Lignans from the Roots and Rhizomes of *Clematis manshurica*

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Two new lignans, clemomanshurinane A and B, along with ten known compounds have been isolated from the roots and rhizomes of *Clematis manshurica*. The structures were elucidated on the basis of their physicochemical and spectroscopic evidence. Clemomanshurinane A and B, (+)-dihydrodehydrodiconiferyl alcohol and (+)-lariciresinol 4,4'-*O*-bis-β-D-glucopyranoside indicated inhibitory effects on COX-2 at final concentration of 10^{-5} mol/l, with the inhibition rates 65.8%, 71.8%, 53.3%, and 60.3%, respectively.

Key words: *Clematis manshurica* Rpur., Lignans, Clemomanshurinane A, Clemomanshurinane B, COX-2

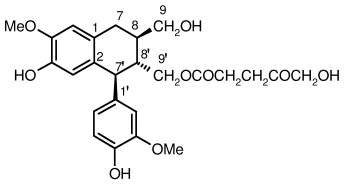
Introduction

Clematis manshurica Pupr., the roots and rhizomes of the plant named "*Weilingxian*" in traditional Chinese medicine (TCM), is widely distributed in the northeast of China. It is popularly used as an anti-inflammatory, antitumor, analgesic agent in TCM. Up to date, only four triterpene saponins (clematoside A-C and clematoside A') have been isolated from the roots and rhizomes of *C. manshurica* [1]. Our preliminary investigations suggested that the 95% ethanol extract of the roots and rhizomes of *C. manshurica* indicated significant inhibitory activities on COX-2, so the investigations on the chemical constituents of the extract were performed. Two new lignans together with ten known compounds have been isolated and elucidated. Herein we reported the isolation and structure elucidation of the new compounds. The preliminary investigations of the biological activities on COX-2 of all these compounds are also presented.

Results and Discussion

A 95% ethanol extract of the dried roots and rhizomes of *C. manshurica* was suspended in water, then extracted by petroleum, ethyl acetate and *n*-butanol successively. The ethyl acetate extract was subjected to silica gel, Sephadex LH-20 column chromatography and purified by prep. HPLC to yield three known lignans, (+)-pinoresinol (**1**) [2], (+)-isolariciresinol (**2**) [3], and (+)-dihydrodehydrodiconiferyl alcohol (**5**) [4], together with two new lignans, clemomanshurinane A (**3**) and clemomanshurinane B (**4**). The *n*-butanol extract was subjected to D101 porous polymer resin, Sephadex LH-20 and silica gel column chromatography, purified by prep. HPLC to yield seven known lignans, (+)-5'-methoxyisolariciresinol 9'-*O*-β-D-glucopyranoside (**6**) [5], (+)-lariciresinol 4,4'-*O*-bis-β-D-glucopyranoside (**7**) [6], (+)-pinoresinol 4-*O*-β-D-glucopyranoside (**8**) [7], (+)-lyoniresinol 9'-*O*-β-D-glucopyranoside (**9**) [8], (+)-lariciresinol 4'-*O*-β-D-glucopyranoside (**10**) [9], (+)-(7'*S*,8*R*,8'*S*)-4,4',9-trihydroxy-3,3',5-trimethoxy-2,7'-lignan-9-*O*-β-D-glucopyranoside (**11**) [10], and (+)-pinoresinol 4,4'-*O*-bis-β-D-glucopyranoside (**12**) [6].

Compound **3** was obtained as a pale yellow syrup. The positive mode HRFABMS showed a $[M]^+$ ion peak at m/z = 474.1898, in accordance with an empirical molecular formula of $C_{25}H_{30}O_9$, which was supported by the ^{13}C NMR spectrum and various DEPT data. The IR spectrum of **3** indicated the absorptions of hydroxyl groups at 3422 cm^{-1}, aromatic rings at 1605 and 1512 cm^{-1} and carbonyl groups at 1721 cm^{-1}. The UV spectrum exhibited maximum



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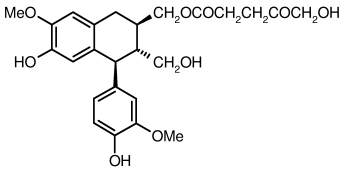

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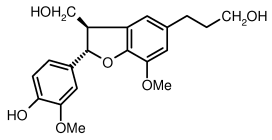

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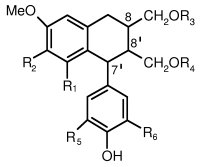
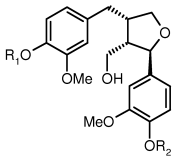
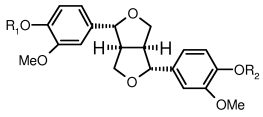
absorptions at 241 and 283 nm. The ^{1}H NMR spectrum showed the presence of two aromatic protons at $\delta = 6.69$ (1H, s) and 6.19 (1H, s), an ABX system at δ = 6.76 (1H, d, $J = 2.0$ Hz), 6.78 (1H, d, $J = 8.0$ Hz) and 6.60 (1H, dd, $J = 2.0$, 8.0 Hz). The ^{13}C NMR spectrum indicated 12 aromatic carbons, three aliphatic methylene carbons ($\delta = 33.3$, 64.5 and 63.9), and three aliphatic methane carbons ($\delta = 39.2$, 44.1 and 48.0). All these suggested that **3** might be a tetrahydroarylnaphthalene-type lignan. Moreover, the ^{1}H and ^{13}C NMR spectra of **3** also exhibited the signals due to a 5-hydroxy-4-oxo-pentanoyl group at δ_H = 2.66 (2H, t, $J = 6.5$ Hz), 2.79 (2H, *overlapped*) and 4.23 (2H, s); δ_C = 209.9, 172.9, 68.6, 33.6 and 28.2 [11], two methoxy groups at δ_H = 3.80 (3H, s) and 3.79 (3H, s), δ_C = 56.3 and 56.2. In the HMBC spectrum of **3**, the protons of H-9' [$\delta = 4.15$ (1H, dd, $J = 2.0$, 11.0 Hz), 3.91 (1H, dd, $J = 2.0$, 11.0 Hz)] showed long range correlations with the carbonyl carbon ($\delta = 172.9$), which suggested that the 5-hydroxy-4-oxo-pentanoyl group was linked to C-9'. In the NOESY spectrum of **3**, the signals of the methoxy groups showed cross peaks with H-6 at $\delta = 6.69$ (1H, s) and H-2' at 6.76 (1H, d, $J = 2.0$ Hz), which indicated that the methoxy groups



	R_1	R_2	R_3	R_4	R_5	R_6	Config.
2	H	OH	H	H	H	OCH_3	(7'*S*, 8*R*, 8'*R*)
6	H	OH	H	Glc	OCH_3	OCH_3	(7'*S*, 8*R*, 8'*R*)
9	OCH_3	OH	H	Glc	OCH_3	OCH_3	(7'*S*, 8*R*, 8'*R*)
11	OCH_3	OH	H	Glc	H	OCH_3	(7'*S*, 8*R*, 8'*S*)



	R_1	R_2
7	Glc	Glc
10	H	Glc



	R_1	R_2
1	H	H
8	Glc	H
12	Glc	Glc

Fig. 1. Structures of the isolated compounds **1**–**12**.

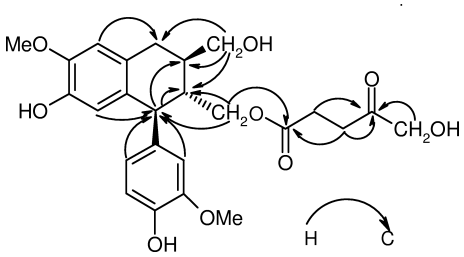


Fig. 2. Key HMBC correlations of **3**.

were linked to C-5 and C-3', respectively. The relative stereochemistry of the tetrahydronaphtalene ring was inferred from the careful analysis of the NOESY spectrum of **3**. The NOESY spectrum of **3** showed no correlation peaks between H-7' and H-8', H-8 and H-8', but cross peaks between H-7' and H-8, H-7' and H-9', H-8' and H-9, which suggested that H-7' had *trans* axial-axial relationship with H-8', but *cis* relationship with H-8. In the ^{1}H NMR spectrum of **3**, the values of the coupling constants between H-7'

and H-8' ($J = 9.0$ Hz) also confirmed that proton H-7' had *trans* axial-axial relationship with H-8'. The absolute stereochemistry of **3** was established by the molecular rotation ($[\alpha]_D^{20} + 39.5^\circ$ ($c = 0.38$, MeOH)) and CD spectrum. The negative Cotton effect at 291 nm and the positive Cotton effect at 276 nm determined the *S*-configuration at C-7' [12, 13], thus C-8 and C-8' must have *R*-configurations. Accordingly, the structure of **3** was elucidated as (+)-(7'*S*,8*R*,8'*R*)-4,4',9-trihydroxy-3',5-dimethoxy-9'-(5-hydroxy-4-oxo-pentanoyl)-2,7'-cyclolignane, named clemomanshurinane A.

Compound **4** was obtained as a pale yellow syrup. The positive mode HRFABMS showed a M^+ ion peak at m/z = 474.1898, in accordance with an empirical molecular formula of $C_{25}H_{30}O_9$, which was similar to **3**. The NMR spectroscopic data of **4** were very similar to those of **3** except for the six aliphatic carbons of the tetrahydronaphthalene ring. In compound **4**, the carbon of C-9 was shifted downfield to $\delta = 67.6$, but C-9' was shifted upfield to $\delta = 61.0$, which suggested the 5-hydroxy-4-oxo-pentanoyl group to be linked to C-9, which was confirmed by the cross peaks between H-9 [$\delta = 4.32$ (1H, dd, $J = 4.0$, 11.0 Hz), 4.14 (1H, dd, $J = 7.5$, 11.0 Hz)] and the carbonyl carbon ($\delta = 173.1$). Detailed analysis of the CD and NOESY spectra of **4** suggested that compound **4** had the similar absolute configuration to **3** and indicated positive rotation, $[\alpha]_D^{20} + 36.7^\circ$ ($c = 0.35$, MeOH). Thus the structure of **4** was determined as (+)-(7'*S*,8*R*,8'*R*)-4,4',9'-trihydroxy-3',5-dimethoxy-9-(5-hydroxy-4-oxo-pentanoyl)-2,7'-cyclolignane, named clemomanshurinane B.

Compounds **1** – **12** were evaluated for inhibitory effects on COX-2 activity induced by lipopolysaccharide (LPS) in murine peritoneal macrophages. The preliminary research results showed that compounds **3**, **4**, **5** and **7** indicated inhibitory effects on COX-2 at a final concentration of 10^{-5} mol/l with the inhibition rates 65.8%, 71.8%, 53.3% and 60.3%, respectively. The other compounds indicated weak inhibition activities on COX-2 with the inhibitory rates at about 30% – 45% (inhibition rates of the positive controls: Celecoxib at final concentration of 10^{-6} mol/l was 75.8%, Indomethacin at the same concentration was 85.9%).

Experimental Section

General

Melting points were measured on an electrothermal melting point apparatus. Optical rotations were recorded on a Perkin-Elmer 243B digital polarimeter. UV spectra were obtained from a TU-1901 spectrometer. IR spectra were recorded on an AVATER-360 spectrometer. NMR spectra were recorded on Inova 300 (300 MHz for ^{1}H and 75 MHz for ^{13}C) and Inova 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometers with TMS as internal standard. HRFABMS were measured on an Autospec-UltimaETOF spectrometer in positive ion mode. CD spectra were detected on a J-810 spectrometer. prep. HPLC were carried out on ODS columns (Alltech 250 × 10 mm i. d., 5 μm) with a Waters 2996 photodiode array detector. For CC, silica gel (200 – 300 mesh, Qingdao Mar. Chem. Ind. Co. Ltd.), Sephadex LH-20 gel (Pharmacia) and D101 porous polymer resin (Tianjin Chem. Ind. Co. Ltd.) were used.

Plant material

The roots and rhizomes *C. manshurica* Rupr. were collected in August 2002 from Heilongjiang province, northeast China. The identification of the plant was performed by Prof. Peng-Fei Tu, Peking University. A voucher specimen is kept in the herbarium of Peking University Modern Research Center for Traditional Chinese Medicine (CM200208).

Biological assay material

Mice (male, 16 – 18 g), were provided by the Experimental Animal Center, Institute of Experimental Animal, Chinese Academy of Medical Sciences & Peking Union Medical College). Positive controls: Celecoxib and Indomethacin. LPS (Sigma Chemical CO.). PGE_2 radioimmunoassay kit (PLA General Hospital, Beijing, China).

Extraction and isolation

The dried roots and rhizomes (15 kg) of *C. manshurica* were extracted with 95% ethanol for three times. After removal of the solvent under reduced pressure at 60 °C, the residue (1.7 kg) was suspended in water and defatted with petroleum ether. The aq. layer was further extracted with ethyl acetate and *n*-butanol successively. The ethyl acetate extract (40 g) was subjected to silica gel (1000 g) column chromatography and eluted with $CHCl_3$–MeOH (100 : 0 – 1 : 1) to afford fractions I – IV. Fraction I was subjected to silica gel column chromatography and eluted with $CHCl_3$–MeOH (10 : 1), then purified by prep. HPLC (MeOH-H_2O = 39 : 61, 280 nm, 3.0 ml/min) to yield **1** (30 mg). Fraction III was subjected to silica gel column chromatography and eluted with $CHCl_3$–MeOH (10 : 1), then purified by prep. HPLC (MeOH-H_2O = 30 : 70, 280 nm, 3.0 ml/min) to yield compounds **2** (20 mg), **3** (4 mg) and **4** (5 mg). Fraction IV was subjected to silica gel column chromatography and eluted with $CHCl_3$–MeOH (6 : 1), then purified by prep. HPLC (MeOH-H_2O = 40 : 60, 280 nm, 2.5 ml/min) to yield **5** (6 mg). The *n*-butanol extract (250 g) was subjected to D101 porous polymer resin and eluted with H_2O,

Table 1. NMR data of **3** and **4** (in acetone-d_6, 500 MHz for ^{1}H and 125 MHz for ^{13}C).

	3		**4**	
NO.	^{1}H	^{13}C	^{13}C	^{1}H
1	–	128.4	127.8	–
2	–	133.5	133.8	–
3	6.19 (1H, s)	116.7	116.8	6.21 (1H, s)
4	–	145.3	145.3	–
5	–	146.6	146.5	–
6	6.69 (1H, s)	112.1	112.0	6.69 (1H, s)
7	2.76 ∼ 2.89 (2H, overlapped)	33.3	33.4	2.76 ∼ 2.84 (2H, m)
8	2.02 (1H, m)	39.2	36.4	1.83 (1H, m)
9a	3.70 (1H, dd, $J = 3.0$, 10.5 Hz)	64.5	67.6	4.32 (1H, dd, $J = 4.0$, 11.0 Hz)
9b	3.64 (1H, dd, $J = 5.0$, 10.5 Hz)			4.14 (1H, dd, $J = 7.5$, 11.0 Hz)
1’	–	137.4	138.1	–
2’	6.76 (1H, d, $J = 2.0$ Hz)	113.7	113.6	6.79 (1H, d, $J = 2.0$ Hz)
3’	–	148.3	148.2	–
4’	–	145.9	145.8	–
5’	6.78 (1H, d, $J = 8.0$ Hz)	115.7	115.5	6.77 (1H, d, $J = 8.0$ Hz)
6’	6.60 (1H, dd, $J = 2.0$, 8.0 Hz)	122.6	122.8	6.64 (1H, dd, $J = 2.0$, 8.0 Hz)
7’	3.86 (1H, d, $J = 9.0$ Hz)	48.0	47.3	3.93 (1H, d, $J = 10.5$ Hz)
8’	2.04 (1H, m)	44.1	47.0	2.28 (1H, m)
9’a	4.15 (1H, dd, $J = 2.0$, 11.0 Hz)	63.9	61.0	3.64 (1H, br.d, $J = 11.0$ Hz)
9’b	3.91 (1H, dd, $J = 2.0$, 11.0 Hz)			3.42 (1H, br.d, $J = 11.0$ Hz Hz)
Penrnoyl				
1	–	172.9	173.1	–
2	2.79 (overlapped)	33.6	33.5	2.81 (2H, $J = 6.5$ Hz)
3	2.66 (2H, $J = 6.5$ Hz)	28.2	28.2	2.64 (2H, $J = 6.5$ Hz)
4	–	209.9	209.8	–
5	4.23 (2H, s)	68.6	68.7	4.23 (2H, s)
5-OCH_3	3.80 (3H, s)	56.3	56.3	3.80 (3H, s)
3’-OCH_3	3.79 (3H, s)	56.2	56.2	3.78 (3H, s)

10, 30 and 50% MeOH successively. The fraction eluted with 10% MeOH (25 g) was subjected to Sephadex LH-20 column chromatography and eluted with water to afford fractions I – III. Fraction II was applied to a silica gel column chromatography and eluted with $CHCl_3$–MeOH-H_2O (7 : 3 : 0.1), then purified by prep. HPLC (CH_3CN-H_2O = 28 : 72, 280 nm, 3.0 ml/min) to yield **6** (6 mg). Fraction III was subjected to a silica gel column chromatography and eluted with $CHCl_3$–MeOH-H_2O (7 : 3 : 0.1), then purified by prep. HPLC (CH_3CN-H_2O = 25 : 75, 275 nm, 3.0 ml/min) to yield **7** (6 mg). The fraction eluted with 30% MeOH was chromatographed on a Sephadex LH-20 column repeatedly and eluted with H_2O to afford fractions IV – VI. Fraction IV was chromatographed on a Sephadex LH-20 and silica gel column ($CHCl_3$–MeOH = 3 : 1), then purified by prep. HPLC (CH_3OH-H_2O = 38 : 62, 280 nm, 2.0 ml/min) to yield **8** (5 mg). Fraction V was chromatographed on a Sephadex LH-20 column repeatedly to afford subfractions I – II. Subfraction I was purified by prep. HPLC (CH_3OH-H_2O = 33 : 67, 280 nm, 2.5 ml/min) to yield **9** (9 mg); subfraction II was subjected to Sephadex LH-20 and silica gel column chromatography ($CHCl_3$–MeOH = 4 : 1), then purified by prep. HPLC (CH_3OH-H_2O = 42 : 58, 280 nm, 2.5 ml/min) to yield **10** (7 mg). Fraction VI was applied to a silica gel column chromatography and eluted with $CHCl_3$–MeOH-H_2O (7 : 3 : 0.1), then purified by prep. HPLC (CH_3OH-H_2O = 40 : 60, 280 nm, 2.5 ml/min) to yield compounds **11** (4 mg) and **12** (6 mg).

Clemomanshurinane A (**3**): pale yellow syrup. – Mp 153 °C. – $[\alpha]_D^{20} + 39.5°$ ($c = 0.38$ MeOH). – UV/vis (MeOH): λ_{max} ($\log \varepsilon$) = 241 nm (4.18). – IR (KBr): ν = 3422 (OH), 2929, 1721(C=O), 1605, 1512, 1453, 1380, 1273, 1205 cm^{-1}. – CD (MeOH, 0.25) $\Delta\varepsilon_{291} - 59.97$, $\Delta\varepsilon_{275} + 41.62$, $\Delta\varepsilon_{252} + 3.79$, $\Delta\varepsilon_{241} + 55.90$, $\Delta\varepsilon_{231} - 1.00$. – ^{13}C{^{1}H}NMR data see Table 1. – HRFABMS: m/z (%) = exp. 474.1898 (100), calcd. 474.1890 [M]$^+$.

Clemomanshurinane B (**4**): pale yellow syrup. – Mp 158 °C. – $[\alpha]_D^{20} + 36.7°$ ($c = 0.35$ MeOH). – UV/vis (MeOH): λ_{max} log(ε) = 240 nm (4.21). – IR (KBr): ν = 3420 (OH), 2928, 1722 (C=O), 1608, 1513, 1455, 1382, 1270, 1207 cm^{-1}. – CD (MeOH, 0.30) $\Delta\varepsilon_{292} - 68.16$, $\Delta\varepsilon_{276} + 46.73$, $\Delta\varepsilon_{252} + 2.67$, $\Delta\varepsilon_{241} + 45.58$, $\Delta\varepsilon_{232} - 2.76$. – ^{13}C{^{1}H}NMR data see Table 1. – HRFABMS: m/z (%) = exp. 474.1898 (100), calcd. 474.1890 [M]$^+$.

Biological activities on COX-2 assay

C_{57}BL/6J mice were given an i. p. injection with 1 ml of 4% thioglycollate 4 days prior to sacrifice. Peritoneal macrophages were collected by lavaging the peritoneal cavity with 8 ml of HBSS. The cells were collected by centrifugation (1000 r/min for 5 min), washed, and then sus-

pended in RPMI 1640 medium and made the concentration of the cells at 1×10^6 cell/ml. The cells were seeded in 48-well plates (0.5 ml/well), incubated under 37 °C with 5% CO_2 for 2 h, then the medium was removed and the cells were washed by HBSS. After this period, to all compounds (10^{-5} mol/l respectively) isolated from the roots and rhizomes of *C. manshurica* positive controls Celecoxib and Indomethacin (10^{-6} mol/l respectively) were added and incubated under 37 °C with 5% CO_2 for 1 h, then stimulated with LPS (1 mg/l) for 12 h. The level of PGE_2 production from endogenous arachidonic acid was measured in cell culture supernatants of peritoneal macrophages by RIA.

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