A new coumarin from pericarps of Zanthoxylum bungeanum Maxim

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Abstract
A new coumarin, 7-oxo-7H-furo-[3,2-g]chromen-9-yl dimethylcarbamate, is isolated from a methanol extract of Zanthoxylum bungeanum pericarps. The structure of this compound 1 is elucidated based on extensive spectroscopic analysis, including infrared, nuclear magnetic resonance, and mass spectrometry. This new compound is also synthesized by a simple acylation reaction with dimethylcarbamoyl chloride. The inhibitory activity of the isolated compound against HeLa and HepG2 cell lines is described. The protein tyrosine phosphatase 1B inhibitory activity against HeLa and HepG2 cancer cells and the sirtuin 1 inhibitory activity against HepG2 cancer cells are also evaluated.

Keywords
coumarins, inhibitory activity, spectroscopic analysis, Zanthoxylum bungeanum Maxim

Introduction
Zanthoxylum bungeanum Maxim. is a species of the Rutaceae family that is widely used, especially in China, Japan, Korea, and India, due to its medicinal properties and edible nature. The pericarps of Z. bungeanum, which are commonly referred to as Qing Hua-Jiao in China, are used as a food additive with a spicy and hot taste. For many years, they have also been used as a traditional herbal medicine to treat inflammatory diseases, ascariasis, diarrhea, epigastric pain, stomach ache, and dysentery. According to previous studies, the chemical components of Z. bungeanum pericarps consist mainly of essential oils, polysaccharides, and alkylamides. Meanwhile, the extracts of Z. bungeanum pericarps were also reported to show antifungal, bacteriostatic, analgesic, anesthetic, anti-inflammatory, and anticancer activities. In this study, one undescribed coumarin was isolated from a methanol extract of Z. bungeanum pericarps and identified as 7-oxo-7H-furo-[3,2-g]chromen-9-yl dimethylcarbamate. Herein we report the isolation, structural determination, inhibition activity against HeLa and HepG2 cancer cells, the protein tyrosine phosphatase 1B (PTP1B) and sirtuin 1 (SIRT1) inhibitory activity against HepG2 cancer cells, and the PTP1B inhibitory activity against HeLa cancer cells of compound 1.

Results and discussion
A 70% methanol extract of the dried pericarps of Z. bungeanum Maxim. was chromatographed through D101 macroporous resin, silica gel, and C18 reverse-phase silica gel to yield one undescribed compound 1 (Figure 1), the molecular structure of which was determined by diverse spectroscopic methods (Supplemental material).

Compound 1 was obtained as a white amorphous powder. Its molecular formula was identified by electrospray ionization high-resolution mass spectrometry (ESI HRMS) as C_{14}H_{11}NO_{5} based on the mass ion at m/z 296.0540 [M+Na]+ (calcd 296.0529). The infrared (IR) absorptions at 1725, 1594, 1634, 1499, and 1442 cm⁻¹ suggested the presence of a carbonyl, a carbon–carbon double bond, and a benzene ring. The 1H NMR (nuclear magnetic resonance) spectrum of compound 1 showed the presence of a furanocoumarin skeleton with signals being present at δH 6.36 (1H, d, J = 9.6 Hz, H-3), 7.78 (1H, d, J = 9.6 Hz, H-4), and 7.54 (1H, s, H-5); two doublets at δH 6.83 (1H, d, J = 2.2 Hz, H-6) and 7.68 (1H, d, J = 2.2 Hz, H-7); and two methyl singlets at δH 3.08 (3H, s, H-2′) and 3.27 (3H, s, H-3′). The 13C NMR spectrum showed one carbonyl carbon at δC 153.3 (CO, C-1′). The 1H-1H COSY spectrum showed the spin systems of H-3/H-4 and H-6/H-7 (Figure 2). The HMBC correlations from H-3 to C-2/4a, from H-4 to C-2/5/9a, from H-5 to C-4/6/8a/9a, from...
H-6 to C-7/5a/8a, and from H-7 to C-6/5a/8a suggested the presence of a 9-substituted furanocoumarin (Figure 2). In addition, the HMBC correlations from H-2′ to C-1′/3′ and from H-3′ to C-1′/2′ (Figure 2) indicated that it contains a dimethylformamide. Thus, compound 1 was determined to be 7-oxo-7H-furo-[3,2-g]chromen-9-yl dimethylcarbamate.

Moreover, compound 1 was also synthesized by a simple acylation reaction with dimethylcarbamoyl chloride (Scheme 1). First, one molecule of hydrogen was removed from xanthotol by reaction with the acyl chloride. Subsequently, the generated HCl was neutralized by K2CO3 to give the final product.

The new coumarin 1 was also evaluated for its inhibitory activity against HeLa and HepG2 cancer cell lines by using the MTT method. After seeding of the compound at concentrations of 50, 100, and 200 µM for 48 h, compound 1 showed strong inhibitory effects against the cell proliferation of these two types of cancer cells (Figures 3 and 4). As the concentration of the compounds increased, the cell viability decreased significantly. Meanwhile, compound 1 was found to exhibit PTP1B inhibitory activity against HeLa cancer cells (Figure 5), as well as PTP1B and SIRT1 inhibitory activity against HepG2 cells (Figures 6 and 7), both in a concentration-dependent manner.

Conclusion

Z. bungeanum has been widely used in Asian countries for a long time due to its medicinal properties and edible nature. Here, a new coumarin (1) is isolated from a methanol extract of Z. bungeanum pericarps and is also synthesized by a simple acylation reaction with dimethylcarbamoyl chloride. This new compound shows inhibitory activity against HeLa and HepG2 cell lines. Meanwhile, this compound also exhibits PTP1B and SIRT1 inhibitory activity, while the specific mechanism of action needs a further study.

Experimental

General experimental procedures

IR and UV (ultraviolet) spectra were obtained on an IFS120HR 670 Fourier Transform Infrared (FTIR) spectrometer (Karlsruhe, Germany) and a PerkinElmer Lambda 750 S spectrometer (PerkinElmer, USA), respectively. The high-resolution electrospray ionization mass spectrometry (HR-ESIMS) were acquired on a Bruker microTOF-Q II mass spectrometer (Karlsruhe), and the NMR spectra were recorded at 400 MHz for 1H and 100 MHz for 13C on a Bruker Avance III-400 spectrometer (Karlsruhe, Germany). The D101 macroporous resin was purchased from Xi’an Lanxiao Technology New Materials Co. Ltd. (Xi’an, China). Silica gel (Qingdao Marine Chemical Factory of China, Qingdao, China) was used for column chromatography (CC). Preparative thin-layer chromatography (TLC) was conducted on high-performance silica gel preparative TLC plates (HSGF254, glass precoated, Yantai Huanghai Silica Gel Development Co., Ltd., Yantai, China). Xanthotol was obtained from Chengdu PureChem-Standard Co., Ltd. (Chengdu, China). Dimethylcarbamoyl chloride was purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). All reagents and solvents were of analytical grade.

Plant material

The pericarps of Z. bungeanum Maxim. were collected from Longnan City of Gansu Province in China in 2016 and authenticated by Dr. Yin Qiang. A specimen (HJ2016001) was deposited at the School of Pharmacy, Lanzhou University.

Extraction and isolation

The dried pericarps of Z. bungeanum Maxim. (1.0 kg) were chopped and extracted with 70% methanol in water (3 × 1L, 7 days each time at room temperature). The crude extract was concentrated to dryness (93 g) under reduced pressure. Next, the residue was suspended in water (0.3 L), and partitioned sequentially with petroleum ether (PE;
3 × 0.3L) and ethyl acetate (EtOAc) (3 × 0.3L), respectively, three times. After removing the solvent, the EtOAc extract (34 g) was chromatographed through a D101 macroporous resin column and eluted with an ethanol–water gradient system (0:100, 30:70, 60:40, 90:10, 100:0, v/v). The fractions that were obtained from the 60:40 ethanol–water elute were evaporated to obtain the dried residue (16 g), which was chromatographed over silica gel using a PE–acetone gradient system (v/v = 50:1, 40:1, 30:1, 20:1, 10:1, 6:1, 3:1, 1:1) to give fractions 1 to 6 according to TLC analysis. Fraction F4 (3.1 g) was further fractionated by a C18 reverse-phase silica gel CC with a methanol–water gradient system (v/v = 50:50, 60:40, 70:30, 80:20, 90:10, 100:0) to yield eight fractions (F4-1–F4-8). Fraction F4-5 (603 mg) was subjected to silica gel CC using CH2Cl2/EtOAc (10:1, v/v) to obtain compound 1 (26 mg). The yield of the isolated compound from the dried pericarp was 0.0026%.

Scheme 1. Synthesis of compound 1.
Reagents and conditions: (a) K2CO3, room temperature, overnight, 90%.

Figure 3. Inhibition effects of compound 1 against the HeLa cell line.

Figure 4. Inhibition effects of compound 1 against the HepG2 cell line.

Figure 5. Inhibitory effects of compound 1 on the PTP1B enzyme in the HeLa cell line.

Figure 6. Inhibitory effects of compound 1 on the PTP1B enzyme in the HepG2 cell line.
7-oxo-7H-furo-[3,2-g]chromen-9-yl dimethylcarbamate (1): white amorphous powder; UV (CH₃OH): \( \lambda_{\text{max}} \) (log ε) 211 (3.61), 244 (3.66), 292 (3.29) nm; IR (film): \( \nu_{\text{max}} \) 2925, 2855, 1725, 1634, 1543, 1390, 1159, 1145, 1085 cm\(^{-1}\); \(^1\)H and \(^{13}\)C NMR data, see Table 1; HR-ESIMS m/z 296.0540 [M + Na]\(^+\) (calcld for C\(_{14}\)H\(_{11}\)NO\(_5\)Na: 296.0529).

Chemical synthesis

Compound 1 was synthesized according to the literature, with a minor modification. In brief, to a solution of xanthotoxin (50 mg, 0.25 mmol, 1 equiv.) in N,N-dimethylformamide (DMF) (5 mL) was added K₂CO₃ (52 mg, 0.375 mmol, 1.5 equiv.) at 0 °C under an argon atmosphere. After stirring at 0 °C for 1 h, dimethylcarbamoyl chloride (40 µL, 0.375 mmol, 1.5 equiv.) was added. The mixture was stirred at room temperature overnight. The reaction progress was monitored by TLC. After the reaction terminated, the mixture was percolated and concentrated in vacuo. The residue was purified by silica gel chromatography (EtOAc/dichloromethane = 1:10, v/v) to give product 1 as a white amorphous powder (60 mg, 90%).

Biological assays

Cell cultures and reagents. The HeLa and HepG2 cell lines were obtained from the China Center for Type Culture Collection (CCTCC). The cancer cell lines were cultured in Dulbecco’s modified Eagle medium (DMEM; Gibco, USA) with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (Biyuntian Biotechnology Co., Ltd., Shanghai, China), and were maintained at 37°C in a humidified atmosphere with 5% CO₂. Paclitaxel was used as positive control and was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. For evaluation of the inhibitory activities against cancer cells, the test compounds were first dissolved in dimethyl sulfoxide, divided into aliquots, and frozen at −20°C until used. The test compounds were diluted with phosphate-buffered saline (PBS) to give the respective concentrations. In all experiments, DMSO was applied as a solvent control, and the final concentration of DMSO was always kept at ≪0.1%.

Cell viability assay (CCK-8 assay). HeLa/HepG2 cells were seeded onto 96-well plates at a density of 5 × 10³ cells/well. After 24 h of culturing in 100 µL DMEM, the cells were untreated (control) or treated with a solution of 10 µL of compound 1 at concentrations of 50, 100, and 200 µM and incubated for 24 h. Next, the medium was exchanged with 100 µL of DMEM that contained 10 µL of the CCK-8 reagent (Beijing Solarbio Science & Technology Co., Ltd., P.R. China). The plate was incubated at 37°C for 1 h in the dark. The absorbance was measured at 450 nm with a microplate reader (RT-6100, Rayto Life and Analytical Sciences Co., Ltd., Shenzhen, P.R. China).

PTP1B/SIRT1 inhibitory activity assay in cell lysate. A human PTP1B/human SIRT1 enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Fanke Industrial Co., Ltd., Shanghai, P.R. China) was used to determine the PTP1B/SIRT1 inhibitory activity of compound 1. The HeLa/HepG2 cells (with a density of 10 × 10⁴ cells/mL) were treated with test compound 1 (concentrations of 50, 100, and 200 µM) and incubated for 24 h at 37°C in 35 mm × 10 mm style cell culture dishes with 2 mL of DMEM. The cells were treated with trypsin and suspended at a density of 10 × 10⁵ cells/mL in PBS (pH 7.2–7.4). The cells were then subjected to repeated freeze–thaw cycles three times and were centrifuged at 2000 r/min at 4°C for 20 min. Fifty microliter samples of the supernatants (test samples) or the standard sample solution were transferred to the 96-well enzyme-labeled microplates, and 100 µL of an enzyme-labeled antibody complex (horseradish peroxidase (HRP)) was added to each well of the microplates. The plates were sealed with a sealing membrane and incubated at 37°C for 60 min. Next, the microplates were washed 5 times, and 50 µL of developer...
A (H$_2$O$_2$) and developer B (3,3′,5,5′-tetramethylbenzidine (TMB)) were added in turn. The solution in plates was gently shaken to mix evenly. The reactions proceeded in a dark environment at 37 °C for 15 min and were then quenched via the addition of 50 µL of H$_2$SO$_4$ solution. The concentration of the reaction product of TMB was calculated from the UV absorbance at 450 nm, which was measured using an RT-6100 microplate reader (Rayto Life and Analytical Sciences Co., Ltd.). The nonenzymatic reaction of TMB was estimated through a blank analysis, which was utilized to correct the concentration of the reaction product.

**Statistical analysis.** The results are expressed as the means ± standard deviation (SD) of three independent experiments (*p < 0.05, **p < 0.01, compared to the control). Statistical analysis was conducted on IBM SPSS Statistics 22 software (International Business Machines Corporation (IBM), Armonk, USA), and differences between group mean values were determined by one-way analysis of variance (ANOVA).

**Declaration of conflicting interests**
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was financially supported by Natural Science Foundation of Gansu Province (No. 21JR7RA486).

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**Supplemental material**
Supplemental material for this article is available online.

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