The benefits of living together – studying marine symbioses to discover enzymes for biotechnology applications

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Over the past 50 years, more than 15 pharmaceuticals derived from marine organisms have come to the market. Most of these come from filter-feeding invertebrates that contain a high proportion of microbial symbionts. Microbiology and molecular genetic studies have shown that many of these drug-like compounds are produced by the microbial symbiont. The enzymes that produce these compounds are promiscuous meaning they can process a diverse range of related substrates, making them extremely attractive to the biotechnology industry. Determining the structure of these enzymes makes them amenable to engineering, allowing them to process non-natural substrates. Using this approach, synthetic substrates can be treated with a cocktail of enzymes to prepare focused libraries of compounds to hit drug targets such as protein–protein interactions. These targets are involved in a range of diseases from cancer to immune disorders and are hard to modulate using small molecule drugs. Complex modified cyclic peptides produced using a chemoenzymatic process may be a promising approach to address these disease conditions.

Drugs from the deep

I still remember my first collection of my favourite seasquirt, its smooth waxy exterior, with dimples showing the green symbiont living within. It was November 1996, off the coast of Manado, Indonesia, and I had just collected the marine ascidian (seasquirt) *Lissoclinum patella* containing the symbiotic cyanobacterium (blue-green alga) *Prochloron didemnidae* from about 10 m deep off a coral reef (Figure 1). I was interested in seasquirts as they had been shown to be an important source of compounds with the potential to treat human disease. One example is the cancer chemotherapy Yondelis®, derived from the Caribbean ascidian *Ecteinascidia turbinata* and developed by the Spanish company PharmaMar. Yondelis® was made by a semi-synthetic process and was approved for the treatment of soft tissue sarcoma in 2007. This was one of the first approved 'drugs from the sea', a quest for new chemotherapies from marine invertebrates that started in earnest in the late 1960s and has since led to more than 15 approved pharmaceuticals. Marine invertebrates have no obvious defences and no immune system but produce

Figure 1. The author collecting marine invertebrates using SCUBA on the Great Barrier Reef (top). The ubiquitous Indo-Pacific ascidian (seasquirt) *Lissoclinum patella* (bottom).

defensive compounds which are proposed to act as an alternative. These compounds are often structurally unique, and many have potent and selective activity against a range of human diseases.

Living together – who does what?

Many of these invertebrates are filter feeders and contain a high proportion of commensal microorganisms. In the early 1990s, scientists started to ask who was producing the compounds of interest, the invertebrate host or the microbial symbionts. It was noticed that compounds that appeared in beetles were similar to compounds found in sponges, suggesting that a related microorganism was responsible for the production of the compound in both organisms. Attempts were made to answer the question by separating host and symbiont cells and analysing them separately, but this gave inconclusive results. An alternative was to try and cultivate the symbiotic microbes, but this proved challenging as many only seemed to grow in the presence of the host. There was obviously a benefit to living together, but it was hard to determine exactly what the benefits were to the symbiont and to the host. It was long suspected that *P. didemnidae* produced sunscreening pigments called mycosporine-like amino acids that were exported to be taken up by the translucent host. Their uptake by the host had the benefit of screening harmful UV radiation from sunlight for the benefit of the photosynthetic *Prochloron*. It was only with the advent of genome sequencing that the questions of who produced the compounds and how they were produced could be answered with any certainty.

Several approaches were used to identify whether the true producers of these fascinating compounds were the marine invertebrate or symbiont. Given that similar compounds were found in several invertebrates, and also in some isolated microorganisms, the search naturally focused first on the microorganism symbiont. The *Lissoclinum/Prochloron* system proved to be a useful model system as it consistently produced modified cyclic peptides called patellamides (Figure 2). In addition, it was easy to obtain the nearly pure *Prochloron* cells by squeezing the host, and DNA could readily be extracted from these using simple procedures. It was not clear what type of biosynthetic pathway produced the peptidic patellamides – a ribosomal or non-ribosomal one – so two approaches were followed to try and identify the biosynthetic gene clusters. The first was to try a 'shotgun' approach – fragments of DNA were cloned into suitable hosts, and libraries of strains containing different *Prochloron* DNA fragments were grown and screened using mass spectrometry to identify clones producing the compounds. This yielded clones that surprisingly produced two compounds in roughly equal quantities. The alternative approach, whole genome sequencing of *Prochloron* DNA, identified the patellamides were unexpectedly produced via a ribosomal pathway now termed 'ribosomally produced

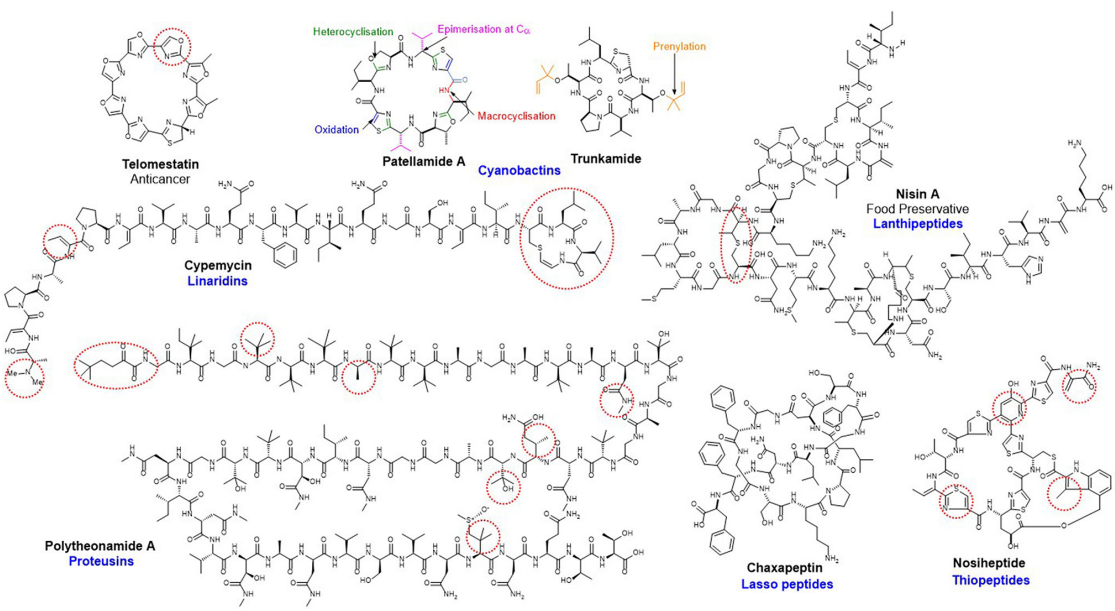

Figure 2. A variety of ribosomally produced and post-translationally modified peptides (RiPPs) showing several different families (blue text indicates RiPP family name). The specific post-translational modifications occurring in the cyanobactins are indicated and some modifications present in other RiPPs circled in red.

and post-translationally modified (PTM) peptides' or RiPPs for short, and that indeed it naturally produced two related compounds as observed in the shotgun work. Work on the sponge *Theonella swinhoei* isolated single bacterial cells, amplified and then sequenced their genomes to show that some of these bacteria (the newly identified *Tectomicrobia* phylum) produced a vast range of structurally unique metabolites. Amongst these were the proteusins, a family of gargantuan RiPP molecules that showed an enormous range and number of post-translational modifications (Figure 2).

Ripping yarns

The RiPP pathway briefly explained in Figures 2 and 3 shows the range of metabolites with a variety of post-translational modifications possible in RiPPs. This huge variety of possible modifications makes it clear why these enzymes are attractive for biotechnological applications. They carry out a great range of complex chemistry without the need for multistep organic synthesis and many of the enzymes are promiscuous, meaning they will accept a range of possible substrates. Some of the enzymes can be engineered to allow them to act on shorter peptide sequences containing non-natural amino acids, without the need for the long leader sequence shown in Figure 3. These enzymes also change the properties of the linear precursor peptides by the formation of large macrocycles, making the cyclic peptide less susceptible to proteolysis, less polar so they are more likely to traverse cell membranes and often having 3D structure that enables them to interact with target binding sites. Other modifications such as heterocycles add conformational constraints and prenylation makes the modified cyclic peptides more lipophilic.

Drugging the undruggable

Cyclic peptides that don't obey standard drug design rules ('Beyond Rule of 5') are now in high demand by the pharmaceutical industry as they can modulate protein–protein interactions (PPIs), large diffuse interactions between proteins involved in a multitude of diseases ranging from immune disorders to cancer. Such compounds can either act as agonists where they behave like one of the binding partners and give rise to a downstream biological effect, or antagonists where they block the protein–protein interaction, thus blocking the biological cascade downstream. There is an urgent need for ways to design modified cyclic peptides against targets, but this is proving to be very challenging, and to then synthesize them in large numbers for testing in disease-focused screens.

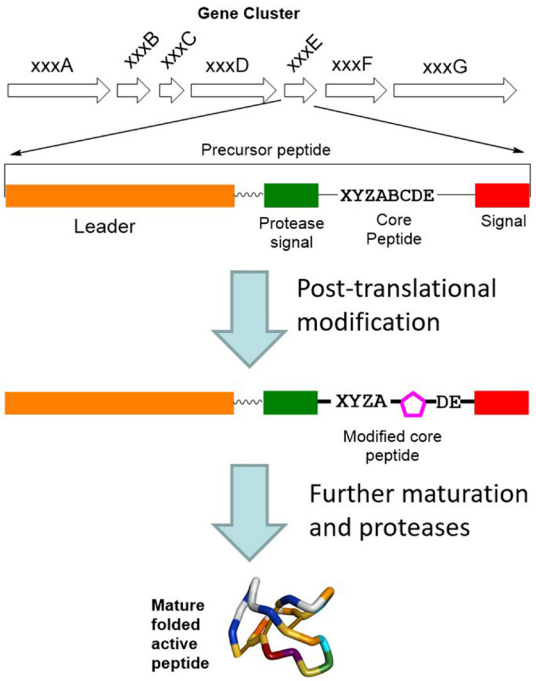

Figure 3. A general biosynthetic pathway for ribosomally produced and post-translationally modified peptides (RiPPs). The precursor peptide contains a leader sequence of 30–40 amino acid residues that is recognized by the post-translationally modifying (PTM) enzymes. Protease/signal sequences flank the core peptide which is modified by the PTM enzymes. Several of these PTM enzymes act on the core peptide creating functionalities such as heterocyclic rings. The 'matured' peptide is subsequently cleaved from the precursor peptide and in some cases a macrocycle is formed, and there is the possibility of further PTMs such as oxidation or prenylation.

Using native and engineered RiPP PTM enzymes makes it possible to rapidly make focused libraries of complex modified peptides as shown in Figure 4. This 'chemoenzymatic' approach uses the best of what synthesis and biosynthesis have to offer, cuts down the use of chemical reagents and organic solvents and shortens the time needed to synthesize these complex cyclic peptides.

Who and how, but not why?

We now return to the question posed at the beginning – what are the benefits of living together in symbiosis? We can answer the 'who' and 'how', but not yet the 'why'. We now know beyond a shadow of a doubt, using genomic and genetic studies that these beautiful metabolites are made by the microbial symbiont. Many research groups that work on RiPPs have shown the elegant biosynthesis that leads to the formation of

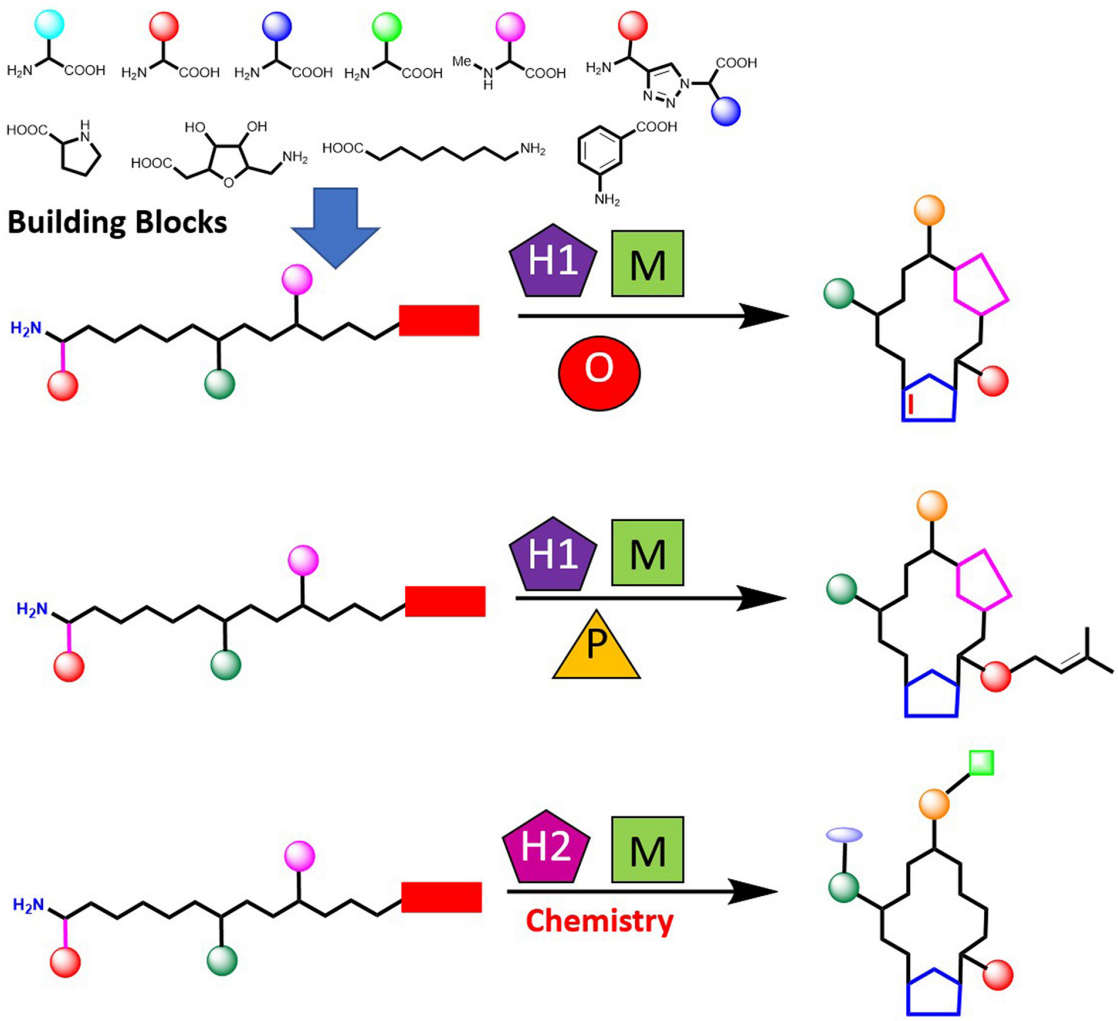

Figure 4. Parallel chemoenzymatic synthesis of complex modified cyclic peptides. Natural and non-natural building blocks are connected using synthetic steps and substrates are subjected to different cocktails of enzymes, or enzymes followed by late-stage chemical modification. This approach makes it possible to create 'focused' libraries of compounds from a single linear peptide substrate. The red 'tag' indicates a minimal peptide sequence that is necessary for the PTM enzyme(s) to recognize the linear peptide. H = heterocyclase; M = macrocyclase; P = prenylase; O = oxidase – see cyanobactin family in Figure 2.

these compounds and how the enzymes involved work at a molecular level. What was not anticipated was how our knowledge of the biosynthesis of these compounds would lead to the possibility of engineering the enzymes and enable them to speed up the synthesis of modified cyclic peptides with the potential to be used in the pharmaceutical industry to try and target diseases with unmet medical need. The questions of why these compounds are being produced and what benefits they confer to the symbiont or host will need further work involving invertebrate and microbial ecologists, experts in the genomics, molecular genetics and biosynthesis of these specialized metabolites and biochemists. ■

Further Reading

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Marcel Jaspars' main expertise is in the discovery, characterization, utilization and biosynthesis of marine natural products. This forms the core of the marine biodiscovery pipeline, and Marcel has frequent contact with people operating at all stages of this pipeline, from the collection and identification of the organisms to their testing in whole animal models. Some of his work on biosynthesis has been spun out as a startup company to discover medicines for complex diseases (www.gyreox.com). Marcel has been active at national and international levels to develop the science, its applications/industrial uptake and associated policy involved in marine biodiscovery and biotechnology.