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Chemical variability within the marine sponge *Aplysina fulva*☆

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Abstract

Dibromotyrosine-derived metabolites are of common occurrence within marine sponges belonging to the order Verongida. However, previous chemical analysis of crude extracts obtained from samples of the verongid sponge *Aplysina fulva* collected in Brazil did not provide any dibromotyrosine-derived compounds. In this investigation, five samples of *A. fulva* from five different locations along the Brazilian coastline and one sample from a temperate reef in the South Atlantic Bight (SAB) (Georgia, USA) were investigated for the presence of bromotyrosine-derived compounds. All six samples collected yielded dibromotyrosine-derived compounds, including a new derivative, named aplysinafulvin, which has been identified by analysis of spectroscopic data. These results confirm previous assumptions that dibromotyrosine-derived metabolites can be considered as chemotaxonomic markers of verongid sponges. The isolation of aplysinafulvin provides additional support for a biogenetic pathway involving an arene oxide intermediate in the biosynthesis of Verongida metabolites. It cannot yet be established if the chemical variability observed among the six samples of *A. fulva* collected in Brazil and the SAB is the result of different environmental factors, distinct chemical extraction and isolation protocols, or a consequence of hidden genetic diversity within the postulated morphological plasticity of this species.

Keywords: *Aplysina fulva*; Dibromotyrosine; Chemical variability; Chemotaxonomy

1. Introduction

Marine sponges belonging to the order Verongida are considered the richest source of brominated natural products biogenetically derived from tyrosine (Bergquist and Wells, 1983; Dembitsky, 2002; Gribble, 1996, 1998, 2000). Due

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to their occurrence in practically all Verongida sponges so far chemically investigated, bromotyrosine-derived alkaloids have been considered as chemotaxonomic markers for sponges of this Order (Bergquist and Wells, 1983; Harper et al., 2001; van Soest and Braekman, 1999). However, the recent isolation of bromotyrosine-derived compounds from sponges belonging to distinct taxa, such as *Agelas oroides* Schmidt, 1864 (Agelasidae, Agelasida, Demospongiae) (König and Wright, 1993), *Oceanapia* sp. (Phloeodictyidae, Petrosina, Demospongiae) (Nicholas et al., 2001), *Jaspis wondoensis* Sim and Kim, 1995 (Ancorinidae, Astrophorida, Demospongiae) and *Poecillastra wondoensis* Sim and Kim, 1995 (Pachastrellidae, Astrophorida, Demospongiae) (Park et al., 2003), indicated that such compounds are not specific chemotaxonomic markers for Verongida (Erpenbeck and van Soest, 2007). Moreover, dibromotyrosine-derived compounds have been isolated from a marine seaweed (Meragelman et al., 2002) and from a crinoid (Shao et al., 2007).

The chemistry of the sponge *Aplysina fulva* Pallas, 1766 (formerly *Verongia fulva* and also *Aplysina fistularis* forma *fulva*) has been the subject of multiple investigations over the last 30 years, and several bromotyrosine-derived metabolites have been isolated from this species (Ciminiello et al., 1994, 1996b; Gopichand and Schmitz, 1979; Gulavita et al., 1995; Rogers et al., 2005; Rogers and Molinski, 2007). Not surprisingly, crude extracts obtained from different *A. fulva* specimens displayed activity in diverse bioassays (Aiub et al., 2006; Kelly et al., 2003, 2005; Waddell and Pawlik, 2000). However, Kelecom and Kannengiesser (1979) reported a pioneering study on the chemistry of Brazilian Verongida sponges focused on *A. fulva* (Kelecom, personal communication) collected in Arraial do Cabo (Rio de Janeiro state), Guarapari (Espírito Santo state) and Abrolhos Archipelago (Bahia state). Interestingly, these authors were unable to isolate or detect dibromotyrosine-derived compounds from these specimens of *A. fulva*, having instead identified only sterols from these sponges. This result was rather astonishing (Munro et al., 1987), considering that virtually all Verongida sponges studied up to the present have yielded bromotyrosine-derived metabolites. Furthermore, it has been reported in the literature that *A. fulva* is likely to be the least well characterized *Aplysina* sponge occurring on the Brazilian coast (Pinheiro and Hajdu, 2001; Pinheiro et al., 2007). This is in contrast to opinions expressed on the basis of study of Caribbean specimens (Zea, 1987). Therefore, we became interested in performing a chemical re-investigation of several specimens of *A. fulva* collected in different locations along the Brazilian coast and the South Atlantic Bight (SAB), USA, in order to search for the occurrence of bromine-containing compounds and, at the same time, verifying any possible chemical heterogeneity among its populations.

2. Material and methods

2.1. Animal material

Sample *BA99ES-69* of *A. fulva* was collected in Salvador, Bahia state (Fig. 1A), in September 1999, immediately immersed in EtOH and stored at −20 °C. Sample *AC01ES-12* of *A. fulva* was collected in Arraial do Cabo, Rio de Janeiro state (Fig. 1B), in April 2001, and immediately immersed in EtOH and stored at −20 °C. The sample *AR02ES-01* of *A. fulva* was collected at Angra dos Reis, Rio de Janeiro state (Fig. 1C), and immediately stored in acetone at −20 °C. Sample *SS02ES-1* has been collected at São Sebastião, São Paulo state (Fig. 1D), and was stored in EtOH at −20 °C. Samples of *A. fulva* collected at the Jiribatuba Mangrove, Bahia (Fig. 1A; sample *BA04ES-84*) were immediately frozen after collection. Vouchers of the *A. fulva* samples *BA99ES-69* and *AC01ES-12* are deposited in the Porifera collection of Museu Nacional (Rio de Janeiro), respectively, under codes MNRJ-2599 and MNRJ-4084. The taxonomic classification scheme adopted is that of Hooper and Van Soest (2002). A sample of *A. fulva* was collected at J Reef (31°36.056 N, 80°47.431 W), a hard bottom area in the South Atlantic Bight located about 32 km off the coast of Georgia (USA), in June 2005 by subsampling 100–300 g samples from large individuals. The sample was bagged, brought to the surface, and placed on ice. These samples were later placed in a −70 °C freezer until extraction. This *A. fulva* sample was identified based on morphological characteristics and thin tissue sections of fibers by Dr. Rob van Soest (University of Amsterdam).

2.2. Isolation of bromotyrosine-derived metabolites

The whole *BA99ES-69* sample (330 g, wet weight) of *A. fulva* was separated from the EtOH and blended twice in MeOH (500 mL). After filtration, both the EtOH and MeOH extracts were pooled and evaporated until the alcohol was completely removed. The remaining aqueous suspension was partitioned against EtOAc (3 × 300 mL). The EtOAc

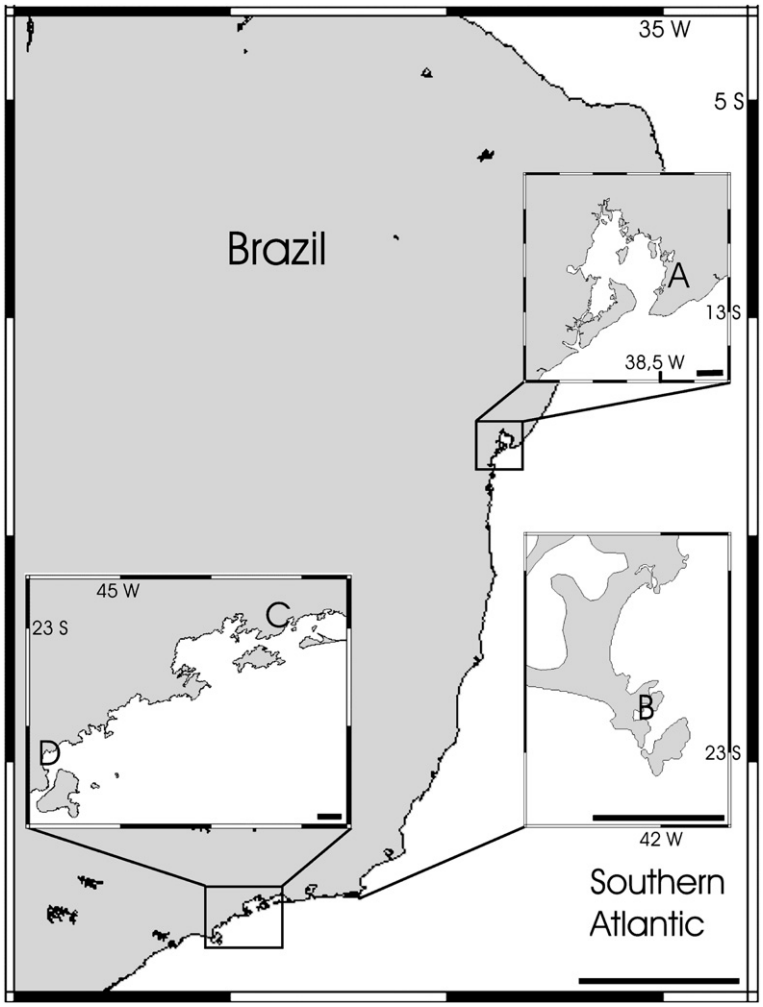


Fig. 1. Map of the Brazilian coast showing the collecting localities. Scale = 500 km. (A) Salvador and Todos os Santos Bay (BA). (B) Arraial do Cabo (RJ). (C) Angra dos Reis (RJ). (D) São Sebastião, Ilhabela and the São Sebastião Channel (SP). Scales for A–D = 10 km.

fraction was evaporated to yield 3.1 g of a brown gum. The whole EtOAc fraction (3.1 g) was subjected to several separations by flash chromatography on silica gel, with gradients of MeOH in CH_2Cl_2. Four fractions were obtained from these separations. The second fraction obtained (Af1.2, 1.546 g) was subjected to a chromatography in a Lichroprep LOBAR® (Merck, size B) SiOH column, with a gradient of MeOH in CH_2Cl_2, to yield several fractions, two of which were subsequently purified by C_{18} reversed phase HPLC (column: μBondapak C_{18} 10 μ, 125 Å, 7.8 × 300 mm; eluent: MeOH/H_2O 8:2) to yield **1** (1.1 mg, 3.3×10^{-4}%) and **2** (1.4 mg, 4.2×10^{-4}%).

Half of the *AC01ES-12* sample of *A. fulva* (545 g wet weight) was separated from the EtOH and blended twice in MeOH (4000 mL). After filtration, both the EtOH and MeOH extracts were pooled, filtered through Celite and evaporated until the MeOH was completely removed. The remaining aqueous suspension was partitioned with EtOAc (3 × 300 mL). The organic layer was evaporated, dissolved in MeOH/H_2O 9:1 and partitioned with hexanes (3 × 300 mL). The MeOH phase was evaporated to yield 4.0 g of crude extract (named AfAC). The AfAC extract was subjected to a flash chromatography on SiOH (gradient of MeOH in CH_2Cl_2), to yield nine fractions (AfAC-1–AfAC-9). Fraction AfAC-5 (0.6255 g) was subjected to a flash chromatography on SiOH with a gradient of EtOAc/MeOH 1:1 in CH_2Cl_2, to give seven fractions (AfAC-5A–AfAC-5G). The fraction AfAC-5B (0.0826 g) was purified by HPLC with a Waters μBondapak C_{18} 7.0 × 300 mm column and 6:4 MeOH/H_2O as eluent, to give 5 mg of subereatensin (**4**). The fraction AfAC-5C (0.0966 g) was separated by flash chromatography on SiOH with

a gradient of 9:1 EtOAc/MeOH in CH_2Cl_2. The second fraction obtained from this separation, AfAC-5C2 (0.0154 g), was purified by HPLC using a Waters μBondapak C_{18} 7.0 × 300 mm column and 9:1 *i*-PrOH/H_2O as eluent, flow rate 1 mL/min., to yield 0.0040 g of a mixture of compounds **2** and **3**. Fraction AfAC-5D (0.4682 g) was further separated by silica gel flash chromatography (gradient of EtOAc in CH_2Cl_2) to give five fractions (AfAC-5D1–AfAC-5D). Fraction AfAC-5D1 was purified by HPLC with a Waters μBondapak C_{18} 7.0 × 300 mm column and 7:3 MeCN/H_2O as eluent, to yield 4 mg of the mixture of compounds **2** and **5**. Fraction AfAC-5D2 was purified by HPLC using a Waters μBondapak C_{18} 7.0 × 300 mm column and 65:35 MeOH/H_2O as eluent, to give 7 mg of 5-[3,5-dibromo-4-[(2-oxo-5-oxazolidinyl)]methoxyphenyl]-2-oxazolidinone (**6**).

The *A. fulva* sample *AR02ES-01* (560 g wet weight) was separated from the acetone and blended in additional acetone (1 L). The whole acetone extract was filtered and evaporated. The residue was dissolved in 1:1 EtOAc/H_2O (500 mL). The organic layer was separated and evaporated to give 4.0414 g of the EtOAc crude extract (AfAR). The AfAR extract was divided in four portions, which were subjected to silica gel chromatography on Waters Sep Pak columns (10 g), using a gradient of EtOAc in CH_2Cl_2, then a gradient of MeOH in CH_2Cl_2. Seven fractions have been obtained from this separation (AfAR-1–AfAR-7). The fraction AfAR-3 (0.4262 g) was separated by silica gel chromatography on a Waters Sep Pak column (10 g) using a gradient of 95:5 EtOAc/MeOH in 9:1 CH_2Cl_2/hexanes as eluent, to give two fractions. The first, AfAR-3A (0.3225 g) was further separated by silica gel chromatography on a Waters Sep Pak column (10 g) with a gradient of 95:5 EtOAc/MeOH in 1:1 CH_2Cl_2/hexanes as eluent, to give four fractions. Fraction AfAR-3A4 was further separated by silica gel chromatography on a Waters Sep Pak column (10 g) with a gradient of 8:2 EtOAc/MeOH in 8:2 CH_2Cl_2/hexanes, to give four fractions. Fraction AfAR-3A4B (42 mg) was purified by HPLC using a Waters μBondapak C_{18} 7.0 × 300 mm column and 6:4 H_2O/MeOH to give 11 mg of a mixture of cavernicolin-1 (**8**) and cavernicolin-2 (**9**). Fraction AfAR-4 (245 mg) was separated by silica gel column chromatography on a Waters Sep Pak (10 g) using a gradient of 9:1 EtOAc/MeOH in CH_2Cl_2 as eluent. Three fractions have been obtained. The fraction AfAR-4B (125 mg) was separated by silica gel chromatography on a Waters Sep Pak column (10 g) using a gradient of 9:1 EtOAc/MeOH in 6:4 CH_2Cl_2/hexanes as eluent. Six fractions were obtained. The fourth, AfAR-4B4 (78 mg) was separated and purified by HPLC Waters μBondapak C_{18} 7.0 × 300 mm column, using 6:4 H_2O/MeOH, to give 14 mg of ($7S^*$,$11R^*$)-5-[3,5-dibromo-4-[(2-oxo-5-oxazolidinyl)]methoxyphenyl]-2-oxazolidinone (**6**) and 2.2 mg of fistularin-3 (**10**).

The *A. fulva* sample *SS02ES-1* (380 g wet weight) was removed from the EtOH and blended in MeOH. Both EtOH and MeOH extracts were pooled, filtered through Celite and evaporated to remove the alcohol until an aqueous suspension was obtained. The H_2O suspension was partitioned against EtOAc (3 × 400 mL) and the organic layer was evaporated until dryness. The EtOAc extract AfSS (2.9243 g) was subjected to a Sephadex LH20 column chromatography (MeOH), to give six fractions, AfSS-1–AfSS-6. Fraction AfSS-4 (0.3559 g) was separated by silica gel flash chromatography using a gradient of 85:15 EtOAc/MeOH in 8:2 CH_2Cl_2/hexanes, to give four fractions. Fraction AfSS-4B (51 mg) was purified by HPLC with a HPLC Waters μBondapak C_{18} 7.0 × 300 mm column, using 45:55 H_2O/MeOH as eluent, to give 2.5 mg of aerothionin (**11**).

The *A. fulva* sample *BA04ES-84* (65 g) was freeze dried and extracted with acetone and MeOH. The MeOH extract was concentrated until 500 mL and partitioned with *n*-pentane. The MeOH fraction was evaporated and dissolved in 3:2 CH_2Cl_2/(1:1 MeOH/H_2O). The CH_2Cl_2/MeOH organic layer was separated. The aqueous layer was further partitioned with 5:1 CH_2Cl_2/MeOH, and the organic layer was collected. Both CH_2Cl_2/MeOH layers were pooled and evaporated to give extract *AfBA04-A* (2.5 g). The acetone extract was evaporated and triturated in 1:1 EtOAc/CH_2Cl_2. The soluble fraction was evaporated to give extract *AfBA04-B* (2.64 g). The insoluble fraction was triturated in MeOH, and the MeOH soluble fraction obtained was evaporated to give the extract *AfBA04-C* (0.11 g).

The extract *AfBA04-B* (2.64 g) was divided in three portions, which were separated by silica gel chromatography on a Waters Sep Pak column (10 g) with a gradient of MeOH in CH_2Cl_2. Eight fractions have been obtained, AfBA04-B1–AfBA04-B8. Fraction AfBA04-B1 (1.521 g) was separated by silica gel column chromatography on a Waters Sep Pak column (10 g) using a gradient of CH_2Cl_2 in hexanes then a gradient of MeOH in CH_2Cl_2. Five fractions were obtained. Half of the fraction AfBA04-B1C (0,330 g) was purified by HPLC using a C_{18} Waters Deltapak 300 × 19 mm column and 75:25 MeOH/H_2O as eluent (flow rate: 2.5 mL/min), to give 3.8 mg of 11-oxoaerothionin (**12**) and 5.8 mg of 11-oxo-12-hydroxyaerothionin (**13**).

The extracts *AfBA04-A* and *AfBA04-C* were pooled (2.61 g), divided in six fractions which were subjected to Sephadex column chromatography (MeOH). Seven fractions were obtained, AfBA04-AC1–AfBA04-AC7. Fractions AfBA04-AC5 and AfBA04-AC6 were pooled (870 mg), divided in three portions which were subjected to silica

gel column chromatography on Waters Sep Pak column (10 g) with a gradient of 9:1 EtOAc/MeOH in 1:1 CH_2Cl_2/hexanes. Seven fractions were obtained. The third, AfBA04-AC56C (71 mg) was purified by HPLC using a C_{18} Waters Deltapak 300 × 19 mm column and 8:2 MeOH/H_2O as eluent (flow rate: 2.5 mL/min), to give 11 mg of fistularin-3 (**10**).

Aplysinafulvin **1**: glassy solid; $[\alpha]_D^{25} = +130^\circ$ (*c* 0.002, MeOH); UV: λ_{max}^{MeOH}/nm (log ε): 230 (3.68), 286 (3.69); CD_{MeOH} (*c* 3.56 mM) $[\theta]_{222}$ −1389, $[\theta]_{251}$ +5371, $[\theta]_{285}$ +2593; IR ν_{max}/cm^{-1}: 3438, 3343, 3194, 2932, 2834, 1665, 1616, 1585, 1436, 1406, 1293, 1217, 1160, 1103, 1078, 975, 831, 771, 716, 603; ^{1}H NMR (400 MHz, acetonitrile-d_3, δ referenced to TMS): 6.41, br s, 1H, NH; δ 6.34, d, 1.4 Hz, 1H, H-5; δ 5.90, br s, 1H, NH; δ 4.77, s, 1H, OH; 3.88, d, 1.4 Hz, 1H, H-1; 3.67, s, 3H, H_3C-10; 3.51, s, 3H, H_3C-9; 2.54, d, 14.6 Hz, 1H, H-7; 2.38, d, 14.6 Hz, 1H, H-7; ^{13}C NMR (100 MHz, acetonitrile-d_3, δ referenced to TMS): 172.8, s, C-8; 149.7, s, C-3; 140.4, d, C-5; 113.6, s, C-4; 109.1, s, C-2; 86.3, d, C-1; 76.5, s, C-6; 60.3, q, H_3C-10; 60.2, q, C-9; 42.1, t, C-7; HREIMS *m/z* $[M + Na]^+$: 393.90752 (calculated for $C_{10}H_{13}Br_2O_4Na$: 393.90836).

2-(3′,5′-dibromo-4′-hydroxyphenyl)acetamide **3**: ^{13}C NMR (100 MHz, 1:1 $CDCl_3$/MeOH-d_4, δ referenced to MeOH residual signal): 173.4, s, C-1; 150.0, s, C-6; 133.0, d, C-4 and C-8; 130.0, s, C-3; 111.0, s, C-5 and C-7; 40.0, t, C-2.

The sample of *A. fulva* (∼50 g) collected at J Reef was extracted twice in 75 mL of a 1:1 mixture of CH_2Cl_2/MeOH, followed by a third extraction in 75 mL of acetonitrile (MeCN). The extracts were combined, filtered, and evaporated to dryness. The crude extract was dissolved in a 6 mL mixture of 1:1 MeCN/H_2O and filtered through a 6 mL Phenomenex Strata-X polymeric sorbent (500 g) column with a plunger to remove contaminants. Fractions collected from this column were evaporated to dryness and dissolved in MeOH at a concentration of 45 mg/mL for analysis by liquid chromatography coupled to mass spectrometry (LC–MS). LC–MS was carried out using a Phenomenex Gemini C_{18} analytical column (4.6 × 250 mm) with a solvent gradient consisting of MeCN and H_2O buffered with 0.1% formic acid. The gradient was 90% water for the first 3 min followed by an increase in the concentration of MeCN to 100% over 28 min with a flow rate of 0.7 mL min^{-1}. Peaks were detected using an Agilent 1100 diode array detector at 254 nm and identified based on their fragmentation patterns and molecular weight in a Micromass quadrapole time-of-flight mass spectrometer using positive electrospray ionization. Once dibromotyrosine derivatives were detected by LC–MS in the J Reef sample of *A. fulva*, these compounds were isolated using a Vydac C_{18} preparative column (10 × 250 mm). Concentrated samples of dissolved crude extract were injected at a volume of 200 μL and separation was achieved using non-buffered MeCN/H_2O with the same gradient as above at 4 mL min^{-1} with monitoring at 254 and 280 nm. This method did not allow for the complete purification of individual compounds. Therefore, standards used in HPLC analyses were mixtures of known dibromotyrosine derivatives. A sample of aeroplysinin-1 was purchased commercially from Axxora for use as a standard. The LC–MS analyses of the fractions obtained indicated the presence of aerophobin-1 (**15**), aerophobin-2 (**16**), aplysinamisin-1 (**17**), aeroplysinin-1 (**18**), 11-hydroxyaerothionin (**13**), 11-oxo-12-hydroxyaerothionin (**12**), homoaerothionin (**14**), aerothionin (**10**), fistularin-3 (**9**) and 2-(3,5-dibromo-1-hydroxy-4-oxocyclohexa-2,5-dienyl)acetamide (**6**) (Table 1).

3. Results

As expected for Verongida sponges, the crude extracts of the five *A. fulva* samples collected in Brazil during this investigation (*BA99ES-69*, *AC01ES-12*, *AR02ES-01*, *SS02ES-1* and *BA04ES-84*) and a sample from J Reef in the SAB (Georgia, USA) contained dibromotyrosine-derived metabolites. The *A. fulva* sample *BA99ES-69* yielded two compounds: the new bromotyrosine derivative aplysinafulvin (**1**), which was identified by analysis of spectroscopic data (see below), and 2-(3,5-dibromo-1-hydroxy-4,4-dimethoxy-2,5-cyclohexadien-1-yl)ethanamide (**2**), identified by analysis of NMR spectra and comparison with data obtained by us for the same compound previously isolated from *Aplysina caissara* Pinheiro and Hajdu (2001) (Saeki et al., 2002).

Aplysinafulvin **1** was isolated as a glassy solid, with a molecular formula of $C_{10}H_{13}Br_2O_4$, established by HREIMS on the sodium adduct molecular ion $[M + Na]^+$ (measured: 393.90752). Analysis of spectroscopic data, including IR [3438, 3343, 3194 (ν_{O-H} and ν_{N-H}) 2932, 2834 (ν_{C-H}) 1665, 1616, 1585 cm^{-1} ($\nu_{C=O}$, amide)], UV (230 and 286 nm), a deceptively simple ^{1}H NMR (see preceding section) and the ^{13}C NMR spectrum (see preceding section), coupled with dereplication with the MARINLIT database (Munro and Blunt, 2007) indicated that **1** was new in the literature. Both ^{1}H and ^{13}C NMR data of **1** were quite similar to data reported for aeroplysinin-1 (Fattorusso et al., 1972), purealidin J (Kobayashi et al., 1995) and an unnamed *Pseudoceratina* sp. compound (Aiello et al., 1995). Indeed, the ^{1}H

Table 1
Occurrence of dibromotyrosine compounds in Brazilian and US samples of *Aplysina fulva*

Compound isolated	*Aplysina fulva* samples					
	BA99ES-69	*AC01ES-12*	*AR02ES-01*	*SS02ES-1*	*BA04ES-84*	J Reef − SAB
Aplysinafulvin (**1**)	×					
Compound **2**	×	×				
Compound **3**		×				
Subereatensin (**4**)		×				
Mixed ketal **5**		×				
Oxazolidinone **6**		×	×			
Cavernicolin-1 (**7**)			×			
Cavernicolin-2 (**8**)			×			
Fistularin-3 (**9**)			×		×	×
Aerothionin (**10**)				×		×
11-Oxoaerothionin (**11**)					×	
11-Oxo-12-hydroxyaerothionin (**12**)						×
11-Hydroxyaerothionin (**13**)						×
Homoaerothionin (**14**)						×
Aerophobin-1 (**15**)						×
Aerophobin-2 (**16**)						×
Aplysinamisin-1 (**17**)						×
Aeroplysinin-1 (**18**)						×
Acetamide **19**						×

and ^{13}C chemical shifts observed for **1** are typical for 2,4-dibromo-1,6-dihydroxy-3-methoxycyclohexa-2,4-diene moiety frequently present in bromotyrosine-derived compounds isolated from Verongida sponges. Analysis of the HMQC and HMBC confirmed our hypothesis. In the HMBC spectrum, we observed the presence of a two proton AB system at δ 2.54 (d, 14.6 Hz) and 2.38 (d, 14.6 Hz) coupled with the carbonyl group at δ 172.8 (C-8) and with the quaternary carbon at δ 76.5 (C-6). Considering these long-range couplings and the IR absorptions at 3438, 3343, 3194 and 1665, 1616, 1585 cm^{-1}, we have been able to establish the position of the acetamide group attached to C-6. The position of the methoxy group at C-1 was established by a long-range coupling observed between the methyl hydrogens (δ 3.51) and C-1 (86.3), in the HMBC spectrum. The carbon C-1 was directly coupled with the doublet hydrogen at δ 3.88 (H-1). Additional long-range couplings observed between H-1 and C-2, C-3, C-5, C-6, C-7 and C-9, between H-5 and C-1, C-3 and C-4, as well as between the hydrogens of H_3C-10 and C-3, enabled us to completely define the planar structure of aplysinafulvin **1**. The absolute stereochemistry of **1** was determined by analysis of the ^1H NMR and circular dichroism spectra. The hydrogens H-1 and H-5 exhibited a W long-range coupling ($J = 1.4$ Hz), which is only possible if the substituents at C-1 and C-6 present a *trans* pseudo-diaxial relative stereochemistry (Fulmor et al., 1970). Since the circular dichroism spectrum of **1** in MeOH presented a positive Cotton effect, the absolute configuration of C-1 and C-6 are 1(*R*), 6(*S*), based on the previous circular dichroism analyses of related bromotyrosine-derived metabolites (Lira et al., 2006). Aplysinafulvin did not display cytotoxic activity against a panel of four human cancer cell lines. To the best of our knowledge, this is the first report of a Verongida bromotyrosine compound with a methoxy group attached to C-1.

The *A. fulva* sample *AC01ES-12* gave a mixture of **2** and 2-(3′,5′-dibromo-4′-hydroxyphenyl)acetamide (**3**), subereatensin (**4**), the mixed ketal **5** as well as 5-[3,5-dibromo-4-[(2-oxo-5-oxazolidinyl)]methoxyphenyl]-2-oxazolidinone (**6**). Identification of the mixture of **2** and **3** was possible by a detailed analysis of spectroscopic data and by comparison with literature data (Chib et al., 1978; Cruz et al., 1990; Sharma et al., 1970). Although no ^{13}C NMR data of **3** have been reported to date (Chib et al., 1978), we have been able to assign all carbon signals observed in the ^{13}C NMR spectrum of the mixture of **2** and **3** by a careful analysis of the direct and long-range couplings observed in the HSQC and in the HMBC NMR spectra. The ^{13}C NMR assignments of **3** are reported in the material and methods section. Subereatensin (**4**) was identified by analysis of spectroscopic data and comparison with literature data (Kijjoa et al., 2002). The mixed ketal **5** was also identified by analysis of spectroscopic data and comparison with literature data (Andersen and Faulkner, 1973). 5-[3,5-Dibromo-4-[(2-oxo-5-oxazolidinyl)]methoxyphenyl]-2-oxazolidinone (**6**) was identified by analysis of spectroscopic data and comparison with data obtained for the same compound recently isolated by us from the ascidian *Clavelina oblonga* (Kossuga et al., 2004).

The *A. fulva* sample *AR02ES-01* yielded the (7*S**,11*R**)-5-[3,5-dibromo-4-[(2-oxo-5-oxazolidinyl)]methoxyphenyl]-2-oxazolidinone (**6**), a mixture of cavernicolin-1 (**7**) and cavernicolin-2 (**8**), and fistularin-3 (**9**). (7*S**,11*R**)-5-[3,5-Dibromo-4-[(2-oxo-5-oxazolidinyl)]methoxyphenyl]-2-oxazolidinone (**6**) was identified by analysis and comparison of spectroscopic and $[\alpha]_D$ data (Kossuga et al., 2004). The relative stereochemistry of (7*S**,11*R**)-5-[3,5-dibromo-4-[(2-oxo-5-oxazolidinyl)]methoxyphenyl]-2-oxazolidinone **6** is herein proposed by comparison of its $[\alpha]_D$ value (+9.3°, *c* 0.9, MeOH) with the $[\alpha]_D$ value measured for the same compound isolated from the ascidian *C. oblonga* (Kossuga et al., 2004). Since both values are practically identical in magnitude, but have opposite signs, we propose the inverse relative configuration for compound **6** isolated from the sample *AR02ES-01* of *A. fulva*. Compounds **7** and **8** were identified by analysis of spectroscopic data and comparison with literature data (D'Ambrosio et al., 1982), fistularin-3 (**9**) was identified by analysis of spectroscopic data and comparison with literature data (Rogers et al., 2005).

The *A. fulva* sample *SS02ES-1* gave aerothionin (**10**), which was identified by analysis of spectroscopic data and by comparison with literature data (Moody et al., 1972; Fattorusso et al., 1970; McMillan et al., 1981; Rotem et al., 1983).

The *A. fulva* sample *BA04ES-84* yielded fistularin-3 (**9**), 11-oxoaerothionin (**11**) (Acosta and Rodriguez, 1992) and 11-oxo-12-hydroxyaerothionin (**12**) (Ciminiello et al., 1994), all compounds were identified by analysis of spectroscopic data and comparison with literature data.

The sample of *A. fulva* collected at J Reef in the SAB, Georgia, USA yielded 10 dibromotyrosine derivatives, identified by LC-MS analysis: fistularin-3 (**9**), aerothionin (**10**), 11-oxo-12-hydroxyaerothionin (**12**), 11-hydroxyaerothionin (**13**), homoaerothionin (**14**), aerophobin-1 (**15**), aerophobin-2 (**16**), aplysinamisin-1 (**17**), aeroplysinin-1 (**18**), and 2-(3,5-dibromo-1-hydroxy-4-oxocyclohexa-2,5-dienyl)acetamide (**19**).

4. Discussion

Our investigation on the occurrence of bromotyrosine derivatives within the marine sponge *A. fulva* indicated that, contrary to the results obtained by Kelecom and Kannengiesser (1979), these metabolites are present in Brazilian samples of this sponge. In addition, this also represents the first report of these metabolites in *A. fulva* sponges from reefs of the SAB. Our results support previous observations that dibromotyrosine-derived metabolites are recurrent in the Verongida, where such compounds are known from all species investigated until the present. Parallel occurrences achieved via diverse factors such as evolutionary convergence, lateral gene transfer, symbiont migration or host switch, are known from *Agelas*, *Dysidea*, *Iotrochota*, *Jaspis*, *Oceanapia* and *Poecillastra* species, spreading five orders across two demosponge subclasses. We believe that Kelecom and Kannengiesser have been unable to isolate or detect such metabolites in the samples of *A. fulva* collected in 1979 due to lack of sensitive methods for isolation and identification of secondary metabolites, unavailable in Brazil at that time.

Previous investigations on the chemistry of *A. fulva* collected in the Caribbean indicated the occurrence of fistularins-1, 2 and 3 (**9**), aerothionin (**10**), 5-[3,5-dibromo-4-[(2-oxo-5-oxazolidinyl)]methoxyphenyl]-2-oxazolidinone (**6**) from a sample of this sponge from the Virgin Islands (Gopichand and Schmitz, 1979), aplysillin A from *A. fulva* collected in the Bahamas (as *A. fistularis fulva*, Gulavita et al., 1995), and a series of 11 dibromotyrosine-derived metabolites also isolated in samples from the Bahamas (Ciminiello et al., 1994; as *A. fistularis* forma *fulva*). Ciminiello et al. (1996b), further reporting on material from the Bahamas, isolated aerothionin (**10**), fistularin-1, fistularin-3 (**9**), aeroplysinin-2, debromoverongiaquinol, 2-(2,4-dibromo-3,6-dihydroxyphenyl)acetamide, homoaerothionin (**14**), aeroplysinin-1 (**18**), 11-hydroxyaerothionin (**13**), 11-ketoaerothionin (**11**), (12*S*)-11-keto-12-hydroxyaerothionin and (12*R*)-11-keto-12-hydroxyaerothionin (both of which are herein indicated as **12**). Moreover, a recent investigation on *A. fulva* from Key Largo, Florida gave two new highly polar dibromotyrosine derivatives (Rogers and Molinski, 2007).

These results differ from ours, since the Brazilian specimens of *A. fulva* yielded metabolites **1**–**5**, **7** and **8**, previously not reported from this species. Nevertheless, compounds **2** and **5** may be considered as artifacts of isolation of MeOH and EtOH addition on 2-(3,5-dibromo-1-hydroxy-4-oxocyclohexa-2,5-dienyl)acetamide (**19**), a compound previously isolated from *A. fulva* (Ciminiello et al., 1994, 1996a,b) and also present in the *A. fulva* sample collected at J Reef (Georgia, USA) herein investigated. Noteworthy is the isolation of compound **1**, which is a new metabolite for the Verongida. Compound **3** has been previously isolated only from *Aplysina archeri* (as *Verongia archeri*) (Chib et al., 1978), while subereatensin (**4**) was known only from *Suberea* aff. *praetensa* (Family Aplysinellidae) (Kijjoa et al., 2002, 2004). Although subereatensin is not derived from a brominated tyrosine, an elegant biogenetic pathway

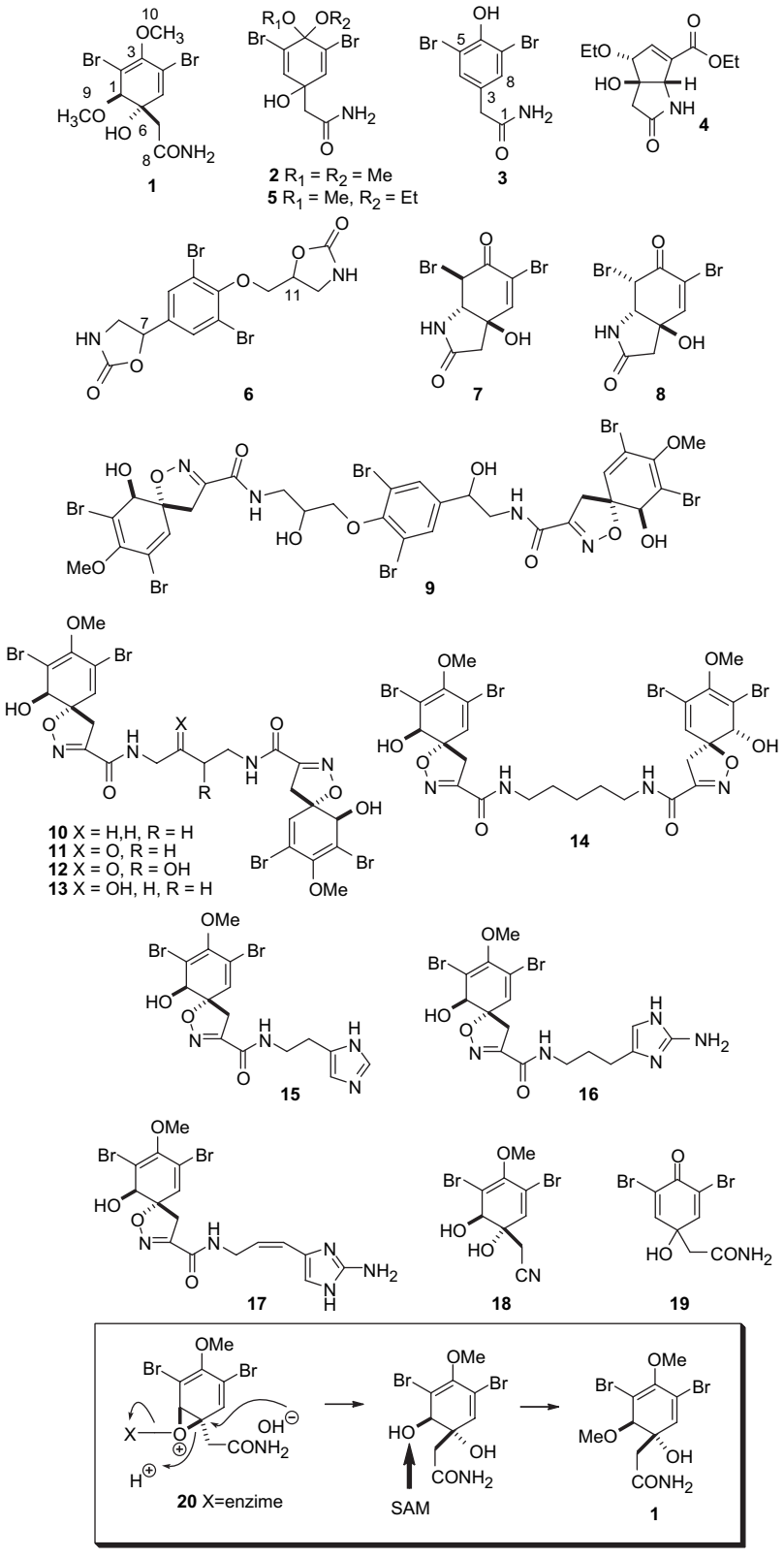
from tyrosine has been proposed for **4** (Kijjoa et al., 2004). Compounds **7** and **8** were known from *Aplysina cavernicola* Vacelet 1959 and have been previously isolated only once (D'Ambrosio et al., 1982). The isolation of compounds **7** and **8** as an epimeric mixture has been previously reported (D'Ambrosio et al., 1982), since both compounds appear to equilibrate to a 3:1 mixture during the chromatographic isolation. Compounds bearing similar dibrominated enone moieties have also been isolated as mixtures from the sponges *A. oroides* (König and Wright, 1993), *S.* aff. *praetensa* (Kijjoa et al., 2002 and 2004), *Aplysina laevis* (Capon and MacLeod, 1987), *A. archeri* (Ciminiello et al., 1996a) and *A. caissara* (Lira et al., 2006). Closely related metabolites have been also isolated from the acorn worm *Ptychodera* sp. (Higa et al., 1987). Compounds **9**–**14**, **18** and **19** have all been previously isolated from *A. fulva* (as *A. fistularis* forma *fulva*; Ciminiello et al., 1994). Aerophobin-1 (**15**) and aerophobin-2 (**16**) have been previously isolated from *Aiolochroia crassa* Hyatt, 1875 (Assmann et al., 1998), while aplysinamisin-1 (**17**) was isolated from *Aplysina cauliformis* Carter, 1882 (Rodriguez and Piña, 1993).

Investigations on the variability of secondary metabolites within a sponge species have not been frequently reported. For instance, it has been observed that the brominated pyrrole alkaloids hymenialdisine, debromohymenialdisine, dibromophakellin and 3-bromohymenialdisin occur in a very similar relative proportion within specimens of the sponge *Axinella carteri* Dendy, 1889 collected in sites distant as far as 2000 km (Supriyono et al., 1995). The composition of diterpenes within the sponge *Rhopaloeides odorabile* Thompson, Murphy, Bergquist and Evans, 1987 varies according to environmental conditions such as light intensity and depth (Thompson et al., 1987).

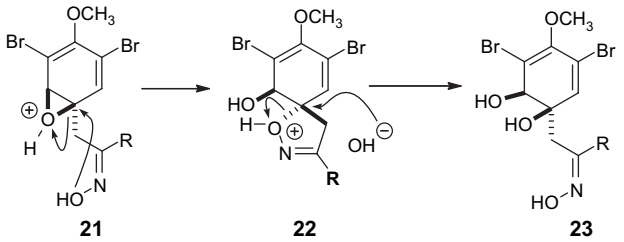
In the case of Verongida sponges, previous studies have suggested that debromoverongiaquinol and aeroplysinin-1 (**18**) are formed through the enzymatic degradation of iso-fistularin-1 and aerophobin-2 (**16**) after injury of *Aplysina aerophoba* Nardo 1843 in aquarium (Weiss et al., 1996). Debromoverongiaquinol is also obtained by exposure of aeroplysinin-1 in alkaline sea water or by extracting frozen-stored sponge specimens (Ebel et al., 1997; Weiss et al., 1996). Specimens of *A. aerophoba* collected in the shallow waters of the Adriatic yielded both debromoverongiaquinol and aeroplysinin-1, while deeper water specimens yielded only debromoverongiaquinol. The biosynthetic capability of the deeper water sample of *A. aerophoba* to produce aeroplysinin-1 is recovered after a few hours in an aquarium. Analysis of the sponge tissues demonstrated that exposed cells contain greater amounts of aeroplysinin-1, while internal, protected tissues, present increased amounts of debromoverongiaquinol (Ebel et al., 1997). Extracts obtained from *A. aerophoba* with damaged tissues present a very strong deterrent activity against the fish *Thalassoma bifasciatum* (Ebel et al., 1997). Both debromoverongiaquinol and aeroplysinin-1 display cytotoxic, algicide and antibacterial activity, while the higher-molecular weight iso-fistularin-3 and aerophobin-2 are biologically inactive in such bioassays (Koulman et al., 1996; Weiss et al., 1996). The results observed indicated that debromoverongiaquinol and aeroplysinin-1 are formed by the sponge from iso-fistularin-3 and aerophobin-2 under stressing conditions, such as phytoalexins in plants (Bailey and Mansfield, 1982). These results have been recently confirmed (Thoms et al., 2006). Nevertheless, distinct results have been obtained by similar investigations carried out with Caribbean samples of *Aplysina insularis* Duchassaing and Michelotti, 1864 and *A. archeri* (Puyana et al., 2003). Analysis by HPLC–MS indicated no changes in the composition of crude extracts obtained from both sponges, either after short (2.5 min) or long (30 and 120 min) periods after injury. The authors measured the concentration changes of debromoverongiaquinol, aeroplysinin-1 (**18**), 11-hydroxyaerothionin (**13**), 11-keto-12-hydroxyaerothionin (**12**), homoaerothionin (**14**), aerothionin (**10**) and fistularin-3 (**9**).

In a parallel investigation on *A. fulva* crude extracts, we observed variations on stereoisomer composition of fistularin-3 (**9**) within the tissues of a single specimen of this sponge (Rogers et al., 2005). Similar observations have been reported in the literature. Terpenes isolated from the sponge *Dysidea herbacea* Keller, 1889 presented variable enantiomeric composition depending on the different geographic locations where specimens were collected (Horton et al., 1990). Related results have been obtained for other metabolites of *D. herbacea* (Molinski and Ireland, 1988; Salomon et al., 1995; Searle and Molinski, 1994), as well as for the composition of terpenes and brominated indole alkaloids of the marine bryozoan *Flustra foliacea* (Peters et al., 2004). Therefore, it seems clear that the secondary metabolite composition of sponge tissues and other marine invertebrates can be influenced by multiple factors, such as depth of collection, light intensity, nutrient availability, exposure to predation, association with or infection by microorganisms, the biosynthetic machinery of a single individual, or even by the chemical procedures used in extractions and purifications.

Our isolation of aplysinafulvin (**1**) raises interesting questions about the biosynthesis of bromotyrosine-derived secondary metabolites of Verongida sponges. Considering that the sample *BA99ES-69* of *A. fulva* studied in the present work was stored in EtOH, it is worthwhile to note that compound **1** presented only methoxy groups, but no ethoxy



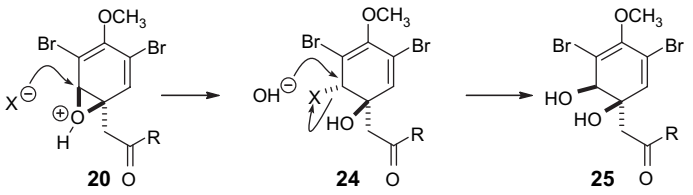
Scheme 1. SAM: *S*-adenosylmethionine.



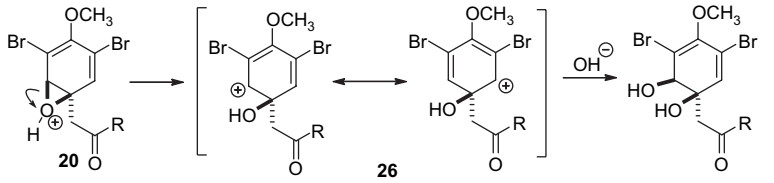
Scheme 2. R = $CONH_2$ or CO_2H.

groups. Since we have been unable to detect related derivatives of compound **1** with ethoxy groups in our samples of *A. fulva*, it is quite possible that compound **1** is a true secondary metabolite rather than an artifact of isolation. The formation of alkoxy and dialkoxy ketals on Verongida metabolites has been of concern for more than 30 years of research on Verongida sponges, since these functional groups are assumed as artifacts of isolation (Andersen and Faulkner, 1973; Kelecom and Kannengiesser, 1979; Minale, 1976). The cyclohexa-2,4-diene system of **1** may arise via the S_N2 attack of MeOH on the arene oxide **20**, but would yield a racemic mixture instead of the optically active **1**. On the other hand, an enzymatically-mediated overture of epoxide **20** by a hydroxyl group would yield an optically active *trans* disubstituted dihydroxy derivative. The dihydroxy intermediate may subsequently incorporate a methyl group via *S*-adenosylmethionine in the hydroxy position attached to C-1, giving aplysinafulvin **1** or its enantiomer (Scheme 1). The overture of the epoxide function may also proceed via an acid catalyzed intramolecular S_N2 attack of the hydroxylamine group as in Scheme 2 (Cruz et al., 1990; Kelecom and Kannengiesser, 1979; Minale et al., 1976). The resulting 1-hydroxy-spiro system **22** is very often found in Verongida sponges (Blunt et al., 2007; Faulkner, 2002). A subsequent overture of the spiro bicyclic system in **22** by a hydroxy group would yield a *cis* dihydroxy derivative such as **23**, instead of the *trans* system observed in **1** (Scheme 1). Therefore, the pathway shown in Scheme 2 seems to be incompatible with the formation of aplysinafulvin. The same result would be observed in the case of the overture of the epoxide ring of **20** by a halogen (X = Cl or Br), giving a haloidrin intermediate (**24**), which would suffer a subsequent S_N2 substitution by a hydroxy group, also leading to a *cis* product (**25**, Scheme 3). Another possible pathway is the formation of a resonance stabilized carbocation (**26**) as in Scheme 4, which can be attacked by a hydroxy nucleophile via S_N1 in either one of the two faces. However, in this case the attack in the face of the acetamide (or acetic acid) substituent will not be favoured by steric hindrance and a *cis* dihydroxy substituted product is expected as well. Thus, a carbocation pathway toward the formation of **1** may also be ruled out. Therefore, the stereoselective overture of an arene oxide intermediate **20** by a direct attack of a hydroxy group seems to be a more plausible biogenetic pathway for the formation of **1** (Scheme 1), as well as for the related formation of the spiroisoxazoline carboxamide moiety frequently found in the structures of several dibromotyrosine-derived metabolites (Rogers and Molinski, 2007). The intermediate **20** can also account for the formation of 3,3-dialkoxyderivatives probably isolated as artifacts (Andersen and Faulkner, 1973), *via* an acid catalyzed S_N' attack such as in Scheme 5. Finally, a dehydration of a reactive intermediate would give the completely aromatized system, as in **3**. A structurally related arene oxide intermediate has been proposed in the biogenetic pathway for the formation of related dibrominated metabolites isolated from the acorn worm *Ptychodera* sp. (Higa et al., 1987).

Lastly, the question of whether bromotyrosine-derived alkaloids are produced by sponge tissues or by bacterial symbionts remains unanswered. Pioneering studies by Thompson et al. (1983) demonstrated that the sponge *A. fistularis* accumulates aerothionin (**10**) and homoaerothionin (**14**) in spherulous cells. Related results have been recently



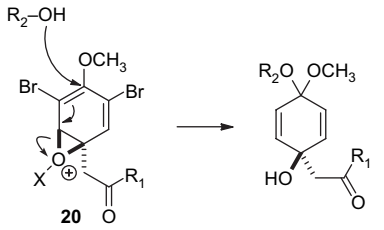
Scheme 3. R = OH or NH_2, X = Cl or Br.



Scheme 4. R = OH or NH_2.

reported for the localization of brominated metabolites within the spherulous cells of *A. aerophoba* (Turon et al., 2000). Although it seems unlikely that metabolites produced by associated microorganisms would be translocated to the host cells, the fact that secondary metabolites are located in sponge cells do not constitute a proof for the actual site where the biosynthesis is achieved. It is known that *A. aerophoba* and *A. cavernicola* possess a high density of a diversified bacterial population (Vacelet, 1975; Friedrich et al., 1999, 2001; Hentschel et al., 2002; Wehrl et al., 2007). These bacteria inhibit the growth of terrestrial Gram negative and Gram positive bacteria, including antibiotic-resistant strains. *A. cavernicola* presents four main bacterial types: *Plactomyces* sp., δ-*Proteobacteria* sp., γ-*Proteobacteria* sp. and *Bacteroides* sp. (Friedrich et al., 1999, 2001; Hentschel et al., 2002), as well as Cyanophyceae and microalgae (Vacelet, 1975). Considering the high bacterial density in *A. cavernicola* tissues, the hypothesis cannot be discarded that one or several of these bacterial strains participate in the biosynthesis of dibromotyrosine-derived secondary metabolites (Friedrich et al., 1999). On the other hand, the bacterial community of the sponges *A. aerophoba* and *Theonella swinhoei* Gray, 1868 are strikingly similar (Friedrich et al., 2001), even though these sponges are taxonomically unrelated, are geographically isolated from each other, and produce secondary metabolites belonging to completely distinct structural classes (*T. swinhoei* specimens typically yield modified peptides and polyketides). Additionally, anaerobic microorganisms associated with *A. aerophoba* promote debromination of aromatic substrates under methanogenic or sulfidogenic conditions (Ahn et al., 2003). Therefore, if associated microorganisms are implied in the biosynthesis of secondary metabolites isolated from Verongida and other sponges, the biochemical expression of microbes may be the result of a long-period of host/parasite association, resulting in very specific metabolite production depending on the species to which the microorganisms are associated. This yet unresolved *scenario* indicates that the biosynthesis of marine invertebrate secondary metabolites, including Verongida bromotyrosine-derived metabolites, justifies further study.

The distinctive chemistry of the *A. fulva* populations in the present investigation suggests that a closer inspection of the morphological plasticity in *A. fulva* may be required. In a recent monographic study of Brazilian *Aplysina*, where a large number of new species were described, Pinheiro et al. (2007) indicated that *A. fulva* may be the least well characterized of all *Aplysina* species in the Tropical western Atlantic. There is a considerable morphological diversity among *A. fulva* specimens along the Brazilian coast, with specimens ranging from variously long single digits, to large bushes where dozens of such digits can be counted. Additional morphotypes include palmate, slightly lamellar, mostly rather irregular forms, as well as repent individuals. Live colour is more frequently a yellowish light-brown (ochre), but this varies also, and specimens located off direct sunlight are most frequently of a bright lemon-yellow colour. In principle, neither Muricy and Hajdu (2006), nor Pinheiro et al. (2007) were capable of distinguishing clear diagnostic features, which permitted the recognition of further distinct *A. fulva* like species along the Brazilian coast. The fascinating chemistry and biology of Verongida sponges clearly deserves additional investigations in order to clarify the origin, biosynthesis and functions of bromotyrosine-derived secondary metabolites, and the considerable richness of



Scheme 5. R_1 = OH or NH_2, R_2 = Me or Et, X = enzyme.

this genus reported by Pinheiro et al. (2007) for the Brazilian coast clearly indicates this as a priority location for pursuing these goals.

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