Isolation and Structure of the Cytotoxic Cycloheptapeptide Phakellistatin 13[⊥]

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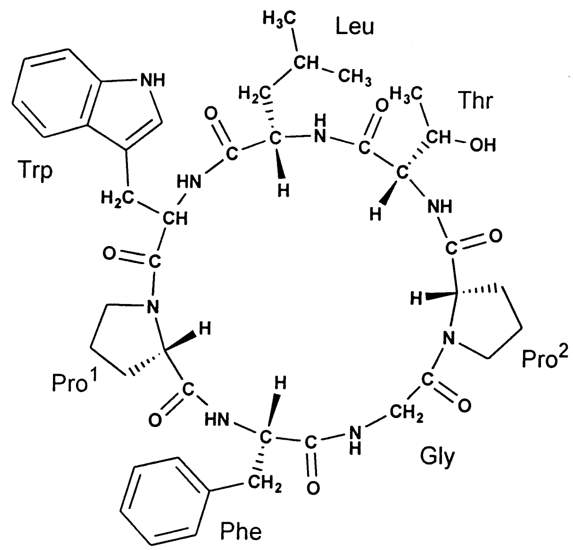
A new cyclic heptapeptide phakellistatin 13 (**1**) had been isolated from the sponge *Phakellia fusca* Thiele, collected at Yongxing Island of China. Its structure was elucidated as cyclo-(Pro[1]-Trp-Leu-Thr-Pro[2]-Gly-Phe) on the basis of MS, UV, IR, and high-field NMR (600 MHz) analysis. The compound was significantly cytotoxic against the human hepatoma BEL-7404 cell line with an $ED_{50} < 10^{-2}$ μg/mL.

Marine sponges continue to be a rich source of new secondary metabolites with unusual architecture and remarkable biological activity.[1] A number of small peptides have been described from marine sponges. They have attracted considerable attention because of their unique structures, rich physiological properties, and thus potential as important drugs.[2] Especially sponges in the genus *Theonella* (order Lithistida) have been a prolific source of structurally diverse, biologically active peptides.[2,3] Recently, phakellistatins (phakellistatins 1−11 and isophakellistatin 3), a series of cyclopeptides, have been separated from *Phakellia* sp. (class Demospongiae, order Axinellida).[4−13] All of known phakellistatins have proline units, a special amino acid, which would make the peptide chain less flexible. Phakellistatins also show medium cytotoxicity.[4−13]

When we screened for in vitro cytotoxicity of extracts from marine invertebrate animals, the crude EtOH extract from the orange sponge *P. fusca* Thiele was found to exhibit cytotoxicity against HL-60 cells ($IC_{50} = 17$ μg/mL). Bioassay-directed solvent partition of the crude EtOH extract yielded an active dichloromethane-soluble fraction. Separation of the active fraction by sequential Sephadex LH-20 permeation, silica gel column chromatographic procedures, followed by reversed HPLC (80% MeOH in H_2O as mobile phase) afforded a new heptapeptide phakellistatin 13 (1).

Phakellistatin 13 showed pseudomolecular ion peaks at *m/z* 799.6 $[M + 1]^+$, 821.7 $[M + Na]^+$, 837.5 $[M + K]^+$ in the positive ion ESIMS spectrum. The ^{13}C NMR and ^1H NMR spectrum revealed resonances consistent with seven amide carbonyls (δ 170.7, 171.7, 169.9, 172.1, 171.1, 168.3, 170.1), seven α-methine carbons (δ 60.0, 53.7, 51.7, 59.0, 61.9, 42.9, 56.6), and monosubstituted phenyl and indole ring systems, suggesting a heptapeptide with phenylalanine and tryptophan units. The seven amino acids were identified by 2D NMR techniques. Two independent spin systems of the type $X{-}CH{-}CH_2{-}CH_2{-}CH_2{-}X'$ were defined using TOCSY, DQFCOSY, and HMQC, indicating the presence of two proline units. The spin systems $X{-}CH_2{-}CH(CH_3)_2$, $X{-}CH_2{-}X'$, and $X{-}CH(OH)CH_3$ were identified,



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suggesting the existence of leucine, glycine, and threonine. The remaining two independent spin systems of the type $X{-}CH_2{-}CH{-}X'$ were attributed to phenylalanine and tryptophan by the HMBC correlations: δ 3.10 (Trp βH)/δ 127.3 (C-11), δ 2.95 (Phe βH)/δ 128.7 (C-38, C-42). The amino acid sequence of phakellistatin 13 was established as cyclo-(Pro[1]-Trp-Leu-Thr-Pro[2]-Gly-Phe) by the following NOE correlations: NH (Trp)/αH (Leu); NH (Leu)/αH, NH (Thr); NH (Thr)/αH, βH (Pro[2]); δH (Pro[2])/αH (Gly); NH (Gly)/αH, βH (Phe); NH (Phe)/αH (Pro[1]) (Figure 1). The amino acid sequence of phakellistatin 13 was further substantiated by the HMBC experiments. The two fragments Trp-Leu-Thr-Pro[2] and Gly-Phe-Pro[1] were assigned by two-bond $^1H{-}^{13}C$ correlation as follows: NH (Trp)/CO (Leu), NH (Leu)/CO (Thr), NH (Thr)/CO (Pro[2]) and NH (Gly)/CO (Phe), NH (Phe)/CO (Pro[1]) (Figure 1). The surprisingly high-field chemical shifts (δ 0.19, 0.32) of γ-H of Pro[1], due to the shielding effect of the phenyl or indole ring system of an adjacent phenylalanine or tryptophan, added further support to this sequence.

The absolute configuration was established at each chiral center by analysis of the *N*-pentafluoropropionyl isopropyl ester derivatives[14] of the acid hydrolysate by means of

[⊥] Pettit group has submitted a phakellistatin 12 manuscript for publication.

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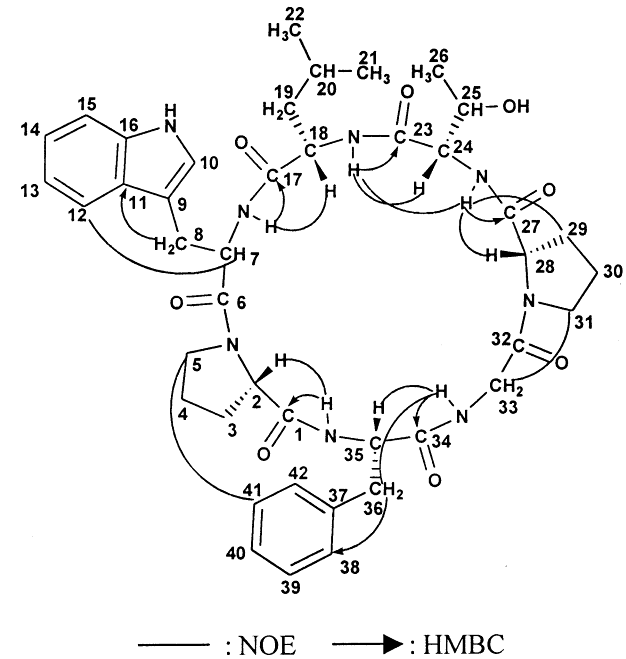

Figure 1. Selected NOE and HMBC correlations.

chiral GC chromatography (Chirasil-Val capillary column, 25 m) methods. All the leucine, threonine, proline, and phenylalanine were found to belong to the L series. However, the configuration of the tryptophan residue, which was destroyed during acid hydrolysis, remains to be determined. Phakellistatin 13 showed strong cytotoxcity against the BEL-7404 human hepatoma cell line, with an $ED_{50} < 10^{-2}$ μg/mL, but was not active against the HL-60 cell line.

Experimental Section

General Experimental Procedures. Optical rotation was measured on a Perkin-Elmer MC-241 polarimeter. Melting points were determined on a RY-2 melting point apparatus (Tianjin Analysis Instruments Manufacturer) and were uncorrected. UV was carried out on a Varian Cary 300 Bio instrument. An IR spectrum was recorded on a Perkin-Elmer 683 infrared spectrometer. The NMR experiments were conducted with a Bruker AM-600 instrument (DMSO-d_6 as solvent); chemical shift values are in ppm (δ) with TMS as internal standard. The ESIMS mass spectra were acquired with a Micromass Quattro mass spectrum instrument.

Silica gel was obtained from Qingdao Ocean Chemical Company. TLC analysis was performed on Merck TLC plates using the solvent system $CH_2Cl_2-MeOH-H_2O$ (90:10:0.5). Components' positions were visualized by 3% ceric sulfate in concentrated H_2SO_4 spray (heating to approximately 150 °C for 10 min). Sephadex LH-20 was obtained from Pharmacia. The preparative HPLC separation was performed on a Whatman Partisil-10 M-9-ODS-2 (C18) HPLC column (9.4 × 500 mm) with 80% MeOH in H_2O. The HPLC instrument was equipped with a Waters UV PDA 996 detector (set for 220 nm from the range 200−600 nm), a Waters 510 pump, and a Millenneum 2000 data station. All solvents were AR grade.

Sponge Material. The sponge *P. fusca* Thiele was collected around Yongxing Island 200 nautical miles off Hainan Province in 1998, air-dried, and identified by L. Jinhe of the Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China. A voucher specimen of the sponge employed in this study is maintained in the Shanghai Nature Museum and in our lab.

Extraction and Purification. The sponge (500 g, dry weight after extraction) was minced and extracted with 85% EtOH (4 × 2000 mL). The combined ethanol extracts were concentrated in vacuo to give 45 g of brown gum. The extract was partitioned between $MeOH-H_2O$ (9:1) and petroleum ether (3 times). The petroleum ether phase was separated and concentrated to give a dark oil (10 g). The $MeOH-H_2O$ phase was diluted 3:2 with water and extracted with CH_2Cl_2. The CH_2Cl_2 extract was concentrated, and the residue (2 g) showed a significant deforming effect [minimum morphological deformation concentration (MMDC) ≤ 100μg/mL, 5-FU as positive control with MMDC ≤ 5μg/mL] against *Pyricularia oryzae* P-2b, a type of rice plant pathogenic fungi. This bioassay method was developed by H. Kobayashi et al. for screening antimitotic and antifungal substances.[15]

Table 1. ^1H (600 MHz) and ^{13}C (150 MHz) NMR Spectral Assignments for Phakellistatin 13 in DMSO-d_6

no.	^{13}C δ	^1H δ (mult., J in Hz)	HMBC ($^1H-^{13}C$)	NOESY
Pro^{1}1	170.7			
2	60.1	3.44(t, 7.2)	C1, C3	
3	30.8	1.52(m), 1.01(m)	C2, C5	
4	28.9	0.19(m), 0.32(m)	C2, C3	
5	45.7	2.91(t, 9.3), 2.37(t, 8.4)		H12, H41, H42
Trp 6	171.7			
7	53.7	4.43(m)	C6	H12, H18
8	26.8	3.10(m)	C11	
9	108.9			
10	124.2	7.32(s)	C8, C11	
NH		10.79(s)		
11	127.3		C16	
12	118.3	7.34(d, 9)	C8, C16	
13	118.5	6.91(d, 7.5)		
14	121.4	7.04(d, 7.5)	C11, C12	
15	111.5	7.34(d, 9)	C11, C12, C13	
16	136.2			
NH		9.33(d, 3)	C17	H18
Leu 17	169.9			
18	51.7	4.48(m)		
19	40.5	1.70(m), 1.50(m)	C18	
20	24.5	1.63(m)		
21	22.1	1.00(d, 6.6)	C19, C22	
22	23.7	0.86(d, 6.6)	C19, C21	
NH		7.13(d, 6.0)	C23	H24
Thr 23	172.1			
24	59.0	4.25(m)	C26	
25	65.7	4.23(m)	C24	
26	20. 7	1.03(d, 6)	C24, C25	
OH		4.87(d, 5.4)		
NH		7.85(d, 9)	C27	H28, H29, H30, H31
Pro^2 27	171.1			
28	61.9	4.26(m)	C29	
29	24.5	1.82(m), 2.27(m)	C30, C31	
30	30.1	1.94(m)	C28	
31	46.3	3.69(m), 3.51(m)	C29, C30	H33
Gly 32	168.3			
33	42.9	4.16(d, 18), 3.94(d, 18)	C32	
NH		7.55(s)	C34	H18, H31, H35, H36
Phe 34	170.1			
35	56.6	4.15(m)	C34	
36	37.3	3.16(t, 12.6), 2.95(t, 12.6)	C38, C42	
37	137.8			
38, 42	128.7	7.10(d, 6.6)	C39, C41, C40	
39, 41	128.5	7.24(t, 7)	C37,C41	
40	126.7	7.19(t, 6.8)		
NH		8.29(d, 6.6)	C1	H2, H7, H33

For the bioassay-guided separation by *P. oryzae* P-2b, the CH_2Cl_2-soluble fraction was chromatographed first on a Sephadex LH-20 column, eluting with $MeOH-CH_2Cl_2$ (2:3) and then $MeOH-CH_2Cl_2-n$-heptane (3:5:1). Eluted fractions were concentrated and tested. A 30 mg active fraction was further separated by silica gel chromatography with $MeOH-CH_2Cl_2$ (0%−10%) step gradient elution to afford an active fraction (3.9 mg), showing one spot by TLC detection; however, it was not pure enough for NMR experiments. Final purification was achieved by preparative HPLC with 80% MeOH in H_2O to afford phakellistatin 13 (3 mg, 6×10^{-4} % of dry specimen wt) as a glassy amorphous solid.

Phakellistatin 13 (1): glassy amorphous solid: mp 198−200 °C; $[\alpha]^{25}_{D}$ −136° (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ϵ) 214 (4.34), 240 (3.50), 283 (3.47) nm; IR (KBr film) ν_{max} 3435, 2960, 2886, 1658, 1629, 1521 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) and ^{13}C NMR (DMSO-d_6, 150 MHz), see Table 1; ESIMS *m*/*z* 827 $[M + K]^+$, 821 $[M + Na]^+$, 799 $[M + H]^+$; HRFABMS *m*/*z* 799.4121 (calcd for $C_{42}H_{55}N_8O_8$, 799.4143).

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