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BIOSYNTHETIC RELATIONSHIP OF CITRAL-*TRANS* AND CITRAL-*CIS* IN *CYMBOPOGON FLEXUOSUS* (LEMONGRASS)

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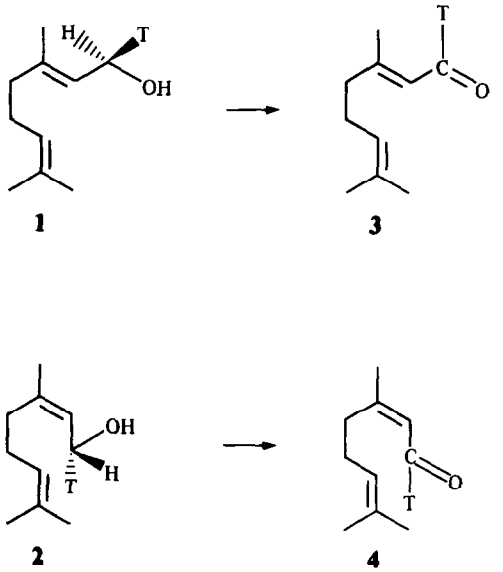
Key Word Index—*Cymbopogon flexuosus*; Gramineae; biosynthesis; monoterpenes; citral-*trans*; citral-*cis*.

Abstract—Use of [^{14}C,^{3}H]-labelled precursors revealed that leaf blades of *Cymbopogon flexuosus* converted geraniol (3,7-dimethylocta-*trans*-2,6-diene-1-ol) into citral-*trans* with loss of pro-(1*S*) hydrogen whereas nerol lost the pro-(1*R*) hydrogen while being converted into citral-*cis*. Secondly, the citral-*trans* is converted into citral-*cis* and vice versa and there is no separate route for the biosynthesis of either of the two aldehyde isomers.

INTRODUCTION

Cymbopogon flexuosus produces an essential oil which consists of α-pinene (0.19%), β-pinene (0.04%), car-3-ene (0.003%), myrcene (14.27%), dipenetene (0.15%), β-phellandrene (0.09%), *p*-cymene (0.13%), methyl heptenone (2.44%) citronellal (0.49%), linalool (1.19%), β-elemene (0.38%), β-caryophyllene (0.32%), citronellyl acetate (1.19%), geranyl acetate (3.00%), citral-*cis* (35.49%) and citral-*trans* (40.34%) [1, 2].

The biosynthesis of citral-*trans* (**3**) and citral-*cis* (**4**) probably involves the biogenetic equivalent of geraniol (**1**). Four different pathways can be hypothetically sketched for the biosynthesis of **3** and **4**. Firstly, compounds **3** and **4** are biosynthesized by different routes from geraniol (**1**) and nerol (**2**), respectively (i.e. **1** → **3** and **2** → **4**, Scheme 1). Secondly, one of the aldehyde isomers is biosynthesized from its alcohol precursor and then converted into another *trans* or *cis* isomer (i.e. **3** → **4** or **4** → **3**). Furthermore, the biosynthesis of **3** and **4** may take place through two different pathways a and b (see Scheme 2). In route a geraniol is converted into citral-*trans* which in turn metabolizes into citral-*cis* (i.e. **1** → **3** → **5** → **6** → **4**) while in route b citral-*cis* is directly biosynthesized from geraniol (i.e. **1** → **7** → **2** → **4**). We have tried to solve these problems by feeding suitably labelled precursors to *C. flexuosus* which produces both the monoterpenes in good yield.



Scheme 1. Geraniol and nerol lose the 1*S*- and 1*R*-hydrogen atom, respectively, during the biosynthesis of citral-*trans* and citral-*cis*.

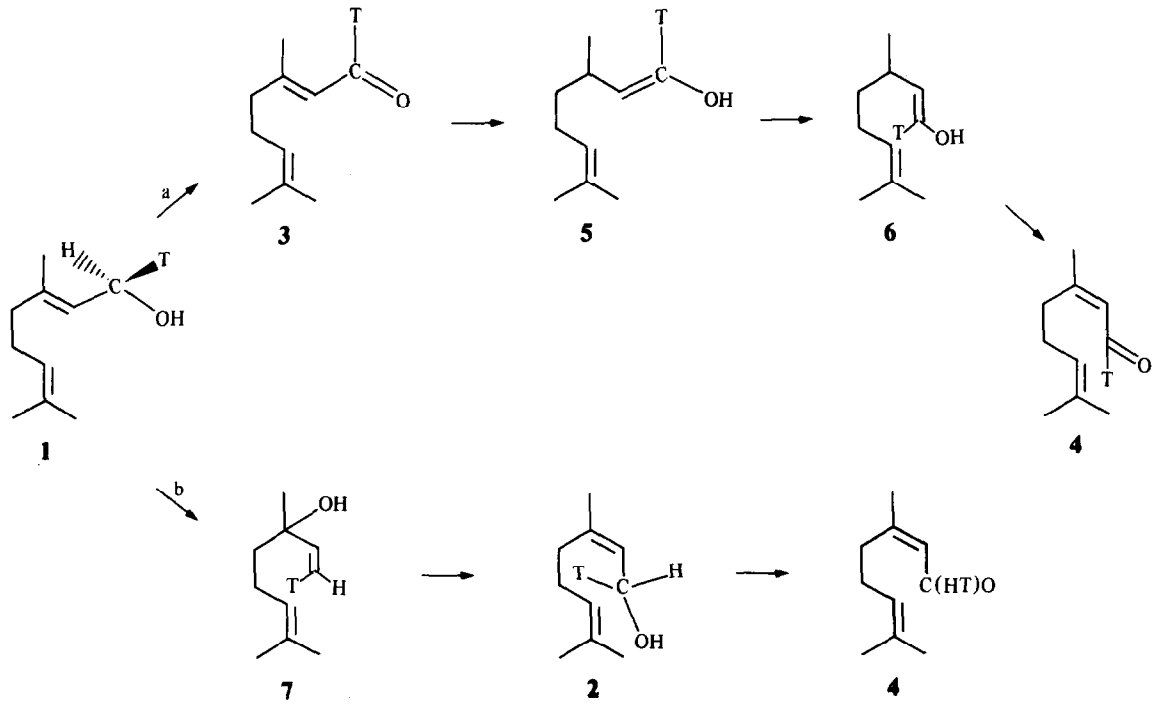
RESULTS AND DISCUSSION

Double-label experiments

The isotope ratios in citral-*trans* and citral-*cis* recovered after feeding leaf blades of *C. flexuosus* with [^{3}H,^{14}C]geraniol and [^{3}H,^{14}C]nerol are presented in Table 1. One general procedure was to purify all biosynthetic products or derivatives to constant specific radioactivity and isotope ratio by crystallization. In the case of aldehydes the 4-phenylsemicarbazone was prepared. Uptake of geraniol and nerol into matched clonal plant material carried out in different months gave good (typically *ca* 0.05%) incorporations into citral which are greater by up to 10 fold than those found from mevalonic acid under the same conditions.

After the metabolism time chosen for our experiments it was observed from experiments 1–4 (Table 1) that geraniol lost the pro-(1*S*) hydrogen during the biosynthesis of citral-*trans* and citral-*cis* whereas nerol lost the pro-(1*R*) hydrogen. Similar results have been obtained in several other higher plants [3, 4]. It is well established from the isotope ratios of citral isomers that both are interconvertible within the plant system and they are not biosynthesised by separate routes as shown in scheme 1. The direct conversion of citral-*trans* (**3**) to citral-*cis* (**4**) is not possible for obvious stereochemical reasons.

Two biogenetic pathways have been postulated (see



Scheme 2. Possible biogenetic routes to citral-*cis*. Geraniol has been represented as the precursor, the actual species may be enzyme bonded, phosphate esters or glucosides, amongst other possibilities.

Table 1. Incorporation of labelled precursors into citral-*trans* and citral-*cis* in *C. flexuosus*

Expt.	Precursor	%*	Isotope ratios†		
			Precursor	Citral-*trans*	citral-*cis*
1.	[^{14}C,1-^{3}H$_1$]-(1*R*)-Geraniol	0.04	1.00	0.98	0.99
2.	[^{14}C,1-^{3}H$_1$]-(1*S*)-Geraniol	0.04	1.00	0.02	0.01
3.	[^{14}C,1-^{3}H$_1$]-(1*R*)-Nerol	0.04	1.00	0.025	0.02
4.	[^{14}C,1-^{3}H$_1$]-(1*S*)-Nerol	0.04	1.00	1.01	1.00
5.	[1-^{3}H]Citral-*trans*	0.06	1.00	0.99	0.99
6.	[1-^{3}H]Citral-*cis*	0.06	1.00	1.00	1.01

*For feeding conditions etc., see Experimental. Percentage incorporation of tracer from ^{14}C in isotope ratio experiments.

†^{3}H:^{14}C standard error (estimated) for ratio, ±0.02. ^{14}C radioactivity was typically 10^3–10^4 dpm. All the experiments were duplicated.

Scheme 2) to explain the mechanism of interconversion of citral-*trans* to citral-*cis* and vice-versa. Once again we go back to the isotope ratios found in Expt. 1 (Table 1). These isotope ratios suggest that geraniol is first converted into citral-*trans* (**3**) with the loss of pro-(1*R*) hydrogen and this is then metabolized into citral-*cis* via a series of reactions (route a, **1** → **3** → **5** → **6** → **4**) maintaining the same isotope ratio as found in **3**. Had the route been different (route b, **1** → **7** → **2** → **4**) some tritium from C-1 of **1** must have definitely been lost during the formation of the carbonyl group thus ultimately bringing down the isotope ratio, which is not observed. A reversal of the sequence of reactions is found after feeding double-labelled nerol. The isotope ratios in Expt. 4 suggest the route **2** → **4** → **6** → **5** → **3** occurs, thus proving that all the reactions occuring between the conversion of citral-*trans* and citral-*cis* are reversible.

Our present results are consistent with the suggestion that the sequence geraniol (**1**) → citral-*trans* (**3**) → **5** → **6** → citral-*cis* (**4**) and nerol (**2**) → citral-*cis* → **6** → **5** → citral-

trans (3) exists in *C. flexuosus*. When radioactive aldehyde isomers were fed to the plant system they did not pass radioactivity into geraniol or nerol.

EXPERIMENTAL

Materials. The specimens of *C. flexuosus* were grown at the experimental farms of CIMAP, Lucknow, India. [1-$^3H_2$]Geraniol and [1-$^3H_2$]nerol were prepared by treatment of citral (7:3 w/w *trans*:*cis*; 0.17 mmole, obtained by oxidation of geraniol with MnO_2) with [$^3H_4$]$NaBH_4$ (25 mCi; 0.085 mmole) in *iso*-PrOH for 12 hr and the alcohols were purified by TLC on silica gel H containing $AgNO_3$ (5%) with AcOH–EtOAc (2:98) as eluant at 2°. Preparation of the silver-impregnated stationary phases by the slurry method sometimes yielded blackened plates after drying and in such cases the dry silica gel plate was sprayed with a satd ethereal soln of $AgNO_3$ immediately before use. The separated alcohols (R_f *ca* 0.44, 0.49; values not reproducible and hence standards were essential) were eluted with Et_2O and the solvent was carefully removed by slow (5 ml hr^{-1}) flash distillation at 40° with monitoring (radio-TLC) to check that no tracer was lost in the distillate; total yield 81%, sp. act. 1.8×10^4 dpm μmol^{-1}. The stereospecifically-labelled alcohols were prepared following the methods developed for the corresponding farnesols [5]. Geraniol (1 mg) and Tween 80 in Pi buffer (0.1 m, pH 8; 0.5 ml) containing EDTA (di Na salt; 330 μg); bovine serum albumin (660 μg), NAD^+ (170 μg) and NADH (170 μg) was added to a soln of liver alcohol dehydrogenase (ex horse liver, 5 units), diaphorase (100 units) in [$^3H_2$]H_2O (200 μl; 1 Ci) and the mixture was emulsified by sonification and incubated (37°; 12 hr). Thereafter the product was extracted (Et_2O; 4 × 2 ml) and after the addition of carrier (100 μl) [1-$^3H_1$]-(1*R*)-nerol (5×10^3 dpm μmol^{-1}) was purified by TLC as described above. [1-$^3H_1$]-(1*R*)-Geraniol (1.6×10^4 dpm μmol^{-1}) was prepared similarly to nerol. [1-$^3H_1$]-(1*S*)-Nerol (1.4×10^4 dpm μmol^{-1}) and [1-$^3H_1$]-(1*S*)-geraniol (2.2×10^4 dpm μmol) were similarly prepared by equilibrating [1-$^3H_2$]geraniol and [1-$^3H_2$]nerol, respectively, in the above described system with H_2O. [^{14}C]-labelled geraniol and nerol were obtained by exposing potted specimens of *Pelargonium graveolens* to an atmosphere of [^{14}C]CO_2 [6]. This labelling procedure is known to lead to generally but not uniformally labelled products and the % incorporation of tracer was *ca* 0.05%. The final products had *ca* 5×10^4 dpm μmol^{-1}.

The ^{14}C- and ^{3}H-labelled geraniol and nerol produced by these methods were shown by a variety of TLC and GLC procedures (described in ref. [7]) to be chemically and radiochemically (99.5%) pure. Before each feeding experiment aliquots of the ^{14}C- and ^{3}H-labelled precursors were mixed and rechromatographed on the silica gel H–$AgNO_3$ system with EtOAc–C_6H_6 (3:17) at 2° and reisolated as described above.

Methods of plant feeding. The leaf blades of *C. flexuosus* were sterilized (0.1 M NaOCl; EtOH) and excised under sterile H_2O. The labelled precursor (typically 50 mg; 0.5 μCi) and Tween 80 (10 μl) was emulsified in Pi buffer (1 ml; pH 7.0; 0.1 M) and fed under conditions of forced transpiration [8]. After uptake of tracer (1 hr), the foliage was maintained on sterile H_2O for 3–4 days under normal conditions of illumination and temperature before harvesting. Preliminary experiments had shown this period to be optimum for passage of tracer into monoterpenes.

Isolation and purification of products. After the period of metabolism, the plant material was steam distilled and carrier essential oil (about 1 ml) was added to it. The essential oil was chromatographed on silica gel H column (10 × 1 cm) and the hydrocarbons were eluted with *n*-pentane (100 ml). The aldehydes were eluted with EtOAc (100 ml). The fractions containing citral were rechromatographed (prep. TLC) on silica gel H containing $AgNO_3$ (5%) with AcOH–EtOAc (2:98) as eluant at 2°. The separated aldehydes (citral-*trans* and citral-*cis*) were eluted with Et_2O and the solvent was carefully removed by slow flash distillation (5 ml/hr) at 40° with monitoring to check that no tracer was lost in the distillate.

The fractions containing citral isomers were finally purified by prep. GLC on Carbowax 20 M (3 m × 0.5 cm, N_2 flow 60 ml/min, 150°), followed by FFAP (same conditions) and then TLC on silica gel H with EtOAc. The final products that have maintained their specific radioactive and isotope ratio through the last two steps of purification 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 specific 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.

Radiochemical methods. These have been described before [6, 9]. Typically samples for assay contained 2000–3000 dpm of ^{14}C and up to 20000 dpm of ^{3}H. 40000 disintegrations were accumulated to ensure that 2σ was $\pm 1\%$. All experiments were carried out in duplicate. A Rack Beta II (LKB Wallac model 1215/1216) liquid scintillation counter was used for determination of radioactivity of the samples.

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