Mechanism of C-2 Hydroxylation during the Biosynthesis of 20-Hydroxyecdysone in Ajuga Hairy Roots

Keiko Nomura and Yoshinori Fujimoto

Department of Chemistry and Materials Science, Tokyo Institute of Technology, Meguro, Tokyo 152–8551, Japan.
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Feeding synthetic [2β-3H]- and [2α-3H]-cholesterols to the hairy roots of Ajuga reptans var. atropurpurea and 3H-NMR analysis of the biosynthesized 20-hydroxyecdysone revealed that hydroxylation at C-2 proceeds with retention of configuration. Feeding [2α,3α-3H]cholesterol followed by 3H-NMR analysis of the 2,3,22-triacetate of the resulting 20-hydroxyecdysone ruled out a mechanism which involves a partial loss of the 2α-hydrogen. The steric course of C-2 hydroxylation in Ajuga hairy roots is identical with that reported in the insect, Schistocerca gregaria.

Key words 20-hydroxyecdysone; Ajuga reptans var. atropurpurea; biosynthesis; hydroxylation; ecdysteroid

20-Hydroxyecdysone (1), a molting hormone of insects, is also distributed in the plant kingdom. We previously demonstrated that the hairy roots of Ajuga reptans var. atropurpurea (Labiatae) are useful for biosynthetic studies of phytocdysteroids. In this transformed biosystem, feeding studies of 3H-labeled substrates have revealed that the 3α-, 4α- and 4β-hydrogens of cholesterol are retained at their original positions after conversion to 20-hydroxyecdysone, and that most of the 6-hydrogen of cholesterol migrates to the C-5 position of 20-hydroxyecdysone. The metabolic fates of these hydrogens are different from those reported in the insects, Schistocerca gregaria and Locusta migratoria, and the fern Polypodium vulgare. In particular, the fate of 3α-hydrogen differs markedly among these species. In contrast to the complete retention of the 3α-hydrogen of cholesterol in Ajuga hairy roots, the hydrogen is reported to migrate to the C-4 position of 20-hydroxyecdysone in P. vulgare. Further, in S. gregaria, 20% of the hydrogen is reportedly retained at positions other than the C-3 of 20-hydroxyecdysone. These differences may be correlated with the mechanism of the introduction of the adjacent C-2 hydroxyl group. It is, therefore, interesting to investigate the mechanism of C-2 hydroxylation in Ajuga hairy roots and compare it with those of other plants and insects.

Results and Discussion

The metabolic fate of the 2β- and 2α-hydrogens of cholesterol during the biosynthesis of 20-hydroxyecdysone was followed up by feeding studies of 3H-labeled substrates, [2β-3H]- and [2α-3H]-cholesterols (2a, 2b). Compound 2a was synthesized according to Chart 1. 6β-Hydroxy-5α-cholest-2-ene (3), prepared from 3,5α-cyclohexan-6-one in two steps (heating with LiBr in N,N-dimethylformamide (DMF) and reduction with NaBH₄), was converted to tetrahydropranyl (THP) ether 4 which was epoxidized to give 2α,3α-epoxide 5, stereoselectively. Reduction of 5 with LiAlH₄ pro-

Chart 1  Synthesis of [2β-3H]cholesterol (2a)

* To whom correspondence should be addressed. 
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Chart 2. Synthesis of [2α-3H]cholesterol (2b)

Fig. 1. 3H-NMR (61 MHz, in Pyridine) Spectra of 20-Hydroxyecdysone Samples Biosynthesized from [2β-3H]Cholesterol 2a (Top) and [2α-3H]Cholesterol 2b (Middle)

3H-NMR spectrum of authentic 20-hydroxyecdysone is given at the bottom.

ceeded in an axial manner to afford [2β-3H]-3α-ol 6. The 3α-alcohol 6 was converted to 3β-alcohol 8 via oxidation leading to 3-ketone 7 and reduction. After protecting 8 as the acetate 9, the THP group was deprotected to give 6β-ol 10 which, upon dehydration, afforded 5-ene 11. Hydrolysis of the acetyl group of 11 gave the desired 2α. Compound 2α exhibited a single signal at δ 1.50 in its 3H-NMR spectrum.

[2α-3H]Cholesterol (2b) was synthesized according to Chart 2. The same starting material 3 was converted to pivaloyl ester 12. Hydroboration of 12 with BD₃ afforded a mixture of four isomeric alcohols, from which 3α-alcohol 13 was separated by silica-gel chromatography. The 3α-alcohol 13 was converted to 3β-alcohol 15 in an oxidation-reduction sequence via 14. Compound 15 was converted to THP ether 16, and then the pivaloyl group was removed by reduction to give 6β-ol 17. Dehydration of 17 gave 5-ene 18. Removal of the THP group of 18 furnished the desired 2b. The α-orientation of the deuterium atom at the C-2 of 2b was established at the stage of the ketone 14. The 1H-NMR spectrum of the non-labeled compound corresponding to 14 exhibited 2α-H and 2β-H signals at δ 2.33 (dt, J=13.8, 2.8 Hz) and 2.41 (td, J=13.7, 7.3 Hz), respectively, whereas that of compound 14 lacked the former signal but exhibited the latter signal at δ 2.39 (dd, J=13.7, 7.3 Hz). Compound 2b exhibited a single signal at δ 1.83 in its 3H-NMR spectrum.

With the two isomeric 3H-labeled cholesterols available, experiments feeding Ajuga hairy roots were performed as described previously. Briefly, 100 mg of the deuteriocholesterol was administered to Ajuga hairy roots which had been preincubated for two weeks, followed by incubation for another two weeks. From the roots, 20-hydroxyecdysone was obtained after chromatographic separation and final purification by HPLC.

The 3H-NMR spectra of the 20-hydroxyecdysones derived from 2a and 2b are shown in Fig. 1. The 20-hydroxyecdysone derived from 2b displayed a deuterium signal at δ 4.15, which corresponds to the 2α-hydrogen of 20-hydroxyecdysone, whereas the 20-hydroxyecdysone derived from 2a did not exhibit any signals in this region. The observed signal could be assigned to the deuterium at C-2 rather than C-3, since the 3α-hydrogen of cholesterol is known to be retained during the conversion to 20-hydroxyecdysone. The assignment was further confirmed by 3H-NMR analysis of the corresponding 2,3,22-triacetate derivative which showed a signal at δ 5.08 due to 2α-3H (Fig. 2). These results indicated that the 2β-hydrogen of cholesterol is stereospecifically removed and the 2α-hydrogen is retained during 20-hydroxyecdysone biosynthesis in Ajuga hairy roots.
The retention of the 2α-hydrogen was shown in the above feeding studies. However, an equilibrium between the 2-hydroxy compound and 2-oxo derivative, during or after the biosynthesis of 20-hydroxycholesterol, would cause partial loss of the 2α-hydrogen. Indeed, characterization of 3-oxo compounds such as 3-dehydrocholesterol and their possible involvement in biosynthesis have been reported. In contrast, no loss of the 3α-hydrogen was reported with the plant, Taxus baccata. A further study was, therefore, undertaken using a doubly labeled compound, [2α,3α-2H]cholesterol (2e). The synthesis of 2e is illustrated in Chart 3. Compound 14 was reduced with LiAlD₄ to give a [2α,3α-2H]-derivative 19, which was converted into 2e, through THP ether 20, 6-ol 21 and 5-ene 22, in the same manner as described for the synthesis of 2b. The 2H-NMR spectrum of 2e exhibited signals at δ 1.82 and 3.50 due to 2α-2H and 3α-2H, respectively.

Compound 2e was fed to Ajuga hair roots in the same manner as described above, and the resulting 20-hydroxycholesterol and its 2,3,22-triace-tate derivative were analyzed by 2H-NMR. The 20-hydroxycholesterol exhibited a broad 2H signal at ca. δ 4.15 (data not shown). The 2H-NMR spectrum of the triacetate is shown in Fig. 2, which displayed partially overlapping signals at δ 5.38 and 5.17 assignable to the C-3α and C-2α deuterium atoms, respectively. The intensity of the two signals was fairly similar. Thus, it is safe to conclude that a mechanism involving partial loss of the 2α-hydrogen does not operate in Ajuga hairy roots.

In conclusion, the present study has established that C-2 hydroxylation during the biosynthesis of 20-hydroxycholesterol in Ajuga hairy roots proceeds with retention of configuration. A possible contribution from a 2-oxo compound was ruled out. Thus, the C-2 hydroxylation is most likely to occur in a direct hydroxylation mechanism. The steric course of the C-2 hydroxylation in Ajuga hairy roots is identical with that reported in the insect, S. gregaria. It is reported that the enzyme responsible for the C-2 hydroxylation of L. migratoria is a monoxygenase which is not a cytochrome P450. We recently reported that the C-25 hydroxylation during the biosynthesis of 20-hydroxycholesterol in Ajuga hairy roots is not stereospecific, but proceeds both via retention and inversion mechanisms.

Experimental

1H-NMR spectra were obtained on a JEOL JNM-LA300 (300 MHz) or LA400 (400 MHz) spectrometer in CDC1₃ solutions and chemical shifts (δ) are reported in ppm downfield from tetramethylsilane (used as an internal reference). The signals for 26-H, 27-H, (6H, d, δ = 7.5—8.1) were assigned at δ 1.86—1.87 for all steroidal compounds, and these data are not described. 2H-NMR spectra were recorded on a JEOL JNM-LA400 spectrometer (61 MHz for 1H) in CHCl₃ (the signal of residual CHCl₃ was at δ 7.26) or in pyridine (the signal of 2H of the solvent was at δ 7.19). HPLC was performed on a Shimadzu LC-6A instrument with an SPD-6A UV detector using a preparative octadecyl silica (ODS) column (Shimadzu STR PREP-ODS column, 25 cm × 20 mm i.d.). Merck Kieselgel 60 and Merck Kieselgel F₂₅₄ plates (20 × 20 cm, 0.5 mm thick) were used for column chromatography and preparative thin-layer chromatography (TLC), respectively.

Chart 3. Synthesis of [2α,3α-2H]cholesterol (2e)
The hydroboration-oxidation reaction using NaBH₄ in place of NaBD₄ afforded four non-labeled products in order of increasing polarity in the ratio of 51:11:12:26:5z-cholestan-2β,6β-diol-6-pivaloyl ester, 1H-NMR δ: 0.67 (3H, s, 18-H), 0.91 (3H, d, J = 6.4 Hz, 21-H), 1.20 (9H, s, pivaloyl), 1.25 (3H, s, 19-H), 4.14 (1H, s, 2-H), 4.94 (1H, s, 4-H), 7.10 (1H, d, J = 2.5 Hz, 6-H). 5z-cholestan-2β,6β-diol-6-pivaloyl ester, 1H-NMR δ: 0.67 (3H, s, 18-H), 0.91 (3H, d, J = 6.3 Hz, 21-H), 1.02 (3H, s, 19-H), 1.19 (9H, s, pivaloyl), 3.80 (1H, m, 2-H), 4.95 (1H, d, J = 2.5 Hz, 6-H). 5z-cholestan-2α,6β-diol-6-pivaloyl ester, 1H-NMR δ: 0.67 (3H, s, 18-H), 0.91 (3H, d, J = 6.4 Hz, 21-H), 1.02 (3H, s, 19-H), 1.19 (9H, s, pivaloyl), 3.80 (1H, m, 2-H), 4.95 (1H, d, J = 2.5 Hz, 6-H). 5α-cholestan-2β,6β-diol-6-pivaloyl ester, 1H-NMR δ: 0.67 (3H, s, 18-H), 0.91 (3H, d, J = 6.4 Hz, 21-H), 1.02 (3H, s, 19-H), 1.19 (9H, s, pivaloyl), 3.80 (1H, m, 2-H), 4.95 (1H, d, J = 2.5 Hz, 6-H).

**[2α,6β]-Pivaloyloxy-5α-cholestan-3β-ol (15)** Oxidation of 13 (584 mg, 11.9 mmol) in the same manner as described for 6 gave 14 (567 mg, 97%), after filtration through Florisil and concentration, as white crystals, mp 118—119°C. 1H-NMR δ: 0.70 (3H, s, 18-H), 0.91 (3H, d, J = 6.8 Hz, 21-H), 1.21 (9H, s, pivaloyl), 1.22 (3H, s, 19-H), 2.11 (1H, d, J = 15.6 Hz, 4-H), 2.10 (1H, d, J = 14.7 Hz, 4-H), 2.39 (1H, dd, J = 13.3, 6.9 Hz, 2β-H), 4.84 (1H, d, J = 2.0 Hz, 6-H). MeOH (10 ml) and NaBH₄ (60 mg, 1.59 mmol) were added to a solution of 14 (567 mg, 1.16 mmol) in THF (5.0 ml), and the reaction mixture was stirred at room temperature for 30 min. An extractive (ether) work-up gave a crude product which was chromatographed on silica-gel (hexane : AcOEt = 7:1) to give 15 (445 mg, 76% from 13) as an amorphous solid. 1H-NMR δ: 0.69 (3H, s, 18-H), 0.91 (3H, d, J = 6.8 Hz, 21-H), 1.04 (3H, s, 19-H), 1.20 (9H, s, pivaloyl), 2.00 (1H, dt, J = 12.5, 2.1 Hz, 4-H), 3.89 (1H, d, J = 2.9 Hz, 6-H) 4.89 (1H, d, J = 2.4 Hz, 6-H) 5α-Cholest-5-en-3β-ol (16) 4-DHP (165 μl, 1.76 mmol) and p-TsOH·H₂O (1.6 mg) were added to a solution of 15 (433 mg, 0.883 mmol) in dry CH₂Cl₂ (2.0 ml) and the reaction mixture was stirred at room temperature for 1 h. An extractive (ether) work-up gave a crude product which was chromatographed on silica-gel (hexane : AcOEt = 7:1) to give 16 (482 mg, 85%) as an amorphous solid. 1H-NMR δ: 0.67 (3H, s, 18-H), 0.91 (3H, d, J = 6.8 Hz, 21-H), 1.05 (3H, s, 19-H), 1.20 (9H, s, pivaloyl), 2.40 (1H, dt, J = 12.5, 1.1 Hz, 4-H), 3.89 (1H, d, J = 2.9 Hz, 6-H). Anal. Calc. for C₂₃H₄₄O₂: C, 78.47; H, 11.52. Found: C, 78.25; H, 11.82.

**[2α,6β]-Pivaloyloxy-5α-cholestan-3β-ol THF Ester (17)** (17) 1H-NMR δ: 0.70 (3H, s, 18-H), 0.91 (3H, d, J = 6.8 Hz, 21-H), 1.21 (9H, s, pivaloyl), 1.22 (3H, s, 19-H), 2.11 (1H, d, J = 15.6 Hz, 4-H), 2.10 (1H, d, J = 14.7 Hz, 4-H), 2.39 (1H, dd, J = 13.3, 6.9 Hz, 2β-H), 4.84 (1H, d, J = 2.0 Hz, 6-H).

**[2α,6β]-Pivaloyloxy-5α-cholestan-3β-ol THF Ester (18)** (18) Dehydration of 17 (325 mg, 0.664 mmol) in the same manner as described for 10 gave a crude product which, upon recrystallization from MeOH, afforded 18 (309 mg, 99%) as white crystals, mp 150—153°C. 1H-NMR δ: 0.68 (3H, s, 18-H), 0.90 (3H, d, J = 6.3 Hz, 21-H), 1.03 (3H, s, 19-H), 3.50 (1H, m, 6-H), 3.62 (1H, m, 3-H), 3.79 (1H, d, J = 2.2 Hz, 6-H), 3.93 (1H, m, 6-H), 4.75 (1H, m, 2-H). Anal. Calc. for C₂₃H₄₄O₂: C, 78.47; H, 11.73. Found: C, 78.47; H, 11.73.

**[2α,6β]-Pivaloyloxy-5α-cholestan-3β-ol THF Ester (19)** 19 (19) 1H-NMR δ: 0.68 (3H, s, 18-H), 0.91 (3H, d, J = 6.6 Hz, 21-H), 1.01 (3H, s, 19-H), 3.50 (2H, m, 3-H, 6-H), 3.91 (1H, m, 6-H), 4.72 (1H, br s, 2-H), 5.35 (1H, s, 6-H). Anal. Calc. for C₂₃H₄₄O₂: C, 78.47; H, 11.73.

**[2α,6β]-Pivaloyloxy-5α-cholestan-3β-ol THF Ester (20)** (20) 1H-NMR δ: 0.68 (3H, s, 18-H), 0.91 (3H, d, J = 6.4 Hz, 21-H), 1.01 (3H, s, 19-H), 1.20 (9H, s, pivaloyl), 4.12 (1H, s, 4-H), 4.84 (1H, d, J = 2.4 Hz, 6-H). Anal. Calc. for C₂₃H₄₄O₂: C, 78.47; H, 11.72.
tection of 19 (503 mg, 1.02 mmol) in the same manner as described for 15
gave, after silica-gel chromatography, 20 (443 mg, 76%) as an amorphous
solid. 1H-NMR δ: 0.67 (3H, s, 18-H3), 0.90 (3H, d, J=6.8 Hz, 21-H3), 1.04
(3H, s, 19-H3), 1.20 (9H, s, pivaloyl), 3.49 (1H, m, 6'-Heq), 3.89 (1H, m, 6'-
Heq), 4.71 (1H, m, 2'-H), 4.89 (1H, m, 6-H). Anal. Caled for C17H33O2:
C, 77.60; H, 11.64.

[2α,3α,5α-H2]-5α-Cholestane-3β,6β,8-diol 3-THP Ether (21) Reduction
of 20 (443 mg, 0.772 mmol) in the same manner as described for 16
gave, after silica-gel chromatography, 21 (350 mg, 93%) as white crystals,
mp 149–151.5 °C (from MeOH). 1H-NMR δ: 0.68 (3H, s, 18-H3), 0.90 (3H, d,
J=6.3 Hz, 21-H3), 1.03 (3H, s, 19-H3), 3.50 (1H, m, 6'-Hax), 3.79 (1H, br s,
6-H), 3.93 (1H, m, 6'-Heq), 4.75 (1H, m, 2'-H). HR-FAB-MS m/z 491.4445
(MH+). Caled for C20H35O2: C, 77.72; H, 11.64.

[2α,3α-H2]-3β-cholesterol 3H-THP Ether (22) Dehydration of 21
(299 mg, 0.609 mmol) in the same manner as described for 17 gave, after
recrystallization from MeOH, 22 (268 mg, 93%) as white crystals, mp 149–
151.5 °C. 1H-NMR δ: 0.68 (3H, s, 18-H3), 0.91 (3H, d, J=7.1 Hz, 21-H3),
1.01 (3H, s, 19-H3), 3.50 (1H, m, 6'-Hax), 3.91 (1H, m, 6'-H), 5.35 (1H, s,

[2β,3β-H2]-Cholesterol (2c) Deprotection of 22 (268 mg, 0.566 mmol)
in the same manner as described for 18 gave, after recrystallization from
MeOH, 2c (190 mg, 86%) as white crystals, mp 149.5–151 °C. 1H-NMR δ:
0.68 (3H, s, 18-H3), 0.91 (3H, d, J=6.6 Hz, 21-H3), 1.01 (3H, s, 19-H3), 5.35
(1H, m, 6-H). 1H-NMR δ: 1.82 (2α-H), 3.50 (3α-H). Anal. Caled for
C20H35O2: C, 78.44; H, 11.93. Found: C, 83.69; H, 12.23.

Incubation with the Hairy Roots of Ajuga reptans var. atropurpurea
The hairy roots, 1200 mg, were transferred steriley into four 500 ml
flasks each containing 250 ml liquid MS medium and precultivated on a
rotary shaker (80 rpm) at 25 °C in the dark as described previously.39 On
day 14, the labeled sterol (100 mg of 2a, 2b or 2c), dissolved in acetone (1 ml)
and Tween 80 (1 ml), was added to the four flasks through a sterile
membrane filter. Incubation was continued for another 14 and the roots were
harvested. The roots (ca. 110 g wet wt) were mixed with sea sand and
CHCl3–MeOH and ground in a mortar with a pestle. The mixture was
sonicated in CHCl3–MeOH (1:1, 300 ml) for 1 h and
filtered. The residue of the tissues was sonicated once again in the same
solvent. The combined filtrate was concentrated in vacuo. The residue was
dissolved in n-BuOH, washed with brine, and concentrated to dryness. The
residue was taken up in CHCl3–MeOH (1:1, 20 ml) and the soluble part was
concentrated. Chromatography of the residue on silica-gel using a CHCl3–
MeOH gradient system give the ecdysteroid fraction (eluted with CHCl3–
MeOH, 7:1—4:1). This (ca. 60 mg) was further separated by preparative-TLC
(developed twice with CHCl3–MeOH=7:1), and finally by HPLC
(solvent, water–MeOH=1:1; flow rate, 6.0 ml/min; detector, 243 nm; retention
time 21.3 min) to give 20-hydroxyecdysone (6.0 mg from 2a, 4.1 mg from
2b and 5.0 mg from 2c).

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