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New Peptolides from the Cyanobacterium *Nostoc insulare* as Selective and Potent Inhibitors of Human Leukocyte Elastase

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Eight new cyanopeptolins (insulapeptolides A–H) were obtained from the cyanobacterium Nostoc insulare. *Their isolation was guided by their bioactivity toward the target enzyme human leukocyte elastase, molecular biological investigations, and MALDI-TOF analysis. These peptides are selective inhibitors of human leukocyte elastase with activities in the nanomolar range. Insulapeptolide D (**4**) was the most potent compound with an IC_{50} value of 85 nM (K_i value of 36 nM).*

Introduction

Cyanobacteria belong to the oldest forms of life on earth and populate nearly every habitat of our planet. Due to the structural diversity of their secondary metabolites and their outstanding bioactivities, cyanobacteria are of interest for pharmaceutical research. In the current study, we focused on the identification of inhibitors for human leukocyte elastase (HLE). HLE is a serine protease that is responsible for the cleavage of elastin, an elastic protein in connective tissues. Excessive activity of this enzyme can result in diseases such as emphysema, arthritis, and cystic fibrosis,[1,2] and HLE inhibitors are of relevance for the therapy of such afflictions. To date, recombinant endogenous elastase inhibitors are already available (for example, secretory leukocyte proteinase inhibitor or α1-proteinase inhibitor). Sivelestat, a sulfonamide, is the only synthetic inhibitor to reach the clinical market,[3,4] and there is a need for further potent drugs. There are two additional serine proteases that are stored in the azurophilic granules of leukocytes, proteinase 3 and cathepsin G. Together with elastase, they are translocated to the surface of the cell membrane and/or released at an inflammatory site after cell stimulation. All three neutrophil serine proteases can act extracellularly in the breakdown of matrix components, but also intracellularly to degrade ingested host pathogens. These proteases are synthesized as inactive zymogens and processed to the active enzymes. Their activity is further regulated by endogenous inhibitors of the serpin and chelonianin families as well as α2-macroglobulin. HLE and proteinase 3 show a similar preference to cleave peptides after a small hydrophobic amino acid. Unlike HLE and proteinase 3, cathepsin G, which is a rather poor proteolytic enzyme, has a primary substrate specificity for aromatic and basic amino acids at this position.[5] Of note is that proteinase 3 is known to be the major autoantigen in Wegener's granulomatosis.[6]

A screening of 17 cyanobacterial strains that belong to eight different genera in a HLE inhibition assay revealed the strain *Nostoc insulare* (Nostocales) to be a potent producer of inhibitors of this enzyme.

The predominant group of cyanobacterial metabolites are peptides of diverse molecular architectures. Many of these peptides are anticipated to be synthesized by nonribosomal peptide synthetases (NRPSs), which operate nucleic acid free at the protein level, and each biosynthetic step requires a respective protein module. A minimal module consists of adenylation, thiolation and condensation domains (named A-, T- and C-domains) for amino acid activation, transfer of activated intermediates, and formation of the peptide bond, respectively.[7–11] NRPS-derived peptides normally contain unusual amino acids like methylproline, which has been described in spumigins (4-methylproline),[12] scytonemin A (3-methylproline),[13] nostopeptolides A1–3 (4-methylproline),[14] and nostocyclopeptides A1–2 (4-methylproline) for example.[15] For nostopeptolides A1–3 and nostocyclopeptides A1–2, the biosynthesis of (2*S*,4*S*)-4-methylproline was recently investigated by feeding experiments and biomolecular investigations.[16,17]

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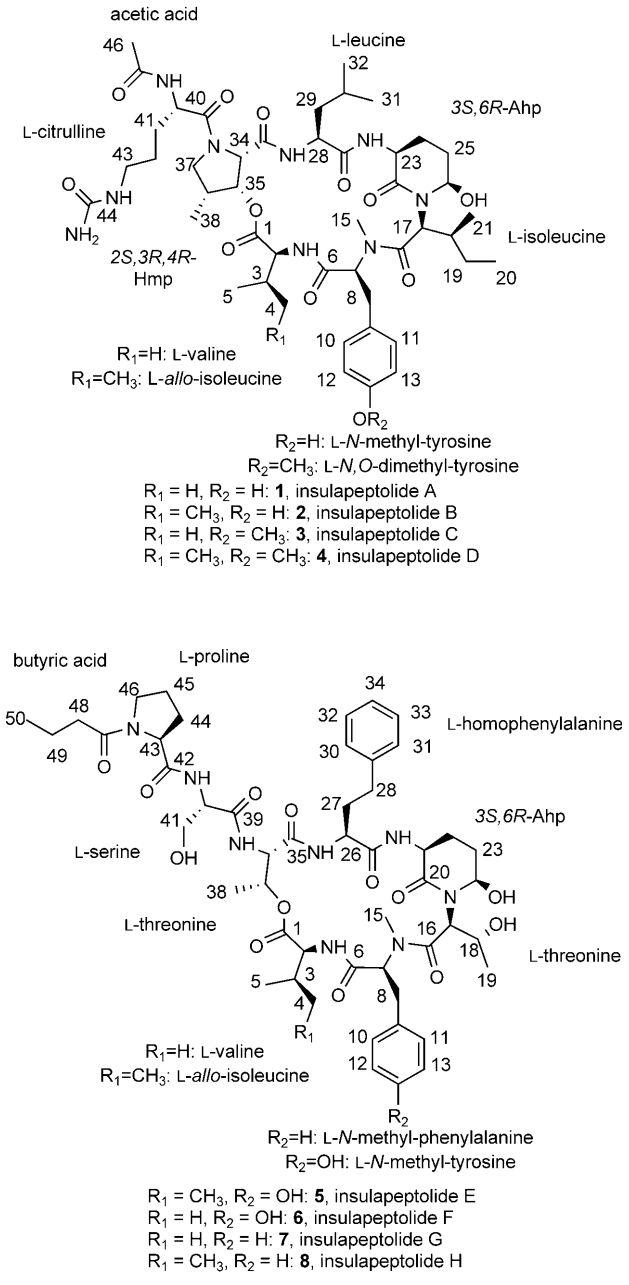
*Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author: NMR spectroscopic data for compounds **1**–**8**; selective ROESY experiments and LC–MS runs (advanced Marfey's method) to determine the relative and absolute configuration of Hmp in compound **1**; chiral GC–MS run for **1**; data of the HLE inhibition assays for the crude extract of N. insulare and the VLC fractions; incubation experiment of HLE with **4**; alignments of the putative NRPS fragment NoiN1 with NosA and NosC; alignment of NoiF with NosF and NcpE; proposal for the biosynthesis of Hmp.*

By making use of the well-documented genetic information of biosynthetic gene clusters for NRPS-type molecules from cyanobacteria (see refs. [17–21]), the metabolic potential of *N. insulare* was investigated by a PCR-based methodology, which showed that this strain is a putative producer of NRPS-derived natural products. Additionally, MALDI-TOF analysis of the extract revealed the presence of unprecedented peptides.

After large-scale cultivation of *N. insulare*, eight new peptides (**1**–**8**, see Scheme 1) with inhibitory activity toward HLE in the micro- to nanomolar range could be isolated by using the bioassay to identify the most-active fractions. Compounds **1**–**4** (insulapeptolides A–D) with the unusual amino acid 3-hydroxy-4-methyl-proline (Hmp) and citrulline (Cit; the latter is extremely rare in cyanobacterial peptides; the only examples are symplocamide A[22] and the nostropeptins E–K[23]) were the most-active compounds with IC_{50} values toward HLE from 85–140 nM. The second group of peptides (**5**–**8**, insulapeptolides E–H), in which the Hmp moiety is exchanged for L-threonine were less potent (IC_{50} values: 1.6–3.5 μM). The inhibitory profile of this class of peptolides was also evaluated with respect to the related neutrophil serine proteases, that is, proteinase 3 and cathepsin G.



Scheme 1. Structures of the insulapeptolides A–H (compounds **1**–**8**).

Results

The crude extracts (MeOH/CH_2Cl_2, 1:2) of 17 cyanobacterial strains that belong to the genera *Tychonema*, *Synechocystis*, *Oscillatoria*, *Fischerella*, *Scytonema*, *Plectonema*, *Tolypothrix*, and *Nostoc* were tested in an HLE activity assay. From all extracts only the one of *N. insulare* (Nostocales) showed a remarkable inhibition of the enzyme with an IC_{50} value of 9 μg mL^{-1} (Supporting Information).

To evaluate the biosynthetic potential of *N. insulare*, a molecular biological analysis that was based on PCR studies was conducted with degenerate primers that were deduced from conserved sequence motifs of adenylation domains of nonribosomal peptide synthetase (NRPS) genes (MTF_2/MTR). This way, it was possible to amplify a putative *nrps* gene fragment of the expected size of 1000 bp from the DNA of *N. insulare*. Several conserved sequence motifs that were typical of adenylate-forming domains (A2–A8) could be identified, and this enabled the PCR amplicon to be classified as a putative peptide synthetase gene fragment that shows homology (on the protein level) to the NRPS sequences *nosA* (60%) and *nosC* (61%) from *Nostoc* sp. GSV224;[18] *nosA* (gene bank no. AAF15891) and *nosC* (gene bank no. AAF17280) belong to a gene cluster that encodes for the biosynthetic enzymes of nostopeptolides A1 and A2.[14] By analyzing the amino acid sequence of the gene fragment, it was possible to determine nine of ten amino acid residues that represent the amino acid binding pocket of one adenylation domain of the NRPS. These specific amino acids, the so-called 'codons' of nonribosomal peptide synthesis, determine which amino acid is selected by the A-domain in question.[10,11] This analysis showed that the amplified *nrps* gene fragment putatively encodes part of an A-domain, which specifically binds leucine (Table 1). To further specify the peptides that are possibly produced by *N. insulare*, we chose a second PCR experiment with a primer pair that was deduced from the

Table 1. Selectivity conferring code of the putative A-domain NoiN1. The obtained codon (first row) is compared with literature data (second row).

Plasmid	Residue position[b]									Activated substrate[c]
	235	236	239	278	299	301	322	330	331	
Noi N1[a]	D	A	W	F	L	G	N	V	V	
	D	A	W	F	L	G	N	V	V	Leu

[a] Plasmid that contained the putative *nrps* gene fragment of *N. insulare* as insert DNA. [b] Position of amino acids (one-letter code) within the A-domain of a NRPS, according to Stachelhaus et al.[11] [c] The amino acid that is specifically adenylated by the A-domain of this module according to literature data.[10,11,18]

amplification experiment of the *nosF* gene (gene bank no. AAF17284).[16,18] This gene encodes for a reductase that is responsible for the last step of the biosynthesis of the rather rare nonproteinogenic amino acid (2*S*,4*S*)-4-methylproline.[16,18] It was possible to amplify a gene fragment of the expected size (about 820 bp), and the deduced amino acid sequence of this amplicon showed 90% identity to NosF. From these experiments, we concluded that the NRPS-derived peptides may contain a C-methylated proline residue.

A MALDI-TOF characterization of the crude extract revealed the presence of several molecular masses (for example, *m/z* 964.5, 978.5, 992.5, 1013.5, 1027.5, 1029.5, 1041.5, 1043.5, and 1057.5) for some of which, no corresponding cyanobacterial compounds could be found in the databases (Antibase 2008, Wiley and database of AnagnosTec GmbH, Potsdam, Germany).

Due to the results of the molecular biological and MALDI-TOF investigations as well as the remarkable inhibitory activity of the crude extract, our strain *N. insulare* was chosen for large-scale cultivation in a 25 L photobioreactor. The obtained biomass was lyophilized and subsequently extracted with dichloromethane/methanol (1:2). The crude extract was separated using silica gel vacuum liquid chromatography (VLC). Eight fractions were subjected to the HLE bioassay (for detailed results see the Supporting Information). Two active fractions, the most polar ones, were purified by preparative reversed-phase HPLC to obtain insulapeptolides A–H (compounds **1**–**8**, see Scheme 1) as white amorphous materials. The structures of these compounds were elucidated by using spectroscopic methods, including 1D (^{1}H, ^{13}C, and DEPT 135) and 2D NMR spectroscopy experiments (HSQC, COSY, HMBC, and NOESY/ROESY). The absolute configurations of the amino acids were determined by chiral GC–MS, chiral HPLC, LC–MS (advanced Marfey's method)[24,25] and combined analysis of homonuclear couplings (^{1}H,^{1}H) and NOE data.

The molecular formula of compound **1** was determined to be $C_{46}H_{71}N_9O_{12}$ based on HRMS (ESI) measurement (calcd: 964.5120 $[M+Na]^+$; found: 964.5115) and NMR spectral data (Table 2). The ^{1}H NMR spectrum showed characteristic resonances for α-CH groups between δ 4.5 and 5.4 ppm. Resonances between δ 6.7 and 7.2 ppm provided evidence for the pres-

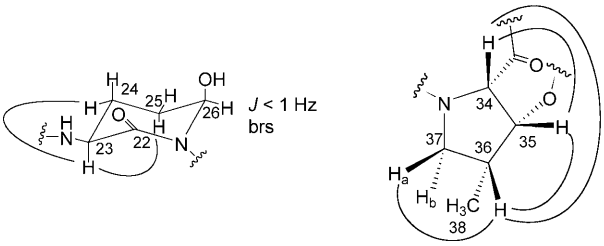
Table 2. NMR spectroscopic data for compound **1** (500 MHz, CD_3OD).

	C/H no.	δ_H (J in Hz)[a]	δ_C[a]	HMBC	ROESY (selected)
Val	1	–	175.1	–	–
	2	4.96, d (4.7)	58.4	1, 3, 4, 5, 6	–
	3	2.33, m	33.2	–	38
	4	0.89, d (6.9)	17.8	2, 3	15, 35
	5	1.03, d (6.9)	20.4	2, 3	15
N-Me-Tyr	6	–	172.0	–	–
	7	5.37, br d (11.4)	62.7	6, 8, 15	17
	8a	3.37, m	34.7	7, 9, 10, 11	–
	8b	2.80, dd (11.4; 14.8)	–	7, 9, 10, 11	17
	9	–	129.2	–	–
	10	7.14, d (8.2)	131.9	8, 12, 13, 14	15, 17, 20, 21
	11	7.14, d (8.2)	131.9	8, 12, 13, 14	15, 17, 20, 21
	12	6.73, d (8.2)	116.8	9, 14	21
	13	6.73, d (8.2)	116.8	9, 14	21
	14	–	157.7	–	–
N-Me	15	2.89, s	31.7	7, 16	4, 5, 10, 11
Ile	16	–	172.8	–	–
	17	4.61, d (11.0)	56.6	16, 18, 21, 22, 26	7, 8b, 10, 11
	18	1.95, m	34.7	–	–
	19a	1.24, m	25.3	–	–
	19b	0.84, m	–	–	–
	20	0.76, t (7.0)	10.8	18, 19	10, 11
	21	0.06, d (6.3)	14.8	17, 18, 19	10, 11, 12, 13
Ahp	22	–	171.5	–	–
	23	4.66, m	50.7	22, 24, 27	24b, 25
	24a	2.99, m	22.2	–	–
	24b	1.97, m	–	–	23
	25	1.96, m	31.1	23, 26	23, 26
	26	5.15, br s	76.0	22, 24	25
Leu	27	–	174.6	–	–
	28	4.67, m	52.5	27, 33	–
	29a	2.02, m	41.1	–	–
	29b	1.58, m	–	–	–
	30	2.12, m	24.6	–	–
	31	1.00, d (6.3)	24.3	29, 30, 32	–
	32	0.93, d (6.3)	21.6	29, 31	34
Hmp	33	–	169.5	–	–
	34	4.53, d (3.2)	66.4	33	32, 35, 36
	35	5.34, t (3.2)	79.2	1	4, 34, 36
	36	2.62, m	39.0		34, 35, 37a, 38
	37a	4.33, br t (8.8)	53.5	34, 35, 36, 39	36, 37b, 40, 42
	37b	3.57, br t (10.4)		36, 38	37a, 38, 40, 42
	38	1.20, d (6.3)	11.4	35, 36, 37	3, 36, 37b
Cit	39	–	173.6	–	–
	40	4.59, m	52.8	39, 41, 42, 45	37a, 37b
	41	1.84, m	29.7	40, 42	46
	42	1.74, m	26.6	–	37a, 37b
	43a	3.26, m	40.5	41	–
	43b	3.08, m		41, 42, 44	–
	44	–	162.3	–	–
Ac	45	–	173.4	–	–
	46	2.00, s	22.2	45	41

[a] Assignments are based on extensive 1D and 2D NMR measurements (HMBC, HSQC, COSY).

ence of an aromatic moiety. Additionally, the ^{1}H NMR spectrum featured two sharp signals at δ 2.00 and 2.89 ppm for an acetylic methyl group (CH_3-46) and an *N*-methyl group (CH_3-15), respectively. The ^{13}C NMR spectrum revealed signals for nine methyls, nine methylenes, 17 methine groups, and eleven quaternary carbons. After assignment of all protons to their directly bonded carbon atoms using ^{1}H,^{13}C HSQC spectroscopy data, it was possible to establish all amino acid residues by ^{1}H,^{1}H COSY and HMBC experiments. HMBC and NOE correlations enabled us to connect the single amino acids of compound **1** to a cyclic structure with a short side-chain. ^{1}H,^{1}H COSY correlations from H-3 (δ_H 2.33 ppm) to H_3-4 (δ_H 0.89 ppm), H_3-5 (δ_H 1.03 ppm) and the α-CH-2 (δ_C 58.4 ppm and δ_H 4.96 ppm) indicated the first residue to be a valine. ^{1}H and ^{13}C NMR spectra showed signals that were characteristic of a 1,4-disubstituted benzene ring (resonances for H-10/11: δ_H 7.14 ppm, d, J=8.2, 2H and H-12/13: δ_H 6.73 ppm, d, J=8.2, 2H). In the HMBC experiment, a strong correlation between H-10/11 and C-8 (δ_c 34.7 ppm) (the proton of the latter being part of the CH-7, CH_2-8 ^{1}H,^{1}H spin system) was detectable. The characteristic downfield shift of C-14 (δ_C 157.7 ppm) indicated a hydroxyl group at this position. A strong HMBC correlation between H_3-15 (δ_H 2.89 ppm) and C-7 (δ_C 62.7 ppm) indicated that the established tyrosine moiety was *N*-methylated. Because H-2 of valine (δ_H 4.96 ppm) exhibited a long-range CH coupling to the carbonyl C-6 (δ_C 172.0 ppm) of *N*-methyl-tyrosine, both amino acids could be identified as directly neighboring. Additionally, the *N*-methyl group CH_3-15 (δ_C 31.7 and δ_H 2.89 ppm) showed an HMBC correlation to the carbonyl C-16 (δ_C 172.8 ppm) of an isoleucine residue, which was assigned by ^{1}H,^{1}H COSY correlations. The direct neighborhood of isoleucine and the aromatic amino acid was furthermore confirmed by the unusual highfield resonance of the methyl group CH_3-21 (δ_H 0.06 ppm), which is caused by the anisotropic effect of the aromatic ring.[26] A further spin system consisting of CH-23, CH_2-24, CH_2-25, CH-26 could be clearly established by ^{1}H,^{1}H COSY correlations. The α-methine proton H-23 (δ_H 4.66 ppm) showed a ^{1}H,^{13}C long-range correlation to the carbonyl group C-22 (δ_C 171.5 ppm). Because the oxymethine proton H-26 (δ_H 5.15 ppm) also had a HMBC correlation to this carbonyl function it was quite clear that C-22 to CH-26 were arranged in a cyclic structure. A comparison with literature data[27] led us to the conclusion that this residue was 3-amino-6-hydroxy-2-piperidone (Ahp). Because of the ^{1}H,^{13}C long-range coupling between H-17 (δ_H 4.61 ppm) of isoleucine and the carbonyl group C-22 (δ_C 171.5 ppm), it was presumed that the Ahp formed a hemiaminal with this isoleucine residue. H_2-29 (δ_H 1.58, 2.02 ppm) showed ^{1}H,^{1}H COSY correlations to H-28 (δ_H 4.67 ppm) and H-30 (δ_H 2.12 ppm). Furthermore, ^{1}H,^{1}H COSY correlations between H_3-31 (δ_H 1.00 ppm) and H-30 as well as between H_3-32 (δ_H 0.93 ppm) and H-30 could be observed. ^{1}H,^{1}H COSY data thus clearly delineated a leucine residue. The α-H-23 of Ahp (δ_H 4.66 ppm) coupled with the carbonyl C-27 (δ_C 174.6 ppm) of this leucine, which prolonged the peptide in the N-terminal direction. The oxymethine function CH-35 (δ_C 79.2 and δ_H 5.34 ppm) exhibited ^{1}H,^{1}H COSY correlations to H-34 (δ_H 4.53 ppm) and H-36 (δ_H 2.62 ppm). H-36 showed additional ^{1}H,^{1}H COSY correlations to H_2-37 (δ_H 3.57, 4.33 ppm) and to the methyl group CH_3-38 (δ_H 1.20 ppm). A HMBC correlation between H-37a (δ_H 4.33 ppm) and α-CH-34 (δ_C 66.4 and δ_H 4.53 ppm) indicated the cyclization of this residue to the unusual amino acid Hmp. H-28 (δ_H 4.67 ppm) of leucine had a weak HMBC correlation to the carbonyl group of Hmp (δ_C 169.5 ppm) and the oxymethine proton of Hmp (H-35; δ_H 5.34 ppm) showed a long-range correlation to C-1 (δ_C 175.1 ppm). Therefore, Hmp is the linkage between the N-terminal leucine and the C-terminal valine, resulting in a 19-membered cyclic depsipeptide. The connectivity between the valine and the Hmp residue was confirmed by ROESY correlations between H-3 (δ_H 2.33 ppm) and the H_3-38 (δ_H 1.20 ppm) of Hmp. ^{1}H,^{1}H COSY correlations allowed us to deduce the presence of a further spin system that began at CH-40 (δ_C 52.8 and δ_H 4.59 ppm) and reached through to CH_2-43 (δ_C 40.5 and δ_H 3.08, 3.26 ppm). A HMBC correlation between H_2-43 and the quaternary C-44 (δ_C 162.3 ppm) suggested the presence of an arginine-like amino acid that did not belong to the cyclic inner core of the peptide. A ROESY correlation between CH_3-46 of the acetyl group (δ_C 22.2 and δ_H 2.00 ppm) and CH_2-41 (δ_C 29.7 and δ_H 1.84 ppm) of this residue determined the amine function to be acetylated. The HMBC coupling between H-37a (δ_H 4.33 ppm) and C-39 (δ_C 173.6 ppm), as well as the strong ROESY correlation between the α-H-40 of the arginine like residue (δ_H 4.59 ppm) and both H_2-37 protons (δ_H 3.57, 4.33 ppm) attached this acetylated amino acid as a "western part" chain to the Hmp amino group. The resulting structural proposal had a mass difference of +1 Da to the measured high-resolution data, which gave rise to the proposal that the arginine-like residue had to be the rarely described citrulline. This assumption was confirmed later by GC–MS analysis.

By chiral GC–MS and chiral HPLC analyses of the hydrolysate, the absolute configuration of several amino acids could be determined as: L-valine, L-leucine, L-isoleucine, L-citrulline and L-*N*-methyl-tyrosine. The relative stereochemistry of Ahp was determined to be (3*S**,6*R**)-Ahp based on NMR spectral data. Due to the very small ^{1}H,^{1}H-coupling of less than 1.0 Hz that was observed for H-26 (br s), the hydroxy group at C26 had to be axially oriented. This axial orientation of the hydroxy group is responsible for the downfield shift of the axially oriented H_a-24 (δ_H 2.99 ppm).[28] The ROESY correlations between H-23 (δ_H 4.66 ppm) and the equatorial H_b-24 (δ_H 1.97 ppm) and between H-23 and H_2-25 (δ_H 1.96 ppm) determined the relative configuration as mentioned above (Scheme 2). By oxidation of compound **1**,[29] following hydrolyzation and advanced Marfey's method[24,25,27] the absolute configuration of the resulting glutamate could be determined as L, and thus the absolute configuration of Ahp as 3*S*,6*R* (=23*S*,26*R* in **1**). The relative configuration of the unusual amino acid Hmp was determined on the basis of a selective ROESY experiment to be (2*S**,3*R**,4*R**)-3-hydroxy-4-methyl-proline (see also Scheme 2). Irradiation of H-36 (δ_H 2.62 ppm) caused, beyond the enhancement of H_3-38 (δ_H 1.20 ppm), enhancements of H-34 (δ_H 4.53 ppm), H-35 (δ_H 5.34 ppm), and H-37a (δ_H 4.33 ppm); this indicates that all these protons are positioned on the same side of the heterocycle. The enhancements of H-34 and H-36



Scheme 2. Selected ROESY correlations, which led to the determination of the relative configuration of Ahp and Hmp in compound **1**.

after irradiation of H-35 further confirmed the relative configuration. To determine the absolute configuration, an advanced Marfey's analysis of the hydrolysate was performed,[24,25,27,30] and was shown to be (2*S*,3*R*,4*R*)-3-hydroxy-4-methylproline (for results of selective ROESY experiments and of Marfey's analysis see the Supporting Information). Compound **1** is named insulapeptolide A.

HRMS (ESI) measurement of insulapeptolide B (**2**) revealed a molecular formula of $C_{47}H_{73}N_9O_{12}$ (*m/z* calcd: 978.5276 $[M+Na]^+$; found: 978.5279). From ^1H, ^{13}C, and DEPT 135 NMR spectroscopic data, it became apparent that compound **2** showed high similarity to **1**. A mass difference of +14 Da and a shift of the protons in the methyl region of the ^1H NMR spectra were the first hint toward an exchange of the valine residue for an isoleucine. Amino acid analysis of the hydrolysate by chiral GC–MS of **2** indeed confirmed the presence of an L-*allo*-isoleucine in addition to L-leucine, L-citrulline, L-*N*-methyl-tyrosine and L-isoleucine. Interpretation of the 2D NMR spectroscopic data confirmed that the L-valine of insulapeptolide A was exchanged with an isoleucine in insulapeptolide B. Assuming that both peptides are derived from the same NRPS complex with the A-domain, which has a relaxed substrate specificity for valine or isoleucine, we propose that the L-valine in **1** is exchanged against L-*allo*-isoleucine in **2**, whereas the rest of the peptide is identical. By using NMR spectroscopic data and by following advanced Marfey's method the configuration of Ahp and Hmp could be shown to be identical to insulapeptolide A.

Compounds **3** (*m/z* calcd for $C_{47}H_{73}N_9NaO_{12}$: 978.5276 $[M+Na]^+$; found: 978.5258) and **4** (*m/z* calcd for $C_{48}H_{75}N_9NaO_{12}$: 992.5433 $[M+Na]^+$; found: 992.5433) could be isolated as two minor metabolites. The ^1H NMR spectra showed an additional signal for a methoxy group (δ_H 3.78 ppm) as compared to the spectra of compounds **1** and **2**, respectively. A HMBC correlation between the methyl protons (R_2) and the quaternary, hydroxylated aromatic carbon (C14) of the *N*-methyl-tyrosine residue confirmed a further methylation of this amino acid in **3** and **4**. Detailed analysis of the 2D NMR (COSY, HSQC, HMBC, and ROESY) spectroscopic data showed that methylation of the aromatic hydroxy functionality was the only difference between insulapeptolide A (**1**) and C (**3**) on one hand, and between insulapeptolide B (**2**) and D (**4**) on the other. The absolute configurations of **3** and **4** were supposed to be the same as in **1** and **2**, as all four structures are congeners of one group of cyanopeptolins.

From the active fractions of the crude extract of *N. insulare* a second group of cyanopeptolins, comprising compounds **5**–**8**, were isolated. The HRMS (ESI) of insulapeptolide E (**5**) showed an ion peak at *m/z* 1043.5084 $[M+Na]^+$ that corresponded to molecular formula of $C_{51}H_{72}N_8NaO_{14}$ (calcd: 1043.5066). A detailed analysis of the 2D NMR spectroscopic data that was obtained from HSQC, COSY, HMBC, and ROESY experiments allowed us to conclude that compound **5** is also a cyclic peptide that belongs to the cyanopeptolin class, but with some differences in the cyclic sequence and the side-chain in comparison to insulapeptolides A–D. By COSY and HMBC experiments the following residues could be established: isoleucine, *N*-methyltyrosine, two threonine, Ahp, homophenylalanine, serine, proline, and butyric acid. By HMBC correlations from the α-CH groups of the single amino acids to the carbonyl groups of the adjacent amino acids, as well as ROESY correlations—for example, between H-28a (δ_H 2.79 ppm) and H-37 (δ_H 5.62 ppm)—the whole amino acid sequence was determined. The connectivity between the N-terminal end of the side-chain (proline) and butyric acid could be shown by the ROESY correlation between H-48a (δ_H 2.40 ppm) and H_2-46 (δ_H 3.63, 3.70 ppm). The absolute configurations were shown to be L-*allo*-isoleucine, L-*N*-methyl-tyrosine, L-threonine, L-homophenylalanine, L-serine, and L-proline by chiral GC–MS and chiral HPLC investigations of the hydrolysate. The configuration of Ahp (3*S*,6*R*) was determined in the same manner as for insulapeptolide A.

In insulapeptolide F (**6**) (*m/z* calcd for $C_{50}H_{70}N_8NaO_{14}$: 1029.4909 $[M+Na]^+$; found: 1029.4907) the isoleucine residue of **5** was exchanged with a valine, as evident from NMR spectroscopic data (Supporting Information).

For compound **7** (*m/z* calcd for $C_{50}H_{70}N_8NaO_{13}$: 1013.4960 $[M+Na]^+$; found: 1013.4953) an obvious change of the resonance signals in the aromatic region of ^1H and ^{13}C NMR spectra was discernable. Further investigations of the 1D and 2D NMR spectroscopic data proved that the only difference between insulapeptolide G (**7**) and insulapeptolide F (**6**) is the replacement of the *N*-Me-Tyr against an *N*-Me-Phe residue.

Mass spectral analysis revealed a molecular formula of $C_{51}H_{72}N_8O_{13}$ (*m/z* calcd 1027.5117 $[M+Na]^+$; found: 1027.5099) for insulapeptolide H (**8**). The NMR spectra were most similar to those of compound **7**, but they lacked the signals for a valine residue and showed signals for an isoleucine moiety instead. Therefore, valine in insulapeptolide G (**7**) is exchanged against an isoleucine in insulapeptolide H (**8**). The absolute configurations of compounds **6**–**8** are assumed to be the same as in compound **5**.

Thus, eight peptides, which correlate to seven of nine initially detected MALDI-TOF signals at *m/z* 964.5 (insulapeptolide A), 978.5 (insulapeptolide B and C), 992.5 (insulapeptolide D), 1013.5 (insulapeptolide G), 1027.5 (insulapeptolide H), 1029.5 (insulapeptolide F) and 1043.5 (insulapeptolide E) were purified from the highly complex crude extract. Two minor compounds, which probably also belong to the cyanopeptolin class, with signals of low intensity at *m/z* 1041.5 and 1057.5 in the MALDI-TOF spectrum could not be isolated. Assuming that the signals, like the others, refer to $[M+Na]^+$ ions, a mass difference of 14 Da to insulapeptolide H and insulapeptolide E, respectively,

suggests a methylation of a further hydroxyl group, for example, of the Ahp residue. Methylations at this position are also known from literature reports, and they support this presumption.[23,31]

Insulapeptolides A–H were evaluated toward the serine proteases human leukocyte elastase (HLE), cathepsin G and proteinase 3 (Table 4). Insulapeptolides A–D (**1**–**4**) were highly potent inhibitors of HLE with IC_{50} values between 85 and 140 nM, whereas insulapeptolides E–H (**5**–**8**) were far less active with IC_{50} values ranging from 1.6–3.5 μM. Toward proteinase 3 and cathepsin G, compounds **1**–**8** were much weaker inhibitors (IC_{50} values >16 μM). Insulapeptolides A–H were thus highly selective for HLE in respect to these enzymes. An IC_{50} value of 85 nM was determined for compound **4** (Figure 1), which corresponds to a K_i value of 30 nM in the case of competitive inhibition. The competitive character of inhibition was exemplarily confirmed for this peptolide by using four different substrate concentrations, and was indicated by the parallel lines of the Hanes–Woolf plot (Figure 2A). This analysis resulted in a K_i of 36 nM as obtained from the secondary plot (Figure 2B). Furthermore, HLE was incubated over 90 min with compound **4**, and enzymatic activity was followed by adding aliquots to the chromogenic substrate. A constant residual HLE activity was observed during this incubation time. This indicates that insulapeptolide D was not degraded by HLE (Supporting Information).

Table 3. NMR spectroscopic data for compound **5** (500 MHz, CD_3OD).

	C/H no.	δ_H (J in Hz)[a]	δ_C[a]	HMBC	ROESY (selected)
Ile	1	–	175.2	–	–
	2	4.74, d (6.3)	57.2	1, 3, 4, 5, 6	–
	3	2.00, m	38.2	2, 4, 5, 51	–
	4a	1.45, m	27.5	2, 3, 5, 51	–
	4b	1.28, m	–	2, 3, 5, 51	–
	51	1.01, t (7.6)	11.6	3, 4	–
	5	0.92, d (6.6)	15.7	2, 3, 4	37
N-Me-Tyr	6	–	172.0	–	–
	7	5.03, dd (3.2; 11.7)	63.3	6, 8, 9, 15, 16	17, 18
	8a	3.39, m	34.5	7, 9, 10, 11	17, 18
	8b	2.79, m		7, 9, 10, 11	17, 18
	9	–	129.1	–	–
	10	7.12, d (8.2)	131.8	8, 12, 13, 14	18, 19
	11	7.12, d (8.2)	131.8	8, 12, 13, 14	18, 19
	12	6.75, d (8.2)	116.8	9, 14	17, 18, 19
	13	6.75, d (8.2)	116.8	9, 14	17, 18, 19
	14	–	157.7	–	–
N-Me	15	2.87, s	31.3	7, 16	10, 11
Thr1	16	–	173.4	–	–
	17	4.58, d (6.6)	56.1	16, 18, 19, 20, 24	7, 8a, 8b, 10, 11, 24
	18	3.70, m	67.4	17, 19	7, 8a, 8b, 10, 11, 12, 13
	19	0.55, d (6.3)	19.6	17, 18	10, 11, 12, 13
Ahp	20	–	171.8	–	–
	21	4.66, dd (6.6; 12.6)	51.0	20, 22, 25	22b, 23b
	22a	2.78, m	22.4	21	–
	22b	1.91, m		21, 24	21
	23a	2.06, m	30.8		
	23b	1.88, m	–	21, 24	21
	24	5.35, brs	77.3	17, 20, 22	17
Hphe	25	–	173.9	–	–
	26	4.40, brd (9.1)	54.5	25	–
	27a	2.49, m	33.4	26, 28	–
	27b	1.98, m	–	–	–
	28a	2.79, m	33.1	26, 27, 29, 30, 31	37
	28b	2.64, m	–	26, 27, 29, 30, 31	–
	29	–	142.2	–	–
	30	7.22, d (7.3)	129.7	29, 34	–
	31	7.22, d (7.3)	129.7	29, 34	–
	32	7.30, t (7.3)	129.5	29, 34	–
	33	7.30, t (7.3)	129.5	29, 34	–
	34	7.20, t (7.3)	127.1	30, 31	–
Thr2	35	–	171.5	–	–
	36	4.80, brs	57.3	35, 37, 38, 39	–
	37	5.62, q (6.6)	73.6	1, 35, 38	5, 28a
	38	1.45, d (6.6)	18.7	36, 37	–
Ser	39	–	173.2	–	–
	40	4.69, t (5.4)	56.8	39, 41, 42	–
	41a	4.00, dd (5.4; 11.4)	63.3	39, 40	–
	41b	3.93, dd (5.4; 11.4)	–	39, 40	–
Pro	42	–	174.9	–	–
	43	4.55, dd (3.8; 8.5)	61.3	42, 44, 45, 46	–
	44a	2.28, m	30.9	42, 43, 45, 46	–
	44b	2.15, m	–	42, 43, 45, 46	–
	45a	2.12, m	25.9	43, 44, 46	–
	45b	2.02, m	–	43, 44, 46	–
	46a	3.70, m	48.8	43, 44, 45	48a
	46b	3.63, m	–	43, 44, 45	48a
Ba	47	–	174.6	–	–
	48a	2.40, m	37.3	47, 49, 50	46a, 46b
	48b	2.28, m	–	47, 49, 50	–
	49	1.68, m	19.2	47, 48, 50	–
	50	1.01, t (7.6)	14.2	48, 49	–

[a] Assignments are based on extensive 1D and 2D NMR measurements (HMBC, HSQC, COSY).

Discussion

Today genetic information that is related to the biosynthesis of natural products is easily retrievable from databases. In

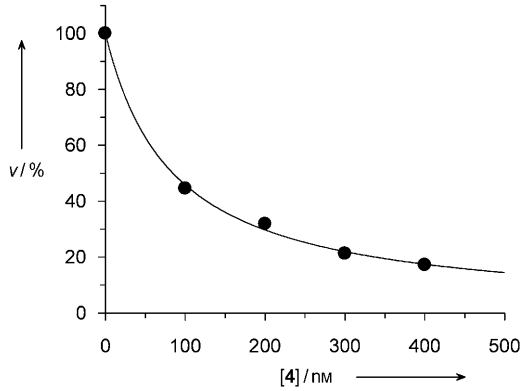


Figure 1. Inhibition of HLE by compound **4** in the presence of 100 μM (= 1.85 K_m) of the chromogenic substrate MeO-Suc-Ala-Ala-Pro-Val-NHNp. The data are the mean values of duplicate measurements. The reactions were followed over 10 min, and the rates, *v*, were determined by linear regression. Rates in the absence of inhibitor, v_0, were set to 100%. Nonlinear regression according to the equation $v = v_0/([I]/IC_{50} + 1)$ gave a value $IC_{50} = 85 \pm 4$ nM.

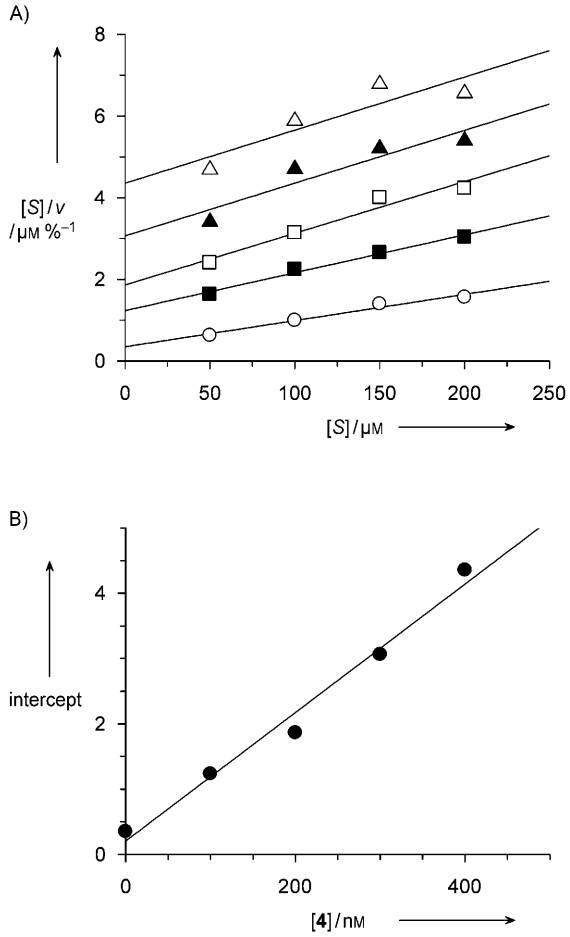


Figure 2. Inhibition of HLE by compound **4** in the presence of different concentrations of the chromogenic substrate MeO-Suc-Ala-Ala-Pro-Val-NHNp. The reactions were followed over 10 min, and the rates, *v*, were determined by linear regression. Rates in the absence of inhibitor and the presence of 100 μM of the substrate were set to 100%. A) Hanes–Woolf plot [S]/*v* versus substrate concentrations, [S]. The data are mean values of duplicate measurements with inhibitor concentrations, [I], of 400 nM (△), 300 nM (▲), 200 nM (□), 100 nM (■) and in the absence of the inhibitor (○). The parallel lines indicate competitive inhibition. Linear regression gave values for vertical intercepts. The intercept for the data in the absence of inhibitor, K_m/V_{max}, was 0.35 ± 0.10 μM %$^{-1}$. B) Plot of vertical intercepts versus the concentrations of **4**. Linear regression according to the equation: intercept = K_m [I]/(K_i V_{max}) + K_m/V_{max}, gave a slope $K_m/(K_i\ V_{max}) = 9.8 \pm 0.8$ %$^{-1}$, that corresponds to a value $K_i = 36 \pm 11$ nM.

Genbank (http://www.ncbi.nlm.nih.gov/Genbank/), more than 450 sequences that are related to NRPS biosynthetic gene clusters are listed; this provides the possibility to deduce appropriate primers and to search for organisms that have the genetic potential to produce such metabolites. Moreover, detailed information on consensus sequences, as are known for adenylation domains (A-domains) of NRPSs, allow one to deduce structural details for the produced natural product.[32,33] The use of molecular biology to screen cyanobacteria (identification of NRPS and PKS genes) has been reported in the literature as an appropriate tool for the identification of the most likely sources of (bioactive) natural products.[34–37] In the current study, a PCR-based screen revealed the ability of *N. insulare* to produce peptides with leucine, methylproline, or derivatives of these two amino acids as subunits. MALDI-TOF investigations of the crude extracts and the results of a HLE inhibition assay proved *N. insulare* to produce bioactive, not yet described, secondary metabolites. Subsequently, the isolation of insulapeptolides A–H (**1**–**8**) was guided by the HLE bioassay.

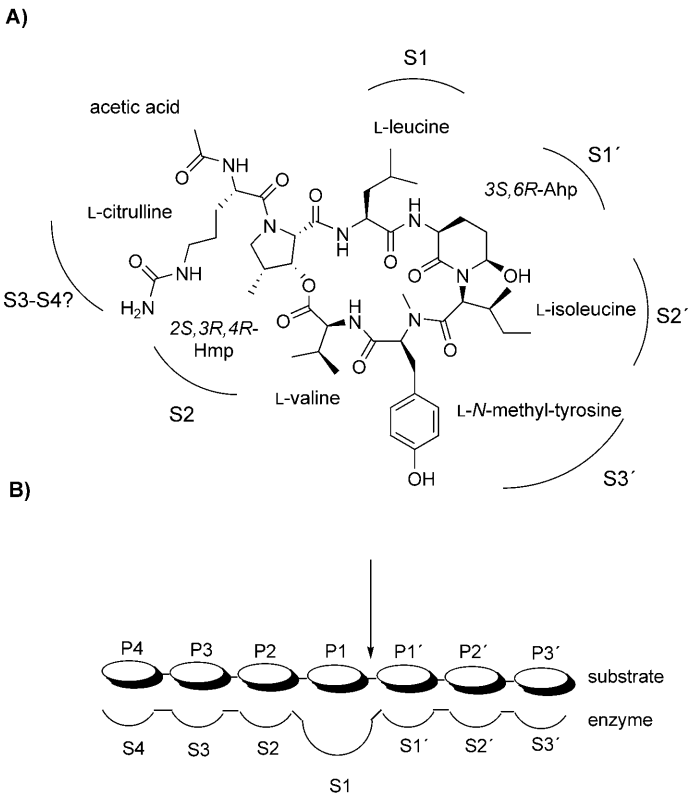
Insulapeptolides belong to the cyanopeptolin group of nonribosomal peptides,[19,38] one of the largest classes of cyanobacterial cyclic peptides to be characterized so far. This group of compounds is defined as 19-membered cyclic depsipeptides that contain the unusual residue Ahp and a side-chain of variable length with a terminal carboxylic acid that is attached to the cyclic core structure.[7,39,40] A striking feature of compounds **1**–**4**, which they share with nostopeptin A and B[41] and the nostropeptins,[23] is the position of the unusual amino acid Hmp, which replaces the threonine residue that is present at this site in all other cyanopeptolins.

The biosynthesis of insulapeptolides A–H (**1**–**8**) probably does not involve eight individual gene clusters of nonribosomal peptide biosynthesis. Two gene clusters that encode the two enzyme systems responsible for the biosynthesis of insulapeptolides A–D and the insulapeptolides E–H are assumed. The observed structural diversity within both groups of insulapeptolides can be explained by incomplete biosynthetic processes and/or relaxed substrate specificities of biosynthetic enzymes. Compounds **1** and **2** contain an *N*-methyltyrosine, whereas compounds **3** and **4** contain a *N,O*-dimethyltyrosine residue; this makes it unlikely that a inaccurately working *O*-methylation domain is responsible for this set of compounds. On the other hand, compounds **1**, **3**, **6**, and **7** contain an L-valine residue at a position at which in **2**, **4**, **5**, and **8** an L-*allo*-isoleucine is located. The occurrence of such pairs of compounds might be due to the presence of adenylation domains with a relaxed substrate specificity for valine or isoleucine, which is in good accordance with literature reports.[10,11,18,32] Also, the incorporation of phenylalanine (in compounds **7** and **8**) instead of tyrosine (in compounds **5** and **6**) could be derived from a relaxed substrate specificity of the respective A-domains. Such "inac-

curacies" in the biosynthesis of cyanobacterial NRPS-derived peptides often result in remarkable libraries of one structural type. For the nostopeptolides from *Nostoc* sp. GSV224[18] and the nostocyclopeptides from *Nostoc* sp. ATCC53789[17] this kind of combinatorial chemistry was proven by characterization of the biosynthetic gene cluster. Furthermore, the brunsvicamides A–C from *Tychonema* sp.,[26] the aurilides B and C from *Lyngbya majuscula*,[42] the wewakpeptins A–D from *Lyngbya semiplena*[43] and the largamides A–C and D–G from an *Oscillatoria* sp.[27] can also be presumed to be part of such naturally derived "libraries."

HLE, proteinase 3 and cathepsin G are homologous serine proteases that are major components of the primary azurophilic granules of polymorphonuclear neutrophils.[5] They play an important role in the proteolytic events associated with inflammation and involved in the development of diseases such as pulmonary emphysema, rheumatoid arthritis, and psoriasis.[2–5] Despite their subcellular localizations and similar 3D structures, these serine proteases have specific biological functions that have not been entirely clarified to date.[5] In a bioassay-guided approach, we have identified new peptolides as potent inhibitors of HLE. The cyanopeptolins **1**–**4** inhibited HLE with IC_{50} values of about 100 nM, whereas compounds **5**–**8** were less active with IC_{50} values in the lower micromolar range. Insulapeptolides A–H (**1**–**8**) were furthermore evaluated toward the closely related serine proteases proteinase 3 and cathepsin G. None of the compounds showed potent activity against these enzymes; this implies a high selectivity for HLE in relation to the two latter serine proteases (Table 4). Although the best compounds (**3** and **4**) exhibited some activity against proteinase 3 and cathepsin G, their IC_{50} values for HLE were 150- or 200-fold lower, respectively. The inhibition values of **1** and **2** for HLE and proteinase 3, which differed by three orders of magnitude, revealed a remarkable preference of these peptolides for HLE. The mode of inhibition of **4** was further characterized kinetically, and a K_i value for competitive inhibition of 36 nM was obtained (Figure 2). It can therefore be concluded that this compound occupies the substrate-binding site of HLE, which is also expected for the other peptides of this study. Serine protease-catalyzed hydrolysis follows an acylation–deacylation mechanism. It was considered that the cyanopeptolins might act as alternate substrates of HLE with a strongly decelerated deacylation step.[44] To test whether the enzyme could proteolytically cleave the peptides, an exemplary incubation experiment was performed with inhibitor **4**. During the time course of incubation, the HLE activity was not regained, which strongly indicates that the peptide inhibitor was not degraded by HLE. This finding is in agreement with results on the inhibition of porcine pancreatic elastase by the cyanopeptolin scyptolin A.[1] The crystal structure of the elastase–scyptolin A complex showed that the Ahp residue occupies a crucial part of the active site, thereby preventing the access of hydrolytic water and cleavage of the inhibitor. On the other hand, the insulapeptolides did not behave as irreversible inhibitors by acylating the active site serine of HLE. This could be concluded because time-independent inhibition was observed for all insulapeptolides, and during incubation with compound **4**, residual activity of HLE was maintained. Thus, we propose that the insulapeptolides act as competitive inhibitors by forming noncovalent enzyme–inhibitor complexes with HLE.

Insulapeptolides A–D (**1**–**4**) are very selective inhibitors of HLE. This selectivity can be explained taking literature reports into account. The enzyme subsites S2, S1′, S2′, and S3′ (standard nomenclature according to Schechter et al.;[45] Scheme 3) are the main determinants of substrate specificity for proteinase 3 and account for its difference from HLE, that is, in proteinase 3 these subsites are considerably more polar. Furthermore, HLE and proteinase 3 accommodate substrates with small, hydrophobic residues at P1 that interact with S1, whereas cathepsin G prefers aromatic or positively charged residues at this position.[2,5] Cyanopeptolins are well known to inhibit several serine proteases.[22,46] For example, scyptolin A and B from *Scytonema hofmanni* inhibit porcine pancreatic elastase with an IC_{50} value of 0.16 μM.[47] Scyptolin A is a cyclic depsipeptide with the sequence *N*-butyryl-Ala-Thr1-Thr2-Leu-Ahp-Thr_3-(3′-chloro-*N*-methyl-Tyr)-Val ((butyryl replaced butyroyl, in which the ester linkage is formed by the hydroxyl group of Thr_2 and the carboxyl group of valine. Matern et al.[1] reported the crystal structure of the scyptolin A–elastase complex showing that scyptolin A binds with its four N-terminal amino acid



Scheme 3. A) Proposed interaction of compound **1** with HLE (see Matern et al.[1] and Linington et al.[22]). B) Schechter and Berger convention for the nomenclature of the enzyme–peptide interaction sites.[2,45] The arrow indicates the scissile bond.

residues (Ala-Thr1-Thr2-Leu) to the subsites S4–S1 of elastase.[1] The interaction with subsites S1′, S2′, and S3′ could not be clearly defined. Scyptolins A and B as well as the insulapeptolides A–D possess leucine at the same position. In the scyptolin A–elastase complex, this residue was accommodated by the S1 pocket. This interaction is in agreement with the primary substrate specificity of elastases. Presumably, the binding of insulapeptolides A–D (**1**–**4**) to HLE is similar to the binding of scyptolin A to porcine pancreatic elastase, and accordingly, the methylproline of compounds **1**–**4** occupies the S2 pocket (Scheme 3). It is postulated that the "eastern parts" of these inhibitors (**1**–**4**) are directed toward the S1′, S2′, and S3′ subsites, and this contributes to their high affinity. For the (albeit much weaker) interactions of the insulapeptolides A–D with proteinase 3, it is also likely that the leucine residue is accommodated by the S1 pocket, because proteinase 3 shares the primary substrate specificity with HLE. The reduced affinity for proteinase 3 might result from less favourable interactions. For example, the hydrophobic moieties methylproline at P2 and isoleucine at P2′ position might interact unfavourably with the corresponding subsites, which are more polar in proteinase 3 than in HLE.

None of the insulapeptolides exhibited a remarkable inhibitory potency toward cathepsin G. This enzyme has a substrate specificity for aromatic or positively charged amino acids at P1. It was presumed that insulapeptolides A–D (**1**–**4**) interact in the same way with this enzyme as with HLE and proteinase 3, by occupying the P1 binding site with the leucine residue. The exchange of leucine in compound **1**–**4** against the aromatic homophenylalanine in **5**–**8** should have improved the affinity to cathepsin G; however, no enhancement of inhibition was observed. This could be due to the nonproteinogenic character of the homophenylalanine residue. In this context it is interesting that largamides D–G were evaluated as inhibitors of bovine chymotrypsin,[27] a serine protease with a similar preference for aromatic amino acids at P1 position as cathepsin G. The putative P1 residues in these peptides are leucine (largamides D and E), tyrosine (largamide F) and the nonproteinogenic homotyrosine (largamide G). Largamide F was somewhat more active (IC_{50} value of 4 μM) than the largamides D/E (10 μM) and largamide G (25 μM). Thus, in support of our proposal, exchanging leucine with the aromatic but nonproteinogenic amino acid homotyrosine did not result in stronger inhibition of chymotrypsin, but replacement by the aromatic and proteinogenic amino acid tyrosine led to the most potent inhibitor within this group of compounds.

There are several structural differences between insulapeptolides A–D (**1**–**4**) and insulapeptolides E–H (**5**–**8**), and we consider two of them to be important for the different potential of HLE inhibition. First, compounds **1**–**4** contain leucine at P1; compounds **5**–**8** contain the more voluminous homophenylalanine and HLE accommodates substrates with small, hydrophobic residues at this position. Second, because the importance of the amino acids that occupy the S2–S4 subsites was emphasized,[1] we suggest that the "western" substructure Ac-Cit-Hmp of **1**–**4** facilitates a stronger affinity to HLE than Bu-Pro-Ser-Thr in **5**–**8**.

Experimental Section

General procedure: A Waters system, controlled by Waters Millenium software that consisted of a 717 plus autosampler, 600 controller pump with in-line degasser and a 996 photodiode array detector was used for performing the purification of compounds **1**–**8**. Chiral HPLC was performed on a Merck–Hitachi system equipped with an L-6200A pump, an L-4500A photodiode array detector, a D-6000A interface with D-7000 HSM software, and a Rheodyne 7725i injection system. ^{1}H,^{13}C, COSY, HSQC, HMBC, NOESY and ROESY NMR spectra were recorded in deuterated methanol either by using a Bruker 300 DPX or a 500 DRX spectrometer operating at 300 or 500 MHz for proton and at 75 or 125 MHz for ^{13}C NMR spectroscopy, respectively. Spectra were calibrated to residual solvent signals with resonances at $\delta_{H/C}$ 3.35/49.0 ppm (CD_3OD). Chiral GS–MS analyses were performed on a Perkin–Elmer AutoSystem XL gas chromatograph that was linked to a Perkin–Elmer Turbomass mass spectrometer and that was equipped with an Alltech Capillary Chirasil-Val column. UV and IR spectra were measured on Perkin–Elmer Lambda 40 and Perkin–Elmer Spectrum BX instruments. Optical rotations were obtained by using a Jasco DIP 140 polarimeter. The enzymatic assays were performed on a Varian Cary 100 Bio spectrophotomter. Human leukocyte elastase (HLE), proteinase 3, and cathepsin G were obtained from Calbiochem, Darmstadt, Germany. The chromogenic substrates MeOSuc-Ala-Ala-Pro-Val-NHNp (Np: 4-nitrophenyl), MeOSuc-Lys(2-picolinoyl)-Ala-Pro-Val-NHNp, and Suc-Ala-Ala-Pro-Phe-NHNp were obtained from Bachem, Bubendorf, Switzerland.

The high-resolution mass spectra were recorded on a microTOF-Q (Bruker Daltonik) equipment.

The MALDI-TOF characterization of the crude extract was done by Dr. Marcel Erhard, AnagnosTec GmbH, Potsdam/OT Golm, Germany by using an Applied Biosystems Voyager DEPRO MALDI-TOF instrumentation (matrix: 2, 5-dihydroxybenzoic acid).

Biological material: The 17 cyanobacterial strains were obtained from different sources. The cyanobacterial strains *N. insulare* Borzi (stock-ID: 54.79), *Synechocystis pevalekii* (stock-ID: 91.79), *Nostoc* sp. (stock-ID: 29.90), *Tolypothrix* sp. (stock-ID: 33.92), *Plectonema* sp. (stock-ID: 42.90), *Scytonema bohneri* Schmidle (stock-ID: 255.80) and *Scytonema myochrons* (stock-ID: 46.87) were obtained from the Culture Collection of Algae (SAG), University of Göttingen, Germany. The strains *Fischerella* sp. 8703 (PCC8703) and *Fischerella* sp. (PCC7909) were obtained from the Pasteur Culture Collection of Cyanobacteria, Paris, France. The strains *Oscillatoria* sp. and *Synechocystis* sp. and one strain of unknown taxonomy (internal code Fä28) were obtained from Dr. B. Heyduck-Söller, Section of Marine Biology, University of Bremen, Germany. The strains *Tychonema* sp. and two strains of unknown taxonomy (internal codes: CTU and CC198) were collected from the waste water pond of a sugar factory in Brunswick, Germany by Olaf Papendorf. The unclassified strain with the internal code Van (probably: *Oscillatoria* sp.) was isolated from a hot spring on Vancouver Island, Canada by Dr. Frithjof C. Kuepper, Head of Culture Collection of Algae and Protozoa, Oban, Scotland. The unclassified sample Fo802 was collected by Dr. Anthony Wright (College of Pharmacy, University of Hawaii) on the island of Formentera, Spain. All strains were kept in 100–150 mL sterile BG-11 medium (Sigma Aldrich, C3061 BG-11) in 300 mL Erlenmeyer flasks for screening purpose. The cyanobacterium *N. insulare* Borzi was cultivated in a 25 L photobioreactor (Planktotec System Pluto) with sterile, filtered BG-11 medium (Sigma Aldrich, C3061 BG-11) over a period of 11 months. During the cultivation at 25 °C, the culture was illuminated with white fluorescent light

(Osram L 58W/11-869). Oxygen was supplied by a continuous stream of sterile air from the lower end of the bioreactor.

***nrps* PCR screening approach**: Chromosomal DNA was isolated according to Smoker and Barnum[48] by using approximately 1 g of cell material. Parts of the protocol were modified. Polymerase chain reaction (PCR) was used to amplify putative *nrps* gene fragments from chromosomal DNA by using the primer pair MTF2 (GC-(AGCT)GG(CT)GG(CT)GC(AGCT)TA(CT)GT(AGCT)CC) and MTR (CC-(AGCT)CG(AGT)AT(CT)TT(AGCT)AC(TC)TG).[49] PCR was carried out by using *Taq* polymerase (Promega, Mannheim, Germany). The PCR products were extracted from agarose gels by using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and ligated into pGEM-T vector (Promega, Mannheim, Germany). Transformation was performed by using competent *E. coli* XL-1 Blue cells. Recombinant plasmid DNA was prepared from 3 mL overnight cultures of transformed *E. coli* XL-1 Blue cells by using the QIAprep Spin Miniprep Kit (Qiagen). Sequencing was conducted in both directions by using standard primers T7 (TAA TAC GAC TCA CTA TAG GG) and M13RP (CAC ACA GGA AAC AGC TAT GAC) and ABI-PRISM Big-Dye™ cycle Sequencing Kit (Applied Biosystems, USA) according to the standard protocols. Analysis of the labeled strands was performed by applying an ABI Prism 310 Genetic Analyzer (Applied Biosystems/Perkin–Elmer, Weiterstadt, Germany). Separation of the DNA fragments with different chain length was performed via capillary electrophoresis (module: "SEQ-POP6 FSE 1 mL", capillary: 61 cm × 50 μm). Data were processed with Sequence Analysis 3.0 software. The resulting sequences were analysed by using the National Center for Biotechnology Information (NCBI) BLAST server (http://www.ncbi.nlm.nih.gov).

PCR Amplification and Cloning of *noiF*: The coding sequence for NoiF was amplified by PCR by using *Taq* polymerase (Promega). The oligonucleotide sequences of the primer pair were deduced from *nosFFor, nosFRev* as described in Luesch et al. (**2003**):[16] *noiF-For* 5′-CTC GAA GAT TTA CAA ATT GC-3′; *noiFRev* 5′-TTA ACT GAT ATT ACC TAA TTG TTG AG-3′. The obtained PCR product was ligated into pGEM-T vector (the kit was obtained from Promega) and cloned in *E. coli* XL1 Blue. The plasmid purification was done by using Gene JET™ Plasmid Miniprep Kit (Fermentas, St. Leon-Rot, Germany). Sequencing was performed at GATC GmbH (Konstanz, Germany). The obtained sequences were online-analyzed by using the National Center for Biotechnology Information (NCBI) BLAST server (http://www.ncbi.nlm.nih.gov).

Isolation procedure: The lyophilized biomass from culture (275 L, 280 g) was exhaustively extracted with MeOH/CH_2Cl_2 (11 L, 2:1) in several steps. The dried extract amounted to 14.7 g. The extract (4.24 g) was fractionated by silica gel vacuum liquid chromatography by using a stepwise gradient solvent system of increasing polarity starting with 100% PE to 100% MeOH. The last two (most polar) fractions showed activity against HLE. Furthermore, both showed similar ^{1}H NMR spectra that indicated the presence of peptidic compounds. To further purify these two fractions, we chromatographed them on RP_{18} solid-phase extraction cartages (Baker-bond Spe™, Octadecyl C_{18}, 1000 mg sorbens) by using a gradient solvent system of decreasing polarity starting from MeOH/H_2O (3:1; fraction 1) to MeOH (fraction 2). Fraction 1 was purified by HPLC (Knauer C_{18} Eurospher-100, 5 μm, 250×8 mm, solvent: MeOH/H_2O (1:1) to MeOH in 60 min, flow: 1 mL min^{-1}) to give compounds **1** (9.7 mg), **2** (7.5 mg), **4** (3.3 mg), **5** (9.5 mg), **7** (6.0 mg) and **8** (10.0 mg). Compounds **3** (1.6 mg) and **6** (5.0 mg) were obtained after further purification by analytical HPLC (Waters C_{18} Xterra, 5 μm, 250×4.6 mm, solvent: isocratic MeOH/H_2O (55:45), flow: 0.7 mL min^{-1} for **3**; MeOH/H_2O (60:40) to MeOH/H_2O (68:32) in 20 min, flow: 0.7 mL min^{-1} for **6**).

Insulapeptolide A (1): white amorphous solid; $[\alpha]_D^{23} = -98.8$ (*c* 0.22; MeOH); UV (MeOH) λ_{max} (log ε) = 204 (4.48), 225 (sh) (4.01), 279 nm (3.11); IR (ATR) ν_{max} = 3372, 2924, 1737, 1631, 1548, 1443, 1333, 1262, 1182, 1149, 1040 cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{46}H_{71}N_9NaO_{12}$: 964.5120 $[M+Na]^+$; found: 964.5115; for NMR spectroscopic data see Table 2.

Insulapeptolide B (2): white amorphous solid; $[\alpha]_D^{23} = -54.7$ (*c* 0.54; MeOH); UV (MeOH) λ_{max} (log ε) = 205 (4.30), 225 (sh) (3.88), 279 nm (3.12); IR (ATR) ν_{max} 3372, 2961, 1738, 1631, 1536, 1443, 1331, 1261, 1205, 1148, 1025 cm^{-1}; HR-ESI MS: *m/z*: calcd for $C_{47}H_{73}N_9NaO_{12}$: 978.5276 $[M+Na]^+$; found: 978.5279; for NMR spectroscopic data see the Supporting Information.

Insulapeptolide C (3): white amorphous solid; $[\alpha]_D^{23} = -77.7$ (*c* 0.11; MeOH); UV (MeOH) λ_{max} (log ε) = 202 (4.44), 225 (sh) (3.98), 277 nm (3.08); IR (ATR) ν_{max} 3368, 2926, 1738, 1645, 1540, 1445, 1374, 1249, 1038 cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{47}H_{73}N_9NaO_{12}$: 978.5276 $[M+Na]^+$; found: 978.5258; for NMR spectroscopic data see the Supporting Information.

Insulapeptolide D (4): white amorphous solid; $[\alpha]_D^{23} = -34.5$ (*c* 0.22; MeOH); UV (MeOH) λ_{max} (log ε) 203 nm (4.26), 225 (sh) (3.84), 277 (2.98); IR (ATR) ν_{max} 3366, 2960, 1738, 1634, 1540, 1443, 1385, 1348, 1247, 1028 cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{48}H_{75}N_9NaO_{12}$: 992.5433 $[M+Na]^+$; found: 992.5433; for NMR spectroscopic data see the Supporting Information.

Insulapeptolide E (5): white amorphous solid; $[\alpha]_D^{23} = -70.2$ (*c* 0.42; MeOH); UV (MeOH) λ_{max} (log ε) = 203 (4.48), 225 (sh) (3.89), 279 nm (3.00); IR (ATR) ν_{max} 3372, 2964, 1726, 1659, 1631, 1547, 1530, 1444, 1332, 1252, 1205, 1067 cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{51}H_{72}N_8NaO_{14}$: 1043.5066 $[M+Na]^+$; found: 1043.5084; for NMR spectroscopic data see Table 3.

Insulapeptolide F (6): white amorphous solid; $[\alpha]_D^{23} = -101.9$ (*c* 0.29; MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.76), 225 (sh) (4.17), 279 (3.32); IR (ATR) ν_{max} 3374, 2964, 1734, 1659, 1634, 1547, 1518, 1446, 1339, 1255, 1206, 1070 cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{50}H_{70}N_8NaO_{14}$: 1029.4909 $[M+Na]^+$; found: 1029.4907; for NMR spectroscopic data see the Supporting Information.

Insulapeptolide G (7): white amorphous solid; $[\alpha]_D^{23} = -83.1$ (*c* 0.26; MeOH); UV (MeOH) λ_{max} (log ε) = 205 (4.46), 258 nm (2.60); IR

Table 4. Inhibitory activity of compounds **1**–**8** against the serine proteases HLE, proteinase 3, and cathepsin G.

Compound	IC_{50} value [μM][a]		
	HLE	proteinase 3	cathepsin G
1	0.14 ± 0.01	> 100	69
2	0.10 ± 0.01	> 100	35
3	0.090 ± 0.001	16	46
4	0.085 ± 0.004	18	17 ± 4
5	3.2 ± 0.2	> 100	> 100
6	1.6 ± 0.1	46	64
7	3.5 ± 0.1	82	46
8	2.7 ± 0.1	98	55

[a] Values with standard error were calculated from duplicate experiments at five different inhibitor concentrations, those without standard error are values or limits calculated from duplicate inhibition experiments at a single inhibitor concentration, 50 μM (proteinase 3) or 25 μM (cathepsin G), according to the equation: $IC_{50} = [I]/(v_o/v-1)$.

(ATR) ν_{max} 3364, 2964, 1736, 1631, 1530, 1443, 1333, 1254, 1204, 1067 cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{50}H_{70}N_8NaO_{13}$: 1013.4960 $[M+Na]^+$; found: 1013.4953; for NMR spectroscopic data see the Supporting Information.

Insulapeptolide H (8): white amorphous solid; $[\alpha]_D^{23} = -56.9$ (*c* 0.39; MeOH); UV (MeOH) λ_{max} (log ε) = 205 (4.14), 261 nm (2.23); IR (ATR) ν_{max} 3371, 2964, 1727, 1658, 1631, 1547, 1530, 1443, 1413, 1331, 1276, 1205, 1067 cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{51}H_{72}N_8NaO_{13}$: 1027.5117 $[M+Na]^+$; found: 1027.5099; for NMR spectroscopic data see the Supporting Information.

Amino acid analysis by chiral GC: Compounds **1, 2** and **5** (0.2–1 mg) were dissolved in HCl (6 N, 1 mL) and heated to 110 °C for 16 h in a closed vial. The acid was removed by using a stream of N_2. The dried hydrolysate was treated with isopropanol (500 μL) and acetyl chloride (150 μL) at 110 °C for 1 h to create the isopropyl-esters of the amino acids. After removal of the solvent, the dry residues were acylated using trifluoroacetic anhydride (0.4 mL) in CH_2Cl_2 (0.4 mL) and heating for 15 min at 110 °C). The reaction mixture was dried and dissolved in EtOAc (100 μL). This solution (1 μL) was analyzed by GC–MS by using an Alltech Capillary Chirasil-Val column (25 m × 0.25 mm; 0.16 μm; program rate: column temperature held at 50 °C for 3 min; 50 °C–180 °C at 4 °C min^{-1}; flow: 0.6 mL min^{-1}; injector temperature: 250 °C). Derivatization of the amino acids was performed by using D- and L-amino acids (5–10 mg; for isoleucine and threonine the configurations D, D-*allo*, L and L-*allo* were taken) and processed as described above. Before analysis by GC–MS, the solutions of standard amino acids were diluted with EtOAc (1:50). The retention times of the *N*-trifluoroacetyl isopropyl ester of the amino acids were compared with those of the derivatized standards. In the case of L-*allo*-isoleucine and D-isoleucine, the samples were spiked (because of the small difference of the retention times: L-*allo*-isoleucine: 15.97 min; D-isoleucine: 15.88 min) to show that the L-*allo*-isoleucine was present. (standards: L-Cit 33.24 min, D-Cit 32.90 min, L-Hphe 31.24 min, D-Hphe 30.97 min, L-Ile 16.30 min, L-*allo*-Ile 15.97 min, D-Ile 15.88 min, D-*allo*-Ile 15.47 min, L-Leu 18.04 min, D-Leu 17.22 min, L-Ser 16.63 min, D-Ser 16.23 min, L-Thr 14.19 min, L-*allo*-Thr 17.15 min, D-Thr 13.71 min, D-*allo*-Thr 16.68 min, L-Val 14.01 min, D-Val 13.54 min; compound **1**: L-Cit 33.26 min, L-Ile 16.40 min, L-Leu 17.92 min, L-Val 14.00 min; compound **2** : L-Cit 33.25 min, L-Ile 16.39 min, L-*allo*-Ile 16.00 min, L-Leu 17.95 min; compound **5** : L-Hphe 31.26 min, L-*allo*-Ile 15.99 min, L-Ser 16.61 min, L-Thr 14.19 min).

Chiral HPLC: Compounds **1**, **2**, and **5** (~0.5 mg), respectively, were hydrolyzed in HCl (6 N) at 110 °C for 16 h. The hydrolysates were dried with a stream of N_2. The residues were dissolved in the mobile phase that was used for the chiral HPLC (1 mg/100 μL).

Mobile phase I: $CuSO_4$ (2 mM) in H_2O/MeCN (95:5), flow rate: 1 mL min^{-1} (Phenomenex Chirex 3126 D-penicillamine; 250× 4.60 mm; detection at 254 nm). Mobile phase I elution times (t_R, min) of standards: L-Pro 9.0 min, D-Pro 19.8 min.

Mobile phase II: $CuSO_4$ (2 mM) in H_2O/MeCN (9:1), flow rate: 1 mL min^{-1} (Phenomenex Chirex 3126 D-penicillamine; 250× 4.60 mm; detection at 254 nm). Mobile phase II elution times (t_R, min) of standards: L-*N*-Me-Tyr 27.5 min, D-*N*-Me-Tyr 24.5 min.

Mobile phase III: $CuSO_4$ (2 mM) in H_2O/MeCN (88:12), flow rate: 1 mL min^{-1} (Phenomenex Chirex 3126 D-penicillamine; 250× 4.60 mm; detection at 254 nm). Mobile phase III elution times (t_R, min) of standards: L-*N*-Me-Tyr 21.5 min, D-*N*-Me-Tyr 18.6 min.

The hydrolysates were analyzed alone and by co-injection with standard amino acids. For compound **1** and **2** the presence of L-*N*-Me-Tyr (mobile phase III) could be shown. For compound **5** L-*N*-Me-Tyr (mobile phase II) and L-Pro (mobile phase I) were assigned.

Oxidization of compounds 1, 2 and 5: Compound **1, 2**, and **5** (0.5–1 mg) were dissolved in AcOH (500 μL) and combined with CrO_3 (2 mg). After stirring for 3 h at room temperature, the reaction mixture was subjected to a disposable ODS column (Bakerbond Spe™, Octadecyl C_{18}, 1000 mg sorbens; H_2O/MeOH). The oxidized product was eluted in the MeOH fraction.

Preparation of 1-fluoro-2,4-dinitrophenyl-5-D/L-leucinamide (FDLA) derivatives: Compounds **1**, **2** (0.5–1 mg), and the oxidized compounds **1, 2**, and **5** were hydrolyzed at 110 °C for 16 h in HCl (6 N, 0.5 mL). The hydrolysates were dried with a stream of N_2 and washed once with H_2O (0.5 mL). The dried residues were dissolved in H_2O (100 μL). The obtained solutions were divided into two portions. After adding $NaHCO_3$ (1 M, 40 μL) and L- or D-FDLA (1%) in acetone (100 μL), respectively, the reaction mixtures were heated at 70 °C for 40 min. The reactions were quenched by the addition of HCl (1 M, 20 μL) and dried. The obtained residues were dissolved in MeCN (250 μL). The L-FDLA derivatives (5 μL) and an equal mixture of the L- and D-FDLA derivatives were analyzed by ESI-LC–MS.

ESI-LC–MS conditions: The analysis of the derivated hydrolysates was performed on a C18 column (Macherey–Nagel Nucleodur 100, 125 mm × 2 mm, 5 μm). Aqueous CH_3CN containing TFA (0.01 M) was used as the mobile phase, and was eluted with a linear gradient of CH_3CN (25–70%) in 45 min (then 70% CH_3CN for 15 min) at a flow rate of 0.2 mL min^{-1}. A mass spectrometer detector was used for detection in ESI (negative) mode. The ion source was kept at 400 °C. The ion spray voltage was −4000 V. A mass range from 300–1000 was scanned in 1.5 s.

Preparing of the crude extracts for the HLE inhibition assay: For screening purposes, the biomass of all cyanobacteria was separated from the medium by filtration and subsequently lyophilized by using a Christ Beta 1–16 freeze dryer (Osterode, Germany). The dried biomass was extracted exhaustively with a mixture of MeOH/CH_2Cl_2 (1:2). Extract and biomass were separated by filtration. The crude extracts were dried on a rotavapor with a vacuum pump. For testing in the HLE inhibition assay, the crude extracts were suspended in MeCN (10 mg mL^{-1}). These suspensions were mixed for 15 min at 600 rpm in an Eppendorf Thermomixer Comfort. Subsequently, the suspensions were placed for 30 min in a Sonorex TK 52 ultrasonic bath at room temperature. Afterwards, the suspensions were filtered, and the solutions were used in the enzyme inhibition assays.

HLE inhibition assay: Human leukocyte elastase was assayed spectrophotometrically at 405 nm at 25 °C.[50] Assay buffer was 50 mM sodium phosphate buffer (500 mM NaCl, pH 7.8). A stock solution of the chromogenic substrate MeOSuc-Ala-Ala-Pro-Val-NHNp was prepared in DMSO and diluted with assay buffer. Final concentration of DMSO was 1.5%, the final concentration of the chromogenic substrate MeOSuc-Ala-Ala-Pro-Val-NHNp was 100 μM, unless stated otherwise. Assays were performed with a final HLE concentration of 50 ng/mL, which corresponded to an initial rate of 0.7 μM min^{-1}, when 100 μM of the substrate was used. An inhibitor solution (10 μL) and the substrate solution (50 μL) were added to a cuvette that contained the assay buffer (890 μL), and the solution was thoroughly mixed. The reaction was initiated by adding the HLE solution (50 μL) and was followed over 10 min. IC_{50} values were calculated from the linear steady-state turnover of the substrate. The HLE inhibition by the crude extracts was determined in the presence of DMSO (0.5%) and acetonitrile (1%).

Proteinase 3 inhibition assay: Human proteinase 3 was assayed spectrophotometrically at 405 nm at 25 °C.[51] Assay buffer was sodium phosphate buffer (50 mM sodium phosphate, 100 mM NaCl, pH 7.8). Inhibitor stock solutions were prepared in DMSO. A 100 mM stock solution of the chromogenic substrate MeOSuc-Lys(2-picolinoyl)-Ala-Pro-Val-NHNp was prepared in DMSO and diluted with a 1:1 mixture of assay buffer and DMSO. Final concentration of DMSO was 3.5%, final concentration of the chromogenic substrate MeOSuc-Lys(2-picolinoyl)-Ala-Pro-Val-NHNp was 100 μM. Assays were performed with a final proteinase 3 concentration of 5.33 mU mL^{-1}, which corresponded to an initial rate of 0.24 μM min^{-1}. An inhibitor solution (10 μL) and the substrate solution (50 μL) were added to a cuvette that contained the assay buffer (890 μL), and the mixture was thoroughly mixed. The reaction was initiated by adding the proteinase 3 solution (50 μL) and was followed over 15 min. IC_{50} values were calculated from the linear steady-state turnover of the substrate.

Cathepsin G inhibition assay: Human cathepsin G was assayed spectrophotometrically at 405 nm at 25 °C.[52] Assay buffer was Tris–HCl (20 mM), NaCl (150 mM, pH 8.4). Inhibitor stock solutions were prepared in DMSO. A stock solution (50 mM) of the chromogenic substrate Suc-Ala-Ala-Pro-Phe-NHNp in DMSO was diluted with assay buffer. The final concentration of DMSO was 1.5%, final concentration of the substrate Suc-Ala-Ala-Pro-Phe-NHNp was 500 μM. Assays were performed with a final concentration of 2.5 mU mL^{-1} of cathepsin G, which corresponded to an initial rate of 0.7 μM min^{-1}. Inhibitor solution (5 μL) and substrate solution (100 μL) were added to a cuvette that contained assay buffer (882.5 μL), and the mixture was thoroughly mixed. The reaction was initiated by adding the cathepsin G solution (12.5 μL) and was followed over 10 min. IC_{50} values were calculated from the linear steady-state turnover of the substrate.

Keywords: cyanobacteria · enzymes · natural products · peptides · structure elucidation

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