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Short communication

Diketopiperazine indole alkaloids from hemp seed

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ABSTRACT

Hemp seeds from non-drug varieties of *Cannabis sativa* L. are an important source of food and medicine. In continuation of our ongoing study on hemp seed, two pairs of stereoisomers of diketopiperazine indole alkaloid (12S, 22R)-Dihydroxyisoechinulin A (**1**), (12S, 22S)-Dihydroxyisoechinulin A (**2**) and (12R/S)-Neoechinulin A (**3**) were isolated. Their structures were elucidated with UV, IR, NMR, MS, CD spectra, ECD and chiral HPLC analysis techniques. This type of alkaloid is more often reported from fungi, such as *Aspergillus* and *Eurotium,* than from plant sources. Since common molds can contaminate herbal medicines, various hemp seed samples and the metabolites of the main fungi isolated from hemp seed were analyzed using HPLC. The data suggested that the isolated compounds are rare constituents of hemp seed, rather than fungal contamination. Thus, in this study, these compounds were isolated from hemp seed for the first time, and a previous study's prediction of endogenous indole alkaloids in hemp was confirmed. Meanwhile, Neoechinulin A could promote SIRT1 expression in HEK293 cell lines. SIRT1 is becoming an important drug target for new therapies in the treatment of neurodegenerative diseases.

1. Introduction

Hemp seed, the seed from non-drug varieties of *Cannabis sativa* L., has been an important source of oil, food, fiber, animal feed and medicine for thousands of years. Phytochemical studies have revealed that the major constituents of hemp seed are fatty acids and esters, steroids, terpenes, lignanamides, and cannabinoids (Flores-Sanchez and Verpoorte 2008; Yan et al., 2015). A handful of quaternary bases have also been reported (Turner et al., 1980). Although the possible presence of indole alkaloids in Cannabis was discussed by Samrah et al. (Turner et al., 1980), there are no published reports of indole alkaloids from Cannabis to date.

In continuation of our ongoing study of hemp seed, two pairs of stereoisomers of diketopiperazine indole alkaloids were isolated for the first time, named (12S, 22R)-Dihydroxyisoechinulin A (**1**), (12S, 22S)-Dihydroxyisoechinulin A (**2**), (12R)-Neoechinulin A (**3a**) and (12S)-Neoechinulin A (**3b**). Although these kinds of compounds have been reported in plants such as *Pulsatilla cernua* (Yu et al., 2013), *Cyrtomium fortunei* (Yang et al., 2013), *Inula hupehensis* (Fei et al., 2012) and *Opuntia dillenii* (Wu et al., 2013), such diketopiperazine indole alkaloids belonging to the echinulin family, are often reported from the metabolites of fungi such as *Aspergillus* (Marchelli et al., 1977; Li et al., 2004a,b), *Eurotium* (Li et al., 2004a,b; Wang et al., 2007) and other genera (Li et al., 2008; Du et al., 2012). Coincidentally, *Aspergillus* and *Eurotium* are common molds that can contaminate herbal medicines. Thus, the question arises whether the isolated indole alkaloids are from hemp seed itself or from the fungi contaminating hemp seeds. In order to answer the question, HPLC-UV techniques were used to confirm the presence of these molecules in hemp seed samples collected from different regions. Simultaneously, the dominant fungi obtained from this hemp seed material were cultured, and their metabolites were also analyzed by HPLC-UV too.

To further explore the bioactivity of the isolated alkaloids, the SIRT1 (silent information regulator two homologue 1) promotion activities of Neoechinulin A was tested using HEK293 cell lines. SIRT1 is a member of the sirtuin family that possesses NAD^+-dependent deacetylase activity and regulates a variety of cellular processes such as energy metabolism, cell-cycle progression and aging (Raghavan and Shah, 2012). SIRT1 is becoming an important drug target for new therapies in the treatment of neurodegenerative diseases, and small molecule SIRT1 activators or compounds that promote its expression could be candidates for the treatment of aging and age-related diseases (Zhang et al., 2015).

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2. Results and discussion

2.1. Structural identification

The ethyl acetate-soluble portion of the 75% EtOH extract of hemp seed was fractionated using silica gel, reverse-phase silica gel, MCI gel and Sephadex LH-20 column chromatography and was further purified by HPLC to afford four diketopiperazine indole alkaloids.

Compounds **1** and **2** were isolated as colorless amorphous powder from the same subfraction by semi-preparative high performance liquid chromatograph at different retention time (t_R 11.5 min and 12.2 min, respectively) (HPLC chromatogram see Fig. S7). The molecular formula of compound **1** was established as $C_{24}H_{31}N_3O_4$ by positive HR-ESI–MS data (*m/z* 448.2206 $[M+Na]^+$, calcd. 448.2207, for $C_{24}H_{31}N_3O_4Na^+$), indicating eleven degrees of unsaturation. The UV absorption at λ_{max} (MeOH): 335 nm (log ε 4.13) and 286 nm (log ε 3.84) showed the presence of conjugated indole chromophores (Li et al., 2004a,b). The 1D (Table 1) and 2D NMR spectra of **1** showed signals ascribable to methyl-substituted diketopiperazine [δ_H 4.21 (H-12), 1.48 (CH_3-20); δ_C 126.0 (C-9), 160.9 (C-10), 51.3 (C-12), 167.4 (C-13), 19.5 (C-20)], a trisubstituted indole [δ_H 7.12 (H-4), 7.02 (H-6), 7.30 (H-7); δ_C 144.8 (C-2), 102.7 (C-3), 126.0 (C-3a), 118.9(C-4), 131.9 (C-5), 123.1 (C-6), 110.9 (C-7), 134.1 (C-7a)], an isopentenyl group [δ_H 6.06 (H-16), 5.06, 5.04 (CH_2-17), 1.49, 1.50 (CH_3-18/19); δ_C 39.1 (C-15), 144.8 (C-16), 111.1 (C-17), 26.7 (C-18), 26.8 (C-19)], a dihydroxyisopentanyl group [δ_H 3.02 (Ha-21), 2.50 (Hb-21), 3.48 (H-22), 1.19, 1.16 (CH_3-24/25); δ_C 37.7 (C-21), 80.2 (C-22), 72.4 (C-23), 23.4 (C-24), 24.7 (C-25)], and a trisubstitited double bond [δ_H 7.17 (H-8); δ_C 113.1 (C-8), 126.0 (C-9)]. Fig. 1 shows the key HMBC correlations. The spectral data are similar to those of Dihydroxyisoechinulin A (Chen et al., 2015; Li et al., 2004a,b). Compound **2** and **1** have very similar NMR data (Table 1). However, their optical rotation $[\alpha]^{20}_D$ (*c* 0.1 MeOH) were obviously different: −11.4° for **1** and −46.8° for **2**. Compound **1** and **2** were tentatively assigned as a pair of epimers. To determine their absolute configuration, ECD spectra for **1** and **2** and their enantiomers were calculated using the TDDFT method. The experimental CD spectrum of **1** matched well with the calculated ECD of 12S, 22R-Dihydroxyisoechinulin (Fig. 2, left). The experimental CD spectrum of **2** matched well with the calculated ECD of 12S, 22S-Dihydroxyisoechinulin (Fig. 2, right). Thus, **1** was determined to be 12S, 22R-Dihydroxyisoechinulin (Li et al., 2004a,b) and **2** was determined to be 12S, 22S-Dihydroxyisoechinulin (Rubrumline A, Chen et al., 2015).

Table 1
^{1}H NMR Data for **1** and **2** (CD_3OD, 600 MHz) and ^{13}C NMR data of **1** (CD_3OD, 150 MHz).

Position	**1**		**2**
	δ_H, multip. *J* in Hz	δ_C	δ_H, multip. *J* in Hz
2		144.8	
3		102.7	
3a		126.0	
4	7.12, s	118.9	7.11, s
5		131.9	
6	7.02, d, 8.2	123.1	7.02, d, 8.2
7	7.30, d, 8.2	110.9	7.30, d, 8.2
7a		134.1	
8	7.17, s	113.1	7.17, s
9		126.0	
10		169.0	
12	4.21, q, 6.8	51.3	4.18, q, 6.8
13		167.4	
15		39.1	
16	6.06, dd, 17.3, 10.6	144.8	6.06, dd, 17.3, 10.6
17	5.06, d, 10.6	111.1	5.06, d, 10.6
	5.04, d, 17.3		5.04, d, 17.3
18	1.49, s	26.7	1.49, s
19	1.49, s	26.8	1.49, s
20	1.48, d, 6.8	19.5	1.51, d, 6.8
21	3.02, dd, 13.9, 1.3	37.7	3.02, d, 13.9
	2.50, dd, 13.9, 10.3		2.50, dd, 13.9, 10.3
22	3.48, dd, 10.3, 1.3	80.2	3.48, dd, 10.3, 1.3
23		72.4	
24	1.19, s	23.4	1.19, s
25	1.17, s	24.7	1.17, s

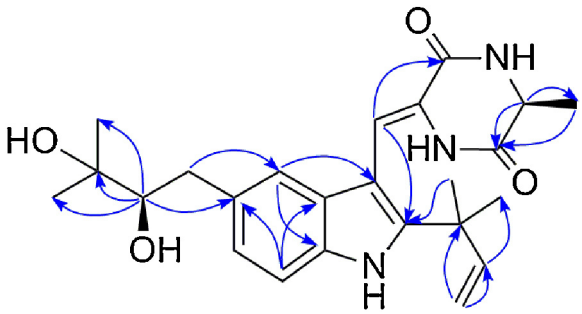

Fig. 1. Key HMBC correlations of compound **1**.

Compound **3** was isolated as a colorless amorphous powder. The molecular formula was established as $C_{19}H_{21}N_3O_2$ by positive HR-ESI–MS data (*m/z* 324.1710$[M+H]^+$, calcd. 324.1712, for $C_{19}H_{22}N_3O_2^+$). The 1D NMR spectra of **3** were similar to Neoechinulin A (Yagi and Doi 1999; Du et al., 2012), except for paired ^{13}C NMR signals appearing at C-15 (δ_C 39.2/39.3), C-12 (51.7/51.6), C-3 (102.8/102.9), C-9 (123.8/123.9), C-3a (125.9/126.0), C-7a (134.3/134.4), C-2 (143.9/144.0), C-13 (159.9/160.0) and C-10 (165.7/165.8), and different optical rotations: **3**, $[\alpha]^{20}_D$ −2.65 (*c* 0.2 MeOH); and (12S)-Neoechinulin A $[\alpha]^{24}_D$ −28.0 (*c* 0.53 MeOH) (Du et al., 2012). Thus **3** was further analyzed by HPLC using a chiral column (CHIRALPAK AS-H, 5 μm, 4.6 × 250 mm), with Hexane/isopropanol/diethylamine (4:1:0.05, v/v/v) as the mobile phase. Two peaks were detected at t_R 53 min and t_R 57 min, and their area ratio was 1:2 (Fig. S14). Thus compound **3** was identified as a pair of enantiomers: (12R)-Neoechinulin A (**3a**) (Yagi and Doi, 1999) and (12S)-Neoechinulin A (**3b**) (Wang et al., 2007; Du et al., 2012) by comparison of their spectral data with literature reports.

2.2. Searching for Neoechinulin A in hemp seed samples using HPLC-UV analysis

To determine the existence of diketopiperazine indole alkaloids in hemp seed, samples collected from the previously studied region (Guangxi-1) and from a different (Shanxi) region were purchased. Neoechinulin A was used as reference (Fig. 3A). HPLC chromatograms of the defatted extracts from the two regions showed that the corresponding chromatographic peaks for Neoechinulin A at 36 min in the shanxi materal (Fig. 3D), and the previously studied Guangxi sample (Guangxi, Fig. 3B). No Neoechinulin A peak was initially observed in the Guangxi-1 materal (Fig. 3C). However, when an additional polyamide column chromatography pretreatment step was performed on the defatted Guangxi-1 material, a Neoechinulin A peak was detected in 30% EtOH/H_2O fraction (Fig. 3F).

2.3. Searching for Neoechinulin A in fungal metabolites using HPLC-UV analysis

In order to further confirm the origin of these four compounds, three major fungi were isolated from the previously studied hemp seed from Guangxi and cultured. The fungi were identified as *Aspergillus Niger*, *Aspergillusa Sojae* and *Penicillium* respectively