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Pseudoceratidine, A Marine Natural Product with Antifouling Activity: Synthetic and Biological Studies

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Abstract: *Syntheses of pseudoceratidine and several analogs were developed in order to explore structure-activity relationships responsible for antifouling and antimicrobial activity.*

INTRODUCTION AND BACKGROUND

Antifouling paints are commonly used to protect ships' hulls, marine pilings, and harbor construction equipment from colonization by microscopic, as well as the propagules of macroscopic, organisms. Besides causing corrosion problems, the attachment of such organisms retards vessels and unnecessarily increases fuel consumption by as much as 40%. The development of effective, low-cost antifouling agents for incorporation into marine coatings has had a major impact on both the recreational and commercial maritime industries, with worldwide demand for such paints now approaching $2 billion.[1]

Copolymers formulated with tributyltin (TBT) methacrylate are the most common antifoulants, used by approximately 80% of the world's commercial shipping fleet. Copper-releasing coatings comprise the bulk of other antifouling paints, after lead and mercury-based coatings were discontinued because of persistent toxicity. When submerged, antifouling paints release toxic compounds at levels (~4 μg/sq cm of surface per day) that can cause adverse environmental effects.[2,3] For example, tin-based paints have been linked to the deaths of dolphins, porpoises, and whales in North Atlantic waters since the 1980s. Vessels painted with conventional TBT-based coatings can have a substantial environmental impact while anchored for long periods in an enclosed harbor. Recent concerns about these effects on marine mammals and other organisms have led to a ban on TBT-containing coatings in the United States, Japan, and other countries.

Dedicated to Madeleine M. Joullié in celebration of her distinguished teaching and research career at the University of Pennsylvania.

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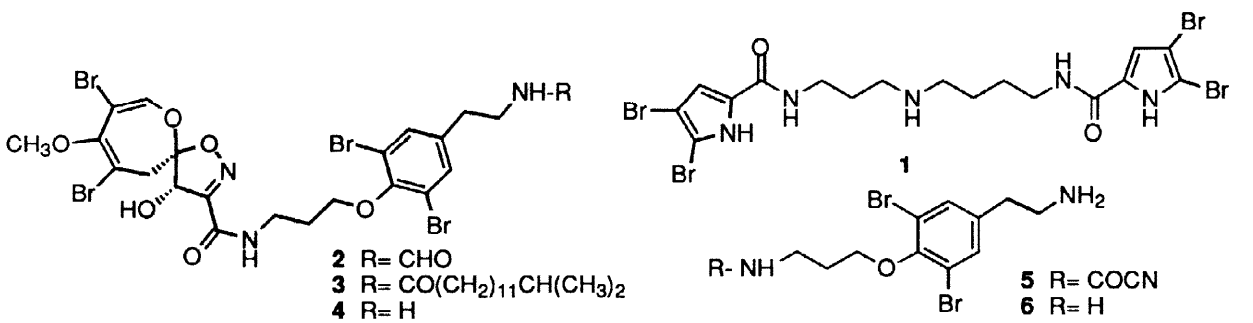
Many benthic organisms have developed chemical defense systems against predators whose larvae settle and become attached to their prey. Since that process is closely related to biofouling, compounds that block settlement might provide new leads for new antifouling coatings. Studies reported by the Fusetani Biofouling Project in Japan have uncovered a wide spectrum of settlement-inhibiting compounds of marine origin. Among the most potent of these antifouling agents are the spermidine diamide pseudoceratidine **1** derived from a marine sponge (Figure),[4] as well as dibromotyrosine derivatives such as ceratinamide A[5] **2** (Figure), ceratinamide B **3**, psammaplysin A[6] **4**, ceratinamine[7] **5**, and moloka'iamine[8] **6**.



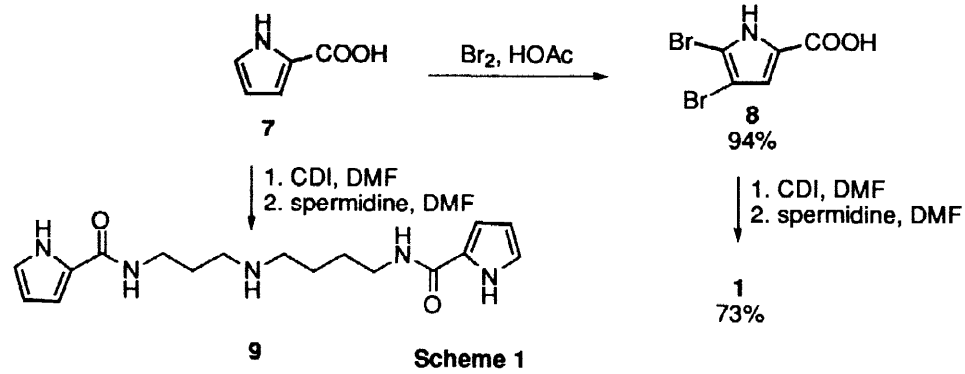
Figure

We[9a] and others[9b] have recently described a total synthesis of pseudoceratidine **1**, which was reported to inhibit metamorphosis of the barnacle *Balanus amphitrite*. To help delineate structural requirements for this bioactivity, we also synthesized the corresponding N^1 and N^8 mono-(4,5-dibromo-2-pyrrolyl) amides of spermidine. Naturally-occurring conjugates of various brominated pyrrole carboxylic acids display a wide range of bioactivities, including antimicrobial and immunosuppressive properties,[10,11,12] and in preliminary assays against a variety of microorganisms, discrimination of the two ends of the polyamine chain was found to be a significant factor in potency.[9a] Here we expand our earlier study of structure-activity relationships in pseudoceratidine with the synthesis of several additional, closely related mono- and bis-(4,5-dibromo-2-pyrrolecarboxamido) residues to test the importance of the pyrrole's substituents and the positively charged secondary amine group in **1**. We also describe the results of biological screening indicating the antimicrobial and antifouling potential of these compounds.

RESULTS AND DISCUSSION

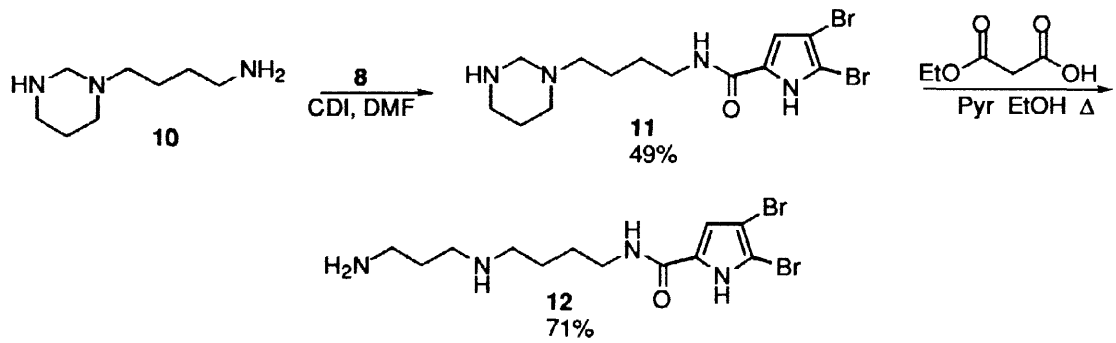
Earlier syntheses of 4,5-dibromopyrrole-2-carboxylic acid **8** (Scheme 1) involved bromination of methyl 2-pyrrolecarboxylate followed by deesterification.[13,14] We developed a more direct route by heating pyrrole-2-carboxylic acid **7** with bromine (2 equiv, 60 °C, HOAc, 1 h), which furnished **8** in 94% yield. The synthesis of pseudoceratidine was completed using the selective acylation procedure of Joshua and Scott.[15]

Activation of acid **8** with 1,1'-carbonyldiimidazole (CDI) in DMF followed by treatment with 0.5 equiv of spermidine gave **1** in 73% yield after chromatography. The nonbrominated analog **9** was synthesized from **7** in similar fashion.



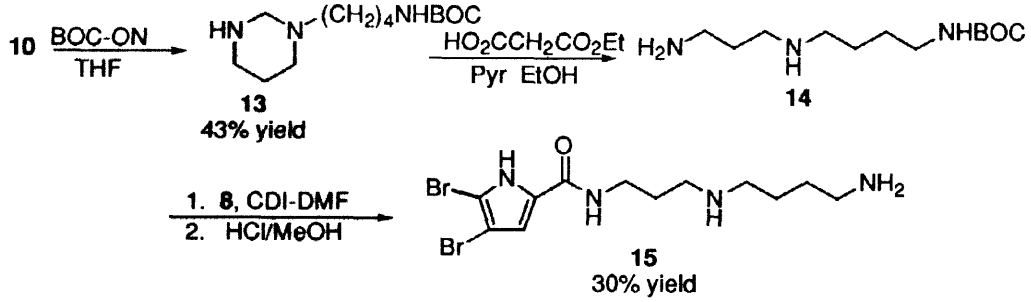
Scheme 1

To synthesize the N^1 and N^8 monoacylated analogs of **1** for structure-activity assays, spermidine was first reacted with formaldehyde to afford hexahydropyrimidine **10** (Scheme 2),[16] which was makes possible selective functionalization at the primary nitrogen. Selective acylation of **10** with **8**, using CDI in DMF, furnished **11** in 50% yield. Deprotection of the hexahydropyrimidine under Knoevenagel condensation conditions (ethyl hydrogen malonate, pyridine, 78 °C in EtOH) afforded N^8-(dibromopyrroloyl)-spermidine **12** in 71% yield.



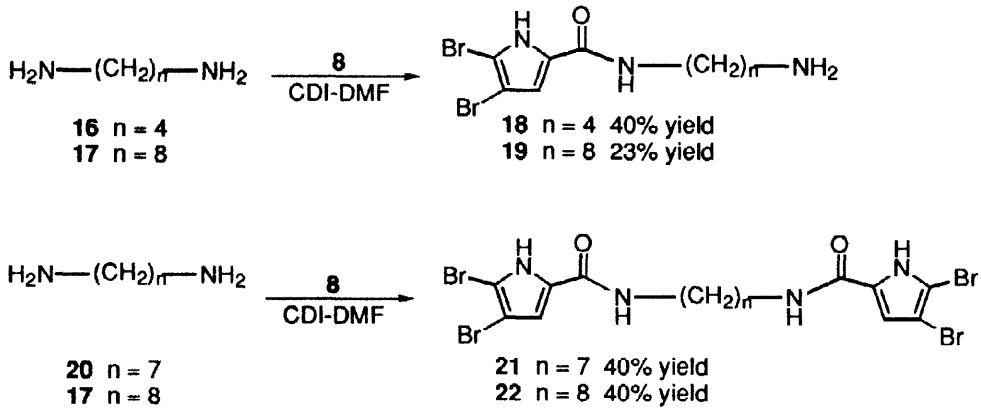
Scheme 2

To synthesize N^1-(dibromopyrroloyl)-spermidine, hexahydropyrimidine **10** was first reacted with 2-(t-butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC-ON, 0.95 equiv, THF) to generate the carbamate-protected hexahydropyrimidine **13** (Scheme 3) in 43% yield. Deprotection of **13** using ethyl hydrogen malonate as before gave N^8-BOC-protected spermidine **14**, which previously had been described in the patent literature.[17,18] Selective acylation of **14** with **8** followed by deprotection with HCl afforded N^1-(dibromopyrroloyl)-spermidine **15** in good yield from **10**.



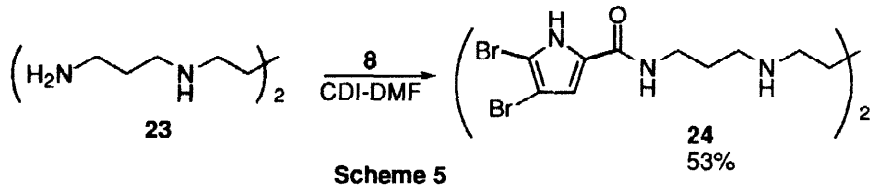
Scheme 3

To evaluate the importance in biological activity of pseudoceratidine's positive charge, as well as the significance of the diamide backbone's overall length, several mono- and diacyl derivatives of 1,ω-diamines were prepared, as indicated in Scheme 4. Monoacylated analogs **18** and **19** were synthesized by coupling diamines **16** and **17** with **8** using CDI in unoptimized yields of 40% and 23%, respectively. Reaction of **20** and **17** with an excess of **8** afforded the diacylated analogs **21** and **22** each in 40% yield. Compounds **18**, **19**, **21**, and **22** were slightly soluble in CH_2Cl_2, CH_3OH, and DMF, and significant product losses were experienced during chromatographic purification.



Scheme 4

Finally, diacylspermine **24** (Scheme 5) was synthesized to examine the effect of an additional positive charge on bioactivity. Coupling of spermine **23** with **8** (2 equiv) afforded **24** in 53% yield.



Scheme 5

Examples of other marine-derived spermidine amides, as well as guanidino-polyamines and polyamine-sterol conjugates displaying cytotoxic,[19] antimicrobial,[20] or antifungal[21] activity, prompted us to screen **1** and its analogs against a panel of pathogenic microorganisms. Data summarized in columns 1-4 of the Table indicate that all three spermidine analogs prepared in this study (**9**, **12**, **15**) were significantly less potent than pseudoceratidine **1**. Only the N^8-monoacylspermidine **12** displayed modest activity against *S. aureus* and *E. coli*. The importance of the pyrrole ring's bromine substituents was underscored by the absence of activity in the nonhalogenated analog **9**. Besides being poorly soluble, mono- and diamides of the 4, 7, and 8-carbon diamines were essentially devoid of activity, with one exception. Octanediamine monoamide **19**, whose overall structure resembled N^8-monoacylspermidine **12**, displayed marginal antimicrobial behavior against *S. aureus* and *E. coli*. Compound **24**, embodying the spermine analog of pseudoceratidine, exhibited broad-spectrum antimicrobial activity, with potency comparable to **1**. Taken as a whole, the biocidal data in the Table indicate that activity requires the 4,5-dibromopyrrolyl appendage, and is most pronounced with bis-amides of polyamines carrying at least one positive charge.

Table
Biological Activity of Pseudoceratidine and Related Polyamines

	Antibiotic				*Antifouling*
Compound	*S. aureus*[a]	*E. coli*[a]	*P. aeruginosa*[a]	*C. albicans*[a]	*B. amphitrite*[b]
1	4	32	128	32	15 (8.0, Ref. 4)
8	n.d.[c]	n.d.	n.d.	n.d.	15
9	>256	>256	>256	>256	n.d.
12	64	128	256	128	inactive
15	>256	256	>256	>256	inactive
18	256	>256	>256	>256	inactive
19	128	128	>256	>256	n.d.
21	>256	>256	>256	>256	0.1
22	>256	>256	>256	>256	n.d.
24	8	32	64	32	45

[a] *Antibiotic activity reported as minimum inhibitory concentration (MIC) in μg/mL;* [b] *Antifouling activity reported as* EC_{50} *in μg/mL;* [c] *n.d. = not determined.*

Several assays based on the surface or swimming behavior of barnacle larvae in flowing systems have been developed to ascertain the effectiveness of antifoulant compounds. Alternatively, by measuring the attachment of larvae to a solid surface, approximate values of EC_{50} (effective concentration corresponding to 50% maximal response) were determined for settlement inhibition (column 5 of the Table). Results with

pseudoceratidine **1** were in good agreement with published data. Noteworthy was the finding that 4, 5-dibromopyrrole-2-carboxylic acid **8** itself was a potent antifouling agent. The corresponding monoamides of **8** with spermidine and butane-1,4-diamine showed no effect in the barnacle settlement assay. Of special interest, however, was the significant level of antifouling activity displayed by 1,7-diaminoheptane diamide **21**. In contrast, spermine diamide **24** was significantly less potent than **21** and less active than the natural product **1**. Thus it would appear that the overall length of the diamide chain, and not the net molecular charge, is a powerful determinant of antifouling behavior.

EXPERIMENTAL SECTION

General: Proton-NMR spectra were taken on a Bruker AF-300 or Varian VXR-400S spectrometer. All chemical shifts were reported on the δ scale in parts per million downfield from Me_4Si (0.00 ppm). Carbon-13-NMR spectra were taken on a Varian VXR-400S (100 MHz) or Bruker AF-300 (75 MHz) spectrometer. **NOTE:** *Reference quality NMR spectra are available on request.* Infrared spectra were taken on a Mattson Galaxy Model infrared spectrometer. Mass spectra were acquired using a Finnigan 3300 mass spectrometer at Cornell or at the University of Illinois Mass Spectrometry Laboratory using a VG ZAB-SE or VG 70-VSE instrument. Melting points were determined with a Thomas-Hoover apparatus and are uncorrected.

Synthesis of 4,5-dibromopyrrole-2-carboxylic acid **8**: A solution of bromine (1.47 g, 9.22 mmol) in glacial acetic acid (HOAc, 6.2 mL) was added dropwise at rt to a stirred solution of pyrrole-2-carboxylic acid **7** (0.512 g, 4.6 mmol) in AcOH (25 mL). Upon completion of addition, the solution was heated to 60 °C for 1 h, then cooled to rt and decolorized with Norit. After filtration through Celite, the solvent was removed in vacuo and the resulting solid was crystallized from H_2O/EtOH to afford the known[13,14] 4,5-dibromopyrrole-2-carboxylic acid **8** (1.17 g, 94%): mp 170 °C (d); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 10.40 (b, 1 H), 6.82 (d, 1 H, J = 2.7 Hz); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 160.4, 125.09, 117.0, 106.8, 98.9; IR (film) 3120, 1695, 1430, 1210, 1180, 760 cm^{-1}; CIMS *m/z* 270 (M^+, 100%).

Coupling of acid **8** with spermidine to form pseudoceratidine **1**: A solution of acid **8** (79 mg, 0.29 mmol) in DMF (0.58 mL) was treated with CDI (48 mg, 0.29 mmol) at rt. The suspension that formed was stirred at rt for 30 min, then treated with spermidine (21 mg, 0.145 mmol) in DMF (0.5 mL), and the reaction stirred at rt for 2 d. After removing the solvent in vacuo, the residue was partitioned between aqueous saturated $NaHCO_3$ (1.5 mL) and EtOAc (1.5 mL), and the aqueous phase was extracted with EtOAc (3 x 1.5 mL). The combined organic layers were dried (Na_2SO_4), filtered, and concentrated in vacuo. The residue was chromatographed (75:25:3 CH_2Cl_2:MeOH:NH_4OH) to afford **1** (69 mg, 73%) as a solid: mp 62-65 °C; R_f 0.44 (75:25:3 CH_2Cl_2:MeOH:NH_4OH); ^{1}H-NMR (300 MHz, DMSO-d_6, 380 °K) δ 7.92 (b, 1 H), 7.75 (b, 1 H), 6.86 (s, 1 H), 6.84 (s, 1 H), 3.33 (q, 2 H, J = 4.4 Hz), 3.29 (q, 2 H, J = 6.6 Hz), 2.97 (q, 4 H, J = 7.0 Hz), 1.90 (p, 2 H, J = 6.7 Hz), 1.59-1.73 (m, 4 H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 159.3, 159.0, 128.3, 128.0, 113.0, 112.8, 104.7, 104.4, 98.0, 97.9, 46.7, 44.9, 38.0, 35.9, 26.4, 26.2, 23.2; HRFAB-MS for $C_{17}H_{22}{}^{79}Br_2{}^{81}Br_2N_5O_2$ (M+1), calc 647.8466, found 647.8433.

Acylation of **10** with **8** to form hexahydropyrimidine **11**: A solution of acid **8** (80 mg, 0.30 mmol) in DMF (0.6 mL) was treated with CDI (52 mg, 0.32 mmol) at rt. The resulting suspension was stirred at rt. for 30 min, then treated with a solution of hexahydropyrimidine **10** (47 mg, 0.30 mmol) in DMF (0.5 mL) and stirred at rt for 3 d. The solvent was removed in vacuo, and the residue was partitioned between aqueous saturated $NaHCO_3$ (1.5 mL) and EtOAc (1.5 mL). The aqueous layer was extracted with EtOAc (3 x 1.5 mL), and the combined organic phases were dried (Na_2SO_4), filtered and concentrated. The residue was flash chromatographed (9:1 CH_2Cl_2:NH_3-saturated MeOH) to afford **11** as an oil (60 mg, 50%): R_f 0.22 (9:1 CH_2Cl_2:NH_3-saturated MeOH); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.30 (br, 1 H), 7.90 (br, 1 H), 7.31 (br, 1 H) 7.14 (br, 1 H), 3.48-3.58 (br, 4 H), 2.96 (br, 1 H), 2.79 (br, 3 H), 2.51 (br, 2 H), 1.74-1.76 (br, 6 H).

Conversion of **11** to N^8-monoacylspermidine **12**: A solution of **11** (61 mg, 0.15 mmol) in EtOH (2.5 mL) and pyridine (48 μL, 0.60 mmol) and ethyl hydrogen malonate (82 μL, 0.74 mmol) was stirred at rt for 30 min, then heated at 78 °C for 3 h. After solvent removal in vacuo, the residue was flash chromatographed (7:3:1 CH_2Cl_2:MeOH:NH_4OH) to afford impure **12** contaminated with ethyl hydrogen malonate. To remove the impurity, the residue was dissolved in aqueous HCl (pH 3), extracted with EtOAc (3 x 1.5 mL), and the aqueous layer was freeze dried. The residue was dissolved in H_2O, heated to 100 °C for 1 h, then freeze dried to afford pure **12** (49 mg, 71%): R_f 0.22 (7:3:1 CH_2Cl_2:MeOH:NH_4OH); ^{1}H-NMR (300 MHz, D_2O) 8.49 (s, 1 H), 7.27 (d, 1 H, J = 1.1 Hz), 6.55 (s, 1 H), 3.13 (t, 2 H, J = 6.7 Hz), 2.82-2.99 (m, 6 H), 1.42-1.57 (m, 4 H); ^{13}C-NMR (75 MHz, D_2O, dioxane) 160.9, 126.5, 113.5, 105.5, 98.9, 47.3, 44.4, 38.4, 36.5, 25.8, 23.7, 23.0; HRFAB-MS for $C_{12}H_{21}{}^{79}Br^{81}BrN_4O$ (M+1), calc 397.0062, found 397.0045.

Conversion of **10** to BOC-hexahydropyrimidine **13**: To an ice-cold solution of **10** (0.52 g, 3.3 mmol) in THF (3.5 mL) was added BOC-ON (0.78 g, 3.2 mmol) in THF (13.3 mL), and the solution stirred at 0 °C for 30 min. After concentrating the solvent in vacuo, the residue was dissolved in Et_2O (50 mL) and washed with 5% aqueous NaOH (2 x 10 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated in vacuo. The residue was flash chromatographed (9:1 CH_2Cl_2:NH_3-saturated MeOH) to afford **13** (0.36 g, 43%) as an oil: R_f 0.39 (9:1 CH_2Cl_2:NH_3-saturated MeOH); ^{1}H NMR (300 MHz, DMSO-d_6, 380 °K) δ 6.1-6.3 (br, 1 H), 3.27 (s, 2 H), 2.95 (dt, 2 H, J = 5.7, 6.5 Hz), 2.67 (t, 2 H, J = 5.5 Hz), 2.51 (t, 2 H, J = 5.4 Hz), 2.27 (br, 1 H), 2.21 (t, 2 H, J = 6.7 Hz), 1.39-1.53 (m, 13 H); ^{13}C NMR ($CDCl_3$, 100 MHz) 156.1, 78.7, 69.6, 55.0, 52.9, 44.8, 40.4, 28.4, 28.0, 26.5, 24.1; IR (film) 3330, 3220, 2940, 1710, 1175 cm^{-1}; FABMS *m/z* 258 (M+1, 100%).

Preparation of N^8-BOC-spermidine **14**: A solution of hexahydropyrimidine **13** (100 mg, 0.39 mmol) in EtOH (6 mL) containing pyridine (125 μL, 1.5 mmol) and ethyl hydrogen malonate (215 μL, 1.9 mmol) was stirred at rt for 30 min, then heated to 78 °C for 4.5 h. The cooled solution was concentrated in vacuo, and the residue flash chromatographed (7:3:1 CH_2Cl_2:MeOH:NH_4OH) to afford impure **14** (177 mg), which was converted to **15** (see below) without further purification.

An analytical sample of **14** was obtained by flash chromatography (7:3:1 CH_2Cl_2:MeOH:NH_4OH): R_f 0.24 (7:3:1 CH_2Cl_2:MeOH: NH4OH); ^{1}H-NMR (300 MHz, DMSO-d_6, 360 °K) δ 2.95 (t, 2 H, J = 6.8 Hz), 2.73 (br. s, 3 H), 2.68 (t, 2 H, J = 6.7 Hz), 2.59 (t, 2 H, J = 6.8 Hz), 2.53 (t, 2 H, J = 6.6 Hz), 1.53 (q, 2 H, J =

6.7 Hz), 1.37-1.47 (m, 13 H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 155.6, 79.2, 59.7, 48.9, 46.9, 31.3, 28.3, 27.4, 26.6; IR (film) 3370, 2930, 1700, 1540, 1370, 1280, 1250, 1175 cm^{-1}.

Conversion of **14** to N^1-monoacylspermidine **15**: To a solution of **8** (103 mg, 0.39 mmol) in DMF (1.3 mL) was added CDI (62 mg, 0.39 mmol), and the resulting suspension was stirred at rt for 30 min, then treated with a solution of **14** (94 mg, ca 0.4 mmol) in DMF (1.3 mL). The mixture was stirred at rt for 2 d, then concentrated in vacuo, and the residue partitioned between $NaHCO_3$ (1.5 mL) and EtOAc (1.5 mL). The aqueous layer was extracted with EtOAc (3 x 1.5 mL) and the combined organic phases were dried (Na_2SO_4), filtered, and concentrated. The residue was chromatographed (9:1 CH_2Cl_2:NH_3-saturated MeOH, then 75:25:3 CH_2Cl_2:MeOH:NH_4OH) to furnish the intermediate N^1-monoacyl-N^8-BOC-spermidine (71 mg, 36%), which was deprotected by stirring with 1 M HCl in MeOH (0.5 mL) at rt for 3 h. After solvent removal in vacuo, the residue was chromatographed (7:3:1 CH_2Cl_2:MeOH:NH_4OH) to give N^1-monoacylspermidine **15** (55 mg, 83%): R_f 0.19 (7:3:1 CH_2Cl_2:MeOH:NH_4OH); ^{1}H-NMR (300 MHz, D_2O) 6.61 (s, 1 H), 3.20 (t, 2 H, J = 6.5 Hz), 2.74-2.82 (m, 6 H), 1.70 (q, 2 H, J = 7.4 Hz), 1.48-1.50 (m, 4 H); ^{13}C-NMR (75 MHz, D_2O, dioxane) 162.3, 127.7, 114.0, 108.0, 98.1, 47.1, 45.2, 39.0, 36.2, 26.5, 24.6, 23.5; IR (KBr) 3410, 3270, 3050, 2920, 2850, 1635, 1560, 1520, 1410, 1380, 1320, 1240 cm^{-1}; FABMS *m/z* 397 (M+1, 20%).

Coupling of **8** with 1,4-diaminobutane to form monoamide **18**: To a solution of **8** (108 mg, 0.40 mmol) in DMF (0.5 mL) was added CDI (101 mg, 0.62 mmol), and after stirring the resulting suspension at rt for 30 min, a solution of diaminobutane **16** (387 mg, 0.44 mmol) in DMF (0.5 mL) was added. The mixture was stirred at rt for 4 d, and solvent was removed in vacuo. The residue was partitioned between saturated $NaHCO_3$ (3 mL) and EtOAc (1.5 mL). The aqueous layer was extracted with EtOAc (2 x 1.5 mL), and the combined organic layers were dried (Na_2SO_4), filtered, and concentrated. The residue was chromatographed (4:1 CH_2Cl_2:NH_3-saturated MeOH) to give amide **18** (54 mg, 40%): R_f 0.32; ^{1}H-NMR (300 MHz, DMSO-d_6, 360 °K) δ 7.54 (br, 1 H), 6.73 (s, 1 H), 5.02 (br, 4 H), 3.22 (q, 2 H, J = 4.2 Hz), 2.68 (t, 2 H, J = 6.6 Hz), 1.42-1.60 (m, 4 H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 162.1, 131.52, 112.0, 109.2, 93.9, 48.6, 37.6, 26.8, 26.4; HRFAB-MS for $C_9H_{14}{}^{79}Br^{81}BrN_3O$ (M+1), calc 339.9483, found 339.9452.

Coupling of **8** with 1,8-diaminooctane to form monoamide **19**: Following the procedure for **18**, the reaction of acid **8** (50 mg, 0.19 mmol), CDI (43 mg, 0.27 mmol), and diaminooctane **17** (27 mg, 0.19 mmol) afforded monoamide **19** (17 mg, 23%): R_f 0.28 (17:3 CH_2Cl_2:NH_3-saturated MeOH); ^{1}H-NMR (300 MHz, DMSO-d_6, 360 °K) δ 7.54 (br, 1 H), 6.75 (s, 1 H), 4.15 (br, 8 H), 3.20 (q, 2 H, J = 6.4 Hz), 2.65 (t, 2 H, J = 7.0 Hz), 1.39-1.53 (m, 4 H), 1.31 (s, 8 H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 161.3, 130.19, 112.0, 108.1, 94.6, 38.1, 29.5, 28.6, 26.3, 25.9; FABMS *m/z* 396 (M+1, 100%).

Coupling of **8** with 1,7-diaminoheptane to form diamide **21**: Following the procedure for **18**, the reaction of **8** (52 mg, 0.19 mmol), CDI (32 mg, 0.20 mmol), and diaminoheptane **20** (25 mg, 0.19 mmol) afforded diamide **21** (24 mg, 40%): R_f 0.57 (9:1 CH_2Cl_2:NH_3-saturated MeOH); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 6.75 (s, 2 H), 4.85 (s, 6 H), 3.23 (t, 4 H, J = 7.2 Hz), 1.51 (br, 4 H), 1.30 (br, 6 H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 158.7, 128.3, 112.3, 104.2, 97.7, 38.5, 29.2, 28.5, 26.4; FABMS *m/z* 633 (M+1, 38%).

Coupling of **8** with 1,8-diaminooctane to form diamide **22**: Following the procedure for **18**, the reaction of **8** (172 mg, 0.64 mmol), CDI (103 mg, 0.64 mmol), and diaminooctane **21** (45 mg, 0.32 mmol) afforded diamide **22** (27 mg, 40%) as a tan solid; mp 165-167 °C; R_f 0.11 (3:97 MeOH:CH_2Cl_2); ^{1}H-NMR (300 MHz, DMSO-d_6, 360 °K) δ 7.59 (s, 1 H), 6.83 (s, 1 H), 3.21 (q, 2 H, J = 6.8 Hz), 1.52 (m, 2 H, J = 7.1 Hz), 1.32 (s, 4 H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 158.7, 128.3, 112.3, 104.2, 97.7, 38.5, 29.2, 28.7, 26.4; IR (KBr) 3110, 2920, 2860, 1640, 1570, 1530, 1420, 1340, 1250; HRFAB-MS for $C_{18}H_{23}{}^{79}Br_2{}^{81}Br_2N_4O_2$ (M+1), calc 646.8514, found 646.8502.

Coupling of **8** with spermine to form diamide **24**: To a solution of **8** (172 mg, 0.64 mmol) in DMF (2.4 mL) was added CDI (103 mg, 0.64 mmol) and the resulting suspension was stirred at rt for 30 min, then treated with spermine **23** (66 mg, 0.33 mmol). The mixture was stirred at rt for 4 d, then concentrated in vacuo. The residue was flash chromatographed (7:3:1 CH_2Cl_2:MeOH:NH_4OH) to afford impure **24** containing acid **8**. The mixture was suspended in aqueous HCl (2 mL, pH 3) and filtered to afford spermine **24** as a tan solid (122 mg, 53%): R_f 0.58 (7:3:1 CH_2Cl_2:MeOH:NH_4OH); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.01 (s, 2 H), 8.58 (s, 1 H), 7.00 (br. s, 1 H), 3.57 (br. s, 2 H), 2.92 (br. s, 4 H), 1.85 (br. s, 2 H), 1.78 (br. s, 2 H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 159.0, 128.1, 113.1, 104.5, 97.9, 46.0, 44.7, 35.7, 25.9, 22.6; IR (KBr) 3430, 3270, 2950, 2800, 1620, 1580, 1420, 1330, 1250; FABMS *m/z* 705 (M+1, 8%).

Antimicrobial Assays: Minimum inhibitory concentrations (MICs) for *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were determined using a modified version of the National Committee for Clinical Laboratory Standards (NCCLS) approved standard for testing aerobic bacteria in a microdilution broth assay.[22] The modification involved using a final volume in each microtiter plate well of 200 μL rather than 100 μL. For *Candida albicans* ATCC 90028, MICs were determined using the NCCLS tentative standard for testing antifungal activity of yeasts.[23]

Antifouling Assays: A simple settlement assay, developed using the barnacle *Balanus amphitrite*, was used.[24] Working solutions of test compounds were prepared by spreading a thin film of each substance in organic solvent over the bottom of a glass container, evaporating the solvent under vacuum, then mixing the residue with seawater filtered to remove molecules and particulates >100 kDa and aged septically. Triplicate assays on each compound were then repeated on a different batch of larval barnacle cultures to check reproducibility.

Suspensions of three to six-day-old cyprids were pipetted into plastic petri dishes (20-30 cyprids each) containing the test substance at 25, 5, 1, and 0.2 μg/mL (5 mL final volume). Dishes were incubated at 28 °C for 24 h (12 h light, 12 h dark), and checked for signs of toxicity. The assays were terminated by adding 10% formalin solution. Unattached larvae were rinsed away using deionized water and collected by suction filtration. Attached and free larvae were counted using a microscope. Values of EC_{50} (effective concentration corresponding to 50% maximal response) for settlement inhibition were estimated by probit analysis[25] from counts of a log distribution series of each test compound.

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