

Article

Isolation and Synthesis of Laxaphycin B-Type Peptides: A Case Study and Clues to Their Biosynthesis

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Abstract: The laxaphycin B family constitutes a group of five related cyclic lipopeptides isolated from diverse cyanobacteria from all around the world. This group shares a typical structure of 12 amino acids from the L and D series, some of them hydroxylated at the beta position, and all containing a rare beta-amino decanoic acid. Nevertheless, they can be differentiated due to slight variations in the composition of their amino acids, but the configuration of their alpha carbon remains conserved. Here, we provide the synthesis and characterization of new laxaphycin B-type peptides. In doing so we discuss how the synthesis of laxaphycin B and analogues was developed. We also isolate minor acyclic laxaphycins B, which are considered clues to their biosynthesis.

Keywords: non ribosomal peptides; cyclic lipopeptide; solid phase peptide synthesis; *Anabaena*; *Lyngbya*

1. Introduction

Among marine organisms, filamentous cyanobacteria occupy a special place and/or are of great interest for chemists because they produce a wide range of bioactive molecules, mainly cyclic lipopeptides [1–3]. Interestingly, they produce this class of secondary peptide metabolites via a non-ribosomal pathway that is responsible, for example, for the modification of natural amino acids into D-, N-methyl, β -hydroxylated, or dehydrated amino acids. These non-ribosomal peptide synthases (NRPS) are often associated with polyketide synthases (PKS) that allow fatty amino acids to be inserted within the peptide sequence [4,5]. The concomitant effects of these two multi-domain enzymes contribute to the vast diversity of structure observed in these secondary cyclopeptide metabolites [6].

Laxaphycins are cyclic lipopeptides synthesized through a hybrid PKS/NRPS biosynthetic pathway by different marine or freshwater cyanobacteria. They contain amino acids of alternate stereochemistry (L or D) and feature a rare fatty β -amino acid with a linear chain of up to 12 carbons [7]. Several studies have reported structural variants and likely biosynthetic derivatives of laxaphycins that can be separated into two groups, the laxaphycin A-type peptides, which are cyclic

undecapeptides, and the laxaphycin B-type peptides, which are cyclic dodecapeptides. Laxaphycin A-type and laxaphycin B-type peptides are generally found in the same cyanobacteria.

Anabaena laxa [8], *A. torulosa* [9], *Lyngbya confervoides* [10], *Trichormus* sp. [11], and *cf. Oscillatoria* sp. [12] express laxaphycins B, B2, B3, and D, lobocyclamides B and C, and trichormamides B and C. Furthermore a *Lyngbya* sp. strain produces lyngbyacyclamides A and B [13] (Figure 1). Horizontal gene transfer between cyanobacteria has been suggested as an explanation for the presence of all these closely related compounds in diverse species [14].

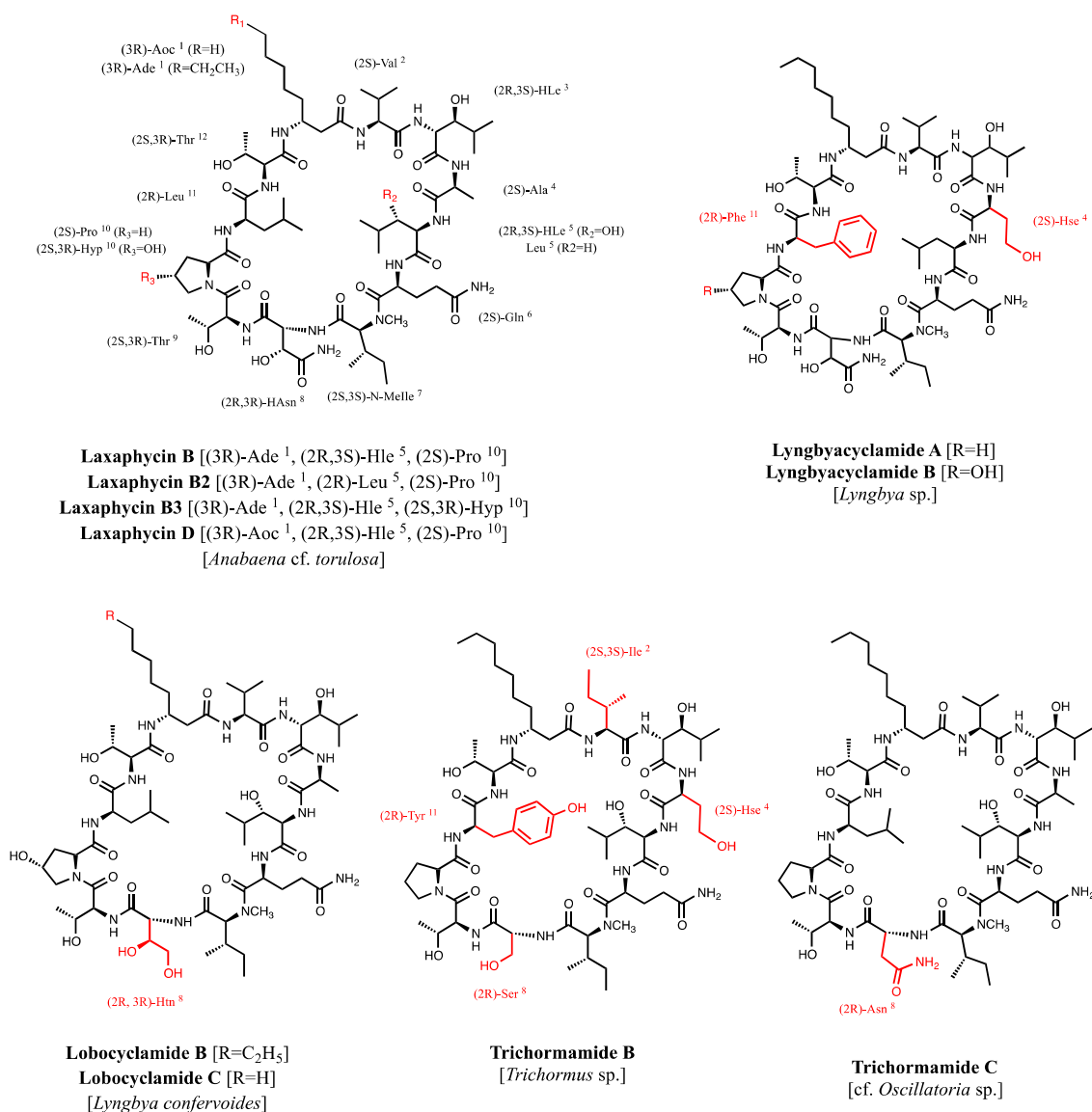


Figure 1. Laxaphycins B, B2, B3, and D and their analogs lyngbyacyclamides A–B, lobocyclamides B–C and trichormamides B–C. Differences between laxaphycins and their homologs are highlighted in red.

In the present study we were interested in the study of laxaphycin peptides from *A. cf. torulosa*, compounds that have already been found to reduce damage by consumers [15]. More specifically, we focus on both total synthesis and structural characterization of laxaphycin B-type peptides; total synthesis being the ultimate way to confirm or revise the proposed structure of such dodecapeptides possessing non-commercial amino acids.

We recently published the first total synthesis of laxaphycin B (short form: laxaB) and lyngbyacyclamide A [16]. Here we describe the synthesis of two new laxaB analogs, including the different steps and strategies that were taken to revise the initially reported structure. We also describe the structure of two new acyclic laxaB-type peptides. The presence of these acyclic

dodecapeptides, named acyclolaxaphycin B (11) and acyclolaxaphycin B3 (12), together with the other laxaphycins in the extract of *A. cf. torulosa*, provide valuable information for the biosynthesis of laxaphycins. Understanding how these non-ribosomal peptides can be chemically synthesized and how such intriguing structures can be synthesized in nature is challenging.

2. Results and Discussion

2.1. Synthesis of Laxaphycin B Analogs

The synthesis of lipocyclopeptides of marine origin is not straightforward, firstly because synthetic chemists have to make a choice among the different potential stereochemistry options for the isolated compounds. Secondly, they need to prepare non-natural amino acids and use them sparingly when developing the synthesis or even have recourse to use related analogs, before extending the synthesis to the natural compound. Taking these limitations into account, especially the uncertainty concerning the stereochemistry of the residue in position 3, we proposed an analog of the ((2*S*,3*S*)-Hle³)laxaphycin B, the compound initially described for laxaB, in which the 2-aminodecanoic acid (Ade), the 3-hydroxyleucines (Hle), and the 3-hydroxyasparagine (HAsn) were replaced by the simplest and commercially available β -alanine, threonine, and asparagine, respectively, leading to analog 1 (Figure 2). The replacement amino acids were selected with respect to the stereochemistry of the alpha carbon of the published compound. These peptides can be obtained either by adopting an in-solution synthetic strategy or by solid phase peptide synthesis (SPPS). Due to its numerous advantages including the avoidance of repetitive purification steps and flexibility, SPPS was used preferentially to the in-solution strategy. Furthermore, we make use of an instrumentation combining the advantages of automation and microwave heating that speeds up synthesis and increases overall yield and purity for difficult peptide sequences [17–20].

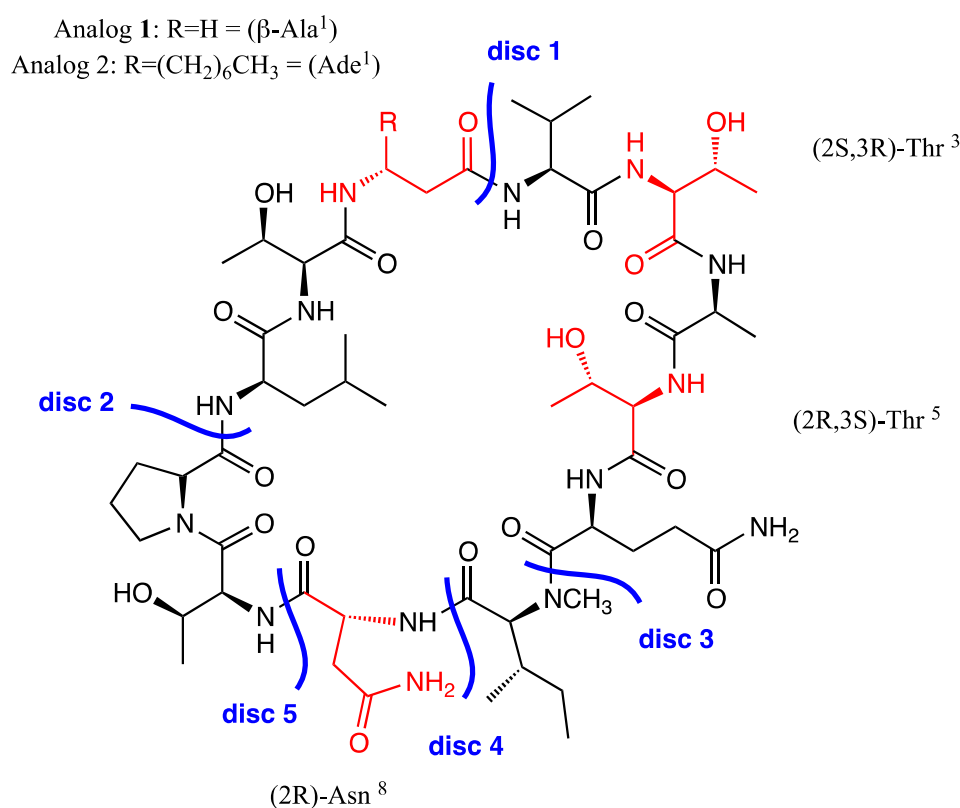


Figure 2. Proposed simplified analogs 1 and 2 of [(2*S*,3*S*)-Hle³]laxaphycin B and feasible disconnections.

2.1.1. Retrosynthetic Analysis of the Laxaphycin B Analog 1

Five disconnections were envisaged for the cyclization of a linear precursor obtained by a Fmoc/*t*Bu SPPS strategy. Depending on the position of the disconnected amide bond, the cyclization can be performed in solution or on the resin [21]. Thus disconnections 1 and 2 (Figure 2) would imply the use of chlorotrityl resin and a subsequent cleavage in dilute acidic conditions of a completely protected linear peptide that has to be cyclized in solution in the last step. Firstly, this option was discarded because a long reaction time is prone to generate dimerization or oligomerization even in dilute conditions and this option requires sophisticated setups involving syringe pumps to deliver the reagents [22,23]. Secondly, the use of microwave irradiation during peptide elongation was recently shown to be deleterious because of the sensitivity of 2-Cl trityl-resin to extended heating periods [24]. Subsequently, we considered the side chains of an aspartic or a glutamic acid as possible anchoring points onto a low loading Rink amide resin since the final cleavage from the resin would produce the corresponding asparagine or glutamine. Indeed, this would allow the entire synthesis to be performed on the resin due to the temporary allyl protection of the C-terminal α -carboxyl-group [25]. Direct use of glutamic acid was inconvenient since the ultimate step of the synthesis, the cyclization, would occur at an impractical coupling site between its carboxylic function and the *N*-methylated group of the isoleucine (disconnection 3, Figure 2).

Alternatively, we proposed grafting the FmocGlu-*N*-Melle(Oallyl) dipeptide (**3b**) on the resin (Figure 2, disconnection 4) that was conveniently prepared by successively coupling compounds **4** and **5** using COMU [26] with final deprotection under acidic conditions of the tertbutylester (Figure 3). Unfortunately, after the addition of the dipeptide onto the resin support and deprotection of the Fmoc protective group, diketopiperazine **6** forms immediately and precludes further extension of the peptide [27]. This was confirmed after acidic release of compound **6** from the resin, and analysis of the crude by LC-MS, which showed a major peak with the expected mass $[M + H]^+$ m/z 256.

Finally, among the remaining possibilities our choice was dictated by the presence of the non-commercially available (2*R*,3*R*) β -OH-Asn⁸, a constituent of natural laxaphycin B. In order to test its potential for future use, we had to develop a protocol for its synthesis starting from a surrogate, thus (2*R*)-aspartic acid was chosen as an anchoring point to the resin (disconnection 5). It is noteworthy that one advantage of this strategy resides in the possibility of accessing trichormamide C analogues.

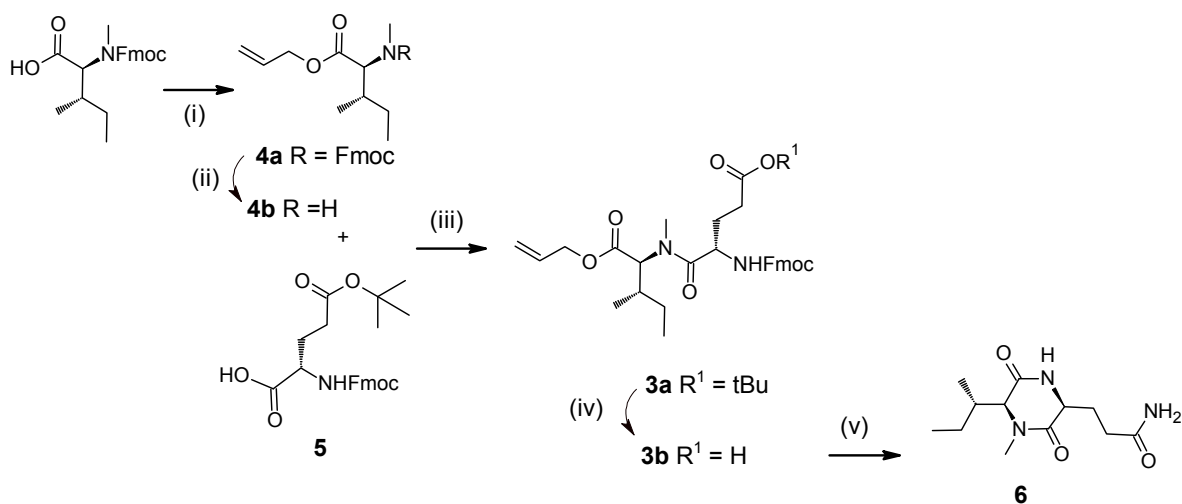


Figure 3. Proposed dipeptide **3b** as a starting material for the synthesis of ((2*S*,3*S*)-Hle³)laxaphycin B analogs and formation of undesired diketopiperazine **6**. Reagents and conditions: (i) Cs(CO₃)₂, DMF, Allylbromide, 76%; (ii) piperidine, 70%; (iii) DIEA, COMU, DMF, 70%; (iv) TFA, CH₂Cl₂; (v) Rink amide resin, HATU, DIEA, piperidine.

2.1.2. Solid Phase Synthesis Preliminary Assay of ((2*S*,3*S*)-Hle³)laxaphycin B Analogs

The ((2*S*,3*S*)-Hle³)laxaphycin B analog **1** was assembled by stepwise SPPS starting with the introduction of the *N*-Fmoc-aspartic acid α -allyl ester onto a low loading resin amide MBHA resin (0.36 mmol/g) using the effective 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate HATU as a coupling reagent and a fivefold excess of standard amino acids with regard to resin capacity (Figure 4). The choice of this relatively expensive reagent was guided by preliminary results, which revealed the presence of a difficult coupling site located at the Gln and *N*-Me-Ile junction, requiring a triple coupling and a capping step. After synthesis of the linear precursor **7**, the α -carbonyl allyl protecting group of Asn was removed using Pd(PPh₃)₄. Head to tail cyclization of the resin-bound peptide was accomplished after Fmoc removal using DIC/oxyma (3 \times 15 min), a base-free condition known to reduce epimerization [28]. Final acidic cleavage followed by HPLC-purification produced the desired peptide **1** (Tables 1 and S1).

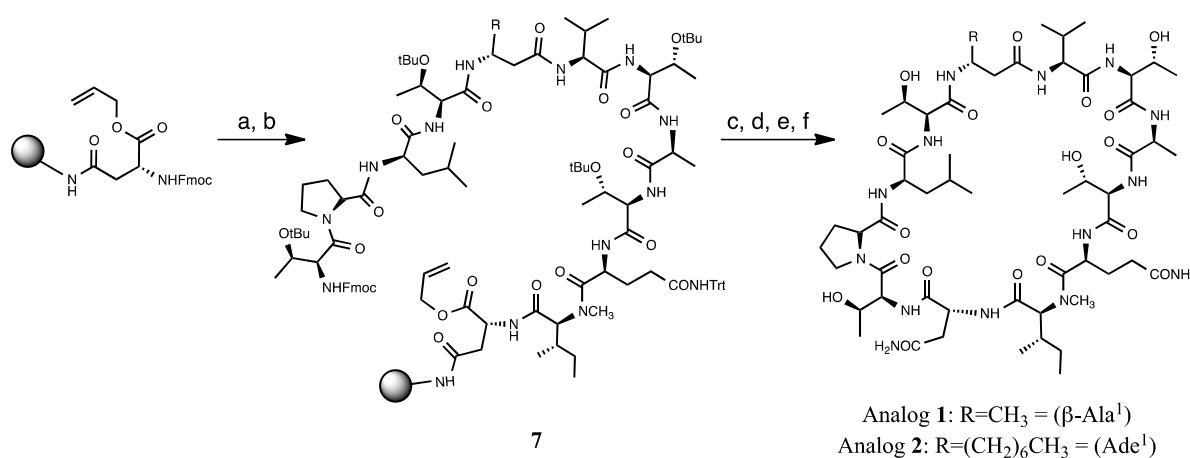


Figure 4. Syntheses of the laxaphycin B analogs **1** and **2**. Reagents and conditions: (a): (i) 20% *v/v* piperidine, DMF (ii) Fmoc-*N*-Melle, HATU, DMF, MW 25 W, 70 °C, 5 min; (b) repetition of conditions (i) and (ii) for Fmoc-Gln-(Trt) 2 \times 10 min, Fmoc-D-Thr(*t*Bu)-OH, Fmoc-Ala-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Val-OH, Fmoc- β -Ala-OH or Fmoc Ade 2 \times 10 min, Fmoc-Thr(*t*Bu)-OH, Fmoc-D-Leu-OH, Fmoc-Pro-OH, Fmoc-Thr(*t*Bu)-OH; (c) Pd(PPh₃)₄, CHCl₃/AcOH/NMM, 3.7/0.2/0.1, *v/v/v* (d) 20% *v/v* pipéridine, DMF, rt, 2 \times 2 min (e) DIC, oxyma, DMF, MW 25 W, 70 °C 3 \times 15 min; (f) TFA/TIS/H₂O 9.5/0.25/0.25, *v/v/v*, TA, 3 h.

Table 1. Retention time (Rt) in minutes and observed mass peaks of the analogs **1**, **2**, **8**, and laxaB.

Compound	1	2	((2 <i>S</i> ,3 <i>S</i>)-Hleu ³)laxa B (8)	Laxaphycin B (9)
Mass Peaks	[M + H] ⁺ <i>m/z</i> 1225	[M + H] ⁺ <i>m/z</i> 1323	[M + H] ⁺ <i>m/z</i> 1395	[M + H] ⁺ <i>m/z</i> 1395
Rt (min)	8.64	16.01	16.3	Natural: 14.8 Synthetic: 14.8

The next step on the way to laxaphycin B synthesis was the introduction of the (2*R*)-2-aminodecanoic acid (Ade) obtained using an already described procedure and subsequent protection with a Fmoc group [29]. Indeed, due to the high hydrophobic character of both Ade and the peptide itself, aggregation of the peptide growing chain was expected even if counterbalanced by microwave irradiation [17]. As expected, a drop in the coupling efficacy of Ade was observed by UV monitoring of the Fmoc group deprotection and was corrected for with a double coupling procedure. Thus analog **2** was obtained and characterized by ¹H-NMR and LC-MS analysis (Tables 1 and S1).

¹H-NMR COSY experiments allowed the chemical shift of all amino acids to be assigned, but ambiguity remained for the four threonines that could not be distinguished. Nevertheless, a

comparison of NMR spectra of the closely related analogs **1** and **2** revealed considerable consistency in the observed chemical shift (Figures S1 and S2).

2.1.3. Laxaphycin B Revised Structure

Finally, as recently reported, the above synthesis method was applied to laxaB [16]. Briefly, after introduction of the rare (2*R*,3*R*)-Fmoc- β -hydroxyaspartic α -allyl ester, (2*R*,3*S*)-Fmoc-Hle-(OTBDMS)-OH and (2*S*,3*S*)-Fmoc-Hle-(OTBDMS)-OH, 3 mg of pure peptide (**8**) was obtained [30,31]. The recorded molecular mass of $[M + H]^+$ m/z 1395 and $[M + Na]^+$ m/z 1417 from LC-MS ESI+ analysis corresponds to the one expected for laxaB. Nevertheless, a comparison of the retention time of compound (**8**) with a natural sample of laxaB (**9**) revealed differences that were further confirmed by co-injection (Table 1) and comparison of NMR spectra. Thus, we could conclude from this experiment that the synthesized compound (**8**) is a diastereoisomer of laxaB (**9**). In a last effort to obtain the natural compound, we hypothesized that the hydroxyleucines must have the same configuration as observed for lobocyclamide B. Repetition of the developed synthesis method using (2*R*,3*S*)-Fmoc-Hle-(OTBDMS)-OH produced, after purification, 3 mg of peptide **9** co-eluting with laxaphycin B and presenting the same ¹H-NMR and mass spectra as the natural compound.

2.2. New Natural Acyclolaxaphycin B Analogs

Collection and extraction of *A. cf torulosa* and initial separation of the organic extract were described in a previous paper [9]. Further examination of the more polar flash chromatography fractions obtained from the organic extract, by C18 RP HPLC yielded two HPLC pure peaks, acyclolaxaphycin B (short form: acyclolaxaB) and acyclolaxaphycin B3 (short form: acyclolaxaB3). AcyclolaxaB (2 mg) and acyclolaxaB3 (3 mg) were obtained as colorless amorphous solids and responded positively to a ninhydrin test suggesting a non-blocked *N*-terminus. LC-MS analysis of pure compounds with electrospray positive ionization revealed two different peptides whose m/z values are 18 units higher than both laxaphycins B (**9**) and B3 (**10**).

2.2.1. Acyclolaxaphycin B (**11**): Structure Elucidation

High-resolution electrospray ionization mass spectrometry (HRESIMS) analysis yielded an $[M + H]^+$ pseudomolecular ion at m/z 1413.8595 for a molecular formula of C₆₅H₁₁₆N₁₄O₂₀ that was supported by NMR spectroscopic analysis. A comparison with laxaphycin B (C₆₅H₁₁₄N₁₄O₁₉) revealed that this corresponds to a gain of H₂O.

In the ¹H-NMR spectrum of acyclolaxaB (**11**), recorded at 500 MHz in DMSO-*d*₆, the close structural relationship between the two peptides was clear; the spectrum exhibited, in the NH proton region, signals typical for CONH₂ protons corresponding to Gln (2 bs, δ_{H} 6.79, and δ_{H} 7.14) and Asn (2 bs, δ_{H} 7.26, and δ_{H} 7.30) similar to those observed for laxaB (Figure S3). Only one significant difference was found in the NH proton region: nine NH doublets and one large singlet (2H) were visible in acyclolaxaB ¹H-NMR spectrum, instead of the 10 NH doublets observed between 7.4 and 8.4 ppm for laxaB.

Almost all ¹H and ¹³C resonances of acyclolaxaB (Table 2) could be assigned using extensive 2D NMR analysis including COSY, TOCSY, HSQC, HSQC-TOCSY, and ROESY (Figures S4–S7).

Initially, spin systems in TOCSY spectrum were identified starting from the signals of the backbone amide protons in the region 8.5 to 6.5 ppm. From the characteristic chemical shift and comparison with laxaB, eight amino acids could be identified as Hle (2 \times), Gln, Val, Leu, Thr (2 \times), and HAsn. A β -Ade residue system was identified starting from a doublet at 7.53 ppm and possessing an AA'BB' spin system (2.27 and 2.38 ppm) with additional signals at 4.05, 1.34–1.40, then 1.20–1.23 ppm. One spin system lacking an amide proton was identified as *N*-MeIle due to the correlations of its H α and H β at 4.71 and 1.91 ppm, respectively. One last amino acid, attributed to Ala residue, was identified starting from a broad singlet (two protons) at 8.04 ppm, to H α (δ_{H} 4.03, 1H, overlapped bs) and H β (δ_{H} 1.36, 3H, d).

Table 2. ¹H and ¹³C NMR data for laxaphycins B and B3 and acyclolaxaphycins B and B3 in DMSO-*d*₆.

	Laxaphycin B		Acyclolaxaphycin B		Laxaphycin B3		Acyclolaxaphycin B3	
	¹³ C	¹ H	¹³ C	¹ H	¹³ C	¹ H	¹³ C	¹ H
β Ade¹								
NH	-	7.58	-	7.53	-	7.52	-	7.53
CαH ₂	40.28	2.33/2.40	40.52	2.27/2.38	-	2.30/2.44	40.42	2.28/2.40
CβH	45.93	4.11	46.27	4.05	45.92	4.08	46.13	4.05
CγH ₂	33.45	1.29/1.40	33.62	1.34/1.40	33.41	1.40	33.49	1.33/1.40
CδH ₂	28.67 *	1.24	28.77	1.23	28.69 *	1.24	28.69	1.21
CεH ₂	28.47 *	1.20	28.61	1.20	28.47 *	1.20	28.53	1.21
CζH ₂	25.18 *	1.20	25.28	1.21	25.22 *	1.20	25.20	1.22
CηH ₂	31.11 *	1.20	31.22	1.21	31.10 *	1.20	31.15	1.20
CθH ₂	21.92 *	1.20	22.04	1.20	21.92 *	1.20	21.97	1.24
CιH ₃	13.79	0.84	13.91	0.85	13.79	0.82	13.81	0.83
CO	171.14	-	170.30	-	171.30	-	170.15	-
Val²								
NH	-	8.18	-	7.89	-	8.10	-	7.89
CαH	59.03	4.09	57.64	4.30	58.89	4.12	57.50	4.31
CβH ₂	29.33	1.97	30.59	2.02	29.37	1.98	30.51	2.02
CγH ₃	18.80	0.91	18.85	0.93	18.56	0.88	18.89	0.93
Cγ'H ₃	18.87	0.85	18.95	0.81	18.85	0.84	18.78	0.81
CO	171.05	-	171.27	-	171.30	-	171.15	-
HLe³								
NH	-	7.94	-	7.69	-	7.90	-	7.70
CαH	55.23	4.34	54.30	4.44	55.15	4.37	54.21	4.44
CβH	76.37	3.49	76.06	3.53	76.48	3.50	76.13	3.53
OH	-	4.94	-	—	-	4.90	-	—
CγH	30.54	1.58	30.68	1.51	30.57	1.60	30.84	1.52
CδH ₃	19.22 *	0.89	19.19	0.91	18.76 *	0.89	19.23	0.91
Cδ'H ₃	18.56	0.76	18.74	0.76	18.43	0.76	18.67	0.76
CO	171.35	-	172.40	-	-	-	172.34	-
Ala⁴								
NH/NH ₂	-	7.86	-	8.04	-	7.87	-	8.05
CαH	49.28	4.22	48.30	4.03	49.30	4.22	48.20	4.04
CβH ₃	17.55	1.31	17.43	1.36	17.65	1.32	17.38	1.36
CO	172.33	-	170.02	-	172.47	-	169.87	-
HLe⁵								
NH	-	7.69	-	8.34	-	7.61	-	8.37
CαH	55.52	4.28	55.40	4.44	55.64	4.28	55.27	4.46
CβH	75.80	3.49	76.21	3.53	75.78	3.48	75.94	3.53
OH	-	5.03	-	—	-	5.05	-	—
CγH	29.90	1.56	30.65	1.51	29.84	1.58	30.73	1.51
CδH ₃	18.65 *	0.89	17.58	0.82	18.69 *	0.88	19.14	0.83
Cδ'H ₃	18.56	0.76	19.28	0.81	-	0.74	17.52	0.83
CO	170.50	-	169.74	-	170.60	-	169.64	-
Gln⁶								
NH	-	7.77	-	8.02	-	7.56	-	8.04
CαH	49.16	4.63	48.94	4.69	49.40	4.58	48.78	4.70
CβH ₂	26.39	1.75/1.97	26.90	1.76/1.93	-	1.64/2.00	26.86	1.77/1.94
CγH ₂	30.72	2.04/2.10	30.63	2.12	-	2.15/2.23	30.70	2.13

CON	174.60	-	174.38	-	174.74	-	174.31	-
NH ₂	-	6.85/7.22	-	6.79/7.14	-	6.79/7.17	-	6.80/7.16
CO	172.49	-	172.45	-	172.64	-	172.27	-
<i>N</i> -Melle ⁷								
NCH ₃	30.03	2.97	30.24	2.97	30.15	3.01	30.20	2.98
C α H	59.85	4.72	59.94	4.71	59.87	4.73	59.79	4.73
C β H	31.56	1.90	31.50	1.91	31.80	1.90	31.41	1.92
C γ H ₂	23.88	0.89/1.29	23.98	0.87/1.28	-	0.74/1.27	23.92	0.87/1.27
C γ' H ₃	15.08	0.76	15.25	0.78	14.99	0.74	15.21	0.79
C δ H ₃	10.33	0.78	10.48	0.79	10.31	0.75	10.41	0.78
CO	170.02	-	169.66	-	170.10	-	169.47	-
<i>H</i> Asn ⁸								
NH	-	7.64	-	7.41	-	7.66	-	7.41
C α H	55.52	4.63	55.22	4.67	55.53	4.63	55.13	4.71
C β H	70.44	4.31	71.04	4.36	70.33	4.35	70.99	4.37
OH	-	5.79	-	5.78	-	5.70	-	-
CON	173.37	-	173.20	-	173.37	-	173.20	-
NH ₂	-	7.27	-	7.26/7.30	-	7.17	-	7.27/7.32
CO	169.16	-	168.92	-	169.12	-	168.75	-
<i>Thr</i> ⁹								
NH	-	7.33	-	7.63	-	7.12	-	7.63
C α H	55.61	4.49	55.25	4.57	55.83	4.46	55.56	4.56
C β H	66.23	3.93	66.54	3.98	66.43	3.90	66.50	3.97
OH	-	4.94	-	-	-	4.89	-	-
C γ H ₃	18.87	1.05	18.64	1.05	18.85 *	1.03	18.59	1.05
CO	168.58	-	168.87 *	-	168.70 *	-	169.04 *	-
<i>Pro</i> ¹⁰ / <i>Hyp</i> ¹⁰								
C α H	59.60	4.33	59.90	4.37	58.62	4.43	58.87	4.44
C β H ₂	29.08	1.82/2.04	28.77	1.83/2.03	37.73	1.84/2.01	37.45	1.89/2.05
C γ H ₂	24.00	1.80/1.90	24.16	1.83/1.90	68.50	4.32	68.48	4.31
OH	-	-	-	-	-	5.08	-	-
C δ H ₂	47.16	3.68	47.49	3.64/3.75	55.48	3.58/3.72	55.60	3.60/3.76
CO	171.21	-	171.42	-	171.47 **	-	171.33	-
<i>Leu</i> ¹¹								
NH	-	7.89	-	7.77	-	7.86	-	7.84
C α H	51.36	4.31	51.44	4.30	51.31	4.35	51.36	4.29
C β H ₂	40.82	1.47	40.44	1.47	41.24	1.47	40.51	1.46
C γ H	24.06	1.53	24.09	1.58	24.12	1.52	24.06	1.58
C δ H ₃	22.71	0.87	22.96	0.86	22.75	0.86	22.83	0.86
C δ' H ₃	21.76	0.82	21.42	0.84	21.72	0.80	21.43	0.83
CO	171.67	-	171.83	-	171.41 **	-	171.33	-
<i>Thr</i> ¹²								
NH	-	7.74	-	7.57	-	7.68	-	7.59
C α H	57.85	4.11	58.13	4.10	58.17	4.10	58.12	4.10
C β H	66.19	4.00	66.52	3.97	66.35	3.97	66.50	3.97
OH	-	4.78	-	-	-	4.80	-	-
C γ H ₃	19.46	0.99	19.55	0.99	19.48	0.99	19.45	1.00
CO	168.67	-	168.87 *	-	168.67 *	-	168.99 *	-

*** *Thr*⁹ and *Thr*¹² Chemical shifts may be interchanged.

Sequence-specific assignments were determined from the HMBC correlations (Figure S8) between carbonyl carbons (residue *i*) and NH or NCH₃ protons (residue *i*+1). These data suggested the presence of two fragments consisting of Ala-Hle-Gln-*N*-Melle-HAsn-Thr (fragment 1) and

Pro-Leu-Thr- β -Ade-Val-Hle (fragment 2). These two partial sequences were confirmed by ROESY correlations between H α or H β (residue *i*) and NH or NCH₃ (residue *i*+1). Fragments 1 and 2 were assembled by two inter-residue ROESY correlations between H α ($\delta_{\text{H}}4.57$) and H β ($\delta_{\text{H}}3.98$) of Thr⁹ and H δ ($\delta_{\text{H}}3.64/3.75$) of Pro¹⁰, establishing the complete sequence as Ala-Hle-Gln-*N*-Melle-HAsn-Thr-Pro-Leu-Thr- β -Ade-Val-Hle (Figure 5). MS/MS data for **11** were consistent with the proposed amino acid sequence with the *y* ions at *m/z* 1213.50 (*y*₁₀), 1085.58 (*y*₉), 828.42 (*y*₇), and 727.42 (*y*₆) and the *b* ions at *m/z* 1266.75 (*b*₁₁), 1167.58 (*b*₁₀), and 456.25 (*b*₄).

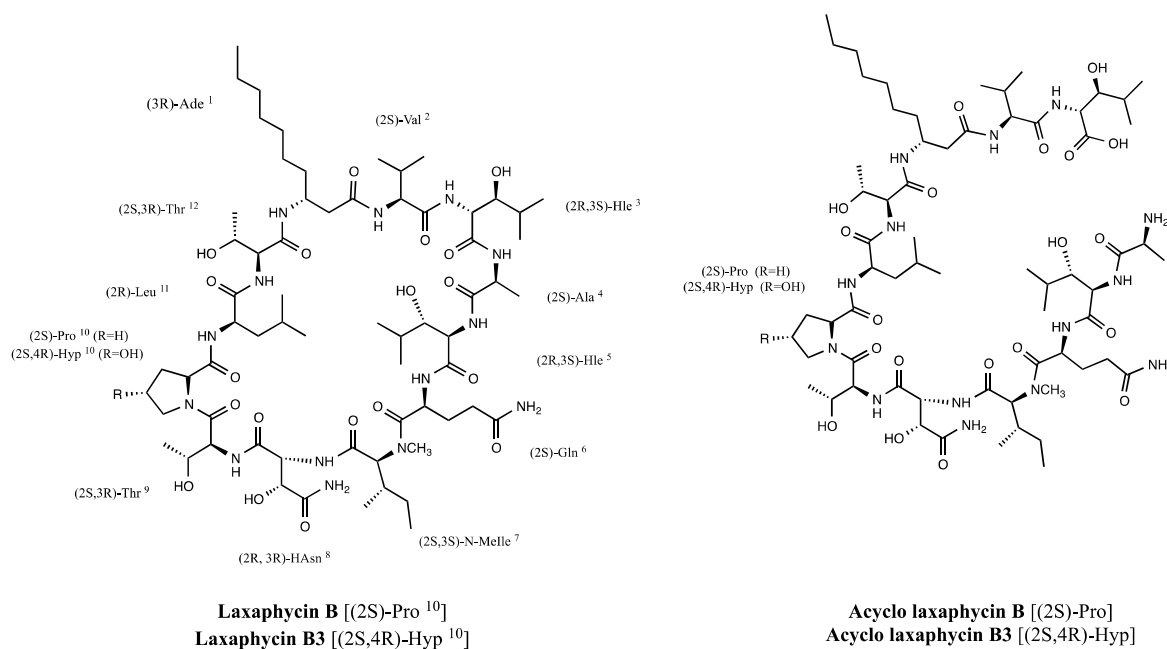


Figure 5. Structures of laxaphycins B (**9**) and B3 (**10**), and acycloaxaphycins B (**11**) and B3 (**12**).

2.2.2. Acycloaxaphycin B3 (**12**): Structure Elucidation

Preliminary spectral data examination, including ¹H and ¹³C-NMR spectroscopy, showed that the new compound was an analog of laxaB3 (**10**) and acycloaxaB (**11**) (Figure S9). HRESIMS analysis yielded a [M + H]⁺ pseudomolecular ion at *m/z* 1429.8482 for a molecular formula of C₆₅H₁₁₆N₁₄O₂₁. In comparison to laxaB3 (C₆₅H₁₁₄N₁₄O₂₀), this corresponds to a gain of H₂O and to acycloaxaB gain of an oxygen atom.

A similar pattern of fragmentation for both compounds **11** and **12** was observed. Comparison of MS/MS spectra showed the same *b*₄ fragment at *m/z* 456.25, the *b*₁₁ (*m/z* 1282.76), *y*₆ (*m/z* 743.49), *y*₇ (*m/z* 844.54), *y*₉ (*m/z* 1101.68), and *y*₁₀ (*m/z* 1229.72) ions being shifted to a higher mass by 16 amu. In the HRESIMS/MS spectra of **12**, *b*₉ (*m/z* 1014.55060, C₄₄H₇₆N₁₁O₁₆⁺, $\Delta_{\text{obs}/\text{calc}} = 0.004$) and *b*₁₁ (*m/z* 1282.76672, C₅₉H₁₀₄N₁₃O₁₈⁺, $\Delta_{\text{obs}/\text{calc}} = 0.005$) fragments were observed. The *y*₆ and *b*₉ fragments in compound **12** shifted by 16 amu compared to **11**, suggesting that the variable residue could be in position 10, 11, or 12, corresponding to the Pro, Leu, or Thr residues, respectively.

The NMR spectral analysis (Figures S9–S14) of acycloaxaB3 showed remarkable similarities with acycloaxaB (**11**) and established the variable residue as Pro/Hyp (Figure 5). The significant difference was the presence of an additional hydroxyl group on proline (H γ at 4.31 ppm *vs.* two H γ at 1.83 and 1.90 ppm for compound **11**; C γ at 68.48 ppm *vs.* 24.16 ppm for compound **11**; C β and C δ were also debled by the presence of the hydroxyl function ($\Delta\delta$ 8.68 and 8.11 ppm, respectively)). HMBC and ROESY correlations established the complete sequence as Ala-Hle-Gln-*N*-Melle-HAsn-Thr-Hyp-Leu-Thr- β -Ade-Val-Hle for compound **12**, and the gross structure of the new compound, acycloaxaphycin B3, differed from acycloaxaphycin B with a replacement of Pro by Hyp.

2.2.3. Acyclolaxaphycins B (11) and B3 (12): Clues to Their Biosynthesis

Acyclolaxaphycins B (11) and B3 (12) are acyclic analogs of laxaphycins B (9) and B3 (10), respectively. They are two novel acyclic structural variants of a core structure (B-type laxaphycins) composed of about 10 cyanobacterial β -amino fatty acid cyclic dodecapeptides, laxaphycins B, B2, B3, and D, lyngbyacyclamides A–B, lobocyclamides B–C, and trichormamides B–C (Figure 1) with conserved amino acid residues. The chemical structure of all these compounds were similar, maintaining a 12-membered ring and sharing the (3*R*)- β -amino fatty acid (β -Aoc or β -Ade), (2*R*,3*S*)-Hle, (2*S*)-Gln, (2*S*)-*N*-MeIle, (2*S*,3*R*)-Thr, and (2*S*,3*R*)-Thr in the positions 1, 3, 6, 7, 9, and 12. In positions 2, 4, 5, 8, 10, and 11, the amino acid residues can vary, but their configuration at each position is strongly conserved.

An important subset of the β -hydroxylation of various amino acid residues observed for non-ribosomal synthesized peptides is catalyzed by cytochrome P450 monooxygenases [32–34]. The same biological machinery is certainly responsible for the β -hydroxylation of leucine and asparagine in laxaphycins and as this reaction is stereospecific, the stereochemistry of hydroxy-leucines, in position 3 and 5, and hydroxy-asparagine, in position 8, must be conserved.

Furthermore, both new peptides showed very similar NMR chemical shifts to laxaphycins B and B3 for the peptidic chain as well as for the side chains, indicating a conservation of the stereochemistry between cyclic and acyclic analogs. An indication of this homology could be seen in the comparison of ^1H and ^{13}C resonances of acyclolaxaphycins B and B3 with the parent compounds laxaphycins B and B3 ($\text{H}\alpha$, $\text{H}\beta$, $\text{C}\alpha$, and $\text{C}\beta$ acyclolaxaphycins B and B3 resonances subtracted from the equivalent ones of laxaphycin B and B3, respectively). With the exception of the structurally modified parts of the molecule, residues 4 and 5 on the NH terminal side and residues 2 and 3 on the COOH terminal side, the maximum difference ($\Delta\delta$) observed was less than 0.1 ppm in ^1H and 0.8 ppm in ^{13}C .

Therefore the complete structure of the two new compounds can be reasonably proposed as: (2*S*)-Ala–(2*R*,3*S*)-HLe–(2*S*)-Gln–(2*S*,3*S*)-*N*-MeIle–(2*R*,3*R*)-HAsn–(2*S*,3*R*)-Thr–(2*S*)-Pro–(2*R*)-Leu–(2*S*,3*R*)-Thr–(3*R*)- β -Ade–(2*S*)-Val–(2*R*,3*S*)-HLe for acyclolaxaphycin B (11) and (2*S*)-Ala–(2*R*,3*S*)-HLe–(2*S*)-Gln–(2*S*,3*S*)-*N*-MeIle–(2*R*,3*R*)-HAsn–(2*S*,3*R*)-Thr–(2*S*,4*R*)-4-Hyp–(2*R*)-Leu–(2*S*,3*R*)-Thr–(3*R*)- β -Ade–(2*S*)-Val–(2*R*,3*S*)-HLe for acyclolaxaphycin B3 (12).

A putative operon encoding the biosynthetic pathway for β -amino fatty acid lipopeptides, the puwainaphycins, was identified in the cyanobacterium *Cylindrospermum alatosporum*; the peptide biosynthesis process is initiated by the activation of a fatty acid residue via fatty acyl-AMP ligase (FAAL) and continued by a multidomain non-ribosomal peptide synthase/polyketide synthetase [35]. The last module incorporates a thioesterase domain in its terminal part that cleaves the finished puwainaphycin chain from the peptidyl carrier protein, thus promoting its cyclization between the NH_2 of β -amino fatty acid and the COOH of a proline residue. The characterization of the two novel acyclic laxaphycin variants 11 and 12 with alanine as NH terminal and Hle as COOH terminal seemed to indicate that in the case of B-type laxaphycins, the biosynthesis process starts with NRPS modules instead of FAAL and acyl carrier protein (ACP) ligase, with the ring closure being performed through a cyclization reaction between the amino group of the alanine residue and the carbonyl of the hydroxyleucine residue. However, one cannot exclude that acyclolaxaphycins B and B3 are enzymatic degradation products formed during cyanobacteria blooms. Enzymatic degradation is often used in resistance mechanisms in the microbial world or in competitive interspecific interactions. Enzymes that degrade or modify natural products provide protection by decreasing toxicity or by regulating the signaling functions of metabolites. Recently, Hoefler *et al.* have observed hydrolysis of cyclic lipopeptides surfactins by bacterial competition using imaging mass spectrometry [36]. However, the ring opening of the cyclic surfactins occurred at the ester functional group, which is not the case in laxaphycin peptides.

3. Experimental Section

3.1. Sampling Sites

The cyanobacterium *A. cf. torulosa* was collected by SCUBA diving at a depth of 1–3 m in the Pacific Ocean, Moorea, French Polynesia. The cyanobacterium sample was sealed underwater in a bag with seawater and then freeze-dried.

3.2. Isolation Procedure

The freeze-dried sample of cyanobacterium *A. cf. torulosa* (600 g) was extracted at room temperature three times with a mixture of MeOH-CH₂Cl₂ (1:1) and ultrasound. The combined extracts were evaporated under reduced pressure to give a greenish organic extract (38 g). The extract was subjected to flash RP18 silica gel column eluted with H₂O (A), H₂O-CH₃CN (2:8) (B), MeOH (C), and MeOH-CH₂Cl₂ (8:2) (D) to afford four fractions (A, B, C, and D). Then, fraction B (2 g) was subjected to flash RP18 silica gel column eluted with a solvent gradient of H₂O-CH₃CN to produce 12 fractions. Fraction 4 was subjected to HPLC purification (UP-50 DB.25M Uptisphere 5 μ) using 62% H₂O-CH₃CN at a flow rate of 3 mL/min to give acyclolaxaphycin B3 (3 mg, *tr* = 28.8 min) and acyclolaxaphycin B (2 mg, *tr* = 31.2 min).

3.3. Mass and NMR Spectroscopies

High-resolution ESI mass spectra were obtained using a Thermo Scientific LTQ Orbitrap mass spectrometer using electrospray ionization in positive mode. 1D-NMR and 2D-NMR experiments of synthetic compounds **1**, **2**, **8**, and **9** were acquired on a JEOL EX 400 spectrometer equipped with a NM-40 dual ¹H, ¹³C probe, whereas those of natural compounds **9**, **10**, **11**, and **12** were acquired on a Bruker Avance 500 spectrometer equipped with a cryogenic probe (5 mm), all compounds in DMSO-*d*₆ (500 μ L) at 303 K. All chemical shifts were calibrated on the residual solvent peak (DMSO-*d*₆, 2.50 ppm (¹H) and 39.5 ppm (¹³C)). The chemical shifts, reported in delta (δ) units, and in parts per million (ppm) are referenced relatively to TMS.

3.4. Solid Phase Peptide Synthesis

Fmoc-protected amino acids, coupling reagents and Rink Amide resin were purchased from Novabiochem (Fontenay-sous-Bois, France) or Iris Biotech (Marktredwitz, Germany). Fmoc-(alloD)Ile-OH and Boc-(L)Lys(Fmoc)-OH were bought from Iris Biotech (Marktredwitz, Germany). DMF for peptide synthesis, and other chemicals were purchased from Aldrich (Saint-Quentin Fallavier, France). Automated solid phase Fmoc-synthesis was performed on a Liberty One synthesizer by CEM (Saclay, France).

Synthesis protocol for automated solid phase peptide synthesis: Automated solid-phase peptide synthesis was performed on a 0.1 mmol scale. 278 mg of Rink amide MBHA LL resin at a 0.36 mmol/g substitution level was pre-swollen for 60 min in DMF and the solvent was drained. Fmoc-removal was achieved with a 20% piperidine solution in DMF, initial deprotection 30 s, 40 W, 75 °C, second step 180 s, 40 W, 70 °C. After washing (3 \times 7 mL DMF), 2.5 mL of a 0.2 M solution of the amino acid in DMF (5 eq relative to resin loading) was added. After addition of a 2 M solution of DIPEA in DMF (0.5 mL, 10 eq) and a 0.45 M solution of HATU in DMF (1 mL, 4.5 eq), the reaction solution was irradiated at 25 W for 5 min reaching a final temperature of 70 °C. All amino acids were coupled using these conditions except Fmoc-Gln(Trt)-OH for which a triple coupling was performed with a prolonged reaction time of 20 min. After full elongation of the peptide, the linear protected peptide linked to the resin was transferred into a batch reactor and the allyl protecting group was cleaved using Pd(P(Ph₃)₄) (m = 0.35 g, 0.3 mmol, 3 eq) with a solution of CHCl₃/AcOH, NMM: 3.7/0.2/0.1 for 4 h at room temperature. After washing the resin using a solution of 0.5% diethyldithiocarbamate in DMF (3 \times 10 mL) and DMF (3 \times 10 mL) and lastly Fmoc removal, the cyclisation was performed with diisopropylcarbodiimide (DIC) and Oxyma, 1 mL, C = 0.5 M, 70 °C 3 \times 20 min, then 120 s at 20 °C between each cycle, followed by a final wash with DMF (3 \times 7 mL). A

Kaiser test was carried out in order to check reaction completion. Finally, the resin was washed with DCM (3×10 mL), transferred to a flask, and the cleavage cocktail (TFA, H₂O, TIS 95/2.5/2.5) added. The resin was shaken for 3 h. The cleaving solution was collected and the resin washed with TFA (2×3 mL). The combined fractions were concentrated in vacuum. The crude peptide was precipitated in cold Et₂O and finally centrifuged. The precipitate was washed with cold ether, extracted with water, and freeze-dried to yield the crude peptide.

Analytical HPLC of peptides: The HPLC analysis was run on a Waters (Guyancourt, France) 2695 HPLC system fitted with an ELS detector 2424 and a PDA 2998 using a Phenomenex Luna 3 μ M C-8 column (150×3 mm). Eluents were water with 0.1% formic acid (buffer A) and acetonitrile with 0.1% formic acid (buffer B). Standard conditions comprised a flow rate of 0.4 mL/min eluting with 40% B to 100% B in 35 min. Standard conditions were applied to all HPLC analyses unless stated otherwise.

Semi-preparative HPLC purification of peptide crudes: A semi-preparative purification of cyclic peptides was performed using a Waters 1525 chromatography system fitted with a Waters 2487 tunable absorbance detector with detection at 214 nm (Guyancourt, France). Purification was performed by eluting solvents A (water) and B (acetonitrile) on a UP50DB C-18 column (250×10 mm) at 3 mL/min.

LC-MS: LC-MS analyses were carried out using a Thermo Fisher Scientific LC-MS device, Accela HPLC coupled to a LCQ Fleet equipped with an electrospray ionisation source and a 3D ion-trap analyzer (Villebon-sur-Yvette, France). The analysis was performed with a Phenomenex Kinetex C-18 column (100×300 mm) using gradient mixture of water with 0.1% formic acid (buffer A) and acetonitrile with 0.1% formic acid (buffer B). Standard conditions were a flow rate of 0.5 mL/min eluting with 20% B to 100% B in 15 min. Standard conditions were applied to all HPLC-MS analysis unless otherwise stated.

Fmoc-NMelle-OH (1.10 g, 3.0 mmol) was dissolved in DMF (7 mL) and cesium carbonate (0.98 g, 3.0 mmol) added. The mixture was stirred for 2 h. Allyl bromide (2.54 g, 1.80 mL, 21.0 mmol) was added to the mixture and stirring continued for 1 h resulting in a milky solution. The mixture was diluted to 20 mL with water and acidified with 1 M HCl. The aqueous layer was extracted with dichloromethane (25 mL, 3×25 mL). The organic layer was washed with brine (15 mL), dried over MgSO₄, and filtered, and the solvent was removed under reduce pressure to give **4a** (Figure 6) as a colorless oil (0.92, 76%). ¹H-NMR (CDCl₃): 7.76 (d, 2H, *J* = 7.5 Hz); 7.59 (d, 2H, *e*, *J* = 8.2 Hz); 7.39 (t, 2H, *c*, *J* = 7.8 Hz); 7.28 (t, 2H, *d*, *J* = 7.5 Hz); 5.89 (m, 1H, *n*); 5.32 (dt, 2H, *m*, *J* = 17.4 Hz, *J* = 10.4 Hz); 4.61 (m, 2H, *i*); 4.51 (m, 1H); 4.25 (m, 1H); 2.90 (s, 3H, *j*); 2.00 (m, 1H, *o*); 1.38 (m, 2H, *r*); 0.90 (d, 3H, *q*, *J* = 7.3 Hz); 0.85 (t, 3H, *s*, *J* = 6.6 Hz). ¹³C-NMR (CDCl₃): 171.6 (C=O); 156.4 (C=O); 144.1 (C_f); 141.4 (C_a); 131.8 (C_nH); 128.4 (C_eH); 127.7 (C_bH); 125.1 (C_dH); 120.0 (C_hH); 118.5 (C_oH₂); 67.7 (C_kH); 65.3 (C_hH₂); 62.5 (C_mH₂); 47.4 (C_gH); 33.6 (C_jH₃); 30.1 (C_pH); 25.0 (C_rH₂); 15.8 (C_qH₃); 11.0 (C_sH₃).

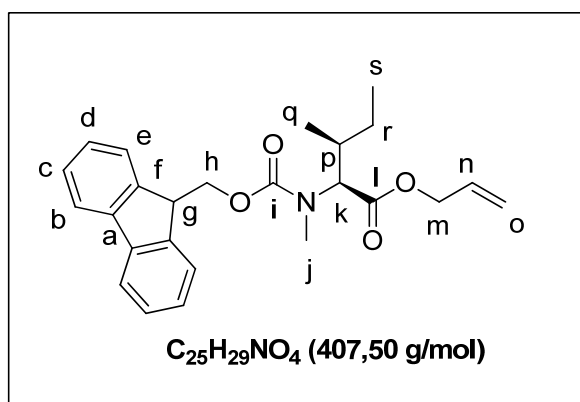


Figure 6. (2S,3S)-allyl 2-(((9H-fluoren-9-yl)methoxy)carbonyl)(methylamino)-3-methylpentanoate (**4a**).

A solution of compound **4a** (1.10 g, 3.0 mmol) in DMF (5 mL) was treated with piperidine (1 mL). The mixture was stirred for 1 h. The mixture was diluted to 20 mL and the aqueous phase was extracted with cHex/AcOEt, 50/50 (3 × 30 mL). The organic layer was washed with brine (15 mL), dried over MgSO₄, and filtered, and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography on silica gel (eluent: cHex/AcOEt 6/4) to yield **4b** (Figure 7) as a colorless oil (0.29 g, 70%). ¹H-NMR (CDCl₃): 5.95 (m, 1H_f); 5.32 (dd, 2H_g, *J* = 22.5 Hz, *J* = 11.3 Hz); 4.65 (m, 2H_e); 3.25 (d, 1H_c, *J* = 5.3 Hz); 2.50 (3 s, 1H_a); 1.92 (m, 1H_h); 1.54 (m, 2H_i); 0.92 (d, 3H_i, *J* = 6.8 Hz); 0.80 (t, 3H_k, *J* = 7.3 Hz). ¹³C-NMR (CDCl₃): 170.6 (C_d=O); 131.5 (C_h); 119.6 (C_gH₂); 66.9 (C_eH₂); 66.1 (C_cH); 37.3 (C_hH); 34.2 (C_aH₃); 26.3 (C_jH₂); 15.3 (C_iH₃); 11.7 (C_kH₃).

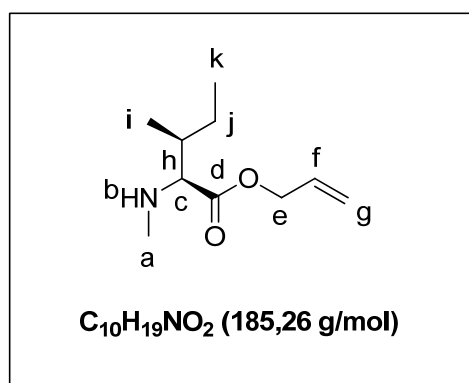


Figure 7. (2*S*,3*S*)-allyl 3-methyl-2-(methylamino)pentanoate (**4b**).

A solution of Fmoc-Glu(OtBu)-OH (0.56 g, 1.30 mmol) in DMF (6 mL) was treated with DIEA (0.23 mL, 1.30 mmol). The mixture was cooled at 0 °C and COMU (0.56, 1.30 mmol) was added. After 5 min, solutions of compound **4b** (0.24 g, 1.30 mmol) in DMF (2 mL) and DIEA (0.23 mL, 1.30 mmol) were added successively at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 48 h. The mixture was extracted with cHex/EtOAc, 50/50 (3 × 10 mL). The organic layer was washed successively with a solution of HCl 1M (10 mL), saturated aqueous NaHCO₃ (10 mL), and brine (10 mL), and dried over MgSO₄. The solvent was removed under reduced pressure. The crude product was purified by chromatography on silica gel using cHex/AcOEt (80/20) as eluent to yield compound **3a** (Figure 8) as a yellow oil (0.50 g, 70%). ¹H-NMR (CDCl₃): 7.75 (d, 2H_b, *J* = 7.3 Hz); 7.60 (d, 2H_e, *J* = 6.2 Hz); 7.40 (t, 2H_c, *J* = 7.3 Hz); 7.22 (m, 2H_d); 5.89 (m, NH_i); 5.65 (m, 1H_v); 5.32 (dd, 2H_w, *J* = 15.6 Hz, *J* = 10.2 Hz); 4.97 (d, 1H_k, *J* = 10.2 Hz); 4.75 (m, 1H_s); 4.60 (m, 2H_u); 4.36 (d, 2H_h, *J* = 7.1 Hz); 4.21 (t, 1H_g, *J* = 6.8 Hz); 3.10 (s, 3H_r); 2.34 (m, 2H_m); 2.03 (m, 2H_i); 1.56 (m, 1H_x); 1.45 (s, 9H_p); 1.29 (m, 2H_z); 0.98 (d, 3H_y, *J* = 6.6 Hz); 0.86 (t, 3H_{z'}, *J* = 7.3 Hz).

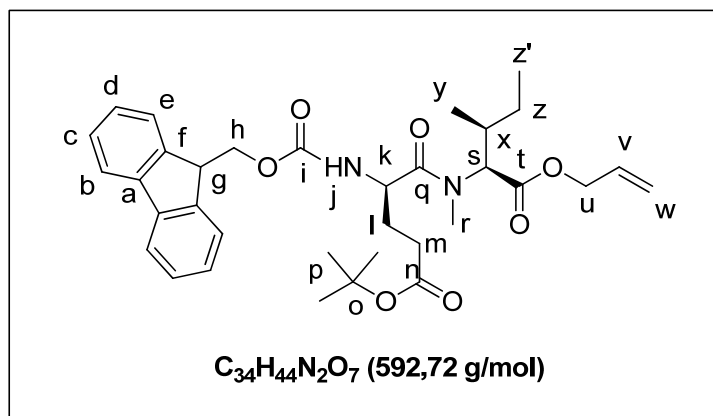


Figure 8. (*R*)-tert-butyl 4-(((9*H*-fluoren-9-yl)methoxy)carbonyl)amino-5-(((2*S*,3*S*)-1-(allyloxy)-3-methyl-1-oxopentan-2-yl)(methyl)amino)-5-oxopentanoate (**3a**).

A solution of compound **3a** (0.5 g, 0.90 mmol) in CH₂Cl₂ (4 mL) was treated with TFA (4 mL). The mixture was stirred for 12 h. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography on silica gel (eluent: cHex/AcOEt 95/5) to yield **3b** (Figure 9) as a white solid (0.43 g, 96%). ¹H-NMR (CDCl₃): 7.75 (d, 2H_b, *J* = 7.3 Hz); 7.60 (d, 2H_e, *J* = 6.2 Hz); 7.40 (t, 2H_c, *J* = 7.3 Hz); 7.22 (m, 2H_d); 5.89 (m, NH_i); 5.32 (dd, 2H_v, *J* = 15.6 Hz, 10.2 Hz); 4.95 (d, 2H_k, *J* = 7.2 Hz); 4.80 (m, 1H_u); 4.60 (d, 2H_r, *J* = 5.9 Hz); 4.36 (m, 2H_h); 4.20 (t, 1H_g, *J* = 7.0 Hz); 3.10 (s, 3H_p); 2.47 (m, 2H_m); 2.03 (m, 2H_i); 1.56 (m, 1H_v); 1.30 (m, 2H_x); 0.98 (d, 3H_w, *J* = 6.6 Hz); 0.86 (t, 3H_y, *J* = 7.3 Hz). (ESI+) *m/z* [M + H]⁺ calcd for C₃₀H₃₇N₂O₇ 536.6, found 536.8.

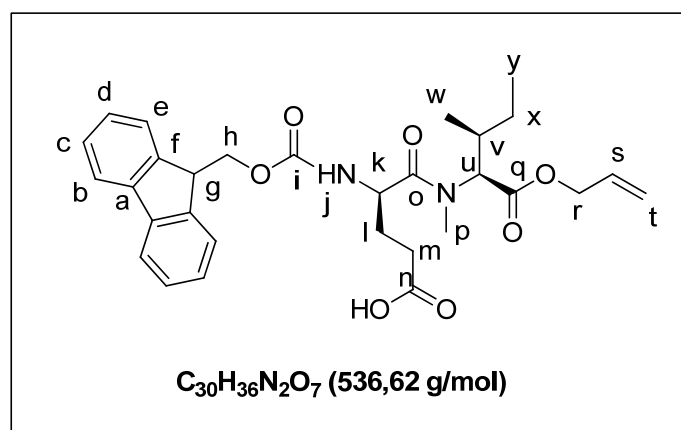


Figure 9 (*R*)-4-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-(((2*S*,3*S*)-1-(allyloxy)-3-methyl-1-oxopentane-2-yl)(methyl)amino)-5-oxopentanoic acid (**3b**).

4. Conclusions

As shown in this work, the preliminary synthesis of the simplified analogs (1) and (2) of the targeted natural compound was a prerequisite for the synthesis of ((2*S*,3*S*)-HLe³)laxaphycin B, the diastereoisomer of the natural compound that was initially described [9]. This initial work resulted in the development of a total synthesis of laxaphycin B and lynngbyacyclamide [19].

This methodological approach is also important for future synthesis of laxaB-type peptides such as the two new linear lipopeptides acyclolaxaphins B and B3 (11, 12) isolated from the tropical marine cyanobacterium *Anabaena cf. torulosa*, the same species in which we have already isolated the related cyclic peptides laxaphins B and B3. The presence of these acyclic laxa B-type compounds together with the cyclized ones in the same extract has, to our knowledge, never been described. The search of other minor acyclic potential biosynthetic precursors will provide valuable information concerning the hybrid PKS/NRPS biosynthetic pathway in this exciting lipophilic cyclic dodecapeptide series. Furthermore, these acyclic peptides suggest another possible disconnection for the future preparation of new analogs of laxaphycin B at the HLe³/Ala⁴ junction. In this case we could expect that the linear peptide would benefit from a pre-organized structure thanks to its almost syndiotactic character [37,38]. Further work is needed to verify this assumption and to develop a synthesis based on the use of a BAL linker [39], which could enable other laxaphycin B type peptides to be synthesised.

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Author Contributions: France Boyaud, Louis Bornancin, and Zahia Mahiout performed the experiments; France Boyaud, Louis Bornancin, Zahia Mahiout, Isabelle Bonnard, Bernard Banaigs, and Nicolas Inguibert

analyzed the data; Isabelle Bonnard, Suzanne Mills, Bernard Banaigs, and Nicolas Inguibert designed the research and wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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