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MINING MICROBIAL SYMBIONTS FOR SPONGE-DERIVED NATURAL PRODUCTS: IMPLICATIONS FOR THE WALLACEA

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Abstract
The Indonesian Coral Triangle is a biodiversity hotspot bisected by the Wallace line. It is becoming clear that ecological and anthropogenic factors are impacting the region. Our research is focused on the biosynthetic products of sponges. These natural products, which are beneficial to human health, will be lost if biodiversity were to decrease. For decades, chemists have looked to marine sponges as a source of novel pharmaceuticals. Over time, there has been growing suspicion these metabolites may actually be produced by microbial symbionts. Herein, we discuss a brief history of sponge natural products chemistry. Sponge associated microorganisms and their likely role in the production of clinically relevant compounds are explored through three case studies. The potential intellectual and pharmaceutical impact locked within the sponges of the Indonesian Coral Triangle is immense. We conclude that conservation, protection and management of this resource are vital from an ecological and human health perspective.

***Keywords*:** Bacteria, bengamide, fijianolide, Indonesian Coral Triangle, psymberin, symbiosis

Background

The Indonesian Coral Triangle is well recognized as a hotspot of biodiversity (Myers *et al*., 2000; Barber, 2009; Carpenter *et al*., 2011). While spanning only 1.6% of the planet's ocean, the Coral Triangle region contains 76% of all known coral species, hosts 37% of all known coral reef fish species, and 53% of the world's coral reefs (Veron *et al*., 2011). No other region of the world contributes this amount of biological diversity. As such, it remains a key area for research, management and preservation.

The first biogeographical studies of the area, however, were terrestrial based. In 1863, Wallace presented a map with a red line starting at the deep strait between Bali and Lombok and passing through the Makassar Strait (Veron *et al.*, 2009). The western region was labeled "Indo-Malayan region" and the eastern "Austro-Malayan region". Renamed "Wallace's line" in 1868, this line has become one of the best known demarcations in the history of biogeography (Veron *et al.*, 2009). Over time, Wallace's Line has been shifted and moved as the result of various studies of terrestrial animal life. Initially, marine life was mostly ignored during these studies. In a 2000 communication to Nature, Barber and coworkers suggested the presence of a localized biogeographic marine boundary, which they termed a "marine Wallace's equivalent" (Barber *et al.*, 2000). Later work on the comparative phylogeography of the Indonesian Coral Triangle revealed clear patterns of species distribution (Carpenter *et al.*, 2011), illustrated in Fig. 1 (pl. 1).

The Indonesian Coral Triangle represents more than a global epicenter of coral species. Together with the soft and stony corals, sponges play an important role in the formation of the complex reef systems. Sponges interact with organisms and their abiotic environments. The rapidly changing conditions of marine ecosystems call into question our abilities to predict the fate of these interactions, as well as sponge distribution and abundance (Wulff, 2012). The results of human interactions and settlements can be extremely complex and difficult to predict (Miller & Hobbs, 2002). Nevertheless, it is clear that human disturbances are negatively affecting the health of the coral reef. Overfishing, air and water pollution events, ocean acidification, deforestation, and coastal development are all factors in reef deterioration (Unsworth, 2013). Sponges have not escaped these effects. For example, sponge diversity in the Spermonde Archipelago has been impacted by anthropogenic-linked increased rates of sedimentation (de Voogd *et al.*, 2006). The protection and preservation of the sponge and other marine biodiversity in the Coral Triangle should be of the utmost importance.

Sponges are among the most primitive multicellar organisms still extant (Blunt *et al.*, 2012). Members of the phylum Porifera (meaning "pore bearing"), sponges have simple bodies and lack specialized organs. Using a collection of cells arranged around a series of canals and chambers, sponges efficiently filter the surrounding water column. This system allows sponges to process volumes of water equal in amount to their own body mass every five seconds (Muller & Muller, 2003). Sponges belong to a diverse taxon and are found in every ocean at all depths. Currently, there are approximately 7,000 taxonomically recognized species of sponges, and new species are continually being discovered (Wörheide *et al.*, 2005). Interestingly, biodiversity studies suggest that this number may be a significant misrepresentation; the actual number of current species could be as much as double what is currently proposed (Wörheide *et al.*, 2005).

As filter feeders, sponges are continually pumping water through their bodies. Bacteria in the surrounding water column are actively taken in by the sponge-driven currents (Imhoff & Stöhr, 2003). The sponge provides an ideal environment for microorganism growth and plays host to a diverse community of microbial symbionts (Taylor *et al.*, 2007a). In some sponges, called "high microbial abundance" sponges, microbes make up as much as 40% of the sponge biomass. In other sponges, referred to as "low microbial abundance" sponges, the microbiota population is relatively sparse (Schmitt *et al.*, 2012). An interesting observation, first described by Hentschel and coworkers, indicated that the composition of the

microbial community of sponges is highly similar, regardless of the host sponge taxa or location (Hentschel *et al*., 2002). A breakdown of microbial sponge symbionts, generated from published literature (Taylor *et al*., 2007b), is shown below in Fig. 2 (pl. 1). The major contributing groups of microorganisms are the Gram-negative beta and gamma proteobacteria. This group includes, among others, bioluminescent bacteria and gliding myxobacteria.

Interest in sponges as a source of novel pharmaceuticals began in the early 1950s with the discovery of the unique nucleosides spongouridine, spongothymid–ine and spongosine (Bergmann & Burke, 1955, 1956). These compounds were the inspiration of the first marine-inspired anti-tumor compound (Ara-A) (Lee *et al*., 1960) and antiviral compound (Ara-C) (Evans *et al*., 1961). By the 1970s, chemists were extracting marine organisms in search of antimicrobial and cytotoxic compounds (Laport *et al*., 2009). Since then, sponges have emerged as the marine Phylum in which most of these compounds have been found. In the past 75 years of marine natural products chemistry, sponge compounds account for ~49% of the total number of compounds isolated from marine animals and 35% of all marine natural product sources (Blunt *et al*., 2012). Molecular structures discovered from sponge taxa have a rich past and increasing future potential. The incredible bioactivity of these compounds have led to numerous clinical trials and approved pharmaceutical products. As illustrated in Fig. 3 (pl. 2), this is an exciting time for the use of sponge-derived compounds. The sponge-derived compounds PM060184 (Martines-Diez *et al*., 2014) and pipecolidepsin A (Coello *et al*., 2014) are currently under evaluation as cancer drugs. The sponge-inspired compound, eribulin mesylate (Towle *et al*., 2001) (sold under the trade name Halaven®) was developed from the sponge compound halichondrin B (Hirata & Uemura, 1986) and approved by the FDA in 2010. E7974 was inspired by the sponge compound hemiasterlin (Talpir *et al*., 1994), and phase I clinical trials have been completed (Rocha-Lima *et al*., 2012).

Academically, the intellectual impact of sponge natural products is immense. A selection of eleven sponge compound classes is shown below in Table 1. To examine the impact of these compounds in the published literature, a Web of Science topic search was conducted on each compound. A total of 2372 publications have appeared on just these eleven compound classes. When search parameters are expanded to include the total number of citations of these publications, the total number of citations is estimated as 77,502. It is a combination of structural characterizations and therapeutic studies that have motivated the substantial intellectual investigations of these sponge-derived natural products. These metrics are not restricted to these compounds alone but are representative of greater trends. Sponge natural product chemistry has been, and will continue to be, an exciting and fulfilling area of scientific research. The contribution to the scientific literature is immense and likely to continue to grow.

Table 1: The intellectual impact of sponge natural products, based on a Web of Knowledge topic search; the list only includes peer reviewed publications as of August 2014. Total publication numbers were generated by completing a topic search using the name of the natural product. The total number of citations was determined through the use of the "citation report" feature within Web of Science, which calculates the number of citations of articles generated in a topic search.

Marine Sponge Natural Product Compound Classes	Wallacea West or East	Total Publications	Total Citations
Jasplakinolides	unknown	517	18039
Fijianolides (aka Laulimalide)	West	163	6613
Milnamide (aka Hemiasterlin)	unknown	73	1985

Bengamides	unknown	56	1467
Psymberin	unknown	45	93
Mycothiazole	unknown	20	400
Plakinidine	unknown	16	297
Latrunculins	West	1309	44780
Aaptamines	both	70	1263
PBDEs	both	29	1159
Fascaplysins	unknown	74	1406

For decades, there has been a growing suspicion that bacteria and other symbiotic microorganisms are the true producers of animal-derived natural products. Natural products are produced by an enzymatic factory with the biosynthetic machinery encoded for in an organism's DNA. It follows that compounds with similar or parallel structural features would be the result of similar biosynthetic pathways. It seems unlikely that a prokaryotic bacterium and a eukaryotic macro organism would have similar gene clusters to make structurally similar compounds. The common hypothesis is that it is likely that symbiotic bacteria are the true producers of many natural products isolated from (marine) animals (Piel, 2011). There are many dramatic examples of this phenomenon in marine sponges; a few of these examples are highlighted in Table 2.

Early review articles explore the phenomenon of structural parallels from a chemical perspective, concluding that the isolated compounds likely originate from microbial symbionts and not the animal hosts (Faulkner, 1992; Kobayashi & Ishibashi, 1993). It wasn't until the dawn of the genomic era, when sequencing technologies advanced and costs decreased, that this hypothesis could be explored on a genetic level. Metagenomic studies of sponges were particularly enlightening; high numbers of bacteria-like polyketide synthase (PKS) genes can be readily detected in the sponge metagenome (Hochmuth *et al*., 2010).

The fijianolides, shown in Fig. 4A (pl. 3), are a class of biologically active sponge-derived compounds with suspected microbial origin. They were isolated nearly concurrently by two separate research groups. The Crews research group isolated fijianolides A and B from the marine sponge *Cacospongia mycofijiensis* (Quinoa *et al*., 1988*).* Scheuer and coworkers isolated identical compounds from an Indonesian sponge *Hyatella* sp. and its nudibranch predator, *Chromodoris lochi*, naming them the laulimalides (Corley *et al*., 1988*).* Extensive synthetic efforts by multiple groups resulted in a large number of published synthetic schemes and analogs (Crimmins, 2002; Mulzer *et al*., 2002; Mulzer & Öhler, 2003), although none have exhibited better activity than fijianolide B. Fijianolide B is especially potent. Initial activity was reported against the KB cell line, with an IC_{50} of 50 ng/mL (Corley *et al*., 1988). Interest in this class of compounds increased when it was shown that it stabilizes microtubules, causing abnormal bundles to form (Mooberry *et al*., 1999). Paclitaxel, the most well-known and successful tubulin stabilizer, binds at a different site compared to the fijianolides (Pryor *et al*., 2002). Cells resistant to paclitaxel therefore respond to the tubulin binding effects of the fijianolides which acts synergistically with paclitaxel. A recent 2014 study utilized x-ray crystallography to define the mechanism of action (Prota *et al*., 2014). Steinmetz and coworkers determined that the compound binds to a unique site on β-tubulin, using its macrolide core to interact with another tubulin dimer. It also stabilizes a part of tublin that is responsible for lateral tubulin contacts in microtubulues.

Table 2: A set of selected examples of sponge-derived vs. bacteria-derived natural products, highlighting the idea that many sponge-derived natural products may be the result of the biosynthetic talents of microorganisms

Sponge metabolite	Sponge Source	Microorganism metabolite	Microorganisms source
Andramid	*Hyatella* sp.	Andramid	*Vibrio* sp.
Bengamide E	*Jaspis* cf. *coriacea*	Bengamide E	*Myxococcu virescens*
Discodermide	*Discodermia dissoluta*	Alteramide A Ikarugamycin	*Alteromonas* sp.
Jasplakinolide	*Jaspis splendens*, *Auletta* sp.	Chondramide D	*Streptomyces* sp.
Latrunculin A	*Cacospongia mycofijiensis* *Negombata magnifica*	Epothilone B	*Chondromyces crocatus*
Maklauvamine A	*Zyzzya* cf. *marsalis*	Makaluvamine A	*Sorangium cellulsoum*
Manzamine A	*Haliclona* sp.	Manzamine A	*Didymium bahiense*
Mimosamycin	*Petrosia* sp.	Mimosamycin	*Streptomyces lavendulae*
Mycalamide A	*Mycale hentscheli*	Pederin	Undescribed symbiont of *Paederus* sp. (beetle)
Okadaic acid	*Halichonbdria okadai* *H. melandocia*	Okadiac acid	Undesribed symbiont bacteria; *Procentrium limai*, *P. concavum*, *Dinophysis* spp.
Renieramycin E	*Reniera* sp.	Saframycin Mx1 Saframycin A	*Myxococcus xanthus*, *Pseudomonas fluorescens*
Salicylihalamide A	*Haliclona* sp.	Apicularen A	*Chondromyces* sp.
Sphinxolide	*Neosiphonia superstes*	Rhizopodin	*Myxococcus stipitus*

When tested *in vivo*, fijianolide B demonstrated significant inhibition of tumor growth in tumor-bearing severe combined immune-deficiency (SCID) mice dosed at 25 mg/kg over five days (Johnson *et al*., 2007). The clear *in vivo* efficacy warranted additional therapeutic evaluations. In another study, fijianolide B was tested in melanoma and fibrosarcoma mice models (Liu *et al*., 2007). This work showed minimal growth inhibition of tumors regardless of dosage and exhibited high toxicity. Further preclinical evaluations are warranted to explore these discrepancies.

What is most interesting about the fijianolides, and the sponge it is isolated from, is the geographical distribution of different chemotypes. Illustrated in Fig. 4B (pl. 3), our 2007 publication outlines the biogeographocal distribution of metabolites and morphology of *C. mycofijiensis* (Johnson *et al*., 2007). Sponges growing in close proximity could have significant chemotypic differences, however, the explanation for these difference were speculative at best. A 2010 study undertook an exhaustive sequencing effort to investigate the *C. mycofijiensis* metagenome (Hochmuth *et al*., 2010). This work demonstrated the extensive number of PKS gene clusters present in this HMA sponge. Consequently, PKS genes were undetectable in LMA sponges. These observations point to the sponge symbionts as the likely source of these biologically significant compounds.

The bengamides (Fig. 5: pl. 4) is another pharmacologically interesting class of sponge derived bioactive compounds with a

microbial connection. They are primarily isolated from the sponge *Jaspis* cf. *coriacea*, a dull orange colored sponge, although other sponges are known to contain the compound. The bengamides all contain a lysine or hydroxylysine derivatized caprolactam with a highly oxygenated polyketide tail. Based on structural clues, these compounds appear to be the result of a hybrid PKS/nonribosomal peptide synthase (NRPS) biosynthetic cluster, although the gene cluster has yet to be identified.

Originally isolated in 1986 as bengamide A (Quinoa *et al*., 1986), there are currently 21 naturally occurring bengamide analogs. The bengamides have received significant attention due to their substantial biological activity. *In vitro* tumor cell cytotoxicity tests indicate IC_{50} values in the low nM range, however poor solubility kept these naturally isolated compounds out of preclinical development. Significant effort was made to exploit the pharmacophore core and develop effective synthetic analogs (Garcia-Ruiz & Sarabia, 2014). One of these analogs, LAF389, was developed at the U.S. Novartis Institute for Biomedical Research (Kinder *et al*., 2001). LAF389 was found to have comparable *in vitro* activity to the potent natural analogs; *in vivo* testing was shown to be effective and tolerable in animals. Phase I clinical trials were initiated in 2000 (Dumez *et al*., 2007). However, adverse effects not seen in animal testing ended the clinical trials of this compound as an anticancer drug.

A more recent study identified the bengamides as a new class of immunomodulators. The bengamides showed significant activity as a nuclear factor - κB (NF-κB) inhibitor (Johnson *et al*., 2012). Dysregulation of NF-κB protein complex is implicated in a wide variety of diseases, and is an attractive area of drug discovery (Karin *et al*., 2004). These observations suggest that the bengamides may have new therapeutic life as a therapy for inflammation, autoimmune disorders and certain cancers.

Like most sponge natural products, it had long been assumed that the bengamide class of compounds was the result of associated microorganisms. In 2005, a group from Sanofi-Aventis submitted a patent that described the isolation of bengamide derivatives from a terrestrial myxobacteria strain, *Myxococcus virescens* (DSM 15898) (Hoffmann *et al*., 2005). The identification of a culturable microorganism as a source of a sponge natural product was a significant advancement, and creates a sustainable source of natural products. The Crews research group at UCSC has successfully used *M. virescens* to generate bengamide analogs for extensive biological screenings (Johnson *et al*., 2012), efforts that would have otherwise been slowed by the need to collect additional sponge material.

Psymberin, and its related analogs, are another fascinating example of connecting a sponge derived natural product with bacterial biosynthetic machinery. Psymberin has a complex polyketide structure that was first isolated from a *Psammocinia* species (Cichewicz *et al*., 2004). An identical structure, named irciniastain A, was also isolated nearly simultaneously from the sponge *Iricinia ramose* (Pettit *et al*., 2004). Psymberin is selectively cytotoxic against certain human cancer cell lines and is remarkably potent. A 2010 study showed that psymberin inhibits protein translation and induces activation of stress-activated protein kinases, likely mediated by mitochondrial-derived reactive oxygen species (Chinen *et al*., 2010). The exact mechanism of cell death was not elucidated. A more recent study, published in 2012, explored the mode-of-action of psymberin through the use of the nematode *Caenorhabditis elegans* (Wu *et al*., 2012). Ethyl methanesulfonate was used to randomly mutate the nematodes. Mutants that

exhibited drug-resistance each contained the same point mutation in the ribosomal large subunit protein. Wu and coworkers showed that the psymberin-resistant mutants were not resistant to mycalamide A, a structurally related compound which binds to a homologus protein in archea, suggesting that the compounds bind differently to the same target. Over a decade after its discovery, exciting work on synthetic analogs and further mechanism of action studies continue on psymberin. Its impressive cytotoxicity and encouraging preclinical efficacy data highlight the need for continued studies.

Interestingly, psymberin and its related analogs also provide one of the first insights into the genetic basis of sponge natural products produced by bacterial associants. Psymberin is a member of the pederin class of compounds. This class consists of a complex polyketide core and variable polyketide or amino acid tails. Pederin was first isolated in 1953 from a collection of 25 million beetles and its structure was later determined in the 1960s (Bielitza & Pietruszka, 2013). Decades later, compounds with remarkably similar structures were discovered. Shown below in Fig. 5 (pl. 4), examples include the mycalamides, onnamides, and icadamide (Perry *et al*., 1988; Sakemi *et al*., 1988; Shinde *et al.*, 2007). This cross phylum structural parallel suggested that a microbial symbiont is responsible for the production of this class of compounds.

To determine the true producer of these compounds, work was begun to isolate and analyze the gene clusters responsible for these metabolites. First, the pederin biosynthetic cluster was isolated from an uncultured symbiont of the *Paederus* sp. beetle in 2002 (Piel, 2002). Later, Piel and coworkers analyzed the metagenome of *Theonella swinhoei*, a Japanese sponge that contains onnamide A (Piel *et al*., 2004a, b, 2005). From this sponge, genes with nearly identical structure to the pederin gene cluster were found. Neither the beetle nor the sponge-derived gene clusters contained any features typical of eukaryotic genes. A 2009 publication later isolated the gene cluster responsible for the production of psymberin from the sponge *Psammocinia* aff. *bulbosa* (Fisch *et al*., 2009). Like the pederin and onnamide A clusters, the gene features were exclusively bacterial in nature. These findings clearly suggest a bacterial symbiont is responsible for the production of this class of compounds. It is intriguing to consider how a marine sponge and a terrestrial beetle could evolve to contain the same bacterial symbiont and what evolutionary advantage is conferred to the sponge.

As the most biologically diverse marine region in the world, the Coral Triangle and the part of it that is represented in Indonesia and Wallacea contains an extensive number of sponge species and in turn, a large number of potentially pharmacologically relevant sponge-derived natural products. Coral reefs and sponge communities are dwindling worldwide due to exposure to ecological and anthropogenic threats. The ecological and pharmacological impacts of a decreased sponge population would be devastating. Continued studies of microbial symbionts as a source of new natural products is increasingly important. By identifying the bacterial producers of sponge-derived natural products sustainable sources of these compounds can be developed with minimal impact on the sponge community. Currently, this process is somewhat limited by the observation that thus far, most sponge-associated bacteria have not yet been cultured in a laboratory setting. Culture independent methods, such as heterologous expression of secondary metabolite producing gene clusters, represent another method of accessing natural products produced by sponge symbionts. These methods are explored in reviews by others (Brady *et al*., 2009; Uria & Piel, 2009; Piel, 2011). Identifying the bacterial sources of sponge-derived natural

products is more than just an intellectual pursuit. This research is vital to the protection and continued health of the sponges in Wallacea and the wider Indonesian section of the Coral Triangle.

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PLATE 1

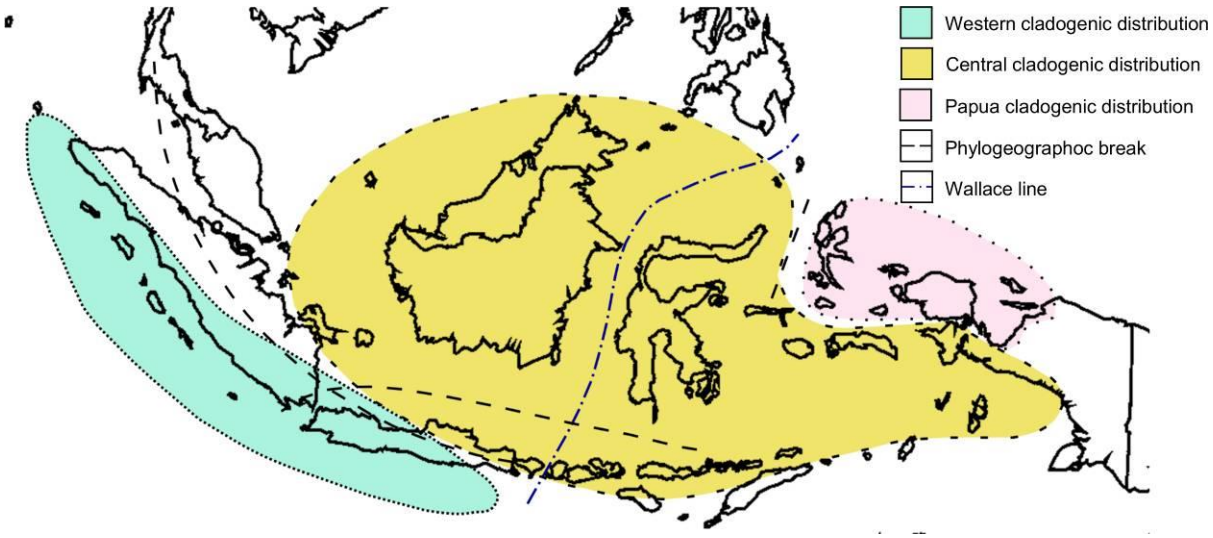


Figure 1: Map of the Indonesia Coral Triangle region with biodiversity hotspots identified. Divisions were based based on the following: data in the literature (Carpenter *et al*., 2011), insights from the Wallace Line, patterns of marine cladogenic distributions, and phylogeographic breaks within the region.

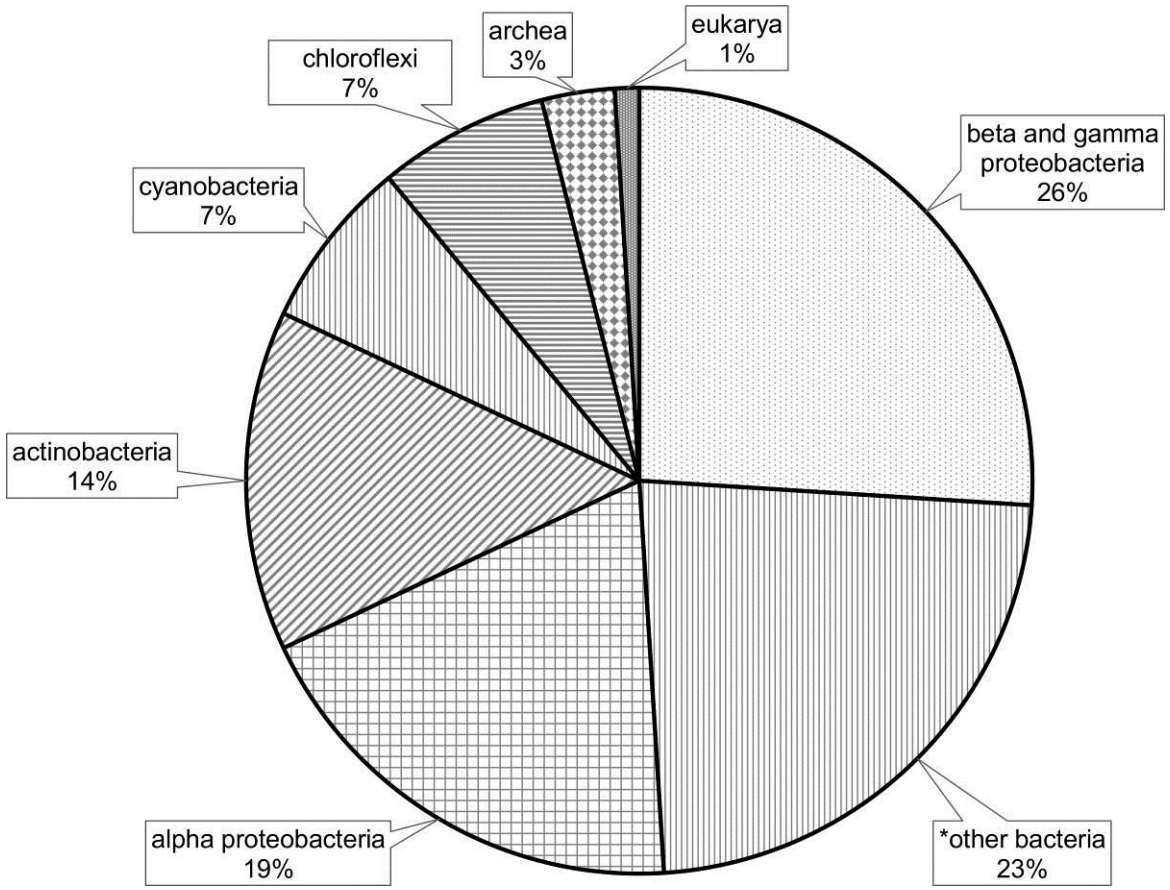


Figure 2: Visual representation of the sponge associated microbial communities. The bacteria listed are grouped according to phylum. *Other bacteria include: Acidobacteria, Bacteroidetes, Deincoccus-Thermus, Gemmatimonadetes, Lentisphaerae, Nitrospira, TM6, and other bacteria of uncertain taxonomy.

PLATE 2

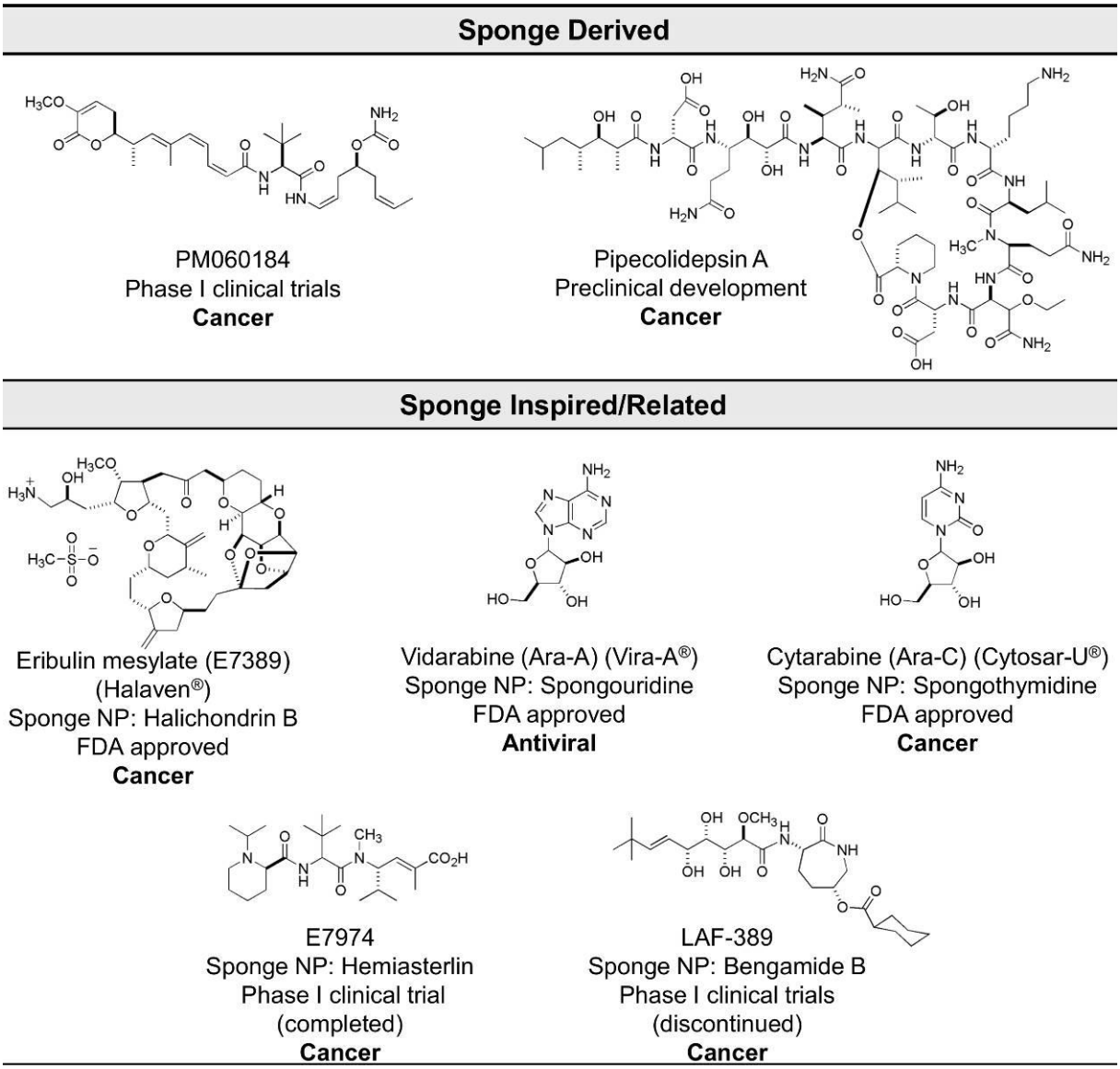
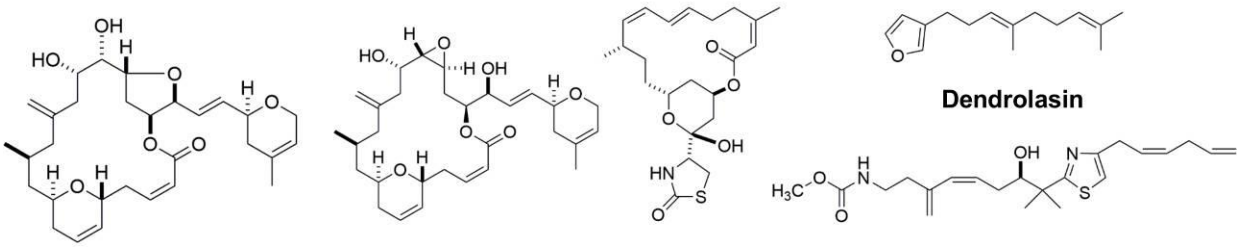


Figure 3: The molecular structures of therapeutics based on the biosynthetic products of sponges. Compounds are grouped according to those actually isolated from sponges or those whose features were inspired by compounds isolated from sponges

PLATE 3

A



Fijianolide A (isolaulimalide) | **Fijianolide B (laulimalide)** | **Latrunculin A** | **Mycothiazole**

B

Collection site	Major constituents	Morphology
Fiji	latrunculin A, dendrolasin	Mushroom
Vanuatu	fijianolide A, fijianolide B, latrunculin A, mycothizaole	Mushroom
Tonga	latrunculin A, mycothiazole	Mushroom
Solomon Islands	latrunculin A, dendrolasin	Tubular
Papua New Guinea	latrunculin A, dendrolasin	Tubular
Indonesia	fijianolide A, fijianolide B, latrunculin A, mycothiazole	Tubular

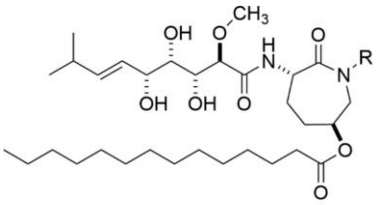
C. Mycofijiensis (mushroom)

C. Mycofijiensis (mushroom)

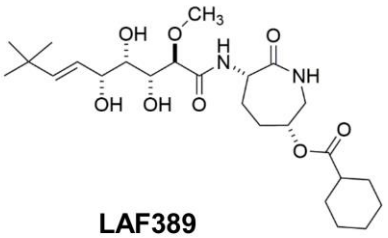
C. Mycofijiensis (tubular)

Figure 4: (**A**) A collection of compounds isolated from *Cacospongia mycofijiensis*; (**B**) Summary of natural products observed from *C. mycofijiensis* broken down by geographic location and sponge morphotype.

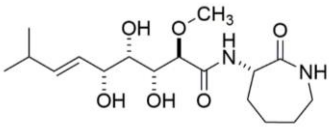
PLATE 4



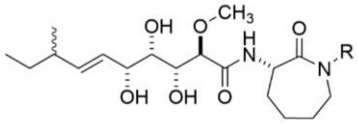
Bengamide A: **R** = H
Bengamide B: R = CH_3
Jaspis coriacea, **1986**
Jaspis digonoxea, **1994**



LAF389
Synthetic analog, **2001**
Entered clinical trials
in 2000 (discontinued)

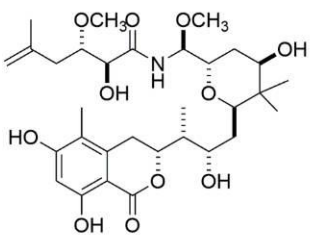


Bengamide E
Jaspis coriacea, **1989**
Myxococcus virescens, **2005**

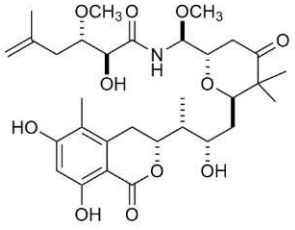


Bengamide E': **R** = H
Bengamide F': **R** = CH_3
Myxococcus virescens, **2005, 2012**

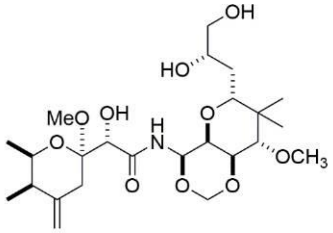
Figure 5: Bengamide class of compounds. Source organisms and year of initial isolation or synthesis indicated where appropriate.



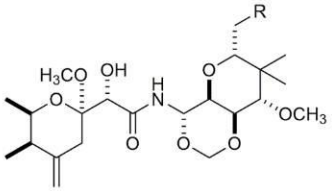
Psymberin (**iraciniastatin A**)
Marine sponges
Psammocinia aff. *bulbosa*
Iricinia ramosa
2004



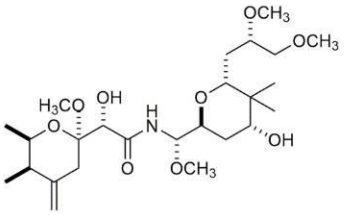
Iraciniastatin B
Marine sponge
Iricinia ramose
2004



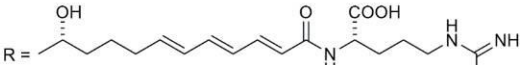
Mycalamide A
Marine sponge
Mycale sp.
2000

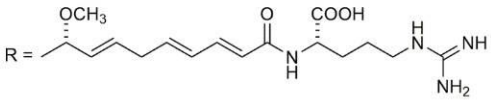


Onnamide A
Marine sponge
Theonella swinhoei
1988



Pederin
Terrestrial beetles
Paederus sps.
1953





Icadamide C
Marine sponge
Discodermia calyx
2007

Figure 5: The onnamide - psymberin class of sponge derived scaffolds. Also shown are selected related metabolites along with their source organisms and the year of their first isolation.