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Review – CORRECTED MANUSCRIPT R1

Marine Drugs: Biology, Pipelines, Current and Future Prospects for Production

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Abstract

The marine environment is a huge reservoir of biodiversity and represents an excellent source of chemical compounds, some of which have large economical values. In the urgent quest for new pharmaceuticals, marine-based drug discovery has progressed significantly over the past several decades and we now benefit from a series of approved marine natural products (MNPs) to treat cancer and pain while an additional collection of promising leads are in clinical trials. However, the discovery and supply of MNPs had always been challenging given their low bioavailability and structural complexity. Their manufacture for pre-clinical and clinical development but also commercialization mainly relies upon marine source extraction and chemical synthesis, which are associated with high costs, unsustainability and severe environmental problems. In this review, we discuss how metabolic engineering now raises reasonable expectations for the implementation of microbial cell factories, which may provide a sustainable approach for MNP-based drug supply in the near future.

Keywords: Marine drugs, pharmaceuticals, production, metabolic engineering, biotechnology

1. Introduction

As with land plants, fungi and bacteria, marine organisms represent an extremely large resource of valuable natural products for humans. The bioactive properties of many of these molecules find applications in a wide range of industrial sectors such as nutrition, biotechnologies, cosmetics, and health (Sigwart et al., 2021). Among the large series of marine natural products (MNPs), those displaying a pharmaceutical potential are of great interest as part of our modern pharmacopeia (Ghareeb et al., 2020). Marine organisms continue to be a source of unique chemical entities with valuable biomedical potentialities (Blunt et al., 2018). This is illustrated nicely by the recent report on turbinicin, a marine microbe sourced polyketide displaying potent antifungal activity, particularly towards pathogenic yeasts that represent a worrisome problem for human health in terms of virulence and drug resistance (Zhang et al., 2020; Zhao et al., 2020).

From an economic viewpoint, the world market of MNPs is considerable with a recent report estimating the market size for marine-derived pharmaceuticals will reach up to USD 10b in 2021 with an expected annual growth rate of more than 8% from 2020 to 2026 (Daniotti and Re, 2021 ; ID: 5123389). As observed for the main anticancer drugs derived from plants, enormous costs of production lead to high cost therapeutics. For instance, the monthly cost for treating breast cancer or liposarcoma with eribulin now reaches USD 10,000 while that of brentuximab for the management of lymphoma exceeds USD 30,000 (Howard et al., 2015 ; Gordon et al., 2018).

From a structural point of view and with few exceptions, MNPs of pharmaceutical importance derive from the so-called secondary metabolism of marine organisms. This means that their biosynthetic routes are diverse in terms of length, starting precursor and characterization level. MNPs also display complex structures that potentially render their total chemical syntheses *de novo* impractical. As a consequence, production strategies relying on marine macroorganism harvesting or culturing coupled with extraction procedures remain sometimes the only alternative for the production of these compounds. In this regard, while their supply can be often implemented with laboratory scale cultures for bacterial, fungal or microalgal sources, this is much more complex to consider for invertebrate sources where secondary metabolites usually naturally accumulate in only very small quantities. Most of the time, the concentrations of the promising MNPs in marine invertebrates may be sufficient for their chemical characterization and preliminary activity evaluation, however, they are far from providing a sustainable supply for pre-clinical and clinical trials, requiring quantities in a gram range, and even less in the perspective of a future commercialization where annual demands usually reach several kilograms (Gomes et al., 2016). For ecological issues, it is inconceivable to think that the supply of these MNPs with promising properties could only depend on wild harvest of marine invertebrates (Heffernan, 2020).

Importantly, similar production limits have been observed for plant-derived pharmaceuticals in past decades (Courdavault et al., 2021). This has promoted the stepwise emergence of alternative strategies

of supply based on metabolic engineering approaches that began to appear alongside the emergence of the synthetic biology era in the early 2000's (Kotopka et al., 2018 ; Nielsen, 2019). This type of strategy relies on the heterologous expression of biosynthetic genes from the source organism in a recipient bacterium and/or yeast to allow the *de novo* synthesis of the expected drugs by the transformed microbe. Likewise, in line with the pioneering success of the antimalarial semi-synthetic artemisinin (Paddon et al., 2013), genetically engineered microorganisms have emerged as suitable cell factories for the production of plant-derived drugs. In this context, we discuss here how all the background gained in recent years concerning the biotechnological production of plant drugs using engineered microbes will undoubtedly pave the way for innovative MNP manufacturing alternatives in the near future.

2. Trends in marine drugs: sources, chemical classes, biological activities and current pipeline

Over five decades of sustained research, more than 30,000 compounds of marine origin have been identified within a diverse palette of biological sources, including both prokaryotic (eubacteria, cyanobacteria) and eukaryotic organisms (fungi, dinoflagellates, algae, sponges, cnidarians, bryozoans, mollusks, ascidians, and echinoderms) (Alves et al., 2018; Caroll et al., 2021). Sponges (porifera) and cnidarians (mainly corals and anemones) for invertebrates as well as marine fungi and actinomycetes for microorganisms have always been the prominent MNP sources in term of quantity and structural diversity (Hu et al., 2015). With the exception of some peptides (e.g. conotoxin), fatty acids (e.g. omega-3-acid ethyl esters) and polysaccharides (e.g. fucoidan, carrageenans, alginates, ulvans, chitins, and chitosans), all others classes of biologically active MNPs arise from the so-called "specialized metabolism" (or secondary metabolism) pathways which means that most of the interesting MNPs are biosynthesized through clade-specific metabolic pathways. The major classes of marine secondary metabolites include alkaloids, terpenoids, polyketides, acetogenins, non-ribosomal peptides, and glycosides (Lu et al., 2021). Figure 1 summarises examples of the structural diversity of MNPs that include approved therapeutics, clinical candidates and bioactive structures that are the focus on ongoing studies. Although these chemical classes are also primarily found in the portfolio of metabolites produced by terrestrial natural sources (mainly plants, fungi and bacteria), recent advances shed light on shared but also on differential features between terrestrial and marine secondary metabolites. While displaying a global lower solubility, MNP scaffolds are substantially larger, containing more long chains and large rings with fewer oxygen but more nitrogen atoms compared to terrestrial natural products. Another distinguishing general trait of MNPs is that a fair proportion of compounds contain bromine and to a lesser extent chlorine, associated with the high concentration of halides in

seawaters and the presence of particular halogenation enzymes (e.g. vanadium bromoperoxidase) in a broad range of marine organisms (Shang et al., 2018; Voser et al., 2022).

About a quarter of the MNPs isolated so far have shown interesting biological activity including antiproliferative, antimicrobial, anti-inflammatory, anti-coagulation, analgesic, neuroprotective, and cardioprotective properties (Hu et al., 2015). To date, fourteen MNP-based drugs are clinically approved by various worldwide health organizations and are currently used to cure/treat some of the main medical pathologies including cancer, cardiovascular diseases, pain, and viral infections (Dyshlovoy and Honecker, 2020). A timeline of their discovery is depicted in Figure 2 and their main characteristics are compiled in Table 1. Beyond these proven pharmaceutical successes, we can anticipate that the number of approved MNP-derived drugs will keep increasing since 33 marine-derived pharmaceutical compounds are currently in clinical trials (5 in phase III, 12 in phase II, and 16 phase I, Table 2). In addition, it is highly likely that MNP research will continue to boom insofar as the recent developments in analytical technology, spectroscopy, machine learning, and high-throughput screening of activities all support the discovery of more than 1,000-1,500 new structures from marine sources annually (Ghareeb et al., 2020; Wilson et al., 2020, Carroll et al., 2021).

As these biomolecules are sourced from nature, bioprospecting researchers in this field need to be aware of the moral and legal obligations on them to ensure equitable benefit sharing with the country from which the biological/genetic material was sourced. In October 2014, parties to the Convention on Biological Diversity (CBD) adopted the supplementary agreement (known as the Nagoya Protocol) which provides a legal framework regarding access and fair and equitable sharing of benefits arising from the utilization of genetic resources. One of the founding aims of the Nagoya Protocol is to put an end to biopiracy, with researchers being required to establish access and benefit-sharing (ABS) agreements before being granted access to a country's national jurisdiction. In a marine context, this jurisdiction covers territorial seas and exclusive economic zones (EEZ), out to a 200 nautical mile boundary (<https://www.cbd.int/abs/>). Thus it is a legal imperative for any bioprospecting in these environments that, in addition to the usual collection permits, ABS agreements are put in place. To date, these close-to-shore and EEZ marine environments are the locations of the overwhelming majority of bioprospecting efforts. There are however ongoing deep-sea bioprospecting efforts, searching the water column and sediment for novel biomolecules. Exploration in ocean territory beyond any national jurisdiction boundaries is explicitly not covered by the Nagoya Protocol and so the concepts of access and benefit-sharing are somewhat vague. The closest regulatory framework is the United Nations Convention on the Law of the Sea (UNCLOS), which contains references to the sustainable use of marine living resources. There is a need for the development of sharing agreements for products arising from open ocean research, beyond bilateral/multilateral agreements, to perhaps extend so that benefits are shared by nations.

3. How approved MNP-based pharmaceuticals are manufactured?

The currently approved MNP-derived pharmaceuticals cover diverse chemical classes encompassing nucleosides, peptides, fatty acids, macrolides, depsipeptides, antibody-drug conjugates, hydroxamic acids, and alkaloids. As a consequence, their production process and cost differ sharply from one compound to another. In this section, we summarize how pharmaceuticals companies currently ensure the supply of these important medicines at an industrial scale.

The process for production of the lipid-regulating omega-3-acid ethyl esters (Lovaza® and Vascepa®) (Figure 2) is among the simplest and cheapest since triglyceride biosynthesis inhibitors are made from fish body oils that are purified and esterified (Cholewski et al., 2018). The anticancer drugs cytarabine (Cytosar-U®) and nelarabine (Arranon®/Atriance®), and the antiviral compound vidarabine (Vira-A®), are analogs of the marine nucleosides spongothymidine and spongouridine (Figure 1), which were originally isolated from the Caribbean sea sponge *Tectitethya crypta* (Figure 2). Due to their fairly simple chemical structures, these DNA/RNA polymerase inhibitors are currently manufactured by classical chemical synthesis (Shelton et al., 2016). The powerful analgesic agent ziconotide (Prialt®) (Figure 2) is ω -contoxin MVIIA (Figure 1), a hydrophilic peptide originally isolated from the venom of the Pacific fish-hunting marine mollusk *Conus magus* (Figure 2). This N-type calcium channel blocker (Gao et al., 2021) is produced by solid phase peptide synthesis (Schroeder and Craik, 2012). The anticancer agent eribulin mesylate (Halaven®) (Figure 2) corresponds to a simplified analogue of the marine polyether macrolide, halichondrin B (Figure 1), which was originally isolated from the marine sponge *Halichondria okadai* (Figure 2). This microtubule-targeted anticancer drug is nowadays considered as the most complex synthetic drug and above all a perfect demonstration of how far the limits of total synthesis of natural products have been pushed on an industrial scale (Bauer, 2016). Another relatively new and efficient class of marine drug-derived pharmaceuticals corresponds to antibody-drug conjugates (ADC), combining a cytotoxic agent of marine origin with a selective monoclonal antibody. Four examples are currently approved to treat specific types of cancer including brentuximab vedotin (Adcetris®), enfortumab vedotin (PADCEV™), polatuzumab vedotin (Polivy™), and belantamab mafodotin (Blenrep™) (Figure 2). These MNP-derived ADC commonly consist of (i) a monoclonal antibody specific to surface antigens present on particular tumor cell types, (ii) a cleavable or non-cleavable linker, (iii) and a microtubule disrupting MNP derivative (either monomethyl auristatin E, MMAE, or monomethyl auristatin F, MMAF) (Joubert et al., 2020). Monoclonal antibodies are classically produced by mammalian (Chinese hamster ovary) cells while linkers are prepared with commercially available chemicals. MMAE and MMAF are synthetic analogs of the highly cytotoxic dolastatin 10 (Figure 1) that was originally isolated from the Indian Ocean sea hare *Dolabella auricularia* (Figure 2). To date, MMAE and MMAF are manufactured by classical chemical synthesis (Zhou et al., 2017). The anticancer MNPs trabectedin (Yondelis®) (Figure 1) and its derivative lurbinectedin

(Zepzelca™) are tetrahydroisoquinoline alkaloids – trabectedin is ecteinascidin 743 while lurbinectin is a modified analog of ET 736, both natural products having been originally identified in the Caribbean sea squirt *Ecteinascidia turbinata* (Figure 2). Although recent advancements provide new scalable total synthesis protocols, the production of these two complex alkaloids still comprises semisynthetic steps performed on the precursor cyanosafracin B produced by fermentation of the bacterium *Pseudomonas fluorescens* (Cuevas and Francesch, 2009; He et al., 2019). Finally, Plitidepsin (Aplidin®) (Figure 1) is a cyclic depsipeptide anticancer drug isolated from the marine tunicate *Aplidium albicans* (Figure 2). Since its approval, this eEF1A2 inhibitor is manufactured by total chemical synthesis (Alonso-Álvarez et al., 2017).

4. Anticipating the development of efficient production processes for future approved MNPs

More than 30 MNP-derived pharmaceutical are currently in clinical trials (Table 2), about a hundred are in preclinical development each year, and the discovery of new compounds with interesting biomedical potentialities and novel mechanisms of action continues with little sign of slowing down (Mayer et al., 2010 ; Newman and Cragg, 2016 ; Mayer et al., 2021 ; Daniotti and Re, 2021). As a consequence, whether for preclinical and clinical development or marketing, it seems important to be able to anticipate efficient processes of sustainable supply for promising MNP-derived drugs. In this respect, all the background accumulated in the past decades that have allowed implementing such industrial production strategies for many different classes of natural products from highly diversified sources may combine to benefit the supply of new marine drugs.

As established with eribulin mesylate (Halaven®), advances in chemical synthesis continuously stretch the boundaries of our capacities of manufacturing pharmaceuticals with increasingly complex structures (Bauer 2016). We can thus expect that this powerful process of supply may continue to stand out as a prominent strategy to produce future approved MNP-derived drugs at an industrial scale. However, we have to admit that this production process is still poorly environment-friendly and very costly insofar the methods require a lot of different reagents and deliver overall low yields. Hence, especially in the case of new MNPs of great potential but displaying structures far too complex for a profitable production by chemical synthesis, alternative biotechnological approaches could be considered for which we summarize some of these key features hereafter.

For instance, concerning MNPs originally biosynthesized by a single cultivable marine microbe such as a bacterium or a fungus, axenic macroscale cultures (in a bioreactor) could be a relevant strategy (Quian et al, 2020). This type of production process has proved its worth for the industrial scale supply of many different antibacterial (erythromycin, vancomycin, streptomycin, penicillins, cephalosporins), antifungal (griseofulvin, amphotericin B) antiparasitic (avermectins, fumagillin), and immunosuppressant

(tacrolimus, cyclosporine, mycophenolic acid) natural products from a broad palette of terrestrial bacterial and fungal sources (Panter et al., 2021). In line with this statement, Zhao and colleagues recently identified a highly oxidized type II polyketide from the marine actinomycetes *Micromonospora* sp., turbinmicin, which displays antifungal activity by inhibiting the vesicle-mediated trafficking pathway of many life-threatening fungi (Zhao et al., 2020 ; Zhang et al., 2020). This promising new antifungal marine drug demonstrated mouse-model efficacy toward multiresistant yeasts and molds. We can thus anticipate that, in case of clinical success, axenic macrocultures would represent the most efficient process strategy to reach an industrial scale production of turbinmicin.

It is also important to remember that, in some specific cases, complementary semi-synthesis steps can be developed to complete the scaffold of some complex microbial secondary metabolites, as illustrated for the anticancer MNPs trabectedin (Yondelis®) and lurbinectedin (Zepzelca™) that are semisynthetic derivatives of cyanosafracin B (produced by axenic cultures of *P. fluorescens*) (Cuevas and Francesch, 2009). A similar approach is currently achieved for the industrial production of the antifungals caspofungin (Cancidas®), micafungin (Mycamine®), and anidulafungin (Ecalta®) that are obtained following additional semisynthetic steps on the precursor pneumocandin B₀ (produced by axenic cultures of the ascomycetes mold *Glarea lozoyensis*) (Balkovec et al., 2014). It is highly likely that such semisynthetic processes would continue to be a procedure of choice for the industrial production of many different classes of clinically used secondary metabolites, as observed for some plant-derived anticancer drugs (Courdavault et al., 2020).

By contrast, production processes for complex MNPs that are originally biosynthesized by marine invertebrates are more difficult to implement. As currently performed for several drugs or their precursors that are extracted from terrestrial medicinal plants, random sampling of the source material from sessile marine organisms directly from the natural environment is not acceptable. Aside from ecological impact considerations, the overall naturally-available bulk mass would usually be insufficient to supply an industrial demand. Most importantly, such a practice is known to directly endanger biodiversity (Heffernan, 2020). By analogy to what has been achieved in the context of the monoculture-based production of some plant drugs such as anticancer vinca-alkaloids (vincristine, vinblastine, vinorelbine) from the Madagascar periwinkle *Catharanthus roseus* or the antimalarial artemisinin from the sweet wormwood *Artemisia annua*, aquaculture of medicine-producing marine invertebrates has progressively been considered since the 1980's (Benkendorff, 2009). This trend is a logical extension of current commercial aquaculture practices, including for instance corals and shells (for tourism souvenir gifts), sponges (personal hygiene and cleaning), seaweeds (production of proteins, lipids and carbohydrates for food and cosmetics) and shellfish farming (mainly oysters for food or mother-of-pearls, clams, nutshells and mussels) (Naylor et al., 2021). To date, there are two main aquaculture methods including mariculture (also termed "*in situ*" aquaculture) and captive breeding (also referred to

as “*ex situ*” aquaculture) and both types of aquaculture display advantages and drawbacks from a MNP production perspective (Leal et al., 2013 ; García-Poza et al., 2020).

On the one hand, *in situ* techniques represent a low cost and simple methodology that relies entirely on the natural environment to supply essential light and nutrients for marine species growth. Importantly, it is nowadays well established in the literature that the production of a plethora of bioactive secondary metabolites initially identified in sponges, cnidarians, bryozoans, mollusks, ascidians, and echinoderms does not exclusively rely on the intrinsic biosynthetic pathways evolved by these marine metazoan species, but rather derives from associated micro-organisms. (Gomes et al., 2016). In other words, there is evidence that culturing marine species in their natural habitats through *in situ* aquaculture promotes holobiont (this means for instance a marine invertebrate associated to different microorganisms) homeostasis and this equilibrium requires the correct orchestration of the different metabolic fluxes necessary for the production and accumulation of specific MNPs (Leal et al., 2013 ; McCauley et al., 2020). However, in practice, monitoring growth conditions is fairly limited and the developing marine invertebrates are usually threatened by environmental stresses (predation, eutrophication, thermal anomalies) (Sheridan et al., 2013). As a consequence, and in the perspective of production of a specific MNP, both the biotic or abiotic stresses could have potential uncontrolled effects on the regulation of MNP biosynthetic pathways. Most importantly, the annual production of a specific metabolite by *in situ* aquaculture may be impeded by climatic variations or even natural disasters leading to a significant reduction in bulk mass necessary for extraction.

In contrast, since a proximate source of natural seawater is not mandatory, *ex situ* practices have also been considered in the framework of future industrial production of valuable MNPs. Captive breeding, although more expensive to implement, prevents occurrence of perturbing abiotic and biotic factors, but also allows the monitoring of optimal developmental conditions that are considerably species specific. All these previously mentioned aspects have thus favored an improved control of MNP production. However, with the exception of corals, cultivation of whole marine organisms *ex situ* has been globally less successful compared to mariculture. In addition, it is important to keep in mind that while *ex situ* aquaculture allows the fine tuning of growth conditions, this practice may change the holobiont composition and causes either the disruption of important microorganisms when cultured in their natural sites or promotes uncontrolled development of undesired microorganisms. As a consequence, although farming has been undertaken for preliminary studies of several compound classes including the halichondrins, ecteinascidins and mycalamides, experimentation aimed at producing valuable MNPs by captive breeding have never been reported hitherto (Leal et al., 2013 ; Gomes et al., 2016).

On the collective basis of these issues, there is an urgent need to implement alternative supply paths for future medically important classes of MNPs to prevent recurrent shortages and to limit the impact of their production on nature. In such a perspective, we discuss in the coming section, how the use of

engineered microbes could progressively stand out as a promising production strategy to overcome potential sourcing limitations of complex MNPs belonging to a broad palette of secondary metabolite classes, as recently emerged for plant-derived pharmaceuticals (Kotopka et al., 2018 ; Nielsen, 2019 ; Courdavault et al., 2021).

5. Marine drug production: a metabolic engineering perspective

The pioneering landmark production of the antimalarial artemisinin in yeast successfully achieved in the 2000's has inspired many subsequent developments in the field. Indeed, a boom in microbial engineering procedures for the production of different types of complex plant natural products (PNPs) has been observed in the past decades, particularly among the major families of polyphenols, terpenes, taxanes, indole alkaloids, opioids, cannabinoids, and tropane alkaloids (Courdavault et al., 2021). On this basis, now is the time to reflect on these outstanding achievements to guide future research in this domain to open new windows for sustainable production processes of complex pharmaceutical MNPs (Figure 3).

A rapid overview of the literature illustrates that the capacity to develop optimized microbial cell factories heterologously producing PNPs has accelerated thanks to very recent technological advancements. These consist in (i) a marked acceleration in the elucidation of the biosynthetic pathways of secondary metabolites (Lau and Sattely, 2015 ; Medema and Osbourn, 2016 ; Caputi et al., 2018 ; Nett et al., 2020 ; Dugé de Bernonville et al., 2020), (ii) an improved rationalization of the reconstruction stages of the biosynthetic pathway in the host microbe (Luo et al., 2019 ; Srinivasan and Smolke, 2020, Guirimand et al., 2021 ; Srinivasan and Smolke, 2021), (iii) the identification of new routes for optimizing the microbial production to reach industrial titers (Pyne et al., 2020 ; Zhang et al., 2020; Carqueijeiro et al., 2020).

As an obvious starting point, reconstructing biosynthetic pathways in heterologous hosts to create a MNP cell factory requires a comprehensive knowledge of the enzymatic reactions involved in the biosynthesis of the targeted compound in the native host organisms (Figure 3A). In bacteria and fungi, this step is facilitated by the genomic clustering of genes encoding enzymes from the biosynthetic pathways of most the secondary metabolites (Keller, 2019 ; Chevette et al., 2020 ; Lee et al., 2021 ; Zhang et al., 2019). However, in the context of a valuable MNP produced by an holobiont such as for instance a marine invertebrate associated to various microorganisms, the research is more challenging since the elucidation of the complete biosynthetic pathway requires the identification of highly specific enzymes encoded by genes present in multiple species and belonging to distinct superfamilies containing several tens to hundreds of members such as reductases, oxidases, methyltransferases, and cytochrome P450s among others.

Fortunately, the arrival of massive omics resources and tools could accelerate such investigations (Hautbergue et al., 2018 ; Dugé de Bernonville et al., 2020 ; Ramzi et al., 2020) (Figure 3B). In that sense, an emerging and powerful strategy relies on identifying a first set of candidate genes through metabolomic and transcriptomic analysis. More specifically, various metabolomics approaches can now lead to the accurate detection of plausible reaction intermediates while recent advancements in molecular networking provide new efficient opportunities in the processing of the acquired metabolomics data (Fox Ramos et al., 2019 ; Stuart et al., 2020). Once compounds are connected and a reaction sequence has been postulated *in silico* (reflecting for instance a metabolic branch), bioinspired organic chemistry and literature data may then help in identifying a type of enzymatic activity required for each step. At this stage, the availability of the holobiont metagenome and massive transcriptomics data are of primary importance since searching the specific functional conserved domains may lead to define first sets of candidate genes.

At this step, identifying the respective genes encoding specific enzymes involved in each catalytic reaction of the investigated pathways requires further prioritization procedures (Figure 3C). The various possibilities include first the screen for homology to orthologous gene sequences of already characterized biosynthesis steps in other models. A BLAST search for protein sequences from multiple species coupled with reconstruction of phylogenies statistically fitted on evolutionary models represent a basic approach to this aim (Farrow et al., 2018). Another possibility consists in prioritizing candidate genes by searching physical gene clusters. Some specific branches of the studied biosynthetic pathway, notably those achieved by microflora species associated to the holobiont, can indeed involve enzymes encoded by genes physically clustered in the same genomic region (Keller, 2019 ; Chevette et al., 2020). Once again in this context, the knowledge of the metagenome sequence from the investigated holobiont is mandatory and the search for gene cluster is particularly efficient with more complete genome sequences composed of very long DNA sequence assemblies (McCauley et al., 2020 ; Robinson et al., 2021). It is interesting to mention that such a global strategy has already begun to bear fruit since it has allowed deciphering of the biosynthetic origin of the anticancer drug trabectedin (Rath et al., 2011; Schofield et al., 2015). Above all, the most powerful and global approach that have recently emerged to rationally prioritize gene sequences in metabolic engineering relies on gene co-expression analysis (Dugé de Bernonville et al., 2020 ; Ramzi et al., 2020). Briefly, these computational strategies are based on the hypothesis that metabolites accumulated specifically in an organism or a holobiont, even in a tissue-specific manner, should be produced through a chain of enzymes displaying a similar spatio-temporal expression pattern (Ambrosino et al., 2019 ; Paul et al., 2019). Several major approaches can now be used for browsing such co-expression data and guiding identification of biosynthetic enzymes. This includes the statistical comparison of contrasted samples (e.g. MNP accumulating sample versus a non-accumulating sample) to identify differentially expressed genes (DEG), the construction of gene co-expression networks (GCNs) to provide a more exhaustive view of

co-expression relationships among genes (Liesecke et al., 2018), and on the use of unsupervised machine learning (UML) that assists in grouping similarly expressed genes into a specific expression cluster (Prihoda et al., 2021). All these concepts have been extensively reviewed elsewhere (Dugé de Bernonville et al., 2020 ; Ramzi et al., 2020).

While new computational approaches now facilitate the identification of candidate biosynthetic genes by browsing both genomic and transcriptomic data, a laborious but essential step consists of validating the correct activity of each predicted genes/enzymes (Figure 3D). To this aim, biochemical characterization of recombinant proteins heterologously expressed in bacteria or yeasts still represents a basic but reliable approach. These functional assays are coupled with metabolomic techniques to detect product formation. Importantly, this approach is sometimes impeded by unknown, unpurifiable, or commercially unavailable reaction substrates. In this specific case, this requires the use of bulk mass extraction from the marine source. It is noteworthy that the validation of multiple genes from the same metabolic branch can be achieved following biosynthetic precursor feeding of the host microbe (expressing bacteria or fungi) co-overexpressing simultaneously several candidate genes. In this respect, tremendous advancement has been recently achieved since scalable processes for the expression of large numbers of full-length biosynthetic genes in a same vector are now accessible (Clevenger et al., 2017 ; Harvey 2018).

Once the complete set of biosynthetic genes identified are biochemically validated, engaging the reconstruction of the pathway in a microbial chassis becomes possible (Figure 3E). A key factor of success to this aim is the choice of an appropriate heterologous host. Indeed, the high number of enzymatic steps, complex species, tissue, differential subcellular localization of intermediates or enzymes, and interconnected regulation networks in the MNP-producing holobiont could dramatically challenge the whole pathway reconstruction in the microbial host. Furthermore, the choice of carbon sources for feeding and the ease of final product purification must be considered to ensure the sustainability of the production process. On this basis, two main microbial models are usually preferred including the bacterium *Escherichia coli* and the baker's yeast *Saccharomyces cerevisiae* (Carqueijeiro et al., 2020 ; Guirimand et al., 2021). The specific requirement of post-translational protein modifications and the presence of membrane-bound proteins condition the selection of the host organism towards the use of a prokaryotic (*E. coli*) or eukaryotic chassis (*S. cerevisiae*). Fortunately, extensive synthetic biology toolboxes are available for both microbial models such as genomic integrative constructs, including CRISPR-Cas9 technologies, and auto-replicative plasmids (Cravens et al., 2019). These versatile genetic tools now allow rapid heterologous pathway prototyping. The bacterium *E. coli* displays advantages in metabolic engineering such as a well-understood native metabolism and fast doubling time. However, this microbial model shows significant physiological differences with eukaryotic cells including the lack of subcellular compartmentation and protein post-translational modification mechanisms, which can alter the metabolic flux of the heterologous pathway and reduce product titer.

By contrast, the yeast *S. cerevisiae* displays a canonical eukaryotic cell architecture allowing the expression of membrane-bound proteins, as usually observed in biosynthetic pathways (e.g. cytochrome P450). However, there is still very few information on the heterologous expression of enzymes from marine invertebrates, in particular relating to post-translational modifications such as protein glycosylation (Zhu et al., 2019). An important and recent advancement in this field is the development of minimal synthetic genome organisms that only include documented genetic elements (Richardson et al., 2017 ; Fredens et al., 2019 ; Glass et al., 2017 ; Lu et al., 2019 ; Lloyd et al., 2018). These microbial chassis have the advantage to significantly decrease the risk of metabolic flux shifting.

While recent breakthroughs now facilitate the development of a prototypical microbial strain producing the μg -low mg range per liter, optimizing MNP synthesis in the microbial host up to industrial scale represents a last but considerable step (Figure 3F). While global approaches usually provide the lead for the main backbone compounds, production of the most effective bioactive molecule will require multiple iterative refinements using the Design-Built-Test-Learn cycle (Figure 3G). The mining of the synthesis pathways of natural biological products is indeed a primary application of system biology, however, the latter has also a great potential in the transformation and optimization of the metabolic network of microbial cell factories. In this regard, genome-scale metabolic network model, metabolic network optimization based on omics technologies now sustains optimization strategies for the efficient synthesis of active natural products in microbial cell factories (Srinivasan and Smolke, 2019). Whereas optimization of media and culture conditions as well as implementation of bioreactors can increase the production titers of the desired MNP, recent advances provide complementary solutions and strategies for improving production yields in the prototypical engineered microbes (Pyne et al., 2019 ; Zhang et al., 2020; Carqueijeiro et al., 2020) (Figure 3E). For instance, it is now well documented that optimizing the microbe native metabolism, notably by increasing production of enzyme co-factors, is a first efficient way to revise metabolic fluxes in the microbial host. In addition, it was found that the timely induction of transgene expression and/or product capture during yeast culture can lead to an improvement of host tolerance to the produced drug. Interestingly, low heterologous gene expression can now be solved by increasing gene copy number using the CRISPR/Cas9 technology (Kulagina et al, 2021). Finally, recent advancements also firmly demonstrate that the identification and expression of more active orthologues is indispensable to reach high production levels (Srinivasan and Smolke, 2020). Taken together, all these optimizations may be required to prevent undesired accumulation of metabolic intermediates and to reroute critical native precursors into the desired MNP biosynthetic pathways without overly impacting cellular fitness. This is an essential prerequisite to follow to reach an industrial scale production.

6. Concluding remarks

As recently successfully achieved for some bacterial, fungal and PNP-derived drugs (Lee et al., 2021), the prospect of exploiting the potential offered by microbial metabolic engineering for developing alternative supply processes for promising MNP-derived pharmaceuticals is now within our grasp (Figure 4). While no MNPs have been produced by metabolic engineering to date, it will be important, in the near future, to carry out the first proof-of-concept studies in this field, notably by attempting to implement pioneering prototypical microbial strains in which biosynthetic pathways of several lead MNP are reconstructed. However, to this aim, it is highly likely that one will have to face some new difficulties of using systems biology methods to synthesize MNPs compared to PNPs. For MNPs that are biosynthesized (at least in part for a complex pathway) by marine invertebrates, the implementation would be requiring extensive research into the specialized metabolic routes in marine macroorganisms. In addition, there is no reported experience on the heterologous expression of marine invertebrate enzymes in bacterial or yeast platforms. Indeed, while the reconstruction of a whole PNP biosynthetic pathway in microbial chassis is feasible, what about the subcellular targeting and activity of MNP enzymes, the metabolic fluxes, the toxicity of MNP intermediates or final products in these recipient cells? It behooves the research community to pursue production of a MNP in this way, particularly one with a complex scaffold, which currently suffers from a high cost of production, and for which the biosynthetic pathways are fully elucidated (Table 3). It is anticipated that such implementations will benefit from the recent advancements made concerning the production of PNP in microbes, notably for MNPs belonging to chemical classes for which benchmark and successful examples were achieved for PNPs (Carqueijeiro et al., 2020). For instance and in such a perspective, producing trabectedin by microbial metabolic engineering could be supported by the recent development of a yeast platform for the synthesis of tetrahydroisoquinoline alkaloids at a gram per liter scale (Pynes et al., 2020). These technological developments seem ripe for future advancement in marine drugs and raise unprecedented expectations for the implementation of engineered cell factories, which may provide in a near future a sustainable approach for MNP pharmaceutical supply.

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Table 1. The currently approved MNP-derived pharmaceuticals.

Drug Name	Chemical class	Marine origin	Disease area	Trademark	Company
Omega-3-acid ethyl esters	Omega-3 fatty acids	Many fish species	Hyper-triglyceridemia	Lovaza®	Glaxo Smith Kline
Eicosapentaenoic acid ethyl ester	Omega-3 fatty acids	Many fish species	Hyper-triglyceridemia	Vascepa®	Amarin
Cytarabine	Nucleoside	<i>Tectitethya crypta</i> (Sponge)	Cancer	Cytosar-U®	Pfizer
Vidarabine	Nucleoside	<i>Tectitethya crypta</i> (Sponge)	Antiviral	Vira-A®	Mochida Pharmaceutical Co.
Nelarabine	Nucleoside	<i>Tectitethya crypta</i> (Sponge)	Cancer	Arranon® / Atriance®	GlaxoSmithKline
Ziconotide	Peptide	<i>Conus magus</i> (Mollusk)	Pain	Prialt®	Jazz Pharmaceuticals
Plitidepsin	Depsipeptide	<i>Aplidium albicans</i> (Tunicate)	Cancer	Aplidin®	Pharmamar
Trabectedin	Alkaloid	<i>Ecteinascidia turbinata</i> (Tunicate)	Cancer	Yondelis®	Pharmamar
Lurbinectedin	Alkaloid	<i>Ecteinascidia turbinata</i> (Tunicate)	Cancer	Zepzelca™	Pharmamar
Eribulin mesylate	Macrolide	<i>Halichondria okadai</i> (Sponge)	Cancer	Halaven®	Eisai Inc.
Brentuximab vedotin	ADC (MMAE)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	Adcetris®	Seagen
Enfortumab vedotin	ADC (MMAE)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	PADCEV™	Astellas Pharma / SeaGen
Polatuzumab vedotin	ADC (MMAE)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	Polivy™	Genetech / Roche
Belantamab mafodotin	ADC (MMAF)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	Blenrep™	GlaxoSmith Kline

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Table 2. The current pipeline of MNP-derived pharmaceuticals.

Drug Name	Chemical class	Marine origin	Disease area	Trademark	Company
PHASE III					
Lurbinectedin	Alkaloid	<i>Ecteinascidia turbinata</i> (Tunicate)	Cancer	Zepzelka™	PharmaMar
Tetrodotoxin	Alkaloid	<i>Pseudoalteromonas</i> sp., <i>Pseudomonas</i> sp., <i>Vibrio</i> sp.	Pain	Halneuron™	Wex Pharmaceutical Inc.
Plinabulin	Diketopiperazine	<i>Aspergillus</i> sp. (Fungi)	Cancer	-	BeyondSpring Pharmaceuticals
Plitidepsin	Depsipeptide	<i>Aplidium albicans</i> (Tunicate)	Coronavirus	Aplidin®	PharmaMar
Marizomib	β-lactone-γ-lactam	<i>Salinospora tropica</i> (Bacteria)	Cancer	-	Triphase
PHASE II					
Bryostatin	Macrolide	<i>Bugula neritina</i> (Bryozoan)	Alzheimer's Disease	-	Neurotrope BioScienc
Plocabulin	Polyketide	<i>Lithoplocamia lithiostoides</i> (Sponge)	Cancer	-	PharmaMar
GTS-21	Alkaloid	<i>Nemertines</i> sp. (Worm)	Cognitive disorders	-	-
AGS-16C3F	ADC (MMAF)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	Agensys / Astellas Pharm
Tisotumab Vedotin	ADC (MMAE)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	HuMax®-TF-ADC	GenMab
Ladiratuzumab vedotin	ADC (MMAE)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	Seattle Genetics
Telisotuzumab vedotin	ADC (MMAE)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	Abbvie
Enapotamab vedotin	ADC (MMAE)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	Genmab
Disitimab Vedotin	ADC (MMAE)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	RemeGen
CAB-ROR2	ADC (MMAE)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	BioAtla
CX-2029	ADC (MMAE)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	AbbVie / CytomX Therapeutics
W0101	ADC (Auristatin variant)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	Pierre-Fabre
PHASE I					
STRO-002	ADC (Taltobulin)	<i>Hemiasterella minor</i> (Sponge)	Cancer	-	Sutro Biopharm
Griffithsin	Lectin	<i>Griffithsia</i> sp. (Red alga)	HIV	-	Center for Predictive Medicine
FOR-46	ADC (MMAF)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	Fortis Therapeutics
ALT-P7	ADC (MMAE)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	3SBio / Alteogen
MRG003	ADC (MMAE)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	Shanghai Miracogen Inc.
RC-88	ADC (MMAE)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	RemeGen
SGN-B6A	ADC (MMAE)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	Seattle Genetics
SGN-CD228A	ADC (MMAE)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	Seattle Genetics
Cofetuzumab pelidotin	ADC (Auristatin variant)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	AbbVie
PF-06804103	ADC (Auristatin variant)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	Pfizer / AbbVie
ZW-49	ADC (Auristatin variant)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	Zymeworks / BeiGene
ARX-788	ADC (Amberstatin 269)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	Ambrex / Zhejiang Medicine
XMT-1536	ADC (Dolaflexin)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	Mersana Therapeutics
A-166	ADC (Duostatin 5)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Cancer	-	Klus Pharma
STI-6129	ADC (Duostatin 5)	<i>Dolabella auricularia</i> (Mollusk) / <i>Symploca</i> sp. (Cyanobacteria)	Amyloidosis	-	Sorrento
MORab-202	ADC (halichondrin B)	<i>Halichondria okadai</i> (Sponge)	Cancer	-	Eisai

Table 3. Current knowledge on the biosynthetic pathways of the main marine drugs.

Drug Name	Biosynthetic pathway	Reference(s)
Omega-3-acid ethyl esters	Elucidated	Lu et al., 2019
Eicosapentaenoic acid ethyl ester	Elucidated	Lu et al., 2019
Spongothymidine	Unknown	-
Spongouridine	Unknown	-
Nelarabine	Unknown	-
ω-contoxin MVIIA	Elucidated	Jin et al., 2019
Plitidepsin	Elucidated	Xu et al., 2012
Trabectedin	Elucidated	Schofield et al., 2015
Halichondrin B	Unknown	-
Dolastatin 10	Unknown	-
Tetrodotoxin	Unknown	Makarova et al., 2029
Plinabulin	Unknown	-
Salinosporamide A	Elucidated	Eustáquio et al., 2009
Bryostatin	Elucidated	Slocum et al., 2018
Plocabulin	Unknown	-
GTS-21	Unknown	-
Griffithsin	Elucidated	Fuqua et al., 2015
Hemiasterlin	Unknown	-

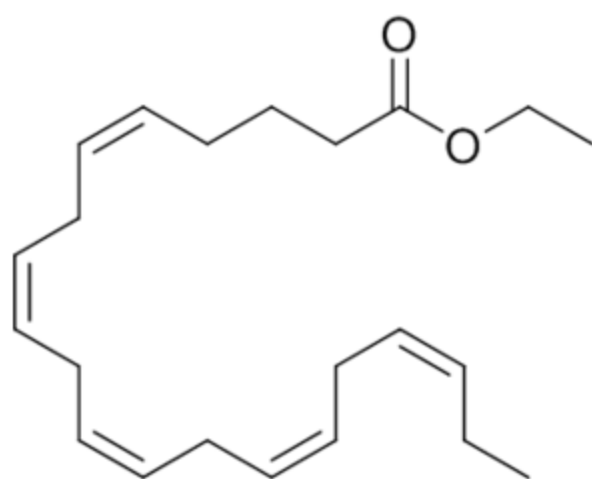
Figure legends

Figure 1. The structures of bioactive MNPs that are either themselves or the inspiration for approved therapeutics and clinical candidates.

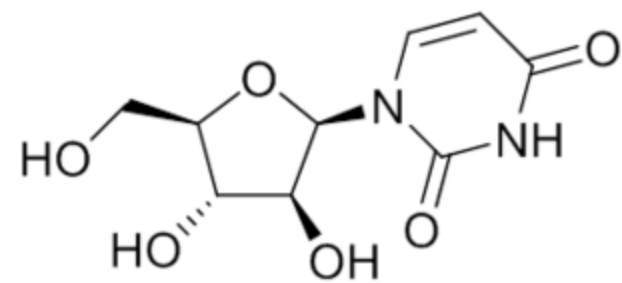
Figure 2. Timeline depicting the successive approval of the currently used MNP-derived pharmaceuticals. Some prominent examples of marine source organisms are indicated. For instance, the anticancer drugs cytarabine and nelarabine, as the antiviral compound vidarabine are analogs of a couple of marine nucleosides, spongothymidine and spongouridine, which were originally isolated from the Caribbean sea sponge *Tectitethya crypta*. The painkiller ziconotide is an ω -conopeptide from the venom of the Pacific fish-hunting marine mollusk *Conus magus*. The anticancer agent eribulin mesylate corresponds to a simplified analogue of the marine polyether macrolide, halichondrin B, which was originally isolated from the sea sponge *Halichondria okadai*. Antibody-drug conjugate (ADC) combining a cytotoxic agent of marine origin with a selective monoclonal antibody such as brentuximab vedotin, enfortumab vedotin, polatuzumab vedotin, and belantamab mafodotin. These ADC's notably include in their structures a microtubule disrupting MNP derivative, i.e. either monomethyl auristatin E or monomethyl auristatin F that are synthetic analogs of the highly cytotoxic dolastatin 10 that was historically isolated from the Indian Ocean sea hare *Dolabella auricularia*. The anticancer MNPs trabectedin and its derivative lurbinectedin are tetrahydroisoquinoline alkaloids originally identified in the Caribbean sea squirt *Ecteinascidia turbinata*. Plitidepsin is a cyclic depsipeptide anticancer drug historically isolated from the marine tunicate *Aplidium albicans*.

Figure 3. A benchmark procedure to implement MNP production in microbial cell factories. A. Theoretical pathway reconstruction. Powerful metabolomics profiling approaches lead to the accurate detection of plausible reaction intermediates while recent advancements in molecular networking provide new efficient opportunities in the processing of the acquired metabolomics data. **B. Identifying a first set of candidate genes.** Once compounds are connected and a reaction sequence has been postulated in silico, bioinspired organic chemistry and literature data may then help in identifying a type of enzymatic activity required for each step. At this stage, the availability of the holobiont metagenome and massive transcriptomics data are of primary importance since searching the specific functional conserved domains may lead to define first sets of candidate genes. **C. Prioritization procedures.** The various possibilities include the screen for homology to orthologous gene sequences of already characterized biosynthesis steps in other models or the search of physical gene clusters. The most powerful and global approach that have recently emerged to rationally prioritize gene sequences in metabolic engineering relies on gene co-expression analysis. **D. Validating the correct activity of each predicted genes/enzymes.** To this aim, biochemical characterization of recombinant proteins heterologously expressed in bacteria or yeasts still represents a basic but reliable approach. **E. Pathway reconstruction in a microbial chassis.** A key factor of success to this aim is the choice of an appropriate heterologous host (a bacterium or a yeast for instance). In such a perspective, extensive synthetic biology toolboxes are available for both microbial models such as genomic integrative constructs, including CRISPR-Cas9 technologies, and auto-replicative plasmids. **F. MNP synthesis in the microbial host.** Main strategies include optimization of media and culture conditions but also implementation of bioreactors, screening of enzyme variant with improved activities, adaptation of the host metabolism, and optimization of enzyme targeting. **G. Optimizing MNP synthesis with multiple iterative refinements based on the Design-Built-Test-Learn cycle.**

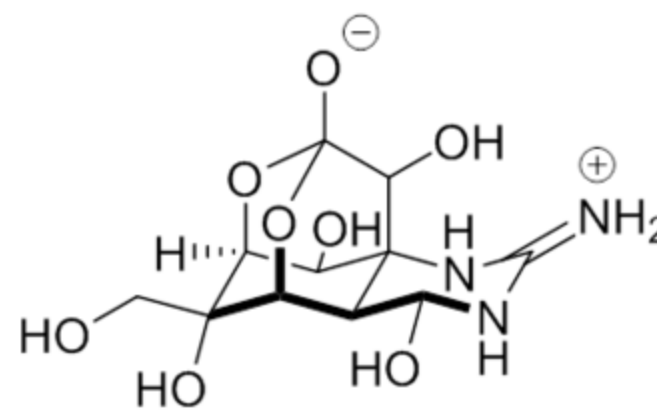
Figure 4. Current (A) and future (B) application fields of metabolic engineering and microbial cell factories.



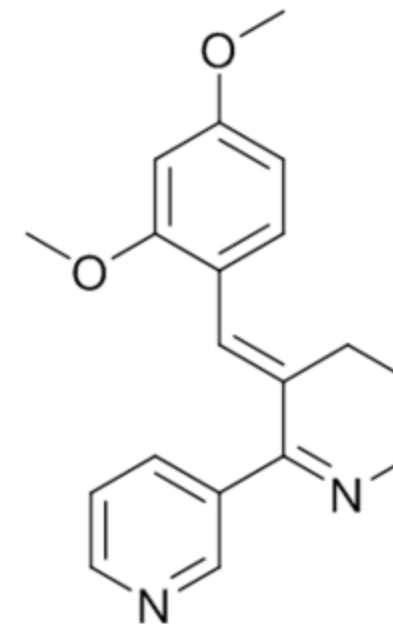
omega-3-acid ethyl ester



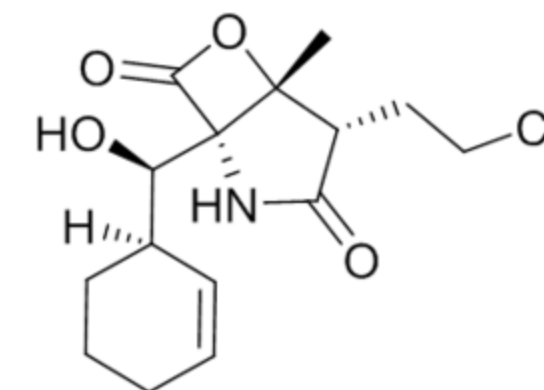
spongouridine



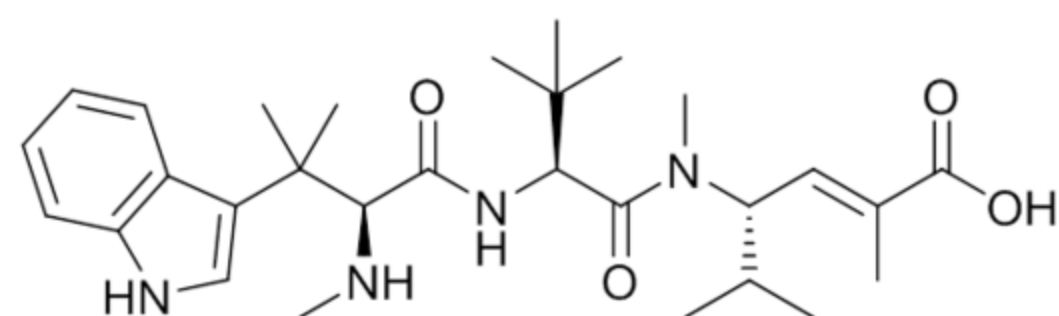
tetrodotoxin



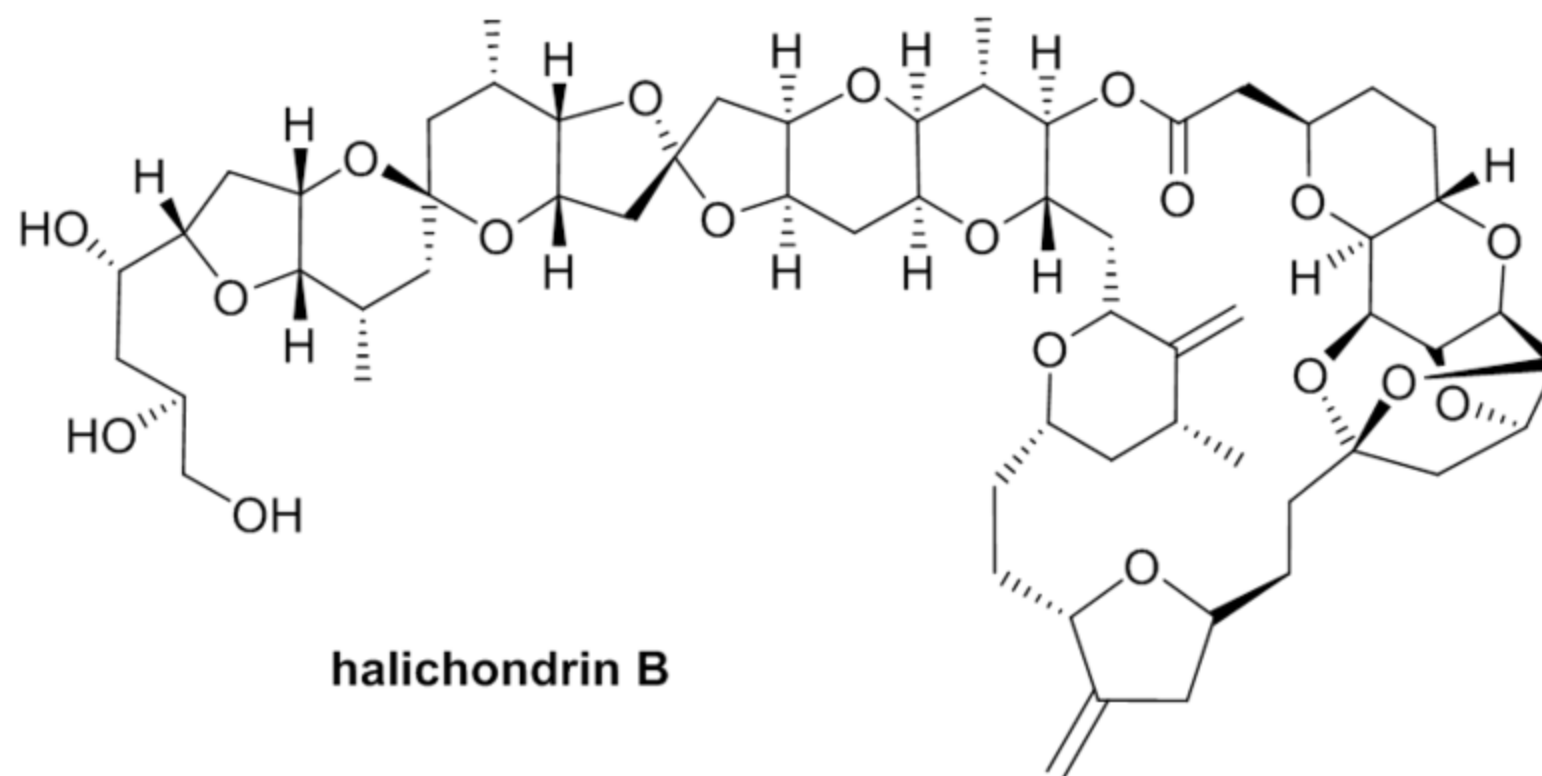
GTS-21



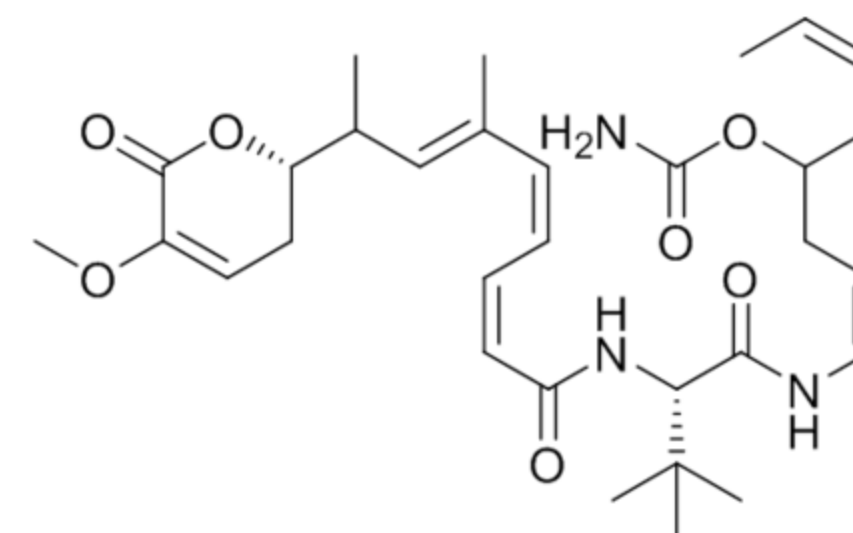
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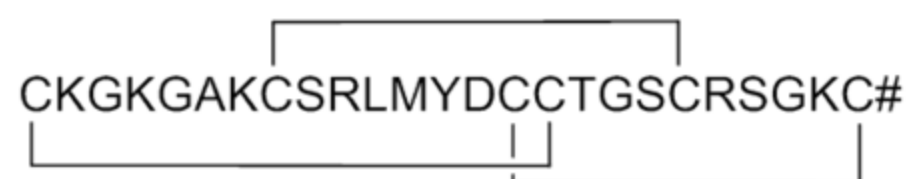
hemiasterlin



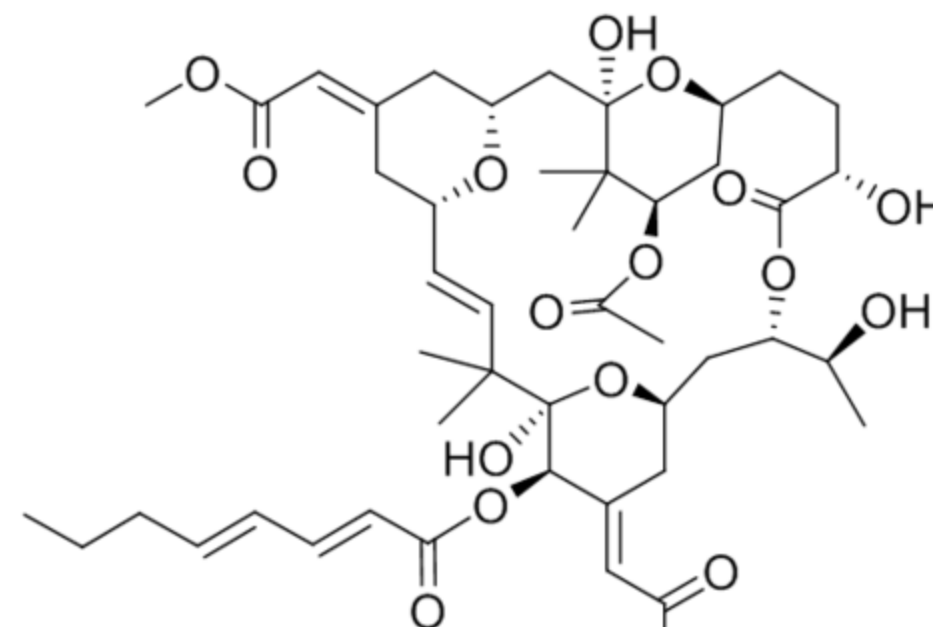
halichondrin B



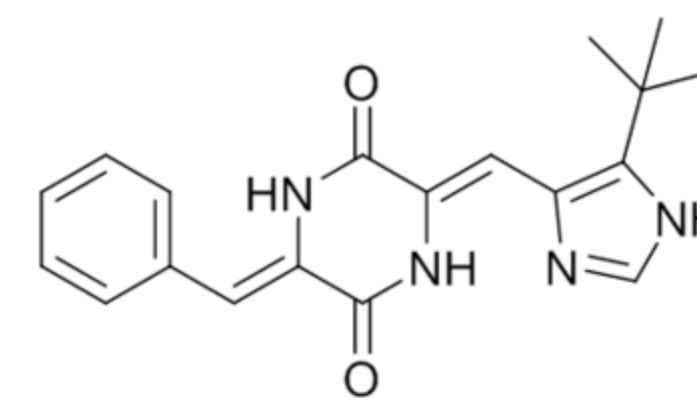
plocabulin



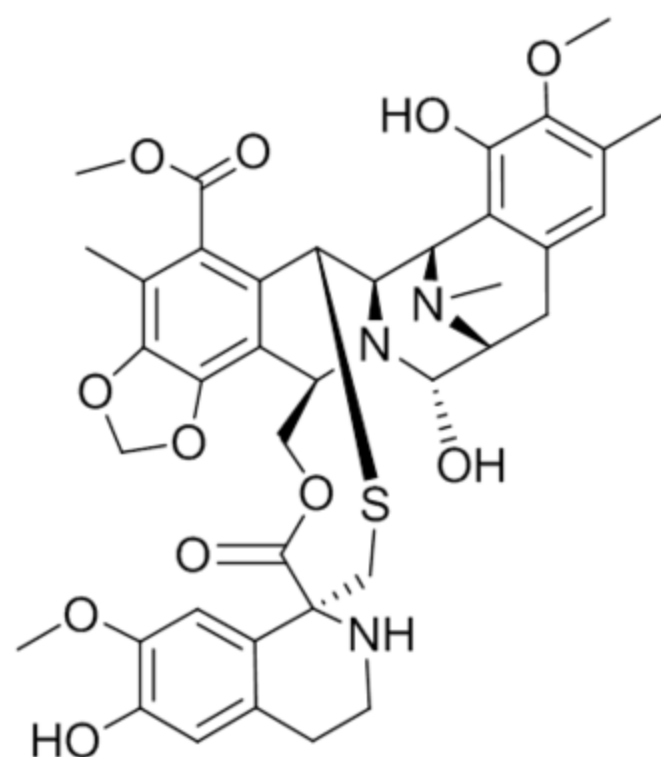
ω-conotoxin MVIIA



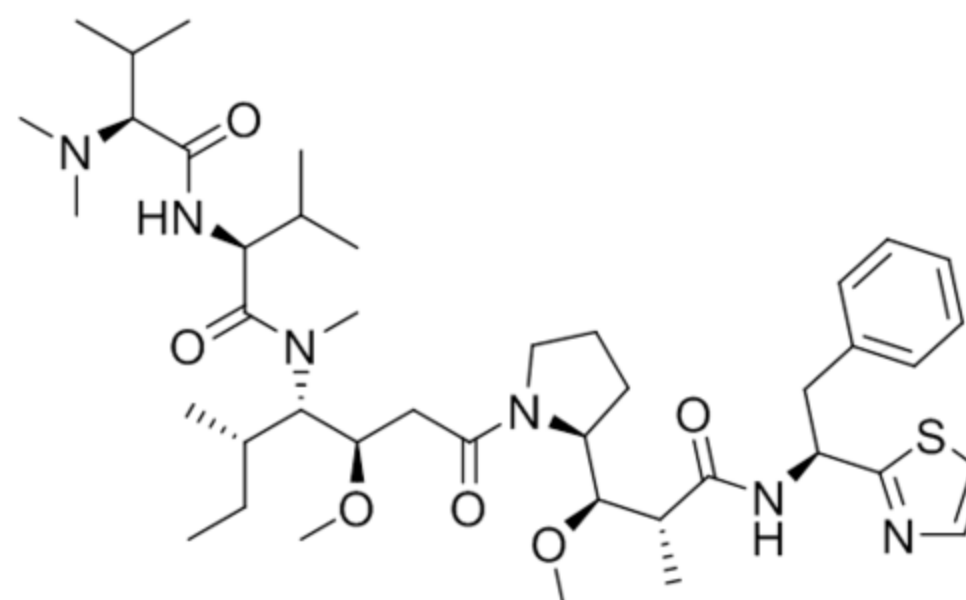
bryostatin 1



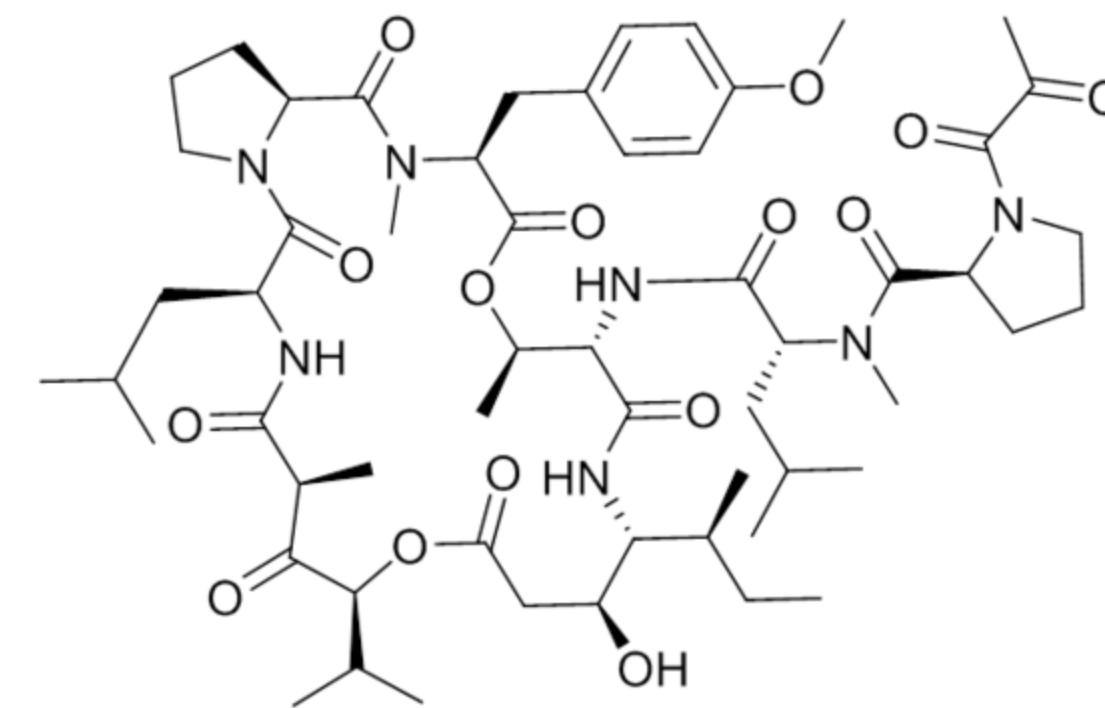
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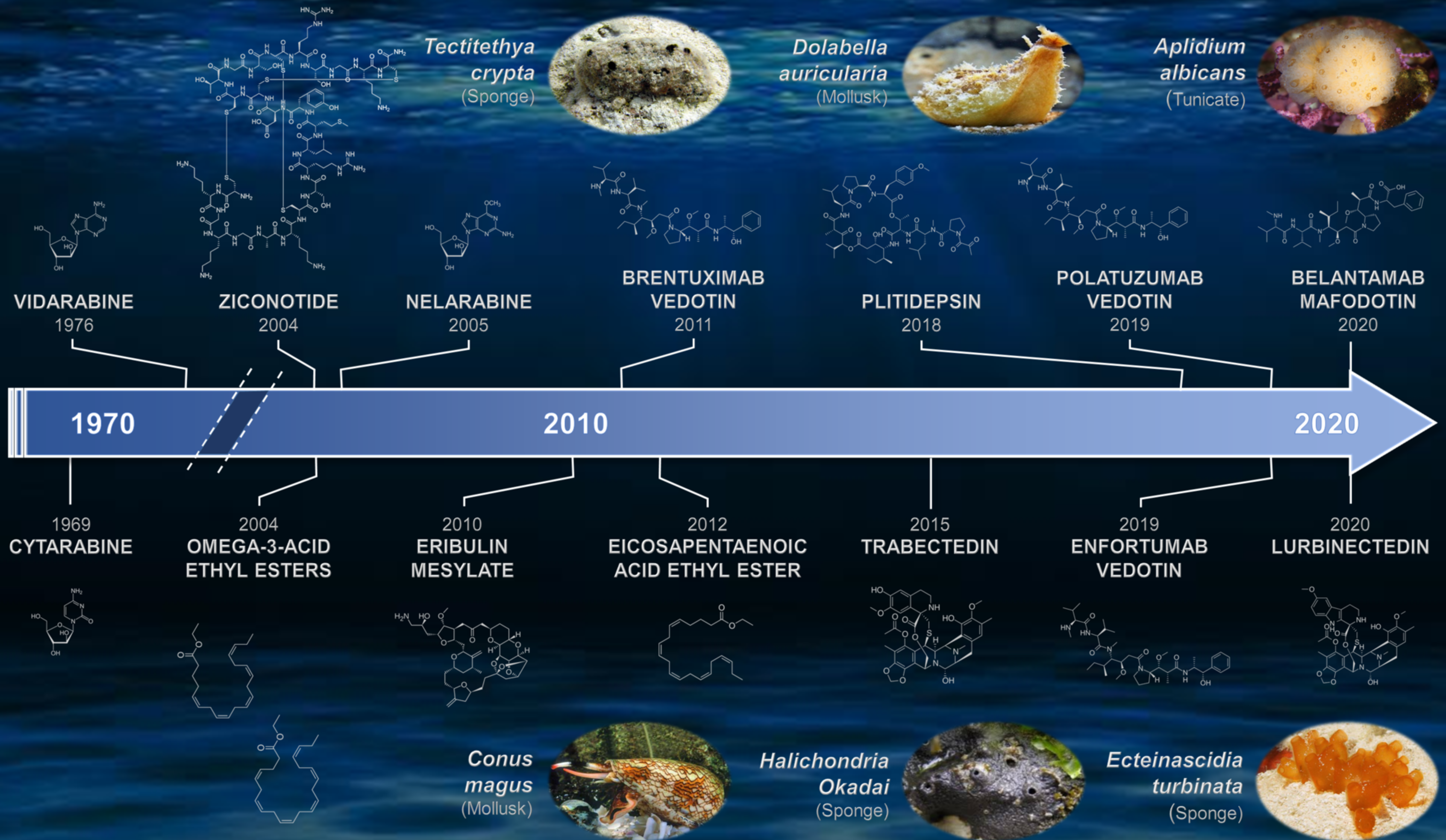
trabectedin

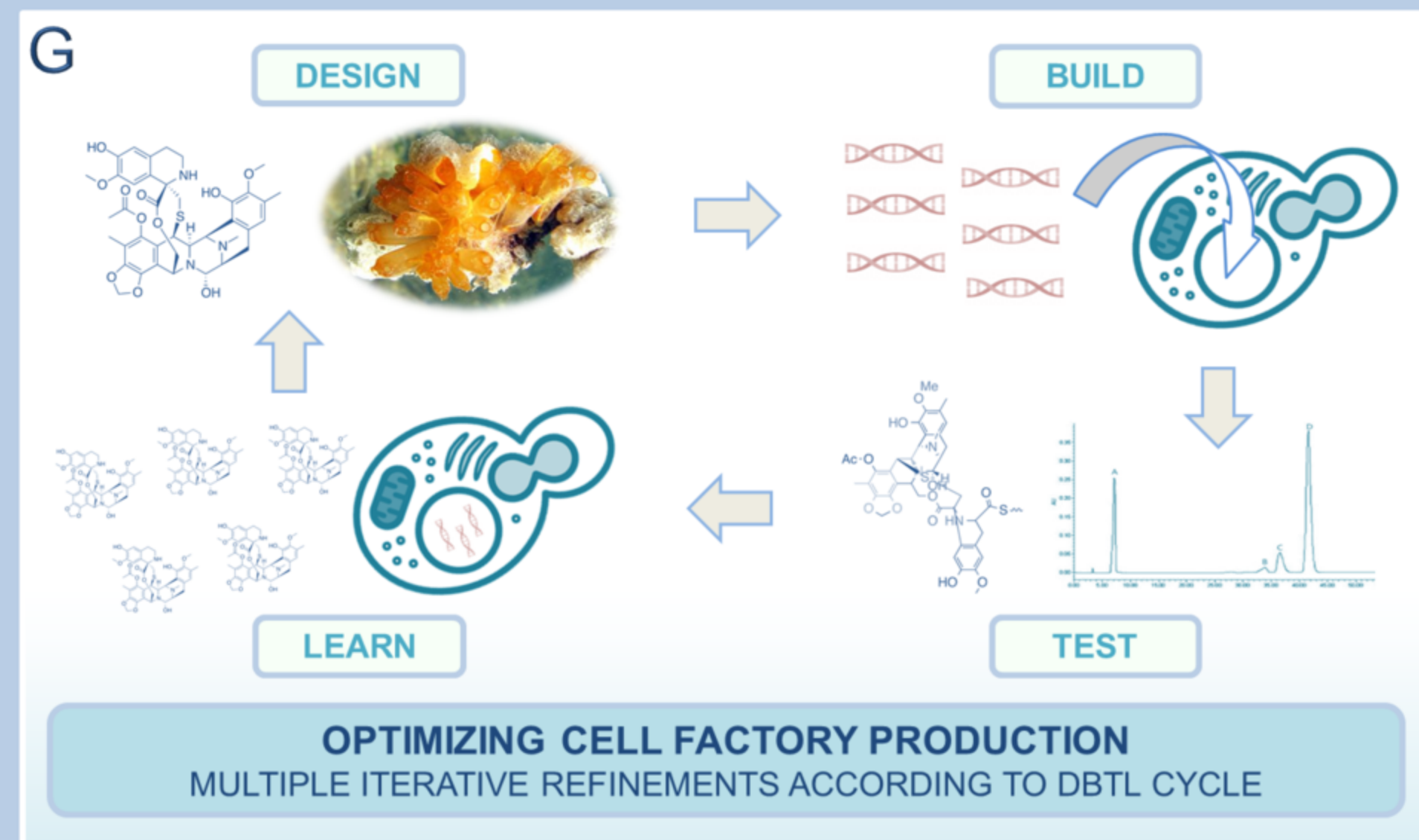
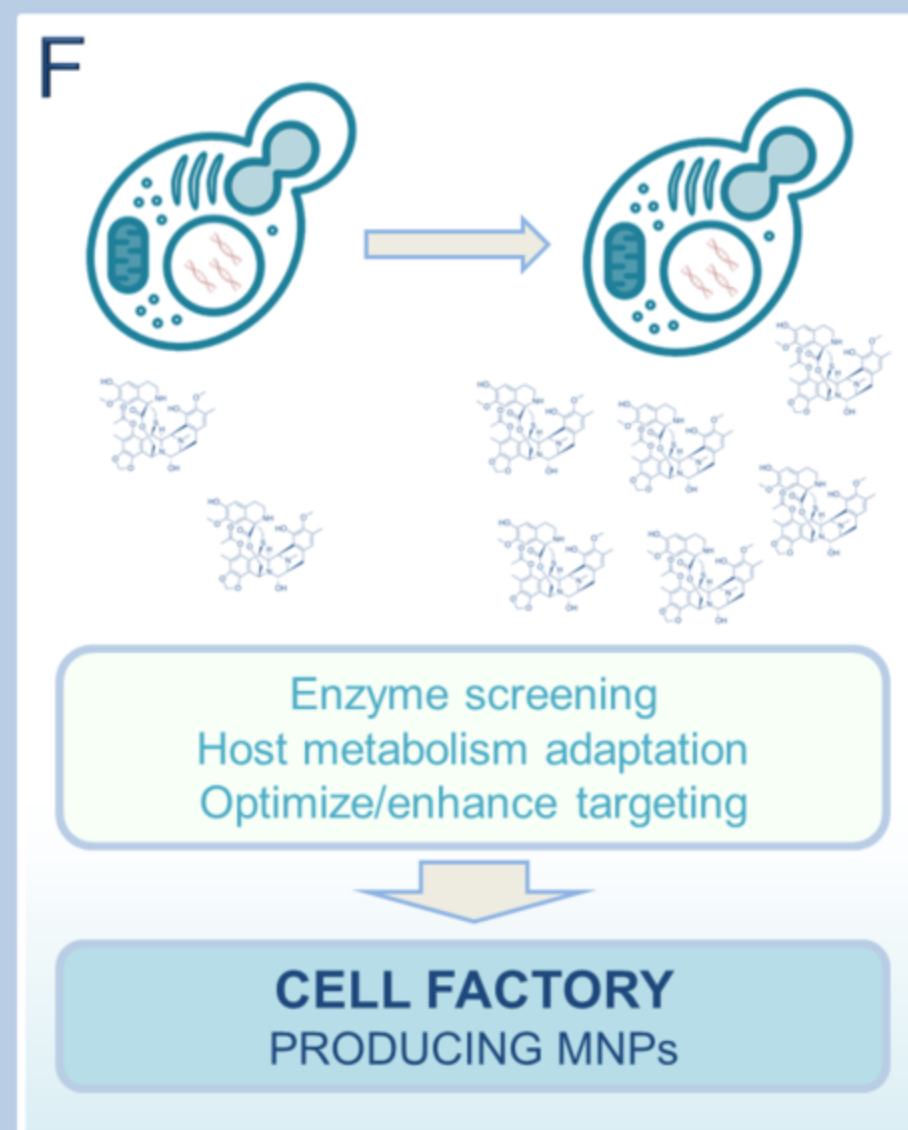
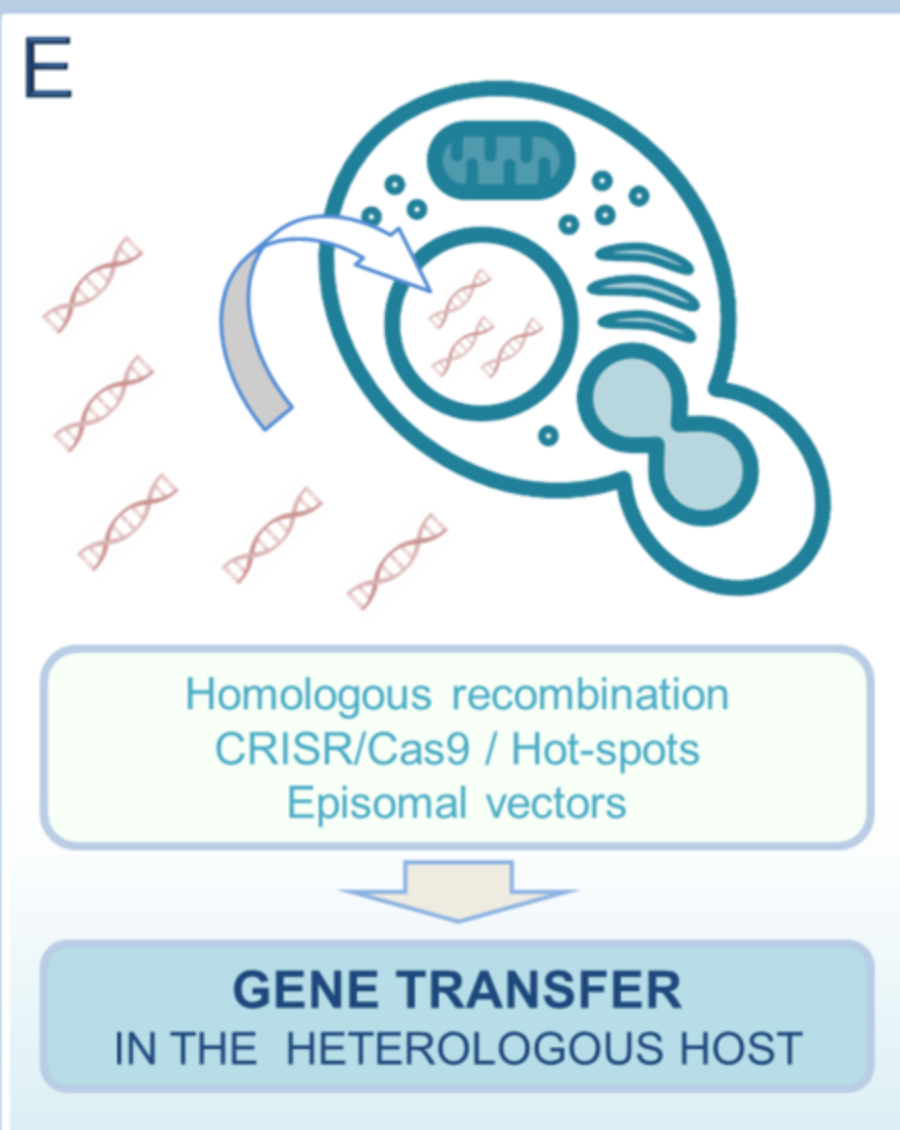
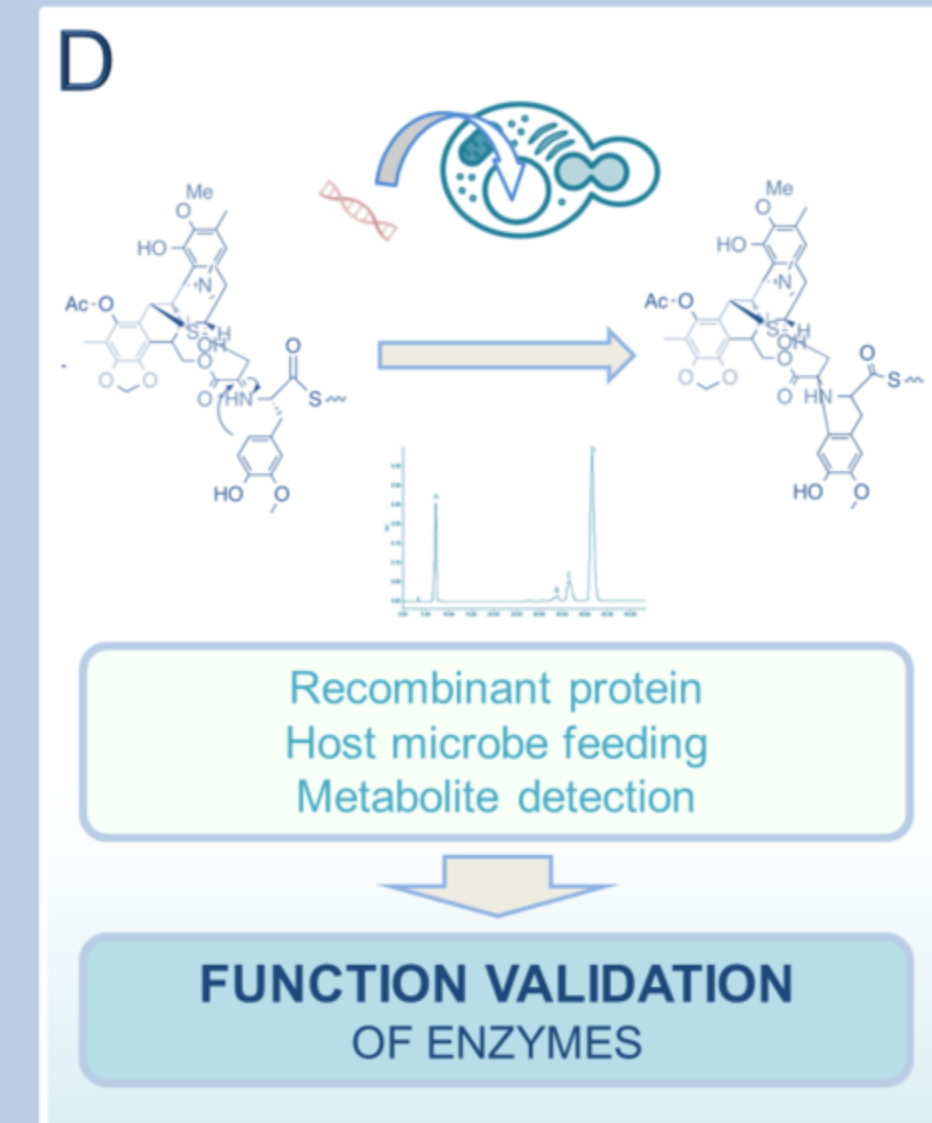
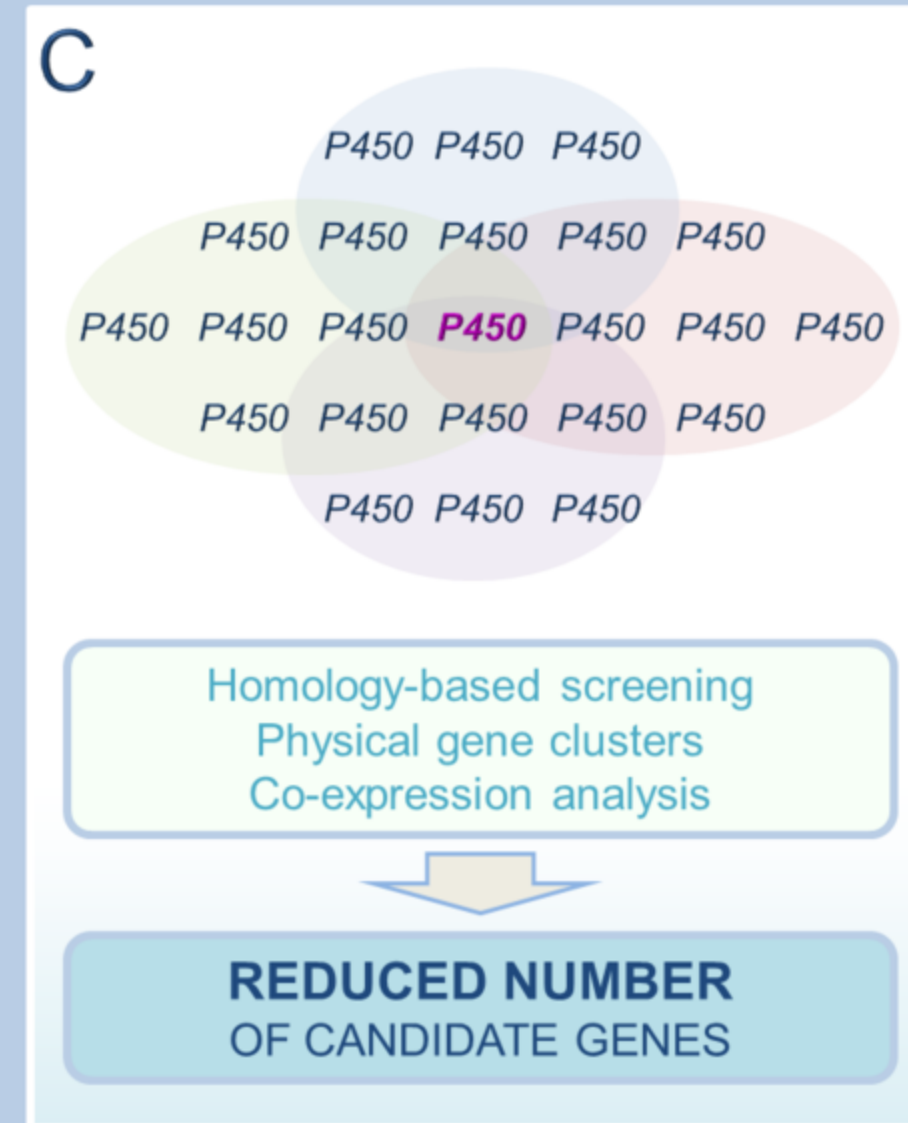
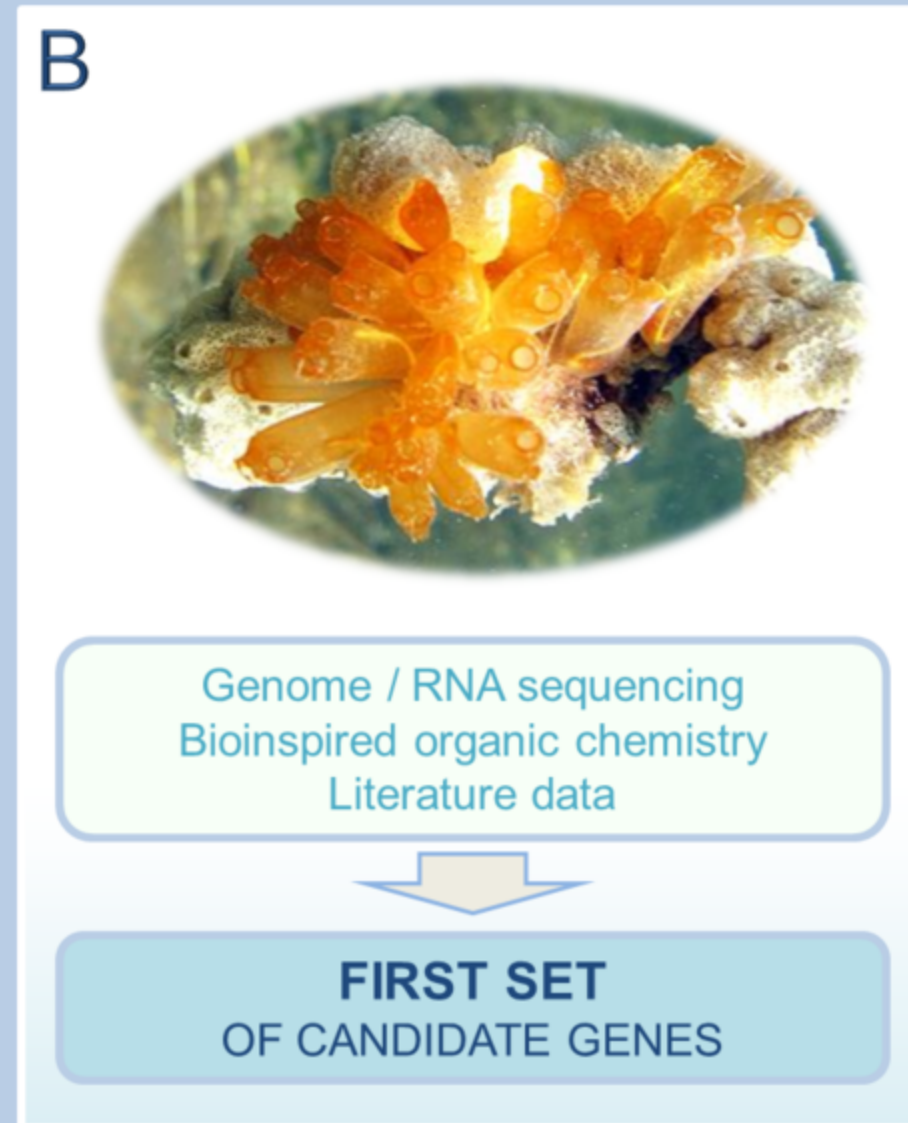
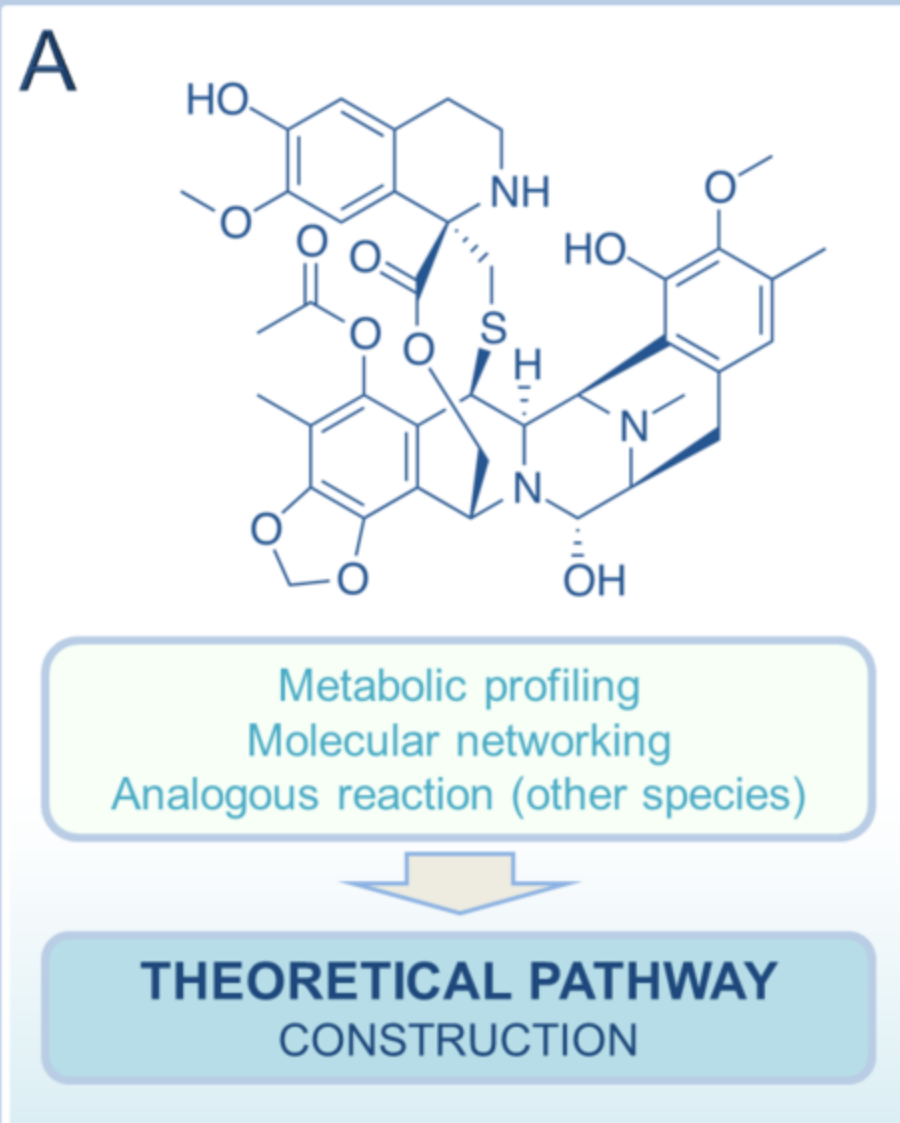


dolastatin 10



plitidepsin

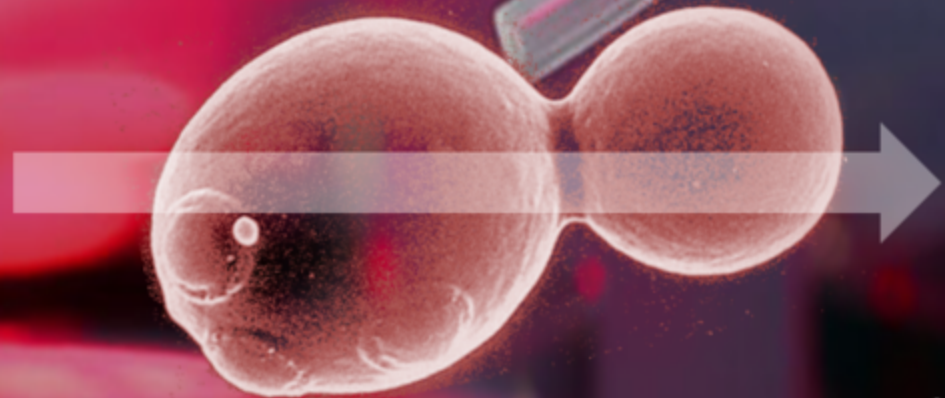




A RED/WHITE BIOTECHNOLOGIES

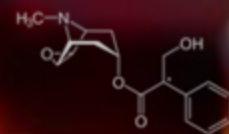
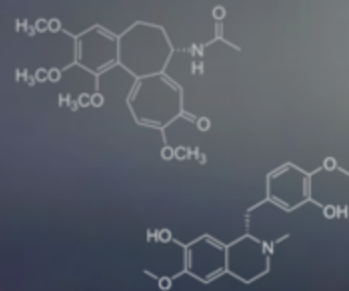
MICROBIAL CELL FACTORIES

CARBON SOURCES



SPECIALIZED METABOLITES

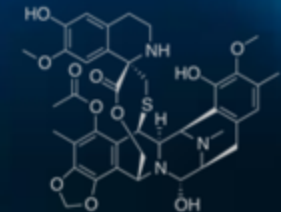
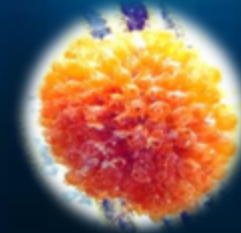
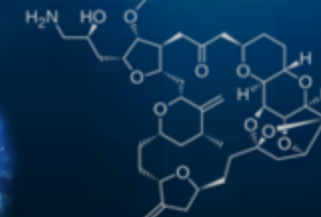
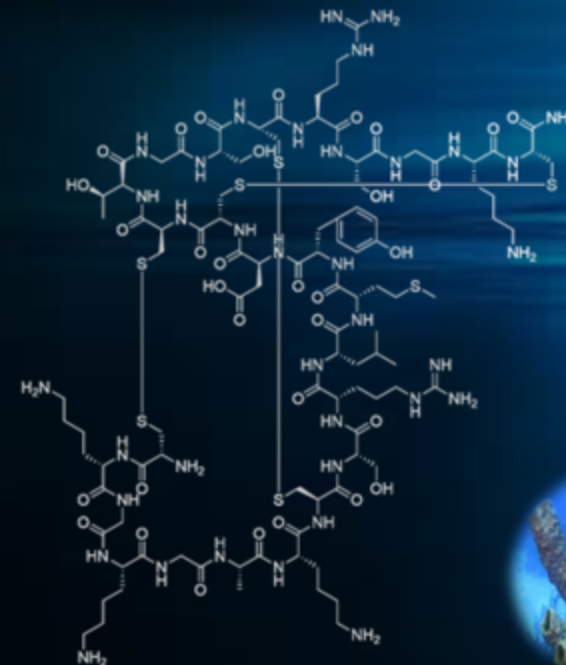
METABOLIC ENGINEERING
SYNTHETIC BIOLOGY
SYSTEM BIOLOGY



B

BLUE BIOTECHNOLOGIES

MARINE NATURAL PRODUCTS



MARINE RESOURCES

Bacteria

Green algae

Red algae

Ascidians

Bryozoans

Cyanobacteria

Porifera

Echinoderms

Brown algae

Mollusks

Dinoflagellates

Cnidarians

Fungi