Isolation and Crystal Structure of Stylopeptide 1, A New Marine Porifera Cycloheptapeptide[1a]

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Stylopeptide 1 (**1**) was isolated respectively from the South and Western Pacific Ocean sponges *Stylotella sp.* and *Phakellia costata*. Structural determination was accomplished by utilizing high-field (500 MHz) 2D-NMR experiments and confirmed by an X-ray crystal structure determination to provide the assignment cyclo(Pro-Leu-Ile-Phe-Ser-Pro-Ile). The absolute configuration of the new cycloheptapeptide was established by employing results of chiral gas chromatographic analyses.

Marine Porifera indigenous especially to tropical ocean areas are rapidly increasing in importance as sources of potentially important new drugs. Illustrative of such promising constituents are the spongistatin[2a,b] and halichondrin/halistatin series[2c,d] of spiroketal/perhydropyrans with remarkable activity against a variety of murine and human cancer experimental systems. Presently, evidence is increasing that certain marine sponges also contain amino acid derivatives, usually as trace constituents, with significant cancer cell growth inhibitory properties such as the new cyclic peptides we discovered in Western Pacific and Indian Ocean Porifera.[3] Other noteworthy examples include new biochemical probes of the discodermin (inhibits tumor promotion) and calyculin (tumor promoting) classes and the antithrombin cyclotheonamides.[4]

In 1983 we collected a *Stylotella* sp. of marine sponge in the Bismarck Archipelago of Papua New Guinea (PNG). A 1986 re-collection (500 kg wet wt using SCUBA) was employed to isolate and characterize the murine P388 lymphocytic leukemia (PS system) cell line inhibitory cycloheptapeptide stylostatin 1 [cyclo(Pro-Phe-Asn-Ser-Leu-Ala-Ile)].[3b] Meanwhile Scheuer and colleagues have nicely determined the structure of the proline-derived PS cell growth inhibitory and immunosuppressive antibiotic Palou'amine from the Western Pacific *Stylotella agminata*.[5] Continuation of a detailed investigation of the PNG *Stylotella* sp. led to the isolation and structural elucidation of the cycloheptapeptide designated stylopeptide 1 (**1**).

A 0.14 g PS active hexane–methanol–toluene (3:1:1) fraction from Sephadex LH-20 column chromatography obtained during isolation[3b] of stylostatin 1 was finally separated by fractional crystallization from aqueous methanol. The principal product (2×10^{-5}% yield) was the new biosynthetic product designated stylopeptide 1 (**1**). The new peptide exhibited a high-resolution molecular ion peak at m/z 768.4647 corresponding to the molecular formula $C_{40}H_{61}N_7O_8$. Amino acid analyses indicated the presence of phenylalanine, proline (×2), leucine, isoleucine (×2), and serine. Presence of the amino acids was confirmed by 2D-NMR employing ^{1}H–^{1}H-COSY, HMQC,[6a] and HMBC[6b] experiments. The amino acid sequence was identified as cyclo(Pro^1-Leu^2-Ile^3-Phe^4-Ser^5-Pro^6-Ile^7) from the HMBC (Figure 1a) and NOESY/ROESY (Figure 1b) experiments. The complete ^{1}H- and ^{13}C-NMR assignments for stylopeptide 1 have been summarized in Table 1. Subsequently, we also isolated (1.3×10^{-5}% yield) stylopeptide 1 from the Western Pacific Ocean sponge *Phakellia costata*.

Interestingly the γ-carbon of Pro^6 resonated upfield at δ 22.11 compared to that of Pro^1 at δ 24.69, which suggested that the peptide bond between the Ile^7 and Pro^1 units might be *trans* and that between Ser^5 and Pro^6 *cis*.[7] Further support for this assumption was provided by differences in the chemical shifts of the β- and γ-carbons ($\Delta\delta\beta\gamma$) in the two proline units. Proline *cis–trans* isomers can be distinguished in solution by the chemical shift differential of the β- and γ-carbons.[8] In a *cis* X-Pro, these signals are further separated than in a *trans* X-Pro. With stylopeptide 1 (**1**) the chemical shifts of the β- and γ-carbons in Pro^6 differ by 8.58 ppm. The corresponding signals of Pro^1 differ by 4.65 ppm, indicating that the former peptide bond is *cis* and the latter *trans*. Strong NOESY/ROESY crosspeaks between the Ser^5 α-proton and the Pro^6 α-proton further supported this observation.

For the dual purposes of gaining further knowledge of well-defined but conformationally restricted cyclic peptide backbones, and to confirm the proposed structure, a

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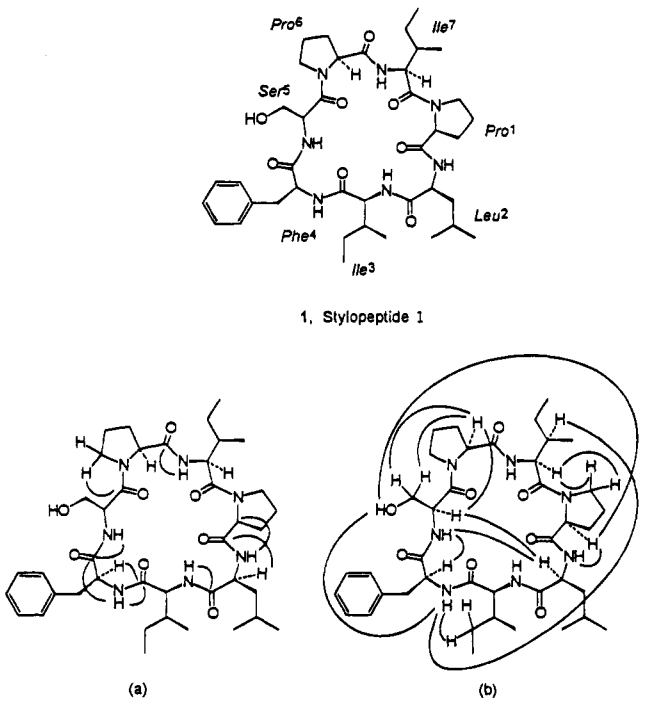

Figure 1. Stylopeptide 1 with (a) some important HMBC correlations (b) interresidue NOESY correlations.

single-crystal X-ray crystallographic analysis of stylopeptide 1 (**1**) was considered essential. Colorless crystals of the peptide were produced with difficulty from a methanol solution. The crystals were extremely unstable, losing solvent of crystallization readily at ambient temperatures. Immersion of the crystals in the mother liquor from crystallization in a sealed glass capillary provided sufficiently stable conditions for rapid data collection. Each molecule of stylopeptide 1 was found to be associated with a single molecule of methanol. On the basis of the A···B bond length observed in an A-H···B hydrogen bond, stylopeptide 1 appears to be involved in both intermolecular and intramolecular H-bonding. Generally, peptide N–H···O bonds have an A···B length in the range of ~2.79 ± 0.12 Å and O–H···O bonds have an A···B length in the range of 2.76 ± 0.09 Å.[9] Thus, intramolecular H-bonding seems probable between the N10···O29 (2.841 Å) heteroatoms. From the bond distances, intermolecular H-bonding between N4···O50 (Ser^5 amide–Leu^2 carbonyl at 2.841 Å), O31···O57 (Ser^5 hydroxyl–CH_3OH at 2.733 Å), O32···O57 (Ser^5 carbonyl–CH_3OH at 2.725 Å), and N16···O55 (Leu^2 amide–Ile^3 carbonyl at 2.883 Å) seems predictable. A computer-generated perspective drawing depicting the absolute configuration and conformation of the cyclic peptide, as well as the sites of proposed H-bonding, appears in Figure 2.

The absolute configuration of the *N*-pentafluoropropionyl-amino acid isopropyl ester derivative[10] of isoleucine prepared from the acid hydrolysate of stylopeptide 1 was ascertained using chiral gas chromatographic analysis and found to be *S*. Thus, from the relative stereochemistry obtained from the X-ray data, all the amino acids present were assigned the *S* absolute configuration. In the solid state, stylopeptide 1 (Figure 2) proved to contain *all-trans*, undistorted peptide bonds except for a *cis* bond between Ser^5 and Pro^6 (suggested by the NMR studies). Whereas the pyrrolidine ring of Pro^6 has a C^γ-endo conformation (χ^1 = 32.0, χ^2 = −41.3, χ^3 = 34.2, χ^4 = −13.5), that of Pro^1 has a C^γ-exo conformation (χ^1 = −11.7, χ^2 = 21.9, χ^3 = 21.3, χ^4 = 11.1).[11] A type VI (a)[12] β-turn occurs at the Ser^5–Pro^6 juncture incorporating the *cis* peptide bond. All values for the pairs ϕ,ψ fall within the allowed regions for L-amino acid units except for Leu^2 which presents the values 69.8°, 0.5°, appreciably higher in energy near the left-handed helical region.

The first samples of stylopeptide 1 carefully isolated and characterized were considered quite pure based on chromatographic and high-field NMR studies. These samples, especially from *P. costata* exhibited strong (ED_{50} ≈ 10^{-2} μg/mL) cancer cell (murine P388 lymphocytic leukemia and human cancer cell lines) growth inhibitory properties. However the profile of activity against the U.S. National Cancer Institute's human cancer cell line panel was reminiscent of the perhydropyran-type[2c,d] antineoplastic agents. Furthermore, the samples of stylopeptide 1 from the *Stylotella* sp. were found only marginally active to inactive in both the NCI and our laboratories. Later, specimens of stylopeptide 1 from other sources were found to be inactive in these cancer cell line systems. Hence it is now clear that certain cyclic peptides isolated from cancer cell growth inhibitory marine animal fractions can complex with or otherwise retain a chemically undetectable amount of a very potent (*cf.* ref 2) antineoplastic substance only detectable by biological methods. Obviously this study has revealed an important potential trap in our otherwise exceptionally useful bioassay guided separation methods and in the general biological evaluation of presumed chemically pure natural products. Because of the trace occurrence of stylopeptide 1 (**1**) and considering the conformational aspects, we plan extended biological evaluations of this cyclic peptide to possibly ascertain reasons for its presence in geographically remote Porifera species.

Experimental Section

For general methods, see ref 3b.

Isolation of Stylopeptide 1 (1). A. From *Stylotella* sp. A 500 kg (wet wt) re-collection (1986, by SCUBA) of the Papua New Guinea marine sponge *Stylotella sp.* (Class Desmospongiae, Order Hadromerida) was extracted and fractionated (PS bioassay) as previously reported.[3b] Partition chromatography of fraction B[3b] on a Sephadex LH-20 column (225 × 4.5 cm) with 3:1:1 hexane–toluene–methanol as eluent provided active fraction C (0.14 g). Final purification of fraction C was achieved by fractional crystallization from aqueous methanol. Stylopeptide 1 (**1**) was isolated as a colorless crystalline solid (0.1 g, 2 × 10^{-5}% yield): mp 228–229 °C (capillary tube uncorrected); $[\alpha]^{25}_D$ −128 (*c* 0.2, CH_3OH); HRFABMS *m/z* 768.4647, calcd 768.4640 for $C_{40}H_{61}N_7O_8$; IR (KBr) 3325, 3295, 2965, 2936, 2876, 1653, 1616, 1532, 1449, 1385, 1331, 1242, 1067, 748, and 702 cm^{-1}; and refer to Table 1 for the ^1H- and ^{13}C-NMR data.

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Table 1. High-Field (500 MHz) NMR Assignments (in DMSO-d_6) for Stylopeptide 1 (1)

position no.	^{1}H (δ)	^{13}C (δ)	HMBC[a]	NOESY/ROESY[b,c]
Pro^1				
1		170.74	2-H, 3-H, Leu^2 N-H	
2	4.01 (t, J = 7.1 Hz)	60.68	3-H, 4-H, Leu^2 N-H	3-H, 4-H, Leu^2 N-H
3	1.72, 2.04 (m)	29.34	2-H, 5-H	
4	1.74, 2.00 (m)	24.69	2-H, 3-H, 5-H	
5	3.58, 4.38 (m)	48.13	3-H, 4-H	
Leu^2				
1		171.72	2-H, 3-H, Ile^3 N-H	
2	3.50 (m)	54.08	N-H, 3-H	3-H
3	1.54, 2.14 (m)	36.96	N-H, 4-H, 2-H	
4	1.40 (m)	24.64	3-H, 5-H, 5′-H	
5	0.77	20.80	3-H	
5′	0.82	23.11	3-H	
NH	8.64 (br s)			2-H, (4-H), Pro^1 2-H
Ile^3				
1		169.65	2-H, Phe^4 N-H, Phe^4 2-H	
2	4.13 (m)	59.12	N-H, 3-H	3-H, 4-H
3	1.40 (m)	36.70	2-H, 4-H, 4′-H, 5-H	
4	1.38, 1.06	23.85	2-H, 4′-H, 5-H	
4′	0.80	14.42	2-H, 3-H, 4-H	
5	0.82	9.46	4-H, 3-H	
NH	7.21			2-H, 3-H
Phe^4				
1		170.90	2-H, 3-H, N-H, Ser^5 N-H	
2	4.13 (m)	55.23	N-H, 3-H	3-H
3	2.82 (dd, J 9.8 and 12.7 Hz), 3.13 (m)	36.90	2-H, *ortho*-H	(*ortho*-H)
4		136.90	*meta*-H	
ortho	7.30	129.79		
meta	7.26	127.93		
para	7.20	126.32		
NH	7.16			2-H, (3-H, Ser^5-OH), Ile^3 4-H, Ile^7 3-H
Ser^5				
1		168.60	2-H, 3-H, N-H, Pro^6 5-H	
2	3.89 (t, 7.1 Hz)	55.07	N-H, O-H, 3-H	(OH), 3-H, Ile^7 N-H, Leu^2 2-H, Pro^6 2-H
3	3.43 (m)	60.74	N-H, 2-H, O-H	(OH, Pro^6 2-H)
OH	5.08 (t, 5.8 Hz)			Pro^6 2-H, Pro^6 5-H, Pro^1 2-H
NH	8.64 (br s)			(OH, 2-H), 3-H, Phe^4 2-H, (Leu^2 2-H)
Pro^6				
1		171.02	2-H, 3-H, Ile^3 N-H	
2	4.70 (d, J 7.6 Hz)	60.50	3-H, 4-H	3-H, (4-H)
3	1.90, 2.36	30.69	2-H, 4-H, 5-H	
4	1.90, 1.50	22.11	2-H, 3-H, 5-H	
5	3.50, 3.34	46.24	2-H, 3-H	
Ile^7				
1		171.20	2-H	
2	4.31 (dd, J = 8.6 and 10.7 Hz)	54.96	3-H, 4′-H, N-H	3-H, Pro^1 5-H
3	2.02 (m)	34.73	2-H, 4-H, 4′-H, N-H	
4	1.13, 1.51	24.19	4-H, 3-H, 4′-H, 5-H	
4′	0.85	14.82	4-H	
5	0.82	10.05	4-H	
NH	9.41 (d, J = 8.3 Hz)			3-H, 2-H, Ser^5 2-H

[a] Mixing time = 60 μs. [b] Mixing time for NOESY = 200 μs. For ROESY = 50 μs. [c] Entries in parentheses refer only to ROESY peaks.

B. From *Phakellia costata*. A P388 cell line active methylene chloride soluble fraction prepared from 500 kg (wet wt) of *Phakellia costata*[3a] collected (1987) in the Federated States of Micronesia (Chuuck) was chromatographed using a series of gel permeation and partition Sepahadex LH-20 columns employing methanol, methylene chloride–methanol (3:2), hexane–toluene–methanol (3:1:1), and hexane–methylene chloride–methanol (8:1:1 → 3:1:1) eluent techniques to yield a more concentrated active fraction. Final separation was accomplished by HPLC (on silica gel 60 with hexane–methylene chloride–methanol 10:5:1, as mobile phase) to furnish stylopeptide 1 (63.5 mg, 1.3 × 10^{-5}% yield).

Stylopeptide 1 Chiral Assignments. The peptide (1 mg) was hydrolyzed with 12 N propionic acid–hydrochloric acid (1:1) at 160 °C for 15 min.[10a] The derived amino acids were converted to *N*-pentafluoropropionyl isopropyl ester derivatives, and the configuration of isoleucine (t_R 18.17 min, *vs* standard L-Ile 18.17 and D-Ile 17.17 min) was determined by chiral capillary chromatography.[10b]

Stylopeptide 1 X-ray Crystal Structure.[21] Crystals of peptide **1** prepared with difficulty from methanol solution were quite unstable and readily lost solvent of crystallization at ambient temperatures. Consequently a crystal (0.30 × 0.30 × 0.40 mm) of peptide **1** crystallized from methanol was mounted inside a sealed glass capillary tube with the crystal immersed in mother liquor. Data collection was performed at 26 ± 1 °C. Crystal data: $C_{40}H_{61}N_7O_8 \cdot CH_3OH$, monoclinic space group $P2_{1'}$ with a = 13.660(3), b = 10.059(3), and c = 16.713(3) Å, β = 95.054(17)°, V = 2287.54 Å^3, λ(Cu Kα) = 1.54184 Å, ρ_o = 1.172 g cm^{-3}, ρ_c = 1.162 g cm^{-3} for Z = 2 and FW = 800.01, F(000) = 864. All reflections corresponding to a complete quadrant, with $2\theta \leq 140°$ were measured using the $\omega/2\theta$ scan technique. After Lorentz and polarization corrections, merging of equivalent reflections, and rejection of systematic absences, 3144 unique reflections remained, of which 2405 were considered observed ($I_o > 2\sigma(I_o)$) and were used in the subsequent structure determination and refinement. Linear and anisotropic decay corrections were applied to the intensity data as well as an empirical absorption correction (based on a series of ψ-scans).[13] Concerted efforts directed at solving the structure using the direct-methods

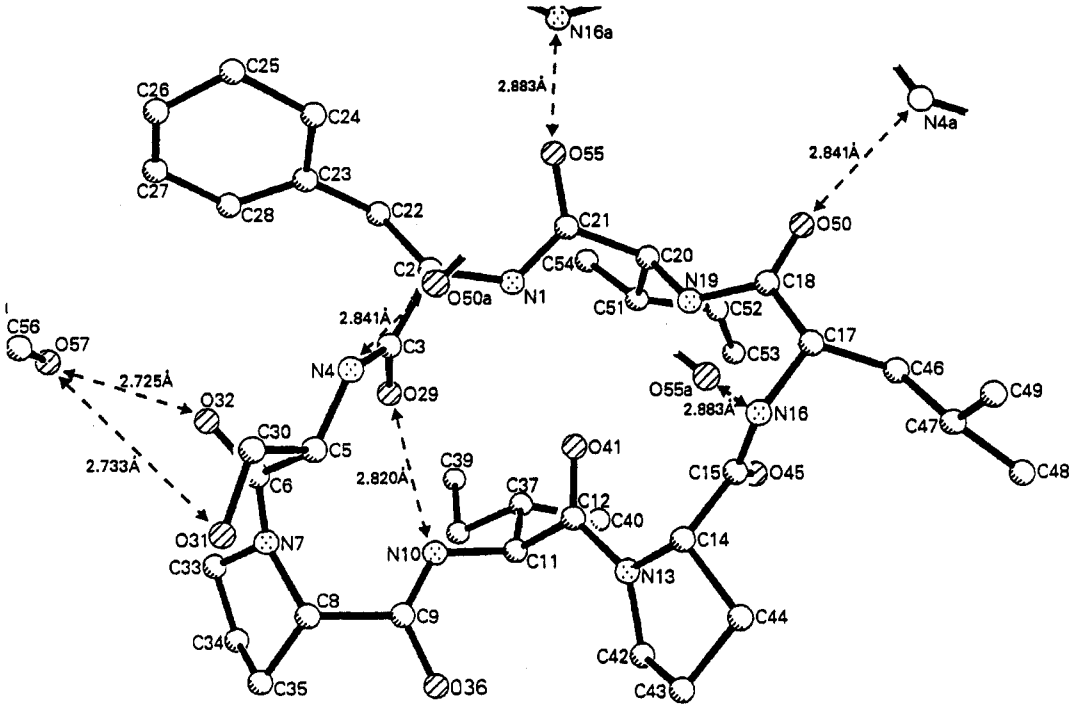

Figure 2. Perspective view of stylopeptide 1 (**1**, less hydrogens).

programs MULTAN[14] and SHELXS-86[15] failed. Similarly, the vector search program PATSEE[16] also failed to locate any proline or phenylalanine fragments when coordinates for these amino acid moieties were used as search input fragments. In each case, the absence of any recognizable fragments precluded further structure expansion. Structure determination was eventually accomplished with the direct-methods program SIR88.[17]

All non-hydrogen atoms, with the exception of one aromatic ring atom of the phenylalanine residue and one terminal methyl atom of an isoleucine moiety, were located on the first run of SIR88, using the default settings. Subsequent difference Fourier maps revealed the remaining atoms as well as the presence of an additional molecule of methanol associated with each molecule of peptide **1**. The remaining hydrogen atoms (calculated at optimum positions for the parent peptide and methanol solvate using the program SHELXL-93)[18] were assigned thermal parameters equal to 1.5 of the U_{iso} value of the atom to which they were attached and then both coordinates and thermal values were forced to ride that atom during final cycles of refinement. The atoms of the cyclic peptide structure, in addition to the solvate atoms of methanol, were refined in a full-matrix least-squares process with SHELXL-93. The final cycles of refinement included 517 variable least-squares parameters (anisotropic refinement on all non-hydrogen atoms). Near the conclusion of the least-squares refinement, the model exhibited some instability, and restraints were applied to some of the bond lengths in the two proline rings (C33–C35 and C43–C44) and the isoleucine unit (C51–C54). Examination of the principal mean square atomic displacement data of the U thermal parameters for C24, O36, C42–C43, C48–C49, and C53 all suggested possible splitting of the assigned atomic sites into two alternate positions for each. However, attempted refinement of a number of these alternate sites with partial occupancies failed to yield a better model. The final model for stylopeptide 1 converged to a standard crystallographic residual R_1 of 0.141 (for observed data) and 0.159 (for all data). The corresponding Sheldrick R values were 0.375 and 0.403, respectively. Bond distances and angles were all within acceptable limits.

The poor R value obtained (for diffractometer data) suggests that the model is not entirely satisfactory. The basic structure proposed for stylopeptide 1 is certain, but some disorder is suspected in the model involving the atoms indicated above. Additional disordered solvent molecules are possibly present but unaccounted for in the unit cell, as suggested by the slightly larger measured density *vs* calculated crystal density, as well as the slightly elevated residual electron density indicated in the final difference map (largest difference peak and hole −0.482 and −0.731 e/Å^3). Finally, analysis of the diffractometer data indicates that the crystal did not diffract well at high angles of θ. A sharp drop in the number of observed reflections occurred at angles of $2\theta > 100°$, limiting the effective resolution of the data collection to ~1.0 Å. These factors presumably contributed to the high R factor observed.

The final structure of stylopeptide 1 was assigned the heptapeptide sequence cyclo[Pro-Leu-Ile-Phe-Ser-Pro-Ile]. The absolute stereochemical assignment of stylopeptide 1 was based upon the known absolute stereochemistry of Ile. Thus, the absolute configuration at the nine chiral centers of peptide **1**, using the numbering shown in Figure 2,[20] follows: 2*S*,5*S*,8*S*,11*S*,14*S*,17*S*,20*S*,37*S*,51*S*. Stylopeptide 1 appears to be in-

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(20) Preparation of Figure 2 was accomplished with SHELXTL-PLUS, G. Sheldrick, Siemens Analytical X-Ray Instruments, Inc., Madison, WI 53719.

(21) The author has deposited atomic coordinates for this structure with the Cambridge Crystallographic Data Centre. The coordinates can be obtained, on request, from the Director, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, UK.

volved in both intermolecular and intramolecular H-bonding, based upon the total A···B bond length observed in an A–H···B hydrogen bond. A computer-generated perspective drawing depicting the absolute configuration and proposed H-bonding sites of stylopeptide 1 appears in Figure 2.

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