The Biosynthesis of Geraniol and Citronellol in *Pelargonium roseum* Bourbon[1]

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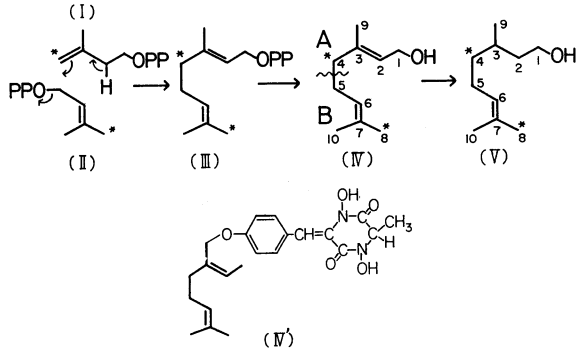
Geraniol and citronellol were biosynthesized from [2-^{14}C]mevalonic acid by the leaves of *Pelargonium roseum* Bourbon. The degradation of the radioactive specimens revealed that the major radioactivity (70—80%) was located at C-4 in the skeleton derived presumably from isopentenyl pyrophosphate and the rest of the activity at C-8 (and/or C-10) in the moiety from 3,3-dimethylallyl pyrophosphate. The unbalance of labeling was distinct from the result observed for the monoterpenes biosynthesized from the same labeled precursor by the flowers of the rose. A discussion is given on factors causing such a discrepancy in the labeling pattern owing to the difference of the plant and/or the organ. Time-course experiments with the use of [2-^{14}C]mevalonic acid indicated the formation of geraniol prior to citronellol both in the intact plant and in the cell-free extract.

The biosynthesis of monoterpenes is said to involve the conversion of mevalonic acid (MVA) into isopentenyl pyrophosphate (IPP) (I) and 3,3-dimethylallyl pyrophosphate (DMAPP) (II), followed by the condensation of IPP (I) with DMAPP (II) directed toward the formation of geranyl pyrophosphate (III), the presumed precursor of monoterpenes. Incorporations of [2-^{14}C]MVA into geraniol (IV) and nerol and their β-glucosides in the petals of *Rosa dilecta*[2,3] and into pyrethrins in the flower heads of *Chrysanthemum cinerariaefolium*[4] have been reported to result in the almost balanced pattern of labeling in the parts generated from IPP (I) and DMAPP (II) respectively. The geranyl moiety of the fungal product, mycelianamide (IV'), has been shown to possess a labeling pattern resulting from the equilibration of a tracer from [2-^{14}C]MVA between the C-5 units.[5] On the other hand, the biosynthesis of cyclic monoterpenes, thujone,[6] camphor,[7] and pulegone,[8] in the leaves and stems of several higher plants has been reported to localize the tracer mainly in the part derived from IPP (I), although one exception has been observed for linalool, an acyclic monoterpene, which had the almost symmetrical pattern of labeling.[9,10] Recently, the symmetrical labeling was also observed for geraniol (IV) biosynthesized in the leaves of *Pelargonium graveolens* Ait.[3] We now have tested the labeling pattern in geraniol (IV) biosynthesized from [2-^{14}C]MVA in the leaves of the geranium, *Pelargonium roseum* Bourbon, as well as the labeling pattern of citronellol (V), the main component of an essential oil of the plant. Contrary to Banthorpe's result,[3] we observed the unbalanced pattern of labeling for both geraniol (IV) and citronellol (V).



PPO = a pyrophosphate group; * = ^{14}C

Scheme 1.

Results

Time-course of the Incorporation of a Tracer into Geraniol (IV) and Citronellol (V). Feeding experiments were carried out on the intact plant tissue and the cell-free extract obtained from *P. roseum* Bourbon. The incorporation of [2-^{14}C]MVA to the small branches of *P. roseum* Bourbon for periods of 2 hr to 2 days resulted in an accumulation of the tracer in the plant oil. The radioactivity of the monoterpenes in the plant oil was determined by thin-layer plate radiochromatography. Table 1 shows the radioactivities of geraniol (IV) and

TABLE 1. INCORPORATIONS OF [2-^{14}C]MVA INTO GERANIOL (IV) AND CITRONELLOL (V) IN THE INTACT PLANT OF *Pelargonium roseum* Bourbon

Time of feeding (hr)	Radioactivities (cpm)		A_V/A_{IV}[a]
	Geraniol (IV)	Citronellol (V)	
2	29	42	1.45
4	57	93	1.63
8.5	60	132	2.20
24	120	490	4.08
48	88	450	5.11

a) A_V and A_{IV} denote the radioactivities of citronellol (V) and geraniol (IV) respectively.

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Table 2. Incorporations of [2-^{14}C]MVA into geraniol (IV) and citronellol (V) in the cell-free extract from *Pelargonium roseum* Bourbon

Time of incubation (hr)	Radioactivities (cpm) Geraniol (IV)	Citronellol (V)	A_V/A_{IV}[a)]
2	156	82	0.53
24	100	84	0.84

a) A_V and A_{IV} denote the radioactivities of citronellol (V) and geraniol (IV) respectively.

citronellol (V) and the ratio (A_V/A_{IV}) of the activities of IV and V at predetermined time intervals after feeding. The contents of geraniol (IV) and citronellol (V) were estimated to be 23.6 and 33.3% of the total oil by means of gas chromatography respectively.

The cell-free extract prepared from *P. roseum* Bourbon was found to transform [2-^{14}C]MVA into the monoterpenes, geraniol (IV) and citronellol (V). Table 2 shows the radioactivities of IV and V and the ratio (A_V/A_{IV}) of their radioactivity at 2 hr and at 24 hr after feeding of the tracer.

Labeling Patterns. By means of elution chromatography on a silica gel column, geraniol (IV) and citronellol (V) were isolated from the leaf oil of *P. roseum* Bourbon to which [2-^{14}C]MVA had been administered. Incorporations of the MVA into IV and V were 0.038 and 0.041%, respectively, of the administered tracer.

Radioactive geraniol (IV) was degraded to acetone containing a tracer from C-7, C-8, and C-10 and to levulinic acid containing a tracer from C-3~C-6 and C-9 by permanganate-periodate oxidation.[11-13] By hypoiodite oxidation, then, the acetone was degraded to iodoform containing a tracer from C-8 (and/or C-10) and to acetic acid originated from C-7 and C-8 (and/or C-7 and C-10), while the levulinic acid to iodoform originated from C-9 and to succinic acid from C-3~C-6. This acid was further cleaved into carbon dioxide originated from C-3 and C-6 and into ethylenediamine from C-4 and C-5 by the Schmidt reaction.[14] The all degradation products, after the conversion into the solid derivative when the product is a liquid, were purified by recrystallization or sublimation. The purified degradation products and geraniol (IV) were converted to barium carbonate by Van Slyke-Folch oxidation[15] in order to determine their specific radioactivities. The results are shown in Table 3.

By permanganate-periodate oxidation,[11-13] radioactive citronellol (V) was degraded to acetone containing a tracer from C-7, C-8, and C-10 and to 6-hydroxy-4-methylhexanoic acid (VI) originated from C-1~C-6 and C-9. Acetone was degraded to iodoform

Table 3. Specific activities of geraniol (IV) biosynthesized from [2-^{14}C]MVA by the intact plant of *Pelargonium roseum* Bourbon and of its degradation products

Compounds (Carbons originated from IV)	Specific activities, dpm × 10^{-3}/mmol	(%)
Geraniol (C-1~C-10)	271	(100)
Levulinic acid (C-3~C-6 and C-9)	220	(81.3)
Iodoform (C-9)	4.2	(1.9)
Succinic acid (C-3~C-6)	210	(77.5)
Ethylenediamine (C-4 and C-5)	197	(72.7)
Carbon dioxide (C-3 and C-6)	14.5	(5.4)
Acetone (C-7,C-8, and C-10)	—	—
Iodoform (C-8 and/or C-10)	25.7	(9.5)
Acetic acid (C-7 and C-8, and/or C-7 and C-10)	23.6	(8.7)

Table 4. Specific activities of citronellol (V) biosynthesized from [2-^{14}C]MVA by the intact plant of *Pelargonium roseum* Bourbon and of its degradation products

Compounds (Carbons originated from V)	Specific activities, dpm × 10^{-3}/mmol	(%)
Citronellol (C-1~C-10)	701	(100)
6-Hydroxy-4-methylhexanoic acid (C-1~C-6 and C-9)	474	(67.6)
Acetone (C-7, C-8, and C-10)	72.3	(10.3)
Iodoform (C-8 and/or C-10)	78.2	(11.2)

containing a tracer from C-8 (and/or C-10) by hypoiodite oxidation. The radioactivities of the degradation products were assayed by counting aliquots of barium carbonate which was prepared from the products, after purified similarly, in the same manner as above. The results are shown in Table 4.

Discussion

The maximum incorporation of a tracer from [2-^{14}C]-MVA into geraniol and nerol of the petals of *Rosa dilecta* has been reported to be observed within 1 hr of feeding.[16] On the other hand, the incorporation of a tracer from the ^{14}C-labeled MVA into geraniol (IV) and citronellol (V) by the intact plant tissues of *P. roseum* Bourbon resulted in the increase in their radioactivities with time of feeding and then the maximum activity in 24 hr, as is shown in Table 1. The results of the incorporations are, although low, similar to those found in analogous experiments.[6-10,17] The low incorporation probably is not due to a rapid passage of the tracer through geraniol (IV) and citronellol (V), since the maximum in the radioactivity was observed for the compounds in the time-course examination, as is shown in Table 1. The increase in the activity ratio of A_V/A_{IV} with time of feeding of a tracer was observed for incubations not only in the intact plant

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tissue but also in the cell-free extract, as is shown in Tables 1 and 2. The results indicate the formation of geraniol (IV) prior to that of citronellol (V) and subsequently the transformation of IV to V. This biosynthetic sequence may be supported by the fact that a geraniol-nerol reductase, which converts both geraniol and nerol into citronellol (V), has been isolated from the rose petals.[18]

The extensive degradation of geraniol (IV) biosynthesized from [2-^{14}C]MVA in the leaves of *P. roseum* Bourbon revealed, as is shown in Table 3, that more than 90 per cent of the total radioactivity was detected in ethylenediamine and iodoform derived respectively from C-4 and C-5 and from C-8 (and/or C-10) of IV. Since C-1, C-2, C-3, and C-9 were almost unlabeled and the rest of the C_5-isoprene unit is nothing other than C-4, the main site of labeling on geraniol (IV) is considered to be C-4 rather than C-5. Thus, the pattern of labeling observed for geraniol (IV) is consistent with the biosynthetic pathway depicted in the Scheme, which shows the formation of geraniol (IV) *via* geranyl pyrophosphate generated from the condensation of IPP (I) with DMAPP (II). Although the sites of labeling accord with the biosynthetic pathway shown in the Scheme, the radioactivities of C-4 and C-8 derived respectively from IPP (I) and DMAPP (II) were unbalanced; the former contained more than 70% of the total activity and the latter did only 20%. Such unbalanced labeling that the tracer predominantly resided in the moiety A of geraniol (IV) was in contrast to the balanced pattern of labeling in the moieties A and B of IV biosynthesized in the petals of the rose.[2,3]

The results of the degradation of citronellol (V) revealed that acetone and 6-hydroxy-4-methylhexanoic acid (VI) contained respectively about 10 and 70% of the total activity, as is shown in Table 4. The acetone and the hydroxy acid (VI) contain a tracer from C-8 (and/or C-10) and C-4, respectively, which should be labeled by a tracer from [2-^{14}C]MVA as shown in the Scheme. The unbalanced pattern of labeling in two positions observed in citronellol (V) is similar to that in geraniol (IV) described above.

The unbalanced labeling thus found in geraniol (IV) and citronellol (V) biosynthesized by the leaves of *P. roseum* Bourbon is of interest, since the phenomenon has not been observed yet for the polyisoprenoids, triterpenes and steroids;[19,20] these are said to be biosynthesized *via* the same precursor, geranyl pyrophosphate, as that in geraniol (IV). Such an unbalanced pattern of labeling may be rationalized in terms of the operation of several factors: a) a pool of DMAPP (II) in the plant may exist which can react with IPP (I) generated from exogenous radioactive MVA before the labeled IPP (I) can be isomerized to DMAPP (II), b) DMAPP (II) may not be the direct mevalonoid origin, c) compartmentation effects may intervene, and d) the excess of MVA unavoidably used may inhibit IPP-isomerase.[21] The incorporation of [2-^{14}C]-MVA into geraniol and nerol[2,3] in the petals of *Rosa dileca* and into pyrethrins[4] in *Chrysanthemum cinerariaefolium* resulted in the nearly balanced pattern of labeling in the moieties derived from IPP (I) and DMAPP (II). In the biosynthesis in leaves of several higher plants, on the contrary, the unbalanced pattern of labeling has been observed in thujane derivatives,[6] camphor,[7] pulegone,[8] and artemisia ketone,[22] as well as in geraniol (IV) and citronellol (V) as described above. These findings related to the labeling pattern suggest that the nature of the synthetic site in the leaves of the higher plants may be different from that in the petals. The presence of an oil gland in the leaves seem to be responsible for the unbalanced labeling. Our presently reported examples of the asymmetrical pattern of labeling differ from a recent report for geraniol (IV) biosynthesized by *P. graveolens*.[3] This discrepancy seems to be due to differences in the plants used, although both the plants belong to Geraniaceae, in the growth stage of the plants employed, and in feeding conditions of the precursor.

Experimental

Materials. Specimens of *P. roseum* Bourbon were grown from a young shoot obtained from the experimental farm of the Soda Perfumery Co., Ehime Prefecture, Japan. The small terminal branches (*ca.* 7 cm long) of the 15 week-old plants, grown outdoors from April to October, were used for feeding experiments. 3-RS-[2-^{14}C]MVA was obtained from the Daiichi Pure Chemicals Co., Ltd. (Tokyo) as the dibenzylethylenediamine salt. Adenosine triphosphate (ATP) was purchased from the Sigma Chemical Co., (St Louis, Mo., U.S.A.) as Sigma Grade Products.

Chromatographic Analyses. Tlc plates were prepared as follows: the end of a glass plate coated with silica gel G (0.25 mm; Merck) were dipped into 10% silver nitrate solution to permeate the solution through the plate and then the plate was dried at 105 °C for 45 min. Upon developing with a mixture of *n*-hexane and ethyl acetate (85: 15 or 50: 50 by volume, respectively), the plate resulted in the good separation of geraniol (IV) (R_f value 0.10 or 0.43) and citronellol (V) (R_f value 0.33 or 0.70). Column chromatography of the steam-volatile oil was performed on silica gel impregnated with 3% (w/w) silver nitrate, using a mixture of *n*-hexane with successively increasing amounts of ethyl acetate (0—25% by volume). Gas-liquid chromatographic analyses were made using a Hitachi Perkin-Elmer F6-D instrument attached with a column (1 m×3 mm) packed with 20% PEG-6000 on Celite (60—80 mesh) at 145 °C.

Measurements of Radioactivities. Thin-layer plate radiochromatograms were taken on an Aloka (Tokyo) JTC-203 instrument at a slit width of 1.5 mm. Radioactivities of geraniol (IV) and citronellol (V), shown in Tables 1 and 2, were determined by cutting the corresponding areas on the thin layer radiochromatogram obtained in the manner as mentioned above to weigh their pieces of paper and then by comparing the peak areas with those obtained with geraniol having the known radioactivity. In order to assay the specific activity, geraniol, citronellol, and all their degradation products were converted into barium carbonate by Van Slyke-Folch oxidation,[15] and aliquots of the barium carbonate

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were counted on planchets at an infinite thickness under an Aloka (Tokyo) 2π-gas-flow low background counter, Model TDC R1361. The counting error for the values shown in Tables 3 and 4 is about $\pm 3\%$.

Incorporations of Tracer. *a) With Intact Plant Tissue*: The stems in the nearly same size (each 6 g) were cut under water, and a phosphate-buffered solution (5 ml, pH 7.3) of [2-^{14}C]MVA (20 μCi, 4 μmol) and ATP (4 mg) was fed through a cut-stem into twigs. Just after the tracer solution had been absorbed, two aliquots (each 0.5 ml) of water were given for completely sucking up all the tracer in the twigs. The time required for the complete administration of the tracer was *ca.* 1 hr. The twigs were then maintained under outdoor conditions on water. At predetermined intervals, two twigs were ground with solid carbon dioxide in a mortor and the crushed plant tissues were then steam-distilled. The yield of the volatile oil was 0.3—0.4% (w/w) of the plant employed, and the contents of geraniol (IV) and citronellol (V) were estimated by glc to be 23.6 and 33.3% of the total plant oil respectively. The oil was chromatographed on the tlc plate coated with silica gel G impregnated with silver nitrate. The plate was then subjected to thin-layer plate radiochromatography for counting radioactivities of the separated constituents. The results are shown in Table 1.

b) With Cell-free Extract: The leaves (30 g) of the geranium were ground with 0.1 M phosphate buffer (pH 7.3, 30 ml) and solid carbon dioxide in a mortar. The resulting slurry was filtered through a cheese cloth to give a green homogenate (30 ml). The homogenate was centrifuged for 30 min at 14000$\times$g and 0 °C. The supernatant film and the precipitate were discarded and the clear middle layer (20 ml) was used as a cell-free extract for incubations. [2-^{14}C]MVA (2 μCi, 0.4 μmol) dissolved in 1 ml of 0.1 M phosphate buffer (pH 7.3) containing 8 mg of ATP was incubated with the cell-free extract at 25 °C for 2 hr and for 24 hr with shaking in the air. The enzymic reaction was stopped by adding 2 M hydrochloric acid and then by heating the mixture for 3 min at 70 °C. The extraction of the mixture with *n*-hexane afforded, after removal of the solvent, an oily product; this was subjected to thin-layer plate radiochromatography. The results are shown in Table 2.

Labeling Patterns. Radioactive geraniol (IV) and citronellol (V) used for determining the labeling patterns were obtained as follows. A phosphate-buffered solution (4 ml, pH 7.3) of [2-^{14}C]MVA (0.1 mCi, 17 μmol) and ATP (5 mmol) was fed through a cut-stem into the small terminal branches (60 g) of *P. roseum* Bourbon for 4 hr. The leaves and stems were then subjected to steam distillation. The steam-volatile oil thus obtained (260 mg) was separated by a combination of column chromatography and tlc to give geraniol (IV) (24 mg) and citronellol (V) (33 mg). These were judged to be chemically and radiochemically pure on the basis of glc, tlc, and thin-layer plate radiochromatography.

a) Degradation of Geraniol (IV): Radioactive geraniol (206 mg) diluted 29 times with the carrier was oxidized with a permanganate-periodate reagent for 24 hr following the method reported in the literatures.[11-13] After the residual oxidant had been decomposed with sodium bisulfite, the weakly alkaline solution was subjected to steam distillation. After the residual alkaline solution was acidified with diluted sulfuric acid, the solution was extracted with ether by a continuous extractor to afford crude levulinic acid (70 mg). This acid was chromatographed repeatedly on a thin layer plate coated with silica gel G by a mixture of chloroform, acetone, methanol, and formic acid (45: 5: 5: 1 by volume) to give pure levulinic acid (39 mg; its *p*-bromophenacyl ester, mp 83—84 °C; lit,[23] mp 84 °C). On the other hand, the aqueous distillate was subjected to hypoiodite oxidation. Iodoform (290 mg) filtered off was purified by sublimation under reduced pressure. The filtrate was acidified with diluted sulfuric acid and then treated with silver sulfate to remove iodine liberated. The aqueous mixture was then steam-distilled. The evaporation of the neutralized distillate afforded acetic acid as the sodium salt, which was further converted into the silver salt; this salt was purified by recrystallization from ethanol–water.

The levulinic acid (100 mg) was oxidized with potassium hypoiodite, which was prepared by adding sufficiently a solution of potassium iodide and iodine (2: 1) to 14 ml of 5% potassium hydroxide until the color persisted. Iodoform (189 mg) filtered off was purified by sublimation under reduced pressure. The filtrate was acidified with diluted sulfuric acid and treated with silver sulfate to remove iodine. The continuous extraction of the aqueous solution with ether afforded crude succinic acid (84 mg). After washed with chloroform, the acid was recrystallized from water and then it melted at 184—186 °C. The succinic acid was degraded to ethylenediamine and carbon dioxide by a modified method[14] of the Schmidt reaction. The carbon dioxide was trapped as barium carbonate.

b) Degradation of Citronellol (V): Radioactive citronellol (220 mg) diluted 20 times with the carrier was oxidized with a permanganate-periodate reagent[11-13] for 6 hr as above. After the same treatment es in geraniol (IV), the mixture was subjected to steam distillation. The continuous extraction of the residual solution, after it was acidified with diluted sulfuric acid, with ether afforded crude 6-hydroxy-4-methylhexanoic acid (VI) (195 mg). The methyl ester of the acid was converted to its 3,5-dinitrobenzoate, mp 145—146 °C (lit,[11] mp 145—147 °C). A half of the aqueous distillate was subjected to hypoiodite oxidation. The yellow precipitate filtered off was sublimated under reduced pressure to give pure iodoform, mp 118—119 °C. From another half of the aqueous distillate, acetone was isolated as the 2,4-dinitrophenylhydrazone derivative, mp 125—126 °C.

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