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Article

Truncated Equinin B Variants Reveal the Sequence Determinants of Antimicrobial Selectivity

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Abstract

Equinin B (GQCQRKCLGHCSKKCPKHPQCRKRCIRRCFGYCL), a marine peptide from *Actinia equina* exhibits antibacterial activity against both Gram-positive and Gram-negative bacteria. To identify a smaller active region, the peptide was cleaved into three fragments: GQCQRKCLGHCS (EB-1), KKCPKHPQCRK (EB-2) and RCIRRCFGYCL (EB-3). Only the 11-residue C-terminal fragment showed selective activity against Gram-positive bacteria, including *Staphylococcus epidermidis*, *Bacillus subtilis*, and *Enterococcus hirae*, while remaining inactive against *Escherichia coli*. Peptide modifications, achieved by replacing cysteine residues with arginine, generally did not enhance activity, but in the C-terminal fragment they reduced hemolytic activity and increased bacterial specificity. Membrane depolarization assays confirmed that the unmodified fragment strongly disrupts bacterial membranes, whereas the modified variant showed minimal depolarization, highlighting its markedly reduced membrane-disruptive potential. *In silico* modelling revealed that the unmodified fragment (EB-3) can adopt multiple membrane-disruption modes, from transient shallow pores to carpet-like mechanisms, while the cysteine-to-arginine variant interacts mainly via partial insertion anchored by arginine residues. Phenylalanine appears to interact with the membrane, and reducing hydrophobicity by its removal abolished antibacterial activity. These findings highlight the 11-residue C-terminal fragment as a tunable, membrane-targeting motif with mechanistic novelty, offering a blueprint for developing safer, selective antimicrobial peptides with reduced cytotoxicity.

Keywords: marine peptides; solid phase peptide synthesis; structure-function relationship; membrane permeability; peptide specificity; peptide-membrane interactions; Gram-positive bacteria

1. Introduction

Even though the discovery of antibiotics revolutionized medicine, their inappropriate use has enabled the spread of resistant bacteria and rendered commercially available antibiotics ineffective. Antibiotic resistance (AR) is one of the greatest threats, and the development of new molecules with antibacterial activity has become a global priority [1–3]. Antimicrobial peptides (AMPs), naturally occurring defense molecules found across many species, represent a powerful alternative antibacterial strategy against antibiotic-resistant pathogens [4]. As part of the innate immune

response, they have low antigenicity and exhibit antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, viruses, and parasites. Because AMPs are molecules with multiple mechanisms of action, they have a low potential for inducing resistance [5–7]. Terrestrial environments have been primarily studied for natural peptides useful to the pharmaceutical industry, even though the marine environment covers 70% of the earth's surface [8,9]. Only in recent decades has the proven therapeutic and anti-infective potential of peptides from marine sources opened a wide field of interest for researchers and companies seeking valuable bioactive compounds from marine fauna and flora [10]. Peptides derived from marine sources have different functions and structures than those isolated from terrestrial sources, due to the distinct adaptive pressures these organisms have experienced during evolution. Most bioactive compounds isolated from marine environments originate from symbiotic microorganisms and organisms, especially mollusks and cnidarians [11,12]. One of these is *Actinia equina* (Linnaeus, 1758), a little-studied anthozoan coelenterate of the *Actiniidae* family, widespread from the Indo-Pacific to the Mediterranean and the Atlantic. It is exposed to harsh conditions since it lives mainly in intertidal zones but also up to a few meters deep, and has developed the capability of to remain out of the sea for hours [13]. From this cnidarian's tentacle, two peptides called equinin A and B were isolated [13]. Equinin B (EB) showed promising antibacterial activity (minimum inhibitory concentrations (MIC) and bactericidal concentrations of 1 mg/mL and 0.25 mg/mL, respectively). The amino acid sequence: GQCQRKCLGHCSKKCPKHPQCRKRCIRRCFGYCL with 34 amino acids, shows a similarity of 37.78% with aurelin, a 40-residue AMP isolated from the mesoglea of the jellyfish *Aurelia aurita* which exhibits activity against Gram-positive and Gram-negative bacteria [14]. It was also found that truncated peptides derived from aurelin and other AMPs could have significant antimicrobial activity [15,16]. We set ourselves the challenge of preparing truncated peptides from equinin B and investigating their antimicrobial activity. The advantage of peptide-based drugs is that they can be easily designed and rapidly synthesized with optimal purity and high yield [17]. This is possible thanks to the development of solid-phase peptide synthesis (SPPS) by Merrifield back in 1963 [18]. This methodology has been improved and automated over the years, allowing the preparation of peptide sequences in large quantities and within short timescales [19]. The synthesis of smaller peptides is easier and faster than that of longer peptides, and truncated parts of bioactive peptides that still retain activity are valuable.

In this paper, we divided equinin B into three parts: the first part contains 12 residues, and the second and third parts contain 11 residues each: GQCQRKCLGHCS (**EB-1**), KKCPKHPQCRK (**EB-2**) and RCIRRCFGYCL (**EB-3**), respectively. In the second series, all Lys residues (if present in the peptide) were replaced by Arg (**EB1-K** and **EB2-K**), and in a third series, all Cys residues were replaced by Arg (**EB1-C**, **EB2-C** and **EB3-C**) (Figure 1). Some amino acids were removed from the peptides, resulting in **EB1a-K** (C3) and **EB3a-C** (F7). After evaluating the antimicrobial activity against *Escherichia coli* and *Staphylococcus epidermidis*, two active peptides, **EB-3** and **EB3-C**, were identified with **EB-3** displaying broad, non-specific activity, whereas **EB3-C** was active only against bacteria and showed no hemolytic effects. Their distinct activities appear to arise from structural differences, with **EB3-C** likely inducing only small membrane defects that selectively destabilize bacterial membranes.

2. Results and Discussion

2.1. Structure-Function Guided Design of the Peptide Library

In order to identify biologically active regions in the equinin B sequence, we generated a set of peptides and applied structure–function–driven modifications to enhance their antimicrobial activity, as shown in Figure 1. In the initial set of peptide syntheses, we prepared three truncated peptides, **EB(1–3)**, derived from EB (Figure 1). The residues are identical to those in equinin B, but the sequence was cleaved twice: after 12 (Ser) and after 23 (Lys), resulting in three peptides, **EB-1**, **EB-2** and **EB-3** (Figure 1). We first evaluated their antimicrobial activity against *Escherichia coli* and

Staphylococcus epidermidis over a concentration range of 0.8 to 100 μ M (Table 1). Determination of their MICs in cation-adjusted Mueller–Hinton broth (MHB) revealed that none of the peptides exhibited inhibitory activity against *E. coli* or *S. epidermidis* within this range, except for peptide **EB-3**, which had a MIC of 25 μ M against *S. epidermidis*. Assuming the **EB-3** region is associated with antimicrobial activity, we analyzed its amino acid composition. Since it lacked lysines, we replaced all lysines in **EB-1** and **EB-2** with arginine, a modification expected to enhance hydrogen-bonding capacity and strengthen interactions with membrane phospholipids, thereby improving membrane-disruptive activity—a hallmark of classical antimicrobial peptides. This step led to the generation of the modified peptides **EB1-K** and **EB2-K**. However, this modification did improve activity against either *E.coli* nor *S.epidermidis*. To further enhance membrane interactions, we replaced all cysteines with arginines in all three peptides. Cysteines can form disulfide bridges, which may constrain peptide flexibility and affect membrane interactions; replacing them with arginine was expected to increase positive charge, reduce disulfide bond formation, and enhance peptide–membrane interactions. Despite these modifications, the antimicrobial activity of all peptides remained unchanged. Nevertheless, only **EB3-C** retained antimicrobial activity, and this activity was observed solely against *S. epidermidis*. We also investigated whether removing the cysteine at position 3 in **EB1-K** would affect activity, resulting in the peptide **EB1a-K**; however, this modification did not improve antimicrobial activity. Notably, the **EB-3** peptide had the highest hydrophobic content at 27%, whereas all other peptides ranged between 8% and 18%. To evaluate whether hydrophobicity contributes to antimicrobial activity, we removed the phenylalanine at position 7 in **EB3-C**, generating the peptide **EB3a-C**. This modification abolished activity against *S. epidermidis*, confirming that hydrophobicity is likely the driving force behind the antimicrobial activity of **EB-3**.

Peptides were synthesized using an automated Fmoc-SPPS approach from the C-terminus to the N-terminus.

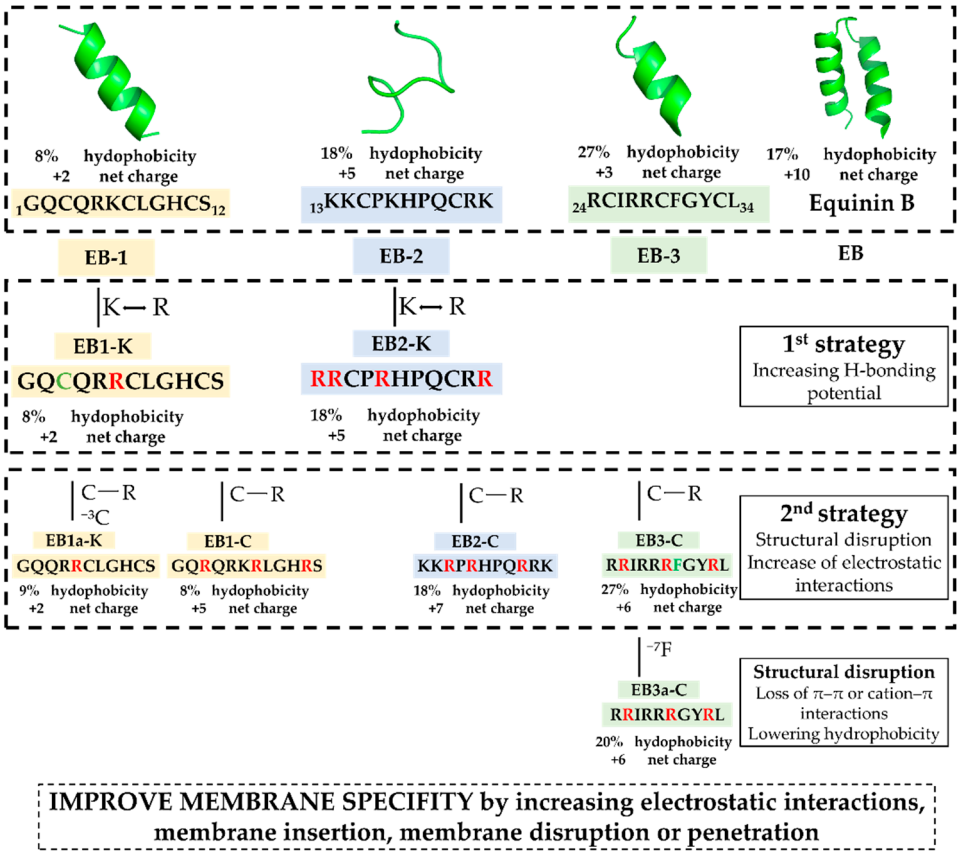


Figure 1. Schematic representation of the design strategy.

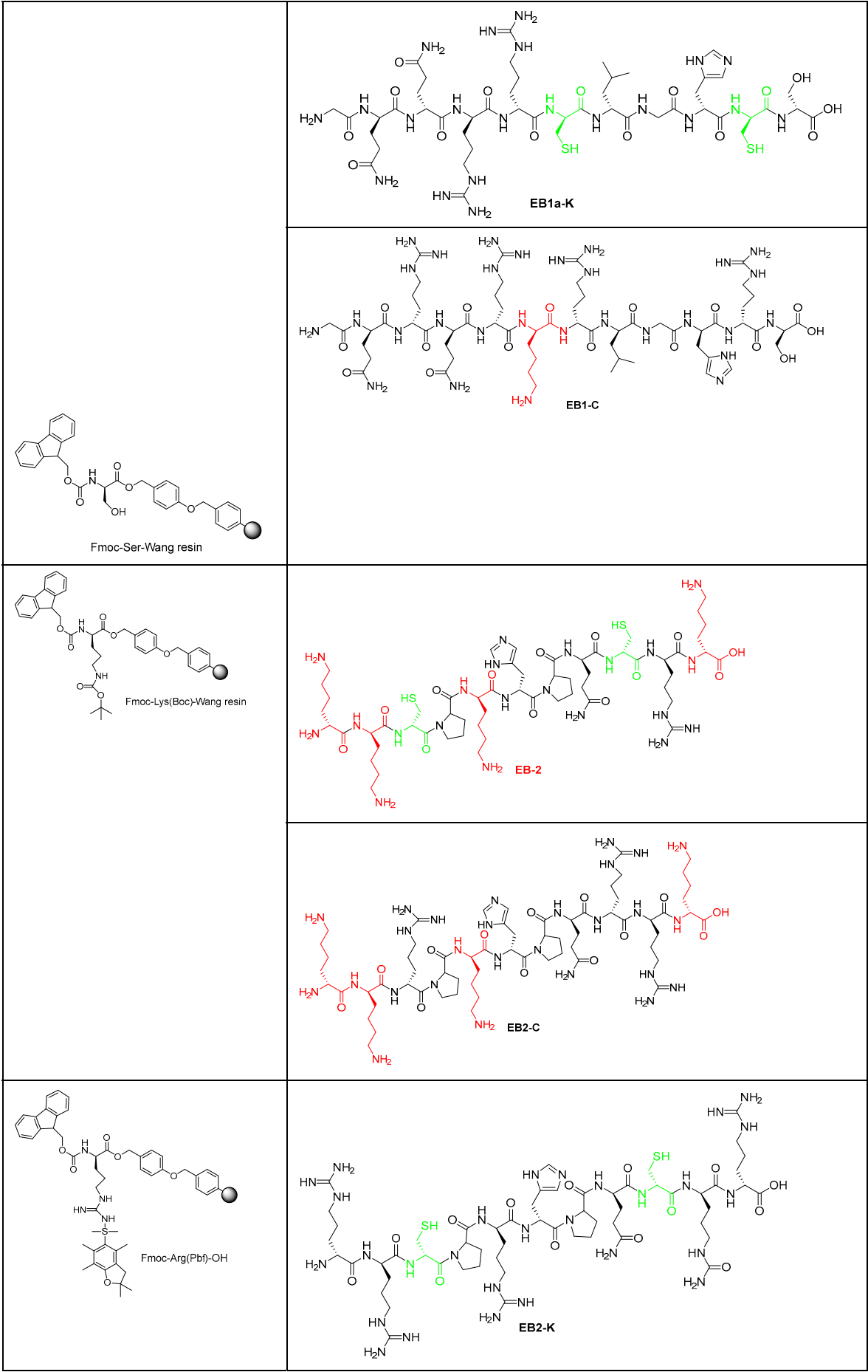
Table 1. Antimicrobial activity of compounds against *E.coli* and *S.epidermidis*.

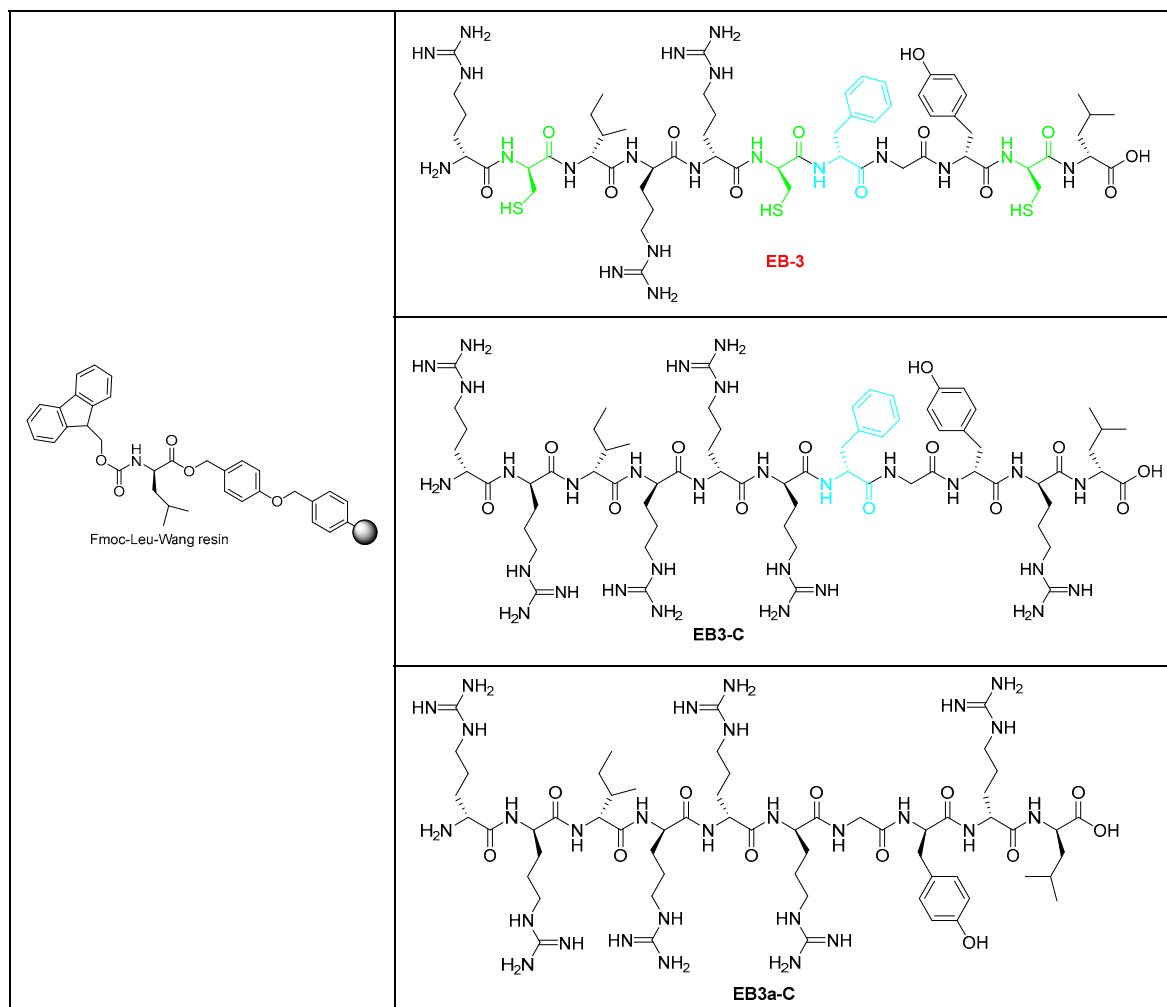
Compound	Sequence	<i>Escherichia coli</i>	<i>Staphylococcus epidermidis</i>
		MIC ₉₀ /μgmL ⁻¹	MIC ₉₀ /μgmL ⁻¹
EB-1	GQCQRKCLGHCS	>100	>100
EB-2	KKCPKHPQCRK	>100	>100
EB-3	RCIRRCFGYCL	100	25
EB1a-K	GQQRRCLGHCS	>100	>100
EB-1K	GQCQRRCLGHCS	>100	>100
EB-2K	RRCPRHPQCRR	>100	>100
EB1-C	GQRQRKRLGHR	>100	>100
EB2-C	KKRPKHPQRRK	>100	>100
EB3a1-C	RRIRRRFGYRL	>100	>100
EB3-C	RRIRRRFGYRL	>100	25-50
EB3a-C	RRIRRRGYRL	>100	>100

The peptides were synthesized on Wang resin with the C-terminal amino acid attached as follows: Fmoc-Ser-Wang resin, Fmoc-Lys(Boc)-Wang resin, Fmoc-Arg(Pbf)-Wang resin, and Fmoc-Leu-Wang resin (Table 2). Synthesis was performed in dimethylformamide using HBTU/OxymaPure® as coupling reagents. The Fmoc group from the first amino acid and subsequent residues after coupling was removed with 20% piperidine in DMF. For activation of carbonyl groups, 0.4 M *N*-methylmorpholine (NMM) was used. After completing the peptide sequence, the peptide was cleaved from the resin and the protecting groups were removed with 95:2.5:2.5 TFA:TIS:H₂O. After deprotection, peptides were precipitated in diisopropyl ether and centrifuged. Following HPLC purification, the compounds were desalted on SPE C18 columns and lyophilized.

Table 2. Starting resins with first anchored amino acids and final peptides obtained with SPPS.

[illegible]





*The amino acids in the truncated peptides where changes were made to the original equinin B sequence are colored differently from the other amino acids. In the derivatives, the new amino acids (Arg) are shown in black. The changes are made to Lys (red) and Cys (green), which are substituted with Arg, or some amino acids are simply removed (Cys in **EB1a-K**, Phe (blue) in **EB3a-C**).

2.2. Evaluation of Peptide Specificity

To assess whether the peptides exhibit activity against other cell types, such as human erythrocytes, and to estimate their specificity and therapeutic index, we performed hemolysis assays (Figure 2). None of the peptides showed hemolytic activity up to 400 μM , except for **EB-3**, which caused significant lysis at concentrations lower than its MIC. Interestingly, replacing cysteine in **EB-3** improved its cytotoxic profile, as **EB3-C** did not induce hemolysis. This modification resulted in a notable therapeutic index of higher than 16, calculated as 400 μM (no hemolysis) divided by 25 μM (MIC against *S. epidermidis*).

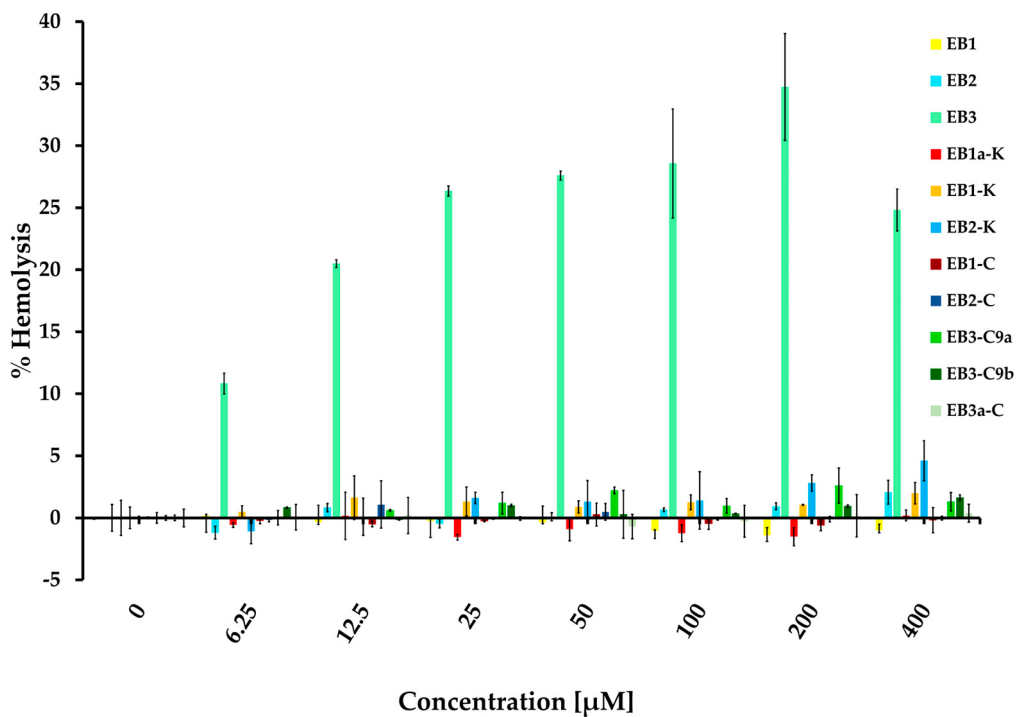


Figure 2. Hemolysis of peptides. Hemolytic activity of peptides was estimated in 2.5 % human erythrocytes derived from pooled whole human blood of at least 10 donors.

2.3. *In Silico* Structural Profiling

In silico structural profiling revealed poorly defined secondary structures in most of the peptides, with a general tendency toward alpha-helix formation (Figure 3). Among them, **EB-3** adopts the most well-defined alpha-helix, featuring less flexible termini and a distinct segregation of charged and hydrophobic residues—hallmarks of an amphipathic helix. This structural arrangement is reflected in its high amphipathic character, as indicated by the highest hydrophobic moment (μ) among the peptides analyzed. Moreover, **EB-3** exhibits the lowest ΔG for partitioning from water to octanol, suggesting a strong potential for membrane interaction (Table 3). Altogether, these features are consistent with key characteristics commonly observed in antimicrobial peptides [20,21]. High μ values are often associated with increased cytotoxicity toward eukaryotic membranes due to stronger and less selective membrane interactions. In this context, it is noteworthy that EB3 exhibited hemolytic activity, whereas its analog EB3a did not. This suggests that reducing amphipathicity may enhance peptide specificity toward bacterial membranes. Interestingly, the modification from **EB-3** to **EB3-C** also resulted in a complete loss of α -helical structure when modeled with AlphaFold [22], whereas PepFold predicted only a small remaining α -helical segment. Whether this difference in secondary structure contributes to the observed increase in selectivity remains an open question. Nevertheless, the larger, contiguous hydrophobic patch in the **EB-3** peptide compared with the more distributed hydrophobicity of the **EB3-C** may explain why the **EB-3** shows activity against both bacteria and erythrocytes, whereas the **EB3-C** remains selective for bacteria.

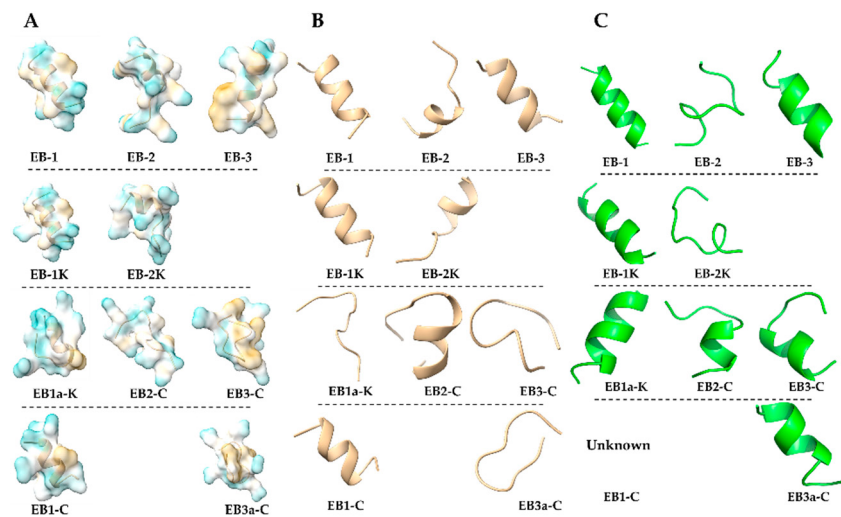


Figure 3. Structural surface characteristics of the conformational assemblies of peptides A) AlphaFold predictions of secondary structure exposing charged (blue) and hydrophobic regions (beige). B) AlphaFold predictions illustrating the folding topology. C) PepFold predictions illustrating the folding topology.

Table 3. Presentation of the difference in Gibbs energies and the moment of hydration for the prepared peptides, Wimley–White partitioning parameters (amphipathicity = hydrophobic moment, μ ; Δ GW-OCT, free energy transfer from water to bilayer).

Compound	ΔG	Total Hydr. Moment
EB-1	9.93	5.3
EB-2	16.35	4.48
EB-3	1.73	6.97
EB1a-K	16.86	5.47
EB1-K	16.84	4.42
EB2-K	20.29	2.17
EB1-C	15.42	4.59
EB2-C	20.01	4.01
EB3-C	7.22	5.58
EB3a-C	8.93	5.47

The bilayer partitioning free energy (Δ G) describes the energetic cost of transferring peptide residues from an aqueous environment into the hydrophobic core of a membrane. It is typically estimated using the water-to-octanol scale, which serves as an experimental proxy for membrane partitioning. Lower (more negative) Δ G values indicate a higher tendency of the peptide to insert into the lipid bilayer, promoting membrane association and potential disruption. Conversely, higher (less negative or positive) Δ G values suggest limited membrane affinity and lower antimicrobial potential [23]. Accordingly, the more favorable (negative) Δ G values observed for **EB-3** and its analogs suggest a potential for stronger membrane activity compared to **EB-1** and **EB-2**.

2.4. Evaluation of Membrane Permeability and Activity

To further evaluate the antimicrobial activity and specificity of **EB-3** and **EB3-C**, we determined their MIC values against *Bacillus subtilis* and *Enterococcus hirae*, using LL-37 as a reference peptide (Figure 4A). Both **EB-3** and **EB3-C** exhibited antimicrobial activity against these strains. The MIC was

in the range of 25.6-102.4, showing the following order of activity for both peptides: *S. epidermidis* > *B. subtilis* > *E. hirae* for both peptides. To investigate whether their mode of action involves membrane permeabilization, we performed propidium iodide (PI) uptake assays (Figure 4B), in which the fluorescent dye penetrates cells only when the membrane integrity is compromised. While LL-37 caused extensive membrane permeabilization in *B. subtilis*, the EB peptides did not induce significant membrane damage even at concentrations up to 400 μ M. A slight increase in permeability was observed for both EB peptides, representing only about 20% of the level induced by LL-37. Interestingly, none of the peptides permeabilized *E. hirae* or *S. epidermidis*. On one hand, these results suggest that the EB peptides may act through a more specific, possibly non-lytic mechanism, and that *B. subtilis* may be more sensitive to their activity. On the other hand, considering that LL-37, a peptide with a well-established membrane-disruptive mechanism, was also unable to permeabilize *E. hirae* and *S. epidermidis*, it is likely that smaller or transient membrane perturbations, undetectable by PI staining, could still be sufficient to cause bacterial death. To test whether small defects in the membrane contribute to the antimicrobial effect, we performed a membrane depolarization assay using the voltage-sensitive dye DiSC₃(5). The results revealed that all peptides induced slight depolarization of the bacterial membrane, with **EB-3** showing a markedly stronger signal than the other variants. These data support the idea that EB-3's antimicrobial effect is closely tied to its ability to disrupt membrane potential. Nevertheless, although some degree of membrane depolarization is detectable for **EB3-C**, the effect is relatively modest, and it remains unclear whether **EB3-C** alters or depolarizes the membrane sufficiently for its antimicrobial activity to be directly attributed to membrane disruption. This pattern points toward a non-membranolytic or intracellular mode of action for **EB3-C**.

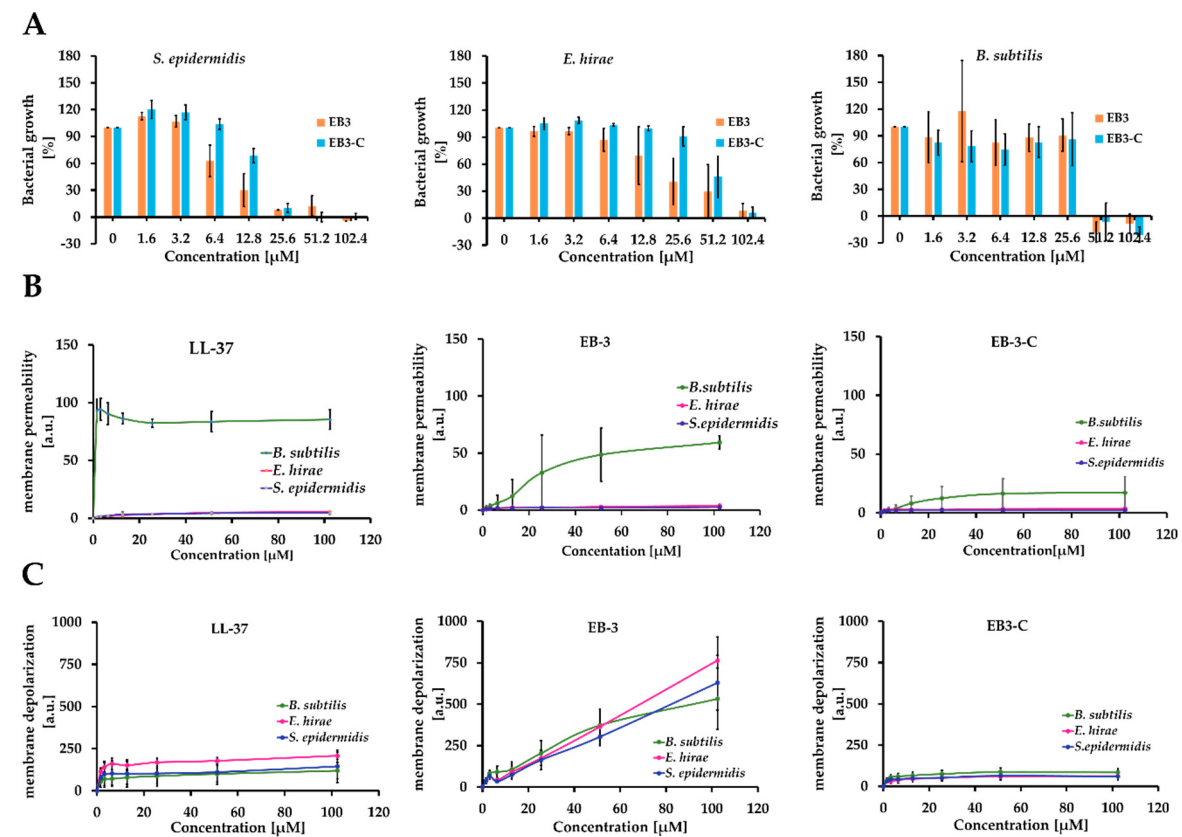


Figure 4. Impact on the Membrane Integrity by EB peptides. A) Bacterial growth, B) membrane permeability and C) Membrane depolarization of *B. subtilis*, *E. hirae* and *S. epidermidis* exposed to **EB-3** and **EB3-C**. Data of A) and B) are mean results of three independent experiments performed in duplicates. Data of C) are mean results of two independent experiments performed in duplicates.

2.5. In Silico Evaluation of Peptide-Membrane Interactions

To predict the molecular interactions of the peptides with bacterial membranes, we performed AlphaFold modeling using membrane compositions resembling *B. subtilis*, *S. epidermidis* and *E. hirae* (Figure 5). For **EB-3** interacting with *Bacillus* membranes, the peptide exhibited transient pore-like insertion accompanied by the formation of a short α -helix. This insertion was relatively shallow, correlating with limited membrane permeability observed experimentally. Notably, hydrogen bonds formed between the backbone of residues R1 and R5, where the N-terminal NH_3 group interacts with the carbonyl oxygen of R5, alongside similar interactions involving the amide nitrogen and residue C2. In contrast, interaction with *E. hirae* membranes showed peptide helix formation with surface association but no membrane insertion. Here, binding was primarily to phosphate (PO_4) groups on the membrane surface, with residue Y9 oriented outward. For *S. epidermidis*, the peptide appeared to act via a detergent-like carpet mechanism, forming short aliphatic helices. Residue F7 inserted into the lipid bilayer along with L11 and I7, while R1 and R5 residues interacted with surface PO_4 groups. Although no similar interactions were observed for R4, they remain possible. Consistent with *E. hirae*, Y9 faced outward, suggesting a conserved orientation.

EB3-C interacts with all Gram-positive membrane mimics through a consistent mechanism involving the formation of a short amphipathic helix, followed by membrane insertion primarily mediated by residue F7 and stabilized through interactions between R4/R6 and phosphate groups. In *B. subtilis* mimics, insertion occurs via F7 alone, whereas in *E. hirae* membranes both F7 and Y9 contribute to anchoring the peptide more deeply. For *S. epidermidis* mimics, insertion is supported by F7 together with L11, again combined with the characteristic R4/R6-phosphate interactions. These conserved electrostatic contacts, together with slight variations in hydrophobic anchoring residues, likely underpin **EB3-C**'s selective and non-hemolytic membrane-destabilizing activity.

Overall, the modeling indicates that **EB-3** lacks membrane selectivity because it does not adopt a stable, deeply inserted conformation in any of the Gram-positive membrane mimics. Instead, it interacts in multiple, non-specific ways—ranging from shallow, transient insertion in *B. subtilis*, to purely surface binding in *E. hirae*, and a carpet-like mode in *S. epidermidis*. This variability, together with its inconsistent hydrophobic anchoring and reliance on general phosphate interactions, prevents **EB-3** from targeting a specific membrane architecture and explains its broad, non-selective activity. Interestingly, **EB-3** also displays greater peptide-peptide interactions during membrane simulations, which along with its larger hydrophobic patch may underlie its reduced specificity and ability to disrupt both bacterial and erythrocyte membranes.

In addition, we performed structure predictions for **EB3a-C** to assess whether the F residue in **EB-3C** is important for membrane interaction. Modelling with *Bacillus* membranes revealed fish-hook-like structures on the surface, characterized by a backward-bent end forming a short β -sheet, with no deep membrane insertion. The peptide showed strong binding to phosphoglycolohydroxamic acid (PGH) on the membrane surface. For *E. hirae* and *S. epidermidis*, **EB3a-C** appeared mostly unfolded, with deep insertion of the C-terminal leucine (L10) into the membrane and association of arginine residues with phosphate (PO_4) groups; notably, R1 was oriented outward. These observations suggest that **EB3a-C** interacts predominantly via surface binding and shallow insertion, relying on specific lipid interactions rather than deep membrane penetration. The structural differences from **EB-3**, including the β -sheet formation and altered insertion depth, likely contribute to its lack of activity.

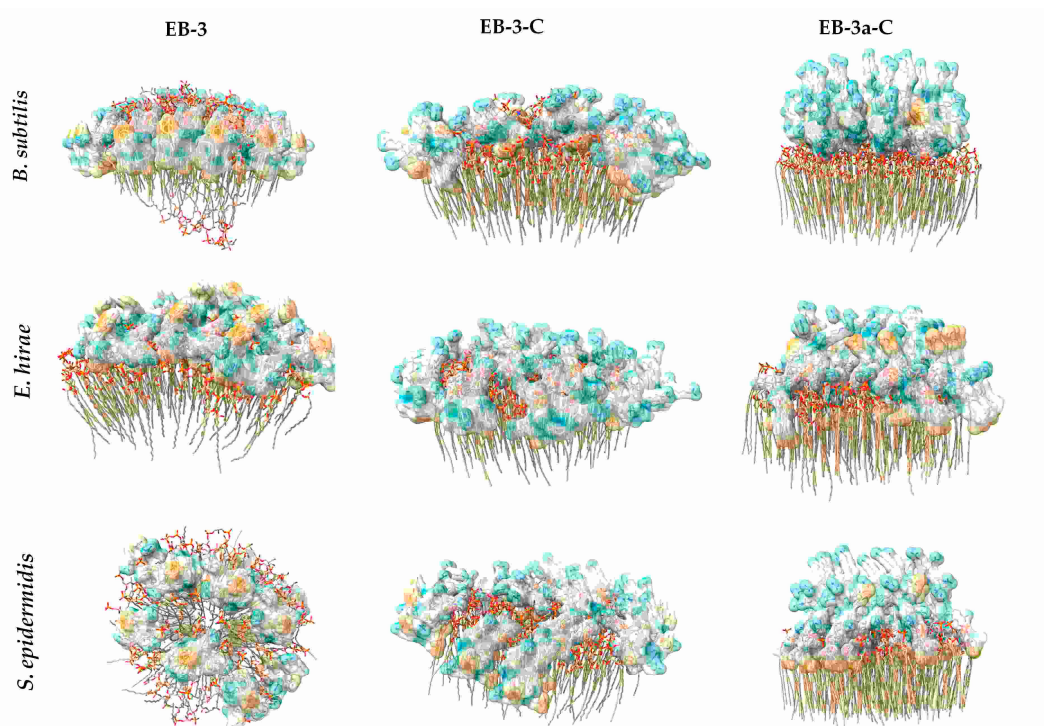


Figure 5. Prediction models of EB-3, EB3-C and EB3a-C interacting with membranes mimicking *B. subtilis*, *E. hirae* and *S. epidermidis* cytoplasmic membranes.

3. Materials and Methods

3.1. General

Commercial reagents and solvents were obtained as follows: Fmoc-Ser(tBu)-Wang resin, Fmoc-Lys(Boc)-Wang resin, Fmoc-Leu-Wang resin, Fmoc-Cys(tBu)-OH, Fmoc-Gly-OH, Fmoc-Phe-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Pro-OH and Fmoc-Ile-OH all purchased from Merck (Darmstadt, Germany).

NMR spectra were recorded on a Bruker AV 600 spectrometer, operating at 150.91 MHz for ^{13}C and 600.13 MHz for ^1H nuclei. The spectra were measured in deuterated dimethyl sulfoxide ($\text{DMSO}-d_6$) at 25 °C. Chemical shifts in parts per million (ppm) were referenced to tetramethylsilane (TMS). Spectra were assigned based on one dimensional ^1H , ^{13}C and APT (Attached Proton Test) and 2D homonuclear COSY (Correlation Spectroscopy) and heteronuclear HMQC (Heteronuclear Multiple-Quantum Correlation) and HMBC (Heteronuclear Multiple Bond Correlation) experiments.

Solid phase peptide synthesis was carried out on SPPS PS3 (Protein Technologies, Inc., Tucson, Arizona, USA) in dimethylformamide (DMF) as solvent, and 20 % piperidine as deprotecting reagent and 0.4 M NMM as activating reagent in combination of coupling reagents HBTU and OximePure.

RP-HPLC analysis and purification was performed on analytical column Zorbax C18 (4.6×150 mm, 5 μm , Agilent Technologies, Waldbronn, Germany) and preparative column Zorbax (21.2×150 mm, 5 μm , Agilent Technologies, Waldbronn, Germany). The analyses were performed on an Agilent 1200 Series System (Agilent Technologies, Waldbronn, Germany), equipped with a vacuum degasser, a quaternary pump, a thermostated column compartment, an autosampler and a variable wavelength detector. The method used was gradient A as follows: 0.1% TFA/water (eluent 1); MeOH (eluent 2); 0-30 min 100 % eluent 1 to 100 % eluent 2; 30-35 min 100% eluent 2; 35-35.1 min 100 % eluent 2 to 100 % eluent 1; 35.1-40 min 100 % eluent 1; flow rate: 1 mL min $^{-1}$ (analytical) and 10 mL min $^{-1}$ (preparative); detection wavelength: 210 and 215 nm; injection volume: 20-50 μL (analytical), 5 mL (preparative).

3.2. Peptide Synthesis

All peptides were synthesized on Wang resin by automated SPPS on a PS3 peptide synthesizer (Protein Technologies, Inc., Tucson, Arizona, USA), following the Fmoc-strategy. The following Fmoc-amino acids were used: Fmoc-Ser(tBu)-Wang resin, Fmoc-Lys(Boc)-Wang resin, Fmoc-Leu-Wang resin, Fmoc-Cys(tBu)-OH, Fmoc-Gly-OH, Fmoc-Phe-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Pro-OH and Fmoc-Ile-OH. Fmoc deprotections were performed with a solution of 20% piperidine in DMF. Peptide assembly was performed with the SPPS standard coupling cycle using commercial Wang resin with anchoring first (C-terminus) amino acid and following Fmoc-protected amino acids (4 eq in DMF), OxymaPure (4 equiv in DMF), and HBTU (4 equiv in DMF). Briefly, the resin charged with the all-protected Fmoc-peptide was treated with a mixture of TFA/TIS/H₂O (95:2.5:2.5, *v/v/v*) at room temperature for cleavage from resin and deprotection of side amino acid chains. After 3 h, the resin was filtered off. The peptides were precipitated with cold diisopropyl ether, centrifuged and lyophilized. Purification and subsequent analysis of peptides was performed using HPLC as explained in Section 3.1. NMR spectra are shown in Supplementary Materials (SM): Figure S1.1.-S1.20.

EB-1, Gly-Gln-Cys-Gln-Arg-Lys-Cys-Leu-Gly-His-Cys-Ser (GQCQRKCLGHCS)

Yield: 86%; C₅₀H₈₆N₂₀O₁₈S₃. *t_R* (A) = 20.23 min. ¹H NMR (600 MHz, DMSO) δ 8.58 - 7.31 NH, 6.94 (s, 0.3H) NH-His, 6.83 (s, 0.7H) NH-His, 4.53 (dd, *J* = 12.8, 7.5 Hz, 1H) α-Gln, 4.42 (dt, *J* = 9.8, 6.4 Hz, 1H) α-Gln, 4.39 - 4.33 (m), 4.31 - 4.22 (m, 3H) α-His, α-Ser, α-Leu, 3.73 (d, *J* = 6.2 Hz, H) α-Gly, 3.70 (dd, *J* = 11.0, 5.4 Hz, 1H) β-Ser, 3.64 (dd, *J* = 11.0, 4.5 Hz, 1H) β-Ser, 3.60 (s, 2H) δ-Arg, 3.08 (d, *J* = 5.9 Hz, 2H) δ-Arg, 3.02 - 2.96 (m) β-His, 2.94 - 2.89 (m) β-Ser, 2.89 - 2.82 (m) β-His, 2.78 - 2.73 (m) ε-Lys, 2.71 - 2.66 (m) ε-Lys, 2.18 - 2.06 (m, 4H) γ-Gln, 1.95 - 1.85 (m, 2H) β-Cys, 1.79 - 1.68 (m, 4H) β-Cys, 1.60 (dd, *J* = 13.6, 6.9 Hz, 2H) γ-Leu, 1.56 - 1.42 (m, 8H) β-Lys, β-Leu, β-Cys, 1.37 - 1.28 (m, 2H) γ-Lys, 0.86 (d, *J* = 6.6 Hz, 3H) δ-Leu, 0.81 (d, *J* = 6.5 Hz, 3H) δ'-Leu. ¹³C NMR (151 MHz, DMSO) δ 174.08 - 165.85 CO, 156.8 ε-Arg, 134.4 ε-His, 132.5 γ-His, 118.10 δ-His, 61.23 Ser-β, 54.9 α-His, 53.3 α-Ser, 53.0 α-Gln, 52.8 α-Cys, 52.3 α-Gln, 52.2 α-Cys, 52.1 α-Cys, 52.1 α-Lys, 52.0 α-Arg, 51.2 α-Leu, 42.0 α-Gly, 41.9 α-Gly, 41.87, 40.6 β-Leu, 40.4 δ-Arg, 40.1 δ-Arg, 40.0 - 39.0 DMSO, 38.7 ε-Lys, 31.6 β-His, 31.4 γ-Gln, 31.2 γ-Gln, 30.63, 30.6 *t*-Bu-OH, 29.9 β-Arg, 29.7 β-Arg, 29.1 β-Cys, 28.5 β-Cys, 27.9 β-Cys, 26.6 β-Lys, 24.9 γ-Arg, 24.0 γ-Leu, 23.1 Leu-δ, 22.1 γ-Lys, 21.4 Leu-δ' MS: *m/z* [M + H]⁺ (calc: 1318.58); [M+2H]²⁺ calc. *m/z* 660.2925; exp. *m/z* 660.2924.

EB-2, Lys-Lys-Cys-Pro-Lys-His-Pro-Gln-Cys-Arg-Lys (KKCPKHPQCRK)

Yield: 77%; C₅₇H₁₀₁N₂₁O₁₃S₂. *t_R* (A) = 14.52 min. ¹H NMR (600 MHz, DMSO) δ 8.42 - 7.10 NH, 4.75 (s, 1H), 4.62 (d, *J* = 7.1 Hz, 1H), 4.46 - 4.32 (m, 4H) α-Pro, α-Lys 4.28 (m, 1H) α-Gln, 4.20 (dd, *J* = 13.5, 8.2 Hz, 2H) α-Lys, 3.84 (t, *J* = 6.5 Hz, 2H) α-Lys, 3.61 (m, 2H) δ-Pro, 3.40 (m) δ-Pro, 3.15 - 3.03 (m) δ-Arg, 2.93 (dd, *J* = 12.9, 5.9 Hz, 2H) β-Pro, 2.89 - 2.84 (m) β-Pro, 2.77 (m) β-Pro, ε-Lys, 2.68 (dd, *J* = 13.0, 7.3 Hz, 1H) β-Pro, 2.20 (t, *J* = 7.6 Hz, 2H) γ-Gln, 2.09 (m, 1H) β-Gln, 1.99 (m) β-Cys, 1.91 (m) β-Gln, 1.84 (m, 2H) β-Cys, 1.76 (m, 4H) δ-Lys, 1.64 (m, 2H) δ-Lys, 1.63 - 1.49 (m) β-Lys, 1.46 - 1.31 (m) δ-Lys, γ-Lys. ¹³C NMR (151 MHz, DMSO) δ 174.3 - 169.9 CO, 157.11 ε-Arg, 59.9 α-Pro, 59.6 α-Pro, 53.3 α-His 52.6 α-Arg, 52.5 α-Gln, 52.4, 52.13 α-Cys, α-Cys, 52.1 α-Lys, 51.8 α-Lys, 51.5 Lys, 50.5 α-Lys, 47.00 δ-Pro, 40.6 δ-Arg, 40.0 - 39.4 DMSO, 38.8 ε-Lys, 38.7 ε-Lys, 38.6 ε-Lys, 31.5 γ-Gln, 31.4 β-Gln, 31.0 β-His, 30.7 *t*-Bu-OH, 30.6 -δ-Lys, 30.5 δ-Lys, 30.0 δ-, 29.6 β-Pro 29.1 β-Pro, 27.5 β-Cys, 26.6 β-Lys, 26.5 β-Lys, 26.5 β-Lys, 26.4 β-Lys, 24.7 γ-Arg, 24.4 γ-Pro, 24.3 γ-Pro 22.3 γ-Lys, 22.1 γ-Lys, 21.0 γ-Lys. MS: *m/z* [M + H]⁺ (calc: 1352.74); [M+2H]²⁺ calc. *m/z* 676.8743; exp. *m/z* 676.8730.

EB-3, Arg-Cys-Ile-Arg-Arg-Cys-Phe-Gly-Tyr-Cys-Leu (RCIRRCFGYCL)

Yield: 77%; C₅₉H₉₆N₂₀O₁₃S₃. *t_R* (A) = 23.34 min. ¹H NMR (600 MHz, DMSO) δ 8.94 NH-Leu, 8.05-7.50 NH, (8.16 d, NH-Arg, NH-Cys; 7.99 d, NH-Arg; 7.94 dd, NH-Gly; 7.92 d, NH-Tyr; 7.89 NH-Ile; 7.84 d, NH-Phe; 7.79 d, NH-Cys; 7.74 d, NH-Leu; 7.24- 7.17 (m) δ-Phe, ε-Phe, ξ-Phe, 7.16- 7.10 NH, 7.02-7.00 (d, *J* = 8.4 Hz) δ-Tyr; 6.65-6.63 (d, *J* = 8.4 Hz) ε-Tyr, 4.54-4.47 (m) α-Cys, α-Arg, 4.42-4.38 (m) α-Arg, 4.36-4.30 (m) α-Tyr, α-Phe, 4.25-4.21 (m) α-Ile, α-Leu, 3.78-3.71 (m) α-Gly, 3.67-3.62 (m) α-Gly, 3.59-3.58 α-Arg, 3.14-3.05 δ-Arg, β-Phe, 2.97-2.86 (m) β-Tyr, β-Phe, β-Cys, β-Arg, 2.80-2.66 (m) β-Arg, β-Cys, β-Tyr, β-Ile, 1.81-1.65 (m) β-Ile, 1.62-1.41 (m) γ-Ile, γ-Leu, γ-Arg, γ-Ile, 1.19-1.08 (m) γ-Ile, 0.95-

0.81 (m) δ -Leu, δ -Ile, CH₃-(β -Ile). ¹³C NMR (151 MHz, DMSO) δ 171.1 CO-Phe, Tyr, 170.8 CO-Ile, Arg, 170.8 CO-Arg, 169.9, 169.7 CO-Cys, 169.5 CO-Leu, 168.2 CO-Gly, 157.0 ϵ -Arg, 156.9 ϵ -Arg, 156.9 ϵ -Arg, 155.8 ξ -Tyr, 137.6 γ -Phe, 130.1 δ -Phe, 129.2 ϵ -Phe, 127.9 δ -Tyr, 127.6 γ -Ty, 126.2, 114.8 ϵ -Tyr, 57.0 α -Ile, 54.3 α -Cys, 54.2 α -Phe, 53.3 α -Arg, 53.2 α -Tyr, 53.0 α -Arg, 52.3 α -Cys, 52.2 α -Cys, 51.9 α -Arg, 51.2 α -Leu, 42.0 β -Gly; 40.3 δ -Arg; 37.3 β -Phe, 37.0 β -Tyr, 36.5 β -Ile, 30.0 β -Cys, 29.9 β -Cys, 29.3 β -Arg, 29.0 β -Arg, 24.9 γ -Arg, 24.3 γ -Arg, 24.1 γ -Leu, 22.9 δ -Leu, 21.8 δ -Leu, 15.3 CH₃-(β -Ile), 10.9 δ -Ile. MS: m/z [M + H]⁺ (calc: 1389.67); [M+2H]²⁺ calc. m/z 695.3392; exp. m/z 695.3413.

EB1a-K, Gly-Gln-Gln-Arg-Arg-Gly-His-Cys-Ser (GQQRRLGHCS)

Yield: 92%; C₄₇H₈₁N₂₁O₁₅S₂. t_R (A) = 18.23 min. ¹H NMR (600 MHz, DMSO) δ 8.55 - 6.88 NH, 4.59 (dd, J = 13.2, 7.6 Hz, 1H) α -Arg, 4.44 (td, J = 8.4, 5.2 Hz, 1H), 4.36 (dt, J = 13.8, 6.9 Hz, 2H), 4.34 - 4.29 (m, 2H) α -Gln, 4.29 - 4.25 (m, 2H) α -His, 4.23 (dd, J = 14.0, 7.9 Hz, 1H) α -Gln, 3.75 - 3.69 (m, 4H) β -Ser, α -Gly, 3.66 - 3.62 (m, 2H) β -Ser, α -Gly 3.59 (d, J = 2.8 Hz, 2H) δ -Arg, 3.11 - 3.05 (m, 5H) δ -Arg, 3.04 - 2.99 (m, 2H) β -His, 2.92 (dd, J = 12.9, 5.1 Hz, 2H) β -His, 2.84 (dd, J = 12.6, 6.1 Hz, 1H) β -Cys, 2.69 (td, J = 13.3, 8.5 Hz, 2H), 2.19 - 2.05 (m, 4H) γ -Gln, 1.93 - 1.82 (m, 2H) β -Gln, 1.70 (d, J = 5.4 Hz, 5H) β -Gln, 1.63 - 1.56 (m, 2H) γ -Leu, β -Arg, 1.47 (m, 8H) β -Leu, β -Arg, γ -Arg, 0.94 (d, J = 18.4 Hz, 4H), 0.86 (d, J = 6.6 Hz, 3H) δ -Leu, 0.81 (d, J = 6.5 Hz, 3H) δ' -Leu. ¹³C NMR (151 MHz, DMSO) δ 174.0 - 165.8 NH, 156.8 ϵ -Arg, 134.6 ϵ -His, 132.3 γ -His, 116.1 δ -His, 61.2 β -Ser, 54.9 α -His, 53.0 α -Cys, 52.8 α -Ser, 52.3 α -Gln, 52.2 α -Arg, 52.0 α -Arg, 51.9 α -Arg, 51.2 α -Leu, 42.0 α -Gly, 41.9 α -Gly, 40.6 β -Leu, 40.4 δ -Arg, 40.1 δ -Arg, 40.0 - 39.0 DMSO, 31.4 γ -Gln, 31.3 γ -Gln, 30.6 t -Bu-OH, 29.9 β -Arg, 29.9 β -His, 29.4 β -Arg, 29.0 β -Gln, 28.3 β -Arg, 27.7 β -Cys, 24.9 γ -Arg, 24.8 γ -Arg, 24.0 γ -Leu, 23.0 δ -Leu, 21.4 δ' -Leu. MS: m/z [M + H]⁺ (calc: 1244.58) [M+2H]²⁺ calc. m/z 622.7909; exp. m/z 622.7914.

EB1-K, Gly-Gln-Cys-Gln-Arg-Arg-Gly-His-Cys-Ser (GQCQRRLGHCS)

Yield: 46%; C₅₀H₈₆N₂₂O₁₆S₃. t_R (A) = 19.80 min. ¹H NMR (600 MHz, DMSO) δ 8.58 - 6.83 NH, 4.59 - 4.55 (m) α -Arg, 4.45 - 4.41 (m) α -Arg, 4.40 - 4.31 (m) α -Gln, 4.29 - 4.24 (m) α -Leu, α -His, α -Ser, 3.74 - 3.73 (m) α -Gly, 3.72 - 3.69 (m) β -Ser, 3.65 - 3.63 (m) β -Ser, 3.60 δ -Arg, 3.09 - 3.07 (m) δ -Arg, 3.02 - 2.89 (m) β -His, 2.87 - 2.70 (m) δ -Arg, 2.68 - 2.61 (m) β -His, 2.52 - 2.38 DMSO, 2.14 - 2.08 (m) γ -Gln, 1.91 - 1.88 (m) β -Gln, 1.74 - 1.72 (m) β -Gln, 1.68 - 1.64 (m) β -Arg, 1.62 - 1.58 (m) γ -Leu, 1.50 - 1.47 (m) β -Leu, 1.36 - 1.12 t -Bu-OH, 0.86 (d, J = 6.6 Hz, 1H) δ -Leu, 0.81 (d, J = 6.5 Hz, 1H) δ' -Leu. ¹³C NMR (151 MHz, DMSO) δ 174.2 δ -Gln, 173.8 δ -Gln, 172.1 CO-Leu, 171.7 CO-Gln, 171.1 CO-Gln, 170.9 CO-Arg, 170.1 CO-Arg, 170.1 CO-His, 170.0 CO-Gly, 170.0 CO-Ser, 168.5 CO-Cys, 165.8 CO-Cys, 156.7 ϵ -Arg, 156.7 ϵ -Arg, 61.2 β -Ser, 54.9 α -His, 53.3 α -Cys, 53.0 α -Ser, 52.8 α -Arg, 52.2 α -Cys, 52.1, 52.04, 51.8 α -Arg, 51.2 α -Gln, 42.1 α -Gly, 41.9 α -Gly, 41.9, 41.85, 40.5 β -Leu, 40.4 δ -Arg, 40.1 δ -Arg, 40.0 - 39.0 DMSO, 31.4 γ -Gln, 31.2 γ -Gln, 30.6 β -His, 30.6 β -Arg, 29.9 β -Arg, 29.9 β -Cys, 29.7 β -Cys, 29.3 β -Cys, 28.5 β -Gln, 27.9 β -Gln, 24.9 γ -Arg, 24.8 γ -Arg, 24.0 γ -Leu, 23.0 δ -Leu, 21.4 δ' -Leu. MS: m/z [M + H]⁺ (calc: 1244.58); [M+2H]²⁺ calc. m/z 674.2955; exp. m/z 674.2955.

EB2-K, Arg-Arg-Cys-Pro-Arg-His-Pro-Gln-Cys-Arg-Arg (RRCPRHPQCRR)

Yield: 77%; C₅₇H₁₀₁N₂₉O₁₃S₂. t_R (A) = 15.94 min. ¹H NMR (600 MHz, DMSO) δ 8.72 - 7.12 NH, 4.70 (dd, J = 13.7, 6.6 Hz, 1H) α -Arg, 4.67 - 4.62 (m, 1H) α -Arg, 4.40 (dd, J = 11.7, 6.1 Hz, 2H) α -Pro, α -Cys, 4.37 - 4.32 (m, 2H) α -Pro, α -Cys, 4.31 - 4.20 (m, 3H) α -Arg, 3.83 (t, J = 6.3 Hz, 1H) α -Arg, 3.64 (m) δ -Pro, 3.68 - 3.58 (m) δ -Pro, 3.37 - 3.31 (m) δ -Pro, 3.17 - 3.10 (m) δ -Arg, 3.08 - 3.02 (m) β -Pro, 2.95 - 2.89 (m) β -Pro, 2.77 - 2.71 (m) β -Pro, 2.20 (t, J = 7.6 Hz, 2H) γ -Gln, 2.12 - 2.06 (m) β -Arg, 2.04 - 1.95 (m) β -Cys, 1.93 - 1.89 (m) β -Arg, 1.88 - 1.82 (m) β -Cys, 1.80 - 1.75 (m) β -His, 1.76 - 1.70 β -His, β -Arg, 1.67 - 1.61 (m) β -Arg, 1.59 - 1.48 (m) γ -Arg. ¹³C NMR (151 MHz, DMSO) δ 174.4 - 168.7 CO, 157.2 ϵ -Arg, 157.1 ϵ -Arg, 157.1 ϵ -Arg, 116.3 δ -His, 60.1 α -Pro, 59.7 α -Pro α , 53.4 α -Arg, 52.7 α -His, 52.2 α -Cys, 52.0 α -Arg, 51.8 α -Arg, 51.7 α -Arg, 47.1 δ -Pro, 46.9 δ -Pro δ 40.6 δ -Arg, 40.5 δ -Arg, 40.5 δ -Arg, 40.3 δ -Arg, 40.2 δ -Arg, 40.0 - 39.4 DMSO, 31.5 γ -Gln 30.6 t -Bu-OH, 29.9 β -Pro, 29.4 β -Pro, 29.1 β -Arg, 28.9 β -Cys, 28.8 β -Arg, 28.5 β -Arg, 28.3 β -His, 27.4 β -Cys, 25.1 γ -Arg, 24.9 γ -Arg, 24.7 γ -Arg, 24.4 γ -Pro, 24.3 γ -Pro, 24.0 γ -Arg. MS: m/z [M + H]⁺ (calc: 1465.75); [M+2H]²⁺ calc. m/z 732.8866; exp. m/z 732.8958.

EB1-C, Gly-Gln-Arg-Gln-Arg-Lys-Arg-Leu-Gly-His-Arg-Ser (GQRQRKRLGHRS)

Yield: 60%; C₅₉H₁₀₇N₂₉O₁₆. t_R (A) = 10.66 min. ¹H NMR (600 MHz, DMSO) δ 8.58 - 6.85 NH, 4.59 - 4.55 (m, 1H) α -Arg, 4.35 (dd, J = 13.7, 7.9 Hz, 1H) α -Gln, 4.28 (dd, J = 14.1, 9.6 Hz, 2H) α -Arg, α -Gln,

4.24 (m, 5H) α -His, α -Gln, α -Leu, α -Ser, α -Lys, 3.80 – 3.71 (m, 2H) α -Gly, β -Ser, 3.70 – 3.62 (m, 2H) α -Gly, β -Ser, 3.62 – 3.56 (m) δ -Arg, 3.09 (m) δ -Arg, ϵ -Lys, 3.03 – 2.98 (m, 1H) β -His, 2.94 – 2.89 (m, 1H) β -His, 2.76 – 2.73 (m) ϵ -Lys, 2.12 (m) γ -Gln, 1.92 – 1.82 (m) β -Gln, 1.76 – 1.71 (m) β -Gln, 1.69 – 1.64 (m) β -Arg, 1.60 (m, 2H) γ -Leu, 1.48 (m, 69H) β -Leu, γ -Arg, β -Lys, 1.30 (m, 2H) γ -Lys, 0.86 (d, J = 6.6 Hz, 3H) δ -Leu, 0.82 (d, J = 6.5 Hz, 3H) δ' -Leu. ^{13}C NMR (151 MHz, DMSO) δ 174.1 – 167.2 CO, 156.8 ϵ -Arg, 134.0 ϵ -His, 132.5 γ -His, 118.0 δ -His, 61.2 β -Ser, 54.7 α -His, 53.2 α -Lys, α -Arg, 52.2 α -Leu, 41.9 α -Gly, 41.0 β -Leu, 40.1 δ -Arg, 40.0 – 39.0 DMSO, 38.7 ϵ -Lys, 31.7 β -His, 31.3 γ -Gln, 27.6 β -Gln, β -Arg, 26.6 β -Lys, 24.8 γ -Arg, 24.1 γ -Leu, 23.0 δ -Leu, 22.1 γ -Lys, 21.4 δ' -Leu. MS: m/z $[\text{M} + \text{H}]^+$ (calc: 1478.86); $[\text{M} + 2\text{H}]^{2+}$ calc. m/z 739.9304; exp. m/z 739.9287.

EB2-C, *Lys-Lys-Arg-Pro-Lys-His-Pro-Gln-Arg-Arg-Lys* (KKRPKHPQRRK)

Yield: 52%; $\text{C}_{63}\text{H}_{115}\text{N}_{27}\text{O}_{13}$. t_{R} (A) = 9.53 min. ^1H NMR (600 MHz, DMSO) δ 8.42 – 7.14 NH, 4.75 (dd, J = 14.3, 7.5 Hz, 1H) α -Lys, 4.51 (dd, J = 12.6, 7.0 Hz, 1H) α -Arg, 4.40 – 4.35 (m, 2H) α -Pro, 4.32 (dd, J = 13.4, 7.3 Hz, 3H) α -Lys, α -Gln, α -Arg, 4.27 (dd, J = 14.5, 9.0 Hz, 1H) α -Arg, 4.18 (td, J = 13.7, 8.3 Hz, 2H) α -Lys, α -His, 3.83 (t, J = 6.5 Hz, 1H) α -Lys, 3.69 – 3.64 (m, 1H) δ -Pro, 3.64 – 3.60 (m, 1H) δ -Pro, 3.60 – 3.53 (m, 1H) δ -Pro, 3.41 (m, 1H) δ -Pro, 3.13 (m) δ -Arg, 3.03 (m) ϵ -Lys, 2.92 (m) ϵ -Lys, 2.79 (m) ϵ -Lys, 2.19 (t, J = 7.6 Hz, 2H) γ -Gln, 2.12 – 2.02 (m, 2H) γ -Pro, 2.02 – 1.94 (m, 1H) γ -Pro, 1.91 (t, J = 6.9 Hz, 1H) β -Gln, 1.90 – 1.81 (m, 2H) β -Gln, γ -Pro, 1.76 – 1.70 (m) δ -Lys, β -Arg, 1.66 – 1.48 (m) β -His, δ -Lys, β -Arg, γ -Arg, 1.37 (m) γ -Lys. ^{13}C NMR (151 MHz, DMSO) δ 174.3 – 168.5 CO, 157.2 ϵ -Arg, 157.2 ϵ -Arg, 157.1 ϵ -Arg, 59.9 α -Pro, 59.3 α -Pro, 52.6 α -Arg, 52.5 α -Arg, 52.3 α -Lys, 52.2 α -Gln, 52.1 α -Lys, 51.9 α -Lys, 50.2 α -Lys, 47.0 δ -Pro, 46.9 δ -Pro, 40.7 δ -Arg, 40.6 δ -Arg, 40.5 δ -Arg, 40.2 – 39.4 DMSO, 38.8 ϵ -Lys, 38.7 ϵ -Lys, 38.6 ϵ -Lys, 31.5 γ -Gln, 31.3 γ -Gln, 31.1 β -His, 30.6 δ -Lys, 30.4 δ -Lys, 29.2 β -Arg, 29.1 β -Arg, 29.0 β -Gln, 28.9 β -Gln, 28.2 β -Pro, 27.5 β -Pro, 26.6 β -Lys, 26.5 β -Lys, 26.3 β -Lys, 24.8 γ -Arg, 24.8 γ -Arg, 24.5 γ -Pro, 24.4 γ -Pro, 22.3 γ -Lys, 22.2 γ -Lys, 22.1 γ -Lys, 21.0 γ -Lys. MS: m/z $[\text{M} + \text{H}]^+$ (calc: 1458.93); $[\text{M} + 2\text{H}]^{2+}$ calc. m/z 729.9662; exp. m/z 729.9648.

EB3-C, *Arg-Arg-Ile-Arg-Arg-Arg-Phe-Gly-Tyr-Arg-Leu* (RRIRRRFGYRL)

Yield: 74%; $\text{C}_{68}\text{H}_{117}\text{N}_{29}\text{O}_{13}$. t_{R} (A) = 17.24 min. ^1H NMR (600 MHz, DMSO) δ 8.97 – 7.57 NH, 7.24 – 7.20 (m) δ -Phe, ϵ -Phe, ζ -Phe, 7.18 – 7.02 NH, 7.01 – 7.00 (d, J = 8.6 Hz) δ -Tyr, 6.65 – 6.64 (d, J = 8.4 Hz) ϵ -Tyr, 4.57 – 4.53 (m) α -Arg, 4.51 – 4.48 (m) α -Arg, 4.40 – 4.38 (m) α -Arg, 4.32 – 4.26 (m) α -Tyr, 4.25 – 4.20 (m) α -Leu, α -Ile, 3.75 – 3.63 (m) α -Gly, 3.14 – 3.02 (m) δ -Arg, β -Phe, 2.97 – 2.94 (m) β -Phe, 2.87 – 2.83 (m) β -Tyr, 2.74 – 2.70 (m) β -Tyr, 2.51 – 2.49 DMSO, 1.79 – 1.43 (m) β -Ile, β -Leu, γ -Ile, γ -Leu, γ -Arg, 0.92 – 0.81 (m) δ -Leu, δ -Ile, CH_3 -(β -Ile). ^{13}C NMR (151 MHz, DMSO) δ 174.1 – 168.4 CO, 159.0 ϵ -Arg, 158.8 ϵ -Arg, 157.2 ϵ -Arg, 157.1 ϵ -Arg, 155.9 ζ -Tyr, 137.4 γ -Phe, 129.9 δ -Tyr, 129.1 ϵ -Phe, 127.9 δ -Tyr, 127.6 γ -Tyr, 126.2 ζ -Phe, 115.0 ϵ -Tyr, 57.0 α -Ile, 54.4 α -Phe, 54.0 α -Tyr, 52.7 α -Arg, 52.4 α -Arg, 51.0 α -Arg, 42.1 α -Gly, 40.7 δ -Arg, 40.6 δ -Arg, 40.5 δ -Arg, 40.5 δ -Arg, 40.2 – 39.4 DMSO, 37.7 β -Phe, 36.9 β -Tyr, 36.7 β -Ile, 29.2 β -Arg, 29.1 β -Arg, 29.0 β -Arg, 28.9 β -Arg, 25.0 γ -Arg, 24.9 γ -Arg, 24.8 γ -Arg, 24.4 γ -Arg, 24.3 γ -Arg, 24.3 γ -Leu, 22.7 δ -Leu, 21.7 δ -Leu, 15.2 β -Ile, 10.8 δ -Ile. HRMS: m/z $[\text{M} + \text{H}]^+$ (calc: 1548.95); $[\text{M} + 2\text{H}]^{2+}$ calc. m/z 774.9771; exp. m/z 774.9764.

EB3a-C, *Arg-Arg-Ile-Arg-Arg-Arg-Gly-Tyr-Arg-Leu* (RRIRRRGYRL)

Yield: 24%; $\text{C}_{59}\text{H}_{108}\text{N}_{28}\text{O}_{12}$. t_{R} (A) = 15.70 min. ^1H NMR (600 MHz, DMSO) δ 8.02 – 7.58 NH, 7.32 – 7.04 (m) NH, 6.99 (d, J = 8.5 Hz, 2H) δ -Tyr, 6.68 – 6.62 (d, J = 8.5 Hz, 2H) ϵ -Tyr, 4.49 (dd, J = 13.2, 8.3 Hz, 1H) α -Tyr, 4.43 – 4.38 (m, 1H) α -Leu, 4.32 (dd, J = 13.8, 8.0 Hz, 2H) α -Arg, 4.30 – 4.25 (m, 3H) α -Arg, 4.22 (dd, J = 15.3, 7.6 Hz, 1H) α -Ile, 3.83 (t, J = 6.4 Hz, 1H) α -Arg, 3.72 (d, J = 6.8 Hz, 2H) α -Gly, 2.94 (dd, J = 14.1, 4.7 Hz, 2H) β -Tyr, 2.71 (dd, J = 14.1, 8.7 Hz, 1H) β -Tyr, 1.72 (ddd, J = 19.9, 14.6, 6.5 Hz, 9H) β -Ile, β -Arg 1.67 – 1.48 (m) β -Arg, γ -Leu 1.45 (ddd, J = 13.4, 7.5, 3.5 Hz, 2H) β -Leu, 1.16 – 1.08 (m, 1H) γ -Ile, 0.91 (d, J = 6.6 Hz, 3H) δ -Leu, 0.87 (d, J = 6.5 Hz, 3H) δ' -Leu, 0.85 (d, J = 6.8 Hz, 3H) CH_3 -(β -Ile), 0.82 (t, J = 7.4 Hz, 3H) ϵ -Ile. ^{13}C NMR (151 MHz, DMSO) δ 173.6 – 168.4 CO, 157.2 ϵ -Arg, 157.1 β -Arg, 155.8 ζ -Tyr, 120.0 δ -Tyr, 127.6 γ -Tyr, 115.0 ϵ -Tyr, 57.1 α -Ile, 54.3 α -Tyr, 52.6 α -Arg, 52.3 α -Arg, 52.2 α -Arg, 52.1 α -Arg, 50.6 α -Leu, 41.9 α -Gly, 40.6 δ -Arg, 40.5 β -Leu, 40.5 δ -Arg, 40.3 δ -Arg, 40.3 δ -Arg, 40.2 δ -Arg, 40.0 – 39.4 DMSO, 36.9 β -Tyr, 36.6 β -Ile, 29.2 β -Arg, 28.9 β -Arg, 28.8 β -Arg, 28.6 β -Arg, 24.9 γ -Arg, 24.8 γ -Arg, 24.7 γ -Arg, 24.3 γ -Ile, 24.3 γ -Arg, 24.0 γ -Ile, 22.7 δ -Leu, 21.5 δ' -Leu, 15.3 CH_3 -(β -Ile), 10.8 ϵ -Ile. MS: m/z $[\text{M} + \text{H}]^+$ (calc: 1401.88); $[\text{M} + 2\text{H}]^{2+}$ calc. m/z 701.4429; exp. m/z 701.4415.

3.2. Biological Activity

3.3.1. Minimum Inhibitory Concentration (MIC) Assay

For these studies we used reference strains *Escherichia coli* ATCC25922, *Bacillus subtilis* 168c, *Staphylococcus epidermidis* DSM 20044 (DSMZ GmbH, Germany) and *Enterococcus hirae* DSM 3320 (DSMZ GmbH, Germany). Bacterial cells were inoculated into Mueller-Hinton Broth II (MHB II; Sigma, Austria) and grown to the mid-logarithmic phase, washed in sodium buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.4). The culture was adjusted to $\sim 1 \times 10^6$ CFU/mL and dispensed into 96-well microtiter plates. Peptides were tested in two-fold serial dilutions across a concentration range of 0.4–100 μ M, each concentration assayed in duplicate. Growth control wells (bacteria + medium, no peptide) and sterility control wells (medium only) were included. In parallel, the inoculum density and purity were verified by plating serial dilutions of the bacterial culture on MHB agar. After incubation at 37 °C for 20 h under static conditions, bacterial growth was quantified by measuring optical density at 620 nm (OD 620). The MIC was defined as the lowest peptide concentration at which OD 620 showed no increase compared to the growth-control baseline. The experiment was repeated in three independent biological replicates.

3.3.2. Hemolysis Assay

The hemolysis assay was performed using pooled human blood of at least 10 healthy volunteers obtained from healthy donors at the Medical University of Graz (ethical approval: 1142/2025). Erythrocytes were isolated by centrifuging the blood for 10 min at 2100 rpm and subsequently washed several times with PBS. A 2.5% erythrocyte solution in PBS was exposed 10 EB peptides (Figure 2.) (final concentrations: 6.25–400 μ M). After 1 h at 37 °C under static conditions, the erythrocyte suspensions were centrifuged for 10 min at 4000 rpm and the absorbance of the supernatants was determined at 405 nm. The percentage of hemolysis was calculated relative to the positive control (1% Triton X-100), according to the following equation

$$\text{Hemolysis (\%)} = (\text{Absorbance at peptide} \times \text{concentration} / \text{Absorbance at Triton}) \times 100$$

3.3.3. In Silico Analysis of Peptides Structural Properties

Peptide structures were predicted using AlphaFold3 (Beta Server, standard settings, 11 Nov 2024) [22]. Molecular graphics and analyses were performed with UCSF ChimeraX [24]. In addition, peptide sequences were submitted to the PEP-FOLD server (<https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/>) to generate 3D models using a coarse-grained force field, which were then clustered with Apollo for secondary structure prediction [15,25,26]. The basic structural parameters of the peptides, including hydrophobicity, acidity, and charge, were calculated using the Peptide 2.0 hydrophobicity/hydrophilicity analysis tool [https://www.peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php] (https://www.peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php). Hydrophobic moment (μ), and bilayer partitioning free energy (ΔG) were obtained using Totalizer, a tool within Membrane Protein Explorer (mPEX) [27], [<https://blanco.biomol.uci.edu/mpex/>] (<https://blanco.biomol.uci.edu/mpex/>). Totalizer computes peptide hydropathy plots based on whole-residue hydrophobicity according to the Wimley–White scales [28], allowing evaluation of the energetic cost of inserting H-bonded peptide bonds of α -helices into membranes. Bilayer partitioning free energy was assessed using the octanol scale, representing the free energy change for transferring residues from water to the hydrophobic core of the bilayer.

3.3.4. Membrane Permeability Assay

Bacterial membrane permeabilization was evaluated using propidium iodide (PI) uptake. PI is a membrane-impermeable dye: it cannot cross intact bacterial cytoplasmic membranes, but when the

membrane integrity is compromised (for example via peptide-mediated permeabilization), PI enters the cell, intercalates into nucleic acids (DNA and RNA) and fluorescence increases markedly. *B. subtilis*, *E. hirae* and *S. epidermidis* cells were grown to mid-logarithmic phase, harvested by centrifugation, washed once with PBS and diluted to a concentration of 1×10^7 cells/mL using PBS instead of growth medium. The cell suspension was incubated with peptides (Table 1) at various concentrations in the presence of PI (final concentration 2.5 $\mu\text{g/mL}$). Fluorescence (excitation 535 nm, emission 617 nm) was monitored every 2 min for 120 min at 37 °C using a microplate reader (GloMax®, Promega). The time-course fluorescence curves were recorded for each peptide concentration (in duplicate for each time point, and the entire experiment was repeated three independent times). From each time-course fluorescence curve (for a given peptide concentration), a constant time-window (for example minutes 10 to 20) was selected where the fluorescence signal was stable or in the linear uptake phase. The fluorescence values within that window were averaged to provide a representative fluorescence uptake value for that peptide concentration. These averaged fluorescence values (mean of duplicates) were then plotted as a function of peptide concentration (for each independent experiment) to generate a dose-response curve (fluorescence uptake vs peptide concentration). The three independent biological replicates were averaged and standard deviations (or standard errors) calculated and plotted.

3.3.5. Membrane Depolarization Assay

The assay was performed as previously described [23] and in analogy to PI assay described above. Briefly, the mid-log phase cells (1×10^7 CFU/mL) were stained with 1 μM DiSC₃(5) in PBS containing 100 mM KCl for 30 min to allow dye self-assembly and K⁺ equilibration. Peptides of different concentration were added, and fluorescence was recorded at 622/685 nm using was monitored every 2 min for 120 min at 37 °C using a microplate reader (GloMax®, Promega). Further analysis was performed analogously to the PI uptake assay described in Section 3.3.4.

3.3.6. *In Silico* Peptide-Membrane Interactions

Structure of the peptides were predicted by AlphaFold and Pepfold programs [22]. Bacterial membrane models were composed as follow: for *B. subtilis*, 35% 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG), 35% 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG), 12% phosphatidylethanolamine (PE), 3% cardiolipin (CL), 5% phosphatidylglycerol (PGH), 2% lysyl-mono-galactosyldiacylglycerol (LMG), 4% digalactosyldiacylglycerol (DGD), and 2% bis(monoacylglycerol)phosphate (B5S); for *E. hirae*, 30% DSPG, 30% DPPG, and 20% CL; and for *S. epidermidis*, 8% PE, 26% DPPG, 26% DSPG, and 20% CL. These models closely reflect membrane compositions reported in earlier studies [29]. The prediction analysis yielded a ranking score of 0.84, ipTM and pTM scores of 0.34 and 0.35, respectively, and an ipTM+pTM value of about 0.34, and these results were consistent across all models.

3.4. Nano-Ultra-Performance Liquid Chromatography-Electrospray Ionization-Quadrupole Mass Spectrometry Analysis

Peptides were desalted on the AssayMAP Bravo Platform (Agilent Technologies, Waldbronn, Germany) using in-house packed mixed-cation exchange (MCX) cartridges. Cartridges were conditioned with methanol (100 μL), equilibrated with ultrapure water (100 μL), and loaded with 100 μL of 0.1 mg/mL peptide solution. Peptides were eluted with 25 μL of 10% ACN in 1% ammonium hydroxide, vacuum-dried, and reconstituted in 0.1% formic acid to a final concentration of 0.1 mg/mL. They were separated on a nanoAcquity UPLC system equipped with a nanoAcquity UPLC 2G-V/M symmetry C18 trap column (100 Å, 5 μm , 180 $\mu\text{m} \times 20$ mm) and a nanoAcquity UPLC BEH C18 analytical column (130 Å, 1.7 μm , 100 $\mu\text{m} \times 100$ mm) (Waters, Milford, MA, USA). The column temperature was set to 40 °C and injection volume was 1 μL . Mobile phase A consisted of aqueous 0.1% formic acid, and mobile phase B consisted of 0.1% formic acid in 95% ACN. Isocratic delivery

of solvent A into the trap column was performed at a flow rate of 15 $\mu\text{L}/\text{min}$ for 2 min. The samples were eluted under gradient elution conditions at a flow rate of 1 $\mu\text{L}/\text{min}$ and a run time of 32 min. The following elution gradient was used: 0–3 min, 80% solvent A; 3–24 min, 45% solvent A; 24–27 min, 1% solvent A; 27–29 min, 80% solvent A; and 29–32 min, 80% solvent A. The modifier solution of 1 mM ethyl methanoate in isopropanol was introduced from a Synapt A channel by a “T” connector at a flow rate of 0.4 $\mu\text{L}/\text{min}$.

A nanoUPLC system was coupled to a nanoESI-QTOF Synapt G2-Si mass spectrometer (Waters, Milford, MA, USA), and instrument parameters were set using MassLynx software (v4.1, Waters, Milford, MA, USA). Acquisition mode for both MS and MS/MS was set to positive polarity and resolution analyzer mode with the following parameters: nitrogen flow was 1.1 bar with a source temperature of 80 $^{\circ}\text{C}$, capillary voltage was set to 4.2 kV, cone voltage was set to 40 kV and spectral acquisition time was 1 s. Collision energy during MS/MS was set to 40 V for all peptides. The mass accuracy of the raw data was achieved by infusing 1 ng/ μL of leucine enkephalin in isopropanol and 0.1% formic acid. Table A1. contains the list of selected precursor ions.

Data analysis The obtained MS/MS spectra were deconvoluted using *ExDViewer* software (v4.6.31, Agilent Technologies). The assignment of y - and b -ion series, as well as sequence coverage calculation, was performed within the same software. MS analysis results are shown in SM: Figure S2.1. – S2.30. and Table S2.1. – S2.10:

4. Conclusions

To identify biologically active regions in the Equinin B (EB) sequence, we generated a set of peptides and applied structure–function-driven modifications to enhance their antimicrobial activity. The sequence was cleaved twice: after 12 (Ser) and after 23 (Lys), resulting in three peptides **EB-1**, **EB-2** and **EB-3**. In the first derivatization all Lys residues in **EB-1** and **EB-2** were replaced with Arg to increase hydrogen bonding (**EB1-K**, **EB2-K**). In the second derivatization all Cys residues were replaced with Arg (**EB1-C**, **EB2-C**, **EB3-C**). All compounds were tested for antimicrobial activity against *E. coli* and *S. epidermidis*. Only peptides from the third part of Equinin B, **EB-3** (MIC 25 μM), and **EB3-C** (MIC 25–50 μM) showed antimicrobial activity only against the Gram-positive *S. epidermidis*. When Phe was removed from **EB3-C**, the antimicrobial activity was lost in the peptide **EB3a-C**. This removal reduced hydrophobicity from 27% to 20%, suggesting that ~20% represents the minimal threshold for antimicrobial activity, consistent with the inactivity of all peptides below this level. Since **EB-3** shows high hemolytic activity, **EB3-C** is the leading peptide for potential drug design. *In silico* structure analysis predicts a better helical structure for **EB-3**, while **EB3-C** shows a less folded structure. To verify that the peptides’ effects were not artifacts and not limited to *S. epidermidis*, they were also tested against two additional Gram-positive species - both showing similar potency against *B. subtilis* (MIC ~25–50 μM) and somewhat lower activity against *E. hirae* (MIC ~50–100 μM), suggesting a more general target. *In silico* analysis of their physicochemical properties indicated that **EB-3** had the lowest bilayer partitioning free energy (ΔG) and higher amphipathicity, suggesting a greater potential for membrane interaction. **EB3-C** exhibited a slightly higher ΔG and lower amphipathicity, consistent with its predicted reduced membrane affinity, highlighting how these properties may contribute to differences in antimicrobial potency. Evaluation of membrane effects using PI and DiSC₃(5) assays revealed that **EB-3** significantly depolarized the membranes of all tested Gram-positive bacteria, but only markedly increased permeability in *B. subtilis*. In contrast, **EB3-C** induced minimal depolarization and negligible permeabilization, supporting a non-lytic mechanism or one that does not involve lethal disruption of the membrane. Structure–function modeling indicates that **EB-3** most likely lacks membrane selectivity due to its ability to adopt multiple non-specific conformations across Gram-positive membranes, including shallow insertion in *B. subtilis*, surface binding in *E. hirae*, and a carpet-like mechanism in *S. epidermidis*. **EB3-C** exhibits consistent, selective interactions via a short amphipathic helix and conserved R4/R6–phosphate contacts, with hydrophobic residues fine-tuning membrane anchoring, which explains its non-hemolytic, targeted antimicrobial activity. In contrast, removal of the phenylalanine residue in **EB3a-**

C disrupts proper membrane engagement, leading to primarily surface-bound interactions and loss of antimicrobial function. These findings demonstrate that **EB-3** is the core antimicrobial region of equinin B against Gram-positive bacteria, that substituting C with R preserves antimicrobial activity while reducing hemolysis, and that subtle structural changes can dramatically alter peptide–membrane interactions and biological activity.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1.1.-S1.20.: NMR spectra of prepared peptides EB derivatives; Figure S2.1. – S2.30. and Table S2.1. – S2.10: MS analysis of peptides EB derivatives.

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Abbreviations

The following abbreviations are used in this manuscript:

AR	Antibiotic resistance
AMPs	Antimicrobial peptides
HBTU	<i>N,N,N',N'</i> -Tetramethyl- <i>O</i> -(1 <i>H</i> -benzotriazol-1-yl)uronium hexafluorophosphate
NMM	<i>N</i> -methylmorpholine
TFA	Trifluoroacetic acid
TIS	Triisopropylsylene
SPE	Solid Phase Extraction
SPPS	Solid Phase Peptide Synthesis
MIC	Minimum Inhibitory Concentration
PI	Propidium iodide
MHB	Mueller-Hinton Broth
DMSO	Dimethyl sulfoxide
DMF	Dimethylformamide

Appendix A

Table A1. List of selected precursor ions and their respective retention times (Rt) used in MS/MS analysis.

Peptide ID		Peptide sequence	Precursor m/z	Rt/min
1	EB-1	GQCQRKCLGHCS	[660.2925+2H] ²⁺	11.53
2	EB-2	KKCPKHPQCRK	[676.8743+2H] ²⁺	09.17
3	EB-3	RCIRRCFGYCL	[695.3392+2H] ²⁺	18.06
4	EB1a-K	GQQRRLGHCS	[622.7909+2H] ²⁺	10.17
5	EB1-K	GQCQRRCLGHCS	[674.2955+2H] ²⁺	10.77
6	EB2-K	RRCPRHPQCRR	[732.8866+2H] ²⁺	09.17
7	EB1-C	GQRQRKRLGHRS	[739.9304+2H] ²⁺	09.76
8	EB2-K	KKRPKHPQRRK	[729.9662+2H] ²⁺	08.76
9	EB3-C	RRIRRRFGYRL	[774.9771+2H] ²⁺	09.07
10	EB3a-C	RRIRRRGYRL	[701.4429+2H] ²⁺	09.23

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