

Halocytin and papillosin, two new antimicrobial peptides isolated from hemocytes of the solitary tunicate, *Halocynthia papillosa*

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We report here the screening of five marine invertebrate species from two taxa (tunicates and echinoderms) for the presence of cationic antimicrobial peptides (AMP) in defence cells (hemocytes). Antimicrobial activities were detected only in the two tunicates *Microcosmus sabatieri* and *Halocynthia papillosa*. In addition, we report the isolation and characterization of two novel peptides from *H. papillosa* hemocytes. These molecules display antibacterial activity against Gram-positive and Gram-negative bacteria. Complete peptide characterization was obtained by a combination of Edman degradation and mass spectrometry. The mature molecules, named halocytin and papillosin, comprise 26 and 34 amino acid residues, respectively. Their primary structure display no significant similarities with previously described AMP. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: tunicate; innate immunity; antimicrobial peptide

Introduction

Like other invertebrates, marine invertebrates lack acquired immunity. Nevertheless, they have developed an effective innate immune system to defend themselves against pathogenic microorganisms. Antimicrobial peptides (AMP) play a key role in this efficient defence system (see [1] for review). They were characterized from hemocytes in several marine invertebrate taxa including arthropoda [2], urochordata [3–7], and mollusca [2,8]. They are synthesized even in absence of challenge, mature precursors and the peptides are stored in granules. In mollusca and arthropoda, the peptides can exert their microbicidal activity on engulfed bacteria or/and can be released into the plasma upon stimulation by microbial substances [2,8,9]. This differs from insects, especially *Drosophila melanogaster*, that synthesize AMP rapidly after septic injury, principally in the fat body, which are released immediately into the hemolymph where they participate in a systemic response [10].

The interest in AMP reflects both their relevance to intrinsic host defence, and their potential development as therapeutics [11–13]. Marine invertebrate are expected to be an important source for antimicrobial molecules. The field of marine invertebrate AMP was not extensively studied yet, and several taxa like echinoderms were not explored. To enrich data in this field, we investigated in the present report the presence of cationic AMP in hemocytes from two tunicate and three echinoderm species. We report here the purification and characterization of two peptides isolated from the tunicate *Halocynthia papillosa*. We report also their activity spectrum and bactericidal effect.

Materials and Methods

Species Collection

Studies were performed on two tunicate species, *Microcosmus sabatieri* and *H. papillosa*, and three echinoderms, *Holothuria tubulosa*, *Cucumaria* sp., and *Paracentrotus lividus*. All samples were collected in Mediterranean Sea along the Catalan coast.

Preparation of Hemocyte Acid Extracts

Specimens were briefly bathed in ethanol, dried and transected peribasally. The hemolymph was passed through a 100- μ m apertures filter into 50-ml tubes containing 150 mg of EDTA powder. The hemolymph (approximately 5 ml/animal) was immediately centrifuged at 800 g for 15 min at 4 °C. The cell pellet was then resuspended in 0.34 M sucrose and then recentrifuged as above.

The cell pellet was then resuspended in 10 volumes of 5% acetic acid and homogenized (30 strokes) using Dounce apparatus

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(100 μm). Thereafter, 20 ml of 5% acetic acid was added and the mixture was stirred overnight at 4 °C. Subsequently, it was centrifuged at 20 000 g for 30 min at 4 °C and supernatant was processed immediately.

Solid Phase Extraction

After washing with acidified water, two successive elutions were performed with 5 and 40% acetonitrile in acidified water. Hemocyte acid extracts were loaded onto Sep-Pak C₁₈ Vac cartridges (Waters Associates) equilibrated in acidified water [0.05% TFA in ultrapure water (UPW): UPW/TFA]. After washing with UPW/TFA, three successive elutions were performed with 10, 60, and 80% acetonitrile in UPW/TFA. The eluted fractions were lyophilized and dissolved in 0.6 ml UPW and tested for antimicrobial activity as described below. Only fractions eluted with 60% acetonitrile were active and submitted to reverse-phase HPLC separation.

HPLC Purification

All HPLC purification steps were carried out on a Waters Breeze system (Waters 1525, Binary HPLC Pump) equipped with a UV detector (Waters 2487, Dual λ Absorbance Detector). Column effluent was monitored by UV absorption at 224 and 280 nm.

Step (1): Aliquots of 150 μl of the 60% Sep-Pak fractions were subjected to reverse-phase HPLC on a Symmetry C₁₈ column (250 mm \times 4.6 mm, Waters). Elution was performed with a linear gradient of 5–65% acetonitrile in acidified water over 90 min at a flow rate of 1 ml/min. Fractions corresponding to absorbance peaks were collected in polypropylene tubes, freeze dried, reconstituted in 0.2 ml UPW, and tested for antimicrobial activity as described below.

Step (2): Active fractions from the first step were further loaded onto a Symmetry C₈ column (250 mm \times 4.6 mm, Waters). Elution was performed with a linear gradient ranging from –10 to +10% acetonitrile of the previous elution acetonitrile percentage, in acidified water over 40 min at a flow rate of 1 ml/min. Fractions were collected and treated as above.

Step (3): The peptides were purified on a Symmetry C₁₈ column (150 mm \times 2.1 mm, Waters) using the acetonitrile gradient described in step 2 at a flow rate of 0.3 ml/min.

Step (4): To ascertain the purity of the peptides, one additional step was performed on a Symmetry C₈ column (150 mm \times 2.1 mm, Waters) at a flow rate of 0.3 ml/min using the acetonitrile gradient described in step 2.

Peptide Primary Structure Determination

Enzymatic digestion

As much as 20 pmoles of peptide were submitted to 25 ng of endoproteinase Lys-C (sequencing grade, Boehringer) in 10 μl of Tris Buffer (25 mM, pH 8.5) containing 1 mM EDTA at 37 °C during 12 h. Desalting of peptides was performed on ZipTip C₁₈ (Millipore) following the protocol supplied by the manufacturer. The digest products were directly analyzed by mass spectrometry and Edman degradation as described below.

Mass spectrometry

As much as 1 μl of purified peptides, corresponding to 5 pmol, were dissolved in UPW and analyzed by Sandwich method on a Matrix-assisted Laser Desorption/Ionization mass spectrometer (Voyager

System 4188, Applied Biosystems) as described by Kussmann *et al* [14]. Molecular masses were calculated from a series of multiple-charged protonated molecular ions.

Sequencing

Purified peptides (20 pmol) were submitted to automated Edman degradation on a pulse-liquid automatic peptide sequencer (Applied Biosystem 492 cLC Protein sequencer).

Peptide synthesis

Five milligrams of each peptide were chemically synthesized without any modification by the company GENPEP (Prades le Lez, France). The HPLC purities of the peptides were 95% for papilloisin and 97% for halocytin.

Microorganisms

Bacteria, *Micrococcus luteus* (A270), *Aerococcus viridans* (54.145 T), *Bacillus megaterium* (66.20 T), *Staphylococcus aureus* (65.8 T), *Pseudomonas aeruginosa* (100 720 T), *Enterococcus faecalis* (103 015 T), *Enterobacter aerogenes* (30.86 T), *Salmonella thyphimurium* (60.62 T), *Klebsiella pneumoniae* (82.91 T), *Neisseria gonorrhoeae* (103 711) were purchased from the Pasteur Institute (Collection de l'Institut Pasteur). *Escherichia coli* DH5 α strain was purchased from Invitrogen.

Antimicrobial assays

After each purification step, antibacterial activity was monitored by a liquid growth inhibition assay [15]. Briefly, 10 μl aliquots from each resuspended fraction were incubated in microtiter plates with both 100 μl of a *M. luteus* suspension (starting OD₆₀₀ of 0.001) and 100 μl of an *E. coli* suspension (starting OD₆₀₀ of 0.001) in Poor-Broth nutrient medium (PB: 1% Bactotrypton, 0.5% NaCl w/v, pH 7.5). Bacterial growth was assayed by measurement of OD₆₀₀ after 12-h incubation at 30 °C for *M. luteus* strain and 37 °C for *E. coli* strain.

The minimal bactericidal concentration (MBC) was determined according to the method of Hancock [16]. Peptides were dissolved in a solution containing 0.01% acetic acid and 0.2% bovine serum albumin (BSA) and then serial doubling dilutions of the stock solution were made in 0.01% acetic acid, 0.2% BSA. As much as 10 μl aliquots from each dilution were incubated in sterile 96 well polypropylene microtiter plates with 100 μl of a suspension of bacteria (starting OD₆₀₀ of 0.001) in Mueller Hinton Broth (MHB) medium (Sigma). Bacterial growth was checked after 18-h incubation under agitation at 30 °C or 37 °C, depending of the bacterial strain used. The MBC was determined by plating the contents of the first three wells showing no turbidity visible growth of bacteria onto MH agar plates and incubating at 30 °C or 37 °C for 18 h. The lowest concentration of peptide that prevented any residual colony formation corresponded to the MBC.

Bactericidal assay

Purified peptide (10 μl at a concentration 10 times higher than the MIC) was mixed with 90 μl of an exponential phase culture of *M. luteus* or *E. coli* (starting OD₆₀₀ of 0.01) in PB and incubated respectively at 30 °C or 37 °C. Aliquots of 10 μl were plated after 0, 3, 10 and 30 min, 2, 6 and 24 h of incubation on nutrient agar and number of colony-forming units was counted after overnight incubation at 30 °C or 37 °C. Controls consisted in bacterial culture incubated with 10 μl of sterile water.

Hemolytical assay

Synthetic peptides were dissolved in a solution containing 0.01% acetic acid and 0.2% BSA in order to reach a concentration of 500 μM . Serial doubling dilutions were made from these peptide stock solutions in 0.01% acetic acid and 0.2% BSA. As much as 20 μl of each dilution were added to 180 μl of a solution containing 2.5% v/v of a sheep erythrocytes suspension in PBS. As positive control of hemolysis, 20 μl of a 10% Triton X-100 in PBS were used instead of peptide dilution. The negative control of hemolysis consists of 20 μl of a 0.01% acetic acid and 0.2% BSA solution. The tests were then incubated for 30 min at 37 °C and centrifuged for 3 min at 10 000 g. The supernatants were finally collected and their absorbance measured at 600 nm (AD 340, Beckman Coulter). The hemolytic percentage was calculated as follow:

$$\% \text{ hemolysis} = \frac{(A_{600} \text{ sample} - A_{600} \text{ negative control})}{(A_{600} \text{ positive control} - A_{600} \text{ negative control})} \times 100.$$

Circular dichroism spectroscopy analysis

All CD spectra were recorded at 20 °C on a Chirascan dichrograph (Applied Photophysics) in a quartz cell with an optical path of 0.5 mm. The peptides were initially dissolved in water at a concentration of 0.2 mg ml⁻¹ for halocytin (0.74 10⁻⁴ M l⁻¹) and 0.33 mg ml⁻¹ for papillosin (1.0 10⁻⁴ M l⁻¹). Spectra were recorded both in water and in presence of increasing amounts of TFE up to 70%. The spectra are an average of three scans recorded from 180 to 260 nm with a scan speed of 0.7 nm s⁻¹. The percentages of the various secondary structures were calculated with the DICHROWEB software [17].

Helical wheel representation of an amino acid sequence

The amino acid side chains are projected down the axis (the axis of an alpha helix, orthogonal to the paper plane). As an ideal alpha helix consists of 3.6 residues per complete turn, the angle between two residues is chosen to be 100 degrees and thus there exists a periodicity after five turns and 18 residues. The figure is a snapshot of a Java Applet written by Edward K. O'Neil and Charles M. Grisham (University of Virginia in Charlottesville, Virginia). The applet is accessible at <http://cti.itc.Virginia.EDU/~cmg/Demo/wheel/wheelApp.html>.

Results

Isolation of Antimicrobial Peptides from Hemocytes

Acid extracts were prepared with 160 ml of hemolymph from 17 *M. sabatieri*, 30 *H. papillosa*, 6 *H. tubulosa*, 23 *Cucumaria* sp. and 24 *P. lividus* specimens. After a first fractionation step on Sep-Pak cartridges (see Section on Materials and Methods), only fractions eluted with 60% acetonitrile were active and submitted to reverse-phase HPLC separation. For the three echinoderm species, no antimicrobial activity was detected in the eluted fractions from the first round of reverse-phase HPLC purification, and no subsequent purification steps were performed. For both tunicate species, several active fractions were obtained after reversed-phase HPLC. Activities against *M. luteus* and *E. coli* were assayed all along the elution gradient (5–65%) revealing several fractions with antibacterial activities (Figure 1(a) and (b)). Nineteen antibacterial fractions were obtained for *Microcosmus sabatieri* and five for *H. papillosa*, ranged between 19 and 40% acetonitrile, and between

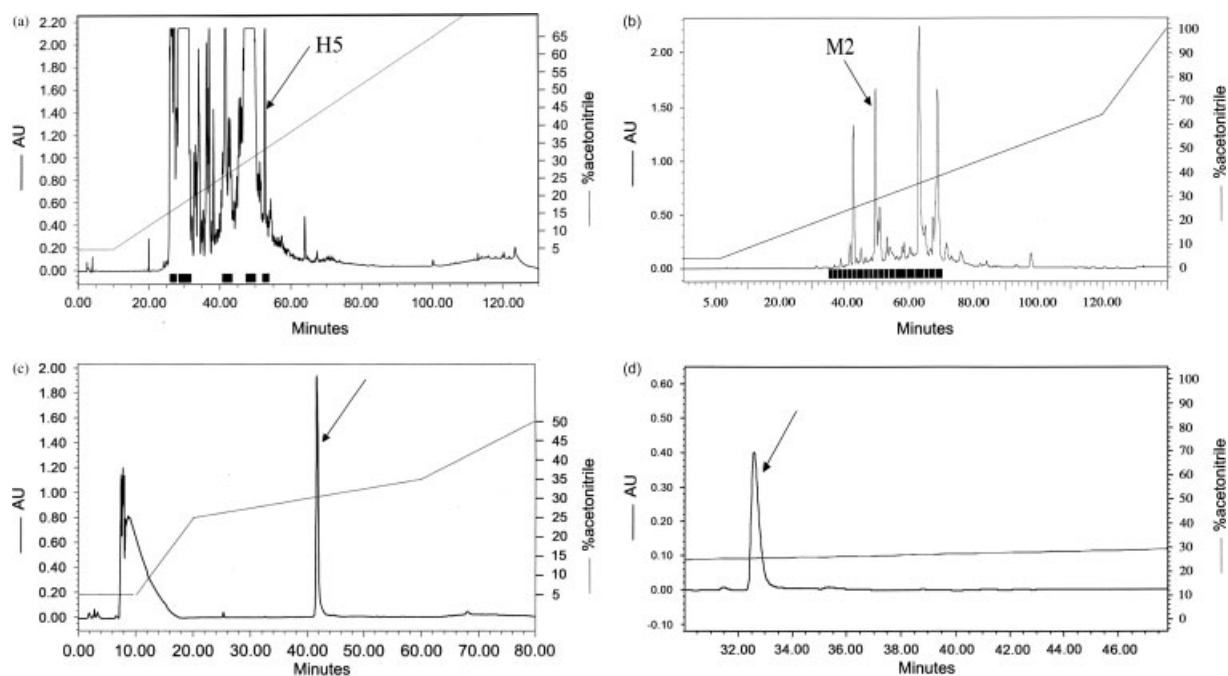


Figure 1. Reverse-phase HPLC of acidic extracts obtained from tunicate hemocytes. After prepurification by solid phase extraction, the material present in the fraction eluted with 60% acetonitrile was loaded onto a reverse-phase C₁₈ column. Elution for this first step HPLC purification was performed with a linear gradient (dotted line) from 5 to 65% acetonitrile over 90 min at a flow rate 1 ml/min. Absorbance peaks were monitored at 224 nm (solidfull line). Chromatograms from *Halocynthia papillosa* (a) and *Microcosmus sabatieri* (b) are represented. Black squares indicate fractions with antimicrobial activities. Fraction H5 contain the future purified halocytin and papillosin antimicrobial peptides (a), whereas fraction M2 contains the isolated but non characterized antimicrobial peptide from *M. sabatieri* (b). Chromatograms from last reverse-phase purification steps on C₈ column of halocytin (c) and papillosin (d) are also presented. Arrows indicate fractions containing the antimicrobial peptide of interest.

16 and 34% acetonitrile, respectively. Concerning *M. sabatieri*, the initial active fractions were also submitted to the three additional purification steps, and one peptide was completely purified from initial fraction M2 (Figure 1(b)). However, the amount was not sufficient for biochemical characterization. The present study focuses exclusively on the antibacterial molecules eluted around 30–32% acetonitrile fraction from *H. papillosa* (Figure 1(a), fraction H5). These fractions were submitted to three additional reverse-phase HPLC purification steps. For other fractions, activities were lost during subsequent purification steps. Finally, two peptides were purified from the fraction H5 of these hemocyte acidic extracts (Figure 1(c) and (d)).

Primary Structure of Isolated Antimicrobial Peptides

Mass spectrometry and analysis of the wide-scan spectrum revealed deduced molecular masses of 2731.55 and 3318.44 Da for the two molecules isolated from the fraction H5 (Figure 1(a)). For each peptide, only one mass was obtained, illustrating the purity of the molecules. These peptides were sequenced by Edman degradation.

An incomplete 22 residues NH₂-terminal sequence was obtained for the first peptide: FWGHIWNAVKRVGANALHGAVT. The complete biochemical characterization was achieved after enzymatic digestion of this peptide with an endoproteinase Lys-C. After digestion, the analysis by mass spectrometry revealed two cleavage products of 1257.48 and 1492.68 Da. These two subpeptides were also sequenced by Edman degradation and gave respectively the sequences: FWGHIWNAVK and RVGANALHGAVTGALS. The combination of all these data gives the complete sequence of this first peptide: FWGHIWNAVKRVGANALHGAVTGALS. This 26 amino acid sequence has a calculated monoisotopic mass of 2731.45 Da, which is in good agreement with the mass measured for the isolated peptide (2731.55 Da). Searches in sequence databases did not yield any homology with known peptides and we propose the name of halocytin for this novel cationic antibacterial peptide.

For the second peptide, the following 34 amino acid sequence was obtained by Edman degradation: GFWKKVGSAAWGVKAAAKGAAVGGLNALAKHIQ. This sequence has a calculated monoisotopic mass of 3318.85 Da, which is also in agreement with the mass measured for the purified peptide (3318.44 Da). As no homology with known peptide was found in databases, we have chosen the name of papillosin for this second cationic antibacterial peptide.

Antimicrobial Activity Spectrum and Bactericidal Assay of Halocytin and Papillosin

Activity spectrum against a variety of bacterial strains of both native and synthetic peptides was investigated (Table 1). Whereas enough native halocytin (104 nmol) was available to perform these tests, only 28 nmol of native papillosin was obtained after the purification steps. Consequently, native papillosin tests were restricted to *M. luteus*, *S. aureus* (Gram-positive bacteria) and *E. Coli* (Gram-negative bacteria). All other strains were tested with synthetic papillosin. For comparison, the activity spectrum of synthetic halocytin was also determined (Table 1). The differences between synthetic and native peptides are probably due to approximative estimation of native peptide amount given by Edman degradation protocol. Indeed, synthetic halocytin is always four fold more active than the native form, while synthetic papillosin is two fold less active than the native one. These differences in activity are identical for all bacterial strains.

Table 1. Minimal bactericidal concentration (MBC) of halocytin and papillosin

Bacteria	Halocytin MBC (μM)		Papillosin MBC (μM)	
	Native	Synthetic	Native	Synthetic
Gram-positive bacteria				
<i>M. luteus</i>	1.56–3.13	0.39–0.78	0.13–0.25	0.19–0.39
<i>S. aureus</i>	6.25–12.50	1.56–3.13	0.5–1.00	1.56–3.13
<i>B. megaterium</i>	0.75–1.56	0.39–0.78	ND	0.05–0.10
<i>A. viridans</i>	3.13–6.25	0.78–1.56	ND	0.19–0.39
<i>E. faecalis</i>	6.25–12.50	1.56–3.13	ND	0.78–1.56
Gram-negative bacteria				
<i>E. coli</i> DH5α	6.25–12.50	6.25–12.50	0.25–0.50	0.78–1.56
<i>S. typhimurium</i>	50–100	12.50–25	ND	0.78–1.56
<i>P. aeruginosa</i>	>100	25–50	ND	3.13–6.25
<i>E. aerogenes</i>	25–50	6.25–12.50	ND	0.78–1.56
<i>K. pneumoniae</i>	12.50–25	1.56–3.13	ND	0.39–0.78
<i>N. gonorrhoeae</i>	25–50	ND	ND	ND

MBC were determined by testing different concentrations of both native and synthetic peptides in liquid growth inhibition assays against different bacterial strains according to the Hancock method. MBC values are expressed in μM. ND is used for Not Done.

In liquid growth inhibition, halocytin had marked activity against Gram-positive strains, and more particularly *M. luteus*, *B. megaterium*, and *A. viridans* (0.39 μM < MBC < 1.56 μM). Activities against *S. aureus* and *E. faecalis* were also found (1.56 μM < MBC < 3.13 μM). Halocytin activity against Gram-negative bacteria was significantly lower with MBC between 6.25 and 50 μM, except against *K. pneumoniae* (1.56 μM < MBC < 3.13 μM).

Papillosin showed important antibacterial activities against both Gram-positive and Gram-negative bacteria. Growth inhibition of Gram-positive bacteria was particularly strong for *M. luteus*, *B. megaterium*, and *A. viridans* (0.05 μM < MBC < 0.39 μM), whereas *S. aureus* growth was less affected (1.56 μM < MBC < 3.13 μM). In contrast to halocytin, papillosin also has a strong activity against Gram-negative bacteria (0.39 μM < MBC < 1.56 μM), except for *P. aeruginosa* (3.13 μM < MBC < 6.25 μM).

In summary, papillosin possess potent activities against Gram-positive and Gram-negative bacteria. Halocytin is active against all the Gram-positive bacteria tested but its activity against Gram-negative bacteria is effective but much less potent. In addition, for all strains tested, papillosin present a higher antibacterial activity than halocytin.

Hemolytical Activity

Peptides hemolytical activity was assessed toward sheep red blood cells (Figure 2). Integrity of red blood cells was not affected by papillosin at all concentrations tested. In contrast, hemolytical activity was detected for halocytin concentrations higher than 3.13 μM, reaching 22% hemolysis at 50 μM.

Circular Dichroism Study

CD spectra of halocytin and papillosin were recorded in water and in presence of various percentages of TFE (Figure 3). The two peptides displayed a very similar behavior. In water, a typical random coil spectrum was obtained. In presence of TFE, their spectra were progressively modified with characteristic bands at

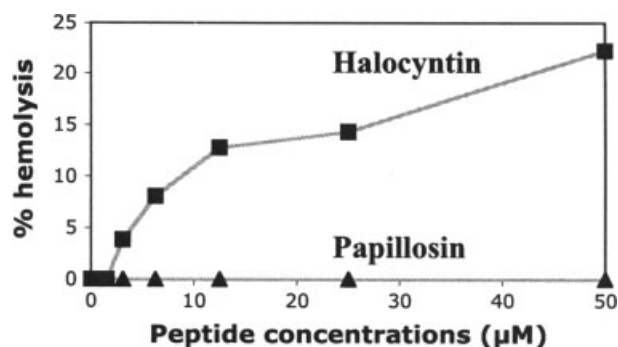


Figure 2. Peptides hemolytic activity. Triton-X100 10% was used as control of 100% hemolysis and an acid acetic/BSA solution as negative control. Hemolytic activity was assessed toward sheep erythrocytes. Percentage hemolysis was calculated with the following equation: % hemolysis = $(A_{600} \text{ sample} - A_{600} \text{ negative control}) / (A_{600} \text{ positive control} - A_{600} \text{ negative control}) \times 100$. Percentage hemolysis values are represented by black squares for halocytin and black triangles for papillosin.

190, 208, and 222 nm (50% TFE spectrum) and an increase of the mean residue ellipticity at 222 nm consistent with an α -helical structure. With 50% of TFE, 60 and 74% of helix were observed for halocytin and papillosin, respectively. At 70% of TFE, the two spectra remained very similar, indicating that there are no significant conformational changes for a higher percentage of TFE. The 204-nm isodichroic point suggests an equilibrium between random coil and α -helical conformations.

Discussion

In a context of new bioactive molecules with antimicrobial activity research, marine environment is probably the one with the greatest potential. Indeed, its biodiversity is unique as it is estimated that between 50 and 80% of all life forms are present only in oceans. On 33 known animal phyla, 32 are present in aquatic environment, among which 21 are exclusively marine.

AMP have been isolated from bacteria, viruses, plants, and animals. They are also called 'natural antibiotics', and they are of extraordinary pharmaceutical interest. Around 700 AMP have already been isolated until now [13] and several originate from marine invertebrates [1].

We report here the screening for antibacterial peptides in five marine invertebrate species. We focused on cationic peptides because it represents the largest group of AMP. Previous works have shown that hemocytes are a tissue of choice for screening in marine invertebrates [4,6,7,18–20]. Our screening for AMP was then not exhaustive, but voluntary oriented on cationic peptides in hemocytes.

Our results show that all tested species possess antimicrobial activity in crude extracts. For the three echinoderm species, these activities were rapidly lost after first purification steps. This indicates that the detected antimicrobial activities could be associated to other molecules than cationic peptides in the extracts. This is in accordance with previous work that revealed the presence of many different antimicrobial nonpeptidic compounds in echinoderms. These molecules are naphthoquinone pigments [21], steroidal glycosides [22], complement like substances [23], lysozymes [24], or polyhydroxylated sterols [25]. They are present in various tissue from the coelomocytes to the body walls [26]. Only one work indicate the potential presence of an AMP in

the echinoderm *Cucumaria frondosa* [27], but it has not been characterized.

In contrast, we revealed antimicrobial activities on the basis of cationic peptides in both tunicate species. We purified one cationic AMP from hemocytes of *M. sabatieri*. However, the small quantity of pure peptide did not allow for complete biochemical characterization. In addition, we fully characterized two new antibacterial peptides from the hemocytes of the solitary tunicate *H. papillosa*, that we named halocytin and papillosin.

AMP, also known as cationic host defense peptides are diverse in their sequence and structure. They are small in size, contain rarely more than 45 residues, they have at least two positive charges and are generally amphipathic with hydrophilic and hydrophobic residues spatially well-separated. Most AMP have more than 50% hydrophobic amino acids and a lower proportion of both neutral polar and negatively charged residues.

Several AMP have already been isolated and characterized from hemocytes of different tunicate species. Halocytin A and B were the first, isolated from the solitary ascidian *Halocynthia roretzi* [28]. These two small tetrapeptides are reported to have antimicrobial activity against both Gram-negative and Gram-positive bacteria, but also to have cytotoxic activities against different eucaryote cell strains. Another short AMP was characterized: an octapeptide named plicatamide and isolated from *Styela plicata* [29]. Despite its small size and its modest cationic properties, its antibacterial activity is very potent. It forms cation-selective channels in model lipid bilayers composed of bacterial lipids. *S. aureus* respond to plicatamide exposure with a massive potassium efflux that begins within seconds [30]. Moreover, like halocytinamines, it is characterized by an oxidatively decarboxylated aromatic C-terminal residue, conferring proteolytic resistance. Several AMP were also characterized from the solitary ascidian *Styela clava*. A first group of five AMP named styelin A–E was characterized [4,31]. The styelins are highly basic polypeptides, encoded as prepropeptides, and are effective against Gram-negative and Gram-positive bacteria, with low MICs. Styelin D and E differed significantly from styelins A–C, and it has been shown that these molecules could be subjected to different post-translational modifications probably involved in the control of activity under low-pH or high-salt conditions [32]. A second group of AMP, clavanins A–E was also isolated from *S. clava* hemocytes [6,33]. The clavanins are a family of α -helical, amphipathic, histidine-rich AMP of 23 amino acids that exhibit C-terminal amidation. Also expressed as prepropeptides, the molecules are broadly effective against Gram-positive bacteria, and also against some Gram-negative bacteria. It was shown that their mode of action is pH-dependent [34]. At neutral pH, clavanins interact nonspecifically with the bacterial membrane as well as artificial phospholipid bilayers and form an ionophore across the membrane. Under acidic conditions, the membrane is still the target, but clavanins do not interact with phospholipids. They seem to target membrane proteins that generate transmembrane ion gradients. It has been suggested that clavanins could adopt a flexible hydrophobic conformation allowing interaction with transmembrane protein, disturbing the ion flux across the membrane. Clavaspirin was also purified from the same species [7]. If the preproclavaspirin cDNA presents exactly the same structure than those of clavanins, the mature peptide displays weak similarities with clavanins. It has been suggested that clavaspirin nucleotides have been inserted into a preexisting clavanin gene by some recombination event [7]. Such recombination events have participated in expanding the diversity of cathelicidins, a family of AMP from the innate

immune system of mammals [35]. Finally, a fourth tunicate species, *Halocynthia aurantium*, provided two other AMP. The first, dicynthaurin, is composed of two 30-residue monomers without any sequence homology to other tunicate AMP [5]. Molecules are C-terminally amidated and linked covalently with a single cysteine disulfide bond. In a membrane mimetic system, it adopts α -helical conformation. It possesses a broad activity spectrum against Gram-negative and Gram-positive bacteria. The second, halocidin, is composed of two subunits containing 18 and 15 amino acid residues that are linked by a single disulfide bond [3]. It has been demonstrated that the 18 residues monomer was more active than the heterodimer or the 15 residue monomer against different bacterial strains.

Analysis of halocytin and papillosin sequence properties (Table 2) clearly shows that both peptides present physical and chemical characteristics of AMP. Papillosin is longer than halocytin and has also a higher net charge (Table 2). Theoretical data suggest that halocytin is slightly more hydrophobic than papillosin. This was confirmed by HPLC data: halocytin is eluted later (28% acetonitrile) than papillosin (25% acetonitrile) during the last reverse-phase purification step on a C_8 column (Figure 1(c) and (d)). Moreover, halocytin possesses an aromatic residue (phenylalanine) in N-terminal position that is considered to play a role in antimicrobial activity [36]. Their bacteriolytic activity was assessed *in vitro*. Both molecules are strongly active against Gram-positive bacteria. However, papillosin presents an activity until 8 times higher than halocytin, depending on the bacteria. Papillosin also possess a high antibacterial activity against a panel of Gram-negative bacteria, while halocytin only present a limited activity against these bacterial strains. The MBC determined for papillosin are more often around or below $1 \mu\text{M}$. Moreover, this last peptide presents no hemolytic activity toward sheep red blood cells (in contrast to halocytin). In conclusion, papillosin seems to be a promising candidate for future studies aimed to evaluate its putative use as therapeutics.

Cationic host defense peptides can be classified in four groups, based on their structure: (i) amphiphatic α -helical, (ii) β -sheet structures stabilized with several disulphide bonds, (iii) extended structures, and (iv) loop structures with one disulphide bond [13]. Analyses by the secondary structure prediction software GOR 4 [37], revealed an α -helical structure for both peptide, from residue 7 to 17 for halocytin, and from residue 9 to 30 for papillosin. Both for halocytin and papillosin, the predicted α -helix was experimentally supported by CD experiments performed on synthetic peptides (Figure 3). Indeed, the two synthetic peptides which are in random coil in water displayed a high propensity to generate α -helical structures in presence of TFE. The structure prediction tool of the antimicrobial peptide database (APD) [38] free online at <http://aps.unmc.edu/AP/main.php>, also confirmed the possibility to form α -helices for both peptides. It also revealed that such helices might be amphiphatic as at least 11 residues for a total of 26 may be situated on the same hydrophobic surface for halocytin, and at least 14 from 34 for papillosin. The amphiphatic nature of both peptides which mean the segregation of hydrophobic and nonhydrophobic residues is represented by wheel diagrams (Figure 4).

Moreover, alignments with the most similar peptide in the APD, find pleurocidin (AP00166) for halocytin and adenoregulin (AP00001) for papillosin, two amphiphatic α -helical structure peptides. This kind of peptide is assumed to destabilize the cytoplasmic membrane, causing its permeabilization. The selectivity for microbial membrane is thought to be due to their high content of anionic lipids on the surface, the high electrical-potential gradient across this membrane, and their lack of cholesterol, supporting a good interaction with cationic peptides [39].

In conclusion, this study shows that hemolymphs of the three echinoderm species *H. tubulosa*, *Cucumaria* sp. and *P. lividus* contain no cationic peptide host defence, which is in agreement with previous attempts to isolate this kind of molecules from other echinoderm species [26]. However, this study reveals the

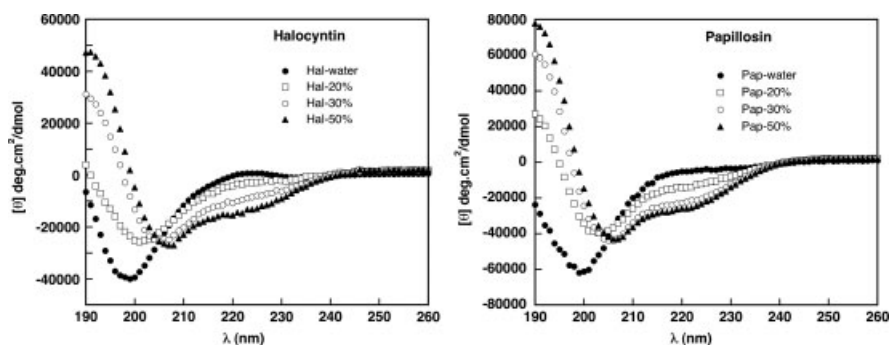


Figure 3. Circular dichroism spectra for halocytin ($0.74 \cdot 10^{-4} \text{ M l}^{-1}$) and papillosin ($1.0 \cdot 10^{-4} \text{ M l}^{-1}$). The spectra were obtained in water, and in presence of 20, 30, and 50% of TFE.

Table 2. Halocytin and papillosin amino acid sequences and physico-chemical properties

Peptide	Amino acid sequence	Total residues	Charge	Isoelectric point	Total hydrophobic ratio(%)
Halocytin	FWGHIWNAVKKRVGANALHGAVTGALS	26	+4	11	53
Papillosin	GFWKVGSAAWGGVKAACKGAAGVGLNALAKHIQ	34	+6	10.6	52

Net charges and total hydrophobic ratio were calculated from ADP web site (<http://aps.unmc.edu/AP/main.php>). Isoelectric points were defined thanks to the Compute pI/Mw tool of the ExPASy website (http://www.expasy.org/tools/pi_tool.html).

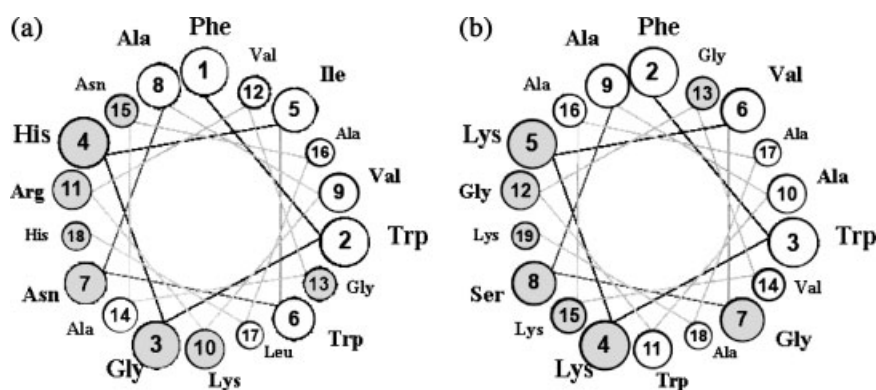


Figure 4. Helical wheel diagrams. Residues 1–18 of halocytin (a) and residues 2–19 of papillosin (b) are shown, with the polar residues shaded gray. The clearly evident clustering of polar and apolar residues imparts amphipathicity.

presence of AMP in both tunicate species, *M. sabatieri* and *H. papillosa*. We characterized two peptides, halocytin and papillosin from *H. papillosa*. An α -helical structure was predicted for each peptide, and we found that both molecules were active either against a panel of Gram-positive and -negative bacteria, with a higher antimicrobial activity for papillosin. The protein-binding potential or Boman index [40] is 0.06 for halocytin and -0.27 for papillosin. These two low values indicate that both peptides selectively interact with the lipids of the microbial membrane, and not with proteins. It means that both peptides represent a good antibacterial drug candidate without many side effects. In combination with the lack of hemolytic activity toward sheep red blood cells for papillosin and also for halocytin at low concentration, these data indicate that the two peptides represent potential candidates for pharmaceutical assays. These molecules are protected by two international patents (WO 2004/081 024 A2 and WO 2004/081 214 A1).

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References

1. Tincu JA, Taylor SW. Antimicrobial peptides from marine invertebrates. *Antimicrob. Agents Chemother.* 2004; **48**: 3645–3654.
2. Bachere E, Gueguen Y, Gonzalez M, de Lorgeril J, Garnier J, Romestand B. Insights into the anti-microbial defense of marine invertebrates: the penaeid shrimps and the oyster *Crassostrea gigas*. *Immunol. Rev.* 2004; **198**: 149–168.
3. Jang WS, Kim KN, Lee YS, Nam MH, Lee IH. Halocidin: a new antimicrobial peptide from hemocytes of the solitary tunicate, *Halocynthia aurantium*. *FEBS Lett.* 2002; **521**: 81–86.
4. Lee IH, Cho Y, Lehrer RI. Styelins, broad-spectrum antimicrobial peptides from the solitary tunicate, *Styela clava*. *Comp. Biochem. Physiol., B Biochem. Mol. Biol.* 1997; **118**: 515–521.
5. Lee IH, Lee YS, Kim CH, Kim CR, Hong T, Menzel L, Boo LM, Pohl J, Sherman MA, Waring A, Lehrer RI. Dicynthaurin: an antimicrobial peptide from hemocytes of the solitary tunicate, *Halocynthia aurantium*. *Biochim. Biophys. Acta* 2001; **1527**: 141–148.
6. Lee IH, Zhao C, Cho Y, Harwig SS, Cooper EL, Lehrer RI. Clavanins, alpha-helical antimicrobial peptides from tunicate hemocytes. *FEBS Lett.* 1997; **400**: 158–162.
7. Lee IH, Zhao C, Nguyen T, Menzel L, Waring AJ, Sherman MA, Lehrer RI. Clavaspilin, an antibacterial and haemolytic peptide from *Styela clava*. *J. Pept. Res.* 2001; **58**: 445–456.
8. Mitta G, Vandenbulcke F, Roch P. Original involvement of antimicrobial peptides in mussel innate immunity. *FEBS Lett.* 2000; **486**: 185–190.
9. Kurata S, Arika S, Kawabata S. Recognition of pathogens and activation of immune responses in *Drosophila* and horseshoe crab innate immunity. *Immunobiology* 2006; **211**: 237–249.
10. Hoffmann JA, Reichhart JM. *Drosophila* innate immunity: an evolutionary perspective. *Nat. Immunol.* 2002; **3**: 121–126.
11. Hancock RE. Cationic antimicrobial peptides: towards clinical applications. *Expert. Opin. Investig. Drugs* 2000; **9**: 1723–1729.
12. Hancock RE. Host defence (cationic) peptides: what is their future clinical potential? *Drugs* 1999; **57**: 469–473.
13. Mookherjee N, Hancock RE. Cationic host defence peptides: innate immune regulatory peptides as a novel approach for treating infections. *Cell. Mol. Life Sci.* 2007; **64**: 922–933.
14. Kussmann M, Lassing U, Sturmer CA, Przybylski M, Roepstorff P. Matrix-assisted laser desorption/ionization mass spectrometric peptide mapping of the neural cell adhesion protein neuroligin purified by sodium dodecyl sulfate polyacrylamide gel electrophoresis or acidic precipitation. *J. Mass Spectrom.* 1997; **32**: 483–493.
15. Bulet P, Cociancich S, Dimarcq JL, Lambert J, Reichhart JM, Hoffmann D, Hetru C, Hoffmann JA. Insect immunity. Isolation from a coleopteran insect of a novel inducible antibacterial peptide and of new members of the insect defensin family. *J. Biol. Chem.* 1991; **266**: 24520–24525.
16. Amsterdam D. Susceptibility testing of antimicrobials in liquid media. In *Antibiotics in Laboratory Medicine*, 4th edn, Loman V (ed.). Williams and Wilkins: Baltimore, 1996; 52–111.
17. Lobley A, Whitmore L, Wallace BA. DICHROWEB: an interactive website for the analysis of protein secondary structure from circular dichroism spectra. *Bioinformatics* 2002; **18**: 211–212.
18. Khoo L, Robinette DW, Noga EJ. Callinectin, an Antibacterial Peptide from Blue Crab, *Callinectes sapidus*, Hemocytes. *Mar. Biotechnol. (NY)* 1999; **1**: 44–51.
19. Mitta G, Hubert F, Noel T, Roch P. Myticin, a novel cysteine-rich antimicrobial peptide isolated from haemocytes and plasma of the mussel *Mytilus galloprovincialis*. *Eur. J. Biochem.* 1999; **265**: 71–78.
20. Miyata T, Tokunaga F, Yoneya T, Yoshikawa K, Iwanaga S, Niwa M, Takao T, Shimonishi Y. Antimicrobial peptides, isolated from horseshoe crab hemocytes, tachyplesin II, and polyphemusins I and II: chemical structures and biological activity. *J. Biochem. (Tokyo)* 1989; **106**: 663–668.
21. Service M, Wardlaw AC. Echinochrome-A as a bactericidal substance in the coelomic fluid of *Echinus esculentus* (L.). *Comp. Biochem. Physiol., B* 1984; **79**: 161–165.
22. Andersson L, Bohlin L, Iorizzi M, Riccio R, Minale L, Moreno-Lopez W. Biological activity of saponins and saponin-like compounds from starfish and brittle-stars. *Toxicol.* 1989; **27**: 179–188.

23. Leonard LA, Strandberg JD, Winkelstein JA. Complement-like activity in the sea star, *Asterias forbesi*. *Dev. Comp. Immunol.* 1990; **14**: 19–30.
24. Canicatti C, Roch P. Studies on *Holothuria polii* (Echinodermata) antibacterial proteins. I. Evidence for and activity of a coelomocyte lysozyme. *Experientia* 1989; **45**: 756–759.
25. Iorizzi M, Bryan P, McClintock J, Minale L, Palagiano E, Maurelli S, Riccio R, Zollo F. Chemical and biological investigation of the polar constituents of the starfish *Luidia clathrata*, collected in the Gulf of Mexico. *J. Nat. Prod.* 1995; **58**: 653–671.
26. Haug T, Kjuul AK, Styrvoid OB, Sandsdalen E, Olsen OM, Stensvag K. Antibacterial activity in *Strongylocentrotus droebachiensis* (Echinoidea), *Cucumaria frondosa* (Holothuroidea), and *Asterias rubens* (Asteroidea). *J. Invertebr. Pathol.* 2002; **81**: 94–102.
27. Beauregard KA, Truong NT, Zhang H, Lin W, Beck G. The detection and isolation of a novel antimicrobial peptide from the echinoderm, *Cucumaria frondosa*. *Adv. Exp. Med. Biol.* 2001; **484**: 55–62.
28. Azumi K, Yokosawa H, Ishii S. Halocyclamines: novel antimicrobial tetrapeptide-like substances isolated from the hemocytes of the solitary ascidian *Halocynthia roretzi*. *Biochemistry* 1990; **29**: 159–165.
29. Tincu JA, Craig AG, Taylor SW. Plicatamide: A lead to the biosynthetic origins of the tunichromes? *Biochem. Biophys. Res. Commun.* 2000; **270**: 421–424.
30. Tincu JA, Menzel LP, Azimov R, Sands J, Hong T, Waring AJ, Taylor SW, Lehrer RI. Plicatamide, an antimicrobial octapeptide from *Styela plicata* hemocytes. *J. Biol. Chem.* 2003; **278**: 13546–13553.
31. Zhao C, Liaw L, Lee IH, Lehrer RI. cDNA cloning of three cecropin-like antimicrobial peptides (Styelins) from the tunicate, *Styela clava*. *FEBS Lett.* 1997; **412**: 144–148.
32. Taylor SW, Craig AG, Fischer WH, Park M, Lehrer RI. Styelin D, an extensively modified antimicrobial peptide from ascidian hemocytes. *J. Biol. Chem.* 2000; **275**: 38417–38426.
33. Zhao C, Liaw L, Lee IH, Lehrer RI. cDNA cloning of Clavanins: antimicrobial peptides of tunicate hemocytes. *FEBS Lett.* 1997; **410**: 490–492.
34. van Kan EJ, Demel RA, Breukink E, van der Bent A, de Kruijff B. Clavanin permeabilizes target membranes via two distinctly different pH-dependent mechanisms. *Biochemistry* 2002; **41**: 7529–7539.
35. Tomasinsig L, Zanetti M. The cathelicidins—structure, function and evolution. *Curr. Protein Pept. Sci.* 2005; **6**: 23–34.
36. Jacobs RE, White SH. The nature of the hydrophobic binding of small peptides at the bilayer interface: implications for the insertion of transbilayer helices. *Biochemistry* 1989; **28**: 3421–3437.
37. Garnier J, Gibrat JF, Robson B. GOR method for predicting protein secondary structure from amino acid sequence. *Methods Enzymol.* 1996; **266**: 540–553.
38. Wang Z, Wang G. APD: the antimicrobial peptide database. *Nucleic Acids Res.* 2004; **32**: D590–D592.
39. Hancock RE, Lehrer R. Cationic peptides: a new source of antibiotics. *Trends Biotechnol.* 1998; **16**: 82–88.
40. Boman HG. Antibacterial peptides: basic facts and emerging concepts. *J. Intern. Med.* 2003; **254**: 197–215.