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Penchinones A–D, two pairs of cis-trans isomers with rearranged neolignane carbon skeletons from Penthorum chinense†

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Penchinones A–D (1–4), two pairs of novel cis-trans lignan isomers with rearranged neolignane carbon skeletons, together with six known flavonoids (5–10), were isolated from the ethyl acetate-soluble portion of a hepatoprotective water decoction of Penthorum chinense. Their structures were determined by extensive spectroscopic data analysis, ECD calculation, and single-crystal X-ray diffraction. Penchinones C and D (3 and 4) featured an unprecedented 7,3-neolignane carbon skeleton. Penchinone A (1) exhibited protective activity against acetaminophen-induced damage to the HL7702 hepatocyte and selective cytotoxicity against the human cancer ovarian cell line, Hey, in vitro.

Introduction

Lignans are a class of naturally occurring phenylpropanoid dimers that may play an important role in plant growth and plant defense against various biological pathogens and pests.1–3 In addition to their biological functions in plants, lignans have significant pharmacological effects, including antibacterial, antiviral, antitumor, antioxidant, anti-inflammatory, immunosuppressive, cardiovascular, and hepatoprotective activities.1–3 Although the molecular backbone consists only of two phenylpropane (C6–C3) units, lignans exhibit an enormous range of structural diversity because of the various linkage patterns of their C6–C3 units. Under IUPAC nomenclature, lignans are classified into three types: classical lignans, neolignans, and oxyneolignans. If the two C6–C3 units are linked by a direct carbon–carbon bond other than an 8-8’ linkage, the compounds are defined as neolignans.4 In the past few decades, phytochemical investigations have led to the isolation of hundreds of neolignans of 21 subtypes: 8,3’-neolignane, 3,3’-neolignane, 8,1’- neolignane, 1,3’-neolignane, 1,7’-neolignane, 8,7’-neolignane, 2,9’- neolignane, 9,9’-neolignane, 8,9’-neolignane, 3,4’-neolignane, 3,4’- neolignane, 5,7-cyclo-8,3’-neolignane, 1’,7-cyclo-8,3’-neolignane, 6’,9-cyclo-8,3’-neolignane, 7’,9-cyclo-8,1’-neolignane, 3’,7-cyclo- 8,1’-neolignane, 2’,7-cyclo-8,7’-neolignane, 2’,6’-cyclo-1,3’-neolignane, 1’,7:2’,8-dicyclo-8,3’-neolignane, and 1’,3:2’,2’:6,6’-tricyclo-1,3’-neolignane.5–4

Penthorum, a genus originally in the family Crassulaceae, was moved to the family Saxifragaceae in 1981, but has been considered a separate family by the APG (Angiosperm Phylogeny Group) system since 1998. Penthorum chinense Pursh is one of the only two species in the family Pentagonaceae, and is widely distributed and cultivated in Sichuan Province, China.5 In this region, the plant is commonly used as a folk medicine for the treatment of hepatic and gall diseases, and as an ingredient of various comestibles.6,7 Our previous investigation of the hepatoprotective portion of the water decoction of P. chinense led to the identification of several lignans. Some exhibited protective activities against acetaminophen-induced hepatocyte injury.7 As continuous search for hepatoprotective secondary metabolites from P. chinense, the bioactive portion was further studied, affording two pairs of cis-trans isomers with unprecedented rearranged neolignane skeletons (1–4) and six known flavonoids (5–10) (Fig. 1). We present herein the details of isolation, structure elucidation, hypothetical biogenesis, and bioactivities of 1–4.

Fig. 1 Structures of compounds 1–10.

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*Electronic Supplementary Information (ESI) available: 1D, 2D NMR, and HRESIMS IR spectra for compounds 1–4, X-ray data for 2, and ECD calculation details for 3. See DOI: 10.1039/x0xx00000x
Results and discussion

Penchinoine A (1) exhibited IR absorptions characteristic of hydroxyl (3317 cm⁻¹), carbonyl (1652 and 1614 cm⁻¹), aromatic ring and olefinic bond (1580, 1503, and 1433 cm⁻¹) functionalities. The molecular formula of 1 was C₃₀H₄₀O₆ with 11 degrees of unsaturation, as indicated by HRESIMS (m/z 365.1002 [M + Na]⁺, calcd 365.1001). The 1H NMR spectrum of 1 exhibited resonances attributable to a 1,2,3,4-tetrasubstituted phenyl (δH 6.27 (d, J = 8.4 Hz) and 6.72 (d, J = 8.4 Hz)), a 1,2,4,5-tetrasubstituted phenyl (δH 8.05 (s) and 6.68 (s)), a trans-propenyl (δH 6.72 (dq, J = 15.6, 1.2 Hz), 5.50 (dq, J = 15.6, 6.6 Hz), and 1.94 (dd, J = 6.6, 1.2 Hz)), and an aromatic acetyl group (δH 2.50), together with an aromatic methoxy (δH 3.88) (Table 1). The 13C NMR and DEPT spectra of 1 revealed 19 carbon resonances assignable to the above protonated units and 10 quaternary carbons, including two ketone carbonyl (δC 203.8 and 199.0) and eight aromatic quaternary carbons (four of which are oxygenated).

To determine the structure of 1, a comprehensive analysis of the 2D NMR data was conducted. In the 1H-1H gCOSY spectrum of 1, the homonuclear coupling correlations between H-5/H-6 and H-7/H-8/H-9 verified the presence of both the 1,2,3,4-tetrasubstituted phenyl and the propenyl (Fig. 2). The connection of the two phenols via the carbonyl C-7 could be deduced from the key HMBC correlations of H-6 and H-5’ with C-7, together with the weak four-bond W-type correlations of H-5 and H-2’ with C-7. The acetyl group was assigned to C-3’, based on the HMBC correlations from H-2’ to C-8, H-9 to C-8/C-3’, and H-5’ to C-3’. Meanwhile, the HMBC peak of H-7 with C-2’/C-6’/C-9’ and H-8’ with C-1’ revealed that the trans-propenyl was located at C-1’. Furthermore, the HMBC correlations of H-5 with C-1’/C-3/C-4, H-6 with C-2/C-4, OMe with C-3, H-2’ with C-4’/C-6’/C-7’, and H-5’ with C-1’/C-3’, together with their chemical shifts and molecular formula, indicated that the methoxy and three hydroxyl groups were substituted at C-3, C-2, C-4, and C-6’, respectively. Thus, the structure of 1 was established.

The spectroscopic data of penchinoine B (2) indicated that it was an analog of 1 with the same molecular formula: C₃₀H₄₀O₆. A comparison of the NMR data of 2 and 1 revealed a cis-propenyl in 2, replacing the trans-propenyl unit in 1 (Table 1). This was confirmed by 2D NMR data analysis. Since some weak four- and five-bond correlations could also be found in the HMBC spectrum, the structure of 2 was further confirmed using single-crystal X-ray crystallographic analysis. An ORTEP drawing, with the atom numbering indicated, is shown in Fig. 3.

Penchinoine C (3) also exhibited spectroscopic resonances for a 1,2,3,4-tetrasubstituted phenyl, a 1,2,4,5-tetrasubstituted phenyl, a trans-propenyl, and an acetyl, similar to those of 1. However, the 1H NMR, 13C NMR, and DEPT spectra revealed that an obviously deshielded isolated methine (δH 4.69, δC 54.2) in 3 replaced the ketone carbonyl in 1. The HRESIMS data for 3 indicated that it had a molecular formula of C₃₀H₄₀O₆. These data suggested that 3 was a lignane with two C₆-C₆ units, which was confirmed by the 2D NMR experiments. In the HMBC spectrum, correlations from H-5 to C-1/C-3/C-4, H-6 to C-2/C-4/C-7, and OMe to C-3, verified the presence of the 1,2,3,4-tetrasubstituted phenyl group with a methoxy unit at C-3 and two oxygenated substituents at C-2 and C-4. The HMBC correlations of H-2’ with C-4’/C-6’/C-7’, H-5’ with C-
Penichone D (4) has the same molecular formula as that of 3, as indicated by the HRESIMS analysis. The 1D NMR data (Table 1) were similar to those of 3, with the exception of the substitution of the resonances for the trans-propenyl moiety in 3 by those attributable to a cis-propenyl unit in 4 [δH 6.44 (dd, J = 12.0, 1.8 Hz), 5.77 (dq, J = 12.0, 6.6 Hz), and 1.80 (dd, J = 6.6, 1.8 Hz); δC 125.6 (C-7), 127.1 (C-8'), and 14.8 (C-9')]. The 2D NMR data and [α]D value of 4 confirmed that it was 7Z-isomer of 3.

The biosynthetic precursors of 1–4 were proposed to be penthorin A (11) and B (12), as the two compounds were previously isolated from the same extract of P. chinense.7 In addition, their analogs have been detected in this species by other researchers.8 As shown in Scheme 1, penthorin A and B are enolized first, after which two possible biosynthetic pathways (routes I and II) are postulated for 1/2 and 3/4, respectively. In route I, the oxidation of the enol intermediate would open the 7,8-bond, liberating a carbonyl group at C-1 and an acetyl group at C-3. Then, an enzyme-catalyzed reduction reaction occurs to produce an aldehyde intermediate. Finally, we determined that the key biochemical reaction would be a free radical reaction combined with a proton transfer, which reconstructs a C-7/C-4 carbon bond to produce 1/2. As indicated in route II, the protonation of 7-OH in the enol unit and the loss of H2O generate a carbocation center at C-7. Subsequently, an enzymatic rearrangement could lead to the migration of the 8,3'-bond to C-7. Then, the carbocation center created at C-8 would be neutralized by H2O, with the subsequent loss of a proton forming an enol intermediate and producing 3/4.

Table 1 NMR spectroscopic data of 1–4

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* NMR data (δ) were measured at 600 MHz for 1H and at 150 MHz for 13C in CD3OD. Proton coupling constants (J) in Hz are given in parentheses. The assignments were based on 1H-1H gCOSY, gHSQC, and gHMBC experiments.

Scheme 1 Hypothetical biogenetic pathway for 1–4

The six known compounds were identified by comparison of spectroscopic data with those reported in the literatures as pinocembrin (5),11 pinostrobin (6),12 pinocembrin-7-O-β-D-glucopyranoside (7),13 5-methoxy-pinocembrin-7-O-β-D-glucoside (8),13 (E)-3-phenyl-1-(2,4,6-trihydroxyphenyl)prop-2-en-1-one (9),15 and pinostrobin chalcone (10).16
Based on the linking position of the two C₆–C₃ units, the reported neolignans were divided into 21 carbon skeletons (Fig. 5). The majority of the neolignans were 8,3'-neolignanes, 8,1'-neolignanes, and 3,3'-neolignanes, especially 4',7-epoxy-8,3'-neolignanes (benzofurans). In recent years, more than one hundred 5'-7-cyclo-8,3'-neolignanes, 5'-7-cyclo-8,3'-neolignanes, and 7,8-cyclo-8,7'-neolignanes have been also detected in several families, including the Lauraceae, Magnoliaceae, and Bignoniaceae. However, other types of neolignanes are comparatively rare, according to the literature of the past few decades. This study discovered two unprecedented rearranged neolignane carbon skeletons from the plant kingdom.

Since new compounds 1–4 were isolated from the hepatoprotective water decoction of *P. chinense*, they were evaluated further to determine their protective activities against acetaminophen (AP)-induced damage to HL7702 hepatocytes in vitro, with the exception of compound 4, because the available sample quantity was too small to conduct the analysis. Compounds 2 and 3 exhibited weak activity, while a significant increase in the viability of HL7702 cells examined via a MTT colorimetric assay showed that 1 provided effective protection against AP-induced damage (Fig. 6). Furthermore, Image-iT Green viability stain and Hoechst 33342 staining were used to demonstrate that 1 could protect damaged HL7702 cells, using a high-content screening (HCS) experiment (Fig. 7). The survival rate of acetaminophen-treated HL7702 cells rose from 35.7% in the model group to 91.0% and 86.4% in two test groups containing 1 at 10 μM and 1 μM, respectively. Interestingly, in the assay of cytotoxic activity against human hepatocellular carcinoma cell line HePG-2, lung cancer cell lines A549 and NCI-H1975, breast cancer cell lines MDA-MB-231 and MCF-7, and ovarian cancer cell line Hey, compound 1 also exhibited selective cytotoxicity against Hey with IC₅₀ value of 13.7 μg/mL.

![Fig. 5](image_url) The reported carbon skeletons of neolignanes.

![Fig. 6](image_url) Protective activity of 1 against AP-induced HL7702 cell damage (*p < 0.05 vs Control, **p < 0.05 vs Model*).

![Fig. 7](image_url) Nuclear DNA staining of the HL7702 cells, where Hoechst 33342 stained live and dead cells blue, image-iT Green viability stain stained dead cells green.

**Experimental**

**General experimental produces**

Optical rotations were measured using a Perkin-Elmer 341 plus. CD spectra were recorded on a JASCO J-815 CD spectrometer. IR spectra were recorded on a Nicolet 5700 FT-IR microscope instrument. NMR spectra were obtained using a Bruker-AVIIIHD-600 spectrometer with the solvent peaks used as the references. HRESIMS spectra were measured using a Waters Synapt G2 HDMS. Column chromatography (CC) was performed using silica gel (200–300 mesh, Yantai Institute of Chemical Technology, Yantai, China), MCI gel CHP 20P (75–150 μm, Mitsubishi Chemical, Co., Japan), and Sephadex LH-20 (Amersham Pharmacia Biotech AB, Uppsala, Sweden). HPLC separation was performed using an instrument equipped with a Cometro 6000LDS pump, a Cometro 6000PVW UV/VIS detector, and an Ultimate (250 × 10 mm²) preparative column packed with C18 (5 μm). TLC was performed using glass precoated silica gel GF254 plates (Qingdao Marine Chemical Inc., Qingdao, China).

**Plant material**
P. chinense was collected in July of 2012 from the culture field in Guling, Sichuan Province, China. Plant identity was verified by Prof. Min Li (Chengdu University of TCM, Sichuan, China). A voucher specimen (SGHC-20120725) was deposited at the School of Pharmacy, Chengdu University of TCM, Chengdu, China.

Extraction and isolation

The air-dried plant material (16 kg) was decoced with H2O (130 L; 3 × 1 h). The aqueous extracts were combined and evaporated under reduced pressure to yield a dark-brown residue (1.8 kg), which was suspended in H2O (2.0 L) and then successively partitioned with EtOAc (8 × 2.0 L) and n-BuOH (8 × 2.0 L). The EtOAc extract (470 g) was chromatographed over a D-101 macroporous adsorbent resin column. Successive elution of the column with 30% EtOH, 50% EtOH, 75% EtOH, and 95% EtOH (15 L each) yielded four fractions (3). The third portion (65 g) eluted by 75% EtOH was subjected to silica gel CC using a gradient elution of increasing MeOH (0–100%) in CH2Cl2 to give 11 fractions (F1 – F11). F2 was separated over the Sephadex LH-20 column eluted with petroleum ether–CHCl3–MeOH (5:5:1) to afford five subfractions (F2.1 – F2.5). The subfraction F2.3 was subjected to silica gel CC using a gradient elution of increasing MeOH (0–100%) in CH2Cl2 to give six parts (F2.3.1 – F2.3.6). Further purification of F2.3.4 with preparative TLC (CHCl3–MeOH, 30:1) followed by reversed-phase semipreparative HPLC (68% MeOH in H2O) yielded 3 (2.7 mg) and 4 (0.7 mg). F2.3.4 was further separated on the preparative TLC (CHCl3–MeOH, 40:1) and reversed-phase semipreparative HPLC (80% MeOH in H2O) to afford 1 (7.5 mg) and 2 (11.8 mg).

Physical-chemical properties of 1–4

Penchonine A (1). Pale yellow powder; UV (MeOH) λmax (logε) 214 (4.05), 252 (4.23), 289 (4.11) nm; IR (KBr) νmax 3138, 2941, 1652, 1614, 1581, 1503, 1433, 1359, 1301, 1159, 1091, 1043, 969, 814, 794 cm⁻¹; 1H NMR (MeOH-d4, 600 MHz) data and 13C NMR (MeOH-d4, 150 MHz) data, see Table 1. (+)-HR-ESIMS m/z 365.1002 [M + Na]+ (calcd for C19H19O2Na, 365.1001).

Penchonine B (2). Pale yellow crystals (MeOH); UV (MeOH) λmax (logε) 215 (4.14), 243 (4.21), 288 (4.17) nm; IR (KBr) νmax 3295, 2974, 2936, 1653, 1602, 1568, 1504, 1427, 1362, 1278, 1161, 1092, 1044, 971, 812, 792, 757 cm⁻¹; 1H NMR (MeOH-d4, 600 MHz) data and 13C NMR (MeOH-d4, 150 MHz) data, see Table 1. (+)-HR-ESIMS m/z 365.1003 [M + Na]+ (calcd for C19H19O2Na, 365.1001).

Penchonine C (3). Colorless gum; [α]D20 +22.7 (c 0.12, MeOH); UV (MeOH) λmax (logε) 224 (4.33), 268 (3.96), 308 (3.53) nm; CD (MeCN) 244 (Δε −1.46), 290 (Δε +0.64) nm; IR (KBr) νmax 3364, 2927, 2853, 1706, 1500, 1480, 1460, 1314, 1262, 1183, 1108, 1072, 802 cm⁻¹; 1H NMR (MeOH-d4, 600 MHz) data and 13C NMR (MeOH-d4, 150 MHz) data, see Table 1. (+)-HR-ESIMS m/z 325.1075 [M – H]− (calcd for C15H14O3, 325.1076).

Penchonine D (4). Colorless gum; [α]D20 +14.9 (c 0.04, MeOH); UV (MeOH) λmax (logε) 222 (4.32), 259 (3.98), 301 (3.55) nm; 1H NMR (MeOH-d4, 600 MHz) data and 13C NMR (MeOH-d4, 150 MHz) data, see Table 1. (+)-HR-ESIMS m/z 325.1073 [M – H]− (calcd for C15H14O3, 325.1076).

X-ray crystallography of compound 2

C22H22O2, M = 360.35, orthorhombic, a = 9.9508(3) Å, b = 19.0262(6) Å, c = 19.3662(6) Å, α = 90.0°, β = 90.0°, γ = 90.0°, V = 3666.52(2) Å3; T = 100(2) K, space group Pbcn, Z = 8, μ(CuKα) = 0.840 mm⁻¹, 19511 reflections measured, 3394 independent reflections (Rint = 0.0362). The final R1 values were 0.0389 (I > 2σI). The final wR2 (F2) values were 0.0999 (I > 2σI). The final R1 values were 0.0389 (all data). The final wR2 (F2) values were 0.0999 (all data). The goodness of fit on F2 was 1.123.

The data were collected using a Bruker APEX DUG diffractometer with Cu Kα radiation. The crystal structures were solved by direct methods using the SHELXS-97 program and refined anisotropically by least-squares method using the SHELXL-97 refinement package. 28 CDC 1414939 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CDC, 12 Union Road, Cambridge CB21EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk).

ECD calculation of compound 3

Conformational analysis of the S-enantiomer was carried out via Monte Carlo searching with the MMFF94 molecular mechanics force field using the Spartan 10 software. 29 The lowest-energy conformers having relative energies within 2 kcal/mol (Electronic Supplementary Information, Fig. S32 and Table S8) were re-optimized using DFT at the B3LYP/6-31+G(d, p) level in vacuum with the Gaussian 09 program. 30 The B3LYP/6-31G+(d, p) harmonic vibrational frequencies were further calculated to confirm their stability. The energies, oscillator strengths, and rotational strengths of the electronic excitations were calculated using the TDDFT methodology at the B3LYP/6-311+G (2d, 2p) level in vacuum. Then, the ECD spectra were simulated by the Gaussymm 2.25 program (n=0.3 eV). The final ECD spectra of (S)-3 was obtained according to the Boltzmann distribution theory and their relative Gibbs free energy (ΔG).

Hepatoprotective activity assay

Cell culture. HL7702 cells were obtained from the Cell Bank of Shanghai Institute of Chinese Academy of Sciences and maintained in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA). All cells were cultured in a humidified atmosphere with a 5% CO2 incubator at 37 °C.

MTT colorimetric assay. Cell viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. HL7702 cells were seeded in 96-well culture plates at a density of 1 × 10⁴ cells with 100 μl culture medium per well and cultured for 12 h in 5% CO2 at 37°C. Then, the cultured cells were exposed to 35 mM acacetaminophen for
hours. After fresh medium (100 µL) containing test sample was added, the cells were cultured for 24 h. Then, the medium was changed into a fresh one containing 5 mg/mL MTT. After 4 h incubation at 37 °C, the medium was aspirated and the cells lysed by addition of 150 µL DMSO. The optical density (OD) value of the formazan solution was measured on a microplate reader at 570 nm.

Image-iT® DEAD Green™ viability stain and Hoechst 33342 staining. Hoechst 33342 staining was used to visualize nuclear DNA in live and dead cells. And the Image-iT® DEAD Green™ viability stain could form highly fluorescent and stable dye-nucleic acid complexes in dead cells. At the end of the treatment of acetaminophen and compounds, remove the medium containing test sample and add fresh blank medium. Then, add Image-iT® DEAD Green™ viability stain to each well and incubate the plate under normal cell culture condition for 30 min. After the medium was removed, add 100 µL of counterstain/fixed solution (6 µL of Hoechst 33342 and 3 mL of 16% paraformaldehyde in 9 mL PBS) and incubated for 15 min at room temperature. The counterstain/fixed solution was removed and the wells were washed with PBS. After adding PBS to each well, the cells were observed under a microscope (ImagExpress micro XLS).

Cytotoxic activity assay

The cytotoxic effects were determined by a MTT colorimetric assay in human hepatocellular carcinoma cell line HepG-2, lung cancer cell lines A549 and NCI-H1975, breast cancer cell lines MDA-MB-231 and MCF-7, and ovarian cancer cell line Hey. Each cell suspension of 1×10³ cells/mL in DMEM containing 10% fetal bovine serum (v/v) was seeded in 96-well plates and cultured for 24 h in 5% CO₂ at 37 °C. After fresh medium (100 µL) containing test sample was added, the cells were cultured for 72 h. Then, the medium was changed into a fresh one containing 5 mg/mL MTT. After 4 h incubation at 37 °C, the supernatant was removed and 150 µL of DMSO was added to dissolve formazan crystals. The optical density (OD) value of the formazan solution was measured on a microplate reader at 490 nm. Each assay was replicated three times. The effect of the compounds on tumor cells viability was calculated and expressed by IC₅₀ of each cell line.

Conclusions

Although lignan molecule is small and contains only two C₆–C₃ units, many subtypes of classical lignans, neolignans, and oxynoeolignans have been found in stems, bark, leaves, seeds, fruits, roots, and rhizomes of plants. In this study, two other subtypes of neolignans were obtained from a hepatoprotective water decoction of P. chinense. We suggested that the unprecedented neolignane skeletons were likely derived from 8,3′-neolignanes. Significantly, the co-occurrence of these unusual neolignans (1–4) in the genus Penthorum, whose taxonomy is still in dispute, may constitute evidence that Penthorum should be considered a distinct family in the APG system. In addition, only compound 1 displayed hepatoprotective activity against acetaminophen-induced damage and selective cytotoxicity against Hey cancer cell. These findings prompt us to pay more attention to these unusual types of neolignans in Penthorum and their structure-activity relationships in future.

Acknowledgements

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Notes and references


29 *Spartan 10*, Wavefunction, Inc.: Irvine, CA.

30 *Gaussian 09*, Revision C.01, Gaussian, Inc.: Wallingford CT, 2010. A full list of authors can be found in the Electronic Supplementary Information.