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Out in the Green: Biologically Active Metabolites Produced by Cyanobacteria

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Abstract: Dried cyanobacteria ('Spirulina') are sold as a nutraceutical for their high content of proteins, essential fatty acids and vitamins. Beyond spirulina, other genera of cyanobacteria produce interesting small molecules that could find use in nutraceutical or pharmaceutical applications. This account presents recent research efforts on antimalarial nostocarboline and the aerucyclamides, as well as on potent toxins such as cyanopeptolin 1020 and microcystins. Combinations of spectroscopic, computational, chemical and biological studies investigated the mechanism of action of these compounds. Their application potential with regard to nutraceuticals or pharmaceuticals is discussed.

Keywords: Chemical biology · Mechanism of action · Natural products · Organic synthesis

Cyanobacteria (formerly called blue-green algae) are prokaryotic photoautotrophs that have been suggested to be present on earth for the past 2.5 billion years.[1] They populate a large variety of different habitats all over the globe, demonstrating their great ability for adaption. In addition, these primary producers are under constant ecological pressure from grazers (insects and crustaceans), competing phototrophs, fungi and bacteria. Therefore, a large portion of the cyanobacterial genome is devoted to the production of biologically active small molecules (natural products, 'secondary metabolites').[2] Some strains produce over twenty different compounds, and major structural classes such as peptides, polyketides, terpenes, alkaloids, lipids and carbohydrates have been isolated from these sources.[3a–f] In addition, these prokaryotic organisms are amenable to genetic modification, and thus higher yields could be obtained through such approaches.

Since ancient times, cyanobacteria have been consumed directly as microbial food in at least two locations separated by over 10000 km, *i.e.* in central America and in Northern Central Africa.[3g] This well-documented tradition reaches well in the 21st century, as 'spirulina' is currently sold in *e.g.* Europe and the US as a nutraceutical and marketed for its ingredients. While 'spirulina' encompasses several genera and species, in general, these are characterized by high protein content, as well as containing essential fatty acids and vitamins. One potential problem arises from the biotechnological production of 'spirulina', as contamination by other cyanobacteria potentially producing toxins has to be considered. In this account, I will discuss our recent efforts to identify additional toxins from cyanobacteria and to elucidate their mechanism of action. In addition, other cyanobacteria than 'spirulina' could provide benefits to human nutrition and health, and therefore, another part of this account is devoted to our recent efforts towards the isolation, structural characterization and chemical and biological studies of biologically active compounds from cyanobacteria with respect to potential nutraceutical or pharmaceutical applications. The interested reader is also referred to the original publications and the references cited therein as documented in the bibliography.

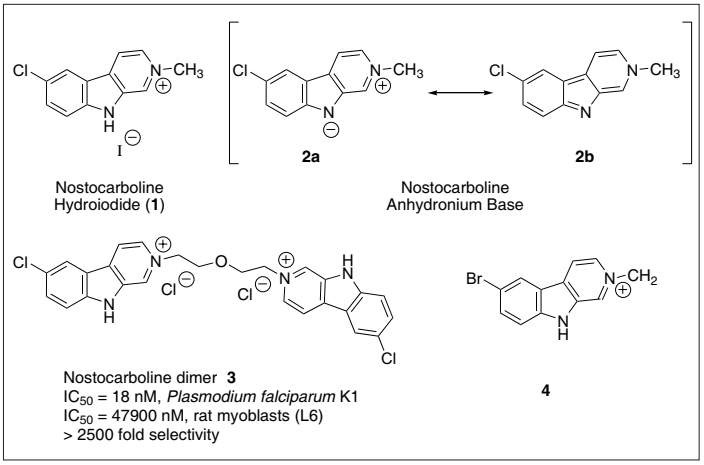

Fig. 1.

In collaboration with the group of Prof. Dr. Jüttner at the Limnological Station of the University of Zurich, we became engaged in spectroscopic and chemical studies of nostocarboline (**1,** Fig. 1), isolated from the freshwater cyanobacterium *Nostoc* 78-12a.[4] Nostocarboline (**1**) is a so-called anhydronium base, a term coined by Robinson[5] for zwitterionic compounds exemplified by the resonance structures **2a** and **2b**. While the initial proof for structure was given by total synthesis,[4] we were later able to crystallize the nostocarboline anhydronium base from a MeOH/Et_2O/hexane mixture and determine its X-ray crystal structure.[6] Interestingly, nostocarboline is a *chlorinated* carbolinium alkaloid found in a cyanobacterium originally isolated from a freshwater pond and cultured in freshwater medium. The source of halogen and the mechanism of halogenation[7] remain unclear.

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Initial biological studies focussed on nostocarboline (**1**) as inhibitor of butyryl- and acetylcholinesterase,[4,8] a target which is addressed by currently used drugs such as galanthamine in early stages of Alzheimer's disease.[9] Similar biological potency was found for both nostocarboline and galanthamine in enzyme assays.[4,8] Regarding the potential ecological role of nostocarboline (**1**), two different possibilities were evaluated: Either a function as a deterrent against grazers such as crustaceans could be possible, or this compound could be acting as an allelochemical against competing phototrophs. The first possibility was examined, and although some trypsin inhibition could be determined,[8] the toxicity of nostocarboline against the sensitive freshwater crustacean *Thamnocephalus platyrus* was found to be very weak.[8] The allelochemical activity as an algicide acting against competing phototrophs was studied next,[10] and strong activity against both prokaryotic and eukaryotic phototrophs could be demonstrated. A structure–activity relationship study demonstrated that the positively charged methyl carbolinium was found to be important for activity.[10] The mechanism of action was studied using chlorophyll-*a* fluorescence imaging in tobacco leaves, and it was demonstrated that a decrease in photosynthesis precedes cell death.[11] Algicidal compounds that do not show significant ecotoxity against crustaceans (or toxicity in general) are also interesting for a completely different application: The malaria parasite *Plasmodium falciparum* contains an organelle of algal (or cyanobacterial descent): the apicoplast.[12] Targeting this apicoplast with selective herbicidal or algicidal compounds has been suggested to constitute a viable approach for the development of new active compounds against malaria.[13] We have shown that nostocarboline is active against *P. falciparum*, while displaying only little toxicity against rat myoblasts.[14] A series of SAR studies were conducted,[6] which resulted in the following conclusions: i) Replacing the N-Me group by larger substituents decreased antiplasmodial activity while increasing cytotoxicity; ii) dimeric structures linked *via* N(2) displayed very good inhibitory (18 nM for dimer **3**) activity with large selectivity (SI >2500); iii) the bromo analogues such as **4** (related to the eudistomin series) retain or even show better *in vitro* activity against *P. falciparum*.[6] We have also tested several compounds in the *Plasmodium berghei* mouse model, and a 50% reduction of parasitaemia vs untreated control group was observed after 4 × 50 mg/kg dosing.[6] These studies suggest nostocarboline (**1**) as interesting structure for further study as an antimalarial agent.[15]

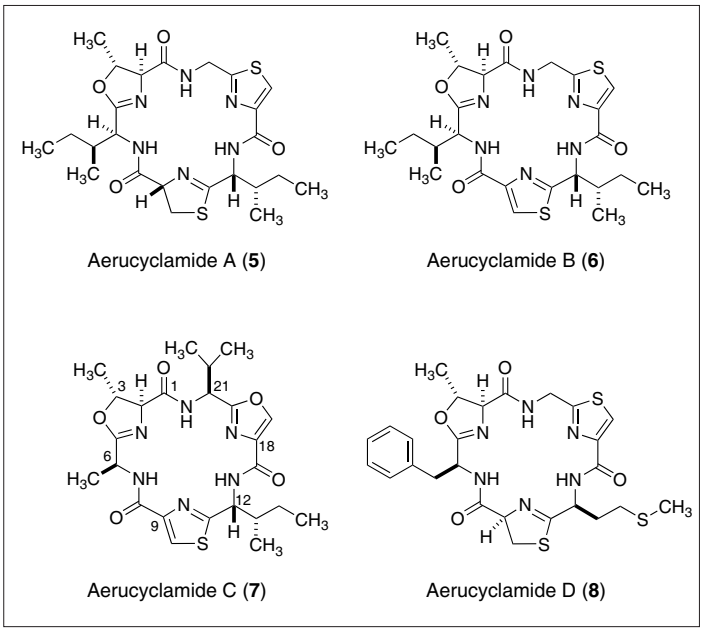

Fig. 2.

We have also investigated the precursor-directed biosynthesis of nostocarboline derivatives in *Nostoc* 78-12A.[11] This research problem constituted a significant challenge for several reasons: i) being photoautotrophs, the uptake of xenobiotic chemicals is heavily regulated and in many cases difficult to carry out in cyanobacteria; ii) the generation of phytotoxic metabolites in a phototroph is difficult; iii) the promiscuity of the enzymes involved in the biosynthesis is difficult to predict *a priori*. As a consequence, there are only a few studies demonstrating the potential of precursor-directed biosynthesis in cyanobacteria.[16] We were able to feed several modified tryptophan precursors and to isolate fluorinated or methylated derivatives, which are difficult to access synthetically, in yields up to 1.2 mg/L of culture medium.[11]

In addition to nostocarboline, we have investigated other antiplasmodial compounds, the aerucyclamides A–D isolated from the cyanobacterium *Microcystis aeruginosa* PCC 7806.[17] The aerucyclamides are members of a large class of macrocyclic hexapeptides (the cyclamides) featuring thiazole, thiazoline, oxazole and oxazoline rings complemented by hydrophobic amino acids. Whereas aerucyclamide A (**5,** Fig. 2) features a thiazoline ring, aerucyclamide B (**6**) represents the thiazole derivative, and consequently, cyclamide **6** can be prepared *via* chemical oxidation from compound **5**. Aerucyclamide C (**7**) contains an oxazole ring instead of a thiazole, and Val and Ala instead of Ile and Gly amino acid residues. The last derivative **8** features Phe, Met and Gly as acyclic amino acids. The large variation of amino acids on this cyclic template stems from the interesting biosynthesis of these compounds *via ribosomal peptide synthesis*.[18] First identified by Schmidt and coworkers and Jaspers and coworkers for related cyclopeptides,[19] it was shown that a hypervariable cassette encoding for the primary sequence of the cyclamide is flanked by processing enzymes responsible for tailoring this precursor peptide into the final metabolite *via* macrocylization, heterocyclization and oxidation (and even possibly epimerization).[18] Dittmann and coworkers established the genetic basis of the biosynthesis for the microcyclamides[20] (= aerucyclamides) but their prediction of chemical structure from genetic data led to a misassignment of structure for microcyclamide 7806A that was later corrected based on chemical, physical and spectroscopic data.[17a]

The aerucyclamides are active against *P. falciparum*: The most active compound is aerucyclamide B (**6**) with a determined IC_{50} value of 0.7 μM and large selectivity (120 μM against L6 rat myoblasts).[17a] Surprisingly, saturation of one double bond to give the thiazoline-containing aerucyclamide A (**5**) resulted in a tenfold loss of activity. The other aerucyclamides C and D also show single digit IC_{50} values against *P. falciparum* with very little toxicity to rat myoblasts. Interestingly, aerucyclamide C (**7**) was the most active compound in this series against *Trypanosoma brucei rhodesiense* STIB 900, the parasite causing African sleeping sickness, with an IC_{50} value of 9.2 μM.[17a] These values were in line with earlier studies by Gerwick and coworkers on different cyclamides, the venturamides.[21]

While the above examples highlight the potential of cyanobacteria for the discovery of potential pharmaceutical agents, many strains of cyanobacteria contain vicious

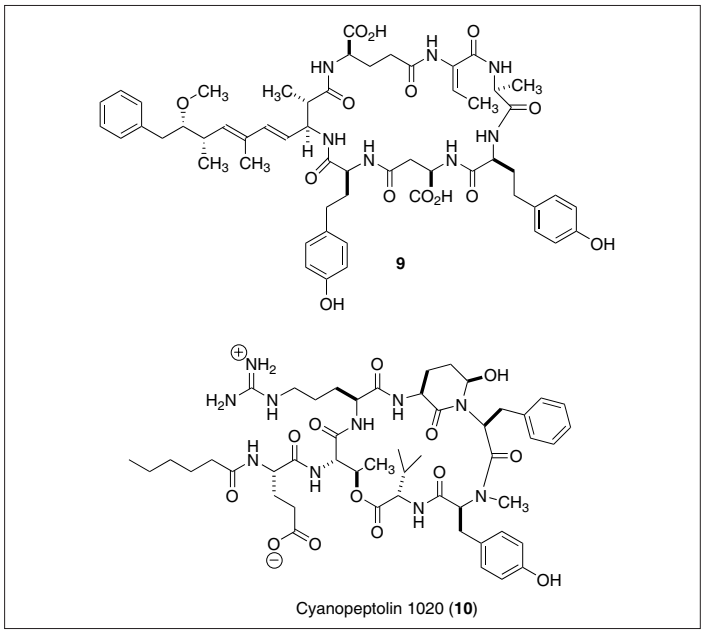

Fig. 3.

toxins that have caused many incidents of poisoning to livestock and even humans.[3a] Therefore, research on cyanobacterial toxins with regard to water quality and water supply still constitutes an important line of research, and in particular as recently more outbreaks of toxin-producing cyanobacteria have been observed. The major class of toxins, which have been heavily studied over the last decades, are the microcystins, prominent hapatotoxins from cyanobacteria.[3a,3f] In a collaboration with scientists from Austria, Uganda, the United States, Switzerland and Japan, we have been active in the determination of new microcystin variants from both European lakes and freshwater bodies of Uganda. For example, we have characterized a didehydrobutyrate microcystin **9** (Fig. 3) containing two homo tyrosine units,[22] a rare amino acid of which the biosynthesis has not been fully elucidated. Other new microcystin variants have been characterized by MS experiments from Uganda freshwater bodies,[23] which are distinctly different from microcystins observed in European or American lakes.

Since large efforts of the research community were dedicated to understanding microcystin toxicity in *Microcystis*, the perception arose in some studies that microcystin is the only toxin present in *Microcystis*. This invalid conclusion is of potential harm, as microcystin detection kits are a major assay for cyanobacterial toxicity. We have demonstrated that the structurally unrelated cyanopeptolin 1020 (**10**) is also a toxin found in *Microcystis* UV-006.[24] This peptide is a member of the cyanopeptolin class of natural products, featuring a hydroxypiperidone residue as a key structural motif. We have determined very potent inhibitory activity of this peptide against a series of serine proteases, and IC_{50} values as low as 670 pM against trypsin have been found.[24] In addition, a range of other serine proteases such as factor XIa (3.9 nM) and kallikrein (4.5 nM) are strongly inhibited by depsipeptide **10**, thus rendering cyanopeptolin 1020 (**10**) a 'dirty' protease inhibitor. We have also determined a LC_{50} value against *Thamnocephalus platyurus* of 8.8 μM, which is in the same range as some of the well-known microcystins.[24] These values thus render the cyanopeptolins a potential second class of toxins in *Microcystis*, which might hold true for other species as well.[25]

Natural products can also provide structural inspiration for the identification of molecular strategies unrelated to the presumed biological purpose of the parent compound. For example, we have analyzed a cyanobacterial siderophore and delineated key features for the generation of a molecular platform for surface modification. The cyanobacterial siderophore anachelin, isolated first by Budzikiewicz and coworkers[26] and independently by Itou *et al.*,[27] from the cyanobacterium *Anabaena cylindrica* displays an interesting structure merging different structural subunits such as an alkaloid part, a peptide fragment and a polyketide. We have investigated the total synthesis and configuration of anachelin,[28] its biogenesis[29] and its mechanism of action as an iron chelator.[30] The latter property proved to be interesting, as earlier studies both from surface chemistry and geochemistry suggested that siderophores are able to bind to surfaces.[31] In addition, the catecholate fragment of anachelin resembles structural units of mussel adhesive proteins.[32] Therefore, we set out to test whether the anachelin chromophore can be used for surface modification. In line with our hypothesis, the anachelin chromophore was an efficient anchor to functionalize metal oxide surfaces,[33] and several applications such as the generation of protein resistant surfaces[33,34] or antimicrobial surfaces[35] have been realized. Key to the efficient binding of the anachelin chromophore is likely to reside in the presence of the ammonium group, which modulates the pKa value of the hydroxy groups and protects the catechol from facile oxidation.[36] Second generation analogs have been developed[36,37] and in particular the nitrodopamine residue[36,38] is a readily available and efficient anchor for surface functionalization.[36] The nitrodopamine anchor has been utilized by us and others for a variety of different applications ranging from drug delivery to nanoparticle functionalization.[36,38,39]

The last example highlights an important role of modern natural products chemistry. Studying all the different aspects of an iron chelator natural product led to completely a different application as an anchor used in surface functionalization. It is therefore of great importance to investigate all the different aspects of a natural product, from its biological origin, its physical and chemical properties to its biological applications, in order to uncover the many secrets of these natural treasures.

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