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Eusynstyelamides A, B, and C, nNOS Inhibitors, from the Ascidian *Eusynstyela latericius*

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Eusynstyelamides A–C (**1**–**3**) were isolated from the Great Barrier Reef ascidian *Eusynstyela latericius*, together with the known metabolites homarine and trigonelline. The structures of **1**–**3**, with relative configurations, were elucidated by interpretation of their spectroscopic data (NMR, MS, UV, IR, and CD). The NMR data of **1** were found to be virtually identical to that reported for eusynstyelamide (**4**), isolated from *E. misakiensis*, indicating that a revision of the structure of **4** is needed. Eusynstyelamides A–C exhibited inhibitory activity against neuronal nitric oxide synthase (nNOS), with IC₅₀ values of 41.7, 4.3, and 5.8 μ M, respectively, whereas they were found to be nontoxic toward the three human tumor cell lines MCF-7 (breast), SF-268 (CNS), and H-460 (lung). Compounds **1** and **2** displayed mild inhibitory activity toward *Staphylococcus aureus* (IC₅₀ 5.6 and 6.5 mM, respectively) and mild inhibitory activity toward the C₄ plant regulatory enzyme pyruvate phosphate dikinase (PPDK) (IC₅₀ values of 19 and 20 mM, respectively).

Nitric oxide (NO) acts as an important cellular messenger involved in a large number of physiological and pathological process.^{1–4} In the brain NO plays an important multifunctional role in the modulation of many neurotransmitters,² whereas the overproduction of NO has been associated with neuropathological disorders, such as stroke, Alzheimer's disease, Parkinson's disease, AIDS, and dementia.⁵ Thus nNOS (neuronal nitric oxide synthase) represents an important therapeutic target for inhibitors. As part of a screening program to detect inhibitors of nNOS, the organic extract of the colonial encrusting ascidian, *Eusynstyela latericius*, collected from the central region of the Great Barrier Reef (GBR), was found to have strong nNOS inhibitory activity.

There have only been two previous reports of chemistry from the genus *Eusynstyela*. Eusynstyelamide (**4**), an optically active modified tryptophan-arginine dipeptide dimer, was reported, without specified configuration, by Ireland and co-workers⁶ from *E. misakiensis* in 1994, and the styelsamines A–D, pyridoacridine alkaloids exemplified by **5**, were reported from *E. latericius* in 1998.⁷ The styelsamines were found to be mildly cytotoxic (IC₅₀ 1.6–89 μ M) toward the human colon tumor cell line HCT-116, whereas eusynstyelamide was found to be less active (IC₅₀ 100 μ M) toward the same cell line.

In the current study, bioassay-guided fractionation of a large-scale extract of *E. latericius* led to the isolation of three metabolites, **1**–**3**, all of which exhibited nNOS inhibitory activity. The spectral data of eusynstyelamide A (**1**) was almost identical to that reported for eusynstyelamide (**4**), although **1** and **4** possessed opposite specific rotations. We believe that the antipode of **1** better represents the compound originally isolated as eusynstyelamide.⁶ The known compounds homarine and trigonelline⁸ were also isolated. In the remainder of this publication, the isolation and structure elucidation of **1**–**3** and their associated biological activities are discussed.

Results and Discussion

The CH₂Cl₂/MeOH extract of the ascidian tissue was partitioned between water and hexane. The dried water-soluble extract was

desalted with cold MeOH, and the MeOH-soluble portion was fractionated, using RP-C₁₈ vacuum liquid chromatography (VLC), to give nine fractions, four of which exhibited nNOS inhibitory activity. The ¹H and ¹³C NMR spectra of the active fractions were very similar, indicating them to be mixtures of similar compounds in varying proportions. The positive mode ESIFTMS of these four fractions were essentially identical, each having an isotope cluster at *m/z* 787/789/791 (1:3:1), indicative of molecules containing two bromine atoms. Separation of a small portion of one of these fractions using RP-C₁₈ HPLC afforded three new compounds, **1**–**3**, as oils.

The molecular formula of **1** was established as C₃₂H₄₀Br₂N₁₀O₄ by ESIFTMS and showed the molecule to have 17 degrees of unsaturation. The IR spectrum in **1** displayed a strong broad absorption at 3464 cm^{−1} for OH and/or NH functionalities and absorption bands at 1680, 1659, 1640, and 1205 cm^{−1} for amide and/or imine groups. From the ¹³C NMR spectral data of **1**, it was possible to discern the presence of 32 carbon atoms: 20 sp², two quaternary sp³, one sp³ methine, and nine methylene sp³ carbons. Most of the signals in the ¹³C NMR spectrum appeared to be paired, pointing toward **1** possibly being dimeric. Further analysis of the NMR data of **1**, measured in both CD₃OD and DMSO-*d*₆, revealed the presence of two disubstituted bromoindole residues, a deduction supported by UV absorption maxima at 234 and 285 nm. The gHMBC NMR data of **1** confirmed the positions of the bromine atoms at C-6 and C-23 and allowed each indole unit to be extended and joined together as shown in partial structure **I** (Figure 1).

The presence of two guanidine groups in the molecule was indicated by comparison of the carbon chemical shifts measured in DMSO-*d*₆ for C-17 (δ_C 156.8) and C-34 (δ_C 156.6) with those reported in the literature,^{9,10} exchangeable proton signals at δ_H 7.64 (br, t) and 7.47 (br, t), and two very broad baseline signals centered on δ_H 7.32 and 6.92 [−C(=NH)NH₂]. Further analysis of the gCOSY and gHMBC spectra allowed the two guanidine groups to be extended to include two C₄H₈ aliphatic chains: subunits **II** and **III** (Figure 1). Partial structures **I**, **II**, and **III** could be connected through the two amides as follows. The NH-28 resonance was clearly present as a triplet at δ_H 8.19 (*J* = 6.1 Hz) in the ¹H NMR spectrum measured in DMSO-*d*₆ and showed gCOSY correlations to both protons at C-29. The gHMBCs from NH-28 to both C-27 and C-29 confirmed that N-28 was connected to C-29. A gHMBC from both H₂-12 protons to C-10 allowed the connectivity between N-11 and C-12 to be established. There were no other exchangeable proton resonances observed in the ¹H NMR spectrum measured in DMSO-*d*₆, indicating the second amide to be tertiary. Sixteen of

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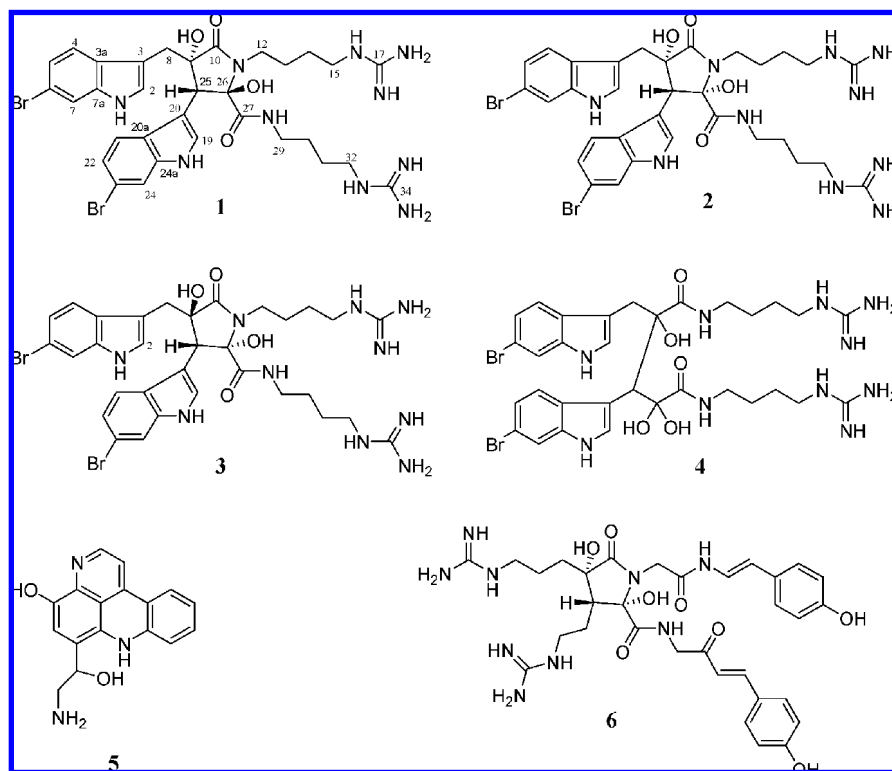
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Chart 1



the 17 degrees of unsaturation implied by the molecular formula were thus accounted for, and as there were no additional multiple bonds, **1** had to contain another ring. Further analysis of the gHMBC spectra of **1** in both CD₃OD and DMSO-*d*₆ showed correlations between H₂-12 (δ_{H} 3.27 in CD₃OD and δ_{H} 3.08 in DMSO-*d*₆) and C-26 (δ_{C} 93.7 in CD₃OD and δ_{C} 91.8 in DMSO-*d*₆), which established the connectivity between N-11 and C-26 and so enabled the fifth and final ring within **1** to be deduced and its planar structure finalized.

Comparison of the NMR, MS, UV, and IR data for **1** with that reported for eusynstyelamide (**4**) showed them to be virtually identical; there were however some anomalies between the published NMR data (shown in Table 1 for comparison) and the structure proposed for **4**.⁶ Structure **4** as it stands does not account for the deshielded nature of the signals for H₂-13 and H₂-14 (DMSO-*d*₆: δ_{H} 1.36 and 1.39 in **1**; δ_{H} 1.38 and 1.38 in **4**) compared to the signals for H₂-30 and H₂-31 (DMSO-*d*₆: δ_{H} 1.09/1.04 and 0.97/0.93 in **1**; δ_{H} 1.10/0.93 and 1.11/0.93 in **4**). Furthermore, C-26 was reported to exist as an α -keto amide hydrate in both protic and aprotic solvents (CD₃OD and DMSO-*d*₆) and the observed two-bromine cluster at *m/z* 787/789/791 in the MS to be an [M + H - H₂O]⁺ ion. Here it is contended that the *m/z* 787/789/791 ion is in fact the [M + H]⁺ ion, as indicated earlier. The small but key gHMBC correlations between the H₂-12 signals and the resonance for C-26 can only arise if both C-12 and C-26

bond with N-11. In the structure proposed for **4** these would need to be six-bond correlations, rather than the much more plausible three-bond correlations seen in **1**. Furthermore, the structure proposed for **1** better explains the downfield shifts of the H₂-13 and H₂-14 signals compared to the H₂-30 and H₂-31 signals and also accounts for the complete absence of an H-11 proton signal as noted by Swersey et al.⁶

To avoid any confusion in the literature, we suggest **1** be called eusynstyelamide A so as to clearly distinguish it from eusynstyelamide. The relative configuration of **1** will be discussed later.

Accurate mass measurement of **2** indicated it to have the molecular formula C₃₂H₄₀Br₂N₁₀O₄, identical to that of **1**, showing **1** and **2** to be isomeric. The ¹H NMR data of **2** (Table 2) were very similar to that of **1**, but there were some clear differences, in particular, the deshielded nature of the H-25 signal in **2** (δ_{H} 4.02, DMSO-*d*₆) compared to the equivalent resonance in **1** (δ_{H} 3.57, DMSO-*d*₆) and the shielded signal for H-21. Likewise, the ¹³C NMR data of **2** (Table 2) were similar to those of **1**, although again there were some clear differences, especially for the resonances associated with C-9, C-10, C-25, and C-26. These observed differences in the ¹H and ¹³C NMR data of **1** and **2** were mainly associated with the three asymmetric centers C-9, C-25, and C-26, suggesting the most likely difference between **1** and **2** to be stereo- rather than regiochemical. Analysis of the gCOSY, gHSQC, and gHMBC data of **2** (Table 2) revealed all of the expected correlations for the regiochemistry as shown; clearly **2** was a stereoisomer of **1**, for which the name eusynstyelamide B is proposed. As for **1**, its relative configuration will be discussed later.

Mass spectral analysis of **3** showed it to have the molecular formula C₃₂H₄₀Br₂N₁₀O₄, and therefore it was isomeric with both **1** and **2**. The UV and IR data of **3** were virtually identical to those of **1** and **2**. The NMR data of **3** (Table 2) were also very similar to those of **1** and **2**, and differences, such as the deshielded nature of H-25 (δ_{H} 4.24 in **3** compared to δ_{H} 3.57 in **1**, DMSO-*d*₆), were associated with centers C-9, C-10, C-25, and C-26. Analyses of the gCOSY, gHSQC, and gHMBC spectra of **3** in both CD₃OD and DMSO-*d*₆ (Table 2) showed the expected correlations for it to have the same regiochemistry as in **1** and **2**. These data together

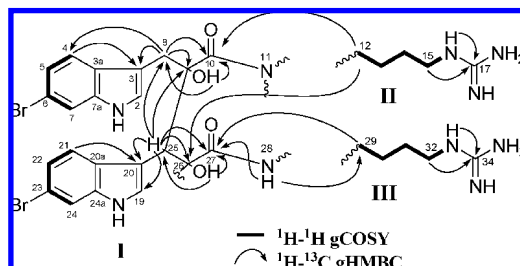


Figure 1. Partial structures **I**, **II**, and **III** of eusynstyelamide A (**1**), showing selective gCOSY and gHMBC correlations.

Table 1. NMR Data for Eusynstyelamide A (**1**)^a and Eusynstyelamide (**4**)^b

pos.	eusynstyelamide A (1)			eusynstyelamide A (1)			eusynstyelamide (4)	
	CD ₃ OD δ _C , mult.	δ _H (J in Hz)	gHMBC ^c	DMSO- <i>d</i> ₆ δ _C , mult.	δ _H (J in Hz)	gHMBC ^c	DMSO- <i>d</i> ₆ δ _C , mult.	δ _H (J in Hz)
1NH								
2	127.1, CH	6.92, s	3, 3a, 7a	125.9, CH	11.04, br s	2, 3, 3a, 7a	126.0, CH	11.2, br s
3	110.4, qC			109.0, qC	6.79, br s	3, 3a, 7a	108.3, qC	6.83, br s
3a	128.3, qC			126.8, qC			126.9, qC	
4	121.4, CH	6.83, d (8.5)	3, 5, 6, 7a	120.5, CH	7.04, d (8.5)	3, 6, 7a	120.7, CH	7.00, d (8.5)
5	122.8, CH	6.66, dd (8.5, 1.7)	3a, 6, 7	120.56, CH	6.78, dd (8.5, 1.8)	3a, 6	120.8, CH	6.75, dd (8.2, 1.7)
6	115.7, qC			113.3, qC			113.4, qC	
7	114.78, CH	7.41, d	3a, 5, 6	113.4, CH	7.47, d (1.8)	3a, 5, 6	113.6, CH	7.47, d (2.1)
7a	138.5, qC			136.7, qC			136.7, qC	
8	29.3, CH ₂	3.46, d (14.4) 2.99, d (14.4)	2, 3, 3a, 9, 10, 25 2, 3, 3a, 9, 10, 25	28.3, CH ₂	3.24, d (14.5) 2.68, d (14.5)	2, 3, 9, 10, 25 2, 3, 9, 10, 25	28.5, CH ₂	3.20, d (14) 2.65, d (14)
9	81.6, qC			78.9, qC	6.86, s, OH	3, 8, 9	78.9, qC	OH not observed
10	177.2, qC			174.0, qC			174.1, qC	
12	39.5, CH ₂	3.27, m	9, 10, 13, 26	37.7, CH ₂	3.08, m	10	38.1, CH ₂	3.15, m 3.12, m
13	26.4, CH ₂	1.51, m	12, 14, 15	24.8, CH ₂	1.36, m		26.0, CH ₂	1.38, m
14	27.2, CH ₂	1.56, m	12, 13, 15	25.7, CH ₂	1.39, m		25.12, CH ₂	1.38, m
15	42.0, CH ₂	3.23, m 3.16, m	14, 17	40.3, CH ₂	3.04, m 3.08, m 7.64, br t	14, 17	40.4, CH ₂	3.05, m 7.89, br s
16NH						15 ^d		
17	158.6, qC			156.8, qC			157.0, qC	
18NH								
19	126.8, CH	7.64, s	20, 20a, 24a	125.4, CH	11.14, br s	19, 20, 24a	125.6, CH	11.4, br s
20	107.0, qC			105.5, qC	7.44, br s	20, 20a, 27a	105.4, qC	7.40, br s
20a	129.1, qC			127.8, qC			127.9, qC	
21	122.3, CH	7.23, d (8.5)	20, 23, 24a	121.0, CH	7.32, d (8.5)	20, 23, 24a	121.3, CH	7.30, d (9)
22	122.9, CH	7.03, dd (8.5, 1.8)	20a, 23, 24	120.63, CH	6.98, dd (8.5, 1.4)	20a, 23	120.8, CH	6.94, dd (8, 1.5)
23	115.9, qC			113.3, qC			113.4, qC	
24	114.82, CH	7.55, d (1.8)	20a, 22, 23	113.4, CH	7.56, d (1.4)	20a, 23, 22	113.6, CH	7.56, d (1.5)
24a	137.8, qC			135.9, qC			135.9, qC	
25	49.8, CH	3.59, s	8, 9, 19, 20, 20a, 26, 27	47.3, CH ₂	3.57, s		47.4, CH	3.59, s
26	93.7, qC			91.8, qC	7.14, s, OH	26, 27	91.9, qC	7.14, br s, OH
27	173.4, qC			171.6, qC			171.6, qC	
28NH								
29	39.7, CH ₂	3.15, m 2.78, ddd (13.4, 6.7, 6.7)	26, 27, 30	38.1, CH ₂	8.19, br t (6.1) 2.94, m 2.77, m	27, 29, 29 ^d 27, 30	38.4, CH ₂	8.17, dd (2, 2) 2.95, m 2.80, m
30	26.8, CH ₂	1.09, m 1.04, m	29, 31, 32	24.9, CH ₂	1.09, m 1.04, m	29, 31	25.34, CH ₂	1.10, m 0.93, m
31	26.3, CH ₂	0.93, m	29, 30, 32	25.0, CH ₂	0.97, m 0.93, m	30, 32	25.32, CH ₂	1.11, m 0.93, m
32	41.9, CH ₂	2.88, t (7.4)	30, 31, 34	40.1, CH ₂	2.89, m 7.47, br t (5.9)	31, 34	40.2, CH ₂	2.94, m 7.80, br s
33NH						32 ^d		
34	158.5, qC			156.6, qC			156.9, qC	
C(=NH)NH ₂					7.32, br 6.92, br			

^a Measured at 150/600 MHz. ^b Shown for comparison purposes, measured at 125/500 MHz. ^c HMBC correlations are from proton(s) stated to the indicated carbon. ^d COSY correlation between NH and protons on stated carbon.

with all of the other physical and spectroscopic data recorded for **3** were consistent with it being a stereoisomer of **1** and **2**, for which the name eusynstyelamide C is proposed.

The relative configuration around the pyrrolidone ring present in **1–3** was deduced from selective 1D-gNOESY experiments. In **1**, NOEs were observed between H-25 and C-26-OH, H-21 and H-25, and C-9-OH and H-19, indicating H-25 and C-26-OH to be *cis* and both to be *trans* to C-9-OH. Thus the chiral centers were assigned as 9*S**, 25*R**, 26*R** (Supporting Information). For **2**, NOEs were observed between H-19 and both of the OH protons at C-9 and C-26 and between H-25 and both H-2 and H-21, indicating both OH groups to be *cis* to each other and on the opposite side of the pyrrolidone ring to H-25. The stereocenters were thus assigned as 9*R**, 25*S**, 26*R**. In **3**, NOEs were observed between H-25 and C-9-OH, H-21, and N-28-H, between C-9-OH and H-2, and between C-26-OH and H-19. These data established H-25 and C-9-OH to be *cis* to each other and *trans* to C-26-OH, and thus the centers were assigned as 9*S**, 25*S**, 26*R**.

The specific rotations of eusynstyelamides A–C (**1–3**) measured at 589 nm were determined to be −29, ±0, and +17, respectively. The CD spectrum of eusynstyelamide B (**2**) exhibited a negative Cotton effect, indicating it not to be racemic, whereas the CD spectra of **1** and **3** exhibited positive Cotton effects (Supporting

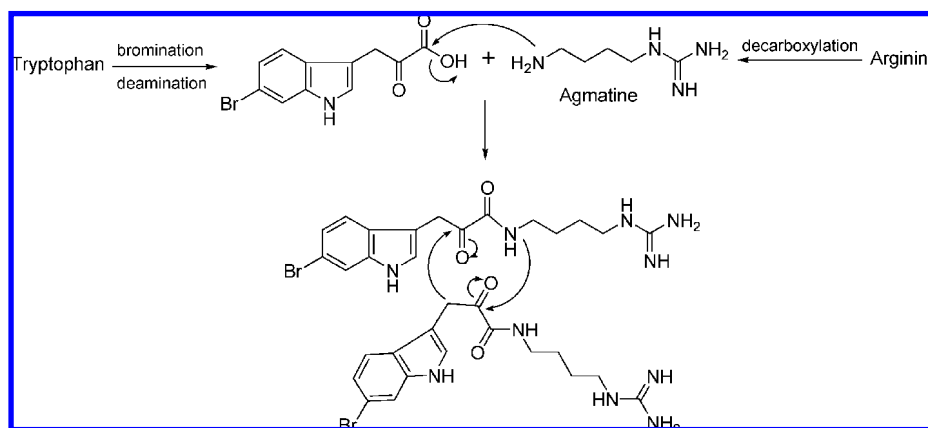
Information). Attempts to determine the absolute configuration of **1–3** from their CD spectra, the octant rule, and the exciton chirality method were not conclusive. The specific rotation of **4** measured at 405 nm was reported to be +125.6 (c 0.226, MeOH), suggesting that it has the opposite absolute configuration to that of **1**. A larger magnitude for the specific rotation of **4** as compared to **1** would be expected due to the shorter wavelength of light used in the measurement. The possibility that **4** could be the fourth possible isomer in the series (9*R**, 25*R**, 26*R**), which would have H-25 and the OH groups at C-9 and C-26 on the same side of the pyrrolidone ring, is very unlikely due to the almost identical NMR spectra of **1** and **4**. As can be seen with **1–3**, the change in the relative configuration around the pyrrolidone ring has a significant effect on the chemical shifts of H-21, H-25, C-9-OH, and C-26-OH, and if **4** possessed a different relative configuration compared to **1**, a significant difference would be expected in the chemical shifts for these protons.

There was no evidence in the NMR spectra of the crude extract or subsequent fractions for the fourth possible isomer in the series. Biogenetically the eusynstyelamides most likely originate from the dimerization of two modified dipeptides, formed from an α-keto acid, derived from tryptophan, and agmatine, derived from the decarboxylation of arginine, as in Figure 2.

Table 2. ^{13}C and ^1H NMR Data (600/175 Hz, $\text{DMSO}-d_6$) for Eusynstyelamides B (2) and C (3)

pos.	eusynstyelamide B (2)			eusynstyelamide C (3)		
	δ_{C} , mult. ^a	δ_{H} (J in Hz)	gHMBC ^b	δ_{C} , mult. ^a	δ_{H} (J in Hz)	gHMBC ^b
1NH		11.00, s	2, 3, 7a		10.85, s	2, 3, 3a, 7a
2	125.4, CH	6.87, d (1.9)	3, 3a, 7a	125.2, CH	6.94, br s	3, 3a, 7a
3	108.9, qC			109.0, qC		
3a	126.4, qC			127.4, qC		
4	120.3, CH	7.01, d (8.5)	3, 6, 7a	120.4, CH	7.18, d (8.4)	3, 4a, 6
5	120.5, CH	6.77, dd (8.5, 1.7)	3a, 6	120.2, CH	6.96, dd (8.4, 1.7)	3a, 6
6	113.8, qC			112.8, qC		
7	113.3, CH	7.46, d (1.7)	3a, 5, 6	113.1, CH	7.42, d (1.7)	3a, 4, 6
7a	136.3, qC			136.4, qC		
8	29.8, CH ₂	3.24, d (15.1) 2.76, d (15.1)	2, 3, 9, 10, 25	31.0, CH ₂	3.25, d (15.1) 2.66, d (15.1)	2, 3, 3a, 10, 26
9	78.1, qC	6.23, s, OH	8, 9, 10, 25	79.1, qC	5.28, s, OH	8, 9, 10, 25
10	175.1, qC			175.5, qC		
12	39.5, CH ₂	3.21, m 2.91, m	13, 26	40.0, CH ₂	3.22, m 2.91, m	10, 13, 26
13	24.9, CH ₂	1.47, m	12, 14	25.0, CH ₂	1.54, m	12, 14
14	25.7, CH ₂	1.38, m	13, 15	26.0, CH ₂	1.42, m	13, 15
15	40.3, CH ₂	3.04, m	14, 17	40.3, CH ₂	3.07, m	14, 17
16NH		7.62, m	15 ^c		7.73, m	15 ^c
17	156.7, qC			156.6, qC		
18NH		11.20, s	19, 20, 24a		11.21, s	19, 20, 20a, 24a
19	127.1, CH	7.66, d (2.2)	20, 20a, 24a	126.0, CH	7.53, br s	20, 20a, 24a
20	105.6, qC			106.4, qC		
20a	127.2, qC			127.7, qC		
21	120.0, CH	6.81, d (8.5)	20, 23, 24a	121.0, CH	7.64, d (8.5)	20, 23, 27a
22	120.4, CH	6.92, dd (8.5, 1.7)	23, 24a	120.9, CH	7.10, dd (8.5, 1.7)	20a, 23
23	113.6, qC			113.4, qC		
24	113.3, CH	7.51, d (1.7)	20a, 22, 23	113.3, CH	7.53, d (1.7)	20a, 21, 23
24a	135.9, qC			136.1, qC		
25	43.7, CH	4.02, s	8, 9, 19, 20, 26, 27	50.4, CH	4.24, s	8, 19, 20, 20a, 27
26	89.1, qC	5.55 (s), OH	26, 27	91.0, qC	6.21, s, OH	9, 26, 27
27	168.8, qC			169.6, qC		
28NH		7.78, br t (5.8)	27, 29, 29 ^c		8.45, br t	27, 29 ^c
29	38.1, CH ₂	2.88, m 2.72, m	30	38.5, CH ₂	3.03, m	27, 30
30	25.2, CH ₂	1.10, m	31	25.5, CH ₂	1.30, m	29, 31
31	25.2, CH ₂	1.10, m	30	25.5, CH ₂	1.25, m	30, 32
32	40.0, CH ₂	2.87, m	34	40.0, CH ₂	2.94, m	31, 34
33NH		7.48, m	32 ^c		7.65, m	32 ^c
34	156.6, qC			156.5, qC		
C(=NH)NH ₂		7.35, br 6.91, br			7.36, br 7.00, br	

^a Carbon assignments obtained from HSQC and HMBC experiments. ^b HMBC correlations are from proton(s) stated to the indicated carbon. ^c COSY correlation between NH and protons on stated carbon.

**Figure 2.** Possible biogenetic formation of the eusynstyelamides A–C (1–3).

A search of the literature for marine natural products containing a pyrrolidone ring highlighted anchinopeptolides A–D from the sponge *Anchinoe tenacior*,^{11,12} which displaced specific ligands from somatostatin, human B2 bradykinin, and neuropeptide Y receptors. The anchinopeptolides, exemplified by anchinopeptolide D (6), are thought to be derived from the dimerization of two modified tripeptide (arginine, glycine/alanine, and tyrosine) halves¹²

with the α -keto acid most likely derived from arginine, thus leading to a different substitution pattern around the pyrrolidone ring compared to the eusynstyelamides. The relative configuration around the pyrrolidone ring in the anchinopeptolides was shown to be the same and matches the relative configuration found in eusynstyelamide B (2). (\pm)-Anchinopeptolide D has been synthesized by the aldol dimerization of a tripeptide and the relative

configuration confirmed.¹³ Two minor adducts with the same relative configurations of **1** and **3** were also produced. Heating a solution of either of the two pure isomers with opposite configuration at the hemiaminal center (equivalent to **1** and **2**) in MeOH at reflux for 1 h resulted in an equilibrium of the two, whereas treating either of these pure isomers with KOH in 1:1 THF/MeOH at 25 °C for 17 h resulted in an equilibrium mixture of the three isomers (equivalent to **1**, **2**, and **3**).¹³ The relative ease with which these isomers were able to equilibrate poses the question of whether the eusynstyelamides are natural or were formed during extraction. Two additional samples of *E. latericius* and a second portion of the original collection (23140) were analyzed by ¹H NMR for the presence of the eusynstyelamides in an attempt to answer this question. Sample 23157 was collected in the same region (Hixson Island, central GBR) as sample 23140, from which eusynstyelamides A–C were isolated, and the second sample was collected at Rib Reef, in the northern region of the GBR. The sample from Rib Reef was extracted with MeOH for <1 min, and the ¹H NMR spectrum indicated that eusynstyelamides A and B (**1**, **2**) were present in a 1:1 ratio. Sample 23157 was extracted with CD₃OD for <1 min, and the ¹H NMR was obtained immediately. A second portion of this same sample was extracted twice with MeOH, the solvent removed with heating, and the ¹H NMR acquired in CD₃OD. In both cases the ¹H NMR spectra indicated that only eusynstyelamide A (**1**) was present. Furthermore, when the MeOH extract of 23157 was further heated at reflux for 1 h, the ¹H NMR spectrum indicated that **1** was still the only isomer present. A second portion of the original sample 23140 was extracted with CD₃OD for <1 min and the ¹H NMR spectrum acquired immediately. Again eusynstyelamides A–C (**1**–**3**) were present in a similar ratio (approximately 2:6:1) to what was seen in the original extraction, which had been carried out within two weeks of sample collection. From these results (Supporting Information) and the fact that there was no evidence of equilibration of the three isomers during prolonged storage in MeOH or CD₃OD, we believe that eusynstyelamides **1**–**3** are indeed natural products.

Eusynstyelamides A–C (**1**–**3**) were tested for their activity against nNOS. Eusynstyelamides B and C showed similar activities with IC₅₀ values of 4.3 and 5.8 μM, respectively, whereas eusynstyelamide A was 10-fold less active, with an IC₅₀ of 41.7 μM. Eusynstyelamides A–C were found to be nontoxic toward the three human tumor cell lines MCF-7 (breast), SF-268 (CNS), and H-460 (lung) at concentrations up to 32 mM. Compounds **1**–**3** were also tested for their antimicrobial activity toward *Escherichia coli*, *Staphylococcus aureus*, *Vibrio harveyi*, *Mycobacterium smegmatis*, and *Candida albicans*. Compounds **1** and **2** displayed mild activity toward *S. aureus* (IC₅₀ 5.6 and 6.5 mM, respectively). Compounds **1** and **2** were also evaluated for their inhibitory activity toward the C₄ plant regulatory enzyme pyruvate phosphate dikinase (PPDK)^{14,15} and demonstrated IC₅₀ values of 19 and 20 mM, respectively.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a JASCO P-1020 polarimeter. UV spectra were measured using a Shimadzu SPD-M10AVP PDA detector. CD spectra were recorded in MeOH on a JASCO J-715 spectropolarimeter. A Nicolet Nexus FTIR spectrophotometer was used to record all IR spectra. ¹H and ¹³C NMR spectra were recorded in CD₃OD and in DMSO-*d*₆ employing a Bruker Avance 600 MHz NMR spectrometer with cryoprobe. NMR spectra were referenced to residual ¹H and ¹³C resonances in the deuterated solvents. Both 1D and 2D NMR spectra were recorded using standard Bruker pulse sequences. Mass spectra were measured with a Bruker BioApex 47e FT-ICR mass spectrometer fitted with an Analytica of Branford electrospray source; ions were detected in positive mode within a mass range *m/z* 200–1000. Direct infusion of MS samples (0.2 mg/mL) was carried out using a Cole Palmer 74900 syringe pump at a flow rate of 80 μL/h. Radioactivity was measured on a Microbeta scintillation counter (Wallac, Finland). HPLC was undertaken using a

Shimadzu LC10-AT pump coupled to a Shimadzu SPD-10A UV/vis detector. HPLC columns were purchased from Phenomenex (Australia). RP-C₁₈ vacuum liquid column chromatography was performed using material from Alltech (USA), and AG50W-X8 (Na⁺ form) cation exchange resin was sourced from BioRad (Australia). L-[2,3-³H]Arginine ([³H]arginine) was purchased from Dupont NE (USA). Optiphase Supermix scintillation fluid was obtained from Wallac (Finland). Compressed gases were supplied by BOC Gases (Townsville, Australia) and were at least 99.99% pure. Purified water was obtained from a Milli-Q water purification system (Millipore, MA). All other solvents used were HPLC grade (Mallinckrodt, MO). All other chemicals were sourced from Sigma-Aldrich (Australia).

Animal Material. The specimens of *Eusynstyela latericius* (family Styelidae, order Pleurogona) were collected by hand using scuba from the waters around Hixson Island (21°45.0' S, 150°17.5' E; Percy Island Group in the Central Great Barrier Reef, Queensland, Australia: 23140 and 23157) and Rib Reef (18°28.7' S, 146°52.3' E) under the collection permit GBRMPA G00/506 and kept frozen (–20 °C) until workup. A voucher specimen (AIMS 23140) is lodged at AIMS.

Extraction and Isolation. The frozen ascidian sample (220 g wet weight, 21 g after extraction) was exhaustively extracted with CH₂Cl₂/MeOH. The solvents were removed under reduced pressure, and the remaining aqueous extract was partitioned with hexane. The water-soluble material was dried and desalted by trituration with cold MeOH (×3) then dried to give 6.5 g of MeOH solubles. This material was fractionated employing gradient RP-C₁₈ vacuum liquid chromatography, using increasing proportions of MeOH in H₂O from 20% MeOH to 100% MeOH, to give eight fractions, followed by a column wash with 1% AcOH/MeOH to give a ninth fraction. Fractions 4–6 and 9 exhibited nNOS inhibitory activity and had similar ¹H and ¹³C NMR spectra and identical MS data. Fractions 1–3 did not exhibit any nNOS inhibitory activity, but were shown to contain the known compounds homarine and trigonelline by comparison of their NMR data with published values.⁸ A portion of fraction five (60% MeOH/H₂O) was further purified by RP-C₁₈ HPLC (Luna C₁₈, 5 μm, 10 × 250 mm) using a gradient of 0.5% aqueous TFA/CH₃CN (7:3) to 0.5% aqueous TFA/CH₃CN (1:1) over 50 min to give **1** (7 mg), **2** (8 mg), and **3** (5 mg.) as oils.

Eusynstyelamide A (1): pale yellow oil, [α]_D¹⁹ –29 (c 0.1, MeOH); CD (Δε₂₁₂ –33, Δε₂₃₀ +8); UV (PDA, MeOH) λ_{max} (rel abs) 235 (1.00), 285 (0.65), 294 (sh) (0.54); IR (film) ν_{max} 3464, 1680, 1659, 1640, 1205 cm^{–1}; ¹H and ¹³C NMR, see Table 1 and Figure 1; HRESIMS *m/z* 787.1676 [M + H]⁺ (calcd for C₃₂H₄₁⁷⁹Br₂N₁₀O₄, 787.1674).

Eusynstyelamide B (2): pale yellow oil, [α]_D¹⁹ ±0.0 (c 0.1, MeOH); CD (Δε₂₀₆ +13, Δε₂₂₄ –11); UV (PDA, MeOH) λ_{max} (rel abs) 235 (1.00), 283 (0.85), 294 (sh) (0.68); IR (film) ν_{max} 3434, 1677, 1645, 1203 cm^{–1}; ¹H and ¹³C NMR, see Table 2; HRESIMS *m/z* 787.1680 [M + H]⁺ (calcd for C₃₂H₄₁⁷⁹Br₂N₁₀O₄, 787.1674).

Eusynstyelamide C (3): pale yellow oil, [α]_D¹⁹ +17 (c 0.1, MeOH); CD (Δε₂₁₂ –22, Δε₂₃₀ 12); UV (PDA, MeOH) λ_{max} (rel abs) 233 (1.00), 285 (0.52), 294 (sh) (0.44); IR (film) ν_{max} 3422, 1677, 1642, 1192 cm^{–1}; ¹H and ¹³C NMR, see Table 2; HRESIMS *m/z* 787.1652 [M + H]⁺ (calcd for C₃₂H₄₁⁷⁹Br₂N₁₀O₄, 787.1674).

nNOS Inhibitory Bioassay. Rat cerebella were excised, snap frozen in liquid nitrogen, and stored at –80 °C. Cerebella were homogenized in 10 volumes of buffer containing 50 mM Tris, 1 mM EDTA, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 1 μg/mL pepstatin A, and 1 μM phenylmethylsulfonylfluoride at pH 7.4. The homogenate was centrifuged at 20000g for 30 min and the supernatant decanted. The pellet was resuspended in the same volume and the process repeated. The two supernatants were combined and stored at –80 °C until needed. Protein concentration was determined with a bicinchoninic acid protein kit following the manufacturer's protocol. nNOS activity was measured by the conversion of [³H]arginine to [³H]citrulline. The experiments were carried out in microtiter plates (total volume = 150 μL) in reaction buffer (33 mM HEPES, 0.65 mM EDTA, 0.8 mM CaCl₂, 3.5 μg/mL calmodulin, 670 μM βNADPH, 670 μM dithiothreitol, pH 7.4) containing 20 nM [³H]arginine (5.75 pmol [³H]arginine = 2.4 × 10⁵ cpm). The assay was performed in duplicate using a 10 μL volume, and background was measured in the presence of 1 mM unlabeled *N*-nitro-L-arginine. Reactions, initiated by adding NOS preparation (equivalent 0.01–0.02 units, where 1 unit is the amount of enzyme that will produce 1 nmol of [³H]citrulline/min under the above conditions), were terminated after 10 min with the addition of 50 μL of 0.3 M EGTA. An aliquot (50 μL) of this quenched reaction mixture

was transferred to 50 μ L of 500 mM HEPES (pH 5.5), and AG50W-X8 (Na^+ form) resin (100 μ L) was added to separate the [^3H]arginine from the [^3H]citrulline. This mixture was vortexed 8–10 times with the resin settling under gravity between mixing steps. Samples were centrifuged at 1200g for 10 min, and 70 μ L of supernatant was removed and added to scintillation fluid and counted on a Microbeta scintillation counter. Assays were conducted at room temperature.

PPDK Inhibition Bioassay. Compounds **1–3** were dissolved in DMSO and screened in the PPDK assay according to a previously described method.¹⁴

Cytotoxicity Assay. Compounds **1–3** were tested for their cytotoxicity against MCF-7 (breast; pleural effusion adenocarcinoma, ATCC HTB-22), SF-268 (central nervous system; glioblastoma), and H460 (lung; large-cell carcinoma, ATCC HTB-177) according to a previously described method.¹⁶

Antimicrobial Testing. Compounds were tested against *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Mycobacterium smegmatis*, *Vibrio harveyi* (strain C071),¹⁷ and *Candida albicans* using the previously described method.¹⁸

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Supporting Information Available: An underwater photograph of the ascidian, selected 1D and 2D NMR spectra, key NOE correlations for **1**, tabulated NMR data for **2** and **3** in CD_3OD , and the CD spectra

of **1–3**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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