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Biosynthesis of 20-Hydroxyecdysone in *Ajuga* Hairy Roots: Hydrogen Migration from C-6 to C-5 during *cis*-A/B Ring Formation†

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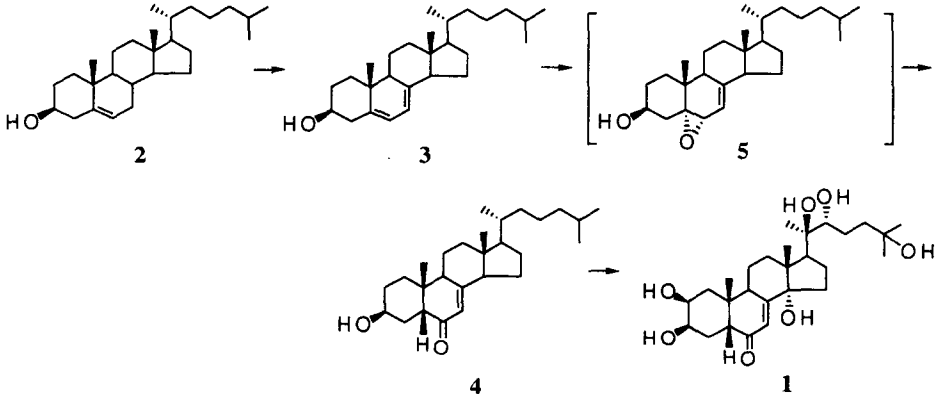
Abstract: Feeding of deuterium labeled cholesterols including [6-^{2}H]cholesterol and [3α,6-$^2H_2$]-cholesterol to hairy roots of *Ajuga reptans* var. *atropurpurea* followed by ^{2}H-NMR analysis of the biosynthesized 20-hydroxyecdysone revealed that most of the deuterium atom located at C-6 of cholesterol migrated to the C-5 position of 20-hydroxyecdysone. © 1997 Elsevier Science Ltd.

20-Hydroxyecdysone (**1**) is the molting hormone of most arthropods and it is also distributed in the plant kingdom. The structure of the steroidal hormone is characterized by a *cis*-A/B ring junction, a 7-en-6-one conjugated system, and polyhydroxyl groups. In insects, accumulated evidence suggests that 20-hydroxyecdysone is biosynthesized from cholesterol (**2**) *via* 7-dehydrocholesterol (**3**) and 3β,14α-dihydroxy-5β-cholest-7-en-6-one (5β-ketodiol).[1] However, the mechanism of the earlier stage of this transformation, *i.e.*, formations of the *cis*-A/B ring junction and 7-en-6-one system, remains unclear. The possibility of a 5α,6α-epoxide intermediate, *e.g.*, 7-dehydrocholesterol 5α,6α-epoxide (**5**), has been suggested repeatedly, without conclusive experimental evidence.[2-6]



Scheme 1. Proposed biosynthetic pathway of 20-hydroxyecdysone in *Ajuga* hairy roots.

We demonstrated previously that hairy roots of *Ajuga reptans* var. *atropurpurea*[7] is a suitable tool for biosynthetic studies of phytoecdysteroids,[8] and subsequently reported on the possible intermediary role of 3β-hydroxy-5β-cholest-7-en-6-one (5β-ketol, **4**) in 20-hydroxyecdysone biosynthesis, based on its positive

incorporation and the behavior of 3α-, 4α- and 4β-hydrogens of cholesterol (they were all retained at their original positions).[9] Scheme 1 shows a postulated biosynthetic pathway of 20-hydroxyecdysone in this tissue culture.[9,10] In this paper, we report on the origin of 5β-hydrogen of 20-hydroxyecdysone in *Ajuga* hairy roots. The finding presented herein strongly supports the involvement of a 5α,6α-epoxide in the *cis*-A/B ring formation in plants.

Since neither 4α- nor 4β-hydrogen was found to migrate to the C-5 position of **1** in *Ajuga* hairy roots, the possible source of the 5β-hydrogen of **1** appeared to be limited to the following three: (a) C-6 hydrogen of cholesterol, (b) hydrogen atom from water, and (c) hydrogen atom from a reducing cofactor such as NADPH. To examine these possibilities, [6-^{2}H]cholesterol (~99% labeled at the C-6 position)[11] was first fed to *Ajuga* hairy roots and the biosynthesized 20-hydroxyecdysone was isolated as described previously.[8] The ^{2}H-NMR spectrum (Fig. 1) of the 20-hydroxyecdysone showed a peak at δ 2.9, which corresponds to the signal of H-5[12] of 20-hydroxyecdysone. The result clearly indicated that H-6 of cholesterol migrated to the C-5 position of **1** during the bioconversion. The 1,2-hydrogen shift seems likely to occur at the stage between 7-dehydrocholesterol and 5β-ketol, *i.e.*, during *cis*-A/B ring formation of **1**, since H-5 of 5β-ketol was previously shown to be retained there during the conversion into 20-hydroxyecdysone.[9]

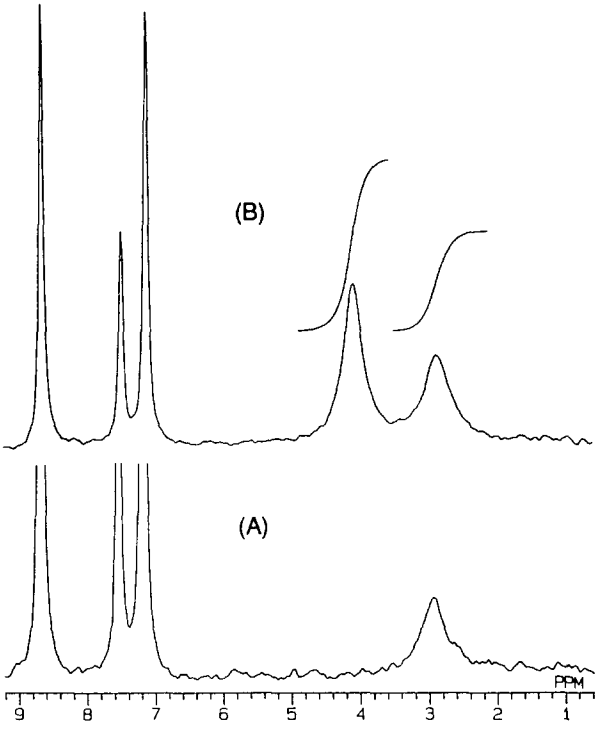


Fig. 1 ^{2}H-NMR spectra (77 MHz, pyridine) of 20-hydroxyecdysone obtained by feeding [6-^{2}H]cholesterol (A) and [3α,6-$^2H_2$]cholesterol (B) to *Ajuga* hairy roots. The signal of Hβ of pyridine was referenced as δ=7.19.

To confirm the hydrogen migration and to examine the relative efficiency of the incorporation, a 1:1 mixture of [4β-^{2}H]-cholesterol (~99% labeled at the C-4 position)[13] and [6-^{2}H]cholesterol was then fed to the hairy roots. The ^{2}H-NMR spectrum of the resulting 20-hydroxyecdysone showed two peaks at δ 2.0 (corresponding to the signal of H-4β of **1**) and δ 2.9 in a *ca.* 3:2 ratio of signal intensity (data not shown). This data further verified the hydrogen migration from the C-6 to C-5 position. However, unexpectedly, *ca.* 1/3 of the 5β-hydrogen of **1** was shown to arise from another hydrogen source. The possibility of a deuterium isotope effect (provided that the C-H bond cleavage at C-6 is involved in a rate-determining step) may be raised to explain the behavior of H-6 of cholesterol. Thus, a doubly-labeled compound, [3α,6-$^2H_2$]cholesterol, was chosen as the next substrate and synthesized from 5α-cholestane-3,6-dione in four steps (reduction with $LiAlD_4$, protection of the 3β-ol as benzoate, dehydration of the 6β-ol with $POCl_3$, and deprotection of the benzoate, overall yield 35%). The ^{2}H-NMR spectrum of **1**, obtained after feeding the doubly-labeled cholesterol, is illustrated in Fig. 1, which exhibited two peaks at δ 4.2 (corresponding to H-3 of **1**) and δ 2.9 in a *ca.* 3:2 ratio. The upper-field signal again confirmed that most of the C-6 deuterium atom of the substrate migrated to the C-5 position. It should be noted that the behavior of the C-6 deuterium is essentially the same as that found in a 1:1 mixture of [4β-^{2}H]- and [6-^{2}H]cholesterols. The postulated deuterium isotope effect is, therefore, unlikely to be the main factor for the partial loss of the C-6 deuterium atom of **2**; rather, the presence of an unidentified mechanism is suggested. A preliminary study indicated that 5β-hydrogen of **1** is not exchanged by an exogenous proton source during extraction and isolation of **1** *via* enolization. Although it is not clear whether an alternative mechanism exists for the formation of the *cis*-A/B ring or the partial loss takes place during the 1,2-hydrogen migration in an enzyme cage, the partial disappearance of the C-6 deuterium atom of cholesterol is likely to occur during the formation of the *cis*-A/B ring.

In conclusion, we have offered unambiguous evidence for the origin of 5β-hydrogen of 20-hydroxyecdysone in *Ajuga* hairy roots, although the non-stoichiometric behavior of H-6 of cholesterol remains an open question. The observed 1,2-hydrogen shift agrees with the postulated 7-dehydrocholesterol 5α,6α-epoxide intermediate (**5**), since it reasonably explaines the generation of 5β-stereochemistry and 6-oxo function of 5β-ketol as well as 20-hydroxyecdysone. Attempted feeding of **5** was not successful since this compound rapidly decomposed under incubation conditions.[14]

The origin of 5β-hydrogen in *Ajuga* hairy roots is in sharp contrast with that reported in the fern *Polypodium vulgare* in which 4β-hydrogen of cholesterol migrates to the C-5 position.[15] We previously reported that H-6 of cholesterol was lost during the formation of ecdysone and 2-deoxyecdysone in *Locust migratoria*.[3] Further, Goodwin *et al.* reported that 4β-hydrogen of cholesterol is lost in Locust, *Sistocerica migratoria*.[16] Quite interestingly, these data suggest that the *cis*-A/B ring of ecdysteroids is biosynthesized not by a single mechanism but at least three mechanisms depending on the species of plants and insects.

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REFERENCES AND NOTES

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