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Antifouling Properties of Simple Indole and Purine Alkaloids from the Mediterranean Gorgonian *Paramuricea clavata*

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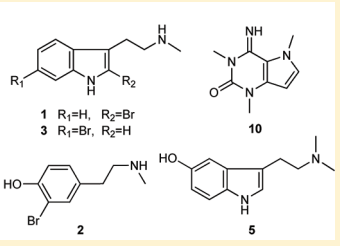
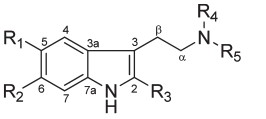
Supporting Information

ABSTRACT: Chemical investigation of the Mediterranean gorgonian *Paramuricea clavata* resulted in the isolation of two new alkaloids, 2-bromo-*N*-methyltryptamine (**1**) and 3-bromo-*N*-methyltyramine (**2**), together with nine known compounds (**3**−**10** and linderazulene). The bromoindole derivative **3** is reported herein for the first time from a natural source. The chemical structures of these compounds were assigned by spectroscopic analyses and comparison with literature values. The antifouling activity and toxicity of compounds **1**−**10** were assessed using three marine biofilm bacteria and the Microtox assay. In contrast to commercial antifoulants, bufotenine (**5**) and 1,3,7-trimethylisoguanine (**10**) showed significant antiadhesion activity against one bacterial strain while being nontoxic.

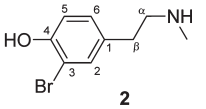


Marine invertebrates have been known in the traditional Mediterranean pharmacopeia since ancient times for their therapeutic potential.[1] Contributing to the Mediterranean benthic biodiversity, octocorals are well known for their ability to biosynthesize a wide array of bioactive secondary metabolites.[2] Numerous chemical investigations of gorgonians have shown that these organisms produce mainly a large variety of cyclized diterpenes together with sesquiterpenes, steroids, prostanoids, and more rarely alkaloids.[2] Some of these compounds have been shown to possess ecological roles[3] and biological activities.[4] The Mediterranean gorgonian *Paramuricea clavata* is a slow-growing and long-lived species typically found within the coralligenous community. This emblematic octocoral is a keystone species that is affected by recurrent disease events.[5] From a chemical point of view, previous studies of *P. clavata* resulted in the isolation of indole alkaloids,[6] caffeine,[7] and linderazulene, a guaiazulene-related pigment.[8]

Within the framework of the ECIMAR program (French National Research Agency) dealing with the inventory of the biodiversity and the chemodiversity of Mediterranean marine invertebrates and in the course of our continuing search for marine natural compounds with antifouling properties, we have investigated the chemical composition of specimens of *P. clavata* collected from two locations in the western Mediterranean Sea (Marseilles, France, and Ceuta, Spain). A careful LC-MS analysis of these samples allowed the detection of brominated derivatives; such compounds have great potential as biofilm inhibitors due to their antibacterial properties.[9] Extracts of *P. clavata* were fractionated by reversed-phase flash chromatography, and selected fractions were purified by semipreparative HPLC, yielding compounds **1**−**10** and linderazulene.



1 $R_1 = R_2 = R_4 = H$, $R_3 = Br$, $R_5 = CH_3$
3 $R_1 = R_3 = R_4 = H$, $R_2 = Br$, $R_5 = CH_3$
4 $R_1 = OH$, $R_2 = R_3 = R_4 = R_5 = H$
5 $R_1 = OH$, $R_2 = R_3 = H$, $R_4 = R_5 = CH_3$
6 $R_1 = R_2 = R_3 = R_4 = H$, $R_5 = CH_3$



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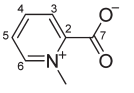

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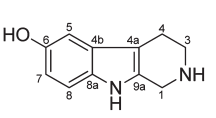

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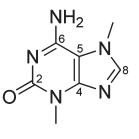

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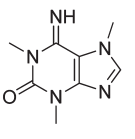

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Table 1. NMR Spectroscopic Data (D_2O, 400 MHz) for Compounds 1 and 2

	2-bromo-*N*-methyltryptamine (**1**)			3-bromo-*N*-methyltyramine (**2**)			
position	δ_C, mult.[a]	δ_H, mult., *J* (Hz)	HMBC[b]	position	δ_C, mult.[a]	δ_H, mult., *J* (Hz)	HMBC[b]
2	119.1, C						
3	109.6, C						
3a	124.3, C			1	125.1, C		
4	117.9, CH	7.58, d (7.5)	6, 7a, 3a, 3	2	128.6, CH	7.43, d (2.0)	β, 3, 4, 6
5	118.3, CH	7.10, t (7.5)	7, 3a	3	104.9, C		
6	120.5, CH	7.19, t (7.5)	4, 7a	4	146.9, C		
7	112.1, CH	7.44, d (7.5)	5, 6, 3a	5	112.0, CH	6.90, d (8.5)	1, 3, 4
7a	135.4, C			6	124.7, CH	7.09, dd (8.5, 2.0)	β, 2, 4
α	49.8, CH_2	3.25, t (7.0)	3, β, *N*-CH_3	α	45.3, CH_2	3.19, t (7.5)	β, 1
β	22.1, CH_2	3.08, t (7.0)	3a, 3, α	β	25.8, CH_2	2.86, t (7.5)	α, 1, 2, 6
N-CH_3	33.4, CH_3	2.59, s	α	*N*-CH_3	28.1, CH_3	2.63, s	

[a] Multiplicities inferred from DEPT and HSQC experiments. [b] HMBC correlations are from proton(s) stated to the indicated carbon.

Compound **1** was obtained as a dark brown solid. Its (+)-ESIMS spectrum showed two pseudomolecular ion peaks $[M + H]^+$ at *m/z* 253 and 255 in an intensity ratio of 1:1, which indicated the presence of one bromine atom in the molecule. In addition, the accurate HRESIMS spectrum of **1** allowed the determination of its molecular formula as $C_{11}H_{13}BrN_2$ requiring six degrees of unsaturation. Its UV spectrum (MeOH) was characteristic of an indole chromophore with absorption maxima at 225, 254, and 282 nm. The ^{13}C NMR (DEPT) spectrum revealed 11 signals including one methyl, two methylenes, four methines, and four quaternary carbons. In the ^1H NMR spectrum of **1** (Table 1), two doublets at δ_H 7.44 (*J* = 7.5 Hz, H-7) and 7.58 (*J* = 7.5 Hz, H-4) and two triplets at δ_H 7.19 (*J* = 7.5 Hz, H-6) and 7.10 (*J* = 7.5 Hz, H-5) were attributed to an ABCD aromatic system. A three-proton singlet signal at δ_H 2.59 was assigned to an *N*-methyl group. Triplets at δ_H 3.25 (*J* = 7.0 Hz, H_2-α) and 3.08 (*J* = 7.0 Hz, H_2-β) were assigned to the methylene protons α and β to a nitrogen, respectively. These data together with those obtained from 2D NMR experiments established the assignment of **1** as a tryptamine derivative. The ^1H and ^{13}C NMR spectra of **1** were found to be closely related to those of the known compound **6**, the main differences being the lack of the aromatic H-2 hydrogen signal as well as the multiplicity and shielding of the signal for C-2 (from δ_C 125.0 in **6** (CH-2) to 119.1 in **1** (C-2)). Thus, the structure of the new compound **1** was established as 2-bromo-*N*-methyltryptamine.

Compound **2** was isolated as a light brown residue. The (+)-ESIMS spectrum showed a 1:1 isotopic cluster of $[M + H]^+$ ions at *m/z* 230/232, suggesting the presence of one bromine atom. The $[M + H]^+$ ion in the (+)-HRESIMS spectrum at *m/z* 230.0176 allowed the molecular formula $C_9H_{12}BrNO$ to be assigned to **2**. The ^1H NMR spectrum showed characteristic signals at δ_H 7.43 (d, *J* = 2.0 Hz, H-2), 7.09 (dd, *J* = 8.5 and 2.0 Hz, H-6), and 6.90 (d, *J* = 8.5 Hz, H-5), which suggested the presence of a 1,2,4-trisubstituted benzene ring. Triplets at δ_H 3.19 (*J* = 7.5 Hz, H_2-α) and 2.86 (*J* = 7.5 Hz, H_2-β) were attributed to a 1,2-disubstituted ethylene fragment, while the singlet at δ_H 2.63 could be assigned to an *N*-methyl group. These fragments were confirmed by the observation of ^{13}C NMR signals corresponding to three aromatic methines (δ_C 128.6, 124.7, and 112.0), three aromatic quaternary carbons (δ_C 146.9, 125.1, and 104.9), two alkyl methylenes (δ_C 45.3 and 25.8), and one methyl (δ_C 28.1). Strong HMBC correlations ($^3J_{CH}$) from both H-2 and H-6 to C-4 and C-β allowed the connection between the aromatic part and the ethylene fragment. Additional HMBC correlation between *N*-methyl hydrogens and C-α supported the assignment of the nitrogen and secured the structure of **2** as 3-bromo-*N*-methyltyramine.

Compound **3** appeared to be an isomer of **1** on the basis of a molecular formula of $C_{11}H_{13}BrN_2$ determined from the HRESIMS data. The ^1H and ^{13}C NMR spectra were similar to those of **6** except for the loss of one aromatic hydrogen on the indole benzene ring (Supporting Information). More precisely, ^1H NMR signals at δ_H 7.68 (d, *J* = 1.5 Hz, H-7), 7.54 (d, *J* = 8.5 Hz, H-4), and 7.27 (dd, *J* = 8.5 and 1.5 Hz, H-5) were consistent with the occurrence of a 1,2,4-trisubstituted benzene ring. Thus, compound **3** was defined as a brominated derivative of *N*-methyltryptamine (**6**). The location of the bromine atom at C-6 was established through specific HMBC correlations, and the structure of **3** was determined to be 6-bromo-*N*-methyltryptamine. This is the first report of **3** as a natural product though it has been previously synthesized.[10,11]

Our study also confirmed the previous reports[6,8] of the tryptamine derivatives serotonin (**4**), bufotenine (**5**), and *N*-methyltryptamine (**6**) and the sesquiterpenoid pigment linderazulene in the extracts of *P. clavata*, while compounds **7**−**10** are described for the first time from this gorgonian. 6-Hydroxytetrahydro-β-carboline (**7**) is supposed to be an artifact produced during the extraction process by a Pictet−Spengler condensation between **4** and formaldehyde, an oxidation product of methanol.[12] The betaine homarine (**8**) is a metabolite commonly found in marine organisms,[13] which has been reported to have antifouling properties.[14] The purine derivative 3,7-dimethylisoguanine (**9**) has been previously isolated from two marine sponges.[15,16] The last known compound, 1,3,7-trimethylisoguanine (**10**), has been described from a New Zealand ascidian, *Pseudodistoma cereum*.[17]

The antifouling activities of compounds **1**−**10** isolated from *P. clavata* were first evaluated by measuring the capacity of these compounds to inhibit biofilm formation of three marine bacterial strains, D41 and TC8, which are *Pseudoalteromonas* spp., and 4M6, which is a *Paracoccus* sp. (Table 2). These data were compared to the results obtained with two commercial antifouling biocides, bis(tri-*n*-butyltin) oxide (TBTO) and Seanine 211.[18] TBTO is a powerful antifoulant, but its toxicity against a wide range of

Table 2. Antiadhesion and Microtox Activities for Compounds 1−10 and TBTO

	bacterial adhesion % ADH[a] ± SD[b]			
compound	D41	TC8	4M6	Microtox EC_{50} μg/mL (μM)
1	28 ± 5	10 ± 10	20 ± 5	
2	70 ± 50	45 ± 1	70 ± 10	
3	45 ± 35	70 ± 10	70 ± 15	372 (1470)
4	110 ± 10	90 ± 10	50 ± 60	>1000 (>5000)
5	30 ± 30	60 ± 20	105 ± 15	>1000 (>5000)
6	90 ± 10	75 ± 10	35 ± 5	104 (597)
7	80 ± 2	50 ± 45	90 ± 25	>1000 (>5000)
8	60 ± 3	110 ± 10	60 ± 20	>1000 (>5000)
9	75 ± 15	60 ± 5	100 ± 15	49 (274)
10	5 ± 20	40 ± 10	90 ± 15	55 (284)
TBTO	0 ± 0	0 ± 0	0 ± 0	<6 (<10)

[a] Percentage of adhered bacteria (100% adhesion is set for the control) at 500 μM. [b] Standard deviation.

organisms, including nontargeted ones, has led to its total ban in antifouling coatings. Compounds **1**−**10** and TBTO were preliminarily tested at 500 μM. Three of the natural compounds (**1**, **5**, and **10**) inhibited at least 70% of the bacterial adhesion for at least one strain. Compound **1**, which showed the highest activity against all the strains, was not isolated in sufficient amount so as to determine its EC_{50}. When compared to TBTO, compounds **5** and **10** showed moderate activity against the D41 strain with EC_{50} values of 95 and 90 μM, respectively, and were inactive against the 4M6 strain (EC_{50} > 250 μM). However, these two compounds demonstrated similar activity to that of Seanine 211 on the D41 strain[18] and were found to be less toxic (IC_{50} > 250 μM vs 6 μM for Seanine 211), suggesting a specific antiadhesion mechanism. At the same time, their toxicity assessed by the Microtox assay (*Vibrio fischeri*), which was selected because of its high sensitivity, precision, and repeatability,[19] gave us additional information. The lack of toxicity was confirmed for compound **5** (EC_{50} > 1000 mg/L or 5000 μM), but weak toxicity was noticed for **10** (EC_{50} = 55 mg/L or 284 μM) in contrast to the result obtained with the D41 strain.

Previous searches for putative natural antifouling agents from gorgonians were mainly conducted on extracts, whereas only a few pure compounds were studied. For example, 3,7,9-trimethyl-6,8-purinediol, batyl alcohol, and 1,2,3,4-tetrahydro-β-carboline, isolated from *Scripearia gracillis*, showed good activity against several marine bacterial species.[20] Compounds **1**, **5**, and **10** add to the list of putative antifouling agents from marine invertebrates, and although the antiadhesion activity of bufotenine (**5**) and 1,3,7-trimethylisoguanine (**10**) is strongly dependent on the bacterial strain, their low toxicity could grant them good potential as environmentally friendly antifoulants.

EXPERIMENTAL SECTION

General Experimental Procedures. Ultraviolet spectra were recorded in MeOH on a Shimadzu UV-2501PC spectrophotometer. 1D and 2D NMR spectra were obtained at 400 and 100 MHz for ^1H and ^{13}C, respectively, on a Bruker Avance 400 MHz NMR spectrometer. Data were acquired with the Brüker XWIN-NMR package and processed with MestReC. Chemical shifts were referenced to the solvent peaks (MeOD, δ_H 3.31, δ_C 49.05; D_2O δ_H 4.79; DMSO-d_6, δ_H 2.50, δ_C 39.43), and formic acid was used as internal standard for ^{13}C NMR spectra in D_2O (δ_C 165.19).[21] Low-resolution mass spectra were measured on an ion trap mass spectrometer fitted with an ESI interface (Esquire 6000, Bruker Daltonics). High-resolution mass spectra (HRESIMS) were conducted on a LTQ/FT-Orbitrap mass spectrometer (Thermo Fisher Scientific). LC-DAD-ELSD-ESI/MS^n analyses were carried out on a LaChrom Elite HPLC (VWR-Hitachi) composed of a L-2130 quaternary pump, a L-2200 autosampler, and a L-2300 column oven. Detection was performed with a L2455 DAD and an ELSD (Chromachem model, Eurosep) coupled to an Esquire 6000 spectrometer. A Flash Spot system (Armen Instruments) was used for flash-chromatography experiments, and a Bio-Tek system (Kontron 525 pump, Bio-Tek 582 column thermostat and Bio-Tek 560 autosampler) with a UV detector with variable-wavelength monitoring (LDC Analytical SpectroMonitor 3100X) for HPLC. Fluorescence measurements for the bioassay were made using an Infinit 200 microplate fluorescence reader (Tecan). All solvents were of HPLC grade and were purchased from Sigma-Aldrich. Bis(tri-*n*-butyltin) oxide came from Acros (Thermo Fisher Scientific), and Seanine 211 was purchased from Rohm & Haas.

Biological Material. Specimens of the gorgonian *Paramuricea clavata* were collected by scuba diving at two distinct sites of the western Mediterranean coast, Marseilles (43°12′34″ N, 5°20′7″ E) in January 2008 and Ceuta (35°53′55″ N, 5°18′29″ W) in February 2008 on the western Mediterranean coasts. The samples from Marseilles were immediately frozen at −20 °C and lyophilized, while the samples from Ceuta were fixed in EtOH (100 mL per 5 g wet wt). These samples were identified by one of the authors (T.P.). A voucher specimen (no. 080124Ma7-01) was deposited at the COM.

Extraction and Purification. The lyophilized material of the gorgonian *P. clavata* collected from Marseilles (35 g dry wt) was extracted with a mixture of CH_2Cl_2/MeOH (1:1; 3 × 250 mL) at room temperature. The concentrated extracts were combined to yield, after solvent removal, 3.5 g of a dark brown lipophilic extract.

This extract was subjected to flash chromatography (UV detection: 205 nm; flow rate: 20 mL/min) on a RP18 column (Merck Super-VarioFlash D26-RP18 model, 15−40 μm, 37 g) with a linear solvent gradient from 100% H_2O to 100% MeCN (25 min) and then from 100% MeCN to 100% CH_2Cl_2 (5 min) followed by an isocratic step with 100% CH_2Cl_2 (5 min), leading to 39 fractions. In order to determine the most interesting ones and the appropriate UV wavelength for purification, these fractions were analyzed by LC-DAD-ELSD-ESI/MS^n. Fraction 2 (eluted with 100% H_2O; 460 mg) was further purified by repeated semipreparative HPLC (Phenomenex Gemini C_6-phenyl, 5 μm; 10 × 250 mm; flow rate: 3 mL/min; UV detection: 292 nm) with a binary solvent gradient (from 100% H_2O to 88% H_2O in MeCN in 16 min) to afford homarine (**8**, 100 mg) and bufotenine (**5**, 5.1 mg). In the same way, fraction 9 (eluted with 67% H_2O in MeOH; 20 mg) was purified by repetitive semipreparative HPLC (gradient H_2O/MeCN: from 95% H_2O to 70% H_2O in 15 min; UV detection at 274 nm) to yield 6-bromo-*N*-methyltryptamine (**3**, 4.5 mg) together with 2-bromo-*N*-methyltryptamine (**1**, 0.8 mg) and *N*-methyltryptamine (**6**, 1 mg). Fraction 8 (eluted with 63% H_2O in MeOH; 230 mg) was purified on a reversed-phase semipreparative HPLC column (Phenomenex Synergi Polar-RP; 4 μm; 10 × 250 mm; flow rate: 3 mL/min; UV detection: 292 nm) with a H_2O/MeOH binary solvent gradient (from 100% H_2O to 90% H_2O in 11 min, then to 80% H_2O in 8 min, and to 50% H_2O in 6 min with a final isocratic step for 5 min) to afford 1,3,7-trimethylisoguanine (**10**, 3.5 mg) and 3-bromotyramine (**2**, 0.9 mg).

The samples collected in Ceuta were immediately fixed in EtOH, and after filtration the eluate was stored at +4 °C. After one week, the solvent was filtered and concentrated under reduced pressure to yield a dark brown residue (912 mg).

This extract was subjected to flash chromatography using the same experimental procedure previously described, and the most interesting fraction (fraction 3 eluted with 100% H_2O; 85 mg) was purified by repeated semipreparative HPLC (Phenomenex Gemini C6-phenyl; flow rate: 3 mL/min; gradient H_2O/MeCN: from 100% H_2O to 88% H_2O in 16 min; UV detection: 292 nm) to afford 6-hydroxytetrahydro-β-carboline (**7**, 3 mg), 3,7-dimethylisoguanine (**9**, 7.5 mg), and serotonin (**4**, 1.2 mg). Linderazulene (1.5 mg) was obtained as the major compound of a fraction obtained after flash chromatography (fraction 28 eluted with 100% MeCN; 6.9 mg).

2-Bromo-*N*-methyltryptamine (1): dark brown solid; UV (MeOH) λ_{max} (log ε) 225 (4.31), 254 (3.34), 282 (3.60) nm; NMR data, see Table 1 and Supporting Information; HRESIMS m/z 253.0338 $[M + H]^+$ (calcd for $C_{11}H_{14}{}^{79}BrN_2$, 253.0340).

3-Bromotyramine (2): light brown solid; NMR data, see Table 1 and Supporting Information; HRESIMS m/z 230.0176 $[M + H]^+$ (calcd for $C_9H_{13}{}^{79}BrNO$, 230.0181).

6-Bromo-*N*-methyltryptamine (3): dark brown solid; UV (MeOH) λ_{max} (log ε) 224 (4.33), 254 (3.34), 286 (3.61) nm; NMR data, see Supporting Information; HRESIMS m/z 253.0337 $[M + H]^+$ (calcd for $C_{11}H_{14}{}^{79}BrN_2$, 253.0340).

Bacterial Adhesion Assay (adapted from Camps et al.[18]). Three strains isolated from natural marine biofilms were used for antiadhesion assays.[18] Each strain was grown in VNSS (Vaatanen nine-salt solution) at 25 °C and sampled in the stationary phase. After centrifugation, cells were suspended in sterile artificial seawater (ASW) until an optical density (OD) of 600 nm, optimized for each strain (between 0.4 and 0.6), was achieved. Then 100 μL of ASW was inoculated into the border-row wells of the microplate (sterile black polystyrene Nunc, Thermo Fisher Scientific), and 100 μL of the bacterial suspension into the other wells using an eight-channel pipet. A 100 μL sample of diluted standard biocide (TBTO) and purified molecules (compounds **1**−**10**) was added in the latter wells for antiadhesion assays and to the ASW wells for nonspecific staining control. All of the concentrations were tested in triplicate. A 100 μL portion of ASW was added to six wells to constitute the bacterial adhesion control. After an optimized time for adhesion (between 15 and 24 h), the nonadhered bacteria were removed by three successive washings (36 g/L sterile NaCl solution, 200 μL). Then bacteria were stained by adding 200 μL of Syto Red 61 (Invitrogen) at 0.5 μM in a 36 g/L NaCl sterile solution. After 10 min under dark conditions, the excess stain was removed by three washes (36 g/L NaCl solution), and the fluorescence intensity was then directly measured in the last NaCl wash (λ_{exc} = 628 nm, λ_{em} = 645 nm) using a microplate fluorescence reader. All compounds were preliminarily tested at 500 μM using the three strains, and the most active ones were then tested at eight different concentrations in triplicate using only D41 and 4M6 (because they showed the highest and lowest inhibition, respectively, for the two compounds selected). A sigmoid dose−response curve was obtained when % of adhesion (relative to the adhesion control) was plotted with the log of compound concentrations, and the EC_{50} (concentration corresponding to 50% of the bacterial adhesion control) was determined.

Bacterial Growth Inhibition Assay. Compounds **5** and **10** were tested according to the method described by Camps et al.[18] The D41 and 4M6 strains were grown on VNSS at 20 °C under shaking conditions (120 rpm) and collected during the exponential phase. After centrifugation, cells were suspended in sterile VNSS ($OD_{600\ nm}$ = 0.1). Each tested compound (180 μL) at eight concentrations was added into four wells of the microtiter plates (sterile transparent polystyrene Nunc, Thermo Fisher Scientific). Then 20 μL of the bacterial suspension was inoculated into all of the wells except the border-row wells, and all of the wells were filled to 200 μL with VNSS. Turbidity ($OD_{600\ nm}$) was measured every hour for 8 h. The growth rate, μ (h^{-1}), was calculated during the exponential phase for each strain, at each concentration. The percent of growth inhibition was calculated relative to the growth control. Percent of inhibition was obtained and then plotted against the log of compound concentrations. A sigmoid dose−response curve was obtained, and the IC_{50} (inhibitory concentration for 50% of the bacteria) determined.

Microtox Toxicity Assay. The standardized Microtox bioassay[22] (Strategic Diagnostics) was used in order to assess the bioactivity of compounds **3**−**10** and TBTO as positive control (see Supporting Information).

■ ASSOCIATED CONTENT

Supporting Information. Experimental details for the Microtox toxicity assay, spectroscopic data for compounds **1**−**10** together with 1D and 2D NMR spectra for compounds **1**−**3**, key $^1H-^1H$ COSY and HMBC correlations for compound **3**, and antiadhesion and antibacterial activities of standard biocides and compounds **5** and **10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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