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Stylissadines A and B: The First Tetrameric Pyrrole–Imidazole Alkaloids†

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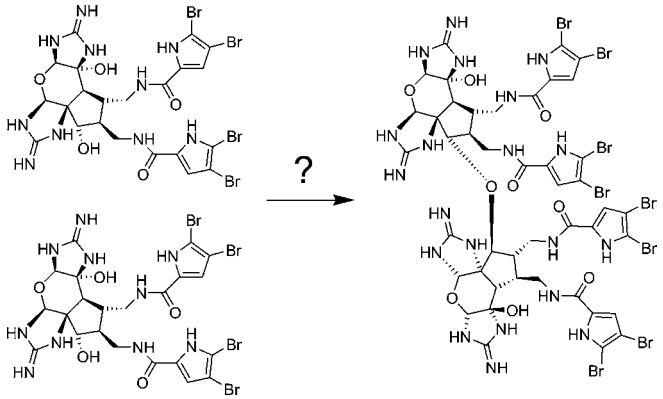
ABSTRACT



Pyrrole–imidazole alkaloids are widely distributed in marine sponges of the orders Halichondrida and Agelasida. Chemical investigation of the Caribbean sponge *Stylissa caribica* led to the isolation of the first tetrameric pyrrole–imidazole alkaloids. The so-called stylissadines are the largest and most complex pyrrole–imidazole alkaloids discovered so far and are therefore a major challenge for the structure determination by NMR spectroscopy. Their isolation and structure elucidation are discussed in detail.

Pyrrole−imidazole alkaloids are widely distributed in the sponge families Agelasidae, Axinellidae, Dictyonellidae, and Hymeniacidonidae.[1] The most prominent member of this family is oroidin (**1**), which was first isolated in 1971 from *Agelas oroides* (see Figure 1).[2] Oroidin (**1**) is the biosynthetic precursor of a whole family of intra- or intermolecular-cyclized derivatives.[3] So far, about 100 pyrrole−imidazole alkaloids have been described in the literature.[4] Examples for dimeric derivatives are sceptrin (**2**),[2c] ageliferin (**3**),[5] and massadine (**4**).[6] Here, we describe the isolation and structure elucidation of the largest and most complex pyrrole−imidazole alkaloids discovered so far.

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 (74 ft depth, July 2000).[7] In an HPLC−HRMS screening of all fractions obtained from Sephadex LH-20 chromatography two highly brominated substances with a mass of m/z = 1630 were detected.[8] Final purification of the compounds was achieved by preparative RP_{C18} HPLC ($MeCN/H_2O$/TFA gradient) to yield massadine (**4**) (31.8 mg, 0.03% of dry weight), **5** (28.9 mg, 0.03% of dry weight),

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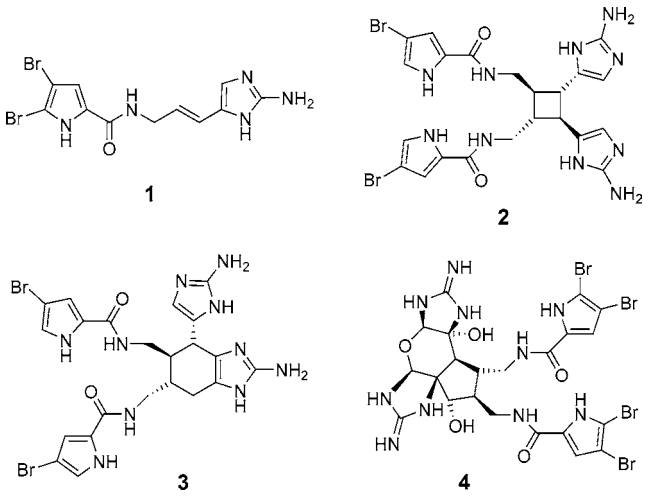

Figure 1. Structural formulas of oroidin (**1**), sceptrin (**2**), ageliferin (**3**), and massadine (**4**).

and **6** (66.8 mg, 0.07% of dry weight). The molecular formulas of **5** and **6** were established by UV,[9] 2D NMR,[10] HR-MS, and API-CID-MS/MS techniques.[11]

The positive electrospray mass spectra of compounds **5** and **6** displayed clusters of ion peaks $[M + H]^+$ at m/z = 1630/1632/1634/1638/1640/1642/1644/1646, which was consistent with a Br_8 isotope pattern. The two substances showed very similar retention times on HPLC. Under API-CID-MS/MS conditions, the loss of guanidine groups[12] and water was detected. Furthermore, a typical fragment arising from the dibromopyrrolecarboxylic acid moiety at m/z = 251.8 was obtained.[13] The high-resolution masses (m/z = 819.8599 $[M + 2H]^{2+}$ and 819.8645 $[M + 2H]^{2+}$) indicated the molecular formula $C_{44}H_{46}N_{20}O_9Br_8$ in both cases corresponding to the 2-fold mass of massadine (**4**) with the difference of one water molecule. The fragmentation pattern under API-CID-MS/MS conditions for **5** and **6** starting from m/z = 828.8 ($[M + H]^+$ of **4**) was identical to massadine (**4**). Therefore, it was very probable from the MS analysis that **5** and **6** were condensation products of two massadine (**4**)[6] molecules which were also isolated from the same sponge specimen.

(8) 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 (0.1% in water), 30 min: 60% MeCN/40% HCOOH (0.1% in water)) with a flow rate of 0.4 mL/min. UV detection was performed with a DAD (Agilent) at a wavelength of 280 nm.

(9) UV spectra were recorded while HPLC separation with a DAD (Agilent). UV of **5**: λ_{max} 275 and 235 nm, **6**: λ_{max} 275 and 235 nm.

(10) ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer (Bruker BioSpin). All NMR experiments were measured at 298 or 300 K (sample concentration stylissadine A: 13.7 mg/600 μL; stylissadine B: 34.0 mg/600 μL). All NMR spectra were also run on a Bruker Avance 700 spectrometer with cryo probe in order to confirm the assignments.

(11) Mass spectrometric analyses were performed on a ESI-oTOF spectrometer (microTOF$_{LC}$, Bruker Daltonics).

(12) The loss of guanidine groups was observed for all pyrrole−imidazole alkaloids which include an oxidized aminoimidazole moiety, e.g., massadine (**4**) and axinellamines or cyclized pyrrole−imidazole alkaloids of the phakellin and isophakellin type.

(13) This fragment was observed for all pyrrole−imidazole alkaloids which include a noncyclized dibromopyrrolecarboxylic acid moiety, e.g., oroidin (**1**), sceptrin (**2**), ageliferin (**3**), and massadine (**4**).

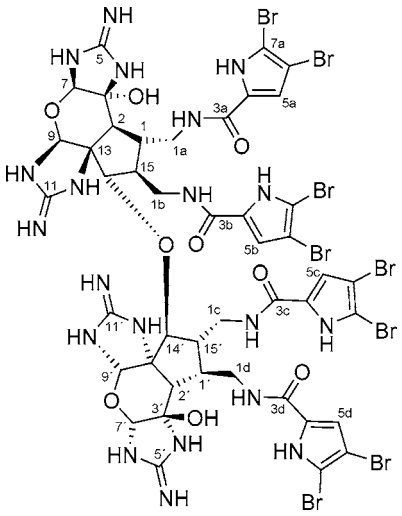

Figure 2. Structural formula of stylissadines A (**5**) and B (**6**). For stylissadine B (**6**) the configuration at center C-2′ is inverted. Both massadine units are shown in the absolute configuration as published by Fusetani et al.[6]

In the 1D ^{13}C NMR spectrum of **6** 44 signals were identified, whereas only 22 were observed for **5**, indicating a symmetric dimeric structure of massadine (**4**) in the latter case. The carbon skeletons of the new compounds were elucidated using correlations from ^{1}H,^{1}H-COSY, ^{1}H,^{13}C-HMBC, and ^{1}H,^{15}N-HMBC experiments. This yielded a structure with two core ring systems and four side chains. The side chains were identified on the basis of the chemical

Table 1. NMR Spectroscopic Data for the Two Tetracyclic Substructures of Stylissadine A (**5**) in DMSO-d_6[a]

no.[b]	δ(^{13}C)	δ(^{1}H) (mult, J in Hz)	δ(^{15}N)[c]
1	40.8	2.12 (m)	
2	45.3	2.22 (d, 11.7)	
3	86.8		
3-OH		7.76 (s)	
4		9.44 (s)	106
5	157.1		
5-NH_2		8.22 (br s)	75
6		9.26 (s)	100[d]
7	89.8	5.37 (d, 2.2)	
9	81.9	5.46 (s)	
10		9.26 (s)	96[d]
11	157.3		
11-NH_2		8.22 (br s)	75
12		9.17 (s)	90
13	69.1		
14	81.6	3.49 (m)	
15	47.9	2.00 (m)	

[a] Chemical shifts δ are given in ppm. ^{1}H and ^{13}C chemical shifts are referenced to the DMSO-d_6 signal (2.50 and 39.5 ppm, respectively). [b] Since **5** is C_2-symmetric, 1 stands for 1 and 1′; 2 for 2 and 2′ etc. [c] ^{15}N NMR spectra were not calibrated with an external standard. δ(^{15}N) has an accuracy of about 1 ppm in reference to NH_3 (0 ppm). [d] Assignment may be interchanged.

shifts which are almost identical to the dibromopyrrole moiety of oroidin (**1**). The core moiety represents the tetracyclic structure of massadine (**4**) as suggested by the MS results. The two massadine units of **5** and **6** are linked through an ether bridge which was proven by the $^{1}H,^{13}C$-HMBC correlations from H-14 to C-14′ and H-14′ to C-14. The ^{13}C NMR chemical shift of C-14 in **5** and **6** (downfield shift of about 5 ppm compared to **4**) confirmed the presence of an ether oxygen at this position. Furthermore, no OH signals were observed in the range of 5.5 ppm for **5** and **6** as it is known from massadine (**4**). This proved that **5** and **6** are stereoisomers (see Figure 2). Tables 1 and 2 summarize the most important NMR data of **5** and **6**.

Table 2. NMR Spectroscopic Data for the Two Tetracyclic Substructures of Stylissadine B (**6**) in DMSO-d_6[a]

no.	$\delta(^{13}C)$	$\delta(^{1}H)$ (mult, J in Hz)	$\delta(^{15}N)$[b]
1	40.9	2.08 (m)	
2	45.8	2.16 (m)	
3	87.0		
3-OH		7.95 (s)	
4		9.301 (s)	106[c]
5	157.4		
5-NH_2		8.20 (br s)	74
6		9.46 (s)	96
7	90.0	5.42 (d, 1.8)	
9	82.4	5.98 (s)	
10		9.304 (s)	101[c]
11	157.3		
11-NH_2		8.32 (br s)	75
12		9.29 (m)	90
13	69.8		
14	83.7	3.78 (s)	
15	50.1	1.84 (m)	
1'	38.8	2.21 (m)	
2'	42.4	2.88 (d, 8.4)	
3'	84.7		
3-OH'		7.45 (s)	
4'		9.34 (s)	113
5'	156.9		
5'-NH_2		8.32 (br s)	75
6'		9.39 (s)	97
7'	86.5	5.24 (s)	
9'	79.2	5.61 (s)	
10'		9.24 (s)	99
11'	156.7		
11'-NH_2		8.43 (br s)	75
12'		8.59 (s)	97
13'	68.3		
14'	84.7	3.70 (d, 5.1)	
15'	45.7	1.94 (m)	

[a] Chemical shifts δ are given in ppm. ^{1}H and ^{13}C chemical shifts are referenced to the DMSO-d_6 signal (2.50 and 39.5 ppm, respectively). [b] ^{15}N NMR spectra were not calibrated with an external standard. The δ value has an accuracy of about 1 ppm in reference to NH_3 (0 ppm). [c] Assignment may be interchanged.

The first step in the assignment of the relative stereochemistry of **5** and **6** was the comparison of the ^{13}C chemical shifts of the stereogenic centers to massadine (**4**). The differences at C-14 are due to the change from a secondary alcohol in massadine (**4**) to an ether functionality in stylissadines A (**5**) and B (**6**). Therefore, it can be concluded from the $\delta(^{13}C)$ values that the relative stereochemistry of stylissadine A (**5**) as well as the first massadine unit (B) of stylissadine B (**6**) is identical to massadine (**4**). The second massadine unit of (B′) stylissadine B (**6**) showed changes in $\delta(^{13}C)$ especially for the pyran positions C-3, C-7, C-9, and C-13 (see Figure 3).

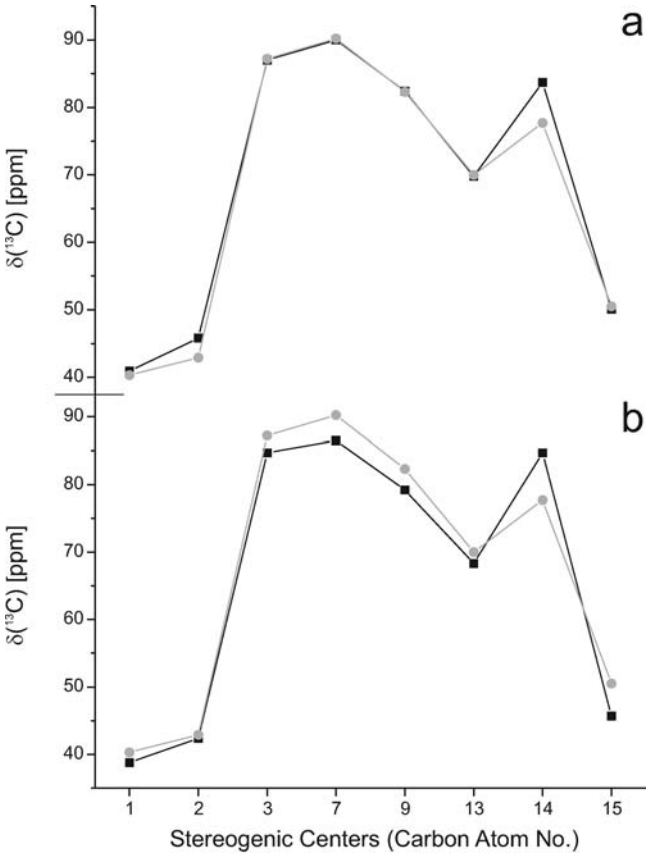

Figure 3. Comparison of the ^{13}C NMR chemical shifts of the eight stereogenic centers of massadine (**4**) to stylissadine B (**6**) ((a) unit with the massadine configuration (B); (b) unit with the 2-*epi*-massadine configuration (B′)). The values for massadine (**4**) are represented as circles and for stylissadine B (**6**) as squares.

Table 3. Selected Interproton Distances [pm] for **5** and **6**[a]

position		stylissadine A (**5**)	stylissadine B (**6**) B'	stylissadine B (**6**) B
H1	H4	230	-	strong signal[b]
H1	H12	240	-	strong signal[b]
H2	H4	260	-	weak signal[b]
H2	H12	270	230	weak signal[b]
H2	H7	270	400	280
H2	H9	210	370	250

[a] The interproton distances are in general too short. Therefore, they were used in a relative manner to differentiate between the massadine and 2-*epi*-massadine configuration. [b] Overlap of ROESY signals.

Interproton distances for stylissadine A (**5**) and stylissadine B (**6**) were obtained from ROESY spectra with different mixing times (150 and 200 ms). Major changes in the interproton distances for the massadine units B and B′ of stylissadine B (**6**) were observed for H-2 to H-4, H-7, H-9, and H-12 as well as for H-1 to H-4 and H-12 (see Table 3). Furthermore, the H-1/H-2 distance is quite short (210 pm) for the second massadine unit (B′) of **6** which indicates a syn orientation of both protons. This change is also confirmed by the different $^3J_{HH}$ coupling constants between the two protons (11.7 versus 8.4 Hz, see Tables 1 and 2). This observation requires a configurational change at C-1 or C-2. The interproton distances are only in agreement with an inversion at C-2 of the massadine unit B′ in stylissadine B (**6**).

The configuration at centers C-3, C-7, C-9, and C-13 for the massadine units B and B′ of stylissadine B (**6**) are identical to massadine (**4**) according to the ROESY analysis. To assign the configuration at centers C-14 and C-15, all distances between these positions were analyzed (see Table 4). These values clearly indicate that the configuration of

Table 4. Interproton Distances [pm] for Stylissadine B (**6**) between the Two Massadine Moieties

position	H15	H15'
H14	270	250
H14'	220	270

centers C-14 and C-15 are identical in both massadine units (B and B′) of stylissadine B (**6**). The pyran ring of the massadine and 2-*epi*-massadine configuration exist in boat conformations (see Figure 4).

The absolute stereochemistry of massadine (**4**) was determined as 1*R*,2*S*,3*R*,7*R*,9*S*,13*S*,14*S*,15*S* (see Figure 1).[6] On the basis of our NMR analysis, four stereoisomers are possible for stylissadines A (**5**) and B (**6**). Since stylissadine A (**5**) shows only half of the signals it must be C_2 or C_s symmetrical. The optical rotation ($\alpha_D = -15.2$)[14] for **5** only allows stereoisomers with C_2 symmetry. Therefore, stylissadine A (**5**) is the formal condensation product of two massadine or two *ent*-massadine moieties. There is no symmetry argument for stylissadine B (**6**). Accordingly, four

(14) Optical rotation was measured using a Perkin-Elmer 241 polarimeter at 20 and 22 °C. **4**: $[\alpha]_D^{20} = -18.5$ (*c* 0.45, MeOH). **5**: $[\alpha]_D^{22} = -15.2$ (*c* 1.215, MeOH). **6**: $[\alpha]_D^{22} = -20.0$ (*c* 0.625, MeOH).

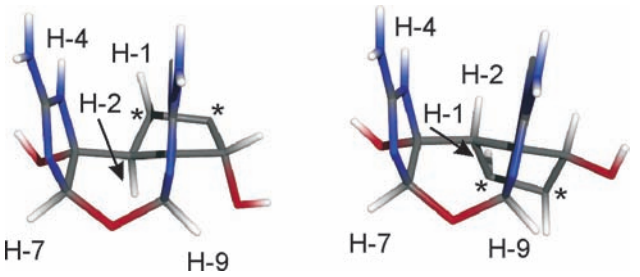

Figure 4. Conformations of the central pyran ring of massadine (left) as observed for stylissadine A (**5**) and one-half (B) of stylissadine B (**6**) and 2-*epi*-massadine (right) as observed for the second half (B′) of stylissadine B (**6**). The asterisks indicated that the molecule was cut at these positions. The simulation results were obtained for non-charged guanidines. When the charges are considered the conformation of the pyran ring changes from one boat to another boat conformation.

stereoisomers (diasteromeric pairs of enantiomers) are possible: (a) **4** + 2-*epi*-**4**, (b) **4** + *ent*-(2-*epi*-**4**), (c) *ent*-**4** + 2-*epi*-**4**, and (d) *ent*-**4** + *ent*-(2-*epi*-**4**).

Stylissadines A (**5**) and B (**6**) are the first tetrameric and therefore largest pyrrole−imidazole alkaloids isolated so far. With 16 stereogenic centers, they represent the most complex structures within the oroidin alkaloid family. The compounds showed minor activities against several pathogenic bacteria, fungi, and cultures of mice fibroblasts (details see Supporting Information).

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Supporting Information Available: A detailed description of the MS and NMR analysis of stylissadines A (**5**) and B (**6**), as well as more information on the extraction and isolation procedure, and additional information for biological tests. This material is available free of charge via the Internet at http://pubs.acs.org.

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