Letters in Peptide Science, 4 (1997) 289–292
KLUWER/ESCOM

LIPS 191

Total structure and biological properties of laxaphycins A and B, cyclic lipopeptides from the marine cyanobacterium *Lyngbya majuscula*

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Received 14 March 1997
Accepted 17 May 1997

Keywords: Antifungal; Cytotoxic; Synergism; Marfey's reagent; *E*-didehydroaminobutyric acid; β-Aminodeca(octa)noic acids

SUMMARY

The tropical marine cyanobacterium *Lyngbya majuscula* produces a series of cytotoxic and antimicrobial cyclic peptides. The total structure of the two major components, laxaphycins A and B, was determined by interpretation of physical data, principally high field NMR, FAB MS and MS/MS, in combination with chemical derivatization and degradation schemes. Absolute stereochemistries of the natural and 'exotic' amino acids were determined. The two cyclic peptides exhibited an unusual biological synergism when tested for antifungal or cytotoxic effects.

INTRODUCTION

Some of the most exciting natural products discovered in recent years, cyclosporin A, FK-506, cyclotheonamides, didemnins, dolastatins and microcystins are strongly modified amino acid-derived metabolites. A large number of these peptides have been isolated from microorganisms or from symbiotic association between marine invertebrates and microorganisms. The characteristic features of these peptides are their cyclic nature and the inclusion of unusual amino acids such as D-amino acids, N-methyl amino acids, β-amino acids or α-β dehydro amino acids in the macrocycle.

From a filamentous cyanobacterium, *Lyngbya majuscula*, collected at Moorea atoll (French Polynesia), we isolated laxaphycins A and B. The gross structures of these lipopeptides were previously described by Frankmölle et al. [1] from a terrestrial cyanobacterium *Anabaena laxa*. In addition to ribosomal (S)-amino acids, these two cyclopeptides contain several exotic amino acids including didehydroaminobutanoic acid, Leu(3-OH), Asn(3-OH) or 3-aminoocta(deca)noic acids. We describe here the complete structure elucidation of laxaphycins A and B, including absolute stereochemistry, by means of a combination of mass spectrometry, NMR and amino acid analysis as well as cytotoxicity and antifungal evaluations.

RESULTS AND DISCUSSION

Structural elucidation

Examination of a peptidic lowbar silica gel chromatography fraction, obtained from the Et_2O extract, by C_8 RP-HPLC yielded two pure HPLC peaks.

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These two compounds were isolated as colourless amorphous solids and were negative to the ninhydrin test, suggesting a blocked N-terminus. NMR studies indicated that these two peaks were laxaphycins A and B.

The positive ions in the FAB mass spectrum of laxaphycin A gave some structural information, in addition to the parent ion ($M+H^+$) at m/z 1196.6. The FAB MS spectrum of laxaphycin B showed only the pseudomolecular ion m/z 1395.6, consistent with the molecular formula $C_{65}H_{115}N_{14}O_{19}$. Sequencing of residues in laxaphycins A and B was achieved by using the combined FAB MS/MS approach. In laxaphycin A, cleavage of the peptide bonds to each side of the α-β unsatured Dhb residue occurred preferably. The two generated acylium ions were decomposed further by both clockwise and counterclockwise successive residue losses. For laxaphycin B, preferential cleavages of Pro, *N*-Me-Ile and Asn(3-OH) amide bonds generated three series of weak and partial fragmentation pathways.

The NMR spectra of laxaphycins A and B were taken at 400 MHz in DMSO-d_6. It was obvious from the well-resolved ^{1}H NMR spectra that one conformation strongly dominated in this solvent; some minor peaks, especially in the N-methyl region (2.6–3 ppm), were apparent, indicating the presence of another minor conformer in slow exchange. Amino acid units of both peptides were characterized by detailed interpretation of 2D NMR spectra which included ^{1}H-^{1}H COSY, HOHAHA, ^{1}H-^{13}C COSY, HMQC and HMBC. The assignment of almost all ^{1}H and ^{13}C resonances was based on connectivity information transmitted via ^{1}H-^{1}H DQF-COSY, HMBC and HMQC. The sequential assignment was confirmed by interpretation of ROESY and HMBC data. The geometry of the tri-substituted olefin (Dhb residue) was shown to be E by NOE experiments: NOE's were found between the NH and vinyl proton as well as between the vinyl methyl and the OH proton of the adjacent Pro(4-OH). Laxaphycin A was shown to be cyclo-[βAoc-Hse-E-Dhb-Pro(4-OH)-Hse-Phe-Leu-Ile-Ile-Leu-Gly], and laxaphycin B to be cyclo-[βAde-Val-Leu(3-OH)-Ala-Leu(3-OH)-Gln-NMeIle-Asn(3-OH)-Thr-Pro-Leu-Thr].

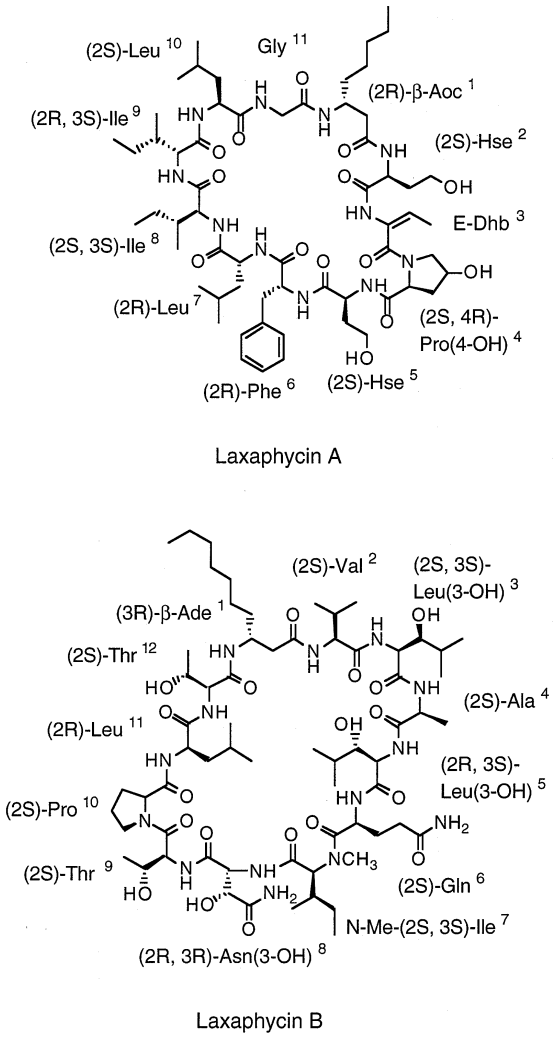

Fig. 1. Structures of laxaphycin A and laxaphycin B.

Hydrolysis of laxaphycins, followed by HPLC analysis of diastereoisomers formed on reaction of the amino acids with 1-fluoro-2,4-dinitrophenyl-5-(L-alaninamide) (FDAA or Marfey's reagent [2]), assigned the chirality of the amino acids. The absolute stereochemistry of the 'exotic' amino acids was determined in the same way after chiral synthesis of homologues and was confirmed by NMR and circular dichroism. D and D-allo-isoleucines were methylated according to the Freidinger procedure [3]. β Aoc and β Ade residues were synthesized according to the Gerwick method [4]. The four isomers

of 3-hydroxyleucines were stereospecifically synthesized via heterocyclic intermediates [cyclo(L-Val-Gly) and cyclo(D-Val-Gly)], following the Schöllkopf method [5]; co-injection of the four FDAA derivative synthetic standards with the FDAA derivatives from laxaphycine B acid hydrolysate defined the stereochemistry of one Leu(3-OH) as (2*R*,3*S*) and the other as (2*S*,3*S*). The relative and absolute stereochemistry of 3-hydroxyasparagine was deduced as follows: native Asn(3-OH) from laxaphycin B hydrolysis was collected as Asp(3-OH) after chromatographic separation on a cation exchange column. It was found to be threo by ^{1}H NMR comparison with standard LD-threo- and LD-erythro-3-hydroxyaspartic acids. Measurement of circular dichroism indicated D-configuration [6].

The location of the two isomers of Leu(3-OH) in the peptidic sequence in laxaphycin B was deduced by a combination of spectral analysis (3D molecular models of partial sequences based on NMR data) and chemical studies (partial hydrolysis of laxaphycin B and identification of progressive liberated amino acid units by the Marfey procedure).

We have shown that the structure of laxaphycin A is cyclo-[(3*R*)-βAoc-(2*S*)-Hse-(E)-Dhb-(2*S*,4*R*)-Pro(4-OH)-(2*S*)-Hse-(2*R*)-Phe-(2*R*)-Leu-(2*S*,3*S*)-Ile-(2*R*,3*S*)-Ile-(2*S*)-Leu-Gly], an [(E)-Dhb^3]-analog of hormotamnin A, described by Gerwick et al. [4]. The structure of laxaphycin B is cyclo-[(3*R*)-βAde-(2*S*)-Val-(2*S*,3*S*)-Leu(3-OH)-(2*S*)-Ala-(2*R*,3*S*)-Leu(3-OH)-(2*S*)-Gln-*N*-Me-(2*S*,3*S*)-Ile-(2*R*,3*R*)-Asn(3-OH)-(2*S*)-Thr-(2*S*)-Pro-(2*R*)-Leu-(2*S*)-Thr].

Biological evaluation

Table 1 summarizes the IC_{50} values obtained for laxaphycins A and B during screening against three cell lines. The cytotoxicity of laxaphycins was evaluated for the parent drug-sensitive CCRF-CEM human leukemic lymphoblasts, the CEM/VLB_{100} vinblastine-resistant subline which presents an MDR phenotype [7], and the CEM/VM-1 subline, usually referred to as atypical MDR cells [8]. The IC_{50} values for adriamycin were determined in these cell lines to provide a standard with which laxaphycins could be compared. Laxaphycin A was not active when tested at a concentration of 20 μM. Laxaphycin B showed pronounced cytotoxic activities on the drug-sensitive cells, with IC_{50} = 1.1 μM, and was practically equally active against the drug-sensitive cells and the drug-resistant cells. Both sublines showed no resistance to laxaphycin B, whereas those lines showed a 62- and 9-fold resistance to adriamycin. So, unlike the clinically used antitumor antibiotic adriamycin, laxaphycin B preserved equal cytotoxicity on Pgp-MDR cells and altered DNA-topoisomerase II-associated MDR cells.

TABLE 1
CYTOTOXIC PROPERTIES OF LAXAPHYCINS MEASURED WITH DRUG-SENSITIVE AND MULTIDRUG RESISTANT HUMAN TUMOUR CELL LINES IN VITRO

Drug	IC_{50} (μM)[a]		
	CCRF-CEM	CEM/VLB100	CEM/VM-1
Adriamycin	0.06 ± 0.02	3.71 ± 1.30 (65)[b]	0.55 ± 0.14 (10)
Laxaphycin A	> 20	> 20	> 20
Laxaphycin B	1.11 ± 0.15	1.02 ± 0.05	1.37 ± 0.15

[a] Data represent mean values ± SD for three separate experiments.
[b] Relative resistant index. Ratio between the IC_{50} value measured with the MDR cell line and that measured with the parent sensitive cell line.

At a concentration at which it is inactive, laxaphycin A exerted a synergistic effect with laxaphycin B, both in the biological activity against *Candida albicans* and in the growth inhibition of lymphoblastic cell lines. This was also observed by Frankmölle and co-workers [9] in the inhibition of *Aspergillus orizae* and in the cytotoxicity assays with KB cells.

Laxaphycin A was not active on *Candida albicans*, but potentialized the activity of laxaphycin B. With 1 μM of laxaphycin A, the IC_{50} of laxaphycin B on human lymphoblastic cells was threefold reduced. Furthermore, this synergistic effect was observable both for drug-sensitive cells and the drug-resistant cell lines.

CONCLUSIONS

Laxaphycins A and B are two lipopeptides coproduced by the same strain of the cyanobacterium *Lyngbya majuscula*, but these two cyclopeptides are structurally different. Laxaphycin A is a cyclic undecapeptide in which the sequence shows an interesting

segregation of hydrophobic and hydrophilic residues. Laxaphycin B is a cyclic dodecapeptide in which hydrophobic and hydrophilic residues are alternated. These two compounds are characterized by the presence of a C_8-C_{10} aliphatic D-β-amino acid. This β-amino fatty acid is a common structural characteristic of a number of lipopeptides with antifungal activity extracted from bacteria.

It is interesting to note that hormothamnin A, a cyclic peptide closely related to the inactive laxaphycin A, differing just in the stereochemistry of the Dhb unit, was described with antimicrobial and cytotoxic activities. According to Gerwick's results [10], hormothamnin A was found to be highly cytotoxic to a variety of cancer cell lines in tissue culture. These results suggest that the configuration of the Dhb residue is a major feature in the cytotoxicity of hormothamnin A. The location of the Dhb unit, which is sandwiched between the three hydrophilic residues in the molecule, may be important to the cytotoxic, antimicrobial and possible chemical defense properties of hormothamnin A. Synthesis of the two peptides, in progress in our laboratory, will allow us to determine the effect of the stereochemistry of Dhb in the toxicity of these compounds.

Synergism between two compounds coproduced by the same organism has rarely been described in the past. The synergistic effect of surfactin on iturin A, two related lipopeptides isolated from the same strain of *Bacillus subtilis*, was pointed out in 1992 [11]. Iturin A and surfactin are cyclooctapeptides containing a C_{14}-C_{17} aliphatic β-amino or β-hydroxy acid. The natural occurrence of these lipopeptide associations in the same organism suggests that the complex might be involved in the cell growth regulation of the producer microorganism or in the cell growth inhibition of competitor microorganisms.

ACKNOWLEDGEMENTS

This work was supported in part by research grants from INSERM. I.B. was supported by La Ligue Nationale Française Contre le Cancer. We wish to thank Dr. Danielle Promé for accurate mass measurements. The lymphoblastic leukemia cell lines were obtained from Dr. W.T. Beck, St. Jude Children's Research Hospital, Memphis, TN, U.S.A. The cyanobacterium, Lyngbya majuscula, was collected by Dr. Bernard Salvat (EPHE).

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