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Note

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Twilight Zone Sponges from Guam Yield Theonellin Isocyanate and Psammaplysins I and J

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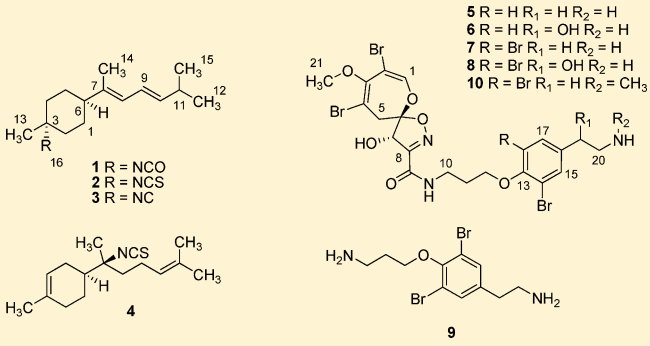
Supporting Information

ABSTRACT: From the organic extracts of two Guam sponges, *Rhaphoxya* sp. and *Suberea* sp., determined to have cytotoxic and chemopreventive activities, three new compounds, theonellin isocyanate (**1**) and psammaplysins I and J (**5**, **6**), and six previously reported compounds (**2**–**4**, **7**–**9**) were isolated and characterized spectroscopically (^{1}H and ^{13}C NMR, MS, IR, UV, $[\alpha]_D$). The two new metabolites (**5** and **6**) isolated from the *Suberea* sp. sponge are rare examples of compounds containing a bromotyramine moiety rather than the more usual dibromo analogue. For the compounds isolated from the *Rhaphoxya* sp., this is the first report of the known compounds **2**–**4** being found in a single sponge. For previously reported compounds **2**–**4** complete unambiguous ^{1}H and ^{13}C NMR data are provided.



It is well accepted that the marine environment is a proven source of molecules without structural precedent,[1,2] of therapeutic agents,[1−3] and of products employed by the cosmetics, agricultural, and chemical industries.[4,5] Over the past three decades in excess of 22 000 natural products have been isolated from marine organisms.[6] The likely reason for this wealth of chemical biodiversity is the fact that they are often produced by sessile life forms found in the oceans. Being fixed or pedestrian yields an organism extremely vulnerable to attack by highly mobile predators if it is not adequately protected. Typically, such organisms, including algae, sea grasses, sponges, tunicates, soft corals, and gorgonians, defend themselves chemically. Given that some of these organisms like sponges are among the most ancient metazoan (multicellular) animals, dating back at least 600 million years, it comes as no surprise that these chemical defenses are highly evolved. Although still to be defined, it might be that in some cases these defense chemicals may be tailored to defend a given organism from a specific predator, meaning those organisms with more than one predatory species can often have a suite of such chemicals.[7]

With this premise in mind, screening of extracts derived from twilight or disphotic zone organisms, those living between 50 and 1000 m depth, was undertaken to test the theory that those organisms from relatively extreme environments would be at least as chemically productive as, if not more than, their counterparts found in more accessible regions of the oceans. This prior investigation in our laboratories of the extracts of 65 Twilight Zone sponges, gorgonians, hard corals, and sponge associated bacteria resulted in an extremely high hit rate of 42% of active extracts,[8] with a hit rate for sponge and gorgonian extracts being an astonishing 72%.[8] On the basis of these screening results, two of 15 sponges were chosen at random for further chemical investigation. Each sponge extract was retested to confirm their bioactivity profiles[8] and subsequently chemically screened for the presence of so-called nuisance compounds, these being usually too toxic or generalist for further development. With this prescreening completed, compounds were then isolated and assessed to determine which were implicated in, or possibly responsible for, the

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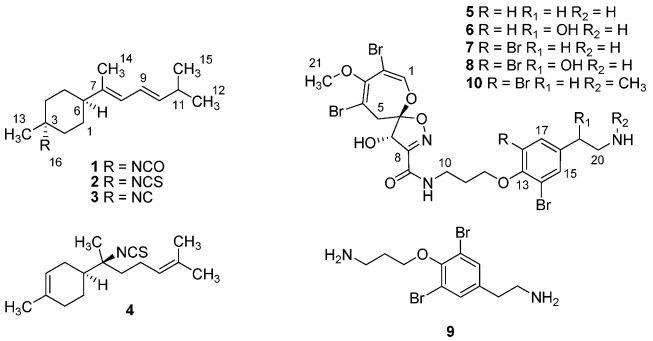
observed activity with the expectation of finding new natural products chemistry that might warrant further development.

The organic extracts of each of the two biologically active sponges[8] were fractionated to yield, in the case of the *Rhaphoxya* sp. sample, one new (**1**) and three known (**2**–**4**) sesquiterpenes substituted with either isocyanato, isothiocyanato, or isocyano (isonitrile) functionalities. The first compound isolated in this study, **1**, was the least abundant and also relatively unstable. The molecular formula of **1** was determined to be $C_{16}H_{25}NO$ by accurate mass measurement. From the ^{1}H and ^{13}C NMR data of **1** (Table 1) only 21 proton and 14 carbon resonances were observed, indicating that at least one if not two of the proton and carbon resonances were degenerate. From the ^{1}H and ^{13}C NMR and UV data of **1** it was evident that the molecule contained a conjugated diene [^{13}C NMR: 139.4 (C-7, C), 123.6 (C-8, CH), 123.5 (C-9, CH), and 140.5 ppm (C-10, C); UV-PDA 240 nm] and an isocyanato moiety [57.9 (C-3, C); 122.4 ppm (C-16, NCO, br)] as the only multiple bonds within the molecule, showing it to be monocyclic and very similar in structure to **2** and **3**, the significant differences between them being the isocyanato moiety in **1**, which also accounted for all of the heteroatoms within the molecule. HMBC correlations observed between the resonance for H_3-13 and those for C-2, C-3, C-4, and C-16 showed C-2, C-4, C-13, and the nitrogen to bond directly with C-3 (see Supporting Information). From the magnitude of the ^{1}H coupling constant between H-9 and H-10 (J = 15.3 Hz) it was evident that Δ^9 had *E* geometry. The C-7/C-9 double-bond also had *E* geometry based on a 2D NOESY cross-peak between H-6 and H-8. Furthermore, comparison of the ^{13}C NMR data for **1** at C-3, C-6, and C-13 with the corresponding data for **2** and **3** (Tables 1 and S1) showed the three molecules to have the same relative configurations. Thus, **1** is the C-3 isocyanato derivative of theonellin isothiocyanate (**2**)[9] and, as such, is best described as theonellin isocyanate. Compounds **1**–**3** are all optically inactive due to the plane of symmetry passing through C-3 and C-6.

Table 1. ^{1}H (400 MHz $CDCl_3$) and ^{13}C NMR (100 MHz $CDCl_3$) Spectroscopic Data for 1 and 2[a]

no.	δ_C, mult. (**1**)	δ_H (J in Hz) (**1**)	δ_C, mult. (**2**)	δ_H (J in Hz) (**2**)
1	27.6, CH_2	1.41, m; 1.64, m	26.9, CH_2	1.43, m; 1.72, m
2	40.0, CH_2	1.68, m; 1.84, m	38.5, CH_2	1.78, m; 1.89, m
3	57.9, C		60.9, C	
4	40.0, CH_2	1.68, m; 1.84, m	38.5, CH_2	1.78, m; 1.89, m
5	27.6, CH_2	1.41, m; 1.64, m	26.9, CH_2	1.43, m; 1.72, m
6	45.6, CH	1.92, m	45.0, CH	1.96, m
7	139.4, C		138.8, C	
8	123.6, CH	5.81, brd, (10.8)	123.8, CH	5.80, brd, (10.7)
9	123.5, CH	6.21, ddd, (1.2, 10.8, 15.3)	123.4, CH	6.20, ddd, (1.2, 10.7, 15.0)
10	140.5, CH	5.59, dd, (7.0, 15.3)	140.7, CH	5.59, dd, (7.1, 15.0)
11	31.4, CH	2.35, dqq, (7.0, 6.7, 6.7)	31.4, CH	2.35, dqq, (7.1, 6.8, 6.8)
12	22.5, CH_3	1.01, d, (6.7)	22.5, CH_3	1.01, d, (6.8)
13	26.0, CH_3	1.35, brs	25.0, CH_3	1.42, brs
14	15.1, CH_3	1.72, s	15.2, CH_3	1.72, s
15	22.5, CH_3	1.01, d, (6.7)	22.5, CH_3	1.01, d, (6.8)
16	122.4, NCO (br)	129.0, NCS		

[a]All assignments are based on interpretation of extensive 1D and 2D NMR measurements.



Together with **1** the three known compounds **2**–**4**[9–11] were also isolated and fully characterized by NMR. For each of these compounds complete and unambiguous ^{1}H and ^{13}C NMR data are provided in Tables 1 and S1. This is the first report of **2**–**4** co-occurring in the same organism. From our previous research[12,13] it is known that compounds with isocyanato, isothiocyanato, or isocyano (isonitrile) functionalities are often biologically active and in the present case are the components responsible for at least some of the observed bioactivity of the original sponge extract. Unfortunately, the instability of **1**–**4** precluded us from proving this contention.

From the organic extract of the *Suberea* sp. sample five bromotyramine derivatives (**5**–**9**) were isolated, two of which were new compounds (**5** and **6**). The known compounds were psammaplysins A (**7**) and B (**8**)[14] and moloka'iamine (**9**).[13] Compound **5** analyzed for $C_{21}H_{24}Br_3N_3O_6$ by mass spectrometry. From the ^{1}H and ^{13}C and 2D NMR data of **5** (Table 2) it was evident the molecule contained 12 sp^2-hybridized carbons, six of which were present as methines, the remainder being quaternary carbons. From these data it was also clear that there were nine sp^3-hybridized carbons, indicating one methyl, six methylenes, one methine, and one quaternary carbon. These deductions meant **5** had to contain four protons bound to oxygen and/or nitrogen and that the molecule was tricyclic. The ^{1}H NMR data showed the presence of a 1,2,4-trisubstituted phenyl group [δ 7.52, d, 2.2 (H-15), δ 7.24, dd, 2.2, 8.5 (H-17), δ 7.05, d, 8.5 (H-18)]. The two proton resonances at δ 3.09 and 3.42 ($C\underline{H}_2$-5) displayed a large 16.0 Hz coupling, similar to that found in the 4,5-dihydro-1,2-oxazole moiety of fistularins[16,17] and in the 4,6-dibromo-5-methoxy-2,3-dihydrooxepine moiety of psammaplysins.[14,18–22] Closer inspection of the ^{13}C NMR data of **5** clearly showed it was characteristic of a psammaplysin skeleton rather than a fistularin, particularly the characteristic resonance for the spiro-carbon C-6 (121.7 ppm). In fact, the C-1 to C-12 part of **5** was found to be identical to that of psammaplysins A (**7**)[14] and F.[20] The molecular formula of **5** indicated it differed from **7** by having one less bromine atom in the phenyl ring and from psammaplysin F by missing the same bromine as well as the methyl of the *N*-CH_3 group. All of the physical and spectroscopic data of **5** supported this deduction, in particular the NOEs between the resonances for H_2-12 and H-18, and those between H_2-19 and H-15 and H-17, which confirmed the regiochemistry of the phenyl ring. This is the ninth reported psammaplysin, psammaplysin I (**5**), and the first to contain a monobromotyramine moiety. Both biosynthetically and synthetically, monobrominated tyramines are considerably more challenging to make than the equivalent 1,3-dibromo

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Table 2. ^{1}H (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) Spectroscopic Data for Psammaplysin I (5) and Psammaplysin J (6) and COSY and gHMBC NMR Data (400 MHz, CD_3OD) for Psammaplysin I (5)[a]

no.	δ_C, mult. (5)	δ_H (J in Hz) (5)	COSY (5)	gHMBC (5)[b]	δ_C, mult. (6)	δ_H (J in Hz) (6)
1	147.6, CH	7.17, s		2, 3, 6	147.6, CH	7.17, s
2	105.2, C				105.2, C	
3	150.7, C				150.7, C	
4	105.4, C				105.4, C	
5a	39.2, CH_2	3.09, d (16.0)	H-5a	3, 4, 6	39.2, CH_2	3.09, d, (16.0)
5b		3.42, d, (16.0)	H-5b	3, 4, 6		3.42, d, (16.0)
6	121.7, C				121.7, C	
7	81.3, CH	5.01, s		6, 8	81.3, CH	5.01, s
8	159.6, C				159.6, C	
9	161.7, C				161.7, C	
10	38.8, CH_2	3.59, m	H-11	9, 11	38.8, CH_2	3.59, m
11	30.9, CH_2	2.13, m	H-10, 12	10, 12	30.9, CH_2	2.13, m
12	69.1, CH_2	4.14, t, (6.0)	H-11	10, 11, 13	69.1, CH_2	4.15, t, (6.0)
13	156.9, C				157.2, C	
14	114.6, C				113.7, C	
15	135.4, CH	7.52, d, (2.2)	H-17	13, 15, 18	132.3, CH	7.66, d, (2.0)
16	132.5, C				136.8, C	
17	132.8, CH	7.24, dd, (2.2, 8.5)	H-15, 18	13, 15, 18	127.6, CH	7.38, dd, (2.0, 8.5)
18	115.8, CH	7.05, d, (8.5)	H-17	13, 14, 16, 17	115.1, CH	7.09, d, (8.5)
19	34.2, CH_2	2.91, t, (7.2)	H-20	15, 16, 17, 20	70.2, CH	4.86, m
20	42.8, CH_2	3.17, t, (7.2)	H-19	16, 19	48.0, CH_2	3.01, m
						3.15, m
21	60.2, CH_3	3.68, s		3	60.2, CH_3	3.68, s
NCH_3						
CONH						
NH						
OH						

[a]All assignments are based on interpretation of extensive 1D and 2D NMR measurements. [b]HMBC correlations are from proton(s) stated to the indicated carbons.

analogues, and an obvious result of this is the much lower occurrence of the monobrominated class in nature; in this sense they should be considered quite special.

Compound **6** was characterized only on the basis of its ^{1}H and ^{13}C NMR data (Table 2), as it could not be successfully separated chromatographically from **5** due to the very small amount of material (1.2 mg of an approximately 3:2 mixture of **6** and **5**). As a result, other unambiguous data (MS, IR, UV, $[\alpha]_D$) could not be recorded; however, by applying subtraction methods a complete and unambiguous set of ^{1}H and ^{13}C NMR data was obtained for future reference (Supporting Information and Table 2). Inspection of the ^{1}H NMR data of the mixture indicated **6** was yet another psammaplysin derivative very similar to **5**. When ^{1}H and ^{13}C NMR spectroscopic data for the two compounds were closely compared and subtraction spectra generated (Supporting Information), it was evident that the differences between the two were in the phenyl ring region (C-13 to C-20) and that these differences could be explained only by the presence of an OH group at C-19, rather than, for example, chlorine, bromine, or sulfate, making **6** the 18-debromo derivative of psammaplysin B,[16] psammaplysin J. All of the NMR data recorded for **6** were consistent with this deduction.

Upon completion of the planar structure analysis of **5** and **6**, their relative configurations required assignment. Literature research revealed that stereochemical assignments for psammaplysins reported to date, A–H,[14,18–22] were based on data comparisons made with psammaplysin A, whose relative configuration was determined by single-crystal X-ray crystallographic analysis.[14] In the 2D NOESY data of **5** it was apparent that there were no interactions between either of the protons attached to C-5 and H-7. On examining minimized structures of **5** with 6*R**, 7*R** and 6*R**, 7*S** configurations, it was evident that the 6*R**, 7*R** configured molecule would be the one expected to show no NOE interactions between H_2-5 and H-7. This information, combined with comparable specific rotation data as well as comparable ^{13}C NMR chemical shifts for C-5, C-6, and C-7 and ^{1}H NMR shifts for H_2-5 and H-7 to that of the other psammaplysins,[14,18–22] indicated **5** and **6** to have the same relative configurations as the ones previously reported. On the basis of our data it was not possible to make deductions concerning the configuration of C-19 in **6**.

On the basis of previously reported biological activity data for psammaplysins[14,18–22] and the fact that polybrominated compounds are unlikely to ever be progressed further than preliminary laboratory screening efforts due to their instability, no further biological evaluations of our materials were undertaken.

The results obtained from this study clearly supported our original contention that Twilight Zone organisms will almost certainly yield interesting and biologically active metabolites that are new to science. What was particularly interesting was that the two sponges selected at random from the group of organisms that were found to have a much higher than expected hit rate in prescreening actually did yield new and known compounds that could, based on similar activities of already known compounds,[12–15,18–22] be generally associated with the observed activities of their original extracts.

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EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotation data were collected employing a Rudolph Research Analytical Autopol IV automatic polarimeter. IR spectra were measured using a Thermo Scientific Nicolet iS10 FTIR spectrophotometer fitted with a Smart iTR. NMR spectra for all compounds were measured on a Bruker Avance DRX 400 MHz NMR spectrometer. All NMR spectra were referenced to NMR solvent signals as follows (δ 7.26 and 77.0 ppm for $CDCl_3$ and δ 3.34 and 49.9 ppm for CD_3OD). FTICR-MS measurement, of **1**, was performed on an unmodified Bruker BioAPEX 47e mass spectrometer equipped with an Analytica of Branford model 103426 (Branford, CT, USA) electrospray ionization (ESI) source in the positive mode. Direct infusion of the sample (0.2 mg/mL in MeOH) was carried out using a Cole Palmer 74900 syringe pump at a rate of 100 μL/h. The instrument was calibrated using a methanolic solution of CF_3COONa (0.1 mg/mL MeOH). HRESIMS, of **5**, was measured on an Applied Biosystems Mariner Biospectrometry TOF workstation using positive electrospray ionization, mobile phase 1:1 MeOH/H_2O. HPLC separations were undertaken employing a Waters HPLC system with binary pumps (Waters 1525), a Waters 717 autosampler, and a Waters 2996 PDA detector together with a Phenomenex Gemini C18, 5 μm, 250 × 10 mm HPLC column.

Animal Material. The sponge *Suberea* sp. (family Aplysinellidae) was collected in October 2005 at Black Coral Kingdom, Guam (N 13.31.98, E 144.63.93), from a depth of 60 m. The sponge is light tan colored, and its growth form is massive, spreading across the substrate. The surface is smooth with pronounced conules; the consistency is rubbery and spongy. The sponge colors darken after preservation. The skeleton is composed of very sparse dendric fibers in relation to the soft tissue. The fibers are in cross-section composed of both bark and pith elements. Thirteen species have been assigned to the genus *Suberea*. All of these, with the exception of *Suberea creba*, are clearly different from the Guam specimen by growth form and/or color. The Guam specimen is most related to *S. creba* from New Caledonia. The growth form and color are similar, but the surface, consistency, and fiber diameter differ. We believe that the current specimen is an undescribed species.

The sponge *Rhaphoxya* sp. (family Dictyonellidae) was collected near Blue Hole, Guam (N 13.26.20, E 144.37.42), at a depth of 90 m, along a drop-off, in July 2006. The sponge is tan colored, and the growth form is massive to subspherical; the consistency is spongy and compressible. The skeleton is composed of vague diverging meandering spicule tracts ascending toward the surface with many broken spicule fragments lying loose in the heavily collagenous soft tissue. The majority of the megascleres are flexuous strongyles, although the terminations of the spicules vary from symmetrical to hastate tapering ends or more telescoped ends. The dimensions of the spicules are 350−550 × 2−5 μm. Seven other species are presently assigned to the genus *Rhaphoxya*, but are known from Southern Australia with the exception of *R. laubenfelsi* (Mexico) and *R. systremma* (New Caledonia). The Mexican species is a branching sponge, with a different spicule composition. The most related species is *R. systremma*, with a known distribution on the Great Barrier Reef and New Caledonia. It differs mainly from the Guam specimen in the dimensions of the spicules. We believe that the Guam specimen is an undescribed species. Both sponges were kept at ambient temperature in seawater in a cooler during transport to the laboratory, where they were immediately frozen at −20 °C. Collection was carried out according to local (Guam) legislation. Voucher specimens (accession number RMNH POR. 3005 and 3014, respectively) have been lodged with the NCB Naturalis, Leiden, The Netherlands.

Extraction and Isolation. Freeze-dried *Rhaphoxya* sp. material (60 g) was exhaustively extracted with MeOH/EtOAc (1:1) to yield 13.2 g of extract. This extract was redissolved in 300 mL of MeOH/H_2O (9:1) and partitioned with an equivalent volume of *n*-hexane. The resultant bioactive *n*-hexane fraction (520 mg) material was dried and further separated employing flash chromatography (silica gel 60, 40−63 μm, 0−100% EtOAc/*n*-hexane) to yield 12 semipure fractions, each of 200 mL. Fractions were evaporated under reduced pressure, and aliquots tested against the whole assay panel.[8] Active fractions were further purified by HPLC employing a silica column (Phenomenex column Luna 3 μm, 250 × 4.6 mm) and isocratic elution with *n*-hexane/EtOAc (98:2) and a flow rate of 0.5 mL/min, affording compounds **1** (0.9 mg, 0.002%), theonellin isothiocyanate (**2**)[9] (18.5 mg, 0.03%), theonellin isocyanide (**3**)[10] (26.1 mg, 0.044%), and 7-isothiocyanato-7,8-dihydro-α-bisabolene (**4**)[11] (2.6 mg, 0.004%); $[\alpha]^{25}_D$ +54 (*c* 0.02, CH_3OH) [lit. +60.5 (*c* 6.8, $CDCl_3$)[11]].

*Theonellin isocyanate (**1**):* clear oil; UV (PDA, CH_3OH/H_2O) λ_{max} 240 nm; ^1H (400 MHz, CD_3OD) and ^{13}C (100 MHz, CD_3OD) NMR data, see Table 1; FT-ICR-HRESIMS *m/z* 270.1836 $[M + Na]^+$ (calcd for $C_{16}H_{25}NONa$, 270.1828).

The sponge *Suberea* sp. was freeze-dried, and the dry material (30 g) exhaustively extracted with MeOH/EtOAc (1:1) to yield 4.0 g of extract. This extract was redissolved in 200 mL of MeOH/H_2O (9:1) and partitioned with an equivalent volume of *n*-hexane. The resultant bioactive aqueous MeOH fraction (2.45 g) was dried under reduced pressure and subjected to reversed-phase (RP) C_{18} vacuum liquid column chromatography (40%, 45%, 50%, 55%, 60%, 65%, 70%, 80%, 90%, 100% MeOH/H_2O) to yield 10 fractions, each of 400 mL. Testing of the fractions in the whole assay panel indicated the 45% and 50% MeOH fractions to be active. These were combined and further separated by C_{18} vacuum liquid column chromatography (5−100% MeOH/H_2O). Final purification of these semipure fractions was achieved by RP HPLC (250 × 10 mm, Phenomenex Gemini C18 column, 5 μm) using a flow rate of 3 mL/min and gradient elution from 60% MeOH (+0.1% formic acid) to 100% MeOH (+0.1% formic acid) in 15 min to yield compounds **5** (5.0 mg, 0.008%); **6**/**5** (3:2 mixture; 1.2 mg, 0.002%); psammaplysin A (**7**)[14] (1.3 mg, 0.002%), $[\alpha]^{25}_D$ −48 (*c* 0.05, CH_3OH) [lit. −65 (*c* 0.05, CH_3OH)[14]]; psammaplysin B (**8**)[14] (5.4 mg, 0.009%), $[\alpha]^{25}_D$ −61.5 (*c* 0.04, CH_3OH) [lit. −60.2 (*c* 0.632, CH_3OH)[14]]; and moloka'iamine (**9**)[15] (4.0 mg, 0.006%).

*Psammaplysin I (**5**):* light yellow, amorphous powder; $[\alpha]^{25}_D$ −90 (*c* 0.35, CH_3OH); UV (PDA, CH_3OH/H_2O); λ_{max} 280 sh, 250 sh, 218 nm; IR (neat) ν_{max} 3387, 3243, 2926, 1670, 1200, 1140 cm^{-1}; ^1H (400 MHz, CD_3OD) and ^{13}C (100 MHz, CD_3OD) NMR data, see Tables 1 and 2; HRESIMS *m/z* 655.9247 $[M]^+$ (calcd for $C_{21}H_{25}{}^{81}Br_2{}^{79}BrN_3O_6$, 655.9252).

*Psammaplysin J (**6**):* 3:2 mixture with psammaplysin I (**5**); light yellow, amorphous solid; UV (PDA, CH_3OH/H_2O); λ_{max} 280 sh, 250 sh, 218 sh nm; ^1H (400 MHz, CD_3OD) and ^{13}C (100 MHz, CD_3OD) NMR data, see Tables 1 and 2.

ASSOCIATED CONTENT

Supporting Information

^1H NMR spectral data for all isolated compounds together with selected 2D NMR spectra of some of the known compounds, and ^1H and ^{13}C NMR (via HMBC for **6**) as well as selected 2D NMR spectra for compounds **1**, **5**, and **6**, together with complete unambiguous ^1H and ^{13}C NMR data for compounds **3**, **4**, and **10** in Table S1, as well as laboratory photographs of the two sponges. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

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DEDICATION

Dedicated to Dr. Gordon M. Cragg, formerly Chief, Natural Products Branch, National Cancer Institute, Frederick, Maryland, for his pioneering work on the development of natural product anticancer agents.

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