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ANTINEOPLASTIC AGENTS, 325.[1] ISOLATION AND STRUCTURE OF THE HUMAN CANCER CELL GROWTH INHIBITORY CYCLIC OCTAPEPTIDES PHAKELLISTATIN 10 AND 11 FROM *PHAKELLIA* SP.

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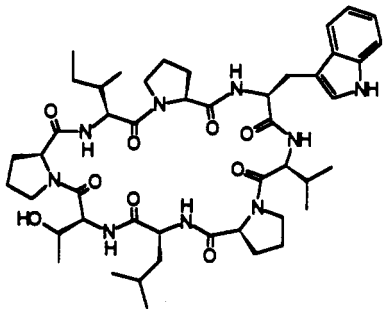
ABSTRACT.—The two new marine sponge (*Phakellia* sp., western Pacific Ocean) constituents, phakellistatin 10 [**1**] and 11 [**2**], were found to be cyclic octapeptides that significantly inhibited growth of the murine P-388 lymphocytic leukemia (ED_{50} values of 2.1 and 0.20 μg/ml, respectively) and human cancer cell lines. The structures were established based on results of extensive tandem ms/ms and high-field (500-MHz) 2D ^{1}H- and ^{13}C-nmr analyses. All of the amino acid units (except Trp, not determined) were found to correspond to the (*S*)-configuration.

Because certain peptides can profoundly influence control of cell proliferation and differentation, it appears likely that anticancer drugs based on naturally occurring peptides will become increasingly important (2,3). Such approaches to improving human cancer treatment are already well under way (1,4,5), especially with modified human peptide hormones (6–9). In 1990 we reported the discovery of hymenistatin 1, the first cell-growth-inhibitory (murine P-388 lymphocytic leukemia) cyclic octapeptide from a marine sponge (*Stylotella aurantium*) (10). Recently, Kobayashi and colleagues described four new cyclic octapeptides from the Okinawan marine sponge *Hymeniacidon* sp., of which two (hymenamides H and J, ED_{50} values of 6.3 and 2.6 μg/ml, respectively) were found to be active against the murine L-1210 lymphocytic leukemia cell line (11). We report herein the isolation and structural elucidation of two new Porifera cyclic octapeptides designated phakellistatins 10 [**1**] and 11 [**2**] with significant activity against the P-388 leukemia cell line (ED_{50} values of 2.1 and 0.20 μg/ml, respectively).

In 1986–87 the yellow-orange sponge *Phakellia* sp. (class Demospongiae, order Axinellida) was collected (500 kg wet wt) at depths of 25 to 40 m in the Federated States of Micronesia (Chuuk). An initial MeOH/sea water extract was concentrated and separated by a solvent partition and gel permeation (Sephadex LH-20) sequence (12). Further separation (P-388 bioassay-guided) was accomplished using partition cc (Sephadex LH-20) employing, successively, hexane-CH_2Cl_2-MeOH (5:5:1), hexane-toluene-MeOH (3:1:1), and hexane-*i*-PrOH-MeOH (8:1:1) as eluents. Final separation was realized by reversed-phase hplc [C_8 column, MeOH-MeCN-H_2O (3:3:4) and MeCN-H_2O (2:3) as eluents]. Phakellistatin 10 (**1**,

[1]For part 324 of this series, see Pettit *et al.* (1).



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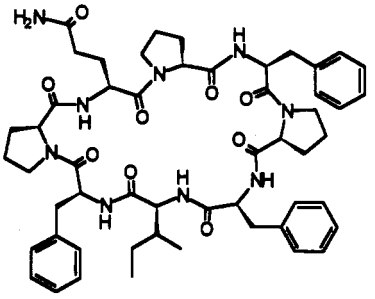

2

14 mg, yield 2.8×10^{-6}%) was obtained as a colorless amorphous powder from MeOH, mp 217–219°; $[\alpha]^{25}D$ −128° (c=0.19, CH_3OH). Phakellistatin 11 (**2**, 34 mg, yield 6.8×10^{-6}%) was also isolated as a colorless amorphous solid, mp 194–196°; $[\alpha]^{25}D$ −163° (c=0.08, CH_3OH).

The high-field (500-MHz) 2D ^{1}H-nmr, APT, HMQC, and ^{13}C-nmr spectra (in CD_2Cl_2, Table 1) of phakellistatin 10 [**1**] indicated the presence of seven CH_3, twelve CH_2, three C-CH-C, and nine C-CH-X groups, as well as eight aromatic carbons and four separated carbonyl signals, which suggested that **1** was a peptide with a tryptophan unit. Extensive application of ^{1}H,^{1}H COSY and HMBC nmr techniques were used for further structure determination. A low-field methyl signal (δ 1.10,d,J=6.0 Hz) was attributed to a threonine unit, two methyls (δ 0.45, d, J=4.5 Hz; δ 0.80, t, J=8.0 Hz) to an isoleucine unit, the methyls at δ 0.76 (d,J=6.5 Hz) and 0.87 (d,J=6.0 Hz) to valine, and two at δ 0.79 (d,J=7.0 Hz) and 0.87 (d,J=6.0 Hz) to leucine. Three -CH_2-CH_2-CH_2- chains corresponding to proline were uncovered. The existence of tryptophan was recognized by a moderate absorption at 281 nm in the uv spectrum.

High-resolution fab mass measurements of the protonated molecular ion of **1** established an exact mass of m/z 904.5271 corresponding to a molecular formula of $C_{47}H_{70}N_9O_9$ (calcd m/z 904.5297) and indicated a cyclic peptide structure. Tandem mass spectrometry of the $(M+H)^+$ ion of m/z 904 produced a number of lower mass fragment ions which were the immonium ions of the amino acids present in the peptide. The presence of these ions of m/z 70 (Pro), 72 (Val), 74 (Thr), 86 (Leu or Ile), and 159 (Try), when combined with the exact mass and nmr information, established the amino acid content as 3×Pro, 2×Leu or Ile, Val, Thr, and Tyr. Protonation at each of the three proline residues followed by ring opening produced three different acylium ions. The position of the Leu and Ile residues was established by the presence of low abundance ions at m/z 466 and 523 that involve fragmentation of the amino acid side-chain. The acylium ions, formed by fragmentation at the amide bonds, can undergo further decomposition to eliminate CO. Such fragment ions that have either Leu or Ile at the C-terminus can undergo further degradation to eliminate C_3H_6 (−42 atomic mass units) in the case of Leu or C_2H_4 (−28 atomic mass units) in the case of Ile. The amino acid sequence of **1** was determined to be *cyclo*-(Pro-Leu-Thr-Pro-Ile-Pro-Tyr-Val).

Structure elucidation of phakellistatin 11 [**2**] was also achieved by 2D nmr and hrfabms analyses. The high-resolution fabms (m/z 974.5135 $[M+H]^+$, Δ +0.5 ppm) established the molecular formula as $C_{53}H_{67}N_9O_9$ (calcd 974.5140). The ^{1}H-nmr spectrum of **2** in DMSO-d_6 (Table 2) showed signals between 7.46 ppm and 8.72 ppm (amide protons which were not visible in MeOH-d_4) suggesting a peptide. Amino acid analysis showed the presence of Pro (3×), Phe (3×), Ile, and Gln units. The twenty-five sites of unsaturation required by the molecular formula and a negative reaction to the ninhydrin-collidine reagent indicated a cyclic peptide.

TABLE 1. ^{1}H- and ^{13}C-Nmr Data of Phakellistatin 10 [**1**] Recorded in CD_2Cl_2.[a]

Carbon	^{13}C	HMQC (500 MHz)	HMBC (500 MHz)
isoleucine			
CO	171.76 p		H-α
NH		8.15 (1H, br)	
α-CH	55.80 n	4.08 (1H, t, J=9 Hz)	H-γ1a, H-γ2
β-CH	35.76 n	1.80 (1H, m)	H-α, H-γ1a, H-γ1β, H-γ2, H-δ
γ1-CH_2	25.66 p	1.46 (1H, m) 1.00 (1H, m)	H-α, H-γ2
γ2-CH_3	15.10 n	0.45 (3H, d, J=4.5 Hz)	H-γ1a, H-γ1b
δ-CH_3	10.65 n	0.80 (3H, t, J=8.0 Hz)	H-γ1a, H-γ1b
leucine			
CO	170.68 p		
NH		4.56 (1H, br)[b]	
α-CH	54.81 n	3.32 (1H, m)	H-βa
β-CH_2	36.40 p	2.16 (1H, m) 1.72 (1H, m)	H-δ1, H-δ2
γ-CH	25.48 n	1.37 (1H, m)	H-δ1, H-δ2
δ1-CH_3	23.52 n	0.87 (3H, d, J=6 Hz)	H-δ2
δ2-CH_3	21.12 n	0.79 (3H, d, J=7 Hz)	H-βa, H-δ1
threonine			
CO	170.68 p		H-α
NH		7.47 (1H, d, J=9 Hz)	
α-CH	57.44 n	5.04 (1H, dd, J=9, 3.5 Hz)	H-γ
β-CH	68.97 n	4.18 (1H, dq, J=9, 6.5 Hz)	
γ-CH_3	19.37 n	1.10 (3H, d, J=6.0 Hz)	
OH		5.14 (1H, br)[b]	
valine			
CO	171.03 p		H-α
NH		7.73 (1H, br)	
α-CH	55.22 n	4.69 (1H, t, J=10 Hz)	H-γ1, H-γ2
β-CH	29.44 n	2.15 (1H, m)	H-α, H-γ1, H-γ2
γ1-CH_3	19.57 n	0.87 (3H, d, J=6.0 Hz)	H-γ2
γ2-CH_3	18.83 n	0.76 (3H, d, J=6.5 Hz)	H-α, H-γ1
tryptophan			
CO	170.68 p[b]		
NH		9.06 (1H, br)[b]	
α-CH	54.81 n	4.58 (1H, m)	H-βa
β-CH_2	25.75 p	3.61 (1H, m)	
		3.38 (1H, dd, J=15, 3.0 Hz)	H-α
1-NH		6.45 (1H, br s)	
2-CH	123.80 n	7.04 (1H, s)	H-βb, H-1
3-C	111.35 p		H-βa, H-2
4-C	127.98 p		H-βa, H-2, H-6, H-8
5-CH	118.75 n	7.56 (1H, d, J=7.5 Hz)	H-7
6-CH	119.69 n	7.09 (1H, t, J=7.5 Hz)	H-8
7-CH	122.33 n	7.16 (1H, t, J=7.5 Hz)	H-5
8-CH	112.14 n	7.39 (1H, d, J=8.0 Hz)	H-6
9-C	136.86 p		H-2, H-5, H-7
proline[1]			
CO	171.03 p		H-β
α-CH	61.49 n	3.65 (1H, m)	
β-CH_2	30.47 p	1.80 (2H, m)	H-γ
γ-CH_2	25.27 p	2.04 (2H, m)	H-β, H-δb
δ-CH_2	49.04 p	3.97 (1H, t, J=4.5 Hz)	
		3.64 (1H, m)	
proline[2]			
CO	171.03 p[b]		
α-CH	63.02 n	4.08 (1H, t, J=9 Hz)	H-γb
β-CH_2	30.06 p	2.12 (2H, m)	H-δa
γ-CH_2	30.47 p	2.14 (1H, m) 1.80 (1H, m)	
δ-CH_2	48.33 p	4.08 (1H, m) 3.33 (1H, m)	
proline[3]			
CO	172.18 p		H-βa, H-βb
α-CH	60.56 n	4.63 (1H, dd, J=9, 6 Hz)	H-βa, H-βb, H-γ, H-δa, H-δb
β-CH_2	29.42 p	2.23 (1H, m) 2.04 (1H, m)	H-γ, H-δa, H-δb
γ-CH_2	25.52 p	1.92 (2H, m)	H-βa, H-βb, H-δa, H-δb
δ-CH_2	48.24 p	3.74 (1H, m) 3.59 (1H, m)	H-βa, H-βb, H-γ

[a]TMS was used as internal standard. The n and p notations correspond to APT results in which n indicates CH or CH_3 and p indicates C or CH_2.

[b]Signals may be interchangeable.

The amino acid sequence of cyclic peptide **2** was first determined by HMBC and ROESY nmr experiments. The -Pro[1]-Gln[2]-Pro[3]-Phe[4]-Pro[5]- segment was deduced by correlations between NH (Gln[2])/CO(Pro[1]) and NH (Phe[4])/CO(Pro[3]) in the HMBC spectrum and by nOe connection between H-2 (Pro[3]) and α-H (Gln[2]) in the ROESY spectrum (in DMSO-d_6). The chemical shift difference ($\Delta\delta$ 4.11 ppm) between the β- and γ-carbons in Pro[3] indicated a *trans* Gln[2]-Pro[3] amide bond. The -Phe[6]-Ile[7]-Phe[8] sequence was suggested by HMBC cross-peaks between NH (Ile[7])/CO(Phe[6]), α-H(Ile[7])/CO(Phe[6]) and NH(Phe[8])/CO(Ile[7]) in DMSO-d_6. In addition, the HMBC spectrum in CD_3CN showed a cross signal for NH (Phe[6]) and CO(Pro[5]). Thus, the structure of phakellistatin 11 was determined to be *cyclo*-(Pro-Gln-Pro-Phe-Pro-Phe-Ile-Phe). When combined with the data on immonium ions produced in tandem mass spectrometry experiments, the amino acid content established by nmr was confirmed. Furthermore, upon collisional activation, the $(M+H)^+$ ions of **2** fragment by a route that confirmed the amino acid sequence to be *cyclo*-(Pro-Gln-Pro-Phe-Pro-Phe-Ile-Phe).

The absolute configurations of both peptides were elucidated by chiral gc analysis (13,14) of the perfluoropropionyl isopropyl ester derivatives of the peptide hydrolysates. Except for tryptophan (which decomposed during the acid hydrolysis) all of the amino acids were found to possess the (*S*)-configuration. With the Ile units, we have assumed the usual stereochemistry at C-3 rather than that of *allo*-Ile.

Cyclic peptides **1** and **2** were tested (10^{-5} M high test concentration; $\log_{10}$ dilutions) in the NCI's 60-cell line human tumor in vitro screen (15–17) and a variety of data

TABLE 2. ^{1}H- and ^{13}C-Nmr Data for Phakellistatin 11 [**2**].[a]

Carbon	^{13}C Nmr	^{1}H Nmr	Carbon	^{13}C Nmr	^{1}H Nmr
pro[1]			pro[5]		
CO	171.16 (s)		CO	169.53 (s)	
α-CH	59.79 (d)	2.85 (1H)	α-CH	59.67 (d)	2.86 (1H)
β-CH_2	30.08 (t)	0.94 (1H), 1.81 (1H)	β-CH_2	29.37 (t)	0.71 (1H, m), 1.65 (1H)
γ-CH_2	21.54 (t)	1.35 (1H), 1.58 (1H)	γ-CH_2	20.81 (t)	0.50 (1H, m), 1.32 (1H)
δ-CH_2	46.21 (t)	3.30 (2H)	δ-CH_2	45.85 (t)	2.87 (1H), 3.12 (1H)
gln[2]			phe[6]		
NH		8.67 (1H, d, J=8.1 Hz)	NH		8.72 (1H, d, J=8.8)
CO	168.49 (s)		CO	168.14 (s)	
α-CH	51.23 (d)	4.16 (1H, br t, J=11.2 Hz)	α-CH	53.64 (d)	4.23 (1H, ddd, J=3.3, 8.8, 12.1 Hz)
β-CH_2	24.76 (t)	1.88 (1H), 1.94 (1H)	β-CH_2	35.43 (t)	2.92 (1H, t, J=12.1 Hz)
γ-CH_2	30.82 (t)	2.00 (1H), 2.10 (1H)	1-C	139.68 (s)	
γ-CO	173.09 (s)		2,6-CH	128.59 (d)	7.24 (2H)
NH_2		6.62 (1H, br s), 7.20 (1H)	3,5-CH	127.57 (d)	7.19 (2H)
			4-CH	125.55 (d)	7.10 (1H)
pro[3]			ile[7]		
CO	172.13 (s)		NH		7.46 (1H, d, J=8.8 Hz)
α-CH	57.84 (d)	4.36 (1H)	CO	171.45 (s)	
β-CH_2	28.87 (t)	1.91 (1H), 2.13 (1H)	α-CH	56.61 (d)	4.07 (1H, d, J=7.7 Hz)
γ-CH_2	24.76 (t)	1.83 (1H), 1.91 (1H)	β-CH	36.09 (d)	1.65 (1H)
δ-CH_2	46.45 (t)	3.46 (td, J=4.2, 10.1 Hz)	β-CH_3	14.89 (q)	1.07 (3H, d, J=6.7 Hz)
		3.66 (td, J=4.3, 10.1 Hz)	γ-CH_2	25.13 (t)	1.17 (1H), 1.57 (1H)
			δ-CH_3	10.82 (q)	0.88 (3H, t, J=7.2 Hz)
phe[4]					
NH		8.45 (1H)			
CO	169.53 (s)		phe[8]		
α-CH	53.92 (d)	4.37 (1H)	NH		8.43 (1H)
β-CH_2	37.25 (t)	2.85 (1H)	CO	169.58 (s)	
		3.07 (dd, J=4.4, 12.5 Hz)	α-CH	53.92 (d)	4.38 (1H)
1-C	135.99 (s)		β-CH_2	37.55 (t)	2.84 (1H), 3.14 (1H)
2,6-CH	129.25 (d)	7.17–7.20 (2H)	1-C	135.99 (s)	
3,5-CH	128.59 (d)	7.20–7.36 (2H)	2,6-CH	129.25 (d)	7.17–7.20 (2H)
4-CH	127.09 (d)	7.29–7.33 (1H)	3,5-CH	128.59 (d)	7.20–7.36 (2H)
			4-CH	127.09 (d)	7.29–7.33 (1H)

[a] ^{1}H nmr 400 MHz, ^{13}C nmr 100.6 MHz. TMS was used as internal standard.
[b] Recorded in DMSO.

analyses (16,18) were performed. Phakellistatins 10 and 11 (**1** and **2**) gave overall panel-averaged GI_{50} concentrations of $7.90\pm2.63 \times 10^{-7}$ M, and $1.32\pm0.49\times10^{-7}$ M, respectively. TGI-COMPARE correlation analyses (18) of the differential cytotoxicity profile of cyclic octapeptide **1** showed Pearson correlation coefficients of 0.81, 0.96, and 0.77 with the profiles of phakellistatin 4 (5), phakellistatin 11 [**2**], and the standard agent vinblastine, respectively. Likewise, similar analyses of the profile of cyclic octapeptide **2** showed Pearson correlation coefficients of 0.83, 0.96, and 0.87 with the profiles of phakellistatin 4, phakellistatin 10 [**1**] and vinblastine, respectively.

Interestingly, **1** is the Pro-Ile-Pro counterpart of the hymenamide Pro-Leu-Pro sequence (11). Hymenistatin 1 bears the same (Ile vs. Leu) relationship to hymenamide G (11). In turn this suggests that these cell growth-inhibitory cyclic peptides have a common microorganism genesis or are growth regulatory/defensive substances produced by closely related marine Porifera. Assessment of the phakellistatins as potentially useful antineoplastic agents is in progress.

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