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REDOX INTERCONVERSIONS OF GERANIOL AND NEROL IN HIGHER PLANTS*

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Key Word Index—*Tanacetum vulgare*; Compositae; *Mentha spicata*; *M. piperita*; Labiatae; *Eucalyptus globulus*; Myrtaceae; *Pinus pinaster*; Pinaceae; monoterpenes; geraniol; nerol; biosynthesis.

Abstract—Use of ^{14}C, ^{3}H-labelled precursors showed that for feedings carried out in winter, isothujone (*trans*-thujan-3-one) was formed in *Tanacetum vulgare* from nerol (3,7-dimethyl-octa-*cis*-2,6-dien-1-ol) without loss of hydrogen from C-1 of the precursor. In contrast, formation from geraniol (the corresponding *trans*-isomer) involved stereospecific loss of the pro-(1S) hydrogen. This suggests that geraniol and nerol were interconverted by a redox system. Similar studies at other seasons with *T. vulgare* and on the biosynthesis of α- and β-pinenes (pin-3-ene; pin-2-(10)-ene) in *Pinus pinaster*; 1,8-cineole (1,8-oxidomethane) in *Mentha piperita* and *Eucalyptus globulus*; and carvone (menth-6,8(9)-dien-2-one) in *M. spicata* did not lead to such unambiguous conclusions. The results may be rationalized if (i) the redox system was reversible and/or (ii) tracer at C-1 of the phosphate esters of the precursors was scrambled by action of a phosphatase that induced C—O bond fission.

INTRODUCTION

Studies of the biosynthetic routes from mevalonate to isothujone in foliage and cell-free extracts of *Tanacetum vulgare* L. indicated that geraniol and nerol were obligatory precursors which were interconverted by a redox system [1]. However these conclusions were dependent largely on data from cell-free extracts that synthesized geraniol and nerol but not the ketone, and it could be argued that geraniol thus produced was formed by enzyme systems normally involved in the biosynthesis of higher terpenes. These systems might be compartmentalized *in vivo* from the systems that form monoterpenes [cf. 2]. Consequently it was desirable to demonstrate the proposed redox systems to be implicated in monoterpene biosynthesis *in vivo*. We now report experiments to achieve this, to characterize the stereospecificity of hydrogen loss, and to explore the incidence of the redox systems in the synthesis of other monoterpenes in other plants. In the event, our results also confirmed our previous experience that the successful outcome of biosynthetic experiments often depends on the season of the year in which feedings took place.

RESULTS AND DISCUSSION

Preparation of precursors

The preparations of (1R)-geraniol-[$1^{3}H_1$], its (1S) epimer and the corresponding nerol compounds were based on methods developed for the corresponding farnesols [3: this includes a penetrating analysis of the stereochemical and enzymic problems involved]. Geraniol is known to exchange its pro-(1R) hydrogen on treatment with liver alcohol dehydrogenase (LADH; EC 1.1.1.1.) [4] and under these conditions is also isomerized to nerol. LADH also catalyses the exchange of the pro-(1R) hydrogen of 2-*trans*-6-*trans* farnesol and its 2-*cis*-6-*trans*-isomer [3] and also of ethanol [5] and probably other alcohols [6]. In the present work it was thus assumed [cf. 6] that the enzyme also exchanged the pro-(1R) atom of nerol. Thus our stereospecifically-labelled precursors were prepared in the following way [cf. 3]: (1R)-nerol-[$1\text{-}^{3}H_1$] and (1R)-geraniol-[$1\text{-}^{3}H_1$] were formed by treatment of geraniol and nerol respectively with NADH-[$^{3}H_1$] and LADH, and (1S)-nerol-[$1\text{-}^{3}H_1$] and (1S)-geraniol-[$1\text{-}^{3}H_1$] resulted from similar incubations of geraniol-[$1\text{-}^{3}H_2$] and nerol-[$1\text{-}^{3}H_2$] with NADH. NADH-[$^{3}H_1$] was formed *in situ* by carrying out the exchange in the presence of H_2O-[$^{3}H_2$] and lipoamide dehydrogenase (diaphorase; EC 1.6.4.3) [7, 8]. Preparation of [^{14}C] and [$^{3}H_2$]-labelled substrates were routine (see Experimental).

Double-label experiments on T. vulgare

Normalized isotope ratios in isothujone biosynthesized from [$^{14}C_1$ ^{3}H]-labelled substrates are given in Table 1. The starting materials were rigorously purified (see Experimental) and the products were recrystallized, in every case, as the 4-phenylsemicarbazone to constant specific radioactivity and isotope ratio. Uptake of geraniol and nerol into matched specimens of clonal plant material carried out in different months all gave good (typically *ca* 0.1 %) incorporations into isothujone which are greater by up to 10^2 fold than those found for MVA under the same conditions [9]. The isotope ratios

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Table 1. Isotope ratios in isothujone biosynthesized in *T. vulgare*

Precursor		Data	$^3H/^{14}C$*		
			Precursor	Product	% I†
(1R)-Geraniol-[^{14}C, 1-$^3H_1$]		Dec. 1976	1.00	1.08	0.12
(1S)-Geraniol-[^{14}C, 1-$^3H_1$]		Dec. 1976	1.00	0.03	0.04
(1R)-Nerol-[^{14}C, 1-$^3H_1$]		Dec. 1976	1.00	1.04	0.16
(1S)-Nerol-[^{14}C, 1-$^3H_1$]		Dec. 1976	1.00	0.97	0.03
Geraniol-[^{14}C, 1-$^3H_2$]	(A)	Sept. 1975	1.00	0.48	0.27
Nerol-[^{14}C, 1-$^3H_2$]	(A)	Sept. 1975	1.00	0.63	0.08
Geraniol-[^{14}C, 1-$^3H_2$]	(B)	Mar. 1976	1.00	0.47	0.50
Nerol-[^{14}C, 1-$^3H_2$]	(B)	Mar. 1976	1.00	0.87	0.08
Geraniol-[^{14}C, 1-$^3H_2$]	(C)	June 1976	1.00	0.29	0.10
Nerol-[^{14}C, 1-$^3H_2$]	(C)	June 1976	1.00	1.02	0.09

* Normalized values. In practice $^3H/^{14}C$ in substrate varied from 0.61 to 1.80 S.e. (estimated) for $^3H/^{14}C$ is ±0.01. ^{14}C radioactivity was typically 10^3 to 10^4 dpm.
† Incorporation of ^{14}C.
A, B, C: See text.

for the matched set carried out in December (first rows, Table 1) with stereospecifically-labelled precursors clearly indicates that nerol was then incorporated into the ketone with no loss of ^3H, whereas geraniol was so incorporated with loss of its pro(1S) hydrogen. This is consistent with the route [cf. 1]: geraniol → nerol → isothujone; where the first two are linked via redox scheme involving their aldehydes (or the biogenetic equivalents of the latter). It has been mentioned *en passant* (with no details) that geraniol and nerol are interconverted in *Menyanthes trifoliata* with stereospecific loss of one hydrogen from C-1 [10], and the interconversion of 2-*trans*-farnesol and its *cis*-isomer in extracts from *Andrographis paniculata* involves stereospecific loss of the pro-(1S) hydrogen of the substrate [3]: in contrast, the epimeric hydrogen is lost for the same conversion in the fungus. *Helminthosporium sativum* [11]. The remaining entries in Table 1 refer to experiments on matched-pairs from the same clone carried out in other seasons. We are confident that these results are real, but they do not lead to simple conclusions as do the experiments that were performed in winter. However, the general pattern was that geraniol was incorporated with loss of more ^3H relative to the ^{14}C-content than was nerol; the possible significance of this is discussed later.

Double-label experiments on other plants

Incorporation experiments were carried out in October 1976 using [^{14}C, 1-$^3H_2$]-labelled geraniol and nerol and foliage of (a) *Pinus pinaster* Ait. (Pinaceae); (b) *Mentha spicata* L. (Labiatae), (c) *Mentha piperita* L. and (d) *Eucalyptus globulus* L. (Myrtaceae). The major monoterpenes of these species are [cf. 12]; (species a) α and β-pinenes, (species b) carvone, and (species c and d) 1,8-cineole: all of which (being cyclic compounds) may be presumed to be derived from nerol, but not directly so from geraniol. Use of high specific activities in precursors ($^3H/^{14}C$ 3.70 to 8.92, s.e. ±0.01) and adequate incorporations (% ^{14}C, 0.01 to 0.14) allowed high counts (10^6 to 10^7 dpm) in products. The latter were rigorously purified, all via solid derivatives that were recrystallized to constant specific radioactivity and isotope ratio, and we are confident that all artefacts were eliminated and impurities removed. The results, as for the feedings of *T. vulgare* in seasons other than winter, did not yield a simple conclusion, but for all four monoterpenes the same pattern was obtained: nerol was incorporated into the products with loss of 20–30% of ^3H (as measured by the $^3H/^{14}C$ ratio), whereas geraniol was incorporated with a corresponding loss of 81–87%.

The above anomalous results, and also those from *T. vulgare* could possibly result from degradation of geraniol and nerol to smaller (C_2 or C_5?) fragments that were reassembled to form monoterpenes. This situation could certainly result in the loss of ^3H relative to that of ^{14}C. We have not proved position-specific incorporation of tracer in our products as ^3H or ^{14}C derived (without scrambling) from C-1 of geraniol and nerol are located at positions in the thujane, pinane and menthane skeletons that are singularly difficult to isolate by degradation. Nevertheless, we think that incorporation *is* probably position-specific for three reasons: (a) such specificity occurs for incorporation of mevalonate under a variety of conditions for a range of monoterpenes in a large number of plants [9, 13]: (b) it is similarly found for the incorporation of ^{14}C-labelled geraniol and nerol in several examples [14–16]; and (c) the isotope ratios that we have found in our anomalous cases all fall in a regular and self-consistent pattern that would be most unlikely if a degradation–reincorporation process were involved.

We suggest that our anomalous results are the consequence of direct incorporations of geraniol and nerol but that the latter intermediates are linked in a redox system which is perturbed (in a seasonally-dependent manner) in two possible ways. (i), the geraniol → nerol interconversion is reversible, the retro-step being comparable in rate with the cyclisation of nerol to isothujone. If this occurs the retro-step could involve loss of hydrogen at C-1 epimeric to that removed in the initial oxidation of geraniol. There is some evidence for analogous situations in other systems [3, 6]. And (ii), equilibria between both geraniol and nerol and their phosphate or pyrophosphate esters may occur that are more rapidly established than the redox system involving the free alcohols. If the phosphatases involved in the

cleavage of the esters to the free alcohols can promote C—O bond fissions and consequent inversion of configuration at C-1, epimerisation of any ^3H that was stereospecifically-positioned at this carbon could occur. Pyrophosphatases from *Menyanthes trifoliata* do cleave geranyl and neryl pyrophosphates with such bond fission [10] and such cleavage results in inversion at C-1 when the porcine liver enzyme was used [17]. Either type of perturbation of the simple (geraniol → geranial → neral → nerol) scheme could account for our observed results.

The data for *T. vulgare* in the latter half of Table 1 reveal an interesting pattern that can be rationalized on the basis of the above suggestions, if, in addition, we postulate a 'conveyor belt', viz. geraniol → nerol → isothujone (together with their appropriate intermediate compounds), in which the monoterpenes are held and so prevented from becoming kinetically free. Exogenously-supplied intermediates consequently have to enter this conveyor belt at the appropriate site. Under conditions when geraniol could efficiently enter the system (pairs A and B; incorporations 0.27, 0.50%) the isothujone produced had the 'expected' isotope ratio, but the system was now effectively saturated and such nerol as could enter (0.08, 0.08%) yielded isothujone with anomalous ratios: presumably this nerol before uptake had been involved in the redox and phosphatase-mediated side reactions that we have previously proposed. In contrast, when geraniol was less effectively taken up into the biosynthetic system (pair C, incorporation 0.10%) it fell prey to the side reactions and yielded isothujone with an anomalous isotope ratio, whereas exogenously-supplied nerol could now more readily enter the system, did not succumb to the side reactions, and yielded isothujone with the 'expected' isotope ratio. The results from the corresponding feedings on the *Pinus* and other plant species suggest that here both geraniol and nerol were, in all examples, subject to ^3H loss through the side reactions, before either was taken up onto the conveyor belt.

If these suggestions are correct, the experiments carried out in mid-winter with *T. vulgare* (previous section) could not have been perturbed in this fashion, i.e. redox conversion of geraniol into nerol was essentially irreversible and subsequent entry of nerol into the pathway leading to isothujone was more rapid than significant reversible formation of the phosphate esters of the alcohols. Such a situation is quite feasible—the redox system is presumably controlled by the availability of NADH and NAD^+ or other cofactors and by the activities of the appropriate enzyme systems, and it has been demonstrated that the activities of several enzymes concerned with the formation of monoterpenes in *T. vulgare* vary widely at different times of the year [9, 18–21]. In fact, the 'successful' outcome of many biosynthetic experiments on higher plants may depend on a fortuitous choice of season for carrying out the feedings. This point does not seem to have been generally explored.

EXPERIMENTAL

Materials. The specimens of *T. vulgare* were from the clone that we have previously used [9]. The other species were obtained from the Royal Botanic Gardens, Kew.

Geraniol and nerol-[1-$^3H_2$] were prepared by treatment of citral (7:3 w/w *trans*:*cis*; 0.17 mmol; obtained by oxidation of geraniol with MnO_2) with $NaBH_4$-[$^3H_4$] (25 mCi; 0.085 mmol) in i-PrOH for 12 hr and the alcohols were purified by TLC on Si gel H containing $AgNo_3$ (5%) with AcOH–EtOAc (2:98) as eluant 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 Si Gel plate was sprayed with a sat. ethereal solution of $AgNO_3$ immediately before use. The separated alcohols (R_f *ca.* 0.44, 0.49; values irreproducible and hence standards were essential) were eluted with Et_2O and the solvent was carefully removed by slow (5 ml h^{-1}) flash-distillation at 40° with monitoring (radio-TLC) to check that no tracer was lost in the distillate; total yield 81% spec. radioactivity 1.8×10^4 dpm μmol^{-1}.

The stereospecifically-labelled alcohols were prepared following methods developed for the corresponding farnesols [3]. Geraniol (1 mg) and Tween 80 (2 mg) in Pi buffer (0.1 m; pH 8.0; 0.5 ml) containing EDTA (diNa salt; 330 μg), bovine serum albumin (660 μg), NAD^+ (170 μg) and NADH (170 μg) was added to a solution of liver alcohol dehydrogenase (ex horse liver; 5 units; Boehringer Corp. (London) Ltd) and diaphorase (100 units; Boehringer Corp. (London) Ltd) in H_2O-[$^3H_2$] (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), (IR)-nerol-[1-$^3H_1$] (5×10^3 dpm μmole^{-1}) was purified by TLC as described above. (IR)-Geraniol-[1-$^3H_1$] 1.6×10^4 dpm μmole^{-1}) was prepared similarly from nerol. (1S)-Nerol-[1-$^3H_1$] (1.4×10^4 dpm μmole^{-1}) and (1S)-geraniol-[1-$^3H_1$] (2.2×10^4 dpm μmole^{-1}) were similarly prepared by equilibrating geraniol-[1-$^3H_2$] and nerol-[1-$^3H_2$] respectively in the above described system with H_2O.

Geraniol and nerol-[^{14}C] were obtained by exposing potted specimens of *Pelargonium graveolens* Ait. (30 cm) to an atmosphere of CO_2-[^{14}C] generated by treatment of $BaCO_3$-[^{14}C] (2 m Ci) with lactic acid (40% aq.) in a 10 l. closed vessel [22, 23]. After exposure to bright sunlight (6 hr), the foliage (20 g) was frozen in liquid N_2, pulverized, mixed with an equivalent volume of anhydrous $MgSO_4$ and extracted (Soxhlet) with Et_2O (12 hr). The extract was reduced in volume (to 5 ml; flash distillation, 37°) and the monoterpene fraction was isolated by passage through a column (1 × 10 cm) of MgO with Et_2O (500 ml) as eluant. After concentration, and addition of carrier (100 μl) geraniol and nerol were isolated by preparative GLC (Carbowax 20 M; 5 m × 0.5 cm o.d.; 150°) and purified by TLC on Si gel H–$AgNO_3$ as described previously and then on Si Gel H with C_6H_6–EtOAc (85:15 v/v). This labelling procedure is known [22] to lead to generally but not uniformly labelled products and the % incorporation of tracer was *ca* 0.05%. Final products had *ca* 5×10^4 dpm μmole^{-1}. Geraniol-[^{14}C] and nerol-[^{14}C] were also prepared by feeding glucose-[U-^{14}C] (1.3 μCi) to specimens of *P. graveolens* and harvesting the oil, as described above, after 3 days: now the % incorporation of tracer was 3.6%. Another method was to incubate acetate-[1-^{14}C] (20 μCi) with a cell-free extract from *P. graveolens* that was prepared by a procedure previously used for *T. vulgare* [20]: a surprisingly efficient (*ca* 0.2%) incorporation of tracer into geraniol and nerol was here obtained.

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 [20]) to be chemically (>99.7%) 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 Si Gel H–$AgNO_3$ system with EtOAC–C_6H_6 (3:17) at 2° and eluted and reisolated as described above.

Methods of plant feeding. Shoots containing several fully-expanded leaves 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 (100 μl) was emulsified in Pi buffer (1 ml; pH 7.0; 0.1 M) and fed under conditions of forced transpiration [9]. 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 (in a bright south-facing aspect) before harvesting. Preliminary experiments (with rough assay of labelled products using a radio-TLC scanner) had shown this period to be optimum for passage of tracer into the monoterpenes.

Purification of products. After the period of metabolism, the plant material was pulverized in liquid N_2, mixed with an equivalent volume of anhydrous Na_2SO_4 and extracted with Et_2O (Soxhlet; 500 ml) for 2 days. The volume was then carefully reduced to *ca* 5 ml by flash distillation and worked up. Isothujone was purified as previously described [9], converted into its 4-phenylsemicarbazone, mp 184°, and recrystallized (Me_2CO; EtOH aq.). α- and β-Pinenes (after addition of 200 μl each of carrier) were separated by TLC on Si Gel H–$AgNO_3$ (5%) with hexane at 2°, followed by TLC on Si Gel H with hexane–EtOAC (93:17) also at 2° (α-pinene, β-pinene; R_f 0.68 and 0.49 in the latter system). The purified compounds were then each converted into their adducts with 2-mercaptoacetic acid and these in turn were derivatized as *S*-benzylisothiuronium salts which were recrystallized mps 162, 158°; (ex Me_2CO; MeOH) [24]. The extract containing carvone, after dilution with carrier (200 μl) was passed through a column of MgO (10 × 1 cm) with hexane and then separated by TLC on Si Gel H with C_6H_6–$CHCl_3$ (1:1) at 2°. The carvone was eluted, converted into the 4-phenylsemicarbazone and recrystallized; mp 178° (ex MeOH). The extracts containing 1,8-cineole, again after addition of carrier (200 μl), were passed through a similar column of MgO or separated on Si Gel H with hexane at 2°: the band containing the ether was then eluted and the 1:1 adduct with *o*-chlorophenol was prepared [25] and recrystallized, m.p. 58° (ex petrol, bp 60–80°).

Radiochemical methods. These have been described in some detail [9]. Especial care was taken to ensure that the biosynthetic precursors and products were radiochemically pure, and after recrystallization of solid derivatives to constant specific radioactivity and isotope ratio, the final products were rerun on TLC (Si Gel H; various solvents) and the chromatograms were scanned (using a Tracerlab radiochromatography scanner) to demonstrate that essentially all (>99%) tracer occurred at the appropriate R_f value. Isotope ratios were determined by standard channels-ratio techniques.

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