Total Structure and Inhibition of Tumor Cell Proliferation of Laxaphycins

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From a mixed assemblage of *Lyngbya majuscula* rich marine cyanobacteria, we isolated a series of cell growth inhibitory cyclic peptides. The structures of the two major components, laxaphycins A (**1**) and B (**2**), and of two minor peptides, laxaphycins B2 (**3**) and B3 (**4**), were determined by spectroscopic methods and degradative analysis. Absolute configurations of natural and nonproteinogenic amino acids were determined by a combination of hydrolysis, synthesis of noncommercial residues, chemical derivatization, and HPLC analysis. The organism producing the laxaphycins was identified as the cyanobacterium *Anabaena torulosa*. The antiproliferative activity of laxaphycins was investigated on a panel of solid and lymphoblastic cancer cells. Our results demonstrate that in contrast to laxaphycin A, laxaphycin B inhibits the proliferation of sensitive and resistant human cancer cell lines and that this activity is strongly increased in the presence of laxaphycin A. This effect appears to be due to an unusual biological synergism.

Introduction

Cyanobacteria (blue-green algae) have been identified as one of the most promising groups of organisms for the discovery of novel, biologically active natural products. Members of the orders Oscillatoriales, mainly *Lyngbya majuscula*, and Nostocales have proven to be the most prolific sources.[1] Cyanobacteria produce many active cyclic peptides and depsipeptides. Most contain unusual amino acid residues. However, those with cycles of 10 to 14 amino acids are not so frequent. Nevertheless, some (about fifteen) have been characterized among more than two hundred peptides found in marine cyanobacteria. Peptides with a 10-residue ring have been found in *Calothrix fusca* (calophycin),[2] *Anabaena* sp. (puwainaphycin E),[3,4] and in different species of *Nostoc* and *Oscillatoria* (microviridins).[5] Kawagushipeptin A[6] and B[7] (*Microcystis aeruginosa*, *Anabaena laxa*, *Hormothamnion enteromorphoides*), scytonemin A[8] (*Scytonema* sp.), and schizothrin A[9] (*Schizotrix* sp.) have 11 amino acids cycles. A dodeca cyclic peptide, wewakazole,[10] has been isolated from *Lyngbya majuscula*. Tolybyssidins A and B[11] (*Tolypothrix byssoidea*), and malevamide B[12] (*Symploca laetaviridis*) have a 13-residues cycle, and malevamide C (*Symploca laetaviridis*) is a 14-residue cyclic peptide. All these peptides contain several structural features that are common to many cyanobacterial peptides, including *N*-methylation, *β*-amino acids, and α-hydroxyacids.

In 1992, Frankmölle et al.[13,14] described peptide sequences[15] of laxaphycins A and B isolated from the freshwater cyanobacterium *Anabaena laxa*. The same year, Gerwick et al.[16,17] isolated and entirely described hormothamnin A, a (*Z*)-α,*β*-didehydro-α-aminobutyric acid [*Z*-Dhb] analog of laxaphycin A from the marine cyanobacteria *Hormothamnion enteromorphoides*. In 1997, our group published preliminary results[18] on the total structure elucidation of laxaphycins A and B isolated from a *Lyngbya majuscula* rich assemblage collected in French Polynesia. More recently, MacMillan et al.[19] described the structure of lobocyclamides A−C from a cryptic cyanobacterial mat containing *Lyngbya confervoides*. These lipopeptides are closely related to laxaphycins. It seems[20] that lobocyclamide A is the [L-Ser[2], D-Tyr[6], L-*allo*-Ile[9]]laxaphycin A analog and lobocyclamide B, [(2*R*,3*S*)-3-OHLeu[3], (2*R*,3*R*)-3-OHThr[8], (2*S*,3*R*)-3-OHPro[10]]laxaphycin B.

In this study, we report the complete structure of laxaphycins A (**1**) and B (**2**), and of two minor peptides related to laxaphycin B, laxaphycin B2 (**3**), and laxaphycin B3 (**4**; Figure 1), as determined by means of a combination of mass spectrometry, NMR spectroscopy, and degradative analysis. Furthermore, we demonstrated that the source of laxaphycins was the marine cyanobacterium *Anabaena torulosa*.

The antiproliferative activity of the two major compounds, laxaphycins A and B, was investigated on a human metastatic malignant melanoma line (M4Beu), which is highly resistant to usual pro-apoptotic drugs, on five human solid cancer cell lines representative of the most common cancers in Western countries [nonsmall-cell lung carcinoma (A549), breast carcinoma (MCF7), ovarian carcinoma (PA1), prostatic carcinoma (PC3), and colon carcinoma (DLD1)], on human normal fibroblasts used as human normal cells and, on L-929 murine immortalized cells. This latter cell line is commonly used in screening programs designed to evaluate the cytotoxicity of chemicals. The antiproliferative and cytotoxic activity of all isolated compounds was also tested on a drug-sensitive human leukemic cell line (CCRF-CEM) and related sublines that express drug resistance associated with overexpression of Pgp (CEM/VLB$_{100}$) or with alteration of DNA topoisomerase II (CEM/VM-1). Furthermore, it was assessed that laxaphycin A at unactive concentration may potentiate the anticancer effect of laxaphycin B. In brief, our results demonstrate that laxaphycin B inhibits the proliferation of sensitive and resistant human cancer cell lines at the micromolar level and that this property is strongly enhanced in the presence of laxaphycin A.

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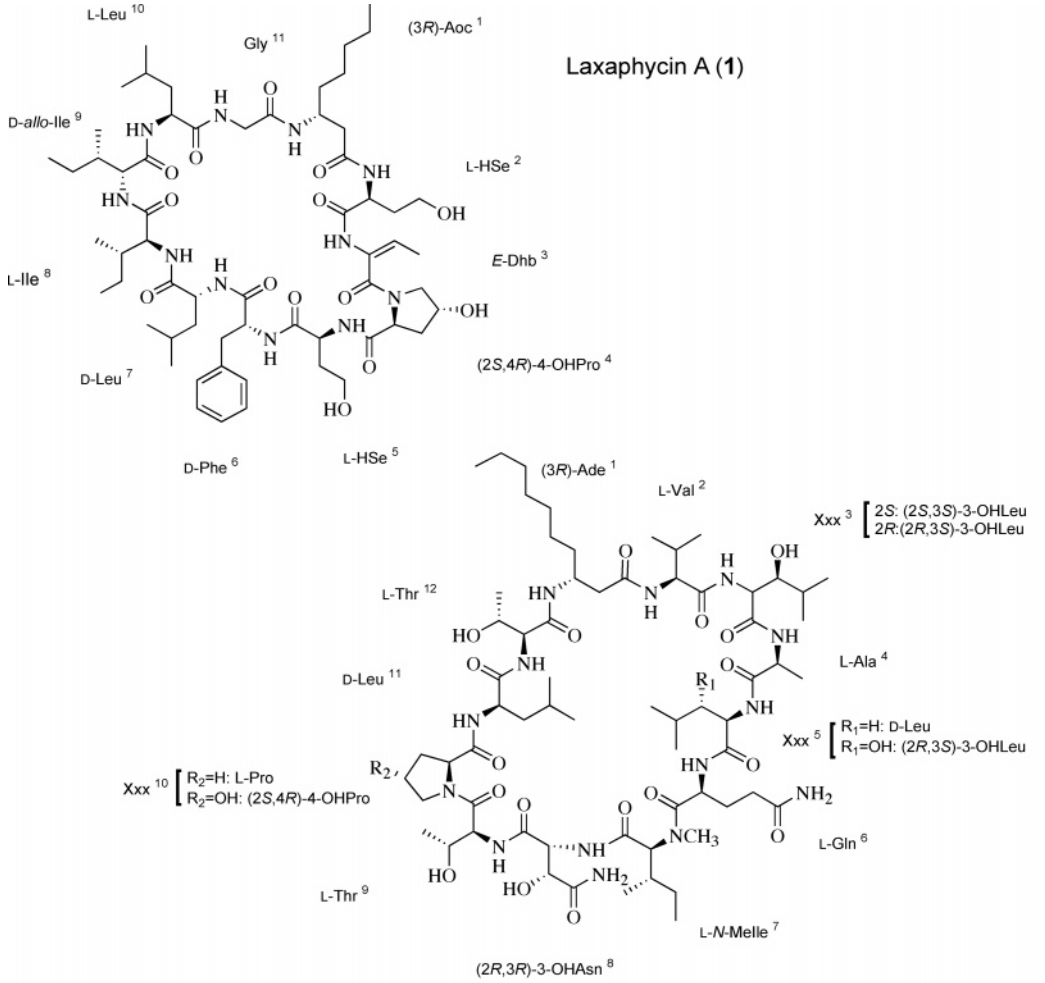

Laxaphycin B (**2**)	(2*S*,3*S*)-3-OH Leu [3], (2*R*,3*S*)-3-OH Leu [5], L-Pro [10]
Laxaphycin B2 (**3**)	(2*R*,3*S*)-3-OH Leu [3], D-Leu [5], L-Pro [10]
Laxaphycin B3 (**4**)	(2*S*,3*S*)-3-OH Leu [3], (2*R*,3*S*)-3-OH Leu [5], (2*S*,4*R*)-4-OHPro [10]

Figure 1. Structure of the lipopeptides isolated from the marine cyanobacterium *Anabaena torulosa*.

Structure of Laxaphycins

Isolation of Laxaphycins. In a preceding study, laxaphycins A and B were isolated and described from an assemblage of 80% *Lyngbya majuscula* together with two other cyanobacterial species: *Anabaena* sp. and *Oscillatoria* sp.[18] The high rate of *L. majuscula* in this assemblage together with the relative high concentration of laxaphycins purified (>0.4% dry weight of the assemblage) suggested that *L. majuscula* was the source of the metabolites. But subsequent collections of *L. majuscula* from the same site over a period of 10 years did not show the laxaphycins, whereas we could reisolate laxaphycins from *Anabaena torulosa* collected in the same place at snorkeling depth.

The mixed cyanobacterial assemblage (ww 1.3 kg), collected as floating wreckage at Moorea atoll (French Polynesia) in October, 1993, was preserved in ethanol. The ethanol extract of cyanobacteria was concentrated and partitioned between a series of solvents of increasing polarity. Repeated silica gel chromatography of the dichloromethanic-soluble fraction, followed by reversed-phase HPLC, yielded four peptides: two major peptides, laxaphycin A and laxaphycin B, and two minor laxaphycin B-type peptides, laxaphycin B2 and laxaphycin B3. Laxaphycins were obtained as colorless amorphous solids and were negative to the ninhydrin test, suggesting a blocked N-terminus.

Molecular ions obtained in FAB-MS and spectral data of ^{1}H and ^{13}C 1D NMR showed immediately that the two major peptides isolated from the marine cyanobacterial assemblage were similar to laxaphycins A and B isolated from the fresh water cyanobacterium *Anabaena laxa*, by Frankmölle et al.[13,14] They described peptide sequences but did not establish amino acid stereochemistry. From the marine cyanobacteria *Hormothamnion enteromorphoides*, Gerwick et al.[16,17] isolated and entirely described hormothamnin A, a (*Z*)-α,*β*-didehydro-α-aminobutyric acid [(*Z*)-Dhb] analog of laxaphycin A.

Laxaphycin A: Structure Elucidation. Almost all the abundant positive ions in the FAB MS/MS spectrum could be assigned to fragments that resulted directly from the cleavage of the peptide backbone. Nomenclature[21] and mechanisms of formation[22] for the peptide fragment ions observed had been already discussed. A pattern of cyclic peptide fragmentation was established[23] that was described by the initial protonation of an amide nitrogen, scission of the *N*-acyl bond, and subsequent fragmentation by loss of amino acid residues (successively or competitively) from the ring-opened acylium ion. The parent

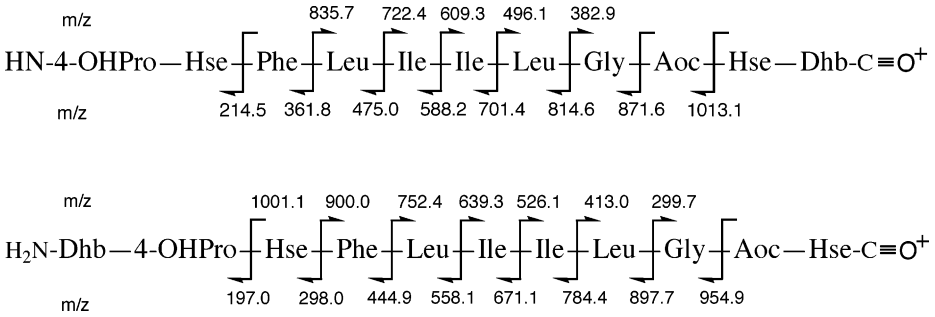

Figure 2. FAB MS/MS fragmentation of laxaphycin A.

ion at *m*/*z* $[M + H]^+$ 1196.4, one of the most prominent ions in the spectrum, was consistent with the formula $C_{60}H_{97}N_{11}O_{14}$. In the higher mass region, ions resulting from the losses of H_2O and CO (characteristic of a cyclic peptide) were observed in very weak abundance (<2%).

Owing to the increased basicity of amide nitrogen atoms of the *N*-alkylated 4-hydroxyproline (4-OHPro[4]) and of the α,*β*-insatured α,*β*-didehydro-α-aminobutyric acid (Dhb[3]) in laxaphycin A, protonation and cleavage of these amide bonds were a highly favored process. Thus, protonation on the peptide nitrogen of 4-OHPro[4] and Dhb[3] was followed by ring cleavage to form the two linear acylium ions (Figure 2) with Dhb[3] and 4-OHPro[4] at the N-terminus. These acylium ions underwent C-terminus fragmentation and N-terminus fragmentation. We could notice the presence of the *m*/*z* 970.6 ion resulting in the α,*β*-bond cleavage of the *β*-aminooctanoic acid (Aoc).[24] Wn and Dn ion-type were not observed for Leu and Ile residues. FAB mass spectrum of acetylated laxaphycin A showed a *m*/*z* $[M + H]^+$ 1322.5 pseudomolecular ion, indicating that laxaphycin A possesses three esterifiable functionalities.

The ^{1}H NMR spectra of laxaphycin A were taken at 400 MHz in DMSO-d_6. It was obvious from these well-resolved spectra that one conformation strongly predominated in this solvent. The assignment of almost all ^{1}H resonances (Table 1) was based on connectivity information transmitted via DQF ^{1}H−^{1}H COSY and HOHAHA spectra. In the ^{13}C NMR spectrum, 60 carbon resonances were apparent. The multiplicity of each carbon resonance was determined by means of the usual DEPT technique. Assignment of the ^{13}C NMR resonances (Table 1) was performed by using HMQC and HMBC experiments. Detailed interpretation of the different 1D and 2D NMR spectra confirmed the presence of 11 residues and established laxaphycin A as cyclo[Aoc−Hse−Dhb−4-OHPro−Hse−Phe−Leu−Ile−Ile−Leu−Gly−]. Information concerning the assignment of the two sets of ^{1}H and ^{13}C signals for homoserine (Hse), Leu, and Ile residues to Hse[2], Hse[5], Leu[7], Ile[8], Ile[9], or Leu[10] was obtained by HOHAHA and ROESY experiments. Strong NOE between the NH and H*β* in the Dhb unit indicated that the geometry of the double bond was *E*. Comparison of C*β*Dhb chemical shift for our peptide (119.0 ppm in DMSO-d_6, 298 K), Frankmölle's [(*E*)-Dhb]-laxaphycin A (119.0 ppm in DMSO-d_6, 308 K), and Gerwick's [(*Z*)-Dhb]-hormothamnin A (121.7 ppm in DMSO-d_6, 298 K) confirmed the geometry of this residue.

Hydrolysis of laxaphycin A followed by Marfey's derivatization[25] and HPLC analysis assigned 4-OHPro as (2*S*,4*R*) and both Hse, one Leu, and one Ile (or *allo*-Ile) as L. Phe, one Leu, and one Ile (or *allo*-Ile) were found to be D. The *β*-amino acid Aoc was determined as 3*R*. *β*-aminooctanoic acid (and *β*-aminodecanoic acid, Ade, in laxaphycin B) residues were synthesized according to Gerwick's procedure.[17] We can notice that elution of DAA-*trans*-4-hydroxyproline constitutes an exception to Marfey's rule because D-*trans*-4-hydroxyproline [(2*R*,4*S*)-4-OHPro] is eluted before L-*trans*-4-hydroxyproline [(2*S*,4*R*)-4-OHPro]. A contrario, L-*cis*-4-hydroxyproline [(2*S*,4*S*)-4-OHPro] is eluted before D-*cis*-4-hydroxyproline [(2*R*,4*R*)-4-OHPro]. The configuration of the C*β*Ile (D-Ile or D-*allo*-Ile) could not be determined according to Marfey's procedure. This was resolved by amino acids OPA derivatization.[26,27] This experiment showed the presence of one Ile (D or L) and one *allo*-Ile (D or L). The chirality of laxaphycin A amino acid units was identical to hormothamnin A. Information concerning the assignment of the two sets of ^{1}H and ^{13}C signals for the Leu residues to Leu[7] or Leu[10] and that concerning the two sets of Ile residues to Ile[8] or Ile[9] was obtained by HOHAHA and ROESY experiments, with the cross-peaks between HαPhe[6]/HNLeu[7], HαLeu[7]/HNIle[8], HαIle[8]/HNIle[9], and HαIle[9]/HNLeu[10]. Sequential assignment of laxaphycin A, determined by interpretation of ROESY and HMBC data, was found to be identical to hormothamnin A. According to Gerwick's results[17] and to the similarity of chemical shifts between laxaphycin A and hormothamnin A ($\Delta\delta H < 0.2$ ppm and $\Delta\delta C < 1$ ppm except for C*β*Dhb), the two peptides seemed to have the same stereochemistry, except for the Dhb unit. Laxaphycin A was determined to be cyclo-[(3*R*)-Aoc−L-Hse−(*E*)-Dhb−(2*S*,4*R*)-4-OHPro−L-Hse−D-Phe−D-Leu−L-Ile−D-*allo*-Ile−L-Leu−Gly-].

Laxaphycin B: Structure Elucidation. The FAB mass spectrum recorded on laxaphycin B showed a $[M + H]^+$ pseudomolecular ion at *m*/*z* 1395.6, consistent with the formula $C_{65}H_{114}N_{14}O_{19}$. A $[M + H]^+$ pseudomolecular ion at *m*/*z* 1605.5, when recorded on acetylated laxaphycin B, allowed us to suppose the presence of five esterifiable positions. Protonation on the amide nitrogen of Pro[10], *N*-methylisoleucine (*N*-MeIle[7]), and 3-hydroxyasparagine (3-OHAsn[8]) generated amide bond cleavage to form three linear acylium ions and produced series of C- and N-terminus fragmentation (Figure 3). This FAB MS/MS study established the amino acid sequence as cyclo[Ade−Val−3-OHLeu−Ala−3-OHLeu−Gln−*N*-MeIle−3-OHAsn−Thr−Pro−Leu−Thr].

The ^{1}H NMR spectra of laxaphycin B were taken at 400 MHz in DMSO-d_6. One set of resonances was observed for each of the residues, indicating that one conformation dominates in this solvent. Some minor peaks, especially in the *N*-methyl and amide proton regions, were apparent, indicating the presence of another conformation in slow exchange. The assignment procedures were very similar to those described for laxaphycin A. The twelve amino acid units were characterized by detailed interpretation of ^{1}H and ^{13}C 2D NMR spectra (DQF ^{1}H−^{1}H COSY, HOHAHA, and HMQC). The peptidic sequence was also supported by deductions from HMBC and NOESY NMR experiments. Information concerning the assignment of the two sets of ^{1}H and ^{13}C signals of Thr and 3-OHLeu residues to Thr[9],

Table 1. ^{1}H and ^{13}C NMR Data of Laxaphycin A (318 K) and Hormothamnin A (298 K) in DMSO-d_6

		laxaphycin A			hormothamnin A[17]	
		^{13}C	^{1}H		^{13}C	^{1}H
		δ (ppm)	δ (ppm)	J (Hz)	δ (ppm)	δ (ppm)
β Aoc[1]	NH		6.82	8.4		6.88
	CαH$_2$	39.92	1.69/1.97		39.90	1.68/1.90
	CβH	44.86	4.27	8.4	44.80	4.28
	CγH$_2$	34.76	1.34	9.5	35.10	1.35
	CδH$_2$	28.76	1.23	9.5	30.80	1.25
	CϵH$_2$	24.98[a]	1.23	9.5		
	CζH$_2$	30.72	1.23	9.5		
	CηH$_3$	13.68	0.84	7.5	13.90	0.89
	CO	169.06				
Hse[2]	NH		7.10	7.1		7.09
	CαH	49.06	4.54	7.1 8.6	49.00	4.70
	CβH$_2$	33.78	1.76	8.6 8.4	33.40	1.80
	CγH$_2$	56.97	3.46	8.4	56.90	3.55
	OH		4.42			4.59
	CO	172.89				
Dhb[3]	NH		10.75			10.69
	CαH	130.79			131.80	
	CβH	118.34	5.57	7.3	121.70	5.76
	CγH$_3$	11.95	1.69	7.3	12.50	1.75
	CO	167.25			166.70	
4-OHPro[4]	CαH	59.06	4.51	9.0, 5.0	59.60	4.48
	CβH$_2$	37.84	1.92/2.27	9.0, 10.2/5.0, 10.2	38.00	1.83/2.25
	CγH	67.90	4.28	9.0, 5.0, 3.6	68.40	4.30
	OH		5.03			5.19
	CδH$_2$	56.97	3.34/3.59	5.0, 11.4/3.6, 11.4	57.60	3.32/3.61
	CO	170.09				
Hse[5]	NH		7.22	6.9		7.04
	CαH	48.90	4.27	6.9 8.7	48.70	4.31
	CβH$_2$	33.78	1.88/1.96	8.7, 8.7, 10.2/7.4, 4.4, 10.2	33.90	1.91/2.01
	CγH$_2$	56.97	3.31/3.45		56.90	3.27/3.42
	OH					4.39
	CO	171.97				
Phe[6]	NH		7.79	8.2		7.65
	CαH	56.05	4.28	8.2, 4.4, 10.8	56.80	4.23
	CβH$_2$	36.99	2.94/3.04	4.4, 13.6/10.8, 13.6	37.00	2.96/3.04
	Cγ	137.82			138.00	
	CδH$_2$	126.11	7.34	8.5	129.10	7.21
	CϵH$_2$	127.95	7.24	8.5 7.4	128.20	7.26
	CζH	128.95	7.18	7.4	126.30	7.39
	CO	171.86				
Leu[7]	NH		7.22	10.5		7.22
	CαH	51.55	4.28		51.40	4.32
	CβH$_2$	42.24	1.18/1.34		39.50	1.03/1.28
	CγH	23.94	1.58		24.70	1.57
	CδH$_3$	22.70	0.80	7.0	21.10	0.75
	Cδ'H$_3$	20.31	0.73	6.6	22.80	0.81
	CO	171.54				
Ile[8]	NH		6.61			6.49
	CαH	55.95	4.63	9.2, 4.0	53.60	4.76
	CβH	38.40	1.76		37.10	1.80
	CγH$_2$	21.92[a]	1.18		26.30	1.24/1.26
	Cγ'H$_3$	15.25	0.76	6.7	14.50	0.75
	CδH$_3$	11.32	0.75	7.4	11.10	0.75
	CO	172.18				
Ile[9]	NH		8.68	9.2		8.44
	CαH	53.85	4.63	9.2, 4.0	53.60	4.68
	CβH	36.73	1.97		37.10	2.07
	CγH$_2$	26.08[a]	1.18		26.30	1.12/1.19
	Cγ'H$_3$	14.34	0.80	7.0	14.50	0.81
	CδH$_3$	11.04	0.84	7.5	11.10	0.84
	CO	172.35				
Leu[10]	NH		8.34	5.70		8.49
	CαH	52.59	4.03	5.70, 4.40	53.10	4.02
	CβH$_2$	42.24	1.58/1.59		39.20	1.36/1.56
	CγH	23.94	1.56		24.10	1.63
	CδH$_3$	21.24	0.83	6.8	21.40	0.85
	Cδ'H$_3$	22.53	0.89	6.5	22.60	0.92
	CO	172.69				
Gly[11]	NH		8.56			8.84
	CαH$_2$	42.24	3.81/3.22	8.1, 16.8/4.8, 16.8	42.30	3.26/3.81
	CO	166.77				

[a] Chemical shifts may be interchanged.

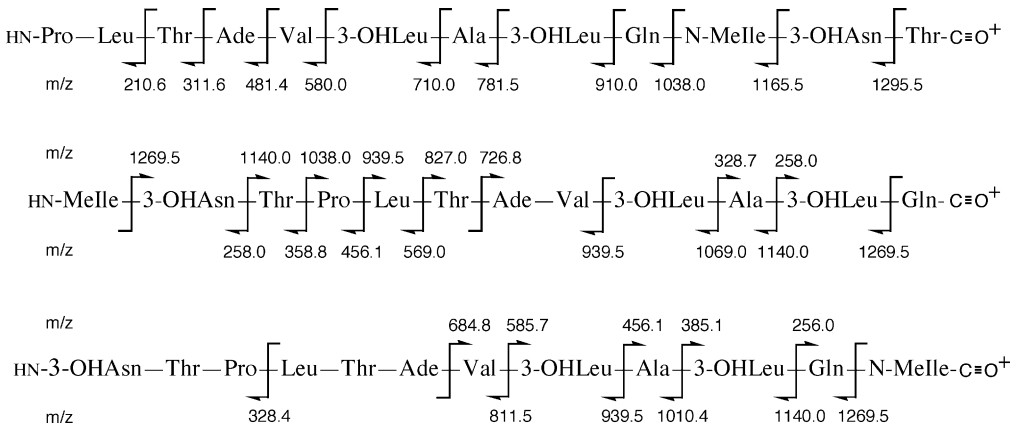

Figure 3. FAB MS/MS fragmentation of laxaphycin B.

Thr[12], 3-OHLeu[3], or 3-OHLeu[5] was obtained by ROESY experiments (Table 2).

As for laxaphycin A, hydrolysis of laxaphycin B, followed by Marfey's derivatization and HPLC analysis assigned Val, Ala, Gln, Pro, and the two Thr units as L and Leu as D. The β-amino acid Ade, synthesized according to Gerwick's procedure,[17] was determined as 3*R*. Noncommercial residues were synthesized to perform HPLC analysis. D- and D-*allo*-isoleucines were *N*-methylated according to the procedure of Freidinger et al.[28] The four isomers of 3-hydroxyleucine were synthesized via heterocyclic intermediates [cyclo(L-Val-Gly) and cyclo(D-Val-Gly)] following Schöllkopf's method.[29−33] Absolute configuration of 3-hydroxyleucines was determined to be (2*R*,3*S*) and (2*S*,3*S*) and *N*-methylisoleucine was determined to be (2*S*,3*S*).

The relative and absolute stereochemistry of 3-hydroxyasparagine was deduced by a combination of spectral and chemical studies. In spectral studies, a coupling constant $J_{\alpha b}$ of 2.5 Hz defined the 3-hydroxyasparagine in a preferential rotamer. The strong intraresidue NOEs, $H_2N/H\alpha$ and $H_2N/H\beta$, observed in the ROESY spectrum of laxaphycin B and inter-residue NOEs between 3-hydroxyasparagine and threonine (medium HNThr/Hα3-OHAsn and HαThr/Hβ3-OHAsn, weak OHThr/Hα3-OHAsn] eliminated erythro configurations (Figure 4).

For chemical studies, native 3-OHAsn from laxaphycin B hydrolysis was collected after a chromatographic separation on a cation exchange column and characterizated by ^{1}H NMR and circular dichroism. 3-Hydroxyaspartic acid was found threo by ^{1}H NMR comparison with standard L/D-*threo*-3-hydroxyaspartic acids, and a measurement of CD of this amino acid indicated D configuration (see experimental). Coinjection of native 3-OHAsp and standard L/D-*threo*-3-hydroxyaspartic acids, according to Marfey's procedure, indicates that the native (2*R*,3*R*)-3-OHAsp coelutes with the first threo isomer. So 3-OHAsp elution is another exception to Marfey's rule because the D isomer is eluted before the L isomer.

The presence of two 3-OHLeu with different configurations introduced another question: the localization of the two residues in the peptide sequence. This localization was deduced as follows: partial hydrolysis of laxaphycin B at 120 °C with a 10% solution of TFA in water and for 30, 40, and 45 min showed a progressive liberation of amino acid units (identificated by Marfey's procedure). At 30 min, Leu, 3-OHAsp, and *N*-MeIle appeared in the chromatogram pattern. Then at 40 min, Glu, (2*R*,3*S*)-3-OHLeu, and Ala were liberated. Finally, at 45 min, (2*S*,3*S*)-3-OHLeu and Val appeared. This experiment lead us to localize (2*R*,3*S*)-3-OHLeu between Gln and Ala and (2*S*,3*S*)-3-OHLeu between Ala and Val. Finally, structure of laxaphycin B was determined as cyclo[(3*R*)-Ade−L-Val−(2*S*,3*S*)-3-OHLeu−L-Ala−(2*R*,3*S*)-3-OHLeu−L-Gln−L-*N*-MeIle−(2*R*,3*R*)-3-OHAsn−L-Thr−L-Pro−D-Leu−L-Thr-].

Laxaphycin B2: Structure Elucidation. Preliminary spectral data examination, including FAB MS/MS, ^{1}H, and ^{13}C NMR spectroscopy, showed that the new metabolite was a lower homologue of laxaphycin B. The molecular weight of laxaphycin B2 (**3**) at *m*/*z* 1378.5 $[M + H]^+$ agreed with a protonated molecular formula of $C_{65}H_{114}N_{14}O_{18}$, which was 16 amu smaller due to the loss of a hydroxyl group in the 3-OHLeu[5] residue. Comparison of the FAB MS/MS spectrum with those obtained for laxaphycin B showed the same fragmentation pattern with the formation of the three linear acylium ions (Figure 5). The N-terminus fragmentation of the acylium ion with 3-OHAsn[8] at the N-terminus led to an acylium ions series (*m*/*z* 569.5, 440.2, 368.8) shifted to a lower mass by 16 amu, in comparison to that of laxaphycin B, just before the *m*/*z* 256 (Gln-*N*-MeIle), suggesting that the variable residue could be 3-OHLeu[5].

The 1D and 2D NMR spectra were complex due to the presence of a mixture of conformers in slow exchange. But the absence of the Hβ3-OHLeu[5] resonance and the presence of two methyl resonances (Cδ at 22.7 and 21.2 ppm) and new signals at 1.52 and 1.58 ppm, corresponding to HβLeu, were easily detected (Table 2). Information concerning assignment of the two sets of ^{1}H and ^{13}C Leu signals to Leu[5] and Leu[11] was obtained by HOHAHA and ROESY experiments. Cross-peaks in the ROESY spectrum between HαVal[2]/HN3-OHLeu[3], HαLeu[5]/HNGln[6], HαLeu[5]/HαGln[6], HαPro[10]/HNLeu[11], and HαLeu[11]/HNThr[12] linked the Leu and Ile residues leading to the sequence cyclo[Ade−Val−3-OHLeu−Ala−Leu−Gln−*N*-MeIle−3-OHAsn−Thr−Pro−Leu−Thr]. Laxaphycin B2 gross structure differs from laxaphycin B by replacement of 3-OHLeu[5] with Leu[5] residue.

Hydrolysis of laxaphycin B2 followed by Marfey's derivatization and HPLC analysis assigned Val, Ala, Gln, *N*-MeIle, Pro, and the two Thr units as L, Ade as 3*R*, 3-OHLeu as (2*R*,3*S*), and the two Leu units as D. Finally, laxaphycin B2 differs from laxaphycin B with respect not only to the substitution of (2*R*,3*S*)-3-OHLeu[5] by D-Leu[5] but also to the inversion of the configuration of the Cα3-OHLeu[3] residue. The structure of laxaphycin B2 was determined as cyclo[(3*R*)-Ade−L-Val−(2*R*,3*S*)-3-OHLeu−L-Ala−D-Leu−L-Gln−L-*N*-MeIle−(2*R*,3*R*)-3-OHAsn−L-Thr−L-Pro−D-Leu−L-Thr-].

Laxaphycin B3: Structure Elucidation. FABMS data established the formula for laxaphycin B3, $C_{65}H_{114}N_{14}O_{20}$, as a higher homologue of laxaphycin B. A similar pattern of fragmentation for both laxaphycin B and B3 was observed on the FAB MS/MS spectrum (Figure 6). The N-terminus frag-

Table 2. ^{1}H and ^{13}C NMR Data of Laxaphycins B, B3 (308 K), and B2 (318 K) in DMSO-d_6

		laxaphycin B			laxaphycin B2			laxaphycin B3		
		^{13}C	^{1}H		^{13}C	^{1}H		^{13}C	^{1}H	
		δ (ppm)	δ (ppm)	J (Hz)	δ (ppm)	δ (ppm)	J (Hz)	δ (ppm)	δ (ppm)	J (Hz)
β-Ade[1]	NH		7.58	8.8		7.58			7.52	9.2
	CαH$_2$	40.28	2.40/2.33	8.8, 14.9/4.0, 14.9		2.43/2.33			2.44/2.30	
	CβH	45.93	4.11	8.8 4.0	46.08	4.08	11.2, 6.0	45.92	4.08	7.5, 3.3
	CγH$_2$	33.45	1.40/1.29			1.54/1.50		33.41	1.40	
	CδH$_2$	28.67[a]	1.24		28.64[a]	1.50/1.38		28.69[a]	1.24	
	CϵH$_2$	28.47[a]	1.20		28.40[a]	1.29	7.1	28.47[a]	1.20	
	CζH$_2$	25.18[a]	1.20			1.29	6.6	25.22[a]	1.20	
	CηH$_2$	31.11[a]	1.20		31.03[a]	1.29	7.1	31.10[a]	1.20	
	CθH$_2$	21.92[a]	1.20		21.83[a]	1.34		21.92[a]	1.20	
	CιH$_3$	13.79	0.84	7.3	13.68	0.92	7.2	13.79	0.82	6.8
	CO	171.14			171.70			171.30		
Val[2]	NH		8.18	7.0		8.11	7.3		8.10	7.6
	CαH	59.03	4.09	7.0	59.20	4.04		58.89	4.12	7.0
	CβH$_2$	29.33	1.97		29.37	2.10		29.37	1.98	
	CγH$_3$	18.80	0.91	6.7	18.67	1.00	6.7	18.56	0.88	7.2
	Cγ'H$_3$	18.87	0.85		18.45	0.95		18.85	0.84	7.2
	CO	171.05			172.18[a]			171.30		
3-OHLeu[3]	NH		7.94	9.1		7.98			7.90	8.4
	CαH	55.23	4.34	9.1 2.0	55.37	4.33	10.3	55.15	4.37	8.4
	CβH	76.37	3.49		76.42	3.51	8.3, 2.4	76.48	3.50	
	OH		4.94	4.4		4.71			4.90	7.2
	CγH	30.54	1.58	14.7, 6.8	30.01	1.70		30.57	1.60	
	CδH$_3$	19.22[a]	0.89	6.8	18.67	1.02	6.7	18.76[a]	0.89	6.8
	Cδ'H$_3$	18.56	0.76	6.8	18.98[a]	0.88	6.7	18.43	0.76	6.8
	CO	171.35			170.94					
Ala[4]	NH		7.86	6.0		7.80			7.87	
	CαH	49.28	4.22	6.0, 7.6	48.79	4.22		49.30	4.22	6.4
	CβH$_3$	17.55	1.31	7.6	17.55	1.33	7.0	17.65	1.32	7.6
	CO	172.33			171.61[a]			172.47		
OHLeu[5]/ Leu[5]	NH		7.69	8.8		7.77	6.5		7.61	8.8
	CαH	55.52	4.28	8.8, 2.9	51.50	4.17	6.5 7.5	55.64	4.28	8.8 2.0
	CβH	75.80	3.49		40.20	1.58/1.52		75.78	3.48	
	OH		5.03	5.4					5.05	5.6
	CγH	29.90	1.56	14.7, 6.8	23.99	1.67		29.84	1.58	14.7, 6.8
	CδH$_3$	18.65[a]	0.89	6.8	22.67	0.95		18.69[a]	0.88	7.2
	Cδ'H$_3$	18.56	0.76	6.8	21.21	0.92	6.7		0.74	7.2
	CO	170.50			171.79[a]			170.60		
Gln[6]	NH		7.77	7.4		7.81			7.56	4.4
	CαH	49.16	4.63	9.2, 4.0	48.79	4.58		49.40	4.58	
	CβH$_2$	26.39	1.97/1.75	14.8, 7.0/-		2.05/1.84			2.00/1.64	
	CγH$_2$	30.72	2.10/2.04	14.8, 8.2/-	30.45	2.21			2.23/2.15	
	CON	174.60			174.56			174.74		
	NH$_2$		7.22/6.85			7.16/6.74			7.17/6.79	
	CO	172.49			171.54[a]			172.64		
N-MeIle[7]	NCH$_3$	30.03	2.97		29.68	2.93		30.15	3.01	
	CαH	59.85	4.72	11.0	59.93	4.72	10.8	59.87	4.73	11.2
	CβH	31.56	1.90			2.02		31.80	1.90	
	CγH$_2$	23.88	1.29/0.89		23.90	-/0.87	6.7		1.27/0.74	
	Cγ'H$_3$	15.08	0.76		15.14			14.99	0.74	6.4
	CδH$_3$	10.33	0.78	7.4		0.99	7.5	10.31	0.75	6.4
	CO	170.02			170.00			170.10		
3-OHAsn[8]	NH		7.64	8.5		7.45	8.7		7.66	
	CαH	55.52	4.63	8.5, 2.5	55.37	4.64	8.7 2.2	55.53	4.63	8.0, 2.0
	CβH	70.44	4.31	2.5, 6.0	70.39	4.36	7.1	70.33	4.35	
	OH		5.79	6.0		5.78	7.1		5.70	6.0
	CONH$_2$	173.37			173.26			173.37		
	NH$_2$		7.27			7.22			7.17	
	CO	169.16			169.11			169.12		
Thr[9]	NH		7.33	8.0		7.34	7.3		7.12	8.0
	CαH	55.61	4.49	8.0, 6.0	55.39	4.49		55.83	4.46	
	CβH	66.23	3.93		66.15	3.95		66.43	3.90	
	OH		4.94	4.4		4.84	9.6		4.89	5.2
	CγH$_3$	18.87	1.05	6.4	18.67	1.14	6.5	18.85[a]	1.03	6.4
	CO	168.58			168.67			168.70[a]		
Pro[10]/ OHPro[10]	CαH	59.60	4.33	4.0	59.69	4.35		58.62	4.43	8.4
	CβH$_2$	29.08	2.04/1.82		28.64	2.15/1.92		37.73	2.01/1.84	
	CγH$_2$	24.0	1.90/1.80	8.3/-		2.02		68.50	4.32/5.08 OH	-/4.0
	CδH$_2$	47.16	3.68	3.9	47.14	3.66		55.48	3.72/3.58	5.6, 10.0/5.6, 10.0
	CO	171.21			171.32[a]			171.47[a]		

Table 2. Continued

		laxaphycin B			laxaphycin B2			laxaphycin B3		
		^{13}C	^{1}H		^{13}C	^{1}H		^{13}C	^{1}H	
		δ (ppm)	δ (ppm)	J (Hz)	δ (ppm)	δ (ppm)	J (Hz)	δ (ppm)	δ (ppm)	J (Hz)
Leu[11]	NH		7.89	7.5		7.92	7.2		7.86	
	CαH	51.36	4.31	7.5	51.50	4.84	7.2	51.31	4.35	
	CβH$_2$	40.82	1.47		40.20	1.60		41.24	1.47	7.2
	CγH	24.06	1.53	7.6, 6.0	23.99	1.61		24.12	1.52	
	CδH$_3$	22.71	0.87		22.67	0.95		22.75	0.86	6.8
	Cδ'H$_3$	21.76	0.82	6.0	21.54	0.92	6.7	21.72	0.80	6.8
	CO	171.67			171.41[a]			171.41[a]		
Thr[12]	NH		7.74	8.9		7.61			7.68	4.0
	CαH	57.85	4.11	8.9, 3.4	57.99	4.09	6.0, 4.0	58.17	4.10	
	CβH	66.19	4.00		66.15	4.00		66.35	3.97	
	OH		4.78	5.2		4.73			4.80	5.6
	CγH$_3$	19.46	0.99	6.5	19.35	1.09	6.5	19.48	0.99	6.8
	CO	168.67			168.67			168.67[a]		

[a] Chemical shifts may be interchanged.

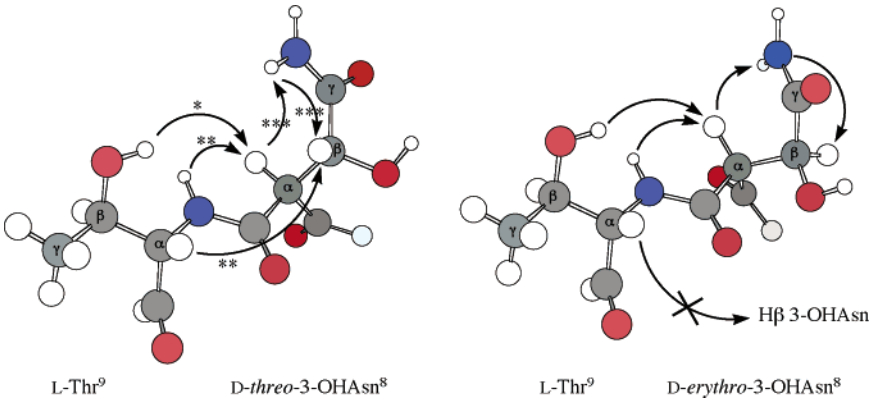

Figure 4. Inter- and intraresidue NOEs observed for the partial structure 3-OHAsn[8]−L-Thr[9]. Arrows indicate small (*), medium (**), and strong (***) dipolar interactions.

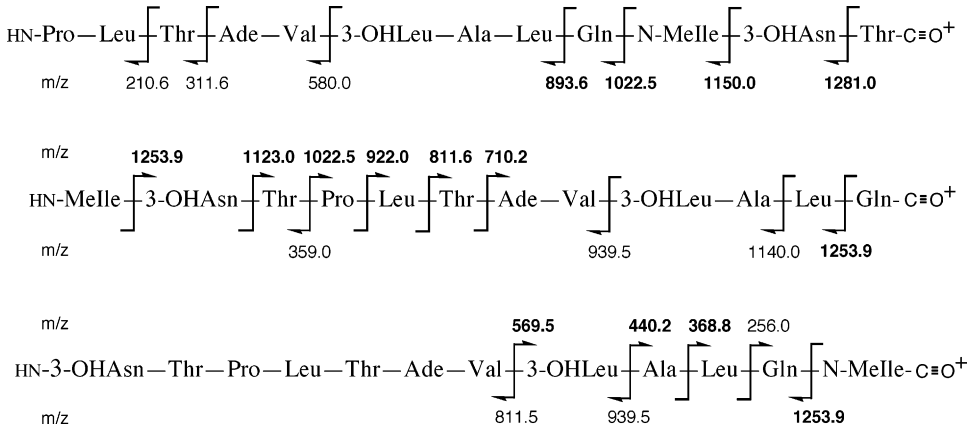

Figure 5. FAB MS/MS fragmentation of laxaphycin B2.

mentation of the acylium ion with Pro at the N-terminus led us to an acylium ion series shifted to a higher mass by 16 amu in comparison to that of laxaphycin B, suggesting that the variable residue could be Pro or Leu. The other series of fragmentation not being complete, it was not possible to conclude in which residue lay the difference according to mass analysis.

The NMR spectral analysis established the variable residue as 4-hydroxyproline (Table 2). Laxaphycin B3 showed remarkable NMR spectral similarities to laxaphycin B, the most significant differences being the presence of an additional hydroxyl group on proline [OH 5.08 ppm and Hγ at 4.32 ppm vs two Hγ at 1.90 and 1.80 ppm for laxaphycin B; Cγ at 68.5 ppm vs 24.0 ppm for laxaphycin B; Cβ and Cδ were also deblinded by the presence of the hydroxyl function ($\Delta\delta$ 8.6 and 8.3 ppm, respectively)].

Marfey's analysis of laxaphycin B3 indicated a (2*S*,4*R*) configuration for 4-OHPro and no change for the other residues. A similarity of the chemical shifts between laxaphycin B3 and laxaphycin B led us to conclude that both peptides have the same peptidic sequence. Thus, the structure of laxaphycin B3 was established as cyclo[(3*R*)-Ade−L-Val−(2*S*,3*S*)-3-OHLeu−L-Ala−(2*R*,3*S*)-3-OHLeu−L-Gln−L-*N*-MeIle−(2*R*,3*R*)-3-OHAsn−L-Thr−(2*S*,4*R*)-4-OHPro−D-Leu−L-Thr-].

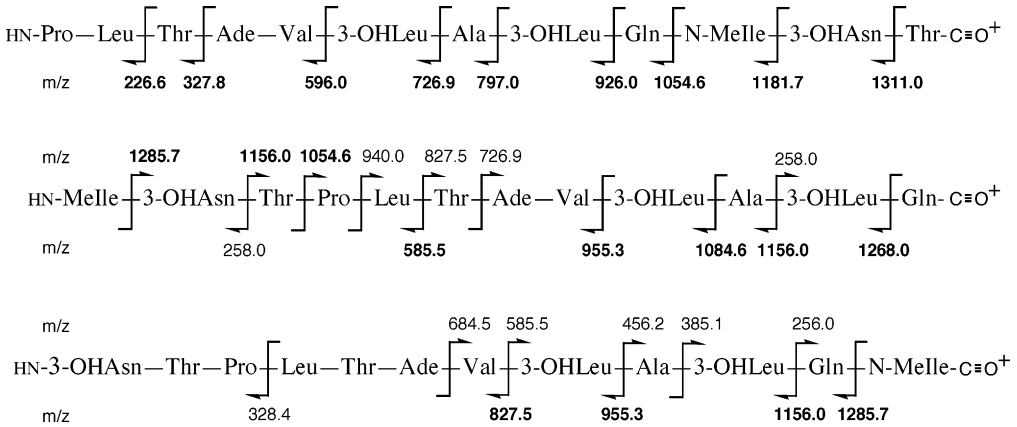

Figure 6. FAB MS/MS fragmentation of laxaphycin B3.

Biological Evaluation of Laxaphycins

Growth-Inhibitory Effect of Laxaphycins on Solid Cancer Cell Lines. To provide information on their antiproliferative activity against solid cancers, laxaphycin A (0−50 μmol) and laxaphycin B (0−20 μmol) were tested versus cisplatin (0−50 μmol) on five human carcinoma cell lines: A549 (non small cell lung), MCF7 (breast), PA1 (ovary), PC3 (prostate), and DLD1 (colon) and a human pigmented malignant melanoma line (M4Beu). To obtain additional information on their specificity for human cell lines and their specificity for cancer versus normal cell lines, each peptide was tested versus L-929 murine immortalized cells and human normal fibroblasts. The growth inhibitory effect was determined by measuring first the metabolic activity of treated and control cultures by the resazurin reduction test (RRT) and then the amount of cell biomass in each well with Hoechst 33342 (Ho). A dose-dependent inhibition of cell proliferation was observed in all cell lines. The IC_{50} of each chemical varied with the cell lines and the used test. However, the data clearly demonstrated the low toxicity of laxaphycin A (IC_{50} > 20 μM on all cell lines) as compared to laxaphycin B (IC_{50}< 2 μM on all cell lines, DLD1 excepted). The low toxicity of laxaphycin A observed in our model is in agreement with data by Frankmölle et al.[13] who reported a lack of toxicity on human KB cells (epidermoid carcinoma) and human LoVo cells (colorectal adenocarcinoma). It has been reported that contrary to laxaphycin A, hormothamnin A is highly toxic on a variety of solid cancer cell lines. Gerwick et al.[16] calculated the IC_{50} values of hormothamnin A as, respectively, 0.17 and 0.13 μM on SW1271 and A529 lung carcinoma cells as 0.11 μM on B16-F10 murine melanoma cells and as 0.60 μM on HCT-116 human colon carcinoma cells. As the only difference between laxaphycin A and hormothamnin A is a different configuration of the Dhb residue, it is likely that the configuration of this residue plays a critical role in the higher cytoxicity of hormothamnin A.

The individual susceptibility of each tested cancer (immortalized and normal) cell line to laxaphycin B and cisplatin is presented in Figure 7. Laxaphycin B (0.19 μM < IC_{50} < 6.0 μM) appeared markedly more toxic than the clinically active drug (2.0 μM < IC_{50} < 23.9 μM) on all tested human lines. In the human cancerous cell lines, the IC_{50} values of laxaphycin B varied between 0.19 and 1.0 μM except on DLD1 colon carcinoma cells, which appeared markedly more resistant to this peptide. The cells exhibiting the highest susceptibility were human PA1 ovary carcinoma cells (IC_{50} 0.19 ± 0.03 μM), M4Beu (IC_{50} 0.3 μM ± 0.03 in Ho test and 0.4 ± 0.03 μM in RRT test), and to a lesser extent androgen-resistant PC3 prostate carcinoma (IC_{50} 0.58 ± 0.03 in Ho test and 0.8 ± 0.1 μM in RRT) and MCF7 breast carcinoma (IC_{50} 0.5 ± 0.07 in Ho test and 1.0 ± 0.12 μM in RRT) cells. Laxaphycin B has, therefore, the capacity to block not only the proliferation of highly proliferative carcinoma cells (PA1) but also that of strongly invasive malignant melanoma cells (M4Beu). Regarding the increasing number of deaths relative to malignant melanoma in the European Union and North America, this finding is of interest. Together with the significant cytotoxicity on PC3 and MCF7 cells, which are representative of two types of cancer whose incidence is still in progression in Western countries, the tumoricidal effect on M4Beu metastatic melanoma justifies further investigations to assess the mechanism of action of this peptide and to determine whether the cytotoxicity of laxaphycin B is cell-type or tissue-dependent. Because this peptide has only

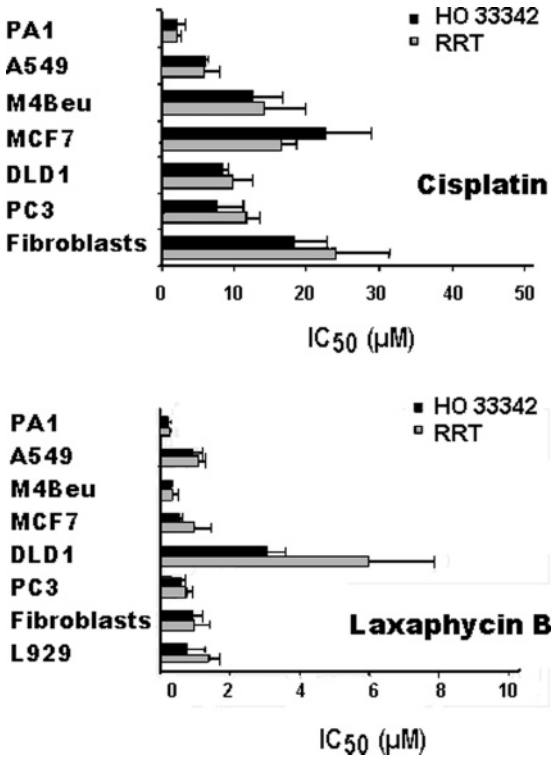

Figure 7. In vitro cell toxicity of laxaphycin B as compared to cisplatin against human solid cancer cells, human normal fibroblasts, and L929 murine immortalized cells. Cell toxicity was evaluated with the Hoechst 33342 (Ho), and the RRT assay on cultures submitted to a 48 h continuous contact with specified concentration of drugs. Results were expressed as the percentage of cells surviving the treatment versus control cells treated with the solvent alone (DMSO 0.5%). Two independent experiments were made in triplicate and averaged (±SD).

Table 3. In Vitro Cytotoxicity of Laxaphycins against Drug-Sensitive and Multidrug-Resistant Tumor Cell Lines

	CCRF-CEM IC_{50}[a] (μM)	CEM/VLB_{100} IC_{50}[a] (μM)	resistance factor[b]	CEM/VM-1 IC_{50}[a] (μM)	Resistance factor[b]
adriamycin	0.06 ± 0.02	3.71 ± 1.30	62	0.55 ± 0.14	9
vinblastine	0.004 ± 0.001	0.356 ± 0.040	89	0.008 ± 0.003	2
laxaphycin A	>20	>20		>20	
laxaphycin B	1.11 ± 0.15	1.02 ± 0.05	0.9	1.37 ± 0.15	1.2
laxaphycin B2	4.15 ± 0.31	3.33 ± 0.08	0.8	4.14 ± 0.09	1
laxaphycin B3	1.50 ± 0.06	1.35 ± 0.05	0.9	1.45 ± 0.11	1

[a] A 50% inhibitory concentration in a 72 h growth inhibition assay ± SD. Values shown are the means of three separate experiments. [b] Resistance factors calculated as IC_{50} for drug-resistant cells/IC_{50} for drug-sensitive cells.

a limited specificity for certain cancer cells in vitro, experiments in animal model bearing xenograft tumors would be useful to assess the therapeutic index of laxaphycin B in vivo.

Growth-Inhibitory Effect of Laxaphycins on Multidrug-Resistant versus Sensitive Human Leukemic Cell Lines. We also screened the cell toxicity of laxaphycins A, B, B2, and B3 against lymphoblastic drug-sensitive and multidrug-resistant cell lines. The cytotoxicity of the different laxaphycins was evaluated for the parent drug-sensitive CCRF-CEM human leukemic lymphoblasts and sublines that express drug resistance associated with either overexpression of the plasma membrane protein P-glycoprotein (Pgp) for CEM/VLB_{100} cells (MDR phenotype)[34] or with altered DNA topoisomerase II for the CEM/VM-1 cells. This latter cell line exhibits resistance to teniposide (VM-26) and is usually identified as an atypical MDR cell line.[35] Results are summarized in Table 3. In all these experiments, adriamycin and vinblastine were used as positive standards and references of anticancer activity.

Laxaphycin A exhibited no activity on acute lymphoblastic leukaemia cell lines when tested for 72 h at a concentration of 20 μM. On the drug-sensitive CCRF-CEM human leukemic lymphoblasts, IC_{50} values of laxaphycins B, B2, and B3 ranged from 1.1 μM to 4.2 μM. Interestingly, these results give some new information on SAR. They indicate that if the γ-hydroxylation of Pro^{10} in laxaphycin B3 have a weak impact on cell toxicity, substitution of 3-$OHLeu^5$ by Leu^5 in laxaphycin B2 and inversion of the configuration of the Cα 3-$OHLeu^3$ resulted in a less toxic compound.

The growth-inhibitory effect of each laxaphycin B was then evaluated on the two resistant leukemic cell lines. The resistance factors of each laxaphycin are presented as the ratio of IC_{50} for drug-resistant cells/IC_{50} for parental cells. Interestingly, the two tested MDR cell lines which are characterized by a 62- and 9-fold resistance to adriamycin and an 89- and 2-fold resistance to vinblastine exhibited similar susceptibility to laxaphycin B-type peptides than the parental cell line. The lack of resistance of CEM/VLB_{100} cells to laxaphycins B, B2, and B3 seems to indicate that none of these peptides is a substrate for P-glycoprotein (Pgp). This energy efflux pump, which extrudes many chemotherapeutic drugs out of cancer cells, plays a key role in the development of multidrug resistance by cancer cells following chemotherapy.[36] Pgp located in intestinal lumen may also drive compounds back into the intestinal lumen, preventing their absorption into blood.[37] Although this has to be confirmed by further studies, our results suggest that laxaphycin B presents the dual advantage of not contributing to MDR encoded by the MDR1 gene and of being unaffected by a decreased bioavailability related to Pgp.

Interaction of Laxaphycins A and B on Growth Inhibition of Human Tumor Cell Lines. Two drugs used in combination may produce enhanced (synergism), additive, or reduced (antagonism) effects. Frankmölle et al.[13] reported a synergistic activity of laxaphycins A and B against the growth of *Aspergillus orizae* and KB cells. Our group has previously observed a synergistic effect between these peptides against *Candida albicans*.[18] The antiproliferative effects of laxaphycin B on cancer cells was evaluated in combination with laxaphycin A. Results in Figure 8 and Table 4 show that, at inactive concentration, laxaphycin A strongly potentiates the cell toxicity of laxaphycin B. A significant synergistic effect was observed on DLD1 colon carcinoma cells and on multidrug-resistant leukemic cell lines. A total of 90% of the DLD1 cells survived to a 48 h continuous treatment with 2 μM of laxaphycin B.

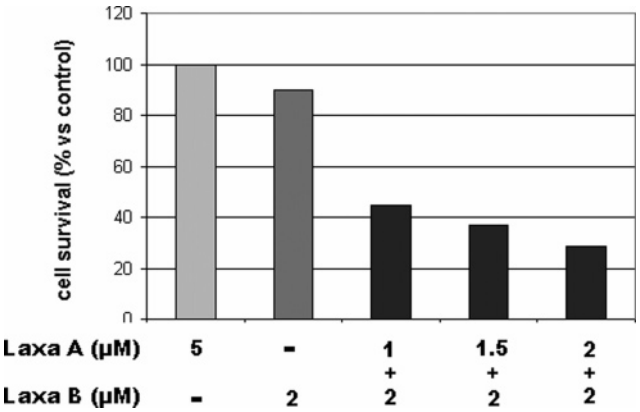

Figure 8. Enhancement of laxaphycin B cytotoxicity (2 μM) by laxaphycin A on DLD1 colon cancer cells. Cell toxicity was evaluated with the RRT assay on cultures submitted to a 48 h continuous contact with specified concentration of drugs. Results were expressed as the percentage of cells surviving the treatment versus control cells treated with the solvent alone (DMSO 0.5%). Data are the average of two independent experiments made in triplicate.

Table 4. Potentialization of Cell Growth Inhibition of Laxaphycin B by Laxaphycin A: Determination of Interaction Index γ

	laxaphycin A dose (μM)	laxaphycin B IC_{50}[a] (μM)	A/B ratio[b]	γ[c]
CCRF-CEM	0	1.11	0	
	0.2	0.62	0.3	0.56
	0.4	0.50	0.8	0.45
	1.0	0.41	2.4	0.41
	2.0	0.35	5.6	0.32
CEM/VM-1	0	1.37	0	
	0.2	0.72	0.3	0.53
	0.4	0.60	0.7	0.44
	1.0	0.43	2.3	0.32
	2.0	0.41	4.9	0.30
CEM/VLB_{100}	0	1.02	0	
	0.2	0.92	0.2	0.90
	0.4	0.57	0.7	0.56
	1.0	0.36	2.8	0.35
	2.0	0.33	6	0.32

[a] A 50% inhibitory concentration in a 72 h growth inhibition assay. Values shown are the means of three separate experiments. [b] A/B ratio is the ratio laxaphycin A concentration/laxaphycin B IC_{50}. [c] Interaction indexes (γ) were calculated by the following formula: $\gamma = (IC_{50}$ B combinated/IC_{50} B alone); $\gamma > 1$ indicates antagonism, $\gamma = 1$ indicates additivity, $\gamma < 1$ indicates synergism, and $\gamma < 0.5$ indicates strong synergism.

Singly, laxaphycin A at 5 μM induced no antiproliferative activity on this cell line. However, in combination at 1, 1.5, or 2 μM, with 2 μM of laxaphycin B, it significantly increased the cytotoxicity of laxaphycin B on DLD1 cells (Figure 8). This indicates a synergistic effect whose efficacy increases with increasing concentration of laxaphycin A.

As it is uncommon to encounter synergistic combinations in which one of the two compounds lacks efficacy, we assessed the degree of synergism from the measure of the interaction index (γ), a quantity that indicates the changed potency of a combination of two drugs.[38] In the case in which laxaphycin A is inactive ($IC_{50} > 20$ μM), γ is the ratio of the IC_{50} values of laxaphycin B in combination and alone. IC_{50} values of laxaphycin B with different concentrations of laxaphycin A, from 0.2 to 2.0 μM, have been determined on the three leukemic cell lines (CCRF-CEM, CEM/VM-1, CEM/VLB_{100}) to estimate the optimal combination mixture. The A/B ratio (laxaphycin A concentration/laxaphycin B IC_{50}) was tested in the range of 0−6. As indicated in Table 4, laxaphycin A potentiates the activity of laxaphycin B as well as on sensitive and on resistant leukemic cell lines. The IC_{50} of laxaphycin B decreases with increasing concentrations of laxaphycin A on the three cell lines. On sensitive CCRF-CEM cells, the IC_{50} value of laxaphycin B was reduced by 2-fold in the presence of 0.2 μM of laxaphycin A (A/B ratio: 0.3) and by 3-fold when laxaphycin A was used at 1 μM (A/B ratio: 2.4) or 2 μM (A/B ratio: 5.6). A 3-fold potentialization of the cell toxicity of laxaphycin B was also observed on resistant CEM/VM-1 and CEM/VLB_{100} cells when laxaphycin A was used at a A/B ratio in the range 2.5−6. In all three cell lines, γ values decrease slowly when the A/B ratio is >2.5. The optimal combination mixture seems to be obtained for A/B ratio = 2.5−3.

Finally, it was observed that the synergistic activities of laxaphycin A and laxaphycin B are expressed on the growth of all leukemic and solid cancer cells tested and on the growth of two fungal strains, *Aspergillus orizae* and *Candida albicans*. It was recently suggested that laxaphycin B may have a different mechanism of action when it is administered in the presence of laxaphycin A.[39] It is noteworthy that the synergistic effect between two small cyclic peptides is not frequent. Very few examples of synergy have been shown, the association being at a molecular level (example of iturins and surfactins from a *Bacillus* strain) or the peptides acting on separate receptors (example of pristinamycins and virginiamycins from two different *Streptomyces* strains).

Conclusion

Laxaphycins A and B are lipophilic cyclopeptides coproduced by the fresh water or marine filamentous cyanobacteria *Anabaena laxa* and *A. torulosa*. It is interesting to note that lobocyclamides A and B, closely related to laxaphycins A and B, were isolated from a mixture of *Lyngbya confervoides* and coral sand with minor amounts (up to 10%) of *Anabaena* sp. and *Oscillatoria* spp.[19] We cannot rule out the possibility that the source of lobocyclamides may be the strain *Anabaena* sp. In addition to ribosomal L-amino acids, these unusual peptides contain several "exotic" amino acids, including α,β-didehydro-α-aminobutyric acid, 3-hydroxyleucine, 3-hydroxyasparagine, or 3-aminoocta(deca)noic acids. Laxaphycin A-type peptides are cyclic undecapeptides in which the sequence shows a segregation between hydrophobic and hydrophilic residues. The hydrophobic face is characterized by a sequence of an alternating D- and L-leucine/isoleucine residue tetrad. The laxaphycin B-type peptides are cyclic dodecapeptides also with a sequence of nonpolar alternating D and L residues (Pro^{10}-Leu^{11}-Thr^{12}-Ade^{1}-Val^{2}). This arrangement, common to laxaphycins A and B, is a structural property found in peptides with the ability to become inserted into lipid bilayer membranes. The sequence of laxaphycins B shows a less pronounced segregation between hydrophobic and hydrophilic residues. The laxaphycin B-type peptides significantly inhibited the proliferation of human solid cancer cells and of sensitive and resistant leukemic cells at micromolar levels. Although laxaphycin A exhibited weak antiproliferative effect on leukemia and solid cancer cells, it presented the great advantage to strongly potentiate the anticancer effect of laxaphycin B on sensitive and resistant cancer cells. In brief, these data suggest that a combination of laxaphycins A and B may be of great interest in human chemotherapy. Studies are underway to assess this hypothesis and to identify the mechanism(s) involved in the synergistic effect of the active mixtures.

Experimental Section

General Instrumentation. Nuclear magnetic resonance (NMR) spectra were recorded on a Jeol EX 400 spectrometer. IR and UV spectra were recorded, respectively, on a Perkin-Elmer 1600 FTIR spectrometer and a Perkin-Elmer 551 spectrometer. Liquid secondary ion mass spectra were performed on a Autospec instrument (Fisons, VG analytical, Manchester, U.K.) fitted with a liquid secondary ion source (LSIMS). The Cesium gun worked at 30 kV, with the ion source voltage being 8 kV. Samples were dissolved in a few μL of 20% aqueous acetic acid, and 1 μL was mixed on the target with 1 μL of a 1:1 (v/v) glycerol thioglycerol mixture acidified with 1 μL of 1% trichloroacetic acid in water. For CID measurements, the collision cell fitted in the first field-free region was filled with helium so as to reduce the precursor ion beam to 50% of its original value. Constant B/E linked scans were performed, with both electrostatic analyzers being scanned in parallel. High performance liquid chromatography (HPLC) was performed with Jasco 880-PU pumps, 7125 Rheodyne injectors, and either a Merck (LMC systeme) differential refractometer detector or a Waters 996 photodiode array detector. OPA-derived amino acids were analyzed by Gilson automatic injector and HPLC system, detected on a Shimazu RF530 fluorimeter.

Isolation of Laxaphycins. A sample (1.24 kg) of *Lyngbya majuscula* collected off the coast of Tahiti in 1993 was stored in EtOH until workup. The extract obtained by repetitive steeping in $CHCl_3$/EtOH was separated by solvent partition. The chloroformic extract (4 g) was subjected to flash chromatography and silica gel column chromatography (solvent: 100% CH_2Cl_2 to 100% MeOH), then the peptide mixture was purified by using C_{18} reversed-phase flash column chromatography with CH_3CN/H_2O (50:50). The individual laxaphycins were separated and purified by repetitive reversed-phase HPLC (SFCC Ultrabase UB 535 C_8 column, 250 × 10 mm, 5 μm particle size, flow rate 1.5 mL/min, differential refractometry detection) using CH_3CN/H_2O (50:50). Laxaphycin B3 (15 mg) was eluted at 16.8 min, laxaphycin B (184 mg) at 20.0 min, laxaphycin B2 (11 mg) at 23.9 min, and laxaphycin A (110 mg) at 34.0 min.

NMR Measurement Conditions. All spectra were obtained with a NM-40TH5 dual ^1H, ^{13}C probe in a JEOL EX400 operating at 400 MHz for proton and 100.53 MHz for carbon-13, at 308 K for laxaphycin B, 318 and 298 K for laxaphycin A, 318 K for laxaphycin B2, and 308 K for laxaphycin B3. ^1H and ^{13}C NMR chemical shifts are referenced to solvent peaks: δ_H 2.49 ppm (residual DMSO-H_6) and δ_C 39.5 ppm for DMSO-d_6.

Two-dimensional (2D) homonuclear correlated experiments DQF-COSY, HOHAHA, and ROESY were all acquired by using standard procedures with a spectral width of about 4000 Hz in both columns F1 and F2. HOHAHA and ROESY were acquired in the phase-sensitive mode. The time domain matrix consisted of 256 points in t_1 and 1024−2048 points in t_2 with 32 acquisitions for 256−300 experiments in t_1. Data sets were zero-filled to 512 points

in t_1 prior to Fourier transformation to obtain a frequency domain matrix of 512 × 1024−2048 real data points. Squared sine bell apodization functions were used. The HOHAHA spectra were recorded with a mixing time of 80 ms. ROESY spectra were measured with mixing times of 150, 250, and 350 ms. Heteronuclear correlated experiments were performed in ^{1}H -detected mode using the standard pulse programs HMQC and HMBC, with a spectral width of about 20 000 Hz in F1 and 4000 Hz in F2. The time domain matrix consisted of 256−512 points in t_1 and 2048 points in t_2, with 128−200 acquisitions for 256 experiments in t_1. Data sets were zero-filled to 512 points in t_1 prior to Fourier transformation to obtain a frequency domain matrix of 512 × 2048 real data points. The evolution delay was set to optimize 140 Hz couplings for HMQC and 8 Hz couplings for HMBC. Squared sine bell apodization functions were used. The *J*-resolved spectra were recorded using a standard pulse sequence to obtain a 4096 × 256 matrix in t_2 and t_1, respectively, with a spectral width of 50 Hz in the t_1 dimension.

Laxaphycin A (1). White amorphous solid; IR ($CHCl_3/CH_2Cl_2$ 1%) 3400, 3325, 2964, 2933, 2875, 1657, 1525 cm^{-1}; UV (MeOH) λ_{max} 224 nm (ϵ 13 820); FABMS *m*/*z* (rel.intensity in %), 1196.6 (100), 1180.0 (15), 588.2 (7), 475.1 (10), 445.0 (9), 383.0 (15), 362.0 (49); FABMS of acetylated laxaphycin A, *m*/*z* (rel. intensity in %), 1360.5 (8), 1344.5 (65), 1322.5 (100), 785.4 (5), 672.3 (8), 559.2 (14), 446.0 (27), 430.0 (19), 386.0 (15), 370.0 (19), 310.0 (18); FAB MS/MS data are shown in Figure 2; NMR data (DMSO-d_6 at 318 K) are shown in Table 1.

Laxaphycin B (2). White amorphous solid; IR ($CHCl_3/CH_2Cl_2$ 1%) 3350, 3328, 3053, 2980, 2932, 1667, 1628, 1535 cm^{-1}; UV (MeOH) λ_{max} 230 nm (ϵ 2360); FABMS *m*/*z* (rel. intensity in %) 1395.6 (100), 1380.0 (22); FABMS of acetylated laxaphycin B *m*/*z* (rel. intensity in %) 1605.5 (100), 1548.0 (21), 1563.0 (23), 810.4 (6), 621.0 (7), 593.2 (18), 521.0 (9), 455.0 (10), 370.0 (13), 357.0 (20), 355.0 (8), 340.0 (31), 310.0 (85); FAB MS/MS data are show in Figure 3; NMR data (DMSO-d_6 at 308 K) are shown in Table 2.

Laxaphycin B2 (3). White amorphous solid; IR ($CHCl_3/CH_2Cl_2$ 1%) 3334, 2960, 2930, 1672, 1642, 1530 cm^{-1}; UV (MeOH) λ_{max} 230 nm (ϵ 4810); FABMS, *m*/*z* (rel. intensity in %) 1424.6 (18), 1379.6 (100), 1363.6 (15); FAB MS/MS data are shown in Figure 5; NMR data (DMSO-d_6 at 318 K) are shown in Table 2.

Laxaphycin B3 (4). White amorphous solid; IR ($CHCl_3/CH_2Cl_2$ 1%) 3338, 3291, 1666, 1629, 1538 cm^{-1}; UV (MeOH) λ_{max} 230 nm (ϵ 2440); FABMS *m*/*z* (rel. intensity in %) 1411.6 (100), 1395.5 (15), 1367.5 (10), 553.1 (8), 456.0 (18), 369.0 (60); FAB MS/MS data are show in Figure 6; NMR data (DMSO-d_6 at 308 K) are shown in Table 2.

Acetylation of Laxaphycins A and B. An amount equal to 2 mg of each laxaphycin was mixed with a few drops of pyridine and acetic anhydride overnight at room temperature. After evaporation, the reaction mixture was purified on a silica gel column with CH_2Cl_2/MeOH 90:10 (v/v).

Synthesis of DL-β-Amino Octanoic and Decanoic Acid (as Described Previously by Gerwick et al.). Malonic acid (2.5 g) was dissolved in 6 mL of dried pyridine, and then 2.5 mL of hexanal (3.2 mL of octanal) and 0.25 mL of distilled pyrrolidine were added. After refluxing for 30 min, the products were poured into 100 mL of chilled diluted HCl solution. The solution was extracted with ether (3 × 100 mL), and the ether layer was washed with distilled water, dried over $MgSO_4$, and evaporated to give 2.4 g of (*E*)-octa-2-enoic acid (3 g of (*E*)-deca-2-enoic acid). The acids (1 g) in methanol (30 mL) were methylated by refluxing for 12 h with Amberlite IR-120 (H^+) resine. After filtration, the reaction mixture was concentrated in vacuo to give a residue that was dissolved in 30 mL of CH_2Cl_2. The CH_2Cl_2 layer was washed with $NaHCO_3$ solution, water, and brine, dried with $MgSO_4$, and evaporated. Each ester (0.50 g) was mixed with 0.62 g of phtalimide in 20 mL of dried pyridine. The mixture was refluxed for 24 h in the presence of EtONa (0.34 g). After cooling to room temperature, the reaction was poured into ice water and the resultant solution was extracted with $CHCl_3$ (3 × 30 mL). The extract was fractionated by silica gel column chromatography to yield 163 mg of methyl 3-phtalimidooctanoate (128 mg of methyl 3-phtalimidodecanoate). ^{1}H NMR data of methyl 3-phtalimidooctanoate ($CDCl_3$, 400 MHz): δ 7.82 (2H, m), 7.71 (2H, m), 4.60 (1H, dd, *J* = 9.8 Hz, 5.4 Hz), 3.58 (3H, s), 3.15 (1H, dd, *J* = 9.8 Hz, 16.0 Hz), 2.75 (1H, dd, *J* = 5.4 Hz, 16.0 Hz), 2.05 (1H, m), 1.7 (1H, m), 1.25 (6H, m), 0.8 (3H, m). ^{1}H NMR data of methyl 3-phtalimidodecanoate ($CDCl_3$, 400 MHz): δ 7.81 (2H, m), 7.69 (2H, m), 4.61 (1H, m), 3.59 (3H, s), 3.15 (1H, dd, *J* = 9.6 Hz, 16.0 Hz), 2.75 (1H, dd, *J* = 5.2 Hz, 16.0 Hz), 2.04 (1H, m), 1.68 (1H, m), 1.22 (8H, m), 0.8 (3H, m). ^{13}C NMR data of methyl 3-phtalimidooctanoate ($CDCl_3$, 100 MHz): δ 172.0, 168.5, 134.0, 132.5, 123.0, 52.0, 48.0, 37.2, 32.5, 31.5, 26.0, 22.5, 14.0. ^{13}C NMR data of methyl 3-phtalimidodecanoate ($CDCl_3$, 100 MHz): δ 171.5, 168.2, 134.0, 133.0, 123.0, 51.6, 48.0, 37.2, 32.5, 31.5, 25.8, 22.5, 22.4, 22.1, 13.8. The methyl 3-phtalimidooctanoate (methyl 3-phtalimidodecanoate) product was hydrolyzed (3 N KOH, 50 °C, 4 h), and the resultant solution was acidified and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with water and dried with $MgSO_4$ and evaporated to give DL-β-amino octanoic and decanoic acid contaminated by a small amount of phtalic acid. ^{1}H NMR data of DL-β-amino octanoic acid (acetone-d_6, 400 MHz): δ 4.40 (1H, m), 2.75 (1H, dd, *J* = 6.0 Hz, 17.0 Hz), 2.55 (1H, dd, *J* = 9.0 Hz, 17.0 Hz), 1.65 (2H, m), 1.50 (1H, m), 1.42 (1H, m), 1.30 (4H, m), 0.88 (3H, t, *J* = 7.0 Hz). ^{1}H NMR data of DL-β-amino decanoic acid (acetone-d_6, 400 MHz): δ 4.41 (1H, m), 2.74 (1H, dd, *J* = 5.8 Hz, 16.0 Hz), 2.55 (1H, dd, *J* = 8.9 Hz, 16.0 Hz), 1.65 (2H, m), 1.50 (1H, m), 1.42 (1H, m), 1.28 (6H, m), 0.87 (3H, t, *J* = 7.0 Hz). ^{13}C NMR data of DL-β-amino octanoic acid (acetone-d_6, 100 MHz): δ 173.5, 47.5, 40.0, 35.0, 32.5, 26.0, 23.0, 14.5. ^{13}C NMR data of DL-β-amino decanoic acid (acetone-d_6, 100 MHz): δ 174.0, 48.0, 40.0, 35.0, 32.4, 26.5, 23.5, 14.4. These β-amino octanoic and decanoic acids were reacted directly with FDAA for HPLC analysis. L-β-Amino acids were eluted before D isomers (cf. Gerwick).

Synthesis of D-*N*-Methylisoleucine and D-*allo*-*N*-Methylisoleucine (Freidinger's Method). Fmoc-D-Ile (0.37 g; Fmoc-D-*allo*-Ile) was suspended in 100 mL of toluene, and 0.24 g of paraformaldehyde and a catalytic amount of *p*-toluene sulfonic acid were added. The mixture was refluxed for 30 min with azeotropic water removal. The solution was cooled, washed with 1 N aqueous $NaHCO_3$ (3 × 25 mL), dried over $MgSO_4$, and concentrated in vacuo to yield 370 mg of a colorless oil. The oxazolidinone was dissolved in 3 mL of CH_2Cl_2, 3 mL of trifluoroacetic acid, and 0.8 mL of triethylsilane were added. The solution was stirred overnight at room temperature, followed by concentration in vacuo to an oil. The residue was dissolved in CH_2Cl_2 and reconcentrated three times. The resultant crystalline product was washed with a mixture of 10% ether in pentane and dried, and the fluoromethyloxycarbonyl group was removed with piperidine in CH_2Cl_2 to yield D-*N*-MeIle and D-*allo*-*N*-MeIle. ^{1}H NMR data of D-*N*-MeIle (DMSO-d_6, 400 MHz): δ 8.80 (1H, br), 8.36 (1H, br), 3.80 (1H, m), 2.50 (3H, m), 1.84 (1H, br), 1.40 (1H, m), 1.21 (1H, m), 0.80 (6H, m). ^{1}H NMR data of D-*allo*-*N*-MeIle (DMSO-d_6, 400 MHz): δ 8.90 (1H, br), 8.80 (1H, br), 3.90 (1H, m), 2.60 (3H, m), 1.90 (1H, m), 1.50 (1H, m), 1.30 (1H, m), 0.90 (3H, t, *J* = 6 Hz), 0.89 (3H, d, *J* = 7.2 Hz). ^{13}C NMR data of D-*N*-MeIle (DMSO-d_6, 100 MHz): δ 169.3, 64.2, 35.0, 32.2, 26.0, 14.2, 11.7. ^{13}C NMR data of D-*allo*-*N*-MeIle (DMSO-d_6, 100 MHz): δ 168.9, 63.7, 34.7, 31.7, 25.5, 13.7, 11.2. D-*N*-MeIle and D-*allo*-*N*-MeIle were derivatized directly with FDAA for HPLC analysis.

Synthesis of (2*R*,3*S*)-3-Hydroxyleucine and (2*R*,3*R*)-3-Hydroxyleucine (Adapted from Schollkopf's Method). An amount equal to 3.75 mL of a 1.6 M solution of butyllithium in hexane was added at −70 °C to 1.03 g of (2*S*)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine [(+)-Schollkopf's reagent] in anhydrous THF (15 mL). After 30 min of stirring at −70 °C, 0.6 mL of isobutanal in THF (5 mL) was added and stirring was continued for 8 h. The reaction mixture was allowed to warm up to 0 °C, neutralized by a 1 mol NH_4Cl solution, and extracted with diethyl ether (3 × 30 mL). The combined ether layers were dried over magnesium sulfate and concentrated under reduced pressure to yield a crude extract that was purified by silica gel column chromatography (eluent:

pentane/ethylacetate 90:10 v/v). We obtained 420 mg of (1*S*,2′*R*,5′*S*)-carbonyl adduct (yield 40.8%) and 62 mg of (1*R*,2′*R*,5′*S*)-carbonyl adduct (yield 6%). The carbonyl adducts were hydolyzed following the general procedure as described for standard peptide hydrolysis. The (1*S*,2′*R*,5′*S*)-carbonyl adduct gave a mixture of (2*R*,3*S*)-3-hydroxyleucine and L-valine, and the (1*R*,2′*R*,5′*S*)-carbonyl adduct gave a mixture of (2*R*,3*R*)-3-hydroxyleucine and L-valine, which were used as the standard for amino acid analysis. ^{1}H NMR data of (1*S*,2′*R*,5′*S*) and (1*R*,2′*R*,5′*S*)-carbonyl adducts ($CDCl_3$, 400 MHz): δ 4.06 (1H, dd, J = 1.6 Hz, 3.2 Hz), 3.96 (1H, dd, J = 3.2 Hz, 3.2 Hz), 3.69 (3H, s), 3.65 (3H, s), 3.59 (1H, br), 2.22 (1H, dsept., J = 3.2 Hz, 6.8 Hz), 1.97 (1H, dsept., J = 1.6 Hz, 6.8 Hz), 1.67 (1H, br), 1.00 (6H, d, J = 6.8 Hz), 0.95 (3H, d, J = 6.8 Hz), 0.67 (3H, d, J = 6.8 Hz). ^{13}C NMR data of ($CDCl_3$, 100 MHz): δ 165.62, 162.48, 77.36 (C_1S) or 77.01 (C_1R), 60.74, 57.21, 52.53, 52.42, 31.88, 30.88, 19.37, 18.97, 16.74. ^{1}H NMR data of (2*R*,3*S*)-3-hydroxyleucine (DMSO-d_6, 400 MHz): δ 3.88 (1H, d, J = 2.4 Hz), 3.54 (1H, dd, J = 2.4 Hz, 8.0 Hz), 3.36 (1H, br), 1.75 (1H, m), 0.91 (3H, d, J = 6.8 Hz), 0.85 (3H, d, J = 6.8 Hz). ^{1}H NMR data of (2*R*,3*R*)-3-hydroxyleucine (DMSO-d_6, 400 MHz): δ 3.52 (1H, br), 3.37 (1H, br), 3.36 (1H, br), 1.86 (1H, m), 0.91 (3H, d, J = 6.8 Hz), 0.96 (3H, d, J = 6.8 Hz). ^{1}H NMR data of L-valine (DMSO-d_6, 400 MHz): δ 3.70 (1H, d, J = 4.0 Hz), 2.18 (1H, m), 0.96 (3H, d, J = 6.8 Hz), 0.98 (3H, d, J = 6.8 Hz). ^{13}C NMR data of (2*R*,3*S*)-3-hydroxyleucine (DMSO-d_6, 100 MHz): δ 170.22, 74.23, 54.84, 29.90, 19.22, 18.6. ^{13}C NMR data of (2*R*,3*R*)-3-hydroxyleucine (DMSO-d_6, 100 MHz): δ 170.05, 74.85, 54.85, 29.90, 19.50, 18.16. ^{13}C NMR data of L-valine (DMSO-d_6, 100 MHz): δ 170.09, 57.26, 29.02, 18.16, 17.70.

Synthesis of (2*S*,3*R*)-3-Hydroxyleucine and (2*S*,3*S*)-3-Hydroxyleucine. These compounds were synthesized as described above from (2*R*)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine [(−)-Schollkopf's reagent]: yield 390 mg (54.5%) of (1*R*,2′*S*,5′*R*)-carbonyl adduct and 64 mg (8.9%) of (1*S*,2′*S*,5′*R*)-carbonyl adduct. ^{1}H NMR data of (1*R*,2′*S*,5′*R*) and (1*S*,2′*S*,5′*R*)-carbonyl adducts ($CDCl_3$, 400 MHz): δ 4.03 (1H, dd, J = 3.6 Hz, 3.2 Hz), 3.93 (1H, dd, J = 3.2 Hz, 3.2 Hz), 3.66 (3H, s), 3.62 (3H, s), 3.56 (1H, br), 2.19 (1H, dsept., J = 3.2 Hz, 6.8 Hz), 1.95 (1H, dsept., J = 1.2 Hz, 6.8 Hz), 1.74 (1H, br), 0.98 (3H, d, J = 6.8 Hz), 0.97 (3H, d, J = 6.8 Hz), 0.93 (3H, d, J = 6.8 Hz), 0.65 (3H, d, J = 6.8 Hz). ^{13}C NMR data of (1*R*,2′*S*,5′*R*) and (1*S*,2′*S*,5′*R*)-carbonyl adducts ($CDCl_3$, 100 MHz): δ 165.51, 162.42, 77.32 (C_1R) or 77.15 (C_1S), 60.67, 57.21, 52.44, 52.35, 31.79, 30.80, 19.30, 19.00, 16.67. ^{1}H NMR data of (2*S*,3*R*)-3-hydroxyleucine (DMSO-d_6, 400 MHz): δ 13.60 (1H, br), 3.90 (1H, d, J = 2.6 Hz), 3.54 (1H, dd, J = 2.6 Hz, 8.8 Hz), 3.36 (1H, br), 1.75 (1H, m), 0.91 (3H, d, J = 6.4 Hz), 0.85 (3H, d, J = 6.4 Hz). ^{1}H NMR data (2*S*,3*S*)-3-hydroxyleucine (DMSO-d_6, 400 MHz): δ 3.67 (1H, d, J = 2.0 Hz), 3.37 (1H, br), 3.36 (1H, br), 1.86 (1H, m), 0.95 (3H, d, J = 6.4 Hz), 0.88 (3H, d, J = 6.4 Hz). ^{1}H NMR data of d-valine (DMSO-d_6, 400 MHz): δ 13.60 (1H, br), 3.71 (1H, br), 2.19 (1H, m), 0.97 (3H, d, J = 7.2 Hz), 0.95 (3H, d, J = 7.2 Hz). ^{13}C NMR data of (2*S*,3*R*)-3-hydroxyleucine (DMSO-d_6, 100 MHz): δ 170.22, 74.17, 54.90, 29.90, 19.20, 18.5. ^{13}C NMR data of (2*S*,3*S*)-3-hydroxyleucine (DMSO-d_6, 100 MHz): δ 170.19, 74.96, 54.84, 29.88, 19.27, 18.45. ^{13}C NMR data of D-valine (DMSO-d_6, 100 MHz): δ 170.19, 57.22, 29.00, 18.20, 17.70.

Hydrolysis of Laxaphycins. For standard hydrolysis, laxaphycin A, laxaphycin B, laxaphycin B2, or laxaphycin B3 (0.5 to 1.2 mg) in 1 mL 6 N HCl was heated at 120 °C for 15 h in a sealed vial under nitrogen atmosphere. Microwave hydrolysis was also performed by using a Teflon flask and heating for 10 min to decreased degradation of β-hydroxy-amino acid and β-amino acid. The cooled reaction mixture was evaporated to dryness, and traces of HCl were removed from the residual hydrolysate by repeated evaporation from water. All the workup procedure was made under nitrogen atmosphere.

Isolation of 3-Hydroxyaspartic Acid from Laxaphycin B. An amount equal to 21 mg of laxaphycin B was dissolved in 4 mL 6 N HCl and heated at 120 °C for 15 h in a sealed vial under nitrogen atmosphere. The cooled reaction mixture was evaporated to dryness, and traces of HCl were removed from the residual hydrolysate by repeated evaporation from water. The residue was chromatographed on a 35 × 1 cm column of Dowex 50 × 2−400 resin (200−400 mesh, hydrogen form) by eluting with a linear gradient between 0.01 N HCl (150 mL) to 3 N HCl (150 mL). Fractions were pooled on the basis of Rf on silica gel TLC (*n*-BuOH/HOAc/H_2O, 4:1:1, v/v/v, ninhydrin detection) and vacuum-dried to give the following amino acids as their hydrochloride salts in order of elution: 3-hydroxyaspartic acid (0.7 mg), L-threonine (2 mg), L-glutamic acid + L-alanine + maleic acid or fumaric acid from dehydration of 3-hydroxyaspartic acid (10.6 mg), (2*R*,3*S*)-3-OHLeu + maleic acid or fumaric acid (2.1 mg), (2*S*,3*S*)-3-OHLeu + L-proline (7.5 mg), L-proline + L-valine (3.4 mg), L-*N*-methylisoleucine + D-leucine (7.8 mg). Identification of these amino acids was established by HPLC analysis with Marfey's derivatization. 3-Hydroxyaspartic acid was found threo by ^{1}H NMR comparison with standard LD-*threo*-3-hydroxyaspartic acids. The δ value for LD-*threo*-3-hydroxyaspartic acids (DCl + internal dioxane reference at 3.75 ppm, 400 MHz): Hα = 4.674 ppm and Hβ = 5.045 ppm. The δ value for LD-*erythro*-3-hydroxyaspartic: Hα = 4.770 ppm and Hβ = 4.915 ppm (obtained from hydrolysis of threo isomers at 120 °C during 20 h in HCl 6 N under an O_2 atmosphere). Measurement of the CD of this amino acid indicated D-configuration according to the literature: $[\theta]_{200} = -3400$; $[\theta]_{216} = -17\,000$; $[\theta]_{239} = 0$ (*c* 0.0117, 0.5 N HCl) [value for L-*threo*-3-hydroxyaspartic acid:[40] $[\theta]_{200} = +5320$; $[\theta]_{205} = +6970$; $[\theta]_{245} = 0$ (*c* 0.0652, 0.5 N HCl)].

Derivatization of Amino Acids with 1-Fluoro-2,4-dinitrophenyl-5-L-alanine Amide (FDAA). For the FDAA (Marfey's reagent) derivatization procedure, the previously obtained crude hydrolysate or small amounts of standard free amino acid were solubilized in 50 μL of a mixture of H_2O/acetone and mixed with 100 μL of a 1% solution of FDAA in acetone. Sodium bicarbonate solution (1 mol; 20 μL) was added to this mixture, and the resultant solution was heated at 40 °C for 1 h and allowed to cool. After addition of 10 μL of 2 mol HCl, the resulting solution was evaporated, dissolved in 0.5 mL of DMSO, and then analyzed by HPLC. The following conditions were used: solvent A, 0.05 mol Et_3N in water, adjusted at pH 3 with H_3PO_4, with 5% of solvent B; solvent B, acetonitrile/methanol (40:60 v/v); gradient with flow rate at 0.8 mL/min, solvent B from 30% to 40% in 20 min and from 40% to 100% in 30 min; column, Interchim Spherisorb OD2 5μ, 250 mm × 4 mm; UV detector at 340 nm. The peaks were identified by co-injection with a DL-mixture of standard amino acids. This procedure established the presence of (2*S*,4*R*)-4-OHPro, L-Hse, Gly, L-Ile, or L-*allo*-Ile, L-Leu, D-Phe, D-Leu, D-Ile, or d-*allo*-Ile, D-βAoc for laxaphycin A; (2*R*,3*R*)-3-OHAsp, L-Thr, L-Glu, L-Ala, L-Pro, (2*S*,3*S*)-3-OHLeu, L-Val, (2*R*,3*S*)-3-OHLeu, L-*N*-MeIle, D-Leu, D-βAde for laxaphycin B; (2*R*,3*R*)-3-OHAsp, L-Thr, L-Glu, L-Ala, L-Pro, L-Val, (2*R*,3*S*)-3-OHLeu, L-*N*-MeIle, D-Leu, D-βAde for laxaphycin B2; (2*R*,3*R*)-3-OHAsp, L-Thr, L-Glu, L-Ala, (2*S*,4*R*)-4-OHPro, (2*S*,3*S*)-3-OHLeu, l-Val, (2*R*,3*S*)-3-OHLeu, L-*N*-MeIle, D-Leu, D-βAde for laxaphycin B3.

Derivatization of Amino Acids with Orthophtaldialdehyde and 2-Mercapto-ethanol (OPA-MCE). An amount equal to 250 μL of a 10 nmol/mL H_2O solution of peptide hydrolysate or standard amino acid was mixed with 5 μL of internal standard (*N*-acetylcysteine) and 400 μL of iodo acetic acid (0.4 mol, pH 11.5). To this solution were added 10 μL of the OPA-MCE solution (125 mg of OPA in 2.5 mL of MeOH and 125 mL of MCE) and 10 μL of H_3BO_3 (0.8 mol, pH 12.5). After 2 min, the mixture was injected in the HPLC system. The following conditions were used: solvent A, sodium acetate/methanol/tetrahydrofurane (80:19.2:0.8 v/v/v); solvent B, sodium acetate/methanol (20:80 v/v), pH 10.5; gradient with flow rate at 0.8 mL/min, solvent B from 0% to 10% in 3 min, first step at 10% during 12 min, then from 10% to 14% in 3 min, second step at 14% during 5 min, from 14% to 50% in 5 min, third step at 50% during 4 min and from 50% to 100% in 18 min; column, C_{18} 5μ, 250 mm x 4 mm with a 25 mm x 4 mm precolumn, temperature at 38.2 °C; fluorimetric detection (λ_{ex} 360 nm, λ_{em} 455 nm). Retention times (min) are given in parentheses: from standard

hydrolysis of laxaphycin A, Hse (17.30), Gly (20.18), Phe (41.99), Ile (44.68), *allo*-Ile (44.90), Leu (45.63), βAoc (49.59).

Evaluation of Antiproliferative Activity on Lymphoblastic Cell Lines. The acute lymphoblastic leukemia cell line CCRF-CEM, the subline CEM/VLB_{100} selected for resistance to vinblastine, and the subline CEM/VM-1 selected for resistance to teniposide (VM-26) were obtained from Dr. W. T. Beck, St. Jude Children's Research Hospital, Memphis, Tennessee. Cells were grown in plastic tissue culture flasks containing RPMI 1640 medium supplemented with 10% fetal calf serum (v/v) and antibiotics (penicillin 1000 U/mL; streptomycin 100 mg/mL) and maintained at 37 °C in a humidified atmosphere containing 5% carbon dioxide. Serial dilutions of laxaphycins were prepared in the culture medium. The drug at the appropriate concentration was added to cell cultures (2×10^5 cells/mL) for 3 days without renewal of the medium. Cells surviving the treatment were then counted by means of a Coulter counter model ZME. Assays were carried out in triplicate, and the results were averaged. The concentration of drugs required to inhibit growth of cells by 50% (IC_{50}) in 72 h was determined for each cell line. Cross-resistance was calculated by dividing the IC_{50} obtained with the resistant cell line by that measured for the parent cell line. The interaction index (γ) was calculated by the following formula: $\gamma =$ (IC_{50} of B combined/IC_{50} of B alone). As indicated in ref 38, when one of the two compounds has no efficacy, γ is the ratio of IC_{50}s of the active component in the two situations, with and without the inactive component. Theoretically, an interaction index of 1 indicates an additive effect; greater than 1 means antagonism and any value less than 1 denotes synergism.

Evaluation of Antiproliferative Activity on Human Carcinoma and Melanoma Cell Lines. Human MCF7 breast adenocarcinoma, PC3 prostate adenocarcinoma, A-549 nonsmall-cell lung (NSCL) carcinoma, PA1 ovarian adenocarcinoma, DLD-1 colon adenocarcinoma cell lines, and L929 murine immortalized cells were purchased from the European Collection of Cell Cultures (ECACC, Salibury, United Kingdom). The M4Beu melanoma line was established in the laboratory of Dr. J. F. Doré (INSERM U-218, Lyon, France) from metastatic biopsy specimen and has been maintained in cell culture for almost 15 years. Normal human fibroblasts were purchased from Biopredic International (Rennes, France) as a frozen culture. All cells were cultured as previously described[41] in Glutamax Eagle's minimum essential medium supplemented with Earle's salts (Gibco-BRL, Paisley, U.K.), fetal calf serum, vitamins, sodium pyruvate, nonessential amino-acids, L-glutamine, and gentamycin base and maintained at 37 °C in a humidified atmosphere containing 5% carbon dioxide. Cell survival was assayed after a 48 h continuous contact with the solvent (DMSO 0.5%), with or without laxaphycin A (0−50 μmol) or laxaphycin B (0−20 μmol) by two cytotoxicity assays (RRT and Hoechst 33342) done in 96-well microplates (Nunclon, Nunc, Roskilde, Denmark), as described in ref 40. The RRT measures the metabolic activity of cultures, while the Hoechst assay (Ho) determines the DNA cellular content after cell lysis. In each assay, the cells were seeded with 5×10^3 cells per well and allowed to adhere for 16 h before treatment with laxaphycin A or laxaphycin B alone or in combination in 0.5% DMSO.

RRT Assay. At the end of treatment (48 h), 150 μL of a 25 μg/mL solution of resazurin in MEM was added to each well. The plates were incubated for 1 h at 37 °C under 5% CO_2 before measurement of fluorescence at 530/590 nm with a fluorescent Ascent FL (Labsystems). Under the conditions used, the fluorescence was proportional to the number of living cells in the well. IC_{50} values were calculated from the concentration-dependent survival curve defined as the fluorescence in experimental wells compared with fluorescence in control wells, after substraction of blank values. Results are the mean ± S.D. of two independent assays done in triplicate.

After reading, the cells were prepared for intracellular DNA quantitation with Ho 33342. Resazurin solution was eliminated with an automatic microplate washer, and the cells were rinsed with PBS (3-fold) and stored at −80 °C until Ho assay.

Ho Assay. On the day of assay, plates were thawed at room temperature for 10 min and 100 μL of a 30 μg/mL solution of Hoechst 33342 in hypersaline buffer containing 10 mmol Tris HCl pH 7.4, 1 mmol EDTA, and 2 mmol NaCl was added to each well. After a 1 h incubation at 37 °C under 5% CO_2, fluorescence was measured at 360/460 nm. In the assay conditions, the DNA content measured with Ho 33342 is proportional to the biomass content. IC_{50} values were calculated as above. Results are the mean ± S.D. of two independent assays done in triplicate.

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