Antibiotic and Cytotoxic Activity of Brominated Compounds from the Marine Sponge *Verongia aerophoba*

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Analysis of the marine sponge *Verongia aerophoba* from the Canary Islands afforded eight brominated secondary metabolites including the small molecular weight compounds aeroplysinin-1 (**5**) and the dienone (**7**) which were previously shown to arise by enzymatically catalyzed degradation of aerophobin-2 (**4**) and isofistularin-3 (**1**) following breakdown of the cellular compartmentation of the sponge. All compounds were identified from their NMR and mass spectra. Aeroplysinin-1 as well as dienone which arise from isofistularin-3 or aerophobin-2 by biotransformation within the sponge showed a significantly higher antibiotic as well as cytotoxic activity than their biogenetic precursors. Antibiotic activity was studied with respect to several gram-positive and gram-negative bacteria including *B. subtilis, S. aureus* and *E. coli*. The MICs (MBCs) of aeroplysinin-1 (**5**) and the dienone (**7**) varied between $12.5-25$ $(50-100)$ µg/ml respectively. Cytotoxicity was tested *in vitro* towards HeLa cells, a human cervix uteri tumour cell line. Aeroplysinin-1 (**5**) and the dienone (**7**) displayed pronounced cytotoxic activity with IC_{50}s of 3.0 and 3.2 µM respectively. A five-fold increase in cytotoxicity was observed after O-acetylation of the dienone (**7**). The IC_{50} of the dienone O-acetate (0.6 µM) was comparable to that of the clinically used anticancer drug cisplatin (0.7 µM).

Introduction

Secondary compounds from marine organisms are often characterized by pronounced biological activities (*e.g.* cytotoxic activity) which suggest potential value as primary structures for the development of new pharmaceuticals [1, 2]. In the marine environment sponges (Porifera) are one of the most interesting groups of organisms with regard to the accumulation of bioactive natural products [2, 3]. Sponges are primitive filter-feeders that constitute some 5000 taxa with the majority being found in the marine environment [4]. Whereas they have a world-wide distribution their greatest diver-

sity is usually found in the tropics where they form conspicuous inhabitants, for example, on coral reefs. It is generally assumed that the ecological success of sponges depends largely upon effective defense mechanisms that rely heavily on the accumulation of toxic or deterrent secondary compounds [2, 5, 6]. The frequent occurrence of antibiotic and/or cytotoxic metabolites in sponges [1] can be interpreted in this context.

The brightly yellow coloured sponge *Verongia aerophoba* Schmidt (syn. *Aplysina aerophoba*) is frequently found in the Mediterranean Sea [7], as well as around the Canary Islands where it is one of the dominating sponges accessible by SCUBA diving [8]. Like other members of the family Verongidae *V. aerophoba* is chemically characterized by unusual brominated secondary compounds (Fig. 1) [2] presumably derived from dibromotyrosine [9]. In continuation of our studies

on the secondary compounds from *V. aerophoba* [8, 10] we report here on the antibacterial and cytotoxic activities of the major brominated constituents.

Materials and Methods

Origin of material

V. aerophoba was collected in September and October 1991 by SCUBA diving or by snorkeling during a scientific cruise of the research vessel "Heincke" (Biologische Anstalt Helgoland) to the Canary Islands [8]. *V. aerophoba* was identified by Dr. R. Haroun and by R. Herrera (Universidad de Las Palmas de Gran Canaria, Facultad de Ciencias del Mar). A voucher specimen is kept at Würzburg.

Extraction and isolation of brominated compounds

See ref. [8].

HPLC analysis

For HPLC analysis 100 mg of lyophilized sponge tissue was ground and incubated in 100% MeOH or in citrate buffer (0.1 M, pH 5.77) at 30 °C for 5 h. Following incubation with buffer the supernatant was decanted and the remaining tissue extracted with 100% MeOH overnight. Supernatant and MeOH extract were subsequently combined and subjected to HPLC analysis. The HPLC-system has been described [8].

Antimicrobial activity

For the determination of antimicrobial activities standard strains of *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa* and *S. cerevisiae* were used. The *S. pyogenes* and *S. faecalis* strains were isolated in the Institute of Hygiene of the University of Würzburg. The strains are listed in Table I. The microorganisms were grown in tryptose-soy broth (Sigma, F.R.G.). The agar diffusion assay was performed following cultivation on Müller-Hinton-Agar plates (Difco, U.S.A.) according to the Bauer-Kirby-Test (DIN 58940); the determination of the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) were performed by standard assays (DIN 58940) [12].

Cytotoxicity

HeLa cells, a human cervix uteri cancer cell line, were used for cytotoxicity testing. This line was grown in suspension culture in Joklik's modification of MEM (Minimum Essential Medium; Flow Laboratories, Irvine, U.K.), supplemented with 10% heat-inactivated fetal calf serum (Gibco, Paisly, U.K.) plus 50 μg/ml streptomycin and 50 IU/ml penicillin G. It was cultured routinely at 37 °C in a humidified incubator gassed with 5% CO_2. The doubling time of the cells was *ca.* 24 h. All experiments were initiated with exponentially growing cells with a viability >95%, as determined with trypan blue.

The cytotoxicity of the test compounds was determined using the microculture tetrazolium (MTT) assay, and compared to untreated controls. This assay is based on the metabolic reduction of soluble tetrazolium salts into insoluble coloured formazan products by mitochondrial dehydrogenase activity of the tumour cells. The enzyme activity and the amount of formazan formed were proportional to the number of living cells, under the test conditions used in the precent study [13].

Of the test compounds, stock solutions of 20 mM in ethanol 96% (v/v) were prepared immediately before use in the cytotoxicity experiments. Exponentially growing cells were harvested, counted and diluted appropriately with culture medium. Of the cell suspension, 50 μl of a solution of the test compounds, obtained by diluting the stock solution with culture medium, was added to each well. The concentration range was from 0.1–100 μM (0.1–100 nmol/ml). The test plates were incubated at 37 °C in a humidified incubator with 5% CO_2 for a short-culture-period of 4 days.

A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, U.S.A.) was prepared at 5 mg/ml in phosphate buffered saline (PBS; 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4). Of this solution 20 μl was pipetted to each well. After an incubation period of 3 h 45 min at 37 °C in a humidified incubator with 5% CO_2, the medium was removed after centrifugation (15 min, 20 °C, 210 g). The formed formazan product was dissolved in 200 μl dimethylsulfoxide. After thorough mixing, the absorbance was measured at 520 nm using a scanning microtiterwell spectrophotometer (Titertek Multiscan, Flow Laboratories).

Cell survival was calculated using the formula: Survival (%) = (absorbance of treated cells – absorbance of culture medium)/(absorbance of untreated cells – absorbance of culture medium) × 100. For each compound tested, the IC_{50} value (drug concentration causing 50% growth inhibition of the tumour cells, compared to controls (100% survival = 0% growth inhibition) was determined from the survival data.

As a reference compound the clinically used anticancer drug cisplatin (Platinol, Bristol-Myers Squibb BV, Woerden, The Netherlands) was used.

Spectroscopy

^1H NMR and ^{13}C NMR spectra were recorded on Bruker AM-300 or WM-400 spectrometers. All 1 D- or 2 D-spectra were obtained using the standard Bruker software. Mass spectra (FAB, glycerol as matrix; EI, probe, 70 eV; DCI, NH₃ as reactant gas) were measured on a Finnigan MAT 8430 or a Finnigan MAT 90 mass spectrometer. GC-MS spectra were recorded on a Finnigan MAT 4515 instrument using a DB-1 capillary column (J & W scientific, California), EI, 45 eV.

1: ^1H NMR (CD₃OD) δ 7.67, 7.66 (s × 2; H-15, H-17); 6.47, 6.46 (d × 2; H-2, H-2'; $J = 0.9$); 4.81 (dd; H-19; $J = 7.3, 4.9$); 4.26 (dddd; H-11; $J = 7.4$, 5.4, 5.4, 4.6); 4.14, 4.14 (d × 2; H-6, H-6'; $J = 1.0$, 1.1); 4.09 (dd; H-12 A; $J = 9.2, 5.3$); 4.04 (dd; H-12 B; $J = 9.4, 5.9$); 3.84, 3.80 (d × 2; H-7 A, H-7'A; $J = 18.3, 18.3$); 3.77 (s × 2; OMe × 2); 3.77 (dd; H-10 A; $J = 13.8, 4.6$); 3.54 (dd; H-10 B; $J = 13.8, 7.5$); 3.54 (dd; H-20 A; $J = 13.5, 4.8$); 3.45 (dd; H-20 B; $J = 13.6, 7.3$); 3.16, 3.11 (d × 2; H-7 B, H-7'B; $J = 18.3, 18.3$). MS (FAB, glycerol) cluster at (m/z) 1107, 1109, 1111, 1113, 1115, 1117, 1119 (M − H)⁻.

2: ^1H NMR (CD₃OD) δ 7.69 (s; H-15, H-17); 6.46 (s; H-2); 5.66 (dd; H-19; $J = 8.6, 7.1$); 4.26 (dddd; H-11; $J = 7.5, 5.2, 5.2, 4.9$); 4.15 (s; H-6); 4.10 (dd; H-12 A; $J = 9.2, 5.4$); 4.06 (dd; H-12 B; $J = 9.2, 5.4$); 4.03 (t; H-20 A; $J = 9.0, 9.0$); 3.84 (d; H-7 A; $J = 18.2$); 3.77 (s; OMe); 3.77 (dd; H-10 A; $J = 13.6, 4.9$); 3.55 (dd; H-10 B; $J = 13.9, 7.5$); 3.48 (dd; H-20 B; $J = 9.2, 7.0$); 3.15 (d; H-7 B; $J = 18.2$). MS (FAB, glycerol) cluster at (m/z) 794, 796, 798, 800, 802 (M + Na)⁺.

3: ^1H NMR (CD₃OD) δ 7.86 (s; H-14); 7.01 (s; H-13); 6.45 (d; H-2; $J = 0.9$); 4.11 (d; H-6; $J = 0.8$); 3.80 (d; H-7 A; $J = 18.3$); 3.76 (s; OMe); 3.57 (t;

H-10; $J = 7.0$); 3.12 (d; H-7 B; 18.3); 2.90 (t; H-11; $J = 7.0$). MS (FAB, glycerol) cluster at (m/z) 475, 477, 479 (M + H)⁺.

4: ^1H NMR (CD₃OD) δ 6.59 (s; H-14); 6.46 (d; H-2; $J = 0.9$); 4.13 (d; H-6; $J = 0.8$); 3.82 (d; H-7 A; $J = 18.5$); 3.76 (s; OMe); 3.63 (t; H-10; $J = 7.0$); 3.14 (d; H-7 B; $J = 18.5$); 2.59 (t; H-12; $J = 7.0$); 1.9 (pentet, H-11). MS (FAB, glycerol) cluster at (m/z) 504, 506, 508 (M + H)⁺.

5: ^1H NMR (CD₃OD) δ 6.37 (d; H-2; $J = 1.2$); 4.15 (d; H-6; $J = 1.2$); 3.76 (s; OMe); 2.88 (d; H-7 A; $J = 17.0$); 2.82 (d; H-7 B; $J = 17.0$). MS (DCI, NH₃) (m/z, rel. int.) 355, 357, 359 (M + NH₄)⁺; 338, 340, 342 (M + H)⁺; 337, 339, 342 (M + NH₄ − H₂O)⁺.

6: ^1H NMR (CD₃OD) δ 6.52 (d; H-2; $J = 0.7$); 5.19 (d; H-6; $J = 0.7$); 3.81 (s; OMe); 2.95 (s; H-7); GC-MS cluster at 338, 340, 342 (M)⁺.

7: ^1H NMR (CD₃OD) δ 7.57 (s; H-2, H-6); 2.70 (s; H-7). MS (DCI, NH₃) (m/z) 341, 343, 345 (M + NH₄)⁺.

8: ^1H NMR (CD₃OD) δ 6.89 (s; H-2, H-6); 3.23 (s; OMe); 3.18 (s; OMe); 2.58 (s; H-7); MS (EI) (m/z) 338, 340, 342 (M − OMe)⁺; 320, 322, 324 (M − OMe − H₂O)⁺.

Results and Discussion

Extraction of different collections of *V. aerophoba* from the Canary Islands yielded eight brominated secondary compounds including isofistularin-3 (**1**), fistularin-1 (**2**), aerophobin-1 (**3**), aerophobin-2 (**4**), aeroplysinin-1 (**5**), aeroplysinin-2 (**6**), the dienone (**7**) as well as the dimethoxyketal (**8**) (Fig. 1). All compounds were unambiguously identified from their NMR and mass spectra, and by comparison with previously published data (for references see [8]).

As shown recently [10] the small molecular weight constituents of *V. aerophoba* such as aeroplysinin-1 (**5**) or the dienone (**7**) are biotransformation products originating from precursors of higher molecular weight such as isofistularin-3 (**1**) or aerophobin-2 (**4**) by enzymatically catalyzed conversions. These biotransformations result in a remarkable increase of the antibiotic as well as of the cytotoxic activity of the originating products **5** and **7** (Fig. 1) compared to their substrates **1** or **4** (Fig. 1). In the agar diffusion assay the methanolic extract of freeze dried *V. aerophoba* (characterized
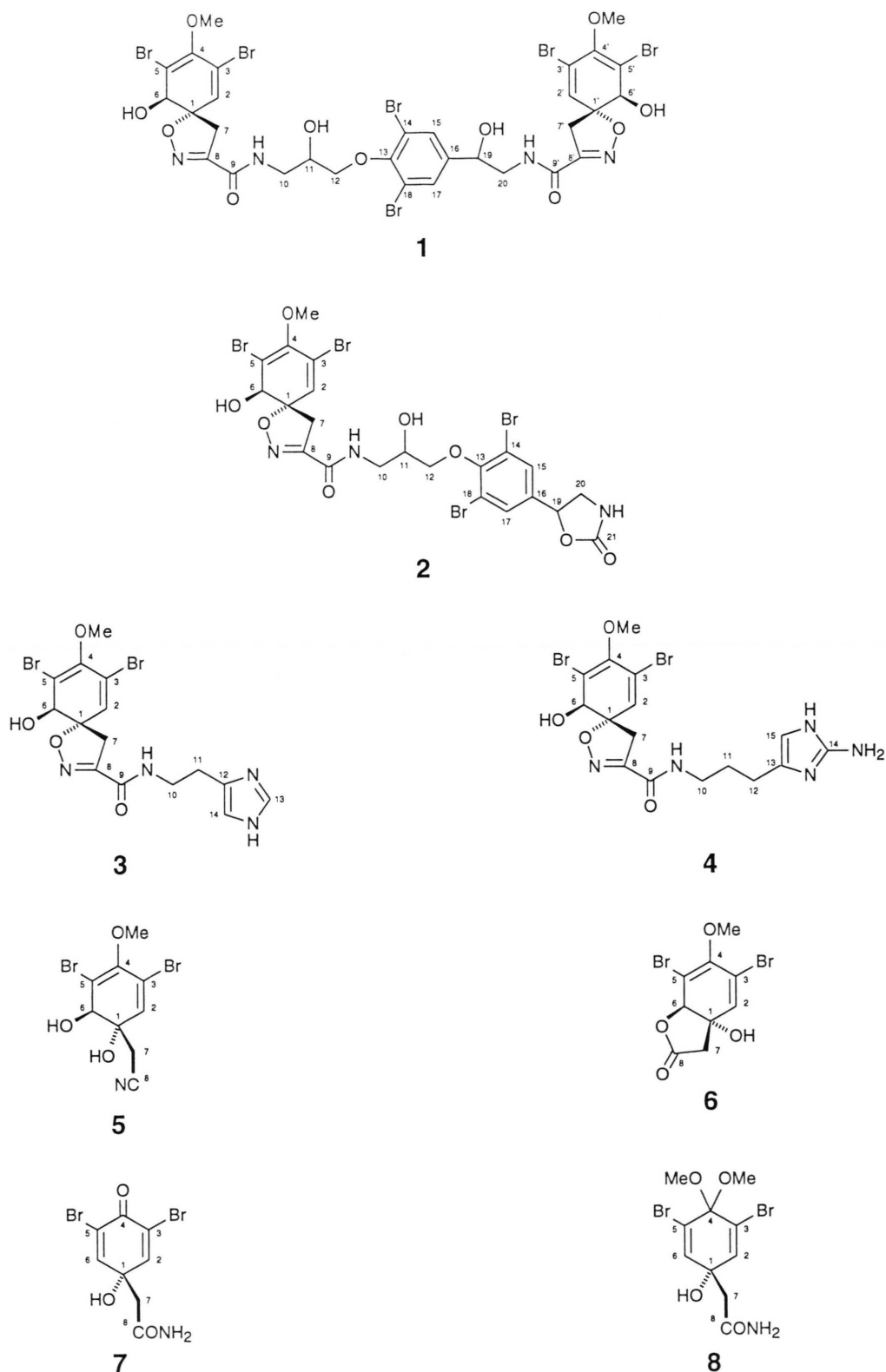
Fig. 1. Structures of brominated compounds (absolute configurations unknown) isolated from *V. aerophoba*.

Table I. Antimicrobial activity of brominated compounds from *V. aerophoba* in the agar diffusion assay.

Microorganisms tested	1	2	4	5	5 O-Ac	6	7	7 O-Ac	8
					(Diameter of growth inhibition in mm)				
Bacillus subtilis 168	–	–	–	24	–	–	21	15	–
Staphylococcus aureus ATCC 25923	–	–	–	30	–	–	20	17	–
Streptococcus faecalis 368	–	–	–	–	–	–	17	n.d.	–
Streptococcus pyogenes 157	–	–	–	–	–	–	24	n.d.	–
Escherichia coli ATCC 25922	–	–	–	24	–	–	17	8	–
Escherichia coli HB 101	–	–	–	30	–	–	23	14	–
Pseudomonas aeruginosa ATCC 27853	–	–	–	–	–	–	–	–	–
Saccharomyces cerevisiae HK 51	–	–	–	–	–	–	–	–	–

Numbers of compounds follow Fig. 1; 100 µg of each compound/disc were tested for growth inhibition; data shown are means of 3 independent experiments; n.d. = not determined.

only by compounds **1**, **3** and **4**) caused no growth inhibition of the test bacterium *Bacillus subtilis* (strain 168) whereas the aqueous extract (dienone (**7**) as dominating compound) showed pronounced antibacterial activity.

For comparative studies all brominated compounds isolated from *V. aerophoba* (except aerophobin-2 (**3**) which was not available in sufficient quantities) were screened for antimicrobial activity towards several gram-positive and gram-negative bacteria, as well as towards a yeast, using the agar diffusion assay (Table I). All of the brominated products tested except aeroplysinin-1 (**5**) and dienone (**7**) were inactive. The latter compounds, however, caused significant growth inhibition of *Bacillus subtilis, Staphylococcus aureus* as well as of two strains of *Escherichia coli* (Table I). The gram-negative bacterium *Pseudomonas aeruginosa,* as well as the yeast *Saccharomyces cerevisiae,* were not susceptible. Derivatization of aeroplysinin-1 (**5**) by acetylation of both hydroxy substituents nullified the antibiotic activity (Table I).

The antimicrobial activity of dienone (**7**), however, was retained following acetylation even though the O-acetyl derivative was somewhat less active towards *E. coli* compared to the underivatized dienone (**7**) (Table I).

The minimum inhibitory concentrations (MICs) of aeroplysinin-1 (**5**) were *ca.* 25 µg/ml (74 µM) towards *B. subtilis* (strain 168) and *S. aureus* (ATCC 25923) whereas dienone (**7**) was slightly less active with MICs of 25–50 µg/ml (68–136 µM) towards both bacteria (Table II). For *E. coli* (ATCC 25922) the MICs of both compounds were comparable (12.5 µg/ml equivalent to 37 or 34 µM for **5** or **7** respectively) (Table II). The minimum bactericidal concentrations (MBCs) of **5** and **7** towards the latter bacteria were in the range of 25–50 µg/ml (74–148 µM) for compound **5** and 50–100 µg/ml (136–272 µM) for compound **7** respectively (Table II).

Cytotoxicity of the brominated secondary compounds from *V. aerophoba* (except aeroplysinin-2 (**6**) and aerophobin-1 (**3**) which were not available

Table II. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of aeroplysinin-1 (**5**) and of dienone (**7**) towards selected bacteria.

Microorganisms tested	(5)		(7)	
	MIC	MBC	MIC	MBC
		(in µg/ml)		
Bacillus subtilis 168	25	100	25–50	100
Staphylococcus aureus ATCC 25923	25	100	25–50	100
Escherichia coli ATCC 25922	12.5	50	12.5	25–50

Numbers of compounds refer to Fig. 1; data shown are means of 3 independent experiments.

in sufficient quantities) was studied *in vitro* against the human HeLa cervix uteri tumour cell line, using the microculture tetrazolium assay. As shown in the antimicrobial studies the biotransformation products aeroplysinin-1 (**5**) and dienone (**7**) were significantly more active than their substrates isofistularin-3 (**1**) or aerophobin-2 (**4**). The IC_{50}s (inhibitory concentrations which reduce cell growth by 50% compared to controls) of aeroplysinin-1 (**5**) and dienone (**7**) were similar for both compounds and varied between 3.0–3.2 µM (equivalent to approximately 0.9–1.0 µg/ml) whereas the IC_{50}s of isofistularin-3 (**1**) or aerophobin-2 (**4**) amounted to 8.5 µM (9.4 µg/ml) or 99.3 µM (50 µg/ml) respectively (Table III). Aeroplysinin-1 (**5**) had previously been shown to display pronounced cytotoxic activity against several *in vitro* systems including L 5178 y mouse lymphoma cells (IC_{50} 0.5 µM), Friend erythroleukemia cells (IC_{50} 0.7 µM) and human mamma carcinoma cells (IC_{50} 0.3 µM) [11].

Acetylation of the free hydroxy groups of aeroplysinin-1 (**5**) resulted in a pronounced decrease of cytotoxic activity towards HeLa-cells (Table III). Similarly, a decrease in activity was observed when acetylating the four hydroxyl groups of isofistularin-3 (**1**) (Table I). Acetylation of dienone (**7**), however, caused a five-fold increase of the cytotoxic activity compared to the underivatized compound (Table III). The dienone O-acetate was comparable in activity to the therapeutically used anticancer drug cisplatine (IC_{50}s of 0.6 and 0.7 µM respectively) (Table III). We suggest that the formation of reactive oxygen radicals may, at least partially, underlie the cytotoxicity of aeroplysinin-1 (**5**) and dienone (**7**). Such radicals can be formed at both the hydroxyl and keto groups, and are stabilized through delocalization, due to the conjugated double bonds in the ring. In addition, large substituents such as bromine may also stabilize the radicals. Following acetylation of (**5**), both hydroxyl groups as centers for radical formation, are blocked. This may explain the ten-fold decrease in cytotoxicity as compared to aeroplysinin (**5**). In contrast, after acetylation of dienone (**7**), a center for radical formation remains present. The increase in cytotoxicity following acetylation of **7** compared to the underivatized compound (Table III) is possibly due to increased lipophilicity resulting in better penetration through cell membranes and higher intracellular concentrations of the acetylated compound. Further studies on the mode of action of these interesting cytotoxic natural products are underway.

Table III. *In vitro* cytotoxicity of brominated compounds from *V. aerophoba* measured towards HeLa tumour cells.

Brominated compounds	IC_{50} [µM]
1	8.5 ± 0.2
1 O-Ac	>100
2	84.3 ± 7.1
4	99.3 ± 14.5
5	3.0 ± 0.3
5 O-Ac	31.6 ± 1.6
7	3.2 ± 0.5
7 O-Ac	0.6 ± 0.2
8	>100
Cisplatin	0.7 ± 0.1

Numbers of compounds follow Fig. 1; data shown are means of 3 independent experiments.

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