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Stereochemical Assignment of C-24 and C-25 of Amarasterone A, a Putative Biosynthetic Intermediate of Cyasterone

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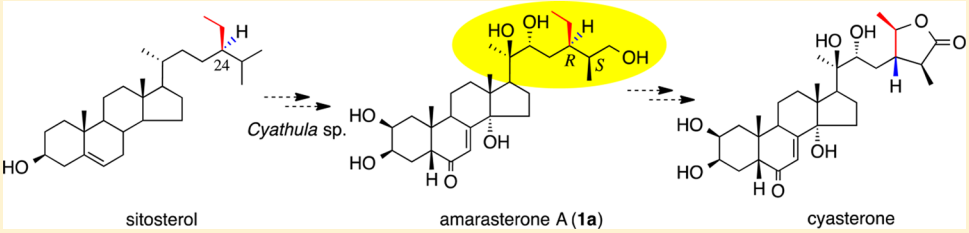
Supporting Information



ABSTRACT: A C_{29} phytoecdysteroid named amarasterone A (**1**) has been isolated from *Cyathula capitata* (Amaranthaceae), *Leuzea carthamoides* (Asteraceae), and *Microsorum scolopendria* (Polypodiaceae). We recently isolated amarasterone A from *C. officinalis*. Amarasterone A has been postulated as a biosynthetic intermediate of cyasterone in *Cyathula* sp. The stereochemistry at the C-24 and C-25 positions of these amarasterone A samples was investigated by comparing the NMR spectroscopic data with those of stereodefined model compounds, (24*R*,25*S*)-, (24*R*,25*R*)-, (24*S*,25*S*)-, and (24*S*,25*R*)-isomers of (20*R*,22*R*)-3β-methoxystigmast-5-ene-20,22,26-triol (**2a**−**d**), which were synthesized in the present study. Amarasterone A isolated from *Cyathula officinalis* was determined to be the (24*R*,25*S*)-isomer (**1a**), while amarasterone A from *L. carthamoides* was found to be the (24*R*,25*R*)-isomer (**1b**). Amarasterone A from *M. scolopendria* was found to be a mixture of **1a** and **1b**. The biosynthesis of cyasterone in *Cyathula* sp. is discussed on the basis of the identical C-24 configuration of sitosterol and amarasterone A.

INTRODUCTION

Cyasterone is a well-known C_{29} phytoecdysteroid first isolated from *Cyathula capitata* (Amaranthaceae) by Japanese researchers in 1968.[1] They further reported the isolation of amarasterone A (**1**), amarasterone B,[2] capitasterone,[3] and precyasterone[4] from the same species and proposed potential biosynthetic correlations (Figure 1). Cyasterone, 28-*epi*-cyasterone, and 25,28-di-*epi*-cyasterone were isolated from *C. officinalis*, and the structures of the former two ecdysteroids were unambiguously established by X-ray crystallography.[5,6] Interestingly, the occurrence of cyasterone has been reported in various *Ajuga* species such as *A. reptans* of the Labiatae family.[7−14]

In general, it can be assumed that a C_{29} phytoecdysteroid is biosynthesized from a C_{29} plant sterol. This implies that the C-24 configuration of the sterol precursor is the same as that of the C_{29} phytoecdysteroid. For example, the C-24 configuration of makisterone C (lemnisterone, podecdysone A, 24-ethyl-20-hydroxyecdysone) is suggested to be *R* simply by analogy to the C-24 stereochemistry of a typical C_{29} plant sterol, sitosterol, although there has been no research reported concerning the stereochemistry.[15−17]

The rather rare sterol, clerosterol ((24*S*)-stigmasta-5,25-dien-3β-ol), is a major sterol in *Ajuga* species,[18,19] and this sterol was established to be a biosynthetic precursor of cyasterone and 28-*epi*-cyasterone in a feeding study using hairy roots of *Ajuga reptans* var. *atropurpurea*.[20] To our knowledge, this is the only report clarifying the biosynthetic relationship between a C_{29} sterol and a C_{29} phytoecdysteroid. Notably, the C-24 configuration of clerosterol is the same as that of cyasterone; thus the configuration at C-24 of clerosterol is not inverted during the biosynthesis.

Our analysis of the sterol fraction of *C. officinalis* indicated that the major sterols were sitosterol (59% of total sterol) and stigmasterol ((22*E*,24*S*)-stigmasta-5,22-dien-3β-ol) (31%); 22,23-dihydrospinasterol ((24*R*)-stigmast-7-en-3β-ol) (4%) and 24-methylcholesterol (5%) were also present.[20,21] This raised a question as to the identity of the sterol precursor of cyasterone in *Cyathula* species. Radioactively labeled sitosterol and fucosterol ((*E*)-stigmasta-5,24(28)-dien-3β-ol) were not incorporated into cyasterone in *C. capitata*.[22,23] We prepared several lines of hairy roots of *C. officinalis* to find an effective experimental system, but none of them were capable of producing ecdysteroids (unpublished results).

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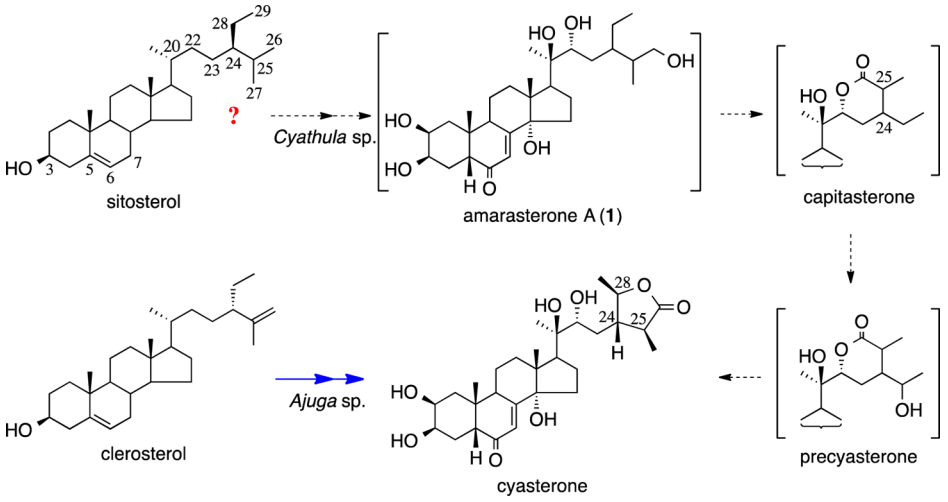

Figure 1. Structures of ecdysteroids isolated from *Cyathula* species and their tentative biosynthetic relationships.

To elucidate the structure of a sterol that serves as the precursor of cyasterone in *Cyathula* sp., we took an indirect approach. As described above, amarasterone A is presumed to be a biosynthetic precursor of cyasterone. It is therefore reasonable to assume that amarasterone A from *Cyathula* species should retain the C-24 configuration of the precursor sterol, as the side chain of amarasterone A is functionalized only at the C-20, C-22, and C-26 positions, most likely by the action of P450 enzymes.[24,25] Thus, it is critical to determine the C-24 configuration of amarasterone A.

Our recent isolation of amarasterone A from *C. officinalis*[26] prompted us to pursue this stereochemical study. Furthermore, amarasterone A was isolated as a mixture of two diastereomers (designated as epimer 1 and epimer 2) from the fern *Microsorum scolopendria* (Polypodiaceae).[27] Unfortunately, the NMR data for the epimer 1 and the epimer 2 were recorded in D_2O, which hindered comparison with our sample of amarasterone A. These samples seemed to be isomeric at the C-24 and/or C-25 positions from the available NMR data.[27] Amarasterone A was also previously isolated from *Leuzea carthamoides* (Asteraceae).[28] The reported NMR data (in CD_3OD) were different from those of our material. Thus, it is also worthwhile to determine the stereochemistry at C-24 and C-25 of the amarasterone A samples to determine which stereoisomers occur in plants.

In the present study, we investigated the configuration of C-24 and C-25 of amarasterone A samples and established that only the (24*R*,25*S*)- and (24*R*,25*R*)-isomers have been observed thus far. In addition, the sterol precursor for cyasterone and the biosynthesis of cyasterone in *Cyathula* species are discussed.

RESULTS AND DISCUSSION

The amount of amarasterone A available from *C. officinalis* (and also from other plant sources) was very small; thus determining the stereochemistry from a natural sample would be difficult. We therefore pursued a strategy to synthesize stereodefined model compounds and determine the configuration of amarasterone A by comparing the NMR data. (20*R*,22*S*)-3β-Methoxystigmast-5-ene-20,22,26-triols (**2a**–**d**) were selected as model compounds (Figure 2).

Four diastereomers **2a**–**d** could most likely be conveniently synthesized by coupling the known 20-hydroxy-22-aldehyde **3**

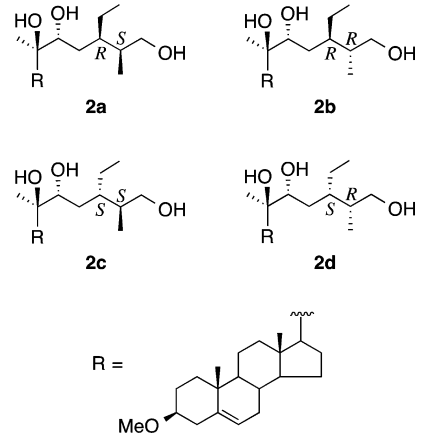

Figure 2. Structures of synthesized stereodefined model compounds **2a**–**d**.

with an organo metal species derived from a C_7 fragment halide. It has been reported that such a coupling reaction yields the desired (20*R*,22*R*)-glycol product.[29] Racemic 2-ethyl-3-methylbut-3-enyl bromide in which the C-27 hydroxyl group was masked as an olefin was used as the C_7 unit. It was anticipated that a mixture of the C-24 epimeric coupling products would be separable. Hydroboration–oxidation of the 25-olefins would complete the synthesis of **2a**–**d** (Figure 3).

Aldehyde **3** was prepared from pregnenolone in three steps:[30] protection of the C-3 hydroxyl group as the methyl ether, addition of 2-lithio-1,3-dithiane, and hydrolysis of the resulting adduct with NCS.[31] (±)-2-Ethyl-3-methylbut-3-enyl bromide (**4**) was synthesized from ethyl 3-methylcrotonate in

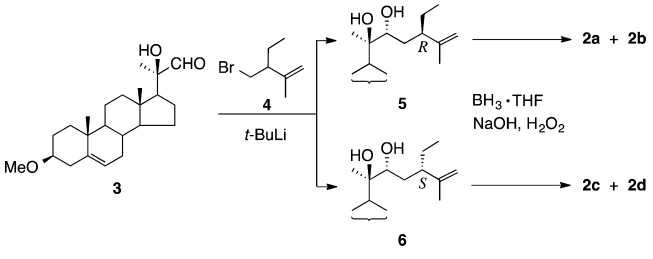

Figure 3. Synthesis of model compounds **2a**–**d**.

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four steps. Reaction of the enolate generated by the action of LDA with EtI gave ethyl 2-ethyl-3-methylbut-3-enoate. Reduction of the ethylated ester with $LiAlH_4$ afforded 2-ethyl-3-methylbut-3-en-1-ol. The alcohol was converted to 2-ethyl-3-methylbut-3-enyl bromide (**4**) via the tosylate.

Attempted coupling of aldehyde **3** with the Grignard reagent prepared from the bromide did not afford the desired product but instead yielded a product resulting from methyl migration (22-hydroxy-3β-methoxy-24-norchol-5-en-20-one).[29] The organo lithium species prepared from the bromide with 2 equiv of *t*-BuLi[32] in pentane−ether at −78 °C gave a product resulting from attack of the *t*-Bu anion on the aldehyde. When the amount of *t*-BuLi was reduced to 1 equiv, the resulting C_7 lithium species smoothly reacted with the aldehyde to give the desired coupling product as a mixture of the C-24 epimers, which were separated by MPLC using a silica gel column to yield the less polar epimer **5** (32%) and the more polar epimer **6** (48%). Crystalline compound **6** was determined to have the 24*S* configuration by X-ray crystallography, as shown in the ORTEP drawing (Figure 4). Compound **5** was thus the 24*R*-epimer.

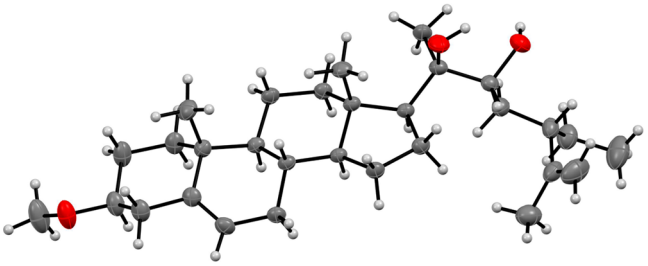
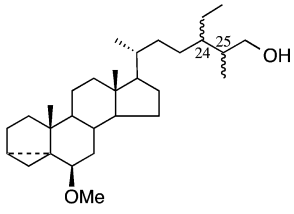


Figure 4. ORTEP drawing of compound **6**.

Hydroboration of compound **5** yielded a mixture of (24*R*)-C-25 epimeric alcohols, which were separated by p-TLC into the less polar **2a** (36% from **5**, R_f 0.35 with hexane/EtOAc 1:2) and the more polar **2b** (20%, R_f 0.33) as crystalline products. Similar hydroboration of **6** yielded a mixture of (24*S*)-C-25 epimeric alcohols, which were separated by silica gel column chromatography to give the less polar alcohol **2c** (38%, R_f 0.53 with hexane/EtOAc 1:2) and the more polar alcohol **2d** (21%, R_f 0.28). Compounds **2a**−**d** were sparingly soluble in D_2O but well soluble in CD_3OD.

The configurations at C-25 of diastereomers **2a**−**d** were deduced from the comparison of their ^{13}C NMR data with those of the stereodefined 6β-methoxy-3,5-cyclo-5α-stigmast-5-en-26-ols **2a′**−**d′** (Figure 5).[33] It has been reported that the



2a′: 24*R*,25*S*
2b′: 24*R*,25*R*
2c′: 24*S*,25*S*
2d′: 24*S*,25*R*

Figure 5. Structures of stereodefined sterols **2a′**−**d′**.

chemical shifts of the C-28 and C-23 signals (recorded in CD_3OD) of **2a′**−**d′** allowed **2a′**/**2d′** to be differentiated from **2b′**/**2c′**, that is, the C-24/C-25 relative configuration. The C-28 signals of (24*R*,25*R*)/(24*S*,25*S*)-isomers (**2b′**/**2c′**) (δ 24.9 and 25.2, respectively) resonated at a lower field compared with those of their (24*R*,25*S*)/(24*S*,25*R*)-counterparts (**2a′**/**2d′**) (δ 23.6 and 23.7, respectively), while the C-23 signals of **2b′**/**2c′** resonated at a higher field (δ 27.0 and 27.3, respectively) compared with those of **2a′**/**2d′** (δ 28.3 and 28.4, respectively). Thus, compounds **2a** and **2b** could be assigned as the (24*R*,25*S*)- and (24*R*,25*R*)-isomers, respectively, and compounds **2c** and **2d** were determined to be the (24*S*,25*S*)- and (24*S*,25*R*)-isomers, respectively. Comparison of the other side chain signals (C-24, C-25, C-26, C-27, and C-29) supported the above stereochemical assignments (Table 1). The 25*S* configuration of **2a** was further verified by X-ray analysis of **2a** (Figure 6). The configurations at C-24 and C-25 of **2a**, **2b**, **2c**, and **2d** were thus established as described above.

With the configuration of the model compounds **2a**−**d** having been established, the NMR data of **2a**−**d** were compared with those of natural amarasterone A samples. Comparison of the ^1H NMR data of amarasterone from *C. officinalis* with those of **2a**−**d** indicated that the natural amarasterone is neither the (24*R*,25*R*)- nor the (24*S*,25*R*)-isomer, as the signal of H_3-27 (δ 0.93) was significantly different from those of **2b** and **2d** (δ 0.77 and 0.80, respectively). However, the unambiguous assignment of the amarasterone sample to either the (24*R*,25*S*)- or the (24*S*,25*S*)-isomer was problematic, although the ^1H NMR values compared more favorably with those of **2a** than **2c**. Similarly, the amarasterone sample form *L. carthamoides* was determined to be either the (24*R*,25*R*)- or the (24*S*,25*R*)-isomer.

However, comparison of the ^{13}C NMR data allowed us to assign the stereochemistry of amarasterone samples. The ^{13}C NMR values for the side chain of amarasterone A from *C. officinalis* were very similar to those of **2a** and significantly different from the other three stereoisomers **2b**−**d** (Table 1). A similar comparison of the ^{13}C NMR data of amarasterone A from *Leuzea carthamoides* showed that they matched with those of the (24*R*,25*R*)-isomer **2b**. The ^1H (Table 2) and ^{13}C NMR values for amarasterone A/epimer 1 and amarasterone A/epimer 2 from *M. scolopendria*, as recorded in CD_3OD solvent in the present study, agreed with those of amarasterone A from *C. officinalis* and those of amarasterone A from *L. carthamoides*, respectively, thus establishing that the epimer 1 and epimer 2 correspond to the (24*R*,25*S*)- and the (24*R*,25*R*)-isomers.

CONCLUSIONS

The present study established that amarasterone A obtained from *C. officinalis* (and presumably the amarasterone A originally isolated from *C. capitata*) has a 24*R*,25*S* configuration (**1a**). In contrast, amarasterone A from *L. carthamoides* has a 24*R*,25*R* configuration (**1b**). Furthermore, *M. scolopendria* was found to contain both the (24*R*,25*S*)- and (24*R*,25*R*)-isomers (**1a** and **1b**). Until now, neither the (24*S*,25*S*)- nor the (24*S*,25*R*)-isomer has been reported. This may reflect the abundance of the major sterol sitosterol rather than its C-24 epimer in the plant kingdom, including the Amaranthaceae, Asteraceae, and Polypodiaceae families. The occurrence of **1a** and **1b** in *M. scolopendria* is reminiscent of the report of the isolation of inokosterone from *Achylanthes fauriei* (Amaranthaceae), which is a C-25 epimeric mixture.[34] It is conceivable that the specificity of the C-26/C-27 hydroxylation depends on the

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Table 1. ^{13}C NMR Data (125 MHz) for 2a–d, 2a′–d′, and Amarasterone A in CD_3OD

no.	2a	2a′[a]	2b	2b′[a]	2c	2c′[a]	2d	2d′[a]	1a[b]	1b[c]
1	38.3		38.3		38.4		38.3		37.4	37.4
2	29.0		29.0		29.0		29.0		68.7	68.7
3	82.0		82.0		82.0		82.0		68.5	68.5
4	39.7		39.7		39.7		39.7		32.9	32.9
5	141.8		141.8		141.8		141.8		51.8	51.8
6	122.8		122.8		122.8		122.8		206.4	206.4
7	33.0		33.0		33.0		33.0		122.1	122.1
8	32.7		32.7		32.7		32.7		168.0	167.9
9	51.7		51.7		51.7		51.7		35.1	35.1
10	38.0		38.0		38.0		38.0		39.3	39.3
11	22.1		22.1		22.1		22.1		21.5	21.5
12	41.6		41.6		41.6		41.7		32.5	32.5
13	44.3		44.3		44.4		44.4		48.6[e]	48.7[e]
14	58.2		58.2		58.2		58.3		85.2	85.2
15	23.0		23.0		23.2		23.2		31.8	31.8
16	25.1		25.1		25.1		25.1		21.5	21.5
17	56.2		56.2		56.2		56.2		50.3	50.3
18	14.0		14.0		14.0		14.0		18.0	18.1
19	19.8		19.8		19.8		19.8		24.4	24.4
20	78.1		78.1		78.2		78.0		78.0	78.0
21	20.7		20.7		20.6		20.7		20.9	20.9
22	75.8		75.6		76.1		74.6		75.9	75.7
23	34.1	28.3	32.1	27.0	33.4	27.3	33.7	28.4	34.1	32.0
24	39.8	43.1	37.9	42.5	41.1	43.2	38.4	42.8	39.7	37.9
25	39.1	40.0	37.6	38.5	37.9	38.7	37.6	38.7	39.1	37.6
26	66.1	66.7	66.7	66.9	66.1	66.8	67.2	67.0	66.1	66.8
27	14.7	13.6	11.5	12.3[d]	14.3	13.5	11.5	12.7[d]	14.8	11.5
28	23.8	23.6	25.4	24.9	25.4	25.2	23.1	23.7	23.7	25.4
29	12.1	12.5	12.3	13.0[d]	12.8	12.6	12.8	13.0[d]	12.1	12.3
MeO	55.8		55.8		55.8		55.8			

[a]Adopted from ref 33. Pertinent data are included in the table. [b]Amarasterone A from *C. officinalis*. The data were identical with those of epimer 1 from *M. scolopendria*. [c]Amarasterone A from *L. leuzea*. Adopted from ref 27. The data were identical with those of epimer 2 from *M. scolopendria*. [d]An HMBC correlation was observed from H_3-27 to C-26 for **2d**. The signals for C-27 and C-29 in ref 33 were interchanged. [e]The signal, which was overlapped by solvent, was assigned based on the HMBC correlation from H_3-18.

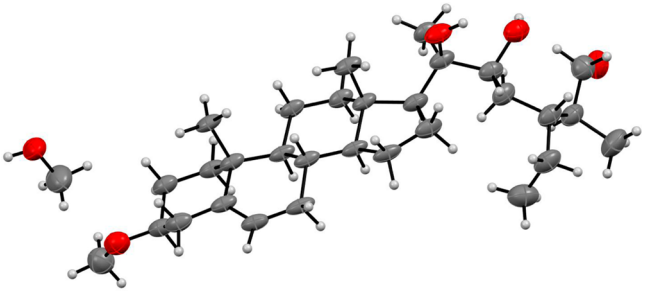

Figure 6. ORTEP drawing of compound **2a**.

plants and *M. scolopendria* hydroxylates both C-26 and C-27 in non-regiospecific manner. We recommend the names of *amarasterone A1* for the (24*R*,25*S*)-isomer (**1a**) and *amarasterone A2* for the (24*R*,25*R*)-isomer (**1b**) (Figure 7).

The finding that amarasterone A1 from *C. officinalis* has a 24*R* configuration, which is the same as that in sitosterol, strongly suggests that sitosterol is a biosynthetic precursor of amarasterone A in *Cyathula* sp. This implies that stereochemical inversion at C-24 must occur in a reaction step subsequent to the formation of amarasterone A1. One possibility is that the $\Delta^{24(25)}$ intermediate is involved in the inversion mechanism. 24-Dehydroprecyasterone (the $\Delta^{24(25)}$ derivative of precyasterone) is possibly a candidate for the $\Delta^{24(25)}$ intermediate, even though it has been isolated from *Ajuga* sp. and not from *Cyathula* sp.[35] 24-Dehydroprecyasterone is likely an intermediate placed between capitasterone (most likely 24*R*) and precyasterone (presumably 24*S*) in cyasterone (24*S*) biosynthesis. Thus, capitasterone should have the same C-24 stereochemistry as amarasterone A1; therefore, the C-24 configuration of precyasterone should be investigated.

The previously observed negligible conversion of sitosterol into cyasterone is most likely due to insufficient uptake of the sterol substrate, as the authors noted.[22,23] It has been well-documented that cholesterol is incorporated into a typical C_{27} phytoecdysteroid, 20-hydroxyecdysone, in plants.[36−39] In contrast, the biosynthetic study of C_{29} and C_{28} phytoecdysteroids has been hampered by the lack of a suitable plant culture system. It is essential to develop an appropriate culture system for *Cyathula* or other plant species to study the hypothesis described above.

EXPERIMENTAL SECTION

General. ^1H and ^{13}C NMR spectra were recorded at 500 MHz for ^1H and 125 MHz for ^{13}C, using $CDCl_3$ or CD_3OD as the solvent. For some compounds, the spectra were recorded with a 400/100 MHz spectrometer when it was specified. ^1H chemical shifts are reported in reference to the internal standard TMS (0.00 ppm) or the residual proton signal of CD_3OD (3.30 ppm). ^{13}C chemical shifts are referenced to $CDCl_3$ (77.0 ppm) or CD_3OD (49.0 ppm). HRMS

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Table 2. ^{1}H NMR (500 MHz) Data for 2a–d and Amarasterone A in CD_3OD[a]

no.	2a	2b	2c	2d	1a[b]	1b[c]
18	0.90 (s)	0.90 (s)	0.90 (s)	0.91 (s)	0.88 (s)	0.89 (s)
19	1.01 (s)	1.01 (s)	1.01 (s)	1.01 (s)	0.96 (s)	0.97 (s)
21	1.18 (s)	1.17 (s)	1.18 (s)	1.19 (s)	1.17 (s)	1.18 (s)
22	3.40 (m)	3.41 (brd, 10.0)	3.43 (brd, 10.5)	3.43 (m)	3.43 (brd, 8.4)	3.45 (dd, 10.4, 2.3)
26	3.34 (dd, 10.5, 7.5)	3.38 (dd, 11.5, 7.0)	3.35 (dd, 10.5, 7.0)	3.40 (dd, 10.5, 7.0)	3.35 (dd, 10.8, 7.6)	3.40 (dd, 11.0, 6.8)
	3.55 (dd, 11.0, 5.0)	3.48 (dd, 11.0, 7.5)	3.54 (dd, 10.5, 5.5)	3.49 (dd, 10.5, 6.5)	3.56 (dd, 10.8, 5.1)	3.50 (dd, 11.0, 7.3)
27	0.92 (d, 7.0)	0.77 (d, 7.0)	0.90 (d, 7.0)	0.80 (d, 7.0)	0.93 (d, 6.9)	0.79 (d, 7.0)
29	0.92 (t, 7.0)	0.92 (t, 7.0)	0.90 (t, 7.0)	0.91 (t, 7.0)	0.93 (t, 7.5)	0.94 (t, 6.4)

[a]The chemical shifts not listed in the table are described in the Experimental Section. [b]Amarasterone A from *C. officinalis*. The data were identical with those of epimer 1 from *M. scolopendria*. [c]Amarasterone A from *L. leuzea*. From ref 27. The data were identical with those of epimer 2 from *M. scolopendria*.

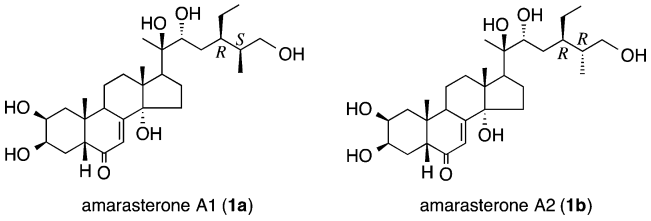

Figure 7. Structures of naturally occurring amarasterone A.

(FAB) spectra were obtained on a double-focusing magnetic sector mass spectrometer. IR spectra were recorded with a Fourier transform (FT-IR) spectrometer. Optical rotations were measured on a polarimeter using a 5 cm cell at approximately 25 °C. TLC and p-TLC were performed on precoated silica gel 60 F254 glass plates (0.25 mm thickness). Silica gel 60 (spherical neutral, 40–100 μm) was used for column chromatography. MPLC was carried out using a silica gel-packed glass column.

Isolation of Amarasterone A1 (1a) from *Cyathula officinalis*. The chips of roots and stems of *Cyathula officinalis* (300 g dry wt) were ground using a mill. The residue was extracted with benzene (900 mL) by heating at 80 °C for 1 h, and the benzene extract was discarded. The defatted residue was extracted with methanol (900 mL) twice by heating at 80 °C for 1 h. The combined methanol solutions were concentrated in vacuo. The residue (45 g) was dissolved in *n*-butanol, washed with brine, and concentrated in vacuo. The residue was chromatographed on silica gel ($CHCl_3$/MeOH 7:1–4:1) to give an ecdysteroid-enriched fraction (101 mg). HPLC analysis (solvent, MeOH/H_2O 1:1; flow rate, 1.0 mL/min; UV detection, 243 nm) of the fraction revealed a peak at 14.5 min, which was different from cyasterone (11.2 min), 25,28-di-*epi*-cyasterone (12.3 min), and 28-*epi*-cyasterone (13.4 min). HPLC separation of the peak afforded **1a** (2.5 mg, 0.0084% based on the dried chips): amorphous solid: ^{13}C NMR data, see Table 1; ^{1}H NMR (CD_3OD) δ 1.43 (t, J = 12.7 Hz, Ha-1), 1.78 (dd, J = 12.7, 4.4 Hz, Hb-1), 3.83 (ddd, J = 12.7, 4.4, 3.6 Hz, H-2), 3.94 (m, H-3), 1.70–1.75 (m, Ha-4, Hb-4), 2.37 (dd, J = 12.7, 4.8 Hz, H-5), 5.80 (d, J = 2.4 Hz, H-7), 3.14 (m, H-9), 1.70 (m, Ha-11), 1.80 (m, Hb-11), 2.11 (ddd, J = 13.0, 13.0, 4.8 Hz, Ha-12), 1.87 (m, Hb-12), 1.95 (m, Ha-15), 1.60 (m, Hb-15), 1.99 (m, Ha-16), 1.74 (m, Hb-16), 2.35 (t, J = 10.0 Hz, H-17), 1.27–1.35 (m, Ha-23, Hb-23), 1.56 (m, H-24), 1.76 (m, H-25), 1.41–1.35 (m, Ha-28, Hb-28); the remainder of the ^{1}H shifts are listed in Table 2; MS (FAB) m/z 509 $[M + H]^+$.

(3β,20*R*)-22-Formyl-3-methoxypregn-5-en-20-ol (3). Sodium hydride (60%, dispersion in paraffin oil) (800 mg, 20 mmol) was added to a solution of pregnenolone (3.16 g, 10 mmol) in dry THF (40 mL) at 0 °C under N_2, and the mixture was warmed to 50 °C and stirred for 30 min at the same temperature. MeI (6.4 mL, 15 mmol) was added to the mixture, and stirring was continued for 2 h at the same temperature. Ether and saturated NH_4Cl (aq) were added, and the organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was chromatographed on silica gel (hexane/EtOAc 10:1) to afford pregnenolone methyl ether (2.4 g, 7.3 mmol, 73%): white crystals, mp 117–119 °C (hexane/AcOEt) (lit.[40] 124–125 °C); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.63 (s, H_3-18), 1.00 (s, H_3-19), 2.12 (s, H_3-21), 2.53 (t, J = 9.0 Hz, H_1-17) 3.06 (m, H-3), 3.36 (s, OCH_3), 5.36 (m, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.2, 19.3, 21.0, 22.8, 24.4, 28.0, 31.5, 31.8, 31.8, 36.9, 37.2, 38.6, 38.8, 43.9, 50.0, 55.5, 56.9, 63.7, 80.2, 121.2, 140.8, 209.4. Anal. Calcd for $C_{22}H_{34}O_2$: C, 79.95; H, 10.37. Found: C, 79.85; H, 10.33.

n-BuLi (1.6 M *n*-hexane solution, 5.0 mL, 8.0 mmol) was added dropwise to a solution of 1.3-dithiane (873 mg, 7.26 mmol) in dry THF (9.5 mL) at −20 °C under N_2, and the mixture was cooled to −78 °C. The methyl ether (2.40 g, 7.26 mmol) in THF (25 mL) was added, and the reaction was stirred for 30 min at the same temperature. The solution was warmed to −50 °C for 1 h. Ether and saturated NH_4Cl (aq) were added, and the organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was chromatographed on silica gel (hexane/EtOAc 8:1 containing 10% $CHCl_3$) to give the dithiane adduct (2.63 g, 5.85 mmol, 79%): white crystals (hexane/AcOEt), mp 185–186 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (s, 3H), 1.00 (s, 3H), 1.44 (s, 3H), 2.75–3.00 (m, 4H), 3.06 (m, 1H), 3.35 (s, 3H), 4.14 (s, 1H), 5.35 (brs, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.3, 19.3, 20.9, 21.6, 23.7, 24.1, 26.0, 27.9, 30.8, 31.3, 31.5, 31.7, 36.8, 37.1, 38.6, 40.1, 42.9, 50.0, 55.1, 55.5, 56.8, 61.2, 76.7, 80.3, 121.4, 140.9. Anal. Calcd for $C_{26}H_{42}O_2S_2$: C, 69.28; H, 9.39; S, 14.23; Found: C, 69.30; H, 9.68; S, 13.96.

A mixture of the dithiane adduct (1.2 g, 2.7 mmol) and *N*-chlorosuccinimide (936 mg, 7.0 mmol) in CH_2Cl_2/H_2O (10:1, 40 mL) was stirred for 3 h at room temperature under N_2.[31] Ether and aqueous $Na_2S_2O_3$ were added, and the separated organic layer was washed with $NaHCO_3$ and brine, then dried over Na_2SO_4, and concentrated in vacuo. The residue was chromatographed on silica gel (hexane/EtOAc 8:1) to afford the aldehyde (647 mg, 67%): colorless needles, mp 128–130 °C (hexane/AcOEt) (lit.[41] 168–170 °C); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.80 (s, 3H), 1.00 (s, 3H), 1.35 (s, 3H), 3.06 (m, 3H), 3.26 (brs, OH), 3.35 (s, 3H), 5.35 (br, 1H), 9.57 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.8, 19.3, 20.9, 22.1, 23.0, 24.1, 27.9, 31.4, 31.7, 36.9, 37.1, 38.6, 40.0, 43.3, 50.1, 55.5, 55.6, 56.5, 79.5, 80.3, 121.2, 140.9, 203.5. Anal. Calcd for $C_{23}H_{36}O_3$: C, 76.62; H, 10.06. Found: C, 76.53; H, 10.35.

2-Ethyl-3-methylbut-3-en-1-ol. *n*-BuLi (1.60 M *n*-hexane solution, 20 mL, 32.0 mmol) was added dropwise to a solution of diisopropylamine (4.5 mL, 32 mmol) in dry THF (40 mL) at −78 °C under N_2. The solution was stirred and allowed to warm to 0 °C and then stirred at the same temperature for 1 h. A solution of ethyl 3-methylcrotonate (3.3 mL, 24 mmol) in dry THF (6.0 mL) was added dropwise to the LDA solution at −78 °C, and the mixture was stirred for 30 min. EtI (2.6 mL, 32 mmol) was added dropwise, then the mixture was allowed to warm to 0 °C for 45 min, and finally stirred for 30 min at room temperature. Ether and saturated aqueous NH_4Cl were added, and the separated organic layer was washed with 2 N HCl, saturated aqueous $NaHCO_3$, and brine, dried over Na_2SO_4, and concentrated in vacuo to give the ethylated ester as a pale yellow oil

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(3.7 g, 98%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.89 (t, J = 7.5 Hz, 3H), 1.25 (t, J = 7.1 Hz, 3H), 1.60−1.84 (m, 2H), 1.75 (s, 3H), 2.91 (t, J = 7.5 Hz, 1H), 4.14 (dd, J = 7.1 Hz, 2H), 4.88 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 12.0, 14.2, 20.2, 23.3, 54.9, 60.4, 113.6, 142.5, 173.8. Anal. Calcd for $C_9H_{16}O_2$: C, 69.19; H, 10.32. Found: C, 69.15; H, 10.32.

$LiAlH_4$ (972 mg, 25.6 mmol) was added in several portions to a solution of the ester (3.7 g, 23.7 mmol) in dry ether (45 mL) at 0 °C under N_2, and the mixture was then allowed to warm to room temperature. After being stirred for 3 h at the same temperature, moist ether and saturated aqueous NH_4Cl were added to the reaction mixture, and the organic layer was washed with aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated in vacuo to give the alcohol as a pale yellow oil (2.4 g, 89%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (t, J = 7.5 Hz, 3H), 1.31−1.45 (m, 2H), 1.67 (s, 3H), 2.18 (m, 1H), 3.51 (m, 2H), 4.83 (brs, 1H), 4.95 (brs, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 11.7, 18.7, 22.1, 51.8, 63.8, 113.8, 145.0. Anal. Calcd for $C_7H_{14}O$: C, 73.63; H, 12.36. Found: C, 73.57; H, 12.62.

2-Ethyl-3-methylbut-3-enyl Bromide (4). TsCl (1.5 g, 7.9 mmol) was added to a solution of the alcohol (755 mg, 6.6 mmol) in pyridine (2.0 mL) at 0 °C. The mixture was stirred for 2 h at the same temperature. Ice chips were added, and the mixture was stirred for 5 min. Ether and saturated aqueous NH_4Cl were added to the reaction mixture, and the organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated in vacuo to give the tosylate (1.4 g, 78%) as a pale oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.80 (t, J = 7.3 Hz, 3H), 1.20−1.50 (m, 2H), 1.56 (s, 3H), 2.27 (m, 1H), 2.45 (s, 3H), 3.96 (m, 2H), 4.69 (brs, 1H), 4.83 (brs, 1H), 7.34 (d, J = 8.4 Hz, 2H), 7.78 (d, J = 8.4 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 11.2, 19.3, 21.6, 22.0, 47.6, 71.9, 113.7, 127.9, 129.7, 133.1, 142.9, 144.6. Anal. Calcd for $C_{14}H_{20}O_3S$: C, 62.66; H, 7.51; S, 11.95. Found: C, 62.56; H, 7.50; S, 11.67.

LiBr (1.4 g, 16.1 mmol) was added to solution of the tosylate (1.4 g, 5.2 mmol) in dry acetone (7 mL), and the mixture was stirred at 60 °C for 5 h. Ether and brine were added to the reaction mixture, and the separated organic layer was washed with brine, dried over Na_2SO_4, and concentrated on a rotary evaporator under reduced pressure (200 mmHg). The residue was passed through a short silica gel column with hexane as an eluent. Removal of the solvent, as described above, afforded the bromide **4** (655 mg, 3.7 mmol, 71%) as a pale yellow oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (t, J = 8.0 Hz, 3H), 1.42−1.60 (m, 2H), 1.67 (s, 3H), 2.32 (m, 1H), 3.39 (d, J = 6.8 Hz, 2H), 4.78 (brs, 1H), 4.91 (brs, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 11.7, 18.8, 24.5, 36.4, 50.9, 113.4, 144.5. Anal. Calcd for $C_7H_{13}Br$: C, 47.48; H, 7.40. Found: C, 47.57; H, 7.12.

(3*β*,20*R*,22*S*,24*R*)-3-Methoxystigmasta-5,25-diene-20,22-diol (5) and (3*β*,20*R*,22*S*,24*S*)-3-Methoxystigmasta-5,25-diene-20,22-diol (6). *t*-BuLi (1.60 M *n*-pentane solution, 2.68 mL, 4.29 mmol) was added to solution of **4** (760 mg, 4.29 mmol) in dry ether (17 mL) and *n*-pentane (26 mL) under argon at −78 °C, and the solution was stirred at the same temperature for 20 min. A solution of **3** (301 mg, 0.859 mmol) in dry ether (8.6 mL) and *n*-pentane (2.9 mL) was added dropwise to the organolithium solution at −78 °C. The mixture was stirred at the same temperature for 5 min and diluted with ether and saturated aqueous NH_4Cl. The organic layer was washed brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was chromatographed on silica gel (eluting with hexane/EtOAc/$CHCl_3$, 6:1:0.7) to obtain a mixture of **5** and **6** (335 mg). Separation of the mixture by MPLC (eluting with hexane/EtOAc 5:1) using a silica gel-packed column (LiChroprep Si-60, mesh 40−63, Merck) gave the less polar **5** (125 mg, 32%) and the more polar **6** (189 mg, 48%).

Compound **5**: colorless needles, mp 142−144 °C (MeOH); R_f 0.41 (hexane/EtOAc 4:1, developed twice); ^{1}H NMR ($CDCl_3$) δ 0.81 (t, J = 7.5 Hz, 3H), 0.90 (s, 3H), 1.01 (s, 3H), 1.19 (s, 3H), 1.67 (s, 3H), 3.06 (m, 1H), 3.35 (s, 3H), 3.50 (brd, J = 10.5 Hz, 1H), 4.80 (brd, J = 1.0 Hz, 2H), 5.36 (brd, J = 5.5 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 11.6, 13.5, 18.5, 19.4, 20.4, 21.0, 22.0, 24.0, 25.2, 28.0, 31.3, 31.8, 35.9, 36.9, 37.2, 38.7, 40.3, 43.2, 47.3, 50.2, 54.6, 55.6, 56.8, 75.3, 76.9, 80.3, 111.6, 121.4, 140.9, 149.3; HRMS (FAB) m/z 459.3814 [M + H]$^+$ (calcd for $C_{30}H_{51}O_3$, 459.3838).

Compound **6**: colorless needles, mp 169 °C (MeOH); R_f 0.38 (hexane/EtOAc 4:1, developed twice); ^{1}H NMR ($CDCl_3$) δ 0.83 (t, J = 7.5 Hz, 3H), 0.88 (s, 3H), 1.01 (s, 3H), 1.21 (s, 3H), 1.61 (s, 3H), 3.06 (m, 1H), 3.35 (s, 3H), 3.38 (brd, J = 10.5 Hz, 1H), 4.78 (brd, J = 2.0 Hz, 1H), 4.84 (brd, J = 2.5 Hz, 1H), 5.36 (brd, J = 5.0 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 12.0, 13.5, 17.7, 19.4, 20.4, 21.0, 21.8, 23.9, 27.0, 28.0, 31.3, 31.8, 34.9, 36.9, 37.2, 38.7, 40.2, 43.2, 46.0, 50.2, 54.7, 55.6, 56.8, 73.5, 77.0, 80.3, 112.8, 121.4, 140.9, 146.6; HRMS (FAB) m/z 459.3814 [M + H]$^+$ (calcd for $C_{30}H_{51}O_3$, 459.3838).

(3*β*,20*R*,22*S*,24*R*,25*S*)-3-Methoxystigmast-5-ene-20,22,26-triol (2a) and (3*β*,20*R*,22*S*,24*R*,25*R*)-3-Methoxystigmast-5-ene-20,22,26-triol (2b). BH_3/THF (1 M solution in THF, 100 μL, 0.10 mmol) was added to a solution of the 25-ene **5** (25 mg, 55 μmol) at room temperature, and the mixture was stirred for 2 h at the same temperature. Water (100 μL), 3 M NaOH (100 μL), and 30% H_2O_2 (100 μL) were added, and the mixture was stirred for 30 min at 40 °C. An extractive workup with $CHCl_3$ yielded a crude product, which was chromatographed on silica gel to give a 3:2 mixture of **2a** and **2b** (10 mg, eluting with hexane/EtOAc 4:1). The mixture was further separated by p-TLC with benzene/EtOAc 4:3 as the developing solvent (developed five times) to afford the less polar **2a** (9.2 mg, 36%) and the more polar **2b** (5.6 mg, 20%).

Compound **2a**: colorless needles, mp 168−170 °C (MeOH); R_f 0.35 (hexane/EtOAc 1:2); $[\alpha]_D^{25}$ −8.0 (c 0.5, $CHCl_3$); ν_{max} ($CHCl_3$) 3434, 3003, 2939, 2875 cm^{-1}; ^{1}H and ^{13}C NMR (CD_3OD) data, see Tables 1 and 2; compounds **2a**−**d** exhibited common ^{1}H signals at δ 3.05 (m, 1H) and 5.36 (m, 1H) for 3α-H and 6-H, respectively; HRMS (FAB) m/z 477.3964 [M + H]$^+$ (calcd for $C_{30}H_{53}O_4$, 477.3944).

Compound **2b**: colorless needles, mp 163−165 °C (MeOH); R_f 0.33 (hexane/EtOAc 1:2); $[\alpha]_D^{25}$ −3.8 (c 0.5, $CHCl_3$); ν_{max} ($CHCl_3$) 3434, 3003, 2939, 2875 cm^{-1}; ^{1}H and ^{13}C NMR (CD_3OD) data, see Tables 1 and 2; HRMS (FAB) m/z 477.3953 [M + H]$^+$ (calcd for $C_{30}H_{53}O_4$, 477.3944).

(3*β*,20*R*,22*S*,24*S*,25*S*)-3-Methoxystigmast-5-ene-20,22,26-triol (2c) and (3*β*,20*R*,22*S*,24*S*,25*R*)-3-Methoxystigmast-5-ene-20,22,26-triol (2d). Compound **6** (48 mg, 105 μmol) was subjected to hydroboration and oxidation as described above to give the less polar **2c** (19.1 mg, 38%, eluting with $CHCl_3$/EtOAc 3:1) and the more polar **2d** (10.3 mg, 21%, eluting with $CHCl_3$/EtOAc 5:4) after silica gel chromatography.

Compound **2c**: amorphous solid; R_f 0.53 (hexane/EtOAc 1:2); $[\alpha]_D^{25}$ +5.0 (c 0.04, $CHCl_3$); ν_{max} ($CHCl_3$) 3434, 3003, 2939, 2875 cm^{-1}; ^{1}H and ^{13}C NMR (CD_3OD) data, see Tables 1 and 2; HRMS (FAB) m/z 477.3953 [M + H]$^+$ (calcd for $C_{30}H_{53}O_4$, 477.3944).

Compound **2d**: amorphous solid; R_f 0.28 (hexane/EtOAc 1:2); $[\alpha]_D^{25}$ +3.6 (c 0.5, $CHCl_3$); ν_{max} ($CHCl_3$) 3434, 3003, 2939, 2875 cm^{-1}; ^{1}H and ^{13}C NMR (CD_3OD) data, see Tables 1 and 2; HRMS (FAB) m/z 477.3943 [M + H]$^+$ (calcd for $C_{30}H_{53}O_4$, 477.3944).

X-ray Crystal Data of (3*β*,20*R*,22*S*,24*S*)-3-Methoxystigmasta-5,25-diene-20,22-diol (6). Crystallographic data: colorless, $C_{30}H_{50}O_3$, FW 458.70, monoclinic, space group $C2$, a = 29.1632(11), b = 6.6782(3), c = 14.5294(6) Å, β = 98.666(1)°, V = 2797.4(2) Å^3, Z = 4, D_{calcd} = 1.089 g cm^{-3}, T = 173 K, λ = 0.71075 Å, μ = 0.068 mm^{-1}, 13 745 measured reflections, 3461 independent reflections, 310 parameters, 1 restraint, $F(000)$ = 1016, $R1$ = 0.0360, $wR2$ = 0.0950 [3213 $I > 2\sigma(I)$], $R1$ = 0.0388, $wR2$ = 0.0964 (all data), max and min residual density 0.250 and −0.159 e·Å^{-3}, and goodness of fit (F^2) = 1.052. The absolute structure was deduced from the known chirality of pregnenolone, which was used for the synthesis. Crystallographic data for the structure have been deposited with CCDC (Deposition No. CCDC 978812).

X-ray Crystal Data of (3*β*,20*R*,22*S*,24*R*,25*S*)-3-Methoxystigmasta-5,25-diene-20,22,26-triol (2a). Crystallographic data: colorless, $C_{31}H_{56}O_5$, FW 508.75, monoclinic, space group $P2_1$, a = 12.7540(4), b = 6.03911(17), c = 19.9264(7) Å, β = 95.3800(15)°, V = 1528.03(8) Å^3, Z = 2, D_{calcd} = 1.106 g cm^{-3}, T = 173 K, λ = 0.71075 Å, μ = 0.569 mm^{-1}, 17 466 measured reflections, 5124 independent

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reflections, 325 parameters, 1 restraint, $F(000)$ = 564, $R1$ = 0.0564, $wR2$ = 0.1452 [3166 $I > 2\sigma(I)$], $R1$ = 0.0844, $wR2$ = 0.1772 (all data), max and min residual density 0.234 and −0.149 e·Å^{-3}, and goodness of fit (F^2) = 1.021. The absolute structure was deduced from the known chirality of pregnenolone, which was used for the synthesis. Crystallographic data for the structure have been deposited with CCDC (Deposition No. CCDC 978811).

■ ASSOCIATED CONTENT

Supporting Information

^{1}H and ^{13}C NMR spectra of amarasterone A (**1a**) isolated from *Cyathula officinalis* and compounds **2a**, **2b**, **2c**, and **2d**, and CIF files for compounds **2a** and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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