Dibromotyrosine Derivatives, a Maleimide, Aplysamine-2 and Other Constituents of the Marine Sponge *Pseudoceratina purpurea*

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A collection of the marine sponge *Pseudoceratina purpurea* from the Gulf of Thailand furnished aplysamine-2, two new bromotyrosine derivatives purpuroceratic acids A and B, two known bromotyrosine derivatives, 3-maleimide-5-oxime and common sponge constituents. Aplysamine-2, purpuroceratic acid A and 3-maleimide oxime were evaluated for their *in vitro* anticancer activity against three cancer cell lines, but only aplysamine-2 exhibited moderate dose dependent growth inhibitory effects.

Key words: Pseudoceratina purpurea, Purpuroceratic Acids A and B, Aplysamine-2, Bromotyrosine Derivatives

Introduction

Sponges of the genus *Pseudoceratina* (order Verongida, family Aplysinellidae) have furnished a variety of brominated tyrosine or heterocyclic derivatives [1 – 8] while unusual glycerides have been isolated from one collection of *Pseudoceratina crassa* [9, 10]. More specifically, a dibromopyrrole-substituted spermidine, a brominated cyanoformamide and dibromotyrosine derivatives have been described from collections of *Pseudoceratina purpurea* collected in the seas surrounding Japan [11 – 13] while *Pseudoceratina purpurea* collected in Okinawa contained zamamistatin, a compact molecule derived from the condensation of two dibromotyrosine units [14]. A recent report [15] described the isolation and biological properties of the psammaplins, monobromotyrosine derived bisulfides obtained from Papua New Guinea collections of *Pseudoceratina purpurea* (see also [16]). We now report isolation of two new bromotyrosine derivatives **1** and **2**, aplysamine-2 (**3**) [17], 3-maleimide-5-oxime (**4**), the antimicrobial tyrosine derivatives (**5**) [18] and (**6**) [19], clionasterol and 1-tetradecene from *Pseudoceratina purpurea* collected in the Gulf of Thailand. Aplysamine-2 exhibited a moderate inhibitory effect against three human cancer cell lines.

Materials and Methods

^{1}H and ^{13}C NMR spectra were recorded at ambient temperature on a Bruker AMC instrument operating at 300.13 and 75.47 MHz, respectively. Rotations were determined on a Polax-2 L instrument. EI mass spectra were measured on a Hitachi Perkin-Elmer RMV-6M instrument. HRMS spectra were run using FAB+

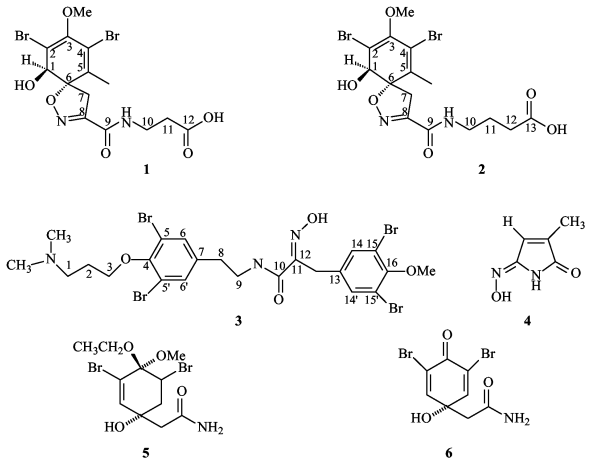

Table 1. ^{1}H and ^{13}C NMR spectra of compound **1**[a].

Position	δH	δC	COSY	HMBC
1	3.90 *dd* (8.1, 0.8)	73.60		C-3, C-4, C-5, C-6
2		120.83		
3		147.16		
4		113.12		
5	6.58 *s*	131.26		C-1, C-2, C-3, C-4, C-7
6		90.16		
7[a]	3.60 *d* (18.2)	40.21	H-7b	C-1, C-5, C-8
7[b]	3.20 *d* (18.2)	40.21	H-7a	C-1, C-5, C-6, C-8
8		154.42		
9		158.90		
10[c]	3.35 *q* (7.2)	35.06	H-11	C-9, C-11, C-12
11[c]	2.45 *t* (7.2)	33.41		C-10, C-12
12	12.16 *brs*	172.73		
NH	8.53 *t* (5.7)		H-10	C-9
OH	6.39 *d* (8.1)		H-1	C-1, C-4, C-5
OMe[b]	3.64 *s*	59.65		C-3

[a] In DMSO-d_6 at 500 MHz resp 125 MHz; [b] intensity 3 protons; [c] intensity 2 protons.

ionization with Xe gas at GKV on a KRATUS CONCEPT III, 2 sector mass spectrometer. The accelerating voltage was 8 KV. FT-ICR mass spectra were run on a 9.4 Tesla instrument. Silica gel for column chromatography was Si Gel 60 (0.2 – 0.5 mm Merck), for analytical and preparative TLC Si gel G-60 254 Merck.

Animal material

Pseudoceratina purpurea Carter, order Verongida, family Pseudoceratinidae, was collected by scuba dives in the Gulf of Thailand near Kho Chang Island, Trad province, Thailand, in November 2001 and frozen immediately at -20° prior to extraction. The sponge was identified by one of us (R.V.S., voucher registered as ZMA POR 17099, Section Invertebrates, Zoologisch Museum, University of Amsterdam).

Extraction, isolation and characterization of the constituents

The sample (2.3 kg net weight) was thawed, homogenized with EtOH (2 l), allowed to stand for 24 h in a dark chamber and filtered. The residue on the filter paper was again extracted with EtOH (3×500 ml), the aqueous alcoholic extracts were combined, evaporated at reduced pressure to ca. 300 ml and extracted with EtOAc (3×500 ml). The EtOAc extracts were combined and concentrated at reduced pressure to give a residue (19 g). The latter was chromatographed over Si gel (100 g) and eluted with petrol-$CHCl_3$ and $CHCl_3$-Me_2O, 150 ml frs being collected as follows: Frs 1-105 (petrol-$CHCl_3$, 2:3 v/v, 106 – 155 (petrol-$CHCl_3$, 1:4 v/v), 156 – 213 ($CHCl_3$), 214 – 264 ($CHCl_3$-Me_2O, 9:1 v/v), 265 – 300 ($CHCl_3$-Me_2O, 4:1 v/v). Recrystallization of frs 11 – 13 (3.25 g) from MeOH gave clionasterol (300 mg) identified by MS, ^{1}H NMR spectrometry and comparison with an authentic sample. Frs. 116 – 148 (690 mg) were purified by TLC (Si gel, $CHCl_3$-Me_2CO-HCO_2H, 85:15:0.1) to give 430 mg of **5** (see below) identified by MS, ^{1}H and ^{13}C NMR spectrometry and 17 mg of **6** [18] identified by MS, ^{1}H and ^{13}C NMR spectrometry and comparison with material isolated earlier from *Suberea* aff. *praetensa* [20]. Frs 164 – 180 (53 mg) on purification by TLC (Si gel, $CHCl_3$-Me_2CO-HCO_2H, 7:3:0.1) gave **4** (16.3 mg). Frs. 181 – 188 (129 mg) on purification by TLC (Si gel, $CHCl_3$-Me_2O-HCO_2H, 8:2:01) gave 1-tetradecene (30 mg), identified by MS, ^{1}H and ^{13}C NMR including HMBC, and **4** (10.3 mg). Frs 197 – 213 (78 mg) on purification by TLC (Si gel, $CHCl_3$-Me_2O-HCO_2H, 8:2:01) furnished more **4** (43 mg). Frs. 230 – 236 (92 mg) on purification by TLC (Si gel, $CHCl_3$-Me_2O-HCO_2H, 8:2:01) gave 27 mg of a 2:1 mixture of **2** and **1**. Frs. 259 – 269 (280 mg) on purification by TLC (Si gel, $CHCl_3$-Me_2O-HCO_2H, 6:4:0.1) furnished **1** (25 mg) and **3** (57 mg).

Purpuroceratic acid A (**1**)

Gum; MS (EI) M + H *m/z* 453; FT-ICR MS M + H 452.9294, calcd. for $C_{13}H_{16}O_6N_2Br_2$-H, 452.9291, $[\alpha]_D^{20}$ – 27.7° (MeOH, C = 0.55g/100 ml). ^{1}H and ^{13}C NMR spectra are listed in Table 1, assignments being based on decoupling, COSY and HMBC correlations. That the substance contained the spirocyclohexadienylisoxazole ring system derived from 3,5-

Table 2. ^{1}H and ^{13}C NMR spectra of compound **2**[a].

Position	δH	δC	COSY	HMBC
1	3.91 *dd* (8.1, 0.8)	73.60		C-3, C-4, C-5, C-6
2		120.87		
3		147.16		
4		113.12		
5	6.59 *s*	131.32		C-1, C-2, C-3, C-4
6		90.27		
7[a]	3.61 *d* (18.2)	40.21	H-7b	C-1, C-5, C-8
7[b]	3.21 *d* (18.2)	40.21	H-7a	C-1, C-5, C-6, C-8
8		154.55		
9		158.95		
10[c]	3.16 *q* (6.3)	38.21	H-11	C-9, C-11, C-12
11[c]	1.68 *q* (7.1)	24.30		C-10, C-12, C-13
12[c]	2.22 *t* (7.4)	31.02		C-10, C-11, C-13
13	12.16 *brs*	174.18		
NH	8.57 *t* (5.8)		H-10	C-9
OH	6.38 *d* (8.1)		H-1	C-1, C-4, C-5
OMe[b]	3.65 *s*	59.65		C-3

[a] In DMSO-d_6 at 500 MHz resp 125 MHz from mixture with compound **1**; [b] intensity 3 protons; [c] intensity 2 protons.

dibromotyrosine in common with other metabolites isolated from Verongida sponges was clear from the ^{1}H and ^{13}C NMR data listed in Table 1. The relative stereochemistry, *i. e.* the *trans*-geometry of the oxygens on C-1 and C-6, is based on comparisons of ^{1}H and ^{13}C chemical shifts with those of fistularin-3 [21] and dideoxyagelorins A and B previously reported from our laboratories [22] and with the ^{13}C chemical shifts of other compounds possessing the same 4-methoxy-3,5-dibromospirohexadienyl isoxazole system such as the recently described purealidin S [23] and caissarins [24]. The nature of the three carbon fragment attached to the amide nitrogen was also obvious from the ^{1}H and ^{13}C NMR spectra (Table 1). Assignments were verified by decoupling, COSY and HMBC.

Purpuroceratic acid B (**2**)

This substance was obtained only as part of a 2:1 mixture with its lower homolog purpuroceratic acid A as a gum; MS (EI) from mixture *m/z* of **2**, 468 *m/z* of **1** 454 found MS (EI and electrospray) found for $C_{14}H_{16}O_6N_2Br_2$-H, *m/z* 467, for $C_{13}H_{14}O_6N_2Br_2$-H, *m/z* 453; FT-ICR M - H for **1**, 452.9294; calcd. for $C_{13}H_{13}O_6N_2Br_2$, 452.9291; M - H for **2**, 466.9447; calcd. for $C_{14}H_{15}O_6N_2Br_2$, 466.9446. The structure of **2** was established by the ^{1}H and ^{13}C NMR spectra, COSY and HMBC data listed in Table 2 which resembled those of **1** but contained signals characteristic of one extra methylene group in the side chain attached to the amide nitrogen.

Table 3. ^{1}H and ^{13}C NMR spectra of aplysamine-2 (**3**)[a].

Position	δH	δC	COSY	HMBC
1[b]	3.28 – 3.33 *m*	54.44		C-2, C-3, N-Me_2
2[b]	2.15 *ddd* (16.2, 5.8, 5.8)	24.95	H-1, H-3	C-1, C-3
3[b]	3.96 *t* (5.8)	70.25	H-2	C-1, C-2
4		150.28		
5,5’		117.22		
6,6’	7.48 *s*	132.98		C-4, C-5, 5’, C-6, 6’, C-8
7		139.37		
8[b]	2.73 *t* (7.0)	33.37		C-6,6’, C-7, C-9
9[b]	3.34 – 3.37 *m*	39.70	H-8, NH	C-7, C-8, C-10
10		163.21		
11		151.77		
12[b]	3.71 *s*	27.80		C-10, C-11, C-13, C-14
13		130.44		
14	7.38 *d* (2.1)	132.98	H-14’	C-12, C-15, C-16
14’	7.12 *dd* (8.5, 2.1)	129.82	H-14, H-15’	
15		110.23		
15’	6.99 *d* (8.5)	112.58		C-13, C-15, C-16 (weak)
16		153.80		
OMe[c]	3.79 *s*	56.20		C-16
N-Me_2[d]	2.81 *s*	42.50		C-1
NH	8.07 *t* (5.7)			C-9, C-10
N-OH	11.86 *s*			C-11

[a] In DMSO-d_6 at 500 MHz resp 125 MHz; [b] intensity 2 protons; [c] intensity 3 protons; [d] intensity 6 protons.

Aplysamine-2 (**3**)

A third constituent of our collection was aplysamine-2 (**3**) which has been reported previously from an *Aplysina* species collected in Australian coastal waters [17]. That it occurred naturally as a hydrochloride was inferred from the upfield shifts of the N-methyl signals and those of H-1 and H-2 on addition of base although the mass spectra indicated the molecular formula $C_{25}H_{28}Br_3N_3O_4$, apparently as the result of the facile loss of HCl. Chemical shifts in the ^{1}H and ^{13}C NMR spectra of our semisolid sample in DMSO are listed in Table 3 and tally essentially with those reported earlier in MeOH, allowing for the difference in solvent. COSY and HMBC correlations which are included in Table 3 confirmed the conclusions reached earlier by the Australian authors solely on the basis of mass spectral evidence.

3-Maleimide-5-oxime (**4**)

Despite its apparent simplicity this appeared to be a new substance whose structure assignment was based on mass spectrometry and ^{1}H and ^{13}C NMR data; colorless crystals; mp 170 – 172° from MeOH, MS *m/z* 126(100), 83(20), 55(35), HRMS 124.04306, calcd.

for $C_5H_6N_2O_2$ 126.04293; ^{1}H NMR (DMSO) $\delta =$ 11.03 *brs* (NH), 10.61 *brs* (=N-OH), 7.26 *d* ($J =$ 0.9 Hz, vinyl H), 1.73 (3p, *d*, $J = 0.9$ Hz, vinylic methyl); ^{13}C NMR (DMSO) $\delta = 164.94$ (C-2), 151.50 (C-5), 137.74 (C-4), 107.67 (C-3), 11.73 (Me). The stereochemistry assigned to the oxime function- *cis* to the NH- group – is based on the NOESY spectrum which displayed a strong cross peak between the oxime proton at $\delta = 10.61$, and the *brs* of the NH proton at $\delta = 11.03$. Both –OH and –NH protons exhibited only very weak cross peaks with the olefinic doublet at $\delta = 7.26$ which gave a cross peak with the signal of the vinylic methyl group.

2,6-Dibromo-cis-1-methoxy, 4-hydroxy-cis-1-ethoxy-4-acetamido-2,5-cyclohexadiene (**5**)

This substance, first obtained from an unspecified *Verongia* species [19], was originally assumed to be an artifact and a mixture of C-1-epimers arising by reaction of parent dienone with solvent. However it was more recently shown [25] that it and three analogs had the stereochemistry specified in the formula and were therefore naturally occurring substances, not artifacts.

Cytotoxicity assay

a) Cell lines. Human tumor cell lines: MCF-7 (breast), NCI-H460 (lung) and SF-268 (CNS) were provided by the National Cancer Institute, Bethesda, MD.

b) Cell growth assay. The protocol used was described in our earlier publication on *Suberea* aff. *praetensa* (Kijjoa *et al.*, 2002).

Table 4. Effect of purpuroceratic acid A (**1**), aplysamine-2 (**3**) and maleimide 5-oxime (**4**) on the growth of human tumor cell lines.

Compounds	MCF-7 (breast)	NCI-H460 (lung) $GI_{50}(\mu M)$	SF-268 (CNS)
1	> 100.1	> 100.1	> 100.1
3	25.7 ± 1.4	32.8 ± 1.8	40.87 ± 1.5
4	> 396.8	> 396.8	> 396.8

Results are the mean ± SEM of three independent experiments performed in duplicate. Doxorubicin was used as positive control, GI_{50} MCF-7 = 42.8 ± 8.2 nM; GI_{50} NCI-H460 = 94.0 ± 8.7 nM; GI_{50} SF-268 = 93.0 ± 7.0 nM.

Results and Discussion

In vitro effects of compounds **1**, **3** and **4** from *Pseudoceratina purpurea* on the growth of three human cancer cell lines are listed in Table 4. Results are given in concentrations causing 50% cell growth inhibition. (GI_{50}). Only aplysamine-2 (**3**) exhibited, after continuous exposure for 48 hours a moderate dose dependent growth inhibitory effect against all three cell lines; the two others were ineffective as growth inhibitors even when tested at concentrations of 110.1 μM and 396 μM, respectively.

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