Isoechinulin-type Alkaloids, Variecolorins A–L, from Halotolerant *Aspergillus variecolor*

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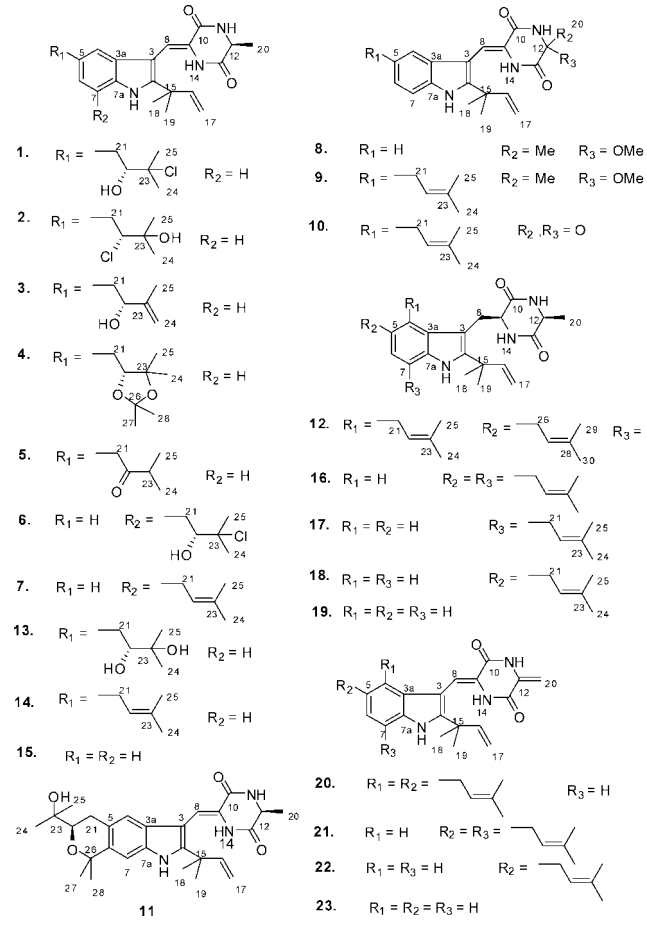
Received May 4, 2007

Twelve new compounds, variecolorins A–L (**1**–**12**), together with eleven known analogues (**13**–**23**) were isolated from the broth of a halotolerant fungus, *Aspergillus variecolor*. The structures of compounds **1**–**12** were determined by chemical and spectroscopic methods. Compounds **1**–**11**, **13**–**15**, and **20**–**23** exhibited weak radical scavenging activity against DPPH, with IC_{50} values from 43 to 103 μM. The new compounds **1**–**12** all were essentially nontoxic against the P388, HL-60, BEL-7402, and A-549 cell lines with IC_{50} values from 70 to 260 μM.

The genus *Aspergillus*, which contains around 180 recognized species, has proved to be a rich source of novel bioactive metabolites.[1,2] Isoechinulin-type alkaloids are one important group found in *Aspergillus* species, and they contain three structural units: an indole, a 2-methyl-3-buten-2-yl, and a diketopiperazine.[3–5] This group consists of about 20 known structures, most of which display radical scavenging activity,[3,4] ultraviolet-A protecting activity, immunosuppressive activity,[6,7] and antibacterial activity.[8] In our search for new isoechinulin type alkaloids, a halotolerant strain of *Aspergillus variecolor* showed UV absorption similar to that of isoechinulin A. Further chemical study led to isolation and structure elucidation of 12 new isoechinulin-type compounds (**1**–**12**) and 11 known ones from the broth of *A. variecolor*. By means of spectroscopic and chemical methods, their structures were determined as **1**–**12**, named variecolorins A−L, dihydroxyisoechinulin A (**13**),[4] isoechinulin A (**14**),[9] neoechinulin A (**15**),[3] echinulin (**16**),[6] tardioxopiperazine B (**17**),[6] tardioxopiperazine A (**18**),[6] preechinulin (**19**),[10] cryptoechinuline G (**20**),[11] alkaloid E-7 (**21**),[12] isoechinulin B (**23**),[9] and neoechinulin B (**23**),[13] respectively. The radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) of these compounds as well as cytotoxic activities of the new compounds are also described in this paper.



Results and Discussion

Variecolorin A (**1**) was obtained as a colorless amorphous powder. The ESIMS molecular ion cluster at *m/z* 466/468 $[M+Na]^+$ (rel int 3:1) indicated the presence of chlorine. The molecular formula of **1** was further determined to be $C_{24}H_{30}N_3O_3Cl$ by HRESIMS: *m/z* 466.1885 $[M+Na]^+$ (calcd 466.1873). Diagnostic IR absorption peaks were observed for hydroxyl, amino, and amide carbonyl groups at 3371, 3274, 1682, and 1633 cm^{-1}, respectively. UV absorptions at λ_{max} 210, 228, and 285, 340 suggested the presence of amide and conjugated indole moieties in **1**.[3] The NMR spectra of **1** displayed signals for two carbonyl, eight quarternary carbons, seven methines, two methylenes, and five methyl groups (Tables 1 and 2). Except for the lack of the 23-O*H* signal at δ 4.24 (s) and the obvious downfield shift (+3.4 ppm) of C-23, the NMR data were quite similar to those of dihydroxyisoechinulin A (**13**), suggesting that **1** was the C-23 chloro-derivative of **13**. This deduction was supported by HMBC correlations between 22-O*H* (δ 4.91, d, J = 6.9 Hz) and C-21 (δ 38.4, CH_2), C-22 (δ 79.8, CH), between H-24 (δ 1.56, 3H, s) and C-22 (δ 79.8, CH), C-23 (δ 75.2, qC), and C-25 (δ 29.5, CH_3).

Variecolorin B (**2**) was a colorless amorphous solid, and HRESIMS suggested the same molecular formula as **1** ($C_{24}H_{30}N_3O_3Cl$). The UV spectrum also suggested that **2** was an analogue of **13**. The NMR spectra of **2** were very similar to those of **13** except for the absence of the 22-O*H* signal at δ 4.17 (d, J = 5.8 Hz) and the noticeable upfield chemical shift of C-22 (−5.7 ppm), consistent with a chlorine at C-22.

Variecolorin C (**3**) was obtained as a colorless amorphous powder. Its molecular formula was determined as $C_{24}H_{29}N_3O_3$ according to the HRESIMS at *m/z* 430.2125 $[M+Na]^+$ (calcd 430.2107), indicating that one molecule of H_2O had been lost from **13**. The two compounds also showed similar UV and NMR spectra. The 23-O*H* and 24-CH_3 signals of **13** were absent in the ^1H NMR spectrum of **3**, and additional methylene signals, at δ 4.74 (br s) and 4.64 (br s), were observed. Accordingly, signals of an oxygenated quaternary carbon and a methyl group were absent in the ^{13}C NMR spectrum of **3**, while two additional sp^2 carbon signals

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10.1021/np070208z CCC: $37.00
Published on Web 09/27/2007

Table 1. ^{1}H NMR Data for Compounds **1**–**12** (Recorded in d_6-DMSO)[a]

position	**1** δ_H (*J*/Hz)	**2** δ_H (*J*/Hz)	**3** δ_H (*J*/Hz)	**4** δ_H (*J*/Hz)	**5** δ_H (*J*/Hz)	**6** δ_H (*J*/Hz)	**7** δ_H (*J*/Hz)	**8** δ_H (*J*/Hz)	**9** δ_H (*J*/Hz)	**10** δ_H (*J*/Hz)	**11** δ_H (*J*/Hz)	**12**[b] δ_H (*J*/Hz)
1(NH)	10.96 (s)	10.99 (s)	10.95 (s)	10.98 (s)	11.01 (s)	10.18 (s)	10.31 (s)	11.12 (s)	10.98 (s)	11.16 (s)	10.84 (s)	10.53 (s)
4	7.06 (br s)	7.04 (br s)	6.99 (br s)	7.05 (br s)	7.03 (br s)	7.07 (d, 7.7)	7.02 (d, 7.8)	7.18 (d, 8.0)	6.98 (br s)	7.20 (br s)	6.92 (s)	
5						6.98 (dd, 7.7, 7.0)	6.95 (dd, 7.8, 6.8)	7.01 (dd, 8.0, 8.1)				
6	7.02 (br d, 8.2)	6.97 (br d, 7.8)	6.95 (br d, 8.3)	7.01 (dd, 8.2, 1.4)	6.91 (dd, 8.3, 1.7)	7.02 (d, 7.0)	6.86 (d, 7.0)	7.09 (dd, 8.0, 8.1)	6.91 (br d, 8.2)	6.93 (dd, 8.4, 1.4)		6.82 (d, 8.1)
7	7.32 (d, 8.2)	7.34 (d, 7.8)	7.29 (d, 8.3)	7.33 (d, 8.3)	7.34 (d, 8.3)			7.43 (d, 8.0)	7.32 (d, 8.2)	7.33 (d, 8.4)	7.16 (s)	7.15 (d, 8.1)
8	6.90 (s)	6.87 (s)	6.88 (s)	6.88 (s)	6.86 (s)	6.88 (s)	6.89 (s)	7.01 (s)	6.99 (s)	7.19 (s)	6.88 (s)	3.46 (dd, 3.7, 14.6) 3.20 (dd, 11.0, 14.6)
9												4.00 (dd, 3.7, 11.0)
11(NH)	8.37 (d, 1.8)	8.37 (d, 1.8)	8.37 (d, 2.0)	8.36 (d, 1.9)	8.37 (d, 1.9)	8.36 (d, 1.8)	8.35 (d, 1.8)	9.10 (s)	9.05 (s)	12.0 (br s)	8.33 (d, 1.8)	6.87 (brs)
12	4.10 (qd, 6.8, 1.8)	4.14 (qd, 6.9, 1.8)	4.13 (qd, 6.9, 2.0)	4.09 (qd, 6.9, 1.9)	4.13 (qd, 6.9, 1.9)	4.17 (qd, 7.0, 1.8)	4.17 (qd, 6.9, 1.8)				4.18 (qd, 6.9, 1.8)	3.86 (br q, 7.0)
14 (NH)	8.57 (s),	8.63 (s),	8.46 (s),	8.60 (s),	8.66 (s),	8.62 (s)	8.67 (s)	9.23 (s)	9.16 (s)	9.82 (s)	8.75 (s)	8.19 (br s)
16	6.07 (dd, 17.4, 10.5)	6.08 (dd, 16.9, 10.5)	6.07 (dd, 17.4, 10.5)	6.08 (dd, 17.4, 10.6)	6.07 (dd, 17.4, 10.5)	6.11 (dd, 17.2, 10.3)	6.14 (dd, 17.0, 10.2)	6.08 (dd, 17.2, 10.9)	6.05 (dd, 17.4, 10.5)	6.06 (dd, 17.6, 10.5)	6.05 (dd, 17.4, 10.6)	6.16 (dd, 17.6, 10.6)
17	5.04 (d, 10.5) 5.01 (d, 17.4),	5.05 (d, 10.5) 5.02 (d, 16.9),	5.05 (d, 10.5) 5.02 (d, 17.4),	5.05 (d, 10.6) 5.03 (d, 17.4),	5.05 (d, 10.5) 5.03 (d, 17.4),	5.07 (d, 10.3) 5.06 (d, 17.2)	5.07 (d, 10.2) 5.06 (d, 17.0)	5.06 (d, 10.9) 5.04 (d, 17.2)	5.04 (d, 10.5) 5.02 (d, 17.4)	5.08 (d, 10.5) 5.05 (d, 17.6)	5.02 (d,17.4) 5.04 (d, 10.6)	5.06 (d, 17.6) 5.02 (d, 10.6)
18	1.48 (3H, s)	1.47 (3H, s)	1.47 (3H, s)	1.48 (3H, s)	1.46 (3H, s)	1.50 (3H, s)	1.51 (3H, s)	1.46 (3H, s)	1.47 (3H, s)	1.47 (3H, s)	1.46 (3H, s)	1.50 (3H, s)
19	1.46 (3H, s)	1.47 (3H, s)	1.46 (3H, s)	1.46 (3H, s)	1.46 (3H, s)	1.50 (3H, s)	1.51 (3H, s)	1.49 (3H, s)	1.49 (3H, s)	1.47 (3H, s)	1.46 (3H, s)	1.47 (3H, s)
20	1.41 (3H, d, 6.8)	1.38 (3H, d, 6.9)	1.40 (3H, d, 6.9)	1.41 (3H, d, 6.9)	1.39 (3H, d, 7.3)	1.37 (3H, d, 7.0)	1.38 (3H, d, 6.9)	1.48 (3H,s,)	1.44 (3H,s,)		1.39 (3H, d, 6.9)	1.31 (3H, d, 7.0)
21	3.08 (br d, 13.7) 2.54 (dd, 13.7, 8.2)	3.47 (br d, 14.2) 2.62 (dd, 14.2, 11.4)	2.74 (dd, 6.2, 13.6) 2.70 (dd, 13.6, 7.1)	2.75 (2H, m)	3.77 (d, 14.7) 3.74 (d, 14.7,)	3.22 (br d, 14.7) 3.01 (dd, 14.7, 7.6)	3.66 (2H, d, 7.3)		3.30 (2H, d, 7.3)	3.32 (2H, d, 7.3)	2.72 (2H, m)	3.27 (br d, 6.4)
22	3.55 (dd, 8.2, 6.9)	3.88 (dd, 11.4, 1.3)	4.08 (ddd, 7.1, 6.2, 4.2)	3.89 (dd, 7.8, 5.0)		3.75 (dd, 6.6, 7.6)	5.42 (br t, 7.3)		5.27 (br t, 7.3)	5.29 (br t, 7.3)	3.49 (dd, 10.1, 3.6)	5.16 (br t, 6.4)
23					2.73 (h, 6.9)							
24	1.56 (3H, s)	1.27 (3H, s)	4.74 (br s) 4.64 (br s)	1.11 (3H, s)	0.97 (3H, d, 6.9)	1.63 (3H, s)	1.75 (3H, s)		1.66 (3H, s)	1.66 (3H, s)	1.18 (3H, s)	1.67 (3H, s)
25	1.54 (3H, s)	1.23 (3H, s)	1.68 (3H, s)	1.09 (3H, s)	0.96 (3H, d, 6.9)	1.63 (3H, s)	1.75 (3H, s)		1.66 (3H, s)	1.65 (3H, s)	1.11 (3H, s)	1.66 (3H, s)
27				1.17 (3H, s)							1.50 (3H, s)	4.89 (dd, 5.2, 5.5)
28				1.32 (3H, s)							1.49 (3H, s)	
29												1.71 (3H, s)
30												1.62 (3H, s)
22-OH	4.91 (d, 6.9)		4.68 (d, 4.6)			5.62 (d, 6.6)						
23-OH		4.88 (s)									4.30 (s)	
12-OCH_3								3.24 (s)	3.24 (s)			

[a] Spectra were recorded at 600 MHz for ^{1}H using TMS as internal standard. [b] The ^{1}H NMR data of H-26 is δ 3.68 (1H, dd, J = 17.2, 5.5 Hz) and δ 3.63 (1H, dd, J = 17.2, 5.2 Hz).

Table 2. ^{13}C NMR Data for Compounds **1**–**12** (Recorded in d_6-DMSO)[a]

position	**1** δ_C	**2** δ_C	**3** δ_C	**4** δ_C	**5** δ_C	**6** δ_C	**7** δ_C	**8** δ_C	**9** δ_C	**10** δ_C	**11** δ_C	**12** δ_C
2	144.0 qC	144.1 qC	143.8 qC	144.2 qC	144.2 qC	143.5 qC	143.8 qC	144.4 qC	144.5 qC	145.9 qC	144.5 qC	141.7 qC
3	103.0 qC	103.1 qC	103.0 qC	103.1 qC	103.2 qC	104.0 qC	104.1 qC	103.6 qC	103.3 qC	103.6 qC	102.7 qC	104.8 qC
3a	126.0 qC	126.0 qC	125.9 qC	126.1 qC	126.2 qC	126.0 qC	126.1 qC	126.2 qC	126.3 qC	126.3 qC	124.9 qC	126.2 qC
4	119.2 CH	119.2 CH	119.1 CH	119.0 CH	119.6 CH	117.0 CH	116.6 CH	119.0 CH	118.1 CH	118.8 CH	118.6 CH	130.4 qC
5	131.1 qC	130.2 qC	130.3 qC	129.5 qC	125.7 qC	119.7 CH	119.8 CH	119.5 CH	132.3 qC	132.9 qC	124.9 qC	129.6 qC
6	122.7 CH	122.3 CH	122.8 CH	122.4 CH	122.4 CH	121.4 CH	120.3 CH	120.8 CH	121.6 CH	121.9 CH	136.1 qC	122.8 CH
7	111.0 CH	111.2 CH	110.9 CH	111.2 CH	111.4 CH	123.7 qC	124.6 qC	111.6 CH	111.4 CH	111.5 CH	107.6 CH	109.2 CH
7a	133.8 qC	133.9 qC	133.7 qC	133.9 qC	134.0 qC	134.4 qC	133.9 qC	135.1 qC	133.6 qC	133.7 qC	134.2 qC	134.6 qC
8	110.4 CH	110.2 CH	110.4 CH	110.3 CH	110.1 CH	110.0 CH	110.5 CH	112.3 CH	112.3 CH	116.4 CH	110.4 CH	30.3 CH_2
9	124.6 qC	125.1 qC	124.7 qC	124.7 qC	125.3 qC	125.1 qC	125.2 qC	124.4 qC	124.1 qC	123.0 qC	124.4 qC	55.6 CH
10	159.8 qC	160.0 qC	159.8 qC	159.8 qC	159.9 qC	159.8 qC	159.9 qC	161.4 qC	161.2 qC	157.4 qC	160.1 qC	167.8 qC
12	50.8 CH	50.6 CH	50.7 CH	50.8 CH	50.6 CH	50.4 CH	50.6 CH	84.0 qC	84.0 qC	152.1 qC	50.5 CH	50.0 CH
13	166.4 qC	166.4 qC	166.3 qC	166.2 qC	166.3 qC	166.4 qC	166.5 qC	163.4 qC	163.2 qC	160.5 qC	166.5 qC	167.5 qC
15	39.2 qC	39.0 qC	39.0 qC	39.0 qC	39.0 qC	39.0 qC	39.3 qC	39.4 qC	39.1 qC	39.3 qC	39.0 qC	39.3 qC
16	145.2 CH	145.2 CH	145.2 CH	145.2 CH	145.2 CH	145.2 CH	145.6 CH	145.1 CH	145.1 CH	144.9 CH	145.1 CH	146.7 CH
17	111.6 CH_2	111.6 CH_2	111.5 CH_2	111.6 CH_2	111.6 CH_2	111.8 CH_2	111.6 CH_2	111.8 CH_2	111.7 CH_2	112.0 CH_2	111.6 CH_2	111.0 CH_2
18	27.5 CH_3	27.5 CH_3	27.6 CH_3	27.5 CH_3	27.4 CH_3	27.5 CH_3	27.6 CH_3	27.4 CH_3	27.4 CH_3	27.7 CH_3	27.5 CH_3	28.6 CH_3
19	27.4 CH_3	27.5 CH_3	27.5 CH_3	27.4 CH_3	27.4 CH_3	27.4 CH_3	27.6 CH_3	27.7 CH_3	27.7 CH_3	27.7 CH_3	27.5 CH_3	28.1 CH_3
20	20.3 CH_3	19.8 CH_3	20.1 CH_3	20.2 CH_3	19.8 CH_3	19.5 CH_3	19.8 CH_3	22.0 CH_3	22.3 CH_3		19.6 CH_3	19.8 CH_3
21	38.4 CH_2	38.8 CH_2	42.3 CH_2	35.5 CH_2	47.4 CH_2	34.0 CH_2	28.9 CH_2		34.2 CH_2	34.1 CH_2	29.6 CH_2	31.3 CH_2
22	79.8 CH	74.2 CH	76.4 CH	84.5 CH	212.1 qC	78.8 CH	122.5 CH		124.5 CH	124.7 CH	75.5 CH	125.0 CH
23	75.2 qC	71.6 qC	147.8 qC	79.7 qC	38.6 CH	75.2 qC	132.0 qC		130.6 qC	130.6 qC	70.3 qC	129.9 qC
24	27.6 CH_3	27.9 CH_3	110.4 CH_2	25.8 CH_3	18.1 CH_3	27.3 CH_3	25.6 CH_3		25.5 CH_3	25.5 CH_3	27.5 CH_3	17.7 CH_3
25	29.5 CH_3	24.4 CH_3	17.7 CH_3	23.0 CH_3	18.1 CH_3	29.5 CH_3	17.8 CH_3		17.6 CH_3	17.7 CH_3	27.3 CH_3	25.6 CH_3
26				105.8 qC							75.3 qC	27.3 CH_2
27				28.6 CH_3							29.0 CH_3	124.5 CH
28				26.7 CH_3							32.5 CH_3	130.4 qC
29												18.1 CH_3
30												25.6 CH_3
12-OCH_3								50.1 CH_3	50.1 CH_3			

[a] Spectra were recorded at 150 MHz for ^{13}C using TMS as internal standard.

at δ 110.4 (CH_2) and δ 147.8 (qC) were observed. These data revealed that **3** is the 23,24-dehydrated derivative of **13**.

The molecular formula of **4**, variecolorin D, was determined to be $C_{27}H_{35}N_3O_4$ by HRESIMS at *m/z* 488.2534 $[M+Na]^+$ (calcd 488.2525). The 1D NMR data and the UV at λ_{max} (log ϵ) 210 (3.6), 229 (3.7), 288 (3.2), and 340 (3.3) nm suggested that **4** was an analogue of **13**. Two proton signals at δ 4.17 (d, $J = 5.8$ Hz) and 4.24 (s) assignable to 22,23-OH in **13** were absent in **4**, and two additional methyl signals at δ 1.17 (s, 3H) and 1.32 (s, 3H) assigned to H-27,28 were observed in **4**. An additional ketal carbon signal at δ 105.8 (qC, C-26) and two additional methyl signals at δ 28.6 (CH_3, C-28) and δ 26.7 (CH_3, C-27) were also observed in **4**. In addition, +4.6 and +7.6 ppm downfield shifts for C-22 and C-23 were observed, respectively. Hydrolysis of **4** with *p*-toluenesulfonic acid[14] yielded compound **13**. Thus, compound **4** was the 22,23-acetonide of **13**.

Variecolorin E (**5**) had molecular formula $C_{24}H_{29}N_3O_3$ as determined by HRESIMS. UV and 1D NMR data suggested that **5** is another analogue of **13**. The NMR data of **5**, except for signals attributed to the side chain, were the same to those of **13**. Two methyl doublet peaks in the ^{1}H NMR spectrum (δ 0.96, 3H, d, $J =$ 6.9 Hz; δ 0.97, 3H, d, $J = 6.9$ Hz) and a multiplet (δ 2.73, 1H) substituted for the corresponding singlet peaks at δ 1.12 and 1.19 and a triplet signal at δ 3.32, respectively. In the ^{13}C NMR spectrum, a methine carbon signal (δ 38.6) and a carbonyl carbon signal (δ 212.1) substituted for the corresponding signals at δ 72.0 (qC) and 79.9 (CH), respectively. A downfield shift for the methylene carbon (+9.4 ppm) and an upfield shift for two methyl carbons (−6.7 and −8.3 ppm) were also observed in the ^{13}C NMR spectrum of **5**. Thus, compound **5** was identified as the 22-dehydro- and 23-deoxy-derivative of **13**.

The ESIMS of variecolorin F (**6**) exhibited a pseudomolecular ion cluster at *m/z* 466/468 $[M+Na]^+$ and the HRESIMS at *m/z* 466.1877 $[M+Na]^+$ was consistent with the molecular formula, $C_{24}H_{30}N_3O_3Cl$, indicating that **6** is an isomer of **1**. The UV spectrum of **6** had the same chromophores as **1**. Except for signals due to the phenyl nucleus, its NMR spectra were very similar to those of **1**. Aromatic proton signals at δ 7.07 (d, 1H, $J = 7.7$ Hz), 6.98 (dd, 1H, $J = 7.7$, 7.0 Hz), and 7.02 (d, 1H, $J = 7.0$ Hz) indicated that a 1,2,3-trisubstituted phenyl nucleus was present. The HMBC experiments showed long-range ^{1}H–^{13}C correlations of H-6 (δ 7.02) with C-21 (δ 34.0), H-21 (δ 3.22) with C-6 (δ 121.4), C-7 (δ 123.7), C-7a (δ 134.4), and H-22 (δ 3.75) with C-7 (δ 123.7). Thus, the structure of **6** was established as 7-(3-chloro-2-hydroxy-3-methylbutyl) neoechinulin A.

Variecolorin G (**7**) had the formula $C_{24}H_{29}N_3O_2$ by HRESIMS. Except for signals due to the phenyl nucleus, its NMR spectra were very similar to those of **14**. Its aromatic proton signals at δ 7.02 (d, 1H, $J = 7.8$ Hz), 6.95 (dd, 1H, $J = 7.8$, 6.8 Hz), and 6.86 (d, 1H, $J = 7.0$ Hz) showed that a 1,2,3-trisubstituted phenyl nucleus was present in **7** rather than the 1,2,4-trisubstituted one in **14**. HMBC experiments showed the key long-range ^{1}H–^{13}C correlations of H-6 (δ 6.86) with C-21 (δ 28.9), H-21 (δ 3.66) with C-6 (δ 120.3), C-7 (δ 124.6), C-7a (δ 133.9), and H-22 (δ 5.42) with C-7 (δ 124.6). Thus, the structure of **7** was established as 7-(3-methyl-2-butene-1-yl) neoechinulin A.

Variecolorin H (**8**) had the molecular formula $C_{20}H_{23}N_3O_3$. The NMR spectra were quite similar to those of neoechinulin A (**15**), except for signals of the diketopiperazine moiety. Compared to the spectra of **15**, an additional methoxyl signal (δ 3.24) instead of the H-12 (δ 4.18) signal was observed in **8**. As expected, an additional methoxyl carbon signal (δ 50.1) and an oxygenated quaternary carbon signal (δ 84.0) were observed the ^{13}C NMR spectrum of **8**. An upfield shift of −3.1 ppm for C-13 and a downfield shift of +2.3 ppm for C-20 were also observed. HMBC experiments showed the key long-range ^{1}H–^{13}C correlations of -OC*H*$_3$ (δ 3.24) with C-12 (δ 84.0) and H-20 (δ 1.48) with C-12 and C-13 (δ 163.4). Thus, the structure of **8** was elucidated as 12-methoxyneoechinulin A.

The molecular formula of variecolorin I (**9**) was determined to be $C_{25}H_{31}N_3O_3$. The NMR spectra were quite similar to those of isoechinulin A (**14**), except for signals of the diketopiperazine moiety. Compared to **14**, an additional methoxyl signal (δ 3.24) instead of the H-12 (δ 4.15) signal was observed in **9** and an additional methoxyl carbon (δ 50.1) and oxygenated quaternary carbon (δ 84.0) signals, instead of a methine carbon signal (δ 50.5), were observed in the ^{13}C NMR spectrum of **9**. The HMBC experiments showed long-range ^{1}H–^{13}C correlations between -OC*H*$_3$ (δ 3.24) and C-12 (δ 80.4) and H-20 (δ 1.44) and C-12 and C-13 (163.2). Thus, compound **9** is 12-methoxyisoechinulin A.

Variecolorin J (**10**) had the molecular formula $C_{23}H_{25}N_3O_3$ (HRESIMS). Except for signals due to a diketopiperazine moiety, its NMR spectra were quite similar to those of **14**. Comparing with those of **14**, the methyl signals (δ 1.45, 3H; δ 19.8, CH_3) and methine signals (δ 4.15; δ 50.5, CH) were absent. Instead, an additional carbonyl carbon signal (δ 152.1) was observed in the 1D NMR of **9**. Shifts of −2.5 and −5.7 ppm for C-10 and C-13 were also observed. These observations indicated that the methyl on C-12 of **14** is substituted by oxygen in **10**.

The molecular formula of variecolorin K (**11**), $C_{27}H_{35}N_3O_4$, indicated 12 unsaturations. This information, coupled with the ^{13}C NMR data, suggested that **11** contained two carbonyl groups, six double bonds, and four rings. Its NMR data and UV data indicated that **11** was an analogue of dihydroxyisoechinulin A (**13**). Carefully comparing the NMR spectra with those of **13**, two methyl singlets (δ 1.49 and 1.50) instead of a 22-O*H* signal (δ 4.17) and two singlets of a 1,2,4,5-tetrasubstituted phenyl group (δ 6.92 and 7.16) instead of the ones of a 1,2,4-trisubstituted phenyl group were observed in the ^{1}H NMR spectrum of **11**. Accordingly, a quaternary carbon signal (δ 136.1) instead of methine (δ 122.9), an additional oxygenated quaternary carbon signal (δ 75.3), and two additional carbon signals (δ 29.0, CH_3; δ 32.5, CH_3) were observed in the ^{13}C NMR spectrum of **11**. These data indicated that a six membered ring was formed between 22-O and C-6 via an isopropylidene. This conclusion was confirmed by the key HMBC correlations between H-7 (δ 7.16, s) and H-22 (δ 3.49, dd, $J = 3.6$, 10.1 Hz) with C-26 (δ 75.3, CH), between H-27 (δ 1.50, s) with C-6 (δ 136.1, qC) and C-28 (δ 32.5, CH_3).

The C-8 (9) double bond geometry of compounds **1**–**11** was determined to be *Z* based on the downfield chemical shift of H-8 due to the deshielding effect of the 10-carbonyl.[13] The configuration at C-12, in compounds **1**–**7**, was assigned as *S* according to literature precedents (1.8–2.0 Hz of $J_{11,12}$).[4,13] This was comfirmed by acidic hydrolysis of compound **1**, as one of the products was identified as L-alanine by chiral HPLC analysis.[15] The configuration at C-22 in **1**, **2**, **3**, **4**, **6**, and **11** was determined to be *R* by comparing the optical rotation and the CD spectrum, as well as the NMR spectrum, with those of **13** (Tables 1 and 2).[4]

The molecular formula of variecolorin L (**12**) was determined to be $C_{29}H_{39}N_3O_2$. Except for signals of a phenyl nucleus, its NMR spectra were similar to those of **16**, indicating they had the same molecular skeleton. The aromatic proton signals at δ 6.82 (d, $J =$ 8.1 Hz) and 7.15 (d, $J = 8.1$ Hz) showed that **12** had a 1,2,3,4-tetrasubstitued phenyl nucleus. The key HMBC correlations between H-26 and C-5 indicated that **12** is 4-(3-methyl-2-butene-1-yl) tardioxopiperazine A. The cis configuration was deduced by the NOESY correlation between H-9 and H-12. The absolute configuration of C-12 was determined as *S* by acidic hydrolysis of compound **12**, one of which products was identified as L-alanine by chiral HPLC analysis.[15] Thus, the configuration at C-9 was also *S*. This was supported by comparing $[\alpha]_D$ (−21) with those of **17** ($[\alpha]_D$ −30) and **18** ($[\alpha]_D$ −15).[6]

The isoechinulin alkaloids are probably biosynthesized via a mixed amino acid–mevalonic acid pathway. Cyclo(Trp-Ala) resulted

Scheme 1. Postulated Biosynthetic Pathway of Compounds **1**–**23**

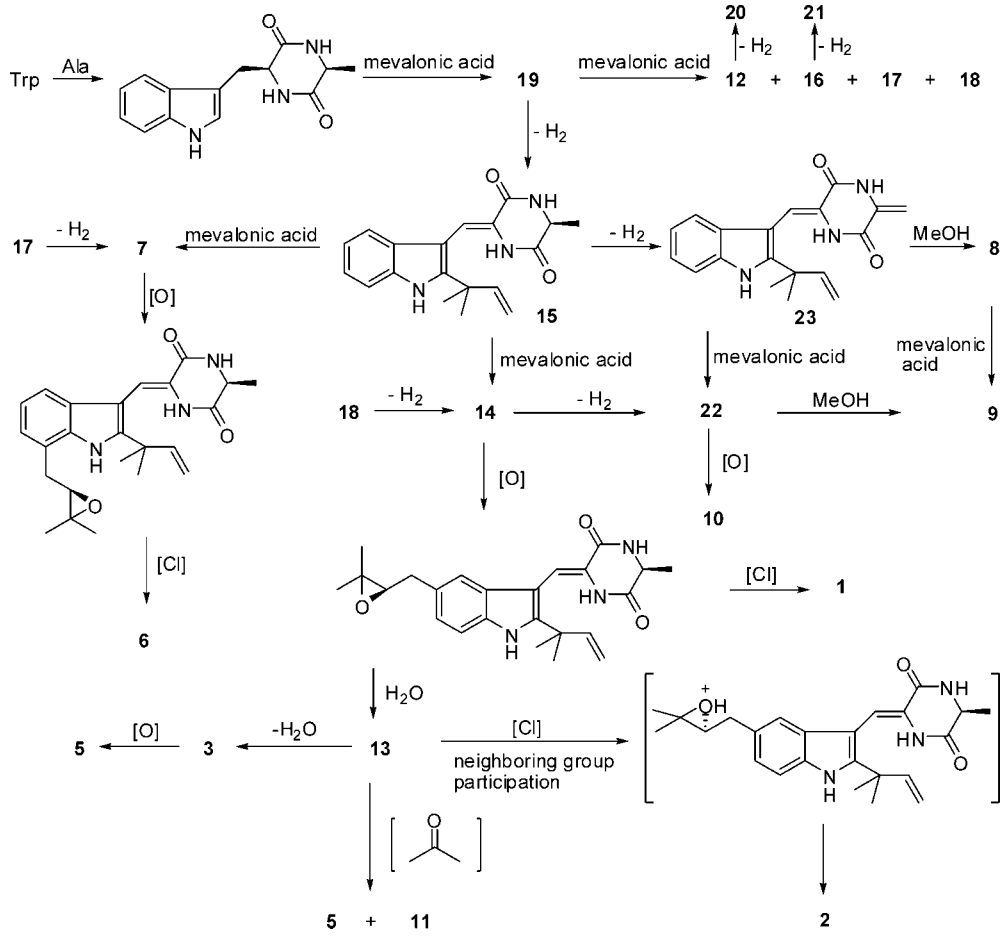

Table 3. Results of Radical Scavenging Activity against DPPH for Compounds **1**–**23**

compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	ascorbic acid
IC_{50} (μM)	79	97	75	88	79	77	86	99	79	102	89	623	79	98	103	569	899	912	1003	51	43	60	65	22

from tryptophan and alanine, which further reacted with mevalonic acid to form isopentenyl-substituted cyclo(Trp-Ala). The latter is postulated to undergo a series of dehydrogenation, oxidation, dehydration, and substitution reactions to form compounds **1**–**23** (Scheme 1). The results indicate that *Aspergillus variecolor* B-17 can use chlorine from the culture medium to synthesis chloro-substituted derivatives.

Compounds **1**–**23** were evaluated for their radical scavenging activity against DPPH.[16] Compounds **1**–**11**, **13**–**15**, and **20**–**23** showed weak activity with IC_{50} values of 79, 97, 75, 88, 79, 77, 86, 99, 79, 102, 89, 79, 98, 103, 51,43, 60, and 65 μM, respectively, while compounds **12** and **16**–**19** were inactive (IC_{50} > 500 μM; ascorbic acid as a positive control, IC_{50} 22 μM; see Table 3).

The new compounds **1**–**12** were also tested for cytotoxic effects on the P388 and HL-60 cell lines using the MTT method[17] and on the BEL-7402 and A-549 cell lines using the SRB method.[18] None of the compounds were cytotoxic against any of the four cell lines (IC_{50} > 50 μM; paclitaxel as a positive control, IC_{50} 0.93 μM).

Experimental Section

General Experimental Procedures. Optical rotations were obtained on a Jasco P-1020 digital polarimeter. UV spectra were recorded on Beckmen DU 640 spectrophotometer. ^{1}H, ^{13}C NMR, and DEPT spectra and 2D-NMR were recorded on a Jeol JNM-ECP 600 spectrometer using TMS as internal standard, and chemical shifts were recorded as δ values. ESI-MS was measured on a Q-TOF Ultima Global GAA076 LC mass spectrometer. Semipreparative HPLC was performed using an ODS column [YMC-pack ODS-A, 10 mm × 250 mm, 5 μm, 4 mL/min].

Fungal Material. The working strain *Aspergillus variecolor* B-17 was isolated from sediments collected in the Jilantai salt field, Alashan, Inner Mongolia, China. It was identified by Prof. Li Tian, the First Institute of Oceanography, SOA, Qingdao, China. The voucher specimen is deposited in our laboratory at −80 °C. The working strain was prepared on potato dextrose agar slants containing 10% NaCl and stored at 4 °C.

Fermentation and Extraction. *Aspergillus variecolor* B-17 was cultured under static conditions at 28 °C for 45 days in 250 1000-mL conical flasks containing the liquid medium (300 mL/flask) composed of glucose (20 g/L), maltose (10 g/L), mannitol (10 g/L), malt extract (3 g/L), monosodium glutamate (10 g/L), NaCl (90 g/L), $MgSO_4$ (5 g/L), and KCl (5 g/L) after adjusting its pH to 6.5. The fermented whole broth (75 L) was filtered through cheese cloth to separate into supernatant and mycelia. The former was extracted three times with ethyl acetate, and the ethyl acetate solution was concentrated under reduced pressure to give a crude extract (97.9 g).

Purification. The crude extract (97.9 g) was subjected to vacuum liquid chromatography on a silica gel column using step gradient elution with $CHCl_3$–petroleum ether (0–100%) and then MeOH–$CHCl_3$ (0–50%). The collected material was combined into nine fractions based on TLC properties. Fractions 3 and 4 were separated by ODS column chromatography (H_2O–MeOH gradient mixtures) into nine subfractions, respectively. Subfraction 3-2 (93 mg), eluted with MeOH:H_2O 1:1, was crystallized from $CHCl_3$:MeOH (1:9) to yield **19** (64 mg). Subfraction 3-3 (2.8 g), eluted with MeOH:H_2O 3:2, was crystallized from $CHCl_3$:MeOH (1:4) to yield **15** (2.1g). Compound **23** (67 mg) was isolated from the mother liquid of subfraction 3-3 by preparative HPLC. Subfraction 3-5 (583 mg), eluted with MeOH:H_2O 3:1, was separated by PHPLC (gradient elution, 55–85% MeOH) to yield compounds **7** (7 mg), **4** (92 mg), **9** (7mg), **14** (15 mg), **17** (8 mg), **18** (16 mg), and

22 (13 mg). Subfractions 3-6 and 3-7, eluted with MeOH:H_2O 9:1, were combined and crystallized from $CHCl_3$:MeOH (2:1) to yield **16** (104 mg). The mother liquid of this subfraction was subjected to PHPLC (gradient elution of 70–100% MeOH) to yield compounds **10** (15 mg), **12** (8 mg), **20** (57 mg), and **21** (18 mg). Subfraction 4-3 (173 mg), eluted with MeOH:H_2O 6:4, was separated by PHPLC (60% MeOH) to yield compounds **8** (15 mg) and **13** (106 mg). Subfractions 4-4 and 4-5, eluted with MeOH:H_2O (7:3 and 3:1), were combined and separated by PHPLC (gradient elution of 60–85% MeOH) to yield compounds **6** (22 mg), **5** (26 mg), and two subfractions 4-4-1 (38 mg) and 4-4-2 (46 mg). Subfractions 4-4-1 and 4-4-2 were purified by PHPLC (45% and 55% MeCN) to yield compounds **1** (16 mg) and **2** (11 mg) and **3** (21 mg) and **11** (29 mg), respectively.

Conversion of 4 to 13. Compound **4** (3 mg) was dissolved in 1 mL of MeOH:H_2O (3:1), and then, *p*-toluenesulfonic acid (1 mg) was added. The mixture was stirred and heated to 50 °C for 24 h. The mixture was poured into water (20 mL) and extracted with EtOAc (3 × 10 mL). The EtOAc layer was evaporated, and the residue was chromatographed over SiO_2 using $CHCl_3$–MeOH to yield compound **13** (1.9 mg) (69% yield).

Acidic Hydrolysis of 1 and 12. Compound **1** (1.0 mg) was dissolved in 6 N HCl, and the mixture was heated at 105 °C for 24 h in a sealed tube. The solution was diluted with 5 mL of H_2O and evaporated to dryness under reduced pressure. The residue was dissolved in 5 mL of H_2O, and the solution was then analyzed by chiral HPLC (Crownpak CR(+), Daicel Chemical, Japan): flow rate 0.4 mL/min; solvent, aqueous $HClO_4$ (pH = 2); detection, 201 nm; temperature 30 °C. The retention time of hydrolyzate was 5.06 min, while the retention times of D- and L-alanine were 4.25 and 5.06 min, respectively. By the same procedure, compound **12** (1.0 mg) gave the same result (hydralyzate, 5.05 min; L-alanine, 5.05 min; D-alanine, 4.25 min).

Biological Assays. In the 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. 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 IPMI-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 at 540 nm.

In the 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.

In the DPPH scavenging assay, 160 μL of reaction mixtures containing test samples and 40 μL DPPH (Sigma) dissolved in MeOH were plated in 96 cell plates incubated in the dark for 30 min. After the reaction, absorbance was measured at 520 nm and percent inhibition was calculated. IC_{50} values denote the concentration of sample required to scavenge 50% of the DPPH free radicals.

Variecolorin A (1): colorless amorphous powder; $[\alpha]^{25}_D$ −39 (*c* 0.1 MeOH); UV (MeOH) λ_{max} (log ϵ) 210 (3.6), 228 (3.6), 285 (3.1), 340 (3.2) nm; CD (MeOH, *c* 1.0), λ_{max} ($\Delta\epsilon$) 212 (−54.7), 238 (+23.2), 255 (+12.6), 264 (+15.2), 274 (+12.9), 284 (+15.2), 337 (−13.8); IR (KBr) ν_{max} 3371, 3274, 2974, 2929, 1682, 1633, 1431, 1382, 1325, 1196, 1029, 905, 759 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, Tables 1 and 2; HRESIMS *m/z* 466.1885 [M+Na]$^+$ (calcd for $C_{24}H_{30}N_3O_3ClNa$ 466.1873).

Variecolorin B (2): colorless amorphous powder; $[\alpha]^{25}_D$ −29 (*c* 0.1 MeOH); UV (MeOH) λ_{max} (log ϵ) 208 (3.5), 228 (3.6), 287 (3.0), 342 (3.2) nm; CD (MeOH, *c* 0.50), λ_{max} ($\Delta\epsilon$) 211 (−16.3), 235 (+11.1), 253 (+4.9), 263 (+5.1), 274 (+4.7), 284 (+4.8), 334 (−4.4); IR (KBr) ν_{max} 3373, 3261, 2965, 2934, 1683, 1631, 1453, 1399, 1341, 1169, 1030, 921, 764 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, Tables 1 and 2; HRESIMS *m/z* 466.1884 [M+Na]$^+$ (calcd for $C_{24}H_{30}N_3O_3ClNa$ 466.1873).

Variecolorin C (3): colorless amorphous powder; $[\alpha]^{25}_D$ −44 (*c* 0.1 MeOH); UV (MeOH) λ_{max} (log ϵ) 209 (3.4), 228 (3.4), 290 (3.1), 345 (3.2) nm; CD (MeOH, *c* 0.50), λ_{max} ($\Delta\epsilon$) 210 (−13.0), 237 (+8.8), 252 (+5.0), 262 (+5.2), 275 (+5.1), 284 (+5.3), 345 (−2.8); IR (KBr) ν_{max} 3380, 3281, 2997, 2955, 1684, 1641, 1519, 1403, 1091, 1043, 923, 834 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, Tables 1 and 2; HRESIMS *m/z* 430.2125 [M+Na]$^+$ (calcd for $C_{24}H_{29}N_3O_3Na$ 430.2107).

Variecolorin D (4): colorless amorphous powder; $[\alpha]^{25}_D$ −51 (*c* 0.1 MeOH); UV (MeOH) λ_{max} (log ϵ) 210 (3.6), 229 (3.7), 288 (3.2), 340 (3.3) nm; CD (MeOH, *c* 0.70), λ_{max} ($\Delta\epsilon$) 212 (−33.3), 239 (+12.8), 253 (+8.6), 265 (+9.9), 273 (+8.6), 280 (+9.9), 341 (−7.5); IR (KBr) ν_{max} 3450, 3206, 2980, 2929, 2863, 1669, 1639, 1433, 1399, 1334, 1311, 1217, 1196, 1091, 931, 809 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, Tables 1 and 2; HRESIMS *m/z* 488.2534 [M+Na]$^+$ (calcd for $C_{27}H_{35}N_3O_4Na$ 488.2525).

Variecolorin E (5): colorless amorphous powder; $[\alpha]^{25}_D$ −20 (*c* 0.1 MeOH); UV (MeOH) λ_{max} (log ϵ) 203 (3.4), 230 (3.4), 285 (3.1), 345 (3.2) nm; CD (MeOH, *c* 0.50), λ_{max} ($\Delta\epsilon$) 212 (−15.4), 236 (+9.7), 253 (+6.4), 264 (+6.6), 275 (+5.0), 284 (+5.1), 327 (−4.4); IR (KBr) ν_{max} 3386, 3206, 3056, 2971, 2930, 1670, 1635, 1418, 1334, 1086, 908, 785 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, Tables 1 and 2; HRESIMS *m/z* 408.2289 [M+H]$^+$ (calcd for $C_{24}H_{30}N_3O_3$ 408.2287).

Variecolorin F (6): colorless amorphous powder; $[\alpha]^{25}_D$ −28 (*c* 0.1 MeOH); UV (MeOH) λ_{max} (log ϵ) 197 (3.7), 228 (3.8), 285 (3.2), 337 (3.3) nm; CD (MeOH, *c* 0.50), λ_{max} ($\Delta\epsilon$) 208 (−15.5), 232 (+8.9), 253 (+2.3), 276 (+2.0), 324 (−3.2); IR (KBr) ν_{max} 3385, 3270, 2978, 2935, 1688, 1635, 1431, 1384, 1330, 1197, 1050, 915, 753 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, Tables 1 and 2; HRESIMS *m/z* 466.1877 [M+Na]$^+$ (calcd for $C_{24}H_{30}N_3O_3ClNa$ 466.1873).

Variecolorin G (7): colorless amorphous powder; $[\alpha]^{25}_D$ −16 (*c* 0.1 MeOH); UV (MeOH) λ_{max} (log ϵ) 201 (3.5), 230 (3.6), 281 (3.1), 338 (3.1) nm; CD (MeOH, *c* 0.50), λ_{max} ($\Delta\epsilon$) 210 (−7.4), 238 (+4.4), 258 (+3.3), 270 (+3.1), 346 (−2.0); IR (KBr) ν_{max} 3355, 3251, 2971, 2923, 1679, 1624, 1436, 1369, 1321, 1168, 1015, 905, 783 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, Tables 1 and 2; HRESIMS *m/z* 392.2350 [M+H]$^+$ (calcd for $C_{24}H_{30}N_3O_2$ 392.2338).

Variecolorin H (8): colorless amorphous powder; $[\alpha]^{25}_D$ 0 (*c* 0.3 MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (3.7), 225 (3.8), 275 (3.2) 352 (3.4) nm; IR (KBr) ν_{max} 3446, 3187, 3067, 2968, 2870, 1698, 1632, 1402, 1325, 1221, 1114, 1043, 927, 746 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, Tables 1 and 2; HRESIMS *m/z* 354.1804 [M+H]$^+$ (calcd for $C_{20}H_{24}N_3O_3$ 354.1818).

Variecolorin I (9): colorless amorphous powder; $[\alpha]^{25}_D$ 0 (*c* 0.1 MeOH); UV (MeOH) λ_{max} (log ϵ) 208 (3.8), 228 (3.7), 285 (3.2), 350 (3.3) nm; IR (KBr) ν_{max} 3420, 3238, 2986, 2977, 1684, 1637, 1425, 1379, 1330, 1208, 1029, 931, 736 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, Tables 1 and 2; HRESIMS *m/z* 444.2264 [M+Na]$^+$ (calcd for $C_{25}H_{31}N_3O_3Na$ 444.2263).

Variecolorin J (10): red amorphous powder; UV (MeOH) λ_{max} (log ϵ) 208 (3.7), 232 (3.7), 285(3.2), 420 (3.1) nm; IR (KBr) ν_{max} 3357, 3181, 3011, 2818, 1738, 1688, 1583, 1394, 1163, 965, 779 cm^{-1};^{1}H NMR and ^{13}C NMR data, Tables 1 and 2; HRESIMS *m/z* 392.1983 [M+H]$^+$ (calcd for $C_{23}H_{26}N_3O_3$ 392.1974).

Variecolorin K (11): colorless amorphous powder; $[\alpha]^{25}_D$ −49 (*c* 0.1 MeOH); UV (MeOH) λ_{max} (log ϵ) 210 (3.8), 228 (3.8), 275 (3.3), 340 (3.2) nm; CD (MeOH, *c* 0.50), λ_{max} ($\Delta\epsilon$) 207 (−11.0), 232 (+7.9), 250 (+4.5), 264 (+5.6), 275 (+5.0), 284 (+5.4), 340 (−2.7); IR (KBr) ν_{max} 3395, 3270, 2973, 2931, 1682, 1630, 1427, 1325, 1217, 1049, 916, 758 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, Tables 1 and 2; HRESIMS *m/z* 488.2506 [M+Na]$^+$ (calcd for $C_{27}H_{35}N_3O_4Na$ 488.2525).

Variecolorin L (12): colorless amorphous powder; $[\alpha]^{25}_D$ −21 (*c* 0.05 $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 197 (3.5), 235 (3.5), 293 (3.1) nm; CD (MeOH, *c* 0.05), λ_{max} ($\Delta\epsilon$) 213 (+3.2), 235 (−7.0), 282 (+0.8); IR (KBr) ν_{max} 3340, 3214, 2968, 2924, 1675, 1447, 1333, 1097 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, Tables 1 and 2; HRESIMS *m/z* 462.3138 [M+H]$^+$ (calcd for $C_{29}H_{40}N_3O_2$ 462.3121).

Acknowledgment. This work was supported by the Chinese National Natural Science Fund (Nos. 30470196 and 30670219). The fungal strain *A. variecolor* B-17 was identified by Prof. Li Tian, First Institute of Oceanography, State Oceanic Administration of China. The cytotoxicity assay was performed at the Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

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NP070208Z