Blue-green treasures

Pedro Leão

A cancer patient treated with a molecule found in algae-eating sea hares native to the Indian Ocean. Jet fuel produced by algae in open urban ponds. A tonne-scale synthesis of pharmaceuticals using enzymes from a green biofilm growing in your backyard. The first example is a reality, but the others are not necessarily confined to a utopian future. All these scenarios can be linked to blue-green algae (cyanobacteria). These talented microbial biochemists generate a vast set of unique secondary (specialized) metabolites. Initially infamous for being potent toxins that have resulted in human deaths, some cyanobacterial secondary metabolites have proven useful and are currently used in the clinic. The enzymes that biosynthesize some of these compounds are likewise remarkable and could find future industrial use. Here, I discuss some aspects of past and current secondary metabolite discovery in cyanobacteria, the potential impact of these small molecules for human activities and how the study of their biosynthesis has unearthed exciting new enzymatic reactions.

Small molecules of natural origin – the so-called natural products – have been used in human medicine for millennia. Without modern technologies through which the active components could be separated from the remaining mixture of compounds, medicinal preparations involved the use of specific parts/organs of plants or animals. Pharmaceutical companies eventually learned to extract, concentrate and separate such components, which gave rise to better treatments. Advances in microbiology and fermentation enabled the exploration of varied bacteria and fungi. Arguably the most impact that natural products had in modern medicine relates to antibiotics. In their majority, the first antibiotics were isolated from fungi and bacteria. These organisms produce antibiotics to wage a chemical war against competitors: the resulting arms race has shaped the structures and refined the potency of their small-molecule arsenal.

Natural product antibiotics are secondary (or specialized) metabolites. Because they have a specialized function, secondary metabolites are typically only shared by organisms that require their functionality and can pay the energetic/ecological cost of their production. This means that even closely related organisms can have a different set of diverse secondary metabolites and that drug discovery advances by carefully and systematically prospecting natural sources. With the success of natural product antibiotics in reducing infectious disease burden, academic and pharmaceutical laboratories launched a global hunt for new natural products, with sampling expeditions in terrestrial environments, as remote as they might have been. Thousands of small molecules were isolated and characterized from organisms collected from desert soils to rainforests. Eventually, the low-hanging fruit had all been picked up and high rediscovery (i.e., the compound being isolated turning out to have been reported previously) rates became commonplace. In a few years, most major pharmaceutical companies dismantled their natural products discovery units, a result of the expensive cost of discovering new natural products and the promise of emerging technologies such as combinatorial chemistry. At the same time, around the 1970s–1980s, some scientists were looking into the oceans as the next biodiversity frontier that was yet to be explored chemically.

Marine natural products

Unsurprising to us now, the marine environment with its exuberant biological diversity proved to be an excellent source of new natural products. Most major classes of compounds known from terrestrial environments, be it alkaloids, terpenes, peptides, phenols, polyketides or glycosides, could also be found in the oceans. But most secondary metabolites isolated from marine organisms were found to contain exclusive structural signatures. This unique chemistry emerged initially from the organisms that were easily harvested in high amounts, either by sampling them in the low tide or by using snorkelling or SCUBA. Macroalgae and marine invertebrates were thoroughly explored initially, with

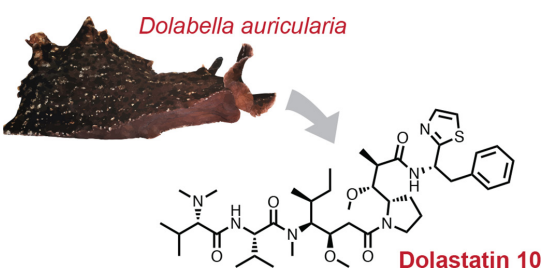

Figure 1. Dolastatin 10 is a potent cytotoxic cyanobacterial secondary metabolite used to treat cancer. It was isolated from a sea hare that feeds on the dolastatin-producing cyanobacteria.

great success. A notable example is that of the sea hare *Dolabella auricularia* which inhabits the Indian Ocean and is well known to people in this region for its bioactive properties. From it, a remarkable compound was isolated: dolastatin 10, a small, modified peptide that was extremely toxic to several types of human cell lines (Figure 1). The potency was so high that it would take less than half a teaspoon of dolastatin 10 diluted in the volume of four Olympic swimming pools to kill the tested cells. Slightly modified versions of this compound are used in four different antibody–drug conjugates currently in clinical use to treat cancer.

Cyanobacterial natural products

As less and less marine algae and invertebrates remained to be explored, natural products researchers turned to microorganisms. Investigations of marine bacteria and fungi, from shallow to deep ocean waters, have brought to light hundreds of new secondary metabolites. Soon, it became evident that the natural products initially found in marine invertebrates were either the same as or very similar to those being isolated from marine bacteria. These invertebrates either enter symbiotic relationships with or feed upon marine bacteria, in any case using the bacterial secondary metabolites for their own defence. That is the case of *Dolabella auricularia*, which feeds on cyanobacteria, the true producers of dolastatin 10.

Blue-green algae, or cyanobacteria, are Gram-negative bacteria responsible for oxygenating the atmosphere of our planet about 3.0 gigayears ago. By performing oxygenic photosynthesis, they have carved out their own ecological niche and have since proliferated and diversified. Today, we find them in virtually all environments on Earth where there is light – marine and freshwaters, soils, deserts or subaerial environments. They can also engage in symbioses with plants, animals and fungi. As primary producers with nitrogen-fixation

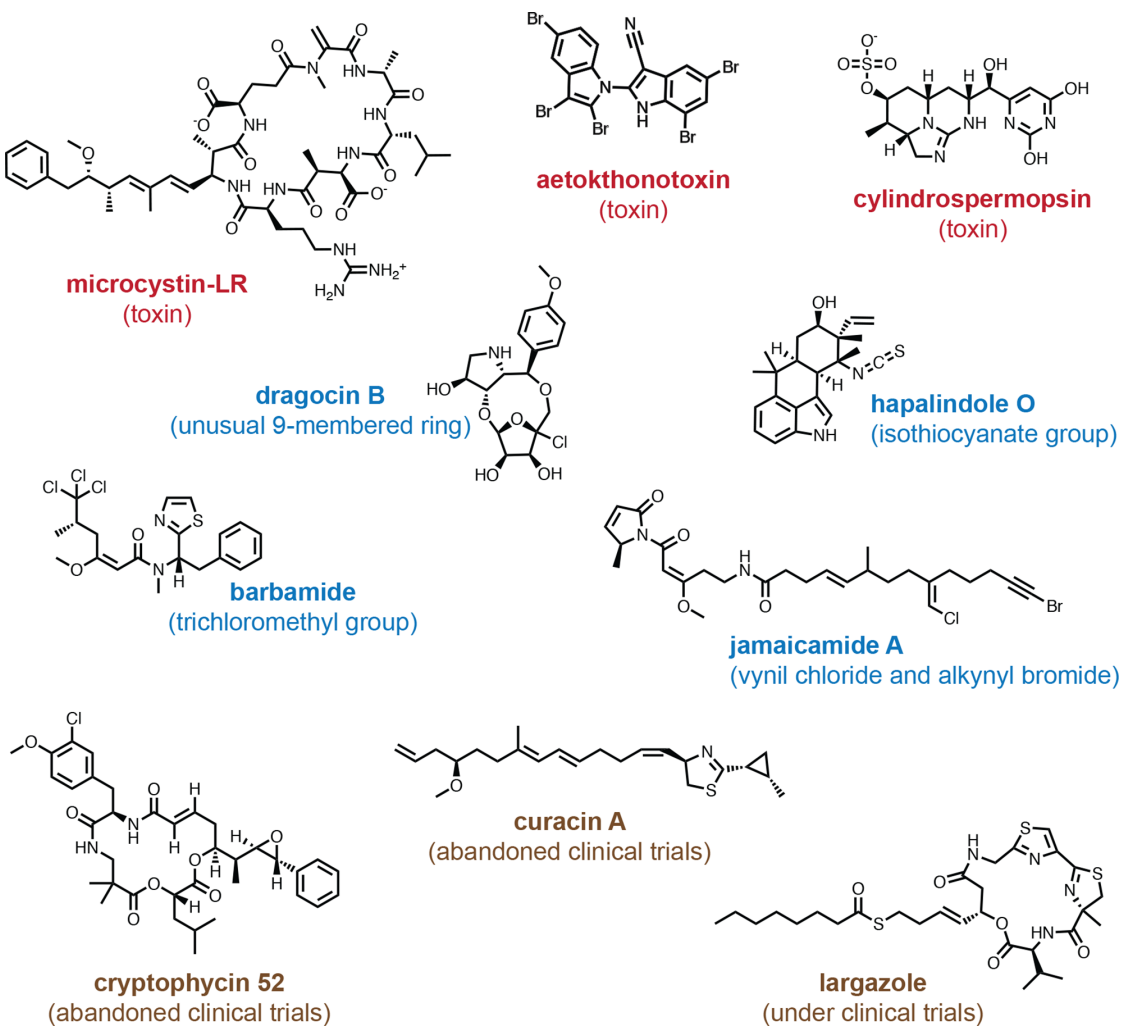

Figure 2. Structures of selected cyanobacterial secondary metabolites

capabilities, cyanobacteria continue to play important roles in major biogeochemical cycles.

A few bacterial groups are extremely talented in terms of the chemistry they can generate. Actinobacteria are outstanding chemical factories, and many of their secondary metabolites have become important drugs. Cyanobacteria are also very rich in secondary metabolites, but their exploration has not been as expeditious. One possible explanation is that cyanobacteria are slow-growing, difficult to isolate and grow axenically (i.e., in pure culture) and also difficult to cultivate in large scales. Still, a lot has been learned about their exquisite secondary metabolites (Figure 2). For one, we know the intricate structures of several toxins – such as microcystins, cylindrospermopsins or aetokthonotoxin – which have led to several poisoning events in animals, including humans. We have also learned about the unusual and curious structural motifs present in marine cyanobacterial secondary metabolites – such as jamaicamide, barbamide or dragocin, or in the terrestrial hapalindole-type compounds (Figure 2). And, aside from dolastatin 10 and its derivatives, some other cyanobacterial metabolites such as the cryptophycins, curacin and largazole have reached clinical trials (Figure 2). Of these, only largazole is still under development.

Next-generation natural products discovery

All these and many other compounds were discovered based on their biological activity, in a process usually termed bioassay-guided isolation that involves following a certain bioactivity through successive rounds of fractionation of an initially complex mixture, until a pure compound is obtained. The purified molecules can then be the subject of structural characterization and biological activity profiling.

Despite bioassay-guided isolation providing a direct route to bioactive compounds, natural products discovery is slowly shifting towards other strategies. The main reason behind it is linked to the emergence of next-generation sequencing technologies which in turned led to a wealth of bacterial genome sequence data. In these data, it is possible to bioinformatically circumscribe

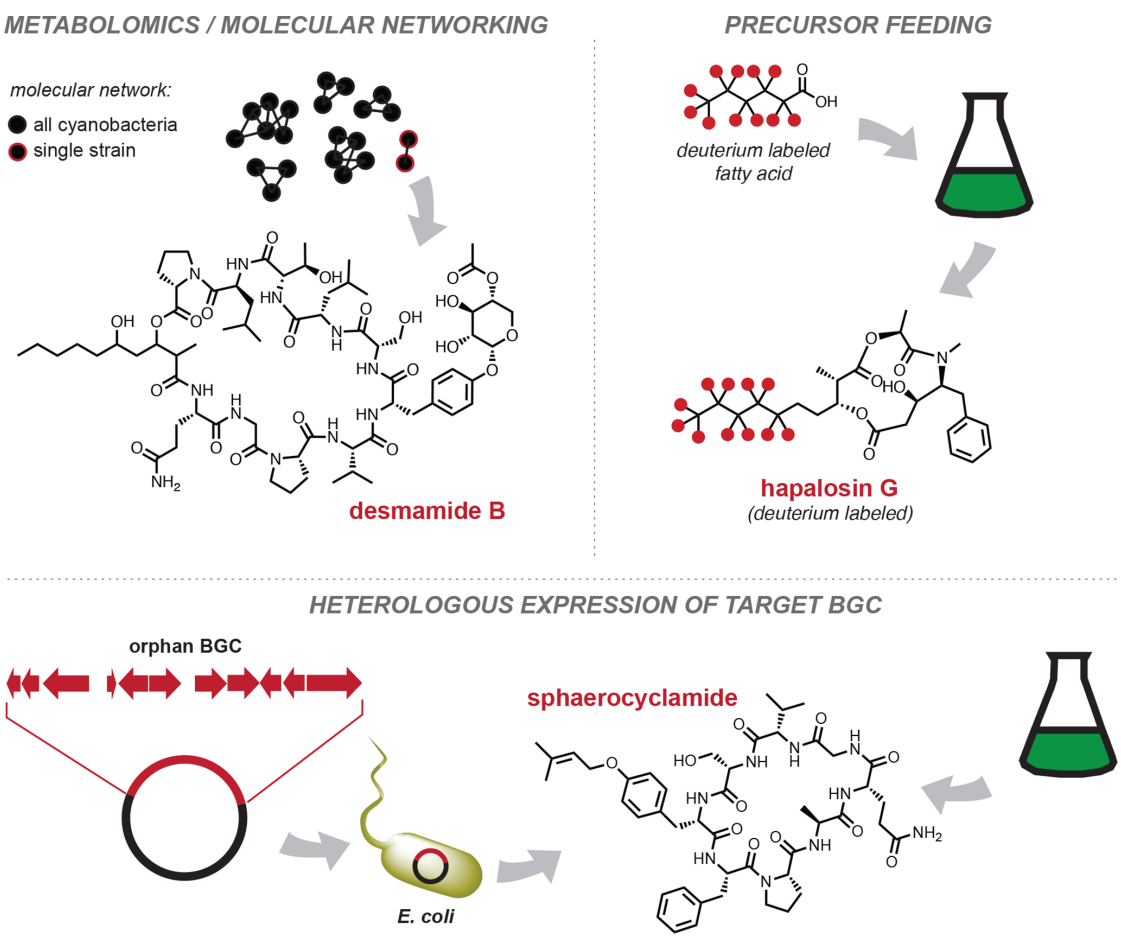

Figure 3. Examples of contemporary strategies to uncover new secondary metabolites from cyanobacteria

biosynthetic gene clusters (BGCs), regions of the genome containing most of the genetic information necessary for the transport and production (and its regulation) of secondary metabolites. One can assume that a single BGC corresponds to one or more (related) secondary metabolites. The analysis of thousands of bacterial genomes, including those extracted from metagenomic datasets and corresponding to uncultured bacteria, made it very clear: we currently have seen just the tip of the iceberg of all the genetically encoded small molecules in nature. Cyanobacteria are no exception. For that reason, many natural products research groups, including ours, have felt reinvigorated in the search for new bacterial chemistry.

A number of strategies have been developed in recent years to allow for the discovery of secondary metabolites from bacteria that have a high number of BGCs or even specifically from specific target BGCs. For example, matching the metabolomes of a large number of (cyano)bacteria can reveal compounds that are unique to a specific strain. We used this strategy to reveal new lipoglycopeptides, a rare class of natural products, from the plant symbiotic cyanobacterium *Desmonostoc muscorum* (Figure 3). In this work, we made use of several metabolomics tools developed for the natural products community by scientists at the University of California, San Diego. We have also recently developed a specific methodology for discovery of new natural products from cyanobacteria. We supply to cyanobacterial cultures exogenous fatty acids that have had all hydrogen atoms replaced by the heavier atom deuterium. These 'labelled' fatty acids eventually find their way into secondary metabolites, which are now heavier on account of deuterium incorporation and can be easily detected using mass spectrometry. We used this strategy to uncover a series of new bioactive compounds – the nocuolactylates and several hapalosin analogues (Figure 3). Perhaps the most promising strategy to reveal the products of target BGCs is their heterologous expression in a suitable host. This methodology has the added advantage of not requiring the target BGCs to originate from cultured organisms. While cyanobacteria are difficult to manipulate genetically, there has been some success in expressing entire BGCs from cyanobacteria in *Escherichia coli*, as, e.g., in our discovery of sphaerocyclamide (Figure 3).

New enzymatic chemistry

Some natural products structures contain unusual scaffolds that are difficult to rationalize according to characterized biochemistry. In other words, one sometimes wonders which enzymatic events lead to the formation of certain structural elements. This intricate relationship between structure and enzymology is ever present in secondary metabolites, which have the most complex structures among natural small molecules. Unusual and intriguing natural products structures are often the starting point for projects that lead to the discovery of unprecedented enzymatic chemistry. The structures of many secondary metabolites are unique to cyanobacteria, and the study of their biosynthesis has revealed novel enzymatic functions. One example relates to alkanes, which are produced by a few organisms, including certain cyanobacteria. The structures of alkanes in cyanobacteria, namely the fact that these contained odd-numbered carbon chains, led to the discovery of two enzymes that transform fatty acids into alkanes (Figure 4). Such enzymes could lead to the bio-based production of hydrocarbon fuels. Another example is the discovery of WelO5, a halogenase that regio- and stereoselectively chlorinates a free-standing substrate at an unactivated carbon centre (Figure 4). This

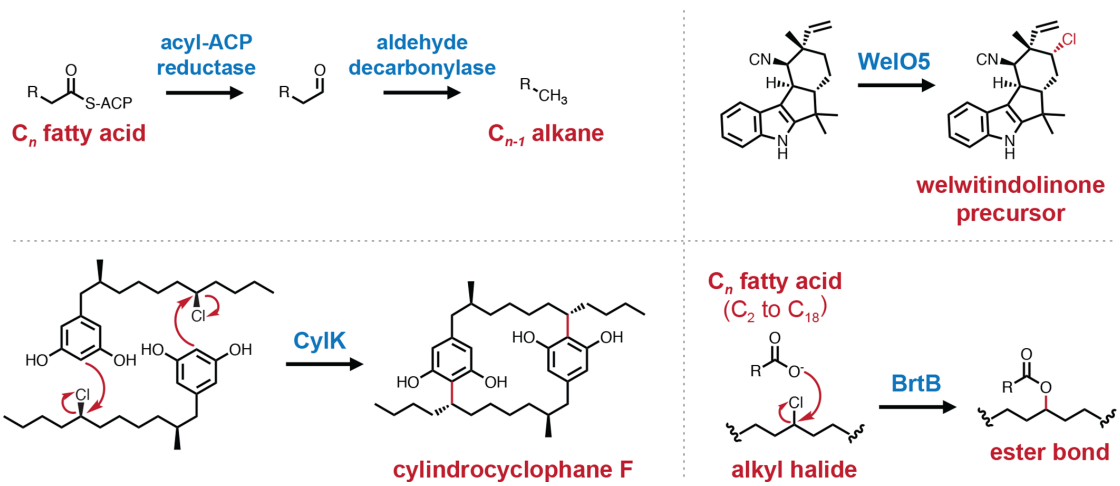

Figure 4. Examples of unusual biochemistry carried out by cyanobacterial enzymes

discovery was made by investigating uncharacterized enzymes in the welwitindolinone BGC. The activity of WelO5 has great potential for biocatalysis, namely for derivatization or late-stage functionalization reactions. The unusual structures of the cyanobacterial cytotoxic cylindrocyclophanes (Figure 4) raised the question of how the carbon–carbon bonds between the aromatic ring and alkyl moieties were generated biochemically. Dissection of the role of the cylindrocyclophane BGC led to the characterization of a striking set of biochemical events that eventually solved the puzzle: (i) a halogenase (CylC) first installs a chlorine atom to activate a C–H bond in the alkyl moiety and (ii) CylK catalyses a nucleophilic substitution reaction by attack of the aromatic ring carbon onto the alkyl halide, generating the C–C bond. This was the first example of a Friedel–Crafts alkylation (a classic synthetic chemistry reaction) in biological chemistry. Akin to this reaction, we characterized a homolog of CylK (BrtB), which catalyses the attack of fatty acid carboxylate onto the alkyl halide, and therefore a new type of biological esterification of fatty acids.

In most cases it is difficult, if not futile, to predict – especially at the time of discovery – which application will secondary metabolites and their biosynthetic enzymes have in a more or less distant future. This is immediately realized when considering that just recently, the anticancer marine natural product plitidepsin was repurposed to fight COVID-19 with extreme potency, or that enzymes are currently used to produce pharmaceuticals (something that would sound unrealistic a few decades ago). But these examples also underline how important it is to keep discovering, characterizing and cataloguing the immense (bio) chemical diversity associated with secondary metabolites. ■

Further reading

- A review on the potential of combining genomics and metabolomics to accelerate secondary metabolite discovery. Hooft, Justin J. J. van der, Hosein Mohimani, Anelize Bauermeister, Pieter C. Dorrestein, Katherine R. Duncan, and Marnix H. Medema. "Linking Genomics and Metabolomics to Chart Specialized Metabolic Diversity." *Chemical Society Reviews* 49, no. 11 (2020): 3297–3314. https://doi.org/10.1039/D0CS00162G
- A review on the unique enzymes from cyanobacterial secondary metabolite biosynthesis. Kleigrewe, Karin, Lena Gerwick, David H. Sherman, and William H. Gerwick. "Unique Marine Derived Cyanobacterial Biosynthetic Genes for Chemical Diversity." *Natural Product Reports* 33, no. 2 (February 4, 2016): 348–64. https://doi.org/10.1039/C5NP00097A.
- A review on the discovery of new enzymology associated with natural products biosynthesis. Scott, Thomas A., and Jörn Piel. "*The Hidden Enzymology of Bacterial Natural Product Biosynthesis*." Nature Reviews Chemistry 3, no. 7 (July 2019): 404–25. https://doi.org/10.1038/s41570-019-0107-1.
- Our study detailing a method for the discovery of new secondary metabolites in cyanobacteria: Figueiredo, Sandra A. C., Marco Preto, Gabriela Moreira, Teresa P. Martins, Kathleen Abt, André Melo, Vitor M. Vasconcelos, and Pedro N. Leão. "Discovery of Cyanobacterial Natural Products Containing Fatty Acid Residues." *Angewandte Chemie International Edition* 60, no. 18 (April 26, 2021): 10064–72. https://doi.org/10.1002/anie.202015105.

Pedro Leão obtained his PhD (2010) from the University of Porto, studying chemically mediated ecological interactions involving cyanobacteria. He carried out postdoctoral studies at CIIMAR (a marine research centre of the University of Porto), with stays at Scripps Institution of Oceanography and Harvard University, where he worked on marine natural products chemistry and biosynthesis. In 2017, he was the recipient of a European Research Council Starting Grant and started his own group at CIIMAR. In 2021, he was appointed as ERA Chair Holder at the same institution. His laboratory focuses on the discovery of natural products and enzymatic chemistry from cyanobacteria. Twitter: @pnleao. E-mail: pleao@ciimar.up.pt

