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Phenanthrenes, 9,10-dihydrophenanthrenes, bibenzyls with their derivatives, and malate or tartrate benzyl ester glucosides from tubers of *Cremastra appendiculata*

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Malate benzyl ester glucoside
Tartrate benzyl ester glucoside

A B S T R A C T

Eleven previously unknown compounds and 23 known compounds, including 20 phenanthrene or 9,10-dihydrophenanthrene derivatives, five bibenzyls, seven malate or tartrate benzyl ester glucosides, adenosine and gastrodin were isolated from tubers of *Cremastra appendiculata*. Among the obtained compounds, two are the first isolated dimers with one phenanthrene or bibenzyl unit connected to C-3 of 2,3,4,5-tetrahydro-phenanthro[2,1-b]furan moiety. In addition, 33 of these compounds were evaluated in vitro for their cytotoxic activity against two cancer cell lines. Among the compounds examined, one compound showed moderate cytotoxic activity, while five showed weak cytotoxic activity against the A549 cell line.

1. Introduction

The tubers of orchidaceous plants, *Cremastra appendiculata* (D. Don) Makino, together with *Pleione bulbocodioides* (Franch.) Rolfe and *Pleione yunnanensis* Rolfe, namely "Shan-Ci-Gu" in traditional Chinese medicine, are used for treatment of various cancers and bacterial infections (Xie et al., 1996). In this regard, Orchidaceae plants can biosynthesis various aromatic compounds, such as bibenzyls, phenanthrenes, 9,10-dihydrophenanthrenes, 9,10-dihydrophenanthrofurans, and tartrate benzyl ester glucosides (Bai et al., 1998; Majumder et al., 1999; Xue et al., 2006; Yang et al., 2007; Zi et al., 2008). Some of these compounds were reported to possess various pharmacological activities (Hahn et al., 2012; Kim et al., 2007; Matsuda et al., 2004; Shim et al., 2004; Toh, 1994; Xue et al., 2006; Yang et al., 2007; Yu et al., 2009). Because *C. appendiculata* availability is far less than market demand resulting in a very high price of the tubers, however, cheaper adulterants such as the tubers of *Tulipa edulis* (Miq.) Baker are sold in the market as the authentic medicines. Mixed use of the title herbal medicine and its adulterants will negatively influence the therapeutic effect though, and could cause even more serious problems, such as hepatic injury and inherent toxicity. Therefore, some researchers have investigated rapid propagation of the tubers of *C. appendiculata* (Mao et al., 2007; Zhang et al., 2010). Although the technique has not been commercialized so far, it is still a promising improved cultivation method. However, the main and active components of the herbal medicines may also change with alteration of the environment (Qi et al., 2006). Thus, it is important to define the chemotaxonomic markers of the tubers of *C. appendiculata* in order to help control its quality.

Previous chemical investigation of the title plant mainly focused on the low-polarity compounds (Dong et al., 2007; Ikeda et al., 2005; Li et al., 2008; Liu et al., 2008; Shim et al., 2004; Xia et al., 2005; Xue et al., 2005, 2006; Zhang et al., 2011), while the constituents of the high-polarity fraction were not investigated to the same extent. In this paper, the isolation and structural elucidation of 11 previously unreported compounds (Figs. 1 and 2), including two 2-isobutylmalate benzyl ester glucosides (**2**, **3**), two 2-benzylmalate benzyl ester glucosides (**5**, **6**), three glycosidic

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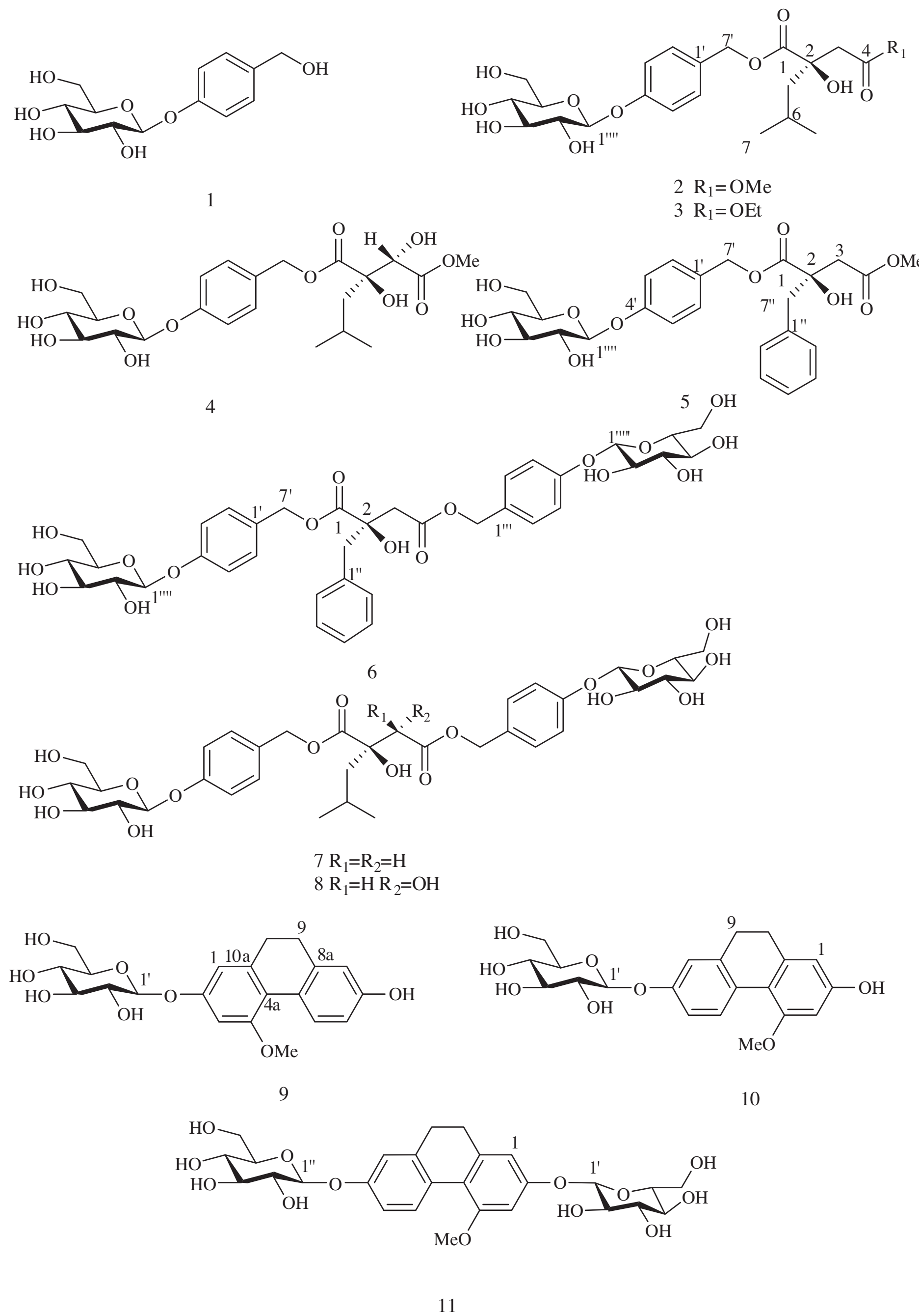

Fig. 1. Structures of compounds **1–11**.

9,10-dihydrophenanthrenes (**9–11**), one biphenanthrene (**27**), and three 2,3,4,5-tetrahydro-phenanthro[2,1-b]furan derivatives (**29**, **32**, **33**) from the ethyl acetate-soluble and water-soluble fractions of *C. appendiculata* are described. The in vitro cytotoxicities of compounds **1–33** are also evaluated against two human cancer cell lines.

2. Results and discussion

Compound **2** was obtained as a colorless gum. Its IR spectrum showed the presence of hydroxyl (3427 cm^{-1}), carbonyl (1736 cm^{-1}), and aromatic ring (1514 cm^{-1}) functional groups, and its molecular formula was determined from HRESIMS as $C_{22}H_{32}O_{11}$. Compound **2** was derived from the analogous compound gastrodin (**1**) (Baek et al., 1999) because part of the ^{1}H and ^{13}C NMR spectra of **2** were similar to those reported for **1.** Moreover, compared with the carbon signals of **1**, the C-7′ in **2** was shifted upfield to δ_C 67.9, which implied an esterification product of gastrodin (**1**). This deduction was supported by the corresponding HSQC and HMBC correlations. It was elucidated that, in comparison to **1**, **2** was esterified by 4-methyl-2-isobutylmalate moiety at C-7′.

In order to determine the absolute configuration of **2**, it was subjected to alkaline hydrolysis with this affording (2*R*)-2-hydroxy-2-(2-methylpropyl) butanedioic acid (Kizu et al., 1999; Yin et al., 2010). Accordingly, structure **2** was elucidated as 1-(4-β-D-glucopyranosyloxybenzyl) 4-methyl (2*R*)-2-isobutylmalate.

Compound **3** was obtained as a colorless gum, with a molecular formula of $C_{23}H_{34}O_{11}$ determined by HRESIMS. The ^{1}H and ^{13}C

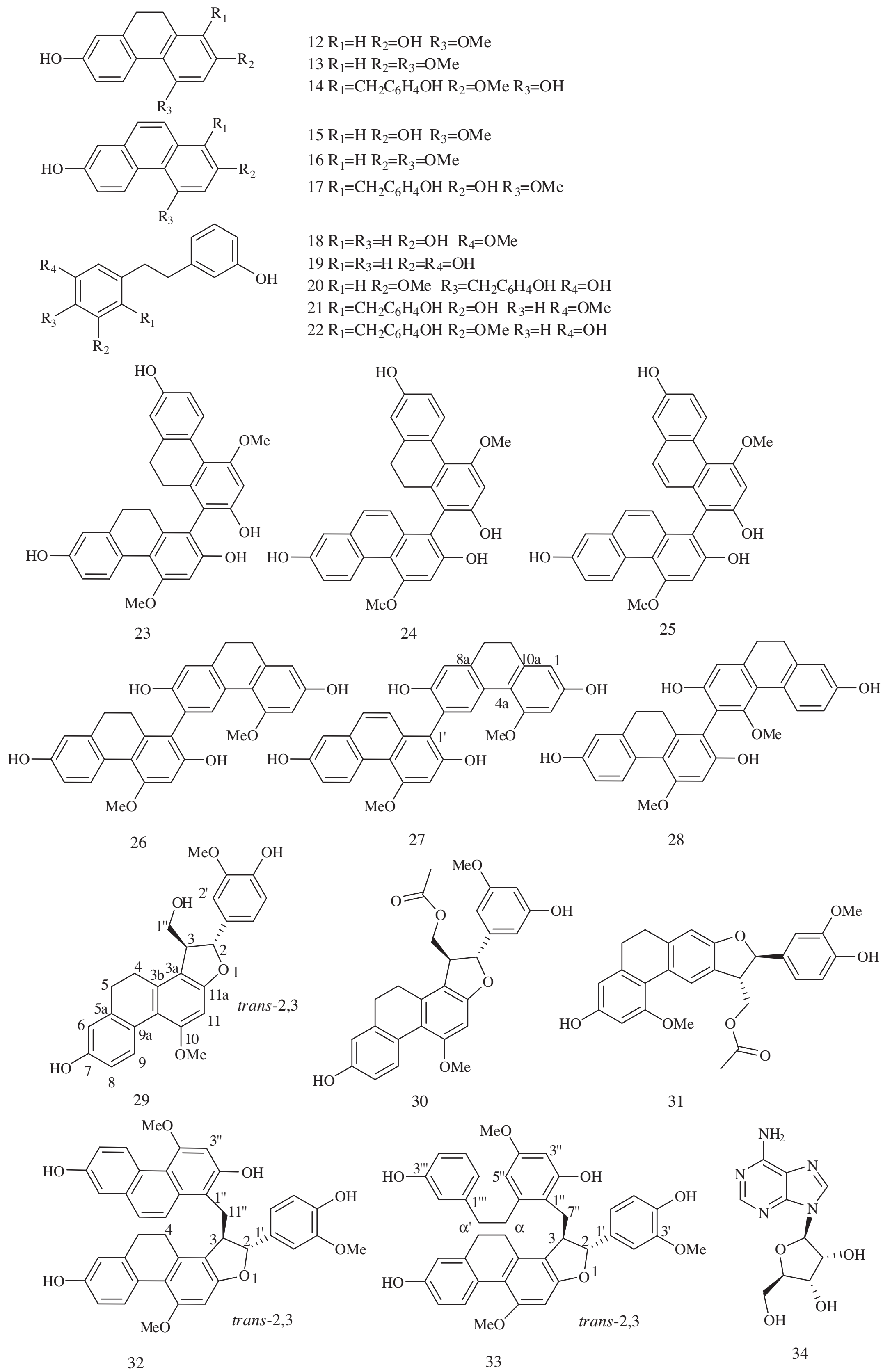

Fig. 2. Structures of compounds **12**–**34**.

NMR spectroscopic data (Table 1) of **3** were very similar to those of **2**. The only difference was the absence of the *O*-methyl moiety, this being replaced by an *O*-ethyl moiety in **3**, showing corresponding signals at δ_H 1.19 (3H, t, J = 7.2 Hz) and δ_H 4.05 (2H, q, J = 7.2 Hz)

Table 1
^1H and ^{13}C NMR spectroscopic data of compounds **2**, **3**, **5**, **6**.[a]

Pos.	**2**[b]		**3**[c]		**5**[d]		**6**	
	δc	δ_H (*J* in Hz)	δc	δ_H (*J* in Hz)	δc	δ_H (*J* in Hz)	δc	δ_H (*J* in Hz)
1	176.0		175.8		175.2		175.2	
2	76.6		76.3		77.1		77.1	
3	45.8	2.62 (d, 15.7) 2.91 (d, 15.7)	45.8	2.60 (d, 15.6) 2.90 (d, 15.6)	44.1	2.62 (d, 15.5) 2.97 (d, 15.5)	44.5	2.64 (d, 16.0) 3.01 (d, 16.0)
4	172.3		171.6		172.3		171.6	
5	49.3	1.57 (dd, 5.6, 14.0) 1.68 (m[f])	48.9	1.57 (d, 5.6, 14.0) 1.67 (m[f])				
6	25.0	1.72 (m[f])	24.8	1.71 (m[f])				
7	24.7	0.91 (3H, d, 6.5)	24.4	0.92 (3H, d, 6.5)				
8	23.9	0.80 (3H, d, 6.5)	23.7	0.80 (3H, d, 6.5)				
1′	130.8		130.6		130.8		130.7	
2′,6′	131.3	7.34 (2H, d, 8.6)	131.0	7.34 (2H, d, 8.7)	131.5 (or 131.4)	7.28 (2H, d, 8.5)	131.5	7.19 (2H, d, 9.0)
3′,5′	117.7	7.10 (2H, d, 8.6)	117.4	7.10 (2H, d, 8.7)	117.7	7.09 (2H, d, 8.5)	117.7	7.07 (2H, d, 9.0)
4′	159.2		159.0		159.3		159.2	
7′	67.9	5.15 (d, 11.6) 5.10 (d, 11.6)	67.6	5.15 (d, 12.0) 5.10 (d, 12.0)	68.0	5.07 (2H, s)	68.0	4.94 (2H, s)
1″					136.6		136.6	
2″,6″					131.5 (or 131.4)	7.10 (2H, dd, 4.0, 7.5)	131.3	7.11 (2H, dd, 6.5, 3.0)
3″,5″					129.1	7.21 (2H, m[f])	129.1	7.22 (2H, m[f])
4″					127.9	7.20 (m[f])	127.9	7.20 (m[f])
7″					46.4	3.01 (d, 13.5)a 2.93 (d, 13.5)b	46.4	3.00 (d, 14.0) 2.92 (d, 14.0)
1‴							130.7	
2‴,6‴							131.0	7.25 (2H, d, 9.0)
3‴,5‴							117.7	7.06 (2H, d, 9.0)
4‴							159.1	
7‴							67.2	4.99 (2H, s)
1⁗ (1⁗′)	102.2	4.92 (d, 7.2)	102.0	4.90 (d, 7.2[e])	102.2	4.93 (d, 7.5)	102.3	4.91 (d, 7.5)
2⁗ (2⁗′)	74.8	3.47 (m[f])	74.6	3.45 (m[f])	74.9	3.46 (m[f])	74.9	3.47 (m[f])
3⁗ (3⁗′)	77.9	3.44 (m[f])	77.7.	3.45 (m[f])	78.0	3.46 (m[f])	77.9	3.47 (m[f])
4⁗ (4⁗′)	71.3	3.41 (m[f])	71.0	3.40 (m[f])	71.3	3.40 (m[f])	71.3	3.41 (m[f])
5⁗ (5⁗′)	78.1	3.44 (m[f])	77.9	3.41 (m[f])	78.2	3.41 (m[f])	78.1	3.45 (m[f])
6⁗ (6⁗′)	62.4	3.89 (dd, 1.8, 12.0) 3.70 (dd, 5.2, 12.0)	62.2	3.89 (dd, 2.0, 12.0) 3.70 (dd, 5.2, 12.0)	62.5	3.90 (dd, 2.0, 12.0) 3.71 (dd, 5.0, 12.0)	62.5	3.89 (m[f]) 3.70 (m[f])

[a] ^1H NMR data were measured in CD_3OD at 400 MHz for **2** and **3**, at 500 MHz for **5** and **6**. ^{13}C NMR data were measured at 125 MHz in CD_3OD.
[b] Data for methoxy: δc 52.2, δ_H 3.57 (3H, s).
[c] Data for ethoxy: δc 61.5, δc 14.2; δ_H 4.05 (2H, q, J = 7.2 Hz), δ_H 1.19 (3H, t, J = 7.2 Hz).
[d] Data for methoxy: δc 52.2, δ_H 3.57 (3H, s).
[e] J were measured in DMSO-d_6.
[f] Overlapping signals.

in the ^1H NMR spectrum. Other resonances in the ^{13}C NMR (Table 1), HSQC and HMBC spectra also supported the structure proposed. The configuration at C-2 in compound **3** was determined following the same procedure as that for **2**. Thus, compound **3** was determined as 1-(4-β-D-glucopyranosyloxybenzyl) 4-ethyl (2*R*)-2-isobutylmalate.

The molecular formula of **5** was determined by HRESIMS as $C_{25}H_{30}O_{11}$. Compared with **2**, signals for the extra benzyl at δc 136.6 (C-1″), 131.5 (or 131.4) (C-2″, 6″), 129.1 (C-3″, 5″), 127.9 (C-4″) and 46.4 (C-7″) in the ^{13}C NMR spectrum and corresponding proton resonances indicated the presence of a benzyl group in **5**, replacing the isobutyl. In the HMBC spectrum, the correlations from H-7″ (δ_H 3.01 and δ_H 2.93) to C-1 (δc 175.2), C-2 (δc 77.1), C-3 (δc 44.1), C-2″ [δc 131.5 (or 131.4)], and C-6″ [δc 131.5 (or 131.4)] further confirmed that the benzyl moiety was positioned at C-2 in **5**. All chemical shifts were assigned by the combination of analyses of the HSQC and HMBC spectra (Table 1). The absolute configuration of **5** was also determined using the same method as that for **2**, with the hydrolytic product identified as (2*R*)-2-benzyl-2-hydroxysuccinic acid (El Bialy et al., 2005). Thus, compound **5** was determined as 1-(4-β-D-glucopyranosyloxybenzyl) 4-methyl (2*R*)-2-benzylmalate.

Compound **6** was obtained as a colorless amorphous powder. Its molecular formula $C_{37}H_{44}O_{17}$ was established by HRESIMS. Signals in the ^1H and ^{13}C NMR spectra (Table 1) of **6** resembled those of **5**, except for resonances for an additional 4-β-D-glucopyranosyloxybenzyl moiety. Correlations from HMBC and HSQC were consistent with the structure proposed and the configuration of **6** was determined using the same method as that for **5**. Hence compound **6** was elucidated as 1,4-bis(4-β-D-glucopyranosyloxybenzyl) (2*R*)-2-benzylmalate.

Compound **9** was obtained as a colorless amorphous powder. The HRESIMS gave a *m*/*z* of 427.135, corresponding to $C_{21}H_{24}O_8$. The UV spectrum showed maximum absorptions at 276 and 290 nm, this being characteristic of a 9,10-dihydrophenanthrene. In the ^1H NMR spectrum (Table 2), the deshielded aromatic proton signal at δ_H 8.03 (1H, d, J = 8.0 Hz, H-5) also indicated that **9** possessed a 9,10-dihydrophenanthrene structure (Majumder and Lahiri, 1990). Seven proton resonances from δ_H 3.37 to δ_H 4.91, together with the coupling constant of H-1′ (7.5 Hz), suggested that **9** was a 9,10-dihydrophenanthrene derivative with one β-D-glucose substituent. The correlation from H-1′ (δ_H 4.91) to C-2 (δc 157.9) in the HMBC spectrum also established unequivocally that the glucose moiety was connected to the 9,10-dihydrophenanthrene moiety

Table 2
^{1}H and ^{13}C NMR spectroscopic data of compounds **9–11**.[a]

Pos.	**9**		**10**		**11**	
	δc	δ_H (*J* in Hz)	δc	δ_H (*J* in Hz)	δc	δ_H (*J* in Hz)
1	109.5	6.62 (d, 2.5)	108.3	6.32 (d, 2.0)	108.5	6.59 (d, 2.0)
2	157.9		158.0		157.2	
3	101.0	6.74 (d, 2.5)	99.2	6.41 (d, 2.0)	100.0	6.68 (d, 2.0)
4	158.8		159.4		157.7	
4a	119.4		116.3		116.8	
4b	125.7		128.9		126.6	
5	130.4	8.03 (d, 8.0)	129.8	8.09 (d, 6.8)	129.0	8.05 (d, 8.4)
6	113.6	6.61 (dd, 3.0, 8.0)	115.0	6.92 (dd, 2.0, 6.8)	114.1	6.88 (dd, 2.8, 8.4)
7	156.5		156.7		156.0	
8	115.1	6.63 (d, 3.0)	116.6	6.94 (d, 2.0)	115.8	6.90 (d, 2.8)
8a	140.8		140.4		139.4	
9	31.1	2.65 (2H, m)	31.2	2.67 (2H, m)	29.8	2.65 (2H, s)
10	31.8	2.67 (2H, m)	31.7	2.66 (2H, m)	30.5	2.65 (2H, s)
10a	141.6		142.2		140.5	
1′	102.6	4.91 (d, 7.5)	102.4	4.91 (d, 7.5)	101.0	4.88 (d, 7.6)
2′(2″)	75.0	3.46 (m[b])	75.0	3.46 (m[b])	73.7	3.24 (m[b])
3′(3″)	78.1	3.47 (m[b])	78.0	3.46 (m[b])	77.1 or 77.2	3.28 (m[b])
4′(4″)	71.6	3.37 (m[b])	71.5	3.40 (m[b])	70.4 or 70.2	3.15 (m[b])
5′(5″)	78.3	3.47 (m[b])	78.2	3.46 (m[b])	77.5 or 77.7	3.33 (m[b])
6′(6″)	62.7	3.70 (dd, 6.0, 12.0)	62.6	3.71 (dd, 5.6, 12.4)	61.2 or 61.3	3.71 (m)
		3.92 (dd, 2.5, 12.0)		3.91 (dd, 2.0, 12.4)		3.47 (m)
1″					101.0	4.85 (d, 7.2)
4-OMe	56.0	3.85 (3H, s)	55.9	3.83 (3H, s)	55.9	3.82 (3H, s)

[a] ^{1}H NMR data were measured at 500 MHz in CD_3OD for **9** and **10**, at 400 MHz in DMSO-d_6 for **11**. ^{13}C NMR data were measured at 125 MHz in CD_3OD.
[b] Overlapping signals.

at C-2. Therefore, structure **9** was determined as 7-hydroxy-4-methoxy-9,10-dihydrophenanthrene-2-*O*-*β*-D-glucopyranoside.

Compound **10** was obtained as a colorless amorphous powder. This compound was identified as an isomer of **9** by its HRESIMS. The ^{1}H NMR and ^{13}C NMR spectra (Table 2) of **10** highly resembled those of **9**. From the correlation from H-1′ (δ_H 4.91) to C-7 (δc 156.7) in the HMBC spectrum, the *β*-D-glucose unit was shown to be located at C-7 of the 9,10-dihydrophenanthrene moiety. Therefore, compound **10** was determined as 7-hydroxy-5-methoxy-9,10-dihydrophenanthrene-2-*O*-*β*-D-glucopyranoside.

Compound **11** was obtained as a colorless amorphous powder and the HRESIMS data established the molecular formula as $C_{27}H_{34}O_{13}$. The ^{1}H and ^{13}C NMR spectra of **11** (Table 2) were more complex than those of **9** and **10**, including signals of two glucose units and one 9,10-dihydrophenanthrene moiety. Correlations from H-1′ (δ_H 4.88) to C-2 (δc 157.2) and from H-1″ (δ_H 4.85) to C-7 (δc 156.0) observed in the HMBC spectrum suggested two *β*-D-glucose units located at C-2 and C-7 of the 9,10-dihydrophenanthrene moiety, respectively. The structure of **11** was therefore determined as 4-methoxy-9,10-dihydrophenanthrene-2,7-di-*O*-*β*-D-glucopyranoside.

Compound **27** was obtained as a brown amorphous powder. The molecular formula $C_{30}H_{24}O_6$ was deduced by HREIMS with $[M]^+$ at *m*/*z* 480.1566 (calcd for 480.1573).

In the ^{1}H NMR spectrum, the deshielded aromatic signals at δ_H 9.44 (1H, d, *J* = 9.2 Hz, H-5′) and δ_H 8.00 (1H, s, H-5) suggested that **27** was a dimer formed from the combination of a phenanthrene unit (Majumder and Basak, 1990; Majumder et al., 1997) and a 9,10-dyhydrophenanthrene unit (Majumder and Lahiri, 1990). The HMBC spectrum showed a correlation from H-5 (δ_H 8.00) to C-1′ (δc 114.9), indicating that the phenanthrene group (C-1′) connected with the 9,10-dyhydrophenanthrene group at C-6. One methoxyl at δ_H 3.68 (3H, s) located at C-4 was determined by correlation from δ_H 3.68 (4-OMe) to δc 159.1 (C-4) in the HMBC spectrum. Although another correlation from δ_H 4.13 to δc 159.9 could also be observed, no further prediction could be made due to the uncertain assignment of the δc 159.9 signal. Therefore, a selective 1D NOE experiment was carried out. Irradiation of the protons at δ_H 3.68 (4-OMe) enhanced the signals of H-3 (2.56%) and H-5 (0.34%), while irradiation of the protons at δ_H 4.13 (4′-OMe) caused enhancement of H-3′ (2.86%) and H-5′ (0.39%). This result was consistent with those of other compounds, in which the methoxyl group was positioned at C-4 (Bai et al., 1998; Yamaki et al., 1990). Therefore, structure **27** was determined as 4,4′-dimethoxy-9,10-dyhydro-[6,1′-biphenanthrene]-2,2′,7,7′-tetraol.

Compound **29** was obtained as a brown amorphous powder. The HREIMS gave an intense parent ion at *m*/*z* 420.1572, corresponding to the molecular formula $C_{25}H_{24}O_6$. The ^{1}H and ^{13}C NMR spectroscopic data of **29** highly resembled those of pleionesin C (**30**) (Dong et al., 2010). It differed from **30** though by the presence of the methylol moiety at C-3, instead of an acetoxymethyl in **30**. Accordingly, signals at δc 172.7 and δc 20.7 from **30** were absent in the ^{13}C NMR spectrum of **29**. The relative configuration of H-2 and H-3 at the dihydrofuran ring was determined to be *trans* by correlation between H-1″ and H-2 in the NOESY experiment (Ali et al., 2003; Dong et al., 2010; Yao et al., 2006). Thus, structure **29** was determined as (2,3-*trans*)-2-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-10-methoxy-2,3,4,5-tetrahydro-phenan thro[2,1-b]furan-7-ol.

Compound **32** was assigned the molecular formula $C_{40}H_{34}O_8$, deduced by negative ion HRESIMS. Signals in the ^{1}H and ^{13}C NMR spectra of **32** could be divided into two groups. One group was very similar to those of **29**, suggesting presence of the 2-(4-hydroxy-3-methoxyphenyl)-10-methoxy-2,3,4,5-tetrahydro-phenanthro[2,1-b]furan-7-ol unit. Besides that, characteristically deshielded aromatic resonances at δ_H 9.42 (1H, d, *J* = 9.3 Hz, H-5″) in the ^{1}H NMR spectrum suggested the presence of an additional phenanthrene unit (Majumder and Basak, 1990; Majumder et al., 1997). In addition, signals in the ^{13}C NMR spectrum including three oxygenated quaternary aromatic carbons, five quaternary aromatic carbons and six methenyl aromatic carbons, together with an HMBC correlation from 4.09 (4″-OMe) to 159.1 (C-4″) confirmed the 2,7-dihydroxy-4-methoxy-phenanthrene as the other unit. The two groups were connected through C-3 and C-1″ with a methylene which was deduced by correlations from δ_H 3.42 (H-11″) to δc 88.6 (C-2), δc 53.1 (C-3), δc 113.4 (C-1″), δc 134.0 (C-10″a) and δc 154.5 (C-2″) in the HMBC spectrum.

The *trans* orientation of H-2 and H-3 at the dihydrofuran ring in **32** was determined by interactions between H-2/H-11″ and H-2′/H-3 in NOESY experiment (Ali et al., 2003; Dong et al., 2010; Iliya et al., 2003; Nozaki et al., 2007; Yao et al., 2006). Therefore **32** was determined as (2,3-*trans*)-3-[(2,7-dihydroxy-4-methoxy-phenanthren-1-yl)methyl]-2-(4-hydroxy-3-methoxyphenyl)-10-methoxy-2,3,4,5-tetrahydro-phenanthro[2,1-b]furan-7-ol.

Compound **33** was isolated as a brown amorphous powder. Positive mode HRESIMS at m/z 669.2470 $[M+Na]^+$ (calcd for 669.2464) indicated its molecular formula as $C_{40}H_{38}O_8$. Signals from the ^{1}H NMR spectrum of **33** could also be divided into two groups, one of which was readily assigned to a 2-(4-hydroxy-3-methoxyphenyl)-10-methoxy-2,3,4,5-tetrahydro-phenanthro[2,1-b]furan-7-ol unit. Resonances in the ^{1}H NMR spectrum of the other group could be attributed to two methylenes [δ_H 2.80 (2H, m, H-α) and δ_H 2.70 (2H, m, H-α')], one 1,2,3,5-tetrasubstituted benzene moiety [δ_H 6.36 (1H, d, J = 2.4 Hz, H-3″) and δ_H 6.34 (1H, d, J = 2.4 Hz, H-5″)], one 1,3-*meta*-substituted benzene moiety [δ_H 6.57 (1H, overlapped, H-2‴), δ_H 6.58 (2H, overlapped, H-4‴, H-6‴), δ_H 7.04 (1H, t, J = 7.6 Hz, 8.4 Hz, H-5‴)] and one methoxyl, which indicated a bibenzyl moiety as the other group. In addition, signals in the ^{13}C NMR spectrum, including three quaternary oxygenated aromatic carbons, three quaternary aromatic carbons, six methenyl aromatic carbons and two methenyl carbons, further confirmed the prediction. Moreover, correlations from δ_H 2.95 (H-7″) to δc 144.0 (C-6″), δc 158.1 (C-2″), δc 89.5 (C-2), and δc 52.2 (C-3) in the HMBC spectrum suggested that the bibenzyl unit and the dihydrophenanthrofuran moiety were connected through C-3 and C-1″ via a methylene.

The relative orientation of H-2 and H-3 was determined as *trans* by the interactions between H-2/H-7″ observed in a NOESY experiment (Ali et al., 2003; Dong et al., 2010; Yao et al., 2006). Consequently structure **33** was determined as (2,3-*trans*)-3-[2-hydroxy-6-(3-hydroxyphenethyl)-4-methoxybenzyl]-2-(4-hydroxy-3-methoxyphenyl)-10-methoxy-2,3,4,5-tetrahydro-phenanthro[2,1-b]furan-7-ol.

Besides the previously unreported compounds, 23 known compounds (Figs. 1 and 2) were identified as gastrodin (**1**) (Baek et al., 1999), (−)-(2*R*,3*S*)-1-(4-β-D-glucopyranosyloxybenzyl)-4-methyl-2-isobutyltartrate (**4**) (Zi et al., 2008), militarine (**7**) (Kizu et al., 1999), loroglossin (**8**) (Kizu et al., 1999), coelonin (**12**) (Majumder and Banerjee, 1988), 7-hydroxy-2,4-dimethoxy-9,10-dihydrophenanthren (**13**) (Krautwurst and Tochtermann, 1981), 4,7-dihydroxy-1-*p*-hydroxybenzyl-2-methoxy-9,10-dihydrophenanthrene (**14**) (Takagi et al., 1983), flavanthrinin (**15**) (Leong et al., 1997), 2-hydroxy-5,7-dimethoxyphenanthrene (**16**) (Broering and Morrow, 1999), 1-*p*-hydroxybenzyl-4-methoxyphenanthrene-2,7-diol (**17**) (Yamaki et al., 1990), batatasin III (**18**) (Leong et al., 1997), 3,3′,5-trihydroxybibenzyl (**19**) (Honda and Yamaki, 2001), 3,3′-dihydroxy-4-(*p*-hydroxybenzyl)-5-methoxybibenzyl (**20**) (Bai et al., 1993), 3,3′-dihydroxy-2-(*p*-hydroxybenzyl)-5-methoxybibenzyl (**21**) (Bai et al., 1993), 3′,5-dihydroxy-2-(*p*-hydroxybenzyl)-3-methoxybibenzyl (**22**) (Bai et al., 1993), blestriarene A (**23**) (Yamaki et al., 1989), blestriarene B (**24**) (Yamaki et al., 1989), blestriarene C (**25**) (Yamaki et al., 1989), gymconopin C (**26**) (Matsuda et al., 2004), blestrianol A (**28**) (Bai et al., 1991), pleionesin C (**30**) (Dong et al., 2010), shanciol H (**31**) (Dong et al., 2010) and adenosine (**34**) (Otsuka et al., 1989).

The cytotoxicities of compounds **1**–**33** were evaluated against A549 and Bel7402 cell lines using the MTT method (Table 3), with bufalin as a positive control. Among the compounds examined, compounds **30**, **32**, **31**, **23**, **24** showed weak cytotoxic activity (IC_{50} 33.6, 38.0, 42.8, 47.5, 48.2 μM respectively); compound **33** showed moderate cytotoxic activity (IC_{50} 16.0 μM) against A549 cell line. All of these compounds were inactive towards Bel7402 cells (IC_{50} > 50 μM).

Table 3
Cytotoxic activities of compounds **23**, **24**, **30**–**33** against A549 cell line.[a]

Compound	IC_{50} value (μM)
23	47.5
24	48.2
30	33.6
31	42.8
32	38.0
33	16.0
Bufalin[b]	0.05

[a] Compounds **1**–**22**, **25**–**29** were inactive against A549 cell line (IC_{50} > 50 μm).
[b] Positive control.

3. Conclusions

This study afforded 11 previously unreported compounds from the tubers of *C. appendiculata*. Among them, the structures of compounds **32** and **33** were unusual as dimers, possessing a phenanthrene or bibenzyl unit connecting to C-3 of the 2,3,4,5-tetrahydro-phenanthro[2,1-b]furan moiety; which is a novel discovery of natural products from Orchidaceae plants. In addition to the compounds described above, 23 known compounds were obtained at the same time of which compounds **4**, **13**, **14**, **20**, **23**, **24**, **26**, **28**, **30** and **31** were isolated from tubers of *C. appendiculata* for the first time.

According to the cytotoxicity assay, six phenanthrenes or 9,10-dihydrophenanthrenes isolated from the herb showed weak or moderate cytotoxic activity against A549 cells. These two types of compounds were also reported to possess cytotoxic activity in previous studies (Guo et al., 2009; Xia et al., 2005; Xue et al., 2006). Moreover, traditional Chinese medicines are always characterized by the synergistic effect of multiple components, thus two classes of bioactive components including phenanthrenes and 9,10-dihydrophenanthrenes should be considered as the chemical markers. Accordingly for quality control it is sensible to determine the total amount of the phenanthrenes and 9,10-dihydrophenanthrenes.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured on a Perkin-Elmer 341 polarimeter, UV spectra were recorded on a Shimadzu UV-2450 spectrometer. IR (4000–400 cm^{-1}) spectra were recorded as KBr disks on a Perkin-Elmer 577 spectrometer. NMR spectra were measured in $CDCl_3$, CD_3OD or DMSO-d_6 on a Bruker AM-400 spectrometer for ^{1}H, 1D NOE NMR spectra (400 MHz), on a Bruker AVANCE III 500 spectrometer for ^{1}H, ^{13}C, DEPT, HSQC and HMBC NMR spectra (500 MHz), and on a Varian Inove-600 spectrometer for NOESY NMR (600 MHz). High-resolution electrospray ionization mass spectra (HRESIMS) were acquired using a Finnigan LCQ^{DECA} mass spectrometer. HREIMS (70 ev) analyses were carried out on a Finnigan-MAT 95 mass spectrometer.

Analytical and semi-preparative HPLC were performed on an Agilent 1100 with an Agilent DAD spectrophotometer and a XDB-C18 column (10 × 250 mm, 5 μm). Column chromatography (CC) separations were carried out on silica gel (200–300 mesh, Qingdao Haiyang Chemical Co., Ltd.) and Sephadex LH-20 gel (Amersham

Biosciences), respectively. TLC was performed on precoated silica gel sheets (GF 254, 100 × 100 mm, 0.15–0.20 mm, Sinopharm Group Co., Ltd.). All solvents used were of analytical grade (Shanghai Sinopharm Chemical Reagent Company, Ltd.). Fractions were monitored by TLC and spots were visualized under UV light or by spraying with 10% H_2SO_4 in EtOH followed by heating.

4.2. Plant material

Tubers of *C. appendiculata* were collected in Guizhou Province, People′s Republic of China, in July 2009. The plant identity was verified by Professor De-an Guo (one of the authors). A voucher specimen (No. 2009016) was deposited at Shanghai Research Center for TCM Modernization, National Engineering Laboratory for TCM Standardization Technology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

4.3. Extraction and isolation

Powdered and air-dried tubers of *C. appendiculata* (30 kg) were successively extracted with EtOH–H_2O (20 L × 3, 95:5 and 70:30, v/v). The dark brown residue resulting (310 g) was partitioned into EtOAc-soluble (120 g) and H_2O-soluble (190 g) fractions. The EtOAc fraction was applied to a silica gel column and eluted with a $CHCl_3$–MeOH gradient (from 100:1–2:1) to give 12 fractions (A_1–A_{12}). Fraction A_6 (50 g) was subjected to a silica gel column washed with a gradient of increasing EtOAc (1–50%) in petroleum ether (PE) (60–90 °C) to give five fractions (A_6B_1 to A_6B_5). Fraction A_6B_1 (5 g) was separated by silica gel CC [CH_2Cl_2–EtOAc (8:1 to 2:1)] producing four fractions ($A_6B_1C_1$ to $A_6B_1C_4$). Fraction $A_6B_1C_2$ was applied to a Sephadex LH-20 column eluted with PE–CH_2Cl_2–MeOH (2:1:1) and further purified by prep. HPLC [MeOH–H_2O (45:55), λ 210 nm] to yield compound **29** (4 mg). Fraction A_7 (10 g) was subjected to silica gel CC eluted with CH_2Cl_2–EtOAc–MeOH (100: 6: 0.8) to give ten fractions (A_7B_1 to A_7B_{10}). Fraction A_7B_2 was applied to a Sephadex LH-20 column [PE–CH_2Cl_2–MeOH (2:1:1)] and further purified by prep. HPLC [MeOH–H_2O (60:40), λ 210 nm] to obtain compound **27** (4 mg). Fraction A_7B_3 was subjected to Sephadex LH-20 CC [PE–CH_2Cl_2–MeOH (2:1:1)] and further separated by prep. HPLC [MeOH–H_2O (30:70), λ 210 nm] to yield compounds **32** (8 mg) and **33** (2 mg). Fraction A_{10} (6 g) was applied to a silica column washed with CH_2Cl_2–EtOAc–MeOH (100:6:0.8) to give four fractions ($A_{10}B_1$ to $A_{10}B_4$). Fraction $A_{10}B_4$ was eluted with MeOH through a Sephadex LH-20 column to afford three subfractions ($A_{10}B_4C_1$–$A_{10}B_4C_3$). Subfraction $A_7B_4C_3$ was further separated by prep. HPLC [MeOH–H_2O (30:70), λ 210 nm] to obtain compounds **9** (8 mg) and **10** (3 mg). The aqueous fraction (190 g) was applied to a MCI resin column with successive elution with EtOH–H_2O (20:80; 50:50; 80:20, v/v). The EtOH–H_2O (50:50, v/v) fraction was separated by reversed-phase silica gel CC eluting with a step gradient of 10%, 20%, 30% and 40% MeOH in H_2O to afford four fractions. Part of the MeOH–H_2O (40:60, v/v) fraction (320 mg) was subjected to Sephadex LH-20 CC [MeOH–H_2O (1:1)] to give three fractions, with the first and second fractions separated by prep. HPLC [MeOH–H_2O (10:90) to (20:80), λ 210 nm] to yield compounds **2** (34 mg) and **3** (5 mg), **5** (28 mg) and **6** (8 mg), respectively. Part of the MeOH–H_2O (20:80, v/v) fraction (64 mg) was subjected to Sephadex LH-20 CC [MeOH–H_2O (1:1)] to give compound **11** (15 mg).

4.3.1. 1-(4-β-D-Glucopyranosyloxybenzyl) 4-methyl (2R)-2-isobutylmalate (**2**)

Colorless gum; $[\alpha]^{20}_D$ −19 (*c* 0.12, MeOH); UV (MeOH) λ_{max} ($\log\varepsilon$) 221 (4.01), 269 (3.00), 275 (2.91) nm; IR (KBr) ν_{max} 3427, 2920, 1736, 1616, 1514, 1439, 1371, 1234, 1172, 1074, 577 cm^{-1}; for ^{1}H NMR and ^{13}C NMR spectroscopic data, see Table 1; HRESIMS at *m*/*z* 495.1833 [M+Na]$^+$ (calcd for $C_{22}H_{32}O_{11}Na$ 495.1842).

4.3.2. 1-(4-β-D-Glucopyranosyloxybenzyl) 4-ethyl (2R)-2-isobutylmalate (**3**)

Colorless gum; $[\alpha]^{20}_D$ −23 (*c* 0.11, MeOH); UV (MeOH) λ_{max} ($\log\varepsilon$) 223 (4.03), 270 (3.25), 276 (3.22) nm; IR (KBr) ν_{max} 3427, 2938, 2843, 1682, 1614, 1520, 1462, 1425, 1325, 1213, 1115, 1059, 991, 829, 702 cm^{-1}; for ^{1}H NMR and ^{13}C NMR spectroscopic data, see Table 1; HRESIMS at *m*/*z* 509.2017 [M+Na]$^+$ (calcd for $C_{23}H_{34}O_{11}Na$ 509.1999).

4.3.3. 1-(4-β-D-Glucopyranosyloxybenzyl) 4-methyl (2R)-2-benzylmalate (**5**)

Colorless amorphous powder; $[\alpha]^{23}_D$ −34 (*c* 0.05, MeOH); UV (MeOH) λ_{max} ($\log\varepsilon$) 215 (3.92), 264 (2.66) nm; IR (KBr) ν_{max} 3427, 2924, 1741, 1614, 1514, 1439, 1367, 1236, 1072, 833, 702, 602 cm^{-1}; for ^{1}H NMR and ^{13}C NMR spectroscopic data, see Table 1; HRESIMS at *m*/*z* 529.1673 [M+Na]$^+$ (calcd for $C_{25}H_{30}O_{11}Na$ 529.1686).

4.3.4. 1,4-Bis(4-β-D-glucopyranosyloxybenzyl) (2R)-2-benzylmalate (**6**)

Colorless amorphous powder; $[\alpha]^{23}_D$ −40 (*c* 0.05, MeOH); UV (MeOH) λ_{max} ($\log\varepsilon$) 219 (4.18), 269 (2.76) nm; IR (KBr) ν_{max} 3427, 2920, 2515, 1797, 1732, 1612, 1514, 1421, 1236, 1169, 1074, 876, 702, 542, 474 cm^{-1}; for ^{1}H NMR and ^{13}C NMR spectroscopic data, see Table 1; HRESIMS at *m*/*z* 783.2495 [M+Na]$^+$ (calcd for $C_{37}H_{44}O_{17}Na$ 783.2476).

4.3.5. 7-Hydroxy-4-methoxy-9,10-dihydrophenanthrene-2-O-β-D-glucopyranoside (**9**)

Colorless amorphous powder; $[\alpha]^{23}_D$ −54 (*c* 0.05, MeOH); UV (MeOH) λ_{max} ($\log\varepsilon$) 217 (4.24), 276 (4.07), 290 (3.98) nm; IR (KBr) ν_{max} 3413, 2920, 1614, 1462, 1271, 1171, 1082, 856, 476 cm^{-1}; for ^{1}H NMR and ^{13}C NMR spectroscopic data, see Table 2; HRESIMS at *m*/*z* 427.1355 [M+Na]$^+$ (calcd for $C_{21}H_{24}O_8Na$ 427.1369).

4.3.6. 7-Hydroxy-5-methoxy-9,10-dihydrophenanthrene-2-O-β-D-glucopyranoside (**10**)

Colorless amorphous powder; $[\alpha]^{23}_D$ −36 (*c* 0.05, MeOH); UV (MeOH) λ_{max} ($\log\varepsilon$) 208 (4.48), 276 (4.24), 290 (4.14) nm; IR (KBr) ν_{max} 3423, 2929, 1614, 1462, 1348, 1259, 1223, 1076, 584 cm^{-1}; for ^{1}H NMR and ^{13}C NMR spectroscopic data, see Table 2; HRESIMS at *m*/*z* 427.1356 [M+Na]$^+$ (calcd for $C_{21}H_{24}O_8Na$ 427.1369).

4.3.7. 4-Methoxy-9,10-dihydrophenanthrene-2,7-di-O-β-D-glucopyranoside (**11**)

Colorless amorphous powder; $[\alpha]^{23}_D$ −28 (*c* 0.05, DMSO); UV (MeOH) λ_{max} ($\log\varepsilon$) 278 (4.14), 292 (4.09) nm; IR (KBr) ν_{max} 3379, 2927, 2519, 2133, 1797, 1612, 1587, 1567, 1462, 1429, 1300, 1086, 1024, 611 cm^{-1}; for ^{1}H NMR and ^{13}C NMR spectroscopic data, see Table 2; HRESIMS at *m*/*z* 589.1895 [M+Na]$^+$ (calcd for $C_{27}H_{34}O_{13}Na$ 589.1897).

4.3.8. 4,4′-Dimethoxy-9,10-dyhydro-[6,1′-biphenanthrene]-2,2′,7,7′-tetraol (**27**)

Brown amorphous powder; $[\alpha]^{20}_D$ +5 (*c* 0.17, MeOH); UV (MeOH) λ_{max} ($\log\varepsilon$) 210 (4.79), 260 (4.84), 310 (4.38), 375 (3.64) nm; IR (KBr) ν_{max} 3427, 2933, 2850, 1614, 1589, 1460, 1437, 1348, 1280, 1224, 1157, 1086, 1020,980, 829, 538 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz) δ 9.44 (1H, d, *J* = 9.2 Hz, H-5′), 8.00 (1H, s, H-5), 7.44 (1H, d, *J* = 9.2 Hz, H-9′), 7.44 (1H, d, *J* = 9.2 Hz, H-10′), 7.12 (1H, d, *J* = 2.8 Hz, H-8′), 7.09 (1H, dd, *J* = 9.2, 2.8 Hz, H-6′), 6.93 (1H, s, H-3′), 6.86 (1H, s, H-8), 6.36 (1H, d, *J* = 2.4 Hz, H-3), 6.35 (1H, d, *J* = 2.4 Hz, H-1), 4.13 (3H, s, 4′-OMe), 3.68 (3H, s, 4-OMe), 2.76 (4H, s, H-9/10); ^{13}C NMR (CD_3OD, 125 MHz) δ 159.9 (C-4′), 159.1 (C-4), 157.6 (C-2), 155.4 (C-7′), 154.3 (C-7), 153.4 (C-2′), 141.9 (C-10a), 140.2 (C-8a), 134.7 (C-10a′), 134.5 (C-8a′), 133.9 (C-5), 130.6 (C-5′), 128.0 (C-9′), 126.5 (C-4b), 126.4 (C-10′), 125.6 (C-4b′), 121.6 (C-6), 117.2 (C-6′), 116.7 (2C, C-4a/4a′), 115.7 (C-8), 114.9 (C-1′), 112.0 (C-8′), 108.4 (C-1), 100.3 (C-3′), 99.3 (C-3), 56.0 (C-4′-OCH_3), 55.9 (C-4-OMe), 31.8 (C-10), 31.0 (C-9); HREIMS at *m*/*z* 480.1566 $[M]^+$ (calcd for $C_{30}H_{24}O_6$ 480.1573).

*4.3.9. (2,3-trans)-2-(4-Hydroxy-3-methoxyphenyl)-3-hydroxymethyl-10-methoxy-2,3,4,5-tetrahydro-phenanthro[2,1-b]furan-7-ol (**29**)*

Brown amorphous powder; $[\alpha]^{20}_D$ +5 (*c* 0.133, MeOH); UV (MeOH) λ_{max} ($\log\varepsilon$) 205 (4.39), 283 (4.00), 306 (3.81) nm; IR (KBr) ν_{max} 3425, 2920, 2850, 1610, 1516, 1454, 1350, 1286, 1238, 1199, 1116, 1028, 548 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz) δ 8.00 (1H, d, *J* = 9.2 Hz, H-9), 6.90 (1H, d, *J* = 2.0 Hz, H-2′), 6.79 (1H, dd, *J* = 8.2, 2.0 Hz, H-6′), 6.74 (1H, d, *J* = 8.2 Hz, H-5′), 6.63 (1H, d, *J* = 2.8 Hz, H-6), 6.62 (1H, dd, *J* = 9.2, 2.8 Hz, H-8), 6.55 (1H, s, H-11), 5.65 (1H, d, *J* = 3.0 Hz, H-2), 3.85 (1H, m, H-1″a), 3.85 (3H, s, 10-OMe), 3.80 (3H, s, 3′-OMe), 3.59 (1H, m, H-1″b), 3.46 (1H, m, H-3), 2.62 (4H, m, H-4/5); ^{13}C NMR (CD_3OD, 125 MHz) δ 160.6 (C-11a), 159.4 (C-10), 156.1 (C-7), 149.0 (C-3′), 147.1 (C-4′), 140.2 (C-5a), 137.5 (C-3b), 135.7 (C-1′), 130.2 (C-9), 126.1 (C-9a), 119.0 (C-6′),117.9 (C-9b), 116.7 (C-3a), 116.1 (C-5′), 114.9 (C-6), 113.7 (C-8), 110.0 (C-2′), 93.8 (C-11), 88.7 (C-2), 64.9 (C-1″), 56.3 (C-3′-OMe), 56.2 (C-10-OMe), 54.6 (C-3), 30.9 (C-5), 27.9 (C-4); HREIMS at *m*/*z* 420.1572 $[M]^+$ (calcd for $C_{25}H_{24}O_6$ 420.1573).

*4.3.10. (2,3-trans)-3-[(2,7-Dihydroxy-4-methoxy-phenanthren-1-yl)methyl]-2-(4-hydroxy-3-methoxyphenyl)-10-methoxy-2,3,4,5-tetrahydro-phenanthro[2,1-b]furan-7-ol (**32**)*

Brown amorphous powder; $[\alpha]^{20}_D$ +5 (*c* 0.175, MeOH); UV (MeOH) λ_{max} ($\log\varepsilon$) 205 (4.84), 250 (4.63), 265 (4.75), 300 (4.39), 315 (4.22) nm; IR (KBr) ν_{max} 3431, 2931, 2840, 1612, 1583, 1516, 1462, 1381, 1352, 1284, 1232, 1200, 1130, 1028, 542, 476 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz) δ 9.42 (1H, d, *J* = 9.3 Hz, H-5″), 8.03 (1H, d, *J* = 9.2 Hz, H-9), 7.95 (1H, d, *J* = 9.3 Hz, H-10″), 7.64 (1H, d, *J* = 9.3 Hz, H-9″), 7.17 (1H, d, *J* = 2.8 Hz, H-8″), 7.11 (1H, dd, *J* = 9.3, 2.8 Hz, H-6″), 6.93 (1H, s, H-3″), 6.66 (1H, d, *J* = 2.4 Hz, H-6), 6.64 (1H, dd, *J* = 2.4, 9.2 Hz, H-8), 6.60 (1H, s, H-11), 6.47 (1H, d, *J* = 8.2 Hz, H-5′), 6.38 (1H, dd, *J* = 8.2, 2.0 Hz, H-6′), 5.55 (1H, d, *J* = 2.4 Hz, H-2), 5.48 (1H, d, *J* = 2.0 Hz, H-2′), 4.09 (3H, s, 4″-OMe), 3.87 (3H, s, 10-OMe), 3.70 (1H, m, H-3), 3.42 (2H, m, H-11″), 2.95 (3H, s, 3′-OMe), 2.80 (2H, m, H-4), 2.67 (2H, m, H-5); ^{13}C NMR (CD_3OD, 125 M) δ 160.3 (C-11a), 159.1 (2C, C-10/4″), 156.1 (C-7), 155.5 (C-7″), 154.5 (C-2″), 148.6 (C-3′), 146.1 (C-4′), 140.3 (C-5a), 137.4 (C-3b), 136.4 (C-1′), 134.2 (C-8″a), 134.0 (C-10″a), 130.6 (C-5″), 130.2 (C-9), 129.0 (C-9″), 126.4 (C-9a), 125.9 (C-4″b), 123.9 (C-10″), 120.6 (C-3a), 117.9 (C-9b), 117.6 (C-6″), 117.0 (C-6′), 116.8 (C-4″a), 115.5 (C-5′), 115.0 (C-6), 113.6 (C-8), 113.4 (C-1″), 112.1 (C-8″), 108.4 (C-2′), 100.2 (C-3″), 93.8 (C-11), 88.6 (C-2), 56.2 or 56.0 (C-10-OMe or C-4″-OMe), 55.4 (C-3′-OMe), 53.1(C-3), 31.0 (C-5), 30.9 (C-11″), 28.2 (C-4); HRESIMS at *m*/*z* 641.2137 $[M-H]^-$ (calcd for $[C_{40}H_{33}O_8]^-$ 641.2175).

*4.3.11. (2,3-trans)-3-[2-Hydroxy-6-(3-hydroxyphenethyl)-4-methoxybenzyl]-2-(4-hydroxy-3-methoxyphenyl)-10-methoxy-2,3,4,5-tetrahydro-phenanthro[2,1-b]furan-7-ol (**33**)*

Brown amorphous powder; $[\alpha]^{23}_D$ −2.04 (*c* 0.049, MeOH); UV (MeOH) λ_{max} ($\log\varepsilon$) 206 (4.66), 281 (4.06), 306 (3.81), 316 (3.66) nm; IR (KBr) ν_{max} 3423, 2921, 2852, 2517, 2158, 1612, 1454, 1286, 1200, 978, 598 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz) δ 8.00 (1H, d, *J* = 8.4 Hz, H-9), 7.04 (1H, t, *J* = 7.6, 8.4 Hz, H-5‴), 6.62 (1H, overlapped, H-8), 6.61 (1H, overlapped, H-5′), 6.60 (1H, overlapped, H-6), 6.58 (3H, overlapped, H-11/H-6‴/H-4‴), 6.57 (1H, overlapped, H-2‴), 6.47 (1H, dd, *J* = 2.0, 8.0 Hz, H-6′), 6.36 (1H, d, *J* = 2.4 Hz, H-3″), 6.34 (1H, d, *J* = 2.4 Hz, H-5″), 6.16 (1H, d, *J* = 2.0 Hz, H-2′), 5.51 (1H, d, *J* = 2.4 Hz, H-2), 3.86 (3H, s, 10-OMe), 3.73 (3H, s, 4″-OMe), 3.64 (1H, m, H-3), 3.60 (3H, s, 3′-OMe), 2.95 (2H, m, H-7″), 2.80 (2H, m, H-α), 2.70 (2H, m, H-α′), 2.68 (2H, m, H-4), 2.48 (2H, m, H-5); ^{13}C NMR (CD_3OD, 125 M) δ 160.4 (C-11a), 160.2 (C-4″), 159.0 (C-10), 158.4 (C-3‴), 158.1 (C-2″), 156.0 (C-7), 148.8 (C-3′), 146.5 (C-4′), 144.7 (C-1‴), 144.0 (C-6″), 140.4 (C-5a), 137.6 (C-3b), 136.3 (C-1′), 130.3 (C-5‴), 130.2 (C-9), 126.3 (C-9a), 120.7 (C-3a), 120.7 (C-6‴), 117.9 or 118.0 (C-9b or C-1″), 117.7 (C-6′), 116.3 (C-2‴), 115.9 (C-5′), 114.9 (C-6), 113.8 or 113.6 (C-8 or C-4‴), 109.1 (C-2′), 107.1 (C-5″), 100.4 (C-3″), 93.8 (C-11), 89.5 (C-2), 56.1 or 56.2 (C-3′-OMe or C-10-OMe), 55.5 (4″-OMe), 52.2 (C-3), 38.7 (C-α′), 36.7 (C-α), 32.1 (C-7″), 30.9 (C-5), 28.1 (C-4); HRESIMS at *m*/*z* 669.2470 $[M+Na]^+$ (calcd for $[C_{40}H_{38}O_8Na]^+$ 669.2464).

*4.4. Alkaline hydrolysis of **2**, **3**, **5**, **6***

To each compound (15–20 mg) was individually added 3% aqueous NaOH solution (5 mL) with the mixture stirred at room temperature for 2 h. The reaction mixture was then acidified to pH 4 by 2 N HCl and partitioned with EtOAc. The EtOAc phase was concentrated under reduced pressure to give (2*R*)-2-hydroxy-2-(2-methylpropyl) butanedioic acid from **2** and **3** (5 mg, 6 mg), (2*R*)-2-benzyl-2-hydroxysuccinic Acid from **5** and **6** (4 mg, 6 mg). The ^{1}H NMR, ESIMS and optical rotation data of (2*R*)-2-hydroxy-2-(2-methylpropyl) butanedioic acid (Yin et al., 2010) and (2*R*)-2-benzyl-2-hydroxysuccinic acid (El Bialy et al., 2005) were identical to those reported in the literature.

4.5. Cytotoxicity assay

Cytotoxicities against the A549 and Bel7402 tumor cell lines were evaluated by the MTT method according to the protocols described (Alley et al., 1988), with bufalin and 0.5% DMSO (aq) as a positive and negative control respectively.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2013.06.001.

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