

ANTIFUNGAL CYCLIC PEPTIDES FROM THE TERRESTRIAL
BLUE-GREEN ALGA *Anabaena laxa*

I. ISOLATION AND BIOLOGICAL PROPERTIES

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Laxaphycins are responsible for the antifungal and cytotoxic activity of crude ethanolic extracts from the cultured blue-green alga *Anabaena laxa*. These cyclic peptides exhibit an unusual biological synergism when tested for antifungal or cytotoxic effects. The isolation procedure for the peptides, their characterization and biological activities are described here along with experiments demonstrating synergism between the two major laxaphycins.

Blue-green algae (cyanobacteria) have been recognized in the last several years as a source of novel cytotoxic and antifungal metabolites. Some of these metabolites are providing potential leads for the development of new pharmaceutical compounds.

Known antifungal compounds are frequently toxic when used at levels high enough to be reliably therapeutic, especially for the systemic infections encountered in immunocompromised patients. New families of compounds that exhibit synergism either with one another or with known antifungal drugs offer the potential for reducing required dosages, thereby mitigating toxicity and providing more effective antifungal treatment regimens.

We report here the isolation of a group of cyclic peptides from a cyanophyte that act synergistically with one another to inhibit the growth of fungi and yeasts. These cyclic peptides also show synergistic cytotoxic effects.

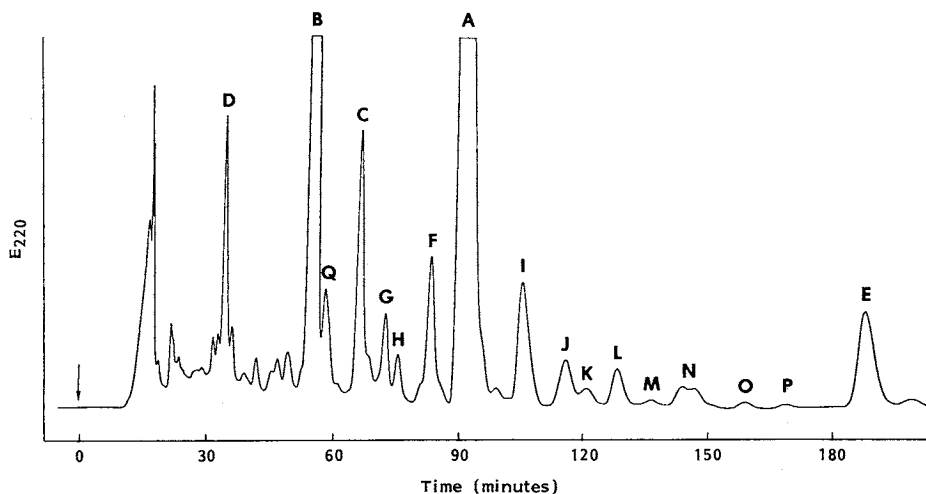
Isolation and Characterization

The cyclic peptides were isolated from the methanol-soluble portion of a crude 7:3 ethanol-water extract of the cyanophyte *Anabaena laxa* Rabenhorst (UH strain FK-1-2) by reversed-phase chromatography. The total yield of peptides based on the dried weight of the alga was 1.3%. The complexity of the mixture of peptides is indicated by the HPLC chromatogram shown in Fig. 1. All marked peaks correspond to cyclic peptides with molecular weights ranging from 1,150 to 1,400 daltons as determined by FAB mass spectrometry. Unmarked peaks probably correspond to additional cyclic peptides that have not yet been characterized. Table 1 lists the yield, relative abundance in the crude extract, and the molecular weight for the individual laxaphycins isolated.

Laxaphycins A, B, C, D, and E have been purified by reversed-phase HPLC, while the remaining characterized peptides have been only partially purified. Peaks marked H, I, and P are known to be mixtures. The two peaks marked N correspond to a laxaphycin in the expected mass range and to another compound which is probably not a cyclic peptide.

All the peptides isolated were found to fall into two groups—those related to laxaphycin A with

Fig. 1. HPLC chromatogram showing the separation of the laxaphycins on C-18 under isocratic conditions.



(CH₃CN - H₂O (48 : 52), YMC RP-C18, 5 μm, 22.5 × 250 mm, 3 ml/minute, UV detection at 220 nm).

Table 1. Yield and molecular weight of laxaphycins isolated from 9.1 g crude extract (from 56.7 g dry alga).

Laxaphycin	mg (% yield)	MW	Laxaphycin	mg (% yield)	MW
A	381.0 (53.0)	1,195	J	7.6 (1.1)	1,211
B	214.2 (29.8)	1,394	K	2.6 (0.4)	1,351
C	45.0 (6.3)	1,378	L	1.2 (0.2)	1,211
D	28.8 (4.0)	1,366	M	6.4 (0.9)	1,197
E	11.3 (1.6)	1,223	N	0.1 (<0.1)	1,179, 789
F	4.5 (0.6)	1,362	O	0.1 (<0.1)	1,151
G	1.9 (0.3)	1,181	P	0.1 (<0.1)	1,223, 1,209
H	3.4 (0.5)	1,378, 1,289	Q	0.1 (<0.1)	1,378
I	10.0 (1.4)	1,367, 1,211, 1,183			

molecular weights between 1,150 and 1,290 and those related to laxaphycin B with molecular weights between 1,350 and 1,400.

Biological Activity

The crude extracts of *A. laxa* were tested for antiproliferative effects using five fungi and two tumor cell lines, human epidermoid carcinoma (KB) and human colorectal adenocarcinoma (LoVo). They were found to be both antifungal in an agar diffusion assay and cytotoxic to KB and LoVo cells. Surprisingly, when the individual peptides were tested in the same assays most of the activity appeared to have been lost, with laxaphycin A showing no activity at all and laxaphycin B showing greatly diminished activity. When the HPLC fractions containing the individual peptides were recombined, however, the level of activity that had been originally seen reappeared. Results of the bioassays are shown in Table 2.

It was noted that in the agar diffusion assay, most markedly against *Aspergillus oryzae*, a zone of inhibition appeared between laxaphycins A or E and B, C, or D when filter paper disks impregnated with these peptides were placed near each other on the agar plate. Fig. 2 demonstrates this phenomenon using laxaphycins A and B. No such zone appeared between laxaphycins having similar molecular weights, i.e. A and E, B and C, B and D, or C and D, under the same conditions. As a result it was decided to

Table 2. Biological activity of cyclic peptides isolated from *Anabaena laxa*.

Laxaphycin	Inhibition zone (mm) in agar diffusion					Cytotoxicity (MIC) ($\mu\text{g}/\text{ml}$)	
	AO	CA	PN	SC	TM	KB	LoVo
Mixture ^a	26	19	26	17	19	nd	nd
A	0	0	0	0	0	>10	>10
B	19 (h)	8 (h)	0	12 (d)	9 (hh)	0.12	0.5
C	14	9	11	12	0	0.1	0.5
D	0	0	0	0	0	5	5
E	0	0	0	0	0	>10	>10

^a Crude mixture of all peptides following separation on HPLC with $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ (75:25).

Test organisms: AO, *Aspergillus oryzae*; CA, *Candida albicans*; PN, *Penicillium notatum*; SC, *Saccharomyces cerevisiae*; TM, *Trichophyton mentagrophytes*.

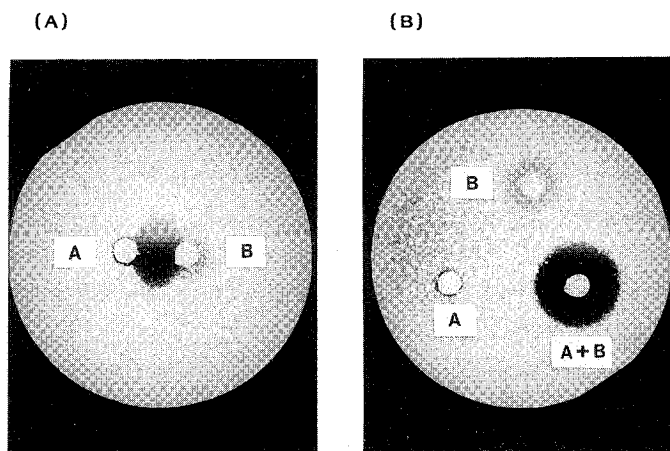
Human cell lines: KB, epidermoid carcinoma; LoVo, colorectal adenocarcinoma.

In agar diffusion the mixture and all compounds were tested at $50 \mu\text{g}/\text{disk}$.

(h), Hazy zone; (hh), very hazy zone; (d), edge of zone diffuse; nd, not done.

Fig. 2. Agar diffusion assay showing possible synergism between laxaphycin A and laxaphycin B ($125 \mu\text{g}/\text{disk}$) in the inhibition of *A. oryzae*.

(A) Laxaphycin A and B on separate disks next to one another. (B) Laxaphycin A and B alone and combined.



determine whether a synergistic reaction might be occurring between type A laxaphycins and type B laxaphycins. These investigations were carried out only with purified A and B, the major peptides present in the alga.

The antifungal agar diffusion assay was repeated using the five fungal strains originally tested. Laxaphycins A and B, both alone and in various combinations were tested. The results are shown in Table 3. All combinations of A and B appeared to be synergistic, but the ratio in which they were combined was not important. All combinations produced similar zones of inhibition. Further studies were conducted in a broth dilution assay adapted from CHMEL and LOURIA¹⁾ using *A. oryzae*, the most susceptible of our five test fungi to the crude algal extract. MICs were determined for peptides A and B individually and for mixtures of the two in various ratios that were diluted in two-fold steps. As in the agar diffusion assay, A alone was not active at the highest concentration tested, $128 \mu\text{g}/\text{ml}$. Laxaphycin B had an MIC of $64 \mu\text{g}/\text{ml}$. Table 4 shows the results obtained when B at its individual MIC was mixed with $128 \mu\text{g}/\text{ml}$ A and then diluted, when a constant dose of A ($128 \mu\text{g}/\text{ml}$) was mixed with a diminishing dose of B, and

Table 3. Antifungal agar diffusion assays demonstrating synergism between purified laxaphycins A and B.

Laxaphycin	$\mu\text{g}/\text{disk}$	Ratio	Inhibition zone (mm)				
			AO	CA	PN	SC	TM
A	50	—	0	0	0	0	0
B	50	—	19 (h)	8 (h)	0	12 (d)	9 (hh)
A+B	25+25	1:1	29	23	30	22	30
A+B	25+50	1:2	27	26	29	20	29
A+B	25+75	1:3	27	25	29	21	29
A+B	50+25	2:1	28	25	29	18	26
A+B	75+25	3:1	27	23	29	18	29

Test organisms and abbreviations as for Table 2.

Table 4. Broth dilution assays with *A. oryzae* demonstrating the extent of synergism between laxaphycins A and B.

Dose B ($\mu\text{g}/\text{ml}$)	Dose A ($\mu\text{g}/\text{ml}$)							
	128	64	32	16	8	4	2	1
128	0	0	0	0	0	0	0	0
64	0	0	0	0	0	0	0	0
32	0	0	0	0	0	0	+/-	+
16	0	nd	0	nd	0	+/-	+	+
8	0	nd	nd	0	+	+	+	+
4	0	0	0	+	+	+	+	+
2	+	nd	nd	nd	nd	+	nd	nd
1	+	nd	nd	nd	nd	nd	+	nd

0, growth inhibition; +, growth; nd, not done.

All combinations tested in duplicate in any one experiment, all combinations tested in at least two experiments.

when a constant dose of B (32 $\mu\text{g}/\text{ml}$) was mixed with a diminishing dose of A. A mixture of A and B in a ratio of 3:2, the ratio in which the two peptides were found in the crude extract, was also tested diluted in two-fold steps (data not shown). Whereas the least amount of an individual laxaphycin (B) necessary to inhibit the growth of *A. oryzae* was 64 $\mu\text{g}/\text{ml}$, only 16 $\mu\text{g}/\text{ml}$ (9.6 μg A + 6.4 μg B) of a mixture of A and B was required to produce inhibition. This combination also resulted in the lowest interaction index (see Table 5) indicating that it was the most synergistic. The interaction index represents the sum of the fractional inhibitory concentrations of the drugs in combination. Theoretically, an interaction index of 1 indicates an additive effect and any value < 1 denotes synergism. The lower the value, the greater the degree of synergism. HALLANDER *et al.*²⁾ found that, because of day-to-day variations in MIC testing procedures, 0.5 was the highest value indicating synergism that could be reliably reproduced.

As in the agar diffusion assays, it did not appear that the ratio of the two peptides played an important role in the synergism since the MICs and interaction indices of the mixtures were similar whether the A : B

Table 5. Interaction indices for combinations of laxaphycins A (MIC > 128 $\mu\text{g}/\text{ml}$) and B (MIC = 64 $\mu\text{g}/\text{ml}$).

Combination ($\mu\text{gA}/\mu\text{gB}$)	Total dose A+B ($\mu\text{g}/\text{ml}$)	Interaction index
128/4	132	< 1.0625
64/4	68	< 0.5625
32/4	36	< 0.3125
16/8	24	< 0.25
8/16	24	< 0.3125
4/32	36	< 0.53125
4/16	20	< 0.28125
2/32	34	< 0.5156
9.6/6.4	16	< 0.175

ratio was 3:2 (9.6 μg :6.4 μg), 2:1 (16 μg :8 μg), or 1:2 (8 μg :16 μg).

The culture medium in which the assay was performed did not appear to affect the level of activity observed. Broth dilution assays were initially performed in Potato Dextrose Broth. Reports in the literature of suppression of antifungal activity when similar undefined culture media was used³⁾ prompted us to repeat the MIC assays for A and B individually in a chemically defined medium, modified yeast nitrogen glucose broth.⁴⁾ The MICs were identical to those determined using Potato Dextrose Broth.

To determine whether the laxaphycins might also act synergistically with known classes of antifungal compounds, broth dilution assays were run on combinations of laxaphycin A or B and amphotericin B, a cell membrane active polyene; miconazole, an imidazole also thought to act by disrupting the cell membrane; 5-fluorocytosine, a nucleic acid synthesis inhibitor; and cycloheximide, a protein synthesis inhibitor. Again the assays were performed using *A. oryzae*. While amphotericin B and miconazole inhibited the growth of this organism at MICs of 1.56 $\mu\text{g}/\text{ml}$ and 0.8 $\mu\text{g}/\text{ml}$, respectively, no synergism was noted when these compounds were combined at concentrations lower than their MICs with laxaphycin A or B. Cycloheximide and 5-fluorocytosine did not inhibit the growth of *A. oryzae* either individually or in combination with either laxaphycin at the concentrations tested.

Cytotoxicity assays with KB cells were also run on purified laxaphycins A and B, both separately and in a mixture identical to that used for the broth dilution antifungal tests where peptides A and B were combined in a 3:2 ratio. Laxaphycin A was not cytotoxic at 10 $\mu\text{g}/\text{ml}$, the highest concentration tested, while the MIC for B was 0.12 $\mu\text{g}/\text{ml}$. The MIC of the mixture was 0.06 μg A + 0.04 μg B (0.1 μg total peptide concentration). A maximum interaction index of 0.339 indicated that the two peptides act synergistically to produce cytotoxicity.

Discussion

In most synergism studies of antifungal or antibiotic compounds reported to date, the synergistic effect appears to result from the combination of drugs that exert their antifungal effects through different mechanisms, one of which facilitates the other. MEDOFF *et al.*⁵⁾ showed that the growth of a variety of yeasts normally resistant to 5-fluorocytosine was inhibited when this drug was administered in combination with amphotericin B. It was hypothesized that the synergistic activity was due to the increased permeability of the yeast's cell membrane induced by amphotericin B which allowed the 5-fluorocytosine to enter the cell in greater concentration to inhibit its nucleic acid synthesis *via* the pyrimidine biosynthetic pathway. Neither 5-fluorocytosine nor amphotericin B acted synergistically with either laxaphycin A or B. Since the type A and type B laxaphycins are structurally very similar to each other, it seems that they probably act on cells *via* the same mechanism and not by one facilitating the other as described above.

Synergistic reactions between structurally similar peptides have been described by LEHRER *et al.*⁶⁾ for some rabbit granulocyte peptides known as defensins. Six of these cysteine-rich peptides were isolated, three of which inhibited the growth of *Candida albicans* alone and one of which was inactive but acted synergistically to increase the activity of the other three. The mechanism for this synergism was not reported.

In addition, a group of antibiotics known as synergistins, which are structurally complex, consisting of a cyclic peptide and a macrocyclic lactone, were shown by ENNIS⁷⁾ to react in a strongly synergistic manner to inhibit Gram-positive bacteria. These compounds act by interfering with bacterial protein synthesis at the amino acid polymerization step. The synergism between synergistins A (the macrocyclic lactone part of the complex) and B (the cyclic peptide part of the complex) results from B acting in some way on the ribosome that permits A to bind to it more avidly.

Studies to elucidate the mechanism of the synergistic action of laxaphycin A with laxaphycin B are currently in progress in our laboratory as are efforts to purify the remaining A-type and B-type minor cyclic peptides. We hypothesize that the antifungal and cytotoxic activity exhibited by these minor

compounds (see Table 2) may result from their contamination with small amounts of laxaphycins from the other subgroup rather than from the compounds' inherent activity.

Experimental

Culture Conditions

Anabaena laxa Rabenhorst (UH strain FK-1-2) was isolated from a mud sample collected on the University of Hawaii at Manoa campus. Clonal cultures were prepared by repeated subculture on solidified media. The alga was cultured in 20-liter carboys containing an inorganic medium, designated A₃M₇.⁸⁾ Prior to autoclaving, the pH of the medium was adjusted to 7.0 with sodium hydroxide. Cultures were illuminated continuously at an incident intensity of 300 $\mu\text{Einstein m}^{-2} \text{s}^{-1}$ from banks of cool white fluorescent tubes, aerated at a rate of 5 liter/minute with a mixture of 0.5% CO₂ in air, and incubated at a temperature of 24 \pm 1 °C. The alga was harvested after a period of six weeks by centrifugation. Yields of lyophilized cells were typically 0.3 to 0.4 g/liter.

Isolation of Individual Laxaphycins

The freeze-dried alga (56.7 g) was extracted with 2 \times 1 liter EtOH - H₂O (7 : 3) to give 9.1 g crude extract. Extraction of the latter with 100% MeOH yielded 6.6 g of methanolic extract, which was subjected to gel filtration on Sephadex LH-20 (active column size 5 \times 45 cm, flow rate 80 ml/hour, fraction size 18 ml, solvent 100% MeOH). The individual fractions were examined by bioassay. Activity was found in three consecutive fractions, which were recombined. After evaporation of the solvent the laxaphycin mixture was further purified using C-18 reversed-phase flash column chromatography with 100% MeOH (active column size 2.5 \times 45 cm, flow rate 80 ml/hour, fraction size 18 ml). The solvent of the active fraction was evaporated and the peptide mixture purified by reversed-phase HPLC (YMC RP-C18, 5 μm , 22.5 \times 250 mm, 75 : 25 CH₃CN - H₂O, 6 ml/minute, UV detection at 220 nm). The individual laxaphycins were then further separated with 48 : 52 CH₃CN - H₂O on C-18 reversed-phase HPLC (same conditions as above). Following this procedure laxaphycins A and E were pure enough for spectral analysis, but to obtain pure compounds, especially for the minor laxaphycins, it was necessary to reinject each of the laxaphycin fractions under slightly modified conditions. Laxaphycin B, for example, was further purified on reversed-phase HPLC with 37 : 63 CH₃CN - H₂O, laxaphycin C with 40 : 70, and laxaphycin D with 35 : 65.

Determination of Biological Activity

Antifungal activity of the individual laxaphycins was determined by an agar diffusion method adapted from SHADOMY *et al.*⁹⁾ employing 6 mm filter paper disks against three filamentous fungi (*Aspergillus oryzae*; *Penicillium notatum*; and *Trichophyton mentagrophytes*, strain A-23) and two yeast-like organisms (*Candida albicans*, strain A-26, and *Saccharomyces cerevisiae*, strain M-25). Any visible zone of growth inhibition was considered a positive reaction.

Cytotoxicity to KB (ATCC CCL 17) and LoVo (ATCC CCL 229) cells was estimated in an assay previously described by PATTERSON *et al.*¹⁰⁾

An adaptation of the broth dilution method described by CHMEL and LOURIA¹⁾ was used to determine the MICs of laxaphycins A and B, amphotericin B (Fungizone) (Gibco Laboratories, Grand Island, NY), miconazole, 5-fluorocytosine, and cycloheximide (all Sigma Chemical Co., St. Louis), against *A. oryzae*. Briefly, 128 μg peptide dissolved in 100 μl MeOH was added to 0.9 ml Potato Dextrose Broth (PDB) (Difco, Detroit) in each of two 13 \times 100 mm test tubes. Serial two-fold dilutions were prepared resulting in peptide concentrations that ranged from 128 $\mu\text{g}/\text{ml}$ to 1 $\mu\text{g}/\text{ml}$. To each tube and to a control tube containing only PDB was added 0.1 ml of a standardized suspension of *A. oryzae* spores obtained by washing the surface of a 4 ~ 7 day old culture with a phosphate buffer of pH 7.4 containing 0.025% Tween 80. Spore counts were adjusted to 1 \times 10⁵ spores/ml as estimated by counting in a hemacytometer.¹¹⁾ The tubes were incubated at 37°C for 48 hours. Fungal growth was estimated visually. The MIC was defined as that concentration of compound causing complete inhibition of growth.

Antifungal synergism was measured using the broth dilution technique described above with mixtures of the two laxaphycins in the following ratios:

- 1) B at its individual MIC (64 $\mu\text{g}/\text{ml}$) plus A at the highest concentration at which it was tested

- individually (128 $\mu\text{g/ml}$) since there was no activity at any concentration tested,
- 2) a constant dose of A (128 $\mu\text{g/ml}$) plus a diminishing dose of B,
 - 3) a constant dose of B at 1/2 of its MIC (32 $\mu\text{g/ml}$) plus a diminishing dose of A,
 - 4) and A plus B in the ratio that they occur in the crude extract ("natural abundance ratio"), 3:2 (154 $\mu\text{g/ml}$:102 $\mu\text{g/ml}$).

The MICs of the mixtures were defined as for the individual peptides.

Interaction indices were calculated by the method of HALLANDER *et al.*²⁾, by the following formula:

$$\text{MIC-A}_{\text{combined}}/\text{MIC-A}_{\text{alone}} + \text{MIC-B}_{\text{combined}}/\text{MIC-B}_{\text{alone}}$$

A sum of 0.5 or less was considered indicative of synergism.

Synergism between the laxaphycins and amphotericin B, miconazole, 5-fluorocytosine, and cycloheximide was measured as for laxaphycin A and B in the ratio described in 1) above. MICs and interaction indices were defined as described.

Cytotoxic synergism in KB cells was determined in the assay described previously by adding mixtures of laxaphycins A and B in the ratios described for the antifungal synergism tests to the KB cells. MICs and interaction indices were defined as described.

Acknowledgments

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