Biosynthesis of Methylcyclopentane Monoterpenoids

IV. VERBENALIN*

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SUMMARY

The biosynthesis of verbenalin (I) was studied by the administration of acetate-1-14C, acetate-2-14C, mevalonate-2-14C, and geraniol-1-14C to Verbena officinalis L. plants. When I which had been formed biosynthetically from mevalonate-2-14C was degraded, randomization of the label between carbon atoms 6 and 9 was not observed; carbon atom 6 retained most of the radioactivity in young and old plants. Randomization between carbon atoms 3 and 8 varied with age of plant. There was complete randomization in young plants, limited randomization in older plants, and essentially nonrandomization in senile plants. The percentage of radioactivity in carbon atom 3 predominated over that in carbon atom 8 as the plant age increased. The ratio of radioactivity found in aglucone to that in glucose is considered to be metabolically significant.

Certain species of Verbena, commonly known as the vervains, some of which were later found to contain verbenalin (I), have a long history of use in folk medicine (1) and perfumes (2). The pharmacological properties of Verbena officinalis L. extracts have been studied (3); however, neither I nor the plants from which it can be isolated have any current physiological importance. The reported physiological activity is possibly due to I, since the methylcyclopentane monoterpenoids possess biological activities of varying kinds (4).

Verbenalin (I), which has been isolated from V. officinalis L. and other species of Verbena (5), is one of the relatively rare methylcyclopentane monoterpenoid glucosides and is of interest because of a structural and biogenetic relationship to other members of the group, which includes asperuloside (6), aucubin (7), catalposide (8), genipin (9), loganin (10), monotropein (11), and plumieride (12). Other related methylcyclopentane monoterpenoids are nepetalactone (13), iridomymecin (14), isoiridomymecin (15), anisomorphalin (16), and a series of related alkaloids of which actinidine (17), skytanthine (18), tecomamine (19), and boeschnikaine (20) are typical examples.

The biosynthesis of I is important since it is both a glucoside and a monoterpenoid and because it may serve as a precursor to the non-indole moiety of several indole alkaloids (21). Studies have established the mevalonoid nature of a number of major indole alkaloids such as vindoline (22-24), reserpine (23), catharanthine (24), and quinine (25). Battersby (26) has reviewed the possibility of monoterpenoids serving as intermediates in their biosynthesis. The biosynthesis of loganin (27, 28), loganic acid (29), and gentiopicroside (30) have been reported. Additional work on loganin as a precursor to the indole alkaloids has also been reported (30-32).

Actinidine, having a pyridine ring, skyanthine, having the same carbon skeleton as actinidine but with a reduced pyridine ring, and nepetalactone were postulated to arise from mevalonic acid (33, 34). Mevalonic acid has been found to be a precursor of skytanthine in Skytanthus acutus M. (35, 36).

The isoprenoid skeleton of the aglucone portion of I, verbenalol (II), suggests that it could arise from an isoprenoid precursor. Selected precursors known to be on the conventional monoterpane biosynthesis pathway were examined as precursors of I.

EXPERIMENTAL PROCEDURE

Gas-Liquid Chromatography—Gas-liquid chromatographic analyses were performed with a Hewlett Packard F and M model 700 dual column gas chromatograph fitted with a thermal conductivity detector system with the use of helium as the carrier gas. Analyses of degradation products were performed on (a) a copper tube, 10 feet × ⅛ inch, packed with 60 to 80 mesh, acid-washed Chromosorb W coated with 25% Union Carbide Carbowax 20M (polyethylene glycol), (b) a stainless steel tube, 6 feet × ⅛ inch, packed with 80 to 100 mesh Gas Pack W coated with 10% General Electric Silicone Rubber SE-30, and (c) a copper tube, 10 feet × ⅛ inch, packed with less than 70 mesh acid washed firebrick coated with 15% phenyl-diethanolamine succinate.
All gas chromatography in which capillary columns were used was done with an Instruments, Inc., model 303 valved gas chromatograph equipped with a hydrogen flame detector system, and with the use of a 10-nl sample injector.

Peak areas were measured either with a planimeter, or by weighing the paper which was under the peak. Standard curves were used for quantitative determination studies.

Administration of Labeled Compounds to Verbena officinalis—
The plants were grown from seeds1 or cuttings.2 The plants used for studies in which age of plants might be a critical factor were all grown from seeds. An aseptic solution of the labeled compound was injected into the hollow internodal stem of the plant at several places with a Hamilton 10-ml syringe. The stem was vented in appropriate places to prevent expulsion of injected material. After 1 week, the plants were harvested, dried in an oven for 48 hours at 40°, weighed, and extracted.

Labeled Compounds Used for Incorporation Studies—dl-Mevalonate-2-14C, purchased from Nuclear Research Chemicals, Orlando, Florida, was converted to the free acid and purified (37).

Chromatographically pure dl-mevalonate-2-14C purchased from New England Nuclear was used for the biosynthesis of I when the labeling patterns were studied as a function of the age of plants. The purity was confirmed as above.

Acetate-1-14C and acetate-2-14C were used as purchased from Calbiochem.

Geraniol-1-14C was not commercially available and was synthesized.3 Its synthesis was accomplished by the chain extension of commercially available 0-methyl-5-heptene-2-one, through a modified Wittig reaction with the use of methyl hromoaacetate-1-14C. The geraniol-1-14C was separated from nerol-1-14C by preparative gas chromatography and was found to be radiochemically pure.4

Isotope Analyses—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. 14C activity was determined by liquid scintillation counting or gas radiochromatography or both. The liquid scintillation counting was performed in a Packard Tri-Carb model 314 liquid scintillation spectrometer, with appropriate corrections for efficiency and quenching (36, 38).

14C activity was also determined by gas radiochromatography with a Perkin-Elmer model 801 gas-liquid chromatography apparatus equipped with a full flow thermal conductivity detector system and with helium as a carrier gas. The total effluent was fed into the counting chamber of a Nuclear-Chicago model 8200 proportional gas flow counter; both the inlet line and the gas counting chamber were held at 25°. Peak areas were measured with a Nuclear-Chicago model 8350 automatic integrator module. A counting efficiency of 34% as calculated from a reference standard of n-heptane-1-14C having a specific activity of 0.2 μCi per ml was obtained. Methane at 75 ml per min was used as the counting gas. The reproducibility of the isotope analyses was such that a ±10% relative error may occur in the distribution of radioactivity as reported in Table III.

Extraction of Verbena (I) from V. officinalis—A 210-g portion of dried powdered plant material was extracted with ethyl ether in a Soxhlet extraction apparatus for 12 days. Some caramalization was observed. The extract was concentrated at room temperature under reduced pressure; the residue was washed with an ethyl ether-acetone (1:1) solution to remove gummy, green plant material from residue. The residue was extracted with hot, moist ethyl acetate, from which white crystals separated upon slow cooling. Several recrystallizations from such ethyl acetate, and from 95% ethanol-ethyl acetate (1:1), gave 252 mg of colorless plates of I, m.p. 151-152°, [α]d 20 -171°, λmax 228 μm and 269 μm (ε 9600 and 100), and Rmax 3400, 2673, 1655, and 1635 cm⁻¹ (59). These measurements were made with an O. C. Rudolph and Sons, Inc., model 50 Universal high precision polarimeter, with a 2-dm tube; a Beckman model DK recording spectrophotometer; and a Beckman IR-5A infrared spectrometer.

Isolation and Purification of Verbena (I) from V. officinalis by Thin Layer Chromatography—Glass plates, 20 x 20 x 0.4 cm, were coated with a 0.75-mm layer of Silica Gel G. The plates were dried and then activated in the oven at 110° for 2 hours.

The dried V. officinalis, weighed and ground, was extracted four times with portions of hot 95% ethanol, each portion of ethanol approximately 30 times the weight of the plant material. The ethanol extracts were combined and concentrated at room temperature under reduced pressure to approximately 5 to 5% of their original volume.

The concentrated extracts were then applied as a narrow stripe to a 0.75-mm layer of recently activated Silica Gel G. The plate was first developed with ethyl ether to remove the abundant ether-soluble lipid material. Adjacent to the strip of crude ethanolic extract an authentic sample of I was placed as a marker for identification. The plate was then developed with a combination of 2,4-dinitrophenylhydrazine and the edge of the plate was acidified 2,4-dinitrophenylhydrazine solution. The portion of the crude stripe corresponding to authentic I was scraped off and crude I was leached from the Silica Gel G with hot 95% ethanol.

The ethanol extract from the leaching operation was then concentrated at room temperature under reduced pressure to approximately the original reduced volume of the ethanolic extract applied to the thin layer plate. This concentrate was then applied to a second layer of Silica Gel G. The plate was then developed with a solution of ethyl acetate-95% ethanol (3:1). A spot having an Rf value of 0.52 was observed.

This general process was repeated, usually a minimum of 8 to 10 times, with different solvent systems, until a single spot was observed for I. Another typical solvent system contained benzene-95% ethanol-ethyl acetate (1:1:4) and gave an Rf value of 0.30 for I. No major differences in the separation were observed when Silica Gel H was substituted for Silica Gel G.

Concentration Determination of Verbena (I) in Plants Used in Biosynthesis Experiments—Verbena (I) was isolated on a microscale as described above, with a relatively small amount of ethanoic extract. The concentration of I was determined by measuring the λmax 228 μm (ε 9600).
determined as above. The radioactivity of the sample was determined by counting aliquots of the ethanolic extract through liquid scintillation counting. The remaining portion of the ethanolic extract was then concentrated and subjected to thin layer chromatographic purification. This process was repeated until constant specific activities were obtained. It should be kept in mind that the determination of specific activity has an inherent relative error of ±10%.

Hydrolysis of Verbenalin (I) to Verbenal (II)—Verbenalin (I) was treated with emulsin (purchased from Mann) in a citrate-phosphate buffer at pH 4.5. After stirring I with emulsin for 4 hours at room temperature, the aglucone II was extracted with several portions of ethyl ether. Drying the ethyl ether solution, filtering, and concentrating at room temperature under reduced pressure gave II, m.p. 130-131.5°, in 50 to 65% yield (99).

Verbenal (II) exhibited $\lambda_{\text{max}}^\text{EthOH} 239$ μm (ε 9000). In 0.01 N sodium hydroxide solution, a bathochromic shift was observed; $\lambda_{\text{max}}$ 272 μm (ε 19,000). The infrared spectrum showed $\lambda_{\text{max}}$ at 3545, 3000, 1750, 1715, and 1640 cm$^{-1}$.

Microscale Purification of Labeled Verbenal (II)—A concentrate of II in ether was obtained as described above and purified by thin layer chromatography. The plate was developed with ethyl acetate-95% ethanol (3:1). The authentic II had an $R_f$ value of 0.83. The amount of II in the stripe was determined from 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 II was concentrated at room temperature under reduced pressure and purified by chromatography on Silica Gel G. The specific activity of II was determined and its radiochemical purity was confirmed.

Kuhn-Roth Oxidation of Verbenalin (I)—Verbenalin (I) was oxidized by heating 30 to 50 mg with 5 to 10 ml of Kuhn-Roth reagent at reflux temperatures for 2 hours (40). 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 C-methyl number, which was 0.48 mole of acid per mole of I used. Aliquots of the previously neutralized steam distillate were evaporated at room temperature under reduced pressure, and radioactivity of the residues was determined by liquid scintillation counting.

Ozonization of Verbenalin (I)—Verbenalin (I) was dissolved (48 mg) in 10 ml of glacial acetic acid and treated at room temperature with a stream of oxygen containing 1 to 3% ozone. The reaction time (approximately 1 min) for I was chosen to cause the disappearance of $\lambda_{\text{max}}$ for I. Shortly after ozonization, the acetic acid solution was concentrated at room temperature under reduced pressure to less than 5 ml; the concentrate was made alkaline with a saturated solution of sodium carbonate and then with sodium hydroxide. Two milliliters of 30% hydrogen peroxide were added and the reaction mixture was stirred for 2 hours. The reaction mixture was then acidified with hydrochloric acid and the excess hydrogen peroxide was decomposed with 5 mg of Adams' platinum oxide catalyst. After filtering out the catalyst and adding salt, the aqueous solution was continuously extracted with ethyl ether for 40 hours. The ethyl ether extract was dried, filtered, and then treated with an ethereal solution of diazomethane. Gas-liquid chromatographic analysis of the resulting methyl esters was performed at 142° with the use of the 10-foot 25% Carbowax 20M column. The major peaks had relative retention times of 25, 42, and 63 min and areas in the ratio of 74:14:12, respectively. These peaks were identified by enrichment with methyl esters of oxalic acid, methylsuccinic acid, and 2-methylglutaric acid in that order, which is the order of their elution. Their identity was confirmed by directly obtaining their mass spectra as they were eluted from the gas-liquid chromatographic column in a combination mass spectrometer-gas chromatograph at 70 eV. The ratio of methyl esters varied with individual ozonizations but the dimethyl oxalate peak in the chromatogram was the largest. These experiments were carried out on 1- to 100-mg samples of I.

Determination of Specific Activity of Ozonization Fragments of Verbenalin (I)—Radioactive I was ozonized as described and the dimethyl esters of the three dibasic acids were formed as before. The yields of these were determined by comparison of peak areas obtained by gas-liquid chromatographic analysis with peak areas of known quantities of pure compounds. The specific activities of the known quantities of the esters were then determined by gas radiochromatography.

Determination of Specific Activity of Oxalic Acid Obtained from Ozonization of Verbenalin (I)—Radioactive I was ozonized as described to obtain the mixture of dibasic acids which was then applied as a thin stripe to a 0.50-mm layer of Merck Silica Gel PF 254+366 for preparative thin layer chromatography. The stripe was tagged with authentic material and the components were located after development by spraying that portion of the plate with bromphenol blue acidified with citric acid. One typical development solvent consisted of benzene methanol acetic acid-p-dioxane-ethyl ether (30:3:2:3:4).

The radioactive oxalic acid was readily separated from the other components of the crude extract by successive preparative thin layer chromatographic purifications as previously described for I and II. It was then esterified with an ethereal solution of diazomethane and its purity was determined 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. Alikeots of the dimethyl oxalate solution of known concentration were then counted by liquid scintillation. The specific activity was readily computed in this manner.

The ozonization of glucose under the same conditions failed to yield oxalic acid or the other dibasic acids previously cited.

RESULTS AND DISCUSSION

Verbenalin (I), a β-D-glucosidic methylcyclopentane monoterpenoid, was obtained in about 0.1% yield by prolonged ether extraction of dried, powdered V. officinalis L. The properties of I agreed well with reports in the literature (39).

Our preliminary studies centered around developing small scale isolation procedures so that practical results could eventually be obtained on a microquantity of radioactive material and thus lessen the need for dilutions. These techniques allow for the purification of as little as 0.1 mg of glucose from V. officinalis plants less than 3 cm tall and having a dried weight of less than 0.1 g. In particular, the absorption maxima of I were useful in developing a microassay of this compound during isolation.

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The concentrations of I within plants varying in age are shown in Table I and were fairly constant (0.12 to 0.15%) until age of 4 months. At flowering time (4½ months) the concentration of I increased to 0.24%. The concentration was always significantly higher for plants harvested in the fall and highest in the flowering plants. Thus formation of I appears to depend upon age and maturity of the plant and the season.

V. officinalis plants were examined for volatile precursors of I. The fresh plant material was steam-distilled and ether extracts of the steam distillate were analyzed by gas-liquid chromatography. No hydrocarbons or oxygenated compounds containing 5 to 10 carbon atoms which might be possible precursors to I were observed. The fresh plant material was also extracted with ether and the extract was directly analyzed by gas chromatography. Volatile materials such as geraniol, citral, and iridodial were not found. Although the presence of these possible precursors in the plant was not established, they may be present in trace quantities and possibly enzyme bound during the biosynthesis of I.

In order to learn more about the biosynthesis of I, several 14C-labeled compounds were tested. The labeled compounds were fed to V. officinalis plants, and the plants were harvested at selected intervals and extracted with ethanol. By repeatedly purifying the crude ethanol extract through preparative thin layer chromatography, it was possible to isolate I and assay it by ultraviolet spectroscopy. The radiochemical purity of I was also determined. Cleavage of I by emulsin permitted isolation of II so that its specific activity could be obtained.

When plants 4 months of age were fed 4.9 µCi of acetate-1-14C, approximately 0.004% of the total radioactivity injected into the plant was found in the aglucone II. A parallel experiment with 3.6 µCi of acetate-2-14C showed that approximately 0.007% of the total radioactivity injected into the plant was incorporated into II. These results are summarized in Table II. The fact that the sugar and aglucone moieties show different incorporation of radioactivity from 14C-labeled acetate supports the concept that their biosynthesis is independent. These data would suggest that I is formed by the reaction of II, or a related compound, with glucose (which may have formed via the tricarboxylic acid cycle and glycolysis) at a late stage of the biosynthesis.

The mean of many feeding experiments showing the incorporation of radioactivity from mevalonate-2-14C into I is presented in Table II. The sugar moiety incorporated radioactivity from mevalonate-2-14C, although the ratio of the activity of the glucose moiety to that of the aglucone moiety was somewhat less than with 14C-labeled acetate. The efficiency of incorporation of mevalonate-2-14C into the aglucone portion is higher than that of 14C-labeled acetate. This result strongly suggests that the aglucone moiety is formed by a conventional terpene biosynthesis pathway.

The methods and precursor molecules used in introducing a label to V. officinalis plants can influence the rate of uptake. When mevalonate-2-14C was injected into the base of the plant from which one or several stems protruded, about 4 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 3 hours were required for the translocation of the radioactivity to the growing tips. Injections were usually made at several sites. When 7.5 µCi of geraniol-1-14C were injected into flowering plants 43 months of age, incorporation (0.006% into II) was observed, but translocation of 14C radioactivity did not seem to occur. One day after feeding geraniol-1-14C into the stems of the plant, no radioactivity could be detected about the growing tips of the plant. Perhaps free geraniol is not on the direct biosynthetic pathway for the formation of I; geranyl pyrophosphate is the more likely biological intermediate and might be expected to yield a much higher percentage incorporation than free geraniol, if it can be translocated to the site of synthesis.

It can be predicted on the basis of the isoprenoid structure of II that the radioactivity incorporated from mevalonate-2-14C should appear in carbon atoms 3 or 8 (or both) and carbon atoms 6 or 9 (or both) if an isoprenoid pathway is followed in its formation. A combination of the Kuhn-Roth oxidation and ozonization reactions was used to determine the location of the labeled carbon atoms.

The verbenalin (I) obtained from mature flowering plants fed mevalonate-2-14C was purified by preparative thin layer chromatography. A portion of the radioactive I was then diluted and the acetone extract was isolated from the Kuhn-Roth oxidation (40) of I. Only 4% of the radioactivity in the aglucone moiety of I was found in carbon atoms 7 and 9. Consequently, less than 4% of the radioactivity should appear in carbon atom 9 of II, and it must be assumed that some randomization is taking place. However, this is probably a maximum.
value. The absence of label at positions other than 3, 6, 7, and 9 of the monoterpane has been assumed in view of the 4% label at C-6 as well as the considerable labeling of the glucose portion of verbenalin. It is not possible at this time to rigorously settle the question of randomization. These data are summarized in Table III and are illustrated in Structure III:

Another portion of radioactive I obtained from plants which had been fed mevalonate-2-14C was likewise diluted, and degraded with ozone to smaller fragments, as shown in Scheme I. The formation of oxalic, methylsuccinic, and 2-methylglutaric acids as products of this oxidative degradation was confirmed by mass spectrometry of their dimethyl esters. The specific activities of these fragments were then determined by gas radiochromatography; the results are summarized in Table III. The dimethyl oxalate obtained from carbon atoms 4 and 8 of I was found to contain 7% of the radioactivity of the aglucone portion of I. Carbon atom 8 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 may be shown that approximately 46% of the radioactivity appeared in carbon atom 6, as illustrated in Structure III. These data are the predicted result if no, or very limited, randomization of 14C label took place during biosynthesis of I. However, a report of random distribution of 14C label in positions 3 and 15 of plumieride (IV) which are equivalent to positions 3 and 8 of III has appeared (41). It has also been reported (42) that essentially complete randomization of 14C label was observed at positions 3 (18 to 20%) and 8 (23 to 26%) for verbenalin (I) when small shoots were used. This report was in contrast to our initial findings which showed little randomization of label. As the plant becomes senile (past flowering (4 to 41 months, 8% at C-8) takes place, a more selective label distribution pattern appears. As the plant becomes senile (past flowering and woody), incorporation of radioactivity decreases.

Verbenalin (I) obtained from each set of plants was hydrolyzed by emulsin to obtain II for specific activity determination. The verbenalin (I) obtained from the 54-month-old plants was not degraded since the low percentage of radioactivity incorporation into II resulted in a low specific activity. Portions of the radioactive glucoside obtained from the four remaining sets of plants were diluted. Each of the four portions was then degraded by Kuhn-Roth oxidation to obtain acetic acid. The radioactivity appearing in carbon atoms 7 and 9 of I contained 3.8, 4.5, 4.6, and 3.6% of the activity in the aglucone portion of the molecule for 1-, 2-, 3-, and 4-month-old plants. Thus, carbon atom 9 had approximately 4% of the radioactivity and by difference, carbon atom 6 would have approximately 46% of the radioactivity in each of the four sets of plants. Thus no change in randomization of radioactivity between carbon atoms 6 and 9 was observed in young, mature, or senile plants. The results are summarized in Table IV.

Additional portions of I obtained from the four sets of plants fed mevalonate-2-14C were diluted and degraded by ozone to determine the randomization of radioactivity at carbon atoms 3 and 8. The four portions were then degraded to obtain oxalic acid. The other acids, although present as oxidation products, were obtained in amounts insufficient to measure their specific activities. The oxalic acid was purifed and converted to the dimethyl ester. The radioactivity appearing in carbon atoms 4 and 8 contained 22, 23, 18, and 8% of the radioactivity of the aglucone of I, as summarized in Table IV. By difference, carbon atom 3 has 28, 27, 32, and 42% of the radioactivity for plants aged 1, 2, 3, and 4 months. These data point to considerable randomization for young plants (1 to 2 months, 23% at C-8). However, as the plant reaches maturity, and flowering (4 to 41 months, 8% at C-8) takes place, a more selective labeling pattern appears. As the plant becomes senile (past flowering and woody), incorporation of radioactivity decreases.

Yeowell and Schmid (41) proposed that randomization between carbon atoms 3 and 15 of IV occurred after ring closure of the 5-membered terpenoid moiety. Randomization of the terminal methyl label (3 and 8 of III or 3 and 15 of V) might occur if these carbon atoms (25% of the radioactivity in each) became part of equivalent methyl, hydroxymethyl, formyl, or carboxyl groups (41, 42). This change in randomization of the
two terminal methyl groups, as a function of age of plant, of an intermediate has also been observed in the biosynthesis of skkyanthine isomers with the use of mevalonate-2-14C as a precursor (36). Randomization between carbon atoms 3 and 8 of V was found in young V. officinalis plants; essentially non-randomization between carbon atoms 3 and 8 was confirmed in older plants as shown in VI.

The metabolic control of randomization of carbon atoms 3 and 8 of I as related to pool size should be considered. The young plant may have a larger pool of monoterpenoid intermediate, and a relatively small amount of methylevelylocopentane monoterpenoid; the two terminal methyl groups have an opportunity to become equivalent. In the old plant, the pool size of the intermediate may decrease as the concentration of the methylevelylocopentane monoterpenoid increases and the two terminal methyl groups (e.g. geraniol or geranyl pyrophosphate) may have less opportunity to become equivalent. It should be noted that young V. officinalis plants have only small amounts of I (approximately 0.1% as shown in Table I); the concentration of I increases with the age of plant and reaches a maximum at flowering (approximately 0.3%). The greatest change in level of enzymes has been reported to occur in pea plants of various ages (43, 44). Our data support the mechanism proposed for the isomerization of isopentenyl pyrophosphate (45). Such isomerization to dimethylallyl pyrophosphate does not result in the loss of identity of the group originally present in isopentenyl pyrophosphate; the methylene group of isopentenyl pyrophosphate retains its identity. In young and old plants of V. officinalis, significant randomization between carbon atoms 6 and 9 of I did not occur, carbon atom 6 retaining most of the radioactivity.

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REFERENCES

2. WAGNER, A., Seisen-Uise-Kette-Wachse, 64, 133 (1957).
3. SAKAI, S., Gifu Kenritsu Ika Daigaku Kiyo, 11, 6 (1963); Chem. Abst., 60, 10384g (1964).