Waiakeamide, a Cyclic Hexapeptide from the Sponge *Ircinia dendroides*[1]

Christina M. S. Mau,[2] Yoichi Nakao, Wesley Y. Yoshida, and Paul J. Scheuer*

Department of Chemistry, University of Hawaii at Manoa, 2545 The Mall, Honolulu, Hawaii 96822

Michelle Kelly-Borges

Department of Zoology, The Natural History Museum, Cromwell Road, London SW7 5BD, U.K.

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From the sponge, *Ircinia dendroides*, collected in Indonesia we isolated a new cyclic hexapeptide, waiakeamide (**1**). Its structure, consisting of three proline residues, two methionine sulfoxides, and one thiazolylphenylalanine, was elucidated by spectral analysis and chemical degradation. Isolation and structural elucidation of waiakeamide is described.

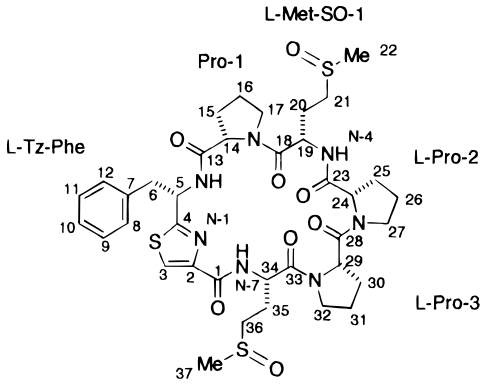
Two linear furanosesterterpenes were reported from a sponge, *Ircinia dendroides*, first collected at Mogan, Grand Canary, Spain.[3] Investigation of an Indonesian collection of *I. dendroides* yielded a new cyclic hexapeptide, waiakeamide (**1**).[4] Its structure, encompassing three proline residues, two methionine sulfoxides, and one thiazolylphenylalanine, was elucidated by spectral analysis and chemical degradation.

The freeze-dried sponge was extracted three times with CH_2Cl_2/2-propanol (1:1), and the residue was further separated by the Kupchan procedure,[5] employing gradient solvent systems of MeOH/hexane (9:1), MeOH/CCl_4 (8:2), and MeOH/CH_2Cl_2 (6:4). A bioassay- and ^{1}H NMR-guided separation scheme led to further separation of the CH_2Cl_2 layer by ODS flash chromatography. Preliminary bioassay data of this fraction showed strong activity against P388 cells at an IC_{50} of 0.054 μg/mL. Therefore, further separation was followed by a sequence of HPLC steps on a reversed-phase column to yield 3.0 mg of a colorless amorphous solid, waiakeamide (**1**). This compound had no biological activity, but the side fractions were active and their purification is in progress.

The molecular formula of waiakeamide (**1**) was established as $C_{37}H_{49}N_7O_8S_3$ by HR-FABMS [*m*/*z* 816.2852 (M + H)$^+$ (Δ −3.1 mmu)]. The ^{1}H NMR spectrum of **1** showed six proton signals between 4.42 and 5.52 ppm, which suggested that **1** was a hexapeptide (Table 1). Analysis of COSY and HMQC spectra[6] established the presence of three prolines and three unidentified α-amino acids.

Preliminary NMR data of two of these residues indicated that they were identical, bearing characteristic methyl singlets at 2.67 and 2.63 ppm in the δ-position, initially assigned to *N*-methyls; however, the downfield chemical shifts of γ-carbons (C-21, 50.5, and C-36, 49.8 ppm) precluded *N*-methyl groups. This pointed to possible methyl sulfoxides with an expected ^{1}H chemical shift of 2.67 ppm,[7] which was supported by IR absorption at 1030 cm^{-1}.[8] Therefore, we identified these two residues as methionine sulfoxide (Met-SO).

The remaining unidentified amino acid had to account for an aggregate of $C_{12}H_{10}N_2OS$, which included the third sulfur atom. The lowest field proton singlet at 8.25 ppm (H-3), bonded to a carbon at 124.8 ppm, showed HMBC[9] correlations to C-1, 2, and 4 (160.9, 149.5, and 171.2 ppm, respectively). These correlations and the carbon chemical shifts are characteristic of a thiazole ring.[10] HMBC correlations between H-5, 6_A, and 6_B/C-4 joined this thiazole to C-5, for a thiazolylphenylalanine (Tz-Phe, **a**), apparently a condensation product of cysteine and phenylalanine. This structural moiety is reminiscent of ulithiacyclamide B and patellamide D, cyclic peptides from the ascidian *Lissoclinum patella*.[11]



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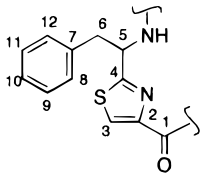

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(1) A preliminary account was presented at the Gordon Research Conference on Marine Natural Products, Ventura, CA, Feb 1996.

(2) MBRS undergraduate researcher, 1994−1996.

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Table 1. Correlated ^{13}C, ^{1}H Data and ROSEY for Waiakeamide (1), Measured in DMF-d_7

amino acid	no.	^{13}C	^{1}H	mult	*J* (Hz)	HMBC	ROESY
Tz-Phe	1	160.9					
	2	149.2					
	3	124.8	8.25	s		C:1, 2, 4	H:22
	4	171.2					
	5	53.1	5.52	dt	7.2, 7.7	C:4, 6, 7	H:8, 12
	6_A	42.6	3.22	dd	12.2, 7.2	C:4, 5, 7, 8, 12	H:8, 12, NH:2
	6_B		3.18	dd	12.7, 6.8	C:4, 5, 7, 8, 12	H:8, 12, NH:2
	7	137.9					
	8	130.1	7.24	m		C:6, 10, 12	H:5, 6_A
	9	129.0	7.29	m		C:7, 11	
	10	127.4	7.24	m		C:8, 12	
	11	129.0	7.29	m		C:7, 9	
	12	130.1	7.24	m		C:6, 8, 10	H:5, 6_A
	N-2		7.80	d	7.7	C:5, 13	H:6_A, 6_B, 14
Pro-1	13	171.4					
	14	61.0	4.42	dd	1.9, 5.5	C:13, 15, 16, 17	H:15_A, 15_B, NH:2
	15_A	28.4	1.99	m		C:13, 17	H:14
	15_B		1.84	m		C:13, 14	H:14
	16_A	25.0	1.92	m		C:17	
	16_B		1.64	m		C:17	
	17_A	48.0	3.83	m		C:15, 16	H:19, 20_B
	17_B		3.75	dt	6.9, 9.7	C:16	H:19, 20_B
Met-SO-1	18	173.0					
	19	52.1	4.78	ddd	4.1, 8.3, 10.2	C:18	H:17_A, 17_B, 20_A, 20_B, 21_A, 21_B
	20_A	24.7	2.56	m		C:18, 19, 21	H:19, NH:4
	20_B		2.40	m		C:18, 19, 21	H:17_A, 17_B, 19
	21_A	50.5	3.15	m		C:19, 20, 22	H:19, NH:4
	21_B		3.01	m		C:19, 20, 22	H:19, NH:4
	22	38.9	2.67	s		C:21	H:3
	N-4		9.16	d	7.7	C:23	H:29
Pro-2	23	172.4					
	24	61.7	4.62	d	7.7	C:23, 25, 27, 28	H:25_A, 25_B
	25_A	31.3	2.46	dd	6.1, 11.9	C:23, 26. 27	H:24, 25_B
	25_B		2.07	m		C:23, 24, 26	H:24, 25_A, 27_B
	26_A	22.6	1.92	m		C:24, 25	H:27_A, 27_B
	26_B		1.70	m			H:27_A, 27_B
	27_A	46.7	3.54	dt	7.2, 11.9	C:26	H:26_A, 26_B
	27_B		3.45	ddd	2.2, 9.4, 11.0	C:25	H:25_B, 26_A, 26_B, 25_B
Pro-3	28	170.7					
	29	60.1	4.55	t	7.6	C:28, 30, 31	H:30_A, NH:4
	30_A	29.1	2.39	m		C:28, 31, 32	H:29, 30_B
	30_B		1.82	m		C:28, 29, 31	H:30_A
	31_A	25.7	2.12	m		C:29, 30	
	31_B		1.93	m		C:30, 32	
	32	48.5	3.83	m		C:30, 31	H:34
Met-SO-2	33	169.5					
	34	50.7	5.07	dt	6.9, 7.2	C:1, 33, 35, 36	H:32, 35, 36_A, 36_B
	35	26.3	2.26	m		C:33, 34, 36	H:34, NH:7
	36_A	49.8	2.98	m		C:34, 35, 37	H:34
	36_B		2.83	m		C:34, 35, 37	H:34
	37	38.4	2.63	s		C:36	
	N-7		7.94	d	7.7	C:1	H:35

Sequencing of the residues was accomplished by HMBC and ROESY[12] analyses. Sequential HMBC correlations were observed between Tz-Phe/Pro-1 (NH-2/C-13), Met-SO-1/Pro-2 (NH-4/C-23), Pro-2/Pro-3 (H-24/C-28), and Met-SO-2/Tz-Phe (H-34 and NH-7/C-1). ROESY correlations between Pro-1/Met-SO-1 (H_2-17/H-19) and Pro-3/Met-SO-2 (H_2-32/H-34) closed the 21-membered ring to complete structure **1**, for waiakeamide.

The absolute stereochemistry for each residue was determined by Marfey analysis.[13] The acid hydrolysate of **1** was derivatized with FDAA and analyzed on ODS HPLC to give L-Pro and L-methionine sulfoxide. Thiazolylphenylalanine isolated from the acid hydrolysate by ODS HPLC was subjected to ozonolysis followed by Marfey analysis yielding L-Phe.

Waiakeamide (**1**) contains two methionine sulfoxides and a thiazolylphenylalanine. This is the first reported methionine sulfoxide from a marine source. Peptides containing thiazole moieties are known from ascidians[14] and from sponges.[15]

Experimental Section

General Procedures. The cell cultures on which cytotoxic activity was tested were murine leukemia (P388), human lung carcinoma (A549), human colon carcinoma (HT29), and human melanoma (MEL28). ^{1}H NMR spectra were recorded on a General Electric GN Omega 500 MHz NMR spectrometer and 125 MHz for ^{13}C. FAB-MS was measured on a JEOL JMX-

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SX102/SX102 tandem mass spectrometer using glycerol + HCl as a matrix.

Extraction and Isolation. The sponge was collected on Oct 1, 1992, at a depth of 3−40 m at Manado, Sulawesi, Indonesia. The sponge forms irregular straplike branches, the surface is conculose with fine protruding fibers, and the sponge is flexible and very difficult to tear. The sponge is deep brownish-green in life and cream in preservative. The skeleton consists of large yellow platelike fibers with embedded detritus, and the sesohyl is permeated with distinctive spongin filaments. The sample is *I. dendroides* (Poléjaeff) (order Dictyoceratida, family Irciniidae) first described from the Philippines. A voucher specimen has been deposited at the Natural History Museum, London, U.K. (BMNG 1992.10.1.1).

The freeze-dried material (153 g) was extracted three times with CH_2Cl_2/2-propanol (1:1) and was partitioned by the Kupchan procedure,[5] employing gradient solvent systems of MeOH/hexane (9:1), MeOH/CCl_4 (8:2), and MeOH/CH_2Cl_2 (6:4). The CH_2Cl_2 layer was further purified by ODS flash chromatography with a stepwise gradient solvent system: MeOH/H_2O (1:1), MeOH/H_2O (7:3), MeOH/H_2O (4:1), 100% MeOH, and $CHCl_3$/MeOH/H_2O (7:3:0.5).

The fraction eluting with MeOH (7:3) was passed through a C-18 BondElut short column, followed by a series of reversed-phase HPLC separation [COSMOSIL $5C_{18}$-AR; MeOH/H_2O (3:2); YMC-Pack ODS-AQ; MeOH/H_2O (3:2)]; it was finally separated by reversed-phase HPLC by two joined columns [COSMOSIL $5C_{18}$-MS and -AR; MeOH/H_2O (7:13)] yielding 3.0 mg (1.96×10^{-3}% yield based on dry weight) of pure peptide. The resulting colorless amorphous solid had the following physical properties: $[\alpha]_D$ −54° (*c* 0.50, MeOH); UV (MeOH) λ_{max} 206.0 nm (ϵ 13 000), 239.0 nm (ϵ 4400); $C_{37}H_{49}N_7O_8S_3$; HR-FABMS, 816.2852 (Δ −3.1 mmu); IR ($CHCl_3$), 1660, 1630, 1030 cm^{-1}.

Hydrolysis of Waiakeamide (1). Waiakeamide (**1**, 1 mg) was dissolved in 500 μL of 5 N HCl and degassed under vacuum for 1 min. In a sealed evacuated ampule, the solution was heated at 105 °C with magnetic stirring for 17 h. The hydrolysate was dried under N_2 and was separated by ODS HPLC [YMC-Pack ODS-AQ; MeCN/H_2O/TFA (23:76:0.05)] to yield the constituent amino acids. Thiazolylphenylalanine was isolated and subjected to ozonolysis; the other fractions were combined and subjected to Marfey analysis.

Derivatization of Amino Acids with Marfey's Reagent. To the combined fractions, a 0.1% solution of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) in acetone was added, followed by the addition of 100 μL of 0.1 N $NaHCO_3$, and the resulting mixture heated at 80 °C for 3 min. After being cooled to room temperature, the reaction mixture was neutralized with 50 μL of 0.2 N HCl, to which was added 100 μL of 50% MeCN containing 0.05% TFA. This solution was analyzed by reversed-phase HPLC (COSMOSIL $5C_{18}$-MS, MeCN/H_2O (25:75) + 50 mM NH_4OAc) yielding only L-Pro (5.6 min; D-Pro 7.5 min). Commercially available methionine sulfoxide resulted in two epimers at the sulfoxide function for the L and D isomers (L, 18.8 and 20.4 min; D, 26.4 and 32.5 min). The hydrolysate of **1** generated two peaks of L-methionine sulfoxide (18.7 and 20.4 min).

Ozonolysis of Thiazolylphenylalanine. A solution of thiazolylphenylalanine (1.2 mg) in MeOH was cooled to −78 °C in a dry ice/acetone bath. A slow stream of ozone was bubbled into the solution for 3 min. After removal of excess ozone by N_2, the solution was dried under a stream of N_2. The residue was oxidized by dissolving it in a solution of 30% H_2O_2/HCOOH (1:2) with stirring at 70 °C, for 20 min. The reaction mixture was dried under N_2, derivatized with FDAA, and analyzed by ODS HPLC [COSMOSIL $5C_{18}$-MS, MeCN/H_2O/TFA (42:57:0.05)] to yield a 3:1 mixture of L- (12.6 min) and D-Phe (17.5 min) derivatives.

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Supporting Information Available: ^1H, ^{13}C, COSY, HMBC, HMQC, and ROSEY NMR spectra of **1** (6 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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