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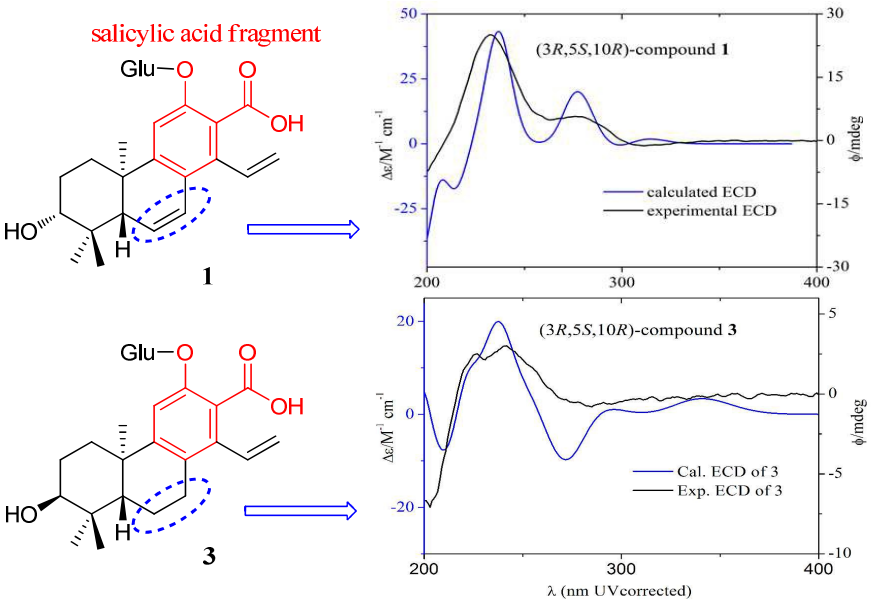

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Stereochemistry of Cleistanthane Diterpenoid Glucosides from *Phyllanthus emblica*

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Jun-Jiang Lv,[a,c] Shan Yu,[a,b] Ying Xin,[a,b] Hong-Tao Zhu,[a] Dong Wang,[a] Rong-Rong Cheng,[a] Chong-Ren Yang,[a] Min Xu,*[a] and Ying-Jun Zhang*[a]

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The time-dependent density functional theory (TDDFT) calculated electronic circular dichroism (ECD) and Mosher's method were applied to establish the absolute configurations of six new highly oxygenated cleistanthane diterpenoid glucosides, phyllanembloids A-F (**1**-**6**), isolated from the roots of *Phyllanthus emblica*. Compounds **1**-**5** featured in the carboxyl group at C-13 adjacent to the hydroxyl group at C-12, which are first examples of cleistanthane diterpenoids with a salicylic acid fragment. The carboxyl group at C-13 can significantly affect the CD spectrum of cleistanthane diterpenoids, and make the excitations of phenylethylene as dominant Cotton effects (240 and 280 nm), rather than the benzene ring excitations dominant Cotton effects at 220 and 240 nm in the 13-methyl analogues. The characteristics of Cotton effects of cleistanthane diterpenoids were summarized according to the experimental and calculated ECD curves, which can be applied as a common method to determine the absolute configurations of this type of diterpenoids.

Introduction

Cleistanthane diterpenoids featuring in a benzene C ring in the skeleton are rare in nature.[1] The first example was cleistanthol isolated from *Cleistanthus schlechteri*.[2] Determination of the absolute configurations of cleistanthane diterpenoids usually depends on the sign of CD cotton effects near 350 nm, generated by the *n*-π* excitation of the conjugated system comprising the benzene ring and the carbonyl group at C-7.[1,3] However, most naturally occurred cleistanthane diterpenoids have no carbonyl group at C-7. Therefore, the application of this method is limited, and most recently published works on cleistanthane diterpenoids did not determine the absolute configurations of these compounds.[4-7]

Phyllanthus emblica L, is an important traditional Chinese plant.[8] Previous studies revealed that its roots present tannins[9] and bisabolane sesquiterpenoid glycosides.[10-13] Further chemical investigation on the titled plant led to the isolation of six new diterpenoid glucosides **1**-**6**. These compounds possess a highly oxygenated cleistanthane skeleton, with a carboxyl group at C-13 (**1**-**5**), an 15-acetyl (**5**), and/or glycosylations on C-12 (**1**-**3**) and C-19 (**5**-**6**). Determination of the absolute configurations of these compounds is a challenge, because it is difficult to get a proper single crystal or carry out Mosher reactions, due to the presence of sugar moiety and carboxyl group. The time-dependent density functional theory (TDDFT) based calculation of electronic circular dichroism (ECD) and optical rotations (ORs) were applied to determine the absolute configurations of **1**-**6**. The theoretical calculated results were confirmed by Mosher's method in the case of **1**. The effects of the substitutions on benzene ring on ECD properties, and the characters of the Cotton effects for cleistanthane diterpenoid were investigated and discussed. In addition, the isolated compounds were evaluated for their anti-heptatitis B virus (HBV) activities and cytotoxicities against human cancer cell lines.

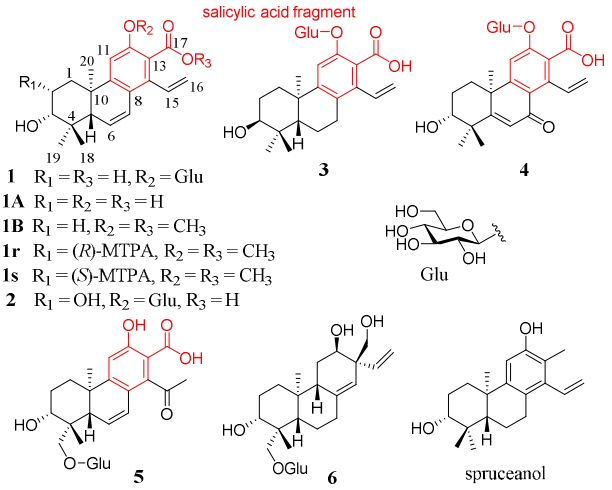

Figure 1. Cleistanthane diterpenoids isolated from *P. emblica*

Table 1. The ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR Spectroscopic data of compounds **1** – **3** and **1A** in CD_3OD (δ in ppm)

No.	**1**		**1A**[a]		**2**		**3**	
	δ_C, mult	δ_H	δ_C, mult	δ_H	δ_C, mult	δ_H	δ_C, mult[a]	δ_H[c]
1	36.0, CH_2	1.68 dt (5.0, 12.5) 2.22 dt (12.5, 3.3)	35.6, CH_2	1.70 dd (10.6, 12.0) 2.13 dt (13.1,2.9)	42.0, CH_2	1.87 dd (2.5, 14.0) 2.56 dd (2.5, 14.0)	33.2, CH_2	1.85 m 2.04 dt (13.1, 3.4)
2	28.6, CH_2	1.77 m 1.82 dd (5.2, 10.0)	29.1, CH_2	2.04 m	72.5, CH	4.21 dd (2.5, 3.5)	27.2, CH_2	1.73 m 2.12 tt (3.2, 13.9)
3	79.4, CH	3.17 dd (5.5,10.5)	78.3, CH	3.57 dd (7.5, 9.0)	79.3, CH	3.18 d (3.5)	76.4, CH	3.43 dd (3.2, 3.2)
4	39.4, C		39.3, C		38.8, C		38.9, C	
5	51.4, CH	1.99 t (3.3)	49.8, CH	2.16 t (3.0)	51.3, CH	2.10 t (2.5)	44.5, CH	1.72 m
6	128.6, CH	5.96 dd (3.0, 10.0)	126.7, CH	6.00 dd (3.0, 10.1)	128.8, CH	6.02 dd (2.5, 10.0)	19.9, CH_2	1.71 m 1.85 m
7	126.6, CH	6.79 dd (3.0, 10.0)	127.3, CH	7.10 dd (3.0, 10.1)	126.4, CH	6.79 dd (2.5, 10.0)	29.6, CH_2	2.57 m 2.82 dd (6.2, 17.7)
8	131.6, C		123.9, C		130.0, C		128.8, C	
9	149.8, C		154.9, C		151.2, C		153.1, C	
10	39.6, C		39.7, C		39.3, C		39.2, C	
11	111.2, CH	7.01 s	111.7, CH	7.10 s	111.5, CH	7.05 s	113.4, CH	7.15 s
12	153.9, C		162.8, C		153.9, C		151.6, C	
13	126.6, C		114.9, C		133.3, C		130.5, C	
14	133.4, C		139.7, C		133.9, C		135.9, C	
15	135.2, CH	6.91 dd (11.4, 17.8)	138.2, CH	7.71 dd (11.5, 16.9)	135.1, CH	6.91 dd (11.5, 17.5)	136.4, C	6.77 dd (11.5, 17.7)
16	121.0, CH_2	5.44 dd (2.0, 11.4) 5.47 dd (2.0, 17.8)	118.8, CH_2	5.30 d (16.9) 5.59 d (11.5)	121.2, CH_2	5.43 d (17.5) 5.47 d (11.5)	119.2, CH_2	5.32 brd (11.5) 5.50 brd (17.7)
17	177.3, C		170.0, C		176.5, C		178.2, C	
18	17.5, CH_3	1.00 s	17.7, CH_3	1.27 s	18.5, CH_3	1.06 s	28.9, CH_3	1.01 s
19	28.5, CH_3	1.04 s	29.1, CH_3	1.31 s	29.8, CH_3	1.23 s	22.9, CH_3	0.94 s
20	20.4, CH_3	0.99 s	20.9, CH_3	1.13 s	22.3, CH_3	1.23 s	25.2, CH_3	1.19 s
1'	104.7, CH	4.81 d (8.0)			104.5, CH	4.85 d (7.5)	105.1, CH	4.78 d (7.5)
2'	75.3, CH	3.49 dd (8.0, 9.0)			75.4, CH	3.48 m	75.2, CH	3.46 m
3'	77.8, CH	3.47 dd (9.0, 9.0)			77.9, CH	3.46 m	77.7, CH	3.44 m
4'	71.8, CH	3.35 dd (9.0, 9.0)			71.6, CH	3.36 dd (8.6, 8.6)	71.6, CH	3.33 m
5'	78.7, CH	3.43 ddd (2.0,7.0,9.0)			78.6, CH	3.44 ddd (2.0,7.0, 8.6)	78.7, CH	3.42 ddd (2.5, 7.0, 9.8)
6'	62.9, CH_2	3.69 dd (7.0, 12.0) 3.91 dd (2.0,12.0)			63.0, CH_2	3.72 dd (7.0, 12.0) 3.93 dd (2.0,12.0)	62.9, CH_2	3.68 dd (7.0, 12.1) 3.90 dd (2.5,12.1)

[a] Data were recorded in pyridine-d_5

Results and Discussion

The HRESIMS (*m/z* 489.2130 [*M*-H]$^-$) analysis returned a molecule formula $C_{26}H_{34}O_9$ for **1**, corresponding to 10 degrees of unsaturation. The NMR data (Table 1) of **1** revealed the signals of one β-glucopyranosyl moiety: an anomeric proton and carbon at δ_H 4.81 (1H, d, J = 7.5 Hz, H-1') and δ_C 104.7 (C-1'). Acidic hydrolysis of **1** afforded D-glucose ($[\alpha]_D^{25}$ +94.3 (c = 0.63, in H_2O) as sugar residue and **1A** as aglycon. Besides, 20 carbon signals due to the aglycon part were observed in the ^{13}C NMR and DEPT spectra, attributed to one carboxyl (δ_C 177.3), 10 sp^2 carbons (δ_C 120-155) including one terminal double bond (δ_C 121.0, CH_2), and nine aliphatic carbons, including three methyls (δ_C 17.5, 28.5, and 20.4) with corresponding protons as singlet signals at δ_H 1.00, 1.04, and 0.99 (s, each 3H) in the ^{1}H NMR spectrum. Moreover, only one aromatic singlet proton at δ_H 7.01 (1H, s) was observed, indicating a penta-substituted benzene ring in **1**. Three mutually coupled olefinic protons at δ_H 6.91 (1H, dd, J = 11.4, 17.8 Hz), 5.44 (1H, dd, J = 2.0, 11.4 Hz), and 5.47 (1H, dd, J = 2.0, 17.8 Hz) belonged to a terminal double bond. Another two olefinic protons at δ_H 6.79 and 5.96 (each 1H, d, J = 10.0 Hz) implied a di-substituted *cis*-vinyl group in **1**. Apart from two double bonds, one benzene ring, one caboxyl and one glucopyranosyl group accounting for eight degrees of unsaturation, it is obvious that there should be another two rings in **1**. The aforementioned data indicated **1** was a cleistanthane type diterpenoid glycoside. The extensive comparison and analysis of 1D NMR (Table 1) data of **1A** suggested that it had the same A, B and C rings to those of spruceanol, a cleistanthane diterpenoid reported from *Croton insularis*.[14] However, instead of the methyl at C-17 and two alphalic methylenes (C-6 and C-7) in spruceanol, a carboxyl carbon (δ_C 177.3) and a di-substituted *cis*-vinyl (δ_C 128.6 and 126.6) group were presented in **1**. The HMBC correlations (Figure 2) from the aromatic proton at δ_H 7.01 (H-11) to carboxyl carbon (δ_C 177.3, C-17) and the vinyl protons at δ_H 5.44 and 5.47 (H_2-16) to C-14 allowed to confirm the direct connectivity between the carboxyl and C-13. The di-substituted *cis*-vinyl group was assigned as C-6 and C-7 in **1**, by the HMBC correlations from H-5 (δ_H 1.99) to C-6/C-7, and δ_H 6.79 (H-7) to C-8/C-9/C-14. The HMBC correlations from the glycosyl H-1' to C-12 (δ_C 153.9) confirmed the locations of the glucosyl on C-12. This was futher confirmed by the ROESY correlation of the anomeric proton H-1' with the aromatic proton H-11. Other 2D NMR correlations confirmed the planar structure of **1** as shown in Figure 2. In the ROESY spectrum of **1**, there were correlations of H-5 with H_3-18/H-1ax (δ_H 1.68), H_3-20 with H-1eq (δ_H 2.22), suggesting that the *trans* fused A/B ring in **1**, and H-5 and Me-20 were diaxial orientated. The large coupling constant of $J_{2b,3}$ (10.0 Hz) indicated an axially orientated H-3, which was further confirmed by the ROESY correlations of H-3 with H-5/H_3-18.

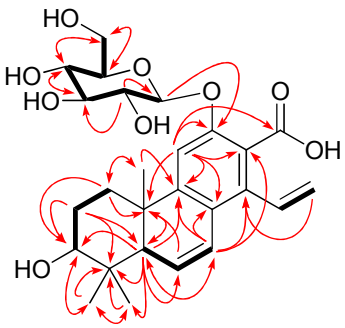

Figure 2. Key ^{1}H-^{1}H COSY(—) HMBC(→) correlations of **1**

The absolute configurations of **1** were established by comparison its experimental ECD spectrum with the theoretically calculated results. The conformational searches were performed by either Monte Carlo search applied MMFF method[15] or potential energy surface (PES) scan. The conformers were optimized using DFT-B3LYP/6-311G (d,p), and the optimized conformers with relative energy < 2 kcal mol^{-1} were selected for ECD calculations using TD-DFT-B3LYP/6-311G (d,p). The final calculated ECD spectrum was obtained by averaging the calculated ECD curves using Boltzmann distribution, respectively.[16]

The aglycon **1A** displayed the similar Cotton effects to its glycoside (**1**) in experimental ECD (Figure 3), suggesting the glycosyl moiety in **1** had little contribution to the ECD spectrum. Both the calculated and experimental ECD spectra displayed a strong positive Cotton effect at 240 nm and a weak Cotton effect at 280 nm. On the basis of the above evidences, the absolute configurations of phyllanembloid A (**1**) were determined to be 3*R*,5*S*,10*R*.

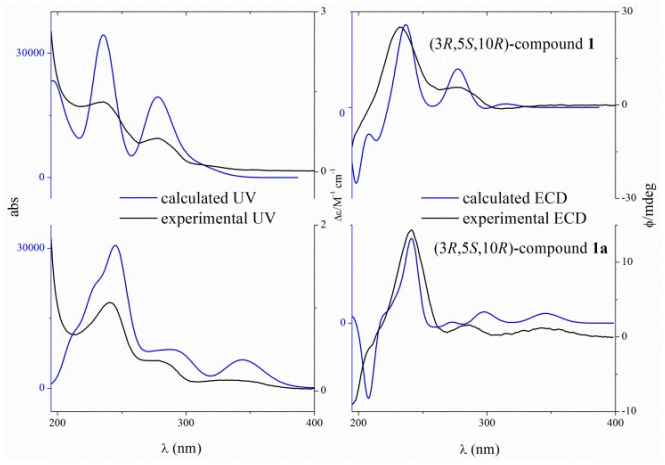

Figure 3. ECD and UV curves of compounds **1** and **1A**

Mosher's method[17,18] was applied to further confirm the absolute configurations of C-3 in **1**. Enzymatic hydrolysis using cellulase for compound **1** was carried out to afford the aglycon **1A**. Then, the carboxyl (C-13) and phenol (C-12) groups were protected by methylation reaction using CH_3I with the acid scavenger K_2CO_3 to give **1B**. Two portions of **1B** were treated

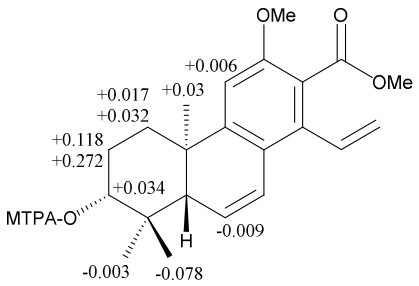

Figure 4. The δ_S - δ_R ($CDCl_3$) values of the MTPA esters of **1B**

with (*S*)-(+)- and (*R*)-(-)- α-methoxy-α-(trifluoromethyl)phenyl-acetyl (MPTA) chloride in pyridine separately, to afford (*R*)- and (*S*)- MTPA ester derivatives **1r** and **1s** of **1B**, respectively. In the ^{1}H NMR spectra of **1r** and **1s**, the undisturbed signals (H-1, H-2, H-3, H-6, CH_3-18, CH_3-19 and CH_3-20) were clearly different (see S1 in Electronic supplementary information (ESI)) and the observed chemical shift differences ($\Delta\delta_{S-R}$, Figure 4) determined the absolute configuration of C-3 in **1B** to be *R*, corresponding to the 3*R* configuration of **1**. This agreed with the results obtained by theoretical ECD calculations.

Phyllanembloid B (**2**) had a molecular formula of $C_{26}H_{34}O_{10}$, as deduced from the HREIMS (*m/z* 506.2133 $[M]^+$). The NMR spectra of **2** (Table 1) were comparable to those of **1**, except that the C-2 methylene signal (δ_C 28.6) in **1** was replaced by an oxymethine signal (δ_C 72.5) in **2**, suggesting that **2** is the C-2 hydroxylated derivative of **1**. The oxymethine carbon at δ_C 72.5 was assigned to be C-2, according to the ^{1}H-^{1}H COSY correlations of its corresponding proton at δ_H 4.21 (H-2) with both H-1 and H-3, and the HMBC correlation from H-2 to C-4 (δ_C 38.8) (see S2 in ESI). The small coupling constants of $J_{2,3}$ (3.5 Hz) and $J_{2,1}$ (2.5 Hz) suggested that H-2 in **2** was equatorially and α-orientated, which was confirmed by the ROESY correlations of H-1a (δ_H 1.87) with both H-2 and H-5 (δ_H 2.10), and H-5 with H-18 (δ_H 1.06). The ROESY correlation of H-5 with H-3 allowed the assignment of H-3 as axial and α-orientation. Compared with compound **1**, the only difference of **2** was the hydroxylation at C-2. Thus, the absolute configuration of **2** could be assigned by comparing of its CD spectrum with that of **1**. The Cotton effects of **2** are comparable to those of **1** (Figure 5). Therefore, the absolute configurations of phyllanembloid B (**2**) were determined to be 2*R*,3*S*,5*S*,10*R*.

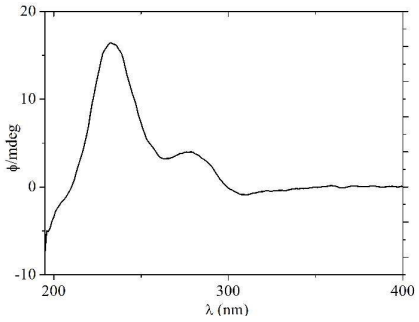

Figure 5. ECD spectrum of compound **2**

Table 2. The ^{13}C (150 MHz) and ^{1}H (600 MHz) NMR Spectroscopic data of compounds **4** – **6** in CD_3OD (δ in ppm)

No.	**4**		**5**		**6**	
	δ_C, mult	δ_H	δ_C, mult	δ_H	δ_C, mult	δ_H
1	36.2, CH_2	1.66 dt (13.9, 3.8) 2.62 dt (13.9, 3.6)	35.8, CH_2	1.73 dt (4.9, 13.5) 2.22 dd (13.5, 3.5)	37.9, CH_2	1.19 m 1.62 m
2	28.0, CH_2	1.97 dq (13.8,3.8) 2.13 dddd (3.6, 12.3, 13.8, 13.8)	28.5, CH_2	1.93 m 1.97 dt (3.3, 13.7)	27.5, CH_2	1.63 m
3	77.1, CH	3.38 dd (3.8, 12.3)	80.0, CH	3.37 dd (4.9, 11.6)	73.1, CH	3.76 t (7.4)
4	44.1, C		43.4, C		43.9, C	
5	172.8, C		51.0, CH	2.12 t (3.2)	46.9, CH	1.77 dd (2.3, 13.0)
6	127.1, CH	6.42 s	129.4, CH	6.16 dd (3.2, 10.0)	23.1, CH_2	1.44 m 1.65 dq (4.9, 13.0)
7	185.0, C		124.1, CH	6.29 dd (3.2, 10.0)	36.2, CH_2	2.24 dt (4.9, 14.0) 2.34 ddd (1.7, 4.5, 14.0)
8	123.5, C		119.9, C		141.8, C	
9	157.1, C		155.0, C		48.1, CH	1.99 dd (6.6, 10.2)
10	43.0, C		39.2, C		39.0, C	
11	112.2, CH	7.36 s	112.1, CH	6.73 s	27.3, CH_2	1.57 ddd (2.2, 10.9, 13.1) 1.65 m
12	157.7, C		162.0, C		71.7, CH	3.90 t (3.9)
13	133.0, C		114.2, C		49.7, C	
14	138.4, C		142.9, C		120.9, CH	5.11 s
15	138.5, CH	7.35 dd (11.5, 17.7)	211.5, C		144.0, CH	5.85 dd (10.2, 17.6)
16	116.6, CH_2	5.27 dd (1.5, 11.5) 5.53 dd (1.5, 17.7)	33.6, CH_3	2.42 s	117.3, CH_2	5.09 dd (2.2, 17.6) 5.14 dd (2.2, 10.2)
17	176.2, C		174.1, C		68.6, C	3.47 d (10.9) 3.60 d (10.9)
18	27.8, CH_3	1.35 s	23.1, CH_3	1.25 s	12.9, CH_3	0.74 s
19	23.4, CH_3	1.31 s	72.3, CH_2	3.68 d (11.9) 4.34 d (11.9)	73.8, CH_2	3.28 d (10.1) 3.82 d (10.1)
20	32.9, CH_3	1.58 s	21.1, CH_3	1.01 s	15.9, CH_3	0.78 s
1'	102.8, CH	5.00 d (7.7)	104.9, CH	4.29 d (7.8)	104.8, CH	4.22 d (7.9)
2'	74.8, CH	3.58 dd (7.7, 9.3)	74.9, CH	3.26 dd (7.8, 9.3)	75.0, CH	3.20 dd (7.9, 9.0)
3'	77.6, CH	3.54 dd (9.3, 9.3)	77.7, CH	3.41 dd (9.3, 9.3)	78.2, CH	3.34 dd (9.0, 9.0)
4'	71.7, CH	3.35 dd (9.3, 9.3)	71.4, CH	3.35 dd (9.3, 9.3)	71.9, CH	3.26 dd (9.0, 9.0)
5'	78.7, CH	3.52ddd (2.2, 7.1, 9.3)	77.7, CH	3.34 m	77.7, CH	3.27 m
6'	62.8, CH_2	3.67 dd (7.1, 12.1) 3.96 dd (2.2,12.1)	62.4, CH_2	3.70 dd (5.2, 12.1) 3.88 dd (1.5,12.1)	63.0, CH_2	3.64 dd (5.7, 11.6) 3.88 dd (2.1,11.6)

Phyllanembloid C (**3**) displayed a quasimolecular ion peak at *m/z* 491.2292 $[M\text{-H}]^-$ in the HREIMS, corresponding to the molecular formula of $C_{26}H_{36}O_9$. The key difference in the NMR spectra of **3** (Table 1) with **1** was the appearance of two aliphatic methylenes at δ_C 19.9 and δ_C 29.6, instead of the $\Delta^{6,7}$ vinyl group in **1**, suggesting that compound **3** was the C-6/C-7 hydrogenated derivative of **1**. This was confirmed by the HMBC correlations (see S2 in ESI) from H-6 (δ_H 1.71 and 1.85) to C-5, C-10, C-18, and from H-7 (δ_H 2.57 and 2.82) to C-5, C-8, and C-9, and as well the ^1H-^1H COSY correlations between H-6 and H-7. In the ROESY spectrum of **3**, correlations of H-20 (δ_H 1.19, 3H, s) with H-19 (δ_H 0.94, 3H, s), and H-5 (δ_H 1.72, 1H, m) with H-18 (δ_H 1.01, 3H, s) indicated the *trans* fused A/B ring, and H-5 and Me-20 were diaxially oriented. The small $J_{2,3}$ (3.2 Hz) indicated an equatorial orientation of H-3, which is at the same face of Me-20 and β-orientated. This was confirmed by the ROESY correlations of H-3 with H_3-18 and H_3-19 (Figure 6). The CD spectum of **3** (Figure 6) demonstrated two weak positive Cotton effects at 220 and 240 nm, which was different from those of compounds **1** and **2**, and the reasons were discussed below. The calculated ECD results of compound **3** with 3*S*,5*S*,10*R* absolute configuration agreed well with the experimental spectrum.

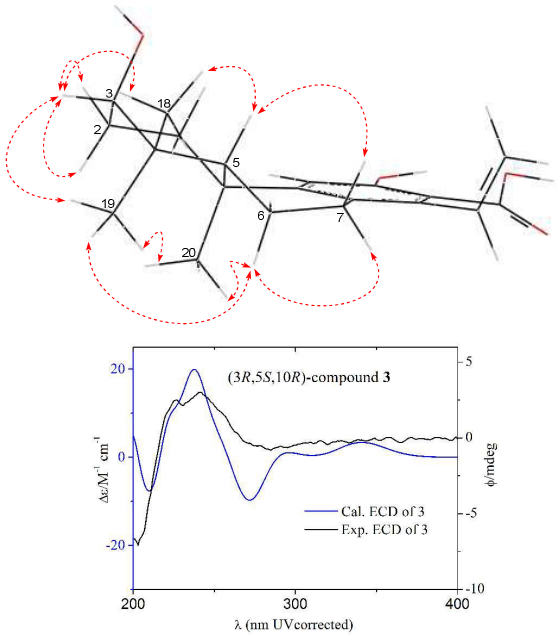

Figure 6. Key ROESY correlations and ECD spectra of **3**

Phyllanembloid D (**4**) had a molecular formula of $C_{26}H_{32}O_{10}$, as established from the HREIMS (*m/z* 504.1998 $[M]^+$). The ^{1}H and ^{13}C NMR spectra of **4** (Table 2) were closely related to those of **1**. However, instead of the di-substituted $\Delta^{6,7}$ and the aliphatic methine at δ_C 51.4 (C-5, CH) in **1**, one ketone (δ_C 185.0, C) and one tri-substituted double bond [δ_C 172.8 (C), 127.1 (CH)] signals appeared in the ^{13}C NMR and DEPT spectra of **4**. The up-field shifted ketone signal at δ_C 185.0 representing an α, β unsaturated carbonyl, was assigned as C-7 based on the key HMBC correlation (see S2 in ESI) from H-11 (δ_H 7.36) to the ketone carbon. The olefinic carbon signals at δ_C 172.8 and 127.6 were assigned to be C-5 and C-6, respectively, according to the HMBC correlations from the olefinic proton H-6 to C-8 and C-10, and from H_3-18, H_3-19, H_3-20 and H-1 to the olefinic quaternary carbon at δ_C 172.8 (C-5). Based on the proton coupling constants and ROESY correlations, the relative configurations of **4** were establised as shown. The calculated ECD spectra of **4** agreed well with the experimental ECD curve of **4**. This determined the absolute configurations of phyllanembloid D (**4**) as 3*R*,10*R* (Figure 7).

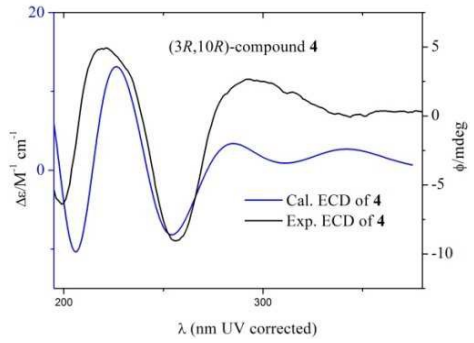

Figure 7. Experimental and calculated ECD curves of **4**

Phyllanembloid E (**5**) had a molecular formula $C_{26}H_{34}O_{11}$, as deduced from the HREIMS (*m/z* 521.2032). The ^{1}H and ^{13}C for the presence of one acetyl (δ_C 211.5, 33.6, and δ_H 2.42) and NMR (DEPT) spectra (Table 3) were close to those of **1**, except that one oxymethylene (δ_C 72.3) appeared in **5**, instead of the $\Delta^{15,16}$ vinyl (δ_C121.0, 135.2) and one methyl (δ_C 17.5) groups in **1**. The acetyl at δ_C 211.5 and 33.6 were assigned respectively to be C-15 and C-16, due to the HMBC correlations (see S2 in ESI) from H-16, H-11 and H-7 to C-14, and from H-16 to δ_C 211.5 (C-15). The oxymethylene at δ_C 72.3 was determined to be C-19 due to the HMBC correlations of H-19 with C-3, C-4, C-19, and C-5. Moreover, the glucosyl moiety was located on C-19 position, taking into account the HMBC correlation from H-19 to C-1'. Other ^{1}H-^{1}H COSY and HMBC correlations confirmed the planar structure of **5**. The large coupling constants of $J_{3,2}$ (12.3 Hz) suggested that H-3 was axially orientated and at the opposite face of 20-methyl. The ROESY correlations of H-3 with H-5 and H-18 indicated that all the three protons located at the same face. Together with the ROESY correlations of H-20 with H-19, the relative configurations of **5** were established.

The Monte Carlo conformer search of **5** produced two major lower energy conformers, **5a** (47.4 %) and **5b** (39.6%) (Figure 8), after optimization. Both **5a** and **5b** were 16-methyl α-oriented conformers. The PES scan using **5a** and **5b** as the starting structure generated another two 16-methyl β-oriented conformers with the same energy level as **5a** and **5b**, respectively (Figure 9 right). The distribution ratio of 16-methyl β- and α-oriented conformers was 67:32, which can explain the split signal of 16-methyl in the ^{1}H NMR spectrum of **5** (Figure 9 left).

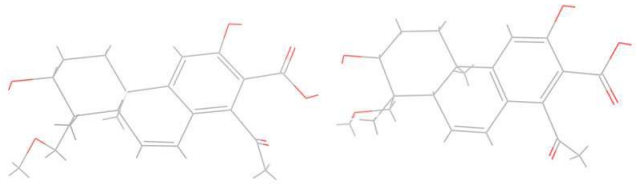

Figure 8. structures of conformers **5a** (left) and **5b** (right).

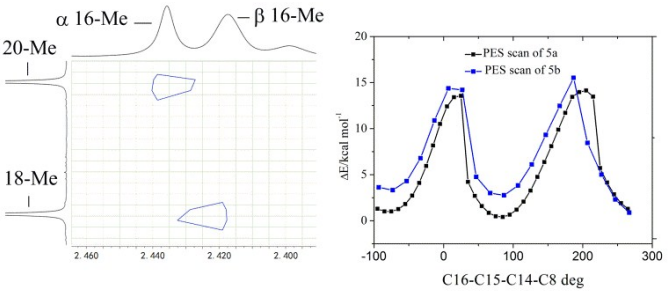

Figure 9. ROESY spectra and PES scan of **5**

The ECD calculation using the optimized lower energy conformers (Figure 10 top) could predict the experimental ECD spectrum of **5** (Figure 10 bottom), which was comparable to those of compounds **1** and **2**, and the absolute configurations of **5** were therefore assigned as 3*R*,4*R*,5*S*,10*R*.

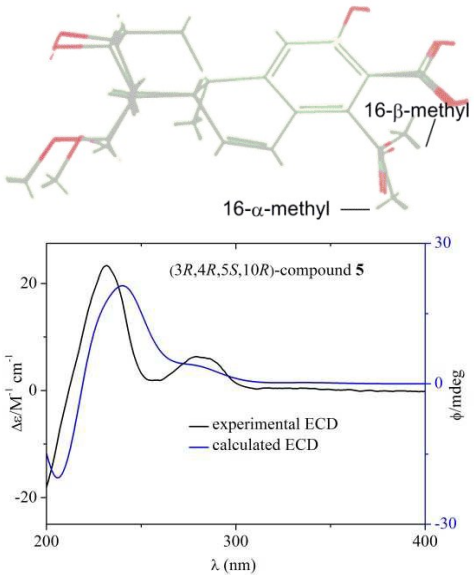

Figure 10. The low energy conformers (top) and ECD spectra of **5** (bottom)

Phyllanembloid F (**6**), a white amorphous powder, had a molecular formula $C_{26}H_{42}O_9$, deduced from the HRESIMS (*m/z* 543.2812 [*M*+HCOO]$^-$). The ^{13}C NMR and DEPT data (Table 2) of **6** showed the presence of one glucosyl moiety, in addition to 20 carbon resonances attributable to the aglycon part of **6**, which was similar to those of ent-isopimara-8(14),15-dien-3β-ol.[3] However, two methyls in ent-isopimara-8(14),15-dien-3β-ol were replaced by two oxymethylenes (δ_C 68.6 and 73.8) in **6**. The oxymethylene signals at δ_C 68.6 and 73.8 were assigned to be C-17 and C-19, respectively, according to the HMBC correlations (see S2 in ESI) from H-16 and H-15 to C-17, from H-17 to C-12, C-13, and C-14, from H-19 to C-3 and C-4. The location of the glucosyl moiety was determined to be at C-19 based on the HMBC correlation from the anomeric proton at δ_H 4.22 (1H, d, J = 7.9 Hz, H-1') to C-19. Thus, the planar structure of **6** was determined. In the ROESY spectrum of **6**, correlations of H-3 with H-5 and H-18, H-5 with H-9 revealed the β orentation for H-3, H-5 and H-9, while correlations of the 20-methyl with H-15 and H-12 indicated the α-orentated C-20, H-12, and $\Delta^{15,16}$ vinyl group. There is no Cotton effect in the CD spectrum used to determine the absolute configurations of **6**, due to the lack of chromophore at the range of 200-400 nm.

The OR of compound **6** was caculated using TDDFT/GIAO methods.[19] The calculated OR (-76.0) agreed well with the experimental value (-47.3), by which the absolute configurations of phyllanembloid F (**6**) were determined to be 3*R*,4*R*,5*S*,9*R*,10*S*,12*R*,13*R*.

The rules of the absolute configurations and Cotton effects of cleistanthane type diterpenoids.

The above mentioned cleistanthane diterpenoids **1-5** featured in a benzene C ring in the skeleton, and this chromophore was adjacent to the stereo centers of C-10 and C-5, of which the Cotton effects are sensitive for the stereo properties of this type of diterpenoids. When a carbonyl group existed in the C-7 position, the absolute configurations of this type of diterpenoid could be determined on the basis of the sign of CD Cotton effects near 350 nm, generated by the *n*-π* exitation of the benzoyl moiety.[1,3] However, most naturally occurred cleistainthane diterpenoids have no carbonyl group at C-7. Herein, we establish a method to determine the absolute configurations directly using CD spectrum without any chemical reactions.

Compounds **1**, **2** and **5** displayed similar strong Cotton effect at 240 nm and weak Cotton effect at 280 nm (Figures 3, 6 and 10). The common characteristics of these compounds were the conjugated system comprising of benzene C ring with a $\Delta^{6,7}$ vinyl group, which was adjacent to the stereo center of C-5. The Cotton effects at 240 and 280 nm were generated by the charge transfer of ^1L_b excitations occuring at the phenylethylene system, respectively. For compound **3** with sp^3 C6-C7 bond, the Cotton effects at 220 and 240 nm were generated by the benzene ring system (Figure 6). Although phyllanflexoid (phy-) B,[20] a cleistanthane diterpenoid isolated from *Phyllanthus flexuosus*, has a $\Delta^{6,7}$ vinyl group in the structure, its CD spectrum was similar to those of **3** and phy-A, whose Cotton effects were generated by the benzene ring rather than the phenylethylene system (Figure 11). It is noted that, there is a 13-carboxyl substitution at the meta-position of the $\Delta^{6,7}$ vinyl group in **1**, **2** and **5**. The induction effect of this electron-withdrawing group enhanced the conjugation of phenylethylene system and made it display strong Cotton effects at 240 and 280 nm. It can be concluded that the carboxyl group at C-13 was able to let the excitations arised from $\Delta^{6,7}$ phenylethylene dominant the Cotton effects of cleistanthane diterpenoids. In other cases, the cleistanthane diterpenoids with alkyl groups at C-13 (phy A and B), whether it has a $\Delta^{6,7}$ vinyl group, the CD spectrum were dominant by the excitations (220 and 240 nm) arised from benzene ring (Figure 11). Together with the absolute configurations determined by experimental and calculated ECD of different structures of compounds **1-5** and the previously reported phy-A, B and C, the CD rules can become a common method to determine the absolute configurations of cleistanthane diterpenoids (Table 3).

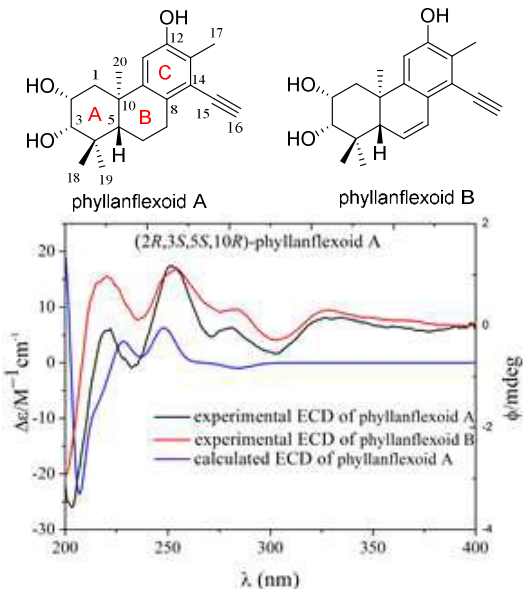

Figure 11. structures and ECD spectra of phyllanflexoids A and B.

Table 3. The CD rules of 5*S*,10*R* cleistanthane diterpenoids

compounds	Functional Group at C-13	Functional Group at C6-C7	Functional Group at A ring	Cotton effects (nm)
1, **2**, **5**	-COOH	CH=CH		240 ++ 280 +
phy-B	-CH_3	CH=CH		220 + 250 +
3	-COOH	CH_2-CH_2		220 + 240 +
phy- A	-CH_3	CH_2-CH_2		220 + 250 +
4	-COOH	O=C-C=C(5)		230 ++ 260 - -
phy- C	-CH_2-OH	CH_2-CH_2	O=C-C=C(4)	220 + 240 + 320 ++

++: strong positive; +: relative weak positive; - -: strong negative

Anti-virus and cytotoxicities of cleistanthane diterpenoids.

Compounds **1** and **5** were evaluated for their anti-heptatitis B virus (HBV) activities and cytotoxicities against five human cancer cell lines [lung cancer (A-549), human myeloid leukemia (HL-60), breast cancer (MCF-7), hepatocellular carcinoma (SMMC-7721), and colon cancer (SW480)], as the previously reported procedures.[21,22] Unfortunately, none of them displayed cytotoxicity at a concentration of 40 μM. Compound **1** was effective to the HBV surface antigen (HBsAg), with IC_{50} value of 0.29 mg/ml.

Conclusions

The absolute configurations of six new highly oxygenated cleistathane diterpenoids, phyllanembloids A–F (**1-6**) from *P. emblica*, were established unambiguously by means of TDDFT calculated ECD, ORs, and Mosher's method in the case of **1**. Using the Monte Carlo search MMFF method, together with the PES scan and TDDFT optimization, the preferential conformations of this type of compounds were figured out. On the basis of the relationship of the CD spectra and the structures of cleistathane diterpenoids, the rules using CD spectra to determine the absolute configurations of cleistathane diterpenoids were summarized. Compounds **1** and **5** were evaluated for their anti-HBV and cytotoxicities against five human cancer cell lines. Only compound **1** exhibited weak HBsAg inhibitory activity.

Experimental Section

General procedures

Optical rotations were performed on a P-1020 polarimeter (JASCO, Tokyo, Japan). IR and UV spectra were measured on a Bruker Tensor 27 spectrometer with KBr pellets and shimadzu UV 2401PC, respectively. 1D and 2D NMR spectra were run on Bruker DRX-500 and AVANCE III-600 NMR spectrometers operating at 500 and 600 MHz for ^{1}H, and 125 and 150 MHz for ^{13}C, respectively. Coupling constants are expressed in Hertz and chemical shifts are given on a ppm scale with solvents as internal standard. ESI-MS and HRESIMS were measured at Bruker HCT/ Esquire and Agilent G6230. ECD and OR were dectected at Applied Photophysics and Jasco P-1020. The apparatus of high performance liquid chromatography was a Agilent 1260 with DAD detector. Column chromatography was performed with Sephadex LH-20 (Pharmacia Fine Chemical Co., Ltd. Uppsala, Sweden), Diaion HP20SS (Mitsubishi Chemical Co., Tokyo, Japan), Rp-8 gel (40-60 μm, Merck, Darmstadt, Germany), Toyopearl HW-40C (TOSOH, Japan), MCI CHP-20P (75-150 μm, Mitsubishi Chemical Co., Tokyo, Japan). Silica gel (200-300 mesh, Qingdao Hailang Group Co., Ltds. Qingdao, People's Republic of China) and a 250 × 9.4 mm, i.d., 5 μm Sunfire C_{18} column (Waters). TLC was carried out on precoated silica gel GF254 plates, which were visualized by ultraviolet and spraying with 10% sulfuric ethanol solution. The quantum chemical calculations were carried out at HPC Center, Kunming Institute of Botany, Chinese Academy of Sciences, China.

Plant materials

The roots of *P. emblica* were collected in Baoshan City, Yunnan Province, People's Republic of China, and identified by Prof. Chong-Ren Yang from Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (KIB-ZL-0100020) has been deposited in State Key Laboratory of Phytochemistry and Plant Resource in West China of Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation

The extraction and pretreatment of the roots of *P. emblica* were carried out as the previously reported procedure.[13] Fr.A was subjected to Sephadex LH-20 (CH_3OH 0-100%) to afford Fr.A1-Fr.A2. Fr.A1 (3.2 g) passaged over MCI CHP-20P column (CH_3OH/H_2O 30% - 90%) to give four fractions (Fr.A1.1-Fr.A1.4). Fr.A1.3 was chromatographed on Toyopearl HW-40C (CH_3OH/H_2O 0-30%) to give three fractions (Fr.A1.3.1-1.3.3), and Fr.A1.3.2 was separated by PHPLC (CH_3CN/H_2O, 10-20%) to afford **2** (8 mg). Fr.A1.4 was purified by Toyopearl HW-40C (CH_3OH 0-30%) to give **1** (220 mg). Fr.B passaged over Sephadex LH-20 (CH_3OH/H_2O 30-100%) to afford Fr.B1-Fr.B2. Fr.B1 was fractioned on MCI CHP-20P column (CH_3OH/H_2O 30%-80%) to afford Fr.B1.1-Fr.B1.6. Fr.B1.4 was purified by Toyopearl HW-40C (CH_3OH/H_2O 0-30%) and Rp-8 (CH_3OH/H_2O 30%-80%) to afford **5** (25 mg). Fr.B1.6 was chromatographed on Toyopearl HW-40C (CH_3OH/H_2O 0-30%) to give Fr.B1.6.1-1.6.4, and Fr.B1.6.1 was purified by PHPLC (CH_3CN/H_2O, 10-20%) to afford **3** (2 mg). Fr.F (24.4 g) passaged over a Sephadex LH-20 (CH_3OH/H_2O 0-100%) to give Fr.F1-Fr.F3. Fr.F1 was fractioned on Rp-8 (CH_3OH/H_2O 30%-80%) to give Fr.F1.1-1.9. Fr.F1.2 was chromatographed on silica gel ($CHCl_3/CH_3OH/H_2O$, 8:2:0.2) to afford Fr.F1.2.1-1.2.4, and Fr.F1.2.2 was purified by PHPLC (CH_3CN/H_2O, 10-20%) afford **6** (2 mg). Fr.F1.3 passaged over Toyopearl HW-40C (CH_3OH 0-30%) to give Fr.F1.3.1-1.3.5, Fr.F1.3.4 was separated by PHPLC (CH_3CN/H_2O, 10-20%) to afford **4** (2 mg).

Phyllanembloid A (1): White amorphous powder; $[\alpha]_D^{25}$ = +65.4 (*c* = 1.6 in methanol); ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) data, see Table 1; IR (KBr) ν_{max} 3429, 2926, 1578, 1384, 1073 cm^{-1}; UV (MeOH) λ_{max} (log ε) 206 (1.23), 235 (1.25), 278 (0.96) nm; ECD (CH_3OH, [nm],ϕ [mdeg]): 233 (25.1), 279 (5.2), 310 (-1.5); MS (ESI): *m/z*: 489 [*M*-H]$^-$; HRMS (EI): *m/z* calcd for: $C_{26}H_{33}O_9$, 489.2130 [*M*-H]$^-$; found: 489.2130.

Phyllanembloid B (2): White amorphous powder; $[\alpha]_D^{25}$ = +17.9 (*c* = 1.1 in methanol); ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) data, see Table 1; IR (KBr) ν_{max} 3422, 2925, 1580, 1385, 1282, 1073 cm^{-1}; UV (MeOH) λ_{max} (log ε) 202 (1.07), 238 (1.14), 278 (0.75) nm; ECD (CH_3OH, [nm], ϕ [mdeg]): 234 (16.5), 277 (3.5), 310 (-1.2); MS (ESI): *m/z*: 506 [*M*]$^-$; HRMS (EI): *m/z* calcd for: $C_{26}H_{34}O_{10}$, 506.2147 [*M*]$^+$; found: 506.2133.

Phyllanembloid C (3): White amorphous powder; $[\alpha]_D^{25}$ = -18.8 (*c* = 1.0 in methanol); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data, see Table 1; IR (KBr) ν_{max} 3425, 2924, 1633, 1416, 1073 cm^{-1}; UV (MeOH) λ_{max} (log ε) 203.2 (1.22) nm; ECD (CH_3OH, [nm], ϕ [mdeg]): 201 (-6.3), 225 (2.6), 242 (2.9), 285 (-1.2); MS (ESI): *m/z* 491 [*M*-H]$^-$; HRMS (ESI): *m/z* calcd for $C_{26}H_{35}O_9$, 491.2287 [*M*-H]$^-$; found: 491.2292.

Phyllanembloid D (4): White amorphous powder; $[\alpha]_D^{25}$ = -42.6 (*c* = 0.45 in methanol); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data, see Table 2; IR (KBr) ν_{max} 3431, 2924, 2854, 1640, 1583, 1463, 1382,1066 cm^{-1}; UV (MeOH) λ_{max} (log ε) 207 (1.40), 246 (1.20), 298 (0.98) nm; ECD (CH_3OH, [nm], ϕ [mdeg]): 218 (5.6), 256 (-9.4), 294 (2.2); MS (EI): *m/z* 622 [*M*-1]$^+$, 606, 379; HRMS (EI): *m/z* calcd for $C_{26}H_{32}O_{10}$, 504.1990 [*M*]$^+$; Found: 504.1998.

Phyllanembloid E (5): White amorphous powder; $[\alpha]_D^{25}$ = +108.1 (*c* = 1.3 in methanol); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data, Table 2; IR (KBr) ν_{max} 3429, 2926, 1622, 1447, 1387, 1358, 1076 cm^{-1}; UV (MeOH) λ_{max} (log ε) 230.2 (1.44), 279 (1.07), 327 (0.64) nm; ECD (CH_3OH, [nm], ϕ [mdeg]): 231 (23.4), 282 (5.7); MS (ESI): *m/z* 521 [*M*-H]$^-$; HRMS (ESI): *m/z* calcd for $C_{26}H_{33}O_{11}$, 521.2028 [*M*-H]$^-$; Found: 521.2032.

Phyllanembloid F (6): White amorphous powder; $[\alpha]_D^{25}$ = -47.3 (*c* = 0.7 in methanol); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data, see Table 2; IR (KBr) ν_{max} 3424, 2950, 1631, 1433, 1385, 1077, 1041 cm^{-1}; UV (MeOH) λ_{max} (log ε) 202.8 (0.97) nm; ECD (CH_3OH, [nm], ϕ [mdeg]): 198 (-21.4), 289 (-0.8); MS (ESI): *m/z* 521 [*M*+Na]$^+$; HRMS (ESI): *m/z* calcd for $C_{27}H_{43}O_{11}$, 543.2811 [*M*+HCOO]$^-$; Found: 543.2812.

Acid hydrolysis of compound 1: Compound **1** (23 mg) in 5% H_2SO_4-EtOH solution (2 mL) was heated at 65 ºC for 4 h. After cooling down to room temperature, the mixture was extracted with $CHCl_3$ (30 mL × 3). The organic layer was dried by Na_2SO_4, and evaporated in vacuum to afford a solid (10 mg), which was subjected to p-HPLC to generate compound **1A** (3 mg). The water layer was neutralized with Amberlite AG IRA-401 and evaporated in vacuum to afford D-glycose (6 mg): $[\alpha]_D^{25}$ = +94.3 (*c* = 0.63 in H_2O)].

Enzymatic hydrolysis of compound 1: compound **1** (20 mg) and cellulase in H_2O (2 mL) was incubated at 37 ºC for 24 h. After cooling down to room temperature, the mixture was passaged over MCI-gel CHP20P column (CH_3OH/H_2O 0% - 90%) to afford **1A** (5 mg).

Compound 1A: White amorphous powder; $[\alpha]_D^{25}$ = +88.6 (*c* = 3.0 in methanol); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data, Table 1; IR (KBr) ν_{max} 3431, 2926, 2855, 1686, 1602, 1456, 1209, 1146 cm^{-1}; UV (MeOH) λ_{max} (log ε) 200 (1.03), 240 (1.24) nm, 281 (0.79), 334 (0.33); ECD (CH_3OH, [nm], ϕ [mdeg]): 241 (14.2), 285 (1.1), 342 (1.0); MS (ESI): *m/z* 327 [*M*-H]$^-$; HRMS (ESI): *m/z* calcd for $C_{20}H_{24}O_4$, 328.1669 [*M*]$^+$; Found 328.1675.

Preparation of compound 1B: 1A (5 mg) was dissolved in 1.5 mL of anhydrous DMF/CH_3CN (2:1) solution, to which K_2CO_3 (5 mg) and CH_3I (500 μL) were added. The reaction was carried out in dark at 40 ºC for 2h. The mixture was poured into H_2O (6 mL) and extracted with EtOAc (6 mL × 3). The organic layers were combined and washed with H_2O (18 mL × 3). After dryness with anhydrous Na_2SO_4, the organic layer was evaporated to dryness. The solid was purified by a silica gel column to afford **1B** (3.7 mg): MS (ESI): *m/z* 357 [*M*+H]$^+$.

Preparation of (*R*)- and (*S*)- MTPA esters (1r and 1s): 1B (1.5 mg) was transferred in a dried tube, to which anhydrous pyridine (300 μL) and (*R*)-(-)-α-methoxy-α-(trifluoromethyl) phenylacetyl (MTPA) chloride (10 μL) were added. The mixture was shaken to make **1B** and MTPA chloride mix evenly, and kept overnight under N_2 gas stream. The mixture was poured into H_2O (1 mL) and extracted with EtOAc (1 mL × 3). The organic layers were combined and washed with H_2O (1 mL × 3). After dryness with anhydrous Na_2SO_4, the organic layer was evaporated to dryness and the solid was monitored by NMR spectrometer. ^{1}H NMR data of (*S*)-MTPA ester derivative **1s** of **1B** (800 MHz, $CDCl_3$; signals were assigned by ^{1}H-^{1}H COSY and ROESY，see S52-S57 in ESI): δ_H 1.806 (1H, dt, *J* = 2.4, 13.0 Hz, H-1a), 2.169 (1H, dt, *J* = 13.0, 3.0 Hz, H-1b), 1.887 (1H, m, H-2a), 2.041 (1H, m, H-2b), 4.759 (1H, dd, *J* = 4.1, 11.8 Hz, H-3), 2.112 (1H, t, *J* = 2.7 Hz, H-5), 5.802 (1H, dd, *J* = 2.8, 9.9 Hz, H-6), 6.635 (1H, dd, *J* = 2.8, 9.9 Hz, H-7), 6.613 (1H, s, H-11), 6.701 (1H, dd, *J* = 11.4, 17.8 Hz, H-15), 5.296 (1H, dd, *J* = 1.0, 17.6 Hz, H-16a), 5.404 (1H, dd, *J* = 1.0, 11.4 Hz, H-16b), 0.955 (3H, s, CH_3-18), 0.856 (3H, s, CH_3-19), 0.976 (3H, s, CH_3-20), 3.771 (3H, s, 12-CH_3O), 3.765 (3H, s, 17-CH_3O). In the manner described for **1s**, another portion of **1B** (1.5 mg) was reacted with (*S*)-(+)-MTPA chloride (10 μL) to give (*R*)-MTPA ester derivative **1r**: ^{1}H NMR (800 MHz, $CDCl_3$; signals were assigned by ^{1}H-^{1}H COSY ROESY): δ_H 1.789 (1H, dt, *J* = 2.4, 13.0 Hz, H-1a), 2.137 (1H, dt, *J* = 13.0 Hz, H-1b), 1.769 (1H, m, H-2a and 2b), 4.725 (1H, dd, *J* = 4.1, 10.9 Hz, H-3), 2.111 (1H, t, *J* = 2.7 Hz, H-5), 5.811 (1H, dd, *J* = 2.8, 9.9 Hz, H-6), 6.634 (1H, dd, *J* = 2.8, 9.9 Hz, H-7), 6.607 (1H, s, H-11), 6.698 (1H, dd, *J* = 11.3, 12.0 Hz, H-15), 5.291 (1H, brd, *J* = 12.0 Hz, H-16a), 5.399 (1H, brd, *J* = 12.0 Hz, H-16b), 0.958 (3H, s, CH_3-18), 0.934 (3H, s, CH_3-19), 0.946 (3H, s, CH_3-20), 3.821 (3H, s, 17-CH_3O), 3.758 (3H, s, 12-CH_3O).

Quantum chemical calculations: The structure models of the compounds were constructed based on NOE analysis. The conformation analysis was carried out using Monte Carlo searching with molecular mechanics MMFF in Sparton'06 (Wavefunction Inc. Irvine, CA). The resulted conformers were re-optimized using DFT at the B3LYP-SCRF/6-311G (d, p) level using the integral equation formalism variant of the polarizable continuum model (IEF-PCM). The free energies and vibrational frequencies were calculated at the same level to confirm their stability, and no imaginary frequencies were found. The optimized low energy conformers with energy < 2 Kcal/mol were considered for ECD calculation. The TD-DFT/ B3LYP-SCRF/6-311G (d, p) method was applied to calculate the excited energies, oscillator strength and rotational strength for 50 states. All the calculations were run with Gaussian '09.[23]

The excited energies and rotational strength were used to simulate ECD spectra of ea1ch conformer by introducing the Gaussian Function. The final ECD spectra of each compound were obtained by averaging all the simulated ECD spectra of all conformers according to their excited energies and Boltzmann distribution. The band shape of the calculated ECD curves were all 0.5 eV if there is no illustration.

Optical rotations (OR) of compound **6** was calculated using TDDFT/GIAO method with B3LYP/6-311++G (2d, p) basis set with the same optimized conformers as ECD calculations. The final optical rotation was obtained by averaging the OR values of each conformer with Boltzmann distribution.

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Notes and references

[a] *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, People's Republic of China..*

[b] *University of Chinese Academy of Sciences, Beijing 100049, People's Republic of China.*

[c] *Chemistry and Chemical Engineering College, Chongqing University, Chongqing, 400030, People's Republic of China.*

* *Tel: +86-871-6522-3235. E-mail: zhangyj@mail.kib.ac.cn (Ying-Jun Zhang) and minxu@mail.kib.ac.cn (Min Xu).*

† Electronic Supplementary Information (ESI) available: Copies of the ^{1}H NMR, ^{13}C NMR, MS, HSQC, HMBC, COSY, and ROESY spectra of **1-6**, **1A**, **1B**, **1r**, and **1s**, and the low energy optimized conformers, calculated ECD curves and ORs of conformers of **1, 1A, 3, 4** and **5**. See DOI: 10.1039/b000000x/

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