Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 13, pp. 1339-1344, 1995
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0960-894X/95 $9.50+0.00

0960-894X(95)00219-7

ISOLATION AND STRUCTURE OF THE HUMAN CANCER CELL GROWTH INHIBITORY CYCLIC DECAPEPTIDES PHAKELLISTATINS 7, 8 AND 9[1,2]

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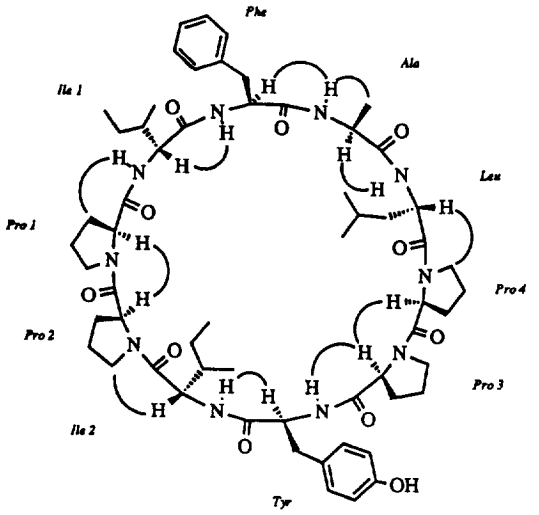
Abstract: Phakellistatins 7-9 respresent the first cancer cell growth inhibitory (P388 ED_{50} 3.0, 2.9 and 4.1 μg/mL respectively) cyclic decapeptides. Each was isolated from the Federated States of Micronesia (Chuuk) marine sponge *Phakellia costata.*

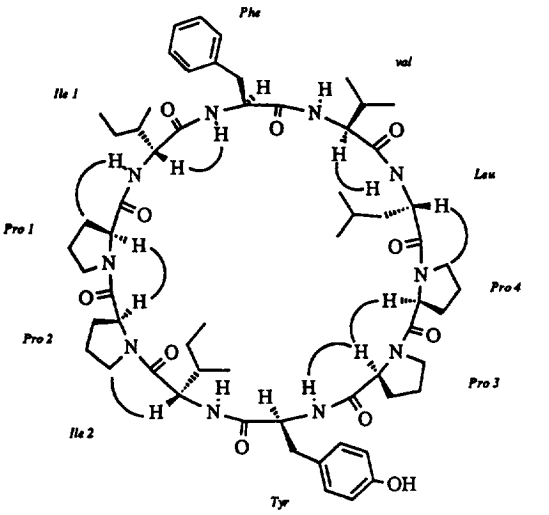
Animals, plants and microorganisms constitute a vast reservoir of peptides with potentially important medicinal properties that range from the partially understood higher animal hormones to the lower organism peptides of generally unknown natural function. Among the latter categories are the diverse series of marine shell-less mollusc antineoplastic[3-5] and cytotoxic[6-7] dolastatins, the cytotoxic marine ascidian peptides,[8] the Australian frog antimicrobal and antiviral coerin-type cyclic peptides,[9] higher plant *(Aster tatoricus,* Compositae) antineoplastic cyclic pentapeptides,[10] fungal antibiotics[11] and the streptomyces antineoplastic antibiotic himastatin.[12] Clearly, discovery and biological evaluation of such valuable naturally occurring peptides is only in a very early phase.[13] We herein report discovery of the first three cancer cell growth inhibitory cyclic decapeptides designated phakellistatins 7, 8 and 9 from a marine sponge and to our knowledge from any marine animal.

Earlier we summarized the isolation and structural elucidation of the cancer cell growth inhibitory cyclic heptapeptide phakellistatins 4-6[1] from the Western Pacific (Chunk, Micronesia) marine sponge *Phakellia costata* (class Demospongia). When the 1987 collection (500 kg wet wt.) of *P. costata* murine P388 lymphocytic leukemia (PS system) active fractions were examined further for trace PS cell line active components we discovered three cyclic decapeptides with significant cancer cell growth inhibitory properties.

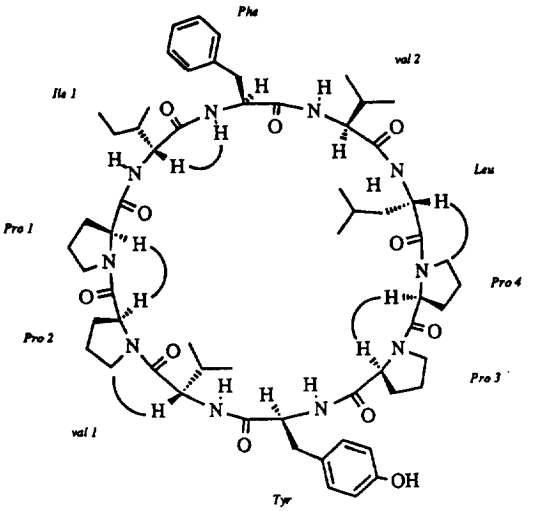
The bioactive dichloromethane soluble fraction prepared from *P. costata* and used to obtain phakellistatins 1 and 4-6 by a solvent partitioning, gel permeation (Sephadex LH-20) and partition chromatographic (LH-20) sequence[1] led to a trace PS active fraction that was further separated (PS bioassay). Continued column chromatographic (partition solvent system) separations on Sephadex LH-20 and final separation by reversed phase HPLC (Phenomenex C8 column and elution with CH_3CN-CH_3OH-H_2O, 10:10:13) afforded phakellistatin 7 (**1**, 6.5 mg, 1.3 x 10^{-6}%), 8 (**2**, 69.8 mg, 1.4 x 10^{-5}%) and 9 (**3**, 7.2 mg, 1.4 x 10^{-6}%).



Structure of Phakellistatin 7 (1) and NOE correlations



Structure of Phakellistatin 8 (2) and NOE correlations



Structure of Phakellistatin 9 (3) and NOE correlations

Phakellistatin 7 (**1**), colorless amorphous powder, mp 192-195°C, $[\alpha]_D$ -106° (c=0.22, CH_3OH), exhibited a pseudomolecular ion peak in the HRFABMS spectrum at m/z 1109.6401 $[M+H]^+$ corresponding to molecular formula $C_{59}H_{84}N_{10}O_{11}$, (Δ + 0.1 mmu). Although interpretation of the high field (500 MHz) ^{1}H-NMR spectra from phakellistatin 7 showed only one conformational form in deuteromethanol solution (Table 1), in deuterochloroform or deuteroacetonitrile at ambient temperatures a complex mixture of conformers was observed. Interpretation of the high-field (500 MHz) 2D-NMR spectra (COSY, HMQC and HMBC) of phakellistatin 7 (**1**) indicated the presence of four Pro, two Ile, and one each of Leu, Phe, Tyr and Ala units (Table 1). The cyclodecapeptide amino acid sequence was established as *cyclo*-(Pro-Pro-Ile-Phe-Ala-Leu-Pro-Pro-Tyr-Ile) by tandem mass spectral studies. Protonation of the four proline residues by FAB mass spectrometry produced four series of fragmentations which were interpreted by MS/MS methods:

m/z:		195	308	455	526	639	736	833	996		
	Pro	Pro	Ile	Phe	Ala	Leu	Pro	Pro	Tyr	Ile	
	m/z:		211	358	429	542	639	736	899	1012	
		Pro	Ile	Phe	Ala	Leu	Pro	Pro	Tyr	Ile	Pro
m/z:		195	358	471	568	665	778	925	996		
	Pro	Pro	Tyr	Ile	Pro	Pro	Ile	Phe	Ala	Leu	
	m/z:		261	374	471	568	681	828	899	1012	
		Pro	Tyr	Ile	Pro	Pro	Ile	Phe	Ala	Leu	Pro

The amino acid sequence of decapeptide **1** was further substantiated by the following NOE correlations (in CD_3OH): NH(Phe)/αH(Ile^1), NH(Ile^1)/βH(Pro^1), αH(Pro^1)/αH(Pro^2), δH(Pro^2)/αH(Ile^2), NH(Ile^2)/αH(Tyr), NH(Tyr)/αH(Pro^3), αH(Pro^3)/αH(Pro^4), δH(Pro^4)/αH(Leu), NH(Leu)/αH(Ala) and NH(Ala)/αH(Phe). Thus, structure **1** was assigned to phakellistatin 7.

Phakellistatin 8 (**2**), colorless crystals from methanol-water, mp 188-191°C, $[\alpha]_D$, -112° (c=0.16, CH_3OH), exhibited a HRFABMS pseudomolecular ion peak at m/z 1137.6714 $[M+H]^+$ leading to molecular formula $C_{61}H_{88}N_{10}O_{11}$, (Δ + 0. 1 mmu). Peptide **2** appeared essentially as one conformer in deuteriochloroform. The presence of Pro, Ile, Leu, Val, Phe and Tyr units in a ratio 4:2: 1: 1: 1: 1 was confirmed by detailed analyses of 2D-nmr spectra (COSY, HMQC and HMBC). The ROESY spectrum of decapeptide **2** provided NOE correlations between NH(Phe)/αH(Ile^1), NH(Ile^1)/βH(Pro^1), αH(Pro^1)/αH(Pro^2), δH(Pro^2)/αH(Ile^2), NH(Tyr)/αH(Pro^3), αH(Pro^3)/αH(Pro^4), δH(Pro^4)/αH(Leu) and NH(Leu)/αH(Val), which inferred the presence of Ile-Pro-Pro-Ile-Phe and Val-Leu-Pro-Pro-Tyr segments. Unsaturation calculations for the molecular formula pointed to a cyclic peptide. While the complete peptide sequence was not unequivocally solved using the available nmr data, the MS/MS analyses did provide additional sequence information and led to the *cyclo*-(Pro-Pro-Ile-Phe-Val-Leu-Pro-Pro-Tyr-Ile) structural assignment for phakellistatin 8 (**2**).

Phakellistatin 9 (**3**), colorless amorphous powder, mp 184-188°C, $[\alpha]_D$, -113° (c=0.68, CH_3OH), exhibited a pseudomolecular ion peak in the FAB-MS at m/z 1123. The molecular formula was determined to be $C_{60}H_{86}N_{10}O_{11}$ by HRFABMS (m/z 1123.6553 $[M+H]^+$, Δ-0.2 mmu). Peptide **3** also appeared as one conformer in deuteromethanol, but not deuterochloroform or deuteroacetonitrile. The high field (500 MHz) ^{1}H-

NMR and APT data in combination with results of extensive COSY, HMQC and HMBC experiments provided evidence that phakellistatin 9 (**3**) was another decapeptide comprised of Pro(x4), Val(x2), Ile, Leu, Phe and Tyr. NOE correlations were found between $\alpha H(Pro^1)/\alpha H(Pro^2)$, $\alpha H(Val^1)/\delta H(Pro^2)$, $\alpha H(Pro^3)/\alpha H(Pro^4)$ and $\alpha H(Leu)/\delta H(Pro^4)$. A tandem MS/MS study (see above for **1**) of peptide **3** gave definitive sequence information. Results of four series of FABMS fragmentations arising from protonation of the four Pro units were indicative of a cyclic peptide structure.

Other internal fragment ions corresponded to the following segments; $[M+H\text{-}Pro\text{-}Pro\text{-}Ile]^+$ at m/z 816, $[M+H\text{-}Pro\text{-}Ile\text{-}Phe]^+$ at m/z 766 and $[M+H\text{-}Pro\text{-}Ile\text{-}Phe\text{-}Val]^+$ at m/z 570. Further analysis of the MS/MS and nmr data completed the structual elucidation of phakellistatin 9 (**3**) as *cyclo*-(Pro-Pro-Ile-Phe-Val-Leu-Pro-Pro-Tyr-Val).

The chirality of the amino acid units of phakellistatins 7 (**1**), 8 (**2**) and 9 (**3**) was determined by GC analysis of *N*-pentafluoropropyl isopropyl ester derivatives of the respective propionic acid-hydrochloric acid hydrolysates.[14,15] All of the amino acids proved to have the (S)-configuration. Furthermore, cross peaks for $\alpha H(Pro^1)/\alpha H(Pro^2)$ and $\alpha H(Pro^3)/\alpha H(Pro^4)$ were observed in all ROESY spectra recorded for phakellistatins 7 (**1**), 8 (**2**) and 9 (**3**) indicating all have *cis*-amide bonds at $CO(Pro^2)/NH(Pro^1)$ and $CO(Pro^4)/NH(Pro^3)$. Additional evidence supporting the *cis* geometries were obtained by the ^{13}C chemical shifts differences ($\Delta\delta\beta\gamma$ 8.19 ~ 9.28 ppm) of the β and γ carbons of Pro^1 and Pro^3 units (Table 1).[16,17]

Interestingly, phakellistatins 7 (**1**), 8 (**2**) and 9 (**3**) obtained from the same source are not only structurally similar, but they also display quite comparable cytotoxicities against the P388 cell *in vitro* (ED_{50} 3.0, 2.9 and 4.1 μg/ml respectively). Against the U. S. National Cancer Institute panel of sixty human cancer cell lines,[18-21] they showed greater differences in overall potency. Phakellistatin 7 (**1**) gave an overall panel-averaged GI_{50} concentration of $5.50 \pm 2.46 \times 10^{-7}$M. In contrast, phakellistatins 8 (**2**) and 9 (**3**) were considerably less potent (mean panel GI_{50}'s for both were $>10^{-5}$M). A TGI-COMPARE correlation analysis[21] of the differential cytotoxicity profile of **1** showed Pearson correlation coefficients of 0.83 with that of phakellistatin 4,[22] and 0.84 and 0.80 with those of standard agents[21] vinblastine and taxol, respectively. The latter correlations suggest that **1** and perhaps all of the other known phakellistatins should be examined in greater detail for possible interactions with cellular tubulin.[21]

Acknowledgment. The research reported herein was made possible by the following financial assistance: Outstanding Investigator Grant CA44344-01Al-06 awarded by the Division of Cancer Treatment, National Cancer Institute, DHHS: the Fannie E. Rippel Foundation, the Arizona Disease Control Research Commission, the Robert B. Dalton Endowment Fund, Virginia Piper, Diane Cummings (The Nathan Cummings Foundation, Inc.), Gary L. Tooker, Polly J. Trautman, and the Eagles Art Ehrmann Cancer Fund. For other very helpful assistance we thank the Federated States of Micronesia (Chunk, D. E. Aten, R. Killion, and A. Amaraich) and Drs. Matthew Suffness, Cherry L. Herald, Zbigniew A. Cichacz, Dennis L. Doubek, Fiona Hogan, Mr. Larry P.

Table 1. The ^{1}H- and ^{13}C-nmr Spectral Assignments for Phakellistatin 7 (1) in CD_3OD or CD_3OH.

	No.	*^{13}C ppm*	*^{1}H ppm*	*HMBC (H to C)*	*nOe*		*No.*	*^{13}C ppm*	*^{1}H ppm*	*HBMC (H to C)*	*nOe*
Ile	1	172.85 s					31	132.15 d	6.97 d	25, 30	
-1	2	58.03 d	4.24 m		Phe-NH		OH		9.02 brs		
	3	34.06 d	2.14 m				NH		9.23 brs		33
	4	26.04 t	1.49 m	5, 6		*Pro*	32	172.85 s			
			1.18 m	5, 6		*-3*	33	62.47 d	4.20 m	32, 34	38
	5	10.23 q	0.83 t	3, 6			34	31.86 t	2.48 m	36	
	6	17.28 q	0.91 d	2, 3, 4					2.05 m	33, 35	
	NH		8.73 brs		9		35	23.35 t	1.70 m		
Pro	7	173.64 s							1.97 m		
-1	8	62.80 d	4.32 m	7, 9, 11	13		36	47.99 t	3.51 m		
	9	32.52 t	2.24 m	7, 8, 10	Ile 1-NH				3.53 m		
			2.50 m	10, 11		*Pro*	37	172.47 s			
	10	23.24 t	1.70 m			*-4*	38	59.98 d	3.24 m		33
			1.97 m				39	29.79 t	1.60 m		
	11	48.07 t	3.52 m						2.08 m	37	
			3.65 m				40	25.61 t	1.80 m		
Pro	12	173.32 s							1.10 m		
-2	13	60.67 d	3.68 m		8		41	47.79 t	3.44 m		
	14	29.11 t	1.82 m	12					3.62 m		
			2.24 m			*Leu*	42	170.72 s			
	15	26.46 t	1.83 m				43	49.97 d	4.66 m	42, 44	41
			2.14 m				44	42.91 t	1.22 m	43	
	16	49.21 t	3.66 m		18				1.49 m		
			3.95 m		18		45	25.74 d	1.58 m		
Ile	17	171.98 s					46	21.96 q	1.01 d	44, 45	
-2	18	56.07 d	4.54 m	17, 20			47	23.98 q	0.87 d	45, 46	
	19	39.70 d	1.68 m	21, 22			NH		7.01 brs		49
	20	25.61 t	1.69 m			*Ala*	48	174.14 s			
			1.08 m				49	50.96 d	4.55 m	48, 50	Leu-NH
	21	11.11 q	0.88 t	19			50	18.06 q	1.36 d	48, 49	Ala-NH
	22	15.18 q	1.02 d	18, 19			NH		8.08 brs	50,52	
	NH		6.99 brs		24	*Phe*	51	174.53 s			
Tyr	23	172.85 s					52	58.03 d	4.22 m		Ala-NH
	24	57.64 d	4.45 m	23, 25	Ile 2-NH		53	39.02 t	2.84 dd	51, 52	
	25	33.40 t	3.42 m						3.06 dd	51,54	
			3.13 m	24, 26			54	137.53 s			
	26	130.44 s					55	130.81 d	7.24 d	53, 57	
	27	132.15 d	6.97 d	25, 28			56	129.96 d	7.34 d	54	
	28	115.64 d	6.52 d	26, 29			57	128.32 d	7.30 m	55	
	29	115.80 s					58	129.32 d	7.34 d	54	
	30	115.64 d	6.52 d	26, 29			59	130.81 d	7.24 m	53, 57	
							NH		7.08 brs		2

Tackett, Ms. Denise Nielsen-Tackett, and Mr. Lee Williams. The four sector tandem mass spectrometer was purchased with funds awarded by the former NSF regional instrumentation program (grant CHE-8620177) and the University of Nebraska-Lincoln. Additional support was provided by the NSF Biology Division (grant DIR-9017262)

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(*Received in USA* 28 *February* 1995; *accepted* 17 *May* 1995)