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Discovery, biosynthesis, and bioactivities of peptidic natural products from marine sponges and sponge-associated bacteria

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Covering 2010 to 2025

Sponges are benthic, sessile invertebrate metazoans that are some of the most prolific sources of natural products in the marine environment. Sponge-derived natural products are often endowed with favorable pharmaceutical bioactivities, and paired with their structural complexity, have long served as title compounds for chemical syntheses. Sponges are holobionts, in that the sponge host is associated with symbiotic and commensal microbiome. Natural products isolated from sponges can be produced by the sponge host, or the associated microbiome. Recent genomic studies have shed light on the sponge eukaryotic host as the true producer of several classes of sponge-derived peptidic natural products. In this review spanning years 2010–2025, we describe peptidic natural products isolated from the sponge hosts and the associated microbiome, detail their biosynthetic processes where known, and offer forward looking insights into future innovation in discovery and biosynthesis of peptidic natural products from marine sponges.

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1. Introduction

Sponges (phylum Porifera) are among the most ancient marine invertebrates. The inventory of sponge species in the ocean continues to expand, with nearly 9000 species described to date.¹ Sponges are multicellular sessile organisms living across diverse marine ecological habitats from tropics to poles, oceans and freshwater, intertidal zones to deep seas.^{2,3} Sponges live by heterotrophic filter-feeding, a process that involves pumping several liters of water per gram of sponge biomass per day.⁴ In addition, carnivorous sponges use specialized appendages to capture prey.^{5,6}

Sponges are holobionts consisting of sponge hosts, symbiotic bacteria, and commensal bacteria, as well as other microbes.⁷ Tightly associated symbiotic bacteria are embedded in the sponge host physiology and the holobiont metabolism with endosymbionts residing within the sponge tissue. Consequently, symbiotic bacteria can be challenging to cultivate (often called “uncultivated”, or less accurately, “uncultivable”) under traditional laboratory culturing conditions.

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Photosynthetic endosymbionts contribute to the nutrient supply to the sponge holobiont.⁸ On the other hand, commensal bacteria that are loosely associated with the sponge host are usually amenable to cultivation.

Secondary metabolites—colloquially referred to as natural products—are cornerstones for pharmaceutical discovery.^{9,10} Among natural products, peptidic natural products (pNPs) are a particularly promising group of bioactive compounds that target a wide range of pathologies including infectious diseases, cancers, diabetes, HIV, chronic pain, among others.¹¹ For the purposes of this review, pNPs are divided according to their biosynthetic origins: ribosomally synthesized and post-translationally modified peptides (RiPPs), and non-ribosomally synthesized peptides (NRPs) (Fig. 1). For the purposes of this review, dipeptide alkaloids are not treated as canonical pNPs (*vide infra*).

The abovementioned organismal complexity of the sponge holobiont has prompted genetic investigations aimed at deciphering which component of the holobiont—the sponge host, or the symbiotic microbiome—is the true producer of the pNPs that are detected in sponge extracts. As many pNPs isolated from sponges share structure similarities with those originating from bacteria, it was proposed that the symbiotic microbiome would be the source of sponge-derived pNPs.¹² This hypothesis

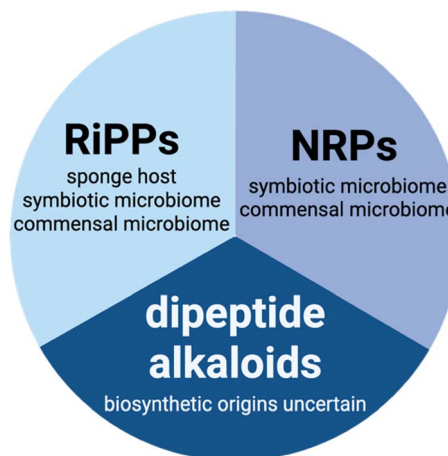
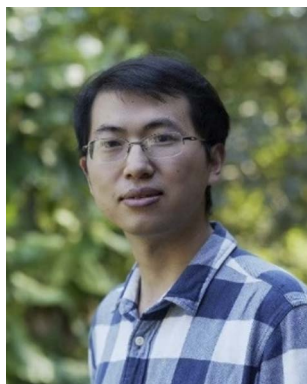


Fig. 1 Chemical classes of pNPs discussed in this review and sites of their biosynthesis within the sponge holobiont.

has indeed borne out (*vide infra*). In addition, the sponge commensal microbiome is now appreciated as a repository possessing numerous biosynthetic gene clusters (BGCs) that encode cryptic pNPs that are not detected in the sponge extracts



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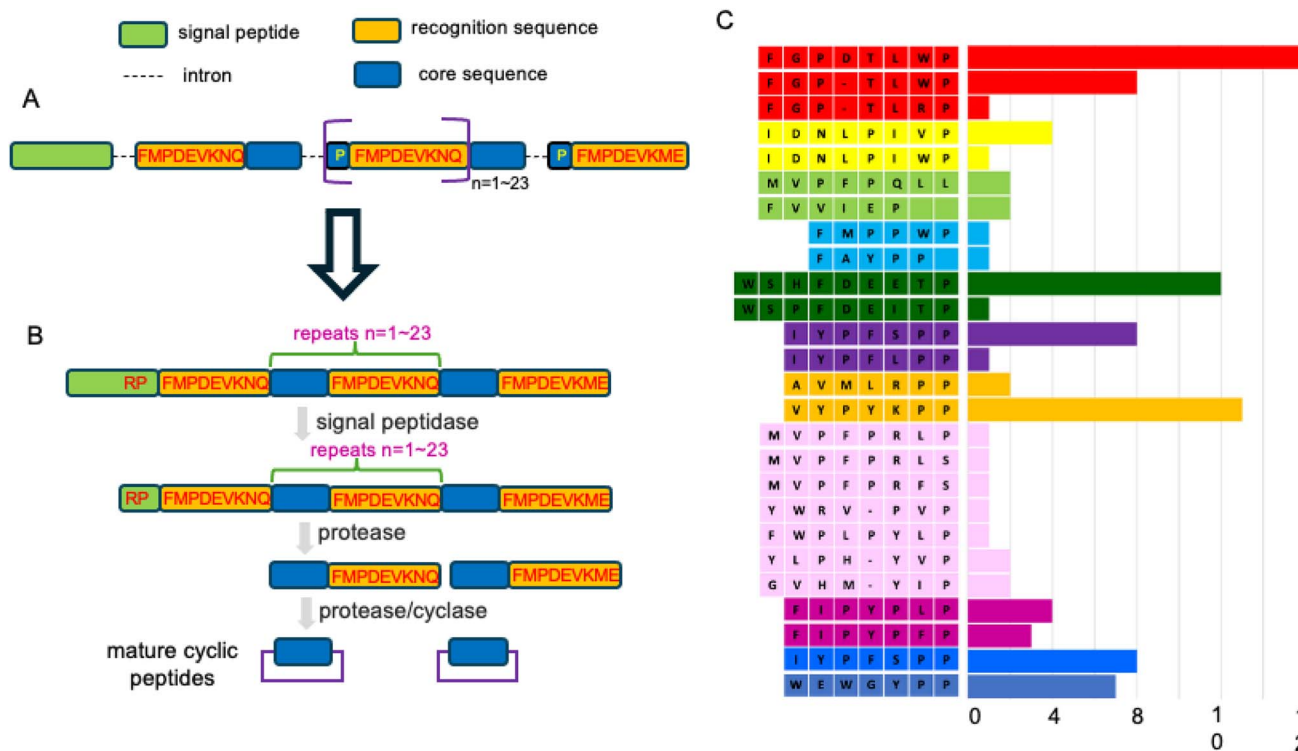


Fig. 2 Sponge genome encodes the precursors for the biosynthesis of PRMPs. (A) General architecture of sponge PRMP precursor genes in the genome. (B) Proposed biosynthetic route of PRMPs. (C) The core peptides found in the intact precursor peptides from genome assembly. Each different color represents a single precursor peptide. The numbers on the x-axis indicate the counts for each core peptide in the corresponding precursor peptide. Figure based on ref. 22.

but can be accessed using microbiology and synthetic biology workflows.^{13–16}

In light of the demonstrated proclivity of the sponge microbiome to furnish pNPs, a question endured if the sponge host itself could also produce pNPs?¹⁷ Certainly, the production of bioactive pNPs—such as venoms and toxins—is widespread in Eukarya, and sponges have been shown to produce other classes of natural products.^{18–20} In recent years, the sponge hosts have themselves shown to be producers of pNPs.^{21,22} These studies provide new avenues to understand the entire sponge holobionts as pNP producers and posit new hypotheses regarding the differential ecological roles of the host-derived and the microbiome-derived pNPs in sponge ecology.²³

With the motivation to juxtapose the sponge holobiont architecture against the biosynthetic routes of sponge-derived pNPs, this review is structured such as to provide a description of the three classes of pNPs according to the site of their biosynthesis within the sponge holobiont—the eukaryotic sponge host itself, the symbiotic microbiome, or the commensal microbiome. Biosynthetic origins for several sponge-derived pNPs have not been deciphered yet; these molecules are discussed separately.

Among pNPs, RiPP biosynthesis starts with the ribosomal synthesis of a precursor peptide. Typically, RiPP precursor peptides are divided into an N-terminal leader and a C-terminal core regions. The leader region is recognized by and binds to the RiPP biosynthetic enzymes which enables post-translational

modification to be affected upon the C-terminal core. Proteolytic removal of the unmodified leader region furnishes the mature RiPP.^{24,25} As described in later sections, the microbes are a well validated source of RiPPs, both from the symbiotic and the commensal microbiomes (Fig. 1). The sponge host has been shown to be a producer of RiPPs as well.

The biosynthesis of NRPs is catalyzed by large megadalton non-ribosomal peptide synthetases (NRPSs) that construct pNPs in an assembly line fashion.^{26–29} Upon ATP-dependent adenylation of the amino acyl carboxylates by the NRPS adenylation domain (A domain), the amino acids are thioesterified to the phosphopantetheinyl arm of thiolation domain (T domain; also referred to peptidyl carrier protein). The NRPS condensation domain (C domain) then catalyzes the peptide bond formation between the downstream T domain-tethered amino acid building block, and the upstream T-domain tethered peptide chain and transfers the growing peptide chain onto the downstream T domain phosphopantetheine arm for the next round of chain elongation by the NRPS A–T–C module. A thioesterase domain (TE domain) terminates chain extension by offloading the mature NRP.^{30,31} These are typical rules; a vast array of other chemical diversity-amplifying modifications have been reported in NRPS assembly lines.³² The symbiotic and commensal microbiomes of marine sponges are sites for biosynthesis of NRPs and various examples are described below. To date, the sponge host has not been found to synthesize NRPs (Fig. 1).



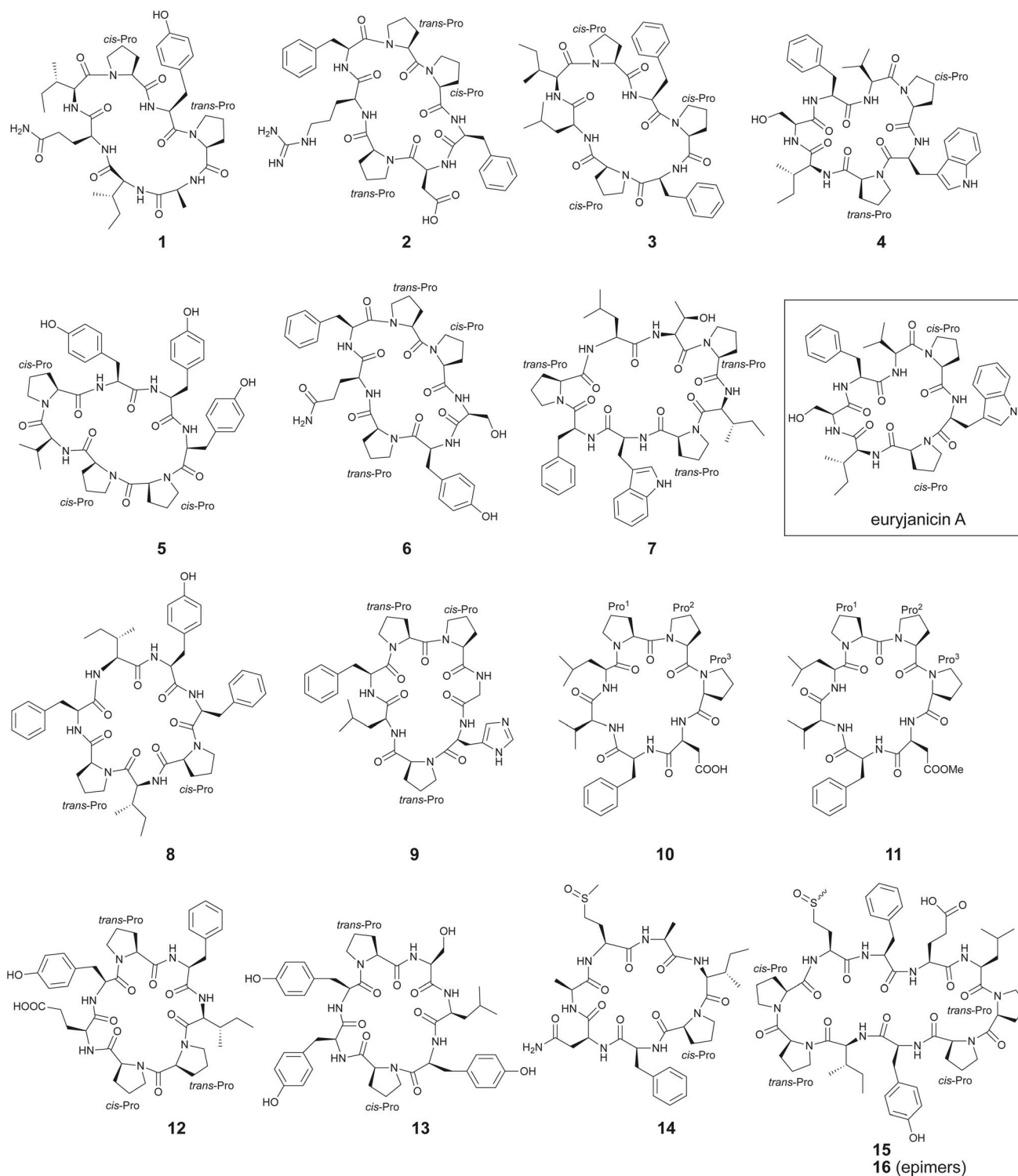


Fig. 3 Structures of the PRMP styllissamides 1–7, styllissatins 8–11, carteritins 12–13 and phakellistatins 14–16 that have been isolated from *Styllissa* spp. sponges. Note the configuration of the prolyl amide bonds in 4 as compared to euryjanicin A (boxed).

Numerous sponge-derived pNPs cannot be classified among RiPPs and NRPs as their biosynthetic origins are unknown. Principal among these are dipeptide alkaloids that belong to the pyrrole aminoimidazole alkaloid (PIA) and the bromotyrosine alkaloid (BTA) chemical classes (Fig. 1).^{33–39} The PIAs and BTAs

possess a dipeptidic structure with conserved proline-derived brominated pyrroles and brominated tyrosine building blocks, respectively. PIAs and BTAs have attracted attention due to their structural complexity that has provided attractive targets for chemical syntheses, as well as due to their chemical diversity



and geographical ubiquity.^{40–42} The biosynthetic routes for sponge-derived PIA and BTA alkaloids have not been deciphered, and hence, the site of their biosynthesis with the sponge holobiont is not known (Fig. 1). In addition to ATP-grasp enzymes and cyclodipeptide synthetases,^{43–47} among other ribosome-independent peptide bond forming modalities,^{48–50} numerous enzymatic routes can be envisaged for the assembly of sponge-derived dipeptide alkaloids. As PIAs and BTAs do not fall under the purview of canonical pNPs, this review does not cover into their discovery, biosynthesis, and progress in deciphering their possible biosynthetic origins.^{51–55} Instead, the reader is directed to excellent review and primary literature articles referenced above.

In this review, we cover the literature published between the years 2010 and 2025, illustrating representative pNPs discovered from marine sponges. We describe the structures of these pNPs, their biological activities, and the biosynthetic routes where known. Finally, we provide a brief description of the chemical and bioinformatic routes that guide pNP discovery and present prospects for pNP discovery from marine sponges and sponge-associated bacteria. The readers are also directed toward other excellent reviews focusing on comprehensive inventory of natural products from sponges and associated microorganisms.^{56–59}

2. pNPs with known biosynthetic origins

2.1. pNPs encoded by the sponge host

2.1.1. Proline-rich macrocyclic peptides (PRMPs). The proline-rich macrocyclic peptides (PRMPs) are a group of pNPs that are head-to-tail macrocyclized by amide bond formation between the peptide main chain N-terminal primary amine and the C-terminal carboxylic acid. The PRMP macrocycle typically comprises of six to eight proteinogenic amino acids with multiple Pro residues. Amino acids with non-polar or aromatic side chains dominate the PRMP sequences with minimal modifications afforded upon the amino acid side chains.⁶⁰ The presence of Pro units constrains the peptide backbone flexibility provides higher affinity and selectivity for binding to their molecular targets.^{61,62}

PRMPs are present in low abundance in sponge extracts, making it challenging to determine their structures using traditional natural product isolation and spectroscopic/crystallographic methods without sacrificing a large amount of sponge tissue biomass. Guided by the propensity of a peptide chain to fragment at the N-terminus of a Pro residue, a mass spectrometry-based sequencing protocol to determine PRMP sequences using sub-gram amounts of dry sponge biomass was developed.⁶³ Supplemented by biomimetic synthesis of head-to-tail macrocyclic peptides, the analytical workflow reliably differentiated between Leu/Ile residues and rapidly recovered the two-dimensional PRMP topologies; note that the *cis/trans* isomerization around the prolyl amide bond could not be discerned using this workflow. In addition to known PRMP sequences, numerous additional PRMPs were detected in

Stylissa and *Axinella* sponge extracts allowing us to posit that the chemical space explored by PRMPs could still be transformatively expanded by analytical innovation despite decades of isolation-based studies by the natural products community;⁶³ similar inferences were made for other classes of sponge-derived natural products as well.^{64–66}

2.1.1.1 Biosynthesis of PRMPs. Querying the microbiome architectures led to the identification that phylogenetically disperse low microbial abundance (LMA) sponges as well as high microbial abundance (HMA) sponges harbored PRMPs.^{63,67–69} To further query the molecular basis for these pNP biosynthesis, Schmidt and coworkers sequenced and assembled draft transcriptomes and draft genomes of *Axinella* and *Stylissa* sponge specimens that were prolific sources of PRMPs. These investigations led to the identification of PRMP-encoding genes in the sponge nuclear genomes and established PRMPs as RiPPs and not NRPs (Fig. 2A).²² Multiple PRMP-encoding cassettes were detected to be embedded within a single open reading frame (ORF), with each cassette flanked by conserved amino acid sequences at both the N- and the C-termini, reminiscent of other multi-cassette RiPP precursor peptide architectures (Fig. 2B).^{70–73} This study also revealed that a vast diversity of PRMPs were encoded within each sponge genome and that the detection and isolation of PRMPs from sponge specimens are likely limited by analytical and compound purification workflows (Fig. 2C).

The finding that the PRMPs are derived from linear precursor peptides indicates that the head-to-tail macrocyclization is a post-translational modification likely catalyzed by a peptidase similar to plant and microbial peptidases that furnish macrocyclic products.^{74–80} The peptidase catalyzed macrocyclization to furnish RiPPs (such as PRMPs) is akin to macrocyclic NRP offloading catalyzed by the NRPS TE domains using an intramolecular nucleophile; both reactions proceed *via* an acyl intermediate generated using a serine side chain hydroxyl or a cysteine side chain thiol and nucleophilic resolution of the acyl intermediate to install the macrocyclizing amide bond (Fig. 2B).^{30,31} The plausible PRMP-furnishing peptidases in marine sponge proteomes have not been reported. In the section below, we highlight some of the recent description of PRMP structures and bioactivities.

2.1.1.2 Recent advances in PRMP discovery. Marine sponges of the genus *Stylissa* sponges are well validated to be prolific sources of PRMPs.⁸¹ Prior to 2010, Köck had described the PRMPs stylissamides A–D from *Stylissa caribica*.⁸² In 2010, the stylissamide family of PRMPs was extended by Köck to include congeners E (1) and F (2) from *S. caribica* collected in the Bahamas (Fig. 3).⁸³ The planar structures of 1–2 were established spectroscopically and using mass spectrometric fragmentation of the peptide chain. The configurations of the prolyl peptide bonds were established by empirical rules correlating with the differences in the ¹³C chemical shifts of their β and γ carbons, and all amino acids were determined to be L-amino acids. No bioactivity was reported. The stylissamide family was further extended in 2014 by Molinski with the description of stylissamides G (3) and H (4) from *S. caribica* collected in the Bahamas (Fig. 3).⁸⁴ Molecule 4 showed mild cytotoxicity against



HCT-116 with EC_{50} of 5.7 μM , while no bioactivity was assigned to **3**. Interestingly, the planar structure of **4** was identical to that of a previously described PRMP euryjanicin A (*vide infra*) with all amino acids possessing L-configuration.⁸⁵ The authors had noted conformational equilibria in solution and the structure of euryjanicin A was established using crystallography. It is thus noteworthy that **4** exhibited a *cisPro-transPro* configuration, while euryjanicin A demonstrated a *cisPro-cisPro* configuration (Fig. 3). Konno and co-workers have synthesized **4**, revisiting its structure using NMR and X-ray diffraction methods.⁸⁶ They demonstrated that, in solution, synthetic **4** has a *cisPro-transPro* configuration, but adopts the *cisPro-cisPro* configuration crystallographically. Therefore, the authors concluded that **4** and euryjanicin A could be identical.

The discoveries of stylissamides I (**5**), L (**6**), and X (**7**) by Gonoï, Mangoni, and Kobayashi have further extended the stylissamide family of PRMPs described from the *Stylissa* genus; noteworthy is the geographical spread of the stylissamides across the Pacific and the Caribbean (Fig. 3).^{87–89} Molecule **5**

exhibited antifungal activity against *Aspergillus niger* with IC_{50} of 4 $\mu\text{g mL}^{-1}$ while **7** exhibited EGF-induced migration of cultured HeLa cells; no bioactivity has been ascribed to **6**.

From the sponge *Stylissa massa*, another family of PRMPs termed the stylissatins have been reported with the congeners A (**8**), B (**9**), C (**10**), and D (**11**) by Kigoshi and Lin described with all amino acids in the L-configuration (Fig. 3).^{90,91} In line with the conformational flexibility of **4** described above, **10** and **11** were isolated as inseparable tautomers, with the three proline residues in either the *cis-trans-cis* or *trans-cis-trans* configurations.⁹¹ Molecule **11** is the aspartyl methyl ester of **10**. Molecule **8** inhibited the production of nitric oxide in LPS-stimulated murine macrophage cells, an activity not previously reported for PRMPs while **9** exhibited moderate inhibitory effects against a panel of human tumor cell lines.^{90,91} The chemical synthesis of **8** and derivatives has been achieved.⁹² The planar structures of stylissatins E–G (cyclo(FVPELWP), cyclo(FPWVPLTP), and cyclo(WLPLTPLP), respectively) were discerned using mass spectrometry based fragmentation.⁶³

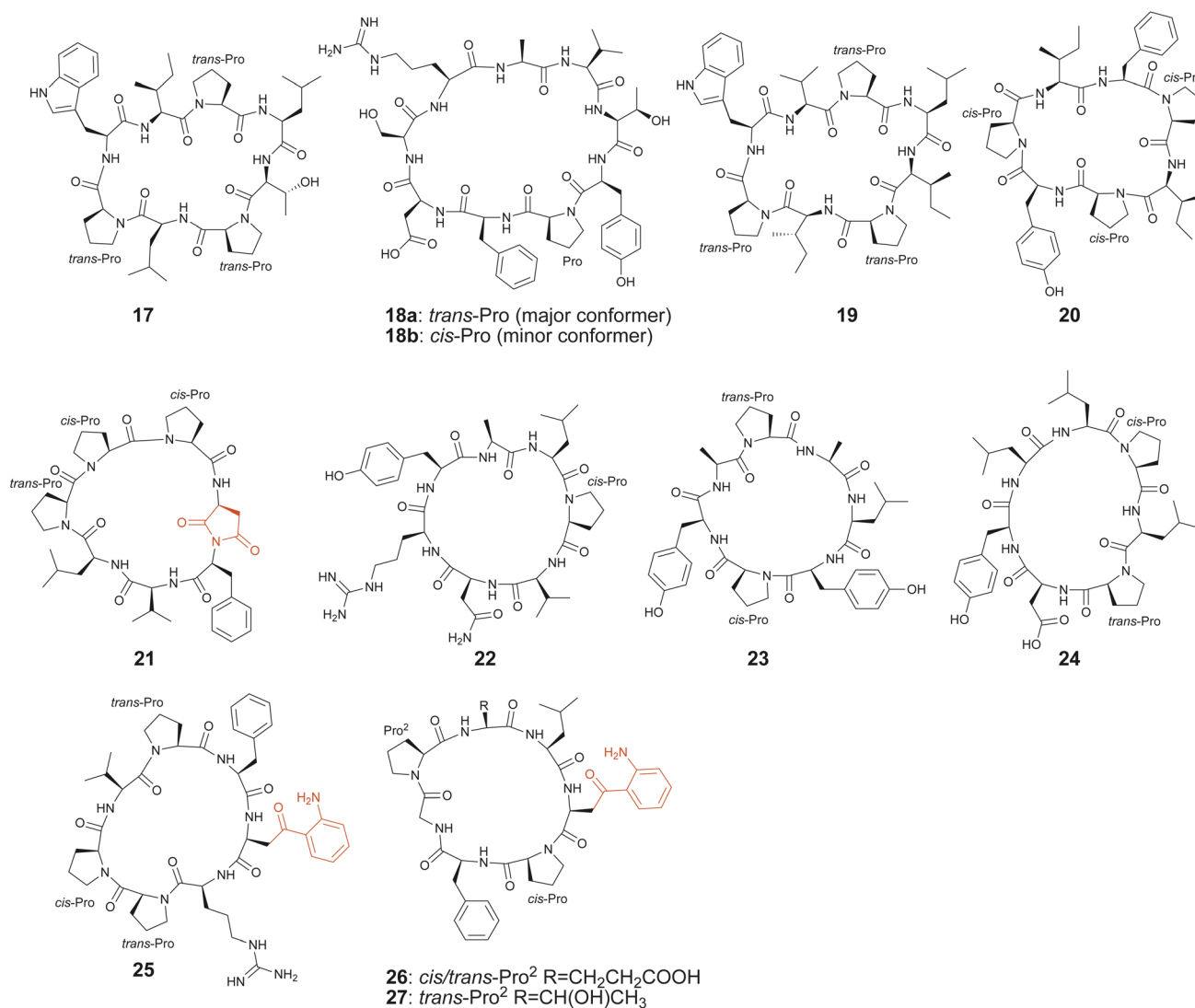


Fig. 4 Structures of phakellistatins **17**–**20**, fuscasins **21**–**24**, and phakefustatins **25**–**27**. Additional PTMs beyond cyclization are highlighted in red.



Yet another family of PRMPs—heptapeptides called carteritins—have been described by Tsukamoto from *Stylissa carteri* with the structures of congeners A (**12**) and B (**13**) deciphered using the usual spectroscopic, fragmentation, and chemical degradation and derivatization procedures (Fig. 3).⁹³ Molecule **12** was cytotoxic against mammalian HeLa, HCT-116, and RAW264 cell lines with IC₅₀ from 0.7 to 1.5 μM. Three new phakellistatin congeners **20–22** (**14–16**) were isolated by Shin from a tropical sponge *Stylissa flabelliformis*.⁹⁴ The same sponge specimen also yielded **12–13**, in addition to several other previously reported PRMPs.⁹⁴ The heptapeptide **14** was isolated as an inseparable mixture of sulfoxide epimers. The decapeptides **15** and **16** were likewise epimers at the methionine side chain sulfoxide (Fig. 3). Molecules **15** and **16** were found to be significantly cytotoxic against several cell lines IC₅₀ ranging from 0.01 to 0.4 μM while **14** was inactive.

Congeners of the family of PRMPs, the phakellistatins, have been isolated from *Phakellia* sponges.⁹⁵ There is no underlying chemical distinction between the different PRMP families, and a PRMP with sequence and topology identical to that of phakellistatin **18** was detected in an *Axinella* sp. sponge specimen, reinforcing a potential lack of sponge species specificity in the PRMPs.⁶³ Proceeding from the description of phakellistatin **1** from Indo-Pacific sponges *Phakellia costata* and *Stylotella aurantium*,⁹⁶ numerous phakellistatin congeners have now been reported.^{97–106} Of these phakellistatins **15–18** (**17–20**, Fig. 4) from *Phakellia fusca* were reported by Lin in 2010. Structures of **17–20** were established using NMR and peptide fragmentation.¹⁰⁶ The absolute configurations of their amino acid residues were

determined to be L. In DMSO-*d*₆, **18** had two sets of NMR signals in a 3 : 1 ratio, corresponding to isomerization around the prolyl amide bond. The Xaa-Pro amide bonds in **17** and **19** were all *trans*; molecule **20** contains all *cis* prolyl amide bonds. Molecule **17** demonstrated cytotoxicity against cancer cell line P388 with IC₅₀ of 8.5 μM, while **18** against P388 and BEL-7402 with IC₅₀ of 5.4 and 14.3 μM, respectively. Several phakellistatins feature successive Pro-Pro residues, leading to a considerable synthetic challenge.¹⁰⁷

Another family of PRMPs, the fuscasins, have been described from *P. fusca*. The heptapeptides fuscasins A–D (**21–24**) were isolated by Lin from *Phakellia fusca* collected in the South China Sea (Fig. 4).¹⁰⁸ Even though PRMPs are RiPPs, posttranslational modifications (PTMs) beyond the head-to-tail macrocyclization are few and far between. Based on the key HMBC correlation of Phe-H α with the γ carbonyl carbon of Asp, a pyrrolidine-2,5-dione fragment formed between the Asp side chain carboxyl and the Phe nitrogen was assigned to **21**.¹⁰⁸ Another PTM was observed for the PRMPs phakefustatins A–C (**25–27**) also isolated by Lin from *P. fusca* wherein a tryptophan side chain was modified to yield a kynurenine residue.¹⁰⁹ Molecule **26** was isolated as a 1 : 1 mixture of *cis/trans*-Pro₂ isomers while **25** selectively exhibited cytotoxicity against cancer cell lines.

Proceeding from the isolation of congeners A–D, heptapeptides euryjanicins E–G (**28–30**) were described by Rodriguez in 2013 from the sponge *Prosuberites laughlini* collected off the west coast of Puerto Rico (Fig. 5).^{85,110,111} As described above for euryjanicin A for which the structure was established using X-ray crystallography,⁸⁵ multiple conformations for **29** and **30**

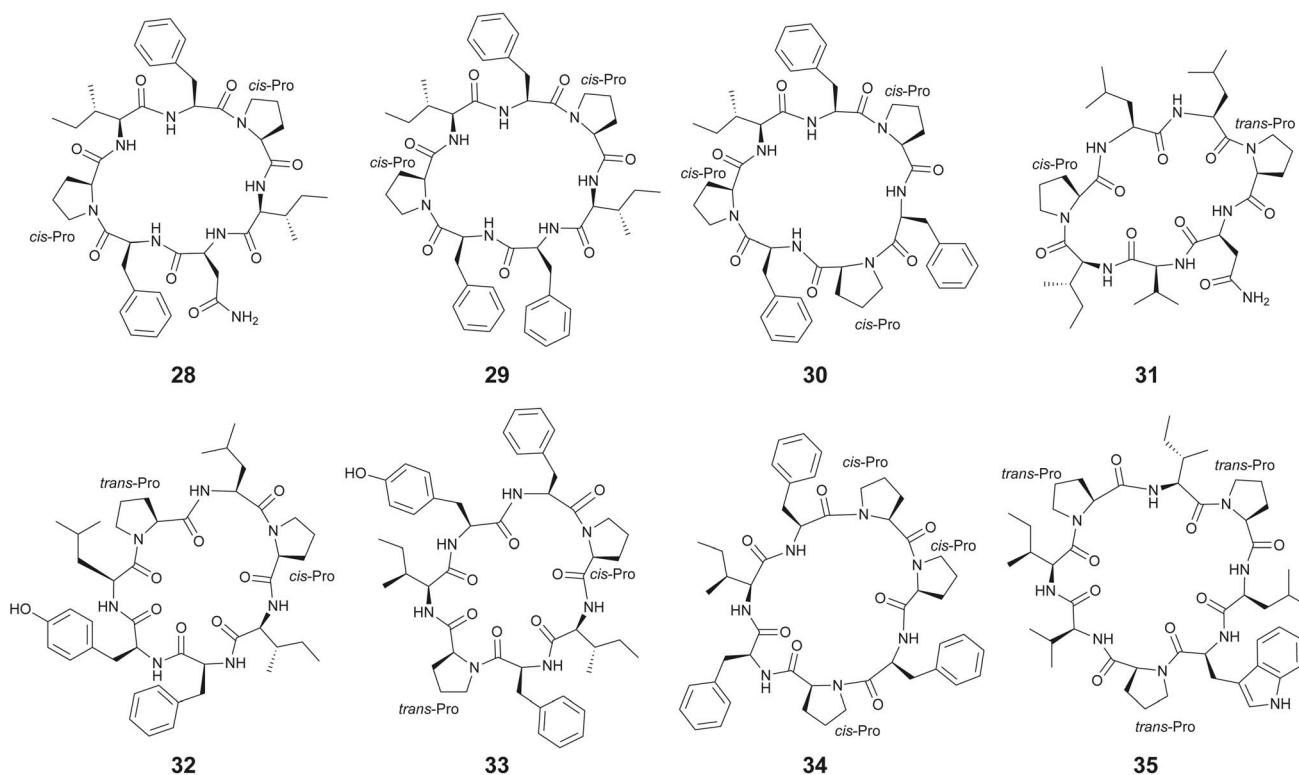


Fig. 5 Structures of euryjanicins **28–30** and reniochalistatins **31–35**.



were observed in solution that were only resolved in pyridine-*d*₅.¹¹¹ Euryjanicins were inactive in a tumor cell line panel. Four heptapeptides reniochalistatins A–D (31–34) and octapeptide reniochalistatin E (35) were isolated by Lin from *Reniochalina stalagmitis* (Fig. 5).¹¹² Only 35 showed moderate activity against tumor cell lines myeloma RPMI-8226 and gastric MGC-803 with IC₅₀ of 4.9 and 9.7 μM. As before and consistent with their biosynthetic origins, all amino acids were determined to possess the L-configuration.

2.1.2. Cysteine bridged peptides (CBPs). For clarity, here, peptides in which cysteine side chain thiols are connected by disulfide bonds are referred to as cysteine bridged peptides (CBPs). CBPs with three or more disulfide bridges are also referred to as knottins. CBPs are commonly detected in Eukarya as neuroactive venoms and toxins.^{113–116} These exceptionally stable peptides attract intense pharmacological interest and are clinically used for the treatment of chronic pain and gastrointestinal disorders, and provide the chassis to develop molecular tools.^{117–121}

2.1.2.1 Biosynthesis of CBPs. There is a rich body of literature describing the discovery of CBPs from marine sponges, and their chemical syntheses. Just as for PRMPs, marine sponge-derived CBPs also comprise of proteinogenic L-amino acids with minimal PTMs other than cyclization, in this instance by disulfide bond formation (PTMs observed in sponge-derived CBPs are discussed below). These factors indicated a ribosomal origin of sponge-derived CBPs, akin to other eukaryotic CBPs.^{19,121} Indeed, by transcriptomic sequencing of the marine sponge *Geodia barretti*, Cárdenas and coworkers recovered precursor peptide sequences originating from the sponge, and not the microbiome, that encoded CBPs belong to the barrettide family (*vide infra*).²¹ In addition to correlating the precursor peptide sequences to previously described chemical structures

of barrettide congeners A (36) and B (37),¹²² Cardenas described the discovery of an additional barrettide congener—barrettide C (38)—by correlating the barrettide sequences unveiled by their transcriptomics data to the mass spectrometry-based profiling of *Geodia barretti* extract (Fig. 6).²¹ The architecture of the barrettide precursor sequences was in line with that of canonical RiPP precursor peptides wherein an N-terminal signal peptide is followed by a leader sequence then followed by the cysteine-rich core sequence.²¹ Peptidases that would conceivably remove the leader sequences from the barrettide cores are not yet known.

2.1.2.2 Recent advances in CBP discovery from marine sponges. As mentioned above, CBPs 36 and 37 were reported by Göransson from *G. barretti* collected in the Swedish Kosterfjord.¹²² Only a single amino acid differentiates the two CBPs. Two disulfide bonds—between the side chain thiols of Cys⁵/

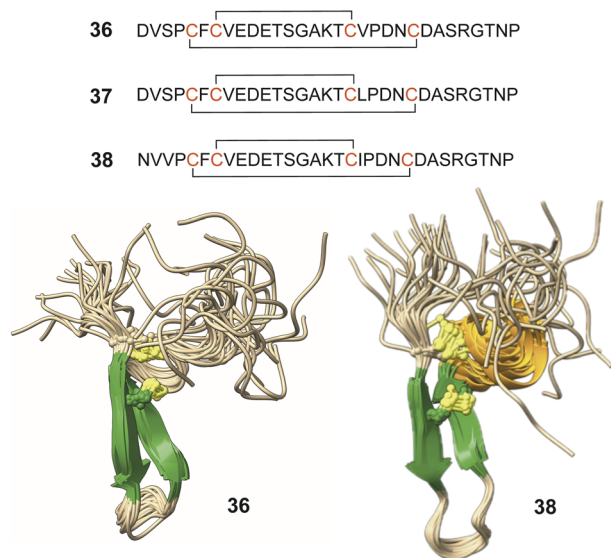


Fig. 6 Barrettide disulfide bond topologies, and ensemble three-dimensional solution structures of 36 and 38 with the cysteine side chains shown in stick ball representation with the sulfur atoms colored yellow.

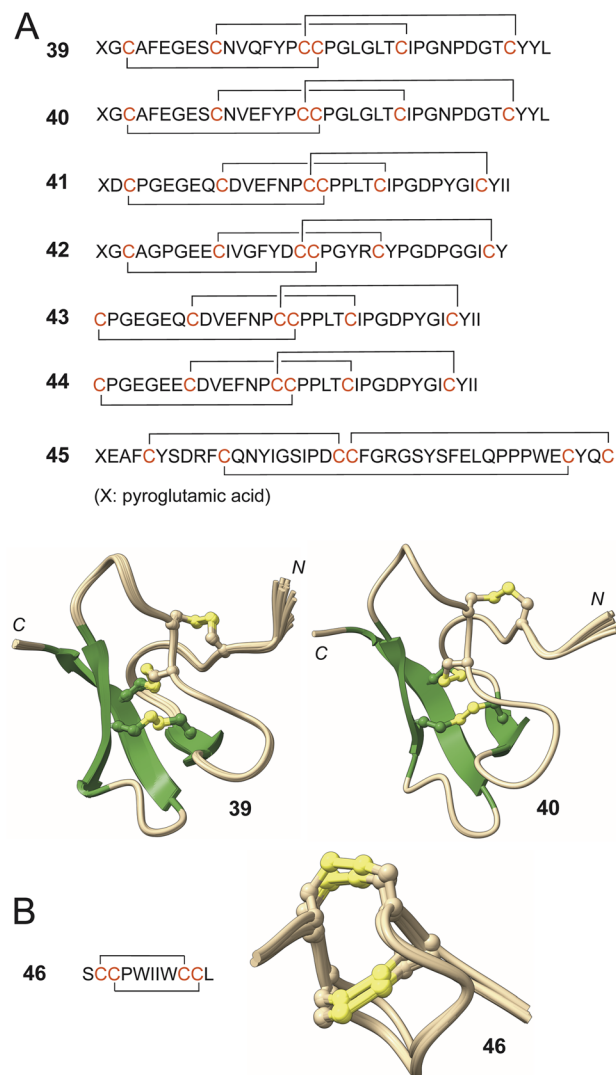


Fig. 7 (A) Asteropsin sequences with disulfide ring topologies denoted. Ensemble solution structures of 39 and 40 demonstrating the conservation of the tertiary core that is rigidified by the three disulfide bonds. The N- and the C-termini are labeled. (B) Topology and tertiary structure of 46.



Cys²³ and Cys⁷/Cys¹⁸—rigidify the barrettide structures. The presence of the disulfide linkages was established by chemical reduction of the pNP and alkylation of the reduced thiols. The topology of the disulfide bond formation and the conformation of the prolyl amide bonds were discerned spectroscopically. The structure of **36** was confirmed by chemical synthesis of the linear peptide and oxidative installation of the disulfide bonds. The authors also established the three-dimensional solution structure of **36** revealing two antiparallel β -sheets each containing five amino acid residues forming a β -hairpin structure, which were braced by two disulfide bonds arranged in a ladder-like mode (Fig. 6). Molecules **36** and **37** did not demonstrate antimicrobial activity but exhibited antifouling activity against barnacle larvae at 0.6 and 6.0 μM , respectively. Guided by the sponge transcriptome mining, the CBP **38** was detected in *G. barretti* extracts by mass spectrometry and the structure was established using a synthetic linear peptide that was oxidatively folded.²¹ The three-dimensional structure of **38** was similar to that of **36** (Fig. 6). Yet another congener, barrettide D, was also detected to be present in sponge extract and the CBP topology was inferred based on sequence similarity with **36**–**38**. Additional barrettide sequences were detected in the sponge transcriptomes which would correlate to congeners not yet detected

and isolated from *G. barretti*. All barrettides, known and bioinformatically predicted, contain two disulfide bonds.

The knottins—CBPs containing three or more disulfide bonds—belonging to the family asteropsins have been described by Jung from the *Asteropus* sp. sponge specimens.^{123–126} To date, the two-dimensional structures of asteropsins A–F (**39**–**44**) have been established. In addition, the three-dimensional structures of all asteropsins except for **42** have been determined using solution NMR spectroscopy, and for **39**, also using X-ray crystallography (Fig. 7). Containing more than thirty all *L*-amino acids, most asteropsins congeners possess an N-terminal pyroglutamate residue and a highly conserved tertiary structure. As would be expected of knottins, all asteropsins exhibited remarkable stability against proteases (trypsin, chymotrypsin, pepsin, elastase) in the gastrointestinal tract and blood plasma.^{123–126} Molecule **44** was non-hemolytic to human and fish red blood cells.¹²⁶ Molecule **39** enhanced veratridine-induced Ca²⁺ influx in murine cerebrocortical neurons, and its heterologous production in bacterial expression systems has been achieved.^{123,127} A solitary N-terminal pyroglutamate residue containing knottin recifin A (**45**) has been described from an *Axinella* sp. sponge that targeted the tyrosyl-DNA phosphodiesterase 1 in an FRET assay with IC₅₀ of 190 nM.¹²⁸

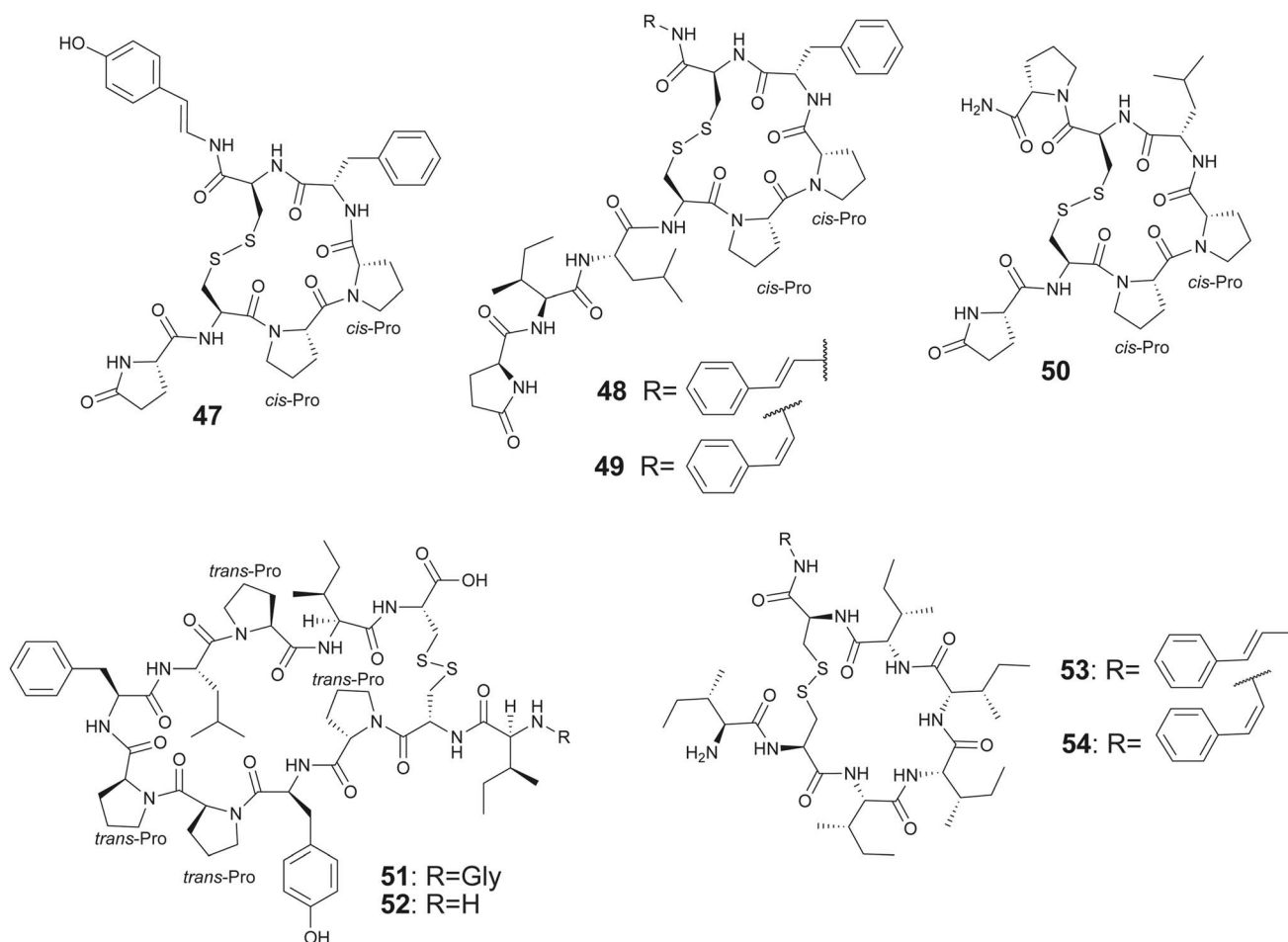


Fig. 8 Structures of gombamides **47**–**50**, chujamides **51**–**52**, and microcionamides **53**–**54**.



Theoretically, for a peptide containing four (barrettides) or six (asteropsins) cysteine residues, multiple different oxidative outcomes are possible. However, in the sponge extract, only a single topological isomer is detected. The originally proposed connectivity of three disulfide bonds in the sponge-derived CBPs neopetrosiamides A and B was revised by comparison to chemically synthesized topological isomers.^{129,130} The topological fidelity of disulfide bond formation in barrettides and asteropsins, and CBPs in general should be considered in context of their three-dimensional structures. Because only one of several theoretically possible outcomes is realized, topology of disulfide bond formation is likely chaperoned by an enzyme active site. Various enzymes catalyze disulfide bond formation. For example, protein disulfide isomerase is a large protein family that can catalyze disulfide formation and isomerization. Peroxiredoxins and glutathione peroxidases are another two widespread protein families that can install disulfide bonds. Quinone and flavin cofactor employing enzymes also oxidize thiols and catalyze disulfide bond formation.^{131,132}

It is conceivable that the chaperoned oxidative formation of the first disulfide bond biases the conformation of the intermediate in a manner that the formation of the subsequent disulfide bonds can proceed in an unguided manner. In such a scenario, an enzyme would be needed to construct only the first disulfide bond with high fidelity.

This plausible scenario was computationally explored for the construction of halichondamide A (**46**), an 11-residue peptide containing all L-amino acids with two disulfide bonds that was discovered in the extracts of the sponge *Halichondria bowerbanki* (Fig. 7).¹³³ The structure of **46** was established using spectroscopy, chemical derivatization, and degradation studies. Molecular dynamics simulations revealed that if disulfide bond between Cys³ and Cys¹⁰ residues would be installed first (conceivably by a biosynthetic enzyme), then the second disulfide bond between Cys² and Cys⁹ residues became highly favorable and could be installed in a non-catalytic manner. The parsimonious biosynthetic scheme presented here is in line with the conservation of disulfide ring topologies of CBPs families such as barrettides and asteropsins described above; a singular dedicated biosynthetic enzyme could construct the entire family of CBP congeners. Molecule **46** was mildly cytotoxic against a panel of cancer cell lines.

The complication of an oxidative cascade is not required for the biosynthesis of CBPs that contain only a single disulfide bond; several such pNPs have been described from marine sponges. Proceeding from the description of the founding member of the gombamide family of CBPs—the heptapeptide gombamide A (**47**) from sponge *Clathria gombawuiensis* by Shin—additional congeners gombamides B–D (**48–50**) were discovered by Proksch from sponge *Clathria basilana* (Fig. 8).^{134,135} The gombamides all possess a single disulfide bond, an N-terminal pyroglutamate residue and two contiguous proline residues in the macrocycle. While **47** terminates in a *p*-hydroxystyrylamide unit which could conceivably arise from the oxidative decarboxylation of a C-terminal tyrosine, **48** and **49** contain *E*- or *Z*-2-phenylethen-1-amine (PEA) residues, respectively, at this position analogously derived from a C-terminal

phenylalanine. The oxidative decarboxylation of C-terminal amino acids in bacterial RiPP biosynthesis has been reported previously.^{136–138} The chemical synthesis of **47** has been realized.^{139,140} Divergent reports for the bioactivity of **47** are available; while the isolated natural product demonstrated moderate cytotoxicity and inhibition of the Na⁺/K⁺-ATPase, these effects were not recovered by the synthetic molecule. Similarly, biological activities of several natural PRMPs have not been replicated following their total synthesis.^{95,134,140}

Another family of proline-rich peptides containing a single disulfide linkage among a constellation of all L-amino acids are the chujamides with congeners A (**51**) and B (**52**) isolated from sponge *Suberites waedoensis* by Shin (Fig. 8).¹⁴¹ Both **51** and **52** demonstrated moderate cytotoxicity against A549 and K562 with IC₅₀ from 10.1 to 55.6 μM with **51** inhibiting Na⁺/K⁺-ATPase with IC₅₀ of 17.2 μM, reminiscent of **47**.

Oxidative decarboxylation of the C-terminal carboxylate is also observed in the sponge-derived single disulfide bridge containing CBPs belonging to the microcionamides family. Proceeding from the description of microcionamides A and B in 2004 that were isolated from the sponge *Clathria abietina*, congeners C (**53**) and D (**54**) were isolated by Ireland, along with the gombamides **48–50**, from *Clathria basilana* collected in Indonesia (Fig. 8).^{135,142} Unlike gombamides, the microcionamides do not possess the N-terminal pyroglutamate or any proline residues. However, the oxidative decarboxylation of the C-terminal residue is conserved. Molecules **53** and **54** demonstrated antibacterial activity against *Staphylococcus aureus* and *Enterococcus faecium* with minimal inhibiting concentration between 6.2 and 12 μM, and induced apoptotic cell death in Jurkat J16 and blocked autophagy in starvation conditions.¹³⁵

In the section above, the biosynthetic routes and recent new molecular discoveries regarding PRMP and CBP pNPs have been presented. Both classes of molecules are produced by marine sponges and possess rather restrictive set of modifications to the peptide chain with macrocyclization being the principal and conserved chemical transformation to the ribosomally synthesized peptide. A much more expanded set of modifications are displayed by pNPs that are synthesized by the symbiotic microbiome associated with the sponge host. These are discussed in the ensuing section.

2.2. pNPs from sponge symbiotic bacteria

Sponges are a rich repository of pNPs that are produced by bacterial symbionts that maintain a close metabolic and physiological relationship with the sponge host.¹⁴³ The BGCs encoding production of these pNPs have been recovered by metagenomic sequencing of the entire sponge symbiont followed by bioinformatically enriching the microbial fraction of the reads and mining the assembled metagenome(s) as guided by the chemical logic of pNP biosynthesis. Another route has been the physical enrichment of bacterial symbionts as guided by their distinguishing physiological traits followed by metagenomic sequencing or single cell sequencing of the enriched bacterial fractions followed by retrobiosynthesis-guided genome mining. Both directions have been successfully



applied for discovery of pNP encoding BGCs from sponge bacterial symbionts.^{8,17,144,145} Examples will be discussed below.

2.2.1. pNPs from *Theonella* spp. The canonical sponge symbiont pNPs arise from a candidate bacterial family, Entotheonellaceae, named because they were first described as inhabitants of the interior of the sponge *Theonella swinhoei*. The sponge *T. swinhoei* and related “lithistid” sponges were famous in the marine natural products family because they were the source of numerous potently bioactive natural products, including pNPs.^{146–153} Faulkner and coworkers initiated pioneering investigations in this field, using tissue dissociation and density gradient centrifugation to fractionate Palauan *T. swinhoei* tissue into unicellular heterotrophic bacteria, filamentous bacteria, cyanobacteria, and sponge cells.^{154,155} Extraction and these fractions found the pNP theopaulamide only in filamentous sponge cells. Sequence analysis of 16S rRNA led to the description of a new candidate genus “*Candidatus*

Entotheonella palauensis”, which was only 87% identical to any sequence in GenBank in the late 1990s.¹⁵⁶ At the time, limited sequencing data led to the assignment of *E. palauensis* as a member of deltaproteobacteria, but as much more sequencing data became available in the late 2000s, it was clear that *Entotheonella* is instead a founding genus of a large Phylum Tectomicrobia, consisting solely of uncultivated bacteria.¹⁵⁷ Piel and co-workers pioneered biosynthetic studies in *Entotheonella*, identifying pathways for many famous marine natural products. Ultimately, the group sequenced the uncultivated filamentous bacteria from *T. swinhoei*, providing a metagenome-assembled genome (MAG) for the symbiont “*Candidatus* Entotheonella factor”, which contains gene clusters encoding many of the pNPs reported from the sponge family Theonellidae.¹⁵⁷ Theonellid sponges are a “talented” pNPs producing factory, especially from the genera *Theonella* and *Discodermia*, discussed below.¹⁵⁸

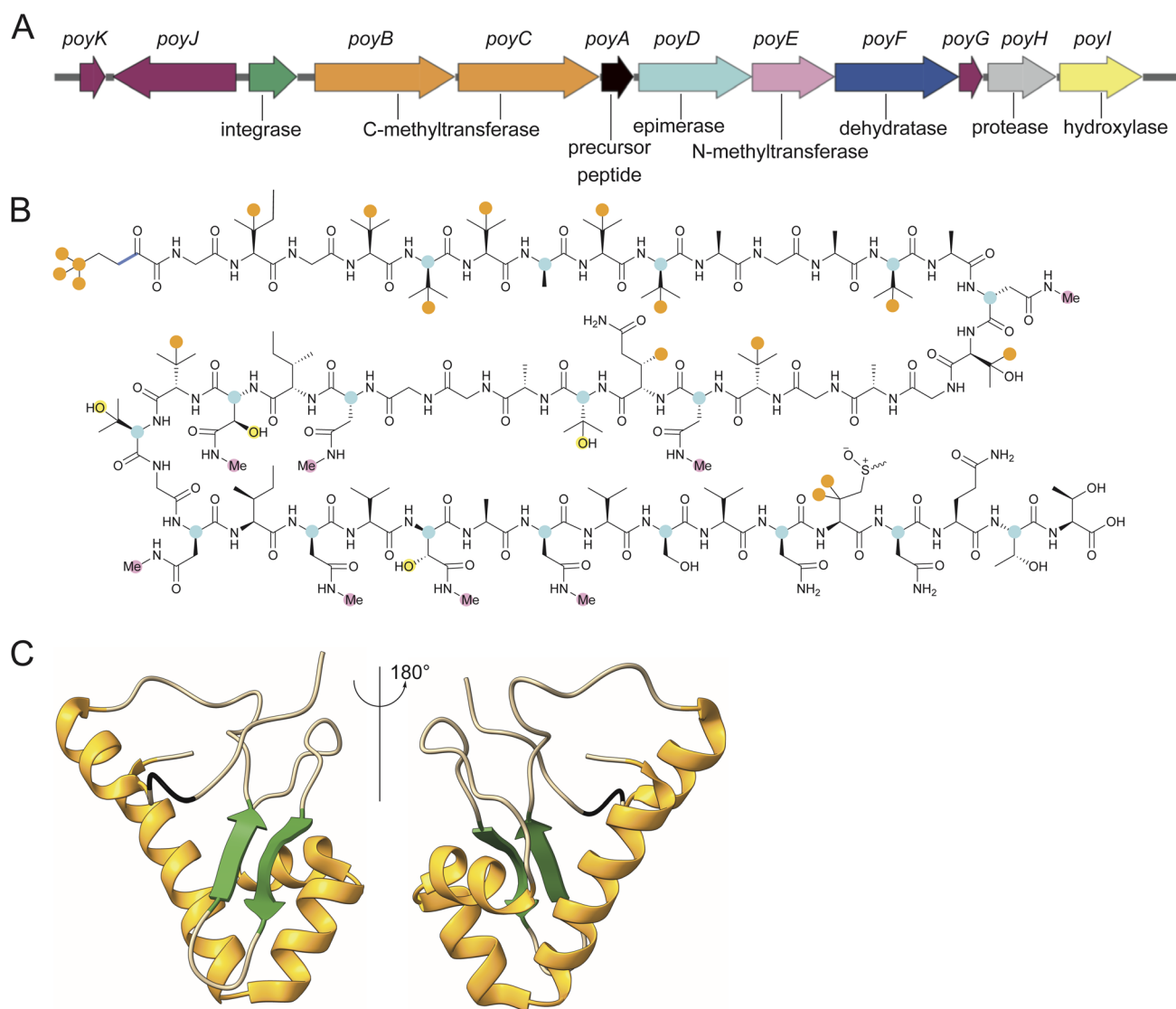


Fig. 9 (A) The *poy* BGC with the functions of the Poy enzymes listed. (B) Structures of polytheonamides A–B (epimers around the sulfoxide) with the chemical modifications catalyzed by the Poy enzymes annotated. (C) Tertiary structure of an NHP possessing RiPP precursor peptide (PDB: 8TB1) colored according to the secondary structural elements.¹⁶⁹ The region separating the leader from the core is colored in black.



Polytheonamides represent the most complex pNPs isolated from *T. swinhoei* with two- and three-dimensional structures established and chemical synthesis realized.^{149,159–161} Polytheonamides were originally proposed to be NRPs due to the presence of many nonproteinogenic and D-amino acid residues. Instead, Piel and co-workers investigated the metagenome of *T. swinhoei*, describing a *poA* BGC demonstrating the biosynthesis of polytheonamides *via* a RiPP pathway.¹⁶² The *poA* locus contains seven genes that install 48 post-translational modifications, including C- and N-methylation, hydroxylation, dehydration, and amino acid epimerization distributed across 49 residues (Fig. 9).^{163–166} The enzymatic activities discovered as a part of unraveling the polytheonamides biosynthetic pathway served as leads for genome mining which further enriched the tapestry of RiPP pNPs by prioritizing the exploration of BGCs using synthetic biology workflows.^{15,167,168}

The architecture of the polytheonamide precursor peptide—PoyA—was intriguing. The PoyA leader peptide was uncharacteristically long as compared to other canonical RiPP leader sequences.^{162,170} Due to its sequence similarity to the sequence of the nitrile hydratase alpha subunits, the PoyA^{leader} belonged to the family of RiPP leader peptides called the nitrile hydratase-like leader peptides (NHLPs) that had been detected

bioinformatically.¹⁷¹ The polytheonamides were the first RiPPs that were functionally connected to NHLP precursor peptides. As mentioned above, PoyA-like RiPP precursor peptides were subsequently mined in genetic database leading to the access to cryptic RiPPs.^{15,167,172,173} The structure of an NHLP has since been determined leading to the appreciation that these leader peptides are akin to proteins rather than peptides wherein they possess rigid tertiary structures leading to new protein/protein interaction modalities underlying RiPP biosynthesis.^{169,174} Microbiomes of geographically and phylogenetically diverse sponges contain an exceptionally high abundance of a cryptic RiPP-encoding BGC with the precursor peptide adopting the NHLP architecture—the ecological and organismal implications of the conservation of this BGC in sponge microbiomes are as yet not clear.¹³

In contrast to the polytheonamides that are RiPPs, two families of NRPS-derived NRPs—the theonellamides and the theopalauamides—have been isolated from *Theonella* sp. sponges. Despite the different names, both families refer to highly similar glycopeptides bridged by a histidinoalanine residue (Fig. 10A).^{14,146–148,175–177} The founding members for these classes of pNPs, theonellamides A–F and theopalauamide were described prior to 2010.^{146–148} Since 2010, theonellamides G–K

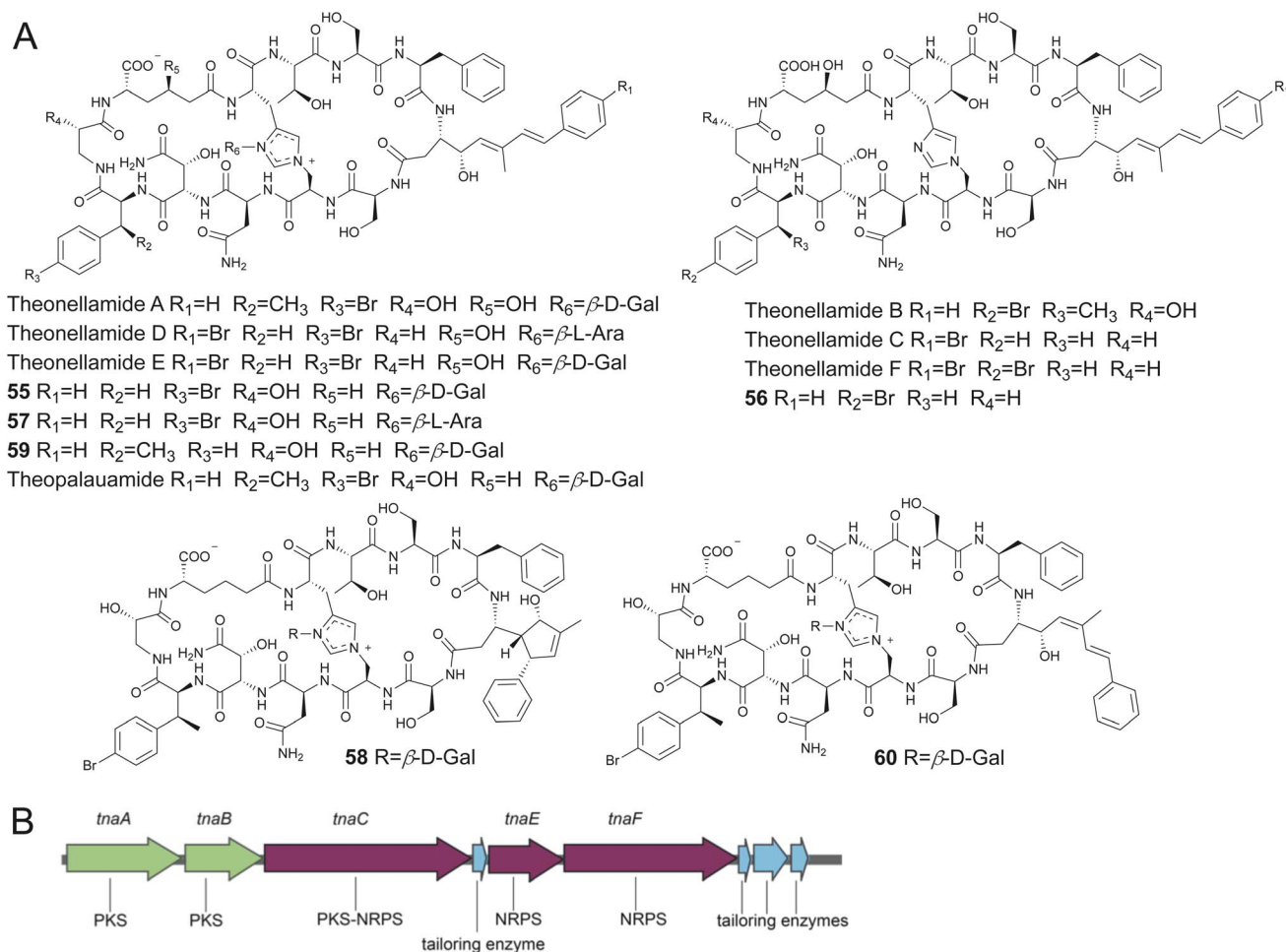


Fig. 10 (A) Structures of theonellamides and theopalauamides described to date. (B) The *tna* BGC.



(55–59) and 5-*cis*-Apoa-theopalauamide (**60**; Apoa: 3-amino-4-hydroxy-6-methyl-8-phenyl-5*E*,7*E*-octadienoic acid) have been described from *Theonella swinhoei* sponges from different locations by different groups with the structure of **56** established by interpretation of mass spectrometry fragmentation spectra.^{14,175–177} The absolute configurations of the sugar moieties were determined by Tanaka's method, involving mild acid hydrolysis and derivatization.¹⁷⁸ Some of these NRPs possess cytotoxic and antifungal activities, possibly because they bind membrane sterols. In fungi, this induces glucan overproduction and activates Rho1-mediated 1,3- β -D-glucan synthesis.^{179,180}

Using single cell sequencing, Piel and co-workers have described the hybrid non-ribosomal peptide synthetase/polyketide synthase (NRPS/PKS) encoding *tna* BGC from the *Theonella* symbiont *Candidatus* Entotheonella sarta that is responsible for the production of theonellamides (Fig. 10B).¹⁴ The NRPS-PKS architecture was rationalized to fit the assembly line biosynthetic pathway furnishing the theonellamide aglycone with a candidate glycosyl transferase enzyme identified to be encoded within the *tna* BGC. Crucially, as rationalized from the adenylation domain specificities encoded within the *tna* BGC, the histidinoalanine ring was proposed to be constructed using histidine and serine amino acid side chains. This transformation would require dehydration of the serine side chain to yield an α/β unsaturated dehydroalanine residue and a stereospecific Michael-type addition of the histidine side chain imidazole nitrogen to the β -carbon of the dehydroalanine residue reminiscent of the lanthionine ring formation in lanthipeptides. Other enzymatic transformations have been realized for the formation of macrocyclic pNPs containing histidine side chain cross links.¹⁸¹ As for theonellamides, a candidate enzyme with similarity to kinases and tetratricopeptide repeat domains was identified in the *tna* BGC and proposed to be involved in the formation of the histidinoalanine cross link; this proposal remains to be experimentally verified.¹⁴

In addition to polytheonamides, theonellamides, and theopalauamides, *Theonella* sponges have been a rich repository of other pNPs, most of which are NRPS-derived NRPs or peptide/polyketide hybrids. These include the keramamides,^{150,182–184} cyclotheonamides,¹⁵² nazumamide A,¹⁵³ and konbamides (Fig. 11). Putative BGCs—*ker* BGC, *cth* BGC, *naz* BGC, and the *kon* BGC, respectively—that were collinear with the assembly line biosynthetic logic of these NRPs were detected in *Entotheonella* symbionts as well.¹⁵⁷ The reader is directed to some excellent review articles on this topic that detail the isolation, chemical syntheses, and bioactivities of these marine natural products.^{185–187} The natural product biosynthetic potential of *Entotheonella* symbionts extends beyond pNPs to include structurally elaborate polyketide natural products. While a discussion of polyketide natural products is outside the scope of this review, the reader is directed to the primary literature ref. 157, 188 and 189.

2.2.2. pNPs from other sponge species. The *Candidatus* Entotheonella symbionts that are profligate in natural product biosynthetic potential are not limited to *Theonella* sponges alone but extend to other sponge genera as well. Recently, the *hcd* BGC was identified from *Ca. Entotheonella halido* symbiont

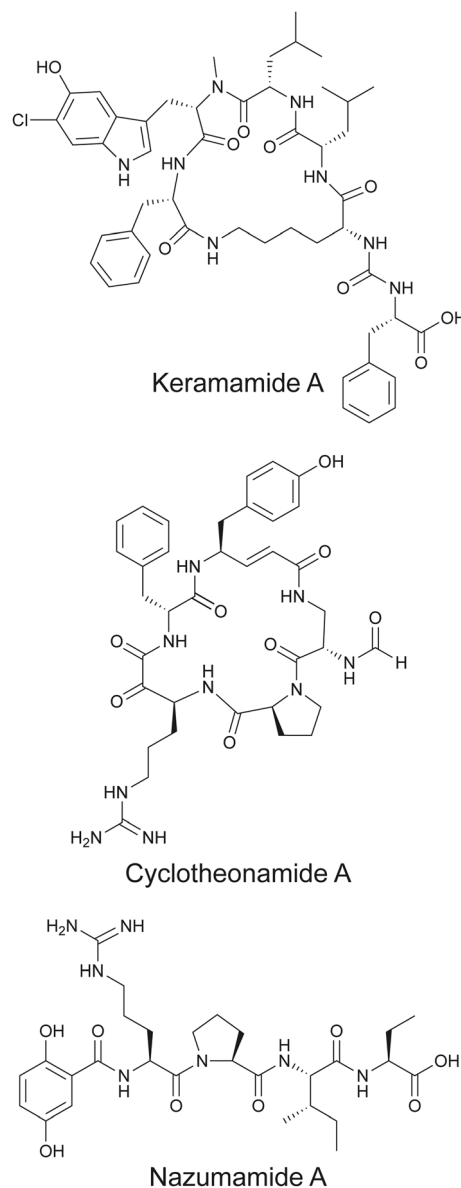


Fig. 11 Representative keramamide and cyclotheonamide congener structures, along with nazumamide A isolated from *Theonella* sponges.

of the sponge *Halichondria dokdoensis* which encoded the production of halicylindramides.¹⁹⁰ Proceeding from the founding members of this family of sponge-derived pNPs—halicylindramides A–E that were isolated from *Halichondria cylindrata*—post 2010, halicylindramide congeners F–H (**61–63**) were isolated by Kang from a *Petrosia* sp. sponge of which molecule **61** demonstrated human farnesoid X receptor antagonistic activity with IC_{50} of 6.0 μ M (Fig. 12).^{191–193} While the description of the *hcd* BGC was indeed in line with the assembly line biosynthesis of the N-formylated halicylindramide deipeptides, the activities of amino acid tailoring enzymes that furnish modifications upon the amino acid side chains—such as bromination of the phenylalanine side chain, methylation of the valine side chain, oxidative tailoring of the tryptophan side



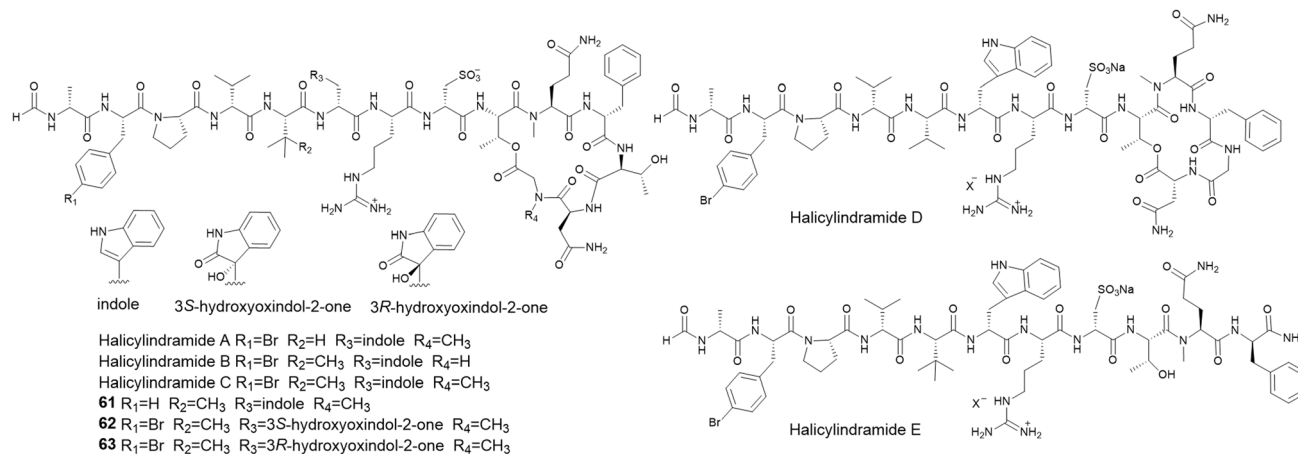


Fig. 12 Structures of halicyclindramides A–H.

chain indole, and cysteine side chain thiol—remain to be described.¹⁹⁰

In the sponge *Discodermia calyx*, a *Ca. Entotheonella* sp. symbiont was shown to be the producer of the polyketide natural product calyculin A diphosphate.¹⁹⁴ The previously described natural product, calyculin A, was furnished upon dephosphorylation of calyculin A diphosphate in response to sponge tissue disruption.¹⁹⁵ Following the identification of the calyculin A diphosphate encoding BGC, Wakimoto, Abe and coworkers detected the presence of kasumigamide (**64**) encoding *kas* BGC in the same *D. calyx* symbiont *Ca. Entotheonella* sp. which directed the isolation and structure elucidation of **64** from marine sponges; **64** had previously been described from marine cyanobacterium *Microcystis aeruginosa* NIES-87 (Fig. 13A and B).^{196–198} The *makas* BGC for the production of **64** in *M. aeruginosa* NIES-87 was subsequently identified.^{196,199}

Another intriguing case of *Ca. Entotheonella* sp.-derived pNPs in *D. calyx* sponges are the N-formylated macrolactams calyxamides A and B (**65–66**) isolated by Abe that demonstrated cytotoxicity against the murine leukemia cell line P388 with IC₅₀ of 3.9 and 0.9 μM, respectively.²⁰⁰ The structures as well as absolute configurations were determined by spectroscopic data and chemical degradation experiments. Structurally, **65** and **66** are a pair of diastereomers differing only in the configuration of the isoleucine residue (Fig. 13C). The calyxamides share several structural features with the keramamides, and as mentioned previously, the *ker* BGC encoding production of the keramamides has been detected in *Ca. Entotheonella* symbionts in *Theonella* sponges.¹⁵⁷ Indeed, microscopic analysis and 16S rRNA encoding gene amplification established the presence of *Ca. Entotheonella* symbionts in the calyxamide harboring *D. calyx* sponge specimens.²⁰⁰ It is thus likely that a *ker*-like BGC remains to be identified in the *D. calyx* *Entotheonella* symbionts. Prior to the studies describing the isolation of pNPs **64–66** from *D. calyx*, the presence of *Entotheonella* symbionts in the sponge *Discodermia dissoluta* had been noted.²⁰¹

There are numerous other families of pNPs isolated from *Theonella*, *Discodermia*, and other sponge species for which the biosynthetic routes have not been identified to date. These

molecules will be highlighted in Section 3. It is likely that metagenomic workflows and retrobiosynthesis-guided genome mining workflows will connect these natural products to the corresponding BGCs in sponge holobionts.

2.3. pNPs encoded by commensal bacteria

In contrast to symbiotic bacteria that are tightly interwoven into the metabolism and physiology of their sponge hosts and are thusly onerous to culture in the laboratory, loosely associated commensal bacteria are amenable for culturing. Recent reports have established that sponge-derived commensal bacteria are likewise sources of novel pNPs. Some of the recent work in this space will be highlighted below.

Aquimarins A–H (**67–74**) are peptide/polyketide hybrid natural products isolated by Piel from a sponge-derived bacterium *Aquimarina* sp. Aq135.^{202,203} Of these, **67–70** and **73–74** are dodecapeptides with a diketide 3-hydroxy-4-methyl pentanoic acid moiety at the N-terminus and a primary amine substituting the C-terminal carboxylate with some congeners possessing chlorinated aliphatic amino acid side chains. Molecules **71** and **72** are truncated analogs. The structures of the molecules were established using spectroscopy, fragmentation, and degradation experiments (Fig. 14A).²⁰⁴ While the molecules were identified guided by their activity against *Mycobacterium tuberculosis*, they were found to possess antibiotic activity against a range of human pathogens such as methicillin-resistant *Staphylococcus aureus* with MIC ranging from 2 to 8 μM. Genome sequencing and mining of the aquimarin producer strain led to the identification of the hybrid NRPS/PKS encoding *aqm* BGC. The *Aqm* assembly line consisted of a bimodular PKS followed by twelve NRPS modules containing three epimerase (E) domains, which was in conformity with the presence of three D-amino acids in the peptide chain. Aliphatic chlorination was experimentally demonstrated to be catalyzed by the Fe^{II}/α-ketoglutarate-dependent halogenase *AqmA*.²⁰⁴ The chemical syntheses of **69** and **70**, and various derivatives was realized.²⁰² Structure activity relationship studies revealed that the primary amine at the peptide C-terminus was critical for activity; the primary amine was conceivably installed by the



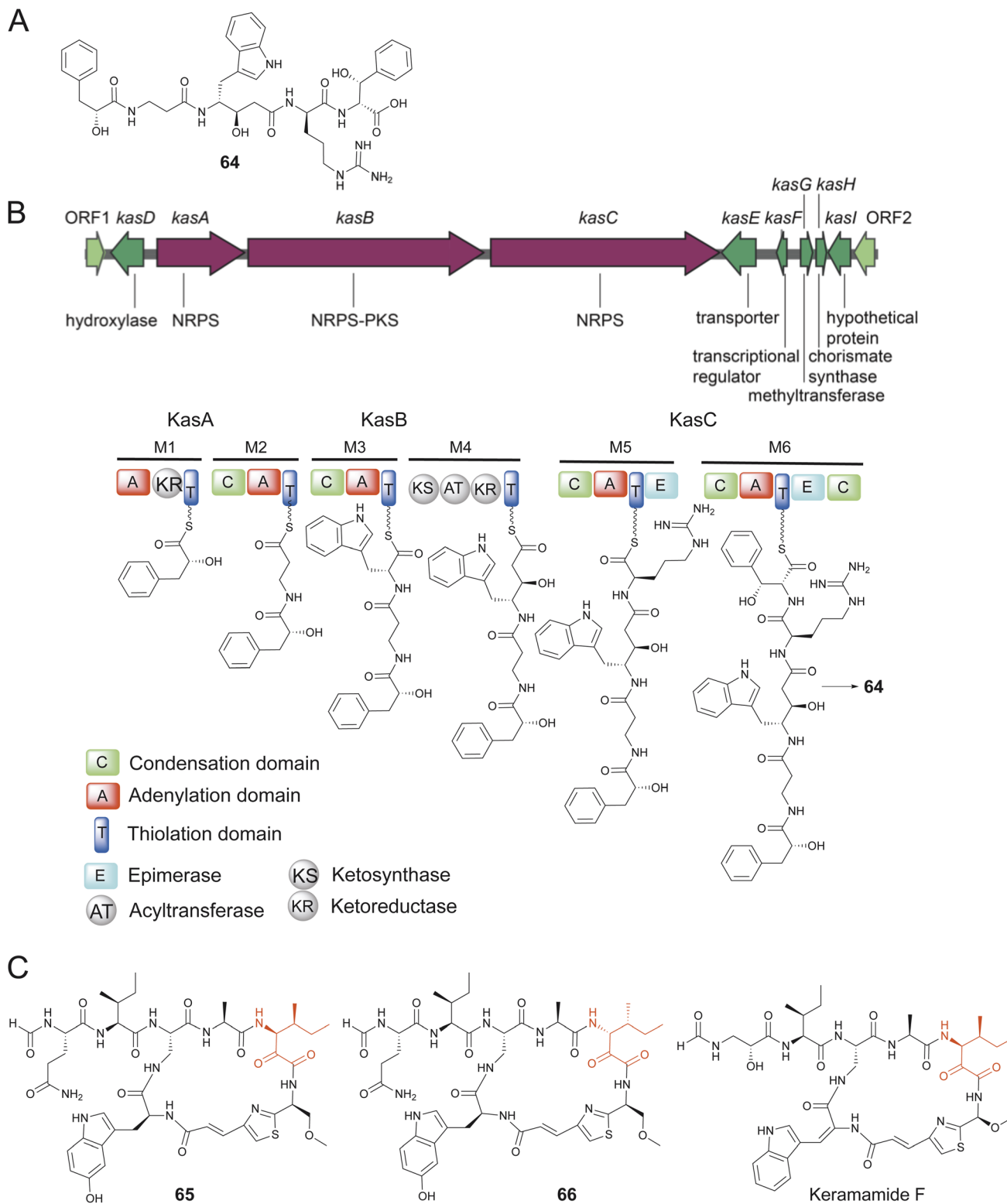


Fig. 13 (A) Structure of **64**. (B) The assembly line biosynthesis of **64** as predicted by the organization of the *kas* BGC. (C) Structures of **65** and **66** and similarity to keramamide F.

reductive offloading of the peptide chain from the NRPS assembly line by the terminal thioester reductase domain of the AqmG NRPS followed by reduction by the NAD-dependent aminotransferase AqmH (Fig. 14B).³⁰

Obligate marine Proteobacteria of the genus *Microbulbifer* are commonly cultured from sponges and other marine invertebrates and are best known for their proclivity to degrade polysaccharides.^{205,206} Concomitant with Igarashi, Agarwal



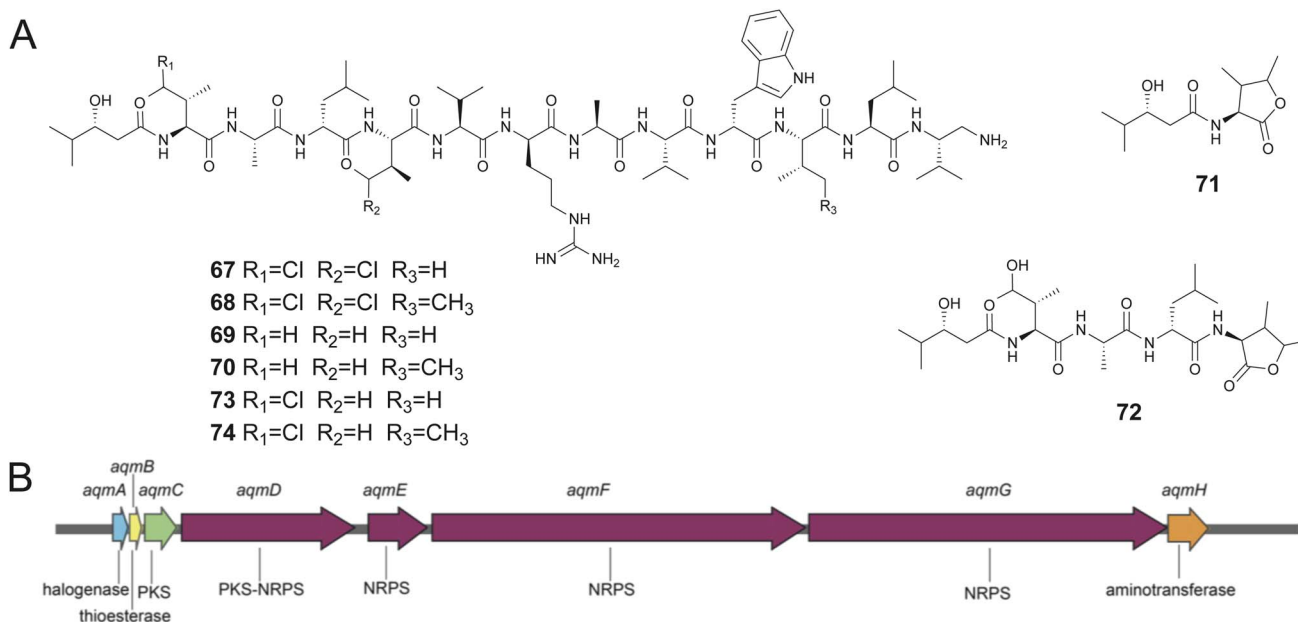


Fig. 14 (A) Structures of aquimarins 67–74. (B) The *aqm* BGC.

reported the discovery of ureido hexapeptidic bulbiferamides A–D (75–78) from sponge- and coral-derived *Microbulbifer* strains.^{207,208} The natural product structures were established using standard spectroscopy and degradation experiments with all amino acids being in the L-configuration. Genome mining led to the identification of the *bulb* BGC encoding a hexamodular NRPS consistent with the bulbiferamide structures (Fig. 15). The presence of the *bulb* BGC was widespread among *Microbulbifer* strains.²⁰⁷ In light of previous studies, the ureido bond in bulbiferamides was postulated to be installed by the C domain of the initiating NRPS module.²⁰⁹

An intriguing structural feature among bulbiferamides was the presence of the macrocyclizing amide bond generated using the tryptophan side chain indole nitrogen. While lysine and ornithine side chain primary amines are commonly observed to participate in macrocyclizing amide bond formation in cyanobacterial ureidopeptide natural products, the bulbiferamides were the first such examples of bacterial natural products possessing a macrocyclizing indolylamide.^{210–212} The terminal TE domain of the BulbE NRPS was experimentally characterized to install the indolylamide bond, making it a unique biocatalytic transformation that potentially complements the synthetically onerous installation of late-stage indolylamides owing to the poor nucleophilicity of the indolic nitrogen.^{213,214} The enzymatic installation of macrocyclizing indolylamides in RiPPs has recently been reported.²¹⁵

The mass spectrometric fragmentation pattern associated with the ureido bond was observed for a different set of pNPs from the marine sponge-derived bacterium *Microbulbifer* sp. MKSA007; this strain also produced bulbiferamides. Isolation of these pNPs followed by spectroscopic and degradation experiments established the structures of heptapeptidic pseudobulbiferamides A–E (79–83) which likewise bear a ureido bond at the N-terminus (Fig. 16).²¹⁶ The pseudobulbiferamides

were highly similar to the heptapeptidic pseudovibriamides A congeners (84–89) that were isolated from the bacterium *Pseudovibrio brasiliensis* strain Ab134 that was cultivated from the marine sponge *Arenosclera brasiliensis*.^{217,218} In addition to pseudovibriamides A congeners, the *P. brasiliensis* strain Ab134 also produced further elaborated hybrid peptide/polyketide pseudovibriamides B congeners 90–95; pseudovibriamides B analogs were not detected in *Microbulbifer* sp. MKSA007 extracts. Genome sequencing enabled identification of the *ppp* BGC in *P. brasiliensis* strain Ab134 encoding production of pseudovibriamides and the *mbp* BGC in *Microbulbifer* sp. MKSA007 encoding production of pseudobulbiferamides.^{216,217} Curiously, the architecture of the *ppp* and the *mbp* BGCs was highly similar, and both BGCs were positioned on plasmids raising the possibility that the genetic potential for making these ureidopeptidic pNPs might be broadly distributed among marine invertebrate associated commensal microbiomes. The pseudovibriamides were also shown to be fundamentally important in modulating the flagellar motility and biofilm formation in *Pseudovibrio* bacteria.²¹⁹

Other pNPs sourced from sponge-derived bacteria include the N-terminal pyroglutamate containing proline-rich linear peptides 96 and 97 isolated from *Microbacterium* sp. V1 which was cultured from the sponge *Petrosia ficiformis* (Fig. 17).²²⁰ The macrocyclic hexapeptide nocardiotide A (98) containing all L-amino acids was isolated from *Nocardioopsis* sp. UR67 which was cultivated from the sponge *Callyspongia* sp. and demonstrated cytotoxicity against a panel of human cancer cell lines of MM. 1S multiple myeloma, HeLa cervix carcinoma, and murine CT26 colon carcinoma with IC₅₀ of 8, 11, and 12 μM mL⁻¹, respectively.²²¹ The macrocyclic heptadepsipeptide xanthostatin B (99) containing a D-valine residue was isolated by Zhang from *Streptomyces* sp. SCSIO 40046 and demonstrated α-glucosidase inhibitory activity.²²²



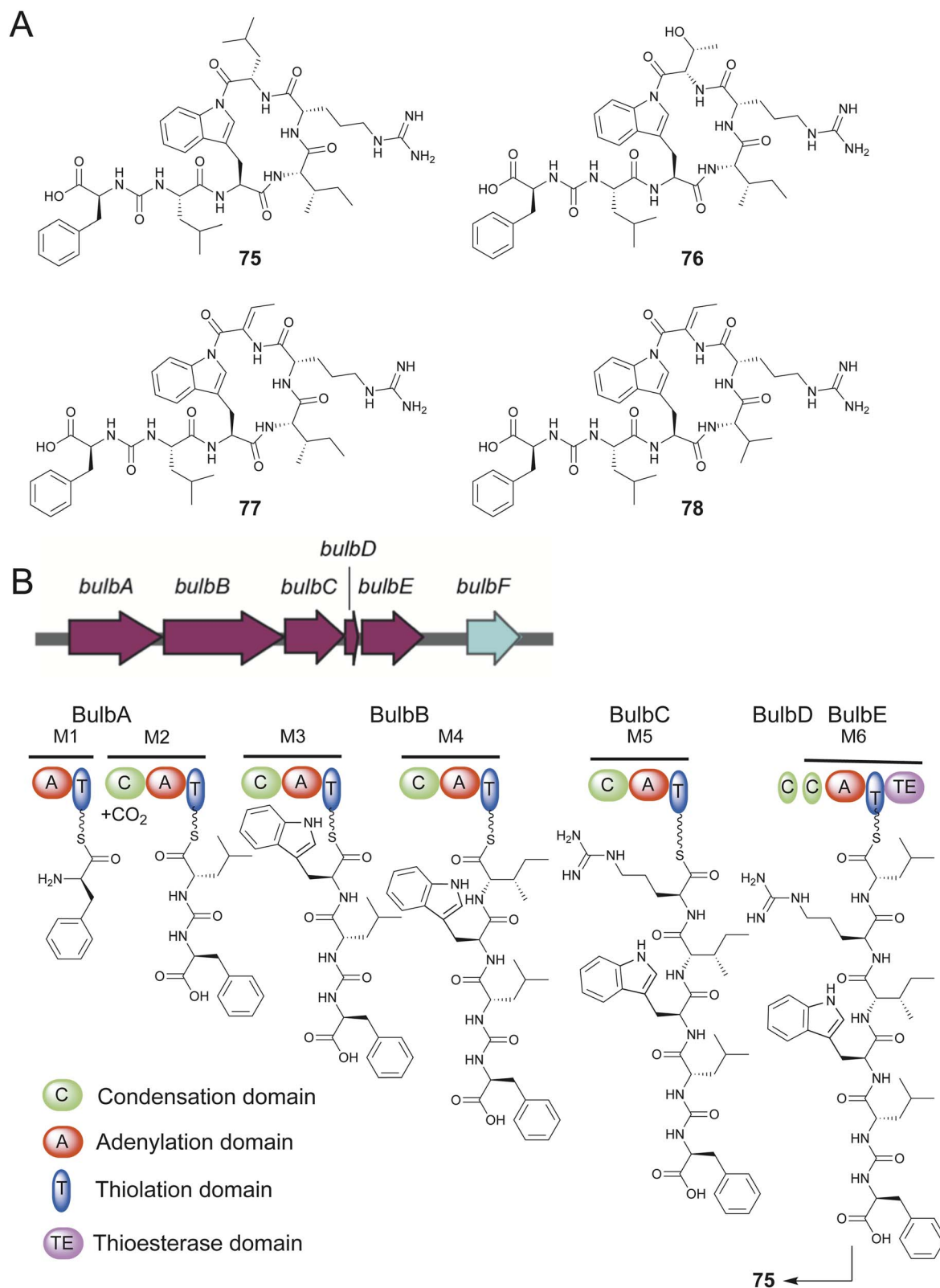


Fig. 15 (A) Structures of bulbiferamides 75–78. (B) The *bulb* BGC and the Bulb assembly line.



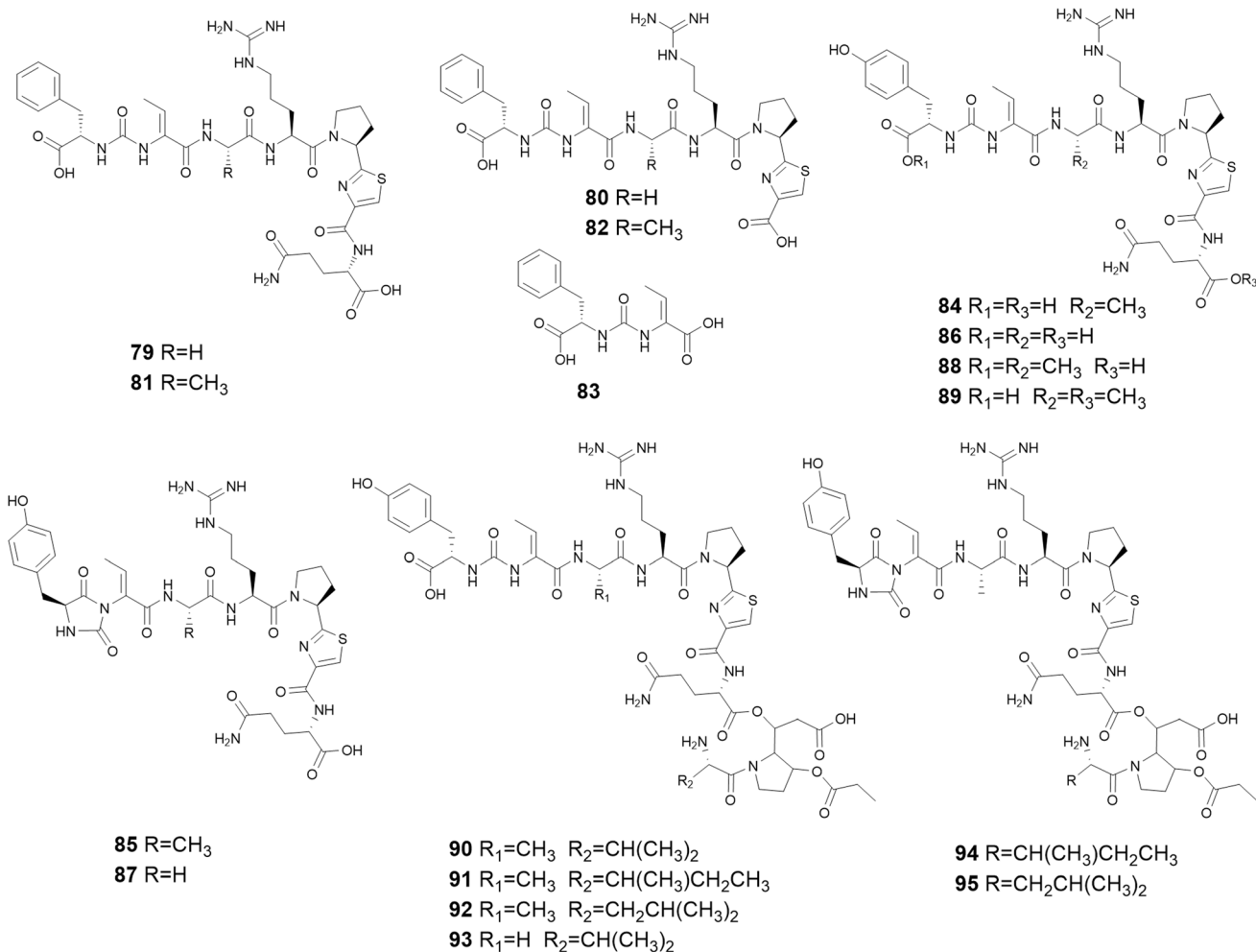


Fig. 16 Structures of pseudobulbiferamides 79–83 and pseudovibriamides 84–95.

3. pNPs with as yet unknown biosynthetic origins

In the sections below, macrocyclic and linear pNPs for which biosynthetic genes, enzymes, and transformations have not been identified are described. While sponge-derived PIAs and BTAs are dipeptidic in nature, they defy characterization as either macrocyclic or linear pNPs; they resemble and can be thought of as alkaloids, rather than canonical pNPs. The reader is directed to some excellent reviews that describe the discovery, synthesis, and pharmacology of PIAs and BTAs.^{33–36,38,40} While the biosynthesis basis for these classes of sponge-derived natural products are as yet unknown, recent metabolomic investigations which build upon prior labeled precursor feeding studies point toward the identities of the core amino acid building blocks.

3.1. Macrocyclic pNPs

Several instances in which the bacterial *Entotheonella* sp. symbionts were responsible for synthesizing pNPs derived from *Theonella* sponges have been described in the previous section.

It is thus instructive to note that several other classes of pNPs have been described from *Theonella* sponges that have yet to be connected to their biosynthetic origins and the corresponding BGCs identified. These are likely targets for future genomic investigations.

Theonellapeptolides are a group of cyclic tri-decadepeptides characterized by the presence of *N*-methyl amino acids, *D*-amino acids, and β -amino acids mainly isolated from *T. swinhoei* with more than 15 congeners reported to date (Fig. 18).^{223,224} Theonellapeptolides show various biological activities, such as cytotoxicity and inhibition of Na⁺ and K⁺ ion transport. Recently, pNPs sulfinyltheonellapeptolide (**100**) and theonellapeptolide If (**101**) from *T. swinhoei* were reported with structures determined using spectroscopy, mass spectrometry, and chemical degradation experiments (Fig. 18).²²⁵ These compounds significantly reduced proliferation rate of HepG2 cells to 30% and 50% at the dose of 10 μ M. A further three analogues theonellapeptolides I Ib (**102**), I Ia (**103**), and I Ic (**104**) were isolated from *T. swinhoei*.²²⁶ As is characteristic of theonellapeptolides, these recently reported congeners contained multiple amino acids with non-polar aliphatic side chains.



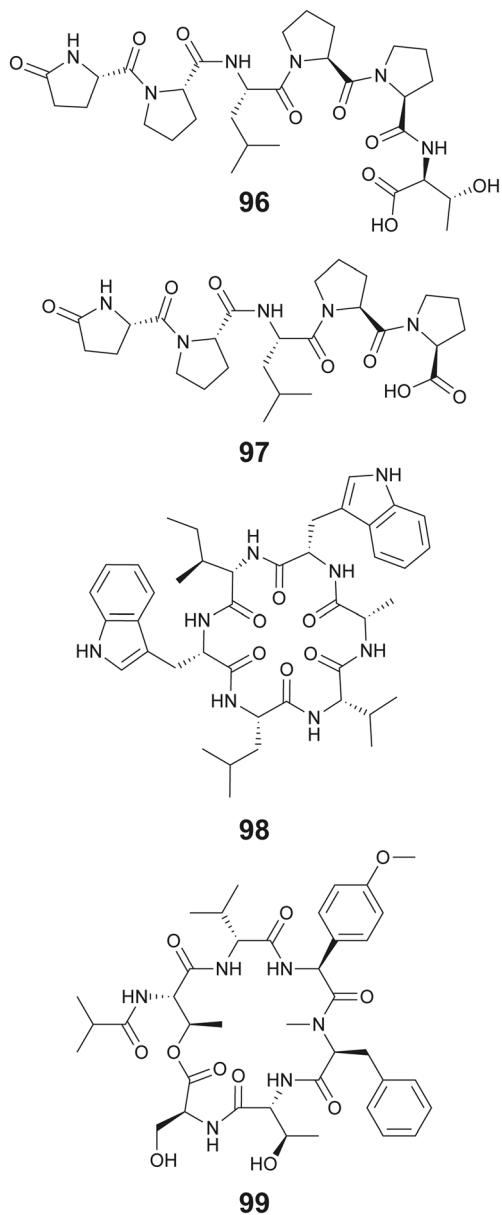


Fig. 17 Structures of pNPs 96–99.

Two recent reports by Carmeli and Carroll described the isolation and structures of the pNPs cyclotheonellazoles A–C (**105–107**) and cyclotheonellazoles D–I (**108–113**) from *Theonella* sponges with the chemical synthesis of **105** now realized (Fig. 19).^{227–229} The cyclotheonellazoles structures resemble the pNP oriamide described from *Theonella* sponges and the calyxamides described from *Discodermia* sponges, and are likely to be biosynthesized by a *Entotheonella*-derived NRPS assembly line.^{200,230} Structural correspondence between cyclotheonellazoles and calyxamides extends to jamaicensamide A (**114**) isolated by Molinski from sponge *Plakina jamaicensis*.²³¹ Molecules **105–107** inhibited chymotrypsin with IC_{50} of 0.62, 2.8, and 2.3 nM, and elastase with IC_{50} of 0.034, 0.10, and 0.099 nM. Molecules **108–113** displayed inhibition of elastase with IC_{50} values ranging from 16.0 to 61.8 nM. The

cyclotheonellazoles were found to be potent protease inhibitors, including the SARS-CoV-2 3-chymotrypsin-like protease. The recurrent theme of pNPs being shared among *Theonella* and *Discodermia* sponges was also realized for the pNP cyclo-lithistide A (**115**). Molecule **115** was originally isolated by Crews from *T. swinhoei* and its structure was revised by Abe upon isolation from *D. japonica* with the configuration of the chloroisoleucine residue in the antifungal cyclodepsipeptide determined by synthesis of chemical standards and comparisons thereof.^{232–234} The presence of a chlorinated pyrrole residue was observed in gunungamide A (**116**), a macrocyclic depsipeptide isolated from *Discodermia* sp.²³⁵ Solomonamide A (**117**) and solomonamide B (**118**) are cyclic tetrapeptides isolated from *T. swinhoei* (Fig. 19).²³⁶ The solomonamide structures were revised upon chemical syntheses which also furnished derivatives to explore their anti-inflammatory activities.^{237–243}

Progressing from the description of neamphamide A, the depsipeptides neamphamides B (**119**), C (**120**), and D (**121**) were described from sponge *Neamphius huxleyi* by Kobayashi and Quinn (Fig. 20).^{244–247} The neamphamides were reported to possess myriad bioactivities which include protective activity against viral infection, anti-mycobacterial activity, and cytotoxicity against human cell lines. Another class of macrocyclic depsipeptides, stellatolides A–G (**122–128**) were isolated from the sponge *Ecionemia acervus* by Cuevas (Fig. 20).²⁴⁸ The chemical synthesis of **122** was realized, and most stellatolides were found to be cytotoxic to A549, HT-29, and MDA-MB-231 human tumor cell lines with GI_{50} from 0.08 to 2.7 μ M except for molecules **127** and **128** that were found to be much less potent.²⁴⁸ Among other structural features, the neamphamides and the stellatolides both possess the β -methoxy tyrosine residue in the peptide macrocycle. The cytotoxic stellatolide H (**129**) was described by Matsunaga from *Discodermia* sp. sponge.²⁴⁹ The cyclodepsipeptides pipecolidepsins A (**130**) and B (**131**) were described from *Homophymia lamellose* with the chemical synthesis of **130** realized.^{250,251} Molecules **130** and **131** displayed cytotoxic against a panel of human tumor cell lines with GI_{50} from 0.01 to 1.12 μ M.²⁵¹ The depsipeptide structures point toward a hybrid NRPS/PKS-involving biosynthetic route that could be encoded within a bacterial symbiont. The biosynthesis of non-proteinogenic amino acid building blocks constituting these depsipeptides, such as the pipecolic residue in **130** and **131**, are well described in bacterial natural product biosynthetic schema.^{252,253} The cyclic sulfated depsipeptide mutremdamide A (**132**) was isolated by Bewley from *Theonella* sp. sponges (Fig. 20).²⁵⁴

Lipodiscamides A–C (**133–135**) are cyclic lipodepsipeptides bearing a dilactone macrocycle described isolated by Abe from the sponge *Discodermia kiensis* (Fig. 21).²⁵⁵ The peptidic macrocycle of lipodiscamides constitutes various non-proteinogenic amino acids such as a dehydronorvaline, ureidoalanine, and citrulline. The citrulline side chain was found to be sulfated in the sulfolipodiscamides A–C (**136–138**) that were isolated from the same sponge.²⁵⁶ Molecules **133–135** demonstrated cytotoxicity against mammalian HeLa cell line with IC_{50} of 18, 26, and 46 μ M, and against P388 with IC_{50} of 23, 20, and 31 μ M, respectively, while molecules **136–138** exhibited



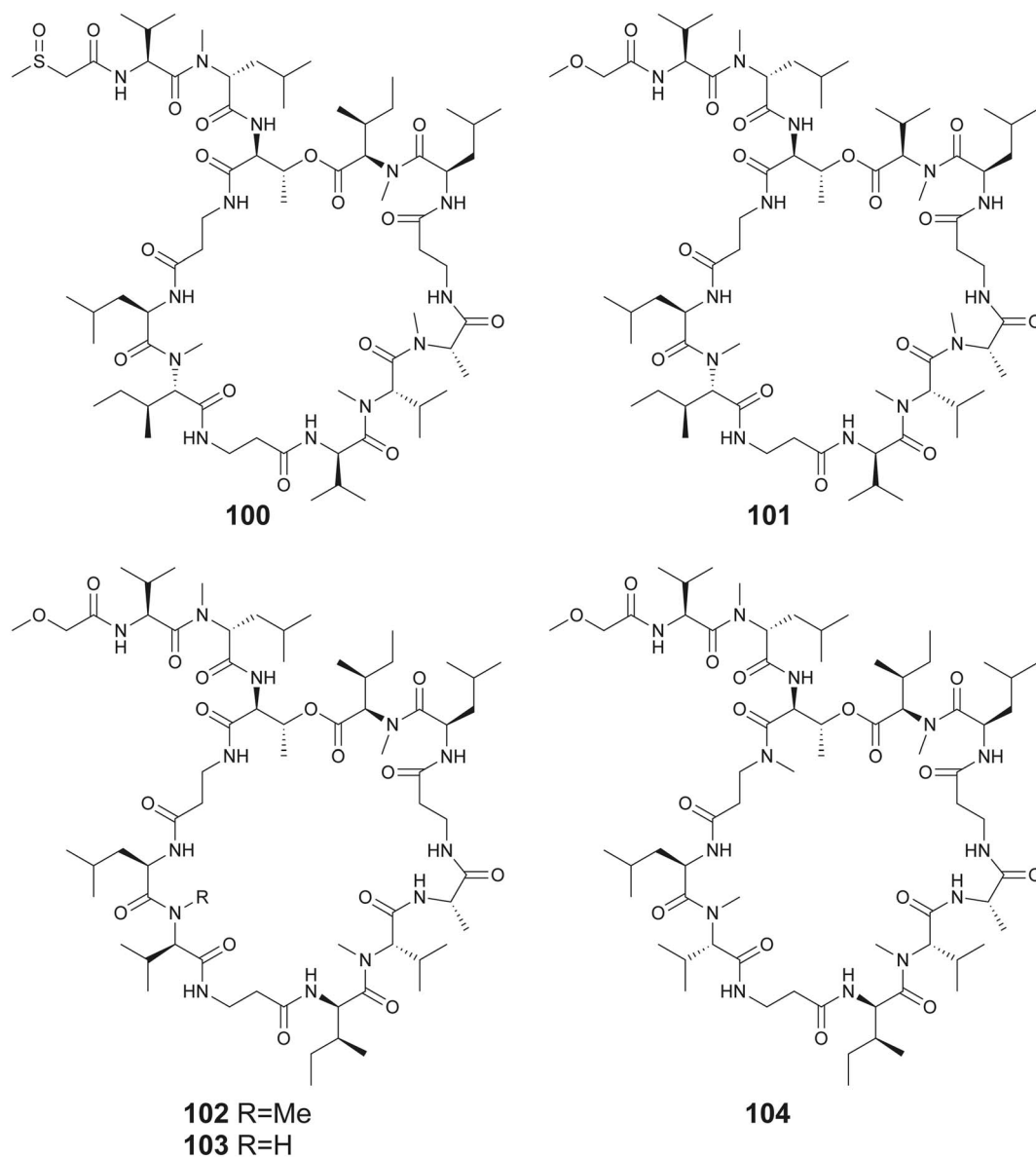


Fig. 18 Structures of recently reported theonellapeptolides 100–104.

cytotoxicity against P388 with IC_{50} of 15, 30, and 29 μM , respectively.

Progressing from Faulkner's description of aciculitin congeners A–C from sponge *Aciculites orientalis*, aciculitin D (139) was described by Matsunaga from the deep-sea sponge *Poecillastra* sp. and found to possess cytotoxic bioactivity against HeLa and HCT-116 cancer cells with IC_{50} of 4.5 and 1.4 μM , respectively.^{257,258} It is tantalizing to propose that the aciculitin peptidic macrocycle to which the glycosylated lipid moiety is appended to arises from a NRPS assembly line. However, a ribosomal route to the peptidic macrocycle can also be envisaged with a bi-radical coupling between the histidine and epimerized tyrosine residue side chains, as have been observed in other RiPP biosynthetic schemes, leading to the formation of the coupled biaryl bridge (Fig. 22).^{181,259–261} Differences in configurational assignments between aciculitins A–C

and 139 suggest that the amino acid configurations should be reinvestigated.

Predicting a plausible biosynthetic route is difficult for the callaerins A–M (140–152), cyclic peptides isolated by Proksch from sponge *Callyspongia aerizusa* (Fig. 23).^{262–264} The chemical synthesis of 140 has been described by Brimble.²⁶⁵ While all amino acids in callaerins are proteinogenic amino acids or derivatives therein that are well represented among RiPPs, an assembly line NRPS-mediated construction cannot be ruled out. The biosynthesis of the macrocyclizing aminoacrylamide moiety is an enzymological novelty. The callaerins demonstrate antitubercular activity by targeting a membrane protein that causes dysregulation of various primary biological processes in the pathogen. Specifically, molecules 140 and 141 exhibited the most potent activity with MIC_{90} of 2 and 5 μM .²⁶⁶

Natural product chemical scaffolds are often shared among marine bacteria and marine sponges with the biosynthesis of



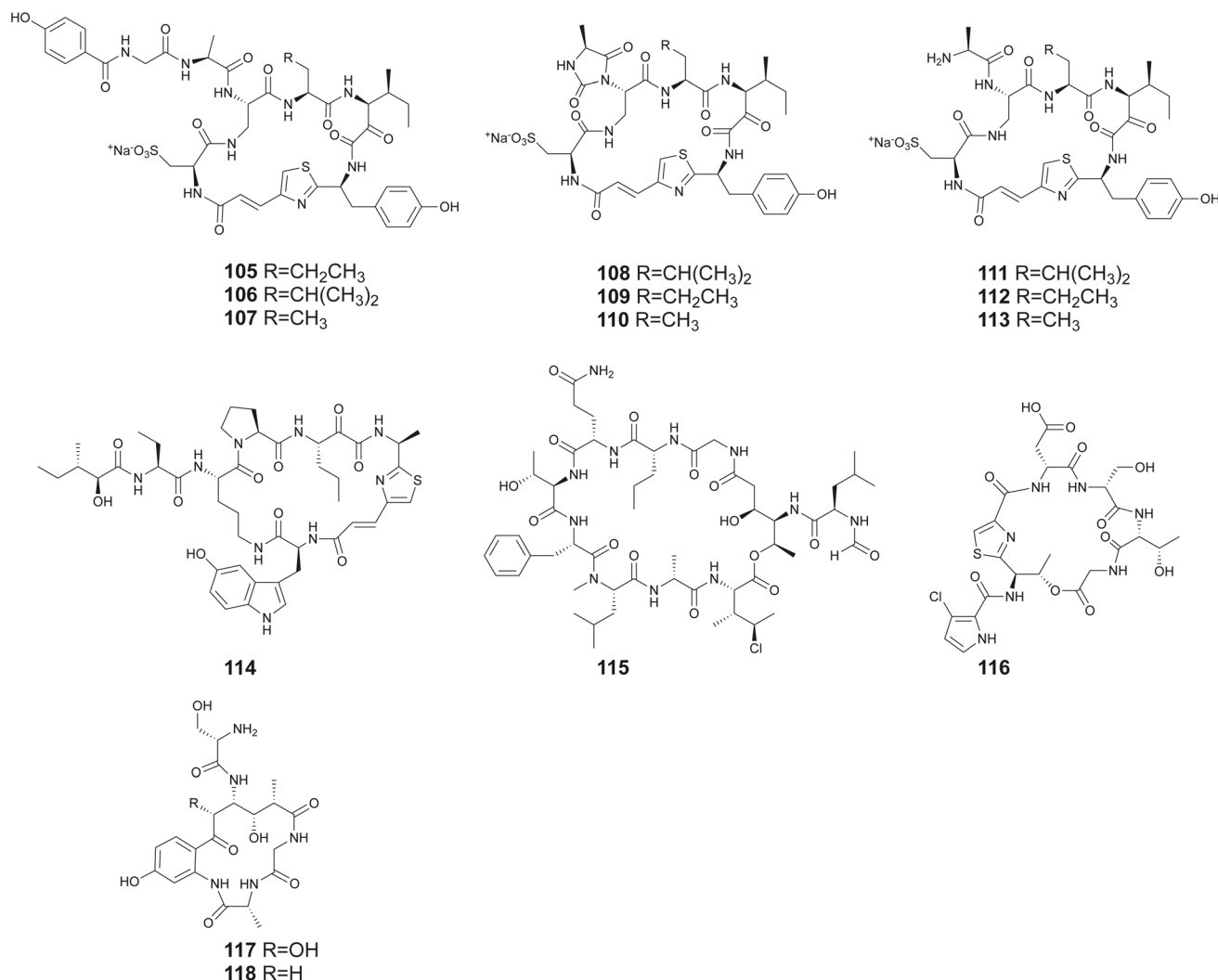


Fig. 19 Cyclotheonellazoles **105**–**113**, jamaicensamide A **114**, cyclolithistide A **115**, gunungamide A **116**, and solomonamides **117** and **118**.

several of these sponge-derived natural products attributed to the symbiotic bacteria residing within the sponge holobiont community.¹² As mentioned previously, marine cyanobacteria are prolific producers of ureidopeptidic molecules that are macrocyclized by the terminal primary amine of lysine or ornithine side chains.^{210–212} From marine sponge *Siliquariaspongia mirabilis*, Bewley described isolation, structure determination, and chemical synthesis of the macrocyclic ureidopeptide namalide (**153**) (Fig. 24).²⁶⁷ Molecule **153** demonstrated inhibition to carboxypeptidase A with IC₅₀ of 250 nM. Subsequently, namalides B (**154**) and C (**155**) were isolated from the freshwater cyanobacterium *Sphaerospermopsis torques-reginae*.²⁶⁸ The namalides were also detected to be produced by cyanobacterium *Nostoc* sp. CENA543 along with the macrocyclic ureidopeptides of the anabaenopeptin family.²¹⁰ These observations perhaps point toward a cyanobacterial symbiont being the possible primary producer of **153** in the sponge *S. mirabilis*. Numerous pNPs isolated from marine sponges have been connected to symbiotic cyanobacterial producers using genomics as well as microscopy and mass

spectrometry-based techniques.^{64,269} Alternatively, akin to konbamide biosynthesis, an *Entotheonella* symbiont could encode production of namalides.^{157,270} These cross-organismal biosynthesis has been observed for swinholides that were isolated from *Theonella* sponges and from marine cyanobacteria, and BGCs for their production have now been detected in terrestrial cyanobacteria as well.^{271–273} Along the same lines, homophymamide A (**156**) was isolated by Matsunaga from the *Homophymia* sp. marine sponge and its chemical synthesis realized (Fig. 24).²⁷⁴

3.2. Linear pNPs

Amide bond *N*-methylation can alter the structure and physicochemical properties of peptides, rendering them resistant to proteolysis and improving upon their bioavailability. Several highly *N*-methylated linear peptides (HNMLPs) have been reported from marine sponges with as yet unknown biosynthetic origins. Note that iterative *N*-methylation in the context of fungal RiPPs is well established.^{275–277}



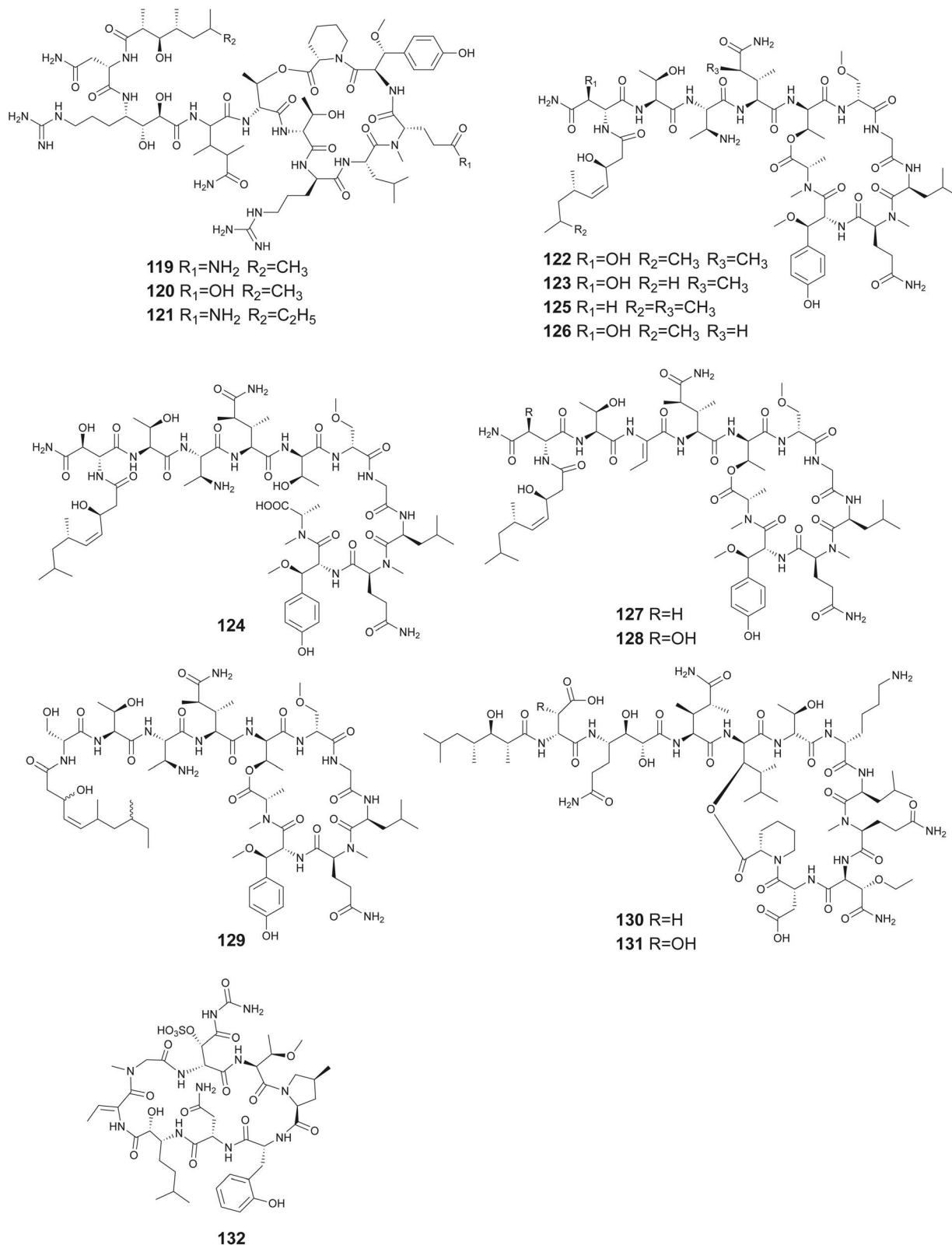
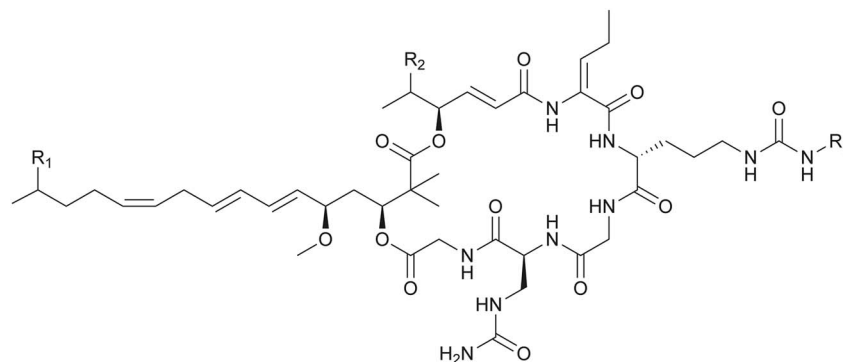


Fig. 20 Sponge-derived cyclic depsipeptides **119**–**132**.

Numerous HNMLPs from marine cyanobacteria have been described. Recently, the linear octapeptide pambamide (**157**) containing all *L*-amino acids was described by Fernández from

the sponge *Cribrochalina* sp. which demonstrated cytotoxicity against cancer cell lines of MDA-MB-231, HT-29, and A549 with GI_{50} of 3.35, 3.80, and 2.46 μM , respectively.²⁷⁸ From the Antarctic





- 133** $R_1=CH_3$ $R_2=CH_3$ $R_3=H$
134 $R_1=H$ $R_2=CH_3$ $R_3=H$
135 $R_1=CH_3$ $R_2=CH_2CH_3$ $R_3=H$
136 $R_1=CH_3$ $R_2=CH_3$ $R_3=SO_3Na$
137 $R_1=H$ $R_2=CH_3$ $R_3=SO_3Na$
138 $R_1=CH_3$ $R_2=CH_2CH_3$ $R_3=SO_3Na$

Fig. 21 Lipodiscamides 133–135 and sulfolipodiscamides 136–138.

sponge *Inflatella coelosphaeroides*, Baker and co-workers described the isolation and structure of friomaramide A (158) which demonstrated inhibition of liver-stage *Plasmodium falciparum* parasite development.²⁷⁹ A second friomaramide congener, friomaramide B (159), was also described by Baker from *I. coelosphaeroides* along with six new HNLMPs termed shagamides A–F (160–165) which inhibited different activities against three blood-stage *P. falciparum* strains, in which molecules 159, 160, and 162 are the most potent ones with IC_{50} values ranging from 1.07 to 8.65 μM .²⁸⁰ The friomaramides possess a C-terminal tryptenamine moiety that is absent in shagamides (Fig. 25).

The linear tridecapeptides yaku'amides A (166) and B (167) were isolated from a deep-sea sponge *Ceratopsion* sp. by Matsunaga and coworkers.²⁸¹ Their structures were initially elucidated by analysis of NMR spectroscopic data and chemical degradation, with complete assignment achieved by total synthesis by Inoue and coworkers.^{282,283} Among the several non-proteinogenic amino acids comprising the yaku'amides are residues bearing α,β -unsaturated as well as β -hydroxylated side chains (Fig. 26). Molecules 166 and 167 exhibited cell-growth inhibitory against P388 cancer cell line. Molecule 167 was further tested against a wide panel of human cancer lines and the molecular target was identified to be the mitochondrial F_0F_1 -ATP synthase.²⁸⁴ Recent efforts have been aimed at streamlining synthetic schemes and furnishing derivatives for biological evaluation.^{285–287}

The linear heptapeptide bearing all L-amino acids, sub-armigeride A (168), was isolated from *Callispongia sub-armigera*.²⁸⁸ Several analogs of 168 were detected to be present in the sponge extract and a cyanobacterial symbiont was proposed to be the producer of the pNP, though that remains to be validated using genomic and biochemical investigations.

4. Future directions in sponge pNP discovery

The already extensive inventory of natural products sourced from marine sponges continues to expand.²⁸⁹ Among these, pNPs have always attracted attention due to their structural complexity and biological activities (*vide supra*). Sponges are also among the most abundant sources of cultivated marine bacteria described in natural products manuscripts, leading to the discovery of many pNPs. This amazing, continued abundance promises many future discoveries. Perhaps more strikingly, metagenomic methods indicate that this is just the beginning. Ongoing research continues to uncover the vast microbial dark matter present in sponge metagenomes, revealing a largely untapped biosynthetic potential.²⁹⁰ For example, Loureiro *et al.* performed metagenomic analysis on four sponge specimens spanning three species and identified 5082 BGCs, with NRPS and RiPP-related BGCs accounting for approximately 30% of the total.²⁹¹ And these are just from bacterial symbionts; relatively little is known about the biosynthetic potential encoded in host sponge genomes, or about the metabolic potential of non-bacterial sponge symbionts such as dinoflagellates (as speculated for halichondrin biosynthesis), archaea, and many other branches of life.^{292,293} Limited data from sponge genomes and transcriptomes also reveals a high potential for undiscovered chemical novelty, with a single sponge potentially encoding 22 cyclic PRMPs, including several new compounds that were then detected by mass spectrometry.²² In addition, the same sponge had more than 23 000 transcripts encoding secretion signals, reinforcing how much completely unknown chemical diversity is out there.

Despite the wealth of potential new compounds, the field faces at least three significant limiting factors: first, as found throughout natural products research, the newly described



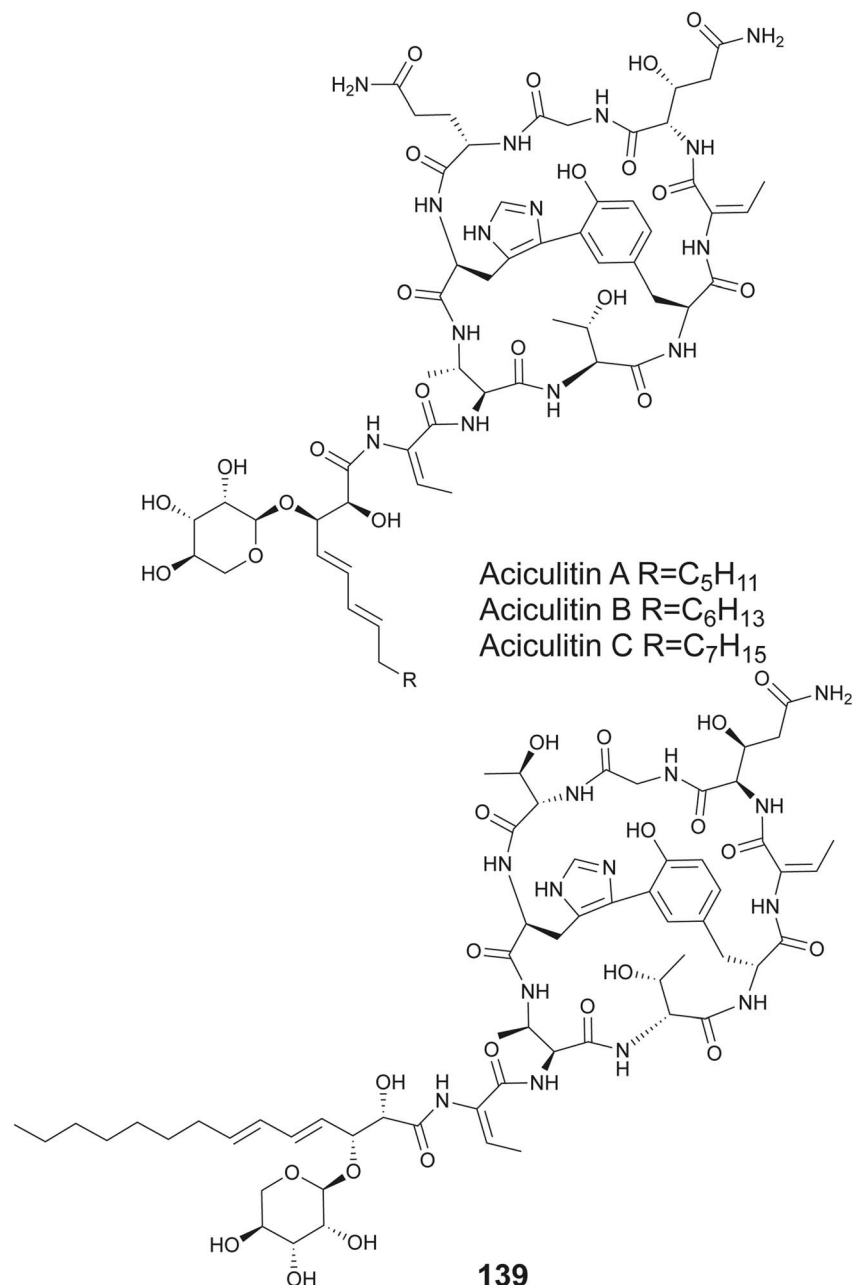


Fig. 22 Acicullitins A–C and acicullitin D (139).

pNPs largely fall into known structural groups previously identified in sponges. In short, structural novelty is much harder to find than in the recent past.²⁹⁴ Second, although thousands of new biosynthetic gene clusters (BGCs) have been discovered in sponge metagenomes, the majority of corresponding molecules have not been accessed in the sponge metabolomes or by synthetic biology approaches.²⁹⁵ It has remained quite hard (with no published examples despite significant effort) to express NRPS biosynthetic pathways from the uncultivated microbial pool in sponges. Third, it is still common that few of the sponge-derived pNPs are sourced in the significant amounts needed to fully validate their biological activities or to take the needed preclinical steps to advance

therapeutic candidates to application. Below, we describe some important considerations and future developments to enhance discovery and development of sponge pNPs.

As a cautionary note, it is important to mention that it has been popular to infer the origin of metabolites based upon cellular localization in host or symbiont, or by structural homology. However, in the modern era, it is important to take an agnostic approach that considers that either, both, or even multiple symbionts might be responsible for making the same compound.¹⁷ Such an approach simultaneously employs the chemical, metagenomic, and transcriptomic methods described below.



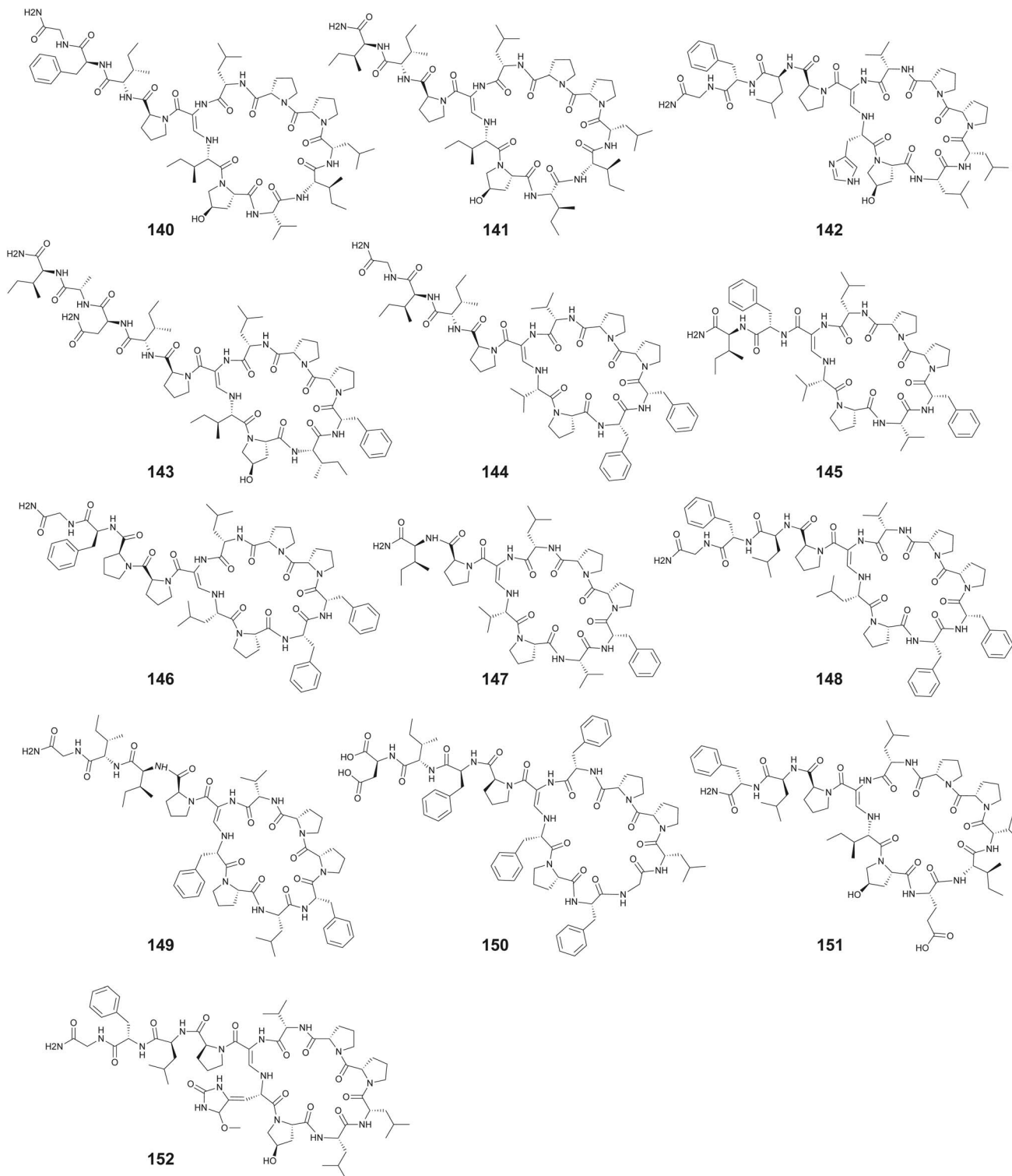


Fig. 23 The callyaerins 140–152.

4.1. Chemical approaches

Traditional approaches, and innovative modifications to those approaches remain the primary tools for discovery. In the initial decades of the field, new compounds were identified from easily accessible sponges found in the shallow water, by snorkeling, or

SCUBA. By the late 1980s or early 1990s, the rate of rediscovery was rapidly increasing. Collection of sponges began to move to difficult-to-reach locales, deep waters (often *via* dredging or submarine collection), or harsher environments such as polar regions.^{296,297} An increased emphasis was placed on cryptic



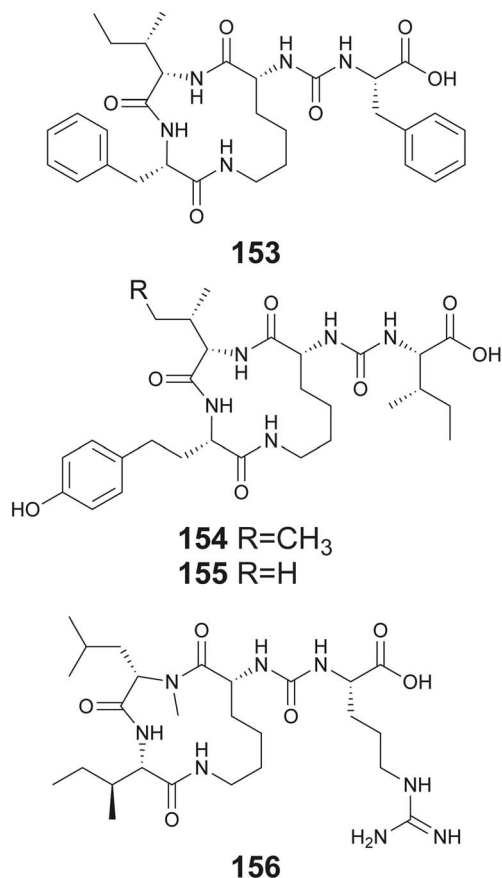


Fig. 24 The namalides 153–155 and homophymamide A (156).

biodiversity (of both host sponges and microbial symbionts), as biodiversity and chemical diversity are often coupled.²⁹⁸

Sponge extracts have traditionally been investigated *via* bioassay-guided fractionation, chemistry-directed methods, or a combination of the two.²⁹⁹ Most of the papers cited in this review used these approaches, demonstrating their continued importance to the field. Reinvestigation of sponges using unique bioassays has been a powerful method of discovery. A limitation to that approach can be that sponges are often chemically defended by major, abundant compounds that can mask the bioactivities of less abundant metabolites, specially as it related to antibiosis bioactivities. Pre-fractionation has been applied to mitigate the problem. This approach is particularly a noteworthy feature of the National Cancer Institute compound library, which has thousands of sponge extracts and fraction libraries, in addition to efforts at other institutes.^{300–302}

Chemistry-directed methods have evolved significantly over the years, with the most recent trend focusing on widespread adoption of mass spectrometry-based metabolomic mining. These methods have continued to increase in importance especially for pNPs that demonstrate highly ordered fragmentation patterns.³⁰³ For instance, the unique fragmentation patterns of the prolyl amide bond and the ureidopeptide bond guided the discovery of novel PRMPs in sponges and bulbiferamides and pseudobulbiferamides in commensal bacteria, respectively.^{22,63,207,216}

4.2. Genomic approaches

4.2.1. pNP discovery from metagenomes.

Metagenomics provides an important method to discover new BGCs encoded in sponge symbionts.¹⁴⁴ Because both RiPP and NRPS pathways exhibit genomic features that are recognizable to a broad array of bioinformatics tools, they are relatively straightforward to identify within sponge metagenomes. The main issue is that those pathways can be difficult to express in heterologous hosts. Although larger biosynthetic enzymes such as NRPSs are still challenging, a few successes with heterologous production of RiPPs reveals the future promise. A notable success in this area was achieved by Piel and co-workers in expressing all enzymes needed to produce polytheonamides and derivatives, enabling synthetic biology approaches to supply the compounds and derivatives and genome mining approaches to identify new natural variants from sponges and other organisms.^{163,166,167}

Recently, Nguyen *et al.* analyzed 13 sponge metagenomes and identified a well-conserved and widely distributed RiPP BGC in marine sponge microbiomes.^{13,16} This BGC encodes a broad-spectrum regiospecific peptidyl halogenase, an enzyme responsible for site-specific halogenation of peptide substrates which provided a valuable biotechnological tool for peptide diversification.^{304,305} The study found that this BGC was present in all queried sponges with high microbial diversity, emphasizing its ecological and functional importance. By applying genome mining and metagenomic sequencing, they established a systematic approach to uncovering cryptic BGCs and discovering new peptide compounds and enzymes from marine sponge metagenomes. This is particularly significant because the integration of metagenomic mining with synthetic biology enables the expression and engineering of BGCs in heterologous hosts. This powerful combination opens new avenues for the discovery and production of new-to-nature pNPs with potential therapeutic value.

In some cases, these types of methods can also be applied to NRPS-derived pNPs and not just to RiPPs. Although not from sponges, Brady and co-workers pioneered the application of direct synthesis of predicted NRPS-derived peptides found in genomes.³⁰⁶ In sponges, there is the unique opportunity to directly query whether those compounds are present, using mass spectrometry in tandem with (bio)synthetic standards.

4.2.2. pNP discovery from transcriptomes.

Genome mining strategies for detection of pNP-encoding BGCs in prokaryotic (meta)genomes are well established. However, analogous detection of BGCs in eukaryotic sequences is still a developing field. If the structure of a host-derived pNP is known, its producing gene and relatives can readily be identified in the sponge transcriptome; this is especially true for RiPPs where the structure and sequence of the pNP correlates with the sequence of the precursor peptide though complications still exist (*vide infra*). An early example of this was described during the discovery of the barrettides from *G. barretti*.²¹ Based upon the known peptide sequence of barrettides A and B, a BLAST search enabled the identification of five new barrettide analogs—barrettides C–G—from genomic and transcriptomic datasets. These analogs exhibited minor sequence variations from



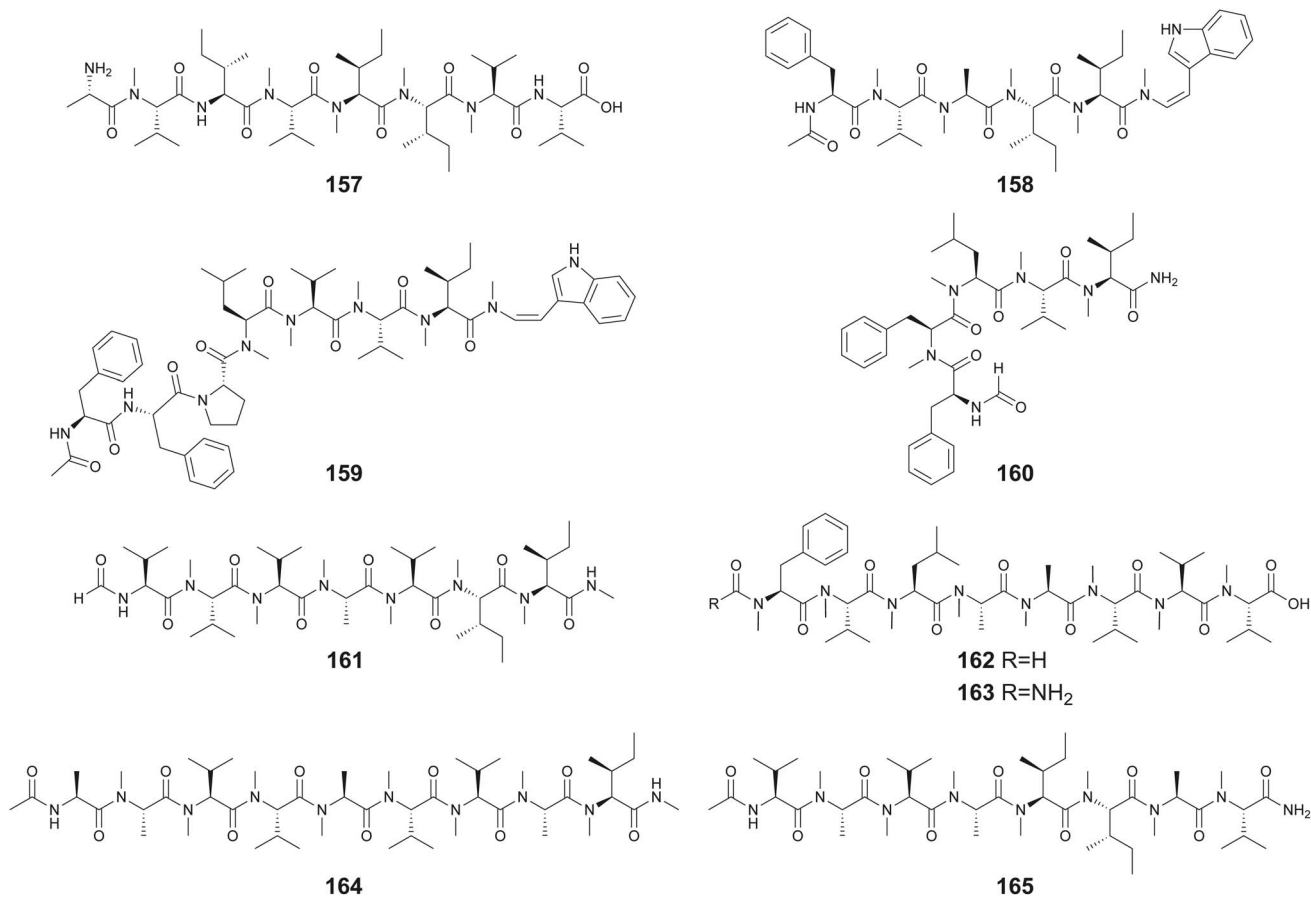


Fig. 25 The HNMLPs 157–165.

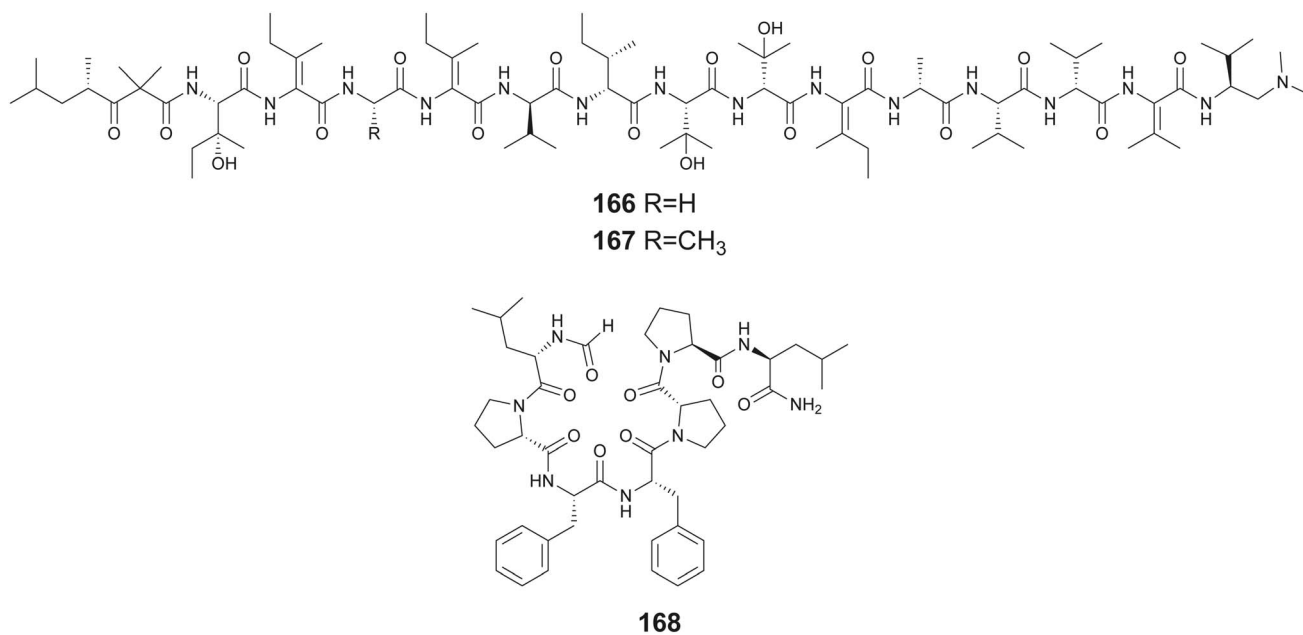


Fig. 26 The yaku'amides 166–167, and subarmigeride A 168.



barrettide A, involving one to four amino acid substitutions. The presence of these newly discovered barrettides was further validated through mass spectrometric analysis of sponge extracts and were readily amenable to production by peptide synthesis.

The above is an example of an ideal case. It is still not trivial to identify sponge-derived pNP transcripts without prior knowledge of the sequence from proteomics or chemical approaches. Often, the high complexity of sponge transcriptomes necessitates additional informatics methods. For example, in the case of discovering PRMP genes from sponges, 79 possible precursor peptides were discovered using BLAST. To determine which peptide(s) was likely to be the correct one, unique characteristics of bioactive peptides were queried.²² Usually, in such peptides, the sequences encoding the pNPs (core peptides) are hypervariable and not sequence conserved, but parts of the precursor peptide that are proteolyzed and discarded (recognition sequences and signal peptides) are highly conserved and often highly repetitive. Such sequences are discoverable using specialized tools such as MEME-ChIP, which was applied to discover the PRMP transcripts.³⁰⁷ Subsequently, many new and known PRMP genes were found. The resulting products are readily amenable to chemical synthesis.

Lack of gene co-localization is another challenge for host-derived pNPs. Although animals often contain true animal-derived BGCs,³⁰⁸ peptides such as the PRMP precursors are so far found distributed across the sponge chromosomes and not co-localized with genes encoding modifying enzymes.²² In better-studied systems, such as cone snails, transcriptomic discovery is aided by a good understanding of prepropeptide regions. Some types of host-derived bioactive peptides are also recognizable because of their doppelgänger nature, in which their sequences converge with important hormones found in their prey organisms.^{116,309} These and other transcriptomic analysis tools provide virtually untapped opportunities for discovery and synthesis of novel host-derived peptides. It should also be noted that sponge genomes encode many NRPSs,³¹⁰ as well as other host-encoded BGCs, which are relatively unexplored.

5. Conclusion and prospects

All components of the sponge holobiont are prolific sources for pNP discovery. Though widely proposed associated microorganisms are the real origin of many pNPs isolated from sponges, the animal hosts indeed can produce pNPs themselves. However, biosynthesis study of sponge animals is still in its infancy with the genetic potential in sponge genomes needs to be unlocked and complemented with molecular and chemical tool development. Furthermore, with principals well established from decades of investigations from microbial systems, currently, most biosynthetic investigations for sponge-derived pNPs are focused on NRPs and RiPPs while other chemical classes of sponge-derived pNPs, such as the PIAs and BTAs have received lesser attention. It is foreseeable that with rapid evolvments in (meta)genome sequencing, bioinformatics, molecular biology, and synthetic biology,

biosynthesis of all types of pNPs and alkaloids from the three components of the sponge holobiont will garner considerable attention and grow significantly. As modern genome mining-based, metabolomics-guided, and traditional bioassay-guided strategies are increasingly more accessible and continue to be refined, it is undoubtable that the sponge holobionts would continue to be cornucopias of pNP discovery. With more chemical entities in hand, not only their biological activities but also their ecological roles will need to be evaluated. We believe mining sponge derived pNPs and alkaloids and investigating their biosynthesis will provide perspective drug leads, new enzymatic and synthetic biology tools, and development in ecological protection.⁹

6. Conflicts of interest

The authors declare no conflicts of interest.

7. Data availability

No primary research results, software or code have been included and no new data were generated or analyzed as part of this review.

8. Acknowledgements

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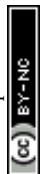
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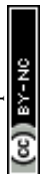
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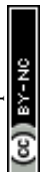
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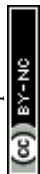
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