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ISOLATION AND STRUCTURE OF PHAKELLISTATIN 2 FROM THE EASTERN INDIAN OCEAN MARINE SPONGE *PHAKELLIA CARTERI*

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Abstract: The marine sponge *Phakellia carteri* indigenous to the Republic of the Comoros was found to contain a new cell growth inhibitory (P388 cell line ED_{50} 0.34 μg/ml) cyclo-heptapeptide named phakellistatin 2.

Interest in marine Porifera as new sources of structurally novel cytotoxic and/or antineoplastic substances has been gaining momentum.[2-7] As part of a long term[8] investigation we have been evaluating species from, especially, the marine sponge class Demospongia for such potentially useful components. Among these cell growth inhibitory constituents we have discovered the first examples of marine sponge cyclo-hepta[7§]- and cyclo-octapeptides.[9] Meanwhile, we have uncovered two new and cytostatic, Demospongia cyclo-heptapeptides[12,13]. The Indo-Pacific Ocean areas have also proven to be a very productive source of sponge cyclopeptide cell growth inhibitors from other marine organisms such as ascidians (towicyclamides A and B,[14]) and cyanobacteria (hormothamnin A,[15]).

We now report the isolation and structural elucidation of a new cyclo-heptapeptide designated phakellistatin 2 (1) with significant activity (ED_{50}, 0.34 μg/ml) against the murine P388 lymphocytic leukemia (PS system) and a "minipanel" of six cell lines from the NCI's primary screening panel of 60 human cancer cell lines.[16,17] Phakellistatin 2 showed $\log_{10}$ GI_{50} values (μg/ml) as follows: OVCAR-3 (1.0); SF-295 (3.0); A498 (2.1); NCI-H460

§Apparently our report (Ref. 7) describing the first sponge cyclo-heptapeptide, axinastatin 1 was missed and this compound has been redescribed as pseudoaxinellin (Ref. 10) and possibly as malaysiatin (Ref. 11).

(2.0); KM20L2 (2.8); SK-MEL-5 (1.0) which are ovarian, brain, renal, lung, colon and melanoma cell lines. Phakellistatin 2 was isolated, utilizing PS guided bioassay, from a methanol-dichloromethane extract of *Phakellia carteri* (order Axinellida, class Demospongiae) collected (1987) in the Eastern Indian Ocean (Republic of Comoros). Application of a series of extraction, solvent partition, gel permeation and partition chromatographic (on Sephadex LH-20) procedures to a 250 kg. (wt. wt.) sample of the orange sponge yielded 0.26 g (9.6×10^{-5}%) of phakellistatin 2 (1) as an amorphous solid: m.p. 199-201°, t.l.c. R_f 0.22 (in 4:2:1 hexane-ethyl acetate-methanol) $[\alpha]_D^{23}$ -148° (c 0.34, CH_3OH); u.v. λ_{max}. nm (log ϵ) in CH_3OH; 224 (3.92) and 275 (2.85); IR $\nu_{max.}$ cm^{-1} (KBr), 3406, 3295, 2963, 2878, 1645, 1447; EIMS m/z (%), 827 (M^+, 100), 771, 736, 720, 713, HRFAB m/z 828.4662 $[M+H]^+$, calc. for $C_{45}H_{62}N_7O_8$ 828.4660.

The initial structural approaches to phakellistatin 2 employing high field 2D-NMR techniques were complicated by the existance of two conformers[18] in deuteriochloroform (1:1 ratio of isomers) and deuteriodichloromethane (3:2 ratio) solutions at ambient temperatures. Fortunately in deuteriomethanol solution phakellistatin 2 appeared in only one conformational form. At that point results of detailed (cf., Table 1) ^1H- and ^{13}C-NMR, APT, ^1H-^1H-COSY, ^{13}C-^1H-COSY and HMBC spectral analyses were found consistent with molecular formula $C_{45}H_{61}N_7O_8$ derived by HRFAB. Interpretation of the ^1H-NMR spin system indicated the presence of proline, isoleucine, phenylananine and tyrosine units in a respective ratio of 3:2:1:1. Hydrolysis of phakellistatin 2 followed by amino acid analyses indicated the same amino acid composition. High resolution MS/MS analyses supported by high field (400 and 500 MHz) NMR experiments led to assignment of peptide 1 as cyclo-(Pro-Tyr-Pro-Phe-Pro-Ile-Ile). The absolute configuration was established at each chiral center by analyzing the acid hydrolysate N-pentafluoropropionyl-isopropyl ester derivatives[19,20] by means of chiral capillary chromatography (Chirasil-Vol. III column) methods.

The structure assigned phakellistatin 2 (1) was firmly substantiated by tandem (MS/MS) mass spectrometry[21] using source-produced fragment ions to confirm interrelationships. Verification of the elemental compositions and assignments for the fragment ions was performed by exact mass measurements. Protonation at the Pro nitrogen atoms accounted for the three main fragmentations observed. In addition, the fragment ion at m/z 731 appeared to arise from ring opening of a protonated Pro followed by its loss. A summary of the three principal mass spectral fragmentation routes appears in Fig. 1.

Interestingly, further separtion of *P. carteri* PS active fractions led to isolation (39 mg, 1.7×10^{-5}% yield) of cyclo-(Pro-Val-Asn-Pro-Phe-Val-Val), axinastatin 1[7], that we

originally discovered in a Western Pacific (Palau) Axinella sp. Phakellistatins 1[13] and 2, hymenistatin 1[9], and axinastatin 1[7] provide initial evidence for what may prove to be the beginning of a potentially important new series of bioactive cyclic peptides.[22]

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REFERENCES AND NOTES

1. Contribution 272 of the series Antineoplastic Agents: For part 271 see Pettit, G. R.; Xu, J.; Williams, M. D.; Christie, M. D.; Doubek, D. L.; Schmidt, J. M.; Boyd, M. R. *J. Nat. Prod.*, submitted.
2. Pettit, G. R.; Tan, R.; Gao, F.; Williams, M. D.; Doubek, D. L.; Boyd, M. R.; Schmidt, J. M.; Chapuis, J-Charles; Hamel, E.; Bai, R.; Hooper, J.N.A.; Tackett, L. P. *J. Org. Chem.*, 1993, *58*, 2538.
3. Pettit, G. R.; Gao, F.; Doubek, D. L.; Boyd, M. R.; Hamel, E.; Schmidt, J. M.; Tackett, L. P.; Hooper, J.N.A. *Gazz. Chim. Ital.*, in press.
4. Fusetani, N.; Sugawara, T.; Matsunaga, S. *J. Org. Chem.*, 1992, *57*, 3828.
5. Thompson, A. M.; Blunt, J. W.; Munro, M.H.G.; Perry, N. B.; Pannell, L. K. *J. Chem. Soc. Perkin Trans. 1*, 1992, 1335.
6. Acosta, A. L.; Rodriguez, A. D. *J. Nat. Prod.*, 1992, *55*, 1007.
7. Pettit, G. R.; Herald, C. L.; Boyd, M. R.; Leet, J. E.; Dufresne, C; Doubek, D. L.; Schmidt, J. M.; Cerny, R. L.; Hooper, J.N.A.; Rützler, K. C. *J. Med. Chem.*, 1991, *34*, 3339.
8. Pettit, G. R.; Day, J. F.; Hartwell, J. L.; Wood, H. B. *Nature*, 1970, *227*,962.
9. Pettit, G. R.; Clewlow, P. J.; Dufresne, C.; Doubek, D. L.; Cerny, R. L.; Rützler, K.

Can. J. Chem., 1990, *68*, 708.

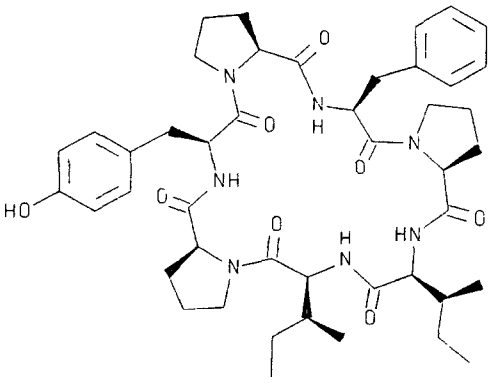
10. Kong, F.; Burgoyne, D. L.; Andersen R. J.; Allen, T. M. *Tetrahedron Lett.*, 1992, *33*, 3269.
11. Fernandez, R.; Omar, S.; Feliz, M.; Quinoa, E.; Riguera, R. *Tetrahedron Lett.*, 1992, *33*, 6017.
12. Pettit, G. R.; Srirangam, J. K.; Herald, D. L.; Erickson, K. L.; Doubek, D. L.; Schmidt, J. M.; Tackett, L. P.; Bakus, G. J. *J. Org. Chem.*, 1992, *57*, 7217.
13. Pettit, G. R.; Cichacz, Z.; Barkoczy, J.; Dorsaz, A.-C.; Herald, D. L.; Williams, M. D.; Doubek, D. L.; Schmidt, J. M.; Tackett, L. P.; Brune, D. C.; Cerny, R. L.; Hooper, J.N.A.; Bakus, G. J. *J. Nat. Prod.*, 1993, *56*, 260.
14. McDonald, L. A.; Foster, M. P.; Phillips, D. R.; Ireland, C. M.; Lee, A. Y.; Clardy, J. C. *J. Org. Chem.*, 1992, *57*, 4616.
15. Gerwick, W. H.; Jiang, Z. D.; Agarwal, S. K.; Farmer, B. T. *Tetrahedron*, 1992, *48*, 2313.
16. Monks, A.; Scudiero, P.; Skehan, P.; Shoemaker, R.; Paull, K.; Vistica, D.; Hose, C.; Langley, J.; Cronise, P.; Vaigro-Wolff, A., Gray-Goodrich, M.; Campbell, H.; Boyd, M. R. *Nat'l Cancer Inst.*, 1991, *83*, 757.
17. Boyd, M. R., "Status of the NCI Preclinical Antitumor Drug Discovery Screen," in: DeVita, V. T., Jr., Hellman, S.; Rosenberg, S. A., eds., Principles and Practice of Oncology, Update, Vol. 3, No. 10, Lippincott, Philadelphia, 1989, pp. 1-12; Boyd, M. R.; Paull, K. D.; Rubinstein, L. R., "*Data Display Analysis Strategies from the NCI Disease-Oriented in vitro Antitumor Screen*," Valeriote, A.; Corbett, T.; Baker, L. eds., *Antitumor Drug Discovery and Development*, Kluwer Academic Press, Amsterdam, 1990, in press; Paull, K. D.; Shoemaker, R. H.; Hodes, L.; Monks, A.; Scudiero, D. A.; Rubinstein, L.; Plowman, J.; Boyd, M. R. *J. Nat'l. Cancer Inst.*, 1989, *81*, 1088.
18. Bean, J. W.; Kopple, K. D.; Peishoff, C. E., *J. Am. Chem. Soc.*, 1992, *114*, 5328.
19. Westall, F.; Hesser, H. *Anal. Biochem.*, 1974, *61*, 610.
20. Shaw, C. J.; Cotter, M. L. *Chromatographia*, 1986, *21*, 197.
21. Cerny, R. L.; Gross, M. L. "Tandem Mass Spectrometry for Determining the Amino Acid Sequences of Cyclic Peptides and for Assessing Interactions of Peptides and Metal Ions" in: Mass Spectrometry of Peptides, Ed. by Desiderio, D. M., CRC Press, Boca Raton, FL, 1990, pp. 289-314.
22. Fusetani, N.; Matsunaga, S. *Chem. Rev.*, 1993, *93*, 1793.

Table 1. Phakellistatin 2 (1) High Field (400 and 500 MHz) NMR Assignments Recorded in CD_3OD.

Carbon	^{13}C-NMR	^{1}H-NMR	^{1}H-^{1}H-COSY	HMBC
Pro*				
CO	172.74p			Ha, Hb
a-CH	59.89n	4.36 (1H, d, 8.1)	Hb, Hb'	
b-CH_2	31.78p	1.86 (1H)	Ha, Hb'	Ha
		2.02 (1H)	Ha, Hb, Hc, Hc'	
c-CH_2	22.56p	1.80 (1H)	Hb', Hc', Hd, Hd'	Ha, Hb, Hb'
		2.03 (1H)	Hb', Hc, Hd, Hd'	
d-CH_2	48.24p	3.32 (1H, m)	Hc, Hc', Hd'	Ha
		3.50 (1H, m)	Hc, Hc', Hd	
Pro**				
CO	172.99p			Ha, Hb, Hb'
a-CH	63.25n	4.46 (1H, brd, 8.6)	Hb, Hb'	Hb', Hc'
b-CH_2	32.77p	2.06 (1H)	Ha, Hc, Hc'	Ha
		2.18 (1H, m)	Ha, Hc, Hc'	
c-CH_2	23.01p	1.58 (1H)	Hb, Hb', Hd, Hd'	Ha, Hd'
		1.82 (1H)	Hb, Hb', Hd, Hd'	
d-CH_2	47.68p	3.36 (1H,m)	Hc, Hc', Hd'	Ha
		3.50 (1H, m)	Hc, Hc', Hd	
Pro***				
CO	173.22p			Ha, Hb, Hb'
a-CH	62.34n	3.12 (1H, d, 8.0)	Hb, Hb'	Hb
b-CH_2	31.69p	0.87 (1H, m)	Ha, Hb', Hc	Ha
		1.82 (1H)	Ha, Hb, Hc, Hc'	
c-CH_2	22.86p	1.30 (1H,m)	Hb,Hb',Hc',Hd,Hd'	Ha, Hb
		1.58 (1H)	Hb', Hc, Hd, Hd'	
d-CH_2	47.68p	3.25 (1H)	Hc, Hc', Hd'	Ha
		3.27 (1H)	Hc, Hc', Hd	
Ile*				
CO	172.99p			Ha, Ha*
a-CH	59.93n	3.68 (1H, d, 10.5)	Hb,	Hb'
b-CH	35.71n	1.81 (1H, m)	Ha, Hb'	Hb', Hc', Hd
b'-CH_3	16.28n	0.64 (3H, d, 6.8)	Hb	Hc'
c-CH_2	27.12p	1.04 (1H, m)	Hc', Hd	Hb', Hd
		1.27 (1H, m)	Hc, Hd	
d-CH_3	10.65n	0.71 (3H, t, 7.2)	Hc, Hc'	Hc'
Ile**				
CO	174.50p			Ha
a-CH	55.80n	4.24 (1H, d, 10.1)	Hb,	Hb',
b-CH	38.35n	1.69 (1H, m)	Ha, Hb'	Hb', Hc', Hd
b'-CH_3	15.16n	0.80 (3H, d, 7.2)	Hb	Hc'
c-CH_2	26.08p	1.09 (1H, m)	Hc, Hd	Hb', Hd
		1.58 (1H)	Hc', Hd	
d-CH_3	11.03n	0.80 (1H, t, 7.2)	Hc, Hc'	Hc'
Phe				
CO	172.78p			Ha, Hb, Hb'
a-CH	55.62n	4.32 (1H, dd, 11.4/4.7)	Hb, Hb'	Hb, Hb'
b-CH_2	38.41p	2.84 (1H, t, 11.9)	Ha, Hb'	Ha, H2, H6
		3.09 (1H)	Ha, Hb	
Ar-C1	136.87p			Hb, Hb',H3, H5
Ar-C2,6	130.64n	7.14 (2H, d, 7.5)	H3, H5	Hb, Hb', H4,
Ar-C3,5	130.06n	7.23 (2H, t, 7.3)	H2, H6, H4	H5, H3
Ar-C4	128.68n	7.22 (1H, t, 7.3)	H3, H5	H2, H6
Tyr				
CO	170.46p			Ha, Hb, Hb'
a-CH	54.33n	4.57 (1H, dd, 6.9/3.6)	Hb'	Hb, Hb'
b-CH_2	37.63p	2.99 (1H)	Hb'	Ha, H2, H6
		3.05 (1H)	Ha, Hb	
Ar-C1	127.35p			Ha,Hb, Hb', H3, H5
Ar-C2,6	131.98n	6.78 (2H, d, 8.3)	H3, H5	Hb, Hb'
Ar-C3,5	116.13n	6.59 (2H, d, 8.5)	H2, H6	H2, H6
Ar-C4	157.77p			H2, H6, H3, H5

*, **, *** Denotes individual amino acid units of the same type without any implied sequence information.

For the coupling constants refer to (Hz) and the n and p notations correspond to APT results in which n indicates one or three protons attached, and p indicates no or two protons attached.



1, Phakellistatin 2

m/z 261 358 505 602 715

1) Pro - Tyr - Pro - Phe - Pro - Ile - Ile

m/z 245 342 455 568 665

2) Pro - Phe - Pro - Ile - Ile - Pro - Tyr

m/z 211 324 421 584 681

3) Pro - Ile - Ile - Pro - Tyr - Pro - Phe

Fig. 1 Phakellistatin 2 (1) peptide sequence determination by tandem MS/MS