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RESEARCH ARTICLE

Prenylated indole diketopiperazine alkaloids from a mangrove rhizosphere soil derived fungus *Aspergillus effuses* H1-1

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Abstract One new prenylated indole diketopiperazine alkaloid, named dihydroneochinulin B (**1**), one known spiro-polyketide-diketopiperazine hybrid cryptoechinuline D (**2**) and three related known metabolites didehydroechinulin B (**3**), neoechinulin B (**4**) and auroglaucin (**5**) were isolated from the mangrove rhizosphere soil derived fungus, *Aspergillus effuses* H1-1. The structures were assigned by detailed spectroscopic analysis. The enantiomers of cryptoechinuline D (**2**) were separated to be (+)-cryptoechinuline D (**2a**) and (−)-cryptoechinuline D (**2b**) by chiral HPLC, and their absolute configurations were determined by ECD analysis. The cytotoxic effects of the compounds were preliminarily evaluated on P388, HL-60, BEL-7402 and A-549 cell lines by SRB or MTT methods, and compounds **2**, **2a** and **3** showed significant activities.

Keywords *Aspergillus effuses* · Mangrove · Metabolite · Alkaloids · Cytotoxic

Introduction

Prenylated indole diketopiperazine alkaloids are hybrid natural products containing both indole diketopiperazine and isoprenoid moieties, which are biosynthesized via prenyl transfer reactions catalysed by indole prenyltransferases (Williams et al. 2000; Li 2009). They are widespread microbial products commonly found in cultures of fungi, such as *Aspergillus* sp., *Penicillium* sp., *Pestalotiopsis* sp., and *Chromocleista* sp. (Sings and Singh 2003). These prenylated indole diketopiperazine alkaloids possess a wide range of biological activities distinct from their non-prenylated precursors (Sings and Singh 2003; Li 2010). In our ongoing search for bioactive novel compounds from marine-derived fungi (Liu et al. 2005; Cai et al. 2011), a strain identified as *A. effuses* H1-1 was isolated from the mangrove rhizosphere soil collected in the coast of Fujian province. Its chemical studies led to the isolation of one new prenylated indole diketopiperazine alkaloid, named dihydroneochinulin B (**1**), (+)-cryptoechinuline D (**2a**), (−)-cryptoechinuline D (**2b**), didehydroechinulin B (**3**) (Gu 2008; Zhou et al. 2010), neoechinulin B (**4**) (Marchelli et al. 1977) and auroglaucin (**5**) (Hamasaki et al. 1981) (Fig. 1). In this article, we reported the isolation, structural elucidation and cytotoxic activities of these compounds.

Materials and methods

General

Specific rotations were obtained on a JASCO P-1020 digital polarimeter. UV spectra were recorded on a Beckman DU 640 spectrophotometer. IR spectra were taken on a Nicolet Nexus 470 spectrophotometer in KBr discs. CD spectra were measured on JASCO J-715 spectropolarimeter. NMR spectra were recorded on a JEOL JNM-ECP 600 spectrometer using TMS as internal standard, and chemical shifts were recorded as δ values. ESIMS were recorded on a Q-TOF Ultima Global GAA076 LC mass spectrometer.

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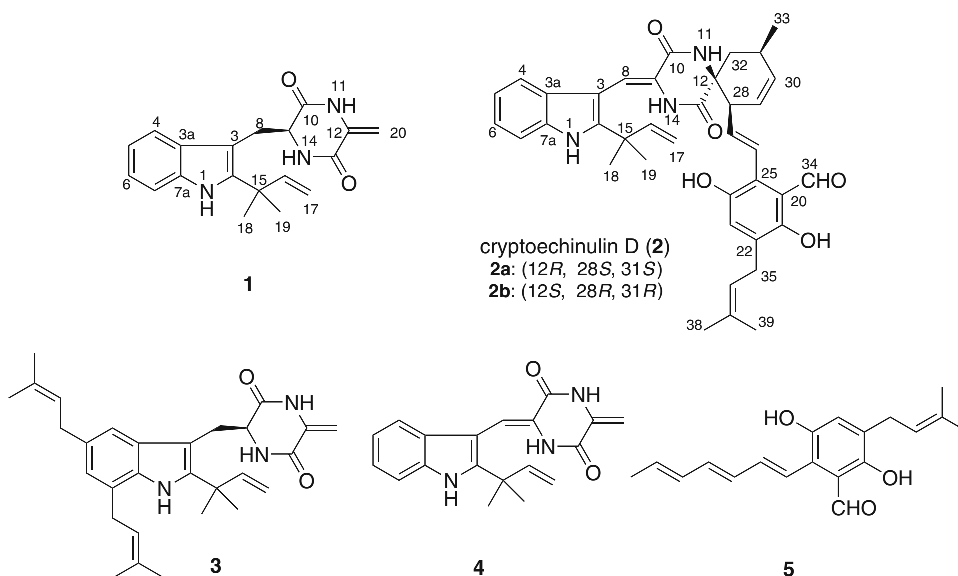

Fig. 1 Structures of compounds **1**–**5**

Semiprepartive HPLC was performed on an ODS column (YMC-pack ODS-A, 10 × 250 mm, 5 µm). Chirl HPLC of **2** was run on a Chiralpak IC column (DAICEL Chiralpak IC, 150 × 4.6 mm, 5 µm). TLC and column chromatography (CC) were performed on plates precoated with silica gel GF254 (10–40 µm, Qingdao Marine Chemical Inc., P. R. China) and over silica gel (200–300 mesh, Qingdao Marine Chemical Inc., P. R. China), respectively.

Fermentation and extraction

The fungus strain was fermented and extracted as previous described (Gao et al. 2012).

Purification

The crude extract (76 g) was subjected to a silica gel (200–300 mesh) column packed in petroleum ether, and was separated into seven fractions (Fr.1–7) using a step gradient elution of petroleum ether-chloroform and chloroform–methanol. The fraction (Fr.4), obtained from the silica gel column eluted with $CHCl_3$–CH_3OH solution (100:1, v/v), was further chromatographed gradiently on silica gel using $CHCl_3$–CH_3OH (100:1–10:1, v/v) as elution and divided into 8 subfractions (Fr.4–1 to Fr.4–8). Subfraction Fr.4–5 was then applied to Sephadex LH-20 using methanol as eluting solvent and yielded compound **1** (22 mg) and **4** (25 mg). The fraction (Fr.3) eluted with $CHCl_3$–CH_3OH (200:1, v/v) solution was further separated on silica gel using petroleum ether-ethyl acetate (5:1, v/v). The subfraction (Fr.3–3) which contained compound **3** was recrystallized in acetone and obtained (6 mg). Subfraction (Fr.3–2) was separated by preparative HPLC on a ODS column using methanol–water (80:20, v/v) as eluting solvent to yield compound **2** (35 mg), and **2** were resolved to **2a** (1.1 mg) and **2b** (1.1 mg) by HPLC on a chiral phase (hexane–isopropanol eluent, 70:30, v/v). Subfraction Fr.3–1 was separated by preparative HPLC on a ODS column using CH_3OH-H_2O (85:15, v/v) as eluting solvent to yield compound **5** (16 mg).

Dihydroneochinulin B (**1**): colorless needles (acetone); mp 213–214 °C; $[\alpha]_D^{20}$ −59.8 (*c*, 0.085 MeOH); UV (MeOH) λ_{max} (log ε) 291 (3.58), 282 (3.64), 223 (4.34) nm; IR (KBr) ν_{max} 3377, 3194, 3085, 2973, 1686, 1632, 1461, 1435, 1330, 1107, 918, 788, 741, 715 cm^{-1}; ^{1}H and ^{13}C NMR data: see Table 1; HR-ESI-MS *m/z* 324.1708 $[M+H]^+$ (calcd for $C_{19}H_{22}N_3O_2$: 324.1712).

Cryptoechinuline D (**2**): yellow solid (acetone); mp 158–160 °C; UV (MeOH) λ_{max} (log ε) 348 (3.75), 284 (3.83), 224 (4.33) nm; IR (KBr) ν_{max} 3344, 3179, 3039, 2969, 2874, 1678, 1635, 1429, 1375, 1282, 1261, 1024, 1004, 917, 745 cm^{-1}; ^{1}H and ^{13}C NMR data: see Table 2; ESI–MS *m/z* 620.30 $[M+H]^+$, 642.29 $[M+Na]^+$.

(+)-cryptoechinuline D (**2a**): $[\alpha]_D^{20}$ +210.3 (*c* 0.15, MeOH); retention time (t_R) 19.0 min (Chiralpak IC, hexane/isopropanol, 70:30, v/v); ECD λ_{max} (ϕ) in methanol: 339 (−1.87), 249 (5,81), 224 (−3.58) nm.

(−)-cryptoechinuline D (**2b**): $[\alpha]_D^{20}$ -199.7 (*c* 0.15, MeOH); retention time (t_R) 21.6 min (Chiralpak IC, hexane/isopropanol 70:30, v/v); ECD λ_{max} (ϕ) in methanol: 342 (2.75), 250 (−9.26), 224 (4.89) nm.

Biological assays

In the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) assay, cell lines were grown in RPMI-1640 supplemented with 10 % FBS under a humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C.

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Table 1 NMR data for compound **1** (DMSO-d_6, 600 MHz, TMS)

No.	δ_C, mult.	δ_H (*J* in Hz)	No.	δ_C, mult.	δ_H (*J* in Hz)
1		10.57, s	10	166.1, C	
2	141.5, C		11		10.51, s
3	104.0, C		12	134.9, C	
3a	128.9, C		13	158.4, C	
4	117.7, CH	7.32, d (7.8)	14		8.02, d (3.2)
5	118.4, CH	6.95, dd (7.8, 7.3)	15	38.8, C	
6	120.4, CH	7.02, dd (7.8, 7.3)	16	146.4, CH	6.15, dd (17.4, 10.5)
7	110.9, CH	7.38, d (7.8)	17	111.0, CH_2	5.07, dd (17.4, 1.2)
					5.02, dd (10.5, 1.2)
7a	134.9, C		18	27.9, CH_3	1.46, s
8	32.1, CH_2	3.28, dd (14.4, 5.5)	19	27.9, CH_3	1.47, s
		3.10, dd (14.4, 8.7)			
9	56.3, CH	4.06, m	20	99.2, CH_2	5.18, s; 4.78, s

Cell suspensions, 200 µL, at a density of 5 × 10^4 cell/mL were plated in 96 well microtiter plates and incubated for 24 h. Then, 2 µL of the test solutions (in MeOH) were added to each well and further incubated for 72 h. Then, 20 µL of the MTT solution (5 mg/mL in RPMI-1640 medium) was added to each well and incubated for 4 h. Old medium containing MTT (150 µL) was then gently replaced by DMSO and pipetted to dissolve any formazan crystals formed. Absorbance was then determined on a SPECTRA MAX PLUS plate reader (Molecular Devices, Sunnyvale, CA, USA) at 540 nm.

In the sulforhodanine B (SRB) assay, 200 µL of the cell suspensions were plated in 96 cell plates at a density of 2 × 10^5 cell/mL. Then 2 µL of the test solutions (in MeOH) was added to each well, and the culture was further incubated for 24 h. The cells were fixed with 12 % trichloroacetic acid, and the cell layer was stained with 0.4 % SRB. The absorbance of SRB solution was measured at 515 nm. Dose response curves were generated, and the IC_{50} values, the concentration of compound required to inhibit cell proliferation by 50 %, were calculated from the linear portion of log dose response curves.

Results and discussion

The ethyl acetate extract (Gao et al. 2012) was concentrated in vacuo and the residue was fractionated by repeated silica column, Sephadex LH-20 and semipreparative HPLC to yield one new compound **1**, together with four known compounds (**2**–**5**).

Compound **1** was isolated as a colorless needle. Its molecular formula, $C_{19}H_{21}N_3O_2$, was obtained from the positive HR-ESI-MS at *m/z* 324.1708 $[M+H]^+$ (calcd. for $C_{19}H_{22}N_3O_2$, 324.1712), indicating 10 degrees of unsaturation. Its UV absorptions at λ_{max} 291, 282 and 223 nm suggested the presence of amide and typical indole moieties in **1** (Li et al. 2004). IR absorption peaks at 3377, 3194, 3085, 1686, 1632, 1461, 1435 cm^{-1} supported the presence of amino and amide carbonyl groups. The NMR spectra data of **1** displayed signals for two amide carbonyls (Wang et al. 2007), six quarternary carbons, six methines, three methylenes, and two methyl groups (Table 1). Except for

Table 2 NMR data for compound **2** (DMSO-d_6, 600 MHz, TMS)

No.	δ_C, mult.	δ_H (*J* in Hz)	No.	δ_C, mult.	δ_H (*J* in Hz)	No.	δ_C, mult.	δ_H (*J* in Hz)
1		11.08, s	14		8.99, s	29	125.0, CH	5.75, dt (10.0, 2.3)
2	144.4, C		15	39.1, C		30	132.8, CH	5.64, dt (10.0, 3.0)
3	103.7, C		16	145.1, CH	6.07, dd (17.4, 10.5)	31	28.5, CH	2.70, m
3a	128.5,C		17	111.7, CH_2	5.03, d (17.4)	32	37.5, CH_2	1.70, m
					5.05, d (10.5)			2.09, m
4	118.9, CH	7.14, d (7.8)	18	27.5, CH_3	1.46, s	33	21.2, CH_2	1.10, d (7.3)
5	119.4, CH	6.99, dd (7.8, 7.3)	19	27.6, CH_3	1.47, s	34	197.4, CH	10.08, s
6	120.7, CH	7.08, dd (7.8, 7.3)	20	117.2, C		35	26.8, CH_2	3.22, d (7.3)
7	111.6, CH	7.42, d (7.8)	21	153.0, C		36	121.4, CH	5.24, bt (7.3)
7a	135.1, C		22	126.1, C		37	132.8, C	
8	111.2, CH	6.96, s	23	124.8, CH	7.01, s	38	17.6, CH_3	1.66, s
9	124.4, C		24	147.0, C		39	25.6, CH_3	1.70, s
10	167.8, C		25	128.5, C		OH-21		11.78, s
11		7.83, s	26	137.2, CH	6.71, d (16.0)	OH-24		9.28, s
12	59.1, C		27	124.4, CH	5.86, dd (16.0, 8.5)			
13	161.6, C		28	45.5, CH	3.59, m			

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the replacement of two olefinic carbon signals (δ_H 6.97, δ_C 111.7 and δ_C 124.5) by two aliphatic ones (δ_H 3.28 and 3.10, δ_C 32.1, CH_2-8; δ_H 4.06, δ_C 56.3. CH-9), the NMR data of **1** were quite similar to those of neoechinulin B (**4**) (Marchelli et al. 1977). Comprehensive analysis of the 2D-NMR (HMQC, and HMBC) correlations (Fig. 2) revealed **1** was a hydrogenated derivative of neoechinulin B. The negative value of optical rotation and the negative Cotton effect in 229 nm suggested the *S*-configuration of C-9 (Kozlovsky et al., 2000). Compound **1** was named as dihydroneoechinulin B.

Cryptoechinuline D (**2**) was firstly obtained from *Aspergillus amstelodami* in 1976 (Gatti et al. 1976), and then from *Eurotium rubrum* in 2008 (Li et al. 2008). Cryptoechinuline D was synthesized by the Diela-Alder reaction and its relative stereochemistry was determined (Inoue et al. 1977), but the absolute configuration was not confirmed and the NMR data were not wholly provided.

The relative stereochemistry of cryptoechinuline D (**2**) was determined to be (12*R**, 28*S**, 31*S**) on the basis of selective NOE difference experiments and its syntheses. In its NOE difference experiments (Fig. 3), when H-31 and H-33 were irradiated, the enhancements of H-32a (δ_H 2.09), and H-32b (δ_H 1.70) and H-11 were observed, respectively. No enhancement of hydrogen signals was observed when H-28 was irradiated, but the relative stereochemistry of C-28 could be deduced to be the same as dihydrocryptoechinuline D from its biosynthetic pathway (Gao et al. 2012).

Cryptoechinuline D (**2**) showed no optical rotation and no CD effects, which indicated that **2** should be an enantiomeric mixture, which was confirmed by the baseline

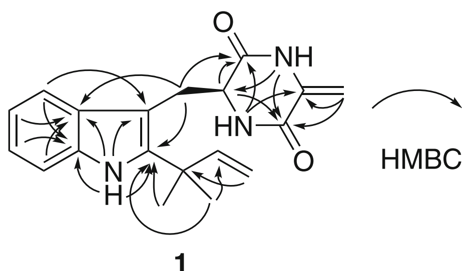

Fig. 2 Key HMBC correlations in compound **1**

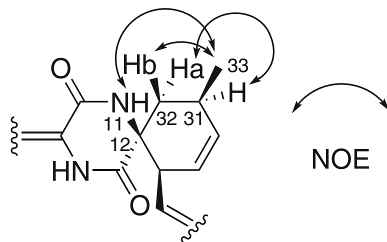

Fig. 3 Key NOE correlations of **2**

separation of its enantiomers by chiral HPLC using Chiralpak IC column. Since the CD spectra (Fig. 4) of **2a** and **2b** were identical with those of (+) and (−) dihydrocryptoechinuline D (Gao et al. 2012), respectively, the absolute configurations of them were assigned as (12*R*, 28*S*, 31*S*) (**2a**) and (12*S*, 28*R*, 31*R*) (**2b**).

Cryptoechinuline D (**2**) could be biosynthesized by an enzyme-catalyzed regiospecific [4 + 2] Diels–Alder reaction. Similar Diels–Alder biosynthetic reaction has already been suggested such as dihydrocryptoechinuline D (Gao et al. 2012), yaoshanenolides (Liu et al. 2012) and lanceolatins (Du et al. 1999; Du et al. 2001). Notably, the two key intermediates neoechinulin B (**4**) and auroglaucin (**5**) were isolated from *A. effuses* H1-1, which supported the biosynthetic hypothesis.

The cytotoxic effects of these compounds were preliminarily evaluated by the SRB (Skehan et al. 1990) or MTT (Mosmann 1983) methods on P388, HL-60, BEL-7402 and A-549, as shown in Table 3. Compound **1** showed weak activity against BEL-7402 and A-549 cell lines, and no activity to the other two tested cell lines. Compound **2** and **2a** exhibited significant inhibitory activity against P388 cell line with IC_{50} values of 3.43 and

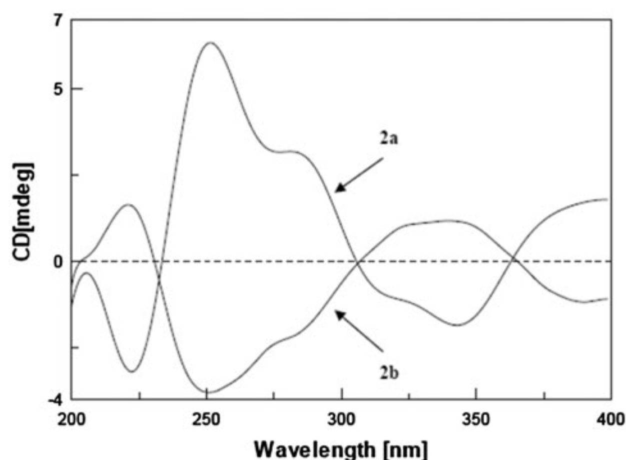

Fig. 4 CD spectra of **2a** and **2b**

Table 3 The cytotoxic activities of compounds **1-3** on cancer cell lines

	IC_{50} (μM)			
	P-388	HL-60	BEL-7402	A-549
1	>100	>100	55.1	30.5
2	3.43	>100	>100	>100
2a	2.50	>100	>100	>100
2b	11.3	>100	>100	>100
3	>100	15.6	4.20	1.43
Doxorubicin	0.33	0.05	0.24	0.08

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2.50 μM, respectively, while compound **2b** showed moderate activity to P388 with IC_{50} values of 11.3 μM. Compound **3** showed significant inhibitory activity against A-549 and BEL-7402 with IC_{50} values of 1.43 and 4.20 μM, respectively.

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