Biosynthesis of the Insect-Moulting Hormone Cyasterone in the Plant Cyathula capitata

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Approximately 30 different closely related compounds possessing moulting hormone activity (ecdysones) have been isolated from plants, but such compounds are by no means universally distributed within the plant kingdom (Hikino & Hikino, 1970; Rees, 1971; Horn, 1971). They include compounds possessing C27, C28 or C29 steroid skeletons, ecdysterone (C27) being probably the most widespread insect-moulting hormone in the plants investigated so far. The incorporation of [14C]cholest-5-en-3β-ol into several C27 ecdysones, including ecdysterone (compound I in Scheme 1), has been demonstrated in various plants (Sauer et al., 1968; Hikino et al., 1970; de Souza et al., 1970). However, it is conceivable that the ecdysone biosynthetic pathway in plants could branch off the normal phytosterol pathway before the 4-desmethyl sterol stage.

We now report studies on the biosynthesis of the C29 moulting hormone, cyasterone (II). This is the major moulting hormone in the plant, Cyathula capitata (Amaranthaceae), where it is accompanied by smaller amounts of other ecdysones, e.g. compounds IV, V and VI (Hikino & Takemoto, 1972). A plausible biosynthetic pathway [(IV) → (V) → (VI) → (II)] to cyasterone (II) has been suggested, based on structural considerations of the ecdysones occurring in the plant (Takemoto et al., 1968). As a preliminary part of our biosynthetic studies, the major sterols of Cyathula capitata were identified as 5α-stigmasta-7,24(28)-dien-3β-ol (VII), 24-ethyl-5α-cholest-7-en-3β-ol (VIII), 24-ethylcholesta-5,22-dien-3β-ol (IX), 24-ethylcholest-5-en-3β-ol (X) and 24-methylcholest-5-en-3β-ol (XI) by g.l.c., mass and nuclear magnetic resonance (n.m.r.) spectroscopy. The configuration of the C-24 ethyl group in compound IX was deduced from its 220 MHz n.m.r. spectrum. For investigation of the biosynthetic pathway of cyasterone, [2-14C, (4R)-4-3H1] mevalonic acid was administered to Cyathula capitata.

A solution of [2-14C, (4R)-4-3H1] mevalonic acid (45 μCi of 14C; 150 μCi of 3H) in ethanol containing 0.01% Nonidet P42 (non-ionic detergent) was administered to the leaves of young seedlings of Cyathula capitata over a 4 week period. After a further week, the plants were harvested and the leaves washed with light petroleum, to remove any unabsorbed mevalonate. The plants were macerated in ethanol and the slurry heated under reflux for 10h. After cooling, the ethanolic solution was filtered and the filtrate evaporated to dryness yielding a gummy residue, which was suspended in water and extracted with light petroleum. The petroleum extracts were combined and washed with water. The original aqueous layer was re-extracted with n-butanol and the combined butanol extracts were then washed with the above water washings. The light petroleum and butanol extracts were then evaporated to dryness.

Purification of sterols. The light petroleum extract was saponified with aqueous ethanolic KOH in the usual manner and the non-saponifiable material was fractionated by alumina column chromatography. The 4-desmethyl sterols were purified by t.l.c. on silica gel, acetylated and the acetates separated by t.l.c. on AgNO3-impregnated silica gel H with chloroform (ethanol-free) for development. 24-Methylcholesterol-5-en-3β-ol (XI) and 24-ethylcholesterol-5-en-3β-ol (X) were separated by preparative g.l.c. The latter sterol was further transformed into 24-ethylcholesterol-4-en-3,6-dione by treatment with Jones reagent (Crabbe et al., 1961). Steryl acetates (except 24-methylcholesterol-5-en-3β-ol) were diluted with carrier material and recrystallized to constant specific radioactivity. The crude squalene fraction from the column chromatography was further purified by t.l.c., hexahydrochloride formation after addition of carrier material, and recrystallization.

Purification of cyasterone. A portion of the butanol extract was diluted with carrier cyasterone and subjected to two successive separations by silica gel t.l.c. in each of two solvent systems [chloroform–methanol, 3:1 (v/v); ethyl acetate–methanol, 10:1 (v/v)].
Scheme 1.
The cyasterone (II) was then partially acetylated (pyridine–acetic anhydride at room temperature for 30min) and the 2-acetoxy-cyasterone (III) purified by two successive separations by silica gel t.l.c. [chloroform–ethanol, 9:1 (v/v)]. The 2-acetoxy-cyasterone was diluted with carrier material and recrystallized to a constant $^3$H/$^{14}$C ratio.

Cycloartenol, the first product of squalene-2,3-oxide cyclization in plants, will be labelled as shown in compound XII, when biosynthesized from [2-$^{14}$C, (4R)-4-$^3$H]-mevalonic acid (Rees et al., 1968). The fate of the tritium at C-24 in cycloartenol during phytosterol biosynthesis will be considered separately after outlining the expected labelling patterns in the nuclear part of the sterols. By analogy with cholesterol bio-

![Chemical structures](image)

Fig. 1. Possible mechanisms of C-24 alkylation in phytosterols
H$_A$ is derived from the 4-pro-R hydrogen of mevalonic acid.
synthesis in animals (Cornforth et al., 1965; Mulheirn & Caspi, 1971) and the known labelling pattern of plant sterols from the above precursor (Smith et al., 1972), \(^{3}\)H should be present in sterols VII and VIII at C-5, C-17 and C-20, the \(^{3}\)H originally present in cycloartenol (XII) at C-8 and C-3 being eliminated during the intermediary formation of a \(\Delta^{8}\) bond and a 3-oxo grouping (associated with C-4 demethylation), respectively. During formation of the sterols IX and X, the \(^{3}\)H originally present at C-5 of compound XII should have been additionally eliminated during introduction of the \(\Delta^{5}\) bond.

The alkyl side chain at C-24 in phytosterols arises by transmethylation from \(S\)-adenosylmethionine. There is now evidence that alternative pathways may operate during alkylation in different organisms (Goad et al., 1974). Some of the possible pathways are outlined in Fig. 1.

The \(^{3}\)H/\(^{14}\)C atomic ratios (Table 1) are based on the known value of 6:6 for squalene. Based on the established operation of route (b) (Fig. 1) involving retention of H\(_{A}\) at C-25 in the biosynthesis of ethylidene sterols (Raab et al., 1968) the sterol VII should have a \(^{3}\)H/\(^{14}\)C atomic ratio of 4:5. It is difficult to offer a satisfactory explanation for the observed lower \(^{3}\)H/\(^{14}\)C ratio (3.63:5) in this compound (Table 1). A \(^{3}\)H/\(^{14}\)C ratio approaching 3:5 for (VIII), clearly demonstrates the elimination of H\(_{A}\) during its biosynthesis, and the possible operation of either routes (a) or (b) [(XVII) \(\rightarrow\) (XX) \(\rightarrow\) (XVIII) \(\rightarrow\) (XIX)] (Fig. 1). The atomic ratios for compounds IX and X indicate the possible operation of similar pathways of alkylation during their biosynthesis. The initially high atomic ratio (2.61:5) for compound X approached 2:5 after further purification by formation of the 4-ene-3,6-dione derivative (XXV, Table 1), and was probably due to a co-crystallizing impurity with a higher \(^{3}\)H/\(^{14}\)C ratio (possibly the corresponding \(\Delta^{7}\) sterol). In summary, it is clear that during formation of the C-24 ethyl sterols (VIII), (IX) and (X), the tritium (H\(_{A}\)) originally at C-24 of cycloartenol (XII) has been eliminated in each case.

The tritium atom retained in cyasterone (II) and its 2-acetoxy derivative (III) (Table 1) is presumably located at C-17. This demonstrates that if cyasterone (II) is formed from a C-24 ethylidene sterol, without the obligatory involvement of a C-24 ethyl sterol, the hydrogen at C-25 (H\(_{A}\)) must be eliminated. Sterols such as compounds VIII and IX having a C-24 ethyl group could be considered as possible precursors of cyasterone.

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**Table 1. Ratios of \(^{3}\)H/\(^{14}\)C in squalene, sterols and cyasterone (and some subsequent chemical transformation products) isolated from Cyathula capitata after administration of \([2-^{14}\text{C},(4R)4-^{3}\text{H}])\text{mevalonic acid}**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Specific radioactivity (d.p.m./mg)</th>
<th>(^{3})H/(^{14})C radioactivity ratio</th>
<th>(^{3})H/(^{14})C atomic ratio (based on squalene)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squalene hexahydrochloride</td>
<td>612</td>
<td>5.61</td>
<td>6:6</td>
</tr>
<tr>
<td>5(\alpha)-Stigmasta-7,24(28)-dien-3(\beta)-ol (VII)</td>
<td>338</td>
<td>4.08</td>
<td>3.63:5</td>
</tr>
<tr>
<td>24-Ethyl-5(\alpha)-cholest-7-en-3(\beta)-ol (VIII)</td>
<td>435</td>
<td>3.32</td>
<td>2.95:5</td>
</tr>
<tr>
<td>(24S)-24-Ethylcholesta-5,22-dien-3(\beta)-ol (IX)</td>
<td>1712</td>
<td>2.42</td>
<td>2.15:5</td>
</tr>
<tr>
<td>24-Ethylcholesta-5-en-3(\beta)-ol (X)</td>
<td>1025</td>
<td>2.94</td>
<td>2.61:5</td>
</tr>
<tr>
<td>24-Ethylcholesta-4-en-3,6-dione (XXV)</td>
<td>315</td>
<td>2.39</td>
<td>2.13:5</td>
</tr>
<tr>
<td>Cyasterone (II)</td>
<td>—</td>
<td>1.20</td>
<td>1.07:5</td>
</tr>
<tr>
<td>2-Acetoxy-cyasterone (III)</td>
<td>154</td>
<td>1.21</td>
<td>1.07:5</td>
</tr>
</tbody>
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The atomic ratios for cyasterone (II) and its 2-acetoxy derivative (III), further demonstrate that the tritium originally located at C-8 of cycloartenol (XIII) must have been eliminated, presumably during \( \Delta^5 \) bond formation. The tritium originally at C-5 in compound XI is also lost, but the mechanism is uncertain. This may involve bond formation, but it is just conceivable that this is not an obligatory step, since the ecdysone pathway in plants (which are capable of sterol synthesis \textit{de novo}) could possibly branch off the normal phytosterol pathway before this stage. The present results rule out certain conceivable pathways of cyasterone formation from ethylidene sterols, but are not inconsistent with the operation of a pathway such as that suggested by Takemoto \textit{et al.} (1968). However, several other possibilities also exist.

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Multimolecular Forms of Phenylalanine–Ammonia Lyase in \textit{Alternaria}

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Phenylalanine–ammonia lyase is a key enzyme in the formation of a variety of phenolic compounds in plants, and in higher plant tissues is widespread and appears to have important regulatory properties. In the case of fungi less is known of this enzyme although it has been reported to occur in several Basidiomycetes (Camm & Towers, 1973).

A study of melanin formation by \textit{Alternaria} sp., a Fungus Imperfectus, led us to examine this fungus for phenylalanine–ammonia lyase activity and hence a possible route from phenylalanine to the pigment.

The fungus was grown in shake culture in an inorganic salts–glucose medium (27°C) and the enzyme was extracted from the washed mycelium by grinding with 0.05 m-Tris–HCl buffer, pH 8.0. After centrifugation of the extract (20000g, 20 min) assays were made by using the method of Zucker (1965). Fig. 1 showed that in dark cultures the appearance of activity in the mycelium was retarded by about 40 h as compared with cultures grown in the light. It is possible that the failure of Nambudiri \textit{et al.} (1970) to detect phenylalanine–ammonia lyase in \textit{Alternaria} was a function of the light regime they