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The toxicity and enzyme activity of a chlorine and sulfate containing aeruginosin isolated from a non-microcystin-producing *Planktothrix* strain

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ABSTRACT

The toxicity of six different *Planktothrix* strains was examined in acute toxicity assays with the crustacean *Thamnocephalus platyurus*. The presence of toxicity in two strains could be explained by the occurrence of microcystins. The other four *Planktothrix* strains were not able to produce microcystins due to different mutations in the microcystin synthetase (*mcy*) gene cluster. In these strains, toxicity was attributed to the presence of chlorine and sulfate containing compounds. The main representative, called aeruginosin 828A, of such a compound in the *Planktothrix* strain 91/1 was isolated, and structure elucidation by 2D NMR and MS methods revealed the presence of phenyllactic acid (*Pla*), chloroleucine (*Cleu*), 2-carboxy-6-(4′-sulfo-xylosyl)-octahydroindole (*Choi*), and 3-aminoethyl-1-*N*-amidino-Δ-3-pyrroline (*Aeap*) residues. Aeruginosin 828A was found to be toxic for *T. platyurus* with a LC_{50} value of 22.4 μM, which is only slightly higher than the toxicity found for microcystins. Additionally, very potent inhibition values for thrombin (IC_{50} = 21.8 nM) and for trypsin (IC_{50} = 112 nM) have been determined for aeruginosin 828A. These data support the hypothesis that aeruginosins containing chlorine and sulfate groups, which were found in microcystin-deficient *Planktothrix* strains, can be considered as another class of toxins.

1. Introduction

Climate change as well as the anthropogenic input of nutrients into freshwaters has resulted in an increase of cyanobacterial biomasses in many lakes and rivers throughout the world during the last decades (Paerl and Huisman, 2008). These organisms are often found to accumulate in very high densities also in lakes that are intensively used as recreational areas or as drinking water reservoirs (Posch et al., 2012). However, cyanobacteria are known to produce and store intracellularly a wide variety of bioactive secondary metabolites, and a collapse of such a bloom might liberate high amounts of these compounds into the water. In recent years, substantial progress has been made to identify possible harmful compounds from different cyanobacterial genera. Most of these substances could be assigned to distinct chemical oligopeptide classes, e.g. microcystins, cyanopeptolins, anabaenopeptins, aeruginosins, cyclamids, microginins and microviridins (Welker and von Döhren, 2006). These peptides exhibit various biological activities such as the inhibition of different phosphatases and proteases (Welker and von Döhren, 2006). Microcystins (MCs) have been found to be responsible for countless animal poisonings all over the world, and even for human toxicity (Sivonen and Jones, 1999), and cyanopeptolins and aerucyclamides received some attention due to their toxicity to *Thamnocephalus platyurus* (Blom et al., 2003; Gademann et al., 2010; Portmann et al., 2008a, 2008b).

There is intense discussion about the possible ecological role of MCs in the environment; however, MCs are still regarded to be the primary defence mechanism of cyanobacteria against grazers (Blom et al., 2001; Kurmayer and Jüttner, 1999). MC-deficient *Planktothrix* genotypes are typically found in rather low proportions of the total cyanobacterial population (Ostermaier and Kurmayer, 2009), but might occasionally exceed 50% of the total abundances. Reasons for the lack of MC production might be various point mutations (e.g. insertions or deletions) or the loss of genes encoding the MC synthetase (*mcy*) gene cluster (Christiansen et al., 2006, 2008). However, the loss or dysfunction of the *mcy*

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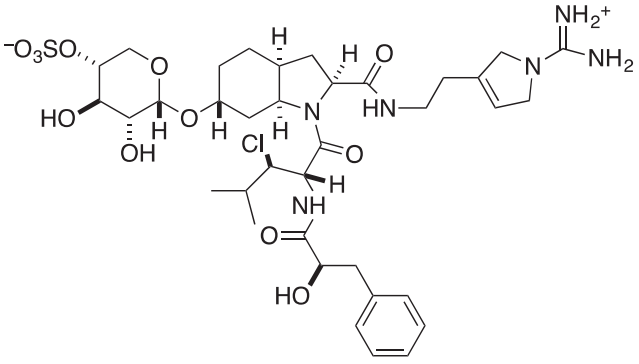
gene cluster did not seem to be disadvantageous for the overall success of the *Planktothrix* genotypes. This is supported by the linear relation of MC deficient genotypes to the total population density of bloom forming *Planktothrix* populations in European lakes (Ostermaier and Kurmayer, 2009). In order to explain these findings it was suggested that an alternative peptide or peptide class might functionally compensate for the lack of MCs. Here we report on a peptide class, the chlorine and sulfate containing aeruginosins, which were found in MC-deficient strains, and which exhibited acute toxicity against the crustacean *Thamnocephalus platyurus*.

2. Material and methods

2.1. General experimental procedures

^{1}H NMR spectra were recorded on a Bruker Avance III 600 MHz with a 5 mm BBFO plus probe or a Bruker Avance III Ascend 700 MHz spectrometer with a 5 mm TCI (H-C/N-D) cryo probe at room temperature. Chemical shifts (δ-values) are reported in ppm, spectra were calibrated related to the solvent residual proton chemical shift (DMSO, $\delta = 2.50$). The coupling constants are specified in Hz. HRMS spectra were recorded on a Bruker maXis 4G instrument. Identification and purification of aeruginosin 828A (**1**) was performed on a Shimadzu 10AVP HPLC system equipped with an automated sample injector, a thermostated column compartment, and photo diode array detector. Mass spectra were recorded on a combined LC–MS (LCQ Duo mass spectrometer, Finningan Thermoquest, USA) equipped with an electrospray ionization source (ESI-MS).



Aeruginosin 828A (**1**)

2.2. Culture, extraction, and bioassay-guided fractionation

A culture collection of six *Planktothrix* strains was established in the laboratory at the beginning of this study. All cyanobacteria were grown in 300 mL Erlenmeyer flasks at 20 °C under constant light conditions at an irradiation of 6 μmol m^{-2} s^{-1} from fluorescent tubes (Osram 930; Lumilux Delux; Warm White 3000K) in 120 mL mineral medium described by Jüttner et al. (1983). Two of the six *Planktothrix* strains were capable of producing MCs, four strains lacked the MC production either due to point mutations or complete loss of the *mcy* gene cluster (summarized in Table S1, Supplementary material). The analysis of the *mcy* gene cluster in *Planktothrix* strains was carried out as part of another study as described elsewhere (Christiansen et al., 2008).

Frozen biomass of all six *Planktothrix* strains was extracted twice with 50% MeOH (10 mL per gram of wet cell biomass) for 2 h in the dark. After centrifugation (25,700 × *g* for 15 min) the supernatants (crude extracts) were fractionated by HPLC equipped using a reversed phase column (Hydrosphere C18, YMC, 4.6 × 250 mm, Stagroma, Reinach, CH) using two solvents: UV-treated deionised water and acetonitrile. The solvents were free of trifluoroacetic acid (TFA) to avoid undesired toxic side effects and unwanted isomerization products (Blom et al., 2001). A linear increase was applied (acetonitrile from 20% to 70% in 50 min, 70–100% in 2 min, isocratic for additional 10 min). Fractions were collected every 3 min; the solvents were evaporated by vacuum centrifugation (SPEEDVAC Plus® SC110A, Savant Instruments Inc., USA). The fractions containing the different cyanobacterial compounds were transferred to a 24 well plate, and re-dissolved in 1% MeOH (500 μL). The toxicity of the fractions of the six crude extracts was tested in a 24-h acute toxicity assay performed using instar II-III larvae (Thamnotoxkit F; MicroBioTests Inc.) of the sensitive crustacean *Thamnocephalus platyurus*, which can be easily hatched from cysts (Blom et al., 2003, 2001). About 20–30 crustaceans were transferred to each well; after 24 h, the mortality rate was determined for each fraction. Each fraction represented the extract of an equivalent of 0.05 μM chlorophyll a of the biomass of the six *Planktothrix* strains.

2.3. Isolation of aeruginosin 828A

Fresh biomass (32 g) of *Planktothrix* strain 91/1 was extracted with 50% MeOH. Crude extracts were obtained after centrifugation and were separated by HPLC using a reversed phase column (Hydrosphere C18, YMC, 4.6 × 250 mm, Stagroma, Reinach, CH) under the following conditions: solvent A was UV-treated deionised water (+0.05% TFA), solvent B: HPLC-grade acetonitrile (+0.05% TFA); a linear increase was applied (as described above). Under the conditions applied aeruginosin 828A (**1**) eluted after 16.0 min. TFA was removed before evaporating the solvents to prevent undesirable isomerization products by applying sequentially the combined HPLC fractions on a C18 cartridge (10 g; Mega Bond Elute, Varian, Agilent Technologies, Basel, CH; conditioned with 10% MeOH). The cartridge was flushed with water to remove the TFA, and aeruginosin 828A (**1**) was eluted with 80% MeOH. Subsequently, the aqueous methanol was evaporated (35 mbar, 40 °C) to achieve a colorless, amorphous solid. After purification 533 μg of pure aeruginosin 828A (>99% HPLC) could be achieved.

Aeruginosin 828A (**1**): UV (47% acetonitrile in water with 0.05% TFA λ_{max} 277 nm; ^{1}H and ^{13}C NMR data (DMSO-d_6), see Table 1; HRMS-ESI: calcd. for $C_{36}H_{53}{}^{35}ClN_6O_{12}SNa^+$ $[M+Na]^+$: 851.3023; found: 851.3019.

2.4. Acute toxicity of aeruginosin 828A

The highly purified aeruginosin 828A was tested in a 24 h acute toxicity assay with *Thamnocephalus platyurus* in six concentrations ranging from 0.5 to 100 μM in triplicates. For every concentration, 20–30 animals were used. The nonlinear regression analysis as well as the LC_{50} value was calculated using Graph Pad Prism 5 for Windows.

2.5. Enzyme inhibition assays

The inhibition of trypsin (0.04 U/200 μL; No. 9471 Fluka, Buchs, Switzerland), and thrombin (5 nM; IHTa Innovative Research, Peary, USA) was tested in microtiter plates. Boc-Gln-Ala-Arg-aminomethylcoumarin (50 μM; Bachem AG, Bubendorf, Switzerland) served as substrate for trypsin, and Boc-Phe-Ser-Arg-aminomethylcoumarin (100 μM; Bachem AG, Bubendorf, Switzerland) as substrate for thrombin according to previously established protocols (Blom et al., 2006; Gademann et al., 2010).