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Chemistry of Verongida Sponges. VII[1] Bromocompounds from the Caribbean Sponge *Aplysina archeri.*

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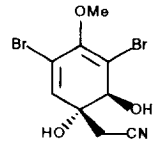
Abstract: A detailed analysis of the secondary liposoluble metabolites of the Verongida sponge *Aplysina archery* has been performed. Ten bromotyrosine derivatives have been identified of which one, **1**, is a novel compound. Structure of **1** has been assigned on the basis of spectroscopic evidence including 2D-NMR experiments. Absolute configuration has been suggested by helicity rule.

Secondary metabolism of Verongida sponges is characterized by typical bromocompounds biogenetically related to tyrosine. All the species belonging to this order so far examined have shown to contain remarkable quantities of such metabolites, whose structures in most cases comprise the unique dibromospyrocyclohexadienyldihydroisoxazole moiety.

On account of the quite interesting bioactivity generally exhibited by these compounds and with the aim of utilizing them as chemotaxonomic markers, we are currently examining a number of Verongida species collected in the Caribbean area during two expeditions in Summer 1990 and 1992. We report here the results obtained from the chemical analysis of a Verongida sponge coming from Bahama Islands: *Aplysina archeri* (Higgin, 1875) belonging to the family Aplysinidae Carter, 1875. The species forms tubes of large size which can grow singly or in clusters. The color of *A. archeri* in life is purplish or vermillion-red outside and light-beige inside and, unlike most other *Aplysina*, it does not darken in the preserved specimens. The consistency of our specimens is soft and elastic, but it seems to exist also a hard form. *A. archeri* is a typical West-Indian species (found in Curaçao, Yucatan, Florida, Jamaica and Bahamas) growing in reef habitat at 2-40 m depth. The three studied specimens (PSS-2105, SS-1606, GB-2505) collected in July 1990 along the coasts of Little San Salvador, San Salvador and Grand Bahama Islands, respectively, at 15-20 m depth, were stored frozen at -20°C until extraction. Sub-samples were incorporated in the collection of the Istituto di Zoologia dell' Università di Genova under the same reference numbers.

The following data are reported on the secondary metabolite composition of this species. In 1981 Makarieva et al.[2], by studying several Verongida from cuban coasts, analyzed two specimens of *A. archeri*: the first, collected at Punta del Este, contained aeroplysinin-2 (**3**) and the dienone **9**[3], while a larger variety of brominated metabolites was shown to be elaborated by the second specimen, coming from Havana, containing, in addition to **3** and **9**, aeroplysinin-1 (**2**), and the oxazolidone **10**. More recently[4] also fistularin-3 (**6**) and lesser quantities of 11-ketofistularin-3 (**11**) were isolated from a Caribbean specimen of *A. archeri*.



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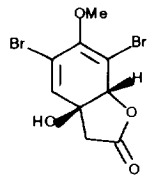

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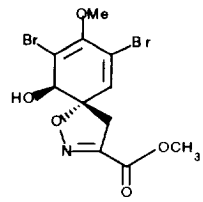

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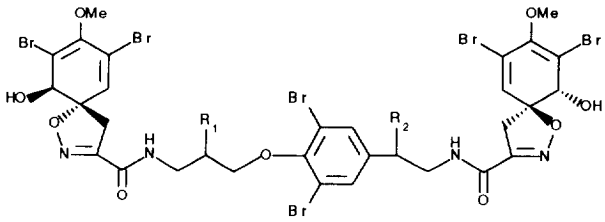
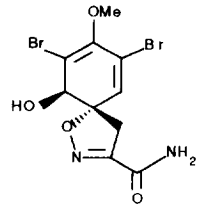


5 $R_1 = R_2 = H$

6 $R_1 = R_2 = OH$

11 $R_1 = O$ $R_2 = H$



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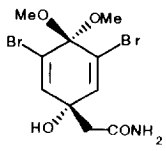

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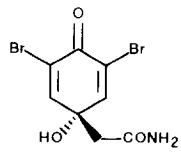

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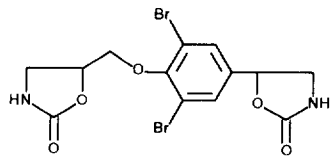

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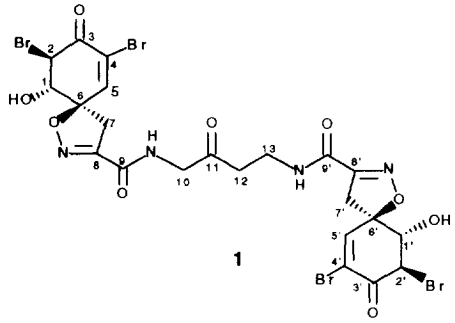
On the basis of a preliminary analysis, our three specimens of *A. archeri* were shown to contain the same secondary metabolites in comparable amounts and therefore an extended analysis was performed only on the larger sample SS-1606. Our investigation has been at moment limited to the less polar compounds present in ethyl acetate solution. It confirmed that compounds **2**, **3**, **6** and **9** are major metabolites of *A. archeri*. In addition our sponge elaborates the bromometabolites **4**, **5** and **7** previously isolated from other Verongida sponges. Remarkable quantities of the new compound **1** were also isolated and this paper describes its structure determination.

Compound **1** is a major metabolite of the sponge; its molecule contains two units quite similar to the usual dibromospyrocyclohexadienyldihydroisoxazole fragments differing from them in having a cyclohenone instead of cyclohexadienyl ring. This structural feature, which is unique among the bromometabolites from Verongida sponges, has been previously found only in Agelorin A and B, two metabolites recently isolated from *Agelas*

oroides[5], a sponge belonging to the order Agelasidae. Compound **1**, tested for antimicrobial activity, resulted to have an antifungal activity. In fact it has a Mic of 64 μg/ml on *Criptococcus neoformans* ATCC90113.



1

Specimens of *A. archeri* were extracted first with a 3:1 mixture of MeOH/toluene and successively with chloroform. The lipophilic material from both extracts was roughly partitioned by SiO_2 column chromatography; selected fractions were successively purified by direct-phase HPLC, affording **1** (1.24% of dry sponge after extraction), **2** (0.21%), **3** (0.26%), **4** (0.088%), **5** (0.18%), **6** (0.44%), **7** (3.1%) and **8** (8.8%) as amorphous solids. Compounds **2**[6], **3**[7], **4**[8], **5**[9], **6**[10], **7**[8] and **8**[11] were identified by comparison of their spectral properties with those reported in the literature.

The fabms of compound **1** showed a 1:4:6:4:1 quintet for the pseudomolecular ion peak $(M+H)^+$ at *m/z* 801, 803, 805, 807 and 809, indicative of the presence of four bromine atoms in the molecule, which was appropriate for the molecular formula $C_{22}H_{20}N_4O_9Br_4$. The UV spectrum (λ_{max} = 252 nm, ε = 7850) indicated the presence of an enone chromophore, while the IR absorption bands showed the presence of alcohol (3360 cm^{-1}), ketone (1713 cm^{-1}), conjugated ketone (1705 and 1600 cm^{-1}) and α-iminoamide (1660 cm^{-1}) groups in the molecule. Both ^1H and ^{13}C-NMR spectra showed a series of split signals, which suggested the presence in **1** of two identical part structures not symmetrically included in the molecule. This is a structural feature commonly found in several Verongida metabolites possessing two spyrocyclohexadienyl units; in our metabolite, however, preliminary consideration of spectral data pointed to a structurally slightly different unit.

Decisive information on the structure of **1** was obtained through some 1D and 2D NMR experiments performed in $(CD_3)_2CO$ on a Bruker AMX-500 spectrometer equipped with a X32 computer using a UXNMR software package. Consideration of the characteristics of the ^1H-NMR spectrum of **1** and ^1H-^1H connectivities observed in a homonuclear shift correlated 2D experiment (COSY) spectrum showed that all the protons in **1** belong to eight isolated spin systems. With the help of HETCOSY data (all the proton bearing carbons were matched) the eight units were confidently identified as the part structures HO-C1-C2 (HO-C1'-C2'), C5 (C5)', C7 (C7)', HN-C10, C12-C13-NH. A long-range carbon-proton correlation experiment (COLOC) together with a series of interproton contacts observed in a ROESY spectrum allowed the above part structures to be combined through the unprotonated carbon atoms C6 (C6') and C11 and through the segments C3-C4 (C3'-C4'), C8-C9 (C8'-C9'). Particularly the following correlations were found to be decisive for assigning structure **1** to the compound under investigation. The methylene hydrogens at C7 (C7') were coupled with the carbons C5 (C5') and C8 (C8'), while long range couplings were observed between H5/C6

(H5'/C6') and H5/C4 (H5'/C4'). These data and the interproton contacts H5/H7$_a$ (H5'/H7$_a$') and H1/H7$_b$ (H1'/H7$_b$') defined the structure of the two dibromospirocyclohexenonyldihydroisoxazole rings. Finally the connection of the segments HN-C10 and C12-C13-NH through an unprotonated carbon atom was evidenced by the long range coupling $H_2$12/C10 observed in the COLOC spectrum and by an intense cross peak $H_2$10/$H_2$12 present in the ROESY spectrum.

Table 1. ^{13}C and ^{1}H Assignment for Compound **1** (CD_3COCD_3)[a]

Carbon	δ_C, mult.		δ_H (mult.*J*/Hz)	
1, 1'	74.73 d	74.78 d	4.38 (dd, 5, 12)	4.40 (dd, 5, 12)
2, 2'	57.11 d	57.07 d	5.08 (d, 12)	5.06 (d, 12)
3, 3'	183.51 s	183.51 s		
4, 4'	122.45 s	122.36 s		
5, 5'	149.09 d	149.17 d	7.63 (s)	7.67 (s)
6, 6'	91.67 s	91.42 s		
7a, 7'a	38.24 t	38.10 t	3.31 (d, 18)	3.29 (d, 18)
7b, 7'b			3.85 (d, 18)	3.86 (d, 18)
8, 8'	154.53 s	154.27 s		
9, 9'	159.76 s	159.64 s		
10	49.17 t		4.22 (d, 6)	
11	204.70 s			
12	39.63 t		2.87 (t, 6)	
13	34.78 t		3.58 (dt, 6, 6)	
NH-9			7.90 (t, 6)	
NH-9'			7.66 (t, 6)	
OH-1, 1'			6.03 (d, 5)	6.08 (d, 5)

[a] Assignment based on DEPT, COSY, HetCOSY and COLOC experiments.

The relative configurations of the chiral centers C1(C1'), C2(C2') and C6(C6') were easily established taking into account the large *J* value H1/H2 (H1'/H2') in the ^{1}H-NMR spectrum (12 Hz) indicative of their *pseudo*-axial orientation and from a series of interproton contacts observed in the ROESY spectrum, particularly those correlating H2/H7$_b$ (H2'/H7$_b$') and OH/H7$_b$ (OH/H7$_b$'); they appeared highly diagnostic in the light of the conformation which the dibromo-spirocyclohexenonyldihydroisoxazole ring preferentially assumes as suggested by a molecular mechanics analysis. Indeed energy minimization calculations on this substructure in **1** revealed that the carbocyclic ring adopts almost exclusively the half-chair like conformation reported in figure 1, which fully agrees with the observed vicinal coupling constant H1/H2 (H1'/H2') (the calculated energy of the next preferred conformation is higher by 2.7 Kcal/mol). All the above data established the relative stereochemistry of the two external cyclic part structures in **1**. The absolute configurations of the chiral centers of the two cyclohexenone rings and therefore of the whole molecule was suggested by its CD spectrum, which showed a positive ellipticity ($\Delta\varepsilon_{252}$ = +1.6) associated with the enone chromophore. This chiroptical property first of all excludes that the two units could be enantiomeric. Moreover, taking into account that the helicity rule can be applied to α,β-unsaturated ketones, it provides evidence that the enone components in both terminal units are positively skewed[12,13]. Inspection of this steric feature in the molecular models of the preferred conformations assumed by the two alternative enantiomeric cyclohexenone rings pointed to the absolute stereochemistry reported in formula **1**. A stereoview of the spirocyclohexenonyldihydroisoxazole moiety having the assigned stereochemistry is reported in figure 2; the calculated value of the dihedral angle O-C3-C4-C5 is 167.5°.

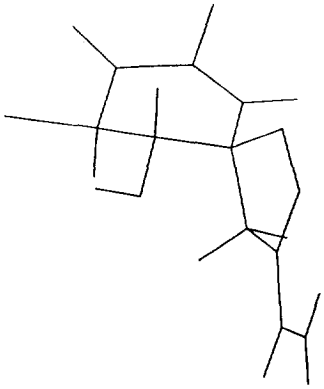


Figure 1. The most stable conformer of the spirocyclohexenonyldihydroisoxazole moiety of **1** obtained from molecular mechanics and dynamic calculations.

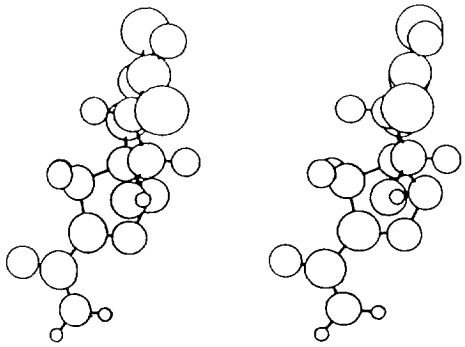


Figure 2. Stereoview of the lowest energy conformation of the spirocyclohexenonyldihydroisoxazole moiety of compound **1**.

EXPERIMENTAL

General methods. ^1H and ^{13}C NMR spectra were determined on a Bruker AMX-500 spectrometer and the solvent was used as an internal standard (CD_3COCD_3: ^1H δ 2.05; ^{13}C δ 205.4 and 30.5). Methyl, methylene and methine carbons were distinguished by a DEPT experiment. FABMS were obtained at 70eV on a Kratos MS50 mass spectrometer. Ft-IR spectra were recorded on a Bruker IFS-48 spectrophotometer using a KBr matrix. UV spectra were performed on a Beckman DU70 spectrometer in aqueous solution. Molecular mechanics and dynamics calculations were carried out on SGI Personal Iris 35G computer using the force field CHARM (QUANTA 4.0 software package). All the force field calculations were carried out *in vacuo* (dielectric constant = 1). Global minimum energy conformations were obtained by performing a high temperature molecular dynamics simulation (HTMDS) followed by energy minimization.[14] By means of a molecular dynamics simulation of 100 ps at 1000 K, 200 conformations of **1** were achieved. All the conformations were then subjected to an energy minimization (1000 steps, conjugated gradient algorithm). Inspection of the minimized structures provided the lowest energy conformation of **1**. Medium pressure liquid chromatographies (MPLC) were performed on a Büchi 861 apparatus using SiO_2 (230-400 mesh). High performance liquid chromatographies (HPLC) were performed on a Varian 2510 apparatus equipped with an RI-3 index detector, using Hibar columns.

Collection, Extraction and Isolation. Specimens of the sponge *A. archeri* were collected as follows: PSS-2105, Summer 1990, Little San Salvador Island, Bahamas,15 m depth; SS-1606, Summer 1990, San Salvador Island, Bahamas, 18 m depth; GB-2505, Summer 1990, Grand Bahama Island, Bahamas, 20 m depth. They were kept frozen at -20° C until used. Reference specimens are deposited at the Istituto di Zoologia dell'Università di Genova, Italy. All the specimens were separately extracted with MeOH/toluene (3x1 liter) and subsequently with $CHCl_3$ at room temperature.

The combined MeOH/toluene solutions, after filtration, were concentrated *in vacuo* to an aqueous suspension which was extracted with EtOAc. Tlc analysis of the combined EtOAc and $CHCl_3$ extracts indicated that the extracts of the three specimens had a very similar metabolic composition and therefore the isolation and quantitation of the metabolites were carried out on the largest specimen SS-1606 (56.58 g dry wt after extraction). The combined EtOAc and $CHCl_3$ extracts of this specimen, after evaporation of the solvent afforded 17.9 g of a dark brown oil, which was chromatographed by MPLC on a Si gel column using a solvent gradient system from *n*-hexane to EtOAc and then to MeOH. Fractions eluted with *n*-hexane/AcOEt (1:1) (fraction A), *n*-hexane/AcOEt (3:7) (fraction B), *n*-

hexane/AcOEt (1:9) (fraction C), AcOET 100% (fraction D) containing bromotyrosines were further separated.

Fraction A, containing aeroplysinins-1 and -2, was chromatographed by HPLC using a Hibar Lichrospher Si60 10 μm (10x250nm) column with a mobile phase of EtOAc/$CHCl_3$ (1:1). It afforded 120 mg of aeroplysinin-1 [**2**][6] and 150 mg of aeroplysinin-2 [**3**][7] identified by comparison of their spectral properties with those reported in literature.

Fraction B was purified by hplc on SiO_2 with a mobile phase of EtOAc/$CHCl_3$ (7:3) to obtain 50 mg of compound **4**[8] and 100 mg of 11,19-dideoxifistularin-3 [**5**][9] as pure compounds identified by comparison of their spectral properties with those reported in literature.

Fraction C, which was further purified using an SiO_2 column with a mobile phase of EtOAc/$CHCl_3$ (1:1) contained fistularin-3 (**6**, 250 mg)[10] in addition of 1,750 g of **7**[8] identified by comparison of their spectral properties with those reported in literature.

Fraction D was chromatographed by hplc on SiO_2 with a mobile phase of EtOAc 100% giving 5 g of compound **8** identified by comparison of its spectral properties with those reported in literature[11] and 700 mg of compound **1** as pure compounds.

Compound 1- FABMS *m/z* $[M+H]^+$ 801, 803, 805, 807 and 809; UV λ max (MeOH) 252 (ε 7850); cd (MeOH) Δε 252 + 1.6; IR (KBr matrix) ν_{max} 3360, 1713, 1705, 1660, 1600 cm^{-1}; ^1H and ^{13}C nmr data, see Table 1.

Determination of antibacterial activity. The *in vitro* antimicrobial activity is given as minimum inhibitory concentration in μg/ml as determined by serial dilution method according to NCCLS in RPMI medium after incubation at 37° C for 24 h with an inoculum size of 10^4 for bacteria and for 48 h with an inoculum size of 10^3 for fungi.

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