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CHEMISTRY OF VERONGIDA SPONGES, III.[1] CONSTITUENTS OF A CARIBBEAN *VERONGULA* SP.

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ABSTRACT.—A detailed analysis of the secondary metabolites of a sponge, *Verongula* sp., has been performed. Eleven compounds have been identified, of which two (**10a** and **11**) are novel bromotyrosine derivatives. Structural assignments of the brominated compounds **10a** and **11** were based on spectroscopic analysis.

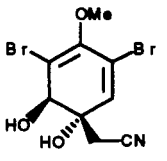
Marine sponges of the order Verongida are of much current biological and chemical interest. A range of unusual secondary metabolites containing up to four bromotyrosine residues has been isolated from sponges belonging to this order which includes, among others, the genera *Aplysina, Ianthella, Psammaplysilla, Pseudoceratina,* and *Verongula*.

Interest in these brominated compounds is prompted by their biological activity such as their in vitro and in vivo activity against Gram-positive bacteria and/or cytotoxicity, as well as the activation of myosin K^+, EDTA-ATPase or inhibition of Na^+, K^+-ATPase (2). Furthermore, bromotyrosine-derived metabolites have long been noted to be distinct markers for marine sponges belonging to the order Verongida and they may be considered as additional distinctive characters helpful in coping with the considerable difficulties inherent in the identification of Verongida species. Such problems arise from the characteristics of the sponges themselves, which are rather variable in color, shape, consistency and often skeletal arrangement. In addition, the Verongidae, being horny sponges, are devoid of spicules. Therefore, the sole anatomical characters that can be used for identification purposes are the type and size of spongin fibres and the skeletal pattern they form. Chemotaxonomy may thus provide assistance in attaining correct identifications and producing consistent taxonomic data. This requires a wider examination of the secondary metabolite content of the sponges.

For this reason we began a study of the secondary metabolites of several Verongida species, which are particularly abundant in the Caribbean area (3). We wish to report here the results obtained from the analysis of one of these species, identified as *Verongula* sp.

The freshly collected sponge was frozen on site using dry ice and kept frozen until examined. The thawed sponge was homogenized in a Waring blender with MeOH-toluene (3:1) and extracted at room temperature for two days. The MeOH/toluene extract was concentrated *in vacuo* and the resulting residue was suspended in H_2O and sequentially extracted with EtOAc and *n*-BuOH. The combined extracts were subjected to Si gel column using a step gradient of EtOAc in *n*-hexane and then MeOH in EtOAc. Further separation by repeated hplc afforded compounds **1** (3), 162 mg; **2** (4) 952 mg; **3** (5), 1.1 g; **4** (6), 428 mg; **5** (7), 120 mg; **6** (2), 945 mg; **7** (2), 30 mg; **8** (8), 646 mg; **9** (8), 35 mg; **10a**, 4 g; and **11**, 12 mg (see Table 1). Compounds **1**–**9**, isolated as amorphous solids, were identified by comparison of their spectral data with those previously reported.

[1]For No. 2 in this series, see P. Ciminiello, *et al.* (1).



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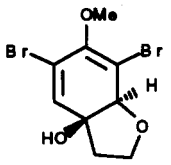

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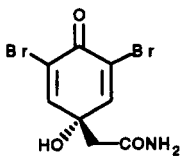

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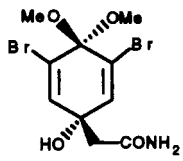

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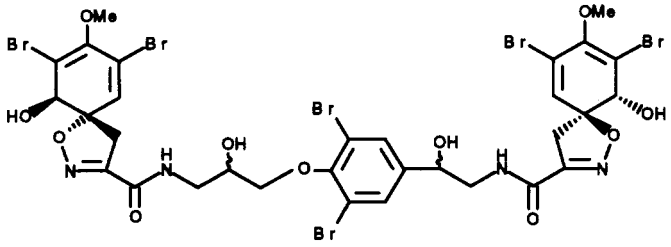

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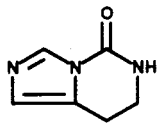

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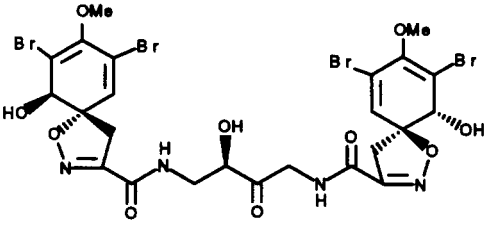

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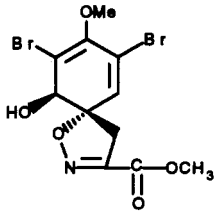

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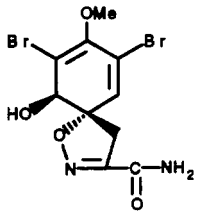

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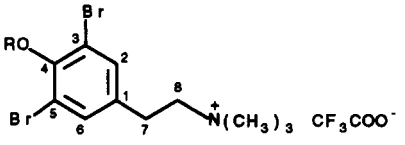
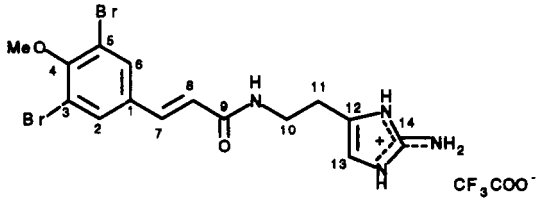


10a R=Me
10b R=H



11

A parent triplet ion in the positive fabms spectrum of **10a** in a ratio of about 1:2:1 at *m/z* 350, 352, and 354 indicated the presence of two bromine atoms in the molecule and suggested the molecular formula $C_{12}H_{18}NBr_2O$, in agreement with the ^{1}H- and ^{13}C-nmr data. The ir spectrum contained bands at ν max 2578 (NC-H stretch) and 1635 (aromatic) cm^{-1}. The ^{1}H-nmr spectrum (CD_3OD) contained a signal at δ 7.69 (2H, s) that was assigned to two protons of a symmetrically tetrasubstituted benzene ring and an AA'BB' system observed at δ 3.67 and 3.17. The latter signal exhibited a long-range correlation to the signal assigned to the aromatic protons of the benzene ring, thus indicating the presence of an Ar-CH_2CH_2-X system. The ^{1}H-nmr spectrum also contained a sin-

TABLE 1. Relative Abundance of Secondary Metabolites in the Sponge *Verongula* sp.

Compound	%[a]	Compound	%[a]
1	0.6	**7**	0.1
2	3.9	**8**	2.7
3	4.6	**9**	0.1
4	1.8	**10a**	16.6
5	0.5	**11**	0.05
6	3.9		

[a]Percentage of the initial solvent extract of the animals.

glet at δ 3.85 (3H), attributable to a methoxy group linked to the benzene ring and a 9H-singlet at δ 3.32, indicative of a trimethylammonium functionality.

All the above data were in good agreement with structure **10a**, which was confirmed by ^{13}C-nmr data (see Table 2); the resonances were assigned on the basis of DEPT and 2D ^{1}H-^{13}C shift correlation experiments via ^{1}J (HETCOSY) and $^{2,3}J$ (COLOC). The following long-range ^{1}H-^{13}C shift correlations established the substitution pattern of the benzene ring: $H_{2,6}/C_4$, $H_{2,6}/C_{3,5}$, $H_{2,6}/C_7$, $H_7/C_{2,6}$, H_7/C_1, $H_8/^{+}N(Me)_3$, OMe/C_4.

that compound **10a** preferentially assumes an anti conformation along the C-7–C-8 bond. This conclusion was supported by molecular modeling analysis, using the CHARMm force field. The energy of the anti and gauche conformers was calculated and a ΔE value of 2.6 kcal/mol was estimated.

Compound **10a** represents the methylated derivative of the sponge metabolite **10b**, previously isolated from *Aplysina fistularis* (9), which has been recognized as being a structural as well as pharmacological hybrid of adrenalin and acetylcholine. Methoxy ethers are common metabolites of marine organisms

TABLE 2. Nmr Spectral Data for Compounds **10a** and **11** (CD_3OD).[a]

Position	10a		11	
	δ_H (mult. J)	δ_C	δ_H (mult. J)	δ_C
1		136.44		136.36
2,6	7.69 (s)	134.84	7.82 (s)	132.98
3,5		119.21		119.48
4		154.46		154.46
7	3.17[b]	29.04	7.42 (d, 15.5)	138.42
8	3.67[b]	67.54	6.57 (d, 15.5)	123.95
9				167.50
10			3.56 (t, 6.6)	39.27
11			2.77 (t, 6.6)	25.92
12				125.89
13			6.59 (s)	110.87
14				146.43
OMe	3.85	61.42	3.91 (s)	61.26
$N(Me)_3^{+}$	3.32	54.11		

[a]Assignment based on ^{1}H-^{1}H and ^{1}H-^{13}C 2D nmr experiments and on DEPT 1D nmr spectra.
[b]AA'BB' system ($J_{AB}=J_{A'B'}=-17.5$ Hz; $J_{A'B}=J_{AB'}=5.5$ Hz; $J_{AA'}=J_{BB'}=12.5$ Hz).

Chemical shifts and coupling constants of protons belonging to the AA'BB' system ($\delta_A=\delta_B=3.57$, $\delta_{A'}=\delta_{B'}=3.17$; $J_{AB}=J_{A'B'}=-17$ Hz, $J_{AB'}=J_{A'B}=5.5$ Hz, $J_{AA'}=J_{BB'}=12.5$ Hz) were determined by computer analysis of the splitting patterns[2] (Figure 1). These data suggested and some of them have been observed to be artifacts resulting from MeOH incorporation during extraction processes. Since we utilized MeOH in the extraction of the sponge, there was indeed the possibility that **10a** was an artifact of MeOH incorporation. This hypothesis was ruled out since we extracted a sample of the sponge with *n*-BuOH after lyophilization and the methoxylated compound was still present, while **10b** could not be detected.

Compound **10a**, which is not yet

[2]The system was simulated on an IBM PS/2 70 with the aid of an unpublished program, NMRSimul by Dr. A. Mangoni. Chemical shifts and coupling constants were determined by visual fitting between experimental and calculated splitting patterns.

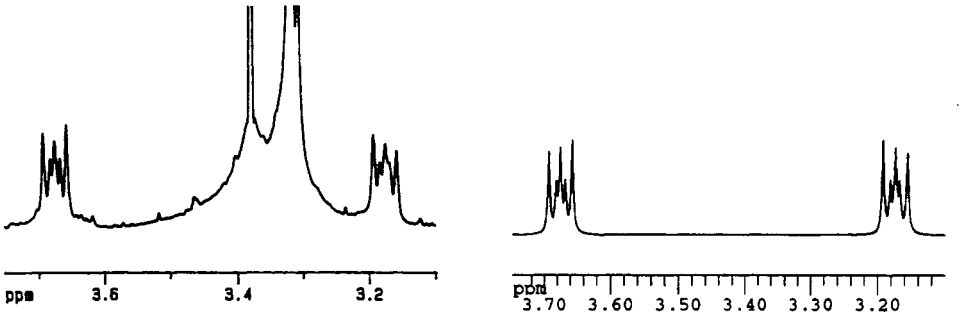


FIGURE 1. Observed (left) and calculated (right) 500 MHz nmr spectra of the methylene protons of **10a**.

known to occur in any other Verongida species, represents by far the most abundant bromo compound of the sponge (1.7% dry wt) and therefore it appears to be a promising candidate as a chemotaxonomic marker for the *Verongula* species being presently studied.

Compound **11** showed intense M^+ ions in the ratio of about 1:2:1 at *m/z* 443, 445, 447 in the positive fabms, which indicated the presence of two bromine atoms in the molecule. Combined analysis of the low-resolution ms and the ^{1}H- and ^{13}C-nmr spectra indicated a molecular formula of $C_{15}H_{17}N_4O_2Br_2$ for compound **11**.

The presence of an amide carbonyl group was indicated by ir absorption at ν max 1690 cm^{-1}. The presence of the usual 1-substituted, 3,5-dibromo-4-methoxy benzene moiety was apparent from both the ^{1}H- and ^{13}C-nmr resonances (see Table 2) which match closely those of **10a**. This partial system must be linked to a trans- oriented vinyl group as indicated by the ^{1}H-^{1}H COSY nmr spectrum which revealed the proton connectivities from the aromatic protons H-2 and H-6 (δ 7.82) to two olefinic protons, H-7 (δ 7.42) and H-8 (δ 6.57).

A 1-(2-aminoimidazole)-2-aminoethane functionality was proposed for the terminal part of the molecule by comparing the remaining ^{1}H- and ^{13}C-nmr signals with those reported for lipopurealin B, a bromotyrosine-derived metabolite of the Verongida sponge *Psammaplysilla purea* (10). Thus structure **11** was established for the compound under investigation; the *E* geometry of the double bond was assigned on the basis of the large coupling constant (15.5 Hz) between H-7 and H-8.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Fabms were obtained at 70 eV on a Kratos MS50 mass spectrometer. Ft-ir spectra were recorded on a Bruker IFS-48 spectrophotometer using a KBr matrix. ^{1}H- and ^{13}C-nmr spectra were determined on a Bruker AMX-500 spectrometer in CD_3OD. ^{1}H-Nmr chemical shifts were referenced to the residual MeOH signal (δ 3.34). ^{13}C-Nmr spectra were referenced to the central peak of CD_3OD at 49.0 ppm. The multiplicities of ^{13}C-nmr resonances were determined by DEPT experiments. Homonuclear ^{1}H connectivities were determined using the COSY experiment. One-bond heteronuclear ^{1}H-^{13}C connectivities were determined with a HETCOSY pulse sequence optimized for $^1J_{CH}$ of 135 Hz. Two- and three-bond ^{1}H-^{13}C connectivities were determined by a COLOC experiment, optimized for $^{2,3}J_{CH}$ of 8 Hz. Molecular modeling studies were performed using the Quanta/CHARMm 3.3 program on a Personal Iris 4D-35G computer. The effect of the solvent was not considered (dielectric constant of 1). The conjugated gradient protocol was used for energy minimization. Mplc was performed on a Büchi 861 apparatus using an SiO_2 (230–400 mesh) column. Hplc was performed on a Varian 5020 apparatus equipped with an RI-3 refractive index detector, using Hibar columns.

ANIMAL MATERIAL.—Two specimens of a *Verongula* sp. were collected on the reefs of San Salvador Island, Bahamas, at 15 m depth, during the summer of 1990. A sub-sample of the same specimen was incorporated in the Porifera collection of the Istituto di Zoologia dell'Università di Genova, under the number SS 14-07. The sponge is massive, with an almost spherical base from which slender, compressed lobes protrude. The

external color is dark brown, but the inner sponge is yellow in vivo. Specimens exposed to air, or preserved, rapidly turn black. The sponge is elastic, with a surface crossed by low lamellar ridges, never forming a polygonal pattern as in other *Verongula* species. Oscules are round, 1–3 mm across, scattered in regular rows, but always restricted to the inner surface of the lobes. The spongin fibers are not abundant, rigid and very brittle. They have a pith which occupies 80–90% of their total thickness, ranging from 100 to 300 μm. Fibers form an irregular reticulation with highly variable meshes, which changes, becoming lamellar, close to the surface. According to this skeletal pattern and to the other characters described, the specimen is here attributed to *Verongula*, but it is remarkably different from the other known species of the genus, which are all from the Caribbean area.

EXTRACTION AND ISOLATION.—The sponge was frozen when still alive at −18° and then dispatched to the laboratory. The collected animals (240 g, dry wt after extraction) were homogenized and the combined homogenates were extracted with MeOH-toluene, 3:1 (500 ml×5) at room temperature. The extracts were evaporated *in vacuo* to give an aqueous phase, which was extracted successively with EtOAc and BuOH. Evaporation of the combined EtOAc and BuOH extracts afforded 24.0 g of an oily residue. Fractionation of this oil was accomplished by mplc on an SiO_2 column, eluted using a step gradient of increasing polarity with *n*-hexane/EtOAc and EtOAc/MeOH mixtures. Fractions eluted with *n*-hexane-EtOAc, 1:1 (fraction A), *n*-hexane-EtOAc, 2:8 (fraction B), EtOAc (fraction C), EtOAc-MeOH, 1:1 (fraction D), and EtOAc-MeOH, 3:7 (fraction E) containing bromotyrosines, and a fraction eluted with EtOAc-MeOH, 9:1, containing compound **6** were further separated.

Isolation of **1**, **2**, *and* **8**.—Fraction A (2.4 g) was chromatographed by hplc using a Hibar LiChrospher Si 60 10 μm (10×250 mm) column with a mobile phase of EtOAc-$CHCl_3$ (1:1), affording 162 mg of aeroplysinin-1 [**1**], 952 mg of aeroplysinin-2 [**2**], and 646 mg of compound **8**.

Isolation of **5** *and* **9**.—Fraction B (280 mg) was purified by hplc on SiO_2 with a mobile phase of EtOAc-$CHCl_3$ (9:1) to obtain fistularin-3 (**5**, 85 mg) and compound **9** (35 mg).

Isolation of **3**, **4**, **5**, *and* **7**.—Fraction C (1.9 g), chromatographed on SiO_2 with a mobile phase of EtOAc-$CHCl_3$ (9:1) further contained fistularin-3 (**5**, 48 mg) in addition to **3** (1.1 g), **4** (428 mg), and **7** (30 mg).

Isolation of **11**.—Further purification of fraction D (269 mg) using a Hibar Superspher RP-18 (10×250 mm) column with a mobile phase of CH_3CN-H_2O-CF_3COOH (1:1:0.2) gave 12 mg of compound **11**.

Isolation of **10a**.—Fraction E (5.67 g) was chromatographed by reversed-phase hplc with a mobile phase of CH_3CN-H_2O-CF_3COOH (6:4:0.2) thus obtaining 4 g of **10a**.

Isolation of **6**.—Fractions eluted with EtOAc-MeOH (9:1) (1.7 g) were rechromatographed by hplc on SiO_2 column with a mobile phase of EtOAc-MeOH (95:5) to obtain pure **6** (945 mg).

Compound **10a**.—Amorphous solid; fabms *m/z* $[M]^+$ 350, 352, 354; ir (dry film) ν max 2578 (NC-H stretch), 1635 (aromatic) cm^{-1}; uv (EtOH) λ max (ε) 277 (1260); ^1H and ^{13}C nmr, see Table 2.

Compound **11**.—Amorphous solid; fabms *m/z* $[M]^+$ 443, 445, 447; ir (dry film) ν max 1690 (C=O, amide) cm^{-1}; uv (EtOH) λ max (ε) 226 (12200), 298 (32000); ^1H and ^{13}C nmr, see Table 2.

Compounds **1**–**9** were identified by comparison of their spectral properties with those previously reported.

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