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Trikoramide A, a Prenylated Cyanobactin from the Marine Cyanobacterium *Symploca hydnoides*

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Keywords: *Symploca hydnoides*, marine cyanobacterium, cyanobactin, cyclic decapeptide

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ABSTRACT

In this study, a new cyclic decapeptide, trikoramide A (**1**), has been isolated from samples of the marine cyanobacterium, *Symploca hydnoides*, collected from Trikora beach, Bintan Island, Indonesia. Trikoramide A (**1**) is the first report of a *C*-prenylated cyclotryptophan-containing cyanobactin from *S. hydnoides*. Its planar structure was deduced by extensive 1D and 2D NMR spectroscopy in conjunction with HR mass spectrometry. In addition, its absolute configuration was determined by Marfey's method as well as 2D NOESY NMR spectral analysis. Compound **1** possessed *in vitro* cytotoxicity against the MOLT-4 human leukemic cancer cell line with IC_{50} value of 5.75 μM as well as weak quorum sensing inhibitory activity based on the *Pseudomonas aeruginosa* lasB-gfp bioreporter strain.

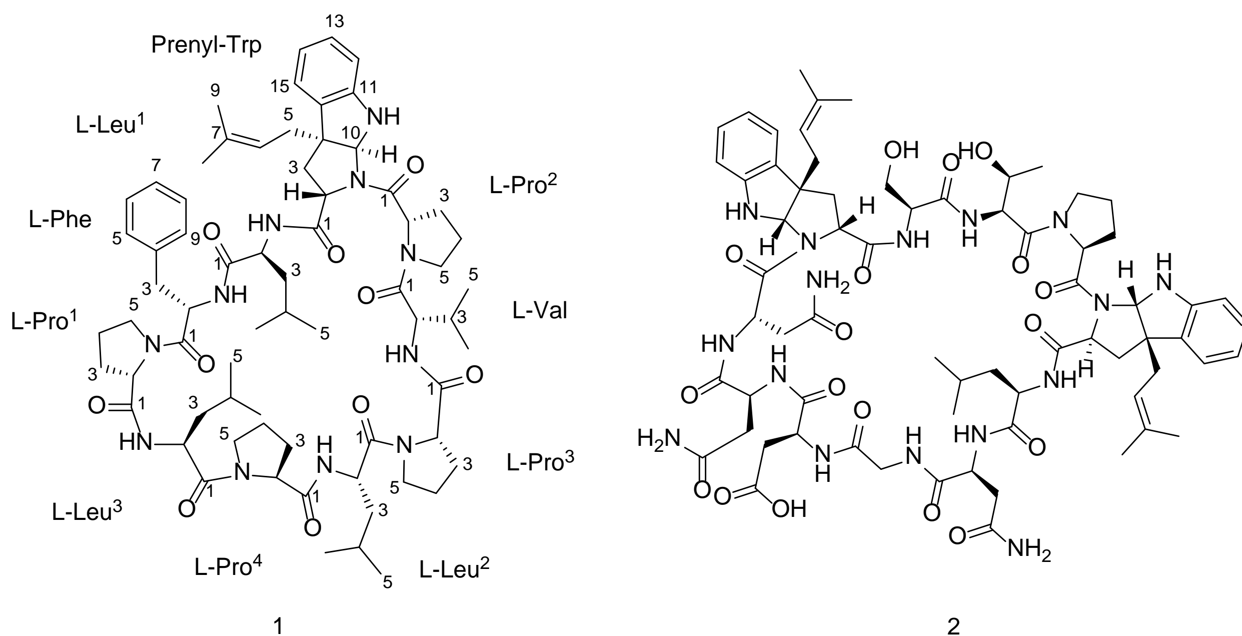
Cyanobacteria are an ancient group of photosynthetic prokaryotes capable of producing a wide variety of bioactive natural products.[1] Many of these active molecules are produced through the polyketide, non-ribosomal peptide (NRP), hybrid polyketide-NRP biosynthetic pathways or by the ribosomally synthesized and post-translationally modified peptide (RiPP) pathway.[1] Among these, RiPPs are one of the emerging groups of bioactive compounds produced by cyanobacteria. Belonging to this group are the cyanobactins, which in recent years have been gaining attention due to their interesting bioactivities.[2] Cyanobactins are defined as ribosomally derived, N–C terminally cyclized peptides, having 6 to 20 amino acids in length, and are biosynthesized by cyanobacteria. Unique modifications, including prenylation by dimethylallylpyrophosphate (DMAPP) and/or heterocyclization of Cys, Ser, or Thr, are also reported in a number of cyanobactins. L-amino acids are usually found in cyanobactins, although rare D-amino acids may be present in the molecules. A majority of the cyanobactins are cyclic peptides with a few examples of linear peptides having rare post-translational modifications.[3]

Although found in various marine animals, such as sponges, mollusks and ascidians, cyanobactins are believed to be primarily produced by cyanobacteria.[4] It has been estimated that up to 30% of all cyanobacteria, including symbiotic and free-living forms, have the genetic capacity to produce cyanobactins.[5] Recently discovered cyanobactins include sphaerocyclamide[6] from *Sphaerospermopsis* sp., piricyclamide[7] from *Microcystis* sp., viridisamide A[3] from *Oscillatoria* sp. and croissamide[8] from *Symploca* sp. Most of the cyanobactins are known for their cytotoxic properties while several others are reported to have antimalarial, allelopathic against competing strains, antifouling, antiviral and anti-multidrug resistant (MDR) activities.[9] Some of the most cytotoxic cyanobactins, including dolastatin 3, trunkamide, ulithiacyclamide, and lissoclinamide 7, are reported to possess potent anti-cancer activities against various human

cancer cell lines.[6, 10-11] In this paper, we report our investigation on the secondary metabolites from the marine cyanobacterium *Symploca hydnoides*, which led to the isolation of a novel bioactive cyanobactin, trikoramide A (**1**), a cyclic decapeptide containing four prolines, three leucines, one valine, one phenylalanine and an unusual *C*-prenylated cyclotryptophan unit.

RESULTS AND DISCUSSION

Marine cyanobacterial samples were collected at Trikora beach, Bintan Island, Indonesia over a period of two years in April of 2018 and 2019. Based on its morphological observations, the cyanobacterium was identified as *Symploca hydnoides* (see Supplementary Data for details). The sample was extracted with CH_2Cl_2:MeOH (2:1) and was subjected to normal phase vacuum liquid chromatography (NP-VLC), where it was separated into eight fractions. The most polar fraction, eluted with 10% MeOH in EtOAc was then subjected to fractionation by reverse-phased solid phase extraction (RP-SPE) followed by a series of reversed-phase HPLC purifications to afford trikoramide A (**1**), as a white amorphous solid (15.1 mg).



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Trikoramide A (**1**) gave a $[M + H]^+$ protonated molecule at *m/z* 1228.7508 by HR-ESI-OrbitrapMS and is consistent with the molecular formula $C_{68}H_{97}N_{11}O_{10}$ and having 26 degrees of unsaturation. The presence of exchangeable doublets between δ_H 6.79 and 8.01 was consistent with amide NH signals, and methine protons between δ_H 3.36 to 4.82 was consistent with presence of α protons in the ^{1}H NMR data, suggesting **1** to be of peptidic nature. ^{1}H and ^{13}C NMR in combination with 2D NMR spectral data, including HSQC, COSY and HMBC, established the presence of nine standard amino acids (4 × Pro, 3 × Leu, 1 × Val and 1 × Phe) and an unusual amino acid, Prenyl Trp, that relates to an identical unit present in kawaguchipeptin A[12] (**2**), which was previously reported from a freshwater cyanobacterium, *Microcystis aeruginosa* (NIES-88).

The structure of the *C*-prenylated cyclotryptophan unit in trikoramide A was confirmed by a combination of ^{1}H-^{1}H COSY and HMBC correlations (Table 1). Six aromatic carbon signals in ^{13}C NMR spectral data were observed at δ_C 148.5, 130.6, 122.6, 128.7, 118.4, 109.5 and the UV absorption (λ_{max} 210 and 295 nm) indicated presence of an indoline moiety.[13-14] The presence of indoline moiety was further evident from the correlations in the ^{1}H-^{1}H COSY correlations between the four aromatic protons (H-12 to H-15). The methine proton, δ_H 5.41, was assigned to C-10 of Prenyl-Trp in comparison with kawaguchipeptin A (**2**).[12] However, trikoramide A is not a structural analogue of kawaguchipeptin A (**2**). There were four HMBC correlations observed for this methine proton as depicted in Figure 1. Using HMBC, HSQC and ^{1}H-^{1}H COSY spectral data, the prenyl group was identified to be connected to C-4 of Prenyl-Trp by the HMBC correlation between H-5 and C-4.

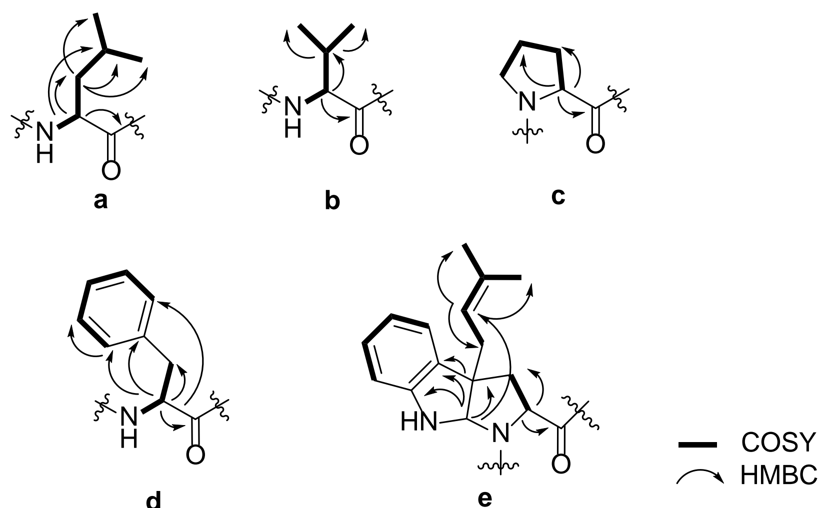

Figure 1. Partial structures of trikoramide A (**1**) as determined by 2D NMR analysis with its key 2D correlations.

Partial structures **a-e** (Figure 1) were first linearly assembled with their sequence determined by a combination of HMBC correlations and MS/MS fragmentation. Five key HMBC correlations, NH of Phe/C-1 of Leu^{1}, NH of Leu^{1}/C-1 of Prenyl-Trp, NH of Val/C-1 of Pro^{3}, NH of Leu^{2}/C-1 of Pro^{4} and NH of Leu^{3}/C-1 of Pro^{1} were observed. Based on these observations, the presence of sequence Pro^{1} – Phe – Leu^{1} – Prenyl Trp – Pro^{2} – Val – Pro^{3} – Leu^{2} – Pro^{4} – Leu^{3} was identified and confirmed based on HR MS/MS fragmentation pattern (Figure 2). The geometries (Figure 3) of the amide bonds at Pro residues are determined based on the ^{13}C NMR chemical shift difference at the β and γ positions ($\Delta\delta_{\beta-\gamma}$).[8, 15] The large difference calculated for Pro^{1} ($\Delta\delta_{\beta-\gamma}$ = 9.9ppm) and Pro^{2} ($\Delta\delta_{\beta-\gamma}$ = 10.1 ppm) indicated that their peptide bond are in *cis* geometry. For Pro^{3} ($\Delta\delta_{\beta-\gamma}$ = 5.3 ppm) and Pro^{4} ($\Delta\delta_{\beta-\gamma}$ = 3.5 ppm), their amide geometries could not be clearly determined from these values alone. Their geometries were subsequently determined to be *trans* based on the following NOESY correlations observed: H2 of Leu^{2} and H5a/5b of Pro^{3}; H2 of Leu^{3} and H5a/5b of Pro^{4}.

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Based on the number of double bonds equivalent calculated from its molecular formula, there was presence of one additional ring, which suggested that **1** must be a cyclic peptide. Therefore, the planar structure of trikoramide A was established as shown in **1**.

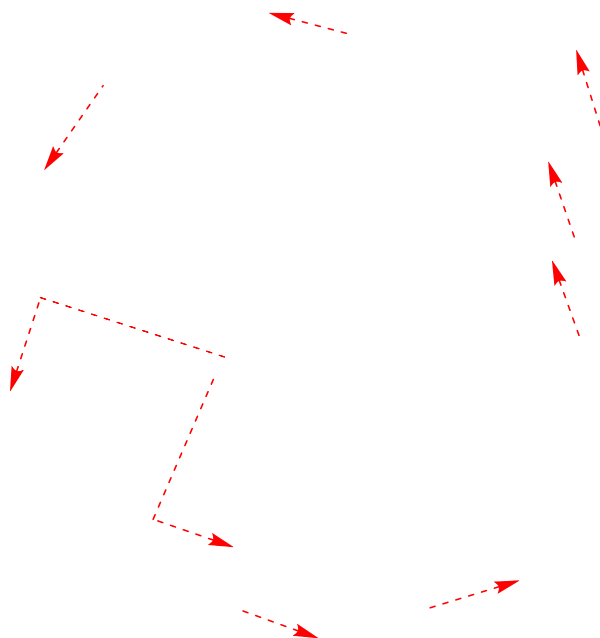

Figure 2. MS/MS fragmentation of trikoramide A (**1**).

The absolute stereochemical configuration of the amino acid units in trikoramide A (**1**) were determined using the Marfey's analysis.[16] The acid hydrolysate of trikoramide A (0.2mg, 6N HCl, 110 °C, overnight) was derivatized with 1-fluro-2,4-dinitro-phenyl-5-L-valine amide (L-FDVA) and analysed with RP C_{18} column on LC-MS system, in comparison with the t_R (min) and *m/z* values of the respective L-FDVA derivatized D- and L-amino acid standards. Based on the results obtained, it was evident that only L-configuration amino acid residues, i.e., L-Pro, L-Val, L-Leu, L-Phe and L-Trp, were present in **1**.

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Table 1. NMR Data for trikoramide A (1) in $CDCl_3$

Unit	Position	δ_C, type	δ_H (J in Hz)	COSY	HMBC[a]	NOESY (selected)
Pro[1]	1	170.0, qC				
	2	60.9, CH	4.03, brd (8.1)	3a, 3b	1, 3, 4, 5	
	3a	31.9, CH_2	2.11, m	3b, 2, 4	2, 4	
	3b		2.42, m	2, 3a, 4	2, 4	
	4	22.0, CH_2	1.94, m	3a, 3b, 5a, 5b	2, 3, 5	
	5a	46.7, CH_2	3.55, m	4, 5b	4	
	5b		3.67, m	4, 5a	4	
Phe	1	172.5, qC				
	2	56.6, CH	3.36, m	3a, 3b, NH	1, 3, 4, 5, 9	
	3a	33.8, CH_2	2.34, m	2, 3b	2, 4, 5	
	3b		3.33, m	2, 3a	2, 4, 5	
	4	139.4, qC			3, 6, 8	
	5 & 9	130.5, CH	7.16, d (7.4)	6, 8	3, 5, 7, 9	
	6 & 8	127.8, CH	7.25, d (7.0)	5, 7, 9	4, 5, 7, 9	
	7	125.3, CH	7.17, d (7.4)	6, 8	5, 6, 8, 9	
	NH		7.49, d (5.3)	2	1 (Leu[1])	
Leu[1]	1	171.7, qC				
	2	50.1, CH	4.82, dd (14.4, 7.5)	3a, 3b, NH	1, 3, 4	
	3a	41.9, CH_2	1.66, m	2, 3b, 4	2, 4, 5	
	3b		1.9, m	2, 3a, 4	2, 4, 5	
	4	23.5, CH	1.88, m	3a, 3b, 5, 6	2, 3, 5, 6	
	5	21.6, CH_3	0.95, d (6.6)	4	2, 3, 4	
	6	23.3, CH_3	0.85, d (7.4)	4	2, 3, 4	
	NH		8.06, brd (7.4)	2	1 (Prenyl Trp)	

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Unit	Position	δ_C, type	δ_H (J in Hz)	COSY	HMBC[a]	NOESY (selected)
Prenyl Trp	1	172.9, qC				
	2	59.2, CH	3.82, dd (10.1, 7.1)	3a, 3b	1, 3	
	3a	36.9, CH_2	2.06, m	2, 3b	2	
	3b		2.48, dd (12.0, 7.2)	2, 3a	2	
	4	58.1, qC			10, 15, NH	
	5	35.6, CH_2	2.41, m	6, 8, 9	4	10
	6	118.3, CH	5.14, t (7.2)	5, 8, 9	5, 8, 9, 10	
	7	135.8, qC			5, 8, 9	
	8	17.9, CH_3	1.50, s	5, 6	5	
	9	25.9, CH_3	1.71, s	5,6	5	
	10	81.4, CH	5.41, s		4, 6, 11, 16	5, 2 (Leu[3])
	11	148.5, qC			NH, 10, 12, 13, 14	
	12	109.5, CH	6.66, d (8.0)	13	13, 14, 15	
	13	128.7, CH	7.08, t (7.6)	12	12, 14, 15	
	14	118.4, CH	6.70, t (7.32)	15	12, 13, 15	
	15	122.6, CH	7.00, d (7.2)	14	12, 13, 14	
	16	130.6, qC			NH, 10	
	NH		7.85, brs		4, 11, 16	
Pro[2]	1	171.5, qC				
	2	60.8, CH	4.24, brd (8.5)	3a, 3b	3, 4, 5	2 (Prenyl Trp)
	3a	32.3, CH_2	2.23, m	2, 3b, 4	2, 4	
	3b		2.42, m	2, 3a, 4	2, 4	
	4	22.2, CH_2	1.75, m	3a, 3b, 5a, 5b	2, 3, 5	
	5a	47.2, CH_2	3.53, m	4, 5b	4	
	5b		3.67, m	4, 5a	4	
Val	1	170.5, qC				

Unit	Position	δ_C, type	δ_H (*J* in Hz)	COSY	HMBC[a]	NOESY (selected)
	2	56.5, CH	4.47, m	3, NH	1, 3	5a, 5b (Pro[2])
	3	30.2, CH	2.21, m	2, 4	2, 4, 5	
	4	18.1, CH_3	0.87, s	3	3, 5	
	5	19.4, CH_3	0.88, s	3	3, 4	
	NH		7.34, brd (9.7)	2	1 (Pro[3])	
Pro[3]	1	170.8, qC				
	2	60.5, CH	4.44, m	3a, 3b	1, 3, 4, 5	
	3a	29.6, CH_2	1.31, m	2, 3b, 4	2, 4	
	3b		2.22, m	2, 3a, 4	2, 4	
	4	24.3, CH_2	2.06, m	3a, 3b, 5a, 5b	2, 3, 5	
	5a	40.6, CH_2	1.62, m	4, 5b	4	
	5b		1.84, m	4, 5a	4	
Leu[2]	1	172.9, qC				
	2	53.6, CH	4.6, m	3a, 3b, NH	1, 3, 4	5a, 5b (Pro[3])
	3a	40.1, CH_2	1.67, m	2, 3b, 4	2, 4, 5	
	3b		1.85, m	2, 3a, 4	2, 4, 5	
	4	25.3, CH	1.56, m	3a, 3b, 5, 6	2, 3, 5, 6	
	5	23.3, CH_3	0.87, d (6.8)	4	2, 3, 4	
	6	23.4, CH_3	0.97, d (6.8)	4	2, 3, 4	
	NH		6.79, brd (8.8)	2	1 (Pro[4])	
Pro[4]	1	170.3, qC				
	2	58.2, CH	3.09, m	3a, 3b	3, 4, 5	
	3a	28.1, CH_2	1.59, m	2, 3b, 4	2, 4	
	3b		2.11, m	2, 3a, 4	2, 4	
	4	24.6, CH_2	2.06, m	3a, 3b, 5a, 5b	2, 3, 5	
	5a	47.8, CH_2	2.83, m	4, 5b	4	

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Unit	Position	δ_C, type	δ_H (J in Hz)	COSY	HMBC[a]	NOESY (selected)
	5b		3.32, m	4, 5a	4	
Leu[3]	1	170.7, qC				
	2	49.9, CH	4.42, m	3a, 3b, NH	1, 3, 4	5a, 5b (Pro[4])
	3a	26.1, CH_2	1.05, m	2, 3b, 4	2, 4, 5, 6	
	3b		2.20, m	2, 3a, 4	2, 4, 5, 6	
	4	24.9, CH	1.53, m	3a, 3b, 5, 6	2, 3, 5, 6	
	5	20.7, CH_3	0.91, d (6.4)	4	2, 3, 4	
	6	21.0, CH_3	1.02, d (6.4)	4	2, 3, 4	
	NH		8.01, d (8.4)	2	1 (Pro[1])	2 (Pro[1])

[a] HMBC correlations optimized for $^{2/3}J_{CH}$ = 8.0 Hz, are from protons stated to the indicated carbon.

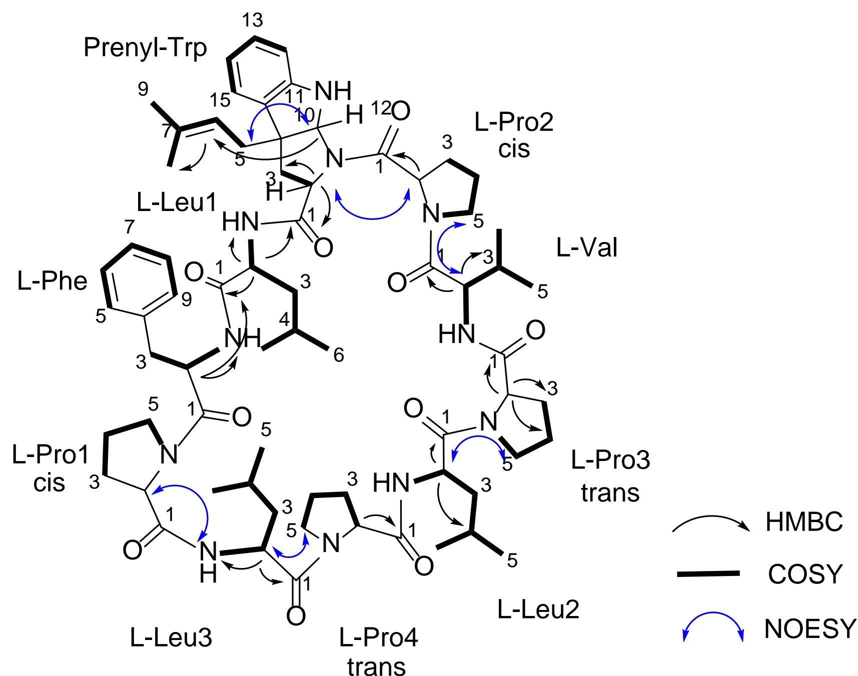

Figure 3. Structure of trikoramide A with key 2D correlations between subunits.

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For the relative and absolute stereochemistry of the unusual Prenyl-Trp residue, it was deduced with the use of NMR spectral and chemical studies. From the Marfey's analysis of the amino acids in the acid hydrosylate of **1**, L-Trp was the degradation product and therefore, C-10 in Prenyl Trp unit was established to be of *S* configuration (Figure 4). A similar approach was used to determine the identical unit in kawaguchipeptin A. Furthermore, from NOESY spectrum of **1**, a cross peak was observed between H-10 and H-6 which suggest the two five membered nitrogen rings to be of cis relation. (Table 1) The coupling constants between C-2 and C-3 (J = 10.1 Hz and 7.1 Hz) of the Prenyl Trp in **1** was also similar to the coupling constants in kawaguchipeptin A[12] (**2**) (J = 11.2 Hz and 6.2 Hz) as well as the Trp derivative in amauromine[14] (J = 11.0 Hz and 6.0 Hz). This suggested the configurations of C-2, C-4 and C-10 of the Prenyl Trp unit in **1** to be of *S* configurations. As such, the absolute stereochemistry of trikoramide A was established as depicted in **1**.

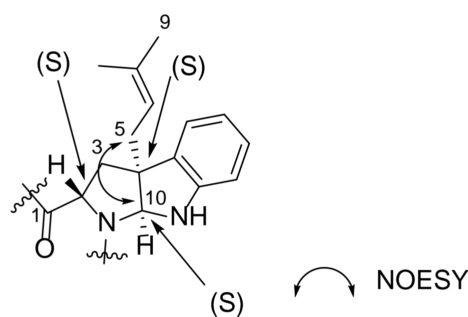

Figure 4. Absolute stereochemistry of the Prenyl Trp residue of **1** with key NOESY correlations.

MS-based metabolomics approaches have been used in natural products research, such as for compound dereplication and detection of new molecules.[17-18] One such metabolomic techniques is the LC-MS/MS molecular networking platform, which relies on the notion that structurally similar molecules share similar MS/MS fragmentation patterns. Molecular networking

comprises of three main steps: Acquiring of LC-MS/MS spectral data of samples, generation of clusters of nodes using the cosine scores logarithms based on relatedness of the MS/MS spectra, and generation of molecular network diagram using Cytoscape.[19] Based on this metabolomics approach, the LC-MS/MS spectral data was obtained for trikoramide A-containing VLC fraction to generate a molecular network to detect related analogues (Figure 5). The edges correspond to the relative relatedness of the nodes and the thickness of the edges indicate the cosine similarity. Based on the node of the precursor ion with *m/z* 1228.56, which was consistent with the $[M + H]^+$ protonated molecule (**1**) by HR-ESI-OrbitrapMS, a cluster containing six nodes was identified. There were five other ions identified within the cluster, such as *m/z* of 1281.50, 1279.00, 1243.01, 1261.03 and 1245.03, which are structurally related to **1**. These analogues were probably present in low quantities and hence, preclude their isolation in this study.

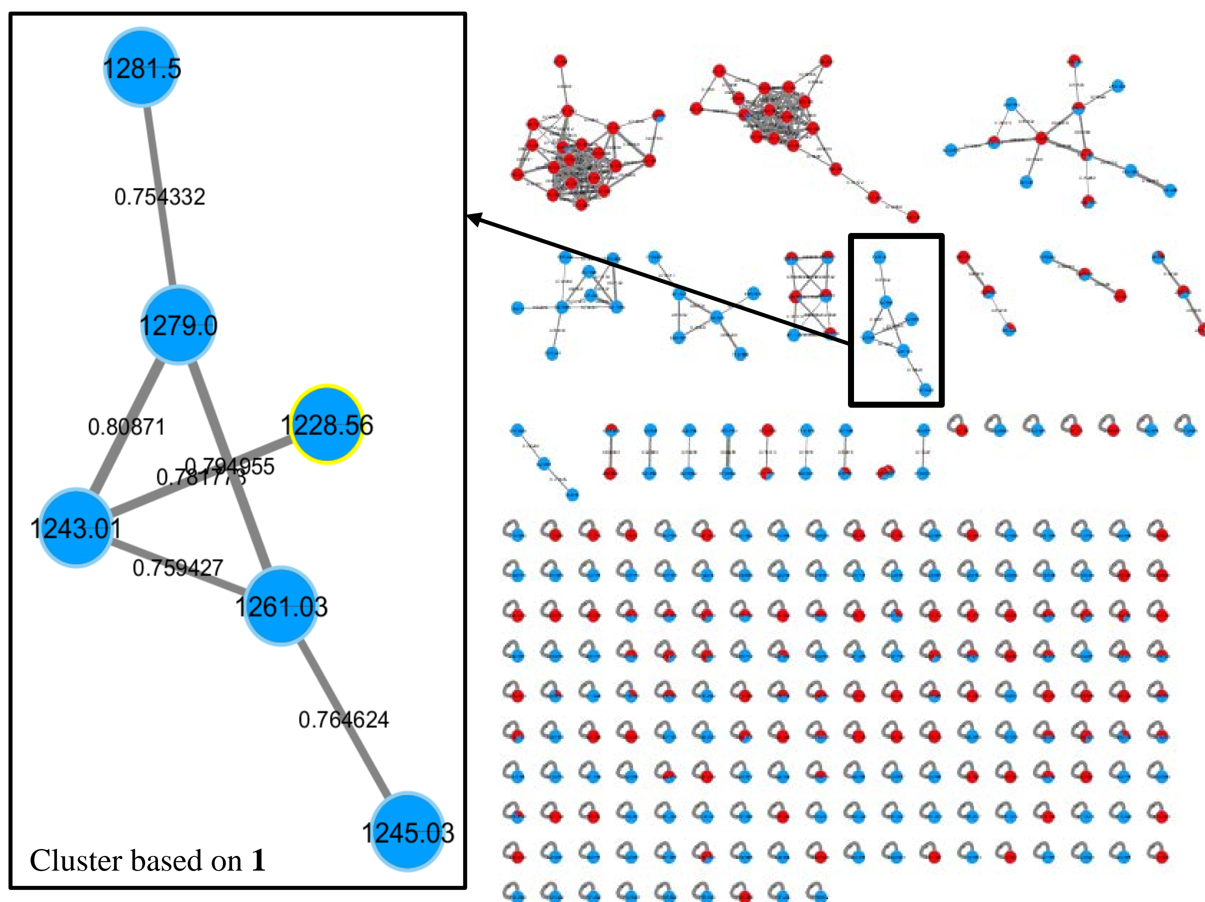

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Figure 5. LC-MS/MS derived molecular network of VLC derived fraction 8. Expanded region on the left panel made to highlight the panel that contain trikoramide A (**1**). Blue with an outer yellow ring node (*m/z* 1228.56) corresponds to the protonated molecule of **1**. Edge thickness corresponds to labeled cosine similarity between nodes.

Trikoramide A (**1**) was found to possess cytotoxic activity with IC_{50} of 5.75 μM when tested against the MOLT-4 leukemia cell line. Compound **1** exhibited weak quorum sensing inhibitory activity in the quorum sensing inhibitory assay based on the *Pseudomonas aeruginosa* lasB-gfp bioreporter strain. It only showed 26% reduction in florescence when compared to DMSO control when tested at 100 μM. Generally, cyanobactin biosynthesis begins with the precursor E-peptide, comprising of the *N*-terminal sequence recognized by the cleaving/modifying enzymes.[20] Subsequently, the precursor peptide or recognition sequence is cleaved, leaving a free amine for macrocyclization and other post modifications, such as oxidation and prenylation of the precursor cyclic molecule.[21] The biosynthesis of the *C*-prenylated cyclotryptophan unit in **1** is intriguing. One hypothesis is that the formation of the cyclotryptophan unit occurs as a post-modification step after macrocyclization of the precursor linear decapeptide containing a Trp unit. Upon macrocyclization to form the cyclic decapeptide, prenylation could occur on C-4 of the Trp unit and alkylation at this carbon atom would facilitate ring closure, giving rise to the 5-membered ring via an indolenine intermediate. To date, prenylated molecules reported from cyanobacteria include *O*-prenylated Ser/Thr/Tyr compounds (e.g. trunkamide[22] and prenylagaramides[23]), *N*-prenylated Trp compounds (e.g. croissamide[8]) as well as *C*-prenylated cyclotryptophan molecules (e.g. kawaguchipeptin A). The report of trikoramide A (**1**) adds to the growing class of cyanobactins from marine cyanobacteria.[2] To our knowledge, trikoramide

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A (**1**) is the first report of a *C*-prenylated cyclotryptophan-containing cytotoxic cyanobactin from marine filamentous cyanobacteria *Symploca hydnoides*.

EXPERIMENTAL SECTION

General Experimental Procedures.

Optical rotations were measured on Anton Paar Polarimeter while UV and IR spectral readings were measured on a PerkinElmer UV-Visible spectrophotometer and a PerkinElmer spectrum 100 FT-IR spectrophotometer, respectively. All NMR spectra were recorded in $CDCl_3$ on a 400 MHz Bruker NMR Spectrometer (400.13 MHz ^{1}H, 100.61 MHz ^{13}C) using residual solvent signals as internal references (referenced to residual CDCl3 observed at δ_H 7.24 or δ_C 77.0) with chemical shifts given in ppm downfield from TMS. Isolation and purification of **1** was conducted on Shimadzu LC-8A preparative LC coupled to a Shimadzu SPD-M10A VP diode array detector HPLC. LC-MS data for Marfey's analysis of Marfey-derivatised acid hydrolysate of **1** and amino acid standards was carried out on an Agilent 1100 series coupled with an Agilent LC/MSD (Liquid Chromatography/Mass Selective Detector) trap XCT mass spectrometer, equipped with an ESI interface system in negative mode. High resolution MS data and MS/MS data were acquired on Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, USA) equipped with a heated electronspray ionization (H-ESI) probe.

Collection, Extraction and Isolation

Marine cyanobacterial samples, with cell morphology resembling that of the genus *Symploca hydnoides*, was collected in April 2018 and 2019 by hand from the intertidal shores of Trikora beach, Bintan Island and stored in 70% EtOH at -20 ^{o}C before workup. The voucher specimen,

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TLT/Tri/22Apr2018/001, is deposited at Natural Sciences and Science Education, Nanyang Technological University, Singapore. The sample (2.0 L, wet weight) was thawed and extracted exhaustively with 2:1 CH_2Cl_2:MeOH. After the solvent was evaporated *in vacuo*, 1.19 g of a crude organic extract was obtained. The extract was then extracted using normal phase Si gel column chromatography using stepwise gradient (100% hexanes, 9:1 hexanes/EtOAc, 4:1 hexanes/EtOAc, 3:2 hexanes/EtOAc, 2:3 hexanes/EtOAc, 1:4 hexanes/EtOAc, 100% EtOAc and 9:1 EtOAc/MeOH). Fraction 8, eluted with 9:1 EtOAc/MeOH, was subjected to solid-phase fractionation on a Sep-Pak C_{18} cartridge (Phenomenex, Torrance, CA, USA) using 100% MeOH to remove pigments. The resulting filtrate was further subjected to semipreparative HPLC separation (Phenomenex Luna 5μm Phenyl-Hexyl, 250 × 10mm, 85% MeOH/H_2O in 30 min at 3.0 ml/min, detected at 210 nm, 230 nm and 290 nm) to yield semipure trikoramide A. A final purification was achieved using semipreparative HPLC (Phenomenex Kinetex 5 μm C_{18}, 250 × 4.6 mm, 85% MeOH/H_2O) to yield pure trikoramide A (**1**, 15.1 mg, t_R = 10.5 min)

Trikoramide A (**1**): white amorphous solid; $[\alpha]_D^{20}$ -109.2 (c 0.29, MeOH); IR (Nujol) ν_{max} 3436, 2953, 1651, 1458, 722 cm^{-1}; UV (MeOH) λ_{max} (log ε) 215 (2.91), 295 (2.84), 425 (2.76) nm; ^1H and ^{13}C NMR data ($CDCl_3$, 400.13 and 100.61 MHz, respectively), see Table 1 and Supplementary Data; HR-ESI-OrbitrapMS *m/z* 1228.7508 $[M + H]^+$ (calcd for $C_{68}H_{98}N_{11}O_{10}$, 1228.7492).

Marfey's Analysis of the Acid Hydrolysate of Trikoramide A (1)

Acid hydrolysis of trikoramide A (**1**, 200 μg) was achieved in 1.0 ml of 6N HCl placed in a sealed reaction vial purged with N_2 gas at 110 ^oC for 24 hr. Trace HCl was then removed *in vacuo* and the resulting hydrolysate was redissolved in 0.2 ml of H_2O. A 1% solution of L-

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FDVA (1000 µL) in acetone and 1N $NaHCO_3$ (50 µL) were added to the aqueous hydrolysate and the mixture subsequently was heated at 40 ºC overnight. Once the resulting mixture was cooled to rt, it was sequentially quenched with 2N HCl (50 µL), then dried under vacuum and resuspended in CH_3CN for LC-MS analysis. Each LC-MS analysis was carried out using a Phenomenex Kinetex C_{18} column (100 × 4.6 mm, 2.6 µm) and a gradient elution of H_2O acidified with 0.1% of HCOOH (A) and CH_3CN (B) at 0.2 ml/min flow rate. The gradient program was set to begin with 20% to 100% of B within 45 min, held at 100% B for 5 min before reconditioning back to the starting composition in 5 min. The detection was optimized at 340 nm and mass range of *m/z* 320 to 500 to eliminate detection of unreacted L-FDVA. The retention times (L- and D-configurations) and ESIMS deprotonated abduct ions (t_{RL}/t_{RD} in min, *m/z* $[M – H]^-$) of the L-FDVA monoderivatized standards were: Pro (23.5/25.3, 394.4), Leu (28.8/32.8, 410.4), Val (26.4/30.2, 396.4), Trp (28.3/30.1, 483.5), Phe (28.9/31.7, 444.5). The derivatized hydrolysate peaks of **1**, identified based on observed *m/z*, gave retention times at 23.6 min, 28.8 min, 26.6 min, 28.9 min and 28.3 min, which corresponded to L-Pro, L-Leu, L-Val, L-Phe and L-Trp, respectively. The analysis was further confirmed by co-injection with L-FDVA derivatized amino acid standards.

Molecular Networking of the Trikoramide-Containing VLC Fraction

VLC-derived fraction 8 was filtered over C_{18} SPE cartridges by application of 1.0 mg sample and elution with elution with 3 ml of CH_3CN. Solvent was removed using a rotary evaporator before being redissolved in 1 ml CH_3CN, then vortex mixing over 5 min and transferred into separate Eppendorf tubes. Tubes were then centrifuged at 10,000 rpm at 4 ºC over 10 min and the supernatant was aliquoted and diluted with ACN to 10,000 × dilution. One-and-a-half microlitres of each 10,000 × dilution sample was subjected to LC-HRMS/MS performed with a

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waters Acquity BEH (C_{18} 50 mm x 2.1 mm, 1.7 µm) column and maintained at a column temperature of 40 ºC and sample temperature of 4 ºC using a step elution program of '1' based on Waters system. Step elution program was as follows: mobile phase of 98% CH_3CN in 0.1% aq. HCOOH/0.1% aq. HCOOH, run time of 14 min and flow rate of 0.5 ml/min. All the mass spectra were collected in the positive ion mode and the data was collected in the data-dependent acquisition mode, where first ten most intense ions of a full-scan mass spectrum were subjected to tandem mass spectrometry (MS/MS) analysis. MS/MS scans were obtained for selected ions with a mass range of 100 to 2000 Da, MS scan time of 0.33 s over 12 min, MS/MS scan time of 0.10 s, and a collision energy ramp of 10-50 V. Lastly, the chromatogram was converted digitally to .mzXML files using freely available MSConvert software and submitted to Global Natural Product Social Molecular Networking (GNPS). A molecular network was then generated to interconnect MS/MS spectra along with blank containing CH_3CN solution.

MOLT-4 Human Leukemia Cell Line Assay

Cytotoxicity of compound **1** was carried out using the MTT bioassay based on the MOLT-4 (T lymphoblast; acute lymphoblastic leukemia) cancer cell line over a 3-day procedure. On the first day, **1** was prepared in 96-well microtiter plate at 10 mM stock concentration dissolved in 100% DMSO, conducted in triplicates. The mixtures were then added with RPMI media, supplemented with fetal calf serum; and serial diluted to give a concentration of 125, 50, 20, 8, and 3.2 µM. To each of the concentration, 10 µL of the diluted extract was added with 70 µL of the MOLT-4 cells. The plate was incubated for 24 hr in a 37 °C, 5% CO_2 incubator. On day 2, each of the wells were added with 20 µL of MTT solution and incubated for 3 hours. Another 100 µL of lysing buffer was added to each well thereafter and incubated overnight. On day 3, the microtiter plate was measured at OD 570 nm and its result tabulated.

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Quorum Sensing Inhibitory Assay

The anti-quorum sensing bioassay was carried out using the *Pseudomonas aeruginosa* reporter strain. Compound **1** was prepared in 96-well microtiter plate at 10 mM stock concentration dissolved in 100% DMSO, conducted in triplicates. The mixtures were then added with ABTGC medium; and serial diluted to give a concentration of 20 µM in the first dilution factor (with 0.2% of DMSO). A total of seven dilution factors, down to 0.3125 µM were done. An overnight culture of PAO1-*lasB-gfp* strain, grown in Luria-Bertani medium at 37 °C, 200 rpm, was diluted in ABTGC medium to an optical density of 0.02 at OD600 which correspond to 2.5×10^7 CFU/mL. An equal amount of the bacterial suspension was added to reach a final test concentration of 10, 5, 2.5, 1.25, 0.625, 0.3125, and 0.1563 µM. DMSO control, media control, and culture control were used and the microtiter plates were incubated at 37 °C in Tecan Infinate 200 Pro plate reader to measure the cell density (OD600) and green fluorescence protein fluorescence (excitation at 483 nm, emission at 535 nm) with 15 min intervals for up to 16 h.

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge on the ACS Publication website. Images showing morphology of *Symploca hydnoides*, ^{1}H NMR, ^{13}C NMR, COSY, HSQC, HMBC, ROESY, NOESY, TOCSY, DEPT 90, DEPT 135 spectra of trikoramide A (**1**) in $CDCl_3$, HRMS spectrum, HR MS/MS fragmentation spectrum of **1** (PDF).

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