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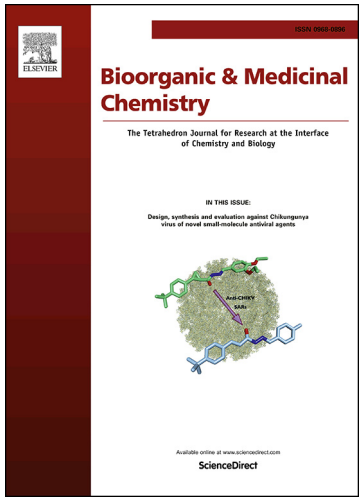

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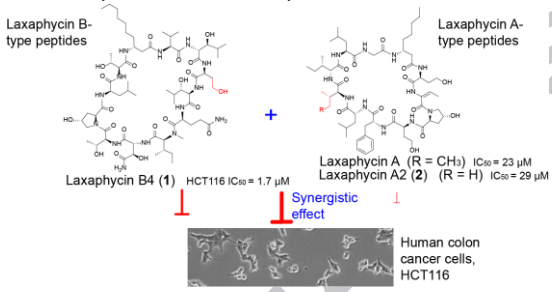

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ABSTRACT

Two new cyclic lipopeptides termed laxaphycins B4 (**1**) and A2 (**2**) were discovered from a collection of the marine cyanobacterium *Hormothamnion enteromorphoides*, along with the known compound laxaphycin A. The planar structures were solved based on a combined interpretation of 1D and 2D NMR data and mass spectral data. The absolute configurations of the subunits were determined by chiral LC-MS analysis of the hydrolysates, advanced Marfey's analysis and 1D and 2D ROESY experiments. Consistent with similar findings on other laxaphycin A- and B- type peptides, laxaphycin B4 (**1**) showed antiproliferative effects against human colon cancer HCT116 cells with IC_{50} of 1.7 µM, while laxaphycins A and A2 (**2**) exhibited weak activities. The two major compounds isolated from the sample, laxaphycins A and B4, were shown to act synergistically to inhibit the growth of HCT116 colorectal cancer cells.

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1. Introduction

Cyanobacteria represent a prolific source of bioactive secondary metabolites with wide pharmaceutical importance.[1,2] A majority of these bioactive molecules are peptides, polyketides or hybrid polyketide-polypeptides being biosynthesized by multi-modular enzymatic systems integrating nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) biosynthetic pathways.[1,3] The intriguing structural features of cyanobacterial compounds allow them to interact with a variety of cellular targets, which results in a broad spectrum of biological activities, including anticancer, antifungal, antimicrobial, protease inhibitory, immunomodulatory and neuromodulatory properties.[4,5]

The laxaphycins are a large family of cyclic lipopeptides found in cyanobacteria, which are characterized by a rare fatty β-amino acid with a linear chain of up to 12 carbons. All laxaphycins can be separated into cyclic undeca- and dodecapeptides, the major representative of each class being laxaphycin A and laxaphycin B, respectively (Figure 1, Supporting Information Figures S1 and S2). Laxaphycins A and B were isolated from the freshwater cyanobacterium *Anabaena laxa* in 1992.[6,7] The absolute configurations of these two compounds were not elucidated until 1997, when laxaphycins A and B were identified again from a collection of a tropical marine cyanobacterium identified as *Lyngbya majuscula*.[8] Within the last two decades, laxaphycin A and B groups have been expanded, with several laxaphycin analogues isolated from marine cyanobacteria (Supporting Information Figures S1 and S2), including hormothamnin A from *Hormothamnion enteromorphoides*,[9,10] laxaphycins B2 and B3 from *Lyngbya majuscula*,[11] lobocyclamides A–C from *Lyngbya confervoides*,[12] and lyngbyacyclamides A and B from *Lyngbya* sp.[13] Related compounds from freshwater cyanobacteria include trichormamides A and B from *Trichormus* sp.,[14] and trichormamides C and D from cf. *Oscillatoria* sp.[15] Laxaphycin A-type peptides are characterized by a segregation of hydrophobic and hydrophilic residues, while laxaphycin B- type peptides have alternating hydrophobic and hydrophilic residues (Figure 1).[8]

Interestingly, laxaphycin A- and B-type compounds have been

frequently co-isolated from the same cyanobacterium.[8,12,14,15] This

phenomenon might be explained from the perspective of their

biological activities. Laxaphycin B-type compounds exhibited

strong to moderate antifungal activities against *C. albicans* and

C. glabrata[12] and anticancer activity towards various cancer cells

(IC_{50} < 2 μM)[11,13–15] as well as antibacterial activities against *M.*

tuberculosis.[15] In contrast, most of the laxaphycin A-type

compounds show weak cytotoxicity (IC_{50} >10 μM) with the

cytotoxic hormothamnin A as an exception.[7,9–11,14,15]

Hormothamnin A is a (*Z*)-α,β-didehydro-α-aminobutyric acid (*Z*-

Dhb) analog of laxaphycin A. It is possible that the geometry of

the Dhb unit in laxaphycin A-type compounds is one of the

contributing factors to its cytotoxicity. Importantly, laxaphycin

A-type and B-type compounds have been reported to act

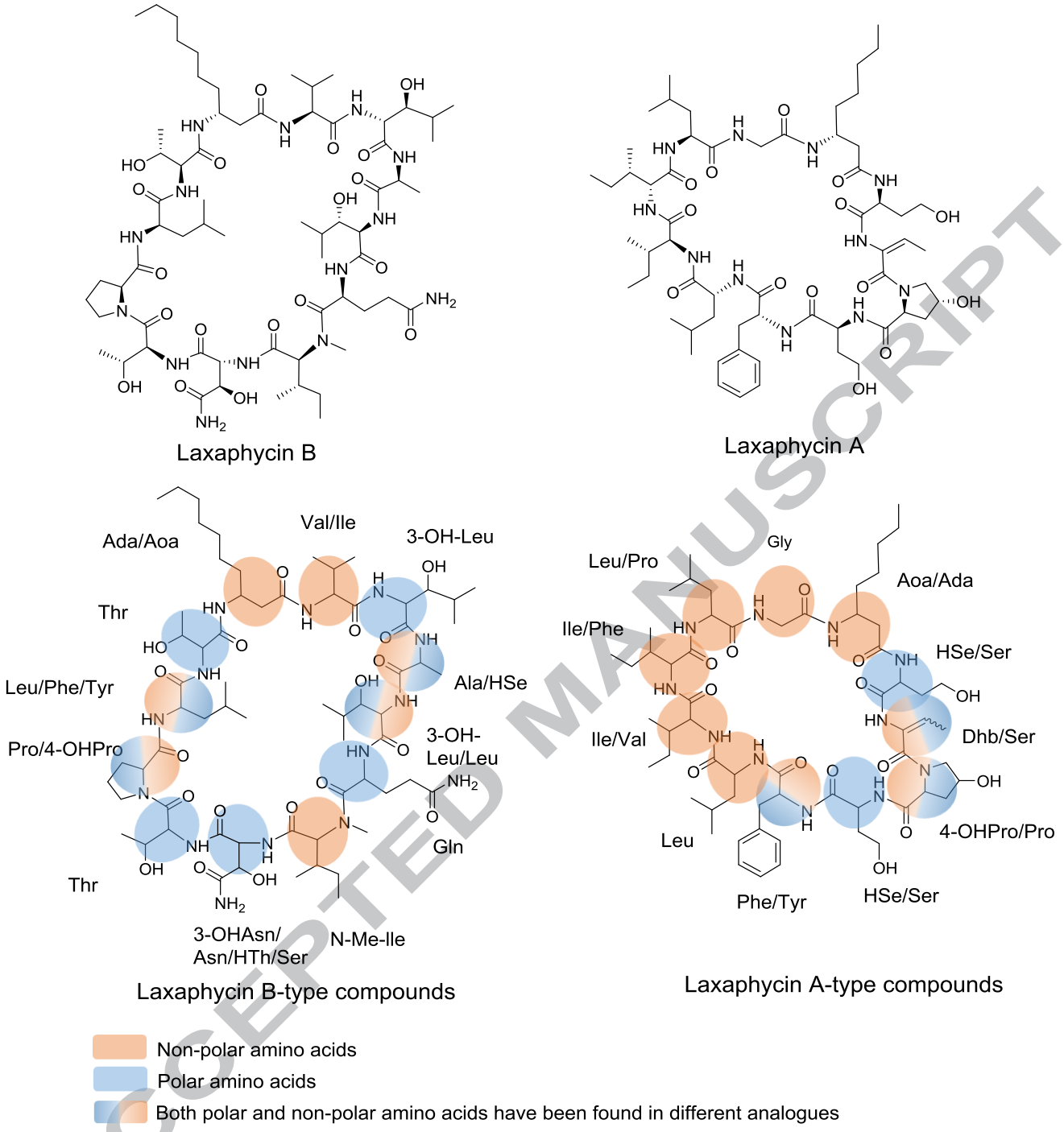


Figure 1. Structures of laxaphycins B and A and characteristic features of B- and A-type compounds. Laxaphycin B- type peptides have alternating non-polar and polar amino acids. Laxaphycin A-type peptides are characterized by a segregation of non-polar and polar amino acids.

synergistically with each other to inhibit fungus and cancer

cells.[11,12] In other words, in order to achieve maximum biological potency, a member of each class of peptide must be present. Although the exact mechanism of action of the synergism remain unknown, the cooperativity provides a possible explanation for the frequently observed coproduction of laxaphycin A-type and B-type peptides from the same cyanobacteria.

In the present study, a field collection of the marine cyanobacterium *Hormothamnion enteromorphoides* has afforded two new laxaphycin analogues: laxaphycin B4 (**1**) and laxaphycin A2 (**2**) (Figure 2). Here, we describe the isolation, total structure determination, and evaluation of their antiproliferative effects in a colon cancer cell line, HCT116, as well as their synergistic effects.

2. Results and Discussion

The cyanobacterium was collected from Garden Key in the Dry Tortugas National Park and extracted with CH_2Cl_2 and MeOH (1:1) to provide the nonpolar extract and EtOH and H_2O (1:1) to provide a polar extract. The nonpolar extract (2.6 g) was subjected to silica chromatography and two rounds of reversed-phase HPLC to yield laxaphycin B4 (**1**) (20 mg), laxaphycin A2 (**2**) (0.4 mg) and laxaphycin A (20 mg).

detailed NMR interpretation of ^{1}H NMR, ^{13}C NMR, HSQC, HMBC, COSY and ROESY spectra (Table 1, Figure 2, Supporting Information Figures S3–S8). The ^{1}H NMR spectrum of **1** exhibited a signal pattern characteristic of a lipopeptide: a group of signals for exchangeable amide protons (δ_H 6.9–8.2), signals of α -protons (δ_H 4.0–5.0), aliphatic methylene signals (δ_H 1.1–1.4) and methyl signals (δ_H 0.7–1.0). Eleven α-amino acid units were characterized by interpretation of COSY, HSQC and HMBC spectrum: two threonines (Thr1/2), two 3-hydroxyleucines (3OH-Leu 1/2), valine (Val), leucine (Leu), 4-hydroxyproline (4-OHPro), *N*-methyl isoleucine (*N*-Me-Ile), homoserine (Hse), glutamine (Gln), 3-hydroxyasparagine (3-OHAsn). The presence of a lipophilic β-amino acid, β-aminodecanoic acid (Ada), was evident by sequential COSY correlations between NH (δ_H 7.64)/ H-3 (δ_H 4.07)/H_2-2(δ_H 2.50; 2.31) as well as sequential COSY correlations from H_2-4 (δ_H 1.38; 1.31) to the region of highly overlapping methylene signals (H_2-5 to H_2-9), which correspond to five carbons in the HSQC spectrum (δ_C 28.7, 28.3, 25.0, 30.9, 21.8) and then to a methyl triplet (δ_H 0.84) (Table 1). The amino acid sequence was assigned based on HMBC and ROESY correlations as 4-OHPro-Leu-Thr2-Ada-Val-3-OHLeu1-HSe-3-OHLeu2-Gln-*N*-Me-Ile-3-OHAsn-Thr1 (Table 1, Figure 2). The 16 degrees of unsaturation and the molecular formula suggested that **1** was a cyclic peptide.

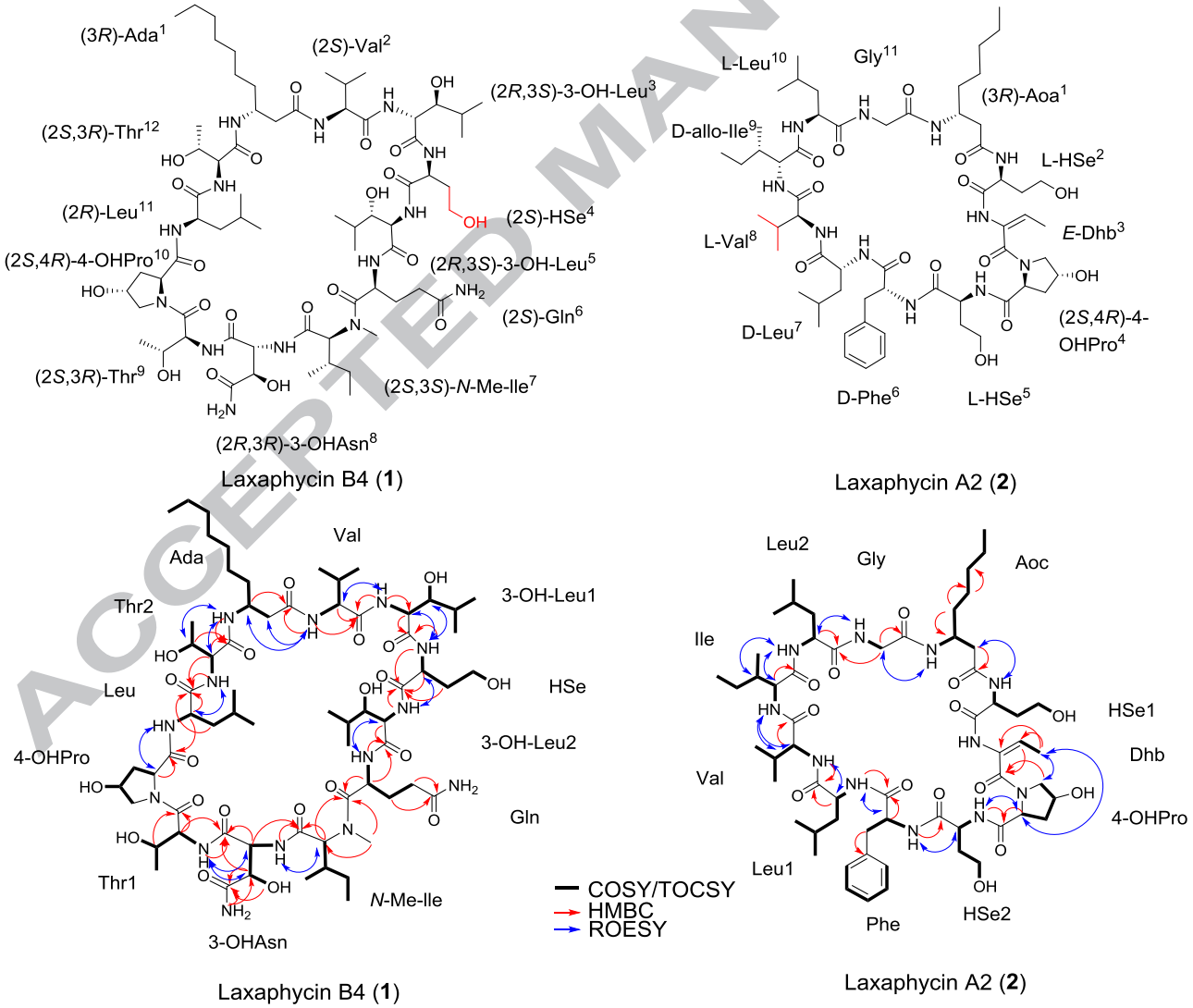


Figure 2. Structures and key NMR correlations of laxaphycin B4 (**1**) and laxaphycin A2 (**2**).

The HR-ESIMS spectrum of compound **1** showed a $[M + Na]^+$ peak at *m/z* 1463.8334, consistent with the molecular formula $C_{66}H_{116}N_{14}O_{21}$. The structure of **1** was established based on a

The chemical shifts of C1 (δ_C 168.6) in Thr1 indicated the presence of an amide or ester moiety, and therefore the cyclic dodecapeptide ring was closed between Thr1 and 4-OHPro.

Compound **1** (0.3 mg) was hydrolyzed with 6 N HCl (110 °C, 20 h) and the hydrolysate was subjected to chiral HPLC-MS, revealing the presence of D-Leu, L-Thr/ L-*allo*-Thr, L-Gln, L-Val, *N*-Me-L-Ile and *trans*-4OH-L-Pro/*cis*-4OH-D-Pro in the molecule (Figure 2, Table 2). The exact assignment for Thr and 4-OHPro and all the other amino acids in **1** was elucidated using advanced Marfey's analysis (Figure 2, Table 3).[16–18] The L-FDLA derivative of the acid hydrolysate of **1** was compared with FDLA derivatives of authentic standards of 3-OHLeu [(2*S*,3*S*)-3-OHLeu-L-FDLA, (2*S*,3*S*)-3-OHLeu-DL-FDLA, (2*S*,3*R*)-3-OHLeu-L-FDLA, (2*S*,3*R*)-3-OHLeu-DL-FDLA], HSe (L-HSe-L-FDLA, L-HSe-DL-FDLA), Thr (L-Thr-L-FDLA, L-*allo*-Thr-L-FDLA) and 4-OHPro (*trans*-4OH-L-Pro-L-FDLA, *cis*-4OH-D-Pro-L-FDLA), 3-OHAsp [L-*threo*-3-OHAsp-L-FDLA, L-*threo*-3-OHAsp-DL-FDLA, D/L-*erythro*-3-OHAsp-L-FDLA], which allowed for assignment of 2*R*,3*S* configurations for 3-OHLeu and L configurations for HSe and Thr and the assignment of *trans*-4OH-L-Pro and D-*threo*-3-OHAsn (Table 3). A comparison of the elution orders of L-FDLA and D-FDLA derivatives of the acid hydrolysate with those reported in the literature assigned 3*R* configuration for Ada in **1** (Table 3).[12,14,16,17,19]

Compound **1** is a laxaphycin B-type peptide featuring a dodecapeptide core and a fatty β-amino acid. It is closely related to laxaphycin B3 with the alanine at position 4 in laxaphycin B3 being replaced by homoserine (Supporting Information, Figure S2). Thus, compound **1** was termed laxaphycin B4 (**1**). Although the configuration of 3-OH-Leu1 in laxaphycin B3 was first deduced as 2*S*,3*S* based on the remarkable NMR spectra similarities between laxaphycins B3 and B, a later study revised the configuration of 3-OH-Leu1 to (2*R*,3*S*)-3-OH-Leu1 in

Table 1. NMR data for **1** in DMSO-d_6 (600 MHz for ^{1}H and 150 MHz for ^{13}C NMR data)

	C/H no	δ_C	δ_H (*J* in Hz)	COSY	HMBC	ROESY
β-Ada	1	171.5, C				
	2a	39.6, CH_2	2.50, m	3, 2b	1, 3, 4	NH (Val)
	2b		2.31, m	3, 2a	1, 3, 4	NH (Val)
	3	45.7, CH	4.07, m	2, 4, NH	1	NH (Val)
	4a	33.4, CH_2	1.38, m	3, 4b, 5	2	
	4b		1.31, m	3, 4a, 5	5	
	5	28.7, CH	1.19, m			
	6	28.3, CH	1.21, m			
	7	25.0, CH_2	1.20, m			
	8	30.9, CH_2	1.20, m			
	9	21.8, CH_2	1.23, m	10		
	10	13.7, CH_3	0.84, t (7.0)	9		
	NH		7.64, d (8.9)	3	3, 1 (Thr2)	2 (Thr2), 3 (Thr2)
Val	1	171.4, C				
	2	58.4, CH	4.16, t (7.2)	3, NH	1, 3, 4	NH (3-OHLeu1)
	3	29.4, CH	1.98, m	2, 4	2, 5	
	4	18.1, CH_3	0.88, d (6.8)	3	3, 5	
	5	18.8, CH_3	0.85, m		2, 3, 4	
	NH		8.17, d (7.6)	2	2, 3, 1 (β-Ada)	2 (β-Ada), 3 (β-Ada)
3-OHLeu1	1	171.6, C				
	2	55.0, CH	4.43, m	3, NH	1, 3	NH (Hse)
	3	76.6, CH	3.50 m	2, 4, OH	4, 6	NH (Hse)
	4	30.5, CH	1.59, m	3, 5, 6	3, 5	
	5	19.0, CH_3	0.92, m	4	3, 4, 6	
	6	18.5, CH_3	0.78, d (6.7)	4	3, 4, 5	
	NH		7.91, d (8.3)	2	2, 1 (Val)	2 (Val)
	OH		4.83, d (7.1)	3	2, 3, 4	
Hse	1	171.8, C				
	2	50.8, CH	4.36, m	NH	3, 4	NH (3-OHLeu2)
	3a	34.3, CH_2	1.86, m	2, 3b, 4a, 4b	1, 2, 4	
	3b		1.82, m	2, 3a, 4a, 4b	1, 2, 4	
	4a	57.4, CH_2	3.49, m	3a, 3b, 4b, OH	2, 3	
	4b		3.42, m	3a, 3b, 4a, OH	2, 3	
	NH		7.96, br d (5.6)	2	1, 2, 3, 1 (3-OHLeu1)	2 (3-OHLeu1), 3 (3-OHLeu1)

	OH		4.50, t (5.2)	4a, 4b	3, 4	
3-OHleu2	1	171.0, C				
	2	55.5, CH	4.31, m	3, NH	1	NH (Gln)
	3	76.0, CH	3.44, m	2, OH		
	4	29.8, CH	1.57, m	3, 5, 6	2, 3, 5	
	5	18.5, CH3	0.90, m	4, 6	3, 4, 6	
	6	18.7, CH3	0.76, m	4, 5	3, 4, 5	
	NH		7.79, d (8.9)	2	2, 1 (Hse)	2 (Hse)
	OH		4.91, d (5.1)	3	3, 4	
Gln	1	173.0, C				
	2	49.2, CH	4.56, m	3a, 3b, NH	1	
	3a	25.4, CH_2	2.00, m	2, 3b, 4a, 4b	4, 5	
	3b		1.67, m	2, 3a, 4a, 4b	4, 5	
	4a	30.4, CH_2	2.29, m	3a, 3b, 4b	2, 3, 5	
	4b		2.17, m	3a, 3b, 4a	2, 3, 5	
	5	174.8, C				
	NH		7.84, d (7.3)	2	2, 1 (3-OHLeu2)	2 (3-OHLeu2)
	NH2a		7.37, br s	NH2b	5	
	NH2b		6.96, br s	NH2a	4, 5	
N-Me-Ile	1	170.2, C				
	2	59.7, CH	4.74, d (10.8)	3, 6	1, 3, 4, 6, N-Me	NH (3-OHAsn)
	3	31.5, CH	1.92 m	2, 6		
	4a	23.5, CH_2	1.29, m	3, 4b, 5	3, 6	
	4b		0.91, m	4a, 5	3, 6	
	5	10.0, CH_3	0.77, m	4a		
	6	14.8, CH_3	0.75, m	3	1, 2, 3, 4	
	N-Me	30.0, CH_3	3.02, s		2, 1 (Gln)	
3-OHAsn	1	169.1, C				
	2	55.3, CH	4.64, dd (8.3, 1.4)	H-3, NH	1, 3, 4	NH (Thr1)
	3	70.0 CH	4.37, m	H-2, OH	1, 2, 4	NH (Thr1)
	4	173.6, C				
	NH		7.67, d (8.3)	2	2, 3, 1 (*N*-Me-Ile)	2 (*N*-Me-Ile)
	NHa		7.30, NH_2		4	
	NHb		7.27, NH_2		3, 4	
	OH		5.82, d (6.4)	3	2, 3, 4	
Thr1	1	168.6, C				
	2	55.7, CH	4.46, m	3, NH	1, 3, 4	
	3	66.6, CH	3.88, dq (11.5, 6.1)	2, 4, OH,	1, 2, 4	
	4	18.6, CH_3	1.05, d (6.1)	3, OH	2, 3	
	OH		4.93, d (4.6)	3	2, 3, 4	
	NH		7.25, d (8.0)	2	2	2 (3-OHAsn), 3 (3-OHAsn)
4-OHPro	1	171.3, C				
	2	58.5, CH	4.44, m	3a, 3b	1, 3, 4	NH (Leu)
	3a	37.6, CH_2	1.99, m	2, 4, 3b, 5b	1, 2, 4, 5	
	3b		1.85, m	2, 4, 3a	1, 2, 4, 5	

	4	68.4, CH	4.32, m	3a, 3b, 5a, 5b, OH	22	
	5a	55.3, CH_2	3.73, dd (10.6, 3.9)	4, 5b	3	
	5b		3.58, d (10.7)	3a, 4, 5a	4	
	OH		5.08, d (3.3)	4	3, 4, 5	
Leu	1	171.9, C				
	2	51.3, CH	4.33, m	3, NH	1, 3, 4	NH (Thr2)
	3	41.0, CH_2	1.47, t (7.2)	2, 4	1, 2, 4, 5, 6	
	4	24.0, CH	1.53, m	3, 5, 6	2, 3, 5, 6	
	5	22.6, CH_3	0.88, m	4	3, 4, 6	
	6	21.6, CH_3	0.82, d (7.0)	4	3, 4, 5	
	NH		7.95, br d (5.5)	2	1 (4-OHPro)	2 (4-OHPro)
Thr2	1	168.8, C				
	2	58.2, CH	4.09, m	3, NH	1, 3, 4, 1 (Leu)	NH (β-Ada)
	3	66.1, CH	3.99, m	2, 4, OH		NH (β-Ada)
	4	19.4, CH_3	1.02, d (6.3)	3	3, 2	
	NH		7.78, d (9.2)	2	1 (Leu)	2 (Leu)
	OH		4.78, d (5.4)	3	2, 3, 4	

Table 2. Chiral amino acid analysis of **1**

Compound parameters for amino acids [a]									Retention times (min) [b]					
Amino acid	MW	Q1 *m/z*	Q3 *m/z*	DP	EP	CE	CXP	CEP	L	D	L-*allo*	D-*allo*	Measured	Assignment
Val	117	118	72	31	8	15	4	12	8.4	14.1	NA	NA	8.5	L
Glu	147	146	102	-30	-2	-20	-4	-14	6.1	7.5	NA	NA	6.0	L
Thr	119	120	74	21	7	13	4	10	7.7	8.9	7.8	11.0	7.8	L or L-*allo*
Leu	131	132	86	31	8	13	4	10	9.3	17.4	NA	NA	18.1	D
N-Me-Ile	145	146	100	35	7	17	2	10	12.5	49.7	15.1	49.3	12.4	L
4-OHPro	131	132	68	31	5	27	4	12	9.1 (*cis*-L)	10.6 (*cis*-D)	10.9 (*trans*-L)	28.6 (*trans*-D)	10.8	*trans*-L or *cis*-D

[a] MS parameters: Q1 (parent ion); Q3 (product ion); DP (declustering potential); EP (entrance potential) CE (collision energy); CXP (collision cell exit potential); CEP (collision cell entrance potential); Positive and negative values indicate positive and negative ionization modes. [b] Measured by LC-MS selected ion chromatogram on a chiral column (Chirobiotic TAG (250x4.6 mm), Supelco; solvent: MeOH:10mM NH_4OAc (40:60, pH 5.12)); flow rate 0.5 mL/min

Table 3. Advanced Marfey's analysis of **1**

Compound parameters for FDLA derivatives of amino acids									Retention times (min)					
Amino acid	MW	Q1 *m/z*	Q3 *m/z*	DP	EP	CE	CXP	CEP	L	D	L-*allo*	D-*allo*	Measured	Assignment
Ada[a]	482	480	NA	NA	NA	NA	NA	NA	32.0 (*S*)	38.8 (*R*)	NA	NA	38.4	*R*
3-OH-Leu[a]	442	440	162	-30	-9.5	-48	-2	-22	14.1 (2*S*,3*S*)	19.3 (2*R*,3*R*)	21.8 (2*R*,3*S*)	13.9 (2*S*,3*R*)	22.2	2*R*,3*S*
Hse[a]	414	412	176	-45	-5.5	-38	-4	-20	11.0 (*S*)	12.0 (*R*)	NA	NA	11.0	L
4-OHPro[a]	426	426	381	66	4.5	25	8	24	NA	9.4 (*cis*-D)	7.9 (*trans*-L)	NA	8.0	*trans*-L
Thr[b]	414	412	192	-40	-8	-26	-4	-20	19.0 (2*S*,3*R*)	NA	19.9 (2*S*,3*S*)	NA	18.9	L
3-OH-Asp[c]	444	442	398	-45	-3	-24	-4	-22	38.1 (L-*threo*)	37.8 (D-*threo*)	41.0/43.5 (L/D-*erythro*)		37.4	D-*threo*[d]

[a]Measured by LC-MS selected ion chromatogram on a reversed-phase column (Phenomenex Kinetex C18, 100 x 2.10 mm, 2.6 µm, flow rate 0.2 mL/min) with a linear gradient from 25% to 65% aqueous acetonitrile containing 0.1% formic acid over 50 min. [b]Measured by LC-MS selected ion chromatogram on a reversed-phase column (Alltech Alltima C18, 5 µm, 250 × 4.6 mm, 5 µm, flow rate 1.0 mL/min) with a linear gradient from 25% to 65% aqueous acetonitrile containing 0.1% formic acid over 50 min. [c]Measured by LC-MS selected ion chromatogram on a a reversed-phase column (Alltech Altima C18, 5 µm, 250 × 4.6 mm; flow rate, 1.0 mL/min) using a linear gradient of MeOH in 0.1% formic acid (20–50% in 60 min). [d]Confirmed by co-injection.

laxaphycin B by conducting total synthesis.[11,20] Therefore, the configuration of 3-OH-Leu1 in laxaphycin B3 is likely to be 2*R*,3*S* as well, which is the same as laxaphycin B4 (**1**).

The molecular formula of **2** was deduced as $C_{59}H_{95}N_{11}O_{14}$ based on a $[M + Na]^+$ peak at *m*/*z* 1204.6930 in HR-ESIMS spectrum and the NMR spectra. Due to the broad NMR signals observed in DMSO-d_6, 1D and 2D NMR experiments of **2** were conducted in CH_3CN-d_3 (Table 4). The structure of **2** was elucidated based on combined analysis of ^{1}H NMR, HSQC, HMBC, COSY, TOCSY and ROESY experiments (Table 4, Figure 2, Supporting Information Figures S9-S15). COSY and TOCSY correlations of **2** revealed the presence of ten α-amino acid residues, including glycine (Gly), phenylalanine (Phe), two leucines (Leu1/2), valine (Val), 4-hydroxyproline (4-OHPro), isoleucine (Ile), two homoserines (HSe1/2), α,β-didehydro-α-aminobutyric acid (Dhb) and one β amino acid unit β-aminooctanoic acid (Aoa) (Table 4, Figure 2). The presence of β-Aoa was deduced by the COSY, TOCSY and HSQC correlations as described for **1**. The amino acid sequence was assigned based on interpretation of HMBC and ROESY correlations as Dhb-4-OHPro-HSe2-Phe-Leu1-Val-Ile-Leu2-Gly-Aoa-HSe1. The molecular formula and degree of unsaturation indicated the cyclic nature of **2**. Thus, the cyclic undecapeptide ring was closed between Dhb and HSe1.

Compound **2** was hydrolyzed with 6 N HCl (110 °C, 20 h) and the hydrolysate of **2** was subjected to chiral HPLC-MS to reveal the presence of D-Phe, L-Val, D-*allo*-Ile and *trans*-4OH-L-Pro/*cis*-4OH-D-Pro (Figure 2, Table 5). Peaks for both L- and D-Leu were detected by chiral HPLC-MS, preventing unambiguous

Table 4. NMR data for **2** in CH_3CN-d_3 (600 MHz for ^{1}H and 150 MHz for ^{13}C NMR data)

		δ_C[a]	δ_H (*J* in Hz)	COSY	HMBC	ROESY
β-Aoa	1	171.1, C				
	2a	41.4, CH_2	1.65, m	3, 2b	1	NH (HSe1)
	2b	41.4, CH_2	1.98, m	3, 2a		
	3	45.9, CH_2	4.34, m	NH, 2a, 2b, 4		
	4	36.1, CH	1.41, m	2, 3		
	5	28.9, CH_2	1.25, m	6		
	6	32.2, CH_2	1.32, m			
	7	32.5, CH_2	1.25, m			
	8	14.4, CH_3	0.87, m			
	NH		6.92, m	3		2a (Gly)
Hse1	1					
	2	50.0, CH	4.69, m	3, NH		
	3	34.3, CH_2	1.80, m	2, 4		
	4	57.8, CH_2	3.58, m	3, OH		
	OH		4.22, m	4		
	NH		6.81, m	2		2a (β-Aoa)
Dhb	1	169.3, C				
	2	131.9, C				
	3	122.6, CH	5.66, q (7.2)	4	1	
	4	11.0, CH_3	1.76, d (7.2)	3	3, 2	2 (4-OHPro), 5a (4-OHPro), 5b (4-OHPro)
4-OHPro	1	172.1, C				
	2	60.7, CH	4.65, dd (8.0, 10.0)	3a, 3b	1	4 (Dhb), NH (HSe2)
	3a	38.9, CH_2	1.95, m	2	1	
	3b	38.9, CH_2	2.39, d (13.8, 7.9)	2, 4	4	
	4	68.4, CH	4.36, br s	5a, 5b, 3a, 3b		
	OH					
	5a	58.5, CH_2	3.46, d (11.0)	4, 5b	4, 1 (Dhb)	
	5b	58.5, CH_2	3.62, dd (11.0, 2.9)	4, 5a		
Hse2	1	174.2, C				
	2	50.3, CH	4.30, m	NH, 3, 2		NH (Phe)

	3	33.4, CH_2	2.14, m	2	1	
	4a	59.1, CH_2	3.42, m	OH, 3, 4b		
	4b	59.1, CH_2	3.51, m	OH, 3, 4a	2	
	OH		2.74, br s	3a, 3b		
	NH		7.15, m	2	1 (4-OHPro)	2 (4-OHPro)
Phe	1	174.2, C				
	2	58.5, CH	4.31, m	3a, 3b	1	NH (Leu1)
	3a	38.2, CH_2	3.13, dd (13.5, 12.0)	2	2, 5/9	
	3b	38.2, CH_2	3.05, dd (13.5, 3.0)	2	4, 5/9	
	4	139.4, C				
	5, 9	130.8, CH	7.45, d (7.7)	3a, 3b, 6/8	3, 6/8, 7	
	6, 8	129.5, CH	7.29, t (7.5)	5/9, 7	7	
	7	127.8, CH	7.22, t (7.3)	6/8	6/8	
	NH		7.91, d (7.4)	2	1 (HSe2)	2 (HSe2)
Leu1	1	173.5, C				
	2	53.2, CH	4.30, m	NH, 3a, 3b	1	NH (Val)
	3a	40.3, CH_2	1.08, m	2		
	3b	40.3, CH_2	1.33, m	2		
	4	25.2, CH	1.58, m	3a, 3b, 5, 6		
	5	23.7, CH_3	0.82, d (6.9)	4	3, 4, 6	
	6	20.6, CH_3	0.75, d (6.6)	4	3, 5, 6	
	NH		6.98, d (8.0)	2	1 (Phe)	2 (Phe)
Val	1	174.5, C				
	2	56.7, CH	4.80, m	NH, 3	1	NH (Ile)
	3	35.0, CH	2.14, m	2, 4, 5		
	4	19.8, CH_3	0.84, d (7.0)	3	2, 3, 5	
	5	16.0, CH_3	0.73, d (6.9)	3	2, 3, 4	
	NH		6.47, d (9.9)	2	1 (Leu1)	2 (Leu1)
Ile	1	174.9, C				
	2	53.1, CH	4.79, m	NH, 3	1	NH (Leu2)
	3	37.6, CH	1.94. m	2, 4b, 6		
	4a	27.7, CH_2	0.91, m			
	4b	27.7, CH_2	1.23, m	3		
	5	12.7, CH_3	0.90, m	4b		
	6	14.8, CH_3	0.83, m			
	NH		7.09, d (9.5)	2		2 (Val), 3 (Val)
Leu2	1	175.2, C				
	2	55.3, CH	3.92, m	NH, 3a, 3b	1	NH (Gly)
	3a	39.9, CH_2	1.56, m	2, 3b	2	
	3b	39.9, CH_2	1.46, m	2, 3a	2, 4, 5	
	4	25.3, CH	1.63, m	3a, 3b, 5, 6		
	5	22.1, CH_3	0.88, d (6.5)	4	3, 4, 6	
	6	22.8, CH_3	0.94, d (6.5)	4	3, 5, 6	

	NH		7.16, s	2		2 (Ile), 3 (Ile)
Gly	1	168.6, C				
	2a	43.6, CH_2	3.91, m	NH, 2b	1	NH (β-Aoa)
	2b	43.6, CH_2	3.37, m	NH, 2a	1, 1 (Leu)	
	NH		7.43, m	2a, 2b		2 (Leu2)

[a]Deduced from HSQC and HMBC spectra.

Table 5. Chiral amino acid analysis of **2**

Compound parameters for amino acids									Retention times (min)					
Amino acid	MW	Q1 *m/z*	Q3 *m/z*	DP	EP	CE	CXP	CEP	L	D	L-*allo*	D-*allo*	Measured	Assignment
Val[a]	117	117	72	31	8	15	4	12	8.4	14.1	NA	NA	8.5	L
Phe[a]	165	166	120	31	8	19	4	12	13.0	18.2	NA	NA	18.6	D
Ile[b]	131	132	86	31	8	13	4	10	17.3	95.0	19.8	75.1	75.7	D-*allo*
Leu[b]	131	132	86	31	8	13	4	10	19.0	65.7	NA	NA	19.1 and 65.8	L and D
4-OHPro[a]	131	132	68	31	5	27	4	12	9.1 (*cis*-L)	10.6 (*cis*-D)	10.9 (*trans*-L)	28.6 (*trans*-D)	10.8	*trans*-L or *cis*-D

[a]Measured by LC-MS selected ion chromatogram on a chiral column (Chirobiotic TAG (250 × 4.6 mm), Supelco; solvent: MeOH:10mM NH_4OAc (40:60, pH 5.12)); flow rate 0.5 mL/min. [b]Measured by LC-MS selected ion chromatogram on the same chiral column (Chirobiotic TAG (250 × 4.6 mm), Supelco; solvent: MeOH:10mM NH_4OAc (90:10, pH 6.0)); flow rate 0.5 mL/min.

Table 6. Advanced Marfey's analysis of **2**

Compound parameters for FDLA derivatives of amino acids									Retention times (min)[a]					
Amino acid	MW	Q1 *m/z*	Q3 *m/z*	DP	EP	CE	CXP	CEP	L	D	L-allo	D-allo	Measured	Assignment
Aoa	454	452	NA	NA	NA	NA	NA	NA	26.2 (*S*)	32.0 (*R*)	NA	NA	32.0	*R*
Hse	414	412	176	-45	-6	-38	-4	-20	11.0	12.0	NA	NA	11.0	L
4-OHPro	426	426	381	66	5	25	8	24	NA	9.4 (*cis*-D)	7.9 (*trans*-L)	NA	8.0	*trans*-L

[a]Measured by LC-MS selected ion chromatogram on a reversed-phase column (Phenomenex Kinetex C18, 100 × 2.10 mm, 2.6 µm, flow rate 0.2 mL/min) with a linear gradient from 25% to 65% aqueous acetonitrile containing 0.1% formic acid over 50 min.

configurational assignment for the two leucines in **2** (Table 5). Partial hydrolysis was not able to be conducted due to limited supply of **2** (0.4 mg), and thus we proposed that the sequence of the nonpolar tetrapeptide residue is the same as that found in all the other laxaphycin A-type compounds (Supporting Information Figure S1). The L-FDLA derivative of the acid hydrolyzates of **2** was compared with FDLA derivatives of authentic standards of HSe (L-HSe-L-FDLA, L-HSe-DL-FDLA) and 4-OHPro (*trans*-4OH-L-Pro-L-FDLA, *cis*-4OH-D-Pro-L-FDLA), which allowed for assignment of L-HSe and *trans*-4OH-L-Pro (Table 6). LC-MS comparison between L-FDLA and DL-FDLA derivatives of the acid hydrolysate of **2** assigned 3*R* configuration for Aoa, similar to the assignment of Ada in **1** (Table 6). The geometric configuration of Dhb was determined to be *E* based on 1D and 2D ROESY correlations between Dhb H_3-4 (δ_H 1.76) and 4-OHPro H_2-5 (δ_H 3.62; 3.46) as well as between Dhb H_3-4 (δ_H 1.76) and 4-OHPro H-2 (δ_H 4.65) (Table 4, Supporting Information Figures S13, S14).[12,15]

Compound **2** is structurally related to laxaphycin A with the Ile at position 8 in laxaphycin A being replaced by a valine (Figure 1, Supporting Information Figure S1). Therefore, compound **2** was named laxaphycin A2 (**2**).

Due to the known anticancer activities of the laxaphycin familiy,[11] we evaluated the antiproliferative activities of these two new laxaphycins in colon cancer HCT116 cells using the MTT assay. We observed antiproliferative activity of laxaphycin B4 (**1**) in HCT116 cells with an IC_{50} value of 1.7 µM, while laxaphycin A2 (**2**) and laxaphycin A only exhibited weak antiproliferative activity with IC_{50} values of 29 µM and 23 µM, respectively. These results are consistent with the trend observed in other laxaphycins in that laxapycin B-type compounds are more potent than laxaphycin A-type compounds.

To further elucidate the synergistic effect between laxaphycin A- and B-type compounds, we aimed to test the combinatorial effect of laxaphycin B4 (**1**) and laxaphycin A in HCT116 cells. We selected these two compounds for this study based on the fact that they are the two major compounds produced by this collection as well as due to the limited supply of laxaphycin A2 (**2**). To conduct the combination study, HCT116 cells were treated concomitantly with both compounds at fixed ratios (A:B4 =32:1; 10:1) for 48 h. These are at equipotency ratios according to their IC_{50} values (1.7 µM and 23 µM) so that the contribution to the effect of each compound would be about equal.[21] Cell viability was measured using the MTT assay, and the combination index (CI) was calculated as an indicator of the mode of drug-drug interactions using CompuSyn software, a software designed based on the principles of Chou–Talalay method.[21] As shown in Figure 3A,B, strong synergism was observed in HCT116 at both combination ratios. Only one combination at low doses (laxaphycin A = 1 µM, laxaphycin B4 = 0.032 µM) showed an antagonistic effect (Figure 3A), which was further explained by a correlation trend between CI and percentage cell viability generated by computer simulation (Figure 3C). In general, the combination index decreases as the cell viability decreases (Figure 3C). When cell viability is lower than 70 %, synergism is predicted between laxaphycin A and B4 (**1**) under the tested combination ratios. A very strong synergistic

effect (CI < 0.1) is predicted when percentage cell viability is lower than 25 %.

In summary, laxaphycins are cyclic lipopeptides featuring a fatty β-amino acid moiety with a linear chain of up to 12 carbons. In the present study, we have discovered two new laxaphycins from a collection of the marine cyanobacterium *Hormothamnion enteromorphoides*. The total structures were determined using a combined analysis of NMR spectra, mass spectral data, chiral LC-MS analysis and advanced Marfey's analysis. Due to their structural similarity to the known laxaphycin B3 and laxaphycin A, these two new compounds were named laxaphycin B4 (**1**) and laxaphycin A2 (**2**), respectively. Laxaphycin B4 (**1**) exhibited antiproliferative effect against human colon cancer HCT116 cells with IC_{50} value of 1.7 μM, while laxaphycin A2 (**2**) had a much weaker effect. Using the same cancer cell model, we have also elucidated that laxaphycins A and B4 (**1**), the two major compounds co-produced in this collection, work synergistically to inhibit cancer cell growth.

3. Experimental Section

3.1. General Experimental Procedures

^{1}H and 2D NMR spectra in DMSO-d_6 and CH_3CN-d_3 were recorded on a Bruker Avance II 600 MHz spectrometer equipped with a 5 mm TXI cryogenic probe using residual solvent signals (δ_H 2.50; δ_C 39.51 ppm, DMSO-d_6; δ_H 1.94; δ_C 118.69 ppm, CH_3CN-d_3) as internal standards. HSQC experiments were optimized for 145 Hz, and HMBC experiments were optimized for 7 Hz. LC-MS data were obtained using an API 3200 (Applied Biosystems) equipped with a Shimadzu LC system. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. UV was measured on a SpectraMax M5 (Molecular Devices). HRMS data was obtained using an Agilent LC-TOF mass spectrometer equipped with an APCI/ESI multimode ion source detector.

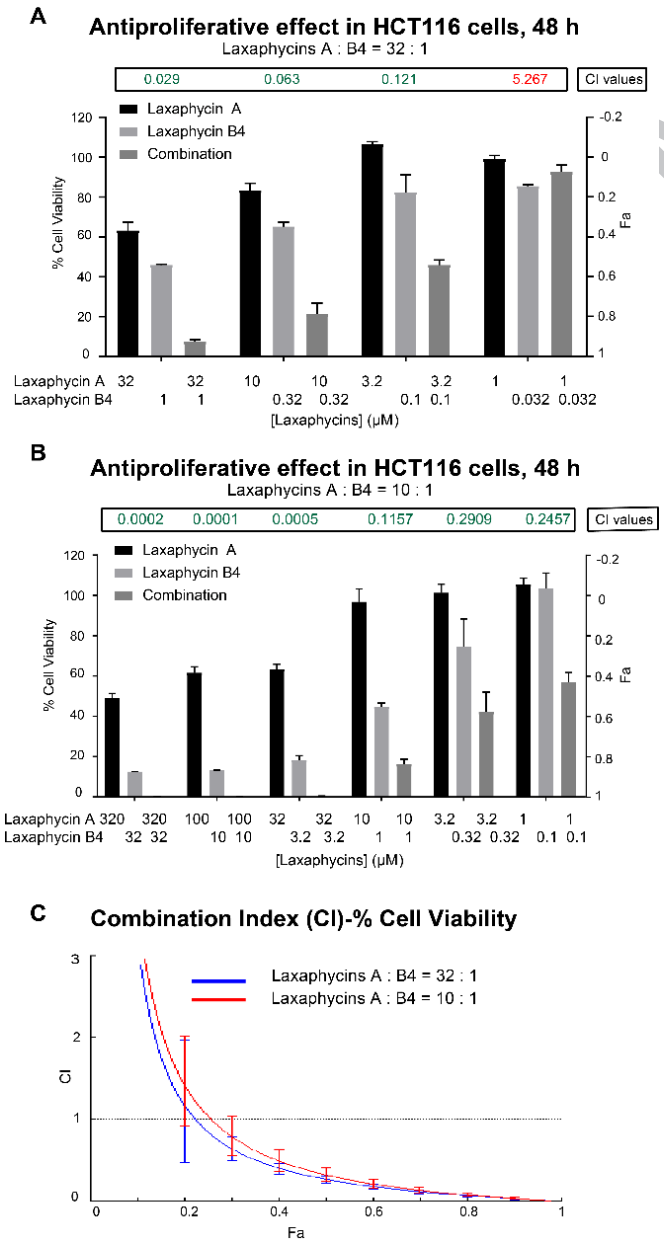


Figure 3. Antiproliferative effect of laxaphycins A and B4 in HCT116 cells. A) Combinatorial effect at a fix ratio of laxaphycin A: laxaphycin B4 = 32:1. B) Combinatorial effect at a fix ratio of laxaphycin A: laxaphycin B4 = 10:1. Cell viability (fraction survival, Fs) values were obtained using MTT assay, 48 h. These data were then converted to the level of cell growth inhibition, or affected fraction (Fa) using the equation of: Fa = 1- Fs. CI values were calculated using CompuSyn software as described in the Experimental Section. A CI value of <1 (in green), 1 and >1 (in red) indicates synergism, additivity and antagonism, respectively. Data are represented by the mean of three independent experiments +SD. C) Effect of the combinatorial ratios of laxaphycins A and B4 (32:1; 10:1) on the Combination Index (CI). CI is plotted against levels of cell growth inhibition or affected fraction (Fa) by computer simulation using CompuSyn software. The vertical bars indicate 95% confidence intervals based on Sequential Deletion Analysis (SDA) using CompuSyn software (see Experimental Section for details).

3.2. Chemicals

Standard amino acids, four isomers of 4-hydroxyproline and L-homoserine were obtained from Sigma-Aldrich, (St. Louis, MO). L-*threo*-3-hydroxyaspartic acid and (2*S*,3*R*)-3-hydroxyleucine was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). (2*S*,3*S*)-3-hydroxyleucine was obtained from AstaTech, Inc (Bristol, PA).

3.3. Biological Material

The cyanobacterium was collected on May 8 and May 9 in 2009 at Garden Key, Dry Tortugas National Park. Voucher specimens (DRTO0000033) are maintained at the Smithsonian Marine Station at Fort Pierce, Florida, and at South Florida Collections Management Center, Everglades National Park. The cyanobacterium corresponds morphologically and chemically with *Hormothamnion enteromorphoides*, which is broadly distributed in tropical waters in Florida, the Caribbean and Pacific and is known to produce hormothamnin A[9] and laxaphycins[22]. It has been suggested that this name should be regarded as a synonym of *Hydrocoryne enteromorphoides*.[23]

3.4. Extraction and Isolation

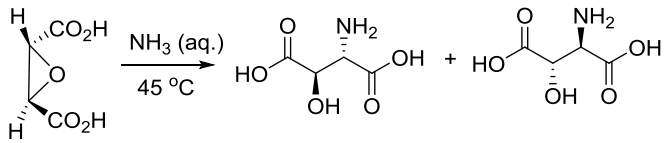
The freeze-dried sample (95 g) was extracted with CH_2Cl_2 and MeOH (1:1) to provide the nonpolar extract and EtOH and H_2O (1:1) to provide a polar extract. The nonpolar extract (2.6 g) was subjected to silica column using a gradient system of increasing i-PrOH in CH_2Cl_2. The fraction eluting with 20% i-PrOH was further purified by reversed-phase HPLC (Phenomenex Luna C18, 250 × 10 mm, 5μm, 2.0 mL/min; PDA detection) using a MeOH–H_2O linear gradient (50–100% MeOH for 30 min and 100% MeOH for 10 min). Fractions were pooled on the basis of retention times, ^{1}H NMR analysis and low-resolution MS measurements to afford laxaphycin B4 (**1**) (t_R 29.5 min), laxaphycin A2 (**2**) (t_R 29.8 min,) and laxaphycin A (t_R 30.5 min). The obtained semi-pure laxaphycins were further purified by the same HPLC column (Phenomenex Luna C18, 250 × 10 mm,

5μm, 2.0 mL/min; PDA detection) using a different solvent system: an CH_3CN–H_2O isocratic method (50% CH_3CN for 30 min) to yield laxaphycin B4 (**1**) (t_R 10.5 min, 20 mg), laxaphycin A2 (**2**) (t_R 20.5 min, 0.4 mg), and laxaphycin A (t_R 26.7 min, 20 mg).

Laxaphycin B4 (**1**). White amorphous solid; $[\alpha]_D^{20}$ −15.6 (*c* 0.09, MeOH); UV (MeOH) λ_{max} 200 nm (ε 20893), 230 nm (ε 2882), 270 nm (ε 362); NMR data, ^{1}H NMR,^{13}C NMR, COSY, HSQC, HMBC and ROESY in DMSO-d_6, see Table 1; HRESI/APCIMS *m*/*z* $[M + Na]^+$ 1463.8334 (calcd for $C_{66}H_{116}N_{14}NaO_{21}$ 1463.8337).

Laxaphycin A2 (**2**). White amorphous solid; $[\alpha]_D^{20}$ +6.9(*c* 0.032, MeOH); UV (MeOH) λ_{max} 200 nm (ε 26788), 230 nm (ε 11346); NMR data, ^{1}H NMR, COSY, HSQC, HMQC, HMBC and ROESY in CH_3CN-d_3, see Table 4; HRESI/APCIMS *m*/*z* $[M + Na]^+$ 1204.6930 (calcd for $C_{59}H_{95}N_{11}NaO_{14}$ 1204.6958).

Laxaphycin A. White amorphous solid. $[\alpha]_D^{20}$ +25.0 (*c* 0.5, MeOH); UV (MeOH) λ_{max} 202 nm (ε 29346), 230 nm (ε 23604) (hormothamnin A from literature $[\alpha]_D^{25}$ + 47.2 (*c* 1.22, MeOH); no data of laxaphycin A available)[10]; NMR data match literature values.[6]



3.5. Synthesis of erythro-3-hydroxy-L/D-aspartic acid.[24]

Ammonia aqueous solution (28–30%) (2 mL) was added to the solid (2*R*,3*R*)-epoxysuccinic acid (100 mg, 0.76 mmol) at 0 °C. The mixture was stirred 48 h at 45–48 °C, then cooled down and concentrated under reduced pressure. Water (3 × 15 mL) was added to the concentrated residue and evaporated again three times to remove traces of ammonia. The crude product was subjected to advanced Marfey's analysis.

3.6. Acid Hydrolysis and Chiral Amino Acid Analysis

Samples of **1** (0.3 mg) and **2** (~0.1 mg) were heated with 6 N HCl (110 °C, 20 h) and the hydrolysates subjected to chiral HPLC-MS [column, Chirobiotic TAG (4.6 × 250 mm), Supelco; solvent, MeOH-10mM NH_4OAc (40:60, pH 5.12): flow rate, 0.5 mL/min; detection by ESIMS in positive or negative ion modes (MRM scan)]. The retention times (t_R, min; MRM ion pair, parent→product) of the authentic amino acids and compound-dependent MS parameters were listed in Table 2 and Table 5. The source and gas-dependent MS parameters were as follows: CUR 50, CAD medium, IS 5500, TEM 750, GS1 65, GS2 65.

3.7. Advanced Marfey's Analysis

Samples of **1** and **2** (30 μg) were subjected to acid hydrolysis and reconstituted in water. Then, 10 μL of 1 M $NaHCO_3$ and 50 μL of 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA, 1% w/v in acetone) or 1-fluoro-2,4-dinitrophenyl-5-DL-leucinamide (DL-FDLA, 1% w/v in acetone) were added to 25 μL of these solutions. After heating at 40 °C for 1 h, with frequent mixing, the reaction mixtures were acidified with 5 μL 2 N HCl, concentrated to dryness and then reconstituted with 250 μL MeCN–H_2O (1:1). Amino acid standards were made into 50 mM stock solutions in water, derivatized with L-FDLA or DL-FDLA in a similar method. Standards and hydrolysates were subjected to reversed-phase HPLC-MS analysis. Details are listed in Table 3 and Table 6. The source and gas-dependent MS parameters were as follows: CUR 40, CAD medium, IS 4500, TEM 450, GS1 40, GS2 40.

3.8. Cell Viability Assay (MTT)

HCT116 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) under a humidified environment with 5% CO_2 at 37 °C. HCT116 (10,000) cells were seeded in 96-well plates. Cells were treated with a series of concentrations of laxaphycins in DMSO (0.5% for both single compound and combinatory studies), 24 h post seeding. Cells were incubated for an additional 48 h before the addition of the MTT reagent. Cell viability (fraction survival, Fs) was measured according to the manufacturer's instructions (Promega, Madison, WI, USA). Treatments were done in triplicate. Nonlinear regression analysis was carried out using GraphPad Prism software for IC_{50} value calculations.

3.9. Combination Index (CI) Calculation

The cell viability (fraction survival, Fs) data was obtained from the MTT assay. For combinatory studies, compound effects were calculated as levels of cell growth inhibition or affected fraction (Fa) of treated verses control cells. Affected fraction (Fa) was obtained using the equation of: Fa = 1- Fs. Dose–effect analyses and calculation of combination index (CI) were performed using CompuSyn software (ComboSyn Inc, Paramus, NJ, USA).[21] CI reflects the extent of synergy or antagonism for two drugs: CI<1, synergy; CI =1, additive effect; CI>1, antagonism. CI is plotted against levels of cell growth inhibition or affected fraction (Fa) by computer simulation. The vertical bars indicate 95% confidence intervals based on Sequential Deletion Analysis (SDA).

Acknowledgments

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Supplementary Data

Structures of laxaphycins and analogues (Figures S1 and S2), NMR spectra (^{1}H NMR, ^{13}C NMR, COSY, HSQC, HMBC, ROESY) for compound **1**, NMR spectra (^{1}H NMR, COSY, TOCSY, HSQC, HMBC, ROESY) for compound **2** and NMR spectra (**(**^{1}H NMR, ^{13}C NMR) for laxaphycin A (Figures S3–S17).

Notes

The authors declare no competing financial interest.

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