CHAPTER - IV

BIOSYNTHESIS OF $\alpha$-ACETOXYROYLEANONE
Plant products can be divided into two major groups, primary and secondary. Polysaccharides, proteins, fats and nucleic acids which are the fundamental building blocks of living matter constitute the primary metabolites and are found in all living organisms. Primary metabolism is the process which gives rise to these essential metabolites.

On the other hand, there are some chemical processes restricted to certain species yielding products which do not play a vital role in the existence of the organism. These are termed as secondary metabolites and are commonly referred to as natural products.

Though these compounds possess no obvious metabolic functions, still they are useful to the plant to some extent. Some of the secondary metabolites like alkaloids from Atropa, Berberis, Cinchona, Colchicum, Ephedra, Holarrhena, Mucuna, Opium, Rauwolfia, Solanum, Vinca etc., and steroidal glycosides (digitoxin derivatives, Ouabain etc.) are of medicinal importance. Compounds like pyrethrins and nicotine have found their use as insecticides. Essential oils are used in perfumery industry and some also in medicine.

The interrelationship between the primary and secondary metabolism is given in scheme 4.1.
Carbohydrates

- Carbon dioxide + Water

Glycolysis

Aromatic compounds

Ammonia

Pyruvic acid

Fatty acids

Acetate-Malonate pathway

Acetyl CoA

Tricarboxylic acid cycle

Amino acids

Proteins

Alkaloids

Nucleic acids

Polyketides

Terpenoids

Aromatic compounds

Steroids

SCHEME 4.1
The distinction between various products is based on their biogenesis. The term refers to the hypothesis postulated to describe the formation of natural products based on the study of compounds possessing a common skeleton and similar functional group pattern. These underlying common features can be used to deduce a reaction scheme by which the entire group can be visualised to have arisen \textit{in vivo}. The oxidised sites, however, could arise by processes which are unrelated to the main biogenetic pathways. Hence, it is essential that, prior to deriving any conclusions from the functional group patterns, a thorough study of a large number of natural products should be made. In fact, the validity of any biogenetic scheme is directly related to the number of compounds whose structures can be deduced in this way. It is striking to note that the organism resorts to the shortest possible path to produce these natural products.

Reactions which have proved successful \textit{in vitro} could be safely assumed to be feasible \textit{in vivo} too. Thus, with the help of appropriate enzymes and under physiological conditions, the organism builds complex molecules from readily available simple precursors. Often more than one hypothesis is suggested for the biogenesis of a molecule. The validity of the hypothesis could be examined by experimental evidences. The actual pathway thus proposed with the support of experimental inferences is called \textquoteleft biosynthesis\textquoteright of the molecule in question.
Use of tracers:

The study of biosynthesis of natural products can be made by the isotopic tracer technique. Till recently, most of the work involved use of precursors containing radioactive carbon ($^{14}$C) or tritium ($^3$H). Since the development of $^{13}$C nuclear magnetic resonance (CMR) spectroscopy, the stable nuclide $^{13}$C has featured prominently, while oxygen ($^{18}$O) and nitrogen ($^{15}$N) tracers have also been used. The radiochemicals used can be generally, uniformly, specifically or doubly labelled. The specific incorporation of the intact substrate into the product could be determined by the use of doubly labelled substrate. For obtaining unambiguous results, care must be taken to ensure radiochemical and chemical purity of these labelled precursors. The chemically pure labelled natural product is then degraded to identifiable fragments in which the position of the label is ascertained. Thus, the metabolic route leading to the formation of the substance under consideration can be unambiguously determined.

Plants are complex multicellular organisms which undergo growth and development accompanied by physiologic specialisation during their entire life time. Depending upon the specific function, the biochemical activity of a cell in one organ of the organism may differ from the activities of cells in other organs of the same organism. In terms of the secondary metabolism, it seems that at a certain time based upon the growth stage attained
by the plant as well as seasonal variation, the synthesis, or even a step in the synthesis, of a particular substance may take place in one part and be elaborated at a later time in another part of the same plant. Consequently, study of such a synthesis requires examination of a suitable part of the plant at an appropriate time. In most cases the problem of selecting the correct site may be bypassed by using the whole plant. To choose the proper time is rather difficult. It is known, however, that the regions of active growth are also the most active centres of biosynthesis in plants. Therefore, one may expect high biosynthetic activity during the active growth period, i.e., flowering and fruiting time.

Several techniques have been employed for administration of labelled precursors to whole plants. In some studies a plant growing hydroponically is used, and the labelled compound is added to the aerated nutrient medium. One cannot assume that the nutrient solution or the plant roots growing therein are free of micro-organisms. Then there is always a possibility that the labelled compound is modified or degraded microbially before it is absorbed by the roots. To eliminate this error, the roots are washed with a germicidal solution before immersing them in the solution of the labelled compound. The disadvantage in this method is that there is a large dilution of the label. To overcome this, use of high specific activity precursors becomes essential.
Gases such as $^{14}$CO$_2$ are administered in sealed polythene bags, or in growth chambers, while solid substrates are dissolved in water or emulsified with the help of surface active agents. The cut ends of the explants can then be dipped into this solution which is rapidly sucked up by the explant because of transpiration through the leaves. Alternatively, the solution can be either injected into the stem or seed pod by means of hypodermic syringe or painted onto the leaves. These methods, being quite tedious and non-quantitative, have been superceded by the 'wick technique'. This is a variation of the injection method and is employed especially in the case of plants having a strong but not too woody stalk. A cotton wick thread is passed through the stem with the help of a fine sewing needle. The ends of the two threads are then kept immersed in the tracer solution contained in a small beaker. The solution diffuses slowly into the plant by capillary action of the thread. After the complete absorption of the precursor solution within a few hours, the container is washed with distilled water to ensure maximum administration of the precursor. The 'wick technique' has many advantages over the other methods. The absorption being slow, it does not disrupt the physiological conditions of the plant to any great extent. It does not involve large dilutions of the precursor solution and hence greater amount of the precursor can be administered in a shorter period of time. Moreover, the method is quite easy, quantitative and does not need strict supervision.
Alternatively, the biosynthetic sequence of a secondary metabolite can also be studied using cell free extracts or crude enzyme systems, auxotrophic methods and plant tissue cultures.

**Major biogenetic pathways:**

The three major biogenetic routes to plant products are the acetate-malonate, acetate-mevalonate and shikimic acid pathways.

**Acetate-malonate pathway:**

The main products of this pathway are the fatty acids and polyketides. Often, these are collectively referred to as 'acetogenins'. Acetyl coenzyme A (1), the precursor of this pathway, undergoes carboxylation with carbon dioxide to produce malonyl coenzyme A (2) with a more reactive methylene group. This can then react with a second molecule of acetyl coenzyme A and then decarboxylate to form a four carbon chain, acetoacetyl coenzyme A (3). The latter can further react with another malonyl unit in a similar way to provide further linear extension of the polyketide chain which may then cyclise via two major routes, path 'a' and 'b', to yield two types of phenol derivatives, viz., the acylphlorogluccinols (4) and the orsellinic acids (5) (Scheme 4.2). Resorcinol (6) and salicylic acid (7) derivatives would be obtained if reduction of an uninvolved ketone occurred prior to cyclisation. In all probability, the polyketide chain being highly reactive would remain enzyme bound throughout its formation and subsequent cyclisation.
\[ \text{CH}_2\text{COSCoA} + \text{CO}_2 \rightarrow \text{HOOCCH}_2\text{COSCoA} \] (1)

\[ \text{CH}_3\text{CH}_2\text{CH}_2\text{COSCoA} \rightarrow \text{CH}_3\text{COCH}_2\text{COSCoA} \] (3)

\[ \text{CH}_3(\text{CH}_2)_{2n}\text{COOH} \text{ Fatty acid} \]

\[ \text{CH}_3\text{CO}(\text{CH}_2\text{CO})_n\text{CH}_2\text{COSCoA} \text{ Polyketide} \]

**Scheme 4.2**
Alternatively, acetyl CoA gives rise to the aliphatic amino acids, like ornithine and lysine via the tricarboxylic acid cycle.

**Acetate – mevalonate pathway:**

Terpenoids and steroids are biosynthesised by the acetate – mevalonate pathway. The biogenesis of these compounds follows the 'Isoprene Rule' according to which they are built up from isoprene units joined by a head to tail linkage. The primary precursor is mevalonic acid (9), which in turn is derived from acetyl CoA (1) through the intermediate formation of acetoacetyl CoA (3) and 3-hydroxy-3-methyl-glutaryl CoA (8) – (Scheme 4.3).

The main difference between the acetate – malonate and this pathway is the addition of the third acetate unit to acetoacetyl CoA. In the former pathway, as shown earlier, the linkage is in a linear fashion, while in the latter pathway, the third acetate molecule is added to the central carbonyl function to yield a branched six carbon unit, viz., 3-hydroxy-3-methyl glutaryl CoA (8). The subsequent reduction of (8) gives mevalonic acid (9) which serves as the precursor for various terpenoidal and steroidal molecules as shown in scheme 4.4.

**Shikimic acid pathway:**

Several important benzenoid compounds in plants are biosynthesised by shikimic acid pathway also. Indeed, it has been shown that a large number of aromatic compounds in higher plants are derived from phenylalanine, tyrosine and tryptophan which
Scheme 4.3

2 CH₃COSCoA ➞ CH₃COCH₂COSCoA
(1) ➞ CH₃COSCoA

\[
\text{HOOCCH₂CCH₂CH} + \overset{\text{NADPH + H}^+}{\longrightarrow} \text{HOOCCH₂CCH₂COSCoA}
\]
(8)

\[
\text{HOOCCH₂CCH₂CH₂OH} \rightarrow \text{HOOCCH₂CCH₂CH₂OH}
\]
(9)
Mevalonic acid
(9)

\[
\begin{align*}
&\text{Dimethylallyl pyrophosphate} \\
&\quad\downarrow \\
&\quad\text{Hemiterpenoids, } C_5 \\
&\text{Geranyl pyrophosphate} \quad\longrightarrow \quad \text{Monoterpenoids, } C_{10} \\
&\quad\downarrow \\
&\text{Farnesyl pyrophosphate} \quad\longrightarrow \quad \text{Sesquiterpenoids, } C_{15} \\
&\quad \downarrow \text{Squalene} \\
&\quad\downarrow \text{Steroids} \quad \text{Triterpenoids, } C_{30} \\
&\text{Geranylgeranyl pyrophosphate} \quad \longrightarrow \quad \text{Diterpenoids, } C_{20} \\
&\quad\downarrow \text{Polyisoprenes} \quad \text{Carotenoids, } C_{40}
\end{align*}
\]

SCHEME: 4.4
are the end products of this pathway.

Erythrose-4-phosphate (10) and phosphophenol pyruvate (11), which are the glycolytic products of glucose-6-phosphate, condense and cyclise to give shikimic acid (12) via quinic and dehydroquinic acids as shown in scheme 4.5. Another molecule of enol pyruvate adds on to shikimic acid-5-phosphate (13) to give chorismic acid (14). The latter acid is a key intermediate in the shikimic acid pathway. It gives rise to tryptophan (16) via anthranilic acid (15). On the other hand, it can rearrange to give prephenic acid (17). Phenylalanine (21) and tyrosine (20) are then obtained from prephenic acid (17) by independent pathways via phenyl - pyruvic acid (19) and its 4-hydroxyderivative (13) respectively (Scheme 4.5). It is interesting to note that the conversion of phenylalanine to tyrosine is rarely observed in dicot plants.

Phenylalanine is the precursor for cinnamic acid (22) and its derivatives like caffeic (23), ferulic (24) and sinapic (25) acids, the corresponding cinnamyl alcohols and benzoic acid derivatives (Scheme 4.6). These phenyl - propanes, generally denoted as C$_6$-C$_3$ compounds, are important intermediates in the biosynthesis of lignins, lignans, neolignans, flavolignans, coumarins, flavonoids and a few alkaloids.
SCHEME 4.5
SCHEME: 4.6
IV.2 BIOGENESIS OF TERPENES

The fundamental knowledge of terpene chemistry is due to the work of Wallach\(^2\) and Ruzicka\(^3\) which led to the proposal of "isoprene hypothesis". Their original idea is, however, not correct for it is not free isoprene which is the ultimate precursor of the terpenes. The search for the true isoprenyl precursor occupied a number of different research groups for many years and several C\(_5\) compounds have been proposed and subsequently discarded\(^4\). The problem was finally solved by groups associated with Bloch\(^5\) and Lynen\(^6\) who elucidated the structure of the precursor and its mode of biosynthesis. The search for a 'biological isoprene unit' met with success in the discovery of mevalonic acid\(^7\) which has now been recognised as the key metabolite in the terpenoid biosynthesis. It is also well established how mevalonic acid is formed in vivo and then transformed into precursors of various classes of terpenes\(^8,9\).

Initially two molecules of acetyl coenzyme A (26) condense to give acetoacetyl coenzyme A (27). The condensation of another molecule of (26) with (27) results in the formation of \(\beta\)-hydroxy-\(\beta\)-methylglutaryl coenzyme A (28) which is reduced virtually irreversibly to mevalonic acid (29) by nicotinamide-adenine dinucleotide phosphate (NADPH). The conversion of mevalonic acid into biological isoprene unit requires the presence of adenosine triphosphate (ATP). The initial steps
involved are the phosphorylation to give the monophosphate (30) and then the pyrophosphate (31) which probably forms the intermediate (32). The dehydration and decarboxylation of the latter gives isopentenyl pyrophosphate (33). Isopentenyl pyrophosphate (33) is then transformed \textit{in vivo} into its isomer, dimethylallyl pyrophosphate (34). Condensation of (33) with (34), eliminating a pyrophosphate grouping, results in the formation of geranyl pyrophosphate (35), the precursor for monoterpenes. Condensation of (35) with another molecule of isopentenyl pyrophosphate (33) in a similar way gives farnesyl pyrophosphate (36), the precursor for sesquiterpenes, which can further condense with another molecule of (33) to generate geranyl pyrophosphate (37), the precursor for diterpenes. Squalene, the precursor of the triterpenoids is formed by tail-to-tail reductive condensation of two molecules of farnesyl pyrophosphate (36) while the carotenoids are derived similarly from geranylgeranyl pyrophosphate. All these transformations are controlled by various enzymes. The above steps are schematically presented in scheme 4.7.

**Monoterpenes:**

These include acyclic, monocyclic, bicyclic and tricyclic types. A large percentage occurs in higher plants as hydrocarbons but alcohols, aldehydes, ketones, acid lactones, oxides, peroxides have also been found in nature.

The biogenesis of monoterpenes is described in scheme 4.8 and summarised below:
Scheme: 4.7. Biogenesis of Terpenoids.
SCHEME 4.8. BIOGENESIS OF MONOTERPENES.
i) Acyclic, e.g., Citronellal\(^{(38)}\), Geraniol (39), nerol (40), linalool\(^{(41)}\).

ii) Cyclic – Geranyl pyrophosphate can undergo cyclisation\(^{(12,13)}\) to give a variety of terpenes like limonene (42), \(\alpha\)-terpinene (43), \(\alpha\)-phellandrene (44).

iii) Non head-to-tail condensation. Artemesia ketone\(^{(14,15)}\) and chrysanthemum carboxylic acid\(^{(15)}\) are examples of irregular terpenes in which the isoprene units are not joined in head-to-tail fashion, unlike most of the terpenes.

iv) Cyclopentanoid, e.g. isoiridomyrmecin\(^{(16,17)}\).

**Sesquiterpenes:**

Sesquiterpenes occur mostly in higher plants and are rarely found in lower plants and animal kingdom (e.g., insects). More than four thousand terpenoidal compounds are known so far and sesquiterpenes numbering around 1200 represent the largest single class. The recent advances in sesquiterpene chemistry pertain not merely to their isolation and characterisation but also include a better understanding of the stereochemistry of the molecules, their reactivity and rearrangements and also their biogenesis, biosynthesis and synthesis.

Structurally, sesquiterpenes possess a cyclic, mono-, bi-, tri- and tetracyclic skeleton. The classification of sesquiterpenes based on Ruzicka's original biogenetic isoprene rule was initially due to Hendrickson\(^{17}\). This was later extended by
Parker et al.\textsuperscript{18} in 1967 in the comprehensive review of the biogenesis of sesquiterpenes. Consequently, the carbon skeleton of virtually all the sesquiterpenes could be derived by suitable cyclisation of cis-trans farnesyl pyrophosphate (48) and trans-trans farnesyl pyrophosphate (49). It is presumed that nero-lidyl pyrophosphate (50) could also serve as a crucial building block.

Removal of pyrophosphate group from (48) yields cations (51) and (52), and its removal from (49) the cation (53). These cations (51), (52) and (53) further cyclise to give two primary cations each. Thus the cations (54) and (55) are formed from (51), (56) and (57) from (52), and (58) and (59) from (53). The primary cations from (54-59) embody sesquiterpenoid structural types directly or by processes involving 1,2 or 1,3-hydride shifts, electronically and sterically controlled cyclisations with the two remaining double bonds (Markownikoff and anti-Markownikoff’s cyclisations, Wagner-Meerwein rearrangements) and 1,2-methyl shifts. Based on these, sesquiterpenes can be generally classified into groups such as farnesanes, bicyclofarnesols, bisabolenes, daucanes, cadinanes, humulanes and caryophyllanes, germacranes, eudesmanes, aremophilanes, aristolanes and aromadendranes etc. (Scheme 4.9). These classes have been extensively reviewed\textsuperscript{19-21}.

**Diterpenoids:**

Diterpenoids contain four isopentane units and can occur with a number of structural variations (Scheme 4.10). Monocyclic
SCHEME 49. BIOGENESIS OF SESQUITERPENES
SCHEME 4.10. PROMINENT MEMBERS OF THE DITERPENE (C_{20}) FAMILY.
forms are rare and this may have some biochemical significance. However, acyclic, bi-, tri-, tetra- and pentacyclic forms are known in large numbers.

Abietic acid (66) and all other diterpenes can hypothetically be derived from the C\textsubscript{20} regular, head to tail polyisoprenoid or geranylgeranyl pyrophosphate units (71). Geranyl linalool (72) isolated from Jasmine oil\textsuperscript{22} is an allylic form of (71). Phytol (60) and Vitamin A (73) are hydroderivatives of geranyl-geraniol\textsuperscript{23-24}. Cyclisation of geranyl-geraniol through three isoprene units, folded as two potential chair cyclohexane rings results in the formation of a molecule which by subsequent oxidation of two substituents yields cataric acid\textsuperscript{25} (74)-(76) (Scheme 4.11). Ionization of terminal nucleophile through a manool skeleton gives rise to pimarane skeleton of tricarboxylic diterpenes such as pimaradiene\textsuperscript{26,27} (79). A potential relationship between diterpenes with the pimarane and those with an abietane skeleton has been given by Ruzicka\textsuperscript{12} (Scheme 4.12).

An interesting feature of a majority of the presently known cyclic diterpenes is the absence of C-3 hydroxyl group and presence of the conventional $5\alpha,10\beta$ configuration of ring A/B as in steroids and triterpenes. Diterpenoid cassaine\textsuperscript{28} is an exception with C-3 hydroxyl group. The generation of furan ring in cafestol (69) and kahweol (70) has been visualized as resulting from a wagner-Meerwein rearrangement of hydroxylated precursors (Scheme 4.13).
Scheme 4.12. Ionic mechanism in the biogenesis of diterpenes.

Scheme 4.13. Biosynthesis of furan ring of kahweol.
The origin of diterpenoid nucleus has received its greatest support from experiments from two mold products. Following the feeding of 2-$^{14}$C-mevalonic acid lactone to Trichothecium roseum L., Birch and Arigoni independently demonstrated that the diterpene rosenonolactone (91) isolated contained the $^{14}$C pattern (Scheme 4.14). The biogenesis presumably proceeds through the hypothetical precursor (87) composed of four isoprenoid units. Rosenonolactone (91) itself does not obey the classical isoprene rule in that it cannot be divided into four regular isopentane units. Both the workers detected the presence of radioactivity in the C-15 methyl group of ring A and its absence in the carbon from the lactone ring. This finding indicated that the C-15 was derived specifically from the C-2 of mevalonic acid. The other mold product which has proved to be of great value in biogenetic studies is gibberellic acid. Geranylgeranyl pyrophosphate formed from mevalonic acid cyclises to a bicyclic intermediate (92) then to the tricyclic diterpenoid (93). Gibberellic acid then arises by formation of ring D from the side chain of (93), contraction of ring B with extrusion of C-7 as the carboxyl group, followed by the loss of angular methyl group and hydroxylation and formation of the lactone group on ring A (Scheme 4.15).

An interesting variation in diterpenoidal forms occurring in nature is well illustrated by the structural formula for pleuromutilin (96) (Scheme 4.16).
SCHEME 4.14. BIOGENESIS OF ROSENOLACTONE FROM 2-\textsuperscript{14}C-MEVALONIC ACID LACTONE.
SCHEME 4.15. BIOSYNTHESIS OF GIBBERELLIN.
SCHEME 4.16. BIOSYNTHESIS OF PLEUROMUTILIN.
Diterpenoid quinones:

These quinones are simply oxidised terpenes. The details about the biogenesis of these quinones is not known. Tymoquinone is the simplest example and the formation of thymol from mevalonate has been confirmed in *Orthodon japonicum* (Labiatae). So far helicobasidin, a fungal metabolite from *Helicobasidium mompa* is the only terpenoid quinone whose origin from mevalonate has been established. Several pathways from farnesyl pyrophosphate to helicobasidin are consistent with the observed labelling pattern.

Bisabolene hypothesis:

The involvement of γ-bisabolene (97), cuparenene (98), cuparene (99) and deoxyhelicobasidin (100) was suggested by Natori et al., without taking into account certain stereochemical consequences. The simplest cyclisation of trans-cis-farnesyl pyrophosphate (101) yields trans bisabolene (102) and similarly cis-cis-farnesyl pyrophosphate yields cis bisabolene (103). Moreover, by further initiation of well recognised rearrangements, both bisabolenes may be derived from either isomer of farnesyl pyrophosphate. If the further intermediates did not allow a randomization of the labelled position in the six membered ring, mevalonate-2-$^{14}$C would give rise to one of the labelled helicobasidins (104) or (105) or to a mixture of both. However, the postulated intermediate, cuparene would lead to randomization between the positions arbitrarily numbered in (104) and (105) as 1 and 3 and between 4 and 5. Another possible labelling pattern in helicobasidin is that represented in (106) (Scheme 4.17).
Farnesyl pyrophosphate $\rightarrow$ (97) $\rightarrow$ (98)

$\rightarrow$ (99) $\rightarrow$ (100) $\rightarrow$ (101) $\rightarrow$ (102) or (103)

$\rightarrow$ (98) $\rightarrow$ (99) $\rightarrow$ (106)

(104) (105)

$\bullet = ^{14}C$

$\times/2 = \text{half the sp. activity of } \bullet$

SCHEME 4.17
IV.3 PRESENT WORK

The tricyclic diterpenoid quinones isolated in the present study have quinonoid 'C' ring. This feature is biogenetically very interesting. It is a well known fact that normal diterpenoids arise from four units of mevalonate, and aromatic rings of other secondary metabolites originate either from acetate or the aromatic amino acids, namely, phenylalanine or tyrosine. The quinonoid moiety of other quinones have been biosynthesized from tyrosine also. Therefore, diterpenoid quinones would either originate from mevalonate or from tyrosine. Consequently, if mevalonic acid-2-^{14}C is incorporated into diterpenoid quinones then the radioactivity is expected to occur at one of the methyls of gem dimethyl, isopropyl methyl, at C (1) and C (7) carbon atoms. Similarly, if L-tyrosine-U-^{14}C is incorporated then the activity should be found in quinonoid moiety only.

The biosynthesis of 7α-acetoxyroyleanone in Salvia moorcraftiana plants was selected for the present study as it was found to be the major compound present when experiments were carried out in the month of June.

Isolation of 7α-acetoxyroyleanone-^{14}C:

To investigate the proposed biogenetic scheme 4.18, mevalonic acid-2-^{14}C (0.5 mci, sp. activity 1.12 x 10^9 dpm/mM) and L-tyrosine-U-^{14}C (0.1 mci, sp. activity 261 mciv/mM) were administered separately to two sets of three to four month old flowering plants by wick technique. During the administration of
SCHEME 4.18. PROPOSED BIOGENESIS AND DEGRADATION OF 7α-ACETOXYROYLEANONE.
L-tyrosine-$^{14}$C, carrier was used resulting in dilution of the tracer. The plants were harvested after 8 days. Following the method similar to the isolation of inactive 7α-acetoxyroyleanone (Ch. II, p. 92), the radiolabelled compound was isolated, purified and assayed till constant activity from each set of plants was obtained. It was observed that only one of these two precursors viz., mevalonic acid-2-$^{14}$C was incorporated into 7α-acetoxyroyleanone. The yield of the compound, activity and its percentage incorporation is given in table 4.1.

Table 4.1. Percentage incorporation of mevalonic acid-2-$^{14}$C and the activity of undiluted 7α-acetoxyroyleanone isolated.

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Yield (mg)</th>
<th>Activity (dpm/mg)</th>
<th>dpm/mM</th>
<th>% incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mevalonic acid-2-$^{14}$C</td>
<td>60</td>
<td>217</td>
<td>$0.81 \times 10^5$</td>
<td>.001</td>
</tr>
</tbody>
</table>

To locate the position of radioactivity in the labelled 7α-acetoxyroyleanone, degradative studies were carried out on this compound. The degradations were first standardised with inactive 7α-acetoxyroyleanone. The products obtained were characterised by spectroscopic methods or by comparison with the naturally isolated compounds. The same degradative methods were then applied to the radiolabelled compound.
The compound 7α-acetoxyroyleanone on alkaline hydrolysis yielded 7α-hydroxyroyleanone (108) which on dehydration with p-toluenesulphonic acid in p-xylene afforded 6,7-dehydroroyleanone (109). Oxidation of the latter compound with alkaline KMnO₄ furnished a mixture of acids which on methylation with ethereal diazomethane yielded a mixture of esters. However, the yield of degraded products even in case of inactive sample was very low thus making the task of isolating the desired acid (11C) from the labelled 7α-acetoxyroyleanone extremely difficult. Therefore, only the first two steps of degradation of the radiolabelled compound were carried out. The activity found in these two steps of reactions was found to be almost constant. This was sufficient to establish that mevalonic acid is the overall precursor for compounds having quinonoid 'C' ring.

It may be mentioned here that 7α-acetoxyroyleanone could be isolated as the major product from S. moorcraftiana collected in the month of June. However, from plants collected in May, the major compound isolated was 6,7-dehydroroyleanone. It appears that the latter is the intermediate from which 7-acetoxy derivative is formed via 6,7-epoxide, 7α-hydroxy compound and followed by its acetylation, which appears to be the last step in the biosynthesis. Thus the low % incorporation in 7α-acetoxyroyleanone is accountable. It is possible that if the biosynthetic experiments had been carried out in the month of May, then 6,7-dehydroroyleanone would have contained higher amount of radioactivity.
Chemicals: The following chemicals were used in the present study: sodium sulphate (anhydrous, AR, Sarabhai), sodium hydroxide (AR, Sarabhai), potassium permanganate (AR, E. Merck), sodium bisulphite (LR, BDH), sodium bicarbonate (LR, BDH), phosphoric acid (LR, BDH), hydrochloric acid (LR, BDH), BBOT (Hewlett - Packard), and p-toluenesulphonic acid (LR, BDH).

Radioactive chemicals: Mevalonic acid-2-$^{14}$C (0.50 mci, 209.4 mg, sp. activity $1.12 \times 10^9$ dpm/mM) and L-tyrosine-U-$^{14}$C (0.1 mci, 0.07 mg, sp. activity 261 mci/mM) were obtained from Isotope Division, Bhabha Atomic Research Centre, Trombay, Bombay-85.

Plant material: Fresh plants growing in Shankaracharya hills of the Kashmir Valley were used for the isolation of diterpenoid quinones. For the biosynthetic studies, these plants were selected from the same region of the valley. Three to four-months old plants were selected in the month of June for these experiments.

Methods:

Chromatography methods: Column and thin layer chromatography were performed using silica gel adsorbent as per the procedure described earlier (Chapter II, p. 34).

Physical methods: The instruments used for recording melting points, UV, IR, NMR, Mass spectra have been specified earlier (Chapter II, p. 30).
Counting of radioactive samples: Radioactive samples were counted on Packard Tri-Carb Liquid Scintillation Spectrometer. The scintillation solution was prepared by dissolving BBOT: 2,5-bis(2-(5-tertbutyl benzoxazoyl) thiophene (4 g), in toluene (AR). Mevalonic acid-2-$^{14}$C was added as an internal standard to correct for quenching. The sample to be counted was dissolved in the minimum amount of a suitable solvent (benzene or methanol, AR) and to this 10 ml of the scintillation solution was added.

Isolation of 7α-acetoxyroyleanone: 7α-Acetoxyroyleanone was isolated from Salvia moorcraftiana plants as per the procedure already described in Chapter IIA, p.

Administration of mevalonic acid-2-$^{14}$C and isolation of $^{14}$C-acetoxyroyleanone: Mevalonic acid-2-$^{14}$C (0.5 mci, 209.4 mg, sp. activity 1.12 x $10^9$ dpm/mM) was dissolved in distilled water (6 ml) and then administered to six flowering plants. A thread was inserted into the stem of each plant (at about one third height), the free ends of which were dipped in 1 ml of the precursor solution contained in a 5 ml beaker tied near the plant on a wooden support. The solution was absorbed by the plant through capillary action within a period of 6-8 hrs. After total take up of the solution each beaker was carefully rinsed with more of distilled water (0.5 - 1 ml) and the washings were allowed to be absorbed. The process was repeated twice to ensure maximum administration of the precursor. The plants were harvested after
8 days and worked up for isolation of radiolabelled 7-α-acetoxyroyleanone by the usual method.

The plant material (200 g, dry weight) was extracted with petroleum ether (60-80°) from which a concentrate (5.1 g) was obtained. This on chromatography using silica gel gave 7α-acetoxyroyleanone (62 mg). It was purified by repeated crystallisation (methanol, m.p. 212°) to constant activity (60 mg, sp. activity 0.81 x 10^5 dpm/mM).

Administration of L-tyrosine-U-14C: L-Tyrosine-U-14C in 0.01N HCl solution (0.1 mci, 0.07 mg, sp. activity 261 mci/mM) was diluted with the carrier compound (10 mg) and was made up to 3 ml with 0.01N HCl. The precursor solution was administered to three plants by 'wick technique' as described above. The harvested plants (110 g, dry weight) yielded pet. ether concentrate (3.5 g). This on column chromatography (silica gel) gave 7α-acetoxyroyleanone (42 mg). The repeated crystallization of the crude compound yielded pure 7α-acetoxyroyleanone (40 mg, m.p. 212°). The isolated 7α-acetoxyroyleanone when assayed for radioactivity was found to be totally inactive.

Degradation of 7α-acetoxyroyleanone: The degradative studies were carried out first with cold 7α-acetoxyroyleanone and then with the radiolabelled compound.

I. Conversion of 7α-acetoxyroyleanone into 7α-hydroxyroyleanone:

To the boiling solution of 7α-acetoxyroyleanone (53 mg) in ethanol (3.5 ml) was added 0.10N NaOH (8 ml). The resulting
magenta coloured solution was refluxed gently for two hours, cooled on an ice bath and acidified with 15% phosphoric acid. The solid that separated out was extracted with chloroform and dried over anhydrous sodium sulphate. After usual work up, the crude 7-acetoxyroyleanone was purified by preparative TLC over silica gel, followed by two crystallisations from aqueous methanol (20 mg, m.p. 172°). It was found to be identical with the isolated natural sample from this plant in all respects (UV, IR, NMR, MS, TLC, Co-TLC).

II. Dehydration of 7α-hydroxyroyleanone to 6,7-dehydoroyleanone:

A mixture of 7α-hydroxyroyleanone (20 mg), xylene (dry 1 ml) and p-toluenesulphonic acid (0.5 mg) was refluxed for 3.5 hrs. The reaction mixture was then concentrated under reduced pressure and purified by preparative TLC followed by repeated crystallisation from methanol to yield pure red rectangular prisms of 6,7-dehydoroyleanone (14 mg, m.p. 171°). Spectral data (UV, IR, MS) was found to be in agreement with natural 6,7-dehydoroyleanone isolated from this plant (Chapter II, p. 95).

III. Alkaline potassium permanganate oxidation of 6,7-dehydoroyleanone:

Aqueous potassium permanganate solution (1%) was added to a solution of 6,7-dehydoroyleanone (14 mg) in 2% aq. NaOH (2 ml) at such a rate that an excess of potassium permanganate solution was maintained in the reaction mixture. The temperature was maintained at 40-45° initially for 2 hrs and then at 65-70° for 1 hr. The solution was then cooled to room temperature and made acid to congo red with dilute HCl. The precipitated MnO₂ was decomposed
with sodium bisulphite. The volume of the solution was reduced to 1 ml and then treated with a saturated solution of sodium bicarbonate. This was then extracted with ether (4 x 5 ml) in order to remove non-acidic impurities. The aqueous phase was then acidified with dil. HCl. The precipitated acid was extracted with ether (4 x 5 ml). The combined organic extracts were washed once with brine and then dried over anhydrous sodium sulphate. Removal of ether yielded a mixture of acids (2.5 mg).

IV. Esterification of acid mixture:

The mixture (2.5 mg) was dissolved in ether and esterified by treating with an excess of ethereal diazomethane. Removal of the solvent yielded a mixture of esters in extremely low amounts and thus could not be separated either by prep. TLC or prep. GLC.

2. Wallach, O., Annalen 239, 1 (1887).


30. Arigoni, D., ibid, p. 231.


