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Characterization of Four Liver-Expressed Antimicrobial Peptides from Antarctic Fish and Their Antibacterial Activity

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Abstract: Liver-expressed antimicrobial peptides (LEAPs) are cysteine-containing cationic peptides. LEAP-1 and LEAP-2 are eight- and four-cysteine containing antimicrobial peptides found in animals, respectively. LEAP-1 is widely known as antibacterial peptide involved in the innate immunity of fish, but the roles of LEAP-1 and LEAP-2 in Antarctic fish species are unknown. In the present study, we synthesized and characterized novel LEAPs with four and eight cysteine residues, derived from Antarctic notothenioid (*Dissostichus mawsoni*) and Antarctic eelpout (*Lycodichthys dearborni*). Circular dichroism spectroscopy of these peptides showed a typical β-sheet conformation. The LEAPs were found to be bactericidal against gram-positive as well as gram-negative bacteria. In the SYTOX green uptake assay, LEAPs did not trigger any significant increase in fluorescence. However, LEAPs competitively bound to DNA and replaced the ethidium bromide (EB) dye. To determine the effect of temperature on the activity of LEAPs, we evaluated the antibacterial activity against *Listeria monocytogenes* at 5, 15, 25, and 35 °C. The results showed that the antibacterial activity of LEAPs increased with a decrease in temperature, which may indicate that the Antarctic fish LEAP are evolutionarily adapted. Taken together, our results suggest that novel Antarctic LEAPs are bactericidal peptides with the likely mode of action being DNA binding and may be evolved to adapt to cold temperature.

Keywords: Antarctic eelpout; Antarctic notothenioid; liver-expressed antimicrobial peptide; disulfide bond; cold-active peptide

1. Introduction

Liver-expressed antimicrobial peptides (LEAPs) are cysteine-containing cationic peptides with antimicrobial activity, which were first discovered in human blood ultrafiltrate and urine samples [1]. In humans, LEAPs with eight and four cysteine residues are termed as hepcidin [1] or LEAP-1 [2] and LEAP-2 [3], respectively. The human LEAP-1 (hLEAP-1) is a cysteine-containing cationic peptide comprising about 20–25 amino acid residues [2]. It has a characteristic β-sheet structure, which is stabilized by eight cysteine residues [2]. These residues form four disulfide bonds, one of which is an unusual vicinal bond between adjacent cysteines at the hairpin turn with an amino terminal Cu(II)- and Ni(II)-binding (ATCUN) motif [4]. The N-terminal region of human LEAP-1 (hepcidin-25) has a metal binding site specific for the coordination of Cu(II) and Ni(II) known as ATCUN motif [5]. The histidine residue at the amino-terminal (XXH) of ATCUN motif in teleosts and mammals are highly conserved, which has been reported to form a putative metal binding motif. It was found that

Ni(II) and Cu(II) interaction with the peptide, results in formation of metal and peptide complex which leads to the oxidative damage of DNA [6]. The trout LEAP with ATCUN motif in the presence of Cu(II) and ascorbate was able to hydrolyze a DNA plasmid. On the contrary, the trout LEAP without ATCUN motif was unable to hydrolyze the plasmid DNA [5]. Thus, ATCUN motif is essential for antimicrobial activity via DNA damage [7]. LEAPs have been identified in many other vertebrates such as fish, chickens, pigs, and mice [8–11]. LEAPs play a significant role in the fish innate immune system [8,12]. The hybrid striped bass was the first fish from which LEAPs were isolated [8]. Since then, LEAPs have been identified in at least 30 fish species [13].

Similar structure of LEAPs are identified in human and fish with respect to their distorted β-sheet shape with a hairpin loop, position of cysteines, and ATCUN motif [14–16]. Nevertheless, sequence analysis revealed the presence of four, six, and seven cysteines in mature LEAPs from fish [13]. There are many differences between the fish and mammalian LEAPs, including the presence of several LEAP variants within a single fish species, such as the seven variants identified in *Acanthopagrus schlegelii* [17]. Moreover, the expression of these isoforms is observed not only in hepatocytes, but also in kidney and hematopoietic tissues [18], contrary to the mammalian LEAPs.

The typical LEAPs with eight cysteine residues show prominent antibacterial activity against bacterial pathogens, fungi, viruses, and some neoplasms [19]. For a potent response against pathogens, fish extensively depend on the production of antimicrobial peptides, which is a part of their first line of defense [20]. The Antarctic notothenioid and Antarctic eelpout were the first Antarctic fish in which four-cysteine LEAP was reported [18]. Recently, synthetic LEAPs with four cysteine residues from *Epinephelus coioides* were reported, which possess antimicrobial and antiviral activities [21]. The existence of several LEAP isoforms in the same organism highlights the need for multiple functions in a complex aquatic environment [22,23].

To date, no studies have addressed whether LEAPs plays a vital role in the innate immunity of Antarctic notothenioid (*Dissostichus mawsoni*) and Antarctic eelpout (*Lycodichthys dearborni*). To determine the potential role of novel four- and eight-cysteine LEAPs in the innate immunity of Antarctic fish, there is a need to evaluate their antibacterial activity. In this study, we attempted to evaluate the antibacterial activity and mechanism-of-action of novel LEAPs with four (4 cys) and eight cysteine (8 cys) residues from *Dissostichus mawsoni* and *Lycodichthys dearborni*. In the Antarctic Ocean, the temperature stays below 2 °C. Therefore, it is important for Antarctic antimicrobial peptides to remain active against cold-loving bacteria at low temperatures. Antarctic antimicrobial peptides such as moronecidin from *Parachaenichthys charcoti* and *Notothenia coriiceps* showed similar levels of antibacterial activity against a cold-loving bacterium (*Psychrobacter* sp.) at low temperature [24]. To determine how innate fish immunity functions at low temperatures, there is a need to investigate the antibacterial activity of LEAPs at low temperatures.

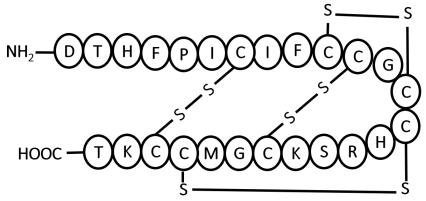
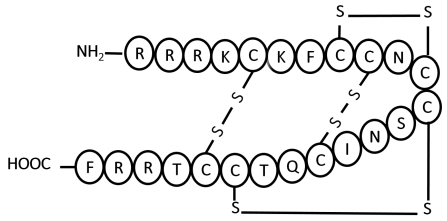
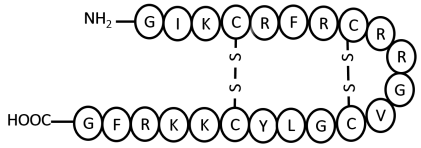
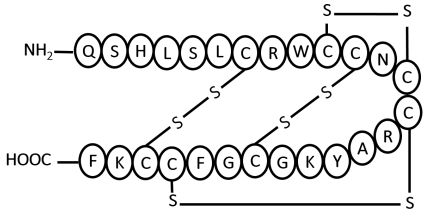
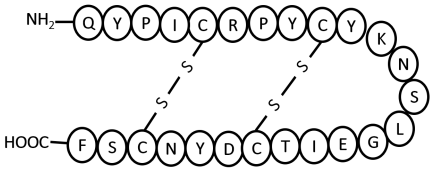
2. Materials and Methods

2.1. Bioinformatic Analysis and Peptide Synthesis

Sequences of various LEAPs were obtained from the NCBI database (the following NCBI accession numbers were used: *Dissostichus mawsoni* (ABY84829.1, ABY84838.1); *Lycodichthys dearborni* (ABY84842.1, ABY84845.1); *Notothenia angustata* (ABY84832.1, ABY84835.1); *Notothenia coriiceps* (XP_010791080.1, XP_010772611.1); *Pogonophryne scotti* (ABY84821.1, ABY84840.1); *Oncorhynchus mykiss* (AAG30029.1, XP_021450828.1); *Epinephelus coioides* (AEK87109.1, ADN06867.1); and Human (AAH20612.1, AAH70199.1)) and aligned using Clustal Omega (1.2.4).

The disulfide-bonded human LEAP (hLEAP-1) (Cat No. AP4601), the disulfide-bonded Antarctic notothenioid LEAP (nLEAP-1 and nLEAP-2), and eelpout LEAP (eLEAP-1 and eLEAP-2) were synthesized by Apeptide (www.apeptide.com, Shanghai, China) (Table 1). The overall net charge of the peptides (at pH 7) was calculated using Innovagen's peptide property calculator (www.pepcalc.com). The peptides were dissolved in a buffer used in each experiment.

Table 1. Liver-expressed antimicrobial peptides (LEAPs): the amino acid sequence and schematic representation of hLEAP-1 (Human liver-expressed antimicrobial peptide), nLEAP-1 and nLEAP-2 (Antarctic notothenioid liver-expressed antimicrobial peptide: *Dissostichus mawsoni*) and eLEAP-1 and eLEAP-2 (Antarctic Eelpout liver-expressed antimicrobial peptide: *Lycodichthys dearborni*), their possible sites of disulfide bond formation and net charge at pH 7.

Peptide	Amino Acid Sequence and Schematic Representation	Disulfide Bonds	Net Charge at pH 7
hLEAP-1	NH_2-DTHFPICIFCCGCCHRSKCGMCCKT-COOH 	7–23, 10–13, 11–19, 14–22	+2.2
nLEAP-1	NH_2-RRRKCKFCCNCCSNICQTCCTRRF-COOH 	5–20, 8–11, 9–16, 12–19	+7
nLEAP-2	NH_2-GIKCRFRCRRGVCGLYCKKRFG-COOH 	4–17, 8–13	+8
eLEAP-1	NH_2-QSHLSLCRWCCNCCRAYKGCGFCCKF-COOH 	7–24, 10–13, 11–20, 14–23	+4.1
eLEAP-2	NH_2-QYPICRPYCYKNSLGEITCDYNCSF-COOH 	5–23, 9–19	0

2.2. Circular Dichroism (CD) Spectroscopy

Circular dichroism (CD) spectroscopy was carried out in the far ultraviolet (UV) range (190–250 nm) at 0.1 nm resolution using a JASCO J-1500 CD Spectrometer, coupled with a Peltier JASCO CDF-426S/15 system for temperature control (Jasco Corp., Tokyo, Japan), using quartz cuvettes of 0.1 cm path length and 1 nm bandwidth. Each spectrum was recorded as an average of five scans in continuous scanning mode, at a scanning speed of 50 nm/min. The baseline was subtracted from each sample spectrum. CD spectra of the peptides were recorded in trifluoroethanol (TFE, 30% *v*/*v* in water). The spectra were recorded at 5, 15, 25, and 35 °C in 30% TFE to analyze the conformational changes in hepcidin peptides

with respect to temperature. The secondary structure of LEAPs was analyzed with BeStSel web server. BeStSel (Beta Structure Selection) is a novel method for the determination of secondary structure and fold recognition from protein circular dichroism spectra.

2.3. Antibacterial Assay

Three strains of gram-negative bacteria (*Escherichia coli*, *Salmonella typhimurium*, and *Vibrio cholerae*) and three strains of gram-positive (*Staphylococcus aureus*, *Bacillus subtilis*, and *Enterococcus facaelis)* were procured from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIBB).

The minimum inhibitory concentrations (MIC) of the peptides against the aforementioned bacterial strains were determined using polystyrene microassay plates (SPL, Pyeongtaek, South Korea). Briefly, a log-phase culture of the bacterial strains was diluted in LB broth and inoculated into the microplates to a final concentration of 1×10^5 cfu/mL. The peptides were diluted two-fold with serial dilution in LB broth and inoculated (9, 6, 4, 1, 0.25, 0.04, and 0.001 μM) into microplates. The microplates were then incubated at 37 °C for 18 h, and the absorbance at 600 nm was measured using a FilterMax™ F5 Multi-Mode Microplate Reader (Molecular Devices, USA). The MIC values, expressed in micromolar concentrations, represent the lowest concentration that inhibited the growth of the target microorganism. To evaluate the effect of temperature on the antibacterial activity, bacterial cultures in the presence of peptides were incubated for 24 h at a temperature below 25 °C, as bacterial growth is slowed down at low temperatures. At 25 and 37 °C, the bacterial cultures were incubated for 18 h. *Listeria monocytogenes* was chosen for this assay, as it can survive and multiply at low temperatures [25,26].

2.4. SYTOX Green Uptake Assay

SYTOX Green uptake assay was performed according to a previously described procedure [27]. Mid-logarithmic phase *S. aureus* were collected and washed thrice with a buffer (5 mM HEPES at pH 7.4 and 20 mM glucose). The bacterial pellet was resuspended in a buffer (5 mM HEPES at pH 7.4, 20 mM glucose, and 100 mMKCl). The SYTOX green dye was added to the bacterial suspension in buffer without the peptides. The bacterial suspension, at a concentration of 1×10^6 cfu/mL, was mixed with 0.5 μM SYTOX Green dye. The 2 × MIC peptides were added to the bacterial suspension with SYTOX green dye. The uptake of SYTOX Green dye was monitored for ~30 min in the dark using FilterMax™ F5 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA), with excitation and emission wavelengths of 485 and 520 nm, respectively. Melittin (a toxin from bee venom) was used as a positive control to obtain the maximal permeabilization values.

2.5. Competitive Dye Displacement Assay

Competitive displacement assay was performed at 25 °C using a fluorescence spectrometer (JASCO FP-6300). The assays were carried out by adding 5 μM of Ethidium bromide (EB) to 20 μM of calf thymus DNA (CT–DNA) solution, and the resulting mixture was titrated with varying concentrations of LEAPs (nLEAP-1, nLEAP-2, eLEAP-1, and eLEAP-2) from 0 to 18 μM at the interval of 10 min. The EB–CT–DNA complex was excited at 485 nm, and its emission spectra were recorded between 520 nm and 750 nm. The binding affinity of LEAPs with CT-DNA was calculated using the Stern–Volmer equation, in terms of the dissociation constant.

Stern–Volmer equation:

$$(F_0 - F)/F = [L]/K_d, \quad (1)$$

Here, F_0 and F are the fluorescence intensities of EB–CT–DNA complex in the absence and presence of LEAP, respectively, [L] is the concentration of the LEAPs, and K_d is the dissociation constant, representing a measure of the binding affinity between LEAPs and CT–DNA.

3. Results

3.1. Bioinformatics and CD Analysis of LEAPs

The amino acid sequences of LEAP-1 and LEAP-2 from *Dissostichus mawsoni* and *Lycodichthys dearborni* were aligned with several other known and predicted LEAPs. As shown in Figure 1, the multiple sequence alignment of mature LEAP sequences from teleost fishes and mammals revealed a highly conserved pattern of eight and four cysteine residues. The alignment also showed the presence of Arginine and lysine amino acids, which are typically associated with high antibacterial activity [28]. The presence of ATCUN motif in the peptides increases the chances of interaction with DNA, thereby promoting antibacterial activity [7]. The ATCUN motif (QSH) was found to be present in nLEAP-1, which could lead to increased interaction with DNA, and consequently, a more potent antibacterial activity. The CD analysis of LEAPs in 30% TFE showed a negative band at about 200nm, characteristic of β-turns loops and distorted β-sheets from 5 to 35 °C (Figure 2). The analysis of CD spectra data with BeStSel software (Beta structure selection) revealed the typical characteristics of β-sheet conformation, including antiparallel strands (antiparallel 1, 2, and 3) and beta turns, among others at different temperatures. Incubation of the nLEAP-2 and eLEAP-2 at different temperatures from 5 to 35 °C showed a change in the CD spectrum at low temperatures, indicating a modification of the peptide structure.

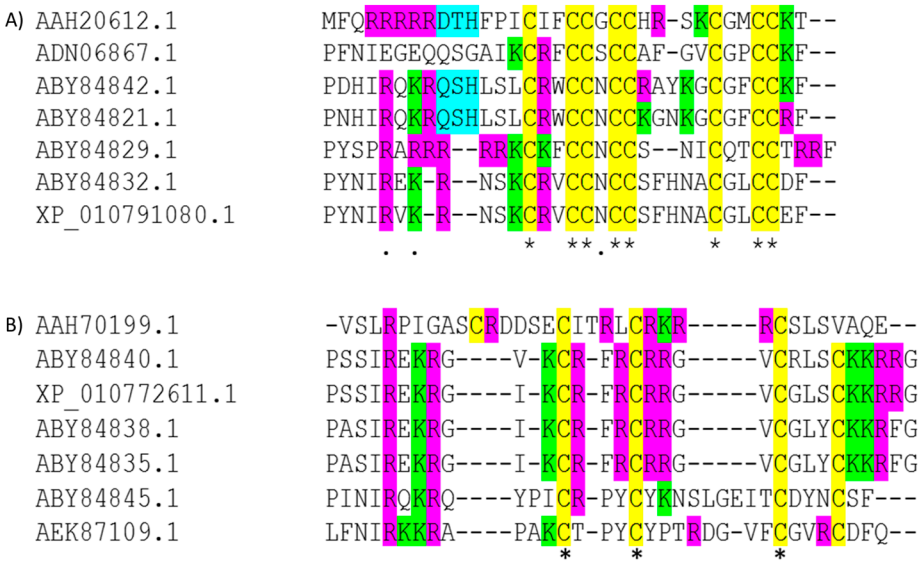


Figure 1. Multiple sequence alignment of Liver-Expressed Antimicrobial Peptides (LEAPs) from human (AAH20612.1, AAH70199.1); *Dissostichus mawsoni* (ABY84829.1, ABY84838.1); *Lycodichthys dearborni* (ABY84842.1, ABY84845.1); *Notothenia angustata* (ABY84832.1, ABY84835.1); *Notothenia coriiceps* (XP_010791080.1, XP_010772611.1); *Pogonophryne scotti* (ABY84821.1, ABY84840.1); and *Epinephelus coioides* (AEK87109.1, ADN06867.1). (**A**) The multiple sequence alignment of LEAP-1 shows highly conserved cysteine region and presence of basic amino acids arginine and lysine residues. The nLEAP-1 (Antarctic notothenioid liver-expressed antimicrobial peptide-1) and eLEAP-1 (Antarctic eelpout liver-expressed antimicrobial peptide-1) contain highly conserved arginine and lysine residues. eLEAP-1 contain ATCUN motif (Amino terminal Cu(II)- and Ni(II)-binding motif) which is also present in human LEAP-1 (Human liver-expressed antimicrobial peptide-1). (**B**) nLEAP-2 (Antarctic notothenioid liver-expressed antimicrobial peptide-2) shows highly conserved cysteine residues with conserved arginine and lysine amino acids and eLEAP-2 (Antarctic eelpout liver-expressed antimicrobial peptide-2) also shows conserved cysteine residues but contain low arginine and lysine amino acids as compared to other peptides. (Cysteine residues (C): highlighted with yellow colour; Arginine residues (R): highlighted with pink colour and lysine residues (K): highlighted with green colour).

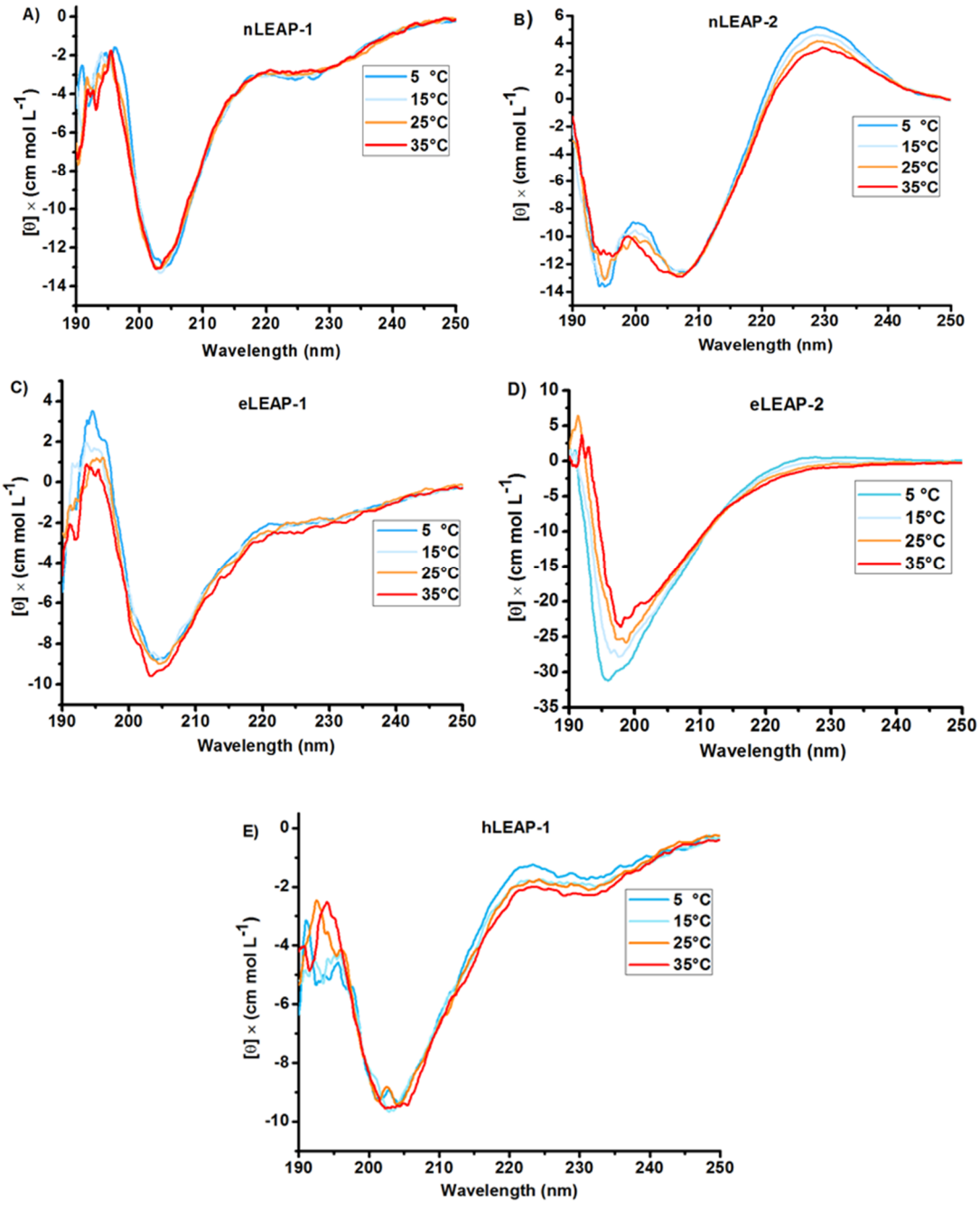


Figure 2. Representative CD spectra of LEAPs with temperature-dependent changes in (**A**) nLEAP-1, (**B**) nLEAP-2, (**C**) eLEAP-1, (**D**) eLEAP-2 and (**E**) hLEAP-1 at 5, 15, 25 and 35 °C. The CD spectra were recorded in structure-promoting solvent system trifluoroethanol, pH 7.4 (TFE, 30% *v*/*v* in water). The values from five independent scans were averaged per sample.

3.2. Antibacterial Activity

LEAP from human displayed broad-spectrum antibacterial activity [2,29]. Consequently, we determined the antibacterial activity of synthetic LEAPs (nLEAP-1, nLEAP-2 from *D. mawsoni*, and eLEAP-1, eLEAP-2 from *L. dearborni*) bearing the amino acid sequences found in Antarctic fishes [18] (Table 1). The antibacterial activity of LEAPs was tested against three gram-positive bacteria (*B. subtilis*, *E. facaelis*, and *S. aureus*) and three gram-negative bacteria (*E. coli*, *V. parahaemolyticus*, and *S. typhimurium*), as described elsewhere [27]. The positive control was human hepcidin-25 (hLEAP-1). MIC values of hLEAP-1, nLEAP-1, nLEAP-2, eLEAP-1, and eLEAP-2 are shown in Table 2. All peptides effectively

inhibited the growth of *V. parahaemolyticus*, with an MIC range of 0.25–4 μM. nLEAP-2 and eLEAP-2 presented lower MIC values than nLEAP-1 and eLEAP-1.

Table 2. Antimicrobial activity of peptides: hLEAP-1 (human), nLEAP-1, nLEAP-2 (*D. mawsoni*), and eLEAP-1, eLEAP-2 (*Lys. dearborni*) against gram-negative and gram-positive bacterial strains. nLEAP-2 effectively inhibited the growth of all bacterial strains with minimum MIC value range of 0.25 to 1 μM concentration. nLEAP-2 inhibited the growth of *B. subtilis*, *S. aureus* and *V. parahaemolyticus* with MIC (Minimum inhibitory concentration) value of 1 μM concentration.

	MIC for Gram-Positive Bacteria (μM)			MIC for Gram-Negative Bacteria (μM)		
Peptides	***E. facaelis***	***B. subtilis***	***S. aureus***	***E. coli***	***S. typhimurium***	***V. parahaemolyticus***
hLEAP-1	9	4	6	6	>9	1
nLEAP-1	9	4	6	9	9	1
nLEAP-2	1	1	1	1	1	0.25
eLEAP-1	9	9	9	>9	> 9	4
eLEAP-2	9	1	1	>9	> 9	1

To determine the effect of temperature, the MIC assay was also performed at 5, 15, 25, and 35 °C against *L. monocytogenes*. The LEAPs effectively inhibited the growth of *L. monocytogenes* at 5 °C (Table 3). Especially, nLEAP-2 and eLEAP-2 inhibited the bacterial growth, with an MIC value of 0.25 μM and 1 μM at 5 °C and 15 °C, respectively. However, at 25 and 35 °C, the MIC values of LEAP-1 and LEAP-2 were >9 μM, indicating that the antibacterial activity of LEAPs decreases with increasing temperature. This result may imply that Antarctic fish LEAPs have evolved to have higher antimicrobial activity at low temperature by reducing disulfide bonds.

Table 3. Temperature-dependent antimicrobial activity of peptides: hLEAP-1 (human), nLEAP-1, nLEAP-2 (*D. mawsoni*), and eLEAP-1, eLEAP-2 (*Lys. dearborni*) against *Listeria monocytogenes* at 5, 15, 25, and 35 °C. The peptides effectively inhibited the growth of *L. monocytogenes* at low temperatures. The nLEAP-2 and eLEAP-2 showed the highest antibacterial activity at low temperatures.

	MIC * Values (μM)			
Peptides	**5 °C**	**15 °C**	**25 °C**	**37 °C**
hLEAP-1	4	9	>9	>9
nLEAP-1	9	>9	>9	>9
nLEAP-2	0.25	1	>9	>9
eLEAP-1	4	>9	>9	>9
eLEAP-2	0.25	1	>9	>9

* MIC is the lowest concentration of an antimicrobial agent needed to kill bacteria.

3.3. Mechanism of Antibacterial Action

SYTOX green uptake assay was performed to investigate the cell penetration property of LEAPs, using a previously described procedure [30]. The SYTOX green dye cannot cross intact bacterial membranes, and it shows a significant increase in fluorescence intensity when bound to nucleic acids [31]. If the peptide causes membrane damage, SYTOX green can cross the bacterial membrane along with the peptide and bind to nucleic acids, resulting in a rise in fluorescence intensity. The bacterial membrane permeability of LEAPs was evaluated at 2 × MIC by monitoring the intracellular influx of SYTOX green dye. Melittin, the positive control, upon addition with bacterial solution, showed a significant increase in the fluorescence intensity of SYTOX green dye. LEAPs, upon addition with bacterial solution, showed a significant decrease in fluorescence intensity of SYTOX green dye, to a value well below the fluorescence intensity of the control without LEAPs (Figure 3). hLEAP-1, nLEAP-1, eLEAP-1, and eLEAP-2 showed a greater decrease in fluorescence compared with nLEAP-2.

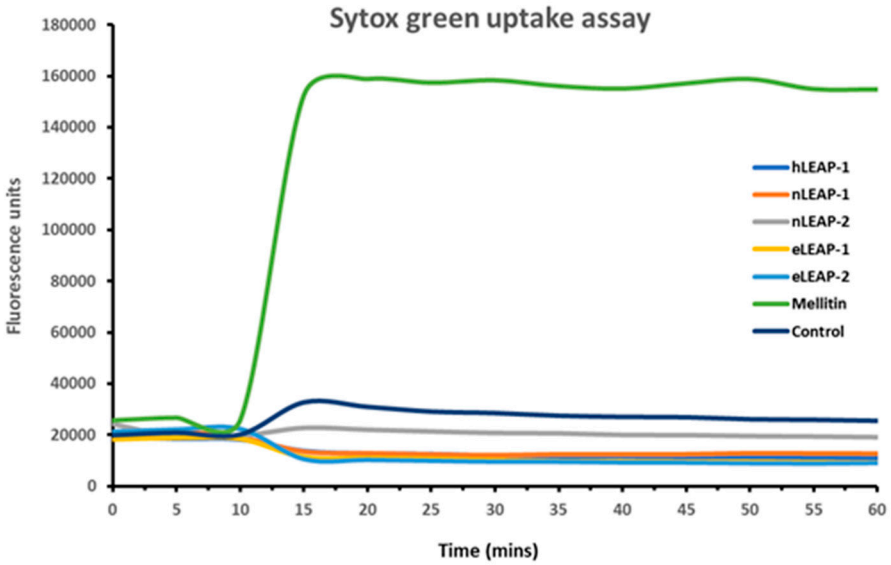


Figure 3. Kinetics of *S. aureus* membrane permeabilization caused by the LEAPs at 2 × MIC. The positive control (melittin) is also shown to provide maximal permeabilization values. *S. aureus* cells suspended in 5 mM HEPES, 20 mM glucose, and 100 mM KCl (pH 7.4) were incubated with 5 μM SYTOX Green and LEAPs. Modifications of the membrane by peptides allow the SYTOX green dye to enter the bacterial cell and bind DNA, resulting in fluorescence signal increase. Fluorescence of SYTOX green dye was monitored, at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

3.4. Competitive Dye Displacement Assay

Competitive displacement assay was performed to study the mechanism of interactions between LEAPs and CT–DNA. EB is a well-known fluorophore with a planar structure, which binds to DNA in an intercalative fashion. EB shows weak fluorescence in aqueous solution, but its fluorescence intensity is enhanced drastically upon intercalation within DNA base pairs. In this assay, any molecule that binds to DNA in a fashion similar to EB is expected to replace EB from the DNA helix in a competitive manner, which results in a decrease in the fluorescence intensity of the EB-DNA complex. With continuous addition of LEAPs to the EB–CT–DNA system, a significant decrease in the fluorescence intensity was observed (Figure S1 in Supplementary Data), likely due to the displacement of EB from the EB–CT–DNA complex by the LEAPs (nLEAP-1, nLEAP-2, eLEAP-1, and eLEAP-2). This suggested that LEAPs replace EB from the DNA helix, thereby indicating that LEAPs bind to DNA in an intercalative mode.

The binding affinity of LEAPs with CT–DNA was calculated in terms of K_d, using the Stern–Volmer plot, as shown in Figure S1 (Supplementary Data), with the help of Equation (1). The K_d values of nLEAP-1, nLEAP-2, eLEAP-1, and eLEAP-2 were found to be 4.1, 6.44, 6.08, and 6.21 μM, respectively. The K_d values of LEAPs were lower than that of the classical intercalator EB (K_d = 7 μM), as calculated previously by Kessissoglou et al. [32], with nLEAP-1 showing the lowest K_d value. The high K_d value of EB compared with nLEAP-1, nLEAP-2, eLEAP-1, and eLEAP-2 indicates that EB has a lower CT–DNA binding affinity than these LEAPs. These results indicate that nLEAP-1, nLEAP-2, eLEAP-1, and eLEAP-2 interact with CT–DNA double-helix, similar to EB, but with a higher CT–DNA binding affinity.

4. Discussion

Cold-adapted organisms have developed networks of defense mechanisms to protect themselves from the harsh aquatic environment [33]. Fish are in close contact with their environment, which contains a large number of pathogenic bacteria. Some of the pathogenic bacteria, such as *Streptococcus iniae*, are capable of even digesting fish tissues [12]. However, fish defend themselves against these pathogens via complex innate defense mechanisms [34,35]. Antimicrobial peptides in fish constitute

the first line of defense against microbial invasions [20]. Hepcidin is one of the most widespread antimicrobial amphipathic peptides involved in fish innate immunity [20]. It was first identified and isolated in human urine, and denominated as LEAP-1 or hepcidin [1], followed by its detection in other mammal and fish species [10,21,29,36–39]. Previous studies had mostly focused on the antimicrobial activities of the hepcidin with eight cysteine residues [2,5,8,40–42], whereas little information is available on the antimicrobial activity of novel, four-cysteine LEAPs isolated from Antarctic fish.

In the present study, we synthesized novel LEAPs derived from Antarctic notothenioid *D. mawsoni* and eelpout *L. dearborni*, with four and eight cysteine residues. The secondary structure of peptides was analyzed by circular dichroism (CD) spectroscopy. Analysis of the CD spectra indicated that the general conformational characteristics were preserved in all the structures of all the LEAPs (Figure 2). All the spectra were typical of β-sheet peptides, stabilized with disulfide bonds that were connected by turn-loop segments [43,44]. In spite of alterations in the disulfide-bonding pattern of trout hepcidin, the disulfide bonds variants show a typical CD spectrum of hairpin structure and maintain the antibacterial activity [5]. Similarly, the CD spectra of our LEAPs at 5, 15, 25, and 35 °C showed the characteristics of a typical beta-sheet structure.

Cationic antimicrobial peptides are defined as peptides with a net positive charge of +2 to +8, and an excess of arginine, lysine, and histidine amino acid residues [45]. These cationic antimicrobial peptides bind specifically to the membrane. Therefore, gram-positive and gram-negative bacteria, viruses, and fungi are the targets of such peptides [46]. nLEAP-1 and 2 have a net positive charge of +7 and +8, with an excess of arginine and lysine amino acids, which likely gives them the ability to bind to the bacterial membrane and efficiently inhibit their growth. The most potent cationic peptides have a charged hydrophilic portion that is segregated from the hydrophobic portion, for example, an amphipathic structure with a hydrophobic core separating two charged segments, which are well suited for undergoing transitions between hydrophilic and hydrophobic environments [46]. nLEAP-1 and nLEAP-2 have an amphipathic structure, which could be the reasoning behind its potent antibacterial activity against gram-positive and gram-negative bacteria. Generally speaking, more than 50% of the amino acids are hydrophobic, a fact reflected in the interaction of such peptides with the membranes as a part of their antibacterial mechanism. The key factors responsible for the antibacterial activity mechanism of action and selectivity toward bacterial cells are the composition of amino acids in the peptide which decides its flexibility, amphipathicity, net charge, hydrogen-bonding capacity, and hydrophobicity [47]. eLEAP-1 has a net +4.1 charge, and thus the peptide bind through the electrostatic interactions between positively charged amino acids on the peptide and negatively charged phospholipids headgroups on bacterial membrane and enter it [48]. eLEAP-2 has a net 0 charge. So, it may have weak electrostatic interaction such as hydrogen bonding with functional group present on the bacterial membrane and the hydrophobic region may help them to cross the outer bacterial membrane.

In the present study, we observed that the LEAPs with four cysteine showed an effective antibacterial activity against gram-positive and gram-negative bacteria, compared with the eight-cysteine peptides. As shown in previous studies, synthetic EC-hepcidin1 (four-cysteine), at a concentration of 18.6 μM or above, inhibits the growth of the gram-negative bacterium *V. vulnificus* and gram-positive bacterium *S. aureus* [21]. As mentioned in previous studies, when common carp (*Cyprinus carpio* L.) was injected intraperitoneally with *V. anguillarum*, the expression level of LEAP-2A was quickly upregulated in the liver, spleen, head kidney, skin, gills, foregut, and hindgut, while the expression level of LEAP-2B was similarly upregulated in the spleen, skin, gills, and hindgut, but not in the liver, head kidney, and foregut [49].

As Antarctic fishes live in a cold environment below 2 °C [50], and AMPs from Antarctic ice-fish have been known to show temperature-dependent antibacterial activity [51], we investigated the effect of low temperature on the antibacterial activity of LEAPs from Antarctic notothenioid and eelpout. We could identify effective antibacterial activity at low temperatures. With MIC values of 0.25 μM at 5 °C and 1 μM at 15 °C, nLEAP-2 and eLEAP-2 were more effective than nLEAP-1 and eLEAP-1, respectively.

To investigate the possible mechanism behind the antibacterial activity, we performed the SYTOX green uptake assay using *S. aureus* [5,27,31]. The LEAPs showed an initial increase, followed by an immediate decrease in the fluorescence intensity of SYTOX green dye, to a level below that of the negative control [5]. As described in a previous study, the trout hepcidin inhibits the bacterial cell growth but does not show cell permeabilization. Further confocal microscopic analysis of trout hepcidin had revealed the intracellular localization of hepcidin peptides [5]. In other studies, human β-defensin peptide HBD4 does not permeabilize the model lipid membranes but damage the inner membrane of *E. coli* cells, and showed the SYTOX green dye accumulation inside the cells by confocal microscopy studies [52]. Therefore, it is possible that LEAPs permeabilized the cells without causing lysis [5]. Another possibility is that LEAPs have a higher DNA binding affinity than the SYTOX green dye. Therefore, initially, the SYTOX green dye bound to DNA, leading to an increase in fluorescence intensity, after which, the LEAPs replace the DNA-bound SYTOX green dye, resulting in a decrease in fluorescence intensity. In previous studies, a similar quenching effect was noticed in the fluorescent intercalator displacement assay [53]. In this assay, the EB binds to DNA, thereby leading to an increase in fluorescence. However, the addition of a DNA-binding compound displaces the DNA-bound fluorescent dye, resulting in a decrease in the fluorescence signal [53]. We performed the competitive displacement assay to investigate whether LEAPs displace the SYTOX green dye from the DNA. It has been shown that cationic AMPs work by entering the cells and interfering with the metabolic functions, hampering the intracellular activities such as DNA and protein synthesis, post-translational folding, enzymatic activity, and cell wall synthesis [54]. LEAPs are β-sheet peptides which may form ion channels in lipid bilayers through aggregation. A short beta-sheet peptide forms porin-like voltage-gated channels in lipid bilayers [55]. Some studies show that the beta-hairpin form barrel-like structures in the lipid bilayer responsible for the peptides lytic action [56].The cationic antimicrobial peptides such as cecropin A2 [57] (+10), buforin II [58] (+7), human lactoferrin1–11 [59] (+4), P7 [60] (+7), APP [61] (+5), human α defensin 5 [62](+4), Human Neutrophil Peptide-I [63] (+3), and CP-1 [64] (+3) show DNA binding as a mechanism of action, as revealed by gel retardation assays and competitive EB displacement assays. Cecropin A2 [57], an α-helix peptide with 36 amino acids residues, isolated from mosquito *Aedes aegypti*, has been shown to inhibit DNA migration at a concentration 128 μM. Similarly, buforin II [58], a random coil peptide with 21 amino acid residues, derived from a natural peptide isolated from Asian toad, inhibits DNA migration at a DNA: peptide ratio of 1:50. Both cecropin A2 and buforin II show a concentration-dependent decrease in the intensity of the DNA band [57,58]. Human lactoferrin, an 11-amino-acid-containing peptide with 24% β-sheet and 41% α-helix structure, isolated from human salivary proteins, inhibits DNA migration at a DNA: peptide ratio of 1:100 [59]. P7 and APP, α-helix peptides with 20 amino acid residues, are analogs of the cell-penetrating peptide (CPP) ppTG2 [60,61,65]. P7 and APP inhibit the migration of DNA at a DNA: peptide ratio of 1:4 and 1:0.08, respectively [61]. Competitive EB displacement assays have shown that P7 and APP bind to DNA in an intercalative manner. Human α-defensin 5 [62], a β-sheet peptide with 32 amino acid residues, localized in the small intestinal Paneth cells in humans, is known to show DNA binding, with a dissociation constant value of 3 μM. Human neutrophil peptide-I [63], a β-sheet peptide, stored in azurophilic granules of polymorphonuclear neutrophils, inhibits DNA migration at a DNA: peptide ratio of 1:50. CP-1, a β-sheet peptide with 9 amino acid residues, obtained from T-cell antigen receptor (TCR) α-chain, inhibits DNA migration at a DNA: peptide ratio of 1:150 [64]. These DNA-binding peptides have varying net charges (+3 to +10) and structures (α-helix, β sheet, and random coil). Furthermore, the DNA-binding property of these peptides is not the outcome of electrostatic interactions between the cationic peptide and the polyanionic DNA. For example, lactoferricin B, a 25-amino-acid-residue peptide with one disulfide bridge and +8 net charge, does not bind to DNA even at a DNA: peptide ratio of 1:200 [66] Human β defensin 4, a β-sheet peptide with 48 amino acid residues shows the considerable damage to *E. coli* membrane [52]. nLEAP-2 with weak DNA binding affinity has high fluorescence intensity of DNA-bound SYTOX green signal, where nLEAP-1, eLEAP-1 and eLEAP-2 with strong DNA binding affinity have low fluorescence intensity.

Taken together, these results strongly support our hypothesis that decrease in the fluorescence intensity of DNA-bound SYTOX green signal is due to the competitive displacement of SYTOX green by LEAPs.

In this study, LEAPs with 4 cys residues (nLEAP-2 and eLEAP-2) showed significant antibacterial activity as compared to their 8 cys counterpart (nLEAP-1 and eLEAP-1) against both gram-positive and gram-negative bacteria. It has been suggested that, the LEAP-1 is involved in both iron regulation and antibacterial activity and LEAP-2 is involved only in antibacterial activity [22,23]. In teleost fish, due to the existence of multiple LEAP genes, functional separation was suggested. In which, the cellular and molecular evidence indicated that, some fish LEAPs may serve only as antimicrobial peptides, other fish and amphibian hepcidins may function as iron regulators [67]. Studies of synthetic human LEAP-1 mutants with 1 disulfide bond removed by pairwise substitution of cysteines with alanine showed only negligible decrease in ferroportin-binding activity and over half of the activity persisted even when 3 of the 4 disulfide bonds were removed leaving only the most terminal disulfide bond intact [44]. If the structure-function relationships of human LEAPs are applicable to Antarctic fish LEAPs, the peptide with 4cys with 2 disulfide bonds intact may have complete bacterial membrane binding ability.

To investigate whether LEAPs had distinct characteristics arising from their origin in fish that inhabit in a cold environment, we investigated the effect of temperature on their structure and antibacterial activity. We analyzed the CD spectra of LEAPs from 5 to 35 °C. The CD analysis of LEAP-2 showed a distinct change in the CD spectrum, among others, indicating a modification of the peptide structure. Thus, the structure of LEAPs are temperature sensitive [4,68]. Interestingly, the antibacterial activity of nLEAP-2 and eLEAP-2 increased at low temperatures as compare to the nLEAP-1 and eLEAP-1, this may indicate that these peptides are adapted to low temperatures with structural modification or that there was a conformational change in the bacterial membrane due to the lower temperature, leading to higher susceptibility to the peptide. Such as piscidin-like antimicrobial peptide, Chionodracine isolated from the *Chionodracohamatus* an icefish species, showed significant antibacterial activity against Antarctic psychrophilic bacteria strains *Psychrobacter* sp. *TAD1* (MIC = 10 μM) and *TA144* (MIC = 15 μM) at 15 °C, the Gram-positive *B. cereus* (MIC = 5 μM) at 25 °C, and the Gram-negative *E. coli* (MIC = 5 μM) at 25 °C. Nevertheless, when the activity of the peptide was tested against *E. coli* and *B. cereus* at 37 °C, the MIC levels increased to 20 μM and 10 μM, respectively [51].

The peptides with reduced number of disulfide bonds may show significant antibacterial activity. Such as the "rocket" analogue-N2 derived from the marine peptide NZ17074 (N1). It is characterized by two disulfide bonds and showed the strongest antimicrobial activity against Gram-negative bacteria with MIC values ranging from 0.25 to 1 mg/mL against *E. coli*, *Salmonella*, and *Pseudomonas* strains compared to other analogues [69].

The reduction to 4 cysteine (nLEAP-2 and eLEAP-2) in the Antarctic notothenioid LEAPs would increase the flexibility of the hairpin structure. In Antarctic and non-Antarctic notothenioid species the structure–function comparisons of lactate dehydrogenase A4 orthologs (A4-LDHs) showed that the amino acid substitution leads to increase in molecular flexibility in small areas of the cold-adapted A4-LDHs that affect the mobility of adjacent active sites resulting in modulation of the enzyme's catalytic rate (Kcat) and substrate-binding affinity (Km) [70]. In the 4 cys LEAPs of Antarctic fish, the most distinct substitutions are the cysteine residues that lead to the reduction of intramolecular disulfide bonds, and this would likely increase structural flexibility and its functional ability.

5. Conclusions

In the present study, we evaluated the antibacterial activities of novel LEAPs from Antarctic notothenioid and eelpout, as well as their mechanisms-of-action, using SYTOX green uptake assay and competitive displacement assay. LEAP-1 and LEAP-2 showed significant antimicrobial activity against gram-positive and gram-negative bacteria. LEAP-2 was more active then LEAP-1 against *L. monocytogenes* at 5 °C. SYTOX green uptake assay revealed that LEAPs do not kill microbial cells by forming pores in the bacterial membrane.

Further, to evaluate the observed decrease in fluorescence after the addition of LEAPs, which could be due to competitive binding to DNA against the dye in the SYTOX green uptake assay, we performed a competitive displacement assay. The assay results suggested that LEAPs competitively replace the dye bound to DNA, which is likely responsible for the observed decrease in the fluorescence of the SYTOX green dye. Therefore, bacterial cell membrane lysis and DNA binding likely constitute the mechanism-of-action of LEAPs in the context of their antibacterial activity.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/9/20/4299/s1, Figure S1: Emission spectra of Ethidium bromide (5 μM) bound to the DNA (20 μM) in the presence of varying concentrations of LEAPs from 0 to 18 μM at the interval of 10 min.

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