THE BIOSYNTHESIS OF VERBENALIN

By

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CHAPTER I

INTRODUCTION

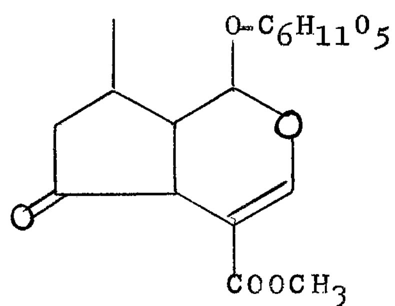
Certain species of Verbena, commonly known as the vervains, which were later found to contain verbenalin(1), have a long history of use as a folk remedy.[1] Ivy poisoning and erysipelas were treated with the root of certain species of Verbena combined with white oak bark. Aborigines used the plant itself as a treatment for ivy poisoning. The Cherokee Indians used the plant for leucorrhea, and also used it as a mild diuretic and emmenagogue. The Meswaki Indians of the Menomini reservation had special names for certain species of Verbena; the white vervain was called "pasankwe yak," "pison kive ak" and "pasankwe uk," meaning "fine hair bloom," "like hair" and "like fine hair," respectively. Indeed, in 1936, Alfred Wagner reported eighteen recipes for various perfumes using Verbena extracts as the active ingredient in each.[2]

In this decade, the pharmacological actions of Verbena officinalis L. extracts have been studied.[3] On the conjunctivitis of rabbits caused by the application of mustard oil, the antiphlogistic action of the alcoholic extracts was more marked than that of the aqueous extracts. Decreasing body weight, or anorexia, was noticed only with

the use of the aqueous extracts. The water soluble portion of the alcoholic extracts had a longer analgesic action than the water insoluble portion of the alcoholic extracts.

Verbenalin(1) which had been isolated from *Verbena officinalis* L. and other species of *Verbena*, is one of the relatively rare methylcyclopentane monoterpenoid glucosides found in plants. Verbenalin(1) is of interest because it appears to be biogenetically related to other members of the group including asperuloside(2),[4] aucubin(3),[5] catalposide(4),[6] genipin(5),[7] loganin(6),[8] monotropein(7)[9] and plumieride(8).[10] Other related methylcyclopentane monoterpenoids are nepetalactone(9),[11] iridomyrmecin(10),[12,13] anisomorphal(11)[14] and a series of related alkaloids of which actinidine(12),[15] skytanthine(13)[16] and tecomamine(14)[17] are typical examples. The glucosides listed in the first group appear to have a direct biogenetic relationship to verbenalin(1). The monoterpenoid alkaloids, of course, appear to have a less direct biogenetic relationship.

Neither 1, nor the plants from which it can be isolated, have any presently important physiological activity, although the weed has been widely used as a folk remedy. This is in direct contrast to many of the other known methylcyclopentane monoterpenoids, most of which possess important biological activity.[18] Nepetalactone(9), the active ingredient of oil of catnip, produced by certain plants of the mint family, has always been a source of



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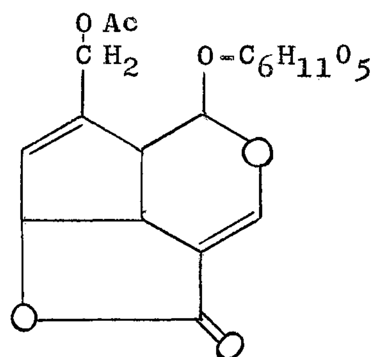

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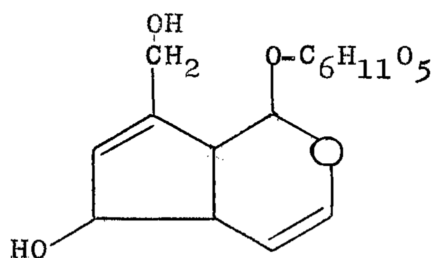

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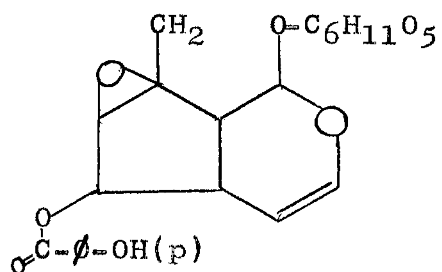

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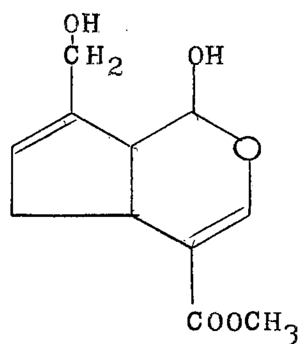

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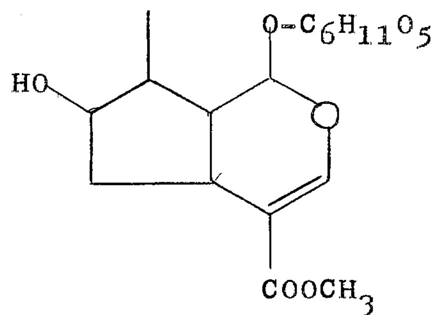

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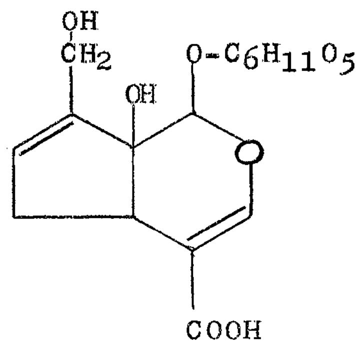
Figure 1. Methylcyclopentane Monoterpenoids



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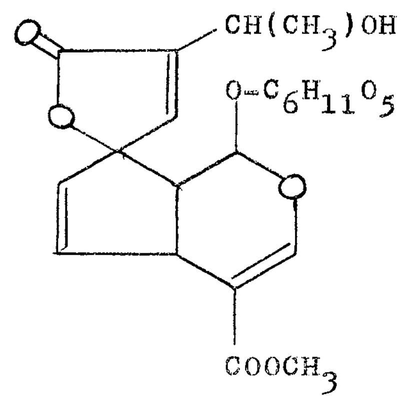

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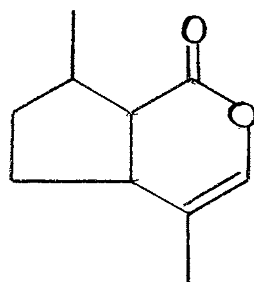

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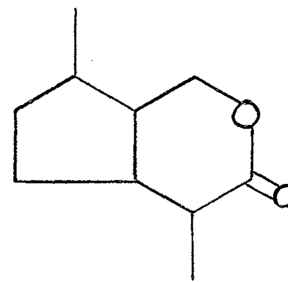

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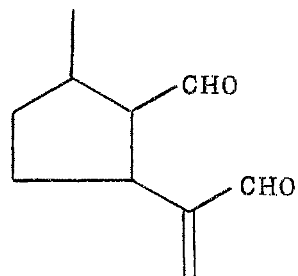

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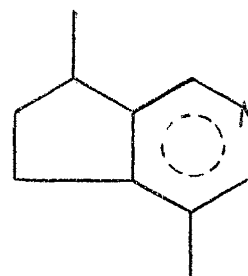

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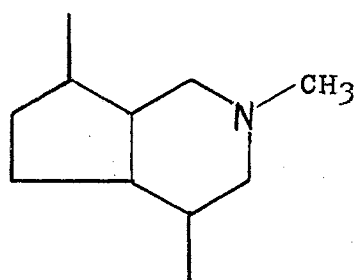


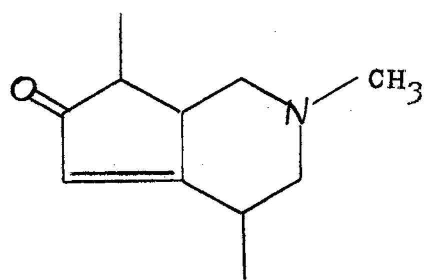


Figure 1. (Continued)

interest because of its ability to attract and excite felines. It has been recently suggested that the function of nepetalactone(9) is to protect the plants that produce it against phytophagous insects.[19] Anisomorphal(11), an ingredient of a defensive spray ejected at predators from the large Southern walking-stick, *Anisomorpha buprestoides*, has been shown to be strongly repellent to ants, beetles, spiders and humans; when inhaled by humans, the terpene dialdehyde is lachrymogenous, and is painfully irritating.[20] The secretion has been reported to cause serious injury to the eyes. Iridomyrmecin(10), an iridolactone isolated in relatively large amounts from a wide range of ants, has considerable insecticidal activity.[21] It is believed to have primarily a defensive role; it may be an alarm secretion which serves as an attractant to members of its own species, and at higher concentrations, it may repel the foe and even stimulate them to exhibit retreat behavior.

The biosynthesis of 1 is important since it may shed some light upon the biosynthesis of other related monoterpenes and because it may serve as a precursor to the non-indole nucleus of the indole alkaloids.[22] Studies have established the mevalonoid nature of a number of indole alkaloids such as vindoline,[23] reserpinine[24] and catharanthine,[25] and certain monoterpenoids have been suggested as important intermediates in the biosynthesis of these substances.[26,27,28] The methylcyclopentane monoterpenoid alkaloids actinidine(12), having a pyridine ring, and

skytanthine(13), having the same carbon skeleton as 12, but with a reduced pyridine ring, are apparently constructed from two isoprene units.[29] The biological precursor of the isoprene unit is mevalonic acid; mevalonic acid has been found to be a good precursor of skytanthine(13) in *Skytanthus acutus* M.[30]

The isoprenoid skeleton of the aglucone portion of 1 suggests that it could arise from an isoprenoid precursor. Several possible precursors were evaluated in this study. Acetate ion should be on the same biogenetic pathway as the isoprenoid precursor, but many steps away from 1. Mevalonic acid would probably be a more efficient precursor for the aglucone moiety of 1 than acetate ion. Geraniol should then be a more efficient precursor than mevalonic acid. Incorporation studies and subsequent degradation of carbon-14 labeled 1 should supply information about the later steps in its biosynthesis.

Verbenalin(1) concentration in plant material, as well as mevalonic acid incorporation in *Verbena officinalis* L. was studied as a function of age of plants. The radioactivity appearing in the sugar moiety of this glucosidic monoterpenoid was examined. This study led to the development of a highly efficient technique for the isolation, identification and purification of milligram amounts of 1.

This study was also undertaken to determine the effect of the age of plants upon the possible biogenetic pathways, using mevalonic acid-2-^{14}C as a precursor. The results of

this study will be compared to and contrasted with other current work in this field.

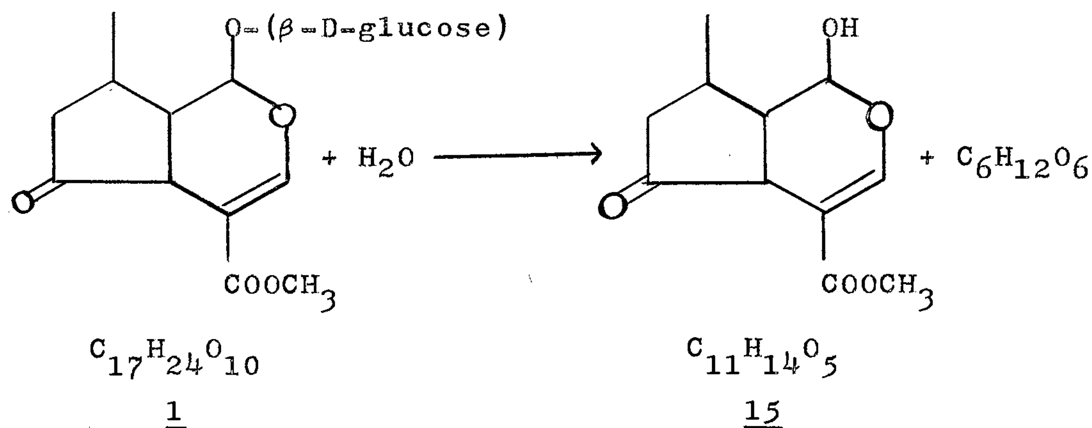
CHAPTER II

THE CHEMICAL HISTORY OF VERBENALIN

The glucoside verbenalin(1), $C_{17}H_{24}O_{10}$, was first isolated from the weed Verbena officinalis L. by L. Bourdier and reported in 1908.[31,32] He subjected the fresh plants to the then usual methods of extraction of glucosides and obtained some crude glucoside which he called verbenalin(1). After purification of the crude glucoside by recrystallization from ethanol and then from ethyl acetate, he established the empirical formula of 1 as $C_{17}H_{25}O_{10}$. The anhydrous, colorless, odorless, crystalline compound having a bitter taste was hydrolyzed with both emulsin and hot dilute sulfuric acid to glucose and an unknown aglucone possessing phenolic properties. Crystalline precipitates were formed after treating the glucoside with phenylhydrazine and hydroxylamine. Verbenalin(1) was found not to be toxic to guinea pigs, but its therapeutic possibilities were not rigorously studied at that time.

Much of the work which followed consisted of sometimes crude attempts to determine the structure of the glucoside. Early investigators confirmed 1 to be a β-D-glucoside, and Reichert, in 1935,[33] reported the empirical formula as $C_{17}H_{24}O_{10}$(believed to be $C_{17}H_{25}O_{10}$ for three decades). He

reported some physical constants, the presence of a methoxyl group in the aglucone verbenalol(15), derived by the hydrolysis of the glucosidic bond in 1.[34]

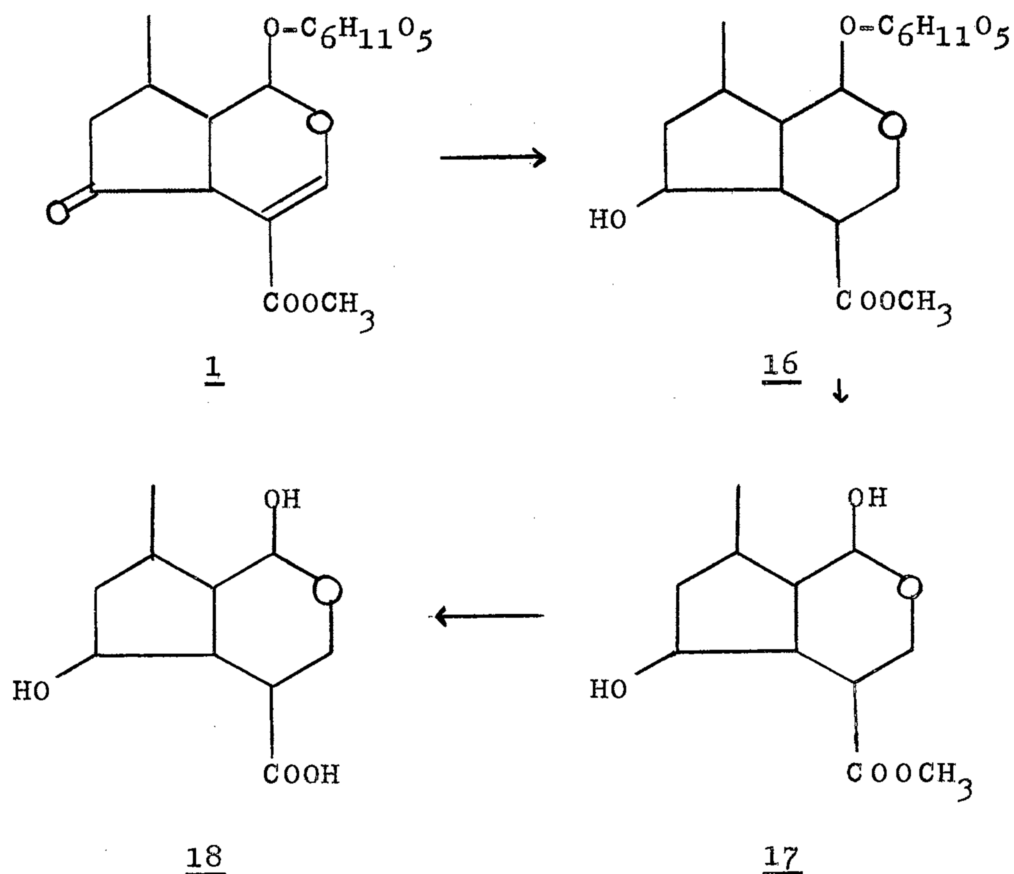


Verbenalin(1) was later isolated from Verbena stricta, and was shown to be identical with the glucoside cornin isolated from Cornus florida L.(common dogwood). In the same decade, Cheymol published a series of studies of the glucoside 1, observing it in other vervains: Verbena hastata, Verbena teucrioides, Verbena littoralis, Verbena supina and Verbena prostrata.[35,36,37,38] The glucosidic content of different parts of these plants was also studied. Many other plants were studied and reported not to contain any glucosides resembling 1. The plants studied were the genetically related species such as Cornus mas L., Cornus sanguinea L., Verbena bonariensis, Verbena hispida and Verbena venosa.

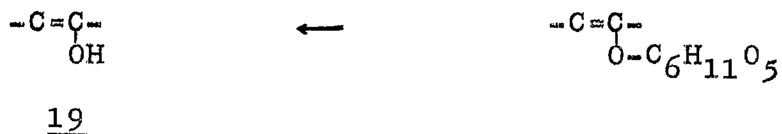
The chemical studies of Cheymol were thought to show that 1 contains a methoxyl group, as determined by Zeisel's method (calculated for $C_{16}H_{21}O_9$-OCH_3: 7.98, calculated for one methoxyl group: found 8.82).[39] Verbenalin(1) was

believed to contain a lactone function because of the progressive hydrolysis by an inorganic alkali. The presence of a double bond was confirmed by the discoloration of solutions of both bromine and potassium permanganate. The presence of a ketone function was confirmed also by the preparation of standard carbonyl derivatives, and the presence of the glucosidic linkage was confirmed by the hydrolysis of 1 to form glucose.

Hydrogenolysis of 1 produced tetrahydroverbenalin(16), $C_{17}H_{28}O_{10}$. Further hydrolysis of 16 produced tetrahydroverbenalol(17), $C_{11}H_{18}O_5$, and the addition of alkali to 17 and subsequent acidification of the product produced verbenalic acid(18).[39]



Cheymol disputed the earlier suggestions that the aglucone was bound to the glucose through a phenolic hydroxyl group; he proposed that the aglucone was apparently linked with the glucose in 1 through an enolic hydroxyl group as shown in structure 19.[39]



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Karrer and Salomon,[40] in 1946, as others had before them, repeated some previous work and tried several new reactions. Verbenalin(1) was extracted from the plants by taking a total ethanolic extract of the dried plant material, concentrating the extract under reduced pressure, and then removing the resulting solid green deposits from the syrup. The remaining green syrup was extracted with several portions of hot ethyl acetate. The combined ethyl acetate extracts, upon standing, on occasion, gradually deposited crystalline 1. Seeding of the combined extracts was often necessary to obtain the crystalline glucoside 1. The methoxyl group values obtained were high, yielding 8.88% instead of the 7.98% required for one methoxyl group.

After hydrolysis of the glucoside by emulsin, Karrer and Salomon[40] thought that they had obtained, as others had previously reported, the aglucone 15. Verbenalol(15) was found to behave very strangely, quite unlike the results described earlier by Cheymol.[38] The melting point was found to vary when the aglucone was recrystallized from

ethyl ether and other solvents. The melting point of 15 often depended upon the number of recrystallizations performed; the melting point usually dropped after several successive recrystallizations. Verbenalol(15) was found to be extremely sensitive to most chemical reagents which Karrer and Salomon used. Karrer and Salomon were unsuccessful in testing for any ketone and hydroxyl groups which were present in the aglucone 15; only brown smears were obtained when the aglucone reacted with phenylhydrazine or hydroxylamine. These researchers found it difficult to prove the presence of a double bond even though they observed a pale yellow color when 15 was treated with tetranitromethane.

Several attempts to hydrogenate the glucoside 1 using platinum catalysts were described as unsuccessful.[40] Verbenalin(1) was hydrogenated with platinized Raney nickel catalysts at room temperature, like the earlier hydrogenations attempted by Cheymol,[38] to yield a mixture of products. Tetrahydroverbenalin(16) was finally produced in about 25% yield by the hydrogenation of the glucoside 1 with platinized Raney nickel catalyst at 100° and 15 atmospheres pressure. Verbenalol(15) was likewise hydrogenated with Raney nickel catalyst at 75° and 15 atmospheres pressure in 50% yield to produce norverbanol(20). Very little information was obtained in this attempt to determine the structure of 20; mixtures evidentally formed during this hydrogenation, resulting in the production of oils. The

analytical data which was obtained about the composition of 20 proved to be unsatisfactory.

OH

COOCH$_3$

15

HO

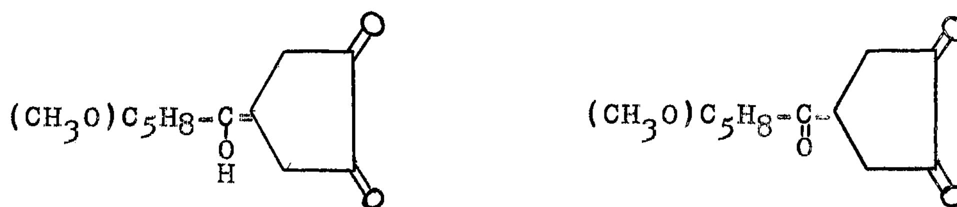
CH$_2$OH

20

Verbenalin(1) was isolated from an American species of vervain, Verbena stricta, by a new and somewhat simplified extraction procedure. This procedure consisted of extracting the dried and powdered plant material with ethyl ether in a Soxhlet extraction apparatus for several weeks at ether reflux temperatures.[41] Chatterjee and Parks then observed many of the same properties of the glucoside which had been previously reported. Verbenalin(1) failed to yield carbonyl derivatives. Verbenalin(1) absorbed one mole of bromine readily, but liberated it slowly upon standing, which they believed to be due to the presence of a labile enolic group. Crystalline tetrahydroverbenalin(16), $C_{17}H_{28}O_{10}$, was prepared from the glucoside 1 by the absorption of two moles of hydrogen in the presence of Adams' catalyst; this is in contrast to the work of Karrer and Salomon.[40] The products obtained by both groups of workers appeared to have the same properties, although the melting points differed by more than 20°. Tetrahydroverbenalol(17),

$C_{11}H_{18}O_5$, was produced by the emulsin hydrolysis of tetrahydroverbenalin(16), but the melting point of 17 was also considerably higher than the melting point of the tetrahydroverbenalol(17) reported earlier by Karrer and Salomon. The hydrogenated aglucone, 17, was found to be unreactive toward standard carbonyl reagents; it did not react with periodic acid, excluding the presence of a vicinal diol group.

Verbenalol(15) was produced by the hydrolysis of the glucoside 1,[41] but its optical rotation differed from that reported previously by both Karrer and Salomon,[40] and Cheymol.[37] The presence of an enolic hydroxyl group in the aglucone was affirmed since the aglucone absorbed and then slowly released one mole of bromine; the aglucone reacted with diazomethane, and the product was then not colored by treatment with ferric chloride. Verbenalol(15), unlike the results obtained with the glucoside 1, yielded a di-2,4-dinitrophenylhydrazone, indicating that 15 contained two carbonyl groups. Finding the aglucone to give a positive iodoform test, to reduce Fehling's solution, and contrary to Karrer and Salomon's[40] observation, to give a deep violet colored solution when treated with ferric chloride, Chatterjee and Parks suggested structure 21 for verbenalol(15), which they hoped would explain their experimental data. Glucose could then be linked to the enolic hydroxyl group in 21 to provide the glucoside 1.



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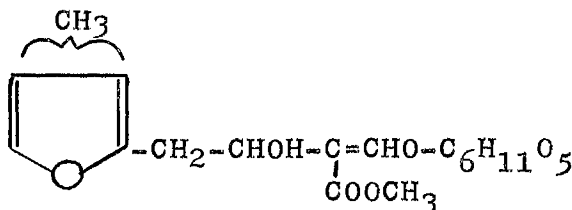
An enol lactone was ruled out as a structural constituent since the iodoform test was positive for the glucoside 1. Further work, to clarify the structure of 1, and to confirm their proposed structure 21, was reported to be in progress.

Several other possible structures for verbenalin(1) were proposed by other groups of researchers. Asano et al. proposed a furan-ring containing structure, 22, on the basis of their chemical studies.[42]



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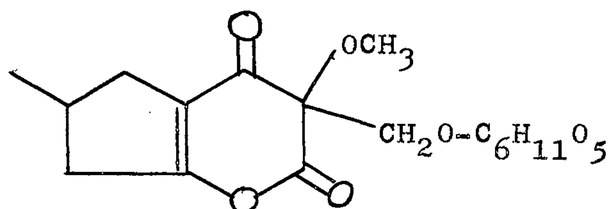
Cohn, Vis and Karrer proposed yet another structure for 1 in 1954 on the basis of some infrared and ultraviolet spectral studies.[43] Structure 23 was formulated since a δ-lactone ring was believed to be responsible for the characteristic absorption band at 1739 cm^{-1} in the aglucone. The absorption band at 1678 cm^{-1} was characteristic of an α,β-unsaturated ketone. A carbon to carbon double bond in conjugation with a carbonyl group was attributed to the absorption band at 1639 cm^{-1}. Along with the fact that the

absorption band at 3450 cm^{-1} probably was due to a hydroxyl group, the presence of other absorption bands led them to believe that the two carbonyl groups were attached to the six-membered ring in structure 23. Infrared spectra in nujol of four derivatives of the glucoside 1 and its aglucone 15 seemed to support their proposed structure.



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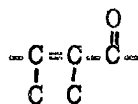
Verbenalin(1) and its aglucone were found to have absorption maxima in the ultraviolet region between 236 and 241 m .[43] These absorption maxima, on the basis of earlier ultraviolet spectral studies of conjugated systems reported by Woodward,[44] were attributed to the chromophore in structure 24, which is part of the structure for the glucoside 23.



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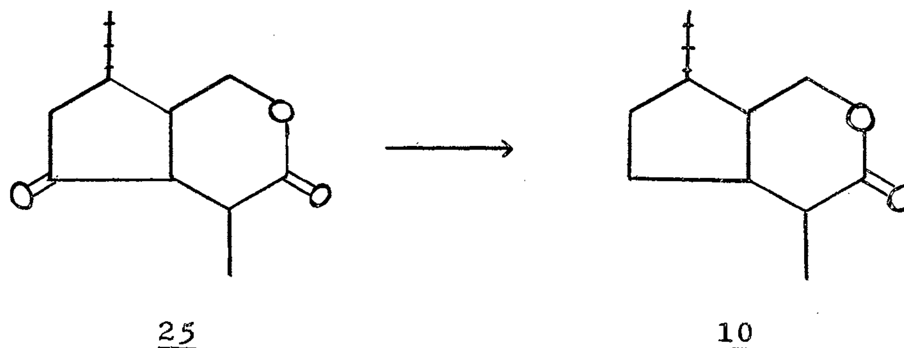
In 1961, Winde et al.[45] studied different methods for the isolation of the glucoside 1, and commented upon Karrer and coworker's earlier proposed structure. By utilizing paper chromatography, Winde et al. showed that the novel

glucoside called verbenin, isolated through the ether extraction of Verbena officinalis L. by Kuwajima in 1939, was not a new compound. Winde et al. experimentally showed that verbenin represented only a crystalline modification of the glucoside 1; verbenin was verbenalin(1). The isolation of several other substances were studied along with their ultraviolet spectra.

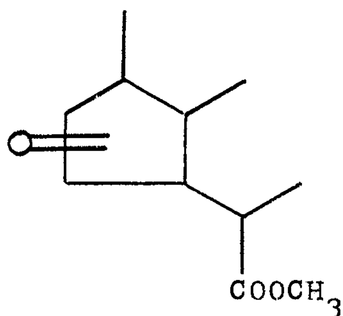
Since none of the previously proposed structures fit all of the available experimental data, Buchi and Manning, in 1960, attempted a new approach.[46,47] Assuming that verbenalin(1), the iridomyrmecins, and the nepetalactones contain a common methylcyclopentane monoterpenoid carbon skeleton, they transformed desoxyverbanone(25) to iridomyrmecin(10) through the Raney nickel catalyst desulfurization of the ethylene thioketal derivative of 25. Desoxyverbanone(25) was formed by the chromium trioxide oxidation in pyridine solution of desoxyverbanol, which can be obtained by the catalytic reduction of 1 over platinized Raney nickel catalyst.



25 10

Verbenalin(1) was shown to contain a methyl ester group through the hydrolysis of 1 with barium hydroxide and

esterification of the product to 1 by treatment with diazomethane. Since 1 contains one carboxyl function, Buchi and Manning suggested part structure 26.[46,47]



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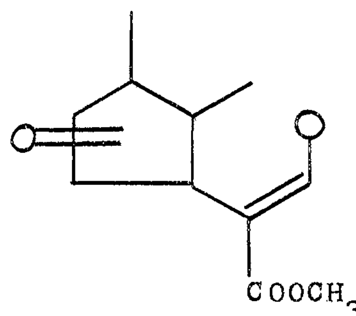

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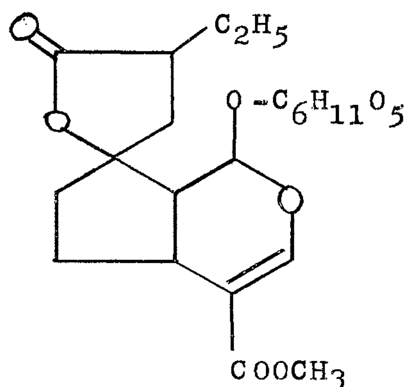
Relating the ultraviolet spectrum of 1 to that of tetrahydrodesoxyplumieride(28) and of bakankosin(29), they expanded their part structure to 27.



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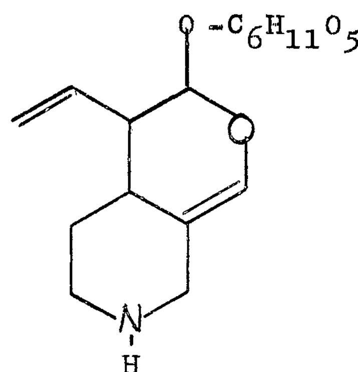

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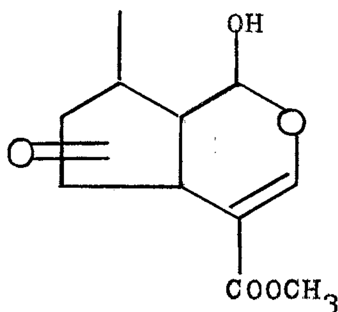
The ultraviolet spectra maxima of 1, 28 and 29 are λ_{max}^{EtOH} 238 mμ (ε 9,600), λ_{max}^{EtOH} 236 mμ (ε 10,000) and λ_{max}^{EtOH} 236 mμ (ε 11,600), respectively.[46,47]

Since verbenalol(15) exhibited positive Fehling and Tollens tests, and having additional ultraviolet spectral data, Buchi and Manning proposed carbon atom one for the

position of the hydroxyl group, giving rise to part structure 30. The structure required to explain the chemical properties of 15 was clarified by the ultraviolet spectrum which showed λ_{max}^{EtOH} 271 mμ (ε 19,000) in 0.01 N sodium hydroxide solution. This bathochromic shift was similar to that observed in ethyl acetoacetate and dihydrobakankogenin. The remaining ketone function was placed at carbon atom five of the cyclopentane ring because the ultraviolet spectrum was typical for planar α,β-unsaturated ketones, and thus they completed the structure of the aglucone 15. Since 1 can be hydrolyzed by emulsin to 15, the structure of verbenalin can be represented by 1.[46,47]



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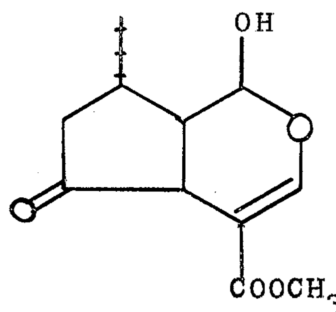
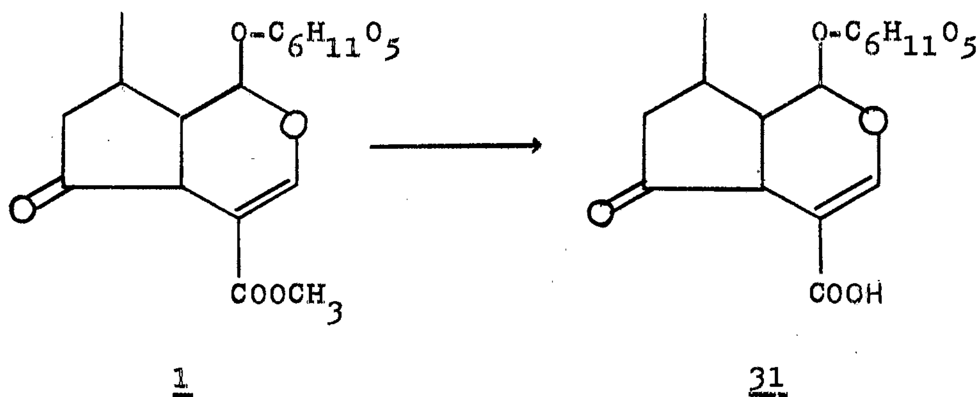


15

The stereochemistry of 1 was determined by relating the configuration of desoxyverbanone(25) to (+)-iridomyrmecin(10); therefore, verbenalin(1) has the configuration shown in structure 15. Reduction of desoxyverbanone(25) to desoxyverbanol with sodium borohydride established the configuration of desoxyverbanol with the exception of carbon atom five. Since catalytic reduction of 1 on Raney nickel catalyst produced desoxyverbanol as well as tetrahydro-

verbenalin(16), the stereochemistry of the carbon atom seven methyl group, and the carbon atom 4a and carbon atom 7a hydrogens in 1 are the same as in (+)-iridomyrmecin(10). Verbenalin(1) was hydrolyzed with barium hydroxide to verbenalinic acid(31), and it was then concluded that the natural product 1 contains the thermodynamically most stable cis-fused ring system.[46,47]



Buchi and Manning believed it unlikely that the configurations were inverted at both carbon atom 4a and carbon atom 7a during their catalytic reductions. Molecular rotation difference tentatively established an α configuration at carbon atom one, when rotational differences between tetrahydroverbenalin(16) and its aglucone, tetrahydroverbenalol(17), were compared with hexahydrodesoxyplumieride and its corresponding aglucone.[46,47]

CHAPTER III

THE BIOGENESIS OF GERANYL PYROPHOSPHATE

Geranyl pyrophosphate(45) is believed to have a pivotal role in the biosynthesis of the methylcyclopentane monoterpenoids and other isoprenoid compounds. Since mevalonic acid(38), which forms the biological isoprene unit, is on the same biogenetic pathway as 45, the formation of 45 from acetate ion through 38 will be described. These three compounds were tested as possible precursors to 1.

The generation of mevalonic acid(38) from acetyl coenzyme A(32) and malonyl coenzyme A(33) is believed to occur by the route shown in Figure 2. Mevalonic acid(38) plays an important role in the biosynthesis sequence and it serves only in the biochemical production of isoprenoid compounds. The intermediates in this sequence are capable of many interconversions; the formation of mevalonate is believed to be essentially an irreversible process. Acetyl coenzyme A(32) and malonyl coenzyme A(33) have been converted, with an enzyme isolated from pigeon liver by Porter et al.,[48] to mevalonic acid(38); the trapping experiments proved that the free coenzyme A ester of hydroxymethylglutaric acid(36) and free acetoacetyl coenzyme A are not obligatory intermediates in the synthesis of mevalonic

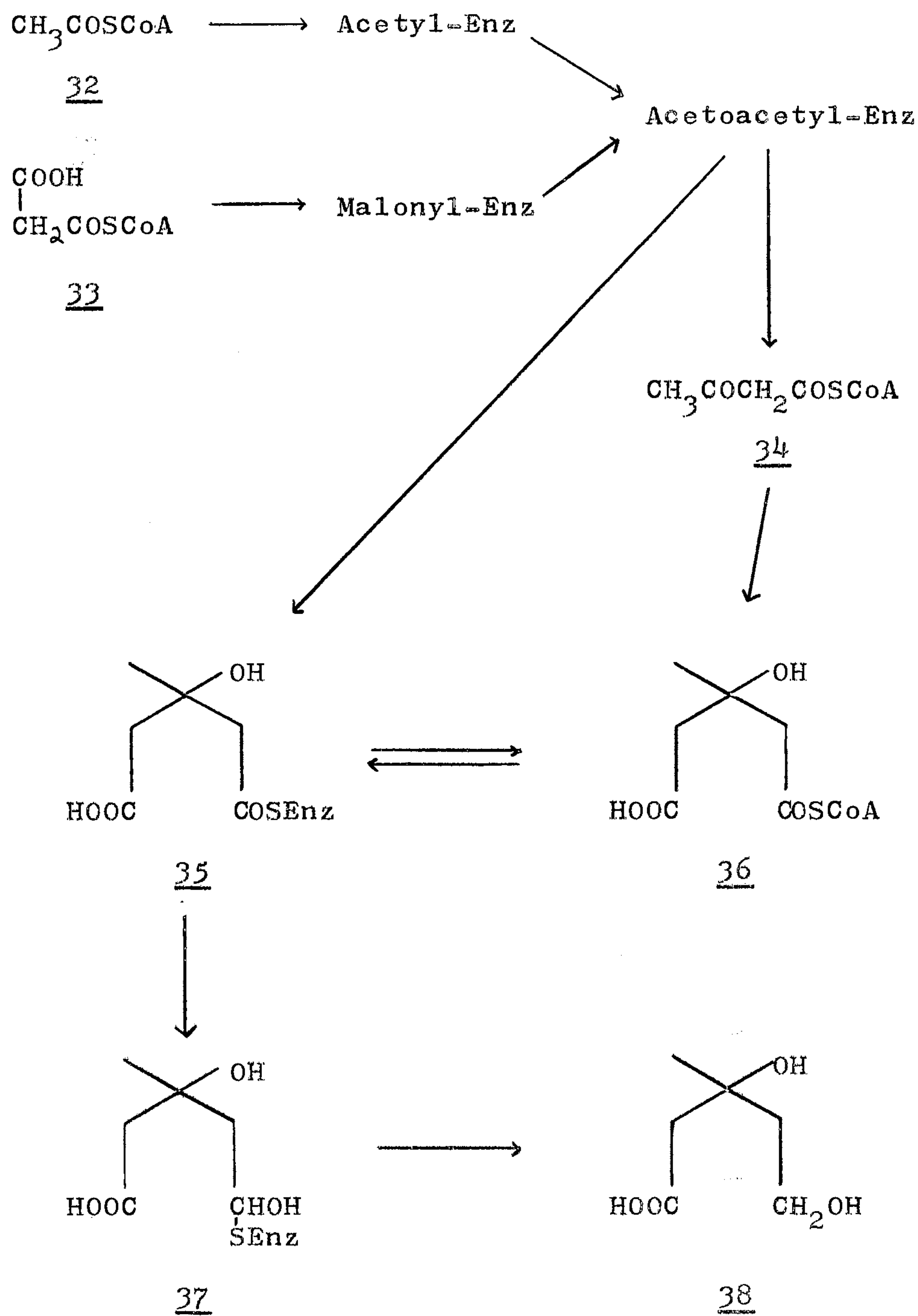


Figure 2. Pathway for the Biosynthesis of Mevalonic Acid

acid(38). The results indicated that the acetoacetyl-enzyme and 2-hydroxy-2-methylglutaryl-enzyme(35) were intermediates in the new pathway.[49] The evidence indicated that the acetate and malonate moieties of acetyl and malonyl coenzyme A were bound to enzyme protein through thioester bonds; the intermediates were bound to the enzyme throughout the conversion.[50] The data were consistent with the proposal that the acetoacetyl-enzyme acts as a key intermediate in the biosynthesis of both cholesterol and isoprenoid compounds, and in the biosynthesis of the fatty acids.

The generation of geranyl pyrophosphate(45) from mevalonic acid(38) through isopentenyl pyrophosphate(42) is believed to occur by the route shown in Figure 3. Mevalonic acid(38) is converted through three successive phosphorylations, phosphorylating the tertiary hydroxyl group after the terminal hydroxyl function is activated as the pyrophosphate ester, and the concerted decarboxylation and loss of the phosphate group of the resulting molecule to form isopentenyl phosphate(42). These phosphorylations are catalyzed by mevalonate kinase, mevalonate-5-phosphate kinase, and mevalonate-5-pyrophosphate kinase, respectively. Geranyl pyrophosphate(45), the ten carbon intermediate formed by the condensation of isopentenyl pyrophosphate(42), and dimethylallyl pyrophosphate(43), may be the precursor of the methylcyclopentane monoterpenoids.

Although on purely structural grounds there may be

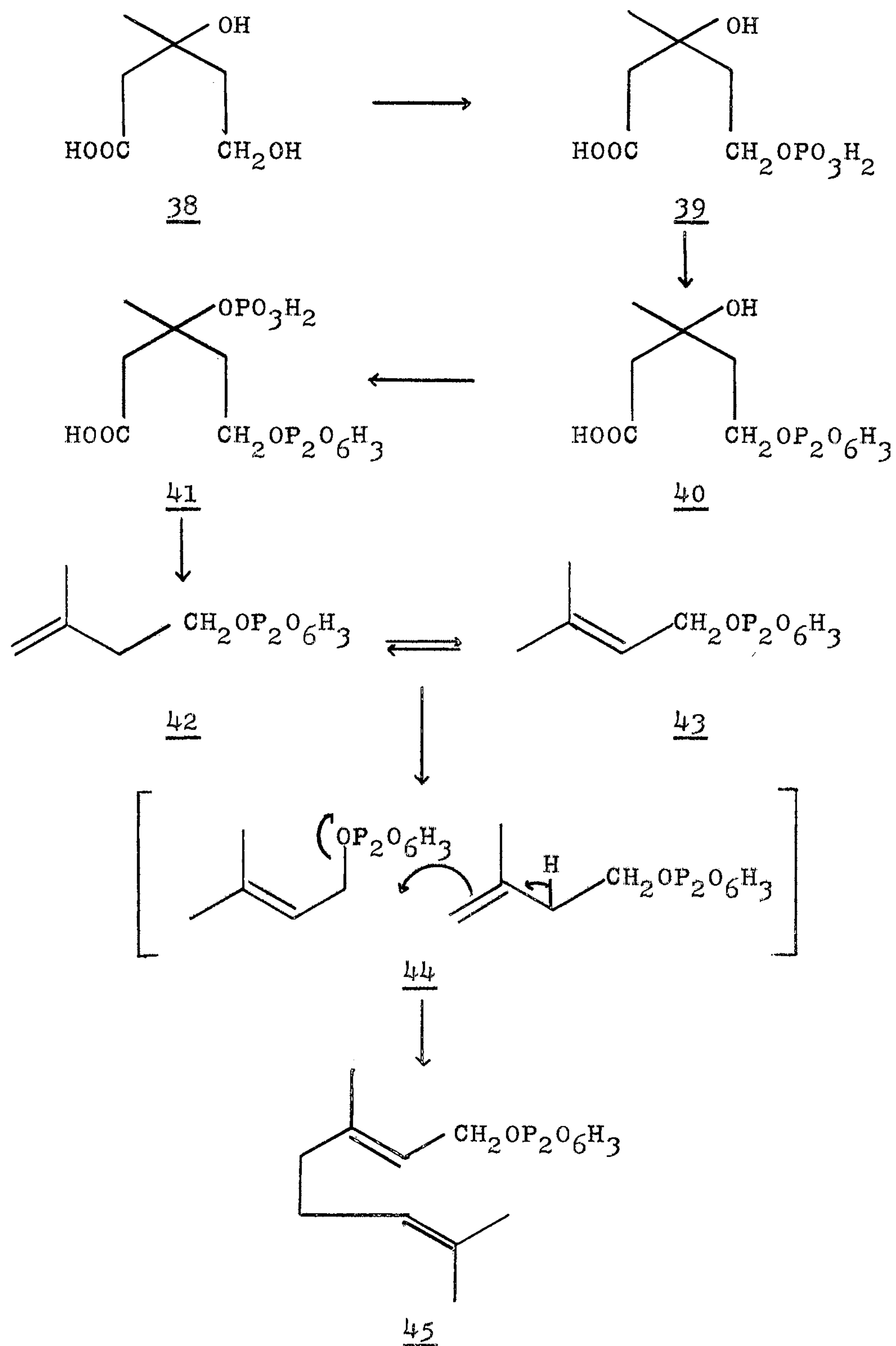


Figure 3. Pathway for the Biosynthesis of Geranyl Pyrophosphate from Mevalonic Acid

several methods by which mevalonate units may be linked together to form terpenes, recent work has made it possible to define in detail the intermediate steps between mevalonate and the biological isoprene unit, isopentenyl pyrophosphate(42). The first in this sequence of reactions is the phosphorylation of mevalonate with adenosine triphosphate catalyzed by mevalonate kinase. The formation of 39, the phosphorylated derivative of mevalonic acid(38), was first reported by Tchen[51] using yeast extract preparations. Mevalonate kinase was partially purified from an extract of yeast and the presence of a divalent metal was shown to be necessary for the enzyme activity.[52] The enzyme was found to be stable and active over a wide pH range. The activity was found to be inhibited by p-chloromercuribenzoate(83% inhibition), indicating the necessity of an SH group; this inhibition was reversed by the addition of reduced glutathione. The presence of mevalonate kinase in this transformation has been demonstrated. Mevalonate kinase has been partially purified from rabbit liver[53] and from hog liver;[54] cell-free preparations of rat testis homogenates[55] have recently been used for the conversion of 38 to 39.

Mevalonate-5-pyrophosphate(40) has been formed by the further incubation of mevalonate. The presence of mevalonate-5-phosphate kinase has been shown to be vital in this second phosphorylation; also required are adenosine triphosphate and divalent metal ions. The presence of this enzyme has been observed and its purification has been

accomplished using baker's yeast[56] and rat liver[57] preparations as the source of the enzyme. Adenosine diphosphate was an additional product detected in this reaction.

The formation of isopentenyl pyrophosphate(42) from mevalonate pyrophosphate(40) proceeds by consuming adenosine triphosphate while producing carbon dioxide and stoichiometric amounts of adenosine diphosphate and inorganic phosphate.[58] It was confirmed that the tertiary hydroxyl group of mevalonate-5-pyrophosphate(40) does react with adenosine triphosphate during the decarboxylation reaction to form isopentenyl pyrophosphate(42).[59] The tertiary hydroxyl function is thus activated for expulsion as its phosphate ester as shown in structure 41, although attempts to isolate the phosphorylated intermediate were unsuccessful.

Lynen et al. found that the isopentenyl pyrophosphate (42) must first isomerize to produce an allylic pyrophosphate in the form of dimethylallyl pyrophosphate(43), before polymerization to yield geranyl pyrophosphate(45) and then higher isoprenoid intermediates.[60,61] They postulated a special role for the enzyme isopentenyl pyrophosphate isomerase which catalyzes this isomerization; the sulfhydryl group of the HS-enzyme may take part in the formation of an enzyme substrate complex. The intermediate, therefore, would be bound to a sulfhydryl group of the enzyme protein. This enzyme apparently contained a free sulfhydryl group since its action was inhibited by both

p-chloromercuribenzoate and iodoacetamide. The partial purification of isopentenyl pyrophosphate isomerase from pig liver has been achieved, and the reversibility of the isomerase reaction was established.[62] An enzyme bound compound was also found; its isolation was in agreement with the proposals developed by earlier work. A mechanism of isomerization by isopentenyl pyrophosphate isomerase was also presented which involved the stereospecific addition or removal of a proton at carbon atom two of isopentenyl pyrophosphate(42).

Geranyl pyrophosphate(45) can then be formed by the condensation, with the elimination of a pyrophosphate ion and a proton, of isopentenyl pyrophosphate(42) and dimethylallyl pyrophosphate(43).[63] The reaction taking place during this condensation may be stereospecific with respect to the elimination of a proton from carbon atom two of isopentenyl pyrophosphate(42).

The methyl groups in the dimethylallyl pyrophosphate (43) thus seem to retain their identity by being either cis or trans with respect to the double bond. The methylene group of isopentenyl pyrophosphate(42) becomes the trans methyl group in dimethylallyl pyrophosphate(43). This phenomenon will be discussed in relation to the results obtained in the studies of the biosynthesis of 1.

It has been recently shown that the stereochemistry of the hydrogen elimination from carbon atom four of the original mevalonate is stereospecific.[64,65,66] During the

synthesis of farnesyl pyrophosphate, the eliminations of three protons from carbon atom two of unstable intermediates resulting in the production of the allylic bonds in dimethylallyl pyrophosphate(43), geranyl pyrophosphate(45) and farnesyl pyrophosphate were presumably stereospecific since the resulting double bonds in farnesyl pyrophosphate were found to have the *trans* configuration.[64]

CHAPTER IV

RESULTS AND DISCUSSION

For the biosynthesis studies performed on verbenalin (1), the plant *Verbena officinalis* L. seemed to be an excellent source of material. Part of the original interest in the glucoside stemmed from the fact that the prolific plant, *Verbena officinalis* L., showed promise in supplying adequate quantities of plant material which could be grown readily. In the early months of work, considerable difficulty was experienced in obtaining viable seeds to be used for the biosynthesis studies. If the seeds of *Verbena officinalis* L. were not fresh, or were not cold-treated during storage, "damping off" occurred frequently during germination. After these initial problems were solved by obtaining viable seeds, a good supply of healthy plants were produced which were rigorously identified before, and at flowering.[67]

Verbenalin(1), a β-D-glucosidic methylcyclopentane monoterpenoid, was obtained by the large-scale ethyl ether extraction of dried, powdered *Verbena officinalis* L. in a Soxhlet extraction apparatus. Authentic samples of 1 were obtained as described and the physical properties of the resulting glucoside agreed well with the documented prop-

erties.[31-47] The scale of these extractions was necessarily large; several kg of plant material was used. The yields were somewhat poor; less than 0.1% of 1 was obtained. Even at ethyl ether reflux temperatures, the glucoside caromelized in the reaction flask; caromelization was noticed after only several days of extraction. This would, of course, reduce the yields of glucoside extracted.

Preliminary studies centered around developing carefully designed isolation procedures on a micro-scale so that practical results could eventually be obtained on a micro quantity of radioactive material and thus perhaps lessen the need for high dilutions. Micro-scale isolation techniques were developed which allow the purification of as little as 0.1 mg of glucoside, from a *Verbena officinalis* L. plant less than three cm tall having a dried weight of less than 0.1 g. The total dried plant material was extracted with portions of hot ethanol; the extract was then purified by preparative thin layer chromatography using silica gel, and different developing solvents until the verbenalin(1) was pure. In particular, the absorption maxima of 1, with its high extinction coefficient in the ultraviolet region, was useful in developing a micro-assay of the glucoside isolated from the plant material. Attempts to perform gas liquid chromatographic analyses of the trimethylsilyl derivative of 1 were not successful.

Verbenalin(1) concentration in freshly dried *Verbena officinalis* L. plants was determined by the above described

ultraviolet assay. The plants varied from one to four and one-half months in age and the analytical results are shown in Table I. It was observed that the concentration of 1 increases from 0.12% to 0.24% as the age of plants increases from one to four and one-half months. This increase in concentration of 1 may coincide with the onset of flowering of the plants which occurred when the plants were four and one-half months of age. In the lower portion of the table, the concentration of 1 was significantly higher for plants harvested in the fall, for both flowering and non-flowering plants. Again, the concentration of 1 was higher in the flowering plants than in the non-flowering plants. The formation of the glucoside appears to be dependent upon both the age and maturity of the plant, and upon the season of the year. This variation may be related to the purpose or the need of 1 by the plant, if such exists.

Because of the availability of live samples of several varieties of *Verbena hybrida*, the glucosidic content of these varieties was examined. Freshly dried samples of Pride, Sutton's Blue, Pink Giant, Lavender, Royal Purple and Sparkle, all of which are *Verbena hybrida*, were extracted with ethyl ether in a large Soxhlet extraction apparatus in an attempt to isolate the glucoside 1. Verbenalin(1) was found not to be present in these plants with this isolation technique. Similar studies with *Cornus mas* L., related to the common dogwood, failed to yield 1.

TABLE I

CONCENTRATION OF VERBENALIN IN DRIED *VERBENA OFFICINALIS* L. PLANTS

Age in Months	No. of Plants	Planting Date	Harvesting Date	Percentage Verbenalin
1	10	January	February	0.12
2.5	5	November	February	0.13
4	4	October	February	0.15
4.5[a]	2	November	March	0.24
2	6	August	October	0.18
4[a]	5	June	October	0.30

[a]Flowering Plants

Freshly dried samples of Pride, Sutton's Blue, Pink Giant, Lavender, Royal Purple and Sparkle were exhaustively extracted with ethanol; it was shown that this ethanolic extract did not contain any 1. Freshly dried samples of Pride, Sutton's Blue, Pink Giant and Royal Purple were also extracted by column percolation with ethyl ether over the plant material. An attempt was made to isolate 1 from the ethyl ether extracts and 1 was not found. It can be concluded that 1 was not formed in sufficient concentration to be found by the large-scale extractive techniques in the varieties of *Verbena hybrida* and the *Cornus mas* L. which were studied.

The structure of the aglucone moiety of the glucoside 1 provides some insight into the chain of events that probably occurred in nature. The structure of the methylcyclopentane compounds previously discussed suggests that the carbon skeleton of the molecules, which can actually be dissected into discrete isoprene units, may be derived from known isoprenoid precursors.

Ruzicka became interested in the biosynthesis of the terpenes in 1920; the important question to be answered then was whether the terpenes were composed of isoprene units.[68] For the compounds which were examined, the answer was positive; the hypothesis grew into what is now known as the "isoprene rule." The "isoprene rule" was a useful tool in the structure elucidation of terpenes; it remains a useful tool in the studies of the biosynthesis of

terpenoids. The "isoprene rule," and thus the generation and utilization of the biological isoprene unit, still provides a base for many theoretical discussions dealing with the biosynthesis of terpenoids. It provides an effective base from which to choose possible precursors in the biosynthesis of 1.

Many proposals have been presented regarding the biogenesis of monoterpenes, but at the outset of this study very little was known about the biosynthesis of the methylcyclopentane monoterpenoids. The biosynthesis pathways for the formation of the methylcyclopentane monoterpenoids and the other isoprenoid compounds must necessarily be branched after the formation of geranyl pyrophosphate(45). Proposals have been offered for the biosynthesis of methylcyclopentane monoterpenoids, but most of these were based upon the biosynthesis of structures with related carbon skeletons. The many proposals do provide the researcher with a working model as a base for new work. This study was undertaken to aid in the elucidation of the biosynthesis pathway used in the formation of 1 by *Verbena officinalis* L. plants.

Verbena officinalis L. plants were examined in an attempt to detect the presence of possible precursors of 1. The fresh plant material was steam distilled and the ethyl ether extracts of the steam distillate were examined by gas liquid chromatography. Hydrocarbons or oxygenated compounds in the five to ten carbon range which might be

possible precursors to 1 were not observed. The fresh plant material was also extracted with ethyl ether. The ethyl ether extracts did not contain compounds which might provoke further interest as precursors. Evidence of compounds such as geraniol, citral and iridodial were not found with either method of analysis.

Geraniol and citral were thought to be intermediates in Sir Robert Robinson's proposed scheme for the biosynthesis of methylcyclopentane monoterpenoids;[69] his proposal was based on related monoterpenoids which occur in nature. Methylheptenone, a ketone found with iridodial in various ants, can be obtained by the reverse aldol reaction of citral.[70] Citral can then be reduced to form L-citronellal; the terminal oxidation of L-citronellal can then yield 2,6-dimethyloct-2-en-1,8-dial. Cyclization of this acyclic precursor, 2,6-dimethyloct-2-en-1,8-dial, would then yield iridodial, which may occupy a key position in the biogenesis of the methylcyclopentane monoterpenoids.[69] The cyclization of 2,6-dimethyloct-2-en-1,8-dial by acetic acid was actually accomplished by Robinson, but this chemical cyclization may not occur in the living plant so the significance of this result is questionable. The oxidation of the lactol tautomer of iridodial might then yield nepetalactone(9), although the chemical oxidation with manganese dioxide in light petroleum produced nepetalinic acid; any nepetalactone(9) which formed was not detected.[71] A Cannizzaro type reaction on iridodial may yield the irido-

lactones, and the reaction of ammonia, or its biological equivalent, on iridodial may produce actinidine(12).

Another route to the methylcyclopentane monoterpenoids must likewise be considered. Citral was cyclized by Cookson utilizing ultraviolet radiation to 2-isopropenyl-5-methylcyclopentane-carboxaldehyde, which contains the same carbon skeleton as the other monoterpenes discussed;[72] this cyclization is illustrated in Figure 4. Although the presence of these possible precursors was not found in Verbena officinalis L., it cannot be concluded from this evidence alone that they do not act as intermediates, present only in minute quantities, in the biosynthesis of 1.

In order to learn more about the biosynthesis of 1, several possible precursors, each labeled with carbon-14, were tested. The carbon-14 labeled precursor was fed to Verbena officinalis L. plants; the plants were then harvested. By taking an exhaustive ethanolic extract and repeatedly purifying the crude extract of 1 by preparative thin layer chromatography, it was possible to isolate the glucoside 1. The concentration of 1 was assayed by virtue of its absorption in the ultraviolet region. The radiochemical purity of 1 was verified. By cleavage of the glucoside 1 with emulsin, it was possible to determine the specific activity of 15.

When plants four months of age were fed 4.9 μc of acetate-1-^{14}C, 0.004% of the total radioactivity injected

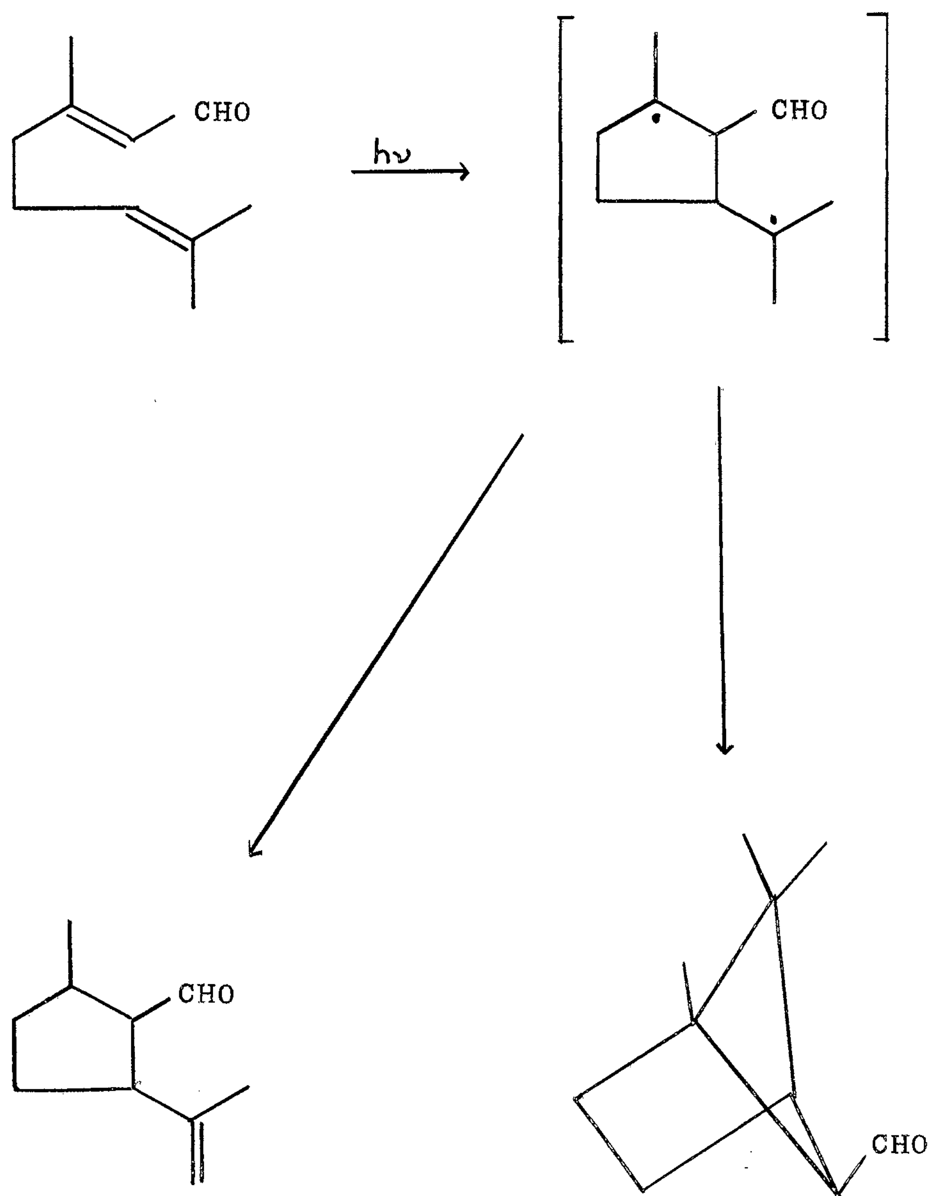


Figure 4. The Photochemical Cyclization of Citral

into the plant was found in the aglucone portion of 1. Verbenalol(15) was found to have a specific activity of 0.055 mμc/mg. The ratio of the activity of the glucose portion of 1 to the activity of the aglucone was four. When plants four months of age were fed 3.6 μc of acetate-2-^{14}C, 0.007% of the total radioactivity injected into the plant was found in the aglucone portion of 1. Verbenalol (15) was found to have a specific activity of 0.066 mμc/mg. The ratio of the activity of the glucose portion of 1 to the activity of the aglucone was five. These results are summarized in Table II.

The percentage incorporation of labeled acetate into the aglucone portion of the molecule is low; the percentage incorporation of labeled acetate into the sugar moiety is somewhat higher. Since the biosynthesis of the sugar and methylcyclopentane monoterpenoid moieties show different amounts of acetate-^{14}C incorporation, their biosynthesis may be independent. These data would suggest that the glucoside 1 is formed by the reaction of the aglucone, or a related compound, with glucose, which was formed via the tricarboxylic acid cycle and glycolysis.

Verbenalin(1) has been shown to incorporate radioactivity from mevalonate-2-^{14}C. The results in this thesis[73] were recently confirmed by Schmid et al.[74] who also succeeded in showing that Verbena officinalis L. specifically incorporates labeled mevalonate-2-^{14}C in the formation of the aglucone moiety of 1.

TABLE II

INCORPORATION OF RADIOACTIVITY INTO VERBENALIN FROM CARBON-14 LABELED PRECURSOR

Isotope	Percentage Radioactivity Incorporated into Aglucone	Specific Activity of Aglucone mμc/mg	Ratio $\frac{\text{Glucose-}^{14}\text{C}}{\text{Aglucone-}^{14}\text{C}}$	Age of Plants in Months	Duration of Experiment in Days
Acetate-1-^{14}C Injected - 4.9 μc Specific Activity - 12.0 mc/mM	0.004	0.055	4	4	8
Acetate-2-^{14}C Injected - 3.6 μc Specific Activity - 35.9 mc/mM	0.007	0.066	5	4	8
Mevalonate-2-^{14}C	0.15	2.0	2	mean of many trials of assorted plants	5-8
Geraniol-1-^{14}C Injected - 7.5 μc Specific Activity - 0.33 mc/mM	0.009	0.17	0.002	4.5	5

The mean of many feeding experiments showing the incorporation of mevalonate-$2^{14}C$ into 1 is shown in Table II. Individual feeding experiments are shown in Table III. Of the total mevalonate-2-^{14}C radioactivity administered to the plant, 0.10%, 0.11%, 0.06% and 0.30% was incorporated into the aglucone portion of the molecule, resulting in specific activities of 4.1, 1.5, 0.80 and 1.6 $m\mu c$/mg verbenalol(15), respectively. One to four times as much radioactivity appeared in the sugar portion of the molecule. The sugar moiety did incorporate radioactivity from mevalonate-2-^{14}C, although the ratio of the activity of the glucose moiety to that of the aglucone moiety was somewhat less than with carbon-14 labeled acetate. The degradation of mevalonate-2-^{14}C to simpler compounds is likely; the simpler compounds can then be reincorporated, leading to the labeled glucose. The percentage incorporation of mevalonate-2-^{14}C in the terpene portion of the molecule is higher than the percentage incorporation of the carbon-14 labeled acetate. This suggests that the aglucone moiety may be formed by a terpene biosynthesis pathway. It should be noted that the percentage incorporation of mevalonate-2-^{14}C is the highest, being 0.30%, in the case of flowering plants.

The methods used in administering the labeled precursors to _Verbena officinalis_ L. plants can influence the rate of uptake of the compound. For ease in injecting the radioisotopes, the total volume of solution administered

TABLE III

INCORPORATION OF MEVALONATE-2-^{14}C INTO VERBENALIN

Radioactive Precursor	Percentage Radioactivity Appearing in Aglucone	Specific Activity of Aglucone mμc/mg	Ratio $\frac{Glucose\text{-}^{14}C}{Aglucone\text{-}^{14}C}$	Age of Plants in Months	Duration of Experiment in Days
Injected - 41 μc Specific Activity 6.27 μc/mg	0.10	4.1	1.5	1	8
Injected - 0.32 μc Specific Activity 6.27 μc/mg	0.11	1.5	2	Assorted plants 1/3 - 3	5
Injected - 1.6 μc Specific Activity 6.27 μc/mg	0.06	0.80	4	3	6
Injected - 0.19 μc Specific Activity 6.27 μc/mg	0.30[a]	1.6	1	2 1/2	5
	0.15	2.0	2	Mean of trials	5-8

[a]Flowering plants

was kept small. Usually, no more than ten μl of solution could be administered to each mature plant per feeding. Several repeated administrations of μl injections seemed to be optimum. When the N,N'-dibenzylethylenediamine salt of mevalonic acid-2-^{14}C was injected into the base of the plant from which the one or several stems protruded, about four hours were required for the translocation of the radioactivity to the growing tips located on the upper portion of the plant. When the radioisotope was injected into the stems near the midsection of the plant (where the stems begin branching), about three hours were required for the translocation of the radioactivity to the growing tips.

Verbenalin(1) has been shown, in this study, to incorporate radioactivity from geraniol-1-^{14}C. When flowering four and one-half month old plants were fed 7.5 μc of geraniol-1-^{14}C, 0.009% of the total radioactivity injected into the plant was found in the aglucone portion of 1. The results are summarized in Table II. For all practical purposes, it can be assumed that the sugar moiety of the molecule did not incorporate radioactivity from geraniol-1-^{14}C. The ratio of the activity of the glucose moiety to that of the aglucone moiety was approximately 0.002. The percentage incorporation of radioactivity into the aglucone moiety of the molecule was much lower than expected, based on the acetate and mevalonate experiments. One day after feeding geraniol-1-^{14}C into the stems of the plant, no radioactivity was detected about the growing tips of the

plant. Translocation of the carbon-14 radioactivity did not seem to occur, and this likely resulted in a low percentage of incorporation. There is another possible explanation for the low percentage of geraniol-1-^{14}C incorporation into the aglucone moiety of 1. Perhaps free geraniol is not on the direct biosynthesis pathway for the formation of 1; geranyl pyrophosphate(45) may be on the pathway and might be expected to yield a much higher percentage incorporation than the free geraniol.

Another significant result obtained from the geraniol-1-^{14}C feeding experiment was the extremely small amount of radioactivity found in the glucose moiety. This confirms the fact that geraniol-1-^{14}C is the least effective of the three precursors tested for the sugar moiety of 1. The ratio of the activity of the glucose moiety to that of the aglucone moiety was the highest with carbon-14 labeled acetate, an expected result. Admittedly, though, it is dangerous to draw conclusions about geraniol-1-^{14}C being an ineffective precursor to the glucose moiety of 1 when the translocation was limited.

It can be predicted, on the basis of the isoprenoid structure of the aglucone portion of 1, that the radioactivity incorporated into the aglucone moiety from mevalonate-2-^{14}C should appear in carbon atoms three and/or eight and carbon atoms six and/or nine if an isoprenoid pathway is followed in its formation. In order to evaluate the significance in the disparity of the results presented,

an attempt was made to locate the specific labeled carbon atoms. A combination of the Kuhn-Roth oxidation and ozonization reactions was used to determine the location of the labeled carbon atoms.

Mevalonate-2-^{14}C was fed to mature, flowering Verbena officinalis L. plants; after a feeding period of five days, the plants were harvested. The verbenalin(1) which was present in these plants was then isolated and purified by preparative thin layer chromatography as described. A portion of the radioactive glucoside was then diluted to a specific activity of 241,000 dpm/m mole. The specific activity of the diluted aglucone 15 was found to be 96,400 dpm/m mole. The acetic acid which was isolated from the oxidation of 1 was found to have a specific activity of 3,760 dpm/m mole. Thus, only 4% of the radioactivity in the aglucone moiety of 1 was found in carbon atoms seven and nine. By difference, less than 4% of the radioactivity could be in carbon atom nine of verbenalol(15). These data are summarized in Table IV and are illustrated in structure 46.

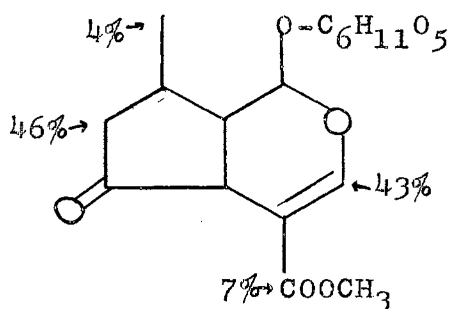


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TABLE IV

DEGRADATION OF CARBON-14 LABELED VERBENALIN

	Specific Activity dpm/m mole	Percentage Distribution of Radioactivity
Kuhn-Roth		
Verbenalin	241,000	---
Verbenalol	96,400	100
Acetate (carbons 7 and 9)	3,760	4
Ozonization		
Oxalate (carbons 4 and 8)	6,960	7
Methylsuccinate (carbons 5, 6, 7, 7a, and 9)	50,800	∿53
2-Methylglutarate (carbons 1 or 4a, 5, 6, 7 7a, and 9)	44,200	∿46

Another portion of the glucoside obtained from plants which had been fed mevalonate-2-^{14}C was likewise diluted. The purified glucoside was degraded with ozone to smaller fragments as shown in Figure 5. Oxalic acid, methylsuccinic acid, and 2-methylglutaric acid were confirmed by mass spectrometry of their dimethyl esters to be products of this oxidative degradation.[47] The dibasic acids formed were esterified by treatment with an ethereal solution of diazomethane. The specific activity of these fragments was then determined by gas radiochromatography; the results are summarized in Table IV. The dimethyl oxalate, obtained from carbon atoms four and eight was found to contain 7% of the radioactivity of the aglucone portion of 1. Carbon atom eight thus had less than 7% of the radioactivity. By comparing the distribution of the radioactivity in the dimethyl methylsuccinate and the dimethyl 2-methylglutarate with the radioactivity in the acetic acid, it was shown that approximately 46% of the radioactivity appeared in carbon atom six, as illustrated in structure 46.

It is of interest at this point to compare these results with the results obtained by Yeowell and Schmid in 1964 from the chemical degradation of plumieride(8).[75] Their results showed that the plumieride(8) formed biosynthetically from mevalonate-2-^{14}C contained 25% of the radioactivity in carbon atom three and 25% of the radioactivity in carbon atom fifteen. They found 44% of the radioactivity in carbon atom seven, and by difference,

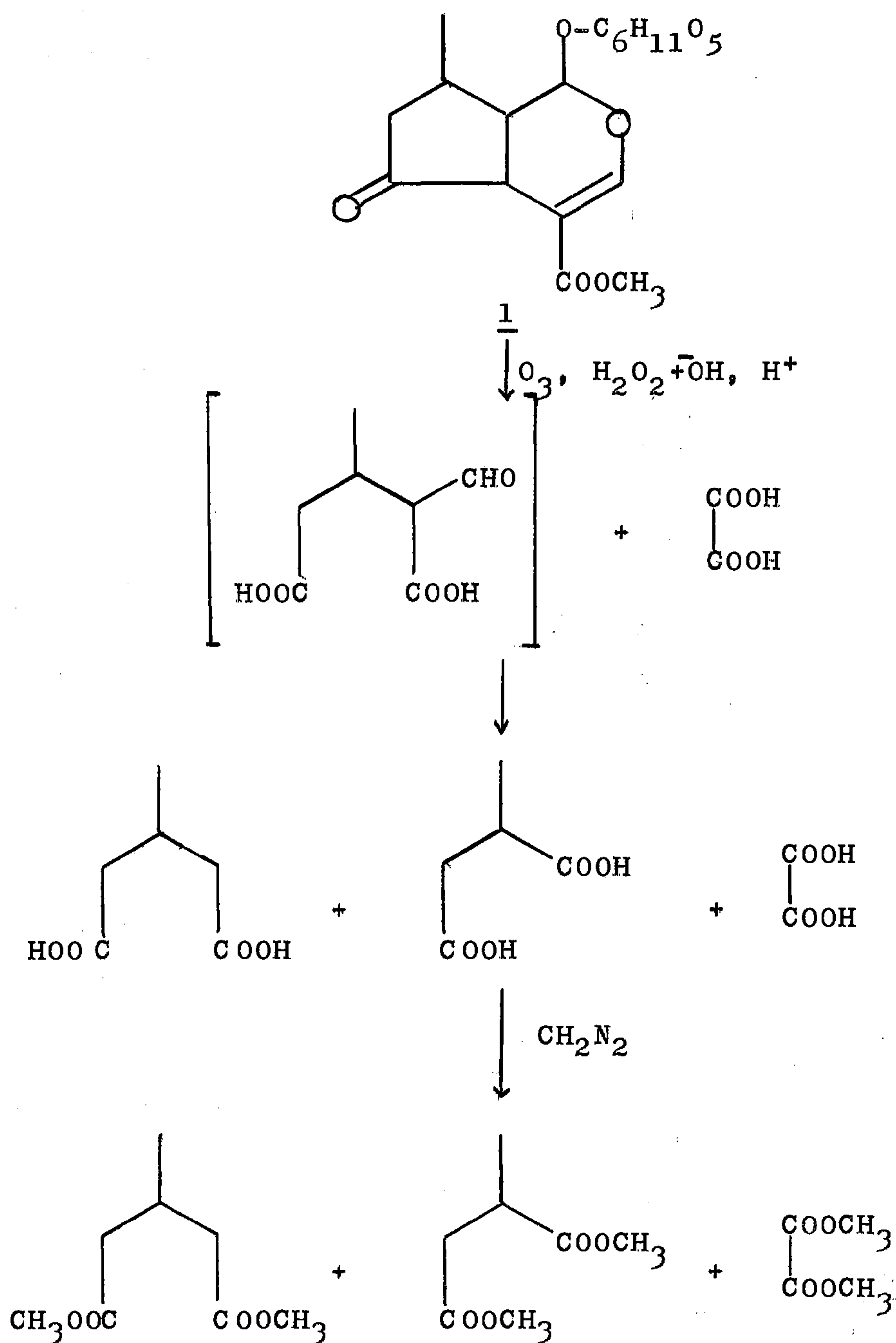
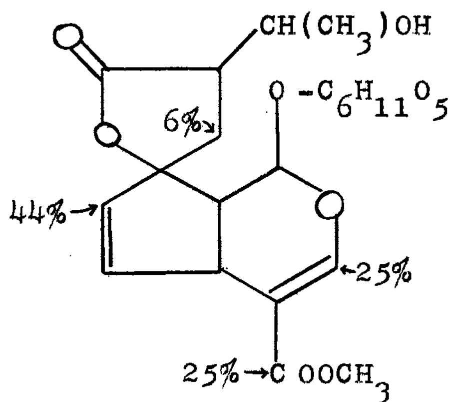


Figure 5. The Ozone Degradation of Verbenalin

concluded that carbon atom ten would contain 6% of the radioactivity as shown in structure 47.



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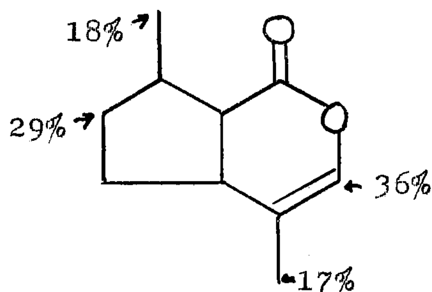
It was proposed that randomization between carbon atoms three and fifteen of plumieride(8) occurred after ring closure of the five-membered terpenoid moiety. Randomization of the terminal methyl label might occur if carbon atoms three and fifteen (25% of the radioactivity in each) became equivalent methyl, alcohol, aldehyde or acid groups.

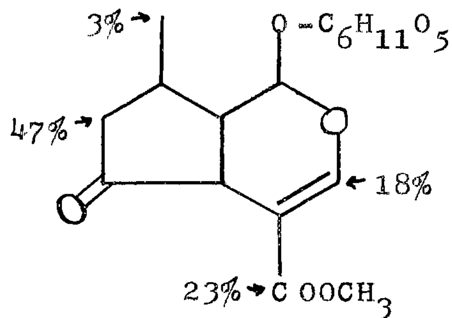
Limited randomization results were recently obtained by Regnier et al.[76] in their study of mevalonate-2-^{14}C incorporation into nepetalactone(9) using Nepeta cataria L. plants. The rate of incorporation was low, but a chemical degradation was developed to permit the determination of the amount of carbon-14 labeling in carbon atoms three and eight; a combination of the haloform and alkaline hydrogen peroxide reactions was used. The Kuhn-Roth oxidation was used to determine the extent of labeling in carbon atoms eight and nine. A limited randomization of radioactivity between carbon atoms three and eight and also carbon atoms

six and nine was observed. Carbon atom three and carbon atom eight contained 36% and 17% of the radioactivity of 9. Carbon atoms six and nine contained 29% and 18% of the radioactivity as illustrated in structure 48.



48

After our work was completed,[73] Schmid et al. reported on the chemical degradation of verbenalin(1) formed biosynthetically from mevalonate-2-^{14}C.[74] The results showed that randomization occurred between carbon atoms three and eight (18% and 23% of the radioactivity residing in each, respectively) and that carbon atoms seven and nine (appearing in the acetic acid fragment of a Kuhn-Roth determination of 49) contained 3% of the radioactivity as illustrated in structure 49.



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The results obtained in our initial study[73] using

mature, flowering Verbena officinalis L. plants indicated that very little randomization occurred between carbon atoms three and eight as shown in structure 46. The results obtained by Schmid et al.[74] using small shoots of Verbena officinalis L. (perhaps one to two months of age) grown during the summer indicated that randomization did occur between carbon atoms three and eight as shown in structure 49. In order to resolve this apparent disparity in labeling patterns using mevalonate-2-^{14}C as a precursor, a study was undertaken to determine the effect of the age of Verbena officinalis L. plants upon the degree of randomization. The specificity or lack of specificity during biosynthesis could then be determined as a function of age of plant.

To determine the effect of age upon possible biosynthesis pathways, groups of Verbena officinalis L. plants (each of the plants were grown from seed so that the age of each plant was accurately known) one, two, three and four months of age were each fed mevalonate-2-^{14}C. The older plants were mature and flowering. The plants were harvested and the crude ethanolic extract of each was treated to obtain radiochemically pure 1. Verbenalin(1) obtained from each set of plants was hydrolyzed by emulsin to obtain the specific activity of the aglucone 15. Portions of the radioactive glucoside obtained from the four sets of plants were diluted to 122,000, 133,000, 394,000 and 388,000 dpm/m mole. The specific activity of the diluted

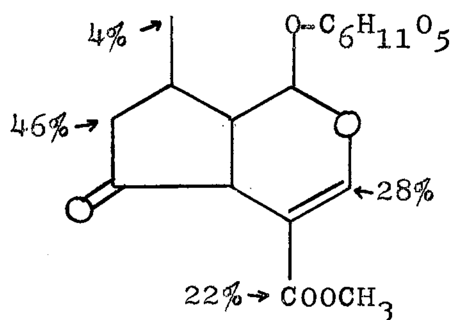
aglucone was 71,000, 69,500, 188,000 and 256,000 dpm/m mole, respectively. Each of the four portions was then degraded by Kuhn-Roth oxidation to obtain acetic acid. The acetic acid formed was found to have specific activities of 2,670, 3,140, 8,700 and 9,050 dpm/m mole. The radioactivity appearing in carbon atoms seven and nine of 1 contained 3.8%, 4.5%, 4.6% and 3.6% of the activity in the aglucone portion of the molecule for one, two, three and four month old plants. Thus, carbon atom nine had approximately 4% of the radioactivity and by difference, carbon atom six would have approximately 46% of the radioactivity in each of the four sets of plants. Randomization between carbon atoms six and nine does not occur in young, mature, or senile Verbena officinalis L. plants. The results are summarized in Table V and are illustrated in Figure 6.

Additional portions of 1 obtained from the four sets of plants fed mevalonate-2-^{14}C were diluted and degraded by ozone to determine the randomization of radioactivity at carbon atoms three and eight. The diluted portions of aglucone had specific activities of 45,000, 42,600, 95,800 and 140,000 dpm/m mole for plants aged one, two, three and four months, respectively. The four portions were then degraded by reaction with ozone to obtain oxalic acid. The methylsuccinic acid and 2-methylglutaric acid, although present as oxidation products, were obtained in insufficient amounts to measure their specific activities. The oxalic acid was purified and converted to the dimethyl

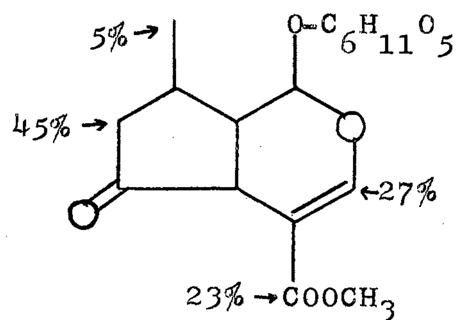
TABLE V

DEGRADATION OF LABELED VERBENALIN OBTAINED FROM PLANTS OF VARIOUS AGES WHICH HAD BEEN FED MEVALONATE-2-^{14}C

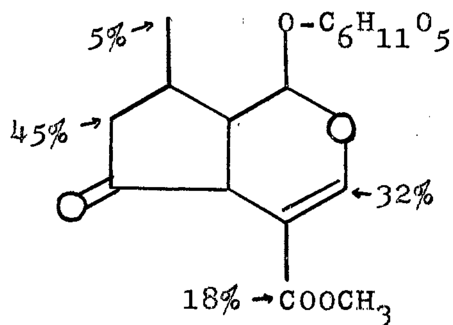
Age in Months	Feeding in dpm/10^6	Percentage Radioactivity Incorporated into Aglucone	Specific Activity of Aglucone in dpm/mM x 10^6	Dilution for Kuhn-Roth Rx.	Specific Activity of HOAc dpm/mM x 10^3	Dilution for Ozonization Rx.	Specific Activity of Dimethyl Oxalate dpm/mM x 10^3
1	36.2	0.04	6.05	x84.5	2.67 (3.8%)	x132	10.2 (22.3%)
2	47.7	0.03	3.66	x52.7	3.13 (4.5%)	x86.0	9.8 (23.1%)
3	38.6	0.09	4.17	x22.2	8.77 (4.6%)	x43.6	16.9 (17.6%)
4	25.8	0.18	6.00	x23.6	9.08 (3.6%)	x43.0	11.8 (8.4%)



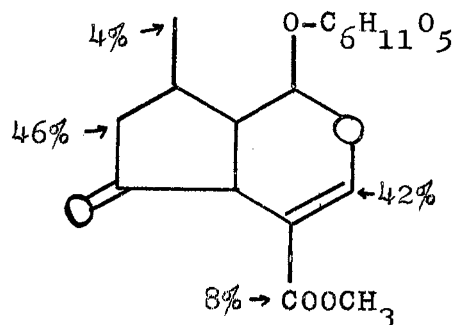
One Month



Two Months



Three Months



Four Months

Figure 6. Labeling Patterns in Verbenalin Using Mevalonate-2-^{14}C as a Precursor

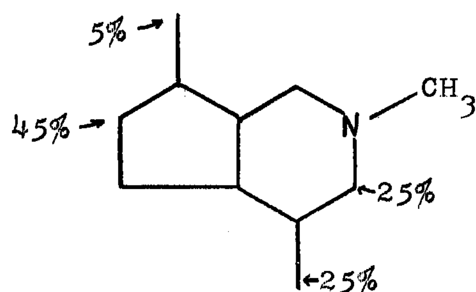
ester. The four portions of dimethyl oxalate were found to have specific activities of 10,200, 9,830, 16,900 and 11,800 dpm/m mole. The radioactivity appearing in carbon atoms four and eight contained 22%, 23%, 18% and 8.4% of the activity of the aglucone portion of 1, as summarized in Table V and illustrated in Figure 6. By difference, carbon atom three would be expected to have 28%, 27%, 32% and 42% of the radioactivity of the aglucone portion of 1 for plants aged one, two, three and four months.

These results obtained from the degradation of 1 provide convincing evidence for the non-randomization of the carbon-14 label in carbon atoms six and nine of 1 formed from mevalonate-2-^{14}C. In young plants and plants past flowering, approximately one-half of the radioactivity of the aglucone portion of 1 is located at carbon atom six. In young plants, one and two months of age, approximately one-fourth of the radioactivity is located at each of carbon atoms three and eight. Plants three months of age exhibit limited randomization; carbon atoms three and eight contain 32% and 18% of the radioactivity. In plants past flowering, 42% and 8% of the radioactivity can be attributed to carbon atoms three and eight. It would thus appear that young shoots of Verbena officinalis L. plants randomize the label from mevalonate-2-^{14}C; perhaps this occurs at the monoterpenoid level when the two carbon atoms which form carbon atoms three and eight in 1 become equivalent. This might occur during the formation of geranyl

pyrophosphate(45) or during the formation of some methyl-cyclopentane monoterpenoid intermediate. In older plants, randomization at carbon atoms three and eight does not occur extensively. Carbon atom three retains most of the radioactivity when mevalonate-2-^{14}C is used as a precursor. It would thus appear that the two terminal methyl groups which form carbon atoms three and eight do not become equivalent during the formation of 1. It would also appear that the two terminal methyl groups in geranyl pyrophosphate(45) or some methylcyclopentane monoterpenoid intermediate retain their identities.

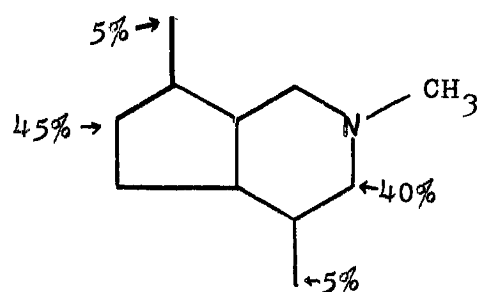
This change in randomization of the two terminal methyl groups, as a function of age of plant, of an intermediate such as geranyl pyrophosphate(45) has been recently noted by Auda et al.[77] When mevalonate-2-^{14}C was used as a precursor, one-fourth of the activity was found in each of carbon atoms three and eight of skytanthine(13) when young plants were used. Approximately 5% of the radioactivity was found in carbon atom nine, as shown in structure 50. The remainder of the radioactivity, 45%, can be assumed to be located at carbon atom six. When old plants were used, randomization did not occur between carbon atoms three and eight as shown in structure 51. Carbon atom three was found to have 40% of the radioactivity; carbon atom eight contained 5% of the radioactivity. The remainder of the radioactivity was found in carbon atoms six and nine, being 45% and 5%, respectively. Randomization

between carbon atoms three and eight was found in young plants; a lack of randomization between carbon atoms three and eight was confirmed in older plants.



1.3 Years

50



3.0 Years

51

It is quite plausible that randomization may occur at the monoterpenoid level in the methylcyclopentane monoterpenoids since carbon atom eight of 1 is highly oxidized, being a carboxylic ester group. Since the biological oxidation proceeds stepwise, an alcohol and an aldehyde group may be intermediates in the formation of the carboxyl group. Randomization occurs during oxidation of carbon atoms three and eight in young plants of Verbena officinalis L. In older plants, perhaps the oxidation of carbon atoms three and eight does not occur at the same time. If it occurs at the same time, perhaps the oxidized methyl groups are held in place by an enzyme surface to give this specificity during the biosynthesis of 1.

Randomization of the terminal methyl label in the isoprenoid portion of certain indole alkaloids has also been reported,[22-28] and the mechanisms suggested are similar to

that proposed by Yeowell and Schmid.[75]

As first suggested by Auda et al.,[77] the extent of specificity during biosynthesis may be subject to control at the enzyme level and by the pool size of the intermediates. Different concentrations of enzymes and of enzyme inhibitors may be present in young, mature and old plants. Perhaps the enzyme necessary for randomization of carbon atoms three and eight in 1 decreases in activity or disappears as the age of the plant increases. This is quite plausible when Verbena officinalis L. plants are considered. The greatest change in randomization occurs during and after flowering; the hormonal changes in the bud may very well exert some control over the enzyme responsible for randomization. Changes in level of enzymes have been reported to occur in pea seedlings; the diamine oxidase rapidly appears and disappears in the plant.[78]

The metabolic control of randomization of carbon atoms three and eight in 1 due to pool size must also be considered. The young plant may have a rather large pool of monoterpenoid intermediate, and a relatively small amount of methylcyclopentane monoterpenoid; the two terminal methyl groups have the opportunity to become equivalent. In the old plant, the pool size of the intermediate may decrease as the concentration of the methylcyclopentane monoterpenoid increases and the two terminal methyl groups may have less opportunity to become equivalent. This may occur in Verbena officinalis L. plants since the young

plants have only small amounts of 1 (approximately 0.1% as shown in Table I); the concentration of 1 increases with age of plant and reaches a maximum at flowering (approximately 0.3%). The greatest change in randomization occurs as the concentration of 1 increases most rapidly.

The data presented in this thesis also supports the mechanism proposed for the isomerization of isopentenyl pyrophosphate(42) which was discussed earlier.[60,62] The isomerization of isopentenyl pyrophosphate(42) to dimethylallyl pyrophosphate(43) does not result in the randomization of the methyl groups originally present in isopentenyl pyrophosphate(42); the methylene group of isopentenyl pyrophosphate(42) retains its identity. In young and old plants of Verbena officinalis L., significant randomization between carbon atoms six and nine of 1 did not occur; carbon atom six retained most of the radioactivity.

Although the pathways for the biosynthesis of 1, and the other methylcyclopentane monoterpenoids are not well understood, one possible sequence is presented in Figures 7, 8 and 9. Each dot shown in the diagrams represents approximately one-fourth of the radioactivity of the aglucone portion of the molecule. The pathway shown in Figure 7 branches to route A as shown in Figure 8, and route B as shown in Figure 9. Route A results in non-randomization of carbon atoms three and eight, since, as illustrated, these carbon atoms do not become equivalent before ring closure

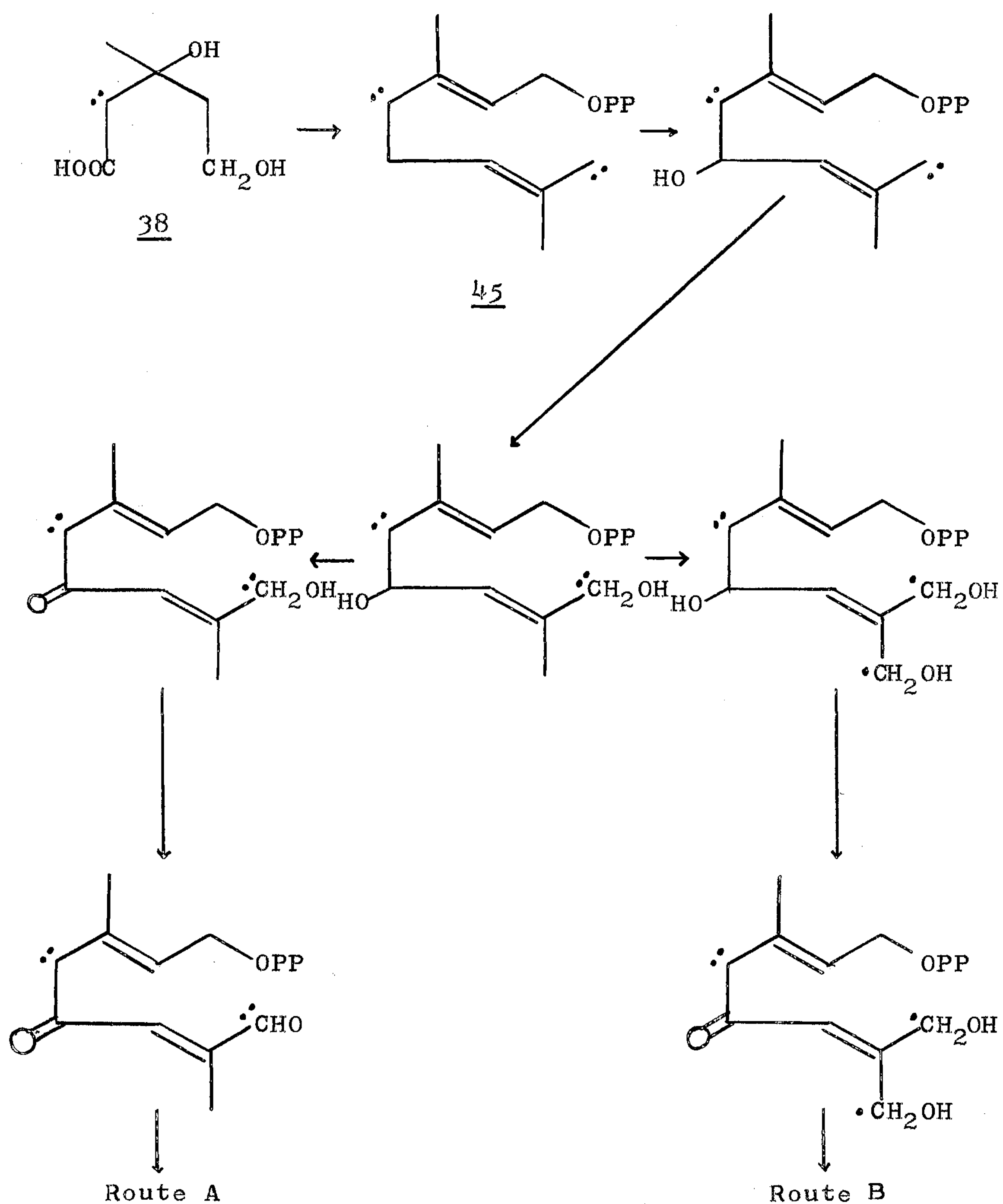


Figure 7. The Biosynthesis of Verbenalin

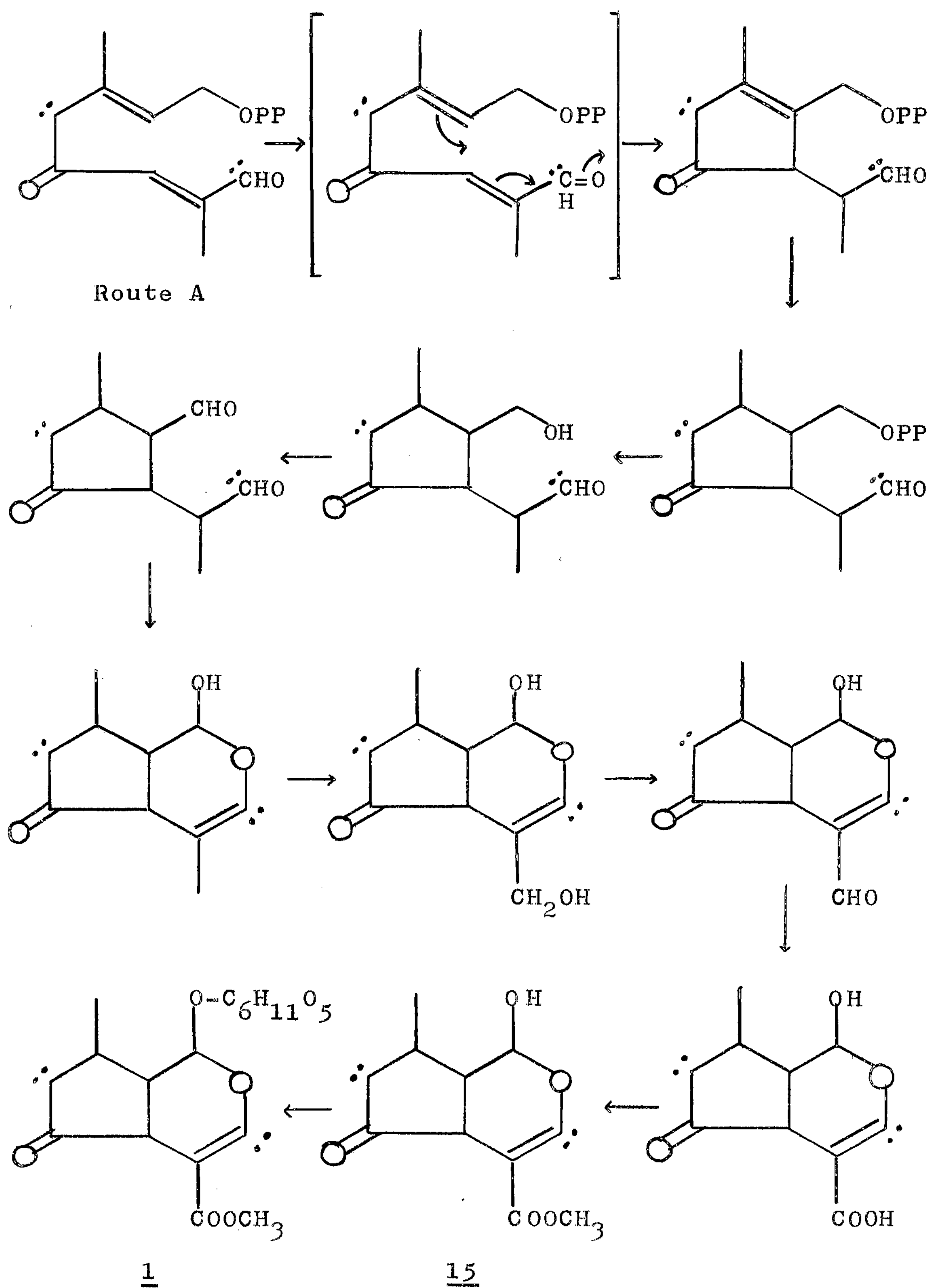


Figure 8. The Biosynthesis of Verbenalin: Route A

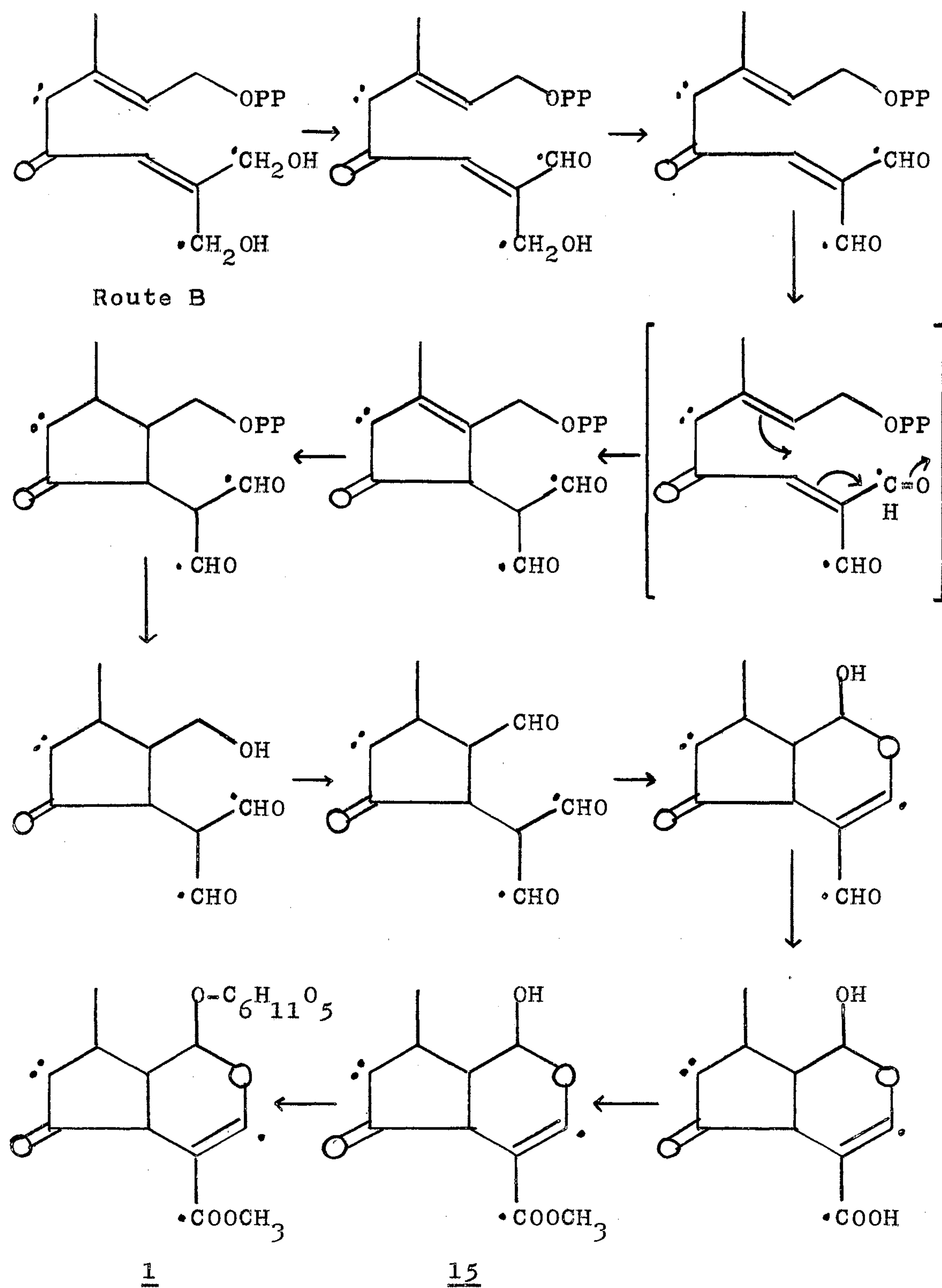


Figure 9. The Biosynthesis of Verbenalin: Route B

of the six-membered ring. Route B results in randomization since carbon atoms three and eight become equivalent early in the biosynthesis scheme.

The scheme proposed is unique in several ways. An allylic hydroxylation reaction is proposed to occur early in the biosynthesis scheme; further oxidation of the hydroxyl group provides the carbonyl group at carbon atom five of 1. Another allylic hydroxylation is proposed to occur at carbon atom three (of 1) to form first a hydroxyl group and then an aldehyde group, which activates the conjugated double bond to aid in closure of the methylcyclopentane ring. The aldehyde group at carbon atom three of 1 is then necessary for the closure of the heterocyclic ring. If carbon atom eight of 1 is oxidized before closure of the heterocyclic ring, randomization may occur between carbon atoms three and eight of 1. The loss of the pyrophosphate function of carbon atom one and its subsequent oxidation to an aldehyde group may occur early or late in the biosynthesis sequence. It is pictured as occurring late since other ring closures could occur if the aldehyde group were present early in the sequence. The stereospecific reduction of the carbon to carbon double bond in the methylcyclopentane ring is pictured as occurring immediately after ring closure of the methylcyclopentane ring; this reduction may occur later in the pathway and still be stereospecific. Other pathways are possible; indeed, the scheme presented in Figures 7, 8 and 9 does not explain all

of the information available, but it is hoped that when used as a model, it may shed some light on the biosynthesis of 1 and the other methylcyclopentane monoterpenoids.

Before the significance of the contrast in randomization and non-randomization of carbon atoms three and eight in 1 for different age Verbena officinalis L. plants can be determined, and before a clear picture of the biosynthesis pathways for the formation of the methylcyclopentane monoterpenoids can be developed, additional study is required.

CHAPTER V

EXPERIMENTAL

Melting points were taken in capillary melting point tubes using a Thomas-Hoover apparatus and are uncorrected. The centigrade scale was used for all temperature measurements.

Optical rotation measurements were made with an O. C. Rudolph & Sons Inc., Model 80 Universal High Precision Polarimeter, using a two dm tube. Distilled water was used as the solvent.

Absorption maxima in the ultraviolet region were measured using a Beckman Model DK Recording Spectrophotometer. The photomultiplier was used and the time constant was set at 0.1 seconds. Infrared spectra were obtained with a Beckman IR-5A Infrared Spectrometer.

Gas liquid chromatographic analyses were obtained with a Hewlett Packard F & M Model 700 dual column laboratory gas chromatograph fitted with a thermal conductivity detector system using helium as the carrier gas, and fitted with a Westronics Model LD11A laboratory type recorder having a 1.1 millivolt range. Analyses of degradation products were performed: (a) on a 10 ft x 1/4 in. copper column packed with 25% Union Carbide Carbowax 20M poly

(ethylene glycol) coated on 60-80 mesh acid-washed Chromosorb W and (b) on a 6 ft x 1/8 in. stainless steel column packed with 10% General Electric Silicone Rubber SE-30 coated on 80-100 mesh Gas Pack W.

Earlier gas liquid chromatographic analyses were obtained with a Beckman Model GC-2A Gas Chromatograph equipped with a thermal conductivity detector system, and a 0-1 millivolt Leeds & Northrup Speedomax H laboratory recorder. Helium was used as the carrier gas.

Gas liquid chromatography was also performed using a Hewlett Packard F & M Model 770 Preparative Gas Chromatograph equipped with a thermal conductivity detector system, using helium as a carrier gas. Small samples were determined with the analytical side of the instrument. Analyses were performed with: (a) a 10 ft x 1/4 in. copper column packed with 20% Carbowax 20M poly(ethylene glycol) coated on acid-washed Chromosorb W and (b) a 10 ft x 1/4 in. copper column packed with 15% phenyldiethanolamine succinate (PDEAS) coated on less than 70 mesh acid-washed Firebrick.

All capillary gas liquid chromatographic analyses were performed with an Instruments Incorporated Model 393 Valved Gas Chromatograph equipped with a hydrogen flame detector system using helium as the carrier gas. The detector temperature was necessarily set at 225°, and the pyrolysis loop was used as the injection port so that the temperature in the vaporization chamber could be regulated to suit the

sample used. Because of the sensitivity of the hydrogen flame detector in the instrument, an analysis could be performed on as little as several nanograms of organic sample, using a nanoliter sample injector.

Peak areas were measured either with a planimeter, or by weighing the paper which was under the peak. Quantitation of the amounts of sample represented by the peak was made by comparison with standard curves obtained at the same time with pure compounds. Reproducible results were not obtained if the gas liquid chromatographic analyses of the known, pure compounds was performed at some time other than the time the analyses were performed on the unknowns.

The stream of oxygen containing 1-3% ozone, used for the oxidative cleavages described, was generated with a Welsbach Model T-23 Laboratory Ozonator using dried oxygen as the gas to be ozonized. The reaction vessel was a long narrow column with a means of dispersing the ozone stream at the bottom. The ozone dispenser terminated in a glass bulb containing several small perforations to release the ozonized oxygen in the form of small bubbles. Since the ozone was readily absorbed by the olefinic compounds oxidized, it was not necessary to use a fritted glass diffuser. Generally, the decomposition of the resulting ozonide was carried out without delay to avoid the possibility of oxidative polymerization.

Mass spectrometric data were obtained on the prototype of the LKB 9000 Mass Spectrometer-Gas Chromatograph

constructed in the Mass Spectrometry Laboratory, Karolinska Instutet, Stockholm, Sweden.

Plant Material Used. Live samples of Pride, Sutton's Blue, Pink Giant, Lavender and Royal Purple, all of which are *Verbena hybrida*, were obtained from S. W. Cobb's Greenhouse, 1707 South Memorial Drive, Tulsa, Oklahoma. Seeds of Sparkle, also a *Verbena hybrida*, were obtained from the George T. Ball Seed Company, West Chicago, Illinois.

Dried *Verbena officinalis* L. weed, used for extractions to obtain working quantities of verbenalin(1) for dilution studies, was obtained from the Product Development Department, S. B. Penick and Company, 100 Church Street, New York 8, New York. Seeds of *Verbena officinalis* L. which were germinated and used for preliminary studies, were obtained from the Botanicka zahrada, University Karlovy, Benatska 2, Prague 2, Czechoslovakia. Seeds of *Verbena officinalis* L. which were germinated and later used for all of the incorporation studies were obtained from the Director, Royal Botanic Gardens, Kew, Richmond, Surrey, England.

Seeds of *Cornus mas* L. were obtained from W. R. Langford, U. S. Department of Agriculture, Agriculture Research Service, Crops Research Division, Plant Introduction, Geneva, New York.

Each of the above mentioned seeds were germinated, and the plants were grown, by the Department of Horticulture, Oklahoma State University.

Attempted Ether Extraction of Verbenalin(1) from Several Varieties of Verbena hybrida. A typical extraction consisted of powdering about 100 g of plant material, which had been dried for 48 hours at 40°, in a mill. The powder was extracted in a Soxhlet extraction apparatus for two or more weeks with freshly distilled ethyl ether. The ether extract was then condensed at room temperature under reduced pressure to reduce its volume to one-fourth. The solid material in the extract was filtered out and washed with a dry ethyl ether and acetone (1:1) solution to remove the gummy plant material. After several 50 ml washings, the residue was extracted with hot moist ethyl acetate. No crystalline 1 was deposited on cooling and allowing the solution to stand. Concentration of the ethyl acetate extracts and seeding also produced no crystals of 1. The ethyl acetate was then completely removed at room temperature under reduced pressure; the slight amount of residue remaining was hydrolyzed with emulsin at pH 4, and the product was tested for reducing sugars with a 4% solution of 2,3,5-triphenyl-2H-tetrazolium chloride in methanol. A red color did not appear. The test was negative and therefore the absence of glucosides was confirmed.

Attempted Extraction of Verbenalin(1) from Several Varieties of Verbena hybrida Using a Total Ethanolic Extract. A typical extraction procedure consisted of powdering 100 g of plant material, which had been dried for 48 hours at 40°, in a mill. The powdered plant material was

refluxed for two hours with 95% ethanol and calcium carbonate boiling chips. The filtrate was concentrated at room temperature under reduced pressure until green gummy plant material formed thick deposits on the inside of the flask. The chlorophyll-containing deposits were washed with hot ethanol; the washings were added to the filtrate. This process of removing solid deposits was repeated approximately ten times until only a thick, green, smooth syrup remained. The small amount of syrup was then extracted with hot, moist ethyl acetate, which was then allowed to cool slowly. No crystals of 1 appeared, even after concentration of the extract. Subsequent seeding produced no 1. A slight amount of residue remained after the ethyl acetate was removed at room temperature under reduced pressure. The residue was hydrolyzed with emulsin at pH 4, and the product was tested for the presence of reducing sugars with a 4% solution of 2,3,5-triphenyl-2H-tetrazolium chloride in methanol. The test was negative.

Attempted Extraction of Verbenalin(1) from Several Varieties of Verbena hybrida by Column Percolation. A typical extraction procedure consisted of powdering one kg of plant material, which had been dried for 48 hours at 40°, in a mill. The powdered plant material was placed into a column percolator, and refluxed ethyl ether was continuously dripped through the plant material for eleven days. The ether extract was concentrated at room temperature under reduced pressure. The solid material which was

extracted was filtered and washed with an ethyl ether and acetone (1:1) solution to remove gummy plant material. Most of the residue dissolved and was washed away. The remaining residue was extracted with several portions of hot, moist ethyl acetate. The combined extracts were filtered and cooled. No crystalline 1 was found. Indeed, with several extractions, no residue remained after the ethyl acetate was removed from the combined extracts.

Extraction of Verbenalin(1) from Verbena officinalis L. A typical example consisted of extracting 210 g of powdered *Verbena officinalis* L., which had been dried for 48 hours at 40°, with ethyl ether in a Soxhlet extraction apparatus. The extraction was allowed to proceed for twelve days. The ethyl ether was concentrated at room temperature under reduced pressure; the residue was washed with an ethyl ether and acetone (1:1) solution to remove gummy, green plant material. White material was observed to be present along with the chlorophyll-containing components. The residue was extracted with hot, moist ethyl acetate, from which white crystals separated upon slow cooling. After several recrystallizations from hot, moist ethyl acetate, and a solution of 95% ethanol and ethyl acetate (1:1), colorless plates were obtained melting at 181-182°. A total of 252 mg of 1 having a specific rotation of - 171° was isolated.

The physical properties of 1 agreed well with literature values.[31-47] The ultraviolet spectrum showed two

absorptions: λ_{max}^{EtOH} 238 mμ (ϵ 9,600) and λ_{max}^{EtOH} 289 mμ (ϵ 100). The infrared spectrum, using a potassium bromide pellet, showed strong absorptions at 3450, 2875, 1685 and 1635 cm^{-1}.

Another macro-scale isolation of 1 consisted of extracting 3.5 kg of dried plant material with ethyl ether in a Soxhlet extraction apparatus. After purification as described, 0.6 g of 1 was obtained. Other similar ethyl ether Soxhlet extractions of the plant material from the same bale of Verbena officinalis L. weed resulted in yields of 1 averaging less than 0.1% recovery. When freshly dried plant material was used for the extraction of the glucoside, the recovery yields increased somewhat. On occasion, evidence of caromelization in the extraction flask was observed during the extraction; this caromelization would lower the total yield of 1. The addition of trace quantities of antioxidants did not appear to reduce caromelization.

The Preparation of the Trimethylsilyl Derivative of Verbenalin(1). The gas liquid chromatographic analysis of the trimethylsilyl derivative of the glucoside 1 was attempted. The formation of the trimethylsilyl derivative in pyridine containing hexamethyldisilazane and trimethylchlorosilane appeared to occur rapidly as evidenced by the formation of a cloudy precipitate, presumably due to the precipitation of ammonium chloride.[79] Attempts to analyze the trimethylsilyl derivative of 1 by gas liquid chroma-

tography did not succeed; even at high column temperatures and at high flow rates, peaks due to the trimethylsilyl derivative of 1 were not observed.

Gas Liquid Chromatographic Analysis of the Steam Distillate of Verbena officinalis L. Two hundred g of dried, powdered *Verbena officinalis* L. were steam distilled until 300 g of distillate were collected. The steam distillate was extracted with four fifty ml portions of ethyl ether. The ethyl ether extracts were combined and were dried over anhydrous magnesium sulfate. The drying agent was removed by filtration, and the major portion of the ethyl ether extract was carefully concentrated at room temperature under reduced pressure to a volume of two ml. The original extract and the concentrated ethyl ether extracts were then analyzed by gas liquid chromatography using a 300 ft x 0.02 in. stainless steel column coated with a film of LAC 886 (ethylene glycol succinate). The gas liquid chromatographic tracings were carefully examined for evidence of any hydrocarbons or oxygenated compounds in the five to ten carbon range. Compounds which might be possible precursors to 1 were not found in the steam distillate.

Gas Liquid Chromatographic Analysis of Ethyl Ether Extracts of Verbena officinalis L. One hundred g of dried, powdered *Verbena officinalis* L. were refluxed with 250 ml of ethyl ether for two hours. The solid plant material was removed by filtration and the major portion of the ethyl

ether extract was carefully concentrated at room temperature under reduced pressure. The original extract and the ethyl ether concentrate were analyzed by gas liquid chromatography using a 300 ft x 0.02 in. stainless steel column coated with a film of Monsanto OS-138 (a six-ring polyphenyl ether, bis[m-(m-phenoxyphenoxy)phenyl]ether). Evidence of hydrocarbons or oxygenated compounds in the five to ten carbon range were not found by the examination of the chromatographic tracings.

<u>Administration of Labeled Compounds to Verbena officinalis L.</u> *Verbena officinalis* L. plants were grown in the greenhouse, Department of Horticulture, Oklahoma State University, from seeds or cuttings. The plants used for studies where age of plants might be a critical factor, were all grown from seeds. An aqueous solution of the labeled compound was injected into the hollow internodular stem of the plant at several places along the stem using a Hamilton ten microliter syringe. The stem was vented in appropriate places to prevent the expulsion of the labeled compounds. Up to ten μl of solution could be injected into each mature plant at one feeding. After a period of five to seven days, the plants were harvested and dried in an oven for 48 hours at 40°. After being dried, the plants were stored in sealed glass containers at 4° until used, although an attempt was made to work with freshly dried plant material.

<u>Labeled Compounds Used for the Incorporation Studies.</u>

For the incorporation studies, DL-mevalonic acid-2-^{14}C-(N,N'dibenzylethylenediamine salt) which had been obtained from Nuclear Research Chemicals, Orlando, Florida, was converted to the free acid by the removal of dibenzylethylenediamine. In order to obtain radiochemically pure material, a known amount of the salt was dissolved in a minimum of distilled water, and 1 N sodium hydroxide solution was added until the pH of the solution reached 10.[80] Two successive extractions of ethyl ether removed the dibenzylethylenediamine. The ethyl ether extracts were washed with 0.5 volume of sodium hydroxide solution adjusted to pH 10, and the washings were added to the aqueous solution of sodium mevalonate. Descending preparative paper chromatography was used to purify the sodium mevalonate-2-^{14}C utilizing Whatman No. 1 paper and 85% isopropyl alcohol as the developing solvent. The purity was confirmed by observing only one spot after paper chromatographic analysis using n-butanol, acetic acid and distilled water (4:1:1) as the developing solvent. The location of radioactivity was made with a Nuclear-Chicago Actigraph III paper strip scanner.

Chromatographically pure DL-mevalonic acid-2-^{14}C-(N,N'dibenzylethylenediamine salt) obtained from the New England Nuclear Corporation, Boston, Massachusetts was used for the biosynthesis of <u>1</u> when the labeling patterns were studied as a function of the age of plants. The purity was confirmed by observing only one spot after paper chromato-

graphic analysis using n-butanol, acetic acid and distilled water (4:1:1) as the development solvent.

Acetate-1-^{14}C and acetate-2-^{14}C were obtained from the California Corporation for Biochemical Research, Los Angeles, California.

Geraniol-1-^{14}C was not commercially available and was synthesized by coworkers in this laboratory.[81] Its synthesis was accomplished by the chain extension of the commercially available 6-methylhept-5-en-2-one, through a modified Wittig reaction using methyl bromoacetate-1-^{14}C. The carbon-14 label was specifically introduced at the terminal function of the resulting ester which was then reduced to geraniol-1-^{14}C. The geraniol-1-^{14}C was found to be radiochemically pure.[82]

Isotope Analysis. The counting techniques used in this study depended upon both the specific activity of the sample in question and the method used in the isolation procedure. Carbon-14 activity of the compounds administered to the plants, the isolated glucoside and corresponding aglucone, and the products formed during the chemical degradation was determined using liquid scintillation counting and/or gas radiochromatography.

The liquid scintillation counting was performed in a Packard Tri-Carb Model 314 liquid scintillation spectrometer, Packard Instrument Co., La Grange, Illinois. The scintillation solvent generally used was composed of 58.7% toluene, 39.3% absolute ethanol, and 2% distilled water.[83]

The added phosphors were 0.5% 2,5-diphenyloxazole and 0.02% p-bis-2-(5-phenyloxazolyl)-benzene. In the Packard Tri-Carb liquid scintillation spectrometer used, this system had an efficiency of 43%.

The other scintillation solvent used for preliminary work consisted of a mixture containing 60 g of naphthalene, 4 g of 2,5-diphenyloxazole (PPO), 200 mg of 2,2-p-phenylenebis(5-phenyloxazole), 100 ml of methanol, 20 ml of ethylene glycol, and enough p-dioxane to make one liter.[84] In the Packard Tri-Carb liquid scintillation spectrometer used, this system had an efficiency of 72%.

Carbon-14 activity was also determined by gas radiochromatography using a Perkin-Elmer Model 801 gas liquid chromatograph equipped with a thermal conductivity detector system and using helium as a carrier gas. The total effluent from the gas liquid chromatograph coming through the detector was fed into the counting chamber of a Nuclear-Chicago Model 8200 proportional gas flow counter, through a heated inlet line held at 250°. The gas counting chamber was also held at 250° to prevent condensation of samples. Peak areas were measured using a Nuclear-Chicago Model 8350 automatic integrator module, equipped with a Nuclear-Chicago Model 8416 laboratory recorder. This instrument had a counting efficiency of 34% as calculated from a reference standard of n-heptane-1-^{14}C, having a specific activity of 0.25 μc/μ mole. The counting gas was methane used at a rate of 75 ml/min.

Isolation and Purification of Verbenalin(1) from Verbena officinalis L. by Thin Layer Chromatography. Glass plates for the thin layer chromatography were made having the following dimensions: 20 x 20 x 0.4 cm. The plates were coated with a 0.75 mm layer of silica gel G (according to Stahl) combined with a calcium sulfate binder and a particle size of 10 - 40 μ. The layer was applied as a slurry consisting of 60 g of silica gel G and 108 ml of distilled water, which had been shaken together for one minute. The plates were allowed to dry for at least two days, and were then activated in the oven at 110° for two hours before use.

The Verbena officinalis L., or other plants used, which had been previously dried, weighed and ground, were extracted with three successive portions of hot 95% ethanol in the presence of calcium carbonate boiling chips. Each portion of ethanol was equal to approximately thirty times the weight of the dried plants extracted. The residue remaining after the extraction was removed by filtration and washed with another portion of hot ethanol. The ethanol extracts and the subsequent washings were combined and then concentrated at room temperature under reduced pressure to approximately 3 - 5% of the original volume of the combined extract.

The concentrated ethanol extracts were then applied as a narrow stripe to a 0.75 mm layer of recently activated silica gel G. The amount of concentrate per plate was

approximately the extract of 0.1 g to 0.2 g of original plant material. The plate was first developed with ethyl ether to remove the abundant ether soluble lipid material. An authentic sample of 1 was placed adjacent to the stripe of crude ethanolic extract as a marker for identification. The authentic spot of 1 was identified by spraying only it with acidified 2,4-dinitrophenylhydrazine solution. The portion of the plate which corresponded to 1 was scraped clean and the crude glucoside 1 was leached from the silica gel G with hot 95% ethanol. Generally, at this stage of purification, the width of the portion of the plate scraped clean was equal to three to four times the width of the spot of authentic 1.

The ethanol extract from the leaching operation was then concentrated at room temperature under reduced pressure to approximately the original reduced volume of ethanolic extract applied to the thin layer plate. The partly purified ethanolic extract was then applied to a second 0.75 mm thick plate of silica gel G as a thin stripe. An authentic sample of 1 was again placed adjacent to the stripe of partly purified 1 to help in identification. The plate was then developed with a solution of ethyl acetate and 95% ethanol (3:1), and the identifying spot of 1 gave an R_f value of 0.52 upon spraying with an acidified ethanolic solution of 2,4-dinitrophenylhydrazine.

This general process was repeated, usually a minimum of eight to ten times using different solvent systems until

a single spot was observed for the glucoside 1. Solvent systems used in the development of the plates contained various combinations of benzene, methanol, ethanol, ethyl acetate, ethyl ether, p-dioxane, methylene chloride and water. Another typical solvent system contained benzene, 95% ethanol and ethyl acetate (1:1:4) resulting in an R_f value of 0.30 for 1 on 0.75 mm thick plates of silica gel G.

For some of the purification steps during the isolation of 1 obtained from the study of labeling patterns versus age of plant, glass plates coated with silica gel H were used. The plates were coated with a 0.75 mm thick layer of silica gel H (according to Stahl) having an average particle size of 10 - 40 μ. The layer was applied as a slurry consisting of 60 g of silica gel H and 120 ml of distilled water. The slurry did not require immediate application since the adsorbant did not contain a calcium sulfate binder. The plates were allowed to dry for at least two days, and were then activated for two hours at 110° before use.

The purification procedure proceeded as described, using the same solvent system for the plates coated with silica gel H as was used for the plates coated with silica gel G. Major differences were not observed. When an authentic sample of 1 was placed adjacent to a stripe of crude ethanolic extract on a 0.75 mm layer of silica gel H and developed with a solution of ethyl acetate and 95%

ethanol (3:1), the R_f value was found to be 0.57 after spraying the appropriate portion of the plate with an acidified ethanolic solution of 2,4-dinitrophenylhydrazine. When the plate was developed with a solution of ethyl acetate, 95% ethanol and benzene (3:1:1), the R_f value for the authentic spot of 1 was found to be 0.39.

Concentration of Verbenalin(1) in the Plants Studied. Verbenalin(1) was isolated on a micro-scale as described above using a relatively small amount of ethanolic extract. The concentration of 1 was determined by measuring the λ_{max}^{EtOH} 238 mμ (ε 9,600). This procedure was used in measuring the verbenalin(1) concentration in plants of different ages to obtain the plot of concentration of 1 versus age of plants.

The Isolation and the Determination of the Radiochemical Purity of Labeled Verbenalin(1) from Plants Which Had Been Injected with Precursors. Verbenalin(1) was isolated on a micro-scale as previously described, and the concentration was determined by measuring the absorption in the ultraviolet region. The radioactivity of the sample was determined by counting aliquots of the above ethanolic extract using liquid scintillation counting. The specific activity was therefore determined for 1. The remaining portion of the ethanolic extract was then concentrated at room temperature under reduced pressure. The extract was then applied to a 0.75 mm thick plate of silica gel G as a thin stripe. An authentic sample of 1 was again placed

adjacent to the stripe of purified 1 to aid in identification. The plate was then developed, and the marker spot of 1 was sprayed with an acidified ethanolic solution of 2,4-dinitrophenylhydrazine; the portion of silica gel G corresponding to 1 was scraped clean. The silica gel G was then leached with several portions of hot ethanol. At this stage of purification, the portion of the plate scraped clean was generally equal to one and one-half times the width of the authentic spot of 1. The concentration of 1 was again determined by taking the ultraviolet spectrum of an aliquot, and its radioactivity was again determined by liquid scintillation counting. The radiochemical purity was thus determined by comparing the values obtained for the specific activity of 1.

Hydrolysis of Verbenalin(1) to Verbenalol(15). Verbenalin(1) was readily cleaved with emulsin in a buffered solution (using a citrate-phosphate buffer) at pH 4.5 to produce 15 in yields of 50-65%. The salt-free, lyophilized, powdered almond emulsin, beta-glucosidase, which was stored in a cool, dry place before use, was obtained from the Mann Research Laboratories Inc., 136 Liberty Street, New York 6, New York. After stirring 1 with emulsin at pH 4.5 for four hours at room temperature, the aglucone was extracted with several portions of ethyl ether after salting out the aqueous solution. After drying the ethyl ether solution with anhydrous magnesium sulfate, filtering it, and concentrating it at room temperature under reduced pressure,

15 was obtained having a melting point of 130-131.5°.[31-47]

Verbenalol(15) exhibited a maximum in the ultraviolet: λ_{max}^{EtOH} 239 mμ (ε 9,000).[43,45,46,47] In 0.01 N sodium hydroxide solution, a bathochromic shift was observed: λ_{max} 272 mμ (ε 19,000). The infrared spectrum agreed well with literature values;[47] the infrared spectrum in chloroform showed strong absorption at 3545, 3000, 1750, 1715 and 1640 cm^{-1}.

Gas Liquid Chromatography of the Trimethylsilyl Derivative of Glucose Obtained from Verbenalin(1). The identification of the glucose obtained by the emulsin cleavage of 1 was accomplished by comparison of the gas liquid chromatogram of the trimethylsilyl derivative[79] of the sugar obtained from 1 and of the corresponding derivative of glucose utilizing a 10 ft x 1/4 in. copper column packed with 5% Wilkens phenyldiethanolamine succinate (PDEAS) coated on Chromosorb P. A 30 mg portion of 1 was hydrolyzed as described to obtain 15. The remaining aqueous solution from which 15 was removed by ethyl ether extraction, was concentrated to dryness at room temperature under reduced pressure. The polyhydroxy compound remaining was dissolved in two ml of anhydrous pyridine (kept over potassium hydroxide pellets). The pyridine solution was treated with 0.2 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane. The reaction mixture was shaken vigorously in a stoppered container for one minute. After ten minutes, the product was analyzed by gas liquid chromatography. The

retention time was compared to those of authentic samples of trimethylsilyl derivatives of glucose obtained by treating 10 mg of glucose with the appropriate amounts of the trimethylsilylation reagents. Upon mixing the authentic glucose derivative with that obtained by the hydrolysis of 1, only one peak was observed on the chromatogram.

Micro-scale Purification of Labeled Verbenalol(15). Verbenalol(15) was obtained as described above. The ethyl ether extract containing the aglucone was concentrated at room temperature under reduced pressure. The concentrated extract was applied as a thin stripe to a 0.75 mm thick plate of silica gel G. An authentic spot of the aglucone was placed alongside the stripe to aid in identification. The plate was developed with ethyl acetate and 95% ethanol (3:1). The spot of authentic 15, which had an R_f value of 0.83, was found and the appropriate portion of the plate was scraped clean of silica gel G. The aglucone 15 was leached with hot ethanol and its concentration was determined by utilizing the ultraviolet absorption in 0.01 N sodium hydroxide solution. The specific activity of the aglucone was then determined through liquid scintillation counting of an aliquot.

The ethyl ether extract containing the aglucone was concentrated at room temperature under reduced pressure. The aglucone 15 was then chromatographed once again on silica gel G and the above process was repeated to determine the specific activity of 15. The radiochemical purity

of the aglucone was thus determined by comparing the values obtained for the specific activity of <u>15</u>.

<u>Kuhn-Roth Oxidation of Verbenalin(1).</u> Verbenalin(<u>1</u>) was oxidized by heating 30 - 50 mg at reflux temperatures for two hours with 5 - 10 ml of Kuhn-Roth reagent prepared by the addition of 250 ml of sulfuric acid (sp gr 1.84) to a solution of 168 g of chromium trioxide in one liter of distilled water.[85] The reaction mixture was steam distilled until at least 50 ml of distillate were collected. The steam distillate was neutralized with 0.07 N sodium hydroxide solution to determine the amount of acetic acid present. The C-methyl number for <u>1</u> was found to be 0.48 using this procedure. The C-methyl number was calculated as the moles of titrable acid per mole of <u>1</u> used. Aliquots of the previously neutralized steam distillate were evaporated at room temperature under reduced pressure, and the activity of the aliquots was determined by liquid scintillation counting. If the specific activity was high enough to warrant it, the samples of sodium acetate (usually only 1 - 3 mg) would have been purified by chromatography on a Celite column.

<u>Ozonization of Verbenalin(1).</u> Verbenalin(<u>1</u>) was dissolved (48 mg) in 10 ml of glacial acetic acid and was treated at room temperature with a stream of oxygen containing 1 - 3% ozone. The reaction time (approximately 1 minute) was controlled by observing the disappearance of the ultraviolet absorption maximum of <u>1</u>, λ_{max}^{HOAc} 248 mμ

(ε 6,900). The volume of the acetic acid solution was reduced to less than five ml at room temperature under reduced pressure; the concentrate was made basic by first treating it with a saturated solution of sodium carbonate and then with sodium hydroxide. To the basic solution, two ml of 30% hydrogen peroxide were added and the reaction mixture was stirred with a Teflon coated stirring bar for two hours. The reaction mixture was then acidified with hydrochloric acid and the excess hydrogen peroxide was decomposed by stirring the solution with five mg of Adam's platinum oxide catalyst. After filtering out the catalyst, and then salting out the solution, the aqueous solution was continuously extracted with ethyl ether for a period of 40 hours. The ethyl ether extract was dried with anhydrous magnesium sulfate, and the drying agent was removed by filtration.

The ethyl ether extract of the ozonization products was then treated with an ethereal solution of diazomethane. Gas liquid chromatographic analysis of the methyl esters was performed at 142° using a 10 ft x 1/4 in. copper column of acid-washed Chromosorb W coated with 25% Carbowax 20M with a flow rate of 100 ml/min of helium. The major peaks had relative retention values of 25:42:63 in the ratio of 74:14:12, respectively. These peaks were identified by enrichment with authentic methyl esters of oxalic acid, methylsuccinic acid and 2-methylglutaric acid, in order of their elution. The assignment of structure was confirmed[47]

by directly obtaining their mass spectra as they were eluted from the gas liquid chromatographic column using the combination Mass Spectrometer-Gas Chromatograph at 70 ev,[86] as shown in Figure 10.

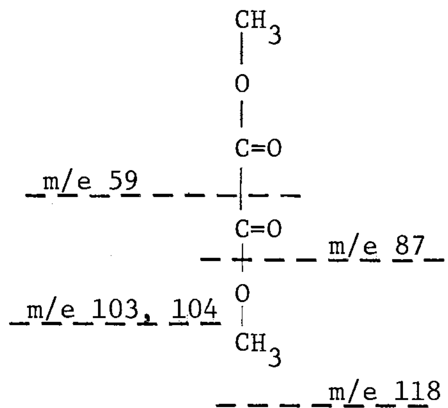
The ratio of methyl esters isolated varied with individual ozonizations, but the dimethyl oxalate was always the most abundant product.

In a subsequent ozonization, the sample size of 1 was reduced to less than one mg and treated with a stream of oxygen containing 1 - 3% ozone. The products were isolated as previously described. The methyl esters were prepared by treatment of the dibasic acids with an ethereal solution of diazomethane and then chromatographed on a 300 ft x 0.02 in. stainless steel capillary column coated with LAC 886 (ethylene glycol succinate). The identity of the products was again confirmed by comparison with known compounds.

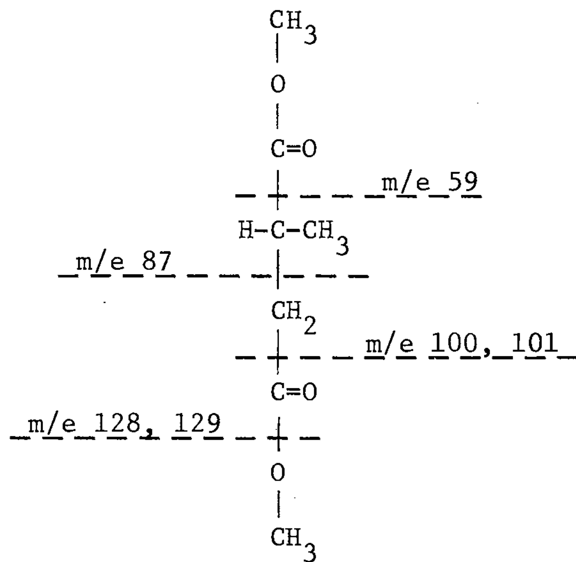
Mass Spectral Identification of Dimethyl Oxalate, Dimethyl Methylsuccinate and Dimethyl 2-Methylglutarate.

Dimethyl oxalate:

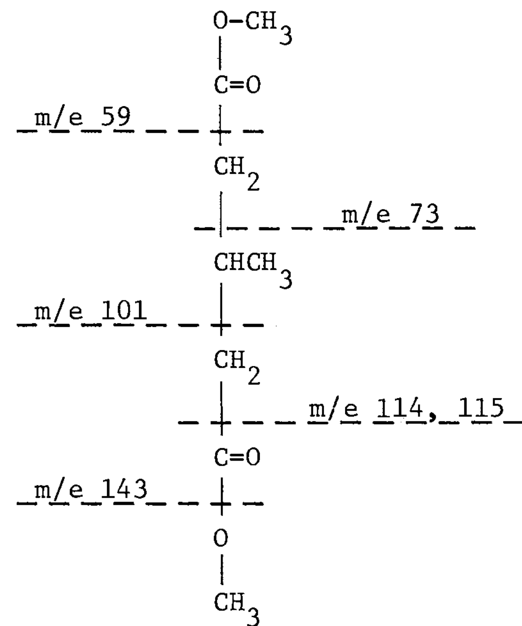
1. The molecular ion was found at m/e 118.
2. Peak at m/e 103 - due to loss of methyl group.
3. Peak at m/e 104 - due to loss of methyl group and subsequent hydrogen rearrangement.
4. Peak at m/e 87 - due to loss of methoxyl group.
5. Peak at m/e 59 - due to a rupturing of the carbon to carbon double bond to form two equal fragments.



Dimethyl oxalate



Dimethyl methylsuccinate



Dimethyl 2-methylglutarate

Figure 10. Mass Spectral Confirmation of Degradation Products

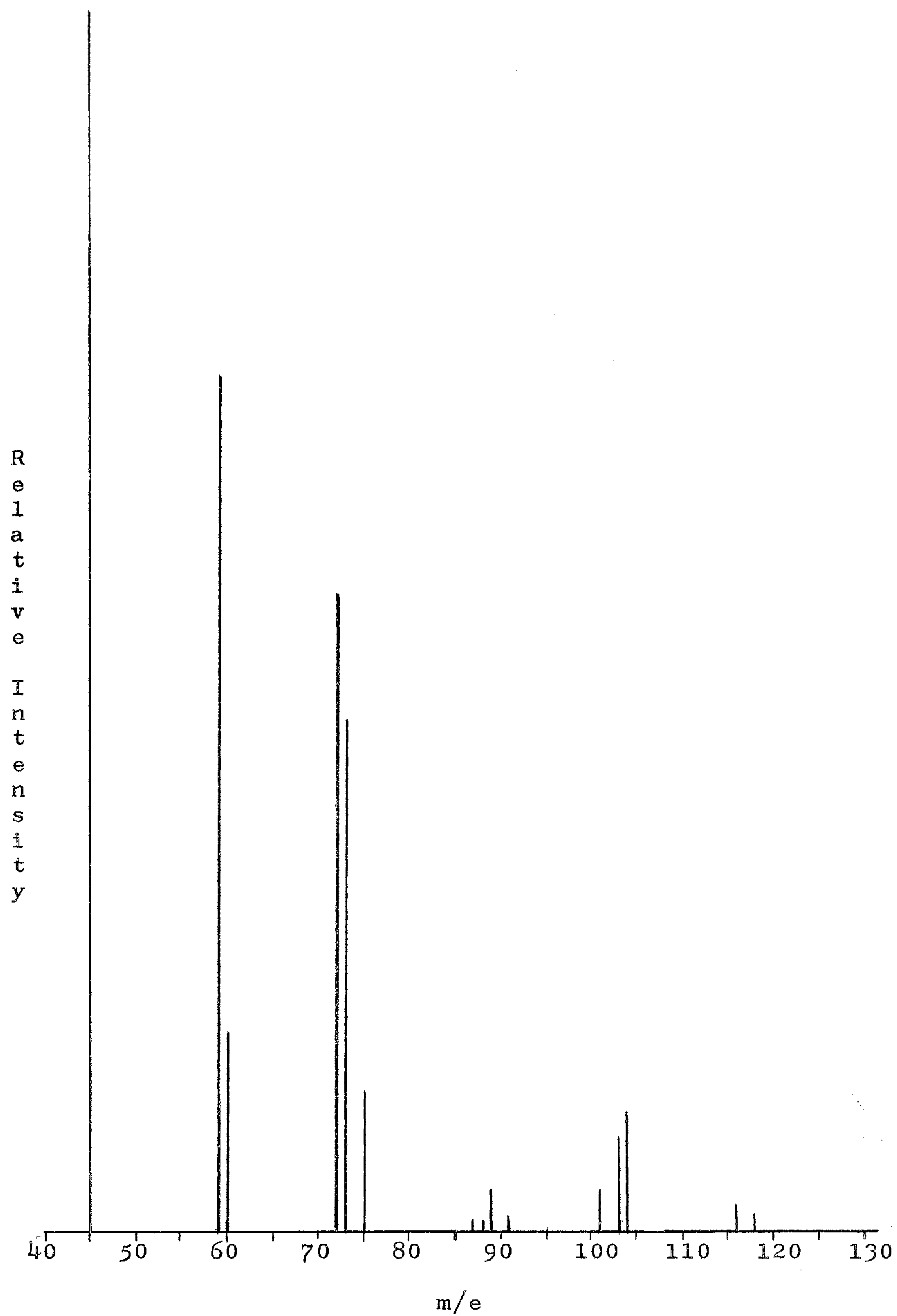


Figure 11. Partial Mass Spectrum of Dimethyl Oxalate

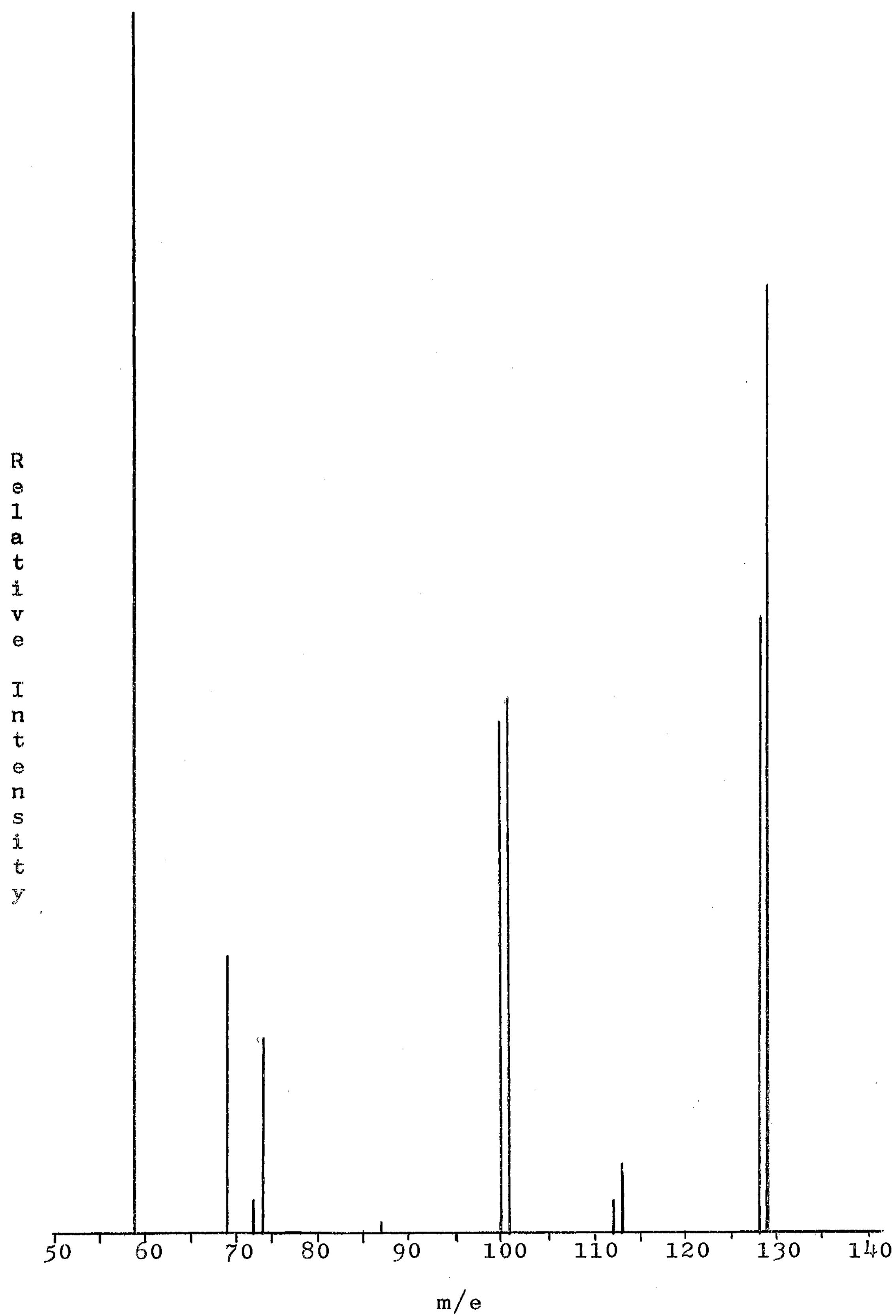


Figure 12. Partial Mass Spectrum of Dimethyl Methylsuccinate

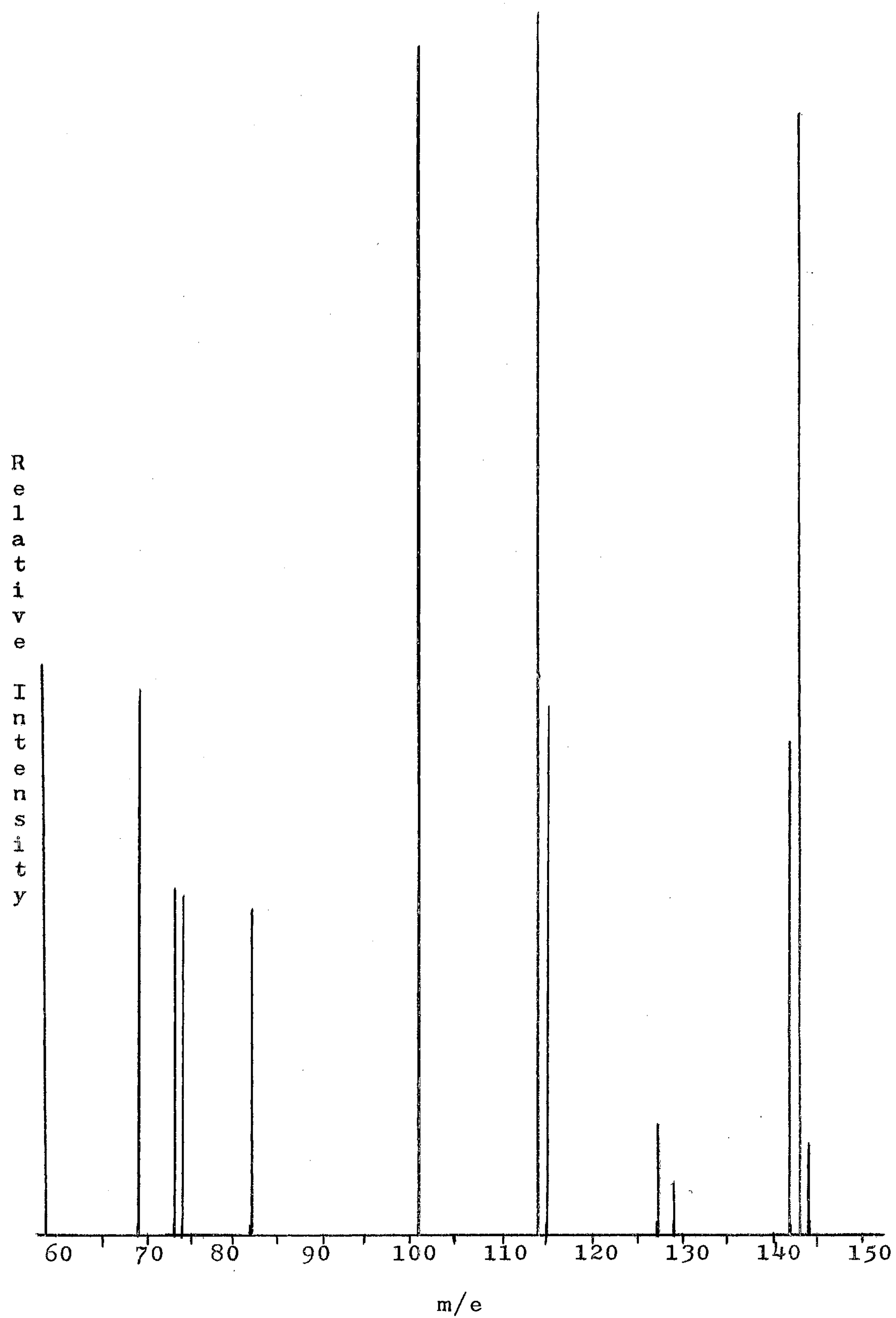


Figure 13. Partial Mass Spectrum of Dimethyl 2-Methylglutarate

Dimethyl methylsuccinate:

1. No molecular ion was found. It is also absent in the mass spectrum of the authentic ester.
2. Peak at m/e 128 - due to loss of methoxyl group.
3. Peak at m/e 129 - due to loss of methoxyl group and subsequent hydrogen rearrangement.
4. Peak at m/e 100 and m/e 101 - due to loss of the carbonyl group.
5. Peak at m/e 87 - due to loss of both the carbonyl and methylene groups.
6. Peak at m/e 59 - due to loss of carbonyl and both methylene groups.

Dimethyl 2-methylglutarate:

1. No molecular ion was found. This is consistent with the mass spectrum of the authentic ester.
2. Peak at m/e 143 - due to loss of methoxyl group.
3. Peak at m/e 114 - due to loss of carbonyl group.
4. Peak at m/e 115 - due to loss of the carbonyl group and subsequent hydrogen rearrangement.
5. Peak at m/e 101 - due to loss of the methylene group.
6. Peak at m/e 73 - due to loss of two methylene groups.
7. Peak at m/e 59 - due to loss of all three methylene groups.

Determination of the Specific Activity of the Ozonization Fragments of Verbenalin(1). Verbenalin(1) was

ozonized as described and the dimethyl esters of the three dibasic acids were made by treatment of the mixture with an ethereal solution of diazomethane. The solvent was removed by evaporation at room temperature under reduced pressure. The concentration of the esters was determined by comparison of peak areas obtained by gas liquid chromatographic analysis with known quantities of pure compounds. The specific activity of the known quantities of the esters was then determined by gas radiochromatography.

Determination of the Specific Activity of Oxalic Acid Obtained from the Ozonization of Verbenalin(1). Verbenalin (1) was ozonized as described to obtain the mixture of dibasic acids. The ethereal solution of the dibasic acids was concentrated at room temperature under reduced pressure. The ethereal extract was applied as a thin stripe to a 0.50 mm layer of silica gel $PF_{254+366}$ for preparative thin layer chromatography. Silica gel $PF_{254+366}$ does not contain a calcium sulfate binder; the medium grain size is 10 - 40μ. The layer was applied as a slurry consisting of 60 g of silica gel $PF_{254+366}$ and 120 ml of distilled water to glass plates having the following dimensions: 20 x 20 x 0.4 cm. The plates were allowed to dry for at least two days, and were then activated at 110° for two hours before use. Authentic samples of the three dibasic acids were placed adjacent to the stripe of crude extract as a marker for identification. The authentic samples were identified after development by spraying the portion of the plate

containing the authentic samples with bromphenol blue acidified with citric acid. One typical development solvent consisted of benzene, methanol, acetic acid, p-dioxane and ethyl ether (30:3:2:3:4). The R_f values obtained using this solvent system were 0.12, 0.32 and 0.35 for oxalic acid, methylsuccinic acid and 2-methylglutaric acid, respectively. Other frequently used solvent systems were benzene, methanol and acetic acid (50:2:1), and benzene, p-dioxane and acetic acid (20:6:1).

The oxalic acid was readily separated from the other components of the extract by successive preparative thin layer chromatographic purifications as previously described for the glucoside <u>1</u> and the aglucone <u>15</u>. The oxalic acid was esterified with an ethereal solution of diazomethane. The purity of the diester was monitored by gas liquid chromatography. The concentration of the dimethyl oxalate was determined by comparison of peak areas obtained by gas liquid chromatography with known quantities of dimethyl oxalate. Aliquots of the dimethyl oxalate solution of known concentration were then counted by liquid scintillation. The specific activity of the dimethyl oxalate was readily computed in this manner.

<u>Ozonization of Glucose.</u> Glucose (200 mg) was dissolved in glacial acetic acid and was treated with a stream of oxygen containing 1 - 3% ozone. Three times as much ozone was passed into the system as would have been used had an equivalent amount of <u>1</u> been ozonized. The acetic

acid solution was concentrated at room temperature under reduced pressure to less than five ml. The concentrate was made basic by first treating it with a saturated solution of sodium carbonate and then with sodium hydroxide. To the basic solution, two ml of 30% hydrogen peroxide were added and the reaction mixture was stirred with a Teflon coated stirring bar for four hours. The reaction mixture was acidified with hydrochloric acid and the excess hydrogen peroxide was decomposed by treating the reaction mixture with a solution of acidified ferrous sulfate. The aqueous solution was exhaustively extracted with ethyl ether. The ether extracts were concentrated at room temperature under reduced pressure and were then treated with an ethereal solution of diazomethane until the yellow color due to the diazomethane persisted. The extract was analyzed by gas liquid chromatography using a 10 ft x 1/4 in. copper column packed with 20% Carbowax 20M coated on acid-washed Chromosorb W. Peaks corresponding to dimethyl oxalate, dimethyl methylsuccinate and dimethyl 2-methylglutarate could not be found when the tracings of the extracts were compared to tracings of the authentic esters which had been previously chromatographed.

BIBLIOGRAPHY

1. C. J. Zufall and W. O. Richtmann, Pharm. Arch., 15, 1 (1944).

2. A. Wagner, Seifensieder-Ztg., 64, 133 (1937).

3. S. Sakai, Gifu Ika Daigak Kiyo, 11, 6 (1963).

4. L. H. Briggs, B. F. Cain, P. W. Le Quesne and J. N. Shoolery, Tet. Letters, 69 (1963).

5. J. Grimshaw and H. R. Juneja, Chem. and Ind. (London), 656 (1960).

6. M. Bobbit, D. W. Spiggle, S. Mahboob, W. von Philipsborn and H. Schmid, Tet. Letters, 321 (1962).

7. C. Djerassi, T. Nakano, A. N. James, L. H. Zalkow, E. J. Eisenbraun and J. N. Shoolery, J. Org. Chem., 26, 1192 (1961).

8. K. Sheth, E. Ramstad and J. Wolinski, Tet. Letters, 394 (1961).

9. H. Inouye, T. Arai, Y. Miyoshi and Y. Yaoi, ibid., 1031 (1963).

10. O. Halpern and H. Schmid, Helv. Chim. Acta, 41, 1109 (1958).

11. R. B. Bates, E. J. Eisenbraun and M. McElvain, J. Amer. Chem. Soc., 80, 3420 (1958).

12. G. W. K. Cavill, Australian J. Chem., 9, 288 (1956).

13. J. F. McConnell, A. McL. Mathieson and B. P. Schoenborn, Tet. Letters, 445 (1962).

14. J. Meinwald, M. S. Chadha, J. J. Hurst and T. Eisner, ibid., 29 (1962).

15. T. Sakan, A. Fryino, F. Murai, Y. Butsugan and A. Suzuki, Bull. Chem. Soc. Japan, 32, 315 (1959).

16. E. J. Eisenbraun, A. Bright and H. H. Appel, Chem. and Ind. (London), 1242 (1962).

17. G. Jones, H. M. Fales and W. C. Wildman, Tet. Letters, 397 (1963).

18. G. W. K. Cavill, Rev. Pure App. Chem., 10, 169 (1960).

19. T. Eisner, Science, 146, 1318 (1964).

20. T. Eisner, ibid., 148, 966 (1965).

21. G. W. K. Cavill, and P. L. Robertson, ibid., 149, 1337 (1965).

22. W. I. Taylor, ibid., 153, 954 (1966).

23. F. McCapra, T. Money, A. I. Scott and I. G. Wright, Chem. Comm., 1, 537 (1965).

24. H. Goeggel and D. Arigoni, ibid., 1, 538 (1965).

25. A. R. Battersby, R. T. Brown, R. S. Kapil, A. G. Plunkett and J. B. Taylor, ibid., 2, 46 (1966).

26. A. R. Battersby, R. T. Brown, J. A. Knight, J. A. Martin and A. O. Plunkett, ibid., 2, 346 (1966).

27. E. S. Hall, F. McCapra, T. Money, H. Fukumoto, J. R. Hanson, B. S. Mootoo, G. T. Phillips and A. I. Scott, ibid., 2, 348 (1966).

28. P. Loew, H. Goeggel and D. Arigoni, ibid., 2, 347 (1966).

29. E. Leete, Science, 147, 1000 (1965).

30. a) C. G. Casinovi and G. B. Marini-Bettolo, Abstract Ab-3, IUPAC Meeting, London, 1963, p. 285. b) C. G. Casinovi, G. Giovannozzi-Sermanni and G. B. Marini-Bettolo, Gazz. Chim. Ital., 94, 1356 (1964).

31. L. Bourdier, J. pharm. chim., 27, 49 (1908).

32. L. Bourdier, ibid., 27, 101 (1908).

33. B. Reichert, Arch. Pharm., 273, 357 (1935).

34. B. Reichert and W. Hoffman, ibid., 275, 474 (1937).

35. J. Cheymol, J. pharm. chim., 25, 581 (1937).

36. J. Cheymol, ibid., 26, 5 (1937).

37. J. Cheymol, Bull. soc. chim. biol., 19, 1608 (1937).

38. J. Cheymol, ibid., 19, 1626 (1937).

39. J. Cheymol, Bull. soc. chim., 5, 633 (1938).

40. P. Karrer and H Salomon, Helv. Chim. Acta, 29, 1544 (1946).

41. A. Chatterjee and L. M. Parks, J. Amer. Chem. Soc., 71, 2249 (1949).

42. J. Asano, Y. Ueno and Y. Tamaki, J. Pharm. Soc. Japan, 62, 7 (1942).

43. M. Cohn, E. Vis and P. Karrer, Helv. Chim. Acta, 37, 790 (1954).

44. R. B. Woodward, J. Amer. Chem. Soc., 63, 1123 (1941).

45. E. Winde, I. Echaust and R. Hansel, Arch. Pharm., 294, 220 (1961).

46. G. Buchi and R. E. Manning, Tet. Letters, 26, 5 (1960).

47. G. Buchi and R. E. Manning, Tetrahedron, 18, 1049 (1962).

48. J. D. Brodie, G. W. Wasson and J. W. Porter, Biochem. Biophys. Res. Commun., 8, 76 (1962).

49. J. D. Brodie, G. W. Wasson and J. W. Porter, J. Biol. Chem., 238, 1294 (1963).

50. J. D. Brodie, G. W. Wasson and J. W. Porter, ibid., 239, 1346 (1964).

51. T. T. Tchen, J. Amer. Chem. Soc., 79, 6344 (1957).

52. T. T. Tchen, J. Biol. Chem., 233, 1100 (1958).

53. K. Markley and E. Smallman, Biochim. Biophys. Acta, 47, 327 (1961).

54. H. R. Levy and G. Popjak, Biochem. J., 72, 35P (1959).

55. R. A. Salokangas, H. C. Rilling and L. T. Samuels, Biochemistry, 4, 1606 (1965).

56. S. Chaykin, J. Law, A. H. Phillips, T. T. Tchen and K. Bloch, Proc. Natl. Acad. Sci. U. S., 44, 998 (1958).

57. A. de Waard and G. Popjak, Biochem. J., 73, 410 (1959).

58. M. Lindberg, C. Yuan, A. de Waard and K. Bloch, Biochemistry, 1, 182 (1962).

59. F. Lynen and U. Henning, Angew. Chem., 72, 820 (1960).

60. B. W. Agranoff, H. Eggerer, U. Henning and F. Lynen, J. Biol. Chem., 235, 326 (1960).

61. F. Lynen, H. Eggerer, U. Henning and I. Kessel, Angew. Chem., 70, 738 (1958).

62. D. H. Shah, W. W. Cleland and J. W. Porter, J. Biol. Chem., 240, 1946 (1965).

63. A. J. Birch, M. Kocor, N. Sheppard and J. Winter, J. Chem. Soc., 1502 (1962).

64. a) G. Popjak, Biochem. J., 96, 1P (1965). b) C. Donninger and G. Popjak, Proc. Roy. Soc. (London), 163, 465 (1966).

65. a) B. L. Archer and D. Barnard, Biochem. J., 96, 1P (1965). b) B. L. Archer, D. Barnard, E. G. Cockbain, J. W. Cornforth, R. H. Cornforth and G. Popjak, Proc. Roy. Soc. (London), 163, 519 (1966).

66. a) J. W. Cornforth, Biochem. J., 96, 3P (1965). b) J. W. Cornforth, R. H. Cornforth, C. Donninger and G. Popjak, Proc. Roy. Soc. (London), 163, 492 (1966).

67. Plants kindly identified by Dr. U. T. Waterfall, Department of Botany, Oklahoma State University.

68. L. Ruzicka, Proc. Chem. Soc. (London), 341 (1959).

69. K. J. Clark, G. I. Fray, R. H. Jaeger and Sir Robert Robinson, Tetrahedron, 6, 217 (1959).

70. A. Verley, Bull. soc. chim., 17, 175 (1897).

71. G. W. K. Cavill and D. L. Ford, Australian J. Chem., 13, 296 (1960).

72. R. C. Cookson, J. Hudec, S. A. Knight and B. R. D. Whitear, Tetrahedron, 19, 1995 (1963).

73. E. J. Eisenbraun, A. G. Horodysky and G. R. Waller, Abstract, Sixth Annual Meeting of the Plant Phenolics Group of North America, Austin, Texas, April 6-8, 1966.

74. J. E. S. Huni, H. Hiltebrand, H. Schmid, D. Groger, S. Johne and K. Mothes, Experientia, 22, 656 (1966).

75. D. A. Yeowell and H. Schmid, ibid., 20, 251 (1964).

76. F. E. Regnier, G. R. Waller, E. J. Eisenbraun and H. Auda, private communications, 1967.

77. H. Auda, H. R. Juneja, E. J. Eisenbraun, G. R. Waller and R. Kays, J. Amer. Chem. Soc., in press, 1967.

78. R. H. Kenton and P. J. G. Mann, Biochem. J., 50, 360 (1952).

79. C. C. Sweeley, R. Bentley, M. Makita and W. W. Wells, J. Amer. Chem. Soc., 85, 2497 (1963).

80. Assistance during purification of isotopes kindly provided by Dr. J. Frost.

81. G. R. Waller and H. R. Juneja, private communications, 1967.

82. Purity kindly confirmed by Dr. G. V. Odell.

83. K. S. Yang, R. K. Gholson and G. R. Waller, J. Amer. Chem. Soc., 87, 4184 (1965).

84. G. A. Bray, Anal. Biochem., 1, 279 (1960).

85. E. J. Eisenbraun, S. M. McElvain and B. F. Aycock, J. Amer. Chem. Soc., 76, 607 (1954).

86. Mass spectrometry kindly performed by Dr. Ragnar Ryhage.

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