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REVIEW

Antifungal peptides in marine invertebrates

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Abstract

A majority of terrestrial and marine organisms use to fend off a wide range of microorganisms, including bacteria and fungi by employing “antimicrobial peptides (AMPs)” that are ribosomally synthesized from proteinogenic amino acids. AMPs are a primary component of innate immune mechanisms in marine invertebrates. In contrast, marine sponges seem to contain no AMPs, but often contain nonribosomal peptides consisting of unusual amino acids that exhibit potent cytotoxic and antifungal activity. Most of these peptides are considered to be of symbiotic bacterial origin. Similarly opisthobranch molluscs sequester unusual bioactive nonribosomal peptides from their prey organisms, cyanobacteria. However, roles of these peptides are unknown.

Key Words: nonribosomal peptide; antifungal activity; antimicrobial peptide; innate immunity; marine invertebrate

Introduction

An increasing number of disease outbreaks have been recorded in marine invertebrates from viral, bacterial and fungal infections, which are largely influenced by environmental conditions, such as pollution and climate warming. Perhaps the most well-known example is “coral breaching” which is partly caused by bacterial and fungal infections (Mydlarz *et al*., 2006). Immunological mechanisms in marine invertebrates are different from vertebrate immune system; they rely solely on innate immune systems that include both humoral and cellular responses, the former of which is performed by antimicrobial peptides contained in the blood and plasma. Cellular immunity also involves antimicrobial peptides secreted into the hemolymph by hemocytes (Tincu and Taylor, 2004).

Naturally-occurring peptides are either synthesized by ribosomal machinery from 20 proteinogenic amino acids or by large enzymes and enzyme complexes called nonribosomal peptide synthases (McIntosh *et al*., 2009). Antimicrobial peptides (AMPs) involved in marine invertebrate immunity are ribosomal peptides (gene-encoded peptides) and classified into: a) linear α-helical peptides, b) peptides with intramolecular disulfide bridges, c) β-sheet and small proteins, and d) peptides with one or two predominant amino acids (Bulet *et al*., 2004; Tincu and Taylor, 2004; Hancock *et al*., 2006; Jenssen *et al*., 2006). The majority of AMPs are amphiphilic and cationic, containing both hydrophilic and hydrophobic surfaces. They show antimicrobial activity by forming pores in microbial membranes or disrupting membranes (Yeaman and Yount, 2003; Brogden, 2005; Jenssen *et al*., 2006). A total of 1,518 AMPs are listed in the second version of Antimicrobial Peptide Database, among which 442 peptides are antifungal (Wang *et al*., 2009).

Nonribosomal peptides found in sponges, molluscs and tunicates are composed of unusual amino acids including D-amino acids and contain organic acids in addition to amino acids as cases of depsipeptides. They exhibit a wide range of biological activities, such as antimicrobial, cytotoxic, and enzyme inhibitory. A majority of them are considered to be derived either from microbial symbionts or cyanobacteria on which opisthobranch molluscs prey as mentioned later.

Surprisingly, a very limited number of peptides found in marine invertebrates have been reported to be antifungal; obviously much more peptides would be antifungal if tested. This review describes only the peptides reported to be antifungal.

Porifera

Sponges are the oldest metazoans and share a common ancestor with other metazoans. They

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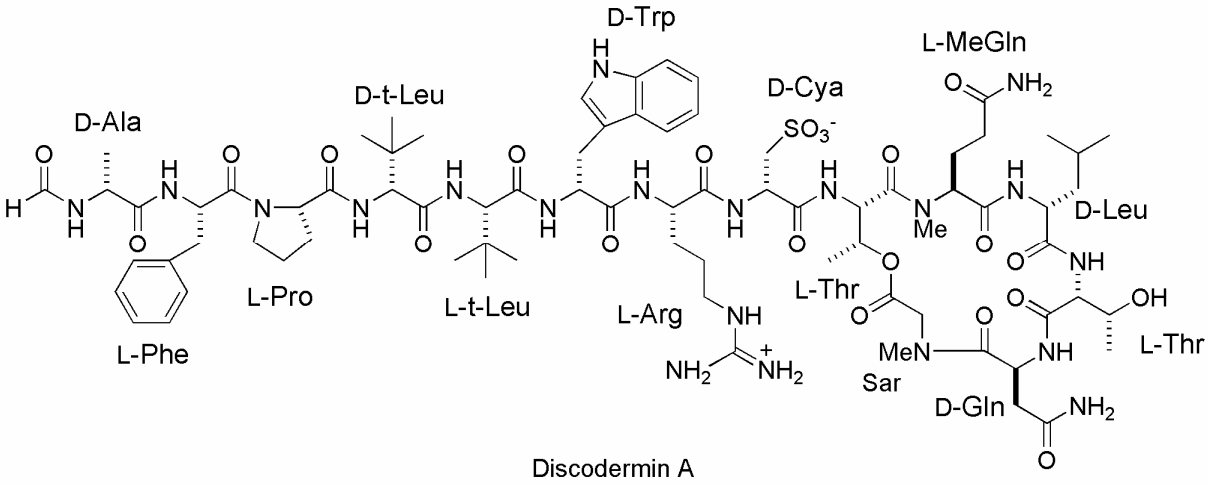


Fig. 1 Structure of discodermin A

possess key molecules which are found in mammalian innate and adaptive immune systems (Müller *et al*., 1999). Even a marine sponge was reported to produce a perforin-like antibacterial protein (Thankur *et al.,* 2003). No antifungal peptides classified as AMPs have been identified in sponges, although antifungal cyclic peptides of ribosomal origin were reported from marine sponges as mentioned later. Instead, sponges contain a large variety of bioactive compounds, including cytotoxc and antimicrobial (Blunt *et al*., 2009), many of which are considered as microbial symbiont origin (Piel, 2004). A number of bioactive unusual nonribosomal peptides were also isolated from sponges, some of which are antifungal (Fusetani and Matsunaga, 1993; Matsunaga and Fusetani, 2003; Blunt *et al*., 2009).

Discodermin A (Fig. 1), the first bioactive sponge peptide isolated from *Discodermia kiiensis*, contains a large number of D-amino acids and such unusual amino acids as *tert*-leucine (*t*-Leu), cysteinoic acid (Cya), and sarcosine (Sar) (Fusetani, 1988; Li *et al*., 1998). It possesses a wide range of bioactivities, including inhibition of various enzymes, antifungal (growth inhibition of *Candida albicans* at 20 μg/disk). A number of its congeners were later isolated from various sponges (Li *et al*., 1998). Jaspamide (= jasplakinolide) (Fig. 2), a cyclic depsipeptide comprising of such unsual amino acids as *N*-methyl-2-bromo-D-tryptophan (Me-BrTrp) and L-β-tyrosine (L-βTyr) isolated from Fijian sponges of the genus *Jaspis*, was fungicidal against *C. albicans* with both an MIC and a minimal lethal concentration of 25 μg/ml (Scott *et al.*, 1988). It is known to promote actin polymerization (Bubb *et al*., 1994). Similar peptides have been reported from various sponges (Li *et al*., 1998; Molinski, 2004).

Marine sponges of the genus *Theonella* are prolific in bioactive metabolites possessing unusual structures (Bewley and Faulkner, 1998). Theonellamide F (Fig. 3) is a bicyclic peptide isolated from a Japanese *Theonella* sp. containing several unusual amino acids, e.g. histidinoalanine, 3-methyl-*p*-bromophenylalanine, (2*S*,4*R*)-2-amino-4-hydroxyadipic acid (L-Ahad), and (3*S*,4*S*,5*E*,7*E*)-3-amino-4-hydroxy-6-methyl-8-(*p*-bromophenyl)-5,7-octadienoic acid (Aboa). It inhibited the growth of *C. albicans* with an MIC 6.3 μg/ml (Fusetani and Matsunaga, 1993; Li *et al*., 1998). A glycosylated peptide of the theonellamide family named theonegramide (Fig. 4) with antifungal activity against *C. albicans* at 10 μg/disk was reported from *T. swinhoei* collected from Palau (Bewley and Faulkner, 1996).

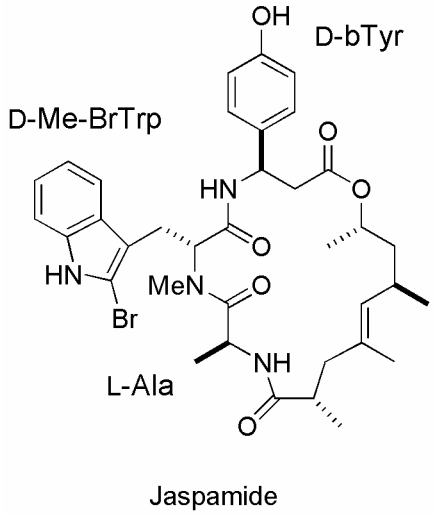


Fig. 2 Structure of jaspamide

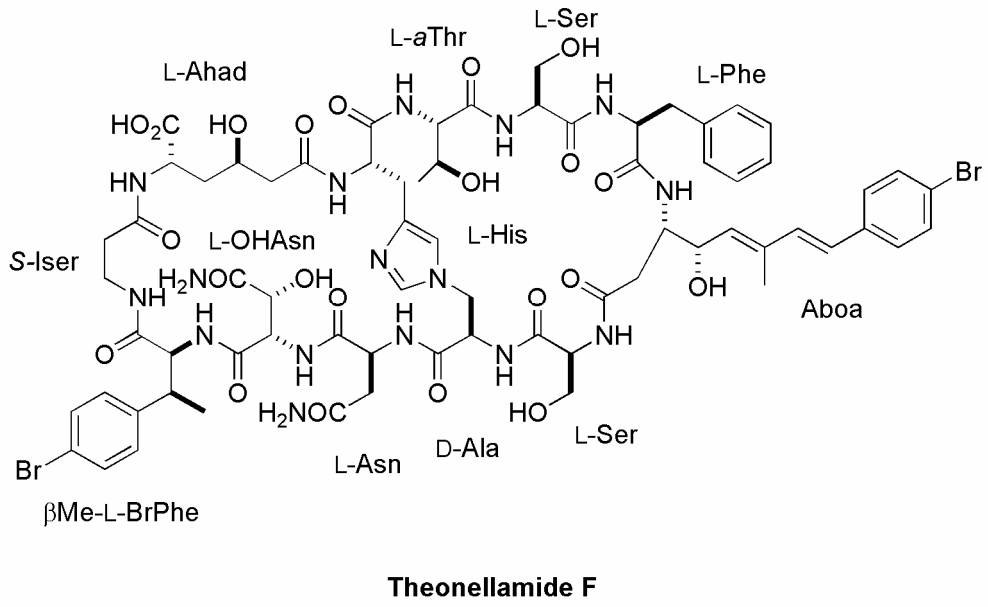


Fig. 3 Structure of theonellamide F

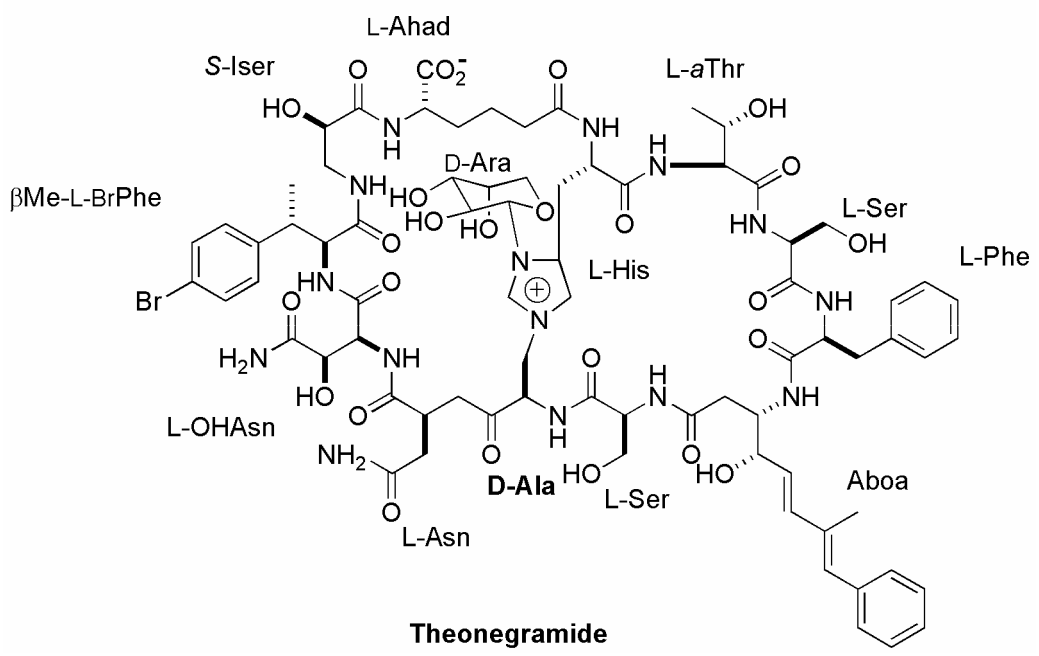


Fig. 4 Structure of theonegramide

Cyclolithistide A (Fig. 5) is another class of cyclic peptide isolated from a Papua New Guinean collection of *T. swinhoei* and contains unusual amino acids, including 4-chloroisoleucine (Cl-Ile), 2-amino-pentanoic acid (D-Ape), and 4-amino-3,5-dihydroxyhexanoic acid (Adha). It showed antifungal activity at 20 μg/disk (Clark *et al*., 1998). Microsclerodermin A (Fig. 6), a highly unusual cyclic hexapeptide isolated from a Palauan *Thenonella* sp., contains new amino acids, e.g., (2*S*,3*R*,4*S*,5*S*,6*S*,11*E*)-3-amino-6-methyl-12-(*p*-methoxyphenyl)- 2,4,5-trihydroxydodec-11-enoic acid (AMMTD), (3*R*)-4-amino-3-hydroxylbutyric acid (GABOB), and 3-hydroxy-4-amino-5-vinylpyrrolidone. It inhibited the growth of *C. albicans* at 1.5 μg/disk (Bewley *et al*., 1994). Several congeners have been reported from lithistid sponges (Molinski, 2004). These peptides were suggested to be produced by a new δ-proteobacterium found in *Theonella* sp. (Bewley and Faulkner, 1998).

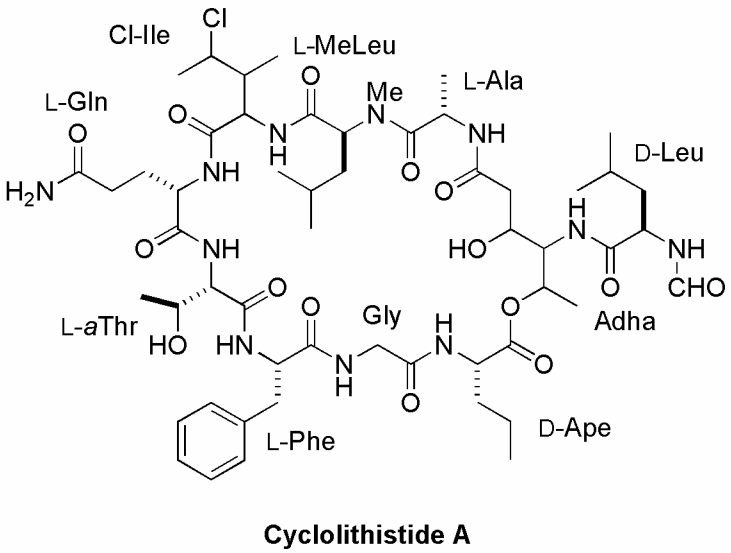


Fig. 5 Structure of cyclolithistide A

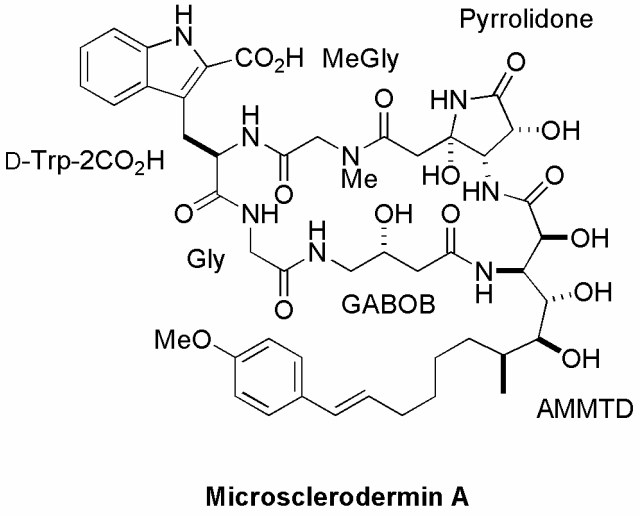


Fig. 6 Structure of microsclerodermin A

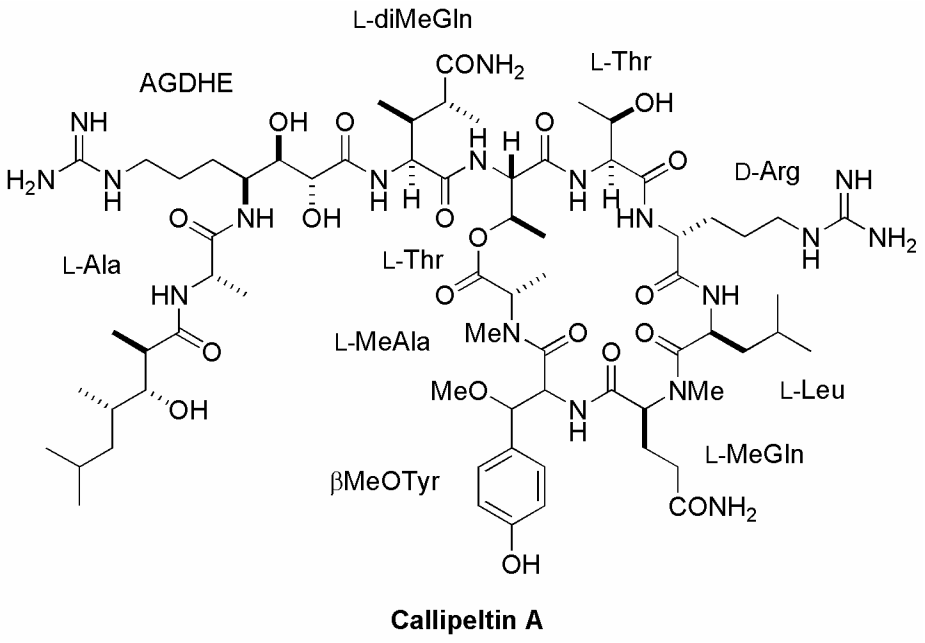
Callipeltin A (Fig. 7) was originally isolated as an anti-HIV cyclodepsipeptide comprising of many unusual amino acids including (2*R*,3*R*,4*S*)-4-amino-7-guanidino-2,3-dihydroxyhep tanoic acid (AGDHE) from a New Caledonian lithistid sponge *Callipelta* sp. It exhibited a 30 mm inhibitory zone at 100 μg/disk (Zampella *et al*., 1996).

More sponge nonribosomal peptides were reported to be antifungal (Li *et al*., 1998; Molinski 2004; Blunt *et al.*, 2009), but modes of antifungal activity of these nonribosomal peptides mentioned above have not been fully elucidated.

Antifungal ribosomal peptides have not been isolated from marine sponges, except for hymenamides, Pro-rich cyclic heptapeptides isolated from *Hymeniacidon* sp. collected in Okinawa (Kobayashi *et al*., 1993). Hymenamide A (Fig. 8) was antifungal against *C. albicans* with an MIC 33 μg/ml as well as cytotoxic.

Cnidaria

Although antifungal activity has been detected in some gorgonian species, no antifungal peptides have been isolated. However, a cytotoxic pentapeptide named gymnangiamide (Fig. 9) similar to dolastatin 10 (Fig.10), a highly antifungal



Callipeltin A

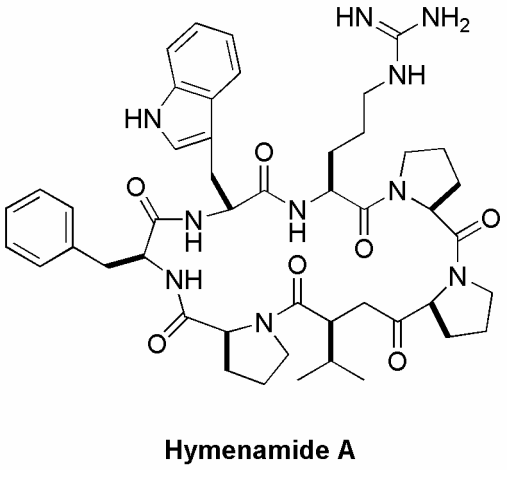
Fig. 7 Structure of callipeltin A

peptide isolated from the sea hare *Dolabella auricularia* mentioned later was reported from the marine hydroid *Gymnangium regae*, though no antifungal activity was described (Milanowski *et al*., 2004). This peptide contains *O*-desmethyldolaproline (Ddap), *N*-desmethyldolaisoleucine (Ddil), L-*threo*-phenylserine (L-Pser), and L-guanidinoserine (Gser). Perhaps this peptide was originated from symbiotic or sequestered cyanobacteria as the case of dolastatins.

A 40-residue AMP named aurelin isolated from the jellyfish *Aurelia aurita* exhibited structural features of defensins and channel-blocking toxins of sea anemone origin, but no antifungal activity was reported (Ovchinnikova *et al*., 2006).

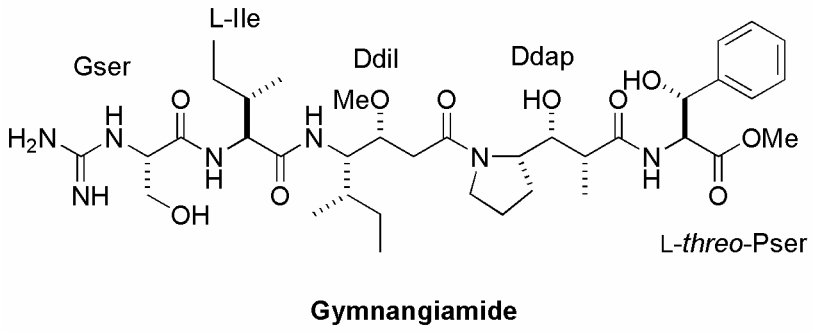
Mollusca

In molluscs, hemocytes are predominantly responsible for innate immune defense and release AMPs (Bulet *et al*., 2004; Tincu and Taylor, 2004). AMPs have been reported from bivalves and opisthobrachs as shown in Table 1; defensins were identified in hemocytes of the mussel *Mytilus galloprovincialis* (MGD-1 and -2) (Mitta *et al.,* 1999b) and in the mantle tissue of the oyster *Crassostrea gigas* (*Cg*-Def) (Gueguen *et al*., 2006; Gonzalez *et al*., 2007), respectively. MGDs and *Cg*-Def inhibited growth of the fungus *Fusarium oxysporum* with an MCI value of 4.5-9 μM, respectively. The solution structure of MGP-1 obtained using ^{1}H NMR spectroscopy consists of a helical part and two antiparallel β-strands. The Cys-stabilized α-β motif is stabilized by 4 disulfide bridges (Yang *et al*., 2000). Similar antibacterial defensins A and B were isolated from hemocytes of *M. edulis*, but their antifungal activity has not been reported (Charlet *et al*., 1996). Defensin A and MGP-1 share some common properties in distribution of hydrophobic and hydrophilic side chains (Yang *et al*, 2000). Molluscan defensins are composed of a β-sheet and 3 disulfide bonds, which is similar to arthropod defensins. Interestingly, an AMP coined *Cg*-Prp (37-residue peptide) remarkably enhanced the antifungal activity of *Cg*-Def, although it is not antifungal (Gueguen *et al*., 2009).



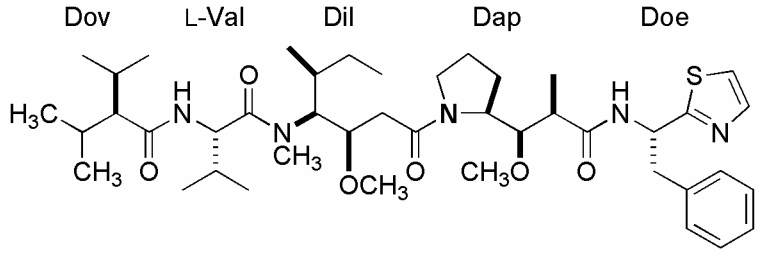
Hymenamide A

Fig. 8 Structure of hymenamide A



Gymnangiamide

Fig. 9 Structure of gymnangiamide



Dolastatin 10

Fig. 10 Structure of dolastatin 10

Ap, a polyproline-type AMP (47 redidues) isolated from the Chilean scallop *Argopecten purpuratus*, showed antifungal activity against *F. oxisporum* and *Saprolegnia parasitica* with IC_{50} values of 2.1 and 0.85 μM, respectively (Arenas *et al*., 2009). It is also highly antibacterial; perhaps Ap enters in lipid bilayer to exhibit antifungal activity. A big defensin named AiBD of the scallop *A. irradians,* similar to arthropod big defensins, has been cloned and expressed; the recombinant AiBD (120 residues) was reportedly not only highly antibacterial, but also strongly fungicidal, though detailed fungicidal activity was not available (Zhao *et al*., 2007).

A novel Arg- and Cys-rich AMP named myticin B (40 amino acids) (Table 1) isolated from hemocytes of *M. galloprovincialis* showed antifungal activity against *F. oxysporum* with MIC 5-10 μM (Mitta *et al*., 1999a). Interstingly, myticin A possessing a similar amino acid sequence to that of myticin B was not antifungal at 20 μM. Mytilin B, a 34-residue AMP containing 4 intramolecular disulfide bonds, purified from hemocytes of the same species exhibited antifungal activity against *F. oxysporum* with MIC 0.7-1.4 μM (Mitta *et al.*, 2000). Mytimycin, a novel antifungal Cys-rich polypeptide of 6.2 kDa that hindered the growth of fungi, was isolated and partially characterized from *M. edulis* (Charlet *et al*., 1996).

Opisthobranch molluscs often sequester bioactive peptides from their prey organisms, especially cyanobacteria and seaweeds (Cimino and Ghiselin, 2001). Dolastatins are highly cytotoxic linear peptides of NRPS metabolites sequested from cyanobacteria of the genus *Lyngbya* by the sea hare *Dolabella auricularia* (Garson, 2001), among which dolastatin 10, a highly unusual pentapeptide comprising of new amino acids, (2*R*,3*R*,4*S*)-dolaproline (Dap), (3*R*,4*S*,5*S*)-dolaisoleucine (Dil), L-dolapherine (Doe), and L-dolavaline (Dov), was shown to be highly antifungal against *Cryptococcus neoformans* with MIC 0.37 μg/ml, but not against other fungi (Pettit *et al*., 1998). Dolastatin 10 is a potent inhibitor of tubulin polymerization. Similarly, kahalalide F (Fig. 11), an unusual cyclic depsipeptide containing (*Z*)-2-amino-2-dehydrobutyric acid (*Z*-Dhb) and L-ornithine (L-Orn) accumulated by the Hawaiian sacoglossan *Elysia rufescens* from the green alga *Bryopsis pennata* is highly cytotoxic as well as strongly antifungal against *C. albicans, C. neoformans* and *Aspergillus fumigatus* with MIC 5-10 μM (Shilabin *et al*., 2007). Kahalalide F is currently under phase II clinical trials as anticancer drugs.

Dolabellin B2, a 33-residue AMP (Table 1) isolated from the body wall of the sea hare *D. auricularia*, is fungicidal against *S. cerevisiae* (IC_{50}~25 μg/ml), while it is fungistatic agaist *C. albicans* (Iijima *et al*., 2003).

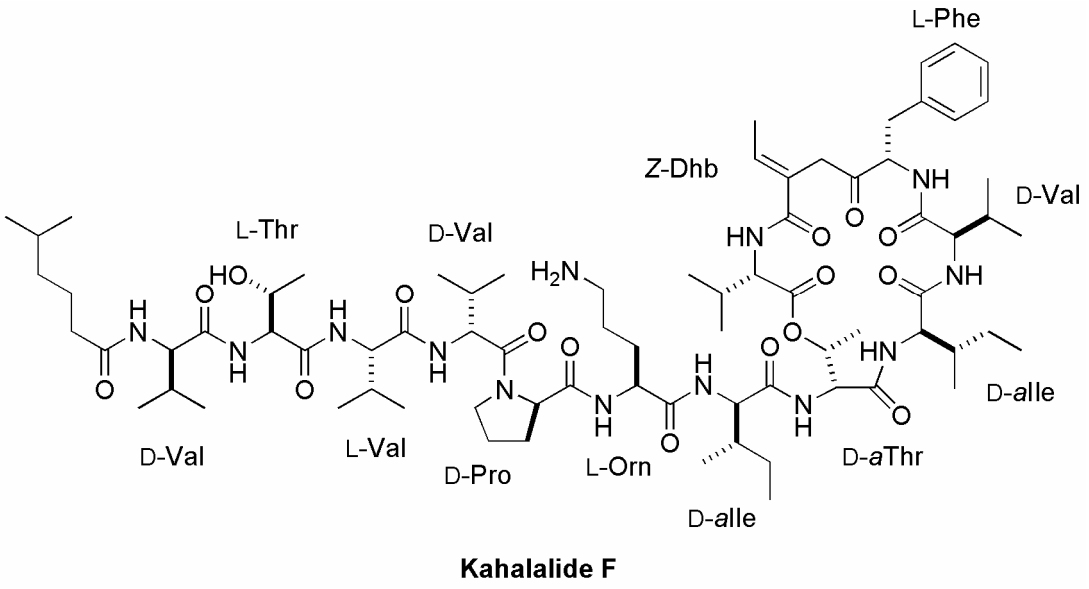


Fig. 11 Structure of kahalalide F

Annelida and Uchiura

Marine worms dwell in sediments, indicating the requirement of antimicrobial strategy for their survival. AMPs have been isolated from polychaete and echiuroid worms. Arenicin-1 and -2 are 21-residue AMPs isolated from coelomoycytes of the polychaete *Arenicola marina* (Table 2) and show no structural similarity to any AMPs reported (Ovchinnikova *et al*., 2004). Arenicins are cationic peptides having two antiparallel β-strands and one disulfide bond between Cys3 and Cys20 (Ovchinnikova *et al*., 2007). Arenicin-1 showed antifungal activity against *C. albicans, C. parasilosis, Malasseria furfur, Trichosporon beigelli* and *Trichophyton rubrum* with MICs of 4.5-9 μM comparable to that of mellitin by disrupting fungal phospholipid membranes (Park and Lee, 2009). It is also antibacterial and hemolytic, which may be interpreted by its permeabilization of model membranes composed of phospholipids or lipopolysaccharides (Andrä *et al*., 2009). Perinerin, a 51 residue AMP isolated from homogenates of the polychaete *Perinereis aibuhitensis*, is a highly cationic, hydrophobic peptide that is not related to any known AMPs (Pan *et al*., 2004). It was antifungal against *Paecilomyces heliothis* with MICs 12.5-25 μg/ml, in addition to bactericidal activity against Gram-negative and –positive bacteria.

Some neuropeptides show potent antimicrobial activity, which is suggested to be involved in innate immunity (Brogden *et al*., 2005). In fact, urechistachynins I and II, five residue neuropeptides found in the echiuroid *Urechis unicinctus* exhibit antibacterial and antifungal activities (MICs 42 and 25 μM against *C. albicans*, respectively) (Sung *et al*., 2008). It was suggested that the plasma membrane of fungi is structurally disrupted by urechistachynins.

Arthropoda

AMPs play a major role in innate immunity of crustaceans and horseshoe crabs. Penaeidins, a family of AMPs of 47-63 residues, were initially characterized from hemocytes of the shrimp *Litopenaeus vannamei* (Destoumieux *et al*., 1997) and later found to be expressed in all penaeid shrimps (Bachère *et al*., 2000; Destoumieux *et al*., 2000; Cuthbertson *et al*., 2004: Gueguen *et al.,* 2006). They are composed of a Pro-rich N-terminal domain, followed by a C-terminal domain stabilized by 3 intramolecular disulfide bonds, which is unique among AMPs (Table 3). Penaeidins exhibit not only antibacterial activity against Gram-positive bacteria, but also antifungal activity against various filamentous fungi, but not against yeasts (e.g. MIC 5-10 μM against *F. oxysporum* that is pathogenic to shrimps) (Destoumieux *et al*., 1999; Bachère *et al*., 2000). The solution structure of penaeidin 3 (*Litvan* PEN3-1) demonstrated that the surface of the Cys-rich domain exhibits an amphipathic character required for antimicrobial properties (Yang *et al*., 2003). A similar result was reported for penaeidin 4 (*Litset* PEN4-1) (Cuthbertson *et al*., 2005). The penaeidin database, PenBase (www.penbase.immunaqua.com), has been constructed; detailed information of 34 penaeidins is contained at moment (Gueguen *et al*., 2006). They are classified into three subgroups based on amino acid sequences: Penaeidin 2 (PEN2), Penaeidin 3 (PEN3) and Penaeidin 4 (PEN4) (Culthbertson *et al*., 2004). Actually, PenBase

Table 1 Amino acid sequences of molluscan antifungal peptides

MGD-1:	**GFGCPNNYQCHRHCKSIPGRCGGYCGGWHRLRCTCYRCG**
MGD-2:	**GFGCPNNYACHQHCKSIRGRCGGYCAGWFRLRCTCYRCG**
Defensin A:	**GFGCPNDYPCKSIPGRXGGYCGGXHRLRCTCYR**
Defensin B:	**GFGCPNDYPCKSIPGRYGGYCGGXHRLRCTC**
Cg-Def:	**GFGCPGNQLKCNNHCKSISCRAGYCDAATLWLRCTCTDCNGKK**
Mytilin A:	**GCASRCKAKCAGRRCKGWASASFRGRCYCKCFRC**
Mytilin B:	**SCASRCKGHCRARRCGYYVSVLYRGRCYCKCLRC**
MyticinB:	**HPHYCTSYYCSKFCGTAGCRRYGCRNLHRGKLCFCLHCSRV**
Ap:	**TYMPVEEGEYIVNISYADQPKKNSPFTAKKQPGPKVDLSGVKAYGPG**
Dolabellanin B2:	**SHQDCYEALHKCMASHSKPFSCSMKFHMCLQQQ**

Each disulfide pair is highlighted in red, pink, green and light blue for MGDs. Cys residues are highlighted in red for the rest of peptides, while Arg in violet for mytilins and myticin and Pro in blue for Ap. X in defensins is a not identified residue.

contains more than 200 entries of penaeidins and the number is growing rapidly due to the active genomic and proteomic research.

Crustins are cationic, Cys-rich antibacterial polypeptides of ca. 7-14 kD occurring in circulating hemocytes of crustaceans that contain a whey acidic protein (WAP) domain in the C-terminus (Smith *et al*., 2008). More than 50 crustin sequences have been reported from a variety of decapods and classified into 3 subgroups, Type I to III. No crustins had been reported to be antifungal until recently when Cru*Pc* of 98 residues belonging to Type II and Cru*Ha*1/2 of 90 residues (Type I) were characterized from the red king crab *Paralithodes camtschaticus* and the spider crab *Hyas araneus*, respectively (Sperstad *et al*., 2009). Cru*Ha*1 inhibited the growth of *S. cerevisiae* with MIC 12.5-25 μM. Hemocytes of *H. araneus* also contain hyastatin, a Gly-rich multi-domain polypeptide of 11.7 kD resembling Type II curstins (Sperstad *et al*., 2009). It exhibited MICs of 12.5 and 6.3-12.5 μM against *S. cerevisiae* and *C. albicans*, respectively.

Horseshoe crabs rely completely on innate immune system which is the first line of inducible host defense against bacterial, fungal and viral pathogens (Iwanaga and Lee, 2005). Quite recently, an excellent review on the molecular basis for innate immune system of horseshoe crabs has been published in this journal (Kawabata *et al*., 2009). The most well-studied species is *Tachypleaus tridentatus* (Japanese horseshoe crab); its hemolymph contains a variety of soluble defense molecules, e.g. hemocyanin, lectins/C-reactive proteins and α_2-macroglobulin, in addition to granular hemocytes that comprise 99 % of total hemocytes. Granular hemocytes consist of large and small granules which are sensitive to lipopolysaccharides (LPS) of Gram-negative bacteria and secret defense substances by stimulation of LPS. Small granules contain various AMPs, including tachyplesins, tachycitin, tachystatins and big defensin (Table 4), while large granules release enzymes, lectins and proteins involved in hemolymph coagulation (Iwanaga *et al.,* 1998; 2005; Kawabata *et al*., 2009).

All AMPs isolated from horseshoe crabs showed binding activity to chitin, a primary target of the innate immune system. Tachyplesins I and II isolated from *T. tridentatus* are composed of 17 amino acids and contain 2 intramolecular disulfide bonds as shown in Table 4 (Nakamura *et al*., 1988;

Table 2 Amino acid sequences of antifungal peptides retrieved in worms

Arenicin 1:	**RWCVYAYVRVRGVLVRYRRCW**
Arenicin 2:	**RWCVYAYVRIRGVLVRYRRCW**
Perinerin:	**FNKLKQGSSKRTCAKCFRKIMPSVHELDERRRGANRWAAGFRKCVSSICRY**
Urechistachykinin I:	**LRQSQFVGSR-NH_2**
Urechistachykinin II:	**AAGMGFFGAR-NH_2**

Table 3 Amino acid sequences of penaeidins from the shrimp *Lytopenaeus vannamei*

Litvan PEN2-1 (PEN2):

EAYRGGYTGPIPRPPPIGRPPFRPVCNACYRLSVSDARNCCIKFGSCCHLVKG

Litvan PEN3-1 (PEN3):

QVYKGGYTRPIPRPPPFVRPLPGGPIGPYNGCPVSCRGISFSQRSCCSRLGRCCHVGKGYSG

Litvan PEN4-1 (PEN4):

HSSGYTRPLPKPSRPIFIRPIGCDVCYGIPSSTARLCCFRYGDCCHRG

Each disulfide pair is highlighted in red, green and light blue; Pro residues are in pink.

Miyata *et al.,* 1989). Similar 18-residue AMPs named polyphemusins I and II were isolated from the American horseshoe crab *Limulus polyphemus* (Miyata *et al.,* 1989). These four AMPs showed similar antimicrobial activity against Gram-negative and –positive bacteria as well as fungi (IC_{50} 0.2 μg/ml against *C. albicans*)(Osaki *et al*., 1999). A big defensin consisting of 79 amino acids was isolated from *T. tridentatus* hemocytes (Saito *et al*., 1995). It is similar to rat defensins and showed potent antimicrobial activity against bacteria, but weak activity against fungi (IC_{50} 20 μg/ml against *C. albicans*)(Osaki *et al*., 1999).

Tachystatin A (44 residues), B (42) and C (41) isolated from *T. tridentatus* exhibited potent antifungal activity against *C. albicans* and *P. pastoris* with IC_{50} values of 0.9-3.0 and 0.1-0.3 μg/ml, respectively (Osaki *et al*., 1999). Tachystatins A and B showed sequence similarity to ω-agatoxin-IVA of a funnel web venom, but tachystatin C, which is no significant sequence similarity to the former two, is similar to insecticidal spider neurotoxins. The solution structure of tachystatin A analyzed by NMR spectroscopy showed an amphiphilic folding found in membrane-interactive peptides (Fujitani *et al*., 2002). Tachystatins contain three disulfide bridges as shown in Table 4. *T. tridentatus* hemocytes contain another AMP named tachycitin which consists of 73 amino acids and five intramolecular disulfides linkages, but showed no significant sequence similarity to known AMPs (Kawabata *et al*., 1996). It showed weak antimicrobial activity against antibacterial and fungi (IC_{50} 52 μg/ml against *C. albicans*)..

It should be noted that the apparent antimicrobial activity of horseshoe crab APMs are significantly reduced under isotonic conditions (0.5 M NaCl for horseshoe crabs) to those under hypotonic conditions (Kawabata *et al*., 2009).

Echinodermata and Urochordata

Starfishes and sea cucumbers are known to contain antifungal saponins named asterosaponins and holothurins, respectively (Fusetani and Kem, 2009). Only a small number of works have been done on AMPs in echinoderms, although antibacterial activity of their coelomocytes were reported; cationic, defensin-like AMPs have been

Table 4 Amino acid sequences of antifungal peptides from horseshoe crabs

Tachyplesin I:	**KWCFRVCYRGICYRRCR-NH_2**
Tachyplesin II	**RWCFRVCYRGICYRKCR-NH_2**
Polyphemusin I:	**RRWCFRVCYRGFCYRKCR-NH_2**
Polyphemusin II:	**RRWCFRVCYKGFCYRKCR-NH2**
Tachystatin A:	**YSRCQLQGFNCVVRSYGLPTIPCCRGLTCRSYFPGSTYGRCQRY**
TachystatinB1:	**YVSCLFRGARCRVYSGRSCCFGYYCRRDFPGSIFGTCSRRNF**
Tachystatin C:	**DYDWSLRGPPKCATYGQKCRTWSPRNCCWNLRCKAFRCRPR**
Tachycitin:	**YLAFRCGRYSPCLDDGPNVNLYSCCSFYNCHKCLARLENCPKGLHYNAYLKVCDWPSKAGCT**

Each sulfide bond is highlighted in red, light blue and green

Table 5 Amino acid sequences of antifungal peptides from tunicates

Clavanin A:	**VFQFLGKIIHHVGNFVHGFSHVF-NH_2**
Clavanin B:	**VFQFLGRIIHHVGNFVHGFSHVF-NH_2**
Clavanin C:	**VFHLLGKIIHHVGNFVYGFSHVF- NH_2**
Clavanin D:	**AFKLLGRIIHHVGNFVHGFSHVF-NH_2**
Clavaspirin:	**FLRFIGSVIHGIGHLVHHIGVAL-NH_2**

recently reported from a sea urchin, but no information about its antifungal activity is available (Li *et al.,* 2008).

Tunicates (ascidians) often contain cytotoxic cyclic peptides, most of which are derived by nonribosaomal peptide synthases, but their antifungal activity have not been examined (Blunt *et al*., 2009). An unusual diketopiperazine named etzionin (Fig. 12) reported from a unidentified Red Sea tunicate inhibited the growth of *C. albicans* and *Aspergillus nidulans* with MIC of 3-12.5 μg/ml (Hirsch *et al*., 1989).

Tunicates employ a prototype of vertebrate innate immune system for host defense; in fact, *Ciona intertinalis* hemocytes are shown to express a number of host defense-related genes involved in innate immune systems (Shida *et al*., 2003). Halocyamines was the first ascidian-derived AMPs isolated from *Halocynthia roretzi* which are post-translationally modified ribosomal peptides. Halocyamine A (Fig. 12) showed weak antifungal activity against *Cryptococcus neoformans* with an MIC of 100 μg/ml (Azumi *et al.,* 1990). Styelin D also contains post-translationally modified residues but its antifungal activity is unknown (Lehrer *et al*., 2003).

Clavanins A-D, α-helical AMPs of 23 residues identified in the solitary tunicate *Styela clava* (Table 5), showed antifungal activity against *C. albicans* with MIC 5-20 μg/ml, in addition to antibacterial activity (Lee *et al*., 1997). A His-rich, 23-residue APM named clavaspirin was identified in pharyngeal tissues of the same species (Lee *et al*., 2001). A synthetic peptide of clavaspirin was antibacterial against Gram-positive and –negative bacteria as well as antifungal against *C. albicans*

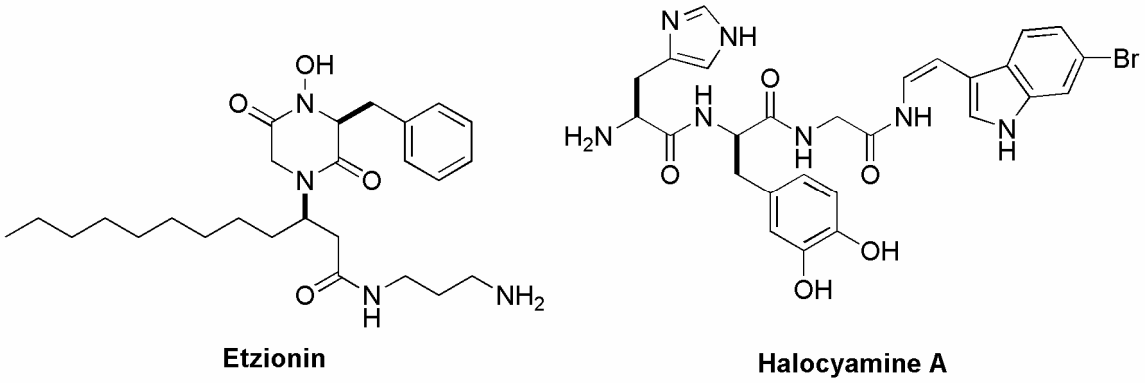


Fig. 12 Structure of etzionin and halocyamine A

with MIC ~5-10 μg/ml. It is highly hemolytic and cytotoxic; α-helical nature of the peptide may responsible for these activities. Recently, search for AMPs using expressed sequence tag (EST) database has been attempted for the tunicate *Ciona intestinalis* on which the genome project was completed, which resulted in identification of gene families coding novel AMPs of α-helical types (Fedders and Leippe, 2008; Fedders *et al*., 2008). A synthetic peptide of C-terminal region consisting 24 amino acids of a putative AMP coded Ci-MAM-A showed potent antifungal activity against *C. albicans* with MIC 3.1 μM, in addition to potent antibacterial activity (Fedders *et al*., 2008). Importantly, its antibacterial activity was retained at high NaCl concentrations (up to 450 mM). Antimicrobial activity and mode of action of the intact peptide are interesting subjects.

Conclusion

A large array of invertebrates live in marine environments harboring high concentrations of pathogenic microorganisms. AMPs are considered to be a major component of the innate immune defense on which marine invertebrates solely rely (Tincu and Taylor, 2004). Therefore, it is surprising that AMPs have been explored for only limited phyla, mainly Mollusca, Arthropoda and Urochordata. Innate immune systems in marine invertebrates which are increasingly important, since emerging numbers of diseases reported for marine invertebrates partly due to seawater warming and water pollution. For better understanding of marine ecosystem, more knowledge should be accumulated for AMPs in a wide range of marine phyla. It is expected that more and more AMPs will be identified in a wide variety of marine invertebrates by genomic and proteomic approaches. These data will contribute to medicinal uses of AMPs (Zasloff, 2002; Jenssen *et al*., 2006). Obviously, more research on modes of action of marine AMPs is required.

Nonribosomal peptides of various bioactivities, most of which are cytotoxic, but those described as antifungal are few, have been discovered from sponges, nudibranchs and tunicates. Although these peptides are considered to be accumulated from symbiotic microbes or prey organisms, their roles in host organisms remain mostly unknown. Ecological roles of nonribosomal peptides in marine invertebrates are interesting subjects.

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