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WHITMANOSIDE A, A NEW α -PYRONE GLYCOSIDE FROM THE LEECH *WHITMANIA PIGRA*

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Abstract – A new α -pyrone glycoside, whitmanoside A (**1**), along with eight known compounds, including two furanones (**2**, **3**) and six proline-derived cyclic dipeptides (**4-9**), were isolated from the dried material of the leech *Whitmania pigra*. The structures of these compounds were established on the basis of their spectroscopic data and acid hydrolysis. This is the first report on the isolation of these compounds from a leech.

The class Hirudinea consists of approximately 680 species of leeches worldwide, which are found on every continent of earth except Antarctica.¹ Previous chemical and biochemical investigations on leeches have led to the discovery of many bioactive proteins and polypeptides,^{2,3} together with several pteridines and lipids.^{4,9} *Whitmania pigra* Whitman belongs to the family Haemopidae and is widely distributed throughout China. It is the major ingredient of several Chinese patent medicines, such as “Naoxuekang Pian” and “Shuxuetong Zhushuye”, which are used to treat paralytic stroke and cerebral thrombus. During our search for bioactive natural products, a new α -pyrone glycoside and eight known compounds were isolated and characterized from the dried material of *W. pigra*.

Chemical investigation on the 95% aqueous ethanol extract of the dried material of *W. pigra* led to the isolation of a new α -pyrone glycoside, whitmanoside A (**1**), and eight known compounds, including two furanones (**2**, **3**) and six proline-derived cyclic dipeptides (**4-9**) (Figure 1). The structure of compound **1** was elucidated by spectroscopic techniques (UV, IR, HR-ESI-MS, together with 1D and 2D NMR) and acid hydrolysis. Compounds **2-9** were identified by comparing their spectroscopic data with the published results as 5-hydroxy-3,4-dimethyl-5-propylfuran-2(5*H*)-one (**2**),¹⁰ 5-hydroxy-3,4-dimethyl-5-pentylfuran-2(5*H*)-one (**3**),^{10,11} cyclo(L-Pro-L-Ala) (**4**),¹² cyclo(L-Pro-L-Val) (**5**),¹³ cyclo(L-Pro-L-Leu) (**6**),¹⁴

cyclo(L-Pro-L-Pro) (**7**),¹² cyclo(L-Pro-L-Phe) (**8**),¹⁵ cyclo(L-Pro-L-Tyr) (**9**),¹³ respectively.

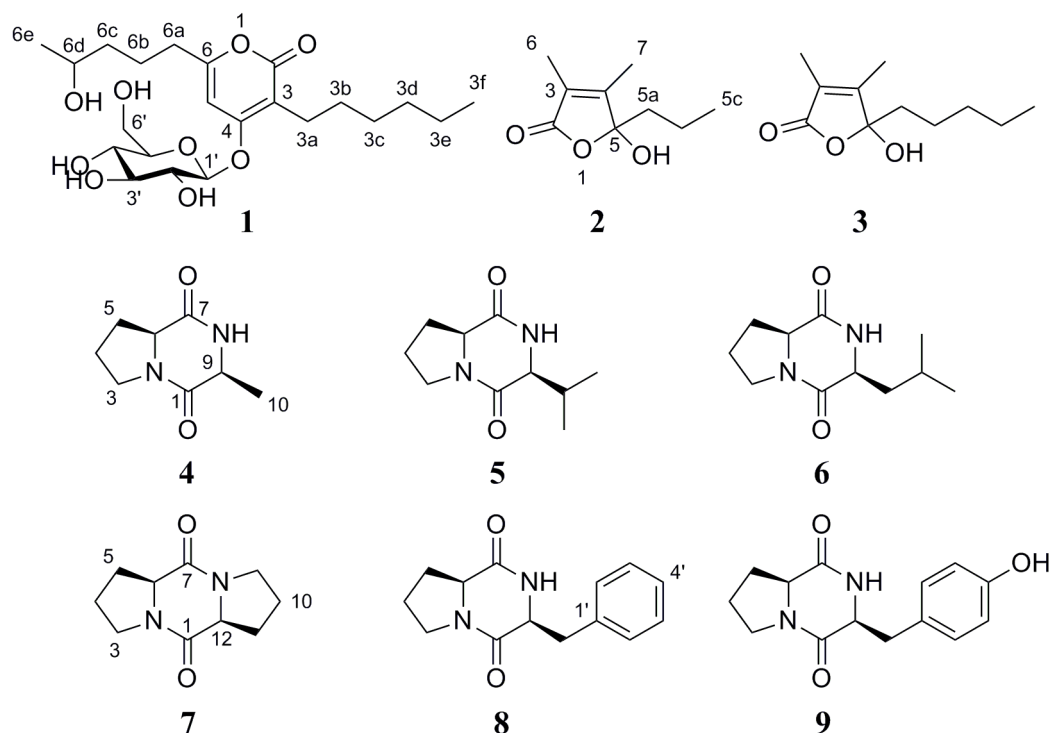


Figure 1. The structures of compounds 1-9

Compound **1** was isolated as a colorless oil. Its molecular formula was determined to be $C_{22}H_{36}O_9$ by a quasi-molecular ion at m/z 467.2235 $[M+Na]^+$ (calcd for $C_{22}H_{36}NaO_9$, 467.2252) in its HR-ESI-MS. The UV spectrum suggested the presence of an α -pyrone chromophore with the absorption maximum at 297 nm.^{16,17} The IR bands at 3448 and 1667 cm^{-1} implied the presence of hydroxy and carbonyl groups. The ^{13}C NMR and DEPT spectra (Table 1) revealed the presence of 22 carbons, including two methyls, nine methylenes, and seven methines along with four quaternary carbons [including one ester carbonyl at δ_C 168.0 (C-2)]. The NMR data suggested the presence of a glucopyranosyl moiety for which the anomeric proton resonated at δ_H 5.04 (1H, d, $J = 7.6$ Hz, H-1'). The NMR data of the aglycone moiety of **1** were similar to those of pseudopyronine A (Figure 2),¹⁷ except the presence of extra signals for an oxygenated methine and the absence of signals for a methylene.

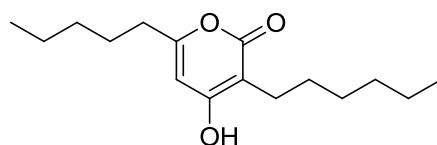


Figure 2. The structure of pseudopyronine A

^1H - ^1H COSY correlations (Figure 3) of H-6a/H-6b, H-6b/H-6c, H-6c/H-6d, and H-6d/H-6e revealed the presence of an alkyl chain in **1**, which was further confirmed by HMBC correlations (Figure 3) of H-6a/C-6b, H-6a/C-6c, H-6c/C-6d, H-6e/C-6c, and H-6e/C-6d. The downfield shift of H-6d (δ_{H} 3.75) in ^1H NMR spectrum and a cross-peak between H-6e (δ_{H} 1.16) and H-6d (δ_{H} 3.75) in ^1H - ^1H COSY spectrum suggested that the alkyl chain was oxygenated at C-6d. HMBC correlations of H-6a/C-5, H-6a/C-6, and H-5/C-6a suggested the oxygenated alkyl chain was located at C-6. The NMR data of **1** indicated the presence of another alkyl chain, which was confirmed by ^1H - ^1H COSY correlations of H-3a/H-3b and H-3e/H-3f as well as HMBC correlations of H-3f/C-3d and H-3f/C-3e. HMBC correlations from H-3a to C-2, C-3, and C-4 suggested the alkyl chain was located at C-3. HMBC correlations from H-5 to C-3 and C-6 confirmed the α -pyrone skeleton in **1**. Full assignment of the NMR data of the aglycone of **1** was accomplished on the basis of ^1H - ^1H COSY, HSQC, and HMBC correlations as well as by comparison with the literature data of its analogues.¹⁷⁻²² The HMBC correlation between H-1' (δ_{H} 5.04) and C-4 (δ_{C} 166.6) suggested that the glucose moiety was connected to C-4. Acid hydrolysis of **1** yielded D -glucose which was identified by reversed-phase HPLC after conversion of the sugar to thiocarbamoyl-thiazolidine derivative.²³ The coupling constant (7.6 Hz) of the anomeric proton at δ_{H} 5.04 indicated the presence of β configuration for the glucopyranosyl moiety. The absolute configuration at C-6d was unable to be determined because only a small amount of **1** was available. Thus, compound **1** was elucidated as 3-hexyl-6-(4-hydroxypentyl)-2*H*-pyran-2-on-4-*O*- β -D-glucopyranoside, and ascribed the trivial name whitmanoside A.

Several naturally occurring α -pyrones had been reported previously,¹⁷⁻²² and all of them were isolated from fungi or bacteria. Therefore, whitmanoside A may be a metabolite of an endophytic fungus or bacterium in leech.

Table 1. NMR data of **1** (300 MHz, in CD_3OD)

Position	δ_{C} , type	δ_{H} (<i>J</i> in Hz)	Position	δ_{C} , type	δ_{H} (<i>J</i> in Hz)
2	168.0, C	-	6a	34.5, CH_2	2.56, m
3	108.4, C	-	6b	24.4, CH_2	1.75, m
4	166.6, C	-	6c	39.2, CH_2	1.47, m
5	98.6, CH	6.40, s	6d	68.1, CH	3.75, m
6	165.6, C	-	6e	23.5, CH_3	1.16, d (6.2)
3a	24.2, CH_2	2.51, m	1'	100.8, CH	5.04, d (7.6)
		2.39, m	2'	74.7, CH	3.45, m
3b	29.0, CH_2	1.46, m	3'	78.6, CH	3.49, m
3c	30.2, CH_2	1.30, m	4'	71.2, CH	3.35, m
3d	32.9, CH_2	1.30, m	5'	78.1, CH	3.45, m
3e	23.7, CH_2	1.30, m	6'	62.5, CH_2	3.90, dd (12.2, 1.9)
3f	14.5, Me	0.90, t (6.6)			3.67, dd (12.2, 6.0)

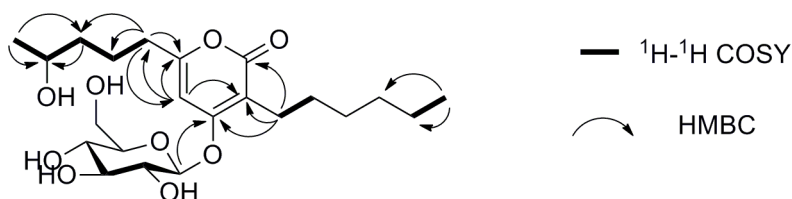


Figure 3. Key ^1H - ^1H COSY and HMBC correlations of **1**.

The specific rotation values of compounds **2** and **3** were almost zero, and thus they were deduced to be racemates. The absolute configurations of the six cyclic dipeptides (**4-9**) were assigned by their specific rotation values and NMR data. According to Adamczeski *et al.*,²⁴ the overall sign of the specific rotation value for a cyclic dipeptide containing proline is depending on the absolute configuration of Pro. More specifically, a cyclic dipeptide with a negative sign of specific rotation value usually has an L -Pro, and vice versa. Once the absolute configuration of Pro is determined, it is possible to conclude the absolute configuration of the whole cyclic dipeptide from the NMR data by comparing with those reported.

Cyclic dipeptides, especially those containing proline, are commonly found in nature.^{25,26} They were reported to possess various biological activities,²⁶⁻²⁸ such as antifungal, antibacterial, and antitumour activities. Further studies on the biological activity of the compounds isolated from leech are in progress.

EXPERIMENTAL

General. Optical rotations were determined by a Jasco P-1020 polarimeter. UV spectra were measured on a Jasco V-550 UV/VIS spectrometer. IR spectra were obtained on a Jasco FT/IR-480 plus spectrometer. MS spectra were recorded on an Agilent 6210 ESI/TOF mass spectrometer and a Thermo Finnigan LCQ Advantage ion trap mass spectrometer. 1D and 2D NMR spectra were recorded on a Bruker AV-300 spectrometer with TMS as internal standard. Silica gel (Qingdao, China) and Sephadex LH-20 (Pharmacia Biotec AB) were used for column chromatographies (CC). All solvents used in column chromatography were analytical grade (Tianjin Damao Chemical Plant, Tianjin, China). High-performance liquid chromatography (HPLC) was performed on an Agilent 1260 Infinity HPLC system (Agilent Technologies Inc., USA) equipped with a photodiode array detector and a C18 reversed-phase column (Cosmosil, 10×250 mm, $5 \mu\text{m}$) at a flow rate of 3 mL/min.

Animal material. The dried material of *W. pigra* was purchased in Guangzhou, Guangdong province of China, in March 2011. The animal material was identified by Prof. Guang-Xiong Zhou of the Jinan University. A voucher specimen (No. 20110325) was deposited in the Institute of Traditional Chinese Medicine and Natural Products, Jinan University, Guangzhou, China.

Extraction and Isolation. The dried material of *W. pigra* (4.0 kg) was powdered and refluxed with 4 L of 95% EtOH (3 × 2 h) at 80 °C. The crude extract (460 g) was suspended in water and successively partitioned with petroleum ether, EtOAc, and *n*-BuOH. The EtOAc extract (43 g) was subjected to a silica gel column (600 g, 100-200 mesh, 6 × 70 cm) with a gradient system of CHCl₃-MeOH (v/v 100 : 0, 99 : 1, 98 : 2, 95 : 5, 9 : 1, 8 : 2, 7 : 3, 1 : 1, each 4000 mL) to give nine fractions (A-I). Fraction B (3.5 g) was separated through a Sephadex LH-20 column (2 × 80 cm) eluting with MeOH to provide seven subfractions (B1-B7). Subfraction B3 (120 mg) was purified by HPLC (MeOH/H₂O, 35 : 65) to give **2** (16.6 mg), **3** (4.3 mg), **5** (10.1 mg), **6** (5.7 mg), **7** (8.3 mg), and **8** (4.2 mg). Fraction C (2.5 g) was fractionated and further purified by HPLC (MeOH/H₂O 2 : 8) to yield **4** (7.4 mg) and **9** (5.4 mg). Fraction E (4.5 g) was subject to silica gel CC (100 g, 200-300 mesh, 3 × 60 cm) with a gradient system of CHCl₃-MeOH (v/v 1 : 0, 95 : 5, 9 : 1, 8 : 2, each 1000 mL) to give six subfractions (E1-E6). Subfraction E4 (32 mg) was purified by HPLC (MeOH/H₂O, 1 : 1) to give **1** (2.8 mg).

Whitmanoside A (1): colorless oil; $[\alpha]_{\text{D}}^{25}$ -11.1 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ): 206 (4.08), 297 (3.54) nm; IR (KBr) ν_{max} : 3366, 2928 1685, 1569, 1385, 1073, 589 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; HR-ESI-MS *m/z* 467.2235 [M+Na]⁺ (calcd for C₂₂H₃₆NaO₉, 467.2252).

5-Hydroxy-3,4-dimethyl-5-propylfuran-2(5H)-one (2): colorless oil; $[\alpha]_{\text{D}}^{25}$ 0 (*c* 1.3, MeOH); ¹³C NMR (DMSO-*d*₆, 75 MHz) δ 171.7 (C-2), 158.5 (C-4), 123.5 (C-3), 107.3 (C-5), 37.8 (C-5a), 16.1 (C-5b), 13.8 (C-5c), 10.6 (C-7), 8.1 (C-6); HR-ESI-MS *m/z* 193.08289 [M+Na]⁺ (calcd for C₉H₁₄O₃Na, 193.08352).

5-Hydroxy-3,4-dimethyl-5-pentylfuran-2(5H)-one (3): colorless oil; $[\alpha]_{\text{D}}^{25}$ 0 (*c* 1.2, MeOH); ¹³C NMR (CD₃OD, 75 MHz) δ 174.7 (C-2), 160.5 (C-4), 125.8 (C-3), 109.3 (C-5), 37.1 (C-5a), 32.9 (C-5c), 23.9 (C-5b), 23.7 (C-5d), 14.4 (C-5e), 10.9 (C-7), 8.4 (C-6); HR-ESI-MS *m/z* 221.11415 [M+Na]⁺ (calcd for C₁₁H₁₈O₃Na, 221.11482).

Cyclo(L-Pro-L-Ala) (4): colorless oil; $[\alpha]_{\text{D}}^{25}$ -6.2 (*c* 1.1, MeOH); ¹³C-NMR (CD₃OD, 75 MHz) δ 172.8 (C-7), 169.2 (C-1), 60.6 (C-6), 52.2 (C-9), 46.6 (C-3), 29.3 (C-5), 23.8 (C-4), 15.9 (C-10); ESI-MS *m/z* 169 [M+H]⁺.

Cyclo(L-Pro-L-Val) (5): colorless oil; $[\alpha]_{\text{D}}^{25}$ -3.2 (*c* 1.2, MeOH); ¹³C-NMR (CDCl₃, 75 MHz) δ 170.2 (C-7), 164.9 (C-1), 60.4 (C-6), 58.8 (C-9), 45.2 (C-3), 28.5 (C-5), 28.4 (C-10), 22.4 (C-4), 19.2 (C-11), 16.1 (C-12); ESI-MS *m/z* 197 [M+H]⁺.

Cyclo(L-Pro-L-Leu) (6): colorless oil; $[\alpha]_{\text{D}}^{25}$ -75.6 (*c* 1.1, MeOH); ¹³C-NMR (CD₃OD, 75 MHz) δ 172.9 (C-7), 169.1 (C-1), 60.4 (C-6), 54.8 (C-9), 46.6 (C-3), 39.5 (C-10), 29.2 (C-5), 25.9 (C-11), 23.8 (C-4), 23.4 (C-12), 22.3 (C-13); ESI-MS *m/z* 211 [M+H]⁺.

Cyclo(L-Pro-L-Pro) (7): colorless oil; $[\alpha]_{\text{D}}^{25}$ -43.1 (*c* 1.0, MeOH); ¹³C-NMR (CD₃OD, 75 MHz) δ 168.8 (C-1, 7), 61.9 (C-6, 12), 46.3 (C-3, 9), 28.9 (5, 11), 24.3 (C-4, 10); ESI-MS *m/z* 195 [M+H]⁺.

Cyclo(L-Pro-L-Phe) (8): colorless oil; $[\alpha]_{\text{D}}^{25}$ -32.9 (*c* 0.9, MeOH); $^{13}\text{C-NMR}$ (CD_3OD , 75 MHz) δ 171.1 (C-7), 167.1 (C-1), 137.5 (C-1'), 131.2 (C-2', 6'), 129.6 (C-3', 5'), 128.2 (C-4'), 60.2 (C-6), 57.8 (C-9), 46.1 (C-3), 38.4 (C-10), 29.5 (C-5), 22.9 (C-4); ESI-MS m/z 245 $[\text{M}+\text{H}]^+$.

Cyclo(L-Pro-L-Tyr) (9): colorless oil; $[\alpha]_{\text{D}}^{25}$ -25.6 (*c* 1.1, MeOH); $^{13}\text{C-NMR}$ (CD_3OD , 75 MHz) δ 170.9 (C-7), 167.1 (C-1), 157.8 (C-4'), 132.3 (C-2', 6'), 127.8 (C-1'), 116.3 (C-3', 5'), 60.2 (C-6), 58.1 (C-9), 46.1 (C-3), 37.8 (C-10), 29.6 (C-5), 22.9 (C-4); ESI-MS m/z 283 $[\text{M}+\text{Na}]^+$.

Acid hydrolysis and HPLC analysis of 1. Compound **1** (2.0 mg) was dissolved in 2 mol/L HCl (10 mL) and heated at 80 °C for 6 h. The mixture was evaporated to dryness, and the residue was partitioned between EtOAc and H₂O. The aqueous phase was concentrated to furnish a monosaccharide residue. After drying under vacuum, anhydrous pyridine (0.6 mL) and D-cysteine methyl ester hydrochloride (2.0 mg) were added to the residue, and the mixture was heated at 60 °C for 1 h. After the reaction mixture was evaporated to dryness, *o*-tolyl isothiocyanate (5 μL) was then added, and the mixture was heated at 60 °C for 1 h. The reaction mixture was directly analyzed by standard C₁₈ HPLC, which was performed on an Agilent 1260 Infinity HPLC system (Agilent Technologies Inc., USA) equipped with a photodiode array detector and a 5C18-MS Cosmosil packed column (4.6 mm \times 250 mm, 5 μm) at 35 °C with isocratic elution of 25% MeCN in 0.1% formic acid solution for 30 min at a flow rate of 0.8 mL/min. The injection volume was 10 μL and peaks were detected at 250 nm. The reaction conditions for authentic D- and L-glucose were the same as described above. The absolute configuration of the monosaccharide in **1** was confirmed to be D-glucose by comparison of the retention time of the thiocarbamoyl-thiazolidine derivative of the acid hydrolysate of **1** (17.43 min) with those of standard samples: D-glucose (17.33 min) and L-glucose (18.91 min), respectively.

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