Chemistry of Verongida Sponges. 9.[1] Secondary Metabolite Composition of the Caribbean Sponge *Aplysina cauliformis*

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A detailed analysis of the secondary metabolites of the sponge *Aplysina cauliformis* has been performed. Eight compounds were identified, two of which (**13** and **14**) are new bromotyrosine derivatives whose structures were determinated from spectroscopic evidence, including 2D NMR. The new compounds were analyzed for cytotoxic activity, and compound **14** was shown to inhibit mammalian protein synthesis and cell proliferation.

Our recent research on the secondary metabolites of *Verongida* sponges has been primarily directed at defining chemical profiles of each species. These could be useful as chemotaxonomic markers and help overcome the difficulties inherent in the identification of *Verongida* sponges. Additional interest in the secondary metabolites of *Verongida* is prompted by the bioactivity they often exhibit.

In this paper, we wish to report the results obtained from a detailed analysis of the Caribbean sponge *Aplysina cauliformis* (Carter, 1882), belonging to the order Verongida, family Aplysinidae Carter, 1875.

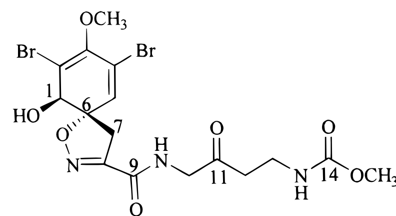
Results and Discussion

The sponge, accurately described by Wiedenmayer (1977)[2], has a creeping or erect habit, with long and rather thin branches starting often from an encrusting base. The sponge surface may be finely conulose to rugose. The external color of our specimens ranges in life from yellow-brown to brown-violet, becoming much darker out of the water. The inside color is always yellow-ochre. The skeleton consists of a reticulation of pithed fibers forming roundish or elongated, rectangular meshes. The stiff consistency, hardly compressible especially in preserved specimens, together with color, may allow *A. cauliformis* to be distinguished from the closely related species *A. fulva* (Pallas, 1776), which is softer and always shows yellow tinges. Both species share the same habitat and are to be found within the rocky bottom reef community in shallow water ranging from 5 to 20 m depth.

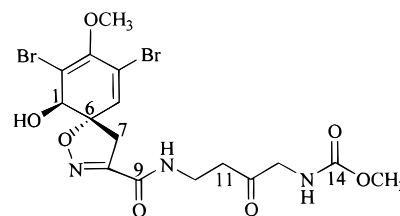
Some data on the sterols and secondary metabolites of *A. cauliformis* have already been reported. In 1981, Makarieva et al.[3] isolated from two specimens of this sponge collected along the Cuban coast the bromo compounds **1**−**4**. In 1993, Rodrìguez et al.,[4] studying specimens collected from the coastal waters of Puerto Rico, isolated, in addition to the known 11-oxoaerothionin (**5**), aplysinamisines I, II, and III (**6**, **7, 8** respectively) (Chart 1).

In July 1990, we collected six different specimens of *A. cauliformis* (SS 14 01, SS 13 11, 1410, 1610, 1902, 3029) along the coasts of the Bahamas Islands (San Salvador, Eleuthera, Grand Bahama, and Andros Islands). They were stored frozen at −20 °C until extraction. Subsamples have been deposited in the sponge collection of the Istituto di Zoologia dell'Università di Genova under the same reference numbers.

Preliminary analysis indicated that compounds **1** and **2** were the major bromometabolites of all six collected specimens (Table 1). A more extended investigation, performed only on the largest specimen (SS 14 01), led to the isolation, in addition to the above compounds, of the bromometabolites **9** and **10**, crasseride (**11**), the imidazotetrahydropyrimidine (**12**), all previously isolated from other *Verongida* sponges, and two novel bromotyrosine derivatives, **13** and **14**. Compounds **1**,[5] **2**,[6] **9**,[7] **10**,[8] **11**,[9] and **12**,[10] were identified by comparison of their spectral data with literature values. It is to be noted that compound **2** is very likely an artifact, formed from the ketone **15** during the extraction procedure, as frequently reported for several *Verongida* sponges.[11]



13



14

The molecular formula $C_{16}H_{19}Br_2N_3O_7$ for the new compound **13** was suggested by the ESI mass spectrum, which shows a 1:2:1 triplet for the molecular ion centered at *m/z* 526 $[M + H]^+$, consistent with the presence of two bromine atoms in the molecule; it was confirmed by the ^{13}C NMR spectrum and DEPT experiment. UV absorption at λ_{max} 280 and 229 nm suggested the presence of a cyclohexadienyl moiety, while the IR bands at 1665 and 3450 cm^{-1} suggested the presence of an α- iminoamide and an alcohol function, respectively. An inspection of ^1H, ^{13}C, and $^1H-^1H$ COSY NMR spectra suggested that the above

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Chart 1

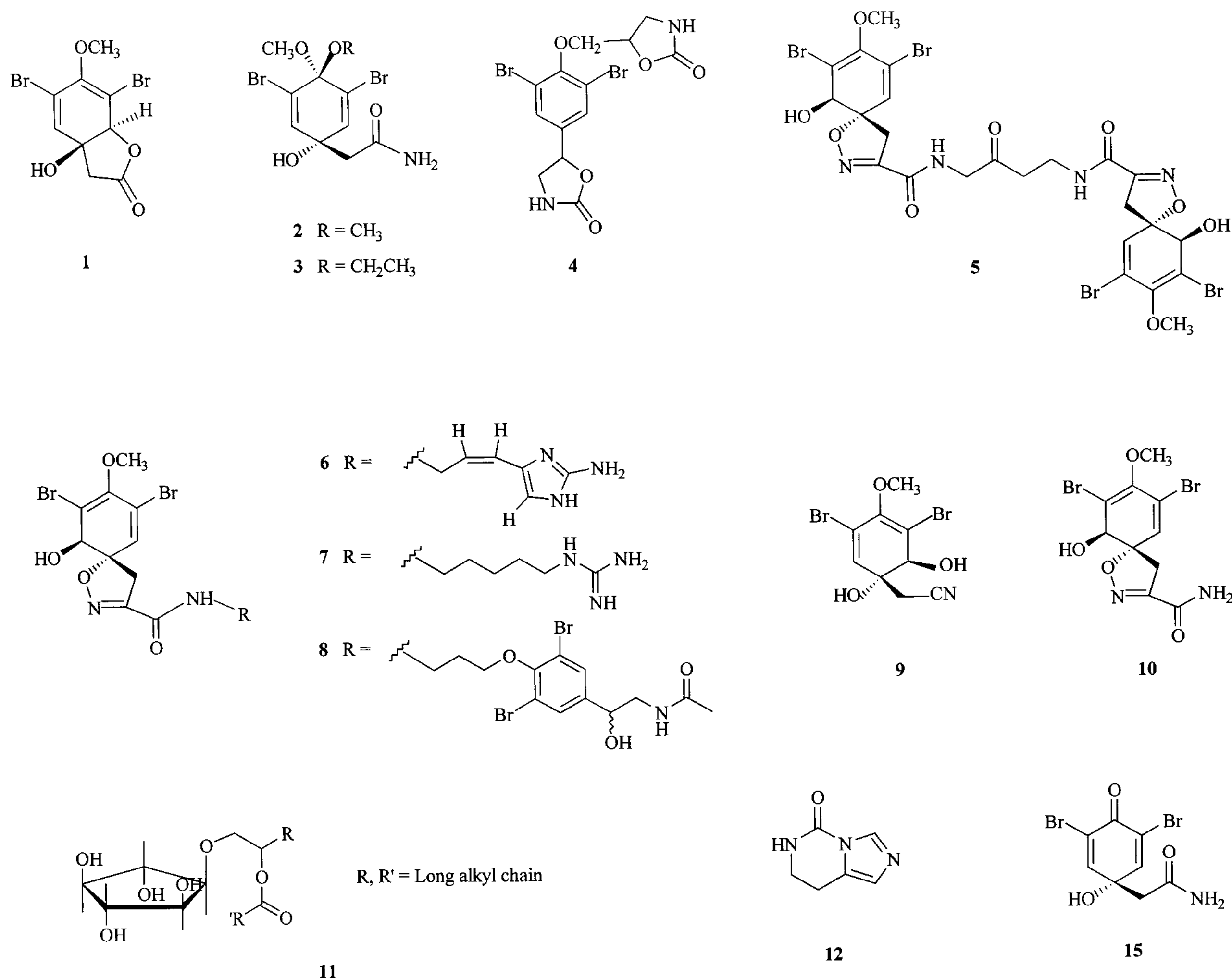

Table 1. Percentage of Metabolites in Specimens of *Aplysina cauliformis*

compd	percentage	compd	percentage
1	1.14	**11**	4.56
2	6.39	**12**	1.82
9	0.82	**13**	0.04
10	0.27	**14**	0.09

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

functionalities were incorporated into one 1-hydroxy-2,4-dibromo-3-methoxy-8-carbamoyl spirocyclohexadienyl isoxazole partial structure. This is a structural feature commonly found in brominated metabolites of *Verongida* sponges.

The remaining acyclic part of the molecule consisted of a ketonic carbonyl, three methylene groups, and one methoxycarbamate group as indicated by the molecular formula, ^{13}C NMR data (δ 205.75, 48.58, 39.75, 35.21, 157.03, and 51.28, respectively), and IR absorptions at 1715 and 1730 cm^{-1}. The sequential arrangement of the above groups was supported by several two-dimensional NMR experiments: the ^{1}H$-^{1}$H COSY NMR spectrum delineated the NH$-$C-10 and the C-12$-$C-13$-$NH segments; the HMBC spectrum indicated a long-range correlation between C-9 (δ 159.13) and the methylene protons at δ 3.99, H_2-10. The chemical shifts of C-10 (δ 48.58) and C-12 (δ 39.75) and those of the related protons H_2-10 (δ 3.99) and H_2-12 (δ 2.61) compared with data reported in the literature [12] pointed to the presence of the ketonic function at C-11. This was confirmed by observation of a ROESY correlation between H_2-10 and H_2-12. The presence and the position of the methoxycarbamate function was suggested by the typical chemical shift in the ^{13}C NMR spectrum of the OCH_3 (δ 51.28) and confirmed by the HMBC correlation between the methoxyl protons (δ 3.50) and C-14 (δ 157.03). The above data supported the assignment of the structure **13**. The trans geometry of the two vicinal oxygen atoms at C-1 and C-6, which is a common feature of all natural spirocyclohexadienyl isoxazole bromo compounds, was indicated by ^{1}H and ^{13}C NMR chemical shift values of the spiro system atoms.[13] The absolute stereochemistry of the chiral centers C-1 and C-6 was suggested by the CD spectrum [(MeOH) $[\theta]_{284}$ +52 500; $[\theta]_{248}$ +61 000], which was analogous to that reported for aerothionin,[14] whose absolute stereochemistry was established by X-ray studies as 1*R*,1′*R*,6*S*,6′*S*.

The remaining new compound **14**, isolated in larger amounts, exhibited UV, IR, and ESI mass spectra identical to those of **13**. Comparison of their ^{1}H and ^{13}C NMR data confirmed the strong similarity between the two compounds. The 1-hydroxy-2,4-dibromo-3-methoxy-8-carbamoylspirocyclohexadienylisoxazole part of the compound **14** was identified as shown for the compound **13**. The acyclic part of the molecule was composed of the same structural units as in **13**, but the ketone was located in a different position. The ^{1}H$-^{1}$H COSY NMR spectrum delineated NH$-$C-10$-$C-11 and C-13$-$NH segments, and therefore, the carbonyl function was assigned to C-12. This was

Table 2. ^{13}C and ^{1}H Assignment for Compounds **13** and **14** (DMSO-d_6)[a]

	13		14	
carbon	δ_C, (mult)	δ_H (mult, *J*/Hz)	δ_C, mult	δ_H (mult, *J*/Hz)
1	73.57 (d)	3.93 (d, 8.57)	73.56 (d)	3.91 (d, 8.23)
2	120.86 (s)		120.79 (s)	
3	147.16 (s)		147.12 (s)	
4	113.11 (s)		113.04 (s)	
5	131.23 (d)	6.60 (s)	131.21 (d)	6.57 (s)
6	90.52 (s)		90.24 (s)	
7a	39.33 (t)	3.62 (d, 18.38)	39.50 (t)	3.61 (d, 18.33)
7b		3.21 (d, 18.38)		3.18 (d, 18.33)
8	154.08 (s)		154.34 (s)	
9	159.13 (s)		158.87 (s)	
10	48.58 (t)	3.99 (d, 5.48)	33.83 (t)	3.34 (dt, 6.51, 5.93)
11	205.75 (s)		38.29 (t)	2.67 (t, 6.51)
12	39.75 (t)	2.61 (t, 6.80)	205.66 (s)	
13	35.21 (t)	3.16 (dt, 6.80, 4.80)	49.89 (t)	3.83 (d, 6.17)
14	157.03 (s)		156.98 (s)	
OCH_3−3	59.79 (q)	3.64 (s)	59.60 (q)	3.65 (s)
OCH_3−14	51.28 (q)	3.50 (s)	51.54 (q)	3.55 (s)
NH-9		8.64 (t, 5.48)		8.43 (t, 5.93)
NH-13		7.09 (t, 4.80)		7.34 (t, 6.17)
OH		6.42 (d, 8.57)		6.33 (d, 8.23)

[a] Assignment based on DEPT, COSY, HMQC, and HMBC experiments.

confirmed by a ROESY correlation between CH_2-11 and CH_2-13. The methoxycarbamate function was indicated by the typical chemical shift of OCH_3 (δ 51.54) and by the HMBC correlation between the methoxyl group at δ 3.55 and C-14 (δ 156.98), which in turn was correlated to H-13. The absolute stereochemistry of the spirocyclohexadienyl-isoxazole moiety, analogous to compound **13**, was deduced from the CD spectrum, which was superimposable with that reported for aerothionin.[14]

It has been reported previously that *Verongida* sponges yield cytotoxic compounds, so we investigated the capability of compounds **13** and **14** to affect cell proliferation. The assay was performed on HeLa cells by estimating the amount of tritiated thymidine incorporated in the DNA according to Kern et al.[15] Compound **14** was shown to have an IC_{50} of 50 μg/mL.

Compound **14** was also found to slightly affect the mammalian protein synthesis in a cell free system obtained by preparing S30 from the hepatoma cell line according to Traub et al.[16] and was completely inactive on a bacterial cell free system.

Experimental Section

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 (DMSO-d_6: ^{1}H δ 2.05, ^{13}C δ 39.7). Methyl, methylene, and methine carbons were distinguished by a DEPT experiment. Electrospray mass spectra were obtained on a VC Prospec Fisons 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 spectrophotometer in MeOH solution. The CD spectra were recorded in MeOH solution on a JASCO J710 spectropolarimeter. Medium-pressure liquid chromatographies (MPLC) were performed on a Büchi 861 apparatus using SiO_2 (230−400 mesh). High-performance liquid chromatography (HPLC) was perfomed on a Varian 2510 apparatus equipped with an RI-3 index detector, using Hibar columns.

Animal Material. Fresh sponge, *A. cauliformis*, was collected in the summer of 1990 along the coast of San Salvador Island (depth −15 m), stored frozen at −20 °C, and identified by Prof. M. Pansini, University of Genova, Italy. Reference specimens (voucher numbers SS 14 01, SS 13 11, 1410, 1610, 1902, 3029) are deposited at the Istituto di Zoologia dell'Università di Genova, Italy.

Extraction and Isolation. The initial procedure employed for the extraction and fractionation of the lipid mixture was perfomed as follows. The homogenized material was extracted with MeOH/toluene (3 × 1 L) and subsequently with $CHCl_3$ (3 × 1 L) at room temperature. The combined MeOH/toluene solution, after filtration, was concentrated in vacuo to give an aqueous suspension that was extracted with EtOAc. The combined EtOAc and $CHCl_3$ extracts (10.96 g of a dark brown oil) were chromatographed by MPLC on a SiO_2 column using a solvent gradient system from *n*-hexane to EtOAc and then to MeOH. Selected fractions were combined on the basis of TLC analyses. Fractions eluted with *n*-hexane/EtOAc (3:7) (fraction A), *n*-hexane/EtOAc (2:8) (fraction B), and EtOAc (fraction C and D) containing bromotyrosines and a fraction eluted with EtOAc/MeOH 8:2 (fraction E) containing compounds **11** and **12** were further separated by HPLC.

Evaporation of fraction A afforded 215 mg of a mixture that was purified by HPLC using a Hibar LiChrospher Si 60 (10 × 250 mm) column with a mobile phase of EtOAc/$CHCl_3$ (1:1) to obtain 90 mg of pure aeroplysinin-1, **9**, and 125 mg of aeroplysinin-2, **1**, identified by comparison of their spectral properties with literature values.[5,7] Fraction B was purified by HPLC using a Hibar LiChrospher Si 60 (10 × 250 mm) column with a mobile phase of *n*-hexane/EtOAc (3:7) and yielded pure compound **10** (30 mg), identified by comparison of its spectral properties with literature values.[8] Fraction C was purified by HPLC on a Hibar LiChrospher Si 60 (10 × 250 mm) column using *n*-hexane/EtOAc (1:9) as eluent. This afforded 700 mg of pure **2** identified by comparing its spectral properties with literature values.[6] Fraction D was chromatographed by HPLC on a Hibar LiChrospher Si 60 (10 × 250 mm) column using EtOAc as the mobile phase, giving 16.3 mg of compound **14** as a pure compound and a mixture containing compound **13**. The latter was rechromatographed by HPLC on a LiChroprep RP-18 column (10 × 250 mm) using a linear gradient system from 100% H_2O containing 0.1% of trifluoroacetic acid to 100% MeOH containing 0.1% of trifluoroacetic acid in 35 min and gave 4.5 mg of pure compound **13**. Fraction E was a mixture containing compounds **11** and **12**. It was resolved by HPLC on a Hibar LiChrospher Si 60 (10 × 250 mm) column using EtOAc/MeOH (9:1) and afforded 50 mg of **11** and 200 mg of **12** identified by comparison of their spectral properties with those reported in the literature.[9,10] The nature and the length of alkyl chains of compound **11** has not been determined.

Compound 13: UV (MeOH) λ_{max} 280 nm (ϵ 10 600), 229 nm (ϵ 18 500); IR (KBr) ν_{max} 3450, 1730, 1715, 1665 cm^{-1}; CD (MeOH) $[\theta]_{284}$ +52 500; $[\theta]_{248}$ +61 000; ^{1}H NMR and ^{13}C NMR

data (DMSO-d_6, 500 MHz) are reported in Table 2; ESIMS [M + H]$^+$ 524, 526, 528.

Compound 14: UV (MeOH) λ_{max} 281 nm (ϵ 10 500), 227 nm (ϵ 19 000); IR (KBr) ν_{max} 3450, 1730, 1715, 1665 cm^{-1}; CD (MeOH) $[\theta]_{284}$ +51 000; $[\theta]_{248}$ +60 000; ^{1}H NMR and ^{13}C NMR data (DMSO-d_6, 500 MHz) are reported in Table 2; ESIMS [M + H]$^+$ 524, 526, 528.

Biological Assays. The in vitro assays on cell proliferation and on inhibition of protein synthesis have been performed according to the methods of Kern et al.[15] and Traub et al.,[16] respectively.

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References and Notes

(1) Part 8: Ciminiello, P.; Fattorusso, E.; Forino, M.; Magno, S. *Tetrahedron* **1997**, *53*, 6565−6572.
(2) Wiedenmayer, F. *Shallow-water sponges of the Western Bahamas*; Birkhaeuser Verlag: Basel und Stuttgart, 1977; p 1−287.
(3) Makarieva, T. N.; Stonik, V. A.; Alcolado, P.; Elyakov, Y. B. *Comp. Biochem. Physiol.* **1981**, *68B*, 481−484.
(4) Rodriguez, A. D.; Pina, I. C. *J. Nat. Prod.* **1993**, *56*, 907−914.
(5) Minale, L.; Sodano, G.; Chan, W. R.; Chen, A. M. *J. Chem. Soc., Chem. Commun.* **1972**, 674−675.
(6) Sharma, G. M.; Vig, B.; Burkholder, P. R. *J. Org. Chem.* **1970**, *35*, 2823−2826.
(7) Roll, D. M.; Chang, C. W. J.; Scheuer, P. J.; Gray, G. A.; Shoolery, J. N.; Matsumoto, G. K.; Van Duyne, G. D.; Clardy, J. *J. Am. Chem. Soc.* **1985**, *107*, 2916−2920.
(8) Ciminiello, P.; Fattorusso, E.; Magno, S.; Mangoni, A.; Pansini, M. *J. Nat. Prod.* **1994**, *57*, 1564−1569.
(9) Costantino, V.; Fattorusso, E.; Mangoni, A. *J. Org. Chem.* **1993**, *58*, 186−191.
(10) Ciminiello, P.; Costantino, V.; Fattorusso, E.; Magno, S.; Mangoni, A.; Pansini, M. *J. Nat. Prod.* **1994**, *57*, 705−712.
(11) Andersen, R. J.; Faulkner, D. J. *Tetrahedron Lett.* **1973**, *14*, 1175−1178.
(12) Acosta, A. L.; Rodriguez, A. D. *J. Nat. Prod.* **1992**, *55*, 1007−1012.
(13) Nishiyama, S.; Yamamura, S. *Bull. Chem. Soc. Jpn.* **1985**, *58*, 3453−3456.
(14) McMillan, J. A.; Paul, I. C.; Goo, Y. M.; Rinehart, K. L.; Klueger, W. C.; Pschigoda, L. M. *Tetrahedron Lett.* **1981**, *37*, 39−42.
(15) Kern, D. H.; Drogemuller, C. R.; Kennedy, M. C.; Hidebrand-Zanki, S. V.; Tanigawa, N.; Sandak, V. K. *Cancer Res.* **1985**, *45*, 5436−5441.
(16) Traub, P.; Mizushima, S.; Lowry C. V.; Nomura, M. *Methods Enzymol.* **1971**, *20*, 391−407.
(17) Ciminiello, P.; Dell'Aversano, C.; Fattorusso, E.; Magno, S.; Carrano, L.; Pansini, M. *Tetrahedron* **1996**, *52*, 9863−9868.
(18) Ciminiello, P.; Fattorusso, E.; Magno, S.; Pansini, M. *Biochem. Syst. Ecol.* **1996**, *24*, 105−113.
(19) Van Soest, R. W. M. *Studies on the Fauna of Curaçao and other Caribbean Islands*; Wagenaar Hummelinck, P., and Van Der Steen, L. J., Eds.; Scien. Res. Surinam and Netherlands Ant.: Utrecht, 1978; Vol. 56, p 1−94.

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