Two new abietane diterpenoid glycosides from *Clinopodium chinense*

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**ABSTRACT**

Two new abietane diterpenoid glycosides, named clinopoditerpenes B (1) and C (2), were isolated from *Clinopodium chinense*. The structures of the new compounds were determined on the basis of extensive spectral analysis. Compound 1 exhibited cardioprotective effect against H_{2}O_{2}-induced apoptosis in H9c2 cells.

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1. Introduction

The genus *Clinopodium* (Labiatae) contains about 20 species in the world. Many species of this genus are traditional medicinal plants with a wide distribution in the tropical and subtropical regions of south-east Asia (Zhong, Xu, et al. 2012). The aerial of *Clinopodium chinense* (Benth.) O. Kuntze, well known as ‘duanxueliu’ in China, has been used as a traditional...
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Folk medicine for the treatment of haematuria, trauma influenza and allergic dermatitis. Previous phytochemical investigations of *C. chinense* led to flavonoids (Aoshima et al. 2012), triterpenoid saponins (Liu et al. 1995; Wang et al. 2013) and phenylpropanoids (Murata et al. 2009), which showed anti-inflammatory and immunity, anti-hyperglycaemic, antitumour and antiradiation pharmacological effects (Tian et al. 2008). During our search for structurally interesting compounds from *C. chinense*, we have reported the isolation of diterpenes and prenylated naphthoquinoids (Zhong, Sun, et al. 2012; Zhong et al. 2014). Further investigations of *n*-BuOH-soluble portion of the extracts from the aerial of *C. chinense*, resulted in the isolation of two new diterpenoid glycosides, clinopoditerpenes B (1) and C (2) (Figure 1). In this paper, we report the isolation and structure elucidation of compounds 1 and 2 and their cardioprotective effects against H₂O₂-induced apoptosis in H9c2 cells.

![Figure 1. Structures of compounds 1 and 2.](image1)

![Figure 2. Protective effects of compound 1 against H₂O₂-induced H9c2 cell injury. H9c2 cells were pretreated with different concentrations of compounds 1 for 24 h before 150 mM H₂O₂ was added into the medium. After incubation for 6 h at 37 °C, cell viability was determined by MTT assay (expressed as the percentage of control). The data are expressed as mean ± SD from three independent experiments. Note: ##p < 0.01 vs. control; *p < 0.05 vs. H₂O₂-treated cell.](image2)
2. Results and discussion

Clinopoditerpene B (1) was isolated as a white amorphous powder ([α]20D = −10.7 (c 0.15, MeOH)). The molecular formula of 1 was established as C26H38O10 from the pseudomolecular [M + Na]+ ion at m/z 533.2356 (calcd 533.2363, Δ−0.001 mmu) in the HR-ESI-MS spectrum, and conjunction with NMR data (Table S1). The IR spectrum showed the presence of hydroxyl (3351 cm⁻¹), carbonyl (1672 cm⁻¹) and phenyl (1601 cm⁻¹) groups. The 1H NMR spectrum showed the presence of a glucopyranosyl unit at δH 4.44 (d, J = 7.8 Hz, H-1′). Acid hydrolysis of 1 (1.0 mg) with 2 N TFA (100 °C, 2 h) afforded D-glucopyranose, which were identified by GC analysis after derivatisation with L-cysteine methyl ester and silylation (Song et al. 2014). The proton and protonated carbon NMR signals of 1 were assigned unambiguously by the 1H-1H-COSY, NOeSY and HSQC experiments. In the HMBC spectrum (Figure S7), the correlation of H-1′ (δH 4.44)/C-12 (δC 148.6) indicated that the glucopyranosyl unit is located at C-12. Vicinal coupling constants, J (1,2), of the anomeric H-atoms (J = 7.8 Hz) indicated a diaxial coupling and β-configuration for the glucose residue. Acid hydrolysis of 1 (1.0 mg) with 2 N TFA (100 °C, 2 h) afforded D-glucopyranose, which were identified by GC analysis after derivatisation with L-cysteine methyl ester and silylation (Song et al. 2014). The HMBC correlations of the H-1′ (δH 5.27)/C-12 (δC 148.6) indicated that the hydroxyl group was located at C-12. Vicinal coupling constants, J (1,2), of the anomeric H-atoms (J = 7.8 Hz) indicated a diaxial coupling and β-configuration for the glucose residue. Acid hydrolysis of 1 (1.0 mg) with 2 N TFA (100 °C, 2 h) afforded D-glucopyranose, which were identified by GC analysis after derivatisation with L-cysteine methyl ester and silylation (Song et al. 2014). The HMBC correlations of the H-1′ (δH 5.27)/C-12 (δC 148.6) indicated that the hydroxyl group was located at C-12. Vicinal coupling constants, J (1,2), of the anomeric H-atoms (J = 7.8 Hz) indicated a diaxial coupling and β-configuration for the glucose residue. Acid hydrolysis of 1 (1.0 mg) with 2 N TFA (100 °C, 2 h) afforded D-glucopyranose, which were identified by GC analysis after derivatisation with L-cysteine methyl ester and silylation (Song et al. 2014). The HMBC correlations of the H-1′ (δH 5.27)/C-12 (δC 148.6) indicated that the hydroxyl group was located at C-12. Vicinal coupling constants, J (1,2), of the anomeric H-atoms (J = 7.8 Hz) indicated a diaxial coupling and β-configuration for the glucose residue. Acid hydrolysis of 1 (1.0 mg) with 2 N TFA (100 °C, 2 h) afforded D-glucopyranose, which were identified by GC analysis after derivatisation with L-cysteine methyl ester and silylation (Song et al. 2014). The HMBC correlations of the H-1′ (δH 5.27)/C-12 (δC 148.6) indicated that the hydroxyl group was located at C-12. Vicinal coupling constants, J (1,2), of the anomeric H-atoms (J = 7.8 Hz) indicated a diaxial coupling and β-configuration for the glucose residue. Acid hydrolysis of 1 (1.0 mg) with 2 N TFA (100 °C, 2 h) afforded D-glucopyranose, which were identified by GC analysis after derivatisation with L-cysteine methyl ester and silylation (Song et al. 2014). The HMBC correlations of the H-1′ (δH 5.27)/C-12 (δC 148.6) indicated that the hydroxyl group was located at C-12. Vicinal coupling constants, J (1,2), of the anomeric H-atoms (J = 7.8 Hz) indicated a diaxial coupling and β-configuration for the glucose residue. Acid hydrolysis of 1 (1.0 mg) with 2 N TFA (100 °C, 2 h) afforded D-glucopyranose, which were identified by GC analysis after derivatisation with L-cysteine methyl ester and silylation (Song et al. 2014).
3. Experimental

3.1. General experimental procedures

Optical rotation data were recorded using a Perkin-Elmer 341 digital polarimeter in MeOH. UV data were obtained using a Shimadzu UV2550 spectrometer in MeOH. IR data were recorded using an FTIR-8400S spectrometer. NMR spectra were recorded on Bruker AV III 600 MHz (the chemical shift values are presented as the δ values with TMS as an internal standard). HR-ESI-MS spectra were measured on a LTQ Orbitrap XL mass spectrometer (Thermo Scientific). Toyopearl HW-40C (75 μm, Tosoh Corporation, Tokyo, Japan), CHP 20P MCI gel (75–150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan), silica gel (100–200 mesh, Qingdao Haiyang Chemical Co., Ltd, Qingdao, China) and C-18 reversed-phase silica gel (50 μm, YMC CO., Ltd., Kyoto, Japan) were used for column chromatography. HPLC separation was performed on a CXTH LC-3000 HPLC system with a CXTH LC-3000 UV spectrophotometric detector and a YMC (250 × 10 mm) semi-preparative column packed with C18 (5 μm, YMC CO., Ltd., Kyoto, Japan) and precoated silica gel GF254 plates (Yantai chemical industry research institute, Yantai, China) were used for TLC. Sugar analysis was performed on an Agilent 6890 N GC equipped with a FID detector; injector temp. 250 °C; detector temp. 250 °C; N2 as carrier gas. D-glucuronidase was purchased from Sinopharm Chemical Reagent Co., Ltd. All solvents employed were analytical grade (Beijing Chemical Works, China).

3.2. Plant material

The plants were purchased from Chinese Medicinal Material Markets (Bozhou, China) in 2013, and identified by Dr Shichun Yu at the Anhui University of Traditional Chinese Medicine, Anhui, China. The voucher specimen (20130312) was deposited in the Key Laboratory of Bioactive Substances and resources Utilization of Chinese Herbal Medicine, Ministry of Education, Beijing, China.

3.3. Extraction and isolation

The dried and powdered aerial parts of C. chinense (15 kg) were extracted twice with 100 L of 70% ethanol (v/v) at 80 °C for 2 h. The combined solution was filtered and evaporated to remove ethanol under reduced pressure completely to yield an extract (3000 g). The extract was suspended in water and successively extracted with petroleum ether, ethyl acetate and n-BuOH. Approximately 310 g residue of n-BuOH was subjected to a D101 column (700 × 250 mm) and then eluted with 20% ethanol, 50% ethanol (v/v), 85% ethanol (v/v) and 100% ethanol (v/v). The fractions of 50 and 85% ethanol were collected and evaporated at 55 °C under vacuum; about 180 g of the 50 and 85% ethanol fractions of the total saponins were obtained. The total saponins (180 g) were loaded on SiO₂ column chromatography with CHCl₃/MeOH. The fraction eluted with CHCl₃/MeOH (100:15) (20 g) was purified on RP-18 with MeOH/H₂O to afford fractions A1–A6. Purification of fraction A2 by Toyopearl HW-40C (MeOH) to give 5 fractions (frs. A2.1–5). Fraction A2.3 was separated by ODS HPLC (YMC ODS-A, 10 × 250 mm; flow rate 2.0 mL min⁻¹; UV detection at 210 nm; eluent MeOH/ H₂O, 78:22) to isolate clinopoditerpene C (2, 10.6 mg). Fraction A2.4 was separated by ODS HPLC (YMC ODS-A, 10 × 250 mm; flow rate 2.0 mL min⁻¹; UV detection at 210 nm; eluent MeOH/H₂O, 70:30) to isolate clinopoditerpene B (1, 4.6 mg).
3.3.1. Clinopoditerpene B (1)
White amorphous powder; [α]D20 = −10.7 (c 0.15, MeOH); UV (MeOH) λmax (log ε) 270 (4.17), 318 (3.75) nm; IR (film) νmax 3351, 2969, 2930, 2871, 1672, 1602, 1426, 1327, 1068, 1023, 834 cm−1; 1H-NMR (600 MHz, DMSO-d6): δ 1.34 (1H, td, J = 12.6, 2.4 Hz, H-1α), 3.24 (1H, m, H-1β), 1.60 (1H, m, H-1α), 1.66 (1H, m, H-1β); 3.11 (1H, dd, J = 11.4, 4.2 Hz, H-3), 1.66 (1H, dd, J = 15.0, 1.8 Hz, H-5), 2.43 (1H, dd, J = 16.8, 1.8 Hz, H-6α), 2.61 (1H, dd, J = 16.8, 1.50 Hz, H-6β), 7.37 (1H, s, H-14), 2.69 (1H, dd, J = 13.2, 7.2 Hz, H-15), 2.82 (1H, dd, J = 13.2, 5.4 Hz, H-15), 1.02 (1H, d, J = 6.6 Hz, H-17), 0.82 (1H, s, H-18), 0.95 (1H, s, H-19), 1.31 (1H, s, H-20), 4.44 (1H, d, J = 7.8 Hz, H-1′), 3.30 (1H, m, H-2′), 3.20 (1H, m, H-3′), 3.21 (1H, m, H-4′), 3.26 (1H, m, H-5′), 3.50 (1H, br d, J = 11.4 Hz, H-6′), 3.50 (1H, br d, J = 11.4 Hz, H-6′); 13C-NMR (150 MHz, DMSO-d6): δ 34.9 (C-1), 75.9 (C-2), 75.9 (C-3), 38.7 (C-4), 49.4 (C-5), 34.8 (C-6), 179.7 (C-7), 127.8 (C-8), 138.5 (C-9), 39.2 (C-10), 147.5 (C-11), 148.6 (C-12), 131.7 (C-13), 119.9 (C-14), 38.9 (C-15), 65.9 (C-16), 23.5 (C-17), 15.6 (C-18), 27.9 (C-19), 17.0 (C-20), 105.8 (C-1′), 73.8 (C-2′), 76.0 (C-3′), 69.5 (C-4′), 75.9 (C-5′), 60.7 (C-6′); HR-ESI-MS: m/z 533.2356 [M + Na]+ (calcd for C26H38O14Na, 533.2363).

3.3.2. Clinopoditerpene C (2)
White amorphous powder; [α]D20 = −25.0 (c 0.08, MeOH); UV (MeOH) λmax (log ε) 282 (3.89) nm; IR (film) νmax 3370, 2928, 2875, 1632, 1420, 1070, 1024, 829 cm−1; 1H-NMR (600 MHz, pyridine-d5): δ 1.42 (1H, br t, J = 12.6 Hz, H-1α), 3.56 (1H, br d, J = 12.6 Hz, H-1β), 1.87 (1H, m, H-2α), 1.95 (1H, m, H-2β), 3.54 (1H, dd, J = 12.6, 3.6 Hz, H-3), 1.30 (1H, m, H-5), 1.63 (1H, m, H-6β), 1.83 (1H, m, H-6β), 2.83 (1H, m, H-7β), 2.92 (1H, dd, J = 11.4, 4.2 Hz, H-7α), 6.77 (1H, s, H-14), 4.45 (1H, m, H-15), 3.97 (1H, m, H-16), 4.26 (1H, m, H-16), 1.36 (1H, d, J = 6.6 Hz, H-17), 1.10 (1H, s, H-18), 1.29 (1H, s, H-19), 1.52 (1H, s, H-20), 5.27 (1H, d, J = 7.8 Hz, H-1′), 4.45 (1H, m, H-2′), 4.36 (1H, m, H-3′), 4.19 (1H, br t, J = 7.8 Hz, H-4′), 3.80 (1H, m, H-5′), 4.33 (1H, br d, J = 10.8 Hz, H-6′), 4.45 (1H, br d, J = 10.8 Hz, H-6′), 5.57 (1H, d, J = 6.6 Hz, H-1′′), 4.25 (1H, m, H-2′′), 4.25 (1H, m, H-3′′), 4.32 (1H, m, H-4′′), 3.97 (1H, m, H-5′′), 4.35 (1H, m, H-6′′), 4.41 (1H, m, H-6′′); 13C-NMR (150 MHz, pyridine-d5): δ 35.7 (C-1′), 29.5 (C-2), 78.5 (C-3), 40.4 (C-4), 53.5 (C-5), 19.9 (C-6), 33.7 (C-7), 134.9 (C-8), 134.7 (C-9), 40.2 (C-10), 149.1 (C-11), 142.7 (C-12), 137.4 (C-13), 118.6 (C-14), 35.1 (C-15), 69.1 (C-16), 19.5 (C-17), 17.2 (C-18), 29.6 (C-19), 20.4 (C-20), 105.8 (C-1′), 85.5 (C-2′), 78.2 (C-3′), 71.1 (C-4′), 79.3 (C-5′), 62.6 (C-6′), 107.3 (C-1′′′), 76.7 (C-2′′′), 78.5 (C-3′′′), 71.6 (C-4′′′), 79.2 (C-5′′′), 62.8 (C-6′′′); HR-ESI-MS: m/z 681.3096 [M + Na]+ (calcd for C32H50O14Na, 681.3098).

3.4. Acid hydrolysis
Clinopoditerpenes B and C (1 mg) were heated in 1 ml TFA (2 N, 95 °C, 2 h). The solutions were extracted three times with 1 mL CHCl3. The aq. phase was dried and the residue redissolved in anhydrous pyridine. The sugars were derivatized with L-cysteine methyl ester hydrochloride (1.5 mg) at 60 °C for 1 h and then silylated with hexamethyldisilzane and chlorotrimethylsilane (Fluka) (2:1, 0.5 mL) at 60 °C for 30 min. GC Analysis on a capillary HP-5 column (30 m x 0.25 mm i.d., 0.25 m; Agilent; column temp. 150 °C for 2 min, then 5 °C/min to 210 °C) using N2 (1 mL min−1) as carrier gas. The splitting ratio was 1/50, and the injection and detector temperature was set at 250 °C. A 3 μL portion of the supernatant was injected for analysis.
3.5. H9c2 cell protection assay

H9c2 cells at a density of 5 x 10^4 cells per well were cultured in DMEM media (Hyclone) supplemented with 10% foetal bovine serum (FBS, Hyclone), and L-glutamine (2 mM). Cultures were maintained at 37°C in 5% CO₂ in a humidified incubator for 24 h. After 6 h of treatment with four different concentrations of drugs, followed by incubation with 150 mM H₂O₂ for 2 h, 20 μL of 5 mg mL⁻¹ MTT solution was added to each well (0.1 mg/well), and incubated for 4 h. The supernates were aspirated, and the formazan crystals in each well were dissolved in 150 μL of DMSO. The absorbance was measured at 570 nm on a microplate reader (BioTek, Vermont). The survival rate of H9c2 cells was evaluated and the inhibition (%) was expressed as the percentage of control (Sun et al. 2011).

Supporting information

Supporting Information as well as MS, NMR, UV, IR spectra of compounds 1 and 2 is available online.

Disclosure statement

No potential conflict of interest was reported by the authors.

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