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STUDIES ON THE BIOSYNTHESIS OF THE CARANE SKELETON*

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Key Word Index—*Pinus sylvestris*; Pinaceae; (+)-car-3-ene; biosynthesis.

Abstract—Measurements of isotope ratios in car-3-ene biosynthesized in *Pinus sylvestris* from (3*RS*)-mevalonate-[2-^{14}C,2*R*-^{3}H$_1$] and [2-^{14}C,4*R*-^{3}H$_1$] and the corresponding *S*-epimers and also from geraniol-[^{14}C,1-^{3}H$_2$] and nerol-[^{14}C,1-^{3}H$_2$] have shown that the carane skeleton is constructed from its presumed monocyclic precursor with migration of an olefinic bond, together with an unexpected 1,2-shift of a proton to the site of the original double bond. The detailed stereochemistry of the processes allows a two-step mechanism to be inferred for the cyclization in which a bonded intermediate is involved. The conversion of geraniol into nerol (*en route* to car-3-ene) probably is a redox process with the intermediacy of the corresponding aldehydes. The present results eliminate a possible mechanism for this isomerization wherein cyclopropane derivatives occur as intermediates.

INTRODUCTION

We have recently shown [1] that the route for the biosynthesis in certain *Pinus* species of (+)-car-3-ene (the most common naturally occurring compound with the carane skeleton) from its presumed precursor geranyl pyrophosphate (**1**, GPP) is not **1** → **2** → **3** → **4**, as had been assumed, but rather may be formally represented by **1** → **2** → **5** → **6** (Scheme 1). This means that a shift of an olefinic bond in the species **5** (the biogenetic equivalent of α-terpineol) must have occurred in the course of the construction of the bicyclic system. We further proved [1] that the pro-2*S* hydrogen of mevalonate (MVA) was lost in the formation of the double bond of **6**.

Such a migration of a double bond is unprecedented in the biosynthesis of monoterpene hydrocarbons [2] (or indeed for sesquiterpenes and higher terpenoids). The question arises as to the reason for its occurrence and in particular, whether it is part of a concerted or similar process linked to the 1,3-elimination that forms the cyclopropyl ring in **6**. We report here the use of dual-labelled precursors to explore further the stereochemistry of these processes, and the results obtained allow us to propose a model for the enzymic cyclization.

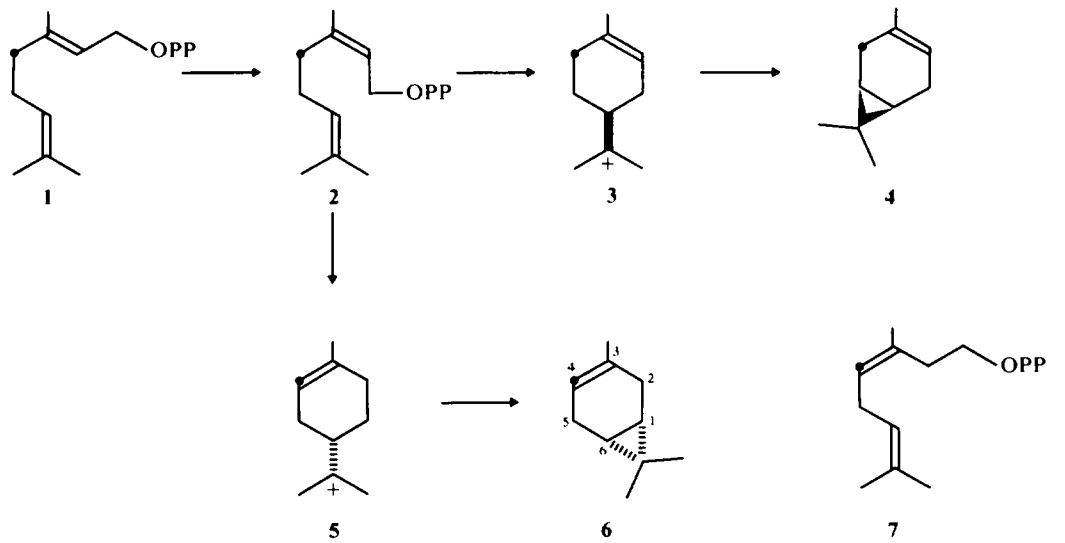
Our plant material was *Pinus sylvestris* (Pinaceae), one of the few good sources of compounds with the carane skeleton. Details of the composition of the oil from this species, the optical purity [*ca.* 100% (+)] of the car-3-ene isolated and times of maximum incorporation of tracer from MVA into monoterpene have been recorded [1].

RESULTS AND DISCUSSION

Firstly, we needed to determine the proportion of the tracer in car-3-ene derived from exogenous MVA-[2-^{14}C] that was located in the moiety of the former derived from isopentenyl pyrophosphate (IPP). This was because interpretations of labelling patterns in monoterpenes biosynthesized from MVA-[2-^{14}C] are bedevilled by the fact that the IPP-derived fragment is generally predominantly (70–100%) labelled, whereas that moiety derived from 3,3-dimethylallyl pyrophosphate (DMAPP) contains much less and sometimes negligible amounts of tracer. This is because of the existence of an endogenous pool of DMAPP or its biogenetic equivalent [3]. In our present work, feeding of MVA-[2-^{14}C] led to an incorporation of tracer into car-3-ene of 0.06% of the 3*R*-isomer: this low value is typical of that found in such feeding experiments [4]. Degradation, as previously described [1], to liberate tracer from C-4 of car-3-ene (cf. **6**) showed that 98 ± 2% of tracer was at this position in the present set of experiments. This additionally confirmed the reality of the previously found double bond shift (i.e. the route **1** → **6**, Scheme 1). Secondly, we confirmed that the pro-2*S* hydrogen of MVA is indeed lost in the formation of the double bond in **6** (Table 1). Thirdly, we had to show that a double bond shift indeed occurred since there is the alternative explanation that **2** is not formed, but that IPP and DMAPP condense to form **7** that in turn bicyclizes to give **6**. This *ad hoc* explanation (that was originally proposed by a referee to accommodate the previous results, cf [1]) can be checked by using MVA-[2-^{14}C,4*R*-^{3}H$_1$] and the corresponding 4*S*-epimer as precursors. Formation of **5** *en route* to car-3-ene would involve [2] loss of the pro-4*S* hydrogen of MVA, whereas formation of **7** would result in incorporation of both hydrogens at C-4 of MVA into product. The results (Table 1) are clear and favour the proposed formation of **5** followed by shift of unsaturation.

Our fourth set of results utilized geraniol (3,7-dimethylocta-*trans*-2,6-dien-1-ol)-[^{14}C,1-^{3}H$_2$] and similarly labelled nerol (the corresponding *cis*-isomer) as precursors. Within the experimental error or close therein (Table 1) one half of the ^{3}H was lost from the former on its

*Part 26 in the series "Terpene Biosynthesis". Reprints of this paper are not available. For Part 24 see Akhila, A. and Banthorpe, D. V. (1979) *Phytochemistry* **18**, 1519.



Scheme 1. Biogenetic routes to car-3-ene. ● is carbon derived from C-2 of MVA. OPP is pyrophosphate.

incorporation into car-3-ene, whereas no such loss occurred from nerol. The loss of one hydrogen from geraniol, but none from its isomer, on being converted into a cyclic monoterpenoid is expected [5] and can be accommodated by a redox interconversion of geraniol and nerol involving the formation of the appropriate aldehydes as intermediates. The whole process reflects the circumstance that nerol, but not geraniol, has the capacity to cyclize to form six-membered rings. However, it is unexpected that no tracer was lost on conversion of nerol into car-3-ene, as one of the hydrogens at C-1 of the former must be eliminated in the construction of the cyclopropane ring. This one hydrogen from C-1 of nerol must, therefore, be intramolecularly transferred to an unexpected site in the carane skeleton, and the only real possibility seems to be a 1,2-shift as shown in Scheme 2 whereby the eliminated hydrogen attaches to one end of the disappearing double bond. Such a shift would provide a driving force for the previously deduced migration of the double bond within the ring system. We checked this theory by oxidizing car-3-ene that had been biosynthesized from geraniol-[1-$^3H_2$] and nerol-[1-$^3H_2$] to the keto acid **10** and subjecting the latter to conditions known (as checked by blanks using ^2H_2O-exchange and monitored by ^1H NMR) to allow exchange of tracer at carbons α to the carbonyl group. The exchange was accompanied by tar formation (due to aldol condensation?) and under the conditions necessary to remove ^3H (allowing for the isotope effect in cleaving C-^3H rather than C-^1H) complete exchange could not be achieved before extensive loss of product had occurred owing to this side reaction. Nevertheless, the results (Table 1) indicate that enolization removed *ca* 40% of one incorporated ^3H from nerol and the loss of tracer was increasing when the experiment had to be discontinued. By contrast less than 10% of tracer was lost under similar conditions with product formed from geraniol. We can interpret these results if nerol is converted into car-3-ene with a proton shift as discussed above. The results for geraniol may be interpreted if the pro-1*S* hydrogen was exchanged in its interconversion to nerol (Scheme 2), as such stereospecificity has been found for this interconversion in several other plant species [5, 6]. Thus, in theory,

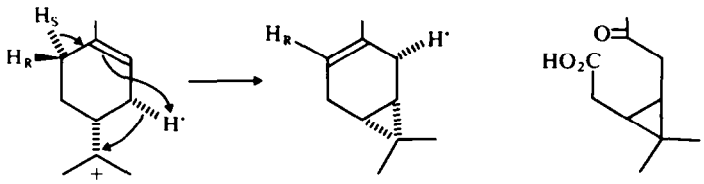
Table 1. ^3H:^{14}C Isotope ratios in car-3-ene formed in *P. sylvestris*

Precursor*	Incorporation†	^3H:^{14}C‡		
		Precursor	Car-3-ene	Keto acid
MVA – [2-^{14}C; 2*R*-$^3H_1$]	0.01	8.23	8.15	—
MVA-[2-^{14}C; 2*S*-$^3H_1$]	0.01	8.25	0.05	
MVA-[2-^{14}C; 4*R*-$^3H_1$]	0.01	6.10	6.18	
MVA-[2-^{14}C; 4*S*-$^3H_1$]	0.05	3.14	1.67	
Geraniol-[^{14}C; 1-$^3H_2$]	0.05	3.14	1.67	1.58
Nerol-[^{14}C; 1-$^3H_2$]	0.05	8.41	8.12	6.43

* For MVA, the biosynthetically utilised isomer; the 3*RS* precursors were fed in each case.

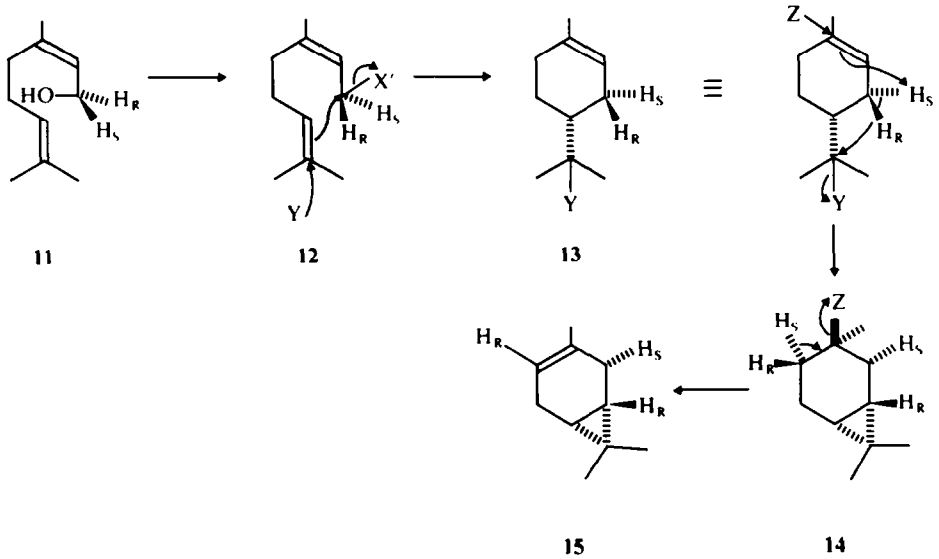
† % incorporation of ^{14}C.

‡ Isotope ratios in MVA, geraniol and nerol, car-3-ene and also the keto acid **10** after equilibration of ^3H with isotopically normal solvent. All values are ±5%.



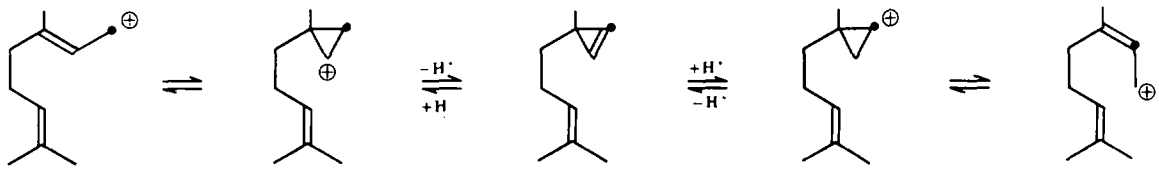
8 9 10

Scheme 2. Concerted formation of the carane skeleton.



11 12 13 14 15

Scheme 3. X-Group mechanism for biogenesis of the carane skeleton.



Scheme 4. Conversion of geraniol into nerol via cyclopropane intermediates. ● is carbon derived from C-1 of geraniol.

the hydrogen that is involved in the 1,2-shift is not labelled when geraniol is the precursor. In practice the 10% exchange of tracer that was found shows that some ^3H was incorporated at C-2 in the product derived from geraniol. This apparent non-specificity of the enzymic redox system is similar to that previously observed and for which various explanations have been proposed [5, 6].

A concerted process of H^+ shift, migration of double bond, and H^+ loss (e.g. **8** → **9**) is forbidden as the usual *anti*-stereoelectronic requirement for the interacting electrons cannot be achieved. Also examination of Dreiding models suggests that in any case the stereochemistry for the (forbidden) *syn* interactions would be difficult to attain. However, an *anti* relationship can be achieved if an 'X-group' mechanism involving two steps for the above electronic transactions is followed (Scheme 3; **13** → **15**). Such mechanisms (X = Enz.?) have been proposed to accommodate stereochemical problems in the biosynthesis of several types of terpenoids and steroids [7] and in the present example it is pleasing to discover that such a two-step mechanism largely relieves the steric restrictions to the reaction that models revealed to be present in the direct cyclization **8** → **9**. If we allow an X-group process, we may reasonably propose a model for the cyclization that involves two binding sites to the enzyme (viz. **12** → **15**). This model would require only one enzyme to catalyse the entire route to the carane skeleton from NPP (or its biochemical equivalent) and the proposed route implies that the biogenetic equivalent of α-terpineol (**13**) never becomes kinetically free. This is consistent with the general experience that exogenously supplied α-terpineol is a poor precursor of monoterpenes [4]. It is interesting to note that

an X-group mechanism has been proposed to accommodate an entirely different set of stereochemical data concerning the biosynthesis of the thujane skeleton from NPP [8]. Also, there is recent evidence from studies using cell-free extracts of various plants [9] that different types of mono- and bi-cyclic monoterpenes may be formed from NPP by the agency of specific cyclases (single enzymes or stable multi-enzyme complexes) each being specific for formation of a particular type of skeleton.

There is an additional inference from the results involving use of geraniol and nerol as precursors. It has been assumed [5] that observation of conversion of geraniol into cyclic monoterpenes with loss of one hydrogen from C-1 under conditions where nerol is converted without loss indicated that geraniol and nerol were linked by a redox system and only the latter could be directly converted into product. Indeed appropriate redox enzyme systems have been isolated from a variety of plant species [6]. An alternative interpretation, proposed for sesquiterpene biosynthesis [10], but obviously generally applicable, suggests that geraniol and nerol (or their biogenetic equivalents) be interconverted via intermediates with cyclopropane and cyclopropene rings (Scheme 4). However, this route would require that ^3H originally at C-1 of geraniol was transferred to C-2 of nerol and ultimately was all located at C-2 (**6**) of car-3-ene but this was not observed. A more satisfactory rejection of this hypothetical mechanism would involve the use of geraniol-[1-^{14}C] and nerol-[1-^{14}C] as precursors. However, location of tracer at C-1 and C-2 of the carane skeleton [6] is difficult to achieve, and we have carried out such experiments on other plant species that produce other, more tractable, types of monoterpenes. These experiments, which also disprove in these particular plants the route in Scheme 4, will be shortly published.

EXPERIMENTAL

Materials. Foliage from young branches of mature specimens of *P. sylvestris* were collected at the Royal Botanic Gardens, Kew, and at the U.C. Botanic Garden at Regent's Park, London. Car-3-ene for use as carrier, was distilled from a commerical sample (ex. Bush Boake Allen and Co. Ltd., London) 121 126°/200 mm Hg, then purified by GLC on FFAP (10%; 6 m × 0.5 cm) at 150°. The final product was >99% pure (TLC; capillary GLC on FFAP, Carbowax 20M). Geraniol-[4,10-$^{14}C_2$] and the corresponding nerol were obtained by feeding *Rosa dilecta* with MVA-[2-^{14}C] as described [11]. Geraniol-[1-$^3H_2$] and the corresponding nerol were available [6].

Isotope studies. Pine needles (*ca* 20 g) were fed with MVA as previously described [1]; in each experiment 2–6 μCi of tracer were used. Geraniol and nerol at similar levels of radioactivity were fed in emulsion generated by sonication of the alcohol (1·5 mg) with Tween 80 (5–10 mg) in H_2O (1 ml). All feedings were carried out within 2–3 days in May (MVA) or July (geraniol, etc.). After a metabolism period of 48 hr, the needles were pulverized in liquid N_2 and extracted (Soxhlet) with Et_2O (200 ml; 16 hr). The extract was concd (3 ml) by flash distillation (37°; 1·5–2 ml hr), car-3-ene (300–500 mg) was added as carrier and the extract was chromatographed on a column of MgO (15 × 1 cm) with C_6H_{12} and successive fractions (3 ml) were monitored by TLC on Si gel H with EtOAc–Et_2O (1:1). The fractions containing car-3-ene were pooled, concd by flash distillation and subjected to prep.-GLC on Carbowax 20M (3 m × 0.5 cm; 150°), followed by FFAP (same conditions) and then TLC on Si gel H with EtOAc. The final product which had maintained its specific radioactivity and isotope ratio through the last two steps of purification was shown to be chemically pure (>99.5%) by capillary GLC on Carbowax 20M and SE-30 and by TLC on Si gel H with a variety of eluants. The specific radioactivity was also constant across the fractions collected from prep.-GLC (Carbowax 20M and FFAP) and across sectors cut from overloaded (and hence smeared) TLC plates. Radiochromatographic scanning (2π-autoscanner) of a variety of TLC separations showed that the product was at least 99% radiochemically pure.

Car-3-ene was ozonized in MeOH at −40° [12]. After removal of MeOH (red. pres.), the ozonide (*ca* 0.7 g) in HOAc (8 ml) was reduced with activated Zn (600 mg) at 95° to yield the keto aldehyde corresponding to **10**: disemicarbazide (ex EtOH), mp 198°. This was converted into **10** with $KMnO_4$ [1]; semicarbazide (ex EtOH) mp 177°. Controls carried out with deuterated solvents with assay of products via ^{1}H NMR indicated that 5%, if any, ^{3}H located in car-3-ene, could have been lost during these procedures. Measurement of ^{2}H exchange to guage the feasibility of ^{3}H exchange was carried out on the keto acid **10** (368 mg) in ^2H_2O (2 ml) with NaOH (0.08 g). After refluxing for 3 hr, extraction and purification of the keto acid and examination by ^{1}H NMR revealed that >95% exchange at carbons α to the carbonyl group had taken place. For the experiment to remove ^{3}H from the keto acid it would have been desirable to extend the time of exchange to at least 30 hr to allow for the unfavourable isotope effect, but in the event, the experiment had to be discontinued after 12 hr of reflux as tar formation was becoming extensive at this time.

Radiochemical methods. These have been described [3]. Typically, samples for assay contained 2000–3000 dpm (^{14}C) and up to 20 000 dpm (^{3}H). 40 000 disintegrations were accumulated to ensure that 2σ was ±1%. All experiments were carried out in duplicate.

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