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Biosynthesis of 20-Hydroxyecdysone in *Ajuga* Hairy Roots: Stereochemistry of C-25 Hydroxylation

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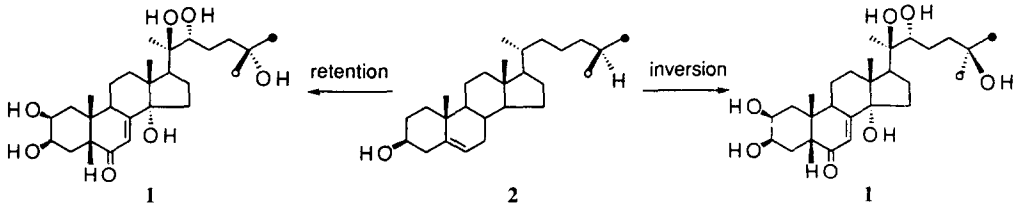
Abstract: Feeding of [$^{13}C_2$]acetate to hairy roots of *Ajuga reptans* var. *atropurpurea* followed by ^{13}C-NMR analysis of the biosynthesized cholesterol and 20-hydroxyecdysone indicated that C-25 hydroxylation in 20-hydroxyecdysone biosynthesis proceeds both in retention and inversion mechanisms. Feeding studies of [26-^{13}C]- and [27-^{13}C]cholesterols established that the ratio of retention and inversion mechanisms is *ca.* 3:1. © 1997 Elsevier Science Ltd.

20-Hydroxyecdysone (**1**) is a molting hormone of most arthropods. 20-Hydroxyecdysone and the related steroidal compounds (phytoecdysteroids) are also distributed in the plant kingdom. However, little has been known regarding the biosynthesis of phytoecdysteroids.[1,2] We found that hairy roots of *Ajuga reptans* var. *atropurpurea*[3] is an excellent tool for biosynthetic studies of phytoecdysteroids[4] and described the results based on the studies using ^{2}H and ^{13}C-labeled compounds.[5-7] It is generally accepted, both in insects[8] and plants,[2,5,7] that the early stage of biosynthesis of **1** from cholesterol (**2**) yields an intermediate having a cis-A/B ring structure, which is then hydroxylated at the C-2, -20, -22 and -25 positions to furnish **1**. It was reported that C-20, C-22 and C-25 hydroxylations were catalyzed by P_{450} mono-oxygenase enzymes[9,10] whereas a different type mono-oxygenase (not inhibited by CO) was responsible for C-2 hydroxylation.[10,11] Further, C-2[12] and C-22[13] hydroxylations were reported to proceed with retention of stereochemistry. In this paper we report the stereochemistry of C-25 hydroxylation in 20-hydroxyecdysone biosynthesis with *Ajuga* hairy roots.



1 **2** **1**

Scheme 1. Two possible stereochemical modes of C-25-hydroxylation

The C-25 position of these sterols is a prochiral center; therefore, it is required to discriminate and correlate the isopropyl pro-*R* and pro-*S* methyl groups of the precursor **2** and product **1**. We thought that ^{13}C-labeling studies can be conveniently employed to chase the metabolic fates of the diastereotopic methyl groups. The ^{13}C chemical shifts for the isopropyl pro-*R* and pro-*S* methyl groups of **2** were assigned previously.[14] However,

the ^{13}C shifts of the corresponding carbons of **1** had not yet been assigned. Thus, our first task was focused on this assignment, which was solved by the synthesis of stereochemically defined model compounds having the same side-chain structure as found in **1**, [26-^{2}H]-(20*R*,22*R*,25)-trihydroxycholesterol 3-methyl ether (one of the hydrogen atoms of the pro-*R*-methyl group is substituted by a deuterium atom) and its [27-^{2}H]-epimer. ^{13}C-NMR comparison of these model compounds and **1** allowed us to assign the signals at δ 28.9 and 29.7 to the pro-*S* and pro-*R* methyl carbons, respectively, of **1**.[15]

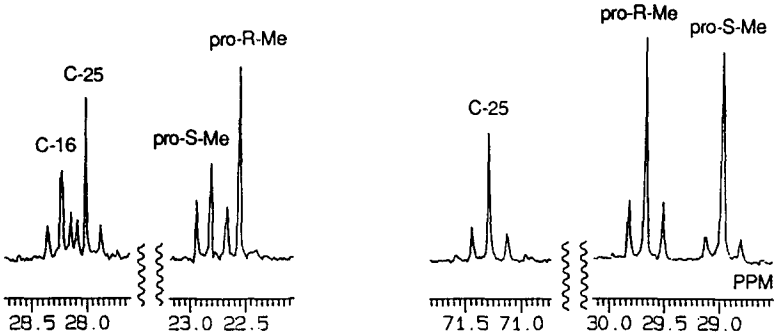
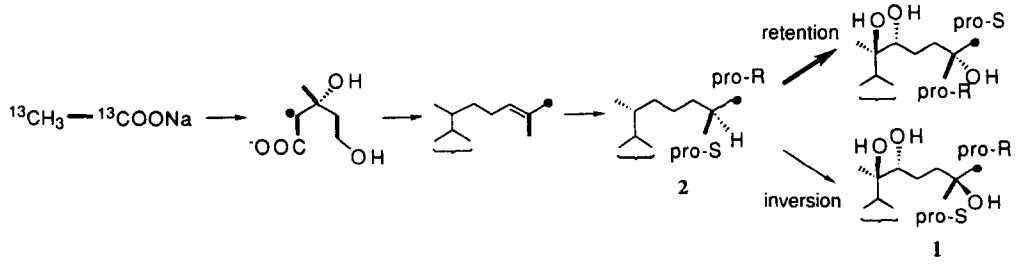


Fig. 1. ^{13}C-NMR spectra (in part) of cholesterol (left, 125 MHz, $CDCl_3$) and 20-hydroxyecdysone (right, 125 MHz, CD_3OD) derived from [$^{13}C_2$]acetate

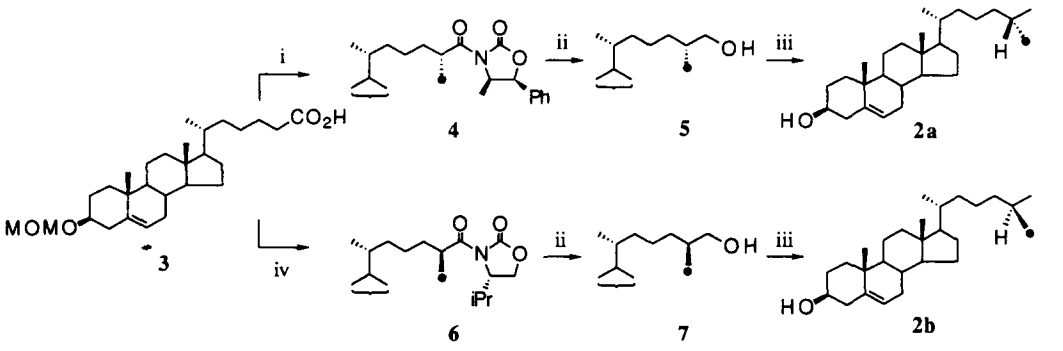
With the unequivocal ^{13}C assignments of the pro-*R* and pro-*S* methyl groups of **1** in hand, the stage was set for feeding studies of ^{13}C-labeled precursors. [$^{13}C_2$]Acetate was fed to *Ajuga* hairy roots (33% ^{13}C labeled acetate, 30 mg/250 ml liquid medium per flask, 8 flasks), and **2** (1.6 mg) and **1** (11 mg) were isolated as described previously.[4] The ^{13}C-NMR spectrum of **2** is shown in Fig. 1. Signals for C-25 (δ 27.9[14]) and the pro-*S* methyl group (δ 22.7[14]) were flanked by doublets (*J*=35.8 Hz) whereas the pro-*R*-methyl signal (δ 22.5) is not accompanied by such satellite peaks. These NMR data indicated that the two-carbon unit of C-25 and pro-*S*-methyl is derived from an intact acetate molecule, while the pro-*R*-methyl carbon arises from a breakdown of the acetate molecule. This implies that the pro-*R*-methyl of **2** is derived from C-2 of mevalonate, while the pro-*S*-methyl comes from C-6 (Scheme 2). These data further suggest that reduction of a Δ^{24}-sterol should take place stereospecifically from the 25-*Si* face.[16]



Scheme 2. Correlation of C-26 and C-27 of cholesterol and 20-hydroxyecdysone

The ^{13}C-NMR spectrum of 20-hydroxyecdysone derived from [$^{13}C_2$]acetate is also included in Fig. 1. The signal of C-25 (δ 71.3) has a flanking doublet (*J*=38.4 Hz). In accord with this pattern, the pro-*R* methyl signal (δ 29.7) was accompanied by a flanking doublet (*J*=38.4 Hz). The signal of the pro-*S* methyl (δ 28.9) is also

accompanied by satellite peaks with a weak, but significant intensity. The observed partial scrambling of the ^{13}C label in 20-hydroxyecdysone, combined with the above finding on cholesterol, suggested the possibility that C-25 hydroxylation during the conversion of **2** to **1** proceeds mostly with retention, but inversion also occurs to a certain extent (Scheme 2). This turned out to be the case as shown by the following studies.



Scheme 3. Synthesis of [pro-*R*-Me-^{13}C]- (**2a**) and [pro-*S*-Me-^{13}C]- (**2b**) cholesterols. Reagent and conditions: i) CDI; (4*R*, 5*S*)-4-methyl-5-phenyl-2-oxazolidinone, *n*-BuLi; LDA, $^{13}CH_3I$ (44%), ii) $LiAlH_4$ (95%), iii) MsCl, Py; $LiAlH_4$; 6N-HCl, THF (93%); iv) CDI; (4*R*)-4-isopropyl-2-oxazolidinone, *n*-BuLi; LDA, $^{13}CH_3I$ (22%).

To get a clear picture of the steric course of the C-25 hydroxylation, [pro-*R*-Me-^{13}C]- (**2a**) and [pro-*S*-Me-^{13}C]- (**2b**) cholesterols were required for incubation. These labeled cholesterols were synthesized according to Scheme 3.[17] Asymmetric methylation ($^{13}CH_3I$) of the oxazolidinone derivatives,[18] prepared from the C-26-oic acid **3**, gave the alkylated products **4** and **6** in good stereoselectivity (the ratio of the depicted product to the C-25 epimer, *ca.* 18:1). Compounds **4** and **6** were converted into the desired **2a** and **2b**, respectively, *via* the alcohols **5** and **7**. Stereochemical purities at the C-25 position of **2a** and **2b** were 22:1 and 18:1, respectively.

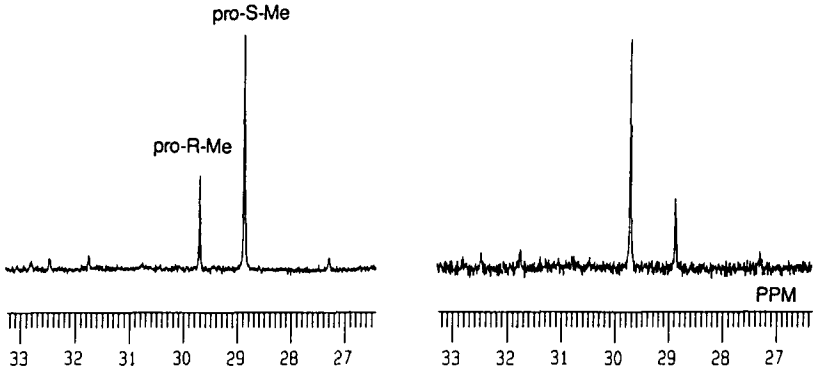


Fig. 2. ^{13}C-NMR spectra (75 MHz, CD_3OD) of 20-hydroxyecdysones derived from **2a** (left) and **2b** (right)

Compounds **2a** (90 mg) and **2b** (102 mg) were separately fed to *Ajuga* hairy roots and the resulting 20-hydroxyecdysone was isolated as described previously.[4] The ^{13}C-NMR spectrum (Fig. 2) of **1** (4.0 mg) derived from **2a** clearly indicates that the pro-*R*-methyl of cholesterol becomes pro-*S*- (*ca.* 75%, estimated by

integration of the two signals) and pro-*R*- (*ca.* 25%) methyl groups of **1**. The ^{13}C-NMR spectrum of **1** (2.3 mg) derived from **2b** showed the reversed labeling pattern as expected (Fig. 2).

It is, therefore, concluded that C-25 hydroxylation during the biosynthesis of 20-hydroxyecdysone from cholesterol in *Ajuga* hairy roots is not stereospecific, but proceeds with *ca.* 3:1 of retention and inversion mechanism. To our knowledge, this is the first elucidation of the stereochemistry of hydroxylation at such an isopropyl cryptic stereocenter. The hydroxylation with inversion of stereochemistry, though not a major pathway, is unprecedented so far.

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