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

II. STRUCTURES OF LAXAPHYCINS A, B, D AND E

WALTER P. FRANKMÖLLE, GEORG KNÜBEL, RICHARD E. MOORE* and GREGORY M. L. PATTERSON

Department of Chemistry, University of Hawaii, Honolulu, Hawaii 96822, U.S.A.

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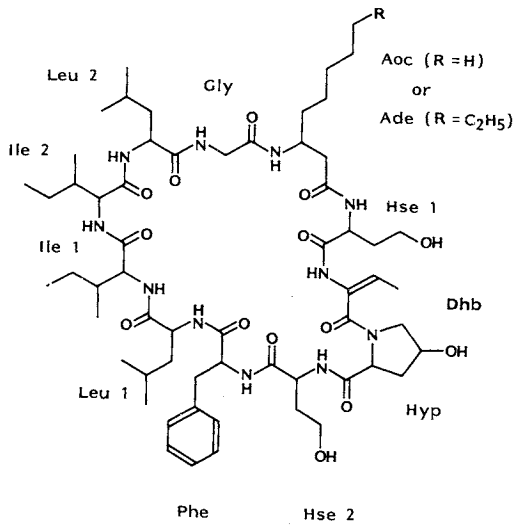
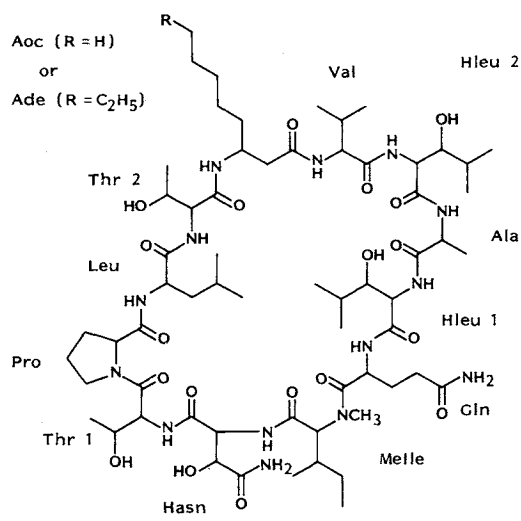
Laxaphycins A and B are the major components in an antifungal mixture of cyclic peptides from the terrestrial blue-green alga *Anabaena laxa* FK-1-2. NMR and MS spectral studies coupled with amino acid analysis indicate that the gross structures of laxaphycins A and B are cyclic (Aoc-Hse-*E*-Dhb-Hyp-Hse-Phe-Leu-Ile-Ile-Leu-Gly) where Aoc is a 3-aminooctanoic acid residue and cyclic (Ala-Hleu-Gln-*N*-MeIle-Hasn-Thr-Pro-Leu-Thr-Ade-Val-Hleu) where Ade is a 3-aminodecanoyl unit, respectively. Laxaphycin E, a minor cyclic undecapeptide, differs in gross structure from laxaphycin A in possessing a 3-aminodecanoic acid unit (Ade) in lieu of Aoc, whereas laxaphycin D, a minor cyclic dodecapeptide, differs from laxaphycin B in possessing a 3-aminooctanoyl unit (Aoc) instead of an Ade unit.

The lipophilic extract of the blue-green alga *Anabaena laxa* Rabenhorst (UH strain FK-1-2) exhibits broad-spectrum antifungal activity.[1] Fractionation of the extract by successive gel filtration and reversed-phase flash column chromatography leads to a mixture of cyclic peptides which accounts for all of the antifungal activity. Further separation by HPLC results in the isolation of two major peptides, laxaphycins A and B, and over fifteen minor laxaphycins.[1] Interestingly, when the individual major peptides are tested in the same assay, laxaphycin A is totally inactive and laxaphycin B shows greatly diminished



laxaphycin A (R = H)
laxaphycin E ($R = C_2H_5$)



laxaphycin B ($R = C_2H_5$)
laxaphycin D (R = H)

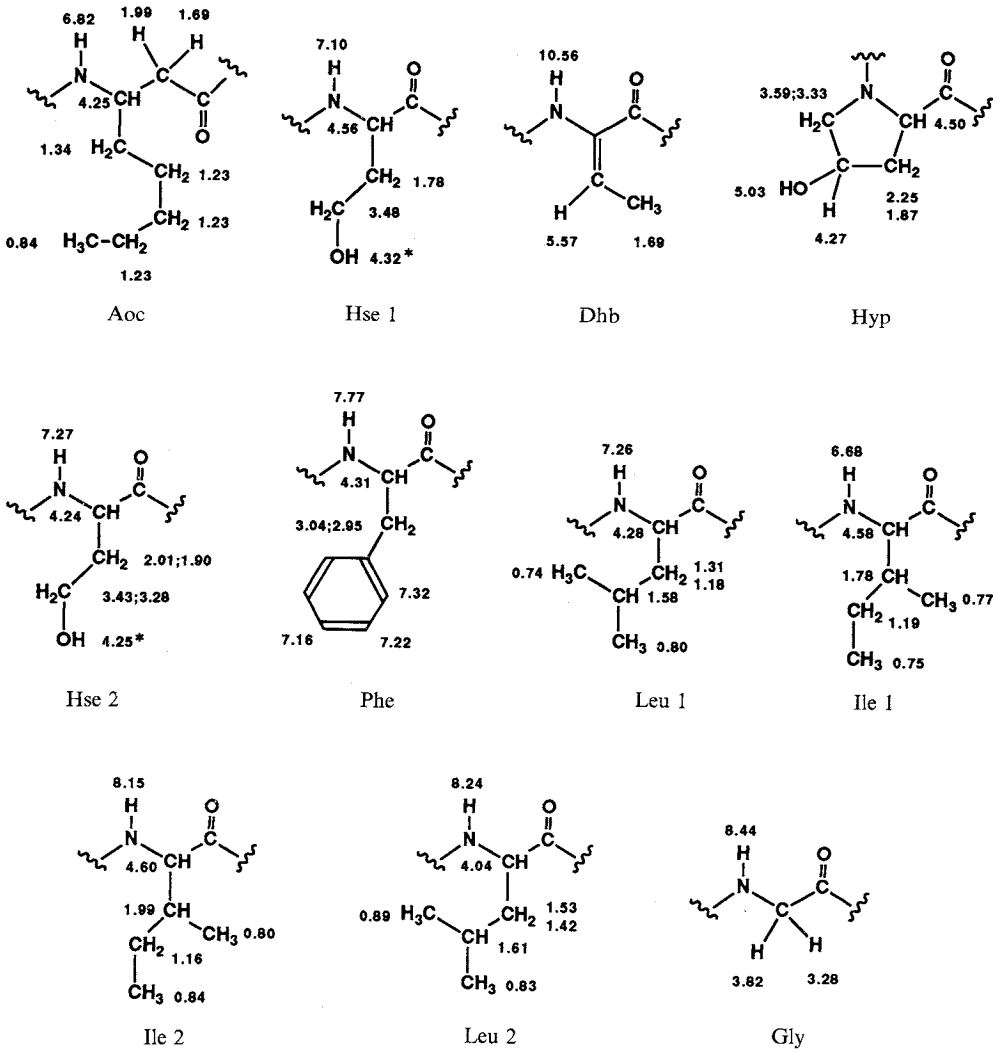
activity. When laxaphycin A, a cyclic undecapeptide, and laxaphycin B, a cyclic dodecapeptide, are recombined, however, potent antifungal activity is synergistically induced.[1] Each of the minor peptides in the alga appears to be structurally and biologically related to either laxaphycin A or B. For example, laxaphycin E belongs to the laxaphycin A class and laxaphycin D belongs to the laxaphycin B class. In the preceding paper we described the isolation and biological properties of the laxaphycins. In this paper we report the gross structures of laxaphycins A, B, D and E.

Laxaphycins A and E

The FAB mass spectrum indicated that laxaphycin A had a molecular weight of 1,195 daltons. Detailed NMR analysis (*e.g.* ^{1}H-^{1}H phase-sensitive COSY and HOHAHA[2]; ^{1}H-^{13}C HMQC[3] and HMBC[4]) in MeOH-d_4, MeOH-d_3, and DMSO-d_6 suggested $C_{60}H_{97}N_{11}O_{14}$ as the molecular formula and established

Fig. 1. Gross structures of amino acid units in laxaphycin A showing ^{1}H chemical shifts in DMSO-d_6 at 60°C.

Chemical shifts for Hse OH signals (*) determined at 35°C.



the presence of the eleven amino acid substructures shown in Fig. 1, *viz.*, two leucine (Leu), two isoleucine (Ile), two homoserine (Hse), phenylalanine (Phe), glycine (Gly), 4-hydroxyproline (Hyp), didehydrobutyrine (Dhb) and 3-aminooctanoic acid (Aoc) residues.

Amino acid analysis confirmed the proposed substructures, and furthermore showed that the two isoleucine units were diastereomeric. Comparison of $J_{2,3}$ in the Ile 1 (3.5 Hz) and Ile 2 (4.4 Hz) units of laxaphycin A in MeOH-d_4 with the coupling constants noted for L-*threo*-isoleucine (3.9 Hz) and L-*allo*-isoleucine (3.6 Hz) in D_2O did not unambiguously differentiate Ile 1 and Ile 2.

Strong NOEs between 2-H and 3-H (2.25 ppm) and between 3-H' and 4-H (1.87 ppm) in the Hyp unit, detected in a 2D-ROESY[5,6] experiment in MeOH-d_4, indicated that Hyp was the *trans* diastereomer, *i.e.* *trans*-4-hydroxyproline. A strong NOE was also seen between the NH and 3-H in the Dhb unit in DMSO-d_6, which meant that the geometry of the double bond was *E*.[7]

Sequencing of the amino acid units into a total gross structure was accomplished with the following three experiments:

1. An HMBC spectrum in MeOH-d_4 allowed us to see ^3J-coupling between the carbonyl-carbon of amino acid unit n and the α-proton of the adjacent amino acid unit n+1. Couplings were detected between C-1 of Dhb and 2-H of Hyp, C-1 of Hyp and 2-H of Hse 2, C-1 of Phe and 2-H of Leu 1, C-1 of Ile 1 and 2-H of Ile 2, C-1 of Leu 2 and 2-H_2 of Gly (both protons), and C-1 of Aoc and 2-H of Hse 1. These data generated partial structures Dhb-Hyp-Hse 2, Phe-Leu1, Ile 1-Ile 2, Leu 2-Gly, and Aoc-Hse 1.

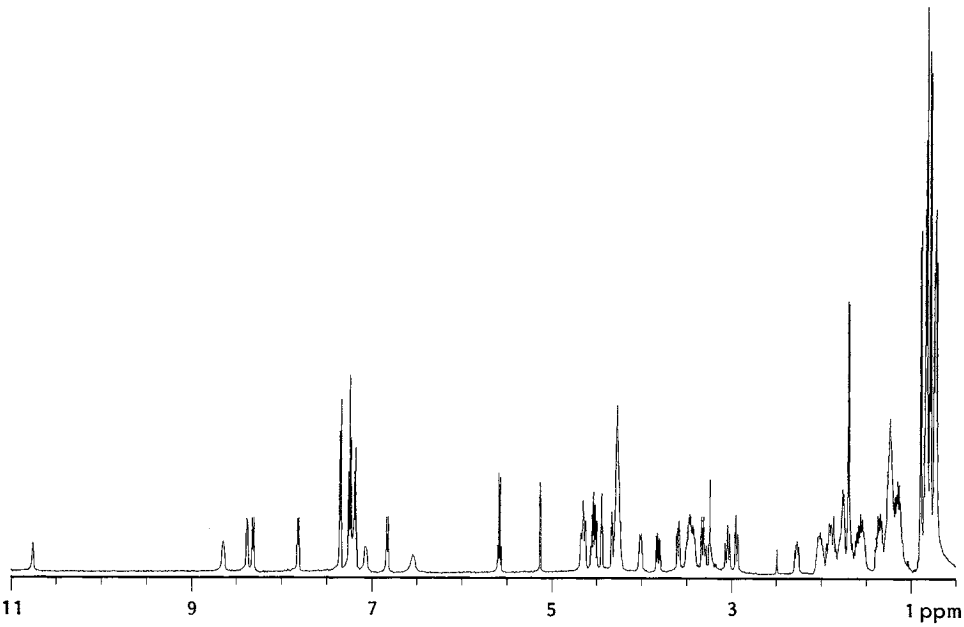
2. An HMBC spectrum in MeOH-d_3 enabled us to detect ^2J-correlations between the carbonyl-carbon of amino acid unit n and the NH-proton of amino acid unit n+1. Cross peaks could be seen between C-1 of Hyp and NH of Hse 2, C-1 of Hse 2 and NH of Phe, C-1 of Phe and NH of Leu 1, C-1 of Leu 1 and NH of Ile 1, C-1 of Ile 1 and NH of Ile 2, C-1 of Ile 2 and NH of Leu 2, C-1 of Leu 2 and NH of Gly, and C-1 of Gly and NH of Aoc. The data verified the partial structures implied from the first HMBC experiment and permitted the linking of these substructures into the linear structure Dhb-Hyp-Hse 2-Phe-Leu 1-Ile 1-Ile 2-Leu 2-Gly-Aoc-Hse 1. Coupling could not be seen between C-1 of Hse 1 and the NH of Dhb, however, to allow an unambiguous connection of these two units into a total gross structure for laxaphycin A. Nevertheless, by process of elimination Hse 1 had to be connected to Dhb.

3. A 2D-ROESY spectrum in MeOH-d_3 allowed us to confirm sections of the gross structure deduced from the HMBC experiments. NOEs could be observed between the α-proton of amino acid unit n and the NH-proton of adjacent amino acid unit n+1, *viz.* between 2-H of Ile 1 and NH of Ile 2, 2-H of Ile 2 and NH of Leu 2, 2-H_2 of Gly (both protons) and NH of Aoc, and 2-H of Aoc and NH of Hse 1. In addition NOEs could be seen between the NH-protons of Hse 2 and Phe and between the NHs of Leu 1 and Ile 1, which were consistent with the proposed sequence. Since the NH signal for Dhb could not be seen in MeOH-d_3, the amide bond between the Dhb and Hse 1 units could not be verified by the ROESY experiment in MeOH-d_3; however, the Dhb NH signal was clearly visible in DMSO-d_6 where it showed a significant NOE in the ROESY spectrum with 2-H of Hse 1.

The gross structure of laxaphycin A was therefore concluded to be cyclic[Aoc-Hse-*E*-Dhb-Hyp-Hse-Phe-Leu-Ile-Ile-Leu-Gly].

The FAB mass spectrum of laxaphycin E indicated a molecular weight (1,223 daltons) that was 28 mass units higher than that observed for laxaphycin A. The ^1H and ^{13}C NMR spectra of the two compounds were essentially identical except for appreciable differences in chemical shift and intensity of signals associated with the β-aminoalkanoic acid unit. When the 500 MHz ^1H NMR spectra of laxaphycins A

Fig. 2. 500 MHz ^{1}H NMR spectrum of laxaphycin A in DMSO-d_6 at 35°C.



(Fig. 2) and E in DMSO-d_6 were compared, the broad, complex signal centered at 1.23 ppm integrated for four additional methylene protons in the case of laxaphycin E, strongly suggesting that the molecular formula of laxaphycin E was $C_{62}H_{101}N_{11}O_{14}$ and that a 3-aminodecanoic acid unit (Ade) was present instead of the Aoc unit. Amino acid analysis and detailed NMR analysis confirmed the proposed structure.

After completion of our work on the gross structure of laxaphycin A, we learned that the GERWICK group at Oregon State University (personal communication) had completed the total structure elucidation of hormothamnin A, the major cytotoxic cyclic peptide from the marine blue-green alga *Hormothamnion enteromorphoides*,[8] and found it to be closely related to laxaphycin A (*Hormothamnion* and *Anabaena* are members of the family Nostocaceae). Hormothamnin A shows a longer retention time (107 minutes) than laxaphycin A (90 minutes) on reversed-phase HPLC with 48:52 acetonitrile-water (YMC RP-C18, 22.5×250 mm column, 6 ml/minute flow rate)[1] and its structure differs in at least one place, *viz.* the geometry of the Dhb unit.

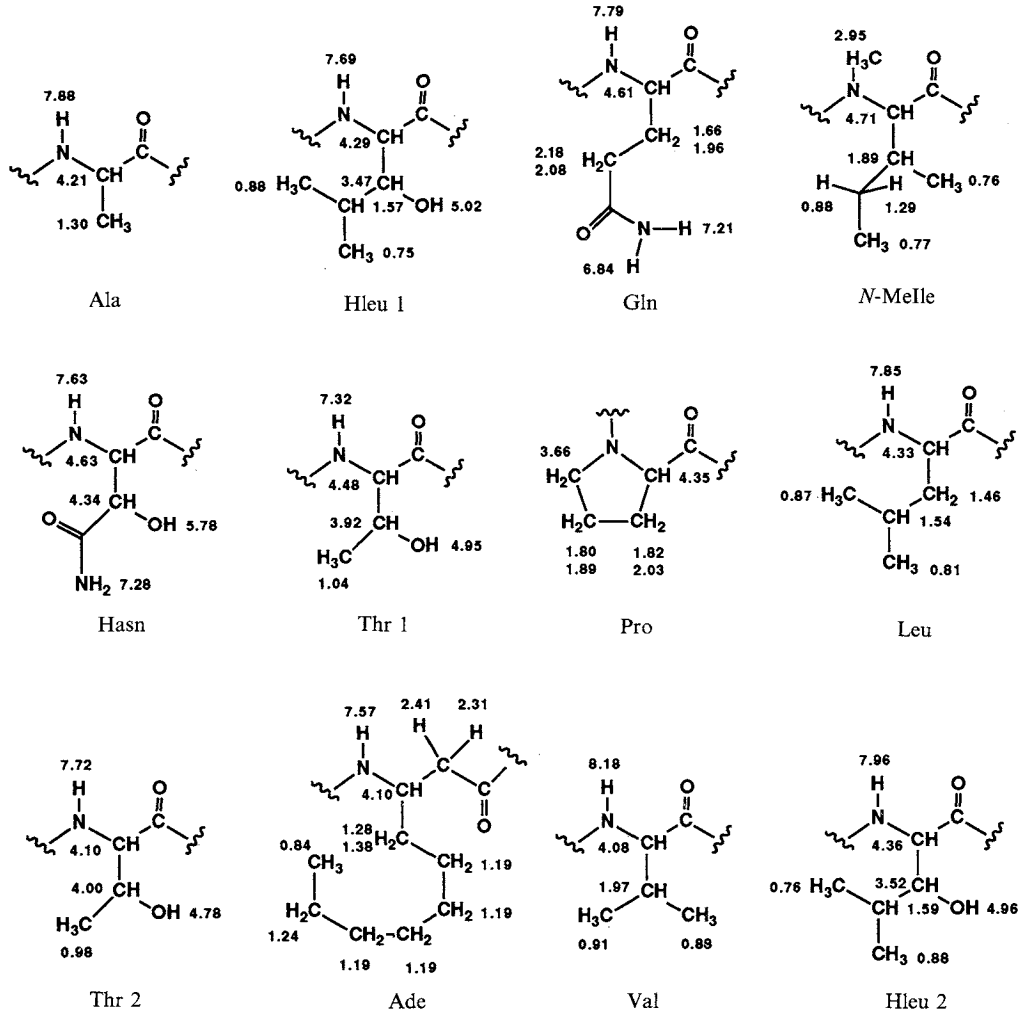
Studies are in progress on the stereochemistry of laxaphycins A and E, but at this writing only the absolute configurations of the common amino acids have been determined.

Laxaphycins B and D

FAB-MS indicated that the molecular weight of laxaphycin B is 1,394 daltons. Amino acid analysis and extensive NMR studies established that laxaphycin B has the molecular formula $C_{65}H_{114}N_{14}O_{19}$ and is comprised of the twelve amino acid substructures shown in Fig. 3, *viz.* alanyl (Ala), 3-aminodecanoyl (Ade), glutaminyl (Gln), hydroxyasparaginyl (Hasn), two hydroxyleucyl (Hleu), leucyl (Leu), prolyl (Pro), two threonyl (Thr), and valyl (Val) units.

Laxaphycin B exists predominately as a 3.5:1 mixture of two conformers in solution as shown by the doubling of signals in both the 500 MHz ^{1}H NMR spectrum (Fig. 4) and the 125 MHz ^{13}C NMR

Fig. 3. Gross structures of amino acid units in laxaphycin B showing ^{1}H chemical shifts in at DMSO-d_6 35°C.



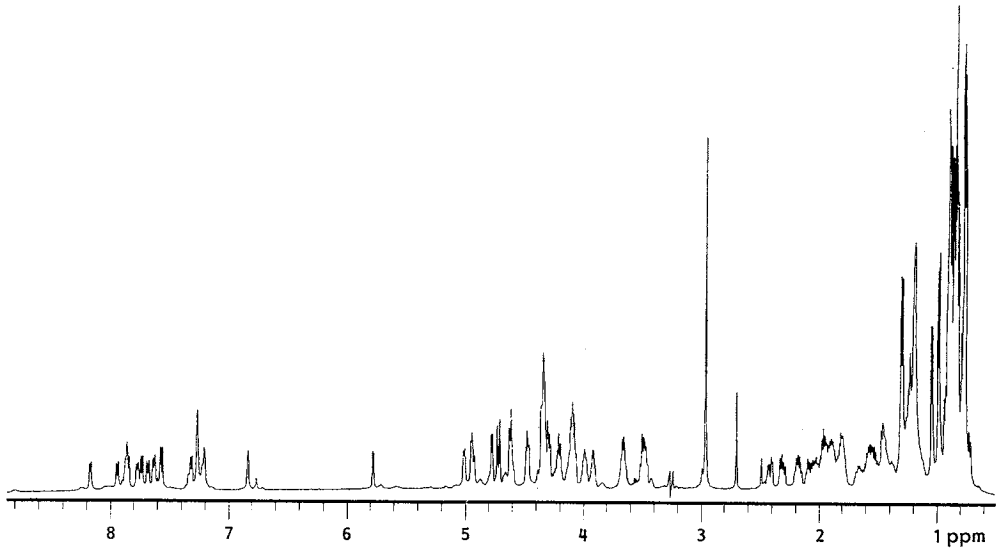
spectrum (Fig. 5). Note for example in the proton spectrum that the *N*-methyl protons of MeIle show signals at 2.95 and 2.65 ppm and one of the protons of the primary amide group of Gln shows signals at 6.82 and 6.78 ppm in a ratio of approximately 3.5 : 1.

The conformational behavior of laxaphycin B in solution presented problems in the unambiguous assignments of ^{13}C signals (Table 1). To rigorously confirm the assignments, two-dimensional ^{13}C-^{13}C DQF-COSY and ^{13}C-^{13}C TOCSY experiments were carried out with uniformly ^{13}C-enriched laxaphycin B.[9]

Sequencing of the amino acid units into a total gross structure was accomplished with HMBC and NOESY experiments.

The HMBC spectrum allowed us to detect ^{2}J-correlations between the carbonyl-carbon of amino acid unit n and the NH-proton of amino acid unit n+1. Cross peaks could be clearly seen between C-1 of Ala and the NH of Hleu 1, C-1 of Hleu 1 and the NH of Gln, C-1 of *N*-MeIle and the NH of Hasn, C-1 of

Fig. 4. 500 MHz ^{1}H NMR spectrum of laxaphycin B in DMSO-d_6 at 35°C.



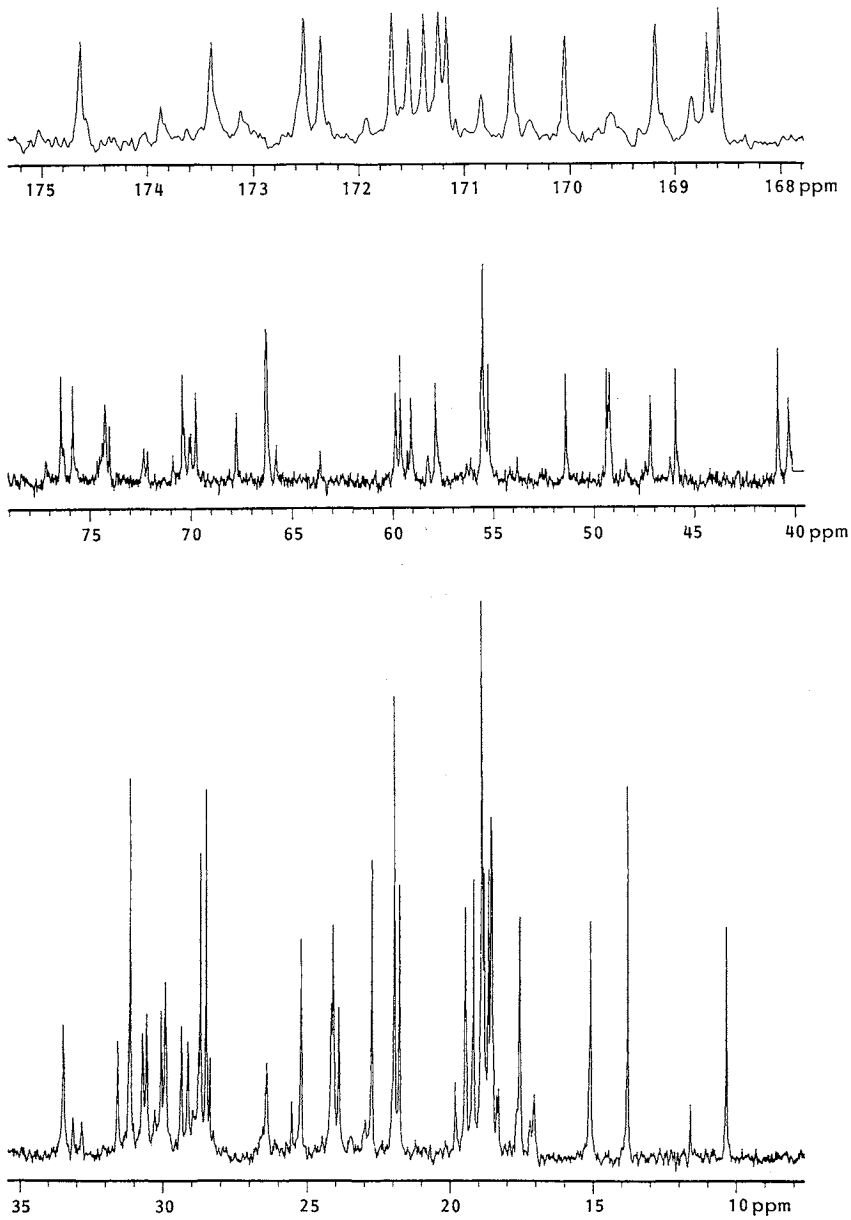
Hasn and the NH of Thr 1, C-1 of Pro and the NH of Leu, C-1 of Leu and the NH of Thr 2, C-1 of Thr 2 and the NH of Ade, C-1 of Ade and the NH of Val, C-1 of Val and the NH of Hleu 2, and C-1 of Hleu 2 and the NH of Ala. Two partial structures, *viz.* Pro-Leu-Thr 2-Ade-Val-Hleu 2-Ala-Hleu 1-Gln and *N*-MeIle-Hasn-Thr 1, could be generated from these data which implied cyclic (Ala-Hleu-Gln-*N*-MeIle-Hasn-Thr-Pro-Leu-Thr-Ade-Val-Hleu) as the gross structure for the peptide.

The HMBC spectrum also enabled us to see ^3J-coupling between the carbonyl-carbon of amino acid unit n and the α-proton of the adjacent amino acid unit n+1. Couplings were detected between C-1 of Ala and 2-H of Hleu 1, C-1 of Hleu 1 and 2-H of Gln, C-1 of Gln and 2-H of *N*-MeIle, C-1 of *N*-MeIle and 2-H of Hasn, C-1 of Ade and 2-H of Val, C-1 of Val and 2-H of Hleu 2, and C-1 of Hleu 2 and 2-H of Ala. Three bond correlations were also observed between C-1 of Gln and the *N*-CH_3 protons of *N*-MeIle and between C-1 of Thr 2 and 3-H of Ade. These data confirmed the presence of partial structure Thr 2-Ade-Val-Hleu 2-Ala-Hleu 1-Gln-*N*-MeIle-Hasn in laxaphycin B.

A NOESY spectrum permitted us to further confirm the gross structure deduced from the HMBC experiments. NOEs could be observed between the α-proton of amino acid unit n and the NH-proton (or *N*-CH_3-protons) of adjacent amino acid unit n+1, *i.e.* between 2-H of Ala and NH of Hleu 1, 2-H of Hleu 1 and NH of Gln, 2-H of *N*-MeIle and NH of Hasn, 2-H of Hasn and NH of Thr 1, 2-H of Leu and NH of Thr 2, 2-H of Thr 2 and NH of Ade, 2-H_2 of Ade (both protons) and NH of Val, 2-H of Val and NH of Hleu 2, and 2-H of Hleu 2 and NH of Ala. In addition NOEs could be seen between both protons on C-5 of Pro and the NH proton of Thr 1. These data were consistent with the proposed gross structure and verified the Thr-Pro linkage in laxaphycin B.

The FAB mass spectrum of laxaphycin D indicated a molecular weight (1,366 daltons) that was 28 mass units lower than the one for laxaphycin B. The ^{1}H and ^{13}C NMR spectra of the two compounds were essentially identical except for differences in chemical shift and intensity of signals associated with the β-aminoalkanoic acid unit. When the 500 MHz ^{1}H NMR spectra of laxaphycins B and D in DMSO-d_6 were compared, the broad, complex signal centered at 1.19 ppm integrated for four additional methylene

Fig. 5. 125 MHz ^{13}C NMR spectrum of laxaphycin B in DMSO-d_6 at 35°C.



protons in the case of laxaphycin B, strongly suggesting that the molecular formula of laxaphycin D was $C_{63}H_{110}N_{14}O_{19}$ and that a 3-aminooctanoic acid unit (Aoc) was present instead of an Ade unit. Amino acid analysis and detailed NMR analysis confirmed the proposed structure.

Experimental

Spectral and Chemical Analysis

500 MHz ^1H NMR and 125 MHz ^{13}C NMR spectra were determined on a 11.75 Tesla instrument. ^1H and ^{13}C NMR chemical shifts are referenced to solvent peaks: δ_H 3.30 (residual CHD_2OD/CHD_2OH)

Table 1. ^{13}C-Chemical shifts for laxaphycin B in DMSO-d_6 at 35°C.

Amino acid unit	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
Ala	172.37	49.37	17.57							
Hleu 1	170.56	55.51	75.84	29.92	18.58					
					18.90					
Gln	172.53	49.21	26.39	30.71	174.64					
N-MeIle[a]	170.06	59.85	31.57	23.88	10.36					
Hasn	169.20	55.51	70.41	173.41						
Thr 1	168.60	55.60	66.28	18.82						
Pro	171.39	59.62	29.14	24.16	47.20					
Leu	171.70	51.40	40.84	24.09	22.74					
					21.78					
Thr 2	168.71	57.87	66.22	19.19						
Ade	171.18	40.31	45.95	33.47	25.20	28.67	28.50	31.13	21.95	13.80
Val	171.54	59.10	29.37	18.55						
				18.37						
Hleu 2	171.26	55.25	76.42	30.57	18.90					
					18.89					

[a] 15.10 (Me on C3), 30.06 (*N*-Me).

and δ_C 49.0 for MeOH-d_4/MeOH-d_3; δ_H 2.49 (residual DMSO-d_5) and δ_C 39.5 for DMSO-d_6. ^{1}H and ^{13}C NMR assignments were determined with the following NMR experiments: DEPT, DQF-COSY, TOCSY (HOHAHA),[2] HMQC[3] and HMBC.[4] Mass spectra were determined on a VG-70SE mass spectrometer operating in the EI or FAB mode.

Amino acid analysis was performed, after standard HCl hydrolysis of the peptides, on a Beckman 6300 Amino Acid Analyzer.

Culture Conditions and Isolation of Laxaphycins A, B, D and E

Mass cultivation of *Anabaena laxa* (strain FK-1-2) and isolation of the peptides was carried out as previously described.[1]

Physical Data for Laxaphycin A

^{13}C NMR (MeOH-d_4): amino acid unit, δ (multiplicity, carbon position) Gly 170.3 (s, 1), 43.5 (t, 2); Dhb 169.7 (s, 1), 132.0 (s, 2), 122.1 (d, 3), 12.8 (q, 4); Hyp 173.3 (s, 1), 61.1 (d, 2), 39.2 (t, 3), 70.3 (d, 4), 58.9 (t, 5); Ile 2 175.0 (s, 1), 55.9 (d, 2), 39.4 (d, 3), 28.1 (t, 4), 12.0 (q, 5), 14.8 (q, CH_3 on C-3); Ile 1 174.4 (s, 1), 58.1 (d, 2), 42.0 (d, 3), 23.7 (t, 4), 12.1 (q, 5), 16.2 (q, CH_3 on C-3); Aoc 171.6 (s, 1), 42.0 (t, 2), 47.0 (d, 3), 36.4 (t, 4), 26.9 (t, 5), 32.7 (t, 6), 23.8 (t, 7), 14.4 (q, 8); Leu 1 174.1 (s, 1), 53.6 (d, 2), 40.5 (t, 3), 25.8 (d, 4), 23.7 (q, 5), 20.9 (q, 5'); Leu 2 175.8 (s, 1), 55.3 (d, 2), 40.5 (t, 3), 25.9 (d, 4), 22.3 (q, 5), 23.0 (q, 5'); Hse 1 174.6 (s, 1), 51.0 (d, 2), 35.0 (t, 3), 59.4 (t, 4); Hse 2 175.0 (s, 1), 51.0 (d, 2), 35.4 (t, 3), 58.9 (t, 4); Phe 175.6 (s, 1), 58.6 (d, 2), 38.5 (t, 3), 139.0 (s, 1'), 130.3 (d, 2' and 6'), 129.5 (d, 3' and 5'), 127.8 (d, 4'). ^{13}C NMR (DMSO-d_6) at 35°C: δ 173.2 (s), 172.9 (s), 172.6 (s), 172.3 (s), 172.1 (s), 172.0 (s), 171.5 (s), 170.2 (s), 169.0 (s), 167.5 (s), 166.8 (s), 137.9 (s), 130.8 (s), 129.1 (d, 2C), 128.1 (d, 2C), 126.3 (d), 119.0 (d), 68.0 (d), 59.2 (d), 57.1 (t), 57.0 (t), 56.9 (t), 56.3 (d), 55.9 (d), 53.6 (d), 52.9 (d), 51.6 (d), 49.0 (d), 48.9 (d), 44.7 (d), 42.3 (t), 40.2 (t), 39.3 (t), 39.2 (t), 39.1 (d), 37.9 (t), 37.0 (t), 36.9 (d), 34.9 (t), 33.8 (t), 33.7 (t), 30.8 (t), 26.2 (t), 25.1 (t), 24.02 (d), 24.97 (d), 22.8 (q), 22.6 (q), 22.1 (t, 2C), 21.3 (q), 20.3 (q), 15.3 (q), 14.4 (q), 13.8 (q), 12.1 (q), 11.5 (q), 11.1 (q).

^{1}H NMR (MeOH-d_4 with exchangeable NH observed in MeOH-d_3): amino acid unit, δ (assignment, multiplicity, ^nJ) Gly 3.38 (2-H, d, $^2J=-17.1$ Hz), 4.07 (2-H', d, $^2J=-17.1$ Hz); Dhb 5.74 (3-H, q, $^3J=7.5$ Hz), 1.80 (4-H_3, d, $^3J=7.5$ Hz); Hyp 4.71 (2-H, dd, $^3J=10.5$ and 7.5 Hz), 2.01 (3-H, m), 2.47 (3-H', dd, $^2J=-13.2$ Hz, $^3J=7.5$ Hz), 4.41 (4-H, m), 3.49 (5-H, m), 3.77 (5-H', dd, $^2J=-11.4$ Hz, $^3J=3.1$ Hz); Ile 1 4.80 (2-H, d, $^3J=3.5$ Hz), 1.84 (3-H, m), 1.00 (4-H, m), 1.18 (4-H', m), 0.83 (5-H_3, m), 0.88 (CH_3 on C-3, m), 6.60 (NH, d); Ile 2 4.71 (2-H, d, $^3J=4.4$ Hz), 1.98 (3-H, m), 1.28 (4-H_2, m), 0.92 (5-H_3, m), 0.88 (CH_3 on C-3, m), 8.32 (NH, m); Aoc 1.78 (2-H, m), 2.06 (2-H', dd, $^2J=-15.4$ Hz, $^3J=2.2$ Hz), 4.45 (3-H,

m), 1.52 (4-H_2, m), 1.77 (5-H_2, m), 1.33 (6-H_2, m), 1.25 (7-H_2, m), 0.92 (8-H_3, m), 7.33 (NH, d); Leu 1 4.45 (2-H, m), 1.42 (3-H, m), 1.15 (3-H', m), 1.65 (4-H, m), 0.86 (5-H_3, m), 0.81 (5'-H_3, m), 7.24 (NH, m); Leu 2 4.00 (2-H, m), 1.62 (3-H, m), 1.51 (3-H', m), 1.65 (4-H, m), 0.91 (5-H_3, m), 0.96 (5'-H_3, m), 8.36 (NH, m); Hse 1 4.48 (2-H, m), 2.19 (3-H_2, m), 3.49 (4-H, m), 3.61 (4-H', m), 7.47 (NH, d); Hse 2 4.63 (2-H, dd, $^3J=6.2$ and 8.4 Hz), 1.98 (3-H_2, m), 3.65 (4-H_2, m), 7.20 (NH, m); Phe 4.41 (2-H, m), 3.07 (3-H, dd, $^2J=-14.0$ Hz, $^3J=3.2$ Hz), 3.20 (3'-H, dd, $^2J=-14.0$ Hz, $^3J=11.8$ Hz), 7.40 (Ph 2'/6', d, $^3J=7.5$ Hz), 7.28 (Ph 3'/5', t, $^3J=7.7$ Hz), 7.19 (Ph 4', t, $^3J=7.2$ Hz), 8.15 (d, NH).

Uniform ^{13}C-Enrichment of Laxaphycin B

The alga was grown in a 20-liter bottle on $Na^{13}CO_3$ using a previously described procedure[7,10] to give 7.8 g of dried alga from which 6.6 mg of labeled laxaphycin B, estimated by ^{13}C NMR analysis to be uniformly enriched to 80% ^{13}C, was isolated.

Acknowledgment

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