Biotechnology and Biochemistry of Marine Natural Products

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Marine ecosystems are a source of biologically active compounds, many of which are currently in clinical use. With the goal of increasing the availability and the chemical diversity of these important compounds, more researchers are applying the tools of biotechnology to the discovery and production of marine natural products. This review summarizes the recent efforts made towards the characterization of the biochemical pathways that result in the production of marine natural products, with an emphasis on the work aimed at understanding the enzymatic activity involved in the biosynthesis of marine natural products.

Key words: Marine biotechnology, Natural products, Polyketides, Diterpenes, Polyunsaturated fatty acids

Natural products isolated from marine samples have a wide spectrum of biological activities and numerous therapeutic applications. Many compounds with antiviral, antibiotic and antitumor activity have been isolated from marine samples and tested in cell culture for their ability to specifically target and kill pathogenic or cancerous cells by disrupting diverse cellular processes (1-2). A number of these compounds, or synthetic analogues based on natural compounds, have entered clinical trials and some are currently administered as therapeutics (3).

Despite the great promise and the wide spectrum of biological activities of marine natural products, their subsequent development as therapeutic products has been slowed down due to several factors including the low availability of biologically active compounds (4), their high chemical complexity (5) and, in some cases, their high toxicity at therapeutic doses. Some compounds are produced by organisms which comprise only a minority component within the diverse population in the sample. In fact, many promising natural products associated with marine sponges and corals have been found not to be made by the invertebrate itself, but rather by symbiotic bacteria which are difficult to isolate, characterize and cultivate.

Several commercial ventures have been created precisely to apply the ethos of biotechnology to the development of marine drugs by devising ways to overcome some of the aforementioned difficulties in order to bring some of the more promising compounds closer to the clinic and to the market (6). Companies such as PharmaMar (Madrid, Spain) and Nereus Pharmaceuticals (San Diego, USA) have developed strategies for identifying cultivable organisms, optimizing growth, maximizing compound production, and increasing chemical diversity through synthetic modification (7) (Figure 1). This approach has yielded the first generation of biotechnology-derived marine natural products. PharmaMar currently makes and sells the natural anticancer compound trabectedin (Yondelis ®), which is produced by a semi-synthetic method in which a natural fermentation product from *Pseudomonas fluorescens* is chemically modified to form the final product (8). Another biotech company developing marine drugs is Nereus Pharmaceuticals, which is currently conducting Phase I clinical trials for proteasome inhibitor, salinosporamide A, for the treatment of solid tumors, lymphomas and multiple myeloma (9). This active compound is isolated directly from the fermentation of marine actinomycete, *Salinispora tropica*, a species first identified and characterized at the Scripps Institution of Oceanography by Paul Jensen and William Fenical (10).

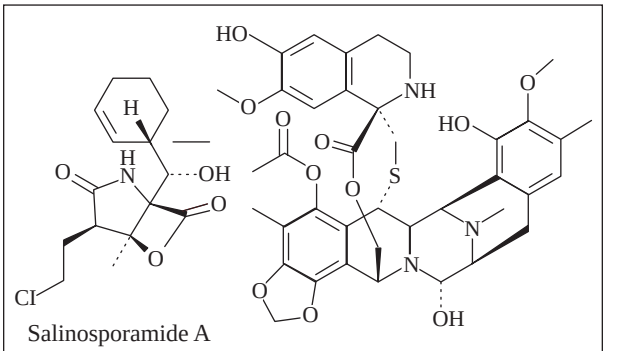

Figure 1. Salinosporamide A and Trabectedin are two compounds derived from marine biotechnology. Salinosporamide is currently in Phase I clinical trials, while Trabectedin has received orphan drug status for the treatment of soft tissue sarcoma.

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The successful application of biotechnology to the development of marine-derived pharmaceuticals has been met with an increased drive towards elucidating the enzyme pathways that lead to the biosynthesis of such complex and biologically active molecules. These recent efforts involve the cloning and characterization of multienzyme complexes, such as polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS), terpene cyclases and halogenases (11-12). Additional efforts involve the mechanistic and structural characterization of some of these biosynthetic enzymes as well as their expression and activity in heterologous hosts. This review attempts to provide a general view of the recent work aimed at understanding the enzyme pathways involved in the biosynthesis of marine natural products.

Marine actinobacteria and salinosporamide A

Actinobacteria are a group of gram-positive, GC-rich bacteria which are well-known producers of biologically active compounds. Many antibiotics, immunosuppressants and anticancer drugs are made from the cultivation of actinobacteria, many of them originally identified in tropical soils. The antibiotics erythromycin and rifamycin are still produced commercially from the fermentation cultures of soil actinobacteria.

Actinobacteria have also been found in marine sediments (10). Both *Salinispora arenicola* and *Salinispora tropica* are marine actinobacteria which were initially identified in sea sediments from the Bahamas. They both require seawater for cultivation and produce a number of natural products. In fact, analysis of the genome of *S tropica* reveals that approximately 9.9% of the DNA sequence is dedicated to the assembly of secondary metabolites (13). One of these secondary metabolites is Salinosporamide A, a potent inhibitor of the proteasome which is currently in Phase I clinical trials for the treatment of solid tumors, lymphomas and multiple myeloma.

The biosynthetic route to Salinosporamide A was proposed on the basis of feeding experiments in which *S tropica* in cell culture was fed labeled precursors, in this case acetate and shikimate (14). From the labeling pattern of the isolated final product the authors deduced the enzyme-catalyzed reactions that lead to its biosynthesis from three building blocks (Figure 2). It was proposed that a polyketide synthase (PKS) catalyzes the condensation of a chlorobutanoyl intermediate with an acetate unit, and subsequently with the clyclohexenyl-alanine amino acid arising from the shikimate pathway which is incorporated into the product by the activity of a non-ribosomal peptide synthetase (NRPS).

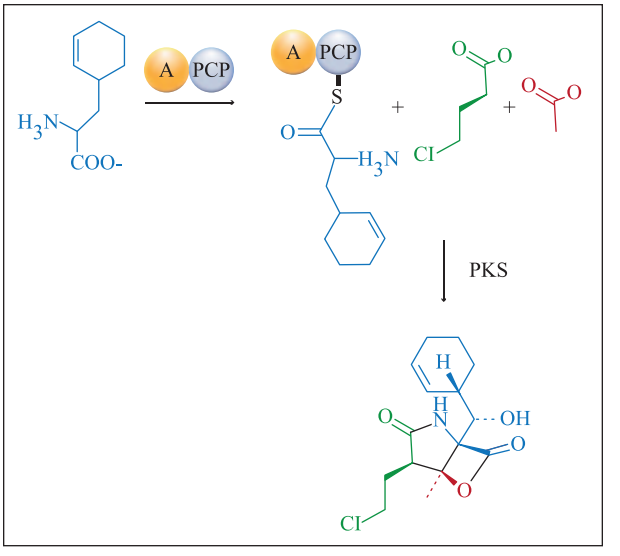

Figure 2. Salinosporamide A is made from three building blocks (14): an amino acid containing a cyclohexene side chain (blue) from the shikimate pathway, a chlorobutyl from S-adenosyl-methionine (11) (green) which is condensed with an acetate unit (red) by a polyketide synthase.

Based on this proposed biosynthetic pathway, a number of analogues of salinosporamide A have been generated using the tools of biotechnology (Figure 3). The inactivation of the Sal X gene, which is required for the biosynthesis of the amino acid,cyclohexenyl-alanine, resulted in the production of antiprotealide, a novel biologically active compound in which the amino acid leucine occupies the place of the original cyclohexenyl amino acid (15). Additionally, chemical complementation experiments in which other non-proteinogenic amino acids were fed to the SalX$^-$ strain, resulted in the incorporation of the exogenous amino acids into the final product, thus demonstrating the capacity of a "mutasynthetic" approach for the discovery of new compounds not easily accessible by conventional chemical methods.

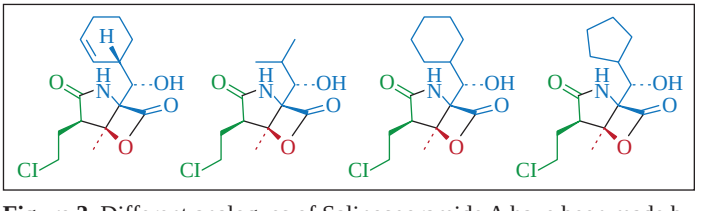

Figure 3. Different analogues of Salinosporamide A have been made by mutasynthesis (15). The approach consists of feeding different amino acids to a strain of *S tropica* that cannot produce the amino acid that gives rise to the cyclohexenyl moiety (blue). Leucine, cyclohexyl alanine and cyclopentylalanine have been incorporated into the final product in the absence of the preferred amino acid.

Enterocin and the reconstitution of biosynthetic pathways

The marine actinobacteria *Streptomyces maritimus* produces a diverse family of antibacterial compounds known as the enterocins and wailupemycins. These compounds are made by a Type II polyketide synthase (PKS) which catalyzes successive condesations of a benzoyl-CoA starter unit with seven malonyl-CoA building blocks by the proteins encA, encB, encC and encD (16). In an attempt to generate novel polyketides, the biosynthetic genes for the benzoyl-CoA starter unit in *S maritimus* were introduced into a streptomyces strain that produces truncated versions of erythromycin. The resulting Streptomyces strain produced novel PKS-derived triketides, containing the benzoate starter unit prescribed by the *S maritimus* genes (17). This result not only confirmed the tolerance of terrestrial Type I PKS toward alternative starter units, but also highlighted the enormous potential of using genes from a marine organism to modify known polyketides.

More recently, the entire biosynthetic pathway for enterocin was reconstituted in vitro from recombinantly expressed enzymes (18). While most enzymes were expressed in *Escherichia coli*, some enzymes were insoluble or inactive when expressed in that host. In those cases, the enzymes were overexpressed in well characterized hosts for the expression of streptomycete proteins, Streptomyces lividans or Streptomyces coelicolor. Efforts to reprogram the biosynthesis of enterocins and wailupemycins include the mutasynthesis of analogues of enterocin with non-natural starter units in which the authors fed aryl acids to a strain of *S maritimus* that was incapable of producing benzoate-primed polyketides. The result was a number of enterocin analogues with a different aryl starter unit (19). This general strategy has been taken a step further with the in vitro reconstituted enterocin enzyme complex (20). In this paper, Kalaitzis, et al. directly prime the corresponding biosynthetic enzyme with an alternative priming unit, other than the natural benzoate. The result is a number of novel enterocin and wailupemycin analogues obtained by means of recombinant technology.

Curacin biosynthesis in cyanobacteria

Cyanobacteria are another source of biologically active marine natural products many of which result from the activity of hybrid PKS:NRPS enzyme complexes (21). This is the case for hybrid molecules barbamide and curacin, both of which result from a biosynthetic route that includes both PKS domains and NRPS domains (22-23). The biosynthesis of barbamide, a molluscicidal compound, involves the condensation of three amino acids (trichloroleucine, phenylalanine and cysteine) with a malonate unit (Figure 4). The ability of the barbamide enzyme complex to specifically incorporate those amino acids has been confirmed in vitro using purified adenylation domains from the barbamide gene cluster over-expressed in *E coli* (22).

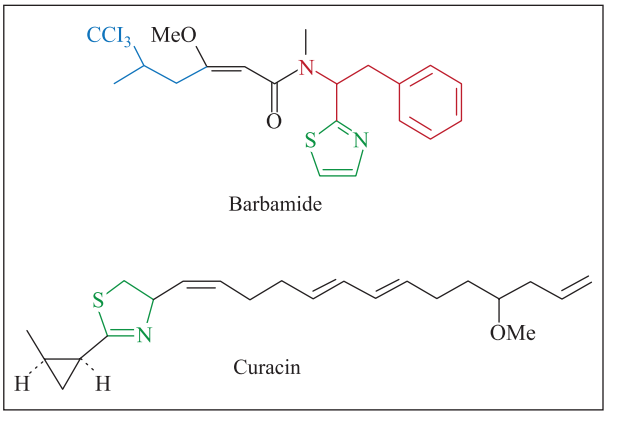

Figure 4. Barbamide and curacin are made by the activity of hybrid PKS-NRPS enzymes. The precursor for the barbamide starter unit is trichloroleucine (blue), followed by malonate condensation and the sequential incorporation of Phenylalanine (red) and cysteine (green). The backbone of curacin is mostly of polyketide origin, except for the thiazole ring (green) which comes from cysteine.

The biosynthesis of curacin, a potent anticancer compound from the cyanobacterium *Lyngbya majuscula*, involves the incorporation of a rare cyclopropyl moiety into the polyketide chain. The initial steps of this enzymatic transformation have been explored using two purified enzymes from the curacin gene cluster, CurE and CurF (24). Both sequences were found to align with the enoyl-CoA hydratase (ECH) family of enzymes which typically catalyze the hydration of double bonded intermediates during the beta-oxidation of fatty acids. Assays using purified enzymes showed that CurE catalyzes the dehydration of HMG-ACP while CurF catalyzes the subsequent decarboxylation to form the 3-methyl intermediate that gives rise to the cyclopropyl moiety. This work opens the possibility of using this CurE-CurF enzyme pair to engineer priming units for other PKS systems as a way to introduce cyclopropanes into known polyketide compounds.

Bryostatin in a symbiont of Bugula neritina

Marine bacteria are not the only source of biologically active natural products. There are many reports of natural products isolated from marine invertebrates. However, many of these natural products have been since found to be made not by the invertebrate animal but rather by a

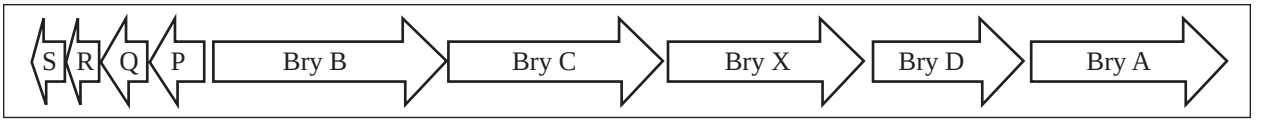

Figure 5. The gene cluster responsible for the production of bryostatin is composed of five Type I PKS multienzymes. According to the proposed biosynthetic route, only four of these multimodular proteins are used. BryX is comprised of enzymatic domains not arranged in the conventional order for Type I PKS.

symbiotic microorganism. This is the case of bryostatin 1, a potent anticancer compound found in the organism *Bugula neritina*, a common and abundant member of the fouling community in harbors and bays. Studies to characterize the genes required for the biosynthesis of bryostatin 1 in *B neritina* revealed that the PKS genes required for the production of bryostatin 1 were located not in the bryozoan but rather in a symbiont *Endobugula sertula* (25).

The benefit of using DNA technology to enhance the production of marine drugs is particularly important in the case of a compound like bryostatin. Bryostatin is an inhibitor of protein kinase C and is currently in clinical trials for the treatment of various cancers, Alzheimers disease and strokes. Yet, the abundance of this polyketide in the producing organism is fairly low. In order to obtain 1 g of bryostatin, over 1500 pounds of *B neritina* are required. For this reason, finding the gene cluster responsible for the production of bryostatin in *E sertula* will be essential in the development of a sustainable source of this important compound.

A gene cluster for a Type I PKS has been cloned and sequenced in *E sertula* strains collected at both deep and shallow waters (26). The genes, named BryA,B,C,D and X, are the only large open reading frames in an otherwise degenerate genome and the only genes for Type I PKS found in *E sertula*, suggesting that they are, in all likelihood, responsible for the production of bryostatin (27). According to the proposed biosynthetic route for this compound, only BryA,B,C and D are necessary for building the polyketide backbone, while BryX is an inactive or seemingly superfluous gene. However, the fact that BryX is present in both deep and shallow strains together with the fact that RT-PCR data shows transcription of the BryX gene, both suggest that the BryX gene is utilized by the organism.

Recently, the first biochemical evidence of biochemical activity in the bry gene cluster came from work with the bryP protein (28). BryP is a discrete acyltransferase domain which loads malonate units onto the ACP domains located in the elongation modules. This activity is required in this type of cluster because there is no acyltransferase present in the modular PKS. Results show BryP to be active against other ACP domains in "AT-less" PKS modules, as well as against excised ACP domains from other Type I PKS.

Pseudopterosins in corals

Pseudopterosins are diterpene molecules isolated from corals with anti-inflammatory and analgesic properties. These compounds have been found to significantly inhibit phorbol myristate acetate-induced topical inflammation in mice and the methyl ether of this natural product has shown promise as a treatment for contact dermatitis (29-30). They are made by the cyclization of geranylgeranyl diphosphate (GGPP) to form the intermediate elisabethatriene, which is in turn converted to a variety of final products (Figure 6). The enzyme that catalyzes such cyclization has been purified directly from the source organism and assayed for cyclase activity (31). The purified enzyme, the first diterpene cyclase from a marine organism ever characterized, has similar properties and kinetic parameters as other diterepene cyclases, such as pI, pH optimum, and Km.

In more recent work, the purified elisabethatriene synthase was challenged with alternative shorter substrates, geranyl diphosphate and farnesyl diphosphate,

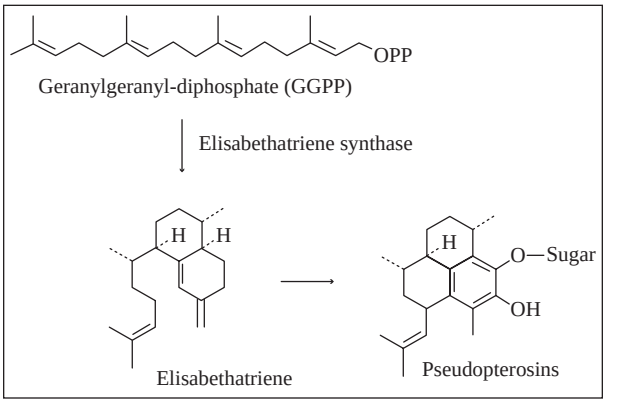

Figure 6. The biosynthesis of pseudopterosins starts with the conversion of GGPP to intermediate elisabethatriene which can be further converted to different pseudepterosins which differ in the nature of the glycosyl (R-group) moiety attached, presumably by a glycosyl transferase.

resulting in the production of alternative diterpenes (32). This result highlights the potential application of this family of enzymes in the generation of novel compounds derived from known diterpene precursors.

Omega-3 Fatty Acids from deep-sea bacteria

Long chain omega-3 fatty acids are essential nutrients in the human diet. They are involved in brain and eye development as well as in the maintenance of good cardiovascular health (33-34). Most of the long chain omega-3 in human diet comes either directly from the consumption of oily fish or indirectly from the relatively inefficient elongation of the medium-chain omega-3 present in some vegetable oils. Fish, in turn, also have an inefficient mechanism for producing their own long-chain omega-3 and must obtain it from a diet rich in microalgae and bacteria, both major sources of these important nutrients.

The biosynthesis of omega-3 in deep-sea bacteria takes place not by the desaturation and elongation of saturated fatty acids, but rather by a polyunsaturated fatty acid (PUFA) synthase system which resembles a PKS in that it builds the fatty acid one acyl unit at a time, introducing the double bonds as the chain grows attached to an acyl carrier protein (35). The PUFA synthase genes have been identified in different organisms and the organization of genes within the clusters show some commonalities (35-36) (Figure 7). For instance, all gene clusters for PUFA synthase sequenced so far have a number of between 4-6 ACP domains in tandem. Also, all PUFA synthase clusters dehydratase (DH) domains, presumed to be responsible for the formation of double bonds in the final product, are organized in pairs. This organization of DH domains is unlike that of DH domains normally found in modular PKS.

The PUFA synthase genes have been introduced into *E coli*, resulting in the production of omega-3 fatty acids in a heterologous host (37-39). These results indicated that PUFA synthase genes alone were all that was required for the biosynthesis of long-chain omega-3 in deep sea bacteria and that no elongase-desaturase enzyme was required. These results also highlight the potential of using *E coli* as a heterologous hosts for the production and for the expression of PUFA synthase proteins.

The biochemical activity of some of the PUFA synthase enzymes has also been reported. In order to determine how many of the tandem ACP domains are required for product assembly, the ACP domains from *Shewanella japonica* were systematically inactivated and expressed in *E coli* with the other components of the PUFA gene cluster (40). Results confirmed the presence of ACP domains that were functional as revealed by the fact that

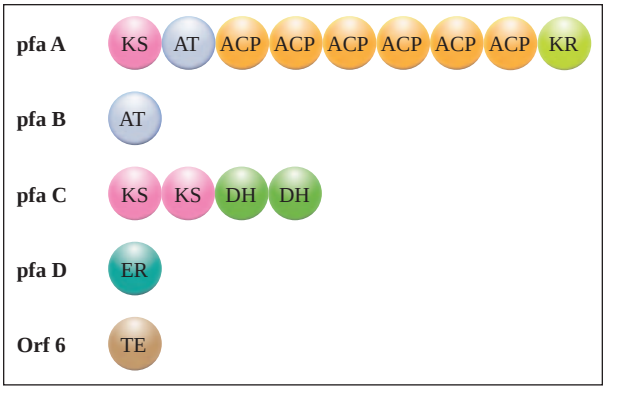

Figure 7. The gene cluster for the EPA-producing PKS in *Photobacterium profundum* consists of 5 open reading frames36. This domain organization is conserved among marine bacteria that produce polyunsaturated fatty acids.

they are recognized as substrates for phosphopantetheinyl transferases (PPTases). More importantly, results showed fatty acids can still be produced if all but one ACP domain has been inactivated by mutagenesis. These results answer the question of how many ACP domains are required for product assembly, but it does not explain the prevalence of tandem ACP domains in all known PUFA clusters, while virtually absent in other modular PKS systems.

The activity of the enoyl reductase enzyme in the PUFA gene cluster, pfaD, has also been measured in vitro (41). In this first measurement of enzymatic activity of a purified PUFA synthase component, the pfaD was expressed, purified and assayed for its ability to catalyze the reduction of the 2-butenoyl-ACP, a predicted authentic intermediate of the reaction. High-resolution mass spectrometric measurements showed a 2 Da increase in the molecular weight of the holo ACP in the presence of NADPH, demonstrating that pfaD is indeed an enoyl reductase as had been predicted by sequence alignments.

Summary

Biotechnology is being applied successfully to the study of marine natural products and to their subsequent development as therapeutics. In the examples discussed in this review, the biosynthetic routes for some of the more interesting and promising marine compounds are studied by a combination of approaches which include genetics, bioorganic chemistry and enzyme biochemistry. Two underlying goals can be discerned from these efforts: 1) finding more sustainable and economically viable ways to produce marine compounds and 2) increasing

the biochemical diversity of compound families. Biotechnology is driving the steady advancement of both of these goals as it promises to open doors in the quest for new medicines and new treatments.

Resumen

El ecosistema marino tropical es una fuente de compuestos con actividad biológica de un valor incalculable. Son varias las iniciativas que ya están en pie con el propósito de explorar tanto la diversidad biológica y química de los océanos como el uso de compuestos de origen marino en el tratamiento de enfermedades mortales. Algunos de estos esfuerzos recientes se centran en el uso de las herramientas de la biotecnología y la bioquímica para el descubrimiento de nuevos compuestos. Otras iniciativas buscan emplear la biotecnología con el propósito de generar diversidad química alrededor de compuestos prometedores así como para generar fuentes renovables para la producción industrial de estos compuestos. Es evidente que el alcanzar estas metas ambiciosas requerirá un conocimiento más completo de los procesos enzimáticos y moleculares que dan pie a la producción de compuestos activos en el ambiente marino. En este artículo se resumen los hallazgos más recientes en la identificación y caracterización de rutas enzimáticas de biosíntesis de compuestos marinos con énfasis particular en el trabajo realizado con enzimas purificadas.

Abbreviations and acronyms

PKS = Polyketide Synthases
NRPS = Non-ribosomal peptide synthetase
ACP = Acyl carrier protein
AT = Acyltransferase
PUFA = Polyunsaturated fatty acids
ECH = Enoyl CoA hydratase
GGPP = geranylgeranyl diphosphate
DH = dehydratase
PPTase = phosphopantetheinyl transferase

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