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ORIGINAL PAPER

Investigations into the ability of the peptide, HAL18, to interact with bacterial membranes

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Abstract Halocidin was isolated from hemocytes, *Halocynthia aurantium* as a heterodimeric peptide consisting of two α-helical subunits, Hal15 and Hal18. Hal18 was shown to have antibacterial properties against *Bacillus subtilis* (MLC = 15 μM) and *Escherichia coli* (MLC = 100 μM). The peptide was shown to produce stable monolayers, which were characteristic of α-helical peptides predicted to orientate parallel to the surface of the interface. Constant area assays showed that Hal18 was surface active (4 μM) inducing surface pressure changes >30 mN m^{-1} characteristic of membrane interactive peptides. The peptide induced stable surface pressure changes in monolayers that were mimetic of *B. subtilis* membranes (circa 7 mN m^{-1}) and *E. coli* membrane-mimics (circa 4 mN m^{-1}). Hal18 inserted readily into zwitterionic DOPE and anionic DOPG monolayers inducing surface pressure changes circa 8 mN m^{-1} in both cases, providing evidence that interaction is not headgroup specific. Thermodynamic analysis of compression isotherms showed that the presence of Hal18 destabilised *B. subtilis* membranes ($\Delta G_{Mix} > 0$), which is in contrast to its stabilising effect on *E. coli* lipid extract implying the differential antimicrobial efficacy may be driven by lipid packing.

Keywords Antimicrobial peptide · Monolayer stability · Peptide monolayer · Lipid–peptide interactions · Thermodynamic analysis

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Abbreviations

α	Interaction parameter
ΔH	Mixing enthalpy
CL	Cardiolipin
C_s^{-1}	Compressibility modulus
DOPE	Dioleoylphosphatidylethanolamine
DOPG	Dioleoylphosphatidylglycerol
α-AMPs	Alpha helical antimicrobial peptides
ΔG_{Mix}	Gibbs free energy of mixing

Introduction

In recent years overuse of antibiotics has led to the emergence and spread of drug-resistant pathogens. For example, there are now widespread reports of methacillin-resistant *Staphylococcus aureus* (MRSA), and bacterium that are no longer sensitive to antibiotics of last resort such as vancomycin (Hiramatsu et al. 1997). The pharmaceutical industry is therefore, constantly searching for new and effective antimicrobial agents, especially for the treatment of multiple drug-resistant bacteria (Diamond 2001; Reddy et al. 2004; Schroder and Harder 2006; Toke 2005; Zasloff 2002). In recent years considerable attention has been given to α-helical antimicrobial peptides (α-AMPs) that exhibit broad spectrum antimicrobial activity (Boman 1995; Zasloff 2002) against Gram-positive bacteria, Gram-negative bacteria, and fungi. These agents seem to have limited susceptibility to drug resistance yet despite intensive study there remains a lack of understanding of their structure/function relationships.

α-AMPs may utilise multiple modes of action. Those with even hydrophobicity distribution along the helical long axis have been postulated to support a mode of action

based on the carpet-type mechanism. In this case the peptide inserts into the outer leaflet of the bilayer causing asymmetric expansion and leading to lysis. Alternatively such a peptide may form transmembrane pores leading to permeabilisation of the bilayer and cell death. Theoretical and experimental studies have also suggested that many α-AMPs have a hydrophobicity gradient along their helix and in this case may invade bacterial membranes via the use of oblique orientated α-helical structure (Dennison et al. 2005a). Most recently, biophysical studies have supported this view and shown that the amphibian α-AMP citropin 1.1, destabilises membranes via angled bilayer penetration (Marcotte et al. 2003). This structural feature causes oblique orientated α-helices to penetrate membranes at a shallow angle of between 30° and 60° resulting in membrane destabilisation thereby promoting lysis (Brasseur 2000; Harris et al. 2000; Rahman et al. 1997; Thomas and Brasseur 2006).

Halocidin, a heterodimeric peptide consisting of two α-helical subunits containing 15 (ALLHHGLNCAKGVLA) and 18 (WLNALLHHGLNCAKGVLA) amino acid residues were isolated from hemocytes of invertebrates, *Halocynthia aurantium* (Jang et al. 2002). Halocidin is a powerful antimicrobial peptide with potent bactericidal action on a wide variety of antibiotic resistant bacteria including *Staphylococcus aureus* and *Pseudomonas aeruginosa.* It has also shown activity against pathogenic aspergillus and candida fungal species (Jang et al. 2002). Interestingly, this peptide exhibits low haemolytic activity indicating it could have potential for use as an antimicrobial (Jang et al. 2002). There appears to be little research into the mode of action used by Halocidin. In this study, we have therefore confirmed the antibacterial properties of Hal18 and investigated the mode of action by use of theoretical and biophysical analysis to gain an insight into its membrane interaction.

Materials and methods

Materials

The synthetic peptide, Hal18 (WLNALLHHGLNCAKGVLA) was synthesised by solid state synthesis and purified by HPLC to purity greater than 95%, confirmed by Electrospray isonisation mass spectrometry (Servern Biotech, UK). Dimyristoyl phosphatidylglycerol (DOPG) and dimyristoyl phosphatidylethanolamine (DOPE) were purchased from Alexis (Birmingham, UK). Buffers and solutions for monolayer experiments were prepared from Milli-Q water with a specific resistance of 18 M cm. All other reagents were purchased from Sigma (UK).

Antimicrobial activity assay

Overnight cultures of *E. coli* strain W3110 and *B. subtilis* strain NGMB 8054 in 3% tryptic soya broth (TSB: Difco) were subcultured in TSB and grown to the mid-exponential phase ($OD_{600} = 0.6$) at 37°C with shaking at 250 rpm. A radical diffusion assay was then undertaken according to the protocol previously described (Wei et al. 2005).

Whole lipid extracts of *B. subtilis* and *E. coli* membranes

The Bligh and Dyer (1959) method of lipid extraction was used to extract membrane lipids from cultures of *B. subtilis* and *E. coli*. In summary, cultures of *B. subtilis* and *E. coli* were established in nutrient broth according to the protocol previously described (Dennison et al. 2006). A 1 ml exponential phase culture ($OD = 0.6$; $\lambda_{nm} = 600$ nm) was washed twice in 25 mM Tris buffer at pH 7.5 and centrifuged using a Jouran bench top centrifuge at 14,000 rpm to form a cell pellet. After the final centrifugation, the cell pellet was suspended in 1 ml Tris buffer and to a 0.4 ml aliquot of this cell suspension, 1.5 ml chloroform/methanol (1:2) was added. This suspension was then vortexed vigorously for 5 min. An additional 0.5 ml of chloroform was added and the suspension was vortexed for 5 min. Finally 0.5 ml of water was added before vortexing for a further 5 min to produce two phases. The suspension was centrifuged, using a bench top centrifuge, at 3,000 rpm for 5 min to ensure separation of the phases. The lower organic layer was transferred to a new centrifuge tube and concentrated using a Jouran speed vac. The extracted lipids were stored at −20°C under N_2.

The surface activity of Hal18

To determine the surface activity of Hal18, surface activity assay was measured in the absence of lipid. These measurements were carried out in a custom milled Teflon trough 5 × 5 cm (NIMA Technology) and the surface tension was monitored by the Wilhelmy method using a Whatman's CH1 paper plate in conjunction with a microbalance. Peptide solution was injected into the stirred 10 mM Tris buffer subphase through a lateral hole using a Hammilton syringe. The concentrations of Hal18 ranged from 1 to 10 μM in the subphase. After injection, the surface pressure increased and continued to do so up to 30 min. The maximal values of these surface pressure changes were then plotted as a function of Hal18 final subphase concentration.

The ability of Hal18 to form a stable monolayer was also investigated using a 601 M Langmuir trough (Nima Technology, Coventry). Hal18 in methanol (2.5 mM) was

spread onto a 10 mM Tris buffer subphase to give 1.19×10^{15} peptide molecules. The peptide monolayer was allowed to settle for 30 min before compression of the barriers at a speed of 41.98 molecules min^{-1}. All experiments were carried out at 21°C.

The phase state of monolayers was studied using the compressibility modulus (C_s^{-1}). This parameter provides a measure of the compressional elasticity of a lipid monolayer, thereby providing information about lipid packing within the monolayer (Alminana et al. 2004), and is given by:

$$C_s^{-1} = -A\left(\frac{\Delta\pi}{\Delta A}\right) \quad (1)$$

where (π) represents monolayer surface pressure and (A) is the area per molecule in the monolayer.

Monolayer studies on the lipid interactions of Hal18

Insertion studies using Langmuir monolayers is a sensitive tool for studying lipid-peptide interactions (Hanakam et al. 1996). Cell membranes have different lipid compositions and so to develop a better understanding of peptide membrane interactions, the ability of Hal18 to penetrate bacterial lipid extract at constant area was studied. Monolayers were formed drop wise by spreading chloroform solutions of DOPG, DOPE, total lipid extract from *B. subtilis*, and *E. coli*, respectively, onto a 10 mM Tris (pH 7.5) subphase. After spreading, the solvent was allowed to evaporate off the subphase surface over 30 min and then the lipid monolayer compressed at a velocity of 10 cm^2 min^{-1} to give a surface pressure of 30 mN m^{-1} where the lipid packing density is equivalent to that of the outer leaflet of a cell membrane (Seeling 1987). Once the desired surface pressure was reached, the barrier position was kept constant. The film was then left to equilibrate for 5 min. In all experiments Hal18 was used to inject into the subphase to give a final peptide concentration of 4 μM in the subphase.

Thermodynamic analysis of Hal18 interactions with lipid monolayer isotherms

The ability of Hal18 to interact with lipid monolayers was also investigated using compression isotherms. Monolayers were formed by spreading onto a 10 mM Tris buffer subphase to give 2.5×10^{15} phospholipid molecules in chloroform solutions. Monolayers were formed from DOPG, DOPE, Cardiolipin and synthetic mixes were also prepared to mimic the membrane composition of *E. coli* and *B. subtilis* as described by Lohner and Prenner (1999). The lipid monolayers were allowed to settle for 30 min before compression of the barriers at a speed of 22.18 molecules min^{-1} until monolayer collapse pressure was achieved. These experiments were repeated in the presence of 4 μM Hal18. For all compression isotherms, changes in lipid monolayer surface pressure with changes in monolayer area were recorded.

The thermodynamic stability of the monolayers was investigated using the Gibbs free energy of mixing (ΔG_{Mix}). This parameter provides a measure of the relative stability of a monolayer by considering the energetics of miscibility of its pure lipid components and is given by:

$$\Delta G_{Mix} = \int_0^{\pi} \left[A_{1,2} - (X_1A_1 + X_2A_2)\right] d\pi \quad (2)$$

where $A_{1,2,\ldots n}$ is the molecular area occupied by the mixed monolayer, A_1, A_2 are the area per molecule in the pure monolayers of component 1 and 2, respectively, X_1 are X_2 are the molar fractions of the components and π is the surface pressure. Numerical data were calculated from the compression isotherms according to the mathematical method of Simpson (Todd 1963).

The interactions between component lipid molecules of monolayers were examined using the interaction parameter (α). This parameter relates the interaction of each molar fraction of lipid in a monolayer to the energy gain through mixing of the monolayer and is given by:

$$\alpha = \frac{\Delta G_{Mix}}{RT\left(X_1X_n^n + X_1^nX_n\right)} \quad (3)$$

where X are the molar fractions of the monolayer lipid components, $R = 8.314$ J mol^{-1} K^{-1} and $T = 294$ K.

The stability and binding interactions of monolayers were further investigated using the mixing enthalpy (ΔH), which is given by:

$$\Delta H = \frac{RT\alpha}{Z} \quad (4)$$

where R and T are as defined in Eq. 3, and Z is the packing fraction parameter, which is calculated using the Quikenden and Tan model (Quickenden and Tan 1974).

Results and discussion

Previous studies have shown the action of antimicrobial peptides involves membrane destabilisation by the use of lipid-interactive oblique orientated α-helices (Dennison et al. 2005a). This structural feature allows the peptide to penetrate the membrane at angle between 30° and 60°, thereby promoting the destabilisation (Dennison et al. 2005a). Hal18 shows structural characteristics possessed by many α-helical antimicrobial peptides. For example, the

helical wheel representation of Hal18 shows that the peptide has the potential to form an amphiphilic α-helix with a large hydrophobic apolar arc of 180°, rich in leucine residues (Fig. 1), which is characteristic of antimicrobial α-helical peptides (Dennison et al. 2005a). When the surface activity of Hal18 was investigated at the air/buffer interface using a Langmuir trough (Fig. 2) increasing peptide concentration caused increases in the surface activity up to 4 μM levels, which is again characteristic of membrane interactive peptides (Dennison et al. 2007). The surface pressure increased immediately after peptide injection and continued to do so until the maximal surface pressure was reached after 30 min. Hal18 also was able to form stable monolayers on the buffer subphase (Fig. 3), where the extrapolated area of the isotherm implies a molecular area for the peptide of 4.2 nm^2. This area is comparable to the predicted area of 4.05 nm^2 (Vie et al. 2000), which is in agreement with values obtained for other α-helical peptides that are predicted to orientate parallel to the surface of the interface (Dennison et al. 2005b). A standard radical diffusion assay was therefore used to investigate the antimicrobial activity of Hal18, against *E. coli* and *B. subtilis*. Hal18 exhibited a MIC of 15 μM for *B. subtilis* NGMB 8054 and 100 μM in the case of *E. coli* W3110. The data can be seen to support characterisation of Hal18 as an α-helical antimicrobial peptide and show it to exhibit differing levels of efficacy against *E. coli* and *B. subtilis.*

It is well established that antimicrobial peptides use membrane invasion as a primary killing mechanism (Brogden 2005) and that antimicrobial peptides can differ in their selectivity for different bacteria (Tossi et al. 2000). It is thought that cationic α-AMPs undergo electrostatic interaction with the target organism and it is recognised that *B. subtilis* has a much greater level of anionic DOPG

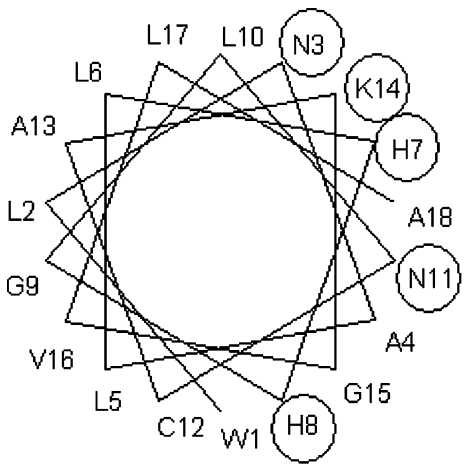

Fig. 1 The primary structure of Hal18 represented as two-dimensional axial projections according to Schiffer and Edmundson (1967). It can be seen that each peptide possesses a narrow hydrophilic face composed of polar and charged residues (*circled*)

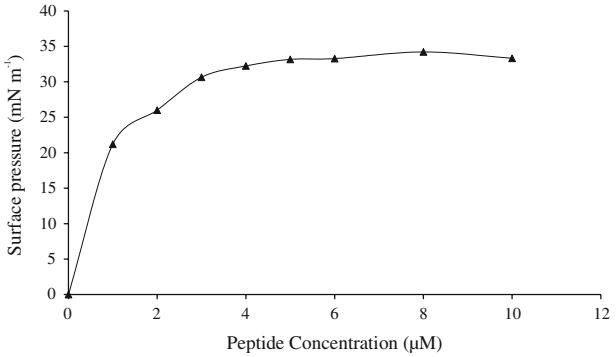

Fig. 2 Surface activity of Hal18 for an air–buffer interface is plotted against peptide concentration

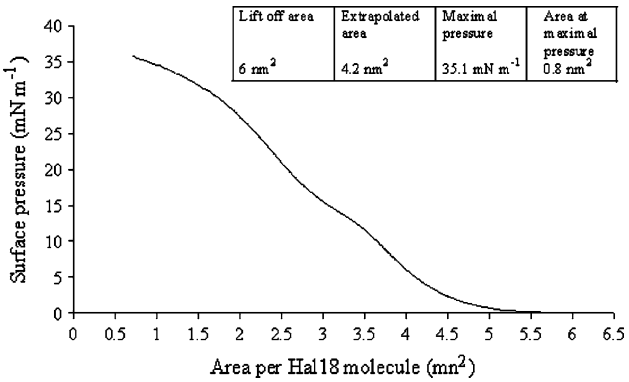

Fig. 3 Compression isotherm of Hal18 showing a pressure-area isotherm for a Hal18 monolayer which was spread onto a subphase of 10 mM Tris pH 7.5

in comparison to *E. coli,* which may be responsible for the differing susceptibility of the organism to Hal18 activity. When tested against DOPG and DOPE monolayers (Fig. 4) though Hal18 showed comparable pressure change within a comparable time frame indicating that interaction does not

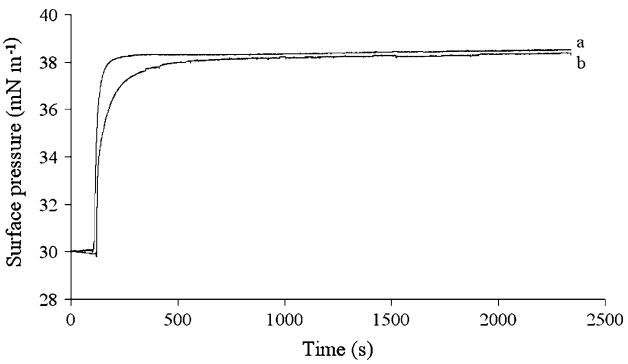

Fig. 4 Time course of Hal18 with lipid monolayer, which were formed DOPG (*a*) and DOPE (*b*). Monolayers were set at an initial surface pressure of 30 mN m^{-1}, mimetic of naturally occurring membranes and after 120 s Hal18 was introduced into the Tris buffer subphase

appear head group specific and is more likely to be driven by surface activity.

The reduced level of DOPE in *B. subtilis* membranes is compensated for by a large increase in DOPG and CL, which is known to support a preference towards non-lamellar formation (H_{II} phase). It may therefore be that varying efficacy is driven by differences in lipid packing and bilayer behaviour. The ability of Hal18 to insert into monolayers that were mimitic of *B. subtilis* and *E. coli* membranes was therefore investigated at constant area (Fig. 5). Hal18 induced stable increases in surface pressures for both *B. subtilis* and *E. coli* bacterial lipid membranes. In the case of *B. subtilis* the maximal surface pressure reached 37 mN m^{-1}, however, in the presence *E. coli* membrane mimics, the peptide induced maximal surface pressure 34 mN m^{-1} implying differences in membrane association. Given these differences do not appear due to headgroup variation (Fig. 4) this would support the theory that the lipid composition is able to effect the nature of membrane association via variation in packing constraints and overall stability, this in turn will lead to variation in preference for carpet-based insertion versus other mechanisms.

It is well established that the packing characteristics of component lipids is an important factor in determining the stability of membrane bilayers (Ishitsuka et al. 2006). To try and gain a further understanding of these differing effects, the stability and lipid packing of the monolayer was investigated using thermodynamic analysis of Hal18 interactions with synthetic *B. subtilis* and *E. coli* membrane isotherms. Isotherms (data not shown) were used to calculate the Gibbs free energy of mixing (ΔG_{Mix}). Table 1 shows that for both organisms, $\Delta G_{Mix} \ll RT = 2444.316$ J mol^{-1}, indicating that deviations from ideal mixing behaviour in these model membranes are small (Sospedra et al. 2001). Isotherm analyses of *B. subtilis* membranes showed that ΔG_{Mix} was positive in the absence of Hal18 but ΔG_{Mix} was greater in the presence of Hal18 (Table 1), which in combination indicate that the peptide had a thermodynamically destabilising effect on these membranes. Further thermodynamic analysis of isotherms was undertaken and the interaction parameter (α) and mixing enthalpy (ΔH) were calculated for these monolayers using Eqs. 3 and 4. Table 2 shows that in the absence of Hal18, both α and $\Delta H > 0$ for *B. subtilis* model membranes. However, in the presence of Hal18, α and ΔH increased confirming that the peptide had a destabilising effect on *B. subtilis* lipid monolayers. The monolayer data (Fig. 5) showing deep penetration by Hal18 and subsequence destabilisation of the system would support the suggestion that Hal18 promotes toxicity in *B. subtilis* using an oblique orientated α-helix to disrupt the bilayer structure. Similar mechanisms of action have been proposed by Boland and Separovic (2006) for antimicrobial peptides of comparable sequence length and characteristics. This structural feature allows the peptide to penetrate the membrane at angle between 30° and 60°, thereby promoting membrane destabilisation (Dennison et al. 2005a).

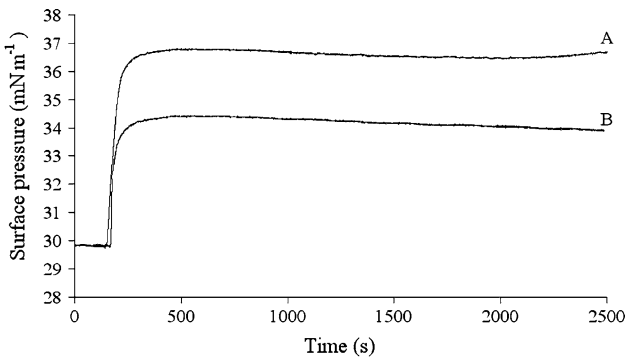

Fig. 5 Time course of Hal18 with lipid monolayers, which were formed from *B. subtilis* (*A*) and *E.coli* (*B*) lipid extract. Monolayers were set at an initial surface pressure of 30 mN m^{-1}, mimetic of naturally occurring membranes and after 120 s Hal18 was introduced into the Tris buffer subphase

Table 1 The Gibbs free energy of mixing (ΔG_{Mix}) of lipid monolayers at varying surface pressure (π)

π (mN m^{-1})	ΔG_{Mix} (J mol^{-1})			
	B. subtilis		*E. coli*	
	−Hal18	+Hal18	−Hal18	+Hal18
5	305.85	378.46	−160.21	−287.43
10	543.72	764.46	−310.63	−629.97
15	848.55	1054.26	−413.27	−880.15
20	1160.17	1640.98	−513.57	−1068.72

In contrast, isotherm analyses of *E. coli* membranes showed that ΔG_{Mix} was negative in the absence of Hal18 indicating higher levels of stability than in the case of *B. subtilis*. In further contrast to *B. subtlils*, the addition of Hal18 appears to have a thermodynamically stabilising effect on *E. coli* membranes. In addition thermodynamic analysis indicates that in the absence of Hal18, both α and $\Delta H < 0$ for *E. coli* model membranes. However, in the presence of Hal18, α and ΔH was more negative indicating that these membranes were thermodynamically more stable in the presence of Hal18. This would imply the interaction of the peptide with the membrane is thermodynamically favourable. The lower pressure change (Fig. 5) may be due to the more stable system limiting peptide insertion. Such an interpretation would fit if in this case antibacterial activity was driven by a carpet mechanism. In this model the peptide inserts into the outer leaflet in a stable manner expanding the area related to the inner leaflet. Similar

Table 2 The interaction parameter (α) and enthalpy of mixing (ΔH) of lipid monolayers at varying surface pressure (π)

π (mN m^{-1})	α				ΔH (J mol^{-1})			
	B. subtilis		*E. coli*		*B. subtilis*		*E. coli*	
	−Hal18	+Hal18	−Hal18	+Hal18	−Hal18	+Hal18	−Hal18	+Hal18
5	14.62	18.10	−16.08	−28.85	17876.71	22120.60	−19659.66	−35270.06
10	26.00	36.56	−31.19	63.24	31779.77	44681.65	−38116.57	−77301.40
15	40.58	50.42	−41.49	−88.36	49596.25	61619.94	−50710.90	−107999.90
20	55.48	78.48	−51.56	−107.30	67809.91	95912.53	−63018.05	−131138.70

mechanisms of antibacterial action have been proposed for VP1 and aurein 1.2 (Dennison et al. 2008).

Conclusion

In summary Hal18 appears effective against both *B. subtilis* and *E. coli* but has higher level of efficacy against *B. subtilis*. Analysis implies membrane interaction is not specific to lipid head group but that the differing efficacy could relate to different modes of action and be driven through surface activity. In the case of *B. subtilis* the data support a lytic mechanism driven by oblique structure, in contrast to *E. coli* where the data are more supportive of a carpet based mechanism. It is recognised that oblique orientated helices area able to generate membrane disruption and lysis at low concentrations in line with the MIC values seen in the case of *B. subtilis*. The data confirm previous studies (Dennison et al. 2008) which show the importance of target membrane composition as well as amino acid composition in determining the mode of action for α-AMPs.

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