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Two new bufadienolides from the rhizomes of *Helleborus thibetanus* with inhibitory activities against prostate cancer cells

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Two new bufadienolide glycosides (**1** and **2**) with an A/B *trans* ring fusion together with nine known compounds (**3**–**11**) were isolated from the rhizomes of *Helleborus thibetanus*. The structures of new compounds were elucidated by extensive spectroscopic analyses in combination with single-crystal X-ray diffraction. The bufadienolides **1** and **3**–**6** exhibited potent cytotoxic activities against the prostate cancer cells.

Keywords: *Helleborus thibetanus*; bufadienolide glycosides; X-ray crystallographic analyses; cytotoxic activity

1. Introduction

Helleborus thibetanus (Ranunculaceae family) is a Chinese special local plant (Tie-Kuai-Zi in Chinese), distributed along the Qinling Mountain, northwest of China. The rhizome part of this plant has been locally used for the treatment of asthma, cystitis, urethritis, sores and traumatic injury (Nanjing University of Traditional Chinese Medicine 2006). In recent years, the chemical constituents and biological activities of the *Helleborus* genus have attracted wide attention because of the existence of diverse array of natural products. Previous phytochemical investigations on the *Helleborus* distributed in Southeast Europe and West Asia have led to the isolation of bufadienolides (Watanabe et al. 2003; Bassarello et al. 2008), spirostanol saponins (Muzashvili et al. 2006; Bassarello et al. 2008), ecdysteriods (Akin & Anil 2007), furostanol saponins (Braca et al. 2004), phytosterol (Bingol & Sener 1986) and flavonoids (Males & Medic-Saric 2001). Pharmacological studies suggested that this plant possesses antitumour (Kerek 2004), antibacterial (Rosselli et al. 2007), immune-regulation (Littmann et al. 2008) and cytotoxic properties (Rosselli et al. 2009). Especially, the bufadienolides were found to exhibit potent positive iontropic, surface anaesthetic, blood pressure elevation properties and antitumour activities *in vitro* and *in vivo* (Gao et al. 2011).

During our continuing search for structurally unique and biologically interesting bufadienolides from the plant kingdom, two new bufadienolide glycosides **1** and **2**, together with nine known compounds **3**–**11** (Figure 1) were isolated from *H. thibetanus*, we reported the isolation, structural elucidation and inhibitory activities against the prostate cancer cells herein.

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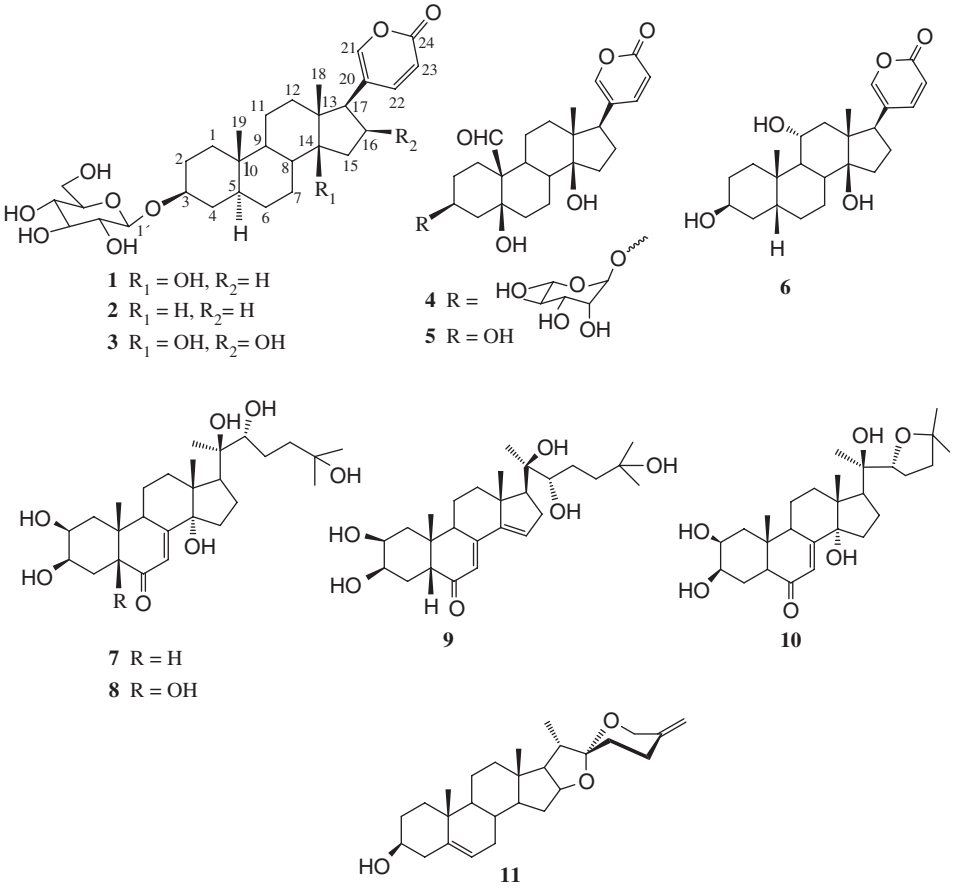

Figure 1. Structures of compounds **1**–**11**.

2. Results and discussion

Compound **1** was obtained as needle-shaped crystals from MeOH solution. The molecular formula was determined to be $C_{30}H_{44}O_9$ based on its HR-ESI-MS data (*m/z* 549.3063 $[M + H]^+$, calculated for 549.3058). Its IR spectrum revealed absorption bands for hydroxyl (3437 cm^{-1}) and carbonyl groups (1708 cm^{-1}). The ^1H NMR spectrum of **1** indicated the presence of a 2H-pyran-2-one unit [δ_H 6.38 (1H, d, $J = 9.9$ Hz, H-23), 7.49 (1H, br s, H-21) and 8.25 (1H, dd, $J = 2.4$, 9.9 Hz, H-22)], two angular methyl resonances at δ_H 0.65 (3H, s, H-19) and 0.89 (3H, s, H-18) and a number of saturated methine and methylene signals at δ_H 0.60–2.80. The ^{13}C NMR revealed a total of 30 carbon signals including 2 methyls, 10 methylenes, 13 methines and 5 quaternary atoms. The four olefinic carbon signals at δ_c 123.3 (C-20), 150.6 (C-21), 147.9 (C-22), 115.3 (C-23) and a carbonyl signal at δ_c 162.4 (C-24) were typical for a 2H-pyran-2-one moiety. These evidences indicated that compound **1** possessed a bufadienolide skeleton (Tian et al. 2010).

The five oxygenated signals δ_c 75.6, 78.9, 72.0, 78.7 and 62.9 together with the anomeric methine signal at δ_c 101.9 (C-1′) suggested the presence of one hexose moiety in the molecule of **1**, which was confirmed by the fragment ion at *m/z* 387 $[M + H\text{-}162]^+$ corresponding to the loss of glucosyl unit in the ESI-MS spectrum. The sugar moiety was further corroborated as D-glucose by co-TLC of the hydrolysed product with an authentic sample. Large coupling constant of the anomeric proton at δ_H 5.05 (1H, d, $J = 7.8$ Hz, H-1′) suggested the

β-configuration of the glycosidic bond. The HMBC (Supplementary Figure S13) correlation between H-1′ (δ_H 5.05, d, $J = 7.8$ Hz, H-1′) and C-3 (δ_c 77.3) confirmed that the glucose moiety was linked to the glycone at C-3. Similarly, HMBC correlations H-18 (δ_H 0.89), H-8 (δ_H 1.67) and H-15b (δ_H 1.86) to the oxygenated quaternary carbon C-14 (δ_c 84.7) confirmed that the hydroxyl group was attached at C-14.

The NMR spectra for the aglycone of compound **1** were very similar to that of bufalin; however, in the ROESY spectrum, the NOE correlations between H-3 (δ_H 4.04) and H-5 (δ_H 0.87) suggested that H-5 was α-oriented and the A/B ring fusion was *trans* (Supplementary Figure S13) because the α-oriented H-3 was commonly observed for all natural bufadienolides (Steyn & van Heerden 1998; Gao et al. 2011). Accordingly, compound **1** was identified as 14β-hydroxy-3β-(β-D-glucopyranosyloxy)-5α-bufa-20,22-dienolide.

The molecular structure and stereochemistry were further confirmed by X-ray crystallographic analysis (Figure 2), which clearly revealed a skeleton of bufadienolide glucoside. The asymmetric unit contained one molecule of **1** and two water molecules linked by hydrogen bonds. The glycone unit consisted of three cyclohexane rings (A, B and C), a five-membered ring (D) and a six-membered lactone ring (E). Rings A, B and C had normal chair conformations, ring D adopted an envelope conformation, while ring E was planar. The ring junctions were A/B *trans*, B/C *trans* and C/D *cis*. In addition, X-ray analysis confirmed the β-configuration of the glycosidic bond, α-oriented H-3, β-oriented 14-OH, and revealed that the glucose unit adopted a normal chair conformation. The small Flack parameter 0.0 (2) together with the known configurations at C-10 and C-17 indicated the absolute configuration of **1** as shown in Figure 2.

Compound **2**, an amorphous powder, exhibited HR-ESI-MS data (*m/z* 533.3109 $[M + H]^+$, calcd for 533.3109), corresponding to a molecular formula $C_{30}H_{44}O_8$ with one oxygen less than that of **1**. The ^1H, ^{13}C and DEPT NMR spectra of **2** were similar to those of **1**, except for the replacement of the oxygenated quaternary carbon atom (C-14) with a methine group (δ_c 51.6), indicating that **2** was the 14-dehydroxy analog of **1**. The full assignments of NMR data were achieved by extensive analyses of 2D spectra (COSY, HSQC, HMBC and ROESY) as shown in Supplementary Figure S14. Accordingly, the structure of **2** was established as 3β-(β-D-glucopyranosyloxy)-5α,14β-bufa-20,22-dienolide.

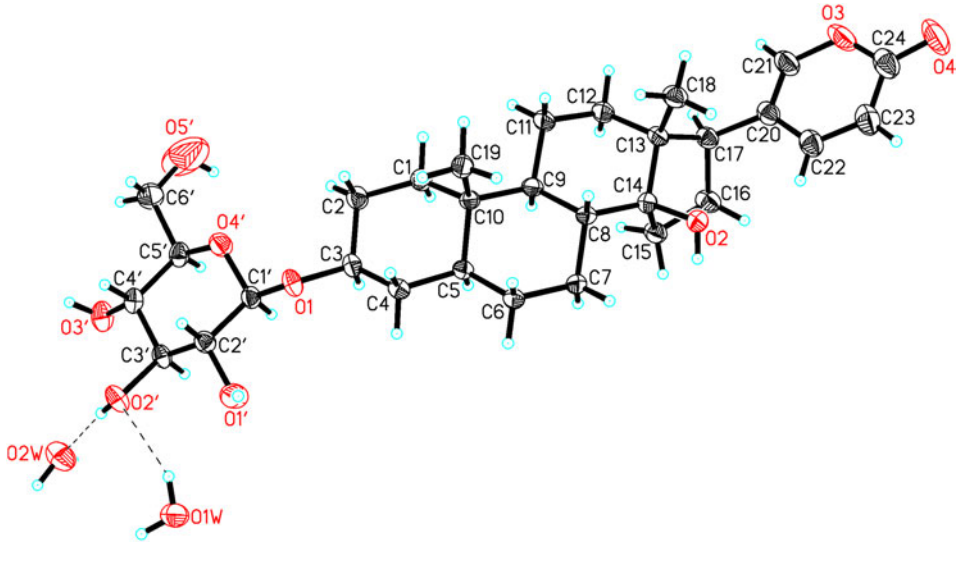

Figure 2. X-ray structure of **1** with atom labelling scheme. The dashed lines represent hydrogen bonds in the asymmetric unit.

The known compounds were identified by comparing their spectral data with reported values in the literatures, and identified to be 14β,16β-dihydroxy-3β-(β-D-glucopyranosyloxy)-5α-bufa-20,22-dienolide (**3**) (Yang et al. 2010), deglucohellebrin (**4**) (Watanabe et al. 2003), 3β,5β,14β-trihydroxy-19-oxo-bufa-20, 22-dienolide (**5**) (Watanabe et al. 2003), gamabufotalin (**6**) (Tashmukhamedov et al. 1995), 20-hydroxyecdysone (**7**) (Lafont et al. 2002), polypodine B (**8**) (Bassarello et al. 2008), stachysterone B (**9**) (Simon et al. 2007), shidasterone (**10**) (Sena Filho et al. 2008) and schidigeragenin A (**11**) (Petricic 1974), (Figure 1). Compounds **6**, **9** and **10** were isolated from *Helleborus* for the first time.

Bufadienolides have been found to exist in both animals and plants. In animals, all the bufadienolides demonstrate an A/B *cis* ring fusion. In the plant kingdom, bufadienolides are found in a number of families, for example Crassulaceae, Hyacinthaceae, Iridaceae, Melianthaceae, Ranunculaceae and Santalaceae. Unlike the animals, plant-derived bufadienolides have both A/B *cis* and A/B *trans* ring fusion modes (Yang et al. 2010). *Helleborus* is the only genus in Ranunculaceae family that was found to contain bufadienolides with both A/B *cis* and A/B *trans* ring fusion modes (Kren & Kopp 1998), which might serve as a chemotaxonomic marker for the genus *Helleborus*.

MTT colorimetric assay was performed to test the antitumour activities of **1**, **3**–**11** in prostate cancer cells (DU145 and PC3) as described earlier. As shown in Table 1, bufadienolides **1** and **3**–**6** exhibited potent cytotoxic effect against DU145 (IC_{50} values in the range 0.02–0.29 μM) and PC3 (IC_{50} values in the range 0.17–2.3 μM), which were comparable to the cytotoxic effects of similar bufadienolides from Venom of *Bufo bufo gargarizans* (Tian et al. 2013). It can be observed that the bufadienolide glycones **4**–**6** exhibited five fold more potent activities than those of bufadienolide glycosides **1** and **3**. The sterol **9** with a long conjugated system exhibited moderate cytotoxic effect with IC_{50} values of 15.0, 49.2 μM against the DU145 and PC3 cancer cells. In contrast, the sterols **7**, **8**, **10** and **11** were not active with IC_{50} values over 100 μM.

3. Experimental

3.1. *General experimental procedures*

Optical rotations were carried out using a JASCO P-1030 automatic digital polarimeter (JASCO Analytical Instruments, Easton, Maryland, USA). IR spectra were measured on a JASCO FT/IR-480 plus infrared spectrometer (JASCO Analytical Instruments, Easton, Maryland, USA) with KBr pellets. 1D and 2D NMR spectra were recorded on Bruker AV-400 spectrometer (Bruker Corporation, Billerica, Massachusetts, USA) with TMS as the internal standard, and chemical shifts were expressed in δ values (ppm). HR-ESI-MS data were detected on an Agilent 6210 LC/MSD TOF mass spectrometer (Agilent Technologies, Santa Clara, California, USA). Silica gel (200–300 mesh, Qingdao Marine Chemical, Inc., Qingdao city, China), ODS silica gel (50 μm, YMC, Kyoto, Japan) and Sephadex LH-20

Table 1. Inhibitory activities of compounds **1** and **3**–**11** against the prostate cancer cells (IC_{50} in μM).

No.	DU145 (μmol/L)	PC3 (μmol/L)	No.	DU145 (μmol/L)	PC3 (μmol/L)
1	0.15 ± 0.02	2.35 ± 0.17	**7**	>100	>100
3	0.29 ± 0.03	1.20 ± 0.14	**8**	>100	>100
4	0.02 ± 0.003	0.33 ± 0.02	**9**	14.85 ± 1.20	49.6 ± 2.6
5	0.06 ± 0.01	0.48 ± 0.03	**10**	>100	>100
6	0.03 ± 0.005	0.14 ± 0.05	**11**	>100	>100
Taxol	0.10 ± 0.01	0.12 ± 0.02			

(Pharmacia, Uppsala, Sweden) were used for column chromatography. Preparative HPLC was carried out on a WUFENG instrument equipped with a UV detector (WUFENG, Shanghai city, China) and a reverse phase C-18 column (5 μm, 20 × 250 mm; Cosmosil, Kyoto, Japan) TLC was performed using precoated silica gel plates (GF254, Yantai Institute of Chemical Industry, Yantai city, Shandong province, China). X-ray crystallographic data collection was performed on an Agilent Gemini S Ultra Sappharie CCD (Agilent Technologies, Yarnton, Oxfordshire, United Kingdom) using CuKα radiation.

3.2. *Plant material*

The herbal materials of *H. thibetanus* were collected in Taibai County, Shanxi Province, China in 2012. The roots were identified by Prof. Guang-Xiong Zhou (Jinan University, China). A voucher specimen (Tkz-001) has been deposited with the College of Pharmacy, Jinan University.

3.3. *Extraction and isolation*

The air-dried rhizomes of *H. thibetanus* (20.0 kg) were extracted twice using 95% EtOH and 60% EtOH, consecutively. The ethanol extract was evaporated to obtain a residue that was suspended in water and then extracted successively with petroleum ether, EtOAc and *n*-BuOH. The EtOAc extract (82.0 g) was subjected to silica gel column (200–300 mesh, 1.5 kg) eluting with gradient $CHCl_3$–MeOH (100:1 to 70:30) and 14 fractions (fractions 1–14) were obtained. Faction 4 (8.0 g) was chromatographed on a silica gel column (200–300 mesh, 165 g) eluted with cyclohexane–acetone (10:1 to 6:4) to afford seven subfractions (4_1–4_7). Subfraction 4_2 was subjected to an ODS column using gradient mixtures of MeOH–H_2O (70:30 to 95:5) as eluents to yield compounds **3** (7.2 mg) and **5** (10.0 mg). Fraction 6 (7.5 g) was chromatographed on a silica gel column (200–300 mesh, 160.0 g) eluted with $CHCl_3$–MeOH (15:1 to 7:3) to afford six subfractions (6_1–6_6). Subfraction 6_2 was purified by recrystallisation in acetone to yield compound **4** (30.0 mg). Subfraction 6_3 was subjected to Sephadex LH-20 ($CHCl_3$–MeOH, 1:1) chromatography followed by preparative HPLC (MeOH–H_2O, 60:40) to yield compounds **2** (5.3 mg) and **9** (15.0 mg). Compound **6** (18.0 mg) was obtained from subfraction 6_4 by recrystallisation. Similarly, fraction 8 (5.0 g) was fractionated by a silica gel column (200–300 mesh, 100.0 g) chromatography eluted with $CHCl_3$–MeOH (10:1 to 7:3) to afford five subfractions (8_1–8_5). Subfraction 8_3 was purified by RP-18 column (MeOH–H_2O, 45: 55 to 100% MeOH) followed by preparative HPLC (MeOH–H_2O, 50:50) to yield compounds **1** (13.0 mg), **11** (8.0 mg), **10** (12.0 mg) and **8** (8.5 mg). Fraction 10 (4.1 g) was subjected to an ODS column using gradient MeOH–H_2O (30:70 to 95:5) as eluents to afford six subfractions (10_1–10_6). Compound **7** (40.0 mg) was obtained from subfraction 10_4 by recrystallisation in methanol.

3.3.1. *14β-hydroxy-3β-(β-D-glucopyranosyloxy)-5α-bufa-20,22-dienolide (**1**)*

$C_{30}H_{44}O_9$, white needle crystal. HR-ESI-MS: (*m/z* 549.3063 $[M + H]^+$, calcd for 549.3058). ^1H NMR (C_5D_5N) δ: 7.49 (1H, br s, H-21), 8.25 (1H, dd, $J = 2.4$, 9.9 Hz, H-22), 6.38 (1H, d, $J = 9.9$ Hz, H-23), 0.89 (3H, s, H-18), 0.65 (3H, s, H-19), 1.85 (1H, m, H-17), 0.87 (1H, m, H-9), 1.67 (1H, m, H-8), 0.87 (1H, overlapped, H-5), 4.04 (1H, m, H-3), 0.86 (1H, overlapped, H-1a), 1.61 (1H, overlapped, H-1b), 1.64 (1H, m, H-2a), 2.16 (1H, overlapped, H-2b), 1.40 (1H, overlapped, H-4a), 1.86 (1H, m, H-4b), 0.97 (1H, m, H-6a), 1.23 (1H, m, H-6b), 1.10 (1H, overlapped, H-7a), 2.35 (1H, overlapped, H-7b), 1.15 (1H, m, H-11a), 1.39 (1H, m, H-11b), 1.26 (1H, overlapped, H-12a), 1.38 (1H, overlapped, H-12b), 1.99 (1H, m, H-15a), 1.86 (1H, overlapped, H-15b), 2.07 (1H, overlapped, H -16a), 1.86 (1H, m, H-16b), 5.05 (1H, d,

$J = 7.8$ Hz, H-1′), 3.98 (1H, m, H-2′), 4.29 (1H, dd, $J = 3.0$, 9.0 Hz, H-3′), 4.25 (1H, dd, $J = 3.0$, 9.0 Hz, H-4′), 4.01 (1H, m, H-5′), 4.61 (1H, brd, $J = 11.0$ Hz, H-6′a), 4.41 (1H, dd, $J = 11.5$, 5.5 Hz, H-6′b); ^{13}C NMR (C_5D_5N) δ: 37.6 (C-1), 30.1 (C-2), 77.3 (C-3), 34.8 (C-4), 44.4 (C-5), 29.4 (C-6), 28.3 (C-7), 42.3 (C-8), 50.2 (C-9), 36.2 (C-10), 22.0 (C-11), 40.8 (C-12), 49.0 (C-13), 84.7 (C-14), 32.9 (C-15), 29.8 (C-16), 51.6 (C-17), 17.5 (C-18), 12.4 (C-19), 123.3 (C-20), 150.6 (C-21), 147.9 (C-22), 115.3 (C-23), 162.4 (C-24), 101.9 (C-1′), 75.6 (C-2′), 78.9 (C-3′), 72.0 (C-4′), 78.7 (C-5′), 62.9 (C-6′).

3.3.2. *3β-(β-D-glucopyranosyloxy)-5α,14β-bufa-20,22-dienolide (**2**)*

$C_{30}H_{44}O_8$, amorphous white powder. HR-ESI-MS: (*m/z* 533.3109 $[M + H]^+$, calcd for 533.3109). ^{1}H NMR (C_5D_5N) δ: 7.49 (1H, br s, H-21), 7.31 (1H, dd, $J = 2.4$, 9.6 Hz, H-22), 6.44 (1H, d, $J = 9.6$ Hz, H-23), 0.65 (3H, s, H-18), 0.44 (3H, s, H-19), 1.16 (1H, m, H-17), 1.18 (1H, m, H-9), 1.25 (1H, m, H-8), 0.92 (1H, m, H-5), 4.05 (1H, m, H-3), 2.15 (1H, m, H-14), 0.92 (1H, overlapped, H-1a), 1.58 (1H, overlapped, H-1b), 1.68 (1H, m, H-2a), 2.12 (1H, overlapped, H-2b), 1.39 (1H, m, H-4a), 1.88 (1H, overlapped, H-4b), 1.15 (1H, m, H-6a), 1.76 (1H, m, H-6b), 1.16 (1H, overlapped, H-7a), 1.61 (1H, overlapped, H-7b), 1.20 (1H, overlapped, H-11a), 1.45 (1H, overlapped, H-11b), 0.99 (1H, m, H-12a), 1.45 (1H, m, H-12b), 2.10 (1H, overlapped, H-15a), 1.85 (1H, m, H-15b), 2.06 (1H, overlapped, H-16a), 1.82 (1H, m, H-16b), 5.07 (1H, d, $J = 7.8$ Hz, H-1′), 3.99 (1H, t, $J = 7.8$ Hz, H-2′), 4.27 (1H, dd, $J = 3.0$, 9.0 Hz, H-3′), 4.19 (1H, dd, $J = 3.0$, 9.0 Hz, H-4′), 3.97 (1H, m, H-5′), 4.56 (1H, dd, $J = 11.5$, 2.0 Hz, H-6′a), 4.41 (1H, dd, $J = 11.5$, 5.5 Hz, H-6′b); ^{13}C NMR (C_5D_5N) δ: 37.6 (C-1), 30.3 (C-2), 77.3 (C-3), 35.1 (C-4), 45.1 (C-5), 26.1 (C-6), 25.2 (C-7), 36.3 (C-8), 54.7 (C-9), 36.3 (C-10), 21.5 (C-11), 37.6 (C-12), 44.5 (C-13), 51.6 (C-14), 32.6 (C-15), 29.5 (C-16), 56.0 (C-17), 12.6 (C-18), 13.2 (C-19), 119.0 (C-20), 149.6 (C-21), 146.1 (C-22), 115.5 (C-23), 162.3 (C-24), 102.3 (C-1′), 75.7 (C-2′), 79.1 (C-3′), 72.2 (C-4′), 78.8 (C-5′), 63.4 (C-6′).

3.4. *Acid hydrolysis of 1 and 2*

Methanol solutions of **1** (5 mg) and **2** (4 mg) were added to 2 M HCl and refluxed for 2 h. After cooling, the reaction mixture was diluted with H_2O and extracted with ethyl acetate. The aqueous layer was neutralised with Na_2CO_3 and then concentrated to 2 mL. The D-glucose in the aqueous layer was identified by co-TLC with authentic sugar samples using $CHCl_3$–MeOH–H_2O (16:9:1) as the mobile phase.

3.5. *X-ray crystallographic analysis of 1*

Data were collected using an Agilent Sapphire CCD with a CuKα radiation, $\lambda = 1.54184$ Å at 173.0(2) K. Crystal data were $C_{30}H_{44}O_9{\cdot}2H_2O$, monoclinic, and space group $P2_12_12_1$; unit cell dimensions were determined to be $a = 6.34339(13)$ Å, $b = 21.4039(5)$ Å, $c = 21.9012(6)$ Å, $V = 2973.61(12)$ Å^3, $Z = 4$, $D_x = 1.306$ g/cm^3, F (000) = 1264, μ (CuKα) = 0.817 mm^{-1}. A total of 6375 reflections were collected until $\theta_{max} = 62.76°$, in which 3434 independent, reflections were observed [$F^2 > 4\sigma(F^2)$]. The structure was solved by direct methods using the SHELXS-97 program. The final refinement gave $R = 0.0510$, $R_W = 0.0640$ and $S = 1.074$. Crystal data of compound **1** have been deposited with the Cambridge Crystallographic Data Centre (CCDC 955228).

3.6. *MTT assay*

The MTT assay was carried out as described previously (Mosmann 1983), with taxol serving as the positive control. Briefly, the prostate cancer cells were seeded into the 96-well plates at a

density of 3×10^3 cells per well DU145 and PC3 cancer cell lines overnight. Then, the cells were treated with a series concentration of compounds **1** and **3**–**11** (compound **2** was not tested due to the limited sample). Following incubation for 48 h, 20 μL of the MTT solution (5 mg/mL in phosphate buffered saline) was added to each well, and the cells were further incubated for 4 h. Then, the medium was removed and replaced by 150 μL of DMSO in each well to dissolve the formazan crystals. The relative cell viability was determined by measuring the optical densities at 570 nm on microplate reader (SPECTRAmax 250, Molecular Devices, Sunnyvale, California, USA). The experiments were performed three times, each in triplicate.

Supplementary material

Supplementary material relating to this article is available online, alongside ^{1}H, ^{13}C, ^{1}H–^{1}H–COSY, HSQC, HMBC and ROESY spectra of compounds **1** and **2**.

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Note

1. These authors contributed equally to this work.

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