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EVIDENCE FOR SEPARATE INTERMEDIATES IN THE BIOSYNTHESIS OF 24α- AND 24β-ALKYLSTEROLS IN TRACHEOPHYTES

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Abstract—When mevalonate-[2-^{14}C] was incubated with seeds of *Pinus pinea*, 23% of the label in sterols was found in *trans*-24-ethylidenecholesterol, 12% in a mixture of 24α- and 24β-methylcholesterol, and 65% in 24α-ethylcholesterol. However, when the radioactive substrate was lanosterol-[24-^{3}H], label appeared only in the 24-ethylidene- (85%) and the epimeric 24-methylsterols (15%). From the ratios of labels in the ethylidene- and methyl-sterols it was possible to show that the tritium in the 24-C_1-mixture was incorporated only into the 24β-methyl epimer. The labelling patterns are consistent with a pathway to 24β-alkylsterols via $\Delta^{25(27)}$-sterols bypassing 24-ethylidenesterols and to 24α-alkylsterols via $\Delta^{24(28)}$-sterols which are isomerized to $\Delta^{24(25)}$-sterols prior to reduction.

INTRODUCTION

Using doubly labelled (^{14}C/^{3}H) mevalonate, several groups of investigators have recently brought forward substantial proof for the intermediacy of $\Delta^{24(25)}$-sterols in the biosynthesis of 24-ethylesterols in several plant genera [1–6]. In one of these (*Spinacea oleracea*) the two dominant sterols have been shown by PMR spectroscopy to have an α-ethyl group at C_{24} uncontaminated by the epimeric configuration [7, 8]. It is known that most of the families of tracheophytes which have been examined, have sterols with exclusively this 24α-ethyl stereochemistry (thirteen families) but there are those in which only the epimeric configuration is present [8–10]. The latter, consisting so far of genera (*Clerodendrum* and *Kalanchoe*) in only two families (Verbenaceae [11, 12] and Crassulaceae, respectively [8]), contain 24β-ethylsterols with a $\Delta^{25(27)}$-bond which has never been observed in 24α-ethylsterols. Furthermore, in *Clerodendrum campbelli* it has been shown that the H-atom originally at C-24 is retained in the $\Delta^{25(27)}$-sterol [12] and there is some evidence for the existence of 24-ethyl-24(25)-dehydrolathosterol in sunflower [13] and pumpkin seeds [8]. It is, therefore, reasonable to conclude from the evidence that 24α-ethyl- and, probably more generally, 24α-alkyl-sterols, arise through a $\Delta^{24(25)}$-sterol, while 24β-alkyl-sterols arise through $\Delta^{25(27)}$-sterols. In the former case the configuration at C-24 would be determined by reduction (of the $\Delta^{24(25)}$-bond) and by alkylation (at C-24 or C-28) in the latter.

Evidence for the pathway at the 24-methyl level consists of the loss of the 24-H-atom in the 24-methyl component of *Spinacea* and *Medicago* species [3] and the existence of 24β-methyl-25(27)-dehydrocycloartanol (cyclolaudenol) in opium [14], bananas [15], and ferns [16] and of 24-methyl-24(25)-dehydrocholesterol (24-methyldesmosterol [17]) in *Withania somnifera*. In all tracheophytes studied [8–10, 18] the 4,4,14-trisdesmethyl-24-methylsterol (regardless of nulcear double bond position) is a mixture of the epimers at C-24 [8–10]. At the level of the Filicopsida and higher (Pteropsida gymnosperms and angiosperms) the principal component has the α-orientation. Work with *Spinacea* and *Medicago* utilized double labelling with mevalonate-[2-^{14}C, 4-$^{3}H_1$] and dealt with ^{14}C/^{3}H ratios (in Δ^{7}-sterols) of 3/5 indicating loss of the 24-H atom, contrasting to 4/5 indicating retention [3]. Since such ratios could not discriminate between loss in the major component (presumably 24α-) and retention in the minor one (presumably 24β-), we examined a case (*Pinus pinea*) already known to contain a mixture of epimers at the 24-methyl level) using lanosterol-[24-^{3}H] as substrate. The reverse of what had been found earlier [3], i.e., retention of label in the epimeric 24-methylcholesterols, should have been observed if 24β-methylsterols arise via $\Delta^{25(27)}$-sterols. At the same time it was possible to examine the origin of 24α-ethylcholesterol with lanosterol-[24-^{3}H] which contained only one instead of the six ^{3}H-atoms which are present when doubly labelled mevalonate was used [1–6]. We have previously demonstrated [19] that lanosterol is efficiently incorporated into the sterol pathway even though cycloartenol is the normal product of cyclization of squalene oxide [20].

The results of our work have confirmed the intermediacy of $\Delta^{24(25)}$-sterols in the 24α-ethyl case (label was lost) and for the first time they constitute labelling evidence (label was retained) for the absence of $\Delta^{24(25)}$ intermediates (which is consistent with the presence of $\Delta^{25(27)}$ intermediates) in the biosynthesis of 24β-methylcholesterol. Consequently, it seems highly probable that there are separate and distinct intermediates in the biosynthesis of the epimeric sterols of tracheophytes and that each of the configurations is determined by a different kind of reaction (reduction for α, alkylation for β).

RESULTS AND DISCUSSION

When mevalonate-[2-^{14}C] was fed to germinating seeds of *Pinus pinea*, it gave a sterol mixture in which 24α-ethylcholesterol contained most of the label (Table 1). 24-Ethylidene- and the epimeric 24-methyl-cholesterols

Table 1. Distribution of label in identified sterols

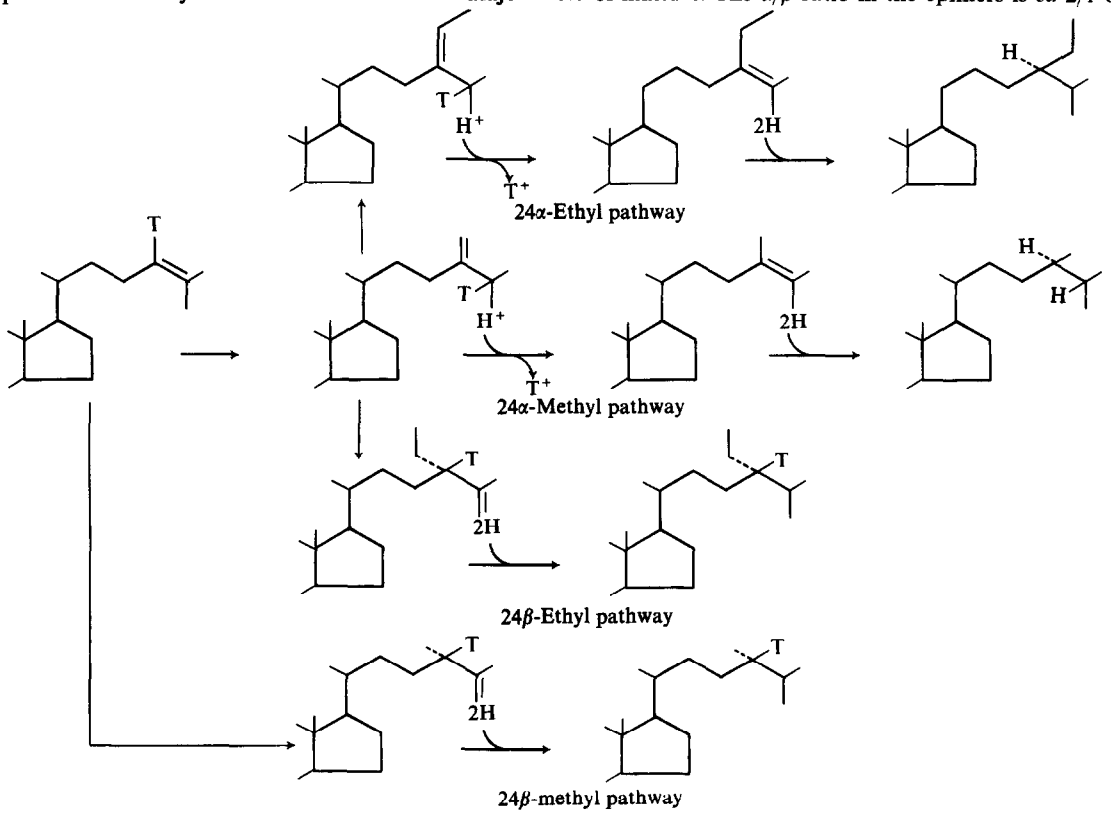
	Label incorporated dpm × 10^{-5}	
Δ^5-Sterol	^{3}H from lanosterol-[24-^{3}H]	^{14}C from MVA-[2-^{14}C]
trans-24-ethylidene	120.0 (85%)*	2.3 (23%)*
24α- and 24β-methyl	21.0 (15%)*	1.2 (12%)*
24α-ethyl	0.59 (0.4%)†	6.5 (65%)†

* Total radioactivity in chromatographically purified (sequentially: Al_2O_3 and Lipidex-5000 columns and argentation TLC) samples. † Total radioactivity in chromatographically purified (sequentially: Al_2O_3 and Lipidex-5000 columns) samples calculated from samples which had been further crystallized 2 (^{14}C) or 3 (^{3}H) times.

contained, in descending order, the remainder of the radioactivity except for a few percent in unidentified material. This demonstrated the biosynthetic capability of the tissue under the conditions used and functioned as a quantitative standard of the relative rates of biosynthesis. The distribution of the label in the 24-methyl- and 24-ethyl-sterols (1:5) also closely approximated their distribution by mass (1:6) which proved the kinetically regulatory steps operating at the time of the experiment to be essentially the same as earlier in the life of the seed when the bulk of the sterol was formed. On the other hand, the incorporation of label into *trans*-24-ethylidenecholesterol was much higher (23% of the total) than its contribution (4%) to the mass. This apparent anomaly is explicable if the ethylidenesterol itself is on the major pathway. In consequence of the significant pool present, radioactivity would be expected to increase in it as a result of a trapping effect. *trans*-24-Ethylidenecholesterol has actually been converted in *Pinus pinea* to 24α-ethylcholesterol [21], and in *Hordeum vulgare* four instead of five ^{2}H-atoms from methyl labelled ^{2}H-methionine are incorporated into the ethyl group [5]. There can be little or no doubt, therefore, that the 24-ethylidene group is precursor to the 24α-ethyl group in tracheophytes.

When the substrate was changed from MVA-[2-^{14}C] to lanosterol-[24-^{3}H], the distribution of label in the metabolites was considerably different (Table 1). Radioactivity appeared as before in the ethylidenesterol and in the methylsterol. However, for all practical purposes no label was present in the ethylsterol. The very small amount (0.4%) detected could have been due either to a slight amount of label at, e.g., C-23 in the substrate or to a minor sterol not eliminated by the purification procedures. Since the ^{14}C work demonstrated active biosynthesis of the 24α-ethylcholesterol, the 24-H-atom must have been lost from the ^{3}H-substrate in agreement with the previous work [1–6].

The incorporation of substantial label (15% of the amount in 4-desmethyl-sterols) into the epimeric 24-methylcholesterols is in agreement with its presence in the 24β-methyl component. No way is available to separate the epimers on a microscale. There is, nevertheless, indirect evidence for the label being present only in the β-epimer. The ratio of label in the ^{14}C case is 23/12 or 1.9/1 for the ethylidenesterol to the epimeric methylsterol mixture. The α/β-ratio in the epimers is *ca* 2/1 by



Scheme 1. Pathways to 24α- and 24β-alkyl-steroids in tracheophytes which are consistent with the data of the past and present investigations. The symbol T is used for tritium.

PMR [9], so if the label were all in the α-epimer the ^3H-ratio should be 1.9/0.67 or 2.8/1; alternatively it would be 1.9/0.33 or 5.8/1 if the label were only in the 24β-epimer. The observed ratio (85/15 or 5.7/1) is in remarkably good agreement with the label residing only in the β-epimer.

The data presented are consistent with Scheme 1 showing the pathways and their implied mechanisms, e.g., a proton addition–elimination without 1,3-transfer in double bond migration ($\Delta^{24(28)}$ to $\Delta^{24(25)}$) in the 24α-alkyl pathways. There is no evidence so far derived from tracheophytes which is in disagreement with that shown in the Scheme except for the sequencing relative to the introduction of the Δ^{22}-bond which is ignored. The Δ^{22}-bond can unequivocally be introduced prior to reduction of the $\Delta^{25(27)}$-bond in the 24β-ethyl routes, since the $\Delta^{22,25(27)}$-dienic side chain is well known to be present in sterols of Cucurbitaceae seeds [21, 23] and mature parts of Verbenaceae [11, 12], and Crassulaceae [8] plants. The Scheme also shows an end-product (the 24β-ethylsterol lacking a $\Delta^{25(27)}$-bond) which has been detected [24] but not yet documented by PMR. Thus, no firm proof for the existence of the $\Delta^{25(27)}$-reductase in tracheophytes exists, although there is every reason to believe it is present in some and perhaps most algae. It is worth noting that the pathway to 24β-methylsterols shown in Scheme 1 is markedly different from that demonstrated in fungi where the configuration is determined by reduction of a $\Delta^{24(28)}$-sterol which appears to be prohibited in tracheophytes. Prior demonstration [25] of the conversion of a $\Delta^{24(28)}$-sterol (24-methylenecholesterol) in *Pinus pinea* to what we now know [9] to be an epimeric mixture of 24-methylcholesterols is best interpreted, in light of the present data, as conversion only to the 24α-methyl epimer via 24-methyldesmosterol (which bears the $\Delta^{24(25)}$-bond).

EXPERIMENTAL

Fresh, intact seeds of *Pinus pinea*, the Italian stone pine, were obtained from Herbst Brothers Seedsmen, Inc., 1000 N. Main St., Brewester, NY 10509. Racemic mevalonate-[2-^{14}C] as the dibenzylethylenediamine salt (0.5 mCi in 24.6 mg) and NaB^3H_4 (293 mCi/mmol) were purchased from New England Nuclear Corporation and Amersham-Searle Corporation, respectively.

Lanosterol-[24-^{3}H]. 24-Ketolanosteryl acetate (mp 133–135°) prepared as previously described [19] was reduced with NaB^3H_4 followed by dehydration of the resulting alcohol to yield lanosteryl-[24-^{3}H] acetate [19]. Saponification of the ester and crystallization from MeOH yielded lanosterol-[24-^{3}H] with a sp. act. of 4.46×10^8 dpm/mg (mp 133.5–135.5°).

Incorporation of MVA-[2-^{14}C] or lanosterol-[24-^{3}H]. *P. pinea* seeds were sterilized and prepared for incubation as described previously [25, 26]. 10 ml of sterile H_2O containing 22.5×10^6 dpm MVA-[2-^{14}C] (0.5 mg) was then distributed between 10 beakers each containing 20 seeds, and an additional 1 ml H_2O was added to each beaker. The following day (when almost all the soln had been absorbed) each lot of seeds was transferred to a sterile, glass Petri dish lined with moistened filter paper. Sterile H_2O was added during the 10-day germination period as necessary. On day 9 an additional 45×10^6 dpm (1 mg) of MVA-[2-^{14}C] dissolved in 10 ml of sterile H_2O was distributed among the dishes. The seeds were germinated in the light at ambient temp. In the lanosterol-[24-^{3}H] incubation 4.0 mg of the sterol (17.8×10^8 dpm/mg) were dissolved in 5 ml of a 0.5% soln of Tween-20 in Me_2CO. To this mixture was added, with shaking, 10 ml of a 0.1% soln of Tween-20 in H_2O to yield a milky suspension. Additional Tween-20 in Me_2CO cleared the soln Me_2CO was removed under a N_2 stream to leave an aq. suspension of lanosterol-[24-^{3}H]. The substrate soln was distributed between 10 beakers each containing 30 shelled *P. pinea* seeds. The following day the seeds were transferred to sterile Petri dishes and maintained under the same conditions as above for 7 days.

Isolation and identification of sterols. After incubation the seeds were homogenized for 1 min in 95% EtOH, saponified, and the neutral lipids extracted with Et_2O. The 4-desmethylsterols, from Al_2O_3 chromatography of the neutral lipids (4% deactivation developed with a gradient of Et_2O in hexane), were further purified on a column of lipophilic Sephadex (Lipidex 5000) eluted with 5% hexane in MeOH. Two fractions were obtained containing white, crystalline solids, one of which was the epimeric 24-methylsterols together with *trans*-24-ethylidenecholesterol and the other was 24α-ethylcholesterol. 24α-Ethylcholesterol was crystallized from MeOH to constant mp and sp. act. (mp 134.5–135.5°). The fraction containing *trans*-24-ethylidenecholesterol and the epimeric 24-methylcholesterols were acetylated (Ac_2O/Py). Argentation TLC of the steryl acetates on Si gel-G (0.35 mm layers) impregnated with 10% $AgNO_3$ and developed once in CH_2Cl_2–hexane–HOAc (75:25:0.5) provided a separation of *trans*-24-ethylidenecholesteryl acetate and the epimeric 24-methylcholesteryl acetates. The individual steryl acetates eluted from the Si gel with Et_2O had properties on GLC identical with authentic standards. All three sterols, 24α-ethylcholesterol, *trans*-24-ethylidenecholesterol, and the epimeric 24-methylcholesterols have been previously isolated from *P. pinea* and characterized [9, 25]. Radioactive measurements were performed on a Nuclear Chicago Mark I Liquid Scintillation Counter, Model 70003.

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