α-Tetralonyl Glucosides from the Green Walnut Husks of *Juglans mandshurica* and Their Antiproliferative Effects

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ABSTRACT

Two new α-tetralonyl glucosides, (4*S*)-4,5,8-trihydroxy-α-tetralone-5-*O*-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranoside (**1**) and (4*S*)-4,8-dihydroxy-α-tetralone-4-*O*-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranoside (**2**), together with eight known compounds (**3**–**10**) were isolated from the green walnut husks of *Juglans mandshurica*. The structural characterization of all compounds was performed by spectroscopic analyses, including 1D and 2D NMR and HR-ESI-MS experiments. The isolated compounds were assayed for their cytotoxicity against two human cancer cell lines, A549 and HeLa. Four compounds (**7**–**10**) exhibited inhibitory effects against two human cancer cell lines with GI_{50} values between 1.3 and 5.8 μM.

Introduction

Juglans, a genus of the family Juglandaceae, comprises about 20 species mainly distributed in the temperate and subtropical regions of the northern hemisphere, extending into South America [1]. *Juglans mandshurica* Maxim. grows in the north of China in the Heilongjiang, Jilin, Liaoning, Hebei, and Shanxi Provinces [2]. Previous phytochemical studies of *J. mandshurica* led to the identification of naphthoquinones, diarylheptanoids, and flavonoids (as well as their glycosides) [3–5]. The biological screening of these compounds showed promising bioactivity, including cytotoxic and antimicrobial activities [5–9].

In the course of our studies on the genus *Juglans*, including *J. mandshurica*, we have analyzed the chemical content of the green walnut husks of *J. mandshurica*. In particular, our attention has been focused towards the *n*-butanol-soluble fraction of the 70% ethanol extract containing naphthoquinone and diarylheptanoid components. This study led us isolate ten compounds including two new α-tetralonyl glucosides, (4*S*)-4,5,8-trihydroxy-α-tetralone-5-*O*-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranosie (**1**) and (4*S*)-4,8-dihydroxy-α-tetralone-4-*O*-β-D-glucopyranosyl (1 → 6)-β-D-glucopyranoside (**2**), and a series of known compounds (**3**–**10**), some of which have not been reported before from *J. mandshurica* (▶ **Fig. 1**). These compounds were screened

for antiproliferative activity against two human cancer cell lines, A549 and HeLa. Herein, details of the isolation, structural elucidation, and antiproliferative activities of these compounds are described.

Results and Discussion

The 70% ethanol extract of the dried the green walnut husks of *J. mandshurica* was subjected to multiple chromatographic steps over silica gel, Sephadex LH-20, and recycling preparative HPLC. Ten compounds were obtained, including two new compounds, (4*S*)-4,5,8-trihydroxy-α-tetralone-5-*O*-β-D-glucopyranosyl (1 → 6)-β-D-glucopyranoside (**1**) (4*S*)-4,8-dihydroxy-α-tetralone-4-*O*-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranoside (**2**), and eight known compounds comprising of four naphthoquinones (**3**–**6**) and four diarylheptanoids (**7**–**10**). The structures of **1**–**10** were identified by physical data analyses, including 1D and 2D NMR and HR-ESI-MS. The sugar residues were identified by GC analyses after hydrolysis.

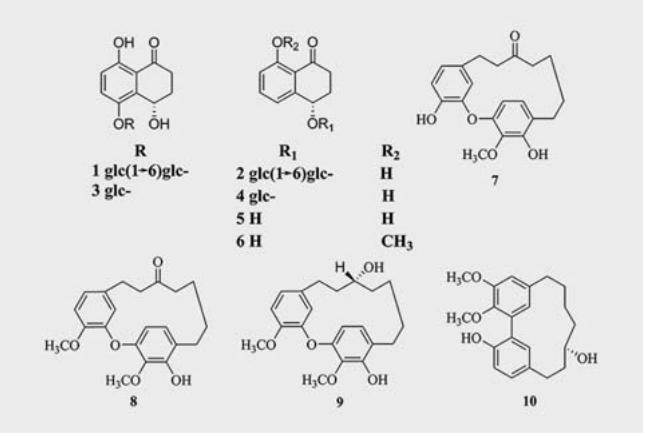
Constituents **1** and **2** are new α-tetralonyl glucosides never described before in the literature. The full assignments of ^{1}H NMR and ^{13}C NMR spectroscopic data of the new isolated compounds (**1** and **2**) are listed. Their key ^{1}H-^{1}H COSY and HMBC correlations are presented in ▶ **Fig. 2**. The respective NMR and HR-ESI-MS spectra are provided in the Supporting Information.

Compound **1** was obtained as a white amorphous powder. The molecular formula of $C_{22}H_{30}O_{14}$ was determined on the basis of the HR-ESI-MS (*m/z* 541.1524, $[M + Na]^+$). Acidic hydrolysis of **1** gave D-glucose as the mono sugar residue. The ^{1}H NMR and ^{13}C NMR data of **1** exhibited signals arising from a CO group at δ_C 206.5, a benzene ring at δ_H 7.60 (1H, d, J = 9.0 Hz, H-6), 6.93 (1H, d, J = 9.0 Hz, H-7), two β-glucopyranosyl moieties [anomeric H-atom at δ_H 4.83 (1H, d, J = 7.7 Hz, H-1′), 4.40 (1H, d, J = 7.7 Hz, H-1″) and C-atom signals at δ_C 104.8 (C-1″), 104.2 (C-1′), 78.0 (C-3″), 77.9 (C-3′), 77.8 (C-5″), 77.4 (C-5′), 75.2 (C-2′), 75.1 (C-2″), 71.6 (C-4″), 71.3 (C-4′), 70.0 (C-6′), and 62.7 (C-6″)]. These NMR characteristics resembled those observed for **3** [10]. However, instead of a β-glucopyranosyl moiety in **3**, compound **1** had two β-glucopyranosyl moieties in view of the anomeric H-atom at δ_H 4.83 (1H, d, J = 7.7 Hz, H-1′), 4.40 (1H, d, J = 7.7 Hz, H-1″). The linkage of the second sugar moiety at OH-C(6′) of the first sugar moiety was established from the HMBC correlations between δ_H 4.40 (1H, d, J = 7.7 Hz, H-1″) and δ_C 70.0 (C-6′). The absolute configuration of the saccharides was determined to be D-glucose by GC analysis of chiral derivatives in the hydrolysate of this compound. As for the absolute configuration of the chiral carbon, (4*S*)-**1** was determined on the basis of a negative Cotton effect at ca. 220–240 nm in the CD spectrum of **1** (**Fig. 2S**, Supporting Information) and comparison of its optical rotation ($[\alpha]_D^{20}$: − 45 in MeOH) with that of literature [11].

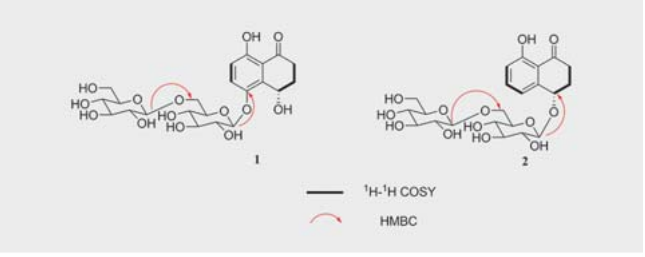
After further comprehensive analysis of its NMR spectra (**Figs. 3S**–**8S**, Supporting Information), compound **1** was identified as (4*S*)-4,5,8-trihydroxy-α-tetralone-5-*O*-β-D-glucopyranosyl (1 → 6)- β-D-glucopyranoside.

Compound **2** was obtained as a white amorphous powder. The molecular formula of $C_{22}H_{30}O_{13}$ was determined on the basis of the HR-ESI-MS (*m/z* 525.1563, $[M + Na]^+$). Acidic hydrolysis of **2** gave D-glucose as the mono sugar residue. The ^{1}H NMR and ^{13}C NMR data of **2** exhibited the signals arising from a CO group at δ_C 206.6, a benzene ring at δ_H 7.53 (1H, t, J = 8.4 Hz, H-6), 7.21 (1H, d, J = 7.4 Hz, H-5), 6.92 (1H, dd, J = 8.4, 1.0 Hz, H-7), two β-glucopyranosyl moieties [anomeric H-atom at δ_H 4.47 (1H, d, J = 7.8 Hz, H-1″), 4.39 (1H, d, J = 7.7 Hz, H-1′)] and C-atom signals at δ_C 105.1 (C-1″), 102.7 (C-1′), 78.1 (C-5″), 78.0 (C-3′), 78.0 (C-3″), 77.1 (C-5′), 75.2 (C-2′), 75.1 (C-2″), 71.7 (C-4″), 71.6 (C-4′), 70.1 (C-6′), and 62.8 (C-6″). These NMR characteristics resembled those observed for **4** [12]. However, instead of a β-glucopyranosyl moiety in **4**, compound **2** had two β-glucopyranosyl moieties in view of the anomeric H-atom at δ_H 4.47 (1H, d, J = 7.8 Hz, H-1″), 4.39 (1H, d, J = 7.7 Hz, H-1′). The linkage of the second sugar moiety at OH-C(6′) of the first sugar moiety was established from the HMBC correlations between δ_H 4.47 (1H, d, J = 7.8 Hz, H-1″) and δ_C 70.1 (C-6′). The absolute configuration of the saccharides was determined to be D-glucose by GC analysis of the chiral derivatives in the hydrolysate of this compound. As for the absolute configuration of the chiral carbon, (4*S*)-**2** was determined on the basis of a negative Cotton effect at ca. 210–230 nm in the CD spectrum of **2** (**Fig. 10S**, Supporting Information) and comparison of its optical rotation ($[\alpha]_D^{20}$: − 51 in MeOH) with that of the literature [11].

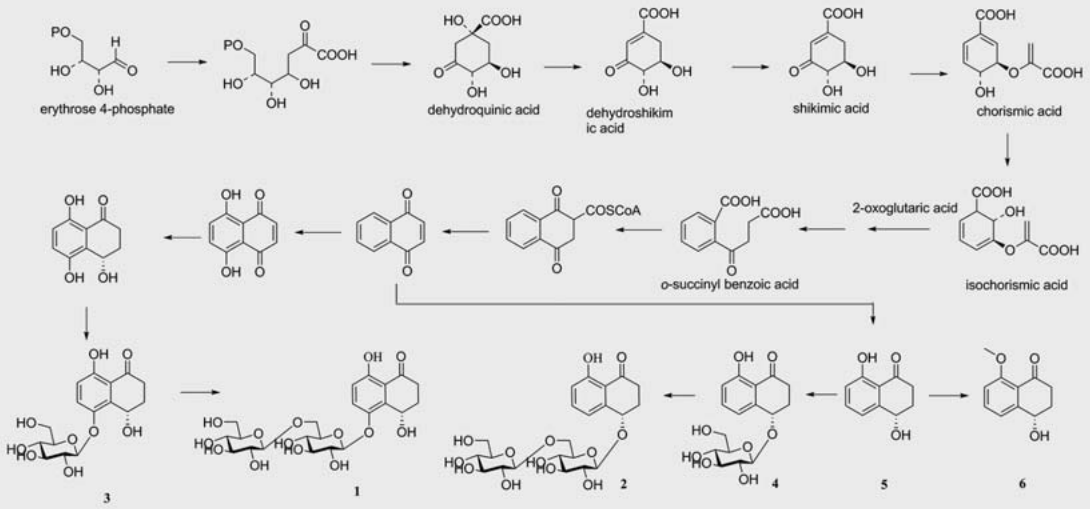
After further comprehensive analysis of its NMR spectra (**Figs. 11S**–**16S**, Supporting Information), compound **2** was identified as (4*S*)-4,8-dihydroxy-α-tetralone-4-*O*-β-D-glucopyranosyl (1 → 6)- β-D-glucopyranoside.



▶ **Fig. 1** Chemical structures of compounds **1**–**10** from the green walnut husks of *J. mandshurica*.



▶ **Fig. 2** The key ^{1}H-^{1}H COSY and HMBC of compounds **1**–**2**.



▶ **Fig. 3** Plausible biosynthetic pathway of new compounds.

α-Tetralonyl glucosides with one β-glucopyranosyl moiety have been reported from the green walnut husks of *J. mandshurica* before. But the naphthoquinone with two β-glucopyranosyl moieties has not been described in the literature so far. The putative biosynthetic pathway begins from erythrose-4-phosphate, which was derived through a series of reactions, including intermolecular cyclization, oxidation, hydroxylation, and glycosylation (▶ **Fig. 3**). The eight known compounds identified by comparing their spectroscopic and spectrometric data (NMR and HRESIMS) with those in the literature are juglanoside E (**3**) [10], berchemiaside A (**4**) [12], regiolone (**5**) [13], berchemiaside B (**6**), 2-oxatrycyclo[13.2.2.13,7]eicosa-3,5,7-(20),15,17,18-hexaen-10-one (**7**), juglanin A (**8**), 2-oxatrycyclo[13.2.2.13,7]eicosa-3,5,7(20),15,17,18-hexaen-10–16-diol (**9**), and (11*S*)-11,17-dihydroxy-3,4-dimethoxy-[7,0]-metacyclophane (**10**) [3, 14, 15]. Copies of the original spectra for the known compounds are obtainable from the corresponding author.

All *J. mandshurica* components isolated were tested for their cytotoxic activity against A549 and HeLa cell lines, with etoposide as the positive control (▶ **Table 1**). Compounds **1**–**6** showed no or weak cytotoxic activity, whereas compounds **7**–**10** exerted strong cytotoxicity with GI_{50} values in the range of 1.3–5.8 μM. Based on the available data, it seems that diarylheptanoids (compounds **7**–**10**) are favorable for the observed cytotoxic activity.

Diarylheptanoid is a group of compounds that bears the 1,7-dihenylheptane skeleton as a special characteristic in the natural product estate [16]. Since the first diarylheptanoid isolated in 1815, approximately 400 such materials have been obtained from natural resources [17]. Hirsutanone and oregonin, from *Alnus japonica* Steud, exhibited potent antiproliferative activity against two colon cancer cell lines (HCT-15 and Colo205) [18]. Tian et al. had suggested that a diarylheptanoid, 1-(4-hydroxy-3-methoxyphenyl)-7-(3,4-dihydroxyphenyl)-4*E*-en-3-heptanone, was isolated from *Alpinia officinarum* and provided new insight into neuroblastoma chemotherapy [19]. Furthermore, diarylheptanoids have been reported by numerous studies to possess diverse bioactivities, including anti-inflammatory [20] and antiemetic [21].

▶ **Table 1** Cytotoxicity data of isolated compounds **1**–**10**.[a]

Compound	GI_{50} (μM)	
	A549	HeLa
1	51.1	73.6
2	67.6	51.9
3	> 100	70.2
4	> 100	> 100
5	36.4	> 100
6	61.6	81.4
7	1.6	2.1
8	5.8	3.3
9	2.4	1.9
10	1.3	2.7
Etoposide[b]	4.4	0.7

A549: human lung cancer cell lines. HeLa: human cervical carcinoma cancer cell lines. [a]Data expressed as GI_{50} values (μM); [b]positive control

In summary, two new α-tetralonyl glucosides (**1** and **2**) and eight known compounds (**3**–**10**) were isolated from the green walnut husks of *J. mandshurica*, and the structures were determined by MS, 1D NMR, and 2D NMR. The results obtained in this study show that diarylheptanoids (**7**–**10**) exhibited inhibitory effects against the two human cancer cell lines (A549 and HeLa) with GI_{50} values below 6 μM, while naphthoquinones (**1**–**6**) were inactive.

Material and Methods

General experimental procedures

The NMR spectra were measured in methanol-d_4 on a Bruker ARX-400 or AV600 instrument with TMS as an internal standard. The CD spectrum was tested using a JASCO pu-2080 spectrometer. IR spectra were taken on a Bruker IFS-55 infrared spectrophotometer with a KBr disk. Optical rotations were measured on a Peking-Elmer 241 MC Spectropolarimeter at 20 °C. ESI-MS spectra were recorded on a Waters Quattro micro API LC/MS/MS spectrometer (Waters). HR-TOF-MS spectra were performed on an Agilent LC/MS spectrometer (Agilent). HPLC was performed on JAI LC9103 Recycling preparative HPLC (Japan Analytical Industries) equipped with JAIGEL-ODS-AP-P column and JAIGEL-GS310 column using a JAI refractive index detector and a JAI UV-3702 detector with MultiChro 2000 workstation. TLC was performed on precoated GF_{254} plates (Merck) and detected by spraying with 10% H_2SO_4 followed by heating.

Plant material

The green walnut husks of *J. mandshurica* were collected in October 2014 at Yichun, Heilongjiang, China, and authenticated by Professor Jin-cai Lu (The School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University). A voucher specimen (No. 1014023) is stored at the key laboratory of Modern Analysis of TCM, School of Pharmacy, Shenyang Pharmaceutical University, Shenyang, China.

Extraction and isolation

The green walnut husks of *J. mandshurica* (20 kg) were extracted with 70% (v/v) aqueous ethanol three times (3 × 80 L) to give 1 kg of crude extract. The crude extract was suspended in 3 L of water. The suspension was successively partitioned with EtOAc (3 × 3 L) and *n*-BuOH (3 × 3 L). The *n*-BuOH-soluble fraction (120 g) was subjected to silica gel chromatography, eluting with gradients of CH_2Cl_2:MeOH (100:13 000 mL, 100:33 000 mL, 100:73 000 mL, 100:153 000 mL, 100:303 000 mL, 100:703 000 mL) on a silica gel column (column dimension: 6.0 × 80 cm) to give six fractions, Fr. A–F (4.7 g, 6.3 g, 12.2 g, 8.4 g, 8.1 g, and 3.0 g). Fraction A was purified by silica gel CC (column dimension: 2.5 × 40 cm) with CH_2Cl_2:MeOH (300:11 000 mL, 150:3800 mL, 100:11 000 mL) to afford 230 mg of Fr. A-1 and 130 mg of Fr. A-2. Fraction A-1 was purified by recycling preparative HPLC with MeOH-H_2O (85:15) as the eluent to obtain pure compound **7** (23.1 mg, t_R 43 min). Fraction A-2 was purified by recycling preparative HPLC with MeOH-H_2O (85:15) as the eluent to obtain pure compounds **8** (15.3 mg, t_R 34 min) and **9** (30.1 mg, t_R 38 min). Fraction B was purified by silica gel CC (column dimension: 2.5 × 40 cm) with CH_2Cl_2:MeOH (100:11 000 mL, 100:21 000 mL, 100:31 000 mL) to afford 400 mg of Fr. B-1. Fraction B-1 was purified by recycling preparative HPLC with MeOH-H_2O (70:30) as the eluent to obtain pure compounds **5** (9.1 mg, t_R 29 min) and **6** (6.2 mg, t_R 35 min). Fraction C was purified by silica gel CC (column dimension: 2.5 × 40 cm) with CH_2Cl_2:MeOH (100:31 000 mL, 100:51 000 mL, 100:81 000 mL) to afford 210 mg of Fr. C-1 and 310 mg of Fr. C-2. Fraction C-1 was purified by recycling preparative HPLC with MeOH-H_2O (55:45) as the eluent to obtain pure compounds **1** (10.5 mg, t_R 21 min) and **3** (9.0 mg, t_R 25 min). Fraction C-2 was purified by recycling preparative HPLC with MeOH-H_2O (55:45) as the eluent to obtain pure compounds **2** (17.7 mg, t_R 24 min), **4** (8.7 mg, t_R 31 min), and **10** (7.9 mg, t_R 35 min).

(4*S*)-4,5,8-Trihydroxy-α-tetralone-5-*O*-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranoside (1)

White amorphous power, $[\alpha]_D^{20}$ = − 45 (*c* 0.6, MeOH); HR-ESI-MS: *m/z* 541.1524, $[M + Na]^+$ (calcd. for $C_{22}H_{30}O_{14}Na$, 541.1523). ^1H NMR (600 MHz, methanol-d_4) δ 7.60 (1H, d, *J* = 9.0 Hz, H-6), 6.93 (1H, d, *J* = 9.0 Hz, H-7), 5.37 (1H, d, *J* = 2.7 Hz, H-4), 4.83 (1H, d, *J* = 7.7 Hz, H-1′), 4.40 (1H, d, *J* = 7.7 Hz, H-1″), 4.21 (1H, dd, *J* = 11.7, 1.9 Hz, H-6′$_a$), 3.88 (1H, dd, *J* = 11.9, 2.1 Hz, H-6″$_a$), 3.83 (1H, dd, *J* = 11.7, 6.2 Hz, H-6′$_b$), 3.66 (2H, m, H-5′/H-6″$_b$), 3.56 (1H, t, *J* = 9.0 Hz, H-2′), 3.49 (1H, t, *J* = 9.0 Hz, H-5″), 3.44 (1H, t, *J* = 9.0 Hz, H-4′), 3.37 (1H, t, *J* = 9.0 Hz, H-3′), 3.31 (1H, d, *J* = 9.0 Hz, H-4″), 3.26 (2H, m, H-2″/H-3″), 3.08 (1H, ddd, *J* = 17.4, 12.7, 6.0 Hz, H-2a), 2.56 (1H, d, *J* = 17.4 Hz, H-2b), 2.26 (1H, m, H-3a), 2.24 (1H, m, H-3b). ^{13}C NMR (100 MHz, methanol-d_4) δ 206.5 (C-1), 159.0 (C-8), 148.7 (C-5), 134.5 (C-4a), 128.5 (C-6), 119.2 (C-7), 116.1 (C-8a), 104.8 (C-1″), 104.2 (C-1′), 78.0 (C-3″), 77.9 (C-3′), 77.8 (C-5″), 77.4 (C-5′), 75.2 (C-2′), 75.1 (C-2″), 71.6 (C-4″), 71.3 (C-4′), 70.0 (C-6′), 62.7 (C-6″), 61.3 (C-4), 33.6 (C-2), 30.2 (C-3).

(4*S*)-4,8-Dihydroxy-α-tetralone-4-*O*-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranoside (2)

White amorphous power, $[\alpha]_D^{20}$ = − 51 (*c* 0.6, MeOH); HR-ESI-MS: *m/z* 525.1563, $[M + Na]^+$ (calcd. for $C_{22}H_{30}O_{13}Na$, 525.1570). ^1H NMR (600 MHz, methanol-d_4) δ 7.53 (1H, t, *J* = 8.4 Hz, H-6), 7.21 (1H, d, *J* = 7.4 Hz, H-5), 6.92 (1H, dd, *J* = 8.4, 1.0 Hz, H-7), 5.07 (1H, t, *J* = 6.3 Hz, H-4), 4.47 (1H, d, *J* = 7.8 Hz, H-1″), 4.39 (1H, d, *J* = 7.7 Hz, H-1′), 4.23 (1H, dd, *J* = 11.7, 2.0 Hz, H-6′$_a$), 3.92 (1H, dd, *J* = 11.8, 1.8 Hz, H-6″$_a$), 3.83 (1H, dd, *J* = 11.7, 6.5 Hz, H-6′$_b$), 3.71 (1H, dd, *J* = 11.8, 5.1 Hz, H-6″$_b$), 3.51 (1H, ddt, *J* = 8.4, 4.2, 1.9 Hz, H-5′), 3.39 (1H, m, H-3″), 3.34 (4H, m, H-3′/H-4′/H-4″/H-5″), 3.28 (2H, t, *J* = 9.0 Hz, H-2′/H-2″), 3.11 (1H, ddd, *J* = 17.9, 9.6, 5.0 Hz, H-2a), 2.69 (1H, ddd, *J* = 17.9, 6.3, 4.8 Hz, H-3b), 2.40 (1H, m, H-3a), 2.34 (1H, m, H-3b). ^{13}C NMR (100 MHz, methanol-d_4) δ 206.6 (C-1), 163.8 (C-8), 144.1 (C-4a), 137.5 (C-6), 120.7 (C-5), 118.6 (C-7), 116.8 (C-8a), 105.1 (C-1″), 102.7 (C-1′), 78.1 (C-5″), 78.0 (C-3′), 78.0 (C-3″), 77.1 (C-5′), 75.2 (C-2′), 75.1 (C-2″), 74.7 (C-4), 71.7 (C-4″), 71.6 (C-4′), 70.1 (C-6′), 62.8 (C-6″), 35.2 (C-2), 31.1 (C-3).

Acid hydrolysis of compounds 1 and 2

Each compound (2 mg) was treated with 1 M HCl (4 mL) at 90 °C for 2 h. Then the reaction mixture was extracted with $CHCl_3$ (3 × 5 mL). The aqueous layer was collected and the water was evaporated under vacuum with the repeated addition of MeOH to remove the solvent completely. The residue was redissolved in anhydrous pyridine (2 mL) and mixed with a pyridine solution of *L*-cysteine methyl ester hydrochloride (2 mL). After the mixed solution was heated at 60 °C for 1 h, trimethylchlorosilane (0.5 mL) was added and the resulting mixture was stirred at 60 °C for another 30 min. Then, the solution was concentrated to dryness

and taken up in water (3 × 1 mL), followed by extraction with *n*-hexane (3 × 1 mL). The supernatant was analyzed by GC. Separations were carried out on HP-5 columns (320 µm × 30 cm, 0.25 µm). Highly pure N_2 was employed as a carrier gas (1.0 mL/min), and the FID detector operated at 280 °C (column temperature 160–200 °C). The retention time of the monosaccharide derivative was as follows: *D*-glucose (14.43 min).

Cell culture

Human lung cancer cells (A549) and human cervical carcinoma cancer cells (HeLa) was provided by the ATCC. The cells were cultured in medium (RPMI 1640 for A549 and DMEM for HeLa) supplemented with 10% heat-inactivated FBS and antibiotics/antimycotics (PSF; 100 units/mL penicillin G sodium, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B). The cells were incubated at 37 °C and 5% CO_2 in a humidified atmosphere. Etoposide (purity > 98%; Sigma) was used as a positive control.

In vitro cell proliferation assay

Cell viability was determined by the sulforhodamine B (SRB) protein staining method. Cells were seeded in 96-well plates and incubated for 24 h, and then fixed (for zero day controls) or treated with the test compounds for 72 h. All compounds were solved in DMSO (final concentration of 0.1% [v/v]), stored at − 20 °C, and diluted to the desired concentration (0.01, 0.1, 1, 10, 100 µM) in normal saline immediately prior to each experiment. Each concentration was tested thrice. At least three experiments were performed. Data leading to the determination of GI_{50} values are provided as dose-response curves in **Fig. 17S**, Supporting Information. After incubation, cells were fixed with 10% trichloroacetic acid (TCA), dried, and stained in 0.4% SRB in 1% acetic acid solution. Unbound dye was washed, and stained cells were dried and dissolved in 10 mM Tris (pH 10.0). Absorbance was measured at 515 nm and cell proliferation was determined as follows: cell proliferation (%) = (average $absorbance_{compound}$ – average $absorbance_{zero\ day}$)/(average $absorbance_{control}$ – average $absorbance_{zero\ day}$) × 100%. GI_{50} values were calculated by nonlinear regression analysis using Table Curve 2D software (Version 5.01, Systat Software Inc.).

Supporting information

The NMR and HR-ESI-MS spectra of compounds **1** and **2** and graphs showing dose-response curves for the investigated compounds are provided as Supporting Information.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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