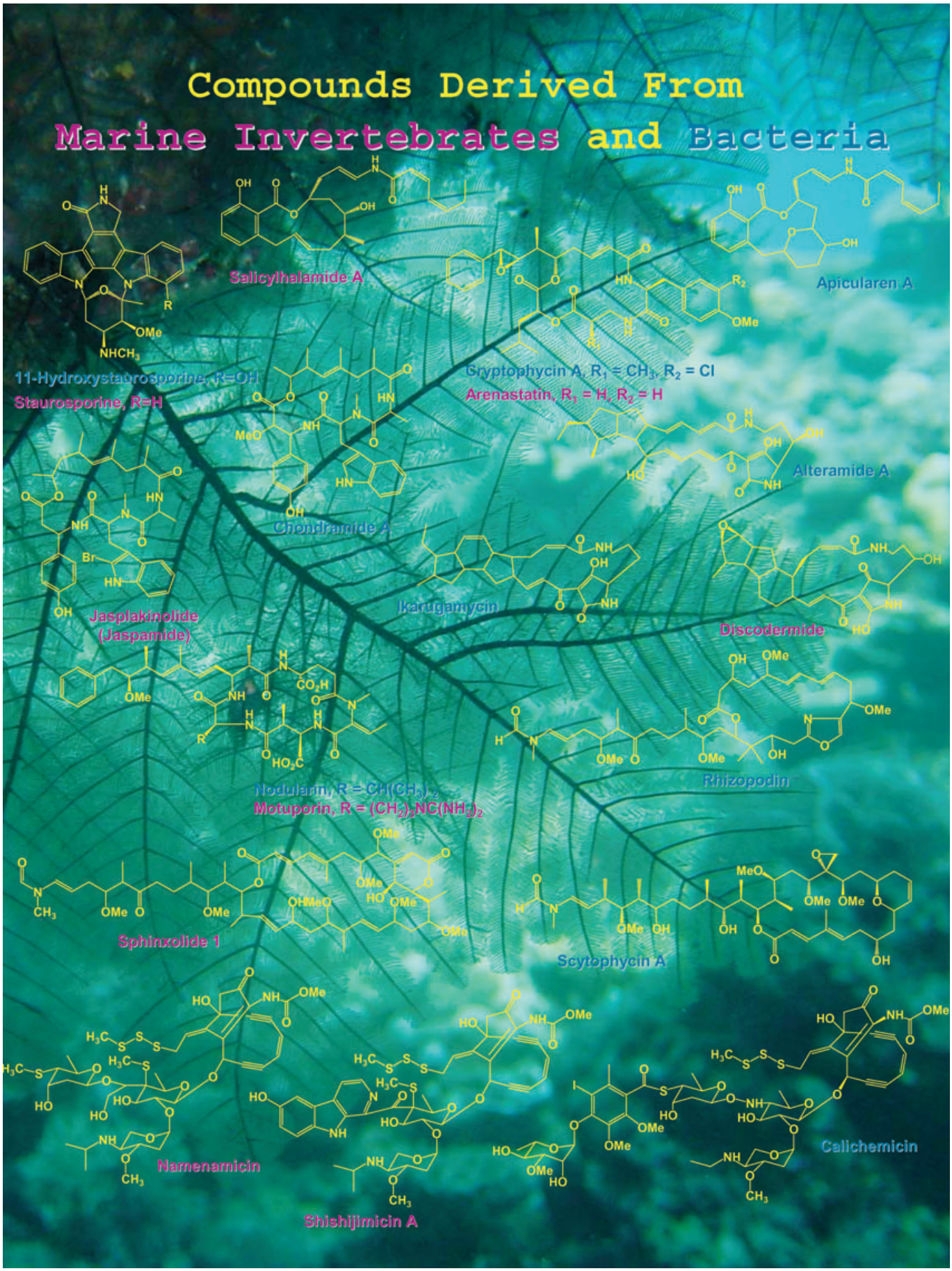

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Utilizing the Power of Microbial Genetics to Bridge the Gap Between the Promise and the Application of Marine Natural Products

J. L. Fortman[a, b] and David H. Sherman*[a]

Marine organisms are a rich source of secondary metabolites. They have yielded thousands of compounds with a broad range of biomedical applications. Thus far, samples required for preclinical and clinical studies have been obtained by collection from the wild, by mariculture, and by total chemical synthesis. However, for a number of complex marine metabolites, none of these options is feasible for either economic or environmental reasons. In order to proceed with the development of many of these promising therapeutic compounds, a reliable and renewable source must be found. Over the last twenty years, the study of microbial secondary metabolites has greatly advanced our understanding of how nature utilizes simple starting materials to yield complex small molecules. Much of this work has focused on polyketides and nonribosomal peptides, two classes of molecules that are prevalent in marine micro- and macroorganisms. The lessons learned from the study of terrestrial metabolite biosynthesis are now being applied to the marine world. As techniques for cloning and heterologous expression of biosynthetic pathways continue to improve, they may provide our greatest hope for bridging the gap between the promise and application of many marine natural products.

1. Introduction

Over the past twenty years, the sea has yielded thousands of bioactive metabolites.[1–3] The most recent *Natural Products Reports* annual review of marine natural products indicates that 677 novel structures were elucidated in 2002 alone.[4] These compounds possess a wide range of potential clinical and commercial applications and have been isolated from both marine micro- and macroorganisms. Currently, detailed analysis and subsequent application is often limited to compounds derived from culturable microorganisms. The gap between potential and application is therefore often a direct result of the resource limitations of marine microorganisms. Some of the compounds isolated are found in concentrations in the order of μg per kg of tissue. This, combined with the limited availability of parent organisms, can result in extremely low supplies of many compounds. To bridge the gap between therapeutic potential and commercial application, several approaches are generally considered. Increased compound supply through the techniques of traditional chemical synthesis and semisynthesis, fermentation, cell culture, mariculture, and biosynthetic pathway cloning are each being advanced by laboratories around the world. Each approach has its merits and limitations. For example, as metabolite complexity increases, the practicality of total chemical synthesis to solve the supply problem decreases. Fermentation of metabolite-producing organisms is only ideal when the metabolite of interest has been isolated from a pure and stable microbial culture. The culture of invertebrate cells has progressed, but it remains a science in its infancy. Mariculture has proven to be tenable for some organisms, but it is somewhat unreliable and cost prohibitive. Advances in the study of microbial secondary-metabolite biosynthesis and metagenomics continue to improve the prospects of cloning and subsequent expression of biosynthetic pathways in heterologous hosts. As the fundamental understanding of secondary-metabolite biosynthesis grows, so does the potential for heterologous pathway expression in overcoming the supply problems for a large percentage of the more structurally complex compounds. This review will show how these techniques are being applied and how the challenges might best be overcome.

2. Applications

Marine sources continue to yield novel compounds with a broad array of bioactivity. These compounds have shown promise in treating cancer, pain, inflammation, allergies, and viral infections.[5] However, at present, few marine natural products have achieved full commercial application. Notable exceptions are the nucleoside analogues Ara-A and Ara-C, which are currently in use as antiviral and anticancer agents.[6] These compounds were discovered in a synthetic library inspired by the activity of the *Cryptotethia crypta* derived nucleoside analogues spongothymidine and spongouridine.[7–9] Pseudopterosin, a terpenoid isolated from the sea whip *Pseudopterogorgia elisabethae*,[10] is active as a topical antiinflammatory agent[11]

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and is currently in use in cosmetic dermatotopical applications.[12] The *Conus magus* (cone snail) derived peptide SNX-111 (Ziconotide) continues to do well in clinical trials as an analgesic agent[13] and appears to be on the cusp of clinical approval. A recent paper highlights the potential of a number of marine metabolites as herbicides and pesticides.[14] Despite this potential, Nereistoxin, originally isolated from the marine worm *Lumbriconereis heteropoda*, and its analogues are the only marine-derived compounds in agricultural use.[15–17]

Table 1 lists the marine-derived compounds currently in clinical trials or preclinical development as anticancer agents. These compounds are representative of the field as a whole, as they are from many different chemical classes and are pure natural products, semisynthetic derivatives, or synthetic analogues based on marine natural products. These medically important compounds will be used to highlight the promise and limitations of a microbial-genetics approach for improved production.

3. Natural Product Biosynthesis

Studies of secondary-metabolite biosynthesis have accelerated in recent years. The field has thus far been dominated by studies on culturable terrestrial bacteria. However, a handful of secondary-metabolite pathways have been described from marine bacteria. The pioneering work promises to move marine microbiology forward in parallel with its terrestrial counterpart and collectively provides a foundation of knowledge to enable a researcher to predict both the content and organization of the genes encoding the biosynthetic enzymes responsible for constructing several families of compounds. These predictive qualities can be utilized to design rational experiments to identify, clone, and subsequently express many secondary-metabolic pathways.

Marine invertebrates have been and continue to be a tremendous source of novel bioactive compounds. The true biosynthetic source of many of these metabolites remains undefined. Given the large numbers of microorganisms living in close association with marine invertebrates, it is very difficult to assign a true biosynthetic origin for any given compound without specific investigation. The similarity of marine-macroorganism-derived compounds to many bioactive compounds isolated from terrestrial microbes (Table 2) is often cited as evidence of a microbial origin.

Localization of these compounds to a specific cell type (for example, sponge choanocytes) is the basis for some arguments that utilize the site of compound storage as a spatial indicator for compound production. These studies have only been conducted on a small percentage of invertebrate-derived compounds (Table 3). Results primarily obtained from sponge-tissue studies show no definitive pattern of localization by compound class. However, as additional studies are completed, such a pattern may emerge. In addition, localization does not necessarily correlate to the site of production. On one hand, many terrestrial microbes secrete their bioactive secondary metabolites into the local environment. On the other hand, given the dynamic nature of the marine environment it is likely that an excreted compound would be effectively washed away, thereby leaving little selective advantage to the producing organism. Conversely, given that sponges have existed for hundreds of millions of years,[112] it is reasonable to propose that coevolution with bacteria may have selected for a symbiotic relationship, which results in sequestering of microbial metabolites in Porifera cells. However, such a relationship has yet to be proven.

Genetic work that focuses on searching for polyketide biosynthesis genes from macroorganisms has suggested that this class of metabolites is produced by symbiotic bacteria.[131,132] It must be noted that the number of these studies remains very limited in comparison to the number of marine-invertebrate-derived polyketides, and thus no set of general principles has yet emerged. However, some evidence involving the role of symbionts in secondary-metabolite production is extremely compelling. Recent work by Piel and co-workers strongly suggests that the beetle-derived toxin pederin (**21**) is of microbial origin. Pederin (**21**) shares a common carbon skeleton with the sponge-derived metabolites mycalamide A (**22**) and onnamide A (**23**).[100,131] Moreover, the biosynthetic gene cluster isolated from the *Paederus fuscipes* metagenome contains the genetic architecture predicted for the much larger onnamide A (**22**) molecule (Scheme 1).[100] This work provides evidence that a known sponge metabolite is produced by a microbial symbiont. While chemical structure similarity and genetic studies

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Table 1. Status of marine-derived natural products in clinical and preclinical trials.[a]

Compound	Source	Status	Biosynthetic Origin	References
bryostatin 1 (**1**)	*Bugula neritina* symbiont *Endobugula sertula* (bacteria)	phase II	PKS	[18–22]
TZT-1027 (**2**)	synthetic dolastatin 10 (sea hare/ cyanobacteria)	phase II	NRPS	[23–26]
cematodin (LU103793; **3**)	synthetic derivative of dolastatin 15 (sea hare)	phase I/II	NRPS	[27–29]
ILX 651 (synthatodin; **4**)	synthetic derivative of dolastatin 15 (sea hare)	phase I/II	NRPS	[27]
ecteinascidin 743 (ET-743; **5**)	*Ecteinascidia turbinata* (tunicate)	phase II/ III	NRPS	[21, 30, 31]
aplidine (**6**)	*Aplidium albicans* (tunicate)	phase II	NRPS	[32–34]
E7389 (**7**)	*Lissodendoryx* sp., first isolated *Halichondria okadai* (sponge)	phase I	PKS	[35, 36]
discodermolide (**8**)	*Discodermia dissoluta* (sponge)	phase I	PKS	[37, 38]
kahalalide F (**9**)	*Eylsia rufescens/Bryopsis* sp. (mollusc/ alga)	phase II	NRPS	[39, 40]
ES-285 (spisulosine)	*Spisula polynyma* (clam)	phase I	fatty acid	[41]
HTI-286 (Hemiasterlin derivative SPA110; **10**)	*Auletta* cf. *constricta*, *Hemiasterella minor* (sponges)	phase II	NRPS	[42–44]
KRN-7000 (**11**)	*Agelas mauritianus* (sponge)	phase I	glycosphingolipid	[45, 46]
squalamine	*Squalus acanthias* (shark)	phase II	terpenoid	[47, 48]
EA-941 (neovastat)	shark	phase II/ III	unpublished mixture	[49, 50]
NVP-LAQ824	synthetic compound based on bacterial and sponge products	phase I	synthetic	[51, 52]
laulimalide (fijianolide; **12**)	*Hyattella* sp., *Cacospongia mycofijiensis* (sponges)	preclinical	PKS	[53–55]
curacin A (**13**)	*Lyngbya majuscula* (cyanobacteria)	preclinical	PKS/NRPS	[56, 57]
vitilevuamide (**14**)	*Didemnum cucliferum, Polysyncraton lithostrotum* (ascidians)	preclinical	NRPS	[58, 59]
diazonamide A (**15**)	*Diazona angulata* (ascidian)	preclinical	NRPS	[60–62]
eleutherobin	*Eleutherobia* sp. (soft coral)	preclinical	terpenoid	[63–65]
sarcodictyin	*Sarcodictyon roseum* (soft coral)	preclinical	terpenoid	[66]
peloruside A (**16**)	*Mycale* sp. (sponge)	preclinical	PKS	[67, 68]
salicylihalimide A (**17**)	*Haliclona* sp. (sponge)	preclinical	PKS/NRPS	[69, 70]
thiocoraline (**18**)	*Micromonospora marina* (bacteria)	preclinical	NRPS	[71]
bryologs (**19**)	synthetic analogues of bryostatin	preclinical	PKS	[18, 72–75]
ascididemin	*Didemnum* sp. (tunicate)	preclinical	pyridoacridine	[76, 77]
variolins	*Kirkpatrickia varialosa* (sponge)	preclinical		[78, 79]
dictyodendrins	*Dictyodendrilla verongiformis* (sponge)	preclinical		[80]
salinosporamide	*Salinospora* CNB-392 (bacteria)	preclinical	amino acid derivative	[185]

[a] Adapted from ref. [5].

circumstantially support a microbial origin for many of these compounds, recent findings indicate that some secondary-metabolite genes are borne on large plasmids and may therefore be highly mobile.[133–135] Studies with dinoflagellates[136] and the discovery of a type I polyketide synthase (PKS) cluster in a terrestrial protist[137] also suggest that these pathways may have evolved in or been horizontally transferred to eukaryotes. Therefore, the possibilities of lateral gene transfer and homologous evolution should always be considered when pursuing a genetic approach to metabolite production.

The vast geographical and phylogenetic differences between macroorganisms producing identical or very similar compounds are also cited as circumstantial evidence of a common symbiont. For example, the polyketide toxin latrunculin A was originally isolated from the Red Sea sponge *Latrunculia* (*Negombata*) *magnifica*.[138] This compound has subsequently been found in the western Pacific ocean in collections of the distantly related sponges (*Caco*) *Spongia mycofiliensis* (Vanauatu),[54] *Fasciospongia rimosa* (Okinawa),[139] and a novel *Thorecidae* genus (American Samoa).[140] This distribution, combined with the lack of genetic or biochemical evidence that sponges are able to synthesize polyketides, suggests that latrunculin A is of microbial origin. Interestingly, localization studies on the related compound, latrunculin B, show accumulation primarily in the vacuoles of some *N. magnifica* cell types,[123] a fact suggesting that it might be a sponge-derived compound. This example serves to illustrate the complexity of the evidence available with regard to the true origin of an invertebrate-derived compound.

3.1. Polyketides and nonribosomal peptides

Nearly two thirds of the potential clinical agents listed in Table 1 are polyketides, peptides, or a combination of the two (Scheme 2). These compounds are often quite large and difficult to access efficiently by traditional synthetic means. Therefore, this review will focus much of its attention on these two classes of chemicals and how current technologies may provide a path to their stable and sustainable production.

Polyketides are polymers of acetate, propionate, and other selected short-chain carboxylic acid precursors. Type I polyketides follow a conserved pattern of biosynthesis that results in a diverse pool of final products. The enzymes involved in this biosynthesis contain conserved sets of catalytic domains. Each set of domains involved in an individual polyketide elongation and subsequent β-carbonyl reduction is designated as a module. In general, biosynthesis of a polyketide is initiated by loading of an acyl starter unit by the first module of the enzymatic system. Once loaded, the starter unit is then extended by Claisen condensations with either malonate or (methyl)malonate from their coenzyme A (CoA) derivatives (Scheme 3 A). After extension, the β-carbonyl moiety can be subjected to re-

Table 2. Marine-derived compounds and their terrestrial counterparts.

Compound	Marine invertebrate source	Related compound	Microbial source
11-hydroxystaurosporine	*Eudistoma* sp. (ascidian)[81]	staurosporine	*Streptomyces staurosporeus*[82]
arenastatin	*Dysidea arenaria* (sponge)[83]	cryptophycin	*Nostoc* sp.[84]
discodermide	*Discodermia dissoluta* (sponge)[85]	ikarugamycin	*Streptomyces phaeochromogenes*[86]
		alteramide	*Alteromonas* sp.[87]
ET-743 (**5**)	*Ecteinascidia turbinata* (ascidian)[30]	saframycin	*Streptomyces lavendulae*[88]
		saframycin	*Myxococcus xanthus*[89]
		cyanosafracin B (**20**)	*Pseudomonas fluorescens*[90]
jasplakinolide/ jaspamide	*Jaspis* sp. (sponge)[91,92]	chondramide	*Chrondramyces crocatus*[93]
motuporin	*Theonella swinhoei* (sponge)[94]	nodularin	*Nodularia spumigena*[95]
mycalamide A (**22**)	*Mycale* sp. (sponge)[96]	pederin (**21**)	endosymbiont of *Paederus fuscipes*[97–100]
namenamicin	*Polysyncraton lithostrotum* (ascidian)[101]	calicheamicin	*Micromonospora echinospora*[102]
onnamide A (**23**)	*Theonella* sp. (sponge)[103]	pederin (**21**)	endosymbiont of *Paederus fuscipes*[97–100]
renieramycin	*Reniera* sp. (sponge)[104]	saframycin	*Streptomyces lavendulae*[88]
		saframycin	*Myxococcus xanthus*[89]
		cyanosafracin B (**20**)	*Pseudomonas fluorescens*[90]
salicylihalamide A (**17**)	*Haliclona* sp. (sponge)[69]	apicularen	*Chrondramyces robustus*[105]
shishijimicin	*Didemnum proliferum* (ascidian)[106]	calicheamicin	*Micromonospora echinospora*[102]
sphinxolide	*Neosiphonia superstes* (sponge)[107,108]	scytophycin	*Scytonema pseudohofmanni*[109]
		rhizopodin	*Myxococcus stipitatus*[110]
theopederin (**24**)	*Theonella* sp. (sponge)[111]	pederin (**21**)	endosymbiont of *Paederus fuscipes*[97–100]

duction by one, two, or all three of a conserved group of domains (Scheme 3 B). Each subsequent elongation of the polyketide chain follows the same pattern, thereby resulting in variability in the reductive state of each carbonyl group. Once the final extension and reduction(s) are carried out, the polyketide chain is cleaved from the enzyme, often through lactonization, by a thioesterase (TE) domain.

In marine-derived polyketides, the addition of an exomethylene moiety is also somewhat common. This is an extremely rare moiety in terrestrial polyketides and one of the few chemical distinctions between metabolites derived from these differing environs. A mechanism involving hydroxymethyl glutamate coenzyme A (HMG-CoA) synthase has been proposed as the source of these functional groups.[141] This enzyme has subsequently been uncovered by homology in the marine cyanobacterial biosynthesis pathways for curacin A (**13**) and jamaicamide.[142,143] It has also been found in the mupirocin biosynthesis cluster in the terrestrial bacteria *Pseudomonas fluorescens*.[144] In these three cases, the exomethylene moiety putatively incorporated by HMG-CoA synthase is further processed to yield a different chemical moiety in each pathway (Scheme 4). This pattern suggests that the HMG-CoA synthase provides a new genetic "handle" by which a subset of marine biosynthesis pathways may be identified from metagenomic libraries (see section 4).

Extensive work in the field of polyketide biosynthesis has shown that the majority of genes that encode these biosynthetic enzymes are clustered together within the genome. These genetic clusters are most often organized in such a manner that their expression sequence is colinear with the assembly of the final product. A number of excellent reviews are available on these medically important secondary metabolites.[145–147]

Nature developed a similar system to provide additional diversity to the pool of bioactive molecules. Nonribosomal peptide synthetase (NRPS) biosynthetic systems polymerize both proteinogenic and nonproteinogenic amino acids into short, linear or cyclic peptides. These systems are also composed of conserved domain types arranged into modules specific for a single extension of the growing peptide. The amino acids are specifically selected and activated by the adenylation (A) domain. They are then loaded onto the phosphopantethienyl arm of a peptidyl carrier protein (PCP), which serves an analogous function to the acyl carrier protein (ACP) in PKS systems. The addition of the amino acid to the nascent peptide is catalyzed by the condensation (C) domain. As with polyketides, a number of auxiliary domains can introduce additional diversity to the final molecule. These include cyclization (Cy), epimerization (E), methyltransferase (MT), and oxidation (Ox) domains. The final product is released from the enzyme complex by a thioesterase (TE) domain (Scheme 5). NRPS enzymes are also generally expressed in an order that is colinear with assembly of the final peptide. A number of excellent reviews on these enzymatic systems are available.[148,149]

In addition to producing polymers of amino acids, NRPS systems can also be found integrated with PKS systems; this results in molecules containing elements of both, such as curacin A (**13**). The biosynthesis of this hybrid molecule illustrates how PKS/NRPS-derived systems have evolved to be functionally compatible and how metabolite assembly and genetic architecture are colinear (Scheme 6).[142]

Table 3. Localization of marine natural products.

Compound	Source	Localization	Chemical class	References
13-demethylisodysidenin	*Dysidea herbacea* (sponge)	*Oscillatoria spongeliae* (cyanobacteria)	chlorinated peptide	[113]
ascidiacyclamide	*L. patella* (ascidian)	*Prochloron* sp. (bacteria)	peptide	[114a, b]
avarol	*Dysidea avara* (sponge)	sponge cells	terpenoid	[115, 116]
bistramide A[a]	*Lissoclinum bistratum* (ascidian)	*Prochloron* sp. (bacteria)	peptide	[114a, b]
bistratene A[a]	*L. bistratum* (ascidian)	ascidian cells	polyketide/peptide	[114a, b]
bryostatin	*Bugula neritina*	*E. sertula* (bacterial symbiont)	polyketide	[20]
crambines	*Crambe crambe* (sponge)	sponge cells	guanidine alkaloid	[117]
dercitamide	*Oceanapia sagittaria* (sponge)	sponge cells	pyridoacridine alkaloid	[118]
didechlorodihydrodysamide C	*D. herbacea* (sponge)	*O. spongeliae* (cyanobacteria)	diketopiperazine	[119]
diisocyanoadociane	*Amphimedon terpenensis* (sponge)	sponge cells	terpenoid	[120]
haliclonacyclamines A & B	*Haliclona* sp. (sponge)	lightest cell fraction	alkaloids	[121]
herbadysidolide	*D. herbacea* (sponge)	sponge cells	terpenoid	[113]
kuanoniamine	*Cystodytes dellechiajei* (ascidian)	pigment cells of the ascidian	pyridoacridine alkaloid	[122]
latrunculin B	*Negombata magnifica* (sponge)	vacuoles within sponge cells	polyketide/peptide	[123]
lissoclinamide 4 & 5	*L. patella* (ascidian)	*Prochloron* sp. (bacteria)	peptide	[114a, b]
P951	*T. swinhoei* (sponge)	filamentous heterotrophic bacteria	peptide	[124]
patellamide D	*L. patella* (ascidian)	*Prochloron* sp. (bacteria)	peptide	[114a, b]
patellamides	*L. patella* (ascidian)	ascidian cells	peptide	[125]
plicatamide	*Styela plicata* (ascidian)	ascidian blood cells	peptide	[126]
pseudoptersin	*Pseudopterogorgia elisabethae* (soft coral)	*Symbiodinium* sp. (dinoflagellate)	terpenoid	[127]
shermilamine	*C. dellechiajei* (ascidian)	pigment cells of the ascidian	pentacyclic alkaloid	[122]
spirodysin	*D. herbacea* (sponge)	lightest cell fraction	terpenoid	[119]
swinolide A	*Theonella swinhoei* (sponge)	unicellular heterotrophic bacteria	polyketide	[124]
tambjamines	*Atapozoa* sp. (ascidian)	ascidian cells	pyrole derivative	[128]
theopalauamide	*T. swinhoei* (sponge)	*Candidatus* Entotheonella palauensis (bacteria)	peptide	[28, 129]
ulithiacyclamide	*L. patella* (ascidian)	ascidian cells	peptide	[125]
ulithiacyclamide	*L. patella* (ascidian)	*Prochloron* sp. (bacteria)	peptide	[114a, b]

[a] The name bistramide A is used here to denote the peptide described by Degnan et al.[114a,b] However, that name has also been used to denote the macrocyclic ether described by Degnan et al. as bistratene A.[130]

3.2. Terpenoids

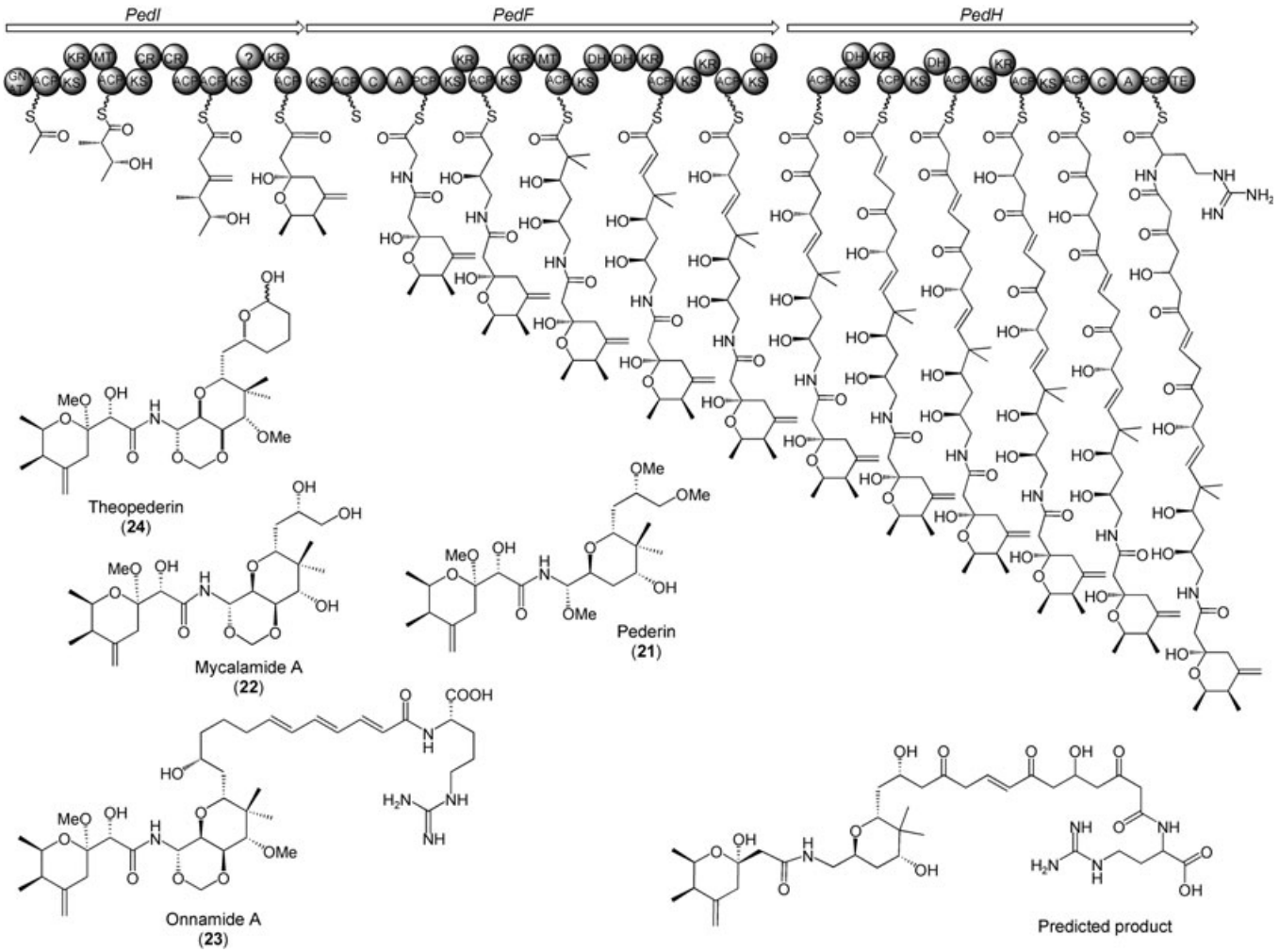
Another class of secondary metabolites that has yielded a large number of bioactive compounds from marine systems is the terpenoids.[4, 150–152] These compounds are derived by the cyclization and subsequent oxidation of repeating isoprene units. The biosynthesis of these important compounds has been studied extensively from both basic science and biotechnology perspectives.[153–156]

In addition to the anticancer terpenes sarcodictyin, eleutherobin, and squalamine (highlighted in Table 1), pseudopterosin, another marine-derived member of this class of compounds, is already in commercial use as a topical antiinflammatory. The pseudopterosins are terpenoid glycosides that were originally isolated from the soft coral *Pseudopterogorgia elisabethae*.[10] However, recent evidence indicates that the pseudopterosins are produced by a microbial symbiont.[127] These compounds are currently produced by mariculture,[157] but their biosynthesis has been studied extensively with an eye toward alternative sustainable production methods.

Studies on marine-derived terpenoids have been accomplished by incorporation of the radiolabeled metabolic precursors geranyl-geranyl diphosphate (GGPP) and xylose.[158] The incubation of these precursors in a cell-free protein lysate from *P. elisabethae* has enabled isolation of the key biosynthetic intermediates in pseudopterosin biosynthesis.[159] The diterpene cyclase responsible for the core structure of pseudopterosin has recently been identified.[160] Even limited amino acid sequence information of this enzyme could provide the key to the cloning and analysis of the corresponding gene by using a "reverse genetics" approach. Reverse genetics utilizes the amino acid sequence of a target enzyme to design PCR primers in order to amplify a portion of the encoding gene for use as a genetic probe. Once characterized, all component genes of the biosynthetic pathway may be transferred to an appropriate heterologous host to facilitate the production of pseudopterosin.

The work described above illustrates how reverse genetics can be used as an alternative to the purely genetic approach utilized to isolate secondary-metabolite genes. This method may be generally applicable to finding enzymes involved in the production of any compound containing a distinct chemical moiety. When the origin of a compound is unknown, as is the case with many of the marine-invertebrate-derived compounds, cell fractionation followed by cell-free assays with radiolabeled key intermediates may provide biochemical evidence to narrow the scope of genetic probing.

Additional inspiration for using biochemical approaches in combination with homology-based gene cloning can be drawn



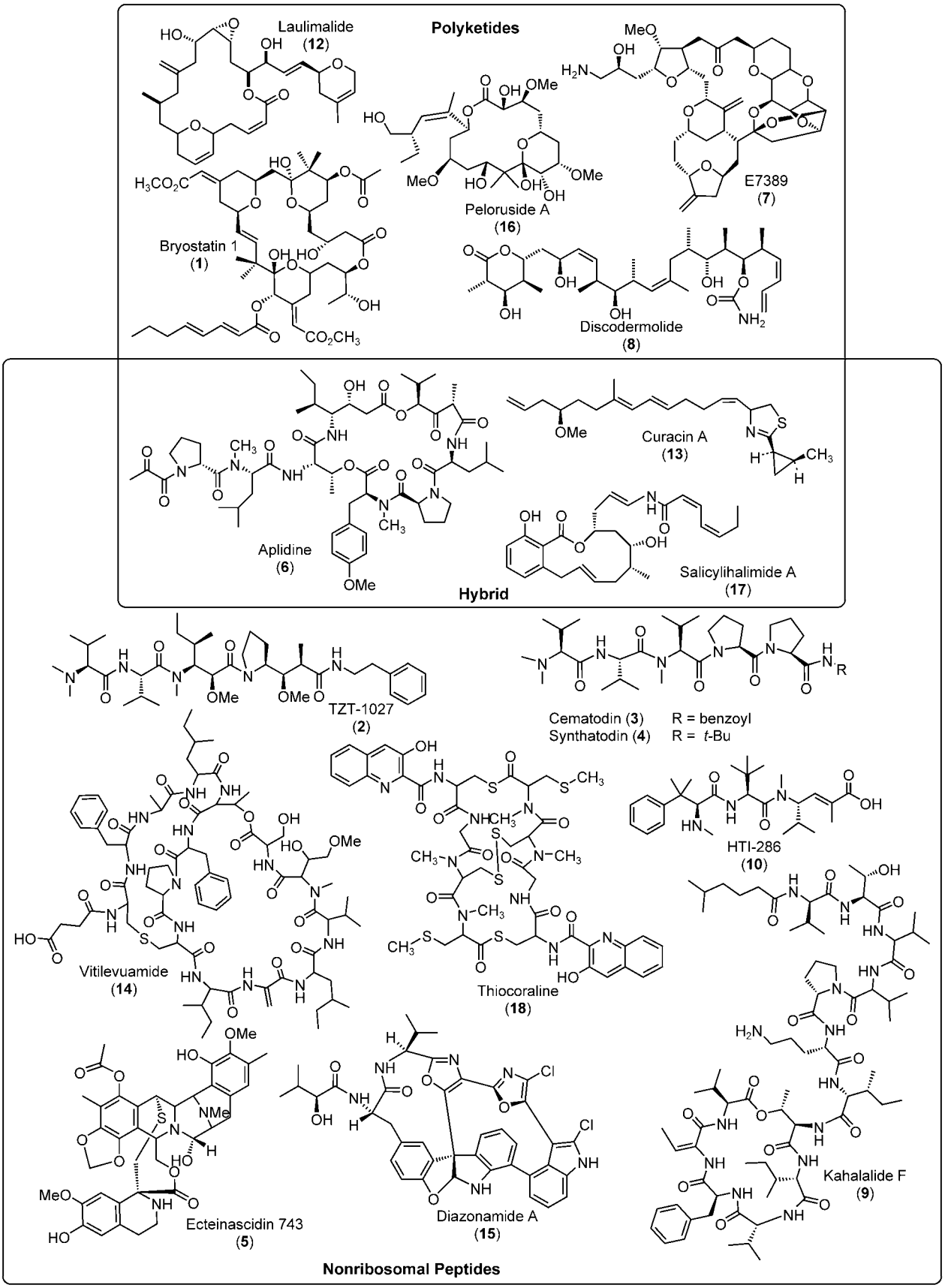
Scheme 1. Pederin (**21**), a microbially derived polyketide, is structurally similar to the sponge-derived natural products theopederin (**24**), mycalamide A (**22**), and onnamide A (**23**). The genes encoding pederin biosynthesis are followed downstream by additional genes that would be predicted to biosynthesize a compound more closely related to onnamide A than pederin. The three core open reading frames are represented by the block arrows above the catalytic scheme. Several additional putatively identified modifying enzymes, including a monooxygenase potentially responsible for the release of pederin between modules 2 and 3, have been omitted for clarity. (Adapted from refs. [100, 131].)

from the extensive work done to elucidate the biosynthetic machinery responsible for the production of the terrestrial terpenoid taxol.[161] Recent studies of one of the P450 hydroxylases involved in taxol biosynthesis shows how providing key intermediates to a microorganism heterologously expressing tailoring enzymes can result in specific processing to the next intermediate.[162] This illustrates the potential value of cloning even partial biosynthetic pathways. It also highlights the fact that secondary-metabolite genes isolated from eukaryotes can be cloned and heterologously expressed in a manner similar to their bacterial counterparts.

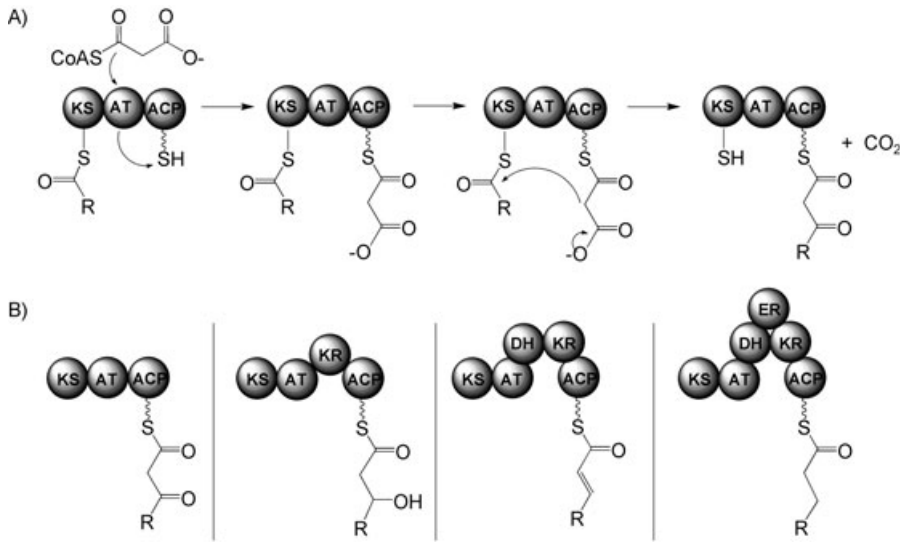
4. Metagenomics

In order to exploit the knowledge that has accumulated about secondary-metabolite biosynthesis, a method for screening genetic material is required. Due to the limitations of culturing marine invertebrates and their associated bacteria, this method needs to be applicable to mixed cells from native sources. Over the last few years, the field of metagenomics has emerged as a valuable tool for the study of complex microbial communities. The term metagenome is used to describe the multiorganism-derived pool of genomic DNA isolated from a defined source.[163] The field of metagenomics has emerged as a promising tool for finding biosynthetic pathways from complex mixtures of terrestrial microorganisms from the soil, and it may be applicable to marine invertebrates. Several studies have involved isolation of mixed genomic DNA from a target source followed by cloning into an appropriate vector. These vectors were then transformed into established bacterial host strains, and the resulting libraries were screened by using a variety of methods to yield secondary-metabolite biosynthetic genes.[164–166]

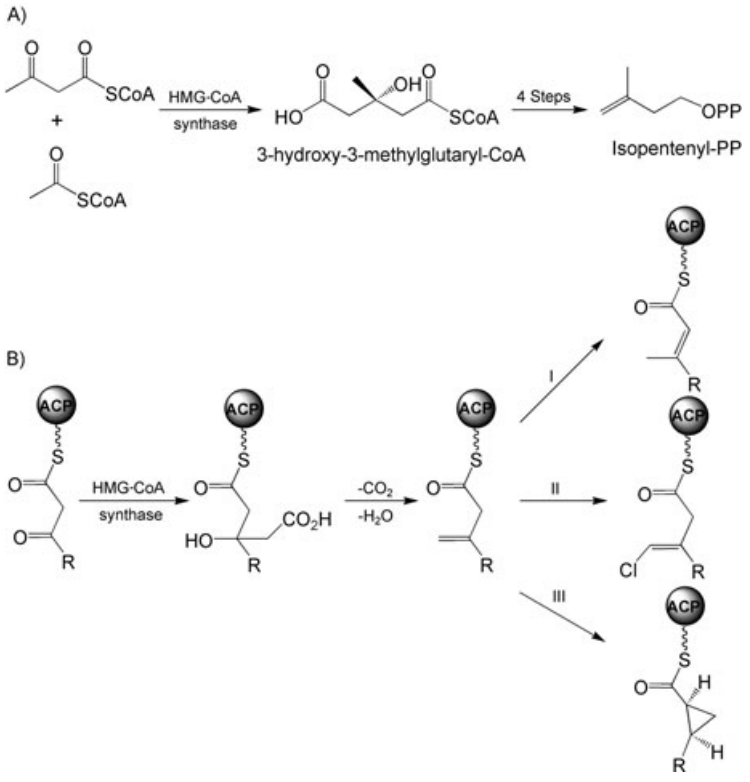
Metagenomic techniques have also been applied in a targeted manner to explore the diversity of PKS genes present in a soil sample. Novel polyketides have been produced by expressing type I PKS clusters from a soil-metagenome sample.[167] In this case, the library was constructed by using an



Scheme 2. Marine-derived polyketides, nonribosomal peptides, and related structures currently in clinical and preclinical development as anticancer agents.



Scheme 3. A) One round of polyketide elongation illustrated with malonyl CoA. B) The four possible reductive states of the β-carbonyl moiety.



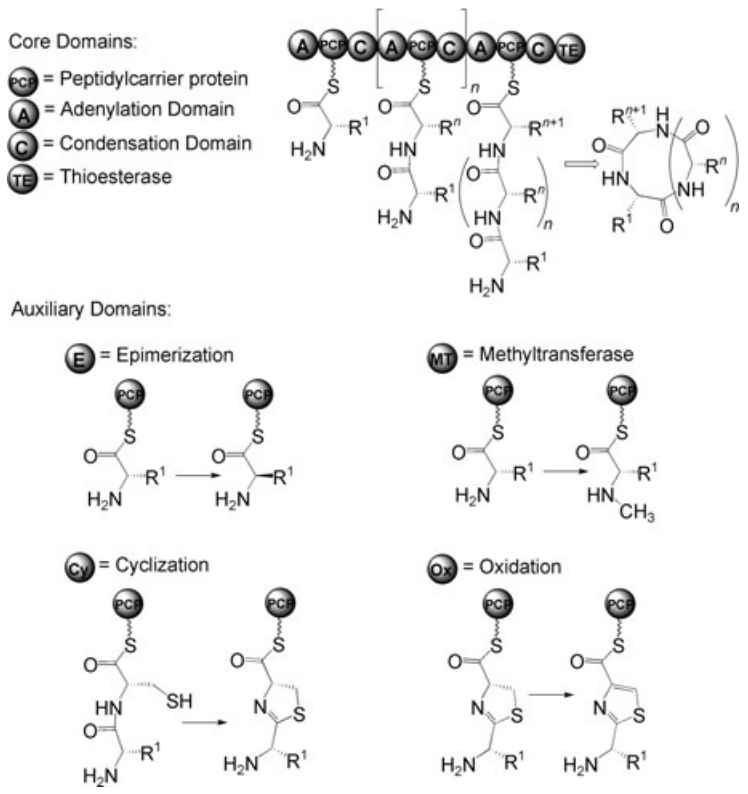
Scheme 4. A) HMG-CoA synthase catalyzes the condensation of acetyl CoA and acetoacetyl CoA to form HMG-CoA. This intermediate can be further processed through the mevalonate pathway to isopentenyl pyrophosphate, a key starting material for sterol biosynthesis. B) This enzyme has also been implicated in the introduction of novel moieties in various PKS pathways: I) Mupirocin, II) jamaicamides, and III) curacin A.

Escherichia coli/Streptomyces shuttle vector, thus enabling the investigator to screen for PKS genes in the more genetically amenable *E. coli* and subsequently express them in *S. lividans*.

Metagenomic cloning techniques have shown utility in an even more specific manner. As mentioned earlier, the biosynthetic gene cluster for the antitumor compound pederin (**21**) was isolated from a metagenomic library.[100,131] In this case, the metagenome was composed of DNA from the beetle *Paederus fuscipes* and its single unculturable bacterial symbiont. The libraries used in this work were generated with DNA extracted from homogenized eggs of toxin-producing females.

Biosynthetic studies on the bryostatin family of anticancer agents, derived from the marine invertebrate *Bugula neritina*, have an evident parallel with pederin biosynthesis.[18] Larvae of the animal have a relatively high number of the bryostatin-producing symbionts but relatively few other microbes commonly associated with adult tissues.[168] Bryostatin 1 (**1**) is a complex polyketide synthesized by the Gram-negative marine bacterium *Endobugula seratula*, an unculturable symbiont of the bryozoan *B. neritina*.[19,20] Recent studies have unequivocally identified *E. sertula* as the producer of the bryostatins,[19,20] a result making the corresponding biosynthetic pathway an ideal target for cloning efforts and (meta)genomic studies. The putative model for the biosynthetic architecture of bryostatin 1 (**1**) following the "rules" of colinearity (Scheme 7) could then be utilized to create a rational probing strategy to isolate bryolactone biosynthetic genes from the *B. neritina* library. The putative involvement of HMG-CoA synthase might provide a specific genetic "handle" for probing of the *E. sertula* library.

Metagenomics can be applied to other marine macroorganisms in a similarly targeted manner. Given our ever-increasing knowledge of secondary-metabolite biosynthesis, an experimental approach can be tailored to target any given molecule by utilizing all available evidence. Library construction can also be directed by enrichment of specific cell types, if localization or other data strongly suggest a specific route of biogenesis. This effort can be followed by PCR screening for the genes of interest. Given that PKS and NRPS genes contain highly conserved motifs, degenerate primers designed to amplify these sequences can be used to probe for the desired classes of genes in a mixture of DNA. However, the ubiquity of these types of genes within prokaryotes limits the specificity of this screen. A carefully chosen sequence of probing techniques can be designed to identify likely candidates



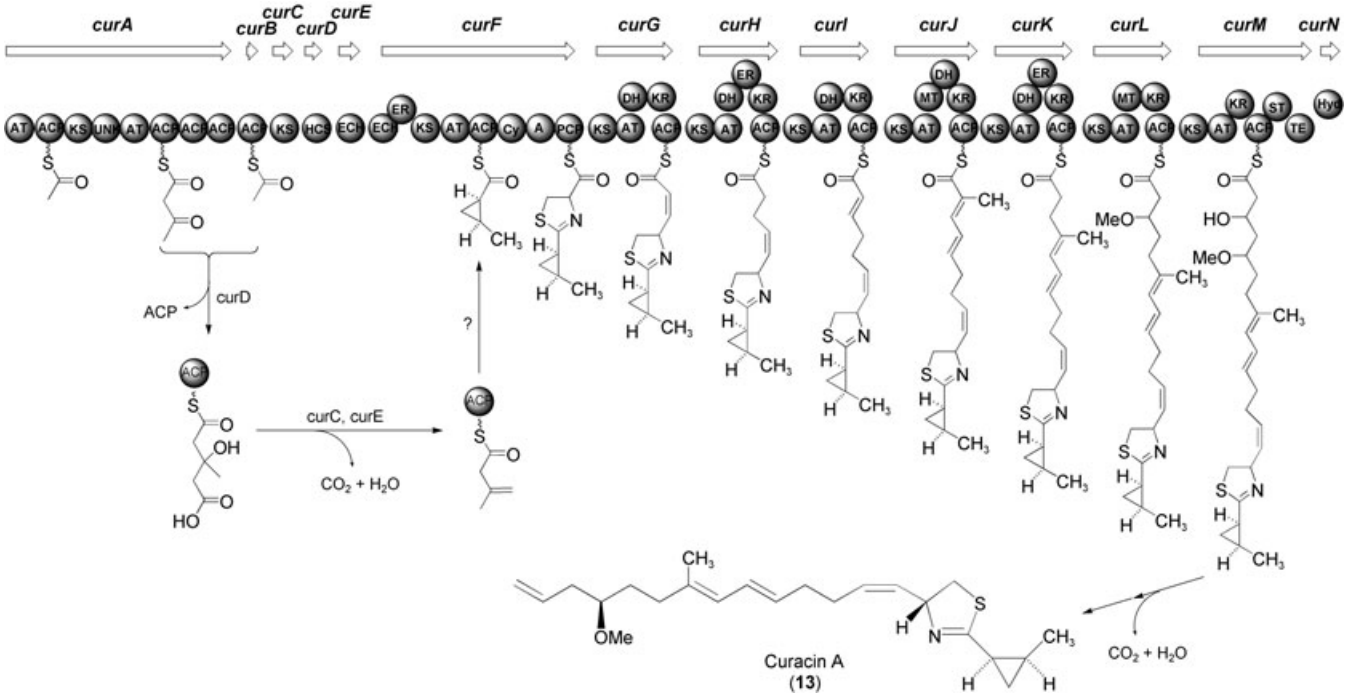
Scheme 5. The core NRPS domains catalyze the building of the peptide backbone. The TE domain releases the nascent peptide from the enzyme complex, usually by cyclization (as shown). This cyclization can also utilize a hydroxy group to form the depsipeptide. Auxiliary domains can further diversify the output of these systems. The examples shown are not inclusive of the entire range of catalytic potential of these systems. For example, the cyclization domains can catalyze the formation of oxazole rings. The methyl transferase domains exist to utilize carbon or oxygen as methyl acceptors.

for a given gene cluster among many clones positive for conserved portions of the genes of interest. Secondary screening of initial candidates with probes for genes encoding conserved modification enzymes, such as methyltransferases, glycosyl transferase, or P450 hydroxylases can quickly narrow the number of candidates in a library. Also, probing for conserved domains can be done semiquantitatively. A polyketide with a 14-membered macrolactone ring will most likely be derived from a biosynthetic system that contains 6 ketosynthase domains.[147] To illustrate how metagenomics may be applied to a specific target molecule, a proposed experimental design for the isolation of the discodermolide (**8**) biosynthesis cluster is outlined (Scheme 8).

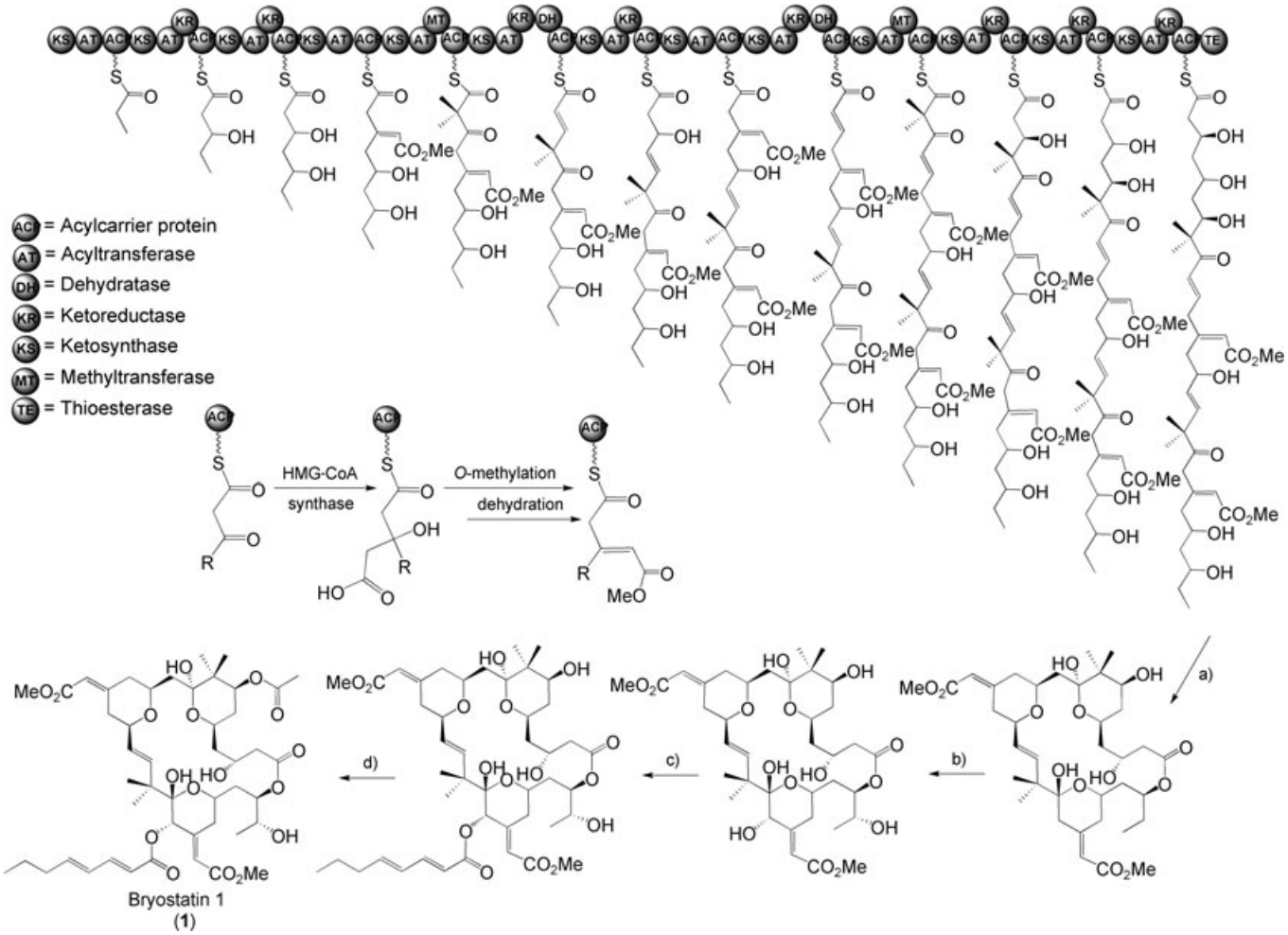
The promise of metagenomic DNA cloning and gene expression for the discovery of new bioactive metabolites has yet to be fully realized. The potential is particularly significant for clusters of PKS and NRPS genes that have evolved within marine microbial systems and invertebrate symbionts. However, although the PKS and NRPS enzymes are similar in function, target genes from marine sources may have diverged to a point where they are not always detectable by using the degenerate PCR primers established from terrestrial systems.

5. Heterologous Expression

An additional hurdle in moving from genetic source material to final product is heterologous expression of the biosynthetic genes in a productive manner.



Scheme 6. The biosynthetic pathway of curacin A (adapted from ref. [142]). This pathway illustrates both the common pattern of colinearity and how PKS and NRPS systems often hybridize to form molecules derived from both acetate and amino acids.

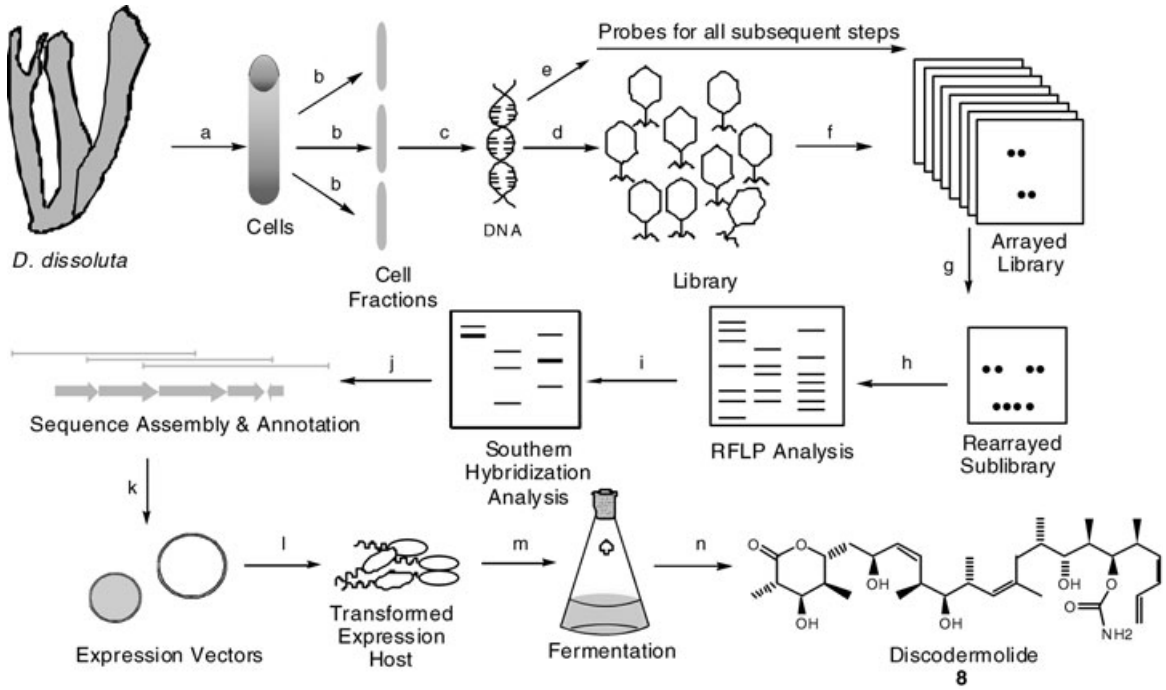


Scheme 7. A biosynthetic scheme for bryostatin 1 based on the standard colinear model of polyketide biosynthesis. a) Additional enzymatic steps, b) cyclization by the TE domain, c) hydroxylation by cytochrome P450, d) ligation of medium-chain unsaturated fatty acid, e) acetylation.

The key to production lies in the selection of an appropriate host. Analysis of the DNA composition can be used to guide the choice of a host strain, including %GC content and codon usage of the genes of interest, along with availability of appropriate metabolic building blocks. There are currently a number of potential host strains that are amenable to genetic manipulation. Implementation of a genetically similar host strain maximizes the potential for productive transcription, translation, and metabolite production by providing a close approximation to the native cellular environment. Transferring secondary-metabolite production between related strains has been accomplished with several gene clusters from phylogenetically distinct bacteria. The biosynthetic pathway for bacitracin was successfully transferred from *Bacillus licheniformis* to the related species *B. subtilis*.[169] The polyketide biosynthesis pathway for the marine-derived telomerase inhibitor griseorhodin A was productively transferred to *Streptomyces lividans* from an environmental *Streptomyces* isolate.[170] The antifungal polyketide 2,4-diacetylphloroglucinol genes have been successfully transferred from *Pseudomonas fluorescens* to a related *Pseudomonas* species,[171] and genes for epothilone biosynthesis have been moved from *Sorangium cellulosum* into the related *Myxococcus xanthus*.[172] The transfer and functional expression of these pathways highlight the high potential for success when utilizing an appropriate host strain.

While these examples highlight the successful transfer of biosynthetic pathways between related strains, there are also notable examples of secondary-metabolite production exchange between unrelated organisms. *Streptomyces coelicolor* has been engineered to produce the myxobacterial metabolite epothilone.[173] *E. coli* has also proven to be a productive host for the cloning and heterologous expression of the gene clusters for violacein and turbomycin from soil-derived metagenomic DNA.[164,165] Also, some particularly effective work has been done to engineer an *E. coli* strain capable of producing 10-deoxyerythronolide B, the aglycone core of erythromycin.[174] This research involved manipulation of propionate catabolism to produce (methyl)malonyl-CoA, a metabolic building block not normally present in *E. coli*.

Development of effective genetic tools for the expression of diverse secondary-metabolic DNA sequences is an essential component of fulfilling the potential of heterologous hosts as production vehicles. Recently, a group reported the engineering of a new bacterial artificial chromosome (BAC) vector that



Scheme 8. Overview of the proposed biosynthetic-pathway cloning process, with discodermolide as an example. a) Tissue dissociation, b) cell fractionation, c) metagenomic DNA extraction, d) library construction, e) probe generation by PCR, f) array library and probe for primary target, g) rearray positive clones and probe for secondary genes, h) restriction fragment length polymorphism (RFLP) analysis to identify overlapping and repeat cosmids, i) Southern hybridization for semiquantitative analysis of ketosynthase (KS) domains, j) sequence assembly and annotation, k) reassembly of biosynthetic pathway in expression vectors, l) transformation of appropriate host strain, m) fermentation, and n) extraction and purification of discodermolide.

can be transferred through conjugation from *E. coli* and incorporated into the genome of recipient organisms containing the *Streptomyces* phage φC31 phage-attachment site *attB*.[175] The resulting system enabled library construction in *E. coli* and transfer by conjugation into two additional host strains. The host strains used in this study included *S. lividans*, with the endogenous antibiotic biosynthesis genes disrupted, and a strain of the nonantibiotic-producing *Pseudomonas putida* engineered to contain the *attB* phage-attachment site. This group constructed three control vectors, each containing a secondary-metabolite biosynthesis cluster known to be productive in each of their original hosts. The results of this set of experiments are summarized in Table 4 (adapted from ref. [175]). The

Table 4. Heterologous metabolite production in three host strains.

Metabolite	*E. coli*	*S. lividans*	*P. putida*
indigo blue + indirubin	+	–	–
granaticin	–	+	–
diacetylphloroglucinol (DAPG)	–	–	+

production of each of the antibiotics in only one strain highlights the importance of host selection for heterologous expression. It also illustrates the potential limitations of gene expression from distantly related strains.

It remains of some concern that the metabolites produced by metagenomic efforts have been relatively simple, including those produced by type I PKS systems.[167] This is probably due to several contributing factors in addition to the genetic compatibility of the host. Complex PKS- and NRPS-derived compounds are generally derived from relatively large gene clusters. The inclusion of all biosynthesis genes is necessary to obtain metabolite production. For the host cell to survive, it may also be essential that one or more resistance genes be functionally expressed. Although many resistance genes reside within biosynthetic gene clusters, there are a growing number of examples where multiple resistance determinants are involved, one or more of which may be unlinked on the bacterial chromosome.[176,177] These factors might limit the opportunities for generating some secondary metabolites through metagenomic library construction and expression.

These evident limitations affirm the need for genetic, as well as functional (for example, biological activity), screening of natural products obtained from the expression of secondary-metabolite genes derived from metagenomic libraries. The fact that many or even most of the biosynthesis genes isolated from marine invertebrates will come from previously uncharacterized microorganisms suggests that great care will be necessary in selecting and developing heterologous hosts. Even intragenus transfer of genes may require additional modifications, such as promoter replacement, before productive expression is achieved. The power of genetic screening as a complement to screening based on biological activity is highlighted by probing a library of environmental *Streptomyces* isolates for genes with high homology to conserved regions of enediyne PKSs.[178] The study found homologues to the genes

involved with the production of this highly reactive class of natural product molecules in approximately 15% of the microbes evaluated.

6. Marine Microbiology

Many therapeutic compounds currently in use have been isolated from terrestrial microbes. There has been a sharp increase in the study of both strain and bioactive-metabolite isolation from microorganisms isolated from marine sources.[179] Innovations in culturing methods continue to enable the cultivation of new microorganisms.[180–183] A new genus of *Actinomycetes* isolated from marine sediments, the *Salinospora*, has been reported to contain hundreds of unique compounds, based on bioactivity.[184] Salinosporamide A represents the first published structure characterized from the *Salinospora*.[185] Our laboratory has recently isolated a number of new actinomycetes,[183] some of which represent an additional branch of the *Micromonospora* genus, independent of the *Salinospora*. The fact that new genera are being described with relative frequency suggests that the field of marine microbiology is still in its infancy.

The culturing of symbionts of marine invertebrates is potentially a great step forward toward procuring a regular and commercially scalable supply of invertebrate-derived compounds. However, the current estimate that $<1\%$ of these microorganisms are amenable to laboratory culturing suggests that significant additional basic research will be required to elucidate the metabolic requirements of these organisms, before a high rate of success can be achieved. While there are great strides being made in the culturing of marine bacteria, the resulting cultures are largely microscale and not yet amenable to industrial applications.[180] However, the techniques used to transfer the epothilone pathway from a slow-growing organism to a related fast-growing organism[172] may be applied to targeted pathways from low-density and slow-growing new isolates. In addition, the genetic investigation of marine-bacteria-derived biosynthetic pathways may provide tools and insight to improve the efficiency of cloning and expression of similar genes derived from metagenomic sources.

Thiocoraline (**18**), salinosporamide, and curacin A (**13**) are the only compounds included in Table 1 that are unmodified metabolites produced by bacterial fermentation (curacin will be discussed in the next section). The yield of thiocoraline (**18**) is approximately 9 mg per liter.[71] While capable of providing a stable and virtually unlimited supply, this titer is low when compared to estimates of industrial antibiotic production that, for example, provide 10–50 g of erythromycin per liter from *Saccharopolyspora erythraea*. To improve secondary-metabolite productivity through the use of molecular techniques, there are two options. First, advances in random genetic manipulation have shown great promise in achieving a substantial boost in secondary-metabolite biosynthesis in *Streptomyces*.[186] Given that thiocoraline (**18**) is produced by the Actinomycete *Micromonospora* sp. L-13-ACM2-092[187] it is reasonable to expect that this approach can be applied successfully to the native producer. Alternatively, the corresponding biosynthetic pathway could be cloned and transferred into *Streptomyces lividans* and further manipulated in this well-established heterologous host. The fact that there are terrestrial *Streptomyces* species[188,189] producing similar compounds suggests that this approach is likely to succeed.

6.1. Cyanobacteria

The cyanobacteria deserve special consideration due to their unique duality. On one hand, they often grow as dense, macroscopic masses. This characteristic enables collection and chemical investigation in a manner normally utilized with sponges and other macroorganisms. On the other hand, they are prokaryotes and as such contain relatively small genomes that can be analyzed with relative ease. As a class, marine cyanobacteria have been shown to contain a vast array of interesting bioactive metabolites.[190] Taken together, these elements make cyanobacteria an extremely interesting and important class of organisms for further study.

The majority of bioactive metabolites isolated from cyanobacteria have been polyketides, nonribosomal peptides, or a hybrid of these two classes.[191] As outlined in Section 3.1., these classes of compounds have been studied for decades in terrestrial microbes, to yield a vast pool of knowledge regarding specific genetic and biochemical origins. This information can now be exploited for biosynthetic analysis relating to cyanobacterial natural products. A single strain of *Lyngbya majuscula* has been shown to produce a number of interesting compounds, including curacin A (**13**), barbamide, antillatoxin, and carmabins A and B.[56,192,193] This strain has been the subject of intense genetic study, which has resulted in the cloning of the barbamide,[194] curacin,[142] jamaicamides,[143] lyngbyatoxin,[195] and carmabin (unpublished results, J. Jia and D. H. Sherman) biosynthetic gene clusters. These represent the first efforts to clone and characterize secondary-metabolite gene clusters from marine cyanobacteria.

Curacin A (**13**) is produced by total synthesis in a 2.6% yield over 15 steps.[57] While initial levels of production clearly do not match estimates of industrial fermentation yields, it is important to consider that those levels are the result of decades of strain and culture-conditions optimization. In fact, conditions for the culturing of *Lyngbya majuscula* to enhance metabolite production have already been explored.[196] However, the recent cloning of the curacin biosynthetic pathway[142] provides an opportunity to attempt the first heterologous expression of a marine cyanobacterial type I PKS cluster.

The dolastatins, originally isolated from the sea hare *Dolabella auricularia*, have been an extremely productive class of lead compounds for anticancer therapy.[197,198] Significantly, the dolastatin derivatives TZT-1027 (**2**), cematodin (**3**), and ILX 651 (**4**) represent 20% of current marine-derived compounds in anticancer clinical trials. Although originally isolated from sea hares, the dolastatins were later found to be produced by cyanobacteria,[25,199] therefore again highlighting the importance of this group of microorganisms.

In addition to the vast chemical diversity found in some cyanobacteria, there is evidence of even greater potential diversity

in the form of cryptic pathways.[191] This is borne out from genome sequencing of the terrestrial cyanobacteria *Nostoc punctiforme* that has revealed a secondary metabolome rich in natural-product pathways. The 9.5 megabase genome of *N. punctiforme* contains at least 53 homologous open reading frames (ORFs) relating to secondary-metabolite biosynthesis arranged in at least 7 clusters.[200] Developing effective technologies for cloning and heterologous expression of these pathways may provide new therapeutic lead compounds, as well as fundamental information about cyanobacterial secondary-metabolite biosynthesis.

7. Other Means of Production

While the focus of this review is molecular biology and marine bacteria, it is important to consider that this is but one path toward the common goal of elucidating the fascinating genetics and biochemical mechanisms of these systems and applying that knowledge toward the creation and discovery of bioactive small molecules. Given the vast diversity of compounds and sources that fall under the heading "marine natural products", it is clear that no one method of production will be optimal for all target molecules. Total synthesis, semisynthesis, and mariculture are currently the only proven sources of marine-invertebrate-derived compounds. Cell culture and biosynthetic-pathway cloning hold enormous potential, but they remain unproven.

Traditional chemical synthesis continues to be the best option for the production of many marine-derived metabolites and the major source of the small amounts of compounds needed for initial therapeutic investigations. Thus, application of total synthesis has allowed the transition from a lead compound to a highly effective clinical agent. Of the compounds listed in Table 1, TZT-1027 (**2**), cematodin (**3**), and ILX 651 (**4**), as well as E7389 (**7**), HTI-286 (**10**), KRN7000 (**11**), and the bryologs (**19**), are synthetic derivatives of marine-natural-product leads and, therefore, have no native organism that can be cultivated or genetically harnessed. These compounds are shown in Scheme 9 alongside their corresponding parental compounds. The number of marine metabolites that have been synthesized continues to grow. The synthesis of the polyether ciguatoxin CTX3C illustrates the high level of complexity that can be achieved through synthetic chemistry.[201] However, the complexity of the majority of target molecules usually requires a multistep process that may ultimately prove to be too inefficient and costly for commercial application. Total synthesis has also proven useful for producing small quantities for clinical studies or aiding in structural elucidation.

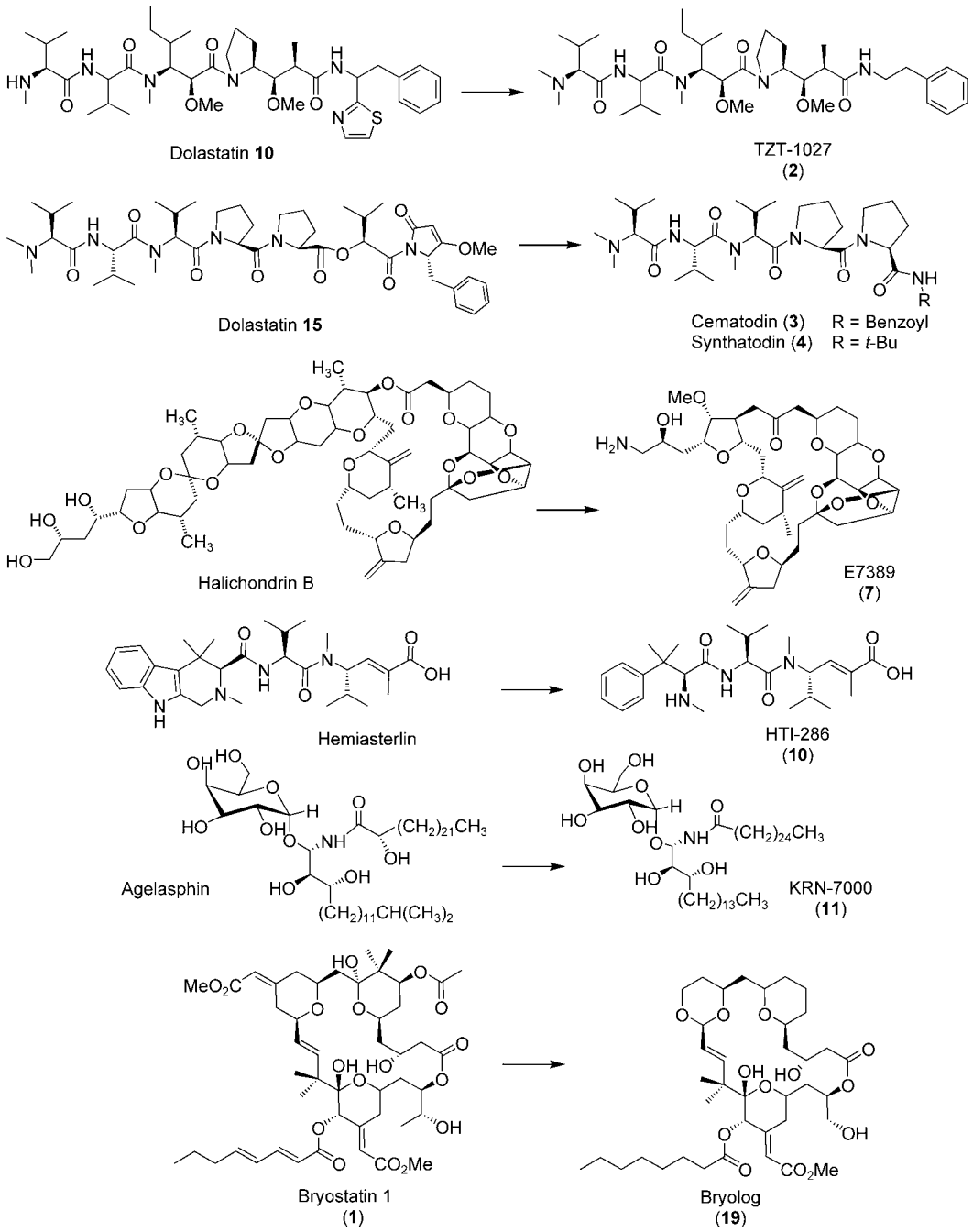
Discodermolide (**8**), a polyketide isolated from the sponge *Discodermia dissoluta*, has shown great promise as a microtubule-stabilizing agent.[37,202] However, this compound is present in low yields (0.002% of wet weight) and the organism has a relatively low distribution in the environment. Therefore, isolation of material from collections of the parent organism is not a viable option. Novartis has recently provided details of a 60-gram synthesis of discodermolide (**8**).[203–207] This synthetic tour de force combined aspects of previously published syntheses and involved more than 43 chemists over a period of 20 months.[207] The final yield of this impressive 39-step effort was 0.65%.[38] While this undertaking has produced sufficient amounts of compound for clinical trials, the overall yield and man hours involved are far from optimal.

The antitumor compound Ecteinascidin 743 (**5**, ET-743) is currently produced by two methods. It is the product of mariculture[21] and semisynthesis.[31] The synthetic route utilizes the core structure from the related microbial metabolite cyanosafracin B (**20**, Scheme 10). Availability of this complex core structure from microbial fermentation is critical for production of this compound. The synthesis from cyanosafracin B (**20**) to ET-743 (**5**) requires 21 steps with an overall yield of approximately 1%.[31] In the absence of the natural-product intermediate, traditional synthetic methods would not be an option for this compound.

Mariculture has had some notable successes in the production of important marine natural products. The yields of compound per kilogram of tissue have improved from initial reports but still remain low. Bryostatin 1 (**1**) is currently being produced for clinical testing by using a sophisticated mariculture system.[21,208] In order to obtain the 18 grams of bryostatin 1 (**1**) used for clinical trials, 28000 pounds (12700 kg) of *Bugula neritina* were extracted (yields are noted at 10^{-3}–10^{-8}% of wet weight).[22,209] Mariculture trials and improvements of extraction and purification protocols have increased the average yield to 5.4 mg per kg (wet weight).[21] The initial yield of ET-743 (**5**) from the tunicate *Ecteinascidia turbinata* is reported to be 1×10^{-4}% of wet weight or about 1 mg per kg.[30,210] Yields reported from mariculture were as high as 8.6 mg per kg.[21] Sarcodictyin and eleutherobin are structurally related isoprenoid compounds isolated from soft corals in disparate regions of the world.[63,66] Eleutherobin is currently being produced by mariculture of the gorgonian *Erythropodium caribaeorum*, with a yield of 0.0012% of wet weight.[65] While productive, mariculture is vulnerable to potential destruction by pests, infection, or weather. These problems are exemplified by the three-year delay in a bryostatin mariculture pilot program, due to El Niño warming of the California coastal waters and loss of ET-743 (**5**) production due to a hurricane that destroyed *E. turbinate* cultures off the coast of Florida.[21] However, the development of methods for increased mariculture-based production of bioactive metabolites continues to add promise to this field.[211,212] Advances are also being made in the development of an aquarium-based invertebrate culture system.[213–215]

Mariculture provides a proven method for the production of theoretically unlimited amounts of complex marine metabolites. While the final cost per gram of material remains quite high,[21] these methods provide a valuable bridge between discovery and efficient production methods.

Taxol (paclitaxel) is a widely used anticancer agent first isolated from the Pacific yew tree *Taxus brevifolia*.[216,217] As is the case with marine invertebrates, collection of this compound from native sources is not a viable long-term option. To overcome this problem, culturing of *Taxus* cells in vitro has been explored and advanced, thereby allowing a shift from semisynthesis to cell culture as a primary means of production.[218] The

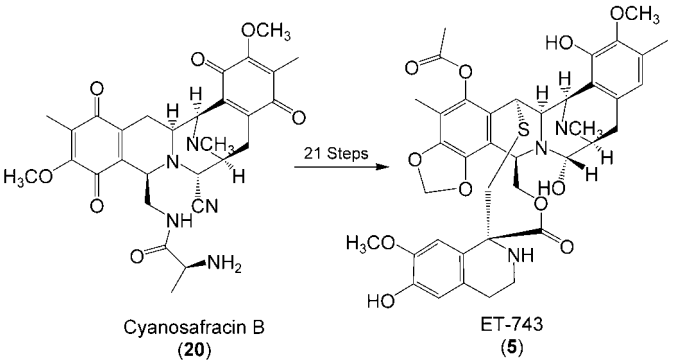


Scheme 9. Potential anticancer therapeutics developed from marine natural products highlight the potential of these compounds as leads.

taxol example provides inspiration for the development of eukaryotic cell cultures for small-molecule production. The culturing of marine invertebrates for the production of secondary metabolites has been challenging due to the complex nutritional and environmental requirements of these organisms, but this field continues to move forward and may ultimately provide a robust means of production for select marine natural products.[219–222]

8. Summary and Outlook

The seas have thus far yielded thousands of bioactive metabolites. Complete exploration and application of these compounds continues to be hampered by supply issues. While there are many means to this end, cloning and heterologous expression of the biosynthetic genes responsible for the assembly of these metabolites holds the greatest promise for affordable, stable, and sustainable production. Successful endeavors will result in a virtually unlimited source of materials with relatively low production costs.



Scheme 10. Fermentation of the terrestrial bacteria *Pseudomonas fluorescens* provides cyanosafracin B (**20**), a complex starting material utilized in the synthesis of ET-743 (**5**).

Additional dividends from this approach include the means for producing engineered structural analogues by combinatorial biosynthesis, chemoenzymatic synthesis, and de novo synthesis through induction of cryptic pathways. Straightforward genetic manipulations in polyketide and nonribosomal peptide systems have yielded novel analogues of a number of compounds.[148,223–226] Typical combinatorial biosynthetic libraries are constructed by introducing genetic elements from two or more pathways to produce a hybrid metabolite. Several detailed reviews exist on this topic.[227,228] Metagenomic libraries constructed to isolate the pathways of specific compounds will also almost certainly reveal cryptic gene clusters and those that are effectively cryptic due to very low expression or parent-cell-population levels. The wealth of new microbial genome sequences from microbes that produce secondary metabolites will unveil huge numbers of new biosynthetic systems. This highlights a bright future for new gene cluster and metabolite discovery through a combination of both genetic and biological-activity screening.

Screening of metagenomic libraries can also be expanded to probe for industrially important enzymes, such as the lipases and proteases used in detergent production. In this respect, a soil-metagenome strategy provided proof of concept with the random cloning of 4-hydroxybutyrate degradation enzymes.[229] This methodology can be further exploited for isolation of individual enzymes with any of a wide range of catalytic applications for utilization in chemoenzymatic synthesis.[230–234] Isolated genes can be manipulated for new functions and increased efficiency by directed evolution.[235–237] These potential value-added aspects of a genetic approach to compound production may well yield significant rewards for both fundamental knowledge and application in enzymology and evolution.

The processes used to produce marine natural products illustrate how several methodologies can be employed to obtain sufficient material to carry a compound through clinical trials. However, the fact that only a small number of these compounds are being pursued for clinical development through traditional chemical synthesis suggests that structural complexity is inhibiting drug development on all but the most promising compounds. While methods for the total synthesis of marine natural products continue to advance and remain the most reliable method of production, it is unlikely that synthetic-chemical-based production will be more cost effective than a robust microorganism capable of generating a therapeutic product.

While it is clear that technology for achieving pathway cloning and heterologous expression is a science in its infancy, the technical challenges of this process continue to be overcome. Along with these challenges come great opportunities for the discovery of new biologically active metabolites. Each discovery, in turn, provides an additional scaffold for bridging the gap between the promise and the application of marine natural products.

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Keywords: biosynthesis · genomics · heterologous expression · marine natural products · secondary metabolites

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