# Multiple Toxin Production in the Cyanobacterium *Microcystis*: Isolation of the Toxic Protease Inhibitor Cyanopeptolin 1020

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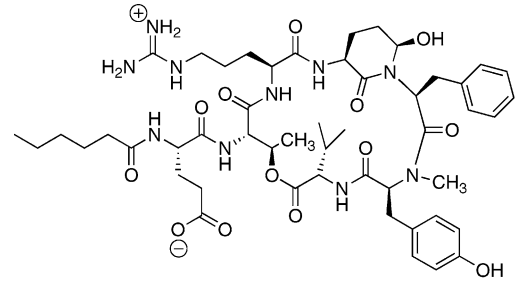
The isolation and structure of cyanopeptolin 1020 (hexanoic acid-Glu-N[-O-Thr-Arg-Ahp-Phe-N-Me-Tyr-Val-]) from a *Microcystis* strain is reported. Very potent picomolar trypsin inhibition ($IC_{50}$ = 670 pM) and low nanomolar values against human kallikrein (4.5 nM) and factor XIa (3.9 nM) have been determined for cyanopeptolin 1020. For plasmin and chymotrypsin, low micromolar concentrations were necessary for 50% inhibition. Cyanopeptolin 1020 was found to be toxic against the freshwater crustacean *Thamnocephalus platyurus* ($LC_{50}$ = 8.8 $\mu$M), which is in the same range as some of the well-known microcystins. These data support the hypothesis that cyanopeptolins can be considered as a second class of toxins in addition to the well-established microcystins in *Microcystis*.

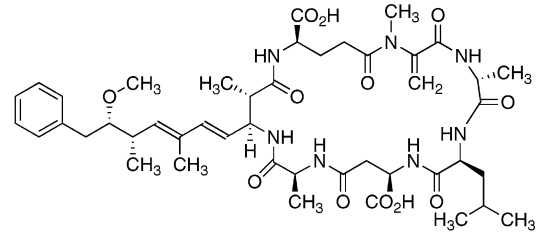
Cyclic depsipeptides containing the structural element 3-amino-6-hydroxy-2-piperidone (AHP) (so-called cyanopeptolins) are widely distributed secondary metabolites in cyanobacteria.[1] The structural diversity of previously described compounds is large, but all can be considered inhibitors of serine proteases of crustaceans and mammals.[1] Although preferentially mammalian enzymes in the context of pharmacological research have been tested, the evolutionary primary target enzymes can be assumed to be those of aquatic invertebrates that are important grazers of these cyanobacteria.[1] Digestive serine proteases of these invertebrates are strongly inhibited[2] and could thus render the cyanobacteria unsuitable food for them. The conserved feature of serine proteases in evolution allows these inhibitors to be successfully applied also against mammalian enzymes. Most of the AHP-containing compounds are active in the micromolar range,[1c] but some fit so perfectly in the reaction center of mammalian serine proteases that nanomolar concentrations were shown to be sufficient for inhibition.[3] Co-crystallization experiments have been conducted to analyze in atomic detail the binding properties of such an inhibitor to the reaction center of mammalian trypsin.[4] While the inhibition of digestive serine proteases of various organisms is well established, the acute toxicity of some of these congeners is not understood. Microcystilide A when applied intraperitoneally to mice induced convulsions and spasms but not death.[5] Cyanopeptolin SS caused the death of crustaceans[6] as did oscillapeptin J for *Thamnocephalus platyurus*.[7]

While little is known about the toxicity of cyanopeptolins, the most studied cyanobacterial toxins are the microcystins, which exert their hepatotoxicity through protein phosphatase inhibition[8] and are produced by species of the genera *Microcystis, Anabaena, Oscillatoria, Planktothrix,* and *Nostoc*. Microcystins are suspected of countless animal poisonings throughout the globe and also thought to be responsible for a severe incident in Brazil, where several persons died of contaminated water.[9] As a direct consequence, the World Health Organization set guidelines for drinking water and defined threshold values for microcystin concentration.[9] However, cyanobacteria of the genus *Microcystis* are known to produce several classes of compounds in addition to the microcystins frequently totaling more than 10 distinct secondary metabolites. For example, *Microcystis aeruginosa* PCC 7806 is known to produce cyanopeptolins A−D, cyanopeptolin 963A, [D-Asp$^3$]microcystin-LR and microcystin-LR, and the aerucyclamides A−D.[10] While most of the attention on toxic cyanobacterial peptides is focused on the microcystins, the above-mentioned reports on toxic effects of cyanobacteria might imply the presence of additional toxins related to cyanopeptolins in *Microcystis*.

Here we report on an extremely potent, picomolar trypsin inhibitor, cyanopeptolin 1020 (**1**), which also binds in the low nanomolar range to factor XIa and human kallikrein. In addition, this compound exhibits acute toxic properties against the crustacean *T. platyurus*.



Cyanopeptolin 1020 (**1**)



Microcystin-LA

The 60% methanolic extract of the biomass of *M. aeruginosa* UV-006 was fractionated and assayed against the sensitive freshwater crustacean *T. platyurus* for acute toxicity. Overall, more than 20 different compounds were present in these fractions, and out of a total of 16 fractions, nine were found to display acute toxicity in this assay. Among these nine fractions, eight compounds were assigned as known or new microcystins (Table S1, see Supporting Information). So far microcystins of all *Microcystis* strains have

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been shown to contain the structural element *N*-methyldehydroalanine rather than the isomeric dehydrobutyrine. Under this presumption, we found the very common and typical microcystin congeners [Asp$^3$]-MC-LR and MC-LR. MC-LR and MC-LA have already been described in a previous study on this strain,[11] and we could confirm both components. In addition, we found the lipophilic congeners MC-LAba, MC-LA, MC-LV, and MC-LL, which were only rarely reported before.[12] Additionally, two new variants were found (assigned based on typical absorption and mass spectra), which have not been described yet (Table S1). However, the amounts available were too small to allow the determination of the structures.

Interestingly, one compound displayed similar toxicity to MC-LA, but on the basis of the UV spectrum, it was hypothesized that this compound is not a microcystin, but a structurally different peptide. In order to elucidate the structure of this toxin, this fraction was purified and analyzed by NMR spectroscopy and chemical degradation (amino acid analysis). The molecular formula of **1** was determined to be $C_{50}H_{72}N_{10}O_{13}$ on the basis of HRMS data complemented by isotope labeling studies that established the presence of 10 N atoms. This molecular formula was consistent with the NMR data (Table 1). The $^1$H NMR spectrum displayed the typical pattern of a peptide. The different amino acids were identified on the basis of $^1$H,$^1$H COSY, 1D-TOCSY, and HMBC data as Glu, Thr, Arg, Ahp, Phe, N-Me-Tyr, Val, and hexanoic acid (HA). The sequence was obtained by $^1$H−$^{13}$C long-range correlations: from Glu-NH to HA-C1, from Thr-NH to Glu-C1, from Arg-NH to Thr-C1, from Thr-H3 to Val-C1, from Val-NH to N-Me-Tyr-C1, from N-Me-Tyr-NCH$_3$ to Phe-C1, and from Ahp-NH to Arg-C1. The macrocyclic ring was established by a ROESY interaction between Ahp-H5 and Phe-H3a and between Ahp-NH and Arg-H2 (Figure 1). This sequence assignment was corroborated by elaborate MS experiments of both natural abundance and $^{15}$N-enriched peptides. The mass fragmentation pattern of cyanopeptolin 1020 (**1**) has been found previously in complex mixtures by MALDI-TOF MS.[2b] The fragment ion at $m/z$ = 904 results from the loss of the hexanoyl fragment (Hex) and water. Moreover, the detection of a peak at $m/z$ = 794 (containing 9 N atoms) corresponds to the loss of the Hex-Glu side chain. The fragment ion at $m/z$ = 467 (6 N atoms) was assigned to the fragment Hex-Glu-dehydrobutyrate-Arg, resulting from cleavage of the peptide bond C-terminal at Arg and dehydration of the Thr residue. The complementary fragment Ahp-Phe-N-Me-Tyr-Val-OH(-H$_2$O) ($m/z$ = 534) could also be detected. The mass detected at $m/z$ = 420 (containing 3 N atoms) was assigned to the Phe-N-Me-Tyr-Val-OH (-H$_2$O) fragment. On the basis of this evidence resulting from MS and NMR experiments, we assign the structure as shown for **1** to cyanopeptolin 1020.

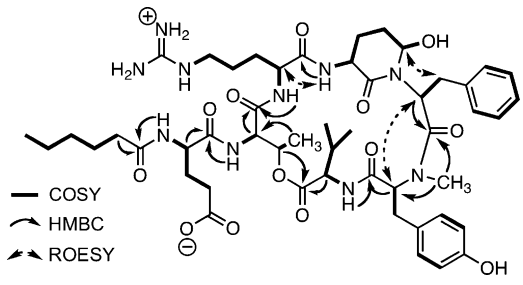


**Figure 1.** Key COSY, ROESY, and HMBC correlations in cyanopeptolin 1020 (**1**).

The configuration of **1** was determined by hydrolysis and derivatization of the amino acids following Marfey's method. This procedure established the configuration of the amino acids as L-Glu, L-Thr, L-Val, L-N-Me-Tyr, L-Phe, and L-Arg. For the Ahp residue, the relative configuration was assigned on the basis of ROESY interactions between Ahp-NH and Ahp-H3a, between Ahp-H3a and Ahp-OH, between Ahp-H2 and Ahp-H3b, and between Ahp-H2 and Ahp-4a. Therefore, the OH group is positioned in a pseudoaxial position, which can be explained by the anomeric effect. The absolute configuration of the Ahp residue was established by oxidation with $CrO_3$, followed by hydrolysis and Marfey's derivatization to yield L-Glu.

Cyanopeptolin 1020 (**1**) belongs to a large group of related cyclodepsipeptides that feature the Ahp residue as a key structural unit.[1] These peptides have been described in the literature as protease inhibitors, where basic amino acids adjacent to Ahp such as Arg or Lys convey selectivity for trypsin, and hydrophobic amino acids result in chymotrypsin selectivity. We have evaluated the inhibitory activity of cyanopeptolin 1020 for trypsin, chymotrypsin, and several other proteases involved in blood coagulation (Table S2). Cyanopeptolin 1020 inhibited trypsin with an $IC_{50}$ value of 0.67 nM. This potent inhibitory power against trypsin is remarkable, and it represents one of the strongest and most potent Ahp-containing inhibitors that have been isolated to date. When compared to A90720A[3] ($IC_{50}$ = 10 nM against bovine trypsin), cyanopeptolin 1020 (**1**) features a Phe residue instead of a Val, and the side chain D-Leu/sulfated glycerate is replaced by a L-Glu/hexanoic acid combination. On the basis of the crystal structure of the A90720A/bovine trypsin complex (PDB code 1TPS),[4] we docked cyanopeptolin 1020 into the active site of bovine trypsin (AutoDock 4.0).[13] Conformational searching followed by docking converged to the general conformation shown in Figure 2 (cyanopeptolin 1020, green; A90720A, gray). The basic interactions of **1** with bovine trypsin match the one found in the crystal structure: the Arg side chain recognizes the P1 binding site, and the Ahp residue modulates the conformation of the side chains of the catalytic triad Asp-His-Ser. It has been suggested from structural work by two groups[3,14] that Ahp-containing inhibitors prevent a water molecule from being close to the scissile bond. The sulfated glycerate residue is replaced by Glu in **1** and the Phe residue occupies the same position as Val in the 1TPS structure. However, the presence of the bulky Phe residue in lieu of Val forces the N-Me-Tyr residue inward, which might result in a more favorable interaction with trypsin and, thus, in increased binding.

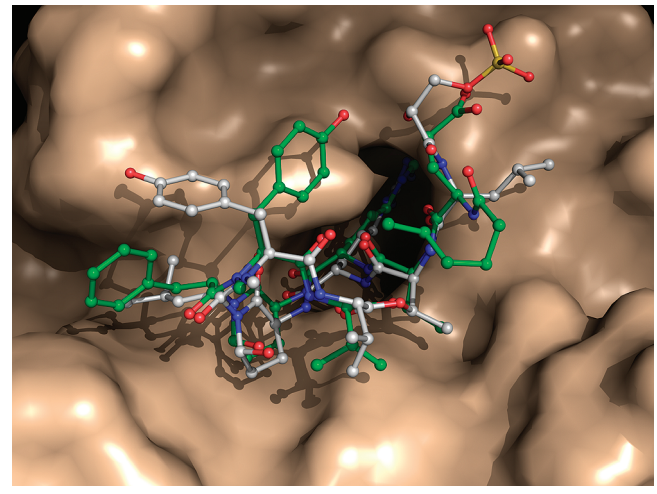

**Figure 2.** Crystal structure of A90720A (gray)/bovine trypsin complex (PDB code 1TPS) with cyanopeptolin 1020 (**1**, green) docked into the active site.

In addition to the $IC_{50}$ value of depsipeptide **1** against trypsin in the picomolar range, low nanomolar $IC_{50}$ values against factor XIa (3.9 nM) and kallikrein (4.5 nM) were determined. For plasmin and chymotrypsin, $IC_{50}$ values of 0.49 and 1.8 $\mu$M, respectively, could be measured. We did not find inhibitory properties against the human plasminogen activator, thrombin, and human LMW

**Table 1.** NMR Spectroscopic Data (600 MHz, DMSO-$d_6$) for Cyanopeptolin 1020 (**1**)

| residue | position | $\delta_C$ | $\delta_H$ ($J$ in Hz) | HMBC[c] | ROESY |
|---|---|---|---|---|---|
| HA | 1 | 172.5, C | | | |
| | 2 | 34.7, $CH_2$ | 2.13, m | HA-1, HA-3, HA-4 | |
| | 3 | 24.6, $CH_2$[a] | 1.51, m | HA-1, HA-2, HA-4, HA-5 | HA-4 |
| | 4 | 30.5, $CH_2$ | 1.23, m | HA-5 | |
| | 5 | 21.6, $CH_2$ | 1.27, m | HA-4, HA-6 | |
| | 6 | 13.6, $CH_3$ | 0.85, t (7.0, 7.0) | HA-4, HA-5 | |
| Glu | 1 | 172.1, C | | | |
| | 2 | 51.5, CH | 4.39, ddd (9.1, 7.8, 5.1) | Glu-1, Glu-3 | Glu-3b, Glu-4 |
| | 3a | 26.7, $CH_2$[b] | 1.88, m | | |
| | 3b | | 1.73, m | | |
| | 4 | 30.0, $CH_2$ | 2.26, m | Glu-3, Glu-5 | Glu-3a |
| | 5 | 173.9, C | | | |
| | NH | | 8.02, d (7.8) | HA-1, Glu-2 | Glu-2, Glu-3b, Glu-4, HA-2 |
| Thr | 1 | 168.8, C | | | |
| | 2 | 54.3, CH | 4.57, d (9.3) | Glu-1, Thr-1 | Thr-4 |
| | 3 | 71.7, CH | 5.42, m | Val-1, Thr-4 | Thr-4 |
| | 4 | 17.5, $CH_3$ | 1.16, d (6.6) | Thr-2, Thr-3 | |
| | NH | | 7.85, d (9.3) | Glu-1 | Thr-2, Thr-4, Glu-2 |
| Arg | 1 | 169.4, C | | | |
| | 2 | 51.1, CH | 4.23, m | | Arg-3a |
| | 3a | 27.0, $CH_2$[b] | 1.88, m | | Arg-3b |
| | 3b | | 1.38, m | | |
| | 4 | 24.6, $CH_2$[a] | 1.38, m | | |
| | 5 | 39.9, $CH_2$ | 3.05, m | | |
| | 6 | 156.4, C | | | |
| | NH | | 8.49, d (8.9) | Thr-1 | Ahp-NH, Thr-2, Thr-3 |
| | NH2 | | 7.46, m | | Arg-5 |
| Ahp | 1 | 168.6, C | | | |
| | 2 | 48.4, CH | 3.62, m | Ahp-1 | Ahp-3b, Ahp-3a, Ahp-4a |
| | 3a | 21.3, $CH_2$ | 2.40, m | | Ahp-3b |
| | 3b | | 1.60, m | | |
| | 4a | 29.0, $CH_2$ | 1.68, m | | |
| | 4b | | 1.56, m | | |
| | 5 | 73.5, CH | 5.07, m | Ahp-1 | Ahp-4a, Ahp-4b, Phe-3a |
| | NH | | 7.11, d (9.2) | Arg-1 | Ahp-2, Ahp-3a, Thr-3, Arg-2 |
| | OH | | 6.05, d (3.0) | | Ahp-3a, Ahp-4a, Ahp-5, Val-5 |
| Phe | 1 | 170.1, C | | | |
| | 2 | 50.0, CH | 4.75, dd (11.8, 3.7) | Phe-1 | Phe-3b |
| | 3a | 35.0, $CH_2$ | 2.90, dd (13.9, 11.8) | Phe-1′, Phe-2′/6′ | Phe-3b |
| | 3b | | 1.80, dd (13.9, 3.7) | Phe-2′/6′ | |
| | 1′ | 136.5, C | | | |
| | 2′/6′ | 129.2, CH | 6.84, d (7.3) | Phe-3, Phe-2′/6′, Phe-5′ | Phe-2, Phe-3a, Phe-3b, Ahp-4b, Ahp-2, Ahp-5 |
| | 3′/5′ | 127.5, CH | 7.18, dd (7.3, 7.3) | Phe-2′/6′, Phe-3′/5′, Phe-4′ | Phe-2′/6′, N-Me-Tyr-2′/6′, N-Me-Tyr-3′/5′ |
| | 4′ | 125.9, CH | 7.14, d (7.3) | Phe-2′/6′ | |
| N-Me-Tyr | 1 | 169.0, C | | | |
| | 2 | 60.7, CH | 4.89, dd (11.6, 2.3) | N-Me-Tyr-1 | N-Me-Tyr-3a, Phe-2 |
| | 3a | 32.5, $CH_2$ | 3.11, dd (14.2, 2.3) | N-Me-Tyr-1′, N-Me-Tyr-2′/6′ | N-Me-Tyr-3b |
| | 3b | | 2.72, dd (14.1, 11.6) | N-Me-Tyr-1′, N-Me-Tyr-2′/6′ | |
| | 1′ | 127.3, C | | | |
| | 2′/6′ | 130.1, CH | 7.00, d (8.4) | N-Me-Tyr-3, N-Me-Tyr-2′/6′, N-Me-Tyr-4′ | N-Me-Tyr-2, N-Me-Tyr-3′/5′, Phe-2, Phe-3a, Phe-3b |
| | 3′/5′ | 115.1, CH | 6.78, d (8.4) | N-Me-Tyr-1′, N-Me-Tyr-3′/5′, N-Me-Tyr-4′ | Ahp-OH (very weak) |
| | 4′ | 156.0, C | | | |
| | $NCH_3$ | 30.0, $CH_3$ | 2.76, s | Phe-1, N-Me-Tyr-2 | |
| | OH | | 9.34, s | N-Me-Tyr-3′/5′, N-Me-Tyr-4′ | N-Me-Tyr-3′/5′ |
| Val | 1 | 171.8, C | | | |
| | 2 | 55.5, CH | 4.71, dd (9.6, 4.5) | Val-1, Val-3, Val-5, N-Me-Tyr-1 | Val-3, Val-4 |
| | 3 | 30.4, CH | 2.07, m | | Val-4, Val-5 |
| | 4 | 18.9, $CH_3$ | 0.87, d (6.8) | Val-2, Val-3, Val-5 | |
| | 5 | 16.9, $CH_3$ | 0.72, d (6.8) | Val-2, Val-3, Val-4 | |
| | NH | | 7.43, d (9.6) | N-Me-Tyr-1 | Val-2, Val-5, N-Me-Tyr-2, N-Me-Tyr-$NCH_3$, Ahp−OH |

[a] These two carbons can be interchanged. [b] These two carbons can be interchanged. [c] HMBC correlations are given from proton(s) stated to the indicated carbon atom.

urokinase at concentrations below 2.5 $\mu$M. Although these values cannot be directly compared, as different enzymes, different substrates, and different concentrations have been used in the experiments, generally very potent inhibition values have been observed.

Additionally, we wanted to confirm the toxicity of cyanopeptolin 1020 (**1**) against the sensitive freshwater crustacean *T. platyurus* using the highly purified cyanopeptolin 1020 (**1**). *T. platyurus* serves as a benchmark organism for the evaluation of the ecotoxicological potential of natural products.[15] We have determined an $LC_{50}$ value of 8.8 $\mu$M in the 24 h acute toxicity assay (Figure S14, Supporting Information). This value is comparable to those determined for microcystins, which represent the prototypical example of cyanobacterial toxins.[16] For example, $LC_{50}$ values of 10.8, 4.7, and 7.0 $\mu$M have been measured for MC-LR, MC-YR, and MC-RR, respectively.[16] The low $LC_{50}$ value of cyanopeptolin 1020 (**1**) thus clearly establishes that this compound constitutes another toxin in *Microcystis* of similar potency to several microcystins. Such toxicity might also be unveiled for other cyanopeptolins, and one related natural product from *Planktothrix rubescens*, oscillapeptin J, has

been described as having similar acute toxic activity.[7] It can be speculated that these toxic effects are related to the very potent serine protease inhibition as described above, in particular as the large family of trypsin-like proteases[17] are involved in many key processes related to the viability of organisms. More detailed toxicological studies on mammals are thus encouraged by this study.

In this note, we have reported the structure elucidation of cyanopeptolin 1020 (**1**) extracted from *M. aeruginosa* UV-006, which inhibits trypsin in the picomolar range and factor XIa and kallikrein in the low nanomolar range. This compound was shown to be toxic to the freshwater crustacean *T. platyurus*, and its toxicity was determined to be comparable to commonly found microcystins, which are frequently described as biotoxins from cyanobacteria. This report thus suggests the presence of multiple toxins in *Microcystis aeruginosa* UV-006 and encourages further evaluation of toxicity of cyanopeptolin 1020 (**1**) in mammals. The co-occurrence of toxic cyanopeptolin 1020 (**1**) along with several microcystins in *Microcystis* might also suggest a re-evaluation of drinking water guidelines and public health concerns currently solely focusing on microcystins to include other relevant toxins such as the presented cyanopeptolin as well.

## Experimental Section

**General Experimental Procedures.** NMR spectra were acquired on a Bruker DRX-600 equipped with a cryoprobe and are referenced to residual solvent proton and carbon signals ($\delta_H$ 2.50, $\delta_C$ 39.5 for DMSO-$d_6$). Accurate mass ESI spectra were recorded on a Bruker FTMS 4.7T BioAPEX II at the University of Fribourg. The identification and the purification were performed on a HPLC Shimadzu 10AVP system equipped with an photodiode array detector and an LCQ Duo ESI mass spectrometric detector (Finnigan Thermo). HPLC analyses for Marfey's experiments were performed on a Dionex HPLC system equipped with a P680 pump, an ASI-100 automated sample injector, a TCC-100 thermostated column compartment, a PDA-100 photodiode array detector, and a MSQ-ESI mass spectrometric detector.

**Culture, Extraction, and Isolation.** The *Microcystis aeruginosa* strain UV-006 was an isolate from Hartebeespoort Dam near Pretoria, South Africa.[11] *M. aeruginosa* UV-006 was grown in mineral medium at 26 °C under constant light conditions ($40 \pm 5$ $\mu$mol $m^{-2}$ $s^{-1}$). Glass tubes with a diameter of 7 cm and a height of 1.5 m containing 5 L of media were used. The tubes were aerated with 200 mL $min^{-1}$ compressed air containing 1000 ppm $CO_2$ and continuously stirred with a magnetic stirring bar. Cell biomass was harvested using a flow-through centrifuge (Westfalla Separator AG, Germany) and subsequently stored at −23 °C. To obtain high percentage labeled cyanopeptolin 1020 (**1**), nitrate was replaced by $^{15}NO_3^-$ in the medium (final concentration 4.0 mM).

Cyanopeptolin 1020 (**1**) was extracted by the addition of 10 mL of 60% methanol per gram of wet cell biomass, overnight incubation at 1 °C in the dark, and centrifugation for 10 min at 27500*g*. Compounds in the supernatant were brought to dryness in a rotatory evaporator, dissolved in 2 mL of 60% MeOH, and filtered (0.2 $\mu$m pore size PTFE syringe filter). The solvent was removed under a moderate stream of nitrogen, and the mixture was stored in glass vials at −23 °C. Compound **1** was then purified by HPLC with a reversed-phase column (C-18 Grom-Sil 120 ODS-4 HE, 250 × 4.6 mm, 5 $\mu$m particle size, Stagroma) with a flow rate of 1 mL $min^{-1}$ and a column temperature of 30 °C. Separation and identification were performed with a binary solvent gradient of UV-treated deionized $H_2O$ (solvent A) and $CH_3CN$ (solvent B), both acidified with 0.05% TFA (trifluoroacetic acid). The following gradient was used: 0 min, 30% B; 10 min, 35% B; 40 min, 70% B; 42 min, 100% B; 50 min, 100% B; compound **1** eluted after 13.0 min. TFA was removed before evaporating the solvents by applying sequentially the combined HPLC fraction on a $C_{18}$ SPE cartridge (5 g, conditioned with 10% MeOH). The cartridge was then washed with $H_2O$ to remove TFA, and **1** was finally eluted with 100% MeOH. Subsequently, the MeOH was removed under reduced pressure and $H_2O$ was removed by lyophilization to afford **1** as a colorless, amorphous solid.

Cyanopeptolin 1020 (**1**): UV (47% acetonitrile in water with 0.05% TFA) $\lambda_{max}$ 277 nm; $^1H$ and $^{13}C$ NMR data (DMSO-$d_6$), see Table 1; HRESIFTMS *m/z* $[M + H]^+$ 1021.5343 (calcd for $C_{50}H_{73}N_{10}O_{13}$, 1021.5353).

**Marfey's Analysis.** Compound **1** (0.1 mg, 0.1 $\mu$mol) was hydrolyzed in a sealed tube with 6 N HCl (0.3 mL) at 105 °C for 24 h. The solution was concentrated to dryness with a stream of $N_2$. The hydrolyzed compound was treated with 60 $\mu$L of a solution of 1% $N_\alpha$-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (FDAA) in acetone and 60 $\mu$L of 6% triethylamine in a sealed vial at 50 °C for 1 h. To the mixture was added 60 $\mu$L of 5% acetic acid, and the mixture was dried under a moderate stream of $N_2$. The residue was dissolved in 200 $\mu$L of MeOH and analyzed by RP-HPLC using an Agilent Zorbax SB-C18 column (150 × 2.1 mm, 3.55 $\mu$m). Mobile phase A was 5% acetic acid in $H_2O$, B was $CH_3CN$/MeOH (9:1), and C was $CH_3CN$. The linear gradient started with 5% B to reach 50% B over 50 min. The column was then washed for 10 min with 100% B, followed by 20 min with 100% C. The column was stabilized before the next injection with 5% B for 20 min and maintained at 50 °C. The flow rate was set at 0.2 mL/min. The configuration was determined by comparison with the retention time of derivatives from commercially available amino acids. The retention time for each amino acid derivative was obtained by extracting the mass of the derivative in the HPLC-MS chromatogram. Retention times (min) for the standard amino acid derivatives were determined as follows: L-Thr 25.2; L-*allo*-Thr 26.4; D-Thr 32.3; D-*allo*-Thr 29.5; L-Arg 21.6; D-Arg 22.3; L-Phe 44.8; D-Phe 50.1; L-Val 40.5; D-Val 46.7; L-N-Me-Tyr (di-FDAA derivative) 53.7; D-N-Me-Tyr (di-FDAA derivative) 54.4; L-Glu 27.6; D-Glu 30.8. The configuration of cyanopeptolin 1020 (**1**) was unambiguously assigned as L-Thr 24.9; L-Arg 21.6; L-Phe 44.9; L-Val 40.0; L-N-Me-Tyr (di-FDAA derivative) 53.7; and L-Glu 27.6; see also the Supporting Information for chromatograms.

**Configuration of Ahp.** The oxidation was realized according to the procedure developed by Itou et al.[18] Cyanopeptolin 1020 (**1**, 0.1 mg, 0.1 $\mu$mol) was oxidized in a solution (0.5 mL) of $CrO_3$ in AcOH (1 mg/mL) at room temperature for 2 h. The mixture was then applied onto a $C_{18}$ SPE cartridge conditioned with 10% MeOH. After applying the mixture, the cartridge was first washed with $H_2O$, and the material was eluted with MeOH. After evaporation of the MeOH the resulting oxidized material was hydrolyzed, derivatized, and analyzed as described above. Only the Marfey's derivative of L-Glu was observed, thus establishing the configuration of position 2 of the Ahp residue to be *S*; see also Supporting Information for chromatograms.

**Acute Toxicity Assay.** The highly purified cyanopeptolin 1020 (**1**) was tested in a 24 h acute toxicity assay with *T. platyurus* (Thamnotoxkit F; MicroBioTests Inc.) in a multiwell plate using instar II and III larvae hatched from cysts. The cyanopeptolin was tested in six concentrations ranging from 0.1 to 100 $\mu$M. For every concentration, 20 to 30 animals were used. The nonlinear regression analysis and determination of the $LC_{50}$ value were calculated with Graph Pad Prism 4 for Windows.

**Protease Inhibition Assays.** The details of the proteases and their respective fluorogenic substrates are summarized in Table S2. The enzyme inhibition assays were carried out in using modified literature procedures.[19] The kinetic fluorescence measurements were realized with transparent microplates (Nunc) using a fluorescence microplate reader (Tecan infinite M1000) set at $\lambda_{ex}$ = 355 nm, $\lambda_{em}$ = 480 nm and measuring a data point every 30 s for 1 h. To obtain the initial reaction rate, linear regression was performed with Microsoft Excel. The enzymes were dissolved in a buffer containing 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% BSA, and 0.01% Triton X-100. To facilitate the dissolution of the substrates, 7.5% DMSO was added. The DMSO amended buffer was also used as a blank. Optimal enzyme concentrations were determined by measuring kinetics with different enzyme concentration. The substrate solution (50 $\mu$L) was added to a mixture of 50 $\mu$L of the enzyme solution and 50 $\mu$L of the blank. The enzyme concentrations were assayed between 0.1 and 10 nM (final concentration). The optimal protease concentrations are listed in Table S2. For the inhibition assays, a mixture of 50 $\mu$L of protease solution and 50 $\mu$L of inhibitor solution was incubated at RT for 30 min. The substrate solution (50 $\mu$L) was then added, and the measurement was directly started. The fractional activity was obtained by dividing the inhibited rate by the uninhibited rate obtained with a blank reaction. Chymotrypsin inhibition assays were performed as stated recently.[7] The inhibition curves and the 50% inhibition values ($IC_{50}$)

were calculated using the nonlinear regression sigmoidal four-parameter formula with Prism 5.0b for Mac OS X.

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**Supporting Information Available:** Detailed structure elucidation, $^1$H NMR, COSY, ROESY, HSQC, and HMBC spectra, chromatograms, and data of the enzyme assays are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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