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BIOSYNTHESIS OF (+)-CAR-3-ENE IN *PINUS* SPECIES*

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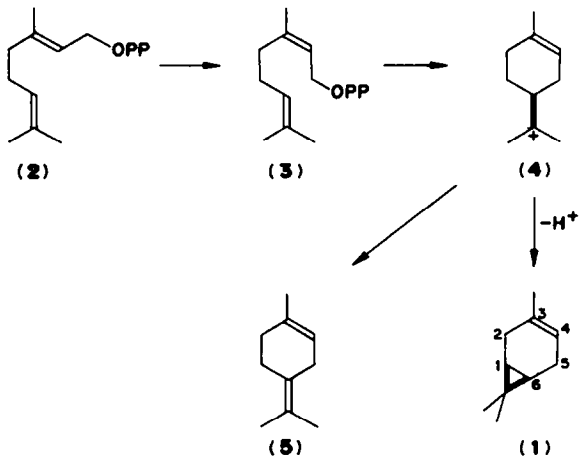
Key Word Index—*Pinus palustris*; *Pinus sylvestris*; (+)-car-3-ene; biosynthesis; labelling patterns.

Abstract—Degradation of (+)-car-3-ene biosynthesized from MVA-[2-^{14}C] in *Pinus palustris* or *Pinus sylvestris* proved that the C-4 atom of the monoterpene is derived from C-2 of MVA rather than C-4 as has been hitherto assumed. The *pro*-2S hydrogen of MVA is stereospecifically lost in the formation of the Δ^3-double bond. These results delineate possible routes for the biosynthesis of the carane skeleton.

INTRODUCTION

(+)-Car-3-ene (**1**) is the only commonly-occurring monoterpene with the carane skeleton. Although it is fairly widely distributed, mainly in the *Coniferae*, nothing is known of the mechanism of its biosynthesis and Ruzicka's classical hypothetical scheme [1] shown in a modernized form in Scheme 1, is usually considered to apply. Support for this route has been claimed as a consequence of statistical analysis of the compositions of plant oils which revealed a high correlation for co-occurrence of car-3- and terpinolene (**5**) which were thus presumed to be both derived from the biogenetic equivalent of the α-terpinyl ion (**4**) by alternative modes of proton-loss [2,3]. Other hypothetical mechanisms have involved nonclassical ions [4] or carbenes [5] derived from NPP (**3**) as intermediates. We now report the results of tracer studies on the formation of (+)-car-3-ene in *Pinus palustris* Miller and *Pinus sylvestris L.* (both *Pinaceae*) which are its most abundant sources.



Scheme 1. Ruzicka's scheme (modified) for formation of the carane skeleton. PP = pyrophosphate. Intermediate (**4**) is formally shown as a carbonium ion: it may be an ester or protein-bonded.

RESULTS

Sources. Analyses of the oils from needles of the *Pinus* species are given in Table 1. These oils comprised 1·0 and 0·2% (w/w) of the foliage for *P. palustris* and *P. sylvestris* respectively and the car-3-ene from the two species had $[\alpha]_D^{25}$ + 10·6 and + 10·9 (c5, $CHCl_3$). The percentage compositions recorded are the mean of 3 analyses and the figures are reproducible to about ± 1% of the quoted values.

Tracer studies. Time-incorporation measurements were made to find the period for optimum incorporation of tracer into car-3-ene. For *P. palustris*, MVA-[2-^{14}C] was maximally incorporated (0·070%) into car-3-ene within 24 hr of pulse-feeding in June–July 1973: this level steadily fell to 0·002% after 168 hr. Incorporations of tracer into longifolene, caryophyllene and the unidentified sesquiterpenes were 0·077, 0·082 and 0·071% at 24 hr, increasing to 0·20, 0·25 and 0·16% at 49–72 hr and falling to 0·002, 0·003 and 0·002% by 168 hr. In *P. sylvestris*, tracer from MVA-[2-^{14}C] was maximally incorporated (0·005%) at 48 hr in March 1974: other monoterpenes and sesquiterpenes were labelled 0·003 and 0·010%. These assays involved radioscanning of chromotograms and so could be expected [cf. 6] to be only semi-quantitative; however the values obtained for car-3-ene were very close to those subsequently obtained when the monoterpene was purified (as a derivative) to constant specific radioactivity (cf. Table 2).

(+)-Car-3-ene biosynthesized in both *Pinus* species from MVA-[2-^{14}C] in the above months was degraded as shown in Scheme 2. Tracer in the monoterpene was assayed after oxidation to the ketoaldehyde (**6**) and purification to constant specific radioactivity of a derivative

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Abbreviations used: MVA, mevalonic acid; IPP, isopentenyl pyrophosphate; DMAPP, 3,3-dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; NPP, neryl pyrophosphate; I-unit, IPP-derived moiety of monoterpene skeleton; D-unit, DMAPP-derived moiety *ditto*.

Table 1. Components of oils of *Pinus* species

Compound	*P. palustris**	*P. sylvestris**
Car-3-ene	31·7	77·1
α-Pinene	26·4	19·9
Camphene	10·3	0·0
β-Pinene	22·0	0·0
Limonene	2·1	0·3
α-Phellandrene	3·3	0·0
Unidentified C_{10}†	1·0	0·8
Longifolene	2·1	0·0
Caryophyllene	0·3	0·0
Unidentified C_{15}†	0·1	0·9
Total	99·3	99·0

* % of component (w/w). † Hydrocarbons (GC–MS analysis).

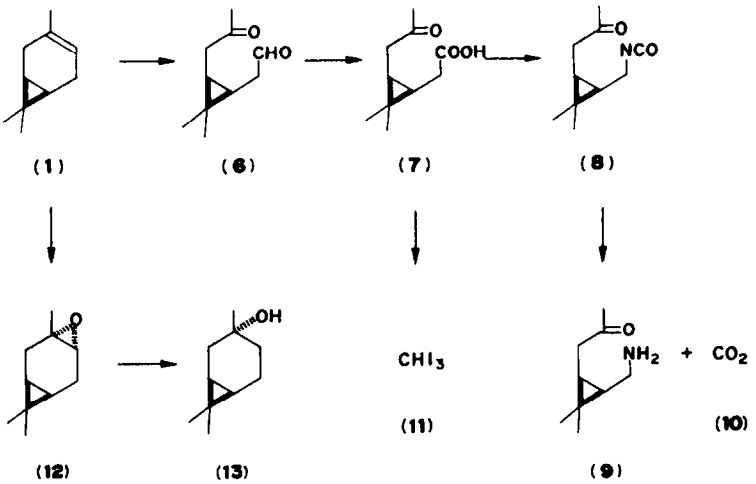
of this. Radioactivities of the degradation products, all of which (in the form of solid derivatives, if necessary) were similarly purified, are in Table 2.

Isotope ratios in car-3-ene (purified as caran-3-ol, **13**) that had been biosynthesized from MVA-[^{3}H; ^{14}C] are given in Table 3. The last column gives the calculated $^3H/^{14}C$ ratios if one ^{3}H atom is lost *per* I-unit but none is lost *per* D-unit and the I and D units are labelled with ^{14}C in the proportion 86:14 (see Table 2). Incorporations in these experiments were similar to those recorded in Table 2 and products typically contained 10^3 to 10^4 dpm of ^{14}C.

DISCUSSION

Most reports of the natural occurrence of car-3-ene refer to *P. sylvestris* but there is some confusion over the other main source described as *P. longifolia* as the latter is a synonym for both *P. palustris* Miller and *P. roxburghii* Sargent [7]. We obtained excellent yields of car-3-ene (77 and 31%) with $[\alpha]_D^{25}$ + 10·6 and + 10·9; from *P. sylvestris* and *P. palustris* respectively; previous reports gave 'best' optically-pure samples with $[\alpha]_D^{25}$ + 7·7 and + 7·0 [8,9]. The patterns of products from our specimens resembled those reported for *P. sylvestris* [10,11] and *P. longifolia* [12,13], and the absence of oxygenated mono- or sesqui-terpenoids was striking.

(+)-Car-3-ene obtained in the biosynthetic experiments and its degradation products were purified to constant specific activity (as solid derivatives if necessary) and CO_2 was purified to the criteria previously utilized [14]. 94–97% of the initially incorporated tracer was accounted for in the degradation products.



Scheme 2. Schemes for degradation and for double-label assay of (+)-car-3-ene.

Table 2. Tracer patterns from degradation of car-3-ene biosynthesized in *Pinus* species after feeding MVA-[2-^{14}C]

Species	t(hr)*	Sp Act †	%I‡	Products (%)¶
P. palustris	24	2008	0·070	**9**(14); **10**(82)
P. palustris	139	979	0·028	**9**(2); **10**(92)
P. sylvestris	48	3023	0·005	**9**(11); **10**(86); **11**(0)

* Metabolic period after uptake of tracer. † Sp act of car-3-ene (dpm $mmol^{-1}$); counted as the disemicarbazone of **6**). Values cannot be compared in different experiments as different quantities of tracer and/or carrier were generally used. ‡ % incorporation of tracer into car-3-ene. ¶ Degradation products (cf. diagram 2: counted if necessary as solid derivatives, see Experimental) and % of tracer in these. Each value is an independent determination.

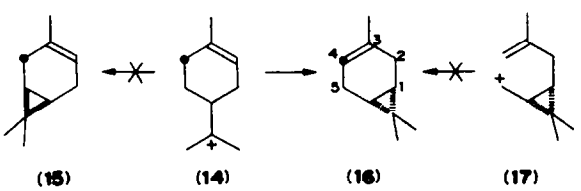
Table 3. Isotope ratios in car-3-ene formed in *Pinus* species after uptake of MVA-[^{3}H : ^{14}C]

Species	Precursor (MVA)*	t(hr)†	^{3}H:^{14}C‡		
			MVA	Car-3-ene	Calc.
P. Sylvestris	[3R-^{14}C;2R-$^3H_1$]-	48	2·98	2·85	—
P. sylvestris	[3R-^{14}C;2RS-$^3H_2$]-	48	1·83	1·04	1·04
P. palustris	[3R-^{14}C;5-$^3H_2$]-	47	0·84	0·45	0·48

* The biosynthetically-utilized isomer: the 3RS-precursors were fed in each case. † Metabolic period after uptake of tracer.‡ Isotope ratios in MVA, car-3-ene (counted as the 3,5-dinitrobenzoate of the derived caran-3-ol), and values calculated on basis of loss of 1 atom of ^{3}H per I-unit, 0 per D-unit, and asymmetric labelling (see text). All ratios are ± 5%.

Several conclusions can be drawn from the tracer experiments. Firstly, the pilot studies indicated that car-3-ene was the most heavily labelled monoterpene and that it was involved in metabolic turn-over in the 168 hr observation period. Also, although the sesquiterpenes composed only *ca* 2·5% of the oil of *P. palustris* (the residue was monoterpenes) the former were labelled at maximum incorporation some five-fold greater than the monoterpenes. A similar disparity in efficiency of incorporation was found in *Mentha piperita* [15] and was attributed to compartmentation of the sites of biosynthesis for mono- and sesqui-terpenes with MVA only being readily accessible to the latter.

Secondly, (+)-Car-3-ene biosynthesised from MVA-[2-^{14}C] in three experiments with both *Pinus* species contained the bulk (82–92%) of the incorporated tracer in the fragment cleaved as CO_2 [Table 2]: i.e. this tracer was in the I-unit of the monoterpene. This asymmetric pattern is similar to that generally found for the biosynthesis of various monoterpenes from a variety of [^{14}C]-labelled precursors [14] and has been attributed to the existence of a protein-bonded pool of the D-unit [16]. More interestingly, the position of the tracer shows that if the biogenetic equivalent of the α-terpinyl ion (**14**: Scheme 3) is an intermediate, the cyclisation proposed by Ruzicka to form the cyclopropyl ring (**14** → **15**) is, at best, quite unimportant. Our results show that the C_4 of (+)-car-3-ene is very predominantly derived from C_2 of MVA-[2-^{14}C] and so the process **14** → **16** involving shift of a double bond must occur. Several routes involving hypothetical exotic precursors (e.g. the *cis*-isochrysanthemyl ion, **17**: Scheme 3) for (+)-car-3-ene may readily be invented, but the invariable co-occurrence in plant oils with (+)-car-3-ene of the normal pattern of mono- and bi-cyclic monoterpenes generally accepted to be derived from **14** [5] and the complete absence of irregular monoterpenes and other exotics suggest that **14** is the precursor of the carane skeleton. The 1,3-elimination to form the cyclo-propyl ring in **14** → **16** has analogues in the formation of cycloartenol, presqualene



Scheme 3. Routes for construction of the carane skeleton. Dot represents ^{14}C in the I-unit derived from MVA-[2-^{14}C].

alcohol, prephytoene and (possibly) chrysanthemyl alcohol. It may be noted, however, that the formation of the cyclo-propyl ring in thujane derivatives is believed to occur by transannular addition of the positive centre to the double bond in the ion derived from terpinen-4-ol [5]. A referee has suggested that our labelling results and also the non-occurrence of car-2-ene (see below) could be explained by the intermediacy of the pyrophosphate of 3,7-dimethyl-octa-3,6-dien-1-ol. Although the formation of this hypothetical compound would require only a minor change in the normal biosynthetic sequence to GPP and NPP there is no direct or inferred evidence for the existence of such a compound in terpene metabolism; furthermore, tracer studies on the biosynthesis of members of the pinane, thujane and camphane series decisively rule out such an intermediate in these cases [5]. The direct formation of this compound from DMAPP and IPP could be tested by the use of 4S-[^{3}H]-MVA.

Thirdly, the results (Table 3) involving MVA-[2R-$^3H_1$] and [2RS-$^3H_2$] show that when allowance is made for ^{3}H introduced into the D-unit, the isotope ratios in (+)-car-3-ene prove that the *pro*-2S hydrogen of MVA is stereospecifically lost, within the experimental error, in the construction of the double bond.

And fourthly, isotope ratios in (+)-car-3-ene formed after feeding MVA-[5-$^3H_2$] indicate, within the experimental error, that half the ^{3}H is lost in the I-unit of the monoterpene. Two explanations are consistent with this pattern. One envisages NPP (**3**), that is the precursor of the α-terpinyl ion (**4**), retaining both hydrogens at C_5 of MVA but one of these subsequently being removed in the cyclisation to give the carane skeleton. The other requires one hydrogen at C_5 of MVA to be lost in the conversion of GPP (**2**) into NPP (**3**) but the remaining hydrogen to be retained in the carane skeleton. Enzyme systems from *Andrographis paniculata* convert 2-*trans*-6-*trans*-farnesyl pyrophosphate into its 2-*cis*-6-trans isomer with the stereospecific loss of a *pro*-1S hydrogen of the substrate [17] presumably in a redox process involving aldehyde-formation and a similar process may be involved in the formation of NPP from GPP.

The cyclisation **14** → **16** could involve the intermediate formation of (+)-car-2-ene but as the latter was not detected in our plant oils the bicyclisation and rearrangement to form **16** from the monocyclic precursor may occur when this is protein-bonded. (+)-Car-2-ene is reported to occur in *P. sylvestris* and a few other higher plants [8]: however a recent reinvestigation of the monoterpenes of this species also failed to detect the compound [11] and analyses of oils from numerous *Pinus* [18–22], *Abies* [23–25], *Cupressus* [24] and *Picea* [26]

species that contain car-3-ene in 10–50% (w/w) have also not reported its ocurrence. The older identifications may well be artefacts of isolation: during conventional steam-distillation the acidity of the pot may rise to *ca* pH 2 [5] and this could cause isomerisation of car-3-ene [cf. 27]. Formation of sylvestrene by ring-scission of car-3-ene has long been recognized to occur during work-up of the same pine oils that are claimed to contain car-2-ene [8].

EXPERIMENTAL

Materials. Foliage from young branches of mature specimens of *P. sylvestris* and *P. palustris* were collected at the Royal Botanic Gardens, Kew, and at the National Pinetum, Bedgebury, Kent, respectively. A commercial sample of car-3-ene (for use as carrier) was distilled (Abegg spinning-band column, 1 m × 0·5 cm), bp 125–6°/200 mmHg and pyrogallol (0·01% w/w) was added to prevent autoxidation.

Oil analysis. A pool of needles (200g) of different ages was steam-distilled in the presence of $NaHCO_3$ to keep the pH greater than 5·0. The volatile oil was collected at −78° and analysed by GC–MS after it had been separated into two fractions by column chromatography on silicic acid-Carbowax 20M [28]. GLC columns (5 m × 0·5 cm) were Carbowax 20M, FFAP and silicone oil (all 20% w/w on acid-alkali washed G-Cel; temperature programmed at 90 to 190° and operated at 5–6 l. hr^{-1} N_2 with FID. The eluent from the columns was directly fed into the gas separator of a MS902 mass-spectrometer and the terpenes were identified by computer-matching of spectra and RR_t with those held (*ca* 6000) in the memory store.

General procedures. The technique of feeding pine needles (20g) with tracer, the extraction (after addition of carrier) and chromatographic purification of car-3-ene, the methods for assaying tracer and determining isotope ratios and the counting statistics employed were outlined in related studies [29–30].

Functionalisation and degradation of (+)-car-3-ene. Most of the reactions have been previously described [8] and were scaled down to 50–100 mg. In all cases, compounds possessed the expected IR, MS and [^{1}H]-NMR spectra and elemental analysis. The purity of each compound was routinely checked by GLC on two capillary columns (Carbowax 20M; SE-30; 50m × 0·04mm both WCOT) and TLC (Si gel or Al_2O_3; Et_2O-C_6H_6; Et_2O-EtOAc mixtures). Unless noted, the mp agreed ± 1° with literature values. Ozonolysis of car-3-ene [cf. 8] led to the keto-aldehyde **6**; 60%, disemicarbazone mp 198° (ex. EtOH–H_2O); which was oxidized with $KMnO_4$ [31] to give the keto acid **7**; 60% semicarbazone mp179° (ex. MeOH–H_2O), lit. 183° [31]. This acid was converted by standard procedures [32] into the keto-isocyanate **8** which was decarboxylated in a Curtius rearrangement [cf. 32] to give the amine **9**, BPh_4-derivative, mp 183° dec. (ex aq MeOH; aq Me_2CO). The overall yield for **7** → **9**, was 55% and the recovery of CO_2 was 92%. **7** was also cleaved to give CHI_3 (**11**) 62%. Oxidation of car-3-ene with *m*-chloroperbenzoic acid in $CHCl_3$ yielded *trans*-car-3-ene oxide **12**; 92% $[\alpha]_D^{20}$ + 14·1 (c10; $CHCl_3$) and this was cleaved with $LiAlH_4$ [33] to give *trans*-caran-3-ol, **13**; 92%, $[\alpha]_D^{20}$ + 25·6 (c10, $CHCl_3$), 3,5-dinitrobenzoate mp 129° (ex. EtOH–H_2O). Radioactive degradation products or their solid derivatives were recrystallized (usually at least thrice) to constant sp act: CO_2 was purified as previously described [33].

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