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Structures and Solution Conformational Dynamics of Stylissamides G and H from the Bahamian Sponge *Stylissa caribica*

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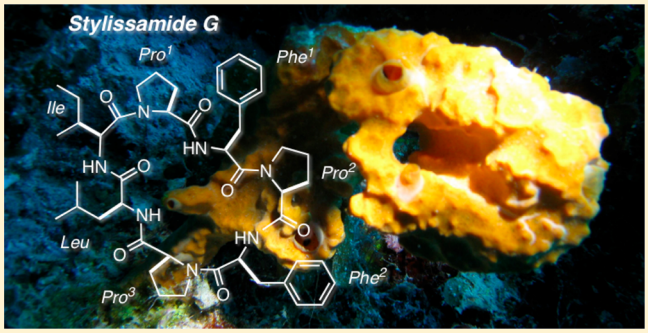
Supporting Information

ABSTRACT: Two new peptides, stylissamides G and H, were isolated from extracts of a sample of *Stylissa caribica* collected in deep waters of the Caribbean Sea. A single sample of *S. caribica* among a collection of 10 samples that were examined by LC-MS appeared to be a different chemotype from the others in that it lacked the familiar pyrrole-2-aminoimidazole alkaloids, stevensine and oroidin, and contained peptides of the stylissamide class. The structures of the title compounds were solved by integrated analysis of the MS and NMR spectra and chemical degradation. The solution conformation of stylissamide G was briefly examined by electronic circular dichroism and temperature-dependent ^{1}H NMR chemical shifts of amide NH signals, which supported a conformationally rigid macrocycle.



The marine sponge *Stylissa caribica* Lehnert & van Soest, 1998 is found at depths in excess of −25 m in the Caribbean Sea. The cyclic heptapeptides stylissamides A−F (**1a**−**d**,[1] **e**, **f**;[2,3] see Supporting Information for structures) have recently been described from *S. caribica* from the Bahamas. Many related peptides exhibit biological activity; for example, stylissamide 'X', from an unidentified *Stylissa* species from Indonesia, inhibited EGF-induced migration of cultured HeLa cells.[4] The cyclic peptides are minor components of these sponges with the major secondary metabolites being, largely, brominated pyrrole 2-aminoimidazole alkaloids[5] (PAIs) such as oroidin (**2a**),[6,7] hymenidin (**2b**),[8] and stevensine (**3**)[9] and their higher-order oligomeric congeners.

Recently, we demonstrated *de novo* biosynthesis of ^{15}N-labeled PAIs (nagelamide H and benzosceptrin C) from ^{15}N-**2a**,[10] providing the first experimental evidence that oroidin is a biosynthetic precursor of higher-order PAIs and participates in single-electron C−C bond forming reactions catalyzed by a metallo-enzyme (or enzymes) with molecular oxygen as the terminal oxidant.[11] The provenance and biosynthetic origins of the stylissamides, however, are less certain.

RESULTS AND DISCUSSION

The secondary metabolite profile of *S. caribica* has been investigated previously by HPLC.[12] In our screening for antifungal and cancer cell-inhibitory marine secondary metabolites, we examined several samples (N = 10) of *S. caribica* collected from the Bahamas in 2007 and 2008. Most showed in vitro antifungal activity against fluconazole-resistant *Candida albicans*, *Can. krusei*, *Cryptococcus neoformans* var. *grubbii*, and *Cryp. neoformans* var. *gatti*. A single sample of *S. caribica* (08-045Q) collected from Sweetings Cay, with a reversed-phase LCMS profile different from other samples, lacked antifungal activity, but showed significant cytotoxic activity toward cultured human colon tumor cells (HCT-116). While mainly **3** and smaller amounts of **2a** were evident in most samples of *S. caribica*,[11] these two compounds were not detected in 08-045Q (LCMS analysis). Instead, the latter sample contained hymenidin (**2b**) as the major PAI along with late-eluting components that lacked Br and with m/z values in the range expected for stylissamides. Sequential solvent partitioning of a MeOH extract of the sample separated the constituents by polarity, to give an *n*-BuOH-soluble fraction that was further purified by preparative HPLC to provide a cytotoxic fraction (IC_{50} = 5.5 $\mu g \cdot mL^{-1}$). Rechromatography of the latter gave pure samples of two new heptapeptides, stylissamides G (**1g**) and H (**1h**).

The molecular formula of **1g**, $C_{45}H_{61}N_7O_7$, was established from HRESIMS data. The high N content of **1g** and preliminary analysis of ^{1}H NMR data (Table 1) suggested a heptapeptide; this was further supported by the presence of cross-peaks in the HSQC spectrum consistent with seven α-CH signals (δ 51.6−61.6 ppm) and seven amide carbonyl signals (δ 170.6−171.7 ppm). Analysis of COSY and HMBC spectra of **1g** provided assignments of spin systems due to one each of Leu and Ile, two Phe, and three Pro residues (Table 1). All three Pro residues could be assigned *cis* conformations based on empirical rules[13] that correlate with ^{13}C NMR chemical shifts

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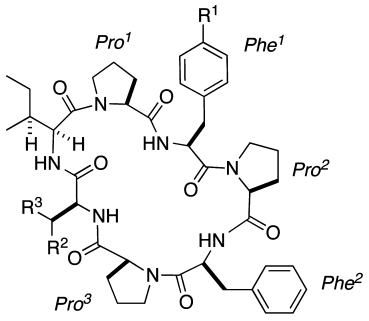
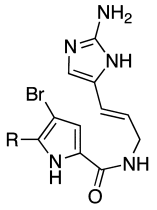
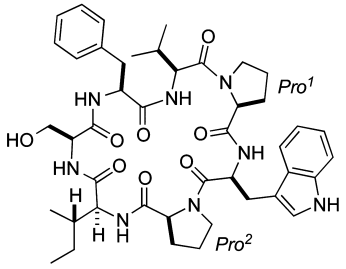


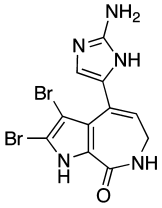
1g *stylissamide G* R^1= R^2= H, R^3=*i*-Pr
4 *phakellistatin*-2 R^1= OH, R^2= Me, R^3= Et



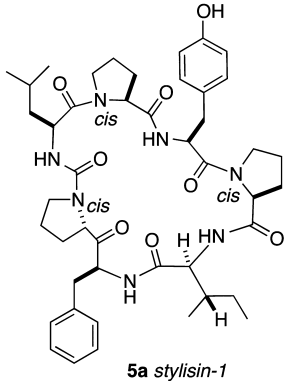
2a *oroidin* R = Br
2b *hymenidin* R = H



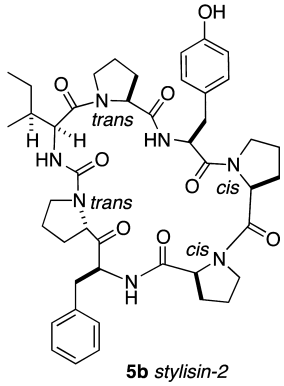
1h *stylissamide H* *trans*-Pro^2
6 *euryjanicin* *cis*-Pro^2



3 *stevensine*



5a *stylisin-1*



5b *stylisin-2*

(δ C_γ < 23.3 ppm) and differences between the β and γ carbons [$\Delta\delta(C_\beta - C_\gamma)$ > 8.0 ppm].

Sequence assignments of **1g** were obtained by analysis of HMBC and NOESY data. A band-selective constant-time HMBC[14] experiment ($\Delta\delta_C$ 161−181 ppm, optimized for J = 8 Hz) showed correlations from δ 4.37 ($Phe^1{}_{H\alpha}$) to δ 169.9 ($Pro^1{}_{C=O}$), δ 4.34 ($Pro^1{}_{H\alpha}$) to δ 171.7 ($Ile_{C=O}$), δ 7.88 (Ile_{NH}) and δ 4.26 ($Ile_{H\alpha}$) to δ 170.6 ($Leu_{C=O}$), δ 8.83 ($Leu_{H\alpha}$) to δ 169.9 ($Pro^3{}_{C=O}$), δ 3.27 ($Pro^3{}_{H\alpha}$) to δ 168.8 ($Phe^2{}_{C=O}$), and δ 8.94 ($Phe^2{}_{NH}$) to δ 171.4 ($Pro^2{}_{C=O}$). The foregoing data were sufficient to define the sequence Pro^1-Phe^1-Pro^2-Phe^2-Pro^3-Leu-Ile. Support for this sequence was also obtained from NOESY data. Cross-peaks in the NOESY spectrum of **1g** were observed between the following pairs of amino acid residue NH and α-CH NMR signals: $Phe^2{}_{NH}$/$Pro^2{}_{H\alpha}$, $Pro^2{}_{H\alpha}$/$Phe^1{}_{H\alpha}$, $Pro^1{}_{H\kappa}$/$Ile_{H\alpha}$, Ile_{NH}/$Leu_{H\alpha}$, Leu_{NH}/$Pro^3{}_{H\alpha}$, and $Pro^3{}_{H\alpha}$/$Phe^2{}_{H\alpha}$ (Figure 1).

Stylissamide G (**1g**) is a variant on the heptapeptide motif detected in cyclic peptides, from not only *Stylissa* (family Dictyonellidae) but other sponges in the family Axinellidae. Cytotoxic phakellistatins constitute a family of over a dozen peptides characterized from different samples of *Phakellia* spp. from the Indian Ocean,[15] Western Pacific,[16] and the South China Sea[17] mostly with the typical heptapeptide motif of

Table 1. ^{1}H and ^{13}C NMR[a] Data for 1g (DMSO-d_6, 600 MHz)

	position	^{13}C, type	^{1}H, mult (J in Hz)
Ile	C=O	171.7, C	
	α	53.1, CH	4.26, t (9.3)
	β	37.1, CH	1.59, m
	βCH_3	14.5, CH_3	0.75, m
	γ	23.8, CH_2	1.98, m
			1.45, m
	δCH_3	10.6, CH_3	0.77, t (7.3)
	NH		7.88, d (9.3)
Pro^1	C=O	169.6, C	
	α	61.0, CH	4.34, brd (9.1)
	β	31.0, CH_2	2.00, m
			2.18, m
	γ	21.3, CH_2	1.32, m
			1.72, m
	δ	45.6, CH_2	3.31, m
Phe^1	C=O	167.7, C	
	α	51.8, CH	4.37, m
	β	35.2, CH_2	3.36, m
			2.98, dd (13.6, 0.6)
	Ph	129.6 (o)	6.91, d (6.4)
		127.7 (m), CH	7.22, m
		126.4 (p), CH	7.21, m
		135.5, C	
	NH		6.55 brs
Pro^2	C=O	171.3, C	
	α	57.1, CH	4.61, d (8.0)
	β	30.4, CH_2	2.16, m
			1.92, m
	γ	21.1, CH_2	1.89, m
			1.81, m
	δ	46.7, CH_2	3.52, m
			3.31, m
Phe^2	C=O	168.8, C	
	α	52.9, CH	4.41, ddd (9.2, 6.4, 1.8)
	β	36.0, CH_2	3.09, dd (13.5, 6.4)
			2.95, dd (13.5, 9.2)
	Ph	128.9 (o), CH	7.28, m
		128.5 (m), CH	7.37, t (7.6)
		126.7 (p), CH	7.28, m
		135.7, C	
	NH		8.94, s
Pro^3	C=O	169.9, C	
	α	60.1, CH	3.27, m
	β	30.0, CH_2	1.89, m
			1.04, m
	γ	21.4, CH_2	1.62, m
			1.27, m
	δ	45.8, CH_2	3.27, m
			3.18, t (9.8)
Leu	C=O	170.6, C	
	α	51.1, CH	4.06, m
	β	38.0, CH_2	1.52, m
			1.10, m
	γ	24.5, CH	1.51, m
	δ	20.3, CH_3	0.73, d (6.3)
	δ'	23.1, CH_3	0.81, d (6.4)
	NH		8.83, d (7.1)

[a]See structures of **1g** and **1h** for the amino acid residue key. ^{13}C NMR shifts from indirect 2D NMR (HSQC, HMBC, and *bsct*HMBC).

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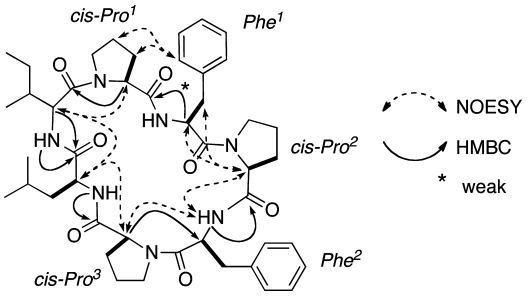

Figure 1. Selected NOESY and HMBC correlations for stylissamide G (**1g**).

stylissamides. In terms of sequence and amino acid composition, peptide **1g** most closely resembles phakellastatin-2 (**4**)[15] and stylisins-1 (**5a**) and -2 (**5b**) from *S. caribica* collected in Jamaica.[18]

The molecular formula of **1h**, $C_{44}H_{58}N_8O_8$, was established from HRESIMS data. The ^{1}H NMR spectrum of cyclopeptide **1h** exhibited conformational heterogeneity in several solvents ($CDCl_3$, DMSO-d_6, and CD_3OD) that significantly complicated interpretation and assignment. Similar solvent-dependent complexity has been observed in the NMR spectra of **4**.[19] A relatively well-resolved ^{1}H NMR spectrum of **1h** was obtained in CD_2Cl_2, and analysis of the spin systems by proton, COSY, HMQC, and HMBC NMR (Table 2) showed the presence of Trp, Ser, Ile, Val, Phe, and two Pro residues. Pro^1 was assigned the *cis* conformation, while Pro^2 was assigned as *trans* according to empirical rules. Attempts to sequence **1h** by HMBC and NOESY failed due to relatively weak carbonyl signals dispersed between multiple conformers. Alternative MS^4 analysis also failed to deliver interpretable fragment ions. Gratifyingly, partial hydrolysis of **1h** (2 M HCl in CH_3CN–H_2O, 60 °C, 90 min) gave a linear peptide from single amide bond hydrolysis that was identified as Pro^1-Trp-Pro^2-Ile-Ser-Phe-Val by LC/MS/MS (Figure 2). The composition and amino acid sequence of **1h** are identical to those of euryjanicin A (**6**),[20] which was isolated by Rodriguez and co-workers from the Caribbean sponge *Prosuberites laughlini* (Díaz, Alvarez & Van Soest, 1987). However, peptides **1h** and **6** are configurational isomers: Pro^2 is in the *trans* configuration in **1h**, while both proline residues in **6** adopt the *cis* configuration. No interconversion of **1h** into **6** was observed upon heating the former in DMSO (70 °C, 30 min), suggesting that **1h** is the thermodynamically favored isomer.

The amino acid residues in all *Stylissa* peptides reported to date are of the L-configuration. Pure samples of **1g** and **1h** were separately hydrolyzed (6 M HCl, 95 °C, 15 h), and, after removal of volatiles, the residues were subjected to Marfey's analysis.[21] The configurations of all amino acid residues in both peptides (except Trp in **1h**, which decomposed during hydrolysis) were also found to be L.[22]

Natural product cyclic heptapeptides and octapeptides are presumed to adopt rigid, conformationally constrained structures ordered by minimized torsional strain, especially those imposed by *cis* and *trans* Pro residues. Aside from computational modeling studies that determined the minimized energy structures,[18] no experimental data have been reported on the conformational dynamics of the stylissamides. We briefly examined the solution conformational dynamics of **1g** and **1h** by temperature-dependent CD spectra (Figure 4) and amide signal (NH) ^{1}H NMR chemical shifts (Table 3).

Table 2. ^{1}H and ^{13}C NMR[a] Data for 1h (CD_2Cl_2, 600 MHz)

	position	^{13}C, δ, type	^{1}H δ, mult (J in Hz)
Ile	C=O	168.9, C	
	α	59.7, CH	3.88, t (5.1)
	β	35.8, CH	1.39, m
	βCH$_3$	15.5, CH$_3$	0.77, d (6.9)
	γ	25.3, CH$_3$	1.16, m
	δ	11.3, CH$_3$	0.63, t (7.4)
	NH		5.63, d (4.8)
Ser	C=O	171.9, C	
	α	54.2, CH	4.75, d (9.9)
	β	63.22, CH$_2$	4.64, d (11.8)
			3.42, d (11.8)
	NH		7.21, m
Phe	C=O	171.7, C	
	α	63.2, CH	3.79, dd (15.5, 7.9)
	β	36.1, CH$_2$	3.67, m
			3.53, dd (14.0, 7.9)
	NH		8.36, s
Val	C=O	N.D.	
	α	56.0, CH	4.30, m
	β	33.0, CH	1.86, m
	γ	17.1, CH$_3$	0.87, d (6.9)
	γ'	20.1, CH$_3$	0.84, d (6.9)
	NH		8.54, d (7.7)
Pro^1	C=O	N.D.	
	α	61.2, CH	4.29, m
	β	30.8, CH$_2$	2.02, m
			1.95, m
	γ	20.9, CH$_2$	1.57, m
			0.99, m
	δ	46.5, CH$_2$	3.35, m
			3.34, m
Trp	C=O	N.D.	
	α	50.0, CH	5.23, m
	β	27.8, CH$_2$	3.29, m
			3.11, dd (15.7, 7.5)
	NH		6.80, d (9.0)
Pro^2	C=O	172.9, C	
	α	63.1, CH	4.26, t (8.3)
	β	29.7, CH$_2$	2.42, m
			1.86, m
	γ	25.4, CH$_2$	2.07, m
			1.84, m
	δ	47.9, CH$_2$	3.93, m
			3.67, m

[a]See structures of **1g** and **1h** for the amino acid residue key. ^{13}C NMR chemical shift assignments from indirect 2D NMR (HSQC, HMBC, and *bsct*HMBC.

At room temperature (23 °C), the CD spectrum of **1g** (CH_3CN, Figure 3) was characterized by two Cotton effects (CEs) (λ 196 ($\Delta\varepsilon$ +23.2), 223 ($\Delta\varepsilon$ −7.2) nm), while that of **1h** showed more complex band structure due to the presence of Trp. At elevated temperatures (50 and 70 °C, Figure 4), little difference was observed in the CD spectra of **1g** and **1h**, although at lower temperature (0 °C) moderate increases in the magnitudes of both CEs of **1g** were observed.

In their study of temperature-dependent ^{1}H NMR amide NH signals and exchange rates in proteins, Baxter and Williamson concluded that temperature coefficients ($\Delta\delta/\Delta T$) greater than −4.5 ppb/K were correlated with hydrogen-

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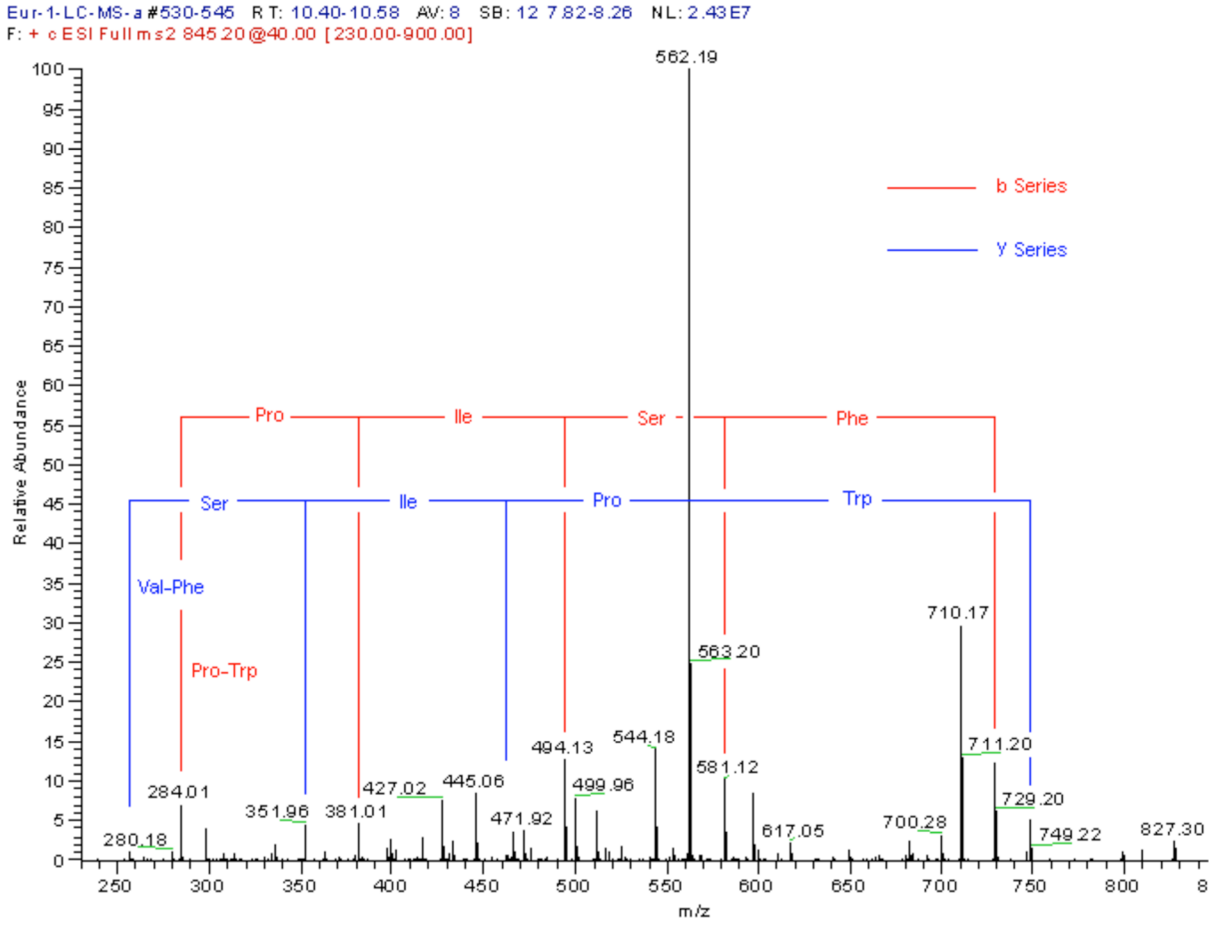

Figure 2. LC/MS/MS analysis of the partial hydrolysis product of stylissamide H (**1h**).

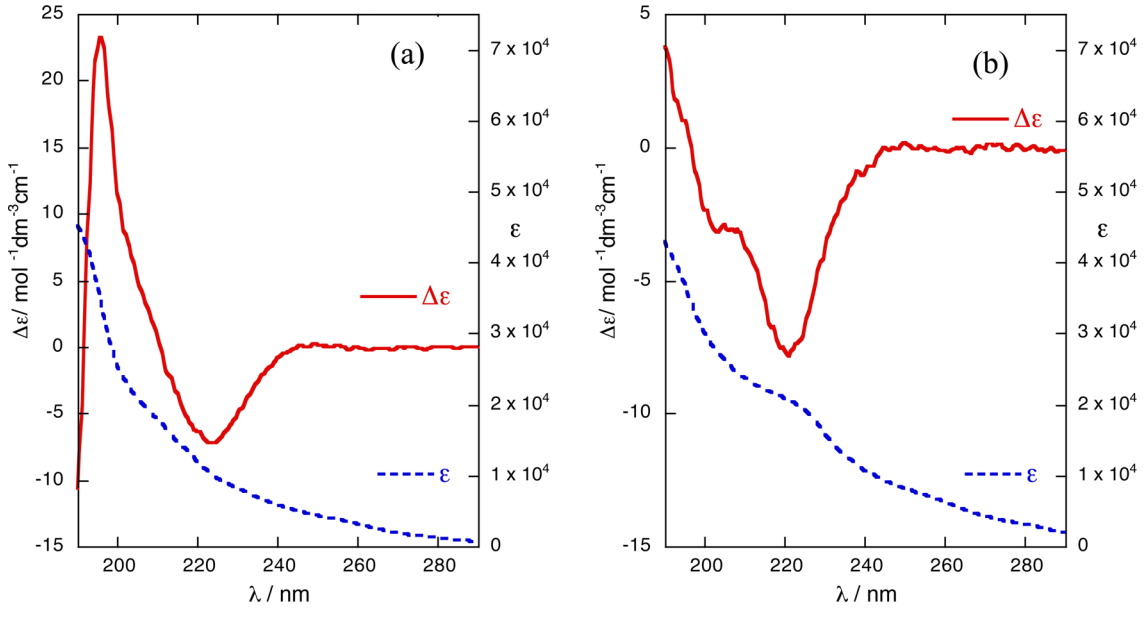

Figure 3. CD and UV spectra (CH_3CN, 23 °C): (a) stylissamide G (**1g**) and (b) stylissamide H (**1h**).

bonded amide protons, while NH protons with values less than −4.5 ppb/K were not hydrogen-bonded.[23] Measurement of temperature-dependent ^{1}H NMR amide NH chemical shifts of **1g** (T = 25, 50, 70 °C) gives $\Delta\delta/\Delta T$ coefficients (Table 3) that are greater than −4.5 ppb/K for Phe^1 and Leu, but lower than −4.5 ppb/K for Phe^2 and Ile, showing that NH of Phe^1 and Leu are strongly hydrogen bonded. These temperature coefficients support a generally tightly constrained amide backbone for **1g** with weaker hydrogen bonding at the Phe^2 and Ile residues. Taking both CD and $\Delta\delta/\Delta T$ measurements into consideration, the data support conformations of **1g** and **1h** with relatively

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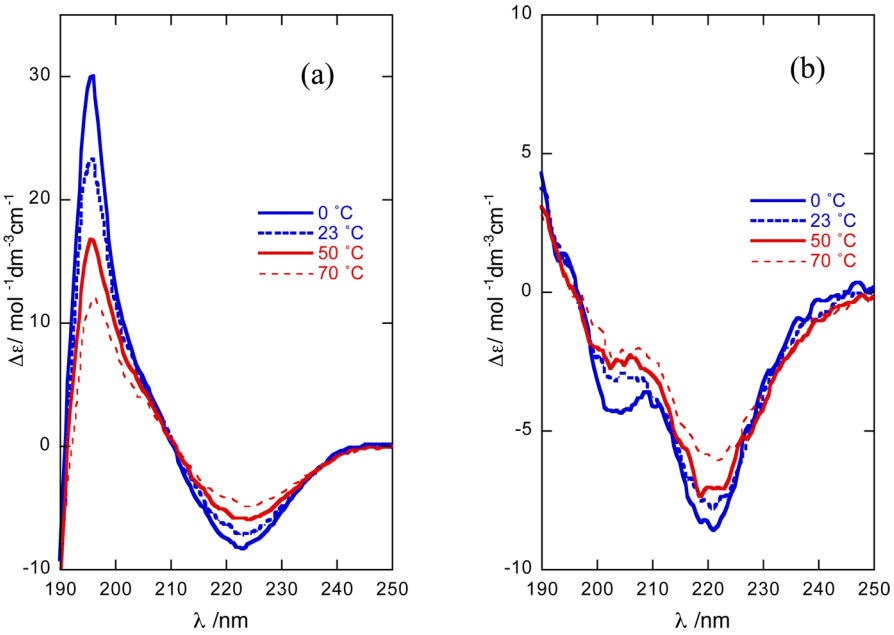

Figure 4. Temperature-dependent CD spectra (CH_3CN): (a) stylissamide G (**1g**) and (b) stylissamide H (**1h**).

Table 3. Temperature Coefficients ($\Delta\delta/\Delta T$) of Amide NH ^{1}H NMR Signals (δ, parts per billion) for 1g

	T (K)			
	298	323	343	
	δ (ppm, N–H)			$\Delta\delta/\Delta T$ (ppb/K)
Ile	7.88	7.73	7.61	−6.0
Leu	8.83	8.80	8.76	−1.5
Phe^1	6.55	6.58	6.61	1.3
Phe^2	8.94	8.82	8.72	−4.9

rigid amide backbones, constrained by intramolecular hydrogen bonds, but higher mobility of the Ile and Phe residues.

Pettit and others have reported potent cytotoxicity of cyclic heptapeptides related to **1g** and **1h**, for example, phakellistatin-2,[15] related phakellistatins,[16] and hymenistatins G, H, and J.[17] Kobayashi and co-workers showed that stylissamide 'X' inhibited EGF-induced migration of HeLa cells in the concentration range 0.1 to 10 μM.[4] In the present work, the fraction of *S. caribica* extract containing stylissamides **1g** and **1h** was cytotoxic to HCT-116 cells. Peptide **1h** exhibited modest in vitro cytotoxicity against HCT-116 (EC_{50} 5.7 μM) while **1g** was essentially inactive (EC_{50} >200 μM).

In conclusion, two new heptapeptides, stylissamides G (**1g**) and H (**1h**), were isolated from a cytotoxic fraction of the sponge *S. caribica* from the Bahamas and characterized by integrated spectroscopic methods. Studies of the solution dynamics of **1g** and **1h** by temperature-dependent CD and amide NH ^{1}H NMR temperature coefficients suggested peptide conformations ordered by a relatively rigid, hydrogen-bonded backbone structure with limited mobility.

EXPERIMENTAL SECTION

General Experimental Procedures. UV–vis spectra were recorded on a Jasco UV V630 dual beam instrument in 1 cm quartz cells. CD spectra were recorded on a Jasco 810 spectropolarimeter in quartz cells (1 or 2 mm path length). ^{1}H NMR and 2D NMR spectra of submilligram samples were measured on a Bruker 600 NMR spectrometer under control of an Avance III console and equipped with a 1.7 mm {^{13}C/^{15}N}^{1}H microcryoprobe, operating at 599.5556 MHz. Additional ^{1}H and ^{13}C NMR spectra were measured on a Jeol ECA 500 spectrometer operating at 500.1599 and 125.6940 MHz, respectively, with samples dissolved in CD_3OD or DMSO-d_6 (99.8 atom % D, Cambridge Isotopes). Low-resolution MS spectra were recorded on a ThermoFisher MSQ Plus Surveyor single quadrupole mass spectrometer, operating in positive ion ESI mode, coupled to a Thermo Fisher Accela ultra-high-performance liquid chromatograph (UHPLC). HRMS measurements were made using an Agilent 6230 TOFMS under positive ion ESI TOFMS conditions and provided by the UCSD Small Molecule MS Facility and the Torrey Pines Scripps Research Institute, La Jolla, California. Semipreparative HPLC was carried out using an Agilent 1100 HPLC under specified columns and gradient conditions.

Animal Material, Extraction, and Isolation. A sample of *Stylissa caribica* (accession 08-08-045) was collected at Sweetings Cay, Bahamas (49.646′ S, 77°54.054′ W) using scuba from a depth of −27 m in June 2008 and kept frozen (−20 °C) until required. The frozen sponge (wet wt 107.1 g) was cut into ~3 cm cubes and extracted with 1:1 CH_2Cl_2–MeOH with stirring (rt, overnight, 2 × 500 mL). Following removal of the solvent under reduced pressure, the residue was partitioned between hexanes (3 × 250 mL) and 9:1 MeOH–H_2O (250 mL). The aqueous MeOH layer was concentrated and further partitioned between *n*-BuOH (3 × 250 mL) and H_2O (250 mL). Removal of the volatile solvent from the upper layer gave a brown semisolid (1.68 g), a portion of which was adsorbed onto a solid-phase extraction cartridge (C_{18}) and eluted with MeOH. The MeOH eluate was concentrated under reduced pressure to give a solid (870 mg), which was separated by semipreparative reversed-phase HPLC (C_{18}, gradient from 10:90 to 60:40 CH_3CN–H_2O + 0.1% TFA) to give 10 fractions. A portion (8.6 mg) of the eighth fraction (36.8 mg) was further purified by semipreparative HPLC (C_{18}, gradient 34:8:58 CH_3CN–*i*PrOH–H_2O + 0.1% TFA) to give didebromonagelamide A[11] (0.4 mg). The tenth fraction (72.3 mg) was found to be cytotoxic against HCT-116 cells (IC_{50} = 5.5 μg.mL^{-1}). A portion of the tenth fraction (22 mg) was further purified by reversed-phase HPLC (C_{18} Phenomenex 250 × 10 mm 5 μm, 100 Å column, 3.0 mL·min^{-1} under the following gradient profile (A = H_2O–0.1% CF_3COOH, B = CH_3CN; 40% B, t = 0 min; 40% B, t = 5 min; 60% B, t = 20 min) and gave stylissamide H (t_R = 12.6 min, 0.50 mg, **1h**, 0.015% wet wt), stylissamide G (**1g**, t_R = 15.1 min, 1.6 mg, 0.049% wet wt), and known compounds stylisin-1 (**5**, t_R = 9.78 min), stylissamide A (**1a**, t_R = 18.5 min), and stylissamide C (**1c**, t_R = 18.5

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min) as colorless solids. See Supporting Information for complete structures.

*Stylissamide G (**1g**):* colorless solid; UV (CH_3CN, 23 °C) λ_{max} (log ε) 258 (2.88), 264 (2.87) nm; CD (0.322 mM, CH_3CN, 23 °C) λ_{max} ($\Delta\varepsilon$) 196 (+23.2), 223 (−7.2) nm, see also Figures 3 and 4; ^1H and ^{13}C NMR, see Table 1; HRESIMS m/z 812.4704 $[M + H]^+$, 834.4522 $[M + Na]^+$ (calcd for $C_{45}H_{62}N_7O_7^+$ 812.4705; calcd for $C_{45}H_{61}N_7NaO_7^+$, 834.4525).

*Stylissamide H (**1h**):* colorless solid; UV (CH_3CN, 23 °C) λ_{max} (log ε) 272 (3.39), 289 (3.25) nm; CD (0.189 mM, CH_3CN, 23 °C) λ_{max} ($\Delta\varepsilon$) 203 (−3.2), 221 (−7.8) nm, see also Figures 3 and 4; ^1H and ^{13}C NMR, see Table 2; HRESIMS m/z 827.4417 $[M + H]^+$ (calcd for $C_{44}H_{59}N_8O_8^+$ 827.4456).

Band-Selective Constant Time HMBC of Stylissamide G (1g) (ref 14). A band-selective constant-time HMBC spectrum of a sample of **1g** (~1.0 mg) in $CDCl_3$ was recorded under the following conditions: the band-selective pulse was a G3 Gaussian cascade of 500 μs duration centered at δ 172 ppm. The ^{13}C spectral width was set to 20 ppm, and ^1H-detected FIDs (64 t_1 increments) were collected at 600 MHz for a total experiment time of 84 min. The delay for selection of long-range coupling was set to J = 8 Hz, and the 2-fold low-pass J filter was optimized to suppress one-bond couplings between J = 120 and 180 Hz.

■ ASSOCIATED CONTENT

Supporting Information

^1H NMR and 2D NMR spectra of **1g** and **1h**, MS/MS/MS spectrum of **1g**, procedures for Marfey's analysis of **1g** and **1h**, bioassay procedures and structures of all stylissamides (**1a**−**1h**). This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

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