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Two new prenylated coumarins from roots of *Zanthoxylum nitidum*

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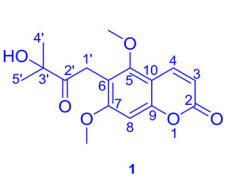
ABSTRACT

Two new prenylated coumarins, 3′-hydroxytoddanone (**1**), and isotoddalolactone (**2**), along with four known analogues (**3**–**6**) were isolated from the roots of *Zanthoxylum nitidum*. Their chemical structures were elucidated based on extensive spectroscopic interpretation and HR-ESI-MS analysis. The absolute configuration of compound **2** was determined by comparing experimental ECD spectrum with that calculated by the time-dependent density functional theory (TDDFT) method. Compounds **4–6** were isolated from the *Zanthoxylum* genus for the first time. The two new compounds were tested for antiproliferative activities *in vitro* on the HL-60, K562 and THP-1 cell lines. Compounds **1** and **2** exhibited moderate cell growth inhibitory activities *in vitro* against human leukemic HL-60 cell lines, with IC_{50} values of 32.64 and 33.15 μM, respectively.

Zanthoxylum nitidum



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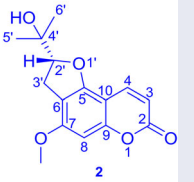

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1. Introduction

Zanthoxylum nitidum (Roxb.) DC (Rutaceae), locally called "*Liang-Mian-Zhen*" in Chinese, is a morphologically variable species found as a liane in rain-forest and as a shrub in dryer habitats [1]. It is widely distributed from India to northern Queensland in Australia, and throughout the southeastern part of China [2]. Its barks and roots have been extensively used in Traditional Chinese Medicine (TCM) for the treatment of rheumatic arthritis, traumatic injury, and pyogenic infections. Previous phytochemical investigations of this plant have resulted in the isolation and identification of a number of bioactive chemical constituents including a series of alkaloids, coumarins and benzenoids [3–9]. Pharmacological investigations of *Z. nitidum* have revealed its anti-tumor [10, 11], antioxidant [12], analgesic [13], and anti-ulcer [14] activities. In the course of our search for anti-cancer agents from natural sources, we launched a systematic study to investigate the chemical constituents in the ethanol extract of *Z. nitidum*. In this paper, we report the isolation, structure elucidation, and antiproliferative activities of the two new coumarins, along with four known analogues isolated from *Z. nitidum*.

2. Results and discussion

Compound **1** was obtained as a colorless needle crystal (in MeOH). Its molecular formula $C_{16}H_{18}O_6$ with eight degrees of unsaturation was determined by HR-ESI-MS at *m/z* 307.1178 $[M+H]^+$. The UV absorption at 326 and 201 nm indicated a coumarin derivative [15]. The IR absorption bands at 3435, 1631, and 1608 cm^{-1} revealed the presence of a hydroxy and two carbonyls. The ^{1}H-NMR spectrum (Table 1) suggested the presence of two pairs of doublets at δ 6.27 and 7.86 (each 1H, d, $J = 9.6$ Hz), which are identical with the characteristic signals of H-3 and H-4 of a coumarin, an aromatic proton singlet at δ 6.64 (1H, s, H-8), two methyl singlet at δ 1.55 (6H, s, H-4′, 5′), and a methylene singlet at δ 3.93 (2H, s, H-1′). In addition, two methoxyl singlets at δ 3.78 (3H, s, 5-OCH_3) and 3.83 (3H, s, 7-OCH_3) were shown in the ^{1}H

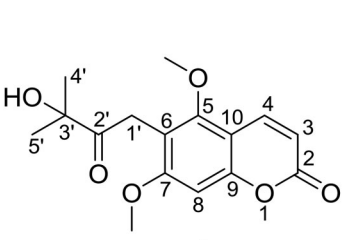
Table 1. The ^{1}H NMR and ^{13}C NMR spectral data of compounds **1** and **2** in $CDCl_3$ (600 and 150 MHz, respectively).

	1		2	
No.	δ_C	δ_H (mult, *J* in Hz)	δ_C	δ_H (mult, *J* in Hz)
2	161.1	—	161.6	—
3	112.9	6.27, 1H, (d, 9.6)	111.5	6.17, 1H, (d, 9.5)
4	138.8	7.86, 1H, (d, 9.6)	138.3	7.79, 1H, (d, 9.5)
5	156.4	—	157.2	—
6	113.8	—	109.3	—
7	161.2	—	159.0	—
8	95.7	6.64, 1H, (s)	92.1	6.36, 1H, (s)
9	155.8	—	156.3	—
10	107.3	—	99.4	—
1′	31.5	3.93, 2H, (s)	—	—
2′	212.1	—	92.1	4.79, 1H, (t, 9.0)
3′	76.9	—	28.1	3.13, 2H, (d, 9.0)
4′	27.0	1.55, 3H, (s)	72.0	—
5′	27.0	1.55, 3H, (s)	26.2	1.37, 3H, (s)
6′	—	—	24.3	1.24, 3H, (s)
5-OCH_3	63.6	3.78, 3H, (s)	—	—
7-OCH_3	56.2	3.83, 3H, (s)	56.0	3.86, 3H, (s)

NMR spectrum. The ^{13}C NMR (Table 1) spectrum displayed sixteen carbon signals containing two carbonyls at δ 161.1 (α-lactone carbonyl) and δ 212.1 (ketone carbonyl), eight olefinic/aromatic carbon signals including three oxygenated aromatic carbon signals. Based on above ^{1}H NMR data, compound **1** was determined to possess a 3-hydroxy-3-methyl-2-oxobutyl side chain in conjunction with the presence of three carbon signals at δc 212.1 (C-2′), 76.9 (C-3′), and 31.5 (C-1′), which was also consistent with the unit of known compound peucedanone [16]. Moreover, the key correlations in the HMBC spectrum (Figure 2) further confirmed the moiety. The assignment of all protonated carbons was determined by HSQC spectrum.

The structure of **1** was elucidated by 2D NMR experiments. In the HMBC spectrum (Figure 2), the long-range correlation between a methoxy proton at δ_H 3.78 and C-5 (δ_C 156.4) determined the location of the methoxy group at the C-5 position. The typical methoxy carbon signal appeared at δ_C 63.6, which is shifted significantly downfield (ca. +7 ppm) due to steric effects. This result suggested that both of the *ortho*-positions of this methoxy group should be substituted [17]. The other methoxy group at δ_H 3.83 was assigned to C-7 (δ_C 161.2) due to the HMBC cross-peak between the methoxy protons and C-7. The side chain was linked to C-6 based on the observed HMBC correlations of H-1′ with C-5, C-6, and C-7. The comparison of the ^{1}H and ^{13}C NMR spectral data of **1** (Table 1) with those of toddanone (**4**) [18] showed that the two compounds are closely related analogues in which the only notable difference is that C-3′ of **1** is substituted with a hydroxy group. Therefore, the structure of compound **1** was deduced as shown in Figure 1 and named as 3′-hydroxytoddanone.

Compound **2** was obtained as a colorless needle crystal (in MeOH). Its molecular formula $C_{15}H_{16}O_5$ with eight degrees of unsaturation was determined by HR-ESI-MS at *m/z* 277.1063 $[M+H]^+$. The UV absorption at 317 nm indicated a coumarin derivative [15]. The IR absorption bands at 3443 and 1630 cm^{-1} revealed the presence of a hydroxy and a carbonyl. The ^{1}H NMR spectrum (Table 1) suggested the presence of two pairs of doublets at δ 6.17 and 7.79 (each 1H, d, J = 9.5 Hz), which are identical with the signals of H-3 and H-4 of a coumarin, an aromatic proton singlet at δ 6.36 (1H, s, H-8), one methoxyl singlet at δ 3.86 (3H, s, 7-OCH$_3$), as well as a 2-(2-hydroxypropan-2-yl)-2,3-dihydrofuran ring signals which was deduced from the following characteristic proton signals at δ_H 4.79 (1H, t, J = 9.0 Hz, H-2′), 3.13 (2H, d, J = 9.0 Hz, H-3′), 1.37 and 1.24 (each 3H, s, H-5′, 6′). The ^{13}C NMR (Table 1) and HSQC spectra displayed fifteen signals, containing one carbonyl at δ 161.6 (α-lactone carbonyl), eight olefinic/aromatic carbon signals including three oxygenated aromatic



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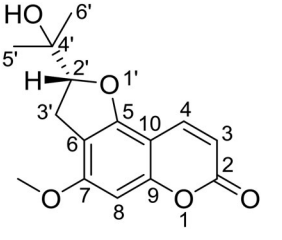

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Figure 1. Structures of compounds **1** and **2** isolated from *Zanthoxylum nitidum*.

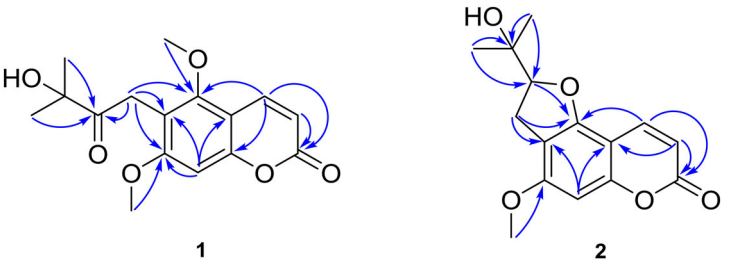

Figure 2. Key HMBC correlations of compounds **1** and **2**.

carbons at δ 157.2 (C-5), 159.0 (C-7), and 156.3 (C-9), one oxygenated methyl carbon signal at δ 56.0, one quaternary carbon signal at δ 72.0 (C-4′), one methylene carbon signal at δ 28.1 (C-3′) and one oxygenated methine carbon signal at δ 92.1 (C-2′). The assignment of all protonated carbons was determined by HSQC spectrum. Most of the NMR data mentioned before was similar to those of·ptilostol [19], but the major difference between them was the chemical shifts of the methylene of the dihydrofuran moiety. Differences were observed in δ_C values ($\triangle\delta_C$ ca.+1 ppm) of the C-3′ signals of the dihydrofuran units in the ^{13}C NMR spectrum (in $CDCl_3$) and in the δ_H values ($\triangle\delta_H$ ca.+0.3 ppm) of H-3′ proton signals in the ^{1}H NMR spectrum (in $CDCl_3$), suggesting that the substitution location of the dihydrofuran moiety of the two products on the coumarin skeleton was different.

The planar structure of **2** was elucidated by the HMBC spectrum (Figure 2). The correlations of H-2′ with C-5 (δ_C 157.2) as well as that of H-3′ with C-5 and C-6 (δ_C 109.3) indicated that the dihydrofuran ring was fused to C-5 and C-6 of the coumarin nucleus in an angular arrangement with an ether linkage at C-5. The C-7 location of the methoxy group was based on the HMBC correlations between a methoxy proton at δ_H 3.86 and C-7 (δ_C 159.0). To determine the absolute configuration of C-2′, ECD calculation of **2** was performed by the TDDFT method. Comparison of the theoretically calculated and experimental ECD curves of **2** (Figure 3) led to determination of the absolute configuration of **2** as 2′*R*. Therefore, the structure of compound **2** was deduced as shown in Figure 1 and named as isotoddalolactone.

By comparing physico-chemical and spectroscopic properties with those reported in the literature, four known coumarins were elucidated as toddalolactone (**3**) [20], toddanone (**4**) [18], 5-methoxysuberenone (**5**) [21], and toddaculine (**6**) [22], respectively. Compounds **4**–**6** were isolated from the *Zanthoxylum* genus for the first time.

The two new compounds were tested for antiproliferative activities *in vitro* on the human leukemia HL-60, K562 and THP-1 cell lines using 5-Fu as the positive control (IC_{50} = 7.54, 23.65, and 5.23 μM, respectively). Compounds **1** and **2** exhibited moderate cell growth inhibitory activities *in vitro* against human leukemic HL-60 cell lines, with IC_{50} values of 32.64 and 33.15 μM, respectively.

3. Experimental

3.1. General experimental procedures

Optical rotation was measured on an Anton Paar MCP 200 polarimeter (Anton Paar GmbH, Ostfildern, Germany). UV-1700 spectrophotometer (Shimadzu Corporation,

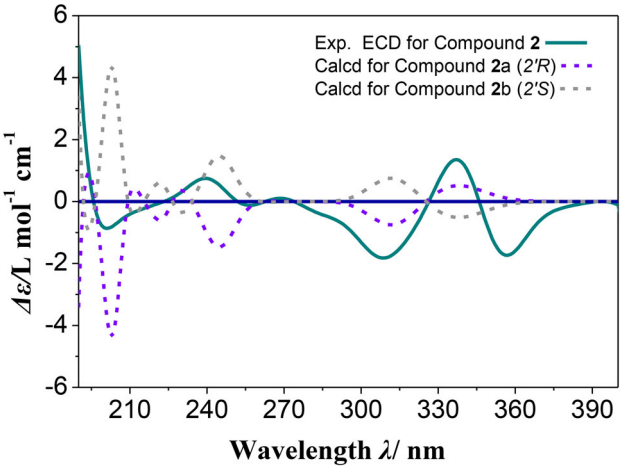

Figure 3. Calculated ECD spectra of (2′*R*)-**2** and (2′*S*)-**2** and experimental ECD spectrum of **2**.

Tokyo, Japan) was used to record UV spectra. The FT-IR Spectra of samples in KBr discs were recorded on a BrukerTensor-27 spectrometer (Bruker, Karlsruhe, Germany) with KBr pellets. Waters AutoSpec Premier P776 spectrometer (Waters Corp., MA, USA) was used to acquire HR-ESI-MS data. ECD spectrum was obtained using MOS-450 detector from BioLogic. NMR (^{1}H NMR, ^{13}C NMR, HMBC, and HSQC) spectra were obtained on a Bruker-AV-600 NMR spectrometer (Bruker Corporation, Bremen, Germany). Qingdao Ocean Chemical Factory provided Silica gel (Qingdao, China). GE Healthcare offered Sephadex LH-20 (Sweden) and ODS was purchased from YMC Co. Ltd. (Kyoto, Japan). Semi-preparative HPLC was conducted on an Agilent 1260 (Agilent Technologies Inc., CA, USA) with a DAD detector equipped with a C_{18} column (10 × 250 mm, 5 μm, YMC Co. Ltd., Japan). Molecular Device microplate reader was performed on a SpectraMax plus 384 (MD, USA).

3.2. Plant material

Dried roots of *Z. nitidum* were purchased in May 2017 from Anguo Market of Hebei Province, China. The plant material was authenticated by Prof. Wei Ning (College of Horticulture, Shenyang Agricultural University). A voucher specimen (ZN-2017112803) was deposited at the Department of Animal Pharmacy of Shenyang Agricultural University.

3.3. Extraction and isolation

The air-dried powdered roots of *Z. nitidum* (3.5 kg) was extracted three times with 95% EtOH under reflux. The EtOH extract was concentrated *in vacuo* to yield a brown-yellow gum (53 g). The residue was then suspended in water (5 L) and partitioned successively with CH_2Cl_2 (3 × 5 L) and *n*-BuOH (3 × 5 L). The CH_2Cl_2 extract (26 g) was separated by chromatography over a silica gel column using a gradient system of increasing polarity *n*-hexane – acetone (100:0–0:100, v/v). The collected

fractions were combined based on their TLC characteristics using UV light to yield seven fractions (Fr. A–Fr. G). Fr. B (4.5 g, *n*-hexane–acetone/20:1) was subjected to Sephadex LH-20 column chromatography (CC) and eluted with CH_2Cl_2–MeOH (1:1) to yield ten fractions (Fr. B1–Fr. B10). Fr.B8 was further separated by HPLC on a semi-preparative YMC C-18 column using MeOH–H_2O (70:30) as the mobile phase to provide **1** (7.2 mg, t_R = 18.2 min). Repeated chromatography of Fr. D (5.2 g, *n*-hexane–acetone/20:1) on a column of silica gel which was eluted with a gradient system of *n*-hexane-acetone (100:3–10:3, v/v) afforded Fr. D1–Fr. D8. Fr. D3 (1.3 g, *n*-hexane–acetone/100:7) was repeatedly recrystallized in *n*-hexane-EtOAc (10:1) to give **2** (2.3 mg). Fr. D5 (1.1 g, *n*-hexane–acetone/10:1) was purified by ODS column eluted with MeOH-H_2O system (80:20) to obtain **4** (2.2 mg). Fr. E (8.3 g, *n*-hexane–acetone/10:1) was purified by Sephadex LH-20 CC eluted with MeOH to afford five fractions (Fr. E1 − E5). Fr. E2 was further separated by semi-preparative HPLC (2.0 ml/min) on a YMC C-18 column using MeOH-H_2O (80:20, v/v) as the mobile phase to yield **3** (2.4 mg, t_R = 13.1 min). Fr. E4 was repeatedly recrystallized in *n*-hexane-acetone (10:1) to give **6** (2.6 mg). Fr. E5 was separated by a preparative TLC with a developing solvent system of *n*-hexane–acetone (3:1) to provide **5** (2.3 mg), which were further purified on Sephadex LH-20 CC in methanol.

3.3.1. 3′-Hydroxytoddanone (1)

Colorless needle crystal; UV (MeOH) λ_{max}: 201, 326 nm; IR (KBr) ν_{max}: 3435, 1631, 1608, 1492, 1441, 1174, 1005, 799 cm^{-1}; ^1H and ^{13}C NMR spectral data are shown in Table 1. HR-ESI-MS: *m/z* 307.1178 $[M+H]^+$ (calcd for $C_{16}H_{19}O_6$, 307.1176).

3.3.2. Isotoddalolactone (2)

Colorless needle crystal; $[\alpha]^{20}_D$ −36.0 (*c* 0.05, MeOH); ECD (MeOH): $\Delta\varepsilon_{205\,nm}$ +1.76, $\Delta\varepsilon_{211\,nm}$ −1.85, $\Delta\varepsilon_{223\,nm}$ +1.79, $\Delta\varepsilon_{306\,nm}$ −1.68, $\Delta\varepsilon_{339\,nm}$ +3.29, $\Delta\varepsilon_{351\,nm}$ −2.04; UV (MeOH) λ_{max}: 317 nm; IR (KBr) ν_{max}: 3443, 1715, 1630, 1368, 1143, 1009, 800 cm^{-1}; ^1H and ^{13}C NMR spectral data are shown in Table 1. HR-ESI-MS: *m/z* 277.1063 $[M+H]^+$ (calcd for $C_{15}H_{17}O_5$, 277.1071).

3.4. Cell growth inhibition assays

The cell growth inhibitory activities of the compounds were evaluated using the human leukaemia HL-60, K562 and THP-1 cell lines by MTT method. The detailed methodology for the cell growth inhibition test has been described in a previous paper [23], with 5- fluorouracil used as the positive control.

3.5. Computational details

The Spartan 14.0 searches based on molecular mechanics with MMFF94S force fields were performed for **2**. Selected meaningful conformers were further optimized by the time-dependent density functional theory (TDDFT) method at the B3LYP/6-31G(d) level in Gaussian 09 program package, which were further checked by frequency calculation and resulted in no imaginary frequencies. Theoretical ECD curves were

calculated at the B3LYP/6-31++G(d,p) level with the CPCM model and simulated using SpecDis 1.51 according to Boltzmann distributions [24].

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Disclosure statement

No potential conflict of interests was reported by the authors.

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