Chem. Pharm. Bull. **48**(3) 344—348 (2000) Vol. 48, No. 3

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

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Received August 31, 1999; accepted November 11, 1999

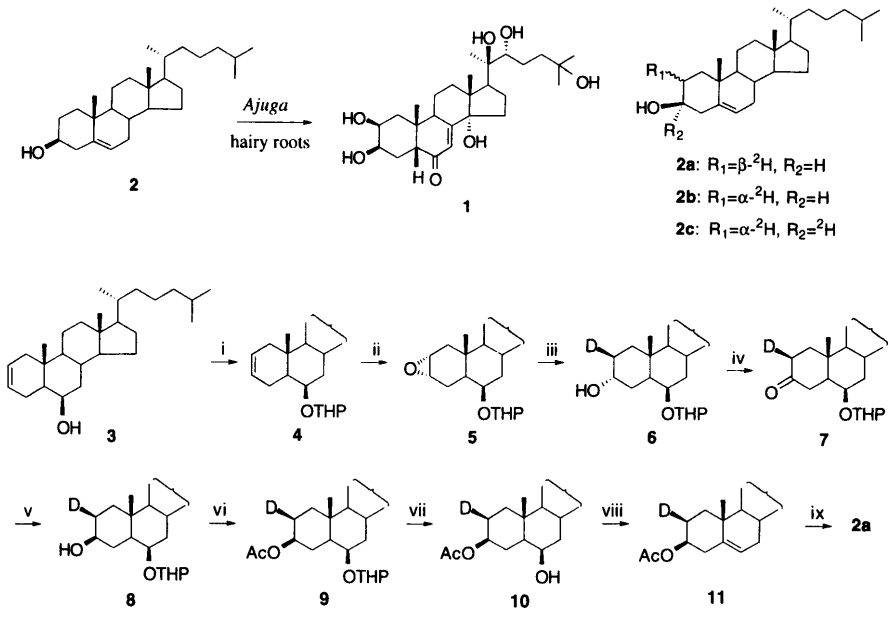
Feeding synthetic [2β-^{2}H]- and [2α-^{2}H]-cholesterols to the hairy roots of *Ajuga reptans* var. *atropurpurea* and ^{2}H-NMR analysis of the biosynthesized 20-hydroxyecdysone revealed that hydroxylation at C-2 proceeds with retention of configuration. Feeding [2α,3α-^{2}H$_2$]cholesterol followed by ^{2}H-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.[1] We previously demonstrated that the hairy roots of *Ajuga reptans* var. *atropurpurea* (Labiatae)[2] are useful for biosynthetic studies of phytoecdysteroids.[3] In this tranformed biosystem, feeding studies of ^{2}H-labeled substrates have revealed that the 3α-, 4α- and 4β-hydrogens of cholesterol are retained at their original positions after conversion to 20-hydroxyecdysone,[4] and that most of the 6-hydrogen of cholesterol migrates to the C-5 position of 20-hydroxyecdysone.[5] The metabolic fates of these hydrogens are different from those reported in the insects, *Schistocerca gregaria*[6] and *Locusta migratoria*,[7] and the fern *Polypodium vulgare*.[8] 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 ca.* 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 hydroxy 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 ^{2}H-labeled substrates, [2β-^{2}H]- and [2α-^{2}H]-cholesterols (**2a**, **2b**). Compound **2a** was synthesized according to Chart 1. 6β-Hydroxy-5α-cholest-2-ene (**3**), prepared from 3,5α-cyclocholestan-6-one in two steps (heating with LiBr in *N*,*N*-dimethylformamide (DMF)[9] and reduction with $NaBH_4$), was converted to tetrahydropyranyl (THP) ether **4** which was epoxidized to give 2α,3α-epoxide **5**, stereoselectively. Reduction of **5** with $LiAlD_4$ pro-



Reagents: i) DHP, *p*-TsOH, CH_2Cl_2 (83%), ii) *m*CPBA, Na_2CO_3, CH_2Cl_2 (80%), iii) $LiAlD_4$, THF (93%), iv) PCC, MS4A, NaOAc, CH_2Cl_2, v) $LiAlH_4$, THF (85% for 2 steps), vi) Ac_2O, Py (91%), vii) H^+, MeOH–THF (76%), viii) $POCl_3$, Py, ix) 5% KOH–MeOH, THF (85% for 2 steps).

Chart 1. Synthesis of [2β-^{2}H]cholesterol (**2a**)

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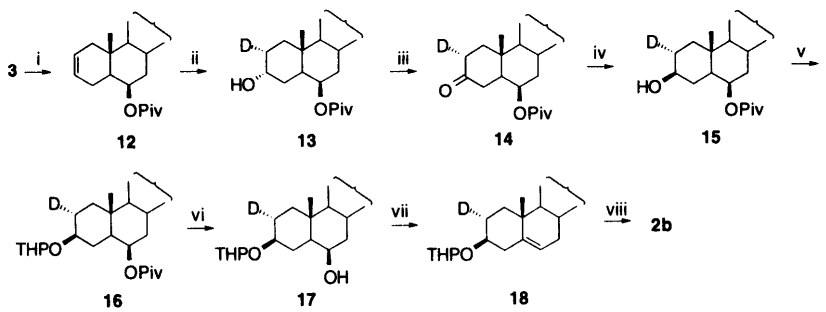


Reagents: i) PivCl, Py (74%), ii) BD_3·THF, THF (23%), iii) PCC, NaOAc, CH_2Cl_2, iv) $NaBH_4$, MeOH–THF (76% for two step), v) DHP, *p*-TsOH, CH_2Cl_2 (85%), vi) $LiAlH_4$, ether (91%), vii) $POCl_3$, Py (99%), viii) H^+, MeOH–THF (79%).

Chart 2. Synthesis of [2α-^{2}H]cholesterol (**2b**)

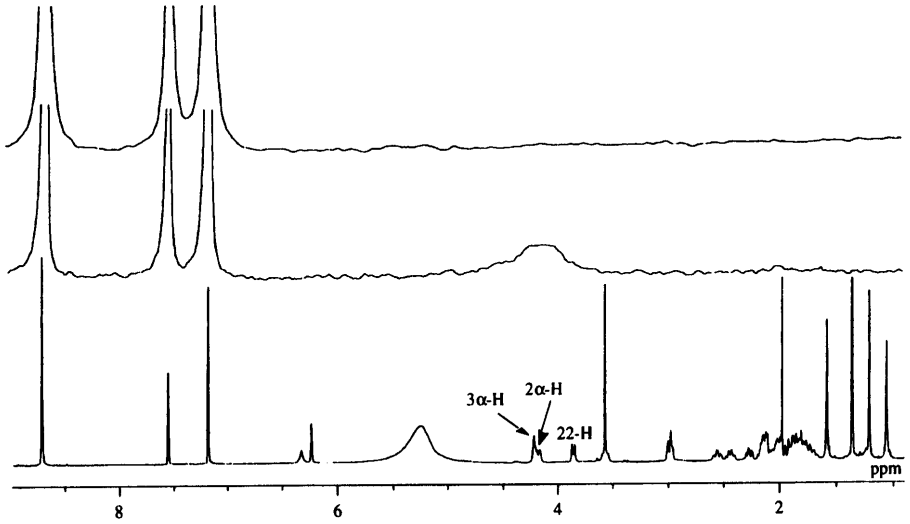


Fig. 1. ^{2}H-NMR (61 MHz, in Pyridine) Spectra of 20-Hydroxyecdysone Samples Biosynthesized from [2β-^{2}H]Cholesterol **2a** (Top) and [2α-^{2}H]Cholesterol **2b** (Middle)

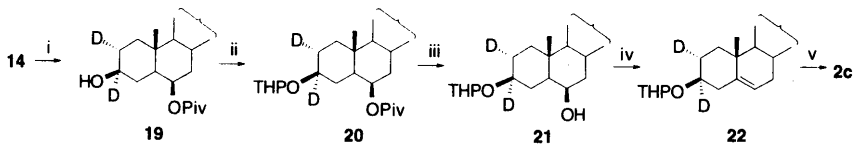
^{1}H-NMR spectrum of authentic 20-hydroxyecdysone is given at the bottom.

ceeded in an axial manner to afford [2β-^{2}H]-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 **2a**. Compound **2a** exhibited a single signal at δ 1.50 in its ^{2}H-NMR spectrum.

[2α-^{2}H]Cholesterol (**2b**) was synthesized according to Chart 2. The same starting material **3** was converted to pivaloyl ester **12**. Hydroboration of **12** with BD_3 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 ^{1}H-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 ^{2}H-NMR spectrum.

With the two isomeric ^{2}H-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 ^{2}H-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.[4] The assignment was further confirmed by ^{2}H-NMR analysis of the corresponding 2,3,22-triacetate derivative which showed a signal at δ 5.08 due to 2α-^{2}H (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.



Reagents: i) $LiAlD_4$, THF (75%), ii) DHP, *p*-TsOH, CH_2Cl_2 (76%), iii) $LiAlH_4$, ether (93%), iv) $POCl_3$, Py (93%), v) H^+, MeOH–THF (86%).

Chart 3. Synthesis of [2α,3α-$^2H_2$]cholesterol (**2c**)

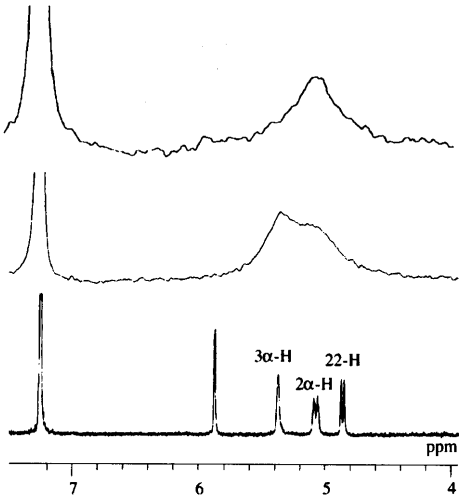


Fig. 2. ^{2}H-NMR (61 MHz, in Pyridine) Spectra of 2,3,22-Triacetate Derivatives of 20-Hydroxyecdysone Biosynthesized from [2α-^{2}H]Cholesterol **2b** (Top) and [2α,3α-$^2H_2$]Cholesterol **2c** (Middle)

^{1}H-NMR spectrum of authentic 20-hydroxyecdysone 2,3,22-triacetate is given at the bottom.

The retention of the 2α-hydrogen was shown in the above feeding studies. However, an equilibration between the 2-hydroxy compound and 2-oxo derivative, during or after the biosynthesis of 20-hydroxyecdysone, would cause partial loss of the 2α-hydrogen. Indeed, characterization of 3-oxo compounds such as 3-dehydroecdysone[10] and their possible involvement in biosynthesis[8] have been reported. In contrast, no loss of the 3α-hydrogen was reported with the plant, *Taxus baccata*.[11] A further study was, therefore, undertaken using a doubly labeled compound, [2α,3α-$^2H_2$]cholesterol (**2c**). The synthesis of **2c** is illustrated in Chart 3. Compound **14** was reduced with $LiAlD_4$ to give a [2α,3α-$^2H_2$]-derivative **19**, which was converted into **2c**, through THP ether **20**, 6-ol **21** and 5-ene **22**, in the same manner as described for the synthesis of **2b**. The ^{2}H-NMR spectrum of **2c** exhibited signals at δ 1.82 and 3.50 due to 2α-^{2}H and 3α-^{2}H, respectively.

Compound **2c** was fed to *Ajuga* hairy roots in the same manner as described above, and the resulting 20-hydroxyecdysone and its 2,3,22-triacetate derivative were analyzed by ^{2}H-NMR. The 20-hydroxyecdysone exhibited a broad ^{2}H signal at *ca.* δ 4.15 (data not shown). The ^{2}H-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 simmilar. 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-hydroxyecdysone 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*.[12] It is reported that the enzyme responsible for the C-2 hydroxylation of *L. migratoria* is a monooxygenase which is not a cytochrome P450.[13,14] We recently reported that the C-25 hydroxylation during the biosynthesis of 20-hydroxyecdysone in *Ajuga* hairy roots is not stereospecific, but proceeds both *via* retention and inversion mechanisms.[15]

Experimental

^{1}H-NMR spectra were obtained on a JEOL JNM-LA300 (300 MHz) or LA400 (400 MHz) spectrometer in $CDCl_3$ solutions and chemical shifts (δ) are reported in ppm downfield from tetramethylsilane (used as an internal reference). The signals for 26-H_3 and 27-H_3 (6H, d, J=7.5—8.1 Hz) were observed at δ 1.86—1.87 for all steroidal compounds, and these data are not described. ^{2}H-NMR spectra were recorded on a JEOL JNM-LA400 spectrometer (61 MHz for ^{2}H) in $CHCl_3$ (the signal of residual C^2HCl_3 was at δ 7.26) or in pyridine (the signal of 2-^{2}H 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 F_{254} plates (20×20 cm, 0.5 mm thick) were used for column chromatography and preparative thin-layer chromatography (TLC), respectively.

5α-Cholest-2-en-6β-ol THP Ether (4) Dihydropyran (DHP) (0.44 ml, 4.88 mmol) and *p*-toluenesulfonic acid monohydrate (*p*-TsOH·H_2O) (2 mg) were added to a solution of compound **3** (940 mg, 2.44 mmol, obtained by $NaBH_4$ reduction of 5α-cholest-2-en-6-one which was prepared from 3,5α-cyclocholestan-6-one by the method of Aburatani *et al.*[9]) in dry CH_2Cl_2 (20 ml), and the reaction mixture was stirred at room temperature for 30 min. Addition of saturated $NaHCO_3$ followed by an extractive (AcOEt) work-up gave a crude product which was chromatographed on silica-gel (hexane : AcOEt=30 : 1) to give **4** (950 mg, 83%) as a colorless oil. ^{1}H-NMR δ: 0.68 (1.3H, s, 18-H_3), 0.70 (1.7H, s, 18-H_3), 0.93 (3H, d, J=5.4 Hz, 21-H_3), 0.96 (3H, s, 19-H_3), 3.52 (1H, d, J=2.0 Hz, 6′-Hax), 3.60 (0.65H, d, J=2.7 Hz, 6-H), 3.77 (0.35H, d, J=2.6 Hz, 6-H), 3.90 (1H, m, 6′-Heq), 4.60 (0.65H, t, J=3.3 Hz, 2′-H), 4.68 (0.35H, s, 2′-H), 5.55 (1H, m, 3-H), 5.57 (1H, m, 2-H). *Anal.* Calcd for $C_{32}H_{54}O_2$: C, 81.64; H, 11.56. Found: C, 81.85; H, 11.59.

2α,3α-Epoxy-5α-cholestan-6β-ol THP Ether (5) Na_2CO_3 (160 mg) and *m*-chloroperbenzoic acid (*m*CPBA) (492 mg, 2.00 mmol) were added to a solution of **4** (940 mg, 2.00 mmol) in dry CH_2Cl_2 (6 ml) and the reaction mixture was stirred at room temperature for 30 min. An extractive (CH_2Cl_2) work-up gave a crude product which was chromatographed on silica-gel (hexane : AcOEt=20 : 1) to give **5** (781 mg, 80%) as an amorphous solid. ^{1}H-NMR δ: 0.65 (1.3H, s, 18-H_3), 0.69 (1.7H, s, 18-H_3), 0.90 (3H, d, J=5.4 Hz, 21-H_3), 0.95 (3H, s, 19-H_3), 3.11 (1H, m, 3-H), 3.24 (1H, m, 2-H), 3.49 (1H, m, 6′-Hax), 3.66 (0.65H, d, J=2.6 Hz, 6-H), 3.83 (0.35H, d, J=2.6 Hz, 6-H), 3.89 (1H, m, 6′-Heq), 4.59 (1H, m, 2′-H). *Anal.* Calcd for $C_{32}H_{54}O_3$: C, 78.96; H, 11.18. Found: C, 79.05; H, 11.46.

[2β-^{2}H]-5α-Cholestane-3α,6β-diol 6-THP Ether (6) $LiAlD_4$ (80 mg, 1.91 mmol) was added to a solution of **5** (767 mg, 1.57 mmol) in dry tetrahydrofuran (THF) (15 ml) at room temperature and the reaction mixture was stirred at 60 °C for 30 min. An extractive (ether) work-up gave a crude product which was chromatographed on silica-gel (hexane : AcOEt=4 : 1) to give **6** (716 mg, 1.46 mmol, 93%) as white crystals, mp 62—64 °C (from hexane). ^{1}H-NMR δ: 0.65 (1.3H, s, 18-H_3), 0.69 (1.7H, s, 18-H_3), 0.90 (3H, d, J=5.4 Hz, 21-H_3), 0.95 (3H, s, 19-H_3), 3.48 (1.35H, m, 6-H, 6′-Hax), 3.62 (0.65H, d, J=2.6 Hz, 6-H), 3.86 (1H, m, 6′-Heq), 4.09 (1H, m, 3-H), 4.56 (0.65H,

br s, 2′-H), 4.63 (0.35H, br s, 2′-H). *Anal.* Calcd for $C_{32}H_{55}{}^2HO_3$: C, 78.47; H+^{2}H, 11.52. Found: C, 78.47; H, 11.94.

[2β-^{2}H]-5α-Cholestane-3β,6β-diol 6-THP Ether (8) NaOAc (48 mg, 0.58 mmol), pyridinium chlorochromate (PCC) (624 mg, 2.90 mmol) and molecular sieves 4A (MS4A) (powder, 6.0 g) were added to a solution of **6** (711 mg, 1.45 mmol) in dry CH_2Cl_2 (20 ml) and the reaction mixture was stirred at room temperature for 10 min. Dry ether was added and the mixture was filtered through a short column of Florisil. Concentration of the filtrate gave **7** (721 mg) as a colorless oil. ^{1}H-NMR δ: 0.70 (1.3H, s, 18-H_3), 0.72 (1.7H, s, 18-H_3), 0.90 (3H, d, J=5.4 Hz, 21-H_3), 2.71 (0.65H, dd, J=14.5 Hz, 4β-H), 2.94 (0.35H, dd, J=14.5 Hz, 4β-H), 3.51 (1.65H, m, 6-H, 6′-Hax), 3.68 (0.35H, d, J= 2.6 Hz, 6-H), 3.83 (1H, m, 6′-Heq), 4.55 (0.65H, br s, 2′-H), 4.67 (0.35H, br s, 2′-H).

$LiAlH_4$ (86 mg, 2.27 mmol) was added to a solution of **7** (721 mg) in THF (11 ml) and the reaction mixture was stirred at room temperature for 20 min. An extractive (ether) work-up gave a crude product which was chromatographed on silica-gel (hexane : AcOEt=3 : 1) to afford **8** (601 mg, 85% from **6**) as white crystals, mp 100—104 and 120—124 °C (from hexane). ^{1}H-NMR δ: 0.70 (1.3H, s, 18-H_3), 0.72 (1.7H, s, 18-H_3), 0.90 (3H, d, J=5.4 Hz, 21-H_3), 2.28 (1H, br s, 2α-H), 3.49 (1H, m, 3-H), 3.57 (0.65H, J=1.9 Hz, 6-H), 3.63 (1H, m, 6′-H), 3.70 (0.35H, d, J= 2.6 Hz, 6-H), 3.87 (1H, m, 6′-Heq), 4.59 (0.65H, br s, 2′-H), 4.69 (0.35H, br s, 2′-H). HR-FAB-MS *m/z* 472.4261 ($MH^+ - H_2O$). Calcd for $C_{32}H_{53}{}^2HO_2$, 472.4265.

[2β-^{2}H]-5α-Cholestane-3β,6β-diol 3-Acetate (10) Ac_2O (2.0 ml) was added to a solution of **8** (601 mg, 1.22 mmol) in pyridine (2.0 ml), and the reaction mixture was stirred at room temperature overnight. Addition of ice chips and an extractive (ether) work-up gave a crude product **9** (597 mg, 91%) as an amorphous solid. ^{1}H-NMR δ: 0.70 (1.3H, s, 18-H_3), 0.72 (1.7H, s, 18-H_3), 0.90 (3H, d, J=5.4 Hz, 21-H_3), 2.04 (3H, s, Ac), 3.48 (1H, m, 6′-Hax), 3.55 (0.6H, d, J=2.0 Hz, 6-H), 3.70 (0.4H, d, J=2.6 Hz, 6-H), 3.85 (1H, m, 6′-Heq), 4.56 (0.6H, br s, 2′-H), 4.65 (0.4H, br s, 2′-H).

MeOH (5.0 ml) and a drop of 2 N HCl were added to a solution of **9** (590 mg) in THF (3.0 ml), and the reaction mixture was stirred at room temperature for 6 h. An extractive (ether) work-up gave a crude product which was chromatographed on silica-gel (hexane : AcOEt=4 : 1) to give **10** (415 mg, 76%) as white crystals, mp 140—143 °C (from MeOH) (lit.[16] 141—142 °C for non-labeled sample). ^{1}H-NMR δ: 0.69 (3H, s, 18-H_3), 0.91 (3H, d, J=6.8 Hz, 21-H_3), 1.04 (3H, s, 19-H), 2.04 (3H, s, Ac), 3.80 (1H, m, 6-H), 3.73 (1H, m, 6-H). *Anal.* Calcd for $C_{29}H_{49}{}^2HO_3$: C, 77.80; H+^{2}H, 11.26. Found: C, 77.89; H, 11.67.

[2β-^{2}H]Cholesterol (2a) $POCl_3$ (103 μl, 1.13 mmol) was added to a solution of **10** (337 mg, 0.753 mmol) in pyridine (4.0 ml), and the reaction mixture was stirred at room temperature for 3 h. An extractive (ether) work-up gave a crude product **11** (412 mg) as white crystals, mp 117—119 °C. ^{1}H-NMR δ: 0.67 (3H, s, 18-H_3), 0.91 (3H, d, J=5.4 Hz, 21-H_3), 1.02 (3H, s, 19-H_3), 2.17 (1H, d, J=8.6 Hz, 4β-H), 5.37 (1H, d, J=5.3 Hz, 6-H).

5% MeOH/KOH (4.0 ml) was added to a solution of **11** (412 mg) in THF (2.0 ml), and the mixture was stirred at room temperature overnight. An extractive (ether) work-up gave a crude product which was chromatographed on silica-gel (hexane : AcOEt=3 : 1) and then recrystallized from MeOH to give **2a** (220 mg, 75% from **10**) as white crystals, mp 149—152.5 °C. ^{1}H-NMR δ: 0.68 (3H, s, 18-H_3), 0.91 (3H, d, J=6.6 Hz, 21-H_3), 1.00 (3H, s, 19-H_3), 3.53 (1H, m, 3-H), 5.35 (1H, m, 6-H). ^{2}H-NMR δ: 1.51 (2β-^{2}H). *Anal.* Calcd for $C_{27}H_{45}{}^2HO$: C, 83.65; H+^{2}H, 11.96. Found: C, 83.45; H, 12.30.

5α-Cholest-2-en-6β-ol Pivaloate (12) Pivaloyl chloride (6.6 ml, 54.7 mmol) was added to a solution of **3** (3.02 g, 7.81 mmol) in pyridine (10 ml) at 0 °C, and the reaction mixture was stirred at 60 °C over night. An extractive (ether) work-up gave a crude product which was chromatographed on silica-gel (hexane : AcOEt=20 : 1) to give **12** (2.71 g, 74%) as white crystals, mp 78—78.5 °C (from MeOH). ^{1}H-NMR δ: 0.67 (3H, s, 18-H_3), 0.91 (3H, d, J=6.6 Hz, 21-H_3), 0.98 (3H, s, 19-H_3), 1.21 (9H, s, pivaloyl), 4.96 (1H, m, 6-H), 5.60 (2H, m, 2-H, 3-H). *Anal.* Calcd for $C_{32}H_{54}O_2$: C, 81.64; H+^{2}H, 11.56. Found: C, 81.39; H, 11.64.

[2α-^{2}H]-6β-Pivaloyloxy-5α-cholestan-3α-ol (13) $BF_3 \cdot Et_2O$ (0.74 ml, 6.01 mmol) was added to a solution of **12** (2.49 mg, 5.28 mmol) and $NaBD_4$ (188 mg, 4.49 mmol) in THF (20 ml) dropwise at 0 °C, and the reaction mixture was stirred at room temperature for 30 min. Water (2.0 ml), 3 N NaOH (2.0 ml) and 30% H_2O_2 (2.0 ml) was added and the mixture was stirred for 30 min. An extractive (AcOEt) work-up gave a crude product which was chromatographed on silica-gel (hexane : AcOEt=6 : 1) to give **13** (584 mg, 23%) as an amorphous solid. ^{1}H-NMR δ: 0.68 (3H, s, 18-H_3), 0.90 (3H, d, J=6.4 Hz, 21-H_3), 1.01 (3H, s, 19-H_3), 1.20 (9H, s, pivaloyl), 4.12 (1H, s, 3-H), 4.84 (1H, d, J=2.4 Hz, 6-H). *Anal.* Calcd for $C_{32}H_{55}{}^2HO_3$: C, 78.47; H+^{2}H, 11.52. Found: C, 78.49; H, 11.72.

The hydroboration–oxidation reaction using $NaBH_4$ in place of $NaBD_4$ afforded four non-labeled products in order of increasing polarity in the ratio of 51 : 11 : 12 : 26; 5α-cholestane-2β,6β-diol 6-pivaloyl ester, ^{1}H-NMR δ: 0.67 (3H, s, 18-H_3), 0.91 (3H, d, J=6.4 Hz, 21-H_3), 1.20 (9H, s, pivaloyl), 1.25 (3H, s, 19-H_3), 4.14 (1H, s, 2-H), 4.94 (1H, d, J=2.9 Hz, 6-H); 5α-cholestane-3α,6β-diol 6-pivaloyl ester, ^{1}H-NMR δ: 0.67 (3H, s, 18-H_3), 0.91 (3H, d, J=6.3 Hz, 21-H_3), 1.01 (3H, s, 19-H_3), 1.20 (9H, s, pivaloyl), 4.12 (1H, s, 3-H), 4.84 (1H, d, J=2.0 Hz, 6-H); 5α-cholestane-2α,6β-diol 6-pivaloyl ester, ^{1}H-NMR δ: 0.67 (3H, s, 18-H_3), 0.91 (3H, d, J=6.3 Hz, 21-H_3), 1.02 (3H, s, 19-H_3), 1.19 (9H, s, pivaloyl), 3.80 (1H, m, 2-H), 4.95 (1H, d, J=2.5 Hz, 6-H); 5α-cholestane-3β-,6β-diol 6-pivaloyl ester, ^{1}H-NMR δ: 0.67 (3H, s, 18-H_3), 0.91 (3H, d, J=6.4 Hz, 21-H_3), 1.05 (3H, s, 19-H_3), 1.20 (9H, s, pivaloyl), 3.64 (1H, m, 3-H), 4.89 (1H, d, J=2.4 Hz, 6-H).

[2α-^{2}H]-6β-Pivaloyloxy-5α-cholestan-3β-ol (15) Oxidation of **13** (584 mg, 1.19 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. ^{1}H-NMR δ: 0.70 (3H, s, 18-H_3), 0.91 (3H, d, J=6.8 Hz, 21-H_3), 1.21 (9H, s, pivaloyl), 1.22 (3H, s, 19-H_3), 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 $NaBH_4$ (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. ^{1}H-NMR δ: 0.69 (3H, s, 18-H_3), 0.91 (3H, d, J=6.8 Hz, 21-H_3), 1.04 (3H, s, 19-H_3), 1.20 (9H, s, pivaloyl), 2.00 (1H, dt, J=12.5, 2.1 Hz, 4-H), 3.65 (1H, m, 3-H), 4.89 (1H, d, J=2.9 Hz, 6-H). *Anal.* Calcd for $C_{32}H_{55}{}^2HO_3$: C, 78.47; H+^{2}H, 11.52. Found: C, 78.25; H, 11.82.

[2α-^{2}H]-6β-Pivaloyloxy-5α-cholestan-3β-ol THP Ether (16) DHP (165 μl, 1.76 mmol) and *p*-TsOH · H_2O (1.6 mg) were added to a solution of **15** (433 mg, 0.883 mmol) in dry CH_2Cl_2 (2.0 ml) and the reaction mixture was stirred at room temperature for 1 h. An extractive work-up (ether) gave a crude product which was chromatographed on silica-gel (hexane : AcOEt=7 : 1) to afford **16** (428 mg, 85%) as an amorphous solid. ^{1}H-NMR δ: 0.67 (3H, s, 18-H_3), 0.91 (3H, d, J=6.8 Hz, 21-H_3), 1.05 (3H, s, 19-H), 1.20 (9H, s, pivaloyl), 3.49 (1H, m, 6′-Hax), 3.62 (1H, m, 3-H), 3.89 (1H, m, 6′-Heq), 4.71 (1H, m, 2′-H), 4.89 (1-H, m, 6-H). *Anal.* Calcd for $C_{37}H_{63}{}^2HO_4$: C, 77.43; H+^{2}H, 11.42. Found: C, 77.36; H, 11.71.

[2α-^{2}H]-5α-Cholestane-3β,6β-diol 3-THP Ether (17) $LiAlH_4$ (20 mg, 0.527 mmol) was added to a solution of **16** (421 mg, 0.733 mmol) in dry ether (10 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) and recrystallized from MeOH to afford **17** (325 mg, 91%) as white crystals, mp 150—152 °C. ^{1}H-NMR δ: 0.68 (3H, s, 18-H_3), 0.90 (3H, d, J=6.3 Hz, 21-H_3), 1.03 (3H, s, 19-H_3), 3.50 (1H, m, 6′-Hax), 3.65 (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.* Calcd for $C_{32}H_{55}{}^2HO_3$: C, 78.47; H+^{2}H, 11.73. Found: C, 78.30; H, 11.88.

[2α-^{2}H]Cholest-5-en-3β-ol THP Ether (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. ^{1}H-NMR δ: 0.68 (3H, s, 18-H_3), 0.91 (3H, d, J=7.1 Hz, 21-H_3), 1.01 (3H, s, 19-H_3), 3.50 (2H, m, 3-H, 6′-Hax), 3.91 (1H, m, 6′-H), 4.72 (1H, br s, 2′-H), 5.35 (1H, s, 6-H). *Anal.* Calcd for $C_{32}H_{53}{}^2HO_2$: C, 81.47; H+^{2}H, 11.54. Found: C, 81.57; H, 11.94.

[2α-^{2}H]Cholesterol (2b) 2 N HCl (100 μl) was added to a solution of **18** (306 mg, 0.649 mmol) in THF (7.0 ml) and MeOH (5.0 ml), and the reaction mixture was stirred at room temperature for 30 min. An extractive (ether) work-up and recrystallization of the crude product from MeOH gave **2b** (197 mg, 79%) as white crystals, mp 148—151 °C. ^{1}H-NMR δ: 0.68 (3H, s, 18-H_3), 0.91 (3H, d, J=6.6 Hz, 21-H_3), 1.01 (3H, s, 19-H_3), 3.51 (1H, m, 3-H), 5.35 (1-H, m, 6-H). ^{2}H-NMR δ: 1.83 (2α-^{2}H). *Anal.* Calcd for $C_{27}H_{45}{}^2HO$: C, 83.65; H+^{2}H, 11.96. Found: C, 83.35; H, 12.17.

[2α,3α-$^2H_2$]-6β-Pivaloyloxy-5α-cholestan-3β-ol (19) $LiAlD_4$ (32 mg, 0.772 mmol) was added to a solution of **14** (678 mg, 1.39 mmol) in THF (10 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 **19** (437 mg, 75%) as an amorphous solid. ^{1}H-NMR δ: 0.69 (3H, s, 18-H_3), 0.91 (3H, d, J=6.8 Hz, 21-H_3), 1.04 (3H, s, 19-H_3), 1.20 (9H, s, pivaloyl), 2.00 (1H, dt, J=12.5, 2.1 Hz, 4-H), 4.89 (1H, d, J=2.9 Hz, 6-H). *Anal.* Calcd for $C_{32}H_{54}{}^2H_2O_3$: C, 78.31; H+^{2}H, 11.50. Found: C, 78.24; H, 12.94.

[2α,3α-$^2H_2$]-6β-Pivaloyloxy-5α-cholestan-3β-ol THP Ether (20) Pro-

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. ^{1}H-NMR δ: 0.67 (3H, s, 18-H$_3$), 0.90 (3H, d, J=6.8 Hz, 21-H$_3$), 1.04 (3H, s, 19-H$_3$), 1.20 (9H, s, pivaloyl), 3.49 (1H, m, 6′-Hax), 3.89 (1H, m, 6′-Heq), 4.71 (1H, m, 2′-H), 4.89 (1-H, m, 6-H). *Anal.* Calcd for $C_{37}H_{62}{}^2H_2O_4$: C, 77.30; H+^{2}H, 11.22. Found: C, 77.20; H, 11.64.

[2α,3α-$^2H_2$]-5α-Cholestane-3β,6β-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). ^{1}H-NMR δ: 0.68 (3H, s, 18-H$_3$), 0.90 (3H, d, J=6.3 Hz, 21-H$_3$), 1.03 (3H, s, 19-H$_3$), 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* 491.4454 (MH$^+$). Calcd for $C_{32}H_{55}{}^2H_2O_3$; 491.4433.

[2α,3α-$^2H_2$]Cholest-5-en-3β-ol 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. ^{1}H-NMR δ: 0.68 (3H, s, 18-H$_3$), 0.91 (3H, d, J=7.1 Hz, 21-H$_3$), 1.01 (3H, s, 19-H$_3$), 3.50 (1H, m, 6′-Hax), 3.91 (1H, m, 6′-H), 5.35 (1H, s, 6-H). *Anal.* Calcd for $C_{32}H_{52}{}^2H_2O_2$: C, 81.29; H+^{2}H, 11.51. Found: C, 81.37; H, 12.00.

[2α,3α-$^2H_2$]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. ^{1}H-NMR δ: 0.68 (3H, s, 18-H$_3$), 0.91 (3H, d, J=6.6 Hz, 21-H$_3$), 1.01 (3H, s, 19-H$_3$), 5.35 (1-H, m, 6-H). ^{2}H-NMR δ: 1.82 (2α-^{2}H), 3.50 (3α-^{2}H). *Anal.* Calcd for $C_{27}H_{44}{}^2H_2O$: C, 83.44; H+^{2}H, 11.93. Found: C, 83.69; H, 12.23.

Incubation with the Hairy Roots of *Ajuga reptans* var. *atropurpurea* The hairy roots, maintained in solid Murashige–Skoog (MS) medium, were transferred sterilely into four 500 ml flasks each containing 250 ml liquid MS medium and preincubated on a rotary shaker (80 rpm) at 25 °C in the dark as described previously.[3] 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 $CHCl_3$–MeOH and ground in a mortar with a pestle. The mixture was sonicated in $CHCl_3$–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 $CHCl_3$–MeOH (1 : 1, 20 ml) and the soluble part was concentrated. Chromatography of the residue on silica-gel using a $CHCl_3$–MeOH gradient system give the ecdysteroid fraction (eluted with $CHCl_3$–MeOH, 7 : 1—4 : 1). This (*ca.* 60 mg) was further separated by preparative-TLC (developed twice with $CHCl_3$: 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**).

Acknowledgments We are grateful to Dr. T. Matsumoto, Daicel Chemical Industries Ltd., for providing *Ajuga* hairy root cultures. This work was partially supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture.

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