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Biosynthesis of 20-hydroxyecdysone in *Ajuga* hairy roots: the possibility of 7-ene introduction at a late stage

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Abstract

Administration of [3α-^{2}H]-3β-hydroxy-5β-cholestan-6-one to hairy roots of *Ajuga reptans* var. *atropurpurea* followed by ^{2}H-NMR spectroscopic analysis of the resulting 20-hydroxyecdysone so formed revealed that the substrate was efficiently incorporated into the latter. Additionally, [5β,7α,7β-$^2H_3$]-2β,3β-dihydroxy-5β-cholestan-6-one was converted into 20-hydroxyecdysone. These findings clearly indicate that *Ajuga* hairy roots are capable of introducing a double bond at the 7-position at a late stage of 20-hydroxyecdysone biosynthesis, suggesting the possibility of an alternative biosynthetic pathway which does not involve 7-dehydrocholesterol as an obligatory intermediate. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: 20-Hydroxyecdysone; Ecdysteroid; Biosynthesis; 3β-Hydroxy-5β-cholestan-6-one; *Ajuga reptans* var. *atropurpurea*; Labiatae

1. Introduction

20-Hydroxyecdysone (**1**), a molting hormone of arthropods, is also distributed in the plant kingdom, being biosynthesized from cholesterol (**2**) in both insects and in plants (Koolman, 1990; Grieneisen, 1994). However, it has been proposed that the mechanism of the construction of the characteristic 5β-H-7-en-6-one structure is different between insects and plants (Davis, Lockley, Boid, Rees & Goodwin, 1980; Davis, Dinan, Lockley, Rees & Goodwin, 1981; Nagakari et al., 1994b). It is generally accepted that 7-dehydrocholesterol (**3**) is derived from cholesterol in insects (Milner, Nali, Gibson & Rees, 1986; Sakurai, Yonemura, Fujimoto, Hata & Ikekawa, 1986), whereas its presence in plants remains to be determined. We recently reported the conversion of 7-dehydrocholesterol in to 20-hydroxyecdysone in hairy roots of *Ajuga reptans* var. *atropurpurea* (Labiatae) (Ohyama, Kushiro, Nakamura & Fujimoto, 1999). On a relative topic, Adler et al. reported a precursor-product relationship between lathosterol (cholest-7-en-3β-ol) and 20-hydroxyecdysone in spinach leaves (Adler & Grebenok, 1995), and the conversion of lathosterol into 20-hydroxyecdysone was subsequently also demonstrated in *Ajuga* hairy roots (Ohyama et al., 1999). In the later investigation, it was suggested that lathosterol might be transformed into 7-dehydrocholesterol. These data thus appeared to support the intermediacy of 7-dehydrocholesterol during 20-hydroxyecdysone biosynthesis in plants (Scheme 1, Path A). However, an alternative biosynthetic pathway which involved a simple transformation of cholesterol into 3β-hydroxycholestan-6-one (**4** for 5β-H and **5** for 5α-H) could not be eliminated. In this regard, the reported isolation of 7,8-dihydroecdysteroids from plants was of interest to us (Faux, Galbraith, Horn, Middleton & Thomson, 1970; Pomilio, González & Eceizabarrena, 1996; Choi, Kim & Choi, 1999). Additionally, compound **5** had been isolated from the plant, *Mandevilla pentlandiana* as well (Cabrera, Palermo, Seldes, Gros & Oberti, 1991) and the involvement of a 5α-H-6-oxo sterol (7,8-dihydro form) in the biosynthesis of a brassinolide, had also been

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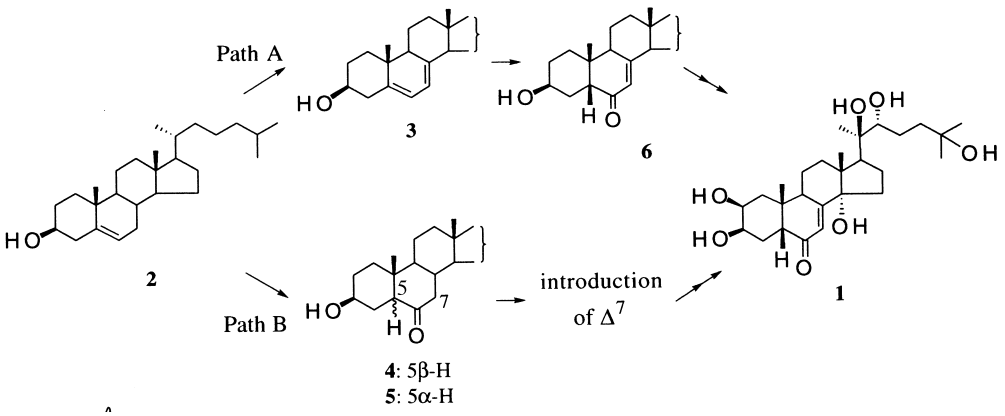
reported (Suzuki et al., 1995). Taken together, these data suggested a capability in plants for transforming a Δ^5-double bond into a 5α-H-6-one. Moreover, 3β-hydroxy-5α-cholestan-6-one (**5**) has been characterized as a metabolite derived from [^{14}C]-cholesterol when administered to prothoracic glands of the silkworm, *Bombyx mori* (Sakurai, 1977); however it was apparently not converted into ecdysteroids in *Schistocerca gregaria* (Milner et al., 1986). These observations, therefore, prompted us to investigate the possibility of the formation of a 6-oxo structure prior to the introduction of the Δ^7-double bond; in other words, to examine the possible involvement of either the 5β or 5α-isomers of 3β-hydroxycholestan-6-one. We describe here the conversion of 3β-hydroxy-5β-cholestan-6-one (**4**) and 2β,3β-dihydroxy-5β-cholestan-6-one (**7**) into 20-hydroxyecdysone (**1**) with *Ajuga* hairy roots.

2. Results and discussion

The incorporation of potential precursors into 20-hydroxyecdysone (**1**) can be examined by administering ^{2}H-labeled substrates to *Ajuga* hairy roots followed by ^{2}H-NMR spectroscopic analysis of the resulting 20-hydroxyecdysone (**1**) as previously described (Nagakari et al., 1994b; Ohyama et al., 1999). We first examined whether 3β-hydroxy-5β-cholestan-6-one (**4**) and 3β-hydroxy-5α-cholestan-6-one (**5**) were converted into 20-hydroxyecdysone. Thus, the requisite ^{2}H-labeled compounds, [3α-^{2}H]-3β-hydroxy-5β-cholestan-6-one (**4a**) and [3α-^{2}H]-3β-hydroxy-5α-cholestan-6-one (**5a**), were prepared according to the published method (Julia, Neuville & Kévorkian, 1964) from [3α-^{2}H]-3β-cholesterol 5,6-epoxide. The structures of **4a** and **5a** were fully characterized by comparison of ^{1}H-NMR and other spectroscopic evidence to those previously reported (Sakurai, 1977).

Compound **4a** was administered to *Ajuga* hairy roots and 20-hydroxyecdysone (**1**) was isolated as previously described (Nagakari, Kushiro, Matsumoto, Tanaka, Kakinuma & Fujimoto, 1994a; Ohyama et al., 1999). The ^{2}H-NMR spectrum of the biosynthesized 20-hydroxyecdysone (**1**) (Fig. 1, spectrum A) exhibited a strong ^{2}H-signal at δ 4.18 assignable to 3α-^{2}H of **1**, indicating that **4a** was converted into **1**. A similar administration of the 5α-H epimer **5a** resulted in a negligible conversion into **1**, as can be seen in Fig. 1 (spectrum B). The marked difference between the observations made with compounds **4a** and **5a** agrees with previous findings with the corresponding 7-ene compounds; that is, 3β-hydroxy-5β-cholest-7-en-6-one (**6**), but not its 5α-isomer, was converted to 20-hydroxyecdysone (**1**) in *Ajuga* hairy roots (Nagakari et al., 1994b). These finding thus seemed to rule out the possibility of any involvement of the 5α-isomer **5** in 20-hydroxyecdysone (**1**) biosynthesis in *Ajuga* hairy roots.

It has also been reported that 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one is converted into **1** in the plant, *Achyranthes fauriei* (Tomita & Sakurai, 1974). Thus, 2β,3β-dihydroxy-5β-cholestan-6-one (**7**), 3β,14α-dihydroxy-5β-cholestan-6-one and 2β,3β,14α-trihydroxy-5β-cholestan-6-one might also serve as intermediates in the biosynthesis of **1**. Since deuterium labeled 2β,3β-dihydroxy-5β-cholest-7-en-6-one, but not 3β,14α-dihydroxy-5β-cholest-7-en-6-one, can be converted into 20-hydroxyecdysone (**1**) in *Ajuga* hairy roots (Ohyama and Fujimoto, unpublished data), 2β,3β-dihydroxy-5β-cholestan-6-one **7** was next evaluated is a potential substrate. Thus, its $^2H_3$ labeled derivative with deuterium atoms at the C-5 and C-7 positions (**7a**) was synthesized by alkaline equilibration (MeOD/NaOH) with concomitant hydrolysis of the known 2β,3β-diacetoxy-5α-cholestan-6-one (Wiechert et al., 1966); the location and content of the deuterium atoms in **7a** was determined to be 85, 85 and 44% at C-5, C-7β and C-7α positions, respectively, on the basis of ^{1}H- and ^{2}H-



Scheme 1. Proposed biosynthetic routes to 20-hydroxyecdysone (**1**) in *Ajuga* hairy roots. Path A: a route involving 7-dehydrocholesterol (**3**). Path B: hypothetical pathway via 3β-hydroxycholestan-6-one.

NMR spectral analyses. EI-MS analysis also indicated that the dideuterio-compound was the most abundant species (49%) accompanied with tri-deuterio (30%) and mono-deuterio (21%) species.

Compound **7a** was next administered to *Ajuga* hairy roots and 20-hydroxyecdysone (**1**) was again isolated as described above. Its ^{2}H-NMR spectrum (Fig. 1, spectrum C) showed two peaks at δ 2.93 and δ 6.25 in a ratio of ca. 2:1 due to 5β-^{2}H and 7-^{2}H, respectively, and hence these results clearly indicated that compound **7a** was converted into 20-hydroxyecdysone (**1**). This further substantiated that a substrate possessing a 5β-H-6-one structure could be dehydrogenated at the C-7 position to furnish 20-hydroxyecdysone (**1**). Since the observed ratio (ca. 2:1) of the remaining deuterium atoms at the C-5 and C-7 postion of 20-hydroxyecdysone (**1**) was essentially identical with that (85:44) of the 5-^{2}H and 7α-^{2}H labelling in the substrate **7a**, it can be concluded that the 7β-hydrogen is stereospecifically eliminated during the conversion of **7a** into 20-hydroxyecdysone (**1**). A *syn*-elimination mechanism (elimination of 7β and 8β-hydrogens) during Δ^{7}-double bond formation is consistent with that described for conversion of cholesterol into ecdysone and 20-hydroxyecdysone (**1**) in insects and plants respectively (Cook, Lloyd-Jones, Rees & Goodwin, 1973). The ^{1}H-NMR spectrum of 20-hydroxyecdysone (**1**) is shown in Fig. 1, spectrum D for comparative purposes.

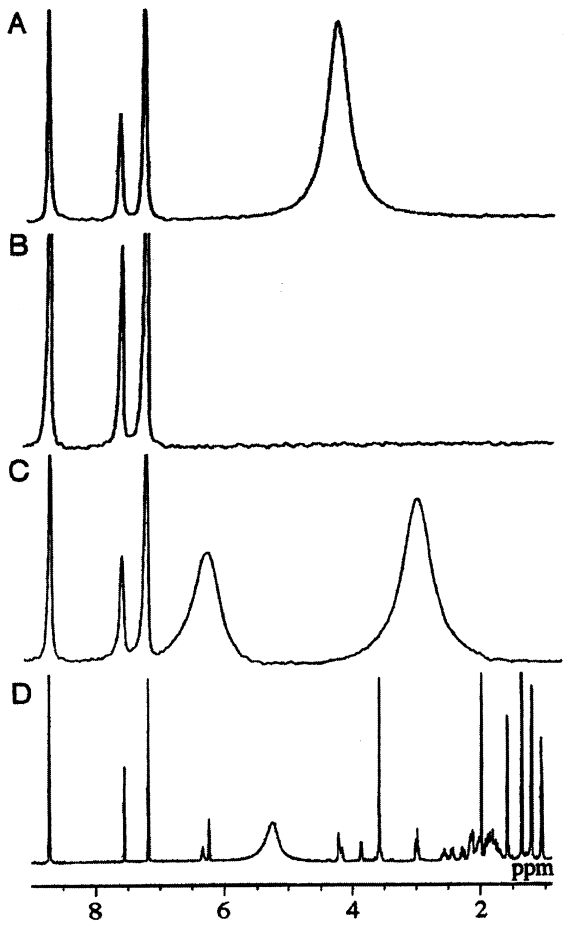
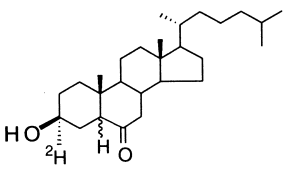
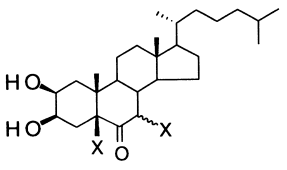


Fig. 1. ^{2}H-NMR spectra (61 MHz, pyridine) of 20-hydroxyecdysone (**1**) samples. A: derived from [3α-^{2}H]-3β-hydroxy-5β-cholestan-6-one (**4a**); B: derived from [3α-^{2}H]-3β-hydroxy-5α-cholestan-6-one (**5a**); C: derived from [5β,7α,7β-^{2}H$_3$]-2β,3β-dihydroxy-5β-cholestan-6-one (**7a**); and D: ^{1}H-NMR spectrum of authentic natural abundance sample.

In summery, in the present study, we have demonstrated that two precursors having 7,8-dihydro-6-one structures (**4** and **7**) can be incorporated into 20-hydroxyecdysone (**1**) in *Ajuga* hairy roots, thus suggesting a biosynthetic route to 20-hydroxyecdysone (**1**) which involves compound **4** (Path B in Scheme 1). Alternatively, it can be considered that compounds **4** and **7** were converted into 20-hydroxyecdysone (**1**) via an enzyme which introduces a Δ^{7}-double bond via a 6-oxo-sterol. However, to our knowledge, there are no reports on the conversion of cholesterol into compound **4**. Accordingly, the biological significance of the present findings must await demonstration of a substrate-product relationship between cholesterol and compound **4**. It should be noted, however, that the H-6 proton of cholesterol must migrate to the C-5 position of compound **4** during such a transformation (Fujimoto, Kushiro & Nakamura, 1997). Preliminary administration studies demonstrated that ^{2}H-labeled cholesterol 5α,6α-epoxide and 5β-cholestan-3β,6β-diol were, however, not incorporated into 20-hydroxyecdysone (**1**) (Fujimoto, Hyodo, unpublished results).



4a: 5β-H
5a: 5α-H



7: X=H
7a: X=^{2}H

3. Experimental

3.1. General

^{1}H- and ^{13}C-NMR (400 and 100 MHz, respectively) spectra were acquired on a JEOL Lambda-400 spectrometer in $CDCl_3$ solutions, with chemical shifts (δ) expressed in reference to TMS signal (δ 0.0) for both ^{1}H and solvent signals (δ 77.0) for ^{13}C. ^{2}H-NMR (61 MHz) spectra were obtained on the same instrument in $CHCl_3$ solutions with chemical shifts referenced to δ 7.26 for $CHCl_3$. ^{2}H-NMR spectra for 20-hydroxyecdy-

sone (**1**) were recorded in pyridine solution and referenced to δ 7.19 for 2,5-H_2 of pyridine. EIMS spectra (70 eV) were recorded with a JEOL JMS-AX505HA spectrometer. [3-^{2}H]Cholesterol, mp 146–147°C, was prepared in a two-step sequence (Jones oxidation followed by $LiAlD_4$ reduction) from cholesterol. 2β,3β-Diacetoxy-5α-cholestan-6-one, 192–194°C, was prepared by the published method (Wiechert et al., 1966).

3.2. *[3-^{2}H]-3β-Hydroxy-5β-cholestan-6-one (**4a**) and [3-^{2}H]-3β-Hydroxy-5α-cholestan-6-one (**5a**)*

m-Chloroperbenzoic acid (401 mg, 2.33 mM) was added to a solution of **2** (600 mg, 1.49 mM) in dry CH_2Cl_2 (20 ml) at 0°C and the mixture was stirred for 30 min. Addition of sat. aq. Na_2CO_3 and subsequent extraction (Et_2O) gave [3α-^{2}H]-5,6-epoxycholestanol (566 mg, 94%) as a mixture of stereoisomers (5α,6α:5β,6β = 5:1). Ethyl bromoacetate (210 μl, 1.90 mM) and activated Zn (167 mg, 2.55 mmol) (Julia et al., 1964) was added to a solution of the epoxide (500 mg, 1.24 mM) in dry benzene (2.0 ml) and the mixture was heated at reflux for 2.5 h under N_2 atmosphere. Sat. aq. NH_4Cl and ether were added and the ether layer was washed with brine, dried over Na_2SO_4 and concentrated to dryness. The residue was subjected to chromatography on a silica gel Lobar column with hexane–AcOEt (3:1) as eluent to give **4a** (148 mg, 30%) as a white solid, mp 113–114.5°C. ^{1}H-NMR δ: 0.66 (*s*, 18-H_3), 0.86 (*d*, J = 6.6 Hz, 26-H_3, 27-H_3), 0.87 (*s*, 19-H_3), 0.92 (*d*, J = 6.6 Hz, 21-H_3), 1.96 (*t*, J = 13.5 Hz, 4-Heq), 2.47 (*dd*, J = 12.7, 4.14 Hz, 5-H). ^{2}H-NMR δ: 4.12 (3α-^{2}H). ^{13}C-NMR δ: 11.97 (C-18), 18.64 (C-21), 21.17 (C-11), 22.54 (C-26), 22.79 (C-27), 23.77 (C-19), 23.79 (C-23), 23.97 (C-15), 27.09 (C-2), 27.99 (C-25), 28.11 (C-16), 28.64 (C-1), 32.89 (C-4), 35.68 (C-20), 36.08 (C-22), 36.84 (C-8), 38.11 (C-10), 39.45 (C-24), 39.51 (C-9), 39.72 (C-12), 43.00 (C-7), 43.06 (C-13), 54.43 (C-5), 56.18 (C-17), 56.97 (C-14), 215.37 (C-6), a signal for C-3 could not be detected. EI-MS m/z (rel. %): 403 (M^+, 32), 388 (8), 384 (57), 369 (17), 332 (100), 331 (52), 291 (10), 290 (10), 273 (13), 271 (12). Anal. Calcd. for $C_{27}H_{45}D_1O_2$: C, 80.34%; H + D, 11.74%. Found: C, 80.53%; H + D, 11.88%.

Continued elution with the same solvent afforded **5a** (154 mg, 31%) as a white solid, mp 138–140°C (from MeOH). ^{1}H-NMR δ: 0.66 (*s*, 18-H_3), 0.75 (*s*, 19-H_3), 0.86 (*d*, J = 6.6 Hz, 26-H_3, 27-H_3), 0.91 (*d*, J = 6.4 Hz, 21-H_3), 2.20 (*dd*, J = 12.3, 2.57 Hz, 7-H), 2.32 (*dd*, J = 13.0, 4.3 Hz, 5-H). ^{2}H-NMR δ: 3.56 (3α-^{2}H). ^{13}C-NMR δ: 11.97 (C-18), 13.09 (C-19), 18.60 (C-21), 21.48 (C-11), 22.51 (C-26), 23.78 (C-27), 23.76 (C-23), 23.94 (C-15), 27.96 (C-25), 28.01 (C-16), 29.87 (C-4), 30.53 (C-2), 35.65 (C-20), 36.34 (C-22), 36.62 (C-1), 36.89 (C-8), 39.42 (C-12), 39.45 (C-24), 40.92 (C-10), 42.93 (C-13), 46.69 (C-7), 53.87 (C-9), 56.07 (C-17), 56.70 (C-5 or C-14), 56.74 (C-14 or C-5), 211.06 (C-6), a signal for C-3 could not be detected. EI-MS m/z (rel. %): 403 (M^+, 100), 388 (11), 385 (14), 370 (16), 332 (7), 331 (5), 290 (30), 272 (8), 248 (41). Anal. Calcd. for $C_{27}H_{45}D_1O_2$: C, 80.34%; H + D, 11.74%. Found: C, 80.60%; H + D, 12.04%.

3.3. *[5β,7α,7β-$^2H_3$]-2β,3β-Dihydroxy-5β-cholestan-6-one (**7a**)*

NaOH (0.52 g, 12.9 mM, 4.08 eq.) dissolved in MeOD (13 ml) was added to a solution of 2β,3β-diacetoxy-5α-cholestan-6-one (Wiechert et al., 1966) (1.59 g, 3.16 mM) in THF (2 ml) and the mixture was stirred for 1 h at 40°C. Sat. aq. NH_4Cl and Et_2O were added to the reaction mixture, the resulting aqueous phase was then extracted with AcOEt, and the combined organic layer was washed with sat. aq. $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated to dryness. Purification of the residue by silica gel column chromatography with hexane–AcOEt (1:1) as an eluant afforded a white solid, which was recrystallized from MeOH to give **6** (625 mg, 39%) as white plates, mp 178–180°C. ^{1}H-NMR δ: 0.66 (*s*, 18-H_3), 0.87 (*δ*, J = 6.2 Hz, 26-H_3, 27-H_3), 0.90 (*s*, 19-H_3), 0.92 (*d*, J = 6.8 Hz, 21-H_3), 2.09 (*brt*, J = 14.8 Hz, 7α-H), 2.20 (*dd*, J = 14.8, 4.4 Hz, 7β-H), 2.43 (*dd*, J = 13.2, 4.4 Hz, 5β-H), 3.79 (*m*, 2α-H), 4.05 (*m*, 3α-H). ^{2}H-NMR δ: 2.37 (5β-H), 2.15 (7-H). ^{13}C-NMR δ: 11.97 (C-18), 18.64 (C-21), 21.31 (C-11), 22.53 (C-26), 22.78 (C-27), 23.55 (C-19), 23.79 (C-23), 23.92 (C-15), 27.98 (C-25), 28.07 (C-16), 31.98 (C-1), 35.66 (C-20), 36.07 (C-22), 37.02 (C-4), 37.16 (C-8), 39.44 (C-24), 39.61 (C-12), 40.47 (C-10), 40.60 (C-9), 43.04 (C-13), 56.16 (C-17), 56.86 (C-14), 67.23 (C-3), 67.93 (C-2), 215.04 (C-6), signal intensities for 53.37 (C-5) and 42.89 (C-7) were weak. EI-MS m/z (rel. %): 422 (4), 421 (11), 420 (13), 419 (6), 404 (6), 403 (16), 402 (20), 401 (8), 388 (3), 387 (5), 386 (4), 375 (5), 374 (5), 373 (3), 372 (3), 369 (2), 335 (21), 334 (80), 333 (100), 332 (43). The ratio of deuterium substituted species (D_3:D_2:D_1 = 30:49:21) was calculated on the basis of M^+ and M-H_2O ions.

3.4. *Administration of ^{2}H-labeled sterols to Ajuga hairy roots*

Administration experiments were carried out as described previously (Nagakari et al., 1994a; Ohyama et al., 1999). A solution of the labeled sterol **4a**, **5a** or **7a** (100 mg each) dissolved in acetone (2 ml) and Tween 80 (2 ml) was added to four 500 ml-flasks containing pre-incubated hairy roots. Following incubation for two weeks, hairy roots weighing ca. 90 g (wet wt.) were harvested and processed to eventually

furnish 13.2, 3.0 and 13.7 mg of **1** from **4a**, **5a** and **7a**, respectively.

Acknowledgements

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