Structure of debromo-carteramine A, a novel bromopyrrole alkaloid from the Mediterranean sponge *Axinella verrucosa*

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Abstract

The butanol extract of the Mediterranean sponge *Axinella verrucosa* was fractionated *via* a bioactivity-guided procedure based on an antibacterial assay. This approach led to the isolation of the novel alkaloid debromo-carteramine A **2** co-occurring with known hymenidin **1**, the main antibacterial agent of the extract, and related inactive carteramine A **3**.

Keywords: Marine natural products, bromopyrrole alkaloids, sponge, antibacterial activity

Introduction

Marine sponges of genus *Axinella* are a well-known source of brominated pyrrole alkaloids.[1-7] Historically, these compounds, which have been found only in the marine environment to date, have attracted the attention of natural product chemists because of their structural complexity. Today, the interest is sparked not only by the wide structural diversity, ranging from simple molecules like oroidin[8,9] to complex structures like palau'amine[10,11] and stylissadine A and B,[12] but also by their organic synthesis,[13] bio-synthesis,[14] and pharmacological activities.[6,7,15] Furthermore, these compounds have a chemo-ecological interest due to their role in chemically mediated interactions such as in the chemical defense against predators[16,17] and fouling organisms.[18-20] The crude extracts of different *Axinella* species have also been reported to inhibit the growth of several environmental bacteria.[21]

Using a bioassay-guided isolation approach, we have analyzed the butanol extract of a sample of *A. verrucosa* collected during the spring 2008 off Massa Lubrense, Bay of Naples. The extract showed strong antibacterial activity against the marine bacterium *Lysinibacillus* sp. It was found that the main component of the extract, hymenidin **1** (Figure 1), previously isolated from *Hymeniacidon* sponge,[22] was also the main compound responsible of the observed antimicrobial activity. Analysis of the fractions exhibiting a weak/moderate activity led to the isolation of a

novel alkaloid of the palau'amine class, debromo-carteramine A **2** (Figure 1). Interestingly, the same fraction also contained the known related compound carteramine A **3** (Figure 1), recently isolated from *Stylissa carteri*[23] and identical with the compound named tetrabromostyloguanidine reported at the same time from *Stylissa caribica*.[24] Carteramine A did not inhibit the bacterium of choice. In this paper, we report the isolation and structure elucidation of the novel compound **2**.

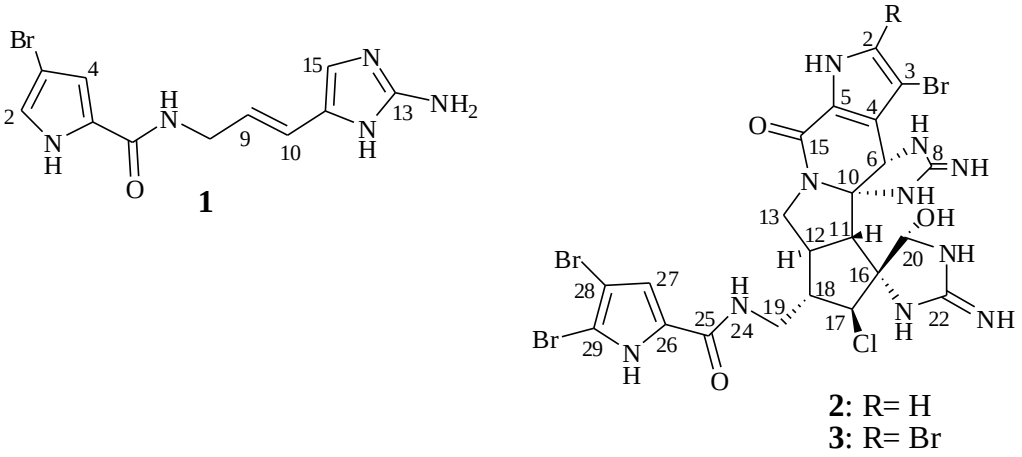

Figure 1. Structures of bromopyrrole alkaloids isolated from the sponge *Axinella verrucosa.*

Results and Discussion

An aliquot (185 mg) of the butanolic soluble portion (690 mg) of the acetone extract of the sponge (5.6 g, dry weight after extraction) was submitted to molecular exclusion chromatography (Sephadex LH-20, $CHCl_3$/MeOH, 1:1) to give two distinct sets of active fractions. The fraction eluted first contained pure hymenidin **1** whereas the second fraction was a mixture that was further purified by reverse-phase HPLC with a linear gradient system to give a pure active compound, debromo-carteramine A (**2**, 4.0 mg), and the inactive related carteramine A (**3**, 3.7 mg). Compounds **1** and **3** were identified by comparison of their spectroscopic data with those reported in the literature. [22-24]

Debromo-carteramine A **2** showed a sodiated-molecular peak at *m/z* 746.9163 in the HRESIMS spectrum, which indicated the molecular formula $C_{22}H_{22}ClBr_3N_{10}O_3$ differing from that of the co-occurring **3** in the presence of a hydrogen atom instead of a bromine. Compound **2** displayed in the ^{13}C NMR spectrum characteristic signals consistent with the presence of two guanidine moieties (δ_C 157.6 and 158.6), and two units of pyrrole-2-carboxylic acid amide exhibiting bromine substituents [δ_C 161.2 (s, C-25), 128.1 (s, C-26) 114.7 (d, C-27), 99.0 (s, C-28), 106.7 (s, C-29) and 152.8 (s, C-15), 126.1 (d, C-2), 122.0 (s, C-5), 121.5 (s, C-4), 94.9 (s, C-3).

Table 1. ^{1}H and ^{13}C NMR spectral data[a] in CD_3OD of debromo-carteramine A **2**

Position	δ_H(mult, *J* in Hz)	δ_C	m	HMBC
2	7.12 (s)	126.1	d	-
3	-	94.9	s	H-2
4	-	121.5	s	-
5	-	122.0	s	H-6, H-2
6	5.79 (s)	55.8	d	-
8	-	157.6		H-6
10	-	83.1	s	H-6
11	2.93 (d, 15)	57.9	d	H-13a, H-6
12	2.59 (m)	42.5	d	H-13b
13	3.17 (app. t, 10)	47.2	t	-
	3.97 (dd, 8, 10)			
15	-	152.8	s	-
16	-	71.9	s	H-17; H-11
17	4.32 (d, 9)	74.5	d	H-20; H-19a
18	2.32 (m)	51.3	d	H-17
19	3.52 (br dd, 8, 14)	40.4	t	H-17
	3.75 (m)			
20	5.98 (s)	84.1	d	H-17, H-11
22	-	158.6	s	H-20
25	-	161.2	s	H-19a
26	-	128.1	s	-
27	6.87 (s)	114.7	d	-
28	-	99.0	s	-
29	-	106.7	s	H-27

[a]Assignments made by ^{1}H-^{1}H COSY, HSQC and HMBC (*J*=10 Hz) experiments.

In the ^{1}H NMR spectrum of **2**, an additional 1H singlet at δ 7.12 (H-2) was present with respect to **3** whereas the remaining part of the spectrum was almost identical. This signal was attributed to an isolated proton in the α-position of a pyrrole ring thus indicating, in agreement with the molecular formula, that **2** lacked the bromine substituent at C-2 with respect to **3**. Further evidence was obtained from the ^{13}C NMR spectrum of **2** containing the CH sp^2 signal in the place of a C sp^2 signal (C-2). The presence of the chlorinated 7-azabicyclo [3.3.0.]octane ring system characterizing all members of the palau'amine class was also evident by analysis of the ^{1}H-^{1}H COSY spectrum. In fact the typical spin-system from H_2-13 to H_2-19 was easily detected. Analysis of 2D-NMR experiments (^{1}H-^{1}H COSY, HSQC, and HMBC) allowed complete proton and carbon assignments as reported in Table 1 and confirmed that **2** was the 2-debromo-derivative of carteramine A[23] (≡ tetrabromostyloguanidine).[24]

The relative stereochemistry of the eight chiral centres of **2** was suggested to be the same as **3** by comparison of their ^{13}C-NMR data[23] and was further supported by analysis of a series of NOE difference and NOESY experiments (Figure 2) recorded in CD_3OD and d_6-DMSO, respectively. Analogous to the data reported for carteramine A,[23] steric effects were observed for compound **2** between H-11 and both H-6 and H-20 according to the reported stereochemistry for both cyclic guanidine moieties. The absence of NOE interactions between H-11 and H-12 was in agreement with the *trans*-11,12-junction of the 7-azabicyclo[3.3.0.]octane ring, analogous to carteramine A. Diagnostic NOE effects were observed between H-12 and H-17, H-11 and H-18, and 20-OH and both H-6 and H-11 that inferred the relative configuration at C-17, C-18, and C-20, respectively.

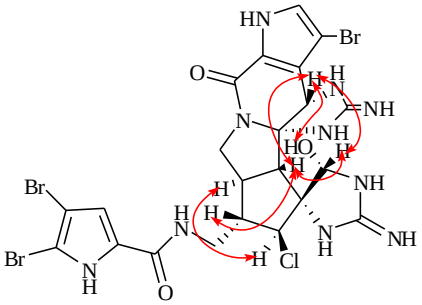

Figure 2. Significant NOE effects in debromo-carteramine A (**2**) by NOE difference and NOESY experiments.

Thus 2-debromo-carteramine A **2** exhibited the same relative configuration as carteramine A[23] (≡ tetrabromostyloguanidine),[24] the stereochemistry of which was determined by NOESY analysis[26] and computational methods,[24] and differed from those reported for palau'amine and related compounds.[10,11] However, in both these recent papers[23,24] the relative configuration of palau'amine congeners was suggested to need revision.

Compounds **1** and **2** showed activity against the environmental marine bacterium *Lysinibacillus* sp. ESY 9 (GenBank accession no. GU059941) in the disc diffusion assay with an inhibition zone diameter of 12.5 mm at a concentration of 100 µg/disc and of 8 mm at a concentration of 200 µg/disc, respectively. It is interesting to note that co-occurring carteramine A (**3**) was found to be inactive in the same test suggesting that free C-2 position is an essential requirement for this activity.

Experimental Section

General. Optical rotations were measured on a JASCO DIP 370 digital polarimeter. The UV spectra were recorded on JASCO 710 spectropolarimeter. ^{1}H and ^{13}C NMR spectra were recorded on DRX 600, AVANCE 400, and DPX 300 MHz Bruker spectrometers in CD_3OD and in d_6-DMSO, with chemical shifts reported in ppm referred to CH_3OH (δ 3.34 for proton and δ 49.9 for carbon) and to DMSO (δ 2.54 for proton and δ 40.5 for carbon), respectively, as internal standards. ESIMS and HRESIMS were measured on a Micromass Q-TOF Micro™coupled with a HPLC Waters Alliance 2695. The instrument was calibrated by using a PEG mixture from 200 to 1000 MW (resolution specification 5000 FWHM, deviation <5 ppm RMS in presence of a known lock mass). Silica gel and exclusion chromatography were performed using precoated Merck F_{254} plates and Sephadex™ LH-20 (Amersham Biosciences), respectively. HPLC purifications were carried out on a Thermo Electron chromatograph coupled with P4000 pumps and a UV2000 double wavelength detector.

Collection and extraction of the animal material

Specimens of *A. verrucosa* were collected by Scuba during the Spring 2008 off Massa Lubrense, Bay of Naples at 25 m depth. The biological material was immediately transferred to the ICB laboratory, where it was cleaned from epibionts, rinsed once and frozen at -20 ºC, until its extraction.

The frozen material was cut into pieces of about 1 cm^3 and extracted with acetone (250 mL × 3) under grinding and sonication. The three phases were combined after filtration and the organic solvent was removed under reduced pressure. The residual water was partitioned three times with Et_2O and subsequently with *n*-butanol. The combined Et_2O phases (461 mg) and the *n*-butanol (690 mg) phase were dried under reduced pressure to give the initial two extracts.

Purification of compounds

An aliquot of *n*-butanolic extract (185 mg) was fractionated on a Sephadex LH-20 column. The collected fractions were re-combined based on their TLC pattern resulting in 17 fractions. All fractions were tested for their antibacterial activity, observing two active fractions, A (72 mg) and B (9 mg), which were further analyzed by ^{1}H NMR. Fraction A contained compound **1** pure, whereas fraction B resulted a mixture, which was purified on reverse-phase HPLC (Phenomenex: Kromasil 5µ C18, 250x10 mm, 40 min gradient from 50% to 100% CH_3OH in H_2O with 0.1% of TFA, flow 2 mL/min, UV detector) to obtain compounds **2** and **3**.

Compound 1. Light yellow powder; ^{1}H NMR (CD_3OD, 400 MHz) δ 4.09 (d, J = 4 Hz, 2H, H_2-8), 6.15 (dt, J = 4, 16 Hz, 1H, H-9), 6.33 (d, J = 16 Hz, 1H, H-10), 6.78 (s, 1H, H-15), 6.85 (d, J =1 Hz, 1H, H-4), 6.96 (d, J = 1 Hz, 1H, H-2); ^{13}C NMR (CD_3OD, 300 MHz) δ 162.4 (s, C-6), 149.1 (s, C-13), 128.7 (d, C-9), 127.4 (s, C-5 or C-11), 127.3 (s, C-11 or C-5), 122.9 (d, C-2), 117.5 (d, C-10), 113.4 (d, C-4), 111.8 (d, C-15), 97.5 (s, C-3), 42.5 (t, C-8). ESIMS *m/z* (rel. intensity) 310/312 (1:1) $[M+H]^+$.

Compund 2. Light yellow powder; $[\alpha]_D$ -9.0 (*c* 0.2, MeOH); UV (MeOH) λ_{max} 277 nm (ε 13,400); ^{1}H NMR (d_6-DMSO, 600 MHz) δ 12.67 (br s, 1H, H-1), 9.38 (s, 1H, H-21), 8.70 (s, 1H, H-23), 9.18 (s, 1H, H-9), 9.00 (s, 1H, H-7), 8. 37 (t, *J* = 6 Hz, 1H, H-24), 7.55 (d, *J* = 5 Hz, 1H, -OH), 7.22 (d, *J* = 2 Hz, 1H, H-2), 6.95 (s, 1H, H-27), 5.71 (d, *J* = 5 Hz, 1H, H-20), 5.52 (s, 1H, H-6), 4.28 (d, *J* = 9, 1H, H-17), 3.76 (dd, *J* = 8 and 10 Hz, 1H, H-13a), 3.52 (m, 1H, H-19a), 3.32 (m, 1H, H-19b), 2.99 (dd, *J* = 10, 10 Hz, 1H, H-13b), 2.82 (d, *J* = 14 Hz, 1H, H-11), 2.45 (m, 1H, H-12), 2.11 (m, 1H, H-18). Selected ^{13}C NMR values (d_6-DMSO, 300 MHz) δ 124.7 (d, C-2), 113.0 (d, C-26), 82.3 (d, C-20), 74.0 (d, C-17), 55.7 (d, C-11), 53.5 (d, C-6), 49.3 (d, C-18), 44.6 (t, C-13), 40.6 (d, C-12), 39.1 (t, C-19). HRESIMS *m/z* 746.9163 $[M+H]^+$ (calcd for $C_{22}H_{23}{}^{35}Cl^{79}Br_3N_{10}O_3$, Δ -3.0).

Compound 3. Light yellow powder; $[\alpha]_D$ -37.0 (*c* 0.1, MeOH), $[\alpha]_D$ lit.[23] -42.0 (*c* 1.26, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) δ 6.86 (1H, s, H-27), 5.98 (1H, s, H-20), 5.75 (1H, s, H-6), 4.33 (1H, d, *J* = 9 Hz, H-17), 3.97 (1H, dd, *J* = 8 and 10 Hz, H-13a), 3.73 (1H, dd, *J* = 4 and 14 Hz, H-19a), 3.52 (1H, dd, *J* = 8 and 14 Hz, H-19b), 3.16 (1H, dd, *J* = 10 and 10 Hz, H-13b), 2.93 (1H, d, *J* = 14 Hz, H-11), 2.57 (1H, m, H-12), 2.31 (1H, m, H-18). ESIMS *m/z* (rel. intensity) 825/827/829/831/833 (2:6:10:7:4:1) $[M+H]^+$.

Antibacterial assays

Disc diffusion assays were performed using the environmental marine bacterium *Lysinibacillus* sp. strain ESY 9 (GenBank accession no. GU059941). The bacterium was isolated on LB plates (10 g L^{-1} Tryptone, 5 g L^{-1} Yeast Extract, 5 g L^{-1} NaCl, 15 g L^{-1} Bactoagar) from sediment collected at 30 m depth close to the sponge *A. verrucosa* at the reef in front of Sdot Yam, Israel.

Test extracts were transferred to blank paper discs (diameter 6 mm) and left until the solvent completely evaporated. For the assay 250 µL of an overnight culture grown in liquid LB medium at 30 ºC were plated onto a LB plates until the surface was dry. The paper discs with the applied extracts were then transferred to the seeded plates. Plates were wrapped with Parafilm and incubated for one day at 30 ºC after which the diameter of inhibition zones, visible as clear zones around the paper discs, was measured to the next half millimeter. Solvent controls were always run in parallel and never showed inhibition zones. Assays were performed for the initial Et_2O (dissolved in chloroform) and *n*-butanol (dissolved in MeOH) extracts, all Sephadex LH-20 fractions of the *n*-butanol, and all reverse-phase HPLC fractions of the second active Sephadex LH-20 fraction including the baseline collection and the column washes.

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