Trichormamides A and B with Antiproliferative Activity from the Cultured Freshwater Cyanobacterium *Trichormus* sp. UIC 10339

Shangwen Luo, † Aleksej Krunic, † Hahk-Soo Kang, ‡ Wei-Lun Chen, † John L. Woodard, ‡ James R. Fuchs, ‡ Steven M. Swanson, † and Jimmy Orjala* †

†Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, 833 S. Wood Street, Chicago, Illinois 60612, United States
‡Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, Ohio 43210, United States

Supporting Information

**ABSTRACT:** Two new cyclic lipopeptides, trichormamides A (1) and B (2), were isolated from the cultured freshwater cyanobacterium *Trichormus* sp. UIC 10339. The strain was obtained from a sample collected in Raven Lake in Northern Wisconsin. The planar structures of trichormamides A (1) and B (2) were determined using a combination of spectroscopic analyses including HRESIMS and 1D and 2D NMR experiments. The absolute configurations of the amino acid residues were assigned by the advanced Marfey’s method after acid hydrolysis. Trichormamide A (1) is a cyclic undecapeptide containing two D-amino acid residues (D-Tyr and D-Leu) and one β-amino acid residue (β-aminodecanoic acid). Trichormamide B (2) is a cyclic dodecapeptide characterized by the presence of four nonstandard α-amino acid residues (homoserine, N-methylisoleucine, and two 3-hydroxyleucines) and one β-amino acid residue (β-aminodecanoic acid). Trichormamide B (2) was cytotoxic against MDA-MB-435 and HT-29 cancer cell lines with IC₅₀ values of 0.8 and 1.5 μM, respectively.

Many structurally diverse lipopeptides, characterized by the presence of a lipid tail connected to a linear or cyclic oligopeptide, have been discovered from bacteria and fungi to date. As a class, lipopeptides are known to possess a wide range of biological activities including antimicrobial, antiproliferative, immunosuppressant, and surfactant properties. Studies into the modes of action of lipopeptides such as daptomycin, syringomycin, and syringopeptin revealed that the lipid tail plays a key role in their interactions with plasma membranes, which leads to ion pump activation, causing membrane depolarization and eventually gives rise to cell death. The cyclic forms of lipopeptides are thought to possess restricted conformational freedom and improved stability compared to their linearized forms. These characteristics are important in the interactions with relevant biological targets. There has been a considerable interest in the discovery, biosynthesis, and mechanisms of action studies of cyclic lipopeptides for their potential pharmaceutical applications.

Cyanobacteria are prolific producers of cyclic lipopeptides, and many structurally diverse cyclic lipopeptides have been reported. Their activities include antimicrobial, antiproliferative, antitumor, 20S proteasome inhibitory, and allelopathic activities. A common feature of the lipid portion of cyanobacterial cyclic lipopeptides is the presence of a β-amino or a β-hydroxy group, which is incorporated into the cyclic peptide backbone. The α position of the lipid unit is either hydroxylated, methylated, or left as a methylene. The chain of the lipid unit often shows modifications such as methylation, hydroxylation, carbonylation, and chlorination.

In our continuing search for antiproliferative natural products from cultured freshwater cyanobacteria, we evaluated the antiproliferative extract from *Trichormus* sp. UIC 10339. Morphological characterization and phylogenetic analysis using a partial 16S rDNA gene sequence identified this strain to be a *Trichormus* sp. (Supporting Information S1–S3). Herein we report the isolation, structure elucidation, and biological activity of two new cyclic lipopeptides, named trichormamides A (1) and B (2), each containing a β-aminodecanoic acid unit as part of their cyclic backbones.

**RESULTS AND DISCUSSION**

*Trichormus* sp. UIC 10339 was obtained from a sample collected from Raven Lake in Northern Wisconsin in August 2010 and cultured in Z media. It was harvested after 8 weeks of growth. The lyophilized cells were extracted with CH₂Cl₂ and MeOH (1:1) and dried in vacuo. This extract showed antiproliferative activity against the human colon cancer cell line HT-29 and was subjected to a bioassay-guided
fractionation scheme using Diaion vacuum liquid chromatography. The fraction eluting with 60% isopropyl alcohol (IPA) displayed antiproliferative activity. LC/MS and $^1$H NMR dereplication indicated the presence of two potentially new peptides with molecular weights of 1184 and 1446 Da. Subsequent reversed-phase HPLC separations yielded trichormamides A (1) and B (2).

Trichormamide A (1) was obtained as a white, amorphous powder. The molecular formula of 1 was deduced as $C_{58}H_{93}N_{11}O_{15}$ based on HRESIMS analysis ($m/z$ 1184.6978 [M + H]$^+$$^+$). The $^1$H NMR spectrum of 1 in DMSO-$d_6$ showed broad signals. The $^1$H NMR spectra of 1 in CD$_3$OD with suppression at 4.9 ppm exhibited sharper peaks, but the resonances close to 4.9 ppm were also suppressed. Therefore, $^1$H NMR, COSY, and TOCSY experiments were conducted in both CD$_3$OD (without signal suppression, but NH signals absent) and CD$_3$OH (suppressed at 4.9 ppm, but NH signals present), respectively, to obtain both NH signals and clear signals close to 4.9 ppm (Supporting Information S4–S12).

The values of chemical shifts were reported using 1D and 2D NMR spectra obtained in CD$_3$OD. The $^1$H NMR spectrum of 1 exhibited a signal pattern characteristic of a lipopeptide, including exchangeable amide signals ($\delta_H$ 6.7–8.9), signals typical of $\alpha$-protons ($\delta_H$ 4.1–5.0), aliphatic methylene signals ($\delta_H$ 1.1–1.4), and several doublet and triplet methyl signals ($\delta_H$ 0.7–1.0). The two downfield doublets ($\delta_H$ 7.25, $J = 8.7$ Hz; $\delta_H$ 6.71, $J = 8.7$) were attributed to a para-substituted phenyl moiety. Analysis of COSY and TOCSY correlations of 1 revealed the presence of 11 amino acid residues, including 10 standard $\alpha$-amino acid residues: three serines (Ser1, Ser2, Ser3), two prolines (Pro1, Pro2), two isoleucines (Ile1, Ile2), tyrosine (Tyr), leucine (Leu), and glycine (Gly). Sequential COSY correlations between NH ($\delta_H$ 7.44), methine H-3 ($\delta_H$ 4.36), and methylene H$_2$-2 ($\delta_H$ 1.59; 2.06), as well as COSY correlations between methine H-3 ($\delta_H$ 4.36) and methylene H$_2$-4 ($\delta_H$ 1.50; 1.58), suggested the presence of a $\beta$-amino acid residue. Sequential COSY correlations from H$_2$-4 ($\delta_H$ 1.50; 1.58) to the region of highly overlapped methylene signals ($\delta_C$ 32.9, 30.3, 30.2, 27.2, and 23.6), and finally to the methyl triplet H-10 ($\delta_H$ 0.86) identified this $\beta$-amino acid residue as $\beta$-aminodecanoic acid (Ada).

Assignment of the amino acid sequence was carried out by a combination of HMBC, ROESY, and ESIMS/MS analysis (Figure 1). HMBC correlations from Ile2-NH ($\delta_H$ 8.84) to Ile1 C-1 ($\delta_C$ 174.1), from Ile1-NH ($\delta_H$ 6.95) to Leu C-1 ($\delta_C$ 173.6), and...
from Leu-NH ($\delta_{H} 7.71$) to Tyr-C-1 ($\delta_{C} 175.1$), from Tyr-NH ($\delta_{H} 7.30$) to Ser3-C-1 ($\delta_{C} 173.0$), and from Ser3-NH ($\delta_{H} 7.57$) to Pro1-C-1 ($\delta_{C} 172.2$). Suggested the partial sequence of Ile2-Ile1-Leu-Tyr-Ser3-Pro1. HMBC correlation from Pro1 H2-5 ($\delta_{H} 3.60$; 3.88) to Ser2-C-1 ($\delta_{C} 174.3$) extended this sequence to Ile2-Ile1-Leu-Tyr-Ser3-Pro1-Ser2. Similarly, the HMBC correlations from Ser1-NH ($\delta_{H} 6.79$) to Ada-C-1 ($\delta_{C} 172.2$), from Ada-NH ($\delta_{H} 7.44$) to Gly-C-1 ($\delta_{C} 170.4$), and from Gly-NH ($\delta_{H} 8.75$) to Pro2-C-1 ($\delta_{C} 176.1$) established the partial sequence of Ser1-Ada-Gly-Pro2. This partial sequence was confirmed by ROESY correlations between Ser1-NH ($\delta_{H} 6.79$) and Ada H2-2 ($\delta_{H} 1.59$; 2.06) and between Ada-NH ($\delta_{H} 7.44$) and Gly H2-2 ($\delta_{H} 3.42$; 3.97). A ROESY correlation between Ser2-NH ($\delta_{H} 7.88$) and Ser1 H2-3 ($\delta_{H} 4.00$; 4.27) linked the two partial sequences together into Ile2-Ile1-Leu-Tyr-Ser3-Pro1-Ser2-Ser1-Ada-Gly-Pro2. The 18 degrees of unsaturation and the molecular formula suggested that 1 was a cyclic peptide. The chemical shifts of Ile2 C-1 ($\delta_{C} 173.8$), C-2 ($\delta_{C} 172.2$), Ada C-1 ($\delta_{C} 45.9$), H2-5 ($\delta_{H} 4.07$; 4.13) suggested the presence of an amide moiety at Ile2-C-1 and Pro2 N. Therefore, the cyclic undecapeptide ring was closed between Ile2 and Pro2. Mass fragmentation analysis using a quadrupole-time-of-flight (qTOF) tandem mass spectrometer confirmed the sequence of amino acid residues determined by NMR analysis. The parent ion [M + H]$^+$ 1184.6 was fragmented using a 40 eV collision energy. As expected, protonation and breakage of the proline amide bond is a highly favored process, and the macro ring of 1 opened between Ile2 and Pro2, forming a linear acylium ion. The acylium ion was continuously fragmented to generate fragment ions as illustrated in Figure 2. The ESIMS/MS fragments were in complete agreement with the structure determined by NMR studies, and the planar structure of 1 was determined as cyclo[Ile2-Ile1-Leu-Tyr-Ser3-Pro1-Ser2-Ser1-Ada-Gly-Pro2].

The absolute configurations of the amino acids were determined using acid hydrolysis followed by the advanced Marfey’s method (Supporting Information S14). The chromatographic comparison between Marfey’s derivatives of the hydrolysate of 1 and appropriate amino acid standards assigned the configurations for Ser1/2/3 and Pro1/2 and the configuration for Tyr. The absolute configuration of Ada was determined as 3R by comparison of the elution order of hydrolysate Ada L- and D-FDLA derivatives with those reported in the literature. The stereoisomers of leucine and isoleucine standards were analyzed by Marfey’s method, and retention times were compared with Marfey’s derivatives of hydrolysate of 1. As leucine and isoleucine isomers have identical molecular weight and very similar retention behavior in HPLC, a rigorous co-injection scheme was utilized to assign Leu as D and Ile1/2 as L (2S, 3S) (Supporting Information S14). The final structure of 1 was determined as cyclo[1-Ile-L-Ile-D-Leu-D-Tyr-L-Ser-L-Pro-L-Ser-L-Ser-(3R)-Ada-Gly-L-Pro].

Trichormamide B (2) was obtained as a white, amorphous powder. The molecular formula was determined as C$_{69}$H$_{115}$N$_{13}$O$_{20}$ (m/z 1446.8508 [M + H]$^+$) by HRESIMS analysis. The $^1$H NMR spectrum of 2 in DMSO-$d_6$ (Table 2) also exhibited signals characteristic of a lipopeptide. Signal doubling was observed for some of the signals in both the $^1$H NMR and DEPTQ spectra, indicating the presence of two conformers of 2 in solution (integration ratio 2:1 in DMSO-$d_6$ according to integration of the phenyl protons from tyrosine). This phenomenon has been reported for cyclic peptides with one or more N-methylated amino acid residues. An elevated-temperature NMR experiment was attempted to generate one average set of signals. However, the coalescence temperature of 2 was higher than the highest operating temperature permitted by a cryogenically cooled probe (T = 328 K), and no signal coalescence was observed. Nevertheless, the elevated-temperature NMR experiment showed improved peak shapes and better resolved coupling patterns as compared to the room-temperature experiments. Thus, the structure elucidation of 2 was performed using the signals from the major conformer at elevated temperature (T = 328 K). Combined analysis of the COSY and TOCSY spectra identified the structures of seven standard amino acids: isoleucine (Ile), glutamine (Glu), serine (Ser), proline (Pro), tyrosine (Tyr), and two threonines (Thr1 and Thr2) (Figure 1 and Table 2). An N-methylated isoleucine (NMelle) was deduced by TOCSY and the HMBC correlation observed from N-Me ($\delta_{H} 3.00$) to NMelle C-2 ($\delta_{C} 59.8$). The presence of homoserine (Hse) was evident by sequential COSY correlations from NH ($\delta_{H} 8.01$) to Hse H$_4$-4 ($\delta_{H} 3.42$; 3.50). The downfield chemical shifts of the Hse C-4 methine ($\delta_{C} 57.3$) and H$_4$-4 ($\delta_{C} 3.42$; 3.50) indicated the attachment of a hydroxy group to C-4. A broad hydroxy signal ($\delta_{H} 5.05$) with a COSY correlation to a downfield methine ($\delta_{H} 3.52$), as well as sequential COSY correlations between NH ($\delta_{H} 8.16$)/H-2 ($\delta_{H} 4.39$)/H-3 ($\delta_{H} 3.52$)/H-4 ($\delta_{H}$ 1.61)/H$_2$-5 ($\delta_{H}$ 0.93) combined with HMBC correlations from 4-Me ($\delta_{H} 0.81$) and H$_2$-5 ($\delta_{H}$ 0.93) to both C-3 ($\delta_{C}$ 76.3) and C-4 ($\delta_{C}$ 30.5), indicated the presence of a 3-hydroxyleucine (3-OHLeu). An additional 3-hydroxyleucine (3-OHLeu2) was identified using a similar approach (Table 2). The presence of a $\beta$-aminodecanoic acid was suggested from COSY, TOCSY, and HSQC correlations as described for trichormamide A (1).
Table 1. NMR Spectroscopic Data of Trichormamide A (1) in CD$_3$OH, $T = 300$ K

| position | $\delta_C$ a, mult. | $\delta_H$ b, mult. (J in Hz) | COSY | HMBC | ROESY |
|----------|---------------------|-------------------------------|-------|-------|--------|
| Ada      |                     |                               |       |       |        |
| 1        | 172.2, C            |                               | 3     | 1     |        |
| 2        | 42.8, CH$_2$        | 1.59, m                       |       |       |        |
|          |                     | 2.06, m                       |       |       |        |
| 3        | 47.7, CH            | 4.36, m                       | 2, 4, NH | 4, 5 |
| 4        | 35.9, CH$_2$        | 1.50, m                       | 3     | 1, 3  |        |
|          |                     | 1.58, m                       |       |       |        |
| 5        | 32.9, CH$_2$        | 1.28, m                       |       |       |        |
| 6        | 30.3, CH$_2$        | 1.29, m                       |       |       |        |
| 7        | 30.2, CH$_2$        | 1.32, m                       |       |       |        |
| 8        | 27.2, CH$_2$        | 1.33, m                       |       |       |        |
| 9        | 23.6, CH$_2$        | 1.31, m                       | 10    |       |        |
| 10       | 12.1, CH$_3$        | 0.86, t (7.4)                 | 9     | 9     |        |
| NH       |                     | 7.44, d (9.9)                 | 3     | 3, 1$_{Gly}$ | 2$_{Gly}$ |
| Ser1     |                     |                               |       |       |        |
| 1        | 175.5, C            |                               |       |       |        |
| 2        | 53.5, CH            | 4.94, dd (6.2, 3.0)           | NH, 3 |       |        |
| 3        | 63.1, CH$_2$        | 4.00, t (9.7)                 | 2     | 1, 2  |        |
|          |                     | 4.27, m                       |       |       |        |
| OH       |                     | 6.79, d (7.6)                 | 2     | 2, 3, 1$_{Ada}$ | 3, 2$_{Ada}$ |
| NH       |                     |                               |       |       |        |
| Ser2     |                     |                               |       |       |        |
| 1        | 174.3, C            |                               |       |       |        |
| 2        | 59.4, CH            | 4.34, m                       | NH, 3 | 1     |        |
| 3        | 61.9, CH$_2$        | 3.91, m                       | 2     | 1, 2  |        |
| OH       |                     | nd$^d$                        |       |       |        |
| NH       |                     | 7.88, br                      | 2     |       | 3$_{Ser}$ |
| Pro1     |                     |                               |       |       |        |
| 1        | 172.2, C            |                               |       |       |        |
| 2        | 62.4, CH            | 4.13, m                       | 3     | 3     |        |
| 3        | 30.1, CH$_2$        | 1.95, m                       | 2, 4  | 4     |        |
|          |                     | 2.14, m                       |       |       |        |
| 4        | 26.2, CH$_2$        | 2.21, m                       | 3, 5  | 3     |        |
|          |                     | 2.28, m                       |       |       |        |
| 5        | 48.6, CH$_2$        | 3.60, m                       | 4     | 3, 1$_{Ser2}$ | 3.88, m |
| Ser3     |                     |                               |       |       |        |
| 1        | 173.0, C            |                               |       |       |        |
| 2        | 55.5, CH            | 4.51, m                       | NH, 3 | 1     |        |
| 3        | 62.4, CH$_2$        | 3.78, m                       | 2     | 1, 2  |        |
|          |                     | 4.13, m                       |       |       |        |
| OH       |                     | nd$^d$                        |       |       |        |
| NH       |                     | 7.57, d (9.8)                 | 2     | 2, 1$_{Pro}$ | 3, 2$_{Pro}$ |
| Tyr      |                     |                               |       |       |        |
| 1        | 175.1, C            |                               |       |       |        |
| 2        | 59.9, CH            | 4.25, m                       | NH, 3 | 1, 3  |        |
| 3        | 37.7, CH$_2$        | 3.06, dd (13.9, 3.2)          | 2     | 1, 2  |        |
|          |                     | 2.98, dd (13.9, 10.8)         |       |       |        |
| 4        | 129.2, C            |                               |       |       |        |
| 5/9      | 131.2, CH           | 7.25, d (8.7)                 | 6/8   | 3, 4, 6/8, 7 |
| 6/8      | 116.4, CH           | 6.71, d (8.7)                 | 5/9   | 4, 5/9, 7 |
| 7        | 157.4, C            |                               |       |       |        |
| OH       |                     | nd$^d$                        |       |       |        |
| NH       |                     | 7.30, d (5.7)                 | 2     | 2, 1$_{Ser3}$ | 3, 2$_{Ser3}$ |
| Leu      |                     |                               |       |       |        |
| 1        | 173.6, C            |                               |       |       |        |
| 2        | 52.9, CH            | 4.53, m                       | NH, 3 | 1, 3, 4 |        |
| 3        | 40.1, CH$_2$        | 1.12, m                       | 2, 4  | 1, 2, 4, 4-Me, 5 |
|          |                     | 1.45, m                       |       |       |        |
| 4        | 26.0, CH            | 1.73, m                       | 3, 4-Me, 5 | 4-Me, 5 |
| 4-Me     | 23.6, CH$_2$        | 1.00, d (6.5)                 | 4     | 3, 4, 5 |
| 5        | 20.6, CH$_2$        | 0.84, d (6.3)                 | 4     | 3, 4, 4-Me |
| NH       |                     | 7.71, d (8.7)                 | 2     | 2, 3, 1$_{Tyr}$ | 2$_{Tyr}$, 3$_{Tyr}$ |
| Ile1     |                     |                               |       |       |        |
| 1        | 174.1, C            |                               |       |       |        |
| 2        | 58.1, CH            | 4.70, d (3.7)                 | NH, 3 | 1     |        |
| 3        | 41.6, CH            | 1.92, m                       | 2, 3-Me, 4 | 2, 3-Me, 4 |
| 3-Me     | 16.2, CH$_2$        | 0.91, d (6.3)                 | 3     | 2, 3, 4 |
| 4        | 24.1, CH$_2$        | 1.19, m                       | 3     | 3, 5  | 5     |

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Thr2 C-1 (δC 170.8), from Ile-NH (δH 8.16) to Ada C-1 (δC 171.2), from Ada-NH (δH 7.52) and Ada H-3 (δH 4.08) to Thr2 C-1 (δC 169.6), from Thr2-NH (δH 7.60) to Tyr C-1 (δC 170.8), and from Tyr-NH (δH 7.97) to Pro C-1 (δC 171.5) indicated a partial sequence of 3-OHLeu1-Ile-Ada-Thr2-Tyr-Pro. The molecular formula and degree of unsaturation indicated the cyclic nature of 2. The chemical shifts of Thr1 C-1 (δC 168.7) and C-2 (δC 55.6) suggested the presence of an amide moiety at Thr1 C-1.16 Similarly, the chemical shifts of Pro C-2 (δC 59.5), C-S (δC 47.0), and H2-5 (δH 3.61; 3.67) also indicated that Pro N was part of an amide bond.17 Therefore, the cyclic dodecapeptide ring was closed between Thr1 and Pro. Mass fragmentation analysis confirmed the sequence assignment of 2 (Figure 3). Protonation on the amide nitrogen at Pro generated amide bond cleavage to form a linear acylium ion, which produced series of fragments (Figure 3). These observed fragments were consistent with the amino acid sequence determined by NMR analysis, and the planar structure of 2 was assigned as cyclo[Thr1-Ser-NMeIle-Gln-3-OHLeu2-Hse-3-OHLeu1-Ile-Leu2-Thr2-Tyr-Pro] (2).

Acid hydrolysis of trichormamide B (2) followed by Marfey’s derivatization with L- and D-FDLA and LC/MS analysis assigned the L configurations for Gln, Pro, Ile (2S, 3S), Thr1/2 (2S, 3R), and the nonproteinogenic amino acid Hse, D configurations for Tyr and Ser, and 3R configuration for Ada. The four stereoisomers of NMeIle were synthesized according to the literature.28,29 On the basis of these standards, the absolute configuration of NMeIle was determined to be L (2S, 3S). The 2S, 3R and 2S, 3S isomers of 3-hydroxysebacic acid were synthesized following Bonnard’s procedure16 and were derivatized with L- and D-FDLA respectively. Because enantiomers exhibit identical retention behavior under non-chiral HPLC conditions, the (2S,3R)-d-FDLA derivative shows the same retention time as (2R,3S)-l-FDLA, and the (2S,3S)-d-FDLA derivative shows the same retention time as (2R,3R)-l-FDLA (detailed explanations can be found in Supporting Information S26). By comparing the above four retention times with retention times of 3-OHLeu1 and 3-OHLeu2 l-FDLA derivatives, both 3-OHLeu1 and 3-OHLeu2 were assigned as 2R, 3S. The complete structure of 2 was determined as cyclo[Thr1-Ser-NMeIle-Gln−3-OHLeu2-Hse−3-OHLeu1-Ile-Ada-Thr2-Tyr-Pro].

Trichormamide A (1) is a cyclic undecapeptide characterized by the presence of β-aminoacidic acid and two d-amino acid

### Table 1. continued

| position | δC | δH | mult. (J in Hz) | COSY | HMBC | ROESY |
|----------|----|----|----------------|------|------|------|
| 5        | 14.3, CH3 | 0.89, t (10.2) | 4 | 3, 4 | 1Leu | 2Leu |
| Ile2     | 173.8, C  | 6.95, d (9.8) | 2 | 1Leu | 2Leu |
|          | 52.1, CH | 4.65, dd (12.2, 2.4) | NH, 3 | 1, 3-Me, 4 |
|          | 26.0, CH  | 1.44, m | 2, 3-Me, 4 |
| 3-Me     | 90.5, CH3 | 0.96, d (6.5) | 3 |
| 4        | 40.5, CH3 | 1.43, m | 3, 5, 5 |
|          | 173.8, C  | 0.85, t m | 4 | 3, 4 |
| Pro2     | 88.4, d (7.9) | 2 | 2, 1Pro2 |
| Gly      | 170.4, C  | 3.42, d (17.3) | NH | 1, 1Pro2 |
|          | 43.9, CH2 | 3.97, d (17.3) | NHAds |
| Gly      | 8.75, br | 2 |

aCarbon chemical shifts were assigned from the DEPTQ spectrum recorded at 226 MHz. bRecorded at 600 MHz. cCarbon chemical shifts are interchangeable. dnd: not detected.
| position | δ_C a mult | δ_H b mult (J in Hz) | COSY | HMBC | ROESY |
|----------|------------|-----------------------|------|------|-------|
| Ada      | 1          | 171.2, C              |      |      |       |
|          | 2          | 40.2, CH_2            | 2.31, m | 3 | 1 | 3, NH_{Ile} |
|          | 3          | 46.0, CH_2            | 4.08, m | 2, 4, NH | 1 | 2, 4 |
|          | 4          | 33.4, CH_2            | 1.36, m | 3 |      |      |
|          | 5          | 28.8, CH_2            | 1.22, m overlapped | | | |
|          | 6          | 28.4, CH_2            | 1.22, m overlapped | | | |
|          | 7          | 25.1, CH_2            | 1.21, m overlapped | | | |
|          | 8          | 31.0, CH_2            | 1.22, m overlapped | | | |
|          | 9          | 21.8, CH_2            | 1.24, m 10 | | | |
|          | 10         | 13.6, CH_2            | 0.85, t (7.2) 9 | 8, 9 | | |
| Ile      | 1          | 170.8, C              |      |      |       |
|          | 2          | 57.6, CH_2            | 4.19, m | NH, 3 | 1, 3, 4 | 3 |
|          | 3          | 35.7, CH_2            | 1.80, m | 2, 3-Me, 4 | 2 |
|          | 4          | 15.1, CH_2            | 0.92, d (7.0) 3 | 2, 3, 4 | | |
|          | 5          | 24.4, CH_2            | 1.16, m | 3, 5 | | |
|          | 6          | 10.2, CH_3            | 0.91, m | 4 | 3, 4 | | |
|          | 7          | 8.16, d (7.4) | 2 | | | |
| 3-OHLeu | 1          | 172.3, C              | | | | |
|          | 2          | 55.3, CH_2            | 4.39, m | NH, 3 | 1, 4 | 3 |
|          | 3          | 76.3, CH_2            | 3.52, m | 2, 3-OH, 4 | 2, 4-Me, 5 |
|          | 4          | 30.5, CH_2            | 1.61, m | 3, 4-Me, 5 | | |
|          | 5          | 18.9, CH_2            | 0.81, d (6.5) | 4 | 3, 4, 5 | 3 |
|          | 6          | 18.5, CH_3            | 0.93, d (6.2) | 4 | 3, 4, 4-Me | 3 |
|          | 3-OH       | 5.05, s | | | | |
|          | 7          | 8.01, d (7.4) | 2 | | | |
| Hse      | 1          | 171.3, C              | | | | |
|          | 2          | 50.9, CH_2            | 4.35, m | NH, 3 | 1 | 3 |
|          | 3          | 34.3, CH_2            | 1.83, m | 2, 4 | | |
|          | 4          | 57.3, CH_3            | 3.42, m | 3 | | |
|          | 5          | 3.50, m nd | | | | |
|          | 6          | 7.95, d (9.6) | 2 | | | |
| 3-OHLeu2| 1          | 171.3, C              | | | | |
|          | 2          | 55.6, CH_2            | 4.32, m | NH, 3 | 1 | 3 |
|          | 3          | 75.9, CH_2            | 3.47, m | 2, 3-OH, 4 | 2, 4-Me, 5 |
|          | 4          | 29.7, CH_2            | 1.58, m | 3, 4-Me, 5 | 4-Me, 5 |
|          | 5          | 18.8, CH_2            | 0.90, d (6.4) | 4 | 3, 4, 5 | 3 |
|          | 6          | 18.5, CH_3            | 0.78, d (6.8) | 4 | 3, 4, 4-Me | 3 |
|          | 3-OH       | 4.93, s | | | | |
|          | 7          | 7.76, d (9.1) | 2 | | | |
| Gln      | 1          | 172.3, C              | | | | |
|          | 2          | 49.3, CH_2            | 4.59, m | NH, 3 | 3, 4, N-Me\textit{NMeIle} | | |
|          | 3          | 26.1, CH_2            | 1.75, m | 2, 4 | | |
|          | 4          | 30.6, CH_2            | 2.12, m | 3 | 5 | 2 |
|          | 5          | 174.4, C              | | | | |
|          | 6          | 7.93, d (9.1) | 2 | | | |
|          | 7          | 6.76, s | | | | |
|          | 8          | 7.17, s | | | | |
| NMelle   | 1          | 169.8, C              | | | | |
|          | 2          | 59.8, CH_2            | 4.73, d (11.0) | 3 | 1, 3 | N-Me |
|          | 3          | 31.5, CH_2            | 1.95, m | 2, 3-Me, 4 | 2, 3-Me | | |
|          | 3-Me      | 15.1, CH_2            | 0.83, m | 3 | 2, 3, 4 | | |
|          | 4          | 23.8, CH_2            | 1.31, m | 3, 5 | | |
|          | 5          | 10.6, CH_2            | 0.84, m | 4 | 3, 4 | | |
residues (D-Tyr and D-Leu). Trichormamide A (1) is a lipid-containing compound and displays amphiphilic character with half of the amino acid sequence being hydrophobic (Ada-Gly-Pro2-Ile2-Ile1-Leu) and the other half of the sequence hydrophilic (Tyr-Ser3-Pro1-Ser2-Ser1). The amphiphilic nature of lipopeptides has been found to be strongly correlated with their plasma membrane interactive potential.5−7 Trichormamide A (1) is structurally related to hormothamnin A, lobocyclamide A, and laxaphycin A with conserved amino acid residues of Leu, Ile1, Ile2, and Gly.14−16 The amino acid residues homoserine, 4-hydroxyproline, α,β-didehydro-α-amino butyric acid, and β-aminooctanoic acid in the east portion of the three related compounds were replaced by structurally related residues of Ser, Pro, Ser, and Ada in trichormamide A (1) (Supporting Information S27). Trichormamide B (2) is a cyclic dodecapeptide characterized by the presence of a β-

| position | δC^a | δH b mult. (J in Hz) | COSY | HMBC | ROESY |
|----------|------|---------------------|------|------|-------|
| Ser      |      |                     |      |      |       |
| N-Me     | 30.0 | CH3                 | 3.00 | s    | 2, 12n |
| Ser      | 1    | 170.6, C            |      |      |       |
| 2        | 549, CH | 4.33, m           | NH, 3 | 1    |       |
| 3        | 60.8, CH2 | 3.60, m        | 2    | 1    |       |
| OH       | ndf  | NH                  | 7.83, d (6.6) | 2 | 1
| NH       |      |                     |      |      |       |
| Thr1     |      |                     |      |      |       |
| 1        | 168.7, C | 4.42, dd (8.5, 3.4) | NH, 3 | 3    |       |
| 2        | 55.6, CH | 3.93, m           | 2, 4 |       |       |
| 3        | 66.2, CH2 | 1.08, d (3.9)     | 3    | 2, 3 |       |
| 4        | 188.8, CH | 1.08, d (3.9)     | 3    | 2, 3 |       |
| 3-OH     | ndf  | NH                  | 7.51, d (7.2) | 2 | 2
| Thr2     |      |                     |      |      |       |
| 1        | 171.5, C | 170.8, C          |      |      |       |
| 2        | 59.5, CH | 1.78, m           | 2, 4 |       |       |
| 3        | 28.6, CH2 | 1.78, m        | 2, 4 |       |       |
| 4        | 240.4, CH2 | 3.61, m      | 3, 5 |       |       |
| 5        | 47.0, CH2 | 3.67, m        | 4    |       |       |
| Ser      |      |                     |      |      |       |
| 1        | 170.5, C | 2, 12n           |      |      |       |
| 2        | 54.5, CH | 4.45, m           | 2    | 1, 2, 5/9 | 2 |
| 3        | 36.6, CH2 | 2.72, m      | 2    | 1, 2, 5/9 | 2 |
| 4        | 127.4, C | 6.99, d (8.3)    | 6/8  | 3, 5/9, 7 | 2 |
| 5/9      | 129.8, CH | 6.03, d (8.3)    | 5/9  | 3, 5/9, 7 | 2 |
| 6/8      | 114.7, CH | 6.63, d (8.3)    | 5/9  | 3, 5/9, 7 | 2 |
| 7        | 155.6, C | 129.8, CH        | 6/8  | 3, 5/9, 7 | 2 |
| Thr2     |      |                     |      |      |       |
| 1        | 169.6, C | 7.97, d (8.6)    | 2    | 12n  | 2 |
| 2        | 58.0, CH | 4.05, m           | NH, 3 | 1, 3 |       |
| 3        | 66.0, CH2 | 3.97, m        | 2, 4 |       |       |
| 4        | 192.4, CH2 | 0.87, d (6.6) | 3    | 2, 3 |       |
| 3-OH     | ndf  | NH                  | 7.60, d (8.1) | 2 | 1
| Thr2     |      |                     |      |      |       |
| 1        | 170.5, C | 170.8, C          |      |      |       |
| 2        | 54.5, CH | 1.78, m           | 2, 4 |       |       |
| 3        | 36.6, CH2 | 1.78, m      | 2, 4 |       |       |

^aCarbon chemical shifts were assigned from the DEPTQ spectrum recorded at 226 MHz. ^bRecorded at 600 MHz. ^Carbon chemical shifts are interchangeable. ^nd: not detected.

Figure 3. ESIMS/MS fragmentation of trichormamide B (2).
amino acid residue (β-aminodecanoic acid), two ω-3 amino acid residues (ω-Tyr and ω-Ser), and four nonstandard amino acid residues (3-OHLeu1, Hse, 3-OHLeu2, NMeIle). Trichormamido B (2) is also an amphiphilic molecule containing a hydrophobic Ada residue and seven hydroxy groups. Trichormamide B (2) is structurally related to the cyclic dodecapeptides lobocyclamides B and C, laxaphycins B, B2, and B3, and lyngbyacyclamides A and B.151–171 A comparison of 2 to these structures revealed that the presence and location of 3- OHLeu1, Gln, NMeIle, Thr1, and Thr2 are conserved in all these peptides, indicating these amino acid residues may be crucial for the biological functions of these molecules.

Trichormamides A (1) and B (2) were evaluated for their antiproliferative activities against the human melanoma cell line MDA-MB-435 and the human colon cancer cell line HT-29. Trichormamide B (2) displayed significant cytotoxic activity against MDA-MB-435 and HT-29 cell lines with IC50 values of 0.8 and 1.5 μM, respectively. Trichormamide A (1) was less active than 2 and showed IC50 values of 9.9 and 16.9 μM against MDA-MB-435 and HT-29, respectively. Compounds structurally related to 1 and 2 were reported to have 2-3-fold increased synergistic activities in antimicrobial and cytotoxicity assays.15,16,26,30 However, the limited supply of 1 stopped us from obtaining statistically significant data in synergistic assays.

In summary, we have obtained two new cyclic lipopeptides, trichormamides A (1) and B (2), each containing the β-amino acid residue β-aminodecanoic acid, from the cultured freshwater cyanobacterium Trichormus sp. UIC 10339. Their planar structures were determined by analysis of the HRESIMS, MS/MS, and 1D and 2D NMR experiments. The stereoconfigurations of the amino acid residues were assigned by the advanced Marfrey’s method using synthesized amino acid standards. Compounds 1 and 2 are not analogues, but both exhibited amphiphilic properties. Trichormamide B (2) was found to be more active against MDA-MB-435 and HT-29 human cancer cell lines than trichormamido A (1), with IC50 values of 0.8 and 1.5 μM, respectively.

**Experimental Section**

**General Experimental Procedures.** Optical rotations were measured on a PerkinElmer 241 polarimeter at 22 °C in MeOH. UV spectra were recorded on a Shimadzu UV spectrophotometer UV2401. IR spectra were acquired on a PerkinElmer 787 IR spectrophotometer. 1D and 2D NMR spectra including 1H NMR, COSY, TOCSY, HSQC, HMBC, and ROESY were obtained on a Bruker Avance DRX 600 MHz spectrometer. A Bruker AvII 900 MHz NMR spectrometer was used to acquire the DEPTQ spectra. 1H and 13C NMR chemical shifts were referenced to the DMSO-d6 (δH 2.50 and δC 39.51, respectively) and CD3OD/CD3OD (δH 3.31 and δC 49.0, respectively) residual solvent signals. The TOCSY experiments were conducted using a 60 ms mixing time. The ROESY spectra were acquired with a 200 ms mixing time. The HMBC spectrum was recorded with an average JCH of 8 Hz, and the HSQC spectrum was recorded with an average JCH of 145 Hz. High-resolution ESI mass spectra and LC-MS data were obtained using a Shimadzu HPLC-HT-TOF spectrometer. The HPLC experiments were performed using an Agilent 1100 series instrument equipped with a diode array detector, and a Waters instrument equipped with a Waters 600 pump and a Waters 2487 detector.

**Biological Material.** Trichormus sp. UIC 10339 was isolated from a sample collected at Raven Lake, Wisconsin, in 2010 (N 45°58.7’, W 89°51.5’). The unialgal (not axenic) strain UIC 10339 was obtained from the UIC strain collection. The unialgal (not axenic) strain UIC 10339 was obtained from the UIC strain collection. The unialgal (not axenic) strain UIC 10339 was obtained from the UIC strain collection.

Cultures were illuminated with fluorescent lamps at 1.03 klx with an 18/6 h light/dark cycle. The temperature of the culture room was maintained at 22 °C. The biomass of UIC 10339 was harvested after 8 weeks of growth by centrifugation and freeze-dried.

**Morphological Identification.** Initial taxonomic identification was performed using a cultured UIC 10339 cyanobacterium. Morphological observation was performed using a Zeiss Axiosstar Plus light microscope equipped with a Canon PowerShot A620 camera. The following characters were utilized for identification: structure of colony, morphology of thallus, size, shape, and motility of trichomes, shape of vegetative cells, presence or absence of mucilage or sheath, presence and arrangement of heterocytes, presence and relative size of akinetes, and morphology of the end cell (Supporting Information S1, S2). The morphological characterization and initial taxonomical designation of UIC 10339 were made according to the system by Komárek et al.

**DNA Extraction, 16S rDNA PCR Amplification, and Sequencing.** Prior to DNA extraction, 8 mL of a static culture of Trichormus sp. UIC 10339 was combined with 1.5 mL of lysozyme buffer and 0.5 mL of 20 mg/mL lysozyme stock solution and incubated at 35 °C for 1 h. After incubation, the cell mass was centrifuged and transferred to a 2 mL microcentrifuge tube. A Wizard Genomic DNA purification kit (Promega) was used to extract the genomic DNA. A partial sequence of the 16S rDNA gene was PCR-amplified using the cyanobacteria-specific primers 106F and 1509R.31 For a total volume of 25 μL, the reaction mixture contained DNA (1 μL, approximately 300 ng), GoTaq reaction buffer (5 μL, 5X), dNTP mix (0.5 μL, 10 mM), upstream and downstream primer (1 μL each, 10 μM), GoTaq DNA polymerase (0.25 μL, 2.5 u/μL), and H2O (16.25 μL). The reaction was performed in a Bio-Rad C1000 thermal cycler with the following reaction program: initial denaturation for 30 s at 98 °C, 35 amplification cycles of 10 s at 98 °C, 30 s at 53 °C, 30 s at 72 °C, and a final extension for 10 min at 72 °C. PCR products were purified using a MinElute PCR purification kit (Qiagen) and sequenced using the cyanobacteria-specific primers 106F, 599F, and 1509R.33 The resulting 16S rDNA gene sequence was deposited in the NCBI GenBank under the accession no. KF444210.

**Extraction and Isolation.** Lyophilized biomass (6.14 g) from eight 2 L cultures of UIC 10339 was extracted with 1:1 CH3Cl/MeOH three times and concentrated in vacuo to yield 650.9 mg of extract, which was fractionated using Diaion HP-20SS resin with an H2O/IPA step gradient (1:0, 4:1, 3:2, 2:3, 3:7, 1:4, 1:9, 0:1 v/v) to yield eight fractions. The fraction eluting with H2O/IPA (2:3 v/v) (58.8 mg) displayed significant antiproliferative activity against the human melanoma cell line MDA-MB-435 with 100% growth inhibition at a concentration of 25 μg/mL. LC-MS combined with HPLC revealed that this fraction revealed the presence of novel lipopeptides. The fraction was subjected to semi preparative reversed-phase HPLC (Varian C8 semipreparative column, 10 × 250 mm, 3 mL/minute) with a linear gradient from 80% to 100% MeOH over 20 min, and the subfraction eluting from 9 to 10 min was collected and dried in vacuo. H NMR and MS flow injection analysis revealed that this subfraction consisted of two compounds. The subfraction was subjected to semi preparative reversed-phase HPLC (Varian C18 semipreparative column, 10 × 250 mm, 3 mL/minute) using isocratic 50% aqueous CH3CN. Trichormamide A (1, 0.8 mg, 0.01% of dry weight) was eluted at 8 min, and trichormamide B (2, 3.7 mg, 0.06% of dry weight) was eluted at 9 min.

**Trichormamide A (1):** white, amorphous powder; [α]23D = +20 (c 0.06, MeOH); UV (MeOH) λmax (log ε) 240 (3.07), 292 (2.95) nm; IR (neat) νmax 3311 (br), 2984, 2942, 2831, 1449, 1414 cm−1; 1H and 13C NMR see Table 1; HR-ESI-TOF-MS (+) m/z 1184.6978 [M+H]+ (calcd for C64H55O15N28 1184.6931).

**Trichormamide B (2):** white, amorphous powder; [α]23D = −25.9 (c 0.32, MeOH); UV (MeOH) λmax (log ε) 240 (3.16), 278 (2.97) nm; IR (neat) νmax 3317 (br), 2943, 2831, 1449, 1414, 1114 cm−1; 1H and 13C NMR see Table 2; HR-ESI-TOF-MS (+) m/z 1446.8508 [M + H]+ (calcd for C68H62N16O26S 1446.8460).

**Synthesis of N-Methylisoleucine, ω-N-Methylisoleucine, ω-αllo- N-Methylisoleucine, and ω-αllo- N-Methylisoleucine** (Based on Shendage’s and Malkov’s Methods28,29). The amino group of...
t-Ile was Boc-protected using a previously published protocol.28 The methylation of NBoc-t-Ile was carried out using Malkov’s method.29 The Boc-protected NMeIle was subjected to acid hydrolysis using the general peptide hydrolysis scheme as described below, to yield t-N-methylisoleucine (73.11 mg), which was analyzed by the advanced Marfey’s method. Although citric acid from the workup was observed as a minor impurity in the t-H NMR spectrum of t-N-methylisoleucine, it did not interfere with the advanced Marfey’s analysis. t-N-Methylisoleucine, t-allo-N-methylisoleucine, and t-allo-N-methylisoleucine were synthesized using the same protocol with t-Ile, t-allo-Ile, and t-allo-Ile as starting compounds. All were analyzed using the advanced Marfey’s method as described for the peptide hydrolysate. t-N-Methylisoleucine: [α]2O +17.6 (c 0.39, MeOH); t-H NMR data (DMSO-d6, 400 MHz) δ 3.84 (1H, d, J = 3.0 Hz), 2.59 (3H, s), 1.95 (1H, m), 1.50 (1H, m), 1.32 (1H, m), 0.91 (3H, d, J = 8.5 Hz), 0.90 (3H, t, J = 7.1 Hz); DEPTQ data (DMSO-d6, 400 MHz) δ 169.5, 64.6, 35.3, 32.5, 26.4, 14.5, 12.0. NMR spectra of D-Ile can be found in Supporting Information S24.

Synthesis of (2S,3R)-3-Hydroxylycine and (2S,3S)-3-Hydroxylycine. These compounds were synthesized using the procedures by Bonnard et al.16 We obtained 148.0 mg of major precursor (R)-1-((2S,3R)-3-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)-2-methylpropan-1-ol and 54.5 mg of minor precursor (S)-1-((2S,3S)-3-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)-2-methylpropan-1-ol. The hydroxylycine precursors were hydrolyzed following the general peptide acid hydrolysis process as described below. The major precursor gave a mixture of (2S,3R)-3-hydroxylycine and t-valine, and the minor precursor gave a mixture of (2S,3S)-3-hydroxylycine and t-valine. These two mixtures were derivatized with t-FDLA and t-FDLA, respectively, and the resulting four derivatization mixtures were analyzed using the general LC-MS method as described for peptide hydrolysate analysis. t-H NMR data of minor precursor (CDCl3, 300 MHz): δ 4.17 (1H, dd, J = 4.3 Hz, 4.0 Hz), 3.98 (1H, dd, J = 3.7 Hz, 3.6 Hz), 3.71 (3H, s), 3.70 (3H, s), 3.65 (1H, br), 2.27 (1H, m), 1.82 (1H, m), 1.68 (1H, br), 1.04 (3H, d, J = 6.9 Hz), 0.90 (3H, d, J = 6.9 Hz), 0.89 (3H, d, J = 6.9 Hz), 0.69 (3H, d, J = 6.9 Hz).

Acid Hydrolysis of Trichormides A (1) and (2). Approximately 0.3 mg of 1 and 2 was hydrolyzed with 1 mL of 6 N HCl for 20 h at 110 °C in separate Teflon-sealed Ace pressure tubes. The cooled hydrolysate mixtures were evaporated to dryness, and traces of HCl were removed from the reaction mixtures by repeated evaporation.

Absolute Configurations of Amino Acids by the Advanced Marfey’s Analysis. t- and d-FDLA (1-fluoro-2,4-dinitrophenyl-5-lysine) were purchased from TCI America. Each acid hydrolysate (approximately 0.3 mg) was separated into two equal portions for derivatization with either t-FDLA or d-FDLA. Each portion was dissolved in 110 μL of acetonitrile and 50 μL of H2O, followed by mixing with 20 μL of 1 N NaHCO3. Finally, 20 μL of t-FDLA or d-FDLA (10 mg/mL in acetonitrile) was added, and the mixtures were heated to 40 °C for 1 h. The reaction mixtures were then cooled to room temperature, and 20 μL of 1 N HCl was added to quench the reaction. The cooled reaction mixtures were evaporated to dryness and redissolved in 300 μL of CH3CN. LC-MS analysis was carried out using a reversed-phase column (Phenomenex Kinetex C18 250 × 4.6 mm, 5 μm, 1.0 mL/min) with a linear gradient from 25% to 65% aqueous CH3CN containing 0.1% formic acid over 50 min. The selective ion chromatograms of t-FDLA and d-FDLA derivatives of each amino acid were compared for the assignment of amino acid configurations. Detailed reports on retention times of each amino acid can be found in Supporting Information S14 and S23.

Antiproliferative Assay. Cytotoxicity assays against the MDA-MB-435 and HT-29 cancer cell lines were conducted according to established protocols.34

■ ASSOCIATED CONTENT

Supporting Information

Supplementary data (morphological and phylogenetic analysis of Trichormus sp. UIC 10339; 1H NMR, DEPTQ, COSY, TOCSY, HMQC, HMBD, and ROESY spectra of 1 and 2; Marfey’s and advanced Marfey’s analysis of 1 and 2; MS/MS spectra of 1 and 2) associated with this article are available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +1-312-996-5583; Fax: +1-312-996-7107. E-mail: orjala@uic.edu.

Notes

The authors declare no competing financial interest.

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