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BIOSYNTHESIS OF MONOTERPENES IN *CYMBOPOGON WINTERIANUS*

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Key Word Index—*Cymbopogon winterianus*; Gramineae; biosynthesis; monoterpenes; geraniol; citronellol; citronellal.

Abstract—The isotope ratios in geraniol, citronellol and citronellal biosynthesized in *Cymbopogon winterianus* from ^{3}H- and ^{14}C-labelled mevalonate indicate that geraniol is first converted into citronellol which in turn is converted into citronellal.

INTRODUCTION

The essential oil of a non-Indian sample of *Cymbopogon winterianus* Jowitt contains citronellal (32.7 %), citronellol (12.5 %), geraniol (23.9 %), limonene (1.3 %), linalool (1.5 %), linalyl acetate (2.0 %), caryophyllene (2.1 %), citronellyl acetate (3.0 %), geranyl formate (2.5 %), geranylacetate (3.2 %), nerol (7.7 %), elemol (6.0 %), methyl isoeugenol (2.3 %), farnesol (0.6 %), and trace amount of methyl heptenone, citronellyl butyrate and methyl eugenol [1]. The chemical compositions of essential oils in aromatic plants vary tremendously and the percentages shown above are included to give an idea of the relative amounts of the constituents present in the oil of this plant species. The essential oils of species cultivated in India have a very similar composition.

The biosynthesis of citronellol and citronellal in higher plants, probably involves the biogenetic equivalent of geraniol. Four main routes are possible (Scheme 1), i.e. geraniol (**3**) → citronellol (**6**); **3** → citronellal (**5**) → **6**; **3** → citral (**4**) → **5** → **6**; **3** → **6** → **5**. I have attempted to establish which of these pathways operate for the biosynthesis of **3**, **5** and **6** in the aerial parts of *C. winterianus*. A number of biosynthetic investigations have been carried out on geraniol [2–5], citronellol [5–7] and citronellal [7], but the conversions of these monoterpenes in one plant species has not been reported so far.

RESULTS AND DISCUSSION

In general, geraniol (and monoterpenes derived therefrom) formed in the higher plants from [2-^{14}C]MVA is predominantly labelled at C-4, i.e. only the C_5 unit derived from isopentenyl pyrophosphate (IPP) is appreciably labelled. This is believed to be due the presence of a metabolic pool of 3,3-dimethylallylpyrophosphate (DMAPP) [8]. This asymmetry of labelling was also found for geraniol, citronellol and citronellal in *C. winterianus* (Table 1, Expt 1). This phenomenon of asymmetric labelling can be exploited for specifically labelling monoterpenes at C-1, C-2, C-3, C-4 and C-10. In order to elucidate the problems outlined it was necessary to supply exogenous MVA that was labelled with ^{14}C, or in some cases ^{3}H, at C-2, C-4 or C-5. Radioactive citronellol and citronellal were also obtained by feeding radioactive MVA to *C. winterianus*. [1-$^{3}H_2$]Citronellol was obtained by reducing citronellal with sodium boro[^{3}H]hydride.

The reason for the particular choice of labelled precursors becomes apparent when possible mechanisms for the biosynthesis of citronellol and citronellal are considered.

Experiment 1 proved that geraniol biosynthesized in *C. winterianus* was asymmetrically labelled from [2-^{14}C]MVA. Although the appropriate degradations were not performed, it is very likely that the ^{14}C was located at C-4 as had been found in studies conducted earlier [9]. The isotope ratios (^{3}H : ^{14}C) in **3**, **5** and **6** in experiments 2 and 3 proved that the 4*S* hydrogen from MVA was specifically lost during the condensation of IPP and DMAPP units and established that the 4*R* hydrogen from MVA remained at C-2 of compounds **3**, **5** and **6** at all stages of biosynthesis. In the experiment with [5-$^{3}H_2$,2-^{14}C]MVA (Expt 4, Scheme 2) geraniol and citranellol had the same isotope ratio as the precursor whereas citronellal had half the ratio of the precursor. This showed that one of the ^{3}H atoms at positions C-1 in geraniol or citronellol had been lost during the oxidation at C-1. This proved that geraniol is converted directly to citronellol. Had the conversion been via citronellal to citronellol the isotope ratio would have fallen to half in citronellol too. This also establishes that either geraniol is directly converted to citronellal or route **3** → **6** → **5** is followed. The isotope ratios in compounds **3**, **5** and **6** in experiment 5 completely ruled out the possibility of direct condensation of IPP and DMAPP to form **1** (Scheme 1), since this would have given citronellol with the same isotope ratio as in the precursor.

In Expt 6 the ^{14}C from citronellol was not incorporated into geraniol, thus eliminating any possibility of citronellol being converted into geraniol in this plant. On the other hand the isotope ratio (^{3}H : ^{14}C) was reduced to half in citronellal, thus proving that one ^{3}H from C-1 of citronellol was lost during its oxidation to citronellal. Experiments 7 and 8 support the results obtained by earlier experiments that citronellol is converted into citronellal and that there is no active enzyme to convert the aldehyde back to the alcohol.

The above experiments provide good evidence that

Table 1. Incorporation of labelled precursors into monoterpenes by *C. winterianus*

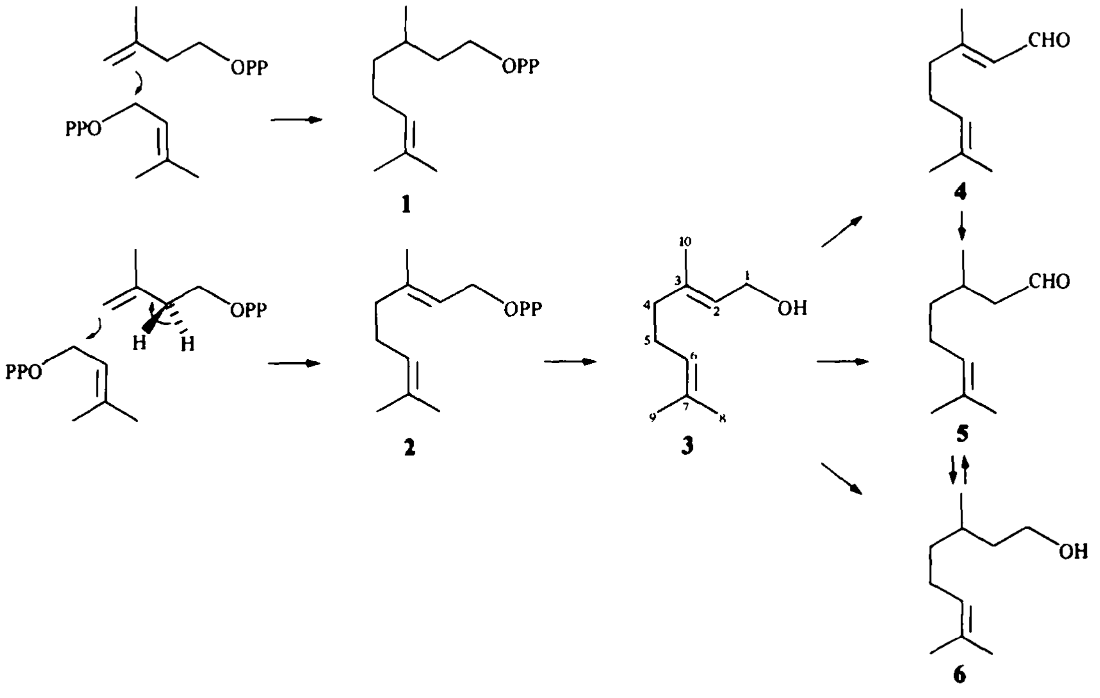
Expt	Precursor	Incorporation* (%)	Sp. activity (dpm/mmol)			
			Geraniol	Degradation products†		
1	[2-^{14}C]MVA	0.1	88638	**7**, 88446 **8**, 15 **9**, 19		
			Isotope ratios (^{3}H:^{14}C)‡			
			Precursor	Geraniol	Citronellol	Citronellal
2	[4*R*-^{3}H$_1$,2-^{14}C]MVA	0.1	1.70	1.68	1.67	1.67
3	[4*S*-^{3}H$_1$,2-^{14}C]MVA	0.1	1.70	0.12	0.17	0.15
4	[5-^{3}H$_2$,2-^{14}C]MVA	0.1	2.00	1.98	1.95	0.98
5	[4-^{3}H$_2$,2-^{14}C]MVA	0.1	2.00	1.02	1.01	1.03
6	[1-^{3}H$_2$,4-^{14}C]Citronellol	0.05	2.00	0.12	1.99	0.98
			Specific activity (dpm/mmol)			
			Precursor	Product		
7	[4-^{14}C]Citronellol	0.05	868	Citronellal, 854		
8	[4-^{14}C]Citronellal	0.05	854	Citronellol, 16		

*In Expts 1–5, incorporation of (3*R*)-[2-^{14}C]MVA.

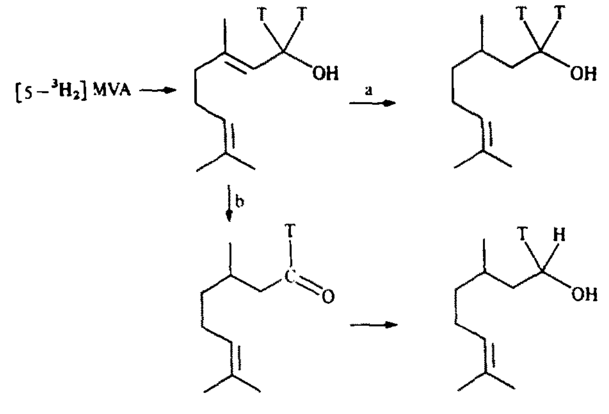
†See Scheme 3 and Experimental.

‡Standard error (estimated) for ratio, ± 0.02.

^{14}C radioactivity was typically 10^3–10^4 dpm. All the experiments were duplicated.



Scheme 1. Possible biogenetic routes to citronellol and citronellal. Geraniol has been represented as the precursor, the actual species may be phosphate esters, glucosides or may be enzyme bonded, amongst other possibilities.

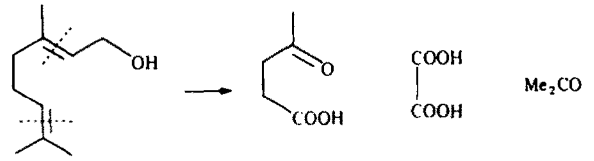


Scheme 2. The possible routes to citronellol from geraniol after feeding [5-$^3H_2$]MVA.

geraniol is converted to citronellol which in turn is converted into citronellal (i.e. route 3 → 6 → 5). The possibility that some conversion of geraniol (3) to citronellal (5) takes place by an enzyme-catalysed double bond rearrangement to give the enol which then rearranges spontaneously to form citronellal (5) cannot be eliminated because this mechanism would also result in the loss of one ^{3}H atom when 5 is biosynthesised from [5-$^3H_2$,2-^{14}C]MVA (Table 1, Expt 4, Scheme 4).

EXPERIMENTAL

Materials. C. winterianus was grown in the experimental farms of CIMAP, Lucknow, India. [2-^{14}C]MVA, [4*R*-$^3H_1$]MVA, [4*S*-$^3H_1$]MVA, [5-$^3H_2$]MVA, [4-$^3H_2$]MVA and NaB^3H_4 were purchased from the Radio-Chemical Centre, Amersham, England and Bhabha Atomic Research Centre, Bombay, India. Labelled geraniol, citronellol and citronellal were obtained by feeding (July) the leaf blades of *C. winterianus* with [2-^{14}C]MVA (100 μCi). [1-$^3H_2$]Citronellol was obtained by reducing citronellal with NaB^3H_4 (25 mCi, 0.0085 mmole) in *iso*-PrOH for 12 hr. The alcohol was purified by TLC on silica gel H as described below.



3 7 8 9

Scheme 3. Degradation products of geraniol.

Feeding methods and isolation of products. Leaf blades of *C. winterianus* (*ca* 100 g) were administered labelled MVA under forced transpiration as described earlier [9], in each case 25 μCi of tracer was used. Citronellol and citronellal (5 μCi each) were administered in an emulsion generated by exposure to ultrasound of a mixture of monoterpene (1–5 mg), Tween 80 (1–2 mg) and H_2O (1 ml). All incubations were carried out within 2–3 days in July–August. Forty eight hr after administration of the radio substrate, the leaf blades were steam distilled. Carrier essential oil (about 1 ml) was added to the steam distilled material, and the essential oil was subjected to CC on a silica gel H column (15 cm) eluted with EtOAc. The fractions containing the monoterpene alcohols and aldehyde were rechromatographed (prep. TLC) on silica gel H containing $AgNO_3$ (5%) with HOAc–EtOAc (1:49) as eluent at 2°. Preparation of the Ag^+-impregnated stationary phases by the slurry method sometimes yielded blackened plates after drying and in these cases the dry silica gel plate was sprayed with a satd ethereal soln of $AgNO_3$ immediately before use. The separated alcohols (geraniol and citronellol) were eluted with Et_2O and the solvent was carefully removed by slow (5 ml/hr) flash distillation at 40° with monitoring (radio TLC) to check that no tracer was lost in the distillate. Pure citronellal was obtained in similar fashion.

The geraniol, citronellol and citronellal recovered from TLC were finally purified by prep. GLC on Carbowax 20 M (3 m × 0.5 cm, N_2 60 ml/min, 150°), followed by FFAP (same conditions) and then TLC on silica gel H with EtOAc. The products maintained their sp. radioactivities and isotope ratios through the last two steps of purification. They were shown to be chemically pure (99.5%) by capillary GLC on Carbowax 20 M and SE-30 and by TLC on silica gel H with a variety of eluants. The sp. radioactivity was also constant across the fractions collected from prep. GLC (Carbowax 20 M and FFAP) and across sectors cut from overloaded TLC plates. Radiochromatographic scanning of a variety of TLC separations showed that the products were at least 99% radiochemically pure.

The degradation of geraniol (see Scheme 3, Expt 1, Table 1) into 7, 8 and 9 was carried out as described earlier [2].

Radiochemical methods. These have been described [3, 10]. The samples for assay by liquid scintillation spectrometry contained 2000–3000 dpm as ^{14}C and up to 20000 dpm as ^{3}H. 40000 disintegrations were accumulated to ensure that 2σ was ±1%.

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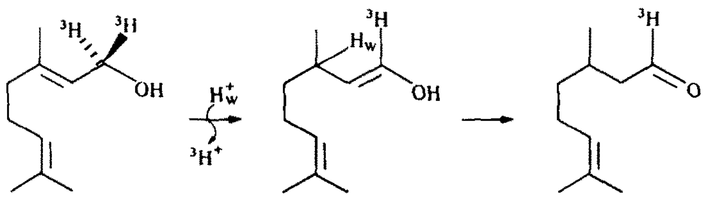

3 5

Scheme 4. Conversion of geraniol into citronellol via enol formation and oxidation of citronellol into citronellal.

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