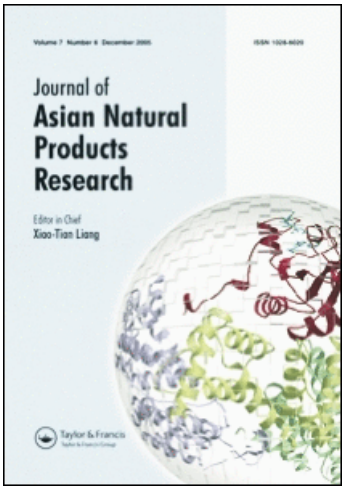

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Shuai Li[a]; Zhen Xue[a]; Su-Juan Wang[a]; Yong-Chun Yang[a]; Jian-Gong Shi[a]
[a] Key Laboratory of Bioactive Substances and Resources Utilization of Chinese Herbal Medicine, Ministry of Education, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College,, Beijing, China

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Terpenoids from the tuber of *Cremastra appendiculata*

Shuai Li, Zhen Xue, Su-Juan Wang, Yong-Chun Yang and Jian-Gong Shi*

Key Laboratory of Bioactive Substances and Resources Utilization of Chinese Herbal Medicine, Ministry of Education, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China

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Two new terpenoids including a cadinane sesquiterpene (**1**), and an *ent*-kaurane diterpene diglycoside (**2**), together with a known triterpene containing 32 carbons (**3**), have been isolated from the ethanolic extract of *Cremastra appendiculata*. Their structures were established by the spectroscopic methods including the IR, MS, 1D-, and 2D-NMR experiments as (−)-cadin-4,10(15)-dien-11-oic acid (**1**), (−)-*ent*-12β-hydroxykaur-16-en-19-oic acid, 19-*O*-β-D-xylopyranosyl-(1 → 6)-*O*-β-D-glucopyranoside (**2**), and (+)-24,24-dimethyl-25,32-cyclo-5α-lanosta-9(11)-en-3β-ol (**3**). Compounds **1–3** were evaluated against several human cancer cell lines. Compound **3** showed *in vitro*-selective cytotoxicity against human breast cancer cell lines (MCF-7) with an IC_{50} of 3.18 μM, but **1** and **2** were inactive ($IC_{50} > 10$ μg/ml).

Keywords: *Cremastra appendiculata*; Orchidaceae; terpenoids; cytotoxic activities

1. Introduction

The tuber of an orchidaceous plant, *Cremastra appendiculata* (D. Don) Makino, is used as 'Shan-Ci-Gu' (Chinese name) in traditional Chinese medicine for the treatment of various cancers [1]. As a part of our program to assess the chemical and biological diversities of several cultivated traditional Chinese medicines, from the ethanolic extract of the tubers of this plant, we have reported more than 15 aromatic metabolites with a variety of structural types including simple phenylethanol derivatives, 9,10-dihydrophenanthrenes, as well as mono-, di-, and trimeric phenanthrenes, together with β-sitosterol, daucosterol, sucrose, and adenosine [2–4]. In a continuation of this work, two new minor terpenoidal metabolites **1–2** were isolated by a careful isolation procedure from the remaining EtOAc- and H_2O-soluble nonaromatic fractions of the same material. Compounds **1** and **2** (Figure 1) were the first cadinane sesquiterpene and *ent*-kaurane diterpene from the orchidaceous plants, and **3** having an unusual triterpene skeleton containing 32 carbons was obtained as the most abundant component from the EtOAc-soluble nonaromatic fraction; its structure was reported recently [5]. This paper deals with the isolation and structural elucidation of **1** and **2**, as well as the cytotoxicities of **1–3** against several human cancer cell lines.

2. Results and discussion

The EtOH extract of the tubers of *C. appendiculata* was partitioned between water and EtOAc. The EtOAc phase was concentrated under vacuum and then subjected to repeated column chromatography over silica gel and Sephadex LH-20 to yield **1** and **3**. The H_2O phase was passed through a

*Corresponding author. Email: shijg@imm.ac.cn

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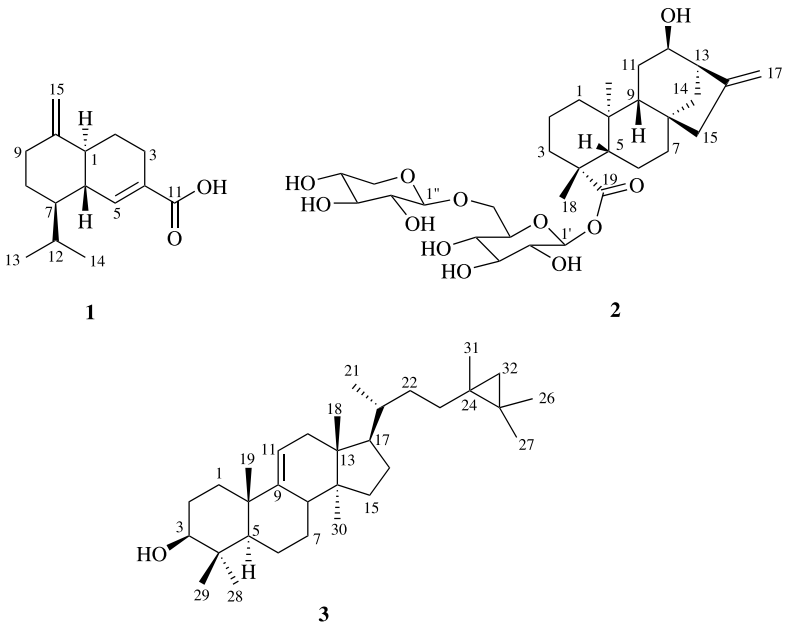

Figure 1. Structures of compounds **1**–**3**.

column packed with HPD-100 resin and then eluted with H_2O, 50% EtOH, 95% EtOH, and acetone to afford four respective fractions. Subsequent separation of the 95% EtOH fraction by column chromatography over silica gel yielded **2**.

Compound **1** was obtained as colorless needles from acetone, mp 139–140°C, $[\alpha]_D^{20}$: −188.2 (*c* 0.09, $CHCl_3$). The IR spectrum showed absorption bands for hydroxyl (3188 cm^{-1}), conjugated carbonyl (1684 cm^{-1}) groups, and double bonds (3080, 1645, and 1425 cm^{-1}). The EI-MS of **1** gave a molecular ion peak at *m/z* 234, and the HR-EI-MS at *m/z* 234.1644 indicated the molecular formula of **1** as $C_{15}H_{22}O_2$. The ^1H NMR spectrum of **1** showed two methyl doublets at δ 0.80 (3H, d, $J = 7.0$ Hz, H_3-13) and 0.97 (3H, d, $J = 7.0$ Hz, H_3-14), a pair of characteristic olefinic proton singlets for an exocyclic double bond at δ 4.60 (1H, s, H-15a) and 4.72 (1H, s, H-15b), and another olefinic proton singlet at δ 7.12 (1H, s, H-5), in addition to a broad exchangeable singlet assignable to a carboxyl proton at δ 10.54 (1H, br s), as well as several multiplets with complex coupling patterns attributed to methylenes and methines between δ 1.10–2.50 (Table 1). The ^{13}C NMR and DEPT spectra of **1** displayed 15 carbon signals including two methyls, five methylenes (one sp^2 hybrid), five methines (one sp^2 hybrid), two olefinic quaternary carbons, and one carbonyl carbon (Table 1). These spectroscopic data in combination with five degrees of unsaturation required by the molecular formula suggested that **1** was a bicyclic sesquiterpene containing one exocyclic double bond and one endocyclic double bond conjugating with the carbonyl.

In order to unambiguously establish the structure, 2D-NMR experiments of **1** were carried out. The proton and protonated carbon signals in the NMR spectra of **1** were assigned unequivocally by the HMQC experiment (Table 1). In the $^1H-^1H$ COSY spectrum, cross-peaks between H_2-2 and H_2-3, between H-12 and both H_3-13 and H_3-14, and correlations from H-7 through H_2-8 to H_2-9 revealed vicinal spin coupling relationships of these protons, indicating the presence of three partial structural units as illustrated by thick bonds in Figure 2. In the HMBC spectrum of **1**, long-range correlations from

Table 1. ^{1}H and ^{13}C NMR spectral data for compound **1**.

No.	δ_H	δ_C
1	1.82 ddd (*ca.* 12.5, 12.5, 3.5)	43.2
2	α 2.02 dddd (12.5, 12.5, 4.5, 3.5)	24.9
	β 1.47 dddd (12.5, 12.5, 12.5, 5.5)	
3	α 2.22 ddd (18.5, 12.5, 3.5)	24.3
	β 2.47 ddd (18.5, 6.0, 4.5)	
4		129.9
5	7.12 s	142.7
6	1.83 dd (*ca.* 12.5, 12.5)	45.8
7	1.38 dddd (12.5, 12.5, 7.0, 3.5)	46.1
8	α 1.85 dddd (*ca.* 12.5, 5.0, 4.0, 3.5)	26.6
	β 1.16 dddd (12.5, 12.5, 12.5, 4.0)	
9	α 2.06 ddd (13.0, 12.5, 4.0)	36.0
	β 2.41 ddd (13.0, 4.0, 4.0)	
10		151.9
11		172.4
12	2.20 m	26.4
13	0.80 d (7.0)	21.4
14	0.97 d (7.0)	15.2
15	a 4.60 s; b 4.72 s	104.1
COO**H**	10.54 br s	

^{1}H NMR spectral data were obtained at 500 Hz in acetone-d_6. ^{13}C NMR spectral data were obtained at 125 Hz in $CDCl_3$. Proton coupling constants (*J*) in Hz are given in parentheses. The assignments were based on the DEPT, $^{1}H-^{1}H$ COSY, HMQC, and HMBC experiments.

H_2-3 to C-1, C-4, C-5, and C-11; H-5 to C-1, C-3, C-4, C-7, and C-11; H_2-15 to C-1, C-9, and C-10; and both H_3-13 and H_3-14 to C-7 and C-12 (indicated by arrows in Figure 2), together with the chemical shift values of these carbons (Table 1), suggested that the planar structure of **1** was cadin-4,10(15)-dien-11-oic acid [6]. A careful comparison of the NMR spectral data of **1** with those of the known isomer γ-muurolen-15-oic acid having a cis ring fusion [7] indicated that there were significant differences between them, especially those data attributed to protons and carbons around three chiral centers. The relative configuration of **1** was elucidated by a detailed analysis of proton coupling constants in the ^{1}H NMR spectrum of **1** in acetone-d_6 with a higher resolution than that in $CDCl_3$. Although the coupling constant between H-1 and H-6 was not recognized due to overlapping of the signals of H-1, H-6, and H-8α in the ^{1}H NMR spectrum of **1**, split patterns of H-2β (dddd, $J_{1,2\beta} = J_{2\alpha,2\beta} = J_{2\beta,3\alpha} = 12.5$ Hz, $J_{3\beta,2\beta} = 5.5$ Hz), H-7

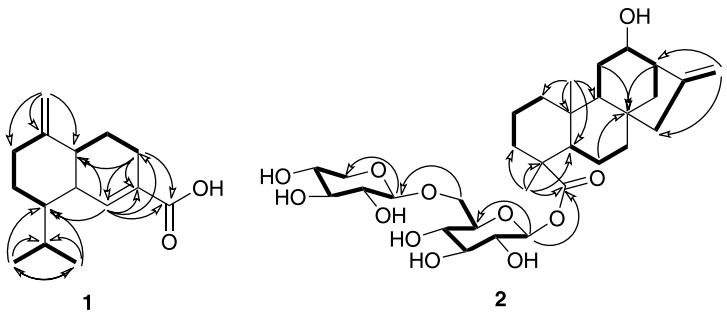

Figure 2. Main COSY (—) and HMBC (→) correlations of compounds **1**–**2**.

Table 2. ^{1}H and ^{13}C NMR spectral data for compound **2**.

No.	δ_H	δ_C	No.	δ_H	δ_C
1	α 1.85 ddd (13.0, 4.5, 3.5) β 0.83 ddd (13.0, 13.0, 3.5)	41.1	17	a 4.77 (br s); b 4.85 (br s)	105.9
2	α 1.40 m β 1.93 m	19.5	18	1.20 (s)	28.6
3	α 2.18 br d (13.0) β 1.05 ddd (13.0, 13.0, 4.0)	38.2	19		176.4
4		44.2	20	0.94 (s)	16.2
5	1.11 br d (11.0)	57.4	1′	5.44 d (8.0)	94.7
6	α 1.78 ddd (13.0, 11.0, 3.0) β 1.97 ddd (13.0, 3.0, 2.0)	22.1	2′	3.40 m	73.4
7	α 1.44 ddd (13.0, 12.0, 3.0) β 1.56 br d (12.0)	40.7	3′	3.51 m	78.0
8		44.2	4′	3.43 m	70.8
9	1.15 br d (10.5)	56.6	5′	3.55 m	76.8
10		39.6	6′	a 4.04 dd (11.0, 2.0); b 3.71 dd (11.0, 5.5)	68.5
11	α 1.27 dddd (14.0,11.5, 10.5, 3.0) β 1.77 dd (14.0, 5.5)	29.5	1″	4.40 d (6.5)	104.0
12	3.74 br dd (11.5, 5.5)	71.0	2″	3.27 m	73.4
13	2.52 br s	51.7	3″	3.39 m	75.9
14	1.16 br d (14.0); 2.06 br d (14.0)	38.9	4″	3.47 m	70.4
15	a 2.01 br s; b 2.01 br s	49.5	5″	a 3.87 dd (11.5, 4.0); b 3.22 dd (15.0, 9.0)	65.2
16		151.2			

Spectral data were obtained in acetone-d_6 at 500 MHz for proton and 125 MHz for carbon. Proton coupling constants (J) in Hz are given in parentheses. The assignments were based on the DEPT, $^{1}H-^{1}H$ COSY, HMQC, HMBC, and phase-sensitive $^{1}H-^{1}H$ COSY experiments.

(dddd, $J_{7,8\beta} = J_{6,7} = 12.5$ Hz, $J_{7,8\alpha} = 3.5$ Hz, $J_{7,12} = 7.0$ Hz), and H-8β (dddd, $J_{7,8\beta} = J_{8\alpha,8\beta} = J_{8\beta,9\alpha} = 12.5$ Hz, $J_{8\beta,9\beta} = 4.0$ Hz) showed large vicinal coupling constants of 12.5 Hz diagnostic for trans pseudo-diaxial relationships between H-1 and H-2β, H-6 and H-7, and H-7 and H-8β. This unambiguously demonstrated that H-1, H-6, and H-7 were pseudo-axial orientations. Therefore, the two six-membered rings in **1** were fused in a trans form, and the isopropyl group at C-7 was a pseudo-equatorial orientation. The trans ring fusion of **1** was further supported by H-5 appeared as a singlet [7–10]. Thus, the structure of **1** was determined as (−)-cadin-4,10(15)-dien-11-oic acid.

Compound **2** was obtained as colorless needles (acetone), mp 183–184°C, $[\alpha]_D^{20}$: −55.8 (*c* 0.11, MeOH), and showed the IR absorption bands for hydroxyl (3408 and 1063 cm^{-1}) and carbonyl (1743 cm^{-1}) functional groups. The FAB-MS of **2** gave a pseudomolecular ion peak at *m/z* 635 $[M + Na]^+$ and the HR-FAB-MS at *m/z* 635.3059, indicating that the molecular formula is $C_{31}H_{48}O_{12}$. The ^{1}H NMR spectrum of **2** showed two methyl singlets at δ 0.94 (3H, s, H_3-20) and 1.20 (3H, s, H_3-18), and two characteristic broad singlets of an exocyclic double bond at δ 4.77 (1H, br s, H-17a) and 4.85 (1H, br s, H-17b), in addition to the partially overlapped multiplets ascribed to aliphatic methylene and methine protons between δ 0.80–2.55, as well as the partially overlapped signals assignable to oxymethylenes, oxymethines, and hydroxyl groups between δ 3.10 and 5.50. The ^{13}C NMR and DEPT spectra of **2** showed 31 carbon signals consisting of 2 methyls, 11 methylenes (1 olefinic and 2 oxygenated), 13 methines (2 anomeric and 8 oxygenated), and 5 quaternary carbons (1 olefinic and 1 carbonyl) (Table 2). These spectroscopic data in combination with eight degrees of unsaturation required by the

molecule suggested that **2** is a diterpene diglycoside possessing a tetracyclic diterpene parent nucleus.

The structure of **2** was finally established by the $^{1}H-^{1}H$ COSY, HSQC, and HMBC experiments. The signals of protonated carbons and their corresponding protons in the NMR spectra were assigned unambiguously by the HSQC experiment. In the higher field region of the $^{1}H-^{1}H$ COSY spectrum of **2**, homonuclear vicinal coupling correlations between H_2-2 and both H_2-1 and H_2-3, between H_2-6 and both H-5 and H_2-7, and from H-9 through H_2-11, H-12, H-13, to H_2-14, as well as cross-peaks between H_2-15 and the exocyclic double bond protons (H-17a and H-17b) demonstrated the presence of partial structural units as depicted by thick bonds in Figure 2. In the HMBC spectrum, long-range heteronuclear correlations from H_3-18 to C-3, C-4, C-5, and C-19; H_3-20 to C-1, C-5, C-9, and C-10; H_2-6, H-11, and H-13 to C-8; and H_2-17 to C-13 and C-15 (indicated by arrows in Figure 2), together with the chemical shift values of these carbons and the quaternary nature of C-4, C-8, C-10, and C-16, unequivocally revealed that the planar structure of the tetracyclic diterpene skeleton of **2** was *ent*-12-hydroxykaur-16-en-19-oic acid or *ent*-12-hydroxykaur-16-en-18-oic acid. A further comparison of the ^{13}C NMR spectral data of the diterpene skeleton with those of the related compounds reported in the literature [11,12] indicated that the ^{13}C NMR spectral data of the diterpene skeleton were in good agreement with those of peniculoside IV and cussoracoside B [11], confirming that the aglycone of **2** was *ent*-12β-hydroxykaur-16-en-19-oic acid. In addition, in the $^{1}H-^{1}H$ COSY spectrum, a hexosyl and a pentosyl unit were readily recognized by homonuclear coupling correlations from H-1′ through H-2′, H-3′, H-4′, and H-5′ to H_2-6′ and from H-1″ through H-2″, H-3″, and H-4″ to H_2-5″. Meanwhile, the HMBC correlations from H-1′ to C-5′, H-1″ to C-5″, and H_2-6′ to C-1″ in combination with the coupling constants of the anomeric protons H-1′ ($J = 8.0$ Hz) and H-1″ ($J = 6.5$ Hz) revealed the presence of a β-pentopyranosyl-(1 → 6)-*O*-β-hexopyranosyl moiety in **2**. Although two monoglycosyl units could not be determined by a coupling constant analysis of the diglycosyl proton signals due to the overlapping of the signals in the ^{1}H NMR spectrum of **2**. However, the ^{13}C NMR spectral data of the diglycosyl moiety were in good agreement with those of β-D-xylopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl moiety of thalictoside IV [13] and β methyl-D-xylopyranoside [14]. Furthermore, acid hydrolysis of **2** yielded xylose and glucose identified by TLC comparisons of the hydrolysis products with authentic sugars. This confirmed that the diglycosyl moiety of **2** was a β-xylopyranosyl-(1 → 6)-*O*-β-glucopyranosyl. Finally, the linkage between the diterpene skeleton and the diglycosyl moiety was unambiguously established by a HMBC correlation from H-1′ to C-19, while a β-configuration was assigned to the sugar units on the basis of high abundance of β-xylopyranoside and β-glucopyranoside in the nature. Therefore, the structure of **2** was determined as (−)-*ent*-12β-hydroxykaur-16-en-19-oic acid 19-β-D-xylopyranosyl-(1 → 6)-*O*-β-D-glucopyranoside.

Compounds **1**–**3** were evaluated against several human cancer cell lines including human colon cancer (HCT-8), human hepatoma (Bel7402), human stomach cancer (BGC-823), human lung adenocarcinoma (A549), human breast cancer (MCF-7), and human ovarian cancer (A2780) cell lines. Compounds **1** and **2** were inactive ($IC_{50} > 10$ μg/ml), and **3** showed *in vitro*-selective cytotoxicity against MCF-7 cells with an IC_{50} of 3.18 μM.

3. Experimental

3.1 General experimental procedures

Melting points were determined on an XT-4 micro melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 spectropolarimeter. UV spectra were taken on Shimadzu UV-260 spectrophotometer. IR spectra were recorded

as KBr disks on a Nicolet Impact 400 FT-IR spectrophotometer. 1D- and 2D-NMR spectra were obtained at 500 and 125 MHz for ^{1}H and ^{13}C, respectively, on an Inova 500 MHz spectrometer in acetone-d_6 or $CDCl_3$ with solvent peaks as references. EI-MS, HR-EI-MS, FAB-MS, and HR-FAB-MS data were measured with a Micromass Autospec-Ultima ETOF spectrometer. Column chromatography was performed with silica gel (200–300 mesh) and Sephadex LH-20. TLC was carried out with glass-precoated silica gel GF_{254} plates. Spots were visualized by spraying 7% H_2SO_4 in 95% EtOH followed by heating.

3.2 Plant material

The tubers of *Cremastra appendiculata* were collected at a cultivating field of Xinbang TCM Limited Company in the eastsouth district of the Qian, Guizhou Province, China, in September 2003. The plant identification was verified by Mr Ding-Xiang He (Xinbang TCM Limited Company, the eastsouth district of the Qian, Guizhou Province 556000, China). A voucher specimen (no. 200366) has been deposited at the Herbarium of the Department of Medicinal Plants, Institute of Materia Medica, Beijing, China.

3.3 Extraction and isolation

The air-dried and powdered tubers of *Cremastra appendiculata* (5 kg) were extracted with 95% EtOH for three times (each 48 h) at room temperature. After the solvent was removed under reduced pressure at <45°C, a dark brown residue (116 g) was obtained. The residue was suspended in water and then partitioned with EtOAc. The EtOAc phase was concentrated to give a residue (37 g) that was separated by column chromatography over silica gel eluting with a gradient of increasing acetone (5–100%) in petroleum ether (60–90°C) followed by elution with MeOH to give 23 fractions (a_1–a_{23}) on the basis of the TLC analyses. Fraction a_4 was chromatographed over Sephadex LH-20 eluting with petroleum ether–$CHCl_3$–MeOH (5:5:1) to give three subfractions, and the second subfraction was purified by column chromatography over silica gel eluting with petroleum ether–ethyl acetate (4:1) to yield **3** (1216 mg). Fraction a_6 was subjected to column chromatography over silica gel eluting with petroleum ether–acetone (8:1) to give **1** (6.5 mg). After evaporation to remove dissolved EtOAc, the water phase was passed through a column packaged with macroporous resin HPD-100 and eluted successively with H_2O, 50% EtOH, 95% EtOH, and acetone to give four respective fractions (C1–C4). The fraction C3 (1.0 g) was chromatographed over silica gel eluting with $CHCl_3$–MeOH (4:1) to yield **2** (12.0 mg).

3.3.1 (−)-Cadin-4,10(15)-dien-11-oic acid (*1*)

Obtained as colorless needles from Me_2CO, 6.5 mg, mp 139–140°C; $[\alpha]_D^{20}$: −188.2 (*c* 0.09, $CHCl_3$); UV (MeOH) λ_{max} (log ε): 220.0 (3.87) nm; IR(KBr) ν_{max}: 3188, 3080, 2960, 2858, 1684, 1647, 1425, 1387, 1282, 1217, 1078, 949, 887, 739, 710, 654, 592, 542 cm^{-1}; ^{1}H NMR (500 MHz, acetone-d_6) and ^{13}C NMR (125 MHz, $CDCl_3$) spectral data: see Table 1; EI-MS *m/z* (%): 234 (17) $[M]^+$, 191 (12), 189 (4), 173 (15), 171 (22), 165 (5), 157 (7), 145 (15), 143 (7), 135 (9), 129 (20), 121 (3), 119 (9), 116 (11), 115 (100), 109 (9), 105 (19), 101 (22), 97 (10), 93.1 (17), 91 (20), 87 (13), 82 (10), 81 (26), 79 (20), 77 (14), 75 (13), 71 (20), 69 (24), 67 (27); HR-EI-MS *m/z*: 234.1643 $[M]^+$ (calcd for $C_{15}H_{22}O_2$, 234.1619).

3.3.2 (−)-ent-12β-Hydroxykaur-16-en-19-oic acid 19-O-β-D-xylopyranosyl-(1 → 6)-O-β-D-glucopyranoside (*2*)

Obtained as colorless needles from Me_2CO, 12.0 mg, mp 183–184°C; $[\alpha]_D^{20}$: −55.8 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ε) 205.0 (3.80) nm; IR (KBr) ν_{max}: 3408, 2927, 2843, 1743, 1655, 1462, 1444, 1325, 1227, 1165,

1063, 989, 881, 771, 683, 609 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) and ^{13}C NMR (125 MHz, acetone-d_6) spectral data: see Table 2; FAB-MS *m/z* (%): 635 (80) $[M + Na]^+$, 301 (10), 207 (10), 185 (5), 145 (5), 133 (15), 123 (8), 115 (100), 105 (25), 93 (35), 85 (8), 81 (15), 73 (25), 69 (10), 57 (20), 53 (8); HR-FAB-MS *m/z*: 635.3059 $[M + Na]^+$ (calcd for $C_{31}H_{48}O_{12}Na$, 635.3043).

3.4 Bioassays

Cells and culture condition and cell proliferation assay, see previous report [4].

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