Stylissamides E and F, Cyclic Heptapeptides from the Caribbean Sponge *Stylissa caribica*

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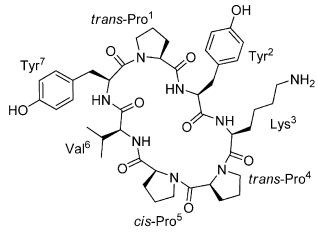
In addition to the four known stylissamides, A (**1**), B (**2**), C, and D, two new cyclic heptapeptides, stylissamides E (**3**) and F (**4**), were isolated from the Caribbean sponge *Stylissa caribica*. The structures of **3** and **4** were elucidated from a combination of mass spectrometric and NMR spectroscopic data as cyclo-(*cis*-Pro^1-Tyr^2-*trans*-Pro^3-Ala^4-Ile^5-Gln^6-Ile^7) for stylissamide E (**3**) and cyclo-(*trans*-Pro^1-*cis*-Pro^2-Phe^3-Asp^4-*trans*-Pro^5-Arg^6-Phe^7) for stylissamide F (**4**).

Marine sponges are known to be a rich source of non-ribosomal cyclic peptides with seven to ten amino acids that show various biological activities.[1] The cyclic nature of these metabolites often provides more lipophilicity and therefore membrane permeability because of a reduced zwitterionic character. Furthermore, proline units lead to a less flexible backbone conformation and result in a higher affinity and selectivity for protein binding.[2] Examples of proline-rich cyclopeptides are the axinastatins (7 or 8 amino acids with 2 or 3 proline residues),[3] axinellins (7−8 aa, 2−3 Pro),[4] hymenamides (7−8 aa, 1−3 Pro),[5] phakellistatins (7−10 aa, 1−4 Pro),[6] and stylopeptides (7−10 aa, 2−4 Pro).[7] Several cyclic heptapeptides have been recently described from the marine sponge *Stylissa caribica*. Stylisins 1 and 2 have been isolated from a Jamaican specimen,[8] while its Bahamanian counterpart[9] contained the stylissamides A (**1**), B (**2**), C, and D.[10] In addition to the four known stylissamides, the two new proline-rich cyclic heptapeptides stylissamides E (**3**) and F (**4**) were detected in a further specimen of *S. caribica*.[11] Herein we report the structure elucidation of **3** and **4** with a combined approach of mass spectrometric and NMR spectroscopic techniques.



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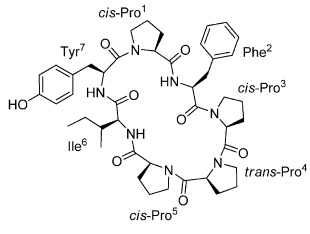

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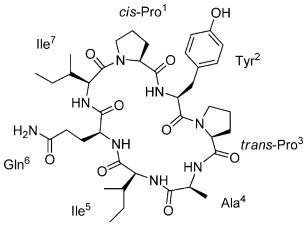

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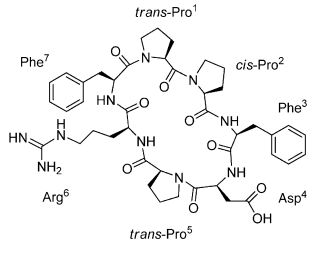

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During our search for bioactive metabolites from Caribbean sponges, we have investigated the crude extract from specimens of *S. caribica* collected in July 2000 in the Bahamas. The freeze-dried sponge tissue was extracted with a 1:1 mixture of MeOH/CH_2Cl_2, and the resulting extract was further purified by liquid/liquid partitioning between *n*-hexane, *n*-BuOH, and H_2O. The *n*-BuOH phase was purified by size exclusion chromatography with Sephadex LH-20.

Using HPLC-MS screening four proline-rich cyclic heptapeptides, stylissamides A (**1**), B (**2**), C, and D, were detected. In addition, this "peptide-containing fraction" showed two unknown substances assigned as stylissamide E (**3**) and stylissamide F (**4**). The final purification of these compounds was achieved by preparative reversed-phase HPLC.

The molecular weight of stylissamide E (**3**) (*m*/*z* 783.4417 [M + H]$^+$) was obtained from ESI mass spectrometry (HR-ESIMS) and indicated the molecular formula $C_{39}H_{59}N_8O_9$. The ^{1}H NMR spectrum of **3** showed seven amide protons at 8.52, 8.31, 7.93, 7.51, 7.42, and 6.85 (2 H) ppm, while the ^{13}C NMR spectrum contained eight carbonyl signals at 174.4, 173.9, 171.4 (2 C), 170.3, 170.1 (2 C), and 168.2 ppm. The investigation of the 2D NMR spectra identified seven amino acids. Two independent spin systems of the type X−CH−CH_2−CH_2−CH_2−X′ were defined using ^{1}H,^{13}C-HSQC-TOCSY, ^{1}H,^{1}H-COSY, and ^{1}H,^{13}C-HMBC and indicating the presence of two proline units. Two spin systems of the type X−CH−CH(CH_3)−CH_2−CH_3 suggested the amino acid isoleucine, while the further spin systems X−CH−CH_3, X−CH−CH_2−CH_2−X′, and X−CH−CH_2−X′ were attributed to alanine, glutamine, and tyrosine. The seven amino acids alanine, glutamine, isoleucines (2×), prolines (2×), and tyrosine complied with the number of carbonyl and amide signals of the 1D NMR data and defined the molecule as a heptapeptide. The amino acids implied 14 degrees of unsaturation, while the number of unsaturations indicated by the molecular formula is 15. This difference suggested a cyclic structure for the molecule.

The sequence of the seven amino acid residues in **3** was elucidated by a detailed analysis of the ^{1}H,^{13}C-HMBC data, which showed correlations between the Hα and the amide proton or in the case of proline the Hδ protons and the carbonyl carbon of the previous amino acid in the backbone of the peptide. The similar chemical shifts of the carbonyl carbons of tyrosine and one proline (170.1 ppm) as well as of glutamine (171.4 ppm) and one isoleucine were differentiated by a semiselective ^{1}H,^{13}C-HMBC experiment. The resulting correlations showed the following separated shifts in the semiselective ^{1}H,^{13}C-HMBC: 170.11 ppm for Tyr^2, 170.14 ppm for Pro^3, 171.38 ppm for Ile^5, and 171.42 ppm for Gln^6. The correlations Tyr^{2}NH/Pro^{1}CO and Tyr^{2}Hα/Pro^{1}CO assigned the fragment Pro^1-Tyr^2, and Ala^{4}NH/Pro^{3}CO and Ala^{4}Hα/Pro^{3}CO provide the segment Pro^3-Ala^4. The correlations Ile^{5}Hα/Ala^{4}CO, Gln^{6}NH/Ile^{5}CO, and Ile^{7}Hα/Gln^{6}CO elongated the second sequence to Pro^3-Ala^4-Ile^5-Gln^6-Ile^7. The analysis of the NOESY spectrum connected both partial structures with the cross peak Tyr^{2}NH/Pro^{3}Hα. Further NOE cross peaks supported the sequence Pro^1-Tyr^2-Pro^3-Ala^4-Ile^5-Gln^6-Ile^7 for **3** (see Figure 1). The final proof of the cyclic structure of stylissamide E (**3**) was achieved by analysis of the MS/MS spectra.

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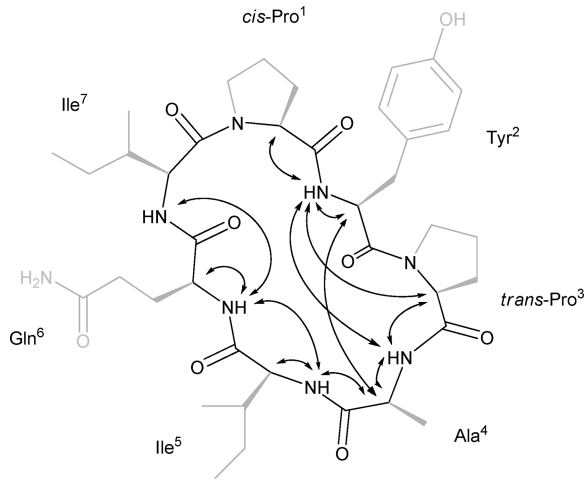

Figure 1. Backbone NOE cross peaks for stylissamide E (**3**).

The configuration of the peptide bond preceding both proline units was established by the difference in the chemical shifts of the β and γ carbons ($\Delta\delta_{\beta,\gamma}$).[12] The differences $\Delta\delta_{\beta,\gamma}$ (9.9 and 3.2 ppm) indicated one *cis* and one *trans* configuration of the proline residues in **3** (see Table 1). The similar chemical shifts of Pro^{1}Hα and Ala^{4}Hα and likewise Pro^{3}Hα and Ile^{7}Hα caused an overlapping of the signals of the NOE spectrum, and observed correlations could not confirm the configuration of the prolines.[13]

The proposed sequence of the seven amino acids was verified by tandem mass spectrometry.[14] The first step in the fragmentation of a cyclic peptide is the ring-opening, which results in a mixture of protonated linear molecules with the same molecular weight but different sequences of amino acids. The investigation of MS/MS experiments of cyclic peptides showed that the amide bond between proline and the preceding amino acid is a preferred position for ring-opening.[15] The second step is the fragmentation of the linear structure of the peptide, generally by successive loss of the C- or N-terminal amino acid in the MS/MS experiments. The isolation and fragmentation of the resulting MS/MS ions in MS^n experiments give additional information about the sequence of the amino acids.

The MS/MS fragmentation of **3** showed the loss of the amino acids alanine, isoleucine, glutamine, and proline directly from the parent ion and proved the cyclic structure of the heptapeptide with a possible ring-opening at several positions. The differentiation of leucine and isoleucine resulted from the NMR spectra, in which only the spin systems for isoleucine were observed. The main fragmentation pathway of *m/z* 670.3865, 542.3615, 429.2596, and 358.2480 showed the losses of isoleucine, glutamine, isoleucine, and alanine. These results confirmed the proposed amino acid sequence with a ring-opening between Ile^7 and Pro^1 and an elimination of the single amino acids Ile^7, Gln^6, Ile^5, and Ala^4 from the C-terminal end of the peptide (see Figure 2 and Table 2). A weak signal for the loss of Pro^3 extended this sequence to Pro^3-Ala^4-Ile^5-Gln^6-Ile^7. Besides the main fragments, there were ions with a reduced molecular weight of *m/z* 17, 28, and 45, which correspond to the elimination of ammonia, carbon monoxide, and both together (NH_3 + CO) from the main fragments (for example: *m/z* 765.4238, 755.4285, and 738.4422). The fragment at *m/z* 582.3304 indicated the loss of the amino acids Ile^5 and Ala^4 combined with an elimination of ammonia. The fragment *m/z* 429.2596 lost proline and alanine under MS^3 conditions. The C-terminal elimination of Ala^4 and N-terminal elimination of Pro^1 determined the sequence Pro^1-Tyr^2-Pro^3-Ala^4. The sequence of **3** was finally established by a combined approach of NMR and mass spectrometric data as cyclo-(*cis*-Pro^1-Tyr^2-*trans*-Pro^3-Ala^4-Ile^5-Gln^6-Ile^7).

The molecular weight of stylissamide F (**4**) (*m/z* 857.4266 [M + H]$^+$, HR-ESIMS) indicated the molecular formula $C_{43}H_{57}N_{10}O_9$.

Table 1. Chemical Shifts of Stylissamide E (**3**) (600 MHz)[a]

entry	residue	position	δ_C/δ_N	δ_H, mult. (*J*/Hz)
1	Pro^1	N	127	
2		CO	170.3	
3		α	60.2	4.38, m
4		β,β'	30.9	1.99[c]; 1.83[c]
5		γ,γ'	21.0	1.53[c]; 0.72[c]
6		δ,δ'	46.0	3.19, m; 2.91, m
7	Tyr^2	NH	121	7.93, d (7.3)
8		CO	170.11[b]	
9		α	56.8	4.08, m
10		β,β'	36.2	3.06, dd (3.9, 13.5); 2.81, t (13.2)
11		1	127.4	
12		2,6	115.1	6.66, d (7.5)
13		3,5	129.5	6.98, d (5.5)
14		4	156.1	
15		OH		9.27, s
16	Pro^3	N	141	
17		CO	170.14[b]	
18		α	59.1	4.30[c]
19		β,β'	28.0	2.19[c]; 1.63[c]
20		γ,γ'	24.8	1.92[c]; 1.85[c]
21		δ,δ'	46.8	3.44[c]; 3.39[c]
22	Ala^4	NH	114	7.51, d (6.8)
23		CO	173.9	
24		α	47.3	4.40, m
25		β	19.5	1.36, d (7.1)
26	Ile^5	NH	120	8.52, br
27		CO	171.38[b]	
28		α	61.4	3.61, dd (2.5, 7.1)
29		β	34.9	1.70[c]
30		βMe	15.3	0.88[c]
31		γ,γ'	25.7	1.56[c], 1.19[c]
32		δ	11.1	0.86[c]
33	Gln^6	NH	112	8.31, d (6.2)
34		CO	171.42[b]	
35		α	54.4	4.02, m
36		β,β'	26.1	1.95[c]; 1.91[c]
37		γ	31.7	2.19[c]
38		δ	174.4	
39		Nϵ	109	7.42, s; 6.85[c]
40	Ile^7	NH	110	6.85[c]
41		CO	168.2	
42		α	54.3	4.32, m
43		β	36.6	1.83[c]
44		βMe	15.8	0.95, d (6.5)
45		γ,γ'	24.3	1.50[c]; 1.09[c]
46		δ	11.9	0.85[c]

[a] ^{1}H and ^{13}C chemical shifts are referenced to the DMSO-d_6 signal (2.50 and 39.5 ppm, respectively). ^{15}N chemical shifts were not calibrated with an external standard. Therefore, the δ value has an accuracy of about 1 ppm in reference to NH_3 (0 ppm), and the ^{15}N chemical shifts are given without decimals. [b] ^{13}C NMR shifts of carbonyl carbons are given with two decimals if one decimal did not allow for differentiation of two different carbonyl carbons. [c] Overlapped signals.

The ^{1}H and ^{13}C NMR spectra of **4** showed five amide signals at 8.25, 7.59, 7.45, 7.38, and 7.35 ppm and eight carbonyl signals at 172.7, 171.9, 171.1, 171.0, 170.8 (2 C), 170.4, and 168.5 ppm (see Table 3). 2D NMR techniques assigned seven amino acids including two different types of spin systems. The repeated existence of the spin system X−CH−CH_2−CH_2−CH_2−X′ indicated three proline and one arginine residue within the peptide, while X−CH−CH_2−X′ were attributed to two phenylalanine units and one aspartic acid. A difference in the degree of unsaturation − 19 in the amino acids and 20 in the calculated molecular formula − suggested a cyclic structure for the heptapeptide. The sequencing of the amino acids was complicated due to the overlap of two proline Hα signals and similar chemical shifts for some carbonyl carbons. The amide proton and Hα region of the semiselective ^{1}H,^{13}C-HMBC experiments showed seven correlations between the Hα or the amide proton and the carbonyl carbon of the previous amino acid. The signals Phe^{3}Hα/Pro^{2}CO, Asp^{4}Hα/Phe^{3}CO, Pro^{5}Hα/Asp^{4}CO, Arg^{6}Hα/Pro^{5}CO, and Phe^{7}Hα/Arg^{6}CO determined the segment Pro^2-Phe^3-Asp^4-Pro^5-Arg^6-Phe^7, while the correlations Asp^{4}NH/Phe^{3}CO and

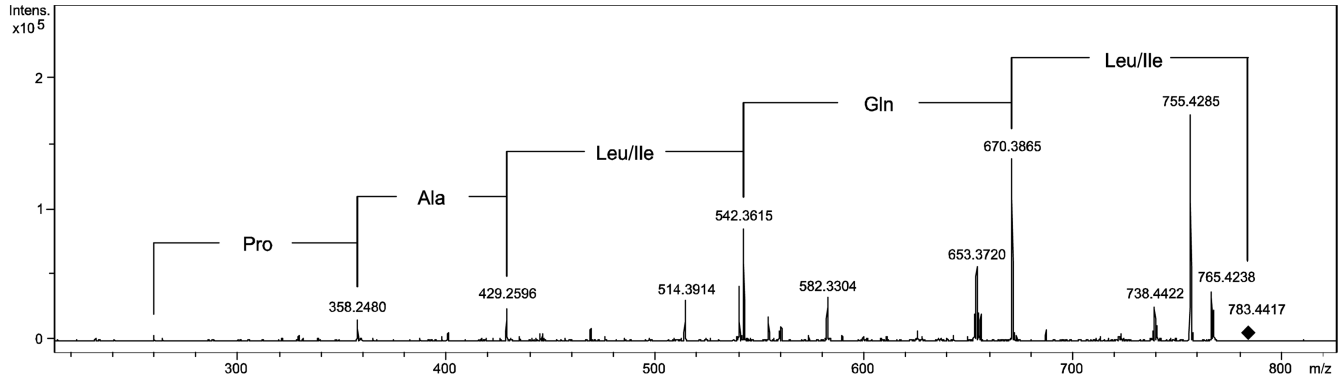

Figure 2. HR-MS/MS spectrum of stylissamide E (**3**). The precursor ion is marked with a solid square. Only the main fragmentation pathway is indicated.

Table 2. Fragments Observed in HR-MS/MS and MS^n Experiments on Stylissamide E (**3**)

m/z	formula	sequence	MS^3 loss
261.1	$C_{14}H_{17}N_2O_3$	Pro-Tyr/Tyr-Pro	
358.2	$C_{19}H_{24}N_3O_4$	Pro-Tyr-Pro	Pro
429.3	$C_{22}H_{29}N_4O_5$	Pro-Tyr-Pro-Ala	Ala, Pro
471.3	$C_{25}H_{35}N_4O_5$	Ile-Pro-Tyr-Pro	
542.4	$C_{28}H_{40}N_5O_6$	Ile-Pro-Tyr-Pro-Ala/ Pro-Tyr-Pro-Ala-Ile	Ile, Ala
670.4	$C_{33}H_{48}N_7O_8$	Pro-Tyr-Pro-Ala-Ile-Gln	Pro, Gln

Phe^{7}NH/Arg^{6}CO confirmed this sequence. The similar chemical shifts of Pro^{1}Hα and Pro^{2}Hα caused an overlap of the potential signals Pro^{2}Hα/Pro^{1}CO and Pro^{1}Hα/CO, but the correlation Pro^{2}Hδ/Pro^{1}CO elongated the proposed fragment to Pro^1-Pro^2-Phe^3-Asp^4-Pro^5-Arg^6-Phe^7. A weak correlation existed between Pro^{1}Hδ and Phe^{7}CO, which indicated the ring-closure of **4**.

The $\Delta\delta_{\beta,\gamma}$ values (3.2, 9.9, and 4.0 ppm for Pro^1, Pro^2, and Pro^5) indicated a *cis* configuration for the peptide bond preceding Pro^2 and a *trans* configuration for Pro^1 and Pro^5 (see Table 3). Similar chemical shifts of Pro^{1}Hα and Pro^{2}Hα caused an overlapping of the signals with the diagonal of the NOESY spectrum.

Tandem mass spectrometry data verified the NMR results. The main fragmentation pathway of **4** under MS/MS conditions implied the loss of C-terminal and N-terminal amino acids. The cyclic structure of stylissamide F (**4**) was confirmed by the loss of aspartic acid, phenylalanine, and proline after ring-opening at different amide bonds directly from the parent ion of **4**. The presence of three proline and two phenylalanine residues in the heptapeptide complicated the sequence elucidation of the single fragments with MS/MS and MS^n experiments. For example, the fragment *m/z* 401.2412 was assigned as Pro^5-Arg^6-Phe^7 and as Arg^6-Phe^7-Pro^1 due to the loss of N- and C-terminal prolines from Pro^5-Arg^6-Phe^7-Pro^1. Similarly, *m/z* 360.2310 indicated Pro^2-Phe^3-Asp^4 and Phe^3-Asp^4-Pro^5, both secondary fragments of Pro^2-Phe^3-Asp^4-Pro^5. In addition to proline as a common position for ring-opening **4** contained the sequence Asp^4-Pro^5. Protonation of the proline amide with the acidic proton of aspartic acid caused the preference for a cleavage of this amide bond. A cyclization of the aspartic acid side chain with the new C-terminal carbonyl group[16] possibly reduced the C-terminal fragmentation of the peptide. After ring-opening at position Asp^4 and Pro^5, the precursor ion successively lost N-terminal Pro^5, Arg^6, Phe^7, and Pro^1 and C-terminal Asp^4 and gave the fragments *m/z* 760.5267, 604.4318, 457.2371, 360.2310, and 245.1250 (see Figure 3). The signals at *m/z* 254.1376 and 195.0779 indicated the fragments Pro^5-Arg^6 and Pro^1-Pro^2. The elimination of ammonia, carbon monoxide, and both together from the main fragments was also observed (for example: *m/z* 840.4581, 829.4737, and 812.4870). The fragment *m/z* 457.2371, assigned as Pro^1-Pro^2-Phe^3-Asp^4 and Pro^2-Phe^3-Asp^4-Pro^5, lost proline and aspartic acid under MS^3 conditions. The sequence of **4** was finally established by a combined

Table 3. Chemical Shifts of Stylissamide F (**4**) (600 MHz)[a]

entry	residue	position	δ_C/δ_N	δ_H, mult. (*J*/Hz)
1	Pro^1	N	141	
2		CO	170.4	
3		α	59.2	4.30[c]
4		β,β'	27.7	2.16, m; 1.66, m
5		γ,γ'	24.5	1.91, m; 1.84[c]
6		δ,δ'	46.3	3.50, m; 3.36[c]
7	Pro^2	N	127	
8		CO	170.82[b]	
9		α	59.8	4.31[c]
10		β,β'	30.6	2.02, m; 1.82[c]
11		γ,γ'	20.7	1.51, m; 0.62, m
12		δ,δ'	45.8	3.17[c]; 2.81, t (10.0)
13	Phe^3	NH	122	8.25, br
14		CO	171.1	
15		α	57.2	4.12, m
16		β,β'	36.5	3.15[c]; 3.09, d (12.8)
17		1	137.2	
18		2,6	128.5	7.20[c]
19		3,5	128.1	7.25[c]
20		4	126.5	7.21[c]
21	Asp^4	NH	109	7.59, d (4.5)
22		CO	170.83[b]	
23		α	48.5	4.72, m
24		β,β'	34.4	3.21[c]; 2.89, d (15.1)
25		γ	172.7	
26		OH		
27	Pro^5	N	132	
28		CO	171.9	
29		α	63.3	4.04, m
30		β,β'	28.9	2.23, m; 1.71, m
31		γ,γ'	24.9	2,01, m; 1,83[c]
32		δ,δ'	46.9	3.77, m; 3.70, m
33	Arg^6	NH	110	7.38, br
34		CO	171.0	
35		α	53.3	3.98, m
36		β,β'	27.3	1.65, m; 1.56, m
37		γ,γ'	25.0	1.39, m; 1.31, m
38		δ,δ'	40.1	3.05, m
39		Nϵ	85	7.45[c]
40		ζ		
41		Nη		
42	Phe^7	NH	114	7.35, br
43		CO	168.5	
44		α	51.1	4.69, m
45		β,β'	36.6	3.22[c]; 2.76 dd (6.3, 14.0)
46		1	137.9	
47		2,6	129.3	7.30, d (5.5)
48		3,5	127.6	7.23[c]
49		4	125.6	7.18[c]

[a] ^{1}H and ^{13}C chemical shifts are referenced to the DMSO-d_6 signal (2.50 and 39.5 ppm, respectively). ^{15}N chemical shifts were not calibrated with an external standard. Therefore, the δ value has an accuracy of about 1 ppm in reference to NH_3 (0 ppm), and the ^{15}N chemical shifts are given without decimals. [b] ^{13}C NMR shifts of carbonyl carbons are given with two decimals if one decimal did not allow for differentiation of two different carbonyl carbons. [c] Overlapped signals.

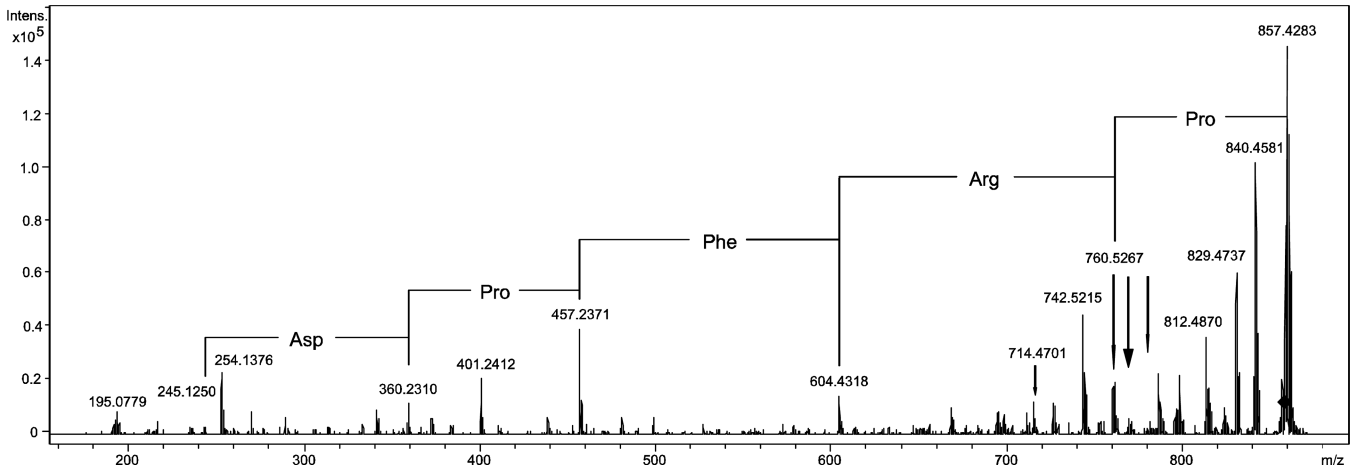

Figure 3. HR-MS/MS spectrum of stylissamide F (**4**). The precursor ion is *m/z* 857.4283. Only one possible fragmentation pathway is indicated.

approach of NMR and mass spectrometric data as cyclo-(*trans*-Pro[1]-*cis*-Pro[2]-Phe[3]-Asp[4]-*trans*-Pro[5]-Arg[6]-Phe[7]).

The absolute configuration of the amino acid residues of stylissamides E and F (**3** and **4**) was determined by Marfey's[17] and the OPA method[18] after hydrolysis of **3** and **4**. All amino acids had the L-configuration.

Conclusion

The family of cyclic heptapeptides from the sponge *S. caribica* is extended by the stylissamides E (**3**) and F (**4**). Stylissamide E (**3**) includes the segment Ile-Pro-Tyr-Pro, which is known from stylisin 2 as well as stylissamides C and D. The amino acids alanine and glutamine in **3** rarely appear in this family of peptides. Stylissamide E (**3**) is the first member of this family with only two proline residues. Compared with the other stylissamides, stylissamide F (**4**) is a rather polar peptide due to the coexistence of the basic amino acid arginine with aspartic acid. As in stylissamides A (**1**), C, and D, stylissamide F (**4**) has three proline residues, including two successive prolines as in stylissamide A (**1**). The structure elucidation of the new cyclic peptides was established by a combined approach of mass spectrometry and NMR spectroscopy.

Experimental Section

General Experimental Procedures. ^{1}H, ^{13}C, and ^{15}N NMR spectra were conducted on a Bruker Advance 600 MHz NMR spectrometer. All experiments were measured at 303 K with DMSO-d_6 as solvent. The DQF-^{1}H,^{1}H-COSY, ^{1}H,^{13}C-HSQC, ^{1}H,^{13}C-HSQC-TOCSY, ^{1}H,^{13}C-HMBC, semiselective ^{1}H,^{13}C-HMBC (pulse: Gaus1.1000, pulse length: 1.4 ms, spectral range: 165−178 ppm), ^{1}H,^{15}N-HSQC, ^{1}H,^{15}N-HMBC, and ^{1}H,^{1}H-NOESY experiments were carried out using standard parameters. The mixing time for NOESY spectra was 200 ms, and the delay for the ^{1}H,^{13}C-HMBC experiment was 80 ms. HPLC-MS analysis were performed with an Agilent 1100 HPLC system and Bruker Daltonics microTOF$_{LC}$. Analytical chromatography: Waters XTerra RP$_{18}$ column (3.0 × 150 mm, 3.5 μm) with a MeCN/H$_2$O/HCOOH gradient [0 min: 10% MeCN/90% HCOOH (0.01%); 30 min: 60% MeCN/40% HCOOH (0.01%) with a flow rate of 0.4 mL min^{-1}]. Preparative chromatography: Prontosil Eurobond C$_{18}$ column (20 × 250 mm, 5 μm) with a MeCN/TFA (0.1%) gradient. UV spectra were recorded during HPLC analysis with a DAD (Agilent). ESIMS/MS spectra were recorded with an Exquire 3000plus ion trap (Bruker Daltonics).

Animal Material. The marine sponge *Stylissa caribica* was collected by scuba diving on July 29 and 30, 2000, at Little San Salvador in the Bahamas (75 to 91 ft depth). The samples were immediately frozen after collection and kept at −20 °C until extraction. The sponge material was compared with previously investigated material of *S. caribica*[19] and was found to match closely (Dr. M. Assmann, personal communication). A detailed taxonomic description of the sponge is given in ref 20, and a voucher fragment of *S. caribica* has been deposited in the collections of the Zoological Museum of Amsterdam under reg. no. ZMA POR. 15607.

Extraction and Isolation. The freeze-dried sponge tissue of *S. caribica* (50.09 g) was crushed with a mill and extracted exhaustively at room temperature with a 1:1 mixture of MeOH/CH$_2$Cl$_2$. The crude extract (23.29 g) was partitioned between *n*-hexane (4 × 400 mL) and MeOH (300 mL). The MeOH extract was then partitioned between *n*-BuOH (5 × 300 mL) and H$_2$O (300 mL). The resulting *n*-BuOH phase (11.56 g) from the solvent partitioning scheme was purified by gel chromatography on Sephadex LH-20 (Pharmacia) using MeOH as the mobile phase. The final purification of the isolated compounds was achieved by preparative RP$_{18}$ HPLC on a Prontosil Eurobond C$_{18}$ column (20 × 250 mm, 5 μm) applying a MeCN/TFA (0.1%) gradient to afford **3** (2.0 mg, 0.004% of dry weight) and **4** (0.7 mg, 0.001% of dry weight).

Stylissamide E (3): light yellow powder; UV (DAD) λ_{max} = 221, 280 nm; CD (1.1 mg/10 mL, MeOH) λ_{max} ($\Delta\varepsilon$) 222 nm (−1.035); ^{1}H and ^{13}C NMR data in Table 1; HPLC/HR(+)ESI-MS t_R = 16.1 min, *m/z* = 783.4417 [M + H]$^+$ (calcd for C$_{39}$H$_{59}$N$_8$O$_9$, 783.4400), Δm = 2.1 ppm.

Stylissamide F (4): light yellow powder; UV (DAD) λ_{max} = 278 nm; CD (0.3 mg/5 mL, MeOH) λ_{max} ($\Delta\varepsilon$) 218 nm (−0.670); ^{1}H and ^{13}C NMR data in Table 2; HPLC/HR(+)ESI-MS t_R = 17.2 min, *m/z* = 857.4266 [M + H]$^+$ (calcd for C$_{43}$H$_{57}$N$_{10}$O$_9$, 857.4304), Δm = 4.4 ppm.

Determination of the Absolute Configuration of Proline by a Modified Marfey's Method:[17] Stylissamides E and F (**3** and **4**, 600 μg each) were placed in 1 mL conical vials containing HCl (16%, 0.5 mL), and the sealed vials were heated at 100 °C for 12 h. After evaporation of the solvent under N$_2$, H$_2$O (100 μL) was added. A 40 μL aliquot of this solution was used for the OPA method. To the remaining hydrolysis solution (60 μL) were added NaHCO$_3$ (0.1 M, 100 μL) and 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (L-FDAA. 0.1%, 50 μL) in acetone, and the sealed vials were heated at 80 °C for 5 min. To the reaction mixture were added HCl (0.2 M, 50 μL) and 50% aqueous MeCN (containing 0.1% HCOOH, 90 μL). The mixture was subjected to HPLC analysis [Waters XTerra RP$_{18}$ column (3.0 × 150 mm, 3.5 μm); MeCN/H$_2$O/HCOOH gradient: 0 min: 30% MeCN/70% HCOOH (0.01%); 30 min: 60% MeCN/40% HCOOH (0.01%) with a flow rate of 0.4 mL min^{-1}]. UV detection was performed at a wavelength of 340 nm.

Determination of the Absolute Configuration of Amino Acids by the OPA Method:[18] A 40 μL aliquot of the hydrolysis solutions was used for the OPA method. In an HPLC vial, 80 μL of *o*-phthaldialdehyde (OPA) solution and 80 μL of *N*-isobutyrylcysteine (0.1%) were added to this solution, and after a reaction time of 2 min 20 μL of the reaction mixture was subjected to HPLC analysis [Phenomenex Hyperclone BDS C$_{18}$ column (4.0 × 250 mm, 5 μm); MeOH/NaOAc gradient: solution A: 125 mM NaOAc in H$_2$O and 20 mL of MeOH, adjusted to pH 6.8 using diluted AcOH solution B: MeOH].

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Supporting Information Available: HPLC-MS spectrum of *Stylissa caribica*; NMR and CD spectra of compounds **3** and **4**. This material is available free of charge via Internet at http://pubs.acs.org.

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