Highly Brominated Antimicrobial Metabolites from a Marine *Pseudoalteromonas* sp.

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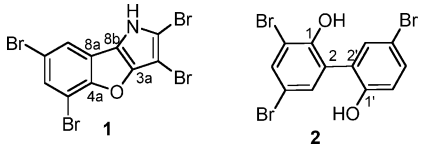
Extracts of a marine *Pseudoalteromonas* sp. (CMMED 290) isolated from the surface of a nudibranch collected in Kaneohe Bay, Oahu, displayed significant antimicrobial activity against methicillin-resistant *Staphylococcus aureus*. Bioassay-guided fractionation of the lipophilic extract led to the isolation and structure elucidation of two new highly brominated compounds, 2,3,5,7-tetrabromobenzofuro[3,2-*b*]pyrrole (**1**) and 4,4′,6-tribromo-2,2′-biphenol (**2**). In addition, we have identified the known compounds pentabromopseudilin and bromophene. We describe the isolation and structure elucidation of the compounds **1** and **2** together with their antimicrobial activities against methicillin-resistant *Staphylococcus aureus*.

The continuing emergence of antibiotic resistance in clinically relevant pathogens has placed considerable pressure on the pharmaceutical industry to develop novel drug candidates.[1] A prime example of the urgent need for novel antibiotic compounds is the Gram-positive bacterium methicillin-resistant *Staphylococcus aureus* (MRSA). In recent years, several reports of clinical resistance to traditional drugs of last resort for the treatment of MRSA infections, such as vancomycin and teicoplanin, have appeared.[2] The development of MRSA infection from an originally hospital-acquired disease (HA-MRSA) to a community-associated problem (CA-MRSA) provides additional reason for concern and makes this pathogen a primary target of antibiotic discovery programs.[3]

Proteobacteria isolated from diverse marine environments have been shown to be prolific producers of secondary metabolites.[4] As part of our screening program aimed at the discovery of pharmaceutical lead compounds for the treatment of infective and neoplastic diseases, we have previously reported on the isolation of metabolites from marine Proteobacteria belonging to the genus *Pseudoalteromonas* associated with marine invertebrates.[5] Another marine bacterial isolate streaked from the surface of a nudibranch collected in shallow waters of Kaneohe Bay, Oahu, and deposited in our collection as CMMED 290 was shown to be a *Pseudoalteromonas* sp. based on its 16S rDNA gene sequence. The EtOAc extracts of cultures grown in marine broth displayed significant broad-spectrum biological activity against *Staphylococcus aureus* (ATCC 25923), methicillin-resistant *Staphylococcus aureus* (ATCC 43300), *Escherichia coli* (ATCC 25922), and *Candida albicans* as well as the human ovarian adenocarcinoma cell line SKOV-3 (ATCC HTB-77).



Large-scale fermentation, extraction, and bioassay-guided fractionation of CMMED 290 led to the identification of two highly brominated compounds, **1** and **2**, that had not previously been reported. These were accompanied by pentabromopseudilin (Figure 1), which had previously been isolated from *P. bromoutilis*,[6] *P. luteoviolacea*,[7,8] and *Chromobacterium*[9] and is structurally related to the new metabolite **1**. It displays a wide range of biological activities (cytotoxic, antibacterial, phytotoxic) including inhibition of both human 12- and 15-lipoxygenases[10] and potent inhibition of myosin-dependent processes.[11] The new compound **2** is a structural analogue of bromophene (Figure 1) with three bromines on the biphenolic core structure. Bromophene has been previously identified in cultures of *P. phenolica* and has been shown to exhibit anti-MRSA activities as low as 1 μg/mL on several clinical isolates, with killing rates higher than that of vancomycin.[12]

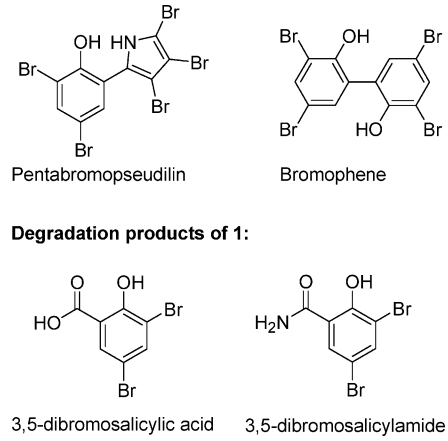


Figure 1. Known compounds isolated from CMMED 290: Pentabromopseudilin and bromophene, structural analogues of **1** and **2**, with 3,5-dibromosalicylic acid and 3,5-dibromosalicylamide, the degradation products from the photo-oxidation of **1**.

Fractionation of the EtOAc extract of CMMED 290 by reversed-phase column chromatography, reversed-phase HPLC, and several recrystallization steps yielded 1.1 mg of pure **1**. The negative mode HRESIMS spectrum of **1** established a molecular formula of $C_{10}H_3{}^{79}Br_4NO$ with the $[M - H^+]^-$ monoisotopic pseudomolecular peak at *m/z* 467.6877 and a characteristic isotopic pattern (2:7:10:7:2) indicative of the presence of four bromine atoms.

The molecular formula required eight degrees of unsaturation, of which five were accounted for by the 10 distinct signals ranging from 81.7 to 155.0 ppm in the ^{13}C NMR spectrum. This suggested that only unsaturated carbon atoms were present in the structure, which had to contain three rings. One of the rings together with three unsaturations was assigned to a 1,2,3,5-tetrasubstituted phenyl ring on the basis of two proton doublets in the ^1H NMR spectrum at 7.63 and 7.52 ppm, respectively, with characteristic *meta*-coupling constants of 1.8 Hz (Table 1). Two of the substituents were assigned to be bromine atoms and one as an oxygen atom, the latter on the basis of a typical ^{13}C chemical shift of 155 ppm for the phenolic carbon atom. The substitution pattern of the benzene ring was

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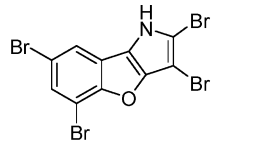
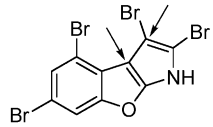
Table 1. NMR Spectroscopic Data (500 MHz, CD_3OD) for 2,3,5,7-Tetrabromobenzofuro[3,2-*b*]pyrrole, **1**

position	δ_C, mult.	δ_H (J [Hz])	HMBC[a]
2	107.2, C		
3	81.7, C		
3a	149.1, C		
4a	155.0, C		6, 8
5	106.4, C		6
6	129.0, CH	7.55, d (1.8)	8
7	117.0, C		6, 8
8	120.0, CH	7.67, d (1.8)	6
8a	120.1, C		8
8b	122.6, C		

[a] HMBC correlations are from proton(s) stated to the indicated carbons.



a) Tetrabromobenzofuro(3,2-b)pyrrole



b) Tetrabromobenzofuro(2,3-b)pyrrole

Figure 2. Tetrabromobenzofuro[3,2-*b*]pyrrole core structure (a) and the alternative furo(2,3-*b*)pyrrole ring fusion (b). Arrows indicate carbons with large errors for calculated ^{13}C chemical shift values.

suggested by 2D-NMR spectra displaying three-bond HMBC correlations from both aromatic protons to the oxygenated carbon atom resonating at 155.0 ppm. The alternative arrangement of the hydrogen and bromine atoms would have allowed for only one such correlation to be observed. ^{13}C NMR chemical shift analysis suggested that the oxygen atom was attached to another carbon atom resonating at 149.1 ppm. This required the third proton represented in the ^1H NMR spectrum by a broad singlet resonance at 8.46 ppm (in $CDCl_3$) to be an exchangeable hydrogen atom attached to nitrogen. This left three additional quaternary olefinic carbon atoms, two of which had to be brominated, and a heteroaromatic nitrogen atom to be assigned with formation of two additional rings. This suggested the presence of a dibrominated pyrrole ring bearing an oxygen atom, which was linked to the carbocyclic ring via the oxygen atom and a carbon−carbon bond. However, the connectivity between the resulting benzofuran ring and the pyrrole ring remained unclear with both orientations, benzofuro(3,2-*b*)-pyrrole as well as benzofuro(2,3-b)pyrrole, being possible (Figure 2).

As only three protons were present in the structure, including a broad NH singlet at 8.46 ppm, 2D-NMR experiments relying on heteronuclear correlations were insufficient to distinguish between these two possible structures. We therefore turned to ^{13}C NMR shift calculation using the ACD Laboratories C+H NMR predictor program (v. 12.01) and compared the computed chemical shifts for structures a and b (Figure 2) to the recorded values. The results summarized in Table 2 strongly suggested the regiochemistry of **1** to be that of a benzofuro(3,2-*b*)pyrrole based on an average $\Delta\delta_C = 2.8$ for the calculated ^{13}C NMR shifts. The alternative structure is characterized by a larger average $\Delta\delta_C = 5.1$, with the largest deviations calculated for carbon atoms 3 ($\Delta\delta_C = 8.8$) and 8b ($\Delta\delta_C = 21.5$), respectively. The observed ^{13}C NMR shift for C-8b agreed well with literature

Table 2. Comparison of Calculated ^{13}C Chemical Shifts for the Proposed (3,2-*b*) Ring Fusion with the Alternative (2,3-*b*) Orientation

position	δ_C, mult.	$\Delta\delta_C$ (3,2-*b*)[a]	$\Delta\delta_C$ (2,3-*b*)[a]
2	107.2, C	0.7	1.3
3	81.7, C	4.1	8.8
3a	149.1, C	3.9	3.9
4a	155.0, C	2.9	2.6
5	106.4, C	2.8	2.8
6	129.0, CH	5.1	5.1
7	117.0, C	3.1	1.0
8	120.0, CH	1.0	0.5
8a	120.1, C	4.2	3.0
8b	122.6, C	0.2	21.5

[a] ^{13}C shifts were calculated with ACD Laboratories C+H NMR predictor program (v. 12.01).

Table 3. NMR Spectroscopic Data (500 MHz, $CDCl_3$) for 4,4′,6-Tribromo-2,2′-biphenol, **2**

position	δ_C, mult.	δ_H (J [Hz])
1	152.3, C	
2	126.1, C	
3	133.0, CH	7.36, d (2.4)
4	113.7, C	
5	134.4, CH	7.39, d (2.4)
6	111.9, C	
1′	148.0, C	
2′	125.5, C	
3′	133.4, CH	7.70, d (2.4)
4′	114.0, C	
5′	134.0, CH	7.42, dd (8.7, 2.4)
6′	119.3, CH	6.92, d (8.7)
OH (1)		5.66, br s
OH (2)		6.08, br s

values, placing this carbon atom within a range of 116−124 ppm.[13] In addition, biosynthetic considerations suggest that 2,3,5,7-tetrabromobenzofuro[3,2-*b*]pyrrole (**1**) may well be a derivative of pentabromopseudilin[14] arising from intramolecular substitution involving positions 3a and 4 with formal loss of hydrobromic acid, forming the new core structure. Lastly, 3,5-dibromosalicylic acid and 3,5-dibromosalicylamide were isolated as two photo-oxidation degradation products from an NMR sample kept in solution for an extended period of time (Figure 1). These compounds cannot arise from a sample of benzofuro[2,3-*b*]pyrrole. Therefore, we report the structure of the new metabolite **1** as shown.

From the active fraction eluted from the C18 flash column with 80% MeOH, 4,4′,6-tribromo-2,2′-biphenol (**2**) was isolated, a compound closely related to bromophene. The negative mode HRESIMS spectrum of **2** revealed a molecular formula of $C_{12}H_7{}^{79}Br_3O_2$, as deduced from the $[M - H^+]^-$ monoisotopic pseudomolecular ion peak at $m/z = 418.7940$ with an isotopic distribution pattern of 3:10:10:3 characteristic for the presence of three bromine atoms. The eight degrees of unsaturation required by the molecular formula were accounted for by 12 aromatic ^{13}C NMR resonances in the range 111.3 to 152.3 ppm and two rings corresponding to a biphenolic structure (Table 3). The ^{13}C NMR signals at 152.3 and 148.0 ppm were indicative of two hydroxy substituents. The ^1H NMR spectrum showed resonances for five aromatic and two exchangeable protons. At this point all substituents had been identified with two hydroxy groups, five protons, and three bromines. Subsequently, the substitution pattern of the two rings was easily deduced from coupling constant analysis. Additional proof for the structure comes from chemical shift comparison of the new compound 4,4′,6-tribromo-2,2′-biphenol (**2**) with the parent bromophene in which the 6′-position is brominated.

In the course of the bioassay-guided fractionation of the highly active EtOAc extract, compounds **1** and **2** were shown to be active

Table 4. IC_{50} Values on MRSA (ATCC 43300)

compound	IC_{50} value
2,4,9,10-tetrabromobenzofuro[3,2-*b*]pyrrole, **1**	1.93 (±0.05)
4,4′,6-tribromo-2,2′-biphenol, **2**	2.19 (±0.08)
bromophene	2.14 (±0.06)
pentabromopseudilin	0.1 (±0.18)
vancomycin	0.91 (±0.09)

against *S. aureus* (ATCC 25923), methicillin-resistant *S. aureus* (ATCC 43300), and *C. albicans*. Due to the limited availability of compounds **1** and **2**, we decided to evaluate their antimicrobial activity against methicillin-resistant *S. aureus* (ATCC 43300) only. Table 4 shows the observed IC_{50} values and those of the known compounds pentabromopseudilin, bromophene, and vancomycin for comparison. Both new metabolites exhibited IC_{50} values in the μM range, with formation of the tricyclic core unit in 2,3,5,7-tetrabromobenzofuro[3,2-*b*]pyrrole (**1**) resulting in a drop of the activity against MRSA by 1 order of magnitude when compared to the parent pentabromopseudilin. Lack of bromination of bromophene at the 6′-position did not appear to influence the activity against the tested MRSA isolate, as both **2** and bromophene were equipotent, with IC_{50} values of 2.1−2.2 μM. The mechanism of antimicrobial activity of bromophene has been demonstrated to originate from its ability to permeabilize the bacterial cell membrane,[12] while not lysing human erythrocytes at antimicrobially effective doses.[12] The mode of antimicrobial activity of **2** is likely the same as that of bromophene owing to the structural similarity, as is the case with other lipophilic, low molecular weight phenols.[15] The lack of phenolic residues in the structure of **1** suggests that its mode of action is likely different. This is the subject of ongoing work to be published in due course.

In conclusion, we have isolated two new and two known highly brominated compounds from a marine *Pseudoalteromonas* sp. Although synthetic derivatives are well known, the benzofuro(3,2-*b*)-pyrrole core structure is hitherto unprecedented in natural products. The structural similarities between **1** and pentabromopseudilin are suggestive of a biosynthetic connection.

Experimental Section

General Experimental Procedures. UV/vis spectra were recorded on a Beckman DU 7400 spectrophotometer. IR spectra were obtained on a ThermoElectron Corp. Nicolet 380 FT-IR system. NMR spectra were recorded in a Shigemi tube and a 5 μL capillary tube on a Varian Inova 500 instrument equipped using a 3 mm microprobe and a Protasis CapNMR probe, respectively. Negative ion HRESITOFMS data were recorded on an Agilent 6210 TOFMS instrument equipped with an Agilent 1100 chromatography module and an Agilent ESI source. Calculation of ^{13}C NMR chemical shift values was carried out using the ACD Laboratories C+H NMR predictor program (v. 12.01).

Isolation of CMMED 290. Isolate CMMED 290 was obtained from the surface of a nudibranch collected while snorkeling in shallow waters in Kaneohe Bay off Oahu, HI. The nudibranch was sampled with a 10 μL bacterial inoculation loop, and the recovered liquid was streaked onto a bacterial agar plate (Difco Marine agar 2216, MA2216). After incubation for 48 h at 31 °C the plate was examined for growth of bacterial colonies. Colonies were picked and restreaked onto clean MA2216 plates for isolation. The isolate was deposited in the culture collection of the Center for Marine Microbial Ecology and Diversity at the University of Hawaii under the accession number CMMED290.

Taxonomy of CMMED 290. DNA was extracted from a culture of the isolate in marine broth. An aliquot was removed, diluted 1:100 in distilled water, and boiled at 98 °C for 5−8 min. Amplification of the16S rDNA gene was carried out in duplicate using primers (Biosynthesis Inc.) 8f and 543r, where r and f indicate reverse and forward, respectively, and numbers are based on the *E. coli* gene.[16] The amplification was performed in 30 cycles of denaturation at 94 °C for 180 s, annealing at 60 °C for 60 s, and extension at 72 °C for 60 s. The product was purified using the Qiagen PCR purification kit (Qiagen Inc.) in accordance with the manufacturer's instructions. Sequencing was performed at the Greenwood Molecular Biology Facility at the University of Hawaii. Sequences were analyzed, aligned, and combined by using Sequencher version 4.8 (Gene Codes Co.). An NCBI and GenBank database search using the BLAST algorithm[17] for highly similar sequences identified the culture as belonging to the Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Pseudoalteromonadaceae; Pseudoalteromonas. Within the *Pseudoalteromonas* genus the culture was shown to have 100% sequence identity to several *Pseudoalteromonas* spp., including *Pseudoalteromonas* sp. D5047, with the accession number FJ161290.1.

Isolation of 1 and 2. CMMED 290 was grown without agitation at RT in 30 × 3 L Erlenmeyer flasks, each containing 1 L of marine broth (MA2216). After a 12-day growth period the cultures were extracted with an equal volume of EtOAc. The combined extract was concentrated *in vacuo* to yield 1.2 g of dry solid. Reversed-phase chromatography (YMC gel) of the extract with a stepwise gradient of 50% to 100% MeOH/H_2O yielded fractions A to H. After removal of pentabromopseudilin and bromophene by recrystallization, C18 RP-HPLC (Phenomenex Luna C18(2); 250 × 10 mm, 5 μm) of fraction F with a linear gradient of 60% to 90% CH_3CN/H_2O (0.5% TFA) gave 1.1 mg of pure compound **1**. Silica gel column chromatography of fraction D with a stepwise gradient of 5−10% CH_2Cl_2/MeOH and subsequent purification on a C18 RP-HPLC column (Phenomenex Luna C18(2); 250 × 10 mm, 5 μm) with a linear gradient of 60% to 90% CH_3CN/H_2O yielded 1.4 mg of compound **2**.

Antimicrobial Activity. Broth microdilution was used to determine antimicrobial activity of pure compounds against methicillin-resistant *Staphylococcus aureus* (ATCC 43300). From 10 mg/mL stock solutions (in DMSO) triplicate 2- to 4-fold serial dilutions of the test samples were prepared in sterile 96-well plates (Corning) with concentrations ranging from 25 μg/mL to 0.4 ng/mL in 100 μL of bacterial cell suspension. Plates were incubated at 37 °C for 12 h. Turbidity values were measured on a Multiskan MCC/340 well plate reader at 405 and 620 nm, and IC_{50} values determined on the basis of the resulting inhibition curves.

2,3,5,7-Tetrabromobenzofuro[3,2-*b*]pyrrole (1): colorless solid; UV (MeOH) λ_{max} (log ε) 315 (1.2), 293 (1.3); ^1H NMR and ^{13}C NMR data (CD_3OD, 500 MHz), see Table 1; negative mode HRESITOFMS $[M - H]^-$ *m/z* 467.6877 (calcd for $C_{10}H_2Br_4NO$, 467.6875).

4,4′,6-Tribromo-2,2′-biphenol (2): colorless solid; UV (MeOH) λ_{max} (log ε) 344 (1.3), 298 (1.4); ^1H NMR and ^{13}C NMR data ($CDCl_3$, 500 MHz), see Table 3; negative mode HRESITOFMS $[M - H]^-$ *m/z* 418.7940 (calcd for $C_{12}H_6Br_3O_2$, 418.7923).

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Supporting Information Available: ^1H NMR and ^{13}C NMR spectra of **1** and **2** and spectroscopic data for the known compounds pentabromopseudilin, bromophene, 3,5-dibromosalicylic acid, and 3,5-dibromosalicylamide. This material is available free of charge via the Internet at http://pubs.acs.org.

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