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CHEMISTRY OF VERONGIDA SPONGES, IV.[1] COMPARISON OF THE SECONDARY METABOLITE COMPOSITION OF SEVERAL SPECIMENS OF *PSEUDOCERATINA CRASSA*

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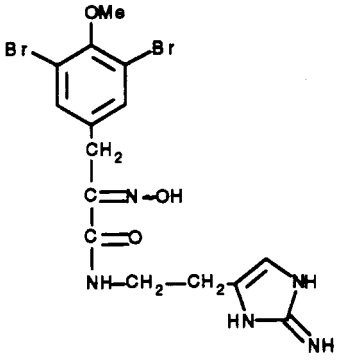
ABSTRACT.—Caribbean specimens of a sponge possessing the morphological characters of *Pseudoceratina crassa* have been analyzed. They were shown to contain a number of known brominated metabolites and three novel compounds (**13–15**), whose structures have been determined on the basis of spectral data. The results obtained have been discussed in the context of either the possible existence of another species of *Pseudoceratina* not morphologically distinguishable from *P. crassa* or, alternatively, the existence of strains *P. crassa* with slight genetic differences.

Pseudoceratina crassa (Hyatt), a marine sponge of the order Verongida, has been reported in the literature under different names, such as *Aiolochroia crassa* (Hyatt, 1875), *Ianthella ianthella* (De Laubenfels 1949), and *Ianthella ardis* (De Laubenfels 1950), which are considered synonymous. Some considerations on the phenotypic variability of Verongida species and on the difficulties of identifying them have been made in a previous paper (2).

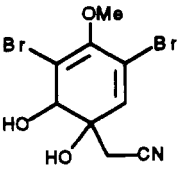
Pseudoceratina crassa is the sole species of this genus that has been described from the Caribbean Sea where the specimens we examined were collected. The sponge is massive, with lobate or, more rarely, ramose processes, yellow to violet in color. The surface is smooth, with blunt or acute conules that may be interconnected by raised ridges. The choanosome is extremely dense and infiltrated by collagen that makes the sponge hard and almost incompressible, especially when preserved in alcohol. The skeleton, as is the rule in the family Aplysinellidae, is organized on a dendritic plane. The fibers are sparse (often a few mm apart) and follow erratic courses, anastomosing here and there. The pith occupies from 40% to 80% of the total thickness of the fiber, which is rather rigid and very stout (150–530 μm in diameter).

The secondary metabolites of *P. crassa* have been extensively analyzed and the results obtained, which appear to be quite inconsistent, can be briefly summarized as follows. Makarieva *et al.* identified aeroplysinin-1, aeroplysinin-2, and 3,5-dibromo-4-ethoxy-1-hydroxy-4-methoxy-2,5-cyclohexadien-1-yl-9-ethanamide as the major compounds in a sample of *P. crassa* collected along the Cuban coast (3). In 1986, a specimen of *Ianthella ardis* (=*P. crassa*) from the Bahamas was shown to contain large quantities of ianthellin and lesser amounts of aeroplysinin-1 by Litaudon and Guyot (4). In 1991, Kassuhlke and Faulkner isolated ethyl-3,5-dibromo-4-(3′-*N*,*N*-dimethylaminopropyloxy)-cinnamate and its free acid from a Caribbean sample of *P. crassa* (5). Recently, we have extensively analyzed a specimen of *P. crassa* collected in summer 1990 along the coasts of San Salvador Island (2). This specimen was shown to elaborate, in addition to crasserides, which are unique five-membered cyclitol glycolipids (6), a number of bromotyrosines [**1–6**], reported in Table 1 (specimen a). Our results were in good agreement with those of Litaudon and Guyot (both specimens accumulate ianthellin, **1**, as the major product,

[1]For Part III in this series, see Ciminiello *et al* (1).



1



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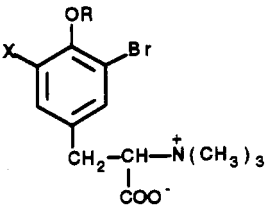

3 R=Me; X=H
4 R=H; X=H
5 R=Me; X=Br
6 R=H; X=Br

and lesser quantities of aeroplysinin-1, **2**), but appeared to be quite unlike those reported by the other authors.

RESULTS AND DISCUSSION

In order to gain information to clarify this problem of compound variability, we investigated several Verongida sponges collected during two expeditions to the Caribbean sea during the summers of 1990 and 1992. They were identified using traditional taxonomic criteria and analyzed chemically. Five specimens collected from the coasts of Little San Salvador (PSS 21-02 and PSS 19-11), San Salvador (SS 14-08), and Grand Bahama Islands (1608 and 1611) conform to the characteristics of *P. crassa*, and apart from slight variations in the external color, they may be considered homogenous from the morphological point of view.

The specimens of *P. crassa* were stored at −20° until needed. On the basis of a preliminary analysis, all specimens were shown to contain the same secondary metabolites in comparable amounts, and an extensive analysis was thus performed on the largest specimen (reference number SS 14-08) collected along the coasts of San Salvador Island in summer 1990. This sponge was shown to contain, in addition to common primary metabolites, the compounds listed in Table 1 (specimen b). Compounds **2–4** and **7–12** are known compounds, previously isolated from Verongida species, and compounds **13–15** are new natural products, although **14** has been previously described as a synthetic product (7).

The lipophilic material from the MeOH extract of the sponge was chromatographed on a Si gel column followed by hplc to give compounds **2** (8), **7** (9), **8** (10), **9** (11), **10** (9), **12** (12), **13**, and **14**. Compounds **2**, **7–10**, and **12** were identified by comparison of their spectral properties with those reported in the literature.

Compound **13** had a molecular formula of $C_{14}H_{15}BrN_4O_3$ as deduced from fabms and ^{13}C-nmr data. A comparison of overall ^{1}H- and ^{13}C-nmr spectral data revealed remarkable similarities (see Table 2) between **13** and the known brominated compound **12** isolated from *Verongula gigantea* (12). Compound **12** differs from **13** in having a 4-methoxy group instead of a 4-hydroxy group. Also the stereochemistry of the oxime group is the same in both compounds, as suggested by the ^{13}C-nmr chemical shift of C-7 (δ 28.7), which is in good agreement with the reported data for *E* oximes of related compounds. In the *Z* stereoisomers the corresponding carbon resonates at higher values (δ ca. 35) (13). Compound **13** was thus identified as the *O*-demethyl derivative of **12**.

Compound **14** had the molecular formula C_9H_8BrNO as determined by hreims. The presence of a -CN group was readily inferred from the intense absorption band at ν max 2250 cm^{-1} in the ir spectrum (14). The ^{1}H-nmr spectrum, which appeared quite simple,

TABLE 1. Percentages of Halogenated Metabolites in Specimens of *Pseudoceratina crassa*.

Sample	Collection	Depth	Compound %[a]														
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Specimen a[b] . . .	San Salvador Island	15 m	1.4	0.01	0.007	0.001	0.005	0.005	—	—	—	—	—	—	—	—	—
Specimen b[c] . . .	San Salvador Island	15 m	—	0.29	0.04	0.02	—	—	0.14	0.04	0.05	0.03	0.03	0.04	0.015	0.005	0.007

[a]Of dry wt of the specimens after extraction.
[b]Data are reported in Makarieva *et al.* (3).
[c]Specimen SS 14-08.

TABLE 2. Nmr Spectral Data of Compounds **12–15**.

Position	Compound							
	12[a]		**13**[a]		**14**[b]	**15**[b]	
	δ_H (mult., *J*)	δ_C	δ_H (mult., *J*)	δ_C	δ_H (mult., *J*)	δ_H (mult.)[c]	δ_C
1		130.3		130.6			
2	7.41 (d, 2.1)	134.6	7.45 (d, 2.2)	134.3	7.49 (d, 2.1)	4.71[d]	81.5
						3.46[e]	
3		111.9		110.4			31.2
						3.37[f]	
4		155.9		153.6			123.5
5	6.90 (d, 8.4)	113.1	6.81 (d, 8.5)	117.1	6.92 (d, 8.4)	7.11 (s)	109.8
6	7.18 (dd, 8.4; 2.1)	130.4	7.06 (dd, 8.5; 2.2)	130.4	7.23 (dd, 8.4; 2.1)		151.5
7	3.79 (s)	28.7	3.78 (s)	28.7	3.81 (s)		141.2
8		152.9		153.1		7.03 (s)	104.7
9		165.7		166.1	3.85 (s)		149.4
10	3.58 (t, 7.1)	39.2	3.57 (t, 7.0)	39.0			171.2
11	2.92 (t, 7.1)	26.2	2.94 (t, 7.0)	25.7		3.38 (s)	54.2
12		132.4		132.8		3.76 (s)[g]	51.5
13	7.25 (br s)	117.5	7.25 (br s)	117.5		3.92 (s)	57.3
14	8.72 (br s)	134.7	8.70 (br s)	134.6			
15	3.82 (s)	56.7					

[a]0.2% TFA-H in CD_3OD.
[b]CD_3OD.
[c]Assignment based on DEPT spectrum and incremental estimations [Pretsch *et al.* (16)].
[d]X part of an ABX system; J_{AX}=7 Hz, J_{BX}=10.5 Hz.
[e]B part of an ABX system; J_{AB}=16 Hz.
[f]A part of an ABX system.
[g]cis to H-2.

revealed the presence of a 1,3,4-trisubstituted phenyl ring (δ 7.49, d, *J*=2.1 Hz; δ 7.23, dd, *J*=8.4 and 2.1 Hz; δ 6.92, d, *J*=8.4 Hz), a methoxyl group (δ 3.85, s), and an isolated deshielded methylene group (δ 3.81, s), which on the basis of its chemical shift value must be positioned between the phenyl ring and the nitrile function. Thus, on the basis of the spectral properties, **14** was formulated as a bromomethoxyphenylacetonitrile. The substitution pattern on the benzene nucleus was deduced through a ROESY nmr experiment, which showed correlations between the aromatic signals at δ 7.49 and 7.23 and the singlet at δ 3.81 (H_2-7), and between the methoxy signal at δ 3.85 and the doublet at δ 6.92 (H-5). The structure **14** was therefore assigned to this compound, which was synthesized in 1938 by Naik and Wheeler (7).

The *n*-BuOH-soluble material was chromatographed on a Si gel column followed by reversed-phase hplc and gave **3** (2), **4** (2), **11** (15), and **15**. Compounds **3**, **4**, and **11** were identified by comparison of their spectroscopic data with those reported in the literature.

The new compound **15** had the molecular formula $C_{12}H_{15}NO_4$ as revealed by hrms (see Experimental). The ^{13}C-nmr spectrum contained six low-field signals at δ 151.5 (C), 149.4 (C), 141.2 (C), 123.5 (C), 109.8 (CH), and 104.7 (CH), which suggested the presence of a tetrasubstituted benzene ring. This was confirmed by its ^1H-nmr spectrum, which showed two singlets in the aromatic region, indicating that the protonated carbon atoms are para. The presence of the phenolic OH group was apparent from the uv spectrum, where the absorption maximum at λ max 284 (pH 7.0) nm was shifted upfield by addition of OH^- (λ max 302 nm).

Further structural information was obtained from the ^1H-nmr spectrum which showed a 1H double doublet at δ 4.71, coupled with signals at δ 3.46 (B part of an ABX

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12 R=Me
13 R=H

14

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system) and δ 3.37 (A part of an ABX system; J_{AX}=7 Hz; J_{BX}=10.5 Hz; J_{AB}=16 Hz), indicative of a CH-CH_2 group, whose carbon atoms resonated in the ^{13}C-nmr spectrum at δ 81.5 and δ 31.2, respectively. The remaining part of the molecule had to comprise a COO^- (ir ν max 1633 cm^{-1}; δ_C 171.2), a methoxyl [δ_H 3.92 (3H), δ_C 57.3], and an $^+N(CH_3)_2$ [δ_H 3.76 (3H) and 3.38 (3H); δ_C 51.5 and 54.2] function.

The overall data indicated above delineated **15** as a 1,1-dimethyl-2-carboxy-2H-indole having a methoxy group and a hydroxy group on the benzene nucleus at positions 6 and 7, respectively, or vice versa. The unequivocal positioning of these two groups was accomplished by a nOe difference nmr experiment, which showed the spectral proximity of the methoxy group resonating at δ 3.92 and H_2-3 (δ 3.39) with the aromatic proton at δ 7.11, and that of the two *N*-methyl protons (δ 3.76 and 3.38) with the aromatic proton resonating at δ 7.03. A nOe effect was also observed between the signal of the N-CH_3 at δ 3.76 and that of H-2 (δ 4.71), which pointed to their *cis* relationship, allowing the stereochemical assignment of the two N-CH_3 groups.

Structurally, compound **15** could be considered a trimethyl derivative of leucodopochrome (2-carboxy-5,6-dihydroxy-2H-indole), a key intermediate in the biogenesis of melanins derived from tyrosine by hydroxylation and oxidative ring closure. It appears reasonable to assume a biogenetic origin of **15** from tyrosine, which would confirm once again the leading role that this amino acid plays in the secondary metabolism of Verongida sponges.

Our data confirm the metabolic variation of *P. crassa*. This can be observed from Table 1, where the secondary metabolites of the specimen under investigation (SS 14-08, specimen b) are compared with those previously reported for another specimen of *P. crassa* (specimen a) collected in the same area (3). In this latter sample, as well as in that studied by Litaudon and Guyot, ianthellin [**1**] was the predominant metabolite. This compound was completely absent in specimen b, which in turn, elaborated aeroplysinin-1 [**2**] and fistularin-3 [**7**] as the major brominated metabolites.

The metabolic composition of sponges has commonly been reported to vary among specimens of the same species, but this normally occurs when they come from different areas, and the variations are generally ascribed to different environmental conditions. In this case we found striking differences in the biochemistry of specimens of *P. crassa* collected during the same expedition, in the same area, and at almost the same depth, and therefore the results obtained cannot be related to environmental or geographical factors. These results suggest the existence of two sub-specific groups of *P. crassa*, with the first characterized by the presence of large quantities of ianthellin [**1**], while the second group lacks ianthellin, but elaborates fistularin-3 [**7**] and aeroplysinin-1 [**2**] as the major compounds. Two hypotheses may be formulated to interpret these results: a) the possible existence of another species of *Pseudoceratina* (not morphologically distinguishable from *P. crassa*); or b) the possibility that different strains of *P. crassa*, with slight genetic differences, are able to elaborate different metabolites.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^{1}H- and ^{13}C-nmr spectra were determined on a Bruker AMX-500 spectrometer and the solvent was used as an internal standard (CD_3OD: ^{1}H δ 3.34; ^{13}C δ 49.0). Methyl, methylene, and methine carbons were distinguished by a DEPT experiment. Mass spectra were recorded on a Kratos MS-80 mass spectrometer at 70 eV. Fabms were obtained at 70 eV on a Kratos MS-50 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 MeOH solution. Optical rotations were measured on a Perkin-Elmer 192 polarimeter in MeOH solution, using a Na lamp at 589 nm and a 10-cm microcell. Mplc was performed on a Büchi 861 apparatus using SiO_2 (230–400 mesh). Hplc was performed on a Varian 2510 apparatus equipped with an RI-3 index detector, using Hibar columns.

ANIMAL MATERIAL.—Specimens of *P. crassa* were collected as follows: SS 14-08, summer 1990, San Salvador Island, Bahamas, 15 m depth; PSS 19-11, summer 1990, Little San Salvador Island, Bahamas, 18 m depth; PSS 21-02, summer 1990, Little San Salvador Island, Bahamas, 20 m depth; 1608, summer 1992, Grand Bahama Island, Bahamas, 12 m depth; 1611, summer 1992, Grand Bahama Island, Bahamas, 12 m depth. They were kept frozen at −20° until used. Reference specimens are deposited at the Istituto di Zoologia dell'Università di Genova, Italy.

EXTRACTION AND ISOLATION.—All the specimens were separately extracted with MeOH-toluene (3:1) and subsequently with $CHCl_3$ at room temperature. The combined MeOH/toluene solutions were concentrated *in vacuo* to give an aqueous suspension that was extracted with EtOAc and subsequently with *n*-BuOH. Tlc analysis of the combined EtOAc and $CHCl_3$ extracts and of the *n*-BuOH-soluble material indicated that the extracts of the five specimens had a very similar metabolic composition and therefore the isolation and quantitation of the metabolites were carried out on the largest specimen SS 14-08 (90 g dry wt). The combined EtOAc and $CHCl_3$ extracts (6 g) of this specimen were chromatographed by mplc on a Si gel column with solvents of increasing polarity from *n*-hexane to EtOAc and MeOH.

Compound **2**.—Fractions eluted with *n*-hexane-EtOAc (1:1) (475 mg) were purified by hplc using a Hibar LiChrospher Si 60 (10×250 mm) column with EtOAc-$CHCl_3$ (1:1) as eluent, giving 264 mg of pure compound **2**, identified by comparison of its spectral properties with those reported in the literature (8).

Compounds **7–10**.—Evaporation of fractions eluted with 100% EtOAc afforded a mixture (388 mg) which was purified by hplc using a Hibar LiChrospher Si 60 (10×250 mm) column with a mobile phase of EtOAc-$CHCl_3$ (9:1) to obtain compounds **7** (9) (130 mg), **8** (10) (35 mg), **9** (11) (48 mg), and **10** (9) (25 mg), identified by comparison of their spectral properties with those reported in the literature.

Compounds **12–14**.—Fractions emerging from the mplc column with EtOAc-MeOH (8:2) (138 mg) were purified by hplc on a LiChroprep RP-18 column (10×250 mm) using a linear gradient from 100% H_2O (containing 0.2% of trifluoroacetic acid) to 100% CH_3CN (containing 0.2% of trifluoroacetic acid) in 20 min, thus affording the pure compounds **12** (24 mg), **13** (14 mg), and **14** (5 mg). Compound **12** was identified by comparing its spectral properties with those reported in the literature (12). Compound **13**: Uv (MeOH) λ max (ε) 387 (620), 289 (2600), 278 (3020), 207 (31000) nm; ir (KBr) ν max 3151, 1675 cm^{-1}; fabms *m/z* $[M+H]^+$ 367 and 369; ^1H- and ^{13}C-nmr data are reported in Table 2. Compound **14**: Uv (MeOH) λ max (ε) 220 (1050), 277 (1260) nm; ir (KBr matrix) ν max 3151, 1675 cm^{-1}; hreims *m/z* 224.9785 and 226.9764 (M^+ calcd for C_9H_8NOBr 224.9788 and 226.9767); ^1H- and ^{13}C-nmr data are reported in Table 2.

Compound **11**.—The *n*-BuOH-soluble fraction was chromatographed on a Si gel column using a stepwise gradient from 0 to 100% MeOH in EtOAc. Fractions eluted with EtOAc-MeOH (9:1) (227 mg) were rechromatographed by hplc on a SiO_2 column with a mobile phase of EtOAc-MeOH (95:5) to obtain pure **11** (32 mg), identified by comparison of its spectral properties with those reported in the literature (15).

Compounds **3** *and* **4**.—Evaporation of the fractions eluted with 100% MeOH produced a mixture of compounds **3** and **4**, which was separated by hplc on a Hibar LiChrospher RP-18 column (10×250 mm) using MeOH-H_2O (1:1) as eluent, thus obtaining 34 mg of **3** and 18 mg of **4**, which were identified by comparison of their spectral properties with those previously reported (2).

Compound **15**.—Fractions eluted with EtOAc-MeOH (3:7) (128 mg) were further purified by hplc on a Hibar LiChrospher RP_{18} column (4×250 mm) using a linear gradient system from 100% H_2O (containing 0.2% of trifluoroacetic acid) to 100% CH_3CN (containing 0.2% of trifluoroacetic acid) in 20 min, thus affording 6.4 mg of pure **15**; $[\alpha]^{25}D$ −12° (*c*=0.001, MeOH); uv (MeOH) λ max 284 (pH 7.0, ε 1100), 225 (pH 7.0, ε 1320), 204 (pH 7.0, ε 5260), 302 (pH 10.0, ε 1295), 248 (pH 10.0, ε 1965), 210 (pH 10.0, ε 8750) nm; ir (KBr) ν max 3400 (OH), 1633 (COO^-) cm^{-1}; hreims *m/z* 237.0993 (M^+, calcd for $C_{12}H_{15}NO_4$ 237.0997); ^1H- and ^{13}C-nmr data are reported in Table 2.

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