# 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 $IC_{50}$ values of 41.7, 4.3, and 5.8 $\mu$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* ($IC_{50}$ 5.6 and 6.5 mM, respectively) and mild inhibitory activity toward the $C_4$ plant regulatory enzyme pyruvate phosphate dikinase (PPDK) ($IC_{50}$ 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-workers[6] 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 ($IC_{50}$ 1.6−89 $\mu$M) toward the human colon tumor cell line HCT-116, whereas eusynstyelamide was found to be less active ($IC_{50}$ 100 $\mu$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 trigonelline[8] 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 $CH_2Cl_2$/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-$C_{18}$ vacuum liquid chromatography (VLC), to give nine fractions, four of which exhibited nNOS inhibitory activity. The $^1$H and $^{13}$C 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-$C_{18}$ HPLC afforded three new compounds, **1**−**3**, as oils.

The molecular formula of **1** was established as $C_{32}H_{40}Br_2N_{10}O_4$ 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 $^{13}$C NMR spectral data of **1**, it was possible to discern the presence of 32 carbon atoms: 20 sp$^2$, two quaternary sp$^3$, one sp$^3$ methine, and nine methylene sp$^3$ carbons. Most of the signals in the $^{13}$C NMR spectrum appeared to be paired, pointing toward **1** possibly being dimeric. Further analysis of the NMR data of **1**, measured in both $CD_3OD$ 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 ($\delta_C$ 156.8) and C-34 ($\delta_C$ 156.6) with those reported in the literature,[9,10] exchangeable proton signals at $\delta_H$ 7.64 (br, t) and 7.47 (br, t), and two very broad baseline signals centered on $\delta_H$ 7.32 and 6.92 [−C(=NH)NH$_2$]. Further analysis of the gCOSY and gHMBC spectra allowed the two guanidine groups to be extended to include two $C_4H_8$ 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 $\delta_H$ 8.19 ($J$ = 6.1 Hz) in the $^1$H 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 $H_2$-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 $^1$H NMR spectrum measured in DMSO-$d_6$, indicating the second amide to be tertiary. Sixteen of

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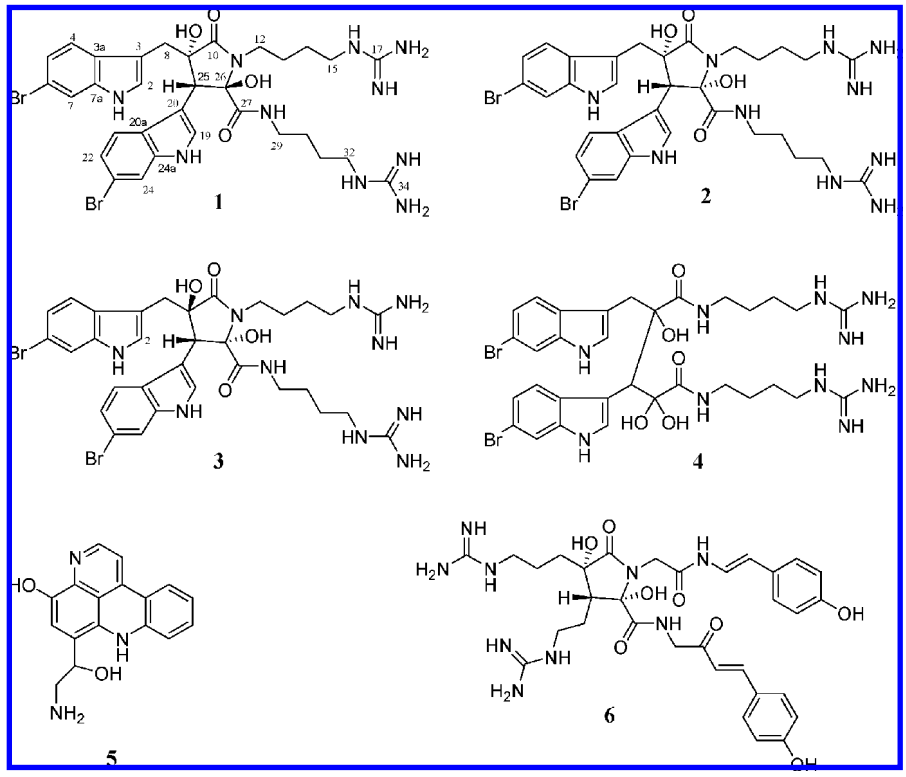
**Chart 1**




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 $CD_3OD$ and DMSO-$d_6$ showed correlations between $H_2$-12 ($\delta_H$ 3.27 in $CD_3OD$ and $\delta_H$ 3.08 in DMSO-$d_6$) and C-26 ($\delta_C$ 93.7 in $CD_3OD$ and $\delta_C$ 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 $H_2$-13 and $H_2$-14 (DMSO-$d_6$: $\delta_{H2}$ 1.36 and 1.39 in **1**; $\delta_{H2}$ 1.38 and 1.38 in **4**) compared to the signals for $H_2$-30 and $H_2$-31 (DMSO-$d_6$: $\delta_H$ 1.09/1.04 and 0.97/0.93 in **1**; $\delta_H$ 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 ($CD_3OD$ and DMSO-$d_6$) and the observed two-bromine cluster at *m/z* 787/789/791 in the MS to be an $[M + H - H_2O]^+$ 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 $H_2$-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 $H_2$-13 and $H_2$-14 signals compared to the $H_2$-30 and $H_2$-31 signals and also accounts for the complete absence of an H-11 proton signal as noted by Swersey et al.[6]

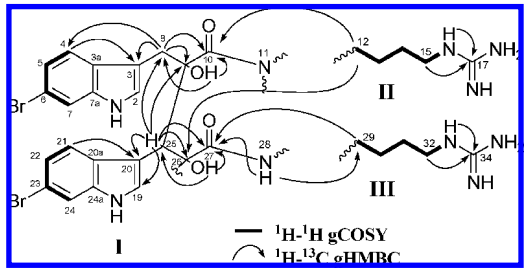



**Figure 1.** Partial structures **I**, **II**, and **III** of eusynstyelamide A (**1**), showing selective gCOSY and gHMBC correlations.

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 $C_{32}H_{40}Br_2N_{10}O_4$, identical to that of **1**, showing **1** and **2** to be isomeric. The $^1$H 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** ($\delta_H$ 4.02, DMSO-$d_6$) compared to the equivalent resonance in **1** ($\delta_H$ 3.57, DMSO-$d_6$) and the shielded signal for H-21. Likewise, the $^{13}$C 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 $^1$H and $^{13}$C 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 $C_{32}H_{40}Br_2N_{10}O_4$, 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 ($\delta_H$ 4.24 in **3** compared to $\delta_H$ 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 $CD_3OD$ 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

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

| | eusynstyelamide A (**1**) | | | eusynstyelamide A (**1**) | | | eusynstyelamide (**4**) | |
|---|---|---|---|---|---|---|---|---|
| pos. | $CD_3OD$ $\delta_C$, mult. | $\delta_H$ ($J$ in Hz) | gHMBC[c] | DMSO-$d_6$ $\delta_C$, mult. | $\delta_H$ ($J$ in Hz) | gHMBC[c] | DMSO-$d_6$ $\delta_C$, mult. | $\delta_H$ ($J$ in Hz) |
| 1NH | | | | | 11.04, br s | 2, 3, 3a,7a | | 11.2, br s |
| 2 | 127.1, CH | 6.92, s | 3, 3a, 7a | 125.9, CH | 6.79, br s | 3, 3a, 7a | 126.0, CH | 6.83, br s |
| 3 | 110.4, qC | | | 109.0, qC | | | 108.3, qC | |
| 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, 3, 3a, 9, 10, 25 | 28.3, $CH_2$ | 3.24, d (14.5) | 2, 3, 9, 10, 25 | 28.5, $CH_2$ | 3.20, d (14) |
| | | 2.99, d (14.4) | 2, 3, 3a, 9, 10, 25 | | 2.68, d (14.5) | 2, 3, 9, 10, 25 | | 2.65, d (14) |
| 9 | 81.6, qC | | | 78.9, qC | 6.86, s, O<u>H</u> | 3, 8, 9 | 78.9, qC | O<u>H</u> 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 | 14, 17 | 40.3, $CH_2$ | 3.04, m | 14, 17 | 40.4, $CH_2$ | 3.05, m |
| | | 3.16, m | | | 3.08, m | | | |
| 16NH | | | | | 7.64, br t | 15[d] | | 7.89, br s |
| 17 | 158.6, qC | | | 156.8, qC | | | 157.0, qC | |
| 18NH | | | | | 11.14, br s | 19, 20, 24a | | 11.4, br s |
| 19 | 126.8, CH | 7.64, s | 20, 20a, 24a | 125.4, CH | 7.44, br s | 20, 20a, 27a | 125.6, CH | 7.40, br s |
| 20 | 107.0, qC | | | 105.5, qC | | | 105.4, qC | |
| 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, O<u>H</u> | 26, 27 | 91.9, qC | 7.14, br s, O<u>H</u> |
| 27 | 173.4, qC | | | 171.6, qC | | | 171.6, qC | |
| 28NH | | | | | 8.19, br t (6.1) | 27, 29, 29[d] | | 8.17, dd (2, 2) |
| 29 | 39.7, $CH_2$ | 3.15, m | 26, 27, 30 | 38.1, $CH_2$ | 2.94, m | 27, 30 | 38.4, $CH_2$ | 2.95, m |
| | | 2.78, ddd (13.4, 6.7, 6.7) | | | 2.77, m | | | 2.80, m |
| 30 | 26.8, $CH_2$ | 1.09, m | 29, 31, 32 | 24.9, $CH_2$ | 1.09, m | 29, 31 | 25.34, $CH_2$ | 1.10, m |
| | | 1.04, m | | | 1.04, m | | | 0.93, m |
| 31 | 26.3, $CH_2$ | 0.93, m | 29, 30, 32 | 25.0, $CH_2$ | 0.97, m | 30, 32 | 25.32, $CH_2$ | 1.11, m |
| | | | | | 0.93, m | | | 0.93, m |
| 32 | 41.9, $CH_2$ | 2.88, t (7.4) | 30, 31, 34 | 40.1, $CH_2$ | 2.89, m | 31, 34 | 40.2, $CH_2$ | 2.94, m |
| 33NH | | | | | 7.47, br t (5.9) | 32[d] | | 7.80, br s |
| 34 | 158.5, qC | | | 156.6, qC | | | 156.9, qC | |
| C(=N<u>H</u>)N<u>H</u>$_2$ | | | | | 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 N<u>H</u> 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-O<u>H</u>, H-21 and H-25, and C-9-O<u>H</u> and H-19, indicating H-25 and C-26-O<u>H</u> to be *cis* and both to be *trans* to C-9-O<u>H</u>. 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-O<u>H</u>, H-21, and N-28-<u>H</u>, between C-9-O<u>H</u> and H-2, and between C-26-O<u>H</u> 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-O<u>H</u>, and C-26-O<u>H</u>, 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 $^{1}H$ NMR Data (600/175 Hz, DMSO-$d_6$) for Eusynstyelamides B (**2**) and C (**3**)

| | eusynstyelamide B (**2**) | | | eusynstyelamide C (**3**) | | |
|---|---|---|---|---|---|---|
| pos. | $\delta_C$, mult.[a] | $\delta_H$ ($J$ in Hz) | gHMBC[b] | $\delta_C$, mult.[a] | $\delta_H$ ($J$ 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)<br>2.76, d (15.1) | 2, 3, 9, 10, 25 | 31.0, $CH_2$ | 3.25, d (15.1)<br>2.66, d (15.1) | 2, 3, 3a, 10, 26 |
| 9 | 78.1, qC | 6.23, s, O<u>H</u> | 8, 9, 10, 25 | 79.1, qC | 5.28, s, O<u>H</u> | 8, 9, 10, 25 |
| 10 | 175.1, qC | | | 175.5, qC | | |
| 12 | 39.5, $CH_2$ | 3.21, m<br>2.91, m | 13, 26 | 40.0, $CH_2$ | 3.22, m<br>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), O<u>H</u> | 26, 27 | 91.0, qC | 6.21, s, O<u>H</u> | 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<br>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(=N<u>H</u>)N<u>H</u>$_2$ | | 7.35, br<br>6.91, br | | | 7.36, br<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 N<u>H</u> and protons on stated carbon.

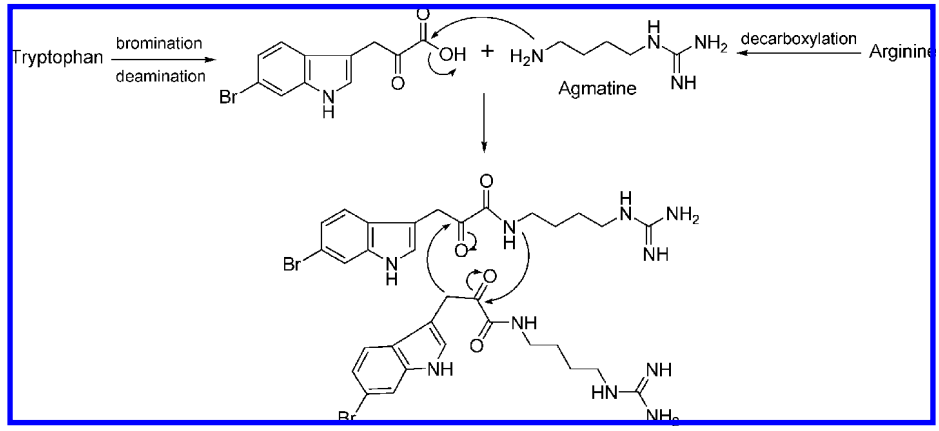



**Figure 2.** Possible biogenetic formation of the eusynstyelamides A−C (**1**−**3**).

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) halves[12] 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**). (±)-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 $^1$H 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 $^1$NMR spectrum indicated that eusynstyelamides A and B (**1**, **2**) were present in a 1:1 ratio. Sample 23157 was extracted with $CD_3OD$ for <1 min, and the $^1$H NMR was obtained immediately. A second portion of this same sample was extracted twice with MeOH, the solvent removed with heating, and the $^1$H NMR acquired in $CD_3OD$. In both cases the $^1$H 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 $^1$H NMR spectrum indicated that **1** was still the only isomer present. A second portion of the original sample 23140 was extracted with $CD_3OD$ for <1 min and the $^1$H 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 $CD_3OD$, 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 $IC_{50}$ values of 4.3 and 5.8 $\mu$M, respectively, whereas eusynstyelamide A was 10-fold less active, with an $IC_{50}$ of 41.7 $\mu$M. 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* ($IC_{50}$ 5.6 and 6.5 mM, respectively). Compounds **1** and **2** were also evaluated for their inhibitory activity toward the $C_4$ plant regulatory enzyme pyruvate phosphate dikinase (PPDK)[14,15] and demonstrated $IC_{50}$ 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. $^1$H and $^{13}$C NMR spectra were recorded in $CD_3OD$ and in DMSO-$d_6$ employing a Bruker Avance 600 MHz NMR spectrometer with cryoprobe. NMR spectra were referenced to residual $^1$H and $^{13}$C 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 m/L) was carried out using a Cole Palmer 74900 syringe pump at a flow rate of 80 $\mu$L/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-$C_{18}$ 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-$^3$H]Arginine ([$^3$H]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 $CH_2Cl_2$/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-$C_{18}$ vacuum liquid chromatography, using increasing proportions of MeOH in $H_2O$ 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 $^1$H and $^{13}$C 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/$H_2O$) was further purified by RP-$C_{18}$ HPLC (Luna $C_{18}$, 5 $\mu$m, 10 × 250 mm) using a gradient of 0.5% aqueous TFA/$CH_3CN$ (7:3) to 0.5% aqueous TFA/$CH_3CN$ (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, $[\alpha]^{19}_D$ −29 (*c* 0.1, MeOH); CD ($\Delta\varepsilon_{212}$ −33, $\Delta\varepsilon_{230}$ +8); UV (PDA, MeOH) $\lambda_{max}$ (rel abs) 235 (1.00), 285 (0.65), 294 (sh) (0.54); IR (film) $\nu_{max}$ 3464, 1680, 1659, 1640, 1205 cm$^{-1}$; $^1$H and $^{13}$C NMR, see Table 1 and Figure 1; HRESIMS *m/z* 787.1676 [M + H]$^+$ (calcd for $C_{32}H_{41}{}^{79}Br_2N_{10}O_4$, 787.1674).

**Eusynstyelamide B (2):** pale yellow oil, $[\alpha]^{19}_D$ ±0.0 (*c* 0.1, MeOH); CD ($\Delta\varepsilon_{206}$ +13, $\Delta\varepsilon_{224}$ −11); UV (PDA, MeOH) $\lambda_{max}$ (rel abs) 235 (1.00), 283 (0.85), 294 (sh) (0.68); IR (film) $\nu_{max}$ 3434, 1677, 1645, 1203 cm$^{-1}$; $^1$H and $^{13}$C NMR, see Table 2; HRESIMS *m/z* 787.1680 [M + H]$^+$ (calcd for $C_{32}H_{41}{}^{79}Br_2N_{10}O_4$, 787.1674).

**Eusynstyelamide C (3):** pale yellow oil, $[\alpha]^{19}_D$ +17 (*c* 0.1, MeOH); CD ($\Delta\varepsilon_{212}$ −22, $\Delta\varepsilon_{230}$ 12); UV (PDA, MeOH) $\lambda_{max}$ (rel abs) 233 (1.00), 285 (0.52), 294 (sh) (0.44); IR (film) $\nu_{max}$ 3422, 1677, 1642, 1192 cm$^{-1}$; $^1$H and $^{13}$C NMR, see Table 2; HRESIMS *m/z* 787.1652 [M + H]$^+$ (calcd for $C_{32}H_{41}{}^{79}Br_2N_{10}O_4$, 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 $\mu$g/mL leupeptin, 2 $\mu$g/mL aprotinin, 1 $\mu$g/mL pepstatin A, and 1 $\mu$M phenylmethylsulfonylfluoride at pH 7.4. The homogenate was centrifuged at 20000*g* 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 [$^3$H]arginine to [$^3$H]citrulline. The experiments were carried out in microtiter plates (total volume = 150 $\mu$L) in reaction buffer (33 mM HEPES, 0.65 mM EDTA, 0.8 mM $CaCl_2$, 3.5 $\mu$g/mL calmodulin, 670 $\mu$M $\beta$NADPH, 670 $\mu$M dithiothreitol, pH 7.4) containing 20 nM [$^3$H]arginine (5.75 pmol [$^3$H]arginine = 2.4 × 10$^5$ cpm). The assay was performed in duplicate using a 10 $\mu$L 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 [$^3$H]citrulline/min under the above conditions), were terminated after 10 min with the addition of 50 $\mu$L of 0.3 M EGTA. An aliquot (50 $\mu$L) of this quenched reaction mixture

was transferred to 50 $\mu$L of 500 mM HEPES (pH 5.5), and AG50W-X8 ($Na^+$ form) resin (100 $\mu$L) was added to separate the [$^3$H]arginine from the [$^3$H]citrulline. This mixture was vortexed 8−10 times with the resin settling under gravity between mixing steps. Samples were centrifuged at 1200*g* for 10 min, and 70 $\mu$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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