar.

Figure 2

(a) Colonies of Aspergillus versicolor (3) on Czapek's agar.

(b) Colonies of Aspergillus versicolor (5) on malt, steep, and potato dextrose agar.

EXPERIMENTAL

Isolation of the organisms from the infected bark

The organisms were isolated from scrapings of the infected part of the bark by dilution and streak plate techniques. Approximately 1 g. of the scrapings were suspended in 10 ml. sterile distilled water and 0.1 ml. of the suspension was plated out on Sabouraud agar. After incubation for a period of seven days at 28°, small colonies with varied colors were evident. By streaking on plates of the same medium, six fungal cultures were isolated in pure form. Of these, three cultures appeared to be similar except for minor variations in their cultural appearances.

Taxonomy, morphology and cultural characteristics of the above fungal isolates

Isolate 1: This is a species of Penicillium nearest to the species P.steckii and has the following characters.[29]

"Colonies on Czapek's agar have restricted growth attaining a diameter of 2 cm. in ten days at room temperature with a velvety surface, and shallow radial furrows in the marginal areas; often more or less zonate in the outer areas with growing margin 1 to 2 mm., turning quickly to yellow green colour with the development of the conidial

areas. Colonies medium to heavy sporing, particularly good in potato dextrose and malt agar. In the latter, they do not develop ridges, but the zonations are characteristic, growth thin and spreading, reverse uncolored or nearly so, exudate lacking."

Conidiophores abundant arising from the substrate, variable in length but usually comparatively short within 200μ, smooth walled, unbranched typically biverticillate. It would belong to the series Penicillium citrinum of the subsection velutina in the asymmetric penicillia.

Isolate 2: A species of Geotrichum, very fast growing on yeast extract agar and rather slow growing in Czapek-Dox medium. Although, the mycelium is formed it breaks up into rectangular long cells within a few hours, so that on examination the whole colony although retaining in gross morphology mycelial habit, appears under the microscope fully split up into cells. Further growth follows a similar pattern. Conidia hyaline, one celled cylindrical, formed by fragmentation of the hyphae. The species belongs to the order moniliales of the group fungi imperfectii.[30]

Isolates 3,4 & 5: All these isolates are Aspergillus sp. and probably are variants of the same species Aspergillus versicolor of the series of the same name.[31]

Colonies on Czapek-Dox's agar rather slow growing in the strain 5, almost velvety, but floccose in both the isolates 3 and 4. While isolate, 5, shows a persistently greenish colony, the isolates 3 and 4 are initially white with outer edges, gradually pass through shades of green with age. However, the reverse in all these cases show a bright red pigment diffusing out in to the medium and the colonies also produce a clear red exudate. The color of the pigment changes to bright yellow in acid pH (5.0 and below).

Heads roughly hemispherical, conidiophores colorless, smooth, vesicle diameter nearly 10 μ, sterigmata, in two series, conidia globose, finely echinulate.

Isolates 5 and 3 show great difference in colony morphology when grown on different media like Czapek-Dox's, potato-dextrose and on malt agar. In Czapek-Dox's they persist in light (almost white) color, while in the other two, they become green in appearance although the dimensions and characters of the conidial apparatus remain unchanged.

Isolate 6: This is a species of Cladosporium with dark conidiophores, branched variously near the apex or the middle portion, clustered or single with

conidia, dark, one celled initially, but becoming two celled with age, ovoid in shape. Reverse of the colony in Czapek-Dox's medium is black and the upper surface is dull greenish black.[30]

Selection of the Microorganism

The ability of the organisms isolated from the infected bark and of some of the common strains available in the NCIM collection to utilize α-pinene in shake cultures was determined in a series of experiments. Determination of the extent of α-pinene decomposition after 24 hours of incubation was assayed by weighing the residual product after solvent extraction of the fermented culture. The object of these series of experiments was mainly to establish the ability of the strains to utilise α-pinene and not for investigating the capacity of the strain to give any specific metabolised product.

In a preliminary experiment five - 500 ml. Erlenmeyer flasks containing 100 ml. Czapek-Dox medium enriched with 0.5% corn-steep liquor were seeded with each of t different fungal inocula, incubated on a rotary shaker for 24 to 48 hours depending upon the rate of growth of the mould. At this stage 0.5% α-pinene was added to each flask and incubation was continued for a further period of 24 hours. After this period the flasks were removed from the shaker, filtered separately and the fermented culture (both mycelia

and the medium) extracted with ether. The combined ethereal extracts were washed with distilled water, dried over anhydrous sodium sulphate and the solvent distilled. The residual liquid was then transferred to a sample tube and weighed.

The results of this experiment with twelve strains of fungi are presented in Table I. In addition to the six isolates from the infected bark, four cultures of A.niger and two cultures of Rhizopus species from the NCIM collection were examined.

T A B L E I

Utilization of -pinene by different strains of fungi

No	Fungi	Species	-Pinene added (g)	Extracts recovered after 24 hr. (g)
Control			2.15	1.677
1	Aspergillus	niger(612-NCIM)	2.15	0.302
2	Penicillium	steckii (1)	2.15	0.351
3	Aspergillus	versicolor(5)	2.15	0.408
4	Geotrichum	sp. (2)	2.15	0.416
5	Cladosporium	sp. (6)	2.15	0.438
6	Aspergillus	niger(621-NCIM)	2.15	0.622
7	Rhizopus	nigricans	2.15	0.742
8	Aspergillus	versicolor(4)	2.15	0.846
9	Aspergillus	niger(517-NCIM)	2.15	0.921
10	Aspergillus	niger(516-NCIM)	2.15	0.932
11	Rhizopus	arrhizus	2.15	1.278
12	Aspergillus	versicolor (3)	2.15	1.658

The results summarized in Table I reveal that the majority of the cultures tested are capable of utilizing α-pinene, but their capacity to decompose the hydrocarbon differs considerably. Since A.niger(612-NCIM) showed a better efficiency in utilizing α-pinene in a 24-hour period it was selected for further investigation and was used exclusively in all the transformations that have been reported here.

This strain of A. niger has also been used to hydroxylate the steroidal substrate, progesterone at the 11 α position in an yield of 70%.[32]

CHAPTER IV

TRANSFORMATION OF α - PINENE

Transformation of α-Pinene

DISCUSSION

α-Pinene (I), $C_{10}H_{16}$, one of the most important monoterpene hydrocarbons, is widely distributed in nature. α-Pinene occurs in almost all essential oils of the Coniferae family and forms the main constituent of the oil of turpentine.[33] The hydrocarbon fraction of the oil of turpentine distilling between 155 to 165° was designated "Pinene" by Wallach[34] and its final structure was suggested by Von Baeyer.[35] α-Pinene is of considerable commercial interest, being the basic raw material for the syntheses of camphor, borneol, terpineol, etc. The chemistry and constitution of the hydrocarbon have been reviewed by Simonsen,[36] Rodd[37] and Guenther.[38]

This chapter deals with the microbiological transformations of this hydrocarbon by Aspergillus niger (612-NCIM) and the isolation and identification of oxygenated derivatives from the fermentation mixture.

As optically pure (d) α-pinene with $[\alpha]_D$ + 51.8° was difficult to obtain, the investigations were carried out on the commercially available material, (BDH), ($[\alpha]_D$ + 23.4°) fractionally purified by batch stripping through an efficient distillation column. The fraction distilling at 155-57°/715 mm. Hg was utilized for the studies; the rotation of the sample corresponded to 70% d and 30% l α-pinene. In vapour phase chromatogram the compound exhibited a single peak.

FERMENTATION

Determination of the optimum conditions for the fermentation

1. Concentration of α-pinene in the medium.
2. Period of conversion.
3. Temperature of conversion.

The optimal conditions for the fermentation were established by trial runs in shake cultures. In these runs, sterile Czapek-Dox media enriched with 0.5 % cornsteep liquor were inoculated with A.niger spores from PDA slants grown for a week at 28°. The organism was allowed to grow for 24 hours and the substrate α-pinene was added at the end of this period. The conversion was studied at different concentrations, different time intervals and at different temperatures.

Concentration of α-pinene in the medium

Optimum concentration of α-pinene which might be a limiting factor in these transformation was studied in shake cultures. Experiments run with graded levels of α-pinene (0.2; 0.4; 0.6; 0.8; 1.0 and 2 percent, V/V) indicated that the yields of oxygenated products reach a maximum with 0.6 % α-pinene and go down considerably at higher concentrations. It is probable that the hydrocarbon is toxic to the mould at higher concentrations.

Therefore, in later experiments a concentration of 0.5% α-pinene was adopted as optimum.

Period of conversion

The optimum time for incubation was determined by running an experiment of six sets, each run to a different length of time. It was found that the optimum conversion was obtained in about 8 hours, a time period which gave the maximum yield as well as a reasonable material balance. Longer periods of fermentation resulted in the disappearance of both the substrate and the transformed products.

Temperature of conversion

The transformations were conducted at two temperatures, 27-28° and 30-31°. The metabolism of α-pinene showed a surprising degree of sensitivity to temperature; at higher temperatures and inspite of the profuse vegetative growth almost no transformation took place. Added α-pinene was recovered almost unchanged from fermentations run at 30-31°.

Based on the above preliminary investigations, conditions were established for fermentations on a large scale. This consisted in adding α-pinene in two lots at four hour intervals to vigorously growing 24 hour old shake cultures of the mould incubated at 28°. The fermentation was allowed to proceed for four hours after the addition of the second instalment of α-pinene. This

procedure although not very satisfactory from the point of view of utilization of α-pinene, gave better overall yields of the oxygenated conversion products.

EXTRACTION

The preliminary procedure for extracting the unreacted substrate and the oxygenated transformation products was based on that devised by Peterson and Murray[39] for the microbiological transformation of steroids. It was found that the transformation products were present both in the mycelial pellets as well as in the medium. Acetonization of the mycelium rendered it more easily accessible to solvents such as ether and n-butanol. The mycelia as well as the fermentation broth were extracted repeatedly with ether and subsequently with n-butanol to ensure complete extraction of the polar transformation products of fermentation.

The acidic transformation products were removed as their water soluble sodium salts from the solvent extracts by washing with aqueous sodium carbonate, leaving only the neutral compounds in the solvent layer.

The acidic fraction obtained from the sodium carbonate washings on acidification and extraction with ether yielded very small amounts of an acid after removal of the solvent. Since the acid could be isolated only in

very small amounts, no further investigations were carried out on this fraction.

Recovery of the neutral oxygenated products

The separation of the relatively polar transformation products from the non-polar unreacted α-pinene was effected by partitioning between n-hexane and 90% aqueous ethanol. A modified Craig four-transfer distribution where each of the n-hexane layers was equilibriated in succession with the four alcoholic layers and pooled, ensured a clearcut and complete separation of the oxygenated products from α-pinene. There were, however, losses in the recovery particularly of α-pinene, on evaporation of the n-hexane layers.

The alcoholic layers on evaporation yielded sweet smelling oils. To save losses, the evaporation was discontinued when practically all the alcohol was distilled. The aqueous emulsion, left as a residue, was extracted into chloroform and the chloroform extracts were dried over anhydrous sodium sulphate. The solvent was distilled out to yield an oily substance.

From a fermentation involving forty shake flasks, with 34 g. added α-pinene, 16-18 g. of the starting material (Fraction A) was recovered unchanged (48-55%). The oily transformation product (Fraction B) weighed 3-4 g. representing a yield of 10-12% based on the initial added

α-pinene and 20-25% on the basis of pinene utilized. The semicrystalline solid material (Fraction C) from the n-butanol extracts weighed about 600-800 mg. accounting for a yield of 2-3% on the basis of pinene utilized.

Fraction, B, on fractional distillation under reduced pressure yielded two clearcut fractions (i) a colorless oil (D) distilling at 120-25° (bath temperature) at 16 mm. Hg and (ii) a viscous oil (E) distilling at 140-45° (bath temperature) at 0.6 mm. Hg, which solidified on standing. This fraction (E) was identical with fraction C obtained from butanol extractions.

The elementary analysis and infrared spectra of the oily Fraction,D, indicated that it was possibly a mixture of two components - a ketone (II) and an alcohol (III).

Ketone,II, was separated from Alcohol, III, through its crystalline bisulphite addition product and was liberated from the bisulphite complex with cold dilute acetic acid. It was then extracted with ether and purified by distillation under reduced pressure; 120-25° (bath temperature)/16 mm. Hg.

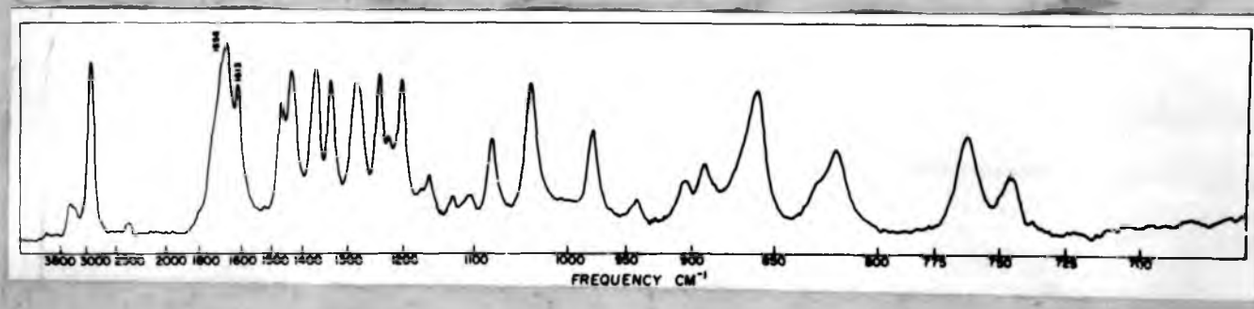
The unreacted Alcohol, III, was recovered from the aqueous layer by repeated extraction with ether and removal of the solvent by evaporation.

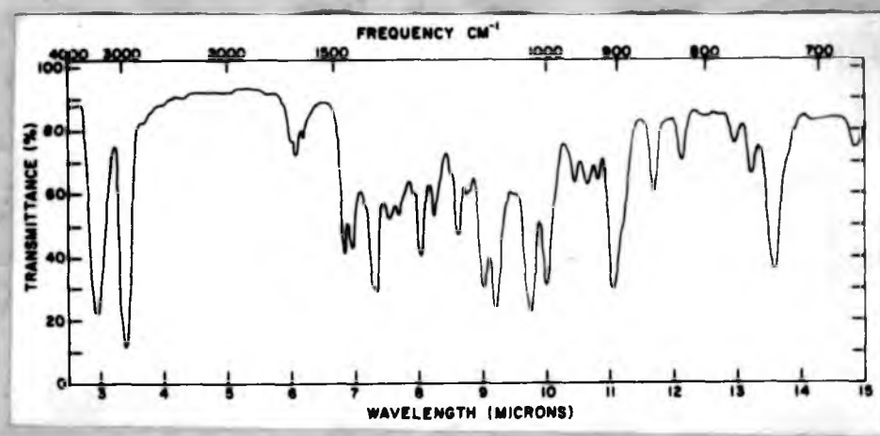
The ketone and alcohol were also separated by column chromatography using grade II neutral alumina. Although, separation by this method was clearcut, the recovery was not quantitative on account of the volatility of the oxygenated products. The recovery through bisulphite complex procedure was preferred to the chromatographic procedure as the overall yields were better.

IDENTIFICATION OF THE PRODUCTS

Ketone, II, analysed for $C_{10}H_{14}O$ and indicated the presence of an α- β unsaturated carbonyl grouping in the I.R. spectrum (1658 cm^{-1} for C=O and 1613 cm^{-1} for the conjugated double bond; page 36). It showed an absorption maximum in the u.v. (λ_{max} 253. $\log \epsilon$ 3.83) and gave a crystalline semicarbazone, melting at 203-205°. These data indicated that the ketone might be d-verbenone.

For comparison d-verbenone was synthesised by chromium trioxide catalysed autoxidation of α-pinene according to the method of Marius Badoche.[40] A comparison of the physical properties, ultraviolet spectra and infrared spectra of verbenone and of its semicarbazone with those of Ketone, II, established conclusively the identity of Compound, II. The mixed melting points of the two semicarbazones showed no depression.





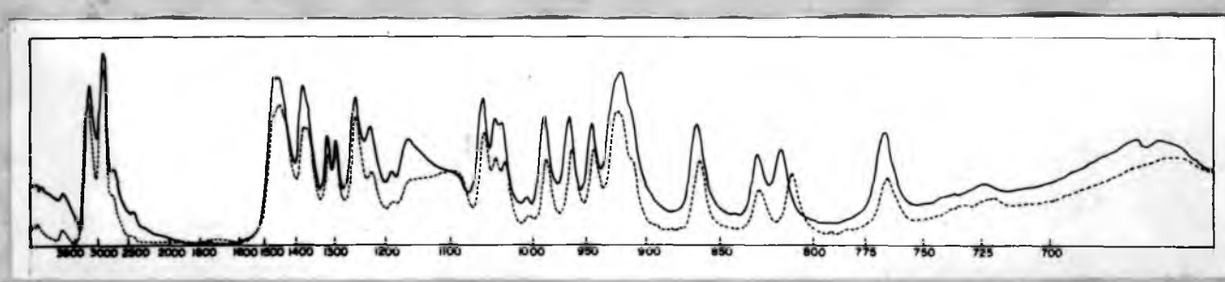
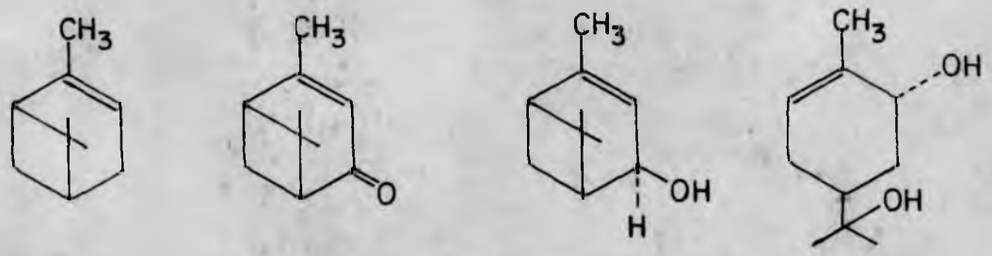


Figure 1

a) Infrared absorption spectra of the ketone, Verbenone.
b) Infrared absorption spectra of the alcohol, Verbenol.
c) Comparative infrared spectra of synthetic sobrerol (- - - - -) with that of isolated sobrerol, (——).

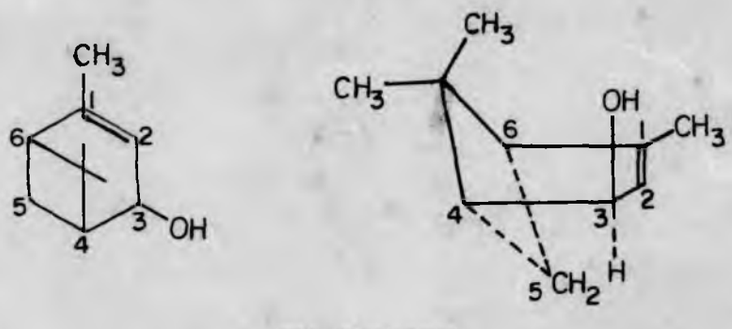


I II III IV

Alcohol, III, purified by distillation under reduced pressure analysed for $C_{10}H_{16}O$. Infrared spectrum exhibited a band at 3360 cm^{-1} indicating the presence of a hydroxyl group. It gave a crystalline p-nitrobenzoate, m.p. 88-90°.

On mild chromic acid oxidation in pyridine or by oxidation with solid manganese dioxide prepared according to the method of Turner[41] in chloroform, Alcohol, III, yielded Verbenone, II, in very good yields.

From the above data, Alcohol, III, was characterised as d-verbenol and its configuration was assigned as <u>cis</u>- on the basis of the rotational data ($[\alpha]_D$ + 17°) of its derivative, p-nitrobenzoate.[42]



<u>Cis</u>- Verbenol

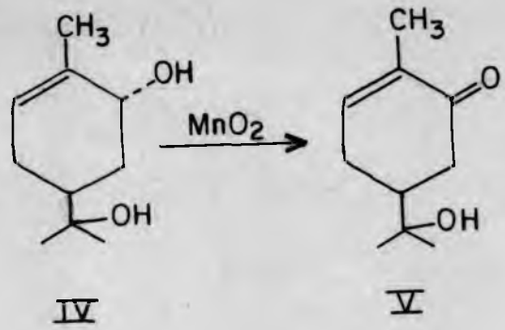
Fraction, E, distilling at 140-45°/0.6 mm. Hg and the n-butanol extract (Fraction F) yielded the same solid compound, IV. The solid analysed for two oxygen functions and I.R.spectrum (page 36) indicated the presence of a strong hydroxyl band in the region 3240 cm^{-1} and the absence of any carbonyl absorption. It showed the presence of a trisubstituted double bond (829 cm^{-1}) although it gave rather a feeble coloration with tetranitromethane in chloroform.

The elementary analysis and physical data indicated that compound, IV, may be a diol. It was finally identified as (d) trans-sobrerol on the basis of the following properties:

(a) It showed no depression in the mixed melting point with a synthetic sample of trans-sobrerol prepared by acid catalysed rearrangement of α-pinene epoxide.[43]

(b) The physical properties and I.R. spectra of IV were identical with those of the synthetic product.

(c) Both were converted to the identical ketone, hydroxycarvatonacetone (V) on oxidation with manganese dioxide in the usual manner.



IV V

Verbenone, verbenol and sobrerol have been identified amoung the autoxidation products of α-pinene. The major product of the autoxidation is, however, verbenone with only small amounts of the alcohol, whereas verbenol is the major metabolite obtained by microbiological oxidation. Furthermore, the observed inhibition of α-pinene transformation at slightly higher temperature indicates that verbenol and verbenone are likely to be true transformation products of the mould and not merely autoxidation products. The optical purity of the products indicated that they may be derived by stereospecific oxygenation processes presumably from d α-pinene. In this connection it may be mentioned that the experimental strain of A. niger hydroxylates the steroid nucleus almost exclusively at the 11 α position in about 70% yield.[32]

There was no significant difference in the rotation of the α-pinene recovered from the fermentation mixture. It may be possible that while d-α pinene was metabolised to the oxygenated intermediates, l-α-pinene was converted presumably through other products to carbon dioxide and water at the same rate by a different pathway. The possible mechanism of the formation of these oxygenated products from α-pinene will be discussed in the concluding chapter.

Under optimum conditions the yield of these products on the basis of the pinene added were 10-15%

verbenol, 2-3 % verbenone, and 2-3 % sobrerol. The major fraction of α-pinene appears to have been metabolised to smaller fragments and probably oxidised to carbondioxide and water.

EXPERIMENTAL

Purification of α-pinene

α-Pinene (B.D.H.grade) fractionally distilled at atmospheric pressure through a 2" x 4' diameter column packed with glass spiral packings and surrounded by an electrically heated jacket, was used in the different fermentation investigations. The physical properties were, b.p.155-57°/710-715 mm. Hg; $[\alpha]_D$(+)23.4°, $n_D^{25.5}$ 1.4650.

The homogeneity of the liquid was checked by vapour phase chromatography when only a single non-resolvable peak with retention time 6.5 min. was obtained. A 1.23 m. silicone column at 144° with a column pressure of 708 m.m and a flow rate of 31.66 ml./min. was employed. Nitrogen was used as the carrier gas and 0.0025 ml. sample was injected.

FERMENTATION

Determination of the optimum concentration of α-Pinene in the medium

Six separate sets of fermentations were carried out with 10 flasks each at 28°, the concentrations of α-pinene being 0.2; 0.4; 0.6; 0.8; 1.0 and 2.0 percent (V/V) in each set. Eight hours after the addition of α-pinene the cultures were extracted and the extracts were separated into unreacted α-pinene and oxygenated products by the procedure detailed in pages 46-50.

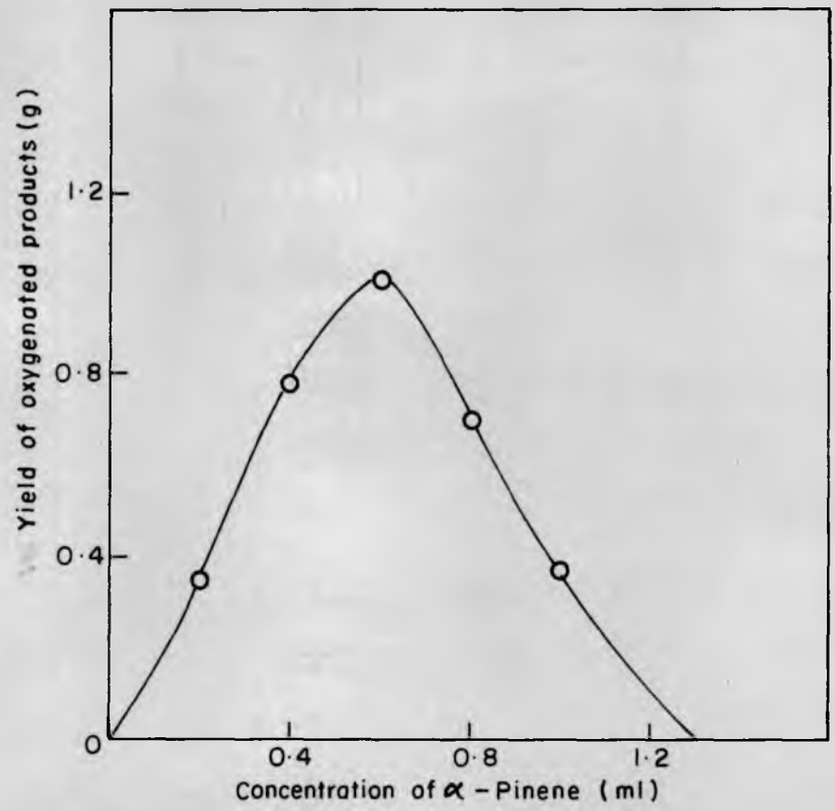


Fig. 1. EFFECT OF CONCENTRATION OF α - PINENE ON THE FORMATION OF OXYGENATED PRODUCTS

T A B L E I

Effect of concentration of α-pinene on the formation of oxygenated products.

No. of flasks	Conc/flask (ml)	Unreacted pinene (g)	Oxygenated products (g)
10	0.2	1.15	0.350
10	0.4	2.2	0.789
10	0.6	3.0	1.010
10	0.8	5.1	0.700
10	1.0	7.2	0.378
10	2.0	12.2	0.050

The results tabulated in Table I (graphically represented in Fig.1) indicate that the oxygenated products formed are proportional to the concentration of α-pinene in the medium upto 0.6 %. At higher concentrations of α-pinene, the transformation was negligible and completely ceased when 2% α-pinene was added.

Determination of the optimum period of conversion

Six sets of fermentations each with 10 flasks were carried out.

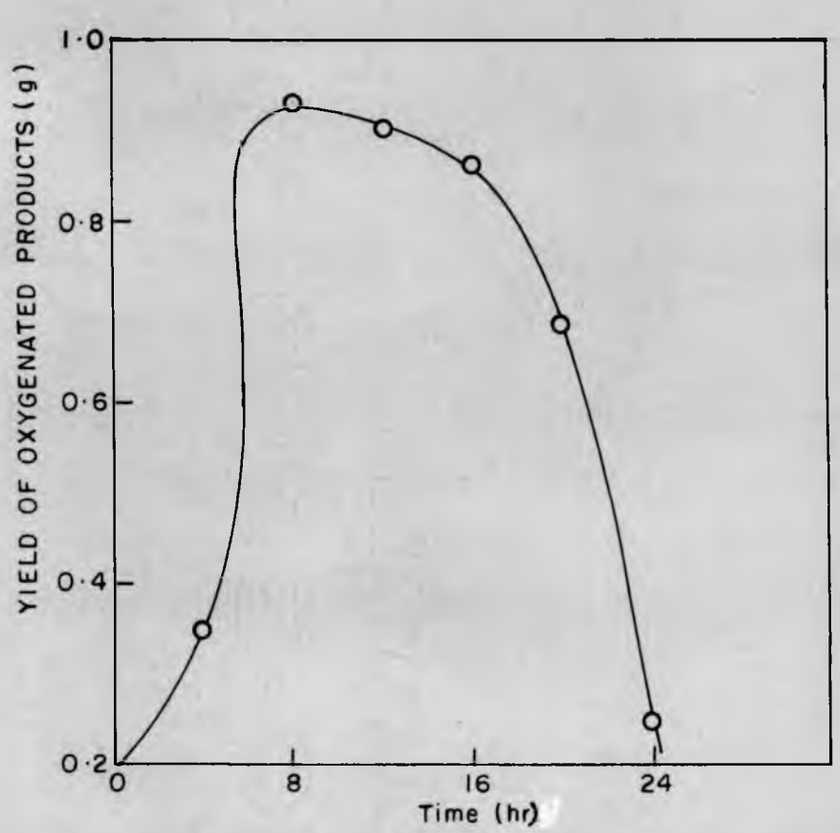


Fig.2. EFFECT OF TIME ON THE YIELDS OF OXYGENATED PRODUCTS

T A B L E II

Effect of conversion period on the yields of oxygenated products

No. of flasks	Time (hr)	Conc/flasks (ml)	Unreacted pinene (g)	Oxygenated products (g)
10	4	0.5	3.80	0.350
10	8	0.5	3.21	0.928
10	12	0.5	3.003	0.902
10	16	0.5	2.87	0.862
10	20	0.5	1.57	0.680
10	24	0.5	1.05	0.250

The results tabulated in Table II (graphically in Fig.2) indicate that the conversion is maximum at 8-hour period, giving a maximum yield and reasonable material balance. After 20 hr there was very little of both the oxygenated products and the substrate, α-pinene. Controls were run side by side and 20 to 30% loss of α-pinene due to evaporation was accounted for during 24 hours.

Determination of the optimum temperature of conversion

The conversion of α-pinene at two temperatures, 27-28^{o} and 30-31^{o} was studied.

T A B L E III

Effect of temperature on the transformation.

No. of flasks	Time (hr)	Conc/flask (ml)	Temp-erature	Unreacted α-pinene (g)	Oxygenated products (g)
10	8	0.5	28.5°	3.15	0.878
10	8	0.5	31.0°	3.9	0.087

The results tabulated in Table III indicate that above 30° the mould was inactive towards this hydrocarbon in spite of the profuse vegetative growth.

Typical fermentation (small scale)

In a typical fermentation, the spores of Aspergillus niger (612-NCIM) sporulated on PDA slants for a week at 28° were used as inoculum for 100 ml. lots of sterile Czapek-Dox medium in 500 ml. Erlenmeyer flasks. The flasks were incubated on a rotary shaker at 28-29° for 24 hours to ensure good growth. α-Pinene (0.5% V/V) purified by distillation was steam sterilised and added to the culture at this stage. The fermentation was allowed to continue for further 4 hours. A second lot of purified α-pinene was added at the end of this period and the fermentation continued for another 4 hour period.

Fifty flasks were employed per batch including ten flasks which served as two sets of controls; in one set, medium and the substrate were incubated and in the other set medium and the inoculum without the substrate were incubated. Table IV illustrates the schedule of operations.

T A B L E IV

Fermentation of α-pinene in shake cultures

Time (hr)	Stage of fermentation	Temperature	pH.
0-24	Mycelial growth	28.5°	4.8
24	Addition of α-pinene, Ist lot (0.5%)	-	-
24-28	Ist transformation period	28.5°	-
28	Addition of α-pinene, 2nd lot (0.5%)	-	-
28-32	2nd transformation period	28.5°	-
32	Harvesting the fermented broth	-	4.4

EXTRACTION

Contents of the experimental flasks (40 in no.) were pooled together and filtered. The mycelium was extracted thrice with acetone using each time an amount slightly more than the volume of the mycelium. It was further extracted consecutively with ether and n-butanol,

(three extractions with each solvent) and finally discarded. The acetone washings were then pooled with the culture filtrate.

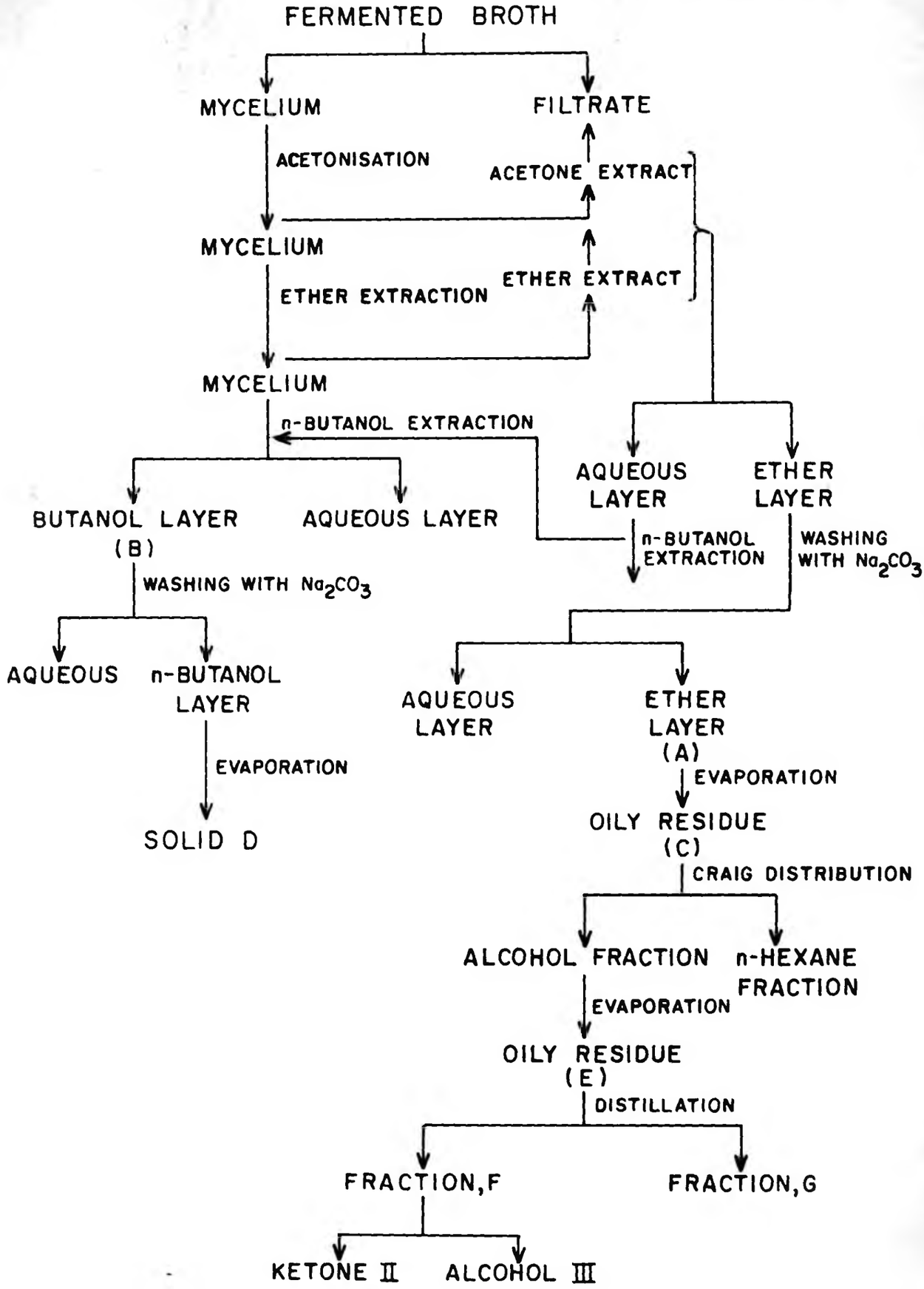
The filtrate was extracted consecutively with ether and n-butanol (three extractions with each solvent) using each time an amount equal to 1/10 of the volume of the filtrate. The extracts were pooled with the corresponding extracts from the mycelium. The acidic products from the combined ether extracts were separated by washing thrice with 5% aqueous sodium carbonate and subsequently twice with water. The ethereal layer (A) was then dried over 4 g. of anhydrous sodium sulphate per liter of solvent. Evaporation of the ether yielded an oily residue, C. (20.5 g.).

Similarly, the combined n-butanol extracts (B) were washed with a 5% sodium carbonate solution, distilled water and evaporated under reduced pressure (30-35 mm.) to yield a crystalline mass, D. (575 mg.).

The first series of controls (without α-pinene) were also filtered and extracted. The extracts from these controls were found to contain common mould fats, sterols etc., entirely different in nature from the oxygenated products obtained from the flasks containing α-pinene.

The second series of controls without the inoculum but with addition of α-pinene were also worked up in the

same manner. The added pinene could be recovered from the ether extracts as α-pinene except for losses due to evaporation (25 to 30%). The extraction procedure has been shown in the following process diagram(Scheme I).


FERMENTED BROTH
MYCELIUM
FILTRATE
ACETONISATION
ACETONE EXTRACT
MYCELIUM
ETHER EXTRACTION
ETHER EXTRACT
MYCELIUM
n-BUTANOL EXTRACTION
AQUEOUS LAYER
ETHER LAYER
BUTANOL LAYER (B)
AQUEOUS LAYER
n-BUTANOL EXTRACTION
WASHING WITH Na_2CO_3
WASHING WITH Na_2CO_3
AQUEOUS
n-BUTANOL LAYER
AQUEOUS LAYER
ETHER LAYER (A)
EVAPORATION
EVAPORATION
SOLID D
OILY RESIDUE (C)
CRAIG DISTRIBUTION
ALCOHOL FRACTION
n-HEXANE FRACTION
EVAPORATION
OILY RESIDUE (E)
DISTILLATION
FRACTION, F
FRACTION, G
KETONE II
ALCOHOL III

Large scale fermentation

A large scale fermentation was carried out with 25 liters of Czapek-Dox medium in a 40 liter certified stainless steel fermentor provided with baffles and a variable speed stirrer. The medium in the fermentor was sterilized at 120° for 45 min. and cooled to room temperature. Spores from a week old culture of the microorganism were inoculated as a water suspension through the inoculating porthole under aseptic conditions. The incubation temperature was 28° and sterile air at a rate of 500 ml/min. was passed through a sparger while stirring the medium at 200 r.p.m.

After a 24 hours growth period, steam sterilised and purified α-pinene (100 ml) was added to the culture. The agitation was continued for a further period of 4 hours. A second instalment of α-pinene (100 ml) was added at the end of this period and the fermentation allowed to proceed for another 4 hours.

After the total transformation period of 8 hours the broth and the mycelium were extracted as previously described and the extracts were concentrated.

On account of the vigorous aeration most of the α-pinene was lost during fermentation and only 3-4% of oxygenated products and 1-2% of unreacted α-pinene were recovered from the fermentation mixture.

Purification of the residues C and D

a) Fractionation of the oily residue 'C'

The oily Residue, C, (20.5 g.) obtained from the ether extracts was distributed in a modified counter-current distribution between n-hexane and 90% ethanol in 4 transfers using 75 ml. of each solvent in four consecutive stages. The ethanol and the n-hexane fractions were pooled together separately.

The n-hexane fraction gave (15 g.) unreacted α-pinene on evaporation. The pooled alcoholic layers were evaporated to 1/5 of its original volume under reduced pressure (30-35 mm.) and extracted with ether. The ether extract on drying and evaporation gave a sweet smelling yellow oily Residue, E, (3.5 g.). Residue, E, yielded two clearcut fractions on distillation under reduced pressure, (i) a colourless Oil, F, distilling at 120-125° (bath temperature)/16 mm. Hg (2.5 g.) and (ii) a highly viscous Oil, G, distilling at 145-150°/ 0.6 mm. Hg (525 mg.) which crystallised out on standing at room temperature.

b) Separation of the Fraction 'F' into Ketone (II) and Alcohol (III)

(i) Bisulphite method - Ketone, II, was separated from Alcohol, III, through its crystalline bisulphite addition product.

Fraction, F, (2.5 g.) was added to a saturated solution of sodium bisulphite in 80% ethanol (8 g. in 15 ml. 80% ethanol) with vigorous stirring for 30 min. The reaction mixture was allowed to stand for 2 hours in the cold. The bisulphite complex which separated out was filtered and Ketone, II, was liberated from the precipitate by decomposing the bisulphite complex with 30-40 ml. of cold dilute acetic acid (0.1 N). The aqueous emulsion was extracted thrice with ether. The combined ether extract was washed with 2% sodium carbonate solution, distilled water and dried over anhydrous sodium sulphate. On evaporation of the solvent a fragrant oil was obtained (500 mg.) which was further purified by distilling under reduced pressure 120-125° (bath temperature)/16 mm. Hg.

The turbid filtrate from the precipitated bisulphite was diluted with water and extracted three times with 25 ml. portions of ether. The ethereal extracts were dried over anhydrous sodium sulphate and evaporated to yield 1.5 g. of Alcohol, III. The product was purified by distillation under reduced pressure, 125-130°/14 mm. Hg.

(ii) Chromatography - Fraction, F, (2 g.) was chromatographed over 50 g. of neutral, grade II alumina (acid treated, water washed and heated at 140° for 6 hours) and was eluted using 100 ml. portions of n-hexane,

n-hexane + 5% benzene; n-hexane + 10% benzene; n-hexane + 50% benzene; benzene; benzene + 5% ether; benzene + 10% ether; benzene + 50% ether; ether; ether + 10% ethanol; ether + 50% ethanol and ethanol.

This procedure resolved Fraction,F ,into Ketone,II, and Alcohol, III. The various fractions were combined on the basis of weight curve for further purification as shown in Fig.3.

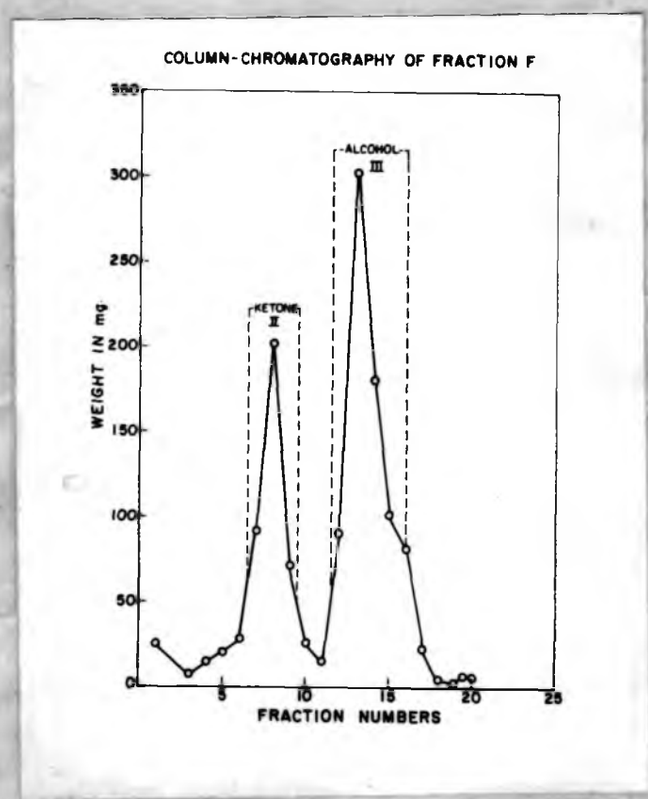


Fig. 3.

T A B L E V

Properties of Ketone, II, and Alcohol, III.

	Ketone, II	Alcohol, III
Molecular formula	$C_{10}H_{14}O$	$C_{10}H_{16}O$
Microanalysis		
Found:	C, 79.54; H, 9.21%	C, 78.99; H, 10.41%
Calcd:	C, 79.95; H, 9.39%	C, 78.89; H, 10.59%
b. p.	110°/16 mm.Hg	112°/14 mm. Hg
$[\alpha]_D$	+ 223° (C, 9.2)	+ 64° (C, 17.4)
$n_D^{25.5}$	1.4960	1.4929
u. v.	max 253 mu log 3.81	-
I. R.	ν_{max} 1658 cm^{-1} for C=O; ν_{max} 1613 cm^{-1} for conjugated double bond	ν_{max} 3360 cm^{-1} for OH absorption.
Derivatives:	Semicarbazone m.p. 203-205°	p-Nitrobenzoate m.p. 88-90° $[\alpha]_D$ + 17° (ethanol).

The semicarbazone of Ketone, II, was prepared by refluxing an aqueous alcoholic (80%) solution of 500 mg. of semicarbazide hydrochloride and 1 g. of sodium acetate with 100 mg. of Ketone, II, on a water bath for 45 to 60 min. On evaporation of excess of alcohol, crystals of semicarbazone separated which melted at 203-205° after repeated crystallisation from ethyl acetate.

Analysis Found: C, 63.29; H, 8.02; N, 19.69%

Calcd. for $C_{11}H_{17}ON_3$ C, 63.74; H, 8.27; N, 20.27%.

u. v. λ_{max} 275 mμ, log ε 4.27.

p-Nitrobenzoate of Alcohol, III, was prepared by refluxing 500 mg. of Alcohol, III, and 1 g. of freshly prepared p-nitrobenzoyl chloride in presence of 5 drops of pyridine for 30-45 min. on a steam bath. A few drops of water were added to the mixture and after standing for 15 min. at room temperature it was stirred into 10 ml. of 5% sodium bicarbonate solution. The precipitated solid was filtered, washed with a little aqueous sodium bicarbonate then with distilled water and sucked dry. The crude derivative was recrystallised from n-hexane to the constant m.p. 88-90°; ($_D$ + 17°; C, 27.75).

Characterisation of Ketone, II

From the physical properties of Ketone, II, and its semicarbazone it was suspected that it might be identical with d verbenone. Verbenone was then synthesised according to Badoche[40] by autoxidation of α-pinene in the presence of chromium trioxide as catalyst.

Humid oxygen was bubbled through a mixture of 20 g. of α-pinene and 1 g. pulverized chromium trioxide contained in a 500 ml. Erlenmeyer flask with continuous stirring for a period of 10 days, when further absorption of oxygen ceased.

The reaction mixture on extraction with chloroform and subsequent evaporation yielded a highly viscous, colored oil (18 g.). This on further steam distillation yielded a colorless liquid (15 g.). The ketone, verbenone was separated from this liquid as asemicarbazone (3.6 g.) melting at 205-207°. Hydrolysis of this semicarbazone by dilute hydrochloric acid gave d-verbenone quantitatively (3 g.; $[\alpha]_D$ + 121°).

Ketone, II, and verbenone were found to be identical in every respect including comparative I.R. spectra and thus, II was characterised as d-verbenone. There was no depression in the mixed melting point of the semicarbazones (204-206°).

Characterisation of Alcohol, III

From the physical properties, Alcohol, III, was identified as d-cis-verbenol. The identity was conclusively established by oxidising it to the known ketone, verbenone.

Oxidation of Alcohol, III, to Verbenone

(i) Manganese dioxide oxidation - A solution of Alcohol, III, (500 mg) in chloroform was stirred with active manganese dioxide for 24 hours at room temperature (27-28°). The manganese dioxide was removed by filtration and washed thrice with hot chloroform. Removal of the solvent from the filtrate gave a yellowish, oily residue (400 mg.) which was purified by distillation under reduced pressure, 140-145° (bath temperature)/16 mm. Hg.

Analysis	Found:	C, 79.74; H, 9.47 %.
	Calcd.for $C_{10}H_{14}O$	C, 79.95; H, 9.39 %.
u. v.	λ_{max} 252.5 mμ	log ε 3.832.

The product was identified as d-verbenone by a comparative I.R. spectra with Ketone, II.

(ii) Chromic acid oxidation - Alcohol, III, (100 mg.) was added to a complex of 50 mg. of chromium trioxide in 5 drops of pyridine. The mixture was kept overnight at room temperature (28-29°) and decomposed

with a little water. It was then steam distilled in a semi-micro steam distillation apparatus when d- verbenone (40 mg) was obtained.

Analysis	Found:	C, 79.80; H, 9.51 %
	Calcd. for $C_{10}H_{14}O$	C, 79.95; H, 9.39 %.

Characterisation of Fraction, D (G)

The solid compound from Fractions D (575 mg) and G (500 mg) was chromatographed over 30 g. of neutral alumina (acid treated, water washed and activated at 140° for 6 hours). The column was developed with 100 ml. portions of solvents in a sequence previously described for the fractionation of Fraction, F. Benzene chloroform mixture (1:1) eluted a white, needle-shaped, crystalline solid (IV; 800 mg.) which was dissolved in 10 ml. of hot benzene and filtered. After standing at 0° overnight, 562 mg. of Compound, IV, was obtained, m.p. 140-145°. Recrystallisation from 10 ml. of benzene yielded the pure compound (475 mg), m.p. 145-147°. Physical properties of Compound, IV, are listed in Table VI.

T A B L E VI

Physical constants of Compound, IV.

	Compound IV
Molecular formula	$C_{10}H_{18}O_2$
Microanalysis:	
Found	C, 70.66; H, 10.7%
Calcd.	C, 70.54; H, 10.66%
m. p.	145-47°
$[\alpha]_D$	+ 152°(C, 12.8)
I.R.	ν_{max} 3240 cm^{-1} - OH absorption
Tetranitromethane test in chloroform	very feeble

The I.R. spectrum and microanalysis indicated that Compound, IV, has two hydroxyl groups. To establish the identity it was necessary to obtain sobrerol by an acid catalysed rearrangement of α-pinene epoxide.

Preparation of authentic sobrerol

a) Epoxidation of α-pinene to pinene epoxide

α-Pinene (5 g.) was added dropwise to freshly prepared monoperphthalic acid solution (20 g. in 100 ml. of ether) with stirring at 0° and the resulting mixture was kept at 4° until the reaction was complete (48 hours)

as indicated by iodometric titration. (The amount of peracid in the reaction mixture was determined by adding 2 ml. aliquot of the ethereal solution to 30 ml. of 20% potassium iodide and titrating the liberated iodine after 10 min. with 0.1 N sodium thiosulphate).

The solution was filtered and the residue as well as the filtrate were extracted thrice with ether. The ether extract was then washed with aqueous sodium carbonate, distilled water and dried over sodium sulphate. On removal of the solvent α-pinene epoxide (2.85 g.) was obtained which was purified by distillation under reduced pressure, b.p. 78-80°/ 20 mm., $[\alpha]_D$ + 51° (C, 20.72).

b) Acid catalysed rearrangement of α-pinene epoxide

α-Pinene epoxide (2 g.) was stirred for 15 min. with 10 ml. of 0.1 N sulphuric acid. The mixture warmed spontaneously and formed crystals of sobrerol immediately. n-Hexane (5 ml.) was added to the cooled mixture to facilitate separation of sobrerol from the liquid. The diol (1.5 g.) was recovered by filtration and purified by repeated crystallisation from benzene to a constant melting point 145-47°. The mixed melting point of sobrerol with Compound, IV, was undepressed and the I.R. spectra of the two samples were identical.

Manganese dioxide oxidation of Compound, IV

A solution of the solid Diol, IV (200 mg.) in 50 ml. of chloroform was stirred for 24 hours at room temperature with 20 g. of active manganese dioxide. The solid catalyst was removed by filtration and the ketone hydroxycarvatonacetone (V) was recovered after removal of the solvent, λ_{max} 235 mμ log ε 3.97.

The synthetic sobrerol also yielded an identical product by manganese dioxide oxidation.

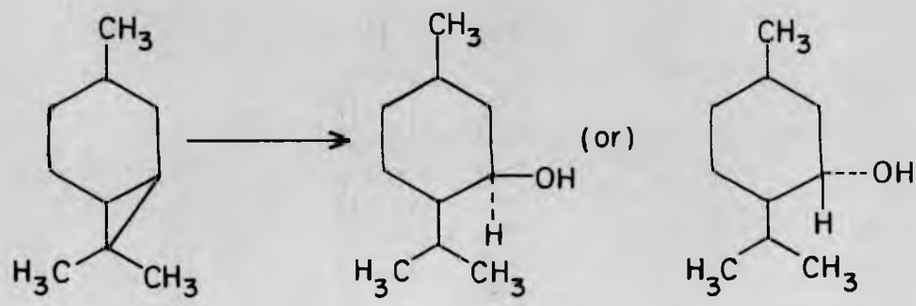
C H A P T E R V

TRANSFORMATION OF Δ^3-CARENE, CARANE AND HUMULENE

Transformations of Δ^3-Carene, Carane and Humulene

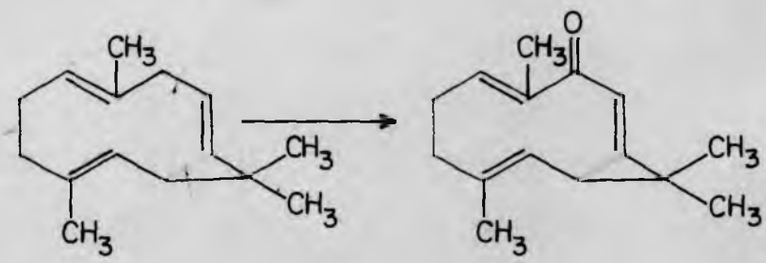
DISCUSSION

In the preceeding chapter the stereospecific conversion of α-pinene into oxygenated products such as cis-verbenol, verbenone and trans-sobrerol by A.niger was described. It was considered necessary to determine whether α-pinene alone is uniquely oxygenated microbiologically, or if such transformations are also possible with other terpenoid hydrocarbons. The action of the experimental strain of A.niger (612) on Δ^3-carene (I), carane (II) and humulene (III) was, therefore, studied. The studies on carane and Δ^3 carene had, moreover, the objective that a biological hydroxylation of the compounds (I and II) at position 6, i.e., at the cyclopropane and cyclohexane ring juncture, could possibly establish a convenient synthesis of the pharmaceutically important compound, menthol (IV) or its isomer, isomenthol (IVa).



II IV IV a

Encouraged by the finding that allylic oxygenation was one of the chief microbiological transformations of α-pinene, the large ring sesquiterpene hydrocarbon, humulene was subjected to the action of the mould with the anticipation that oxygenation at position allylic to both the double bonds would provide a pathway to zerumbone (V). It should be mentioned here that a convenient chemical synthesis of zerumbone from humulene has not been worked out as yet.



V

Δ^3-Carene (I), $C_{10}H_{16}$ is distributed fairly wide in nature and occurs in turpentines from Pinus longifolia and various other sources.[44,45] Simonsen and his collaborators[44] were the first to isolate this hydrocarbon as a fraction distilling at 165-70° from turpentine. After extensive studies the constitution of carene was established by a number of investigators.[46-48]

The occurrence of the saturated bicyclic hydrocarbon, carane, $C_{10}H_{18}$, has not been reported as yet in natural sources. Carane was prepared by Krestinski and

Solodki[49] by catalytic hydrogenation of Δ^3-carene. It has also been obtained by distillation of pulegone hydrazone with potassium hydroxide by Kistner and Zawadrosky.[50]

Fermentation conditions and extraction procedures employed were essentially the same as those used for α-pinene. Compared to α-pinene both Δ^3-carene and carane were found to be resistant to oxygenation by the experimental strain of A.niger.

In the case of Δ^3-carene, however, 0.5-1% of an oxygenated product was isolated by solvent extraction, solvent-solvent distribution and distillation from the 72-hours fermentation mixture. This liquid showed a weak C=O absorption in the I.R. spectrum. Further studies were not carried out since the yields were very low and in some batches none of the product could be isolated.

In the case of carane the substrate could be recovered unchanged from the fermentation mixture, even after a prolonged period of 72 to 96 hours of fermentation, (amounting for losses due to evaporation). I.R.spectra of the crude oily fraction obtained after fermentation indicated the complete absence of any hydroxyl or carbonyl functions.

These studies would suggest that the presence of the cyclopropane ring in Δ^3-carene and carane probably

prevents the microbial oxidation of the hydrocarbons by the strain of A.niger , even though these hydrocarbons are readily autoxidised, both in air and in oxygen.[51] Whether this resistance is generally true for other fungal strains can only be determined after a wider screening of several microorganisms.

The sesquiterpene hydrocarbon, humulene(III), $C_{15}H_{24}$, was first isolated from oil of hops (Humulus lupulus) by Chapman[52] and was found later by Bhattacharyya and coworkers[53] in the oil of wild ginger (Zingiber zerumbet). Its structure has been established conclusively by Šorm[54] and Sukh Dev[55] on the basis of physicochemical evidence.

This hydrocarbon was also found to be relatively resistant towards A.niger . On prolonged fermentation (72 hours) 0.2-0.5% of a solid alcohol (m.p.140-43°) was obtained from the neutral fraction. However, further work on this hydrocarbon was not taken up since it was observed that under the acidic conditions of fermentations namely at pH 4.5, the hydrocarbon was susceptible to rearrangement even in the absence of the mould.

EXPERIMENTAL

The fermentations were carried out in batches of forty shake flasks at a time. Δ^3-carene, carane or humulene was added in concentrations of 0.5 ml/100 ml., 0.5 ml/100 ml. and 0.2 ml/100 ml., respectively, to each of the forty flasks containing a 24 hours culture of A.niger in 100 ml. Czapek-Dox medium enriched with 0.5% corn steep liquor at pH 4.5. The mixture was incubated at 28° for 24-72 hours depending upon the rate of conversion. After the necessary transformation period, the fermentation mixture was filtered and the mycelium and the broth were extracted with ether and n-butanol as described in the previous chapter. The n-butanol fraction on evaporation yielded 0.5 to 0.7% of products which consisted mainly of normal mould products.

Oxygenated products from Δ^3-carene and carane

The concentrates after removal of ether did not show any appreciable change in the I.R.spectra with those of the starting hydrocarbons. However, in the case of Δ^3-carene, 0.5-1% of a viscous high boiling liquid (VI) was obtained after fractional distillation of the concentrate.

I.R.spectra: 1653 cm^{-1}(weak band) and a shoulder at 1613 cm^{-1} (α-β unsaturated C=O).

Oxygenated product from humulene

The ether extract from the pooled cultures to which humulene was added after washing with aqueous sodium carbonate, water and drying (7.1 g.) was chromatographed over (200 g.) neutral alumina (grade II). The column was developed with petroleum ether (60-80°), benzene and chloroform. Unreacted humulene was obtained from the petroleum ether fraction. The benzene and chloroform mixture eluates gave 32 mg. of a solid alcohol which was recrystallised from petroleum ether to the constant melting point, 140-43°.

The product was probably an artifact due to an acid catalysed cyclisation of humulene, since a similar product was obtained in traces from control flasks.

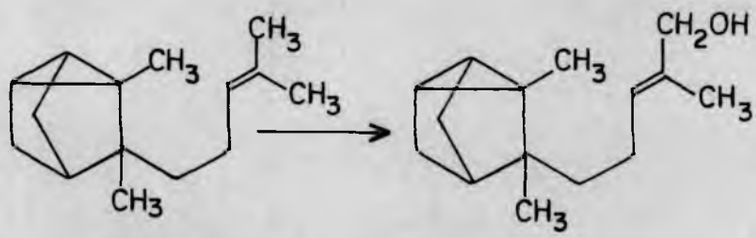
CHAPTER VI

TRANSFORMATION OF α-SANTALENE

Transformations of the Sesquiterpene Hydrocarbon-α-Santalene

DISCUSSION

In the purification of sandalwood oil, 10-15% of the essential oil from the plant is obtained as hydrocarbons of little or no perfumery value, α-santalene comprising 40-50% of this hydrocarbon fraction. Since it has been established from work reported in Chapter IV that a hydroxyl group could be introduced in a position allylic to a double bond atleast in case of α-pinene, it seemed desirable to find out whether α-santalene (I) could be converted by this strain of A.niger to the commercially useful allylic alcohol, α-santalol (II).



I II

It should be mentioned here that a chemical transformation of this hydrocarbon to α-santalol has not been reported yet in literature.

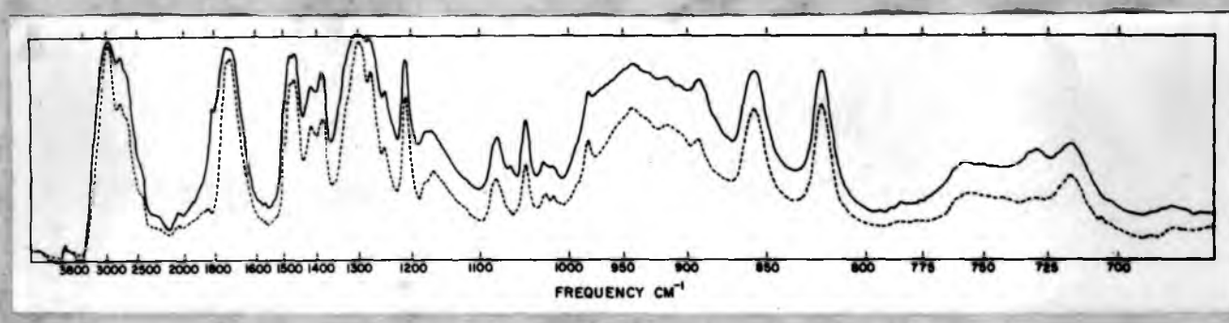
The sesquiterpenic hydrocarbon, α-santalene(I), $C_{15}H_{24}$, is one of the constituents of East Indian sandalwood

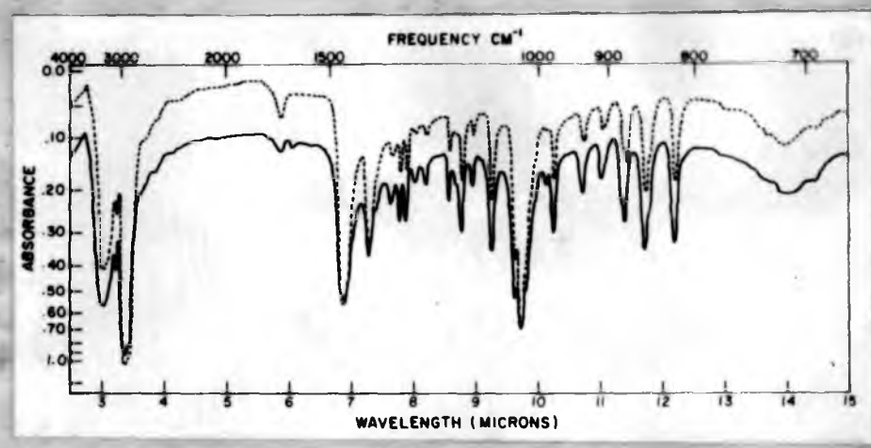
oil, obtained from the wood of Santalum album.[56] It is tricyclic and a derivative of tere-santalic acid. Its constitution has been conclusively established by the extensive investigations of Semmler and collaborators.[56]

The general procedure and the techniques for the fermentation and the extraction processes were similar to those already mentioned in the preceeding chapters.

Briefly, incubation of A.niger with α-santalene after a 30-hour growth period, followed by extraction of the culture filtrate and the acetonised mycelium with ether yielded a mixture of both acidic and neutral products. The acidic products were removed from the neutral as the soluble sodium salt which on further acidification, extraction and solvent distillation yielded a brownish solid mass (III), which was difficult to crystallise from the usual organic solvents. Purification by the sublimation technique was, therefore, resorted to.

Compound, III, obtained pure (m.p. 152-54°) after repeated sublimation in vacuo at 140-45° (bath temperature)/30-35 mm. pressure, analysed for $C_{10}H_{14}O_2$. The I.R.spectrum indicated the presence of a carboxyl group (2660 cm^{-1} and 1706 cm^{-1}).





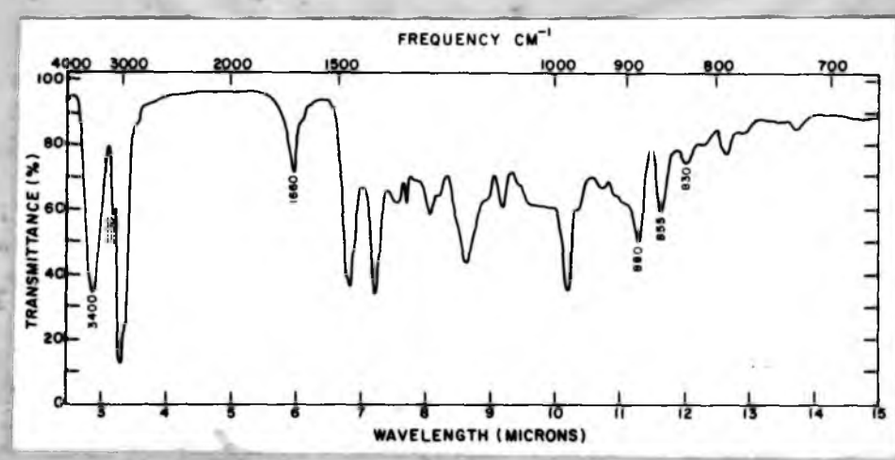


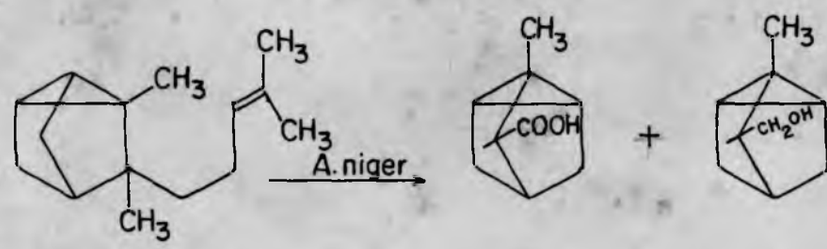
Figure 1

(a) Comparative infrared spectra of isolated tere-santalic (---) acid with that of authentic sample (——).

(b) Comparative infrared spectra of isolated tere-santalol(- - -) with that of synthetic tere-santalol(——).

(c) Infrared spectra of the liquid alcohol, V.

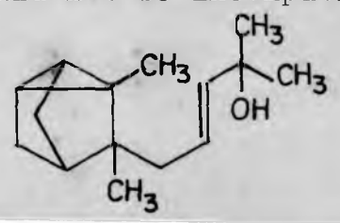
From these characteristics, it was possible to identify Compound, III, as tere-santalic acid, by comparing its physical constants and I.R. spectrum with those of an authentic sample. The mixed melting point of both the samples was undepressed.



I III IV

The neutral fraction from the fermentation mixture yielded a small amount (0.5-1%) of solid alcohol, $C_{10}H_{16}O$ (IV) which was identified as tere-santalol by its mixed melting point with an authentic sample and by comparative I.R. spectra.

The neutral fraction further yielded a liquid C_{15} alcohol (1-2%) which indidated the presence of a hydroxyl group (3400 cm^{-1}), a double bond (1660 cm^{-1}) and a tricycline system (855 and 880 cm^{-1}) in the I.R. spectrum. Eventhough the microanalytical data were in good agreement with those of α-santalol (II), the I.R.spectrum showed marked differences in the finger print region. From these data the tentative conclusion was made that the hydroxylation is on the terminal double bond in the side chain leading to a tertiary alcohol. A probable structure which would not be incompatible with the physical data is:



The above experiments were started with the intention of converting α-santalene to α-santalol. No trace of α-santalol could be detected amongg the transformation products. Perhaps, A.niger may not be a suitable organism for this conversion and a study of vafious other microorganisms in this respect may yield the desired result.

EXPERIMENTAL

The fermentation and extraction procedures were the same as those used for α-pinene. Forty 500ml. Erlenmeyer flasks, each containing 100 ml. growth medium were inoculated with A.niger spores and incubated on a rotary shaker at 27.5°. After a 30-hours growth period, α-santalene 121-123°/ 10 mm. Hg $[\alpha]_D$ + 10.8° was added (0.2 ml/100 ml. medium) and the transformation was allowed to proceed for 24 hours.

The contents of the forty flasks were pooled, filtered, the mycelium was acetonised and the mycelium and the filtrate were extracted with ether. The ethereal extracts were washed with 5% sodium carbonate solution to separate neutral and acidic components from the extracts. The ether extract was washed with distilled water, dried over anhydrous sodium sulphate and the solvent distilled off to give a residue (A1) (4.25 gm.), which was further fractionated into different fractions by chromatography on grade II alumina.

The aqueous fraction was acidified with concentrated hydrochloric acid, extracted with chloroform, washed with distilled water and dried over sodium sulphate. On evaporation of the chloroform extract a solid mass (III) (825 mg.) was obtained.

Solid, III, was best purified by sublimation at 140-145° under water pump pressure (30-35 mm.) to give the pure product (705 mg.) with a constant melting point, 152-154°. The physical characteristics are shown in Table I.

T A B L E I

Properties of Solid, III

	Solid III
Molecular formula	$C_{10}H_{14}O_2$
Microanalysis:	
Found	C, 71.86; H, 8.5%
Calcd.	C, 72.26; H, 8.49%
m. p.	152-54°
neutral equivalent	161(± 3)
$[\alpha]_D$	— 63.17° (C,3.32 in benzene)
I.R. spectrum	ν_{max} 2660 cm^{-1}
	ν_{max} 1706 cm^{-1}

From microanalysis, I.R.spectra and the neutral equivalent data the solid Compound, III, was identified as tere-santalic acid. Mixed melting point with an authentic sample was undepressed (152-54°).

Separation and identification of the neutral fraction

Residue, A_1 (4.25 g.) was adsorbed on a neutral Grade II alumina column (175 g.) and was eluted with graded solvent mixtures. n-Hexane, benzene:ether(1:1) mixture and ether eluted out unreacted α-santalene(I) (3.25 g.), solid alcohol (IV) (25 mg.) and a liquid alcohol (V) (100 mg.), respectively.

The solid Alcohol, IV, (25 mg.) was purified by sublimation at 90-95° under water pump pressure(30-35 mm) to the constant m.p. 108-110°.

I.R. spectra: ν_{max} 3300 cm^{-1} -OH- absorption.

Compound, IV, was identified as tere-santalol by comparing its I.R.spectra and melting point with those of an authentic sample prepared by lithium aluminium hydride reduction of tere-santalic acid.

The liquid Alcohol, V,(100 mg.) purified by distillation under reduced pressure (140-45°/6 mm.) had the following characteristics:

Molecular formula		$C_{15}H_{24}O$
Analysis:	Found	C, 81.24; H, 11.29%
	Calcd.	C, 81.76; H, 10.98%.
b.p.		140-45°/6 mm.
tetranitromethane test in chloroform.		positive

I.R.spectra: ν_{max} 3400 cm^{-1}(OH absorption); 1660 cm^{-1}(tri or tetrasubstituted double bond); 855 & 880 cm^{-1}(a tricycling ring system).

C H A P T E R VII

TRANSFORMATION OF CAMPHENE AND β-SANTALENE

Transformations of Camphene and β-Santalene

DISCUSSION

Camphene, (I), $C_{10}H_{16}$, is the only crystalline bicyclic monoterpene hydrocarbon occurring in nature. The l-isomer was first isolated by Gobolov[57] from the essential oil Abies sibiria, the racemic and the two optical isomers were later shown to be widely distributed in nature.[58]

The bicyclic sesquiterpene, β-santalene, II, $C_{15}H_{24}$, occurs in East Indian sandalwood oil, obtained from the wood of santalum album.[56] This isomer always occurs together with the α-isomer and can be separated from the latter by repeated fractional distillation through an efficient column.

The fermentations were usually carried out by adding the substrates (100 mg camphene/100 ml. medium and 180 mg β-santalene/100 ml. medium) to a 30-hour culture of the fungus. A 24 hour transformation period was allowed and the oxygenated products were isolated by the usual solvent extraction procedure as described in the previous chapters.

With camphene as the substrate, the experimental strain of A.niger produced two new compounds (a) a neutral

compound (III) in 5-7% yield and (b) an acidic compound (IV) in 30% yield. It is interesting to note that the same acid (IV) was also obtained from the fermentation of β-santalene in 35% yield. However, in this case no other neutral compound could be isolated.

The crude Acid, IV, could be isolated only as its corresponding Anhydride, V, since the free acid was highly unstable even under mild conditions. Anhydride, V, was purified by distillation under reduced pressure (80-88°/3 mm.).

Controls run under strictly comparable conditions without the addition of camphene or β-santalene did not yield any of the transformation products, but only small amounts of high boiling substances possibly mould acids, which solidified on standing at room temperature. The latter could be eliminated by fractional distillation taking advantage of its high boiling point in the region of 180-190°/3 mm. On the other hand, the transformation product distilled in the region 80-88°/3 mm. and remained as a liquid. Thus the latter could be isolated free from contaminating mould products.

Structure of the liquid Anhydride, V

The constitution of Anhydride, V, was established by oxidative degradation and by its eventual synthesis.

Compound, V, analysed for $C_{11}H_{16}O_3$. The neutral equivalent, determined by dissolving in sodium hydroxide followed by titration with standard acid, was 96 (± 2). The compound was optically inactive and found to be unsaturated as shown by decolorisation of bromine solution in carbon tetrachloride and a weakly positive tetranitromethane test.

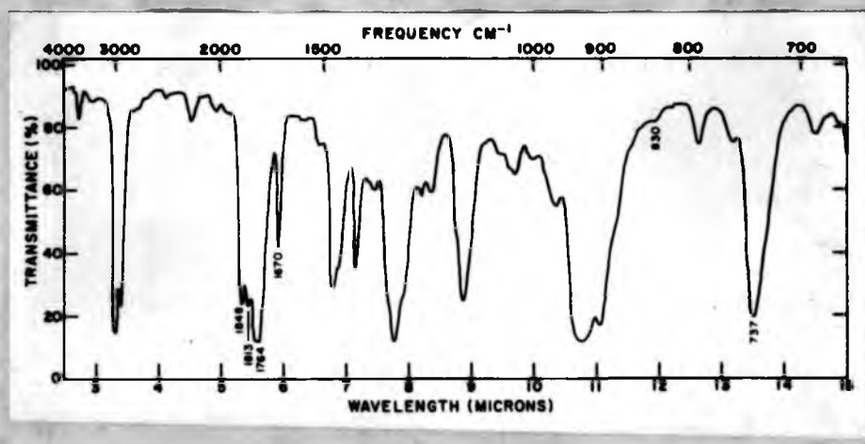
The I.R.spectra indicated the presence of

(i) a five membered ring anhydride (1848 cm^{-1}, 1813 cm^{-1} and 1764 cm^{-1});

(ii) a tri or tetra substituted double bond(1670 cm^{-1} and a weak band at 830 cm^{-1}) possibly conjugated with a carbonyl system, and

(iii) a straight chain containing at least four methylene groups (735 cm^{-1}).

The u.v. spectra (λ_{max} 249 mμ, log ε 3.81; end absorption, λ_{max} 213 mμ, log ε 4.21) also supported the existence of a tri or tetra substituted double bond in conjugation with the carbonyl system. In agreement with this conclusion, the liquid anhydride failed to consume perbenzoic acid showing that the double bond is inert to acid catalysed epoxidation due to conjugation with the carbonyl group of the anhydride moiety.



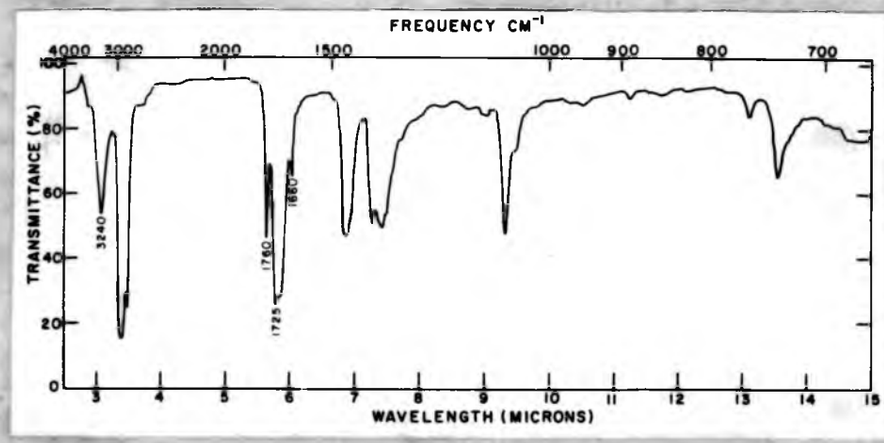


Figure 1

(a) Infrared spectra of Anhydride, V (as liquid film).

(b) Infrared spectra of Imide, VIII (in Nujol mull).

Kuhn-Roth determination indicated the presence of at least two C.methyl groups. On catalytic hydrogenation in glacial acetic acid in the presence of platinum oxide catalyst, Liquid, V, absorbed one mole of hydrogen and yielded a saturated liquid, VI, ($C_{11}H_{18}O_3$). The absence of 1670 cm^{-1} absorption in the I.R.spectra and negative tetranitromethane test indicated that the double bond had disappeared. The liquid Anhydride, V, on the other hand, failed to absorb any hydrogen when hydrogenation was carried out in alcohol medium showing that the double bond is placed in a hindered and rigid position.

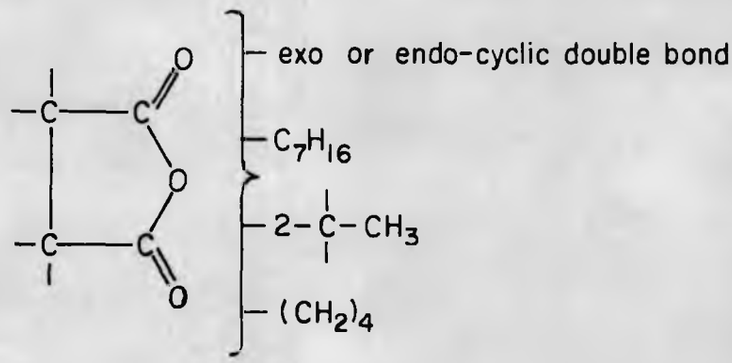
Attempts to dehydrogenate Compound, V, with selenium were unsuccessful indicating the absence of any 6-membered cyclic system in the liquid anhydride.

On reduction with excess of lithium aluminium hydride, the liquid anhydride,(V) yielded a liquid diol, $C_{11}H_{22}O_2$, (VII) still containing one double bond, (positive tetranitromethane test and bands at 1670 cm^{-1} and 829 cm^{-1} in the I.R. spectra).

Compound, V, gave a mono-2,4-dinitrophenyl-hydrazone (m.p. 148-50°) which showed strong ultraviolet absorption at 325 mu characteristic of non-conjugated phenyl-hydrazones showing that either the carbonyl reacting with phenyl hydrazine is not in conjugation

with the double bond or, the double bond may have shifted out of conjugation during the reaction.

A partial structure can be assigned to Anhydride, V, on the basis of the above experimental evidence.



Partial structure of Anhydride, V

The position of the double bond and the paraffin side chain and the nature of the five membered cyclic ring system were further determined by ozonolysis studies on both the anhydride and its crystalline derivative, Imide, VIII.

Anhydride, V, yielded an ammonium salt on treatment with aqueous ammonia which on distillation under reduced pressure afforded a crystalline Imide, VIII, ($C_{11}H_{17}O_2N$; m.p. 60-61°). The same imide was obtained in somewhat poorer yields but in a purerform by heating the anhydride with urea. Compound, VIII, showed characteristic I.R.absorption(p. 78) of a five membered ring

imide (3140 cm^{-1}; 1760 cm^{-1} and 1725 cm^{-1}) and the absence of any trisubstituted double bond at 820-840 cm^{-1} region.

On saponification and acidification, the pure imide (VIII) yielded Anhydride, V. The similarities of the physical constants and superimposability of the I.R.spectra of the original anhydride and that derived from the imide indicated conclusively the homogeneity of the original liquid anhydride,(V) and that no basic structural rearrangements had occurred during the treatment.

Ozonolysis of Anhydride, V, resulted in the formation of a mixture of neutral and acidic carbonyl compounds which were converted to their 2:4-dinitrophenylhydrazones. From the acidic fraction, 2:4-dinitrophenylhydrazone of keto-caprylic acid, IX, (128-30°) was isolated and identified by microanalysis, melting point, mixed melting point and comparative I.R.spectra with an authentic sample.

Ozonolysis of the imide, VIII, afforded a stable crystalline Ozonide, X, (m.p. 46-48°) which showed the presence of an ozonide system (1056 cm^{-1}) in I.R.spectra.

Criegee[59] has shown after studying more than 20 ozonides of various types that this peak is due to C=O stretching frequency of the ozonide system and has

also reported that five membered substituted cyclic imides form stable ozonides.

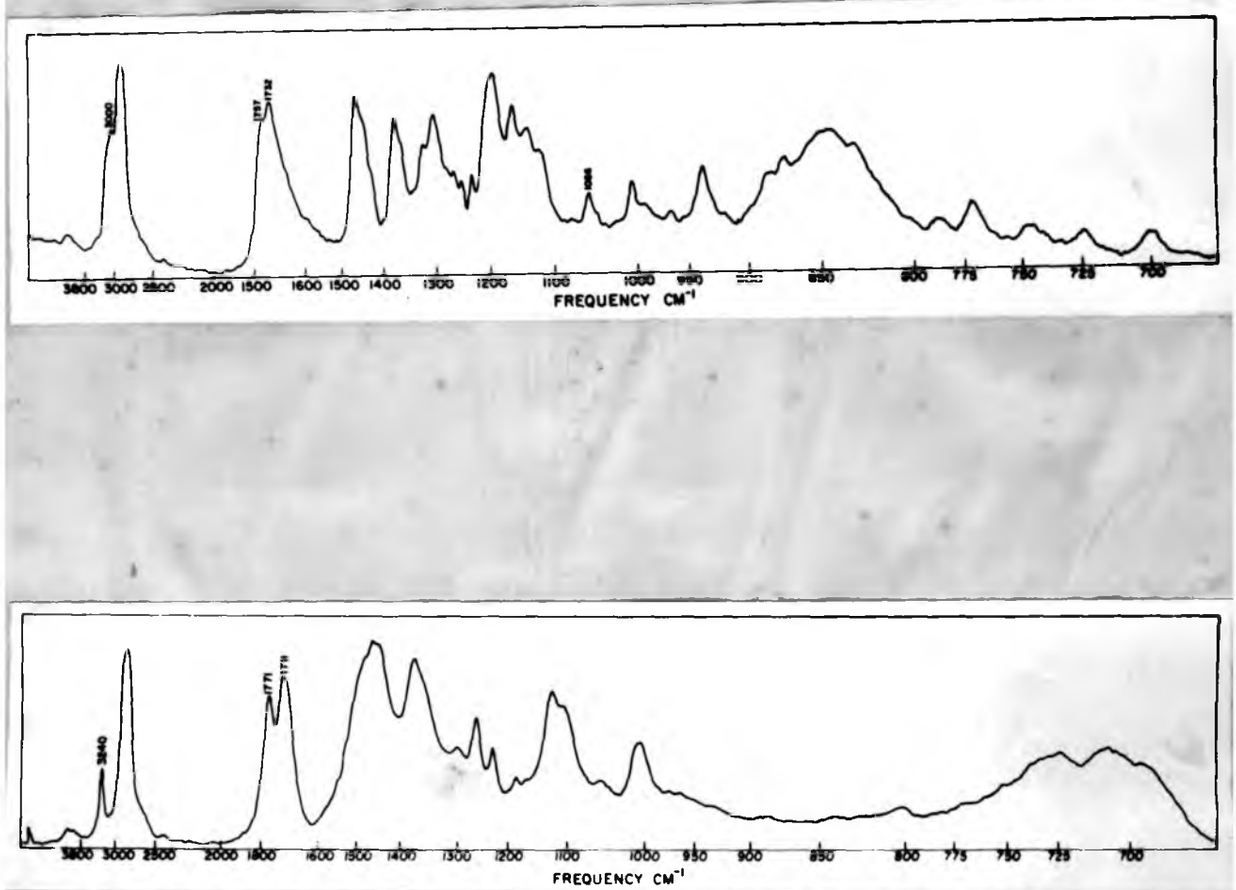


Figure 2

(a) Infrared spectra of Ozonide, X (in nujol mull).

(b) Infrared spectra of Diketoimide, XI (in nujol mull).

Owing to the relatively greater stability of this ozonide, milder methods of decomposition such as addition of water, reduction with zinc and hydrochloric acid proved unsuccessful. More drastic methods of reductive decomposition had, therefore, to be resorted to. On hydrogenation with active palladium-calcium carbonate the ozonide yielded Diketo imide, XI, ($C_{11}H_{17}O_4N$; m.p. 92-95°). The I.R.spectrum showed the presence of -NH($3240\ cm^{-1}$); CO-NH-CO ($1761\ cm^{-1}$) and C=O ($1711\ cm^{-1}$) groupings in the molecule.

On alkaline hydrolysis Compound, XI, gave rise to α-ketocaprylic acid (IX) and pyruvic acid, both identified through their 2,4-dinitrophenylhydrazones and comparative paper chromatography with authentic samples.

Ozonolysis of anhydride and imide

Anhydride, V

$$C_{11}H_{16}O_3 \xrightarrow{O_3} CH_3(CH_2)_5\overset{\overset{O}{\|}}{C}\text{-}COOH$$

+ Other products

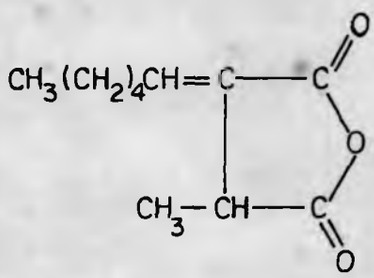
Imide, VIII

$$C_{11}H_{17}O_2N \xrightarrow{O_3} C_{11}H_{17}O_5N \ (46-48^\circ) \xrightarrow{H_2(Pd)} C_{11}H_{17}O_4N \ (92-95^\circ) \xrightarrow{HOH}$$

$$CH_3(CH_2)_5\overset{\overset{O}{\|}}{C}\text{-}COOH + CH_3\text{-}\overset{\overset{O}{\|}}{C}\text{-}COOH$$

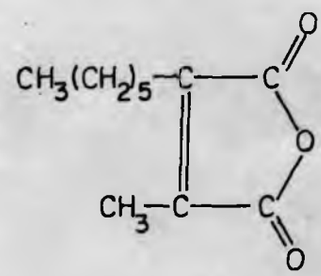
Bhattacharyya and coworkers[60] have reported that Anhydride, V, on chromic acid oxidation give a mixture of acids which could be separated and identified as caproic (XII), heptanoic (XIII), acetic (XIV) and propionic (XV) acids.

In view of the above experimental evidence the liquid Anhydride, V, and the solid Imide, VIII, can be represented by structures V, or Va and VIII, respectively.

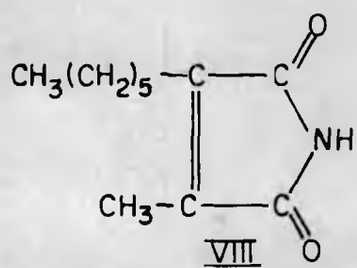


Va

(or)



V

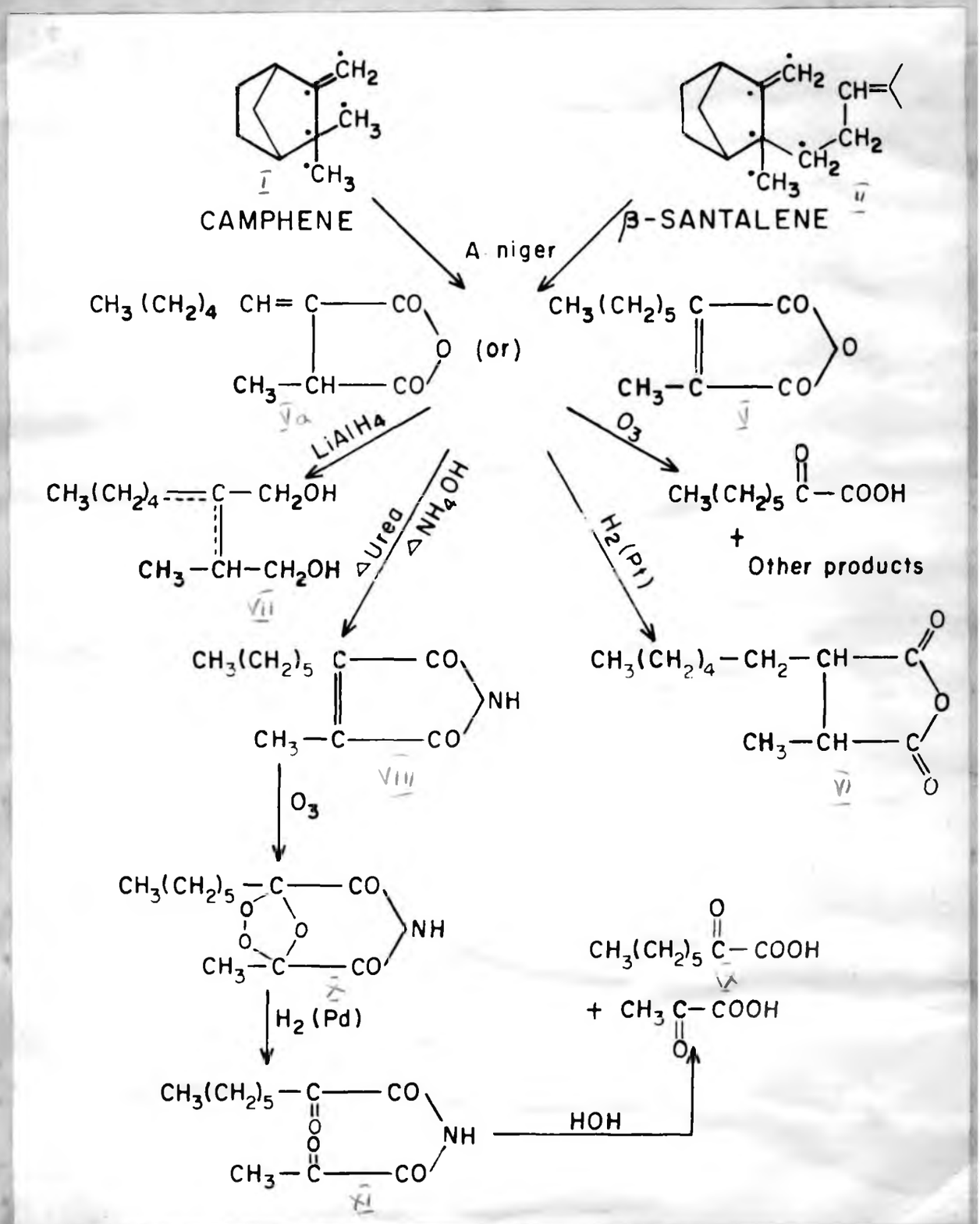


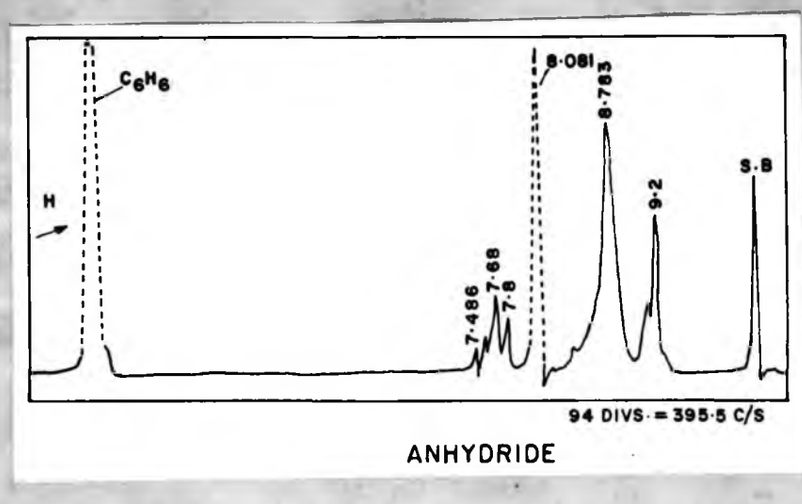
VIII

The proton n.m.r. spectra of the liquid anhydride, however, is in complete accord with the Structure, V, and indicated that both Anhydride, V, and Imide, VIII, have similar structures.

On the basis of the absence of vinyl proton (3-6 τ region) and the presence of $C=C-CH_3$ at 8.1 τ

Structures V and VIII could be assigned to the anhydride and imide, respectively.





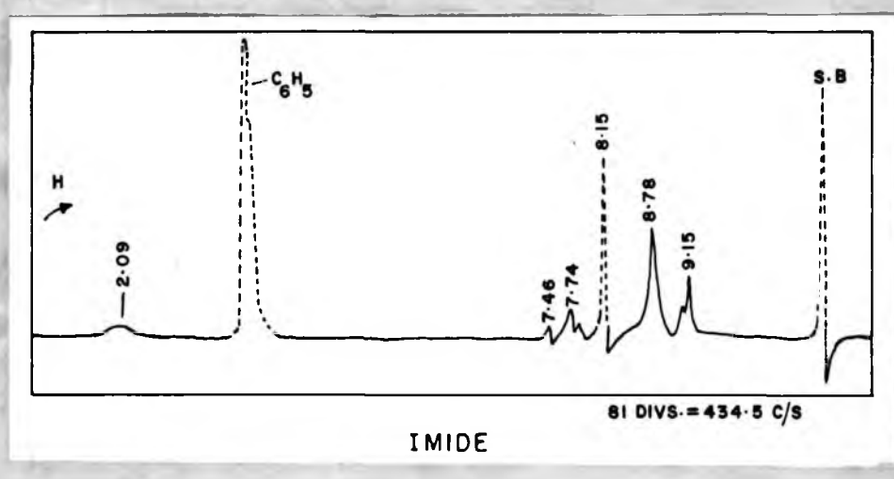


Figure 3

NMR spectra of Anhydride, V and Imide, VIII.

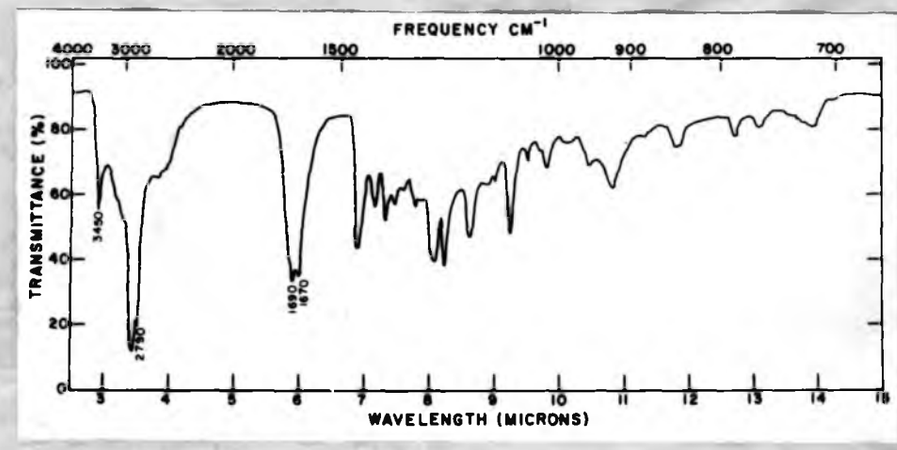
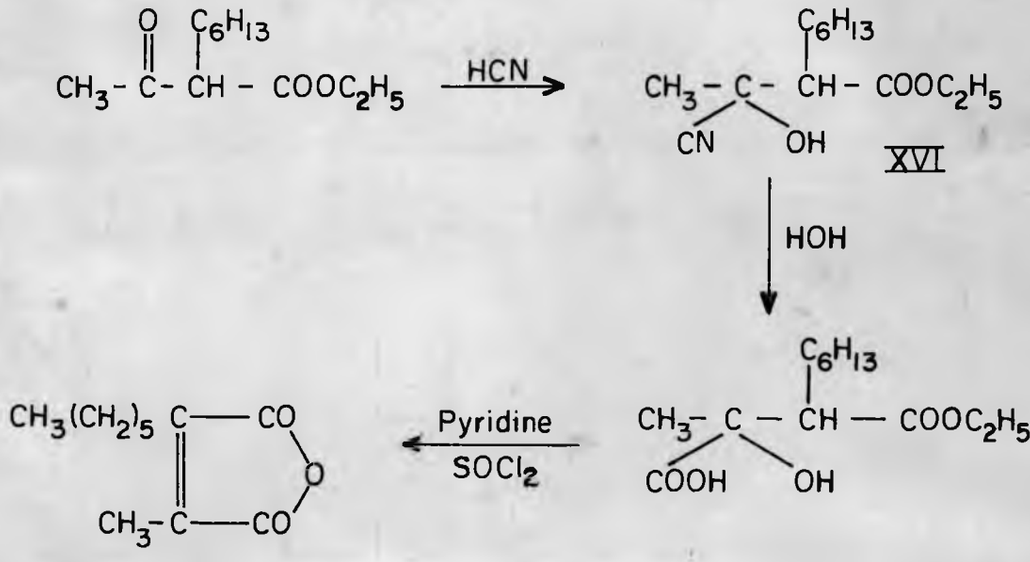


Figure 4

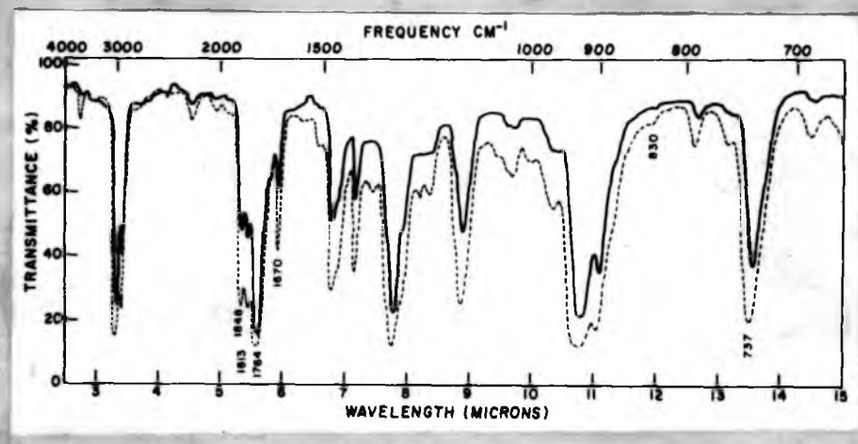
Infrared spectra of Hydroxydicarboxylic acid, XVII (in nujol mull).

Structures V and VIII for the anhydride and imide, respectively, were further confirmed by synthesis. The primary step consisted of a cyanohydrin synthesis on ethyl-n-hexyl-acetoacetate. The cyanohydrin (XVI) was hydrolysed without isolation to the corresponding hydroxy dicarboxylic acid (XVII) ($C_{11}H_{20}O_9$, m.p.132-34°), which on refluxing with thionyl chloride and pyridine yielded the anhydride as characterised through its 2,4-dinitrophenylhydrazone. The yields were, however, low.



V XVII

A comparison of the physical constants and I.R. spectra of the synthetic anhydride with those of Anhydride, V, conclusively established the structure of Anhydride, V, as the anhydride of 2 nonene 2,3-dicarboxylic acid.



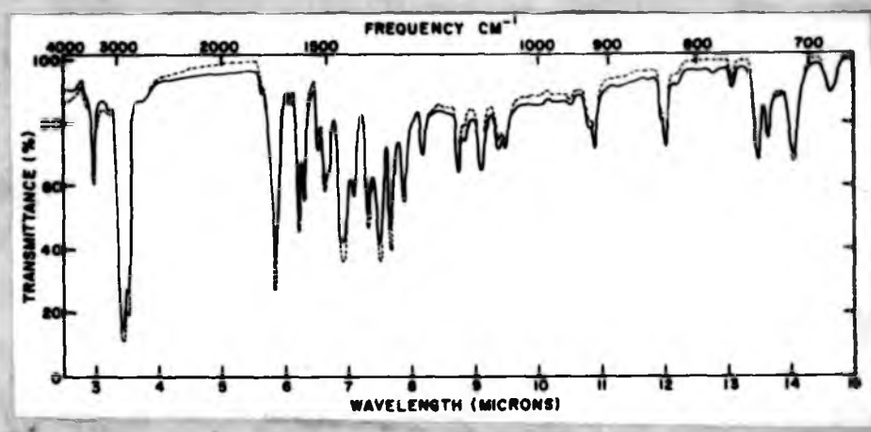


Figure 4

(a) Comparative infrared spectra of synthetic Anhydride, V_1,(—) with that of isolated Anhydride, V.(---)

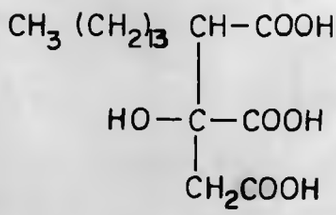
(b) Comparative infrared spectra of 2,4-dinitrophenyl-hydrozone derivatives of both synthetic Anhydride, V1 (—) and isolated Anhydride, V. (---)

The 2,4-dinitrophenylhydrazone of both the mould metabolite and the synthetic compound proved to be identical through analysis, mixed melting point and I.R.spectra.

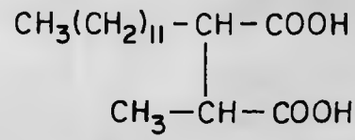
The question arises whether Anhydride, V, is a true transformation product or an artifact. The six carbon side-chain is obviously difficult to derive from any part of the terpenoid hydrocarbon, and presumed to be synthesised from substrates other than terpenes. The citraconic acid carbons may on the other hand have their origin in the (•) marked carbon which are common in both the terpenoid hydrocarbons.

An identical anhydride, methyl hexyl maleic anhydride has been obtained by Dhavlikar[61] of this laboratory on micfobiological degradation of the sesquiterpene hydrocarbon, longifolene which has the same basic ring system as camphene and β-santalene. Work on the rates of transformation of the initial hydrocarbon, has also shown that the rate of formation of the anhydride, closely follows that of the disappearence of longifolene from the medium. However, this cannot be regarded as absolute proof that the transformation products are truly derived primarily from the substrate as there is a possibility that substrates of a camphene type might have induced the organism to produce the anhydride by a metabolic shift.

Biosynthesis of compounds such as caperatic acid (XVIII) and roccellic acid (XIX) by lichens has been reported by Mitzo Asano[62] and Kennedy.[63] This presumably takes place in the case of lichens by an aldol condensation of the fatty acid residues with a suitable acid from the tricarboxylic acid cycle. The condensation of octanoate or its equivalent with pyruvate may be visualised to give rise tc Anhydride, V.



XVIII



XIX

Whether Anhydride, V, is truly a product of terpenoid transformation or an artifact can be settled only by the use of labelled compounds. Such investigations are beyond the scope of the present exploratory studies.

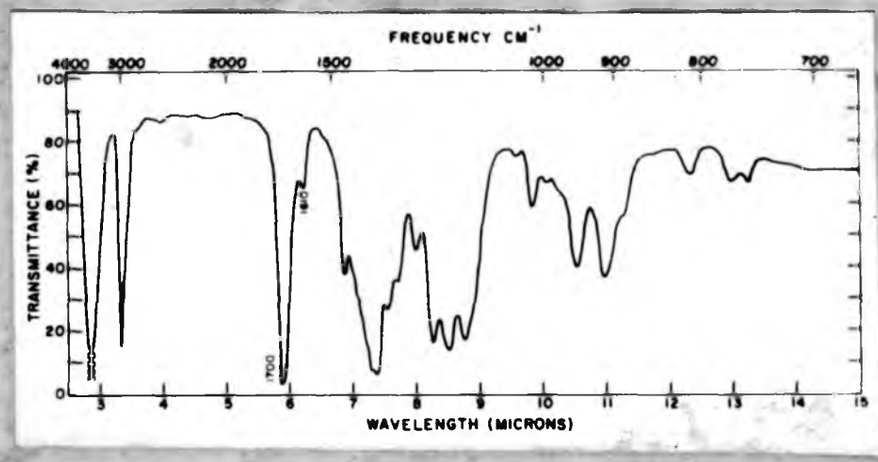
Characterisation of the neutral product

The neutral fraction (III) which was obtained on removal of the solvent was chromatographed on neutral alumina column. The chloroform-alcohol mixture (4:1) eluted a colorless, fragrant liquid (XX) which distilled at 70-74° (bath temperature)/30 mm. Hg. Liquid, XX,which

analysed for $C_6H_{12}O_2$ showed the presence of both hydroxy and carbonyl absorption in the I.R.spectrum(p. 91).

The presence of both the hydroxyl and carbonyl groupings in the molecule was proved by making the benzoate derivative and hydrolysing it. The liquid which was regenerated after hydrolysis of the ester also showed the presence of -OH and -CO groupings in the molecule.

From the I.R. and the microanalysis data, the only structure admissible for this compound is that of an aliphatic acyclic compound with -OH and -CO functions. The yield of this hydroxy carbonyl compound (XX) was small and variable. In some fermentations only traces of XX could be isolated. Further studies on its structure was, therefore, discontinued.



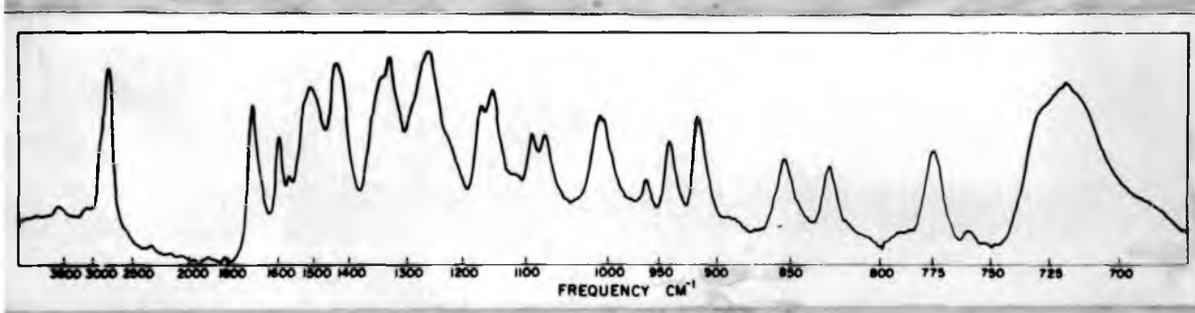


Figure 5

(a) Infrared spectra of Liquid, XX.

(b) Infrared spectra of the benzoate derivative of Liquid, XX.

EXPERIMENTAL

Fermentation

The experimental conditions employed in the present investigation were essentially the same as those used for α-pinene. Briefly, the mould under investigation was cultured in an energy rich synthetic Czapek-Dox medium under aerobic conditions in forty-shake cultures at 28° for 24 to 30 hours. The hydrocarbons camphene (m.p. 51-53°; $[\alpha]_D$ + 85.6°), 100 mg./100 ml., and β-santalene (b.p. 125-30°/7 mm.; $[\alpha]_D$-29.8°), 180 mg./100 ml. were added to the respective cultures and incubated for a further period of 24 hours.

The methods used for determining the optimal conditions for the fermentation correspond to those developed for the microbial transformation of α-pinene. With camphene as the substrate a transformation period of 24 hours was found to be optimum as far as the yield of the acidic material was concerned. The incubation temperature was maintained at 27.5° and the initial concentration of the substrate was 100 mg./100 ml.

With β-santalene which was added after a thirty-hours growth period at 27.5° the rates of oxidation were found to be higher and the mould could metabolise 0.2%(V/V) of added β-santalene in 24 hours. In the actual fermentation experiments a level of 180 mg/100 ml. was used.

Extraction

The method of extraction of the fermentation mixture has been described in detail in the earlier chapter. In short, the acetonised mycelium and the culture filtrate from forty shake cultures were extracted with ether and the constituents of the solvent layer were separated into acidic and neutral components by extraction with 5% sodium carbonate solution.

The ethereal layer (A) containing the neutral fraction was dried over anhydrous sodium sulphate, evaporated and the residual oil (A_1) was fractionated on a neutral alumina column.

The aqueous layer (600 ml.) containing the acidic product as the sodium salt was acidified with concentrated hydrochloric acid and extracted thrice with 100 ml. portions of chloroform. The combined chloroform extracts were washed with distilled water and dried over anhydrous sodium sulphate. Evaporation of the solvent gave in 30% yield, a highly viscous yellow oily liquid (IV; 2.79 g.), which was purified by repeated distillation under reduced pressure to yield the pure Compound, V, (120-25°- bath temperature / 3 mm. Hg).

The sesquiterpene hydrocarbon, β-santalene, also yielded the same Compound, V, in 35% yield from the aqueous sodium carbonate fraction after acidification, chloroform extraction and evaporation of the solvent.

Physicochemical studies on Compound, V

The purified Compound, V, had the following properties as listed in Table I.

T A B L E I

Physical characterisation of Compound V

		Compound, V
Molecular formula		$C_{11}H_{16}O_3$
Microanalysis:	Found	C, 67.32; H, 8.22 %
	Calcd.	C, 67.08; H, 8.12 %
Kuhn-Roth-determination		1.7 moles - C-CH_3
	Found	C_7CH_3 23.42%
	Calcd. for (	2(C-CH_3) 27.04%
b. p.		85-87°/ 3 mm.
$[\alpha]_D$		0
neutral equivalent		98 (± 2)
I.R.spectra	i)	1848, 1813, and 1764 cm^{-1} - a five membered ring anhydride.
	ii)	1670, and 830 cm^{-1} - a tri or tetrasubstituted double bond
	iii)	735 cm^{-1} -a straight chain containing at least four CH_2 groups.
U.V.spectra	i)	λ_{max} 249 mμ; log ε 3.81
	ii)	λ_{max} 213 mμ; log ε 4.21

Hydrogenation

A solution of the liquid anhydride (600 mg.) in glacial acetic acid (10 ml.) was hydrogenated at 23° under atmospheric pressure in the presence of platinum oxide (12 mg.) catalyst. One mole of hydrogen was taken up in two hours indicating the presence of one double bond. After hydrogenation was complete (2 hours), the catalyst was filtered off, the solvent was diluted with water and extracted thrice with 30 ml. portions of ether. The ether layer was thoroughly washed with distilled water till free from acetic acid and dried over sodium sulphate. On distillation an oil(VI) was obtained which analysed for $C_{11}H_{18}O_3$.

Analysis	Found:	C, 66.32; H, 9.01%
	Calcd.for $C_{11}H_{18}O_3$	C, 66.64; H, 9.15%.
I.R.spectra		Absence of 1670 amd 829 cm^{-1} bands.
tetranitromethane test (in chloroform)		negative

Reduction of V, with lithium aluminium hydride

Into a well dispersed suspension of lithium aluminum hydride (500 mg) in anhydrous ether (25 ml.) cooled in an ice-bath, a solution of Anhydride, V, (250 mg.) in ether was introduced with vigorous stirring.

After stirring for one hour in the ice bath the reaction mixture was refluxed at 35° for 1 hour. The mixture was then chilled in an ice bath and cautiously decomposed with cold water. The ether solution was decanted off and the precipitate extracted thrice with small volumes of ether. The combined ether extracts were washed first with 5% sodium carbonate solution, then with distilled water and finally dried over anhydrous sodium sulphate. Evaporation of the ether yielded a colorless liquid Diol, VII, which distilled at 150-53° (bath temperature)/ 1.0 mm. Hg. The liquid analysed for $C_{11}H_{22}O_2$.

Analysis	Found:	C, 71.0; H, 12.0%
	Calcd.for $C_{11}H_{22}O_2$	C, 70.92;H, 11.9%.

I.R.spectra

i) ν_{max} 3350 cm^{-1} - OH absorption

ii) ν_{max} 1670 and 829 cm^{-1} - tri or tetrasubstituted double bond.

Preparation of the imide of V

The liquid anhydride (500 mg.) was dissolved in ammonium hydroxide (3-4 ml.) and heated on a sand bath at 130° for 15 minutes. After evaporation of excess of ammonia, the resultant ammonium salt was distilled under reduced pressure (195-98°- bath temperature/1 mm.). The viscous colorless distillate (415 mg.) was solidified on standing. The product was crystallised from n-hexane to give 350 mg. of the pure Compound, VIII (m.p.60-61°).

Analysis	Found:	C, 67.39; H, 8.69; N,6.89%
	Calcd. for $C_{11}H_{17}O_2N$	C, 67.66; H, 8.78; N,7.17%.

Imide, VIII, was also prepared by heating the anhydride with urea. This procedure yielded a purer product.

Compound, V, (200 mg.) was heated with urea (75 mg.) at 160° until the evolution of ammonia ceased as indicated by a litmus test paper. The residue was then extracted thrice with chloroform, in lots of 20 ml. each time, and the solvent then removed by distillation. The residual material (168 mg) was distilled under reduced pressure (200°- bath temperature/1 mm. Hg) and the product crystallised twice to give 115 mg. of the pure analytical sample (VIII) (m.p. 60-61°). The properties of Compound, VIII, are listed below:

Analysis	Found:	C, 67.71; H, 8.71; N,6.98%
	Calcd. for $C_{11}H_{17}O_2N$	C, 67.66; H, 8.78; N,7.17%.
m. p.		60-61°
$[\alpha]_D$		0
I.R. spectra	i)	ν_{max} 3140 cm^{-1} -(NH).
	ii)	ν_{max} 1760 cm^{-1}(CONHCO)and
	iii)	ν_{max} 1708 cm^{-1}(C=O).

Saponification of Imide, VIII, to Anhydride, V

The imide (250 mg.; m.p. 60-61°) was heated for 4 hours on an oil bath at 140° with a solution of 4 N sodium hydroxide (40 ml.). After saponification the solution was diluted and extracted with chloroform, to remove the unsaponified imide. The aqueous layer was made acidic with concentrated hydrochloric acid and extracted thrice with chloroform. The chloroform extracts on solvent removal followed by distillation furnished Anhydride, V (120-25° - bath temperature/3 mm.).

Analysis	Found:	C, 66.58; H, 9.3%
	Calcd.for $C_{11}H_{16}O_3$	C, 66.64; H, 9.15%.

2,4-Dinitrophenylhydrazone of Anhydride, V

To a refluxing methanolic solution of 2,4-dinitrophenylhydrazine (200 mg.) containing 6-7 drops of concentrated sulphuric acid, Anhydride, V, (150 mg.) was added. The clear yellowish orange colored solution, thus obtained was left at 0° and the crystalline product collected after 2-3 hours (crude m.p. 142-46°) was recrystallised thrice from ethanol to give yellow needles, m.p. 148-50°.

Analysis	Found:	N, 14.72 %.
	Calcd. for $C_{17}H_{20}O_6N_4$	N, 14.89 %.

u.v. absorption $\lambda_{max.}$ 325 mμ, log ε 4.25 (characteristic of non-conjugated phenyl hydrazones indicating that only one of the C=O of the phenyl-hydrazone is in conjugation with the double bond).

Ozonolysis of Anhydride, V

A solution of the anhydride (400 mg.) in purified ethyl acetate (40 ml.) was ozonised at -20° till no more ozone was absorbed (2 hours; potassium iodide test). The solvent was removed under suction at room temperature and the residual syrup was decomposed by adding 25 ml. of distilled water, heated on a water bath for 1 hour and left aside for cooling. The aqueous solution was repeatedly extracted with ether and the combined ethereal extracts were washed with 5% sodium carbonate to separate neutral and acidic fractions. The acidic fraction on acidification with concentrated hydrochloric acid, extraction with chloroform and evaporation of solvent yielded a viscous Liquid, IX. This was converted into its 2,4-dinitro-phenylhydrazone, which after three crystallisations from alcohol was obtained as fine yellow orange needles (128-30°).

The analytical data and the melting point of the 2,4-dinitrophenylhydrazone indicated the identity of IX as α-keto caprylic acid. There was no depression in the mixed melting point of the two 2,4-dinitrophenylhydrozones.

Ozonolysis of the Imide

Imide, VIII, (500 mg.) on ozonolysis in the same manner as described earlier afforded a stable crystalline Ozonide, X, (positive starch iodide test) which was crystallised from petroleum ether (40-60°) to the constant melting point 46-48°.

Analysis	Found:	C, 54.4; H, 7.1; N, 5.5%
	Calcd. for $C_{11}H_{17}NO_5$	C, 54.31; H, 7.04; N, 5.76%.
I.R. spectra	(i)	ν_{max} 3000 cm^{-1} NH- absorption
	(ii)	ν_{max} 1723 and 1700 cm^{-1} absorption due to cyclic ring imide.
	(iii)	ν_{max} 1056 cm^{-1} absorption due to ozonide system.

Decomposition of Ozonide, X

The ozonide (480 mg.) was taken up in ethylacetate (50 ml.) to which freshly prepared active palladium catalyst on calcium carbonate (75 mg.) was added. Hydrogen was bubbled through the mixture at room temperature and atmospheric pressure for 2 hours. On evaporation of ethylacetate in *vacuo* a crystalline product was obtained which was recrystallised four times from *n*-hexane to the constant melting Diketo-imide, XI, (375 mg.).

Analysis	Found:	N, 5.82 %.
	Calcd. for $C_{11}H_{17}O_4N$	N, 6.16 %.
m. p.		92-95°

I.R.spectra i) ν_{max} 3240 cm^{-1} NH absorption

ii) ν_{max} 1761 cm^{-1} -CO-NH-CO- absorption

iii) ν_{max} 1711 cm^{-1} -C=O. absorption.

Hydrolysis of the Diketo imide, XI

The diketoimide (375 mg) was taken up in a saturated bariumhydroxide solution (100 ml.) and stirred for 24 hours at room temperature (27-28°). The solution was acidified and extracted with chloroform. The chloroform extract was evaporated under reduced pressure to yield a mixture of keto acids (300 mg.) which were converted to their corresponding 2,4-dinitrophenylhydrazone derivatives. The 2,4-dinitrophenylhydrazones were separated by fractional crystallisation and were identified as those of α-keto caprylic acid, IX, (128-30°) and pyruvic acid (m.p. 213-15°) by comparing melting points and paper chromatograms[64] (n-butanol-ammonia water) with authentic samples.

Total synthesis of Anhydride, V

The first step consisted in the preparation of ethyl-n-hexyl-acetoacetate.

In a 1 liter two-necked flask fitted with an efficient double surface condenser and a separating funnel, sodium (5 g.) was added slowly to anhydrous ethanol (150 ml.) with occasional cooling of the flask. When the reaction had subsided, ethylacetoacetate (25 ml) was added. Stirring was continued and the flask was heated slowly. Hexyl bromide (35 ml.) was added dropwise through the separating funnel and the mixture was continuously stirred and refluxed till neutral to moist litmus (48-52 hours). The reaction mixture was then cooled and the solution was decanted from the precipitate of sodium bromide. The precipitate was washed with absolute alcohol and the washings were combined with the decanted solution. The excess of alcohol present was distilled off. The residual liquid, ethyl-n-hexyl acetoacetate (15 g.) was distilled in vacuo.

Analysis	Found:	C, 67.17; H, 10.48%
	Calcd.for $C_{12}H_{22}O_3$	C, 67.25; H, 10.35%.
b. p.		120-22°/0.5 mm.

Preparation and hydrolysis of cyanohydrin(XVI)

A solution of ethyl-n-hexyl-acetoacetate (8 g.) in anhydrous ether (15-20 ml.) was added slowly with moderate stirring at -10° to liquid hydrocyanic acid

(3-4 ml.) containing saturated aqueous potassium cyanide (1 ml.) and conessine dimethobromide (10 mg.). The mixture was left at 0° for 30 hours and then at 24° for 7-8 hours. It was then poured into 30% (W/V) hydrochloric acid (70 ml.) previously cooled to 0°. The solution was left for 3 days at 0° and after removal of excess of hydrocyanic acid by aeration was heated first to 50° for 2 hours and then to 100° for 20 hours.

After cooling, the solution was extracted thrice with ether and the combined ethereal extracts were washed with 1N sodium hydroxide to remove any acidic component as its soluble sodium salt. The alkaline extract was then acidified with concentrated hydrochloric acid, extracted with chloroform and the solvent distilled when a viscous liquid (XVII) was obtained. This was then distilled under vacuum (200° - bath temperature/3 mm.) which solidified on standing (250 mg.). On repeated crystallisation from n-hexane, Compound, XVII, was obtained as white, flat, hexagonal crystals, m.p. 130-34°.

Analysis	Found:	C, 56.7; H, 8.84%.
	Calcd. for $C_{11}H_{20}O_5$	C, 56.88; H, 8.6%.
m. p.		132-34°
I.R. spectra	(i) ν_{max} 3450 cm^{-1}	-OH absorption
	(ii) ν_{max} 2790 cm^{-1}	-OH stretching vibrations of COOH-
	(iii) ν_{max} 1690 and 1670 cm^{-1}	- C=O vibrations of COOH.

Dehydration of Hydroxy acid, XVII, to Anhydride, V

The hydroxy acid (XVII) (250 mg.) was heated at 150° in presence of thionylchloride (2 ml.) and pyridine (5 drops) for 4 hours. The residue was extracted with chloroform and washed repeatedly with 1N sodium hydroxide. The alkaline extracts on acidification, extraction with chloroform and removal of the solvent by distillation furnished a liquid (V_l) which was purified by distillation under vacuum (120-25°- bath temperature/ 3 mm.).

Analysis	Found:	C, 67.63; H, 8.12%
	Calcd. for $C_{11}H_{16}O_3$	C, 67.32; H, 8.22%.

It gave a 2,4-dinitrophenylhydrazone derivative, (m.p. 148-50°). Mixed melting point with a sample of 2,4-dinitrophenylhydrazone of Anhydride, V, (m.p.148-50°) was undepressed.

Analysis	Found:	C, 54.6 ; H, 5.2; N,14.48%.
	Calcd.for $C_{17}H_{20}O_6N_4$	C, 54.25; H, 5.36;N,14.89%.

A comparison of the physical properties, u.v. and I.R. spectra of the synthetic Anhydride, V_l, and its derivative with those of Anhydride, V, conclusively proved the structure of the anhydride.

Characterisation of the neutral product(XX)

The neutral fraction (III; 1.9 g.) was chromatographed over a neutral grade II alumina (50 g.) and was eluted using mixtures of graded solvents. n-Hexane and chloroform alcohol mixture (4:1) eluted unreacted camphene (500 mg.) and liquid (XX; 238 mg.) respectively. Liquid, XX, which was further purified by distillation under reduced pressure had the following properties:

Analysis	Found:	C, 61.2; H, 10.7%	
	Calcd.for $C_6H_{12}O_2$	C, 61.1; H, 10.35%.	
b. p.		158-61°/725 mm.	
I.R.spectra	(i)	ν_{max} 3500 cm^{-1}	hydroxyl absorption
	(ii)	ν_{max} 1700 cm^{-1}	Carbonyl(C=O) absorption

3,5-Dinitrobenzoate of Liquid, XX, was prepared by refluxing the Compound, XX (100 mg.) with freshly prepared 3,5-dinitrobenzoyl chloride (150 mg.) in presence of 3-4 drops pyridine on a steam bath for 45-60 minutes. The resultant mixture was cooled and decomposed with saturated aqueous sodium carbonate. The solid material was then filtered, washed with distilled water and recrystallised from aqueous ethanol(80% v/v), m.p. 91-93°.

From the above physical data the only plausible partial structure for this liquid may be that of an aliphatic acyclic compound with -OH and -CO functions.

C H A P T E R VIII

PROBABLE MECHANISMS OF TRANSFORMATION OF TERPENES

Probable mechanisms of transformation of terpenes

From the nature of products obtained by fermentation of terpenoid hydrocarbons with A.niger the primary step in these transformations appears to be the introduction of oxygen function in these hydrocarbons. These oxygenation processes will be discussed with reference to the accepted mechanism of oxygen transfer reactions.

It should be made clear that no systematic enzymic studies have been made to elucidate the actual mechanism of transformations. Some preliminary manometric studies indicated that α-pinene inhibits the rather persistent endogenous respiration of the mould and a long time is needed to overcome this inhibition. The endogenous energy source in the fungal cells is quite large and the addition of the terpenoid hydrocarbon did not enhance the rate of oxygen uptake to any significant extent even after the preliminary inhibition was overcome.

In order to understand the nature of these oxygenation and oxidation processes it might be relevant to recapitulate briefly some of the postulates developed for some similar transformations in aromatic compounds and steroids which have been studied in greater detail.

The oxygenating enzymes

According to Mason[65] the enzymes involving directly or indirectly molecular oxygen can be divided into three broad classes viz., (1) the oxygen transferases (2) mixed function oxidases and (3) electron transferases.

Of these, the first two classes are directly concerned with the oxygenation processes in biological system.

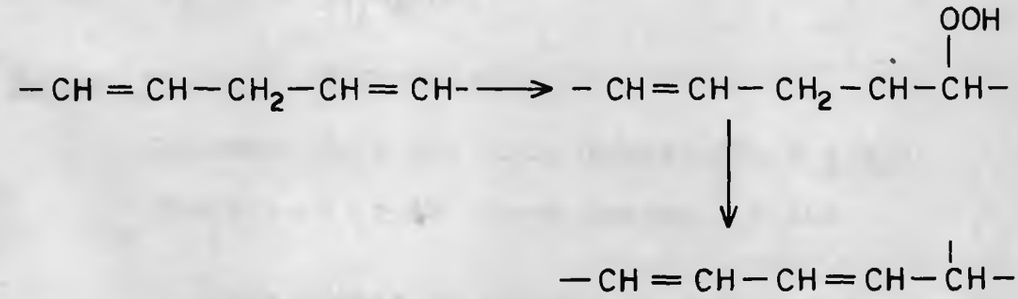
The "oxygen transferases" catalyse the consumption of one mole of oxygen per mole of the substrate in such a manner that both the atoms of the consumed oxygen appears in the product.

$$A + O_2 \xrightarrow{\text{Enzyme}} AO_2$$

or $$2A + O_2 \longrightarrow 2AO \text{ (not observed).}$$

Usually the oxygen transferases are responsible for the oxidative cleavage of aromatic rings, common examples being pyrocatechase, 3-hydroxyanthranilic acid oxidase, protocatechuic acid oxidases etc. The only enzyme of this class which is concerned with a direct oxygenation process is lipoxidase. Lipoxidase occurs widely in nature and is involved in the oxidation of unsaturated fatty acids containing methylene interrupted cis-double bonds. One of the mechanisms proposed for

lipoxidase action[66] is that the primary step consists in reduction of molecular oxygen to the reactive hydroperoxide radical which attacks a double bond in the fatty acid chain. The adduct loses a hydrogen radical to some suitable acceptor leaving the conjugated hydroperoxide which can undergo subsequent transformations.



Lipoxidase also represents a unique case of oxygen transfer where no metals seem to be involved in the process of activation of oxygen.

The mixed function oxidases comprise of two different but interdependent catalytic activity, viz., (i) reduction of one atom of oxygen in the oxygen molecule coupled with (ii) a specific oxygenation with the other atom of oxygen.

Two similar sets of equations can be formulated to explain the overall transformations, depending on, which one of the above activities precedes the other.

Scheme 1

$$\text{Enzyme} + O_2 \longrightarrow \text{Enzyme} \sim O_2$$

$$\text{Enzyme} \sim O_2 + AH \longrightarrow AOH + \text{Enzyme} \sim O$$

$$\text{Enzyme} \sim O + DH_2 \longrightarrow \text{Enzyme} + D + H_2O.$$

where AH is the substrate and DH_2 is the electron acceptor.

Scheme II

$$\text{Enzyme} \sim O_2 + DH_2 \longrightarrow \text{Enzyme} \sim O + D + H_2O$$

$$\text{Enzyme} \sim O + AH \longrightarrow \text{Enzyme} + AOH$$

A large number of enzymes including the steroid hydroxylases, aromatic ring hydroxylating enzymes, squalene oxidocyclase, the phenolase complex, luciferase, etc. belong to this class. The characteristic features of the mixed function oxygenation are that atmospheric oxygen is directly incorporated into the product without exchange with the medium and that the enzymes require the presence of other electron donors such as reduced pyridine nucleotides besides the substrate for functioning.

Hyano and coworkers[67] were able to show by studies with H_2O^{18} and O_2^{18} that the hydroxyl group introduced by the adrenal steroid 11β-hydroxylase into steroidal substrates is derived entirely from O_2^{18} and is not even exchangeable with the hydroxyl of the medium. Identical conclusions have also been drawn in studies with different aromatic hydroxylases,[68] the squalene hydroxylase[69] and steroid 7β and 11α hydroxylases from

moulds.[70] The absolute stereospecificity of the oxygenation processes has been established in the last two systems by the demonstration that the epimeric 7α and 11β hydrogens remain unaffected in these reactions.

It is logical to presume that the hydroxylation of terpenes by A. niger is brought about by mechanisms similar to those observed in the case of steroids. There are some broad similarities in both the oxygenation processes. The experimental conditions necessary are essentially identical. Both these oxygenation processes are characterised by a high degree of stereospecificity. Furthermore, the experimental strain of A.niger has been shown to hydroxylate steroids such as progesterone and 18-hydroxyprogesterone[71] at the 11α position.[72]

No studies have yet been made so far to ascertain whether the enzymes responsible for the oxygenation of terpenes are adaptive or constitutive. In the case of steroids, however, indirect but convincing evidence has been obtained about the adaptive nature of the hydroxylating enzymes.[73] The enzymes in Pseudomonas capable of oxidising camphor also appear to be substrate-induced.[74]

It would, therefore, not be out of place to discuss the terpene transformations reported here in the light of known mechanisms.

Types of oxygenation

Mechanically the types of oxygenation observed so far can be divided into three arbitrary categories.

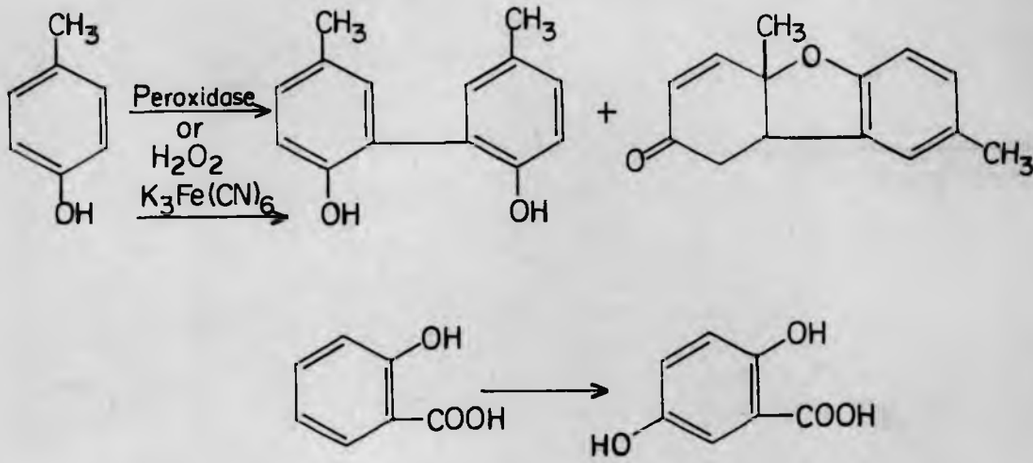
1) Oxygenation at allylic or activated positions; example - formation of verbenol from α-pinene.

2) Oxygenation on a double bond; example - formation of sobrerol from α-pinene.

3) Oxygenation at a chemically inert position; example - formation of 2-nonene-2,3-dicarboxylic acid from camphene.

The nature of active oxygen

It is still not certain whether the reactive species of oxygen in most of the hydroxylating systems is an ion or a free radical. In some cases, however, from the chemical nature of the products a diagnosis is possible. Lipoxidase which utilises molecular oxygen and some peroxidase oxidases[75] which oxidise aromatic compounds using hydrogen peroxide as the electron acceptor obviously operate through free radical intermediates. The products formed in these peroxidative reactions are often identical to those obtained by ferricyanide oxidation,[76] a process known to involve single electron transfers.



The nature of the products in aromatic hydroxylation brought about by the enzymes from liver microsomes[77] indicate that an electrophilic oxygen may be the reactive species.

OH^+ The electrophilic hydroxyl, OH^+, has been implicated in many organic reactions. Although OH^+ is known to exist in the mass spectrograph, Coulson has pointed out from theoretical grounds that OH^+ would be extremely unstable in aqueous sytems.[78]

Enzyme~$Fe^{++}O_2$ and Enzyme~Fe - O^{++}

It has been observed, however, that most of the oxygenating enzymes, excepting perhaps lipoxidase, require a metal ion such as iron and copper as an integral component. Based on the behaviour of iron in autoxidation, Mason has proposed that an electrophilic species such as Enzyme~Fe O^{++} may be the active

hydroxylating species. Evidence for the existence of such higher valence states of iron has been provided by George.[79] The tetravalent oxygenated iron species is presumed to be derived through a two-electron reduction of the pentavalent complex, Enzyme $\sim$ Fe-O_2^{++}, through the following sequence of reactions.

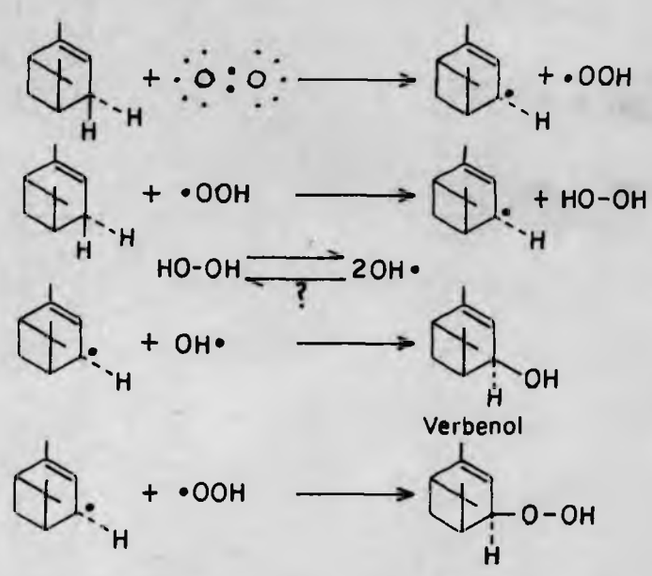
$$\text{Enzyme}\sim Fe^{++} + O_2 \longrightarrow \text{Enzyme}\sim Fe^{++}\ O_2$$

$$\text{Enzyme}\sim Fe^{++}O_2 + DH_2 \longrightarrow \text{Enzyme}\sim Fe^{++}\ O$$

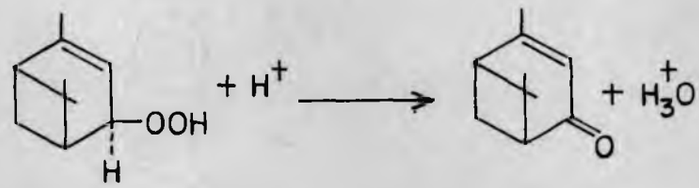
The probable mechanisms for the oxidation of terpenes

Unfortunately from the nature of the products derived in the current investigation no clearcut diagnosis is possible about the nature of active oxygen. Both the types of reactive oxygen-free radical or cationic,-will, therefore, have to be considered.

1) Free radical mechanisms - A free radical lipoxidase type of mechanism for oxygen activation can be postulated to explain the formation of verbenol and verbenene from α-pinene.



The hydroperoxide can be hydrolysed to verbenone by acid,



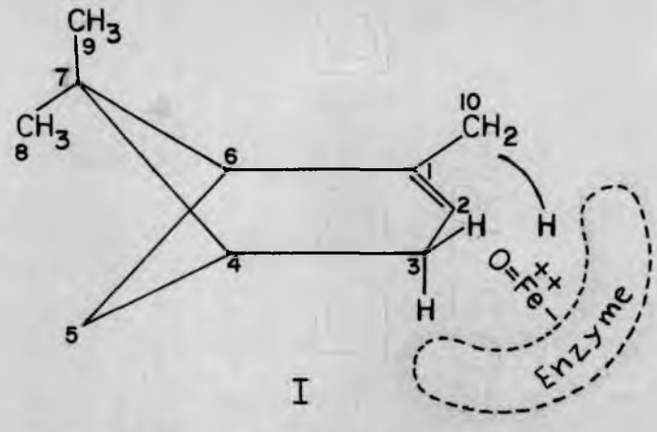
and the ketone stereospecifically reduced to cis-verbenol by an alcohol dehydrogenase type of mechanism.

Mixed mechanism - It is also possible to visualise a mixed mechanism involving a metal which can withdraw from or donate to a free radical to convert it to a cation or an anion.

$$RH + O_2 \longrightarrow R\cdot + OOH\cdot$$
$$OOH\cdot + e \longrightarrow OOH^-$$
$$R\cdot \longrightarrow R^+ + e$$
$$R^+ + H_2O \longrightarrow ROH + H^+$$
$$OOH^- + H^+ \longrightarrow H_2O_2$$

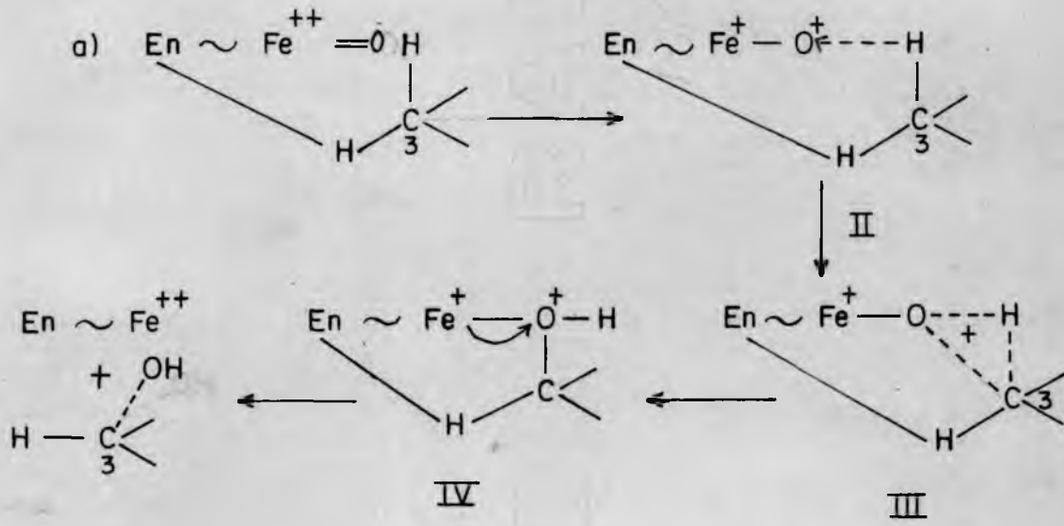
Ionic mechanisms - An ionic mechanism involving an incipient OH^+ or Mason's electrophilic oxygenating species can also be formulated for verbenol formation.

The molecule of α-pinene can be visualised to be held on to the enzyme surface with the two allylic hydrogens at position 10 and 3α as in the figure I.

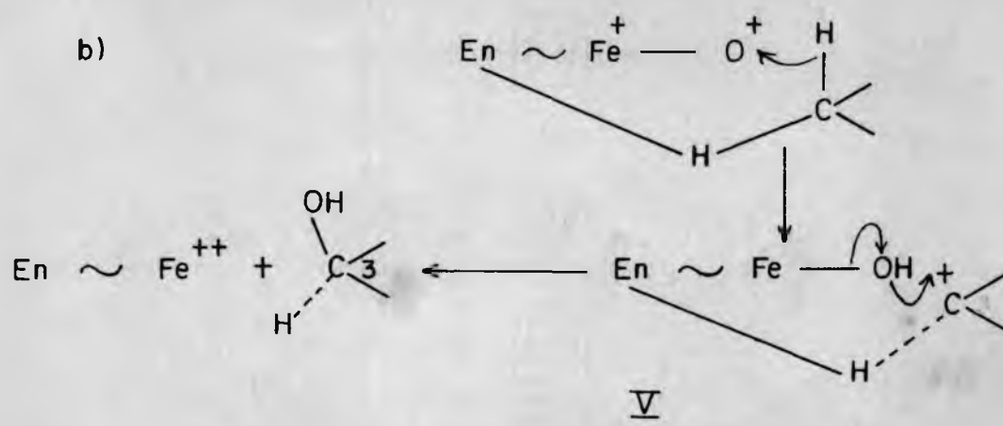


I

In the above model the proximity of the catalytic site of the enzyme (present to be as Fe^{++} =O) to the 3βH may bring about a hydroxylation by either of the two mechanisms.



In this pathway it is presumed that in species III the O --- C bond is formed and the C --- H bond is broken, one following the other.

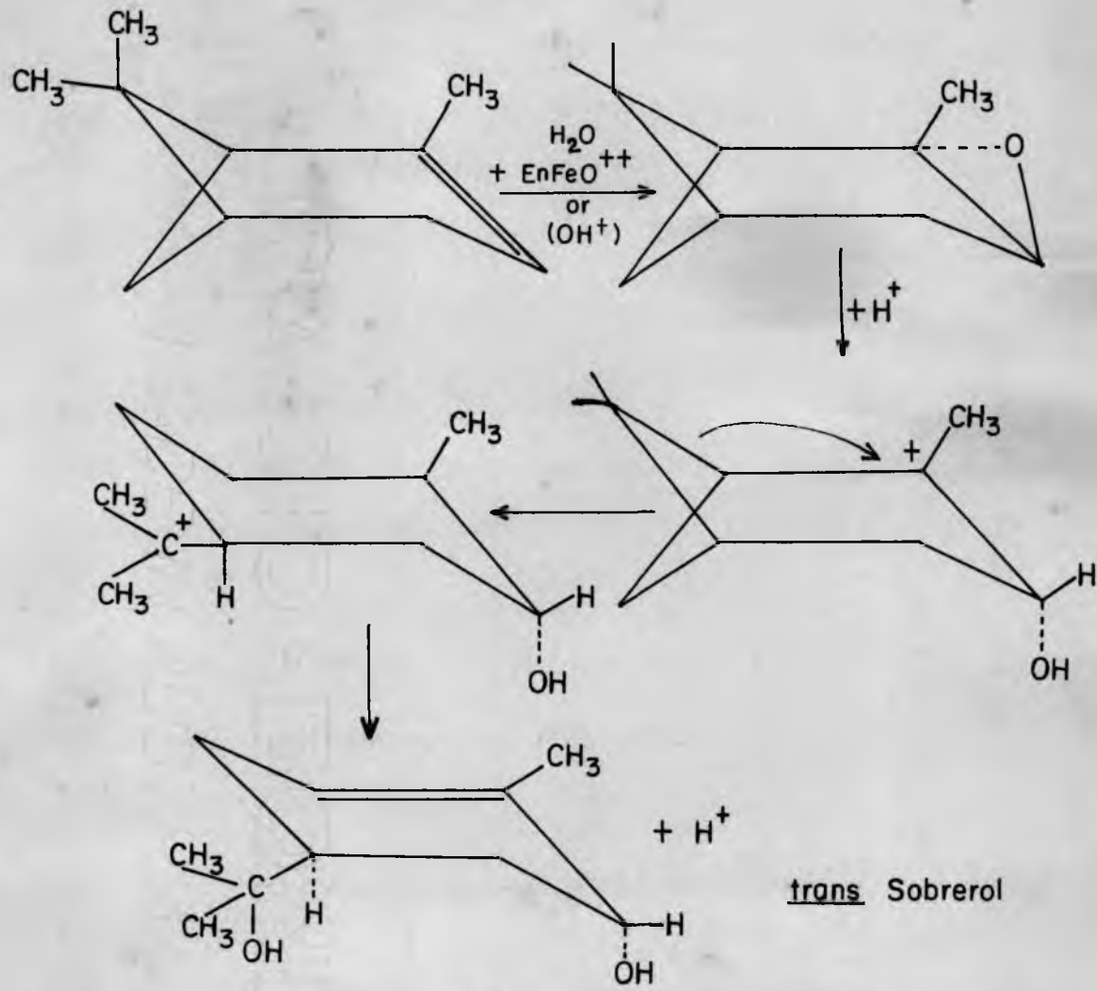


V

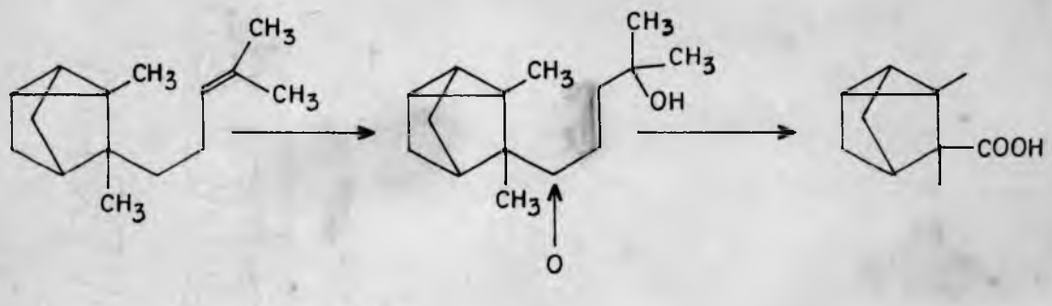
b) In the second pathway the incipient carbonium ion as in(V)reacts preferentially and instantaneously with the incipient hydroxyl liberated from the complexed iron. The collision probability of this vicinal hydroxyl with the carbonium ion would be overwhelmingly greater than any other OH from the medium. This argument can be justified from the observed behaviour in intramolecular catalysis. For instance the hydrolysis of aspirin[80] is independent of hydrogen ion concentration over a wide range of pH (4-8) and is predominantly due to an intramolecular attack by the vicinal carboxylate ion.

It should be noted that the enzyme substrate model (I) also predicts that the more hindered cis-verbenol would be the reaction product from α-pinene. It also explains why atmospheric oxygen is directly incorporated without exchange with the hydroxyls of the medium. Furthermore, this model can be extended to the case of steroid hydroxylation even at an inert position such as 11.

The formation of sobrerol can be explained by postulating an attack of an electrophilic oxygen (or a free radical oxygen followed by a protonation) on the double bond as the initial step. The opening of the epoxide ring and the subsequent rearrangement can then follow by a nonenzymatic acid catalysed process.



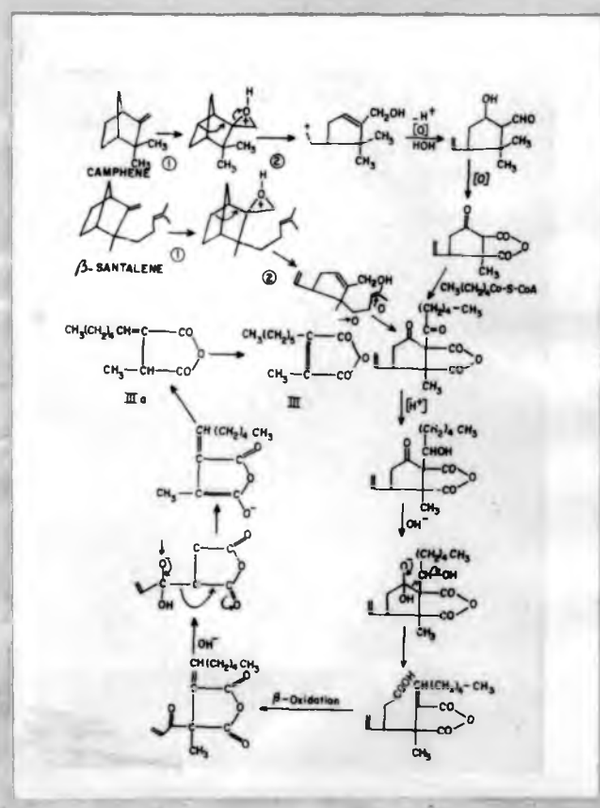
Oxidation of α-santalene - Extending these concepts to the case of α-santalene the primary step can be visualised to be an oxygenation on the double bond followed by further oxygenation eventually leading to tere-santalic acid.



In support of this mechanism the isolation of A C_{15} intermediate with a tertiary hydroxyl such as in (VI) may be taken into consideration.

Oxidation of camphene and β-santalene

It has been mentioned earlier that it was not possible to ascertain whether 2-nonene-2,3-dicarboxylic acid is a true transformation of camphene or β-santalene. However, assuming that the five-carbon dicarboxylic acid portion is actually derived from these terpenoid substrates one can postulate a mechanism for its formation based on the type of reaction involving a bond shift which leads to sobrerol formation from α-pinene oxide.



The only difficulty with this mechanism is that the formation of the primary carbonium ion by a bond shift in step 2 would be a high energy process.

In conclusion, it can be said that the justification of all these mechanistic interpretations would have to wait until more data are available about the nature of enzymes involved and the mode of oxygen activation in these transformations. At the present moment it is not even possible to predict the course of oxygenation and the products obtained by the action of microorganisms on a given terpenoid substrate.

...

C H A P T E R IX

S U M M A R Y

S U M M A R Y

It has been known for sometime that the yields of some essential oils from plant material are affected by the microbial infection of the oil bearing plant. A cause and effect relationship has been suspected between the yield of oxygenated terpenes of importance in the industry and the fungal infection in the case of Agarwood. No clearcut evidence is, however, available about the capacity of the microorganisms to transform the terpenoid hydrocarbons to oxygenated compounds. The present studies were undertaken in order to investigate the ability of microorganisms, particularly fungi, to convert simple terpenoid hydrocarbons, such as α-pinene, Δ^3-carene, carane, humulene, α and β santalenes, and camphene.

1. From a sample of infected agarwood, six strains of fungi, identified as (1) penicillium-steckii, (2) Geotrichum Sp, (3,4,5) Aspergillus versicolor, (6) Cladosporium Sp. were isolated using standard techniques. After a preliminary screening of these six isolates in addition to six strains of fungi from N.C.I.M. collection, with respect to their ability to metabolise α-pinene, a strain of Aspergillus niger was selected for further investigations.

2. Tnis experimental strain of A.niger was found to metabolise α-pinene (0.5%) in 4-8 hours. From the fermentation broth and the mycelium, three oxygenated derivatives were isolated by solvent-extraction, solvent-solvent distribution fractional distillation, bisulphite treatment and chromatogfaphy viz., (i) d-cis-verbenol, $C_{10}H_{16}O$, b.p. 112°/14 mm. Hg $[\alpha]_D$ + 64°, p.nitrobenzoate 88-90°); (ii) d-verbenone, $C_{10}H_{14}O$, b.p. 110°/16 mm. Hg $[\alpha]_D$ + 223°, u.v. absorption λ_{max} 253 mμ log ϵ 3.81); and (iii) d-trans-sobrerol, $C_{10}H_{18}O_2$ (m.p. 145-46°, $[\alpha]_D$ + 152°.

3. Compared to α-pinene, carane and Δ^3-carene as well as the large ring sesquiterpene, humulene, proved to be relatively resistant to oxygenation by the experimental strain of A.niger. However, in the case of Δ^3-carene a volatile oil was obtained from the fermentation mixture in quantities insufficient for further chemical studies.

4. The sesquiterpene hydrocarbon, α-santalene, was degraded mainly to an acid having the formula, $C_{10}H_{14}O_2$ (m.p. 152-54°) which was identified as tere-santalic acid. From the neutral fraction an alcohol $C_{10}H_{16}O$ (m.p. 112-114°) was isolated in poor yields and identified as tere-santalol.

5. Camphene and β-santalene, however, proved to be easily oxidisable by this mould. An identical dicarboxylic acid, which could be isolated only as the liquid anhydride, $C_{11}H_{16}O_3$ (b.p. 89-90°/3 mm.) was obtained from the fermentation mixture. The liquid anhydride absorbed one mole of hydrogen on catalytic hydrogenation in presence of acetic acid and platinum oxide catalyst indicating the presence of one double bond. Kuhn-Roth degradation of the anhydride showed the presence of two C.methyl groups. The crystalline imide (60-61°) prepared from the anhydride by treatment with ammonia gave a crystalline ozonide (46-48°) which could be decomposed only by palladium on calcium carbonate in presence of hydrogen yielding a diketo-imide (92-95°). On further alkaline hydrolysis this compound gave rise to α-keto caprylic acid and pyruvic acid, both identified through their 2,4-dinitrophenylhydrazones and comparative paper chromatography with authentic samples. In view of the above experimental evidence the liquid anhydride was identified as the anhydride of 2-nonene 2,3-dicarboxylic acid. This structure for the anhydride was further confirmed from the n.m.r. spectra and total synthesis.

6. The mode of microbial attack on terpenoid hydrocarbons is discussed with reference to known oxygen transfer mechanisms in other biological systems.

****

BIBLIOGRAPHY

B I B L I O G R A P H Y

1.	Linter, C.J., and Liebig, H.H.,	Z.Physiol.Chem., 72, 449 (1911).
2.	Mamoli,L., and Vercellone, A.,	Ber.,70,470(1937).
3.	Bertand, G.,	Compt.rend., 122, 900 (1896).
4.	Peterson,D.H.,and Murray,H.C.,	J.Am.Chem.Soc., 74, 1871(1952).
5.	Peterson,D.H. in Mosttig, E. (Editor),	Proc.of the 4th Internation Congress of Biochemistry,Vienna, Vol.IV, Pergamon Press, New York, 1958,p.83.
6.	Vischer,V.,and Wettstein, A. in Nord, F.F. (Editor).	Advanc.Enzymology,Vol.20, Interscience Publishers, Inc., New York,1958,p.237
7.	Eppstein, S.H., Meister, P.D., Murray,H.C., and Peterson,D.H., in Harris, R.S.,Marrian,G.F., and Thimann, K.V.,	Vitamins and Hormones, Vol.14, Academic Press, Inc., New York, 1956, p. 359.
8.	Fried, J., Thoma, R.W., Perlman, D., Heiz, J.E., and Borman, in Pincus, G.,	Recent Progr.Horm.Res., Vol.11, Academic Press, Inc., New York, 1955, p. 157.
9.	Talalay, P.,	Physiol,Rev., 37,362 (1957).
10.	Hanson, F.R., Mann, K.M., Nielson, E.D., Anderson,H.V., Brunner, M.P., Karnemaat, J.N., Collingsworth, D.R., and Hainer, W.J.,	J.Am.Chem.Soc., 75, 5369(1953).
11.	Holmlund, C.E.,Feldman, L.I., Rigler, N.E., Nielson, B.E., and Evans, R.H.,	J.Am.Chem.Soc., 83, 2586 (1961).
12.	Vischer, E., Schmidlin, J., and Wettstein, A.,	Experientia, 12, 50 (1956).
13.	Schmidlin, J., Anner, G., Billeter, J.R., and Wettstein, A.,	Experientia, 11, 365 (1955).

14.	Thoma, R.W., Herz, J.E., Fried, J., Perlman, D., and Gerke, J.R.,	Unpublished data cited from Fried. J., (See ref. 8).
15.	Fried, J., Thoma, R.W., Sabo, E.F., and Grabowicz, P.,	Unpublished data. Cited from Fried, J. (See ref. 8).
16.	Nehr, R., and Wettstein, A.,	Helv.Chim.Acta, 39, 2062 (1956).
17.	Prelog, V. in Wolsteinholme, G.E.W., and O'Connor, C.M. (Editors),	Ciba. foundation study. Group No.2, J & A Churchill Ltd., Gloucester Place, 1959, p. 79.
18.	Prelog, V., and Acklin, W.,	Helv.Chim.Acta, 39, 748 (1956).
19.	Acklin, W., Prelog, V., and Zach, D.,	Helv.chim.Acta, 41, 1428 (1958).
20.	Bradshaw, W.H., Conrad, H.E., Corey, E.J., Gunsalus, I.C., and Lednicer, D.,	J.Am.Chem.Soc., 81, 5507 (1959).
21.	Hedegaard, J., Conrad, H.E., and Gunsalus, I.C.,	Bact. Proc., 183(1961).
22.	Werner Seubert,	J.Bact., 79, 426(1960).
23.	Sadgopal, and Varma, B.S.,	Soap, Perfumery and Cosmetics, 25,169(1952).
24.	Tunstall	Report of Assam, 1929-30. Cited from Sadgopal and Varma, B.S. (See ref. 23).
25.	Bose, S.R.,	Science and Culture, 4, No.2, 89 (1938), Tetrahedron Letters, 9, 13 (1959).
26.	Bhattacharyya, S.C., and Jain, T.C.,	Tetrahedron Letters, 9, 13 (1959).
27.	Fred, E.B., and Waksman, S.A.,	Laboratory Manual of general microbiology. McGraw-Hill, New York, 1928, p. 14.

28.	Prescott, S.C., and Dunn,C.G.,	Industrial Microbiology, 3rd ed. McGraw Hill, New York, 1959, p.644.
29.	Raper, K.B., and Thom, C.,	Manual of Penicillia, Williams and Wilkins Co., 1949, p.350.
30.	Clements, F.E., and Shear, C.L.,	in "The Genera of Fungi," Hafner Publishing Co., 1945, pp. 201-205.
31.	Raper, K.B., and Thom, C.,	in Manual of Aspergilli, Williams and Wilkins Co., 1949, p. 189.
32.	Bhattacharyya, P.K., and Prema, B.R.,	Unpublished data.
33.	Gildemeister, E., and Hoffmann, F.,	Die, Atherischen ole, 1, 342 (1928).
34.	Wallach, O,	Ann., 227, 300 (1885).
35.	van. Baeyer, A.,	Ber., 29, 3, 2775(1896).
36.	Simonsen, J.L.,	in The Terpenes, Vol.II, Cambridge University Press, Cambridge, 1952.
37.	Barton, D.H.R. in Rodd, E.H. (Editor),	Chemistry of Carbon Compounds, Vol.II, Part B, Elsevier Publishing Co., Amsterdam, 1953, p.563.
38.	Guenther, E.,	in The Essential Oils, Vol.II, D. Van Nostrand, Co., Inc., New York, 1949, p. 54.
39.	Peterson, D.H., Murray, H.C., Eppstein, S.H., Reineke,L.M., Weintraub, A., Meister, P.D., and Leigh, H.M.,	J.Am.Chem.Soc., 74, 5933 (1952).
40.	Badoche, M.,	Bull. Soc.Chim. France, 764 (1953).
41.	Turner, D.L.,	J.Am.Chem.Soc., 76, 5175 (1954).

42.	Bose, A.K.,	J.Org.Chem., 20, 1010(1955).
43.	Moore,R.N., Golumbic,C., and Fisher, G.S.,	J.Am.Chem.Soc., 78, 1173(1956).
44.	Simonsen, J.C.	Ind. For. Rec., 10, 56(1923).
45.	Rao, B.S., and Simonsen, J.L.,	J.Chem.Soc., 127, 2494(1925).
46.	Perkin, W.H., and Thorpe, J.F.,	J.Chem.Soc., 75, 48(1899).
47.	Owen, J., and Simonsen, J.L.,	J.Chem.Soc., 135, 1225(1933).
48.	Guha, P.C., and Sankaran, D.K.,	Ber., 70, 1688(1937).
49.	Krestinski, V., and Solodki, F.,	J.Pr.Chem., 126, ii , 14(1930).
50.	Kishner, N., and Zavadrosky, A.,	J.Soc.Phys-Chem, Russe., 43, 1132 (1911).
51.	Rao, B.S.,	Ind.For.Rec., 9, 203 (1924).
52.	Chapman, A.C.,	J.Chem.Soc., 67, 54, 780(1895).
53.	Balakrishnan, R.K., Razdan, R.K., and Bhattacharyya, S.C.	P. and E.O.R., 47, No.8, 274 (1956).
54.	Benesova, V., Herent, V., and Sorm, F.,	Coll.Czech.Chem.,Commu., 26, No.7, 1832(1961).
55.	Sukh Dev,	Tetrahedron, 9, 1 (1960).
56.	Semmlu, F.W., and Bode, K.,	Ber., 40, 1120 (1907).
57.	Gobolov, P.G.,	J.Soc.Phys.Chem.Russe., 20, 585 (1888).
58.	Gildemeister, E., and Hoffmann, F.,	Die Ätherischen Öle 1, 357 (1928).
59.	Criegee, R.,	Unpublished data. Cited from Richmond, J.E., and Altman,K.I., J.Am.Chem.Soc., 74, 4368(1952).

60.	Bhattacharyya, P.K., Prema,B.R., Dhavalikar,R.S., and Ramachandran, B.V.,	Proc.Ind.Sci.Congress, 1962.
61.	Bhattacharyya, P.K., and Dhavalikar, R.S.,	Unpublished work.
62.	Mitizo Asano and Zisaku Chta,	Ber., 66B, 1020(1933).
63.	Kennedy,G., Breen,J., Keane,J., and Nolan, T.J.,	Sci.Proc.Roy. Dublin Soc., 21, 557 (1937).
64.	Cavallini, D., Frontali,N., and Toscni, G.,	Nature, 163, 568(1949).
65.	Mason, H.S.,	130th meeting, Am.Chem., Soc., 55c (1956).
66.	Holman, R.T.,	Arch.Biochem., 15, 403 (1947).
67.	Hayano, M., Lindberg, M.C., Dorfman, R.I., Hancock,J.E.H., and Doering W. von E.,	Arch.Biochem.Biophys., 59, 529 (1955).
68.	Mason, H.S., Fowlks, W.L. and Peterson, E.W.,	J.Am.Chem.Soc., 77, 2914 (1955).
69.	Tchen,T.T., and Bloch.K.,	J.Am.Chem.Soc., 78, 1516 (1956).
70.	Hayano,M., Saito, A., Stone, D., and Dorfman,R.I.,	Biochim. et.Biophys. Acta, 21, 380(1956).
71.	Bhattacharyya, P.K., Kulkarni, B.D., Godse, D.D., and Mansaram,	Proc. 49th Ind.Sci. Congress, 1962.
72.	Bhattacharyya, P.K. and Prema, B.R.,	Unpublished data.
73.	Perlman, D.,	Abstr., 130th meeting, Am.Chem.Soc., 33A(1956).
74.	Bradshaw, W.H.,	Federation Proc., 18, No.1, 196(1959).
75.	Chance,B., and Fergusson, R.R., in McElroy, W.D., and Glass, B. (Editors),	The mechanismsof Enzyme action, Johns Hopkins Press, Baltimore, 1954.

76.	Thyagarajan, B.S.,	Chem.Rev., 58, 439 (1958).
77.	Mitoma,C., Posner,H.S., Reitz,H.C., and Udenfriend, S.,	Arch.Biochem.Biophys., 61, 431 (1956).
78.	Coulson, C.A.,	J.Chem.Soc., 778(1956).
79.	George, P.,	Biochem.J., 54, 267(1953).
80.	Bender, M.L.,	Chem.Rev., 60, 53 (1960).