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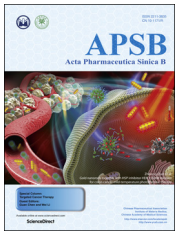

ORIGINAL ARTICLE

Limonoids from seeds of *Azadirachta indica* A. Juss. and their cytotoxic activity

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Abstract Four new limonoid-type nortriterpenoids, 1-detigloyl-1-*O*-methacryloylsalannin (**1**), 28-deoxo-2,3-dihydronimbolide (**2**), 12-acetoxy-3-*O*-acetyl-7-*O*-tigloylvilasinin (**3**) and 12-acetoxy-3-*O*-acetyl-7-*O*-methacryloylvilasinin (**4**), along with five known ones, were isolated from seeds of *Azadirachta indica* A. Juss. Their structures were elucidated by various spectroscopic methods, including UV, IR, MS, NMR, X-ray crystallography, quantum chemical calculation, as well as by comparison of their spectroscopic data with those reported. In the *in vitro* cytotoxic assay, **2** showed inhibitory activity against human breast cancer MDA-MB-231 cell line with IC_{50} value of 7.68 ± 1.74 μmol/L, and **5** inhibited growth of human cervical cancer Hela cell line, melanoma A375 cell line and promyelocytic leukemia HL-60 cell line, with IC_{50} 12.00 ± 2.08, 17.44 ± 2.11, and 13.95 ± 5.74 μmol/L, respectively.

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1. Introduction

Neem tree (*Azadirachta indica* A. Juss.) belonging to the meliaceae family is indigenous to India and Burma[1,2]. Neem has been used as a folk medicinal plant in India for over thousands of years. Almost all parts of this magical tree—leaves, stems, barks, roots, seeds, fruits, flowers, etc.—could be natural drugs in treatment of cough, wounds, leprosy, skin ulcers, intestinal worms, diabetes, cholera, diarrheal, etc.[1,3,4]. Therefore, neem received a bulk of attention of phytochemists and their efforts led to discovery of more than 300 compounds from this plant. Over 130 of these compounds belong to limonoid-type triterpenoids[5].

Limonoids are derived from apotirucallane- or apoeuphane-type triterpenoids, the side chains of which lose four terminal carbons and in the most cases form a furan ring. Over a thousand of limonoids have been found in nature. Due to their structural features, limonoids are classified into many subgroups, including ring intact limonoids, ring-seco limonoids, rearranged limonoids and limonoid derivatives[5]. Limonoids have shown a variety of activities, including anticancer[5], anti-inflammation[6,7], antimalaria[8], antibacteria[9], antiprotozoal[5], etc. Especially in the studies on anticancer activity of limonoids, many of them exhibited inhibitory activity against various human cancer cell lines. For examples, both 1-*O*-deacetylohchinolide B[10] and 15-*O*-deacetylnimbolindin B[11] can remarkably inhibit growth of HeLa S3 human cervical adenocarcinoma cells with IC_{50} of 0.10 μmol/L. Notably, cytotoxic mechanisms of nimbolide and azadirone, two main limonoid constituents in neem, were extensively studied. Nimbolide can inhibit cancer cell growth through inducing ROS-mediated apoptosis[12], inhibiting P13K/Akt signaling[13,14], and upregulating reversion-inducing cysteine-rich protein with Kazal motifs (RECK)[15]. Azadirone can enhance the efficacy of tumor necrosis factor-related apoptosis-inducing ligand against cancer cells[16]. Therefore, limonoids are a group of promising compounds for discovery of new anticancer agents. Herein, we report four new limonoids, together with five known ones (Fig. 1), from neem seeds and their cytotoxic activity.

2. Result and discussion

Compound **1** (Fig. 1) was obtained as a white amorphous powder with $[\alpha]_D^{25}$ +74 (*c* 0.10, MeOH). Its molecular formula of $C_{33}H_{42}O_9$ was determined by the quasi-molecular ion peak at *m/z* 605.2719 $[M + Na]^+$ (Calcd. 605.2721) observed in the HR-ESI-MS. The ^1H and ^{13}C NMR spectra of **1** showed a methacrylyl group [δ_H 6.21 (s, 1H, H-3′a), 5.60 (s, 1H, H-3′b) and 2.05 (s, 3H, H-4′); δ_C 166.1 (C-1′), 136.8 (C-2′), 125.6 (C-3′) and 18.1 (C-4′)], an acetyl group [δ_H 1.96 (s, 3H, 3-OCOMe); δ_C 170.4 (3-OCOMe) and 20.9 (3-OCOMe)] and an oxygen-bearing methyl group [δ_H 3.25 (s, 3H, 12-OMe) and δ_C 51.5 (12-OMe)], which were confirmed by the HMBC correlations from H-4′ to C-1′, C-2′ and C-3′, and from H-3-OCOMe to C-3-OCOMe. The rest NMR data, including a group of characteristic signals assigned to a *β*-substituted furan ring [δ_H 7.24 (brs, 1H, H-21), 6.28 (brs, 1H, H-22) and 7.31 (brs, 1H, H-23); δ_C 127.0 (C-20), 138.8 (C-21), 110.6 (C-22) and 142.9 (C-23)], are very similar to those of the limonoid skeleton of salannin[17,18].

The structure of **1** was finally constructed by its 2D NMR spectra. The proton and protonated carbon resonances in the NMR spectra of **1** were firstly assigned by the HSQC experiment. The ^1H–^1H COSY correlations H-1/H_2-2/H-3, H-5/H-6/H-7, H-9/H_2-11 and H-15/H_2-16/H-17 indicated four structural fragments labeled in red in Fig. 2. In the HMBC spectrum, the correlations from H_3-29 to C-3, C-4, C-5 and C-28, from H_3-19 to C-1, C-5, C-9 and C-10, from H_3-30 to C-7, C-8, C-9 and C-14, and from H_3-18 to C-13, C-14 and C-17 tethered four red fragments together with the blue ones shown in Fig. 2. The HMBC correlation of H-9/C-12, together with the chemical shift of C-12 indicated that C-12 was linked to C-11 and oxidized into a carboxyl group which was methylated due to the HMBC correlation between H-12-OMe and C-12. The methacrylyl group, acetyl group and furan ring located at C-1, C-3 and C-17, respectively, based on the HMBC correlations of H-1/C-1′, H-3/C-3-OCOMe, H-17/C-20, H-17/C-21 and H-17/C-22. Finally, two tetrahydrofuran rings were constructed by the correlations between H-28 and C-6, and between H-7 and C-15 in the HMBC spectrum. Therefore, the planar structure of **1** was elucidated.

The relative configuration of **1** was determined by its NOESY spectrum. The NOE correlations of H-6/H_3-19/H-1, H-6/H_3-29/H-1/H-3, H-6/H_3-30/H-7, H_3-19/H_2-11/H_3-30 and H_3-29/H-28*β* indicated these protons were oriented on the same side of the ring system and arbitrarily assigned as *β*. H-5 was assigned as *α* due to the correlation between it and H-28*α*. H-9 strongly correlated with H-15 in the NOESY experiment, whereas H-11 correlated with both H_3-19 and H_3-30, which indicated that H-9 and H-15 have *α* configurations. Finally, the NOE correlations from H-15 to H-22 indicated that the furan ring has an *α* orientation as well. The absolute configuration of **1** was finally determined by X-ray crystallography as shown in Fig. 3.

Compound **2** was obtained as a white amorphous powder with $[\alpha]_D^{25}$ +102 (*c* 0.20, MeOH). Its quasi-molecular ion peak at *m/z* 477.2240 $[M + Na]^+$ (Calcd. 477.2248) was observed in the HR-ESI-MS, which indicated its molecular formula was $C_{27}H_{34}O_6$. Except for a methoxyl group, there were 26 carbon signals in the ^{13}C NMR spectrum, including four sp^2 carbons [δ_C 126.9 (C-20), 138.9 (C-21), 110.5 (C-22) and 142.9 (C-23)] belonging to a furan ring, which implied that **2** was a limonoid-type nortriterpenoid as well. In the HMBC spectrum of **2**, H_3-19 (δ_H 1.22) correlated to a keto group with a chemical shift at δ_C 213.0,

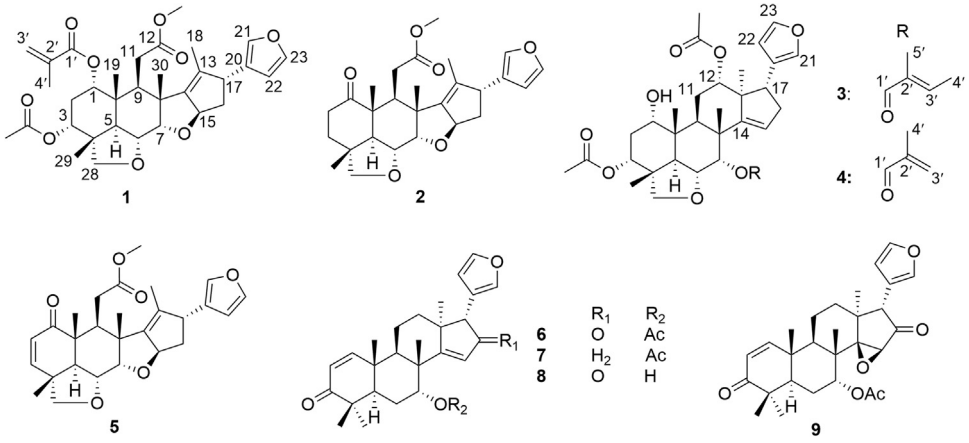

Figure 1 The structures of compounds **1**–**9**.

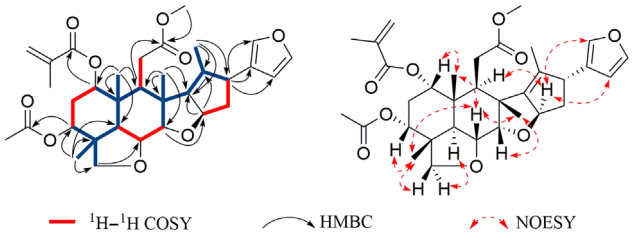

Figure 2 Selective 2D NMR correlations of compound **1**.

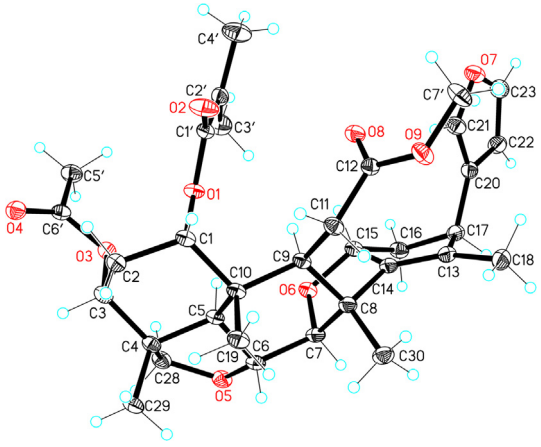

Figure 3 ORTEP drawing of the compound **1**.

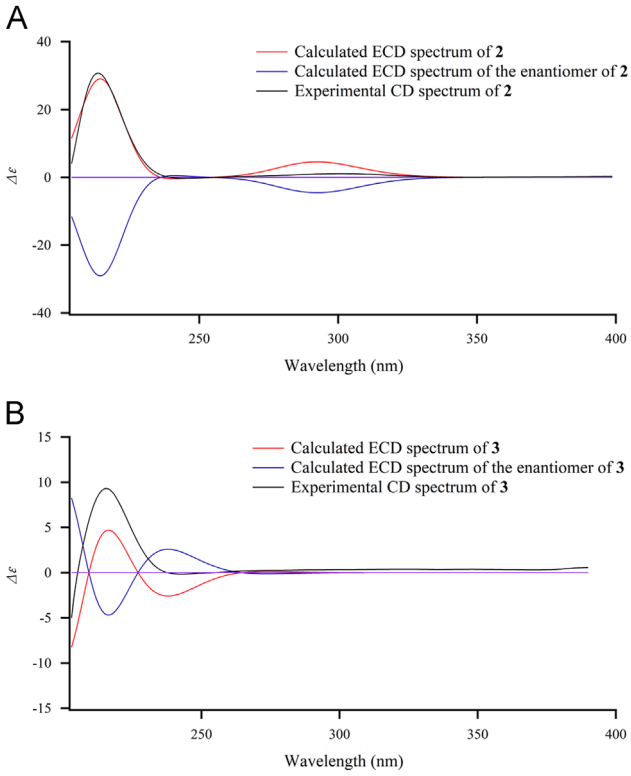

Figure 4 Experimental CD spectra of compounds **2** and **3** (black) overlaid with the calculated ECD spectra of **2** and **3** (red) and their enantiomers (blue).

indicating C-1 was oxidized into a keto group. Based on this clue, it was found that the NMR data of **2** were very similar to those of 2,3-dihydronimbolide[19] except that C-28 (a carbonyl carbon) of 2,3-dihydronimbolide was replaced by an isolated oxygen-bearing methylene [δ_H 3.53 (d, J=7.5 Hz, H-28α) and 3.75 (d, J=7.5 Hz, H-28β), and δ_C 82.8 (C-28)], implying C-28 was reduced into a methylene in **2**. This deduction was confirmed by the HMBC correlations from H_3-29 to C-3, C-4, C-5 and C-28, and from H-28α and 28β to C-5 and C-6. In the NOESY spectrum of **2**, the NOE correlations of H-11a/H_3-19/H_3-29/H-6/H_3-30/H-7/H-16β/H-17, as well as the correlations of H-5/H-9/H-15, indicated that **2** possessed the same relative configuration profile as that of 2,3-dihydronimbolide[19]. And the optical rotation of **2** is positive, which is also same as those of 2,3-dihydronimbolide[19], 28-deoxonimbolide[20] and nimbolide[20]. All these data implied that the absolute stereochemistry of **2** was consistent with those of the known nimbolide-type limonoids. Finally, the absolute stereochemistry of **2** was confirmed by comparison of its experimental CD spectrum with the calculated ones, as shown in Fig. 4. Therefore, the structure of **2** was determined as 28-deoxo-2,3-dihydronimbolide.

Compound **3** was obtained as a white amorphous powder with $[\alpha]_D^{25}$ –25 (c 0.10, MeOH). Its molecular formula, $C_{35}H_{46}O_9$, was also determined by the quasi-molecular ion peak at m/z 633.3032 shown in its HR-ESI-MS spectrum. The ^{1}H and ^{13}C NMR data of **3**, in combination with its HMBC spectrum, indicated that there were two acetyl [δ_H 2.05 (s, 3H), δ_C 169.3 and 21.1; δ_H 1.91, δ_C 171.2 and 21.6] and one tigloyl [δ_H 6.88 (q, J=7.0 Hz, H-3′), 1.79 (d, J=7.0 Hz, H_3-4′) and 1.86 (s, H_3-5′); δ_C 166.8 (C-1′), 128.9 (C-2′), 137.1 (C-3′), 14.7 (C-4′) and 12.4 (C-5′)] groups in **3**. The rest NMR data showed 26 carbons and 33 protons, including a set of the characteristic signals attributed to a furan ring, indicating a limonoid-type nortriterpenoid backbone, too. Comparison of NMR data between **3** and 1,3-diacetyl-12-hydroxy-7-tigloylvilasinin[21] showed the chemical shifts of C-1 and C-11 were shifted 1.0 and 5.7 ppm upfield in **3**, while those of C-2 and C-12 were shifted 2.8 and 1.9 ppm downfield, which indicated that 12-OH was acetylated in **3** instead of acetylation of 1-OH in 1,3-diacetyl-12-hydroxy-7-tigloylvilasinin. This deduction was confirmed by the HMBC correlation from H-12 to C-12-OCOMe. The relative configuration of **3** was approved the same as that of 1,3-diacetyl-12-hydroxy-7-tigloylvilasinin by the NOESY experiment in which the NOE correlations of H-3/H_3-29/H-6/H_3-19/H_3-30, H-1/H_3-19/H-11a and H-7/H_3-30/H-12 assigned them β orientation, while the correlations of H-5/H-9/H_3-18/H-22 indicated these protons and the furan ring possessed α orientation. Finally, the absolute stereochemistry of **3** was confirmed by comparison of its experimental CD spectrum with the calculated ones, as shown in Fig. 4.

Compound **4** was obtained as a white amorphous powder with $[\alpha]_D^{25}$ –87 (c 0.08, MeOH). Based on its HR-ESI-MS spectrum, the molecular formula of **4** was $C_{34}H_{44}O_9$ which was CH_2 less than that of **3**. And the NMR data of **3** and **4** were very similar, except for the presence of a methylacryloyl [δ_H 6.13 (brs, H-3a′), 5.54 (brs, H-3b′) and 1.97 (s, H_3-4′); δ_C 165.9 (C-1′), 136.7 (C-2′), 124.9 (C-3′) and 18.5 (C-4′)] in **4** instead of a tigloyl group in **3**, which was confirmed by the HMBC correlations from H-4′ to C-1′, C-2′ and C-3′. Therefore, **4** was determined as 12-acetoxy-3-*O*-acetyl-7-*O*-methacryloylvilasinin.

By comparison of the NMR data of five known compounds with those reported in the literature, they were identified as 28-deoxonimbolide (**5**)[20] azadiradione (**6**)[22], azadirone (**7**)[23], nimbocinol (**8**)[22] and epoxyazadiradione (**9**)[24], respectively.

In the *in vitro* cytotoxic assay, **2** showed inhibitory activity against MDA-MB-231 cell line with IC_{50} value of 7.68±1.74 μmol/L; and **5** inhibited growth of Hela cell line, A375 cell line and HL-60 cell line, with IC_{50} 12.00±2.08, 17.44±2.11 and 13.95±5.74 μmol/L, respectively. Cisplatin was used as the positive control. It inhibited growth of A375, Hela, HL-60, MDA-MB-231, HepG2, K562 and MCF-7 cells with IC_{50} of 2.50±0.50, 8.96±1.59, 1.54±0.38, 1.70±0.43, 2.46±0.14, 8.17±2.31 and 11.43±2.79 μmol/L, respectively.

3. Experimental section

3.1. General experimental procedures

Optical rotations were measured with a JASCO P-2000 polarimeter (Jasco, Tokyo, Japan). UV spectra were recorded on a JASCO V-550 spectrophotometer (Jasco, Tokyo, Japan). IR spectra were performed on a FT/IR-6600 spectrometer (Jasco, Tokyo, Japan). NMR spectra were acquired on a Bruker Avance III-600 and a Bruker Avance III-300 instruments (Bruker, Bremerhaven, Germany). High-resolution mass spectra were obtained on a LCQ Advantage MAX (Finnign, USA). CD spectrum was measured on a Chirascan spectropolarimeter (Applied Photophysics, Ltd.). X-ray Crystallography was collected at 100 K on a Rigaku Oxford Diffraction Supernova Dual Source, Cu at Zero equipped with an AtlasS2 CCD using Cu Kα radiation. Silica gel (80−100 and 200−300 mesh, Qingdao Haiyang, Qingdao, China), Sephadex LH-20 (Pharmadex), and RP-C18 (AA12S50, YMC) were used for column chromatography. Preparative HPLC was carried out using an Ultimate 3000 instrument (Thermo Scientific, USA) with a Waters XBridge RP-C_{18} column (250 mm × 10 mm). Analytical HPLC was run on using an Agilent 1260 instrument (Agilent, USA) with a Phenomenex Synergi RP-C18 column (250 mm × 4.6 mm).

3.2. Botanical material

The seeds of *Azadirachta indica* A. Juss. were collected from Yunnan province and were authenticated by Prof. Hanhong Xu, College of Resource and Environmental Engineering, South China Agricultural University. A voucher specimen was deposited in the College of Pharmacy, Jinan University.

3.3. Extraction and isolation

The air-dried, powdered seeds of *A. indica* (30 kg) were macerated for 12 h with 150 L of 95% EtOH and refluxed for 2 h in twice. Then the combined EtOH extracts were concentrated under reduced pressure to give a crude residue (2.5 kg). The crude extract was suspended in 8 L distilled H_2O and partitioned with petroleum ether (PE), EtOAc and *n*-BuOH (3 × 8 L for each solvent). After removal of the solvent under vacuum, the EtOAc (431 g) fraction was subjected to a silica gel column chromatography eluted with a gradient of increasing acetone (0–50%) in PE, followed by a gradient of increasing MeOH (5%–100%) in $CHCl_3$, to afford 18 fractions (Fr.1–18). Fr.10 (40 g) was chromatographed on a silica gel column with increasing amounts of EtOAc:PE (1:10–1:0) to provide 26 subfractions (Fr.10a–10z). Fr.10t (2.6 g) was further fractionated *via* an ODS column (RP-18, AA12S50, 200 g) by eluting with a gradient of MeOH (55%–65%–75%–85%–100%) in H_2O to obtain 30 sub-sub-fractions (Fr.10t1–Fr.10t30). Fr.10t10 (276 mg) was purified by reversed-phase semi-preparative HPLC (4 mL/min, 53% MeOH) to obtain compounds **5** (28 mg, t_R 23.35 min), **2** (10 mg, t_R 30.16 min) and **1** (19 mg, t_R 46.56 min). Fr.10t14 (53 mg) was purified by semi-preparative HPLC (4 mL/min, 59% MeOH in H_2O) to yield compounds **3** (3 mg, t_R 55.58 min) and **4** (2 mg, t_R 46.30 min). Fr.10w (1.1 g) was separated by Sephadex LH-20 to afford 6 sub-sub-fractions (Fr.10w1–Fr.10w6), using PE:CH_2Cl_2:MeOH (5:4:1) as mobile phase. Fr.10w1 and Fr.10w5 were two pure compounds, *i.e.* compounds **8** (16 mg) and **6** (24 mg), respectively; Fr.10w2 (108 mg) was purified by semi-preparative HPLC (4 mL/min, 50% MeOH in H_2O) to yield compounds **9** (13 mg, t_R 21.37 min) and **7** (4 mg, t_R 41.54 min).

1-*O*-Detigloyl-1-*O*-methacryloylsalannin (**1**): white amorphous powder; $[\alpha]_D^{25}$ +74 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (logε) 206.5 (4.12) nm; IR (KBr) ν_{max} 2981, 2954, 2873, 1727, 1633, 1434, 1369, 1291, 1242, 1167, 1050, 949, 874 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HR-ESI-MS *m/z* 605.2719 $[M + Na]^+$ (Calcd. for $C_{33}H_{42}O_9Na$, 605.2721).

28-Deoxo-2,3-dihydronimbolide (**2**): white amorphous powder; $[\alpha]_D^{25}$ +102 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (logε) 205 (4.36) nm; IR (KBr) ν_{max} 2947, 2875, 1736, 1709, 1458, 1434, 1209, 1162, 1054, 1033, 950, 754 cm^{-1}; CD (*c* 0.22 mmol/L, MeOH) λ ($\Delta\varepsilon$) 208 (+37.52), 198 (–28.07), 192 (–15.00) nm; ^1H and ^{13}C NMR data, see Table 1; HR-ESI-MS *m/z* 477.2240 $[M + Na]^+$ (Calcd. for $C_{27}H_{34}O_6Na$, 477.2248).

12-Acetoxy-3-*O*-acetyl-7-*O*-tigloylvilasinin (**3**): white amorphous powder; $[\alpha]_D^{25}$ –25 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (logε) 204 (4.91), 221 (4.54) nm; IR (KBr) ν_{max} 3582, 2972, 2924, 2851, 1730, 1709, 1648, 1376, 1248, 1137, 1081, 1036, 876 cm^{-1}; CD (*c* 0.08 mmol/L, MeOH) λ ($\Delta\varepsilon$) 214 (+10.33), 196 (−49.14), 192 (−23.43) nm; ^1H and ^{13}C NMR data, see Table 1; HR-ESI-MS *m/z* 633.3032 $[M + Na]^+$ (Calcd. for $C_{35}H_{46}O_9Na$ 633.3034).

12-Acetoxy-3-*O*-acetyl-7-*O*-methacryloylvilasinin (**4**): white amorphous powder; $[\alpha]_D^{25}$ –87 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (logε) 204 (4.30) nm; IR (KBr) ν_{max} 3568, 2973, 2928, 2888, 2842, 1727, 1373, 1245, 1161, 1079, 1027 cm^{-1}; CD (*c* 0.08 mmol/L, MeOH) λ ($\Delta\varepsilon$) 214 (+6.08), 199 (−47.52) nm; ^1H and ^{13}C NMR data, see Table 1; HR-ESI-MS *m/z* 619.2880 $[M + Na]^+$ (Calcd. for $C_{34}H_{44}O_9Na$ 619.2878).

3.4. Quantum chemical calculation

Conformational analysis was carried out by using the MMFF94 molecular mechanics force field *via* the MOE software package as previously reported[25]. All quantum computations were performed using Gaussian 09 program package, on an IBM cluster machine. Conductor-like polarizable continuum model (CPCM) was adopted to consider solvent effects using the dielectric constant of MeOH (ε=32.6).

3.5. X-ray crystallography

The data of **1** was collected at 100 K on a Rigaku Oxford Diffraction Supernova Dual Source, Cu at Zero equipped with an AtlasS2 CCD using Cu Kα radiation. Data reduction was carried out with the diffractometer's software[26]. The structures were solved by direct methods using Olex2 software[27], and the non-hydrogen atoms were located from the trial structure and then refined anisotropically with SHELXL-2014[28] using a full-matrix least squares procedure based on F^2. The weighted *R* factor, *wR* and goodness-of-fit *S* values were obtained based on F^2. The hydrogen atom positions were fixed geometrically at the calculated distances and allowed to ride on their parent atoms. Crystallographic data for the structure reported in this paper have been deposited at the Cambridge Crystallographic Data Center and allocated with the deposition numbers: CCDC 1589663.

Table 1 NMR data of compounds **1–4** in $CDCl_3$ (δ in ppm, J in Hz).

Position	**1**[a]		**2**[a]		**3**[a]		**4**[b]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	4.79 (t, 2.7)	71.6		213.0	3.50 (t, 2.7)	72.1	3.50 (t, 2.7)	71.8
2α	2.21 (m, 2H)	27.6	2.24 (m)	34.7	2.00 (dt, 16.3, 2.8)	30.3	2.00 (dt, 16.3, 2.8)	30.0
2β			2.85 (td, 14.5, 6.1)		2.31 (dt, 16.2, 3.2)		2.31 (dt, 16.2, 3.2)	
3α	4.96 (t, 2.7)	71.2	1.83 (td, 13.0, 4.4)	35.8	5.09 (t, 2.7)	73.9	5.09 (t, 3.2)	73.6
3β			1.94 (dd, 13.0, 6.1)					
4		42.7		39.3		42.5		42.3
5	2.78 (d, 12.6)	39.9	2.36 (d, 12.0)	52.2	2.40 (d, 12.4)	40.6	2.40 (d, 12.4)	40.3
6	3.98 (dd, 12.6, 3.2)	72.6	4.09 (dd, 12.0, 2.7)	72.3	4.20 (dd, 12.4, 2.9)	72.9	4.22 (d, 2.9)	72.7
7	4.16 (d, 3.2)	85.5	4.13 (d, 2.7)	85.3	5.67 (d, 2.9)	73.7	5.65 (d, 2.9)	73.9
8		49.1		49.9		44.4		44.1
9	2.73 (dd, 8.4, 4.0)	39.4	2.59 (t, 5.1)	41.1	2.98 (dd, 8.4, 4.0)	36.4	2.98 (dd, 8.4, 4.0)	36.1
10		40.5		50.7		40.4		40.1
11a	2.30 (m)	30.6	2.79 (dd, 15.5, 5.1)	33.1	2.21 (m)	24.5	2.21 (m)	24.2
11b	2.19 (m)		2.29 (dd, 15.5, 5.1)		1.46 (m)		1.46 (m)	
12		172.8		173.4	5.05 (dd, 9.1, 7.3)	78.14	5.05 (dd, 9.1, 7.3)	77.9
13		135.0		134.8		51.9		51.6
14		146.4		146.1		155.9		155.6
15	5.42 (t, 7.2)	87.9	5.49 (t, 6.7)	87.8	5.62 (t, 2.4)	122.9	5.63 (t, 2.6)	122.7
16α	2.22 (m)	41.3	2.19 (dd, 12.1, 6.6)	41.4	2.36 (m, 2H)	36.8	2.38 (m, 2H)	36.6
16β	2.11 (m)		2.11 (m)					
17	3.62 (d, 8.8)	49.4	3.63 (d, 8.7)	49.4	2.96 (d, 8.8)	50.6	2.96 (d, 8.8)	50.3
18	1.65 (s, 3H)	13.0	1.67 (s, 3H)	12.9	1.01 (s, 3H)	15.9	1.02 (s, 3H)	15.7
19	0.98 (s, 3H)	15.1	1.22 (s, 3H)	14.5	0.96 (s, 3H)	15.6	0.96 (s, 3H)	15.3
20		127.0		126.9		124.9		124.7
21	7.24 (br s)	138.8	7.24 (br s)	138.9	7.19 (br s)	140.5	7.19 (br s)	140.2
22	6.28 (br s)	110.6	6.33 (br s)	110.5	6.23 (br s)	112.1	6.23 (br s)	111.8
23	7.31 (br s)	142.9	7.31 (br s)	142.9	7.31 (br s)	142.1	7.31 (br s)	141.9
28α	3.66 (d, 7.5)	77.6	3.53 (d, 7.5)	82.8	3.27 (d, 7.6)	78.11	3.27 (d, 7.7)	77.8
28β	3.57 (d, 7.5)		3.75 (d, 7.5)		3.50 (d, 7.6)		3.50 (m)	
29	1.21 (s, 3H)	19.5	1.38 (s, 3H)	18.9	1.16 (s, 3H)	19.1	1.17 (s, 3H)	18.8
30	1.29 (s, 3H)	16.9	1.25 (s, 3H)	17.3	1.18 (s, 3H)	27.3	1.19 (s, 3H)	27.0
1′		166.1				166.8		165.9
2′		136.8				128.9		136.7
3′a	6.21 (s)	125.6			6.88 (q, 7.0)	137.1	6.13 (br s)	124.9
3′b	5.60 (s)						5.54 (br s)	
4′	2.05 (s, 3H)	18.1			1.79 (d, 7.0, 3H)	14.7	1.97 (s, 3H)	18.5
5′					1.86 (s, 3H)	12.4		
3-OCOMe		170.4				169.3		169.1
3-OCOMe	1.96 (s, 3H)	20.9			2.05 (s, 3H)	21.1	2.05 (s, 3H)	20.9
12-OCOMe						171.2		171.0
12-OCOMe					1.91 (s, 3H)	21.6	1.91 (s, 3H)	21.3
12-OMe	3.25 (s, 3H)	51.5	3.55 (s, 3H)	51.6				

[a]600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR;
[b]300 MHz for ^1H NMR and 75 MHz for ^{13}C NMR.

3.6. *Cytotoxic assay*

Human breast cancer cell line (MCF-7), human malignant melanoma cell line (A375), human liver carcinoma cell line (HepG2), human Caucasian chronic myelogenous leukaemia cell line (K562), human promyelocytic leukemia cell line (HL-60), human breast cancer MDA-MB-231 cell line and human cervix epithelioid carcinoma cell line (HeLa) were provided by Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China. As previously reported[29], all cells were cultured in RPMI 1640 medium supplemented with 10% FBS and antibiotics (Penicillin 100 IU/mL, Streptomycin 100 μg/mL), and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cisplatin was used as the positive control.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.apsb.2017.12.009.

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