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NOTE

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Brominated Arginine-Derived Alkaloids from the Red Sea Sponge *Suberea mollis*

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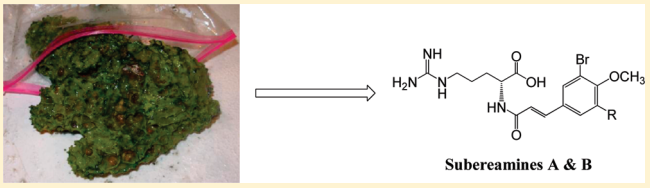
Supporting Information

ABSTRACT: Investigation of a new collection of the Red Sea sponge *Suberea mollis* afforded two new brominated arginine-derived alkaloids, subereamines A (**1**) and B (**2**), a new brominated phenolic compound, subereaphenol D (**3**), and the known compounds dichloroverongiaquinol (**4**), aerothionin (**5**), and purealdin L (**6**). The structures of the isolated compounds were assigned using one- and two-dimensional NMR spectra and HRFABMS data. The absolute configurations of subereamines A (**1**) and B (**2**) were determined by acid hydrolysis followed by chiral-phase LC-MS. The antimicrobial and antioxidant activities of the isolated compounds have been evaluated. Dichloroverongiaquinol and subereaphenol D displayed significant antimicrobial activity. Using the DPPH TLC autographic rapid screen for free radical scavenging effects, subereaphenol D displayed a significant antioxidant effect. In addition, the cytotoxic activities of the isolated compounds were investigated.



Sponges are a prolific source of bioactive substances with antibiotic, anticancer, antiviral, antibacterial, anti-inflammatory, and antihistaminic properties.[1−6] Marine sponges of the order Verongida are of much current biological and chemical interest. They are characterized by the elaboration of brominated metabolites that are biogenetically related to tyrosine. These metabolites are considered as distinct markers for Verongid sponges. Diverse biological activities for these compounds have been reported including antifungal,[7] antibacterial,[8,9] cytotoxic,[10,11] and enzyme-inhibitory effects.[12]

Previous studies of the Red Sea sponge *Suberea mollis* (order Verongida, family Aplysinellidae) resulted in the identification of a number of cytotoxic, antioxidant, and antimicrobial compounds,[13,14] which have prompted us to reinvestigate the sponge. In the present work, a new collection of the sponge resulted in the identification of two new brominated arginine-derived alkaloids, subereamines A (**1**) and B (**2**), and a new brominated phenolic compound, subereaphenol D (**3**), together with the known compounds dichloroverongiaquinol (**4**),[15] aerothionin (**5**),[14] and purealdin L (**6**).[16] Detailed examination of the UV, NMR, and HRFABMS spectroscopic data established the assignment of these compounds.

Subereamine A (**1**) was purified as an optically active, white, amorphous powder. The FABMS spectrum of **1** showed two pseudomolecular ion peaks at *m*/*z* 435 and 437 in an intensity ratio of 1:1, indicating the presence of one bromine atom in the molecule. The HRFABMS allowed the assignment of the molecular formula as $C_{16}H_{21}BrN_4NaO_4$, $[M + Na]^+$ requiring eight degrees of unsaturation. The ^{13}C NMR spectrum of **1** (Table 1) displayed signals for 16 carbons including two carbonyls, four quaternary carbons, three methylenes, six methines, and one methyl as assigned from the HSQC

1 R = H
2 R = Br

3

4

5

6

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Table 1. NMR Spectroscopic Data (DMSO-d_6) for Subereamines A (1) and B (2) and Subereaphenol D (3)

	subereamine A (**1**)			subereamine B (**2**)			subereaphenol D (**3**)		
position	δ_C (mult.)	δ_H [mult., J (Hz)]	HMBC[a]	δ_C (mult.)	δ_H [mult., J (Hz)]	HMBC[a]	δ_C (mult.)	δ_H [mult., J (Hz)]	HMBC[a]
1	129.2, C			124.8, C			128.2, C		
2	131.6, CH	7.63, brs	4, 7	131.3, CH	7.67, s	3, 4, 5	119.2, C		
3	110.9, C			117.7, C			145.0, C		
4	155.9, C			153.6, C			110.4, CH	6.45, d (8.5)	2, 6
5	112.6, CH	7.06, d (8.0)	1, 3, 4	117.7, C			119.8, CH	6.47, d (8.5)	1, 3
6	128.3, CH	7.42, d (8.0)	4	131.3, CH	7.67, s	3, 4, 5	146.0, C		
7	136.1, CH	7.20, d (16.0)	1, 2, 9	134.3, CH	7.17, d (15.5)	1, 2, 9	35.1, CH_2	3.37, s	6, 8
8	121.9, CH	6.64, d (16.0)	1, 9	124.8, CH	6.72, d (15.5)	9	172.7, C		
9	164.0, C			163.4, C					
10		8.11, d (7.0)	9, 17		8.24, d (7.0)	9			
11	53.8, CH	4.08, m	9, 17	53.9, CH	4.08, q (7.0)	9, 17			
12	29.6, CH_2	1.74, 1.63, m	17	29.4, CH_2	1.74, 1.64, m				
13	25.1, CH_2	1.52, 1.44, m		25.0, CH_2	1.52, 1.46, m				
14	40.4, CH_2	3.08, 3.00, m	16	40.3, CH_2	3.09, 3.00, m	16			
15		9.25, brs			9.24, brs				
16	157.3, C			157.2, C					
17	176.1, C			176.0, C					
OCH_3	56.4, CH_3	3.82, s	4	60.5, CH_3	3.80, s	4	59.4, CH_3	3.67, s	3

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

experiment. Interpretation of the ^{1}H and ^{13}C NMR, COSY, HSQC, and HMBC experiments established the assignment of two substructures of **1** (A and B). The NMR signals (Table 1) at δ 129.2 (C-1), 7.63/131.6 (H-2/C-2), 110.9 (C-3), 155.9 (C-4), 7.06/112.6 (H-5/C-5), 7.42/128.3 (H-6/C-6), 7.20/136.1 (H-7/C-7), 6.64/121.9 (H-8/C-8), and 164.0 (C-9) suggested the assignment of fragment A as a 1,3,4-trisubstituted cinnamoyl moiety. The substituents at the phenyl ring were a bromine and a methoxy. The coupling constant value between H-7 and H-8 (J = 16.0 Hz) suggested the E configuration for the C-7/C-8 double bond. Additional NMR data suggested the assignment of fragment B as an arginine moiety. This assignment was confirmed by the presence of NMR signals at δ 4.08/53.8 (H-11/C-11), 1.74, 1.63/29.6 (H_2-12/C-12), 1.52, 1.44/25.1 (H_2-13/C-13), 3.08, 3.00/40.4 (H_2-14/C-14), 9.25 (N*H*-15), and 157.3 (C-16). The connectivity of the fragments A and B through an amide linkage was supported from the HBMC correlations of H-11/C-9 and N*H*-10/C-9. Additional HMBC correlations (Table 1) supported the assignment of the quaternary carbons and secured the structure of **1**. Thus, compound **1** was assigned as (*E*)-2-(3-(3-bromo-4-methoxyphenyl)acrylamido)-5-guanidinopentanoic acid. Interestingly, a very similar compound, L-(−)-(*N-trans*-cinnamoyl)arginine, was recently isolated from a terresterial plant, *Gelius oppositifolius*.[17] The co-occurrence of cinnamoylarginine derivatives in both terresterial and marine sources is noteworthy from the point of view of the biosynthesis of such metabolites.

The FABMS spectrum of **2** displayed three pseudomolecular ion peaks at m/z 512.9, 514.9, and 516.9 in an intensity ratio of 1:2:1, indicating the presence of two bromine atoms in the molecule. In addition, the molecular formula of subereamine B (**2**) was assigned as $C_{16}H_{20}Br_2N_4NaO_4$, $[M + Na]^+$, as established from HRFABMS, indicating the presence of an additional bromine atom in the molecule relative to **1**. Comparison of the ^{1}H and ^{13}C NMR and HMBC data of **2** with those of **1** (Table 1) showed nearly identical signals for both compounds. Moreover, the ^{1}H NMR spectrum of **2** (Table 1) showed a two-proton downfield aromatic methine singlet at δ 7.67 (2H, H-2/H-6, s) instead of three one-proton signals (H-2, H-5, and H-6) in **1**, suggesting 3,5-dibromination of the phenyl ring in **2**. Accordingly, compound **2** was assigned as (*E*)-2-(3-(3,5-dibromo-4-methoxyphenyl)acrylamido)-5-guanidinopentanoic acid.

The absolute configurations of subereamines A (**1**) and B (**2**) were determined by a combination of acid hydrolysis followed by enantioselective analysis. Chiral-phase LC-MS of the acid hydrolysates of **1** and **2** established the presence of D-arginine [(*R*)-(−)-arginine)].

The FABMS spectrum of **3** showed two pseudomolecular ion peaks at m/z 281.9 and 283.9 in an intensity ratio of 1:1, indicating the presence of one bromine atom in the molecule. In addition, accurate HRFABMS of **3** supported the molecular formula of $C_9H_{10}BrNNaO_3$, $[M + Na]^+$. The ^{13}C NMR spectrum of **3** (Table 2) displayed nine signals including six aromatic carbons (δ 146.0−110.4), one carbonyl functionality at δ 172.7, and signals at δ 35.1 and 59.4. The ^{1}H NMR spectrum of **3** (Table 1) displayed two one-proton *ortho*-coupled doublets at δ 6.47 and 6.45 (J = 8.5 Hz, H-4 and H-5), suggesting the 1,2,3,4-tetrasubstitution of the phenyl ring. The substituents were a hydroxy, a methoxy, a bromine, and an acetamide moiety. In addition, ^{1}H NMR signals at δ 3.37 (2H, s) and 3.67 (3H, s, OCH_3) indicated the presence of methylene and methoxy groups. The HSQC data revealed the presence of five quaternary carbons, two aromatic methines, one methylene, and one methyl. The HMBC cross-peaks (Table 1) of H-5/C-1, H_2-7/C-1, H-4/C-2, H-5/C-3, OCH_3/C-3, H-4/C-6, H_2-7/C-6, and H_2-7/C-8 supported the placement of all functionalities on the aromatic moiety of **3**. Accordingly, **3** was assigned as 2-(2-bromo-6-hydroxy-3-methoxyphenyl)acetamide (subereaphenol D).

Marine sponges continue to be a rich source of secondary metabolites with diverse bioactivities. Reinvestigation of the sponge *Suberea mollis* afforded two new brominated cinnamoyl

arginine alkaloids, subereamines A (**1**) and B (**2**), a brominated phenol, subereaphenol D (**3**), and the known compounds dichloroverongiaquinol (**4**), aerothionin (**5**), and purealdin L (**6**).

The isolated compounds were evaluated for their antimicrobial, antioxidant, and cytotoxic activities. With regard to the antimicrobial activity, compounds **1** and **2** were inactive against all tested organisms, whereas **5** and **6** were moderately active against *S. aureus*, with an inhibition zone of 5 mm. Compound **3** was more active and displayed inhibition zones of 10 and 18 mm against *S. aureus* and *E. coli*, respectively, while **4** showed an inhibition zone of 15 mm against *E. coli*. Compound **3** was active against *C. albicans* with an inhibition zone of 20 mm, whereas all the other compounds were inactive against *C. albicans*. Subereaphenol D (**3**) showed a very strong bright yellow color in the antioxidant assay, suggesting a powerful antioxidant activity. The high antioxidant activity of **3** could be attributed to its phenolic nature. Moreover, compounds **3** and **4** were cytotoxic to HeLa cells, with IC_{50} values of 19 and 13 μM, respectively, while other compounds were either inactive or not evaluated in this assay.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were recorded on a JASCO DIP-730 digital polarimeter. UV spectra were recorded on a Hitachi 300 spectrometer. IR spectra were recorded on a Varian 800 FT-IR spectrometer. NMR spectra were obtained in DMSO-d_6 on a Bruker (600 MHz for ^{1}H and 150 MHz for ^{13}C) spectrometer. ^{1}H and ^{13}C NMR chemical shifts were referenced to the DMSO-d_6 solvent signal. Positive FAB mass spectrometric data were obtained with a Q-TOF mass spectrometer using leucine enkephalin at m/z 556.2771 $[M + H]^+$ as the reference mass. Precoated silica gel G-25 UV_{254} plates were used for thin-layer chromatography, and silica gel 60, 230−40 μm mesh (E. Merck), and Sephadex LH-20 (Pharmacia) for column chromatography.

Animal Material. The marine sponge *Suberea mollis* was re-collected off Hurghada at the Red Sea coast at a depth of 19 m, during summer of 2009. The sponge has been described in detail elsewhere.[14] A voucher specimen, measuring 3.5 cm, is incorporated in the collections of the Zoological Museum of the University of Amsterdam under registration number ZMAPOR 16621. Another voucher specimen was deposited in the Red Sea Invertebrates Collection of the Department of Pharmacognosy, Suez Canal University, under the code number 2009DY8.

Extraction and Isolation. The frozen sponge (600 g, wet wt) was sliced into small pieces and homogenized in EtOH. The resulting extract was partitioned between *n*-hexanes/H_2O and EtOAc/H_2O. The cytotoxic/antimicrobial EtOAc extract (900 mg) was subjected to size exclusion chromatography on a Sephadex LH-20 column equilibrated with MeOH to give seven major fractions. The cytotoxic/antimicrobial fractions 4 and 5 were combined, and the mixture (320 mg) was partitioned on a SiO_2 column using CH_2Cl_2/MeOH gradients to give five major fractions. Fraction 2 (170 mg) was purified on a semipreparative C_{18} HPLC column using 50% MeCN at a flow rate of 2 mL min^{-1} to give compounds **1** (11 mg), **2** (13 mg), **4** (7.5 mg), **5** (3.6 mg), and **6** (4.2 mg). Fraction 3 (32 mg) was subjected to final purification on a semipreparative C_{18} HPLC column using 50% MeCN at a flow rate of 2 mL min^{-1} to afford compound **3** (4.5 mg).

*Subereamine A (**1**):* white, amorphous powder; $[\alpha]^{21}_D$ −15 (*c* 0.54, MeOH); UV (MeOH) λ_{max} (log ε) 292 (3.54), 232 (3.91), 221 (3.62) nm; IR ν_{max} (film) 3335, 3166, 3045, 1665, 1400, 1205, 978 cm^{-1}; NMR data, see Table 1; HRFABMS m/z 435.0647 $[M + Na]^+$ (calcd for $C_{16}H_{21}{}^{79}BrN_4NaO_4$, 435.0644).

*Subereamine B (**2**):* white, amorphous powder; $[\alpha]^{21}_D$ −20.9 (*c* 0.33, MeOH); UV (MeOH) λ_{max} (log ε) 296 (3.63), 238 (3.74), 222 (3.76) nm; IR ν_{max} (film) 3333, 3165, 3045, 1668, 1402, 1207, 978 cm^{-1}; NMR data, see Table 1; HRFABMS m/z 512.9753 $[M + Na]^+$ (calcd for $C_{16}H_{20}{}^{79}Br_2N_4NaO_4$, 512.9749).

*Subereaphenol D (**3**):* dark brown powder; UV (MeOH) λ_{max} (log ε) 304 (2.93), 221 (3.52) nm; IR ν_{max} (film) 3430, 1665, 1400, 1206 cm^{-1}; NMR data, see Table 2; HRFABMS m/z 281.9747 $[M + Na]^+$ (calcd for $C_9H_{10}{}^{79}BrNNaO_3$, 281.9742).

Determination of the Antimicrobial Activity Using the Agar Diffusion Method (ref 18). The antimicrobial activities of the isolated compounds were evaluated against *E. coli* (ATCC 35218), *Staphylococcus aureus* (ATCC 6538P), *Pseudomonas aeruginosa* (ATCC 9027), *Klebsiella pneumoniae* (ATCC 10032), and *Candida albicans* (ATCC 2091) using the agar diffusion method. One milligram of each compound was dissolved in 1 mL of DMF, and 100 μL of each solution was inserted in the cups, then incubated at 37 °C for 24 h. The inhibition zones were measured and compared with those produced by the reference compounds erythromycin (8 mm against *S. aureus*@15 μg/disk), ciprofloxacin (8 mm against *P. aeruginosa*@5 μg/disk), chloramphenicol (10, 9, and 7 mm against *K. pneumonia*, *S. aureus*, and *P. aeruginosa*@30 μg/disk), and clotrimazole (28 mm against *C. albicans*@10 mg/mL, 100 μL).

Acid Hydrolysis and Absolute Configurations of Subereamines 1 and 2. Subereamines A (**1**) and B (**2**) (1.0 mg) were treated separately with 2 mL of 6 N HCl and heated at 110 °C for 18 h in ampules previously sealed under N_2 gas, evaporated to dryness, and suspended in H_2O. The configuration of the arginine in the hydrolysates of both **1** and **2** was determined by chiral-phase LC-MS using Chirobiotic T, 250 × 4.6 mm; 20 mM ammonium acetate (pH 4.1)/MeOH (1:1); flow rate, 1 mL/min; detection by UV at 205 nm; and ESIMS in positive ion mode. The retention time of the natural product hydrolysate of both **1** and **2** matched that of D-arginine [(*R*)-(−)-arginine)].

Evaluation of the Antioxidant Activity. Compounds **1**−**6** were examined for their antioxidant activity using TLC autographic assay for DPPH radical scavenging effect.[19] The compounds were dissolved in MeOH at a concentration of 1 mg/mL, and vitamin E was prepared at a similar concentration and used as a positive control. Six micrograms of each compound was applied in the form of a spot of 4 mm in diameter. The radical scavenging effects were detected on a TLC plate, using a spray reagent composed of a 0.2% (w/v) solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) in MeOH. The plate was observed 30 min after spraying.

Determination of the Cytotoxic Activity of the Compounds. The cytotoxic activities of the isolated compounds were evaluated against HeLa cells using the MTT assay.[20] Briefly, HeLa cells were incubated overnight at 37 °C in 5% CO_2/air in microtiter plates. Test materials, adriamycin (positive control), and DMSO (negative control) were added to the top row of a 96-well microtiter plate and serially diluted (1:4) downward. After a 72 h incubation, cell viability was determined colorimetrically using a Molecular Devices Emax microplate reader (490 nm), recording the amount of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) reduced to formazan using the CellTiter 96 AQueous nonradioactive cell proliferation protocol (Promega). Minimum inhibitory concentration (IC_{50}, μg/mL) values were calculated using the program SOFTmax PRO (Molecular Devices).

ASSOCIATED CONTENT

Supporting Information. ^{1}H and ^{13}C NMR spectra of compounds **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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