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NOTE

Chemical constituents from *Ginkgo biloba* leaves and their cytotoxicity activity

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Abstract
One novel neoligan glucoside, Ginkgoside B (**1**), and one new glucose ester, 6-*O*-(4-hydroxyhydrocinnamoyl)-D-glucopyranose (**2**), along with nine known compounds (**3**–**11**) were isolated from the ethanol extract of *Ginkgo biloba* leaves. Their structures were elucidated by combination of spectroscopic analyses and alkaline methanolysis. The absolute configuration of compound **1** was determined by single-crystal X-ray diffraction. All the isolated compounds were evaluated for their cytotoxicity activities, and compound **11** exhibited IC_{50} values of 36.20 and 58.95 μM against 5637 and HeLa cell lines, respectively.

Keywords *Ginkgo biloba* · Alkaline methanolysis · X-ray diffraction · Cytotoxicity

Introduction

Extracts of *Ginkgo biloba* (EGb) are commonly used in traditional Chinese medicine to treat bronchial asthma, stroke, hypertension, hypercholesterolemia, and cerebrovascular diseases [1, 2]. Previous investigations also revealed great potential of EGb in anti-tumor agents development [3, 4]. However, the underlying pharmacodyamic material basis of EGb involved in anti-tumor activity has not been elucidated in detail. In a search for chemical constituents against tumors, the extracts of *Ginkgo biloba* were investigated, leading to the isolation of two new compounds (**1** and **2**) and nine known compounds (**3**–**11**) (Fig. 1). In the subsequent cytotoxicity assay, compound **11** exhibited moderated cytotoxicity against two tumor cell lines.

Results and discussion

Compound **1** was obtained as colorless crystals. The molecular formula $C_{24}H_{28}O_{11}$, with eleven unsaturations, was established based on its quasi-molecular ion peak at *m/z* 515.1526 $[M+Na]^+$ (calcd for $C_{24}H_{28}O_{11}Na$, 515.1529) in the HRESIMS spectrum. The ^1H NMR spectrum of **1** revealed the existence of five aromatic protons, including an ABX spin system at δ 6.85 (dd, $J=8.0$, 1.2 Hz, H-6), 6.81 (d, $J=1.2$ Hz, H-2), 6.77 (d, $J=8.0$ Hz, H-5), and an AB spin system at 7.01 (d, $J=1.2$ Hz, H-2′), 6.92 (d, $J=1.2$ Hz, H-6′) (Table 1). Moreover, the ^1H NMR spectrum showed that resonances ascribe to a methoxyl at δ 3.88 (3H, s), two hydroxymethyls at δ 4.84 (1H, d, $J=11.2$ Hz, H-7′a), 4.62 (1H, d, $J=11.2$ Hz, H-7′b), 3.81 (1H, dd, $J=10.8$, 4.4 Hz, H-9a), 3.72 (1H, dd, $J=10.8$, 7.2 Hz, H-9b), two methines at δ 5.54 (1H, d, $J=5.2$ Hz, H-7), 3.43 (1H, ddd, $J=7.2$, 5.2, 4.4 Hz, H-8), along with a $-OCH_2O-$ group at δ 5.93 (2H, s). In turn, the ^{13}C NMR and DEPT spectra showed resonances for 24 carbons: seven olefinic quaternary carbons (including four oxygenated ones), seven methines (five olefinic and one oxygenated), three oxygenated methylenes, and one methoxyl,

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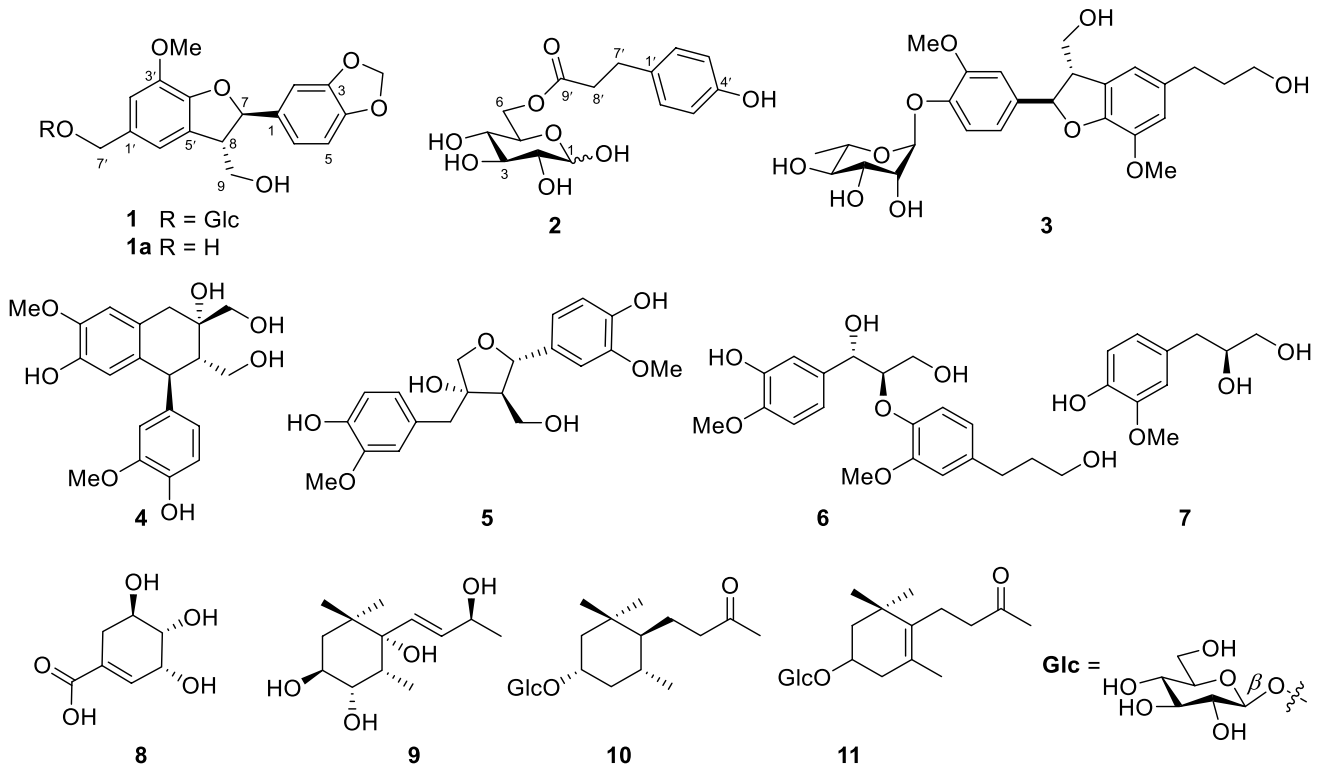

Fig. 1 Structures of compounds **1**–**11**

Table 1 ^{1}H NMR and ^{13}C NMR (CD_3OD) of compound **1**

Position	δ_C	δ_H (mult, *J* in Hz)	Position	δ_C	δ_H (mult, *J* in Hz)
1	137.5		4′	149.2	
2	107.1	6.81 (d, 1.2)	5′	129.6	
3	149.5		6′	118.6	6.92 (d, 1.2)
4	149.0		7′	72.0	4.84 (d, 11.2) 4.62 (d, 11.2)
5	109.2	6.77 (d, 8.0)	3′-OMe	56.9	3.88 (s)
6	120.4	6.85 (dd, 8.0, 1.2)	OCH_2O	102.6	5.93 (s)
7	89.0	5.54 (d, 5.2)	1″	103.0	4.34 (d, 7.6)
8	55.7	3.43 (ddd, 7.2, 5.2, 4.4)	2″	75.3	3.23 (d, 8.4, 7.6)
9	65.0	3.81 (dd, 10.8, 4.4) 3.72 (dd, 10.8, 7.2)	3″	78.2	3.25 (overlap)
1′	132.6		4″	71.9	3.29 (overlap)
2′	114.5	7.01 (d, 1.2)	5″	78.3	3.32 (overlap)
3′	145.5		6″	63.0	3.88 (dd, 12.0, 1.2) 3.67 (dd, 12.0, 4.4)

of which 16 were assigned to the dihydrobenzofuran-type norneolignan skeleton, and the remaining were ascribed to a methylenedioxy, a methoxyl and a sugar moiety. The speculations above were confirmed by the key $^{1}H-{}^{1}H$ COSY, HSQC and HMBC correlations shown in Fig. 2. The sugar moiety was established as *β*-glucose ($J_{1'',2''}$ = 7.6 Hz) and confirmed as the D-sugar by subsequent enzymatic hydrolysis. The HMBC correlations from H-1″ to C-7′ indicated that O-glycosylation should occur at C-7′. The coupling constant between H-7 and H-8 ($J_{7,8}$ = 5.2 Hz) suggested the 7,8-*trans* pattern [5]. This arrangement was further validated by the NOESY correlations between H-7 and H-9, and between H-8 and H-6, H-6′ (Fig. 2).

In addition, the absolute configurations of C-7 and C-8 were proved to be 7*R*, 8*S* by single-crystal X-ray diffraction analysis using the anomalous scattering of Cu K*α*

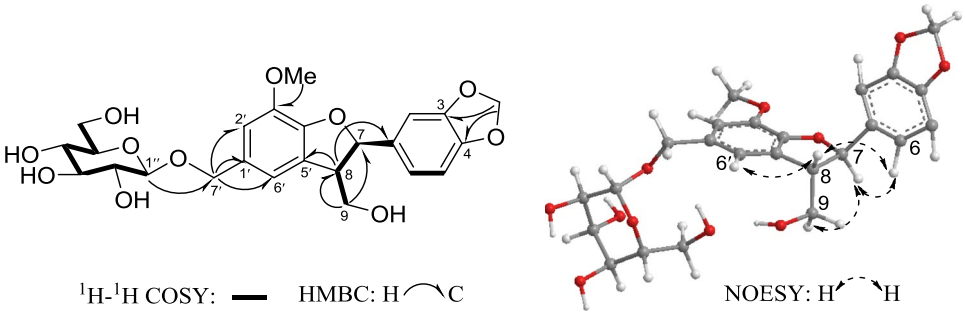

Fig. 2 Key ^{1}H–^{1}H COSY, HMBC and NOESY correlations for compound **1**

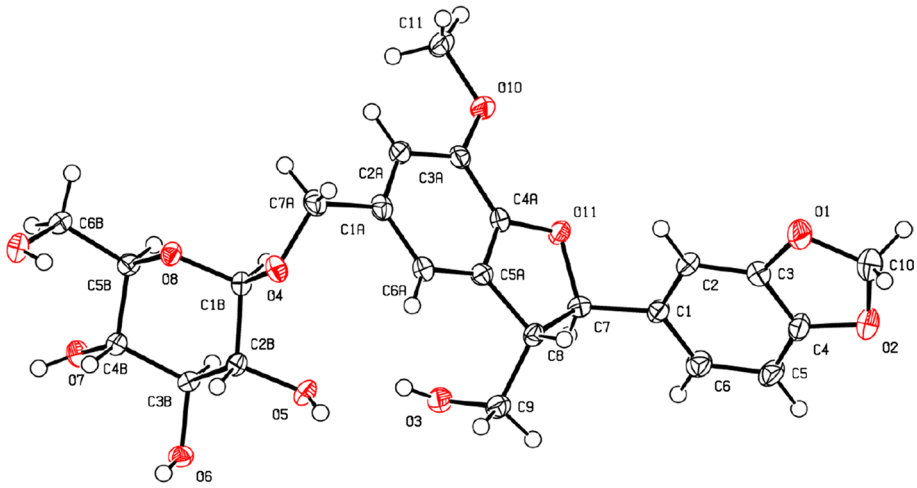

Fig. 3 ORTEP drawing of compound **1**

radiation (Fig. 3). Therefore, the structure of compound **1** was identified as (7*R*,8*S*)-3′ -methoxy-7′,9-dihydroxy-3,4-methylenedioxy-4′,7-epoxy-8′,9′-dinor-8-5′-neolign-7′-ol-7′-*O*-*β*-D-glucopyranoside, named as Ginkgoside B.

Compound **2** was obtained as a white powder. Its molecular formula was established as $C_{15}H_{20}O_8$ based on the quasi-molecular ion peak at *m/z* 351.1064 $[M–H]^-$ (calcd for $C_{15}H_{19}O_8$, 351.1056) in the HRESIMS spectrum, implying six degrees of unsaturation.

The ^{1}H NMR spectrum of **2** exhibited a series of proton signals ranging from 3.0 to 5.1 ppm and the ^{13}C NMR spectrum exhibited corresponding carbon signals ranging from 65.0 to 98.3, which suggested the presence of a sugar moiety (Table 2). The dual peaks in ^{1}H NMR spectrum of **2** indicated the presence of both the *α*- and *β*-anomers, with a ratio of 1:1. Four doublet proton signals of aromatic protons at *δ* 7.02 (2H, d, 8.0 Hz, H-2′, H-6′) and 6.69 (2H, d, 8.0 Hz, H-3′, H-5′) suggested the presence of one *para*-disubstituted benzene group in the structure of compound **2**. Its ^{13}C NMR and DEPT spectra showed the presence of six aromatic carbons (two signals at *δ* 130.4 and 116.3 appearing as double intensity), one quaternary carbons (*δ* 174.8), two methylene carbons (*δ* 37.3, 31.2), which indicated the presence of 4-hydroxy-hydrocinnamoyl group. The HMBC correlations from H-6 to C-9′ indicated that the 4-hydroxyphenyl propionic acid was attached to C-6 position of the sugar moiety (Fig. 4). On alkaline methanolysis with sodium methoxide in methanol, **2** afforded D-glucose and 4-hydroxyphenyl propionic acid. Therefore, compound **2** was determined as 6-*O*-4-hydroxy-hydrocinnamoyl-D-glucopyranose.

Table 2 ^{1}H NMR and ^{13}C NMR (CD_3OD) of compound **2**

Position	*α*-isomer of **2**		*β*-isomer of **2**	
	δ_C	δ_H (mult, *J* in Hz)	δ_C	δ_H (mult, *J* in Hz)
1	94.1	5.09 (d, 3.2)	98.3	4.48 (d, 8.0)
2	73.8	3.36 (dd, 8.0, 3.2)	76.3	3.14 (dd, 8.0, 8.0)
3	74.8	3.67 (t, 9.2)	78.0	3.34 (dd, 9.2, 8.0)
4	71.8	3.30 (dd, 9.6, 9.2)	70.7	3.96 (dd, 9.6, 9.2)
5	72.0	3.28 (ddd, 9.6, 5.6, 1.6)	75.4	3.46 (ddd, 9.6, 6.0, 1.6)
6	65.0	4.39 (dd, 12.0, 1.6) 4.18 (dd, 12.0, 5.6)	65.0	4.34 (dd, 12.0, 1.6) 4.15 (dd, 12.0, 6.0)
1′	132.9		132.9	
2′, 6′	130.4	7.02 (d, 8.0)	130.4	7.02 (d, 8.0)
3′, 5′	116.3	6.69 (d, 8.0)	116.3	6.69 (d, 8.0)
4′	156.9		156.9	
7′	31.2	2.82 (t, 7.2)	31.2	2.82 (t, 7.2)
8′	37.3	2.58 (t, 7.2)	37.3	2.58 (t, 7.2)
9′	174.9		174.9	

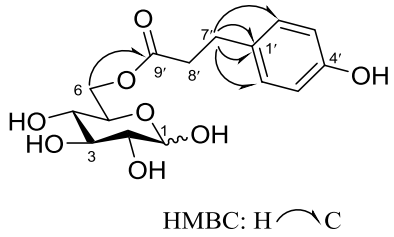

Fig. 4 Key HMBC correlations for compound **2**

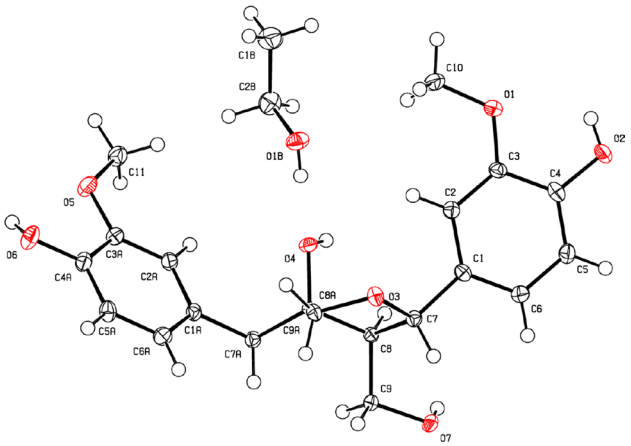

Fig. 5 ORTEP drawing of compound **5**

Based on the NMR data analysis and comparison with the reported spectroscopic data, compounds **3** – **11** were identified as icariside E (**3**) [6] (+)-cycloolivil (**4**) [7] (-)-olivil (**5**) [8], erythro-(7*S*,8*R*)-guaiacyl-glycerol-*β*-*O*-4′-dihydroconiferyl ether (**6**) [9], 2*S*-3-(4-hydroxy-3-methoxyphenyl)propane-1,2-diol (**7**) [10], shikimic acid (**8**) [11], wilsonol A (**9**) [12], alatoside E (**10**) [13], and icariside B_6 (**11**) [14], respectively. Compounds **1** – **3**, **6** – **7** and **9** – **11** were reported from *Ginkgo biloba* for the first time. So far, there is no report about (−)-olivil (**5**) of its crystal data due to the physical properties, although it has been isolated from various plants since 1927 [15]. In this study, compound **5** was recrystallized carefully in ethanol containing a small amount of $CHCl_3$ and the crystal was subjected to single-crystal X-ray diffraction analysis with Cu K*α* radiation. The absolute configuration of compound **5** was assigned as 7*S*, 8*R*, 8′*S* by the calculated Flack parameter of 0.19(9), which was consistent with the previously reported result (Fig. 5) [16].

Compounds **1**–**11** were evaluated for their cytotoxicity against two human cancer cell lines, including 5637 and HeLa cells in vitro. Results showed that compound **11** displayed moderated activity with the IC_{50} values of 36.20 and 58.95 μM, respectively, while the *cis*-platin (positive control) exhibited IC_{50} values of 14.83 and 19.38 μM. The other compounds showed no significant cytotoxicity ($IC_{50} > 100$ μM). The cytotoxicity mechanism of **11** might be similar to that of lauroside B [17], a megastigmane glycoside obtained from *Laurus nobilis* leaves, which was reported to suppress the proliferation of human melanoma cell lines via inhibition of NF-*κ*B activation.

Experimental section

General experimental procedures

Melting points (uncorrected) were measured on a SGW X-4A microscopic melting point apparatus. Optical rotations were determined on a Rudolph Autopol IV polarimeter. UV and FT-IR spectra were determined using Puxi TU-1950 and FTIR-650 instruments, respectively. NMR spectra were recorded on a Bruker AM-400 spectrometer and the ^1H and ^{13}C NMR chemical shifts were referenced to the solvent or solvent impurity peaks for CD_3OD at δ_H 3.31 and δ_C 49.15, for $CDCl_3$ at δ_H 7.24 and δ_C 77.23. High-resolution electrospray ionization mass spectra (HRESIMS) were carried out on a Waters Xevo G2-XS QTof spectrometer or a Thermo Fisher Ultimate 3000 RSLC/Q spectrometer. The X-ray diffraction experiments were carried out on a Rigaku XtaLAB synergy four-circle diffractometer with Cu K*α* radiation ($\lambda = 1.54184$ Å). Column chromatography (CC) was performed using silica gel (Qingdao Marine Chemical Inc., China), ODS (50 μm, Fuji Silysia Chemical Ltd., Japan), and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Sweden). TLC was performed with silica gel 60 F254 (Yantai Chemical Industry Research Institute).

Plant materials

The fresh leaves of *Ginkgo biloba* were collected in Xinyang, People's Republic of China, in August 2017. The botanical identification was made by Prof. Lin Yang, School of Life Science and Engineering, Lanzhou University of Technology. A voucher specimen (SPH2017B) was deposited in the herbarium of School of Chemistry and Chemical Engineering, Xuchang University.

Extraction and isolation

The leaves of *Ginkgo biloba* (5.2 kg) were extracted with 95% EtOH at room temperature (3 × 40 L) to afford a crude extract of 302.8 g after evaporation of the solvent under vacuum. The extract was suspended in distilled H_2O and partitioned with CH_2Cl_2 and EtOAc. The EtOAc soluble portion (87.3 g) was subjected to silica gel CC using petroleum ether–EtOAc (50:1 to 0:100) as eluent to give five fractions F1–F5. Fraction F1 (eluted by petroleum

ether–EtOAc 50:1) was subsequently purified using RP-C_{18} CC (MeOH–H_2O, 50:50–100:0) to afford compounds **5** (6.2 mg), **7** (5.7 mg) and **9** (3.5 mg). Fraction F2 (eluted by petroleum ether–EtOAc 30:1) was further separated using RP-C_{18} CC (MeOH–H_2O, 40:60–90:10) to give compounds **6** (4.1 mg) and **11** (3.8 mg). Fraction F3 (eluted by petroleum ether–EtOAc 20:1) was passed through an RP-C_{18} CC eluted with MeOH-H_2O (30:70–90:10) to give six subfractions (F3-1 to F3-6). Subfraction F3-1 was chromatographed on a Sephadex LH-20 column (MeOH) to give compounds **1** (7.2 mg) and **10** (5.1 mg). Subfraction F3-2 was purified by RP-C_{18} CC (MeOH–H_2O, 40:60) to give compound **4** (3.7 mg) and **8** (9.2 mg). Subfraction F3-4 was purified by silica gel CC (CH_2Cl_2–MeOH–H_2O, 80:19:1) to give compounds **2** (28.2 mg) and **3** (3.7 mg).

*Compound **1***. Colorless crystals. $[\alpha]_D^{20}$–10.3° (c 0.18, MeOH). mp 152 – 155 °C. IR (KBr) ν_{max} 3423, 2913, 1932, 1195, 1132, 1076 cm^{-1}. UV λ_{max} (MeOH) nm (log ε): 205 (4.6), 235 (4.1), 285 (3.8). HRESIMS *m/z* 491.1504 [M – H]$^-$ (calcd for $C_{24}H_{27}O_{11}$, 491.1548). ^{1}H NMR and ^{13}C NMR (CD_3OD), see Table 1.

*Compound **2***. White powder. mp 145 – 147 °C. $[\alpha]_D^{20}$ + 37.3° (c 0.7, MeOH). IR (KBr) ν_{max} 3421, 1742, 1720, 1519, 1272, 1075, 823 cm^{-1}. UV λ_{max} (MeOH) nm (log ε): 225 (4.3), 280 (3.6). HRESIMS *m/z* 327.1074 [M – H]$^-$(calcd for $C_{15}H_{19}O_8$, 327.1053). ^{1}H NMR and ^{13}C NMR (CD_3OD), see Table 2.

Enzymatic hydrolysis of compound 1

Compound **1** (5.8 mg) and β-glucosidase (20 mg, CAS: 9001-22-3) were dissolved in 1 mL of H_2O and stirred at room temperature for 24 h. The resulting aglycone was extracted with EtOAc three times. The combined EtOAc layer was washed with water, dried (Na_2SO_4), concentrated and purified by silica gel CC ($CHCl_3$–MeOH, 20: 1) to give Ginkgol B (**1a**) as a colorless oil. As a new compound gained by enzymatic hydrolysis of compound **1**, Ginkgol B was further identified by HRESIMS and NMR.

Ginkgol B (***1a***). $[\alpha]_D^{20}$ + 34.6° (c 0.07, $CHCl_3$). IR (KBr) ν_{max} 3438, 2917, 2850, 1725, 1143, 1086 cm^{-1}. UV λ_{max} (MeOH) nm (log ε): 288 (3.96). HRESIMS *m/z* 353.0987 [M + Na]$^+$ (calcd for $C_{18}H_{18}O_6Na$, 353.0996). ^{1}H-NMR ($CDCl_3$) δ: 3.55 (1H, ddd, J = 6.4, 6.4, 4.4 Hz, H-8), 3.88 (3H, s, OCH_3), 3.90 (1H, dd, J = 10.8, 4.4 Hz, H-9a), 3.92 (1H, dd, J = 10.8, 6.4 Hz, H-9b), 4.61 (2H, s, H-7′), 5.56 (1H, d, J = 6.4 Hz, H-7), 5.92 (2H, s, –OCH_2O–), 6.74 (1H, d, J = 8.0 Hz, H-5), 6.82 (1H, d, J = 1.2 Hz, H-2), 6.83 (1H, dd, J = 8.0, 1.2 Hz, H-6), 6.84 (2H, overlap, H-2′, 6′). ^{13}C-NMR (CD_3OD) δ: 54.1 (C-8), 56.3 (–OMe), 64.4 (C-9), 65.8 (C-7′), 88.0 (C-7), 101.3 (–OCH_2O–), 106.7 (C-2), 108.4 (C-5), 111.8 (C-2′), 115.6 (C-6′), 119.9 (C-6), 127.9 (C-5′), 134.8 (C-1′), 135.5 (C-1), 144.7 (C-3′), 148.2 (C-3), 148.2 (C-4), 148.2 (C-4′).

Alkaline methanolysis of compound 2

The methanolysis of **2** was conducted according to the literature with slight modifications [18]. To a solution of compound **2** (9.2 mg) in MeOH (1 mL), NaOMe (4.0 mg) was added. The mixture was stirred at room temperature for 1 h and then diluted with EtOAc, washed with water, dried (Na_2SO_4), concentrated and purified by silica gel CC to give 4-hydroxyphenyl propionic acid (**2a**) and D-glucose. D-glucose was identified by comparison with the ^{1}H NMR data [19] and $[\alpha]_D$ value {($[\alpha]_D^{20}$ + 35.4° (c 0.14, H_2O) vs L-glucose, $[\alpha]_D^{20}$–20° (c 0.89, H_2O)} [20].

*4-Hydroxyphenyl propionic acid (**2a**)* [21]: White powder. ^{1}H-NMR (CD_3OD) δ: 2.53 (2H, t, 8.0, H-8), 2.81 (2H, t, 8.0, H-7), 6.69 (2H, d, 8.0, H-3, H-5), 7.02 (2H, d, 8.0, H-2, H-6). ^{13}C-NMR (CD_3OD) δ: 31.4 (C-7), 37.3 (C-8), 116.3 (C-3), 116.3 (C-6), 130.4 (C-2), 130.4 (C-6), 133.1 (C-1), 156.9 (C-4), 177.1 (C-9).

Single-crystal X-ray diffraction analysis of compounds 1 and 5

The single-crystal diffraction data of compounds **1** and **5** were collected on a Rigaku XtaLAB synergy four-circle diffractometer with Cu Kα radiation (λ = 1.54184 Å), with the CrysAlisPro software (version 1.171.39.34b) for data reduction and analysis. The single-crystal diffraction data were collected at 100 K and the structures were solved by direct methods and refined by full-matrix least-squares method on F2 using SHELX algorithms in Olex2. All non-hydrogen atoms were refined with anisotropic displacement parameters. All hydrogen atoms were generated geometrically. The X-ray crystallographic data have been deposited at the Cambridge Crystallographic Data Centre (CCDC, ref. No. 1,940,729, 1,940,730).

*Crystallographic Data of **1**:* $C_{24}H_{28}O_{11}$, M = 492.46, monoclinic, $P2_1$, a = 12.5744 (2) Å, b = 5.0380 (1) Å, c = 17.4645 (3) Å, $\alpha = \gamma = 90°$, β = 96.324(1), V = 1099.64 (3) Å^3, T = 100 K, Z = 2, D_{calcd} = 1.487 g cm^{-3}, μ (Cu Kα) 1.00 mm^{-1}, crystal size 0.2 × 0.2 × 0.2 mm^3, F (000) = 520. The final R_1 value is 0.0363 (wR_2 = 0.0949) for 3711 reflections [$I > 2\sigma(I)$]. Flack structure parameter: – 0.16 (12).

*Crystallographic Data of **5**:* one independent molecule of **5** and a CH_3CH_2OH molecule in the asymmetric unit, $C_{20}H_{24}O_7 \bullet CH_3CH_2OH$, M = 422.46, orthorhombic, $P2_12_12_1$, a = 9.0958 (1), Å, b = 11.7546 (2) Å, c = 19.5329 (3) Å, $\alpha = \beta = \gamma = 90°$, V = 2088.41 (5) Å^3, T = 100 K, Z = 4, D_{calcd} = 1.344 g cm^{-3}, μ (Cu Kα) 0.85 mm^{-1}, crystal size 0.2 × 0.2 × 0.2 mm^3, F (000) = 904. The final R_1 value is

0.0324 ($wR_2 = 0.0830$) for 3975 reflections [$I > 2\sigma(I)$]. Flack structure parameter: 0.19 (9).

Cytotoxicity assay

Two cancer cell lines (5637 and HeLa) were used for cytotoxic activity assays. 5637 and HeLa cells were cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (Hyclone) at 37 °C in a humidified atmosphere with 5% CO_2. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [22]. A volume of 90 μL of adherent cells was seeded into each well of the 96-well culture plates with initial destiny of 1×10^4 cells/mL, and cells were allowed to adhere for 24 h before addition of test compounds. Then, test concentrations of compounds **1**–**11** (10 μL) were added to each well. Each cancer cell line was then exposed to the test compounds at concentrations of 1, 5, 10, 25, 50, and 100 μM in triplicate for 24 h. After 24 h of treatment, MTT solution (15 μL, 5 mg/mL) was added to each well and the plates were incubated for 4 h at 37 °C. The supernatant was then removed from formazan crystals and DMSO (100 μL) was added to each well. The optical density of the lysate was measured at 492 nm in a microplate reader. Results were expressed as a percentage of the control, and half maximal inhibitory concentration (IC_{50}) values were calculated. Cisplatin injection (5 mg/mL, Jiangsu Hansoh Pharmaceutical Group Co., Ltd., Jiangsu, China) was used as a positive control. The final concentration of DMSO used to dissolve the test compounds was less than 0.1% (v/v).

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