Pergamon

Tetrahedron Vol. 50, No. 3, pp. 783-788, 1994

0040-4020/94 $6.00+0.00

0040-4020(93)E0029-F

Chemistry of Verongida Sponges. I. Constituents of the Caribbean Sponge *Pseudoceratina crassa*

Stefania Albrizio, Patrizia Ciminiello, Ernesto Fattorusso*, Silvana Magno

Dipartimento di Chimica delle Sostanze Naturali, Università degli Studi di Napoli "Federico II"
Via D. Montesano 49, 80131 Napoli, Italy

Maurizio Pansini

Istituto di Zoologia, Via Balbi 5, 16126 Genova, Italy

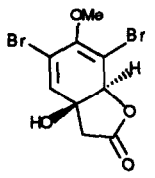
Abstract: A detailed analysis of the secondary metabolites of the sponge *Pseudoceratina crassa* has been performed. The structures of a new triterpene (**7**) and four new bromotyrosine derivatives (**8-11**) were determined by spectroscopic analysis. The bromocompounds of *P. crassa* can be used as chemical markers to support the identification of the sponge based on morphological characters.

Verongida (Bergquist, 1978) are marine sponges chemically characterized by a series of secondary brominated metabolites, biogenetically arising from bromotyrosines, none of which are yet known to occur in any other marine sponge. In comparison with the extensive chemical investigation on these organisms, which led to the isolation of more than one hundred novel bromocompounds, uncertainties exist concerning the identification of the species which these products derive from. The difficulties in identifying the species belonging to this order come from their high phenotypic variability and from the absence of reliable skeletal characteristics. In addition a systematic revision of the Caribbean Verongida is still lacking and is highly needed.

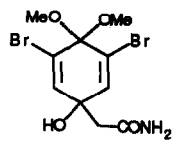
Some discrepancies can be noticed by examing the data reported on the constituents of *Pseudoceratina crassa*, a Verongida species which has been reported in the sponge literature also under different names, such as *Aiolochroia crassa* (Hyatt, 1875) [1], *Ianthella ianthella* (De Laubenfels, 1949), *Ianthella ardis* (De Laubenfels, 1950) that are considered as synonyms. In 1981 Makarieva *& al.* report aeroplysinin-1(**1**), aeroplysinin-2 (**2**) and 2-(3,5-dibromo-4-ethoxy-1-hydroxy-4-methoxy-2,5-cyclohexadien-1-yl) ethanamide (**3**) as the major compounds of a specimen of *Aiolochroia crassa*, collected along the Cuban coast [2]. In 1986 a sample of *Ianthella ardis* (Bahamas) was shown to elaborate large quantities of ianthellin (**4**, 1.4% dry weight), and smaller amounts of aeroplysinin-1 (**1**, 0.01% dry weight) by Litaudon and Guyot, but no mention about the co-occurrence of compounds **2** and **3** was reported[3]. Finally in 1991 Kassuhlke and Faulkner by studying a Caribbean sample of *P. crassa*, collected near Albert Town (Bahamas), observed a single UV-absorbing spot in the organic extract. This material was isolated and identified as ethyl 3,5-dibromo-4-(3'-N,N-dimethylamino-propyloxy) cinnamate (**5**, 0.13 % dry weight). Additional quantities of the free acid (**6**, 0.2 % dry weight), were recovered from the aqueous extract[4]. During the Fenical expedition in July 1990, along the coasts of the Bahama Islands, we collected several specimens of the same sponge species which was identified on the basis of the external morphology (colour, oscules, conules, consistency etc.), observed and photographed *in vivo*, and the peculiar characteristics (few, brittle

fibers, more than 500 μm thick, rarely forming meshes) of the skeleton. We can reasonably suppose that our specimens - which perfectly match with the recent descriptions of Wiedenmayer (1977)[5] and Van Soest (1978)[6] - are conspecific with those studied by some of the authors cited before.

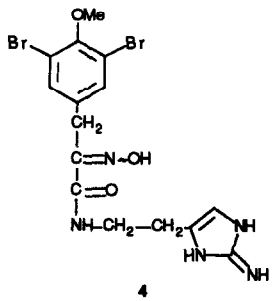
1



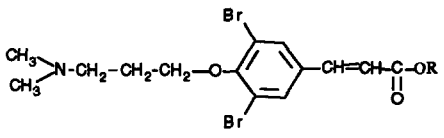
2



3

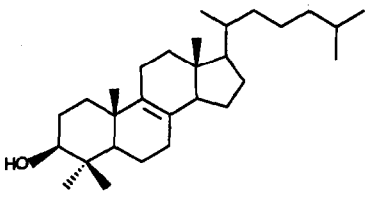


4

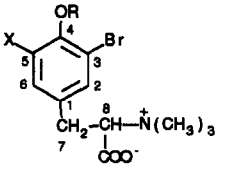


5 R = Et
6 R = H

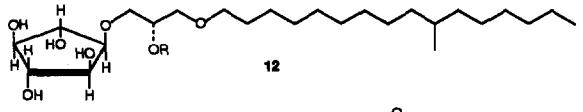
To gain a detailed knowledge on the secondary metabolism of this sponge, which could be usefully utilized to attain unambiguous identification of *P. crassa* species, our Caribbean specimen has been extensively analyzed. In this paper we report the obtained results. A number of secondary metabolites (**1, 4, 7, 8-11, 12**) have been isolated and quantitized. Compounds **1** and **4** are known compounds previously isolated from Verongida sponges [3,7]. The isolation from the same *P. crassa* sample of the six five-membered cyclitol glycolipids (**12**) and their structure determination are reported in a recent paper of our research group[8]. The present communication describes the structure elucidation of the novel compounds **7-11**.



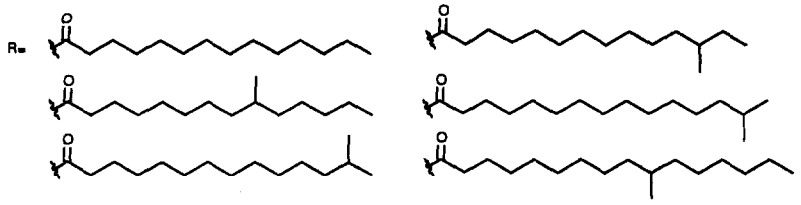
7



8 R=H; X=Br
9 R=Me; X=Br
10 R=Me; X=H
11 R=H; X=H



12



Specimens of *P. crassa* (63.22 g dry weight) collected along the coasts of San Salvador Island were stored frozen and extracted with MeOH-toluene (3:1) and successively with $CHCl_3$. The extracts were combined and partitioned by medium-pressure liquid chromatography on silica gel followed by HPLC to give **1** (7.6 mg), **4** (885.0 mg), **12** (93.0 mg) and a sterol fraction (285.2 mg).

Compounds **1** and **4** were identified as aeroplysinin-1 (racemic mixture) and ianthellin respectively by comparison of their high resolution mass and ^1H and ^{13}C NMR spectra with those reported in literature.

The sterol fraction, chromatographed by HPLC on SiO_2 column showed to contain cholesterol and cholestanol as the major compounds together with lesser quantities of 24-methyl-cholesta-5,7-dien-3-β-ol and 24-ethyl-cholest-5-en-3-β-ol. 4.0 mg of the novel compound **7**, identified on the basis of the following evidences, were also obtained. Its molecular formula $C_{29}H_{50}O$ was determined from interpretation of the M^+ ion in the HREIMS. The sterol-like structure of **7** was indicated by ^1H NMR spectrum which showed the presence of a tertiary methyl group resonating in the high field region (δ 0.61) reminiscent of the 18 methyl group and two methyl doublets at δ 0.92 (3H) and δ 0.86 (6H), characteristic of the 21, 26 and 27 methyls of a saturated side chain respectively.

Further indicative features in ^1H NMR spectrum were the signals for three additional tertiary methyls (δ 0.82, δ 0.99 and δ 1.01) and a double doublet for a proton of a hydroxymethine group at δ 3.25, attributable to H_3-19, H_3-28, H_3-29 and H-3 of a 3β-hydroxy-4,4-dimethyl sterols respectively.

Compound **7**, as indicated by two signals for two quaternary carbons at δ 127.97 and 135.93 in the ^{13}C NMR spectrum, contains a tetrasubstituted double bond, which can only be accomodated in a sterols nucleus at $\Delta^{8(9)}$ or $\Delta^{8(14)}$. The observed chemical shift of H_3-18 at δ 0.61 is in excellent agreement with that reported for $\Delta^{8(9)}$ sterols while remarkably differs from that described for a $\Delta^{8(14)}$ (δ 0.86) [9,10].

Support for the proposed structure was obtained from the analysis of ^{13}C NMR spectrum values; the assigned resonances of the nuclear carbons (see experimental section) match well with the data of the model compound 4,4-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol[11] while the chemical shift values of the remaining carbons fully agree with those reported for an usual sterol side chain[12]. Thus the compound **7** was formulated as 4,4-dimethyl-5α-cholest-8-en-3β-ol.

The *n*-BuOH-soluble material from the extracts of the sponge, partitioned by MPLC on SiO_2 and successively by reversed-phase HPLC, was shown to contain in addition to usual protein aminoacids, and purine and pyrimidine bases, little amounts of four novel brominated tyrosine derivatives, **8**, **9**, **10**, and **11**.

Compound **8** was an amorphous white solid with a molecular formula of $C_{12}H_{15}Br_2O_3N$ as determined by accurate mass measurement of the molecular ion peak in the e.i. mass spectrum.

The infrared spectrum contained bands at ν_{max} 3446 (OH) and 1576 (COO^-) cm^{-1}. The UV spectrum showed absorption maximum at λ_{max} 287 nm at pH 7.0 which shifted at λ_{max} 307 nm at pH 10.0 indicating for **8** a phenol chromophore.

The very simple ^1H NMR spectrum displayed just two singlets and an ABX system (see table). The singlet at δ 3.28 (9 H) was assigned to the methyls of an $^+N(CH_3)_3$ group, while the ABX system suggested the presence of a methylene adjacent to a chiral methine group. The ^{13}C NMR spectrum contained resonances of the pertinent carbon atoms at δ 52.69 (CH_3), 32.75 (CH_2), 81.31 (CH). The 2H singlet resonating in the downfield region of ^1H NMR spectrum at δ 7.46 along with the occurence of four resonances at δ 153.44 (C), 134.22 (CH), 128.81 (C), 112.96 (C) in the aromatic region of the ^{13}C NMR spectrum suggested the existence of a symmetrically 1,2,3,5-tetrasubstituted benzene ring.

These data and the remaining signal at δ 170.61, in the ^{13}C NMR spectrum, attributable to a carboxylic carbon, enabled us to conclude that **8** was a N,N,N-trimethyl dibromotyrosine. The substitution pattern on the benzene

nucleus was deduced by the strong NOE enhancement of the AB signal by irradiation at δ 7.46.

Compound **9** was isolated as an optically active amorphous solid. In the HREIMS of compound **9** the highest peak at *m\z* 333.8839 corresponded to loss of $N(CH_3)_3$ from the molecular formula $C_{13}H_{17}Br_2O_3N$ which was determined by ^{13}C NMR and DEPT measurements.

The ^1H NMR spectrum exhibited a 3H singlet at δ 3.81, while a methyl resonance at δ 61.00 was present in the ^{13}C NMR spectrum. All the other NMR data were similar to those of the co-occurring compound **8** (see table). It follows that **9** is O-methyl-N,N,N-trimethyl-3,5-dibromotyrosine.

Table. NMR spectral data of compounds 8, 9, 10, and 11.

	8		9		10		11	
Pos.	δ_H (mult. *J* in Hz) (CD_3OD)	δ_C (CD_3OD)	δ_H (mult. *J* in Hz) (CD_3OD)	δ_C (CD_3OD)	δ_H (mult. *J* in Hz) (D_2O)	δ_C (CD_3OD)	δ_H (mult. *J* in Hz) (D_2O)	δ_C (CD_3OD)
1		128.81		136.44		130.54		128.04
2	7.46 (s)	134.22	7.30 (s)	135.08	7.22 (d, 2.0)	135.29	7.20	134.87
3		112.96		118.96		112.79		111.41
4		153.44		154.79		156.79		155.80
5		112.96		118.96	6.66 (d, 8.5)	113.91	6.65 (d, 8.5)	117.76
6	7.46 (s)	134.22	7.30 (s)	135.08	6.95 (dd, 8.5, 2.0)	131.04	6.89 (dd, 8.5, 2.0)	130.72
a	3.12 (t, 12.0)		3.12 (t, 12.0)		2.80 (t, 12.0)		2.80 (t, 12.0)	
7		32.75		32.91		33.67		
b	3.29[a]		3.29[a]		2.95[a]		3.01[a]	33.17
8	3.73 (dd, 12.0, 3.5)	81.31	3.74 (dd, 12.0, 3.5)	80.97	3.42 (dd, 12.0, 3.7)	81.91	3.52 (dd, 12.0, 3.7)	81.62
9		170.61		170.61		170.45		170.89
OMe			3.81 (s)	61.00	3.54 (s)	57.04		
$^+N(Me)_3$	3.28	52.69	3.30 (s)	52.74	2.96 (s)	53.17	3.02 (s)	52.67

a. submerged by other signal

Compound **10** was an amorphous solid; the molecular formula $C_{13}H_{18}BrO_3N$ was derived from the exact mass of the ion at *m/z* 255.9720 [M^+ - $N(CH_3)_3$] in the HREIMS coupled with ^{13}C NMR.

Comparison of ^1H and ^{13}C NMR spectra with those of **8** and **9** suggested that **10** differed from **9** in having only one bromine atom on the benzene ring.

The coupling pattern of the ^1H NMR signals at δ 7.22 (d, *J*= 2.0 Hz), 6.95 (dd, *J*= 8.5, 2.0 Hz) and 6.66 (d, *J*= 8.5 Hz) indicated a 1,2,4 trisubstituted benzene nucleus. The location of three substituents (-OCH_3, Br, aminoacidic chain) was deduced by some interproton contacts detected through NOE difference experiments. The most diagnostic enhancements were observed for H-5 upon irradiation of OCH_3, which illustrated their *ortho*-relationship and for H-2 and H-6 on irradiation at δ 2.80 (one of the H_2-7 protons), consistent with the location of these three protons as reported in figure.

The remaining compound **11** ($C_{12}H_{16}BrO_3N$ from HREIMS), obtained in minute amounts, was identical to **10** as evidenced by ^1H and ^{13}C NMR data (see table) and differs simply in having at position 4 of the benzene nucleus an OH group instead of a methoxy group. The phenolic chromophore was also evidenced from the shifts of the UV spectra which showed at pH 7.0 λ_{max} 287 nm shifted at λ_{max} 307 nm at pH 10.0.

The results reported in the present paper indicate that the secondary metabolism of our sponge parallels that of the specimens collected and investigated in 1986 by Litaudon and Guyot[3] Both specimens elaborate large amounts of ianthellin (**4**) and smaller quantities of aeroplysinin 1 (**1**) suggesting that they belong to the same species. It is to be noted that compound **4** can be considered a good chemotaxonomic marker since it is not at all widespread in the Verongida, not having been found until now in any other species.

It appears quite hard to ascribe the striking differences between the constituents of our sponge and those of the specimens, apparently belonging of the same species, investigated by Makarieva & *al.*[2] and by Kassuhlke and Faulkner[4] to variations of the environmental conditions that may affect their metabolism. More probably the reasons must be sought in the difficulties inherent in identifying Verongida sponges exclusively on the basis of morphological characters without a chemotaxonomic support.

Experimental section

General methods. ^1H and ^{13}C NMR spectra were determined on a Bruker AMX-500 spectrometer. Methyl, methylene and methine carbons were distinguished by a DEPT experiment. MS spectra were recorded on a Kratos MS80 mass spectrometer at 70 eV. 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. Optical rotation were measured on a Perkin-Elmer 192 polarimeter in methanol solution,using a sodium lamp at 589 nm and a 10-cm microcell.

High performance liquid chromatographies (HPLC) were performed on a Varian 2050 apparatus equipped with an RI-3 index detector, using Hibar columns.

Collection and extraction. The sponge *P. crassa* was collected along the coasts of San Salvador Island, Bahamas, and kept frozen until used. Reference specimens are deposited at the Istituto di Zoologia dell'Università di Genova (Italy).

The sponge (63.22 g dry weight) was extracted with MeOH/toluene 3:1 (8 x 500 ml) and subsequently with $CHCl_3$ (3 x 500 ml) at room temperature. The combined MeOH/toluene solutions were concentrated *in vacuo* to give an aqueous suspension which was extracted with EtOAc (4 x 300 ml) and subsequently with *n*-BuOH (3 x 250 ml). The EtOAc and $CHCl_3$ extracts were combined (23.16 g) and chromatographed on an SiO_2 column with solvents of increasing polarity from *n*-hexane to AcOEt and MeOH.

Isolation of 1. Fractions eluted with *n*-hexane/EtOAc 6:4 (100 mg), were purified by HPLC using a Hibar LiChrospher Si 60 (10 x 250 mm) column with $CHCl_3$/EtOAc 1:1, giving 7.5 mg of pure compound **1** (optical inactive) identified by comparison of its spectral properties with those reported in literature[7].

Isolation of 4. Fractions eluted with AcOEt/MeOH 1:1, were purified by HPLC using a Hibar LiChrosorb C_{18} (10 x 250 mm) column with a mobile phase of MeOH/H_2O 95:5., giving 885 mg of pure compound **4** identified by comparison of its spectral properties with those reported in literature[3].

Analysis of the sterol fraction. Evaporation of fractions eluted with *n*-hexane/EtOAc 8:2 afforded a mixture (285.2 mg) which was purified by HPLC using a Hibar LiChrospher Si 60 (10 x 250 mm) column with a mobile phase of *n*-hexane/EtOAc 85:15., giving cholesterol (119.0 mg), cholestanol (110.2 mg), 24-methyl-cholesta-5,7-dien-3-β-ol (18.9 mg) and 24-ethyl-cholest-5-en-3-β-ol (33.2 mg) and 4.0 mg of compound **7**.

4,4-dimethyl-5α-cholest-8-en-3β-ol (7): $[\alpha]_D^{25}$ +31.7°; HRMS *m/z* 414.3865 (M^+, calcd. for $C_{29}H_{50}O$, 414.3863); ^1H NMR data ($CDCl_3$): δ 3.25 (H-3; dd; J = 11, 4 Hz); 0.61 (H_3-18; s); 0.98 (H_3-19; s); 0.92 (H_3-21; d; J = 6.5 Hz); 0.86 (H_3-26; d; J = 6.5 Hz); 0.86 (H_3-27; d; J = 6.5 Hz); 0.82 (H_3-28; s); 0.99 (H_3-29; s); ^{13}C NMR data ($CDCl_3$): δ 35.76 (C-1); 28.46 (C-2); 78.98 (C-3); 39.51 (C-4); 50.23 (C-5); 22.05 (C-6); 28.01 (C-7); 127.97 (C-8); 135.93 (C-9); 36.27 (C-10); 22.55 (C-11); 36.97 (C-12); 42.09 (C-13); 51.90 (C-14); 23.78 (C-15); 28.82 (C-16); 54.87 (C-17); 11.29 (C-18); 18.44 (C-19); 36.14 (C-20); 19.85 (C-21); 36.25 (C-22); 23.93 (C-23); 39.51 (C-24); 28.01 (C-25); 22.36 (C-26); 22.83 (C-27); 15.37 (C-28); 27.90 (C-29).

Isolation of compounds 8-11. The *n*-BuOH soluble fraction was chromatographed on an SiO_2 column using a

stepwise gradient from 0 to 100% MeOH in EtOAc.

Fractions eluted with MeOH contained a mixture of compounds **8-11**. The mixture was separated by HPLC on a Hibar LiChrospher C_{18} (4 x 250 mm) column, using MeOH/H_2O 1:1 as eluent.

Compound 8: (3.5 mg); $[\alpha]_D^{25}$ -1.35°; UV (H_2O) λ_{max} 287 (pH 7.0, ε 1260), 307 (pH 10.0, ε 1995), 281(pH 4.0, ε 1250); IR (KBr matrix) 3446 (OH), 1576 (COO^-) cm^{-1}; HRMS *m/z* 378.9406 (M^+, calcd. for $C_{12}H_{15}Br_2O_3N$, 378.9416); ^1H and ^{13}C data are reported in table.

Compound 9: (3.1 mg); $[\alpha]_D^{25}$ -8.33°; UV (H_2O) λ_{max} 277 (ε 1263); IR (KBr matrix) 3446 (OH), 1576 (COO^-) cm^{-1}; HRMS *m/z* 333.8839 [M^+- $N(CH_3)_3$]; ^1H and ^{13}C data are reported in table.

Compound 10: (4.5 mg); $[\alpha]_D^{25}$ -9.00°; UV (H_2O) λ_{max} 277 (ε 1260); IR (KBr matrix) 3446 (OH), 1576 (COO^-) cm^{-1}; HRMS *m/z* 255.9720 [M^+- $N(CH_3)_3$]; ^1H and ^{13}C data are reported in table.

Compound 11: (0.6 mg); $[\alpha]_D^{25}$ -15.00°; UV (H_2O) λ_{max} 287 (pH 7.0, ε 1257), 307 (pH 10.0, ε 1994), 281(pH 4.0, ε 1254); IR (KBr matrix) 3446 (OH), 1576 (COO^-) cm^{-1}; HRMS *m/z* 301.0323 (M^+, calcd. for $C_{12}H_{16}BrO_3N$, 301.0310); ^1H and ^{13}C data are reported in table.

ACKNOWLEDGEMENTS

We wish to thank Prof. W. Fenical for giving us the opportunity to partecipate in an expedition to the Caribbean Sea, during which the sponge *P. crassa* was collected. This work is a result of a research supported by CNR, contr. 91-3242 and by M.U.R.S.T. (40 and 60%), Rome, Italy. Mass spectra were provided by "Servizio Spettrometria di Massa del CNR e dell'Università di Napoli". NMR, UV and IR spectra were performed at "Centro Interdipartimentale di Analisi Strumentale", Università di Napoli "Federico II". The assistance of the staff is gratefully appreciated.

REFERENCES

1. Hyatt A.; *Mem. Boston Soc. Nat. Hist.*, **1875**, *2*, 481-554.
2. Makarieva T. N.; Stonik V. A.; Alcolado P., and Elyakov Y. B.; *Comp. Biochem. Physiol.*, **1981**, *68B*, 481-484.
3. Litaudon M. et Guyot M.; *Tetrahedron Letters*, **1986**, *27*, 4455-4456.
4. Kassuhlke K. E. and Faulkner D. J.; *Tetrahedron*, **1991**, *47*, 1809-1814.
5. Wiedenmayer F.; *Shallow-water sponges of the Western Bahamas*, Birkheauser Verlgas, Basel und Stuttgart, 1977, 1-287.
6. Soest, R. W. M. van; *Stud. Fauna Curacao Caribb. Isl.*, **1978**, *56*, 1-94.
7. Fattorusso E.; Minale L. and Sodano G.; *J: C: S: Perkin I*; **1972**, *35*, 16-18.
8. Costantino V.; Fattorusso E. and Mangoni A.; *J. Org. Chem.*, **1993**, *58*, 186-191.
9. Bhacca N. S. and Williams D. H.; *Applications of NMR Spectroscopy in Organic Chemistry*; Holden-Day, INC; San Francisco; 1966, 19-23.
10. Iida T.; Tamura T., Miura N., Matsumoto T.; *Steroids*, **1977**, *29*, 453-460.
11. Fattorusso E., Giovannitti B., Lanzotti V., Magno S. and Violante V.; *Steroids*, **1992**, *57*, 119-121.
12. Breitmaier E., Voelter W.; *Carbon-13 NMR Spectroscopy*, 3rd ed.; VCH Publishers, New York, 1987, 340-357.

(*Received in UK* 30 *July* 1993; *revised* 8 *October* 1993; *accepted* 15 *October* 1993)