The Biosynthesis of (+)- α -Pinene in *Pinus* Species

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1. The degradation of (+)- α -pinene biosynthesized from 3*RS*-[2- 14 C]mevalonate by *Pinus radiata* or *Pinus nigra* revealed an asymmetrical labelling pattern whereby the moiety derived from isopentenyl pyrophosphate contained at least 90% of the incorporated tracer. This pattern differed both in asymmetry and position of labelling from previous results obtained with *P. nigra*, but is consistent with the generally accepted hypothetical mechanism for the biosynthesis of the pinane skeleton. 2. (+)- α -Pinene biosynthesized in *Pinus attenuata* and in the previously named two species from 3*RS*-[2- 14 C,(4*R*)-4- 3 H₁]mevalonate and its (4*S*)-isomer retained all the 4*R* hydrogen atoms (within the experimental error) but lost all the 4*S* hydrogen atoms of the precursor. This stereospecificity of hydrogen loss is the same as that previously found for the formation of geraniol and nerol in other plant species, and the result may be reasonably inferred to be general for monoterpenes.

Incorporation experiments using 3*RS*-[2- 14 C,(4*R*)-4- 3 H₁]mevalonate and its (4*S*)-isomer have shown that both geraniol and nerol were formed in certain *Rosa* and *Pelargonium* species with almost complete loss of the *pro*-4*S* hydrogen atom of the precursor and with almost complete retention of the epimeric *pro*-4*R* hydrogen atom (Banthorpe *et al.*, 1972*b*). These rather unexpected results were explained on the basis either of nerol being formed from geraniol (free alcohols or the pyrophosphate esters being involved) or of different prenyltransferases directly producing the two isomeric pyrophosphates of the alcohols. Cyclic monoterpenes would be expected to show a stereospecificity of hydrogen loss that was similar to nerol, for the latter is generally considered to be their parent.

We here report the stereochemistry of hydrogen loss in the formation of (+)- α -pinene (I) in three *Pinus* species as well as the elucidation of the labelling pattern resulting from incorporation of [2- 14 C]-mevalonate.

Results

Double-labelling experiments

Preliminary screening of the uptake of tracer from [2- 14 C]mevalonate into α -pinene showed that incorporations were low and rather variable in foliage from all three *Pinus* species. However, in all cases incorporations increased to a plateau value over approx. 5 days.

Table 1 records the $^3\text{H}/^{14}\text{C}$ ratios in (+)- α -pinene

Table 1. $^3\text{H}/^{14}\text{C}$ ratios in (+)- α -pinene formed after uptake of 3*RS*-[2- 14 C,(4*R*)-4- 3 H₁]mevalonate and its (4*S*)-isomer by *Pinus* species

For experimental details see the text. Average values for incorporations are given when two separate feeding experiments were performed. The $^3\text{H}/^{14}\text{C}$ ratio in products was measured after purification to constant specific radioactivity (most experiments were performed in duplicate).

^3H -labelled 3 <i>RS</i> -[2- 14 C]mevalonate	Species	Time before harvesting (days)	Incorporation of 3 <i>R</i> -precursor (%)	$^3\text{H}/^{14}\text{C}$ ratio in precursor	$^3\text{H}/^{14}\text{C}$ ratio in products
[(4 <i>S</i>)-4- $^3\text{H}_1$]	<i>P. attenuata</i>	1	2×10^{-3}	1.56	<0.01
[(4 <i>R</i>)-4- $^3\text{H}_1$]	<i>P. attenuata</i>	1	2×10^{-3}	1.53	1.50, 1.51
[(4 <i>S</i>)-4- $^3\text{H}_1$]	<i>P. radiata</i>	5	5×10^{-4}	2.01	<0.01, <0.01
[(4 <i>R</i>)-4- $^3\text{H}_1$]	<i>P. radiata</i>	5	5×10^{-4}	2.01	1.92, 1.96
[(4 <i>S</i>)-4- $^3\text{H}_1$]	<i>P. nigra</i>	5	1×10^{-1}	2.01	0.02, 0.01
[(4 <i>R</i>)-4- $^3\text{H}_1$]	<i>P. nigra</i>	5	1×10^{-1}	2.01	1.99, 1.98

isolated and purified to constant specific radioactivity after 3*RS*-[2-¹⁴C,(4*R*)-4-³H₁]mevalonate and its (4*S*)-isomer had been fed to the plants for various time-periods. Percentage incorporations rather than dilution factors are calculated for reasons previously described (Banthorpe *et al.*, 1972*b*). The ³H/¹⁴C ratios recorded are means of three assays on each sample of purified material and have standard deviations of ± 0.03 : all feedings except one were carried out in duplicate. Radioactivities in the ¹⁴C channel

varied over the range 3×10^2 – 4×10^4 d.p.m. (above background) in the various experiments.

Incorporation of ¹⁴C: degradation of (+)- α -pinene

(+)- α -Pinene biosynthesized by *Pinus radiata* from [2-¹⁴C]mevalonate was purified to constant specific radioactivity and was degraded through the sequences (I)–(V) and (I)–(VIII) (see Scheme 1). (+)- α -Pinene similarly formed in *Pinus nigra* was also degraded

Scheme 1. Degradation scheme for α -pinene

(I) α -Pinene; (II) pinonic acid; (III) pinic acid; (IV) 2,2-dimethyl-1-aminomethyl-3-aminocyclobutane; (V) CO₂; (VI) 2,2-dimethyl-3-ethyl-1-carboxymethylcyclobutane; (VII) 2,2-dimethyl-3-ethyl-1-aminomethylcyclobutane; (VIII) CO₂.

Table 2. Specific radioactivities of (+)- α -pinene biosynthesized from [2-¹⁴C]mevalonate by *Pinus* species and of its degradation products

For experimental details see the text. α -Pinene was counted for radioactivity as pinonic acid (II, Scheme 1). For structures of products, see Scheme 1.

Species	Incorporation of 3 <i>R</i> precursor (%)	Specific radioactivity (d.p.m./mmol)	
		α -Pinene	Degradation products
<i>P. radiata</i>	2×10^{-4}	840	(III) 832; (IV) 0; (V) 729
<i>P. radiata</i>	3×10^{-3}	9320	(VI) 9115; (VII) 130; (VIII) 8990
<i>P. nigra</i>	5×10^{-2}	17232	(VI) 17012; (VII) 321; (VIII) 16910

by the latter route. Results are shown in Table 2. In the first experiment with *P. radiata*, α -pinene was harvested 24 h after completion of uptake of tracer. In the other two experiments a metabolism time of 5 days was allowed.

In Table 2 the specific radioactivities of the degradation products had standard deviations of approx. $\pm 5\%$ of the quoted values, except for compound (V) in the first degradation, which had a value of approx. $\pm 10\%$. Compound (IV) (first degradation) had a radioactivity indistinguishable from the background, i.e. the specific radioactivity was less than approx. 30 d.p.m./mmol.

Discussion

The double-labelling studies on *P. attenuata* Lemm., *P. radiata* D. Don. and *P. nigra* var. *Austriaca* (Family Coniferae) follow the pattern observed for both geraniol and nerol and their glucosides from *Rosa* and *Pelargonium* species in that, within experimental error, the *pro*-4*R* hydrogen atom of mevalonate was completely retained whereas the analogous *pro*-4*S* hydrogen atom was completely eliminated. The incorporation of tracer was low, but this is typical for leaf monoterpenes (Loomis, 1967; Banthorpe *et al.*, 1972a) and has been reasonably explained (Banthorpe *et al.*, 1970). It has been claimed that [$2\text{-}^{14}\text{C}$]mevalonate is incorporated into α -pinene from *P. attenuata* in 0.5% yield (Stanley, 1958), but the product was not purified to currently required standards and may have contained a radioactively labelled impurity.

Our results confirm the previous findings (Banthorpe *et al.*, 1972b) that there is no correlation

between loss of the *pro*-4*S* and *pro*-4*R* hydrogen atoms of mevalonate and the respective biosynthesis of a *trans*- or *cis*-substituted double bond in monoterpenes, although such a correlation does exist for the formation of rubber (Archer *et al.*, 1966) and for certain polyisoprenoid alcohols (Gough & Hemming, 1967, 1970; Stone & Hemming, 1967). The pattern observed is consistent with the hypothesis that neryl pyrophosphate is a parent of cyclic monoterpenes. A route to these compounds involving isomerization of geranyl pyrophosphate to linaloyl pyrophosphate and cyclization of the latter is feasible, and an enzyme system from orange-juice vesicles that cyclizes linalool to menthane derivatives with the supposed intermediate formation of linaloyl pyrophosphate is known (Potty *et al.*, 1970), but the consensus of opinion is that neryl pyrophosphate is probably the precursor of cyclic monoterpenes (Banthorpe *et al.*, 1972a).

Degradation of (+)- α -pinene biosynthesized from [$2\text{-}^{14}\text{C}$]mevalonate in *P. radiata* and *P. nigra* showed that tracer was almost entirely (at least 90%) located at C-3 (see Scheme 2, structure XI). This pattern of asymmetric labelling is typical of that found in several mono- and sesqui-terpenes after incorporation of [$2\text{-}^{14}\text{C}$]mevalonate (Banthorpe *et al.*, 1972a; Croteau & Loomis, 1972). The location of tracer at C-3 supports the generally accepted hypothesis (Ruzicka, 1953) that the pinane skeleton is constructed by internal addition of a cationic centre to the double bond in the ion (XII) (or its biogenetic equivalent) derived from α -terpineol.

Previous workers have obtained results that differ from those recorded here (Sandermann & Schweers, 1962; Sandermann *et al.*, 1968). α -Pinene of unspecified chemical, optical and radiochemical purity

Scheme 2. Numbering and structure of α -pinene, its adducts and related compounds

(XI) α -Pinene. (XII) Probable mechanism for cyclization of α -terpineol to form α -pinene. (XIII) Previously reported labelling pattern for α -pinene ($\bullet = ^{14}\text{C}$). (XIV) Structure of adduct of α -pinene and 2-thioacetic acid. (XV) (+)-Neoisopinocampheol [derived by modification of the adduct of (+)- α -pinene and 2-thioacetic acid].

was obtained after administration of $[2-^{14}\text{C}]$ mevalonate to *P. nigra* var. *Austriaca*, and on the basis of partial degradation a pattern of labelling (Scheme 2, formula XIII) was reported in which the moieties derived from isopentenyl pyrophosphate and 3,3-dimethylallyl pyrophosphate were equivalently labelled. This 'symmetrical' pattern seemed very reasonable at the time, but the unexpected location of tracer at C-1 (rather than at C-3) was inconsistent with the mechanism proposed by Ruzicka (1953) (see above) and necessitated the postulation of rather unlikely biosynthetic routes involving either cyclization of a monocyclic precursor with the intermediate formation of a novel zwitterion (Sandermann & Schweers, 1962) or an intramolecular Diels-Alder cyclization of an acyclic precursor (Richards & Hendrickson, 1964).

As the optical purity of the α -pinene used in these early experiments was unspecified, it might be argued that the material was mainly the (–)-isomer and that two distinct enzymic routes, giving different labelling patterns, occurred corresponding to the formation of each optical isomer. Two enzymic routes to the pinane skeleton must indeed occur (and frequently occur at the same time) as α -pinene typically exists in an optically impure form, whereas β -pinene is usually optically pure (Kugler & Kovats, 1963), and almost invariably (the predominant) (+)- α -pinene occurs together with (–)- β -pinene of the opposite absolute configuration (Kovats, 1963; Banthorpe & Whittaker, 1966). Such a dichotomy of routes to the pinane skeleton has also been independently deduced from chemotaxonomic studies on numerous *Pinus* species (Zavarin, 1970) and can be inferred from the pattern of time-incorporation of tracer into α - and β -pinene in needles of *P. radiata* (Valenzuela *et al.*, 1966). However, the α -pinene samples obtained from our specimens of *P. radiata* and *P. nigra* both contained (+)- and (–)-isomers in the approximate ratio 4:1, and so any difference in labelling pattern resulting from the formation of skeletons of opposite absolute configuration would have been detected. Our results thus suggest that a similar mechanism was utilized for the biosynthesis of both optical isomers, and it seems likely that the epimeric species formally represented by structure (XII) are the precursors.

A minor conclusion arose from the degradation experiments. α -Pinene was purified through its adduct with 2-thioacetic acid. Although the product had been reported previously, it was ill-characterized and its structure was unknown (Sutherland & Wells, 1956; Buess *et al.*, 1957) and uncertain because of the ready tendency of the pinane skeleton to undergo Wagner-Meerwein rearrangement. Elemental and mass-spectrometric analysis showed that the purified product was a 1:1 adduct and the spectra were consistent with structure (XIV): thus an i.r. band at

2890cm^{-1} was attributable to $\text{R}_3\text{C-H}$, and the n.m.r. spectrum resembled that of α -pinene but with a multiplet (3H) at $6.4\text{--}6.8\tau$ attributable to S-CH_2 and S-CH . In addition, the fragmentation pattern under electron impact (at 70eV) was completely consistent with structure (XIV). This assignment was confirmed, and in addition the sulphur-containing group and the methyl group at C-2 were shown to be α - and β -orientated, by esterification of the adduct followed by *S*-methylation and Hofmann degradation of the resulting -onium salt. This procedure gave α -pinene (22%), an unidentified monoterpene (23%), which probably was δ -pinene (pin-3-ene), and (+)-neoisopinocampheol (XV, 40%).

Experimental

All melting points were uncorrected. N.m.r. spectra were measured in 10% solution in carbon tetrachloride or deuteriochloroform with tetramethylsilane (τ 10.00) as internal standard on a Perkin-Elmer R12 60 MHz spectrometer.

Materials

Pinus species. *P. radiata* and *P. nigra* [0.6 m (2 ft) specimens] were obtained from Hillier and Sons (Winchester, Hants., U.K.) and were cultivated outdoors in Central London; young terminal foliage of *P. attenuata* was obtained from the National Pinetum (Bedgebury, Kent, U.K.) as required.

Feeding with radioactive materials. These experiments were carried out in the period May–September, usually in July or August. The lactones of 3*RS*- $[^{14}\text{C}]$ - or 3*RS*- $[^3\text{H}]$ -mevalonate were converted into the salts of the acids by incubation with aq. 0.1 M- NaHCO_3 (1.0 ml) for 1 h at 37°C , immediately before feeding. 3*RS*- $[2-^{14}\text{C}]$ - and 3*RS*- $[4\text{R}(\text{S})-4-^3\text{H}_1]$ -mevalonate (from The Radiochemical Centre, Amersham, Bucks., U.K.) were used at specific radioactivities of 6.4 and 116 mCi/mmol respectively.

Preliminary experiments to determine the time-course of labelling used batches (20–30 g) of foliage, which were fed with $[2-^{14}\text{C}]$ mevalonate (approx. 20 μCi) in water (1 ml) in sterile conditions under illumination and with forced transpiration. After uptake of tracer the foliage was maintained on sterile nutrient medium for the predetermined times. Full details of these feeding techniques have been reported (Banthorpe & Wirz-Justice, 1969; Banthorpe *et al.*, 1970). Each sample of foliage (approx. 5 g) was extracted with hexane, the extract was separated by column chromatography and g.l.c. (see 'Purification of α -pinene' below) and the α -pinene fraction was assayed for tracer by using a 2π -radiochromatography scanner (Tracerlab Instruments Inc.; Texas Instruments, Houston, Tex., U.S.A.).

In the incorporation experiments proper, similar quantities of needles and terminal meristem of the *Pinus* species were fed with mevalonate (50–100 μ Ci in various experiments) and were harvested after being maintained on nutrient medium for the times recorded in the tables. Most feedings in the experiments with doubly labelled precursor were done in duplicate.

Purification of α -pinene

The foliage was ground in liquid N_2 and the resulting powder plus an equal weight of anhydrous Na_2SO_4 was refluxed with hexane (20ml) for 4h. Samples (0.1–0.5g) of α -pinene (from Bush Boake Allen Ltd., London E.15, U.K.; >98% pure by g.l.c.) were added as carrier; the solution was concentrated to approx. 5ml at $-10^\circ C$, and this residue was chromatographed on a column of silica gel H (from E. Merck, Darmstadt, Germany)– $AgNO_3$ (Norin & Westfelt, 1963) with hexane–ether (2:1, v/v). α -Pinene was obtained from the appropriate concentrated fractions (which were monitored by t.l.c.) by preparative g.l.c. on Carbowax 20M (20%, w/w, on Chromosorb W; 60–80 mesh) in a column (6m $\times$ 1cm external diam.) maintained at $90^\circ C$, with a flow rate of 5.4 litres of N_2 /h. The effluent was trapped with approx. 70% recovery by using a previously described apparatus (Banthorpe *et al.*, 1972b).

The nitrosochloride derivative or the adduct with 2,4-dinitrobenzenesulphenyl chloride (Kharasch, 1956) could not be obtained in a crystalline form in satisfactory yields, and α -pinene was treated (Sutherland & Wells, 1956; Buess *et al.*, 1957) with freshly distilled 2-thioacetic acid, b.p. $90^\circ C$ at $1.24 kN/m^2$ (10mmHg) to give an adduct that was purified by chromatography on a column of silicic acid (Silicar CC7; Merck) with hexane–ether (1:1, v/v) as eluent. The resulting material (yield 71%) had n_D^{25} 1.4660, $[\alpha]_D^{20}$ $-16.8 \pm 1^\circ$, when prepared from α -pinene, $[\alpha]_D^{20}$ $+21.3 \pm 1^\circ$; i.r. bands at 2910, 2890, 1710, 1295, 1125, $930 cm^{-1}$; n.m.r. absorption at 6.4–6.8 τ (multiplet, 3H: S-CH₂, S-CH); mass spectrum, base peak 41 (abundance 100), parent peak 228 (5.6), other main peaks at 173, 169, 109, 145, 137, 136, 91 and 55 with abundances of 5.9, 2.9, 9.1, 5.6, 3.3, 9.1, 9.1 and 39.7; molecular formula (mass spectrometry) $C_{12}H_{20}SO_2$ (Found: C, 63.0, H, 8.9. Calc. for $C_{12}H_{20}SO_2$: C, 63.1; H, 8.9%). The benzylisothiuronium salt of the adduct had m.p. $155^\circ C$ after recrystallization from acetone: samples for radioactive assay were purified by recrystallization of this derivative and were reconverted into the α -pinene-thioacetic acid adduct before radioactivity counting.

To prove the structure of the latter adduct, the compound (prepared by using almost optically pure (+)- α -pinene, $[\alpha]_D^{20}$ $+51 \pm 1^\circ$) was converted into its methyl ester with diazomethane and the crude pro-

duct was methylated with methyl iodide to give the sulphonium iodide as a red oil (Found: I, 31.9. $C_{15}H_{27}SO_2I$ requires I, 31.9%). Treatment of this with a twofold molar excess of NaOH in dioxan–water (3:2, v/v) at $100^\circ C$ gave: (+)-neoisopinocampheol (XV, yield 40%), $[\alpha]_D^{20}$ $+21 \pm 1^\circ$ (c 10 in benzene), α -pinene (yield 22%) and an unidentified hydrocarbon (yield 23%). The products were isolated by preparative g.l.c. on the Carbowax 20M column described above and the first two compounds were identified by comparison of their i.r. spectra with those of authentic samples: the last product had a spectrum very similar to that of α -pinene and was probably δ -pinene (pin-3-ene). No β -pinene was detected and no interconversion of α -, β - and the presumed δ -pinene occurred in controls.

α -Pinene from *P. radiata*, *P. attenuata* and *P. nigra* had $[\alpha]_D^{25}$ (c 5 in ethanol) $+35.2$, $+30.2$ and $+36.7^\circ$ (all $\pm 1^\circ$) after purification by the g.l.c. and t.l.c. procedures outlined above. The yield of oil from the needles was approx. 0.2% (w/wet wt.) in each case.

Degradation schemes

The procedures used are mostly well described in the literature (see Simonsen, 1957) and were scaled down to employ 100–500mg of substrate at each step. Carrier was added if necessary to maintain appropriate quantities. All the well-authenticated products had melting or boiling points in agreement ($\pm 1^\circ C$) with literature values and had elemental analyses and i.r. and n.m.r. spectra consistent with the accepted structures.

α -Pinene was oxidized with $KMnO_4$ (Dupont, 1923) to form *cis*-pinonic acid (II), which was purified by passage, with benzene–ether (1:1, v/v) as eluent, through a column of silicic acid (Silicar CC7; Merck) to which 0.025M- H_2SO_4 (40%, w/w) had been added to maintain a free-running powder (Kinnory *et al.*, 1955). The product, yield 53%, had m.p. $104^\circ C$ after recrystallization from water and formed a tosylhydrazone, m.p. $135^\circ C$ (after recrystallization from ethanol) and a semicarbazone, m.p. $210^\circ C$ (after recrystallization from aq. ethanol). The radioactive assay was made on *cis*-pinonic acid. This acid was also recovered as its tosylhydrazone (yield 58%) after ozonolysis of α -pinene in methanol at $-78^\circ C$ and preparation with performic acid by the standard technique.

cis-Pinonic acid(II) was subjected to the bromoform reaction (Baeyer, 1896) to give pinic acid(III), yield 90%, m.p. $90^\circ C$ (after recrystallization from water); Schmidt reaction of the latter (Wolff, 1946; Hedrick, 1959) gave the diamine (IV) in 58% yield together with CO_2 (V) in 80% yield. The CO_2 was collected from aq. $Ba(OH)_2$ as $BaCO_3$ and the

diamine was purified on columns of alumina and silica gel H (Merck) with methanol-ether (1:1, v/v) and methanol-ethyl acetate (19:1, v/v) as eluents respectively. A diacetyl derivative could not be readily prepared and purification for radioactive assay was effected through the tetraphenylboron salt (Flaschka & Barnard, 1960), m.p. 115°C (decomp.) (after recrystallization from aq. acetone), or the dihydrochloride, m.p. 260°C (after recrystallization from chloroform-ether), or the dipicrate, m.p. 268°C (after recrystallization from aq. ethanol).

In the alternative degradation procedure, Huang-Minlon-type reduction of *cis*-pinonic acid formed compound (VI), m.p. 130°C (after recrystallization from water) in 60% yield. This was decarboxylated by the Schmidt procedure (Wolff, 1946) to form the hitherto unreported *amine* (VII) (yield 72%) and CO₂ (yield 95%), which was collected as described above.

1-Aminomethyl-2,2-dimethyl-3-ethylcyclobutane (VII) was purified as its hydrochloride, m.p. 243°C (after recrystallization from chloroform); reconversion into the free amine gave a product of b.p. 235°C, molecular formula (mass spectrometry) C₉H₁₉N (Found: C, 76.5; H, 13.0; N, 9.9; C₉H₁₉N requires C, 76.6; H, 13.4; N, 9.9%) with n.m.r. spectrum consistent with the assumed structure.

These methods enable tracer at C-2, C-3, and C-10 to be measured. Complete degradation could be achieved if α -pinene was converted into bornyl chloride (IX) in 30% yield by treatment with HCl in chloroform at 0°C (Thurber & Thielke, 1931), and this product further converted into bornene (X, yield 52%) of the same optical purity as the starting material (cf. Borowiecki & Chrétien-Bessière, 1967) by heating with a twofold excess of potassium 2-butylocyclohexoxide (Hanack & Hähnle, 1962) or potassium menthoxide in the corresponding alcohol as solvent at 80°C. These steps were carried out on isotopically normal material and α -pinene was converted into bornene in 15% overall yield. Methods are available (Banthorpe & Baxendale, 1970) for the further degradation of compound (X).

Radiochemical methods

The procedures and the counting statistics used have been described in detail (Banthorpe *et al.*, 1972b). All tracer-containing materials were assayed as solids that had been recrystallized (usually three times) to constant specific radioactivity. α -[¹⁴C,³H]Pinene was counted as the adduct with 2-thioacetic acid, whereas α -[¹⁴C]pinene was purified by g.l.c. and t.l.c. techniques and then was oxidized and counted as the (solid) pinonic acid.

Typically 30–100mg samples of labelled material were assayed, containing 500–5000 d.p.m. above background.

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