BIOSYNTHETIC STUDIES IN PLANT TISSUE CULTURE

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A thesis submitted for the degree of Doctor of Philosophy.

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October 1977.

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To Lesley

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Acknowledgements.

First and foremost I would like to thank my supervisor Professor K.H. Overton for all his help and encouragement throughout the past three years.

I am also extremely grateful to Miss I. Freer for her assistance in culturing the tissues. Without her this work would not have been possible.

Thanks are also due to many other members of the Chemistry Department for spectroscopic services and liquid scintillation counting.

Summary.

The biosynthesis of sesquiterpenoids in Andrographis paniculata callus tissue cultures and in a derived cell-free system has been investigated. The mechanism of trans:cis isomerisation of the C-2, C-3 double bond of farnesol was shown not to involve the aldehydes as intermediates. The mode of cyclisation of farnesyl pyrophosphate in the formation of the bisabolane sesquiterpenoids, paniculides A, B and C, has been elucidated by a combination of ^{13}C and radioactive tracer techniques. Cyclisation of 2-cis, 6-trans-farnesyl pyrophosphate produces Z-γ-bisabolene which is oxygenated by the cultures to the paniculides.

The role of the amino acid leucine in terpenoid biosynthesis has been studied. It was shown that incorporation of radioactivity from labelled leucine into terpenoids takes place via breakdown to acetyl coenzyme A and acetoacetate, and that in this case, isoprenoids are produced by the established route via mevalonate.

The presence of the enzyme leucine 2,3-amino mutase has been demonstrated in the cell-free system. The β-leucine produced by this enzyme has the (3S)-configuration. The reaction is reversible and does not depend on coenzyme B_{12}.

General Introduction.

The origin of plant tissue culture may be traced back to the beginning of this century. In 1902 Haberlandt proposed that it may be possible to culture isolated vegetative cells of higher plants in simple nutrient solutions and that each cell should be genetically capable of giving rise to a complete plant of the type from which the culture had originated (1). He realised that such cultures should give insight into the properties which the cell possesses as a fundamental unit. However, it was not until 1937 that Gautheret and White independently succeeded in establishing cultures of plant cells which were capable of growth for prolonged periods (2,3).

The term tissue culture is normally used as an inclusive term to cover the culture of all types of plant tissues. Two main divisions of the subject may be made: organ culture, where the tissue, when grown in vitro, retains the morphology and organisation of the plant part from which it originated, for example root culture; callus culture, where the tissue grows as a mass of mostly undifferentiated cells in which there is little structural organisation. The present work will be concerned exclusively with callus cultures. Protoplast cultures have also received attention in recent years (4). In this technique, cells are grown without cell walls in a medium whose osmotic potential is carefully controlled. Techniques for the initiation and maintenance of plant tissue cultures are described in a number of excellent monographs and reviews (5-9).

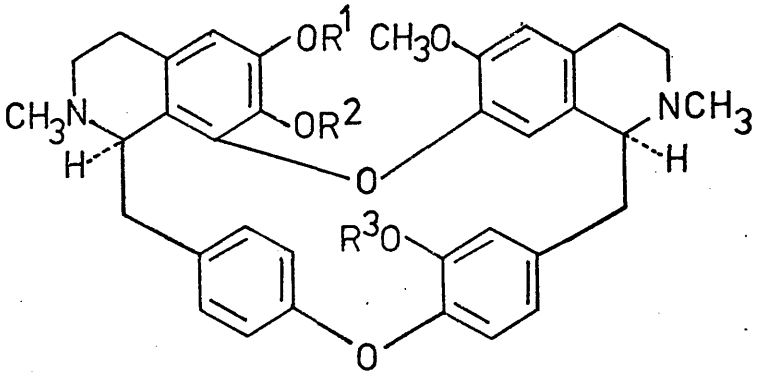
For the chemist, the interest in plant tissue culture lies in two main areas. The production of medicinally

useful secondary metabolites by tissue culture methods, if attainable on an industrial scale, would have several advantages over the traditional methods of plant cultivation (10). Useful compounds might be produced in a controlled environment, independent of climatic conditions. Since there is little seasonal variation in cultured cells, a constant supply of raw material of assured quality would be obtained. Cultures of plants from many different geographical locations could be grown under similar conditions and their metabolism regulated to optimum production of the desired compound. All of these factors should lead to an improvement in productivity and a reduction in the cost of raw materials for the pharmaceutical industry. However, the method has never been used on a significant industrial scale for either the de novo synthesis of medicinal plant products (11,12), or the biotransformation of more advanced but accessible intermediates (13,14).

Cultures which show a sufficiently high growth rate combined with a high yield of metabolite are very rare. Radioimmunoassay techniques have been developed which make possible the rapid quantitative assay of metabolite levels in large numbers of very small cell populations, thus aiding the identification of material which is suitable for further study. The application of these methods to the selection of high yielding strains has recently led to the isolation of cultures in which the level of metabolite is, in some cases, superior to that in the intact plant (15,16). Another major obstacle in applying plant tissue culture on an industrial scale is that cultured plant cells show long-term genetic

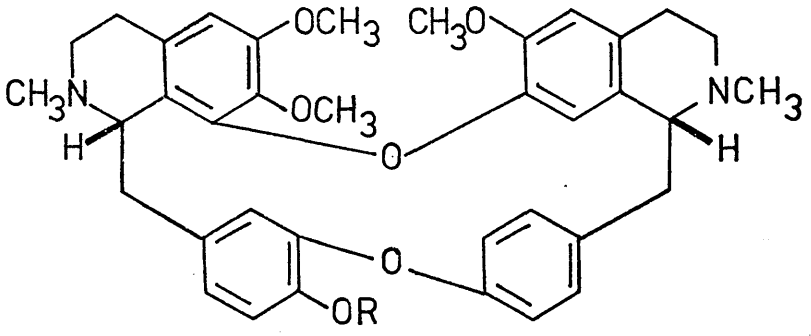
instability (17). This problem could be overcome by establishing banks of stock cultures which could be preserved over a period of some years. A freeze-storage method has been developed which may help solve this problem (18). Despite recent progress, much work remains to be done in this field before plant tissue cultures can be exploited by the pharmaceutical industry on a scale comparable to that possible with microbial fermentations.

The second area of interest for the chemist is in the study of biosynthetic and biodegradative pathways in plant tissue. A major problem in the study of the biosynthesis of secondary metabolites in intact higher plants has been the low level or total failure of precursor incorporation. There are several possible causes including problems of permeability, translocation, and the segregation of metabolic pools. Tissue cultures possess a very much simpler organisation, so that compared with higher plants these problems should be greatly reduced. They can be grown under standard conditions, have short growth cycles and show little seasonal variation. Incorporation and turnover of labelled precursors can be studied over short periods of time since the system is more dynamic than a mature plant. This is of particular advantage in biosynthetic studies. The strict requirement for sterility of both medium and plant tissue ensures that the metabolic activity observed is really a property of the plant tissue and not of microorganisms associated with it. Cell-free systems and purified enzyme preparations are quite easily obtained from cultured cells possibly because of the absence of phenols and quinones which cause inactivation of enzymes during their extraction



(1) $R^1+R^2=CH_2$ $R^3=CH_3$

(3) $R^1=CH_3$ $R^2=R^3=H$

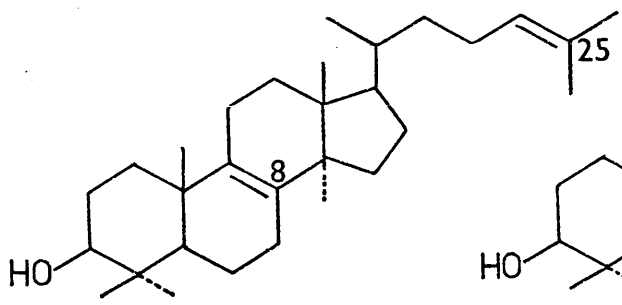


(2) $R=CH_3$

(4) $R=H$

from intact plants. A unique advantage possessed by callus cultures is that under the appropriate hormonal stimulus they may frequently be induced to regenerate a plant organ or a whole plant which is in many respects comparable to the intact plant (19). This capacity offers the opportunity to study metabolic processes at various stages of differentiation.

Despite these advantages, tissue cultures have been relatively little used for biosynthetic studies. The establishment of a culture showing good growth and vigorous metabolism can be a lengthy process requiring perhaps years of largely empirical experimentation to establish the necessary culture conditions. A more serious disadvantage is that tissue cultures sometimes do not synthesise the metabolites characteristic of the intact plant or do so in only minute amounts. It must always be borne in mind that a biosynthetic scheme which has been found to operate in a tissue culture may not be exactly the same as that which exists in the intact plant. The differences between the metabolism of cultures and intact plants are most apparent when different substances are produced. These differences are mostly of a minor nature and involve only a simple structural change of the metabolite. For example, it has been found (20) that cultured cells of Stephania cepharantha lack the enzymes necessary for methylation and methylenedioxy group formation in the final steps of the biosynthetic route leading to the biscoclaurine alkaloids cepharanthine (1) and isotetrandrine (2) and accumulate instead the intermediate alkaloids aromoline (3) and berbamine (4). In such cases it is most unlikely that the early stages of biosynthesis



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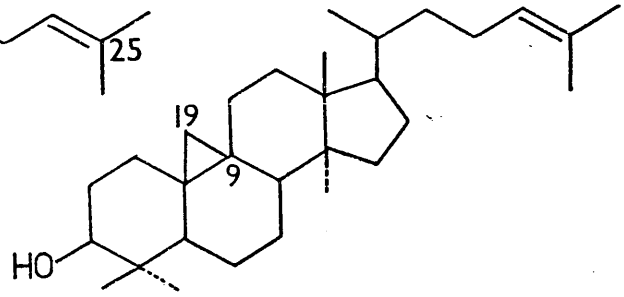

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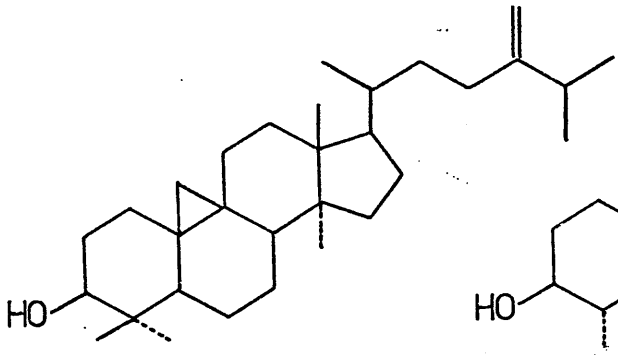

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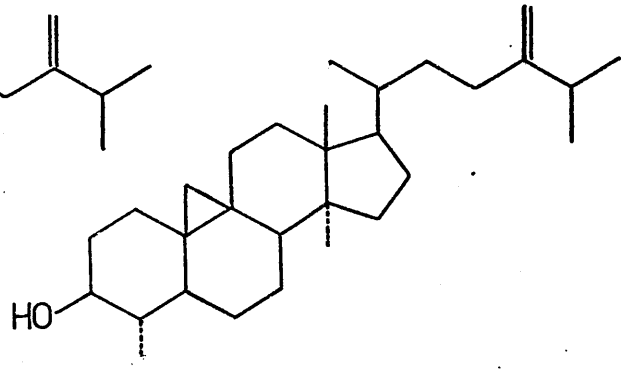

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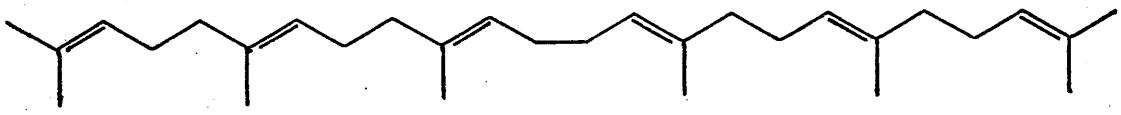

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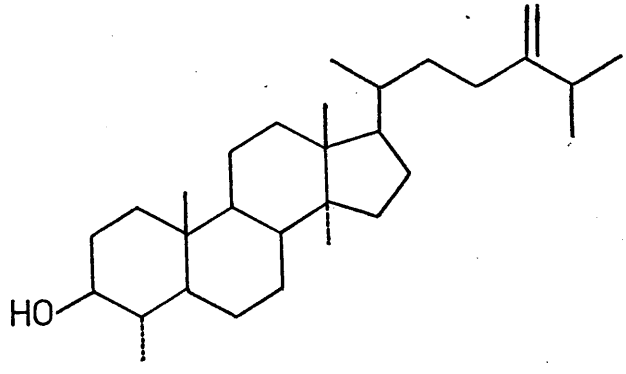


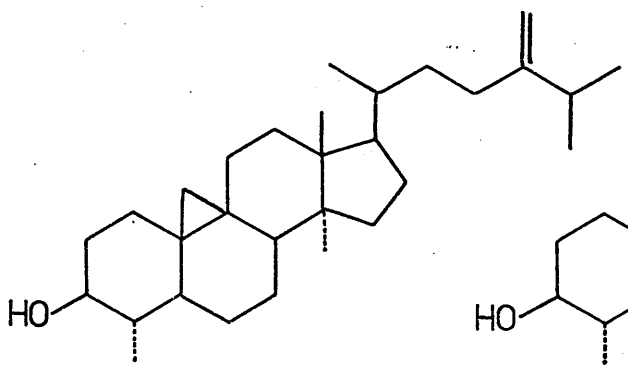
(10)

differ significantly in the two systems. Even if the assumption is wrong, this in no way invalidates biosynthetic studies in such cultures which should be regarded as legitimate biological systems for study in their own right. The usefulness of tissue cultures in the study of secondary metabolism in plants is illustrated in the following selected examples.

In what are probably the most extensive biosynthetic investigations to date with plant tissue cultures, Ourisson, Benveniste and their colleagues have studied the biosynthesis of plant sterols, using tissues of Nicotiana tabacum, (tobacco) and Rubus fruticosus, (bramble).

Lanosterol (5) has been shown to be the precursor of steroids in animals and fungi (21), but it has never been found in higher plants. The frequent occurrence of cycloartenol (6), 24-methylenecycloartanol (7) and cycloeucalenol (8) in photosynthetic plant tissues has led to the suggestion that 9β, 19β-cyclopropylsterols may take the place of lanosterol in plant sterol biosynthesis (22,23).

Incubation of [1-^{14}C]acetate or [$^{14}CH_3$]methionine with tobacco tissue cultures for as short a time as five minutes resulted in incorporation of radioactivity into intermediates of sterol biosynthesis (24,25). Lanosterol could not be detected, while cycloartenol was rapidly and heavily labelled although it was present only in minute concentrations. It was possible to identify, in addition to phytosterols, squalene (9), cycloartenol (6), 24-methylenecycloartanol (7), 24-methylenelophenol (10) and its ethylidene homologue, and to show that the order of labelling was squalene, cycloartenol 24-methylenelophenol and



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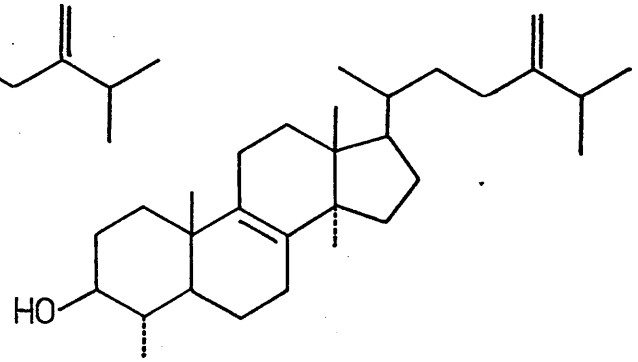

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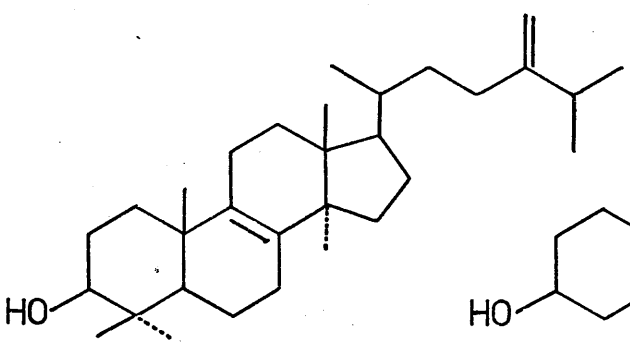

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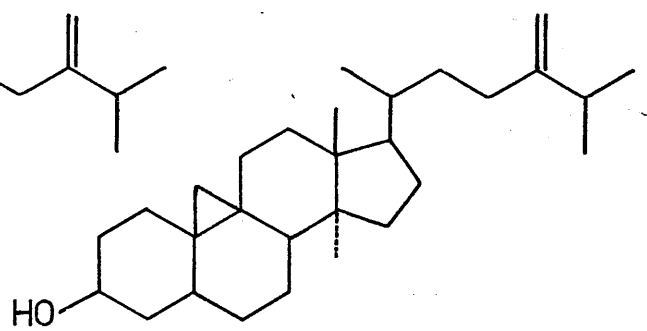


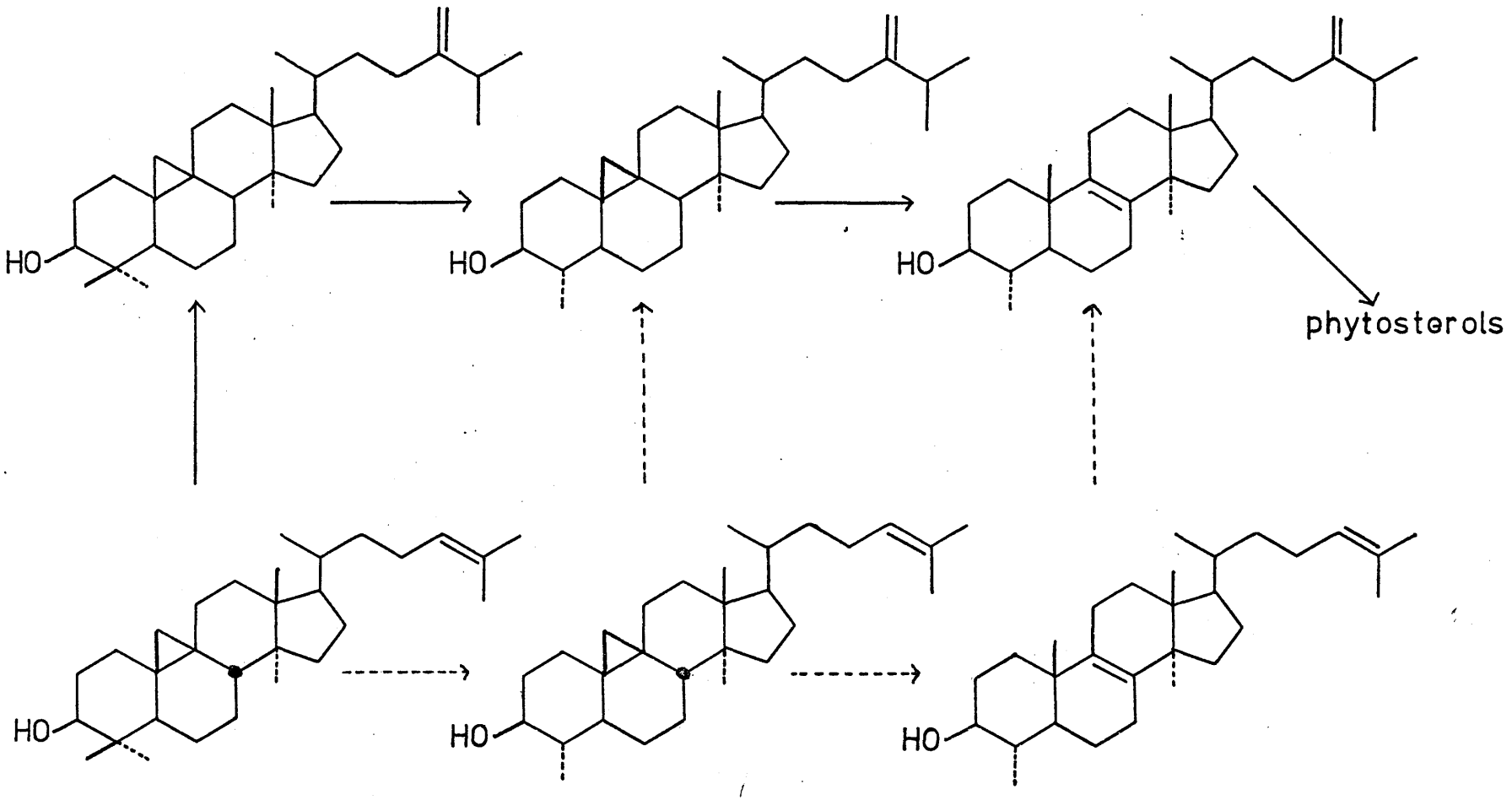
(13)

phytosterols. Cycloeucalenol (8) and obtusifoliol (11) were subsequently also identified in tobacco tissue cultures and were shown (26) to incorporate label from [1-^{14}C]acetate, supporting their possible role as intermediates in phytosterol biosynthesis.

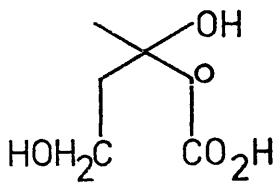
When [25-^{14}C]cycloartenol was incubated with tobacco tissue cultures it was converted into all the phytosterols previously found to be produced from acetate (27). Lanosterol was metabolised to 24-methylenelanost-8-enol (12) and obtusifoliol (11), but this can probably be ascribed to a lack of specificity in the enzymes responsible for C-24 methylation and C-4 demethylation. Thus cycloartenol and not lanosterol must play a central role in plant sterol biosynthesis.

A key step in the biosynthetic pathway must involve cleavage of the 9β, 19β cyclopropane ring. The presence of cycloeucalenol (8) and obtusifoliol (11) suggests that the former may be the substrate in this step. The enzyme which opens the cyclopropane ring has been obtained (28) in microsomal preparations from tobacco and bramble tissues. 4,4-Dimethylsterols are very poor substrates, while cycloeucalenol (8) and 24-methylenepollinastanol (13) are good substrates, which suggests that the 4β-methyl group of 4,4-dimethylsterols inhibits the enzyme. Since neither cycloartenol nor 24-methylenecycloartanol is a substrate, the major pathway leading to phytosterols probably does not involve lanosterol or 24-methylenelanosterol. These results, taken with other relevant information, have led to the conclusion that phytosterol biosynthesis does not follow a unique route but can proceed by a number of

SCHEME 1



o denotes ^{14}C



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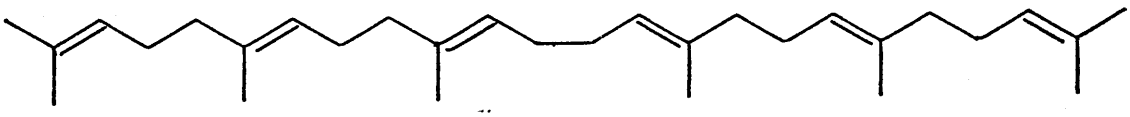

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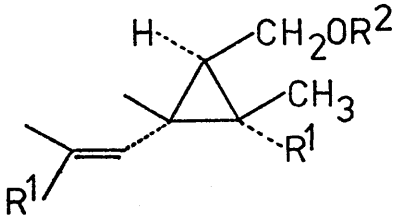
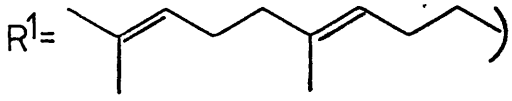
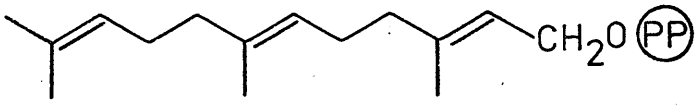




(15) $R^2 = P_2O_6 =$ (PP)

(16) $R^2 = H$



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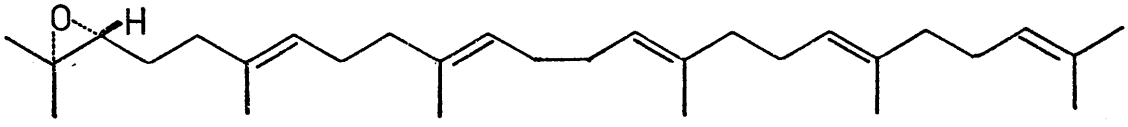

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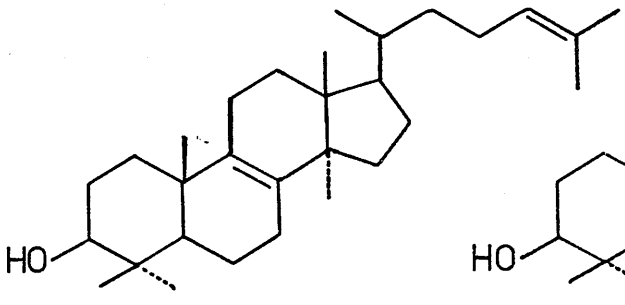
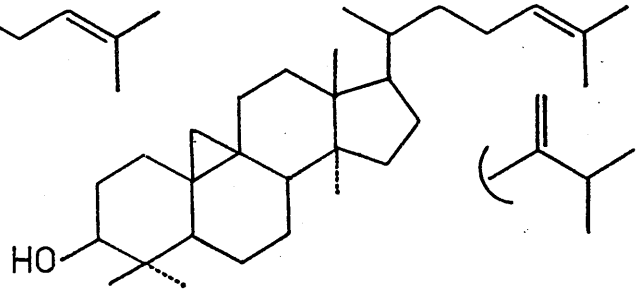




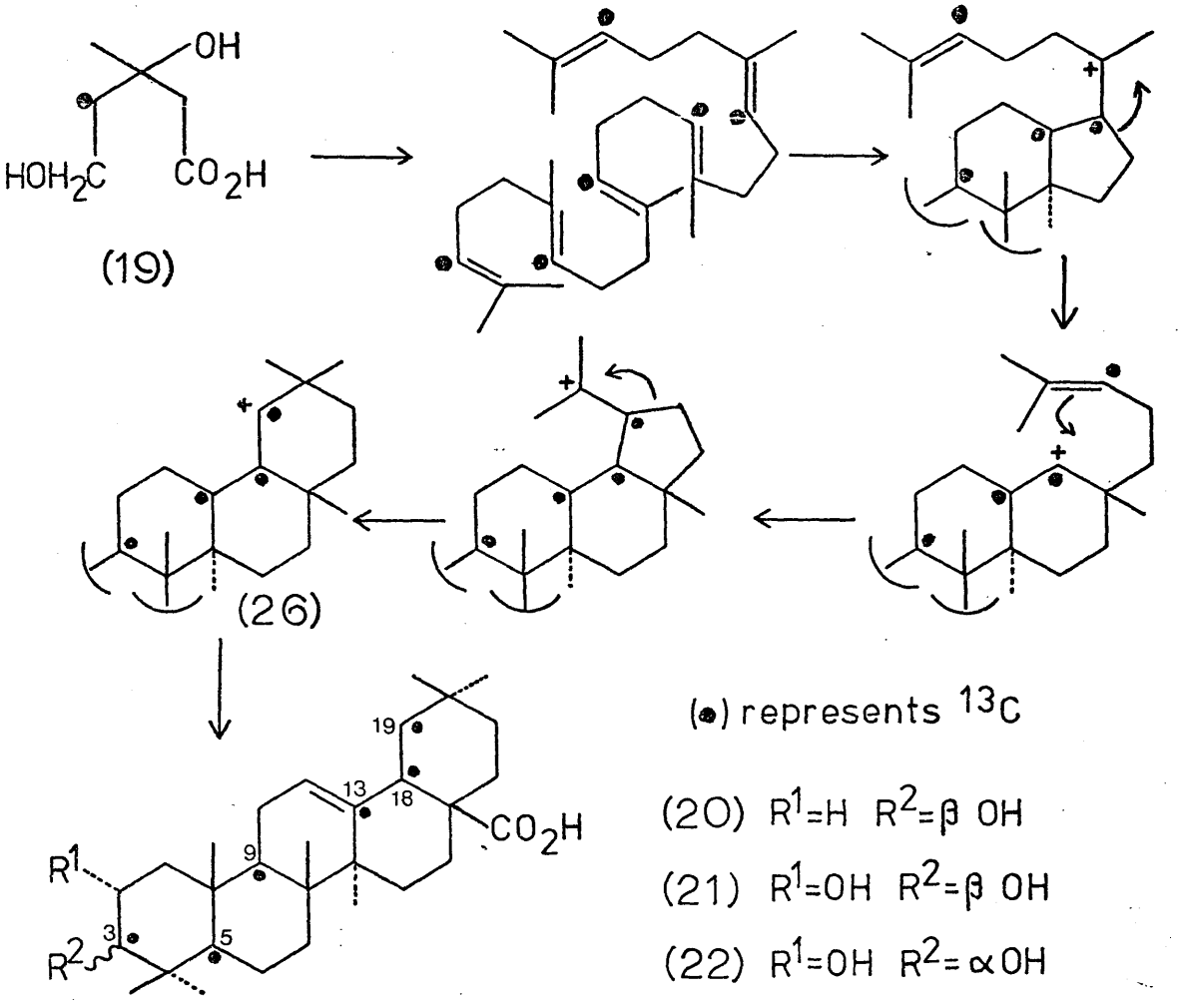
(5) (6) (7)

interrelated routes as shown in scheme 1. A similar metabolic grid has been proposed for yeast sterol biosynthesis (29).

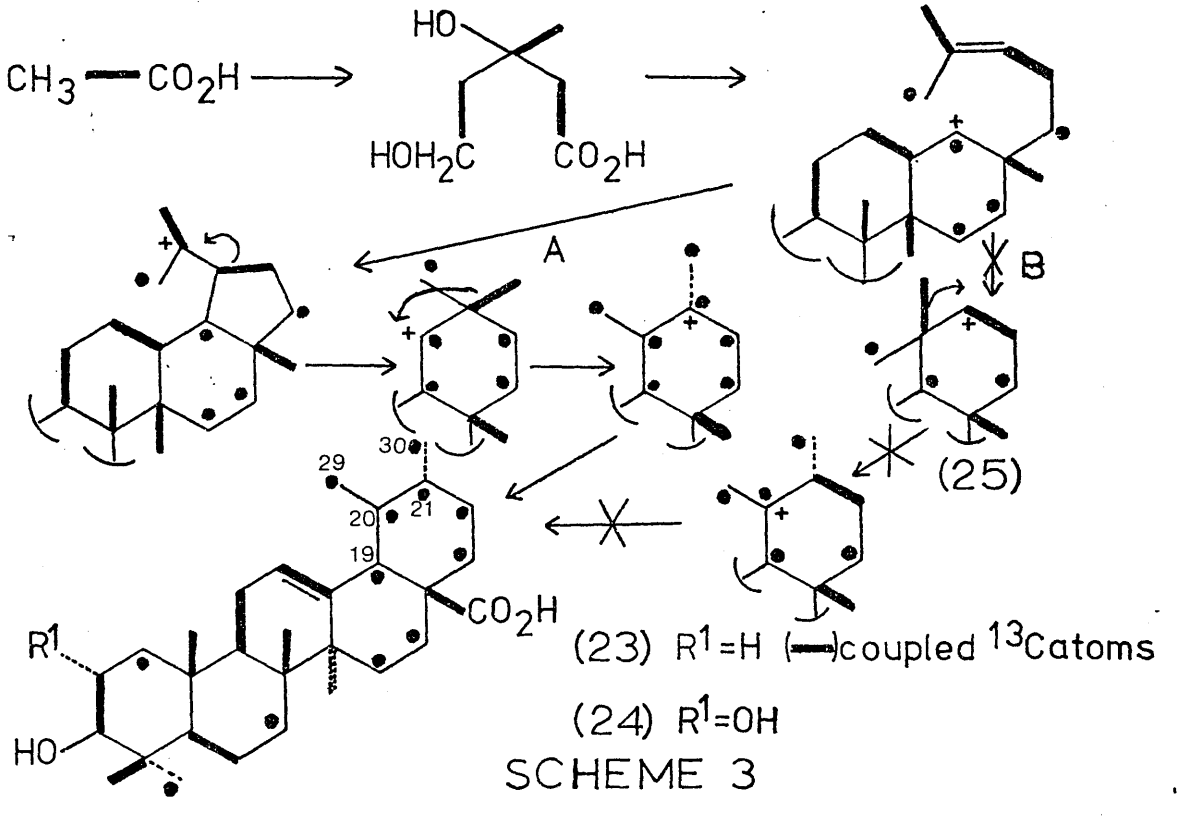
A cell-free system prepared from bramble tissue cultures has been shown (30) to incorporate label from [2-^{14}C] mevalonate (14) into squalene (9) in the presence of a number of cofactors including reduced nicotinamide adenine dinucleotide (NADH). However, when the cofactor was omitted from the incubation, squalene was only poorly labelled. The product of the second experiment was identified as presqualene pyrophosphate (15), which was isolated as the corresponding alcohol (16) after hydrolysis by alkaline phosphatase (E C 3.1.3.1). Presqualene pyrophosphate has also been implicated in animal steroid biosynthesis and it is probably the intermediate between 2-trans, 6-trans-farnesyl pyrophosphate (17) and squalene (9).

The cyclisation of squalene in animal systems is known to proceed via (3R)-2, 3-oxidosqualene (18). The synthesis of this intermediate in tobacco tissues has been demonstrated (31) by trapping experiments in which radioactivity from [1-^{14}C]acetate was detected in added oxidosqualene. It was also shown that 2, 3-oxidosqualene was converted into cycloartenol (6) and 24-methylenecycloartanol (7) but not into lanosterol (5) by both tobacco (32) and bramble (33) cultures.

Triterpenoid biosynthesis has been studied by Tomita and his colleagues using cultures of Isodon japonicus. Their experiments exploit the high incorporations of precursors obtainable with tissue cultures to study the labelling pattern in secondary metabolites of higher plants



SCHEME 2



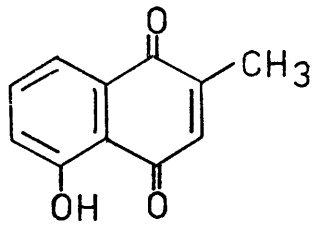
SCHEME 3

by ^{13}C NMR spectroscopy. Incorporation (34) of [4-^{13}C] mevalonate (19) into oleanolic (20), maslinic (21) and 3-epi-maslinic (22) acids showed in the ^{13}C NMR spectra of the methyl ester derivatives enhancement of the signals assigned to C-3, C-5, C-9, C-13, C-18 and C-19. This result is in accordance with the formation of β-amyrin from squalene as indicated in scheme 2.

Later work (35,36) used [1,2-$^{13}C_2$]acetate to investigate ursane biosynthesis in the same cultures. Two pathways (A) and (B) may be considered for the biosynthesis of ursolic (23) and 2α-hydroxyursolic (24) acids (scheme 3). The ^{13}C NMR spectra of the methyl esters of (23) and (24) obtained from Isodon japonicus cultures which had been fed with [1,2-$^{13}C_2$]acetate showed singlets for the signals ascribed to C-19, C-20, C-21, C-29 and C-30. This result excludes an intermediate of type (25) from the biosynthetic pathway which thus proceeds by route (A) and not route (B). The ursanes and oleananes are therefore biosynthesised via a common intermediate (26).

A major route by which aromatic compounds are degraded in plants has been examined in detail by Ellis and by Durand and Zenk. Although the homogentisate pathway for tyrosine degradation was well-known in animals and in microorganisms, its unequivocal demonstration in plant tissues was difficult because of the bacteria which are always associated with plants and which may be able to metabolise tyrosine very rapidly by this route. Tissue cultures offer a ready source of sterile plant tissue which has a vigorous metabolism.

[β-^{14}C]Tyrosine (27) was efficiently incorporated (37) into the naphthaquinones plumbagin (28) and 7-methyljuglone (29)



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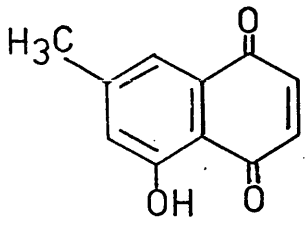

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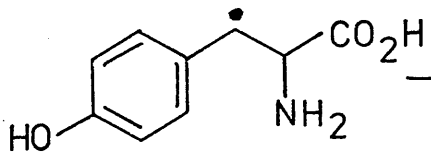

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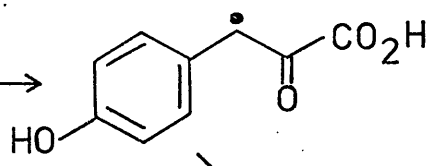
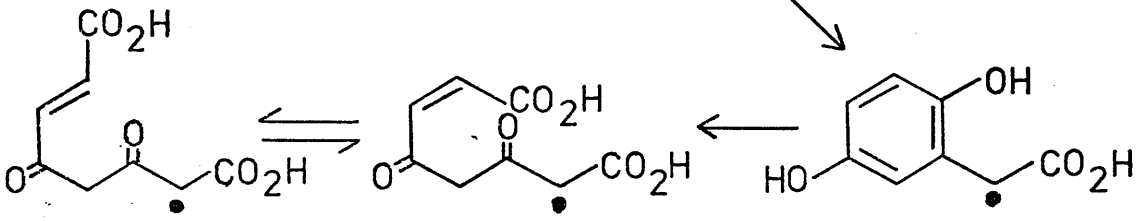
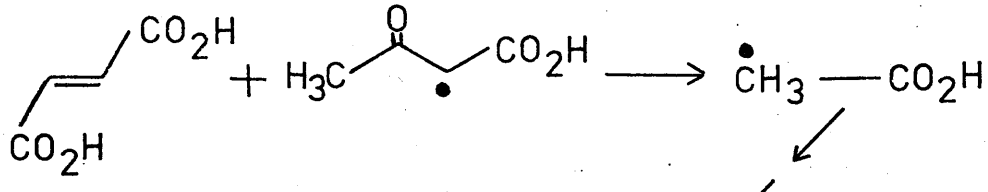
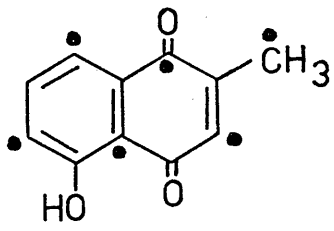




(32) (31) (30)



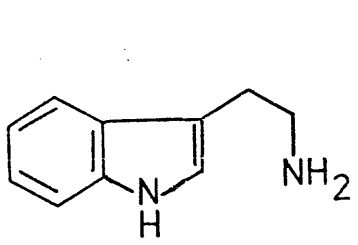


(•) represents ^{14}C

SCHEME 4

by cultures of Drosophyllum lusitanicum. However, degradation revealed that the label from tyrosine appeared at alternate carbon atoms in both metabolites. Label must have been incorporated from acetate via the acetate-malonate pathway which implies that tyrosine must have been degraded to acetate by the homogentisate pathway. [2-^{14}C] Homogentisic acid (30), [2-^{14}C] 4-maleyl- (31) and [2-^{14}C] 4-fumaryl- (32) acetoacetates were all incorporated into plumbagin (28). Administration of the homogentisate oxygenase inhibitor αα'-bipyridyl in the presence of [β-^{14}C] tyrosine resulted in the accumulation of labelled homogentisate. These results are consistent with the degradation of tyrosine by the homogentisate pathway as shown in scheme 4. The presence of all the necessary enzymes has been demonstrated (38) in tissue homogonates. The key enzyme for ring-cleavage, homogentisate oxygenase (E C 1.13.1.5), has been purified 190-fold from cultures of D. lusitanicum and its presence has been detected in tissue cultures of ten other plants. The homogentisate pathway for cleavage of aromatic rings thus appears to be widespread in higher plants.

Callus cultures of Catharanthus roseus have been thoroughly investigated for the production of indole alkaloids, since the plant contains over sixty alkaloids of which four possess moderate anti-tumour activity. Only ten indole alkaloids were detected in the cultures (39), none of which was of the dimeric type characteristic of the plant. The very low levels of incorporation of precursors into indole alkaloids using whole plants of the genera Vinca, Catharanthus and Aspidosperma have severely hampered



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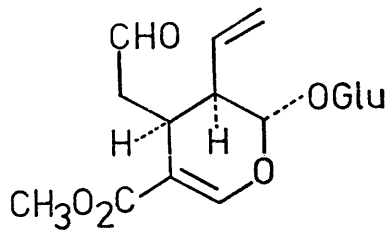

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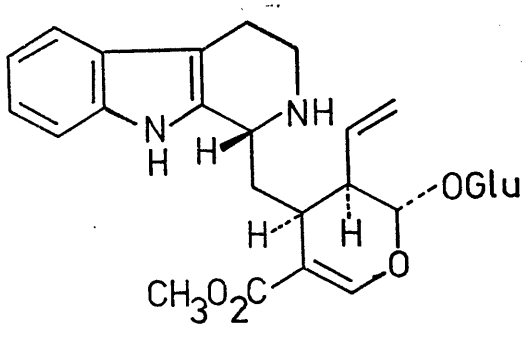

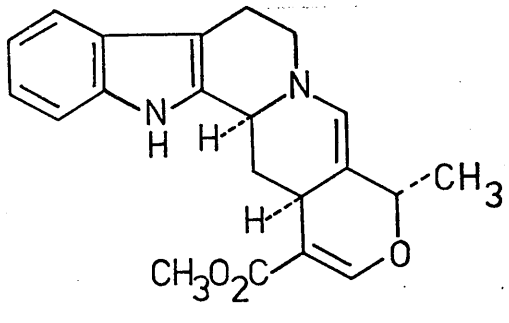

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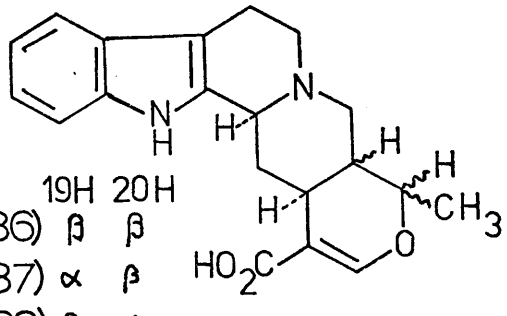
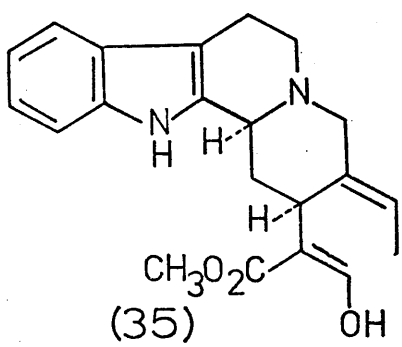


19H 20H
(36) β β
(37) α β
(38) β α



(35)

SCHEME 5

biosynthetic studies. Incubation of cell-free preparations from both seedlings and tissue cultures of Catharanthus roseus with either [2-^{14}C]tryptamine (33) or [OC-$^{3}H_3$] secologanin (34) incorporated (40) label into the Corynanthé alkaloids geissoschizine (35) and ajmalicine (36). The callus-derived system also converted geissoschizine (35) into ajmalicine (36). Incorporations with the cell-free preparation from cultured tissue were much superior to those from seedlings. Other workers have recently reported (41) that a cell-free preparation from fermentor-grown C. roseus cells is able to synthesise ajmalicine (36), 19-epi-ajmalicine (37) and tetrahydroalstonine (38) from tryptamine and secologanin in the presence of either NADPH or NADH. In the absence of reduced pyridine nucleotides, however, precursors of the ajmalicine isomers accumulated. One of these precursors has now been identified (42) as cathenamine (39) on the basis of spectroscopic evidence and it has been shown to be incorporated into (36), (37) and (38) on incubation with the cell-free system in the presence of NADPH. The proposed biosynthetic pathway is shown in scheme 5.

The literature contains many further examples of the use of plant tissue cultures in the study of the biosynthesis of secondary plant metabolites such as steroidal sapogenins, fatty acids, flavonoids, anthocyanidins, ret rochalcones, anthraquinones, coumarins and alkaloids. A review of the subject is in press (43). Reviews are available on compounds isolated from tissue cultures (44) and on the biotransformation of steroids by tissue cultures (45).

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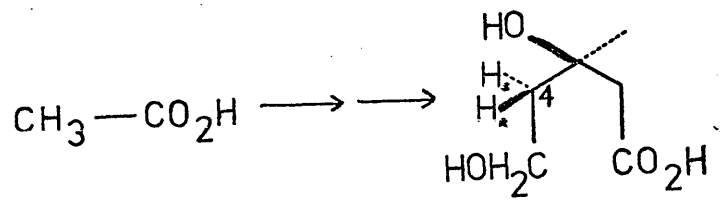
SECTION I

THE BIOSYNTHESIS OF 2-*CIS*, 6-*TRANS*-FARNESOL



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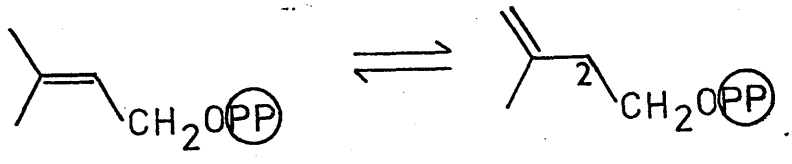

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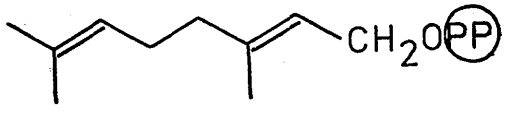

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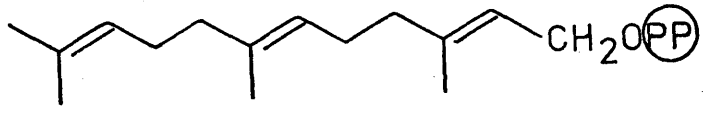

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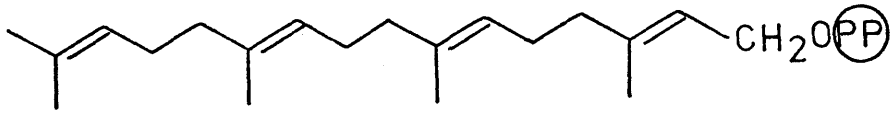

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SCHEME 1

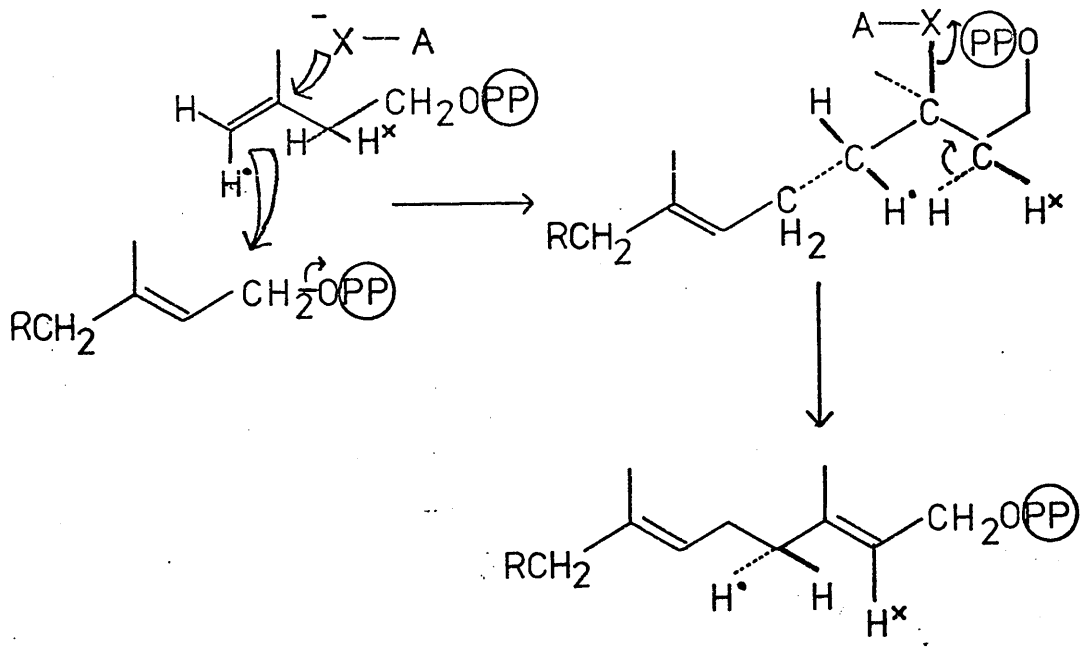
Section 1.

The Biosynthesis of 2-cis, 6-trans-farnesol.

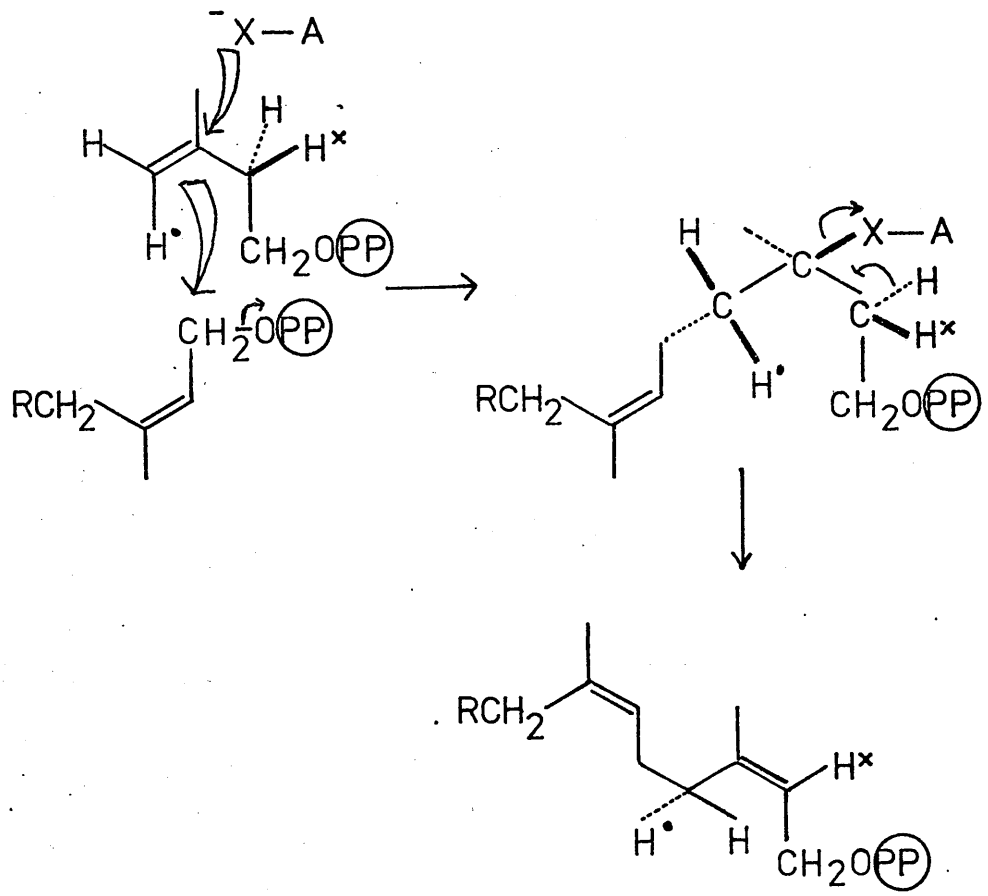
Introduction.

Terpenoid biosynthesis has been the subject of intense investigation for over twenty years and although rapid progress has been made in the area, many intriguing problems remain to be solved. The basic pathway of terpenoid biosynthesis from acetate via (3R)-mevalonate (1) to isopentenyl (2), dimethylallyl (3), geranyl (4), farnesyl (5) and geranylgeranyl (6) pyrophosphates is shown in scheme 1. The pathway is now well-established (1) and provides a mechanistic rationalisation of the biogenetic isoprene rule proposed by Ruzicka (2).

The C_{15} compound farnesyl pyrophosphate (5) is the progenitor of all sesquiterpenoids, and its biosynthesis from mevalonate has been studied in complete stereochemical detail because of its intermediacy in the formation of steroids. This has been made possible by the use of samples of mevalonate in which each prochiral hydrogen atom has been substituted in turn by deuterium or tritium (3). The isoprenoid chain is built up by the enzyme prenyl transferase (E C 2.5.1.1.) which catalyses the condensation of isopentenyl pyrophosphate (2) with the allylic pyrophosphates (3) - (5). In this process, one of the protons from C-2 of isopentenyl pyrophpsphate is lost, corresponding to loss of one of the protons at C-4 of mevalonate. As one would expect for an enzymic process, this has been shown to be stereospecific. It was found that in the formation of trans double bonds the pro-4S proton of mevalonate was lost, while the pro-4R proton was retained (4). Exactly the opposite

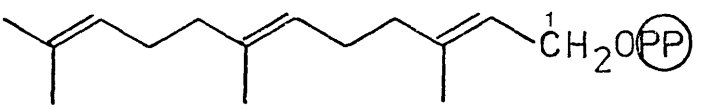


trans-prenyl transferase



cis-prenyl transferase

SCHEME 2



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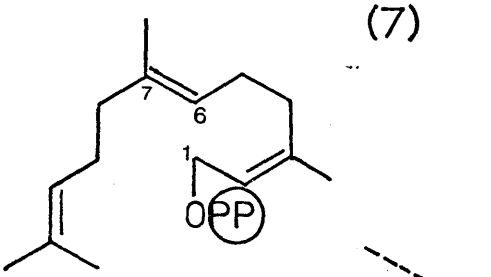

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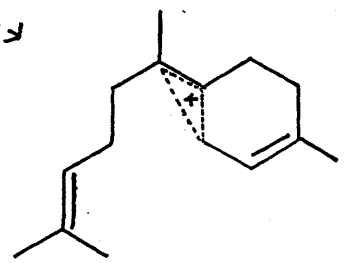

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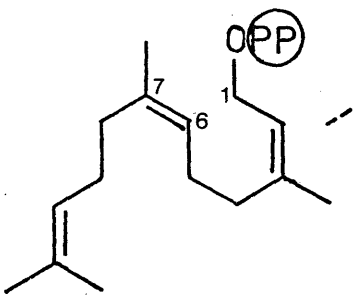

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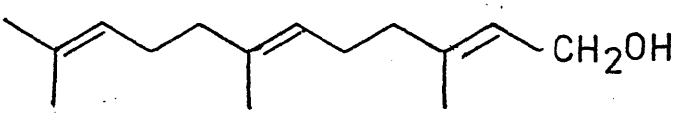

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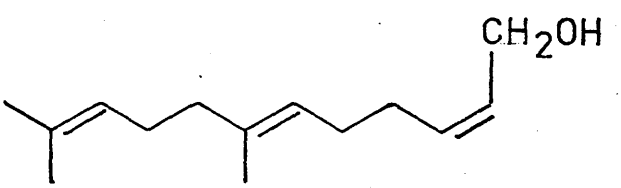
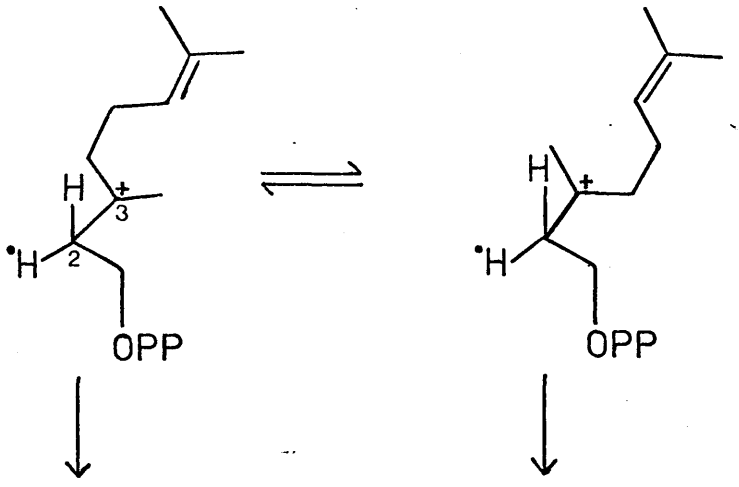


(12)

situation holds in the formation of the cis double bonds in rubber (5) where the pro-4S proton is retained. The mechanisms of action of the two types of prenyl transferase proposed (6) by Cornforth and Popjak are shown in scheme 2.

While 2-trans, 6-trans-farnesyl pyrophosphate (5) is the geometrical isomer which is involved in the formation of squalene (7), it is 2-cis, 6-trans-farnesyl pyrophosphate (8) which on simple geometrical grounds must be the precursor in the initial cyclisation leading to many sesquiterpene skeletons. Clearly, cyclisation which is initiated by nucleophilic attack of the 6,7 double bond at C-1 of farnesyl pyrophosphate, is only geometrically feasible for the cis, trans isomer and not for the trans, trans isomer. It is worth noting that the non-classical carbonium ion (9) which is generated by this process could also be produced in a similar manner from 2-cis, 6-cis-farnesyl pyrophosphate (10).

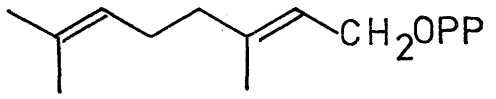
Although cis, trans-farnesyl pyrophosphate had been suggested as a precursor of sesquiterpenoids many years ago, it is only relatively recently that direct information on its biosynthesis has been forthcoming. Both trans, trans-farnesol (11) and cis, trans-farnesol (12) were labelled (7) when [2-^{14}C]mevalonate was incubated with a cell-free system from Pinus radiata seedlings. The pyrophosphate of the trans, trans-isomer was also detected, but there was no evidence of the presence of phosphorylated derivatives of the cis, trans-isomer. No isomerisation of the pyrophosphates (5) and (8) could be detected. Later experiments with the same system and in a cell-free preparation from Citrus sinesis (8) showed that the pro-4S proton of



cis trans

•H derived from pro-4S of mevalonate

SCHEME 3



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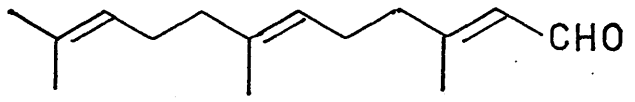

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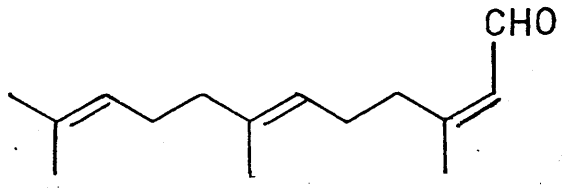
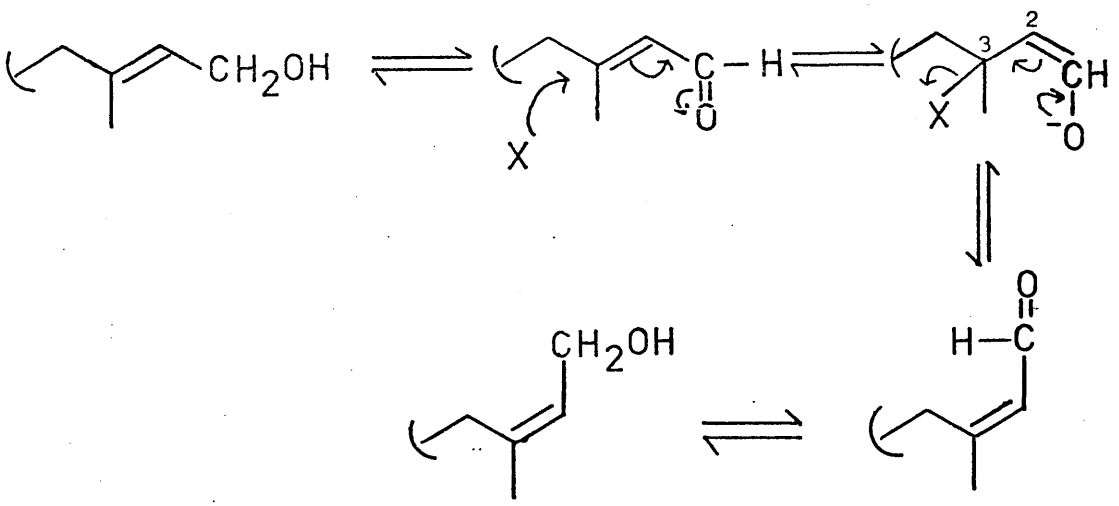


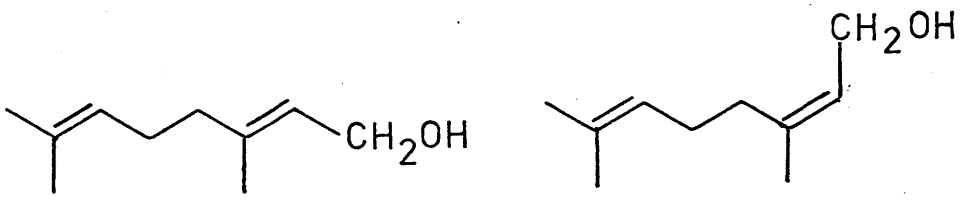
(14)

mevalonate was lost during formation of both the 2-*trans* and the 2-*cis* double bonds of the farnesol isomers (11) and (12). This indicates that a *trans* prenyl transferase is responsible for the formation of both the *trans* and the *cis* double bonds. Because no interconversion of the geometrical isomers could be detected in these experiments, Cori and his co-workers suggested that during formation of farnesyl pyrophosphate, rotation about the 2,3 bond of any intermediate carbonium ion could give rise to both geometrical isomers of the product (scheme 3). In this way, the *pro*-4S proton of mevalonate is lost, but a *cis* double bond is formed. This is equivalent to *syn* elimination of hydrogen and X group according to the mechanism shown in scheme 2. Such a hypothesis is supported by the low specificity (9) which prenyl transferase shows for the long-chain part of the allylic substrate. This suggests that it is only weakly bound to the active site of the enzyme, which may make rotation possible.

In further work by Cori (10), using a cell-free extract of orange flavedo, geranyl pyrophosphate (4) was shown to be a precursor of the sesquiterpene alcohols. In this case, the aldehydes 2-*trans*, 6-*trans*-farnesal (13) and 2-*cis*, 6-*trans*-farnesal (14) were detected in addition to the alcohols. Sequential labelling studies showed that the order of synthesis was *trans*, *trans*-farnesol, *trans*, *trans*-farnesal, *cis*, *trans*-farnesal and *cis*, *trans*-farnesol. Again no isomerisation of the pyrophosphates was observed. This result strongly suggests that the aldehydes (13) and (14) are involved in the interconversion of the geometrical isomers, possibly by Michael addition of a nucleophilic



SCHEME 4



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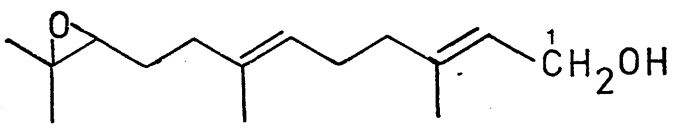

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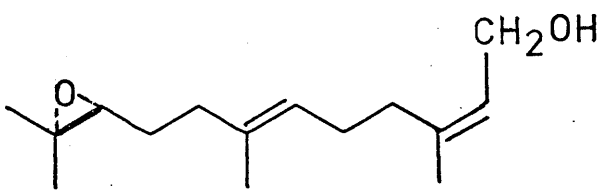

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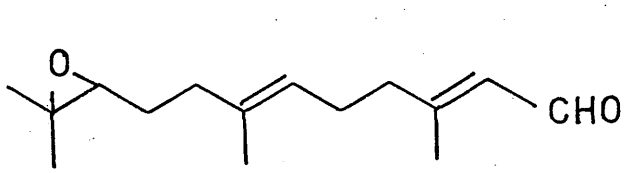

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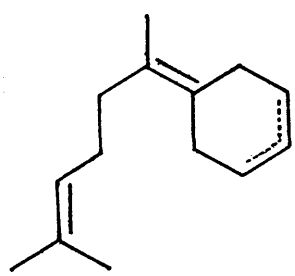

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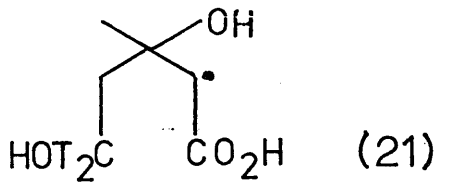

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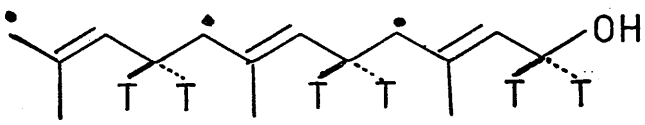

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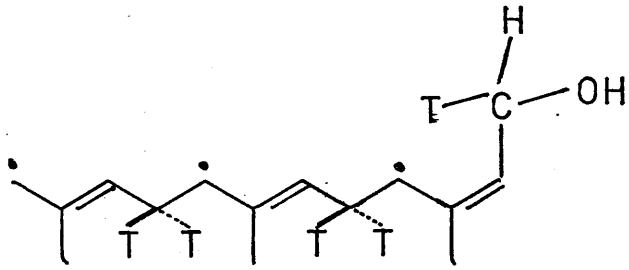


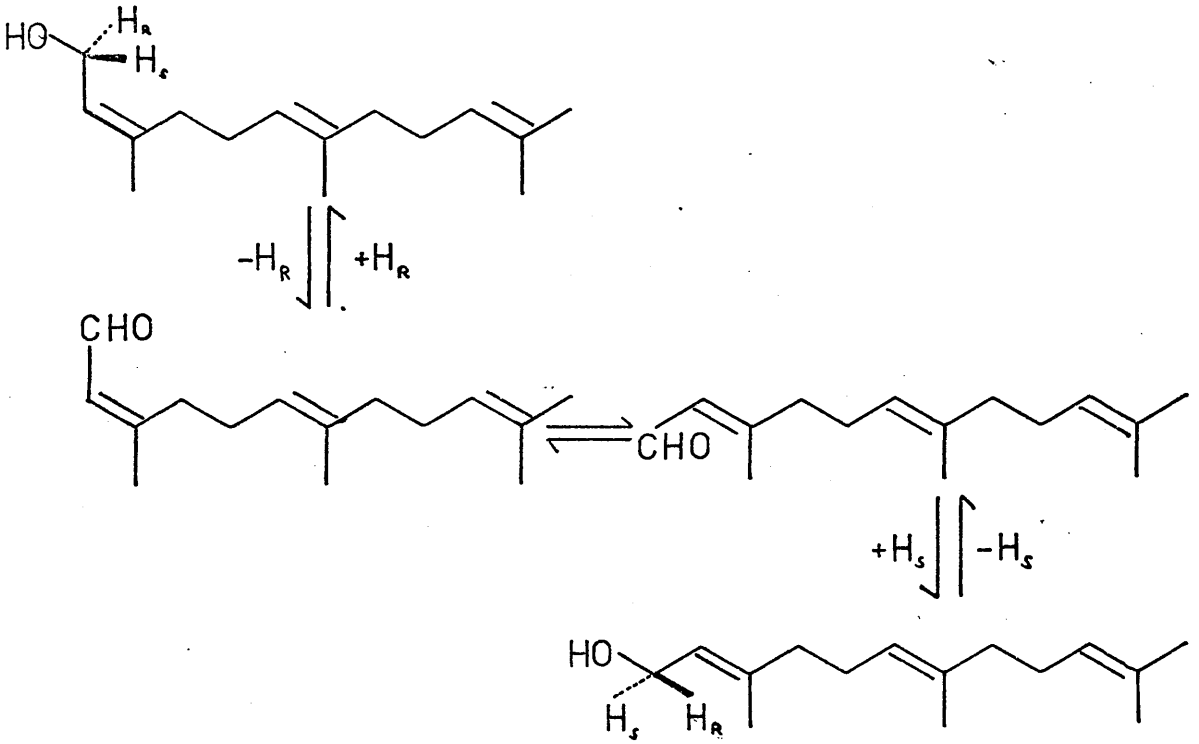
(23)

X group, rotation about the 2,3 bond and subsequent reversal of the X group addition (scheme 4). A similar redox mechanism for trans to cis double bond interconversion has been demonstrated (11) in the case of the monoterpenoids geraniol (15) and nerol (16).

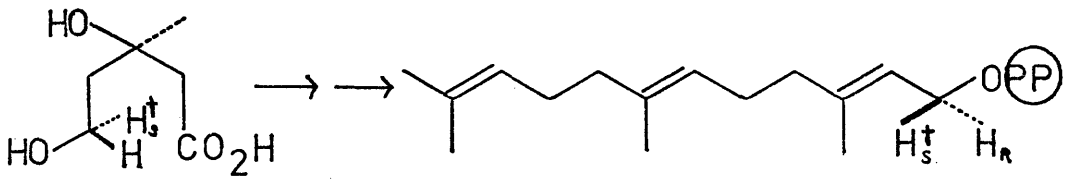
During an examination of the metabolites of 2-trans, 6-trans-10,11-epoxyfarnesol (17) in Helminthosporum sativum Suzuki and Marumo found (12) that one of the products was the corresponding cis, trans isomer (18). It was shown that one of the hydrogen atoms at C-1 of the trans, trans compound (17) was lost in the isomerisation and that 2-trans, 6-trans-10,11-epoxyfarnesal (19) was a precursor of the cis, trans-isomer (18). Similar results were also obtained with farnesol.

Work on the biosynthesis of farnesol has been carried out in these laboratories using a cell-free system prepared from Andrographis paniculata callus tissue cultures. This cell-free system incorporates radioactivity from mevalonate into trans, trans-farnesol, cis, trans-farnesol and ϒ-bisabolene (20). It was found (13) that the pro-4R hydrogen of mevalonate was retained, and the pro-4S hydrogen lost in both geometrical isomers of farnesol, as in the studies of Cori's group. When (3RS)-[2-^{14}C, 5-$^{3}H_2$]mevalonate (21) was incubated with the cell-free system, the trans, trans-farnesol (22) isolated had retained virtually all the tritium present in the mevalonate, while the cis, trans-farnesol (23) had lost one-sixth of the tritium label. This implies that cis, trans-farnesol is formed via the trans, trans-isomer and not the reverse.

The cell-free system was also able to catalyse



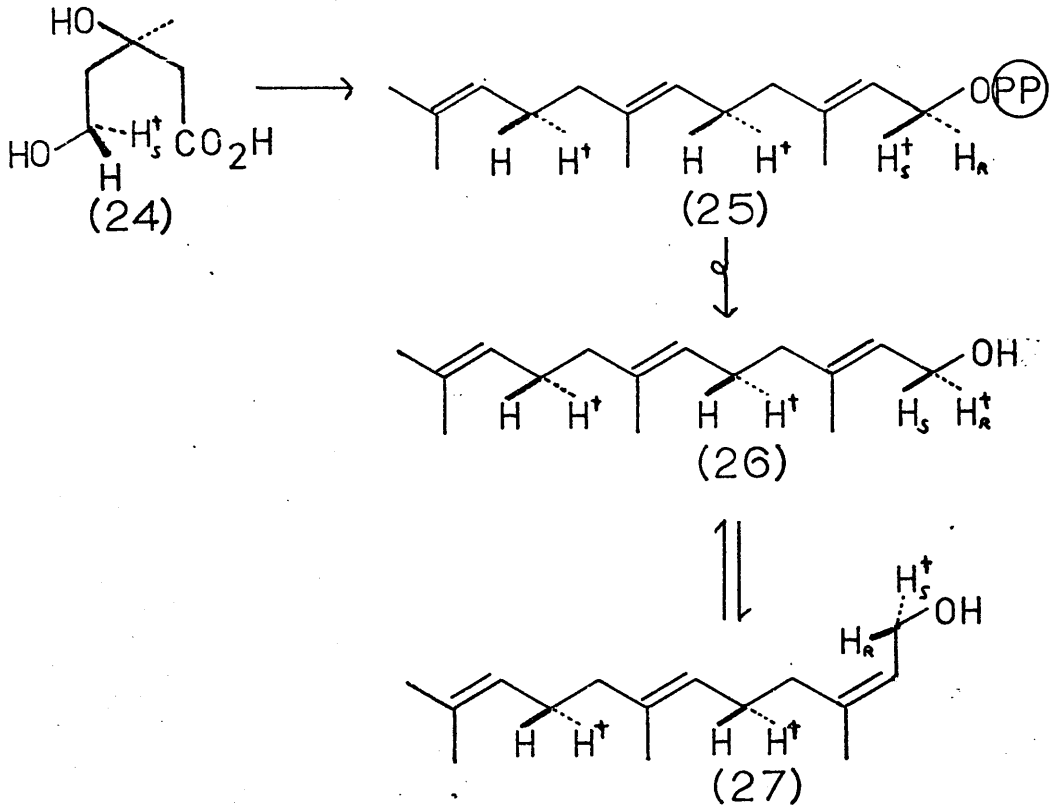
SCHEME 5



SCHEME 6

reversible cis: trans isomerisation of farnesol. When [4,8,12,-$^{14}C_3$, 1-$^{3}H_2$]trans, trans-farnesol was isomerised to cis, trans-farnesol, half of the tritium label was lost (14). The same loss occurred on isomerisation in the opposite direction. These results are in accord with isomerisation via aldehydes as shown in scheme 5. The steroechemistry of hydrogen loss from C-1 during isomerisation was next investigated, using samples of trans, trans- and cis, trans-farnesol, each specifically tritiated at either the pro-1R or the pro-1S position. The unexpected result is that the pro-1S hydrogen is lost in the conversion of trans, trans- into cis, trans-farnesol but the pro-1R hydrogen is lost in the reverse reaction.

Later experiments (15) exposed the possibility that the Andrographis cell-free extract may be able to catalyse trans: cis isomerisation of the pyrophosphates. Cornforth has demonstrated (16) that in mammalian liver preparations the pro-5S hydrogen of mevalonate should become the pro-1S hydrogen of trans, trans-farnesyl pyrophosphate (scheme 6). In the Andrographis system, it was shown that the pro-5S proton of mevalonate was retained in both trans, trans- and cis, trans-farnesol. Thus, the pro-1S hydrogen of trans, trans-farnesyl pyrophosphate survives in cis, trans-farnesol. From the previous work which has been outlined above, the pro-1S hydrogen of trans, trans-farnesol should be lost during isomerisation. One possible explanation of these results is that the cell-free system contains an enzyme which hydrolyses the initially formed trans, trans-farnesyl pyrophosphate with inversion at C-1, which implies that there is C-O bond fission during hydrolysis. Label

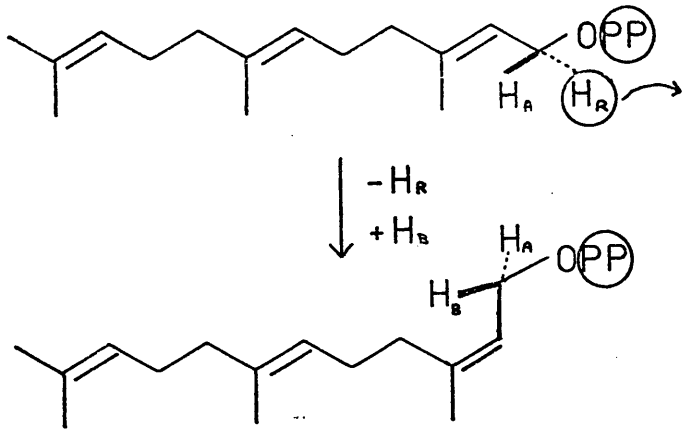

HO
HO
$H_S^{\dagger}$
CO_2H
H
(24)
OPP
H $H^{\dagger}$ H $H^{\dagger}$ $H_S^{\dagger}$ H_R
(25)
OH
H $H^{\dagger}$ H $H^{\dagger}$ H_S $H_R^{\dagger}$
(26)
$H_S^{\dagger}$
OH
H_R
H $H^{\dagger}$ H $H^{\dagger}$
(27)

SCHEME 7

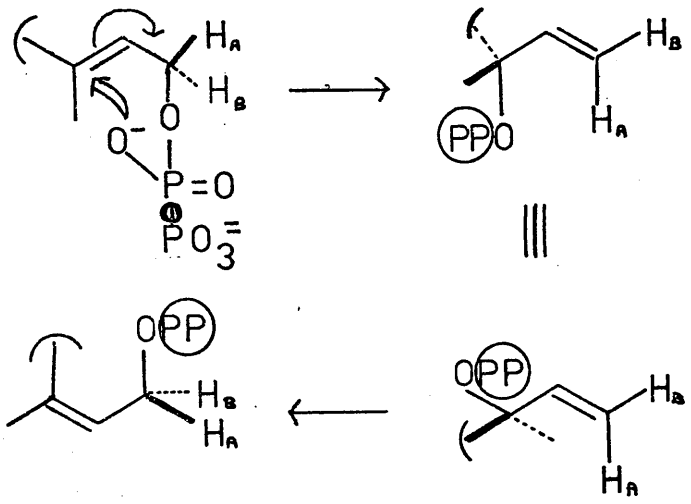
from the pro-5S position of mevalonate (24), which appears at the pro-1S position of trans, trans-farnesyl pyrophosphate (25) would then be located at the pro-1R position of the alcohol (26). The label would then be retained in the isomerised product (27) and would be in the pro-1S position (scheme 7). Phosphatases normally effect hydrolysis with P-O bond fission (17) and thus there is retention of configuration at the carbinol carbon atom. Experiments using (1R)- and (1S)-[1-$^{3}H_{1}$]-trans, trans-farnesyl pyrophosphate showed conclusively that the phosphatase activity present in the Andrographis extract was of the normal type and that hydrolysis did not lead to inversion at C-1. Labelling studies using $H_2{}^{18}O$ have confirmed that hydrolysis proceeds with P-O bond fission.

It has recently been suggested (18) that hydrolysis of geranyl pyrophosphate (4) in Menyanthes trifoliata occurs with inversion at C-1. Poulter and Rilling have shown (19) that crystalline, homogeneous prenyl transferase (E C 2.5.1.1) from pig liver catalyses, to a small extent, the hydrolysis of its allylic substrate and that this activity is inherent in the enzyme. Hydrolysis, which is assumed to occur when the isopentenyl pyrophosphate binding site is occupied by a molecule of water, leads to inversion of configuration at C-1. Thus in a crude enzyme preparation, although phosphatase activity of the normal type may be present, it is conceivable that the prenols isolated may, at least in part, have suffered an inversion of configuration at C-1, since they may have been hydrolysed by prenyl transferase before their release from the enzyme.

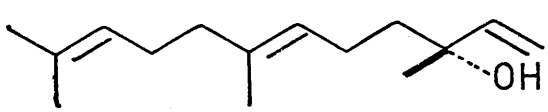
Shine and Loomis have shown (20) in the monoterpenoid



SCHEME 8



SCHEME 9



(28)

case that phosphorylated alcohols can be isomerised without hydrolysis. The isomerase was found to be photoinducible and to require a flavin and a thiol for maximal activity. Direct isomerisation of trans, trans-farnesyl pyrophosphate to the cis, trans-isomer has been observed (21) in a cell-free system derived from Trichothecium roseum. Isomerisation proceeds with loss of the pro-1S hydrogen of the trans, trans pyrophosphate.

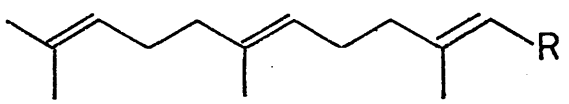
The results in the Andrographis case are most simply rationalised by postulating that there is a farnesyl pyrophosphate trans: cis isomerase which effects loss of the pro-1R hydrogen of trans, trans-farnesyl pyrophosphate and introduces hydrogen from solvent in the pro-1R position of the product as shown in scheme 8.

It has also been suggested (22) that trans: cis isomerisation can occur by processes which do not involve loss of label from C-1. In this case, nerolidol (28) or its pyrophosphate is the intermediate. Isomerisation by means of such a 1,3 shift results in overall inversion of configuration at C-1 (scheme 9).

Table 1.

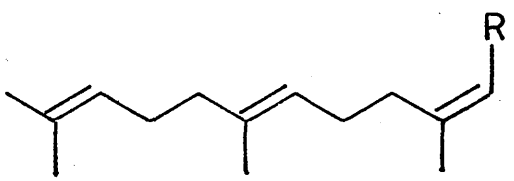
Percentage incorporation of aldehydes into products.

		Product			
	Substrate	trans, trans-aldehyde	cis, trans-aldehyde	trans, trans-alcohol	cis, trans-alcohol
control	[1-^{3}H] trans, trans-farnesal	64.4	19.6		0.72
experiment	[1-^{3}H] trans, trans-farnesal	28.6	15.2	27.0	11.2
control	[1-^{3}H] cis, trans-farnesal	56.0	14.7		2.275
experiment	[1-^{3}H] cis, trans-farnesal	9.6	2.0	22.2	40.0



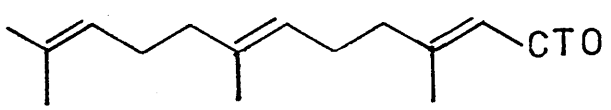
(11) R = CH_2OH

(13) R = CHO

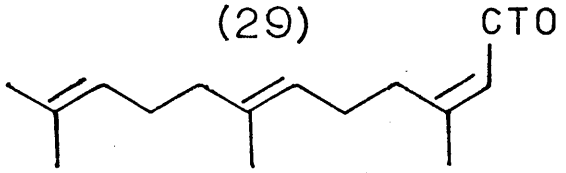


(12) R = CH_2OH

(14) R = CHO



(29)

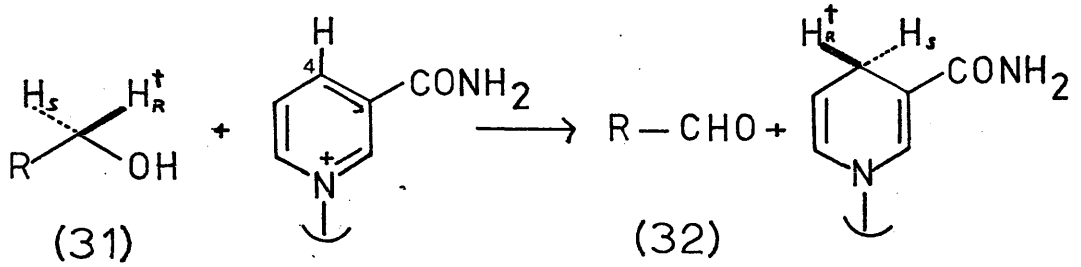


(30)

Discussion.

It has been suggested elsewhere in this thesis (p18), that isomerisation of trans, trans-farnesol (11) to cis, trans-farnesol (12) in the cell-free system derived from Andrographis paniculata proceeds via the corresponding aldehydes (13) and (14). The aldehydes are not isolated as products when either [2-^{14}C]mevalonate or one of the farnesol isomers is the substrate in the incubation. However, this does not necessarily exclude them as intermediates in the isomerisation process. Indeed, it is unlikely that they would be produced in isolable amounts since the enzyme system contains an excess of NAD(P)H.

To see whether the aldehydes might nevertheless function as intermediates, [1-^{3}H]trans, trans- (29) and [1-^{3}H] cis, trans-farnesal (30), prepared in almost quantitative yield by oxidation of the corresponding tritiated alcohols with manganese dioxide in dry benzene, were incubated with the cell-free system. This resulted in incorporation of radioactivity into trans, trans- and cis, trans-farnesol. The results of these incubations and the relevant control experiments are shown in table 1. Considerable non-enzymic isomerisation of the aldehydes was observed under the incubation conditions. In both experiments the composition of the recovered aldehyde mixture was similar to that found in the corresponding control. This is not unexpected, since it has been noted (23) that the aldehydes equilibrate rapidly in very dilute aqueous base and in the presence of thiols, including the thiol group of denatured enzymes. The enzyme system also



(31) (32)

SCHEME 10

contains alcohol dehydrogenase activity as expected.

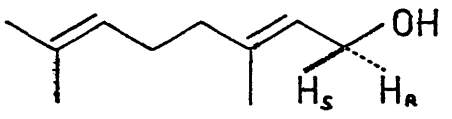
Oxidoreductase activity is universal throughout the plant and animal kingdoms and is central to many vital biochemical processes. Alcohol dehydrogenase (E C 1.1.1.1) catalyses the reversible oxidation of primary alcohols (31) to the corresponding aldehydes (32), using nicotinamide adenine dinucleotide (NAD^+) as co-factor (scheme 10). During oxidation of the alcohol, a hydride ion is transfered from the carbinol carbon atom to C-4 of the pyridinium ring of NAD^+. This process is stereospecific with respect to both substrate and co-factor. In the case of alcohol dehydrogenase from horse liver, it has been shown (24) that the pro-1R hydrogen of the substrate is stereospecifically transfered to the co-factor and that it becomes the pro-4R hydrogen of NADH (25). Such enzymes are said to be A side specific with respect to pyridine nucleotide. Comparative study of a number of oxidoreductase enzymes has led Bently to propose (26) that the stereospecificity of a particular reaction is independent of the source of the enzyme which catalyses it. It has also been noted (27) that when a metabolic sequence involves consecutive oxidoreductase reactions, the enzymes have the same stereospecificity towards pyridine nucleotide.

The stereochemistry of label at C-1 of farnesol can be determined by a method based on the procedure developed by Simon (28) for the preparation of (1R) and (1S) labelled primary alcohols. The incubation mixture contains NADH, NAD^+, liver alcohol dehydrogenase (E C 1.1.1.1) and diaphorase (lipoamide dehydrogenase, E C 1.6.4.3).

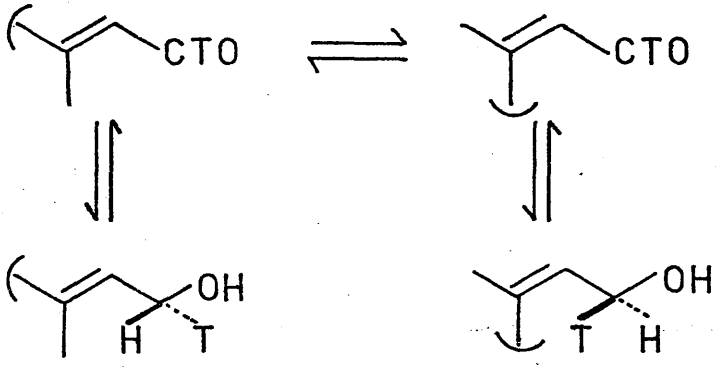
Table 2.

$^{3}H:^{14}C$ ratios of alcohols before and after exchange.

Substrate	Products	% retention
trans, trans-farnesol 5.70	trans, trans-farnesol 5.60	98.2
	cis, trans-farnesol 5.58	98.0
cis, trans-farnesol 6.59	trans, trans-farnesol 6.51	98.7
	cis, trans-farnesol 6.42	97.4



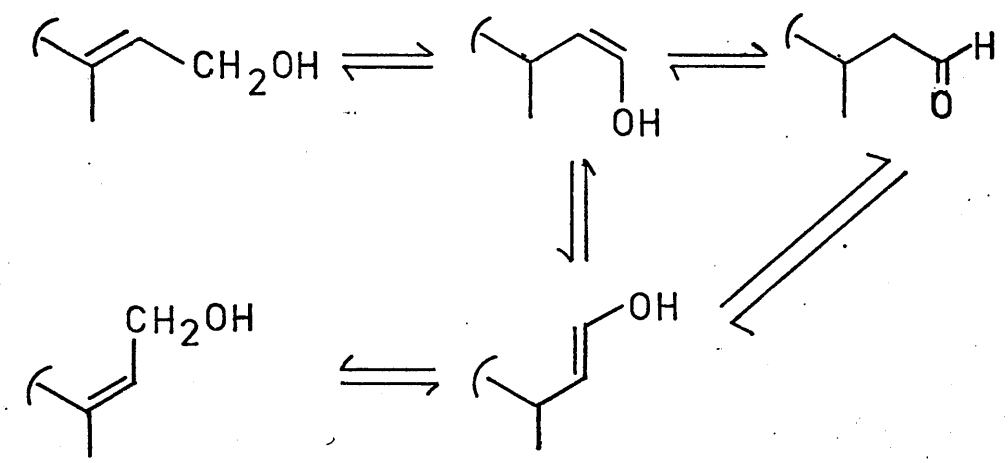
(15)



SCHEME 11

Liver alcohol dehydrogenase catalyses exchange between the pro-1R hydrogen of farnesol and NADH, while diaphorase exchanges hydrogen between NADH and water. Thus, incubation exchanges label out of the pro-1R position of farnesol. By analogy with the established exchange (23) of the pro-1R hydrogen in geraniol (15), it has been assumed (29) that trans, trans-farnesol also exchanges its pro-1R hydrogen with liver alcohol dehydrogenase. It has been shown in these laboratories (14) that this assumption is correct and that the pro-1R hydrogen of cis, trans-farnesol is also exchanged by liver alcohol dehydrogenase.

If aldehydes are intermediates in the cis: trans isomerisation, then incubation of the [1-^{3}H]aldehydes with the cell-free system must give trans, trans-farnesol and cis, trans-farnesol with label in the pro-1R and pro-1S positions respectively, based on the previously established stereochemistry of hydrogen loss from C-1 during isomerisation (p18) as shown in scheme 11. However, incubation with the liver alcohol dehydrogenase/diaphorase system of the farnesol isomers obtained from the [1-^{3}H]aldehyde showed that in each case label was in the non-exchangable pro-1S position (table 2). It appears that the alcohol oxidoreductase in the Andrographis cell-free system reduces both trans, trans-farnesal and cis, trans-farnesal with the same stereochemistry as does liver alcohol dehydrogenase; the Andrographis enzyme also exchanges the pro-1R proton. Thus, although an oxidoreductase system which is potentially capable of catalysing trans, trans- to cis, trans-farnesol isomerisation is present in Andrographis cell-free extract, this cannot be part of



SCHEME 12

the mechanism which is responsible for isomerisation. When one of the farnesol isomers is incubated with the cell-free system, products of isomerisation by this mechanism are not obtained, presumably because of the presence of excess NAD(P)H.

Thus it appears that if trans, trans-farnesal and cis, trans-farnesal are involved at all in the isomerisation of of the farnesol isomers, they are never set free from the enzyme which is responsible for the isomerisation. The isomerisation enzyme is not the alcohol dehydrogenase whose activity is detected when the aldehydes are incubated with the cell-free system. It should be pointed out that loss of hydrogen from C-1 of farnesol does not necessarily imply that the isomerisation mechanism requires overall oxidation at any stage. As shown in scheme 12, isomerisation of allylic alcohols could take place via the isomeric aldehydes or their enolic forms.

Experimental.

General.

All thin layer chromatography on silica was carried out on precoated Merk Kieselgel G plates (20cm×20cm). Silver nitrate-impregnated plates were freshly prepared as required by spraying precoated t.l.c. plates with aqueous silver nitrate (15%, w/v) until they were saturated. The plates were allowed to dry overnight in the dark at room temperature before activation by heating at 100°C in an air oven for two hours prior to use.

Radioactivity was determined by liquid scintillation counting in a Philips model PW-4530 liquid-scintillation analyser, with 2,5-diphenyloxazole (5g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.1g/l) in toluene as scintillant. Counting efficiencies were typically 90% for ^{14}C alone and 75% and 32% respectively for ^{14}C and ^{3}H in experiments using both nuclides. Corrections for counting efficiency and quenching were automatically computed, using a channels-ratio mode of operation with ^{137}Cs as external standard. Chromatograms were scanned for radioactive components using a Panax Thin Layer Scanner RTLS 1A. Radiogas liquid chromatography was performed on a Pye 104/Panax Radiogas detector system operated in either the ^{14}C only or ^{3}H mode. Counting efficiencies were ~40% for ^{3}H and 90% for ^{14}C. Analytical g.l.c. was performed on a Perkin Elmer F11 gas chromatograph with a flame ionisation detector using nitrogen as carrier gas.

^{1}H NMR spectra were recorded at 60MHz on a Varian spectrometer. ^{13}C NMR spectra were obtained on a Varian XL 100 spectrometer at 25.2MHz operated in the Fourier

transform mode using deuterium internal lock. All chemical shifts are reported as parts per million downfield of internal tetramethylsilane reference. Mass spectra were recorded on EMI MS 12 spectrometer. Melting points were obtained on a Koffler hot stage apparatus and are reported uncorrected. High speed centrifugation was carried out in a MSE High Speed 25 centrifuge.

Maintenance of Andrographis paniculata callus cultures.

Procedures for the establishment and maintenance of A. paniculata stock cultures have been described (30). Callus tissue of hypocotyl origin was grown on a medium whose inorganic constituents were those of modified White's root culture medium (31), except that ferric sodium ethylenediamine tetra-acetate (Fe-EDTA) was added as iron source (32). The medium also contained White's vitamin supplements, 2,4-dichlorophenoxyacetic acid (6mg/l), meso-inositol (100mg/l), sucrose (20g/l) and 15% coconut milk. The callus tissue was grown on medium solidified with 0.7% agar (Oxoid no. 3) for twenty-one days after subculture and then inocula (~0.5g fresh weight) were transfered to liquid medium (100ml) contained in 250ml Erlenmeyer flasks. The flasks were kept on a horizontal rotary shaker (140rev./min.) at 25±1°C with illumination (approx. 3200lx.). Stock cultures, subcultured on solid medium as described above, have been maintained in these laboratories for a period of more than ten years.

Preparation of cell-free extracts.

Callus tissue which had been grown in suspension culture for twenty-one days was collected by filtration and homogenized in buffer (1ml/g fresh weight) for two minutes

at 0°C in a precooled glass tissue homogenizer (Kontes Glass Co., Vineland, N.J., U.S.A.) driven by an electric motor. The homogenization buffer contained sucrose (0.35M), reduced glutathione (5mM), nicotinamide (5mM), soluble polyvinylpyrrolidone (mol. wt. 24,500; 1% w/v), manganese chloride (0.13mM) and magnesium chloride (0.13mM) in sodium phosphate buffer (0.1M, pH 7.6). The homogenate was centrifuged at 74,500×g (25,000 rev./min.; fixed angle rotor, M.S.E. Ltd., no. 59584) for ninety minutes at 4°C and the supernatant was used directly as the source of the enzyme system. The protein concentration of the extract was approximately 2.6mg/ml as estimated by the method of Lowry (33) using bovine serum albumin as standard.

Preparation of [1-$^3H_1$]trans, trans-farnesal (29) and [1-$^3H_1$]cis, trans-farnesal (30).

Manganese dioxide, prepared by a modified Attenburrow method (34), was activated by azeotropic removal of water with benzene using a Dean and Stark apparatus. Purified [1-$^3H_2$]trans, trans-farnesol (2mg, 8×10^8dpm) was oxidised to [1-$^3H_1$]trans, trans-farnesal (29) by stirring overnight at room temperature with activated manganese dioxide (50mg) in dry benzene (1ml). Manganese dioxide was removed by filtration through a bed of celite which was throughly washed with benzene, followed by a small portion of ethanol, to remove any adsorbed material. The solution was then evaporated to small volume and purified by preparative t.l.c. (ethyl acetate-hexane, 1:3, v/v) followed by t.l.c. on silver nitrate-silica (ethyl acetate-acetic acid, 499:1, v/v). Radioscanning located radioactivity only in the band corresponding to trans, trans-farnesal. This material was

then eluted from the silica with ethyl acetate and made up to a volume of 10ml with ethyl acetate. Counting of an aliquot showed that the solution contained 3.6×10^7dpm/ml tritium activity.

By exactly the same procedure, [1-$^3H_2$]cis, trans-farnesol (2mg, 9.2×10^7dpm) was oxidised with manganese dioxide in benzene and the aldehyde purified, to yield 10ml of ethyl acetate solution of [1-$^3H_1$]cis, trans-farnesal (30) (4×10^6dpm/ml).

Incubation of [1-^{3}H]trans, trans-farnesal (29) and [1-^{3}H] cis, trans-farnesal (30) with Andrographis cell-free extract.

[1-^{3}H] trans, trans-farnesal (3.6×10^7dpm, ~200µg) was suspended in sodium phosphate buffer (0.1M, pH 7.6, 0.1ml) with Tween 80 (1mg) by sonicating for ten minutes (Mettler Electronics Ultrasonic Cleaner, model ME 1.5). The suspension was then incubated with the cell-free extract (3ml) containing NADH (0.5mM), NADPH (0.5mM), ATP (1mM) and sodium fluoride (1mM) (final volume 4ml) for sixteen hours at 27°C in a 15ml Warburg flask. The flask was evacuated and filled with nitrogen five times before mixing substrate and medium.

[1-^{3}H]cis, trans-farnesal (4×10^6dpm, ~200µg) was similarly incubated. Control experiments were run with boiled extract in both cases.

Incubations were terminated by the addition of ice-cold ethanol (8ml). Denatured protein was then centrifuged down at 4000×g for five minutes and the supernatant extracted with hexane (3×10ml). After drying over anhydrous sodium sulphate, the solution was evaporated to small volume and carrier farnesol (2mg, trans, trans-cis, trans, 2:1) added before preparative t.l.c. (ethyl acetate-hexane, 1:3, v/v),

which allowed separation of alcohol from aldehyde. The geometrical isomers of farnesol and farnesal were then separated by preparative t.l.c. on silver nitrate-silica (ethyl acetate-acetic acid, 499:1, v/v). The bands corresponding to the individual components were scraped off the plates, eluted with ethyl acetate and the radioactivity in each was determined. The results are given in table 1 (p21).

Exchange of alcohols with liver alcohol dehydrogenase-diaphorase.

The tritiated alcohol samples obtained by incubation of [1-^{3}H]trans, trans-farnesal described in the previous experiment were mixed with the appropriate [4,8,12-$^{14}C_3$]farnesol isomers, which had been obtained biosynthetically (14) to give ^{3}H:^{14}C ratios of ~5. This doubly-labelled material was then subjected to preparative t.l.c. (ethyl acetate: hexane, 1:3, v/v) before exchange. After mixing with Tween 80 (1mg) an aliquot of each substrate was ultrasonically dispersed in sodium phosphate buffer (0.1M, pH 8.0, 2.8ml), containing ethylenediamine tetra-acetic acid disodium salt (1mM), bovine serum albumin (2.6mg), NAD^{+} (0.34mM) and NADH (0.34mM). Horse liver alcohol dehydrogenase (E C 1.1.1.1; 100µl suspension, 5 units) and diaphorase (E C 1.6.4.3; 100µl, 100 units) were added and the mixture incubated under nitrogen for twelve hours at 37°C. The incubation mixture was then saturated with sodium chloride and extracted with hexane (3×10ml). After drying the solution over anhydrous sodium sulphate, carrier farnesol (2mg, 2:1 mixture trans, trans-cis, trans) was added. The farnesol isomers were separated and purified as previously described. The

$^{3}H:^{14}C$ ratios for both the substrates and the products were determined as the free alcohol, the acetate and the trimethylsilyl ether. The acetates were prepared by heating the alcohol samples with dry pyridine (100µl) and acetic anhydride (100µl) at 100°C for thirty minutes. Excess reagents were then removed in a stream of nitrogen at -80°C and the residue was subjected to preparative t.l.c. (ethyl acetate:hexane, 1:3, v/v). The O-trimethylsilyl ethers were similarly prepared by heating the alcohol samples with dry dimethylformamide (100µl) and bis (trimethylsilyl) acetamide (100µl) at 100°C for thirty minutes. The results are shown in table 2, (p23) and are the average values.

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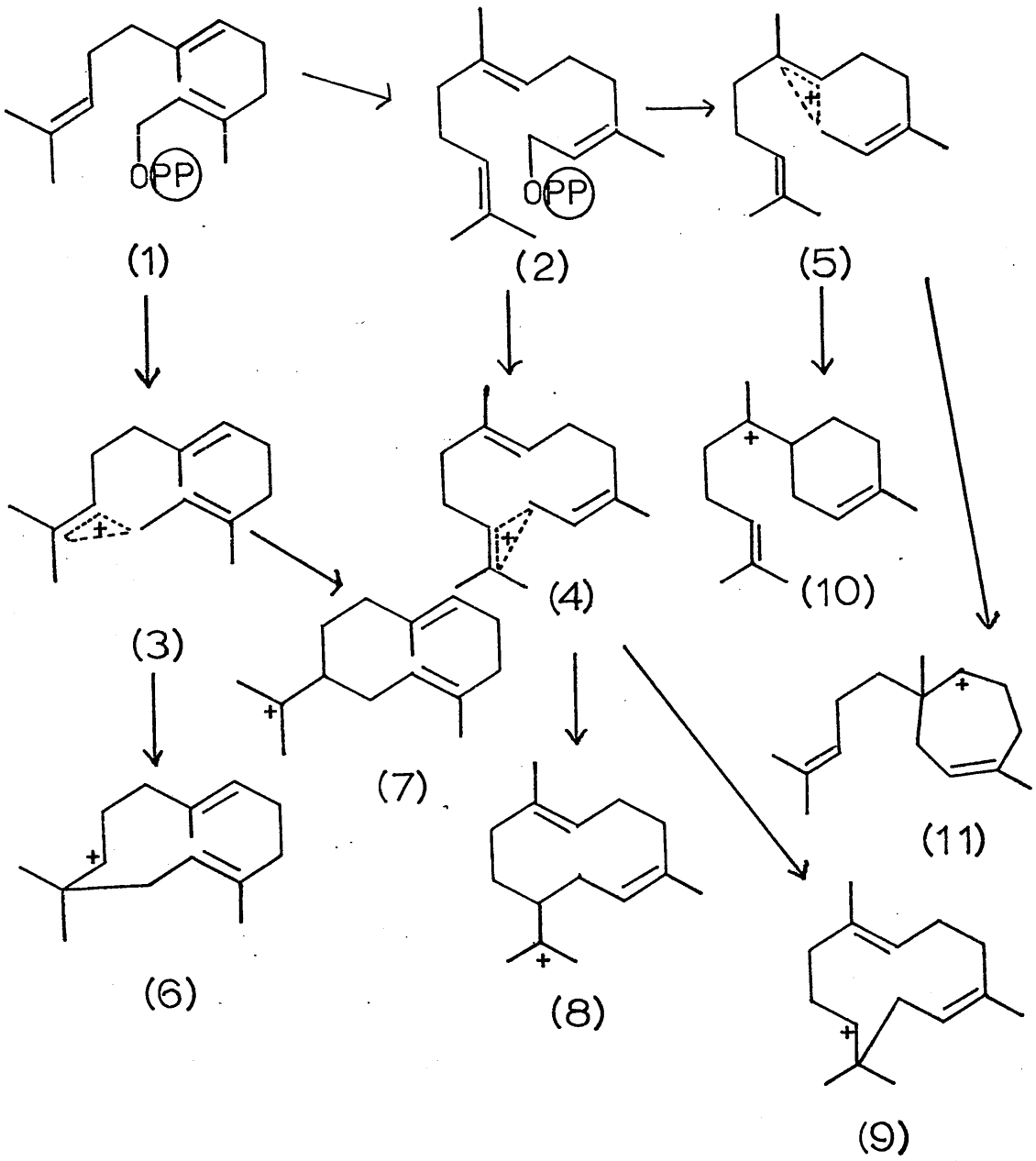
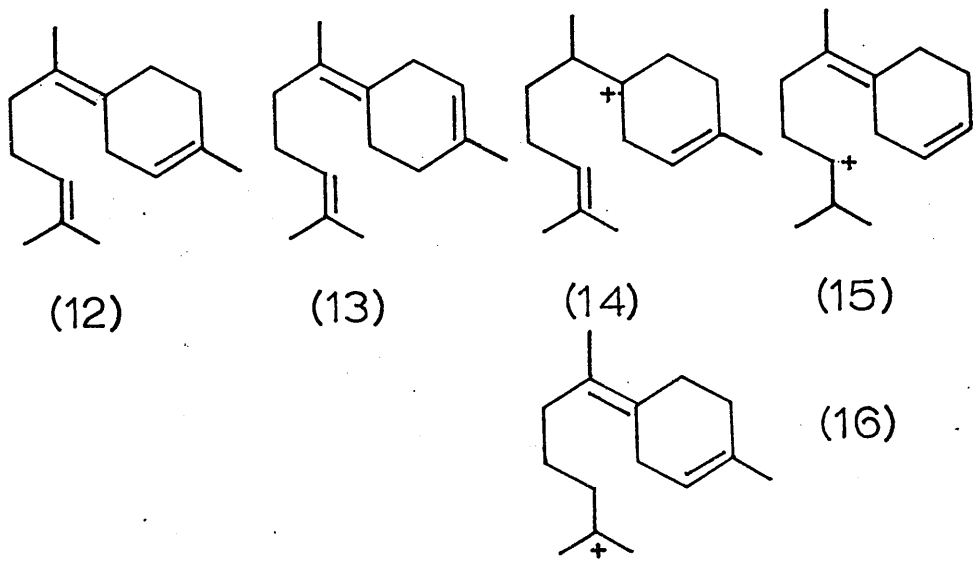
SECTION 2

THE BIOSYNTHESIS OF BISABOLENE



SCHEME 1



Section 2.

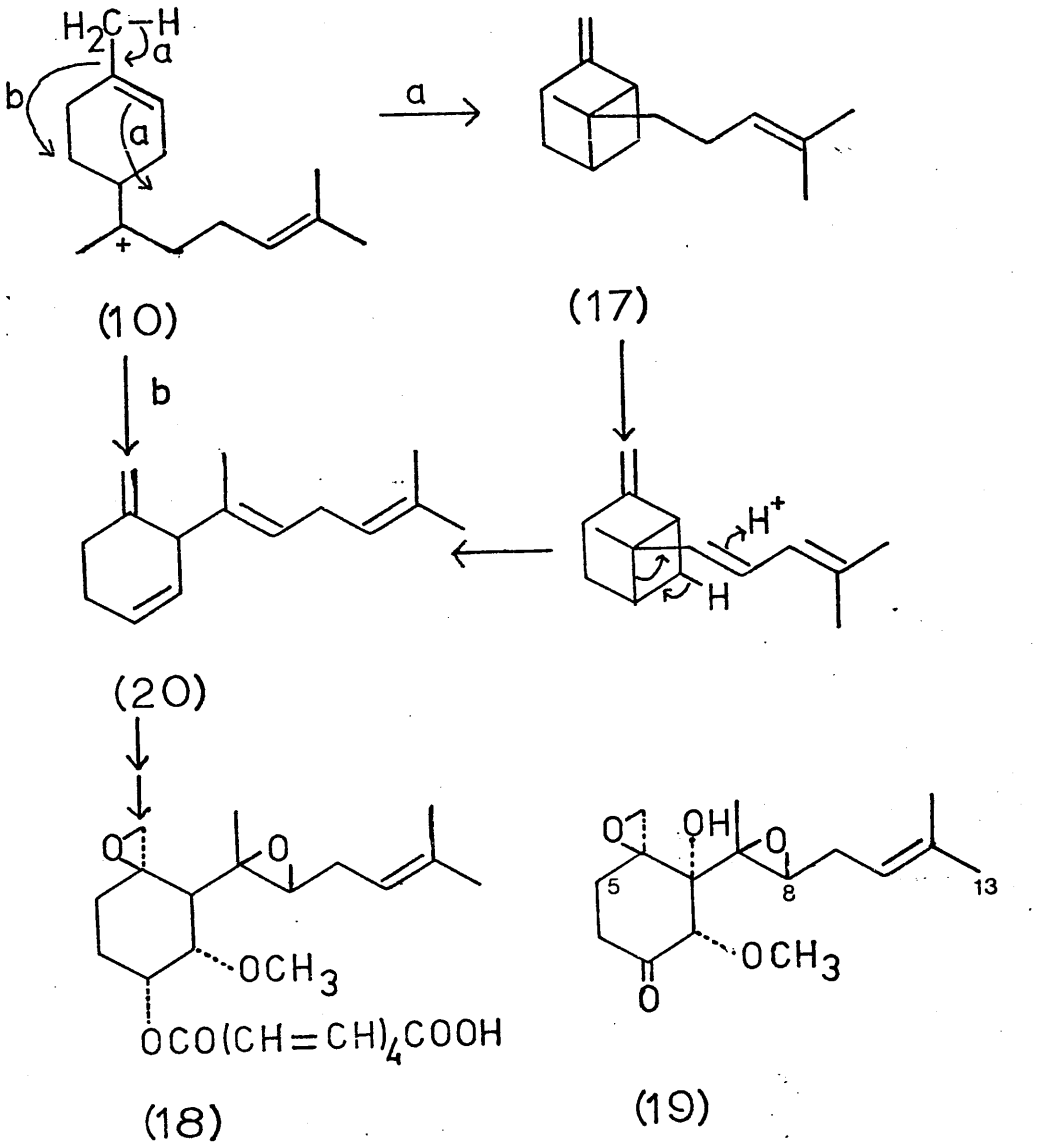
The Biosynthesis of Bisabolene.

Introduction.

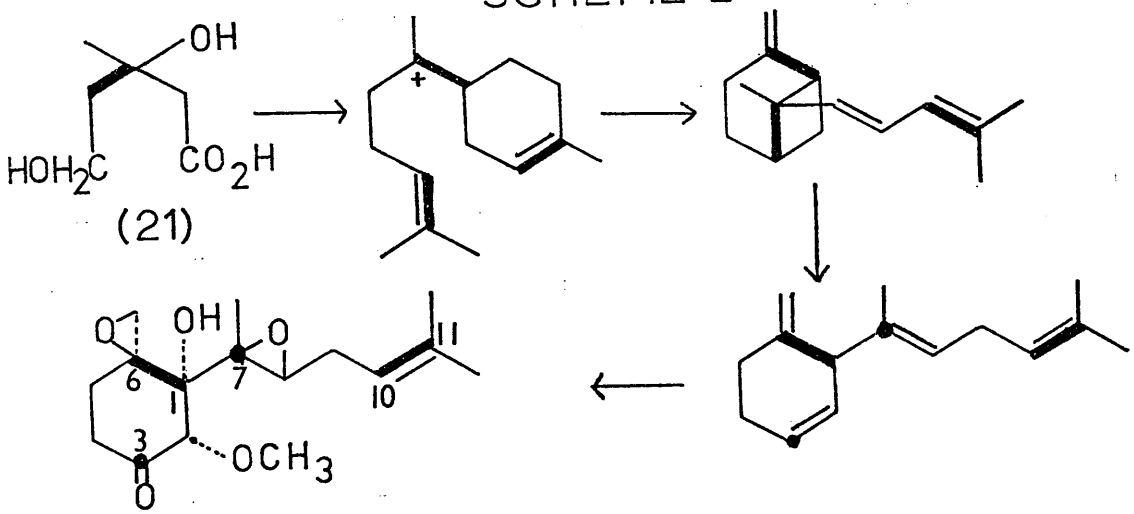
The sesquiterpenoids are a large and diverse group of naturally-occurring compounds encompassing a variety of structural types and functional groups. At the present time, over one hundred sesquiterpene carbon skeletons are known (1). The study of the ways in which Nature can derive such diversity from one molecule, trans, trans-farnesyl pyrophosphate (1) is a fascinating challenge.

The first step in the formation of almost all cyclic sesquiterpenoids (1,2) involves nucleophilic attack of one of the double bonds of the isoprenoid chain on C-1 of either trans, trans-farnesyl pyrophosphate (1) or cis, trans-farnesyl pyrophosphate (2). Some of the possible cyclisation modes, which initially produce the non-classical carbonium ions (3), (4) and (5), are shown in scheme 1. The further rearrangements of the derived cations (6)-(11), by means of stereospecific cyclisations, hydride shifts and methyl shifts, give rise to the profusion of skeletal types. It should be noted that such enzyme-catalysed cyclisations probably do not involve free carbonium ions as illustrated, but rather their enzyme-bound equivalents. However, for the sake of convenience, the biosynthesis of sesquiterpenoids is normally discussed in terms of free carbonium ions.

One of the most important of these ions is the bisabolenyl cation (10) which by loss of a proton generates either Z- or E-γ-bisabolene (12) or (13). Three further cations (14), (15) and (16) could be generated either by protonation of γ-bisabolene or by an intramolecular hydrogen migration in



SCHEME 2

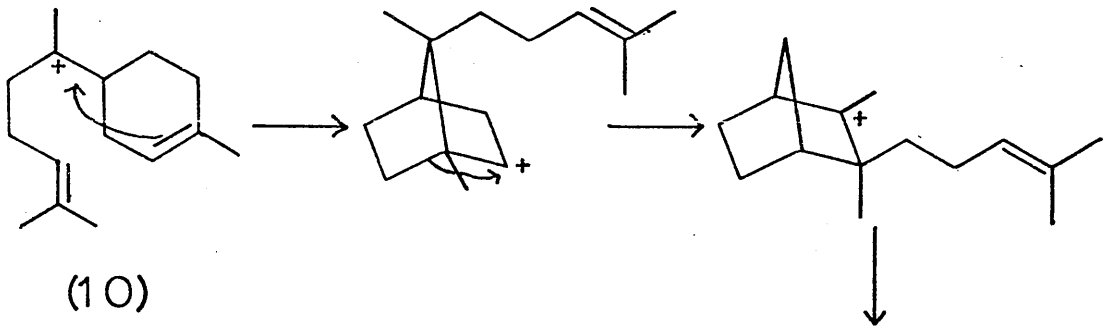


(▬) coupled ^{13}C atoms

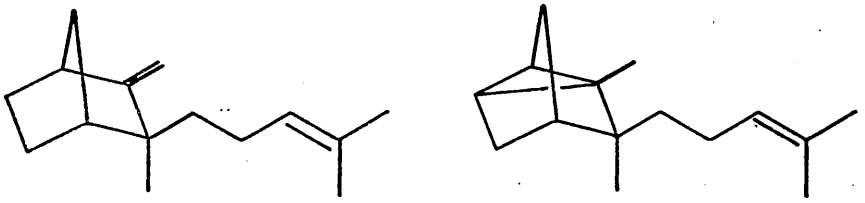
SCHEME 3

ion (10). Bisabolene and the four derived cations have been suggested as possible intermediates in the biogenesis of a large number of sesquiterpenoids.

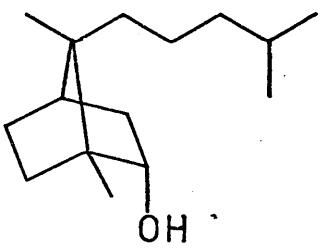
Several groups of sesquiterpenoids can be derived from cation (10) by cyclisation, rearrangement and deprotonation. These include compounds which are structurally isoprenologous monoterpenes such as β-bergamotene (17). Some years ago it was shown (3) in cultures of Aspergillus fumigatus that mevalonate is specifically incorporated into the antibiotic fumagillin (18) thereby establishing its terpenoid nature. Birch proposed (4) a novel mechanism for its formation from cis, trans-farnesyl pyrophosphate (2) via β-bergamotene (17) (scheme 2). The biosynthesis of the structurally similar compound ovalicin (19) has recently been investigated in detail. When [1,2-$^{13}C_2$]acetate was used as a precursor, ^{13}C NMR analysis showed (5) that six intact acetate units had been incorporated into ovalicin, while C-5, C-8 and C-13 although enriched in ^{13}C appeared as singlets, indicating that they were derived from C-2 of mevalonate. The direct route (b) from the bisabolenyl cation (10) to the tetraene intermediate (20) involves a 1,3-shift of the ring methyl group. This route is inconsistent with the finding that C-6 and C-14 originate from the same acetate molecule. Further support for route (a) involving β-bergamotene (17) comes from experiments (6) using [3,4-$^{13}C_2$]mevalonate (21). Carbons 1 and 6 of ovalicin, derived from C-4 and C-3 respectively of the same mevalonate molecule, gave rise to a pair of doublets in the ^{13}C spectrum, as did C-10 and C-11. Carbon atoms 3 and 7 of ovalicin appeared as enhanced singlets, indicating that



(10)

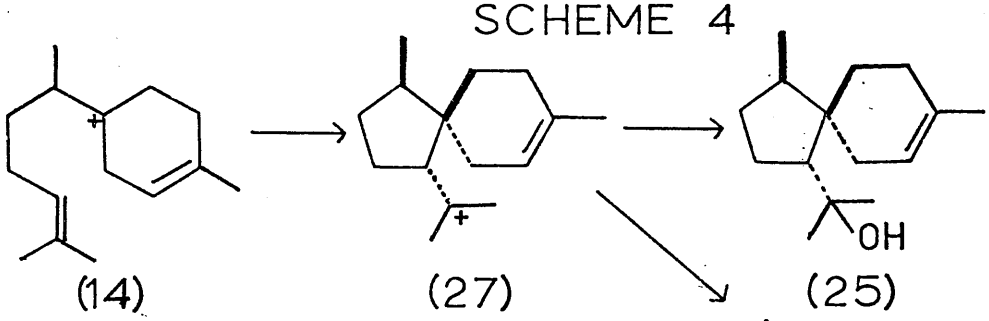


(23) (22)

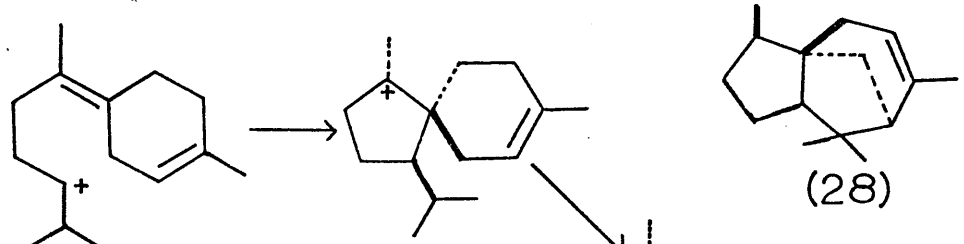


(24)

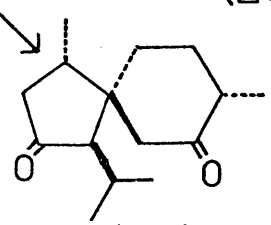
SCHEME 4



(14) (27) (25)

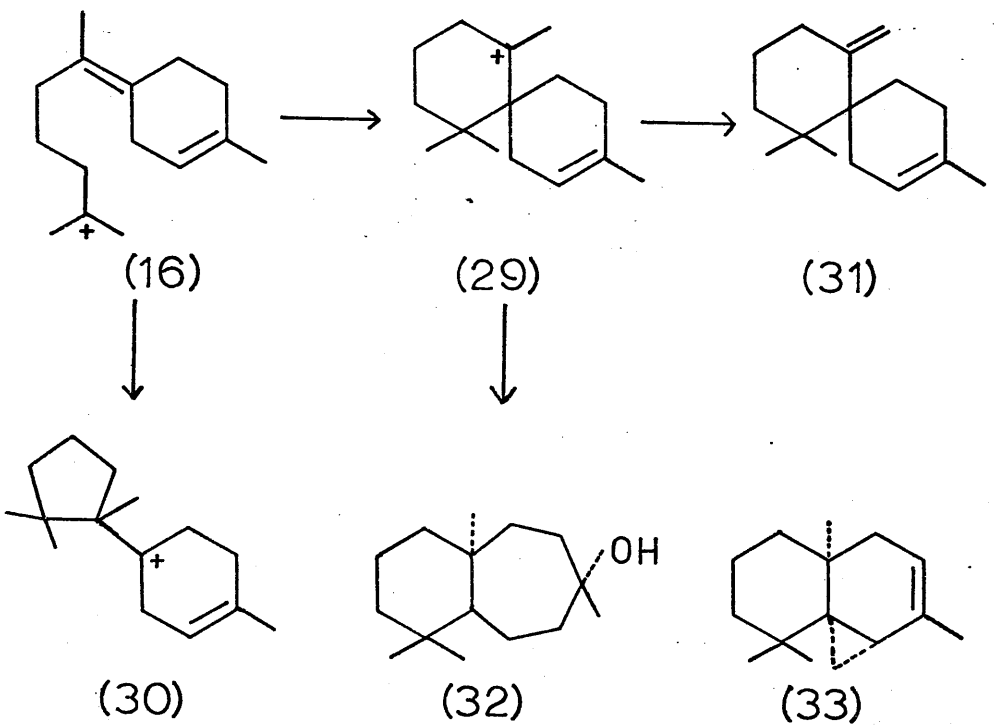


(15) (28)



(26)

SCHEME 5



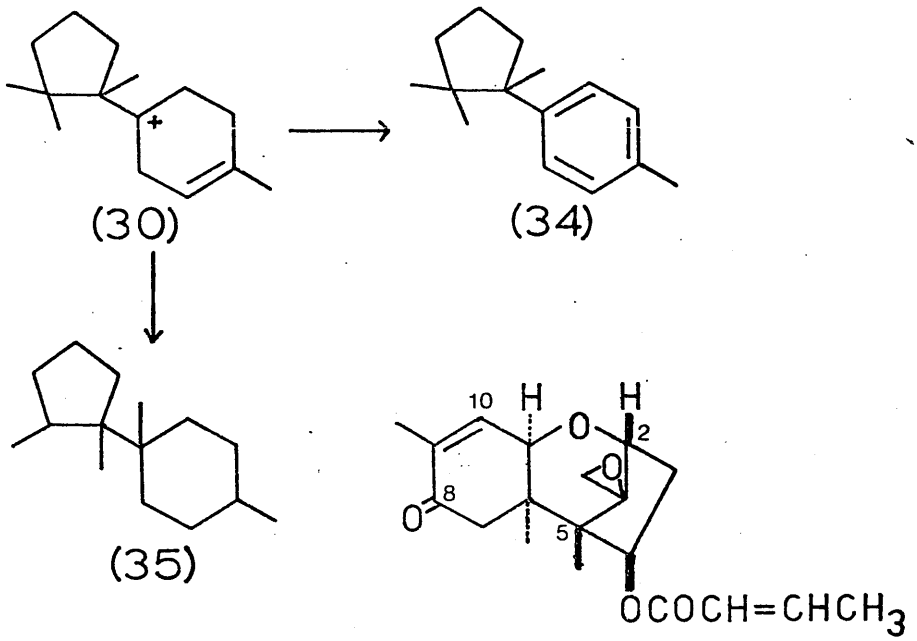
SCHEME 6

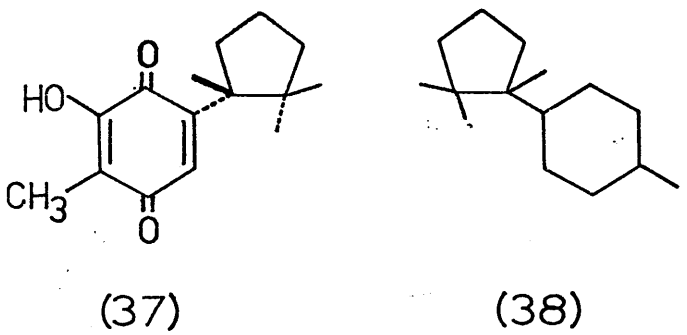
in this prenyl unit the C-3, C-4 bond of mevalonate had been broken (scheme 3). This evidence again supports route (a) and the intermediacy of β-bergamotene in the biosynthesis of ovalicin.

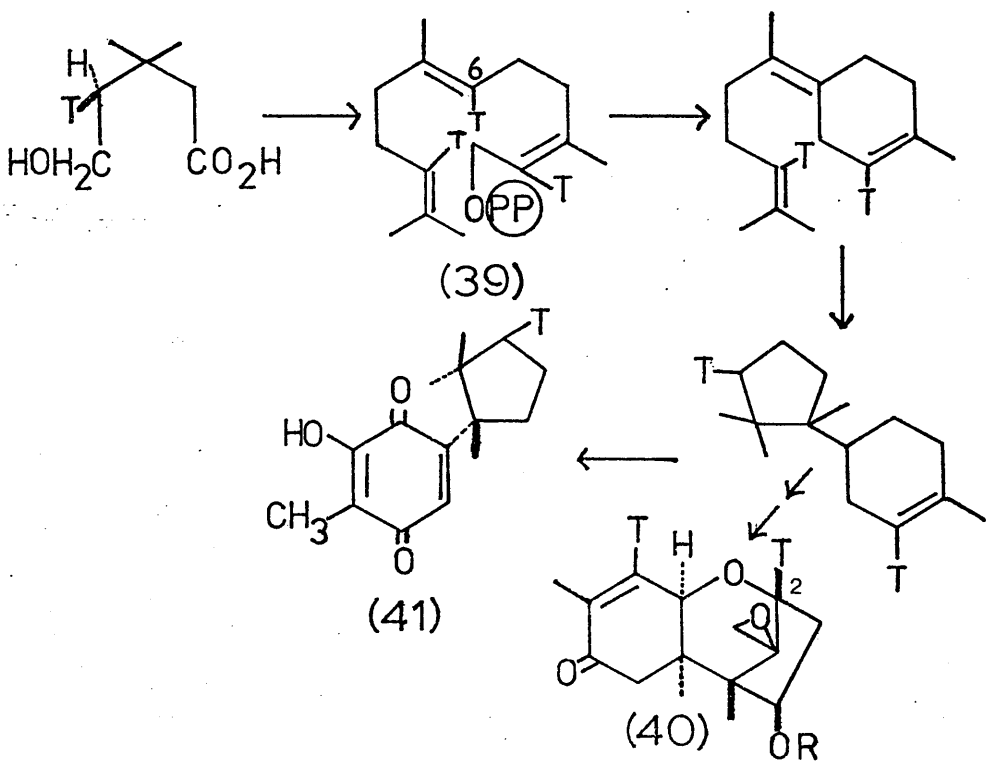
No biosynthetic information is available on the other isoprenologous monoterpenoids such as α-santalene (22), β-santalene (23) and campharenol (24), whose biogenesis is thought to involve cation (10) (scheme 4).

Cations (14) and (15) have been proposed as possible intermediates in the biogenesis of [4.5]spirocyclic sesquiterpenoids such as α-acorenol (25) and acorone (26). It has been suggested that cyclisations of these two different cations give rise to the two enantiomeric series of [4.5]spirocyclic sesquiterpenoids which are found in Nature. The spirocyclic cation (27), which is the supposed intermediate in the formation of α-acorenol (25) has also been invoked (7) in the proposed biogenesis of the tricyclic cedranes such as (-)-α-cedrane (28), as shown in scheme 5. An alternative biogenesis of the cedranes has been proposed (2), which involves concerted cyclisation of γ-bisabolene (12).

Cyclisation of cation (16), as shown in scheme 6, may proceed in two different senses to yield the species (29) or (30). The spirocyclic cation (29), whose formation by direct cyclisation of farnesyl pyrophosphate is also conceivable (8), is thought to be the precursor of the chamigranes such as β-chamigrene (31). Rearrangement of the chamigrane cation results in the formation of the widdrane and thujopsane skeletons as exemplified by widdrol (32) and thujopsene (33), respectively. The


(30)
(34)
(35)
10
H
H
O
2
O
8
O
5
OCOCH=CHCH3
(36)


O
HO
CH3
O
(37)
(38)

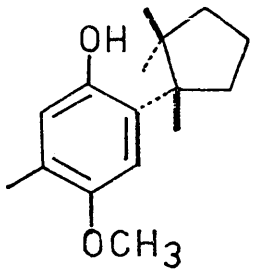

H
T
HOH2C
CO2H
6
T
T
OPP
T
(39)
T
T
T
T
O
HO
CH3
O
(41)
T
H
O
T
2
O
O
(40)
OR

SCHEME 7

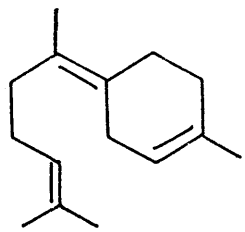
bicyclic cation (30) has been proposed as the precursor of a number of closely related sesquiterpene skeletons. Compounds of the cuparane class such as cuparene (34) are formed from cation (30) without rearrangement, while formation of the trichothecane skeleton (35) requires a 1,3-methyl shift or two 1,2-methyl shifts.

The biosynthesis of the trichothecane antibiotics has been the subject of extensive investigation for many years and must be the most thoroughly studied area of sesquiterpene biosynthesis. Trichothecin (36), which is a metabolite of Trichothecium roseum, is perhaps the best known of this group of compounds. Helicobasidin (37) is one of the two major metabolites of Helicobasidium mompa, and although it is superficially dissimilar, closer inspection reveals that it is a cuparane, based on carbon skeleton (38) while trichothecin is based on the trichothecane skeleton (35).

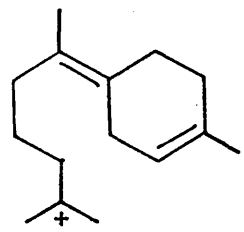
As previously discussed, the pro-4S hydrogen atom of mevalonate is lost in the formation of both trans, trans- and cis, trans-farnesol in all cases which have been investigated. Thus, starting with (4R)-[4-$^{3}H_{1}$]mevalonate, there should be complete retention of tritium in cis, trans-farnesyl pyrophosphate (39), loss of one tritium upon incorporation into trichothecin (40) and loss of two labelled atoms in helicobasidin (41) (scheme 7). Hanson has verified experimentally (9) that two out of three labels are in fact retained in trichothecin derived from (4R)-[4-$^{3}H_{1}$]mevalonate. When the same experiment was carried out in H. mompa (10), an interesting result was observed. Instead of two tritium atoms being lost, two of



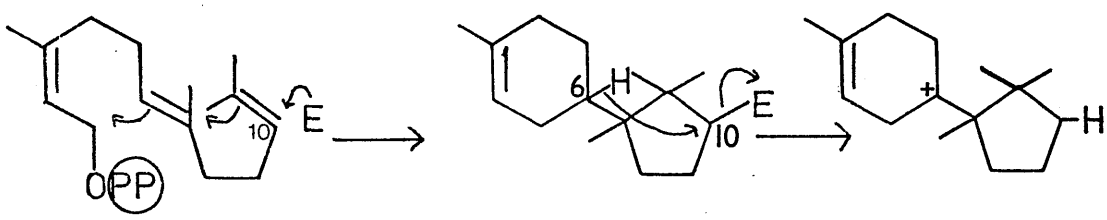
(42)



(12)

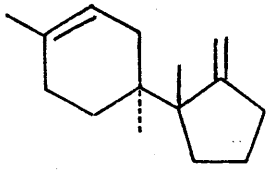


(16)

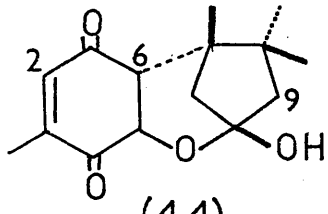


(2)

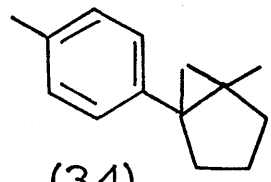
SCHEME 8



(43)



(44)

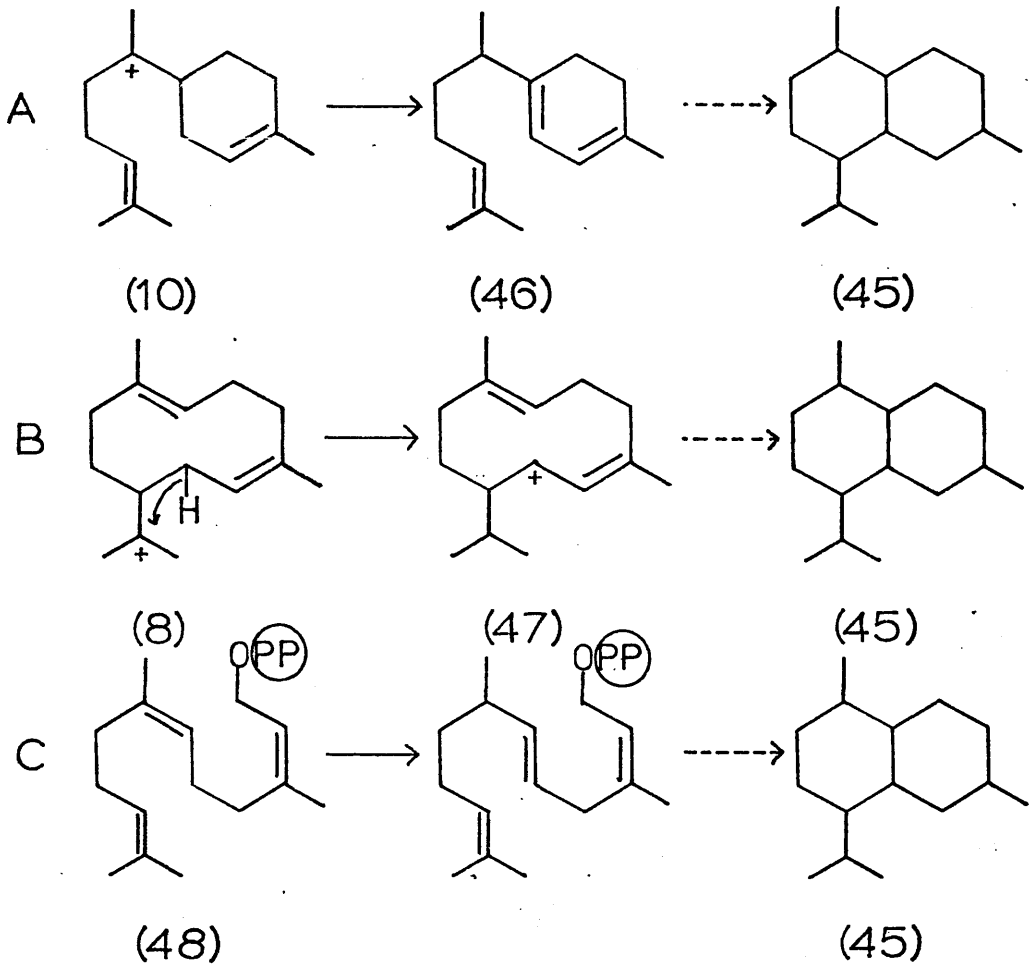


(34)

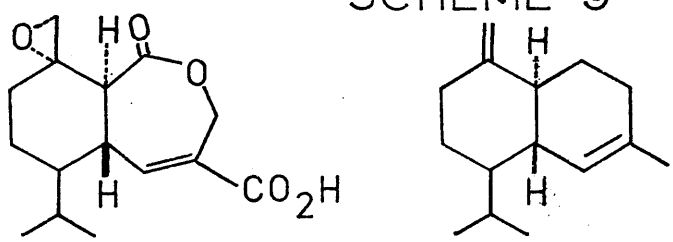
the three were retained. A co-metabolite in H. mompa (42) was found to retain all the tritium activity, indicating that label at C-6 of farnesyl pyrophosphate is somehow transferred to the five-membered ring. This result led Nozoe to propose that bisabolene derivatives such as (12) and (16) are not involved in the biosynthesis of helicobasidin. Later work (11) showed that there was no loss of label from C-6 of farnesyl pyrophosphate during cyclisation to the trichothecane skeleton, and that the hydrogen atom had probably migrated to C-2 of the product.

On this basis Hanson has suggested (12) that cyclisation of cis, trans-farnesyl pyrophosphate (2) may be concerted with attack of the enzyme initiating cyclisation at C-10. Hydride from C-6 of the substrate then migrates to C-10 to displace enzyme as shown in scheme 8. The involvement of cis, trans-farnesyl pyrophosphate (2) in the initial cyclisation was indicated (13) by the observation that the pro-1S hydrogen of the trans, trans isomer was stereospecifically lost on incorporation into trichodiene (43) in a cell-free system from T. roseum.

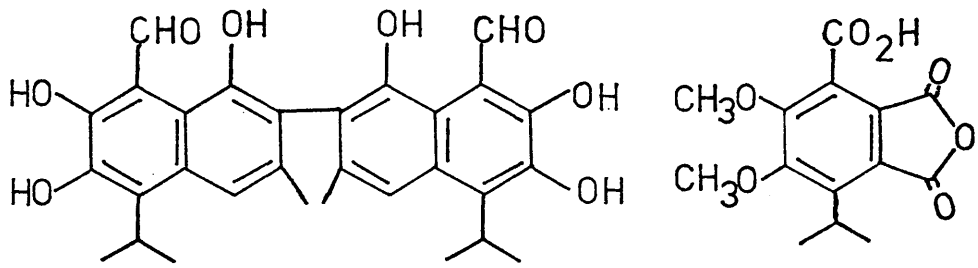
Similar results have been obtained (14) by Arigoni's group in the study of the biosynthesis of the cuparane derivative lagopodin B (44). A number of proposed intermediates, including cuparene (34), were shown to be efficient precursors of lagopodin B. (4R)-[4-$^{3}H_{1}$, 2-^{14}C] Mevalonate was specifically incorporated into lagopodin B, and degradation showed that half a tritium equivalent was located at C-2 while the remaining two equivalents were at C-9. It may be concluded from this result that during cyclisation a 1,4-hydride shift from C-6 to C-9 occurs,



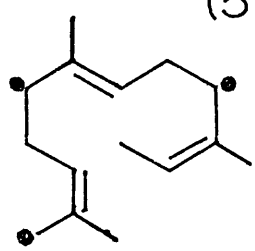
SCHEME 9



(49) (50)



(51) (52)



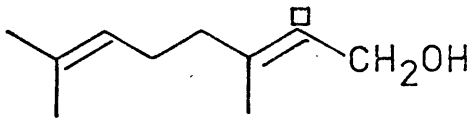
(53) (•) label from [2-^{14}C]mevalonate

excluding a bisabolene derivative such as (12) as a biosynthetic intermediate.

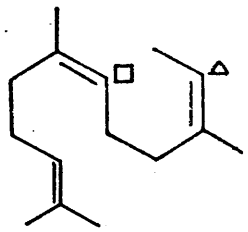
Sesquiterpenoids which have a 1,7-dimethyl-4-isopropyldecalin system as in (45), occur as four series of stereochemical variants. Three proposals have been made for their biogenesis (2,15). Pathway (a) in scheme 9 involves the bisabolenyl cation (10). This leads to formation of γ-curcumene (46), which cyclises to the required system (45). Pathway (b) involves formation of the germacrane cation (8), which undergoes a 1,3-hydrogen shift to give cation (47) and this could cyclise as shown. The third possibility involves the intermediacy of cis, cis-farnesyl pyrophosphate (48) which could isomerise and undergo concerted cyclisation as shown.

The fungal metabolite avocettin (49) is thought to be the end product of extensive oxidative attack of ent-γ-cadinene (50). Recent studies by Arigoni (16) on avocettin and a number of other metabolites derived from the cadinane system have shown that they are all biosynthesised via the germacrane cation (8) and that stereospecific 1,3-hydrogen migration takes place.

Gossypol (51) is a toxic yellow compound isolated from the cotton plant. Biogenetically, it may be considered as having arisen by phenol oxidative coupling of two C_{15} units of the 1,7-dimethyl-4-isopropyldecalin type. [2-^{14}C] Mevalonate was shown to be specifically incorporated into gossypol (17) and no label was lost on degradation to gossic acid (52). This eliminates the mode of cyclisation of cis, trans-farnesyl pyrophosphate implied in (53), which should lead to loss of one third of the activity in gossic



(54)



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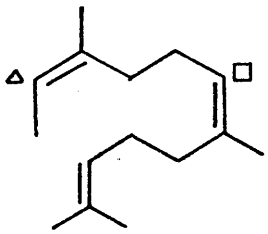
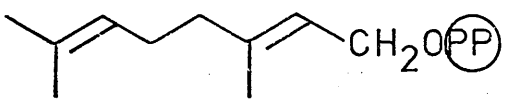


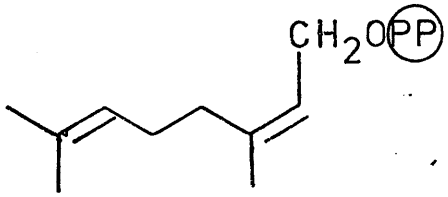
(56)

□ label from [2-^{14}C]geraniol

△ label from [2-^{14}C]farnesol



(58)



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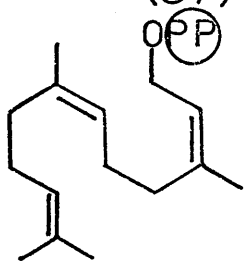
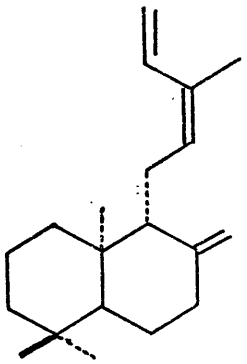


(48)

acid. When [2-^{14}C]geraniol (54) was used as precursor, the gossic acid obtained was radioactively labelled, but no radioactivity was found in gossic acid when [2-^{14}C] farnesol was used. Thus the mode of cyclisation in gossypol formation must be as implied in (55) and not (56).

In addition, all four geometrical isomers of farnesyl pyrophosphate were tested as precursors (18). Only the 2-*cis*-isomers were incorporated and the 2-*cis*, 6-*cis*-isomer was by far the best precursor. Neryl pyrophosphate (57) was a far better precursor than geranyl pyrophosphate (58). All these results imply that 2-*cis*, 6-*cis*-farnesyl pyrophosphate (48) is the precursor which is cyclised as implied in (55). This is the only case in which the 6-*cis* isomer of farnesol has been implicated in sesquiterpenoid biosynthesis. It may be noted that its putative precursor, neryl pyrophosphate (57) has been reported (19) to be essentially unreactive in enzymatic condensation with isopentenyl pyrophosphate.



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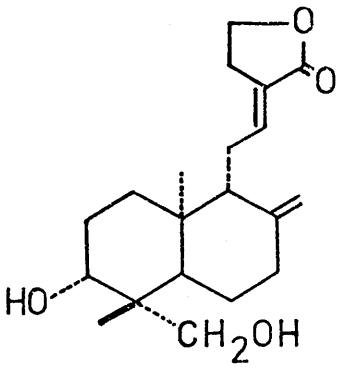

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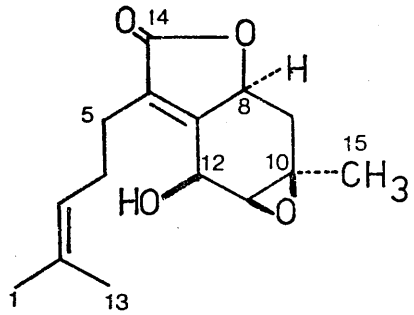

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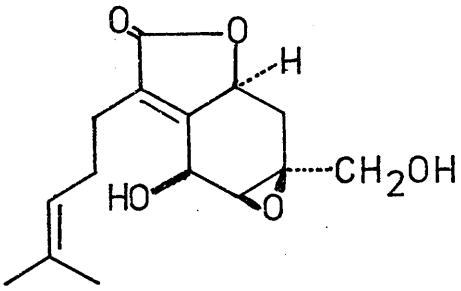
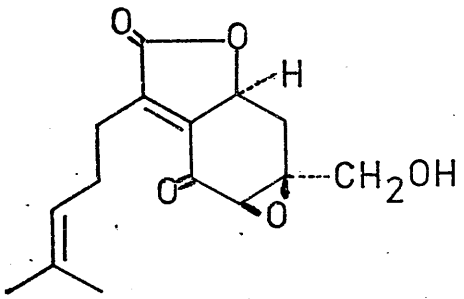


(62)



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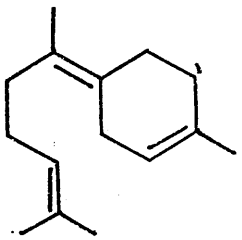
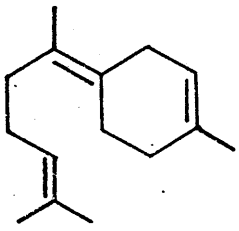


(12)

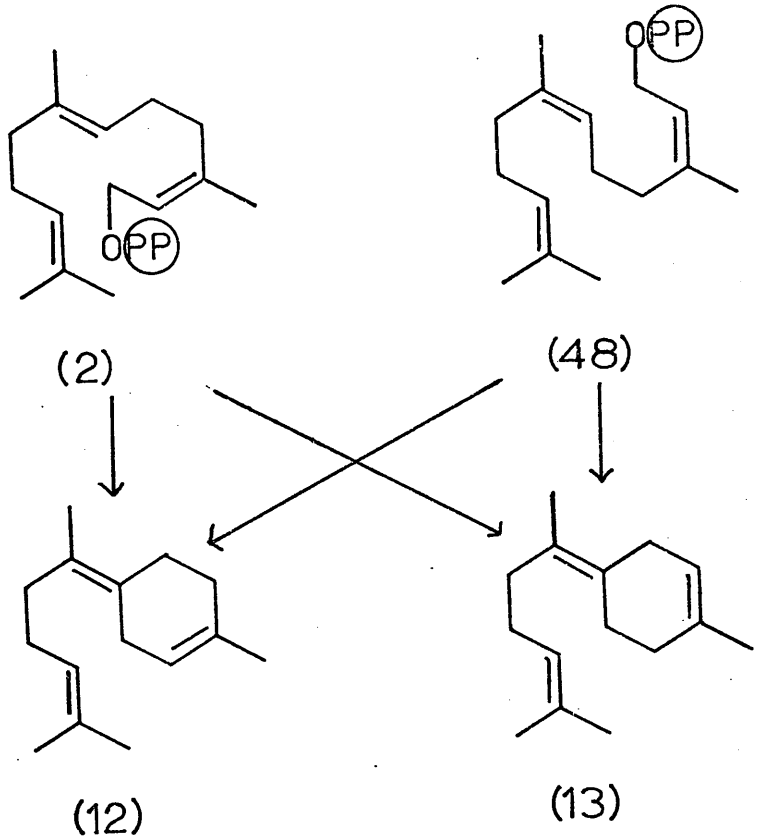


(13)

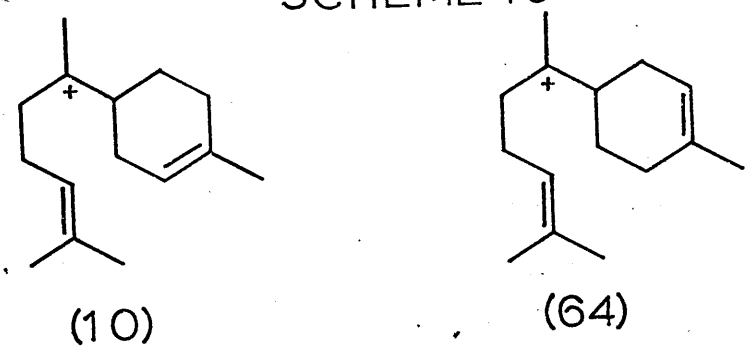
Discussion.

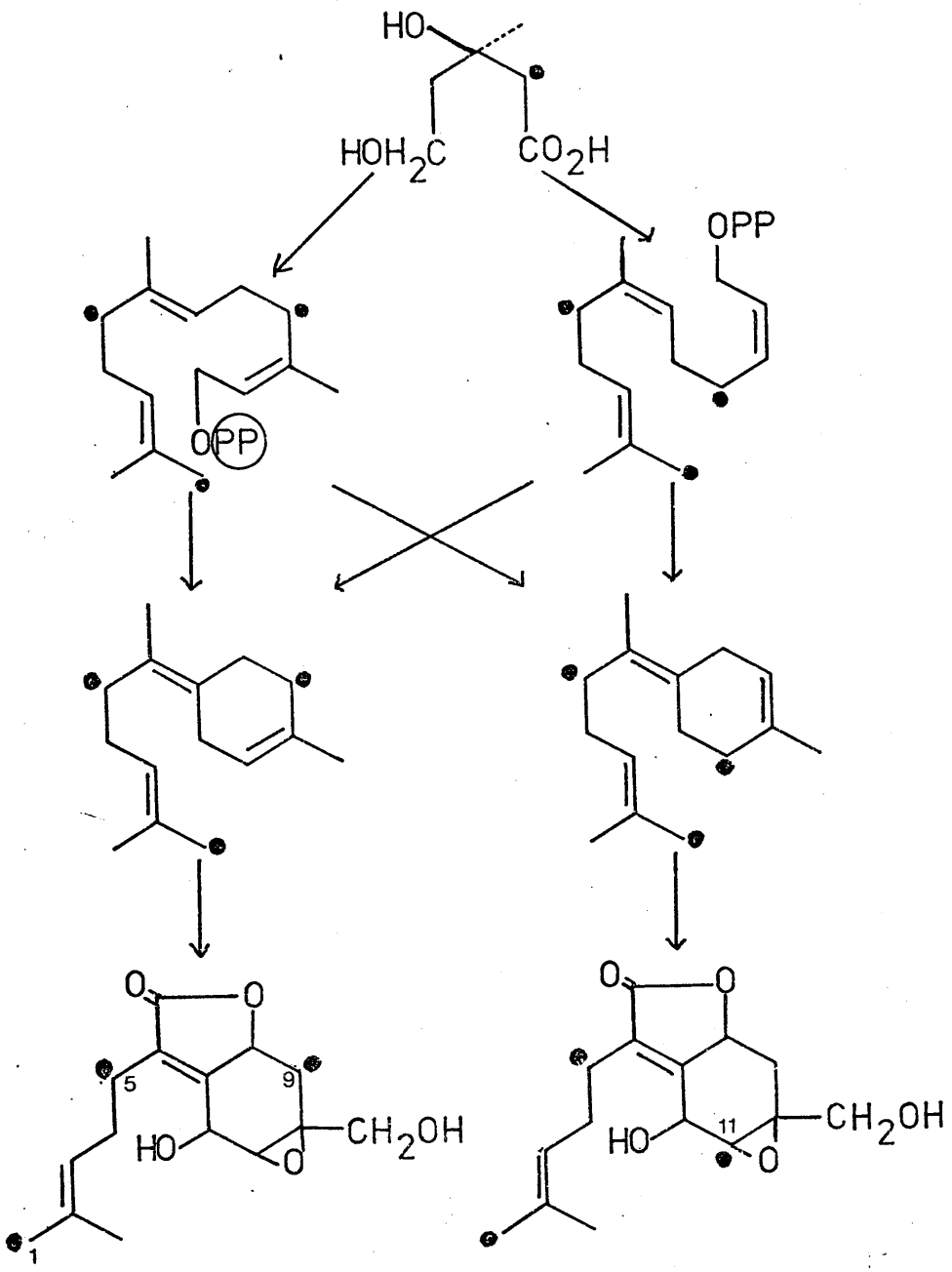
Andrographis paniculata Nees. (Acanthaceae), known in English as the creat plant, is native to India and the tropics. It forms the basis of numerous traditional Indian medicinal preparations (20) and because of this, its constituents have been the subject of several investigations (21). The plant contains a variety of diterpenoids based on ent-labatriene (59) of which andrographolide (60) is typical. However, no diterpenoid substances could be detected (22) in callus tissue cultures of A. paniculata despite the examination of several isolates from different plant parts obtained under various conditions of culture. Instead, three sesquiterpenoid lactones, paniculides A (61), B (62) and C (63) were isolated from the culture medium (23). Paniculide B (62) was consistently produced in large amounts, while paniculide A (61) was produced by cultures which were grown in the dark and the unstable compound paniculide C (63) appeared only sporadically. These metabolites bear functional groups similar to the diterpenoids and appear to be based on γ-bisabolene (12) or (13). On the other hand, none of these sesquiterpenoids is present in the intact plant. This is a rather rare example of a tissue culture which produces metabolite not closely related to substances found in the parent plant. This unexpected result prompted the investigation of the biosynthesis of these sesquiterpenoids in our laboratories.

Despite the importance which has been attached to the role which γ-bisabolene, or cations derived from it, play in the biosynthesis of sesquiterpenoids, the biosynthesis of the parent compound has never been studied in detail.



SCHEME 10



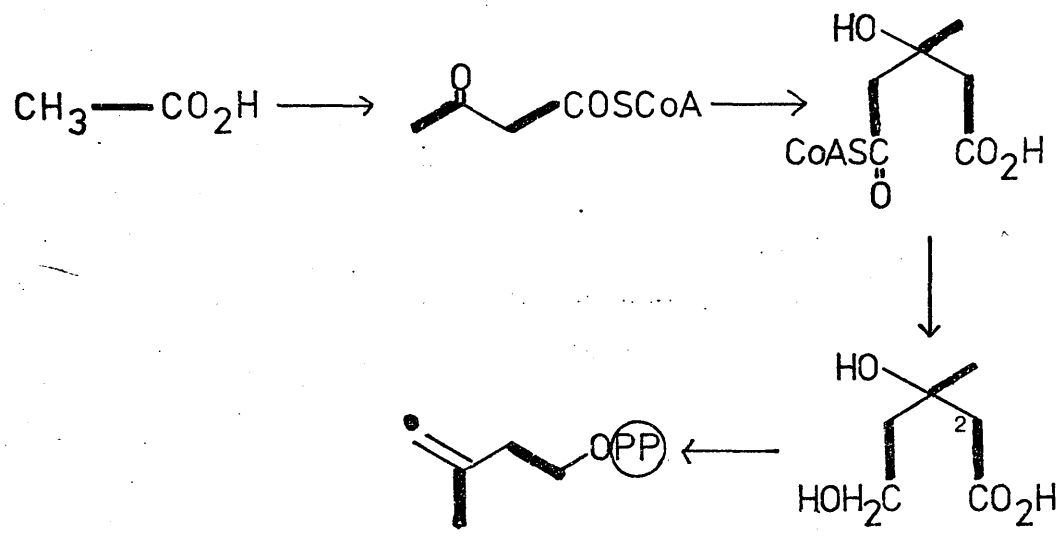


SCHEME 11

In 1962, Ruzicka outlined (24) possible pathways for the formation of ϒ-bisabolene as shown in scheme 10. He proposed that either cis, trans-farnesyl pyrophosphate (2) or the cis, cis-isomer (48) could cyclise, as previously discussed, to give Z-ϒ-bisabolene (12) or E-ϒ-bisabolene (13) respectively. However, a crossover pathway via non-classical cations is possible in principle, as scheme 10 demonstrates. This arises out of the possible interconversion of the intermediate carbonium ions (10) and (64) by rotation about the C-6, C-7 bond. Thus, either geometrical isomer of farnesyl pyrophosphate could be the substrate and either geometrical isomer of ϒ-bisabolene could be the product in the cyclisation.

A series of experiments was devised to resolve these questions by the study of the biosynthesis of paniculide B (62) in A. paniculata tissue cultures. As shown in scheme 11, a preliminary answer to the problem could come from examination of the fate of C-2 of mevalonate in paniculide B. Two of the proposed pathways would lead to C-9 of paniculide B being derived from C-2 of mevalonate, while in the other two pathways C-11 would be derived from C-2 of mevalonate . Since isolation of the diagnostic carbon by degradation would be difficult in the case of paniculide B, the problem was tackled by ^{13}C NMR.

The use of [1,2-$^{13}C_2$]acetate as a precursor of terpenoids readily allows the identification of carbon atoms of the metabolite which are derived from C-2 of mevalonate (25). The acetate precursor used is highly enriched at both carbon atoms and thus shows ^{13}C-^{13}C coupling in the ^{13}C NMR spectrum. When mevalonate is biosynthesised from



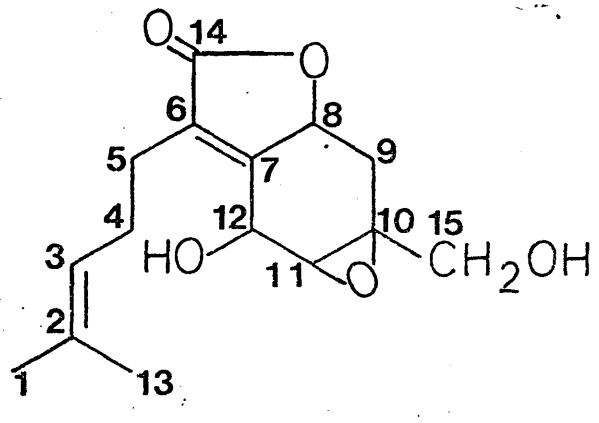
(—) coupled ^{13}C atoms

SCHEME 12

Table 1.

^{13}C NMR spectrum of paniculide B.

Peak no.	Chemical shift δ(ppm)	No. of attached H atoms (off resonance)	Enriched from [1-^{13}C] acetate	Assignment
1	173.3	0	-	C-14
2	161.0	0	-	C-7
3	131.3	0	+	C-2, C-6
4	126.4	0	+	
5	123.5	1	-	C-3
6	75.2	1	+	C-8
7	67.2	1	+	C-12
8	63.6	2	-	C-15
9	61.8	1	-	C-11
10	60.1	0	+	C-10
11	32.7	2	-	C-9
12	27.1	2	+	C-4
13	25.3	3	-	C-1
14	22.8	2	-	C-5
15	17.4	3	-	C-13



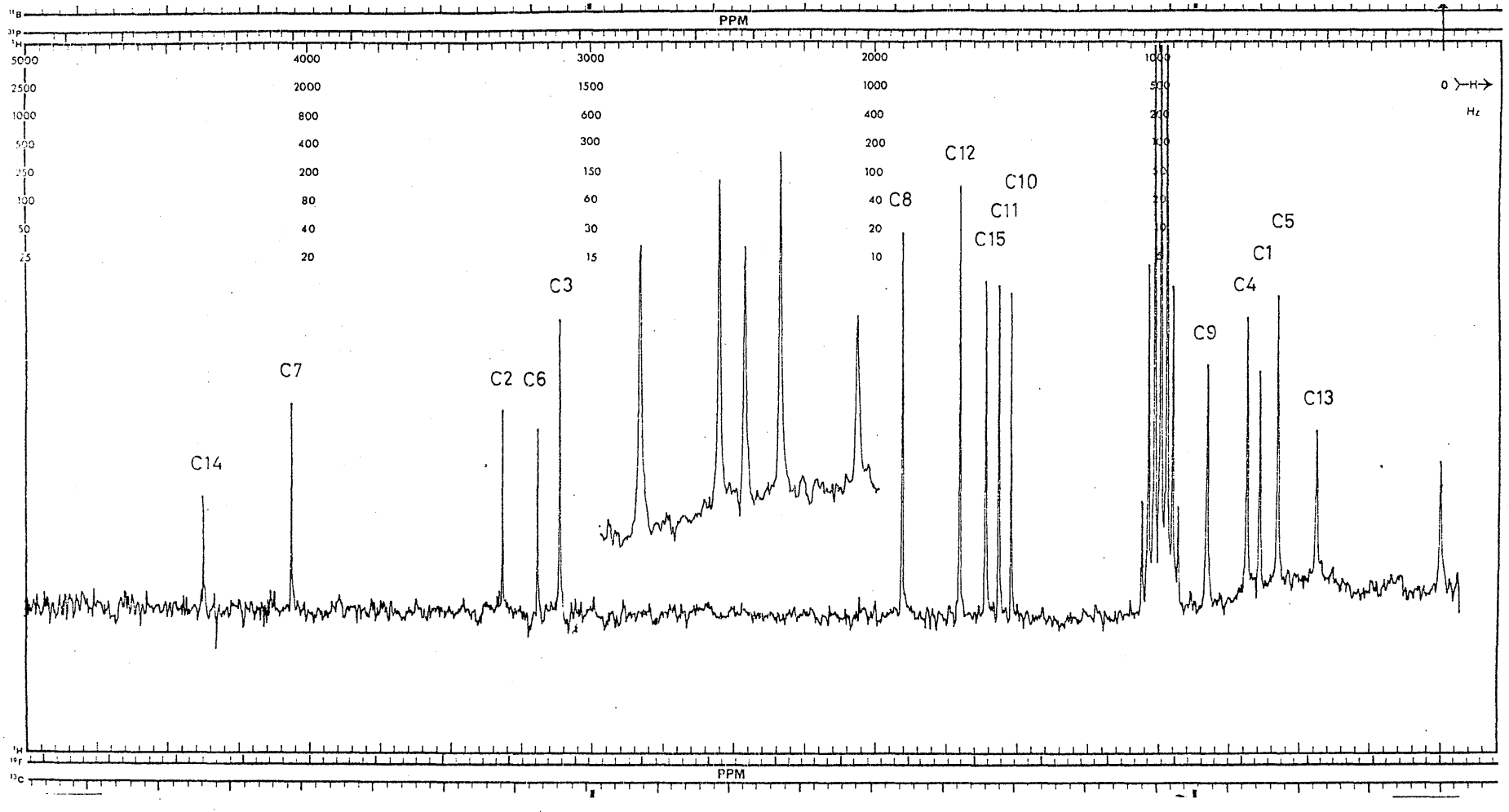
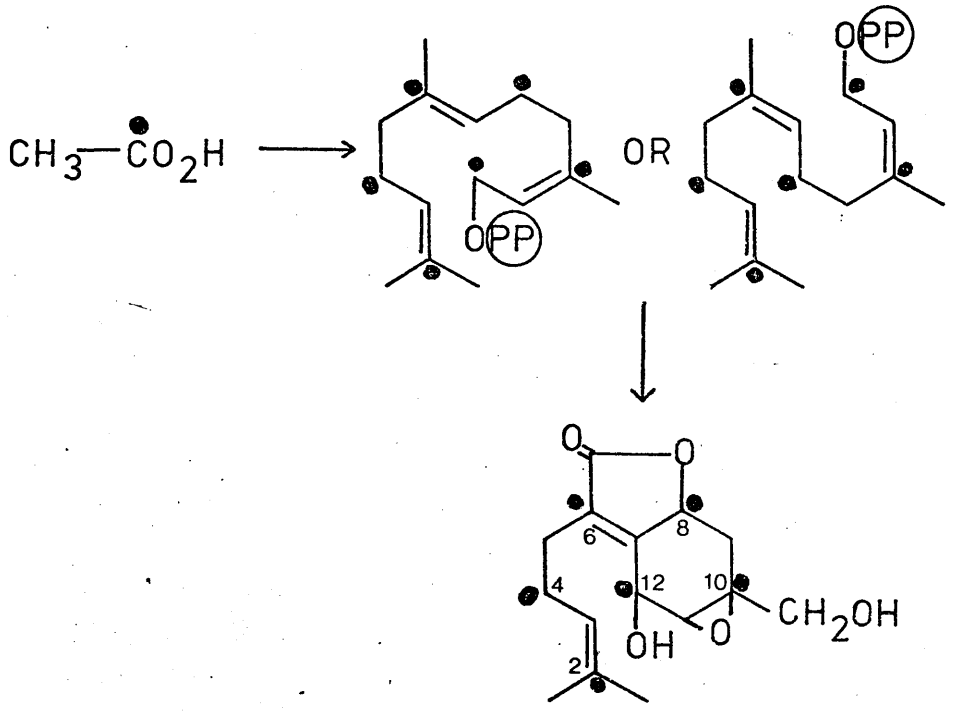


Figure 1 ^{13}C NMR Natural Abundance Spectrum of Paniculide B (proton noise-decoupled)

doubly-labelled acetate as shown in scheme 12, there are three pairs of intact acetate units. Thus in the ^{13}C NMR spectrum of this mevalonate one observes coupling of C-1 with C-2, C-3 with C-3' and C-4 with C-5. No coupling is observed between adjacent acetate units since the precursor is incorporated highly diluted with unlabelled endogenous acetate. Thus there is only a small (but finite) chance that any one molecule of metabolite contains more than one labelled acetate unit. When mevalonate is converted into isopentenyl pyrophosphate, C-1 is lost as carbon dioxide. In this step, C-2 loses its ^{13}C partner and thus carbon atoms derived from C-2 of mevalonate are not coupled in the ^{13}C NMR spectrum of any terpenoid formed from [1,2-^{13}C] acetate. It should be noted that signals from coupled ^{13}C atoms contain two elements, a doublet, produced by molecules which have neighbouring enriched ^{13}C and a singlet, produced by those molecules which do not have neighbouring enriched ^{13}C. Thus all coupled atoms give signals which appear as triplets, while carbon atoms derived from C-2 of mevalonate give singlets whose intensity is enhanced compared with natural abundance.

The ^{13}C NMR spectrum of paniculide B was assigned by means of chemical shift data, off-resonance decoupling of of the natural abundance spectrum and examination of the spectrum of material derived from feeding [1-^{13}C]acetate. The information obtained from these experiments is summarised in table 1. The natural abundance ^{13}C NMR spectrum of paniculide B is shown in figure 1. The lowest field resonance at δ173.3ppm can be assigned on the basis of its chemical shift to the carbonyl group, C-14. The carbonyl



(65)

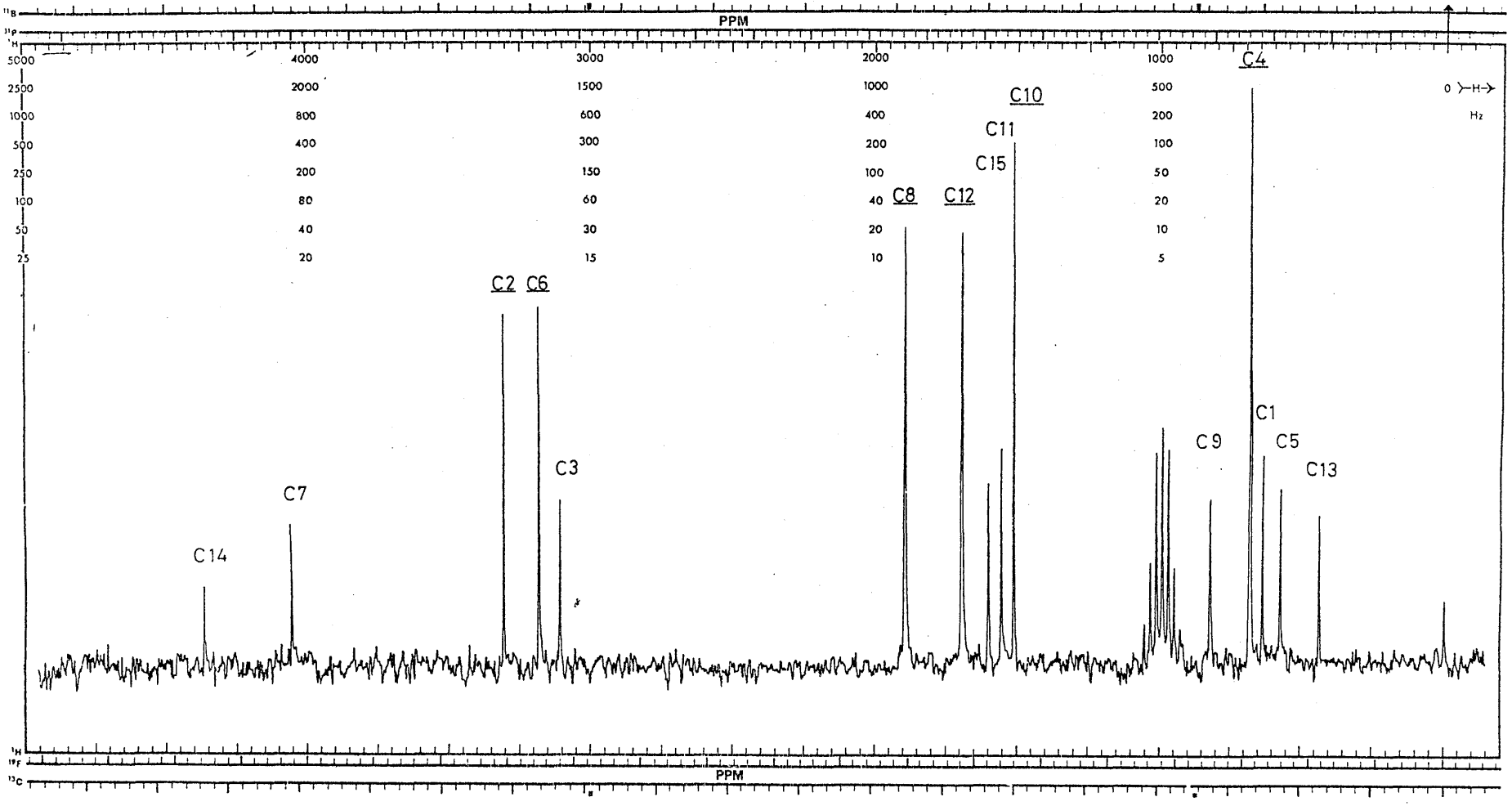
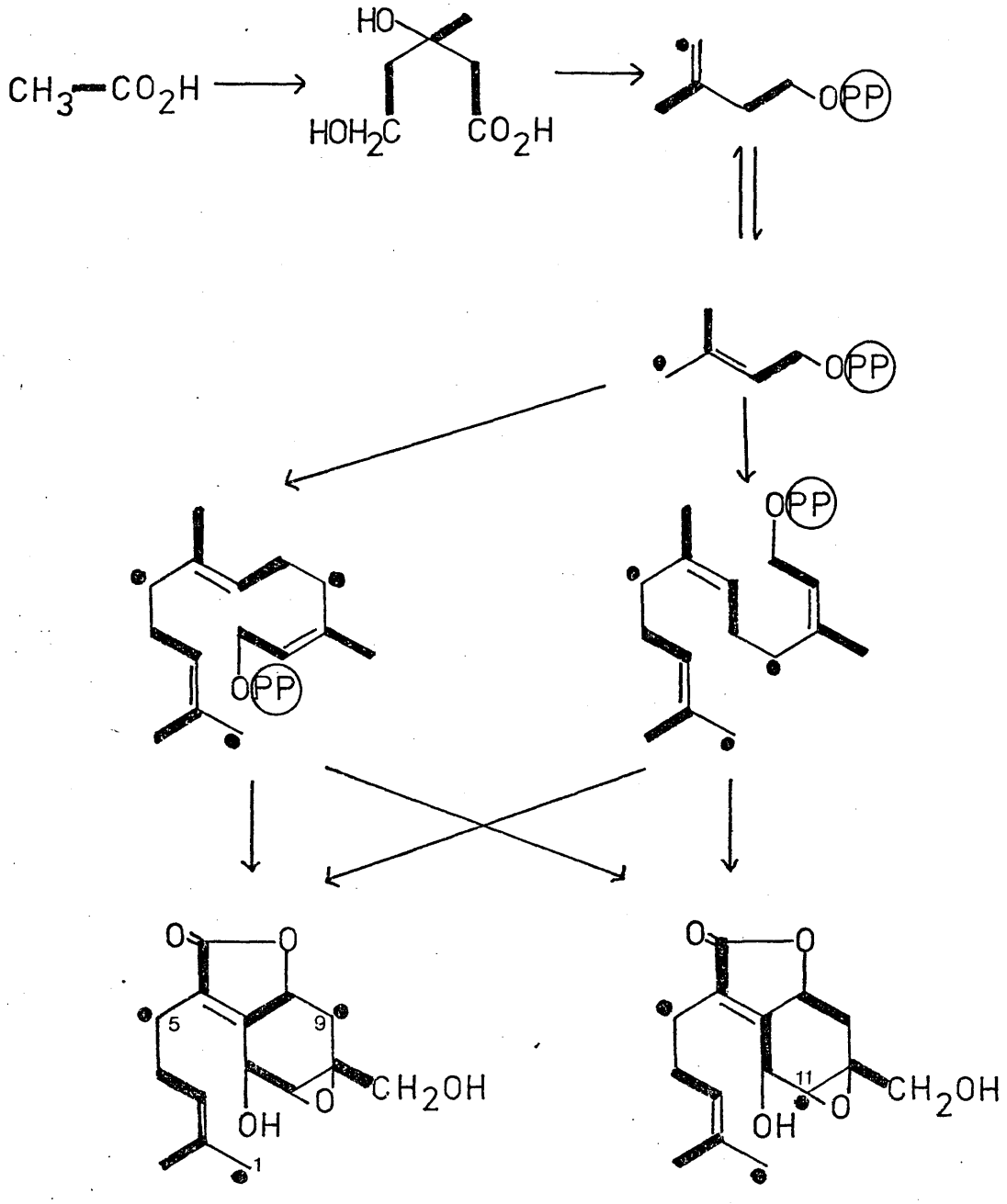


Figure 2 ^{13}C NMR Spectrum of Paniculide B Biosynthesised from [1-^{13}C]Acetate (proton noise-decoupled) Enriched signals are indicated

shielding of the parent system cyclobutanolide is reported to be 178.4ppm (26), and this value would be expected to decrease by about 5ppm on conjugation, as in the case of saturated and unsaturated methyl esters. Peak 2 has been assigned to C-7, which is vinylogously related to the carbonyl group. Calculation of the chemical shift of such a carbon atom from substituent parameters gives a value of 166.9ppm, which is in reasonably good agreement with the observed value of 161.0ppm. The remaining peaks fall into three distinct groups on the basis of chemical shift.

Peaks 3, 4 and 5 clearly correspond to the three remaining sp^2-hybridised carbon atoms of paniculide B, C-2, C-3 and C-6. Off-resonance experiments show that peak 5 is the signal from a carbon atom bearing one hydrogen, which allows it to be assigned to C-3. The shielding of this carbon atom at δ123.5ppm is in good agreement with that of similar carbon atoms in acyclic terpenoids. Peaks 3 and 4 cannot be unambiguously assigned at this stage.

Peaks 6, 7, 8, 9 and 10 are the signals of carbon atoms bearing oxygen i.e. C-8, C-10, C-11, C-12 and C-15. On the basis of off-resonance experiments, peak 8 can be assigned to C-15, while peak 10 corresponds to C-10. The spectrum of paniculide B derived from [1-^{13}C]acetate (figure 2) shows enrichment of peaks 3, 4, 6, 7, 10 and 12. Regardless of the mode of folding farnesyl pyrophosphate, [1-^{13}C]acetate should label paniculide B as shown in (65). Of the peaks in this group which remain unassigned, only peak 9 is not enriched from [1-^{13}C]acetate, and thus must correspond to C-11. Esterification results in a downfield shift of the ^{13}C resonance of a carbinol



(66) (67)

SCHEME 13

(▬▬) coupled ^{13}C atoms

(●) non-coupled ^{13}C atoms

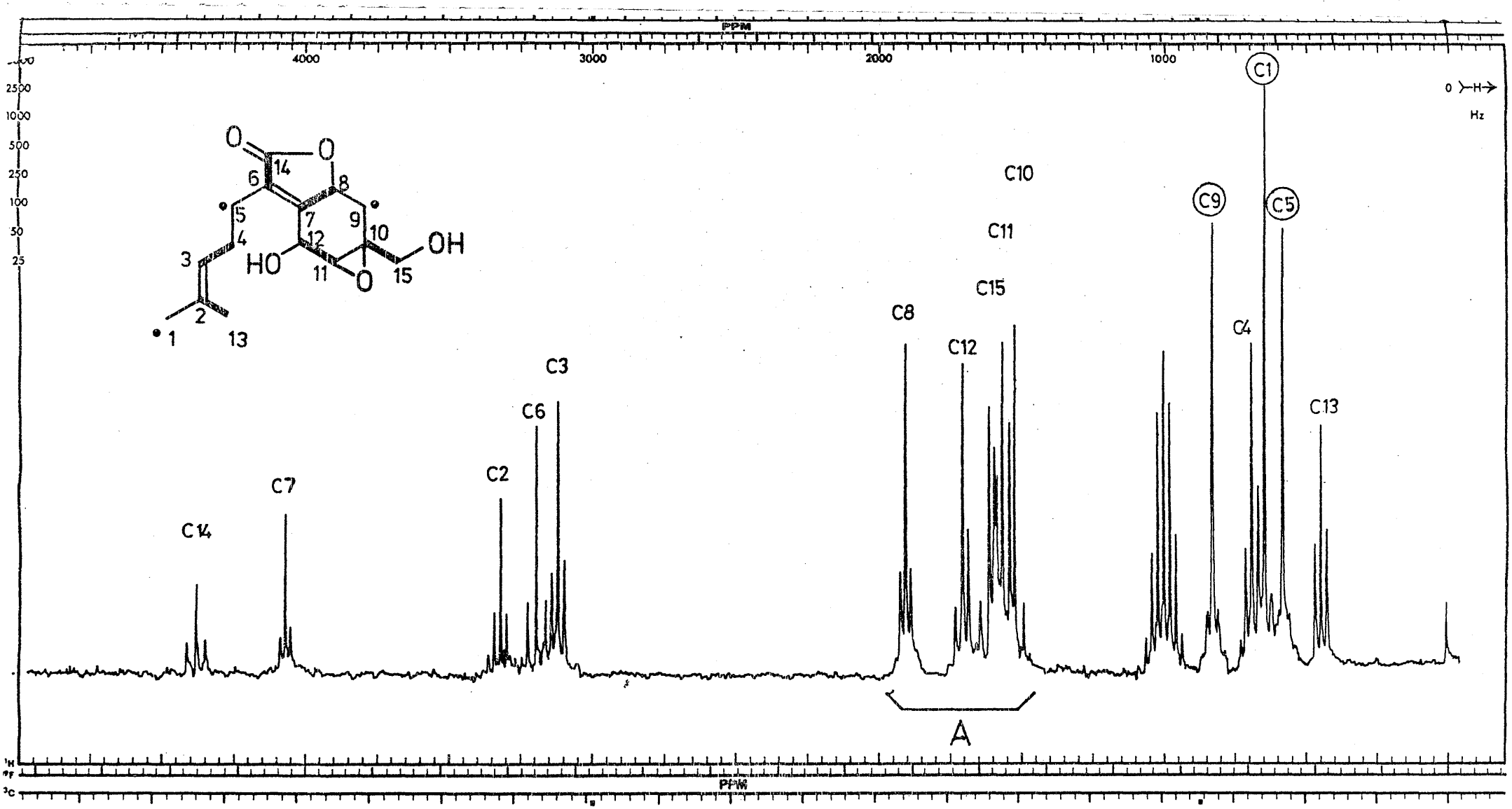


Figure 3 ^{13}C NMR Spectrum of Paniculide B Biosynthesised from [1,2-$^{13}C_2$]Acetate (proton noise-decoupled)

carbon atom. Peak 6 can be assigned to C-8 since it is at lower field than peak 7 which can therefore be ascribed to C-12.

The remaining methyl and methylene carbon atoms of paniculide B i.e. C-1, C-4, C-5, C-9 and C-13 must account for the remaining signals. Peak 12 is enriched when [1-^{13}C]acetate is used as precursor and thus may be assigned to C-4. Off-resonance experiments showed that peaks 11 and 14 both correspond to methylene groups, and consideration of the relative chemical shifts, and the residual coupling observed, allow these signals to be assigned to C-9 and C-5 respectively. Peaks 13 and 15 correspond to the two methyl groups C-1 and C-13. A methyl group which is cis to an alkyl substituent is more highly shielded than one which is trans, as has been shown by the study of the cis- and trans- isomers of but-2-ene and a variety of acyclic terpenoids (27). Thus peak 13 and peak 15 can be assigned to C-1 and C-13 respectively.

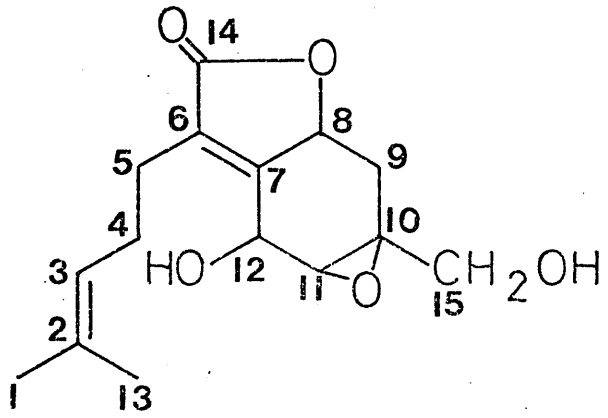
The only remaining ambiguity in the assignment is the identity of peaks 3 and 4, which must be assigned to C-2 and C-6.

Scheme 13 shows the possible pathways to paniculide B, indicating the two different arrangements of acetate units which could be produced, The ^{13}C NMR spectrum of paniculide B derived from [1,2-$^{13}C_2$]acetate (figure 3) shows three signals which are essentially singlets as expected, since three of the carbon atoms of a sesquiterpenoid must be derived from C-2 of mevalonate. These singlet signals correspond to C-1, C-5 and C-9. Paniculide B is thus represented by (66) and not (67). Carbon-11 of paniculide B appears in the spectrum as a triplet which is made up of a

Table 2.

^{13}C-^{13}C coupling constants obtained from the spectrum shown in figure 3.

Carbon	Chemical shift δ(ppm)	$^{1}J(^{13}C-^{13}C)$Hz
1	25.3	-
2	131.2	42
3	123.5	43
4	27.1	44
5	22.8	-
6	126.4	62
7	161.0	35
8	75.2	35
9	32.7	-
10	60.1	49
11	61.8	46
12	67.2	46
13	17.4	42
14	173.3	63
15	63.6	49



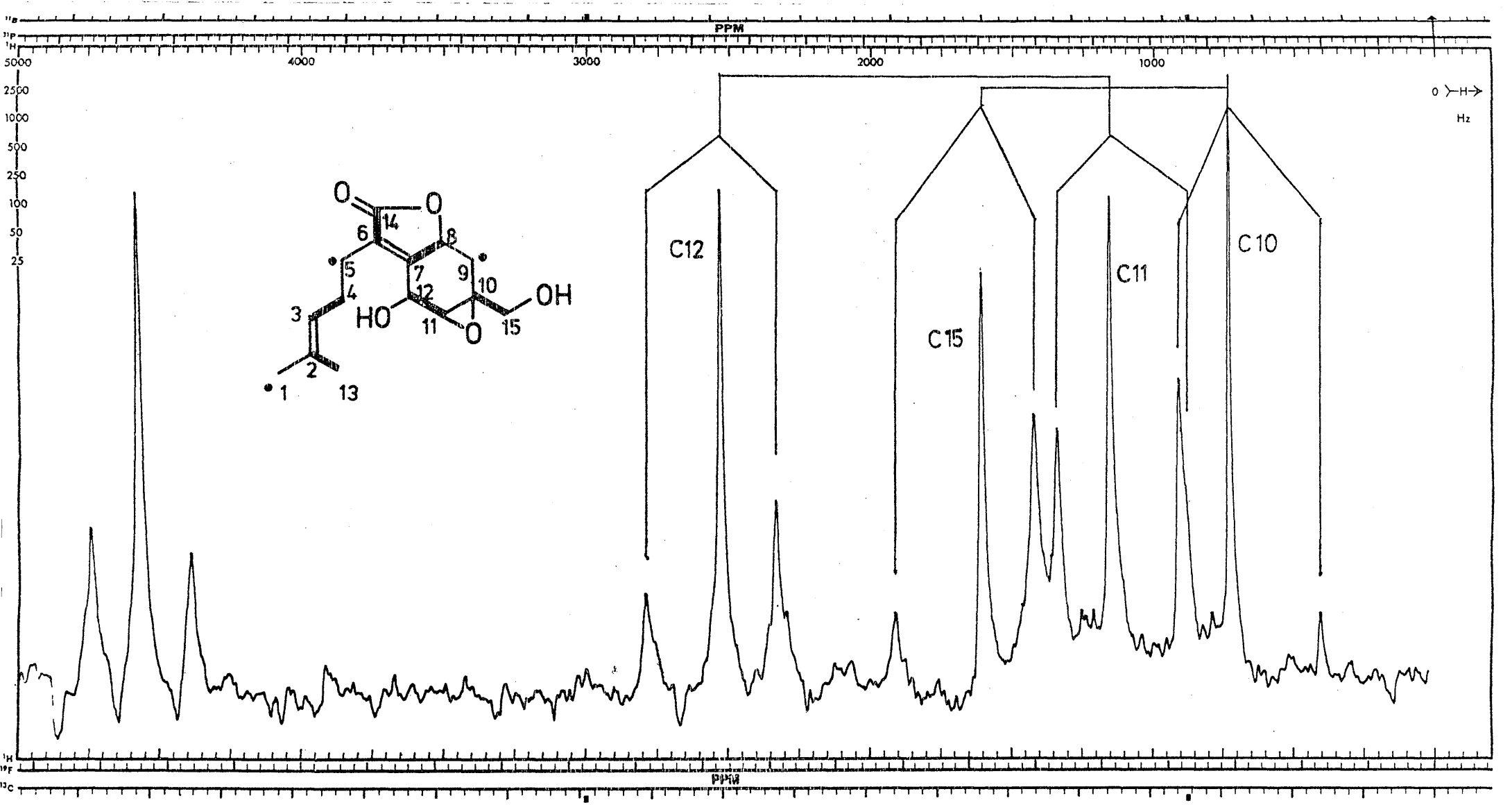


Figure 4 ^{13}C NMR Spectrum of Paniculide B Biosynthesised from [1,2-$^{13}C_2$]Acetate: Segment A of Figure 3 expanded

singlet natural abundance signal on which is superimposed the doublet due to coupling within the acetate unit. The chemical shift of each carbon atom of paniculide B and the coupling constants obtained from the spectrum are given in table 2. The coupling constant observed for peak 3 is 42Hz, while that observed for peak 4 is 62Hz. This now allows their unambiguous assignment as C-2 and C-6 respectively, since they are coupled to C-3 and C-14 which have already been assigned.

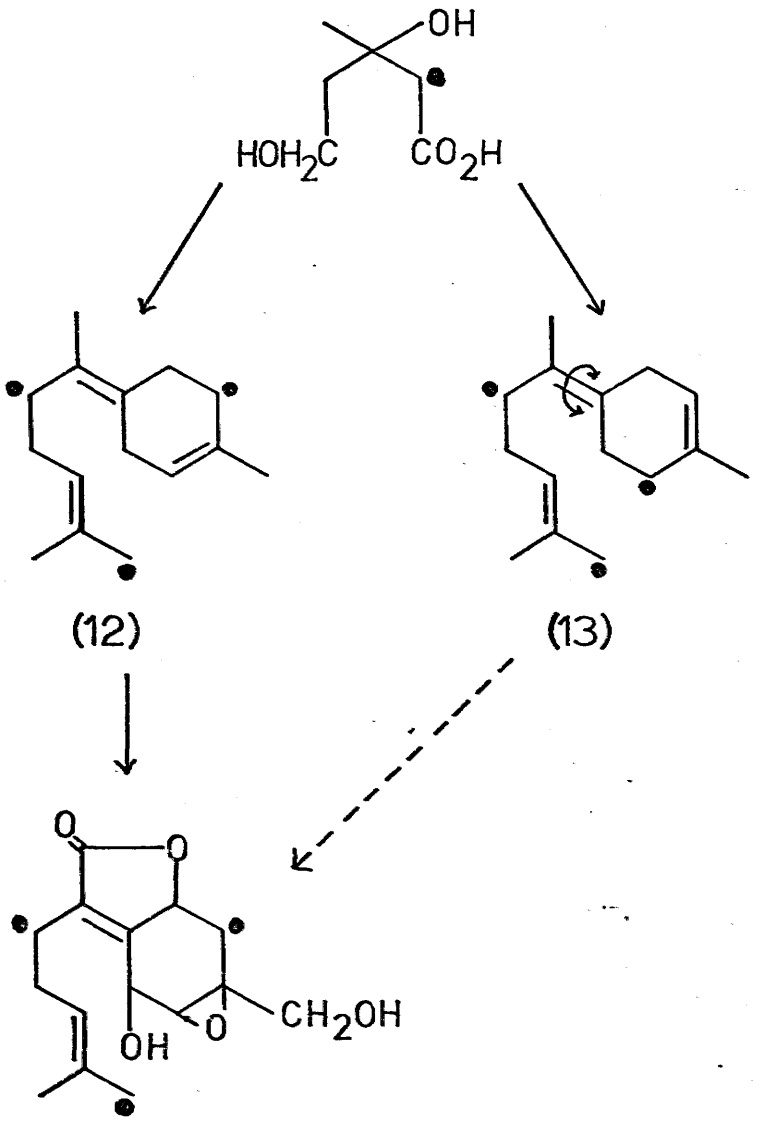
The one bond $^{13}C-^{13}C$ coupling constants J_{CC} are known to be approximately correlated by the s character of the orbitals making up the bond under consideration. Thus, in general, J_{CC} increases in the order $sp^3-sp^3 < sp^3-sp^2 < sp^2-sp^2$. The values of $J_{2,13}$ and $J_{3,4}$ are similar to those quoted in the literature for such bonds (28), while $J_{7,8}$ is considerably lower, possibly because of the influence of the electron withdrawing and electronegative substituents. The value of $J_{6,14}$ is higher, as would be expected for a bond between two sp^2 hybridised atoms. The coupling constants for the sp^3-sp^3 bonds are rather higher than the values normally quoted. However, it has been noted that substitution of sp^3C-CH_3 bonds with electronegative atoms or alkyl groups increases the value of the coupling constant. For example, in ethane J_{CC} is 34.6Hz, while in ethanol it is 37.7Hz. The high coupling constants observed may be a reflexion of the heavily oxygenated cyclohexane ring in paniculide B.

The spectrum shows two AB quartets for coupling of C-12 with C-11 and C-15 with C-10. The approximate portion of the spectrum is shown in figure 4. It should be noted

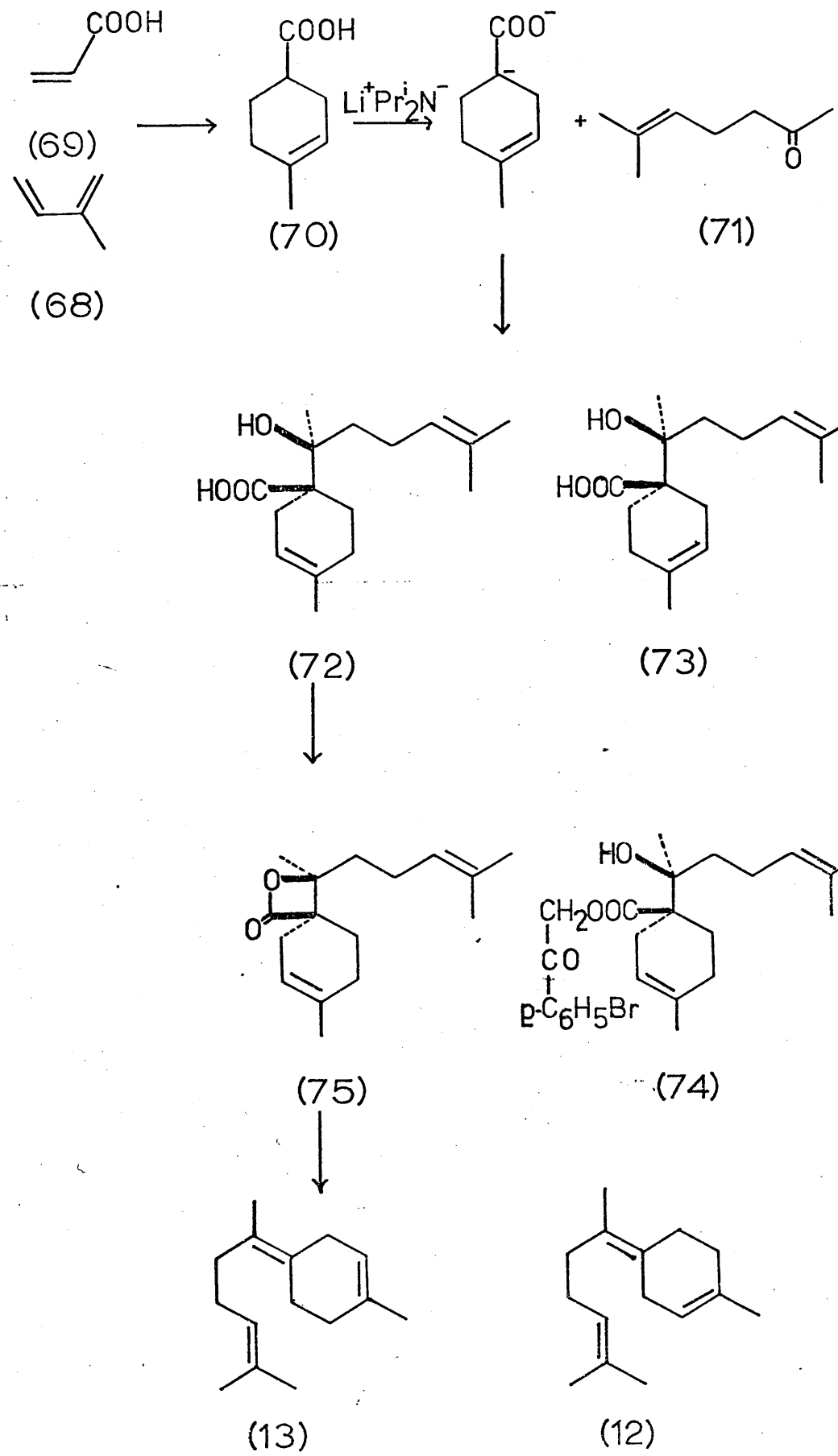
Table 3.

Calculated position of singlet components of AB systems.

	C_{12}	C_{11}	C_{15}	C_{10}
obs (Hz)	236.0	98.9	144.4	57.1
calc (Hz)	236.2	99.5	144.6	57.3



SCHEME 14

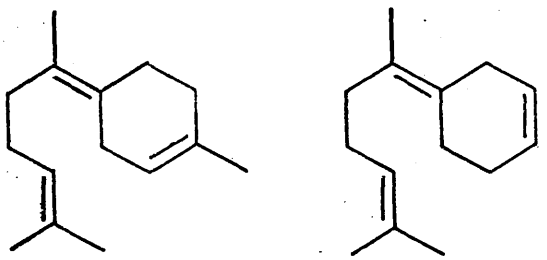

COOH
(69)
(68)
COOH
(70)
Li⁺Pr²₂N⁻
COO⁻
O
(71)
HO
HOOC
(72)
HO
HOOC
(73)
O
O
(75)
HO
CH₂OOC
CO
p-C₆H₅Br
(74)
(13)
(12)

SCHEME 15

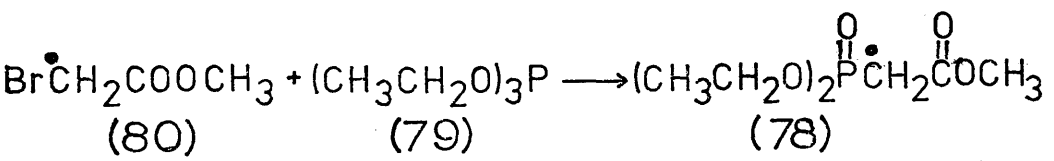
that the singlet (non-coupled) element of each signal is not in the middle of its doublet. Calculation of the chemical shift of each resonance from the positions of the AB quartets gives complete agreement with the position of the observed natural abundance signal (table 3). All calculated shifts were within 0.6Hz of the observed value.

From the labelling pattern in paniculide B, it appears likely that the precursor ϒ-bisabolene is the Z-isomer (12) and not the E-isomer (13). However, since double bond isomerisation of ϒ-bisabolene cannot be excluded, independent evidence on the identification of ϒ-bisabolene as either the E- or Z-isomer is desirable. Isomerisation of the bisabolene skeleton about the tetrasubstituted double bond of E-ϒ-bisabolene during elaboration of paniculide B could lead to the observed labelling pattern arising from this isomer (scheme 14).

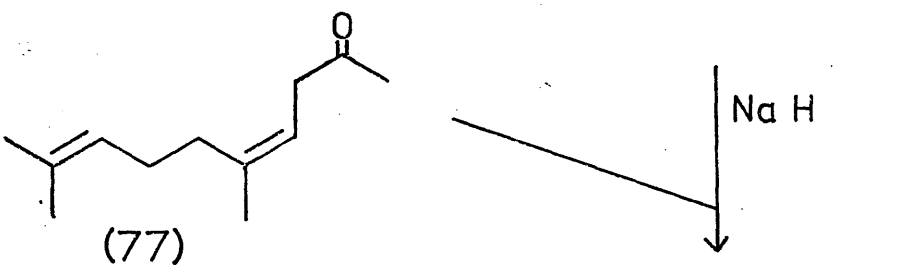
The E- and Z-isomers of ϒ-bisabolene have recently been identified by Faulkner (29), who unambiguously synthesised the two isomers by the route depicted in scheme 15. Diels-Alder condensation of isoprene (68) with acrylic acid (69) yielded 4-methylcyclohex-3-ene carboxylic acid (70). Treatment of the derived di-anion as the lithium salt with 6-methylhept-5-ene-2-one (72) gave the β-hydroxy acid as a mixture of diastereomers (72) and (73) which were separated by fractional crystallisation from chloroform. The relative configuration of the less soluble isomer (72) was determined by X-ray crystal structure determination of the p-bromophenacyl derivative (74). The hydroxy acid (72) was then converted into the corresponding β-lactone (75) by treatment with p-toluenesulphonyl chloride in pyridine,



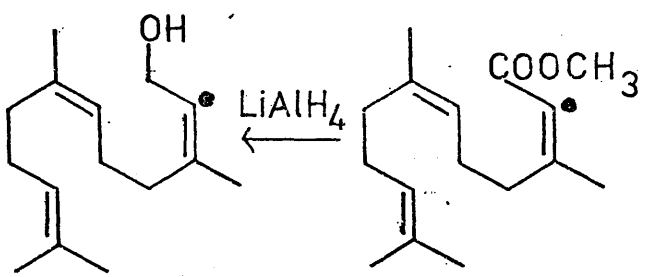
(12) (13)



(80) (79) (78)



(77)



(76)

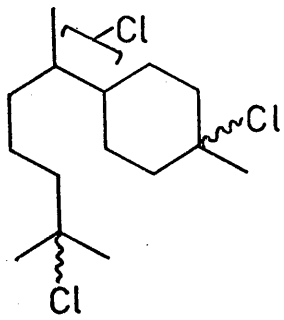
(•) represents ^{14}C

SCHEME 16

a procedure which is known to preserve the configuration at the carbinol carbon atom (30). Pyrolytic elimination of carbon dioxide from the lactone then afforded pure E-γ-bisabolene (13). In the same manner, Z-γ-bisabolene (12) was prepared, starting with the diastereomeric hydroxy acid (73).

The cell-free extract prepared from Andrographis paniculata callus cultures incorporates radioactivity from [2-^{14}C]mevalonate into 2-trans, 6-trans and 2-cis, 6-trans farnesol and into γ-bisabolene. Material biosynthesised by the cell-free system was examined by radio-gas liquid chromatography. Co-injection of γ-bisabolene biosynthesised from [2-^{14}C]mevalonate and a mixture of synthetic Z- and E-γ-bisabolene located radioactivity only in the Z-isomer (12). Thus it appears that Z-γ-bisabolene (12) is the precursor of paniculide B and that there is no transposition of the double bond in its formation. It is of interest to note that Faulkner has found (29) that γ-bisabolene from plant sources contains only the Z-isomer (12), while the E-isomer is present in a species of seaweed (Laurencia).

The only remaining question to be answered is which isomer of farnesol is involved in the cyclisation process. The cell-free system produces only trans, trans- and cis, trans-farnesol. Although neither of the 6-cis-isomers can be detected, this by no means excludes them as intermediates in the biosynthetic process. In order to test the incorporation of cis, cis-farnesol into γ-bisabolene, in the cell-free system, [2-^{14}C]cis, cis-farnesol (76) was prepared (31) by Wittig condensation of neryl acetone (77) with ^{14}C-labelled diethylmethoxycarbonylmethylphosphonate



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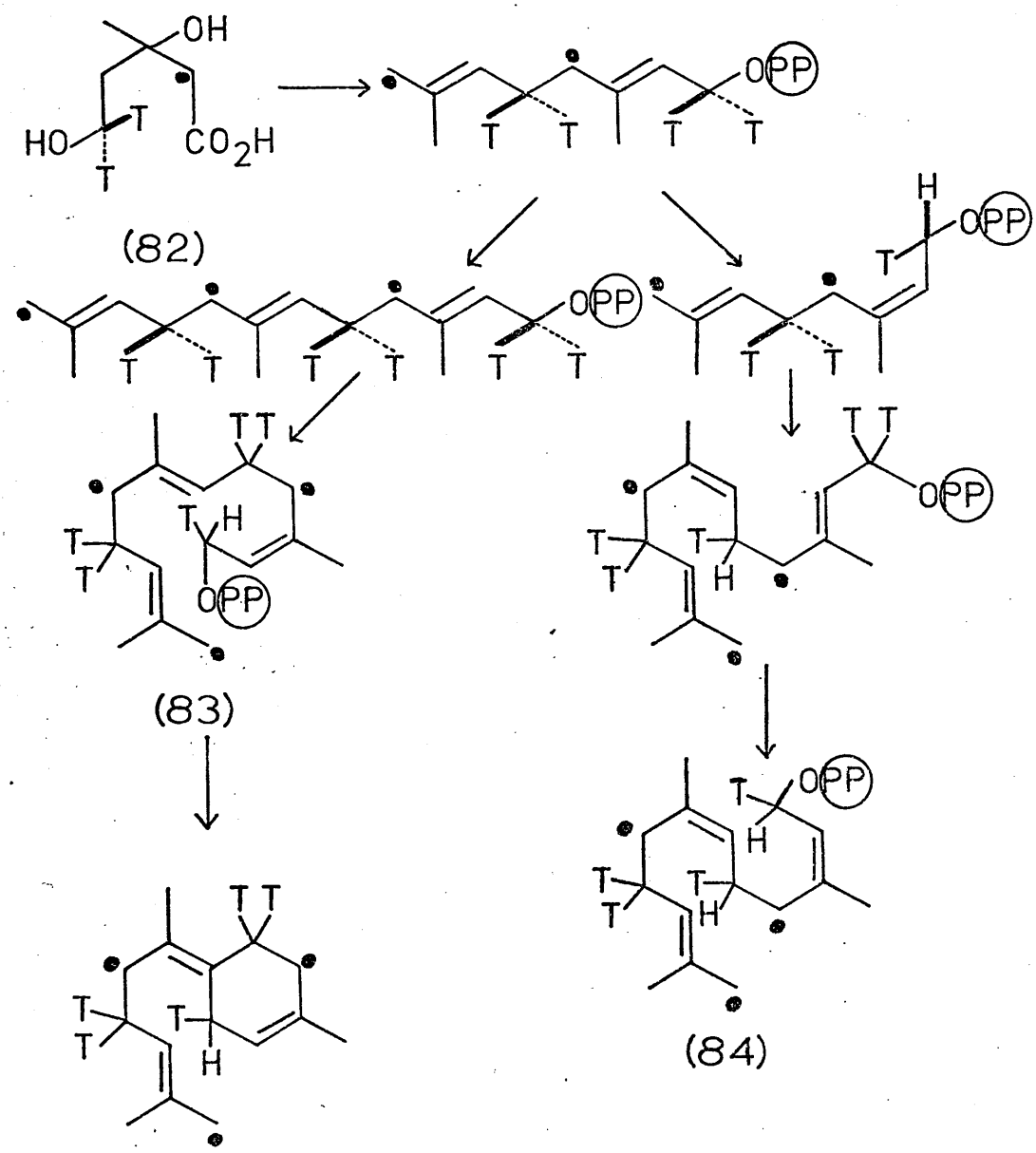
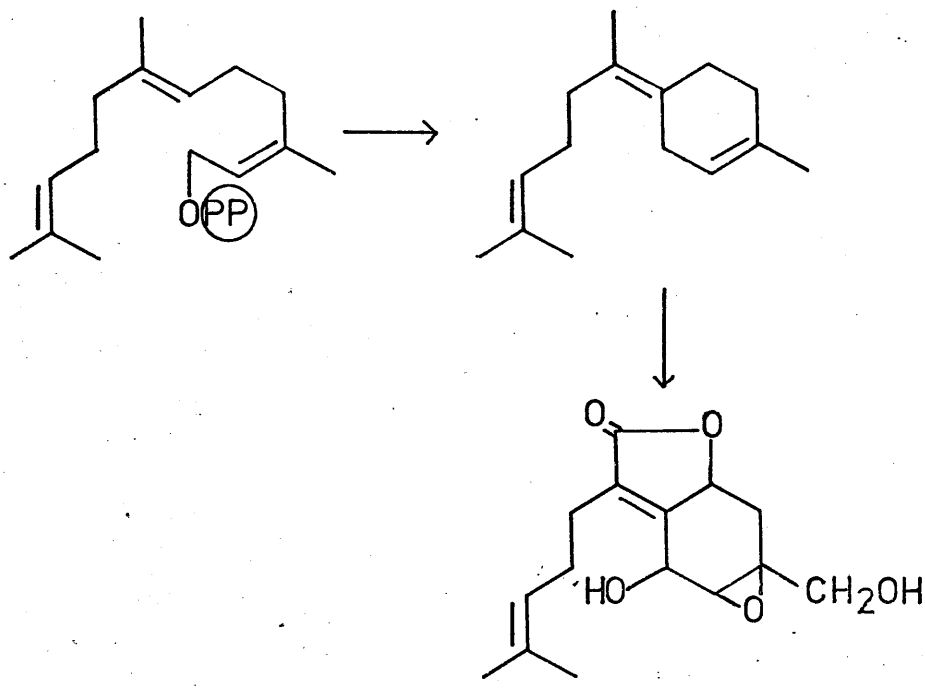


SCHEME 17

(78), which was itself prepared by Michaelis-Arbuzov reaction of triethylphosphite (79) with methyl [2-^{14}C] bromoacetate (80), as shown in scheme 16. For purposes of double labelling, [4,8,12-$^{14}C_3$]cis, trans-farnesol was prepared biosynthetically from [2-^{14}C]mevalonate using the Andrographis cell-free extract. The substrates were incubated with the cell-free system, and the hydrocarbon fraction was isolated by preparative thin layer chromatography after addition of carrier. The crystalline derivative, bisabolene trihydrochloride (81), was then prepared (32) in each case. After crystallisation to constant activity, the incorporation of radioactivity from [2-^{14}C]cis, cis-farnesol was 0.02%, while [4,8,12-$^{14}C_3$] cis, trans-farnesol gave an incorporation of 1.2%. Thus cis, trans- and not cis, cis-farnesyl pyrophosphate appears to be involved in the biosynthesis of Z-γ-bisabolene.

Further support for the intermediacy of cis, trans-farnesyl pyrophosphate in bisabolene biosynthesis comes from an examination of the fate of label from C-5 of mevalonate. Double labelling experiments using [2-^{14}C, 5-$^{3}H_2$]mevalonate (82) showed that bisabolene retained five out of the six labels from mevalonate. This is in accord with biosynthesis via cis, trans-farnesyl pyrophosphate (83) which has lost one of the labels from C-1 of the farnesyl residue during trans to cis isomerisation (33). The intermediacy of cis, cis-farnesyl pyrophosphate (84) would require loss of two of the tritium labels derived from C-5 of mevalonate, one at the C_{10} stage and one at the C_{15} stage (scheme 17).

The stereochemistry of this hydrogen loss has recently

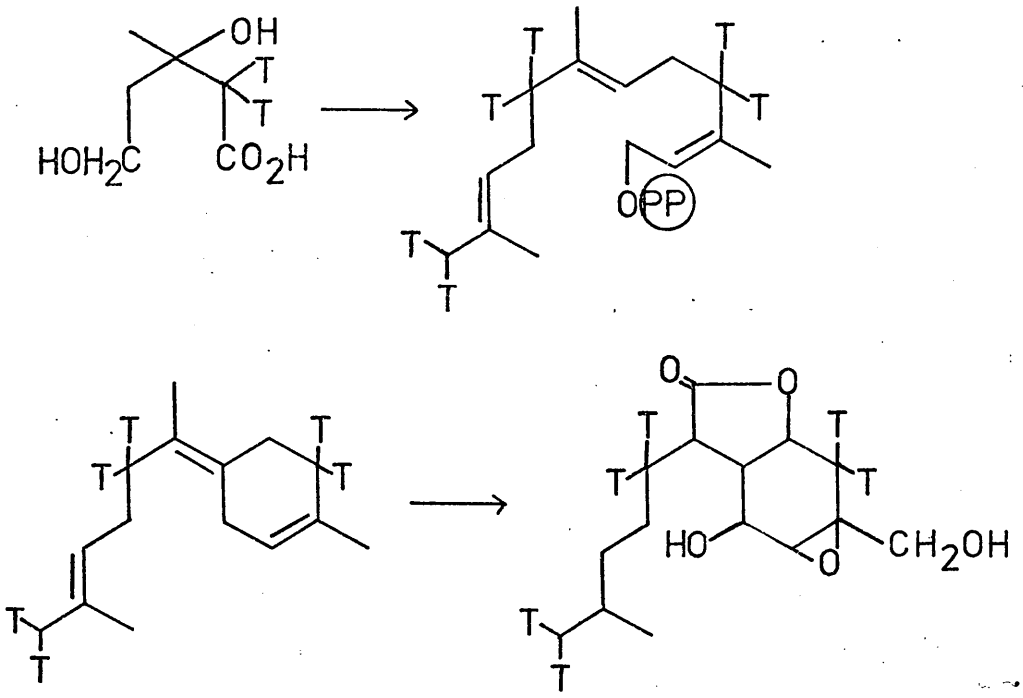


SCHEME 18

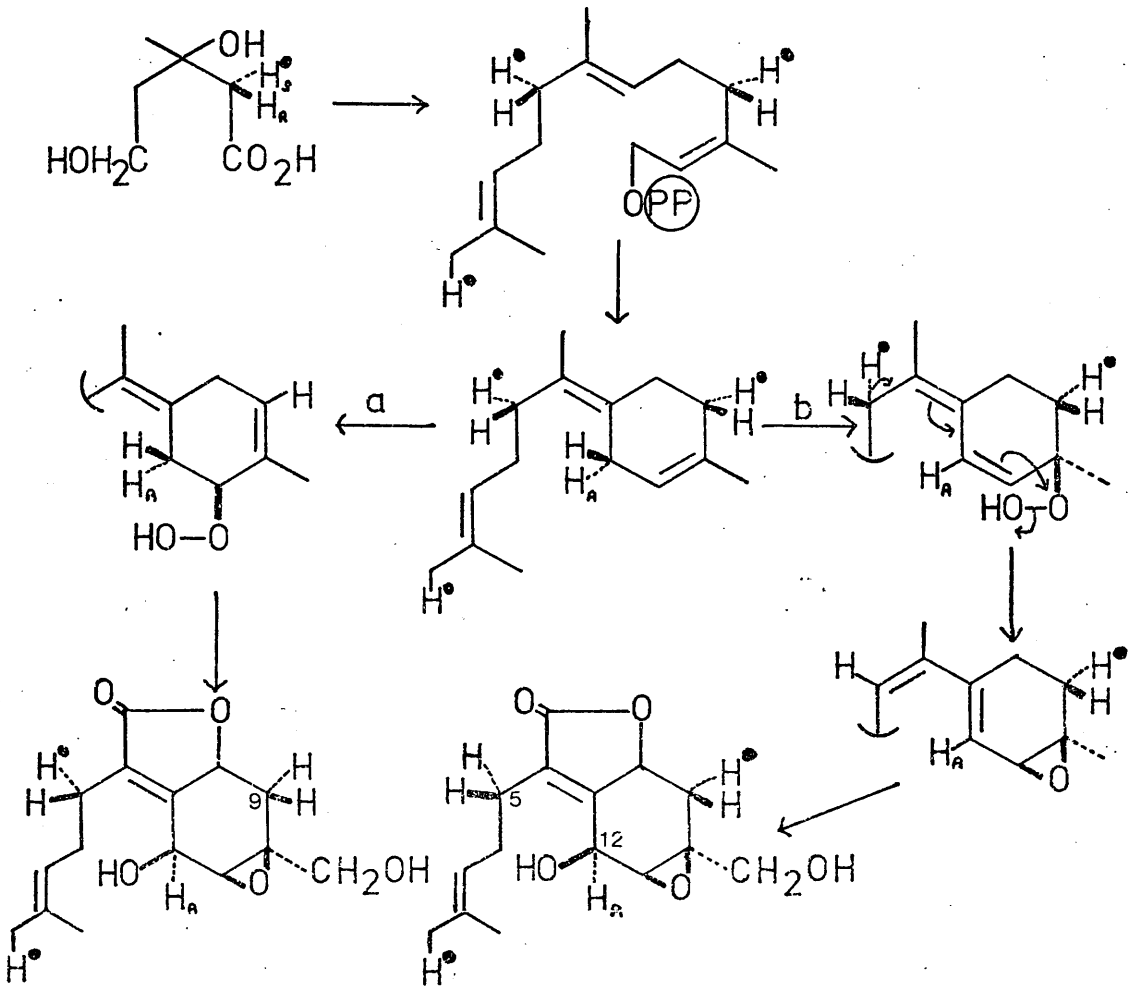
been studied (33) in these laboratories. When (5S)-[2-^{14}C, 5-^{3}H$_1$] mevalonate was converted into γ-bisabolene, there was no loss of tritium label. This implies that the hydrogen which is lost is derived from the pro-5R position of mevalonate. This is in accord with the mechanism proposed for isomerisation of farnesyl pyrophosphate, as previously discussed (p18).

Experiments to study the incorporation of the farnesol isomers, as the alcohols, and bisabolene into paniculide B were unsuccessful because of the insolubility of the substances in water. Farnesol was successfully incorporated with the cell-free system as a suspension using Tween 80 detergent. However, this method was not successful when applied to the intact cultures. An experiment in which [1-^{3}H$_2$]cis, trans-farnesol was incubated with dimethylsulphoxide "permeabilized" tissues (34) was also unsuccessful. A much more satisfactory approach was to use the natural biosynthetic intermediate, farnesyl pyrophosphate, which is water-soluble. A mixture of [4,8,12-^{14}C$_3$]trans, trans- and cis, trans-farnesyl pyrophosphate, which had been prepared biosynthetically by the cultures and isolated by adsorbtion on XAD2 resin, was fed to Andrographis cultures and was found to be incorporated into paniculide B to the extent of 1.6%. This approach has not been pursued further because of the difficulty of preparing pyrophosphate in high yield.

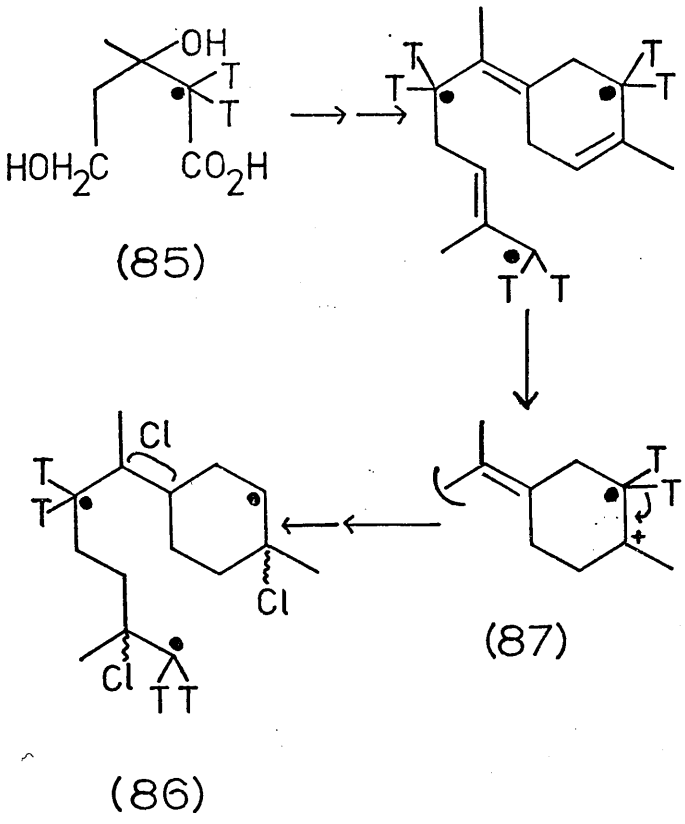
The overall picture of paniculide B biosynthesis as revealed by our experiments is shown in scheme 18. Cyclisation of cis, trans-farnesyl pyrophosphate produces Z-γ-bisabolene which is converted into paniculide B



SCHEME 19



SCHEME 20

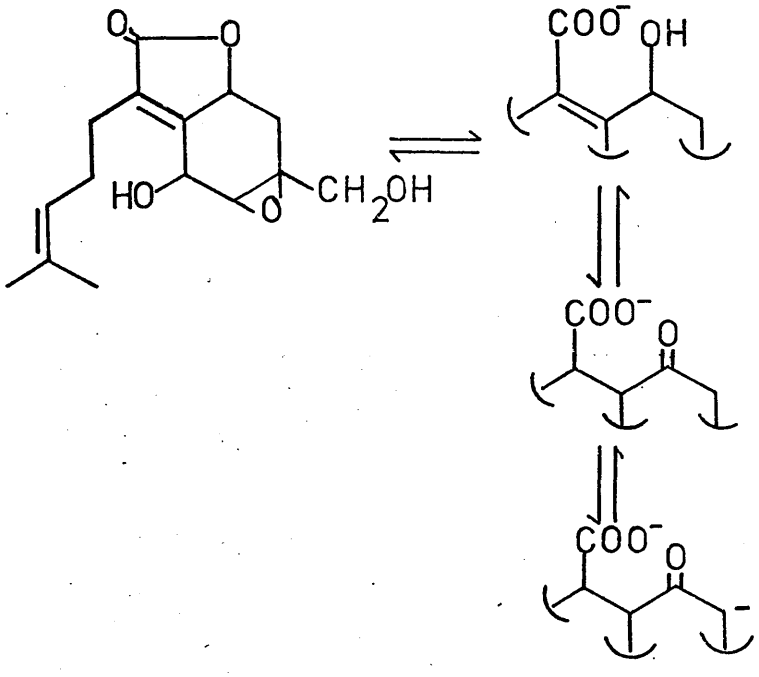


SCHEME 21

without double bond isomerisation. This satisfyingly establishes the most direct path that can be formulated.

The later stages in the biosynthetic pathway have also been studied by the incorporation of various samples of tritiated mevalonate. It has been shown (35) that when [2-$^{3}H_{2}$]mevalonate is used as precursor, one sixth of the label is lost in paniculide B. As implied in scheme 19, no loss of tritium from C-2 of mevalonate is expected in the biosynthesis of paniculide B. When (2R)-[2-^{14}C, 2-$^{3}H_{1}$] mevalonate was incorporated into paniculide B, there was 95% retention of label. Thus, at some stage, one of the hydrogen atoms derived from the pro-2S position of mevalonate is lost. Scheme 20 shows two mechanisms by which this loss could occur. The stereochemistry of hydrogen loss from C-12 of Z-γ-bisabolene, as shown in path (b), is consistent with the observed stereochemistry (36) of allylic hydroperoxide formation by soyabean lipoxygenase (E C 1.13.1.13). Experiments in these laboratories using samples of mevalonate tritiated at C-5 have shown (35) that hydroxylation at C-12 of paniculide B proceeds with retention of configuration consistent with either of the pathways proposed in scheme 20.

In an attempt to verify that the hydrogen loss occurred during oxygenation of Z-γ-bisabolene, [2-^{14}C, 2-$^{3}H_{2}$] mevalonate (85) was incorporated into γ-bisabolene using the cell-free system. However, when the trihydrochloride derivative (86) was isolated, it was found to retain only four of the tritium labels. This loss is explained by acid-catalysed double bond migration via the carbonium ion (87) during formation of the derivative, leading



SCHEME 22

to loss of labelled atoms which are allylic to the ring double bond (scheme 21). Thus at the stage of bisabolene, there has been no loss of hydrogen from the atoms which become C-1 and C-5 of paniculide B.

It was hoped that hydrogen at C-9 of paniculide B could be exchanged under basic conditions (scheme 22). However treatment of paniculide B with potassium carbonate in deuterated water resulted in no incorporation of deuterium. Thus it has not been possible to determine whether hydrogen is lost from C-5 or C-9 during oxygenation.

Part of this work has been published (37).

Experimental.

General procedure for isolation of paniculide B (62).

Suspension cultures of A. paniculata were grown as previously described (p26). The tissue was then removed by filtration and the medium was concentrated by evaporation under reduced pressure at less than 40°C to approximately 25% of its original volume. The resulting concentrate was extracted three times with an equal volume of ethyl acetate. After drying over anhydrous sodium sulphate, the solvent was evaporated and paniculide B (r_f 0.18) isolated by preparative thin layer chromatography over silica (chloroform-methanol, 94:6, v/v). Paniculide B was detected by ultraviolet absorbtion.

Biosynthesis of paniculide B from [1-^{13}C]acetate.

[1-^{13}C]acetate (149.7mg, 90.0 atom %) was administered as a small volume of filter-sterilized aqueous solution to cultures of A. paniculata (15 flasks, 2.55 dry weight callus tissue) which had been grown in suspension culture for twenty days following transfer from solid medium. After a further ten days' growth, paniculide B (118.4mg) was isolated as described above. This material was then recrystallised from chloroform to yield pure paniculide B (86.5mg) which was examined by ^{13}C NMR in d_6-dimethylsulphoxide solution. The spectrum and its assignment are discussed on p44.

Biosynthesis of paniculide B from [1,2-$^{13}C_2$]acetate.

In a parallel experiment under exactly the same conditions, a filter-sterilized solution of [1,2-$^{13}C_2$]acetate (150.8mg, 91.7 atom %) was administered to A. paniculata suspension cultures and paniculide B (117mg) was isolated

by preparative t.l.c. The ^{13}C NMR spectrum of this material is discussed on p45.

Incorporation of [2-^{14}C]mevalonate into γ-bisabolene.

(3RS)-[2-^{14}C]mevalonic acid lactone (5μCi, 0.28μ moles) was converted to its potassium salt by treatment with aqueous potassium hydroxide (1M, 100μl) at 37°C for one hour. The solution was then neutralized by addition of aqueous sodium dihydrogen orthophosphate solution (1M, 100μl) and incubated overnight with the cell-free system containing added co-factors, as previously described (p28).

The incubation was terminated by addition of ice-cold ethanol (8ml). After standing for thirty minutes at 0°C, the denatured protein was removed by centrifugation and the supernatant was extracted with hexane (3×10ml). The dried solution was then evaporated to small volume under reduced pressure and applied to a preparative t.l.c. plate (ethyl acetate-hexane, 1:3, v/v) after addition of carrier bisabolene (200μg, commercial mixture). Radioscanning showed that the bands corresponding to farnesol (r_f 0.23) and bisabolene (r_f 0.8) both contained radioactivity. The incorporations (estimated on non-derivatised material) were 1.2% and 0.4% respectively.

The bisabolene obtained in this experiment was examined by radio-g.l.c. on 1% SE 30 at 110°C, operating in the "^{14}C only" mode. When an aliquot of the biosynthetic material was co-injected with a synthetic mixture of Z- and E-γ-bisabolene, radioactivity was detected only in the peak corresponding to Z-γ-bisabolene (I=1500) and not in E-γ-bisabolene (I=1515). Samples of synthetic Z- and E-γ-bisabolene were kindly supplied by Dr. D.J. Faulkner,

University of California, San Diego.

Preparation of [2-^{14}C]cis, cis-farnesol.

Nerylacetone (Z-6,10-dimethyl-2-keto-5,9-diene) (77) was obtained from a mixture containing the E- and Z-isomers by preparative g.l.c. on 5% SE 30 at 135°C. The Z-isomer has a retention index of 1340, while the E-isomer has I=1380.

Triethylphosphite (15mg, 0.09m mole) was heated at 120°-130°C for five hours with [2-^{14}C]methyl bromoacetate (50μCi, 12mg, 0.08m mole) under an air condenser. Volatile material was then removed by very gentle heating of the resulting [^{14}C]diethylphosphonoacetate in vacuo. The residue (15mg, 0.076m mole) was dissolved in dry tetrahydrofuran (25μl) and added slowly to a stirred suspension of sodium hydride (7.3mg, 50% dispersion, 0.155m mole) in the same solvent (200μl) under nitrogen. Stirring was continued at room temperature for a further forty-five minutes before addition of a solution of nerylacetone (14.8mg, 0.076m mole) in tetrahydrofuran (25μl). After stirring for a further two days at room temperature, water (1ml) was added, and the mixture extracted with ether (3x5ml). The dried solution was then evaporated to ~5ml and added over a period of thirty minutes to a stirred suspension of lithium aluminium hydride (7.5mg, 0.156m mole) in dry ether (1ml) at 0°C. The reaction mixture was stirred for a further thirty minutes and then saturated aqueous ammonium chloride solution (5ml) was cautiously added, followed by water (10ml). The organic layer was separated, and the aqueous layer was extracted with ether (3x5ml). Farnesol was purified by preparative t.l.c. over silica (ethyl acetate-hexane, 1:3, v/v) and cis, cis-farnesol was separated from the trans,

cis-isomer by preparative t.l.c. over silver nitrate-silica (ethyl acetate-acetic acid, 499:1, v/v). Analytical g.l.c. on 1% SE 30 at 150°C showed that the material was >95% cis, cis-isomer. The radiochemical yield of [2-^{14}C]cis, cis-farnesol (76) was 9%.

Incorporation of [4,8,12-$^{14}C_3$]cis, trans-farnesol and [2-^{14}C]cis, cis-farnesol into bisabolene.

[4,8,12-$^{14}C_3$]cis, trans-farnesol (6.2×10^5dpm), which had been prepared biosynthetically from [2-^{14}C]mevalonate by the cell-free system, was ultrasonically dispersed in 0.2ml sodium phosphate buffer (0.1M, pH 7.6) with Tween 80 (1mg). The suspension was incubated anaerobically overnight with the cell-free system and then worked up as previously described. Carrier bisabolene (1mg, commercial material) was added before preparative t.l.c. The radioactive bisabolene which was isolated was dissolved in dry ether (2ml) and the crystalline trihydrochloride derivative was prepared by passing dry hydrogen chloride gas through the solution for fifteen minutes at 0°C. The solution was then allowed to stand at room temperature overnight before addition of carrier bisabolene trihydrochloride (6mg). The ether was then evaporated and the solid residue was recrystallised from a small quantity of methanol. After five recrystallisations, the specific activity was constant at 1062dpm/mg. This represents an incorporation of 1.2%. In a parallel experiment, [2-^{14}C]cis, cis-farnesol (7.7×10^5 dpm), which had been prepared as described above, was incubated with the cell-free system under identical conditions. After the second recrystallisation, the bisabolene trihydrochloride obtained in the experiment was found to have a very

low level of ^{14}C activity, representing an incorporation of only 0.02%.

Incorporation of [2-^{14}C, 5-$^{3}H_2$]mevalonate into bisabolene.

[2-^{14}C]Mevalonic acid lactone (0.5μCi, 0.028μ moles) was converted to the potassium salt as previously described. The dibenzyl ethylenediamine salt of [5-$^{3}H_2$]mevalonate (10μCi, 0.0015μ moles) was converted to the potassium salt with saturated aqueous potassium hydrogen carbonate solution (50μl). The solution was then extracted with ether (2x50μl) to remove the free base. Excess ether was then removed in a stream of nitrogen. The aqueous solutions were mixed and neutralized with 0.2M hydrochloric acid. The final volume of the substrate solution was ca. 500μl. This substrate was incubated with the cell-free system on three times the usual scale; the final volume of the incubation was 12ml. After workup in the usual manner, bisabolene trihydrochloride was prepared. Carrier material (6mg) was added and the trihydrochloride was crystallised to constant ratio. The ^{3}H:^{14}C ratio of [2-^{14}C, 5-$^{3}H_2$]mevalonate fell from 20.06 to 17.1 in bisabolene trihydrochloride. This is a retention of tritium of 85.4%. In a duplicate experiment, the ^{3}H:^{14}C ratio of the substrate was 4.5, while the derived bisabolene trihydrochloride had a ratio of 3.7, representing 82.2% tritium retention. Loss of one sixth of the tritium requires 83.3%.

Incubation of [1-$^{3}H_2$]cis, trans-farnesol with "permeabilized" tissues (34).

Callus tissue (5g fresh weight, grown in suspension culture for twenty-one days) was added to sodium phosphate buffer (0.1M, pH 7.6) containing 2-mercaptoethanol (30mM),

dimethylsulphoxide (10% v/v) and [1-$^{3}H_{2}$]cis, trans-farnesol (6×10^{6}dpm) as substrate. The tissue was incubated aerobically with shaking for twenty hours at 37°C. The incubations were terminated by placing in a boiling water bath for two minutes. Water (5ml) was added and the buffer was extracted with ethyl acetate (5×5ml). No radioactivity could be recovered.

Incorporation of [4,8,12-$^{14}C_{3}$]farnesyl pyrophosphate into paniculide B.

(3RS)-[2-^{14}C]Mevalonate (20μCi, 1.12μ moles) was incubated overnight with the cell-free system under the conditions previously described. The incubation was worked up in the usual manner. The aqueous layer, after extraction with hexane, was partially evaporated under reduced pressure to remove ethanol. Dilute ammonium hydroxide (0.01M) was added, and the solution was evaporated once more. This was repeated several times to ensure that most of the ethanol had been removed. The aqueous solution was then stirred for thirty minutes with XAD-2 resin (5g, previously washed with 0.01M ammonium hydroxide). After transfer of the resin to a chromatography column, and washing with 0.01M ammonium hydroxide, farnesyl pyrophosphate was eluted (0.01M $NH_{4}OH$-methanol, 1:9, v/v). The incorporation of radioactivity from [2-^{14}C]mevalonate into the pyrophosphate fraction was 5.4%. An aliquot was hydrolysed with alkaline phosphatase (E C 3.1.3.1, 100μl) in tris buffer (0.1M, pH 8.6) containing magnesium chloride (0.3mM) overnight at 37°C. Radio-gas liquid chromatography showed that the hydrolysis product contained only trans, trans- and cis, trans-farnesol with slight traces of nerolidol.

This mixture of [4,8,12-$^{14}C_3$] trans, trans- and cis, trans-farnesyl pyrophosphate (2.36×10^6 dpm) was administered to cultures of A. paniculata (4 flasks, 0.68g dry weight callus tissue) which had been grown in suspension culture for sixteen days following transfer from solid medium. Paniculide B was isolated in the usual manner after a further seven days and crystallised to constant activity. Incorporation of [4,8,12-$^{14}C_3$]farnesyl pyrophosphate into paniculide B was 1.6%.

Incorporation of (2R,3R)-[2-^{14}C, 2-$^{3}H_1$]mevalonate into paniculide B.

The substrate was prepared by mixing [2-^{14}C]mevalonic acid lactone (5μ Ci) with (2R,3R)-[2-$^{3}H_1$]+(2S,3S)-[2-$^{3}H_1$] mevalonic acid lactone and conversion to the potassium salt as previously described. The neutralized solution of the potassium salt was diluted with phosphate buffer (0.1M, pH 7.6) to 1ml, sterilized by filtration and administered to twenty-one-day-old suspension cultures (5 flasks, 0.85g dry weight callus tissue) which were isolated in the normal manner and crystallised to constant activity (2.3% incorporation of ^{14}C). The ^{3}H:^{14}C ratio of the substrate was 3.14, while in paniculide B the ratio was 3.00. Thus, there has been 95% retention of tritium.

Incorporation of [2-^{14}C, 2-$^{3}H_2$]mevalonate into bisabolene.

[2-^{14}C, 2-$^{3}H_2$]Mevalonate (5 μCi ^{14}C) was incubated with the enzyme system under the normal conditions, and the hydrocarbon fraction isolated from preparative t.l.c. after addition of carrier bisabolene (5mg, commercial mixture). The trihydrochloride derivative was prepared and crystallised to constant activity. Incorporation (based on ^{14}C) was

0.23%. The $^{3}H:^{14}C$ ratio of the substrate was 5.81, while the bisabolene trihydrochloride had a ratio of 3.87. This indicates that there is 66.1% tritium retention in bisabolene trihydrochloride.

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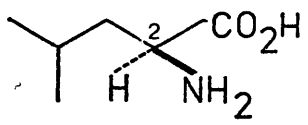
SECTION 3

THE ROLE OF LEUCINE IN TERPENOID BIOSYNTHESIS



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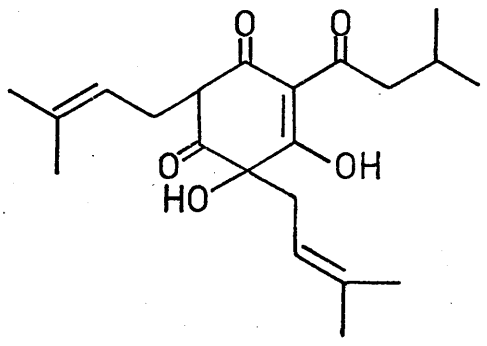

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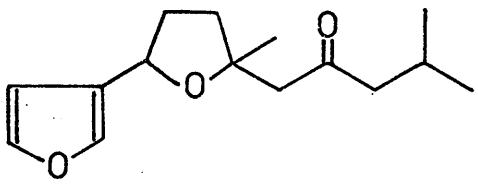

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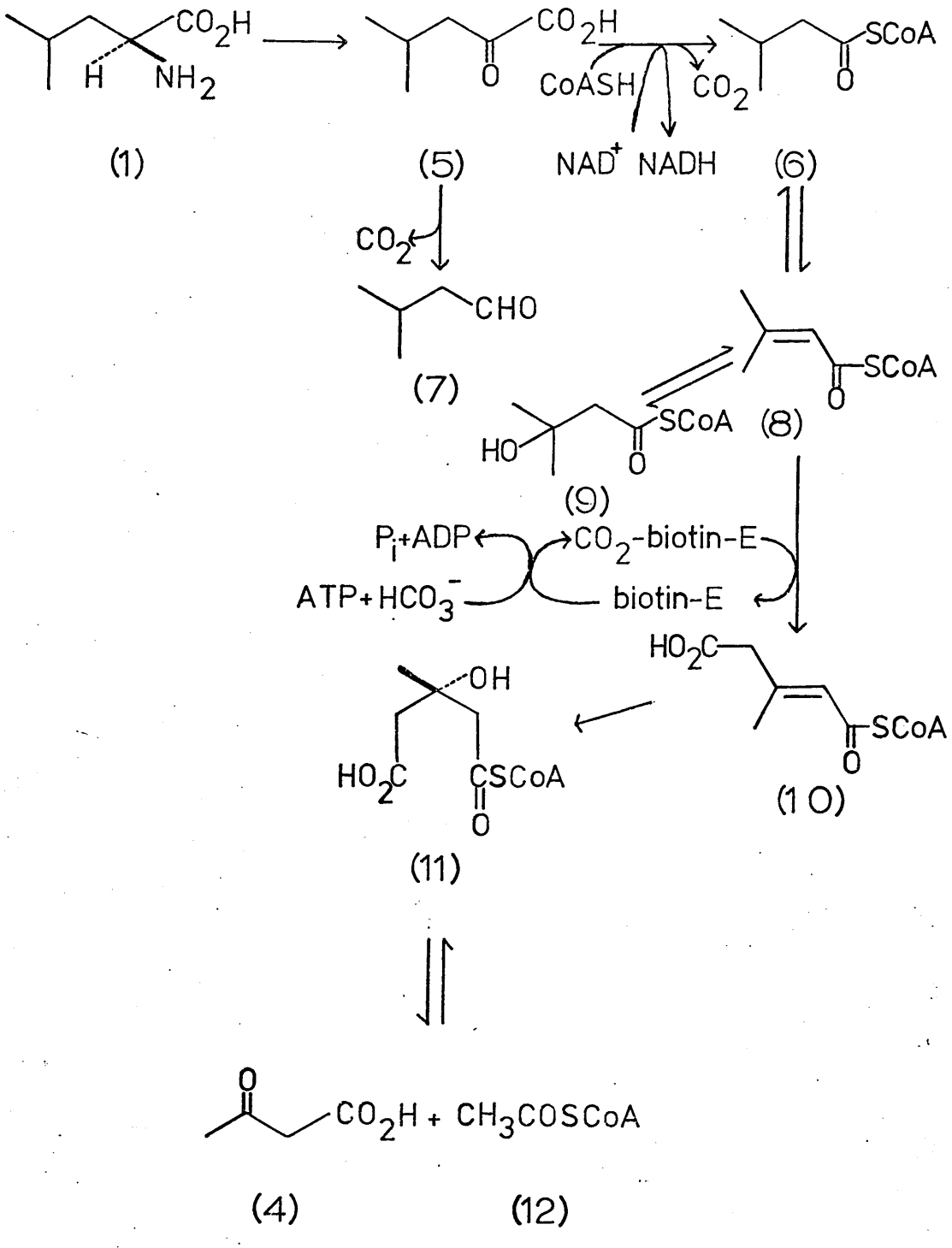
Section 3.

The Role of Leucine in Terpenoid Biosynthesis.

Introduction.

The role which amino-acids play in terpenoid biosynthesis has received intermittent attention in recent years. As long ago as 1950, Bloch showed (1) that (2S)-leucine (1) is incorporated into cholesterol, although at that time the mode of biosynthesis of terpenoids and steroids was largely unknown. Leucine was later shown (2) to act as precursor of β-carotene in the fungus Phycomyces blakesleeanus. The precise route by which incorporation occurred was unclear from these early experiments, but it was shown that C-1 and C-2 of leucine were poorly incorporated compared with the other carbon atoms. Mevalonic acid was first isolated by Folkers and co-workers from distillers' solubles in 1957 and it was shown to be the key intermediate in terpenoid biosynthesis (3). From that time onwards, all major biosynthetic investigations concentrated on the mechanism by which mevalonate acts as a precursor of terpenoids, and leucine was largely forgotten. However, incorporation of leucine into several terpenoids has been reported more recently, among them gibberellins (4) and hop resins (5), which include the meroterpenoid humulone (2). Leucine has also been shown (6) to be a precursor in the biosynthesis of the sesquiterpenoid ipomeamarone (3). This report, which does not include degradative results, is the only previous report of the incorporation of leucine into a sesquiterpenoid.

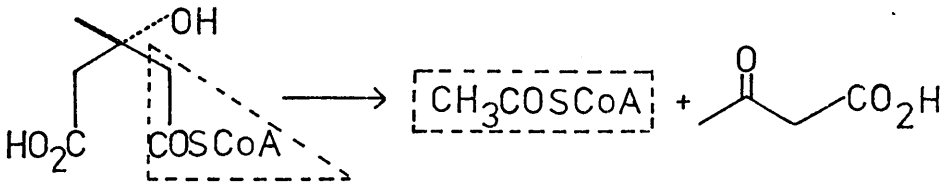
The degradative pathway for leucine in mammals has been established in detail. In 1935, Edson demonstrated (7) the


CO2H
H
NH2
(1)
CO2H
O
CoASH
CO2
SCoA
O
NAD+ NADH
(5)
(6)
CO2
CHO
(7)
SCoA
O
HO
SCoA
O
(9)
(8)
Pi+ADP
CO2-biotin-E
ATP+HCO3-
biotin-E
HO2C
SCoA
O
(10)
OH
HO2C
CSCoA
O
(11)
O
CO2H + CH3COSCoA
(4)
(12)

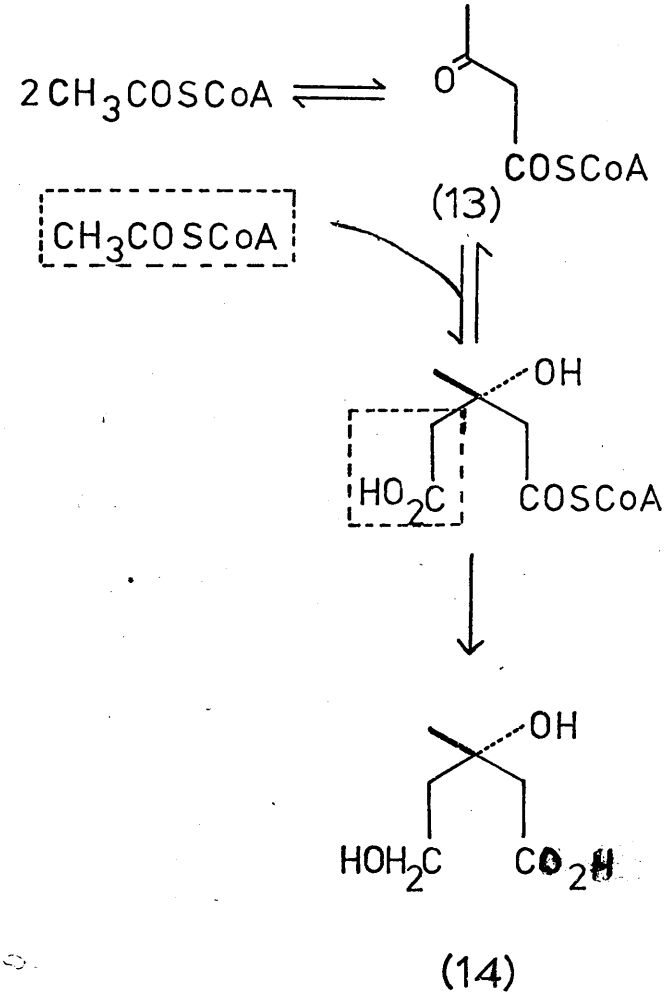
SCHEME 1

conversion of leucine (1) into acetoacetate (4) in liver slices. The steps involved in the degradation of leucine were elucidated by isotope incorporation experiment and later by the enzymatic studies of Coon and his associates (8).

The first step in the pathway (scheme 1) is transamination to the corresponding α-keto acid (5) which is subsequently decarboxylated and converted into 3-methylbutanoyl-CoA (6). It has been postulated that 3-methylbutanal (7) is an intermediate in this process but although enzymatic decarboxylation of the keto acid (5) to the aldehyde has been demonstrated (9) there is no evidence that 3-methylbutanal can then be converted into the CoA ester (6). The next step is desaturation of CoA ester (6) to 3-methylbut-2-enoyl-CoA (8), a process familiar in the metabolism of straight chain fatty acids (10). When the CoA ester (6) was incubated anaerobically in the presence of organic oxidising agents, 3-hydroxy-3-methylbutanoyl-CoA (9) was obtained. Extracts of heart and liver as well as crystalline crotonase (E C 4.2.1.17) catalyse (11) the reversible hydration of the unsaturated ester (8). Although it was originally thought that the CoA ester (9) was the substrate for the next reaction, it was later shown (12) that hydration at this stage is a redundant side reaction. In the major pathway, unsaturated ester (8) is next carboxylated (13) to E-β-methylglutaconyl-CoA (10) in a two-step reaction involving biotin, adenosine triphosphate and magnesium ion (14). The enzymology of this step has been throughly studied and the enzyme has been purified to homogeneity and crystallised from extracts of a species of



(11) (12) (4)



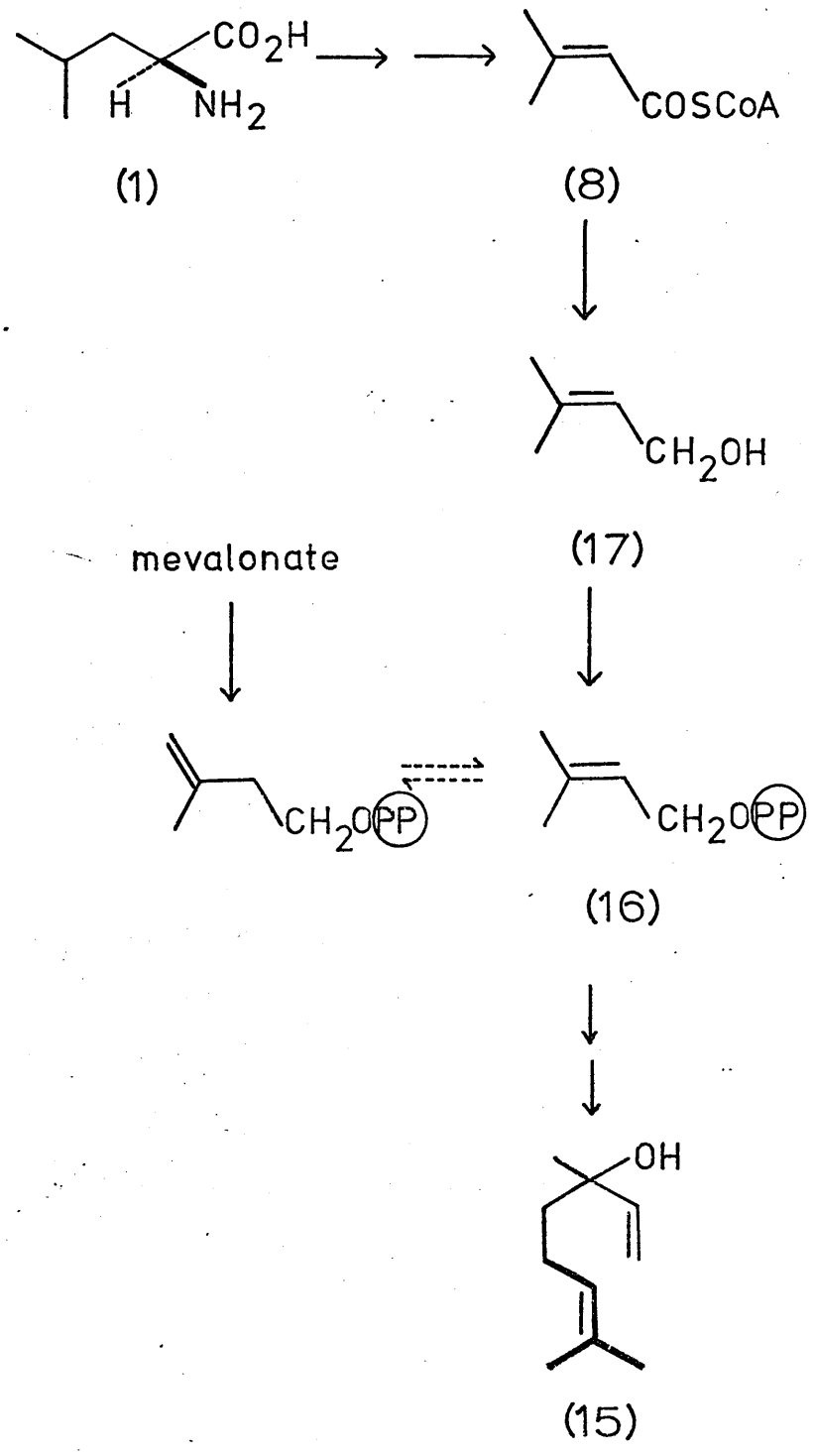
(14)

SCHEME 2

Achromobacter (15). The enzyme methylglutaconyl-CoA hydratase (E C 4.2.1.18) then catalyses stereospecific syn addition of water (16), yielding (3S)-3-hydroxymethylglutaryl-CoA (11). At this point, the leucine degradative pathway and the pathway to mevalonate meet. Under conditions of ketone body formation in animal systems, the CoA ester (11) is cleaved by hydroxymethylglutaryl-CoA lyase (E C 4.1.3.4) to acetyl-CoA (12) and acetoacetate (4). The acetyl-CoA, which is derived from the pro-S acetic acid group of 3-hydroxymethylglutaryl-CoA, is produced with inversion (17).

The biosynthesis of mevalonate from acetate involves, in the initial step, condensation between two molecules of acetyl-CoA, which yields acetoacetyl-CoA (13) (scheme 2). Upon condensation with a further molecule of acetyl-CoA, under the influence of the enzyme hydroxymethylglutaryl-CoA synthase (E C 4.1.3.5), acetoacetyl-CoA (13) produces (3S)-3-hydroxymethylglutaryl-CoA (11). In the synthase reaction, acetyl-CoA forms the pro-R acetic acid group of the product; coenzyme A is liberated from acetyl-CoA during the reaction and acetoacetyl-CoA (13) is attacked at the si-face (18). It has been observed that inversion at the methyl group of acetyl-CoA occurs in this reaction (19). Reduction of the CoA ester by hydroxymethylglutaryl-CoA reductase (E C 1.1.1.34) then leads, essentially irreversibly, to mevalonate (14), the key intermediate in terpenoid biosynthesis.

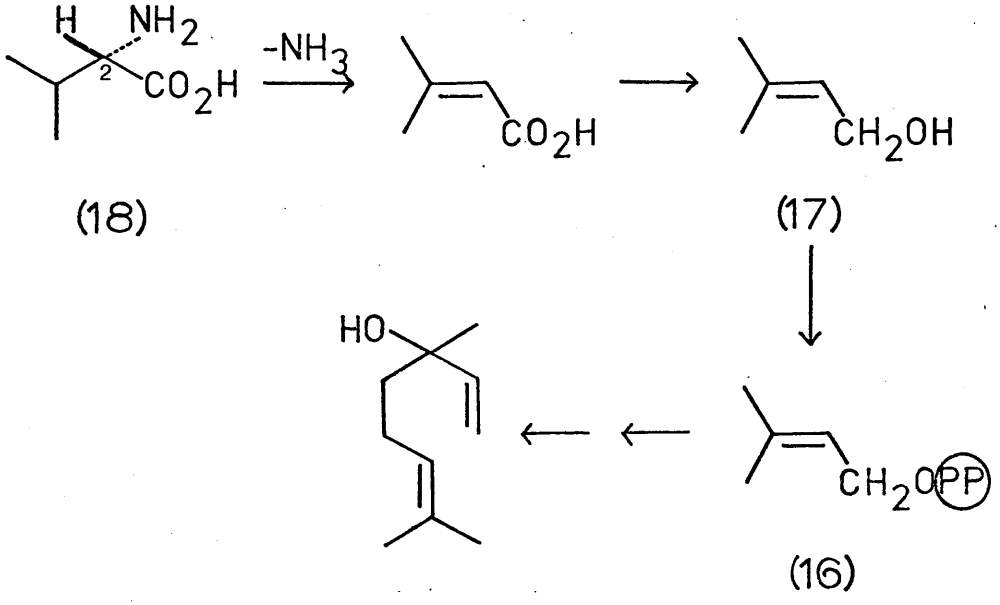
There are several reports that (2S)-leucine (1) is not incorporated into monoterpenoids in higher plants (20), but Suga and his colleagues have recently observed (21)



(1) (8) (17) (16) (15)

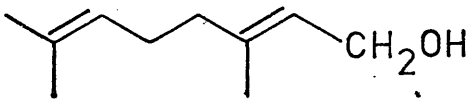
SCHEME 3

its incorporation into linalool (15) in Cinnamomum camphora. The maximum incorporation observed from (2S)-[U-^{14}C]leucine into linalool was only 0.004%, while the maximum incorporation from [2-^{14}C]mevalonate was about ten times greater. The distribution of radioactivity in linalool was examined by degradation and it was found that while leucine labelled predominantly the dimethylallyl-derived moiety, mevalonate labelled predominantly the isopentenyl-derived portion of the molecule. In the biosynthesis of isoprenoids from [2-^{14}C]mevalonate by higher plants, the predominance of tracer in the moiety derived from isopentenyl pyrophosphate has been observed for some monoterpenoids (22) and sesquiterpenoids (23) in contrast to the uniform distribution which is found in di- and triterpenoids. If the pathway by which leucine is incorporated into linalool proceeds *via* mevalonate, the distribution of label should be the same whether leucine or mevalonate is used as the precursor. The complementary labelling patterns from leucine and mevalonate led Suga to propose that leucine is degraded directly to dimethylallyl pyrophosphate (16). It is supposed that 3-methylbut-2-enoyl-CoA (8) is produced by the degradation of leucine, as previously demonstrated in mammalian liver. Reduction of this CoA ester, presumably by reduced pyridine nucleotide, affords 3-methylbut-2-en-1-ol (17) which could be phosphorylated to give dimethylallyl pyrophosphate (16) as shown in scheme 3. Thus the unequal labelling pattern observed when [U-^{14}C]leucine is used as a precursor of monoterpenoids may be explained by direct production of dimethylallyl units of non-mevalonoid origin, provided that the rate of isopentenyl pyrophosphate:



(18) (17) (16)

SCHEME 4



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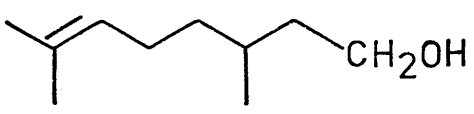
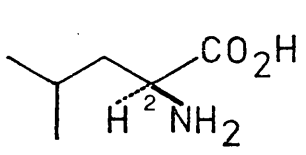


(20)

dimethylallyl pyrophosphate isomerisation is slow.

It has been reported (24) that (2S)-[U-^{14}C]valine (18) is incorporated into linalool in the same plant, and the distribution of label was shown to be similar to that which is found when leucine is used as precursor. The proposed degradative pathway for valine is shown in scheme 4. Similar results have been found (25) for the incorporation of (2S)-[U-^{14}C]leucine and (2S)-[U-^{14}C]valine into geraniol (19) and citronellol (20) in Pelargonium roseum.

Details of recent work (26) on the incorporation of leucine into meroterpenoids will be discussed at a later point (p73).



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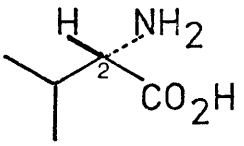
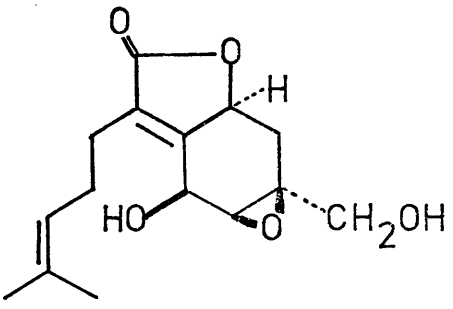


(18)



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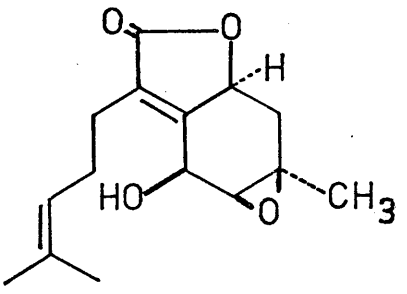

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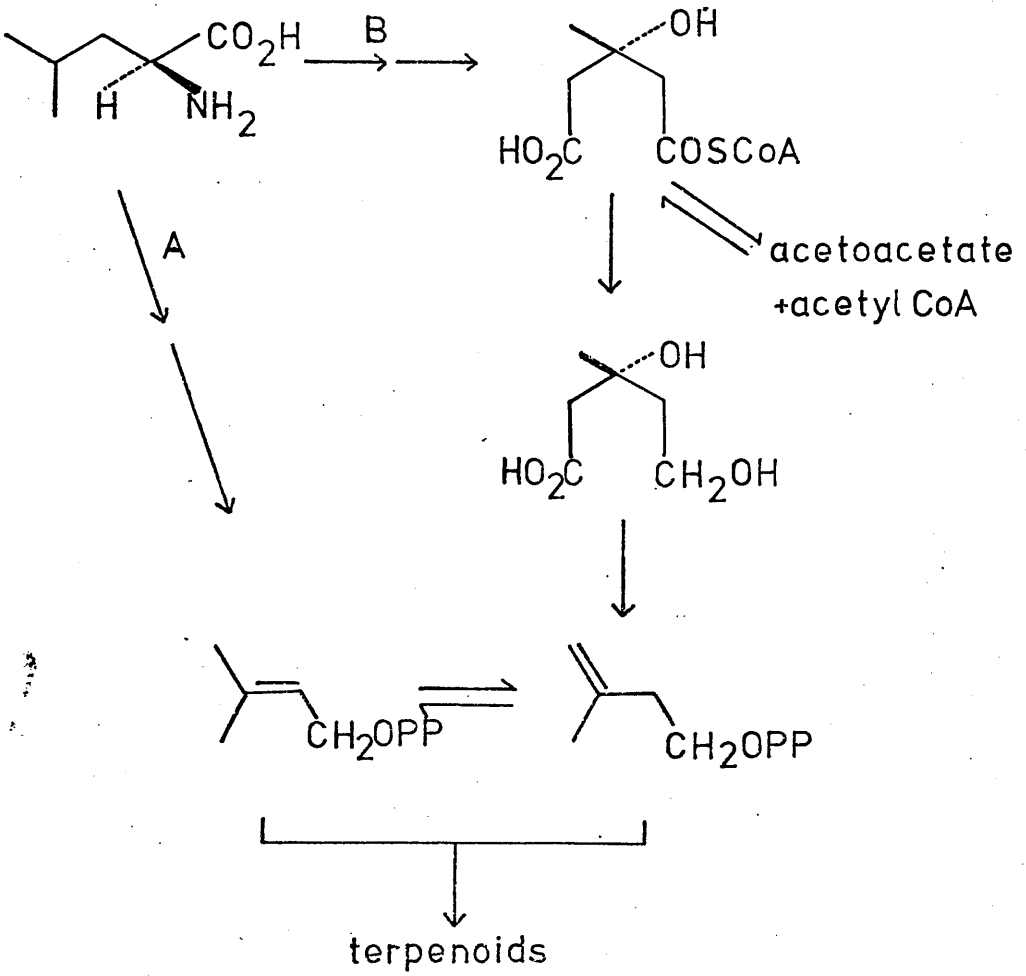
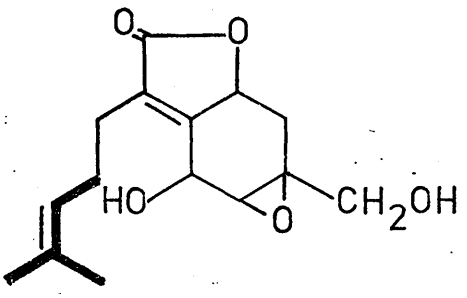


SCHEME 5



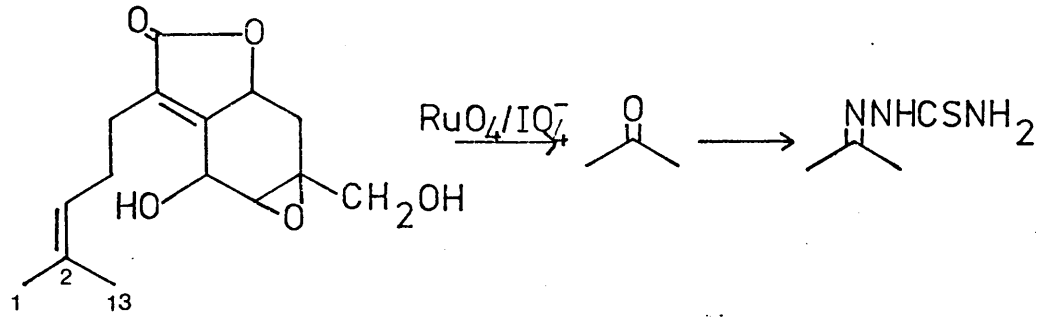
(23)

Discussion.

In view of Suga's recent work (21,24,25) on the incorporation of (2S)-leucine (1) and (2S)-valine (18) into monoterpenoids, it was of interest to test these amino acids as possible precursors of paniculide B (21) in Andrographis paniculata tissue cultures. It was found that while (2S)-[U-^{14}C]leucine was an efficient precursor of paniculide B (0.6% incorporation), (2S)-[U-^{14}C]valine was not incorporated. When the concentration of leucine in the culture medium was greater than ~1mM, paniculide A (22) was the labelled metabolite rather than paniculide B (21). Evidently, either leucine or one of its catabolic products inhibits hydroxylation at C-15. It is of interest to note that the incorporations reported here are much superior to those reported (0.004%) by Suga using cut plant stems for incorporation into linalool.

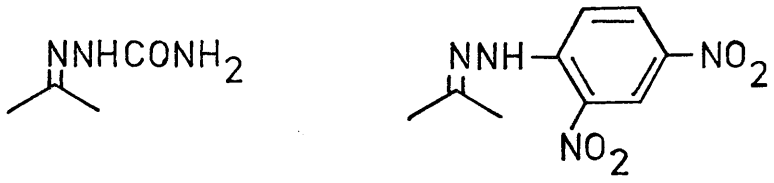
The possible routes for leucine degradation are outlined in scheme 5. If pathway (a) is followed, as has been suggested in monoterpenoid biosynthesis, one would expect that leucine should preferentially label the dimethylallyl-derived portion of the molecule. Thus, (2S)-[U-^{14}C]leucine should label the portion of paniculide B indicated in (23) more heavily than the rest of the carbon skeleton, while the reverse should be true for incorporation of [2-^{14}C]mevalonate.

A procedure was developed to test this hypothesis in which paniculide B, biosynthesised from either leucine or mevalonate, was degraded by ruthenium tetroxide/periodate oxidation. The fragment corresponding to C-1, C-2 and C-13 produces acetone (24) which was isolated as its crystalline

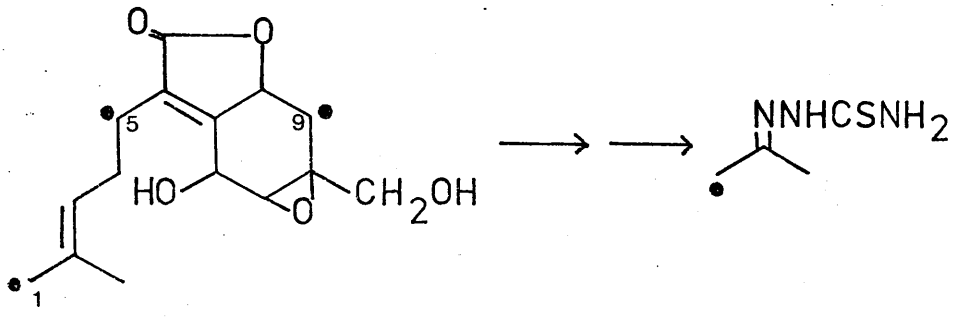


(21) (24) (25)

SCHEME 6



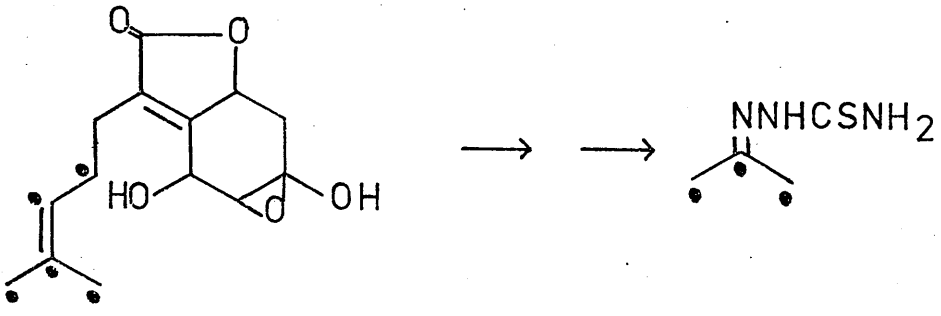
(26) (27)



(28) (29)

(•) represents ^{14}C

SCHEME 7



(30) (31)

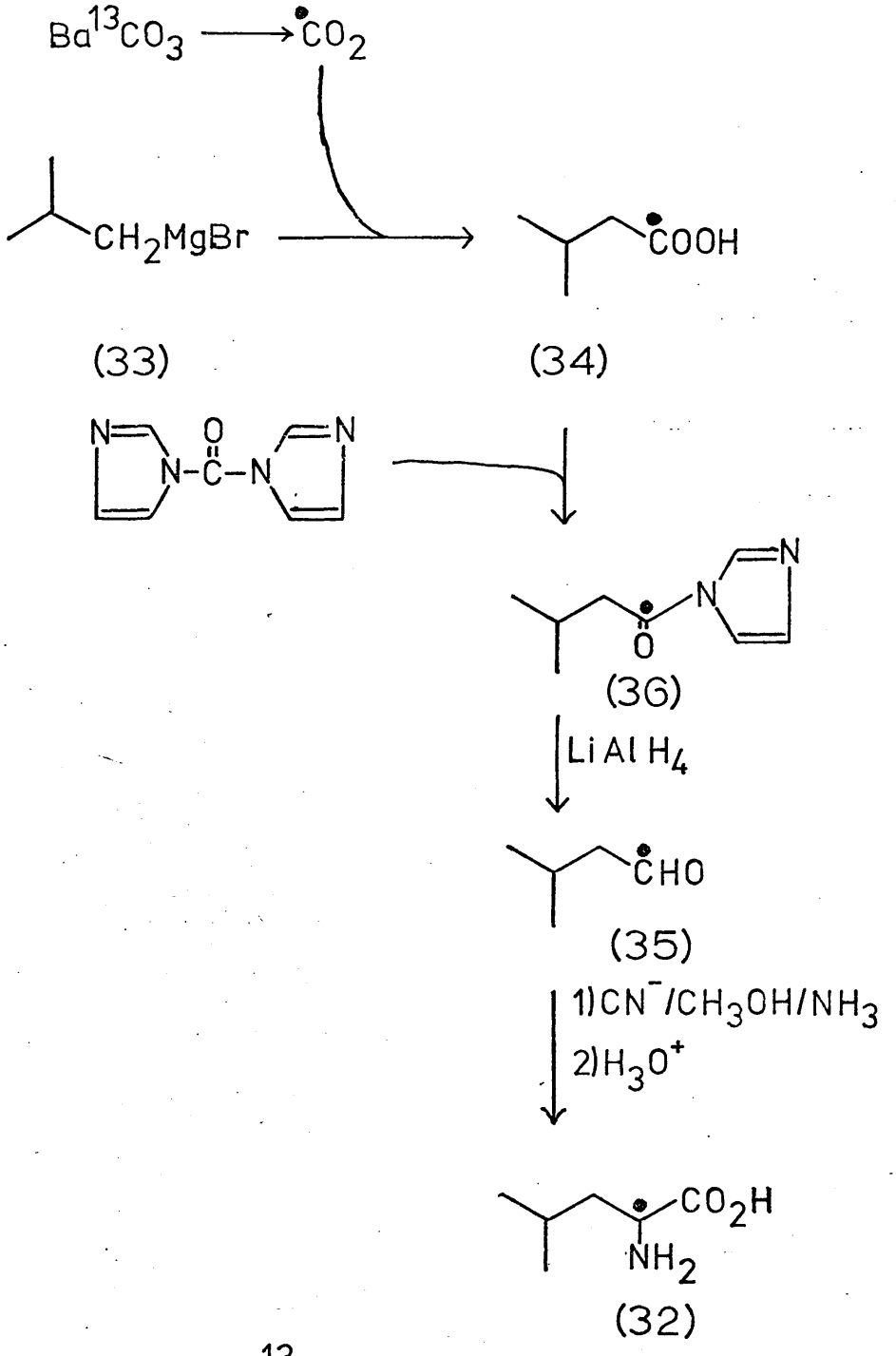
SCHEME 8

thiosemicarbazone (25) as shown in scheme 6. The thiosemicarbazone was chosen rather than the semicarbazone (26) because the sulphur-containing derivative is formed much faster (27), thus ensuring less loss of the volatile acetone. The 2,4-dinitrophenylhydrazone (27) was not used because of the severe colour-quenching which it causes in liquid scintillation counting.

When [2-^{14}C]mevalonate is used as precursor, C-1, C-5 and C-9 of paniculide B (28) should be labelled. Thus if paniculide B, which has been biosynthesised from [2-^{14}C] mevalonate, is degraded to acetone, the specific activity of the derivative (29) should be 33% of the specific activity of the paniculide B from which it was obtained (scheme 7). The experimental value was found to be 28%, indicating that there is little preferential labelling of the isopentenyl-derived portion of paniculide B from [2-^{14}C] mevalonate.

If leucine labelled only the dimethylallyl-derived portion of paniculide B, then when paniculide B (30) is biosynthesised from (2S)-[U-^{14}C]leucine, 60% of the label should be in the acetone derivative (31) as shown in scheme 8. The experimental value is 18%. Clearly, leucine does not label only the dimethylallyl-derived part of the molecule. If (2S)-[U-^{14}C]leucine is incorporated uniformly into paniculide B, then the acetone should contain 20% of the activity.

In an attempt to discover whether (2S)-leucine is the enantiomer which is incorporated into paniculide B, (2S)-[U-^{14}C]leucine was mixed with either (2S)-[4,5-$^{3}H_2$]leucine or (2RS)-[4,5-$^{3}H_2$]leucine and fed to the cultures. The



(•) represents ^{13}C

SCHEME 9

Table 1.

Incorporation of (2S)-[U-^{14}C]leucine into the paniculides.

100mg/l (2S)-leucine.

age	1	2	3	weeks
wt pan A (mg/10 flasks)	8.0	8.2	9.0	
wt pan B (mg/10 flasks)	-	14.0	20.5	
% incorporation into pan A	7.8	7.3	8.4	
% incorporation into pan B	0.3	0.3	0.4	

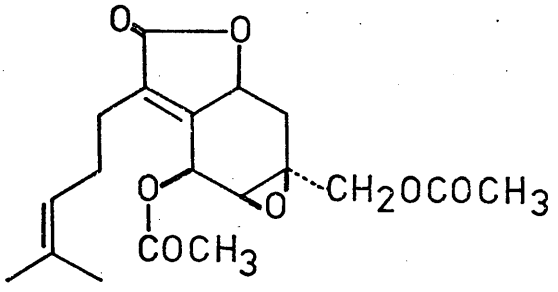
50mg/l (2S)-leucine.

age	1	2	3	weeks
wt pan B (mg/10 flasks)	-	15.0	21.0	
% incorporation into pan B	1.7	1.8	3.1	

results indicate that both enantiomers are used with equal facility, since in both cases there is ~24% retention of tritium in paniculide B. This implies that the cultures contain an amino acid racemase (E C 5.1.1.-). Such enzymic activity has been previously been reported (28) in Nicotiana tabacum tissue cultures, but the enzyme has been well-characterised only from microbial sources (29).

In order to obtain more definitive evidence for the incorporation pattern of leucine into paniculide B, it was decided to resort to ^{13}C NMR spectroscopy. Synthesis of (2RS)-[2-^{13}C]leucine (32) was undertaken, following a route previously described (29) for the synthesis of [2-^{14}C]leucine (scheme 9). Label was introduced by carbonation of the Grignard reagent (33) with $^{13}CO_2$ generated from labelled barium carbonate. The [1-^{13}C]3-methylbutanoic acid (34) was then converted to the corresponding aldehyde (35) by reduction of the imidazolyl derivative (36) with lithium aluminium hybride. Strecker reaction of the aldehyde (35) followed by acid hydrolysis then yielded the desired product, (2RS)-[2-^{13}C]leucine (32).

Feeding conditions were optimised for incorporation of (2S)-[U-^{14}C]leucine into paniculide B using a medium replacement technique to obtain minimum dilution of paniculide B with unlabelled metabolite. The Andrographis cultures were grown for one, two or three weeks under normal conditions. The medium was then removed and replaced with fresh medium containing (2S)-[U-^{14}C]leucine with the addition of various levels of carrier (2S)-leucine. The results are shown in table 1. When the concentration of leucine in the medium was 100mg/l or greater, only

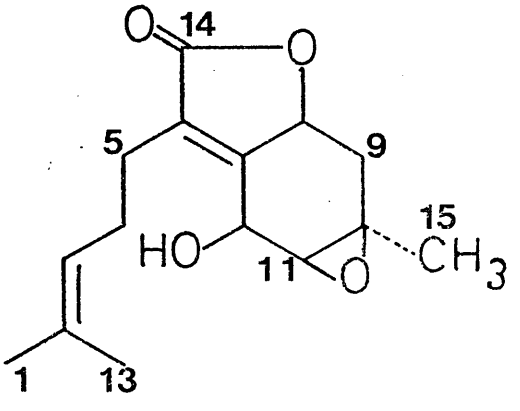


(37)

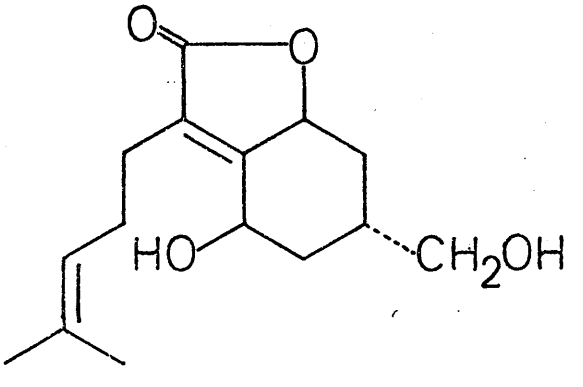
Table 2.

Assignment of ^{13}C NMR spectrum of paniculides A and B.

	A	B
Carbon atom	δ(ppm)	δ(ppm)
1	25.3	25.3
2	131.3	131.2
3	123.5	123.5
4	27.1	27.1
5	22.9	22.8
6	126.4	126.4
7	160.7	161.0
8	75.0	75.2
9	36.6	32.7
10	56.8	60.1
11	64.9	61.8
12	67.3	67.2
13	17.4	17.4
14	173.3	173.3
15	22.9	63.6



PANICULIDE A



PANICULIDE B

paniculide A was labelled. Incubation of (2RS)-[2-^{13}C] leucine (50mg/l) with three week-old A. paniculata cultures failed in two separate experiments to produce incorporation of ^{13}C into paniculide B, even though (2S)-[U-^{14}C]leucine is efficiently incorporated under these conditions. The specific incorporation of (2S)-[U-^{14}C]leucine in these experiments was 15.5%, and the (2RS)-[2-^{13}C]leucine used contained 70 atom % ^{13}C. This allows one to calculate (30) the expected peak enhancements in the metabolites. Even if six carbon atoms of paniculide B were labelled, a peak enhancement of ~50% should be observed for these carbon atoms in the ^{13}C NMR spectrum. However, no deviation from natural abundance intensities could be discerned even after preparation of the diacetate derivative of paniculide B (37) and peak height normalisation on the unlabelled acetate peaks.

In a further experiment, (2RS)-[2-^{13}C]leucine was fed to the cultures under conditions where (2S)-[U-^{14}C]leucine was incorporated into paniculide A. In this case, the specific incorporation of the radioactive tracer was 28.3%, which should lead to a 184% peak enhancement if six carbon atoms were labelled from (2RS)-[2-^{13}C]leucine. Again no significant incorporation of ^{13}C was observed. The chemical shifts and peak assignments of the ^{13}C NMR spectrum of paniculide A are listed in table 2. The spectrum is easily assigned in comparison with that of paniculide B, which has previously been discussed (p43). It should be noted that removal of the hydroxyl group has resulted in an upfield shift of the α carbon atom (C-15) of 40.6ppm, so that this signal now coincides with that of C-5 which

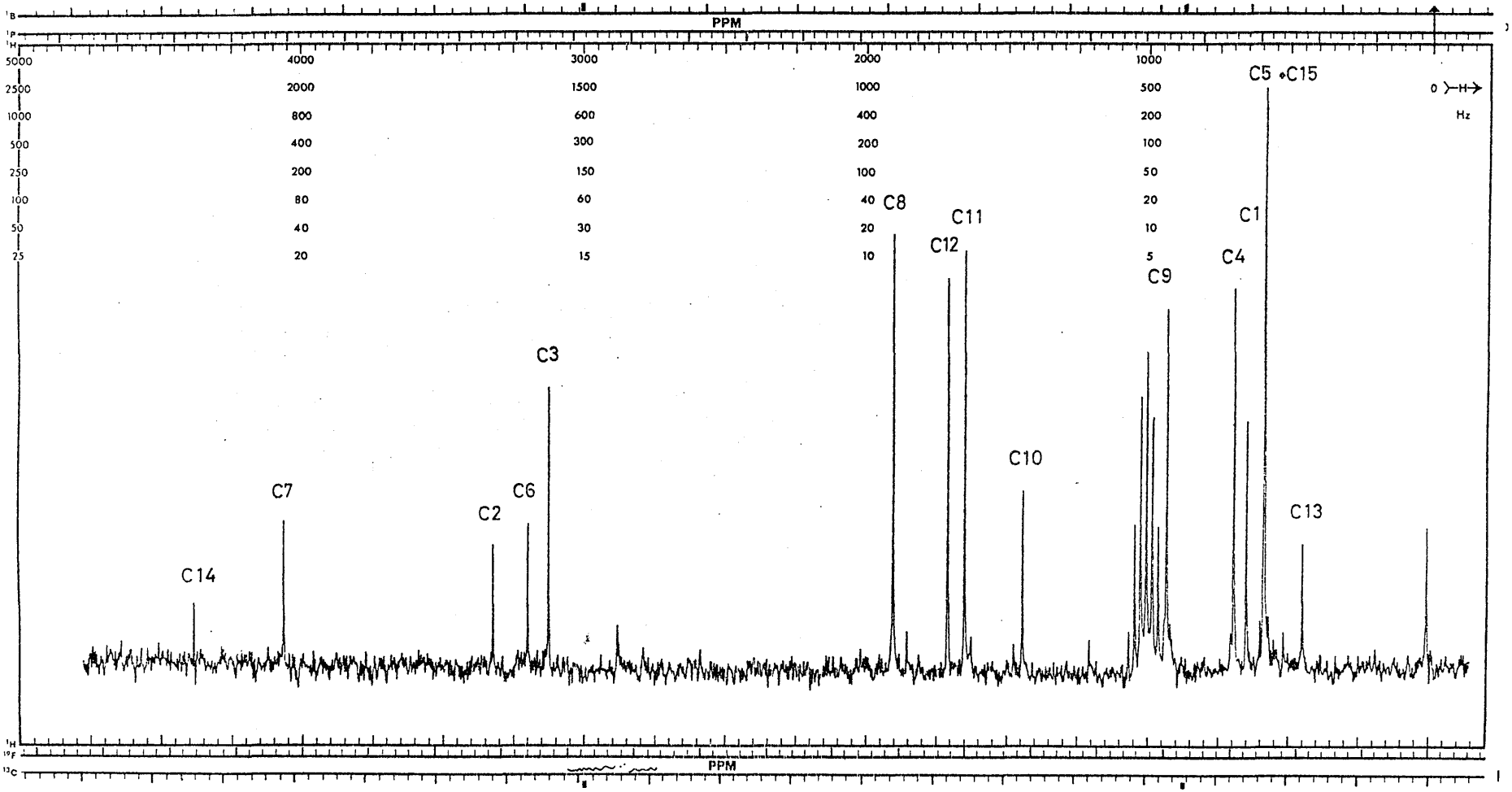
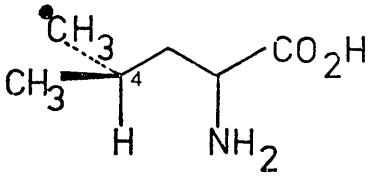


Figure 1 ^{13}C NMR Spectrum of Paniculide A Biosynthesised from (2RS)-[2-^{13}C]Leucine (proton noise-decoupled)



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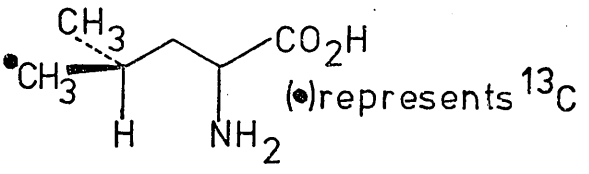

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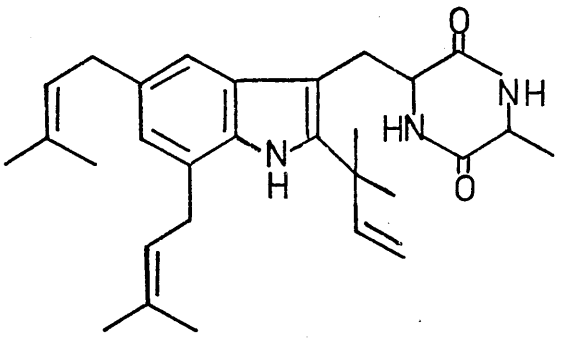

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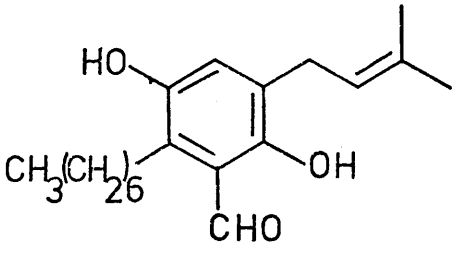

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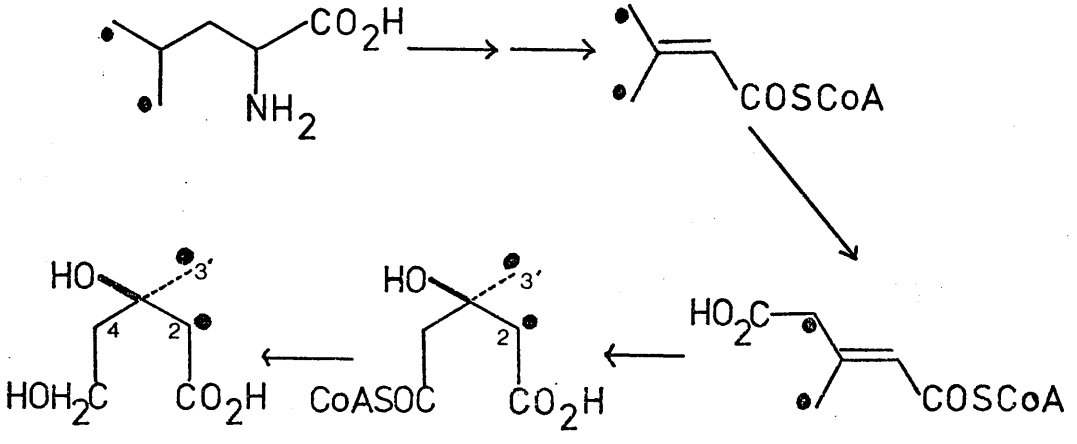
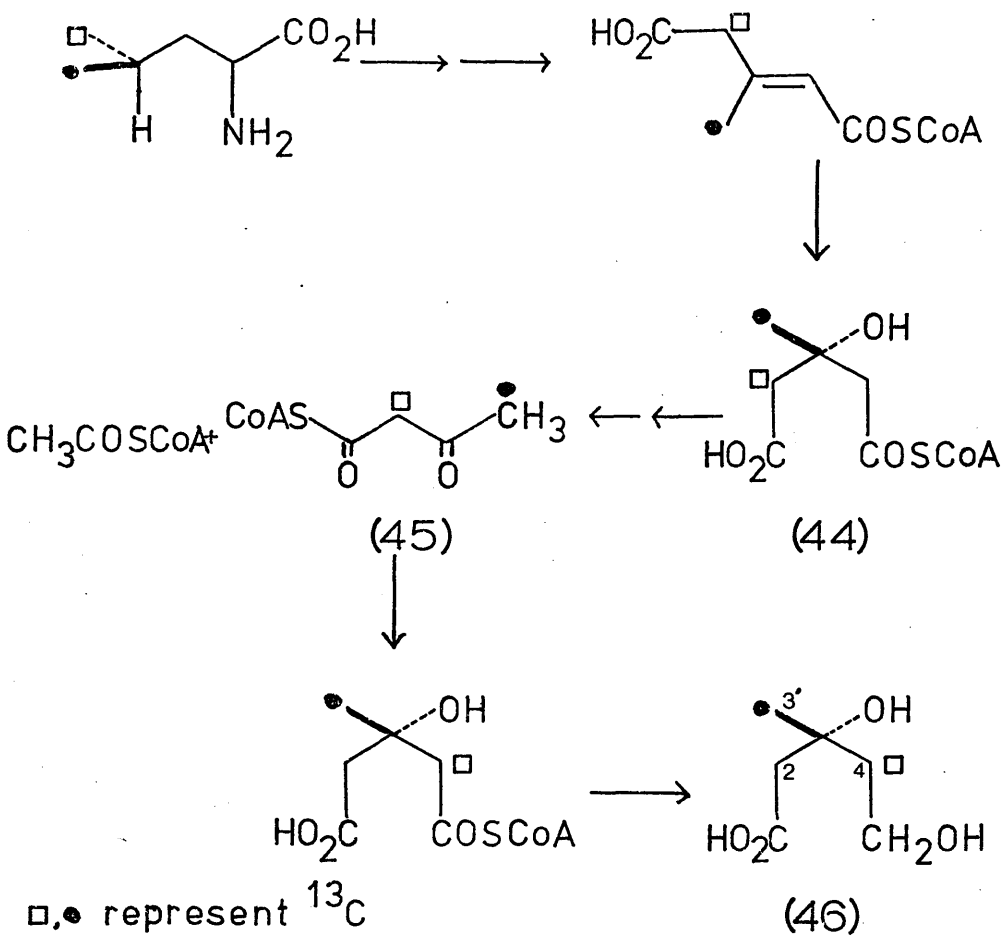


(43) (42)

SCHEME 10

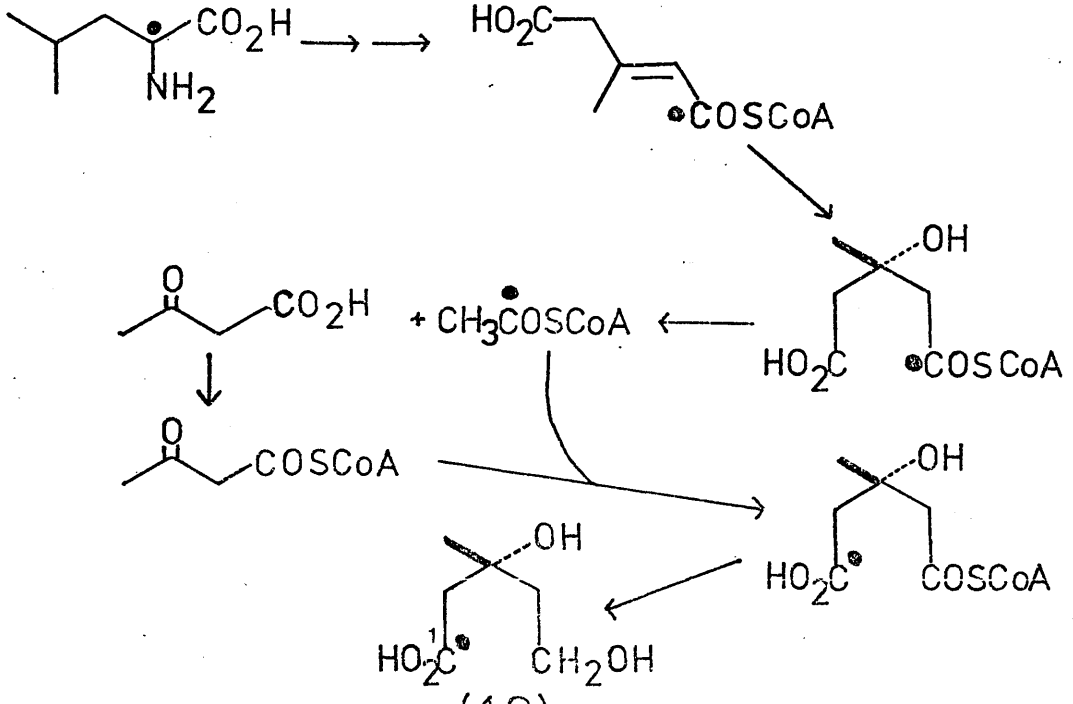
has not shifted. The β carbon atom (C-10) is also more shielded in paniculide A by 3.3ppm. The two γ carbon atoms, C-9 and C-11, are deshielded by removal of the hydroxyl group by 3.9 and 3.2ppm respectively. The sense and magnitude of all these shifts are fully compatible with the established substituent effects for the hydroxyl group (31). The spectrum of paniculide A obtained from feeding (2RS)-[2-^{13}C]leucine is shown in figure 1. It must be concluded from these results that C-2 of leucine is not incorporated into terpenoids in Andrographis paniculata tissue cultures.

A clue to the interpretation of these rather perplexing results is provided by consideration of a recent report by Fuganti and his colleagues (26) who studied the incorporation of (4R)-[5-^{13}C]leucine (38) and the (4S)-isomer (39) into the meroterpenoids echinuline (40) and flavoglaucine (41) in Aspergillus amstelodami. As shown in scheme 10, if degradation of leucine occurs by the pathway previously characterised in mammalian liver, the two methyl groups of leucine should become C-2 and C-3' of (3S)-3-hydroxymethylglutaryl-CoA, as indicated in (42). Direct reduction of (42) by hydroxymethylglutaryl-CoA reductase would give mevalonate (43) in which C-2 and C-3' originate from the two methyl groups of leucine. From the ^{13}C NMR spectrum of echinuline and flavoglaucine biosynthesised from the isomeric labelled leucine samples (38) and (39), it was deduced that there is preferential incorporation of the pro-4S methyl group of leucine into C-3' of mevalonate, while the pro-4R methyl group is incorporated into C-4 of mevalonate. Thus, instead of labelling C-2 of mevalonate,



□,● represent ^{13}C

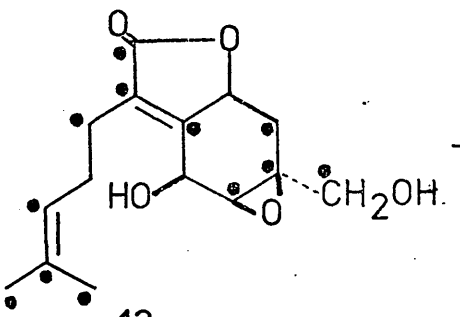
SCHEME 11



SCHEME 12

one of the methyl groups of leucine labels at C-4. The interpretation of these results is shown in scheme 11. Degradation of (3S)-3-hydroxymethylglutaryl-CoA (44) to acetoacetate (45) and acetyl-CoA must have taken place by the action of hydroxymethylglutaryl-CoA lyase (E C 4.1.3.4). In this reaction, acetyl-CoA is derived from the pro-S acetic acid group of the substrate (17). The synthesis of (3S)-3-hydroxymethylglutaryl-CoA is catalysed by hydroxymethylglutaryl-CoA synthase. In the synthase reaction, however, acetyl-CoA forms the pro-R acetic acid group of the product (18), and coenzyme A is liberated from acetyl-CoA during the reaction. Thus, degradation of 3-hydroxymethylglutaryl-CoA to acetyl-CoA and acetoacetate leads to label from the methyl groups of leucine appearing at C-3' and C-4 of mevalonate (46).

Scheme 12 shows the fate of C-2 of leucine when there is degradation to acetyl-CoA (47) and acetoacetate. If acetyl-CoA which is labelled from C-2 of leucine is incorporated into mevalonate (48) by the hydroxymethylglutaryl-CoA synthase system and does not contribute to the acetoacetyl-CoA pool, the label is at C-1 and is thus lost as carbon dioxide in the formation of isopentenyl pyrophosphate. Failure to observe incorporation of label from C-2 of leucine into paniculide B indicates that degradation of leucine to acetyl-CoA and acetoacetate probably occurs in A. paniculata cultures and that under these conditions acetyl-CoA is not incorporated into acetoacetyl-CoA. Presumably acetoacetyl-CoA is not synthesised by the tissues from acetyl-CoA because of product inhibition. Alternatively, there may be a compartmentation effect which



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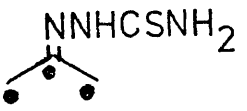
(49)



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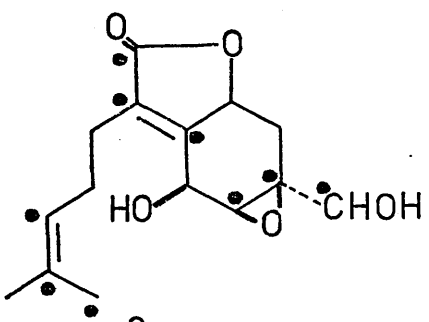

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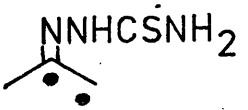

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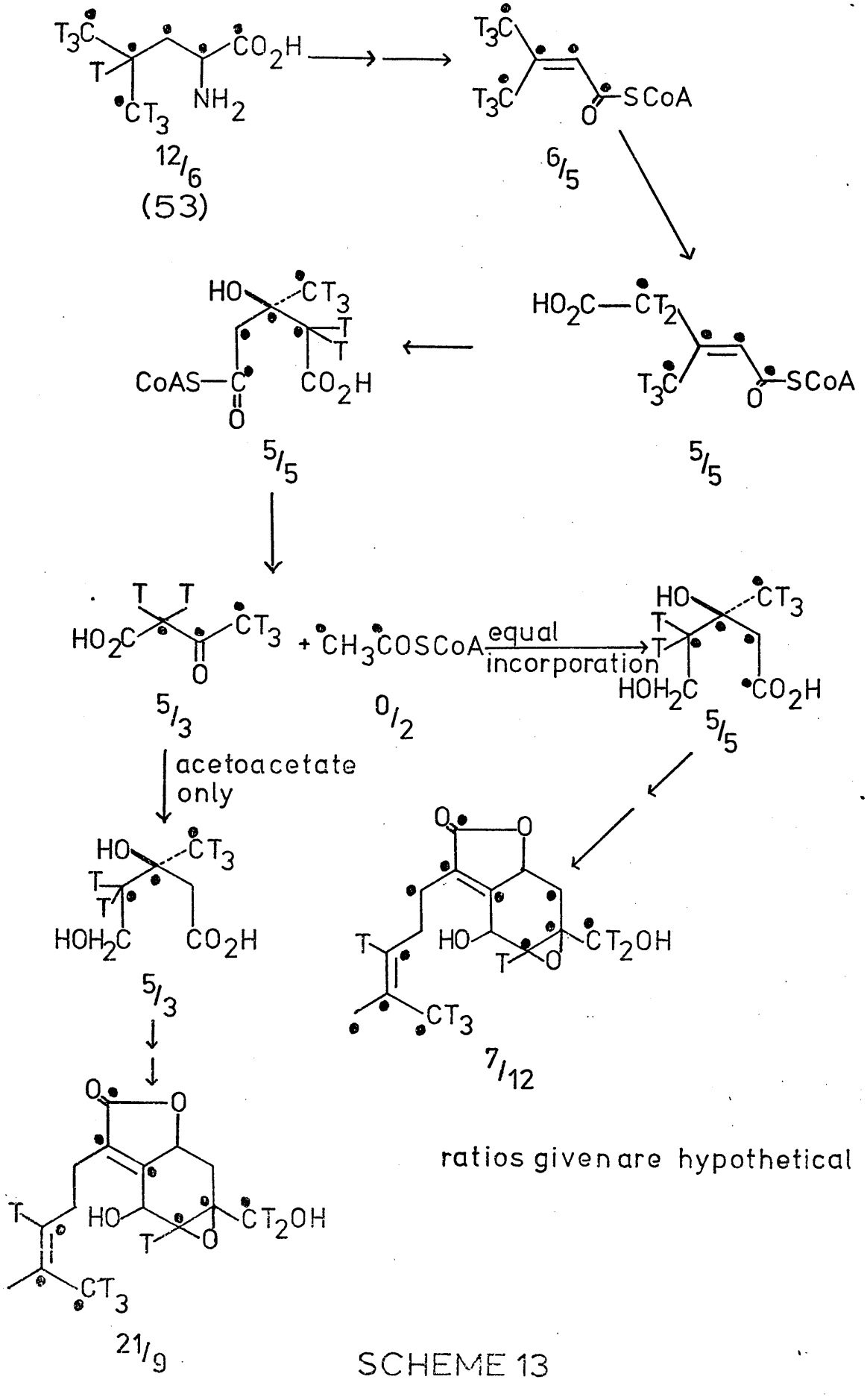
12/6
(53)
6/5
5/5
5/5
5/3
0/2
equal incorporation
5/5
5/3
acetoacetate only
5/3
7/12
ratios given are hypothetical
21/9

SCHEME 13

only allows acetoacetate to be transported to the site of terpenoid biosynthesis, while acetoacetyl-CoA remains on the site of leucine degradation. This is entirely plausible since the transport properties of acetoacetate anion and acetyl-CoA ester will differ markedly. Acetoacetate has been shown many years ago (32) to be incorporated into cholesterol without prior breakdown to acetate.

It is now possible to rationalise the observed pattern of incorporation of (2S)-[U-^{14}C]leucine into paniculide B. If the acetyl-CoA and acetoacetyl-CoA are diluted with endogenous material and re-incorporated to the same extent, then the labelling pattern in paniculide B biosynthesised from (2S)-[U-^{14}C]leucine should be as shown in (49) and the acetone derivative (50) obtained by oxidation should contain 25% of the radioactivity. If the acetyl-CoA is not re-incorporated at all, then paniculide B should be labelled as shown in (51) and the acetone derivative (52) should contain 22% of the radioactivity. The experimental value of 18% does not distinguish between these alternatives.

Now that the degradative pathway has been established in outline, rationalisation of the observed tritium retention in paniculide B biosynthesised from (2S)-[U-^{14}C, 4,5-$^{3}H_2$] leucine (53) may be attempted. Scheme 13 shows the tritium: carbon ratio for each proposed intermediate. Thus, it may be calculated that equal dilution and incorporation of acetyl-CoA and acetoacetate should lead to 29% tritium retention, while if acetyl-CoA is not re-incorporated at all there should be 39% tritium retention in paniculide B. The experimental value is 24%, which tends to support the

Table 3.

	Activity in acetone.	^{3}H retention from (2S)-[U-^{14}C, 4,5-$^{3}H_2$] leucine.
Acetyl-CoA incorporated twice as well as acetoacetate	27%	20%
Equal incorporation of acetyl-CoA and acetoacetate	25%	29%
Only acetoacetate incorporated	22%	39%
Observed	18%	24%

lower figure, implying that both acetyl-CoA and acetoacetate are incorporated. However, it should be noted that there may be considerable (non-enzymic) loss of tritium from acetoacetate and unless the extent of this loss can be determined, it is difficult to come to any definite conclusion. The results and possible conclusions are summarised in table 3. For the purposes of comparison, the expected values are also given for the case in which acetyl-CoA is incorporated with twice the efficiency of acetoacetate. It seems certain that leucine is incorporated into paniculide B via acetyl-CoA and acetoacetate, but the extent of dilution with endogenous material and degree of incorporation of these substances requires further study. In particular, the extent of incorporation of acetyl-CoA could be determined by the use of leucine labelled at C-3.

(2S)-[U-^{14}C]leucine and (2S)-[U-^{14}C]valine were incubated with the cell-free system from A. paniculata tissue cultures which contained all the co-factors necessary for conversion of mevalonate into farnesol. Incorporation of radioactivity was observed into neither farnesol nor γ-bisabolene. The pathway for leucine degradation by the cell-free system must be blocked at a point so far undetermined.

Experimental.

Feeding of (2S)-[U-^{14}C]valine and (2S)-[U-^{14}C]leucine to Andrographis cultures.

(2S)-[U-^{14}C]Valine (10μ Ci, 5μg) was administered as a filter-sterilized aqueous solution (200μl) to cultures of A. paniculata (5 flasks, 0.85g dry weight callus tissue) which had been grown in suspension culture for twenty-one days following transfer from solid medium. After a further seven days' growth, paniculide B was isolated from the culture medium as previously described (p53). Liquid scintillation counting and radio-scanning of the t.l.c. plate showed negligible activity in paniculide B.

When (2S)-[U-^{14}C]leucine (10μ Ci, 4μg) was used as a precursor in a similar experiment, radio-scanning of a chromatogram of the extract showed that paniculide B was labelled. The paniculide B which was isolated was diluted with carrier and crystallised to constant activity from ethyl acetate. The total yield of paniculide B was 27.6mg of specific activity 1.33 10^4dpm/mg, representing an incorporation of 1.6% In a duplicate experiment, the incorporation was 0.78%.

Degradation of paniculide B biosynthesised from [2-^{14}C]mevalonate.

(3RS)-[2-^{14}C]Mevalonate (5μ Ci, 0.29μ mole) was fed to 5 flasks of three-week-old A. paniculata cultures and paniculide B was isolated in the usual way after seven days. Carrier paniculide B was added and the material was crystallised to constant activity (9.6×10^3dpm/mg, 2.69×10^6 dpm/m mole, after four crystallisations from ethyl acetate). This represents an incorporation of 1.8%.

A sample of this paniculide B (14mg, 0.05m mole) was mixed with 2-methylpropan-2-ol (0.2ml). Ruthenium dioxide (1.5mg, 0.011m mole) was then added. A solution of sodium periodate (0.4m mole) and periodic acid (0.01m mole) in water was then added in small portions over a period of three hours. After one hour, a slow stream of nitrogen was passed through the reaction mixture to entrain acetone which was trapped by passing the gas through three test tubes, each containing thiosemicarbazide (30mg, 0.33m mole) and sodium acetate (60mg, 0.75m mole). The nitrogen stream was left on for a further sixteen hours to ensure that all the acetone had been removed from the reaction mixture. The contents of the test tubes were then combined, water (10ml) was added and the product extracted with ethyl acetate (3x10ml). The acetone thiosemicarbazone (r_f 0.35) was isolated (5.7mg, 86% theoretical) by preparative t.l.c. over silica (chloroform-methanol, 94:6, v/v) and crystallised from aqueous methanol to constant activity. The specific activity of acetone thiosemicarbazone was 5.72×10^3dpm/mg (7.497×10^5dpm/m mole). This is 28% of the specific activity of the paniculide B.

Degradation of paniculide B biosynthesised from (2S)-[U-^{14}C]leucine.

Paniculide B (14mg, 0.05m mole) biosynthesised from (2S)-[U-^{14}C]leucine (10μ Ci, 4μg) with 0.3% incorporation was crystallised to constant activity (1.8×10^3dpm/mg, 5.22×10^5dpm/m mole). Degradation of a sample of this paniculide B (14mg, 0.05m mole) to acetone was carried out exactly as described above. The specific activity of the acetone thiosemicarbazone obtained (6mg, 90% theoretical) in this

experiment was 679.1dpm/mg (8.9×10^4dpm/m mole). This is 18% of the specific activity of the paniculide B.

Incorporation of (2S)-[U-^{14}C, 4,5-$^{3}H_2$]leucine and (2S)-[U-^{14}C]leucine (2RS)-[4,5-$^{3}H_2$]leucine into paniculide B.

Andrographis suspension cultures (twenty-four days after subculture, 3 flasks, 0.51g dry weight callus tissue) were fed with (2S)-[U-^{14}C, 4,5-$^{3}H_2$]leucine (1μ Ci ^{14}C, 0.04μg, ^{3}H:^{14}C 12.0). After a further ten days, paniculide B was isolated and purified in the usual manner. The incorporation after crystallisation to constant activity was 0.62% and the ^{3}H:^{14}C ratio was 2.89. This represents a tritium retention of 24%.

In a parallel experiment, (2S)-[U-^{14}C]leucine (1μ Ci, 0.04μg) was mixed with (2RS)-[4,5-$^{3}H_2$]leucine (50μ Ci) and used as a substrate for paniculide B biosynthesis under the conditions described above. The ^{3}H:^{14}C ratio of the substrate was 43.32, while the paniculide had a ratio of 9.98. This represents 23% retention of tritium.

Preparation of (2RS)-[2-^{13}C]leucine.

The synthesis of (2RS)-[2-^{13}C]leucine was performed by the use of vacuum line techniques which have been described by Pichat (29). A solution of Grignard reagent was prepared by reaction of 1-bromo-2-methylpropane (2.74g, 20m mole) with magnesium turnings (490mg, 20m mole) in dry ether (40ml). The flask containing this solution was then transfered to the vacuum line and frozen in liquid nitrogen. Carbon dioxide (5m mole), which was generated by reaction of concentrated sulphuric acid with ^{13}C labelled barium carbonate (0.992g, 5m moles, 87.6 atom % ^{13}C), was

then distilled into the flask containing the Grignard reagent. The reaction flask was sealed off from the rest of the vacuum line and allowed to warm up to -25°C. Vigorous stirring was started as soon as possible. Any carbon dioxide remaining in the vacuum line was condensed by freezing the reaction mixture in liquid nitrogen once more. After stirring for a further ten minutes, dilute sulphuric acid (6N, 20ml) was slowly added with cooling. Silver sulphate (5g) was added and the reaction mixture distilled at atmospheric pressure. The ether, which distils first, was discarded and the product was entrained in the aqueous distillate. The [1-^{13}C]3-methylbutanoic acid (34) was titrated with 1M sodium hydroxide as it distilled using phenolphthalin as indicator. After evaporation of water under reduced pressure, the sodium salt was dried completely by heating *in vacuo* at 110°C overnight.

Dry ether (50ml) was distilled into the flask containing the dry salt at the same time as dry hydrogen chloride (6m moles, generated from sodium chloride and concentrated sulphuric acid). After stirring overnight at room temperature, excess hydrogen chloride was removed by distillation *in vacuo* of part of the ether. The reaction flask was then purged with nitrogen before addition of carbonyl bisimidazole (1.0g, ~6m mole). The reaction mixture was then stirred at room temperature for two hours and refluxed for a further thirty minutes. The imidazole derivative (36) was then reduced at 0°C by careful addition of lithium aluminium hydride (~95mg, ~2.5m mole). Small aliquots of the reaction mixture were removed during reduction to be monitored by analytical g.l.c. on 1% SE 30 at 50°C. Addition

of lithium aluminium hydride was stopped as soon as the first traces of alcohol could be detected. The aluminium salts were decomposed by addition of dilute sulphuric acid (5ml).

The reaction mixture was re-cooled to $0^{o}C$ and saturated with ammonia. Potassium cyanide (390mg, 6m mole), ammonium chloride (321mg, 6m mole) and a saturated solution of ammonia in methanol (12ml) were then added and the mixture was stirred at room temperature for forty-eight hours. Ether was removed under reduced pressure and the residue was acidified with concentrated hydrochloric acid (40ml). The reaction mixture was then left to stand overnight before refluxing for eight hours. The solution was then evaporated to dryness, taken up in a small volume of water and treated with charcoal. The aqueous solution was then applied to a column (35cm×2.5cm) of Dowex 50 W8 cation exchange resin in the H^{+} form. The column was eluted with 0.3M hydrochloric acid. Presence of amino acid in fractions was determined by a ninhydrin test. Fractions containing the product (2RS)-[2-^{13}C]leucine were combined and evaporated to dryness. The residue was dissolved in the minimum quantity of water and applied to a similar column of Dowex 50 in H^{+} form. The column was eluted with 0.3M hydrochloric acid (50ml), washed with distilled water until no trace of chloride ion could be detected (silver nitrate test), and the free amino acid was eluted with 2.0M ammonium hydroxide, yielding (2RS)-[2-^{13}C]leucine (282mg, 43% yield based on barium carbonate).

The ^{13}C NMR spectrum of this material was recorded in D_2O/NH_4OH/dioxan; chemical shift (converted to TMS scale) assignment; 55.1, C-2; 44.3, C-3 (doublet J 35Hz); 25.2,

C-4; 23.2, 22.1, C-5, C-5'; C-1 not observed. The spectrum was similar to that of unlabelled leucine, except that the peak at δ55.1 was greatly enhanced. The chemical shift agrees well with the literature value (33) for the α carbon of leucine (δ54.4ppm) which was obtained in neutral solution. ^{13}C content was ~70 atom %.

Optimisation of feeding conditions for (2S)-[U-^{14}C]leucine.

Culture medium was removed from suspension cultures (5 flasks) which had been grown for seven, fourteen or twenty-one days following transfer from solid medium. The medium was replaced with fresh sterile medium containing (2S)-[U-^{14}C]leucine (1μ Ci) and non-radioactive (2S)-leucine (50mg/l or 100mg/l). Paniculide B was extracted after a further seven days and crystallised. The results are shown in table 1. When the high leucine concentration was used, paniculide B was not labelled, but another less polar compound, which was identified as paniculide A (r_f 0.53) was labelled. The mass spectrum of this metabolite showed M^+ at 264, m.p. 118-120° (lit. 120-21°C) (23).

Feeding of (2RS)-[2-^{13}C]leucine to Andrographis cultures.

Culture medium (1.5l) was prepared which contained (2RS)-[2-^{13}C]leucine (75mg) and (2S)-[U-^{14}C]leucine (1μ Ci), and used to replace the medium of three-week-old Andrographis cultures. After seven days, paniculide B was isolated and crystallised. Absolute incorporation into recrystallised paniculide B (34mg) was 2.8%. The specific incorporation of (2S)-[U-^{14}C]leucine into paniculide B was 13%. Mass spectroscopy failed to detect any incorporation of ^{13}C. No peak enhancements were readily apparent in the ^{13}C NMR spectrum of the material. The diacetate derivative (37) was

prepared by treatment of the paniculide B (34mg) with acetic anhydride (0.2ml) in dry pyridine (0.2ml) at room temperature overnight. Water (5ml) was then added, and the product extracted into ethyl acetate (3x5ml). Pyridine was then removed from the organic phase by washing with saturated copper sulphate solution (3x10ml), followed by water (2x5ml). After drying, the solution was evaporated to small volume and applied to a preparative t.l.c. plate (chloroform-methanol, 96:4, v/v). The band (r_f 0.58) corresponding to paniculide B diacetate (37) was scraped off and eluted. The derivative m.p. 98-99° (lit. 98-99°) (23) was recrystallised from ethyl acetate/hexane. The ^{13}C NMR spectrum was recorded in CD Cl_3:chemical shift, assignment; 172.8, C-14; 170.2, 169.8, C-1 of acetate; 152.4, C-7; 132.9, C-2; 129.9, C-6; 122.7, C-3; 74.7, C-8; 69.1, C-12; 65.6, C-15; 59.1, C-11; 57.7, C-10; 32.7, C-9; 27.1, C-4; 25.7, C-1; 23.9, C-5; 20.6, C-2 of acetate; 17.7, C-13..

In a repeat experiment using once again (2RS)-[2-^{13}C] leucine (75mg) and (2S)-[U-^{14}C]leucine as components of the culture medium (15 flasks), radioactivity was incorporated only into paniculide A (3.0%). Paniculide A (18mg) was isolated and its ^{13}C NMR spectrum examined. The spectrum is shown in figure 1 and its assignment is discussed on p72. No incorporation of ^{13}C into paniculide A was detected in either the ^{13}C spectrum or the mass spectrum despite the specific incorporation of 28.3%.

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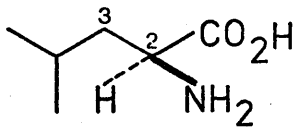
SECTION 4

THE LEUCINE AMINO MUTASE REACTION



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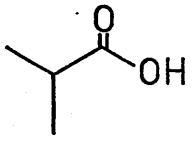

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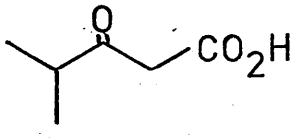

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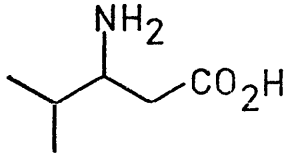

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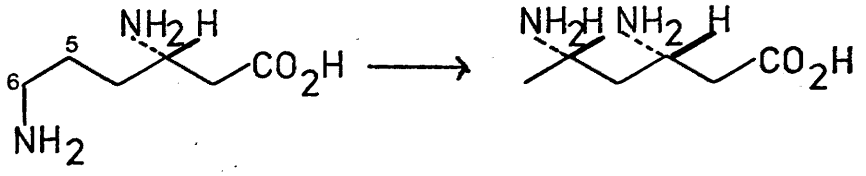
Section 4.

The Leucine Amino Mutase Reaction.

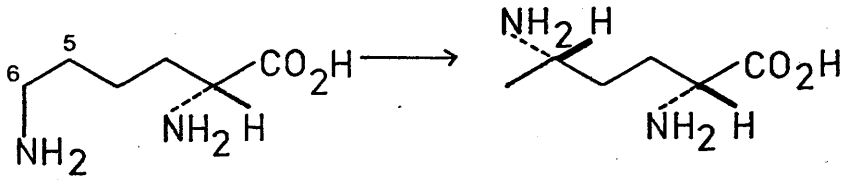
Introduction.

A new degradative pathway for leucine has recently been described by Poston (1). He found that the end products of leucine (1) fermentation by a strain of Clostridium sporogenes were acetate, 2-methylpropanoate (2) and ammonia. Formation of the acid (2) cannot be rationalised by the previously established pathways of leucine degradation. The presence of this acid, along with that of 3-keto-4-methylpentanoate (3) suggested that the initial degradative step in this case may be isomerisation to 3-amino-4-methylpentanoic acid (β-leucine) (4). When (2S)-α-[U-^{14}C]leucine was incubated with a cell-free extract from Cl. sporogenes, radioactivity was detected in added (3RS)-β-leucine. Similarly, when (3RS)-β-leucine was incubated with the cell-free system, α-leucine could be detected by a colorimetric method. This leucine 2,3-amino mutase activity was shown to be dependent on coenzyme B_{12}. Addition of the corrinoid-binding mucoprotein, intrinsic factor, to reaction mixtures containing (3RS)-β-leucine as substrate caused a decrease in the amount of α-leucine formed, while S-adenosylmethionine had no appreciable effect on the reaction. Leucine 2,3-amino mutase activity was shown to be widely distributed in mammalian tissues and in several Clostridia.

The enzyme was later reported to be present in bean seedlings (2). The presence of a coenzyme B_{12}-dependent enzyme in higher plants or tissues derived from them is unprecedented. There are two possible explanations:



SCHEME 1



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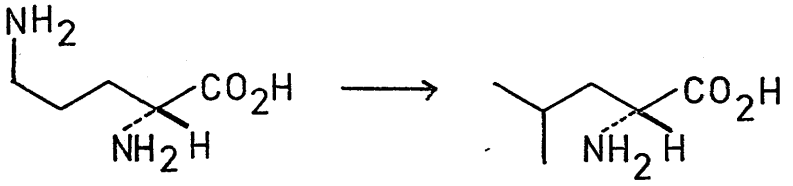
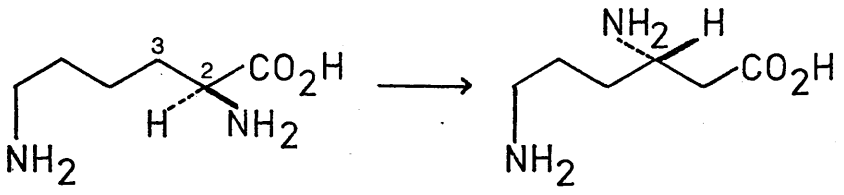


SCHEME 3

coenzyme B_{12} may be acting as a substitute for a naturally occurring co-factor. It would not be unreasonable to assume that this substance may be derived from chlorophyll, although no such co-factor has ever been described. More probably, since whole plants were used in this study, the enzyme may have been derived, not from the plant tissue, but from the bacteria associated with it.

Three coenzyme B_{12}-dependent amino mutases have been described. They all catalyse migration of the ω-amino groups of different diamino acids. They are β-lysine 5,6-amino mutase, D-α-lysine 5,6-amino mutase and ornithine amino mutase, which catalyse the reactions shown in schemes 1, 2 and 3 respectively. The two lysine amino mutase enzymes have been obtained in highly purified form (3) and a number of their co-factor requirements and properties have been studied. Both enzymes are remarkable in their requirement for an unusually large number of co-factors. For maximal activity they require coenzyme B_{12}, a thiol, a monovalent cation, a divalent cation, adenosine triphosphate and a carbonyl compound (pyruvate or pyridoxal phosphate). The mechanism of these reactions is not well understood, but it has been suggested that some of the co-factors participate in reactions which modify the enzyme, rather than being involved in the hydrogen and amino group migrations <u>per se</u>.

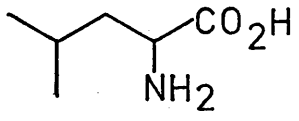
In the α- and β-lysine 5,6-amino mutase reactions, a hydrogen abstracted from C-5 of the amino acid replaces the amino group which migrates from C-6 to C-5. When coenzyme B_{12}, specifically tritiated at the 5'-methylene position of the 5'-deoxyadenosyl moiety, is used as



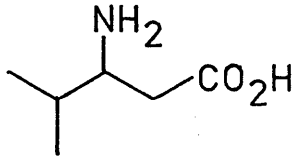
SCHEME 4

co-factor, label is transfered to C-6 of the product in both cases (4,5). In the β-lysine 5,6-amino mutase reaction it has been shown (5) that β-lysine, re-isolated after incubation with tritiated coenzyme, contains label at C-5 and that the labelled atom occupies the same diastereotopic position as the hydrogen atom which is transfered to C-6 of the product. The role which coenzyme B_{12} plays in the migrations catalysed by a number of enzymes has been reviewed (6).

Lysine 2,3-amino mutase catalyses the formation of β-lysine as shown in scheme 4. Although it superficially resembles the action of leucine 2,3-amino mutase, the lysine enzyme is not dependent on coenzyme B_{12} (7). The enzyme, which has been purified to homogeneity, requires pyridoxal phosphate as co-factor and is activated by S-adenosylmethionine, supposedly an allosteric effector. The mechanism of the reaction is not known, but it has been shown that hydrogen from water is not incorporated into lysine or β-lysine during the amino mutase reaction. A review is available dealing with the enzymes of lysine metabolism (8).



(1)



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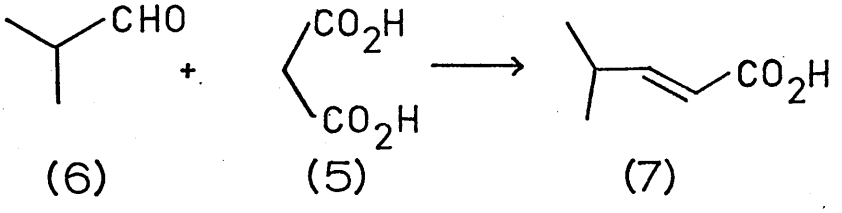
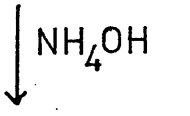
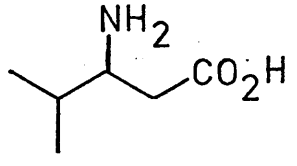


(6) (5) (7)





(4)

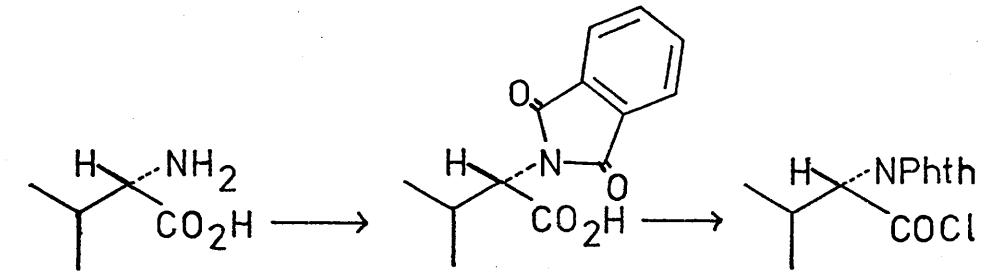
SCHEME 5

Discussion.

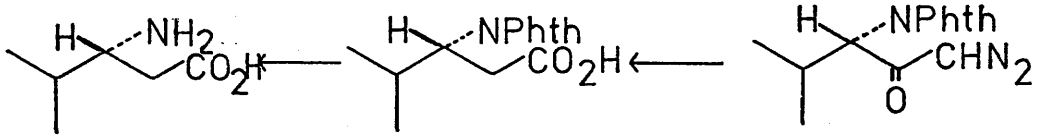
Doubts remain about Poston's work (2) on the coenzyme B_{12}-dependent enzyme leucine 2,3-amino mutase in plants because of the non-sterility of the plant material used. This enzyme catalyses reversible amino group migration of α-leucine (1) to give β-leucine (4). It seemed to us worthwhile to look for such enzymic activity in a tissue culture derived system which has the automatic advantage of being strictly sterile.

Racemic β-leucine (3-amino-4-methylpentanoic acid) (4) was synthesised by the route shown in scheme 5. Malonic acid (5) was condensed (9) with 2-methylpropanal (6) in pyridine to give 4-methylpent-2-enoic acid (7) in moderate yield. Addition of ammonia in the expected sense was then effected by heating the acid (7) in a sealed tube with concentrated aqueous ammonia at 110°-120°C for three days to yield (3RS)-β-leucine (4).

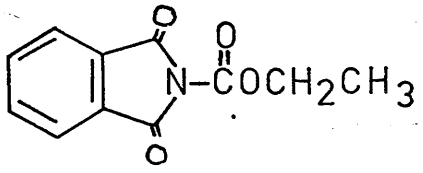
(2S)-α-[U-^{14}C]Leucine was incubated with the Andrographis cell-free system and coenzyme B_{12} in the presence of unlabelled (3RS)-β-leucine. After passage of the incubation mixture through a Dowex 50 cation exchange column, radioscanning of thin layer chromatograms of the first three radioactive fractions eluted from the column located radioactivity in the band corresponding to β-leucine. The ion exchange column does not completely separate α- and β-leucine, so that the fractions contain approximately equal amounts of radioactivity in the two amino acids. The incorporation of (2S)-α-[U-^{14}C]leucine into β-leucine was ~0.4%. No radioactive β-leucine could be detected in a control experiment using boiled extract. Thus, leucine



(8) (9) (11)



(12) (13) (14)



(10)

SCHEME 6

2,3-amino mutase activity appears to be present in the cell-free extract from Andrographis tissue. This confirms that the enzyme can be produced by plant tissue.

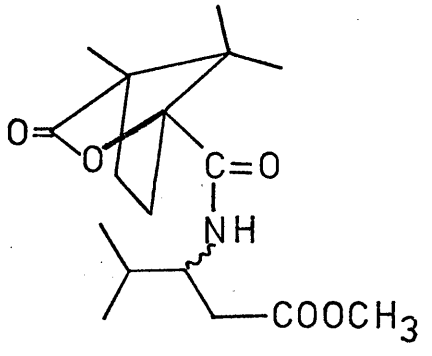
The leucine 2,3-amino mutase reaction raises several stereochemical questions: (a) which enantiomer of α-leucine acts as substrate; (b) which enantiomer of β-leucine is the product; (c) is there inversion or retention of configuration at C-2 and at C-3 during the reaction? Question (b) has been answered definitively in the following way. Chiral β-leucine was prepared from (2S)-valine (8) by Arndt-Eistert homologation, essentially as described by Balenovic (10), as shown in scheme 6. The protected derivative (2S)-N-phthaloylvaline (9), prepared by reaction (11) of (2S)-valine (8) with N-carboethoxyphthalimide (10), was converted to the acid chloride (11) by treatment with thionyl chloride. The diazoketone (12), prepared by reaction of the acid chloride with diazomethane, then underwent Wolff rearrangement with retention of configuration (12) in aqueous dioxane in the presence of silver oxide to give (3S)-N-phthaloyl-β-leucine (13). Removal of the N-phthaloyl protecting group with hydrazine (13) then gave (3S)-β-leucine (14).

A method for the resolution of racemic β-leucine was then developed based on the gas chromatographic separation (14) of a series of N-acyl-β-leucine methyl esters (15). The derivatives were formed by Fischer esterification of the free amino acid with hydrogen chloride in dry methanol, followed by reaction of the methyl ester hydrochloride (16) with the appropriate optically active acid chloride (17) in dry pyridine. The results, which are given in table 1,

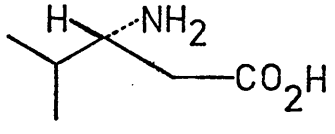
Table 2.

Retention indices of α- and β-leucine methyl ester camphanamides on 1% OV 210 at 170°C.

	I
(2R)-α-leucine	2545
(2S)-α-leucine	2580
(3S)-β-leucine	2610
(3R)-β-leucine	2630



(18) ≡ (15), R=a



(14)

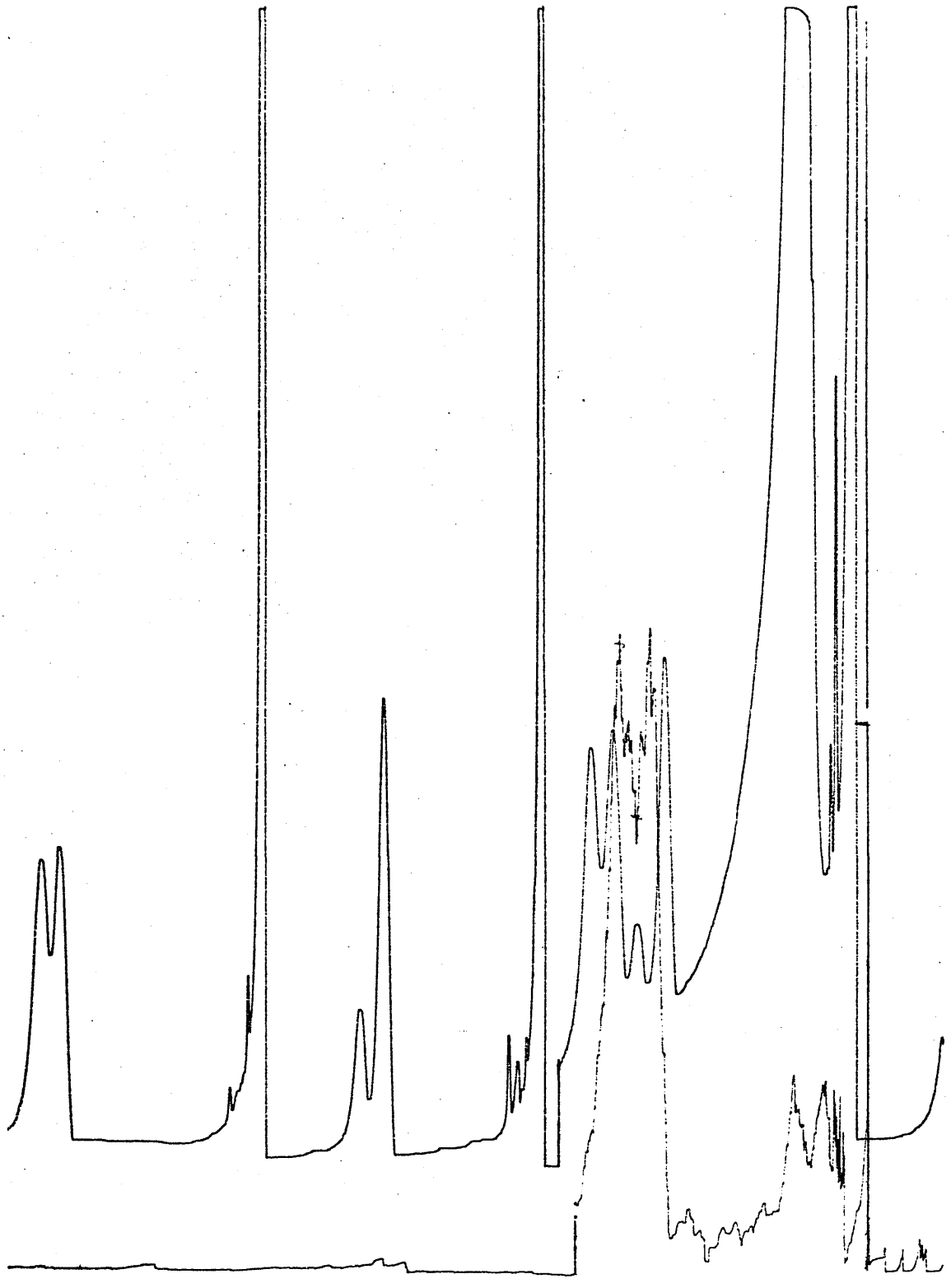
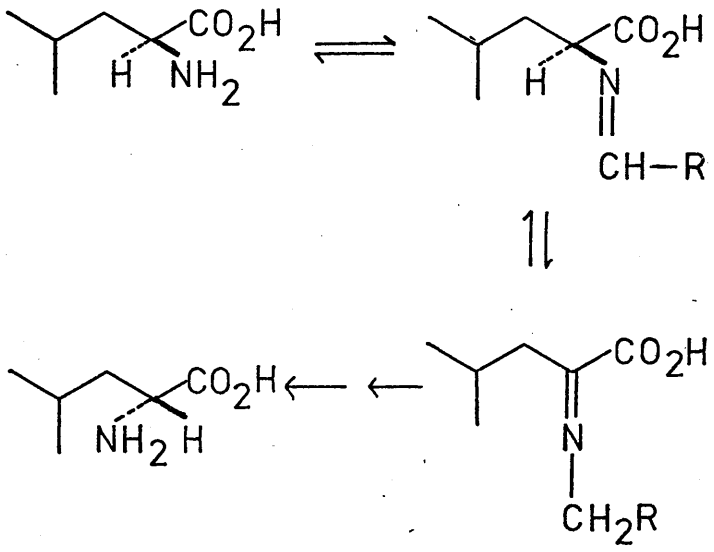


Figure 1 Gas Liquid Chromatography of β-Leucine Biosynthesised from (2S)-[U-^{14}C]Leucine 1% OV210 at 170°
Lower trace marks radioactivity



SCHEME 7

show that the best resolution of β-leucine is obtained when the methyl ester camphanamide (18) is chromatographed on 1% OV 210. The use of this derivative allowed the clear chromatographic resolution of (2R)-α-leucine, (2S)-α-leucine, (3S)-β-leucine and (3R)-β-leucine. The retention indices of the four derivatives are given in table 2. The peaks were identified by preparation of derivatives from (2R)- and (2S)-α-leucine and from (3S)-β-leucine (14), which was prepared as described above.

When the methyl ester camphanamide derivative of β-leucine, which had been biosynthesised from (2S)-α-[U-^{14}C]leucine by the cell-free system, was examined by radio-g.l.c., radioactivity was found in the peak corresponding to (3S)-β-leucine, as shown in figure 1. There was no evidence of radioactivity in (2R)-α-leucine, indicating that racemisation, if it occurs, does not lead to accumulation of this enantiomer. Thus the absolute stereochemistry of β-leucine produced in the Andrographis cell-free system is (3S) as shown in (14). This experiment does not indicate which enantiomer of α-leucine is the substrate for the enzyme. This point could be established by the incubation of (2S)-α-[2-$^{3}H_1$]leucine. If the (2S) enantiomer is the substrate, label should be retained in the β-leucine product, while if the (2R) enantiomer were involved, label would be lost according to the accepted racemisation mechanism via condensation of the amino acid with pyridoxal phosphate and subsequent aldimine-ketimine interconversion (scheme 7).

The reverse reaction has also been demonstrated. When (3RS)-β-leucine is incubated with the cell-free system,

Table 3.

Dependence of α-leucine production on amount of β-leucine used.

β-leucine substrate (μmole)	α-leucine formed (arbitrary units)
0.5	0.042
2.0	0.061
3.0	0.078

Table 4.

Cofactor dependence of leucine 2,3-amino mutase reaction.

Cofactors: FAD (0.5μM), coenzyme A (0.5mM), NAD (0.5mM), pyridoxal phosphate (0.5mM).

Addition	Amount of α-leucine formed (arbitrary units)	
	Experiment (1)	Experiment (2)
none	0.098	0.077
4mM coenzyme B_{12}	0.046	0.071
0.5mM SAM	0.124	0.106
0.5mM SAM+4mM coenzyme B_{12}	0.035	0.091

Addition	Amount of α-leucine formed (arbitrary units)	
	Experiment (1)	Experiment (2)
none	0.087	0.125
4mM coenzyme B_{12}	0.051	0.094
1mg IF	0.281	0.248
4mM coenzyme B_{12} +IF	0.102	0.143

SAM denotes S-adenosylmethionine; IF denotes intrinsic factor.

α-leucine can be detected. The amount of α-leucine produced by the cell-free system is easily measured by a ninhydrin method, since β-leucine does not react with ninhydrin under the conditions of the assay (1). As shown in table 3, the amount of α-leucine produced depends on the amount of β-leucine used.

Up to this point, the only co-factor which was added to the incubation was coenzyme B_{12}. Having established that the ninhydrin assay is suitable for the measurement of leucine 2,3-amino mutase activity, the co-factor dependence of the enzyme was investigated. The results are shown in table 4. Addition of coenzyme B_{12} to the incubation appears to suppress formation of α-leucine, while addition of the corrinoid-binding mucoprotein, intrinsic factor, markedly enhances its formation. Thus coenzyme B_{12} appears to be inhibitory to the leucine 2,3-amino mutase reaction in Andrographis cell-free extract. There is a slight stimulation of activity on addition of S-adenosylmethionine. The dependence of the coenzyme on other co-factors has not yet been tested. The leucine 2,3-amino mutase activity of Andrographis cell-free extract does not appear to resemble that reported by Poston (1,2) and may be more like lysine 2,3-amino mutase which is dependent on pyridoxal phosphate and S-adenosylmethionine (7).

Experimental.

Preparation of 4-methyl-2-enoic acid (7).

Malonic acid (15.6g, 0.156mole) was dissolved in dry pyridine (15ml). The solution was cooled to 0°C and 2-methylpropanal (10.8g, 0.15mole) was slowly added. The mixture was left to stand for three hours and then refluxed overnight. The solution was poured into dilute hydrochloric acid (50ml) and ice. The oily upper layer was removed and the aqueous phase was extracted with ether (2x50ml). The organic solutions were combined with the oily material and extracted with dilute hydrochloric acid (4x50ml), saturated copper sulphate solution (2x50ml) and water (4x50ml). The solution was dried over anhydrous sodium sulphate and the solvent was evaporated under reduced pressure, leaving 4-methylpent-2-enoic acid as a clear viscous oil (6.3g, 55% yield). IR ν(max) 3100 (broad), 1700cm^{-1}.

(3RS)-3-amino-4-methylpentanoic acid (β-leucine) (4).

4-Methyl-2-pentenoic acid (5g, 0.044mole) was mixed with concentrated ammonium hydroxide solution (25ml) and heated in 6 sealed tubes at 110-120°C for seventy-two hours. After cooling, the tubes were opened and the contents removed. Water was removed under reduced pressure and the resulting oil was left aside to crystallise. The product was recrystallised from methanol-ether. The yield of (3RS)-β-leucine was 2.3g (40%) m.p. 198-199°C (literature 197°) (1).

Incubation of (2S)-α-[U-^{14}C]leucine with the cell-free system.

The cell-free extract from A. paniculata cultures was prepared as previously described (p26). The incubation

mixture contained cell-free extract (0.8ml), (2S)-α-[U-^{14}C] leucine (5μCi, 10μ mole), (3RS)-β-leucine (10μ mole) and coenzyme B_{12} (4μ mole) in a final volume of 1ml. After overnight anaerobic incubation in the dark, the reaction was terminated by addition of 20% perchloric acid (0.1ml). Denatured protein was removed by centrifugation and the supernatant was applied to a column of a Dowex 50 ion exchange resin in the H^+ form. The column was washed with 0.3M hydrochloric acid (50ml), then with water until no chloride could be detected, and the amino acids eluted with 2.0M ammonium hydroxide. Fractions (3ml) were collected and radioactivity monitored in each. The first 3 radioactive fractions (6,7 and 8) were found to contain approximately equal amounts of radioactivity in α- and β-leucine, while later fractions contained only α-leucine. Fractions were analysed by t.l.c. over alumina (Merk F_{254} Type T, 20×20cm pre-coated plates) (propan-1-ol-NH_4OH, 3:1, v/v), and the amount of radioactivity in each of the isomers was estimated by radio-scanning of the plates. Both amino acids can be visualised using a spray containing ninhydrin and hydrindantin, while if ninhydrin is used alone, only α-leucine gives a coloured spot (α-leucine r_f 0.14, β-leucine r_f 0.24). The incorporation of radioactivity into β-leucine was 0.4%.

Synthesis of (2S)-N-phthaloylvaline (9).

(2S)-Valine (2.93g, 0.025mole) and sodium carbonate decahydrate (7.15g, 0.025mole) were dissolved in water (45ml), and N-carboethoxyphthalimide (5.48g, 0.025mole) was added, and the mixture was stirred at room temperature for two hours. After brief heating to 40°C, the solution was cooled

and carefully acidified with dilute hydrochloric acid. After standing at 0°C for three days, the precipitated product had not solidified. The aqueous phase was decanted and the oil was dissolved in a minimum quantity of saturated sodium hydrogen carbonate solution and extracted with ether (2×20ml). Traces of ether were removed from the aqueous solution by stirring under reduced pressure at room temperature for one hour.

The solution was then cautiously re-acidified with vigorous stirring and a few seed crystals were added. The product, (2S)-N-phthaloylvaline (9), was filtered off after standing for thirty minutes at 0°C. The yield was 5.18g (84%), m.p. 116° (literature 116°); $[\alpha]_D^{25}$=-68.5° (ethanol) (literature -69°) (15).

Synthesis of (3S)-β-leucine (14).

(2S)-N-Phthaloylvaline (617mg, 2.5m mole) was heated at 60°C with thionyl chloride (3ml) for thirty minutes. Excess thionyl chloride was removed *in vacuo* and the solid residue was dissolved in dry ether (10ml). The solution of acid chloride was slowly added to a stirred ethereal solution of diazomethane (5m mole). After stirring overnight, the ether was removed to give the diazoketone (12) as a yellow oil which was not further purified. The diazoketone was dissolved in dioxan (5ml) and added dropwise to a stirred suspension of freshly prepared silver oxide (250mg) in water (10ml) containing sodium thiosulphate (250mg). After stirring at 60°C for one hour, more silver oxide and sodium thiosulphate (100mg each) were added and the mixture was stirred for a further two hours. Silver oxide was removed by filtration through a bed of celite

which was washed with hot dioxan. The organic solvent was evaporated under reduced pressure and the aqueous residue was partitioned between ether and sodium hydrogen carbonate solution. The aqueous solution was extracted with ether, acidified, and the product extracted into ether. After drying the solution, the solvent was evaporated to yield (3S)-N-phthaloyl-β-leucine (313mg, 45%) as a viscous gum.

Removal of the phthaloyl protecting group was effected by stirring (3S)-N-phthaloyl-β-leucine (313mg, 1.2m mole) with 60% aqueous hydrazine at room temperature for three days. The reaction mixture was basified with a small amount of ammonium hydroxide and applied to a column of Dowex 1 anion exchange resin (formate form). The column was throughly washed with distilled water, and the amino acid was eluted with 1M formic acid (20ml). After evaporation under reduced pressure to a smaller volume, this solution was applied to a column of Dowex 50 (H+ form). The column was throughly washed with distilled water, and (3RS)-β-leucine was eluted with 2M ammonium hydroxide (yield 60mg, 38%); m.p. 198-199°.

Preparation of β-leucine methyl ester amide derivatives.

Small samples (~2mg) (3RS)-β-leucine were dissolved in dry methanol (2ml) and dry hydrogen chloride was passed through the solution at 40°C for twenty minutes. After standing at 40°C for a further three hours, all trace of solvent was removed in vacuo. The residue was dissolved in a small amount of dry pyridine and the appropriate acid chloride (2mg) was added. After fifteen minutes at room temperature, the reaction mixture was warmed to ~80°C and pyridine was removed in a stream of nitrogen. The residue

was then dissolved in ethyl acetate (1ml) and examined by g.l.c. on various columns. The results obtained for a variety of derivatives are given in table 1, p91. The most satisfactory result was obtained when (-) camphanoyl chloride was used as the chiral resolving agent. The individual enantiomers of both α- and β-leucine were separable by this method (table 2, p92).

When the methyl ester camphanamide derivatives prepared from material containing -leucine, which had been biosynthesised by the cell-free system from (2S)-[U-^{14}C]leucine (10μCi, 0.2% incorporation), was examined by a radio-g.l.c. on 1% OV 210 at 170°C (operating in the ^{3}H mode), only (2S)-α-leucine and (3S)-β-leucine were found to be radioactive. Thus, the product of the leucine amino mutase reaction is (3S)-β-leucine (figure 1, p92).

Formation of α-leucine from β-leucine.

The cell-free extract (0.8ml), containing coenzyme B_{12} (4μ mole) as the only cofactor, was incubated with various amounts of (3RS)-β-leucine (0.5, 2.0 or 3.0μ mole, final volume 1ml) as previously described. The amount of β-leucine produced was determined by the ninhydrin assay according to Rosen (16). Optical density was determined on a Perkin Elmer 550 spectrophotometer in 1mm cells at 570μm. Under the conditions of the assay, β-leucine gives only a very poor colour yield (1). Allowance was made for the contribution of β-leucine and endogenous colorogenic material by subtraction of a blank value which was determined from an aliquot taken immediately after mixing the substrate with the enzyme system. The results, which are shown in table 3, are given as the differences between the

blank value and the optical density after overnight incubation. Blank values were typically 0.2.

Cofactor dependence of the leucine 2,3-amino mutase reaction.

(3RS)-β-leucine (10 mole) was incubated overnight in the dark with the cell-free system (0.8ml) containing FAD (0.5 M), coenzyme A (0.5mM), NAD^+ (0.5mM) and pyridoxal phosphate (0.5mM) in a final volume of 1ml. Coenzyme B_{12} (4mM), S-adenosylmethionine (0.5mM) and intrinsic factor (1mg) were added to the incubation as required. Incubations were terminated by addition of 20% perchloric acid (0.1ml) and the amount of α-leucine was determined by the ninhydrin method described above. The results are shown in table 4, p93. Duplicate experiments were run to test for the dependence on all three cofactors.

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