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Structure and Biological Evaluation of New Cyclic and Acyclic Laxaphycin-A type peptides

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Abstract

Five new laxaphycins were isolated and fully characterised from the bloom forming cyanobacteria *Anabaena torulosa* sampled from Moorea, French Polynesia: three acyclic laxaphycin A-type peptides, acyclolaxaphycin A (**1**), [des-Gly^{11}]acyclolaxaphycin A (**2**) and [des-(Leu^{10}-Gly^{11})]acyclolaxaphycin A (**3**), as well as two cyclic ones, [L-Val^{8}]laxaphycin A (**4**) and [D-Val^{9}]laxaphycin A (**5**). The absolute configuration of the amino acids, established using advanced Marfey's analysis for compounds **2-5,** highlights a conserved stereochemistry at the Cα carbons of the peptide ring that is characteristic of this family. To the best of our knowledge, this is the first report of acyclic analogues within the laxaphycin A-type peptides. Whether these linear laxaphycins with the aliphatic β-amino acid on the N-terminal are biosynthetic precursors or compounds obtained after enzymatic hydrolysis of the macrocycle is discussed. Biological evaluation of the new compounds together with the already known laxaphycin A shows that [L-Val^{8}]laxaphycin A, [D-Val^{9}]laxaphycin A and [des-Gly^{11}]acyclolaxaphycin induce cellular toxicity whereas laxaphycin A and des-[(Leu^{10}-Gly^{11})]acyclolaxaphycin A do not affect the cellular viability. An analysis of cellular death shows that the active peptides do not induce apoptosis or necrosis but instead, involve the autophagy pathway.

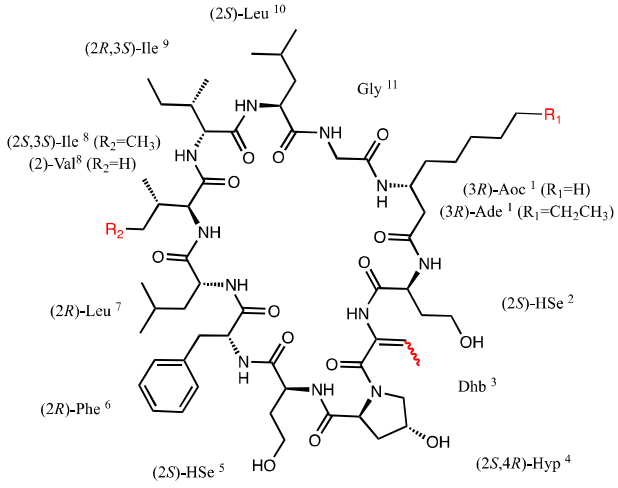
1. Introduction

Marine organisms constitute a prolific source of secondary metabolites that show a range of bioactivities including antibacterial, antitumoral, antifungal or antimalarial activities.[1,2] Among them, filamentous cyanobacteria are recognised as producing a wide range of bioactive molecules, a majority of which are cyclic lipopeptides.[3–5] Laxaphycins and congeners are lipopeptides that have been isolated from several cyanobacteria found

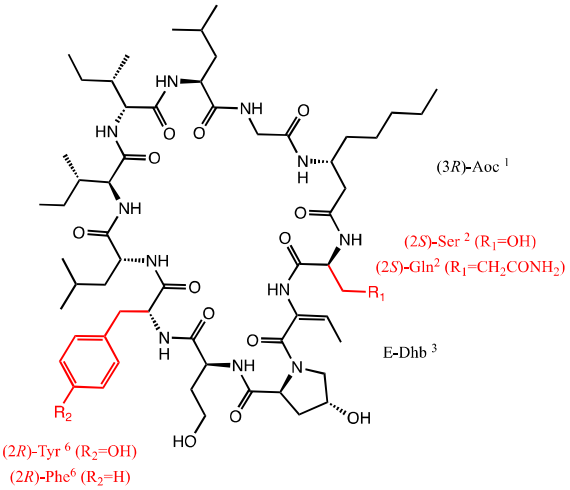
worldwide. One of their most prominent features is the presence of non-proteinogenic amino acids such as a β-amino acid with an aliphatic side chain of 8 to 10 carbon atoms. They are divided into two sub-families; laxaphycin A- and B- type peptides with laxaphycin A, a cyclic undecapeptide, and laxaphycin B, a cyclic dodecapeptide, as the representative compounds of each sub-family, respectively. The sequence of laxaphycin A shows a segregation between hydrophobic and hydrophilic residues, with an α,β-dehydrated amino acid that is inserted between two hydroxylated aminoacids.

The laxaphycin A sub-family contains 8 peptides including laxaphycin A,[6,7] laxaphycin E,[7] hormothamnin A,[8] laxaphycin A2,[9] lobocyclamide A,[10] scytocyclamide A,[11] trichormamides A[12] and D[13] produced by *Anabaena torulosa, A. laxa, Hormothamnion enteromorphoides, Lyngbya confervoides, Scytonema hofmanni, Trichormus* sp. *and Oscillatoria* sp., respectively (Fig. 1). Laxaphycin A is produced by both the freshwater strain *A. laxa* and the marine strain *A. torulosa*. Laxaphycins and congeners share some similarities with those of puwainaphycins, isolated from the freshwater cyanobacteria *Anabaena* sp.[14] and *Cylindrospermum alatosporum.*[15] Puwainaphycins are cyclic decapeptides with a β-amino fatty acid (i position) and an α,β-dehydrated amino acid in a i+2 position as in laxaphycins A.

The biosynthesis of such compounds relies on multifunctional enzymes: non-ribosomal peptide synthases (NRPSs) or hybrid NRPS/polyketide synthases (PKSs).[16–18] These enzymes are organised in modules and are responsible for the biosynthesis of peptides with usual and non-proteinogenic amino acids. NRPS are responsible for the modification of natural amino acids into D-, *N*-methyl, β-hydroxylated, or dehydrated amino acids, whereas PKS, sometimes associated with other enzymes such as FAAL (fatty acyl-AMP ligase), enable the insertion of fatty amino acids into the peptide sequence. The biosynthetic gene cluster encoding a hybrid NRPS/PKS containing FAAL involved in the synthesis of the puwainaphycins has been characterized.[19] The presence of compounds with a comparable biosynthetic pathway in different cyanobacterial strains may be explained by horizontal gene transfer events between cyanobacteria[20] or may suggest an ancient evolutionary origin within cyanobacteria.



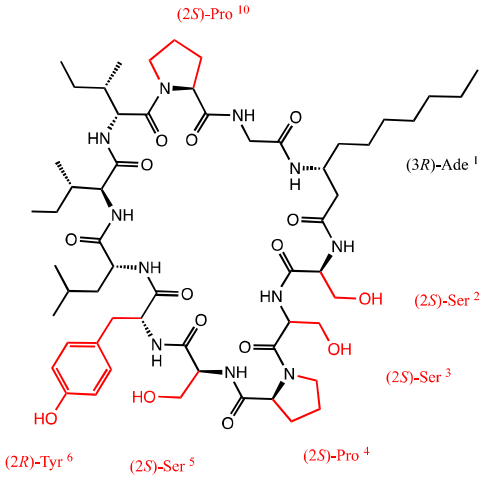
Laxaphycin A [(3*R*)-Aoc[1], E-Dhb[3], (2*S*,3*S*)-Ile[8]]
Laxaphycin E [(3*R*)-Ade[1], E-Dhb[3], (2*S*,3*S*)-Ile[8]]
[*Anabaena* cf. *torulosa*]



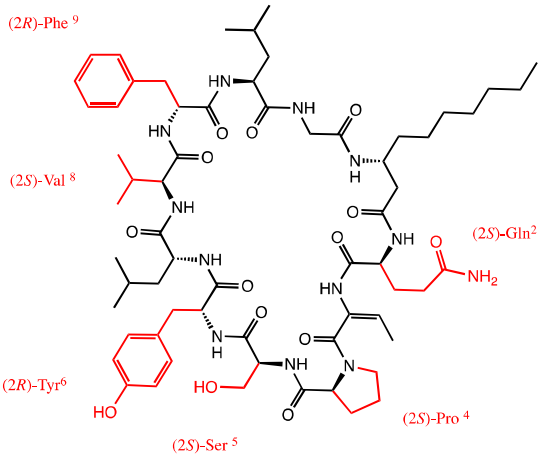
Lobocyclamide A
[*Lyngbya confervoides*]

Hormothamnin A [(3*R*)-Aoc[1], Z-Dhb[3], (2*S*,3*S*)-Ile[8]]
Laxaphycin A2 [(3*R*)-Aoc[1], E-Dhb[3], (2*S*)-Val[8]]
[*Hormothamnion enteromorphoides*]

Scytocyclamide A (R1=H, R2=CH_2CONH_2
[*Scytonema hofmanni*]



Trichormamide A
[*Trichormus* sp.]



Trichormamide D
[cf. *Oscillatoria* sp.]

Fig. 1. Laxaphycins A and E, and the analogues hormothamnin A, lobocyclamide A, scytocyclamide A and trichormamides A and D. Amino acid modifications to the reference compound laxaphycin A are highlighted in red.

The structure characterisation of the acyclic analogues of laxaphycins B and B3, acyclolaxaphycins B and B3, isolated from *A. torulosa* were recently published.[21] In the present study, we describe the isolation, structure determination, and biological evaluation on neuroblastoma cells of five new laxaphycin A-type peptides, three acyclic analogues named acyclolaxaphycin A (**1**), [des-Gly[11]]acyclolaxaphycin A (**2**) and [des-(Leu[10]-Gly[11])]acyclolaxaphycin A (**3**) and two cyclic forms termed [L-Val[8]]laxaphycin A (**4**) (or

laxaphycin A2) [*] and [D-Val[9]]laxaphycin A (**5**), from a new field collection of *A. torulosa*. The planar structures were determined by 2D NMR and high resolution mass fragmentation, and the advanced Marfey's analysis enabled the absolute configurations to be determined. The effect of the new laxaphycins compared to laxaphycin A on the cellular viability of neuroblastoma cells shows that while laxaphycin A and des-(Leu[10]-Gly[11])]acyclolaxaphycin A did not affect cellular viability, the cyclic [L-Val[8]]laxaphycin A and [D-Val[9]]laxaphycin A and the acyclic [des-Gly[11]]acyclolaxaphycin induced cellular damage through autophagic mechanisms.

2. Results and discussion

The cyanobacterium *A. torulosa* was sampled in Moorea's lagoon, French Polynesia, during a bloom. Samples were collected, sealed underwater in a bag, freeze-dried and extracted. HPLC-DAD-ELSD and LC-MS analysis of the crude extract revealed an unusual chromatographic profile. Typically, *A. torulosa* extracts are comprised of three major components: laxaphycins A, B and B3. However, in the analysed extract, an additional polar group of five potentially new peptides, with molecular weights of 1043, 1156, 1213 and 1181 Da (two compounds), were also found (Fig. 2). A comparison with other samples collected in Moorea during the same season, location and depth, could not explain the observed differences. The crude extract was fractionated using flash chromatography and the resulting fractions containing the new peptides were subjected to HPLC purification to yield acyclolaxaphycin A (**1**), [des-Gly[11]]acyclolaxaphycin A (**2**), [des-(Leu[10]-Gly[11])]acyclolaxaphycin A (**3**), [L-Val[8]]laxaphycin A (**4**) and [D-Val[9]]laxaphycin A (**5**). All compounds were obtained as colourless amorphous solids and compounds **1**, **2** and **3** responded positively to a ninhydrin test suggesting a non-blocked *N*-terminus.

[*] While we were preparing the manuscript, Cai et al.[9] published the structure of [L-Val[8]]laxaphycin A (**4**), which the authors named laxaphycin A2.

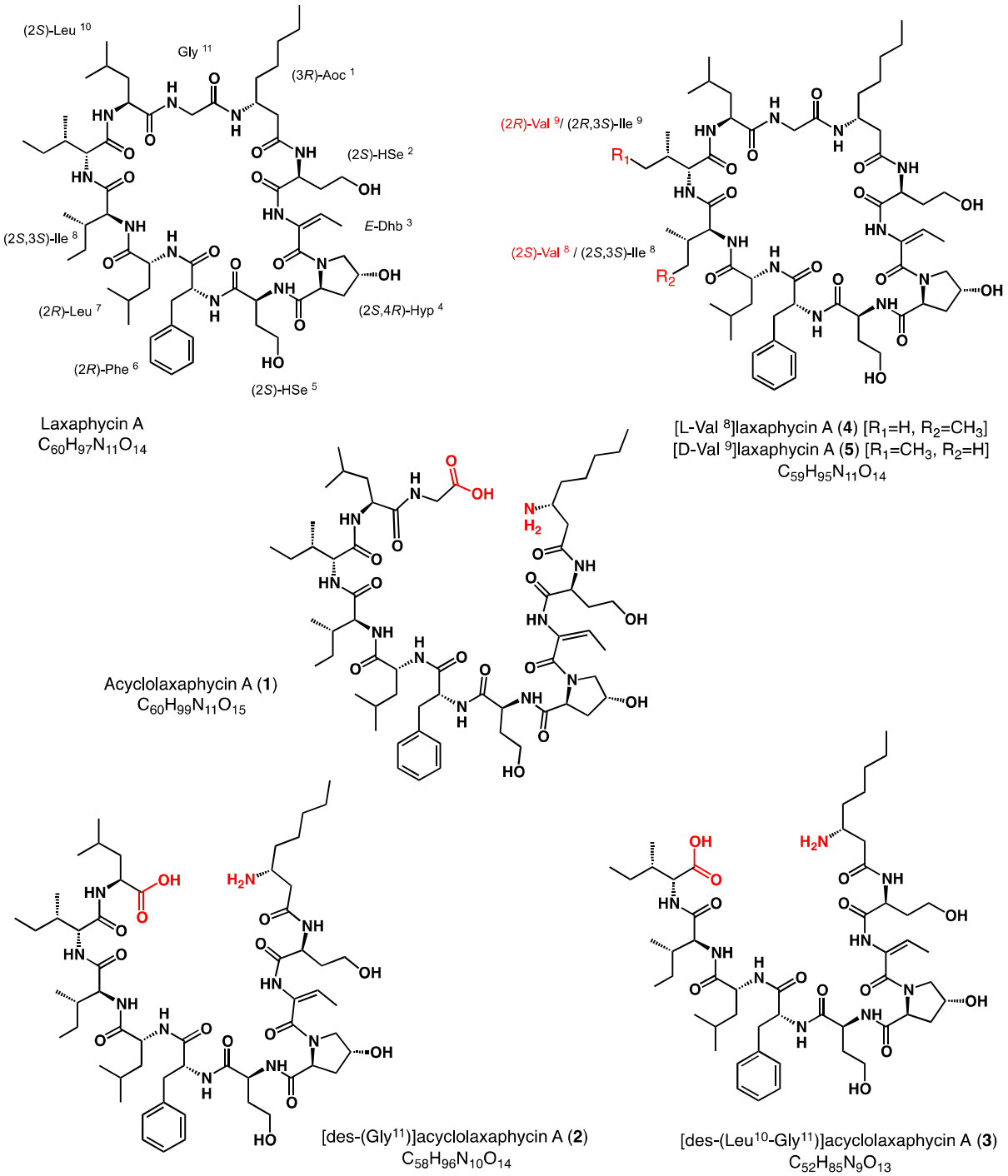


Fig. 2. The structures of compounds **1**-**5** in comparison with laxaphycin A. Modifications to the reference compound laxaphycin A are highlighted in red.

2.1. Structure elucidation of acyclolaxaphycin A (1), [des-Gly^{11}]acyclolaxaphycin A (2), [des-(Leu^{10}-Gly^{11})]acyclolaxaphycin A (3):

The positive high-resolution electrospray ionisation mass spectrometry (HR-ESI(+)-MS) spectrum of compound **1** gave a $[M + H]^+$ pseudomolecular ion at *m/z* 1214.7380, consistent with molecular formula $C_{60}H_{99}N_{11}O_{15}$ while the HR-ESIMS spectra of compounds

2 and **3** showed $[M + H]^+$ pseudomolecular ions at *m/z* 1157.7231 and 1044.6385 respectively, corresponding to molecular formulas $C_{58}H_{96}N_{10}O_{14}$ and $C_{52}H_{85}N_9O_{13}$.

A comparison between the molecular formulas of compound **1** and laxaphycin A ($C_{60}H_{97}N_{11}O_{14}$) suggested an additional H_2O in acyclolaxaphycin A (**1**) while the difference between compounds **2** and **1** corresponded to a loss of a Gly residue (C_2H_3NO), and the difference between compounds **3** and **2** to a sequential loss of a Leu residue ($C_6H_{11}NO$). The signal distribution pattern observed in the ^{1}H-NMR spectrum of compounds **1**, **2** and **3** is characteristic of lipopeptides with amide NH signals resonating at δ_H 7.30–8.50, CαH signals at δ_H 3.50–4.70, aliphatic CH_2 at δ_H 1.10–1.30 and CH_3 signals at δ_H 0.70–0.90. The presence in each spectra of diagnostic signals corresponding to the ethylenic protons of Dhb indicated that the three peptides were related to laxaphycin A. Extensive analyses of NMR and mass spectroscopy spectra allowed for the structure of those peptides to be defined, including acyclolaxaphycin A (**1**), [des-Gly^{11}]acyclolaxaphycin A (**2**) and [des-(Leu^{10}-Gly^{11})]acyclolaxaphycin A (**3**).

2.1.1 Acyclolaxaphycin A (**1**)

The ^{1}H-NMR spectrum of acyclolaxaphycin A (**1**) (Supporting Information S1) revealed a strong structural similarity with laxaphycin A. The spectrum featured typical signals of aromatic protons corresponding to Phe (δ_H 7.15–7.30), as well as NH, Hβ and Hγ that corresponded to Dhb residues (δ_H 9.93, 5.55 and 1.66, respectively). In the amide proton region, eight NH doublets and one singlet were observed instead of the nine doublets and one singlet observed for laxaphycin A.

Almost all ^{1}H and ^{13}C resonances of acyclolaxaphycin A could be assigned using extensive 2D-NMR analyses including COSY, TOCSY, HSQC, HSQC-TOCSY, and ROESY (Table 1) (Supporting Information S 1-7) despite the low amount of compound available (1.5 mg). First, an analysis of the TOCSY spectra enabled the structure of nine amino acids to be established: two leucine (Leu), two isoleucine (Ile), two homoserine (Hse), one phenylalanine (Phe), one glycine (Gly), and one α,β-dehydroaminobutyric acid (Dhb). The structure of the lipophilic beta amino acid, β-aminooctanoic acid (Aoc), was assigned by COSY and TOCSY correlations between Hα (δ_H 2.45), Hβ (δ_H 3.30) and aliphatic protons at 1.50, 1.30, 1.26 and 1.22 ppm. Furthermore, HSQC-TOCSY and HMBC correlations from highly overlapped methylene signals (δ_H 1.22, 1.26 and 1.30) with three carbons (δ_C 21.71, 24.39 and 30.92 respectively) completed the structure of Aoc. The amide protons of Aoc were not observed in the spectrum within the context of the conditions used in the experiment. A spin system without an amide proton was identified as Hyp based on sequential correlations between Hα (δ_H 4.43) and Hβ (δ_H 1.93; 2.09), Hβ and Hγ (δ_H 4.24), Hγ and Hδ (δ_H 3.39; 3.49). The amino acid sequence was deduced by ROESY correlations (Fig. 6), and the $^3J_{CH}$ or $^4J_{CH}$ correlations in HMBC spectrum were not observed. ROESY correlations between Hα and Hβ (residue i) or NH (residue i+1), and between HδPhe/HαHse, HγHse/HγDhb, and HγDhb/HδHyp, suggested the presence of two fragments consisting of Aoc-Hse-Dhb-Hyp-Hse-Phe (fragment 1) and Leu-Ile-Ile-Leu-Gly (fragment 2) (Fig. 3).

The initial protonation of the acyclic peptide and subsequent fragmentation through the loss of amino acid residues of acyclolaxaphycin A by positive ESI-MS/MS revealed the presence of b and y ion fragments. Mass analysis found the presence of b ions at *m/z* 1139.71 (b10), 1026.63 (b9), 913.54 (b8), 800.46 (b7), 687.38 (b6), 540.26 (b5), 326.21 (b3) and 243.17 (b2) as well as y ions at *m/z* 889.54 (y8) and 675.45 (y6) which connect fragments 1 and 2. A second series of fragments (b' and y') was found due to the increased basicity of amide nitrogen atoms of the N-alkylated 4-hydroxyproline (Hyp^4) protonation and cleavage of these amide bonds. The presence of b' ions at *m/z* 814.51 (b'7), 701.43(b'6), 588.34 (b'5), 475.26 (b'4) and 362.17 (b'3) confirmed the partial sequence Hyp-Hse-Phe-Leu-Ile-Ile-Leu-Gly (Fig. 3).

The overall mass fragmentation (Supporting Information S8) analysis established the complete sequence as Aoc-Hse-Dhb-Hyp-Hse-Phe-Leu-Ile-Ile-Leu-Gly supporting the proposed structure of **1** that we named acyclolaxaphycin A (Fig. 2). The gross structure of acyclolaxaphycin A differs from laxaphycin A due to a ring opening between residues 1 and 11.

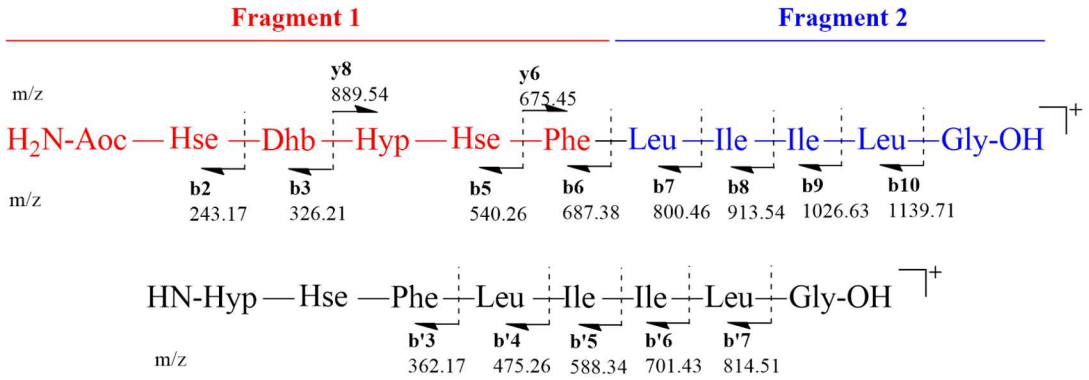


Fig. 3. ESI(+)-MS/MS fragmentation of acyclolaxaphycin A (**1**).

2.1.2 [des-(Gly^{11})]acyclolaxaphycin A (**2**)

Examination of preliminary spectral data, including ESI(+)-MS/MS, ^1H, and ^{13}C-NMR (Supporting Information S9-S10) spectroscopies, indicated that the new metabolite was a lower homologue of compound **1**. The similarity between acyclolaxaphycin A and compound **2** was evident based on NMR spectral analysis (Table 1). The ^1H-NMR spectrum revealed the presence of seven doublets in the amide region, one singlet and a broad singlet (2H) instead of eight doublets and one singlet for acyclolaxaphycin A. TOCSY and ROESY (Supporting Information S12 and S15) analyses showed a correlation between the spread of NH singlet at δH 7.77 and Hα and Hβ of Aoc, which establishes that an N-term is present on the Aoc residue. The absence of Gly in **2** is confirmed by the lack of the carbonyl and Hα signals of Gly. HMBC cross-peaks between carbonyl carbons (residue i) and NH protons (residue i+1) allowed for the assignment of two fragments Aoc-Hse-Dhb (fragment 1) and Hyp-Hse-Phe-Leu-Ile-Ile-Leu (fragment 2) which is confirmed by the analysis of ROESY correlations between Hα or Hβ (residue i) and NH (residue i+1) (Fig. 4). The two fragments (1 and 2) were assembled by two inter-residue ROESY correlations between Hγ (δ_H 1.67) of Dhb and

Hδ (δ_H 3.36; 3.45) of Hyp resulting in a complete sequence of Aoc-Hse-Dhb-Hyp-Hse-Phe-Leu-Ile-Ile-Leu.

MS/MS data (Supporting Information S16) for **2** were consistent with the proposed amino acid sequence (Fig. 4) and established the amino acid sequence to be Aoc-Hse-Dhb-Hyp-Hse-Phe-Leu-Ile-Ile-Leu. The proposed structure of **2** that we named [des-(Gly^{11})]acyclolaxaphycin A (Fig. 2) differs from laxaphycin A in the ring opening between residues 1 and 11 and the subsequent loss of Gly^{11}.

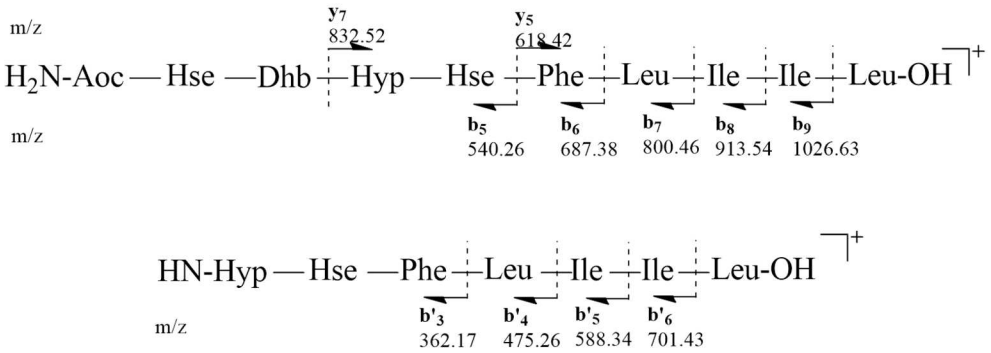


Fig. 4. ESI(+)-MS/MS fragmentation of [des-(Gly^{11})]acyclolaxaphycin A (**2**).

2.1.3 [des-(Leu^{10}-Gly^{11})]acyclolaxaphycin A (**3**)

The ^{1}H and ^{13}C spectra of compound **3** (Supporting Information S 17-S18) were almost identical to those of **2** except for the absence of signals of one amide doublet and one carbonyl carbon, indicating the loss of an amino acid (Table 1). Analysis of TOCSY data (Supporting Information S20) confirmed the lack of a Leu residue as suggested by HR-ESI(+)-MS data. As described for [des-(Gly^{11})]acyclolaxaphycin A, HMBC spectrum provided an assignment for two fragments: Aoc-Hse-Dhb (fragment 1) and Hyp-Hse-Phe-Leu-Ile-Ile (fragment 2) (Fig. 5); the missing residue was identified as Leu^{10} compared to **2**. ROESY correlations (Supporting Information S23) between Hα or Hβ (residue i) and NH (residue i+1) confirmed the structure of the partial sequence and correlations between Hδ (δ_H 3.37; 3.46) of Hyp and Hβ and Hγ (δ_H 1.68) of Dhb defined the complete sequence as Aoc-Hse-Dhb-Hyp-Hse-Phe-Leu-Ile-Ile.

The sequence was confirmed by ESI(+)-MS/MS data analyses (Supporting Information S24), which revealed the presence of y ions at *m/z* 719.43 (y6) and b ions at *m/z* 913.54 (b8), 800.46 (b7), 687.38 (b6), 540.26 (b5), 326.21 (b3), and 701.43 (b'6), 588.34 (b'5), 475.26 (b'4), and 362.17 (b'3) (Fig. 5).

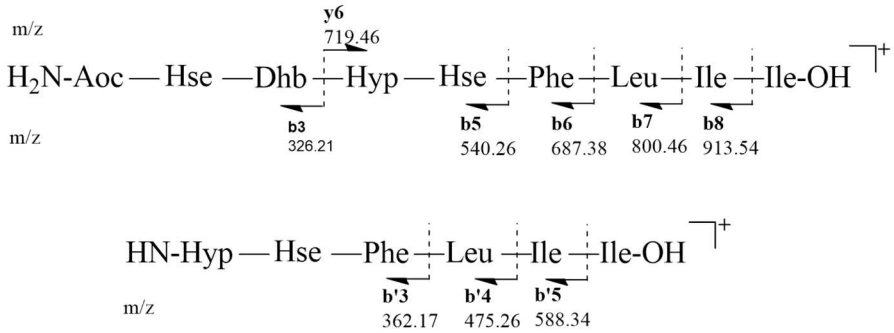


Fig. 5. ESI(+)-MS/MS fragmentation of [des-(Leu[10]-Gly[11])]acyclolaxaphycin A (**3**).

Mass fragmentation and NMR data established the amino acid sequence as Aoc-Hse-Dhb-Hyp-Hse-Phe-Leu-Ile-Ile supporting the proposed structure of **3** that we named [des-(Leu[10]-Gly[11])]acyclolaxaphycin A (Fig. 2). The gross structure of [des-(Leu[10]-Gly[11])]acyclolaxaphycin A differs from **2** by a ring opening between residues 1 and 10, and the subsequent loss of Gly[11] and Leu[10].

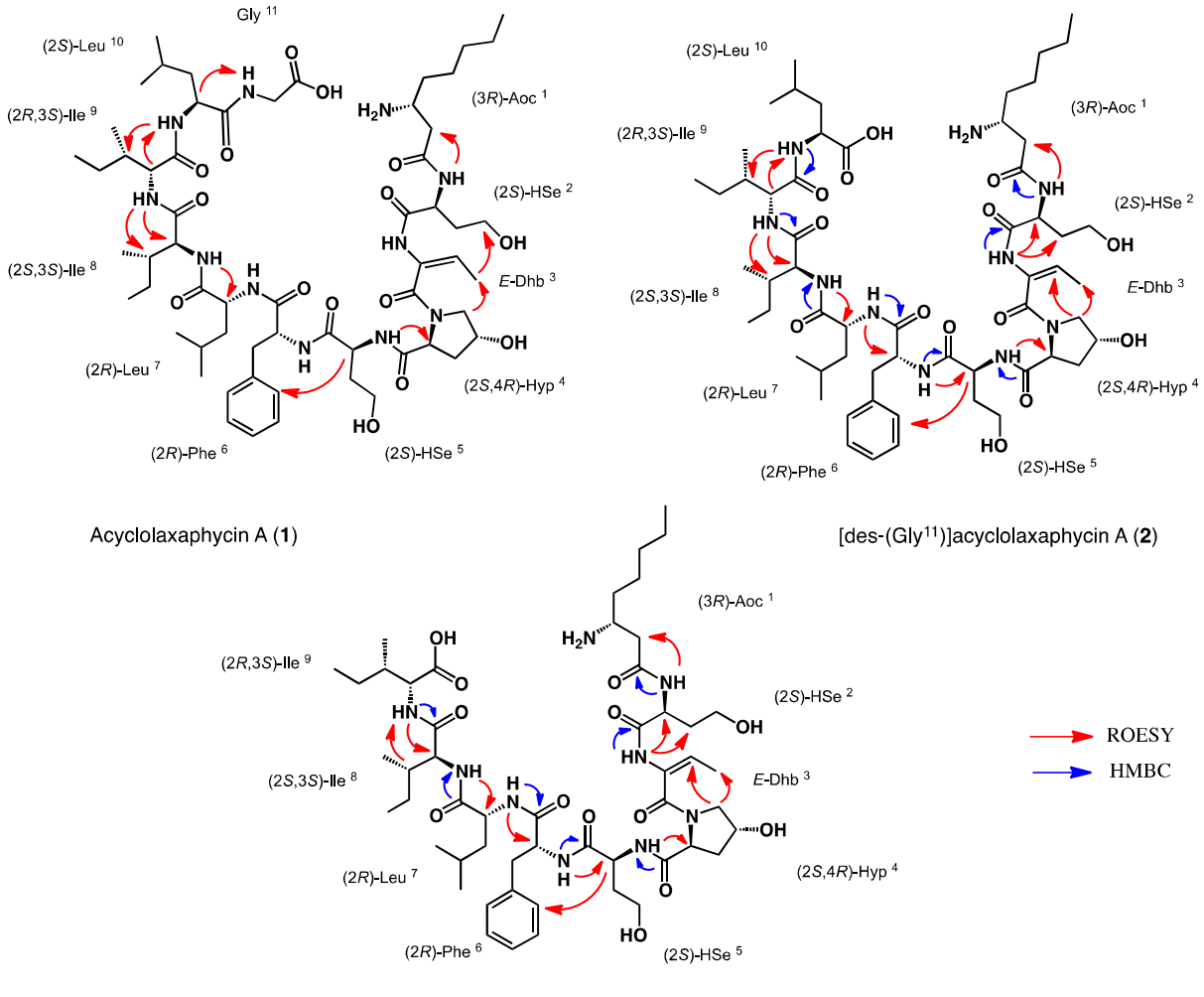


Fig. 6. Structures and key NMR correlations of acyclolaxaphycin A (**1**), [des-Gly[11]]acyclolaxaphycins A (**2**) and [des-(Leu[10]-Gly[11])]acyclolaxaphycins A (**3**). ROESY and HMBC correlations are shown with red and blue arrows respectively.

Table 1. NMR Spectroscopic Data for laxaphycin A (318K), acyclolaxaphycin A (**1**), [des-Gly^{11}]acyclolaxaphycin A (**2**) and [des-(Leu^{10}-Gly^{11})]acyclolaxaphycin A (**3**) (303 K) in DMSO-*d6*.

		Laxaphycin A		**(1)**		**(2)**		**(3)**	
		^{13}C δ (ppm)	^{1}H δ (ppm)	^{13}C δ (ppm)	^{1}H δ (ppm)	^{13}C δ (ppm)	^{1}H δ (ppm)	^{13}C δ (ppm)	^{1}H δ (ppm)
βAoc[1]	NH	-	6.82	-	-	-	7.77	-	7.77
	CαH$_2$	39.92	1.69/1.97	36.13	2.45	36.31	2.54/2.44	36.53	2.53/2.44
	CβH	44.86	4.27	48.26	3.26	48.03	3.35	48.04	3.35
	CγH$_2$	34.76	1.34	31.29	-	31.93	1.50	31.95	1.51
	CδH$_2$	28.76	1.23	24.39	1.30	24.07	1.30	24.07	1.30
	CεH$_2$	24.98	1.23	21.71	1.26	21.71	1.26	21.71	1.27
	CζH$_2$	30.72	1.23	30.92	1.22	30.79	1.23	30.79	1.23
	CηH$_3$	13.68	0.84	13.74	0.86	13.68	0.86	13.69	0.86
	CO	169.06	-	166.54	-	169.50	-	169.51	-
Hse[2]	NH	-	7.10	-	8.31	-	8.31	-	8.31
	CαH	49.06	4.54	49.76	4.43	49.85	4.40	49.84	4.41
	CβH$_2$	33.78	1.76	34.95	1.66/1.81	34.67	1.83/1.69	34.96	1.82/1.67
	CγH$_2$	56.97	3.46	53.35	3.28/3.41	57.31	3.41	57.32*	3.41
	OH	-	4.42	-	-	-	-	-	-
	CO	172.89	-	170.25	-	170.00	-	170.05	-
Dhb[3]	NH	-	10.75	-	9.93	-	9.76	-	9.77
	Cα	130.79	-	131.47	-	131.48	-	131.47	-
	CβH	118.34	5.57	113.67	5.55	113.96	5.53	114.09	5.53
	CγH$_3$	11.95	1.69	12.16	1.66	12.09	1.67	12.15	1.68
	CO	167.25	-	166.71	-	164.72	-	164.74	-
Hyp[4]	CαH	59.06	4.51	58.09	4.43	57.62	4.46	57.82	4.46
	CβH$_2$	37.84	1.92/2.27	37.98	1.93/2.09	37.75	1.92/2.02	37.76	2.02/1.91
	CγH	67.90	4.28	68.17	4.24	68.39	4.24	68.39	4.24
	OH	-	5.03	-	-	-	4.85	-	-
	CδH$_2$	56.97	3.34/3.59	55.46	3.39/3.49	55.46	3.36/3.45	55.50	3.37/3.46
	CO	170.09	-	170.74	-	171.10	-	170.60	-
Hse[5]	NH	-	7.22	-	8.19	-	8.00	-	7.99
	CαH	48.90	4.27	50.03	4.26	50.30	4.19	50.27	4.19
	CβH$_2$	33.78	1.88/1.96	34.95	1.61/1.71	34.67	1.58	34.64	1.58
	CγH$_2$	56.97	3.31/3.45	57.35	3.26	57.35	3.29/3.43	57.36*	3.29/3.43
	OH	-	-	-	-	-	-	-	-
	CO	171.97	-	171.66	-	171.56	-	171.73	-
Phe[6]	NH	-	7.79	-	8.47	-	7.92	-	7.86
	CαH	56.05	4.28	54.68	4.52	53.92	4.46	53.81	4.46
	CβH$_2$	36.99	2.94/3.04	37.67	2.79/3.12	36.93	2.79/3.05	37.04	2.79/3.04
	Cγ	137.82	-	138.07	-	137.72	-	137.65	-
	CδH$_2$	126.11	7.34	129.19	7.26	131.48	7.24	129.08	7.22
	CεH$_2$	127.95	7.24	127.82	7.20	129.03	7.23	127.90	7.22
	CζH	128.95	7.18	125.99	7.15	127.90	7.17	126.07	7.16
	CO	171.86	-	171.28	-	170.67	-	171.05**	-
Leu[7]	NH	-	7.22	-	8.08	-	7.98	-	7.97
	CαH	51.55	4.28	56.06	4.27	51.61	4.35	51.47	4.37
	CβH$_2$	42.24	1.18/1.34	40.56	1.46	40.83	1.47	40.98	1.47
	CγH	23.94	1.58	24.06*	1.60	24.17	1.60	24.13	1.59
	CδH$_3$	22.70	0.80	23.15	0.86	22.86	0.87	22.84	0.87
	Cδ'H$_3$	20.31	0.73	22.85	0.86	21.47	0.83	21.57	0.83
	CO	171.54	-	170.93	-	171.92	-	171.70	-
Ile[8]	NH	-	6.61	-	7.95	-	7.73	-	7.76
	CαH	55.95	4.63	56.31	4.34	57.00	4.31	56.46	4.41
	CβH	38.40	1.76	36.82	1.76	36.80	1.75	37.32	1.77
	CγH$_2$	21.92	1.18	24.15	0.99/1.36	24.00	1.07/1.41	23.73	1.39
	Cγ'H$_3$	15.25	0.76	15.04	0.77	15.32	0.81	15.38	0.81
	CδH$_3$	11.32	0.75	11.27	0.78	11.02	0.78	11.13	0.80
	CO	172.18	-	172.70	-	170.92	-	171.08**	-

Ile[9]	NH	-	8.68	-	7.99	-	7.82	-	7.96
	CαH	53.85	4.63	56.92	4.21	54.90	4.44	54.51	4.38
	CβH	36.73	1.97	35.72	1.80	37.00	1.84	36.36	1.88
	$C\gamma H_2$	26.08	1.18	25.26	1.07/1.29	25.77	1.10/1.26	25.66	1.13/1.28
	$C\gamma'H_3$	14.34	0.80	14.80	0.80	14.33	0.78	14.83	0.85
	$C\delta H_3$	11.04	0.84	10.71	0.83	11.42	0.83	11.40	0.84
	CO(OH)	172.35	-	171.38	-	170.87	-	173.11	-
	(OH)	-	-	-	-	-	-	-	12.51
Leu[10]	NH	-	8.34	-	8.47	-	8.05		
	CαH	52.59	4.03	51.20	4.19	50.08	4.24		
	$C\beta H_2$	42.24	1.58/1.59	39.87	1.53	39.86	1.53		
	CγH	23.94	1.56	24.16	1.60	24.77	1.60		
	$C\delta H_3$	21.24	0.83	21.34	0.81	22.75	0.87		
	$C\delta'H_3$	22.53	0.89	20.72	0.80	21.11	0.83		
	CO(OH)	172.69	-	172.34	-	173.79	-		
	(OH)	-	-	-	-	-	12.37		
Gly[11]	NH	-	8.56	-	7.40				
	CαH	42.24	3.22/3.81	43.54	3.50				
	CO(OH)	166.77	-	173.72	-				
	(OH)		-	-	-				

*,** values can be exchanged

2.2. Structural elucidation of [L-Val[8]]laxaphycin A (4) and [D-Val[9]]laxaphycin A (5)

2.2.1 [L-Val[8]]laxaphycin A

The molecular formula of **4** was deduced as $C_{59}H_{95}N_{11}O_{14}$ based on a $[M + H]^+$ peak at *m/z* 1182.7095 in HR-ESI(+)-MS spectrum and NMR spectra. A comparison between molecular formulas of **4** and laxaphycin A ($C_{60}H_{97}N_{11}O_{14}$) indicated that the new metabolite was a lower analogue (14 amu smaller) of laxaphycin A. The ^{1}H-NMR spectrum revealed strong structural similarity with laxaphycin A (Supporting Information S26). Ten amino acid residues were characterised by an interpretation of COSY, TOCSY, HSQC, HSQC-TOCSY, and ROESY (Supporting Information S28-S32): Aoc, two Hse, Dhb, Phe, two Leu, Gly, Hyp and Ile (Table 2). HSQC analysis revealed the loss of correlations between Cβ and Hβ, Cγ and Hγ, Cδ and Hδ of Ile[8] observed in laxaphycin A. Instead of Ile, the presence of a valine (Val) spin system was deduced from TOCSY, HSQC, HSQC-TOCSY correlations between the amide signal (δ_H 6.53) to a Hα (δ_H 4.67; δ_C 55.59), a Hβ (δ_H 02.12; δ_C 32.31) and two methyl groups (δ_H 0.73; δ_C 19.20 and δ_H 0.64; δ_C 15.27). ROESY correlation connected the amide signal to HαLeu[7]. NOESY correlations linked NH Ile[9] (δ_H 8.33) to Hα (δ_H 4.67), Hβ (δ_H 2.12) and Hγ (δ_H 0.73) of Val. HMBC cross-peaks between NH Ile[9] (δ_H 8.33) and the adjacent carbonyl of Val (δ_C 172.20); Hα (δ_H 4.68) of Val and the adjacent carbonyl of Leu[7] (δ_C 171.65) positioned the valine residue between Ile[9] and Leu[7]. Finally, HMBC correlations between carbonyl carbons (residue i) and NH or Cα protons (residue i+1) and ROESY correlations between Hα (residue i) and NH (residue i+1) completed the cyclic structure of compound **4**. Thus, the sequence was established as cyclo[Aoc-Hse-Dhb-Hyp-Hse-Phe-Leu-Val-Ile-Leu-Gly]. Mass fragmentation analysis confirmed the sequence assignment of **4**. Similar to laxaphycin A, the protonation and cleavage of the amide bond preferentially occurred on the amide nitrogen of Pro. Thus the cyclic ion was opened between Dhb[3] and Pro[4] and formed a linear acylium ion that generated a series of fragments. These observed fragments (Supporting

Information S33) were consistent with the proposed amino acid sequence (Fig. 7) determined by NMR.

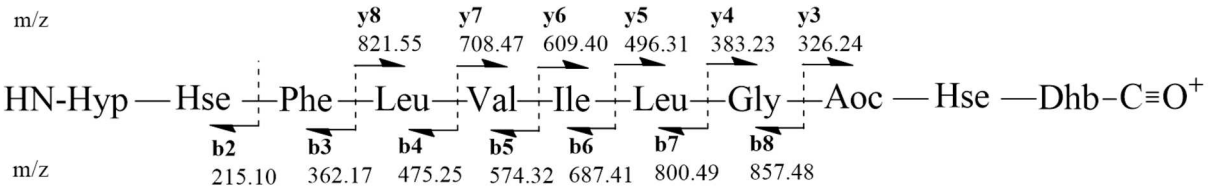


Fig. 7. ESI(+)-MS/MS fragmentation of [L-Val[8]]laxaphycin A (**4**).

2.2.2 [D-Val[9]]laxaphycin A (5)

The molecular formula of [D-Val[9]]laxaphycin A (**5**) was determined as $C_{59}H_{95}N_{11}O_{14}$ by HR-ESI(+)-MS analysis (*m/z* 1182.7095 $[M+H]^+$) and the new metabolite was deduced to be a lower analogue (14 uma smaller) of laxaphycin A. This result supported the presence of two isomers (compounds **4** and **5**). The ^{1}H-NMR spectrum (Supporting Information S35) revealed strong structural similarity with [L-Val[8]]laxaphycin A and 2D-NMR including TOCSY, HSQC, HSQC-TOCSY and ROESY (Supporting Information S37-S41) revealed the presence of eleven amino acid residues: Aoc, two Hse, Dhb, Phe, two Leu, Gly, Hyp, Ile and Val (Table 2). However, the Val residue was located in position 9 since the correlation in Ile[8] between Cβ and Hβ, observable in the HSQC spectrum of laxaphycin A, was also present in the HSQC spectrum of **5**, and the correlation in Ile[9] between Cβ and Hβ was absent. HMBC data confirmed this assumption by revealing correlations between Ile[8] carbonyl (δ_C 172.12) and Val NH (δ_H 8.33), as well as between Val carbonyl (δ_C 172.25) and Leu[10] NH (δ_H 8.35). Additionally, ROESY correlations were observed between Hα (δ_H 4.61) and Hβ (δ_H 1.80) of Ile[8] and NH (δ_H 8.33) of Val and between Hα (δ_H 4.41) and Hβ (δ_H 2.22) of Val and NH (δ_H 8.35) of Leu[10] which confirmed the position of Val. Using HMBC and ROESY correlations, the complete sequence was defined as cyclo[Aoc-Hse-Dhb-Hyp-Hse-Phe-Leu-Ile-Val-Leu-Gly]. The mass fragmentation analysis revealed the preferential opening of the macro ring between Dhb[3] and Pro[4] as in laxaphycin A and [L-Val[8]]laxaphycin A. The fragments (Supporting Information S42) were in complete agreement with the structure proposed above (Fig. 8).

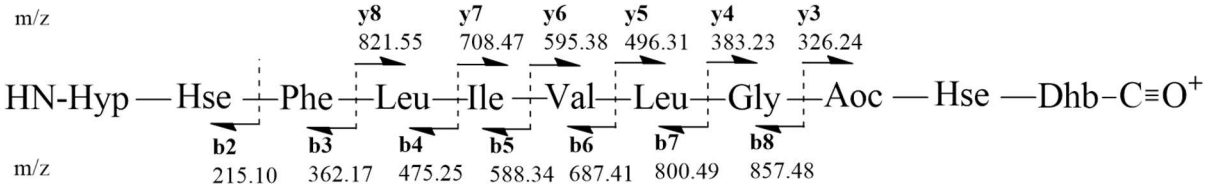


Fig. 8. ESI(+)-MS/MS fragmentation of [D-Val[9]]laxaphycin A (**5**).

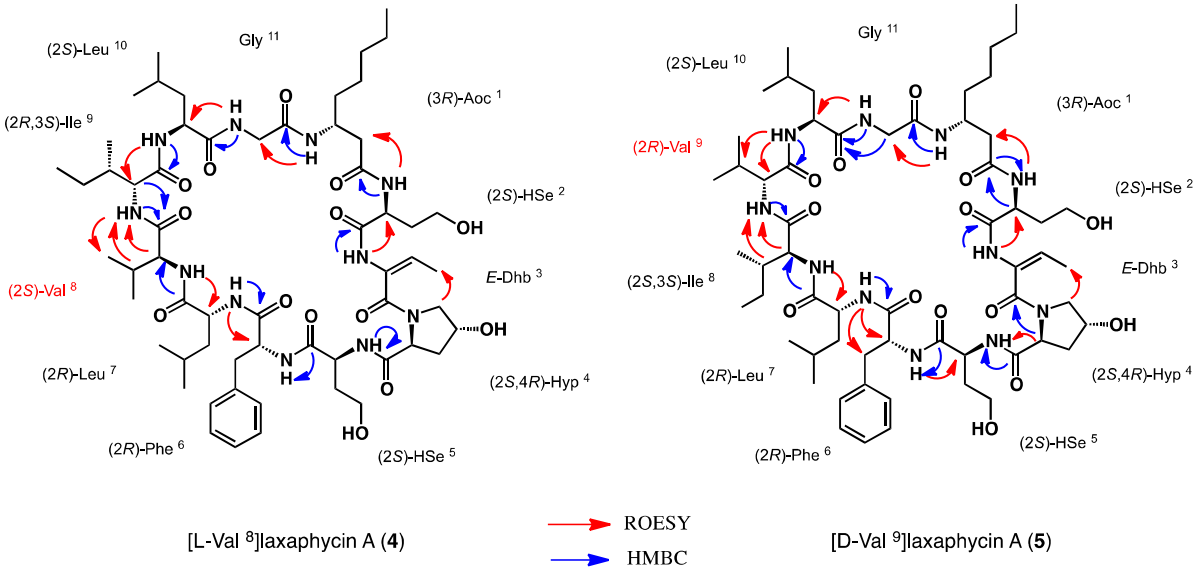


Fig. 9. Structures and key NMR correlations of [L-Val[8]]laxaphycin A (**4**) and [D-Val[9]]laxaphycin A (**5**). ROESY and HMBC correlations are shown with red and blue arrows respectively.

Table 2. NMR spectroscopic data for laxaphycin A (318K), [L-Val[8]]laxaphycin A (**4**) and [D-Va[l9]]laxaphycin A (**5**) (303 K) in DMSO-*d6*.

		Laxaphycin A		[L-Val[8]]laxaphycin A (**4**)		[D-Val[9]]laxaphycin A (**5**)	
		^{13}C δ (ppm)	^{1}H δ (ppm)	^{13}C δ (ppm)	^{1}H δ (ppm)	^{13}C δ (ppm)	^{1}H δ (ppm)
β Aoc[1]	NH	-	6.82	-	6.84	-	6.88
	CαH$_2$	39.92	1.69/1.97	40.09	1.66/1.91	44.93	1.74/1.97
	CβH	44.86	4.27	44.66	4.25	40.11	4.21
	CγH$_2$	34.76	1.34	34.93	1.34	34.70	1.34
	CδH$_2$	28.76	1.23	25.10	1.24	25.10	1.23
	CεH$_2$	24.98	1.23	22.08	1.24	22.04	1.23
	CζH$_2$	30.72	1.23	30.77	1.22	30.82	1.23
	CηH$_3$	13.68	0.84	13.85	0.85	13.81	0.84
	CO	169.06	-	169.00	-	169.18	-
Hse[2]	NH	-	7.10	-	7.15	-	7.14
	CαH	49.06	4.54	49.08	4.55	49.17	4.54
	CβH$_2$	33.78	1.76	33.67	1.76	33.85	1.76
	CγH$_2$	56.97	3.46	56.96	3.31/3.42	57.09	3.45
	OH	-	4.42	-		-	-
	CO	172.89	-	173.49	-	172.87	-
Dhb[3]	NH	-	10.75	-	10.80	-	10.75
	Cα	130.79	-	130.11	-	130.84	-
	CβH	118.34	5.57	118.82	5.59	118.25	5.57
	CγH$_3$	11.95	1.69	12.15	1.69	12.14	1.68
	CO	167.25	-	167.59	-	167.26	-
Hyp[4]	CαH	59.06	4.51	59.33	4.51	59.14	4.51
	CβH$_2$	37.84	1.92/2.27	37.83	1.87/2.29	38.02	1.87/2.26
	CγH	67.90	4.28	68.04	4.29	68.01	4.27
	OH	-	5.03	-		-	5.15
	CδH$_2$	56.97	3.34/3.59	57.14	3.32/3.61	57.02	3.31/3.59
	CO	170.09	-	170.20	-	170.22	-
Hse[5]	NH	-	7.22	-	7.29	-	7.29
	CαH	48.90	4.27	48.83	4.28	49.01	4.25
	CβH$_2$	33.78	1.88/1.96	33.78	1.85/1.99	33.95	1.83/1.99
	CγH$_2$	56.97	3.31/3.45	56.89	3.28/3.43	56.78	3.29/3.43

	OH	-	-				
	CO	171.97	-	178.08	-	172.03	-
Phe[6]	NH	-	7.79	-	7.86	-	7.80
	CαH	56.05	4.28	56.52	4.26	56.14	4.28
	$C\beta H_2$	36.99	2.94/3.04	37.00	2.95/3.06	37.02	2.95/3.01
	Cγ	137.82	-	137.93		137.88	
	$C\delta H_2$	126.11	7.34	129.04	7.36	129.07	7.35
	$C\varepsilon H_2$	127.95	7.24	128.13	7.25	128.06	7.24
	CζH	128.95	7.18	126.28	7.19	126.23	7.18
	CO	171.86	-	171.91	-	171.90	-
Leu[7]	NH	-	7.22	-	7.20	-	7.32
	CαH	51.55	4.28	51.67	4.24	51.58	4.29
	$C\beta H_2$	42.24	1.18/1.34	34.92	1.37	39.41	1.15/1.28
	CγH	23.94	1.58	24.02	1.56	23.97	1.58
	$C\delta H_3$	22.70	0.80	22.85	0.80	22.85	0.80
	$C\delta'H_3$	20.31	0.73	20.28	0.72	20.44	0.73
	CO	171.54	-	171.65	-	171.65	
Ile[8]/Val[8]	NH	-	6.61	-	6.53	-	6.71
	CαH	55.95	4.63	55.59	4.67	56.02	4.61
	CβH	38.40	1.76	32.31	2.12	38.52	1.80
	$C\gamma H_2$	21.92	1.18	19.2	0.73	22.41	1.20
	$C\gamma'H_3$	15.25	0.76	15.27	0.64	15.37	0.77
	$C\delta H_3$	11.32	0.75			11.46	0.73
	CO	172.18	-	172.20	-	172.12	-
Ile[9]/Val[9]	NH	-	8.68	-	8.33	-	8.33
	CαH	53.85	4.63	53.54	4.68	56.78	4.41
	CβH	36.73	1.97	37.07	2.02	30.44	2.22
	$C\gamma H_2$	26.08	1.18	26.26	1.15	19.17	0.83
	$C\gamma'H_3$	14.34	0.80	14.35	0.80	16.62	0.83
	$C\delta H_3$	11.04	0.84	11.31	0.85		
	CO	172.35	-	172.66	-	172.25	-
Leu[10]	NH	-	8.34	-	8.42	-	8.35
	CαH	52.59	4.03	52.97	4.00	52.60	4.04
	$C\beta H_2$	42.24	1.58/1.59	39.35	1.37/1.54	39.39	1.40/1.51
	CγH	23.94	1.56	23.95	1.58	24.02	1.58
	$C\delta H_3$	21.24	0.83	22.59	0.89	22.63	0.89
	$C\delta'H_3$	22.53	0.89	21.69	0.84	21.34	0.83
	CO	172.69	-	172.94	-	172.87	-
Gly[11]	NH	-	8.56	-	8.70	-	8.59
	CαH	42.24	3.22/3.81	42.27	3.22/3.78	42.30	3.30/3.78
	CO	166.77	-	166.71		166.92	

2.3. Absolute configuration of compounds 2-5

The absolute configuration of each amino acid residue in compounds **2**-**5** was established using advanced Marfey's method after hydrolysis (Supporting Information S25, S34, S43).[22,23] LC-MS comparison between the Marfey's derivatives of [des-(Gly[11])]-acyclolaxaphycin A (**2**) assigned *2S* configuration of Hse (x2), *2R* configuration of Phe and *3R* configuration of Aoc (Supporting Information). Stereochemical identification of leucines and isoleucines was elucidated by comparison between L-FDLA and LD-FDLA derivatives of authentic standard stereoisomers and **2**, and indicated the presence of (*2R*)-Leu, (*2S,3S*)-Ile, (*2R,3S*)-Ile and (*2S*)-Leu as found in laxaphycin A. Hydroxyproline constitutes an exception of the Marfey's rule because D-FDLA-(*2S*)-Hyp derivative elutes before the L-FDLA-(*2S*)-Hyp derivative[6]. Thus the absolute configuration of the α carbons of Hyp was assigned as *2S.* Based on the previous stereochemical assignment of the Hyp residue in laxaphycin A, the

identical retention time observed for the Hyp derivative both in laxaphycin A and compound **2** enabled the Cγ configuration to be assigned as *4R*. The geometric configuration of Dhb was determined from ROESY correlations. Strong ROESY cross-peaks between the NH (δ_H 9.76) and the ethylenic proton (δ_H 5.53) of Dhb, and between the Hγ (δ_H 1.67) of Dhb and the Hδ (δ_H 3.36; 3.45) of Hyp were observed, assigning the geometric configuration of the double bond as *E*. Therefore, we established the complete structure of [des-(Gly^{11})]-acyclolaxaphycin A (**2**) as (*3R*)-Aoc-(*2S*)-Hse-(*E*)-Dhb-(*2S,4R*)-Hyp-(*2S*)-Hse-(*2R*)-Phe-(*2R*)-Leu-(*2S,3S*)-Ile-(*2R,3S*)-Ile-(*2S*)-Leu.

[des-(Leu^{10}-Gly^{11})]acyclolaxaphycin A (**3**): the absolute configuration of each amino acid residue was assigned as (*3R*)-Aoc, (*2S*)-HSe (x2), (*2S,4R*)-Hyp, (*2R*)-Phe (*2R*)-Leu, (*2S,3S*)-Ile and (*2R,3S*)-Ile (Supporting Information S25). The geometric configuration of Dhb was defined as *E* with ROESY correlations between the NH (δ_H 9.77) and the ethylenic proton (δ_H 5.53) of Dhb and between Hγ (δ_H 1.68) of Dhb and Hδ (δ_H 3.37; 3.46) of Hyp. The complete structure could be defined as (*3R*)-Aoc-(*2S*)-Hse-(*E*)-Dhb-(*2S,4R*)-Hyp-(*2S*)-Hse-(*2R*)-Phe-(*2R*)-Leu-(*2S,3S*)-Ile-(*2R,3S*)-Ile.

[L-Val^{8}]laxaphycin A (**4**): the absolute configuration of each amino acid residue was defined with Marfey's method (Supporting Information S25). The absolute configuration of Val was determined as *2S* and the geometric configuration of Dhb was assigned as *E* with ROESY correlations between NH (δ_H 10.80) and Hβ (δ_H 5.59) of Dhb and between Hγ (δ_H 1.69) of Dhb and Hδ (δ_H 3.32; 3.61) of Hyp. The complete structure of **4** was defined as cyclo-[(*3R*)-Aoc-(*2S*)-Hse-(*E*)-Dhb-(*2S,4R*)-Hyp-(*2S*)-Hse-(*2R*)-Phe-(*2R*)-Leu-(*2S*)-Val-(*2R,3S*)-Ile-(*2S*)-Leu-Gly-].

[D-Val^{9}]laxaphycin A (**5**): the ROESY correlations between NH (δ_H 10.75) and Hβ (δ_H 5.57) of Dhb and between Hγ (δ_H 1.68) of Dhb and Hδ (δ_H 3.31/3.59) of Hyp determined that the geometric configuration of Dhb was *E*. In contrast with [L-Val^{8}]laxaphycin A (**4**), the Val residue was assigned as (*2R*)-Val, but this result is consistent with the stereochemistry of the Cα backbone of the ring, the (*2R*)-Val replacing the (*2R, 3S*)-Ile. Thus the structure was cyclo-[(*3R*)-Aoc-(*2S*)-Hse-(*E*)-Dhb-(*2S,4R*)-Hyp-(*2S*)-Hse-(*2R*)-Phe-(*2R*)-Leu-(*2S,3S*)-Ile-(*2R*)-Val-(*2S*)-Leu-Gly-].

Acyclolaxaphycin A (**1**): due to the small amount of compound obtained, the stereochemistry of acyclolaxaphycin A was not elucidated, but the configuration of the Cα overall backbone seems to be maintained in laxaphycin A analogues. The configurational analysis with Marfey's procedure gave the same results for laxaphycin A and compounds **2**-**5**. Thus the absolute configuration was not established for compound **1**, but we speculate that the complete structure of acyclolaxaphycin A (**1**) is (*3R*)-Aoc-(*2S*)-Hse-(*E*)-Dhb-(*2S,4R*)-Hyp-(*2S*)-Hse-(*2R*)-Phe-(*2R*)-Leu-(*2S,3S*)-Ile-(*2R,3S*)-Ile-(*2S*)-Leu.

Interestingly, the NMR chemical shifts of laxaphycin A and acyclolaxaphycin A are relatively close. These results were unexpected for a cyclic peptide and its acyclic equivalent. We hypothesise that the secondary structure of both molecules are similar although we are unable to confirm this from intra-molecular ROESY correlations.

2.5. Biosynthesis and biotransformation within the laxaphycin family.

[L-Val^{8}]laxaphycin A and [D-Val^{9}]laxaphycin A are two variants of laxaphycin A characterised by the presence of a Val residue in position 8 or 9 instead of an Ile residue. Interestingly, peptides from laxaphycin-A family, while produced by different cyanobacteria, share the (*3R*)-β-amino fatty acid (Aoc or Ade), (*2R*)-Leu and Gly in positions 1, 7 and 11. It is important to emphasise that other amino acid residues vary through isosteric substitutions, but the absolute configuration of the carbon α is conserved at each position. The presence of a Val residue in compounds **4** and **5**, instead of an Ile residue in laxaphycin A, is consistent with such isosteric substitutions and may indicate a lack of specificity of the amino acid adenylation domains as already mentioned for the biosynthesis of puwainaphycins.[15,19] Acyclolaxaphycins **1**, **2** and **3** are three acyclic analogues of laxaphycin A obtained by a ring opening between Gly^{11} and Aoc^{1} (compound **1**) and the successive loss of one (compound **2**) or two residues (compound **3**), with the stereochemistry of all amino acids being retained from laxaphycin A to **1**, **2** and **3**. Although no study has been published on the biosynthetic pathway of the laxaphycin peptides, characterisation of the minor acyclic acyclolaxaphycins **1**, **2** and **3** could indicate a mechanism similar to the biosynthesis of puwainaphycins. Indeed, the activation of the fatty acid residue, carried out by FAAL and pursued by PKS/NRPS enzymes, leading to the *β*-aminooctanoic acid, could constitute the first step in the biosynthesis of laxaphycin A-type peptides, followed by amino acid assembly starting from Hse^{2} to Gly^{11} and may suggest that a thioesterase (TE) domain promotes the final cyclization between the NH_2 of the *β*-Aoc and the COOH of the Gly residue.

We published the characterisation of acyclolaxaphycins B and B3, two acyclic laxaphycins B-type peptides with a ring opening occurring between OH-Leu^{3} and Ala^{4}. We argued that the biosynthesis process of such compounds could start with the NRPS module instead of FAAL and ACP ligases, and finish with the cyclization between the amino group of the alanine residue and the carboxyl of the hydroxyleucine residue.[21] But given that FAAL enzymes are usually used in starter loading in PKS/NRPS systems, possible mechanisms leading to the acyclic compounds can be considered.

It is not unlikely that both acyclolaxaphycins A **1**-**3** and acyclolaxaphycins B and B3 ensue from an enzymatic degradation as a resistance mechanism from competitive species. A study showed that the hydrolysis of a bacterial lipodepsipeptide surfactin, operating on an ester bond, was carried out by a filamentous bacterium.[24] A similar enzymatic hydrolysis may occur for laxaphycins, though no ester bond is present making them more robust and less inclined to hydrolysis. More recently a mechanism of resistance toward nonribosomal peptide antibiotics based on hydrolytic cleavage by D-stereospecific peptidases has been demonstrated,[25] the hydrolysis occurring at the C-terminal side of D-aa. In the case of laxaphycins B, the C-terminal aminoacid in the linear peptides are D-aa, [(*3R,2S*)-3-OHLeu]. In the case of acyclolaxaphycins **1** and **2**, the C-terminal aminoacids being respectively Gly and L-Leu, we can consider another mechanism. TE-catalyzed cyclorealease may be also in competition with TE-catalyzed hydrolysis, without or with module skipping, leading respectively to compound **1** or to truncated compounds **2** and **3**.[26]

2.6 Biological activities

The effect after 24 h incubation of laxaphycins on the cellular viability was studied by tetrazolium dye (MTT) and lactate dehydrogenase (LDH) assays on neuroblastoma cells (Table 3). Of all compounds, laxaphycin A and [des-(Leu^{10}-Gly^{11})]acyclolaxaphycin A (**3**) did not elicit any cytotoxic effect ($IC_{50} \geq 10\mu M$), while [des-Gly^{11}]acyclolaxaphycin A (**2**) showed decreased viability but did not reach a full viability inhibition. [L-Val^{8}]laxaphycin A (**4**) and [D-Val^{9}]laxaphycin A (**5**) showed a complete cellular viability inhibition after 24 h incubation in both assays. Although the IC_{50} values were lower with MTT assay than with LDH assay, the potency order was the same, (**4**) < (**5**). We also tested the effect on the viability at shorter exposure time. MTT assay after 6 h treatment showed similar IC_{50} (Table 3).

Table 3: IC_{50} of laxaphycins A after 24 or 6 h treatment in neuroblastoma SHSY5Y cells. IC_{50} values (in μM) followed by the 95% confidence interval (CI) and the coefficient of determination (R^2).

Compound name	24h IC_{50} (MTT)	6h IC_{50} (MTT)	24h IC_{50} (LDH)
Laxa A	Non toxic	Non toxic	Non toxic
(2)	Incomplete cellular inhibition	Incomplete cellular inhibition	Non toxic
(3)	Non toxic	Non toxic	Non toxic
(4)	0.6, CI: 0.3-1.2, R^2: 0.94	1.3, CI: 0.3-5.5, R^2: 0.90	3.6, CI:1.2-10.5, R^2: 0.94
(5)	5.6, CI: 1.2-25.0, R^2: 0.93	3.7, CI: 0.4-34.8), R^2: 0.88	12.4, CI: 0.9-170.4), R^2: 0.90

Since the MTT assay, based on the mitochondrial function, shows lower IC_{50} values, we decided to study the influence of laxaphycins A after a 6 h incubation on the mitochondrial membrane potential (Ψm) of neuroblastoma cells. Among the five compounds tested, four affected the Ψm (laxa A, (**2**), (**4**) and (**5**)). Interestingly, [des-Gly^{11}]acyclolaxa A (**2**) decreased the Ψm without affecting the cellular viability (Fig. 11A). As mitochondria are the main producers of reactive oxygen species (ROS), we studied the modulation of ROS release by laxaphycins. Following a 6 h treatment, only two compounds affect ROS production (Fig. 11B). Compounds (**2**) and (**5**) decreased ROS levels. [des-Gly^{11}]acyclolaxa A (**2**) inhibited ROS levels around a 40 % versus control levels but, at the concentrations tested, the viability of the neuroblastoma cells was not affected. This effect was also tested at a shorter incubation time (1h) and the observed effects were identical to 6 h (data not shown).

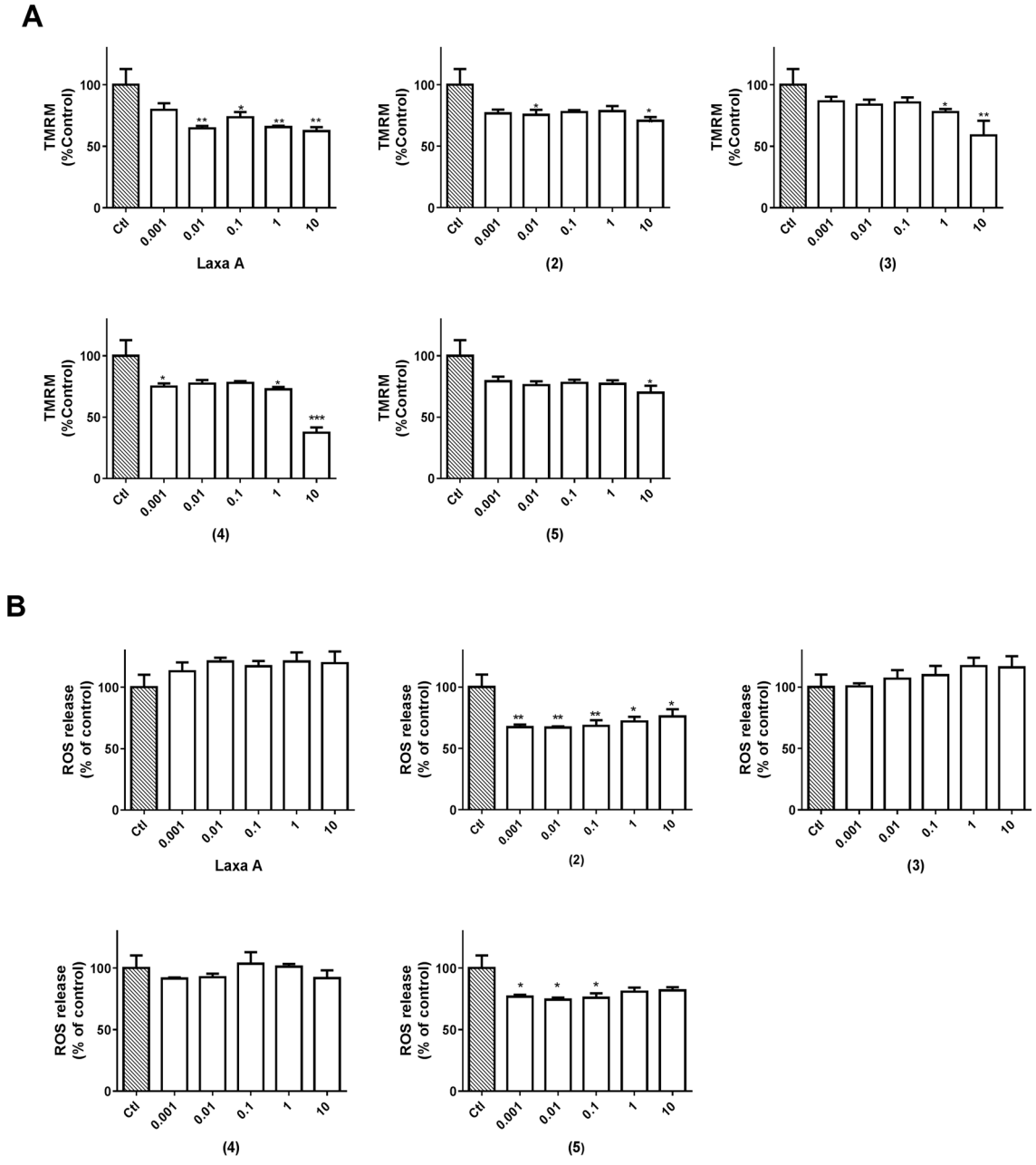


Fig. 11. Modulation of mitochondrial potential and reactive oxygen species release on neuroblastoma cells by laxaphycins. A) Effect of µM concentrations of laxaphycins after 6h incubation over mitochondrial membrane potential measured by TMRM assay. **B)** Effect of µM concentrations of laxaphycins after 6h over ROS release measured by DCFH-DA staining. Data are presented as percentage of untreated cells. Control samples are treated with the same proportion of compounds diluent..Values are mean ± SEM of four independent experiments performed by triplicate. *p<0.05, **p<0.01 and ***p<0.001. Laxaphycins tested are laxa A, [des-Gly^{11}]acyclolaxa A (**2**), [des-(Leu^{10}-Gly^{11})]acyclolaxa A (**3**), [L-Val^{8}]laxa A (**4**), [D-Val^{9}]laxa A (**5**).

We studied what kind of cellular death could be induced by these compounds. For this, we used the annexin V/propidium iodide staining. Cells were treated with 10 µM of each compound and after 6 h flow cytometry analysis revealed that (**4**) and (**5**) showed a significant decrease in the viable cell population but no increase in apoptotic or necrotic cells (Fig. 12A). In view of these data and the effects over the mitochondria, we analysed if the laxaphycins

could be inducing another type of cellular phenomenon different from apoptosis or necrosis, autophagy.[27] Autophagy is a controlled phenomenon of self-degradation of damaged, repetitive or risky cellular components as a self-renewal mechanism that participates in cell survival and death. Under some circumstances such as environmental or external compound-mediated conditions, a continuously or enhanced autophagy activation may lead to cellular death.[28] We first analysed the levels of the autophagosome marker microtubule-associated light chain 3 (LC3), one of the most readily used markers for autophagy related to autophagosome development and maturation. LC3-I is the soluble isoform and LC3-II is the autophagic-vesicle-associated form;[29] the ratio between the two isoforms allowed us to verify the presence of this cellular event. Under cellular stress conditions, LC3 is cleaved by ATG4 family proteins to form LC3-I and subsequently conjugated to phosphatidylethanolamine (PE) to form LC3-II, which is located in autophagosomes. LC3-II is thus an appropriate autophagy marker because its amount correlated with the number of autophagosomes and autophagy-related structures.[30] Among all the compounds, only (**2**), (**4**) and (**5**) induced an increase in the LC3-II/I ratio pointing to an autophagic process and in agreement with the flow cytometry results (Fig. 12B). [D-Val^{9}]laxa A (**5**) and [des-Gly^{11}]acyclolaxa A (**2**) were the two compounds that produced a higher ratio increase with a ratio value of 6.4 ± 0.9 and 6.7 ± 0.7 respectively *versus* the control value of 2.6 ± 0.3 of non treated cells. Additionally, we analysed the mechanistic target of rapamycin (mTOR), a protein kinase complex that regulates cellular responses to stress, nutrient deprivation or extracellular stimulus and negatively modulates autophagy. In physiological conditions, with nutrients and energy availability, mTOR is phosphorylated (active form) and therefore autophagy is inhibited, although a low level of autophagy always occurs to eliminate damaged organelles or misfolded proteins. However, under nutrient deprivation, mTOR is inactivated (appears in its non phosphorylated form) inducing autophagy.[31,32,33] To test whether laxaphycins can induce autophagy, the phosphorylation of mTOR was studied by western blot. Compounds (**2**), (**4**), and (**5**) induced a marked decrease from 100% of phospho-mTOR in non treated cells to 70.3 ± 8.1%, 73.1 ± 10.1%, and 70.7 ± 10.3% respectively in treated cells (Fig. 12C). These results confirmed that [des-Gly^{11}]acyclolaxa A (**2**), [L-Val^{8}]laxa A (**4**), and [D-Val^{9}]laxa A (**5**) induce autophagy in SHSY5Y neuroblastoma cells.

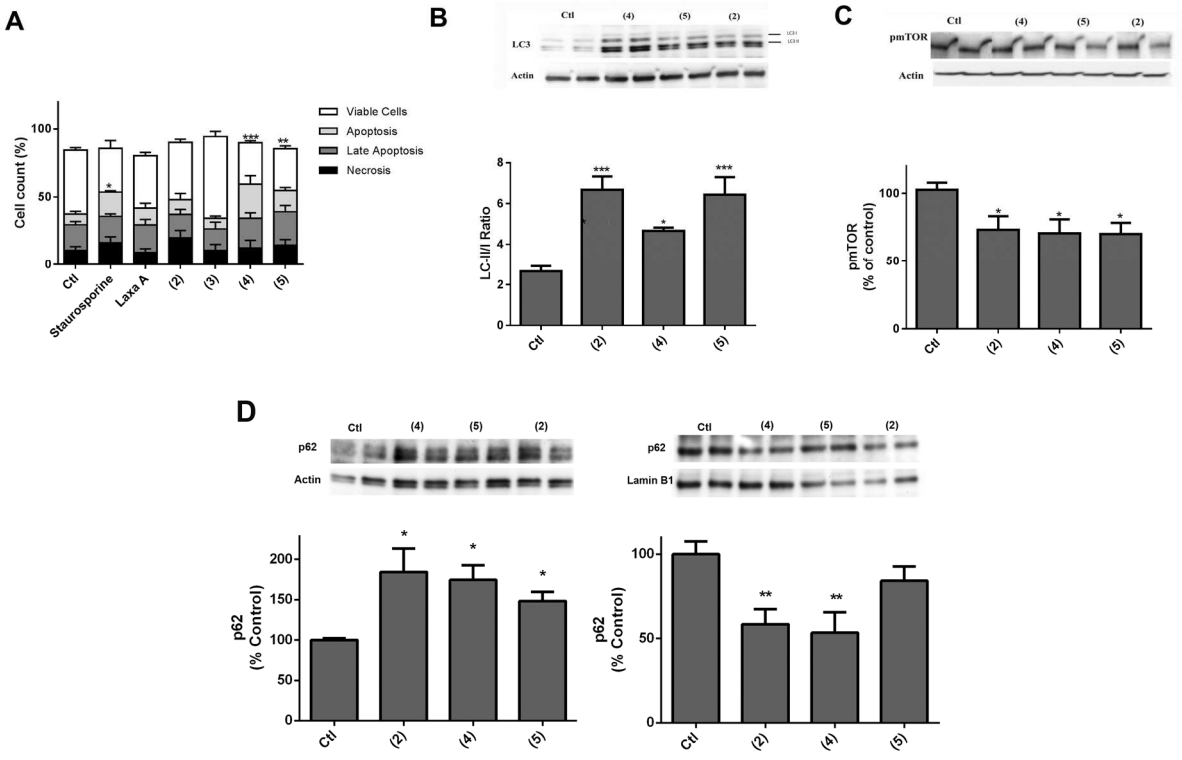


Fig. 12. Laxaphycins induced cell death in SHSY5Y cells. Cells were treated with 10 µM for 6 h before sample processing for cytometry or western blot. **A)** Flow cytometry analysis of viable, apoptotic and necrotic cells by annexin V/ propidium iodide staining. Data are presented as percentage of untreated cells. **B)** LC3 expression measurement by western blot, representative blot and bars histogram of LC3 II/I ratio after 6 h incubation with laxaphycins in culture medium. Data are presented as ratio of LC3II expression /LC3I expression. **C)** mTOR phosphorylation (pmTOR) expression study after 6 h incubation with laxaphycins in culture medium. Representative blot and bar histogram of pmTOR expression. **D**) p62 expression levels after laxaphycins treatment. Representative blot and bar histogram of p62 expression. Data are presented as percentage of untreated cells. Values are mean ± SEM of three independent experiments performed by triplicate. Western blot samples are running in duplicate in each experiment. Control samples are treated with the same proportion of compounds diluent. *p<0.05, **p<0.01 and ***p<0.001.

To confirm autophagy, western blot was running to detect sequestosome-1 (SQSTM1)/ubiquitin-binding protein p62 (p62) levels as a measurement of autophagic flux. After 6 h incubation compounds **2**, **4** and **5** induced an increase of cytoplasmic p62 levels meanwhile membrane levels were decreased after compound **2** and **5** treatment (Figure 12D). Although compound **4** decreased p62 levels in the membrane fraction a 15.8 ± 8.8 % vs control cells, this reduction was not statistically significant. This results, together with the alterations of the Ψm, point to a mitophagic process.

3. Conclusion

In summary, we have identified three new acyclic lipopeptides, termed acyclolaxaphycin A (**1**), [des-(Gly^{11})]acyclolaxaphycin A (**2**) and [des-(Leu^{10}-Gly^{11})]acyclolaxaphycin A (**3**) from the cyanobacterium *Anabaena torulosa*. From the same

species, we also isolated two new cyclic lipopeptides, [L-Val^{8}]laxaphycin A (**4**) and [D-Val^{9}]laxaphycin A (**5**). The two cyclic compounds appear to be close analogues of the known laxaphycin A, previously isolated from the same species of cyanobacterium. [L-Val^{8}]laxaphycin A has been recently isolated from a marine cyanobacterium *Hormothamnion enteromorphoides*.[9] To the best of our knowledge, the presence of cyclic lipopeptides with their acyclic equivalents has never been described within this family and raises several assumptions. We can assume that acyclic laxaphycins A ensued from an enzymatic degradation as a resistance mechanism. Within the laxaphycin B-type peptides we previously described the structure of acyclolaxaphycins B and B3 isolated from *A. torulosa*. But the ring opening within the two family-type peptides does not occur at the same position in the sequence. Thus the isolation of minor acyclic analogues of lipopeptides may lead to the characterisation of novel specific peptidases.

Among the compounds isolated, several differences were also observed in the biological effects. Whereas laxaphycin A did not affect SHSY5Y viability (10 µM), compounds [L-Val^{8}]laxaphycin A (**4**) and [D-Val^{9}]laxaphycin A (**5**) showed an IC_{50} of 0.6 and 5.6 µM respectively and the compound [des-(Gly^{11})]acyclolaxaphycin A (**2**) was less potent with 50 % decrease in viability compared to non treated cells at the highest concentration tested (10 µM). The lack of, or poor, cytotoxic activity of the parent compound laxaphycin A over SHSY5Y cells was in accordance with previous reports in tumoral and non tumoral cell lines.[6,34] The three active compounds did not induce apoptosis or necrosis in neuroblastoma cells as proved with propidium iodide /annexin V assays. However, they induced an autophagy process in these neuronal lines as it was corroborated by the induced alterations in LC3, p62 and mTOR levels. At the tested concentration, these compounds seem to affect mitochondria as observed in Ψm assays and reinforced by p62 expression. This results point to a mitophagic event, in which dysfunctional mitochondria are delivered through autophagosomes in order to maintain an optimal cellular functioning. More assays will be needed to deepen in the effect of these compounds on the mitochondria and clarify if the potential mitochondrial membrane alteration is enough to alter the cellular energy supply and trigger the autophagic process.

Although autophagy is a lysosomal degradation process that can be essential for cellular survival, differentiation and proliferation, it can also result in cellular death. This catabolic pathway is activated under different stimuli such as starvation, infection or hypoxia among others, to degrade damaged organelles or dysfunctional proteins and allows for a proper cellular homeostasis.[35] Several studies have linked this autophagic route with different neuronal pathologies such as stroke, Parkinson´s or Alzheimer´s diseases. In these pathologies, the induction of autophagy is being studied as a therapeutic option to clear aggregated proteins at impaired neurons and the study of compounds with capacity to induce this phenomenon could be of potential interest for the pharmaceutical industry. However, an excessive autophagy induction can also worsen the situation and a balance must be found.[36,37]

Among the laxaphycin A-type peptides only hormothamnin A (the *Z*-Dhb isomer of laxaphycin A) was described as exerting a cytotoxic effect in the micromolar range (IC_{50} value between 0.13 and 0.72 µg/ml).[11] Other members such as trichormamides A and D (IC_{50}

between 9.9 and 16.9 μM),[15,16] laxaphycin[9,12] or laxaphycin A2[12] ($IC_{50} \geq 10\mu M$) are weakly active or inactive. In this study [L-Val^8]laxaphycin A (**4**) and [D-Val^9]laxaphycin A (**5**) showed a complete cellular viability inhibition after 24 h incubation with both tetrazolium dye (MTT) and lactate dehydrogenase (LDH) assays on neuroblastoma cells (IC_{50} value 0.6 and 5.6 μM). These results show that very small structural modifications can induce changes in cytotoxic activity. Interestingly, the acyclic peptide **2** is more biologically active than the cyclic laxaphycin A. The similarity of the NMR spectra between the two compounds led us to believe that **2** retains the same secondary structure as laxaphycin A but that the cleavage of the peptide and the loss of Gly increased the hydrophilic character of **2** which in turn may improve its biological activity. The conservation of the secondary structure in **3** is less evident and may explain the loss of activity. The lack of acyclolaxaphycin A (**1**) did not allow further progress. However, it is difficult to compare all of these results since the cytoxicity evaluations were performed on different cell lines (lung, melanoma, colon, HCT 116 or neuroblastoma cell lines), with different exposure times (24, 48, 72 h). Some research groups involved in the synthesis of non-ribosomal peptides have forged the path toward the total synthesis of laxaphycins and their analogs,[38,39,40] such as the recent total synthesis of trichormamide A, carried by our group.[41] The synthesis of these compounds will enable the establishment of structure-antiproliferative activity relationships and will allow for a better comprehension of their mode of action.

4. Experimental section

4.1. Biological material

The cyanobacterium, *A. torulosa*, was collected on SCUBA at a depth of 1-3 m in the Pacific Ocean in Moorea, French Polynesia (S 17°29'22'', W149°54'17''). The cyanobacterium sample was sealed underwater in a bag with seawater and then freeze-dried.

4.2. Extraction and isolation

Freeze-dried biomass of *A. torulosa* (600 g) was extracted at room temperature 3 times with a mixture of MeOH–CH_2Cl_2 (1:1) and an ultrasound was performed over 10 minutes. The evaporation of the combined extracts under reduced pressure led to a greenish organic extract (38 g) that was subjected to flash RP18 silica gel column eluted with H_2O (A), H_2O–CH_3CN (2:8) (B), MeOH (C) and MeOH–CH_2Cl_2 (8:2) (D) successively resulting in 4 fractions (A, B, C and D). Afterwards, fraction B was subjected to flash RP18 column eluted with a solvent gradient of H_2O–CH_3CN resulting in 12 fractions. Fraction 5 was subjected to reverse-phase HPLC purification (Interchim, UP-50 DB.25M Uptisphere, 250x10 mm, 5μm) using an isocratic elution with 68% H_2O–CH_3CN at a flow rate of 3 mL/min to give compounds **3** (4 mg, rt=22.7 min) and **1** (1.5 mg, rt=31.3 min). Fraction 8 led to compound **2** (5 mg, rt=22.4 min) with 58% H_2O-CH_3CN, while fraction 9 led to compounds **4** (6.5 mg, rt=24.7 min) and **5** (6.5 mg, rt=25.9 min).

*Acyclolaxaphycin A (**1**)*: white amorphous powder; $[\alpha]_D^{22}$ -0.80 (*c* 0.125, MeOH); UV (MeOH) λ_{max} 203 nm (ε 5000); HR-ESI-TOF-MS (+) *m/z* 1214.7380 $[M + H]^+$ (calcd for $C_{60}H_{100}N_{11}O_{15}$, 1214.7356); ^1H and ^{13}C NMR see Table 1.

*[des-Gly^{11}]acyclolaxaphycin A (**2**)*: white amorphous powder; $[\alpha]_D^{22}$ -10.60 (*c* 0.5, MeOH); UV (MeOH) λ_{max} 202 nm (ε 16200); HR-ESI-TOF-MS (+) *m/z* 1157.7231 $[M + H]^+$ (calcd for $C_{58}H_{97}N_{10}O_{14}$, 1157.7141); ^1H and ^{13}C NMR see Table 1.

*[des-(Leu^{10}-Gly^{11})]acyclolaxaphycin A (**3**)*: white amorphous powder; $[\alpha]_D^{22}$ -9.87 (*c* 0.375, MeOH); UV (MeOH) λ_{max} 203 nm (ε 6600); HR-ESI-TOF-MS (+) *m/z* 1044.6385 $[M + H]^+$ (calcd for $C_{52}H_{86}N_9O_{13}$, 1044.6300); ^1H and ^{13}C NMR see Table 1.

*[L-Val^8]laxaphycin A (**4**)*: white amorphous powder; $[\alpha]_D^{22}$ +2.40 (*c* 0.250, MeOH); UV (MeOH) λ_{max} 203 nm (ε 9300); HR-ESI-TOF-MS (+) *m/z* 1182.7095 $[M + H]^+$ (calcd for $C_{59}H_{96}N_{11}O_{14}$, 1182.7094); ^1H and ^{13}C NMR see Table 2.

*[D-Val^9]laxaphycin A (**5**)*: white amorphous powder; $[\alpha]_D^{22}$ +6.40 (*c* 0.125, MeOH); UV (MeOH) λ_{max} 202 nm (ε 16800); HR-ESI-TOF-MS (+) *m/z* 1182.7095 $[M + H]^+$ (calcd for $C_{59}H_{96}N_{11}O_{14}$, 1182.7094); ^1H and ^{13}C NMR see Table 2.

4.3.LC-MS and HPLC-ELSD analyses

LC-MS analyses were carried out using a Thermo Fisher Scientific LC-MS device, Accela HPLC coupled with a LCQ Fleet equipped with an electrospray ionisation source and a 3D ion-trap analyser. HPLC-ELSD analyses were performed with a Waters Alliance HPLC system coupled to an ELS detector. The analyses were performed on a reversed-phase column (ThermoHypersil Gold C-18, 150 x 2.1 mm, 3 µm) employing a gradient of 10% to 100% CH_3CN over 40 min followed by 25 min at 100% CH_3CN (all solvents buffered with 0.1% formic acid) with a flow rate of 0.3 mL/min.

4.4. Mass and NMR Spectroscopies and UV Spectrophotometry

High-resolution ESI mass spectra were obtained on a Bruker Qtof Impact II (compounds **1**, **4** and **5**) and on a Thermo Scientific LTQ Orbitrap mass spectrometer (compounds **2** and **3**) using electrospray ionisation in positive mode. 1D-NMR and 2D-NMR experiments were acquired on a Bruker Avance 500 spectrometer equipped with a cryogenic probe (5 mm), all compounds were dissolved in DMSO-*d*6 (500 µL) at 303 K. All chemical shifts were calibrated on the residual solvent peak (DMSO-*d*6, 2.50 ppm (^1H) and 39.5 ppm (^{13}C)). The chemical shifts, reported in delta (δ) units, and in parts per million (ppm) are referenced relatively to TMS. UV spectra were obtained on a Jasco V-630 spectrophotometer.

4.5. Advanced Marfey's analyses

The Marfey's analyses were carried out on compounds **2**, **3**, **4**, **5** and laxaphycin A. Approximately 0.3 mg of each compound were hydrolysed with 1 mL of 6 N HCl for 20 h at 110 °C in sealed glass vials. The cooled hydrolysate mixtures were evaporated to dryness and

traces of HCl were removed from the reaction mixtures by repeated evaporation. Each hydrolysate mixture was dissolved in H_2O (100 µL). 110 µL of acetone, 20 µL of 1 N $NaHCO_3$, and 20 µL of L-FDLA or DL-FDLA (1-fluoro-2,4-dinitrophenyl-5-L- or DL-leucinamide, 1% w/v in acetone) were added to each 50 µL aliquot. The mixtures were then heated to 40 °C for 1h. The cooled solutions were neutralised with 1 N HCl (20 µL), and then dried in vacuo. The residues were dissolved in 1:1 CH_3CN–H_2O and then analysed by LC-MS. LC-MS analyses were performed on a reversed-phase column (ThermoHypersil Gold C-18, 150 x 2.1 mm, 3 µm) with two linear gradients constituted with solvent A 0.01 M formic acid in water and solvent B CH_3CN: (1) from 20% B–80% A to 60% B–40% A at 0.3 mL/min over 70 min and (2) from 10% B–90% A to 50% B–50% A at 0.3 mL/min over 70 min, then to 80% B–20% A over 10 min. The configuration of the α carbon for each residue can be assigned in accordance with the elution order of the D- and L-FDLA derivatives:[22,23] aminoacids for which the D-FDLA analogue elutes first have a D configuration, while those for which the L-FDLA analogue elutes first have a L configuration. Detailed reports of retention times of each amino acid can be found in Supporting Information. Furthermore, the hydrolysates were compared to those of laxaphycin A.

4.6 Biological assays

4.6.1 Cell culture

Human neuroblastoma SH-SY5Y cell line was purchased from American Type Culture Collection (ATCC), number CRL2266. Cells were cultured in Dulbecco´s Modified Eagle´s medium: Nutrient Mix F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS), 1% glutamax, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were dissociated weekly using 0.05% trypsin/EDTA.

4.6.2 Chemicals and solutions

Plastic tissue-culture dishes and flasks were purchased from JetBiofil (Guangzhou, China). Fetal serum, trypsin and Dulbecco's Modified Eagle's medium (DMEM) were from Thermofisher scientific (Massachusetts, USA). All other chemicals were reagent grade and purchased from Sigma-Aldrich (Madrid, Spain).

4.6.3 Cytotoxicity assay

Cell viability was assessed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) test, as previously described[42,43] and by the lactate dehydrogenase (LDH) test. The assay was performed in cells grown in 96 well plates and exposed to different compound concentrations (0.01 µM, 0.05 µM, 0.1 µM and 1 µM) added to the culture medium. Cultures were maintained in the presence of the compounds at 37 °C in humidified 5% CO_2/95% air atmosphere for 24 or 6 h. For MTT test, Quillaja bark saponin (Sigma) was used as cellular death control and its absorbance was subtracted from the other data. After treatment, cells were rinsed and incubated for 1h with a solution of MTT (500 µg/mL) dissolved in saline buffer. After washing off excess MTT, cells were disaggregated

with 5% sodium dodecyl sulfate and the absorbance of the coloured formazan salt was measured at 595 nm in a spectrophotometer plate reader.

LDH was checked with the LDH-Cytotoxicity assay kit II (Abcam, UK), following the commercial protocol. This kit use LDH release as an indicator of cell survival.

4.6.4 Mitochondrial membrane potential measurement

For mitochondrial membrane potential ($\Delta\Psi m$) measurement, tetramethylrhodamine methyl ester (TMRM) assay was used.[44] Cells were washed twice with saline solution and incubated with 1 µM TMRM for 30 min. Then neuroblastoma cells were solubilized with 50% DMSO–50% water. Fluorescence intensity values were obtained using a spectrophotometer plate reader (535 nm excitation, 590 nm emission).

4.6.5 Determination of ROS production

ROS levels were measured with a fluorescence assay using 2'7'-dichlorofluorescein diacetate (DCFH-DA), as previously described.[45] Briefly, DCFH-DA enters the cell and is de-esterified to the ionized free acid (DCFH). ROS reacts with DCFH, forming the fluorescent 2',7'-dichlorofluorescein (DCF). Upon incubation with the compounds, cells were washed with saline solution and then loaded with 20 µM DCF-DA for 30 min at 37 °C. Cells were washed and kept at room temperature for 30 min to allow a complete de-esterification. DCF level was measured using a fluorescence plate reader where excitation was monitored at 475 nm and emission at 525 nm.

4.6.6 Flow cytometry analysis

The cell death induced by laxaphycins was determined with the Annexin V-FITC Apoptosis Detection Kit (Immunostep, Spain) following manufacturer´s instructions. $1x10^6$ SH-SY5Y cells per well were seeded in 6-well plates and incubated for 6h with laxaphycins. After treatment, cells were washed with PBS and resuspended in Annexin binding buffer containing Annexin V-FITC and Propidium Iodide. SH-SY5Y cells were incubated for 15 min and analysed by flow cytometry using the ImageStreamMKII (Amnis Corporation, Merck-Millipore). The fluorescence of 10000 events was analysed with IDEAS Application 6.0 software (Amnis Corporation, Merck-Millipore).

4.6.7 Western Blotting

Protein levels were analysed by western blot. SHSY5Y cells were incubated with compounds for 6h and afterwards they were washed twice with ice-cold PBS. An ice-cold hypotonic buffer solution composed of 20 mM Tris-HCl pH 7.4, 10 mM NaCl and 3 mM $MgCl_2$, and a 1% Triton X-100 containing a Roche complete phosphatase/protease inhibitors cocktail was added. Then cells were scrapped and finally centrifuged at 3000 rpm at 4 °C for 10 min to obtain the protein cytosolic fraction. Bradford assay was used to quantify total protein concentration. Cell lysates containing 20 µg were resolved in gel loading buffer and lysates were electrophoresed through a 10% sodium dodecyl sulfate polyacrylamide gel (BIORAD) and transferred onto PVDF membranes (Millipore). The membrane blocking and

antibody incubation was performed by Snap i.d protein detection system. The immunoreactive bands were detected using the Supersignal West Pico Chemiluminiscent Substrate or Supersignal West Femto Maximum Sensitivity Substrate (Pierce) and the Diversity 4 gel documentation and analysis system (Syngene, Cambridge, U.K.). Chemiluminiscence was measured with the Diversity Gene Snap software (Syngene). A list with the antibodies and concentrations used in this work can be found in supplemental information.

For protein measurements in membrane fraction, cells were washed with PBS and a hypotonic solution was added (20 mM Tris-HCl pH 7.4, 10 mM NaCl and 3 mM $MgCl_2$, plus phosphatase/protease inhibitors cocktail). Then, cells were incubated 15 min on ice and centrifuged at 3000 rpm, 4 °C for 15 min. The supernatant was saved as the cytosolic fraction and the pellet was resuspended in a nuclear extraction buffer (100 mM Tris pH 7.4, 2 mM Na_3VO_4, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM EGTA, 0.1% SDS, 1 mM NaF, 0.5% deoxycholate, and 20 mM $Na_4P_2O_7$, containing 1 mM PMSF and a protease inhibitor cocktail). Samples were incubated in ice for 30 min, vortexing in intervals of 10 min, and centrifuged at 14000 g, 4 °C for 30 min. The nuclear fraction was quantified with Bradford method.

4.6.8 Statistical analysis

All the results are expressed as means ± SEM of three or more experiments. A statistical comparison was performed with T-Student or Anova one-way with the Graph pad prism software. *P* values <0.05 were considered statistically significant.

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Author contributions: LB, EA, RA, NI, IB, LB and BB designed the research; LB ran chemical experiments; EA and RA performed the biological tests; LB, EA, RA, IB and BB analyzed the data; all authors wrote and revised the manuscript.

Supporting Information

Supplementary data (^{1}H NMR, ^{13}C, TOCSY, HSQC, HSQC-TOCSY, HMBC, and ROESY spectra of **1**,**2**,**3**,**4** and **5** and advanced Marfey's analysis of **2**,**3**,**4**, and**5**) associated available in Supporting Information (S1-S43).

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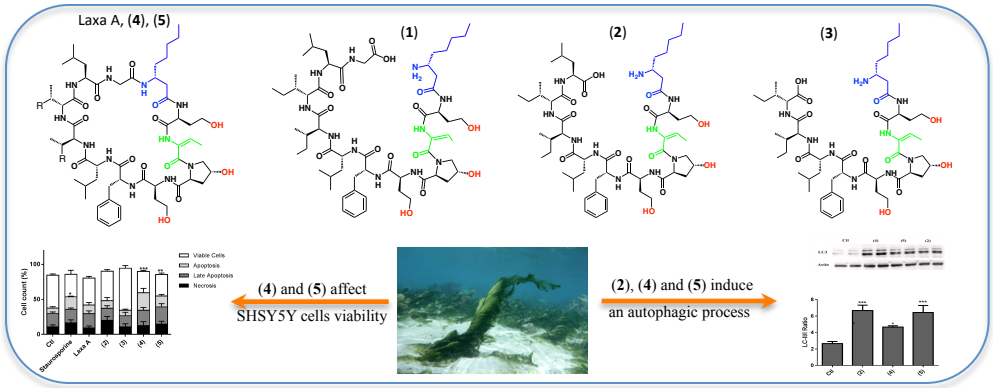
Laxa A, (4), (5)
(1)
(2)
(3)
Viable Cells
Apoptosis
Late Apoptosis
Necrosis
Cell count (%)
Ctl
Staurosporine
Laxa A
(2)
(3)
(4)
(5)
(4) and (5) affect
SHSY5Y cells viability
(2), (4) and (5) induce
an autophagic process
LC-III Ratio