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1,2 HYDROGEN-SHIFTS IN THE BIOSYNTHESIS OF THE THUJANE SKELETON*

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Key Word Index—*Tanacetum vulgare*; Compositae; isothujone; biosynthesis; 1,2-hydrogen-shifts.

Abstract—Degradation of (+)-isothujone (*trans*-thujan-3-one) biosynthesized in *Tanacetum vulgare* from (3*RS*)-mevalonic acid (MVA)-[2-^{14}C, 2-$^{3}H_2$] showed that one hydrogen from C-2 of the precursor was specifically incorporated at C-4 of product whereas the other was lost. Feeding of α-terpineol-[9-^{14}C, 4-$^{3}H_1$, 10-$^{3}H_3$] (*p*-menth-1-en-8-ol) yielded isothujone with the same isotope ratios as in precursor. These results indicate 1,2 hydrogen-shifts at two locations in the construction of the thujane skeleton from α-terpineol or its biogenetic equivalent, and are consistent with a mechanism involving direct cyclization of the latter to a product that by-passes the formation of the biogenetic equivalent of terpinen-4-ol (*p*-menth-1-en-4-ol) as an intermediate. (3*R*)-MVA-[^{14}C, ^{3}H] was more effectively incorporated (up to 1.5%) into (+)-isothujone *in vivo* during autumn or winter than in summer (up to 0.02%).

INTRODUCTION

Ruzicka's hypothetical scheme [1] for the biogenesis of the thujane series of monoterpenes (shown in modernized form in Fig. 1) requires a 1,2 hydride-shift in the interconversion of the ions derived from α-terpineol (**1**) and terpinen-4-ol (**2**) or the biogenetic equivalents of these species. Studies with ^{14}C-labelled precursors [2, 3] have shown the scheme to be correct in outline but a hydrogen shift has never been demonstrated here or in the biosynthesis of any other monoterpene although such rearrangements are well-established in the formation of higher terpenoids [4, 5]. Recently, α-terpineol was demonstrated to be a more effective precursor of (+)-isothujone (**3**) than was terpinen-4-ol in cell-free preparations from *Tanacetum vulgare* (Tansy: fam. Compositae) and it was proposed [6] that the biogenetic equivalent of the former precursor cyclized to form the thujane skeleton in a process that was concomitant with introduction of oxygen into the ring (Fig. 2): this by-passes the involvement of terpinen-4-ol as an obligate precursor and implies two 1,2 hydride-shifts. We now describe experiments on *T. vulgare* to test for the occurrence of such shifts *in vivo*.

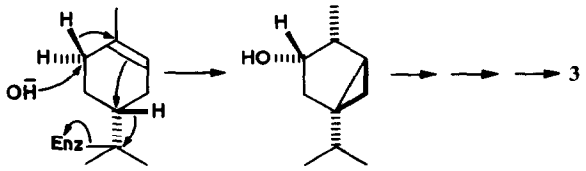

Fig. 2. Hypothetical concerted mechanism for the biogenesis of thujane skeleton. Enz = Enzyme.

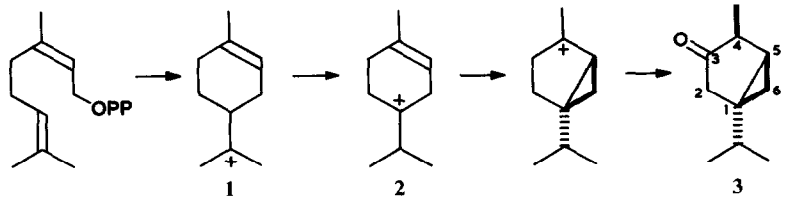

Fig. 1. Ruzicka's scheme for the biogenesis of the thujane skeleton. OPP = pyrophosphate group.

RESULTS AND DISCUSSION

Tracer studies

Feeding of 3*RS*-MVA-[2-^{14}C, 2-$^{3}H_2$] to shoots of *T. vulgare* as described in the Experimental Section gave (+)-isothujone containing the isotope ratios shown in Table 1. In each case products were recrystallized to constant specific radioactivities as the 4-phenylsemicarbazone, the preparation of which was shown, by controls, to proceed without significant enolization and consequent possible loss of ^{3}H. The ratios clearly show that 50 ± 1% of ^{3}H that was incorporated into the notional precursor, α-terpineol, was subsequently lost in the formation of the thujane skeleton.

* Part 19 of series 'Terpene Biosynthesis'. For part 18, see Banthorpe, D. V., Doonan, S. and Gutowski, J. (1977) *Phytochemistry*, **16**, 45.

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Table 1. Isotope ratios in isothujone biosynthesized from (3*RS*)-MVA-[^{14}C, 2-$^{3}H_2$]

Expt.	Date	MVA*†			Isothujone‡§			%I‖
		^{14}C	^{3}H	^{14}C:^{3}H	^{14}C	^{3}H	^{14}C:^{3}H	
1	10.10.75	43.4	22.1	1.96	213	54.9	3.88	1.45
2	12.2.76	64.0	35.0	1.83	140	37.6	3.72	0.65

* (dpm/mmol) × 10^{-6} (S.E. ± 1%). † normalized ratio (for each expt): (^{3}H:^{14}C) = 2:1. ‡ (dpm/mol) × 10^{-6} (S.E. ± 1%). § normalized ratio: (^{3}H·^{14}C): expt 1 = 1.01:1.00: expt 2 = 1.02:1.00. ‖ % Incorporation of (3*R*)-MVA-[2-^{14}C].

We have repeatedly found [2, 3 and unpublished experiments: 32 independent degradations] that essentially all (92–100%) of the tracer in (+)-isothujone biosynthesized in *T. vulgare* from MVA-[2-^{14}C] was located at C-3 (i.e. in the isopentenyl pyrophosphate (IPP)-derived moiety). This pattern, which results [7] from the existence of an endogenous pool of 3,3-dimethylallyl pyrophosphate (DMAPP), was followed in the present examples: degradation (cf. Fig. 3) of isothujone yielded CO which contained >98 and 97% of the incorporated ^{14}C (expts 1 and 2 respectively). Thus it

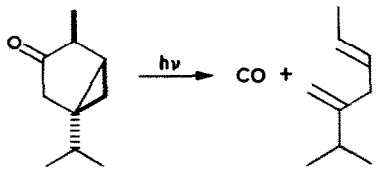

Fig. 3. Degradation of isothujone by photolysis.

seemed likely that the ^{3}H that was retained in product was also associated with the IPP-derived moiety and had undergone a 1,2-shift as in Fig. 2. Such a migration was proved by the following observations: (i) Regeneration of isothujone from the 4-phenyl-semicarbazone by treatment with base yielded, in each case, product that was essentially devoid of ^{3}H (^{14}C:^{3}H) >600). Controls (carried out in medium containing D_2O followed by NMR assay) showed that only exchange at α-carbons to the carbonyl group could have occurred, and so the locations of ^{3}H in the originally biosynthesized products were restricted to C-2 and/or C-4. (ii) No method could be devised to regenerate isothujone from its solid derivative without extensive loss of ^{3}H, and so part of the biosynthetic product from expt 2, was exhaustively purified (GLC, TLC) rather than via derivatization to give a sample with the same (±2%) specific radioactivity and ^{14}C:^{3}H ratio as that of the portion that *was* purified by recrystallization of the 4-phenyl-semicarbazone. This former product (^{14}C:^{3}H, 3.70) was converted into α-thujaketonic acid (**4**; Fig. 4) in conditions known [2, 3] to permit insignificant (<2%) exchange of hydrogen. After purification by formation of derivative and recrystallization (controls showed negligible exchange of hydrogen in these steps also), **4** was essentially devoid of ^{3}H (^{14}C:^{3}H >1000) but all ^{14}C was retained. Thus, for each molecule of 3*R*-MVA-[2-$^{3}H_2$]* incorporated into the IPP-derived portion of isothujone, one atom of tracer was transferred to C-4 of the product, presumably in a hydride shift, and the other was lost in the oxidation process.

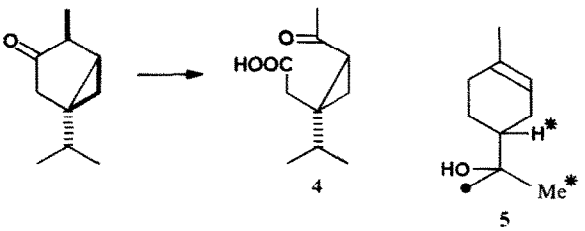

Fig. 4. Degradation of isothujone to α-thujaketonic acid (**4**). Doubly labelled α-terpineol (V): ● = ^{14}C; *H = ^{3}H.

A similar 1,2-shift at the distal end of the thujane skeleton was explored using α-terpineol-[9-^{14}C, 4-$^{3}H_1$, 10-$^{3}H_3$] (**5**). The location of ^{3}H in this molecule, although virtually certain as a result of the synthetic route employed, was confirmed by parallel experiments using D_2O followed by product analysis using NMR. In contrast to the first set of experiments, these feedings were carried out in summer (July, August) and incorporations (^{14}C) of 0.07 and 0.09% were achieved. The isotope ratios (^{14}C:^{3}H) in precursor (2.03 and 4.32) were now essentially unaltered in products (2.15 and 4.39) in the two experiments (all ±2%). The location of ^{3}H in products was not demonstrated, but we have previously shown [8] that α-terpineol-[^{14}C] was incorporated (ca. 0.08%) in clonal material of *T. vulgare* into isothujone in similar conditions without scrambling of tracer, and our results imply that a 1,2-shift occurred as in Fig. 2. We hope to confirm this using geraniol and nerol-[6-$^{3}H_1$] as precursors.

The 1,2 hydrogen-shifts at the two sites are intramolecular and either occur directly or by reversible transfer (e.g. to and from NAD^+ or $NADP^+$) within a strictly compartmentalized system. They may be concerted (cf. Fig. 2) or sequential and may be presumed to be stereospecific: despite considerable study it has not been possible to assign which of these classes of mechanism applies to the analogous cyclizations and hydride shifts that occur in the construction of the steroid skeleton [9–11]. The concerted mechanism in Fig. 2 leads in the cyclization step to a product epimeric at C-4 to (+)-isothujone. A more attractive route is a two stage process involving an 'X'-group as in Fig. 5. Similar 'X'-group mechanisms (X = Enzyme?) have been proposed to accommodate stereochemical details of the biosynthesis of several classes of terpenoids [12–14] and in the present case such a route leads to a product (**8**) with the same configuration at C-4 as (+)-isothujone. In addition, such a route requires the migration of a pro-(2*S*) mevelonoid hydrogen to C-4 of the thujane skeleton, and this stereospecificity is indeed tentatively concluded from tracer studies described in the last section of this Discussion. (The α-hydrogen that migrates in **7** may be inferred to be derived from the pro-(2*S*) hydrogen of MVA using stereochemical corre-

* The 3*RS*-misture was fed, but it is universally accepted that only the 3*R*-isomer of MVA is utilized in terpenoid biosynthesis.

lations deduced for the biosynthesis of farnesyl pyrophosphate [15].) An X-group mechanism also readily accommodates the co-occurrence of (+)-sabinene (**6**), (+)-isothujanol (**8**) and (+)-neoisothujanol (epimeric at C-3 with **8**) in *T. vulgare* [16].

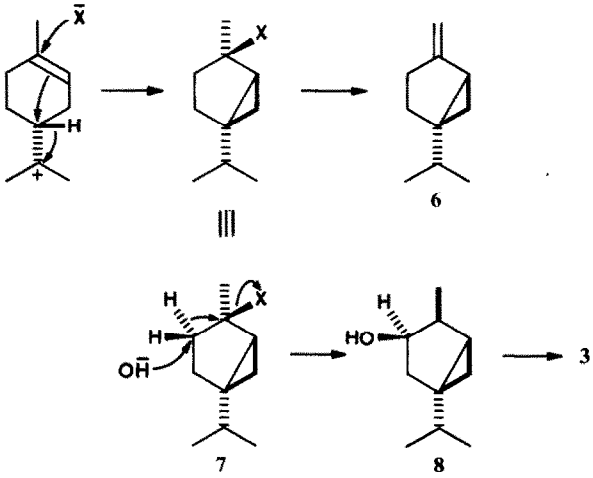

Fig. 5. 'X-Group' mechanism for the biogenesis of (+)-isothujone.

Seasonal dependence of incorporation

The high (0.65, 1.45%) incorporations of 3*R*-MVA-[2-^{14}C] obtained in feeding experiments carried out in autumn and winter (the latter on greenhouse material that was not perceptibly making new growth) were unexpected. The folklore of essential oil production and the limited experience of others [cf. 17], together with knowledge of the seasonal dependence of geraniol synthetase in *T. vulgare* [6] has led us to carry out previous feedings in the period of active growth and flowering (April to early September). In this period incorporations of 3*R*-MVA-[2-^{14}C] of, at best, 0.02% were obtained [2, 3, 7], although IPP and DMAPP were incorporated up to 0.5% under similar conditions [3]. These values are typical for the incorporations of C_5 and C_6 precursors into monoterpenes in foliage of higher plants [17]. We intend to explore the generality of high incorporations in 'off-peak' conditions: it may be that when photosynthesis is at low levels, carotenoid and phytosterol synthesis are correspondingly reduced and exogenous precursor can be channelled into the production of lower terpenoids. As far as we are aware feeding of biosynthetic precursors to plants during their dormant (winter) phase has never been reported.

Experiments with (2R) and (2S)-MVA-[2-$^3H_1$]

Prior to the previously-described studies, we carried out six sets of feedings (June to August) of our clone of *T. vulgare* with (3*RS*, 2*R*)-MVA-[2-^{14}C, 2-$^3H_1$] and its (2*S*) isomer in attempts to define the stereospecificity of any 1,2 hydrogen-shift. Incorporations were typical for summer feedings (*ca* 0.01%) but the isotope ratios (^{14}C:^{3}H; value 1.0 in precursors) varied irregularly within the range 0.6 to 2.3 for rigorously purified samples of isothujone biosynthesized from either isomer of MVA. We consider these to be real values and thus it appeared that ^{3}H in products could be lost *or gained* compared with the precursors in a manner that could not be readily rationalized. Partial degradation showed that these results were not the consequence of significant incorporation of MVA into the DMAPP-derived moiety of the monoterpene. Anomalous results in which ^{3}H from both (2*R*) and (2*S*)-MVA-[2-$^3H_1$] were lost have been frequently observed in studies on terpenoid biosynthesis and have been attributed [18–22] to scrambling and loss of tracer at C-2 due to the reversibility of IPP-isomerase (E.C. 5.3.3.2), and others have found irregular and inexplicable isotope ratios in products biosynthesized from these isomers of MVA [23–25]. We presume that the uptake of ^{3}H into products can only arise from exchange between isothujone and H_2O-[$^3H_1$] produced by the isomerase at the biosynthetic sites but an increase in ^{3}H:^{14}C in products compared with that in the substrate could also be the result of isotope effects (which favour reaction of ^{1}H-containing, rather than ^{3}H-containing compounds) on reactions involving the decomposition of MVA, isothujone or an intermediate between these compounds. In an attempt to circumvent the difficulty in interpretation of such isotope ratios we degraded the isothujone produced in a matched set of feedings from (2*R*)-MVA-[2-$^3H_1$] and its (2*S*) isomer respectively. Conversion of each sample of isothujone into α-thujaketonic acid (**4**) showed that 61% of ^{3}H incorporated from the (2*S*)-isomer resided at C-4 of product whereas only 3% of that from the (2*R*) isomer was so located. The residue of tracer in each sample (1800 and 1670 dpm respectively, corresponding to 39 and 97% of that taken up) was presumably accumulated in exchange processes. This result is consistent with the stereospecific migration of the pro-(2*S*) hydrogen of MVA in the 1,2–shift.

Reversibility of the isomerase only becomes important when the subsequent condensation to form C-10 compounds is sluggish [21]. Our 'winter' feedings of MVA-[2-^{14}C, 2-$^3H_2$] achieved incorporations of up to 145-fold those of 'summer' feedings and it is reasonable to infer that the condensation was not sluggish under the former conditions. The lack of anomalous results in these feedings thus becomes understandable. Unfortunately, 'winter' feedings using the (2*R*) and (2*S*) isomers of MVA-[2-$^3H_1$] could not be made as these precursors are no longer available commercially.

EXPERIMENTAL

Most of the chemical, phytochemical and radiochemical techniques have been described in detail [2, 3, 7, 8].

Sources. T. vulgare was a clone that was cultivated outdoors in central London. All feeding experiments were made on plants directly lifted from the plots except those carried out in February which used plants potted in the previous October and maintained throughout the winter at 20–25° in a bright south-facing window under natural illumination and with weekly watering. (+)-Isothujone, $[\alpha]_D^{22}$ (neat) + 74.3, for use as carrier was extracted from specimens of *T. vulgare* growing on the railway embankment at Bletchley, Bucks. These plants yielded an oil containing essentially only (+)-isothujone (>99% w/w). Our clonal material typically gave oil containing *ca* 83% of this product [16] and so the extracted oil could be used without purification. α-Terpineol-[9-^{14}C, 4-$^3H_1$, 10-$^3H_3$] was prepared as previously described [8] for α-terpineol-[9-^{14}C] using 4-acetyl-1-methylcyclohex-1-ene (50 mg) that had been labelled with tracer at carbons α to to the carbonyl group by treatment (120 hr, 30°) with H_2O-[$^3H_2$] (1 ml; 0.5 mCi) and dioxan (0.2 ml) containing NaOH (2 mg). Controls using H_2O-[$^2H_2$] followed by NMR

analysis of product showed that exchange occurred only at the expected positions. It was similarly checked that no ^3H was lost in the subsequent Grignard reaction used to introduce ^{14}C, and in the work-up. The double-labelled alcohol was purified by GLC (Carbowax 20M at 140°) and TLC on Si gel H and silicic acid with C_6H_6–toluene (1:4); EtOAc–hexane (15:85); and $CHCl_3$ to the criteria that we have previously [2] used for purification of radioactive liquids

Feeding experiments. MVA-[^{14}C, ^3H] (50 μg; total *ca* 150 μCi) was stem-fed to freshly cut shoots of *T. vulgare* that had been sterilized (0.1 M NaOCl; EtOH) and excized under sterile H_2O [cf. 2]. After uptake of tracer (0.5 to 1 hr) the foliage was maintained on sterile H_2O (4 day; 25°) before harvesting. α-Terpineol-[^{14}C, ^3H] (50 mg; total *ca* 300 μCi) was fed in emulsion with Triton X-100 [8] and the foliage was maintained as above and harvested after 25 hr. Subsequently, the foliage was pulverized in liq. N_2 and the residue was mixed with an equal part of dry Na_2SO_4 and extracted with Et_2O (100 ml; 20° for 7 day). Isothujone (300 μl) was then added as carrier and Et_2O was removed by slow flash distillation (bath temperature 45°; 3 ml/hr) to recover a residue (2 ml); some tracer passed into the distillate, and the latter was consequently recycled (40°; 2 ml/hr) to give Et_2O essentially free of radioactivity. The final residue was separated (TLC) on Si gel GF 254 with C_6H_6–EtOAc (85:15) at 4° and isothujone was eluted and converted into its 4-phenylsemicarbazone (78%: mp 184°): this was recrystallized (usually 3 ×) to constant specific radioactivity from EtOH–H_2O; Me_2CO–H_2O and/or MeOH. Controls using D_2O solns and analysis by NMR showed that inappreciable (<2%) exchange of ^3H could have occurred under these conditions. When isothujone had to be purified without derivatization (see Discussion) the extract from TLC was further purified by GLC (Carbowax 20M; 0.5 cm × 3 m; 20% w/w on 60–80 mesh G-Cel; N_2 60 ml/min; 130°) and collected in an empty U-tube at 0°. Collection efficiencies were only *ca* 50%, but use of other traps and temperatures caused mist formation and reduced yields. The product was then rerun on the original TLC system, and then on Si gel G with EtOAc at 4° to give a chromatographically pure [TLC; GLC on Carbowax 20M and Apiezon L capillary (50 m × 0.02 mm) columns] compound with specific radioactivity and ^{14}C-^3H ratio identical (±2%) with that of a control sample that had been purified through its 4-phenylsemicarbazone. Isothujone was converted into the oxime of α-thujaketonic acid [60%; mp 168–9°] which was recrystallized to constant specific activity from EtOH–H_2O. Again it was checked that insignificant exchange could have occurred in these procedures [cf. 3]. In contrast, controls showed that essentially complete exchange of hydrogen at the α-carbons to the carbonyl group occurred when isothujone was regenerated from its 4-phenylsemicarbazone by treatment with 0.5 M KOH in dioxan–H_2O (1:1), reflux for 12 hr]. Isothujone (100 μl) in *iso*-PrOH (1.5 ml) was photolysed (20° for 24 hr) in a water-cooled silica cell and CO evolved was collected and assayed [cf. 2, 3]. The reaction was initially sluggish if the water jacket was below *ca* 12°.

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REFERENCES

1. Ruzicka, L., Eschenmoser, A. and Heusser, H. (1953) *Experientia* **9**, 357.
2. Banthorpe, D. V., Mann, J. and Turnbull, K. W. (1970) *J. Chem. Soc.* (C), 2689.
3. Banthorpe, D. V., Ekundayo, O., Mann, J. and Turnbull, K. W. (1975) *Phytochemistry* **14**, 707.
4. Jayme, M., Schaefer, P. C. and Richards, J. H. (1970) *J. Am. Chem. Soc.* **92**, 2059.
5. Goodwin, T. W. (1971) *Biochem. J.* **123**, 293.
6. Banthorpe, D. V., Bucknall, G. A., Doonan, H. J., Doonan, S. and Rowan, M. G. (1976) *Phytochemistry* **15**, 91.
7. Allen, K. G., Banthorpe, D. V., Charlwood, B. V., Ekundayo, O. and Mann, J. (1976) *Phytochemistry* **15**, 101.
8. Banthorpe, D. V., Doonan, H. J. and Wirz-Justice, A. M. (1972) *J. Chem. Soc.* Perkin I 1764.
9. Cornforth, J. W. (1968) *Angew. Chem. Intern. Ed.* **7**, 903.
10. Corey, E. J., de Montellano, P. R. O. and Yamato, H. (1968) *J. Am. Chem. Soc.* **90**, 6254.
11. Corey, E. J., Lin, K. and Yamato, H. (1969) *J. Am. Chem. Soc.* **91**, 2132.
12. Popjak, G. (1970) *Natural Substances Formed Biologically from Mevalonic Acid* (Goodwin, T. W. ed). p. 17. Academic Press, London.
13. Cornforth, J. W. (1969) *Quart. Rev.* **23**, 125.
14. Rees, H. H., Goad, L. J. and Goodwin, T. W. (1969) *Biochim. Biophys. Acta* **176**, 892.
15. Cornforth, J. W. (1973) *Chem. Soc. Rev.* **2**, 1.
16. Banthorpe, D. V. and Wirz-Justice, A. M. (1969) *J. Chem. Soc.* (C), 541.
17. Banthorpe, D. V., Charlwood, B. V. and Francis, M. J. O. (1972) *Chem. Rev.* **72**, 115.
18. Bimpson, T., Goad, L. J. and Goodwin, T. W. (1969) *J. Chem. Soc. Chem. Commun.* 297.
19. Smith, A. R. H., Goad, L. J. and Goodwin, T. W. (1968) *J. Chem. Soc. Chem. Commun.* 926.
20. Coscia, C. J., Bolta, L. and Guarnaccia, R. (1970) *Arch. Biochem. Biophys.* **136**, 498.
21. Rees, H. H. and Goodwin, T. W. (1972) *Biosynthesis* (Geissman, T. A. ed.). Vol. I, p. 59. Chem. Soc., London.
22. Hanson, J. R. and Nyfeler, R. (1975) *J. Chem. Soc. Chem. Commun.* 171.
23. MacMillan, J. Simpson, T. J. and Yeboah, S. K. (1972) *J. Chem. Soc. Chem. Commun.* 1063.
24. Sliwowski, J. and Kasprzyk, Z. (1974) *Phytochemistry* **13**, 1451.
25. Sliwowski, J. K. and Caspi, E. (1976) *J. Chem. Soc. Chem. Commun.* 196.