Bromoisoxazoline Alkaloids from the Caribbean Sponge *Aplysina insularis*

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An investigation of a specimen of the Caribbean sponge *Aplysina insularis* resulted in the isolation of fourteen bromoisoxazoline alkaloids (**1**–**14**), of which 14-oxo-aerophobin-2 (**1**)* is a novel derivative. Structure elucidation of the compounds have been established from spectral studies and data for **1** are reported. Constituents **2** to **6** and **11** to **14** have not been identified sofar in *Aplysina insularis* species. The presence of the known compounds **7** to **9** in *Aplysina insularis* indicates that their use for chemotaxonomical purposes is questionable.

Introduction

Dienone (**16)** and dimethoxyketal (**17**) were the first compounds to be isolated out of the large group of bromotyrosine derivatives from two marine sponges, *Aplysina cauliformis* and *Aplysina fistularis*, of the family Aplysinidae (Sharma and Burkholder, 1967; Minale *et al.*, 1976) during a search for new sponge constituents with antimicrobial activity. Of these two secondary metabolites only the dienone showed biological activity against Gram-positive and Gram-negative bacteria (Sharma and Burkholder, 1967). Subsequently *Aplysina fistularis* has yielded almost thirty bromotyrosine derivatives with different biological activities. *Aplysina fistularis* Pallas (1766) is a synonym for various other sponges such as *Verongia thiona, Verongia tenuissima* and *Verongia aurea* (Carney and Rinehart, 1995). Detailed investigations of these led to a separation of the species *Aplysina fistularis* into *Aplysina fulva* and *Aplysina insularis* (Pulitzer-Finali, 1986).

During a scientific cruise to the Caribbean Sea in August 1995 we were able to collect 55 sponge specimens all belonging to the Demospongiae class. An initial identification of the sponges on the cruise suggested one species of the Aplysinellidae family namely *Pseudoceratina crassa* and seven different species of the Aplysinidae family namely *Aplysina insularis, Aplysina fulva, Aplysina lacunosa, Aplysina cauliformis, Aplysina archeri, Verongula gigantea* and *Verongula rigida*. Crude methanolic extracts of small freeze dried vouchers of all 55 samples were prepared and analyzed by UV-detected HPLC chromatography. The composition of the HPLC patterns supported the initial classifications as different patterns characteristic of the different species could be recognized, although a detailed analysis of the specimens for chemotaxonomic purposes has not yet been performed. Subsequently *Aplysina insularis* was chosen for further investigation as this showed the largest amounts of compounds and highest diversity of the different peaks in the hydrophilic and lipophilic regions of the chromatogram. We report here the identity of the secondary metabolites isolated from this sponge.

Materials and Methods

Aplysina insularis was collected off the shore of Long Island, Bahamas, by snorkeling (1–2 m depth). The voucher specimen consists of a short tube 5 cm in height and 2 cm in diameter, with

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terminal vent (oscule) 0.7 cm in diameter, and a short side fistule. Originally the exterior was yellow in colour but turned black upon transport of the sponge to the surface; the interior is dark reddish brown. The sponge surface shows some shallow longitudinal depressions but is otherwise smooth. The skeleton consists of laminated amber-coloured spongin fibres 38–100 μm in diameter with a continuous pith of 14–47 μm in diameter (comprising 36–62% of the fibre diameter). The pith is characteristically black or dark-brown in transmitted light. The fibres form an isodictyal reticulation of elongated-polygonal meshes of 800–900 × 1200–1500 μm in expansion. These characteristics conform to the type *Aplysina insularis*. A voucher specimen is deposited under registration no. ZMA POR. 11059 in the Zoölogisch Museum, Amsterdam, The Netherlands.

The sponge samples were immediately frozen after collection and kept at −20 °C until extraction. After lyophilisation the resulting tissue (17.6 g dry weight for the investigated specimen) was ground to a powder and exhaustively extracted with MeOH (3 × 300 ml) at room temperature. Evaporation of the solvent yielded the crude extract, which was partitioned between EtOAc (5 × 200 ml) and water (200 ml). The aqueous layer was subsequently extracted with water-saturated *n*-BuOH (5 × 200 ml). The resulting EtOAc (1.2 g) and *n*-BuOH (1.2 g) fractions were taken to dryness and redissolved in a mixture of CH_2Cl_2 / MeOH (95:5 v/v and 1:1 v/v respectively). The EtOAc fraction was subjected to a silicagel 60 (Merck, Germany) column with a mixture of CH_2Cl_2 / MeOH (95:5 v/v) as eluent and the resulting fractions were collected and monitored by TLC on precoated TLC plates (Si gel 60 F_{254}, Merck, Darmstadt, Germany) using the same solvent mixture. UV detection of the different constituents was performed at 254 and 366 nm. Further purification of the isolated compounds was achieved by column chromatography on Sephadex LH 20 using acetone and a mixture of MeOH/CH_2Cl_2 (1:1 v/v) as eluents. As final purification step column chromatography on reversed phase LiChroprep® RP-18 columns (Merck, Darmstadt, Germany) was used with MeOH/H_2O/TFA mixtures (80:20:0.01, 80:20:0 and 70:30:0 v/v/v) as eluent systems. Examination of the *n*-BuOH fraction started with column chromatography on Sephadex LH 20 using MeOH and a MeOH/CH_2Cl_2 (1:1 v/v) mixture as eluent system. Further purification steps included silicagel 60 (Merck, Germany) column chromatography using a mixture of MeOH/CH_2Cl_2 (80:20 v/v) as eluent followed by Sephadex LH 20 column chromatography with MeOH as eluent. Again final purification of the isolated constituents was achieved using reversed phase LiChroprep® RP-18 columns (Merck, Darmstadt, Germany) and a MeOH/H_2O (1:1 v/v) mixture as eluent. Detection of the isolated secondary metabolites during the isolation process was performed in the same way as described above for the EtOAc fraction.

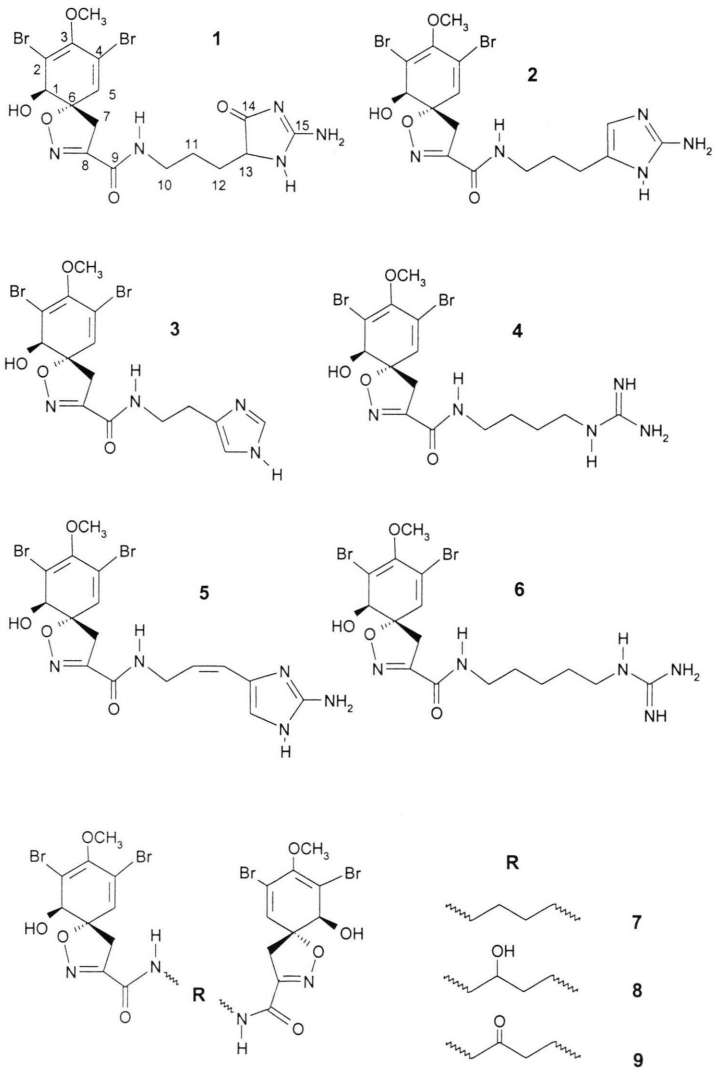
For structure elucidation ^1H (1D and 2D COSY) NMR and ^{13}C (1D and DEPT-135) NMR spectra were recorded on a Bruker AM 300 NMR spectrometer locked to the major deuterium resonance of the solvent, CD_3OD. CD spectra were recorded in methanolic solution on a Jobin Yvon CD 6 spectropolarimeter. High performance liquid chromatography (HPLC) was performed on a Gynkotek HPLC system (Gynkotek, Germany) equipped with photodiode array detector, using a routine detection wavelength of 254 nm. All separations were performed on a prefilled Eurospher C-18 column (Knauer GmbH, Germany, 125 × 4 mm i.d.) applying a gradient starting with 100% H_2O (pH 2, adjusted with phosphoric acid) for the first five min and running up to 100% MeOH within the following 35 min to keep this eluent for another five min. Prior to use all solvents had been distilled, whereas spectral grade solvents were used for all spectroscopic experiments.

Results and Discussion

The isolation of secondary metabolites from the dictyoceratid sponge *A. insularis* yielded fourteen (**1**–**14**) constituents, of which 14-oxo-aerophobin-2 (**1**) (Fig. 1) is a novel bromoisoxazoline derivative. The *n*-BuOH soluble fraction of the crude methanolic extract of *A. insularis* gave seven different bromotyrosine derivatives namely the previously described aerophobin-1 (**3**) (40.3 mg) (Cimino *et al.*, 1983), aerophobin-2 (**2**) (21.7 mg) (Cimino *et al.*, 1983), aplysinamisin-1 (**5**) (3.4 mg) (Rodriguez and Pina, 1993), aplysinamisin-2 (**6**) (9.7 mg) (Rodriguez and Pina, 1993), purealidin L (**4**) (29.3 mg) (Kobayashi *et al.*, 1995), 2-(3,5-di-

brom-4-methoxyphenyl)ethyltrimethylammonium chloride (**13**) (33.7 mg) (Ciminiello *et al.*, 1994) and the novel derivative 14-oxo-aerophobin-2 (**1**) (11.3 mg), while the EtoAc fraction yielded seven more constituents. Except for the quite unusual marine natural product p-hydroxybenzoic acid ethyl ester (ethylparaben) (**14**) (4.2 mg) the remaining six compounds belong to the group of bromoisoxazoline alkaloids, namely the known derivatives aerothionin (**7**) (91.8 mg) (Fattorusso *et al.*, 1970), 11-oxo-aerothionin (**9**) (8.0 mg) (Acosta and Rodriguez, 1992), 11-hydroxy-aerothionin (**8**) (7.4 mg) (Kernan *et al.*, 1990), fistularin-3 (**10**) (72.4 mg) (Gopichand and Schmitz, 1979), 11,19-dideoxyfistularin-3 (**11**) (3.2 mg) (Kernan *et al.*, 1990) and methyl (6*R*, 5*S*)-7,9-dibrom-6-hydroxy-8-methoxy-1-oxa-2-azaspiro[4.5]deca-2,7,9-trien-3-carboxylate (**12**) (3.2 mg) (Ciminiello *et al.*, 1994).

Structure elucidation of the constituents **2–13** was achieved by comparing the spectral data to those reported in the literature. The structure of (**14**) (*p*-hydroxybenzoic acid ethyl ester = ethylparaben) was confirmed by comparison of MS-, UV- and HPLC-data with the commercially available standard substance (Sigma) and by coelution of the isolated constituent and the standard in a HPLC experiment.



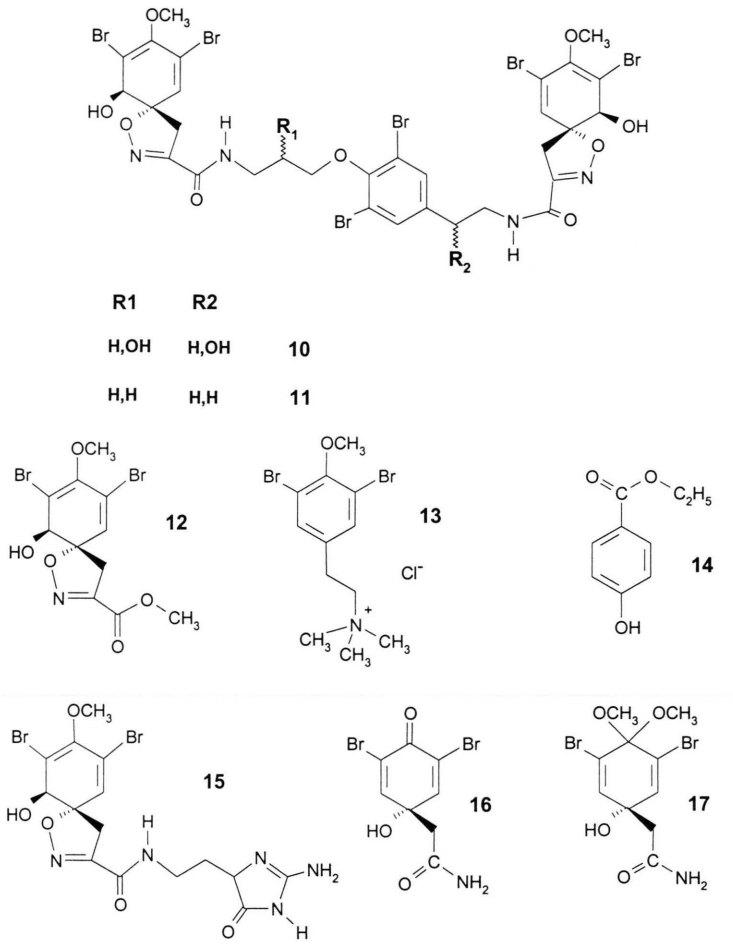


Fig. 1. Secondary metabolites of *Aplysina insularis* and related species.

The bromoisoxazoline derivative 14-oxo-aerophobin-2 (**1**) showed an isotopic cluster of MH^+ ions in the ratio of 1:2:1 at *m/z* 520, 522, 524 in the FAB-MS spectrum, indicating the presence of two bromine atoms in the molecule. Comparing the fragmentation pattern of (**1**) (Fig. 2 and 3) to the previously described aerophobin-2 (**2**) (Cimino *et al.*, 1984) the FAB-MS and the NMR data indicated a molecular formula $C_{16}H_{19}Br_2N_5O_5$. By comparing the ^1H and ^{13}C NMR spectra of (**1**) (Tables I and II) to those of the previously reported aerophobin-2 (**2**) (Cimino *et al.*, 1983) and purealidin K (**15**) (Kobayashi *et al.*, 1995) and by analyzing the ^1H-^1H COSY spectrum of (**1**), the entire structure could be elaborated to be that shown (**1**).

Table I. ^1H NMR spectral data of **1** in CD_3OD.

Position	Chemical shift (δ)
H-1[a]	4.13 (1H, s)
H-5[a]	6.46 (1H, s)
H-7a	3.81 (1H, d, *J* = 18.3)
H-7b	3.13 (1H, d, *J* = 18.3)
H-10	3.34–3.37 (m, under solvent)
H-11	1.65 (2H, m)
H-12a	1.87 (1H, m)
H-12b	1.65 (1H, m)
H-13	4.09 (1H, dd, *J* = 5.2 / 5.2)
3-OCH_3	3.77 (3H, s)

[a] These two signals show a crosspeak in the COSY spectrum.

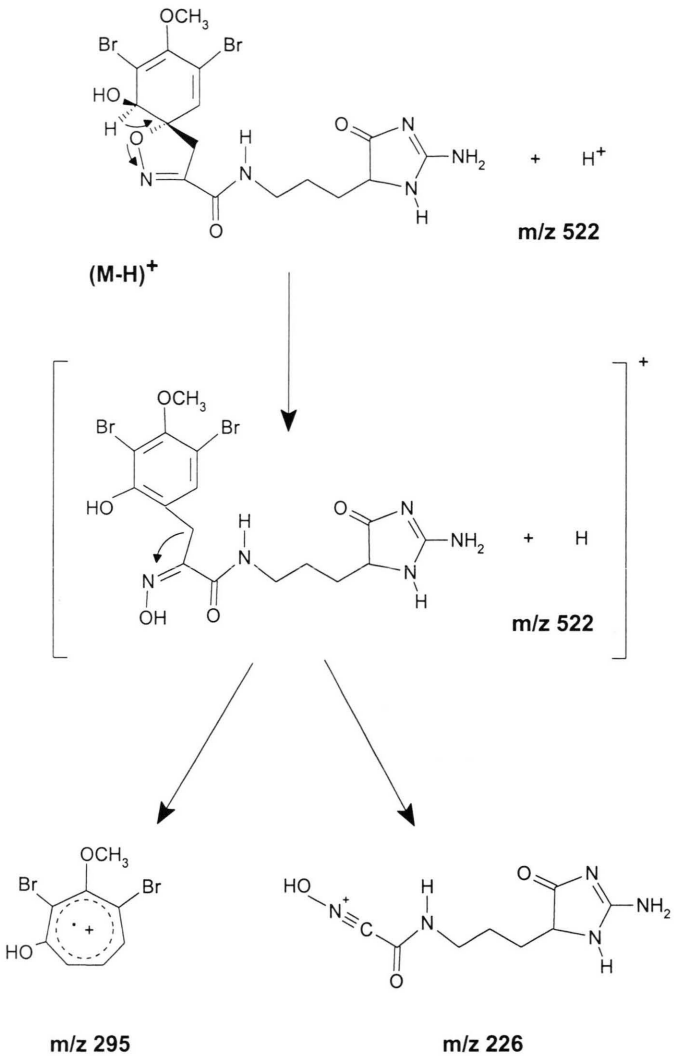


Fig. 2. Fragmentation pattern of **1**.

In the ^{1}H NMR spectrum the new derivative (**1**) showed the same characteristic proton signals of the spirocyclohexadiene-isoxazole ring system as aerophobin-2 (**2**) at 4.13 ppm (1H, s, H-1), 6.46 ppm (1H, s, H-5), 3.13 ppm (1H, d, H-7b, J = 18.3 Hz) and 3.81 ppm (1H, d, H-7a, J = 18.3 Hz). The presence of the spirocyclohexadiene-isoxazole moiety was confirmed by the characteristic ^{13}C signals of carbons 1 to 9 (Table II). The presence of the aliphatic side chain (H-10 to H-13) was evident in the 2D COSY spectrum in which protons 10 to 12 were to higher field compared to aerophobin-2 (**2**) (Cimino *et al.*, 1983). This difference is a result of the loss of the double bond between C-13 and C-14 in (**1**) and the presence of the aliphatic C-13 carbon atom, that is also found in purealidin K (**15**). The loss of the signal of H-14 at 6.47 found in (**2**) indicated a significant difference at this point in the molecule. Taking the molecular mass, characteristic ^{13}C chemical shifts (Tab.II) and comparison of the ^{13}C NMR data with those of purealidin K (**15**) confirmed the presence of the 14-oxo-derivative of aerophobin-2. Positive Cotton effects in the CD-spectrum of **1** ($c=2.1\times10^{-4}$, MeOH) at 245 nm (Δ^{20}: +1.9) and 287 nm (Δ^{20}: +1.7) indicate that the absolute configuration of C-1 and C-6 are 1*R* and 6*S*, respectively (McMillan *et al.*, 1981).

It should be emphasized that the pattern of bromoisoxazoline alkaloids as elucidated in this study

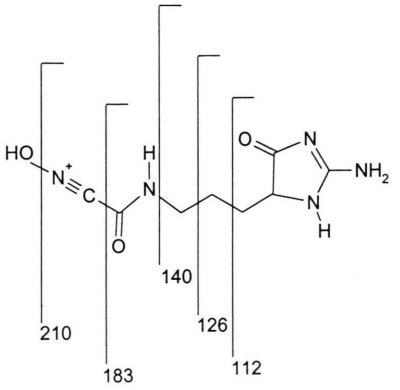


Fig. 3. Decomposition of the ion m/z 226.

Table II. ^{13}C NMR spectral data of **1** in CD_3OD.

Position	Chemical shift (mult.)
C-1	75.5 (d)
C-2	114.1 (s)
C-3	149.3 (s)
C-4	122.8 (s)
C-5	132.3 (d)
C-6	92.3 (s)
C-7	40.2 (t)
C-8	155.3 (s)
C-9	161.6 (s)
C-10	39.9 (t)
C-11	29.8 (t)
C-12	25.6 (t)
C-13	61.9 (d)
C-14	190.6 (s)
C-15	171.4 (s)
3-OCH_3	60.4 (q)

appears to be highly conserved for *A. insularis* as shown by comparative HPLC analysis of an additional 14 specimens of this sponge, that had likewise been collected in the Caribbean in 1995 (data not shown).

Compounds (**2**) to (**6**) and (**11**) to (**14**) have been described previously, although not for this species and thus these are new constituents for *Aplysina insularis*. Aerothionin (**7**) (Fattorusso *et al.*, 1970) and its derivatives 11-hydroxy-aerothionin (**8**) and 11-oxo-aerothionin (**9**) (Ciminiello *et al.*, 1994) have previously been described for *Aplysina fistularis* species (Moody, 1972; Ciminiello *et al.*, 1994) but not specifically for *Aplysina insularis*. On the other hand our isolation of aerothionin (**7**) and its derivatives (**8**) and (**9**) from *Aplysina insularis* contradicts the earlier conclusion of Ciminiello *et al.* (1996) that these special bromoisoxazoline derivatives are chemotaxonomic markers for *Aplysina fulva*. According to our previous studies (Ebel *et al.*, 1997) we have shown that extraction of fresh *Aplysina* sponge tissues or extraction with aqueous solvents gives rise to dienone (**16**) as biotransformation product, while the amounts of genuine constituents such as aerothionin (**7**) and fistularin-3 (**10**) diminish through enzymatic conversion.

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