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ANTINEOPLASTIC AGENTS 315. ISOLATION AND STRUCTURE OF THE MARINE SPONGE CANCER CELL GROWTH INHIBITOR PHAKELLISTATIN 5[1]

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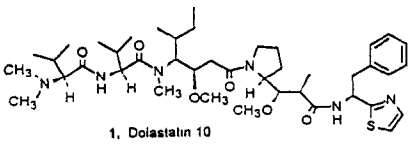
Abstract: The Federated State of Micronesia (Chuuk) marine sponge *Phakellia costada* has been found to contain (9.6×10^{-6} % yield) a new human cancer cell growth inhibitor designated phakellistatin 5 (3). Structural elucidation was accomplished employing high resolution FAB tandem MS/MS and high field (500 MHz) 2D-NMR techniques. Extension of the NMR experiments over the temperature range -25 to 25°C allowed the principal solution conformation of phakellistatin 5 to be assigned (Figure 2). The absolute configuration was found to correspond to S-amino acid units except for R-Asn.

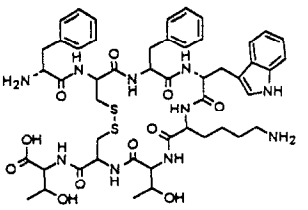
The promising potential of certain naturally occurring peptides, and synthetic modifications, for improving human cancer treatment is becoming more generally recognized.[2,3] Illustrative are dolastatin 10 (1),[4] presently in advanced preclinical development and the somatostatin modification sandostatin (2, octreotide) which is undergoing phase I clinical trials for carcinoid and islet cell-type neuroendocrine cancers.[5] The related somatostatin derivative RC-160-gastrin releasing antagonist RC-3095[6] was found somewhat less effective against this prostate line. The related bombesin$^{6-14}$ nonapeptides led to cytostatic effects with two small cell lung cancer xenografts.[7] Other interesting leads include Ser-Asp-Nε-Ac-Lys that retards Yoshida (ascites) rat liver cancer cells,[8] the marine tunicate cyclic peptide potellamide D[9] that reverses multidrug resistance in the CEM-VLB 100 human leukemia cell line and the potentially important series of marine Porifera cyclic peptides we discovered that inhibit human cancer cell growth.[10] Herein we report the isolation and structural elucidation of a new marine sponge cyclic peptide named phakellistatin 5 (3) that exhibits an unusual pattern of selective growth inhibition against the U. S. National Cancer Institute (NCI) human cancer cell line panel.[11-13]

From 500 kg (wet wt.) of the Western Pacific sponge *Phakellia costata* (recollected 1987 at -15m by SCUBA, on the North Pass of Pis Island, Chuuk) a murine P388 lymphocytic leukemia cell line active methylene chloride soluble fraction[10d] was further separated employing the

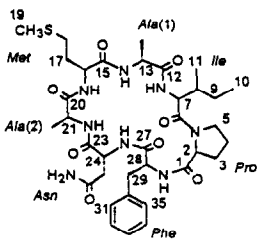
P388 bioassay. Separation of the active fraction by sequential permeation (Sephadex LH-20) and partition (LH-20) column chromatographic procedures, high speed countercurrent distribution techniques[14] followed by HPLC (reversed phase C8, with acetonitrile-methanol-water, 10:10:13, as mobile phase) afforded phakellistatin 5 (3), 47.9 mg, 9.6×10^{-6}% yield, $[\alpha]_D$ -102° (c, 2.28 CH_3OH) as an amorphous powder (P388, ED_{50} 0.23 μg/mL).



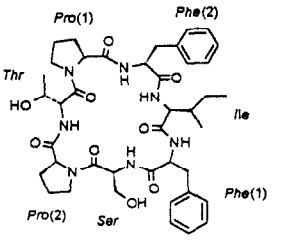
1, Dolastatin 10



2, Octreotide



3, Phakellistatin 5



4, Phakellistatin 4

The molecular formula of phakellistatin 5 (3) was found to be $C_{35}H_{52}N_8O_8S$ by HRFABMS [m/z 745.3706 $(M+H)^+$ for $C_{35}H_{53}N_8O_8S$, Δ-0.1 mmu]. From the molecular formula as well as ^1H- and ^{13}C-NMR data, it became evident that phakellistatin 5 was a cyclic peptide. Complete assignments for the ^1H- and ^{13}C-NMR resonances (in 3:1 CD_3CN-CD_3OD, Table I) were accomplished using a combination of 2D-NMR experiments (^1H,^1H-COSY, HMQC and HMBC). The detailed NMR studies resulted in identification of seven amino acid units, namely Phe, Ile, Pro, Met, Ala (x2) and Asn. Upon hydrolysis with 6N HCl at 105°C for 24 h, five of the same six amino acids and aspartic acid (from Asn) were detected.

The HMBC NMR analyses (Table 1) suggested the seven amino acid sequence presented in structure 3. Two segments, Ile-Ala(1)-Met-Ala(2) and Asn-Phe-Pro were assigned by two-bond ^1H-^{13}C correlations as follows: NH (Ile)/CO (Ala 1), NH (Ala 1)/CO (Met), NH (Met)/CO (Ala 2), NH (Asn)/CO (Phe) and NH (Phe)/CO (Pro). Other useful HMBC correlations were located between αH (Pro)/CO (Pro) and αH (Ala 2)/CO (Ala 2), and between αH (Pro)/CO (Ile) and αH (Ala 2)/CO (Asn). In turn, that clearly identified the (Ile) CO-N (Pro) and (Asn) CO-NH (Ala 2) relationships and the cyclic heptapeptide structure (3). Also, cross peaks involving NH (Ile)/αH (Ala 1) and NH (Ala 2)/αH (Asn) in the ROESY spectrum supported connectivity across the NH (Ile)/CO (Ala 1) and NH (Ala 2)/CO (Asn) amide bonds. The amino acid sequence of cyclic peptide 3 was confirmed by results of FAB-MS/MS studies.

Table 1 : The ^{1}H- and ^{13}C-NMR Spectral Assignments for Phakellistatin 5 (3) in 3:1 CD_3CN-CD_3OD

No.	^{13}C ppm	^{1}H ppm	J (Hz)	HMBC* (^{1}H to ^{13}C)	No.	^{13}C ppm	^{1}H ppm	J (Hz)	HMBC* (^{1}H to ^{13}C)
Pro 1	172.34 s				19	15.76 q	2.07 s		18
2	62.37 d	4.42 d	7.5	1,6	NH		8.12 brs		20
3	31.91 t	1.78 m		1	*Ala* 20	175.14 s			
		2.23 dd	12.3,6.0		*(2)* 21	53.59 d	3.94 q	7.4	20,23
4	22.29 t	0.84 m			22	17.43 q	1.38 d	7.4	
		1.61 m			NH		7.69 brs		
5	47.35 t	2.78 dd	10.0		*Asn* 23	172.68 s			
		3.31 m			24	51.39 d	4.39 dd	6.0,2.8	
Ile 6	173.20 s				25	37.28 t	2.94 dd	15.4,2.8	23,26
7	59.14 d	4.14 dd	7.7,4.1	6			3.18 m		
8	37.48 d	1.74 m			26	174.32 s			
9	26.08 t	1.30 m			NH		7.79 d	4.5	23,27
		1.61 m			*Phe* 27	174.06 s			
10	11.59 q	0.86 t	7.4		28	59.60 d	4.24 m		27
11	15.76 q	0.89 d	7.4		29	38.39 t	3.15 m		28,30,31
NH		7.82 brs		12			3.26 m		
Ala 12	174.32 s				30	138.94 s			
(1) 13	49.20 d	4.54 m		12	31	130.21 d	7.23 m		32,33,34
14	16.47 q	1.25 d	6.5		32	129.98 d	7.32 m		30,31,35
NH		7.60 d	8.0	15	33	128.17 d	7.20 m		
Met 15	173.43 s				34	129.98 d	7.32 m		30,31,35
16	54.35 d	4.52 m			35	130.21 d	7.23 m		32,33,34
17	32.44 t	1.98 m			NH		8.53 d	6.9	1
		2.09 m							
18	31.61 t	2.53 m		19					
		2.45 m		19					

* The HMBC spectrum was measured in CD_3CN-CD_3OH (3:1).

Collisional activation of the $[M + H]^+$ ions of phakellistatin 5 produced immonium ions of Ala (m/z 44), Pro (m/z 70), Leu/Ile (m/z 86), Asn (m/z 87), Met (m/z 104), and Phe (m/z 120). The presence of these amino acids, when combined with the exact mass data, identified the amino acid composition to be 1 x Pro, 1 x Phe, 1 x Asn, 2 x Ala, 1 x Met, and 1 x Leu (Ile). The CAD spectrum[15a] of the $(M + H)^+$ ions of phakellistatin 5 (Fig. 1) contained three series of fragment ions that were used to identify the sequence as *cyclo*-(Pro-Phe-Asn-Ala-Met-Ala-Ile). Other ions observed in the CAD spectrum that provided confirmation of the sequence were assigned Ala-Ile (m/z 185), Ile-Pro (m/z 211), Phe-Asn (m/z 262), Phe-Asn-Ala (m/z 333), and Met-Ala-Ile (m/z 316). In summary this allowed the structure of phakellistatin 5 (3) to be assigned *cyclo* (Pro-Phe-Asn-Ala-Met-Ala-Ile).

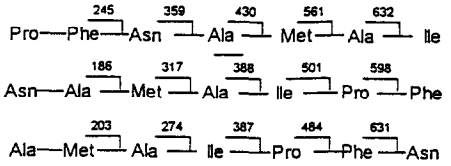


Fig. 1. HRFAB MS/MS derived assignments supporting the amino acid sequence of phakellistatin 5 (3).

The absolute configuration of phakellistatin 5 (3) was deduced by analyzing an acid hydrolysate converted to *N*-Pentafluoropropionyl-isopropyl ester derivatives[16] using chiral gas chromatography (chirasil-Val III column).[17] By comparing the retention time of each amino acid derivative with those of the authentic (S)- and (R)- amino acids, it was ascertained that each contained the (S)-configuration except for the (R)-Asp unit. Attention was next directed to a conformational analysis employing high field NMR over the temperature range -25° to 25°C.

The NMR spectra of phakellistatin 5 (3) indicated predominately a single conformer in CD_3CN-CD_3OH (3:1) solution at ambient temperature. A likely solution conformation of cyclic peptide 3 was deduced from detailed analyses of ROESY and NOESY spectra as a function of temperature dependence of the α-amide proton chemical shifts.[15b] The ROESY spectrum showed a cross peak between αH (Pro) and αH (Ile), indicating *cis* geometry for the amide bond at N (Pro)/CO (Ile). The *cis* Ile-Pro configuration was supported by the difference ($\Delta\delta\beta\gamma$ 9.62 ppm)[18,19] in the Pro β- and γ-carbons ^{13}C chemical shifts. The Phe, Ala (1) and Met α-amide protons showed less temperature dependence ($\Delta\delta/\Delta_T$: -2.3, -0.45 and -3.4, respectively) than those of Ile, Asn and Ala (2) ($\Delta\delta/\Delta_T$: -8.5, -8.0 and -7.4, respectively, suggesting that the three amide protons of Phe, Ala (1) and Met participated in intramolecular hydrogen bonds.[20,21] Also, the NOE correlation between NH (Ala 1) and NH (Met) indicated those amide hydrogens were directed inside the peptide ring.

The amide hydrogen chemical shift of Ile showed a large temperature dependence and NOE correlations were observed between NH (Ile), βH (Ile) and NH (Ile)/βCH_3 (Ala 1) (Figure 2). From this evidence, the Ile amide bond was placed outside the peptide ring. Cross peaks corresponding to NH (Ala 2), αH (Asn) and NH (Ala 2)/βCH_3 (Ala 2) in the ROESY spectrum suggested that the N-H bond of Ala (2) was nearly oriented in the same direction as the α-H bond of Asn. In turn, that served to further confirm the (R)-configuration for Asn and suggested that the Asn-Ala (2) unit formed a type II′ β-turn.[22] Therefore, the backbone of peptide 3 appeared to have one *cis* amide bond at N(Pro)/CO (Ile), a type II′ β-turn at Asn-Ala (2), and three transannular hydrogen bonds at NH (Phe)/CO (Ala 1), NH (Ala 1)/CO (Phe) and NH (Met)/CO (Phe). At temperatures below 5°C, the amide proton $\Delta\delta/\Delta_T$ of Ala 2 decreased to <0.4, pointing to a new intramolecular hydrogen bond at NH (Ala 2)/$CONH_2$ (Asn) arising from the Asn primary amide.

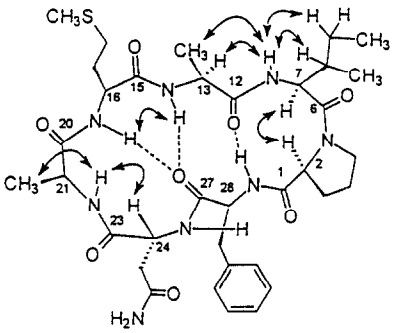


Fig. 2. The NOE (arrows) and intramolecular hydrogen bond (dotted lines) assignments for phakellistatin 5 (3).

Evaluation of phakellistatin 5 (3) against the NCI human cancer cell line panel gave the following interesting results. Quadruplicate testing of phakellistatin 4 (4),[10a] and phakellistatin 5 (3) in the U. S. National Cancer Institute's disease-oriented in vitro primary screen[11-13] revealed distinctive and reproducible GI_{50} mean graph profiles.[12] The approximate mean panel GI_{50} values were 3 μM and 0.6 μM, for peptides 3 and 4 respectively. Comparative analyses (not shown) of these characteristic patterns of differential growth inhibition by cyclic peptides 3 and 4 did not reveal any strong correlations between the two, nor to the profiles produced by any of the "standard agents"[11] for which common mechanisms of action are known or implicated. In further studies it will be of interest to determine if the cytostatic phakellistatins are of a new mechanistic class.

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(15) (a) The FAB and FAB MS/MS spectra were obtained using a VG ZAB-T, a four sector mass spectrometer of BEBE design. Samples were dissolved in methanol, and an aliquot containing 1 to 3 μg was placed on the probe along with 1 μl of matrix, a 1:1 mixture of 3-nitrobenzyl alcohol and glycerol containing 1% (by volume) of trifluoroacetic acid. A Cs^+ ion gun operated at 25 keV was used to desorb the ions from the probe. The instrument accelerating voltage was 8 kV. The VG ZAB-T consists of MS1, a standard high resolution ZAB mass spectrometer, and MS2, a prototype Mattauch-Herzog design, incorporating a standard magnet and an inhomogeneous ESA. This design allows the use of a photodiode array for simultaneous detection of ions over a variable mass range or a single point detector for scanning acquisitions. For experiments reported here, sample quantities were large enough so that the single point detector was sufficient. CAD spectra were obtained at the third field-free region (between MS1 and MS2) by addition of He into the collision cell to attenuate the ion beam by 70%. The collision cell was floated at 4kV. MS1 was operated at a resolution of 1000, MS2 resolution was set to 1200 (1/2 height definition). Ten to fifteen 20 sec scans were signal averaged to produce each spectrum. (b) The ^{1}H-NMR temperature dependence experiments were conducted with a Varian Unity 500 NMR spectrometer at +25, +15, +5, -5, -15 and -25°C.

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