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ANTINEOPLASTIC AGENTS 323. ISOLATION AND STRUCTURE OF PHAKELLISTATIN 6 FROM A CHUUK ARCHIPELAGO MARINE SPONGE[1]

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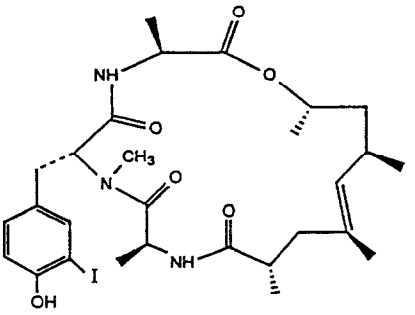
Abstract: Phakellistatin 6 (7), a trace (7.5×10^{-7}% yield) constituent of the marine sponge *Phakellia costata* located in the Federated States of Micronesia has been found to significantly inhibit growth of certain human cancer cell lines (GI_{50} 0.1 to 0.01 μg/ml). The structure of phakellistatin 6 (7) was deduced as *cyclo* (Pro-Trp-Leu-Pro-Ile-Pro-Phe) employing a combination of MS/MS and high field (500 MHz) 2D-NMR procedures followed by chiral (all *S* amino acids) assignments using hydrolysis → derivatisation → chiral chromatographic techniques.

A variety of tropical marine Porifera species have been found to contain cell growth inhibitory peptides. Structurally, these potentially important substances range from relatively small (3 amino acid units) depsipeptides such as geodiamolide A (1)[2,3] and jaspomide[3] to the 48-unit polytheonomides A-C.[4] To date, the sponge cycloheptapeptides we have discovered, such as phakellistatins 1-5 (2-6),[5] the axinastatins,[6] the stylostatins,[7] and Kobayashi's hymenamides A and B[8] have formed the largest and most structurally consistent series. Because the structures of such cytotoxic cycloheptapeptides are revealing potentially important bioactivity vs. structure clues, we have intensified our search among the trace peptide components of the very productive Western Pacific (Chuuk, Federated State of Micronesia) sponge *Phakellia costata*,[5e] collected (500 kg. wet. wt) in 1987.

The P388 active fraction, prepared[5e] from a methanol extract of *Phakellia costata* by solvent partition → gel permeation → partition column chromatographic procedures (using Sephadex LH-20 and silica gel), was subjected to final separation using reversed phase (RP-8) HPLC with acetonitrile-methanol-water (10:10:13) as eluant to afford phakellistatin 6 (7) as a colorless amorphous powder (3.75 mg, 7.5×10^{-7} yield): R_f (silica gel) 0.40 n-hexane-dichloromethane-methanol (10:5:1), $[\alpha]_D$ -128.8 (c=0.37, CH_3OH); FABMS m/z 851 $[M+H]^+$; HRFABMS calcd for $C_{47}H_{62}N_8O_7$; 851.4820, found; m/z 851.4825, 0.5 mmu $[M+H]^+$. From the molecular formula combined with initial results from ^{1}H-NMR and APT data and UV absorptions at λ 289,

280, 273 (sh) and 213 nm, phakellistatin 6 appeared to be a peptide with mono-substituted phenyl and indole ring systems.



1, Geodiamolide A

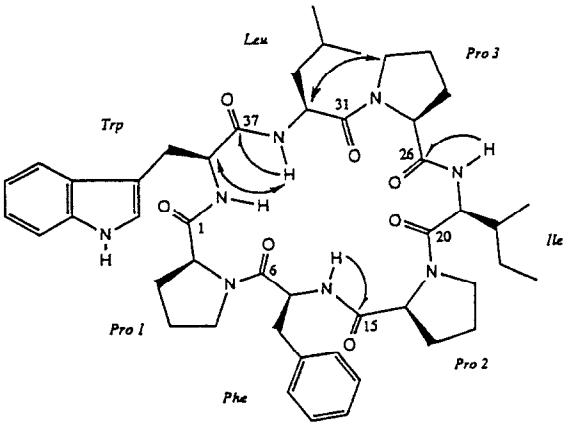
2, Cyclo(Pro-Ile-Pro-Trp-Pro-Phe-Ile), Phakellistatin 1

3, Cyclo(Pro-Tyr-Pro-Phe-Pro-Ile-Ile), Phakellistatin 2

4, Cyclo(Pro-Phe-Gly-Pro-Thr-Ile-trans-photo-Trp), Phakellistatin 3

5, Cyclo(Pro-Thr-Pro-Phe-Ile-Phe-Ser), Phakellistatin 4

6, Cyclo(Pro-Phe-Asp-Ala-Met-Ala-Ile), Phakellistatin 5



7, Phakellistatin 6 with HMBC ——
and NOE ⟷ Correlations

Extensive 2D-NMR (COSY, HMQC and HMBC) spectral interpretations resulting from phakellistatin 6 (7) allowed the signal assignments and relationships recorded in Table 1. These corresponded to seven partial structures; namely, isoleucyl, leucyl, phenylalanyl, tryptophanyl and prolyl (x3) units comprising a heptapeptide. Since the molecular formula required 20 unsaturation sites, satisfied in part (19 total) by Pro (x3), Phe, Trp and seven carbonyls, the remaining unsaturation site was attributed to the cyclic peptide ring. Evidence supporting a cyclic peptide structure was also obtained by MS/MS analyses. The HMBC and NOE correlations provided some useful information about the amino acid sequence. HMBC correlations from amide NH protons to amine carbonyl carbon signals such as NH [Leu] (δ 8.20)/CO [Trp] (δ 172.65), NH [Ile] (δ 6.38)/CO [Pro_3] (δ 170.33) and NH [Phe] (δ 6.50)/CO [Pro_2] (δ 171.66) revealed the presence of Trp-Leu, Pro_3-Ile and Pro_2-Phe segments. Furthermore, the ROESY spectrum yielded two important NOE correlations between α H [Leu] (δ 4.50) and δ H [Pro_3] (δ 3.65), and NH [Leu] (δ 8.20) and α H [Trp] (δ 4.67) which suggested linking these two segments between Trp-Leu and Pro_3-Ile. Because the NH proton of the Trp unit was not observed in the ^{1}H-NMR spectra, the connections between NH (Trp) and either CO (Phe) or CO(Pro) could not be ascertained. The uncertainty introduced was resolved by the results of MS/MS mass spectral studies.

The FAB MS/MS analyses[9] revealed internal fragments due to Pro-Trp at m/z 284, Leu/Ile-Pro (or Pro-Leu/Ile) at m/z 211 and Pro-Phe (or Phe-Pro) at m/z 245. These results not only supported the linkages of amino acid units derived by NMR interpretations established, but also the Trp-Pro and Pro-Phe-Pro bonding. Three series of MS fragmentations resulted from protonation of the three prolyl residues followed by ring opening as shown in Fig. 1 finally confirmed the sequence of phakellistatin 6 (7) as cyclo-(Pro-Trp-Leu-Pro-Ile-Pro-Phe).

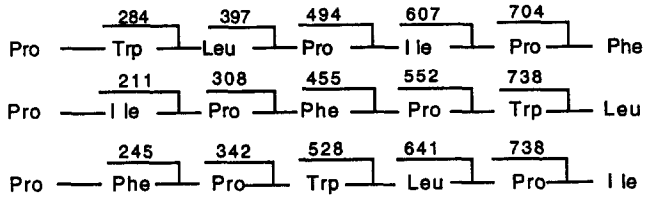


Fig. 1. Phakellistatin 6 (7) fragmentation and tandem MS/MS peptide sequence analyses.

Table 1: The High Field (500MHz) ^{1}H- and ^{13}C-NMR Spectral Assignments for Phakellistatin 6 (7) in CD_2Cl_2

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	170.88 s				24	12.15 q	0.93 t		22, 23
-1 2	62.18 d	4.16 d		1	25	14.25 q	0.87 d		22
3	30.91 t	1.89 m			NH		6.38 d	6.0	26
4	25.94 t	2.02 m			*Pro* 26	170.03 s			
5	46.81 t	3.42 m			*-3* 27	61.34 d	3.64 d		
		3.50 m			28	29.56 t	1.87 m		26, 27
Phe 6	170.54 s				29	22.07 t	1.64 m		27
7	53.14 d	4.67 m					1.77 m		
8	40.01 t	2.82 dd		6, 7, 9	30	47.12 t	3.65 m		
		3.21 m		6, 7, 9	*Leu* 31	172.33 s			
9	136.27 s				32	51.20 d	4.50 m		31, 33
10	129.96 d	7.11 d	7.0	8, 11	33	39.71 t	1.43 m		
11	129.03 d	7.28 dd	6.5	9			1.53 m		
12	127.60 d	7.24 m			34	25.70 d	1.71 m		35
13	129.03 d	7.28 dd	6.5	9	35	20.88 q	0.92 d		33, 34, 36
14	129.96 d	7.11 d	7.0	8, 13	36	23.72 q	1.00 m		33, 34, 35
NH		6.50 d	4.0	15	NH		8.20 d	7.5	37
Pro 15	171.66 s				*Trp* 37	172.65 s			
-2 16	60.69 d	3.35 d			38	54.72 d	4.67 m		
17	31.82 t	1.86 m		15, 16	39	28.04 t	3.21 m		
		1.30 m			40	109.82 s			
18	22.38 t	1.39 m		16	41	124.14 d	7.21 s		40, 42
		1.49 m			NH		6.61 brs		
19	47.35 t	3.28 m			42	127.60 s			
		3.35 m			43	111.73 d	7.38 d	7.5	42, 44
Ile 20	169.56 s				44	119.93 d	7.07 t	7.5	42, 43
21	56.93 d	4.31 m		20	45	122.67 d	7.17 t	7.5	46, 47
22	39.41 d	1.52 m		20	46	119.24 d	7.48 d	7.5	45, 47
23	26.00 t	1.37 m			47	136.66 s			
		1.56 m							

The absolute configuration of the constituent amino acids in peptide 7 was ascertained by analyzing the acid hydrolysate N-pentafluoro-propionyl-isopropyl ester derivatives by means of chiral capillary chromatography (chirasil-Vol. III column) methods.[10,11] Based or direct and indirect comparisons, each amino acid unit in phakellistatin 6 (7) was assigned the L-configuration. Due to the presumed decomposition of Trp, its absolute configuration was not assigned.

Phakellistatin 6 was found to display a reassuring level of cancer cell growth inhibition against the murine P388 lymphocytic leukemia (ED_{50} 0.185 μg/ml) and a series human cancer cell lines: ovarian (OVCAR-3; GI_{50} 0.025 μg/ml), CNS (SF-295; GI_{50} 0.041 μg/ml), renal (A498: GI_{50} 0.078 μg/ml); lung (NCI-H460; GI_{50} 0.019 μg/ml); colon (KM20L2; GI_{50} 0.021 μg/ml) and melanoma (SK-MEL-5; GI_{50} 0.032 μg/ml). The consistent presence of proline in this series of cancer cell growth inhibitory cyclic peptides suggests that the three dimensional structures influenced by the *cis* or *trans* amide bonding preferences is important. Such considerations are presently being pursued by synthetic and X-ray crystal structure determination.

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