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Violacin A, a new chromanone produced by *Streptomyces violaceoruber* and its anti-inflammatory activity

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ABSTRACT

A new chromanone derivative, named violacin A (**1**), was isolated from the fermentation broth of *Streptomyces violaceoruber* as a potential anti-inflammatory compound. The structure of violacin A was established using comprehensive NMR spectroscopic data analysis together with UV, IR, and MS data. The anti-inflammatory effects and action mechanisms of violacin A were investigated *in vitro*. The results demonstrated that violacin A attenuated the production of NO, IL-1β, IL-6, and TNF-α as well as inhibited the expression of iNOS in LPS-induced RAW 264.7 cells. Additionally, Western blot and qRT-PCR results revealed that **1** down-regulated pro-inflammatory cytokines expression correlated with the suppression of NF-κB signaling pathway.

Increasing awareness of inflammation is not confined to that it is a localized protective reaction to injury or infection, but also a spotlight in pathogenesis of many inflammatory disorders.[1] Abundant evidence has demonstrated that inflammation plays a critical role in the development of multiple diseases, including atherosclerosis and coronary artery disease, diabetes, Alzheimer's disease and the like.[2–5] Natural products serve as the basis for providing lead compounds in the search for therapeutic approach of inflammation-associated disorders.[6] Many natural compound, such as triptolide, resveratrol, silymarin have been used as anti-inflammatory agent in clinic.[7,8]

In the process of our continuous search for bioactive secondary metabolites from streptomyces associated with animal feces, *Streptomyces violaceoruber* (YIM 101131), which was isolated from fecal samples excreted by healthy adult *Equus burchelli* (zebra) living in Yunnan Wild Animal Park, was chosen to investigate the chemical constituents. Three new compounds including a tetralone derivative, a tryptophan derivative, and a fatty acid amide as well as a known compound GTRI-02 were obtained from the fermentation broth of *S. violaceoruber* in our previous work and the new tetralone derivative showed inhibition of NO production in LPS-stimulated RAW 264.7 macrophage cells with an IC_{50} value of 51.2 μM.[9] The further separation of the scale-up fermentation broth (70 L) of *S. violaceoruber* by a sequential column chromatographies over Sephadex LH-20, silica gel, and ODS led to yield another new chromanone, violacin A (**1**) (Fig. 1A).[10,11] Herein, we reported the isolation, structure elucidation, anti-inflammatory effects and mechanisms in LPS-stimulated RAW264.7 macrophage cells of violacin A (**1**).

Compound **1** was obtained as a colorless, amorphous solid and its molecular formula was elucidated as $C_{13}H_{14}O_5$ by HRESIMS at *m/z* 273.0743 $[M+Na]^+$. Its IR spectrum showed the presence of hydroxyl group (3182 cm^{-1}), carbonyl groups (1708, 1669 cm^{-1}), and benzene ring (1608, 1582, 1461 cm^{-1}). The ^1H NMR spectrum (Table 1) of compound **1** revealed the presence of two *meta*-coupled aromatic protons at δ_H 6.25 and 6.14, two AB system methylenes at δ_H 3.02 (d, J = 14.9 Hz), 2.98 (d, J = 14.9 Hz) and δ_H 3.04 (dd, J = 15.9, 2.0 Hz), 2.60 (d, J = 15.9 Hz), two singlet methyls at δ_H 2.45 and 2.19. In addition, signals of two active protons were observed at δ_H 10.35 and 7.08. The ^{13}C NMR data (Table 1) of compound **1**, together with its HSQC spectrum, suggested thirteen carbon signals comprising two carbonyl carbons at δ_C 205.6 and 191.0, six aromatic carbons at δ_C 163.1, 161.3, 142.9, 113.0, 112.3, and 102.0, one ketal carbon at δ_C 100.3, two methylenes at δ_C 52.8 and 47.9, and two methyls at δ_C 32.3 and 23.0. Comparison of the ^1H and ^{13}C NMR data of **1** to those of a known compound, 2,7-dihydroxy-5-methyl-2-(2-oxobutyl)chroman-4-one[12] revealed that both had the same chromanone skeleton. The difference between them was one quartet methylene was missing and a

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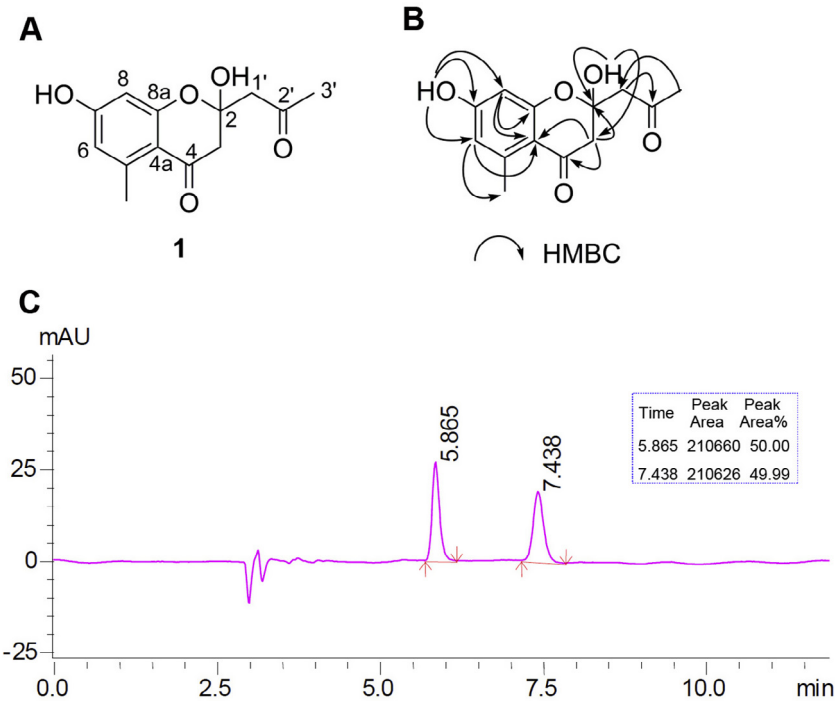

Fig. 1. Structure and chiral analysis of violacin A (**1**). (A) The structure of **1**; (B) The main HMBC correlations of **1**; (C) The chiral analysis of **1**. Column: CHIRALPAK® (0.46 cm I. D. × 25 cm, 5 μm); Mobile phase: *n*-hexane/ethanol/trifluoroacetic acid = 80/20/0.1 (*v*/*v*/*v*); Flow rate: 1.0 ml/min; λ: 220 nm.

Table 1
^{1}H NMR (600 MHz) and ^{13}C (150 MHz) NMR data of compound **1** in DMSO d_6.

No.	δ_C, type	δ_H, mult. (*J* in Hz)	HMBC
2	100.3, C		
3	47.9, CH_2	3.04, dd (15.9, 2.0) 2.60, d (15.9)	C-2, C-4, C-4a
4	191.0, C		
4a	112.3, C		
5	142.9, C		
6	113.0, CH	6.25, d (2.4)	C-4a, C-8, 5-CH_3
7	163.1, C		
8	102.0, CH	6.14, d (2.4)	C-4a, C-6, C-7, C-8a
8a	161.3, C		
1′	52.8, CH_2	3.02, d (14.9) 2.98, d (14.9)	C-2, C-3, C-2′
2′	205.6, C		
3′	32.3, CH_3	2.19, s	C-1′, C-2′
5-CH_3	23.0, CH_3	2.45, s	C-4a, C-5, C-6
2-OH		7.08, d (2.0)	C-2, C-3
7-OH		10.35, brs	C-6, C-7, C-8

singlet methyl replaced a triplet methyl in **1**, these findings hinted the substitute at C-2 is 2-oxopropyl in **1**. HMBC correlations from H-1′ (δ_H 3.02, 2.98) to C-2 (δ_C 100.3), C-3 (δ_C 47.9), and C-2′ (δ_C 205.6), from H-3′ (δ_H2.19) to C-1′ (δ_C 52.8) and C-2′ (δ_C 205.6) confirmed the structure (Fig. 1A, B). **1** was identified as a racemic mixture on the basis of chiral analysis by HPLC with a chiral column (Fig. 1C). Thus, the structure of **1** was determined to be (±)-2,7-dihydroxy-5-methyl-2-(2-oxopropyl)chroman-4-one, and named violacin A.[13]

Macrophages act as innate immune cells which play decisive roles in the primary response to inflammation.[14] Lipopolysaccharide (LPS) activates macrophages to produce a series of pro-inflammatory cytokines and inflammatory mediators such as nitric oxide (NO), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor (TNF-α).[15] Excessive amount of pro-inflammatory cytokines produced by activated macrophages is responsible for a number of inflammatory and infectious diseases.[16] LPS stimulated RAW264.7 cells are often used as a screening model to discover the anti-inflammatory agents.[17] To investigate the anti-inflammatory activities of violacin A (**1**), the NO release and expression of IL-1β, IL-6, and TNF-α in LPS-stimulated RAW264.7 cells were measured after treatment with violacin A through Griess assay and qRT-PCR methods.[18,19] As shown in Fig. 2A, violacin A dose-dependently inhibited NO production in the LPS-stimulated cells. MTT assay at varying concentration of **1** (3.3–90 μM) was carried out to exclude the cytotoxicity of **1** against RAW 264.7 cells.[20] Data (Fig. 2B) indicated that **1** had no effect on cell viability. In addition, the mRNA expression levels of IL-1β, IL-6, and TNF-α were obviously inhibited in a dose-dependent manner in LPS-stimulated cells after treatment with **1** (Fig. 3). The data suggested that **1** could down-regulate pro-inflammatory cytokines expression to present anti-inflammatory potential.

Inducible nitric synthase (iNOS), an isoforms of NO synthases, catalyzes the production of nitric oxide (NO) from L-arginine in the cells. Therefore, inhibitory of overproduction of NO through blocking iNOS expression could be potential therapeutic approach for inflammation-associated diseases. Herein, the experiments were carried out to determine the effects of **1** on the mRNA and protein expression of iNOS.[21] As presented in Fig. 4A, LPS stimulation obviously increased the iNOS protein level, whereas the stimulatory effects were blocked when the cells were pretreated with **1**. Consistent with Western blot analysis, **1** significantly suppressed LPS-induced gene expression of iNOS in a dose-dependent manner in RAW 264.7 cells (Fig. 4B). Results suggested that **1** could down-regulate LPS-stimulated iNOS expression to inhibit the production of NO in RAW 264.7 cells.

Nuclear factor-κB (NF-κB) signaling pathway plays a pivotal role in the progress of inflammation.[22] Under normal physiological conditions, NF-κB p65 is localized in the cytoplasm associated with regulatory proteins called inhibitors of κB (IκB). Upon activation, IκB-α undergoes phosphorylation, ubiquitination and degradation, which results in NF-κB p65 subunit being released and translocated to nucleus, where they bind to specific promoter regions of target genes, including pro-inflammatory genes.[23] The activation