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Natural Product Research

Formerly Natural Product Letters

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/gnpl20

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To cite this article: Ying Wu , Yi-Fan Zhang , Hai-Tao Zhang , Xin-Hua Ma , Na-Na Yang , Shi-Biao Feng , Tian-Hua Zhong & Yong-Hong Zhang (2020): A new cyclopeptide alkaloid from *Clematis Florida* , Natural Product Research, DOI: 10.1080/14786419.2020.1809399

To link to this article: https://doi.org/10.1080/14786419.2020.1809399

NATURAL PRODUCT RESEARCH
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A new cyclopeptide alkaloid from *Clematis Florida*

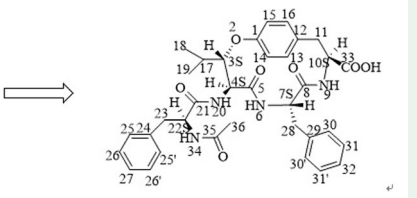
Ying Wu[a], Yi-Fan Zhang[b], Hai-Tao Zhang[a], Xin-Hua Ma[a], Na-Na Yang[a], Shi-Biao Feng[a], Tian-Hua Zhong[c] and Yong-Hong Zhang[a]

[a]Fujian Provincial Key Laboratory of Natural Medicine Pharmacology, School of Pharmacy, Fujian Medical University, Fuzhou, P.R. China; [b]Medical Imaging Department, First Affiliated Hospital of Fujian Medical University, Fuzhou, P.R. China; [c]Key Laboratory of Marine Biogenetic Resources, State Oceanic Administration, Third Institute of Oceanography, Xiamen, P.R. China

ABSTRACT

One new 14-membered ring cyclopeptide alkaloid, plenane A (**1**), along with six known compounds (**2-7**), was isolated from the roots of *Clematis florida*. Their structures were elucidated by means of NMR spectroscopic analysis and mass spectrometry. In addition, their anti- inflammatory effects on lipopolysaccharide-induced RAW264.7 macrophages were evaluated *in vitro*. The compounds **1**, **2** and **6** exhibited potent indirect inhibitory effects, with IC_{50} values of 40.92, 22.88 and 6.32 μM, respectively.

Clematis florida



plenane A

ARTICLE HISTORY
Received 10 April 2020
Accepted 7 August 2020

KEYWORDS
Clematis florida; cyclopeptide alkaloid; anti-inflammatory; RAW264.7 macrophages

1. Introduction

Clematis florida Thumb (Ranunculaceae) is a perennial plant and was once considered to be a cultivated variety from Ranunculaceae *Clematis* (*Clematis florida* Thunb. *var. plena* D. Don) (Editorial Committee of Flora of China 1980). This plant has long been used as a folk remedy in She Ethnopharmacy in southeast China for the treatment of rheumatism, diuretic, laxative, stomachache, and jaundice (Huang et al. 2013). Several triterpene, triterpene glycosides and alkaloids have been reported as constituents of *C. florida* (Sun et al. 2019). Previous studies have reported the *n*-butanal subfraction of *C. florida* exhibited potent anti-inflammatory activity (Sun et al. 2019). In a continued

CONTACT Yong-Hong Zhang zhangyh@fjmu.edu.cn

Supplemental data for this article can be accessed at https://doi.org/10.1080/14786419.2020.1809399.

search for novel active constituents from this plant, a new cyclopeptide alkaloid (**1**), together with six known compounds (**2-7**), was isolated from the root part of the plant. Herein, we describe the isolation and structural elucidation of the new compound, as well as the anti-inflammatory and cytotoxic activities of the isolated compounds *in vitro*. The isolation and characterization of plenane A (**1**) classified as cyclopeptide alkaloid with 14-membered rings similar to justicianene A (Jin et al. 2015) have never been described in previous publications from the genus *Clematis*.

2. Results and discussion

Compound **1** was obtained as a white amorphous powder. Its molecular formula was assigned as $C_{35}H_{40}N_4O_7$ by HR-ESI/MS at *m/z* 627.2829 $[M\text{-}H]^+$. The IR spectrum exhibited bands corresponding to peptide linkages. Resonances attributable to all 35 carbons and 40 protons were observed in the 1 D and 2 D NMR spectra. The presence of five downfield carbon signals for amide or ester carbonyl groups atδ_C 172.6, 169.8, 169.2, 168.9 and 168.5 in the ^{13}C NMR spectrum (Table S1), and four amide proton signals at δH 7.88, 7.65, 7.62, and 7.54 in the ^1H NMR spectrum (Table S1), revealed that **1** could be a peptide alkaloid. HSQC, HMBC, and ^1H-^1H COSY spectral data established the structure with a phenylalanine as a common ring-bonded amino acid residue, and 3-hydroxyleucine as a β-hydroxy amino acid unit, which is connected to the tyrosine fragment via an ether bridge. Attached to the amino group of 3-hydroxyleucine is a phenylalanine amino acid residue with an acetyl group linked to amino group.

The ^1H NMR spectrum (DMSO-d_6, 400 MHz) of **1** showed two sets of methyl doublets at δ_H0.77 (d, $J_{17,\ 18}$=6.2 Hz) and 0.78 (d, $J_{17,\ 19}$=6.3 Hz), which were assigned at C-18 and C-19, respectively. The C-3 and C-4 methine protons appeared at δ_H 5.71 (dd, $J_{3,\ 4}$=8.0, $J_{3,\ 17}$=2.0 Hz) and 4.77(dd, $J_{4,\ 3}$=8.0, $J_{4,\ NH\text{-}20}$=10.1 Hz), respectively. In the ^1H-^1H COSY spectrum, these two doublets had cross-peaks with the signal at δ_H 1.25-1.16 (1H, m), corresponding to H-17. In turn, H-17 showed a cross-peak with H-3 and H-3 showed a cross-peak with H-4. H-4 exhibited another cross-peak with NH-20 at δ_H 7.88 (d, $J_{4,\ NH\text{-}20}$=10.1 Hz). This spin system confirmed 3-hydroxyleucine as the hydroxylated amino acid of the macrocyclic ring. The HMBC correlation of H-4/C-21 indicated the connection between the 3-hydroxyleucine and phenylalanine with an acetyl group linked to amino group. In addition, in the HMBC experiment, cross-peaks of NH-34, H-22, H-36, and C-35 (δ_C 168.5) suggested that the acetyl group was linked to the amino group of phenylalanine. The other phenylalanine moiety was identified from cross-peaks in the ^1H-^1H COSY spectrum, between NH-6 at δ_H 7.54 (d, $J_{NH\text{-}6,\ 7}$=9.6 Hz) and H-7 at δ_H 4.14–4.16 (1H, m), between H-7 and Ha-28 at δ_H 2.49 (dd, $J_{28a,\ 7}$=4.0, J_{gel}=14.0 Hz) and Hb-28 at δ_H 2.24 (dd, $J_{28b,\ 7}$=3.0, J_{gel}=14.0 Hz), together with the correlation between H-30 at δ_H 7.17-7.20 (m) and C-28 at δ_C 36.9 in the HMBC spectrum. The tyrosine fragment was characterized from cross-peaks in the ^1H-^1H COSY spectrum between NH-9 at δ_H 7.62 (d, $J_{NH\text{-}9,\ 10}$=9.1 Hz) and H-10 at δ_H 4.78–4.82 (m), and of the latter with H_a-11 at δ_H 3.24 (dd, $J_{11,\ 10}$=5.6, J_{gel}=13.1 Hz) and H_b-11 at δ_H 2.44 (dd, $J_{11,\ 10}$=4.0, J_{gel}=13.1 Hz). Key HMBC correlations were very crucial for the linkage of these fragments and are shown in Figure S1. The linkage sequence of amino acid residues was determined by the correlations between H-10 and C-8 (δ_C

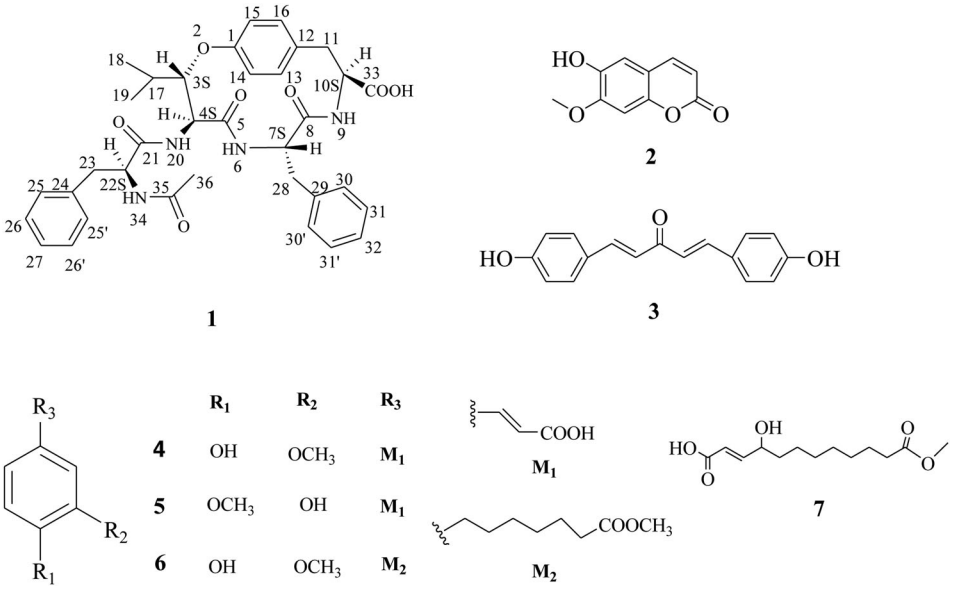


Figure 1. Structures of compounds **1–7** isolated from *C.florida*.

169.2), between H-7 and C-5 (δ_C 168.9), between H-3 and C-1 (δ_C 154.7); thus, the plane structure of the cyclopeptide alkaloid was determined. The NMR spectroscopic assignments and the coupling constants of **1** were summarized in Table S1. The vicinal coupling constant of ca. 8.0 Hz for the methine protons of 3-hydroxyleucine (H-3/H-4) indicated an erythro relative configuration for this residue, whereas threo compounds showed a $J_{3,4}$ of ca. 2 Hz (Morel et al. 1979).

The ^{13}C NMR spectrum played an important role in the stereochemical assignment of ring bonded β-OH amino acid (Païs et al. 1979). For the L-erythro β-OH-amino acid configuration, the resonance for C-4 appeared at ca. 55.0 ppm, whereas for the D-erythro configuration it appeared at ca. 53.0 ppm (Haslinger 1978). A clear difference in the chemical shift was also observed for C-3. In the L-erythro series, the signal appeared at ca. 82.0 ppm, whereas, for the D-erythro, it appeared at a lower field, at ca. 87.0 ppm. Compound **1** showed a signal in ^{13}C NMR spectra at δ_C 79.6 ppm for C-3 and 56.1 for C-4, which suggested that the 3-hydroxyleucine moiety possessed the L-erythro (3*S*, 4*S*) configuration. The stereochemistry of the tyrosine and phenylalanine was determined by analysis of NOESY interactions. In the NOESY spectrum, H-4 exhibited a prominent cross-peak with NH-6, while the latter showed a very weak cross-peak with H-7; thus, the configuration of the phenylalanine unit of **1** was determined as 7*S*. In the same way, H-7 exhibited a prominent cross-peak with NH-9, while the latter showed a very weak cross-peak with H-10 in the NOESY spectrum, indicating a 10*S* configuration for Tyr. In the NOESY spectrum, H-4 exhibited a prominent cross-peak with NH-20, while the latter showed a very weak cross-peak with H-22; thus, the configuration of the side chain phenylalanine unit was determined as 22*S*. This evidence is consistent with the L (*S*) configuration of the amino acid of higher plants. Therefore, the stereochemistry of compound **1** was proposed as 3*S*, 4*S* (L-erythro-β-hydroxyleucine), 7*S*-phenylalanine, 10*S*-tyrosine and 22*S*-phenylalanine, and Chem 3 D drawing

with selected diagnostic NOEs was also shown in Figure S2. Consequently, the structure of 1 was determined as shown (Figure 1) and named plenane A.

Compared the physical and spectral data with the literature data, 6 known compounds (**2–7**) were identified as one coumarin: isoscopolactin (**2**) (Bayoumi et al. 2010); one phenolic compound: kinsenone (**3**) (Wang et al. 2002); three organic acids:, trans ferulic acid (**4**) (Prachayasittikul et al. 2009; Shen et al. 2010), trans isoferulic acid (**5**) (Prachayasittikul et al. 2009), and methyl 6-(4-Hydroxy-3-Methoxy Phenyl) hexanoate (**6**) (Luo et al. 2016); and one fatty acid: (E) − 4-hydroxy-dodec-2-enedioicacid-12-O-methylester (**7**) (Deng et al. 2014), respectively. All compounds were isolated from *Clematis florida* for the first time, and the structures were shown in Figure 1. Table S1–S2 showed the ^{1} H and ^{13} C spectral data of the isolated compounds (**1–7**).

The anti-inflammatory effects of compounds **1–7** on LPS-induced RAW264.7 cells were studied. The results showed that compounds **1** and **2** showed mild indirect inhibitory effects, with IC_{50} values of 40.92 and 22.88 μM, respectively. The compound **6** was the most active molecule with IC_{50} of 6.32 μM (Table S3), indicating that compounds **1**, **2** and **6** play an anti-inflammatory effect by inhibiting the production of NO. SRB analysis was used to examine the cytotoxic effects of compounds **1-7** on seven tumor cell lines. No significant cytotoxic activity was observed for compounds **1-7** (Table S4).

3. Experimental

3.1. Plant material

The roots of *Clematis florida* Thunb. were obtained by Shecao herb store in Ningde, Fujian Province, P.R China, in July 2018, and identified by Prof. Yonghong Zhang, Fujian Medical University. *Clematis florida* Thumb was once considered to be a cultivated variety from Ranunculaceae *Clematis* (*Clematis florida* Thunb*. var. plena* D. Don), and there are obvious difference in the shape of stamens between them. The stamens in *C. florida var. plena* are petal-like, while wide linear in *C. florida* (Editorial Committee of Flora of China 1980). A voucher specimen was deposited in the Laboratory of the Natural Products, Fujian Medical University, P.R. China (*No*. zyh 20180701).

3.2. General experimental procedures

The UV spectrum and data were determined using a *UV-210 A*spectrometer (Hitachi Ltd, Tokyo, Japan). The IR spectrum and data were obtained by using *Nicolet 170SX FT-IR* spectrometer (Thermo Electron Corporation, Waltham, MA). The NMR spectra were recorded using an AVANCE 400 MHz instrument (Bruker Corporation, Switzerland) at 400 (^{1}H) and 100 MHz (^{13}C) with TMS as internal standard. The HR-ESI-MS spectrum were taken on *Bruker APEX II* mass spectrometer and the EI-MS spectrum were recorded by LCMS-2020 mass spectrometer, in *m/z*. GC was performed on silica gel (SiO_2; 100-200 or 200-300 mesh; *Qingdao Marine Chemical Factory*), *MCI* gel CHP-20P (75-150 μm; *Mitsubishi Chemical Co*.), ODS (50 μm, *Canada Quebec*) and C18 Semi-preparative column (250 × 10 mm, 5 μm, Waters). Silica gel *G* (*Qingdao Marine Chemical*

Factory) was used for TLC with visualization under UV light and by spraying with 5% H_2SO_4 in EtOH (v/v) followed by heating.

3.3. Extraction and isolation

Dried and crushed root of *C. florida* (10 kg) were soaked in methanol for three times at room temperature one week each. After evaporation of the solvent under reduced pressure, the residue was suspended in water and extracted with petroleum ether, EtOAc and n-BuOH, successively. The residue of the petroleum ether layer (68.5 g), EtOAc layer (102.1 g) and n-BuOH layer (416.4 g) were obtained after concentrated with recovery solvent.

The EtOAc layer (102.1 g) was fractionated by silica gel CC using a stepwise gradient of petroleum ether/EtOAc (1:0-0:1) to yield fifteen fractions (Fr. A1-A15). Compound **7** was crystallized from Fr.A2 as white granular crystal. Fr. A8 (18.3 g) was subjected to *C-18ODS* (solvent: MeOH/H_2O, 10:95-100:0, v/v) to give eight subfractions (Fr. A8-1 - Fr. A 8-8); each subfraction was separated by *Sephadex LH-20* (CH_2Cl_2/MeOH 1:1) and purified by semi-PHPLC (MeOH/H_2O 20%-100%, 2.0 mL·min^{-1}) to yield compound **1** (13.2 mg), **2** (6.5 mg), **3** (6.7 mg), **4** (8.2 mg), **5** (7.3 mg) and **6** (7.9 mg), respectively.

3.4. Plenane a

$C_{35}H_{40}N_4O_7$, white amorphous powder; $[\alpha]_D^{20}$- 135.2 (c 0.05, DMSO); IR (KBr): v_{max} 3420 (OH), 3262 (NH), 1716, 1641, 1620, 1217 cm^{-1}; for ^{1}H and ^{13}C NMR (DMSO-d_6) spectroscopic data, see Table S1; HR-ESI-MS: *m/z*, 627.2829[M-H]$^+$ (calcd for $C_{35}H_{39}N_4O_7$, 627.2818).

3.5. Anti-inflammatory effect assay

The anti-inflammatory effect assay was measured by Griess reagent colorimetry and the contents of nitric oxide (NO) in the supernatant were detected with indomethacin as positive control (Kim et al. 2020). Briefly, 1×10^4/ml cells were seeded into 96-well plates and allowed to adhere for 40-48 h. Compounds **1–7** and indomethacin dissolved in DMSO and diluted with fresh medium to 6 degrees of concentration (3.125, 6.25, 12.5, 25.0, 50.0, and 100.0 μg·ml^{-1}) were added into each well for 4 h, then 100 μL of LPS (2 μg·ml^{-1}) was added. After incubation at 37 °C for 16-20 h, the content of NO in the supernatant was determined according to the Griess reagent kit and the half maximal inhibitory concentration of cell proliferation (IC_{50}) was calculated with the GraphPad prism program obtained by plotting the inhibition rate versus the concentrations (Table S3).

3.6. Cytotoxic assay

The cytotoxic activity assay was measured by SRB method using 5-fluorouracil (5-FU) as positive control (Keawpradub et al. 1999). Cells (Huh7, HepG2, KB, H460, Hela, A549, B16-F10) were plated at a density of 6×10^3/ml cells on 96-well plates and left to

adhere for 24 h before treatment. Compounds **1–7** and 5-FU dissolved in DMSO and diluted to a 6 degree concentration (3.125, 6.25, 12.5, 25.0, 50.0, 100.0 $\mu g \cdot ml^{-1}$) with fresh medium were added to each well for 48 h. The drug concentration resulting in 50% inhibition of the growth (IC_{50}) was calculated with GraphPad prism program (Table S4).

4. Conclusion

The isolation of the EtOAc yielded one new 14-membered ring cyclopeptide alkaloid plenane A (**1**), along with six known compounds (**2–7**). The result of anti-inflammatory effect on LPS induced RAW264.7 cell showed that compound **1**(cyclopeptide alkaloid) and **2** (coumarin) exhibited potent inhibitory effects, among the four organic acid compounds (**3–6**), only compound **6** (aromatic compound with long fatty acid ester) showed significant inhibitory activity in vitro by inhibiting the production of NO, which indicates that the long fatty acid ester chain may contribute to the anti-inflammatory effect of compound **6**. *Clematis florida* was recognized to be a good source for anti-inflammatory activity may be attributed to these constituents. This study supported the fact the traditional application of *C. florida* by the local communities for inflammatory disorders such as rheumatoid arthritis.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was financially supported by the National Natural Science Foundation of China (*No.* 41806173), the Natural Science Foundation of Fujian Province (*No.* 2016J01369 and *No.* 2018J01847), the Open Project of National Marine Bureau Key Laboratory of Marine Biogenetic Resources (HY201807, HY201604), and the Science and Technology Innovation Platform Project of Fujian Province (2018Y2001).

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