

Planktocylin, a Cyclooctapeptide Protease Inhibitor Produced by the Freshwater Cyanobacterium *Planktothrix rubescens*

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The freshwater cyanobacterium *Planktothrix rubescens* produces the cyclooctapeptide cyclo(Pro-Gly-Leu-Val-Met-Phe-Gly-Val). The chemical structure is new. This homodetic cyclic octapeptide was named planktocylin (**1**). It consists solely of proteinogenic L-amino acids and is a strong inhibitor of mammalian trypsin and α -chymotrypsin and a moderately active inhibitor of human recombinant caspase-8. Mass spectrometric and 2D-NMR spectroscopic data allowed the determination of its structure. Synthetic planktocylin was identical to the natural product.

Planktonic freshwater cyanobacteria have proved to be a rich source of new oligopeptides showing high structural diversity and bioactivity.¹ The two most important genera (numerically, economically, and from the standpoint of toxicology) are *Microcystis* and *Planktothrix*. They colonize in a large number of ecotypes and chemotypes eutrophic (*Microcystis*) and mesotrophic to eutrophic (*Planktothrix*) lakes throughout the world. On the basis of morphological and cytological features, *Planktothrix* has been separated as a new genus from *Oscillatoria*.² In addition to some minor species, *P. agardhii* and *P. rubescens* have been differentiated by morphological features. However, a biochemical trait, such as phycobilins, turned out to allow a much more reliable differentiation of the two species.³ *P. agardhii* possesses only phycocyanins and is therefore blue-green colored, whereas *P. rubescens* additionally contains phycoerythrins, which cause a magenta red color. The strict differentiation of these two species has important implications for research on oligopeptide metabolites because the oligopeptidic compounds differ essentially in both species. Unfortunately, in many publications on bioactive oligopeptides, both species have been confused, and only in the limited cases in which the strains came from a culture collection can the correct name be applied.

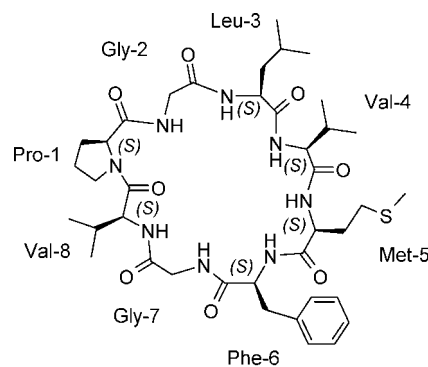
P. rubescens is not ingested by *Eudiaptomus*, an important crustacean grazer that lives in lakes exhibiting mass production of this cyanobacterium.⁴ The ultimate reason for this food avoidance may be the presence of microcystins^{5,6} and protease inhibitors. Protease inhibitors inhibit the digestion of the protein fraction of the ingested food and lead to starvation of the grazers. The efficiency of inhibitors of *P. rubescens* on digestive enzymes of crustaceans has been shown.⁷ Trypsin and chymotrypsin were determined to be the major digestive proteases in the gut of crustaceans,⁸ and it is not surprising that effective inhibitors against both serine proteases are found in all strains of *P. rubescens* so far investigated. Therefore, these inhibitors are particularly important as a chemical defense of *P. rubescens* against potential grazers.

In this context, several serine protease inhibitors have been described for *P. rubescens*. Oscillapeptin G was first isolated as a weak tyrosinase inhibitor.⁹ In subsequent studies, the structure was revised and inhibitory activities were found for chymotrypsin and elastase.¹⁰ Other serine protease inhibitors with properties of

chymotrypsin inhibitors are oscillatorin¹¹ and the planktopeptins isolated from a bloom on Lake Bled, Slovenia.¹²

Oscillarin is an effective inhibitor of thrombin.¹³ Isolates from other cyanobacterial genera are also very effective serine protease inhibitors. Micropeptins are cyclodepsipeptides isolated from different *Microcystis* strains.¹⁴ These compounds contain the 3-amino-6-hydroxy-2-piperidone unit, which is important for their inhibition mechanism. The compound A90720A isolated from the terrestrial *Microchaete lohtakensis* is also a member of this class of inhibitor with strong effects on trypsin.¹⁴ Chymotrypsin and elastase inhibition were found for the cyclic nostopeptin A and nostopeptin B, which were isolated from *Nostoc minutum*.¹⁴

Recently we described oscillapeptin J,¹⁵ which turned out to be a potent inhibitor of trypsin, being active in the nanomolar range. In addition it exhibited properties of a crustacean-specific toxin.¹⁶ Here we describe a further effective trypsin inhibitor of novel structure, which we named planktocylin. The compound belongs to the few known cyclic octapeptides consisting exclusively of proteinogenic L-amino acids and lacks any further amino acid modification.



1 Planktocylin

Results and Discussion

Massive blooms of *P. rubescens* occurred in May 2002 on Lake Hallwilersee, Switzerland. The surface film was collected and stored at -23°C for analysis. Several strains of *P. rubescens* were isolated by picking¹⁷ from this lake and subsequently grown under low light intensity. The 60% aqueous methanolic extracts of bloom samples and of the biomass of a culture designated *P. rubescens* H9 were separated by HPLC on a reversed-phase C18 column, and the elution profiles were studied by diode array and ESIMS detection.

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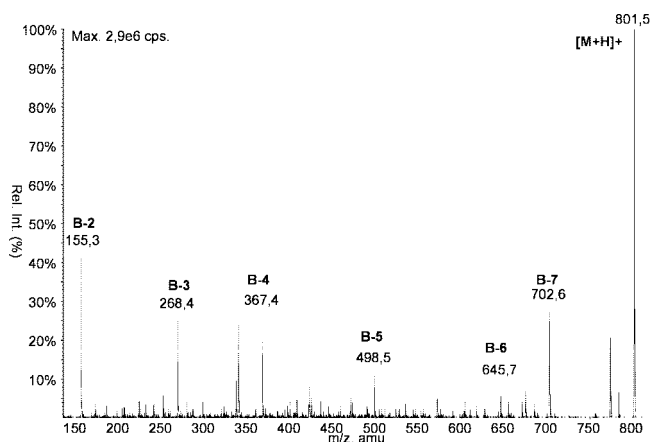


Figure 1. Mass fragmentation of **1** by MS-MS and assignment of fragments.

In both extracts a strong peak appeared at $t_R = 24.7$ min exhibiting a quasi molecular ion at m/z 801. We named this compound planktocyclin (**1**). Because the compound was sensitive to oxygen, a modified separation procedure was applied.

High-resolution electrospray Fourier transform ion cyclotron mass spectrometry (ESI-FT-ICR-MS) of planktocyclin gave a monoisotopic signal at 801.43277 amu, corresponding to $[C_{39}H_{61}N_8O_8S]^+$ (theoretical mass 801.43276, relative mass error Δ_m 0.01 ppm). Therefore, for planktocyclin the molecular formula of $C_{39}H_{60}N_8O_8S$ was deduced.

After derivatization (TFA/ethyl esters) of the hydrolysate of planktocyclin, the constituent amino acids were identified by chiral GC-MS and quantitatively determined by enantiomer labeling. The following amino acids in the corresponding relative molar ratios were detected: Pro (1.00 ref), Val (2.13), Leu (0.96), Met (0.25), Phe (1.07), and Gly (2.06). The configuration of the amino acids was established by comparison of their retention times (first value, in min) with those of standards (in italics) on a Lipodex E (30% in PS255) capillary column as follows: L-Val (15.30/*D-13.45, L-15.26*); L-Leu (19.09/*D-17.14, L-19.00*); L-Pro (24.48/*D-26.18, L-24.44*); L-Met (29.01/*D-28.67, L-29.00*); L-Phe (30.96/*D-30.81, L-30.91*).

In MS-MS experiments, the preferential cleavage site of the macrocycle was, as expected, the secondary amide of Pro,¹⁸ leading to a series of fragment ions originating from the linear acylium ion (Figure 1). The major fragmentation sequence involved the loss of C-terminal amino acids to yield ions of the B-series with m/z 702.6 (B7), 645.7 (B6), 498.5 (B5), 367.4 (B4), 268.4 (B3), and 155.3 (B2). This fragmentation series corresponds to an amino acid sequence of Pro-Gly-Leu-Val-Met-Phe-Gly-Val in the cyclic peptide with a head-to-tail cyclopeptidic ring closure between Pro and Val. In addition, the Y3 (m/z 304), Y4 (m/z 435), and Y5 (m/z 534) fragment ions were detected in correspondence to the structure of **1**. Fragment ions of planktocyclin sulfoxide confirmed the amino acid sequence of **1** by MS-MS experiments (Figure 2).

The structure of *cyclo*(Pro-Gly-Leu-Val-Met-Phe-Gly-Val) (**1**) was confirmed by 1D and 2D NMR spectra. 1H NMR and ^{13}C NMR shifts are given in Table 1. The spin systems of the eight amino acids were identified using COSY and TOCSY experiments (Table 1). ^{13}C NMR signals were assigned from HSQC and HMBC spectra. The peptide sequence of planktocyclin was deduced from NOESY data (sequential walk). NOE correlations between the α proton of Val-8 and both δ and δ' protons of Pro-1 suggest that the Val-8-Pro-1 amide bond is in *trans* configuration. This finding is further confirmed by the γ carbon ^{13}C chemical shift of Pro-1 at 24.7 ppm.¹⁹ To corroborate the structure elucidation, a reference substance was synthesized via Fmoc peptide synthesis strategy on a solid support. The NMR spectra of the isolated and synthetic compound were compared and found to be fully identical (Table 1) and, thus, confirmed the structure of planktocyclin (**1**).

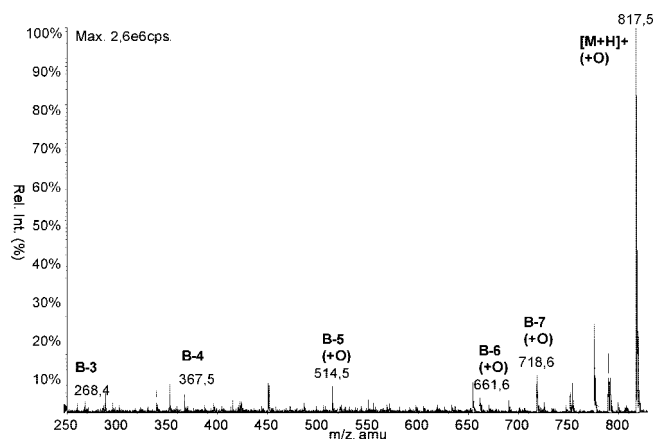


Figure 2. Mass fragmentation of planktocyclin sulfoxide and assignment of fragments.

Protease inhibition assays with isolated planktocyclin showed high activity against trypsin ($IC_{50} = 104$ nM) and α -chymotrypsin ($IC_{50} = 10$ μ M) (Figure 3A). Human caspase-8 was moderately inhibited ($IC_{50} = 93$ μ M), and mammalian cathepsin B showed no inhibition ($IC_{50} > 500$ μ M). Planktocyclin has the rare structure of a cyclic octapeptide consisting solely of proteinogenic L-amino acids and containing only one proline. Agardhiptins, which were described previously from *Planktothrix agardhii* [NIES-204],²⁰ are another example of cyclic oligopeptides containing only proteinogenic amino acids. The agardhiptins are tryptophan-rich oligopeptides, but only agardhiptin A exhibited moderate inhibitory activity against plasmin ($IC_{50} = 78$ μ M). Other cyclic oligopeptides of proteinogenic amino acids have recently been isolated from marine sponges.²¹ The presence of one or more prolines in these peptides and their reduced conformational flexibility was regarded as essential for bioactivity of the molecules.^{22,23}

The seeds of higher plants are also important sources of proline-containing bioactive cyclic peptides consisting of proteinogenic amino acids. Cherimolacyclopeptides A and B are cyclooctapeptides from the seeds of *Annona cherimola*, a small tree native to Ecuador and Peru. Both peptides were found to be cytotoxic against KB tumor cells.²⁴ The seeds of *Linum usitatissimum*, which is cultured worldwide, contain cyclolinopeptides with immunosuppressive activities on human and mouse peripheral-blood lymphocytes.²⁵ Several cyclic peptides from the seeds of *Vaccaria segetalis* (Caryophyllaceae), named segetalins, have estrogen-like activity.²⁶ The seeds have been used as a Chinese drug for invigorating blood circulation, for regulating menstrual disturbance, and to dispel edema.

Many oligopeptides of cyanobacteria are synthesized by giant multienzyme complexes, the nonribosomal peptide synthetases.^{27–29} However, recently a ribosomal biosynthetic pathway was shown for the precursor peptides of the patellamides. The patellamides were isolated from *Prochloron didemni*, the cyanobacterial symbiont of the tunicate *Lissoclinum patella*,³⁰ and are pseudosymmetrical cyclic dimers, each having an unusually fused oxazoline-thiazole unit.³¹ Structurally less complex cyclic oligopeptides such as planktocyclins, which contain only proteinogenic amino acids, are also strong candidates for such ribosomally coded peptide syntheses.

In summary we have shown that *P. rubescens* produces a novel protease inhibitor that belongs to the seldom found structures of all L-configured cyclooctapeptides.

Experimental Section

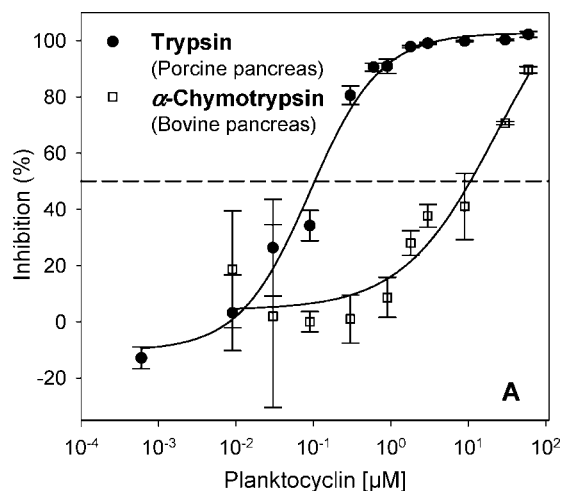
General Experimental Procedures. The 2D-NMR spectra (COSY, TOCSY, NOESY, HSQC, HMBC) were measured on an AMX 600 MHz NMR spectrometer (Bruker) equipped with a 5 mm Z-gradient triple resonance probe head and on a DRX500 NMR spectrometer (Bruker) equipped with a 5 mm Z-gradient broad band inverse probe head. Spectra

Table 1. ^1H and ^{13}C NMR Data for Planktocyclin in $\text{DMSO}-d_6$

position ^a		natural		synthetic	
		^1H	^{13}C	^1H	^{13}C
Pro-1	CO		<i>b</i>		172.3
	αCH	4.08	60.4	4.08	60.6
	βCH_2	1.74	28.5	1.75	28.5
		2.09		2.10	
	γCH_2	1.84	24.7	1.85	24.9
		2.00		2.00	
	δCH_2	3.55	47.0	3.54	47.2
		3.72		3.75	
Gly-2	CO		<i>b</i>		168.5
	NH	8.76		8.70	
	αCH_2	3.39	42.5	3.37	42.5
		3.97		3.95	
Leu-3	CO		<i>b</i>		<i>b</i>
	NH	7.61		7.63	
	αCH	4.58	50.3	4.59	50.2
	βCH_2	1.49	40.2	1.52	40.0
	γCH	1.52	23.7	1.51	23.5
	δCH_3	0.81	21.8	0.81	21.6
	$\delta'\text{CH}_3$	0.78	22.9	0.79	22.7
	CO		<i>b</i>		<i>b</i>
Val-4	NH	8.45		8.30	
	αCH	3.71	61.0	3.71	60.9
	βCH	2.10	28.6	2.13	28.5
	γCH_3	0.89	18.7	0.89	18.7
	$\gamma'\text{CH}_3$	0.92	19.3	0.92	19.2
	CO		<i>b</i>		<i>b</i>
Met-5	NH	8.16		8.10	
	αCH	4.09	52.9	4.09	53.1
	βCH_2	1.72	29.8	1.70	29.8
		2.26		2.25	
	γCH_2	2.34	29.3	2.35	29.3
	δCH_3	1.95	14.0	1.95	14.0
	CO		<i>b</i>		<i>b</i>
Phe-6	NH	8.11		8.13	
	αCH	4.31	54.1	4.27	54.4
	βCH_2	2.89	36.0	2.87	35.8
		3.12		3.24	
	γC		137.9		138.1
	δCH	7.17	128.6	7.16	128.9
	εCH	7.23	127.9	7.23	128.1
	ζCH	7.19	126.4	7.19	126.6
Gly-7	CO		<i>b</i>		168.2
	NH	8.18		8.08	
	αCH_2	3.46	42.5	3.48	42.9
		3.81		3.76	
Val-8	CO		<i>b</i>		<i>b</i>
	NH	7.08		7.09	
	αCH	4.54	54.1	4.54	54.5
	βCH	2.01	31.0	2.03	30.0
	γCH_3	0.77	17.4	0.77	17.1
	$\gamma'\text{CH}_3$	0.87	19.0	0.87	18.9

^a COSY and TOCSY experiments have been used to identify spin systems of amino acids. ^b Assignment ambiguous.

were recorded in and referenced to $\text{DMSO}-d_6$ (2.49 ppm; 39.5 ppm). HPLC-ESIMS experiments were performed on a 2000 Q Trap mass spectrometer (Applied Biosystems/MDS Sciex, Darmstadt, Germany) coupled to an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany). High-resolution FTICR-MS spectra were measured on an APEX II ESI-FTICR mass spectrometer equipped with a 4.7 T magnet (Bruker-Daltonics, Bremen, Germany). LC-MS-MS spectra were acquired in the positive-ion mode as enhanced product ion scans at a scan rate of 1000 amu s^{-1} in high-resolution mode, with 5.5 kV spray voltage, 12 V entrance potential, 350 °C TurboIonSpray source temperature, 40 V declustering potential, 42 V collision cell entrance potential, and 40 V collision energy with a 10 V collision energy spread. The composition of the derivatized hydrolysate was determined by GC-MS (Agilent MSD 5973/6890, Agilent). The mass spectra for the determination of the molar absorption coefficient were obtained on a GC-EIMS (Fison Instruments, GC 8000 Top, MD 800). The fluorescent enzyme-substrates were measured on a fluorescence plate reader (SpectraMAX Gemini XS, Molecular Devices Corp., Sunnyvale, CA).

**Figure 3.** Planktocyclin concentration inhibition curves for trypsin ($R^2 = 0.996$; $P < 0.0001$) and α -chymotrypsin ($R^2 = 0.936$; $P = 0.226$). Mean values of 3 replicates and standard deviation.

Cultivation and Harvest of *Planktothrix rubescens*. *P. rubescens* H9 was isolated from Lake Hallwilersee, Switzerland (47°18'08.28" N; 8°12'39.86" E), and cultivated as a monoxenic batch culture in 300 mL Erlenmeyer flasks. The flasks were irradiated with light from fluorescent tubes ($7 \mu\text{mol m}^{-2} \text{s}^{-1}$). The cyanobacterium was grown at 20 °C on a cyanobacterial mineral medium³² without shaking. To reduce the buoyancy of the filaments, the suspensions were pressurized with N_2 at 20 bar before centrifugation. The biomass was stored frozen at -23 °C. Additional wet biomass (~880 g) of *P. rubescens* was obtained from a bloom collected from Lake Hallwilersee in May 2002. LC-MS and MS-MS analyses of planktocyclin derived from the bloom and the cultured strain H9 were performed to confirm the identity of the compound. The retention times and mass fragmentation patterns of planktocyclin of both sources were so close to each other that they were regarded as identical.

Isolation of Planktocyclin. The thawed biomass of *P. rubescens* was extracted with 60 % (v/v) $\text{MeOH}/\text{H}_2\text{O}$, taking into account the amount of H_2O already present in the biomass. After centrifugation, separation of the supernatant, and evaporation of most of its solvents, the resulting nearly dry residue was diluted in 30% (v/v) $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ and chromatographed on a reversed-phase C18 column (250 × 4.6 mm, 5 μm , 120 Å, Grom-Sil ODS-4 HE, Stagroma, Rottenburg-Hailfingen, Germany). A linear gradient of UV-treated deionized H_2O (A) and CH_3CN (B) was used for separation (30 to 35% B in 10 min, 35 to 70% B in 15 min, 70 to 100% B in 2 min) at 30 °C and a flow rate of 1 mL min^{-1} . Planktocyclin was eluted under these conditions at retention times between 24 and 25 min. Acidification of the eluent, although giving better separations, was avoided in order to minimize hydrolysis and oxidation reactions (sulfur-containing compound) in the subsequent purification steps. The combined eluents containing planktocyclin were diluted with H_2O , freeze-dried, and vented with argon after termination of lyophilization. Under these conditions very little planktocyclin was oxidized to its sulfoxide. Preparative HPLC was repeated twice to obtain pure planktocyclin. The colorless and fluffy powder was stored in 60% (v/v) $\text{CH}_3\text{CN}/\text{H}_2\text{O}$. The yield was about 2.5 mg per 7.0 g fresh weight. (ESI)MS-MS experiments of planktocyclin and its sulfoxide were performed by direct MS infusion on the ESIMS operating in the two-stage full scan type. The eluent consisted of $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{TFA}$ (30:70:0.05), and the supplied voltage for the parent ion was 30 V.

Amino Acid Analysis. The composition and configuration of the amino acids of planktocyclin were determined after hydrolysis in 6 M HCl at 110 °C for 24 h. The dry hydrolysate was derivatized to the *N*-(*O*)-TFA/ethyl esters and analyzed by chiral GC-MS on a 20 m × 0.25 mm Lipodex E/PS255 (30:70) capillary column. Quantitation of amino acids was performed by the method of enantiomer labeling.³³

Determination of the Molar Absorption Coefficient. The molar absorption coefficient of planktocyclin (50 μg in MeOH) was determined by quantitative analysis of L-Phe after acidic hydrolysis (6.0 M HCl at 110 °C for 15 h). L- $^{13}\text{C}_9$ phenylalanine (1 μg) (Cambridge

Isotope Laboratories, Inc., MA) was added as an internal standard before hydrolysis. After derivatization of the dry hydrolysate with MTBSTFA (Fluka, Switzerland) in tetrahydrofuran (THF) and trifluoroacetic acid (TFA) (50/50/0.1; v/v/v) the solution was analyzed on a GC-EIMS. Analyses were conducted on a capillary column (30 m DB-1301, 0.32 mm i.d., 0.25 μ m film thickness) under the following separation conditions: 1 min at 120 °C, 120 to 250 °C at a rate of 10 °C min⁻¹. The integrals of the fragment ions at m/z 234 (30%), m/z 308 (15%), m/z 336 (11%), and m/z 242, m/z 316 and m/z 345 of L-U-¹²C₉ phenylalanine and L-U-¹³C₉ phenylalanine, respectively, were applied to determine the amount of unlabeled amino acids. This value was correlated to the UV absorption of planktocylin at 273 nm in MeOH measured before hydrolysis.

Protease Inhibition Assays. Fluorogenic substrates³⁴ were used to determine the inhibition of proteases (trypsin, α -chymotrypsin, cathepsin B, and caspase-8) by planktocylin. The measurements were performed in black 96-well microtiter plates. The reaction was monitored in 2 min intervals for up to 40 min. The IC₅₀ values agreed independent of whether they were calculated from the kinetics or end point measurements (which were actually used). The IC₅₀ values were calculated using the nonlinear regression sigmoid four-parameter formula: $y = d + a/(1 + (x/c)^b)$ and the program Sigma Plot 8.0. (a = maximum value, b = slope of the curve, c = slope of the curve at the point of 50%, d = minimum value, x = the amount of compound that inhibits the proteases by 50%).

For trypsin-inhibition experiments, dimethylated trypsin from porcine pancreas (proteomics grade, Sigma) was used. The assay consisted of 10 μ L of trypsin (134 mU), 140 μ L of incubation buffer (50 mM Tris/HCl, 150 mM NaCl, 1 mM CaCl₂, 0.1 mg mL⁻¹ bovine serum albumin, pH 8.0), and 30 μ L of planktocylin solution (12 different concentrations between 4.0 nM and 400 μ M in 60% (v/v) CH₃CN/H₂O). The mixture was preincubated for 5 min at 37 °C in a 200 μ L well, and the reaction was started by addition of 20 μ L of the substrate solution (50 μ M Boc-Gln-Ala-Arg-7-amido-4-methylcoumarin in incubation buffer). The fluorescence change (λ_{ex} 380 nm, λ_{em} 440 nm) was monitored for 20 min at 37 °C.

Bovine pancreas α -chymotrypsin (Sigma) was used to determine the inhibition efficiency of planktocylin. The substrate glutaryl-Gly-Gly-Phe-7-amido-4-methylcoumarin (Bachem, Bubendorf, Switzerland) was dissolved in 5% (v/v) DMSO, and a 300 μ M solution in incubation buffer was prepared and measured fluorometrically at λ_{ex} 380 nm, λ_{em} 440 nm. Ten different concentrations of planktocylin (60 nM to 400 μ M) were dissolved in 60% (v/v) CH₃CN/H₂O. The assay consisted of 140 μ L of incubation buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl₂, pH 8.0), 30 μ L of planktocylin solution, and 10 μ L of α -chymotrypsin (1.02 U mL⁻¹ incubation buffer), which was preincubated for 5 min at 37 °C. The reaction was started by adding 20 μ L of substrate solution (300 μ M) and continued for 20 min at 37 °C.

The substrate for cathepsin B (from bovine spleen, ≥ 10 U mg⁻¹ protein, Sigma) was *N*-benzyloxycarbonyl-alanine-arginine-arginine-4-methoxy- β -naphthylamide (Z-Ala-Arg-Arg-4MBNA acetate salt), obtained from Bachem, Bubendorf, Switzerland. The substrate was dissolved in 5% (v/v) DMSO, and a 6.4 mM stock solution was prepared in phosphate incubation buffer (12 mM NaH₂PO₄, 88 mM KH₂PO₄, 1.33 mM Na₂EDTA, 2.7 mM dithiothreitol, 0.03% Brij 35, pH 5.8). The assay containing 70 μ L of phosphate incubation buffer, a 50 μ L solution of cathepsin B (3.8 mU mL⁻¹ incubation buffer), and 30 μ L of the planktocylin solution (eight different concentrations between 6.0 μ M and 1 mM in 60% (v/v) CH₃CN/H₂O) was preincubated for 5 min at 37 °C. To start the reaction, 50 μ L of the substrate solution (0.64 mM) was added and the increase of fluorescence (λ_{ex} 345 nm, λ_{em} 425 nm) was measured for 30 min at 37 °C.

The assay of human caspase-8 (recomb. *E. coli*) (according to the manual provided with the assay kit No. CASP8F-1KT, Sigma) was based on the hydrolysis of the peptide substrate acetyl-Ile-Glu-Thr-Asp-7-amido-4-methylcoumarin and monitored fluorometrically (λ_{ex} 360 nm, λ_{em} 440 nm). The assay consisted of 70 μ L of caspase buffer pH 7.4 (20 mM HEPES, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, and 5% sucrose), 15 μ L of 10 different concentrations of planktocylin (2 μ M to 1 mM in 60% (v/v) CH₃CN/H₂O), and 5 μ L of caspase-8 (10 μ g mL⁻¹). After preincubation for 5 min at 25 °C, the reaction was started by addition of 10 μ L of substrate solution (150 μ M in caspase buffer). The measurements were continued for 40 min.

Planktocylin (1): amorphous, colorless powder; UV (MeOH) λ_{max} 273 nm (ϵ 1040); ¹H NMR, ¹³C NMR data, see Table 1; ESMS m/z

[M + H]⁺ 801.4; ESI-FTICR-MS m/z [M + H]⁺ 801.43277 corresponding to [C₃₉H₆₁N₈O₈S]⁺ (theoretical mass 801.43276, relative mass error Δ_m 0.01 ppm.).

Synthesis of 1. Planktocylin was synthesized by using procedures for Fmoc solid-phase peptide synthesis³⁵ in combination with an alkanesulfonamide safety-catch linker.^{36,37} To prepare the safety catch handle, 3-carboxypropanesulfonamide was coupled to aminomethyl polystyrene resin (100 mg, loading 0.9 mmol/g). Attachment of Fmoc-L-Met-OH as the first amino acid residue was carried out according to an optimized procedure described by Backes and Ellman.³⁶ The peptide chain elongation was performed according to the following protocol: (i) Fmoc removal: 20% piperidine/DMF (v/v), 2 $\times$ 5 min, (ii) wash procedure: 5 $\times$ DMF, 5 $\times$ DCM, 5 $\times$ Et₂O, (iii) coupling procedure: 5 equiv of Fmoc-aa-OH, 5 equiv of HOBt, 5 equiv of DIC, 4 h, RT, (iv) wash procedure as (ii). The last residue was coupled as Boc-L-Phe-OH. For cleavage-cyclization: the Boc-protected peptidyl resin was shaken with anhydrous *N*-methylpyrrolidinone (3 mL) and treated with iodoacetoneitrile (20 equiv) and *N*-ethyldiisopropylamine (5 equiv) for 24 h and was shielded from light. Subsequently the protecting Boc group was removed by treating the resin with a mixture of TFA/phenol/triisopropylsilane/H₂O (88:5:5:2) for 2 h. The peptide was liberated under cyclization from the handle by shaking the peptidyl resin in THF/DIPEA (4:1) for 16 h. The cleavage solution was lyophilized and purified by reversed-phase HPLC, yielding 3.1 mg of planktocylin as a colorless solid.

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Supporting Information Available: ¹H NMR, ¹³C NMR, and HMBC spectra of natural and synthetic planktocylin. Inhibition curves of cathepsin B and caspase-8 assayed with **1**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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