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EVIDENCE FOR GERANIOL AS AN OBLIGATORY PRECURSOR OF ISOTHUJONE*

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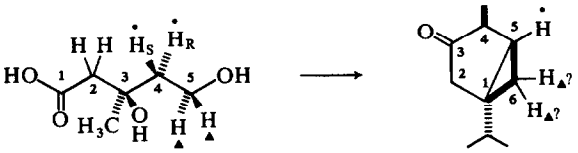
Key Word Index—*Tanacetum vulgare*; Compositae; tansy; monoterpenes; biosynthesis; isothujone; nerol; geraniol.

Abstract—Isothujone (*trans*-thujan-3-one) was formed from MVA-[^{14}C, ^{3}H] in *Tanacetum vulgare* with retention of the pro-(4R) hydrogen of precursor, but with loss of the pro-(4S) hydrogen and of one hydrogen from C-5. Cell-free extracts could not sustain the formation of isothujone from MVA but yielded geraniol and nerol (3,7-dimethylocta-*trans*-2,6-dien-1-ol and its *cis* isomer) with retention of the pro-(4R) and loss of the pro-(4S) hydrogen in each case: no hydrogen was lost from C-5 of MVA in formation of geraniol, but one such atom was lost in the formation of nerol. These results support the sequence: geraniol → nerol → isothujone: in which the first two compounds (or their biogenetic equivalents) are interconverted by a redox process involving their derived aldehydes. They are not consistent with a direct pathway to nerol from C_5 intermediates or with routes involving cyclisation of linalol (3,7-dimethylocta-1,6-dien-3-ol) formed directly from the C_5 compounds or from geraniol. The cell-free preparations could not interconvert geraniol and nerol, their phosphates or pyrophosphates. This may be due to the inability of a prenyltransferase–isomerase multi-enzyme system to accept exogenously-supplied intermediates under these (*in vitro*) conditions.

INTRODUCTION

The metabolic fate of the hydrogens at C-2 of MVA (cf. **1**) in the biosynthesis of (+)-isothujone (**2**) in *Tanacetum vulgare* L. has recently been explored [1]. We now report the fates of the other biochemically-significant hydrogens (at C-4 and C-5) and draw conclusions concerning the roles of geraniol, nerol and linalol as precursors of the bicyclic ketone. The locations of hydrogens derived from C-4 and C-5 of MVA can be inferred to be in the positions shown in isothujone (**2**) as a result of ^{14}C-studies on the construction of the bicyclic skeleton [1].



1 **2**

RESULTS AND DISCUSSION

General

Normalised isotope ratios in isothujone, geraniol and nerol obtained after feeding MVA-[^{14}C, ^{3}H] both *in vivo* and *in vitro* are given in Table 1. The precursors were prepared by mixing (3RS)-MVA-[2-^{14}C] with (a) (3RS)-MVA-[5-$^{3}H_2$]; (b) {(3R, 4R)-MVA-[4-$^{3}H_1$] + (3S, 4S)-MVA-[4-$^{3}H_1$]}; and (c) {(3R,4S)-MVA-[4-$^{3}H_1$] + (3S, 4R(-MVA-[4-$^{3}H_1$]}. In these mixtures, only (3R)-MVA is involved in terpenoid biosynthesis [2]. The percentage incorporations of tracer were typical of those found in previous experiments with this and related plant species [1, 3, 4]. All products were rigorously purified to constant specific radioactivity, by derivatisation and recrystallisation where possible (see Experimental) and, in addition, the procedure for forming the derivative (4-phenylsemicarbazone) of isothujone was shown [1] not to involve loss of ^{3}H by exchange processes.

We have previously found [1] that essentially all (92–100%) tracer incorporated into isothujone from MVA-[2-^{14}C] by *T. vulgare* was located at C-3 [3] and was thus derived from IPP, and this pattern was found in the present work. Thus, photolytic cleavage of the ketone removed C-3 as CO containing 98 ± 2% of the incorporated tracer. Consequently it seemed likely that the incorporated ^{3}H also resided in the IPP-derived moiety. Location of ^{3}H incorporated in a position-specific manner from MVA-[4-$^{3}H_1$] and [5-$^{3}H_2$] into this moiety of the thujane skeleton is extremely difficult to achieve by chemical degradation and was not attempted. Nevertheless, we consider scrambling of ^{3}H from the expected positions in isothujone to be unlikely on two counts: (i) the self-consistent ^{3}H/^{14}C ratios in products, and (ii) the demonstrated lack of scrambling of tracer from MVA-[2-$^{3}H_2$] in almost contemporaneous studies on the same clone [1]. It is noteworthy that rarely have the locations of tracer been demonstrated in studies involving double-isotope labelling of natural products: gen-

* Part 21 of the series "Terpene Biosynthesis". Reprints of this paper are not available. For part 20, see: Banthorpe, D. V., Charlwood, B. V., Greaves, G. M. and Voller, C. M. (1977) *Phytochemistry* **16**, 1387.

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Abbreviations used: MVA, mevalonic acid; IPP, isopentenyl pyrophosphate; DMAPP, 3,3-dimethylallyl pyrophosphate.

Table 1. Isotope ratios in products biosynthesised from MVA-[^{14}C, ^{3}H]

(3R)-MVA*	Product	Source†	^{3}H/^{14}C‡ in MVA	^{3}H/^{14}C‡ in Product	%§
[2-^{14}C, (4R)-4-$^{3}H_1$]	Isothujone	Leaves	1.00	0.94	0.005
[2-^{14}C, (4S)-4-$^{3}H_1$]	Isothujone	Leaves	1.00	0.01	0.004
[2-^{14}C, 5-$^{3}H_2$]	Isothujone	Leaves	1.00	0.53	0.005
[2-^{14}C, (4R)-4-$^{3}H_1$]	Geraniol	Extract	1.00	0.99	0.21
[2-^{14}C, (4S)-4-$^{3}H_1$]	Geraniol	Extract	1.00	0.01	0.20
[2-^{14}C, (4R)-4-$^{3}H_1$]	Nerol	Extract	1.00	0.92	0.03
[2-^{14}C, (4S)-4-$^{2}H_1$]	Nerol	Extract	1.00	0.003	0.04
[2-^{14}C, 5-$^{3}H_2$]	Geraniol	Extract	1.00	0.96	0.32
[2-^{14}C, 5-$^{3}H_2$]	Nerol	Extract	1.00	0.47	0.02

* Metabolically-active form of MVA.

† Leaves and cell-free extracts of *T. vulgare*.

‡ Normalised values. Typically ^{3}H/^{14}C in MVA was 6–8. Estimated s.e. was ±0.01 for MVA and ±0.04 for products. Values are the means of 2 independent experiments carried out under each set of conditions. Typically the ^{14}C in products was 10^3 to 10^4 dpm.

§ % Incorporation of (3R)-MVA-[2-^{14}C].

erally the observation of the expected or of explicable isotope ratios in products has led to the implicit assumption that position-specific incorporations had occurred [2].

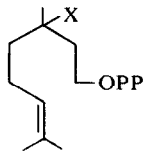
A similar preferential labelling (up to 100% in the IPP-derived moiety) had been found [4] for geraniol and nerol biosynthesised from IPP in extracts of *T. vulgare*. In the present work both asymmetric labelling (of this moiety) and position specific incorporation was proved by the finding that both geraniol and nerol formed *in vitro* from MVA-[2-^{14}C, $^{5}H_2$] retained their ^{14}C but lost 96 ± 3% ^{3}H on being oxidised to their corresponding acids. By reasonable extrapolation, a similar regio- and position-specific incorporation of MVA-[4-$^{3}H_1$] may be presumed.

Fate of hydrogen from C-4 *of MVA*

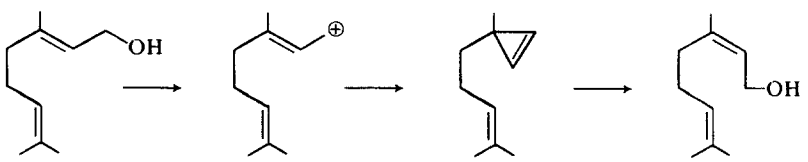
The role of these hydrogens was explored by assay of isothujone formed in leaves of *T. vulgare*, and of geraniol and nerol biosynthesised in cell-free extracts. An extract could not be obtained that could sustain the formation of the ketone from MVA [cf. 4]. Within the limits of the experimental error (or closely thereabouts, cf. Table 1) all three products were formed with the retention of the pro-(4R) hydrogen of MVA and loss of the pro-(4S) hydrogen.

It is generally accepted that nerol (*cis*-$\Delta^{2,3}$) or its biogenetic equivalent is an obligatory precursor of cyclic monoterpenes and so these results indicate that in our systems the construction of either a *cis* or *trans*-$\Delta^{2,3}$ involves stereospecific loss of the pro-(4S) mevalonoid hydrogen. Similar results have been found for the formation of several other monoterpenes and some sesquiterpenes from a variety of plant species [5–12], and construction of *trans*-$\Delta^{2,3}$ in certain other terpenoids has also been inferred to involve loss of the pro-(4S) mevalonoid hydrogen [13-17]: in contrast, formation of a *cis*-Δ in rubber [13] and in certain polyprenols [18–20] has been shown to require the stereospecific loss of the epimeric pro-(4R) atom.

One explanation of the stereospecificity found in our present, and the previous, work is that geraniol and nerol are independently formed in steps each involving direct condensation of IPP and DMAPP (route A). Another possibility is that these C_5-compounds form an intermediate (e.g. **3**) that can undergo proton loss to give either product (route B). Detailed analyses of each of these situations reveal that either could lead to the observed stereospecificity [7, 8]. A very attractive alternative is that the sequence; geraniol → nerol → cyclic monoterpenes; occurs with the first two compounds being interconverted (i) in a redox process involving their aldehydes (route C) [7, 8]; (ii) a 1,3-suprafacial anionotropic rearrangement (geraniol → linalol → nerol) involving phosphate esters (route D) [21] or (iii) a mechanism proposed for the analogous interconversion of farnesols involving cyclopropene intermediates (route E, cf. 4 → 5 → 6 → 7) [22]. Of routes (C) to (E) there is experimental evidence only for the first: the required redox enzymes have been detected in several species of plants [23–25] as have the aldehydes required for the geraniol → nerol and the corresponding interconversions of farnesols [9, 10, 26–28]. There is some



3 (PP = pyrophosphate
X = unspecified group (of enzyme?)



4 **5** **6** **7**

evidence for a direct *trans* → *cis* isomerisation of geraniol and nerol phosphates [29] and for an isomerisation of the corresponding farnesyl pyrophosphates that involves loss of hydrogen from C-1 [30], but as the mechanisms of these tentative processes have remained completely unspecified we shall not consider these further.

Routes (A)–(E) can all accommodate the results obtained by feeding MVA-[4-$^3H_1$]: the crux is whether geraniol is an obligatory precursor of isothujone (routes C, D and E) or is a non-obligatory intermediate (routes A and B). An attempt to decide this solution is in the next section.

Fate of hydrogens at C-5 *of MVA*

The experiments using MVA-[5-$^3H_2$] clearly show that (for the *one* molecule of MVA incorporated per molecule of monoterpene, see above) one ^3H is lost in the formation of isothujone *in vivo*, whereas geraniol is formed *in vitro* with incorporation of both mevalonoid hydrogens and nerol is formed with the loss of one. These results are not consistent with routes A, B and D as these require formation of isothujone and nerol with no loss of mevalonoid hydrogen from C-5. Our results are consistent with routes C and E and in the absence of any experimental evidence whatsoever for the latter, we prefer route C. That is, geraniol (or its biogenetic equivalent) is an obligatory precursor of isothujone in our system.

Linalol has been proposed as an obligatory precursor of cyclic monoterpenes in *Citrus* species [31–34] although modern standards of rigour in purification and identification of products were not achieved and interconversions of linalol, geraniol and nerol were ignored. And indeed others have disputed this role for linalol [35]. Our results exclude the intermediacy of linalol in route D and are also inconsistent with additional routes involving this compound to the exlcusion of nerol: viz.

(IPP + DMAPP) → linalol → isothujone

or

(IPP + DMAPP) → geraniol → linalol → isothujone.

In our opinion the literature contains no compelling evidence for linalol as an obligatory precursor of any cyclic monoterpene.

Attempted interconversion of geraniol and nerol in vitro

Attempts to demonstrate the redox interconversion of geraniol and nerol in cell-free extracts failed. Extracts that converted MVA into geraniol and nerol (0.4 and 2.5%; and 2.3 and 1.2% respectively in two independent experiments) catalysed negligible (<0.1%, if any) interconversion of geraniol and nerol, their phosphates or their pyrophosphates. Others have reported similar results for cell-free systems from *Pinus* and *Citrus* species that produce geraniol and nerol or the analogous farnesols from MVA [8, 10, 26, 36]. We suggest that *in vitro*, a prenyltransferase–isomerase complex that functions as a unit in monoterpene biosynthesis may be able to accept MVA or IPP but not geraniol or nerol or their phosphate esters: furthermore, the intermediates of the redox process may not be released from a C-5 → C-10 conveyor belt [cf. 5, 8]. Tracer studies *in vivo* (see following paper) indicate that exogenously-supplied geraniol or nerol can however intervene in these situations.

EXPERIMENTAL

Detailed descriptions of most of the techniques have been given in previous papers of this series.

Feeding expts. MVA-[^{14}C] and MVA-[^3H] ($^3H/^{14}C$ 6 to 8; 0.1 g; total 10 to 30 μCi) in C_6H_6 were mixed, assayed and stem-fed with forced transpiration to *T. vulgare* (young shoots; 15 cm; 50 g) in June to September. After uptake of tracer (0.5 hr) the plants were kept in distilled H_2O for 48 hr under natural illumination and temperature, harvested, carrier added and the isothujone purified as its 4-phenylsemicarbazone [cf. 1, 3, 37], yield 89%, m.p. 184° ex aq. EtOH. Isothujone was photolysed to yield CO as previously described [3]. Cell-free extracts were prepared by standard methods [4] and geraniol and nerol formed after incubation of the extract with MVA-[^{14}C, ^3H] ($^3H/^{14}C$ *ca* 6; 2 μCi total) were extracted and purified as previously described [4]. It is extremely difficult to obtain a solid derivative of these alcohols that is suitable for purification to constant specific activity. For experiments involving MVA-[4-$^3H_1$], the alcohols were oxidised with MnO_2 [7] to their aldehydes and purified as the semicarbazones (70% yield, mps 162 and 183°. This procedure could not be applied when MVA-[5-$^3H_2$] was used (as the oxidation step would remove ^3H), and here the compounds were purified by TLC on 4 different systems [4].

Radiochemical techniques. These were routine [1, 3–5] and the channels-ratio method used for isotope ratios has been described in detail [7]. Counting efficiencies were *ca* 95 and 40% respectively for ^{14}C and ^3H, and under the conditions used the maximum overlap of ^{14}C into the ^3H channel was *ca* 5%: hence for $^3H/^{14}C$ *ca* 6, the contribution of ^{14}C to the ^3H channel was <1%, and in any case could be allowed for.

Phosphate esters. Geranyl and neryl phosphates and pyrophosphates (>98% pure) were prepared by Dr. J. Gutowski [38].

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