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Stylissamides A–D – New Proline-Containing Cyclic Heptapeptides from the Marine Sponge *Stylissa caribica*

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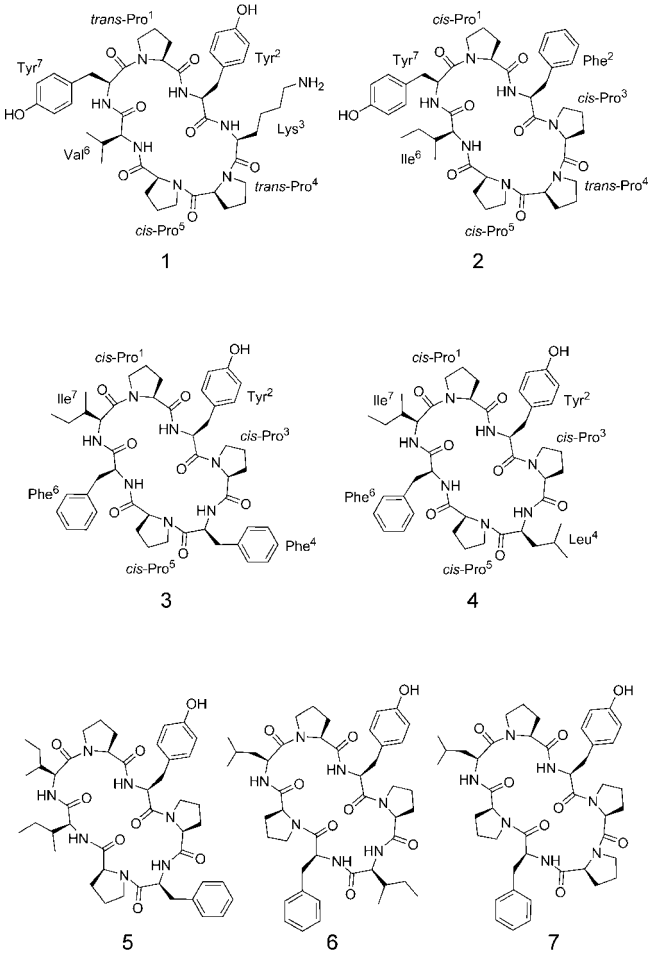
Four new cyclic heptapeptides, stylissamides A–D (**1**–**4**), were isolated from the Caribbean sponge *Stylissa caribica*. The structures of these metabolites were elucidated by NMR and MS/MS methods. The peptides contain three and, in one case, four proline residues. The sequence assignment of **1**–**4** by NMR was supported by fragmentations in HR-MS/MS measurements. The absolute configuration of all amino acid residues was assigned as L using Marfey's method and the OPA method.

Introduction

Sponges of the order Halichondrida are a rich source of non-ribosomal cyclic peptides containing seven to ten amino acids.[1] Sponges of the genus *Phakellia* reveal the substance class of phakellistatins whereas sponges of the genus *Hymeniacidon* contain the hymenamides. The phakellistatins especially, such as phakellistatin 2 (**5**),[2] show cytotoxic effects against a variety of tumour cell lines.[3] Very recently, two cyclic heptapeptides, stylisin 1 (**6**) and 2 (**7**), were isolated from the Jamaican sponge *Stylissa caribica* (see Scheme 1). In contrast to other cyclic peptides they are inactive in antimicrobial, anti-malarial, anti-cancer, anti-HIV-1, anti-Mtb, and anti-inflammatory assays.[4] The Caribbean sponge *Stylissa caribica* (order Halichondrida) was found to be a rich source of pyrrole-imidazole alkaloids.[5] Using HPLC-HRMS screening of the crude extract several new metabolites were isolated: 4-bromopyrrole-2-carboxy-N(ε)-lysine,[6] 4-bromopyrrole-2-carboxyarginine,[6] oxocyclostylidol,[7] and stylissadines A and B.[8] In the course of this investigation a Sephadex LH-20 fraction of the *n*-butanol phase was analyzed. This fraction contained four non-brominated compounds with molecular masses between *m*/*z* 800 and 900. A comparison of the accurate molecular masses with the literature (MarinLIT®) revealed four metabolites with a previously unobserved mass which were separated by preparative HPLC. Herein, we report the structure elucidation of these new compounds by spectroscopic methods (NMR, MS, and MS^n).



Scheme 1. Structural formulae of stylissamides A–D (**1**–**4**), phakellistatin 2 (**5**), stylisin 1 (**6**), and stylisin 2 (**7**).

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Results and Discussion

In our continuous search for new bioactive secondary metabolites of marine sponges from tropical waters, *Stylissa caribica* was collected by SCUBA at Little San Salvador in the Bahamas (23 m depth, July 2000). The freeze-dried sponge tissue was extracted with a 1:1 mixture of MeOH/CH_2Cl_2 and the crude extract was partitioned by liquid/liquid extraction between *n*-hexane, *n*BuOH and H_2O.

The resulting *n*BuOH phase was purified by Sephadex LH-20 chromatography. Final purification of the four metabolites was achieved by preparative RP_{18} HPLC. The fact that the metabolites were not halogenated, the characteristic 1D NMR spectra and the occurrence of cyclic peptides in other sponges of the order Halichondrida suggested the compounds were peptides. The four compounds were elucidated by 2D NMR and MS methods. The NMR spectroscopic data are summarized in Tables 1, 3, 5 and 6 respectively.

For each of the four compounds **1**–**4** (see Scheme 1) seven carbonyl carbons were observed in the ^{13}C NMR spectrum. The ^{1}H NMR spectrum showed four amide protons for **1**, **3**, and **4** and three amide protons for **2**. Together with the results of the HPLC analysis of the hydrolysed metabolites (Marfey's method) which showed that proline was a common amino acid in all four compounds, these results indicated the assignment to the structural class of heptapeptides. Due to the cyclic peptides found in other sponges of the order Halichondrida the degrees of unsaturation for the molecular formulae of **1**–**4** (HR-MS) were compared to the sum of degrees of unsaturation in the amino acids contained in each peptide. The molecular formula of **1** resulted in 19 degrees while the amino acids accounted for 18 degrees of unsaturation, indicating the cyclic nature of this compound. The degrees of unsaturation were compared in the same way for compounds **2** (20 vs. 19), **3** (23 vs. 22) and **4** (19 vs. 18), and suggested a cyclic structure for these compounds as well.

The molecular weight of **1** (*m*/*z* 845.4555 $[M + H]^+$) was obtained from the ESI mass spectrum (HR-ESIMS) and indicated the molecular formula $C_{44}H_{61}N_8O_9$. The ^{1}H NMR spectrum of **1** displayed four amide proton signals at 7.67, 7.62, 7.33 and 7.24 ppm in addition to seven carbonyl signals at 171.5, 171.4, 2 × 170.5, 170.0, 169.1 and 167.9 ppm in the ^{13}C NMR spectrum (see Table 1). All amino acid residues were assigned by 2D NMR techniques as 3 × Pro, Val, Lys, and 2 × Tyr, which was in accordance with the carbonyl and amide proton signals and defined the molecule as a heptapeptide. The amino acid sequence was established by a combination of classical and semi-selective $^{1}H,^{13}C$-HMBC experiments between the NH, Hα, and in the case of proline, Hδ of one amino acid and the carbonyl carbon of the preceding amino acid. The correlations Pro^1-Hα/Tyr^7-CO and Pro^1-Hδ/Tyr^7-CO proved the fragment Tyr^7-Pro^1. The correlations Lys^3-Hα/Tyr^2-CO, Pro^4-Hα/Lys^3-CO, Pro^5-Hα/Pro^4-CO, Val^6-Hα/Pro^5-CO and Tyr^7-Hα/Val^6-CO lengthened this fragment to give the final sequence Tyr^2-Lys^3-Pro^4-Pro^5-Val^6-Tyr^7-Pro^1. In addition to the $H\alpha_{(i)}/CO_{(i-1)}$ correlations the amide $NH_{(i)}/CO_{(i-1)}$ correlations were observed for Lys^3-NH/Tyr^2-CO, Val^6-NH/Pro^5-CO and Tyr^7-NH/Val^6-CO. The ring closure in stylissamide A (**1**) was given by another $^{1}H,^{13}C$-HMBC correlation between Tyr^2-NH and Pro^1-CO and two NOE correlations between Tyr^2-NH and both, Pro^1-Hα and Pro^1-Hγ.

Table 1. ^{1}H, ^{13}C and ^{15}N NMR chemical shifts of stylissamide A (**1**) in $[D_6]$DMSO.[a]

Entry	Residue	Position	δ_C/δ_N	δ_H, mult. (*J* /Hz)
1	Pro^1	N	135	–
2		CO	171.4	–
3		α	62.6	3.97, dd (7.3, 9.0)
4		β, β′	28.3	2.02, m; 1.60, m
5		γ, γ′	24.8	2.02, m
6		δ, δ′	46.7	3.74, m
7	Tyr^2	NH	108	7.67, d (8.1)
8		CO	170.5	–
9		α	54.2	4.32, m
10		β, β′	34.2	3.17, dd (3.1, 14.0); 2.89, dd (11.8, 14.0)
11		1	128.2	–
12		2, 6	129.2	7.00, d (7.6)
13		3, 5	114.6	6.65, d (7.6)
14		4	155.6	–
15		OH	–	9.18, s
16	Lys^3	NH	115	7.33, d (7.0)
17		CO	167.9	–
18		α	50.4	4.40, d (8.4)
19		β, β′	30.5	1.82, m; 1.50, m
20		γ, γ′	22.0	1.32, m; 1.31, m
21		δ, δ′	26.4	1.57, m
22		ε, ε′	38.3	2.76, dd (7.0, 14.3)
23		NH_2	34	7.76, s
24	Pro^4	N	139	–
25		CO	170.0	–
26		α	58.6	4.30, m
27		β, β′	27.7	2.17, m; 1.74, m
28		γ, γ′	24.2	1.91, m, 1.85, m
29		δ, δ′	46.6	3.44, m; 3.34, m
30	Pro^5	N	126	–
31		CO	170.5	–
32		α	60.0	4.42, d (8.4)
33		β, β′	30.9	2.17, m; 2.07, m
34		γ, γ′	21.5	1.85, m; 1.51, m
35		δ, δ′	46.1	3.42, m
36	Val^6	NH	117	7.62, d (8.4)
37		CO	169.1	–
38		α	61.3	3.67, t (8.4)
39		β	29.5	1.77, m
40		γ	18.9	0.77, d (6.7)
41		γ′	18.9	0.45, d (6.7)
42	Tyr^7	NH	112.7	7.24, d (8.7)
43		CO	171.5	–
44		α	51.3	4.90, dt (3.4, 9.8)
45		β, β′	37.0	3.28, m; 2.39, dd (10.7, 14.0)
46		1	126.4	–
47		2, 6	129.9	7.07, d (7.6)
48		3, 5	114.8	6.67, d (7.6)
49		4	155.8	–
50		OH	–	9.23, s

[a] ^{1}H and ^{13}C chemical shifts were referenced to the $[D_6]$DMSO signal (2.50 ppm and 39.5 ppm, respectively). ^{15}N NMR spectra were not calibrated with an external standard. Therefore, the ^{15}N NMR shifts are given without decimals. The δ value has an accuracy of about 1 ppm in reference to NH_3 (δ = 0 ppm).

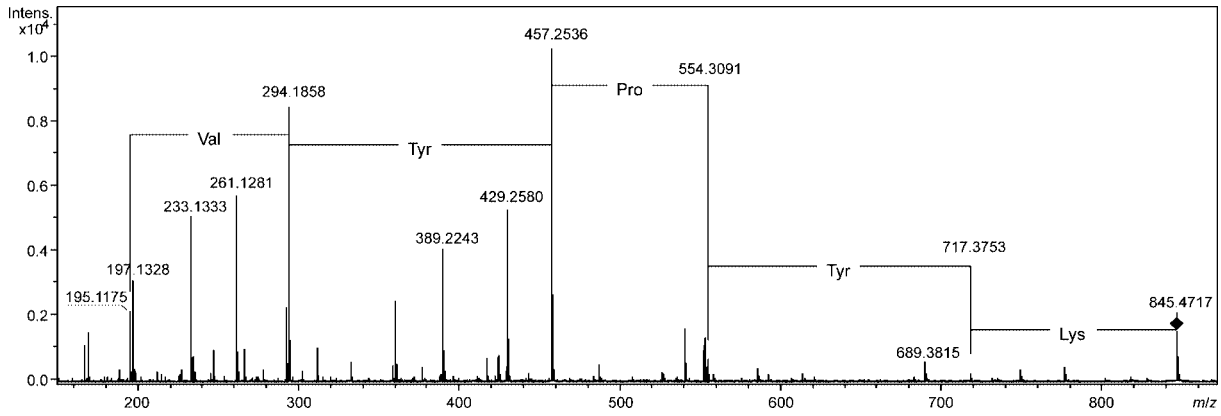

Figure 1. HR-MS/MS spectrum of stylissamide A (**1**). The precursor ion is indicated by the filled square. Only the main fragmentation pathway is indicated. Masses associated with this fragmentation pathway and the corresponding observed masses derived from CO loss (28 amu) are given.

The sequence was supported by other NOE correlations between $NH_{(i)}$ and $H\alpha_{(i-1)}$ and in the case of proline $H\delta_{(i)}$ and $H\alpha_{(i-1)}$ (see Figure 3 and Supporting Information).

The sequence of stylissamide A (**1**) was further supported by the fragmentation pattern obtained through HR-MS/MS and MS^n measurements. Although, the cyclic structure of the peptides **1**–**4** does not provide a definite position for ring-opening reactions the protonated amide nitrogen may be favoured in such reactions.[9] Mass spectrometric studies on cyclic and linear peptides containing proline residues showed a preferential protonation of the proline nitrogen due to a high proton affinity.[10] For stylissamide A (**1**) there are three proline residues which are preferred for ring-opening reactions. The occurrence of at least three proline residues in the stylissamides and the resulting possibilities for protonation and subsequent ring opening prevent the sequencing only by CID-MS^n experiments. However, the combined application of HR-MS/MS and MS^n experiments allows for sequencing the peptide fragments.[11]

For stylissamide A (**1**) the combination of these techniques (see Supporting Information) confirmed the sequence assignments by NMR spectroscopy. After ring opening at the Lys-Pro amide bond successive losses of Lys, Tyr, Pro, Tyr and Val gave the corresponding acylium ions (b_n fragments) at *m/z* 717.3753, 554.3091, 457.2536, 294.1858 and 195.1175 (see Figure 1, Table 2). Other fragments from ring openings at the Tyr-Pro and Pro-Pro amide bonds are found as well and confirmed the results mentioned before. The main peak at *m/z* 457.2536 was determined to be the Pro-Pro-Val-Tyr and Pro-Val-Tyr-Pro fragments.

The *cis*/*trans* configuration of the peptide bond preceding the proline residues was determined on the basis of the Cβ and Cγ shift difference ($\Delta\delta_{\beta\gamma}$)[12] and upon the presence of an NOE correlation between the proline Hα and the Hα of the preceding amino acid, which is present in a *cis* configuration but absent in *trans*-proline.[13] The $\Delta\delta_{\beta\gamma}$ of Pro^1 and Pro^4 were both 3.5 ppm and that of Pro^5 was 9.5 ppm. This indicated a *trans* configuration for Pro^1 and Pro^4 and a *cis* configuration for Pro^5. The NOE correlation Pro^4-Hα/Pro^5-Hα was observed while the correlations Tyr^7-Hα/Pro^1-Hα and Lys^3-Hα/Pro^4-Hα were not present. Thus, the amino acid sequence of **1** was established as cyclo-(*trans*-Pro^1-Tyr^2-Lys^3-*trans*-Pro^4-*cis*-Pro^5-Val^6-Tyr^7).

Table 2. Fragments observed in HR-MS/MS and MS^n experiments of stylissamide A (**1**).

m/z	Fragment formula	Error [ppm]	Sequence	MS^3 loss
195.1175	$C_{10}H_{15}N_2O_2$	24.2	Pro-Pro	–
197.1328	$C_{10}H_{17}N_2O_2$	22.2	Pro-Val	–
261.1281	$C_{14}H_{17}N_2O_3$	18.1	Pro-Tyr	–
294.1858	$C_{15}H_{24}N_3O_3$	15.5	Pro-Pro-Val	Pro, Val
389.2243	$C_{20}H_{29}N_4O_4$	15.3	Pro-Tyr-Lys	Lys
457.2536	$C_{24}H_{33}N_4O_5$	19.8	Pro-Pro-Val-Tyr Pro-Val-Tyr-Pro	Pro, Tyr
554.3091	$C_{29}H_{40}N_5O_6$	21.2	Pro-Pro-Val-Tyr-Pro	–[a]
717.3753	$C_{38}H_{49}N_6O_8$	20.5	Pro-Pro-Val-Tyr-Pro-Tyr	Pro, Tyr

[a] Due to the low intensity of this fragment it was not possible to perform MS^3 measurements.

The molecular weight of **2** (*m/z* 812.4311 $[M + H]^+$) as determined by HR-ESIMS indicated the molecular formula $C_{44}H_{58}N_7O_8$. The ^1H NMR spectrum of **2** showed three amide proton signals at 8.65, 8.24 and 6.45 ppm while the ^{13}C NMR spectrum showed four carbonyl carbons at 171.2, 170.2, 168.5, 167.6 ppm and three carbonyl carbon signals at approximately 169.8 ppm, which could be differentiated into two signals at 169.75 ppm and one at 169.81 ppm (see Table 3). All amino acid residues were assigned by 2D NMR techniques as 4 × Pro, Ile, Phe and Tyr which defined the heptapeptide structure. Sequence assignments by ^1H,^{13}C-HMBC experiments were difficult due to the overlap of the carbonyl carbon chemical shifts of two prolines (Pro^1 and Pro^4) and isoleucine. A semi-selective ^1H,^{13}C-HMBC experiment allowed us to assign the three carbonyl C atoms at 169.8 ppm to two prolines (169.75 ppm) and isoleucine (169.81 ppm). The fragment Pro^5-Ile^6 was assigned from the correlations Ile^6-NH/Pro^5-CO and Ile^6-Hα/Pro^5-CO as well as a weak correlation Ile^6-Hγ/Pro^5-CO and the NOE correlation Ile^6-NH/Pro^5-Hα. The fragments Ile^6-Tyr^7 and Tyr^7-Pro^1 were proven by the correlations Tyr^7-

NH/Ile^6-CO, Tyr^7-Hα/Ile^6-CO, Pro^1-Hα/Tyr^7-CO, Pro^1-Hδ/Tyr^7-CO and the NOE correlations Tyr^7-NH/Ile^6-Hα and Pro^1-Hα/Tyr^7-Hα, thereby establishing the partial sequence Pro^5-Ile^6-Tyr^7-Pro^1. Another fragment Pro^3-Pro^4 was given by the correlation Pro^4-Hα/Pro^3-CO. Pro^1 and Pro^4 showed the same carbonyl carbon shift at δ 169.75 ppm. Associated with this shift were the correlations Phe^2-NH/169.75 ppm, Phe^2-Hα/169.75 ppm, Pro^5-Hα/169.75 ppm and Pro^5-Hδ/169.75 ppm which suggested the fragments Pro^1-Tyr^2 or Pro^4-Tyr^2 and Pro^1-Pro^5 or Pro^4-Pro^5, respectively. Taking into account the partial sequence Pro^5-Ile^6-Tyr^7-Pro^1 a segment Pro^1-Pro^5 was ruled out, therefore establishing the segment Pro^4-Pro^5 and consequently Pro^1-Phe^2. Both segments were supported by the NOE correlations Pro^4-Hα/Pro^5-Hα and Pro^1-Hδ/Phe^2-NH. This defined the final sequence as Pro^3-Pro^4-Pro^5-Ile^6-Tyr^7-Pro^1-Phe^2. The ring closure was given by the two NOE correlations Phe^2-Hα/Pro^3-Hα and Phe^2-Hβ/Pro^3-Hα as well as the two weak HMBC correlations Pro^3-Hα/Phe^2-CO and Pro^3-Hδ/Phe^2-CO. The configuration of the peptide bonds preceding the proline residues was determined to be *cis* for Pro^1, Pro^3, and Pro^5 and *trans* for Pro^4 by the $\Delta\delta_{\beta\gamma}$ values (9.4, 8.3, and 8.6 ppm for Pro^1, Pro^3, and Pro^5, respectively, and 2.6 ppm for Pro^4). The NOE correlations Tyr^7-Hα/Pro^1-Hα, Phe^2-Hα/Pro^3-Hα and Pro^4-Hα/Pro^5-Hα were observed whereas Pro^3-Hα/Pro^4-Hα was not present. This confirmed the configurational assignment. The sequence of **2** was thus established as cyclo-(*cis*-Pro^1-Phe^2-*cis*-Pro^3-*trans*-Pro^4-*cis*-Pro^5-Ile^6-Tyr^7) and confirmed on the basis of MS^n data (see Figure 3 and Supporting Information). The fragment *m*/*z* 308.1979, assigned as Pro-Pro-Ile, lost Ile and Pro under MS^3 conditions. The main fragment at *m*/*z* 405.2511 lost Pro to yield the mass *m*/*z* 308.1979 and Ile to give the mass *m*/*z* 292.2 (Pro-Pro-Pro) under MS^3 conditions. The fragment at *m*/*z* 568.3178 lost Tyr to give the mass *m*/*z* 405.2511 and lost also Pro to give the fragment at *m*/*z* 471.2632 (see Figure 2, Table 4). This fragmentation pathway is only possible for the sequence Pro-Pro-Pro-Ile-Tyr which is in accordance with the NMR spectroscopic data.

The molecular weight of **3** (*m*/*z* 862.4490 $[M + H]^+$, HR-ESIMS) indicated the molecular formula $C_{48}H_{60}N_7O_8$. The ^{1}H NMR spectrum of **3** showed four amide proton signals at 8.96, 8.89, 8.16 and 6.62 ppm while the ^{13}C NMR spectrum displayed five carbonyls at 171.7, 171.5, 169.8, 169.0, 168.3 ppm and two carbonyl carbon signals at approximately 169.6 ppm, which could be differentiated into 169.66 ppm and 169.64 ppm (see Table 5). The amino acid residues were identified by 2D NMR as 3 × Pro, Ile, 2 × Phe and Tyr, which indicated a heptapeptide. Due to the overlapping carbonyl carbon signals a semi-selective ^{1}H,^{13}C-HMBC experiment was needed for the sequence assignment. The correlation Tyr^2-Hα/Pro^1-CO defined the segment Pro^1-Tyr^2. Pro^1-Hα and Pro^1-Hδ displayed correlations to Ile^7-CO. Along with the correlations Ile^7-Hα/Phe^6-CO, Phe^6-NH/Pro^5-CO, Pro^5-Hα/Phe^4-CO and Phe^4-Hα/Pro^3-CO the final sequence Pro^3-Phe^4-Pro^5-Phe^6-Ile^7-Pro^1-Tyr^2 was established. Additional ^{1}H,^{13}C-HMBC correlations between the amide proton and the carbonyl carbon of the preceding amino acid existed for Tyr^2-NH/Pro^1-CO, Pro^4-NH/Pro^3-CO and Ile^7-NH/Phe^6-CO. A weak correlation existed between Pro^3-Hδ and Tyr^2-CO which indicated the ring closure. This weak correlation was supported by an NOE correlation between Pro^3-Hα and Tyr^2-Hβ. The $\Delta\delta_{\beta\gamma}$ values clearly indicated a *cis* configuration for all pep-

Table 3. ^{1}H, ^{13}C and ^{15}N NMR chemical shifts of stylissamide B (**2**) in [D$_6$]DMSO.[a]

Entry	Residue	Position	δ_C/δ_N	δ_H, mult. (*J* /Hz)
1	Pro^1	N	131	–
2		CO	169.75[b]	–
3		α	60.5	3.49, d (8.5)
4		β, β′	30.4	1.68, m; 1.53, m
5		γ, γ′	21.0	1.38, m; 1.29, m
6		δ, δ′	45.4	3.22, m; 3.10, m
7	Phe^2	NH	114	6.45, d (3.9)
8		CO	167.6	–
9		α	52.1	4.14, m
10		β, β′	35.8	3.12, m
11		1	135.9	–
12		2, 6	127.6	7.22, m
13		3, 5	129.3	6.95, d (6.4)
14		4	126.3	7.19, m
15	Pro^3	N	130	–
16		CO	168.5	–
17		α	56.1	4.45, m
18		β, β′	29.1	2.03, m; 1.81, m
19		γ, γ′	20.8	1.79, m
20		δ, δ′	46.4	3.41, m; 3.31, m
21	Pro^4	N	133	–
22		CO	169.75[b]	–
23		α	58.3	4.30, t (7.8)
24		β, β′	27.5	2.28, m; 1.66, m
25		γ, γ′	24.9	2.06, m; 1.91, m
26		δ, δ′	46.7	3.65, m; 3.42, m
27	Pro^5	N	128	–
28		CO	170.2	–
29		α	60.3	4.41, t (8.1)
30		β, β′	30.2	2.33, dd (6.4, 11.6); 1.90, m
31		γ, γ′	21.6	1.82, m; 1.46, m
32		δ, δ′	45.6	3.39, m; 3.21, m
33	Ile^6	NH	122	8.65, d (9.2)
34		CO	169.81[b]	–
35		α	56.8	4.01, m
36		β	34.8	1.83, m
37		β-Me	15.3	0.68, d (7.1)
38		γ, γ′	24.6	1.23, m; 1.04, m
39		δ	10.6	0.72, t (7.4)
40	Tyr^7	NH	122	8.24, d (8.5)
41		CO	171.2	–
42		α	50.9	4.43, m
43		β, β′	37.2	2.78, dd (10.6, 13.0); 2.61, dd (6.7, 13.0)
44		1	126.2	–
45		2, 6	129.8	6.96, d (7.4)
46		3, 5	114.7	6.67, d (7.8)
47		4	155.9	–
48		OH	–	9.28, s

[a] ^{1}H and ^{13}C chemical shifts are referenced to the [D$_6$]DMSO signal (2.50 ppm and 39.5 ppm, respectively). ^{15}N NMR spectra were not calibrated with an external standard. Therefore, the ^{15}N NMR shifts are given without decimals. The δ value has an accuracy of about 1 ppm in reference to NH_3 (δ = 0 ppm). [b] ^{13}C NMR shifts of carbonyl carbons are given with two decimals if one decimal did not allow for differentiation of two or three different carbonyl carbons.

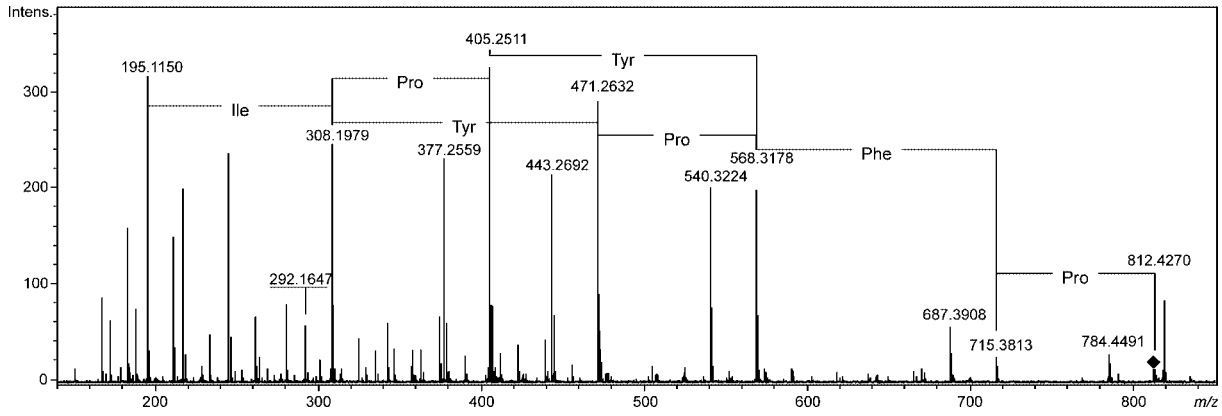

Figure 2. HR-MS/MS spectrum of stylissamide B (**2**). The precursor ion is indicated by the filled square. Only the main fragmentation pathway is indicated. Masses associated with this fragmentation pathway and the corresponding observed masses derived from CO loss (28 amu) are given.

tide bonds preceding the proline residues in **3**, with $\Delta\delta_{\beta\gamma}$ values of 9.6, 9.5 and 9.3 ppm for Pro[1], Pro[3], and Pro[5], respectively. The corresponding NOE correlations between the proline Hα and the Hα of the preceding amino acid were present for Pro[3] and Pro[5], but not for Pro[1] due to overlapping signals. The sequence of **3** was therefore established as cyclo-(*cis*-Pro[1]-Tyr[2]-*cis*-Pro[3]-Phe[4]-*cis*-Pro[5]-Phe[6]-Ile[7]) as shown in Figure 3.

Table 4. Fragments observed in HR-MS/MS and MS^n experiments on stylissamide B (**2**).

m/z	Fragment formula	Error [ppm]	Sequence	MS^3 loss
195.1150	$C_{10}H_{15}N_2O_2$	11.4	Pro-Pro	–
292.1647	$C_{15}H_{22}N_3O_3$	2.8	Pro-Pro-Pro	–[a]
308.1979	$C_{16}H_{26}N_3O_3$	3.3	Pro-Pro-Ile	Pro, Ile
405.2511	$C_{21}H_{33}N_4O_4$	3.6	Pro-Pro-Pro-Ile	Pro, Ile
471.2632	$C_{25}H_{35}N_4O_5$	6.5	Pro-Pro-Ile-Tyr	Tyr, Ile
568.3178	$C_{20}H_{29}N_4O_4$	8.4	Pro-Pro-Pro-Ile-Tyr	Tyr, Pro

[a] Due to the low intensity of this fragment it was not possible to perform MS^3 measurements.

The molecular weight of **4** (*m/z* 828.4657 $[M + H]^+$, HR-ESIMS) indicated the molecular formula $C_{45}H_{62}N_7O_8$. The ^1H and ^{13}C NMR spectra of **4** showed four amide signals at 8.81, 8.70, 8.10 and 6.57 ppm and seven carbonyls at 171.6, 171.2, 170.7, 170.5, 169.5, 169.4 and 168.2 ppm, respectively (see Table 6). The amino acid residues were determined as 3 × Pro, Leu, Ile, Phe and Tyr. The HMBC correlations between Ile[7]-Hα/Phe[6]-CO, Phe[6]-Hα/Pro[5]-CO and Pro[5]-Hα/Leu[4]-CO determined a segment Leu[4]-Pro[5]-Phe[6]-Ile[7]. Leu-Hα and Tyr-Hα showed the same ^1H chemical shift, and both correlated to Pro[1]-CO and Pro[3]-CO which made it difficult to assign a partial sequence Pro[1]-Leu rather than Pro[3]-Leu or Pro[1]-Tyr rather than Pro[3]-Tyr from the Hα-correlations. However, a clear $NH_{(i)}/CO_{(i-1)}$ HMBC correlation established Pro[3]-Leu[4] and consequently Pro[1]-Tyr[2], which defined two segments Pro[3]-Leu[4]-Pro[5]-Phe[6]-Ile[7] and Pro[1]-Tyr[2]. A weak correlation between Pro[3]-Hδ and Tyr[2]-CO connected both segments. Another weak correlation Pro[1]-Hα/Ile[7]-CO indicated the ring closure. NOE correlations supported the sequence; the correlations Ile[7]-CH_3/Pro[1]-Hα and Phe[6]-Hα/Ile[7]-NH proved the sequence Phe[6]-Ile[7]-Pro[1] and the second segment Tyr[2]-Pro[3]-Leu[4]-Pro[5] was given by the correlations Leu[4]-Hβ′/Pro[5]-Hα, Pro[3]-Hα/Leu[4]-NH and Tyr[2]-Hβ′/Pro[3]-Hα. The $\Delta\delta_{\beta\gamma}$ values (9.7, 8.7 and 10.0 ppm for Pro[1], Pro[3], and Pro[5], respectively) clearly indicated a *cis* configuration for all peptide bonds preceding the proline residues in **4**. The corresponding NOE correlations between the proline Hα and the Hα of the preceding amino acid were present only for Pro[5], but were, if present, overlapped for Pro[1] and Pro[3]. The amino acid sequence of **7** was established as cyclo-(*cis*-Pro[1]-Tyr[2]-*cis*-Pro[3]-Lys[4]-*cis*-Pro[5]-Phe[6]-Ile[7]).

The MS^n analysis of stylissamides C (**3**) and D (**4**) was complicated by the occurrence of the tripeptide fragments Pro-Tyr-Pro and Pro-Phe-Ile (see Figure 4). Both fragments have a similar molecular mass of *m/z* 358.1761 and *m/z* 358.2125, calculated for Pro-Tyr-Pro and Pro-Phe-Ile, respectively, which prevent a differentiation of these fragments under standard MS^n conditions. However, the sequences of **3** and **4** could be followed by the HR-MS/MS spectra of each compound. Both peptides successively lost Ile, Phe and Pro to give the fragments at *m/z* 505.2484 (**3**) and *m/z* 471.2645 (**4**). Starting from these fragments stylissamide C (**3**) lost Phe whereas stylissamide D (**4**) lost Leu to give the fragment at *m/z* 358.20. An identical fragmentation pattern for both molecules starting from this fragment indicated the tripeptide sequence Pro-Tyr-Pro. These results confirmed the different composition of **3** and **4** in only one amino acid.

The absolute configuration of the amino acid residues of stylissamide A-D (**1**–**4**) was determined by Marfey's method[14] and the OPA method[15] after hydrolysis of **1**–**4**. All amino acids were found to possess L-configurations.

Conclusions

The isolated stylissamides A-D (**1**–**4**) extend the variety of cyclic and proline-rich peptides from marine sponges. In addition to the stylisins 1 (**6**) and 2 (**7**)[4] these metabolites are the first examples of cyclic peptides from the sponge

Table 5. ^{1}H, ^{13}C and ^{15}N NMR chemical shifts of stylissamide C (**3**) in [D_6]DMSO.[a]

Entry	Residue	Position	δ_C/δ_N	δ_H, mult. (J /Hz)
1	Pro[1]	N	120	–
2		CO	169.8	–
3		α	61.0	4.36, m
4		β, β′	30.9	2.22, m; 2.04, m
5		γ, γ′	21.3	1.78, m; 1.45, m
6		δ, δ′	45.7	3.38, m
7	Tyr[2]	NH	113	6.62, d
8		CO	168.3	–
9		α	52.0	4.33, m
10		β, β′	34.5	3.22, dd (6.1, 13.8); 2.92, m
11		1	125.3	–
12		2, 6	130.3	6.73, d (8.5)
13		3, 5	114.5	6.62, d
14		4	155.9	–
15		OH	–	9.21, s
16	Pro[3]	N	129	–
17		CO	171.5	–
18		α	57.3	4.60, d (8.1)
19		β, β′	30.5	2.17, m; 2.01, m
20		γ, γ′	21.0	2.00, m; 1.89, m
21		δ, δ′	46.5	3.57, m; 3.32, m
22	Phe[4]	NH	124	8.96, d (1.6)
23		CO	169.0	–
24		α	53.3	4.32, m
25		β, β′	35.8	3.10, m; 2.93, m
26		1	135.6	–
27		2, 6	128.9	7.25, m
28		3, 5	128.4	7.32, t (7.3)
29		4	126.7	7.25, t (7.3)
30	Pro[5]	N	132	–
31		CO	169.64[b]	–
32		α	59.6	3.03, d (7.9)
33		β, β′	29.8	1.61, m; 0.84, m
34		γ, γ′	20.5	1.32, m; 0.47, m
35		δ, δ′	45.5	3.09, m; 2.89, m
36	Phe[6]	NH	122	8.89, d (8.5)
37		CO	169.66[b]	–
38		α	54.1	4.22, m
39		β, β′	35.5	2.76, dd (3.5, 13.3); 2.68, t (12.9)
40		1	138.9	–
41		2, 6	128.6	7.23, m
42		3, 5	127.3	7.21, t (7.4)
43		4	125.4	7.12, t (7.4)
44	Ile[7]	NH	115	8.16, d (9.4)
45		CO	171.7	–
46		α	53.0	4.32, m
47		β	37.3	1.64, m
48		β-Me	14.5	0.79, d (6.9)
49		γ, γ′	23.8	1.49, m; 1.02, m
50		δ	10.5	0.80, t (7.1)

[a] ^{1}H and ^{13}C chemical shifts are referenced to the [D_6]DMSO signal (2.50 ppm and 39.5 ppm, respectively). ^{15}N NMR spectra were not calibrated with an external standard. Therefore, the ^{15}N NMR shifts are given without decimals. The δ value has an accuracy of about 1 ppm in reference to NH_3 (δ = 0 ppm). [b] ^{13}C NMR shifts of carbonyl carbons are given with two decimals if one decimal did not allow for differentiation of two or three different carbonyl carbons.

Stylissa caribica. In fact, stylissamide B (**2**) is the first cyclic heptapeptide from marine sponges of the order Halichondrida that includes the tripeptide fragment Pro-Pro-Pro.

The sequences of stylissamides C (**3**) and D (**4**) differ by only one amino acid. Phenylalanine in stylissamide C (**3**) is replaced by leucine in stylissamide D (**7**). In addition, **3** differs by only one amino acid from phakellistatin 2 (**5**), isolated from the marine sponge *Phakellia carteri*.[2] Here, the fragment Phe-Ile in **3** is replaced by Ile-Ile in **5**. The sequence of stylissamide D (**4**) shows a striking resemblance to that of stylisin 1 (**6**) in that it is inverse to the sequence of **6**.

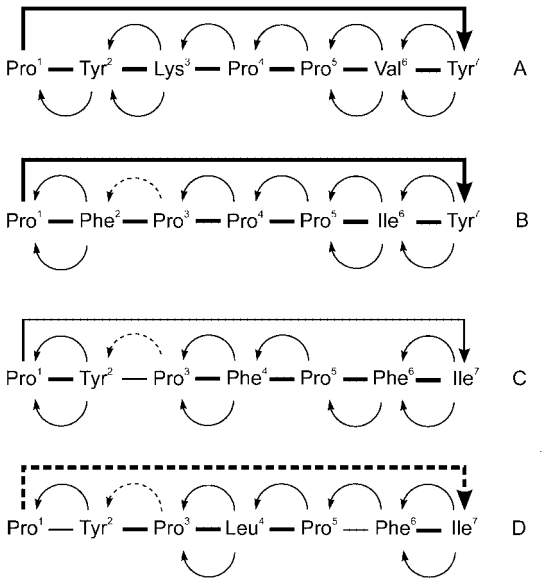

Figure 3. Sequential $^{1}H,^{13}C$-HMBC and $^{1}H,^{1}H$-NOE correlations in stylissamides A–D (**1**–**4**). Solid arrows above the sequence indicate $H\alpha_{(i)}/CO_{(i-1)}$ HMBC correlations, and dashed arrows show $H\delta_{(i)}/CO_{(i-1)}$ HMBC correlations. Arrows below indicate $NH_{(i)}/CO_{(i-1)}$ HMBC correlations. Standard bonds indicate that no NOE correlation was observed. Bold bonds indicate $NH_{(i)}/H\alpha_{(i-1)}$ and $H\delta_{(i)}/H\alpha_{(i-1)}$ NOE correlations between adjacent amino acids.

In contrast to members of the phakellistatin or hymenamide group no antimicrobial or cytotoxic effect was found for stylissamide A (**1**). The biological activities of the remaining metabolites are under investigation.

Experimental Section

General: ^{1}H, ^{13}C and ^{15}N NMR spectra were recorded with Bruker Avance 400 and 600 NMR spectrometers. All experiments were measured at 298 K or 300 K. The DQF-$^{1}H,^{1}H$-COSY, $^{1}H,^{13}C$-HSQC, $^{1}H,^{13}C$-HMBC, $^{1}H,^{15}N$-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 HMBC measurements was 80 ms. HPLC-MS analyses were performed with an Agilent 1100 HPLC system and a Bruker Daltonics microTOF$_{LC}$ mass spectrometer. Separation was achieved by a Waters XTerra RP_{18} column (3.0 × 150 mm, 3.5 μm) applying a MeCN/H_2O/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]. UV spectra were recorded during HPLC analysis with a DAD (Agilent). Accurate mass spectra were acquired using a Bruker microTOF$_{LC}$ mass spectrometer. Accurate MS/MS spectra were acquired using a Bruker microTOF$_{Q}$. Mass calibration was performed using sodium formiate cluster. MS^3 spectra were acquired using a Bruker Esquire 3000plus ion trap. All mass spectrometers were equipped with an ESI-source.

Table 6. ^{1}H, ^{13}C and ^{15}N NMR chemical shifts of stylissamide D (**4**) in [D_6]DMSO.[a]

Entry	Residue	Position	δ_C/δ_N	δ_H, mult. (J /Hz)
1	Pro[1]	N	131	–
2		CO	169.4	–
3		α	60.9	4.31, m
4		β, β′	31.0	2.20, m; 2.00, m
5		γ, γ′	21.3	1.77, m; 1.48, m
6		δ, δ′	45.7	3.37, m
7	Tyr[2]	NH	112	6.57, d (3.9)
8		CO	168.2	–
9		α	51.7	4.41, m
10		β, β′	34.8	3.17, m; 2.91, dd (2.6, 14.3)
11		1	125.3	–
12		2, 6	130.2	6.72, d (7.8)
13		3, 5	114.6	6.61, d (7.5)
14		4	155.7	–
15		OH	–	9.17, s
16	Pro[3]	N	130	–
17		CO	171.6	–
18		α	57.4	4.58, d (7.5)
19		β, β′	30.1	2.11, m; 1.95, m
20		γ, γ′	21.4	2.05, m; 1.86, m
21		δ, δ′	46.6	3.54, m; 3.29, m
22	Leu[4]	NH	125	8.70, m
23		CO	170.5	–
24		α	51.1	4.42, m
25		β, β′	37.9	1.53, t (11.7); 1.28, t (11.7)
26		γ	23.9	1.87, m
27		δ	23.2	0.94, d (6.4)
28		δ′	20.3	0.86, d (6.4)
29	Pro[5]	N	127	–
30		CO	170.7	–
31		α	59.9	4.02, d (8.1)
32		β, β′	31.0	1.99, m; 1.90, m
33		γ, γ′	21.0	1.54, m; 0.82, m
34		δ, δ′	46.1	3.23, m; 3.06, t (9.8)
35	Phe[6]	NH	122	8.81, d (8.4)
36		CO	169.5	–
37		α	54.6	4.25, m
38		β, β′	35.2	2.74, m
39		1	139.2	–
40		2, 6	128.8	7.31, d (7.5)
41		3, 5	127.6	7.24, t (7.5)
42		4	125.7	7.14, t (7.5)
43	Ile[7]	NH	115	8.10, d (9.5)
44		CO	171.2	–
45		α	52.6	4.33, m
46		β	36.9	1.66, m
47		β-Me	14.4	0.77, d (7.4)
48		γ, γ′	23.5	1.45, m; 1.10, m
49		δ	10.1	0.76, t (7.4)

[a] ^{1}H and ^{13}C chemical shifts are referenced to the [D_6]DMSO signal (2.50 ppm and 39.5 ppm, respectively). ^{15}N NMR spectra were not calibrated with an external standard. Therefore, the ^{15}N NMR shifts are given without decimals. The δ value has an accuracy of about 1 ppm in reference to NH_3 (δ = 0 ppm).

Extraction and Isolation: The sponge *Stylissa caribica* was collected by SCUBA at Little San Salvador in the Bahamas (23 m depth, July 2000). The samples were immediately frozen after collection and kept at –20 °C until extraction. The freeze dried sponge samples of *Stylissa caribica* (94.7 g) were crushed with a mill and extracted exhaustively at room temp. with a 1:1 mixture of CH_2Cl_2/MeOH. The orange-colored crude extract of *Stylissa caribica* was partitioned between *n*-hexane (4 × 400 mL) and MeOH (300 mL). The MeOH extract was then partitioned between *n*-BuOH (3 × 500 mL) and H_2O (300 mL). The resulting *n*-BuOH (15.9 g) phase 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 Kromasil RP_{18} column (16 × 250 mm, 10 μm) applying a MeCN/TFA (0.1% in water) gradient to afford **1** (35.3 mg, 0.037% of dry weight), **2** (8.7 mg, 0.009% of dry weight), **3** (12.5 mg, 0.013% of dry weight), and **4** (5.1 mg, 0.005% of dry weight).

Stylissamide A (1): was obtained as a light yellow powder. UV (DAD): λ_{max} = 224, 275 nm. $[\alpha]_D^{20}$ = 86.8 (*c* = 0.46, MeOH); HPLC/HR(+)ESI-MS: R_t = 11.0 min, *m*/*z* 845.4555 $[M + H]^+$ (calcd. for $C_{44}H_{61}N_8O_9$ 845.4556), Δ*m* = 0.1 ppm.

Stylissamide B (2): was obtained as a light yellow powder. UV (DAD): λ_{max} = 275 nm; HPLC/HR(+)ESI-MS: R_t = 21.6 min, *m*/*z* 812.4311 $[M + H]^+$ (calcd. for $C_{44}H_{58}N_7O_8$ 812.4341), Δ*m* = 3.7 ppm.

Stylissamide C (3): was obtained as a light yellow powder. UV (DAD): λ_{max} = 232, 275 nm; HPLC/HR(+)ESI-MS: R_t = 25.2 min, *m*/*z* 862.4490 $[M + H]^+$ (calcd. for $C_{48}H_{60}N_7O_8$ 862.4498), Δ*m* = 0.9 ppm.

Stylissamide D (4): was obtained as a light yellow powder. UV (DAD): λ_{max} = 229, 275 nm; HPLC/HR(+)ESI-MS: R_t = 24.3 min, *m*/*z* 828.4657 $[M + H]^+$ (calcd. for $C_{45}H_{62}N_7O_8$ 828.4654), Δ*m* = 0.3 ppm.

Determination of the Absolute Configuration of Proline by Marfey's Method: The analysis was performed using a modified Marfey′s method.[14] Stylissamides A–D (**1**–**4**, 700 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_2O (100 μL) was added. A 40 μL aliquot of this solution was used for the OPA method. To the remaining hydrolysis solution (60 μL), $NaHCO_3$ (0.1 M, 100 μL) and 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (L-FDAA, 0.1%, 50 μL) in acetone were added, 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% formic acid, 90 μL). The mixture was subjected to HPLC analysis [Waters XTerra RP_{18} column (3.0 × 150 mm, 3.5 μm); MeCN/H_2O/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]. UV detection was performed at a wavelength of 340 nm.

Determination of the Absolute Configuration of Amino Acids by the OPA Method: The analysis was performed using a modification of a previously described method.[15] A 40 μL aliquot of the hydrolysis solution was used for the OPA method. In an HPLC vial, 80 μL of *o*-phthaldialdehyde (OPA) solution and 80 μL of *N*-isobutyryl-cysteine (0.1%) were added to this solution, and after a reaction time of 2 min 20 μL of the reaction mixture were subjected to HPLC analysis [Phenomenex Hyperclone BDS C18 column (4.0 × 250 mm, 5 μm); MeOH/NaOAc gradient: solution A: 125 mM NaOAc in water and 20 mL MeOH, adjusted to pH 6.8 using diluted HOAc; solution B: MeOH].

Supporting Information (see also the footnote on the first page of this article): MS and 1D ^{1}H NMR spectra of compounds **1**–**4**, MS^n spectra of compounds **1** and **2**, $^{1}H,^{1}H$-COSY-, $^{1}H,^{1}H$-NOESY-, $^{1}H,^{13}C$-HMBC and semi-selective $^{1}H,^{13}C$-HMBC data for compounds **1**–**4**, assigned $^{1}H,^{13}C$-HMBC and $^{1}H,^{1}H$-NOESY spectra for compound **2**, $\Delta\delta_{\beta\gamma}$ values and NOE correlations used for the *cis*/*trans* configuration assignments for compounds **1**–**4**.

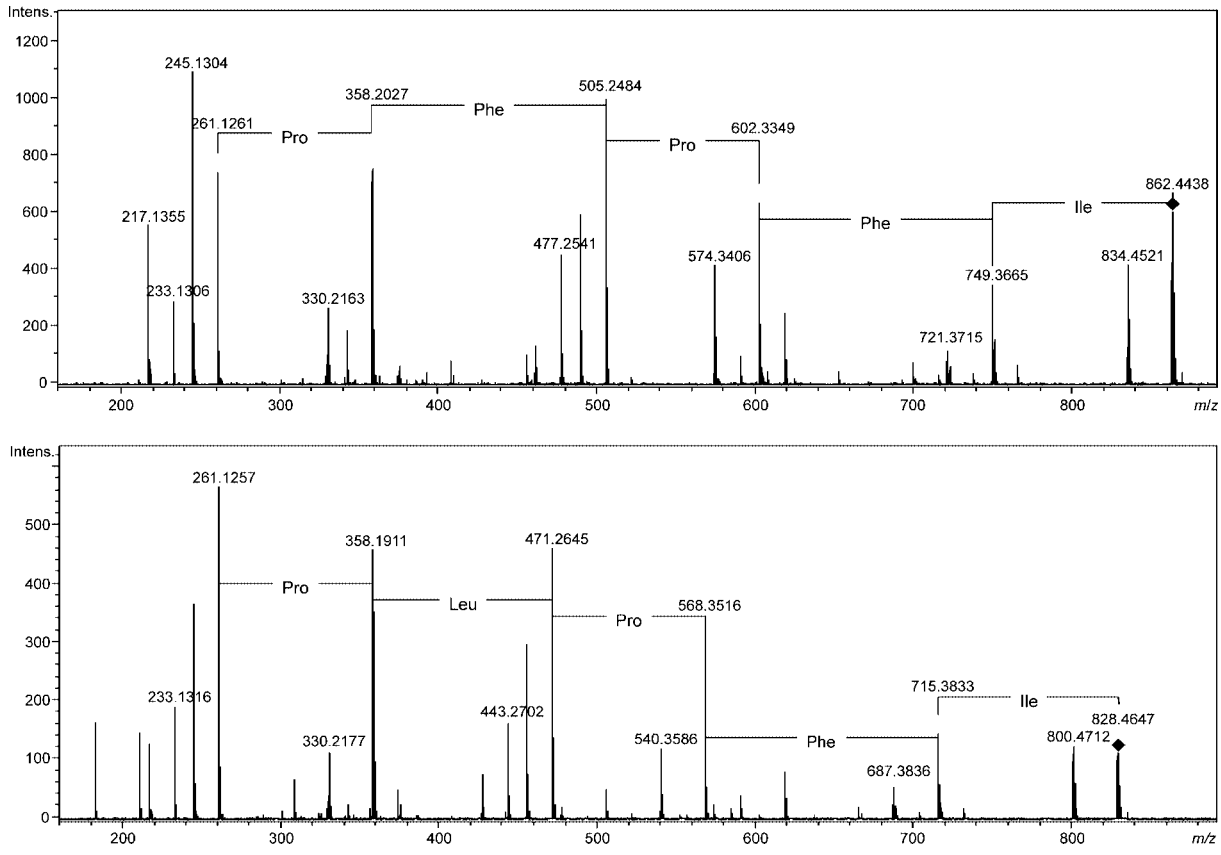

Figure 4. HR-MS/MS spectra of stylissamide C (**3**, above) and stylissamide D (**4**). The precursor ions are indicated by the filled squares. Only the main fragmentation pathways are given. Masses associated with this fragmentation pathway and the corresponding observed masses derived from CO loss (28 amu) are given.

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