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Life in cellulose houses: Symbiotic bacterial biosynthesis of ascidian drugs and drug leads

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Abstract

Ascidians (tunicates; sea squirts) are sources of diverse, bioactive natural products, one of which is an approved drug and many of which are potent drug leads. It has been shown that symbiotic bacteria living with ascidians produce some of the bioactive compounds isolated from whole animals, and indirect evidence strongly implicates symbiotic bacteria in the synthesis of many others. However, for the majority the producing organism has not been identified. In cases where a symbiotic origin has been definitively assigned, the resulting data lead to improved paths to drug discovery and development from marine animals. This review traces evidence for symbiotic production where such evidence exists and describes the strengths and limitations of that evidence.

Introduction

Approximately 1,000 marine natural products have been reported from ascidians. Like sponges, ascidians are soft-bodied, benthic invertebrates that are likely to require chemical defenses for survival in high-predation environments.[1] This rationale has led to more than 35 years of investigation into ascidians as sources bioactive natural products,[2] resulting in the identification of many potent drug leads and even a clinically approved agent (Yondelis; Et-743).[3,4]

Although ascidians (Phylum Chordata) are not closely related to sponges (Phylum Porifera), they live a similar sedentary, filter-feeding lifestyle. As is the case with sponges, many metabolites first isolated from ascidians resemble or are identical to compounds from bacteria (Table 1). For this reason, as with sponges, it has been hypothesized that symbiotic bacteria are the true sources of many “ascidian” metabolites.[5] In addition, there are several ascidian compounds that are apparently found only in marine animals. The presence of these secondary metabolites across distantly related animal taxa is sometimes used as an argument for a potential symbiotic source.

Recent advances add considerably to the evidence in favor of a bacterial origin for many ascidian compounds (Figure 1). A particularly well-studied interaction is the symbiosis between didemnid ascidians and *Prochloron* spp. photosynthetic cyanobacteria that inhabit their cellulose tunics.[6] *Prochloron* is sometimes required for the survival of the animals providing fixed carbon to the host and recycling nitrogen. Many other symbiotic interactions in ascidians exist but are not as well documented. Experimental evidence has shown that

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Prochloron produces some of the metabolites originally isolated from ascidians.[7,8^{**},9-10] In addition, other recent studies directly or indirectly implicate bacteria in the production of ascidian compounds.[11,12^{**}]

A caveat: This field is in its infancy, and much more research is required to fully determine the origin of the diverse natural products isolated from ascidians. Although this review focuses on known or possible symbiotic sources, other possibilities include synthesis by the host animal, by eukaryotic microbes, or by multiple organisms, among others.

1. Polyketides

Sagittamide A was isolated from didemnid ascidians.[13] It was proposed that sagittamide A (**1**) could arise from polyketide extension with hydroxymalonyl CoA, such as is found in the bacterial zwittermicin (**2**) and aflastatins (Figure S1). If so, this could indicate a possible bacterial origin, but as the authors point out there are many other possibilities.

Compelling, but indirect, evidence exists that a series of ascidian polyketides exemplified by iejimalide (eg **3**) may be produced by bacteria (Figures S2 and S3).[14] All of the compounds in this series except for bistramides [15] are strikingly similar in their probable biosynthesis, and they can be structurally aligned. The molecules have very similar putative starter units, followed by 0-2 amino acids. From there, a putative polyketide chain proceeds for 16-26 carbons, including a macrocycle of 13-24 atoms. Bistramides distantly resemble this family of ascidian polyketides, except that the amino acids are at the center of the compounds rather than at the starter side.

These compounds are similar to bacterial polyketides such as rhizoxins (eg **9**) and archazolids (eg **10**).[16,17^{*}] Exact analogs of biselides and haterumalides (eg **11**) have been isolated from soil and epiphytic bacteria, and haterumalides have also been isolated from sponges.[18^{*}] Lobatamides are very similar to salicylhalamides from sponges as well as to oximidines I, II and III (**12**) from bacteria. Iejimalide A was isolated from a marine cyanobacterial mat.[19^{*}] Bistramides more closely resemble sponge polyketides that have been traced to symbiotic proteobacteria, such as mycalamide A (**13**).[20^{**}]

The source of the iejimalide relative, palmerolide A in *Synoicum adareanum* from Antarctica was studied using a metagenomic approach.[21^{*}] Fragments of polyketide synthase genes from the *trans*-AT group were identified by PCR, and proteobacteria were identified using a 16S analysis. Further data are required to tie the reported sequences to palmerolide. Bistramides were identified in tropical ascidians, *Lissoclinum bistratum*, that harbor *Prochloron* cyanobacterial symbionts. Two localization studies have examined the origin of bistramides, both indicating that the bistramides were more concentrated in *Prochloron* than in the ascidian host.[22,23]

A number of C₁₁ polyketides has been reported from several ascidians (Figure S4).[24] All members of the group contain odd-chain, highly oxidized hydrocarbons with a central methyl sidechain. The canonical representative of this class is lissoclinolide (**14**), which is identical to the actinomycete compound tetrenolin. A series of related derivatives (eg **15-18**) has been isolated from sponges, ascidians, and a cyanobacterial mat.[25-28] The ascidian *Diplosoma virens* was separated from symbiotic *Prochloron* cyanobacteria, and C₁₁ compounds could be detected in the resulting bacterial acetone extracts, indicating a potential *Prochloron* origin.[27^{*}]

Intriguingly, ascidian enediynes namenamicin (**19**) and the shishijimicins (**20**) contain core structures that are nearly identical to that of calicheamicin (eg **21**), an actinomycete natural

product that is part of the clinically used Mylotarg (Figure S5).[29-31] Cultivation attempts have not met with reported success.[32]

Enterocins (eg **22**) have been isolated from ascidians and bacteria. (Figure S6).[33] In the original isolation of enterocin derivatives from *Didemnum* sp. ascidians, the authors unsuccessfully attempted to cultivate bacterial producers of enterocins, and they also noted the presence of V-shaped, *Arthrobacter*-like bacteria in fixed tissue samples.[33]

2. Peptides and primarily peptidic compounds

Most of the drug development activity with ascidian compounds has focused on peptides, including both peptides of probable nonribosomal origin and those of probable or demonstrated ribosomal origin.

Ecteinasidin 743 (Et-743; Yondelis; **23**), which is an approved anticancer agent in Europe and in advanced trials in the US, is of possible nonribosomal origin. The ecteinasidins were isolated from the ascidian *Ecteinasidia turbinata* and *E. thurstoni* (Figure S7).[34] They are related to the safracins and saframycins from bacteria, as well as to renieramycins, jorumycins, and similar compounds from sponges. In bacteria, a nonribosomal peptide synthetase (NRPS) encoding either 3 or 4 modules is solely responsible for synthesis of a core tetrapeptide, and for reductive cyclization of the tetrapeptide to form 3 new heterocycles. The bacterial tetrapeptide is composed of alanine or derivative thereof, glycine, and two units of highly modified tyrosine.[35] Et-743 is apparently composed of cysteine (in place of alanine), glycolate, and two subunits of modified tyrosine. There is a twist: an additional dopamine derivative is added to the N-terminal side of the peptide in Et-743.

In *E. turbinata*, molecular methods identified the γ -proteobacterium, *Candidatus* Endoecteinasidia frumentensis, as a potentially vertically transmitted, intracellular bacterium associated with *E. turbinata* in the Mediterranean.[36"] Later, *E. turbinata* collected in 3 different locations in the Caribbean was analyzed for the presence of persistent bacteria, which might be associated with compound production. Only *E. frumentensis* was found in all 3 animals, validating this strain as a persistent symbiont.[37] However, no peer-reviewed data have been presented linking the bacteria to compounds as of this writing (but see [12"]).

The didemnins family of ascidian natural products has been described from several ascidians (Figure S8).[38] Included in this family is aplidine, which was given orphan drug status in Europe for cancer. The didemnins are cyclic depsipeptides of possible nonribosomal origin. In addition, there are 1-2 polyketide-like units, forming isostatine and the statine-like Hip group. Most of the variability in the didemnins family occurs at the side chain, which contains diverse amino acids and acyl moieties. The only exception to this is in the tamandarins, which have a slightly different composition. So far, mixed polyketide-peptide biosynthesis has only been described in bacteria and lower eukaryotes, indicating that the compounds could be derived from symbiotic bacteria. Statines and isostatines are thought to be largely associated with bacteria.[39]

Two peptide families may be either ribosomal or nonribosomal, exemplified by diazonamides (eg **29**), from *Diazonia* sp., and vitilevuamide (**30**), from *P. lithostrotum* and *Didemnum cuculliferum* (Figure S9).[40,41] Both molecules have features that have been previously found only in nonribosomal or only in ribosomal peptides isolated from bacteria.[42,43]

The biggest single class of complex natural products from ascidians is the ribosomal peptide group, with more than 60 representatives (eg, **31-33**). The cyanobactin family includes N-C circular peptides that are further modified by heterocyclization or prenylation at cysteine, serine, and threonine, and several other modifications (Figure S10).[7,44,45] Cyanobactins have been isolated from both free-living cyanobacteria and from ascidians. Virenamides (eg **34**) are biosynthetically similar to cyanobactins.[46] Westiellamide / cyclohexazoline has been isolated both from cyanobacteria and from ascidians, while virenamide-like molecules (eg **35**) have also been isolated from cyanobacteria.[47]

Genetic methods were used to definitively show that uncultivated symbiotic cyanobacteria, *Prochloron* spp., synthesize cyanobactins in ascidians. Genes were identified from coral reef animals by metagenome sequencing or by random library generation and functionally expressed in *Escherichia coli*. [9,10] Cyanobactin biosynthetic genes were localized to the *Prochloron* genome in whole-genome sequencing studies, showing that the compounds originate in *Prochloron*. [9] No such genes were found in the animal host or in other bacteria in the assembly. Representatives of all compound classes have been identified in ascidian symbionts using genetic methods, accounting for about 6% of known ascidian natural products. [7,8**] Taken together, the evidence demonstrates that symbiotic cyanobacteria synthesize this group of ascidian natural products.

The cyanobactins story illustrates the biotechnological application of symbiosis studies (Figure 2). The rare but preclinical lead cyanobactin trunkamide was produced in *E. coli* culture. [7] Many other cyanobactins and cyclic peptides, both natural and unnatural, have been produced *in vivo* in *E. coli* or using recombinant pure enzymes for *in vitro* synthesis. [8,48,49**] The cyanobactin biosynthetic pathway is the most substrate-flexible natural product assembly line so far characterized, and studies of symbiosis were the key to rapidly understanding this flexibility. Briefly, identical enzymes tolerate extremely diverse substrates, catalyzing N-C circularization, heterocyclization, and prenylation through simple genetic engineering of short 18-24 nt DNA cassettes.

Other simple cyclic peptides, cyclo[Leu-Pro]₂, cyclo[Phe-Pro]₂, and cyclo[Val-Pro]₂, have been isolated from ascidians, bacteria, and other organisms. [50] Dragmacidin piperazine derivatives have been isolated both from sponges and from *Didemnum candidum* ascidians. [51]

3. Alkaloids

Tambjamines and staurosporines are the only ascidian alkaloids for which indirect experimental evidence indicates a possible bacterial source (Figure S11). Tambjamines (eg **36**) are pyrrole alkaloids that have been isolated from bryozoans and ascidians as well as their nudibranch predators, where they are likely used as defense against predation by fish. [1,52] In addition, tambjamines have been isolated from actinomycete bacteria, [53] and they are closely related to the prodigiosin-type alkaloids that are common in diverse bacterial strains. [54] A tambjamine derivative (YP1; **37**) was isolated from the marine bacterium, *Pseudoalteromonas tunicata*, which in turn was isolated from the surface of an ascidian, but one that does not contain tambjamines. [11]

Staurosporine (**38**) and related metabolites such as granulatimides (eg **39**) are isolated from diverse taxonomic groups (Figure S11). [55,56] These compounds are extremely common in bacteria, and they have been isolated from a number of ascidians, especially *Eudistoma toalensis* and its predatory flatworm, where they serve as potential feeding deterrents. [57] Although the animal compounds are of presumed bacterial origin, as of this writing there is no direct experimental evidence supporting this view. A potential contrary example is found in the granulatimides, which were identified in ascidian cells of *Didemnum* sp. [58] Cellular

localization does not necessarily indicate the metabolic origin of metabolites, but these data could be interpreted as possibly indicating an animal source for the compounds.

The pyridoacridines (eg **40**) are found in ascidians and sponges, with many identical compounds found in both taxa, but related compounds have not been found outside of these groups or in bacteria (Figure S12).[59,60] There is some indirect evidence based upon sulfur content that ascidian pyridoacridines may be localized in ascidian cells, and bacterial symbionts of these ascidians have also been described.[61] Sponge-derived pyridoacridines were localized to sponge cells, which was interpreted cautiously to indicate a potential eukaryotic origin for the compounds.[62]

The ascidian pibocins (eg **41**) are closely related to fungal festuclavines (eg **42**) (Figure S12).[63] Fascaplysin (**43**) has been isolated from ascidians and sponges.[64]

4. Terpenoids

The only ascidian isoprenoids with data supporting possible symbiotic origin are the ritterazines (eg **44**), dimeric steroidal alkaloids isolated from *Ritterella tokioka* (Figure S13). [65] Because they bear a striking resemblance to the tubeworm-derived cephalostatins, it has been proposed that the compounds might be derived from a microbial source. A challenge to this idea is that cholestane sterols are not known to be made by bacteria

5. Miscellaneous metabolites

There are many other ascidian metabolites that cannot be discussed here. One group includes tubercidin analogs, which have been isolated from *Didemnum voeltzkowi*. [66] Identical compounds have been isolated from algae, while similar compounds are known from diverse sources including actinomycetes.

Conclusion

Although as apparent above the data are often limited and of varying quality, we believe that current data support the hypothesis that most of the potentially bioactive ascidian natural products are made by symbiotic bacteria. Further understanding of the general rules behind these symbioses will thus be greatly useful to biotechnology and drug development, as exemplified by early work on the cyanobactin biosynthetic pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Figure 2.

Table

Ascidian Compound	Closest Bacterial Homolog	Compound Identity Level	Experimental Evidence
1. Polyketides			
A. Lipid-like			
Sagittamides	Aflastatins, zwittermicin	Distant	None
B. Iejimalide group			
Lobatamides	Oximidines I, II, III	Very similar	None
Patellazoles	Archazolid	Very similar	None
Palmerolide	Archazolid	Somewhat similar	<i>trans</i> -AT sequences identified by PCR
Haterumalides / biselides	Biselides	Very similar to identical	None
Iejimalides	Iejimalide A	Identical	None
Bistramides	Pederin / onnamides	Similar motifs	Compounds localized to <i>Prochloron</i> by cell separation
C. Odd-chain hydrocarbons			
Lissoclinolide	Tetrenolin	Identical	None
Other C ₁₁ hydrocarbons	Nakienones	Very similar	Compounds localized to <i>Prochloron</i> by cell separation
D. Eneidyne			
Namenamicin / shishijimicins	Calicheamicin	Very similar	None
E. Rearranged polyketides			
Enterocins	Enterocins	Identical	None
2. Peptides			
A. Nonribosomal			
Didemnins	Statines and numerous nonribosomal depsipeptides	Similar motifs	None
Ecteinascidins	Saframycins and safracins	Very similar	Protein / gene isolation, 16S analysis
B. Uncertain			
Diazonamide	Nonribosomal peptides, microcins, thiopeptides	Unusual, some common motifs	None
Vitilevuamide	Lantibiotics, microcins, nonribosomal peptides, thiopeptides	Unusual, some common motifs	None
B. Ribosomal			
Westiellamide	Cycloxazoline	Identical	None
Other cyanobactins (patellamides, trunkamide, etc.)	Tenuocyclamides, prenylagaramides, others	Very similar	Whole genome sequencing and heterologous pathway expression
Virenamides	Aeruginosamide	Very similar	None
Cyclic (X-Pro) ₂	Cyclic (X-Pro) ₂	Identical	None
3. Alkaloids			
A. Indole alkaloids			

Ascidian Compound	Closest Bacterial Homolog	Compound Identity Level	Experimental Evidence
Staurosporines	Staurosporines	Very similar to identical	None; for granulatimide, microscopy showed concentrated in animal cells
Pibocins	Brominated festuclavine (fungal derivative)	Almost identical to fungal metabolites	None
Fascaplysin	None	None	Also found in sponges
Pyridoacridines	None	None	Also found in sponges; localized to sponge cells
B. Pyrroles			
Tambjamines	Tambjamines	Very similar to identical	Cultivated bacteria from ascidian
4. Terpenes			
Ritterazines	None	None	None
5. Miscellaneous			
Tubericidins	Tubericidins	Identical	None