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Biocatalysis of Cycloastragenol by Filamentous Fungi to Produce Unexpected Triterpenes

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Abstract: The biocatalysis of cycloastragenol, a natural tetracyclic triterpenoid with anti-aging activity, by cultured whole cells of three strains of filamentous fungi, namely *Cunninghamella elegans* AS 3.1207, *Syncephalastrum racemosum* AS 3.264 and *Doratomyces stemonitis* AS 3.1411 produced 15 metabolites. Thirteen of them are new compounds. The structures of these metabolites were fully characterized on the basis of HR-ESI-MS analyses together with 1D and 2D NMR spectroscopy. The three fungal strains exhibited significant biocatalytic preferences: *C. elegans* enabled hydroxylation reactions, particularly on the 28- and 29-CH_3 groups; *S. racemosum* efficiently catalyzed a complicated rearrangement reaction to form the unusual ranunculane skeleton, which was further substituted with diverse side chains at C-19; *D. stemonitis* mainly led to carbonylation reactions, especially on 3-OH. It is particularly noteworthy that *S. racemosum* also catalyzed an unexpected ring expansion reaction to generate the rare 9(10)a-homo-19-nor-cycloartane skeleton. Biocatalysis was proved powerful in the structural diversification of cycloastragenol for future structure-activity relationship studies.

Keywords: biocatalysis; cycloastragenol; filamentous fungi; ring expansion; tetracyclic triterpenoids

Introduction

Structural modification of bioactive natural products is usually necessary to improve solubility, enhance efficacy, or reduce toxicity. Chemical approaches are the routine solution but often encounter challenges when facing regio- or stereo-selectivity problems. Moreover, multi-step reactions often result in low yield of the final products. Biocatalysis, by means of enzyme systems, shows high selectivity and efficiency, and has been widely used in pharmaceutical synthesis.[1] It is generally operated under mild conditions, and the most popularly used biological systems include fungi, bacteria, and cultured plant suspension cells. A number of filamentous fungi was reported to catalyze complex transformations that were difficult to realize by chemical approaches. For example, *C. elegans* AS 3.1207 was able to convert steroidal saponins into pregnenolones,[2] and *S. racemosum* AS 3.264 could convert paeoniflorin into albiflorin.[3] Biocatalysis is attracting more and more interests of researchers.

Cycloastragenol, or (20*R*,24*S*)-3β,6α,16β,25-tetrahydroxy-20,24-epoxycycloartane (CA, **1**), is the genuine sapogenin of astragaloside IV, a major bioactive constituent of *Astragalus* plants.[4–6] Astragaloside IV exhibits various pharmacological properties, such as anti-inflammatory,[7] anti-viral,[8] anti-aging,[9] anti-oxidant,[10] and so on. Cycloastragenol could delay the onset of cellular aging by increasing telomerase activity,[11] up-regulate the immune system by inducing IL-2 release,[12] and enhance the antiviral function of human $CD8^+$ T lymphocytes.[13] CA has been considered as a promising new generation of anti-aging agent. However, few analogues of CA are known to us, so far. Its structural modification is thus of great necessity for further evaluation of structure-activity relationships. Recently, Kuban et al.[14] reported the formation of a complicated rearrangement product of CA with a novel ranunculane framework by *Cunninghamella blakesleeana* NRRL 1369, which demonstrated the power of biocatalysis in structural diversification of cycloastragenol to produce novel derivatives.

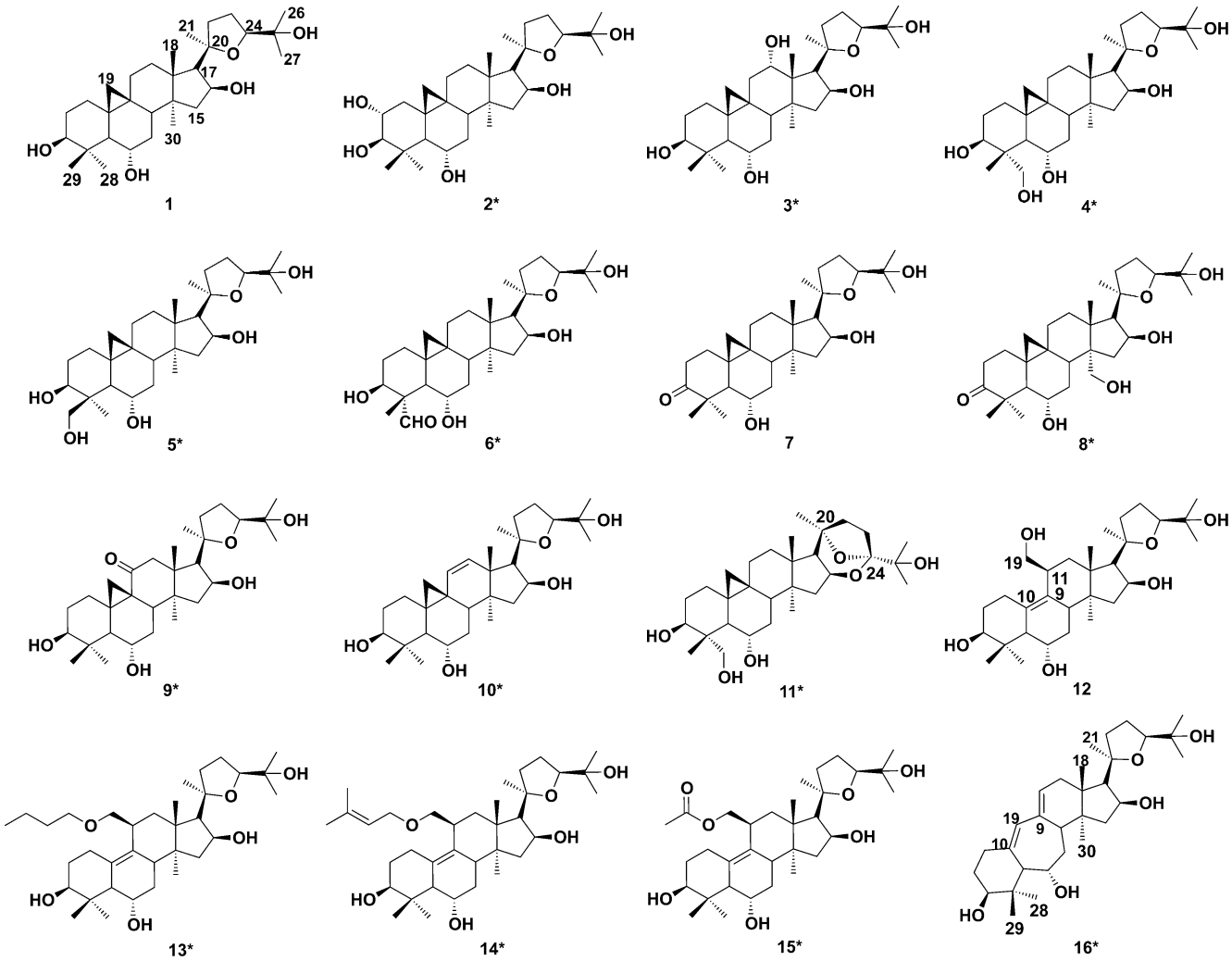


Figure 1. Chemical structures of compounds **1–16** (* new compound).

This paper describes the biocatalysis of cycloastragenol by three strains of filamentous fungi, namely *Cunninghamella elegans* AS 3.1207, *Syncephalastrum racemosum* AS 3.264 and *Doratomyces stemonitis* AS 3.1411, and structure elucidation of the 15 biotransformed products. The biocatalytic features of the three fungal strains were also discussed.

Results and Discussion

Cycloastragenol (**1**) as the substrate for biocatalysis was obtained by Smith degradation of astragaloside IV. A preliminary screening test revealed that CA could be efficiently metabolized by three fungal strains, *Cunninghamella elegans* AS 3.1207, *Syncephalastrum Racemosum* AS 3.264, and *Doratomyces stemonitis* AS 3.1411. Scaled-up biocatalytic fermentations of these three stains were then separately carried out. After 6 days of incubation with CA, the fungal broth was extracted with ethyl acetate. The extract was separated by silica gel column chromatography, and then purified by semi-preparative HPLC-RI to obtain compounds **2** (4 mg), **3** (5 mg), **4** (100 mg), **5** (100 mg), **6** (20 mg), **11** (3 mg) and **12** (12 mg) from the culture broth of *C. elegans*, compounds **3** (10 mg), **9** (31 mg), **10** (3.5 mg), **12** (360 mg), **13** (10 mg), **14** (7 mg), **15** (15 mg) and **16** (5.5 mg) from *S. racemosum*, and compounds **7** (730 mg), **8** (4.5 mg) and **12** (5.5 mg) from *D. stemonitus.* The structures of compounds **1–16** are shown in Figure 1. The NMR spectroscopic data for **16** are given in Table 1, the data for **1–15** are given in Table 2 (^{1}H NMR for **1–8**), Table 3 (^{1}H NMR for **9–15**) and Table 3 (^{13}C NMR for **1–15)**, respectively.

Ring Expansion Product 16

Compound **16** was deduced to possess the molecular formula $C_{30}H_{48}O_5$ based on its HR-ESI-MS data (found: $m/z = 977.70692$, calcd. for $[2M+H]^+$:

Table 1. 1D and 2D NMR[a] spectroscopic data for compound **16** (*J* in Hz).

No.	^{13}C	^{1}H	HMBC	^{1}H-^{1}H COSY	NOESY
1	41.1	2.30, 2H, m	C-3, C-5, C-10, C-19	H-2	H-5, H-19, H-28
2	34.7	1.92, 1H, m (a); 2.18, 1H, m (b)	C-1, C-3, C-4, C-5, C-10	H-1, H-3	H-29; H-3
3	77.9	3.83, 1H, brd (12.0)		H-2	H-2 (b), H-5, H-28
4	43.4				
5	59.8	2.67, 1H, d (5.6)	C-3, C-4, C-6, C-10, C-28, C-29	H-6	H-1, H-3, H-28
6	73.4	4.30, 1H, m		H-5, H-7	H-8, H-28, H-29
7	38.2	2.16, 2H, m	C-5, C-6, C-8, C-9	H-6, H-8	H-15 (a)
8	41.9	2.56, 1H, m	C-6, C-9	H-7	H-6, H-29
9	138.2				
10	135.1				
11	127.6	5.66, 1H, s	C-10, C-14	H-12	H-19, H-12 (a)
12	39.1	2.10, 1H, m (a); 2.25, 1H, m (b)	C-9, C-13, C-15	H-11	H-18, H-21; H-30
13	43.7				
14	45.8				
15	44.6	1.94, 1H, m (a); 2.23, 1H, m (b)	C-8, C-13, C-16, C-17, C-30	H-16	H-7, H-18; H-30
16	72.9	5.08, 1H, brs	C-13	H-17, H-18	
17	57.2	2.53, 1H, d (8.0)	C-13, C-14, C-16, C-18, C-20, C-21, C-22	H-18	H-21, H-30
18	19.0	1.30, 3H, s	C-12, C-14, C-17		H-8, H-12 (a), H-15 (a), H-22
19	127.9	6.18, 1H, s	C-5, C-8, C-9, C-11		H-1, H-11
20	87.0				
21	28.4	1.35, 3H, s	C-18, C-22		H-12 (a), H-17, H-22 (a), H-24
22	34.9	1.68, 1H, m (a); 3.12, 1H, m (b)	C-20, C-21, C-24, C-25	H-23	H-18, H-26; H-18
23	26.4	2.08, 1H, m (a); 2.33, 1H, m (b)	C-22, C-25	H-22, H-24	H-24; H-26
24	81.8	3.93, 1H, dd (5.2, 9.0)	C-25	H-23	H-21, H-23 (a), H-26, H-27
25	71.3				
26	27.1	1.34, 3H, s	C-24, C-25, C-27		H-22 (a), H-23 (b), H-24, H-27
27	28.1	1.62, 3H, s	C-24, C-25, C-26		H-24, H-26
28	26.1	1.79, 3H, s	C-3, C-4, C-5, C-29		H-3, H-5, H-6, H-29
29	15.9	1.20, 3H, s	C-3, C-4, C-28		H-2 (a), H-28, H-6, H-8
30	17.9	0.85, 3H, s	C-8, C-15		H-12 (b), H-15 (b), H-17

[a] ^{1}H and ^{13}C NMR data were recorded in pyridine-d_5 at 400 MHz and 100 MHz, respectively.

977.70782). The ^{1}H NMR spectrum displayed two singlet signals for two tri-substituted olefinic systems (δ_H=5.66, 6.19), four oxygenated tertiary protons (δ_H=5.08, 4.30, 3.93, 3.84), and seven methyl groups in the up-field region. The absence of characteristic *AX* system proton signals in the high field region for 19-CH_2[15] implied cleavage of the 9,19-cyclopropane ring. In the ^{13}C NMR spectrum, four unsaturated carbons (δ_C=127.6, 127.9, 135.1, 138.2), and six oxygen-bearing methines (δ_C=87.0, 81.8, 77.9, 73.4, 72.9, 71.3) were detected. δ_C=71.3 and 87.0 corresponded to two quaternary carbons according to the DEPT spectrum. The major differences in ^{13}C NMR spectra of **16** from CA were signals corresponding to ring B and C carbons (Table 1).

Further analysis of the ^{1}H-^{1}H COSY and HSQC spectra resolved three spin-coupling systems: δ_H= 5.66 (δ_C=127.6)→δ_H=2.10, 2.25 (δ_C=39.1) (I), δ_H= 3.84 (δ_C=77.9)→δ_H=1.92, 2.18 (δ_C=34.7)→δ_H=2.30 (δ_C=41.1) (II), and δ_H=2.67 (δ_C=59.8)→δ_H=4.30 (δ_C=73.4)→δ_H=2.16 (δ_C=38.2)→δ_H=2.56 (δ_C= 41.9) (III). Spin system I was ascribed to a partial section of ring C according to the HMBC correlations of δ_H=2.10 and 2.25 (12-CH_2) with δ_C=44.6 (C-15)/43.7 (C-13), and δ_H=1.30 (18-CH_3) with δ_C=39.1 (C-12); II was attributed to C-3, C-2 and C-1 of ring A due to

Table 2. [1]H NMR[a] spectroscopic data for compounds **1**–**8** (*J* in Hz).

H	**1**	**2***	**3***	**4***	**5***	**6***	**7**	**8***
1	2.01, 1H, m; 2.09, 1H, m	1.90, 1H, m; 1.97, 1H, m	2.01, 1H, m; 2.05, 1H, m	2.12, 1H, m; 2.18, 1H, m	2.17, 2H, m	2.03, 1H, m; 2.16, 1H, m	1.35, 1H, m; 2.08, 1H, m	1.34, 1H, m; 2.04, 1H, m
2	1.30, 1H, m; 1.71, 1H, m	4.20, 1H, dt (4.8, 9.2)	1.37, 1H, m; 1.74, 1H, m	1.26, 1H, m; 1.71, 1H, m	1.30, 1H, m; 1.66, 1H, m	1.70, 2H, m	2.54, 1H, m; 2.78, 1H, m	2.53, 1H, m; 2.74, 1H, m
3	3.71, 1H, dd (4.6, 11.4)	3.63, 1H, d (9.2)	3.70, 1H, m	4.21, 1H, dd (5.0, 11.4)	3.89, 1H, m	4.31, 1H, d (9.2)		
5	1.78, 1H, d (9.6)	1.89, 1H, d (10.0)	1.79, 1H, d (10.0)	2.04, 1H, d (8.0)	1.82, 1H, d (8.0)	2.24, 1H, d (10.0)	2.25, 1H, d (10.0)	2.27, 1H, d (10.0)
6	3.85, 1H, m	3.81, 1H, dt (4.8, 10.0)	2.83, 1H, m	3.84, 1H, dt (3.8, 10.0)	3.97, 1H, m	3.66, 1H, dt (4.8, 11.0)	3.73, 1H, m	3.72, 1H, brs
7	1.71, 1H, m; 1.88, 1H, m	1.70, 1H, m; 1.86, 1H, m	1.81, 1H, m; 1.88, 1H, m	1.66, 1H, m; 1.89, 1H, m	1.63, 1H, m; 1.82, 1H, m	1.61, 1H, m; 1.87, 1H, m	1.66, 1H, m; 1.79, 1H, m	2.13, 2H, m
8	2.00, 1H, m	1.97, 1H, m	1.93, 1H, m	1.89, 1H, m	1.95, 1H, m	1.97, 1H, m	1.90, 1H, m	2.07, 1H, m
11	1.28, 1H, m; 2.04, 1H, m	1.27, 1H, m; 2.04, 1H, m	2.12, 1H, m; 2.44, 1H, dd (5.6, 14.8)	1.21, 1H, m; 2.09, 1H, m	1.20, 1H, m; 2.02, 1H, m	1.22, 1H, m; 2.07, 1H, m	1.05, 1H, m; 2.05, 1H, m	1.11, 1H, m; 2.04, 1H, m
12	1.71, 2H, m	1.63, 2H, m	4.26, 1H, brs	1.71, 2H, m	1.71, 2H, m	1.29, 1H, m; 1.71, 1H, m	1.68, 2H, m	1.67, 1H, m; 1.84, 1H, m
15	1.84, 1H, m; 2.18, 1H, m	1.80, 1H, m; 2.14, 1H, m	1.94, 1H, m; 2.24, 1H, m	1.83, 1H, m; 2.18, 1H, m	1.76, 1H, m; 2.13, 1H, m	1.81, 1H, m; 2.13, 1H, m	1.80, 1H, m; 2.15, 1H, m	1.75, 1H, m; 3.26, 1H, m
16	5.08, 1H, m	5.04, 1H, brs	5.01, 1H, m	5.07, 1H, brs	5.02, 1H, m	5.06, 1H, brs	5.07, 1H, m	5.27, 1H, m
17	2.60, 1H, d (7.8)	2.55, 1H, d (8.0)	3.31, 1H, d (8.0)	2.59, 1H, d (8.0)	2.52, 1H, d (8.0)	2.58, 1H, d (8.0)	2.58, 1H, d (8.0)	2.82, 1H, d (7.6)
18	1.50, 3H, s	1.45, 3H, s	1.83, 3H, s	1.48, 3H, s	1.43, 3H, s	1.47, 3H, s	1.47, 3H, s	1.59, 3H, s
19	0.39, 1H, d (4.1); 0.67, 1H, d (4.1)	0.46, 1H, d (4.1); 0.68, 1H, d (4.1)	0.52, 1H, d (4.1); 0.66, 1H, d (4.1)	0.47, 1H, d (4.1); 0.63, 1H, d (4.1)	0.41, 1H, d (4.1); 0.63, 1H, d (4.1)	0.42, 1H, d (4.1); 0.65, 1H, d (4.1)	0.41, 1H, d (4.1); 0.73, 1H, d (4.1)	0.44, 1H, d (4.1); 0.78, 1H, d (4.1)
21	1.37, 3H, s	1.34, 3H, s	1.87, 3H, s	1.38, 3H, s	1.34, 3H, s	1.38, 3H, s	1.37, 3H, s	1.37, 3H, s
22	1.75, 1H, m; 3.17, 1H, m	1.70, 1H, m; 3.12, 1H, m	2.00, 1H, m; 2.66, 1H, m	1.76, 1H, m; 3.17, 1H, m	1.74, 1H, m; 3.15, 1H, m	1.75, 1H, m; 3.16, 1H, m	1.73, 1H, m; 3.16, 1H, m	1.76, 1H, m; 3.22, 1H, m
23	2.11, 1H, m; 2.37, 1H, m	2.07, 1H, m; 2.35, 1H, m	2,12, 1H, m; 2.35, 1H, m	2.14, 1H, m; 2.37, 1H, m	2.08, 1H, m; 2.34, 1H, m	2.12, 1H, m; 2.38, 1H, m	2.09, 1H, m; 2.37, 1H, m	2.11, 1H, m; 2.38, 1H, m
24	3.93, 1H, dd (5.6, 9.0)	3.91, 1H, dd (5.6, 8.8)	4.07, 1H, m	3.94, 1H, dd (5.6, 9.0)	3.87, 1H, brs	3.94, 1H, dd (5.6, 9.0)	3.94, 1H, dd (5.6, 9.0)	3.94, 1H, dd (5.2, 9.0)
26	1.35, 3H, s	1.34, 3H, s	1.42, 3H, s	1.35, 3H, s	1.31, 3H, s	1.35, 3H, s	1.35, 3H, s	1.34, 3H, s
27	1.63, 3H, s	1.61, 3H, s	1.60, 3H, s	1.64, 3H, s	1.59, 3H, s	1.63, 3H, s	1.64, 3H, s	1.58, 3H, s
28	1.95, 3H, s	1.97, 3H, s	1.99, 3H, s	4.36, 1H, d (11.2); 4.45, 1H, d (11.2)	2.19, 3H, s	10.12, 1H, s	1.81, 3H, s	1.80, 3H, s
29	1.42, 3H, s	1.46, 3H, s	1.44, 3H, s	1.32, 3H, s	4.30, 1H, d (10.4); 4.85, 1H, d (10.4)	1.67, 3H, s	1.51, 3H, s	1.52, 3H,s
30	1.07, 3H, s	1.02, 3H, s	1.41, 3H, s	1.05, 3H, s	1.01, 3H, s	1.02, 3H, s	1.01, 3H, s	4.10, 1H, d (11.6); 4.18, 1H, d (11.6)

[a] The data were recorded at 400 MHz in pyridine-d_5; * new compound.

the correlations of 28-/29-CH_3 with C-3; III belonged to ring B involving three methines and one methylene ($\delta_{C\text{-}5}=59.8$, $\delta_{C\text{-}6}=73.4$, $\delta_{C\text{-}7}=38.2$, and $\delta_{C\text{-}8}=41.9$). The vinyl quaternary carbon at $\delta_C=135.1$ correlated with H-1, H-2, H-5 and $\delta_H=6.18$ was assigned to C-10. The other unsaturated quaternary carbon at $\delta_C=138.2$ correlated with H-8, $\delta_H=6.18$, $\delta_H=2.10$ and $\delta_H=2.25$ was ascribed to C-9. The HMBC correlations of $\delta_H=6.18$ with C-5, C-8, C-9 and $\delta_C=127.6$ indicated that it connected the above three spin-coupling systems. Thus, ring B was deduced as a seven-membered ring ($\delta_C=127.9$, 135.1, 59.8, 73.4, 38.2, 41.9, and 138.2), and the $\delta_H=6.18$ signal was assigned to H-19. It could be assumed that during the biocatalytic process, the 9,19-propane ring of cycloastragenol was cleaved and the B ring expanded to form an unusual 9(10)a-homo-19-nor-cycloartane skeleton. A similar 9,10-seco-cycloartane sapogenin (secomacorogenin B) had been reported from *Astragalus* species by Isaev and co-workers.[16]

Table 3. [1]H NMR[a] spectroscopic data for compounds **9**–**15** (*J* in Hz).

H	**9***	**10***	**11***	**12**	**13***	**14***	**15***
1	2.04, 1H, m; 2.11, 1H, m	1.99, 1H, m; 2.10, 1H, m	2.08, 1H, m; 2.17, 1H, m	1.94, 1H, m; 2.97, 1H, m	1.96, 1H, m; 2.94, 1H, brs	1.97, 1H, m; 2.94, 1H, d (8.4)	1.97, 1H, m; 2.94, 1H, d (8.4)
2	1.43, 1H, m; 2.47, 1H, m	1.53, 1H, m; 1.74, 1H, m	1.26, 1H, m; 2.02, 1H, m	1.94, 1H, m; 2.10, 1H, m	1.97, 1H, m; 2.23, 1H, m	1.97, 1H, m; 2.22, 1H, m	1.97, 1H, m; 2.22, 1H, m
3	3.72, 1H, brs	3.70, 1H, brs	4.23, 1H, dd (4.8, 11.4)	3.80, 1H, brs	3.84, 1H, brs	3.84, 1H, brs	3.84, 1H, brs
5	1.92, 1H, d (9.6)	1.74, 1H, m	2.02, 1H, d (10.0)	2.34, 1H, d (6.2)	2.35, 1H, d (6.4)	2.35, 1H, d (7.4)	2.35, 1H, d (7.4)
6	4.07, 1H, m	3.98, 1H, m	3.85, 1H, m	4.20, 1H, m	4.21, 1H, brs	4.20, 1H, m	4.20, 1H, m
7	1.90, 1H, m; 2.06, 1H, m	1.79, 1H, m; 1.98, 1H, m	1.66, 1H, m; 1.86, 1H, m	1.75, 1H, m; 2.11, 1H, m	1.76, 1H, m; 2.10, 1H, m	1.74, 1H, m; 2.10, 1H, m	1.74, 1H, m; 2.10, 1H, m
8	2.55, 1H, t (8.4)	2.42, 1H, m	1.81, 1H, m	2.67, 1H, m	2.68, 1H, d (11.2)	2.68, 1H, d (11.0)	2.68, 1H, d (11.0)
11		5.48, 1H, d (10.0)	1.17, 1H, m; 2.07, 1H, m	3.38, 1H, brs	3.39, 1H, brs	3.39, 1H, brs	3.39, 1H, brs
12	2.66, 1H, d (16.2); 2.74, 1H, d (16.2)	6.26, 1H, d (10.0)	1.62, 1H, m; 2.36, 1H, m	1.65, 1H, m; 2.67, 1H, m	1.78,1H, m; 2.38, 1H, d (13.8)	1.81, 1H, m; 2.40, 1H, d (13.8)	1.81, 1H, m; 2.40, 1H, d (13.8)
15	1.93, 1H, m; 2.26, 1H, m	1.88, 1H, m; 2.24, 1H, m	1.66, 1H, m; 1.97, 1H, m	1.94, 1H, m; 2.16, 1H, m	1.93, 1H, m; 2.16, 1H, m	1.93, 1H, m; 2.16, 1H, m	1.93, 1H, m; 2.16, 1H ,m
16	5.10, 1H, brs	5.14, 1H, brs	4.54, 1H, brs	5.08, 1H, m	5.07, 1H, brs	5.08, 1H, dd (6.4, 13.8)	5.08, 1H, dd (6.4, 13.8)
17	2.72, 1H, d (7.6)	2.84, 1H, d (8.0)	2.69, 1H, d (8.0)	2.57, 1H ,d (8.0)	2.58, 1H, d (8.0)	2.58, 1H, d (7.8)	2.58, 1H, d (7.8)
18	1.33, 3H, s	1.44, 3H, s	1.41, 3H, s	1.55, 3H, s	1.48, 3H, s	1.48, 3H, s	1.48, 3H, s
19	1.12, 1H, d (4.1); 1.83, 1H, d (4.1)	0.63, 1H, d (3.8); 0.97, 1H, d (3.8)	0.43, 1H, d (4.1); 0.64, 1H, d (4.1)	3.94, 1H, m; 4.05, 1H, brs	3.61, 2H, m	3.66, 2H, m	3.66, 2H, m
21	1.30, 3H, s	1.49, 3H, s	1.54, 3H, s	1.41, 3H, s	1.45, 3H, s	1.41, 3H, s	1.41, 3H, s
22	1.68, 1H, m; 3.07, 1H, m	1.75, 1H, m; 3.22, 1H, m	2.05, 1H, m; 2.69, 1H, m	1.59, 1H, m; 3.12. 1H, m	1.69, 1H, m; 3.17, 1H, m	1.68, 1H, m; 3.16, 1H, m	1.68, 1H, m; 3.16, 1H, m
23	2.06, 1H, m; 2.33, 1H, brs	2.10, 1H, m; 2.38, 1H, m	1.63, 1H, m; 1.70, 1H, m	2.04, 1H, m; 2.29, 1H, m	2.07, 1H, m; 2.33, 1H, m	2.08, 1H, m; 2.34, 1H, m	2.08, 1H, m; 2.34, 1H, m
24	3.91, 1H, dd (5.6, 9.0)	3.96, 1H, brs		3.89, 1H, dd (5.2, 9.0)	3.92, 1H, dd (5.2, 9.0)	3.91, 1H, dd (5.2, 9.0)	3.91, 1H, dd (5.2, 9.0)
26	1.34, 3H, s	1.35, 3H, s	1.61, 3H, s	1.34, 3H, s	1.35, 3H, s	1.34, 3H, s	1.34, 3H, s
27	1.62, 3H, s	1.64, 3H, s	1.69, 3H, s	1.67, 3H, s	1.63, 3H, s	1.63, 3H, s	1.63, 3H, s
28	1.97, 3H, s	2.04, 3H, s	4.37, 1H, d (11.2); 4.46, 1H, d (11.2)	1.86, 3H, s	1.86, 3H, s	2.04, 3H, s	2.04, 3H, s
29	1.40, 3H, s	1.48, 3H, s	1.35, 3H, s	1.16, 3H, s	1.18, 3H, s	1.17, 3H, s	1.17, 3H, s
30	1.26, 3H, s	1.00, 3H, s	0.98, 3H, s	0.89, 3H, s	0.85, 3H, s	0.85, 3H, s	0.85, 3H, s
1′					3.47, 1H, m; 3.59, 1H, m	4.17, 2H, m	4.17, 2H, m
2′					1.66, 2H, m	5.59, 1H, s	5.59, 1H, s
3′					1.50, 2H, m		
4′					0.94, 3H, t (7.3)	1.70, 3H, s	1.70, 3H, s
5′						1.73, 3H, s	1.73, 3H, s

[a] The data were recorded at 400 MHz in pyridine-d_5; * new compound.

The NOE correlations of H-3/28-CH_3, 29-CH_3/H-6, H-6/H-8, H-8/18-CH_3 and 30-CH_3/H-17 confirmed that the *trans*-conjunctions of A/B, B/C and C/D rings of compound **16** were the same as those of cycloastragenol. The NOE correlation between H-24 and 21-CH_3 verified the *S*-configuration of C-24. Based on the above evidences, the structure of compound **16** was established as (20*R*,24*S*)-3β,6α,16β,25-tetraroxy-

Table 4. ^{13}C NMR[a] spectroscopic data for compounds **1**–**15**.

C	1	2*	3*	4*	5*	6*	7	8*	9*	10*	11*	12	13*	14*	15*
1	31.5	41.4	31.5	31.2	31.7	30.4	31.8	31.8	31.2	31.0	31.1	29.3	29.3	29.3	29.3
2	32.8	71.2	33.1	32.7	32.7	33.2	35.8	35.9	28.2	32.7	32.6	33.1	33.2	33.1	33.1
3	78.3	84.0	78.3	72.3	80.2	71.1	216.8	216.8	77.8	77.9	72.3	77.4	77.3	77.3	77.3
4	42.4	42.5	42.5	46.2	45.0	55.8	50.5	50.5	42.6	42.2	46.5	42.3	42.4	42.4	42.4
5	54.0	54.1	54.3	49.7	54.2	50.6	53.5	53.6	53.5	53.6	49.6	57.7	57.7	57.7	57.8
6	68.3	68.2	69.1	67.8	68.7	66.8	69.1	69.2	65.7	68.3	67.5	67.7	67.6	67.7	67.5
7	38.8	38.8	39.6	38.0	39.1	37.5	38.4	38.9	36.4	38.7	38.0	37.4	37.3	37.3	37.3
8	47.3	47.1	48.4	47.4	47.4	47.8	48.2	48.3	41.9	45.8	46.6	41.1	41.0	41.0	40.9
9	21.0	20.2	21.2	20.5	22.0	20.3	21.2	21.3	39.7	25.5	20.2	132.9	132.4	132.5	131.2
10	29.9	28.6	30.0	29.1	29.4	28.5	28.5	28.5	35.0	33.3	29.5	134.9	135.2	135.3	136.8
11	26.3	26.5	38.7	26.0	26.2	26.1	26.1	27.2	210.2	131.7	26.3	39.5	35.8	35.9	34.8
12	33.4	33.3	72.8	33.4	33.3	32.2	33.2	32.6	53.3	135.2	33.5	33.3	33.9	34.0	33.8
13	45.0	45.0	50.7	45.0	45.0	45.0	45.0	45.7	45.6	47.1	44.6	44.9	44.7	44.7	44.5
14	46.2	46.2	46.3	46.5	46.1	46.3	46.0	51.5	45.9	48.2	45.7	45.5	45.4	45.4	45.2
15	46.8	46.7	49.8	47.0	46.8	46.8	47.0	39.9	45.2	45.0	43.3	45.1	45.1	45.1	45.0
16	73.4	73.4	72.8	73.4	73.4	73.3	73.4	73.9	72.9	73.8	74.1	73.4	73.3	73.3	73.2
17	58.4	58.4	52.2	58.5	58.4	58.5	58.5	58.7	56.8	54.1	61.8	58.2	58.2	58.2	58.1
18	21.6	21.6	21.1	21.9	21.7	21.8	22.1	23.3	20.8	21.4	22.6	20.2	20.2	20.2	20.0
19	31.0	31.1	31.7	31.6	31.4	31.2	31.0	30.7	27.7	28.6	32.0	68.6	77.3	76.4	70.1
20	87.2	87.2	87.5	87.2	87.2	87.2	87.2	87.3	86.7	87.5	84.8	87.3	87.3	87.2	87.1
21	28.2	28.2	27.5	28.2	28.2	28.2	28.5	28.5	28.5	28.7	30.4	28.6	28.6	28.7	28.6
22	34.9	34.9	38.3	35.0	34.9	34.9	34.9	35.0	34.9	35.0	31.8	34.7	34.9	34.8	34.8
23	26.4	26.5	26.0	26.4	26.4	26.4	26.4	26.4	26.4	26.4	33.2	26.4	26.4	26.4	26.4
24	81.7	81.7	83.5	81.7	81.7	81.7	81.7	81.8	81.8	81.8	110.7	81.6	81.6	81.6	81.7
25	71.2	71.2	70.8	71.2	71.2	71.2	71.2	71.2	71.2	71.2	72.8	71.3	71.3	71.3	71.3
26	27.1	27.1	27.1	27.1	27.1	27.2	27.1	27.1	27.1	27.1	25.3	27.1	27.1	27.1	27.1
27	28.6	28.7	27.5	28.2	28.2	28.5	28.2	28.1	28.2	28.1	25.6	28.1	28.1	28.1	28.1
28	29.4	29.9	29.7	67.8	24.7	204.1	28.6	28.5	29.0	29.6	67.8	27.3	27.3	27.3	27.2
29	16.1	17.2	16.3	12.4	65.1	8.6	20.4	20.4	15.5	15.8	12.4	15.2	15.2	15.1	15.1
30	20.2	20.2	22.0	20.4	20.2	19.8	20.4	63.1	19.7	22.0	20.1	19.5	19.4	19.4	19.4
1′													70.2	66.9	170.9
2′													32.2	122.6	21.0
3′													19.8	136.3	
4′													14.0	25.7	
5′														18.0	

[a] The data were recorded at 100 MHz in pyridine-d_5; * new compound.

20,24-epoxy-9(10)a-homo-19-nor-cycloartane (Figure 1). It was named neoastragenol. The key HMBC, ^1H-^1H COSY and NOE correlations are illustrated in Figure 2.

Hydroxylation Products 2–5

Four monohydroxylated metabolites (**2**, **3**, **4**, **5**) were identified with the same molecular formula $C_{30}H_{50}O_6$ based on their HR-ESI-MS data.

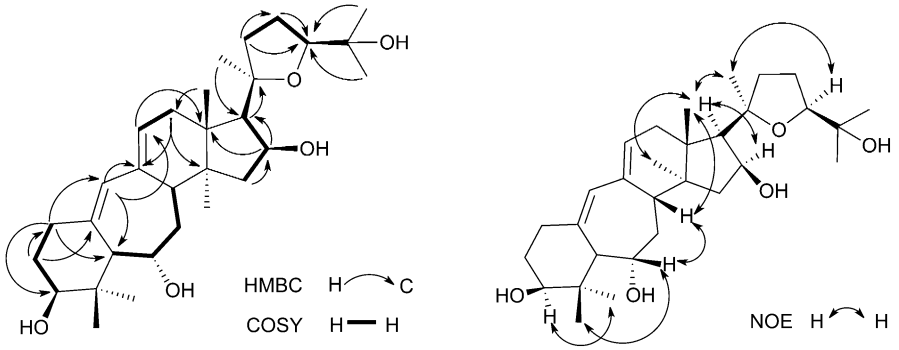


Figure 2. Key HMBC, ^1H-^1H COSY and NOE correlations of compound **16**.

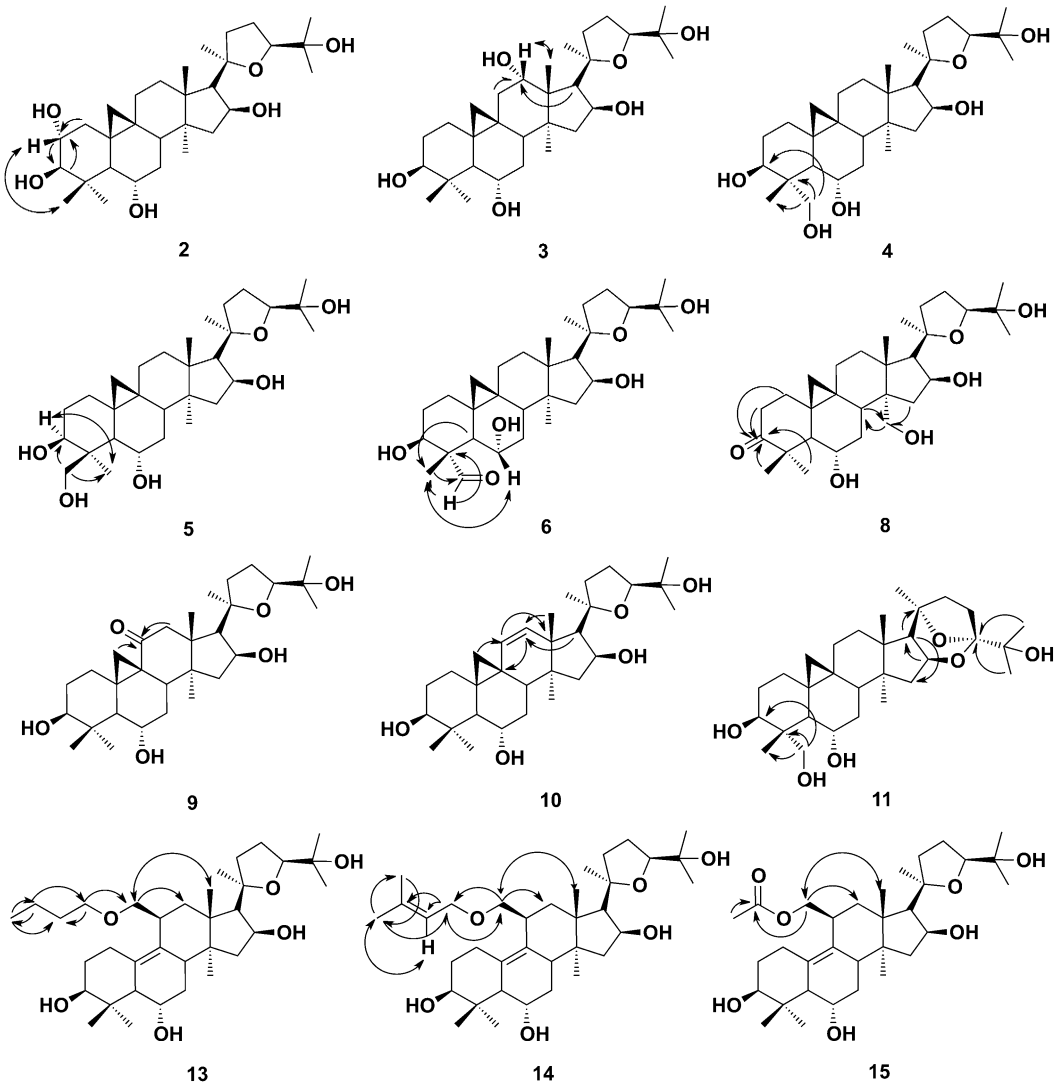


Figure 3. Key HMBC and NOE correlations of compounds **2**–**6**, **8**–**11**, and **13**–**15.**

The ^{1}H NMR spectra of **2** and **3** both displayed one more down-field oxygenated tertiary proton than **1**, suggesting that hydroxylation occurred on a methylene carbon. Further comparison of the ^{13}C NMR data of **2** with those of **1** revealed significant down-field shifts of C-1 (+9.9 ppm) and C-3 (+5.7 ppm), indicating the hydroxylation at C-2 (δ_C = 71.2). This was confirmed by the HMBC correlations of H-2/C-3, H-1/C-2 and H-3/C-2. The NOE correlation of H-2/29-CH_3 suggested the α-configuration of 2-OH (Figure 3). Therefore, compound **2** was characterized as (20*R*,24*S*)-2α,3β,6α,16β,25-pentahydroxy-20,24-epoxy-cycloartane.

The additional hydroxy group of compound **3** was speculated to be at C-12 according to the significant down-field shifts of C-11 (+12.4 ppm) and C-13 (+5.6 ppm) when compared to cycloastragenol. This was confirmed by the HMBC correlations of H-11/C-12, H-17/C-12, and 18-CH_3/C-12. The NOE correlation of H-12/18-CH_3 indicated the α-configuration of 12-OH (Figure 3). Thus, compound **3** was identified as (20*R*,24*S*)-3β,6α,12α,16β,25-pentahydroxy-20,24-epoxy-cycloartane.

Different from **2** and **3**, the ^{1}H NMR spectra of **4** and **5** both showed two additional down-field proton signals which formed AB coupling systems. In the meanwhile, one methyl signal of cycloastragenol has disappeared. The new proton signals of compound **4** appeared at δ_H = 4.36 and 4.45 (J = 11.2 Hz). In the ^{13}C NMR spectrum of compound **4**, significant changes of C-3 (−6 ppm), C-4 (+3.8 ppm) and C-5 (−4.3 ppm) were observed when compared with cycloastragenol. This evidence indicated the presence of 28- or 29-OH, which was also suggested by the HMBC correlations of 28-H_2 with C-3, C-4 and C-5 (Figure 3). According to the NOE correlation of H-6/29-CH_3, the additional hydroxy group was determined to be at C-28. Therefore, compound **4** was identified

as (20*R*,24*S*)-3β,6α,16β,25,28-pentahydroxy-20,24-epoxy-cycloartane. Likewise, the additional hydroxy group of compound **5** was determined to be at C-29 according to the NOE correlation between H-3 and 28-CH_3 ($\delta_H = 2.19$). Therefore, the structure of **5** was established as (20*R*,24*S*)-3β,6α,16β,25,29-pentahydroxy-20,24-epoxy-cycloartane.

Carbonylation Products 6–9

Four carbonylated metabolites (**6**, **7**, **8**, **9**) were identified according to the characteristic down-field carbonyl signals ($\delta_C > 200$ ppm) in their ^{13}C NMR spectra.

The molecular formula of **6** was determined as $C_{30}H_{48}O_6$ based on its HR-ESI-MS data ($m/z =$ 1009.69873, calcd. for $[2M+H]^+$: 1009.69745). The strong IR absorption at 1717 cm^{-1} indicated the presence of a carbonyl group. The ^1H NMR spectrum exhibited an aldehyde proton signal at $\delta_H = 10.12$ (1H, s) together with six methyl signals, indicating that one methyl group of cycloastragenol was converted to a formyl group. When compared to cycloastragenol, the ^{13}C NMR spectrum of compound **6** displayed significant changes for C-3 (−7.2 ppm), C-4 (+13.4 ppm) and C-5 (−3.4 ppm). Thus, it could be deduced that either 28- or 29-CH_3 was converted to aldehyde. This deduction was supported by the HMBC correlations of H-5 ($\delta_H = 1.82$) and 28-/29-CH_3 ($\delta_H = 1.67$) with $\delta_C = 204.1$, as well as $\delta_H = 10.12$ with C-28/29 ($\delta_C =$ 8.6) and C-4 ($\delta_C = 55.8$). The NOE correlation of H-6/29-CH_3 confirmed the presence of the 28-aldehyde group (Figure 3). Therefore, the structure of compound **6** was established as (20*R*,24*S*)-3β,6α,16β,25-tetrahydroxy-20,24-epoxy-cycloartan-28-carbaldehyde.

Compound **7** was characterized as the previously reported cyclopycnanthogenin [(20*R*,24*S*)-6α,16β,25-trihydroxy-20,24-epoxy-cycloartan-3-one] by comparing its NMR data with the literature values.[17]

The HR-ESI-MS of compound **8** provided evidence for the molecular formula of $C_{30}H_{48}O_6$ ($m/z =$ 1009.69461, calcd. for $[2M+H]^+$: 1009.69745). In the ^1H NMR spectrum, the signal for one methyl group of cycloastragenol disappeared and two new signals corresponding to a CH_2 group were observed ($\delta_H = 4.10$, d, $J = 11.6$ Hz; $\delta_H = 4.16$, d, $J = 11.6$ Hz). It could thus be inferred that compound **8** was a derivative of **7**, with a hydroxy group introduced at one methyl group. The ^{13}C NMR spectral data were very similar to those of **7** except for C-14 (+5.5 ppm) and C-15 (−7.1 ppm), indicating that the new hydroxy group was substituted at C-30. The HMBC correlations of H-30/C-8, H-15/C-30 and H-8/C-30 further confirmed this deduction (Figure 3). Based on the above evidence, compound **8** was identified as (20*R*,24*S*)-6α,16β,25,30-tetrahydroxy-20,24-epoxy-cycloartan-3-one.

Compound **9** was characterized as an isomer of **6** according to its HR-ESI-MS data ($m/z = 1009.69791$, calcd. for $[2M+H]^+$: 1009.69745). According to the DEPT spectrum, one CH_2 signal of cycloastragenol disappeared, and it could be converted to a carbonyl group. The ^{13}C NMR spectrum also showed significant changes for C-9 (+18.7 ppm), C-10 (+5.1 ppm) and C-12 (+19.9 ppm) when compared to cycloastragenol, indicating that the carbonyl group ($\delta_C = 210.2$) was located at C-11. The HMBC correlations of H-12/C-11 and H-19/C-11 further confirmed this deduction (Figure 3). In accordance, the characteristic H-19 signals ($\delta_H = 1.83$ and 1.12, $J = 4.1$ Hz) shifted down-field remarkably due to a de-shielding effect of the carbonyl group. Therefore, compound **9** was identified as (20*R*,24*S*)-3β,6α,16β,25-tetrahydroxy-20,24-epoxy-cycloartan-11-one.

Dehydrogenation (10) and Cyclization (11) Products

The molecular formula ($C_{30}H_{48}O_5$) of the dehydrogenation product (**10**) was established by HR-ESI-MS data ($m/z = 1466.05808$, calcd. for $[3M+H]^+$: 1466.05780). Two *cis*-olefinic proton signals ($\delta_H = 5.48$, d, $J = 10.0$ Hz; $\delta_H = 6.26$, d, $J = 10.0$ Hz) in the ^1H NMR spectrum indicated the presence of a double bond. When compared to cycloastragenol, the ^{13}C NMR spectrum showed significant changes for C ring carbons (C-9, C-13, C-14, and C-15). Thus, the double bond should be located at C-11 and C-12. This deduction was further confirmed by the HMBC correlations of H-17/C-12, 18-CH_3/C-12, H-12/C-9 and H-11/C-13 (Figure 3). Therefore, compound **10** was characterized as (20*R*,24*S*)-3β,6α,16β,25-tetrahydroxy-20,24-epoxy-cycloartan-11(12)-ene.

Compound **11** was identified as a 16,24-epoxy derivative of cycloastragenol. The HR-ESI-MS revealed its molecular formula as $C_{30}H_{48}O_6$ ($m/z = 505.35218$, calcd. for $[M+H]^+$: 505.35236). Its ^1H NMR spectrum displayed two new proton signals due to a CH_2 group ($\delta_H = 4.37$ and 4.46, $J = 11.2$ Hz), suggesting that one methyl group of cycloastragenol was hydroxylated. The carbon signals for ring A were almost identical to those of compound **4**, suggesting that the hydroxy group was introduced at C-28. This deduction was confirmed by the HMBC correlations of H-28/C-3 and H-28/C-4, as well as the NOE correlation of 29-CH_3/H-6. When compared to **4**, the signal for H-24 had disappeared. In contrast, a new down-field quaternary carbon signal at $\delta_C = 110.7$ corresponding to an acetal carbon was observed in the ^{13}C NMR spectrum. Considering that **11** had one more unsaturation degree than **4**, it could be deduced that a new ring linking C-16 and C-24 was formed in compound **11**. Although the HMBC correlation of H-16/C-24 was not detected, the ^{13}C NMR data of the 16,24;20,24-di-

epoxy sub-structure complied very well with those of the known cycloalpigenin [(20*R*,24*R*)-16β,24:20,24-diepoxy-cycloartane-3β,7β,25-triol].[18] Therefore, we deduced the *R*-configuration of C-24. Based on the above evidence, compound **11** was identified as (20*R*,24*R*)-3β,6α,25,28-tetrahydroxy-16β,24:20,24-diepoxy-cycloartane.

Rearrangement Products 12–15

The characteristic 9,19-cyclopropane ring of cycloastragenol were cleaved in four biotransformed metabolites (**12**, **13**, **14**, **15**) as the signals corresponding to 19-CH_2 disappeared. They were elucidated to possess the unusual ranunculane skeleton through a complicated rearrangement.[14] To the best of our knowledge, only two compounds with this novel skeleton had been reported so far.[14,19]

Compound **12** possessed the molecular formula of $C_{30}H_{50}O_6$ according to its HR-ESI-MS data (*m/z* = 1013.73266, calcd. for $[2M+H]^+$: 1013.72875). By comparing its ^1H and ^{13}C NMR data with those in the literature, it was identified as the known (20*R*,24*S*)-3β,6α,16β,19,25-pentahydroxy-ranunculan-9(10)-ene (spectra measured in CD_3OD).[14]

The molecular formula of compound **13** was established as $C_{34}H_{58}O_6$ based on its HR-ESI-MS data (*m/z*=1125.85414, calcd. for $[2M+H]^+$: 1125.85395). Its ^1H and ^{13}C NMR data were very similar to those of **12** except that C-19 ($\delta_C = 76.4$) was shifted down-field by 8.7 ppm, and that it showed additional signals corresponding to one methyl ($\delta_H = 0.94$, t, $J = 7.3$ Hz; $\delta_C = 14.0$) and three methylenes [($\delta_H = 1.50$, m; $\delta_C = 19.8$), ($\delta_H = 1.66$, m; $\delta_C = 32.2$), ($\delta_H = 3.47$, m, $\delta_H = 3.59$, m; $\delta_C = 70.2$)]. These carbons were numbered as 4′, 3′, 2′, 1′ from up-field to down-field, respectively. This sub-structure was characterized as an *n*-butyl group linked to an oxygen atom, according to the HMBC correlations of H-4′/C-3′, H-4′/C-2′, H-3′/C-4′, H-3′/C-1′, H-1′/C-3’ and H-1′/C-2′. The HMBC correlations of H-1′/C-19 and H-19/C-1’ indicated that the *n*-butyl group was attached to 19-OH of the ranunculane skeleton. The NOE correlation of H-19/18-CH_3 further confirmed the β-configuration of the side chain at C-11 (Figure 3). Therefore, compound **13** was identified as (20*R*,24*S*)-3β,6α,16β,25-tetrahydroxy-19-butoxy-ranunculan-9(10)-ene.

The HR-ESI-MS data of **14** established its molecular formula as $C_{35}H_{58}O_6$ (*m/z* = 575.43122, calcd. for for $[M+H]^+$: 575.43061). According to its ^1H and ^{13}C NMR spectra, compound **14** should also contain a ranunculane skeleton. Aside from signals attributed to the triterpene skeleton, additional signals corresponding to two methyls [$\delta_H = 1.73$ ($\delta_C = 25.7$), $\delta_H = 1.70$ ($\delta_C = 18.0$)], two vinyl carbons ($\delta_C = 136.3$, 122.6) and one methylene ($\delta_H = 4.17$; $\delta_C = 66.9$) were also observed. They were numbered as 5′, 4′, 3′, 2’ and 1′, respectively. This sub-structure was characterized as an isopentenyl group according to the HMBC correlations of H-4′/C-5′, H-4′/C-1′, H-5′/C-4′, H-1′/C-2’ and H-1′/C-3′. The isopentenyl group was connected to C-19 through an ether bond, according to the HMBC correlations of H-1′/C-19 and H-19/C-1′. The HMBC correlation of H-19/C-12 and the NOE correlation of H-19/18-CH_3 confirmed the 19β-side chain was connected to C-11 (Figure 3). Finally, compound **14** was identified as (20*R*,24*S*)-3β,6α,16β,25-tetrahydroxy-19-isopentenyloxy-ranunculan-9(10)-ene.

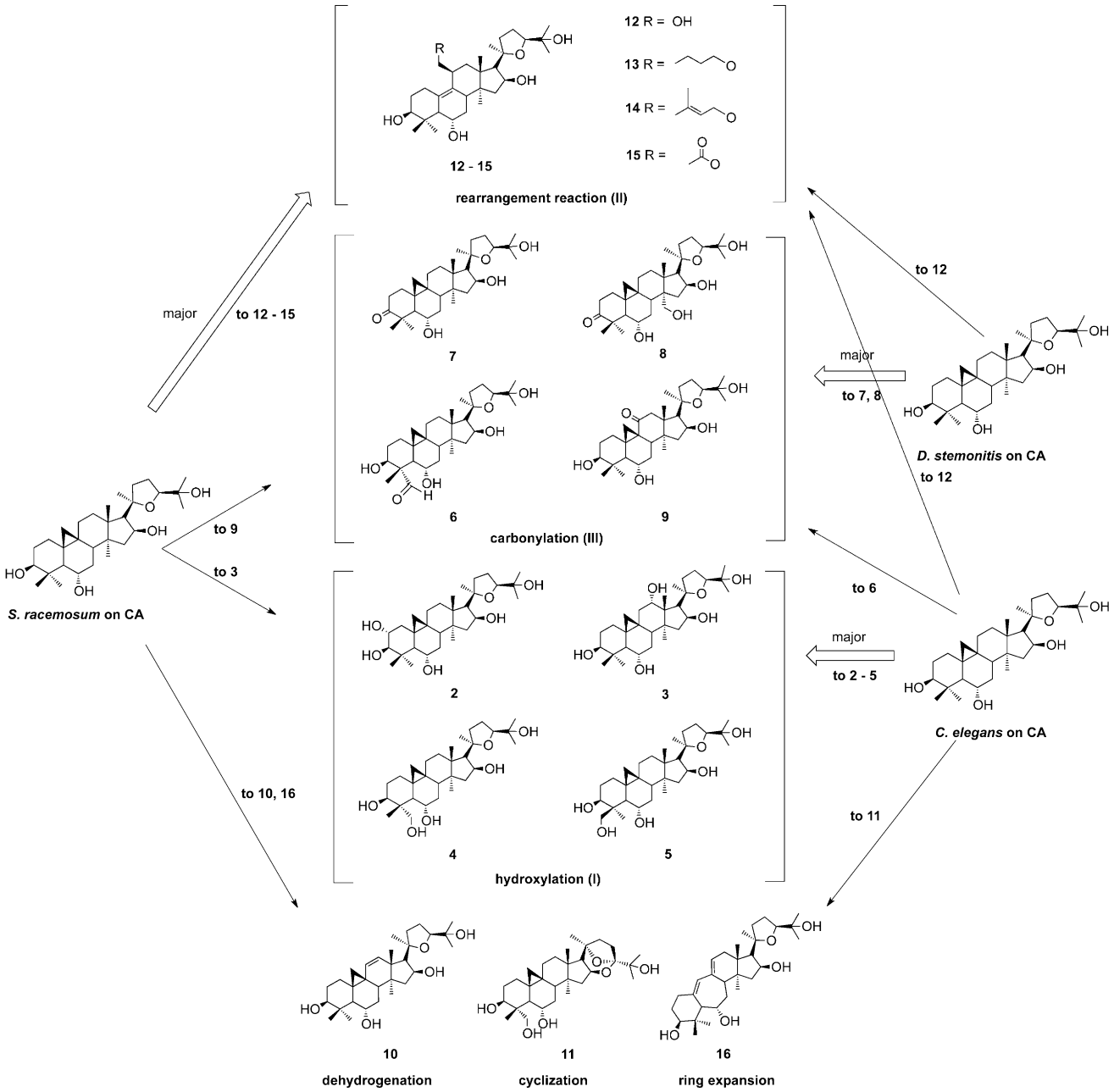
The molecular formula of compound **15** was established as $C_{32}H_{52}O_7$ on the basis of its HR-ESI-MS data (*m/z* = 549.37849, calcd. for $[M+H]^+$: 549.37858). The IR absorption at 1739 cm^{-1} indicated the presence of an ester carbonyl group. The ^1H and ^{13}C NMR spectra showed signals attributed to the ranunculane skeleton, together with signals for one carbonyl group ($\delta_C = 170.9$) and one methyl group ($\delta_H = 2.13$). The latter two signals indicated the presence of an acetoxy group. The acetoxy group was connected to C-19 ($\delta_C = 70.1$) based on the HMBC correlation of H-19/C-1′. Moreover, the 19β-acetoxy substituent attached to C-11 of the triterpene skeleton was confirmed by the HMBC correlation of H-19/C-12 and the NOE correlation of H-19/18-CH_3. Based on the above evidence, compound **15** was characterized as (20*R*,24*S*)-3β,6α,16β,25-tetrahydroxy-19-acetoxy-ranunculan-9(10)-ene.

Biocatalytic Features of the Three Fungal Strains

The biocatalysis of three strains of filamentous fungi (*Cunninghamella elegans*, *Syncephalastrum racemosum* and *Doratomyces stemonitis*) on cycloastragenol showed significant preferences. The biotransformation reactions mainly involved hydroxylation (type I), rearrangement (type II), and carbonylation (type III). In addition, dehydrogenation, cyclization, and a novel ring expansion reaction were also observed (Scheme 1).

C. elegans preferred to catalyze hydroxylation reactions, particularly on 28- and 29-CH_3 (**4**, 10.00% yield; **5**, 10.00%). Hydroxylation on methylene was also observed as minor reactions (C-2 for **2**, 0.40% yield; C-12 for **3**, 0.50%), which showed α-stereoselectivity. The total percentage conversion of hydroxylation reaction added up to more than 20%. In addition, *C. elegans* could also catalyze carbonylation (**6**, 2.00%), rearrangement (**12**, 1.20%), and the rare 16,24-cyclization reaction (**11**, 0.30%).

S. racemosum displayed a strong capacity to catalyze the complicated rearrangement reaction to produce the unusual ranunculane skeleton. Compound **12** was obtained in a yield of 17.14%. Moreover, *n*-



Scheme 1. Biocatalytic reactions of *Cunninghamella elegans* AS 3.1207, *Syncephalastrum racemosum* AS 3.264 and *Doratomyces stemonitis* AS 3.1411 on cycloastragenol (CA, **1**).

butylated (**13**, 0.48%), isopentenylated (**14**, 0.33%) and acetylated (**15**, 0.71%) derivatives of compound **12** were also produced. The total percentage conversion of the arrangement reaction reached 18.7%. *S. racemosum* could also catalyze hydroxylation (**3**, 0.48%), carbonylation (**9**, 1.48%), and dehydrogenation (**10**, 0.17%) reactions on cycloastragenol. It is particularly noteworthy that *S. racemosum* realized the unexpected ring expansion reaction to produce the unusual 9(10)a-homo-19-nor-cycloartane triterpene skeleton (**16**, 0.26%).

D. stemonitis tended to catalyze the carbonylation reaction on cycloastragenol. The carbonylation at 3-OH was the major reaction as compound **7** was obtained in a high yield of 44.24%. The combination of hydroxylation and carbonylation yielded compound **8** (0.27% yield). The rearranged product **12** was also obtained in 0.33% yield.

Some of the above biocatalytic reactions, such as rearrangement, cyclization, and ring expansion could be difficult for chemical approaches. In addition, each of the three fungal strains exhibited its biocatalytic preference, which could be used to synthesize differ-

ent types of cycloastragenol derivatives. It is also noteworthy that all three strains could convert cycloastragenol into compound **12** through a rearrangement reaction. Kuban et al reported the same reaction by a *Cunninghamella* species very recently.[14] It appeared that different fungal strains contained similar enzymes that catalyzed this complicated rearrangement reaction.

Conclusions

In summary, the biocatalysis of three strains of filamentous fungi on cycloastragenol was carried out to obtain 15 transformed products, including 13 new compounds. *Cunninghamella elegans*, *Syncephalastrum racemosum*, and *Doratomyces stemonitis* preferred to catalyze hydroxylation, rearrangement, and carbonylation reactions, respectively. In addition, an unexpected ring expansion product was obtained from *C. elegans*. These biocatalytic reactions could be difficult for chemical approaches. The synthesis of an array of novel cycloastragenol derivatives allows for the future study on telomerase activation activity-structure relationships for the discovery of anti-aging drug candidates.

Experimental Section

Apparatus and Reagents

Melting points were determined on an XT4A apparatus (uncorrected). A Perkin-Elmer 243B polarimeter was used to measure the optimal rotations at 20 °C. IR spectra were obtained with a Thermo Nicolet Nexus 470 FT-IR spectrometer. ^{1}H, ^{13}C and 2D NMR experiments were performed on a Bruker Avance III (400 MHz) NMR spectrometer in pyridine-d_5. The HR-ESI-MS data were acquired on a Bruker APEX II Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. Column chromatography was performed on silica gel (200–300 mesh, Qingdao Marine Chemical Corporation, Qingdao, China), ODS (Fuji Silysia Chemical Ltd., Kasugai, Japan), and Sephadex LH-20 (Pharmacia Biotech AB, Sweden).

Astragaloside IV (purity > 98%, Sanleng Biotechnological Co. Ltd., Guilin, China), sodium periodate (analytical grade, Sinopharm Chemical Reagent Co. Ltd., China), glucose, methanol, acetonitrile (analytical grade, Beijing Chemical Factory, China), ultra-pure water (Millipore, Bedford, MA, USA), and potatoes freshly purchased from the local supermarket were used for the biotransformation experiments.

All the 13 fungal strains were purchased from China General Microbiological Culture Collection Center. Fermentations were carried out in potato media consisting of 20 g of potato extract (prepared from 200 g of potato slices extracted in boiling water for 30 min), 20 g of glucose, and 1000 mL of water. The media were sterilized at 121 °C and 1.06 kg/cm^2 for 30 min.[20] The strains were maintained on potato dextrose agar slants at 4 °C.

Preparation of Cycloastragenol

Cycloastragenol (CA) was prepared from astragaloside IV by Smith degradation.[21,22] Typically, an amount of 1 g astragaloside IV was dissolved in 90 mL methanol, 60 mL water containing 5 g $NaIO_4$ was then added, and the solution was stirred overnight at room temperature. The reaction mixture was concentrated to about 50 mL under reduced pressure, and then extracted by an equal volume of ethyl acetate for three times. The separated ethyl acetate layer was washed with an equal volume of ethyl acetate-saturated water twice, and the organic layer was evaporated to dryness. The residue was dissolved in 80 mL 75% methanol, and 1 g $NaBH_4$ was added. The solution was left to stand for 24 h at room temperature, 80 mL water were added, the pH adjusted to 2.0 with 1 mol/L H_2SO_4, then the mixture was allowed to stand for another 24 h. The reaction mixture was extracted with 150 mL ethyl acetate thrice, and the ethyl acetate layer was washed with ethyl acetate-saturated water thrice. The organic layer was then evaporated to dryness to yield the crude product; yield: 750 mg. The crude product was separated by silica gel column chromatography and eluted with chloroform-methanol (50:1, 30:1, v/v). Following TLC detection, the 30:1 fraction afforded CA; yield: 384 mg. The purity was above 98% as detected by HPLC-RI. The structure was identified by ^{1}H and ^{13}C NMR. Following the above procedure, a total of 5 g CA was prepared for biotransformation experiments.

Screening of Fungal Strains

A total of 13 different fungal strains, including *Alternaria alternata* AS 3.4578, *Doratomyces stemonitis* AS 3.1411, *Fusarium avenaceum* AS 3.4594, *Mucor spinosus* AS 3.3450, *Rhizopus chinensis* CICC 3043, *Syncephalastrum racemosum* AS 3.264, *Cunninghamella elegans* AS 3.1207, *Mucor fragilis* AS 3.2215, *Phyllosticta pirina* AS 3.2886, *Colletotrichum lini* AS 3.4486, *Fusarium sambucinum* AS 3.4602, *Rhizoctonia solani* AS 3.2888, and *Saccharomyces cerevisiae* ACCC 2168 were screened in the preliminary test for their abilities to metabolize cycloastragenol.

Scaled-Up Biotransformation Experiments

The fungal strains (*Cunninghamella elegans*, *Syncephalastrum racemosum* and *Doratomyces stemonitis*) had been sub-cultured for three times on potato dextrose agar slants before use to obtain maximal enzyme activities. The pre-activated fungi were incubated in a 250-mL flask (containing 100 mL potato culture medium) for two days to obtain a stock inoculum. The scaled-up biotransformation fermentations were carried out in 1000-mL Erlenmeyer flasks containing 400 mL potato culture medium. For each flask, 5 mL of the inoculum were added. Two days later, CA in 95% ethanol (12.5 mg mL^{-1}) was added to the cultures at a final concentration of 62.5 μg mL^{-1}. The fungi were incubated at 25 °C on a rotary shaker (120 rpm) in the dark. After 6 days of incubation, the cultures were pooled and filtered, and the supernatant was extracted with an equal volume of ethyl acetate thrice. The organic layer was concentrated to dryness for metabolite isolation.

Isolation of Transformed Products

The isolation process mainly contained three steps, preliminary isolation on a silica gel column with chloroform-methanol as the elution solvent, removing of pigments over Sephadex LH-20 column, and purification by semi-preparative HPLC. An Alltech 426 HPLC system with an RI 2000 detector was used for semi-preparative HPLC. Samples were separated on an Agilent Zorbax SB-C_{18} chromatographic column (9.4×250 mm, 5 μm) eluted by mixtures of methanol (HPLC grade, Mallinkrodt Baker, Phillipsburg, NJ) and water.

Cunninghamella elegans: A total of 1.00 g of CA was added to the cultures. After 6 days of incubation, 2.45 g of ethyl acetate extract were obtained from the culture supernatant. The extract was separated on a silica gel column and eluted with chloroform-methanol (60:1 to 1:1, v/v) to give four fractions. After pigment removal, the fractions were purified by semi-preparative HPLC-RI. Fr. 1 yielded **11** (3 mg, 0.30%) while Fr. 2 yielded **2** (4 mg, 0.40%), **3** (5 mg, 0.50%), **4** (100 mg, 10.00%), **5** (100 mg, 10.00%), **6** (20 mg, 2.00%), and **12** (12 mg, 1.20%).

Syncephalastrum racemosum: A total of 2.10 g of CA was added to the cultures. After 6 days of incubation, 4.51 g of ethyl acetate extract were obtained from the culture supernatant. The extract was separated on a silica gel column and eluted with chloroform-methanol (60:1, 30:1, 20:1, 10:1, 5:1, 2:1, 0:1, v/v) to give 28 fractions. By preparative HPLC-RI purification, **12** (360 mg, 17.14%) was obtained from Fr. 24–26 while compounds **3** (10 mg, 0.48%), **9** (7 mg, 0.33%), and **16** (5.5 mg, 0.26%) from Fr. 20–23; **9** (24 mg, 1.14%), **10** (3.5 mg, 0.17%), **15** (15 mg, 0.71%), **14** (7 mg, 0.33%), and **13** (10 mg, 0.48%) were obatined from Fr. 16–19.

Doratomyces stemonitis: A total of 1.65 g of CA was added to the cultures. After 6 days of incubation, 3.38 g of ethyl acetate extract were obtained from the culture supernatant. The extract was separated on a silica gel column and eluted with chloroform-methanol (60:1, 30:1, 20:1, 10:1, 5:1, 2:1, 0:1, v/v) to give 29 fractions. The fractions were purified with preparative HPLC-RI yielding **7** (730 mg, 44.24%) from Fr. 11–16 while compounds **12** (5.5 mg, 0.33%) and **8** (4.5 mg, 0.27%) were obtained from Fr. 23–27.

Structure Characterization

(20*R*,24*S*)-2α,3β,6α,16β,25-Pentahydroxy-20,24-epoxy-cycloartane (2): white powder (MeOH); mp 228–229 °C; $[\alpha]_D^{20}$: +38.2 (*c* 0.15, MeOH); IR (KBr): ν_{max} = 3413 (ν_{O-H}), 2969, 2935, 2878 (ν_{C-H}), 1450 (δas C−H), 1377 (δs C−H of CH_3), 1057, 1033 (ν_{C-O}) cm^{-1}; HR-ESI-MS: *m/z* = 1013.72970, calcd. for $[2M+H]^+$: 1013.72875, corresponding to $C_{30}H_{50}O_6$; ^{1}H NMR (400 MHz, pyridine-d_5) and ^{13}C NMR (100 MHz, pyridine-d_5) data, see Table 2, Table 3, and Table 4.

(20*R*,24*S*)-3β,6α,12α,16β,25-Pentahydroxy-20,24-epoxy-cycloartane (3): white powder (MeOH); mp 162–163 °C; $[\alpha]_D^{20}$: +35.8 (*c* 0.15, MeOH); IR (KBr): ν_{max} = 3422 (ν_{O-H}), 2967, 2932, 2877 (ν_{C-H}), 1459 (δas C−H), 1381 (δs C−H of CH_3), 1073, 1019 (ν_{C-O}) cm^{-1}; HR-ESI-MS: *m/z* = 507.36772, calcd. for $[M+H]^+$: 507.36801, corresponding to $C_{30}H_{50}O_6$; ^{1}H NMR (400 MHz, pyridine-d_5) and ^{13}C NMR (100 MHz, pyridine-d_5) data, see Table 2, Table 3,and Table 4.

(20*R*,24*S*)-3β,6α,16β,25,28-Pentahydroxy-20,24-epoxy-cycloartane (4): white powder (MeOH); mp 271–272 °C; $[\alpha]_D^{20}$: +34.2 (*c* 0.12, MeOH); IR (KBr): ν_{max} = 3325 (ν_{O-H}), 2966, 2933, 2873 (ν_{C-H}), 1465, 1447 (δas C−H), 1379 (δs C−H of CH_3), 1039 (ν_{C-O}) cm^{-1}; HR-ESI-MS: *m/z* = 1013.72944, calcd. for $[2M+H]^+$: 1013.72875, corresponding to $C_{30}H_{50}O_6$; ^{1}H NMR (400 MHz, pyridine-d_5) and ^{13}C NMR (100 MHz, pyridine-d_5) data, see Table 2,Table 3, and Table 4.

(20*R*,24*S*)-3β,6α,16β,25,29-Pentahydroxy-20,24-epoxy-cycloartane (5): white powder (MeOH); mp 243–244 °C; $[\alpha]_D^{20}$: +32.1 (*c* 0.17, MeOH); IR (KBr): ν_{max} = 3395 (ν_{O-H}), 2935, 2893 (ν_{C-H}), 1443 (δas C−H), 1379 (δs C−H of CH_3), 1031 (ν_{C-O}) cm^{-1}; HR-ESI-MS: *m/z* = 1013.73324, calcd. for $[2M+H]^+$: 1013.72875, corresponding to $C_{30}H_{50}O_6$; ^{1}H NMR (400 MHz, pyridine-d_5) and ^{13}C NMR (100 MHz, pyridine-d_5) data, see Table 2, Table 3, and Table 4.

(20*R*,24*S*)-3β,6α,16β,25-Tetrahydroxy-20,24-epoxy-cycloartan-28-aldehyde (6): white powder (MeOH); mp 241–242 °C; $[\alpha]_D^{20}$: +30.9 (*c* 0.15, MeOH); IR (KBr): ν_{max} = 3399 (ν_{O-H}), 2969, 2938, 2871 (ν_{C-H}), 1717 ($\nu_{C=O}$), 1447 (δas C−H), 1377 (δs C−H of CH_3), 1080, 1035 (ν_{C-O}) cm^{-1}; HR-ESI-MS: *m/z* = 1009.69873, calcd. for $[2M+H]^+$: 1009.69745, corresponding to $C_{30}H_{48}O_6$; ^{1}H NMR (400 MHz, pyridine-d_5) and ^{13}C NMR (100 MHz, pyridined_5) data, see Table 2, Table 3, and Table 4.

(20*R*,24*S*)-6α,16β,25-Trihydroxy-20,24-epoxy-cycloartan-3-one (7): white powder (MeOH); mp 230–231 °C; $[\alpha]_D^{20}$: +30.9 (*c* 0.15, MeOH); IR (KBr): ν_{max} = 3384 (ν_{O-H}), 2987, 2935, 2871 (ν_{C-H}), 1707 ($\nu_{C=O}$), 1463, 1448 (δas C−H), 1380 (δs C−H of CH_3), 1114, 1039 (ν_{C-O}) cm^{-1}; ^{1}H NMR (400 MHz, pyridine-d_5) and ^{13}C NMR (100 MHz, pyridine-d_5) data, see Table 2, Table 3, and Table 4.

(20*R*,24*S*)-6α,16β,25,30-Tetrahydroxy-20,24-epoxy-cycloartan-3-one (8): white powder (MeOH); mp 149–150 °C; $[\alpha]_D^{20}$: +71.2 (*c* 0.07, MeOH); IR (KBr): ν_{max} = 3421 (ν_{O-H}), 2966, 2932, 2883 (ν_{C-H}), 1708 ($\nu_{C=O}$), 1450 (δas C−H), 1380 (δs C−H of CH_3), 1073, 1022 (ν_{C-O}) cm^{-1}; HR-ESI-MS: *m/z* = 1009.69461, calcd. for $[2M+H]^+$: 1009.69745, corresponding to $C_{30}H_{48}O_6$; ^{1}H NMR (400 MHz, pyridine-d_5) and ^{13}C NMR (100 MHz, pyridine-d_5) data, see Table 2, Table 3, and Table 4.

(20*R*,24*S*)-3β,6α,16β,25-Tetrahydroxy-20,24-epoxy-cycloartan-11-one (9): white powder (MeOH); mp 250–251 °C; $[\alpha]_D^{20}$: +86.8 (*c* 0.08, MeOH); IR (KBr): ν_{max} = 3422 (ν_{O-H}), 2966, 2928 (ν_{C-H}), 1462 (δas C−H), 1377 (δs C−H of CH_3), 1110, 1058, 1034 (ν_{C-O}) cm^{-1}; HR-ESI-MS: *m/z* = 1009.69791, calcd. for $[2M+H]^+$: 1009.69745, corresponding to $C_{30}H_{48}O_6$; ^{1}H NMR (400 MHz, pyridine-d_5) and ^{13}C NMR (100 MHz, pyridine-d_5) data, see Table 2, Table 3, and Table 4.

(20*R*,24*S*)-3β,6α,16β,25-Tetrahydroxy-20,24-epoxy-cycloartan-11(12)-ene (10): white powder (MeOH); mp 229–230 °C; $[\alpha]_D^{20}$: +102.0 (*c* 0.05, MeOH); IR (KBr): ν_{max} = 3409 (ν_{O-H}), 3045 (ν_{C-H} of cyclopropane ring), 2979, 2931, 2874 (ν_{C-H}), 1708, 1639 ($\nu_{C=C}$), 1460 (δas C−H), 1376 (δs C−H of CH_3), 1062, 1033 (ν_{C-O}) cm^{-1}; HR-ESI-MS: *m/z* = 1466.05808, calcd. for $[3M+H]^+$: 1466.05780, corresponding to $C_{30}H_{48}O_5$; ^{1}H NMR (400 MHz, pyridine-d_5) and ^{13}C NMR (100 MHz, pyridine-d_5) data, see Table 2, Table 3, and Table 4.

(20*R*,24*R*)-3β,6α,25,28-Tetrahydroxy-16β,24:20,24-diepoxy-cycloartane (11): white needle powder (MeOH); mp 163–164 °C; $[\alpha]_D^{20}$: +34.2 (*c* 0.10, MeOH); IR (KBr): ν_{max} = 3384 (ν_{O-H}), 2928 (ν_{C-H}), 1459 (δas C–H), 1377 (δs C–H of CH_3), 1099, 1037 (ν_{C-O}) cm^{-1}; HR-ESI-MS: *m/z* = 505.35218, calcd. for $[M+H]^+$: 505.35236, corresponding to $C_{30}H_{48}O_6$; ^{1}H NMR (400 MHz, pyridine-d_5) and ^{13}C NMR (100 MHz, pyridine-d_5) data, see Table 2, Table 3, and Table 4.

(20*R*,24*S*)-3β,6α,16β,19,25-Pentahydroxy-ranunculan-9(10)-ene (12): white needle powder (MeOH); mp 309–310 °C; $[\alpha]_D^{20}$: +34.2 (*c* 0.10, MeOH); IR (KBr): ν_{max} = 3478, 3387 (ν_{O-H}), 2972, 2945, 2888 (ν_{C-H}), 1635 ($\nu_{C=C}$), 1453, 1427 (δas C–H), 1379 (δs C–H of CH_3), 1092, 1044, 1027, 1005 (ν_{C-O}) cm^{-1}; HR-ESI-MS: *m/z* = 1013.73266, calcd. for $[2M+H]^+$: 1013.72875, corresponding to $C_{30}H_{50}O_6$; ^{1}H NMR (400 MHz, pyridine-d_5) and ^{13}C NMR (100 MHz, pyridine-d_5) data, see Table 2, Table 3, and Table 4.

(20*R*,24*S*)-3β,6α,16β,25-Tetrahydroxy-19-butoxy-ranunculan-9(10)-ene (13): white powder (MeOH); mp 130–131 °C; $[\alpha]_D^{20}$: +34.7 (*c* 0.09, MeOH); IR (KBr): ν_{max} = 3451, 3421-(ν_{O-H}), 2963, 2932, 2869 (ν_{C-H}), 1628 ($\nu_{C=C}$), 1454 (δas C–H), 1377 (δs C–H of CH_3), 1100, 1057 (ν_{C-O}) cm^{-1}; HR-ESI-MS: *m/z* = 1125.85414, calcd. for $[2M+H]^+$: 1125.85395, corresponding to $C_{34}H_{58}O_6$; ^{1}H NMR (400 MHz, pyridine-d_5) and ^{13}C NMR (100 MHz, pyridine-d_5) data, see Table 2, Table 3, and Table 4.

(20*R*,24*S*)-3β,6α,16β,25-Tetrahydroxy-19-isopentenyloxy-ranunculan-9(10)-ene (14): white powder (MeOH); mp 124–125 °C; $[\alpha]_D^{20}$: +9.6 (*c* 0.13, MeOH); IR (KBr): ν_{max} = 3420 (ν_{O-H}), 2969, 2934, 2871 (ν_{C-H}), 1630 ($\nu_{C=C}$), 1451 (δas C–H), 1379 (δs C–H of CH_3), 1062 (ν_{C-O}) cm^{-1}; HR-ESI-MS: *m/z* = 575.43122, calcd. for $[M+H]^+$: 575.43061, corresponding to $C_{35}H_{58}O_6$; ^{1}H NMR (400 MHz, pyridine-d_5) and ^{13}C NMR (100 MHz, pyridine-d_5) data, see Table 2, Table 3, and Table 4.

(20*R*,24*S*)-3β,6α,16β,25-Tetrahydroxy-19-acetoxy-ranunculan-9(10)-ene (15): white powder (MeOH); mp 211–212 °C; $[\alpha]_D^{20}$: +1.1 (*c* 0.37, MeOH); IR (KBr): ν_{max} = 3407 (ν_{O-H}), 2966, 2933, 2873 (ν_{C-H}), 1739 ($\nu_{C=O}$), 1453 (δas C–H), 1382 (δs C–H of CH_3), 1234 (ν_{C-O} of the ester), 1088, 1034 (ν_{C-O}) cm^{-1}; HR-ESI-MS: *m/z* = 549.37849, calcd. for $[M+H]^+$: 549.37858, corresponding to $C_{32}H_{52}O_7$; ^{1}H NMR (400 MHz, pyridine-d_5) and ^{13}C NMR (100 MHz, pyridine-d_5) data, see Table 2, Table 3, and Table 4.

Neoastragenol [(20*R*,24*S*)-3β,6α,16β,25-tetrahydroxy-20,24-epoxy-9(10)a-homo-19-nor-cycloartane] (16): white powder (MeOH); mp 241–242 °C; $[\alpha]_D^{20}$: +60.0 (*c* 0.11, MeOH); IR (KBr): ν_{max} = 3422 (ν_{O-H}), 2968, 2933, 2902 (ν_{C-H}), 1720 ($\nu_{C=O}$), 1626 ($\nu_{C=C}$), 1447 (δas C–H), 1375 (δs C–H of CH_3), 1179, 1034 (ν_{C-O}) cm^{-1}; HR-ESI-MS: *m/z* = 977.70692, calcd. for $[2M+H]^+$: 977.70782, corresponding to $C_{30}H_{48}O_5$; ^{1}H NMR (400 MHz, pyridine-d_5) and ^{13}C NMR (100 MHz, pyridine-d_5) data, see Table 1.

Supporting Information

The results of the preliminary screening test of 13 fungal strains, and the HR-ESI-mass, ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC, HMBC, NOESY spectra of 15 transformed products together with cycloastragenol are available as Supporting Information.

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