# Aestuaramides, a Natural Library of Cyanobactin Cyclic Peptides Resulting from Isoprene-Derived Claisen Rearrangements

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S *Supporting Information*

ABSTRACT: We report 12 cyanobactin cyclic peptides, the aestuaramides, from the cultivated cyanobacterium *Lyngbya aestuarii*. We show that aestuaramides are synthesized enzymatically as reverse *O*-prenylated tyrosine ethers that subsequently undergo a Claisen rearrangement to produce forward *C*-prenylated tyrosine. These results reveal that a nonenzymatic Claisen rearrangement dictates isoprene regiochemistry in a natural system. They also reveal one of the mechanisms that organisms use to generate structurally diverse compound libraries starting from simple ribosomal peptide pathways (RiPPs).
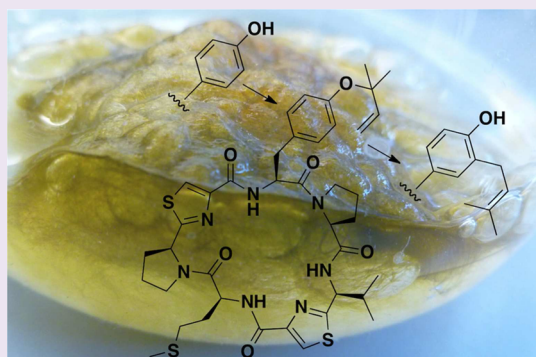
Natural products are important starting points in the process of drug discovery.[1] In part, this is because they present a diversity of scaffolds and other chemical features. A major goal of natural products research has been to understand how enzymes dictate regioselective chemical modifications that underlie this structural diversity. For instance, many natural products are alkylated by isoprene equivalents, such as dimethylallylpyrophosphate (DMAPP), which is installed by a large enzyme class known as prenyltransferases.[2] Prenyltransferases are known to carry out regioselective prenylation of aromatic carbons, hydroxyl groups, amines, and thiols, leading to diverse, bioactive products.

Mechanistically, prenyltransferases function by ionization of prenyl diphosphate, followed by electrophilic alkylation of the substrates.[3] A longstanding hypothesis has been that prenyltransferases dictate regioselectivity by positioning substrates. However, recent biochemical work supports the hypothesis that the final regioselectivity observed may not always be enzymatically controlled. Instead, in some cases a nonenzymatic Claisen rearrangement may dictate the isolated chemistry.[4] In other cases, regioselectivity is at least partly substrate controlled, where different substrates exhibit differences in prenylation pattern by the same enzyme.[5,6]

We have been studying this phenomenon using a family of small, cyclic peptides known as cyanobactins, which present a diverse series of prenylated metabolites.[7−10] Cyanobactins begin as small proteins synthesized on the ribosome. Subsequently, they are posttranslationally modified in a variety of ways. For example, cyanobactins are often prenylated. The isoprene unit usually results from DMAPP, which is installed in a forward or reverse orientation on the amino acids Ser, Thr, Tyr, and possibly Trp by a series of prenyltransferases exemplified by LynF (Figure 1).[4]
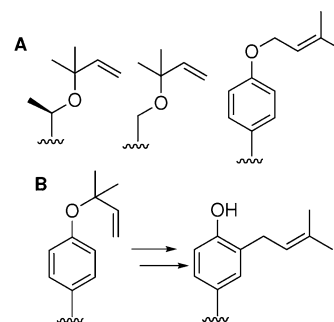

Figure 1. Prenylation in cyanobactin cyclic peptides. (A) Previously identified cyanobactin natural products are reverse *O*-prenylated on Ser/Thr or forward *O*-prenylated on Tyr. (B) LynF reverse *O*-prenylates Tyr, and the product undergoes a spontaneous Claisen rearrangement *in vitro*.

Purified LynF accepts many different Tyr-containing substrates, including cyclic peptides with diverse amino acid sequences and simple Tyr derivatives, such as boc-Tyr.[4] For all of the substrates attempted in *in vitro* reactions, LynF *O*-prenylates Tyr in the reverse orientation, which is an otherwise unprecedented biochemical modification. Surprisingly, we found that this reverse-prenylated intermediate undergoes a spontaneous Claisen rearrangement, even in the absence of enzyme, to yield a forward *C*-prenylated moiety that is very common in natural products (Figure 1B). This result supported earlier hypotheses that, perhaps, spontaneous Claisen rearrangements might underlie many of the observed prenylated

natural products.[11] To the best of our knowledge, this may be the only biological relevant Claisen rearrangement outside of the well-studied shikimate pathway.[12]

A weakness of our previous study was that the actual natural products made by LynF, from the cyanobacterium *Lyngbya aestuarii*, were not known. Instead, we predicted the structures of the natural products based upon the *L. aestuarii* genome sequence and, on that basis, synthesized putative substrate analogues.[4,7] Because it is now known that the regioselectivity of at least some prenyltransferases is substrate controlled,[5,6] it remained possible that this Claisen rearrangement was substrate-dependent and not naturally occurring. Therefore, we sought to isolate these putative new compounds from their natural source. Here, we report isolation of novel compounds, aestuaramides A (**1**) and B (**2**), and we demonstrate that the spontaneous Claisen rearrangement of the natural products is important in their natural structural diversity. These are the first novel compounds reported from *L. aestuarii*, a globally abundant cyanobacterium.[13−15]

The *L. aestuarii* genome sequence contained a cyanobactin pathway that we predicted would build cyclic peptides using the ribosomally derived core sequences, ACMPCYP and VCMPCYP (Figure 2).[7] We obtained the sequenced strain,
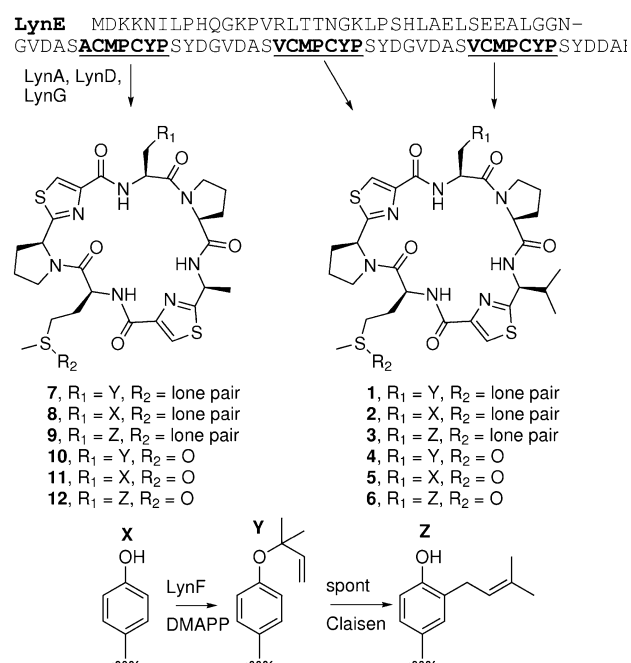

Figure 2. Ribosomal synthesis of aestuaramides. The LynE precursor peptide is shown at top, with the sequence encoding products underlined and bold. Compounds identified in this study are numbered. On the basis of biochemical precedent, LynADG would lead to production of cyclic peptides **2** and **8**. Subsequently, prenylation by LynF would lead to reverse *O*-prenylated Tyr products **1** and **7**.

L. aestuarii PCC 8106, and cultivated it over a six-month period. This led to gradual accumulation of a cyanobacterial broth (20 L) from continuous culture, which was rapidly extracted with methanol to repress isolation artifacts. The crude extract was analyzed by Fourier transform ion cyclotron resonance (FTICR) MS/MS, leading to the identification of 12 cyclic peptides, which we named aestuaramides A−L (**1**−**12**) (Figure 2). Two of these, aestuaramides A and B (**1** and **2**),

resulted from the core sequence VCYMPCYP and were present in sufficient quantity to enable purification and NMR analysis.

Aestuaramide A (**1**) was assigned a molecular formula of $C_{40}H_{51}N_7O_6S_3$ (m/z 822.3160 $[M + H]^+$), as determined by FTICR and MS/MS analyses. This formula, in tandem with the predicted linear sequence VCMPCYP, revealed that **1** was likely isoprenylated. NMR spectroscopy in DMSO-d_6 confirmed the presence of five spin systems that could be assigned to the amino acids (Val, Met, Pro, Tyr, and Pro) and one that could be assigned to isoprene (Figure 3 and Table 1). Additionally, two sharp singlets (δ = 7.77 and 8.03) in the aromatic region were consistent with the presence of two Cys-derived thiazoles. The amino acid sequence was confirmed by a combination of ROESY, MS/MS, and chemical degradation. ROESY data showed that Tyr was adjacent to Pro1, while Met was adjacent to Pro2. MS/MS confirmed that the peptide was cyclic and matched the proposed peptide sequence precisely (Figure 4). Further evidence was obtained by carefully hydrolyzing related peptide **2** and analyzing it by Marfey's method. We optimized hydrolysis following a protocol that leaves thiazole intact.[16] All of the predicted amino acids were present in the molecule, according to this analysis. In particular, the thiazole-Pro and thiazole-Val connectivities were confirmed from this result. Additionally, this method revealed that the configurations of all amino acids were L.

NMR spectroscopy unambiguously showed that **1** contained a reverse *O*-Tyr isoprene moiety. The ROESY spectrum revealed correlations between the methyl groups of the reverse-prenyl moiety and the Tyr ε protons. In addition, the ^1H and ^{13}C chemical shifts of the Tyr aromatic moiety and isoprene were very similar to those previously reported for reverse-*O*-prenylated boc-Tyr.[4] Finally, MS/MS data confirmed this assignment. We previously showed using multiple substrates that reverse *O*-prenylated Tyr readily loses isoprene in the course of mass spectrometry, while the *C*-prenylated Tyr does not lose isoprene under most conditions.[4] Similarly, **1** readily lost isoprene even under mild fragmentation conditions (Figure 4).

On the basis of FTICR-MS, aestuaramide B (**2**) had a molecular formula of $C_{35}H_{43}N_7O_6S_3$ (m/z 754.2153 $[M + H]^+$), indicating that it was likely an unprenylated analogue of **1**. Indeed, comparative analysis of ^1H NMR and MS data revealed that these compounds were nearly identical, except that peaks corresponding to isoprene were absent in **2** (Figure 4 and Table 1). Although the ^1H NMR spectra of **1** and **2** were largely identical, the greatest chemical shift differences observed were localized to the phenol ring of tyrosine (**1**, δ = 6.91 and 6.88; **2**, δ = 6.83 and 6.67). Moreover, the ^1H NMR spectrum of **2** contained a sharp singlet consistent with a phenolic OH (δ = 9.33), while this signal was clearly absent in the ^1H spectrum of **1**. To further confirm the amino acid sequences and position of prenylation of these peptides, we carefully examined their MS/MS data, which were consistent with the proposed structures (Figure 4). A comparison of MS/MS spectra for **1** and **2** was particularly telling since the fragments were similar, but **2** lacked isoprene.

In 1:1 D_2O/CD_3OD at 50 °C, **1** was underwent the Claisen rearrangement over a 21 h period (Figure 5 and Table 2). This rearrangement could be clearly observed by following the chemical shifts of the aromatic residues and comparing them to those for authentic standards of forward *C*-prenylated and reverse *O*-prenylated Tyr that we had previously characterized. The rate of rearrangement was slower than what we had
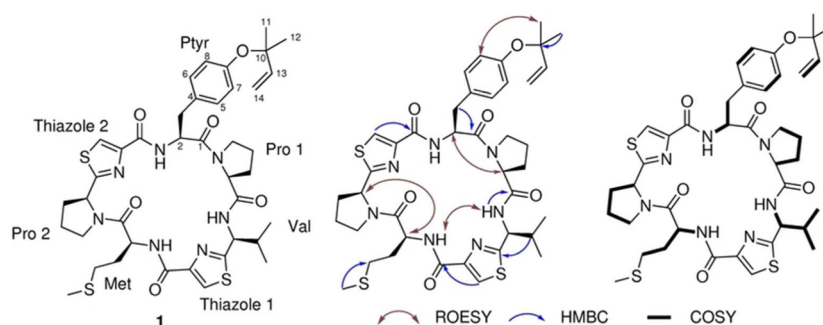
Figure 3. Summary of key NMR data for **1**.

previously observed in aqueous enzyme reaction buffers with LynF,[4] indicating either an important effect of aqueous buffers or substrate dependence in the rearrangement itself. An identical, C-prenylated compound was also found in the freshly harvested *L. aestuarii* extract, and therefore, this compound was named aestuaramide C (**3**).

Compound **3** had a nearly identical ^{1}H NMR spectrum to that of **1** in 1:1 D_2O/CD_3OD (Supporting Information Figure S3K). The major differences observed were in the chemical shifts of the aromatic moiety and in the isoprene unit. In contrast to the *p*-phenol of **1**, **3** had three ^{1}H signals in the aromatic region, representing a 1,3,4-trisubstituted aromatic ring. The chemical shifts of these protons (δ = 7.02 (H-6), 6.91 (H-5), and 6.94 (H-8)) were consistent with a dialkylated phenol. The observed coupling constants showed that an additional alkyl group was present at H-7 (Supporting Information Figure S3J). Chemical shifts of the isoprene moiety had changed from those for a reverse-prenylated compound (**1**) to a forward-prenylated compound. In particular, the isoprene methyls were at δ = 1.54 in **1** and δ = 1.79 and 1.82 in **3**. By FTICR-MS analysis, **3** and its forward-C-prenylated relatives exhibit strikingly different fragmentation patterns than the reverse-O-prenylated series. While the O-prenylated **1** readily loses C_5H_8, this loss is not observed in **3** or in any other member of the C-prenylated series. In the course of analysis, the rearrangement product **3** was spontaneously oxidized. By LC-FTICR and MS/MS, the C-prenylated and Met-oxidized **3** was identical with one of the compounds in the crude extract. The rearrangement and single oxidation product of **1** was identical by LC-FTICR-MS/MS to compound **6** (Supporting Information Figure S5 and see below). Aside from these differences, the only obvious difference in the ^{1}H NMR spectra was that the thiazole-2 proton exhibited a downfield shift from δ = 8.04 to δ = 8.08 during the conversion of **1** to **3**, while the thiazole-1 proton was invariant.

A limitation of the above data is that, owing to the amount of material recovered, ^{13}C data was only available for compound **1** and not **2** or **3**. Assignments thus relied on ^{1}H NMR data and FTICR-MS/MS. The MS/MS spectra of these compounds were identical except for differences directly attributable to prenylation of tyrosine. Moreover, by NMR this prenylation could be clearly observed to occur on tyrosine in both **1** and **3**.

In addition to **1**−**3**, nine other related products could be observed in the *L. aestuarii* crude extract by MS analysis (Figures 1 and S2, Supporting Information). Aestuaramides A−C (**1**−**3**) resulted from posttranslational modification of the precursor peptide sequence, VCMPCYP. In addition to these reverse O-prenylated, unprenylated, and forward C-prenylated compounds, we also observed three further products,

aestuaramides D−F (**4**−**6**), corresponding to the sulfoxide derivative of **1**−**3**, leading to six total cyclic peptides derived from the VCMPCYP sequence. FTICR-MS provided high-resolution masses. Further, the MS/MS fragmentation patterns were virtually identical to those for the nonsulfoxide analogues, except fragments containing Met are larger by 16 Da. We have previously observed a similar pattern of oxidized and nonoxidized Met in other cyanobactins.[17]

Similarly, six related products resulting from the ACMPCYP precursor peptide sequence were identified. These compounds (aestuaramides G−L, **7**−**12**) were reverse O-prenylated, unprenylated, or forward C-prenylated, with or without Met oxidation. The structures were assigned using a genome sequencing-based prediction and very similar MS/MS spectra to those of their NMR-characterized relatives, **1** and **2**.

Characterization of RiPP products remains a challenge in part because low yields are sometimes obtained. Countering this problem, an advantage of working with RiPPs is that the peptide sequence is genomically encoded, so that if a fragment containing the predicted sequence is found with high resolution by mass spectrometry, then based upon the size of the genome of the producer, it is probable (or in some cases virtually certain) that the compound in question is encoded by the RiPP sequence.[18,19] With aestuaramides, standard amino acids Pro, Tyr, and Met were clearly observed. The main problem lies in the assignment of posttranslational modifications. In the case of aestuaramides, biochemical precedent indicated that the products should be N−C cyclic (presence of LynA and LynG) and that they should contain thiazoles (LynD and oxidase). Indeed, in the MS spectra, the major compounds lacked $H_{10}O_3$ in comparison with the predicted linear peptides. The fragmentation methods used here cannot break the thiazole−amino acid linkage, such that Val−thiazole, Ala−thiazole, and Pro−thiazole could be inferred. Fragments for these predictions were present in all spectra. Moreover, when a peptide is cyclic rather than linear, the number of possible fragments greatly increases,[20] and indeed, the evidence here favors cyclization. However, these are hypotheses that require further testing. Fortunately, in the case of **1**, a complete NMR data set confirmed the amino acid assignments and provided additional strong support in favor of the presence of thiazoles and N−C circularization. Additional ^{1}H NMR spectra of **2** and **3** served to further support these structures. Comparison of the fragmentation pattern of **1** to those for **2**−**12** showed that they were very similar to **1**, with the differences being explained by the minor change in peptide sequence and in prenylation pattern, as described above. Because compounds **4**−**12** were elucidated solely on the basis of MS and genome sequence, they are tentatively assigned with a lower level of evidence.

Table 1. NMR Data for 1 and 2 in DMSO-d_6

			1	2
		^{13}C	^{1}H (J in Hz)	^{1}H (J in Hz)
Met	NH		7.02, d (8.6)	7.04, d (8.3)
	1	ND		
	2	49.4, CH	4.78, m	4.77, m
	3	32.3, CH$_2$	1.95, m; 1.75 m	1.97, m; 1.73 m
	4	30.4, CH$_2$	2.40, m	2.41, m
	5	14.4, CH$_3$	2.04, s	2.08, s
thiazole 1	1[a]	169.4, C		
	2	ND		
	3[a]	123.8, CH	7.76, s	7.77, s
Val	NH		9.08, s	9.10, d (9.5)
	1	172.8, C		
	2	54.9, CH	4.75, m	4.75, m
	3	30.6, CH	2.34, m	2.38, m
	4	19.6, CH$_3$	0.58, d (6.5)	0.60, d (6.7)
	5	19.2, CH$_3$	0.78, d (6.5)	0.82, d (6.7)
Pro 1	1	170.4, C		
	2	61.3, CH	3.08, m	3.10, m
	3	27.8, CH$_2$	1.72, m; 1.50, m	1.74, m; 1.56, m
	4	23.4, CH$_2$	1.64, m; 1.57, m	1.65, m; 1.58, m
	5	48.5, CH$_2$	3.90, m; 3.11, m	3.93, m; 3.11, m
Ptyr	NH		7.50, d (7.0)	7.51, d (7.0)
	1	174.5, C		
	2	52.4, CH	4.03, m	3.98, m
	3	38.5, CH$_2$	2.98, m; 2.52, m	2.94, dd (13.0, 4); 2.52, m
	4	133.2, C		
	5/6	129.6, CH	6.91, d (8.0)	6.85, d (8.0)
	7/8	121.6, CH	6.88, d (8.0)	6.69, d (8.0)
	9	158.1, C		
	9-OH			9.33, s
	10	79.3, C		
	11/12	26.4, CH$_3$	1.36, s	
	13	143.9, CH	6.07, dd (18.0, 11.4)	
	14	113.6, CH$_2$	5.12, m	
thiazole 2	1[a]	170.7, C		
	2	ND		
	3[a]	124.7, CH	8.05, s	8.05, s
Pro 2	1	ND		
	2	58.5, CH	5.86, brd (7.3)	5.85, d (7.3)
	3	28.8, CH$_2$	2.41, m; 1.96, m	2.44, m; 2.04, m
	4	21.4, CH$_2$	1.92, m; 1.74, m	1.92, m; 1.73, m
	5	46.4, CH$_2$	3.67, m; 3.52, m	3.70, m; 3.52, m

[a]Assignments may be interchanged with one another.

Previously, we showed that LynF reverse *O*-prenylates Tyr in diverse artificial substrates and that these intermediates spontaneously rearrange to form the forward *C*-prenylated derivatives.[4] We proposed that this Claisen rearrangement might possibly underlie many different types of *C*-prenylated phenolic natural products. Here, we show that LynF reverse *O*-prenylates its natural substrates and that these rearrange to form forward *C*-prenylated products within their natural environments. With these specific substrates, the Claisen rearrangement is quite slow and, in fact, much slower than with the substrate analogues that we previously used,[4] meaning

that all intermediates could be directly observed. The identification of these natural compounds confirms that this spontaneous Claisen rearrangement is important in natural isoprenoid chemistry. Our initial work with purified enzyme itself suggests that it can be difficult to directly observe this transformation,[4] and that the possibility should be considered more seriously in future studies of phenol prenylation.

The presence of both forward- and reverse-prenylated cyclic peptides in a single organism adds to the chemical complexity of ribosomal peptide natural products, which are now referred to as RiPPs.[21] In the aestuaramide biosynthetic gene cluster (*lyn*), a single precursor peptide, LynE, encodes the sequences for both the VCMPCYP and ACMPCYP series of compounds (Figure 2). A heterocyclase, LynD, was proposed to synthesize thiazole from Cys, with the help of an oxidase found in LynG.[22,23] Subsequently, LynA and LynG proteases cleave and circularize the compounds, in reactions that have been characterized in related cyanobactin systems.[24−26] On the basis of our work with purified LynF, it is only at this late stage, when peptides have already been macrocyclized, that prenylation would take place.[4] We previously showed that prenylation is perhaps the least efficient enzymatic step in cyanobactin pathways.[27] In heterologous expression experiments in *Escherichia coli*, the natural cyanobactin trunkamide was prenylated by 0, 1, or 2 DMAPP units. Our results here show that these prenyltransferases can be quite slow even in their native hosts. Indeed, LynF and relatives exhibit exceptionally relaxed substrate specificity,[24] which is likely directly related to this slow rate. Finally, Met autoxidation[17] leads to a further six derivatives, for 12 total compounds resulting from a single precursor peptide. Autoxidation is probably nonenzymatic (spontaneous) since oxidation increases over time even in extracted or purified materials.

This represents a striking structural diversity from a single RiPP precursor peptide. Most commonly, only a single product results from a single precursor peptide, although there are a few cases in which up to four products result. Twelve natural products from a single precursor peptide may represent a record for RiPPs. The production of numerous derivatives from a single pathway is one of the ways in which variable tailoring steps allow RiPP pathways to contribute to chemical diversity in bacteria. Of note, there are two copies of the Val-containing sequence and one of the Ala-containing sequence encoded on LynE. As shown above, we noted a greater amount of Val-containing products in comparison to Ala-containing products. One possibility is that this ratio directly correlates to a different abundance of the two species. In cyanobactin precursor peptides, there are several cases with multiple repeats of product-coding sequences in a single peptide, although repetition of the same coding sequence within a peptide appears to be relatively rare.[17] There are also many cases in which only a single coding sequence is found in a cyanobactin precursor peptide. Our current working hypothesis is that multiple coding sequences within a single peptide result from a duplication of the entire precursor peptide, followed by recombination to eliminate nonidentical sequences. In support of this hypothesis, we have documented several cases in which multiple precursor peptides (up to nine) are found in a single cyanobactin operon.[17] In cases that we have examined, it appears that only some of these precursors lead to isolable products.[17] It seems likely that this repetition may be more common than generally realized since it creates an assembly challenge that can lead to artifacts in shotgun-sequenced
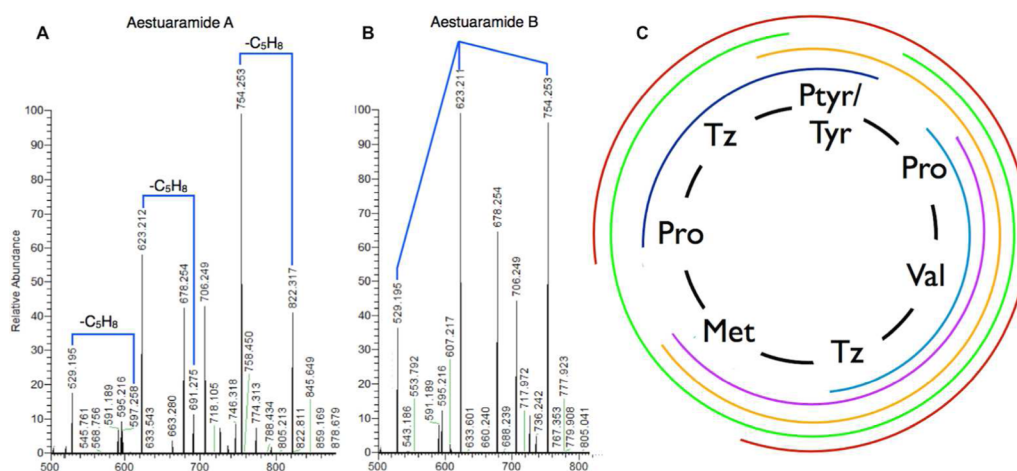
Figure 4. Summary of key MS data. FTICR-MS/MS shows that (A) **1** is *O*-prenylated, while (B) **2** is not; the MS/MS spectra are otherwise identical. (C). MS/MS data provide evidence for the fragments shown, providing unambiguous evidence of peptide sequence (see Supporting Information for full details).
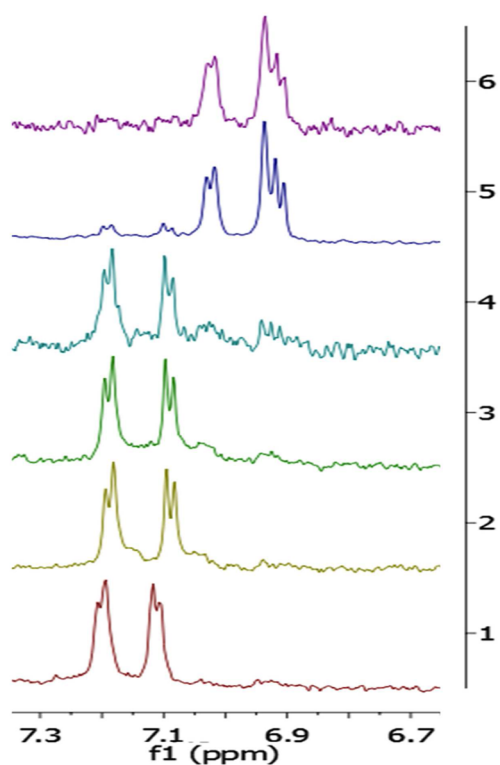

Figure 5. Claisen rearrangement converting compound **1** to **3**. Shown is the Tyr aromatic region of the ^{1}H NMR spectrum as compound **1** subjected to heating as described in the methods section; *y*-axis, 1, time 0, RT; 2, time 0, 50 °C; 3, 0.5 h, 50 °C; 4, 2 h, 50 °C; 5, 19 h, 50 °C; 6, 21 h, 50 °C. At time points 1−4, the starting material **1** dominates the spectrum, while, by time period 6, **1** is completely absent and has been replaced by **3** (see Supporting Information Figure S3K for more details).

Table 2. Key NMR Data for Spectra Shown in Figure 5

No.	1	3
5	7.20, d (8.0)	6.94, s
6	7.20, d (8.0)	7.02, d (7.6)
7	7.11, d (8.0)	
8	7.11, d (8.0)	6.91. d (7.6)
11	1.54, s	1.82, s
12	1.54, s	1.79, s
13	6.24, dd (17.6, 10.9)	
14	5.28, m	5.35, brs

multiple coding sequences in a single precursor peptide has also been found in N−C cyclic peptides from plants.[29]

L. aestuarii is a biologically important, dominant benthic (bottom-dwelling) component of shallow bays in oceans around the world.[13−15] To the best of our knowledge, the only previously reported products of *L. aestuarii* are widely occurring UV-shielding pigments.[30,31] Therefore, aestuaramides represent the first novel compounds from this widespread source. It has been shown that other cyanobactins are components of numerous types of cyanobacteria, including species that are dominant components of the plankton.[7,8,10,17] Cyclic peptides such as aestuaramides may be exceptionally widespread metabolites in natural ecosystems. The natural roles of the compounds are largely unknown, but their abundance, structural diversity, and occasionally potent biological activity in pharmaceutical screens hints that they may be important in the biology of cyanobacteria and other organisms.

◼ METHODS

General Methods. ^{1}H NMR, ^{13}C NMR, and ^{1}H−^{1}H COSY spectra were recorded on a Varian INOVA 500 (^{1}H 500 MHz and ^{13}C 125 MHz) NMR spectrometer with a 3 mm Nalorac MDBG probe at 300 K. HSQC and HMBC spectra were recorded on a Varian INOVA 600 (^{1}H 600 MHz and ^{13}C 150 MHz) NMR spectrometer equipped with a 5 mm ^{1}H[^{13}C,^{15}N] triple resonance cold probe with a *z*-axis gradient, utilizing residual solvent signals for referencing. High-resolution mass spectra (HRMS) were obtained using a Bruker APEXII FTICR mass spectrometer equipped with an actively shielded 9.4 T superconducting magnet, an external Bruker APOLLO ESI source, and a Synrad 50W CO_2 CW laser. Onyx monolithic C_{18} semipreparative columns were used for HPLC, as conducted on a Hitachi Elite Lachrom System equipped with a Diode Array L-2455

genomes. Alternatively, we have previously documented between-strain cyanobactin pathway recombination.[28] This indicates that the environment also serves as a reservoir for coding sequence diversity. This is very similar to the duplication−recombination scenario above, except that multiple precursor peptides would be created in multiple strains, rather than in a single strain. Interestingly, the phenomenon of

detector. C_{18} flash chromatography was performed using silica gel 90 (Fluka).

Cultivation. *L. aestuarii* PCC 8106 was obtained from the Pasteur Culture Collection and maintained in MN marine medium. To produce aestuaramides, the cyanobacteria were cultivated in MN marine medium (~10 mL) in plastic 100 × 15 mm Petri dishes on a windowsill at RT. MN medium (1 L): artificial seawater to 75% strength; $MgSO_4 \cdot 7H_2O$ (40 mg); $CaCl_2$ (20 mg); $NaNO_3$ (750 mg); $K_2HPO_4 \cdot 3H_2O$ (20 mg); citric acid (3 mg); ferric ammonium citrate (3 mg); EDTA disodium potassium salt (0.5 mg); $NaHCO_3$ (2 mg); trace metal mix (1 mL). Trace metal mix (1 L): H_3BO_3 (2.86 g); $MnCl_2 \cdot 4H_2O$ (1.81 g); $ZnSO_4 \cdot 7H_2O$ (222 mg); $NaMoO_4 \cdot 2H_2O$ (390 mg); $CuSO_4 \cdot 5H_2O$ (79 mg); $Co(NO_3)_2 \cdot 6H_2O$ (49.4 mg). Seed cultures of *L. aestuarii* were transferred into the Petri dishes, which were then allowed to grow for a 2-month period.

Extraction and Purification. *L. aestuarii* was removed from the media by filtration to yield a cell mass (33.8 g wet weight), which was flash frozen with liquid N_2 and ground with a mortar and pestle. This material was extracted with methanol (4 × 500 mL). Wet HP-20 resin (Supelco) was added to 75% of the initial crude extract (1.5 L), and the extract was dried onto the resin by rotary evaporation. The resin was extensively washed with water and eluted with a methanol gradient (50%, 75%, and 100%), followed by 75% acetone in water. Fractions eluting in 100% methanol or 75% acetone were dried and resuspended in methanol, to which DMSO was then added yielding 80:20 DMSO/methanol mixtures that were purified by C_{18} flash chromatography. Columns were equilibrated in 1:1 methanol/water, the applied extract was washed with the same mixture, and the desired products were eluted with 75% acetonitrile. Finally, C_{18} HPLC was performed with a flow rate of 3 mL min^{-1} and a gradient from 1% to 60% acetonitrile over 60 min, to yield **1** (0.4 mg, 1.6×10^{-3}% of wet weight) and **2** (0.1 mg, 3.9×10^{-4}% of wet weight). For FTICR of the crude extract, a small-scale methanol extract was used with freshly flash-frozen material.

LC-MS Analysis of D/L-FDLA Derivatives. Compound **2** (20 μg) was dissolved in 6 N HCl (200 μL) and heated in sealed ampule vials at 110 °C for 8 h. The solvent was removed *in vacuo*. The acid hydrolysate of **2** was dissolved in H_2O. To a 50 μL aliquot was added 1 N $NaHCO_3$ (20 μL) and 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (1% solution in acetone, 100 μL), and the mixture was heated to 40 °C for 50 min. The solution was cooled to RT, neutralized with 1 N HCl (20 μL), and then dried *in vacuo*. The residue was dissolved in MeOH and then analyzed by LC-MS. The analysis of the L- and D-FDLA derivatives was performed on a EclipseXDB-18 column (150 × 4.6 mm, 5 μm) employing a linear gradient from 20% to 80% CH_3CN in 0.01 M ammonium formate at 0.5 mL min^{-1} over 50 min. The retention times of the D- and L-FDLA derivatives, respectively, were as follows: L-Ala, 17.90, 16.50 min, m/z 428 $[M - H]^-$; L-Val-Tzl, 31.80, 29.50 min, m/z 493 $[M - H]^-$; L-Pro, 24.18, 20.61 min, m/z 408 $[M - H]^-$; L-Tyr, 17.90, 17.05 min, m/z 474 $[M - H]^-$; L-Pro-Tzl, 26.38, 26.28 min, m/z 491 $[M - H]^-$.

Claisen Rearrangement. Compound **1** (0.4 mg) in 1:1 CD_3OD/D_2O was heated to 50 °C in the NMR probe and followed by ^1H NMR for 21 h.

ASSOCIATED CONTENT

ⓢ Supporting Information

FTICR/IRMPD characterization of **1** and **2**; LC-FTICR and MS/MS characterization; NMR spectra; LC-MS analysis of D/L-FDLA derivatives; identity of thermally produced aestuaramide C. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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