Isolation and Structure of Phakellistatin 14 from the Western Pacific Marine Sponge *Phakellia* sp.[†,1]

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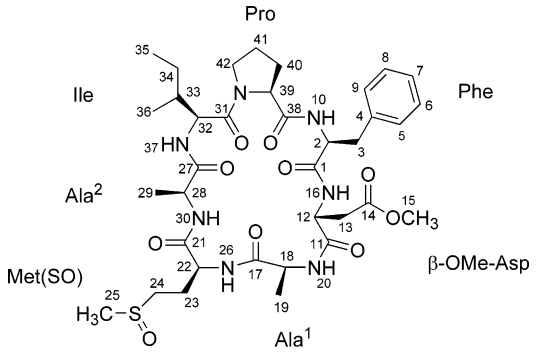
A new cycloheptapeptide, phakellistatin 14, was isolated in 8.8×10^{-7}% yield from *Phakellia* sp., a marine sponge from Chuuk, Federated States of Micronesia. The structure (**1**), cyclo-Phe-β-OMe-Asp-Ala-Met-(SO)-Ala-Ile-Pro, was elucidated by 1D and 2D NMR spectral analyses augmented by HRFABMS. The chirality of each amino acid unit was determined to be *S* using chiral HPLC methods. Phakellistatin 14 showed cancer cell growth inhibitory activity (ED_{50} 5 μg/mL) against the murine lymphocytic leukemia P388 cell line.

As earlier anticipated,[2] marine organisms are now well established as an especially productive source of structurally unique cancer cell growth inhibitors and in vivo active anticancer drugs.[3] Relatively small molecular weight peptides are quite prominent among marine organism constituents. Illustrative are the dolastatins, where the 10th in the series[4] has been undergoing human cancer phase II clinical trials[3] along with the structural modification we synthesized and designated as auristatin PE (a k a TZT-1027).[3a] Structural modifications of the linear marine peptide dolastatin 15 in phase II human cancer clinical trials are Cemadotin and Ilex 651.[3] Another such advance is based on the cyclic peptides kulolides 1−3.[3b] Other more recent reports of such interesting cyclic peptides include homodolostatin 16[5a] from a Kenyan specimen of *Lyngbya majuscula*, ulongapeptin[5b] from a Palau collection of *Lyngbya* sp., leucamide A[5c] from an Australian sponge, and renieramide[5d] from a Vanuatu sponge.

As a result of our previous research directed at discovering new anticancer active peptides, the marine porifera genus *Phakellia* was found to be a productive source of cancer cell growth inhibitory cyclic peptides. Such investigations employing *Phakellia carteri* from Republic of the Comoros and *Phakellia* sp. from Chuuk in the Federated States of Micronesia led to the isolation and structural elucidation of the active cyclopeptides designated phakellistatins 1−12,[6] albeit generally as trace constituents. The present study was focused on an active (murine P388 lymphocytic leukemia ED_{50} 0.0042 μg/mL) fraction from the above Western Pacific Ocean collection of *Phakellia* sp. that led to isolation and structural elucidation of a new cycloheptapeptide herein named phakellistatin 14.[7]

Results and Discussion

On the basis of the bioassay results against the murine P388 lymphocytic leukemia and a panel of human cancer cell lines, the CH_2Cl_2−CH_3OH extract of *Phakellia* sp. was initially separated by a solvent partition sequence followed by a series of gel permeation and column partition chromatographic separations employing Sephadex LH-20[6,8] as previously reported. Further purification of a trace cancer cell line active fraction by reversed-phase HPLC on a Prepex C8 column with acetonitrile−methanol−water (3:3:4) as eluent afforded the new cycloheptapeptide phakellistatin 14 (**1**, 4.4 mg, 8.8×10^{-7}% yield) as a colorless amorphous powder, mp 189−191 °C, $[\alpha]^{25}_D$ −64.86° (*c* 0.28, CH_3OH).



phakellistatin 14 (**1**)

General analyses of ^1H and ^{13}C NMR, APT, and HMQC signals showed characteristic peptide resonances including eight carbonyls, six NH groups (two appeared in two sets of signals, δ_H 7.79/7.68, 7.66/7.28), seven α-CH groups, and a number of CH_2 and CH_3 groups (Table 1). A comprehensive interpretation (TOCSY, COSY, and HMBC) of the 2D NMR spectra recorded in CD_2Cl_2 indicated seven amino acid units: Ala (×2), Ile, Phe, Pro, an Asp-derivative, and a Met-derivative. The Met unit appeared in two sets of frequencies in an approximate 1:1 ratio (Table 1). For this reason, the NMR experiments were also conducted in CD_3CN−CD_3OD, which revealed additional information about the amino acid units. The Met-methyl groups corresponded to two sets of ^{13}C and ^1H signals at δ_C/δ_H 38.27/2.61, 38.13/2.59. Because of the crucial role of the NH groups in determining the amino acid sequence of cycoheptapeptide **1**, subsequent discussion of the structural analyses will concentrate on spectral data obtained employing deuterodichloromethane as NMR solvent.

The Asp-derivative was found to exhibit a downfield methyl singlet (δ_C/δ_H 52.75/3.74), associated with the β-carbonyl group (δ_C 173.32) in the HMBC experiment. Thus, the Asp unit contained a β-methyl ester in place of the usual β-carboxyl group. Structural determination of the Met-derivative focused on a sulfur atom bonded to meth-

[†] Dedicated to the memory of Dr. Cecil R. Smith, Jr. (1924−2004), an outstanding United States Department of Agriculture (USDA) research chemist, who led discovery of the harringtonine series of anticancer drugs.

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Table 1. ^{1}H and ^{13}C Spectral Assignments for Phakellistatin 14 (**1**) Recorded in CD_2Cl_2

position	δ ^{13}C	δ ^{1}H	HMBC	ROESY
Phe				
1-CO	172.54			
2-CH	58.60	4.26 (ddd, 6/12/12)	C1, C3, C4	H16, H3, H10
3-CH_2	37.61	3.22 (m)	C2, C4 C5, C9	H16, H2
		3.08	C1, C2, C4	H16, H2, H10
4-C	137.80			
5, 9-CH	129.29	7.24 (d, 7)	C3, C6, C7, C8	
6, 8-CH	128.95	7.32 (t, 6.5)	C4, C5, C9	
7-CH	127.24	7.24 (d, 7)	C6, C8	
10-NH		8.40 (d, 7)	C38, C2, C3	H32, H16, H2, H3b
β-OMe-Asp				
11-CO	172.06			
12-CH	49.83	4.53 (m)		H20, H13, H16
13-CH_2	36.46	3.24 (m)	C11, C12, C14	H12
		3.09 (m)	C12, C14	H20, H12, H16
14-CO	173.32			
15-OCH_3	52.75	3.74 (s)	C14	
16-NH		7.66 (d, 4.0)	C12	H32, H12, H3b, H10
Ala[1]				
17-CO	172.97			
18-CH	53.53	3.96 (m)	C17, C19	H26, H19
19-CH_3	16.95	1.43 (d, 9.5)	C17, C18	H26, H18, H20
20-NH		7.79 (s, br)	C17	H19, H12
		7.68 (d, 5)		H30, H22, H22′, H19, H13b
Met(SO) (chiral a)				
21-CO	170.69			
22-CH	53.26	4.41 (ddd, 4.5/10/10)		H23
23-CH_2	25.40	2.42 (m)	C22, C24	H22
		2.27 (m)	C22, C24	H22
24-CH_2	50.61	2.95 (m)	C22, C23, C25	
		2.68 (m)	C22, C23, C25	
25-CH_3	38.41	2.56 (s)	C24	
26-NH		7.72 (s, br)	C17	H22′, H18, H19
Met(SO) (chiral b)				
21′-CO	170.69			
22′-CH	53.26	4.51 (m)		H26, H20
23′-CH_2	24.74	2.40 (m)	C22′, C25′	
		2.30 (m)	C22′	
24′-CH_2	50.86	2.85 (m)	C22′, C23′, C25′	
		2.76 (m)	C22′, C23′, C25′	
25′-CH_3	38.41	2.52 (s)	C24′	
26-NH		7.72 (s, br)	C17	H22′, H18, H19
Ala[2]				
27-CO	172.97			
28-CH	48.81	4.57 (q, 7.0)	C27, C29, C21	H37, H29, H30
29-CH_3	16.01	1.30 (d, 8.0)	C27, C28	H28, H30
30-NH		7.44 (d, 7.6)	C28	H28, H29, H20
Ile				
31-CO	170.94			
32-CH	58.18	4.09 (m)	C33, C34	H39, H33, H34, H36, H37, H16, H10
33-CH	36.89	1.75 (m)		H32, H34b, H36, H37
34-CH_2	25.33	1.72 (m)	C32, C35, C36	H32
		1.25 (m)	C33, C35, C36	H32, H33
35-CH_3	11.23	0.90 (t, 8.5)	C33, C34	
36-CH_3	15.39	0.92 (d, 8.0)	C32, C33, C34	H32, H33
37-NH		6.93 (s, br)		H32, H33, H28
Pro				
38-CO	171.06			
39-CH	61.40	4.36 (d, br, 7.5)	C38, C40, C41, C42, C31	H40, H41b, H32
40-CH_2	31.46	2.31 (m)	C38, C41, C42	H39, H41a
		1.75 (m)	C38, C39, C41	H39, H42a
41-CH_2	21.68	1.62 (m)	C39	H40a, H42a
		0.93 (m)		H39
42-CH_2	46.60	3.38 (m)	C41	H40b, H41a
		2.91 (m)	C40	

ylene (δ_{H24} 2.95, 2.68) and methyl (δ_{H25} 2.56) groups, and these signals were close to the regular resonance range of the $-CH_2-S-CH_3$ group.[9] However, in the ^{13}C NMR spectrum, the methylene group appeared at δ_{C24} 50.61 and the methyl at δ_{C25} 38.41 ppm, which evidenced greater deshielding as compared with the normal range for $-CH_2-S-CH_3$ (δ_C ~30 ppm or lower)[10] and pointed to a $-CH_2-S(O)-CH_3$ group.[11] That evidence indicated the Met-derivative unit of phakellistatin 14 was methionine sulfoxide. The IR spectrum with absorption at 1032 cm^{-1} also supported the sulfoxide assignment.[12] The two sets of NMR signals[13a] with a ratio of 1:1 for the Met(SO) unit suggested a 1:1 occurrence of epimer at the sulfoxide chiral center.

The sequence of amino acids in phakellistatin 14 was partially deduced to include Ala-Met(SO)-Ala and Ile-Pro-Phe-β-OMe-Asp by interpretation of HMBC correlations

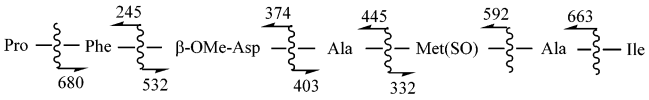

Figure 1. Mass spectral fragmentation of phakellistatin 14.

between carbonyls and protons of adjacent amide and α-positions: δ_C/δ_H 172.97/7.72 (Ala[1]-CO/Met(SO)-NH), 170.69/7.44 (Met(SO)-CO/Ala[2]-NH), 170.94/4.36 (Ile-CO/Pro-Hα), 171.06/8.40 (Pro-CO/Phe-NH), and 172.54/7.66 (Phe-CO/Asp ester-NH). The key sequential information missing from the HMBC spectra was supplied by the ROESY spectrum, which established cross-peaks for the α-protons and adjacent amide protons at δ_H/δ_H 4.53/7.79 (β-OMe-Asp-CH/Ala[1]-NH), 4.57/6.93 (Ala[2]-CH/Ile-NH). That completed the cycle connections for Phe-β-OMe-Asp-Ala[1]-Met(SO)-Ala[2]-Ile-Pro.

On the basis of interpretations of the high-resolution FAB mass spectrum, the ion peak at *m/z* 776.3698 $[M + H]^+$ provided the molecular formula $C_{36}H_{53}N_7O_{10}S$, which was consistent with the amino acid units deduced from its NMR analyses. Furthermore, FABMS/MS analyses (Figure 1) of phakellistatin 14 revealed ions corresponding to the sequential fragmentation losses expected from structure **1**.

The α-carbon stereochemistry of cycloheptapeptide **1** was established as all *S*-configurations by chiral HPLC analyses of the amino acid units found in the peptide hydrolysate. After phakellistatin 14 was hydrolyzed with propionic acid and hydrochloric acid,[14] the resultant amino acid residues were analyzed by chiral HPLC using direct comparison with authentic specimens (except for β-OMe-Asp) bearing the *S*-configuration. The β-OMe-Asp unit of phakellistatin 14 was, as expected, converted to aspartic acid under the above conditions. Treatment of commercial β-OMe-Asp under the same acid hydrolysis conditions yielded *S*-Asp, as identified during the chiral HPLC analysis of the phakellistatin 14 hydrolysate. Both the aspartic acid β-methyl ester and methionine sulfoxide units found in phakellistatin 14 are unique to this series of marine sponge peptides. Other occurrences of methionine sulfoxide in naturally occurring peptides have been reported.[11,13]

Evaluation of phakellistatin 14 (**1**) against the murine P388 lymphocytic leukemia (ED_{50} 5 μg/mL) and a panel of human cancer cells (GI_{50} 0.75−3.4 μg/mL) showed moderate cancer cell growth inhibitory activity. With our previous series of *Phakellia*-derived phakellistatins, we have been investigating whether the cancer cell growth inhibition is a function of the cyclic peptide conformation or the result of a weakly bonded impurity in an amount detectable in our cancer cell line evaluations, but not revealed by chemical and spectroscopic analyses.[15] On the basis of the following important research results, we now believe that conformational changes account for differences in cancer cell line results between various synthetic and natural specimens of the same phakelliastatin. The moderate human cancer cell growth inhibition exhibited by phakelliastatin 14 is considered quite valid.

Jaspars and colleagues[16] isolated our earlier discovered cyclic heptapeptide phakellistatin 2[17] from a Fijian collection of the marine sponge *Stylotella aurantium* and proved the existence of two conformers that could be separated by HPLC and remain stable in methanol solution for a month or longer. By means of very careful NMR analyses, they were able to prove that our original specimen of phakellistatin 2,[17] including the later total synthetic product,[15c] was the cis/cis/cis Pro conformer,[16] which corresponded in turn to the "more polar" conformer isolated from the Fiji specimens.[16] Very importantly, with regard to the "less polar" conformer of phakellistatin 2 as well as the all-cis Pro conformer, the level of cancer cell growth inhibition against the A2780 ovarian cancer cell line (IC_{50} μg/mL in methanol: "polar" 2.7 vs "less polar" 1.1) was found to be quite dependent on the solvent and length of time the conformer remained in the solvent (inactive upon two-month storage).[16] In summary, the phakellistatin cancer cell line results are very sensitive to conformational changes.

Experimental Section

General Experimental Procedures. Specific rotation data were determined with a Perkin-Elmer 241 polarimeter. Ultraviolet spectra were recorded using a Perkin-Elmer Lambda 3β UV/vis spectrophotometer equipped with a Hewlett-Packard laser jet 2000 plotter. IR spectra were obtained with an AVATAR 360 FT-IR instrument with the sample prepared as a $CHCl_3$ film. The NMR experiments were conducted using a Varian Unity Inova 500 spectrometer operating at 500 and 100 MHz for ^1H and ^{13}C NMR, respectively. High-resolution mass spectra were obtained using a JEOL LCMate instrument. FABMS/MS was performed by the Washington University Mass Spectrometry Resource. Reversed-phase HPLC was performed on a Prepex C8 column (250 × 10 mm, 5−20 μm, by Phenomenex) with a Gilson HPLC instrument and detected by a Gilson UV detector. The chiral HPLC of the amino acids was conducted with a Chirex (D)-penicillamine column (250 × 4.6 mm, by Phenomenex) using a Waters Delta 600 HPLC with dual λ UV detector. The commercial amino acids used for chiral analyses were from either Sigma-Aldrich Chemical Co. or Acros Organics.

***Phakellia* sp.** The yellow-orange marine sponge *Phakellia* sp. (500 kg wet wt, class Demospongiae, order Axinellida) was collected at a depth of 25−40 m in the Federated States of Micronesia (Chuuk) between late November of 1986 and early February 1987. Generally, the sponge measured 5−30 cm in height and 3−50 cm in width. The sponge was stored and shipped in a solution of CH_3OH−seawater and identified by Dr. John N. A. Hooper, Sessile Marine Invertebrates, Queensland Museum, South Brisbane, Queensland 4101, Australia. Voucher specimens are retained by Dr. Hooper and in our Institute at Arizona State University.

Separation and Isolation of Phakellistatin 14 (1). The dichloromethane−methanol extract of the sponge was concentrated to dryness, and the residue (2.636 kg) was partitioned between methanol−water (9:1) and *n*-hexane, then methanol−water (3:2) and dichloromethane, to afford 128 g of the CH_2Cl_2 fraction (P388 murine lymphocytic leukemia cell line ED_{50} 0.45 μg/mL). The CH_2Cl_2 fraction was subjected to a series of Sephadex LH-20 column chromatographic separations employing successively CH_3OH, 3:2 CH_2Cl_2−CH_3OH, 3:1:1 *n*-hexane−toluene−CH_3OH, and 8:1:1 *n*-hexane−*i*-ProOH−CH_3OH as eluents. One of the latter active fractions (30 mg, ED_{50} 0.042 μg/mL) was further separated by reversed-phase HPLC to afford phakellistatin 14 as an amorphous powder (4.4 mg, 8.8 × 10^{-7}% yield).

Phakellistatin 14 (1): colorless solid, mp 189−191°C; $[\alpha]^{25}_D$ −64.86° (*c* 0.28, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 220 (3.45), 268 (sh 2.73) nm; IR ($CHCl_3$) ν_{max} 3332, 1647 and 1032 cm^{-1}; NMR data, see Table 1; HRMS (APCI positive) *m/z* 776.3698 $[M + H]^+$ (calcd for $C_{36}H_{54}N_7O_{10}S$, 776.3652).

Hydrolyses of Phakellistatin 14, β-OMe-*S*- and *RS*-Asp. Phakellistatin 14 (1.0 mg) was treated with hydrochloric acid−propionic acid (1:1, v/v, 1 mL) in a sealed tube with heating at 152−155 °C for 15 min. The liquid was evaporated under N_2 and the residue dried under vacuum to afford the hydrolysate as a pale yellow mixture. Using the same method, β-OMe-*S* and *RS*-Asp were separately hydrolyzed to give *S*- and *RS*-aspartic acids, respectively.

Phakellistatin 14 Chiral Assignments. Using a ligand-exchange-type Chirex column (250 × 4.6 mm, *N*,*S*-dioctyl-(D)-

penicillamine complexed with Cu^{2+}), the phakellistatin 14 hydrolysate and authentic *S*- and *RS*-amino acids were analyzed under one or two mobile phase conditions for polarity reasons: (1) aqueous 2 mM $CuSO_4$−CH_3CN (93:7), flow rate at 1.2 mL/min, *S*-Ala (t_R 5.7 min), *R*-Ala (t_R 6.8 min), *S*-Met(SO) (t_R 7.2/7.6 min), *R*-Met(SO) (t_R 9.2/9.8 min), *S*-Pro (t_R 11.0 min), *R*-Pro (t_R 21.3 min); (2) aqueous 2 mM $CuSO_4$−CH_3CN (90:10), flow rate at 1.2 mL/min, *S*-Asp (t_R 13.8 min), *R*-Asp (t_R 18.0 min), *S*-Ile (t_R 20.0 min), *R*-Ile (t_R 22.5 min), *S*-Phe (t_R 60 min), *R*-Phe (t_R 62 min). Co-injection of the *S*-Asp with the hydrolysate sample of β-OMe-*S*-Asp showed the same single peak, which confirmed that β-OMe-*S*-Asp was hydrolyzed under the conditions (HCl−propionic acid). All of the chiral α-carbons of the amino acid units in phakellistatin 14 (**1**) were revealed to correspond to the *S*-configuration by direct co-injection of the authentic amino acids with the peptide hydrolysate.

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