3β-Hydroxy-5β-cholest-7-en-6-one as an Intermediate of 20-Hydroxyecdysone Biosynthesis in a Hairy Root Culture of *Ajuga reptans* var. *atropurpurea*

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[3α-^{2}H]-, [4α-^{2}H]- and [4β-^{2}H]-Cholesterols and [3α-^{2}H]- and [5-^{2}H]-3β-hydroxy-5β-cholest-7-en-6-ones were converted with a hairy root culture of *Ajuga reptans* var. *atropurpurea* into 20-hydroxyecdysone, in which the deuterium atoms retained their original positions, thus strongly suggesting that 3β-hydroxy-5β-cholest-7-en-6-one is an obligatory intermediate in the biosynthesis of ecdysteroids in the plant.

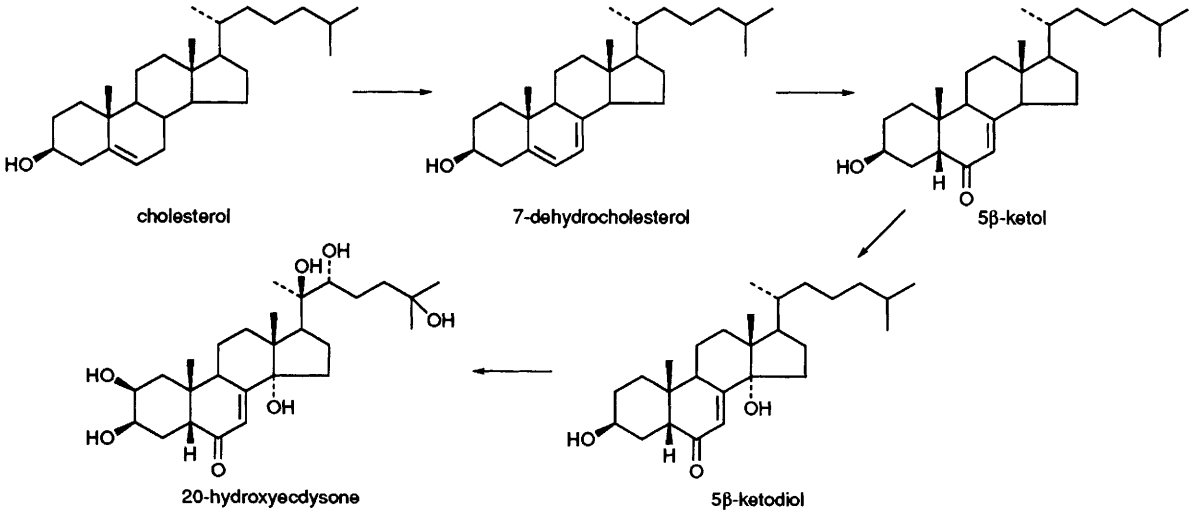
20-Hydroxyecdysone is the moulting hormone of most of the arthropods. Its characteristic *cis* A/B ring junction, 7-en-6-one system, and polyhydroxyl groups are responsible for biological activity. In insects, 20-hydroxyecdysone is biosynthesized from cholesterol *via* 7-dehydrocholesterol and 3β,14α-dihydroxy-5β-cholest-7-en-6-one (5β-ketodiol) (Scheme 1).[1] Ecdysteroids are also distributed in the plant kingdom. In plants, cholesterol is also found to be a precursor of ecdysteroids.[2–4] Although later stages of 20-hydroxyecdysone biosynthesis, *e.g.* hydroxylation at C-2, C-20, C-22 and C-25, have been studied intensively,[1] little is known about the mechanism of earlier stages, especially the formation of the *cis* A/B ring junction and 7-en-6-one system.

Goodwin and coworkers have suggested an intermediary role of a 5α,6α-epoxide in the fern *Polypodium vulgare*[4] and a 3-keto-4-ene in the locust *Schistocerca gregaria*[5] on the basis of the metabolic fate of 3α-, 4α- and 4β-hydrogens of cholesterol. We have recently demonstrated that a transformed hairy root culture of *Ajuga reptans* var. *atropurpurea*[6] is able to convert cholesterol into 20-hydroxyecdysone in appreciable yield and is a suitable tool for biosynthetic studies.[3] With this system the feeding experiments of [3α-^{2}H]- **1**,[7] [4α-^{2}H]- **2**[8,9] and [4β-^{2}H]-**3**[10] cholesterols have now been performed to examine the fate of these hydrogens, and the results obtained are in contrast with those reported by Goodwin *et al.*

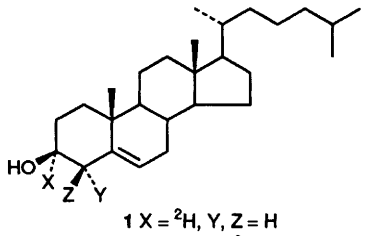
Incubation of the labelled cholesterols were carried out as described previously.[3] The hairy root clone of *Ajuga* was cultured in liquid MS medium supplemented with sucrose (3%) at 25 °C for two weeks in the dark before incubating the labelled cholesterols. Compound **1** (100 mg), dissolved in Tween 80 (2 ml), acetone (4 ml) and distilled water (2 ml), was added through a membrane filter to the hairy root grown in the medium (1000 ml). This was incubated in the dark on a rotary shaker at 25 °C for another two weeks and harvested. The root, weighing 110 g (wet weight), was extracted and separated as described previously[3] to furnish 20-hydroxyecdysone (3 mg). The ^{2}H NMR spectrum of the 20-hydroxyecdysone showed a peak at δ 4.2, which corresponds to the signal of H-2α (δ 4.17) or H-3α (δ 4.21).[11] This signal was unambiguously assigned to that of H-3α by ^{2}H NMR analysis of 2,3,22-triacetate derivative which exhibited a signal only at δ 5.40 (the chemical shifts of H-3α and H-2α are at δ 5.37 and 5.08, respectively).

Compound **2** was similarly incubated and ^{2}H NMR analysis of the resulting 20-hydroxyecdysone (3 mg) showed a signal only at δ 1.7. Although the signals of H-4α (δ 1.80), H_2-11 (δ 1.71 and 1.88), H-15β (δ 1.89), H-23 (δ 1.85) and H-24 (δ 1.81) resonate in this region,[11] the signal was assigned to H-4α since it is highly unlikely that the other hydrogens are derived from 4α-H of the substrate. Similarly, the incubation of compound **3** afforded 20-hydroxyecdysone (4 mg), which exhibited a signal at δ 2.0 in the ^{2}H NMR spectrum. This signal was assigned to H-4β on the basis of biosynthetic consideration, although the signals of H-1β (δ 1.91), H-4β (δ 2.02), H-12β (δ 1.95) and H-16β (δ 2.08) resonate in this region.[11]

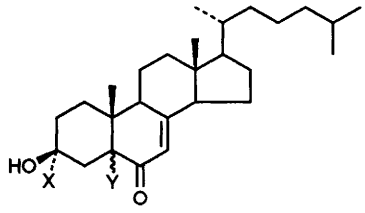
These results clearly indicated that the 3α-, 4α- and 4β-hydrogens of cholesterol are retained at the respective positions of 20-hydroxyecdysone during the biotransformation in *A. reptans* var. *atropurpurea*. Our results were in contrast with the findings reported by Goodwin and coworkers that 3α-H of cholesterol migrates to C-4, and 4β-H migrates to C-5 of 20-hydroxyecdysone in *P. vulgare*.[4] The present observation strongly suggests the possibility that the *cis* A/B ring junction of ecdysteroids is formed *via* a simple mechanism, *i.e.* a modification of 5-ene moiety of 7-dehydrocholesterol to a 6-one moiety of 7-en-6-one structure with the concomitant formation of 5β-stereochemistry, rather than 3-oxo- or 4-ene-steroid intermediate.[4,5] 7-Dehydrocholesterol



Scheme 1



1 X = ^{2}H, Y, Z = H
2 X = H, Y = ^{2}H, Z = H
3 X, Y = H, Z = ^{2}H



4 X = ^{2}H, Y = 5β-H
5 X = H, Y = 5β-^{2}H
6 X = H, Y = 5α-^{2}H

and 5β-ketodiol could be assumed to be an intermediate in 20-hydroxyecdysone biosynthesis in plants from the analogy of insects. These consideration, taken together with the results described above, prompted us to examine the possibility that 3β-hydroxy-5β-cholest-7-en-6-one (5β-ketol) as an immediate precursor of 5β-ketodiol. In this line, further incubation was carried out with three deuterium labelled substrates, [3α-^{2}H]-5β-ketol **4**, [5β-^{2}H]-5β-ketol **5** and [5α-^{2}H]-5α-ketol **6**.

Compound **4** (*ca.* 98% deuterium labelled at the 3α-position) was prepared from 3β-hydroxy-5α-cholest-7-en-6-one[12] in three steps, *i.e.* Swern oxidation leading to 5α-cholest-7-ene-3,6-dione (55%), selective reduction with NaB^2H_4 leading to [3α-^{2}H]-3β-hydroxy-5α-cholest-7-en-6-one (90%), and C-5 epimerization using NaOH–MeOH at 40 °C for 3 min (10%). Compounds **5** and **6** (*ca.* 80% deuterium labelled at C-5 and *ca.* 5% at C-7) were obtained in 5 and 6%, respectively, by the treatment of 3β-acetoxy-5α-cholest-7-en-6-one with MeONa in D_2O–THF (5 min, at room temperature).

Incubation of **4** (100 mg) afforded 20-hydroxyecdysone (3 mg), which exhibited a signal at δ 4.15 in the ^{2}H NMR spectrum. The signal could be assigned to H-3α rather than H-2α. This indicated that 5β-ketol was incorporated into 20-hydroxyecdysone. Compound **5** was similarly incubated to give 20-hydroxyecdysone (4 mg), whose ^{2}H NMR spectrum exhibited a signal at δ 2.9, corresponding to the chemical shifts of either H-5β (δ 3.01) or H-17 (δ 3.00) of 20-hydroxyecdysone.[11] Since it is highly unlikely that 5β-H of the substrate migrates into the C-17 position, the signal was assigned to H-5β. This result confirmed the incorporation of 5β-ketol into 20-hydroxyecdysone, and further indicated that the 5β-hydrogen of the substrate retained its original position. Incubation of **6** and ^{2}H NMR analysis of the resulting 20-hydroxyecdysone (3 mg) showed that **6** was not incorporated.

The present studies have provided definitive evidence that 5β-ketol is metabolized into 20-hydroxyecdysone in *Ajuga* tissue culture. It should be noted that the incorporation yield of 5β-ketol was at the same level or slightly higher than that of cholesterol. The results strongly suggest that 5β-ketol is an obligatory intermediate between 7-dehydrocholesterol and 5β-ketodiol in the biosynthesis of 20-hydroxyecdysone in this plant (Scheme 1). This implies that the formation of *cis* A/B ring junction should occur prior to C-14 hydroxylation. It can be considered that addition of water to Δ^5 of 7-dehydrocholesterol followed by oxidation may lead to 5β-ketol. Alternatively, 5α,6α-epoxycholest-7-en-3β-ol may be transformed into 5β-ketol either by a direct epoxide–carbonyl rearrangement or some other mechanism. We have previously reported that C-6 hydrogen of cholesterol was lost during the transformation into ecdysteroids in *Locust migratoria*,[13] which would rule out the rearrangement mechanism at least in insects. In insects 5β-ketol is not regarded as an intermediate of 20-hydroxyecdysone biosynthesis, although there are several papers which describe some conversion of this compound into ecdysteroids.[1]

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