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Exploring novel bioactive compounds from marine microbes

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The historical paradigm of the deep ocean as a biological 'desert' has shifted to one of a 'rainforest' owing to the isolation of many novel microbes and their associated bioactive compounds. Recently, there has been an explosion of information about novel bioactive compounds that have been isolated from marine microbes in an effort to further explore the relatively untapped marine microbes and their secondary metabolites for drug discovery. The microbes are recovered and purified from the ocean by both conventional and innovative isolation methods to obtain those previously thought to be 'uncultivable'. To overcome the difficulties and limitations associated with cultivation techniques, several DNA-based molecular methods have been developed to bypass the culture-dependent bottleneck. Bioactive compounds isolated using the above strategies have not only shown importance in biotechnological and pharmaceutical applications but have also increased our understanding of the diversity of marine microbiota, ecosystem functions and the exploitable biology.

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Introduction

Although more than 30 000 diseases have been clinically described, less than one-third of these can be treated symptomatically and only a few can be cured. New therapeutic agents are urgently needed to treat medical needs that are currently unmet. Natural products once played a major role in drug discovery [1••,2].The marine environment covers more than 70% of the world's surface. In the past, this has proven to be a rich source of extremely potent compounds [3,4•], which represent a considerable number of drug candidates [5••]. However, to date, the biodiversity of marine microbes and the versatility of their bioactive metabolites have not been fully explored.

Microbes can sense, adapt and respond to their environment quickly and can compete for defense and survival by the generation of unique secondary metabolites. These compounds are produced in response to stress and many have shown value in biotechnological or pharmaceutical applications. The marine environment was once thought to have high salt, poor nutrition and less microbial growth. On the contrary, soil microbes are widely regarded to live in a much more crowded and competitive environment. The ecology of marine natural products actually reveals that many of these compounds are chemical weapons and have evolved into highly potent inhibitors of physiological processes in the prey, predators or competitors of the marine organisms that utilize them for survival. Venter *et al.* [6••] harnessed the power of high-throughput DNA sequencing and computational genomics to produce a massive dataset of large DNA fragments from the total microbial genomes extracted from the subtropical North Atlantic Ocean off the Bermuda coast. They studied the DNA extracted from ~1500 l of surface seawater and within this identified more than 1.2 million new genes. The discovery of such an enormous number of new genes from such a small sample obtained in one of the world's most nutrient-impoverished bodies of water poses significant challenges to the emerging field of marine molecular microbial ecology and evolutionary biology. Marine microbes live in a biologically competitive environment with unique conditions of pH, temperature, pressure, oxygen, light, nutrients and salinity, which is especially rich in chlorine and bromine elements. There is no wonder that marine microbial metabolites exhibit special biological activities compared with 'terrestrial' bacteria [7•,8].

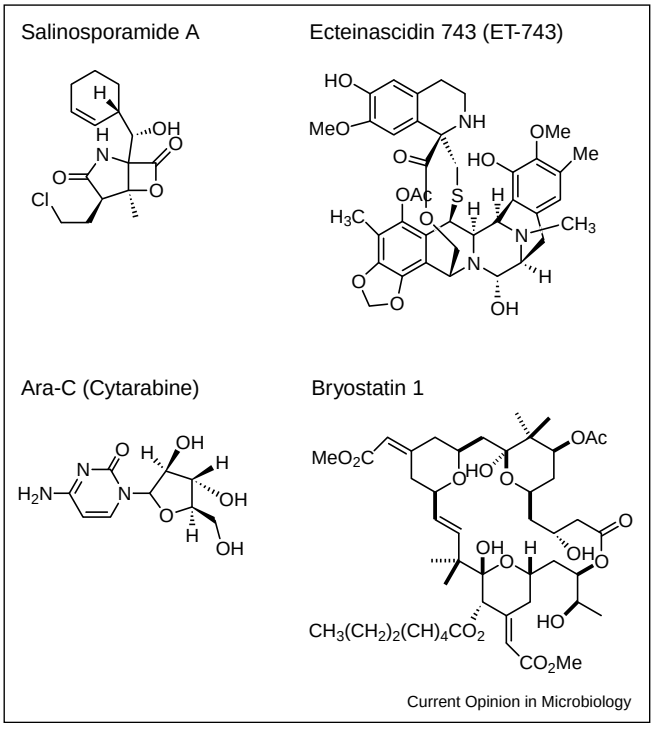
The emphasis of this review is on marine microbial producers and on how to maximize their potential to generate novel biologically active compounds. We highlight three different strategies to directly isolate marine microbes and their biosynthetic gene clusters.

Potent therapeutic agents isolated from marine organisms using a conventional culturing method

Perhaps the first notable discovery of a biologically active marine natural product was reported over 50 years ago by

Bergman [4•]. The discovery of arabinose-based nucleosides was the first demonstration that naturally occurring nucleosides could be found that contain sugars other than ribose and deoxyribose [4•]. Dedemnin B isolated from the tunicate *Tridemnum solidum* was the first defined marine product to enter clinical trials for any major human disease. Since then, there has been an explosion of interest in alternative nucleoside compositions; the antiviral drugs Ara-C (Figure 1) and Ara-A were subsequently developed, with obvious linkages to later antiviral agents such as acyclovir and AZT (also known as zidovudine). Synthetic analog studies led to the development of Ara-C (the drug name of which is cytarabine) as a clinically useful anticancer agent [4•,9•]. A probable biological reason for the presence of such highly bioactive compounds in marine microbes could stem from the necessity of a toxin to be potent because of the diluting effect of seawater. However, such high bioactivity of a potential drug could also lead to toxicity, which is a problem in the drug discovery process. Many drugs could be more effective at a reduced dosage if low doses of synergistic compounds are simultaneously introduced. This synergistic premise might offer a unique method for marine drug discovery and could rescue marine drug candidates that have been abandoned because of inadequate safety profiles [2]. Bryostatin (Figure 1) is likely to be produced by a bacterial symbiont and is now in combination therapy [4•].

Figure 1



Structures of some representative marine bioactive metabolites.

Currently, no other approved marine drugs have been obtained directly from the ocean; however, a significant number of marine-derived compounds have been in clinical or in preclinical trials. More than 720 marine metabolites were reported in the literature during 2003, about half of which demonstrated biological activities. Yondelis, better known as ecteinascidin 743 (Figure 1), is in Phase II and III trials in Europe and in the USA, respectively, for treatment of soft tissue sarcoma, and the Conus toxin, also known as Ziconotide or Prialt, is in Phase III clinical trials for intractable pain [4•].

Table 1 summarizes the current status of representative compounds isolated from marine sources. The marine natural products are classified in the following categories: antiparasitic drugs, antinematodal drugs, antituberculosis agents, antiviral leads and antifungal agents [10••]. They include aureol, puupehenone, sarcophine, palinurin and manzamine alkaloids [11•].

In addition, there are many marine organisms, such as cone snails, whose toxic venom is used for defense and contains up to 50 different peptides that selectively inhibit the function of ion channels involved in the transmission of nerve signals in animals [12•]. As we can see from the table, sponges, together with other marine organisms such as corals, are an important source of biologically active natural products. Several microbes isolated from sponges and from other marine animals produced the same compounds in the laboratory as those originally isolated from their hosts. Because the majority of marine invertebrates have no methods of physical defense against predators, it is reasonable to hypothesize that symbiotic marine microorganisms are the original producers of these bioactive compounds [8].

It is reported that sponges are often associated with symbiotic microbial populations that include archaea, bacteria, actinomycetes, fungi, cyanobacteria and microalgae. In some cases, it is these microorganisms, and not the sponge cells, that are the probable source of the secondary metabolites of interest [13]. For example, the polybrominated biphenyl ether antibiotics isolated from the sponge *Dysidea herbacea* are really produced by the endosymbiotic cyanobacterium *Oscillatoria spongeliae* [13]. Work on isolation of and cultivation of sponge symbionts and the nature of their symbiotic relationships have been reviewed elsewhere [13]. Not surprisingly, many marine microbes have been cultivated and isolated from seawater, sediments and symbiotic hosts by conventional approaches inherited from soil microbiology [14]. In addition to the open ocean, there are diverse and dynamic areas, such as mangroves, coral reefs, hydrothermal vents and deep-sea sediments, in which microbes can be searched for. Natural products have been isolated from marine invertebrates such as sponges, tunicates, mollusks and bryozoans. This not only demonstrates

Table 1

Representative marine metabolites with different therapeutic activities.

Compound	Type	Source
Antifungal activity		
Aurantoside B	Polyketide	*Siliquariaspongia japonica* sponge
Phorboxazole A	Macrolide	*Phorbas* sp. sponge
Halishigamide A	Macrolide	*Halichondria* sp. sponge
Halichondramide	Macrolide	*Halichondria* sp. sponge
Fascaplysin	Bis (indole) alkaloid	*Fascaplysinopsis* sp. sponge
Meridine	Polycyclic alkaloid	*Corticium* sp. sponge
Bengazole A	Oxazole-containing fatty-acid ester	*Jaspis* sp. sponge
Ptilomycalin A	Polycyclic	*Ptilocaulis spiculifer* guanidine alkaloid sponge
Antituberculosis activity		
Alkaloid (+)-8-hydroxymanzamine	Heterocyclic ring system attached to α-carboline moiety	*Pachypellina* sp. sponge and *Petrosiidae* genus
Axisonitrile-3	Cyanosesquiterpene	*Acanthella klethra* sponge
Litosterol	C-19 hydroxysteroid	Okinawan soft coral *Litophyton viridis*
Puupehenones	Shikimate-sesquiterpene	Sponges
Antiviral activity		
Papuamides A	Cyclic depsipeptides	*Theonella mirabilis* and *T. swinhoei* sponges
Avarone	Sesquiterpene hydroquinone	*Dysidea avara* sponge
Gymnochrome D	Brominated phenanthroperylenequinone	Fossil crinoid *Gymnocrinus richeri* pigments
Microspinosamide	Cyclic depsipeptide	*Sidonops microspinosa* sponge
Solenolide A	Diterpene lactone	Gorgonian of the genus *Solenopodium*
Hennoxazole A	Oxazole-containing alkaloid	*Polyfibrospongia* sp. sponge
Thyrsiferol	Triterpene	Red alga *Laurencia venusta*
Spongiadiol	Tetracyclic furanoditerpene	Deep-water *Spongia* sp
Antibacterial activity		
Squalamine	Aminosterol	Dogfish shark *Squalus acanthias*
Cribrostatin 3	Isoquinolines	*Cribrochalina* sp. sponge
Bromosphaerone	Bromoditerpenes	*Sphaerococcus coronopifolius* red alga
Pestalone	Chlorinated benzophenone	*Rosenvingea* sp. brown alga
Jorumycin	Dimeric isoquinoline alkaloid	*Jorunna funebris*
Antiprotozoal activity		
Cyclic peroxides	Peroxide	*Plakortis* aff *angulospiculatus* sponge
Sigmosceptrellin-B	Alkaloid	*Diacarnus erythraeanus* red sea sponge
Plakortide	Cyclic peroxylactone	*Plakinastrella onkodes* sponge
Ascochyta salicorniae	Fungus	*Ulva* sp. green alga
Anthelmintic activity		
Dihydroxytetrahydrofuran	Tetrahydrofuran	*Notheia anomala* south Australian marine brown alga
Amphilactams		*Amphimedon* sp. sponge
Geodin A magnesium salt lactam tetramic acid	Macrocyclic polyketide	*Geodia* sp. sponge

the numerous opportunities that oceans provide for the discovery of new compounds but also validates the pharmacological value of the exploration of oceans for novel compounds.

There are some concerns about the isolation of marine microbes. Some researchers claim that these organisms are hard to maintain in the laboratory environment. However, one successful case was the recent discovery of a new genus of actinomycetes, *Salinospora*, which can only be found in the marine environment [15,16]. In excess of 2,500 strains of this taxon have now been isolated. The potent proteasome inhibitor salinosporamide A (Figure 1) was isolated from a culture of a *Salinospora* sp. that originates from a heat-treated marine sediment sample in the Bahamas. The structure of salinosporamide A, including its absolute stereochemistry, was deduced by way of spectral and X-ray analyses. Salinosporamide A displayed potent and selective *in vitro* cytotoxicity against cell lines in the National Cancer Institue (NCI) panel. It also exhibited inhibition of the chymotrypsin-like proteolytic activity of the purified 20S proteasome. The unique function of the core-fused-lactam–lactone bicyclic ring structure of salinosporamide A appears to contribute to its anti-cancer potency. It is therefore not surprising that this compound showed potent inhibition of cancer growth, including human colon carcinoma, non-small cell lung cancer and breast cancer.

It seems probable that there are many natural products awaiting to be discovered and tested for different medical applications. Indeed, more and more novel marine microbes and novel bioactive secondary metabolites will continue to be isolated [7•].

Innovative ways to isolate less-culturable or uncultivable marine microbes

Microbial community analyses by epi-fluorescence microscopy and rRNA sequencing strategies have revealed that the microorganisms that can be cultured only represent a small fraction of the microbial population and might not be the most prevalent in the natural environment. It is expected that one of the largest efforts in this field of research in the next decade will be the exploration of a means to grow less-culturable marine microbes. It is thought that the reason for the enormous discrepancy between the total viable cell count and the cell count of culturable cells might be because of the following: cell damage by oxidative stress; formation of viable but non-culturable cells; inhibition by high substrate concentrations; induction of lysogenic phages upon starvation; and lack of cell–cell communication in laboratory media. Among the approaches used to enhance the resuscitation of less culturable strains, are the addition of cell signaling molecules and the use of oligotrophic isolation media [17,18].

One method utilized the concept of 'extinction culturing' to isolate cultures in small volumes of low-nutrient media (typically three orders of magnitude less than common laboratory media). Many novel strains among the *Proteobacteria*, *Planctomycetes*, *Bacteroidetes*, *Acidobacteria* and *Verrucomicrobia* were obtained; these included some previously believed to be uncultivable [18]. Several novel bacterial strains from the western Sargasso Sea were successfully isolated using this method [19].

Another development is based on the assumption that uncultivable microorganisms might grow in pure culture if provided with the chemical components of their natural environments. To test this hypothesis, specialized growth chambers were developed that mimic the natural marine environment of the microorganisms to optimize the isolation of pure cultures *in vitro*. Microorganisms in sediment samples were serially diluted with seawater, mixed with agar, placed in diffusion chambers, and incubated in an aquarium that simulated the environmental conditions of the sampling site. The number of microcolonies obtained was in the range of 2–40% of the cells inoculated, which is much higher than the recovery rate for conventional cultivation methods [20].

A high throughput cultivation method based on the combination of single cell encapsulation and a gel microdroplet was also developed. Gel microdroplets are small porous gel matrixes that encapsulate individual cells. This method allows the exchange of metabolites and/or signaling molecules that are produced by other microorganisms. It enables cells to grow with nutrients that are present in their natural environment. After long periods of incubation with a constant flow of a very low concentration of nutrients, flow cytometry was used to separate gel beads containing cells that had grown into small microcolonies from those that had not. Many novel marine bacteria have been isolated in this way, including members of previously uncultured groups that are abundant in seawater [21••,22,23].

One argument against the application of less-culturable strains to a drug discovery program is that they can not be cultured at a high cell density. The counter argument is that, although they might initially require the addition of growth factors or oligotrophic growth conditions, there is evidence that once cultured the organisms can be grown using nutrient-rich media. Using 960 cells cultured in microdroplets, 67% of the cultures were subsequently able to grow to densities of >107 cells per ml [21••,22,23]. This allows the cells to be cultured in a manner that could easily be applied to drug discovery platforms.

The use of metagenomic methods to bypass the culture-dependent bottleneck

It is widely accepted that more than 99.8% of the microbes present in many environments are not readily culturable. Metagenomic technology tries to overcome this bottleneck by the development and the utilization of culture-independent approaches. The major sequencing approach in the Sargasso Sea identified a number of DNA sequences that was by far greater than had previously been detected [6••]. This study is likely to lead to new investigations of the microbial world and its evolution. A recent example gave significant insights into the community structure and the metabolism of a natural acidophilic biofilm that is growing on the surface of drainage flowing from an acid-mine. This was mainly possible through the reconstruction of microbial genomes that are present in this niche. For this purpose, the near-complete genomes of *Leptospirillum* group II and Ferroplasma type II were reconstructed. A detailed analysis of these genomes allowed pathway-reconstruction of carbon fixation and energy generation and provided insights into the survival strategies of microbes within this extreme acidic environment [24••]. Cultivation-independent biochemical studies of microbial mats of a northwestern Black Sea shelf, which oxidize methane under anaerobic conditions, resulted in the identification of a prominent nickel-containing protein. This approach is not only remarkable because of the biochemical and ecological findings but also because it uncovered, for the first time, the function of a novel protein. Metagenomic technologies were subsequently applied to determine the corresponding gene [25••].

The metagenomic approach maximizes the diversity of libraries of marine natural product extracts by accessing

the DNA directly from marine samples [23,26]. Such DNA can be directly isolated, digested into large fragments with restriction enzymes, and cloned into an artificial vector. The vector is then transformed into a surrogate host. Environmental DNA libraries can be prepared that contain large fragments of DNA from a wide range of uncultivated bacteria within an environmental sample. This recombinant approach obviates the need for culturing diverse microorganisms and provides a relatively unbiased sampling of the vastly untapped genetic diversity that is present in various microenvironments. As an additional advantage, the genes that encode biosynthesis of a product of interest can be isolated and analyzed using the bioinformatics tools, thus providing a potential boost to the efforts of analytical chemists to identify the product. Furthermore, the possibility of regulating the expression of isolated environmental gene clusters, or combining them with genes for other pathways to obtain new compounds, furnishes a further advantage over traditional natural product discovery methodologies. However, it must be noted that these biosynthetic and regulatory genes might be dormant in the host and that optimal induction conditions might be required for the production of novel natural products [14]. The screening of metagenomic libraries has resulted in the identification of a significant number of novel genes that encode for biocatalysts or for molecules that have a high potential use in pharmaceutical products or in production processes. The isolation of genes that encode for novel therapeutic molecules is a valuable area of research. The genes of interest within these searches are often type I and type II polyketide synthases (e.g. β-ketoacyl synthetases), which are key genes involved in the synthesis of polyketide antibiotics and antitumor agents by Gram-positive *Streptomyces* and are often part of large biosynthetic gene clusters [27••].

Advances in DNA sequencing and in bioinformatic technologies now make it possible to rapidly identify the clusters of genes that encode bioactive compounds and to make computer predictions of their chemical structure based on gene-sequence information [28,29]. These structure predictions can be used to identify new chemical entities before they are expressed in diversified fermentation broth and provide important physicochemical ''handles'' that guide compound purification and structure confirmation. Genome sequence tags (GSTs) are genes involved in natural product biosynthesis. These GSTs are used as probes to screen for the presence of such genes within a clonal library. Any clone that contains a GST can then be screened for novel natural-product gene clusters. More than 450 natural-product clusters have been identified in this manner [29].

Conclusions

The past three years have seen an explosion of information in the field of novel bioactive compounds that have been isolated from marine microbes. It is therefore timely to review the past successes of marine microbial natural products as medicines and to examine future possibilities that arise from both conventional and new technologies to further explore the biodiversity of marine microbes and their associated secondary metabolites.

However, future success is not a matter of old versus new; it is dependent on learning how to apply the existing methodologies of genomics, proteomics, combinatorial chemistry, DNA shuffling, combinatorial biosynthesis, biodiversity, bioinformatics and high-throughput screening to rapidly evaluate the activities in extracts as well as in purified components derived from marine microbes. The remarkable chemical diversity encompassed by natural products continues to be of relevance to drug discovery. Although today's drug discovery engine operates at an accelerated pace compared with the era in which natural products were pre-eminent sources of drug leads, numerous approaches have been developed to capture the intrinsic value of their drug properties. Biodiversity, creativity and versatility of marine microbial metabolites can now be studied by way of culture-dependant and -independent methods. We are in a position to start probing many major questions that, only a few years ago, were beyond our grasp, among them being the functional role of these microbes and their metabolites in marine habitats as well as the significance of ecological and evolutionary processes of such high microbial diversity in the sea.

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