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A New Butoxy Substituted Indolediketopiperazine from the Marine Derived Fungus *Aspergillus* sp. 66may

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Abstract: A new butoxy substituted indolediketopiperazine (**1**), together with five known indolediketopiperazines (**2−6**), which are derivatives of brevianamides, were isolated from the marine derived fungus *Aspergillus* sp. 66may. Careful analysis and comparison of the HRESIMS, as well as 1D and 2D NMR datasets, identified the structures of the isolated compounds. The subsequent antibacterial and anti-inflammatory activities showed that compound **4** possessed antimicrobial bioactivities against *Bacillus subtilis* ATCC 39620 with minimum inhibitory concentration (MIC) value of 64 μg/mL. Compounds **1**, **2**, **4** exhibited potential inhibitory activities against NO production with IC_{50} value of 28.2, 21.3, 23.6 μM, respectively. It seems that butoxy substitution at C-9 in brevianamides have little effects on their antimicrobial and anti-inflammatory bioactivities.

Keywords: Diketopiperazines; brevianamide; antibacterial activity; anti-inflammatory.

1. Introduction

Diketopiperazines (DKPs) which are characterized by a stable six-membered ring pharmacophore, have attracted much attention for its significant bioactivities for a long time [1-2]. The peptide bonds in DKPs are usually catalyzed by nonribosomal peptide synthetases (NRPSs) or cyclodipeptide synthases (CDPSs) [3]. DKPs have a broad range of promising biological activities, including antimicrobial, antitumor and antiviral activities [4-5]. For example, the marine-derived DKP plinabulin (recently named as NPI-2358), which is an analogue of the natural isolated DKP (NPI-2350) from *Aspergillus* sp., is now in phase III trial for the treatment of non-small cell lung cancer (NCT02504489) [6-7]. DKPs are widely found in fungi of *Aspergillus*, *Penicillium*, and *Eurotium* [4]. Indolediketopiperazines (indole DKPs) are a subclass of DKPs, expect for DKPs core structure, they usually contain one or more indole moieties. As to DKPs, indolediketopiperazines also have a wide spectrum of biological activities [8].

Brevianamides are naturally occurring prenylated indolediketopiperazines alkaloids, which are usually produced by *Penicillium* or *Aspergillus*. Brevianamides A−F were first isolated by Birch and

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Wright from *Penicillium brevicompatum* [9]. Among them, brevianamides A and D are confirmed to have insecticidal activity [10]. The dimeric brevianamides, such as brevianamides J [11] and S [12], were obtained from *Aspergillus versicolor*. Biosynthetic pathway elucidation conducted by Ye Y. unveiled that the enzyme BvnE is an essential isomerase/semipinacolase for the formation of brevianamides A and B, which are occurred through a BvnE-controlled semipinacol rearrangement and hetero-Diels–Alder cycloaddition [13].

Due to the extreme environment of high pressure, high salinity, low temperature, and low oxygen concentration, marine-derived microorganisms have always been a promising source for drug leads discovery [14]. In our continuous investigation for bioactive new compounds from marine microorganisms, we have obtained immunomodulatory activity compounds of amino acid conjugated anthraquinones [15], anti-MRSA agent of equisetin [16]. Recently, HPLC-DAD metabolites analysis of the crude extracts of the marine-derived fungus 66may and subsequent chemical investigations lead to the isolation of six indolediketopiperazines (brevianamides), including a new indolediketopiperazine (**1)** substituted with n-butoxy group, which is rare in nature (Figure 1). We report herein the isolation, structure elucidation, antibacterial and anti-inflammatory activities of these brevianamide derivatives.

2. Materials and Methods

2.1. General Experimental Procedures

Optical rotations were measured on an MCP 500 polarimeter (Anton Paar). UV spectra were obtained by a U-2600 spectrometer (Shimadzu). NMR spectra were recorded with a Bruker Avance III HD at 400 MHz for ^{1}H nuclei and 100 MHz for ^{13}C nuclei. Chemical shifts (δ) are given in ppm with reference to tetramethylsilane (TMS). High Resolution Electrospray Ionization Mass Spectroscopy (HRESIMS) spectra were measured with a Maxis quadruple-time-of-flight mass spectrometer (Bruker). Column chromatography (CC) was performed on silica gel (200−300 mesh, Yantai Jiangyou Silica Gel Development Co., Ltd.). Semi-preparative High Performance Liquid Chromatography (HPLC) was carried out using a Thermo Scientific UltiMate 3000 with a C18 column (250 × 10 mm, 5 µm, YMC). RAW 264.7 cells were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China).

2.2. Strain Material

The strain, which was isolated from the sea sediment at a depth of 35 meter (116°30.202’E, 22°29.355’N), was supplied by professor Jianhua Ju. A vouch of the strain of *Aspergillus* sp. 66may was deposited at School of Pharmacy and Bioengineering, Chongqing University of Technology, Chongqing, China. The ITS (Internally Transcribed Spacer) sequence of 18S rDNA gene (494bp) of the strain 66may was submitted to GenBank under the accession number of OR336351, which showed the highest similarity (99.2%) with that of *Aspergillus versicolor* ATCC 9577 (NR 131277) and *Aspergillus jensenii* (NRRL 58600). Thus, this strain was assigned as *Aspergillus* sp. 66may.

2.3. Fermentation

A single colony of the fungus of *Aspergillus* sp. 66may cultured on potato dextrose agar (PDA) medium (30% potato starch, 2% glucose, 2% agar, w/v) were inoculated into seed medium of TSBY (3% tryptone soy broth, 0.5% yeast extract, 10.5% sucrose) in 250 mL Erlenmeyer flasks and cultured for 2 days at a condition of 200 rpm and 28°C. Then the seed cultures (13%, v/v) were transferred into a 2 L Erlenmeyer flask containing 650 mL PDB medium (30% w/v potato starch, 2% glucose, w/v) suppled with 3% sea salt and cultured for another 7 days at the same condition of 28°C and 200 rpm.

2.4. Extraction and Isolation

A total of 9 L fermented cultures were obtained and subsequently separated by centrifugation immediately after the fermentation of 7 days. Then both the mycelium and liquor were extracted three times with equal ethyl acetate to afford residues after solvent evaporation. The obtained residues (5.8 g) were subjected to silica gel CC to obtain eight fractions (Fr.A1−Fr.A8) eluted with a mixture solvent of petroleum ether/ethyl acetate (100:0, 90:10, 80:20, 70:30, 50:50, 80:20, 30:70, 0:100, v/v, each gradient 100 mL). Fr.A5 and Fr.A6 were subjected to silica gel CC and eluted with the same solvent of petroleum ether/ethyl acetate again to obtain Fr.B1-Fr.B6 and Fr.C1-Fr.C5, respectively. Fr.B3-Fr.B5 were then combined and purified with semi-preparative HPLC equipment with a C18 column (250 × 10 mm, 5 μm), eluting with a mixture of CH_3CN/H_2O (0-20 min, 45:55-80:20) at a flow rate of 3 mL min^{-1} to yield **1** (3.4 mg). Fr.C2-Fr.C3 were combined and purified under the same elution system, to obtain 2 (30.8 mg). Fr.A7 was further purified with semi-preparative HPLC eluting with CH_3CN/H_2O mixture solvent (0-20 min, 30:80-80:20, CH_3CN/H_2O, v/v, 3 mL min^{-1}) to get **3** (12.5 mg). Fr.A8 was further isolated through silica gel CC using a gradient of petroleum ether/ethyl acetate (8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, v/v) to give Fr.D1-Fr.D7. Fr.D3-Fr.D6 was merged and purified by semi-preparative HPLC using a mixture solvent of CH_3CN/H_2O (0-20 min, 30:70-70:30, v/v, 3 mL min^{-1}) to yield **4** (22.1 mg). Fr.A8 was further purified with semi-preparative HPLC to give **5** (28.7 mg), **6** (25.3 mg), using the CH_3CN/H_2O mixture solvent (0-20 min, 30:80-80:20, v/v, 3 mL min^{-1}).

*2.5. HPLC Analysis of Compound **2** After Disolving in n-Butanol for Different Days*

Compound **2** was disolved in n-butanol to a finally concentration of 50 μg/mL, and placed in a bottle at room temperature for 1 and 3 days. Then, compound **2** incubated in n-butanol was analyzed by HPLC on a Prominence LC-20A HPLC equipment with a COSMOSIL 5C18-MS-II C18 column (250 × 4.6 mm) eluting with a linear gradient elution system of CH_3CN/H_2O (0-20 min, 15-85% CH_3CN; 20.1-25 min, 15% CH_3CN) at a flow rate of 1 mL/min.

2.6. Antimicrobial Assays

The antimicrobial activities were measured by the method reported previously [16]. Briefly, the 96-well plates were added with 100 μL sterile 2 x TY medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl, w/v) containing tested strains, then the tested compounds dissolved in DMSO were serially diluted to designed concentrations of 256 μg/mL to 2 μg/mL, after that each of them were added to the corresponding cells with a quantity of 100 μL for incubation at 37℃ for 12−16 hours. Each experiment was repeated three times independently. The strains of *Micrococcus luteus* ATCC 10240, *Bacillus subtilis* ATCC 39620, *Staphylococcus aureus* ATCC 29213, *Acinetobacter baumannii* ATCC 19606, *Klebsiella pneumoniae* ATCC 700603, and *Pseudomonas aeruginosa* ATCC 27853 were used in this study. Apramycin was used as positive controls, while DMSO was used as blank control.

2.7. Anti-inflammatory Activity

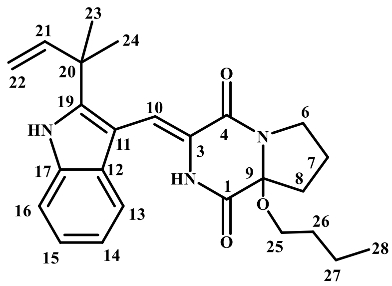
The isolated compounds were tested for their inhibition of NO production in lipopolysaccharide (LPS)-induced RAW 246.7 mouse macrophages according to the method reported in the literature [17]. Briefly, the cells were firstly cultured for 12 h in 96-well plates to a density of 5×10^5 cells/well. LPS (1 μg/mL) and the tested dissolved compounds (different concentrations in DMSO) were then added to the cells. After another 24 h incubation, 50 μL cell culture supernatant was then transferred to a new plate contained 50 μL NO detection reagent I and II. Then, the cells were measured their absorbance at 540 nm. Aminoguanidine was set as the positive control.

3. Results and Discussion

3.1. Structure Identification and Eluciation

The known compounds **2–6** were identified to be brevianamide Q [18], epi-deoxybrevianamide E [19], brevianamide V [20], brevianamide K [21], and brevianamide R [22], by comparing their NMR data with those reported previously, respectively.

Compound **1** was obtained as white power with a molecular formula of $C_{25}H_{31}N_3O_3$ proposed by the $[M-H]^-$ peak at *m/z* 420.2275 (calcd. for $C_{25}H_{30}N_3O_3$, 420.2293) detected by the HRESIMS (Figure S1), indicating twelve degrees of unsaturation. The UV/Vis spectra suggest that it is a derivative of brevianamides with characteristic absorbance at 226, 284, and 341 nm in UV/Vis spectrum (Figure S2). The ^{13}C-NMR spectrum unveiled signals for twenty-five carbons, including two carbonyls, twelve sp^2 carbons, eleven sp^3 carbons. The ^1H-NMR spectrum showed eight olefinic proton signals, three methyl groups at δ_H 0.96, δ_H 1.54, δ_H 1.56, and six methylene proton signals. The ^1H NMR resonances at δ_H 7.04 (1H, t, J = 8.0 Hz), 7.13 (1H, t, J = 8.0, Hz), 7.28 (1H, d, J = 8.0 Hz), 7.43 (1H, d, J = 8.0 Hz), and the sp^2 carbons signals at δ_C 104.8, 112.9, 120.2, 121.2, 122.7, 127.6, 137.0, 146.5, together with the key COSY correlation of H-4/H-5/H-6/H-7; and HMBC correlations of H-13/C-11, 12, 14, 15, 17; H-14/C-12, 13, 16; H-15/C-13, 14, 17; H-16/C-12, 14, 17 unveiled the presence of substituted indole moiety. The signals of two methyl groups at δ_H 1.54 (3H, s), δ_H 1.56 (3H, s); three olefinic proton signals at δ_H 5.10, δ_H 5.12, δ_H 6.12; and the COSY correlation of H-21/H-22, together with the key HMBC correlations of H-21/C-20, 20, 23, 24; H-22/C-20, 21; H-23/C-20, 21, 24; H-24/C-20, 21, 23 suggested the existence of an isopentenyl structure chromophore, which was connected to the indole moiety at C-19 indicated by the HMBC correlation of H-21/C-19. Further key HMBC correlation of H-10/C-3, 4, 11, 12, 19 indicated the presence of a dehydroindole tryptophan residue (Figure 2). Further COSY and HSQC datasets revealed the remaining ^{1}H-^{1}H spin systems consisted with one -CH_2-CH_2-CH_2-CH_3, and one -CH_2-CH_2-CH_2- moiety. Except for the existence of a dehydroindole tryptophan residue and two carbonyls/esters at δ_C 161.8, 165.3, which are contributing ten degrees of unsaturation, there were remaining two degrees of unsaturation undetermined, indicating two rings may be existed in its structure. Further HMBC correlations analyses confirmed the presence of one proline and butoxy residues within the structure of **1**. These two fragments were linked through C-9, aided by the key HMBC correlation of H-7, 8, 25/C-9. Careful analysis and comparison the NMR data with that of brevianamide V, we finally identified and named this compound as n-butoxylbrevianamide V (**1**). The NOESY correlations of δ_H 7.35 (H-13)/δ_H 8.3 (H-2) in $CDCl_3$ (Figure S7−S8), indicate that the geometry of $\Delta^{3,10}$ was cis configuration [18]. The property of rotation with the value of $[\alpha]_D^{25} = 0$, and the absence of Cotton effect in the CD spectrum, suggested that **1** might be a pair of enantiomers.



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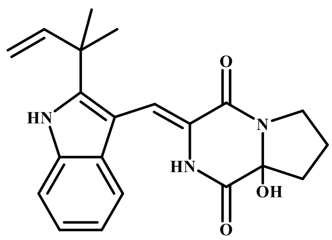

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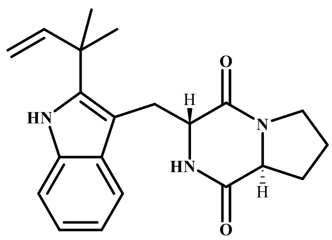

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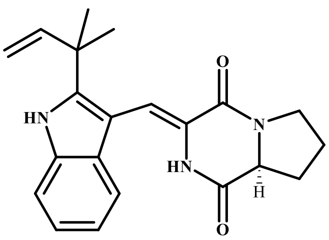

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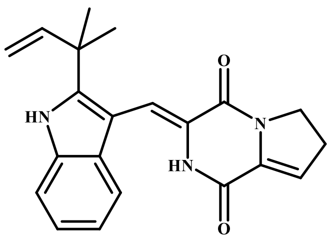

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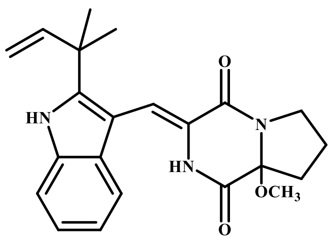

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Figure 1. The chemical structure of isolated compounds

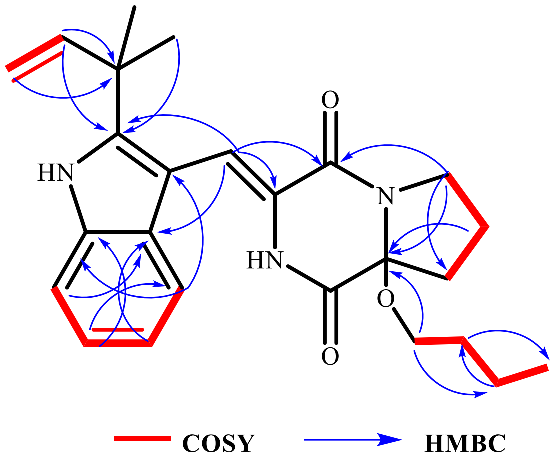

Figure 2. Key COSY and HMBC of n-butoxylbrevianamide V (**1**)

Table 1. The ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for n-butoxylbrevianamide V (**1**) in CD_3OD

Position	δ_H **multi. (*J* in Hz)**	δ_C**, type**
1		165.3, C
3		125.5, C
4		161.8, C
6	3.71, m; 3.81, m	46.7, CH_2
7	2.01, m; 2.12, m	20.5, CH_2
8	2.12, m; 2.44, m	35.1, CH_2
9		92.9, C
10	7.32, (s)	116.3, CH
11		104.8, C
12		127.6, C
13	7.28, (d, 8.0)	120.2, CH
14	7.04, (t, 8.0)	121.2, CH
15	7.13, (t, 8.0)	122.7, CH
16	7.43, (d, 8.0)	112.9, CH
17		137.0, C
19		146.5, C
20		40.6, C
21	6.12, (dd, 17.2,10.8)	146.3, CH
22	5.10, (d, 10.8); 5.12, (d, 17.6)	112.8, CH_2
23	1.56, (s)	28.2, CH_3
24	1.54, (s)	28.5, CH_3
25	3.55, (m)	65.2, CH_2
26	1.63, (m)	33.0, CH_2
27	1.45, (m)	20.7, CH_2
28	0.96, (t, 7.2)	14.3, CH_3

*3.2. Relationship Between Compound **1** and Compound **2***

Compounds with butoxy substitution are rare in nature products. In this study we have isolated an indolediketopiperazine (compound **1**) substituted with butoxy group, together with non substituted one (compound **2**) and other known indolediketopiperazines (compounds **3−6**). In order to decipher the possible relevation between compound **1** and compound **2**, we disolved compound **1** in n-butanol and then analyzed by HPLC using the method above mentioned in different days. Results showed that compound **2** can not convert to compound **1** in the presence of n-butanol in room tempareture (Figure 3).

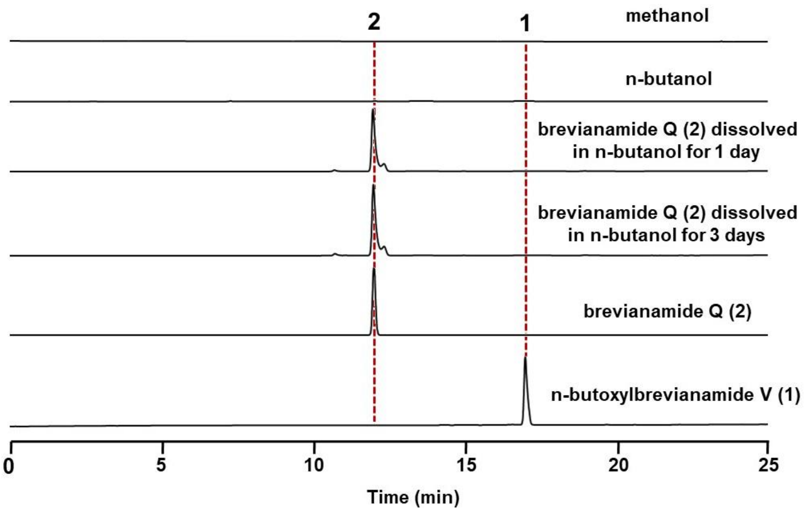

Figure 3. HPLC results of brevianamide Q (**2**) after dissolving in n-butanol for 1 and 3 days, respectively.

3.3. Bioactivity Assay

Table 2. Antibacterial activities of compounds **1**−**6** (MIC, μg/mL), apramycin is the positive control

Compounds	*M. luteus* ATCC 10240	*B. subtilis* ATCC 39620	*S. aureus* ATCC 29213	*A. baumannii* ATCC 19606	*K. pneumoniae* ATCC 700603	*P. aeruginosa* ATCC 27853
4	>128	64	>128	>128	>128	>128
Apramycin	16	16	1	4	1	2

Compounds **1**, **2**, **3**, **5**, and **6** didn't show antibacterial activities under the concentration of 128 μg/mL

Due to the multiple bioactivities of DPKs family, the isolated ones in this study were further assessed for their antibacterial and anti-inflammatory assay. Results unveiled that compound **4** possessed mild antibacterial activity against *Bacillus subtilis* with MIC value of 64 μg/mL (Table 2). Compounds **1**, **2** and **4** exhibited potential anti-inflammatory bioactivities with IC_{50} values of 28.2, 21.3, 23.6 μM against NO production inhibition, respectively (Table 3). The pharmacological intensity of these compounds roughly equals to that of the positive control of aminoguanidine (IC_{50} = 20.1 μM).

Table 3. Inhibitory activities against NO production, aminoguanidine is the positive control

Compounds	IC_{50} (μM)
1	28.2
2	21.3
3	>50
4	23.6
5	>50
6	>50
Aminoguanidine	20.1

3.4. Discussion

Marine fungi are rich resource for DPKs discovery, and numerous DPKs have been reported, along with their bioactivities [22]. Among them, brevianamides are family of indolediketopiperazines alkaloids. In this study a new butoxy substitute brevianamide (n-butoxylbrevianamide V), together with the five known brevianamides, were isolated from the marine-derived fungus *Aspergillus* sp. 66may. Brevianamides usually carry one isopentenyl moieties, sometimes dimers are also observed in

Aspergillus versicolor, however, butoxy substitution brevianamides have not been reported, which is also very rare in nature products. The only similar example is the ethoxy group substitution brevianamides reported by Ding Y, also known as aspamides, which carried the ethoxy group at C-6 or C-9 position [23]. This is the first case of butoxy substituted brevianamide. In order to rule out the possibilities that compound **1** is actually an artificial product derived from n-butanol and compound **2**, compound **2** was dissolved in n-butanol for 1 and 3 days, and then analyzed by HPLC. Results unraveled that compound **2** may not be converted to compound **1** by dissolving in n-butanol at room temperature. However, the biosynthesis origin of the butoxy residue is still unknown in that the rare butoxy substitute nature products.

As to the antibacterial assay, through comparison of the bioactivities of compound **4** with that of **1**, **2**, **5** and **6,** results unveiled that C-9 modifications in the isolates such as hydroxybutylation, hydroxylation, hydroxymethylation may lead to the decrease or loss of antibacterial activity, however this structure-function correlation is needed to be extensively investigated. Meanwhile, anti-inflammatory activities showed that compound **1**, **2**, **4** exhibited potential inhibitory activities against NO production. Bioactivities results indicate that butoxy substitution at C-9 in brevianamides have little effects on their anti-inflammatory bioactivities.

In summary, a new butoxy substituted brevianamide as well as five known brevianamides were isolated and identified from the strain of the marine-derived fungus 66may. Based on the extensively NMR and HRESIMS data analysis, compound **1** was finally identified and named as n-butoxylbrevianamide V, while the NMR data comparison with the known reported analogs of in the literatures revealed that compound **2**−**6** were brevianamide Q, epi-deoxybrevianamide E, brevianamide V, brevianamide K, brevianamide R, respevtively. This is the first report of the butoxy substitute indolediketopiperazine and its anti-inflammatory activities.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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