Haligramides A and B, Two New Cytotoxic Hexapeptides from the Marine Sponge *Haliclona nigra*

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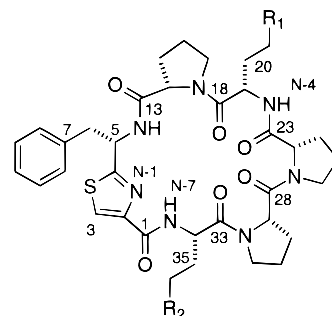
Bioassay-guided fractionation of a cytotoxic aqueous extract of the sponge *Haliclona nigra* provided two new cyclic hexapeptides, haligramides A (**1**) and B (**2**), in addition to the known peptide, waiakeamide (**3**). The structures of peptides **1** and **2** were elucidated by extensive NMR analyses and by comparison of their spectral data with those of waiakeamide (**3**). The identity of haligramide A (**1**) was confirmed by its oxidative conversion to waiakeamide (**3**). Further structural confirmation was provided by oxidation of peptides **1**, **2**, and **3** to the common bis-sulfone derivative **4**.

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,[2−8] macrolides,[9] polyacetylenes,[10,11] polyketides,[12,13] and steroids.[14,15] Our analysis of the aqueous extract of *H. nigra* (*sensu* De Laub, Haliclonidae), collected from Papua New Guinea, was initiated due to the distinctive pattern of differential cytotoxicity it produced in the U.S. National Cancer Institute (NCI)'s 60-cell-line antitumor screen.[16,17] Bioassay-guided fractionation of the extract provided three hexapeptides, two of which were new.

Results and Discussion

The cytotoxic *H. nigra* extract was initially fractionated by VLC on a wide-pore C_4 column eluted with increasing concentrations of MeOH in H_2O. Cytotoxic activity was concentrated into a fraction that, by ^{1}H NMR analysis, was predominantly composed of peptides. Final C_{18} HPLC purification of this material provided two new cyclic peptides, haligramides A (**1**) and B (**2**), and the known compound waiakeamide (**3**), which was identified by comparing its physical and spectral data with previously reported values.[18] HRFABMS of a CsI-doped sample of compound **1** provided a pseudomolecular ion $[M + Cs]^+$ at *m*/*z* 916.2003, which established its molecular formula as $C_{37}H_{49}N_7O_6S$. This differed from the molecular formula of waiakeamide (**3**) ($C_{37}H_{49}N_7O_8S$) only by having two fewer oxygen atoms. The IR spectrum of **1** displayed bands at 3379, 1633 (br), and 1520 cm^{-1}, which are typical of peptides, while UV absorptions at 242, 254, and 260 nm indicated the presence of aromatic residues.[19] The peptidic nature of haligramide A (**1**) was further supported by the presence of six carbonyl signals between δ 160.5 and 173.7 and three amide NH proton resonances between δ 7.68 and 9.16 in the ^{13}C and ^{1}H NMR spectra (Table 1), respectively. The NMR spectral data of haligramide A (**1**) were very similar to those recorded for waiakeamide (**3**),[18] which suggested a close structural relationship between the two compounds. However, methyl group resonances observed at δ_H 2.63 (δ_C 38.4) and δ_H 2.67 (δ_C 38.9), attributable to the two methionine sulfoxide residues in **3**, were significantly shifted upfield in **1**, where they appeared at δ_H 2.15 (δ_C 14.8) and δ_H 2.18 (δ_C 15.4). This spectral change, coupled with the loss of two oxygen atoms in the molecular formula of **1**, revealed that the methionine sulfoxides in waiakeamide (**3**) were substituted by reduced methionine residues in haligramide A (**1**). Upfield shifts observed for the ^{1}H and ^{13}C resonances of the γ-methylene groups (C-21 and C-36) in the two methionine residues in **1**, as compared to **3**,[18] were consistent with this assignment. HMBC (Table 2) and 1D NOESY (Figure 1) data confirmed the identity and sequence of the amino acid residues in haligramide A (**1**), and thus established that it was the bis-methionine analogue of waiakeamide (**3**).



1 $R_1 = R_2$ = S−Me
2 R_1 = S−Me, R_2 = SO−Me
3 $R_1 = R_2$ = SO−Me

The absolute stereochemistry of the amino acid constituents of waiakeamide (**3**) was previously established by a combination of acid hydrolysis, ozonolysis, and Marfey[20] analysis.[18] An oxidative conversion of haligramide A (**1**) to waiakeamide (**3**) would, therefore, allow assignment of the absolute stereochemistry of **1**. To accomplish this, compound **1** was treated with a mixture of dichloromethane, acetic acid, and 30% aqueous hydrogen peroxide for 1 h at room temperature (Scheme 1).[21] After evaporation of the solvents, the ^{1}H NMR spectrum of the reaction product was identical to that of waiakeamide (**3**). When this same reaction was carried out for 6 h, the methionine residues in haligramide A (**1**) were oxidized to the corresponding sulfones and thus yielded compound **4**. The downfield

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Table 1. NMR Spectral Data for Haligramide A (**1**) and Haligramide B (**2**) in DMF-d_7

	haligramide A (**1**)				haligramide B (**2**)		
no.	^{13}C	^{1}H	mult (*J*, Hz)	TOCSY	^{1}H	mult (*J*, Hz)	TOCSY
Tz-Phe							
1	160.5						
2	149.3						
3	124.7	8.25	s		8.26	s	
4	171.1						
5	52.9	5.55	t (7.5)	3.14, 3.18, 7.68	5.55	t (7.0)	3.16, 7.66
6a	43.1	3.14	dd (12.5, 7.5)	3.18, 5.55, 7.68	3.16	d (7.0)	5.66, 7.66
6b		3.18	dd (12.5, 7.5)	3.14, 5.55, 7.68	3.16	d (7.0)	5.66, 7.66
7	137.6						
8, 12	130.0	7.16	br d (7.0)	7.26, 7.29	7.17	d (7.5)	7.25, 7.30
9, 11	129.0	7.29	t (7.0)	7.16, 7.26	7.30	t (7.5)	7.17, 7.25
10	127.5	7.26	t (7.0)	7.16, 7.29	7.25	t (7.5)	7.17, 7.30
N-2		7.68	d (7.5)	3.14, 3.18, 5.55	7.66	d (8.0)	3.16, 5.55
Pro-1							
13	170.7						
14	60.6	4.47	d (8.5)	1.68, 1.80, 1.96, 2.10, 3.77	4.47	d (8.5)	1.66, 1.82, 1.95, 2.10, 3.77
15a	27.9	2.10	m		2.10	m	
15b		1.80	m		1.82	m	1.66, 1.82, 1.95, 3.77, 4.47
16a	25.1	1.96	m		1.92	m	
16b		1.68	m		1.66	m	
17a, b	47.8	3.77	m	1.68, 1.80, 1.96, 2.10, 4.47	3.77	m	1.66, 1.82, 1.95, 2.10, 4.47
Meth-1							
18	173.7						
19	51.5	4.74	ddd (10.5, 7.5, 4.5)	2.19, 2.47, 2.68, 2.76, 9.16	4.75	dd (7.0, 3.5)	2.22, 2.43, 2.70, 2.78, 9.12
20a	31.1	2.47	m		2.43	m	2.22, 2.70, 2.78, 4.75, 9.12
20b		2.19	m		2.22	m	2.43, 2.78, 4.75, 9.12
21a	31.1	2.76	m	2.19, 2.47, 2.68, 4.74, 9.16	2.78	m	2.22, 2.43, 2.70, 4.75, 9.12
21b		2.68	m	2.19, 2.47, 2.76, 4.74, 9.16	2.70	m	2.22, 2.43, 2.78, 4.75, 9.12
22	15.4	2.18	s		2.18	s	
N-4		9.16	d (7.5)	2.19, 2.47, 2.68, 2.76, 4.74	9.13	d (7.0)	2.20, 2.43, 2.70, 2.78, 4.75
Pro-2							
23	172.3						
24	61.6	4.61	d (8.0)	1.68, 1.92, 2.06, 2.45, 3.41, 3.54	4.61	dd (8.0, 2.0)	1.68, 1.91, 2.06, 2.46, 3.43, 3.55
25a	31.3	2.45	m		2.46	m	1.68, 1.91, 2.06, 3.43, 3.55, 4.61
25b		2.06	m		2.06	m	1.68, 1.91, 2.46, 3.43, 3.55, 4.61
26a	22.6	1.92	m		1.91	m	
26b		1.68	m		1.68	m	
27a	46.7	3.54	m	1.68, 1.92, 2.06, 2.45, 3.41, 4.61	3.55	m	1.68, 1.91, 2.06, 2.46, 3.43, 4.61
27b		3.41	m	1.68, 1.92, 2.06, 2.45, 3.54, 4.61	3.43	m	1.68, 1.91, 2.06, 2.46, 3.55, 4.61
Pro-3							
28	170.5						
29	60.1	4.52	t (7.5)	1.82, 1.92, 2.11, 2.39, 3.84	4.52	dd (7.5, 3.0)	1.80, 1.90, 2.11, 3.82, 3.89
30a	29.1	2.39	m		2.39	m	
30b		1.82	m		1.80	m	
31a	25.7	2.11	m		2.11	m	
31b		1.92	m		1.90	m	
32a	48.6	3.84	m	1.82, 1.92, 2.11, 2.39, 4.52	3.89	m	1.80, 1.90, 2.11, 3.82, 4.52
32b		3.84	m	1.82, 1.92, 2.11, 2.39, 4.52	3.82	m	1.80, 1.90, 2.11, 3.89, 4.52
Meth-2							
33	169.7						
34	50.6	5.05	t (8.0)	2.12, 2.64, 7.87	5.05	dd (8.0, 4.0)	2.24, 2.82, 2.91, 7.92
35a, b	33.0	2.12	m		2.24	m	2.82, 2.91, 5.05, 7.92
36a	29.7	2.64	m		2.91	m	2.24, 2.82, 5.05, 7.92
36b		2.64	m		2.82	m	2.24, 2.92, 5.05, 7.92
37	14.8	2.15	s		2.61	s	
N-7		7.87	d (8.0)	2.12, 2.64, 5.05	7.92	d (7.0)	2.24, 2.82, 2.91, 5.05

chemical shifts (δ 3.06 and 3.12) of the methyl group protons in **4** were characteristic of methionine sulfones.[21] Oxidation of waiakeamide (**3**) with H_2O_2 provided a product that, by HPLC, ^{1}H NMR, and MS characterization, was identical to the bis-sulfone **4** derived from oxidation of haligramide A (**1**). Thus, haligramide A (**1**) has the same absolute stereochemistry as waiakeamide (**3**).

The molecular formula of haligramide B (**2**), $C_{37}H_{49}N_7O_7S$, which was deduced by HRFABMS measurements, contained one less oxygen than that of waiakeamide (**3**). The ^{1}H NMR spectrum of compound **2** (Table 1) corresponded closely to both haligramide A (**1**) and waiakeamide (**3**). The most distinctive feature in the ^{1}H NMR spectrum of **2** was the presence of two methyl singlets at δ 2.18 and 2.61. This suggested that haligramide B (**2**) contained both methionine and methionine sulfoxide residues. COSY and TOCSY experiments confirmed the following spin systems: NH-4 (δ 9.13)−H-19 (δ 4.75)−H_2-20 (δ 2.22, 2.43)−H_2-21 (δ 2.70, 2.78) and NH-7 (δ 7.92)−H-34 (δ 5.05)−H_2-35 (δ 2.24, 2H)−H_2-36 (δ 2.82, 2.91). The downfield shifts of the C-36 γ-methylene protons, relative to the C-21 γ-methylene resonances, indicated that the former spin system comprised the methionine sulfoxide residue as in waiakeamide (**3**),[18] and the latter spin system was part of the methionine moiety. Selective 1D NOESY experiments were used to assign the methyl groups of the methionine and methionine sulfoxide moieties and to confirm the sequence of the constituent amino acids. Irradiation of the methyl signal at δ 2.18 (H_3-22) produced significant NOE interactions with H_2-21, while irradiation of the δ 2.61

Scheme 1. Oxidation of Peptides **1**, **2**, and **3**

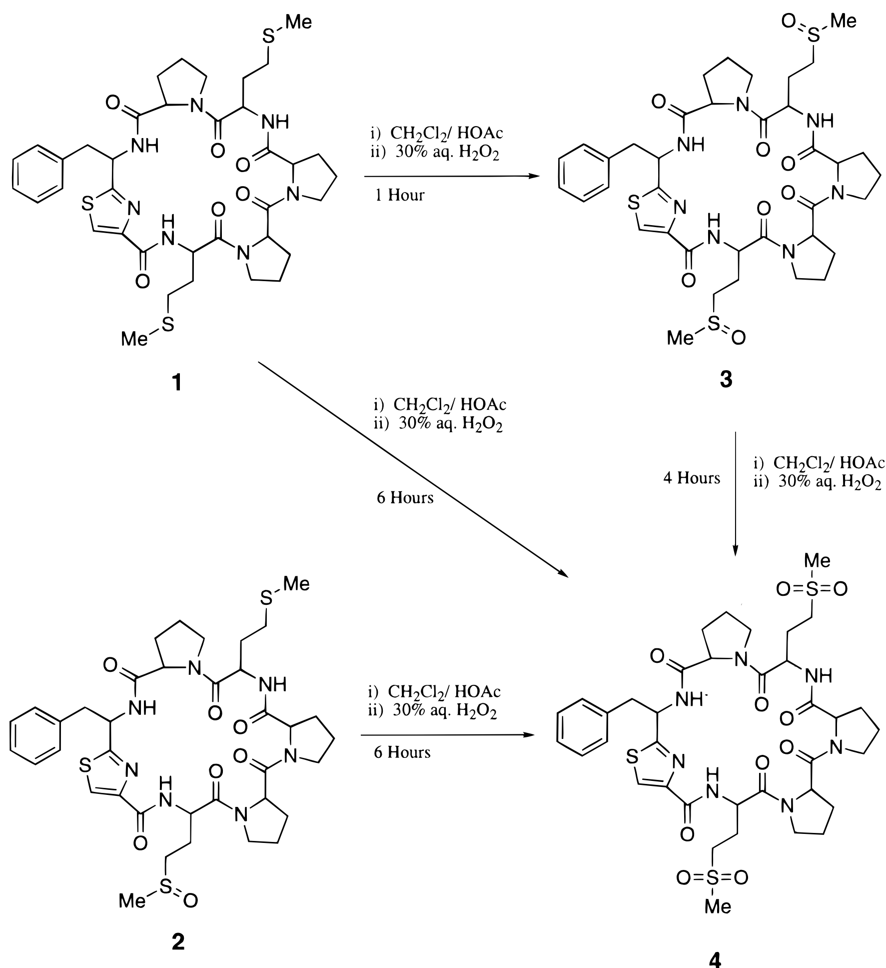

Table 2. Key HMBC Correlations Observed for Haligramide A (**1**) in DMF-d_7

proton	$^1H \rightarrow {}^{13}C$ correlations ^2J	^3J
H-3	149.3 (C-2)	170.7 (C-4)
H-5	43.1 (C-6), 170.7 (C-4)	
H-6b	52.9 (C-5), 137.6 (C-7)	130.0 (C-8, C-12), 170.7 (C-4)
H-8		43.1 (C-6), 127.7 (C-10), 130.0 (C-12)
H-9		129.0 (C-11), 137.6 (C-7)
H-10		130.0 (C-8)
NH-2	52.9 (C-5), 171.1 (C-13)	
H-14	27.9 (C-15), 171.1 (C-13)	25.1 (C-16), 173.7 (C-18)
H_2-17	25.1 (C-16)	
H-20b	31.1 (C-21), 51.5 (C-19)	
H_2-21	31.1 (C-20)	15.4 (C-22)
H_3-22		31.1 9 (C-21)
NH-4	172.3 (C-23)	173.7 (C-18)
H-24	31.3 (C-25), 172.3 (C-23)	22.6 (C-26), 46.7 (C-27)
H-25a		46.7 (C-27), 172.3 (C-23)
H-25b	61.6 (C-24)	172.3 (C-23)
H-26a		61.6 (C-24)
H-27b		31.3 (C-25)
H-29	29.1 (C-30), 170.5 (C-28)	
H-30a		48.6 (C-32)
H_2-32	25.7 (C-31)	29.1 (C-30)
H-34	33.0 (C-35), 169.7 (C-33)	29.7 (C-36), 60.1 (C-29)
H_2-35	29.7 (C-36)	169.7 (C-33)
H_2-36	33.0 (C-35)	14.8 (C-37), 50.6 (C-34)
H_3-37		29.7 (C-36)
NH-7	50.6 (C-34), 160.5 (C-1)	169.7 (C-33)

methyl group (H_3-37) resulted in a NOE with H_2-36. Observation of NOE interactions between H-19 and H_2-17, NH-4, H_2-20 and between H-34 and H_2-32, NH-7, H_2-35 supported the regiochemical assignment of the methionine sulfoxide and methionine residues in **2**. To confirm its structure, compound **2** was oxidized to the corresponding bis-sulfone derivative **4**, which was identical by ^1H NMR and HPLC analysis to the bis-sulfone oxidation products of **1** and **3**.

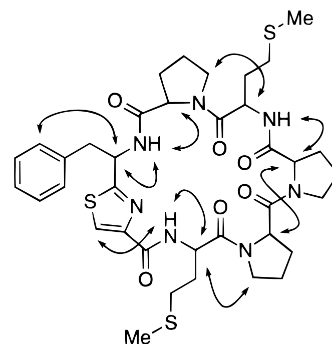

Figure 1. Key NOESY interactions in **1**.

Compounds **1** and **2** were evaluated for cytotoxic properties in a 2-day in vitro assay. The IC_{50} values (μg/mL) for haligramides A (**1**) and B (**2**) against the A-549 (lung), HCT-15 (colon), SF-539 (CNS), and SNB-19 (CNS) human tumor cell lines are listed below with the individual cell line identifiers: haligramide A (**1**), A-549 (5.17), HCT-15 (15.62), SF-539 (9.00), SNB-19 (9.08); haligramide B (**2**), A-549 (3.89), HCT-15 (8.82), SF-539 (5.01), SNB-19 (6.56).

Experimental Section

General Experimental Procedures. HPLC was performed on a Varian Rainin system employing a Dynamax C_{18} column (1 × 25 cm or 0.46 × 25 cm), using a flow rate of 3 mL/min or 0.6 mL/min and UV detection at 220 nm. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. Ultraviolet (UV) and infrared (IR) spectra were obtained on a Beckman DU-640 and a Perkin-Elmer 1600 FTIR spectrometer, respectively. The ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded in DMF-d_7 on a Varian INOVA 500 spectrometer, and the chemical shifts are reported in parts per million (ppm) relative to the residual nondeuterated solvent signals. Inverse-detected heteronuclear correlations were measured using HSQC (optimized for $^1J_{CH} = 140$ Hz) and HMBC (optimized for $^nJ_{CH} = 8.5$ and 3.5 Hz) pulse sequences. HRMS were acquired on a JEOL SX102 mass spectrometer.

Animal Material. Samples of the sponge *Haliclona nigra* were collected from the northern coast of Papua New Guinea in November 1992, by the Coral Reef Research Foundation. A voucher specimen (#0CDN697) for this collection is maintained at the Smithsonian Institute.

Isolation of Peptides. The frozen sponge samples were ground into a coarse powder (100.3 g) and extracted with H_2O. The H_2O-soluble fraction was freeze-dried to provide 24.2 g of aqueous extract. A 20.0 g aliquot of the crude extract was subjected to C_4 VLC eluted with increasing concentrations of MeOH in H_2O. Cytotoxic activity was concentrated into the fraction that eluted with MeOH−H_2O (1:2). Further purification of this fraction by C_{18} HPLC using MeOH−H_2O (80:20) yielded, in order of elution, compound **3** (4 mg; $t_R = 5.5$ min), compound **2** (1.0 mg; $t_R = 6.98$ min), and compound **1** (2 mg; $t_R = 12.05$ min).

Haligramide A (1): white amorphous powder; $[\alpha]_D$ −36.5° (*c* 0.1, MeOH); UV (EtOH) λ_{max} (log ϵ) 206 (4.35), 242 (4.11), 254 (4.15), 260 (3.99) nm; IR (film) υ_{max} 3379, 3263, 1633 br, 1521, 1181, 1070, 756 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRFABMS *m/z* 916.2003 [M + Cs]$^+$ (calcd for $C_{37}H_{49}O_6N_7S_3$-Cs, 916.1961).

Haligramide B (2): white amorphous powder; $[\alpha]_D$ −32.5° (*c* 0.09, MeOH); UV (EtOH) λ_{max} (log ϵ) 206 (4.26), 240 (3.63) nm; IR (film) υ_{max} 3379, 3270, 1630 br, 1531, 1208, 1180, 1074, 1032, 797, 749 cm^{-1}; ^1H NMR, see Table 1; due to the very limited availability of sample, a complete ^{13}C NMR spectrum of **2** was never obtained; HRFABMS *m/z* 932.1933 [M + Cs]$^+$ (calcd for $C_{37}H_{49}O_7N_7S_3Cs$, 932.1910).

Waiakeamide (3): white amorphous powder; $[\alpha]_D$ −82° (*c* 0.2, MeOH) (lit.[18] −54°); UV, IR, MS, ^1H NMR, and ^{13}C NMR data identical to previously reported values.[18]

Oxidation of 1, 2, and 3. Compound **1** (0.2 mg) was dissolved in 300 μL of CH_2Cl_2, and 300 μL of glacial HOAc was added to it. After cooling for 10 min at 4 °C, 10 μL of 30% aqueous $H_2O_2$was added to the reaction mixture, which was stirred for 1 h at room temperature. The solvents were evaporated to dryness to yield compound **3**. Similar reaction with 0.4 mg of haligramide A (**1**) using 400 μL of CH_2Cl_2, 400 μL of HOAc, and 20 μL of 30% aqueous H_2O_2 for 6 h afforded the bis-sulfone **4**. A similar 4−6 h treatment of 0.4 mg each of haligramide B (**2**) or waiakeamide (**3**) also yielded compound **4**: colorless gum; ^1H NMR (500 MHz, DMF-d_7) δ 9.18 (1H, d, J = 8.0 Hz), 8.24 (1H, s), 7.95 (1H, d, J = 7.5 Hz), 7.66 (1H, d, J = 7.5 Hz), 7.31 (2H, d, J = 7.5 Hz), 7.24 (1H, t, J = 7.5 Hz), 7.23 (2H, d, J = 7.5 Hz), 5.52 (1H, dd, J = 7.5, 7.5 Hz), 5.12 (1H, dd, J = 7.0, 7.0 Hz), 4.79 (1H, m), 4.62 (1H, d, J = 7.5 Hz), 4.53 (1H, t, J = 7.5 Hz), 4.44 (1H, d, J = 7.5 Hz), 3.84 (1H, m), 3.82 (2H, m), 3.74 (1H, m), 3.54 (2H, m), 3.51 (2H, m), 3.30 (2H, m), 3.17 (2H, d, J = 7.5 Hz), 3.12 (3H, s), 3.06 (3H, s), 2.61 (2H, m), 2.43 (1H, m), 2.39 (1H, m), 2.34 (2H, m), 2.10 (1H, m), 2.05 (1H, m), 2.03 (1H, m), 1.92 (1H, m), 1.90 (1H, m), 1.87 (1H, m), 1.83 (1H, m), 1.82 (1H, m), 1.69 (1H, m), 1.60 (1H, m); FABMS *m/z* 848 [M + H]$^+$.

HPLC Analysis of the Reaction Products. Semisynthetic **3** and **4**, derived from haligramide A (**1**), were analyzed by C_{18} HPLC using MeOH−H_2O (90:10) as the mobile phase. Retention times of 4.57 and 4.40 min for **3** and **4**, respectively, were identical with those of natural waiakeamide (**3**) and semisynthetic **4** obtained from waiakeamide (**3**). Co-injection of the oxidized peptides confirmed their identity. A similar HPLC analysis was conducted with the bis-sulfone product of haligramide B (**2**).

Cytotoxicity Evaluation. DMSO solutions of chromatography fractions and aliquots of the purified haligramides were assayed for cytotoxic properties in a 2-day in vitro assay, experimental details of which have been reported previously.[22]

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