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BIOSYNTHESIS OF CHIRAL α- AND β-PINENES IN *PINUS* SPECIES

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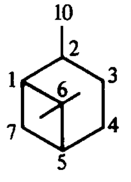
Key Word Index—*Pinus* sp.; Pinaceae; biosynthesis; monoterpenes; α-pinene; β-pinene.

Abstract—C-3 of (+) and (−)-α-pinene and of (−)-β-pinene biosynthesized in several *Pinus* species was derived from C-2 of mevalonate; and the hydrogen at C-5 in all the isomers was derived from that at C-6 in nerol. This pattern is consistent with two routes for bicyclization of the acyclic biosynthetic precursor: one leads to (−)-β-pinene and the other to (+)-α-pinene of opposite absolute configuration. (−)-α-Pinene probably results from subsequent isomerisation of the (−)-β-isomer, and (very small) amounts of (+)-β-pinene result from similar (unfavoured thermodynamically) isomerisation of the (+)-α-isomer.

INTRODUCTION

β-Pinene (pin-2(10)-ene; cf. **1**) occurs in almost all *Pinus* species as the (−)-isomer of high optical purity ($[\alpha]_D$ *ca* −21°; *ca* 96% e.e.), whereas the (+)-isomer is extremely rare. In contrast, α-pinene (pin-2-ene) occurs naturally as both (+) and (−) compounds with a wide range of optical purity ($[\alpha]_D$ *ca* −47 to +48°; *ca* 92% e.e. at limits) [1]. Typically, (−)-β-pinene (**2**) co-occurs with the predominant (+)-α-pinene (**3**) and these two isomers have opposite absolute configurations [1]. A similar situation occurs for *Citrus* and other genera [2, 3]. Chemotaxonomic studies and investigation of the time-incorporation of tracer into the α- and β-isomers in *Pinus* species led to the inference of two separate routes from the acyclic biosynthetic precursor(s) to α- and β-pinenes [4, 5] and this scheme has been somewhat supported by recent enzymic studies on pinene biosynthesis in a *Salvia* species [6]. On the other hand, the disparity between the optical purities of the isomers could be held to support initial formation of the (−)-β-compound and subsequent isomerisation to the thermodynamically more stable α-isomers: several chemically-reasonable mechanisms for the isomerisation may readily be envisaged. It has been shown that (+)-α-pinene [and probably (−)-α-pinene] formed in *P. nigra* and *P. radiata* from [2-^{14}C] MVA was exclusively labelled at C-3 [7] and this contradicted the pattern previously obtained for a sample of α-pinene of unspecified chemical and optical purity from *P. nigra* [8]. We now report tracer experiments on the formation of (+) and (−)-α-pinenes and (−)-β-pinene in several *Pinus* species (Pinaceae) to ascertain the pattern of incorporation of tracer into compounds with the enantiomeric pinane skeletons.



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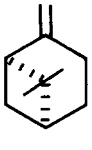

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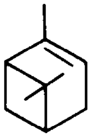

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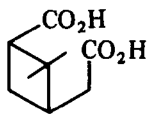

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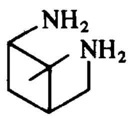

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RESULTS AND DISCUSSION

Chiral pinenes were isolated after feeding four *Pinus* species with [2-^{14}C] MVA and [2-^{14}C, 2-^{3}H$_2$] MVA and were rigorously purified to constant specific radioactivity. Incorporations were low but were typical for such experiments with higher plants [cf. 9]. The resulting (−)-β-pinenes were in > 90% e.e. but the α-isomers had a wide range of optical purities: thus, (+)-α *ca* 34%; (−)-α 38–64% e.e. (Table 1). The proportions of structural and optical isomers broadly agreed with the previous literature values [10–14].

The samples of ^{14}C-labelled (−)-β-pinene were isomerized to the (+)-α-isomer (by a method shown not to alter the optical purity) and this, and the directly isolated α-pinenes, were degraded (**4** → **6**) to liberate C-2 and C-3 as carbon dioxide. The diacid (**5**) contained all the tracer from **4** and the amine (**6**) was always isolated and assayed; isotope balances were always > 98%. The results show that for α- and β-pinenes of whatever chirality over 96% of incorporated tracer was located at C-2 and C-3. However, from biogenetic considerations we can safely

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Abbreviations: MVA, mevalonate; IPP, isopentenyl pyrophosphate; DMAPP, 3,3-dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; NPP, neryl pyrophosphate; e.e., enantiomeric excess; LSR, lanthanide shift reagent.

Table 1. Incorporation of [2-^{14}C] MVA, and [2-^{14}C, 2-^{3}H$_2$] MVA into pinenes

Species	Isomer	%*	$[\alpha]_D^{20}$	% Incorp† C-3	^{3}H/^{14}C‡ I	II	III
P. elliottii Engel.	α	23	−32.2	96	—	—	—
	β	62	−20.3	97	—	—	—
P. ponderosa Laws	α	49	−19.0	—	1.00	0.53	0.01
	β	32	−21.3	—	1.00	0.98	0.00
P. radiata D. Don	α	26	+18.3	98	1.00	0.49	0.01
	β	64	−21.5	100	1.00	0.97	0.02
P. sylvestris L.	α	63	+13.2	99	1.00	0.50	0.02
	β	25	−21.5	—	1.00	0.97	0.00

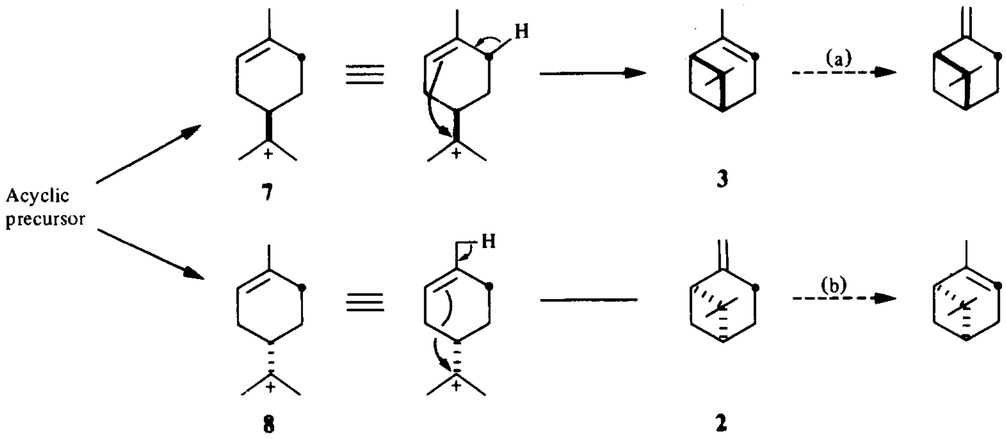
* % of isomer in extractable oil.

† % of ^{14}C incorporated into pinane skeleton located at C-3. (s.e., *ca* ±1%). Total incorporations into the individual monoterpenes were in the range 8 × 10^{-4} (P. *radiata*) to 9 × 10^{-2}% (P. *ponderosa*) of the applied (3*R*)-MVA.

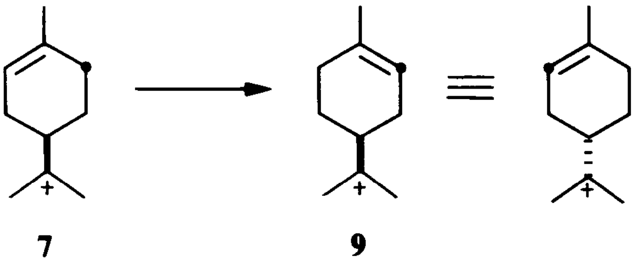
‡Normalized isotope ratios for I, [2-^{14}C, 2-^{3}H$_2$] MVA (precursor); II, α- or β-pinene; III, the 3-oxo-derivatives (pinocamphone and pinocarveol).

assume that the actual location was C-3. The observation that exogenous MVA only labelled the IPP-derived moieties of the pinenes is expected. Such asymmetric labelling is almost unvariably found in such tracer studies on foliage of higher plants and has been attributed to the existence of a metabolic pool of DMAPP or its biogenetic equivalent [15]. The presumed location of ^{14}C at C-3 was confirmed and the work was extended by means of the feedings of [2-^{14}C, 2-^{3}H$_2$] MVA. Table 1 records the normalized ^{3}H: ^{14}C ratios for the precursor, the α- or β-pinenes, and the 3-oxo-derivatives of the latter. In all cases ^{3}H had been specifically (> 98%) incorporated at C-3.

These results are consistent with two distinct routes leading from the acyclic precursor, which may be NPP [16], GPP [6] or myrcene/ocimene [17], to α- and β-pinene. These precursors may bicyclize in a concerted fashion, e.g. myrcene may undergo direct cyclo-addition to β-pinene, but if a monocyclic intermediate is involved as is envisaged in the classical scheme for monoterpene biogenesis [18] two epimeric forms of this must occur, e.g. 7 and 8, Scheme 1. The intermediates may be considered the biogenetic equivalents of these carbocations but their actual nature is unknown. Our results rule out any route, such as that implicitly supported by previous tracer work [8], whereby one enantiomer (e.g. 7) is converted into the epimeric enantiomer (e.g. 9) by double-bond migration (Scheme 2). If the latter occurred, the two enantiomeric pinane skeletons [as in (+)-α and (−)-β-pinenes] would be labelled at C-1 and C-3 respectively after uptake of [2-^{14}C] MVA. The likelihood of the divergence of the routes to (−)-β- and (+)-α-pinene before the formation of the monocyclic intermediate (as in Scheme 1) was investigated



Scheme 1. Hypothetical scheme for the biosynthesis of α- and β-pinenes. Dot represents label from C-2 of MVA.



Scheme 2. Isomerization of intermediate carbocation by shift of C=C. Dot represents label from C-2 of MVA.

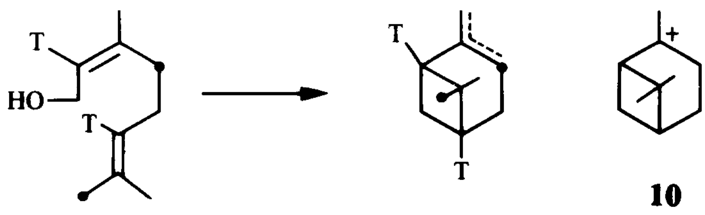
using $[3,10\text{-}^{14}C;\ 2,6\text{-}^{3}H_2]$nerol as precursor. This was readily available in good yield (20% incorporation of 3*R*-isomer) by feeding $[2\text{-}^{14}C;\ (4R)\text{-}4^{3}H_1]$ MVA to flowerheads of *Rosa dilecta*. Uptake is known to be position specific and unusually both IPP- and DMAPP-derived moieties of the monoterpene are equivalently labelled [19]. (−)-β-Pinene and its (+) and (−)-α-isomers biosynthesized from this precursor by *P. radiata* and *P. ponderosa* (incorporation 0.2–0.5%) all retained ^{3}H at C-5 (Scheme 3). This was shown by the normalized $^{3}H:^{14}C$ ratios which for the precursor and the three pinenes were 1.00:1, 1.02:1, 0.96:1 and 0.97:1 (all ±0.3), respectively. An epimerization such as $\mathbf{7} \rightarrow \mathbf{8}$, perhaps proceeding via the diene, would have been most unlikely to have retained the hydrogen at C-5.

If the route outlined in Scheme 1 is correct, the primary products of the two pathways are (−)-β- and (+)-α-pinene. It is understandable that different enzymes are necessary to produce these products. Examination of models shows that for reaction of **8** one of the hydrogens of the ring-linked methyl group can be perfectly positioned to achieve an *anti*-orientation with respect to the departing π-electrons of the double bond as the latter bind to the positive centre of the (formal) carbocation. Thus, a concerted addition-elimination can readily occur to yield β-pinene; whereas a hydrogen of the methylene group in the six-membered ring that has to be lost to form α-pinene cannot achieve this favourable stereochemistry. If the latter compound is to be formed, as it presumably is from **7**, a quite different mechanism, and hence a quite different active site of an enzyme, is required. Now interaction of the π-electrons with the positive centre must predominate and the reaction is probably two-step. First, a pinan-2-yl carbocation (**10**), or more probably a bonded species closely resembling it in electronic structure, is formed. This then breaks down to α-pinene, the factors directing this orientation of elimination being those that govern the much greater thermodynamic stability of α- over β-pinene. If this interpretation is correct, a considerable fraction of the (−)-β-pinene may be subsequently isomerized either chemically or enzymatically to (−)-α-pinene (Scheme 1, reaction *b*). This should be facile (at equilibrium, α:β is *ca* 96:4 [1]), and so this accounts for the wide variation in $[\alpha]_D$ formed for α-pinene as in addition to (+)-α-pinene formed by the direct route varying and perhaps predominating, amounts of the (−)-isomer may be fed in from the 'β-pinene' route. In contrast, the (−)-β-pinene formed in the direct route is optically diluted to a very limited extent by the (+)-β-isomer fed in by isomerization of (+)-α-pinene (Scheme 1, reaction *a*) as the equilibrium for formation of the former from (+)-α-pinene is very unfavourable.

EXPERIMENTAL

Materials. Terminal foliage was used from the *Pinus* species (trees: 1 to 4 m) that were established outdoors. $^{14}C,^{3}H$-labelled MVA was available commercially (0.5–0.7 Ci/mmol). Labelled nerol was obtained by feeding buds of *R. dilecta* with the appropriate MVA (100 μCi) and working up as described [19]; the product (diluted with carrier) had sp. act. 10.2 mCi/mmol.

Feeding of precursors and degradation of products. Feeding techniques, extraction and purification of products and the methods for degradation of α-pinene have been very fully recorded [7]. An additional detail was that α- and β-pinene were very effectively separated by TLC on $AgNO_3$–silica gel H at 4° with elution by (i) hexane–Et_2O (200:1) and (ii) hexane–Et_2O (40:1); R_f values for α- and β-isomers were 0.14 and 0.48, respectively. The α- and β-pinenes that had been purified to constant sp. act. via solid derivatives [7] were chemically (> 99%; TLC various systems; GC on Carbowax 20 M and SE-30, 50 m capillary columns) and radiochemically pure (2π scanning of TLC; several systems). The optical purities were determined conventionally (Ericsson–Bendix electronic polarimeter) and also using a chiral LSR [20]; values from the two methods agreed ±3%. β-Pinene (0.5 g; 2×10^{4} dpm) was converted into α-pinene (90% yield) with Pd–H_2 [21]. β-Pinene was oxidized to pinocar-



Scheme 3. Dot represents label from C-2 of MVA. T represents ^{3}H derived from C-4 of MVA.

vone (62%) with SeO_2 [22]; and pinocamphone (92%) resulted from hydroboronation–oxidation of α-pinene [23].

Radiochemical methods. Tracer-containing materials were assayed as solids that had been recrystallized (usually × 3) to constant sp. act. The exception was CO_2 that was collected in ethanolamine–diglyme. $[^{14}C, ^{3}H]$Pinenes were assayed as their adducts with 2-thioacetic acid [7] whereas $[^{14}C]$ α-pinene was oxidized and counted as pinonic acid. Typically, 50–100 mg samples of material were assayed containing 500–10^4 dpm above background. In all cases at least 4×10^4 disintegrations were accumulated (so that 2σ was ±1%) using 0.8% butyl-PBD in toluene as scintillant.

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