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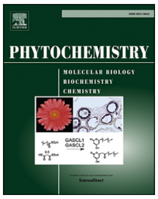

Flavonoids, alkaloids from the seeds of *Crotalaria pallida* and their cytotoxicity and anti-inflammatory activities

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ABSTRACT

Three flavonoids, cropalliflavones A–C, including two homoisoflavonoids with rare skeletons; three previously undescribed alkaloids, usaramine-N-oxide and cropallins A-B; and sixteen known compounds, were isolated from the seeds of *Crotalaria pallida* Ait. The absolute configurations of cropalliflavone A and usaramine-N-oxide were established by an ECD calculation and X-ray crystallography, respectively. Additionally, cropalliflavone B showed anti-proliferative activity against the MCF-7 cell line with an IC_{50} value of 6.77 μM, and cropalliflavone C showed anti-inflammatory activity, with an IC_{50} value of 16.07 μM.

1. Introduction

Traditional medicine plays an important role in the prevention and treatment of diseases in China. The genus *Crotalaria* comprises approximately 550 species worldwide, in which 40 species distributed in China (Editorial Committee of Flora of China, 1998). Twenty-two of these species have been used in Chinese traditional and folk medicines for the treatment of tumour, rheumatoid arthritis, tinea scabies and traumatic injuries (Lian, 1986).

Crotalaria pallida Ait (Leguminosae) was authenticated by Prof. Li-Hong Wu and has been used as folk medicine in southwest and east China. *C. pallida* is also known as "Zhu-Shi-Dou", its roots are used to treat scrofula, mastitis and dysentery; its stems and leaves are used to treat diarrhoea; and its seeds are used to treat neurasthenia, dizziness, leucorrhoea and tumours (Lian, 1986; The National Assembly of Chinese Herbal Medicine Editorial Committee, 1996). Modern pharmacological research has demonstrated that this species displays a variety of biological activities, including anti-inflammatory, antimicrobial, estrogenic and mutagenic activities (Ko et al., 2004; Pelegrini et al., 2009; Weng et al., 2003; Arzt and Mount, 1999; Lin et al., 2006; Boldrin et al., 2013).

In our previous studies, we isolated sixteen flavonoids and two pyrrolizidine-type alkaloids from *C. albida* (Sun and Chou, 2015; Sun et al., 2013) and twenty-one isoflavonoids from *C. sessiliflora* (Fan et al., 2015). In an ongoing search for bioactive natural compounds from the genus *Crotalaria*, the seeds of *C. pallida* were selected for a detailed phytochemical study. This led to the isolation of six previously undescribed compounds, two homoisoflavonoids (**1**–**2**), one flavonoid (**3**), three alkaloids (**11**–**13**), and sixteen known ones (**4**–**10**, **14**–**22**). Additionally, some compounds were evaluated for their anti-inflammatory activity in macrophage RAW 264.7 cells and cytotoxic activities against the MCF-7 and CaCo2 cell lines.

2. Results and discussion

2.1. Chemistry

Compound **1** was isolated as a white amorphous powder. Its molecular formula ($C_{23}H_{22}O_7$) was deduced from an HR-ESI-MS at *m/z* 843.2645 ($[2\,M + Na]^+$, calcd. for 843.2629), an HR-EI-MS at *m/z* 410.1367 ($[M]^+$, calcd. for 410.1366) and ^{13}C NMR data, which

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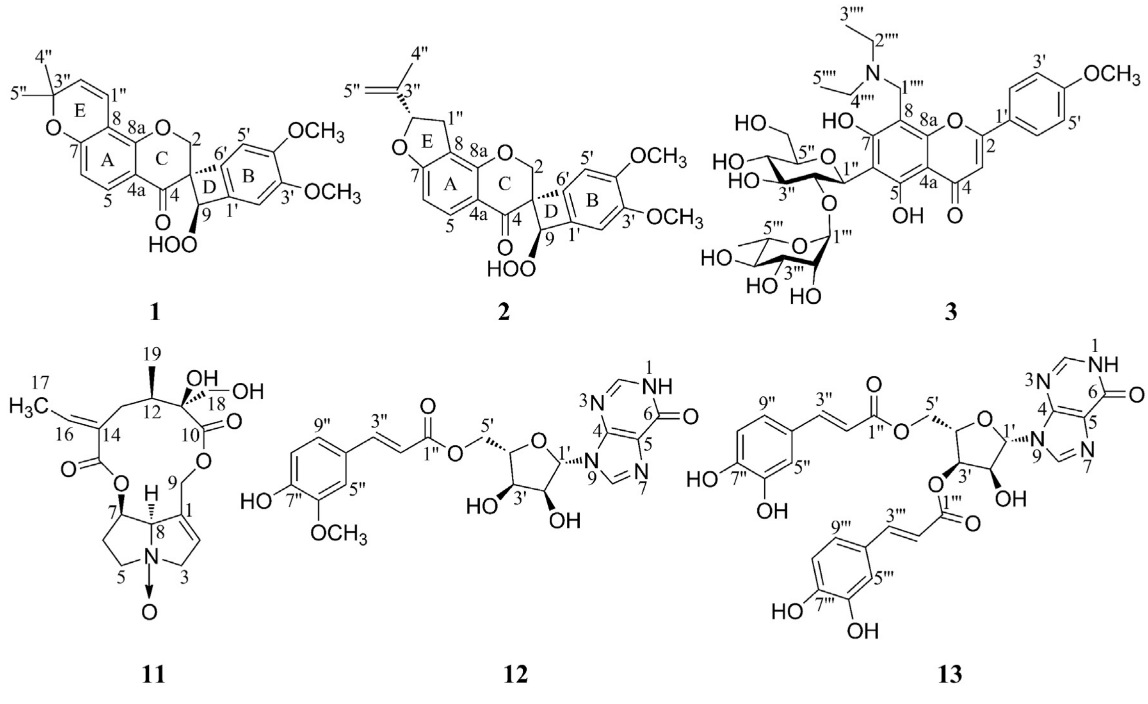
indicated 13 degrees of unsaturation. The ^{1}H NMR data displayed a set of *ortho*-coupled aromatic protons [δ_H 7.73 (1H, d, J = 8.7 Hz, H-5), 6.48 (1H, d, J = 8.7 Hz, H-6), ring A], two other aromatic protons [δ_H 6.49 (1H, s, H-2′), 6.57 (1H, s, H-5′), ring B], two olefinic protons [δ_H 6.61 (1H, d, J = 10.1 Hz, H-1″), 5.56 (1H, d, J = 10.1 Hz, H-2″)] and fifteen aliphatic protons, including an oxygenated methyne [δ_H 4.59 (1H, br s, H-9)], a methylene [δ_H 4.50 (1H, d, J = 12.1 Hz, H-2α), 4.64 (1H, dd, J = 12.1, 2.2 Hz, H-2β)], two methoxyls [δ_H 3.82 (3H, s, 3′–OCH_3), 3.73 (3H, s, 4′–OCH_3)], and two methyls [δ_H 1.45 (3H, s, H-4″), 1.39 (3H, s, H-5″)]. The above spectroscopic data resembled those of 5,7-dihydroxy-3′,4′-dimethoxyspiro {2*H*-1-benzopyran-7′-bicyclo[4.2.0]octa[1,3,5]-trien}-4-one (a homoisoflavonoid with rare scillascillin-type) (Mutanyatta et al., 2003). Additionally, the ^{13}C NMR and DEPT data showed 23 carbons, which included a scillascillin skeleton (16 carbons), and two olefinic carbons, two methyls, two methoxyls and one tertiary carbon. The HMBC cross-peaks (Fig. 2) of H-4"/H-5" with C-3" (δ_C 78.0), C-2" (δ_C 128.8), H-2″ with C-1" (δ_C 115.4), C-4" (δ_C 28.5), and H-1″ with C-7 (δ_C 160.7), C-8 (δ_C 111.1) and C-8a (δ_C 156.6) indicated that a 2,2-dimethyl α-pyran fragment (ring E) was attached to C-7 and C-8. Moreover, the HMBC cross-peaks of H-9 with C-2 (δ_C 63.8), C-3 (δ_C 67.4), C-4 (δ_C 191.3) and C-1' (δ_C 108.6), in combination with the molecular formula and the fragment ion $[M-OH]^-$ at *m*/*z* 393.1 by DART-MS (Figs. S7a–7c, Supporting Information) suggested that an unusual hydroperoxy group located at C-9. The HMBC correlations drawn in Fig. 2 also showed the two methoxyls located at C-3′ and C-4'.

The NOESY correlation (Fig. 2) of H-2β with 9-OO$\underline{H}$ (δ_H 4.51) indicated that the hydroperoxy group (9–OOH) was $\underline{\beta}$-oriented. Moreover, the absolute configuration of compound **1** was confirmed as 3*S*,9*R* by comparing the experimental and calculated ECD spectra of **1** (Fig. 4), which was performed using the time-dependent density functional theory (TDDFT) method at the B3LYP/6-31G (d) level (Zhao et al., 2016). Therefore, the structure of **1** was defined as shown (Fig. 1) and named cropalliflavone A.

Compound **2** was also obtained as a white amorphous powder and had the same molecular formula ($C_{23}H_{22}O_7$) as compound **1**, which was deduced from an HR-ESI-MS at *m*/*z* 843.2242 ($[2M+Na]^+$, calcd. for 843.2629) and an HR-EI-MS at *m*/*z* 410.1370 ($[M]^+$, calcd. for 410.1366) (Figs. S15a–15c, Supporting Information). Furthermore, the 1D-NMR data of **2** were similar to those of **1**, except for a missing methyl signal, two sp^2 methyne signals and one tertiary carbon signal in **1** as well as the appearance of two methylenes [δ_C 31.1, C-1"; δ_C 112.7, C-5"], one methyne [δ_C 87.9, C-2"] and one sp^2 tertiary carbon [δ_C 142.8, C-3"] in **2**. Based on these analyses, it suggested that an isopropenyl dihydrofuran fragment (ring E) may locate at C-7 and C-8 instead of the 2,2-dimethyl α-pyran fragment, as seen in **1**. The HMBC cross-peaks (Fig. 2) of H-4" (δ_H 1.77, 3H, s) with C-2″, C-3″ and C-5″ as well as H-1″α/H-1″β with C-2″, C-7 (δ_C 168.0), C-8 (δ_C 113.2) and C-8a (δ_C 111.7) further verified the planar structure of **2**.

In the NOESY experiment (Fig. 2), correlations of 9-OO$\underline{H}$/H-2β/H-1″β/H-2″ indicated that 9–OOH and H-2″ were β-oriented. Therefore, the structure of **2** was characterized as shown (Fig. 1) and named cropalliflavone B.

Compound **3** was obtained as a yellow amorphous powder. Its molecular formula was determined to be $C_{33}H_{43}NO_{14}$ by an HR-ESI-MS at *m*/*z* 676.2633 ($[M-H]^-$, calcd. for 676.2605). The ^{1}H and ^{13}C NMR data of **3** (Table 2), with the aid of HSQC, HMBC and ^{1}H-^{1}H COSY experiments, indicated the presence of a flavonoid C-glycoside. The ^{1}H NMR data showed two doublets at δ_H 8.12 (2H, d, J = 8.8 Hz, H-2′, 6′) and 7.05 (2H, d, J = 8.8 Hz, H-3′, 5′), corresponding to an AA'BB′ system of the *para*-substituted B-ring, one singlet at δ_H 6.75 (1H, s, H-3) attributed to H-3 typical of a flavone derivative and one methoxyl group located at C-4′ based on the HMBC correlation between δ_H 3.86 (3H, s, 4′–OCH_3) with δ_C 162.5 (C-4′). The absence of the H-6 and H-8 proton signals suggested that compound **3** had a 4′,5,6,7,8-pentasubstituted flavone nucleus. Two anomeric protons at δ_H 4.83 (1H, d, J = 10.0, H-1″) and 4.99 (1H, s, H-1‴) together with a methylene group at δ_H 3.54 (1H, dd, J = 10.7, 5.3 Hz, H-6″a) and 3.75 (1H, d, J = 10.7 Hz, H-6″b) as well as a doublet methyl group at δ_H 0.49 (3H, d, J = 6.1, H-6‴) were also observed in the ^{1}H NMR spectrum of **3**, indicating the presence of



1 **2** **3**

11 **12** **13**

Fig. 1. Structures of compounds **1**–**3** and **11**–**13**.