Proline-Containing Cyclopeptides from the Marine Sponge *Phakellia fusca*

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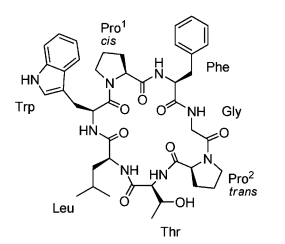
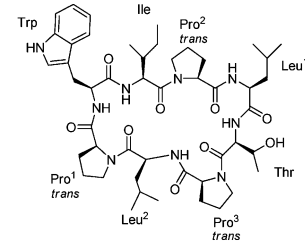
Four new cyclopeptides, phakellistatins 15−18 (**2**−**5**), together with five known cyclopeptides, phakellistatin 13 (**1**), hymenistatin 1, and hymenamides G, H, and J, were isolated from the South China Sea sponge *Phakellia fusca*. Their structures were elucidated by HR-ESIMS, NMR, and MALDI-TOF/TOF sequence analysis. The absolute configurations of the amino acid residues of **2**−**5** were assigned to be L by enantioselective HPLC analysis.

Marine organisms are prolific producers of structurally novel natural products with significant biological activities, which have been considered as promising resources for lead compounds or drug candidates.[1] Marine sponges of the genus *Phakellia* (order Halichondrida, family Axinellidae) have attracted a great deal of attention for having bioactive cyclopeptides (phakellistatins),[2] alkaloids,[3] and polyethers.[4] Besides phakellistatins, some structurally similar cyclopeptides have been obtained from the sponges of the order Halichondrida, such as axinastatins[5] and axinellins[6] (from the genus *Axinella*), hymenistatin 1[7] and hymenamides[8] (from the genus *Hymeniacidon*), stylopeptides,[9] stylostatin,[10] and wainunuamide[11] (from the genus *Stylotella*), and stylisins[12] and stylissamides[13] (from the genus *Stylissa*). These cyclopeptides commonly consist of seven to 10 amino acid residues including at least one proline residue, and some of them showed cancer cell line cytotoxicity and antifungal activity. Studies by Pettit's group showed that phakellistatins were trace secondary metabolites of sponges of the genus *Phakellia*.[2] Our previous studies on the chemical constituents of the marine sponge *Phakellia fusca* (500 g, dry wt) collected from the South China Sea led to the isolation of a cytotoxic cyclopeptide, phakellistatin 13 (**1**).[2b] In our reinvestigation aimed at bioactive cyclopeptides of *P. fusca* on a larger scale (15 kg, dry wt), four new proline-containing cyclopeptides, phakellistatins 15−18 (**2**−**5**), together with five known cyclopeptides, phakellistatin 13 (**1**), hymenistatin 1, and hymenamides G, H, and J, were obtained from the CH_2Cl_2-soluble and *n*-BuOH-soluble extracts. Herein, we report the isolation and structure elucidation of these proline-containing cyclopeptides (**2**−**5**) along with the *in vitro* cytotoxicity of phakellistatins 15 (**2**) and 16 (**3**).



1 (phakellistatin 13)



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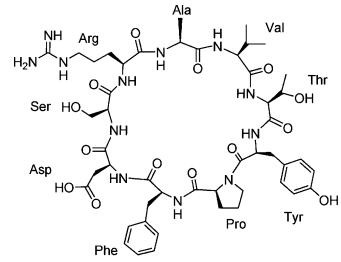
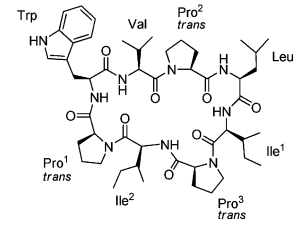


3a *trans*-Pro (major conformer)
3b *cis*-Pro (minor conformer)



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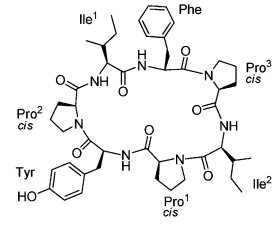

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Results and Discussion

The EtOH extract of dried *P. fusca* was partitioned between EtOAc and H_2O. The EtOAc phase extract was subjected to solvent partitioning to yield a CH_2Cl_2-soluble extract, and the H_2O phase fraction was extracted by *n*-BuOH to give an *n*-BuOH-soluble extract. By column chromatography or vacuum liquid chromatography (on Sephadex LH-20, ODS silica, and silica gel) and RP-HPLC, three new cyclopeptides, **2**, **4**, and **5**, together with four known cyclopeptides (**1**, hymenistatin 1, and hymenamides G and H) were obtained from the CH_2Cl_2-soluble extract. Similarly, one new cyclopeptide (**3**) and the known hymenamide J were isolated from the *n*-BuOH-soluble extract.

Phakellistatin 15 (**2**) was obtained as a glassy amorphous solid from MeOH, and its molecular formula was established as $C_{48}H_{71}N_9O_9$ from the positive ion HR-TOF-ESIMS peak at *m*/*z* 940.5276 and the ^{13}C NMR data. The ^1H and ^{13}C NMR spectra of **2** in DMSO-d_6 were characteristic of a peptide.[14] The ^{13}C NMR spectrum exhibited eight amide carbonyl carbons, as well as eight α-amino acid carbons (Table 1). The ^1H NMR spectrum showed five amide NH signals and one hydroxy proton signal at δ_H 5.22 (1H, d, $J = 12.8$ Hz). Eight aromatic carbons and a NH signal at δ_H 10.76 (br s) suggested the existence of a Trp residue (Table 1). Detailed analysis of the HMQC, COSY, HMBC, TOCSY, and HMQC-TOCSY spectra allowed the identification of eight amino acid residues as Pro (3×), Leu (2×), Ile, Trp, and Thr. According to the restrictions of the molecular formula and the corresponding

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Table 1. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Data for **2** in DMSO-d_6

position	δ_C, mult.	δ_H (J in Hz)	position	δ_C, mult.	δ_H (J in Hz)
Pro^1			γ	24.44, CH_2	a: 2.00, m
CO	171.13, C				b: 1.85, m
α	60.51, CH	3.85, t (7.2)	δ	47.91, CH_2	a: 3.99, m
β	28.71, CH_2	a: 1.81, m			b: 3.59, dt (7.0, 9.4)
		b: 1.63, m	**Leu^1**		
γ	24.38, CH_2	a: 1.99, m	NH		8.76, d (6.8)
		b: 1.81, m	CO	169.74, C	
δ	46.66, CH_2	a: 3.75, m	α	52.96, CH	3.46, m
		b: 3.46, m	β	35.82, CH_2	a: 2.16, m
Trp					b: 1.66, m
NH		7.91, d (7.1)	γ	24.6, CH	1.47, m
CO	170.30, C		δ	20.92, CH_3	0.87, d (6.0)
α	55.66, CH	3.94, m	δ'	23.28, CH_3	0.85, d (7.3)
β	24.58, CH_2	a: 3.47, m	**Thr**		
		b: 3.36, dd (14.4, 3.8)	NH		7.49, d (9.4)
1	110.96, C		CO	168.77, C	
2	123.38, CH	6.97, br s	α	56.12, CH	4.87, dd (9.5, 3.5)
3		10.76, br s	β	67.65, CH	4.13, m
4	136.05, C		γ	18.99, CH_3	1.03, d (6.3)
5	111.3, CH	7.32, d (8.0)	OH		5.22, d (12.8)
6	120.76, CH	7.05, t (8.0)	**Pro^3**		
7	118.1, CH	6.97, t (8.0)	CO	170.93, C	
8	117.93, CH	7.46, d (8.0)	α	59.07, CH	4.46, dd (8.7, 4.4)
9	127.06, C		β	28.31, CH_2	a: 2.06, m
Ile					b: 1.92, m
NH		7.40, d (9.8)	γ	24.54, CH_2	a: 1.83, m
CO	170.11, C				b: 1.72, m
α	54.19, CH	4.67, t (10.5)	δ	46.92, CH_2	a: 3.68, dt (9.8, 6.8)
β	33.01, CH	2.02, m			b: 3.54, dt (9.8, 6.8)
γ	23.31, CH_2	a: 1.36, m	**Leu^2**		
		b: 1.03, m	NH		8.13, d (8.6)
δ	9.52, CH_3	0.76, t (7.4)	CO	170.87, C	
β-Me	15.07, CH_3	0.78, d (6.6)	α	48.14, CH	4.44, m
Pro^2			β	38.02, CH_2	a: 1.70, m
CO	170.55, C				b: 1.05, m
α	60.32, CH	4.10, t (7.9)	γ	24.36, CH	1.54, m
β	29.61, CH_2	a: 2.15, m	δ	20.86, CH_3	0.81, d (6.6)
		b: 1.71, m	δ'	23.02, CH_3	0.86, d (6.6)

degrees of unsaturation, eight peptide bonds were essential for **2**, indicating **2** was a cyclopeptide.

Two fragments, Pro^1-Trp-Ile and Pro^2-Leu^1-Thr-Pro^3-Leu^2, were indicated by the HMBC correlations between Trp-NH/Pro^1-CO, Ile-NH/Trp-CO, Leu^1-NH/Pro^2-CO, Thr-NH/Leu^1-CO, Pro^3-Hδ/Thr-CO, and Leu^2-NH/Pro^3-CO. The ROESY correlations between Ile-Hα/Pro^2-Hδ and Leu^2-Hα/Pro^1-Hδ allowed the establishment of the sequence of **2** as cyclo(Pro^1-Trp-Ile-Pro^2-Leu^1-Thr-Pro^3-Leu^2). The $\Delta\delta_{C\beta-C\gamma}$ values of the Pro residues (4.33, 5.17, and 3.77 ppm for Pro^1, Pro^2, and Pro^3, respectively) and the ROESY correlations between Xaa^{i-1}-Hα/Pro^i-Hδ suggested that all three Xaa-Pro amide bonds were of the *trans*-configuration.[13,15]

The sequence of **2** was further confirmed by MALDI-TOF/TOF sequence analysis. Although there was more than one possible ring-opening position for the cyclopeptide, the preferred ring-opening of **2** occurred at the Leu^2-Pro^1 amide bond due to the proline effect on proline's high proton affinity.[16,17] The immonium and related ions indicated the existence of Leu or Ile (*m*/*z* 86, 44), and Tyr (*m*/*z* 136) residues.[18] A linearized peptide **2**, Pro^1-Trp-Ile-Pro^2-Leu^1-Thr-Pro^3-Leu^2, was demonstrated by a main series of adjacent $b_n(+1)$ ions at *m*/*z* 805, 708, 607, 494, 397, and 284, corresponding to the successive loss of Leu, Pro, Thr, Leu, Pro, Ile, and the terminal dipeptide ion Pro-Trp (Figure 1).[18a,19]

Phakellistatin 16 (**3**) was obtained as a glassy, amorphous solid from MeOH, and its molecular formula $C_{48}H_{68}N_{12}O_{14}$ was deduced from the positive ion HR-TOF-ESIMS peak at *m*/*z* 1059.4883 and the negative ion HR-TOF-ESIMS peak at *m*/*z* 1035.4917, as well as the ^{13}C NMR data. Phakellistatin 16 (**3**) existed as a mixture of two conformers in common NMR solvents (CD_3OH, CD_3CN, C_5D_5N, DMSO-d_6, etc.), and the two sets of NMR signals in the ratio of 3:1 in DMSO-d_6 were relatively well resolved. The ^{1}H and ^{13}C NMR spectra of **3** exhibited two sets of eight amide NH, one hydroxy proton, nine amide carbonyl carbons, and nine α-amino acid carbons, which were indicative of a peptide (Table 2).[14] Extensive inspection of the HMQC, COSY, HMBC, TOCSY, and HMQC-TOCSY spectra allowed the identification of two sets of nine amino acid residues as Pro, Phe, Asp, Ser, Arg, Ala, Val, Thr, and Tyr, of which the Arg (*m*/*z* 100, 112, 129), Phe (*m*/*z* 120), and Tyr (*m*/*z* 136) residues were indicated by their immonium and related ions in the MALDI-TOF/TOF spectrum.[18] For the major conformer **3a**, the HMBC correlations between Ser-NH/Asp-CO, Ala-NH/Arg-CO, Val-NH/Ala-CO, and Tyr-NH/Thr-CO, together with the ROESY correlations between Asp-Hα/Ser-NH, Ser-NH/Arg-NH, Arg-NH/Ala-NH, Ala-NH/Val-NH, Val-NH/Thr-NH, and Thr-NH/Tyr-NH, exhibited the fragment of Asp-Ser-Arg-Ala-Val-Thr-Tyr. The ROESY correlations between Tyr-Hα/Pro-Hδ, Pro-Hα/Phe-NH, and Phe-Hα/Asp-NH extended this fragment as cyclo(Pro-Phe-Asp-Ser-Arg-Ala-Val-Thr-Tyr). The NMR spectra of the minor conformer **3b** exhibited similar HMBC and ROESY correlations, showing the same peptide sequence as that of the major conformer **3a** (Figure 2). The MALDI-TOF/TOF sequence analysis confirmed the connections of amino acid residues of **3** and showed that the preferred ring-opening began at the Tyr-Pro peptide bond (Figure 1).[18a,19]

The $\Delta\delta_{C\beta\text{-}C\gamma}$ value (4.53 ppm) of the Pro residue in the major conformer **3a** and its ROESY correlation between Tyr-Hα/Pro-Hδ indicated that the Tyr-Pro amide bond in **3a** was the *trans*-configuration, while the $\Delta\delta_{C\beta\text{-}C\gamma}$ value (8.77 ppm) of the Pro residue in the minor conformer **3b** and its ROESY correlation between Tyr-Hα/Pro-Hα suggested that the Tyr-Pro amide bond in **3b** was the *cis*-configuration (Table 2 and Figure 2).[13,15]

Table 2. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Data for **3** in DMSO-d_6

	3a (major conformer)		**3b** (minor conformer)	
position	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)
Pro				
CO	171.23, C		170.67, C	
α	60.64, CH	4.00, m	59.83, CH	3.51, m
β	28.74, CH_2	a: 1.77, m	29.88, CH_2	a: 1.56, m
		b: 1.26, m		b: 0.95, m
γ	24.21, CH_2	a: 1.66, m	21.11, CH_2	a: 1.57, m
		b:1.53, m		b:1.41, m
δ	47.13, CH_2	a: 3.63, m	45.74, CH_2	a: 3.18, m
		b: 3.19, m		b: 3.01, m
Phe				
NH		7.23, br s		8.50, br s
CO	169.24, C		170.05, C	
α	54.44, CH	4.22, m	56.01, CH	4.22, m
β	34.87, CH_2	a: 3.33, m	35.54, CH_2	a: 3.02, m
		b: 2.90, m		b: 2.96, m
1	138.56, C		138.11, C	
2, 6	128.88, CH	7.08, d (7.5)	128.88, CH	7.21, d (7.5)
3, 5	128.10, CH	7.23, t (7.5)	128.03, CH	7.23, t (7.5)
4	126.19, CH	7.16, t (7.5)	126.19, CH	7.16, t (7.5)
Asp				
NH		7.00, d (8.8)		7.24, ov
CO	172.72, C		172.72, C	
α	50.28, CH	4.71, br t (10.2)	50.19, CH	4.34, br t (7.5)
β	41.96, CH_2	a: 2.82, m	40.03, CH_2	a: 2.69, m
		b: 2.17, m		b: 2.55, m
β-CO	175.78, C		176.12, C	
Ser				
NH		8.53, d (4.4)		8.44, d (4.4)
CO	169.89, C		170.28, C	
α	57.36, CH	4.01, m	57.24, CH	4.14, m
β	60.63, CH_2	a: 3.78, m	60.53, CH_2	a: 3.76, m
		b: 3.68, br d (7.8)		b: 3.67, m
OH		4.88, d (6.0)		4.89, d (6.0)
Arg				
NH		8.76, d (7.2)		8.26, d (6.4)
CO	171.49, C		172.42, C	
α	53.33, CH	4.04, m	52.79, CH	4.12, m
β	27.80, CH_2	a: 1.79, m	27.60, CH_2	a: 1.99, m
		b: 1.72, m		b: 1.80, m
γ	23.44, CH_2	a: 1.58, m	23.72, CH_2	a: 1.58, m
		b: 1.55, m		b: 1.55, m
δ	40.57, CH_2	a: 3.14, m	40.83, CH_2	a: 3.14, m
		b: 2.94, m		b: 2.94, m
δ-NH		9.36, br s		9.36, br s
guanidine	157.42, C		157.24, C	
Ala				
NH		7.58, d (7.0)		7.93, d (4.8)
CO	172.25, C		172.58, C	
α	47.58, CH	4.44, qui (7.0)	49.94, CH	3.93, qui (6.5)
β	18.87, CH_3	1.28, d (7.0)	16.75, CH_3	1.29, d (7.0)
Val				
NH		8.08, d (6.3)		7.63, d (7.5)
CO	171.31, C		170.94, C	
α	59.96, CH	3.98, t (7.0)	60.53, CH	3.87, t (7.1)
β	29.38, CH	2.07, m	28.96, CH	2.16, m
γ	18.38, CH_3	0.87, d (6.8)	18.57, CH_3	0.87, d (6.8)
γ'	19.43, CH_3	0.89, d (6.8)	19.46, CH_3	0.91, d (6.8)
Thr				
NH		7.63, d (7.5)		7.49, d (7.5)
CO	169.67, C		170.14, C	
α	58.83, CH	3.98, m	57.53, CH	4.27, m
β	65.58, CH	4.15, m	65.70, CH	4.11, m
OH		5.03, br s		5.88, br s
γ	20.63, CH_3	0.98, d (6.4)	20.54, CH_3	0.99, d (6.4)
Tyr				
NH		7.01, d (8.2)		7.83, d (8.2)
CO	170.41, C		169.89, C	
α	51.15, CH	4.81, q (7.4)	53.33, CH	4.59, br s
β	36.57, CH_2	a: 2.83, m	36.94, CH_2	a: 3.04, m
		b: 2.94, m		b: 2.60, m
1	126.92, C		126.19, C	
2, 6	130.06, CH	7.06, d (8.3)	130.41, CH	6.92, d (8.3)
3, 5	115.11, CH	6.64, d (8.3)	115.11, CH	6.62, d (8.3)
4	155.90, C		156.18, C	

Phakellistatin 17 (**4**) was obtained as a glassy, amorphous solid from MeOH, and its molecular formula was determined to be $C_{49}H_{73}N_9O_8$ from the positive ion HR-TOF-ESIMS peak at m/z 938.5482 and the ^{13}C NMR data. Phakellistatin 17 (**4**) appeared essentially as one conformer in DMSO-d_6. Inspection of the HMQC, COSY, HMBC, TOCSY, and HMQC-TOCSY spectra revealed the

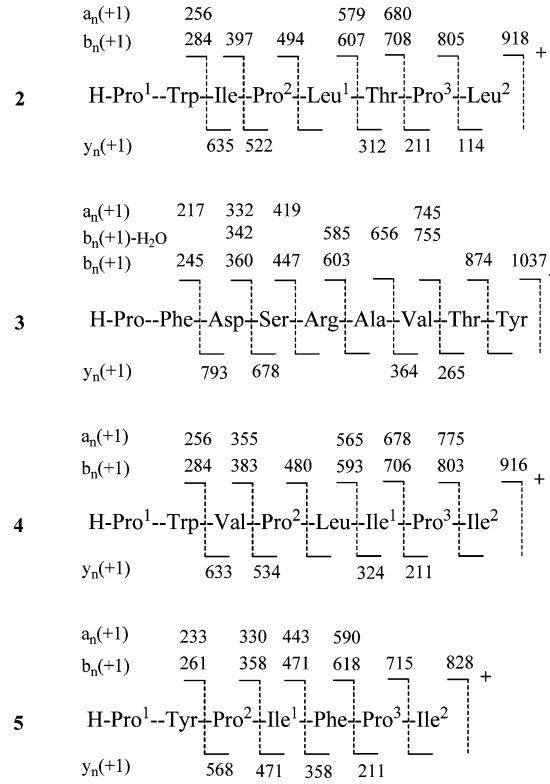

Figure 1. MALDI-TOF/TOF sequence ions (m/z) for protonated molecular $[M + H]^+$ ions of phakellistatins 15−18 (**2**−**5**).

presence of eight amino acid residues as Pro (3×), Trp, Val, Leu, and Ile (2×) (Table 3).

The HMBC correlations between Trp-NH/Pro^1-CO and Leu-NH/Pro^2-CO and the ROESY correlations between Trp-Hα/Val-NH and Val-Hα/Pro^2-Hδ suggested the fragment of Pro^1-Trp-Val-Pro^2-Leu. The ROESY correlations between Ile^1-Hα/Pro^3-Hδ and Pro^3-Hα/Ile^2-NH indicated the existence of Ile^1-Pro^3-Ile^2. The ROESY correlations between Leu-Hα/Ile^1-Hα and Ile^2-Hα/Pro^1-Hδ connected these two fragments, and accordingly the peptide **4** was established as cyclo(Pro^1-Trp-Val-Pro^2-Leu-Ile^1-Pro^3-Ile^2). This sequence was further supported by the MALDI-TOF/TOF sequence analysis, and the preferred ring-opening occurred at the Ile^2-Pro^1 amide bond (Figure 1).[18a,19] The $\Delta\delta_{C\beta-C\gamma}$ values of Pro residues (4.10, 4.70, and 4.02 ppm for Pro^1, Pro^2, and Pro^3, respectively) and the ROESY correlations between Xaa^{i-1}-Hα/Pro^i-Hδ indicated that all three Xaa-Pro amide bonds were of the *trans*-configuration.[13,15]

Phakellistatin 18 (**5**) was obtained as a glassy, amorphous solid from MeOH, and its molecular formula $C_{45}H_{61}N_7O_8$ was established from the positive ion HR-TOF-ESIMS peak at m/z 850.4481 and the ^{13}C NMR data. Phakellistatin 18 (**5**) existed mainly as one conformer in CD_3OH. Examination of the HMQC, COSY, HMBC, TOCSY, and HMQC-TOCSY spectra allowed the identification of seven amino acid residues as Pro (3×), Ile (2×), Phe, and Tyr (Table 4). The HMBC correlations between Tyr-NH/Pro^1-CO, Phe-NH/Ile^1-CO, and Ile^2-NH/Pro^3-CO exhibited the fragments Pro^1-Tyr, Ile^1-Phe, and Pro^3-Ile^2, respectively. There were no further adequate HMBC or ROESY correlations for the complete sequence assignment, which was finished by MALDI-TOF/TOF sequence analysis. A linearized peptide **5**, Pro^1-Tyr-Pro^2-Ile^1-Phe-Pro^3-Ile^2, was indicated by a series of adjacent $b_n(+1)$ ions, which suggested that peptide **5** was cyclo(Pro^1-Tyr-Pro^2-Ile^1-Phe-Pro^3-Ile^2), and the preferred ring-opening occurred at the Ile^2-Pro^1 amide bond (Figure 1).[18a,19] The $\Delta\delta_{C\beta-C\gamma}$ values of the three Pro residues (9.58, 9.24, and 8.99 ppm for Pro^1, Pro^2, and Pro^3, respectively) showed that all three Xaa-Pro amide bonds were of the *cis*-configuration.[13,15]

The absolute configurations of the amino acid residues of phakellistatins 15−18 (**2**−**5**) were determined by enantioselective

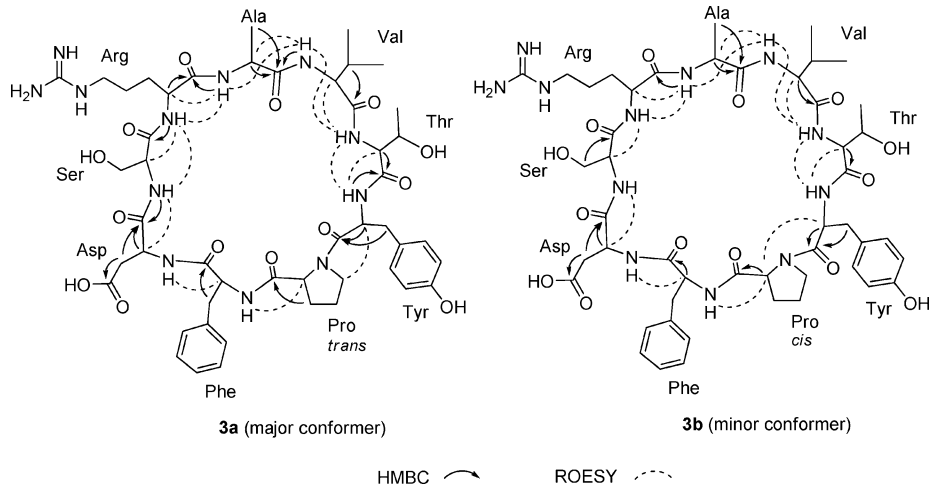

Figure 2. Key HMBC and ROESY correlations for phakellistatin 16 (**3**).

Table 3. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Data for **4** in DMSO-d_6

position	δ_C, mult.	δ_H (J in Hz)	position	δ_C, mult.	δ_H (J in Hz)
Pro[1]			δ	47.29, CH_2	a: 3.87, m
CO	170.90, C				b: 3.59, m
α	60.92, CH	3.88, m	**Leu**		
β	28.83, CH_2	a: 1.94, m	NH		8.25, d (6.8)
		b: 1.78, m	CO	170.11, C	
γ	24.73, CH_2	a: 1.76, m	α	54.09, CH	4.50, m
		b: 1.73, m	β	37.29, CH_2	a: 2.14, m
δ	47.05, CH_2	a: 3.59, m			b: 1.60, m
		b: 3.49, m	γ	24.46, CH	1.47, m
Trp			δ	20.65, CH_3	0.85, d (6.8)
NH		8.20, d (6.8)	δ'	23.23, CH_3	0.87, d (6.8)
CO	169.75, C		**Ile[1]**		
α	56.46, CH	3.75, m	NH		7.55, d (8.0)
β	23.51, CH_2	a: 3.62, m	CO	171.50, C	
		b: 3.40, dd (14.5, 3.1)	α	53.93, CH	4.65, m
1	111.43, C		β	37.96, CH	1.74, m
2	123.49, CH	6.98, br s	γ	23.16, CH_2	a: 1.31, m
3		10.73, br s			b: 1.15, m
4	136.11, C		δ	10.94, CH_3	0.75, t (7.6)
5	111.23, CH	7.32, d (8.0)	β-Me	14.83, CH_3	0.85, d (6.6)
6	120.71, CH	7.05, t (8.0)	**Pro[3]**		
7	117.98, CH	6.96, t (8.0)	CO	169.85, C	
8	118.07, CH	7.47, d (8.0)	α	59.41, CH	4.54, m
9	127.01, C		β	28.83, CH_2	a: 2.12, m
Val					b: 1.71, m
NH		7.71, d (9.0)	γ	24.81, CH_2	a: 1.83, m
CO	169.36, C				b: 1.73, m
α	55.81, CH	4.52, t (10.0)	δ	47.35, CH_2	a: 3.50, m
β	29.85, CH	2.11, m			b: 3.44, m
γ	18.99, CH_3	0.78, d (6.9)	**Ile[2]**		
γ'	19.46, CH_3	0.89, d (6.9)	NH		8.32, br d (6.0)
Pro[2]			CO	169.56, C	
CO	172.01, C		α	54.18, CH	4.39, t (9.1)
α	60.52, CH	4.13, t (7.3)	β	38.40, CH	1.48, m
β	29.35, CH_2	a: 2.09, m	γ	24.23, CH_2	a: 1.32, m
		b: 1.73, m			b: 0.98, m
γ	24.65, CH_2	a: 2.01, m	δ	10.28, CH_3	0.76, t (7.3)
		b: 1.86, m	β-Me	14.14, CH_3	0.88, d (8.0)

HPLC analysis after hydrolysis of **2**−**5**. All amino acids were found to possess the L-configurations.

Phakellistatins 15−18 (**2**−**5**) are new proline-containing cyclopeptides from the South China Sea sponge *P. fusca*. Phakellistatins 15 (**2**) and 17 (**4**), with analogous sequences Pro-Trp-Val/Ile-Leu-Thr/Ile-Pro-Leu/Ile, are structurally similar to hymenamide H, which was originally from the Okinawan sponge *Hymeniacidon* sp.,[8b] supporting the remarkable analogy in the cyclopeptides from the genera *Axinella*, *Hymeniacidon*, *Phakellia*, *Stylotella*, and *Stylissa*.[6b,13] Phakellistatin 18 (**5**) has the same seven residues as those of phakellistatins 1[2j] and 2[2i] and only differs in the sequence. As a matter of fact, cyclopeptides from the genus *Phakellia* were obtained using similar isolation schemes, and most of them were isolated from the CH_2Cl_2-soluble extract.[2] Phakellistatin 16 (**3**), which has a divergent backbone compared with other cyclopeptides from the sponges of the order Halichondrida, is the first example from the *n*-BuOH-soluble extract of the genus *Phakellia*, and its hydrophilicity can be ascribed to the hydrophilic residues Arg, Asp, Ser, and Thr. Phakellistatin 15 (**2**) appears as one conformer in DMSO-d_6, while phakellistatins 16−18 (**3**−**5**) exist as more than one conformer in common solvents. The proline residue of phakellistatin 16 (**3**) bears *trans*- and *cis*-configurations in the major and the minor

Table 4. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Data for **5** in CD_3OH

position	δ_C, mult.	δ_H (J in Hz)	position	δ_C, mult.	δ_H (J in Hz)
Pro[1]			γ	26.75, CH_2	a: 1.43, m
CO	172.23, C				b: 1.14, m
α	62.46, CH	3.51, br d (8.4)	δ	10.72, CH_3	0.83, t (7.5)
β	32.08, CH_2	a: 1.72, m	β-Me	16.11, CH_3	0.78, d (6.8)
		b: 1.30, m	**Phe**		
γ	22.50, CH_2	a: 1.58, m	NH		8.30, d (7.8)
		b: 1.41, m	CO	173.75, C	
δ	47.21, CH_2	a: 3.43, m	α	53.31, CH	4.61, m
		b: 3.12, m	β	39.60, CH_2	a: 2.97, m
Tyr					b: 2.91, m
NH		8.67, d (8.2)	1	137.42, C	
CO	170.44, C		2, 6	130.26, CH	7.18, d (7.5)
α	54.27, CH	4.57, m	3, 5	129.74, CH	7.30, t (7.5)
β	37.37, CH_2	a: 3.03, m	4	128.20, CH	7.25, t (7.5)
		b: 2.98, m	**Pro[3]**		
1	127.27, C		CO	172.53, C	
2, 6	131.71, CH	6.78, d (8.4)	α	59.58, CH	4.48, br d (7.8)
3, 5	115.99, CH	6.62, d (8.4)	β	31.49, CH_2	a: 2.06, m
4	157.60, C				b: 1.91, m
Pro[2]			γ	22.50, CH_2	a: 2.21, m
CO	173.32, C				b: 1.83, m
α	62.72, CH	4.43, br d (8.1)	δ	48.04, CH_2	a: 3.56, m
β	32.13, CH_2	a: 2.41, m			b: 3.36, m
		b: 2.03, m	**Ile[2]**		
γ	22.89, CH_2	a: 1.93, m	NH		8.39, br s
		b: 1.64, m	CO	172.72, C	
δ	47.78, CH_2	a: 3.55, m	α	58.67, CH	4.00, br d (7.8)
		b: 3.44, m	β	37.49, CH	1.77, m
Ile[1]			γ	26.44, CH_2	a: 1.72, m
NH		9.33, br s			b: 1.33, m
CO	172.18, C		δ	11.19, CH_3	0.83, t (7.8)
α	59.91, CH	3.93, t (9.0)	β-Me	15.28, CH_3	0.96, d (7.1)
β	35.89, CH	1.90, m			

conformers, respectively, implying that the proline residue greatly affects the solvent conformations. Although conformers in solution may lead to overlapping signals in the NMR spectra disadvantageous to the structure determination, the presence of the proline residue in phakellistatins 16−18 (**3**−**5**) can facilitate the TOF/TOF sequence analysis.[16,17]

The new cyclopeptides **2**−**5** were tested for cytotoxic activity *in vitro*. Phakellistatin 15 (**2**) exhibited cytotoxicity against cancer cell line P388 with an IC_{50} value of 8.5 μM. Phakellistatin 16 (**3**) showed cytotoxicity against cancer cell lines P388 and BEL-7402 with IC_{50} values of 5.4 and 14.3 μM, respectively. Phakellistatins 17 (**4**) and 18 (**5**) showed no cytotoxicity against the cancer cell lines P388 and BEL-7402 in this assay.

Five known cyclopeptides, phakellistatin 13 (**1**),[2b] hymenistatin 1,[7] and hymenamides G, H, and J,[8b] were determined on the basis of high-resolution TOF-ESIMS, 1D- and 2D-NMR experiments including HMQC, COSY, HMBC, TOCSY, HMQC-TOCSY, and ROESY, and MALDI-TOF/TOF sequence analysis. Notably, hymenamide J existed as a mixture of two conformers in DMSO-d_6, affording complicated NMR spectra, but its sequence could be established adequately by a MALDI-TOF/TOF experiment, whereas the sequence was previously achieved by Edman degradation of its partial hydrolysates.[8b]

Experimental Section

General Experimental Procedures. Optical rotation data were recorded on a JASCO P-1030 polarimeter. Melting points were measured on a SCW X-4 melting point apparatus and were uncorrected. The NMR experiments were conducted with a Bruker AVANCE-600 instrument. High-resolution TOF-ESIMS spectra were acquired with a Waters Q-Tof micro YA019 mass spectrometer. MALDI-TOF/TOF spectra were recorded on a 4700 Proteomics analyzer (Applied Biosystems, USA). Reversed-phase HPLC was performed on a YMC-Pack Pro C18 RS column (250 × 10 mm, 5 μm) using a Waters 1525 HPLC instrument with a Waters 2998 UV detector. Column chromatography (CC) was performed on Sephadex LH-20 (Pharmacia) and YMC ODS-A (50 μm). Vacuum liquid chromatography (VLC) was performed on silica gel (200−300 mesh, Qingdao Ocean Chemical Company); the fractions were monitored by TLC (HSGF 254, Yantai, China), and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in H_2O. The enantioselective HPLC of the amino acids was conducted with a Chirex 3126 (D)-penicillamine column (Phenomenex, 150 × 4.6 mm). The commercial amino acids used for enantioselective analysis were from Sigma-Aldrich Chemical Corporation.

Animal Material. Specimens of *Phakellia fusca* were collected around Yongxing Island and seven connected islets in the South China Sea during June 2007 and were identified by Prof. Jin-He Li (Institute of Oceanology, Chinese Academy of Sciences, China). A voucher sample (No. DS-PF02) was deposited in the Laboratory of Marine Drugs, Department of Pharmacy, Changzheng Hospital, Second Military Medical University, China. The sponge *P. fusca* is of great abundance in the Xisha Islands, and therefore this collection was considered to have no significant adverse ecological effect.

Extraction and Purification. The sponge (15 kg, dry wt) was extracted with 95% EtOH, and combined EtOH extracts were concentrated under reduced pressure. This extract was suspended in H_2O and extracted in turn with EtOAc and *n*-BuOH to afford the EtOAc-soluble extract and the *n*-BuOH-soluble extract. The EtOAc-soluble extract was partitioned between MeOH−H_2O (9:1) and petroleum ether to yield a brownish-red oil (358 g). The MeOH−H_2O (9:1) phase was diluted to 3:2 with water and extracted with CH_2Cl_2 to afford the CH_2Cl_2-soluble extract (55 g). This CH_2Cl_2-soluble extract was subjected to VLC on silica gel using CH_2Cl_2−MeOH (50:1, 20:1, 15:1, 10:1, 5:1, and 2:1) as eluent to give 10 subfractions (A−J). Subfraction D was subjected to CC on Sephadex LH-20 and ODS silica and further purified by HPLC (YMC-Pack Pro C18 RS, 5 μm, 10 × 250 mm, 1.5 mL/min, UV detection at 215 and 280 nm) eluting with CH_3OH−H_2O (90:10) to yield pure peptides **5** (11.5 mg) and **1** (120 mg). Similarly, new peptides **2** (10.8 mg) and **4** (19.4 mg), together with three known peptides, hymenistatin 1(16.8 mg), hymenamide G (18.0 mg), and hymenamide H (21.5 mg), were purified from subfraction E. The *n*-BuOH-soluble extract was subjected to CC on Sephadex LH-20 to afford three subfractions (K−M). Subfraction K was subjected to CC on ODS silica and further purified by HPLC to yield pure peptides **3** (33.8 mg) and hymenamide J (35.2 mg).

Phakellistatin 15 (2, cyclo(L-Pro-L-Trp-L-Ile-L-Pro-L-Leu-L-Thr-L-Pro-L-Leu)): glassy, amorphous solid; mp 213−215 °C; $[\alpha]^{19}_D$ −167 (*c* 0.120, MeOH); NMR data, see Table 1; MALDI-TOF/TOF data, see Figure 1; HR-TOF-ESIMS *m/z* 940.5276 $[M + Na]^+$ (calcd for $C_{48}H_{71}N_9O_9Na$, 940.5272).

Phakellistatin 16 (3, cyclo(L-Pro-L-Phe-L-Asp-L-Ser-L-Arg-L-Ala-L-Val-L-Thr-L-Tyr)): glassy, amorphous solid; mp >300 °C dec; $[\alpha]^{19}_D$ −38 (*c* 0.100, MeOH); NMR data, see Table 2; MALDI-TOF/TOF data, see Figure 1; HR-TOF-ESIMS *m/z* 1059.4883 $[M + Na]^+$ (calcd for $C_{48}H_{68}N_{12}O_{14}Na$, 1059.4876) and *m/z* 1035.4917 $[M - H]^-$ (calcd for $C_{48}H_{67}N_{12}O_{14}$, 1035.4900).

Phakellistatin 17 (4, (cyclo(L-Pro-L-Trp-L-Val-L-Pro-L-Leu-L-Ile-L-Pro-L-Ile)): glassy, amorphous solid; mp 194−196 °C; $[\alpha]^{19}_D$ −126 (*c* 0.055, MeOH); NMR data, see Table 3; MALDI-TOF/TOF data, see Figure 1; HR-TOF-ESIMS *m/z* 938.5482 $[M + Na]^+$ (calcd for $C_{49}H_{73}N_9O_8Na$, 938.5480).

Phakellistatin 18 (5, cyclo(L-Pro-L-Tyr-L-Pro-L-Ile-L-Phe-L-Pro-L-Ile)): glassy, amorphous solid; mp 194−196 °C; $[\alpha]^{19}_D$ −104 (*c* 0.280, MeOH); NMR data, see Table 4; MALDI-TOF/TOF data, see Figure 1; HR-TOF-ESIMS *m/z* 850.4481 $[M + Na]^+$ (calcd for $C_{45}H_{61}N_7O_8$, 850.4479).

Hydrolyses of Phakellistatins 15−18 (2−5). Each of the peptides **2**−**5** (1.0 mg) was dissolved in 6 N HCl (1 mL) in a sealed tube and heated at 110 °C for 24 h. After cooling, the liquid was evaporated under N_2, and then the residue was dried under vacuum to yield the hydrolysates.

Phakellistatins 15−18 (2−5): Configurational Assignments. The hydrolysates of peptides **2**−**5** and authentic L- and D-amino acids were analyzed using a ligand-exchange type Chirex 3126 (D)-penicillamine column (150 × 4.6 mm, *N*,*S*-dioctyl-(D)-penicillamine complexed with Cu^{2+}) with aqueous $CuSO_4$ or $CuSO_4$−MeOH as mobile phase on a Waters 1525/2998 HPLC instrument. All amino acid residues in phakellistatins 15−18 (**2**−**5**) were revealed to correspond to the L-configuration by comparison of retention time values (t_R, min) with those of standard amino acids: (1) aqueous 2 mM $CuSO_4$−MeOH (85:15), flow rate at 1 mL/min, L-Ile (15.7), D-Ile (24.4), L-Leu (17.2), D-Leu (25.7), L-Tyr (18.0), D-Tyr (26.4); (2) aqueous 2 mM

$CuSO_4$−MeOH (70:30), flow rate at 1 mL/min, L-Phe (17.4), D-Phe (23.5), L-Trp (48.8), D-Trp (52.9); (3) aqueous 1 mM $CuSO_4$, flow rate at 1 mL/min, L-Ala (6.0), D-Ala (8.4), L-Arg (4.0), D-Arg (6.1), L-Pro (13.1), D-Pro (30.9), L-Val (19.7), D-Val (34.4); (4) aqueous 1 mM $CuSO_4$, flow rate at 0.5 mL/min, L-Asp (9.2), D-Asp (9.7); (5) aqueous 1 mM $CuSO_4$, flow rate at 0.2 mL/min, L-Ser (30.2), D-Ser (33.5); (6) aqueous 0.5 mM $CuSO_4$, flow rate at 0.5 mL/min, L-Thr (15.6), D-Thr (18.5).

MALDI-TOF/TOF Sequence Analysis of Phakellistatins 15−18 (2−5) and Known Cyclopeptides. The peptides were dissolved in 0.5 μL of matrix solution (α-cyano-4-hydroxycinnamic acid (CHCA) in 0.1% TFA, 50% ACN) before being spotted on the target plate. Samples were analyzed with a 4700 MALDI-TOF/TOF Proteomics analyzer (Applied Biosystems, USA) after air-drying. The UV laser was operated at a 200 Hz repetition rate with a wavelength of 355 nm, and the accelerated voltage operated at 20 kV. Myoglobin digested by trypsin was used to calibrate the mass instrument with internal calibration mode. All acquired spectra of samples were processed using 4700 Explore software (Applied Biosystems) in a default mode. Parent mass peaks ($[M + H]^+$) were picked out for tandem TOF/TOF analysis.

Cytotoxicity Assay. Cytotoxicity was evaluated as IC_{50} values by using the MTT assay as described previously.[20] Compounds were solubilized in DMSO, with the working concentration of test substances ranged from 1 to 100 μg/mL. Cells were inoculated into 96-well plates. After incubation for 24 h, the cells were treated with various concentrations of test substances for 48 h and then were incubated with 1 mg/mL MTT at 37 °C for 4 h, followed by solubilization in DMSO. The formazan dye product was measured by the absorbance at 570 nm on a microplate reader.

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Supporting Information Available: MALDI-TOF/FOF and NMR spectra of new peptides **2**−**5**, and NMR data tables and MALDI-TOF/FOF data for known peptides **1**, hymenistatin 1, and hymenamides G, H, and J. This material is available free of charge via the Internet at http://pubs.acs.org.

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