The Stereochemistry of Betulaprenol Biosynthesis

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It is now well established that in the biogenesis of all-*trans*-polyisoprenoid compounds the 4-*pro-S*-hydrogen of mevalonate is eliminated and the 4-*pro-R*-hydrogen is retained. This has been shown for the biosynthesis of *trans-trans*-farnesyl pyrophosphate and all-*trans*-squalene by a rat liver preparation (Cornforth, Cornforth, Donninger & Popják, 1966), of squalene and phytoene by carrot-root preparations (Goodwin & Williams, 1966), of squalene and phytol by green leaves (Wellburn, Stone & Hemming, 1966) and of squalene and ubiquinone by *Aspergillus fumigatus* Fresenius (Stone & Hemming, 1967). The converse of this holds for the biogenesis of all-*cis*-polyisoprenoid compounds, although the evidence for this is necessarily more limited and has been illustrated only for the biosynthesis of poly-*cis*-rubber. This material retains the 4-*pro-S*-hydrogen of mevalonate and the 4-*pro-R*-hydrogen is eliminated (Archer *et al.* 1966). With polyisoprenoids of mixed stereochemistry the evidence is not quite so conclusive (Stone & Hemming, 1967). Nevertheless the generalization that the biogenesis of *trans*-isoprene units involves the elimination of the 4-*pro-S*-hydrogen and retention of the 4-*pro-R*-hydrogen of mevalonate, but that for *cis*-isoprene units only the 4-*pro-S*-hydrogen is retained, is well founded.

The woody tissue of *Betula verrucosa* contains esters of betulaprenols-6, -7, -8 and -9 (I). In each prenol two of the internal isoprene residues are in the *trans*-configuration (Lindgren, 1965; Wellburn & Hemming, 1966). It has been suggested that these prenols are formed by *cis*-addition to *trans-trans*-farnesyl pyrophosphate (Wellburn & Hemming, 1966). This would require each prenol to contain three biogenetically *trans*-isoprene residues. The now general availability of [2-¹⁴C,(4*R*)-4-³H₁]mevalonate and of [2-¹⁴C,(4*S*)-4-³H₁]mevalonate has provided an opportunity to test this point.

Radiochemicals were purchased from The Radio-

chemical Centre, Amersham, Bucks. A benzene solution of [2-¹⁴C,(4*R*)-4-³H₁]mevalonic acid lactone was prepared by mixing approx. 1 mc of a mixture of 3*R*-[(4*R*)-4-³H₁]- and 3*S*-[(4*S*)-4-³H₁]-mevalonic acid lactone (134 mc/m-mole) with approx. 100 μc of 3*RS*-[2-¹⁴C]mevalonic acid lactone (5.03 mc/m-mole). Similarly [2-¹⁴C,(4*S*)-4-³H₁]mevalonic acid lactone was prepared by mixing approx. 250 μc of a mixture of 3*R*-[(4*S*)-4-³H₁]- and 3*S*-[(4*R*)-4-³H₁]-mevalonic acid lactone (115 mc/m-mole) with approx. 25 μc of 3*RS*-[2-¹⁴C]mevalonic acid lactone (5.03 mc/m-mole). The appropriate amount of each benzene solution was then mixed with a little 60 mM-phosphate buffer, pH 7.4, and the benzene evaporated in a stream of nitrogen to provide solutions of mevalonate. In describing each mixture as 4*R*- or 4*S*-mevalonic acid the 'unnatural' 3*S*-form has been neglected.

In Expts. A and B mevalonate (5 μc of ¹⁴C in approx. 0.5 ml.) was taken up by transpiration over 2–4 hr. into terminal twigs of *Betula verrucosa* about 9 in. long and carrying 8–12 leaves. The twigs were then allowed to take up 1 mg. of unlabelled mevalonate in water over 24 hr. in the same way and were then left in water for a further 24 hr. at approx. 20°.

In Expt. C approx. 1 g. wet wt. of sliced stem (0.25 in. diam.) in 5 ml. of 60 mM-phosphate buffer, pH 7.4, containing 2 μc of mevalonate, was shaken in air at 26° for 16 hr.

Tissue whole lipid was extracted and chromatographed on alumina as described by Stone, Wellburn, Hemming & Pennock (1967). Polyprenyl esters and squalene were eluted by diethyl ether-light petroleum (1:50, v/v), polyprenols by diethyl ether-light petroleum (1:5, v/v) and chlorophyll by diethyl ether. After adding 2 mg. of appropriate diluent (i.e. betulaprenols, squalene or phytol) to each fraction, the unsaponifiable lipid was recovered (Stone *et al.* 1967) and the alumina chromatography was repeated. Any squalene present was again eluted by diethyl ether-light petroleum (1:50, v/v) and free polyprenols and phytol were eluted by diethyl ether-light petroleum (1:5, v/v).

Squalene was further purified by preparative thin-layer chromatography and through the thiourea clathrate (Stone & Hemming, 1967). Betulaprenols-6 to -10 were twice subjected to preparative thin-layer chromatography (silica gel G, with CHCl₃; *R_F* 0.55). This was followed by repeated

(I) Betulaprenols-6 to -9 (*n* = 6–9).

Table 1. Incorporation of [2-¹⁴C,(4*R*)-4-³H₁]- and [2-¹⁴C,(4*S*)-4-³H₁]-mevalonate into the esterified betulaprenols and phytol of the stem of twigs of *Betula verrucosa*

The value for percentage incorporation is based on the total amount of ¹⁴C present in both the natural (3*R*) and unnatural (3*S*) components of each mixture of mevalonates. The corrected ³H/¹⁴C ratio is the ratio (a) assuming the ³H/¹⁴C ratio of the original mevalonate to be 1:1 and (b) having multiplied the answer by the number of C atoms from position 2 of mevalonate expected to be present in each molecule of the corresponding compound. The ³H/¹⁴C ratios, uncorrected for ³H decay but counted at the same time as the fractions below, for the original [2-¹⁴C,(4*R*)-4-³H₁]- and [2-¹⁴C,(4*S*)-4-³H₁]-mevalonic acid lactones were respectively 10.08 and 9.95.

	From [2- ¹⁴ C,(4 <i>R</i>)-4- ³ H ₁]mevalonate		From [2- ¹⁴ C,(4 <i>S</i>)-4- ³ H ₁]mevalonate	
	¹⁴ C incorporation (% of dose)	Corrected ³ H/ ¹⁴ C ratio	¹⁴ C incorporation (% of dose)	Corrected ³ H/ ¹⁴ C ratio
Phytol*	0.0089	4.19:4	0.0047	0.04:4
Betulaprenol-6	0.0140	4.89:6	0.0125	0.63:6
Betulaprenol-7	0.0470	3.19:7	0.0295	4.15:7
Betulaprenol-8	0.0190	2.76:8	0.0080	5.29:8
Betulaprenol-9	0.0005	3.03:9	0.0015	5.78:9

* Esterified as part of the chlorophyll molecule.

preparative thin-layer reversed-phase partition chromatography (Stone *et al.* 1967) with acetone-water (17:3, v/v) as mobile phase. In this system betulaprenols-6, -7, -8 and -9 had *R_F* values of approx. 0.75, 0.62, 0.45 and 0.32 respectively. Phytol was purified via preparative thin-layer chromatography (Wellburn *et al.* 1966) followed by preparative thin-layer reversed-phase partition chromatography with acetone-water (3:1, v/v) as mobile phase (*R_F* 0.40). The prenols and phytol were recovered from the chromatograms and assayed for ³H and ¹⁴C content as described by Stone & Hemming (1967) for hexahydropolyprenols.

The results for the esterified betulaprenols of Expt. A are recorded in Table 1. The phytol ratios were as expected from experiments with leaf tissue (Wellburn *et al.* 1966) and served as an internal control. Three of the four betulaprenols gave results consistent with each prenol containing three biogenetically *trans*-isoprene residues. The ratios for betulaprenol-6 were almost certainly in error owing to contamination with biogenetically all-*trans* material. Insufficient activity remained to make further attempts at purification worth while. However, betulaprenol-6 isolated from the unesterified portion of the lipid gave corrected ³H/¹⁴C ratios of 2.95:6 (4*R*) and 2.86:6 (4*S*). Less radioactivity was recovered in the free prenols (0.003% of the ¹⁴C dose) than in those esterified (0.08% of the ¹⁴C dose).

In Expt. B, the prenols of both stem and leaves were recovered. Leaf betulaprenols-6 to -9 (free plus esterified) were less radioactive (0.04% of the ¹⁴C dose) than were those of the stem (0.18% of the ¹⁴C dose). In each case betulaprenol-7 contained approx. 75% of this radioactivity and the corrected ³H/¹⁴C ratios were 3.04:7 (4*R*) and 4.41:7 (4*S*) for the leaf and 3.00:7 (4*R*) and 4.43:7 (4*S*) for the

stem. The ratios for leaf and stem phytol were respectively 4.05:4 (4*R*), 0.16:4 (4*S*), and 3.97:4 (4*R*), 0.02:4 (4*S*), and those for leaf and stem squalene were respectively 5.60:6 (4*R*), 0.14:6 (4*S*), and 5.80:6 (4*R*), 0.02:6 (4*S*).

The incorporation of radioactivity into betulaprenols-6 to -9 (free plus esterified) by stem slices (Expt. C) was lower (0.04% of the ¹⁴C dose) than in Expts. A and B, but the ³H/¹⁴C ratios were much the same, namely 3.00:7 (4*R*) and 4.21:7 (4*S*) for betulaprenol-7, 3.93:4 (4*R*) and 0.00:4 (4*S*) for phytol and 6.16:6 (4*R*) and 0.31:6 (4*S*) for squalene.

It is clear that the stem and leaf of *Betula verrucosa* are capable of synthesizing betulaprenols-6 to -9 from exogenous mevalonate and that three of the isoprene residues of each prenol are biogenetically *trans*. Assuming that one of these residues is the ω-terminal residue these findings comply with the stereochemical evidence of nuclear-magnetic-resonance spectrometry (Wellburn & Hemming, 1966) and are also consistent with each prenol being formed by *cis*-addition to all-*trans*-farnesyl pyrophosphate.

Very little radioactivity was incorporated into the betulaprenols-10 to -13 of either leaf or stem. When purified in essentially the same way (Wellburn & Hemming, 1966) as the betulaprenols-6 to -9 small quantities of biogenetically all-*trans* material chromatographed between the bands of the individual, negligibly-labelled, betulaprenols. An identical result was obtained with leaves of *Ficus elastica*, in which experiment the trace quantities of highly labelled all-*trans* material were also separated from the unlabelled ficaprenols-10 to -13 by the formation of a thiourea clathrate (K. J. S. Stone, A. R. Wellburn & F. W. Hemming, unpublished work). It would appear that, in both

woody and leaf tissues, exogenous mevalonate is available to betulaprenols-6 to -9 but not to betulaprenols-10 to -13. In the leaf this is most likely due to the localization of the group of longer prenols inside and the shorter prenols outside the chloroplasts, for when leaves were exposed to $^{14}\text{CO}_2$ over 3 days the former became heavily labelled whereas the latter incorporated relatively little radioactivity (F. W. Hemming, unpublished work). A similar difference in intracellular localization may occur in the woody tissue.

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