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PAPER

Pseudoceramines A–D, new antibacterial bromotyrosine alkaloids from the marine sponge *Pseudoceratina* sp.†

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Bioassay-guided fractionation of the CH_2Cl_2/MeOH extract of the Australian marine sponge *Pseudoceratina* sp. resulted in the purification of four new bromotyrosine alkaloids, pseudoceramines A–D (**1–4**), along with a known natural product, spermatinamine (**5**). The structures of **1–5** were determined by spectroscopic methods. Pseudoceramines A (**1**) and B (**2**) feature a rare bromotyrosyl-spermine-bromotyrosyl sequence, and pseudoceramine C (**3**) is the first example of bromotyrosine coupled with an *N*-methyl derivative of spermidine. Compounds **1–5** were screened for inhibition of toxin secretion by the type III secretion (T3S) pathway in *Yersinia pseudotuberculosis*. Compounds **2** and **5** inhibited secretion of the Yersinia outer protein YopE (IC_{50} = 19 and 6 μM, respectively) and the enzyme activity of YopH (IC_{50} = 33 and 6 μM, respectively).

Introduction

Type III secretion (T3S) constitutes a common virulence system present in many Gram-negative bacterial species, including *Yersinia* spp., *Salmonella* spp., *Shigella* spp., *Pseudomonas aeruginosa*, entheropathogenic *Escherichia coli*, enterohemoragic *E. coli*, and *Chlamydia* spp.[1,2] These bacteria depend on their respective T3S systems to invade the host, resist phagocytosis, grow in deep tissues, and cause disease. The well-studied plasmid-encoded Ysc (for Yersinia secretion) T3S system of *Yersinia* represents these common virulence systems. The bacterium adheres to a eukaryotic cell and injects a set of bacterial effector proteins, Yops (Yersinia outer proteins), into the lumen of the target cell, resulting in inhibition of the innate immune response.[3,4] Six different effector Yops have been identified including YopE, which inhibits phagocytosis, pore formation and cytokine production and YopH, a protein tyrosine phosphatase, which also inhibits phagocytosis; and T- and B-cell activation.[5] These Yops are essential for virulence and synthetic small molecules that inhibit Yop secretion have been identified.[6,7] It has been shown that these inhibitors can attenuate virulence without affecting bacterial growth in a number of bacterial species,[7–11] and chemical optimization has resulted in more potent compounds and quantitative structure–activity relationships.[6,12,13] In addition two articles describe activity of T3S inhibitors *in vivo*.[14,15] These findings clearly indicate the possibility to develop novel antibacterial agents that target T3S-based virulence without a detrimental effect upon bacterial growth.[16,17]

High-throughput screening (HTS) of our prefractionated natural product library[18,19] has recently been undertaken in order to discover new compounds that potentially inhibit the T3S system. A primary assay utilizing the bacterial clone *Y. pseudotuberculosis* YPIII(pIB102-Elux),[6] with a luminescent reporter gene under the control of the promoter for the T3S effector protein YopE, detected three fractions derived from a sponge belonging to the genus *Pseudoceratina* (Pseudoceratinidae) that inhibited the luminescent signal. To verify the positive hits a colorimetric assay to detect the protein tyrosine phosphatase enzyme activity of the secreted effector protein YopH was used and suggested a T3S-selective inhibition by these fractions. Bioassay-guided fractionation of the CH_2Cl_2/MeOH extract from *Pseudoceratina* sp. resulted in the purification of four new bromotyrosine alkaloids, pseudoceramines A–D (**1–4**), along with the known, spermatinamine (**5**). Pseudoceramines A (**1**) and B (**2**) feature a rare bromotyrosyl-spermine-bromotyrosyl sequence, and pseudoceramines C (**3**) is the first example of bromotyrosine coupled with an *N*-methyl derivative of spermidine. These compounds were tested in a combined reporter-gene and phosphatase assay and also an *in vitro* antibacterial assay to estimate whole cell survival in the presence of the compounds. Compounds **2** and **5** exhibited potent inhibitory activity in the combination assay and also demonstrated similar levels of activity in an assay for inhibition of the bacterial growth. The inhibitory effect on bacterial growth suggests that the compounds might be general antibacterials and not specific

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T3S inhibitors. It was previously shown that the screening assay identifies both types of compounds.[6]

Marine sponges from the order Verongida have proved to be a rich source of bromotyrosine-derived alkaloids,[20] some of which exhibit potent antibacterial,[21,22] anti-HIV,[23,24] antimalarial[25,26] and cytotoxic activities.[27,28] Chemical modification usually occurs both in the side chain and the aromatic ring of the brominated tyrosine precursors, giving rise to a broad range of biosynthetically related compounds. Previously, we have reported spermatinamine (**5**)[29] from the same sponge material, which was the first natural product inhibitor of the cancer-related target, isoprenylcysteine carboxyl methyltransferase (Icmt). In this paper, we report the isolation and structure elucidation of compounds **1–5** as well as their inhibitory activity against the T3S system in *Yersinia pseudotuberculosis*.

Results and discussion

The freeze-dried and ground marine sponge *Pseudoceratina* sp. was extracted with n-hexanes, CH_2Cl_2/MeOH (4 : 1), and MeOH. The CH_2Cl_2/MeOH extracts were combined and initially chromatographed by reversed-phase C_{18} HPLC (MeOH/H_2O/0.1% TFA) to give 60 fractions that were subsequently screened for T3S inhibition. Fractions 36–41 showed activity in both the reporter-gene (YopE) and phosphatase (YopH) assays and were further purified by C_{18} HPLC (MeOH/H_2O/0.1% TFA) to yield the TFA salts of the bromotyrosine alkaloids **1–5**.

Compound **5**, a symmetrical molecule, was isolated as the major component (70 mg, 1.05% dry wt) and identified as spermatinamine following comparison of the NMR/MS data with literature values.[29]

Pseudoceramine A (**1**) was obtained as an optically inactive powder. The LRESIMS showed 1 : 4 : 6 : 4 : 1 ion cluster peaks at *m/z* 911/913/915/917/919 $[M+H]^+$ and 456/457/458/459/460 $[M+H]^{2+}$, indicating the presence of four bromine atoms. The $[M+H]^+$ ion in the (+)-HRESIMS at *m/z* 911.0007 allowed the molecular formula $C_{31}H_{42}Br_4N_6O_6$ to be assigned to **1**. The ^{1}H-NMR data (Table 1) and HSQC spectrum showed signals for two exchangeable protons [δ_H 12.04 (s) and 11.95 (s)], two amide protons [δ_H 8.18 (t, J = 6.0) and 8.21 (t, J = 6.0)], four aromatic protons [δ_H 7.46 (2H, s) and 7.35 (2H, s); δ_C 132.5 and 132.0], two benzylic methylenes [δ_H 3.77 (2H, s) and 3.71 (2H, s); δ_C 27.6 and 27.3], one aromatic methoxy [δ_H 3.75 (3H, s), δ_C 60.1], two *N*-methyls [δ_H 2.72 (6H, s), δ_C 38.9], and a large number of aliphatic methylene multiplets. The aforementioned data of **1** was similar to those of spermatinamine (**5**) implying that **1** was also a bromotyrosine alkaloid. In comparison with **5**, the NMR spectroscopic data for **1** differed significantly about one of the bromotyrosine moieties, with the absence of an aromatic methoxy and the upfield-shifted aromatic carbon at C-3″(C-5″) and C-1″ (δ_C 117.0 and 136.2 in **5**; δ_C 111.6 and 132.6 in **1**, respectively). This indicated that one aromatic methoxy in **5** was replaced by a hydroxy in **1**.[30] Further evidence came from the presence of two sets of bromotyrosyl signals in the ^{1}H-NMR spectrum of **1**, since the new molecule was unsymmetrical compared with **5**. The *E* configurations for both oximes in **1** were determined by the diagnostic carbon chemical shifts of the benzylic methylenes (C-7′ and C-7″, δ_C 27.6 and 27.3, respectively). The benzylic methylene corresponding to *Z* oximes are known to resonate > δ_C 35.[31] Detailed interpretation of the 2D data (HMBC, ^{1}H-^{1}H COSY and ROESY) confirmed the connectivity between spermine and bromotyrosine moieties and allowed the fully structural assignment of **1** as a 4″-*O*-demethyl derivative of **5** (Fig. 1).

Table 1 NMR data for pseudoceramines A (**1**) and B (**2**) in DMSO-d_6[a]

	1		**2**	
Number	δ_H (mult, J/Hz)	δ_C[b]	δ_H (mult, J/Hz)	δ_C[b]
2	3.21 (2H, t, 5.4)	36.0	3.22 (2H, m)	35.8
3/12	1.81 (4H, m)	23.6	1.84 (4H, m)	23.6
4/11	3.01 (4H, m)	53.2	3.07 (4H, m)	53.4
5/10-NMe	2.72 (6H, s)	38.9	2.73 (6H, s)	39.0
6/9	3.03 (4H, m)	53.3	3.04	53.7
7/8	1.62 (4H, m)	20.3	1.64 (4H, m)	20.3
13	3.21 (2H, t, 5.4)	36.0	3.14 (2H, m)	35.8
1′		136.4		136.2
2′/6′	7.46 (2H, s)	132.5	7.46 (2H, s)	132.6
3′/5′		117.1		117.1
4′		151.5		151.6
4′-OMe	3.75 (3H, s)	60.1	3.78 (3H, s)	60.2
7′	3.77 (2H, s)	27.6	3.77 (2H, s)	27.8
8′		150.8		150.7
9′		163.3		163.3
1″		132.6		no
2″/6″	7.35 (2H, s)	132.0	7.51 (2H, s)	133.4
3″/5″		111.6		117.1
4″		149.0		152.4
4″-OMe			3.78 (3H, s)	60.2
7″	3.71 (2H, s)	27.3	2.98 (1H, m)	34.2
			3.08 (1H, m)	
8″		151.2	3.95 (1H, brs)	61.1
9″		163.3		166.4
8′-NOH	12.04 (1H, s)		12.04 (1H, s)	
8″-NOH	11.95 (1H, s)			
1(N)	8.18 (1H, t, 6.0)		8.21 (1H, t, 6.0)	
14(N)	8.21 (1H, t, 6.0)		8.71 (1H, t, 5.4)	
5/10(N)	9.42 (2H)[c]		9.51 (1H, brs)	
			9.78 (1H, brs)	
8″-N^+H_2Me			2.51 (3H, s)	31.3
			8.95 (1H, brs)	
			9.05 (1H, brs)	

[a] ^{1}H-NMR at 600 MHz and ^{13}C-NMR at 150 MHz. [b] ^{13}C chemical shifts obtained from 2D NMR experiments. [c] Obtained from ^{1}H–^{1}H COSY correlations.

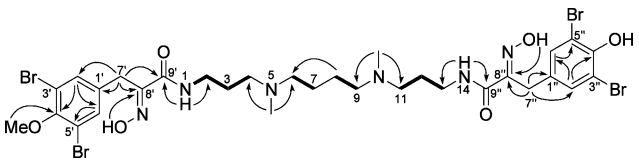
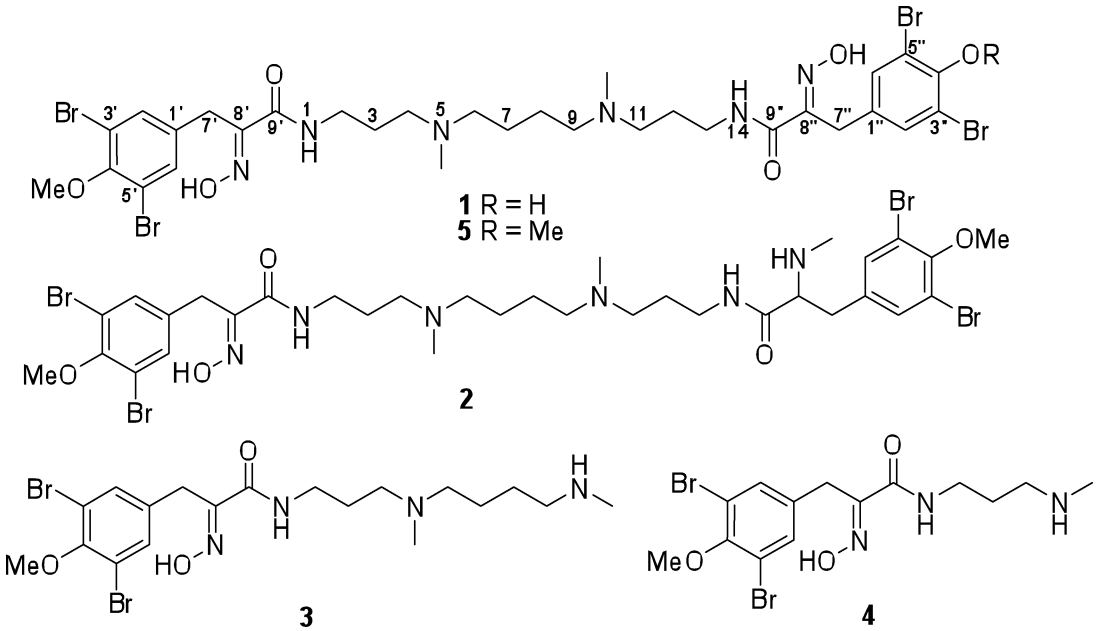


Fig. 1 Selected HMBC (↔) and ^{1}H-^{1}H COSY (—) correlations of **1**.

Pseudoceramine B (**2**) exhibited a molecular formula of $C_{33}H_{48}Br_4N_6O_5$ as determined by HRESIMS at *m/z* 925.0494 $[M+H]^+$ (calcd for $C_{33}H_{49}{}^{79}Br_4N_6O_5$, 925.0492). The ^{1}H NMR spectrum of **2** displayed signals for one oxime proton [δ_H 12.04 (s)], two amide protons [δ_H 8.21 (t, J = 6.0 Hz) and 8.71 (t, J = 5.4 Hz)], four aromatic protons [δ_H 7.46 (2H, s) and 7.51 (2H, s)], two benzylic methylenes [δ_H 3.77 (2H, s) and 2.98 (1H, m)/3.08 (1H, m)], two aromatic methoxys [δ_H 3.78 (6H, s)], two *N*-methyls [δ_H 2.73 (6H, s)], and a large number of aliphatic methylene multiplets. The aforementioned data implied that **2** was an analogue of **5**, with minor structural modification occurring on one of the bromotyrosine moieties. Detailed analysis of HSQC, HMBC, and ^{1}H-^{1}H COSY data revealed that **2** possessed an unusual



N-methyl group at C-8″ instead of an (*E*)-oxime commonly found in bromotyrosine metabolites. The presence and the location of the 8″-*N*H-methyl group were indicated by ^{1}H-^{1}H COSY correlations of 8″-*N*H/*N*-Me and H-8″, as well as the HMBC correlation from *N*-Me to C-8″ (Fig. 2). The ^{1}H-NMR signal of 8″-*N*-methyl, which overlapped with the DMSO-d_6 solvent peak (δ_H 2.51) was distinguished when recorded in CD_3OD (δ_H 2.64, Supporting information S10). Further evidence came from the downfield-shifted carbon signals of C-7″ and C-9″ (from δ_C 27.6 and 163.3 in **5** to 34.2 and 166.4 in **2**, respectively), as the conjugated oxime double bond in **5** was reduced in **2**. The stereochemistry of the chiral center (C-8″) in **2** was not determined, given the small amount of compound, however we note possible correlation with the similar moiety in suberedamine A that has had its absolute stereochemistry determined by chemical means.[32]

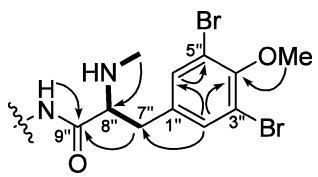


Fig. 2 Selected HMBC (↔) and ^{1}H-^{1}H COSY (—) correlations for partial structure of **2**.

The minor metabolite isolated was pseudoceramine C (**3**), which was obtained as an optically inactive TFA salt. The ESIMS showed an isotopic cluster of $[M+H]^+$ ions in the ratio of 1 : 2 : 1 at *m*/*z* 521, 523, and 525, indicating the presence of two bromine atoms in **3**. The molecular formula of $C_{19}H_{30}Br_2N_4O_3$ was determined by HRESIMS. The ^{1}H-NMR spectrum of **3** displayed one oxime proton [δ_H 12.03 (s)], three exchangeable protons [δ_H 9.25 (1H, brs) and 8.37 (2H, brs)], one amide proton [δ_H 8.21 (1H, t, J = 6.0 Hz)], two aromatic protons [δ_H 7.46 (2H, s)], one benzylic methylene [δ_H 3.77 (2H, s)], one aromatic methoxy [δ_H 3.75 (3H, s)], two *N*-methyls [δ_H 2.72 (3H, d, J = 4.2 Hz) and 2.55 (3H, t, J = 4.8 Hz)], and a large number of aliphatic methylene multiplets (Table 2).

Table 2 NMR data for pseudoceramines C (**3**) and D (**4**) in DMSO-d_6[a]

	3		**4**	
Number	δ_H (mult, J/Hz)	δ_C[b]	δ_H (mult, J/Hz)	δ_C
2	3.20 (2H, m)	35.8	3.21 (2H, m)	35.8
3	1.82 (2H, m)	23.6	1.74 (2H, m)	25.8
4	2.98 (2H, m)	53.4	2.84 (2H, m)	46.1
5-NMe	2.72 (3H, d, 4.2)	39.0	2.54 (3H, t, 5.4)	32.5
6	3.07 (2H, m)	53.4		
7	1.63 (2H, m)	20.3		
8	1.57 (2H, m)	22.2		
9	2.88 (2H, t, 6.0)	47.2		
10-NMe	2.55 (3H, t, 4.8)	32.1		
1′		136.5		136.2
2′/6′	7.46 (2H, s)	132.9	7.46 (2H, s)	132.9
3′/5′		117.2		117.0
4′		151.8		151.7
4′-OMe	3.75 (3H, s)	60.1	3.76 (3H, s)	60.3
7′	3.77 (2H, s)	27.8	3.77 (2H, s)	27.8
8′		150.8		150.9
9′		163.4		163.4
8′-NOH	12.03 (1H, s)		12.02 (1H, s)	
1(N)	8.21 (1H, t, 6.0)		8.21 (1H, t, 5.4)	
5(N)	9.25 (1H, brs)		8.29 (2H, brs)	
10(N)	8.37 (2H, brs)			

[a] ^{1}H-NMR at 600 MHz and ^{13}C-NMR at 150 MHz. [b] ^{13}C chemical shifts obtained from 2D NMR experiments.

2D NMR analysis (HSQC, ^{1}H-^{1}H COSY and HMBC) revealed 19 carbon signals accounted for one bromotyrosine moiety, two proton spin systems (-$CH_2CH_2CH_2$- and -$CH_2CH_2CH_2CH_2$-), and two *N*-methyls. The connectivity of these fragments were achieved by HMBC correlations of N_1-H/C-9′ and C-2, N_5-methyl/C-4 and C-6, and N_{10}-methyl/C-9 (Fig. 3), indicating **3** was one of the N_{10}-C_{11} cleavage derivatives of **5**. Thus pseudoceramine C was assigned to structure **3**. Compound **3** is the first example of a bromotyrosine coupled with a spermidine derivative.

Pseudoceramine D (**4**) was obtained as an optically inactive TFA salt. The molecular formula of $C_{14}H_{19}Br_2N_3O_3$ was established by HRESIMS at *m*/*z* 435.9864 $[M+H]^+$ (calcd for $C_{14}H_{20}{}^{79}Br_2N_3O_3$,

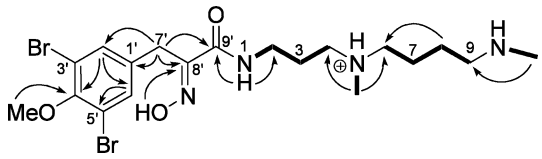


Fig. 3 Selected HMBC (↔) and H–H COSY (—) Correlations of **3**.

435.9866) and NMR data. The ^{1}H-NMR spectrum of **4** was very similar to that of **3** except for the absence of one *N*-methyl and a couple of aliphatic methylene signals. The ^{13}C NMR spectrum of **4** displayed 14 signals that accounted for one amide carbonyl, one oxime carbon, six aromatic carbons, four aliphatic carbons, one *N*-methyl, and one aromatic methoxy. Aforementioned data implied **4** was a fragment of **3**, consisting of a bromotyrosine moiety and 1, 3-propandiamine chain. 2D NMR analysis (HSQC, ^{1}H-^{1}H COSY and HMBC) confirmed the connectivity of the two moieties and illustrated that **4** was one of the N_5-C_6 cleavage derivatives of **3** (Fig. 4).

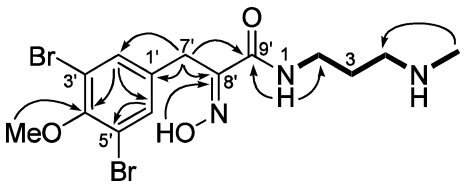


Fig. 4 Selected HMBC (↔) and H-H COSY (—) correlations of **4**.

Compounds **1–5** were tested in the YopE (reporter gene assay), YopH (phosphatase assay) and antibacterial assays (Table 3). The reference compound streptomycin was used as an example of a general antibiotic mode of action. Compounds **2** and **5** exhibited potent inhibitory activity in the reporter-gene assay (IC_{50} = 19 and 6 μM, respectively) and the phosphatase assay (IC_{50} = 33 and 6 μM, respectively), and also demonstrated similar levels of activity in an assay for inhibition of bacterial growth. This suggested **2** and **5** might act as general antibacterials rather than specific inhibitors of the T3S system. These data also suggest that the intact bromotyrosyl-spermine-bromotyrosyl sequence and methoxy groups might be essential for Yop inhibition, as compound **3** and **4** with only one bromotyrosyl moiety and short chains showed a decrease in their activity while **1**, which was missing one methoxy also displayed reduced activity. Further studies would determine if this action was based on toxicity or a real effect on the T3S system and if the compounds have broad antibacterial activity.

Table 3 The YopE, H and antibacterial assay activities of compounds **1–5**

	IC_{50} (μM)*		
Compound	YopE	YopH	Antibacterial assay
1	~29[a]	>100	>100
2	19 ± 6	33 ± 6	~40[b]
3	>100	>100	>100
4	>100	>100	>100
5	6 ± 2	6 ± 1	4 ± 1
Streptomycin	38 ± 3	31 ± 10	36 ± 6

*approximate 50% inhibition shown when IC_{50} could not be determined.[a] 52 ± 4% at 29 μM; [b] 103 ± 15% at 91 μM.

Conclusions

In summary, this paper describes the bioassay-guided isolation and structure elucidation of four new bromotyrosine alkaloids, pseudoceramines A–D (**1–4**), along with a known natural product, spermatinamine (**5**). Pseudoceramines A (**1**) and B (**2**) feature a rare bromotyrosyl-spermine-bromotyrosyl sequence, and pseudoceramine C (**3**) is the first example of bromotyrosine coupled with an *N*-methyl derivative of spermidine. Compounds **2** and **5** were identified as putative T3S inhibitors with YopE IC_{50} values of 19 and 6 μM, respectively. Both compounds also inhibited bacterial growth suggesting that they are general antibacterials rather than specific T3S inhibitors.

Experimental

General procedures

NMR spectra were recorded at 30 °C on either a Varian 500 MHz or 600 MHz Unity INOVA spectrometer. The latter spectrometer was equipped with a triple resonance cold probe. The ^{1}H and ^{13}C NMR chemical shifts were referenced to the solvent peak for DMSO-d_6 at δ_H 2.49 and δ_C 39.5. LRESIMS were recorded on a Waters ZQ mass spectrometer. HRESIMS were recorded on a Bruker Daltronics Apex III 4.7e Fourier-transform mass spectrometer. IR and UV spectra were recorded on a Bruker Tensor 27 spectrometer and a Jasco V650 UV/Vis spectrophotometer, respectively. Optical rotations were recorded on a Jasco P-1020 polarimeter. A BIOLINE orbital shaker was used for the large-scale extraction of sponge material. Alltech Davisil 40–60 μm 60 Å C_{18} bonded silica was used for pre-adsorption work. A Waters 600 pump equipped with a Waters 996 PDA detector and a Waters 717 autosampler were used for HPLC. A ThermoElectron C_{18} Betasil 5 μm 143 Å column (21.2 mm × 150 mm) and a Phenomenex Luna 5 μm C_{18} column (10 mm × 250 mm) were used for semi-preparative HPLC separations. All solvents used for chromatography, UV, and MS were Lab-Scan HPLC grade, and the H_2O was Millipore Milli-Q PF filtered.

Collection and identification of sponge material

The sponge sample *Pseudoceratina* sp. (phylum: Porifera, class: Demospongiae, order: Verongida, family: Pseudoceratinidae) was collected by scuba diving at a depth of 14 m from Erskine Island, Capricorn-Bunker Group, Great Barrier Reef, Queensland, Australia, in August 1996. A voucher sample, QMG307576, was lodged at the Queensland Museum, South Brisbane, Queensland, Australia.

Extraction and isolation

The freeze-dried and ground sponge material (10 g) was transferred to a conical flask (1 L), n-hexane (250 mL) was added and the flask was shaken at 200 rpm for 2 h. The n-hexane extract was filtered under gravity, then discarded. CH_2Cl_2/MeOH mixture (4 : 1, 250 mL) was added to the de-fatted sponge material in the conical flask and shaken at 200 rpm for 2 h. The resulting extract

was filtered under gravity, and set aside. MeOH (250 mL) was added and the MeOH/sponge mixture was shaken for a further 2 h at 200 rpm. Following gravity filtration the marine material was extracted with another volume of MeOH (250 mL), while being shaken at 200 rpm for 16 h. All CH_2Cl_2/MeOH extractions were combined and dried under reduced pressure to yield a dark brown solid (1.5 g). A portion of this residue (1.0 g) was pre-adsorbed to C_{18}-bonded silica, then packed into a stainless steel guard cartridge (10 × 30 mm) that was subsequently attached to a C_{18} Betasil HPLC column. Isocratic HPLC conditions of 90% H_2O (0.1% TFA)/10% MeOH (0.1% TFA) were initially employed for the first 10 min, then a linear gradient to MeOH (0.1% TFA) was run over 40 min, followed by isocratic conditions of MeOH (0.1% TFA) for a further 10 min, all at a flow rate of 9 mL min^{-1}. Sixty fractions (60 × 1 min) were collected from time = 0. Fraction 41 and 39 yielded pure spermatinamine (**5**, 70 mg, 1.05% dry wt) and pseudoceramine B (**2**, 1.5 mg, 0.023% dry wt), respectively. Fraction 38 was further purified by repeating the HPLC conditions described above and yielded pseudoceramine D (**4**, 3.0 mg, 0.045% dry wt) at 40 min. Fraction 40 and fraction 36 were further purified by HPLC using a C_{18} Luna column, using a gradient from 55% MeOH (0.1% TFA)/45% H_2O (0.1% TFA) to MeOH (0.1% TFA) over 20 min at a flow rate of 4 mL min^{-1}. Pseudoceramine A eluted at 12 min (**1**, 2.3 mg, 0.035% dry wt) while pseudoceramine C eluted at 11 min (**3**, 0.9 mg, 0.014% dry wt), respectively.

Pseudoceramine A (1). white powder; $[\alpha]_D^{25}$ 0 (*c* 0.11, MeOH); UV λ_{max} (MeOH)/nm (log ε) 207 (4.99), 285 (3.63); IR ν_{max}/cm^{-1} (film) 3439, 1675, 1472, 1205, 1136, 1000, 724; ^1H (600 MHz) and ^{13}C (150 MHz) NMR, Table 1; (+)-LRESIMS *m/z* (35 eV) (rel int) 456 $[C_{31}H_{42}{}^{79}Br_4N_6O_6+2H]^{2+}$ (15), 457 $[C_{31}H_{42}{}^{79}Br_3{}^{81}BrN_6O_6+2H]^{2+}$ (60), 458 $[C_{31}H_{42}{}^{79}Br_2{}^{81}Br_2N_6O_6+2H]^{2+}$ (100), 459 $[C_{31}H_{42}{}^{79}Br^{81}Br_3N_6O_6+2H]^{2+}$ (90), 460 $[C_{31}H_{42}{}^{81}Br_4N_6O_6+2H]^{2+}$ (40), 911.0 $[C_{31}H_{42}{}^{79}Br_4N_6O_6+H]^+$ (1), 913.0 $[C_{31}H_{42}{}^{79}Br_3{}^{81}BrN_6O_6+H]^+$ (5), 915.0 $[C_{31}H_{42}{}^{79}Br_2{}^{81}Br_2N_6O_6+H]^+$ (12), 917 $[C_{31}H_{42}{}^{79}Br^{81}Br_3N_6O_6+H]^+$ (7), 919 $[C_{31}H_{42}{}^{81}Br_4N_6O_6+H]^+$ (2); (+)-HRESIMS *m/z* 911.0007 (calcd for $C_{31}H_{43}{}^{79}Br_4N_6O_6$, 910.9972).

Pseudoceramine B (2). white powder; $[\alpha]_D^{25}$ +8 (*c* 0.09, MeOH); UV λ_{max} (MeOH)/nm (log ε) 207 (4.57), 275(3.24); IR ν_{max}/cm^{-1} (film) 3435, 1675, 1473, 1410, 1203, 1135, 996, 723; ^1H (600 MHz) and ^{13}C (150 MHz) NMR, Table 1; (+)-LRESIMS *m/z* (35 eV) (rel int) 463.0 $[C_{33}H_{48}{}^{79}Br_4N_6O_5+2H]^{2+}$ (10), 464.0 $[C_{33}H_{48}{}^{79}Br_3{}^{81}BrN_6O_5+2H]^{2+}$ (52), 465.0 $[C_{33}H_{48}{}^{79}Br_2{}^{81}Br_2N_6O_5+2H]^{2+}$ (95), 466.0 $[C_{33}H_{48}{}^{79}Br^{81}Br_3N_6O_5+2H]^{2+}$ (100), 467.0 $[C_{33}H_{48}{}^{81}Br_4N_6O_5+2H]^{2+}$ (49), 925.1 $[C_{33}H_{48}{}^{79}Br_4N_6O_5+H]^+$ (1), 927.1 $[C_{33}H_{48}{}^{79}Br_3{}^{81}BrN_6O_5+H]^+$ (3), 929.1 $[C_{33}H_{48}{}^{79}Br_2{}^{81}Br_2N_6O_5+H]^+$ (5), 931.1 $[C_{33}H_{48}{}^{79}Br^{81}Br_3N_6O_5+H]^+$ (4), 933.0 $[C_{33}H_{48}{}^{81}Br_4N_6O_5+H]^+$ (2); (+)-HRESIMS *m/z* 925.0494 (calcd for $C_{33}H_{49}{}^{79}Br_4N_6O_5$, 925.0492).

Pseudoceramine C (3). white powder; $[\alpha]_D^{25}$ 0 (*c* 0.05, MeOH); UV λ_{max} (MeOH)/nm (log ε) 206 (4.28), 275 (3.03); IR ν_{max}/cm^{-1} (film) 3437, 1669, 1472, 1204, 1136, 996, 722; ^1H (600 MHz) and ^{13}C (150 MHz) NMR, Table 2; (+)-LRESIMS *m/z* (35 eV) (rel int) 261.0 $[C_{19}H_{30}{}^{79}Br_2N_4O_3+2H]^{2+}$ (46), 262.0 $[C_{19}H_{30}{}^{79}Br^{81}BrN_4O_3+2H]^{2+}$ (100), 263.0 $[C_{19}H_{30}{}^{81}Br_2N_4O_3+2H]^{2+}$ (60), 521.0 $[C_{19}H_{30}{}^{79}Br_2N_4O_3+H]^+$ (5), 523.0 $[C_{19}H_{30}{}^{79}Br^{81}BrN_4O_3+H]^+$ (13), 525.0 $[C_{19}H_{30}{}^{81}Br_2N_4O_3+H]^+$ (6); (+)-HRESIMS *m/z* 521.0750 (calcd for $C_{19}H_{31}{}^{79}Br_2N_4O_3$, 521.0757).

Pseudoceramine D (4). white powder; $[\alpha]_D^{25}$ 0 (*c* 0.12, MeOH); UV λ_{max} (MeOH)/nm (log ε) 207 (4.31), 275 (3.18); IR ν_{max}/cm^{-1} (film) 3450, 1676, 1205, 1136, 723; ^1H (600 MHz) and ^{13}C (150 MHz) NMR, Table 2; (+)-LRESIMS *m/z* (35 eV) (rel int) 436.0 $[C_{14}H_{19}{}^{79}Br_2N_3O_3]^+$ (40), 438.0 $[C_{14}H_{19}{}^{79}Br^{81}BrN_3O_3]^+$ (100), 440 $[C_{14}H_{19}{}^{81}Br_2N_3O_3]$+ (63); (+)-HRESIMS *m/z* 435.9864 (calcd for $C_{14}H_{20}{}^{79}Br_2N_3O_3$, 435.9866).

Reporter-gene assay

YPIII(pIB102-Elux)[33] from a culture grown on LB agar containing 25 μg mL^{-1} chloramphenicol (Sigma) for 48 h at 26 °C was used to inoculate a liquid culture, then grown for 12–16 h in Brain Heart Infusion Broth (BHI; Difco, Detroit, MI, USA) containing 25 μg mL^{-1} chloramphenicol (Sigma, St Loius, MO, USA) on an orbital shaker at 26 °C. Liquid cultures were diluted to an OD_{620} of 0.2, then further diluted 1 in 4 in Ca^{2+} depleted media (BHI media with 5 mM EGTA and 20 mM $MgCl_2$) before addition of 30 μL of bacteria to a 384 well white solid lidded Optiplate™ (Perkin Elmer, Meriden CT, USA). Before bacterial addition, 5 μL of fraction or compound/controls were added to the plate, by diluting library plates of fractions or compounds in 100% DMSO with a Minitrak™ (PerkinElmer) liquid handler, by addition of 1 μL of fraction to 4 μL of H_2O.

Plates were incubated at 26 °C for 1 h followed by incubation at 37 °C for 3 h and then transferred back to 26 °C for 15 min. 15 μL of 0.1% decanal (Sigma) emulsified in H_2O was added to each well. Plates were read on a Trilux (PerkinElmer) counter using a luminescence protocol. A dose response of streptomycin was used as an antibacterial control, prepared in a separate assay plate. Bacteria in BHI with 2.5 mM Ca^{2+} was used as an in plate negative control for the assay. Ca^{2+} is known to down regulate Yop production without effecting bacterial growth, whilst Ca^{2+} depletion stimulates Yop production and suppresses growth.[6] The highest dose of compounds in the assay was 286 μM, with the exception of compound **3**, at 143 μM.

Combined reporter-gene and phosphatase assay

To investigate the T3S-specificity a combined reporter-gene and phosphatase assay was used to investigate the secretion of YopH and the expression of YopE at different compound concentration. Before addition of decanal, 5 μL of the final assay volume was added to a clear 384 well plate (Becton Dickinson, Franklin Lakes, NJ, USA) containing 45 μL of YopH substrate (12.5 mM pNPP; Acros Organics, Antwerp, Belgium; 20 mM MES pH 5.0 and 0.8 mM DTT), with a Biomek liquid handler (Beckman Coulter, Fullerton, CA, USA). Plates were incubated for 15 min at 37 °C before addition of 10 μL of sodium hydroxide to stop the reaction. Plates were then read at 405 nm on a VictorII Wallac plate reader (PerkinElmer). To the remainder of the assay volume decanal was added and luminescence was measured as described above. Controls were as described for the reporter-gene assay. The highest dose for any of the compounds in the assay was 286 μM, with the exception of compound **3**, which was tested at 143 μM.

Antibacterial optical density (OD_{620}) assay

YPIII(pIB102-Elux) cultures were grown overnight, then diluted to an OD_{620} of 0.2 in BHI medium, with 2.5 mM Ca^{2+}. A further 1 : 4 dilution in BHI with 2.5 mM Ca^{2+} was prepared before addition to the assay. 50 µL of the diluted bacteria was added to clear, 384 well lidded plates (Becton Dickinson), containing 5 µL of fraction/compound or controls. Plates were incubated for 3 h at 37 °C and then transferred to room temperature for 15 min before reading at 620 nm on a VictorII Wallac plate reader. Streptomycin was used as a negative in plate growth control and an external plate contained a dose response of streptomycin for the estimation of the antibacterial IC_{50}. The highest dose of all compounds in the assay was 182 µM, with the exception of compound **3**, which was tested at 91 µM.

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