A New Antifouling Hexapeptide from a Palauan Sponge, *Haliclona* sp.

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Bioassay-guided fractionation of an extract of the sponge *Haliclona* sp. provided a known hexapeptide, waiakeamide (**1**), and a new sulfone derivative (**2**). The structures of hexapeptides **1** and **2** were elucidated by extensive NMR analyses and the advanced Marfey's method with LC/MS. These compounds showed repellent activity against the blue mussel, *Mytilus edulis galloprovincialis*.

Marine sponges belonging to the genus *Haliclona* have been the subject of extensive chemical studies. Recent investigations of *Haliclona* species have led to the isolation of alkaloids,[1−3] steroids,[4,5] macrolides,[6] polyketides,[7] and polyacetylenes.[8,9] As part of our research aimed at the discovery of new antifoulants from marine invertebrates, we have already isolated several repellent substances[10−14] against such marine fouling organisms as the barnacle, *Balanus amphitrite*, and the blue mussel, *Mytilus edulis galloprovincialis*. The Palauan sponge *Haliclona* sp. (family Haliclonidae, order Haplosclerida) was also collected for this research. This specimen contained the new hexapeptide **2** together with the previously isolated hexapeptide, waiakeamide (**1**).[15,16] In this paper, we report the isolation and the structural elucidation of these hexapeptides.

The EtOAc-soluble fraction of the MeOH extract of the marine sponge *Haliclona* sp. was chromatographed on a silica gel column using eluents of increasing polarity ($CHCl_3$−MeOH and EtOAc−hexane) to yield a mixture of compounds. The fractions containing hexapeptides were further purified by reversed-phase HPLC to yield compounds **1** and **2**. Fractionation was monitored using repellent activity against blue mussels.

HRFABMS analysis of **1** established its molecular formula as $C_{37}H_{49}N_7O_8S_3$. From the six signals corresponding to the α positions of amino acid residues by ^{1}H, ^{13}C, and HSQC NMR spectra, a hexapeptide structure was suggested for **1**. Analysis of the chemical shifts and the 2D NMR correlations indicated the presence of three prolines and two methionine sulfoxides. The remaining amino acid was identified as thiazolyl phenylalanine from HMBC data and the characteristic chemical shifts of four aromatic carbon signals (δ 136.1, 129.5, 128.5, and 127.1) of the phenyl moiety and ^{13}C and ^{1}H signals [(δ 123.5, C-3), (δ 169.0, C-4), and (δ 7.94, H-3)] of the thiazole ring. The HMBC correlations supported the cyclic structure of **1** (see Figure 1). Both the NMR data and HRFABMS data enabled compound **1** to be identified as the previously isolated compound waiakeamide.[15,16]

NMR spectral data for compound **2** were similar to those for compound **1**. The ^{13}C (δ 38.9, C-22) and ^{1}H (δ 2.65, H-22) signals for **1** assigned to the *N*-methyl group of the methionine sulfoxide were shifted to lower field in **2** [(δ 41.5, C-22), (δ 3.00, H-22)]. The HRFABMS data for **2** showed *m/z* 832.2884 $[M + H]^+$, which established its molecular formula as $C_{37}H_{49}N_7O_9S_3$, having one oxygen atom more than **1**. These data indicated that the C-19 substituent of **2** was a methionine-sulfone. HMBC correlations again supported the cyclic peptide structure (Figure 1). Thus, **2** was proposed as the oxidized form of waiakeamide (**1**).

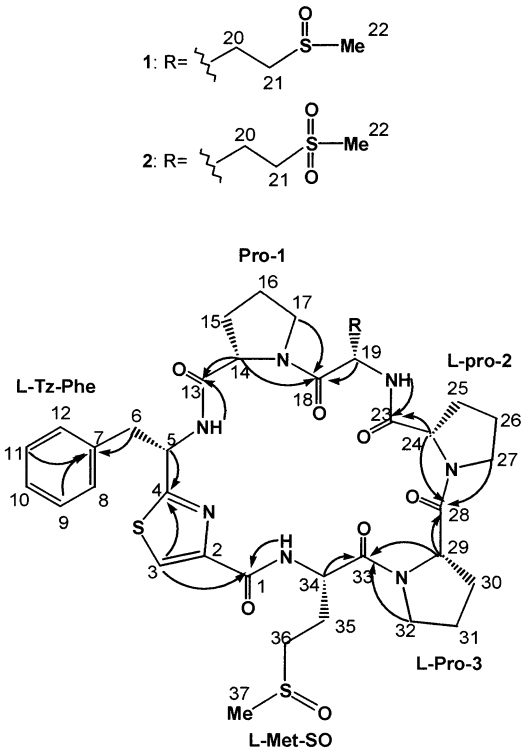

Figure 1. HMBC correlations for isolated compounds.

Rashid et al.[16] have previously reported that waiakeamide, which has two methionine sulfoxide residues, was obtained from haligramide A by chemical oxidation with H_2O_2. However, the bis-sulfone derivative is also formed from haligramide A and waiakeamide by the same method.[16] Thus, no selective oxidation was observed in either case. Interestingly, no bis-sulfone derivative was found in our research, suggesting the possibility that **2** is not an artifact.

The stereochemistry of each component amino acid in **1** and **2** was determined by the advanced Marfey's method with flash hydrolysis;[17,18] on the other hand, the absolute stereochemistry of waiakeamide in the literture[15] was established by a combination of acid hydrolysis, ozonolysis, and the Marfey analysis. Compounds **1** and **2** were hydro-

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lyzed in 6 N HCl at 110 °C for 1 h, and the hydrolysates were derivatized with L- and D-FDLA (1-fluoro-2,4-dinitrophenyl-5-1-leucinamide) and analyzed by LC/MS. The hydrolyzation time was shortened in order to prevent the enantiomerization of the thiazolyl amino acid. It is reported that L-amino acid-L-FDLA derivatives are generally eluted faster than the D-amino acid-L-FDLA derivatives or L-amino acid-D-FDLA derivatives under reversed-phase conditions with a few exceptions.[17,19] This rule has been shown to be applicable to the FDLA derivatives of thiazolyl amino acids (Fujii et al., manuscript in preparation). The Phe-Tzl residue in **1** or **2** was unambiguously determined to be the L-isomer because the major peak of the L-isomer and trace peaks due to the D-isomer (*m*/*z* 543 $[M + H]^+$) were detected by the derivatization with L-FDLA. These results corresponded with the previously reported absolute configuration of waiakeamide (**1**) and supported the structure of **2** given.

The antifouling activities of these compounds were tested by the foot-stimulating method[20] developed at our institute. By using this method, we have previously isolated the hexa- and heptapeptides named haliclonamides A−E;[21,22] this should be removed from the same *Haliclona* sp. In comparison with the haliclonamides, **1** and **2** showed quite similar effectiveness against blue mussels.

Experimental Section

General Experimental Procedures. The optical rotation values were determined with a Horiba SEPA-300 high-sensitivity polarimeter. Infrared spectra were obtained from a KBr pellet with a JASCO FT-IR-7000 spectrophotometer. ^{1}H and 2D NMR data were measured with a Varian Unity Inova 750 NMR spectrometer, and ^{13}C NMR were measured with a Varian Unity Inova 500 NMR spectrometer, using $CDCl_3$. HRFABMS data were measured with a JEOL JMS-SX102 mass spectrometer, and LC/MS were acquired on an Applied Biosystems Qster (ESI-TOFMS) with an Agilent HP1100 HPLC system.

Collection and Extraction. *Haliclona* sp. was collected in Palau in September 1997, frozen on site, and stored at −20 °C before extraction. A voucher specimen is deposited at our laboratory (97P-21). The frozen sample of sponge (1.9 kg) was extracted with MeOH (3 × 2 L). The extract was evaporated under reduced pressure to give a residue, which was partitioned between EtOAc and H_2O and between BuOH and H_2O.

Isolation of Hexapeptides. The EtOAc layer was evaporated to dryness (4.9 g) and chromatographed on a silica gel column eluting with a $CHCl_3$−MeOH gradient system (from 0 to 5% MeOH in $CHCl_3$) to give 17 fractions. Bioactive 10 and 11 were combined (558.5 mg) and further fractionated on a silica gel column with a mixed solvent system of EtOAc−hexanes (from 60% to 100% EtOAc in hexane). Fraction 6 (75.2 mg, crude **1** and **2**) was active in the antifouling assay and was further purified by reversed-phase HPLC (C_{18} column 10 × 250 mm; UV detector at 215 nm; flow 3 mL/min) with a solvent system of 40% MeOH in H_2O to give pure compounds **1** and **2**.

Waiakeamide (1): white amorphous material (12 mg); IR (KBr) ν_{max} 3448, 2928, 1630, 1514, 1340, 1185, and 1023 cm^{-1}; $[\alpha]^{27}_D$ −260 (*c* 0.42, $CHCl_3$); ^{1}H NMR (750 MHz, $CDCl_3$) δ 7.94 (1H, s, H-3), 5.40 (1H, m, H-5), 3.28 (1H, m, H-6), 2.97 (1H, m, H-6), 7.07 (1H, d, *J* = 7.0 Hz, H-8), 7.26 (1H, m, H-9), 7.24 (1H, m, H-10), 7.26 (1H, m, H-11), 7.07 (1H, d, *J* = 7.0 Hz, H-12), 4.32 (1H, m, H-14), 2.60 (1H, m, H-15), 2.06 (1H, m, H-15), 1.97 (1H, m, H-16), 1.79 (1H, m, H-16), 3.64 (1H, m, H-17), 3.55 (1H, m, H-17), 4.80 (1H, m, H-19), 2.52 (1H, m, H-20), 2.39 (1H, m, H-20), 3.00 (1H, m, H-21), 2.82 (1H, m, H-21), 2.65 (3H, s, H-22), 4.73 (1H, m, H-24), 2.52 (1H, m, H-25), 1.99 (1H, m, H-25), 1.98 (1H, m, H-26), 1.82 (1H, m, H-26), 3.86 (1H, m, H-27), 3.66 (1H, m, H-27), 4.44 (1H, t, *J* = 7.5 Hz, H-29), 2.16 (1H, m, H-30), 1.91 (1H, m, H-30), 1.99 (1H, m, H-31), 1.98 (1H, m, H-31), 3.76 (2H, m, H-32), 5.03 (1H, m, H-34), 2.38 (1H, m, H-35), 2.86 (1H, m, H-36), 2.63 (3H, s, H-37), 8.06 (bs, NH-2), 9.24 (d, *J* = 7.5 Hz, NH-4), 7.90 (bs, NH-7); ^{13}C NMR (125 MHz, $CDCl_3$) δ 160.7 (s, C-1), 148.5 (s, C-2), 123.5 (d, C-3), 169.0 (s, C-4), 53.2 (d, C-5), 43.3 (t, C-6), 136.1 (s, C-7), 129.5 (d, C-8), 128.5 (d, C-9), 127.1 (d, C-10), 128.5 (d, C-11), 129.5 (d, C-12), 170.4 (s, C-13), 61.2 (d, C-14), 22.3 (t, C-15), 24.9 (t, C-16), 46.6 (t, C-17), 172.8 (s, C-18), 51.1 (d, C-19), 26.5 (t, C-20), 50.4 (t, C-21), 38.9 (q, C-22), 172.1 (s, C-23), 60.3 (d, C-24), 24.5 (t, C-25), 24.6 (t, C-26), 47.7 (t, C-27), 169.9 (s, C-28), 59.3 (d, C-29), 28.6 (t, C-30), 25.2 (t, C-31), 47.9 (t, C-32), 168.8 (s, C-33), 50.1 (d, C-34), 26.0 (t, C-35), 49.8 (t, C-36), 38.6 (q, C-37); HRFABMS *m*/*z* 816.2884 $[M + H]^+$ (calcd for $C_{37}H_{50}N_7O_8S_3$, 816.2883).

Sulfone derivative (2): white amorphous material (1.9 mg); IR (KBr) ν_{max} 3454, 1626, 1539, 1296, and 750 cm^{-1}; $[\alpha]^{26}_D$ −108 (0.16, $CHCl_3$); ^{1}H and ^{13}C NMR (see Table 1); HRFABMS *m*/*z* 832.2823 $[M + H]^+$ (calcd for $C_{37}H_{50}N_7O_9S_3$, 833.2832).

Table 1. ^{13}C and ^{1}H NMR Data for Compound **2**[a]

amino acid	atom number	δC	δH
L-Phe-Tzl	1	160.8	
	2	148.6	
	3	123.5	7.93 (1H, s)
	4	169.1	
	5	53.2	5.40 (1H, m)
	6	43.3	3.26 (1H, m), 3.01 (1H, m)
	7	136.1	
	8, 12	129.5	7.07 (1H, d, *J* = 7.0)
	9, 11	128.6	7.23 (1H, m)
	10	127.1	7.27 (1H, m)
	NH-2	123.7	8.06 (bs)
L-Pro-1	13	170.1	
	14	60.2	4.65 (1H, bd, *J* = 6.8 Hz)
	15	26.2	2.62 (1H, m), 1.78 (1H, m)
	16	24.6	2.08 (1H, m), 1.98 (1H, m)
	17	46.5	3.83 (1H, m), 3.75 (1H, m)
L-Met-SO_2	18	172.7	
	19	50.7	4.79 (1H, m)
	20	23.7	2.57 (1H, m), 2.42 (1H, m)
	21	50.8	3.28 (2H, m)
	22	41.5	3.00 (3H, s)
	NH-4	11.2	9.27 (d, *J* = 7.0 Hz)
L-Pro-2	23	172.3	
	24	61.2	4.32 (1H, dd, *J* = 7.5, 3.8 Hz)
	25	31.2	2.55 (2H, m), 2.05 (1H, m)
	26	22.4	2.04 (1H, m), 1.81 (1H, m)
	27	47.7	3.89 (1H, m), 3.66 (1H, m)
L-Pro-3	28	169.8	
	29	59.4	4.43 (1H, t, *J* = 7.5 Hz)
	30	28.6	2.20 (1H, m), 1.92 (1H, m)
	31	25.2	2.19 (1H, m), 1.99 (1H, m)
	32	48.0	3.80 (2H, m)
L-Met-SO_1	33	169.2	
	34	50.2	5.06 (1H, m)
	35	25.8	2.37 (2H, m)
	36	50.0	2.90 (1H, m), 2.82 (1H, m)
	37	38.8	2.64 (3H, s)
	NH-7		7.92 (bs)

[a] Run in $CDCl_3$, 125 MHz for ^{13}C and 750 MHz for ^{1}H NMR; chemical shifts are shown in the δ scale with number of protons, mutiplicities, and *J* values (Hz) in parentheses.

The Advanced Marfey's Method. Hexapeptide (500 μg) was dissolved in 6 N HCl (500 μL) in a sealed tube. The reaction mixture was heated at 110 °C for 1 h and evaporated to dryness. The residue was dissolved in H_2O (100 μL) and divided in two portion (50 μL); then 1 M $NaHCO_3$ (20 μL) and 1% L- or D-FDLA acetone solution (100 μL) were added. After incubation at 40 °C for 1 h, the reactions were quenched with 1 N HCl (20 μL) and diluted with CH_3CN (260 μL). One microliter of each solution was injected on LC/MS.

LC/MS analysis of the L- and D-FDLA derivatives was performed on an Agilent HP1100 HPLC system and a Tsk gel Super-ODS column (100 × 2.0 mm i.d.) with a flow rate of 0.2 mL/min. The solvent was 1% formic acid−CH_3CN with a linear gradient from 30% to 80% CH_3CN over 25 min. An Applied

Biosystem QSTAR mass spectrometer was used for detection in the ESI (positive) mode. The eluent from the HPLC was split at a ratio of 1:40 for introduction into the mass spectrometer at a flow rate of 5 μL/min.

Evaluation of Antifouling Activity. Hexapeptides **1** and **2** were diluted with a small amount of MeOH, then diluted in artificial seawater (ASW) to estimate the activity. The influence of the organic media has been investigated previously by Hayashi and Miki,[20] and the solvent alone should not show any activity. MeOH had no effect at up to 5% in ASW. Each potential antifouling substance was individually dripped on the foot of the blue mussel, and the animal's reactions were observed. The activity of each test compound was indicated by the percentage of mussels showing a reaction at each concentration of test solution, this being calculated as follows: number of reacted mussels/(number of reacted mussels + nonreacted mussels) × 100%. Ten mussels were prepared for each sample. One hundred percent antifouling activity was observed at a concentration of 100 ppm, although the test mussels showed no reactivity at concentrations of less than 10 ppm. The foot stimulus activity of these hexapeptides was quite similar to those of copper sulfate and bis(tributyltin) oxide, which have previously been reported by Hayashi and Miki.[20] These inorganic compounds are well known as standard antifouling substances against blue mussels.

Cytotoxicity Testing. The cytotoxicity testing (IC_{50}, μg/mL) was carried out against murine leukemia cells (P-388; ATCC: CCL 46). P-388 cells were seeded to 96-well plates and cultured for 14 h. Various concentrations of samples were added to each well. After a 40 h incubation, cell numbers were measured by the AlamarBlue assay. Haliclonamide A, isolated from the same *Haliclona* sp., showed an IC_{50} of 40 μg/mL concentration against this cancer cell line, but compounds **1** and **2** had no activity at the same concentration; the IC_{50}'s of these compounds were more than 1000 mg/mL.

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