Novel Bromopyrrole Alkaloids from the Sponge *Agelas dispar*

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Further investigation of the Caribbean marine sponge *Agelas dispar* for biologically active constituents has led to the isolation of the novel bromopyrrole alkaloids longamide B (**1**), and clathramides C (**2**) and D (**3**), whose structures have been determined by spectroscopic methods. Isolation of the known keramadine (**4**) and of the ecdysonic sterols ecdysterone (**5**) and ajugasterone C (**6**) is also reported. The antimicrobial activities of the isolated bromopyrrole alkaloids is summarized.

Alkaloids containing a brominated pyrrole moiety constitute by far the commonest class of secondary metabolites in Agelasida sponges. As a proof of this fact, presently more than 25 bromopyrroles have been reported from Agelasida, and the number increases to more than 50 if some alkaloids produced by the taxonomically related order Axinellida (and presumably derived from the same precursors) are added to this list.

In earlier papers we reported the isolation of a series of novel alkaloids with the bromopyrrole nucleus[1−4] from four Caribbean *Agelas* sponges (*A. clathrodes*, *A. conifera*, *A. dispar*, *A. longissima*). Several of these compounds were especially interesting because they are endowed with peculiar biological activity (i.e., agelongine[1] acts as a specific inhibitor of serotonergic receptors; dispacamide A[3] is a potent noncompetitive antihistaminic; and clathramides A and B[4] are antifungal agents).

The variety of pharmacological activities displayed by such metabolites greatly increased our interest in this class of alkaloids, and the need to isolate additional quantities of the above compounds or of their structural analogues for further testing prompted us to reinvestigate the crude fractions obtained in the previous isolation work. This study resulted in the isolation of three additional novel bromopyrrole alkaloids from the sponge *A. dispar* Duchassaing and Michelotti, 1860 (family Agelasidae, order Agelasida): longamide B (**1**), clathramide C (**2**), and clathramide D (**3**), which are the object of the present paper. The methanolic extract of this organism also contained the known alkaloid keramadine (**4**), and the known ecdysonic sterols **5** and **6**.

Specimens of *A. dispar* were collected at −14 m in the lagoon of Little San Salvador Island (Bahamas) during the summer of 1992. Freshly collected animals were stored frozen and then extracted with MeOH. The crude extract was initially partitioned between Et_2O and H_2O, and the H_2O layer was then partitioned with 1-BuOH.

Medium-pressure Si gel cromatography (MPLC) of the Et_2O-soluble fraction, followed by HPLC (eluent EtOAc), yielded longamide B (**1**). The butanol layer was initially subjected to chromatography over a column packed with reversed-phase (RP18) Si gel, and those fractions obtained eluting with MeOH−H_2O 1:1 were composed of polar alkaloids and steroids. These were further purified by MPLC over Si gel using a system of eluents with a gradient of increasing polarity from EtOAc to MeOH. Fractions eluted with MeOH−EtOAc 8:2 afforded clathramides C (**2**) and D (**3**); those eluted with EtOAc−MeOH 7:3 gave keramadine (**4**), while fractions eluted with EtOAc−MeOH 8:2, purified by HPLC, furnished steroids **5** and **6** in the pure state.

Spectroscopic analysis allowed us to readily identify compound **5** as the widespread ecdysterone;[6] whereas sterol **6** was ajugasterone C,[7] a rather uncommon ecdysone steroid first isolated from the leaves of *Ajuga japonica* in 1969. It was identified by comparison of its spectral properties with those reported in the literature. To our knowledge this is the first report of ajugasterone C from a marine sponge.

Longamide B (**1**) was obtained as a colorless amorphous solid. Its FABMS (positive ion) exhibited pseudomolecular ion peaks at *m*/*z* 351, 353, 355 $[M + H]^+$ in the 1:2:1 ratio, suggesting the presence of two bromine atoms. The molecular formula $C_9H_8Br_2N_2O_3$, consistent with six unsaturation units, was secured by HRFABMS mesurements at *m*/*z* 351. In addition, its FABMS showed two intense and diagnostic fragment ions: *m*/*z* 292, 294, 296 for M-CH_2COOH and *m*/*z* 272, 274 for M-Br.

Absorption bands in the UV spectrum of **1** [λ_{max} 210 (ϵ 12 110), 230 (ϵ 8500), and 280 (ϵ 7700) nm] suggested the presence of a pyrrole ring possessing a carbonyl function at the α-position.[8] The analysis of the ^{13}C-NMR spectrum ($CDCl_3$), aided by a DEPT experiment, confirmed that a pyrrole ring with only one proton-bearing carbon was a part of the molecule **1**. The chemical shift values in the ^{13}C-NMR spectrum, compared with literature data,[9] defined the placement of two bromine atoms at C-2 (δ 122.2) and C-3 (δ 104.4) on the pyrrole nucleus.

The resonances of two quaternary carbons were observed at δ 156.5 and 167.8 in the ^{13}C-NMR spectrum. These carbons are assignable to an amide carbonyl and

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Table 1. ^{13}C- (125 MHz) and ^{1}H- (500 MHz) NMR Data of Compounds **2** and **3** in CD_3OD

	2		**3**	
Position	δ_C, mult.	δ_H, mult., int., *J* in Hz	δ_C, mult.	δ_H, mult., int., *J* in Hz
1-NH		9.65[a] (br.s,1H)		9.61[a] (br.s, 1H)
2	124.6, CH	7.06 (d, 1H, 1.7)	124.8,CH	7.08 (d, 1H, 1.7)
3	97.2, C		97.3, C	
4	117.6, CH	6.95 (d, 1H, 1.7)	117.3, CH	6.86 (d,1H, 1.7)
5	124.0, C		124.3, C	
6	160.3, C		160.4, C	
7-NH		7.97[a] (br s, 1H)		7.78[a] (br s, 1H)
8	51.4, CH	4.09 (m, 1H)	52.7, CH	4.16 (m, 1H)
9	31.7, CH_2	2.31 (m, 2H)	33.4, CH_2	2.59 (dd, 1H,14.2, 5.2) 1.94 (dd, 1H,14.2, 11.4)
10	58.3, C		58.0, C	
11	69.2, CH_2	4.25 (d, 1H, 12.1) 4.41 (d, 1H, 12.1)	68.8, CH_2	4.21 (d, 1H 12.1) 4.53 (d, 1H, 12.1)
12-NH		11.83[a] (br s, 1H)		11.92[a] (br s, 1H)
13	154.0, CH	7.96 (br s, 1H)	153.9, CH	8.00 (br s, 1H)
14-NH		9.25 (br s, 1H)		9.27 (br s, 1H)
15	23.5, CH_3	1.45 (s, 3H)	20.9, CH_3	1.50 (s, 3H)
16	174.3, C		174.4, C	

[a] D_2O exchangeable signals recorded in DMSO-d_6.

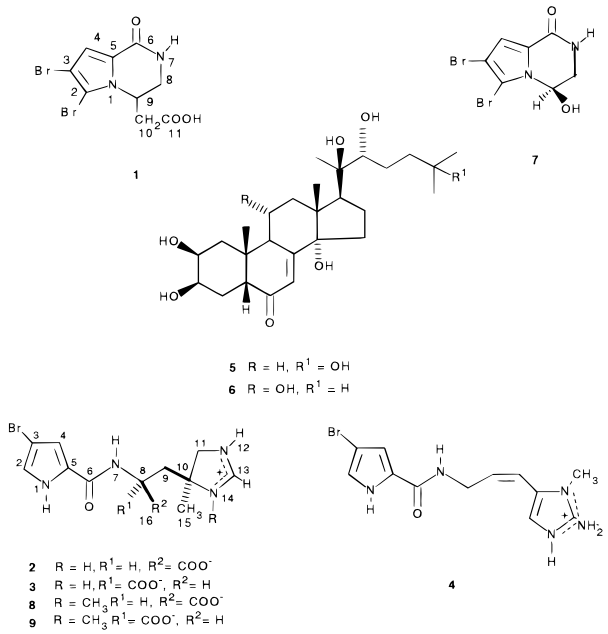
a carboxyl group, respectively. Further support for the presence of these functions was provided by strong absorptions at ν_{max} 1640 cm^{-1} and 1780 cm^{-1} in the FTIR (KBr) spectrum.

An $^{1}H-^{1}H$ COSY NMR experiment ($CDCl_3$) showed the presence of a $NH-CH_2-CH-CH_2-$ system. Unambiguous association of all the proton signals with the resonances of the relevant carbon atoms was established using an HMQC experiment. The above subunit was connected with the remaining part of the molecule as indicated by structure **1**, taking into account the fact that the NH group must be part of the previously identified amide function and considering some key correlation peaks present in the HMBC spectrum. In particular, the aromatic carbon atom resonating at δ 124.0 (C-5) was correlated to the methine proton H-9. Cross peaks were observed also between NH-7 and C-9, and C-11 and H-9. The latter correlation confirmed the position of the carboxyl group on C-10, consistent with the loss of CH_2COOH in the FABMS. All these data were consistent with the structure **1**.

Compound **1** appeared to be present as a mixture of enantiomers at C-9, as suggested by its specific rotation $[\alpha]_D$ 0.0° (*c* 0.004 in MeOH) and by its CD spectrum, which showed no absorptions between 200 and 400 nm. It is the first cyclic bromopyrrole alkaloid found in the sponge *A. dispar*. A structurally related *Agelas* alkaloid, with a hydroxyl group in place of the acetate group of **1**, is longamide (**7**),[2] previously found in *A. longissima*.

Clathramide C (**2**) (molecular formula $C_{12}H_{15}BrN_4O_3$ deduced by HRFABMS analysis) showed a very similar ^{1}H-NMR spectrum (Table 1) to clathramide A (**8**),[4] an alkaloid recently isolated by our research group from a specimen of *A. clathrodes*. The differences between the ^{1}H-NMR spectra of these two compounds were confined to a slight shift of some proton resonances and to the lack of an *N*-methyl signal at δ 3.25 in the ^{1}H-NMR spectrum of **2** present in that of **8**. These data suggested that **2** corresponds to the N-14 nor-derivative of clathramide A (**8**). Further evidence came from inspection of the ^{13}C-NMR spectrum of **2** (Table 1), which exhibited only one methyl resonance.

Comparison of NMR (Table 1) and MS data allowed us to assign the structure of the N-14 nor-derivative of clathramide B (**9**)[4] to the diastereomer clathramide D (**3**).



Stimulated by these findings we reexamined the MeOH extract previously obtained from *A. clathrodes* and found that, in addition to the reported clathramides A and B, minute amounts of clathramides C and D were also present. On the other hand, clathramides A and B were absent in *A. dispar*.

The MPLC fraction eluted with EtOAc−MeOH 7:3 and purified by reversed-phase HPLC (eluent MeOH−H_2O 1:1) furnished keramadine (**4**),[5] identified by comparison of its NMR and MS data with those reported in the literature. Keramadine is a bromopyrrole alkaloid of the oroidin family, first isolated in 1984 as a potent serotonergic receptor antagonist and till now reported only in an unidentified *Agelas* species of the Japanese area. The present finding of the bioactive keramadine in *A. dispar* represents the first report of its isolation from a sponge of the Caribbean area.

The bromopyrrole alkaloids found in *A. dispar* (longamide B, clathramide C, clathramide D, and keramadine) were screened for antibiotic activity against Gram-positive (*Bacillus subtilis* ATCC #6633 and *Staphilococcus aureus* ATCC #6538) bacteria. Moderate antibacterial activities were exhibited by longamide B and keramadine (MIC of about 50 μg/mL), while the two clathramides were inactive in this test. However, clathramides C and D maintained the antifungal activity expressed by their methylated analogues (clathramides A and B) against *Aspergillus niger*.[4] In fact, in an agar disk diffusion assay, 100 μg of the mixture **2**−**3** caused a zone of inhibition of almost 7 mm.

Experimental Section

General Experimental Procedures. Optical rotations were measured in MeOH on a Perkin−Elmer 192 polarimeter equipped with a sodium lamp ($\lambda = 589$ nm) and a 10-cm microcell. IR (KBr) spectra were recorded on a Bruker model IFS-48 spectrophotometer. UV spectra were obtained in CH_3CN using a Beckman DU70 spectrophotometer. LRFABMS and HRFABMS (CsI ions) were performed in a glycerol matrix on a VG Prospec (FISONS) mass spectrometer. ^{1}H- (500 MHz) and ^{13}C- (125 MHz) NMR spectra were measured on a Bruker AMX-500 spectrometer; chemical shifts are referenced to the residual solvent signal ($CDCl_3$ $\delta_H = 7.26$, $\delta_C = 77.0$; CD_3OD $\delta_H = 3.34$, $\delta_C = 49.0$; DMSO-d_6 $\delta_H = 2.50$). The multiplicities of ^{13}C resonances were determined by DEPT experiments. One-bond heteronuclear ^{1}H−^{13}C connectivities were determined with the Bax−Subramanian HMQC pulse sequence using a BIRD pulse of 0.50 s before each scan in order to suppress the signals originating from protons not directly bound to ^{13}C (interpulse delay set for $^1J_{CH} = 140$ Hz). During the acquisition time, ^{13}C broad-band decoupling was performed using the GARP sequence. Two- and three-bond ^{1}H−^{13}C connectivities were determined by HMBC experiments optimized for a $^{2,3}J$ of 10 Hz. MPLC was performed using a Büchi 861 apparatus. HPLC separations were achieved on a Waters 501 apparatus equipped with a UV detector (λ 245 nm) and with Hibar LiChrospher (250 × 4 mm) columns.

Animal Material, Extraction, and Isolation of Compounds 1−6. The sponge *A. dispar* was collected in the summer of 1992 in the lagoon of Little San Salvador Island at a depth of 14 m, and identified by Prof. M. Pansini (Università di Genova). A reference specimen has been deposited at the Istituto di Zoologia, Università di Genova, Italy. The sponge (130 g of dry wt after extraction) was homogenized and extracted with MeOH (4 × 500 mL). The obtained oily aqueous residue was partitioned against Et_2O, and successively against *n*-BuOH. The Et_2O-soluble fraction was concentrated *in vacuo* to give a brown oil (3.2 g) that was subjected to normal-phase MPLC (stepwise gradient elution from hexane to EtOAc to MeOH). Fractions eluted with EtOAc−MeOH 9:1 were further purified by HPLC (eluent EtOAc), yielding longamide B (**1**). The BuOH layer was concentrated *in vacuo* affording 7.5 g of a brown viscous oil. This was subjected to chromatography on a column packed with RP-18 Si gel. Three fractions (A−C) were collected (eluents: MeOH−H_2O 1:1, MeOH−$CHCl_3$ 9:1, and $CHCl_3$, respectively). Fraction A (2.3 g), containing polar alkaloids and polar steroids, was further chromatographed by MPLC (SiO_2 column; solvent gradient system of increasing polarity from EtOAc to MeOH). Fractions eluted with EtOAc−MeOH (8:2), rechromatographed by HPLC (eluent $CHCl_3$−MeOH 85:15, SiO_2 column, flow 1.0 mL/min) furnished steroids **5** and **6**. Fractions eluted with EtOAc−MeOH 7:3 gave pure keramadine (**4**), while fractions eluted with MeOH−EtOAc 8:2, rechromatographed by HPLC (eluent H_2O−MeOH, 6:4, RP18 column, flow 0.8 mL/min) gave a fraction composed by **2** and **3** in the ratio 4:1. Repeated HPLC separations on a RP-18 column, using H_2O−CH_3CN 1:1 as eluent (flow 0.3 mL/min), afforded pure compounds **2** and **3**.

Longamide B (1): colorless amorphous solid; $[\alpha]^{25}_D$ 0° (*c* 0.004 in MeOH); IR (KBr) ν_{max}, 1780, 1640 cm^{-1}; UV (CH_3CN) λ_{max} 210 (ϵ 12100), 230 (ϵ 8500), 280 (ϵ 7700) nm; ^{1}H NMR ($CDCl_3$) δ 7.02 (1H, s, H-4), 5.67 (1H, br s, NH-7), 4.76 (1H, br d, J = 11.2, H-9), 3.94 (1H, dd, J = 13.2, 3.1, H-8a), 3.68 (1H, dd, J = 13.2, 5.1, H-8b), 2.97 (1H, dd, J = 16.8, 11.2, H-10a), 2.60 (1H, dd, J = 16.8, 1.5, H-10b); ^{13}C NMR ($CDCl_3$) δ 122.2 (C-2, s), 104.4 (C-3, s), 113.8 (C-4, d), 124.0 (C-5, s), 156.5 (C-6, s), 40.4 (C-8, t), 47.5 (C-9, d), 32.3 (C-10, t), 167.8 (C-11, s); FABMS (positive ion, glycerol matrix) *m/z* 351, 353, 355 (1:2:1) [M + H]$^+$; 292, 294, 296 (1:2:1); 272, 274 (1:1); HRFABMS *m/z* 349.8739, calcd for $C_9H_8{}^{79}Br_2N_2O_3$, 349.8796.

Clathramide C (2): colorless amorphous solid; $[\alpha]^{25}_D$ −6° (*c* 0.001 in MeOH); IR (KBr) ν_{max} 2448, 1679, 1645 cm^{-1}; UV (CH_3CN) λ_{max} 208 (ϵ 14 900), 230 (ϵ 13 000), 265 (ϵ 10 900) nm; ^{1}H and ^{13}C NMR (CD_3OD), see Table 1; ^{1}H NMR (300 K, DMSO-d_6) δ 11.83 (1H, br s, NH-12), 9.55 (1H, br s, NH-1), 7.85 (1H, s, NH-7), 7.94 (1H, br s, H-13), 7.14 (1H, d, J = 1.5, H-2), 6.83 (1H, d, J = 1.5, H-4), 4.43 (1H, d, J = 10.3, H-11a), 4.21 (1H, d, J = 10.3, H-11b), 4.04 (1H, m, H-8), 2.20 (2H, m, H_2-9), 1.36 (3H, s, H_3-15); FABMS (positive ion, glycerol matrix) *m/z* 343 and 345 (1:1) [M + H]$^+$; HR−FABMS *m/z* 341.0467 [M + H]$^+$, calcd for $C_{12}H_{15}{}^{79}BrN_4O_3$, 341.0484.

Clathramide D (3): colorless amorphous solid; $[\alpha]^{25}_D$ +10° (*c* 0.001 in MeOH); IR (KBr) ν_{max} 2448, 1679, 1645 cm^{-1}; UV (CH_3CN) λ_{max} 208(ϵ 14900), 230 (ϵ 13 000), 265 (ϵ 10 900) nm; ^{1}H and ^{13}C NMR (CD_3OD), see Table 1; ^{1}H NMR (300 K, DMSO-d_6) δ 11.92 (1H, br s, NH-12), 9.62 (1H, br s, NH-1), 7.68 (1H, s, NH-7), 7.90 (1H, br s, H-13), 7.20 (1H, d, J = 1.5, H-2), 6.93 (1H, d, J = 1.5, H-4), 4.38 (1H, d, J = 12.0, H-11a), 4.22 (1H, d, J = 12.0, H-11b), 4.03 (1H, m, H-8), 2.05 (1H, m, H-9a; H-9b was completely submerged by the solvent signal), 1.37 (3H, s, H_3-15); FABMS (positive ion, glycerol matrix) *m/z* 343 and 345 (1:1) [M + H]$^+$.

Keramadine (4): colorless powder; FABMS (positive ion, glycerol matrix) 324 and 326 [M + H]$^+$; ^{1}H NMR (DMSO-d_6), IR (KBr), and UV (MeOH) identical with those reported in the literature;[5] ^{1}H NMR (CD_3OD) δ 7.03 (1H, d, J = 1.7, H-2), 6.97 (1H, s, H-12), 6.78 (1H, d, J = 1.7, H-4), 4.12 (2H, br d, J = 11.2, H_2-8), 6.00 (1H, dt, J = 11.2, 5.6, H-9), 6.28 (1H, d, J = 11.2, H-10), 3.45 (3H, s, H-16).

Bioassays. Gram positive bacteria (*B. subtilis* ATCC #6633 and *S. aureus* ATCC#6538) and the fungus *A. niger* were used for the antimicrobial assays, with the use of the Mueller−Hinton agar test. Longamide B (**1**)

and keramadine (**4**) showed a moderate activity against Gram-positive bacteria, with minimum inhibitory concentrations (MIC) values of 40 μg/mL (*B. subtilis*) and 35 μg/mL (*S. aureus*) for **4** and 45 μg/mL (*B. subtilis*) and 55 μg/mL (*S. aureus*) for **1**. In an agar disk diffusion assay, 100 μg of the mixture of **2**−**3** caused a zone of inhibition of 7 mm against *A. niger*. All compounds were dissolved in DMSO. Zones of inhibition were recorded after 16 h for bacterial culture, and 40 h for fungal cultures. Results presented are the average of two experiments.

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