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Antineoplastic Agents 390. Isolation and Structure of Phakellistatin 12 from a Chuuk Archipelago Marine Sponge†

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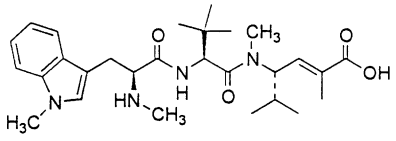
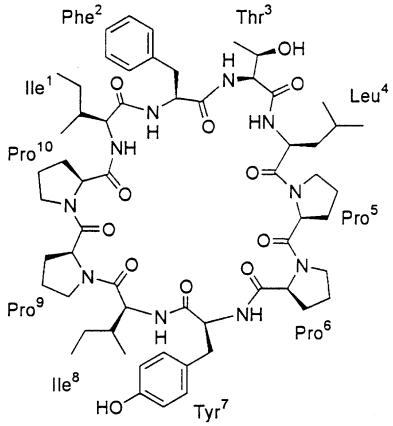
Abstract—A new cancer cell growth inhibitory (P388 lymphocytic leukemia ED_{50} 2.8 μg/mL) cyclodecapeptide designated phakellistatin 12 (**2**) has been isolated as a trace (1.7×10^{-6}% yield) constituent of the Western Pacific Ocean (Federated States of Micronesia-Chuuk) sponge *Phakellia* sp. Employing principally a combination of high resolution FAB with high field (500 MHz) ^{1}H, ^{13}C and 2-D NMR and chiral GC analyses the structure (all S chirality) *cyclo*-Ile-Phe-Thr-Leu-Pro-Pro-Tyr-Ile-Pro-Pro was assigned.

Marine Porifera species of quite diverse taxonomy and geographical origin are proving to be a very productive resource for discovery[2] of new and biologically active peptides. Some[2b–e,g–i] of these are proving to have cancer cell growth inhibitory properties and, for example, hemiasterlin (**1**)[3a] as well as kahalalide[2g] have exhibited substantial antineoplastic activity. Structural modification of such leads[3] should prove useful.

As part of an investigation of the marine sponge genus *Phakellia costata* (class Demospongiae, order Axinellida) focused on cyclic peptide cancer cell growth inhibitory constituents we found the Indo-Pacific (Truk Archipelago) *P. costata* to contain the cycloheptapeptides phakellistatins 1[4a] and 6[4b] cyclodecapeptides phakellistatins 7–9[4c] and, for example, the Western Indian Ocean (Republic of the Comoros) *Phakellia carteri* to produce cycloheptapeptides phakellistatin 2,[4d] 3, and isophakellistatin 3[4e] Later, we discovered the cyclooctapeptide phakellistatins 10 and 11[4f] in the Chuuk (Federated State of Micronesia) *Phakellia* sp. We now report that further detailed study (P388 lymphocytic leukemia cell line bioassay directed) of trace fractions from the latter Western Pacific yellow-orange Porifera species (collected in 1986–1987) has led to a new cancer cell growth inhibitory (P388 ED_{50} 2.8 μg/mL) cyclodecapeptide designated phakellistatin 12 (**2**): 8.6 mg, 1.7×10^{-6}% yield from 500 kg wet wt of *Phakellia* sp., colorless amorphous solid, mp 199–201 °C, $[\alpha]_D^{25}$ −132° (*c* 0.105, CH_3OH) and R_f 0.2 on SiO_2 in 15:1 CH_2Cl_2–CH_3OH. One of the trace P388 active fractions from the final hexane–2-propanol–methanol (8:1:1) partition chromatogram on Sephadex LH-20[4f] was further separated by reversed-phase HPLC (C8, CH_3OH–CH_3CN–H_2O, 3:3:4) to afford phakellistatin 12 (**2**) and the cycloheptapeptides



1, hemiasterlin



2, phakellistatin 12

†See ref 1.

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axinastatins 1[4g] (4.1 mg, 8.2×10^{-7}%) and 3 (in a 5.0-mg mixture, as evidenced by ^{1}H NMR and MS) we previously discovered in the Republic of Palau marine sponge *Axinella* sp. Phakellistatin 12 exhibited UV (CH_3OH) λ_{max} (log ε) 222 (4.31), 254 (3.58) and 269 (3.52) nm and IR (film) V_{max} 3376, 2963, 2878, 1632, 1514 and 1452 cm^{-1}. The molecular formula $C_{60}H_{86}N_{10}O_{12}$ was established by HRFAB MS (*m*/*z* 1161.6298 for $[M+Na]^+$, calcd 1161.6324). Analysis of the UV, IR, MS and NMR data indicated a cyclic peptide structure for phakellistatin 12 with 23 unsaturation units. The overall structure was elucidated employing high field NMR (500 MHz techniques).

The ^{1}H and ^{13}C NMR spectra of peptide **2** revealed the presence of 10 carbonyl components (cf. Table 1) and six protons bonded to amide nitrogen atoms. Further interpretation of the 2-D NMR spectra established a phenylalanine unit by recognition of five aromatic protons in three connected spin systems (δ 7.33, 2H, t, $J=7.5$ Hz; δ 7.31, 2H, d, $J=6.0$ Hz and δ 7.29, 1H, t, $J=7.3$ Hz) and HMBC correlations between Ar–CH (δ 129.94/7.31) and β-CH_2 (δ 38.14/3.16, 3.00). A tyrosine unit was defined by a *para*-substituted aromatic ring with its hydroxyl-bonded carbon shifted down field to δ 154.19 ppm combined with HMBC signals for the aromatic components (δ 130.94/7.02) and the β-CH_2 (δ 32.02/3.51, 3.27).

From examination of the COSY and TOCSY spectra, it was concluded that six of seven methyl groups (four in doublets at δ 1.03, 0.97, 0.90 and 0.83 and two in

Table 1. Phakellistatin 12 (**2**) ^{1}H and ^{13}C NMR data (in $CDCl_3$)

Unit	^{13}C (multiplicity)	^{1}H (*J* in Hz)	HMBC (H to C)	Unit	^{13}C (multiplicity)	^{1}H (*J* in Hz)	HMBC (H to C)
Ile[1]				Pro[6]			
NH		8.62 (br, s)		CO	170.84s		
CO	172.82s[a]			α-CH	61.22d	4.10 (d, 8.0)	CO(Pro[5]), CO(Pro[6]) β-C, δ-C
α-CH	57.53d	4.19 (m)		β-CH_2	31.39t	2.65 (dd, 12/6)	
β-CH	32.88d	2.06 (m)				1.98 (m)	α-C, γ-C
γ-CH_3	16.45q	0.90 (d, 7.0)	α-C, β-C, γ-C	γ-CH_2	22.30t	1.89 (m)	
γ-CH_2	24.91t	1.39 (m)				1.62 (m)	
		1.10 (m)		δ-CH_2	46.38t	3.48 (m)	γ-C
δ-CH_3	9.93q	0.80 (t, 7.5)	β-C, γ-C			3.40 (m)	β-C
Phe[2]				Tyr[7]			
NH		7.06 (br, s)		NH		9.14 (br, s)	
CO	172.27s[a]			CO	170.74s[a]		
α-CH	58.66d	4.19 (m)		α-CH	57.72d	4.28 (m)	
β-CH_2	38.14t	3.16 (dd, 13.5/10)	2-C	β-CH_2	32.02t	3.51 (m)	
		3.00 (d, 11)				3.27 (d, 12)	
1-C	136.78s			1-C	136.75s		
2,6-C	129.94d	7.31 (d, 6.0)	β-C, 2,6-C, 4-C	2,6-C	130.94d	7.02 (d, 8.5)	β-C, 2,6-C, 4-C, 3,5-C
3,5-C	128.70d	7.33 (t, 7.5)	1-C, 3,5-C	3,5-C	114.60d	6.57 (d, 8.5)	4-C, 2,6-C, 4-C
4-C	126.83d	7.29 (t, 7.3)	2,4-C	4-C	154.21s		
Thr[3]				Ile[8]			
NH		7.23 (br, s)		NH		6.63 (br, s)	
CO	170.84s[a]			CO	171.16s		
α-CH	58.70d	4.60 (m)		α-CH	54.63d	4.56 (t, 8.5)	β-C, γ-C, γ′-C
β-CH	67.31d	4.62 (m)		β-CH	38.53d	1.63 (m)	
γ-CH_3	20.03q	1.10 (d, 6.0)	β-C	γ-CH_3	14.92q	1.03 (d, 6.5)	α-C, β-C, γ-C
				γ′′ CH_2	24.36t	1.62 (m), 1.04 (m)	
Leu[4]				δ-CH_3	11.03q	0.86 (t, 8.0)	β-C
NH		7.02 (br)	CO (Thr[3])				
CO	170.74s			Pro[9]			
α-CH	48.97d	4.64 (m)		CO	170.25s[a]		
β-CH_2	41.69t	1.37 (m)		α-CH	58.82d	3.40 (m)	
γ-CH	24.54d	1.55 (m)		β-CH_2	28.27t	2.00 (m)	CO
δ-CH_3	23.77q	0.83 (d, 7.0)	β-C, δ′-C			1.67 (m)	
δ′-CH_3	21.76q	0.97 (d, 6.5)	β-C, γ-C, δ-C	γ-CH_2	25.44t	2.08 (m), 1.77 (m)	
				δ-CH_2	48.10t	3.96 (m), 3.60 (m)	β-C
Pro[5]							
CO	170.25s			Pro[10]			
α-CH	58.73d	3.44 (m)		Co	171.25s		
β-CH_2	28.93t	2.00 (m)	γ-C, CO	α-CH	61.13d,	3.95 (m)	
		1.77 (m)		β-CH_2	31.39t	2.55 (m)	
γ-CH_2	25.30t	2.03 (m)				2.08 (m)	
		2.00 (m)		γ-CH_2	22.24t	1.95 (m), 1.67 (m)	
δ-CH_2	46.87t	3.62 (m), 3.38 (m)	β-C	δ-CH_2	46.66t	3.51 (2H, m)	

[a]Assignments might be exchanged.

triplets, δ 0.86 and 0,80) were divided into three pairs, which corresponded to one leucine and two isoleucine units respectively. The only down field methyl group (δ 1.10, d, $J = 6$ Hz) was assigned to a threonine unit by observing HMBC cross signals from the methyl with both α-CH (δ 58.70/4.60) and β-CH (δ 67.31/4.62). The remaining 12 methylene, four methine and four carbonyl groups were assigned by results of COSY, TOCSY and HMBC analyses to represent four proline units. That left only one of the 23 sites of unsaturation unaccounted for with the missing one satisfied by a cyclic structure.

The amino acid sequence was partially deduced based on ROESY spectrum analysis. With this structure the HMBC experiments provided little useful information as the carbonyl carbon signals were too congested. A series of NOE interactions observed at δ 4.19/7.06 (α-H of Ile^1/NH of Phe^2), 7.06/7.23 (NH of Phe^2/NH of Thr^3), 7.23/7.02 (NH of Thr^3/NH of Leu^4), 4.60/7.02 (α-H of Thr^3/NH of Leu^4), 4.64/3.62, 3.38 (α-H of Leu^4/δ-2H of Pro^5), 3.44/4.10 (α-H of Pro^5/α-H of Pro^6), and 4.10/9.14 (α-H of Pro^6/NH of Tyr^7) suggested partial sequence Ile^1-Phe^2-Thr^3-Leu^4-Pro^5-Pro^6-Tyr.[7] Another segment was assigned to Ile^8-Pro^9 by considering the cross peak at δ 4.56/3.96, 3.60 (α-H of Ile^8/δ-2H of Pro^9). Results of these NMR analyses allowed the structure of phakellistatin 12 to be assigned *cyclo*-Ile^1-Phe^2-Thr^3-Leu^4-Pro^5-Pro^6-Tyr^7-Ile^8-Pro^9-Pro^{10}. With the complete peptide sequence in hand, it became possible to assign cross peaks in the ROESY spectrum at δ 6.63/7.02 (NH of Ile^8/NH of Leu^4), 6.63/4.60 (NH of Ile^8/α-H of Thr^3) and 7.02/4.28 (NH of Leu^4/α-H of Tyr^7). Apparently, these interactions are the result of inner ring hydrogen bonds between the carbonyl and NH groups of Leu^4 and Tyr^7. Furthermore, Leu^4 and Ile^8 were *trans* amide bonded to their adjacent units Pro^5 and Pro^9. That was implied by their nOe results and the differences between the ^{13}C chemical shifts of the β- and γ-carbons ($\Delta_{\beta,7} = 3.63/3.83$ ppm, respectively).[5,6] However, Pro^6 and Pro^{10} were found linked by *cis* amide bonds to the next units (Pro^5/Pro,[6] Pro^9/Pro^{10}). Importantly, the analogous bonding has been confirmed by our recent X-ray crystal structure analysis of the cyclodecapeptide phakellistatin 8[7] where the Thr^3 of phakellistatin 12 is substituted by Val^3.

The absolute configuration of each component amino acid unit in phakellistatin 12 (**2**) was determined by results of chiral GC experiments.[8,9] Both the peptide acid hydrolyate and *S*- and *R*-amino acid standards were converted to *N*-pentafluoropropionylisopropyl ester derivatives. Based on comparisons of the GC data, all the α-amino acid carbons in phakellistatin 12 were assigned the *S*-configuration.

The close structural relationship of the marine invertebrate phakellistatins 8[7] and 12 to the antamanide-type constituents of the mushroom *Amanita phalloides* presents some interesting questions in evolutionary biology and genetics. The cancer cell growth inhibitory activity and other biological properties of phakellistatin 12 are being further evaluated and a total syntheses[10] will be undertaken for purposes of structural and biological[11] confirmation.

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