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Cristatumins A–D, new indole alkaloids from the marine-derived endophytic fungus *Eurotium cristatum* EN-220

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ABSTRACT

Four new indole alkaloids, namely, cristatumins A–D (**1–4**), along with six known congeners (**5–10**) were identified from the culture extract of *Eurotium cristatum* EN-220, an endophytic fungus isolated from the marine alga *Sargassum thunbergii*. The structures of these compounds were established on the basis of extensive spectroscopic analysis. Each of these compounds was evaluated for antimicrobial and insecticidal activity. Compounds **1** and **9** showed antibacterial activity against *Escherichia coli* and *Staphyloccocus aureus*, respectively, while compounds **2**, **6**, and **7** exhibited moderate lethal activity against brine shrimp. Preliminary structure–activity relationships were also discussed.

The fungal genus *Eurotium*, which is the teleomorph of *Aspergillus*, has been proved to be a rich source of novel bioactive metabolites.[1–3] The tryptophan-derived alkaloid, generally characterized by a reverse isoprenic chain in the C-2 position of the indole nucleus, is an important class of secondary metabolites found in *Eurotium* species.[2,3] These compounds were reported to possess radical scavenging,[4] anti HIV-1 replication,[5] and antibacterial activity.[6]

As part of our efforts toward the chemical investigation of marine-derived fungi, a variety of structurally interesting and biologically active compounds were isolated and identified from fungal species derived from marine algae[7,8] and mangrove plants.[9] In the current study, four new indole alkaloids, cristatumins A–D (**1–4**, Fig. 1),[10] along with six known congeners (**5–10**, Fig. 1), were isolated from *Eurotium cristatum* EN-220, an endophytic fungus isolated from the marine alga *Sargassum thunbergii*. This Letter describes the isolation, structure elucidation and biological activity of these compounds (**1**–**10**).

The fungal strain *E. cristatum* EN-220 was cultured on rice solid medium at room temperature under static conditions for 30 days. The rice culture was exhaustively extracted with EtOAc to give a crude extract, which was dried and fractionated by silica gel vacuum liquid chromatography (VLC) using different solvent systems of increasing polarity from petroleum ether (PE) to MeOH to yield 12 fractions (Frs. 1–12) based on TLC analysis. Frs. 7–10 were further purified by a combination of silica gel, Sephadex LH-20, and Lobar LiChroprep RP-18 column chromatography to yield four new indole alkaloids **1–4**, along with six other congeners including neoechinulin A (**5**),[11] isoechinulin A (**6**),[12] variecolorin G (**7**),[4] preechinulin (**8**),[13] tardioxopiperazine A (**9**),[14] and echinulin (**10**).[15]

Compound **1**,[10] a colorless amorphous powder, was detemined to have a molecular formula $C_{19}H_{21}N_3O_3$ on the basis of HRESIMS data. The 1D NMR data (Table 1) and UV absorptions at λ_{max} 223, 284, and 333 nm suggested that **1** was an anologue of neoechinulin A (**5**).[11] However, the methyl signals at δ_C 17.9 (q, C-20) and δ_H 1.42 of **5** were replaced by the oxygenated CH_2 signals at δ_C 65.3 (t, C-20) and δ_H 4.00, 3.89 of **1**. This deduction was supported by the ^1H–^1H COSY correlations from 20-O*H* to H-20 and from H-20 to H-12 (Fig. 2). The above evidence indicated that the alanine residue in the 2,5-diketopiperazine moiety of **5** was replaced by the serine residue in **1**, which was so far not found in this type of indole diketopiperazine. The lower-field-shifted CH at δ_H 7.03 (1H, s, H-8) of **1** implied that H-8 was influenced by the deshielding effect of the C=O group, which suggested the double bond between C-8 and C-9 to have *Z*-geometry.[16] The absolute configuration at C-12 was tentatively assigned as *S* by comparing its specific rotation ($[\alpha]_D^{20}$ = −17.6, *c* 0.34, MeOH) with that of **5** ($[\alpha]_D^{24}$ = −28.0, *c* 0.53, MeOH).[17] Based on the above spectral evidence, the structure of **1** was determined and it was named as cristatumin A.

Compound **2** was obtained as a colorless amorphous powder with the molecular formula, $C_{29}H_{39}N_3O_3$, as established by the HRESIMS analysis. The 1D NMR data of **2** (Table 1) revealed marked similarities to echinulin (**10**)[15] except that the methyl group at C-20 (δ_C 19.9, δ_H 1.47) of **10** disappeared in that of **2**. Instead, the

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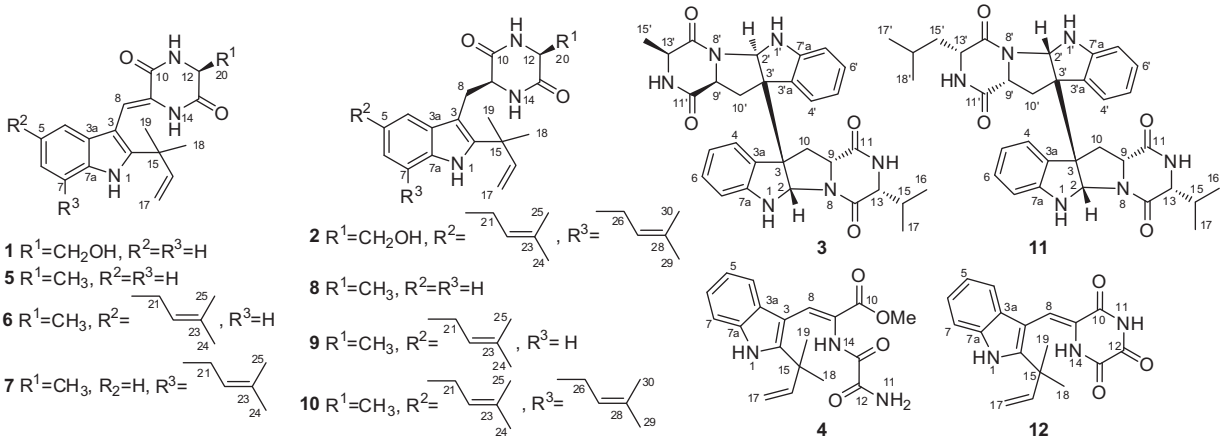

Figure 1. The structures of isolated compounds **1**–**10** and the reference compounds **11** and **12**.

oxymethylene signals (δ_C 63.9 for C-20, δ_H 3.94) were observed in the 1D NMR spectrum of **2** (Table 1). These spectroscopic evidences suggested that the C-20 Me group in **10** was replaced by CH_2OH group in **2**. Further analysis of the ^1H–^1H COSY, HSQC, and HMBC data verified the structure of **2** as shown in Figure 1. The NOE correlations from H-9 to H-12 indicated the *cis* relationship of these two protons. The absolute configuration of **2** was tentatively assigned as 9*S* and 12*S* by comparing its specific rotation with that of **10** ($[\alpha]_D^{20}$ = −11.1 for **2** vs $[\alpha]_D^{20}$ = −39.1 for **10**, both determined in $CHCl_3$).[15,18] Based on the above spectral evidence, the structure of **2** was determined and it was named as cristatumin B.

Cristatumin C (**3**) showed the molecular formula $C_{30}H_{32}N_6O_4$ as determined by HRESIMS. Analysis of the 1D NMR data revealed that, similar to **11**,[19] compound **3** was an almost symmetrical molecule consisting of two indole diketopiperazine moieties. The NMR spectroscopic data of **3** suggested that the isobutyl unit in **11** was replaced by a methyl group in **3**, which was indicated by the fact that the signals corresponding to isobutyl unit of **11** at δ_C 40.7 (t, C-15′), 23.5 (d, C-16′), 22.6 (q, C-17′), and 21.1 (q, C-18′) were not observed in that of **3**. Instead, resonance for a methyl group at δ_C 14.8 (q, C-15′) was present. The above spectroscopic data indicated that the leucine residue in **11** was replaced by an alanine residue in **3**. This deduction was supported by the difference in the molecular formulae of **3** and **11**, as well as the ^1H–^1H COSY, and HMBC correlations (Fig. 2). The observed NOE correlations from H-2 and H-13 to H-9, from H-9 to H-4′/H-5′, from H-9′ to H-4, and from H-2′ and H-13′ to H-9′ established the relative configuration of **3**.[19,20] Chiral HPLC analyses[21] of the acid hydrolysate of **3** revealed that the valine and alanine residues were in D- and L-configuration, respectively (Supplementary material). This result indicated a 2*S*, 3*S*, 9*R*, 13*R*, 2′*R*, 3′*R*, 9′*S* and 13′*S* absolute configuration for **3**.

The HRESIMS data of cristatumin D (**4**) gave a molecular formula of $C_{19}H_{21}N_3O_4$, indicating eleven degrees of unsaturation. Its NMR data was very similar to **12** (neoechinulin E),[12] except one more methoxyl signal at δ_H 3.72 (3H, s, OCH_3-10) and δ_C 51.9 was observed in the 1D NMR spectra of **4**. However, the degrees of unsaturation for **12** were twelve, one more than that of **4**, indicating that **4** was a ring-opened diketopiperazine derivative of **12**. This deduction was supported by the HMBC correlations from H-OCH_3 to C-10 and from 11-NH_2 to C-11. The lower-field-shifted CH at δ_H 7.77 (1H, s, H-8) of **4** suggested the double bond between C-8 and C-9 to have *Z*-geometry.[16] This is the first report of indole alkaloid characterized by the ring open of 2,5-diketopiperazine moiety.

Compounds **1**–**10** were evaluated for the lethality against brine shrimp (*Artemia salina*)[22] and the results indicated that compounds **2**, **6**, and **7** exhibited moderate activity with the LD_{50} values of 74.4,

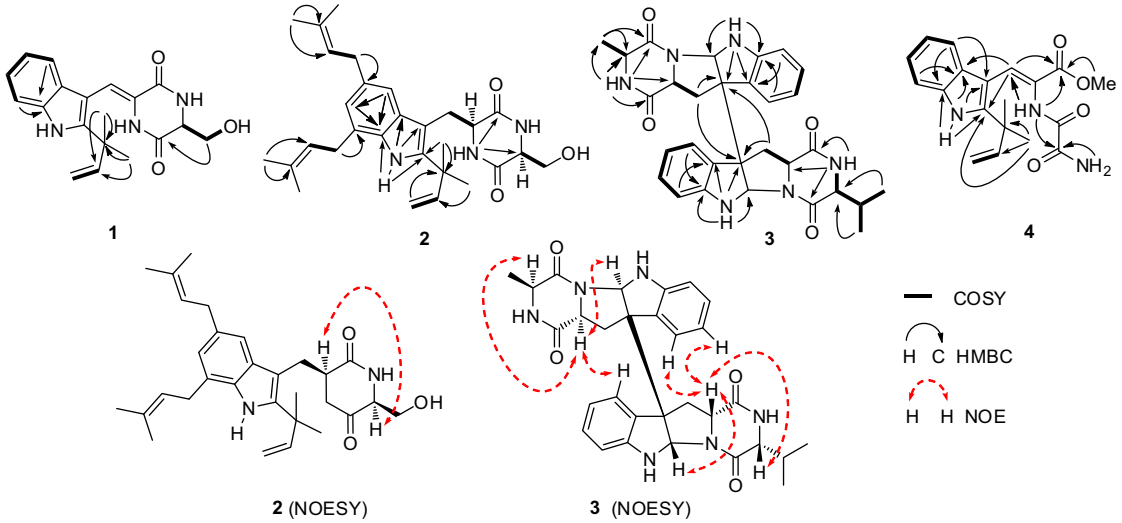

Figure 2. Key HMBC, ^1H-^1H COSY, and NOE correlations of **1**–**4**.

Table 1
^{1}H and ^{13}C NMR data of compounds **1**–**4**

No.	**1**[a]		No.	**2**[b]		No.	**3**[c]		No.	**4**[c]	
	δ_H (J in Hz)	δ_C		δ_H (J in Hz)	δ_C		δ_H (J in Hz)	δ_C		δ_H (J in Hz)	δ_C
1-NH	10.28 (br s)		1-NH	8.05 (br s)		1-NH	6.70 (s)		1-NH	11.23 (br s)	
2		144.7 (s)	2		141.4 (s)	2	4.80 (s)	78.8 (d)	2		146.0 (s)
3		104.5 (s)	3		104.1 (s)	3		59.7 (s)	3		105.7 (s)
3a		127.4 (s)	3a		129.1 (s)	3a		130.3 (s)	3a		125.5 (s)
4	7.40 (d, 8.5)	120.2 (d)	4	7.15 (s)	115.2 (d)	4	7.39 (d, 7.3)	124.3 (d)	4	7.19 (d, 8.0)	119.9 (d)
5	7.02 (m)	120.8 (d)	5		134.0 (s)	5	6.63 (m)	117.9 (d)	5	6.89 (m)	119.4 (d)
6	7.09 (m)	122.2 (d)	6	6.80 (s)	122.9 (d)	6	7.02 (m)	128.6 (d)	6	7.03 (m)	120.8 (d)
7	7.38 (d, 8.6)	112.4 (d)	7		123.4 (s)	7	6.60 (d, 7.8)	108.8 (d)	7	7.37 (d, 8.0)	111.5 (d)
7a		136.3 (s)	7a		132.3 (s)	7a		149.0 (s)	7a		134.9 (s)
8	7.03 (s)	110.2 (d)	8	3.67 (dd, 14.6, 3.5) 3.23 (dd, 14.6, 11.7)	29.9 (t)	9	4.08 (t, 9.5)	55.6 (d)	8	7.77 (s)	129.5 (d)
9		126.9 (s)	9	4.41 (dd, 11.7, 3.5)	54.6 (d)	10	3.04 (m) 2.33 (dd, 13.9, 9.5)	37.2 (t)	9		134.9 (s)
10		160.6 (s)	10		168.4 (s)	11		168.3 (s)	10		165.0 (s)
11-NH	7.21 (br s)		11-NH	6.63 (br s)		12-NH	8.25 (br d, 4.1)		10-OMe	3.72 (s)	51.9 (q)
12	4.14 (m)	59.4 (d)	12	4.09 (br s)	56.2 (d)	13	3.37 (m)	62.4 (d)	11-NH_2	7.97 (br s) 7.66 (br s)	
13		165.3 (s)	13		166.0 (s)	14		167.4 (s)	12		158.1 (s)
14-NH	7.92 (br s)		14-NH	5.81 (br s)		15	1.93 (dq, 13.1, 6.7)	31.8 (d)	13		161.4 (s)
15		40.1 (s)	15		39.0 (s)	16	0.69 (d, 6.7)	18.0 (q)	14-NH	9.62 (br s)	
16	6.14 (dd, 17.4, 10.5)	146.1 (d)	16	6.09 (dd, 17.4, 10.5)	145.8 (d)	17	0.79 (d, 6.7)	18.9 (q)	15		39.5 (s)
17	5.10 (d, 17.4) 5.08 (d, 10.5)	112.3 (t)	17	5.16 (d, 17.4) 5.15 (d, 10.5)	112.3 (t)	1′-NH	6.64 (s)		16	6.14 (dd, 17.6, 10.5)	145.0 (d)
18	1.56 (s)	28.0 (q)	18	1.51 (s)	27.8 (q)	2′	4.97 (s)	78.7 (d)	17	5.13 (d, 17.6) 5.12 (d, 10.5)	111.8 (t)
19	1.56 (s)	28.0 (q)	19	1.51 (s)	27.9 (q)	3′		60.0 (s)	18	1.51 (s)	27.8 (q)
20	4.00 (m) 3.89 (m)	65.3 (t)	20	3.94 (m)	63.9 (t)	3′a		130.6 (s)	19	1.51 (s)	27.8 (q)
20-OH	4.54 (br s)		21	3.39 (d, 7.2)	34.6 (t)	4′	7.38 (d, 7.4)	124.6 (d)			
			22	5.35 (t, 7.2)	124.5 (d)	5′	6.62 (m)	118.1 (d)			
			23		131.6 (s)	6′	7.03 (m)	128.6 (d)			
			24	1.74 (s)	25.8 (q)	7′	6.61 (d, 7.7)	108.9 (d)			
			25	1.80 (s)	17.9 (q)	7′a		149.0 (s)			
			26	3.53 (d, 7.3)	31.3 (t)	9′	4.20 (t, 8.6)	57.0 (d)			
			27	5.42 (t, 7.3)	122.9 (d)	10′	3.07 (m) 2.62 (dd, 14.0, 8.6)	35.3 (t)			
			28		133.0 (s)	11′		169.5 (s)			
			29	1.74 (s)	25.7 (q)	12′-NH	8.06 (s)				
			30	1.86 (s)	17.9 (q)	13′	4.00 (q, 6.9)	50.5 (d)			
						14′		169.3 (s)			
						15′	1.15 (d, 6.9)	14.8 (q)			

[a] Measured in acetone-d_6.
[b] Measured in $CDCl_3$.
[c] Measured in DMSO-d_6, at 500 MHz for ^{1}H and 125 MHz for ^{13}C with reference to the solvent signals, δ in ppm.

16.9, and 42.6 μg/mL, respectively. The antimicrobial activities[23,24] against two bacteria (*Escherichia coli* and *Staphyloccocus aureus*) and five plant-pathogenic fungi (*Alternaria brassicae*, *Valsa mali*, *Physalospora obtuse*, *Alternaria solania*, and *Sclerotinia miyabeana*) were also evaluated. Compounds **1** and **9** displayed potent inhibitory activity against *E. coli* and *S. aureus* with the MIC values of 64 and 8 μg/mL, respectively, while compounds **4** and **10** showed weak activity against *S. aureus*, each giving the inhibition zone of 8 mm at 100 μg/disk (MICs were not determined). Chloramphenicol was used as positive control and showed the MIC value of 4 μg/mL against *E. coli* and *S. aureus*.

The antibacterial activity of **1** appears related to the serine residue in the 2,5-diketopiperazine moiety compared to that of **5** (with the alanine residue). The serine residue was also important to the brine shrimp lethality of **2** compared with that of **10**. The structure difference between **6** and **9** was only at C-8/C-9, indicating that the single bond between C-8/C-9 (compound **9**) was essential to its antibacterial activity, while the double bond at C-8/C-9 (compound **6**) appears important for its brine shrimp lethality. Compound **6** was more active than **5** and **7** in the brine shrimp bioassay, which was probably due to the number and substituted position of the isoprenic chain. This deduction was also proved by the structure differences between compounds **8**, **9**, and **10** compared with their antibacterial activities.

In summary, we identified four new indole alkaloids, cristatumins A–D (**1–4**), along with six congeners (**5–10**) from *E. cristatum* EN-220, an endophytic fungus isolated from the marine alga *S. thunbergii*. Cristatumin A (**1**) displayed moderate activity against *E. coli*, and tardioxopiperazine A (**9**) displayed potent activity against *S. aureus*. This is the first report for the antibacterial activity of **9**. Cristatumin B (**2**), isoechinulin A (**6**), and variecolorin G (**7**) exhibited moderate lethal activity against brine shrimp. This is also the first report for the brine shrimp inhibition activity of **6** and **7**.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.05.088.

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10. Physical and spectroscopic data of new compounds: (a) cristatumin A (**1**): colorless amorphous powder; $[\alpha]_D^{20}$: −17.6 (c 0.34, MeOH); UV (MeOH) λ_{max} (log ε) 223 (4.28), 284 (3.69), 333 (3.79) nm; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 362 [M+Na]$^+$; HRESIMS *m*/*z* 362.1477 [M+Na]$^+$ (calcd for $C_{19}H_{21}N_3O_3Na^+$, 362.1480). (b) Cristatumin B (**2**): colorless amorphous powder; $[\alpha]_D^{20}$: −11.1 (c 0.09, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 228 (4.52), 276 (3.90) nm; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 500 [M+Na]$^+$; HRESIMS *m*/*z* 500.2885 [M+Na]$^+$ (calcd for $C_{29}H_{39}N_3O_3Na^+$, 500.2889). (c) Cristatumin C (**3**): colorless amorphous powder; $[\alpha]_D^{20}$: +315.7 (c 1.21, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.77), 242 (4.02), 301 (3.47) nm; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 563 [M+Na]$^+$; HRESIMS *m*/*z* 563.2377 [M+Na]$^+$ (calcd for $C_{30}H_{32}N_6O_4Na^+$, 563.2382). (d) Cristatumin D (**4**): yellow amorphous powder; UV (MeOH) λ_{max} (log ε) 220 (4.41), 265 (4.02), 350 (4.11) nm; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 378 [M+Na]$^+$; HRESIMS *m*/*z* 378.1425 [M+Na]$^+$ (calcd for $C_{19}H_{21}N_3O_4Na^+$, 378.1429).
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