The Antibacterial ent-Eusynstyelamide B and Eusynstyelamides D, E, and F from the Arctic Bryozoan *Tegella cf. spitzbergensis*

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S *Supporting Information*

ABSTRACT: The brominated tryptophan-derived ent-eusynstyelamide B (**1**) and three new derivatives, eusynstyelamides D, E, and F (**2**−**4**), were isolated from the Arctic bryozoan *Tegella cf. spitzbergensi*s. The structures were elucidated by spectroscopic methods including 1D and 2D NMR and analysis of mass spectrometric data. The enantiomer of **1**, eusynstyelamide B, has previously been isolated from the Australian ascidian *Eusynstyela latericius*. Antimicrobial activities are here reported for **1**−**4**, with minimum inhibitory concentrations (MIC) as low as 6.25 μg/mL for **1** and **4** against *Staphylococcus aureus*. Eusynstyelamides **2** and **3** showed weak cytotoxic activity against the human melanoma A 2058 cell line.
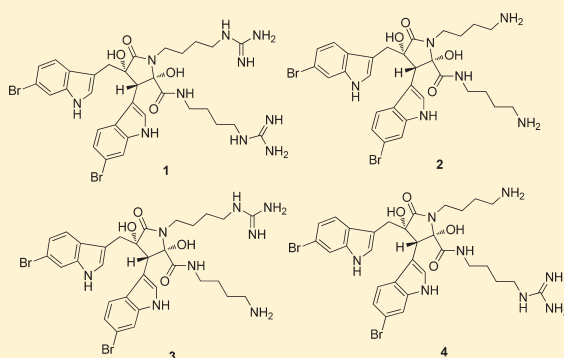
Cold-water bryozoans (moss animals, phylum Ectoprocta) have yielded a significant number of bioactive metabolites and have been the source of 35 published natural products.[1,2] In the present study, an investigation into the chemistry of the Arctic bryozoan *Tegella cf. spitzbergensis* resulted in the isolation and structural determination of ent-eusynstyelamide B (**1**) and three new derivatives, eusynstyelamides D, E, and F (**2**−**4**). Ent-eusynstyelamide B (**1**) is the enantiomer of the known brominated tryptophan metabolite eusynstyelamide B.[3] Metabolites **1**−**4** exhibited antibacterial activity. The structures were elucidated by high-resolution mass spectrometry and NMR techniques. To our knowledge, this is the first report of bioactive metabolites from a *Tegella* species.

Eusynstyelamide B was previously isolated along with its two isomers, eusynstyelamides A and C, from the Australian ascidian *Eusynstyela latericius*.[3] Neuronal nitric oxide synthase (nNOS) inhibition and modest anticancer and antibacterial activities were reported for eusynstyelamides A, B, and C, and mild inhibition of the plant regulatory enzyme pyruvate phosphate dikinase (PPDK) was reported for eusynstyelamides A and B. Eusynstyelamide,[4] which has previously been isolated from the ascidian *Eusynstyela misakiensis* in the Philippines, was reported to contain an open central motif instead of the five-membered ring and an additional hydroxy group. The structure was later amended as eusynstyelamide A.[3]

A number of other brominated tryptophan derivatives have been reported from cold-water bryozoans. The majority of these compounds originate from the bryozoan *Flustra foliacea*, which is mainly found in the North Sea, as well as in Canadian waters. These include flustramides A and B[5] and a series of flustramines,[6−8] for which bioactivity has been reported for flustramine A (muscle relaxant activity,[9] potassium-channel blocking properties,[10] and cytotoxicity[11]) and for flustramine F (antifungal activity).[8] Another series of indole-imidazole alkaloids, securamines A−G, have been isolated from the North Sea bryozoan *Securiflustra securifrons*,[12,13] but the compounds were not investigated for their bioactivities.

Examples are found in the literature of metabolites isolated from species prevalent in temperate climates that have later been reported in cold-water organisms. The alkaloid tambjamine A, which resembles the bacterial prodigiosin,[14] was originally reported in a tropical bryozoan and later discovered in the arctic bryozoan *Bugula longissima*.[1] We have recently shown that the alkaloid 3-dehydroxytubastrine originally isolated from an Australian sponge can also be found in the cold-water ascidian *Dendrodoa aggregata*.[15] These observations point to biosynthesis by microorganisms associated with the organisms rather than the invertebrate species themselves. Instances of metabolites first thought to originate from macroorganisms that are then proposed to be synthesized by symbiotic bacteria are increasing in the literature and include the anticancer bryostatins, which are secondary metabolites reported from bryozoan species.[16] Apart from differences in configuration, the structures of ent-eusynstyelamide B (**1**)

and eusynstyelamide B are identical and the compounds might therefore be synthesized by microorganisms symbiotic to both *T. spitzbergensis* and the ascidian *E. latericius*. However, it has been suggested that different strains of bacteria can produce the same metabolite, as in the case of the cytotoxic macrolide swinholide A.[17] Because **1** and eusynstyelamide B were found in different phyla, located in totally different geographical zones, biosynthesis by different bacterial strains is probable.

Ent-eusynstyelamide B (**1**)

Eusynstyelamide D (**2**)

Eusynstyelamide E (**3**)

Eusynstyelamide F (**4**)
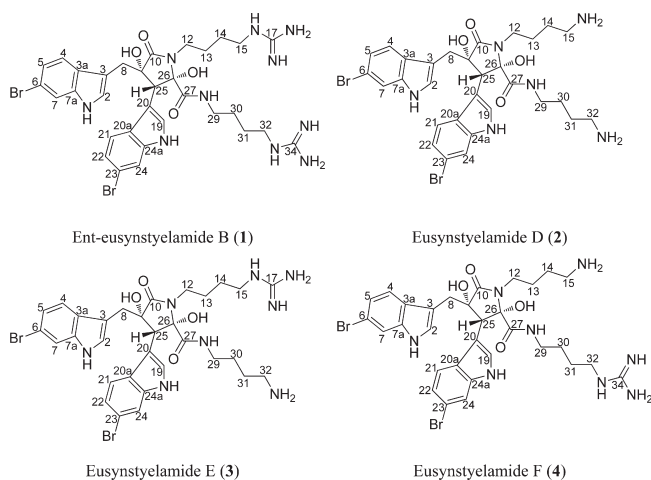
In the current isolation of **1**−**4** the CH_2Cl_2/MeOH extract of *T. spitzbergensis* was first desalted on a SPE RP-C18 column, and the dried eluate further fractionated by RP HPLC on a preparative C18 column. The major fraction was collected for further investigation. An LC-MS analysis showed the presence of four components, each with isotope clusters in a 1:2:1 ratio with peaks two mass units apart, indicative of compounds containing two bromine atoms. Further purification of the major fraction yielded four pure components, **1**−**4**, as oils.

HREIMS revealed a molecular formula of $C_{32}H_{40}Br_2N_{10}O_4$ for **1** (m/z 787.1675 for $[M + H]^+$) and 17 degrees of unsaturation. A survey of the literature based on the mass data revealed that **1** could be identical with eusynstyelamide B.[3] Comparison of the spectroscopic data measured in both CD_3OD and DMSO-d_6 (Table S1 in the Supporting Information) revealed identical structures. A NOESY spectrum further confirmed that **1** and eusynstyelamide B had the same relative configuration, 9S^*, 25R^*, 26S^*.[3] The physical data obtained for **1**−**4** were comparable to those reported in the literature for eusynstyelamide B: no optical activity at 589 nm, UV absorbances of 226 and 284 nm (indicative of indole groups), strong IR absorption around 3300−3400 cm^{-1} (consistent with OH and/or NH groups), an amide carbonyl stretch at 1677 cm^{-1}, and C−N stretching absorptions at 1205 cm^{-1}. Similarities in the NMR shift values of **1**−**4** suggested the same relative configuration for all four compounds. The similarities in the CD spectra of **1**−**4** (see the Supporting Information) supported the same configuration for all of the compounds. The CD spectra of the compounds further exhibited Cotton effects, indicating that they were not racemic. The ^{13}C shift values of **1**−**4** were assigned through gHSQC and gHMBC experiments.

A sample of eusynstyelamide B was obtained, and a CD spectrum was acquired, which exhibited maxima opposite of **1**, revealing that **1** was in fact the enantiomer of eusynstyelamide B (see the Supporting Information). As with the previous report,

Table 1. ^{13}C NMR Shifts for ent-Eusynstyelamide B (**1**) and Eusynstyelamides D (**2**), E (**3**), and F (**4**) (100 MHz, CD_3OD)

position	**1** δ_C, mult.[a]	**2** δ_C, mult.[a]	**3** δ_C, mult.[a]	**4** δ_C, mult.[a]
2	126.5, CH	126.6, CH	126.6, CH	126.4, CH
3	110.2, C	110.0, C	110.1, C	109.7, C
3a	127.9, C	127.7, C	127.7, C	127.8, C
4	121.3, CH	121.3, CH	121.4, CH	121.1, CH
5	122.6, CH	122.1, CH	122.5, CH	122.3, CH
6	115.6, C	115.5, C	115.2, C	115.0, C
7	114.5, CH	114.5, CH	114.5, CH	114.3, CH
7a	138.5, C	138.3, C	138.4, C	138.0, C
8	30.1, CH_2	30.1, CH_2	30.1, CH_2	29.8, CH_2
9	80.7, C	80.9, C	80.6, C	80.8, C
10	178.7, C	178.6, C	178.4, C	178.2, C
12	41.0, CH_2	40.9, CH_2	41.0, CH_2	40.8, CH_2
13	26.3, CH_2	25.8, CH_2	26.3, CH_2	25.5, CH_2
14	26.8, CH_2	25.3, CH_2	26.8, CH_2	26.3, CH_2
15	41.8, CH_2	40.0, CH_2	41.9, CH_2	39.9, CH_2
17	158.6, C		158.3, C	
19	128.0, CH	128.2, CH	128.1, CH	127.8, CH
20	106.8, C	106.6, C	106.6, C	106.2, C
20a	128.9, C	128.7, C	128.6, C	128.4, C
21	121.3, CH	121.3, CH	121.4, CH	121.1, CH
22	122.5, CH	122.5, CH	122.4, CH	122.3, CH
23	115.6, C	115.3, C	115.2, C	115.0, C
24	114.7, CH	114.7, CH	114.8, CH	114.5, CH
24a	137.2, C	137.9, C	137.6, C	137.3, C
25	45.9, CH	46.0, CH	45.9, CH	45.6, CH
26	92.3, C	92.3, C	92.1, C	92.9, C
27	170.2, C	171.3, C	170.8, C	170.7, C
29	39.8, CH_2	39.6, CH_2	39.6, CH_2	39.7, CH_2
30	26.5, CH_2	25.3, CH_2	25.3, CH_2	26.3, CH_2
31	26.5, CH_2	26.4, CH_2	26.4, CH_2	26.3, CH_2
32	41.6, CH_2	39.8, CH_2	39.9, CH_2	41.4, CH_2
34	156.9, C[b]			

[a] Carbon assignments obtained from gHSQC and gHMBC experiments. [b] Only observed in DMSO-d_6.

determination of the absolute configuration of **1** was not successful.[3]

Accurate mass measurements of **2** provided a molecular formula of $C_{30}H_{36}Br_2N_6O_4$ (m/z 703.1242 for $[M + H]^+$). A mass difference of 84 was seen between **2** and **1**, corresponding to a loss of ($-C_2N_4H_4$), resulting in two terminal amino groups instead of the two guanidine groups of **1**. Tables 2 and 3 show the carbon and proton shifts of **2** in CD_3OD. The NMR data of **1** in CD_3OD are also listed for comparison. The chemical shifts were virtually identical, showing that **2** was indeed a derivative of **1**. As expected, the guanidine carbons (C-17 and C-34) were not observed in **2**.

HREIMS analysis of **3** and **4** yielded a molecular formula of $C_{31}H_{38}Br_2N_8O_4$ (m/z 745.1463 for $[M + H]^+$) for both compounds. This corresponded to a mass difference of 42 ($-CN_2H_2$) between these two metabolites and **1**, indicating that **3** and **4** each contained one terminal guanidine group instead of two. The carbon and proton shifts of **3** and **4** are also listed in

Table 2. ^{1}H NMR Shifts for ent-Eusynstyelamide B (1) and Eusynstyelamides D (2), E (3), and F (4) (400 MHz, CD$_3$OD)

	1	2	3	4
position	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)
2	6.89, s	6.89, s	6.88, s	6.92, s
4	6.90, d (8.5)	6.86, d (8.5)	6.89, d (8.5)	6.82, d (8.5)
5	6.69, dd (8.5, 1.7)	6.67, dd (8.5, 1.7)	6.68, dd (8.5, 1.7)	6.65, dd (8.5, 1.7)
7	7.41, d (1.7)	7.41, d (1.7)	7.41, d (1.7)	7.43, d (1.7)
8	3.40, d (14.0)	3.40, d (14.0)	3.40, d (14.0)	3.39, d (14.0)
	2.96, d (14.0)	2.96, d (14.0)	2.96, d (14.0)	2.96, d (14.0)
12	3.37, m	3.38, m	3.37, m	3.40, m
	3.18, m	3.14, m	3.17, m	3.13, m
13	1.65, m	1.62, m	1.65, m	1.64, m
	1.60, m		1.60, m	
14	1.55, m	1.16, m	1.55, m	1.13, m
15	3.15, t (7.0)	2.91, m	3.14, t (7.0)	2.92, m
19	7.75, s	7.74, s	7.75, s	7.74, s
21	6.76, d (8.5)	6.72, d (8.5)	6.75, d (8.5)	6.69, d (8.5)
22	6.94, dd (8.5, 1.7)	6.94, dd (8.5, 1.7)	6.94, dd (8.5, 1.7)	6.91, dd (8.5, 1.7)
24	7.53, d (1.7)	7.54, d (1.7)	7.54, d (1.7)	7.55, d (1.7)
25	3.97, s	3.93, s	3.96, s	3.94, s
29	2.98, m	2.98, m	2.98, m	2.98, m
	2.87, m	2.81, m	2.83, m	2.84, m
30	1.10, m	1.12, m	1.15, m	1.11, m
31	1.09, m	1.10, m	1.10, m	1.10, m
	1.01, m	1.01, m	1.03, m	1.01, m
32	2.83, m	2.58, m	2.58, t (7.0)	2.81, m

Table 3. Antibacterial Activities of ent-Eusynstyelamide B (1) and Eusynstyelamides D (2), E (3), and F (4)

	MIC (μg/mL)			
	1	2	3	4
S. aureus	6.25	12.5	12.5	6.25
C. glutamicum	12.5	12.5	12.5	6.25
MRSA	20	20	20	>50
E. coli	12.5	12.5	12.5	12.5
P. aeruginosa	25	25	25	12.5

Tables 1 and 2, showing that the chemical shifts were comparable and that 3 and 4 were new derivatives of 1. The guanidine carbon C-17 was observed in 3, indicating that the guanidine group at C-32 was missing, while the absence of C-17 in 4 pointed to loss of a guanidine group at this position. The chemical shifts for H-15 in 4 and position H-32 in 3 were similarly shielded compared to 1 and as discussed for 2.

The eusynstyelamides are biogenetically derived from arginine and tryptophan and are similar to other natural products such as anchinopeptolide,[18] barettin,[19] and the leptoclinidamines.[20] Tapiolas et al. suggest a biosynthetic route for the eusynstyelamides involving the dimerization of two modified dipeptides formed from an α-keto acid derived from tryptophan and agmatine, a decarboxylation product of arginine.[3] Recently, eusynstyelamide A was synthesized from 6-bromoindole, methyl glycidate, and Boc-protected agmatine.[21]

The possibility that 2−4 are artifacts formed by acidic degradation of 1 during extraction was tested by treating 1 dissolved in H$_2$O with TFA and incubating for two weeks before an LC-MS analysis

was performed. Analysis of the data showed that 1 had not degraded into metabolites 2−4, suggesting that 2−4 were indeed natural products and not artifacts of 1.

Table 3 shows the antibacterial activity of 1−4. The test bacteria were *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Corynebacterium glutamicum*, and methicillin-resistant *Staphylococcus aureus* (MRSA). Previously, eusynstyelamide B was found to display mild antibacterial activity against *S. aureus* (IC$_{50}$ 6.5 mM), and no activity was reported against *E. coli*.[3] In the present study, 1 was found to have minimum inhibitory concentrations (MIC) of 6.25 μg/mL against *S. aureus* and 12.5 μg/mL against *E. coli*. The data indicated that ent-eusynstyelamide B (1) possessed more potent antibacterial activity than eusynstyelamide B. Eusynstyelamides 1−4 were generally more active against Gram-positive bacteria than Gram-negative bacteria.

Weak antifungal activity against *Candida albicans* was observed with an MIC of 100 μg/mL for 1 and 3 and an MIC of 50 μg/mL for 2 and 4. Weak activity against the melanoma cell line A-2058 was observed for 2 (IC$_{50}$ 57 μM) and 3 (IC$_{50}$ 114.3 μM).

In the present study, the antibacterial metabolite ent-eusynstyelamide B (1) was for the first time isolated from the Arctic bryozoan *T. spitzbergensis*, and three new antibacterial derivatives of 1, eusynstyelamides D, E, and F (2−4), were added to the eusynstyelamide family.

■ EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on an AA-10R automatic polarimeter from Optical Activity Ltd. UV spectra were recorded on a SPEKOL 2000 spectrophotometer

from Analytik Jena, AG. CD spectra were recorded using a Jasco J-810 spectropolarimeter (Jasco International Co., Ltd.). Infrared spectra were obtained on a Varian 7000e FT-IR spectrometer. All NMR data were recorded on a Varian Unity INOVA 400 HMz spectrometer in CD_3OD and DMSO-d_6. The spectra were referenced to the residual ^1H and ^{13}C solvent peaks. Mass spectra were measured using the Thermo Scientific Accela HPLC- LTQ-Ion Trap-Orbitrap Discovery system. Solid-phase extraction was carried out on a Chromabond C18 SPE cartridge from Machrey-Nagel. HPLC was performed on a 1100 series instrument with a binary pump and a photodiode array detector from Agilent Technologies. A C18 column (250×10 mm, 5 μm) from Phenomenex and an Ace 5 C18 HL column (250×10 mm, 5 μm) from Hichrom Ltd. were used for purification.

Animal Material. Specimens of *Tegella cf. spitzbergensis* (Bidenkap, 1897) (phylum Ectoprocta, class Gymnolaemata, order Cheilostomata, family Calloporidae) were collected around Bear Island in the North Atlantic ($74°14.534'$ N, $19°2.114'$ E) using an Agassiz trawl at 59 m depth on May 16, 2007, and kept frozen until used. A voucher specimen (M09JAN0059-7) is kept at Marbank, the national marine biobank in Tromsø, Norway.

Extraction and Isolation. Lyophilized material of the bryozoan was extracted with CH_2Cl_2/MeOH (1:1). The dried sample (175 mg) was dissolved in 10% MeOH and loaded on a SPE cartridge. Retained material was eluted with 100% MeOH, and the cartridge was washed repeatedly with 100% CH_3CN. The MeOH and CH_3CN fractions were combined and dried in vacuo (164 mg) before separation on a RP-HPLC column using a gradient of 10% to 100% MeOH/0.1% formic acid in H_2O. A peak that eluted with 35% MeOH/0.1% formic acid/65% H_2O was collected and repurified employing isocratic elution with 60% CH_3CN/65% H_2O/0.1% TFA. Four peaks eluting at 18 min (**2**), (1.1 mg), 21 min (**3**) (1.2 mg), 22 min (**4**) (2.9 mg), and 27 min (**1**) (2.6 mg) were collected for structural determination.

ent-Eusynstyelamide B (1): pale yellow oil, $[\alpha]^{22}_D \pm 0.0$ (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 226 (4.13), 284 (3.48), 295 (3.42), CD (0.13 M, MeOH) λ_{max} ($\Delta\varepsilon$) 302 (12.8), 292 (9.0), 228 (36.7), 217 (−1.6), 208 (28.9); IR ν_{max} 3380, 1677, 1205 cm^{-1}; HRESIMS m/z 787.1675 [M + H]$^+$ (calcd for $C_{32}H_{41}Br_2N_{10}O_4$, 787.1676).

Eusynstyelamide D (2): pale yellow oil, $[\alpha]^{22}_D \pm 0.0$ (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 226 (4.08), 284 (3.43), 295 (3.37); CD (0.16 M, MeOH) λ_{max} ($\Delta\varepsilon$) 302 (10.7), 291 (8.4), 228 (32.4), 216 (−8.0), 208 (16.7); IR ν_{max} 3332, 1677, 1205 cm^{-1}; HRESIMS m/z 703.1242 [M + H]$^+$ (calcd for $C_{30}H_{37}Br_2N_6O_4$, 703.1247).

Eusynstyelamide E (3): pale yellow oil, $[\alpha]^{22}_D \pm 0.0$ (*c* 0.05, MeOH); UV (PDA, MeOH) λ_{max} (log ε) 226 (4.10), 284 (3.45), 295 (3.39); CD (0.17 M, MeOH) λ_{max} ($\Delta\varepsilon$) 302 (11.7), 291 (8.0), 227 (34.8), 215 (−6.8), 207 (15.4); IR ν_{max} 3380, 1677, 1205 cm^{-1}; HRESIMS m/z 745.1463 [M + H]$^+$ (calcd for $C_{31}H_{39}Br_2N_8O_4$, 745.1470).

Eusynstyelamide F (4): pale yellow oil, $[\alpha]^{22}_D \pm 0.0$ (*c* 0.05, MeOH); UV (PDA, MeOH) λ_{max} (log ε) 226 (4.10), 284 (3.45), 295 (3.39); CD (0.16 M, MeOH) λ_{max} ($\Delta\varepsilon$) 301 (13.3), 292 (9.1), 227 (40.0), 216 (−10.0), 207 (22.5); IR ν_{max} 3348, 1676, 1205 cm^{-1}; HRESIMS m/z 745.1463 [M + H]$^+$ (calcd for $C_{31}H_{39}Br_2N_8O_4$, 745.1470).

Eusynstyelamide B: CD (0.17 M, MeOH) λ_{max} ($\Delta\varepsilon$) 302 (−12.3), 292 (7.7), 227 (−35.1), 217 (2.6), 206 (−26.2).

Antibacterial Assay. Test strains used were *Staphylococcus aureus* (ATTC 9144), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATTC 27853), *Corynebacterium glutamicum* (ATTC 13032), and Methicillin-resistant *Staphylococcus aureus* (ATCC 33591). All isolates were grown at 37 °C in Mueller Hinton broth (MHB; Difco Laboratories, Detroit, MI, USA). Bacterial growth was continuously monitored with an Envision plate reader (Perkin-Elmer). The test was performed in 96-well Nunc microtiter plates, in which 50 μL of test fractions dissolved

in H_2O was incubated with 50 μL of a suspension of an actively growing (log phase) culture of bacteria diluted to a starting concentration of approximately 5×10^5 cells per well. The antimicrobial peptide cecropin B (25 μM) was used as a positive control. The minimum inhibitory concentration was defined as the minimum concentration resulting in no change in optical density after incubation for 24 h at 37 °C. Compounds were tested in duplicates at concentrations ranging from 2 to 100 μg/mL.

Antifungal Assay. *Candida albicans* (ATCC 10231) was cultivated in potato dextrose agar with 2% glucose at room temperature. Fungal spores were dissolved in potato dextrose broth (Difco), and the cell concentration was determined and adjusted after counting in a Bürker chamber. An aliquot of 50 μL of fungal spores (final concentration 2×10^5 spores/mL) was inoculated at 37 °C in 96-well Nunc microtiter plates along with 50 μL of the test compounds, which were dissolved in Milli-Q water. Synthetic cecropin B (6.25 μM) was used as a positive control. MIC was defined as the minimum concentration resulting in no visible growth after 48 h of incubation and was determined microscopically. All compounds were tested in duplicates at concentrations ranging from 2 to 100 μg/mL.

Anticancer Assay. Cell viability assays were performed using the human melanoma cell line A-2058. Briefly, exponentially growing cells were seeded into 96-well microplates. After 24 h of incubation at 37 °C, the cells were exposed to the test compounds for 72 h. Then the living cells were assayed by the addition of 10 μL of CellTiter96 reagent (Promega). The plates were incubated for 1 h for the color development, and the absorbance at 485 nm was measured in a DTX 880 multimode detector from Beckman Coulter. All compounds were tested in duplicates at concentrations ranging from 2 to 100 μg/mL for determination of IC_{50} values.

ASSOCIATED CONTENT

Ⓢ Supporting Information. 1D and 2D NMR data for eusynstyelamides B, D, E, and F (**1−4**) in both CD_3OD and DMSO-d_6, key gHMBC and gCOSY correlations for **1**, and 3D representation of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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