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Lyngbyazothrins A−D, Antimicrobial Cyclic Undecapeptides from the Cultured Cyanobacterium *Lyngbya* sp.

Elmi N. Zainuddin,[†,§] Rolf Jansen,[‡] Manfred Nimtz,[‡] Victor Wray,[‡] Michael Preisitsch,[†] Michael Lalk,[†] and Sabine Mundt*[,†]

Institute of Pharmacy, Ernst-Moritz-Arndt-University, Friedrich-Ludwig-Jahnstrasse 17, D-17487 Greifswald, Germany, and Helmholtz Centre for Infection Research, Inhoffenstrasse 7, D-38124 Braunschweig, Germany

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Four novel cyclic undecapeptides, lyngbyazothrins A (**1**), B (**2**), C (**3**), and D (**4**), were isolated from the cultured *Lyngbya* sp. 36.91 as binary mixtures (**1/2** and **3/4**). Their structures were elucidated by analysis of 1D (^{1}H and ^{13}C) and 2D (COSY, TOCSY, ROESY, NOESY, HMQC, and HMBC) NMR spectra, ESIMSMS, ESITOFMS, and amino acid analyses. Three unusual amino acids were present and identified as 4-methoxyhomophenylalanine in **1** and **3**, homophenylalanine in **2** and **4**, and 3-amino-2,5,7,8-tetrahydroxy-10-methylundecanoic acid (Aound) in all compounds, while **3** and **4** have an additional *N*-acetyl-*N*-methyltyrosine unit. The mixture of lyngbyazothrins A (**1**) and B (**2**) shows only low antimicrobial activity against *Micrococcus flavus*, whereas the mixture of lyngbyazothrins C (**3**) and D (**4**) was active against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Serratia marcescens*. It seems that the acyl residue at C-5 of the Aound unit plays an important role in antimicrobial activity.

The increasing number of bacterial strains resistant to clinically applied antibiotics makes the search for new active compounds of crucial importance. Cyanobacteria are known as useful sources of bioactive natural products such as peptides, alkaloids, macrolides, and derivatives of fatty acids and lipids. Moreover, it has been shown that cyanobacterial metabolites exhibit a diverse spectrum of biological activities including antibiotic, anticancer, and protease-inhibiting activities.[1−4] Cyclic cyanopeptides often contain long-chain β-amino acids, as found for instance in laxaphycins,[5] hormothamnin A,[6] calophycin,[7] puwainaphycins,[8] schizotrin A,[9] nostofungicidine,[10] lobocyclamides,[11] obyanamide,[12] ulongapeptin,[13] homodolastatin 16,[14] guineamides,[15] largamide H,[16] dossypeptolide,[17] and pahayokolides.[18] They are most likely of mixed polyketide synthase/nonribsomal peptide synthetase origin, and most of them have antibacterial, antifungal, or cytotoxic activities.

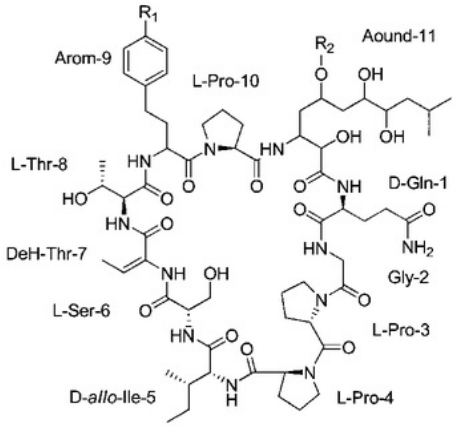
Although the role of secondary metabolites for cyanobacteria themselves is still uncertain, it is generally accepted that cyanobacteria synthesize these compounds as defense against predators and competitors in their particular ecological niche.[2] Consequently, cyanobacterial metabolites are of interest as potential new antibiotics.

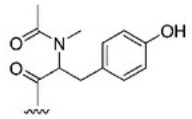
From a screening of 21 lipophilic and hydrophilic extracts obtained from seven cultured strains of cyanobacteria, an extract of *Lyngbya* sp. 36.91 was most active against several bacterial strains and a yeast strain. Bioassay-guided fractionation of the MeOH/H_2O extract finally provided four new cyclic undecapeptides, lyngbyazothrins A/B (**1/2**) and C/D (**3/4**), as binary mixtures. In this paper we report their isolation and structural elucidation.

Results and Discussion

Freeze-dried biomass of laboratory cultured *Lyngbya* sp. 36.91 was extracted with solvent mixtures of increasing polarity. The residue from the MeOH/H_2O extraction was fractionated using a silica gel column with a step gradient from EtOAc to MeOH. Five fractions were collected and tested against *B. subtilis* SUG 14 using the agar diffusion method. Fraction F3, eluted with 50% EtOAc/MeOH, exhibited the strongest antibacterial activity. Further fractionation of F3 was achieved by vacuum silica gel chromatography with the solvent mixture EtOAc/*n*-PrOH/H_2O containing increasing amounts of water. Six fractions were collected and tested against seven bacterial strains and a yeast strain. Fractions F3-2 and F3-3 showed considerable activity. Separation and purification of both fractions by semipreparative HPLC with a gradient system MeCN/H_2O afforded two fractions, F-P1 (5 mg) and F-P2 (10 mg), which were obtained as white, amorphous powders after lyophilization.

A 1D ^{1}H NMR spectrum of fraction F-P1 in 50% aqueous CD_3CN indicated the presence of two sets of aromatic signals in the low-field region 6−8 ppm in a ratio of 2.3:1, corresponding to





R_2=$C_{12}H_{14}NO_3$

	R_1	R_2
Lyngbyazothrin A (**1**)	OCH_3	H
Lyngbyazothrin B (**2**)	H	H
Lyngbyazothrin C (**3**)	OCH_3	$C_{12}H_{14}NO_3$
Lyngbyazothrin D (**4**)	H	$C_{12}H_{14}NO_3$

* Corresponding author. Tel: +49-3834-864869. Fax: +49-3834-864885. E-mail: smundt@uni-greifswald.de.
† Ernst-Moritz-Arndt-University Greifswald.
‡ Helmholtz Centre for Infection Research Braunschweig.
§ Marine Science and Fishery Faculty, Hasanuddin University, Tamalanrea, Km 10, Makassar 90245, Indonesia.

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Table 1. NMR Spectroscopic Data of Lyngbyazothrins A (**1**) and B (**2**) in 50% Aqueous CD_3CN Containing a Trace of CF_3CO_2H[a]

unit	C/H no.	δ_C	δ_H	Unit	C/H no.	δ_C	δ_H	δ_H
Gln-1	H_N		7.97	Thr-8	H_N		7.61	
	CO	174.2			CO	173.0		
	C_α	54.5	4.11		C_α	60.9	4.17	
	C_β	28.3	1.94, 1.87		C_β	67.7	4.17	
	C_γ	32.1	2.23		C_γ	20.0	1.11	
	C_δ	178.3					A (**1**)	B (**2**)
	NH_2		6.87, 5.98[c]	Arom-9	H_N		7.92	7.94
Gly-2	H_N		8.05		CO	172.5[f]		
	CO	169.2			C_α	52.4	~4.3[b]	~4.3[b]
	C_α	42.5	3.91		C_β	32.7	2.57, 2.44	2.63, 2.50
Pro-3	CO	172.5[f]			C_γ	31.1[h]	1.87	1.90
	C_α	59.7	4.58		1	133.9[h]		
	C_β	29.8[f]	2.24, 1.81		2,6	130.6[h]	7.07	7.14
	C_γ	25.3	1.90, 1.90		3,5	114.8[h]	6.81	7.24
	C_δ	47.7	3.46		4	158.5[h]		7.15
Pro-4	CO	175.2						
	C_α	62.3[e]	~4.3[b]		4-OMe	56.1	3.69	
	C_β	28.8[f]	2.20, 1.80	Pro-10	CO	174.5		
	C_γ	26.0	2.05, 1.93		C_α	61.8[e]	4.19	
	C_δ	48.6	3.74, 3.49		C_β	30.3	2.04, 1.78	
allo-Ile-5	H_N		8.20		C_γ	25.2	1.78, 1.78	
	CO	174.4			C_δ	48.3	3.44, 3.22	
	C_α	56.6	4.48	Aound-11[d]	1	173.9		
	C_β	36.2	2.12		2	73.5	4.06	
	C_γ	26.8	1.18		3	50.4	4.20	
	C_γ	14.9	0.77		4	39.7	1.58	
	C_δ	11.9	0.77		5	65.6	3.66	
Ser-6	H_N		8.10		6	38.7	1.49, 1.36	
	CO	172.1			7	72.4	3.58	
	C_α	58.1	4.23		8	73.7	3.48	
	C_β	62.4	3.86, 3.74		9	41.5	1.22, 1.14	
DeH-Thr-7	H_N		9.27		10	24.9	1.64	
	CO	167.1			11	24.0	0.83[g]	
	C_α	128.9			12	21.9	0.78[g]	
	C_β	130.6	5.78		NH-3		7.56	
	C_γ	14.0	1.83					

[a] The residual signals from acetonitrile were used as reference at 1.93 ppm (^1H) and 1.3 ppm (^{13}C). The ^1H chemical shifts are from correlations in the 2D TOCSY spectrum of the binary mixture of **1** and **2**, while the ^{13}C chemical shifts are from correlations in the HMQC and HMBC spectra of **1**. The ^1H data are identical for both peptides apart from the chemical shifts of residue 9 (Arom-9). [b] Signal overlaps with the residual water signal. [c] In 50% aqueous CF_3CD_2OH. [d] Unambiguous ^1H signal assignments were possible from comparison of the intensities of the cross-peaks of this system at different positions in the 2D TOCSY spectrum. [e–g] Interchangeable. [h] Only these carbons could be assigned for B (**2**) at 32.0 (C_γ), 141.6 (1), 129.6 (2,6), 129.6 (3,5), 127.3 (4).

an AA′BB′ system for the major component (**1**) and a phenyl system for the minor component (**2**), suggesting fraction F-P1 was a binary mixture of two closely related compounds. Positive ion ESIMS in the accurate mass mode and high-resolution ESITOFMS confirmed the presence of two new compounds in fraction F-P1, lyngbyazothrins A (**1**) and B (**2**), with protonated molecular ions at *m/z* 1313.6974 $[M + H]^+$ and *m/z* 1283.6848 $[M + H]^+$. These data are compatible with the molecular formulas $C_{62}H_{96}N_{12}O_{19}$ and $C_{61}H_{94}N_{12}O_{18}$, respectively. NMR and MS data suggested a modified peptide structure for the new compounds. Extensive efforts have been made to separate the two components by HPLC using different stationary phases such as C18 alkyl phases and ether-linked phenyl and pentafluorophenyl phases. All stationary phases were tested with several mobile phases such as MeCN/H_2O with 0.1% TFA or 0.1% formic acid and ammonium acetate/MeCN, but pure compounds could not be obtained. Preparative TLC with various solvent systems, one- and two-dimensional, was also not effective.

A detailed structural investigation of the mixture, therefore, was undertaken using homonuclear 1D and 2D NMR techniques. Initially, a number of different solvents, including water, 50% aqueous CF_3CD_2OH, and 50% aqueous CD_3CN, were tested to find the most suitable system. Signals for the amide protons were detectable only upon acidification of the NMR solutions with a trace of CF_3CO_2H. Under these conditions the side-chain signals of the Gln residue were difficult to detect in aqueous CD_3CN but unambiguous in 50% aqueous CF_3CD_2OH.

Subsequently, two full sets of 2D TOCSY, ROESY, and NOESY spectra were recorded in aqueous CD_3CN as well as in CF_3CD_2OH. The spin systems of residues 1 to 10 in the mixture of **1** and **2** were identified by TOCSY and ROESY spectra starting from the amidic protons in the region 7 to 9.5 ppm, and from the characteristic spin system and chemical shifts of proline in the region 3 to 5 ppm. Additional signals of the extended side-chain of residue 11 were readily identified in the high-field region of the TOCSY spectrum between 1.7 and 0.7 ppm and indicated the complexity of this system (Table 1). Careful analysis of the cross-peaks and their respective intensities at different positions in the TOCSY spectra of both solutions indicated this was a 3-amino-2,5,7,8-tetrahydroxy-10-methylundecanoic acid. The *E*-configuration of the dehydrothreonine residue was determined from the observation of an intraresidue NOE between the olefinic and NH protons.

Although there was clear evidence of a mixture of two compounds from the NMR spectra, this did not complicate the analysis too much, as the amidic signals of both systems overlapped closely, indicating both compounds were structurally very similar. Indeed, the presence of the second component was only apparent from the intensities of a set of amidic signals that corresponded to the signal of residue 9, where there was a shift difference of ca. 0.02 ppm between the amide protons of **1** and **2** (Table 1).

Small differences were also evident for the side-chain methylene groups. The assumption that this residue contained the variable aromatic system was confirmed by the observation of long-range correlations in the TOCSY spectra between the γ-CH_2's and the *ortho* protons (H2,6; Table 1) of the aromatic systems of **1** and **2**, as well as from NOE correlations of the same aromatic protons with both the β- and γ-CH_2's in the ROESY spectrum. The ROESY

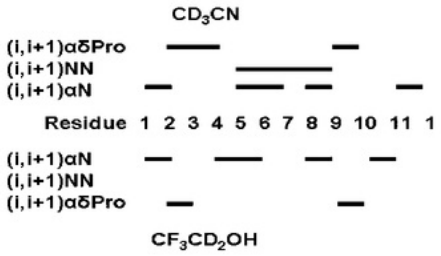

Figure 1. Sequential NOE data determined for lyngbyazothrins A (**1**) and B (**2**) from ROESY spectra recorded in 50% aqueous CD_3CN (upper) and 50% aqueous CF_3CD_2OH (lower).

spectrum also indicated the presence of a 4-methoxy group from correlations with H3,5 in the same aromatic system of **1**.

The sequences of the secondary amides were deduced from a combination of (*i*, *i*+1)αN and (*i*, *i*+1)NN NOE data observed between hydrogen atoms of adjacent residues (*i*, *i*+1) in the ROESY spectra taken in 50% aqueous CD_3CN and in 50% aqueous CF_3CD_2OH (Figure 1). In particular, there was an unambiguous series of *i*(*i*+1)NN NOEs that identified the sequence of residues 5 to 9 between the two prolines 4 and 10. The positions of the proline residues were evident from correlations between the proline δ-CH_2's and the α-CHs of the preceding residue (2−3, 3−4, and 9−10). Finally, the (*i*, *i*+1)αN for residues 10 and 11 found in 50% CF_3CD_2OH and the observation of a NOE between 2-H of residue 11 and the NH of Gln-1 completed the cyclic system.

Subsequently the sequences of **1** and **2** were confirmed by heteronuclear NMR data from 1D ^{13}C and 2D HMQC and HMBC NMR spectra. The combination of intraresidue data from the 2D ^1H and ^{13}C spectra allowed unambiguous ^{13}C assignments of all of the residues (Table 1). In particular, the carbonyl carbons were assigned from correlations with either Hα or H*β* of the same residue. The sequences Pro-4 to Arom-9 and Pro-10 to Gly-2 were then confirmed from their strong two-bond NH−CO correlations in the HMBC spectrum. Thus, the combined heteronuclear and homonuclear NMR data of the binary mixture of **1** and **2** provided unambiguous identification of the amino acid sequences of both cyclic peptides.

Electrospray ionization tandem mass spectrometry (ESIMS/MS) confirmed the amino acid composition and gave some details of the amino acid sequence. The daughter ion spectra of the protonated molecular ions of **1** and **2** were rather complex and did not allow a straightforward sequence assignment, consistent with the presence of cyclic peptides. The fragmentation patterns of both compounds were very similar, a further indication of two closely related structures. The presence of proline, leucine/isoleucine, and both homophenylalanine (HPhe)/4-methoxyhomophenylalanine (Htm) derivatives could be confirmed by detection of their immonium ions at *m/z* 70, 86, 134 (in **2**) and 164 (in **1**), respectively. Finally, the sequence assignment was independently confirmed by daughter ion spectra of two linear fragments obtained by nozzle/skimmer fragmentation of the intact compounds (MS^3). Fragmentation of the daughter ion at 563.3 mass units in the TOF MS/MS of the molecular ion $[M + H]^+$ of **1** afforded the sequence Ser-Ile-Pro-Pro-Gly-Gln (Figure 2), while a second sequence, Thr-DeHThr-Ser-Ile-Pro, was apparent in the fragmentation of the daughter ion at 920.4 mass units.

After acidic hydrolysis, amino acid analysis of the binary mixture of **1** and **2** identified six amino acids, Glx (Gln or Glu), Ser, Gly, Thr, Pro, and Ile, in a ratio of 1:1:1:1:3:1. Several additional peaks, which could not be assigned initially, were observed. These results were confirmed by GC-MS analysis of the same hydrolysate after derivatization with EtOH/HCl followed by trifluoroacetylation. From their electron impact mass spectra (EIMS), three unusual amino acids were identified with masses compatible with 4-methoxyhomophenylalanine (Htm) from **1**, homophenylalanine (HPhe) from **2**, and 3-amino-2,5,7,8-tetrahydroxy-10-methylundecanoic acid (Aound) from both **1** and **2**.

Using the same ^1H NMR and MS techniques, two new compounds, lyngbyazothrins C (**3**) and D (**4**), in a ratio of 2:1, respectively, were also identified in fraction F-P2. As described for the mixture of **1/2**, the separation of **3/4** by HPLC or TLC with different stationary and mobile phases failed. The accurate protonated molecular masses $[M + H]^+$ of both compounds were *m/z* 1532.7875 for **3** and *m/z* 1502.7801 for **4** and are compatible with the molecular formulas $C_{74}H_{109}N_{13}O_{22}$ and $C_{73}H_{107}N_{13}O_{21}$, respectively.

Six amino acids, Glx (Gln or Glu), Ser, Gly, Thr, Pro, and Ile, found in **1** and **2**, were also identified in the binary mixture of **3** and **4** after acid hydrolysis. Besides the three unusual amino acids found in **1** and **2** (Htm, HPhe, and Aound), compounds **3** and **4** had one additional methylated amino acid as a constituent, viz., *N*-methyltyrosine, which was identified as its *N*,*O*-bistrifluoroacetyl ethyl ester by GC-EIMS. Again, the mass difference of 30, previously observed between **1** and **2** in fraction F-P1, was due to the exchange of the homophenylalanine residue for its methoxylated derivative in **3** and **4** in fraction F-P2. The daughter ion spectra of **3** and **4** were also complex, although similar fragmentation patterns were observed to those of the lower molecular weight compounds of fraction F-P1.

The mass difference between the compound pairs from fractions F-P1 and F-P2 was calculated as 219. Since an intense fragment ion generated by the loss of a constituent of 237 Da (219 + 18[H_2O] = 237) from both parent molecular ions of fraction F-P2 was observed, an easy elimination of this additional residue was indicated. The amino acid compositional analysis confirmed the additional amino acid present only in fraction F-P2 must be *N*-methyltyrosine (195 Da). The calculated mass difference of 42 Da compared to the eliminated residue (237 Da) can be convincingly explained by the presence of an additional *N*-acetyl group, which was detected in the ^1H NMR spectrum (Table 2) and whose position followed from the observation of a three-bond correlation in the HMBC spectrum between the *N*-methyl group and the acetyl carbonyl. Therefore, these data suggest the additional presence of *N*-acetyl-*N*-methyltyrosine in both compounds from fraction F-P2 linked to the periphery of the peptide rings of fraction F-P1.

The presence of two new compounds in fraction F-P2 was also confirmed from the NMR data. Lyngbyazothrins C (**3**) and D (**4**) have the same macrocyclic ring system. The sole additional set of signals was readily attributed to *N*-acetyl-*N*-methyltyrosine, which was bound through its carboxyl group to the 5-hydroxyl group of residue 11 according to the substantial low-field shift of 5-H and smaller shifts of 4-H and 6-H of these residues compared to **1** and **2** (Table 2).

Thus the combined NMR spectroscopic and mass spectrometric data provided an unambiguous identification of the structure of the lyngbyazothrins as cyclic undecapeptides. Enantioselective GC-MS analysis of the common amino acids indicated the presence of L-Gln (detected as D-Glu), L-Pro, D-allo-Ile, L-Ser, and L-Thr. Reference material was not available for the two remaining moieties, residues 9 and 11, so the configuration of these residues and the modified tyrosine remain unassigned.

In the agar diffusion test the **3/4** mixture showed modest antibacterial activity against *B. subtilis* SBUG 14 (25 μg/disk: 18 mm diameter of inhibition zone), *E. coli* ATCC 11229 (100 μg/disk: 18 mm), and *E. coli* SBUG 13 (100 μg/disk: 15 mm) and low activity against *P. aeruginosa* ATCC 27853 (100 μg/disk: 8 mm) and *S. marcescens* SBUG 9 (200 μg/disk: 8 mm). At the same concentrations no activity was detected for the **1/2** mixture and only low activity against *M. flavus* SBUG 16 (100 μg/disk: 8 mm). All lyngbyazothrins were inactive against the yeast *Candida maltosa* SBUG 700.

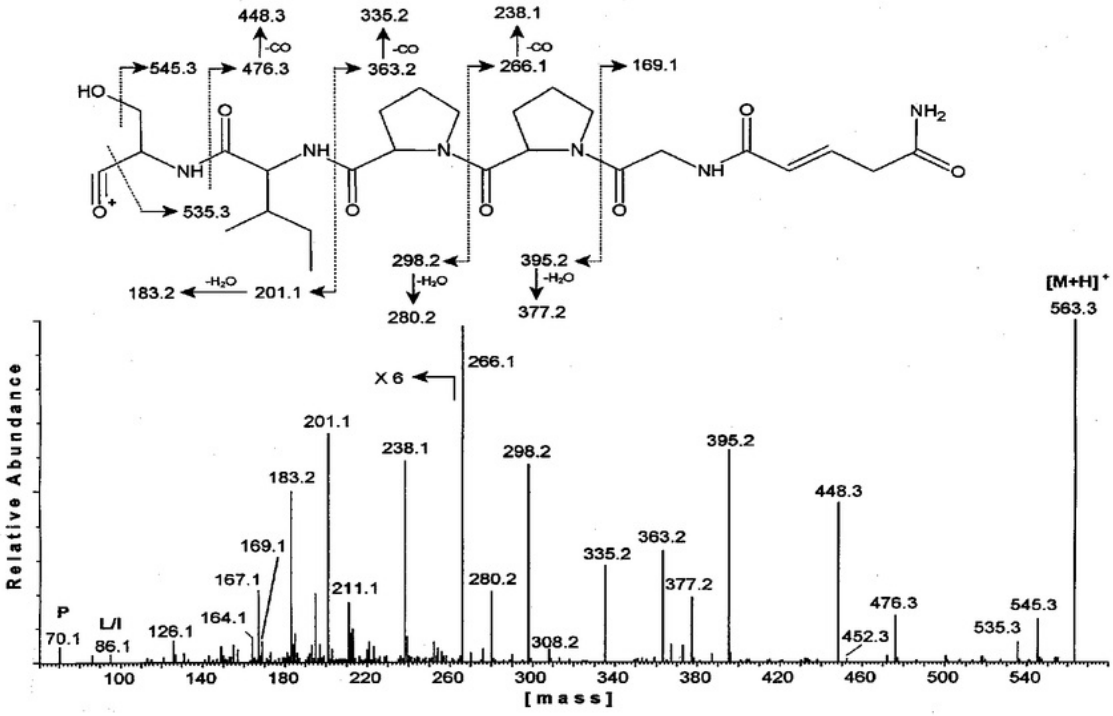

Figure 2. Sequence assignment Ser-Ile-Pro-Pro-Gly-Gln from the nozzle/skimmer fragmentation of the daughter ion at 563.3 mass units in the TOF MS/MS of the molecular ion $[M + H]^+$ of **1**.

Although several undecapeptides have been isolated from cyanobacteria, only schizotrin A[9] from the cultured cyanobacterium *Schizothrix* sp. (TAU in IL-89-2) and pahayokolides A and B[18] from a *Lyngbya* sp. isolated from the periphyton mat from the Florida Everglades have any sequence similarity to our compounds. Schizotrin A has an Htm residue similar to **1** and **3**, which is bound to Pro-Aound-Gln-Gly-Pro, which is common to all our compounds. The same sequence is also found in pahayokolides A and B, but in contrast to schizotrin A, in **1** and **3** it is connected with homophenylalanine. Significant differences are found for the remaining five residues of the cyclic systems (Phe-Val-Ser-DeHThr-Ser in schizotrin A and Phe-Z-Dhb-Ser-E-DhB-Thr in the pahayokolides compared to Pro-allo-Ile-Ser-DeHThr-Thr in **1**−**4**). The free hydroxy group at C-5 of the Aound residue in **1** and **2** is substituted by *N*-acetyl-*N*-methyltyrosine in **3** and **4** instead of the *N*-butyryl-*N*-methylalanine residue in schizotrin A. Pergament and Carmeli[9] suggested that the attachment of proline to the Aound unit is responsible for the biological activity of schizotrin A against *B. subtilis* and several yeasts. More specifically, from our data the activity appears to be due to the linkage of the acyl group at C-5 of the Aound unit, as only the binary mixture of **3** and **4** showed activity against *B. subtilis*, although all lyngbyazothrins (**1**−**4**) have proline residues attached to the Aound unit. This assumption is supported by the structure and activity of pahayokolide A, where the hydroxy group at C-5 is also substituted by an *N*-acetyl residue, however with an *N*-acetyl-*N*-methyl-leucine. Pahayokolide A at 30 μg/disk inhibited the growth of *B. subtilis* with an inhibition zone of 32 mm in the agar plate diffusion assay; the MIC was estimated at 5 μg/mL.[19] The inhibitory activity of the binary mixture of **3** and **4** against *B. subtilis* in the agar diffusion assay was lower; 25 μg caused an inhibition zone of 18 mm, and a MIC of 25 μg/mL was estimated. It seems that the acyl residue at C-5 plays an important role in antimicrobial activity. Probably the nature of the amino acid in the substituent may influence the selectivity of the antimicrobial activity. Thus, schizotrin A and pahayokolide A, containing the aliphatic amino acids alanine and leucine in the residue, did not show activity against *E. coli*, whereas **3** and **4**, which contain the aromatic amino acid tyrosine, were active against this Gram-negative bacterium.

Experimental Section

General Experimental Procedures. ^{1}H (1D and 2D COSY, TOCSY, ROESY, and NOESY) and ^{13}C (1D and 2D HMQC and HMBC) NMR spectra were recorded without spinning on Bruker Avance DMX 600 and Avance^{III} 600 NMR spectrometers, respectively. The peptides were dissolved in either 9:1 H_2O/D_2O, 50% aqueous CF_3CD_2OH, or 50% aqueous CD_3CN, which was acidified with a trace of TFA to active final volumes of 700 μL. Measurements were carried out at 300 K with mixing times of 110 ms for the TOCSY and 500 ms for the ROESY and NOESY spectra. ^{1}H and ^{13}C chemical shifts are given in ppm relative to TMS but were determined relative to the residual signals of the solvent at 1.93 and 1.3 (^{13}C) ppm, respectively.

Positive ion electrospray ionization mass spectra (ESIMS and ESI-MS/MS) were recorded on a Micromass QTOF^2 mass spectrometer. The isotopic composition of the samples was determined in the accurate mass mode using β-cyclodextrin ($[M + H]^+$ = 1135.378 Da) as internal reference compound. After hydrolysis (6 N HCl, 110 °C, 24 h) the resulting amino acids were derivatized with phenylisothiocyanate and analyzed on an automated Applied Biosystems 402 A amino acid analyzer. GC/MS analysis was performed on the same hydrolysate after ethylation of the carboxyl groups followed by trifluoroacetylation of the amino groups on a Thermo/Finnigan GCQ mass spectrometer using a DB5 column.

High-resolution mass spectra were recorded using a Bruker Daltonics microTOF ESI mass spectrometer coupled to an Agilent 1100 HPLC. Mass tuning was performed prior to every analysis using an Agilent ESI-tune mix to a mass deviation lower than 5 ppm. Samples were dissolved in a mixture of MeCN/H_2O (50/50) and separated on a Synergi POLAR-RP column (250 × 4.6 mm, 80 Å, 4 μm, Phenomenex) loaded with 20 μg/run. A solvent gradient of deionized water (Clear UV plus Water preparation and recycling GmbH, Germany) and MeCN (gradient grade ROTH, Germany) from 35% MeCN to 100% MeCN in 30 min was used with a flow rate of 0.5 mL min^{-1}.

Column chromatography was carried out on silica gel Si 60, 0.015−0.04 mm (Merck, Germany); vacuum liquid chromatography

Table 2. ^{1}H NMR Spectroscopic Data of Lyngbyazothrins C (**3**) and D (**4**) in 50% Aqueous CD_3CN Containing a Trace of CF_3CO_2H[a]

unit	C/H no.	δ_C	δ	unit	C/H no.	δ_C	δ C (**3**)	δ D (**4**)
Gln-1	H_N		7.91					
	CO	174.4		Arom-9	H_N		7.80	7.85
	C_α	54.4	4.01		CO	171.9[g]		
	C_β	28.7	1.87		C_α	nd	4.33	4.32
	C_γ	32.1	2.20		C_β	32.8, nd	2.58, 2.45	2.58, 2.44
	C_δ	178.0			C_γ	31.0, 32.0	1.84	1.87
	NH_2		7.46, 6.41[b]		1	133.6, 141.6		
Gly-2	H_N		8.02		2,6	130.6, 129.3	7.07	7.14
	CO	169.0			3,5	114.6, 129.3	6.80	7.24
	C_α	42.3	3.88		4	158.5, 126.9		7.15
Pro-3	CO	172.4			4-OMe	55.9	3.69	
	C_α	59.7	4.58	Pro-10	CO	173.7		
	C_β	29.6[e]	2.24, 1.80		C_α	61.6[d]	4.16	
	C_γ	25.3	1.88		C_β	30.1[e]	2.01, 1.84	
	C_δ	47.6	3.45		C_γ	25.1	1.81	
Pro-4	CO	174.9			C_δ	48.1	3.42, 3.18	
	C_α	62.3[d]	4.31	Aound-11[c]	1	173.3		
	C_β	28.6[e]	2.20, 1.79		2	71.9[f]	4.03	
	C_γ	25.9	2.05, 1.92		3	49.9	4.03	
	C_δ	48.8	3.74, 3.49		4	41.6[h]	1.84, 1.73	
allo-Ile-5	H_N		8.21		5	70.5[f]	5.05	
	CO	174.2			6	41.6[h]	1.48	
	C_α	56.3	4.48		7	72.7	3.33	
	C_β	36.0	2.13		8	73.4	3.48	
	C_γ	26.7	1.18		9	41.6	1.23, 1.10	
	C_γ	14.8	0.77		10	24.9	1.63	
	C_δ	11.8	0.77		11	23.7	0.83	
Ser-6	HN		8.13		12	21.7	0.78	
	CO	172.4[g]			NH-3		7.67	
	C_α	58.2	4.21				C (**3**)	D (**4**)
	C_β	62.4	3.88, 3.76	R_2	CO	172.0		
DeH-Thr-7	H_N		9.29		C_α	60.6, 63.8	4.89	4.66
	CO	166.8			C_β	33.9, 34.4	3.11, 2.89	3.16, 2.87
	C_α	128.7			1	129.3, 128.8		
	C_β	131.2	5.79		2, 6	130.8, 131.0	6.96	7.00
	C_γ	13.9	1.84		3, 5	116.2, 116.4	6.68	6.71
Thr-8	HN		7.50		4	155.8, 156.1		
	CO	173.2			*N*-Me	37.3[h]	2.71	2.67
	C_α	61.0	4.13		CO	174.2		
	C_β	67.7	4.13		Me	21.6	1.86	1.84
	C_γ	20.0	1.11					

[a] The residual ^{1}H signal from acetonitrile was used as reference at 1.93 ppm. The ^{1}H chemical shifts are from correlations in the 2D TOCSY spectrum of the binary mixture of **3** and **4**. The ^{1}H data are identical for both peptides apart from the chemical shifts of residues 9 (Arom-9) and R_2. [b] In 50% aqueous CD_3CN without CF_3CO_2H. [c] Unambiguous ^{1}H signal assignments were possible from comparison of the intensities of the cross-peaks of this system at different positions in the 2D TOCSY spectrum. [d–h] Interchangeable. nd, not determined.

(VLC), on silica gel Si 60, 0.040−0.063 mm (Merck, Germany). Fractions were monitored by TLC developed with *i*-PrOH/EtOH/H_2O (5:4:2) on aluminum sheets precoated with silica gel 60 F_{254} (Merck, Germany) under UV detection at 254 and 366 nm and spraying with anisaldehyde/sulfuric acid. HPLC was performed on a component system (Kontron Instruments, Italy) of pumps 422 and 422S, auto sampler 360, and diode array detector DAD 440 with a Gemini HPLC data system, version 1.91 SST 1.6. For analytical HPLC a Synergi POLAR-RP column (250 × 4.6 mm, 80 Å, 4 μm, Phenomenex) was loaded with 20 μg/run. A solvent gradient of deionized water (Clear UV plus SG Water preparation and recycling GmbH, Germany) and MeCN (gradient grade ROTH, Germany) from 35% MeCN to 100% MeCN in 30 min was used with a flow rate of 1.0 mL/min. Preparative HPLC was performed with the same system using a semipreparative RP column (250 × 10 mm, Synergi Polar-RP 80 A, 4 μm). The column was loaded with 3 mg/run. Separation was achieved with 35% MeCN in 30 min at a flow rate of 4.7 mL min^{-1}. For separation of mixtures of **1/2** and **3/4** besides the Synergi POLAR-RP column a LiChrospher 100 RP-18 (250 × 4 mm, 5 μm, Merck, Germany), a Luna 5 μm C18 (2) (250 × 4.6 mm, Phenomenex), and a MonoChrom MS column (250 × 4.6 mm, 5 μm, Varian) were tested. Different gradient systems were used for improving separation of the mixtures such as H_2O/MeCN with 0.1% TFA or 0.1% formic acid and 41 mM ammonium acetate/MeCN from 10% to 100% MeCN with flow rates of 1, 0.5, and 0.3 mL min^{-1}. For preparative TLC, PSC plates (Si 60 F_{254}, 1 mm, Merck, Germany) were loaded with 2 mg of **1/2** and **3/4**, respectively, developed with mixtures of *i*-PrOH/EtOH/H_2O or *n*-BuOH/MeOH/H_2O and detected at 254 nm.

All chemicals were used as received, and solvents were distilled prior to use except for HPLC.

Culture Conditions. *Lyngbya* sp. 36.91 SAG (Culture Collection of Algae, Göttingen, Germany) was cultured in a glass column containing 40 L of BG11 medium.[19] After 42 days the cells were harvested by filtration using a 200-mesh silkscreen cloth, lyophilized, and stored at −20 °C until use. A yield of 24 g (0.6 g L^{-1}) of dry mass was achieved.

Extraction and Isolation. The freeze-dried cells (5 g) were successively extracted with stirring at room temperature for 1 h with three portions of 250 mL of *n*-hexane, MeOH, MeOH/H_2O, and H_2O, respectively. Cells were separated at 4 °C by centrifugation at 4500 rpm for 20 min, and the supernatants were pooled. After evaporation of the organic solvents the remaining water was removed by lyophilization. The yield of the MeOH/H_2O extract was about 10%. The crude MeOH/H_2O extract (0.5 g) was fractionated by silica gel column chromatography (open column, 3.2 × 40.5 cm, flow rate 0.5 mL min^{-1}) with 450 mL of EtOAc (F-1), 75% EtOAc in MeOH (F-2), 50% EtOAc in MeOH (F-3), 25% EtOAc in MeOH (F-4), and 100% MeOH (F-5), respectively. Fraction F3 (125 mg) showed the highest activity against *B. subtilis* in the agar diffusion assay. Further separation of F3 was done on a VLC column [open column, 1.2 × 30 cm, 5 runs, 25 mg/run, flow rate 1 mL min^{-1} realized by a VAC V-500 vacuum pump (Büchi, Switzerland)]. Elution was started with 100 mL of EtOAc/*n*-PrOH/H_2O (2:6:0.5) followed by 100 mL of these components with increasing amounts of water [(2:6:1), (2:6:1.5), (2:6:2), (2:6:2.5), (2:6:3), respectively]. Six fractions (F3-1 to F3-6) were collected and tested for activity against *B. subtilis*. Activity was detected in fractions

F3-2 (75 mg) and F3-3 (33 mg). Finally 22 mg of F3-2 and 33 mg of F3-3 were separated by semipreparative HPLC using an isocratic mixture of 35% MeCN in H_2O. The main peaks (t_R = 22.3 min) of both (F3-3 and F3-2) fractions were combined to F-P2 (10 mg), containing lyngbyazothrins C (**3**) and D (**4**). A further peak of the separation of F3-3 (t_R 21.1 min) was collected as F-P1 (5 mg), containing lyngbyazothrins A (**1**) and B (**2**).

Enantioselective Analysis of Amino Acids. The sample was hydrolyzed using 6 N HCl at 120 °C for 12 h, conditions that resulted in the conversion of Gln to Glu. After drying, the resulting free amino acids were derivatized with 4 N HCl/propan-2-ol (1 h, 110 °C) and, after removal of reagents, the amino acid isopropyl esters were then acylated with pentafluoropropionic acid anhydride in dichloromethane (150 °C, 12 min). Excess reagents were again removed and the amino acid derivatives analyzed on a Chirasil Val column (50 m) connected to a GCQ ion trap mass spectrometer. The constituent amino acids were identified by their characteristic mass spectra, and their chirality was determined by comparison to standard D,L amino acids.

Lyngbyazothrins A/B (1/2): white, amorphous powder; UV (CH_3CN/H_2O) λ_{max} 254 nm; ^1H NMR and ^{13}C NMR in 50% aqueous CD_3CN (2.3:1 ratio) see Table 1; ESIMS (**1**) *m/z* 1313.697 $[M + H]^+$; HRMS (ESITOFMS) *m/z* 1313.6974 (calcd for $C_{62}H_{97}N_{12}O_{19}$, 1313.6993); ESIMS (**2**) *m/z* 1283.689 $[M + H]^+$; HRMS (ESITOFMS) *m/z* 1283.6848 $[M + H]^+$ (calcd for $C_{61}H_{95}N_{12}O_{18}$, 1283.6887).

Lyngbyazothrins C/D (3/4): white, amorphous powder; UV (CH_3CN/H_2O) λ_{max} 254 and 275 nm; ^1H NMR in 50% aqueous CD_3CN (2:1 ratio) see Table 2; ESIMS (**3**) *m/z* 1532.784 $[M + H]^+$; HRMS (ESITOFMS) *m/z* 1532.7875 (calcd for $C_{74}H_{110}N_{13}O_{22}$, 1532.7888); ESIMS (**4**) *m/z* 1502.775 $[M + H]^+$; HRMS (ESITOFMS) *m/z* 1502.7801 $[M + H]^+$ (calcd for $C_{73}H_{108}N_{13}O_{21}$, 1502.7783).

Evaluation of Antimicrobial Activity. The agar diffusion assay was carried out in 90 mm Petri dishes containing 20 mL of nutrient agar seeded with inocula of the test organisms.[20] The following strains were used: *Bacillus subtilis* (strain SBUG 14: reference ampicillin 10 μg, inhibition zone 14 mm; strain ATCC 6051: reference ampicillin 10 μg, inhibition zone 33 mm), *Staphylococcus aureus* (strain SBUG 511: reference ampicillin 10 μg, inhibition zone 22 mm; strain ATCC 65382: ampicillin 10 μg, inhibition zone 35 mm), *Escherichia coli* (strain SBUG 13: reference ampicillin 50 μg, inhibition zone 17 mm; strain ATCC 11229: reference ampicillin 50 μg, inhibition zone 26 mm), *Pseudomonas aeruginosa* (strain ATCC 27853: reference gentamycin 25 μg, inhibition zone 26 mm), *Proteus mirabilis* (strain SBUG 47: reference ampicillin 10 μg, inhibition zone 25 mm), *Serratia marcescens* (strain SBUG 9: reference ampicillin 10 μg, inhibition zone 28 mm), and *Candida maltosa* (strain SBUG 700: reference amphothericin 100 μg, inhibition zone 20 mm). Different concentrations were used for the tests: 2 mg/paper disk for extracts, 500 μg/paper disk for fractions of the first separation, 250 μg/paper disk for fractions of the second separation, and 25−200 μg/paper disk for pure compounds. All test materials were dissolved in suitable solvents, which were evaporated before applying the paper discs to the surface of the solid agar medium. After 24 h of incubation at 37 °C for bacteria and 28 °C for yeast the diameters of inhibition zones were measured across the whole zone including the paper disk of 7 mm. The negative control was performed by loading the paper discs with an equivalent volume of solvent and the positive control by application of ampicillin or gentamicin for bacteria and amphotericin for yeast. Determination of MIC (minimal inhibitory concentration) against *Bacillus subtilis* ATCC 6051 was done according to the methods of the European Pharmacopoeia.[21]

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Supporting Information Available: Details of the 1D and 2D ^1H (TOCSY, ROESY) and 2D ^{13}C (HSQC, HMBC) NMR spectra of lyngbyazothrins A/B, and 1D and 2D ^1H (COSY, TOCSY, ROESY) and 2D ^{13}C (HMBC) NMR spectra of lyngbyazothrins C/D are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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