ISOLATION AND STRUCTURE OF THE HUMAN CANCER CELL GROWTH INHIBITORY
PHAKELLISTATIN 4 FROM THE WESTERN PACIFIC SPONGE PHAKELLIA COSTATA[1]

George R. Pettit,* Jun-ping Xu, Zbigniew Cichacz, Jean M. Schmidt,
and Ann-Christine Dorsaz

*Cancer Research Institute and Department of Chemistry, Arizona State
University, Tempe, AZ 85287-1604, USA*

Michael R. Boyd

*Laboratory of Drug Discovery Research and Development, Developmental
Therapeutics Program Division of cancer Treatment, National Cancer
Institute, Frederick Cancer Research and Development Center,
Frederick, MD 21702-1201, USA*

Ronald L. Cerny

*Midwest Center for Mass Spectrometry, Department of Chemistry,
The University of Nebraska-Lincoln, Lincoln, Nebraska 685-0362, USA*

Abstract - Structural elucidation by high field 2D nmr and FAB-ms/ms

techniques led to *cyclo*-(Pro-Thr-Pro-Phe-Ile-Phe-Ser) as the

assignment for phakellistatin(4)a new cell growth inhibitory peptide

from the Chuuk Archipelago marine sponge *Phakellia costata*.

Evidence[2-10] is rapidly increasing that certain marine sponges in the class Demospongiae

produce cyclic peptides with cancer cell growth inhibitory properties. In rapid pursuit of

this potentially important source of new antineoplastic biosynthetic products, we have

extended our earlier evaluation of the *Phakellia* genus[5-7] with emphasis on *Phakellia costata*

Kiechnick (order Axinellida) collected (1985-87) in the Chuuk Archipelago (Federated States

of Micronesia). The original murine P388 lymphocytic leukemia cell line active cyclic

peptide lead from this Porifera species was found to be phakellistatin (1)[5] and that was

followed by isolation of phakellistatins (2)[6] and (3).[7]

A P388 cell line active methylene chloride soluble fraction prepared from 500 kg (wet wt.)

of *P. costata* recollected (1987) in Chuuk was separated (murine P388 lymphocytic leukemia

bioassay guided) by a series of gel permeation (Sephadex LH-20) and partition (LH-20 and

silica gel) column chromatographic techniques interspersed by high speed countercurrent-

distribution procedures.[5] Final separation was accomplished by hplc (on C8, with

acetonitrile-methanol-water, 10:10:13, as mobile phase) to afford phakellistatin(4)(45 mg,

$9 \times 10^{-6}\%$ yield, $[\alpha]_D$-97°, c,2.24, CH_3OH) as an amorphous powder (P388, ED_{50} 0.325 μg/ml).

Phakellistatin 4 showed a molecular ion peak at m/z 790 $[M+H]^+$ in the FAB-ms spectrum. The

molecular formula was established as $C_{41}H_{56}N_7O_9$ by HRFAB-ms [m/z 790.4146 $(M+H)^+$, Δ+0.6 mmu].

Two groups of ultraviolet absorption maxima appeared at 210-240 nm and 251-267 nm which were

attributed to a mono-substituted benzene ring. The 500 MHz and 400 MHz ^{1}H, ^{13}C-nmr and APT

spectra gave well separated signals which enabled straightforward interpretation of the COSY

spectra. These data together with results of extensive HMQC and HMBC experiments led to the

signal assignments and relationships noted in Table 1 for phakellistatin(4)and its assignment

as a heptapeptide composed of seven α-amino acid units viz, serine (Ser), isoleucine (Ile),

threonine (Thr), proline (Pro x 2) and phenylalanine (Phe x 2). Those assumptions were

further confirmed by results of amino acid analysis of the phakellistatin(4)acid hydrolysis

product.
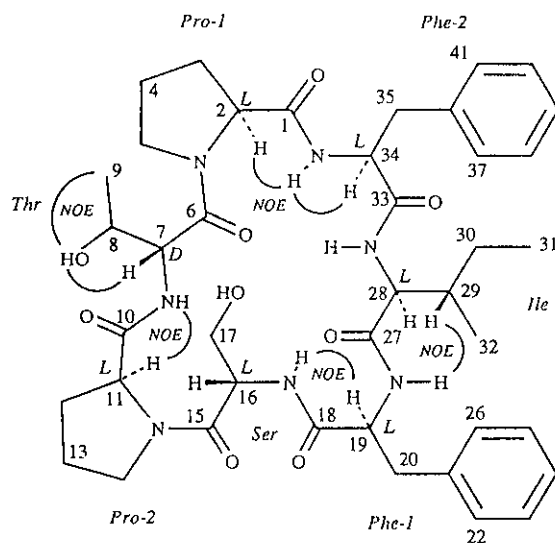
Phakelliastatin(4)and Principle NOE Correlations

HMBC and ROE data provided important information about the amino acid sequence in this

heptapeptide. Specifically, HMBC correlations from amide NH protons to the amide carbonyl

carbon signals such as NH [Thr] (δ 8.67)/C10 [Pro 2] (δ 171.81), NH {Ser} (δ 8.90)/C18 {Phe

1] (δ 173.55), NH [Phe 1] (δ 8.55)/C27 [Ile] (δ 173.50), NH [Ile] (δ 7.60)/C33 [Phe 2] (δ 171.74) and NH [Phe 2] (δ 7.68)/C1 [Pro 1] (δ 170.32) were very useful. The ROESY spectrum afforded the following NOE correlations between four amide NH protons and four amino acid α-methine protons, NH [Thr]/H11 [Pro 2] (δ 5.05), NH [Ser]/H19 [Phe] (δ 4.98), NH [Ile]/H34 [Phe 2] (δ 4.81) and NH [Phe 2]/H2 [Pro 1] (δ 4.37). Furthermore, another ROE correlation between the amide NH proton of Phe 1 and the β-methine proton of Ile (H29, δ 2.10) was found. That result added more support for the Ile-Phe 1 linkage, and revealed that the amide H-N in Phe 1 to be oriented close to the C-Hβ bond of Ile. The correlations from HMBC and ROESY experiments clearly indicated the sequence of the seven amino acid units as Pro 2-Thr-Pro 1-Phe 2-Ile-Phe 1-Ser. Although no HMBC or NOE correlations were found between Pro 2 and Ser, the amide nitrogen atom of Pro 2 was assumed to be bonded to the carboxyl carbon of Ser. Because the molecular formula ($C_{41}H_{56}N_7O_9$) indicated phakellistatin (4) to be a cyclic heptapeptide that assumtpion seemed secure.

Table 1 :   The ^{1}H- and ^{13}C-nmr Spectral Data of Phakelliastatin 4 and Their Assignments (in CDCl3)

No.	^{13}C ppm	^1H ppm	J (Hz)	HMBC (^1H to ^{13}C)	No.	^{13}C ppm	^1H ppm	J (Hz)	HMBC (^1H to ^{13}C)
Pro 1	170.32 s				20	37.95 t	2.82 t	10.5	19
-1 2	60.56 d	4.37 dd	5.2,8 4	1,3,4,5			3.08 m		18,19,21,22
3	29.04 t	1.62 m			21	136.43 s			
		2.10 m		1,2,4	22	129.41 d	7.25 m		
4	24.63 t	1.55 m			23	126 75 d	7.18 m		
		1.82 m			24	128.37 d	7.27 m		
5	47.36 t	3.50 m			25	126 75 d	7 18 m		
		3 75 m			26	129.41 d	7.25 m		
Thr 6	170.15 s				NH		8.55 d	8.4	19,20,27
7	58.49 d	4.80 m	6,8		Ile 27	173 50 s			
8	65.82 d	4.47 m		9	28	66 10 d	3 12 m		27,29,30,32
9	19.16 q	1.35 d	6.3	8	29	32.74 d	2.10 m		
OH		5.32 brd			30	25.23 t	0 52 m		
NH		8.67 d	8.7	10			0.92 m		
Pro 10	171.81 s				31	10.46 q	0 59 t	7.0	29,30
-2 11	61.25 d	5 05 m			32	14.80 q	0.15 d	6.6	29,30
12	29.28 t	1.92 m		10,11,13	NH		7.60 m		
		2.58 m	11.5,6.4	14	Phe 33	171.74 s			
13	22.02 t	1.70 m			-2 34	53 40 d	4.81 m		33,35
		2.05 m			35	37.95 t	2.92 dd	8.0,13.7	33,34,36,37
14	46.18 t	3.35 m		12			3.05 m		33,34,36,37
		4.00 m		12	36	136.98 s			
Ser 15	170.32 s				37	129.30 d	7.24 m		35
16	54.37 d	4.75 m			38	126.56 d	7.17 m		
17	61.40 t	3.55 dd	8.7,9.3	15,16	39	128.37 d	7.27 m		
		3.65 m		15,16	40	126.56 d	7.17 m		
NH		8.90 d	4.6	18	41	129.30 d	7.24 m		35
Phe 18	173.55 s				NH		7.68 d	9.2	1
-1 19	53.68 d	4.98 m		18,20,27					

The sequence was further confirmed by results of FAB-ms/ms analyses.

Collisional activation of the $(M + H)^+$ ions of phakellistatin(4) produced immonium ions

characteristic of Ser (m/z 60), Pro (m/z 70), Thr (m/z 74), Leu/Ile (m/z 86), and Phe (m/z

120) and combined with the determined exact mass of $[M + H]^+$ requires an amino acid

composition of 2 x Pro, 2 x Phe, 1 x Leu (Ile), 1 x Ser, and 1 x Thr. Protonation of the

amide nitrogen of either of the two prolines or of the serine upon FAB results in ring

opening and the formation of three different acyllium ions. The CAD spectrum of the $[M + H]^+$

ions contained three series of fragment ions (Figure 1) that define the sequence as cyclo-

(Pro-Thr-Pro-Phe-Ile-Phe-Ser). Other ions observed in the CAD spectrum provided additional

confirmation for the sequence.   These internal fragments, resulting from two backbone

cleavages proved to be due to Phe-Ile (or Ile-Phe) of m/z 261, Phe-Ser of m/z 235, Phe-Ser-

Pro-Thr of m/z 433, Thr-Pro-Phe of m/z 346, Thr-Pro-Phe-Ile of m/z 459, and Ile-Phe-Ser of

m/z 348. In addition, ions resulting from elimination of CO from acyllium ions (the ion of

m/z 217 arises from the ion of m/z 245, for example) were also observed. Therefore, the

structure of phakellistatin(4) was deduced to be cyclo-(Pro-Phe-Ile-Phe-Ser-Pro-Thr).
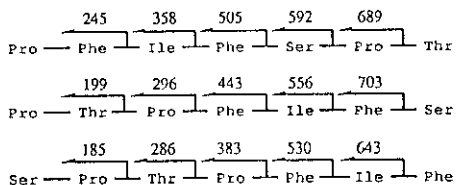
| | 245 | 358 | 505 | 592 | 689 | |
| Pro — Phe | Ile | Phe | Ser | Pro | Thr |

| | 199 | 296 | 443 | 556 | 703 | |
| Pro — Thr | Pro | Phe | Ile | Phe | Ser |

| | 185 | 286 | 383 | 530 | 643 | |
| Ser — Pro | Thr | Pro | Phe | Ile | Phe |

Figure 1 : Phakellistatin 4 sequence determining MS/MS fragment ions

The absolute configuration of phakellistatin (4) was ascertained by analyzing the acid

hydrolysate converted to N-pentafluoropropionyl isopropyl ester derivatives and using a

chiral gc (chirasil-Val III column).[5] By comparing the retention time of each amino acid

derivative with those of the authentic S and R amino acids, evidence was obtained that all

of the Ser, Ile, Pro and Phe units corresponded to the (S)-configuration while the Thr unit

possessed the (R)-configuration.

Phakellistatin (4) displayed significant in vitro growth inhibitory activity against murine

lymphocytic leukemia P388 (ED_{50} 0.32 μg/ml), and a selection of human cancer cell lines: ovarian (OVACR-3: GI_{50} 0.24 μg/ml), CNS (SF-295: GI_{50} 0.5 μg/ml), renal (A498: GI_{50} 0.81 μg/ml), lung (NCI-H460: GI_{50} 0.34 μg/ml, colon (KM20L2: GI_{50} 0.35 μg/ml) and melanoma (SK-MEL-5: GI_{50} 0.19 μg/ml). When phakellistatin(4)was evaluated (quadruplicate experiments) against the U. S. National Cancer Institute (NCI) human cancer cell line panel[11-13] a distinctive and reproducible mean graph profile[12] was obtained. The mean panel GI_{50} values were found to be 0.6 μM. Interestingly, compare analyses did not reveal a strong correlation with any of the NCI standards[11] with known mechanisms of action

Interestingly, phakellistatin(4)is the first marine sponge peptide we have encountered with an (R)-amino acid unit. When our total synthetic studies directed at increasing the availability of such potentially useful marine invertebrate cyclic peptides are further advanced, we will obtain definative evidence for the exact chirality of the Thr unit.

ACKNOWLEDGMENT

REFERENCES

1.   (a) Dedicated to Professor Rolf Huisgen on the occasion of his 75th birthday. (b) Contribution 303 of the series Antineoplastic Agents. For part 302 see G. R. Pettit,

Z. A. Cichacz, F. Gao, M. R. Boyd, C. L. Herald, and J. M. Schmidt, *J. Chem. Soc., Chem. Commun.*, accepted.

2. G. R. Pettit, P. J. Clewlow, C. Dufresne, D. L. Doubek, R. L. Cerny, and K. Rutzler, *Can. J. Chem.*, 1990, *68*, 708.

3. G. R. Pettit, C. L. Herald, M. R. Boyd, J. E. Leet, C. Dufresne, D. L. Doubek, J. M. Schmidt, R. L. Cerny, J. N. A. Hooper, and K. C. Rützler, *J. Med. Chem*, 1991, *34*, 3339.

4. G. R. Pettit, J. K. Srirangam, D. L. Herald, K. L. Erickson, D. L. Doubek, J. M. Schmidt, L. P. Tackett, and G. J. Bakus, *J. Org. Chem.*, 1992, *57*, 7217.

5. G. R. Pettit, Z. A. Cichacz, J. Barkoczy, A-C. Dorsaz, D. L. Herald, M. D. Williams, D. L. Doubek, J. M. Schmidt, L. P. Tackett, D. C. Brune, R. L. Cerny, J. N. A. Hooper, and G. J Bakus, *J. Nat. Prod.*, 1993, *56*, 260.

6. G. R. Pettit, R. Tan, M. D. Williams, L. P. Tackett, J. M. Schmidt, R. L. Cerny, and J. N. A. Hooper, *BioMed. Chem. Lett.*, 1993, *3*, 2869.

7. G. R. Pettit, R. Tan, D. L. Herald, and M. D. Williams, *J. Org. Chem.*, in press.

8. G. R. Pettit, F. Gao, and R. L. Cerny, *Heterocycles*, 1993, *35*, 711.

9. G. R. Pettit, F. Gao, R. L. Cerny, D. L. Doubek, L. P. Tackett, J. M. Schmidt, and J-C. Chapuis, *J. Med. Chem.*, in press.

10. J. Kobayashi, M. Tsuda, T. Nakamura, Y Mikami, and H. Shigemore, *Tetrahedron*, 1993, *49*, 2391.

11. M. R. Boyd, "Status of the NCI Preclinical Antitumor Drug Discovery Screen", in *CANCER: Principles and Practice of Oncology UpdateS*, Vol. 3, No. 10, ed. by V. T. DeVita, Jr., S. Hellman, and S. A. Rosenberg, Lippincott, Philadelphia, 1989, pp. 1-12.

12. M. R. Boyd, K. D. Paull, and L. R. Rubinstein, "Data Display and Analysis Strategies for the NCI Disease-oriented *in vitro* Antitumor Drug Screen", in *Cytotoxic Anticancer Drugs: Models and Concepts for Drug Discovery and Development*, ed. by A. Valeriote, T. Corbett, and L. Baker, Kluwer Academic Publishers, Amsterdam, 1992, pp. 11-34.

13. M. R. Boyd, "The Future of New Drug Development", in *Current Therapy in Oncology*, ed. by J. E. Niederhuber, B. C. Decker, Inc., Philadelphia, 1993, pp. 11-22.

Received, 9th May, 1994