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Biosynthesis of phytoecdysteroids in *Ajuga* hairy roots: clerosterol as a precursor of cyasterone, isocyasterone and 29-norcyasterone

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Abstract—Feeding studies of six ^{13}C-labeled sterols, including clerosterol, to hairy roots of *Ajuga reptans* var. *atropurpurea* have established that clerosterol is a precursor of three phytoecdysteroids, cyasterone, isocyasterone and 29-norcyasterone.

One structural feature of phytoecdysteroids is the presence of a one- or two-carbon substituent at the C-24 position of the C_{27}-ecdysteroid skeleton.[1] Cyasterone **1**, a C_{29}-phytoecdysteroid, was first isolated from *Cyathula capitata* (Amaranthaceae),[2] and subsequently identified in several *Ajuga* species (Labiatae).[3,4] Isocyasterone **2**, the C-25 epimer of **1**, was isolated from *C. capitata*[5] and *Ajuga* genera.[4] Isolation of a C_{28}-ecdysteroid, 29-norcyasterone **3**, was reported from *Ajuga reptans* and its variations.[4,6] The structures of these cyasterones are depicted in Figure 1. It is well established that cholesterol is a precursor of 20-hydroxyecdysone both in insects[7] and in plants.[8] In contrast, little is known about the biosynthetic relationship between C_{28}- or C_{29}-phytoecdysteroids and precursor sterols, although Goodwin et al. reported that radioisotope labeled sitosterol and fucosterol were negligibly incorporated into **1** with *C. capitata*.[9]

Hairy roots of *A. reptans* var. *atropurpurea* contain 20-hydroxyecdysone as well as cyasterones **1**–**3**.[4] We previously demonstrated that *Ajuga* hairy roots are useful for the biosynthetic studies of ecdysteroids, showing that both acetate and cholesterol were converted into 20-hydroxyecdysone in such efficiency that ^{13}C- and ^{2}H-tracers can be used.[10] In these studies, cholesterol was not converted into any of **1**–**3**. Subse-

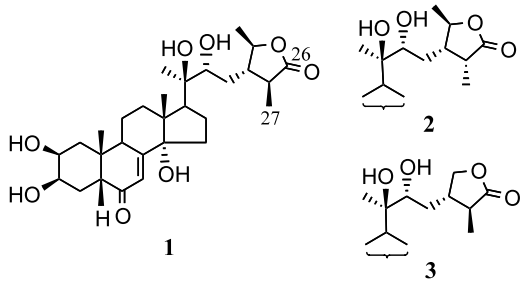

Figure 1. Structures of cyasterone **1**, isocyasterone **2** and 29-norcyasterone **3**.

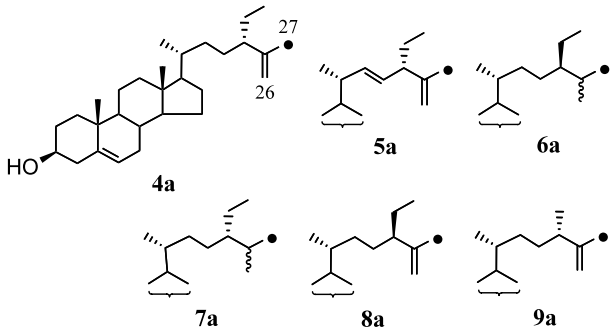

Figure 2. Structures of the ^{13}C-labeled sterols fed to *Ajuga* hairy roots.

Keywords: steroids and sterols; ecdysteroids; cyasterone; isocyasterone; 29-norcyasterone; clerosterol; codisterol; biosynthesis.

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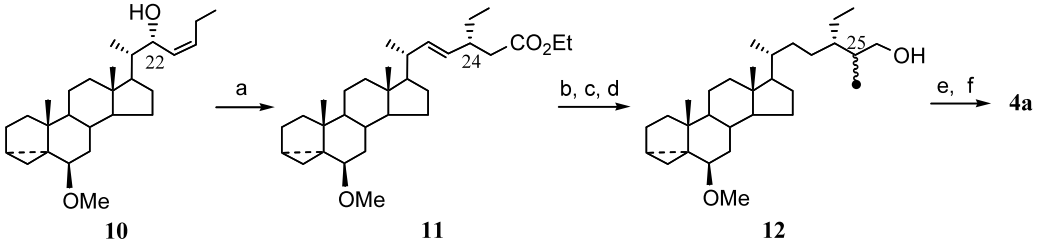

Figure 3. Synthesis of [27-^{13}C]clerosterol **4a**. *Reagents and conditions*: (a) $CH_3C(OEt)_3$, $EtCO_2H$; (b) H_2–Pd/C; (c) LDA, ^{13}C-MeI; (d) $LiAlH_4$; (e) *o*-NO_2–PhSeCN, Bu_3P, then H_2O_2; (f) aq. dioxane, TsOH.

quent feeding studies with [1,2-$^{13}C_2$]acetate and [2-^{13}C]mevalonate established that C-2 and C-6 of mevalonate become the lactone carbonyl (C-26) and the methyl (C-27) carbons of **1**–**3**, respectively.[11,12]

The present studies have focused on the identification of a precursor sterol in the biosynthesis of **1**–**3** with *Ajuga* hairy roots. Feeding studies of six ^{13}C-labeled sterols **4a**–**9a** (Fig. 2) allowed us to establish that clerosterol **4**, a major sterol of this plant, serves as a precursor of **1** and **2**. In addition, evidence for the conversion of **4** into 29-norcyasterone **3** via fission of the C-28–C-29 bond was obtained.

The structures of the ^{13}C-labeled sterols, [27-^{13}C]clerosterol **4a**, [27-^{13}C]dehydroclerosterol **5a**, [26-^{13}C]-(25*RS*)-sitosterol **6a**, [26-^{13}C]-(25*RS*)-clionasterol **7a**, [27-^{13}C]-24-*epi*-clerosterol **8a** and [27-^{13}C]codisterol **9a** are listed in Figure 2. Clerosterol **4** and dehydroclerosterol **5** were tested in view of their occurrence in *Ajuga* hairy roots. Sitosterol **6** is a typical plant sterol in higher plants. Its C-24 epimer, clionasterol **7** and 24-*epi*-clerosterol **8** were chosen to investigate the possible importance of C-24 configuration and Δ^{25}-olefinic functionality. Codisterol **9** is a C_{28}-analogue of **4** and is assumed to be a precursor of **3**. This sterol was previously characterized, albeit in a minute amount, in *Ajuga* hairy roots cultured under certain conditions.[13]

The synthesis of **4a** was carried out according to the route shown in Figure 3, while compounds **5a**–**9a** were prepared in a minor modification of this route.[14] The known (24*R*)-ester **11**,[15] obtained from (22*R*)-allylic alcohol **10** via orthoester Claisen rearrangement, was hydrogenated, and the requisite $^{13}CH_3$ group was introduced using enolate chemistry. Reduction of the methylated compound with $LiAlH_4$ gave the alcohol **12** as a C-25 epimeric mixture. Dehydration of **12** followed by regeneration of the Δ^5-3β-ol system furnished **4a**.

Feeding experiments of **4a**–**9a** to *Ajuga* hairy roots were carried out as described previously.[10] Briefly, **4a** was fed to *Ajuga* hairy roots (150 mg substrate to eight 500 mL-Erlenmeyer flasks, each containing 250 mL liquid medium and hairy roots pre-incubated for 2 weeks). After incubation for 2 weeks, the hairy roots were harvested and subjected to extraction and separation to afford cyasterones **1**–**3** after final HPLC fractionation as described previously.[4,10] The ^{13}C NMR spectra of **1**–**3** biosynthesized from **4a** are illustrated in Figure 4.

In the spectrum (Fig. 4B) of **1**, the signal at δ 181.8 (lactone carbonyl) was more intense (ca. threefold) compared to that of the non-labeled reference (Fig. 4A), whereas the signal at δ 15.93 (C-27) was observed in the same intensity as the reference. The data clearly indicated that **4a** was converted into **1**. Similarly, it is indicated that **4a** was converted into **2** as shown in the spectrum (Fig. 4C) of **2** wherein the incorporated ^{13}C-label resided on the lactone carbonyl carbon (δ 182.0) as observed for **1**.

Rather unexpectedly, the ^{13}C NMR spectrum of **3** (Fig. 4D) also showed ca. threefold enrichment at the lactone carbonyl carbon (δ 182.3). The signal at δ 14.4 (C-27) was not enriched. The results indicated that the regiose-

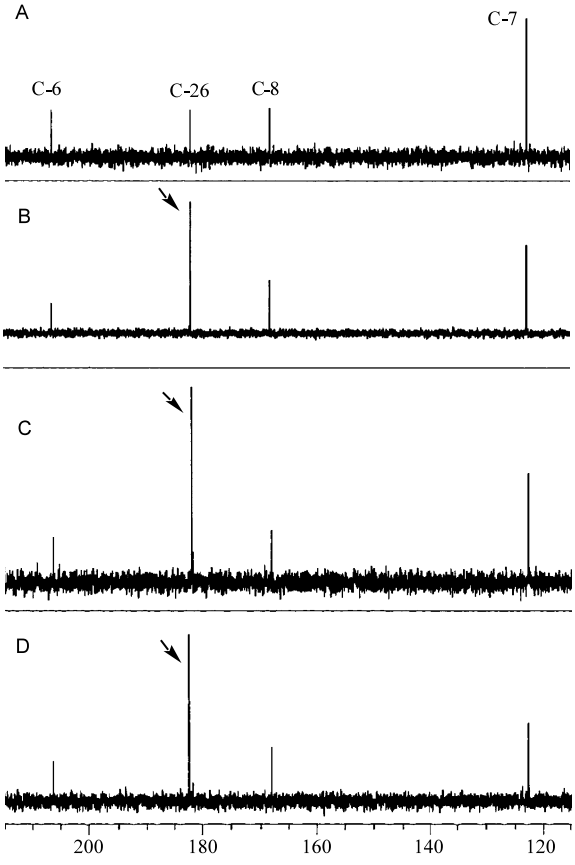

Figure 4. ^{13}C NMR spectra (125 MHz, CD_3OD) of **1** (B), **2** (C) and **3** (D) biosynthesized from **4a**. Spectrum (A) for non-labeled **1**.

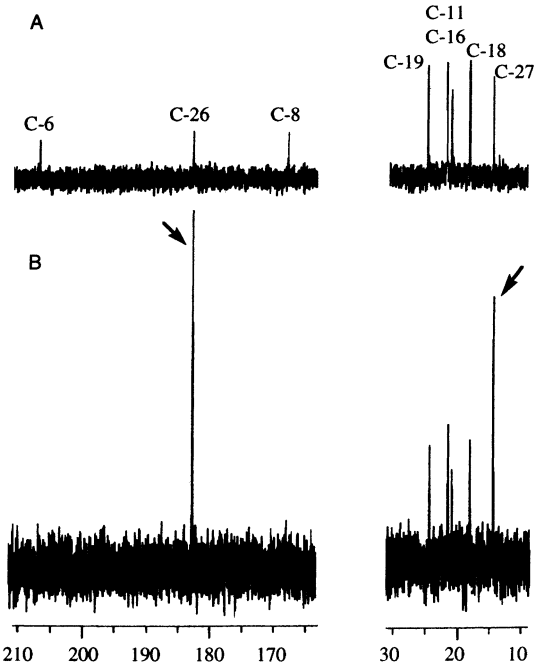

Figure 5. ^{13}C NMR spectrum (125 MHz, CD_3OD) of **3** (B) biosynthesized from **9a**. Spectrum (A) for non-labeled **3**.

lective enrichment on the lactone carbonyl carbon of **3** is in common with **1** and **2**, and, more importantly, that **4** was subjected to C-28–C-29 bond cleavage reaction and converted into **3**.

As expected, the C_{28}-sterol codisterol **9a** was incorporated into the C_{28}-ecdysteroid **3**, but not into the C_{29}-ecdysteroids **1** and **2**. The ^{13}C NMR spectrum of 29-norcyasterone **3** obtained upon feeding of **9a** is shown in Figure 5 in which both the lactone carbonyl carbon (C-26) and the methyl group (C-27) were enriched with the former being more highly enriched. The observed scrambling of the ^{13}C label is not consistent with the aforementioned stereospecific labeling of C-26 and C-27 of **3** when originated from acetate, mevalonate and clerosterol. We are inclined to propose that a principal precursor of **3** would be **4**. The mechanism of the conversion of codisterol **9** to **3**, relevant to the scrambling of the label, must await further study. None of the other tested sterols, **5a**–**8a**, was incorporated into any of **1**–**3**, further supporting the biological significance of clerosterol as a precursor of **1**–**3**. It seems that the Δ^{25}-double bond with a 24β (24*S*)-ethyl group would be a required structural unit for subsequent γ-lactone formation.

We have recently observed that ^{13}C-cycloartenol is converted into 20-hydroxyecdysone as well as into **1** and **3**.[16] Further, ^{13}C-24-methylenecholesterol was shown to be converted into **4**[13] and also into **1**.[16] On the basis of the present study, together with these previous observations, the biosynthetic route of *Ajuga* ecdysteroids can be proposed as shown in Figure 6. Ajugasterone B **13** has been isolated from several *Ajuga* species including *A. reptans*,[17] and the C-24 configuration of **13** is tentatively assigned as depicted (Fig. 6). This ecdysteroid can be regarded as an intermediate to be positioned between clerosterol **4** and cyasterones **1**–**3**. Figure 6 also represents metabolic correlation of C-26 and C-27 of **1**–**3** and the precursor clerosterol **4**, which confirms the previous results obtained from feeding studies with [1,2-$^{13}C_2$]acetate and [2-^{13}C]mevalonate (vide supra).[11–13]

It should be noted that the sterol profiles of *Ajuga* and *Cyathula* are completely different, whereas their ecdysteroid constituents are similar. Especially, the C-24α configurations of *Cyathula* sterols[9,18] (sitosterol **6** and stigmasterol) are opposite to those of the ecdysteroids; *Ajuga* sterols (**4** and **5**) and cyasterones **1**–**3** have the same 24β configuration. The *Cyathula* pathway leading to cyasterones could conceivably branch off from the 24α-sterol biosynthetic route at an early stage, or, alternatively, some minor sterol with 24β-configuration,

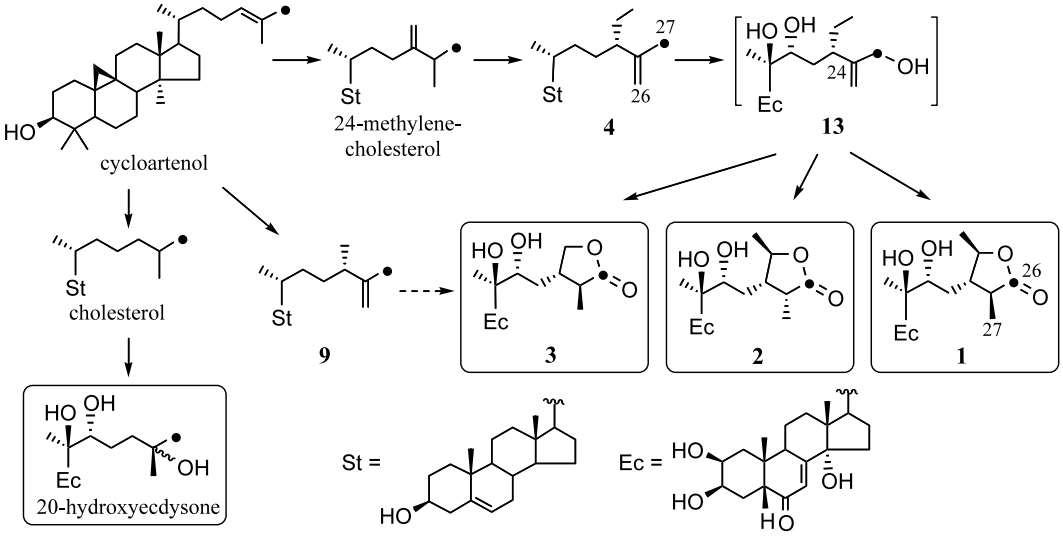

Figure 6. Proposed biosynthetic pathway of *Ajuga* ecdysteroids. Dots refer to carbon atoms biosynthetically correlated to C-2 of mevalonate.

if any, would serve as a precursor of **1** and **2** in *Cyathula* sp.

In conclusion, we have demonstrated that the three cyasterones **1**–**3** are all derived from clerosterol **4** in *Ajuga* hairy roots. This is the first paper to prove the relationships between substrate sterols and C_{29}/C_{28} phytoecdysteroids.

Acknowledgements

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14. For **5a**: hydrogenation step was omitted. For **6a**: analogous to **7a** but starting with the known (22*S*)-epimer of **10**. For **7a**: the 26-ol **12** was mesylated and reduced with $LiAlH_4$. For **8a**: starting with the known (22*S*)-epimer of **10**. For **9a**: starting with the known 26-nor analogue of **10**. The ^{13}C labeled compounds **4a**–**9a** were fully characterized by spectroscopic and elemental analyses. **4a**: mp 129–131°C, ^{13}C NMR δ: 17.81 (C-27). **5a**, mp 147°C, ^{13}C NMR δ: 20.19 (C-27). **6a**, mp 138.5–139°C, ^{13}C NMR δ: 18.98, 19.76 (C-26, C-27). **7a**, mp 138–139°C, ^{13}C NMR δ: 18.94, 19.58 (C-26, C-27). **8a**, mp 136–137°C, ^{13}C NMR δ: 18.06 (C-27). **9a**, mp 149–150.5°C, ^{13}C NMR δ: 18.72 (C-27).
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18. Analysis of the sterol fraction of *C. officinalis* roots, which contain **1** and **2**, confirmed that (24α)-alkylsterols (sitosterol 54% and stigmasterol 33%) are contained exclusively.