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Cytotoxic dibromotyrosine-derived metabolites from the sponge *Aplysina gerardogreeni*

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Abstract—The chemical study of the sponge *Aplysina gerardogreeni* collected at the Gulf of California has led to the isolation of four new dibromotyrosine-derived metabolites, aplysinones A–D, whose structures were determined by spectroscopic analysis and chemical methods. The new compounds and four semisynthetic analogues prepared in this study have shown cytotoxic activity against human tumor cell lines.

1. Introduction

Sponges of the Order Verongida are the most prominent natural source of brominated metabolites biogenetically derived from tyrosine. The various modifications occurring at the aromatic ring and the side chain of tyrosine, together with the combination of these tyrosine-derived moieties with amine residues derived from other amino acids, give rise to an array of structurally diverse metabolites, ranging from simple derivatives as aeroplysinin-1 (**1**),[1] to more elaborated compounds as homoaerothionin (**2**)[2] and psammaplin A (**3**),[3] or macrocycles like the bastadins.[4] Since the earlier studies, bromotyrosine-derived metabolites have frequently been shown to possess antibacterial activities,[1,5] and more recently antimycobacterial[6] and inhibitory activity of the detoxification enzyme mycothiol-*S*-conjugate amidase of *Mycobacterium tuberculosis*.[7] Additionally, antiviral,[8] antihistamine,[9] antifouling,[10] antiplasmodial,[11] and antiangiogenic[12] activities have been described. In the antitumor area, a number of bromotyrosine-derived natural compounds have been ascribed cytotoxic activities.[13] A remarkable example can be found in psammaplin A (**3**), a cytotoxic compound displaying potent inhibitory activity of both histone deacetylase (HDAC) and DNA methyl transferase. Furthermore, the synthetic compound NVPLAQ824, which is based in part on the pharmacophores of **3** and another two HDAC inhibitors, has been advanced for an anticancer phase I clinical trial.[14]

As a part of our research directed toward the characterization of new cytotoxic metabolites from sponges, herein we report the results of the chemical investigation of the cytotoxic extract of the sponge *Aplysina gerardogreeni*, collected at the Gulf of California. A previous account on this species described the isolation of aerothionin (**4**) and calafianin (**5**),[15] whose stereochemistry has recently been revised.[16] On the other hand, specimens collected at the Pacific coasts of Panama have been shown to contain aeroplysinin-1 (**1**), and three additional simple dibromotyrosine-derived metabolites.[11] Our study of *A. gerardogreeni* has led to the isolation of the new cytotoxic compounds aplysinones A–D (**6**–**9**).

2. Results and discussion

Freeze–dried specimens of *A. gerardogreeni* were extracted with acetone/MeOH. After solvent evaporation under reduced pressure, the MeOH soluble portion was subjected to column chromatography. The fraction eluted with $CHCl_3$/MeOH (90:10) was subjected to repeated separations over silica gel column and HPLC to yield aplysinones A–D (**6**–**9**).

Keywords: Natural products; Sponges; Dibromotyrosine-derived metabolites; Structure determination; Cytotoxicity.
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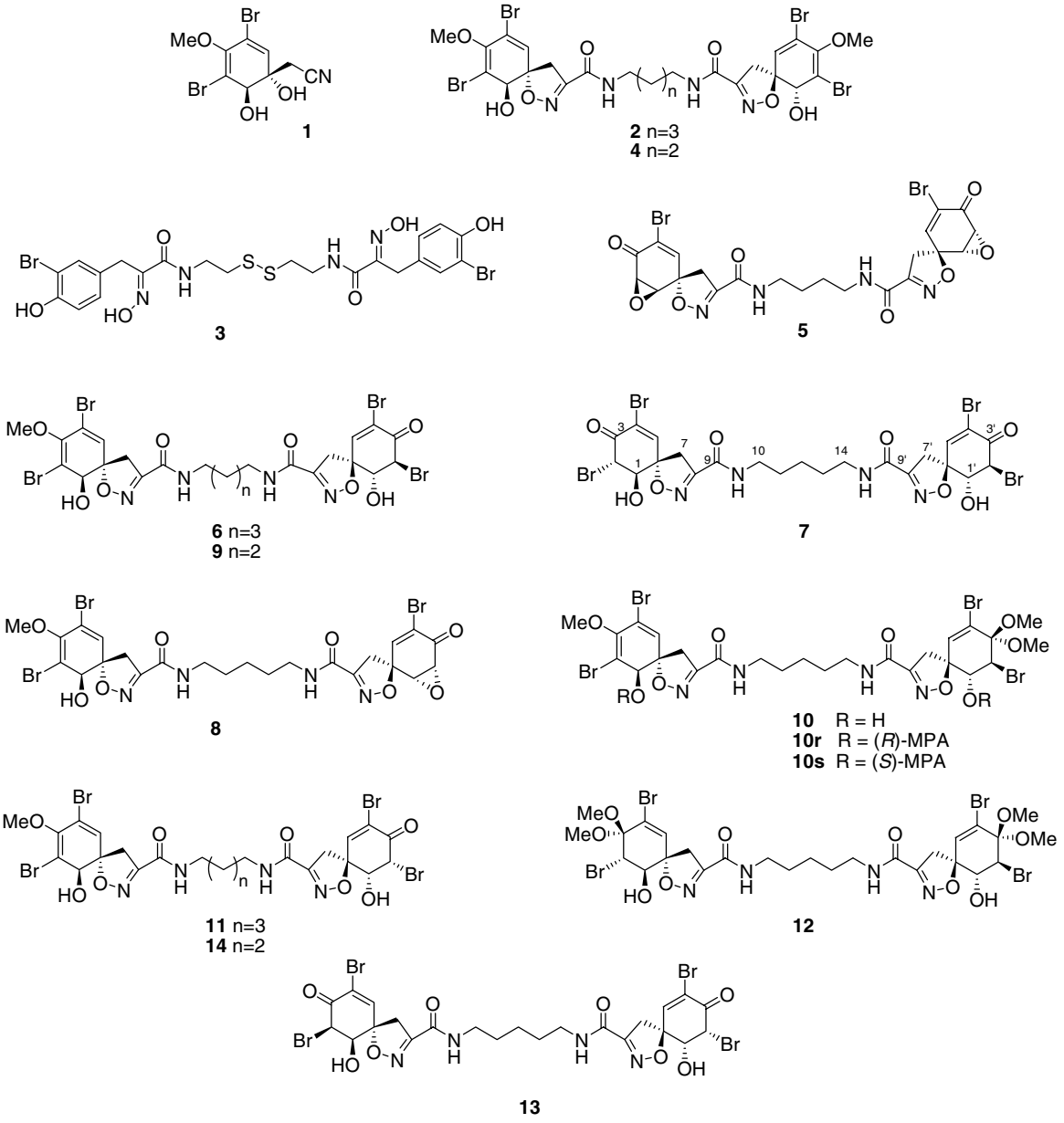

2.1. Aplysinone A (6)

The LRESI(+) mass spectrum of compound **6** exhibited an isotopic cluster for the sodiated molecular ion at *m*/*z* 837:839:841:843:845 (rel. int. 1:4:6:4:1), indicating the presence of four bromine atoms in the molecule. In particular, the molecular formula $C_{24}H_{26}Br_4N_4O_8$ was determined by HRFABMS. The NMR spectra (Table 1) displayed the signals of an *O*-methyl group [δ_H 3.71 (s)], an olefinic proton [δ_H 6.51 (s, H-5)], an oxymethine proton [δ_H 4.16 (d, J = 8.3 Hz, H-1)], and an AB system [δ_H 3.82 (d, J = 18.2 Hz, H-7a) and 3.17 (d, J = 18.2 Hz, H-7b)] that together with an spirocarbon [δ_C 91.5 (s, C-6)] and an amide carbonyl [δ_C 159.9 (s, C-9)] were characteristic of a spirocyclohexadienylisoxazoline moiety derived from dibromotyrosine, such as those present in homoaerothionin (**2**). This proposal was fully confirmed by the HMBC correlations shown in Figure 1.

The NMR spectra also showed the signals of a ketone carbonyl at δ_C 183.7 and a deshielded olefinic methine at δ_C 149.5 (d)/δ_H 7.63 (s) that together with a signal at δ_C 122.5 (s), attributable to a brominated olefinic carbon, were assigned to an α-bromo-α,β-unsaturated ketone moiety. The signals at δ_C 75.1 (d)/δ_H 4.37 (dd, J = 11.5 and 5.6 Hz) and δ_C 57.4 (d)/δ_H 5.07 (d, J = 11.5 Hz) were assigned to two vicinal methines linked to oxygen and bromine atoms, respectively. In the HMBC spectrum, the olefinic proton (H-5′) was correlated with the oxymethine carbon (C-1′), whereas the bromomethine proton (H-2′) showed correlations with the carbonyl carbon (C-3′) and with a fully substituted carbon at δ_C 91.5 (C-6′) (Fig. 1). These data, together with an isolated methylene [δ_C 38.6 (t)/δ_H 3.85 (d, J = 18.1 Hz, H-7′a) and 3.26 (d, J = 18.1 Hz, H-7′b)], an imine carbon [δ_C 154.9 (s, C-8′)], and an amide carbonyl [δ_C 159.7 (s, C-9′)],

Table 1. NMR data for aplysinones A–D (**6**–**9**)[a,b,c]

Position	**6**		**7**		**8**		**9**	
	δ_C	δ_H (m, *J* in Hz)	δ_C	δ_H (m, *J* in Hz)	δ_C	δ_H (m, *J* in Hz)	δ_C	δ_H (m, *J* in Hz)
1	75.3	4.16 (d, 8.3)	75.0	4.37 (dd, 11.5, 5.6)	75.3	4.16 (dd, 8.2, 0.9)	75.2	4.16 (dd, 8.2, 0.8)
2	113.9		57.4	5.07 (d, 11.5)	113.8		113.9	
3	148.7		183.7		149.0		148.7	
4	122.0		122.5		122.0		122.0	
5	132.4	6.51 (s)	149.5	7.62 (s)	132.4	6.50 (d, 0.9)	132.4	6.51 (d, 0.8)
6	91.5		91.4		91.5		91.5	
7	40.3	3.82 (d, 18.2)	38.6	3.86 (d, 18.1)	40.2	3.84 (d, 18.5)	40.2	3.83 (d, 18.3)
		3.17 (d, 18.2)		3.27 (d, 18.1)		3.16 (d, 18.5)		3.17 (d, 18.3)
8	155.4		154.9		155.3		155.3	
9	159.9[d]		159.7		159.3[d]		159.9[d]	
10	39.7	3.31 (m)	39.7	3.31 (m)	39.8[d]	3.31 (m)	39.5	3.34 (m)
11	29.7	1.60 (m)	29.7	1.61 (m)	29.7	1.60 (m)	27.5	1.62 (m)
12	24.7	1.39 (m)	24.7	1.39 (m)	24.7	1.39 (m)	27.5	1.62 (m)
13	29.7	1.60 (m)	29.7	1.61 (m)	29.7	1.60 (m)	39.5	3.34 (m)
14	39.7	3.31 (m)	39.7	3.31 (m)	39.7[d]	3.31 (m)		
1′	75.1	4.37 (dd, 11.5, 5.6)	75.0	4.37 (dd, 11.5, 5.6)	58.1	4.08 (dd, 3.5, 2.6)	75.0	4.37 (dd, 11.4, 5.7)
2′	57.4	5.07 (d, 11.5)	57.4	5.07 (d, 11.5)	54.2	3.84 (d, 3.5)	57.5	5.07 (d, 11.4)
3′	183.7		183.7		186.0		183.7	
4′	122.5		122.5		124.1		122.5	
5′	149.5	7.63 (s)	149.5	7.62 (s)	144.1	7.37 (d, 2.6)	149.5	7.62 (s)
6′	91.5		91.4		85.3		91.5	
7′	38.6	3.85 (d, 18.1)	38.6	3.86 (d, 18.1)	44.5	3.74 (d, 18.2)	38.6	3.85 (d, 18.0)
		3.26 (d, 18.1)		3.27 (d, 18.1)		3.62 (d, 18.2)		3.26 (d, 18.0)
8′	154.9		154.9		155.6		154.9	
9′	159.7[d]		159.7		159.9[d]		159.7[d]	
OCH_3	60.2	3.71 (s)			60.2	3.71 (s)	60.2	3.71 (s)
OH (**1**)		5.40 (d, 8.3)		5.96 (d, 5.6)		5.48 (d, 8.2)		5.41 (d, 8.2)
OH (**1′**)		5.96 (d, 5.6)		5.96 (d, 5.6)				5.96 (d, 5.7)
NH		7.60 (br t, 5.6)		7.61 (br t, 6.7)		7.72 (br s)		7.64 (br s)
NH		7.60 (br t, 5.6)		7.61 (br t, 6.7)		7.62 (br s)		7.64 (br s)

[a] ^1H and ^{13}C NMR spectra recorded at 600 and 150 MHz, respectively.
[b] Assignments aided by COSY, HSQC, HMBC, and NOESY spectra.
[c] Recorded in $(CD_3)_2CO$.
[d] Signals with the same superscript in the same column may be interchanged.

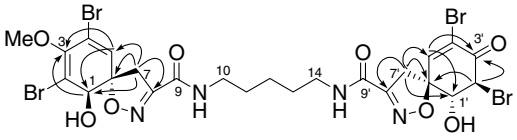

Figure 1. Selected HMBC correlations observed for aplysinone A (**6**).

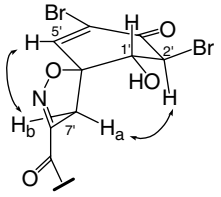

Figure 2. Selected NOESY correlations observed for aplysinone A (**6**).

were accommodated in a second spiro subunit involving a cyclohexenone and a dihydroisoxazole ring. This structural assignment was supported by the HMBC correlations of the methylene protons H-7′ with the spirocarbon C-6′, the oxymethine carbon C-1′, the olefinic carbon C-5′, and the imine carbon C-8′ (Fig. 1). The remaining signals of the NMR spectra were due to a chain of five methylenes that linked the two bicyclic subunits through the amide groups. With regard to the relative configuration of the cyclohexenone–dihydroisoxazole bicycle, the coupling constant (11.5 Hz) between H-1′ and H-2′ indicated that these protons were *trans*-diaxially oriented. In addition, the NOESY cross peak between H-2′ and H-7′a defined the *cis*-relationship between H-2′ and the methylene group of the isoxazoline ring (Fig. 2).

We next tried to assign the absolute configuration of compound **6** by derivatization with (*R*)- and (*S*)-α-methoxy-α-phenylacetic acids (MPA). However, under the reaction conditions an undesired elimination of the oxygenated function at C-1′ was produced, affording the corresponding dibromocyclohexadienone ring. On the other hand, during the purification of fractions containing aplysinone A (**6**) with $CHCl_3$/MeOH mixtures, the acetal derivative **10** was formed. The NMR spectra of **10** were related to those of **6** except for the absence of the signal due to the carbonyl group, showing in turn those of a ketalic carbon [δ_C 97.4 (s, C-3′)] and two additional *O*-methyl groups. Treatment of **10** with (*R*)- and (*S*)-MPA acids led to the diesters **10r** and **10s**, respectively. The observed chemical shift differences ($\Delta\delta = \delta_R - \delta_S$) were positive for H-5, H_2-7, H-5′, and

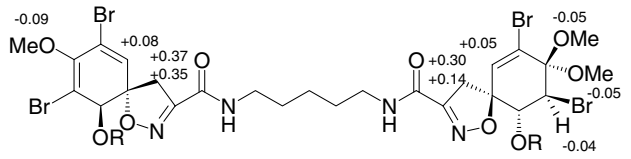

Figure 3. $\Delta\delta$ (δ_R-δ_S) for compounds **10r** and **10s**.

H_2-7′, and negative for H-2′ and the methoxyl groups (Fig. 3). Following the MPA rules,[17] these data indicated an *R* configuration for both C-1 and C-1′, and therefore, an absolute configuration 1*R*,1′*R*,2′*S*,6*S*,6′*S* for **10** and its parent compound aplysinone A (**6**).

The structure of aplysinone A (**6**) is related to that of homoaerothionin (**2**), also isolated from the sponge. Thus, cleavage of one of the enol ether functions of **2** upon treatment with TFA in acetone for 12 h yielded a mixture of aplysinone A (**6**) and its C-2′ epimer **11**, that were separated by HPLC. The most significant differences between the ^{1}H NMR spectra of **6** and **11** were observed in the signals due to H-1′ and H-2′. In compound **11** these protons gave rise to signals at δ 4.54 (br dd, J = 5.6 and 2.9 Hz, H-1′) and 5.26 (br s, H-2′) that were consistent with a *cis*-relationship (axial–equatorial) between H-1′ and H-2′.

2.2. Aplysinone B (7)

The molecular formula of compound **7**, $C_{23}H_{24}N_4O_8Br_4$, was established by HRFABMS. However, the ^{13}C NMR spectrum only displayed 12 signals, indicating that **7** possessed a symmetrical structure. The ^{1}H NMR spectrum exhibited signals attributable to an olefinic proton [δ 7.62 (s)], a proton geminal to a bromine atom [δ 5.07 (d, J = 11.5 Hz)], a proton geminal to a hydroxyl group [δ 4.37 (dd, J = 11.5 and 5.6 Hz)], and an isolated methylene [δ 3.86 (d, J = 18.1 Hz) and 3.27 (d, J = 18.1 Hz)]. These data and the ^{13}C NMR signals due to a ketone carbonyl [δ 183.7 (s)], an amide carbonyl [δ 159.7 (s)], and a spirocarbon [δ 91.4 (s)] matched with a cyclohexenone–isoxazoline subunit such that present in aplysinone A (**6**). The remaining signals of the spectra were due to a chain formed by methylene groups. These data, and taking into account the molecular formula and the symmetrical structure, led to propose that aplysinone B (**7**) possessed two identical cyclohexenone–isoxazoline moieties linked through a central chain of five methylenes.

Similar to **6**, during the isolation of compound **7** the *bis*-ketal derivative **12** was obtained. Aplysinone B (**7**) is also related to homoaerothionin (**2**), from which differs by the functionalization of the six membered rings: ketone carbonyls in **7** and the corresponding enol ethers in **2**. Thus, treatment of homoaerothionin (**2**) with TFA in acetone for 24 h yielded aplysinone B (**7**) and its stereoisomer **13**.

2.3. Aplysinone C (8)

The LRESI(+) mass spectrum of compound **8** showed an isotopic cluster (rel. int. 1:3:3:1) for the sodiated molecular ion at *m*/*z* 757:759:761:763 (M+Na) that indicated the presence of three bromine atoms in the molecule. A comparison of the NMR spectra of **8** with those of aplysinone A (**6**) indicated that **8** contained a spirocyclohexadienylisoxazoline moiety linked to a chain of five methylene groups identical to that present in **6**. The remaining signals of the spectra suggested the presence of a cyclohexenone–isoxazoline subunit also related to that of **6**, although some diagnostic differences were observed. In particular, the signals corresponding to the methines at C-1′ and C-2′ appeared at δ_C 58.1 (d)/δ_H 4.08 (dd, J = 3.5 and 2.6 Hz) and δ_C 54.2 (d)/δ_H 3.84 (d, J = 3.5 Hz), respectively. These data were consistent with the presence of an epoxide function at C-1′, C-2′. In the NOESY spectrum the protons H-1′ and H-5′ were correlated with the methylene protons H-7′a and H-7′b, respectively. However, these correlations were consistent both with a *trans*- and with a *cis*-relationship between H-1′ and the methylene group of the dihydroisoxazole ring. Recently, Nishiyama and coworkers have described diagnostic differences between the NMR data of compound **5** and the stereoisomer displaying a *cis* orientation of H-1 and C-7, H-1′ and C-7′.[16] The NMR data of the epoxycyclohexenone–isoxazoline moiety of compound **8** matched with those of **5**, and therefore a *trans*-relationship between H-1′ and the methylene group of the isoxazoline ring was proposed for aplysinone C (**8**).

2.4. Aplysinone D (9)

The NMR spectra of compound **9** were closely similar to those of aplysinone A (**6**), except for the signals of the central chain of the molecule. In particular, the NMR spectra of **9** displayed signals attributable to four methylene groups. These data, together with the molecular formula $C_{23}H_{24}N_4O_8Br_4$ determined by HRFABMS, led to propose the structure **7** for aplysinone D.

The structure of aplysinone D is related to that of aerothionin (**4**), also isolated from the sponge. Thus, cleavage of one of the enol ether functions of **4** by treatment with TFA gave a compound identical to **9** together with its C-2′ epimer **14**. Since the absolute configuration of **4** has been previously assigned,[18] this chemical correlation defined the absolute configuration of aplysinone D as depicted in formula **9**.

The possibility that aplysinones A–D (**6**–**9**) had been formed from **2** and **4** during the isolation process could be considered. However, a natural origin for **6**–**9** is suggested by the fact that compounds **11**, **13**, and **14**, which are formed in comparable amounts to **6**, **7**, and **9** by nonenzymatic hydrolysis of **2** and **4**, were not obtained from the extract.

2.5. Cytotoxic activity

The new aplysinones A–D (**6**–**9**) isolated from *A. gerardogreeni* together with the semisynthetic analogues **10**–**14** were evaluated for their cytotoxic activity against the human tumor cells MDA-MB-231 (breast adenocarcinoma), A-549 (lung carcinoma), and HT-29 (colon

adenocarcinoma). The measured parameters were GI_{50} (concentration that causes 50% growth inhibition), TGI (concentration that causes total growth inhibition), and LC_{50} (concentration that causes 50% cell killing) (Table 2).

Aplysinones A (**6**), B (**7**), D (**9**), and their respective stereoisomers **11**, **13**, and **14**, displayed significant growth inhibitory activity of the three tested lines, with most of the GI_{50} values lower than 5 μM. Furthermore, compounds **6**, **7**, **11**, and **13** caused total growth inhibition of the three cell lines, in general with TGI values also lower than 5 μM, and compounds **9** and **14** were active as total growth inhibitors of MDA-MB-231 and HT-29 cells. On the other hand, the main effect of aplysinone C (**8**) was observed in MDA-MB-231 growth inhibition. Among the tested compounds, aplysinone B (**7**) and compound **13** were the most potent growth inhibitors of all cell lines. Furthermore, both compounds exhibited significant cell killing activity of the three tested lines, with LC_{50} values ranging from 3.0 to 5.5 μM. In addition, the known compounds homoaerothionin (**2**) and aerothionin (**4**) were tested for comparison purposes, resulting inactive in all cases. From a structural point of view, the cytotoxic compounds **6**, **7**, **11**, and **13** differ from the inactive compound **2** by possessing ketone carbonyls at one or two of the six membered rings instead of enol ether functions. Similarly, **9** and **14** differ from **4** by the carbonyl group at one of the six membered rings. These data suggest that the ketone function is important for the bioactivity within this family of compounds. With regard to the ketal derivatives, compound **12** was inactive, reinforcing the importance of the ketone group for activity. On the contrary, compound **10** was inhibitor of cell growth, although the possibility that **10** could have been partially hydrolyzed during the assay to yield the active compound **6** cannot be ruled out.

3. Experimental

3.1. General procedures

Optical rotations were measured on a Perkin-Elmer 341 polarimeter. IR spectra were recorded with a Perkin-Elmer FT-IR System Spectrum BX. ^1H and ^{13}C NMR spectra were recorded at 600 and 150 MHz, respectively, on a Varian INOVA 600 spectrometer using $(CD_3)_2CO$, $CDCl_3$, or $(CD_3)_2SO$ as solvent. ^1H and ^{13}C NMR chemical shifts were referenced using the corresponding solvent signals [δ_H 2.04 and δ_C 29.8 for $(CD_3)_2CO$, δ_H 7.26 and δ_C 77.0 for $CDCl_3$, δ_H 2.49 and δ_C 39.5 for $(CD_3)_2SO$]. COSY, NOESY, HSQC, and HMBC experiments were performed using standard Varian pulse sequences. Low resolution mass spectra were recorded on a Thermo LCQ spectrometer. High resolution mass spectra were recorded on a Autospec-Q spectrometer. Column chromatography was carried out using Merck Silica gel 60 (70–230 mesh). HPLC separations were performed on a LaChrom-Hitachi apparatus equipped with LiChrospher Si-60 (Merck) columns in normal phase mode and with LiChrosorb RP-18 (Merck) columns in reversed phase mode. All solvents were of spectral grade or distilled prior to use.

3.2. Collection, extraction, and isolation

The sponge *A. gerardogreeni* was collected by hand using SCUBA at the Gulf of California and the freeze–dried specimens (279.3 g) were extracted with acetone/MeOH (1:1, 4 L). After filtration and evaporation of the solvent under reduced pressure, the extract was treated with Et_2O (1.5 L) and the soluble portion was separated. Subsequent addition of MeOH (1.5 L) allowed separation of the material soluble in this solvent. This solution was evaporated to dryness and the residue (11.7 g) was chromatographed over a silica gel column eluted with $CHCl_3$/MeOH mixtures of increasing polarities (90:10 to 50:50) and subsequently MeOH. The fraction eluted with $CHCl_3$/MeOH (90:10) (7.1 g) was chromatographed over a silica gel column using as eluant hexane/EtOAc mixtures of increasing polarities (65:35 to 20:80) and subsequently EtOAc and MeOH. The fraction eluted with hexane/EtOAc (40:60) was separated over a silica gel column eluted with $CHCl_3$/MeOH (99:1) to obtain the known compounds homoaerothionin[2] (**2**, 520 mg), aerothionin[2,18,19] (**4**, 383 mg), and a mixture that was subjected to repeated HPLC separations ($CHCl_3$/MeOH, 98:2 to 95:5; MeOH/H_2O, 65:35) to yield the new compounds aplysinone C (**8**,

Table 2. Cytotoxicity assay results for compounds **2**, **4**, and **6**–**14**

Compound	GI_{50} (μM)			TGI (μM)			LC_{50} (μM)		
	MDA-MB-231	A-549	HT-29	MDA-MB-231	A-549	HT-29	MDA-MB-231	A-549	HT-29
2	na	na	na	na	na	na	na	na	na
4	na	na	na	na	na	na	na	na	na
6	3.3	4.5	2.8	5.4	7.1	4.8	8.2	na	9.1
7	1.6	2.0	1.5	2.1	2.9	2.1	3.0	4.1	3.0
8	5.6	10.7	12.6	5.8	na	na	6.4	na	na
9	3.5	5.7	4.1	5.0	na	5.2	7.6	na	11.3
10	4.2	7.1	4.8	6.0	na	na	9.6	na	na
11	3.5	5.4	4.8	4.9	8.7	5.0	6.7	11.2	5.4
12	na	na	na	na	na	na	na	na	na
13	1.7	2.4	1.6	2.4	3.5	2.5	3.5	5.5	3.7
14	2.9	5.6	3.4	4.7	na	4.9	8.0	na	8.5
Doxorubicin[a]	0.1	0.1	0.1	0.3	0.2	0.3	1.4	1.0	0.4

na, not active (GI_{50}, TGI, or LC_{50} > 10 μg/mL).
[a] Used as positive control.

2.1 mg), aplysinone A (**6**, 74.4 mg), aplysinone B (**7**, 19.0 mg), and aplysinone D (**9**, 2.4 mg).

3.3. Aplysinone A (6)

Amorphous solid; $[\alpha]_D^{25}$ +65.0° (*c* 0.1, acetone); IR ν_{max} (film) 3357, 2927, 2856, 1708, 1659, 1600, 1538, 1440, 911, 754 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; LRESI(+) *m/z* 837, 839, 841, 843, 845 $(M+Na)^+$; HRFABMS(+) *m/z* 838.8315 (calcd for $C_{24}H_{26}N_4O_8{}^{79}Br_3{}^{81}BrNa$, 838.8361), 840.8378 (calcd for $C_{24}H_{26}N_4O_8{}^{79}Br_2{}^{81}Br_2Na$, 840.8341), 842.8305 (calcd for $C_{24}H_{26}N_4O_8{}^{79}Br^{81}Br_3Na$, 842.8320).

3.4. Aplysinone B (7)

Amorphous solid; $[\alpha]_D^{25}$ −4.0° (*c* 0.1, acetone); IR ν_{max} (film) 3382, 2924, 2853, 1706, 1662, 1602, 1539, 1456, 912, 756 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; LRESI(+) *m/z* 823, 825, 827, 829, 831 $(M+Na)^+$; HRFABMS(+) *m/z* 824.8249 (calcd for $C_{23}H_{24}N_4O_8{}^{79}Br_3{}^{81}BrNa$, 824.8205), 826.8218 (calcd for $C_{23}H_{24}N_4O_8{}^{79}Br_2{}^{81}Br_2Na$, 826.8184), 828.8195 (calcd for $C_{23}H_{24}N_4O_8{}^{79}Br^{81}Br_3Na$, 828.8164).

3.5. Aplysinone C (8)

Amorphous solid; $[\alpha]_D^{25}$ +102.0° (*c* 0.1, acetone); IR ν_{max} (film) 3366, 2934, 1701, 1668, 1602, 1540, 1438, 1262, 912, 759 cm^{-1}; ^1H and ^{13}C NMR data in $(CD_3)_2CO$, see Table 1; ^1H NMR (600 MHz, $(CD_3)_2SO$) δ 8.61 (1H, br t, J = 5.8 Hz, NH), 8.50 (1H, br t, J = 5.8 Hz, NH), 7.48 (1H, d, J = 2.6 Hz, H-5′), 6.57 (1H, d, J = 0.9 Hz, H-5), 6.37 (1H, br d, J = 4.6 Hz, OH), 4.12 (1H, dd, J = 3.6 and 2.6 Hz, H-1′), 3.93 (1H, br d, J = 3.4 Hz, H-1), 3.92 (1H, d, J = 3.6 Hz, H-2′), 3.66 (1H, d, J = 17.9 Hz, H-7′), 3.63 (3H, s, OCH_3), 3.61 (1H, d, J = 18.2 Hz, H-7), 3.60 (1H, d, J = 17.9 Hz, H-7′), 3.20 (1H, d, J = 18.2 Hz, H-7), 3.14 (4H, m, H_2-10 and H_2-14), 1.46 (4H, m, H_2-11 and H_2-13), 1.26 (2H, m, H_2-12); ^{13}C NMR (150 MHz, DMSO-d_6) δ 186.1 (s, C-3′), 158.8[a] (s, C-9), 158.2[a] (s, C-9′), 154.9 (s, C-8′), 154.6 (s, C-8), 147.1 (s, C-3), 143.7 (d, C-5′), 131.3 (d, C-5), 122.8 (s, C-4′), 120.8 (s, C-4), 113.1 (s, C-2), 90.0 (s, C-6), 83.9 (s, C-6′), 73.5 (d, C-1), 59.6 (q, OCH_3), 56.8 (d, C-1′), 52.9 (d, C-2′), 43.5 (t, C-7′), 40.0 (t, C-7), 38.8[b] (t, C-10), 38.7[b] (t, C-14), 28.5 (2 × t, C-11 and C-13), 23.7 (t, C-12), [a,b]signals marked with the same superscript may be interchanged; LRESI(+) *m/z* 757, 759, 761, 763 $(M+Na)^+$; HRFABMS(+) *m/z* 758.9125 (calcd for $C_{24}H_{25}N_4O_8{}^{79}Br_2{}^{81}BrNa$, 758.9100), 760.9098 (calcd for $C_{24}H_{25}N_4O_8{}^{79}Br^{81}Br_2Na$, 760.9080).

3.6. Aplysinone D (9)

Amorphous solid; $[\alpha]_D^{25}$ +86.0° (*c* 0.1, acetone); IR ν_{max} (film) 3350, 2925, 1700, 1664, 1542, 1269, 911, 754 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; LRESI(+) *m/z* 823, 825, 827, 829, 831 $(M+Na)^+$; HRFABMS(+) *m/z* 824.8220 (calcd for $C_{23}H_{24}N_4O_8{}^{79}Br_3{}^{81}BrNa$, 824.8205), 826.8224 (calcd for $C_{23}H_{24}N_4O_8{}^{79}Br_2{}^{81}Br_2Na$, 826.8184), 828.8217 (calcd for $C_{23}H_{24}N_4O_8{}^{79}Br^{81}Br_3Na$, 828.8164).

3.7. Compound 10

Amorphous solid; $[\alpha]_D^{25}$ +51.0° (*c* 0.1, acetone); IR ν_{max} (film) 3380, 2938, 1661, 1600, 1540, 1456, 920, 760 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ 6.82 (1H, br t, J = 5.9 Hz, NH), 6.78 (1H, br t, J = 5.8 Hz, NH), 6.45 (1H, s, H-5′), 6.33 (1H, s, H-5), 4.39 (1H, s, H-1), 4.33 (1H, d, J = 9.8, H-1′), 4.21 (1H, d, J = 9.8 Hz, H-2′), 3.92 (1H, d, J = 18.6 Hz, H-7a), 3.79 (1H, d, J = 18.0 Hz, H-7′a), 3.75 (3H, s, OMe-3), 3.56 (3H, s, OMe-3′), 3.37 (2H, m, H-10 and H-14), 3.36 (3H, s, OMe-3′), 3.32 (2H, m, H-10 and H-14), 3.03 (1H, d, J = 18.6 Hz, H-7b), 2.98 (1H, d, J = 18.0 Hz, H-7′b), 1.59 (4H, m, H_2-11 and H_2-13), 1.38 (2H, m, H_2-12); ^{13}C NMR (150 MHz, $CDCl_3$) δ 159.4[a] (s, C-9), 159.3[a] (s, C-9′), 154.2 (s, C-8), 153.7 (s, C-8′), 148.0 (s, C-3), 135.1 (d, C-5′), 131.0 (d, C-5), 124.5 (s, C-4′), 121.3 (s, C-4), 112.6 (s, C-2), 97.4 (s, C-3′), 91.8 (s, C-6), 91.1 (s, C-6′), 73.9 (d, C-1), 71.7 (d, C-1′), 60.2 (q, OMe-3), 54.8 (d, C-2′), 52.2 (q, OMe-3′), 51.8 (q, OMe-3′), 39.2 (t, C-7), 39.1[b] (t, C-10), 38.9[b] (t, C-14), 38.6 (t, C-7′), 28.7[c] (t, C-11), 28.6[c] (t, C-13), 23.6 (t, C-12), [a–c]signals with the same superscript may be interchanged; LRESI(+) *m/z* 883, 885, 887, 889, 891 $(M+Na)^+$; HRFABMS(+) *m/z* 884.8791 (calcd for $C_{26}H_{32}N_4O_9{}^{79}Br_3{}^{81}BrNa$, 884.8780), 886.8774 (calcd for $C_{26}H_{32}N_4O_9{}^{79}Br_2{}^{81}Br_2Na$, 886.8759), 888.8771 (calcd for $C_{26}H_{32}N_4O_9{}^{79}Br^{81}Br_3Na$, 888.8739).

3.8. Synthesis of the (*R*)-MPA derivative 10r

A solution of **10** (2.5 mg, 2.9×10^{-3} mmol) in CH_2Cl_2 (0.25 mL) was cooled at 0 °C and treated with CH_2Cl_2 solutions of *N*,*N*′-dicyclohexylcarbodiimide (10 mg, 0.049 mmol in 0.25 mL), (*R*)-MPA acid (6.0 mg, 0.036 mmol in 0.25 mL), and *N*,*N*-dimethylaminopyridine (1 mg, 7.5×10^{-3} mmol in 0.25 mL). After stirring for 2 h, the mixture was allowed to warm to room temperature and purified on preparative TLC (hexane/EtOAc, 3:7) to yield 2.0 mg of compound **10r**: ^1H NMR (600 MHz, $CDCl_3$): δ (selected data, assignments aided by COSY and NOESY experiments) 6.42 (1H, s, H-5′), 6.30 (1H, s, H-5), 5.83 (1H, d, J = 11.4 Hz, H-1′), 5.81 (1H, s, H-1), 4.08 (1H, d, J = 11.4 Hz, H-2′), 3.66 (3H, s, –OCH_3), 3.60 (1H, d, J = 18.0 Hz, H-7′a), 3.56 (3H, s, –$OCH_3(\beta)$), 3.33 (1H, d, J = 18.5 Hz, H-7a), 3.28 (3H, s, –$OCH_3(\alpha)$), 3.03 (1H, d, J = 18.5 Hz, H-7b), 3.00 (1H, d, J = 18.0 Hz, H-7′b).

3.9. Synthesis of the (*S*)-MPA derivative 10s

A solution of **10** (2.5 mg, 2.9×10^{-3} mmol) in CH_2Cl_2 (0.4 mL) was cooled at 0 °C and treated with CH_2Cl_2 solutions of *N*,*N*′-dicyclohexylcarbodiimide (10 mg, 0.049 mmol in 0.3 mL), (*S*)-MPA acid (6.0 mg, 0.036 mmol in 0.3 mL), and *N*,*N*-dimethylaminopyridine (1 mg, 7.5×10^{-3} mmol in 0.5 mL). After stirring for 1h, the mixture was allowed to warm to room temperature and purified by HPLC ($CHCl_3$/MeOH, 98:2) to yield 1.6 mg of compound **10s**: ^1H NMR (600 MHz, $CDCl_3$): δ (selected data, assignments aided by COSY and NOESY experiments) 6.37 (1H, s, H-5′), 6.22 (1H, s, H-5), 5.92 (1H, s, H-1), 5.85 (1H, d, J = 11.4 Hz,

H-1′), 4.12 (1H, d, J = 11.4 Hz, H-2′), 3.75 (3H, s, –OCH_3), 3.61 (3H, s, –OCH_3(β)), 3.33 (3H, s, –OCH_3(α)), 3.30 (1H, d, J = 18.0 Hz, H-7′a), 2.98 (1H, d, J = 18.6 Hz, H-7a), 2.86 (1H, d, J = 18.0 Hz, H-7′b), 2.66 (1H, d, J = 18.6 Hz, H-7b).

3.10. Compound 12

Amorphous solid; $[\alpha]_D^{25}$ −27.0° (c 0.1, acetone); IR ν_{max} (film) 3356, 2925, 2854, 1654, 1600, 1542, 1458, 1260, 930, 799 cm^{-1}; ^1H NMR (600 MHz, $(CD_3)_2CO$) δ 7.54 (2H, br t, J = 5.7 Hz, NH), 6.60 (2H, s, H-5 and H-5′), 5.42 (2H, d, J = 5.6 Hz, OH), 4.46 (2H, d, J = 11.1 Hz, H-2 and H-2′), 4.26 (2H, dd, J = 11.1 and 5.6 Hz, H-1 and H-1′), 3.70 (2H, d, J = 18.0 Hz, H-7a and H-7′a), 3.54 (6H, s, OMe-3 and OMe-3′), 3.33 (6H, s, OMe-3 and OMe-3′), 3.28 (4H, m, H_2-10 and H_2-14), 3.00 (2H, d, J = 18.0 Hz, H-7b and H-7′b), 1.59 (4H, m, H_2-11 and H_2-13), 1.37 (2H, m, H_2-12); ^{13}C NMR (150 MHz, $(CD_3)_2CO$) δ 160.1 (2 × s, C-9 and C-9′), 154.8 (2 × s, C-8 and C-8′), 137.3 (2 × d, C-5 and C-5′), 124.7 (2 × s, C-4 and C-4′), 98.9 (2 × s, C-3 and C-3′), 92.0 (2 × s, C-6 and C-6′), 72.1 (2 × d, C-1 and C-1′), 57.7 (2 × d, C-2 and C-2′), 52.9 (2 × q, OMe-3 and OMe-3′), 52.0 (2 × q, OMe-3 and OMe-3′), 39.6 (2 × t, C-10 and C-14), 38.9 (2 × t, C-7 and C-7′), 30.0 (2 × t, C-11 and C-13), 24.6 (t, C-12); LRESI(+) m/z 915, 917, 919, 921, 923 $(M+Na)^+$; HRFABMS(+) m/z 916.9057 (calcd for $C_{27}H_{36}N_4O_{10}{}^{79}Br_3{}^{81}BrNa$, 916.9042), 918.9055 (calcd for $C_{27}H_{36}N_4O_{10}{}^{79}Br_2{}^{81}Br_2Na$, 918.9022), 920.9054 (calcd for $C_{27}H_{36}N_4O_{10}{}^{79}Br^{81}Br_3Na$, 920.9001).

3.11. Treatment of homoaerothionin (2) with CF_3COOH

(A) A solution of **2** (50 mg, 0.06 mmol) in acetone (1.5 mL) was treated with TFA (0.2 mL). After stirring at room temperature for 12 h, the mixture was purified by preparative TLC ($CHCl_3$/MeOH, 95:5) to obtain unreacted **2** (17.0 mg) and a mixture that was purified by reversed-phase HPLC (acetone/H_2O, 1:1) to yield aplysinone A (**6**, 9.0 mg) and its isomer **11** (8.5 mg).

Compound **11**: ^1H RMN (600 MHz, $(CD_3)_2CO$) δ 7.64 (1H, br t, J = 6.0 Hz, NH), 7.60 (br t, J = 6.2 Hz, NH), 7.47 (1H, s, H-5′), 6.51 (1H, s, H-5), 5.90 (1H, d, J = 5.6 Hz, OH-1′), 5.40 (1H, d, J = 8.3 Hz, OH-1), 5.26 (1H, br s, H-2′), 4.54 (1H, br dd, J = 5.6 and 2.9, H-1′), 4.16 (1H, d, J = 8.3 Hz, H-1), 3.86 (1H, d, J = 18.3 Hz, H-7′a), 3.83 (1H, d, J = 18.3 Hz, H-7a), 3.71 (3H, s, OCH_3), 3.36 (1H, d, J = 18.3 Hz, H-7′b), 3.31 (4H, m, H_2-10 and H_2-14), 3.17 (1H, d, J = 18.3 Hz, H-7b), 1.60 (4H, m, H_2-11 and H_2-13), 1.39 (2H, m, H_2-12); ^{13}C RMN (150 MHz, $(CD_3)_2CO$) δ 183.6 (s, C-3′), 159.9[a] (s, C-9), 159.6[a] (s, C-9′), 155.6 (s, C-8′), 155.3 (s, C-8), 148.7 (s, C-3), 146.4 (d, C-5′), 132.4 (d, C-5), 124.1 (s, C-4′), 122.0 (s, C-4), 113.8 (s, C-2), 91.5 (s, C-6), 90.5 (s, C-6′), 75.3 (d, C-1), 73.4 (d, C-1′), 54.9 (d, C-2′), 41.6 (t, C-7′), 40.2 (t, C-7), 39.8[b] (t, C-10), 39.7[b] (t, C-14), 29.8 (2 × t, C-11 and C-13), 24.7 (t, C-12), [a,b]signals with the same superscript may be interchanged.

(B) A solution of **2** (50 mg, 0.06 mmol) in acetone (1.0 mL) was treated with TFA (0.4 mL). After stirring at room temperature for 24 h, the mixture was purified by preparative TLC ($CHCl_3$/MeOH, 95:5) followed by HPLC (acetone/H_2O, 1:1) yielding aplysinone B (**7**, 7.0 mg) and compound **13** (4.0 mg).

Compound **13**: ^1H RMN (600 MHz, $(CD_3)_2CO$) δ 7.66 (2H, br t, J = 5.7 Hz, NH), 7.47 (2H, s, H-5 and H-5′), 5.91 (2H, d, J = 5.6 Hz, OH), 5.26 (2H, br s, H-2 and H-2′), 4.54 (2H, br dd, J = 5.0 and 2.7 Hz, H-1 and H-1′), 3.87 (2H, d, J = 18.3 Hz, H-7a and H7′a), 3.36 (2H, d, J = 18.3 Hz, H-7b and H-7′b), 3.32 (4H, m, H_2-10 and H_2-14), 1.61 (4H, m, H_2-11 and H_2-13), 1.40 (2H, m, H_2-12); ^{13}C RMN (150 MHz, $(CD_3)_2CO$) δ 183.6 (2 × s, C-3 and C-3′), 159.7 (2 × s, C-9 and C-9′), 155.7 (2 × s, C-8 and C-8′), 146.4 (2 × d, C-5 and C-5′), 123.7 (2 × s, C-4 and C-4′), 90.5 (2 × s, C-6 and C-6′), 73.4 (2 × d, C-1 and C-1′), 54.9 (2 × d, C-2 and C-2′) 41.6 (2 × t, C-7 and C-7′), 39.8 (2 × t, C-10 and C-14), 29.7 (2 × t, C-11 and C-13), 24.7 (t, C-12).

3.12. Treatment of aerothionin (4) with CF_3COOH

A solution of **4** (50 mg, 0.06 mmol) in acetone (1.5 mL) was treated with TFA (0.2 mL). After stirring at room temperature for 12 h, the mixture was purified by preparative TLC ($CHCl_3$/MeOH, 95:5) to obtain unreacted **4** (34.5 mg) and a mixture that was purified by HPLC (acetone/H_2O, 1:1) to yield aplysinone D (**9,** 1.6 mg) and compound **14** (4.0 mg).

Compound **14**: ^1H RMN (600 MHz, $(CD_3)_2CO$) δ 7.68 (1H, br t, J = 5.8 Hz, NH), 7.63 (1H, br t, J = 5.8 Hz, NH), 7.47 (1H, d, J = 1.0 Hz, H-5′), 6.51 (1H, d, J = 0.8 Hz, H-5), 5.90 (1H, d, J = 5.6 Hz, OH-1′), 5.40 (1H, d, J = 8.3 Hz, OH-1), 5.26 (1H, br s, H-2′), 4.54 (1H, br dd, J = 5.3 and 2.8 Hz, H-1′), 4.16 (1H, dd, J = 8.3 and 0.8 Hz, H-1), 3.87 (1H, d, J = 18.3 Hz, H-7′a), 3.83 (1H, d, J = 18.2 Hz, H-7a), 3.71 (3H, s, OCH_3), 3.36 (1H, d, J = 18.3 Hz, H-7′b), 3.34 (4H, m, H_2-10 and H_2-13), 3.17 (1H, d, J = 18.2 Hz, H-7b), 1.62 (4H, m, H_2-11 and H_2-12); ^{13}C RMN (150 MHz, $(CD_3)_2CO$) δ 183.6 (s, C-3′), 159.9[a] (s, C-9), 159.7[a] (s, C-9′), 155.6 (s, C-8′), 155.3 (s, C-8), 148.7 (s, C-3), 146.4 (d, C-5′), 132.4 (d, C-5), 122.5 (s, C-4′), 122.0 (s, C-4), 113.8 (s, C-2), 91.5 (s, C-6), 90.5 (s, C-6′), 75.2 (d, C-1), 73.0 (d, C-1′), 55.0 (d, C-2′), 41.6 (t, C-7′), 40.2 (t, C-7), 39.5 (2 × t, C-10 and C-13), 27.5[b] (t, C-11), 27.4[b] (t, C-12),[a,b]signals with the same superscript may be interchanged.

3.13. Cytotoxicity assays

Compounds **2**, **4**, and **6**–**14** were tested against the human tumor cell lines MDA-MB-231 (breast adenocarcinoma), A-549 (lung carcinoma), and HT-29 (colon adenocarcinoma). Cytotoxicity assays were performed by PharmaMar. A colorimetric type of assay using sulforhodamine B (SRB) reaction has been adapted for a quantitative measurement of cell growth and viability following the method described in the literature.[20]

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