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Bioactive norditerpenoids from *Flickingeria fimbriata*

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Seven new degraded diterpenoids (**1**−**6** and **11**), four new *ent*-pimarane type diterpenoids (**7**−**10**) and a known analogue (**12**) were isolated from the stem of *Flickingeria fimbriata*. Compounds **1**−**4**, namely norflickinflimiods A−D, possessed a rare 16-nor-*ent*-pimarane skeleton, and **5**−**6**, namely norflickinflimiods E and F, represented an unusual skeleton of 15,16-dinor-*ent*-pimarane. The structures including absolute configuration of **1**–**12** were elucidated using spectroscopic analysis, single-crystal X-ray diffraction, computational method, and chemical correlations. All the isolates were screened for anti-inflammation and antitumor activities, and compounds **5**, **6**, **9**, and **12** showed potent inhibitory activities against lipopolysaccharide (LPS)-induced TNF-α production in RAW264.7 cells with IC_{50} values less than 10 μM, while compounds **8**−**11** exhibited cytotoxicity against MCF-7 cells (9.6, 9.0, 12.0, and 15.6 μM).

Introduction

Flickingeria fimbriata (Bl.) Hawkes, also known as *Dendrobium fimbriatum* and *Ephemerantha fimbriata*, belongs to Orchidaceae family, widely grows in Guangxi, Hainan, and Yunnan Provinces of China. It was used as a substitute of the precious and scarce Traditional Chinese Medicine *Dendrobium candidum* for treating pneumonia, tuberculosis, asthma, and pleurisy.[1] Previous phytochemical investigation of this plant led to the isolation of diterpenes,[2–4] phenanthrenes,[4] steroids,[5] and bibenzyls,[6] and some of them exhibit P-glycoprotein inhibition,[2] anti-inflammation,[7–9] and antimutagenic activities.[10] In the course of our search for structurally intriguing and biologically significant metabolites from medical plants, eleven new and a known diterpenoids were isolated from the stem of *F. fimbriata*. Herein, we described the details of the isolation, structural elucidation, biogenetic synthesis, and bioactivities of the obtained compounds.

Results and discussion

The air-dried powder of the stems of *F. fimbriata* was extracted with 95% EtOH at room temperature (rt) to give a crude extract, which was suspended in H_2O and successively partitioned with petroleum ether, EtOAc, and *n*-BuOH. The EtOAc and *n*-BuOH extracts were subjected to various column chromatography protocols to afford compounds **1–12**.

Norflickinflimiod A (**1**) was obtained as colorless needles ($CHCl_3$−MeOH), and its molecular formula was determined to be $C_{19}H_{30}O_4$ (five degrees of unsaturation) from the quasi-molecular ion peak at *m/z* 345.2039 $[M + Na]^+$ (calcd for $C_{19}H_{30}O_4Na$, 345.2036) in HRESIMS spectrum. The IR absorption bands at 3446 and 1708 cm^{-1} indicated the presence of hydroxyl and carbonyl groups, respectively. The ^1H NMR spectrum of **1** (Table 1) showed the signals of four tertiary methyl groups [δ_H 0.77 (s, H_3-20), 0.89 (s, H_3-18), 1.00 (s, H_3-19), and 1.19 (s, H_3-17)], two oxygenated methines [δ_H 3.35 (d, J = 2.6 Hz, H-3) and 3.90 (ddd, J = 2.6, 6.4, 8.8 Hz, H-2)], one olefinic proton [δ_H 5.41 (s, H-14)], and a series of aliphatic methylene multiplets. The ^{13}C NMR spectrum combined with DEPT experiments resolved 19 carbon resonances attributable to four quaternary methyls, five sp^3 methylenes, five methines (two oxygenated and one olefinic carbon), and five quarternary carbons (including one carboxyl and one olefinic carbons). The double bond and the carboxyl group accounted for two out of the five degrees of unsaturation, and the leftover double-bond

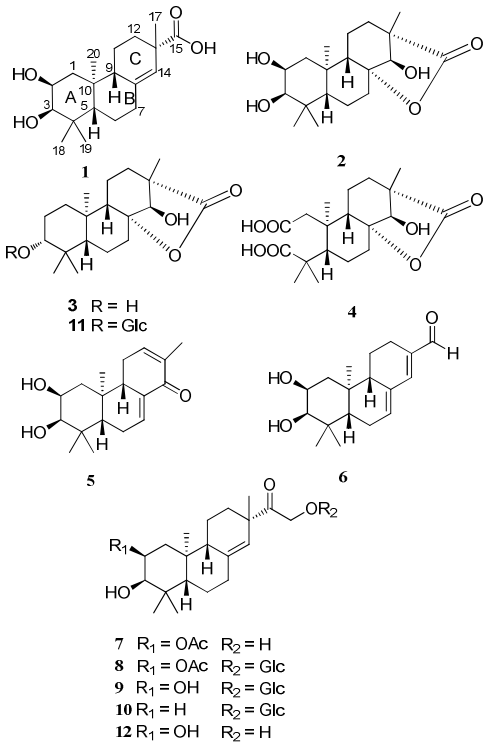


Fig. 1 Structures of compounds **1**−**12**.

Table 1 ^{1}H NMR data for compounds **1–7** (δ in ppm, J in Hz)

position	**1**[a]	**2**[a]	**3**[a]	**4**[b]	**5**[c]	**6**[c]	**7**[c]
1α	1.56, m	1.54, m	1.69, dt (2.8, 13.0)	2.36, dd (4.4, 13.6)	1.52, m	1.85, dd (4.4, 12.0)	1.56, m
1β	1.54, m	1.43, t (12.0)	1.10, dd (5.6, 13.0)	2.63, dd (4.4, 13.6)	1.60, m	1.56, m	1.67, t (12.6)
2α	3.90, ddd (2.6, 6.4, 8.8)	3.91, ddd (2.8, 4.0, 12.0)	1.62, m		3.98, ddd (2.8, 4.4, 11.8)	4.05, ddd (2.8, 4.4, 11.8)	5.14, ddd (2.4, 4.2, 12.6)
2β			1.58, m				
3	3.35, d (2.6)	3.35, d (2.8)	3.18, dd (6.0, 10.4)		3.41, d (2.8)	3.49, d (2.8)	3.51, br s
5	1.48, br s	1.30, d (10.0)	0.89, m	2.54, m	1.76, dd (5.6, 11.6)	1.73, dd (4.4, 12.0)	1.55, d (11.1)
6α	1.45, d (2.0)	1.30, d (10.0)	1.66, m	1.81, m	2.02, m	1.91, m	1.54, br s
6β	1.67, dddd (2.2, 6.8, 10.0, 13.6)	1.67, dd (4.4, 11.2)	1.62, m	1.62, m	2.17, br s	1.95, m	1.37, dd (4.6, 13.3)
7α	2.33, dd (2.0, 13.8)	1.75, dt (4.4, 16.0)	1.86, m	1.71, m	5.90, br s	6.11, d (2.4)	2.37, d (4.6)
7β	2.07, td (2.2, 13.8)	1.88, br s	1.78, t (2.4)	1.94, m			2.12, m
9	1.85, d (6.2)	1.71, d (3.6)	1.58, m	2.83, m	2.62, m	2.08, m	1.90, dd (5.8, 9.2)
11α	1.38, td (3.6, 12.6)	1.53, m	1.28, m	1.29, m	2.52, br s	2.21, d (3.6)	1.64, br s
11β	1.55, d (6.2)	1.53, m	1.27, m	1.60, m	2.48, dd (8.8, 16.8)	2.12, br s	2.10, d (5.8)
12α	1.13, d (3.6)	1.30, d (10.0)	1.29, m	1.89, m	6.83, br s	2.65, dd (3.6, 17.6)	2.31, dt (2.4, 12.0)
12β	2.16, d (12.6)	1.87, br s	1.77, m	1.80, m		1.99, br s	1.15, br s
14	5.41, s	3.39, br s	3.39, br, s	3.41, s		6.83, br s	5.41, s
16a							4.33, d (4.6) (overlap)
16b							4.33, d (4.6) (overlap)
17	1.19, s	1.06, s	1.06, s	1.04, s	2.31, s	9.45, s	1.12, s
18	0.89, s	0.93, s	0.84, s	1.27, s	0.93, s	0.98, s	0.93, s
19	1.00, s	1.02, s	1.00, s	1.26, s	1.00, s	1.04, s	1.03, s
20	0.77, s	1.02, s	0.97, s	1.07, s	0.73, s	0.85, s	0.75, s
CH_3CO							2.07, s

[a] Recorded at 400 MHz, in CD_3OD. [b] Recorded at 500 MHz, in CD_3OD. [c] Recorded at 400 MHz, in $CDCl_3$.

equivalents required a tricyclic nature of **1**. The gross structure was constructed by two-dimensional (2D) NMR analysis. Three fragments (Fig. 2), **a** (C-1 to C-3), **b** (C-5 to C-7), and **c** (C-11 to C-9 and C-12) were readily established by the ^{1}H–^{1}H COSY correlations. The connectivity of these fragments, quaternary carbons and other substitutes was accomplished by the HMBC correlations (Fig. 2). In particularly, the carboxyl group (δ_C 180.1, C-15) was attached at C-13 by HMBC correlations of H_3-17/C-15, and H-14/C-15. Thus, the planar structure of a nor-pimarane-type diterpene was emerged as shown in Fig. 1.

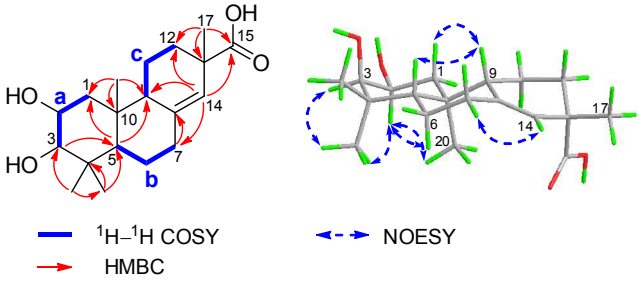


Fig. 2 Selected 2D NMR correlations of **1**.

Analysis of the NOESY correlations (Fig. 2) and coupling constants permitted the determination of the relative configuration of **1**. The NOESY spectrum showed cross peaks between the proton pairs H-2/H_3-19, H-2/H_3-20, and H-2/H-6α, indicating that these protons were cofacial and axial oriented, which were arbitrarily assigned as α orientation. Accordingly, OH-2 and H_3-18 were assigned as β orientation, suggesting a chair conformation for ring A. Based on the small coupling constant (J = 2.4 Hz) between H-2 the H-3, the OH-3 was assigned as β orientation. The β assignment of coplanar protons H-5 and H-9 were deduced from the NOE correlations of H-1β/H-5, H-5/H-7β and H-5/H-9. However, the orientation of H_3-17 could not be assigned by NOE data as the correlation of H-14 with H_3-17 was insufficient to determine its direction. Fortunately, the X-ray diffraction analysis of a crystal of **1** permitted the β configuration of Me-17 (Fig. 3), and also confirmed the relative configuration of other chiral centers.

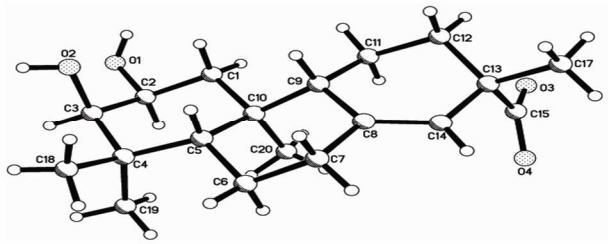


Fig. 3 Single-crystal X-ray structure of **1**.

To determine the absolute configuration of **1**, the CD exciton chirality method was applied on its 2,3-di-*p*-methoxybenzoate derivative (**1a**). As shown in Fig. 4, the negative chirality resulting from the exciton coupling between the two chromophores of *p*-methoxybenzoate at 264 nm ($\Delta\varepsilon$ −2.15, π-π^* transition) and 234 nm ($\Delta\varepsilon$ −0.30, π-π^* transition) indicated that the transition dipole moments of the two

chromophores were oriented in a counterclockwise manner.[11] Thus, the absolute configurations of C-2 and C-3 of **1a** were determined to be 2*S* and 3*R*, respectively. Consequently, the structure of compound **1** was determined to be (2*S*,3*R*,5*S*,8*R*,9*S*,10*S*,13*S*)-16-nor-2,3-dihydroxy-*ent*-pimar-8(14)-en-15-oic acid, as shown in Fig. 1.

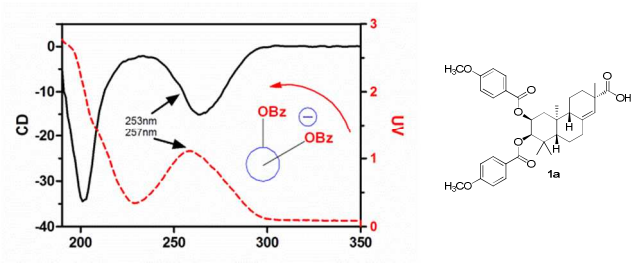


Fig. 4 CD and UV spectra of compound **1a** in MeCN. The arrow denotes the electric transition dipole of the chromophore.

Norflickinflimiod B (**2**), a colorless oil, had the molecular formula $C_{19}H_{30}O_5$ as inferred from the HRESIMS with five degrees of unsaturation. Comparison of the NMR data of **2** with those of **1**, indicating the trisubstituted double bond in **1** was replaced by one oxygenated quaternary carbon (δ_C 87.0, C-8) and an oxygenated methine (δ_C 79.4, C-14) in **2**. The location of the oxygenated carbons were established by the key HMBC correlations of H-6/C-8, H-7/C-14, H-9/C-14, H-14/C-15, and H-15/C-14. As **2** maintained the same degrees of unsaturation as **1**, an additional ring was required, The severely downfield-shifted C-8 (δ_C 87.0) indicated the presence of a γ-lactone between the C-15−O−C-8 bonds.[12] Thus, the gross structure of **2** was deduced as shown in Fig. 1.

The relative configuration of **2** was determined by comprehensive analysis of its NOESY spectrum. To determine its absolute configuration, **2** was transformed to its acetonide derivative (**2a**) to afford a crystal, which was analyzed by X-ray crystallography (Fig. 5). The final refinement of the Cu Kα data resulted in a Flack[13] parameter of −0.11(14) and the Hooft[14] parameter of −0.09(6), which allowed unambiguous assignment of the absolute configuration of **2** as 2*S*,3*R*,5*S*,8*R*,9*S*,10*R*,13*S*,14*R*.

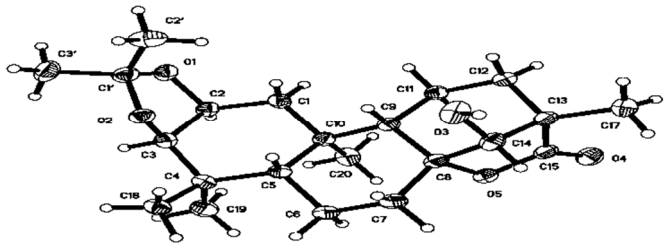


Fig. 5 Single-crystal X-ray structure of **2a.**

HRESIMS analysis of norflickinflimiod C (**3**) showed a molecular formula of $C_{19}H_{30}O_4$ with one oxygen atom less than **2**. The ^{1}H and ^{13}C NMR data of **3** was closely related to **2**, with the main difference being the presence of an sp^3 methylene (δ_C 27.7) in **3** instead of an oxygenated methine (δ_C 66.7) in **2**, which suggested that **3** was a reduced derivative of **2**, and the location of the hydrogenolyzed methine was supported by the crucial ^{1}H–^{1}H COSY correlations of H-1/H$_2$-2. The NOE experiment revealed a β-equatorial orientation of H-3, based on the coupling constants ($J_{3,2}$ = 6.0, 10.4 Hz) and the NOESY correlation of H-3/H-5. The relative configuration of other chiral centers were the same as those of **2**. The CD curve of **3** (see Electronic Supplementary Information S84) matched well with that of **2**, indicating a 13*S* configuration. Thus, the structure of **3** was determined as shown in Fig. 1.

Table 2. ^{13}C NMR data of compounds **1**–**7** (δ in ppm)

position	1[a]	2[a]	3[a]	4[b]	5[c]	6[c]	7[c]
1	40.9	41.4	39.1	41.5	39.9	40.1	36.0
2	67.3	66.7	27.7	175.0	65.9	66.4	70.9
3	80.0	79.7	79.6	182.8	78.9	78.9	76.7
4	39.5	39.3	40.0	47.3	38.1	38.1	38.6
5	48.5	48.0	55.1	49.1	42.8	42.6	47.2
6	21.3	19.3	19.6	22.1	24.6	21.2	21.5
7	36.6	34.8	35.0	34.5	125.2	135.1	35.4
8	139.9	87.0	86.9	87.3	145.4	135.2	141.9
9	51.7	45.8	46.1	39.0	53.6	50.4	50.5
10	40.2	38.3	37.2	41.5	35.3	36.0	39.3
11	22.9	19.0	19.4	19.6	29.5	24.5	20.2
12	34.3	26.6	26.8	26.7	141.7	21.8	32.5
13	43.9	46.9	47.1	46.8	146.1	138.3	46.8
14	127.4	79.4	79.5	79.6	197.4	148.7	123.8
15	181.0	181.0	181.2	181.0			214.3
16							65.7
17	28.2	18.6	18.7	19.0	26.4	193.5	27.3
18	22.8	22.7	16.6	25.4	20.9	21.6	22.0
19	29.3	29.4	29.1	27.4	28.0	28.1	28.5
20	15.9	16.5	15.6	18.5	14.3	14.4	15.2
2-OCOCH$_3$							21.3
2-OCOCH$_3$							170.1

[a] Recorded at 100 MHz, in CD_3OD. [b] Recorded at 125 MHz, in CD_3OD. [c] Recorded at 100 MHz, in $CDCl_3$.

The molecular formula of norflickinflimiod D (**4**) was established as $C_{19}H_{28}O_7$ by HRESIMS. The ^{13}C NMR spectrum of **4** exhibited 19 carbon resonances which were classified by DEPT experiments as four methyls, five methylenes, three methines (an oxygenated one), and seven quaternary (three carbonyl, one oxygenated, and three high-field carbons). Aforementioned data implied that compound **4** possessed most structural features of **2**, except for the major difference occurring on the ring A. The observation of two additional carboxyl groups (175.0, C-2 and 182.8, C-3) in **4**, suggested that a 2,3-*seco* nature of ring A was required. The location of carboxyl groups at C-2 and C-3 were supported by HMBC correlations from H-1 to C-2 and from H-18/19 to C-3, respectively. So, the planar structure of **4** was elucidated as shown. A chemical transformation from **2** to **4** was successfully performed stepwise by periodic acid oxidation and Pinnick oxidation[15] (Fig. 6), which also assigned the stereochemistry of C-5, C-8, C-9, C-13, and C-14 in **4** to be the same as that in **2**. To our best knowledge, compound **4** represents the first example of this unprecedented skeleton of 2,3-*seco*-16-nor-*ent*-pimarane.

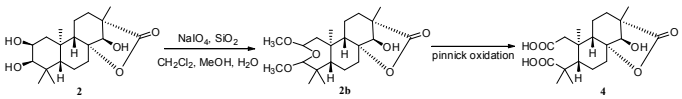


Fig. 6 The transformation of **2** to **4**.

The molecular formula of norflickinflimiod E (**5**), $C_{18}H_{26}O_3$, was evident from the molecular ion peak at *m/z* $[M + H]^+$ of 291.1950 (calcd for $C_{18}H_{27}O_3$, 291.1955) in the HRESIMS spectrum. The NMR data indicated that **5** was related to **1**, except for the absence of the carboxyl (C-15) and the appearance of a cross-conjugated bicyclic dienone.[16] HMBC correlations of H-5/C-7, H-6/C-8, H-7/C-9 and C-14, H-12/C-9, C-14, and C-15, H-17/C-12

and C-14 allowed the assignment of $\Delta^{7(8),\ 12(13)}$ and the C-14 ketone carbonyl. The relative configuration at the chiral centers of **5** was determined to be the same as that of **1** by the NOESY spectrum. Due to the limited amount of **5**, the derivative for CD exciton chirality method and single crystal for X-ray diffraction were unavailable. Therefore, the absolute configuration of **5** was determined by the calculated ECD spectrum, using the Gaussian 09 program at the TD-DFT-B3LYP/6-31(d,p) level in MeOH. The calculated ECD spectrum (Fig. 7) matched the experimental result very well, allowing the assignment of the absolute configuration of **5** as depicted in Fig. 1.

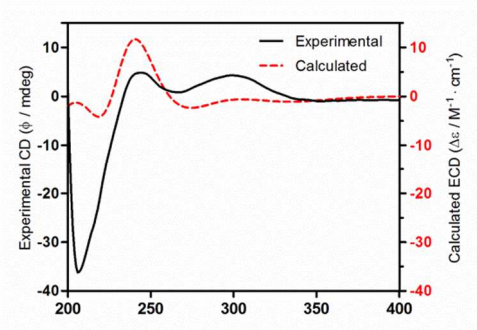


Fig. 7 ECD spectra of **5**.

Norflickinflimiod F (**6**) had the molecular formula of $C_{18}H_{26}O_3$ from HRESIMS. The NMR data of **6** was similar to that of **5** except that the $\Delta^{12(13)}$ double bond in **5** was migrated to $\Delta^{13(14)}$, forming an α, β-unsaturated aldehyde group. This conjugated system was characterized by the red shifted of the UV absorption with respect to that of **5**, and the location was identifieded by HMBC correlations of H-7/C-14, H-14/C-7, H-14/C-17, and H-17/C-14. The relative configuration of **6** was established to consist with **5** by the comprehensive analysis of NOESY spectrum. The absolute configuration of **6** was determined by comparison of the calculated CD spectrum with the experimental result (Fig. 8), as shown in Fig. 1.

The molecular formula of 2-acetyl-flickinflimiod (**7**) was determined as $C_{22}H_{34}O_5$ from HRESIMS data. The ^{1}H and ^{13}C NMR spectra of **7** were very similar to those of flickinflimbrol A (**12**)[17] except for the presence of the signals assigned to the acetyl group [δ_H 2.07 (s), δ_C 21.3, and 170.1],[18] indicating that **7** was an acetylated derivative of **12**.

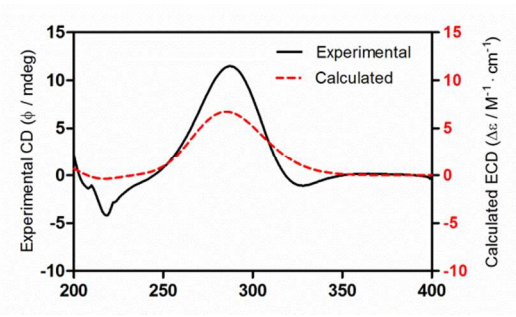


Fig. 8 ECD spectra of **6**.

The acetoxy group was assigned at C-2 by HMBC correlation from H-2 (δ_H 5.14) to the acetyl carbonyl at δ_C 170.1, which was supported by the upfield-shifted C-1 and C-3 signals in **7** with respect to those in **12**. The relative configuration of **7** was assigned to be the same as that of **12** by comparing their NMR data. The observed CD curve for compound **7** exhibited similar Cotton effects with that of **1**, indicating that the absolute configuration of C-13 was the same as that of **1**. Thus, compound **7** was determined to be (2*S*,3*R*,5*S*,9*R*,10*S*,13*S*)-16,3-dihydroxy-2-acetoxy-*ent*-pimar-8(14)-en-15-one, as depicted in Fig. 1.

Flickinflimoside A (**8**) was obtained as an optically active colorless oil, the molecular formula of $C_{28}H_{44}O_{10}$ was determined by the *m/z* of 585.2926 $[M + Na]^+$ (calcd 585.2917) from HRESIMS. The 1D NMR of **8** resembled that of **7** except for the presence of additional signals being attributed to a β-glucopyranose residue [δ_H 4.23 (J = 8.0 Hz, H-1') and δ_C 104.2 (C-1')], indicating **8** was a glycosylated derivative of **7**. The glucopyranose moiety was located at C-16 by the mutual HMBC correlations from H_2-16 to the hemiacetal carbon C-1', and from H-1' to C-16. Furthermore, this was supported by the downfield-shifted carbon signal of C-16 (δ_C 72.5) in **8** with respect to that in **7** (δ_C 65.7). Acid hydrolysis of **8** generated a diterpene and glucose mixture. The diterpene was confirmed to be **7** by NMR data and optical rotation. The absolute configuration of the β-glucose was identified to be D-configuration by HPLC analysis.[19,20] Thus **8** was determined as depicted.

The molecular formula of flickinflimoside B (**9**) was determined to be $C_{26}H_{42}O_9$ by HRESIMS with 42 mass units less than **8**. Its ^{1}H and ^{13}C NMR spectra closely resembled those of **8**, except for the absence of signals for an acetyl group, indicating that **9** was deacetylated derivative of **8**. The relative configuration of **9** was the same as that of **8** according to the NOESY spectrum. Inspection of the CD curve indicated that the absolute configurations of the chiral centers of the aglycon moiety of **9** were congruent with those of compound **1**. Thus, compound **9** was determined to be (2*S*,3*R*,5*S*,9*R*,10*S*,13*S*)-2,3,16-trihydroxyl-15-keto-*ent*-pimar-8(14)-ene-16-*O*-β-D-glucopyranoside.

The molecular formula of $C_{26}H_{42}O_8$ for flickinflimoside C (**10**) was determined by HRESIMS. The ^{1}H and ^{13}C NMR spectra of **10** exhibited high similarity to those of **9**, except for the absence of the signal of the oxygenated methine (C-2) in **9**, and presence of an sp^3 methylene in **10**. Hence, consistent with its molecular formula, compound **10** was proposed to be a dehydroxylated derivative of **9**, which was supported by HMBC correlations. The β-glucose moiety (δ_H 4.23, d, J = 7.6 Hz, H-1′) obtained from acid hydrolysis of **10** was determined to be the D-glucose by HPLC analysis. The relative configuration was determined as shown by the NOE correlations. The CD spectrum of **10** shared the same curve with that of **7**, suggesting that **10** was (2*S*,3*R*,5*S*,9*R*,10*S*,13*S*)-2,3,16-trihydroxyl-15-keto-*ent*–pimar-8(14)-ene-16-*O*-β-D-glucopyranoside.

Norflickinflimoside (**11**) was obtained as a colorless oil, the molecular formula of $C_{25}H_{40}O_9$ was deduced from HRESIMS. The ^{13}C NMR spectrum showed 25 carbon resonances, of which six were characterized of a glucose. Apart from the sugar moiety, the 1D NMR data of the aglycone of **11** were similar to those of **3**, indicating that **11** was a glycosidic derivative of **3**. The linkage between the glucose moiety and the aglycone was established by the HMBC correlation of H-1′/C-3. The coupling constant (J = 7.7 Hz) of H-1′ and H-2′ deduced the glucose to be a β-anomeric configuration. Acid hydrolysis of **11** provided a D-glucose identified by the same means as those of **8**. And the obtained aglycone was found to be identical to **3** by the NMR, MS, and optical rotation data. Thus, the structure of compound **11** was determined to be (3*R*,5*S*,8*R*,9*S*,10*R*,13*S*,14*R*)-16-nor-*ent*-pimar-8,15-olide-3α-*O*-β-D-glucopyranoside.

The known compound flickinflimbrol A (**12**) was identified by comparison of its spectroscopic data with the literature,[17] and the

Table 3 ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data of compounds **8**–**11** in CD_3OD (δ in ppm, J in Hz)

position	**8**		**9**		**10**		**11**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1α	1.48, m	37.2	1.52, m	40.5	1.64, m	38.1	1.59, m	23.8
1β	1.70, t (11.4)		1.55, m		1.19, s		1.75, t (3.5)	
2α	5.08, d (11.4)	72.0	3.87, d (12.0)	67.2	1.59, br s	28.2	1.71, dt (3.5, 13.1)	38.7
2β					1.58, br s		1.05, m	
3	3.46, s	77.2	3.34, m	80.0	3.19, s	77.6	85.7, t (4.4)	85.9
4		40.5		39.5		40.1		39.2
5	1.52, m	48.5	1.48, m	48.5	1.10, s	55.4	0.93, dd (2.7, 11.4)	55.5
6α	1.21, t (12.6)	21.3	1.23, m	21.3	1.20, s	21.2	1.63, m	19.6
6β	1.61, m		1.66, m		1.60, s		1.62, m	
7α	2.39, d (12.6)	36.6	2.40, dd (2.4, 14.2)	36.7	2.40, d (9.6)	36.8	1.86, m	35.0
7β	2.12, m		2.12, ddd (5.4, 12.1, 14.2)		2.09, s		1.77, m,	
8		143.1		143.3		143.5		86.9
9	1.92, t (8.0)	47.1	1.91, t (8.0)	51.9	1.77, t (8.0)	52.0	1.58, dd (3.8, 9.0)	46.0
10		39.9		40.4,		39.5		37.0
11α	1.42, dd, (5.0, 16.0)	22.7	1.42, ddd (4.1, 12.6, 16.6)	22.8	1.43, dd (4.0, 14.0)	23.3	1.28, m	19.3
11β	1.58, m		1.59, m		1.66, s		1.61, m	
12α	1.10, s	33.4	1.09, s	33.5	1.07, s	33.6	1.28, m	26.7
12β	2.29, d (16.0)		2.30, d (12.6)		2.29, d (14.0)		1.86, m	
13		48.6		48.6		48.6		47.0
14	5.54, s	125.5	5.52, s	125.2	5.51, s	124.9	3.39, s	79.4
15		213.5		213.6		214.7		181.1
16a	4.47, d (18.0)	72.4	4.49, d (18.2)	72.4	4.47, d (18.0)	72.5		
16b	4.86, d (18.0)		4.86, d (18.2)		4.74, d (18.0)			
17	1.13, s	27.5	1.13, s	27.5	1.12, s	27.5	1.06, s	18.7
18	1.00, s	29.2	1.00, s	29.3	1.00, s	29.0	1.07, s	29.2
19	0.95, s	22.6	0.89, s	22.7	0.80, s	16.4	0.88, s	17.4
20	0.79, s	15.9	0.74, s	16.0	0.69, s	15.1	0.99, s	15.6
2-OCH_3CO	2.04, s	21.3						
2-OCH_3CO		173.6						
1'	4.24, d (8.0)	104.2	4.24, d (7.6)	104.2	4.23, d (7.6)	104.2	4.32, d (7.6)	101.8
2'	3.25, m	75.0	3.26, d (3.7)	75.0	3.22, m	75.0	3.16, dd (7.6, 9.1)	75.1
3'	3.25, m	78.2	3.27, d (3.2)	78.1	3.25, m	78.2	3.37, dd (2.7, 4.0)	78.1
4'	3.27, s	71.5	3.25, s	71.5	3.26, s	71.5	3.28, d (8.8)	71.8
5'	3.35, m	77.6	3.23, s	77.6	3.35, m	77.6	3.24, dd (2.3, 5.7)	77.6
6'a	3.63, dd (4.0, 12.0)	62.7	3.65, dd (5.0, 12.0)	62.7	3.63, dd (4.0, 12.0)	62.7	3.67, dd (5.7, 11.8)	62.7
6'b	3.85, d (12.0)		3.86, d (12.0)		3.85, d (12.0)		3.86, dd (2.4, 11.8)	

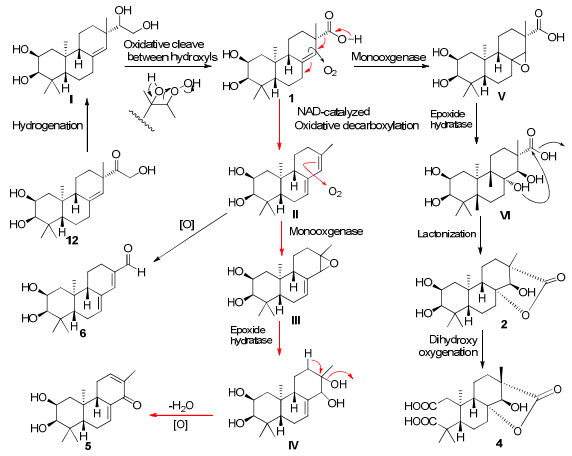
absolute configuration was also determined by the CD spectrum.

As compounds **1**−**2** and **4**−**6** represented rare 15-nor and 15,16-dinor ent-pimarane, respectively. A plausible biogenetic pathway for norditerpenes of them was proposed (Scheme 1), in which the biogenetic precursor was proposed to be compound **12**, a coexisting major compound isolated in this study. After hydrogenation, **12** could be transformed into an intermediate **I**, which would then undergo oxidative cleavage between hydroxyls to give **1**, and **1** undergoes a series of reactions including decarboxylation, double bond epoxidation, epoxide hydrolysis, and lactonization to yield **2** and **4**−**6**.

Compounds **1–12**, **2a**, and **2b** were tested for their inhibitory activities against LPS-induced NO and TNF-α production in RAW264.7 cells. To exclude cytotoxic compounds, the cytotoxicity against the RAW264.7 cells line was first evaluated. The data showed that none of compounds negatively impacted the survival of RAW264.7 cells at 120 μM, and the inhibitory activities as shown in Table 4. All compounds were also tested against the human tumor cell lines MCF-7 and SHSY-5Y. Only four diterpenoid glycosides **8**−**11** exhibited cytotoxicity against MCF-7 cells with IC_{50} values 9.6, 9.0, 12.0, and 15.6 μM in comparison with the positive control of paclixatol (IC_{50} < 0.009 μM), while others were inactive with IC_{50} > 25 μM.

Conclusions

In summary, eleven new diterpenoids including four 16-nor-*ent*-pimaranes, two unique 15,16-dinor-*ent*-pimaranes, three diterpenoid glycosides and one rare 16-nor-*ent*-pimarane glycoside were



Scheme 1 Plausible biogenetic pathway for **1**, **2**, and **4**−**6**.

isolated from *F. fimbriata*, and the structures were elucidated by extensive spectroscopic techniques and chemical means. The absolute configurations of the new compounds were determined by means of X-ray diffraction analysis, CD exciton chirality method, and calculated ECD method. The anti-inflammation and cytotoxicity of all isolates and two derivatives were tested. Compounds **5**, **6**, **9**, and **12** exhibited potent inhibitory activity against TNF-α production in RAW264.7 cells, and **8**−**11** exhibited moderate cytotoxicity against MCF-7 cells.

Table 4 Inhibitory effects of compounds **1**−**12**, **2a**, and **2b** on LPS-induced NO/TNF-α production in RAW264.7 cells.

Compound	IC_{50} (μM)	
	NO	TNF-α
1	>25	>25
2	21.6	>25
3	>25	19.9
4	15.4	>25
5	>25	7.8
6	>25	9.5
7	>25	11.0
8	15.4	7.8
9	18.0	15.6
10	15.4	>25
11	>25	16.8
12	>25	9.5
2a	>25	13.7
2b	>25	21.9

The potent anti-inflammatory compounds isolated in this study may explain the anti-inflammatory efficacy of this plant in Traditional Chinese Medicine, and supported the action of *F. fimbriata* serving as a substituent for *D. candidum* when used for inflammation-related disease. Studies towards their molecular mechanism of anti-inflammation are in progress.

Experimental Section

General experimental procedures

Melting points were measured on an OptiMelt MPA 100 automated melting point system (Stanford Research Systems) and are uncorrected. Optical rotations were measured on a Rudolph Autopol I automatic polarimeter. IR spectra were determined on a Bruker Tensor 37 infrared spectrophotometer. NMR spectra were measured on a Bruker AM-400 spectrometer at 25 °C. EIMS and HREIMS (70 eV) were recorded on a Finnigan MAT 95 mass spectrometer. ESIMS was measured on a Finnigan LC QDECA instrument, and HRESIMS was performed on a Waters-Micromass Q-TOF. A Shimadzu LC-20 AT equipped with a SPD-M20A PDA detector was used for HPLC. A YMC-pack ODS-A column (250 × 10 mm, S-5 μm, 12 nm) was used for semipreparative HPLC separation. Silica gel (300–400 mesh, Qingdao Haiyang Chemical Co., Ltd.), C_{18} reversed-phase silica gel (12 nm, S-50 μm, YMC Co., Ltd), Sephadex LH-20 gel (Amersham Biosciences) and MCI gel (CHP20P, 75–150 μm, Mitsubishi Chemical Industries Ltd.) were used for column chromatography. All solvents used were of analytical grade (Guangzhou Chemical Reagents Company, Ltd.). All cell lines were obtained from the China Center for Type Culture Collection of the Chinese Academy of Sciences.

Plant material

Stems of *F. fimbriata* were collected in July 2010 from Yunnan Province, P. R. China, and were identified by one of the authors (Prof. D. P. Yang). A voucher specimen (accession number: LSJSH201010) has been deposited at the School of Pharmaceutical Sciences, Sun Yat-sen University.

Extraction and Isolation

The air-dried powder of the stems of *F. fimbriata* (5.0 kg) was extracted with 95% EtOH (3 × 10 L) at rt to give 354 g crude extract, which was suspended in H_2O (1.5 L) and successively partitioned with petroleum ether (PE, 3 × 1.5 L), EtOAc (3 × 1.5 L), and *n*-BuOH (3 × 1.5 L). The EtOAc extract (200 g) was subjected to silica gel column chromatography (CC) and eluted with PE / dichloroform / EtOAc / acetone / MeOH successively to afford five fractions (I−V). Fraction III (9.5 g) was chromatographed over an MCI column eluting with MeOH/H_2O (0:10→10:0) to afford five fractions (Fr. IIIa–IIIe). Fr. IIIa (1.2 g) was separated by silica gel CC (CH_2Cl_2/MeOH, 50:1→10:1) to afford five fractions (Fr. IIIaa−IIIae). Fr. IIIac was subjected to Rp-C_{18} CC using a gradient of MeOH/H_2O (v/v from 3:7 to 10:0) to yield **11** (66 mg) and another fraction, which after chromatography on a Sephadex LH-20 column using ethanol as eluent, yielded **7** (93 mg). Fraction IIIc (2.2 g) was subjected to silica gel CC (CH_2Cl_2/MeOH, 50:1→10:1) to give three fractions (Fr. IIIca–IIIcc). Fr.IIIcc was further purified on a Sephadex LH-20 eluting with methanol to yield **4** (5 mg). Fraction IIIe (1.2 g) was subjected to Sephadex LH-20 eluting with methanol to give four fractions (Fr. IIIea–IIIed). Fr. IIIeb was subjected to silica gel CC (CH_2Cl_2/acetone, 50:1→5:1) to yield **5** (8 mg). Fraction IV (13.5 g) was chromatographed over a MCI column eluting with MeOH/H_2O (0:10→10:0) to afford five fractions (Fr. IVa–IVe). Fr. IVb (1.6 g) was separated by Rp-C_{18} CC using a gradient of MeOH/H_2O (v/v from 3:7 to 10:0) to yield **3** (62 mg). Fr. IVc (3.2 g) was subjected to Sephadex LH-20 eluting with methanol to give four fractions (Fr. IVca–IVcd). Fr. IVcb was further purified by silica gel CC (CH_2Cl_2/acetone, 50:1→5:1) to give **6**. Fr. IVd (2.2 g) was subjected to Sephadex LH-20 eluting with methanol to give four fractions (Fr. IVda–IVcc). Fr. IVdb was further purified by silica gel CC (CH_2Cl_2/MeOH, 100:1→10:1) to give **1** (200 mg) and **2** (190 mg). The *n*-BuOH extract (91 g) was chromatographed over an MCI gel column eluting with a gradient of MeOH/H_2O (v/v from 0:10 to 10:0) to give five fractions (Fr. III–IIV). Fr. I was separated over Rp-C_{18} CC using a gradient of MeOH/H_2O (v/v from 1:19 to 4:6) to yield

12 (789 mg), **8** (9 mg), and **9** (38 mg). Fr. IIII was separated over an Rp-C_{18} column using a gradient of MeOH/H_2O (v/v from 1:9 to 4:6) to yield **10** (56 mg).

Compound characteristics

Norflickinflimiod A (1): colorless needle crystal; mp 201.5−202.5 °C; $[\alpha]^{20}_D$ +65.1 (*c* 0.24, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 204 (2.80) nm; CD (*c* 5.6 × 10^{-3} M, CH_3CN), λ_{max} ($\Delta\varepsilon$) 220 (− 0.6), 301 (+ 0.01); IR (KBr) ν_{max} 3446, 3373, 2925, 2859, 1708, 1549, 1462, 1389, 1259, 1129, 1064, 951, and 757 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; positive ESIMS *m/z* 354.5 $[M + Na]^+$; negative ESIMS *m/z* 341.2 $[M - H]^-$; HRESIMS *m/z* 345.2039 $[M + Na]^+$ (calcd for $C_{19}H_{30}O_4Na$, 345.2036).

Norflickinflimiod B (2): colorless oil; $[\alpha]^{20}_D$ −2.7 (*c* 0.3, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 204 (2.79) nm; CD (*c* 5.3 × 10^{-3} M, CH_3CN), λ_{max} ($\Delta\varepsilon$) 220 (− 0.12); IR (KBr) ν_{max} 3364, 2924, 2859, 1753, 1457, 1376, 1126, 1060, 767, and 750 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; positive ESIMS *m/z* 361.1 $[M + Na]^+$, 699.2 $[2M + Na]^+$; negative ESIMS *m/z* 337.2 $[M - H]^-$, 383.3 $[M + HCOO]^-$; HRESIMS *m/z* 361.1987 $[M + Na]^+$ (calcd for $C_{19}H_{30}O_5Na$, 361.1985).

Norflickinflimiod C (3): colorless oil; $[\alpha]^{20}_D$ −13.3 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 204 (2.64) nm; CD (*c* 9.3 × 10^{-4} M, CH_3CN), λ_{max} ($\Delta\varepsilon$) 221 (− 0.31); IR (KBr) ν_{max} 3445, 2956, 2924, 2856, 1747, 1460, 1374, 1119, 1064, 949, 793, and 750 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; positive ESIMS *m/z* 345.3 $[M + Na]^+$, 667.6 $[2M + Na]^+$; negative ESIMS *m/z* 367.3 $[M + HCOO]^-$, 679.5 $[2M + HCOO]^-$; HRESIMS *m/z* 345.2047 $[M + Na]^+$ (calcd for $C_{19}H_{30}O_4Na$, 345.2036).

Norflickinflimiod D (4): colorless oil; $[\alpha]^{20}_D$ −6.5 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 204 (2.50) nm; CD (*c* 8.2 × 10^{-4} M, CH_3CN), λ_{max} ($\Delta\varepsilon$) 219 (− 0.57); IR (KBr) ν_{max} 2956, 2922, 2856, 1814, 1723, 1665, 1594, 1462, 1378, 1142, 1021, 797, and 751 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; positive ESIMS *m/z* 391.1 $[M + Na]^+$; negative ESIMS *m/z* 367.0 $[M - H]^-$; HREIMS *m/z* 391.1739 $[M + Na]^+$ (calcd for $C_{19}H_{20}O_7Na$, 391.1727).

Norflickinflimiod E (5): colorless oil; $[\alpha]^{20}_D$ +19.4 (*c* 0.36, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 242 (2.76) nm, 205 (2.21)nm; CD (*c* 1.7 × 10^{-3} M, MeOH), λ_{max} ($\Delta\varepsilon$) 207 (− 0.67), 242, (+ 0.11), 299 (+ 0.09); IR (KBr) ν_{max} 3441, 2926, 2862, 1705, 1549, 1462, 1387, 1127, 1060, and 767 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; positive ESIMS *m/z* 291.3 $[M+H]^+$; negative ESIMS *m/z* 325.1 $[M + Cl]^-$; HRESIMS *m/z* 291.1950 $[M + H]^+$ (calcd for $C_{18}H_{26}O_3$, 291.1955).

Norflickinflimiod F (6): colorless oil; $[\alpha]^{20}_D$ + 37.8 (*c* 0.16, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 290 (2.29) nm, 203 (2.50)nm; CD (*c* 1.7 × 10^{-3} M, MeOH), λ_{max} ($\Delta\varepsilon$) 289 (+ 0.22); IR (KBr) ν_{max} 3449, 2922, 2857, 1733, 1480, 1379, 1246, 1128, 1060, 798, and 750 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; positive ESIMS *m/z* 291.3 $[M + H]^+$; negative ESIMS *m/z* 325.2 $[M + Cl]^-$; HRESIMS *m/z* 289.1810 $[M - H]^-$ (calcd for $C_{18}H_{26}O_3$, 289.1809).

2-Acetyl-flickinflimiod (7): colorless oil; $[\alpha]^{20}_D$ −12.7 (*c* 0.07, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 264 (1.83) nm, 203 (2.49) nm; CD (*c* 2.6 × 10^{-3} M, CH_3CN), λ_{max} ($\Delta\varepsilon$) 208 (− 0.74), 296, (+ 0.04); IR (KBr) ν_{max} 2958, 2924, 2853, 1462, 1376, 794, and 744 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; negative ESIMS *m/z* 413.3 $[M + Cl]^-$; HRESIMS *m/z* 401.2292 $[M + Na]^+$ (calcd for $C_{22}H_{34}O_5Na$, 401.2298).

Flickinflimoside A (8): colorless oil; $[\alpha]^{20}_D$ −58.0 (*c* 0.22, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 203 (2.52) nm; CD (*c* 1.9 × 10^{-3} M, CH_3CN), λ_{max} ($\Delta\varepsilon$) 208 (− 1.72), 303, (+ 0.08); IR (KBr) ν_{max} 3445, 2931, 2867, 1714, 1646, 1461, 1379, 1257, 1126, 1085, and 950 cm^{-1}; ^1H and ^{13}C NMR data, see Table 3; positive ESIMS *m/z* 563.5 $[M + Na]^+$, 1103.9 $[2M + Na]^+$; negative ESIMS *m/z* 585.4 $[M + HCOO]^-$, 1125.9 $[2M + HCOO]^-$; HRESIMS *m/z* 585.2926 $[M + HCOO]^-$ (calcd for $C_{28}H_{44}O_{10}$, 585.2917).

Flickinflimoside B (9): colorless oil; $[\alpha]^{20}_D$ −3.7 (*c* 0.17, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 203 (2.48) nm; CD (*c* 2.0 × 10^{-3} M, CH_3CN), λ_{max} ($\Delta\varepsilon$) 208 (−2.3), 300, (+ 0.13); IR (KBr) ν_{max} 3366, 2926, 2850, 1712, 1460, 1370, 1128, 1060, and 790 cm^{-1}; ^1H and ^{13}C NMR data, see Table 3; negative ESIMS *m/z* 533.3 $[M + Cl]^-$; HRESIMS *m/z* 521.2726 $[M + Na]^+$ (calcd for $C_{26}H_{42}O_9Na$, 521.2721).

Flickinflimoside C (10): colorless oil; $[\alpha]^{20}_D$ −40.2 (*c* 0.07, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 204 (2.52) nm; CD (*c* 2.1 × 10^{-3} M, CH_3CN), λ_{max} ($\Delta\varepsilon$) 210 (− 3.01), 298, (+ 0.18); IR (KBr) ν_{max} 3371, 2925, 2855, 1712, 1460, 1373, 1121, 1068, and 793 cm^{-1}; ^1H and ^{13}C NMR data, see Table 3; positive ESIMS *m/z* 505.6 $[M + Na]^+$, 987.7 $[2M + Na]^+$; HRESIMS *m/z* 517.2586 $[M + Cl]^-$ (calcd for $C_{26}H_{42}O_8Cl$, 517.2574).

Norflickinflimoside (11): colorless oil; $[\alpha]^{20}_D$ −28.2 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 204 (2.51) nm; CD (*c* 6.2 × 10^{-4} M, CH_3CN), λ_{max} ($\Delta\varepsilon$) 214 (−2.23); IR (KBr) ν_{max} 3442, 2956, 2855, 1460, 1376, 1129, 1065, 795, and 750 cm^{-1}; ^1H and ^{13}C NMR data, see Table 3; positive ESIMS *m/z* 507.3 $[M + Na]^+$; negative ESIMS *m/z* 519.2 $[M + Cl]^-$; HRESIMS *m/z* 519.2344 $[M + Cl]^-$ (calcd for $C_{25}H_{40}O_9Cl$, 519.2366).

X-ray crystallographic data for 1 and 2a

The crystallographic data for norflickinflimiod A (**1**) and **2a** have been deposited at the Cambridge Crystallographic Data Centre (deposition no. CCDC-951382 and CCDC-951383). Copies of these data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.htm.

Chemical transformations

Chemical transformation of **2** to **2a**.[21] 2,2-Dimethoxypropane (5 mL) was added to a stirred solution of **2** (34 mg, 0.1 mmol) in CH_2Cl_2 (1 mL). The mixture was stirred at rt for 3 h and then was worked up with pH 7.00 buffer, CH_2Cl_2 and NaCl respectively. After evaporating the solvent, the resulting crude product was subjected to silica gel CC ($CHCl_3$/MeOH 40 : 1) to afford **2a** (19 mg), which was identified by NMR and MS. ^1H NMR ($CDCl_3$, 400 MHz) δ 4.14, (1H, ddd, *J* = 4.3, 5.7, 10.6 Hz, H-2), 3.71, (1H, d, *J* = 4.3 Hz, H-3), 3.48, (1H, s, H-14), 1.84, (2H, m, H_2-12), 1.83, (2H, m, H_2-7), 1.56−1.68, (5H, m, H_2-6, H_2-11, H-9), 1.47, [3H, s, $(CH_3)_2CO_2$-2(3)], 1.32, [3H, s, $(CH_3)_2CO_2$-2(3)], 1.29, (1H, m, H-5), 1.13, (3H, s, H_3-17), 1.09, (3H, s, H_3-18), 0.96, (3H, s, H_3-19), 0.94, (3H, s, H_3-20); ^{13}C NMR ($CDCl_3$, 100MHz) δ 178.2 (C-15), 107.2 [$(CH_3)_2CO_2$-2(3)], 84.7 (C-8), 82.6 (C-3), 79.0 (C-14), 71.4 (C-2), 48.3 (C-5), 45.7 (C-13), 44.2 (C-9), 41.2 (C-1), 37.2 (C-10), 35.4 (C-4), 33.9 (C-7), 28.9 [$(CH_3)_2CO_2$-2(3)], 28.5 (C-19), 26.5 [$(CH_3)_2CO_2$-2(3)], 25.5 (C-12), 24.4 (C-18), 18.4 (C-11), 18.3 (C-17), 18.1 (C-6), 14.9 (C-20); mp 175.5−176.5 °C; negative ESIMS *m/z* 427.3 $[M + HCOO]^-$, 799.6 $[2M + HCOO]^-$; HRESIMS *m/z* 427.2326 $[M + HCOO]^-$ (calcd for $C_{22}H_{35}O_8$, 427.2337).

Chemical transformation of **2** to **2b**.[22] An aqueous solution of $NaIO_4$ (0.1 M, 1.5 mL) was added dropwise to a vigorously stirred suspension of chromatographic grade silica gel (1.8 g, 0.03 mol) in CH_2Cl_2 (1 mL), resulting in the formation of a flaky suspension. A solution of **2** (34.2 mg, 0.1 mmol) in CH_2Cl_2 (1 mL) and MeOH (1 mL) was then added, and the reaction was stirred for 24 h. The mixture was filtered through sintered glass, and the silica gel was thoroughly washed with CH_2Cl_2. Evaporation of the solvent afforded compound **2b** as a colorless oil, which was identified by NMR and MS. ^1H NMR ($CDCl_3$, 400 MHz) δ 4.57, (1H, dd, *J* = 4.9, 10.2 Hz, H-2), 4.51, (1H, s, H-3), 3.50, (1H, s, H-14), 3.49, (3H, s, OCH_3-2), 3.41, (3H, s, OCH_3-3), 2.00, (1H, dd, *J* = 5.2, 11.6 Hz, H-7), 1.73−1.80, (6H, m, H_2-1, H_2-11, H_2-12), 1.56, (2H, m, H_2-6), 1.52, (1H, dd, *J* = 4.8, 12.0 Hz, H-9), 1.36, (1H, m, H-7), 1.13, (3H, s, H_3-20), 1.12, (3H, s, H_3-17), 0.96, (3H, s, H_3-18), 0.95, (3H, s, H_3-19), 0.94, (1H, m, H-5); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 177.9 (C-15), 102.5 (C-3), 99.4 (C-2), 85.1 (C-8), 79.1 (C-14), 56.9 (C-5), 56.4 (OCH_3-3), 55.9 (OCH_3-2), 45.9 (C-13), 44.1 (C-4), 43.2 (C-7), 42.3 (C-9), 39.7 (C-10), 34.7 (C-1), 27.2 (C-19), 25.7 (C-12), 20.0 (C-6), 19.2 (C-11), 17.9 (C-17), 14.9 (C-18), 14.4 (C-20); positive ESIMS *m/z* 401.4 $[M + Na]^+$, 779.7 $[2M + Na]^+$; negative ESIMS *m/z* 413.2 $[M + Cl]^-$, 791.4 $[2M + Cl]^-$; HRESIMS *m/z* 401.2312 $[M + Na]^+$ (calcd for $C_{22}H_{34}O_5Na$, 401.2298).

Chemical transformation of **2b** to **4**.[15] $NaClO_2$ (19.8 mg, 0.22 mmol), NaH_2PO_4 (14.9 mg, 0.11 mmol), and H_2O (0.5 mL) were added to a solution of compound **2b** (9 mg, 0.0211 mmol) in THF (0.1 mL), followed by *t*-BuOH (1 mL) and 2-methyl-2-butene (2 M solution in THF, 1 mL). The resulting mixture was stirred at rt for 1 h. EtOAc was then added, and the mixture was transferred to a separatory funnel and

evaporated. The crude residue was purified by PTLC ($CHCl_3$:MeOH, 20:1), yielding **4** as a colorless oil (3.5 mg).

Preparation of the 2,3-di-*p*-methoxybenzoate (**1a**) of norflickinflimiod A (**1**).[23] Compound **1** (15 mg) was treated with *p*-methoxybenzoic acid (50 mg), DCC (50 mg), and DMAP (10 mg) in $CHCl_3$ (4 mL) at rt for 48 h. The reaction mixture was purified by silica gel CC (eluting with $CHCl_3$ and MeOH) and then Rp-C_{18}-HPLC using a gradient of MeOH/H_2O (80:20→100:0, 20 min, 4.0 mL/min), which yielded 2 mg 2,3-di-*p*-methoxybenzoate (white amorphous powder). CD (CH_3CN, $\Delta\varepsilon$) 201 (−4.92), 234 (−0.3), 264 (−2.15) nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.49, (1H, s, H-14), 3.67, (1H, m, H-2), 3.49, (1H, s, H-3), 2.29−2.42, (4H, m, H-6, H-7), 1.96−2.14, (4H, m, H-1, H-12), 1.83, (1H, m, H-9), 1.44, (1H, m, H-5), 1.37, (3H, s, H-17), 1.06, (3H, s, H-18), 1.00, (3H, s, H-19), 0.92, (3H, s, H-20), [7.96−7.99, (4H, m), 6.91−6.96, (4H, m), 3.88, (3H, s, CH_3O-*p*), 3.86, (3H, s, CH_3O-*p*), *p*-methoxybenzoyl]; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 181.2 (C-15), 140.8 (C-8), 124.4 (C-14), 76.9 (C-3), 71.4 (C-2), 50.5 (C-9), 47.4 (C-3), 44.4 (C-13), 39.4 (C-10), 38.7 (C-4), 36.4 (C-7), 35.5 (C-12), 32.8 (C-1), 28.6 (C-18), 27.0 (C-17), 22.2 (C-19), 21.7 (C-11), 20.0 (C-6), 15.2 (C-20), (163.5, 132.7, 131.6, 114.1, 113.7, 55.5, 55.6) (*p*-methoxybenzoyl); negative ESIMS *m*/*z* 589.3 $[M - H]^-$.

Determination of sugar configuration. Compounds **8**−**11** (2 mg), respectively, was refluxed with 2 mL of 2 M HCl (dioxane/H_2O, 1:1) at 100 °C for 4 h. After removing the dioxane under vacuum, the solution was then diluted with H_2O and then extracted with EtOAc (3 × 1 mL). The aqueous layer was evaporated under vacuum, diluted repeatedly with H_2O, evaporated under vacuum to obtain neutral residue, and then analyzed by TLC over silica gel (Me_2CO/*n*-BuOH/H_2O, 6:3:1) together with authentic sugar sample (glucose, R_f = 0.49). The remaining residue was dissolved in pyridine (200 μL), to which 2 mg of L-cysteine methyl ester hydrochloride was added. The mixture was stirred at 60 °C for 1 h; then 50 μL of *o*-tolyl isothiocyanate was added, and the mixture was stirred at 60 °C for another 1 h. The reaction mixture was directly analyzed by standard C_{18} HPLC [a YMC-pack ODS-A column (250 × 10 mm, S-5 μm, 12 nm), CH_3CN/H_2O, 25:75, 3 mL/min]. The peak (t_R = 19.0 min) coincided with a derivative of D-glucose, as compared with authentic D-glucose with t_R at 19.1 min.

Biological assays

The following human tumor cell lines were used: MCF-7, and SHSY-5Y, which were obtained from ATCC (Manassas, VA, USA). All cells were cultured in DMEM medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS, Hyclone) at 37 °C in a humidified atmosphere with 5% CO^2. Cell viability was assessed by conducting colorimetric measurements of the amount of insoluble formazan formed in living cells based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA). Briefly, 100 μL of adherent cells was seeded into each well of a 96-well cell culture plate and allowed to adhere for 12 h before test compound addition, while suspended cells were seeded just before test compound addition, both with an initial density of 1×10^5 cells / mL in 100 μL of medium. Each tumor cell line was exposed to the test compound at various concentrations in triplicate for 48 h, with paclitaxel (Sigma) as positive control. After the incubation, MTT (100 μg) was added to each well, and the incubation continued for 4 h at 37 °C. The cells were lysed with 100 μL of 20% SDS−50% DMF after removal of 100 μL of medium. The optical density of the lysate was measured at 595 nm in a 96-well microtiter plate reader (Bio-Rad 680). The IC_{50} value of each compound was calculated by Reed and Muench's method.[24]

Nitric Oxide Inhibitory Assay.[25] Inhibition of NO production was determined in a LPS-stimulated RAW 264.7 macrophage cell line. Murine monocytic RAW 264.7 macrophages were seeded in 96-well plastic plates (1×10^5 cells/well) containing DMEM medium (Hyclone, UT, USA) with 10% FBS and allowed to adhere for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was then replaced with fresh medium containing LPS (2 μg/mL) and test compounds at 10 μM, and the cells were incubated for 24 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant with Griess reagent (0.5% sulfanilamide and 0.05% naphthylene-diamide dihydrochloride in 2.5% H_3PO_4) and then allowed to stand for 5 min at rt. The absorbance at 540 nm was measured using a HTS 7000 microplate reader. The nitrite concentration in the medium was determined from the calibration curve (r = 0.9998) obtained by using different concentrations of sodium nitrite ($NaNO_2$) in the culture medium as the standard. Blank correction was performed by subtracting the absorbance due to medium only from the absorbance reading of each well.

Assay for the production of pro-inflammatory cytokines (TNF-α).[26] Compounds were dissolved in DMSO, and suspensions of RAW264.7 cells were cultured in complete RPMI 1640 medium (Hyclone Laboratories) containing 10% FBS. The cultured cells were incubated with the tested compounds (10 μM) for 24 h, followed by LPS stimulation (2 μg/mL). Supernatants were collected to analyze cytokine levels. The mouse TNF-α ELISA Kit was used to determine the cytokine concentration in the culture supernatants.

Cytotoxic Assay. Cell viability in the presence or absence of the tested compounds was determined using the standard MTT assay.[27]

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Electronic Supplementary Information (ESI) available: [NMR spectra of **1**−**12**, **2a**, and **2b**; the enlarge X-ray structure of **1** and **2a**; CD spectra of **1**−**12** are available free of charge]. See DOI: 10.1039/b000000x/

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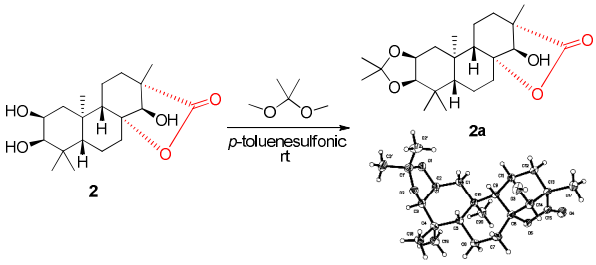
Table of Content:



11 new diterpenoids determined by NMR, X-ray diffraction, calculated ECD and CD exciton chirality methods were isolated from *Flickingeria fimbriata*.