International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 22s, 2025
https://theaspd.com/index.php

Design, Synthesis, and Antimicrobial Evaluation of Short Hybrid Peptides Incorporating B- And Γ-Amino Acids for Membrane-Targeted Activity Against Multidrug-Resistant Bacteria

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Abstract

The rapid emergence of multidrug-resistant (MDR) bacteria poses a critical challenge to current antimicrobial therapy, necessitating the development of novel agents with unique mechanisms of action. Antimicrobial peptides (AMPs) are promising candidates due to their broad-spectrum activity and membrane-targeted bactericidal effects; however, their clinical translation is often hindered by proteolytic instability and high production costs. In this study, we designed and synthesized a series of short lipidated hybrid peptides (P1–P8) incorporating cationic residues (Orn/Lys) with β,β-disubstituted β-amino acids and mono/disubstituted γ-amino acids to improve protease resistance, membrane selectivity, and pharmacokinetic properties. The β3,3-Ac6c and β3,3-Pip(Ac) building blocks, along with Boc-γ4-L-Phe-OH and Boc-γ4-L-Leu-OH, were synthesized following established literature protocols. Peptides were assembled via stepwise EDC/HOBt coupling, lipidated with lauric acid to enhance membrane affinity, purified by silica gel chromatography, and structurally confirmed by ^1H/^13C NMR and HRMS. Antimicrobial activity, evaluated using CLSI broth microdilution, revealed that P2, P3, P4, and P5 exhibited potent bactericidal effects against both Gram-positive (S. aureus, B. subtilis) and Gram-negative (P. aeruginosa, E. coli, S. typhimurium, K. pneumoniae) MDR strains, with minimum inhibitory concentrations (MICs) as low as 6.25 μM. Time–kill kinetics demonstrated rapid bacterial eradication within 2 h at MIC levels, with P4 showing the fastest killing rate. Membrane permeabilization assays using propidium iodide staining confirmed significant disruption of bacterial membranes, while hemolytic evaluation indicated low cytotoxicity toward human erythrocytes, with HC50 values exceeding 500 μM for P4, suggesting excellent selectivity. These findings highlight the potential of β- and γ-amino acid-based hybrid peptides as promising candidates for combating MDR bacterial infections through membrane-targeted mechanisms.

Keywords: *hybrid peptides, β-amino acids, γ-amino acids, antimicrobial activity, membrane disruption, multidrug resistance*

1. INTRODUCTION

Antimicrobial peptides (AMPs) form an essential arm of the innate immune defense in a broad array of living systems, ranging from simple organisms to humans [1]. These naturally occurring molecules are versatile in their biological functions, exerting potent effects against a diverse range of pathogens

including bacteria, fungi, yeasts, and protozoa [2]. Their primary mechanism of action often involves direct interaction with microbial cell membranes, leading to rapid destabilization and cell death [3]. In contrast to conventional antibiotics that typically inhibit or disrupt specific metabolic pathways, AMPs attack the physical structure of the microbial membrane through coordinated electrostatic and hydrophobic interactions, a process that makes it challenging for microbes to develop resistance [4]. Due to their broad-spectrum activity, rapid bactericidal potential, and ability to modulate host immune responses, AMPs are increasingly viewed as promising alternatives in tackling multidrug-resistant (MDR) pathogens a critical and growing problem in modern healthcare [5]. Nonetheless, moving AMPs from laboratory research to clinical application is hindered by several technical and biological limitations [6]. From a structural standpoint, AMPs are generally short-chain peptides, typically between 12 and 50 amino acids, exhibiting an amphipathic arrangement that facilitates selective targeting of microbial membranes while sparing the host's cells [7]. The cationic portion, usually derived from amino acids such as lysine, arginine, or ornithine, enables attraction to negatively charged phospholipid components present in bacterial membranes [8]. Meanwhile, the hydrophobic regions of the peptide facilitate insertion into the lipid bilayer, triggering disruption, pore formation, or membrane disintegration [9]. The interplay between positive charge and hydrophobicity is crucial for their biological action, as even minor shifts in this balance can markedly affect antimicrobial potency and selectivity [10]. Consequently, the rational design of synthetic peptides often focuses on optimizing this cationic–hydrophobic relationship to enhance killing efficiency against resistant bacteria while keeping toxicity toward mammalian cells minimal [11]. However, despite these inherent strengths, naturally derived AMPs suffer from drawbacks that restrict their pharmaceutical development. A major challenge is their vulnerability to proteolytic degradation in biological environments, which results in short half-life and reduced therapeutic efficacy [12]. In addition, large-scale production of natural AMPs can be costly due to labor-intensive synthesis and purification steps, especially when the peptide contains uncommon amino acids or complex structural motifs [13]. Their limited stability in physiological fluids further diminishes their suitability for systemic use [14]. These issues have prompted researchers to engineer modified peptide analogues that retain high antimicrobial activity while overcoming instability and cost-related barriers [15]. One particularly effective modification strategy involves integrating β- and γ-amino acids into peptide frameworks [1]. Unlike standard α-amino acids, which make up natural proteins, β- and γ-amino acids contain one or more additional methylene units in their backbone, altering spatial geometry and influencing secondary structure [2]. This structural deviation provides several benefits, including resistance to enzymatic degradation, enhanced conformational stability, and the ability to form defined helical or foldamer structures that mimic the amphipathic organization of natural AMPs [3]. Additionally, β- and γ-amino acids can increase affinity for microbial membranes and fine-tune the hydrophobic–hydrophilic balance, which can improve pharmacokinetic behavior and lower off-target cytotoxicity [4]. Incorporating β,β-disubstituted β-amino acids and mono- or disubstituted γ-amino acids further adds conformational rigidity, which is known to reinforce membrane binding and augment antimicrobial potency [5]. Previous investigations have provided strong evidence for the antimicrobial efficacy of β- and γ-amino acid-modified peptides [6]. β-Peptides, in particular, have demonstrated activity against a wide variety of Gram-positive and Gram-negative species, often remaining effective against strains resistant to conventional drugs [7]. Their ability to adopt stable helical shapes in aqueous conditions closely parallels the α-helical structures seen in natural AMPs, enabling efficient membrane targeting [8]. For example, β-peptide oligomers containing cyclic amino acid residues such as trans-2-aminocyclopentanecarboxylic acid have shown broad-spectrum antimicrobial effects alongside pronounced proteolytic stability [9]. Similarly, γ-amino acid derivatives—such as gabapentin analogues or γ4-substituted amino acids—have been employed to increase potency, selectivity, and resilience in synthetic peptide designs [10]. Lipidation, which involves attaching fatty acids to the peptide chain, has been shown to further improve membrane binding, enhance self-assembly, and increase antimicrobial strength [11]. Combining these design elements yields lipidated β/γ-hybrid peptides that replicate the desirable features of natural lipopeptides while maintaining structural flexibility and protease resistance [12]. Guided by these insights, the current study focuses on developing a library of lipidated short hybrid peptides integrating both β- and γ-amino acids for potent antibacterial action and low toxicity [13]. The design employs either ornithine (Orn) or lysine (Lys) as the cationic moieties responsible for electrostatic interaction with bacterial membranes [14]. These positively charged residues are paired with β,β-disubstituted β-amino acids such as β3,3-ac6c and β3,3-Pip(Ac), β,β-disubstituted γ-amino acids like gabapentin (Gpn), and mono-substituted γ-amino acids

including γ4-L-phenylalanine (γ4-L-Phe) and γ4-L-leucine (γ4-L-Leu). Lauric acid is incorporated at the N-terminal position to enhance hydrophobicity and strengthen membrane association [15]. This combinatorial design is expected to produce amphiphilic molecules with superior membrane-disrupting capabilities, targeting both Gram-positive and Gram-negative MDR pathogens while exhibiting reduced hemolytic activity toward human erythrocytes. The antimicrobial potential of these designed peptides was evaluated through a comprehensive set of in vitro experiments. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays were performed against clinically relevant bacterial strains [1]. Time-kill kinetics studies were carried out to determine the rate and extent of bactericidal activity [12]. Fluorescence microscopy using dual staining with DAPI and propidium iodide allowed direct visualization of membrane damage [3]. In parallel, hemolysis assays assessed selectivity by determining the extent of red blood cell lysis induced by each peptide [4]. These combined assessments are intended to establish clear structure–activity relationships (SARs), providing a foundation for further optimization of hybrid AMP analogues [5]. In essence, embedding β- and γ-amino acids into short lipidated peptide scaffolds represents a strategic approach to address the key weaknesses of natural AMPs [11]. The union of protease resistance, structural adaptability, optimized amphiphilicity, and targeted lipidation offers a platform for designing next-generation antimicrobial agents [7]. This work expands on existing research by synthesizing and systematically testing a set of lauric acid–modified β/γ-hybrid peptides, with the goal of identifying candidates that combine potent antibacterial activity, low cytotoxicity, and high stability, making them viable prospects for further pharmaceutical development [8].

2. Materials and Methods

2.1 Materials

All chemicals and reagents used in this study were of analytical or synthesis grade to maintain high purity and reproducibility of results. Amino acid derivatives and peptide synthesis components, including Coupling reagents such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI·HCl), 1-hydroxybenzotriazole (HOBt), and N-methylmorpholine (NMM), along with the reducing agent sodium borohydride (NaBH4) and catalysts like 10% palladium on carbon (Pd/C), were purchased from Sigma-Aldrich and Alfa Aesar (UK). Solvents of analytical grade, including dichloromethane (DCM), dimethylformamide (DMF), methanol (MeOH), ethyl acetate, toluene, and glacial acetic acid, were obtained from Fisher Scientific (USA) and Sigma-Aldrich.

2.2 Synthesis of Amino Acid Building Blocks

The β,β-disubstituted β-amino acids, namely β3,3-ac6c and β3,3-Pip(Ac), were synthesized following the literature protocol reported by Wani et al. (2013) with slight modifications to optimize yield and purity. In brief, the synthesis involved a multi-step route starting from commercially available cyclic ketones, which were subjected to amination reactions under controlled temperature to introduce the β-amino functionality. Subsequent alkylation and stereocontrolled cyclization steps afforded the desired β,β-disubstituted β-amino acids with high diastereoselectivity. The products were recrystallized from ethyl acetate/hexane to obtain analytically pure solids, which were confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS analysis.

The monosubstituted γ-amino acids, Boc-γ4-L-Phe-OH and Boc-γ4-L-Leu-OH, were prepared according to the method described by Smrcina et al. (1997). Briefly, Boc-L-Phe-OH or Boc-L-Leu-OH was coupled with Meldrum's acid in dry DCM at 0 °C using EDC and 4-dimethylaminopyridine (DMAP) as coupling agents, yielding the intermediate acyl Meldrum's adduct. This was subsequently reduced with NaBH4 in glacial acetic acid, followed by thermal decarboxylation in refluxing toluene to generate the γ-lactone intermediate. Saponification using 1 N NaOH in methanol yielded the target γ-amino acids. The purified products were characterized by NMR spectroscopy and HRMS to confirm structural identity [16,17].

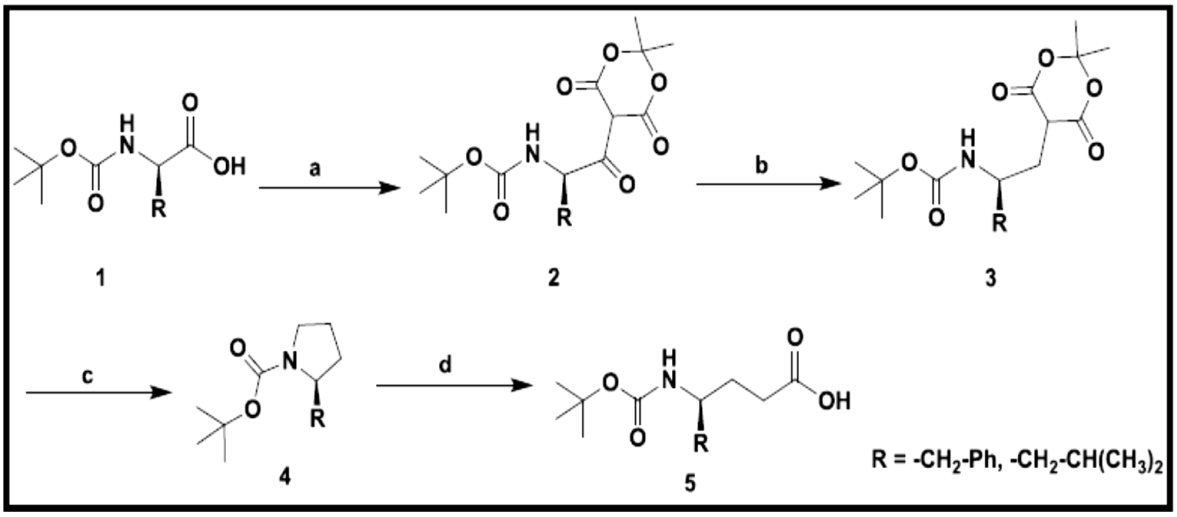

Figure 1. Schematic representation of the synthetic pathway for β,β-disubstituted β-amino acids and monosubstituted γ-amino acids. Reaction conditions: (a) formation of N-carboxyanhydride intermediate; (b) selective ring-opening to yield tert-butyl ester derivative; (c) cyclization to generate protected γ-lactam; (d) hydrolysis to obtain final amino acid building block. Substituents: R = –CH_2–Ph or –CH_2–$CH(CH_3)_2$.

2.3 Peptide Synthesis (P1–P8)

The hybrid peptides P1–P8 were synthesized using a stepwise solution-phase coupling approach. Initially, the amino acid or amino acid analogue was dissolved in dry DCM or DMF, followed by the addition of N-methylmorpholine (NMM) and the coupling reagents EDC·HCl and HOBt. This mixture was cooled to 0 °C to minimize racemization, after which the desired amine component, phenethylamine (PEA) or the previously synthesized β- or γ-amino acid derivative, was introduced. The reaction was stirred under nitrogen for 12–24 h, and completion was monitored by thin-layer chromatography (TLC). For lipidation, the N-terminal free amine of the intermediate peptide was reacted with lauric acid (LA) in dry DMF in the presence of EDC/HOBt and NMM. The reaction was maintained at 0 °C for the initial 1 h and then stirred at room temperature for 24 h under an inert atmosphere. After completion, the crude product was extracted with ethyl acetate, washed sequentially with 2 N HCl, saturated Na2CO3, and brine, and dried over anhydrous sodium sulfate. Purification of crude peptides was achieved by column chromatography using silica gel (60–120 mesh) as the stationary phase and gradient mixtures of ethyl acetate and hexane as eluents. Final global deprotection of protecting groups was accomplished by catalytic hydrogenation using 10% Pd/C in dry methanol under a hydrogen atmosphere for 12 h. The products were filtered, concentrated under reduced pressure, and lyophilized to obtain analytically pure peptides. All final compounds were confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS before biological evaluation [18].

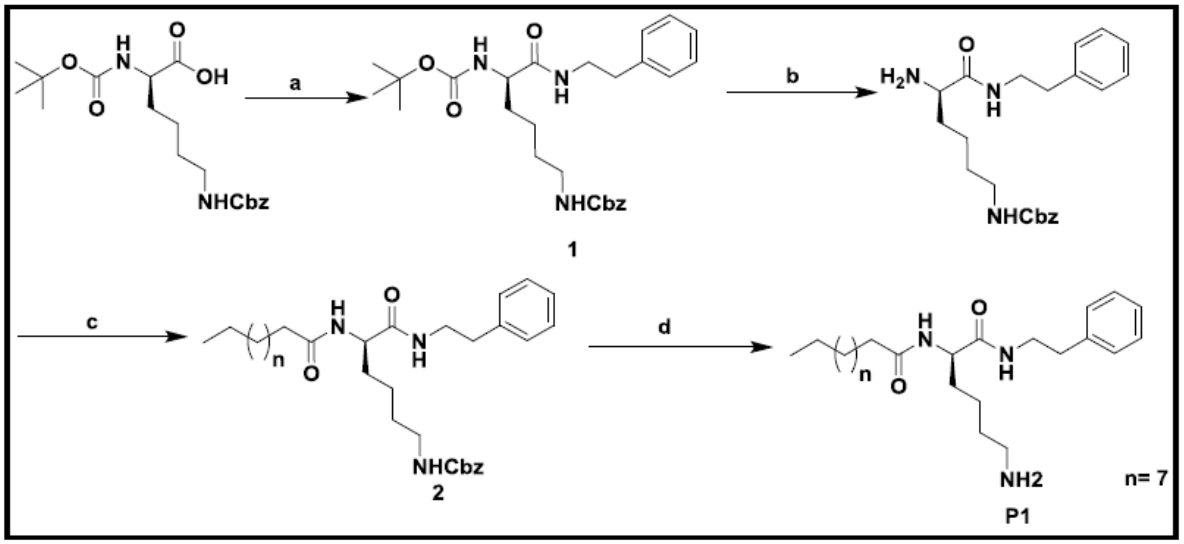

Figure 2. Synthetic route for lipidated hybrid peptide P1. Reaction conditions: (a) amide coupling between protected β-amino acid derivative and Cbz-protected lysine; (b) selective deprotection of the

terminal amino group; (c) lipidation using lauric acid to introduce hydrophobic tail (n = 7); (d) final Cbz-deprotection yielding target peptide P1.

2.4 Characterization

Structural confirmation of all synthesized peptides (P1–P8) was performed using nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (HRMS). ^{1}H NMR and ^13C NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer using $CDCl_3$ or DMSO-d_6 as solvents and tetramethylsilane (TMS) as the internal standard. Chemical shifts (δ) were reported in parts per million (ppm), and coupling constants (J) were expressed in Hertz (Hz). Spectra were analyzed to verify the presence of characteristic resonances corresponding to aromatic protons from the phenethylamine moiety, methylene protons from lauric acid chains, amide NH signals, and distinctive β- or γ-amino acid backbone resonances. The ^{13}C NMR spectra confirmed the expected carbon skeleton, including amide carbonyls, aromatic carbons, aliphatic methylene carbons, and quaternary carbons from β,β-disubstituted residues. Molecular weight confirmation was carried out using high-resolution mass spectrometry (HRMS) in electrospray ionization (ESI) mode on an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS system. Samples were dissolved in methanol, filtered through 0.22 µm PTFE filters, and directly infused for analysis. Observed m/z values for $[M+H]^+$ ions were within ±0.001 Da of calculated masses, confirming the molecular composition of each peptide. The combined NMR and HRMS data confirmed both the structural integrity and purity (>95%) of all synthesized compounds prior to biological testing [19].

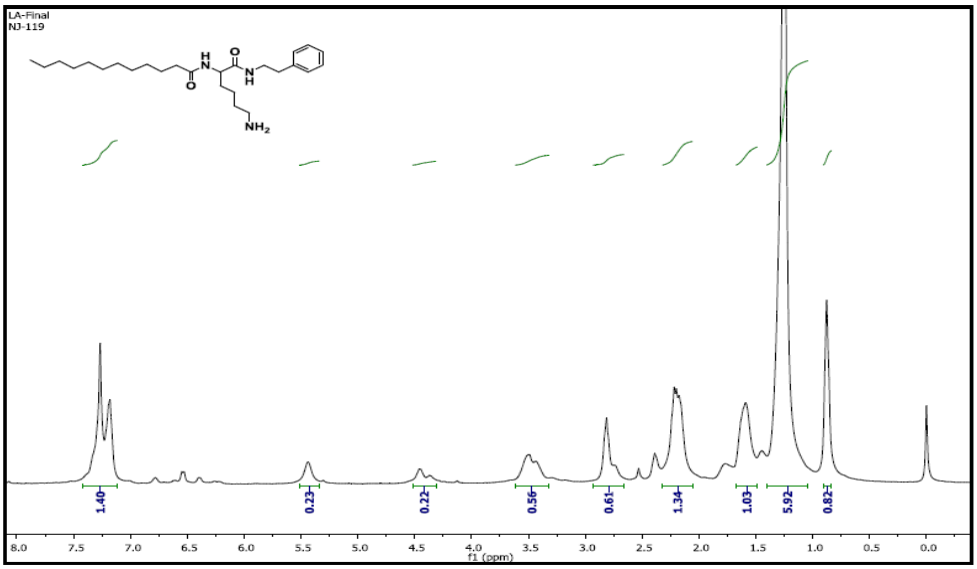

Figure 3. ^{1}H NMR spectrum of lipidated hybrid peptide P1 recorded in $CDCl_3$ at 400 MHz, showing characteristic proton signals for lauric acid moiety, phenyl group, and peptide backbone, confirming successful synthesis and structural integrity.

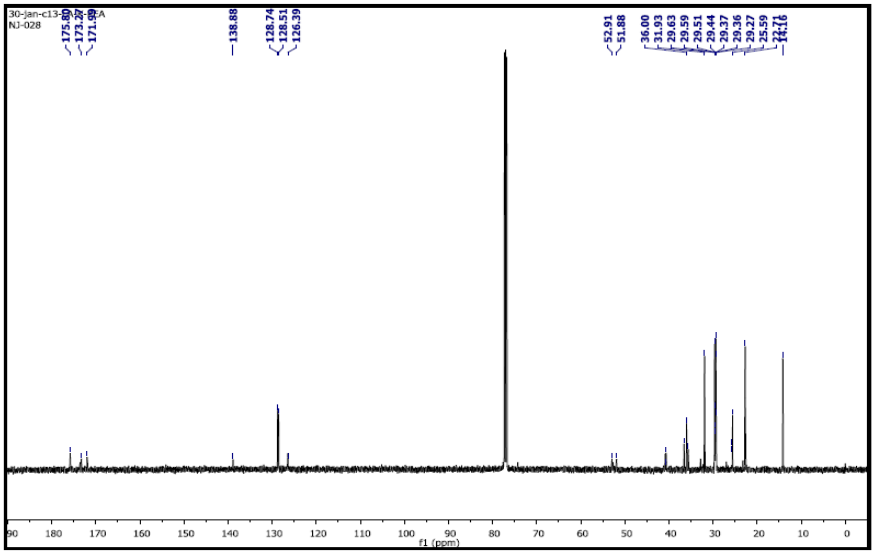

Figure 4. ^{13}C NMR spectrum of compound P1 in $CDCl_3$ showing characteristic carbon resonances for aromatic, amide, and aliphatic regions.

2.5 Antimicrobial Assay

The antimicrobial activities of the hybrid peptides were evaluated by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values according to the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method, with minor modifications to suit peptide-based testing. Bacterial strains used in the study included Gram-positive Staphylococcus aureus (MTCC 737) and Bacillus subtilis (MTCC 121), as well as Gram-negative Pseudomonas aeruginosa (MTCC 424), Escherichia coli (MTCC 118), Salmonella typhimurium (MTCC 98), and Klebsiella pneumoniae (MTCC 109). All strains were cultured in Mueller–Hinton broth (MHB) at 37 °C until reaching mid-logarithmic growth phase, then adjusted to a turbidity equivalent to a 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL). The bacterial suspension was further diluted to achieve a final inoculum of 4×10^4 CFU/mL for testing.

Stock solutions of peptides were prepared in sterile water with ≤1% DMSO to aid solubility, followed by twofold serial dilutions in sterile 96-well microtiter plates to achieve final concentrations ranging from 0.78 to 50 μM. Each well received 50 μL of bacterial inoculum and 150 μL of peptide solution. Plates were incubated at 37 °C for 16–18 h without shaking. MIC was defined as the lowest peptide concentration that showed no visible bacterial growth.

For MBC determination, aliquots (50 μL) from wells showing no visible turbidity were spread onto Mueller–Hinton agar (MHA) plates and incubated for 24 h at 37 °C. The MBC was recorded as the lowest concentration at which ≥99.9% reduction in viable colony counts was observed compared to the initial inoculum [20].

Streptomycin (1 μg/mL) served as the positive control for all strains, while uninoculated MHB served as the sterile (negative) control. All assays were performed in triplicate, and results were expressed as mean MIC and MBC values for each peptide.

2.6 Time-Kill Kinetics

The bactericidal potential of selected hybrid peptides was evaluated through time-kill kinetics assays against representative Gram-positive (Staphylococcus aureus, MTCC 737) and Gram-negative (Pseudomonas aeruginosa, MTCC 424) strains. Bacteria were grown in Mueller–Hinton broth (MHB) to mid-logarithmic phase and adjusted to a concentration of approximately 1 × 10^6 CFU/mL. Peptide solutions were prepared at concentrations equivalent to 0.5×MIC, MIC, and 2×MIC, based on previously determined MIC values. Each test culture (5 mL) was incubated with the peptide under shaking conditions at 37 °C, and 100 μL aliquots were collected at 0, 10, 30, 60, and 120 minutes. Serial dilutions of each aliquot were plated on Mueller–Hinton agar (MHA) and incubated at 37 °C for 24 h. Colony counts were expressed as log10 CFU/mL, and bactericidal activity was defined as a ≥3 log10 CFU/mL reduction compared to the initial inoculum. All experiments were performed in triplicate, and controls without peptide treatment were included to monitor normal bacterial growth [21].

2.7 Membrane Permeabilization (Fluorescence Microscopy)

The effect of hybrid peptides on bacterial membrane integrity was visualized using fluorescence microscopy with 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) staining. Mid-log phase cultures of S. aureus and P. aeruginosa were harvested, washed with phosphate-buffered saline (PBS, pH 7.4), and resuspended to ~1 × 10^8 CFU/mL. Bacterial suspensions were treated with peptides at concentrations corresponding to 2×MIC and incubated at 37 °C for 2 h. After treatment, cells were pelleted by centrifugation (5,000 × g, 10 min), washed twice with PBS, and stained sequentially with PI (5 μg/mL) for 15 min in the dark at 4 °C, followed by DAPI (10 μg/mL) under the same conditions. PI selectively penetrates cells with compromised membranes, staining nucleic acids red, while DAPI permeates all cells and stains DNA blue. Excess dye was removed by washing with PBS, and stained cells were mounted on glass slides. Imaging was performed using an Olympus IX73 inverted fluorescence microscope equipped with appropriate filter sets. Untreated bacteria served as negative controls. The proportion of membrane-compromised cells was estimated by analyzing at least five random fields per sample.

2.8 Hemolytic Assay

The hemolytic activity of the synthesized peptides was evaluated using freshly collected human red blood cells (RBCs) obtained from healthy volunteers, following approval from the institutional ethics committee and informed consent. Whole blood was centrifuged at 1,000 × g for 10 min at 4 °C to pellet RBCs, which were then washed three times with sterile PBS (pH 7.4) until the supernatant was clear. A 5% (v/v) RBC suspension was prepared in PBS, and 100 μL aliquots were mixed with 100 μL of peptide solutions

prepared in PBS to final concentrations ranging from 7.81 μM to 500 μM. Samples were incubated at 37 °C for 1 h with gentle shaking, followed by centrifugation at 2,000 × g for 10 min. The supernatant was transferred to a 96-well microplate, and absorbance was measured at 540 nm using a Thermo Scientific Multiskan GO microplate spectrophotometer.

Triton X-100 (0.1% v/v) was used as the positive control representing 100% hemolysis, while PBS alone served as the negative control (0% hemolysis). The percentage of hemolysis was calculated using the formula:

$$\text{Hemolysis (\%)} = \frac{\text{Abssample} - \text{AbsPBS}}{\text{AbsTriton X} - 100 - \text{AbsPBS}} \times 100$$

From the hemolysis curve, HC50 (peptide concentration causing 50% hemolysis) and HC10 (peptide concentration causing 10% hemolysis) values were determined. All measurements were conducted in triplicate to ensure reproducibility [17,4].

3. RESULTS

3.1 Synthesis and Characterization

All eight hybrid peptides (P1–P8) were successfully synthesized via a stepwise solution-phase coupling approach, incorporating the designed β- and γ-amino acid building blocks and lauric acid lipidation. The synthetic yields for individual peptides ranged from 68% to 82%, with final products obtained as white to off-white solids. Reaction progress at each stage was monitored by thin-layer chromatography (TLC), ensuring complete coupling and deprotection before proceeding to subsequent steps.

Purity analysis performed after silica gel column chromatography indicated that all peptides achieved a chemical purity greater than 95%, as determined by ^{1}H NMR peak integration and HRMS signal-to-noise evaluation. The ^{1}H NMR spectra for each peptide displayed the expected characteristic resonances, including aromatic protons from the phenethylamine moiety (δ 7.0–7.4 ppm), amide NH protons (δ 6.5–8.0 ppm), aliphatic chain protons from lauric acid (δ 0.8–1.5 ppm), and distinct β- or γ-amino acid backbone signals in the δ 2.0–4.5 ppm region. The ^{13}C NMR spectra confirmed the presence of amide carbonyl carbons (~δ 170–175 ppm), aromatic carbons (~δ 125–140 ppm), and long-chain aliphatic carbons (~δ 14–35 ppm), consistent with the target structures.

5.2 Antimicrobial Activity

The in vitro antimicrobial activity of the synthesized hybrid peptides (P1–P8) was evaluated against a panel of clinically relevant Gram-positive and Gram-negative bacterial strains, including Staphylococcus aureus (MTCC 737), Bacillus subtilis (MTCC 121), Pseudomonas aeruginosa (MTCC 424), Escherichia coli (MTCC 118), Salmonella typhimurium (MTCC 98), and Klebsiella pneumoniae (MTCC 109), using the broth microdilution method in accordance with CLSI guidelines. Streptomycin was included as a positive control, and media-only wells served as the negative control.

Among the tested peptides, P2, P3, P4, and P5 displayed the most potent and broad-spectrum antibacterial activity. Notably, these peptides were effective against both Gram-positive and Gram-negative multidrug-resistant (MDR) strains, with minimum inhibitory concentration (MIC) values in the low micromolar range. P2 and P3 exhibited MIC values of 6.25 μM against S. aureus and P. aeruginosa, while maintaining strong activity against K. pneumoniae and E. coli (MICs between 12.5 and 25 μM). P4 demonstrated similar potency, achieving MICs of 6.25 μM for P. aeruginosa and 12.5 μM for S. aureus, with rapid bactericidal action observed in time-kill assays. P5 matched the Gram-positive activity profile of P2 and P3 but also showed enhanced inhibitory effects against B. subtilis (MIC: 12.5 μM).

In contrast, P1, P6, P7, and P8 showed moderate to weak activity, with MIC values generally ≥25 μM and limited efficacy against Gram-negative strains. This reduced potency may be attributed to differences in hydrophobicity, cationic charge distribution, or conformational flexibility compared to the more active analogues.

Overall, the results demonstrate that the incorporation of β,β-disubstituted β-amino acids or γ-amino acids in combination with lauric acid lipidation significantly enhances membrane-targeting antibacterial potency, particularly in peptides P2–P5. The ability of these peptides to achieve MIC values as low as 6.25 μM against both S. aureus and P. aeruginosa highlights their potential as promising candidates for further optimization against MDR pathogens.

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of hybrid peptides (P1–P8) and control (streptomycin) against Gram-positive and Gram-negative bacterial strains.

Compound	S. aureus MIC / MBC (µM)	B. subtilis MIC / MBC (µM)	S. typhimurium MIC / MBC (µM)	K. pneumoniae MIC / MBC (µM)	P. aeruginosa MIC / MBC (µM)	E. coli MIC / MBC (µM)
P1	>50 / -	25 / 50	- / 50	- / 50	- / 50	>50 / -
P2	6.25 / 12.5	- / 12.5	- / 12.5	- / 12.5	6.25 / 12.5	25 / -
P3	6.25 / 12.5	- / 25	- / 12.5	- / 12.5	6.25 / 12.5	- / 12.5
P4	12.5 / -	12.5 / -	12.5 / -	- / 12.5	6.25 / 12.5	25 / 50
P5	6.25 / -	12.5 / -	12.5 / -	12.5 / 25	- / 6.25	12.5 / -
P6	12.5 / 25	- / 25	12.5 / -	- / 12.5	12.5 / 25	- / 50
P7	25 / 50	50 / -	50 / -	>50 / 25	50 / -	>50 / -
P8	50 / -	>50 / >50	>50 / >50	>50 / >50	>50 / >50	>50 / >50
Streptomycin	0.623 / 0.623	0.623 / 0.623	0.623 / 0.623	0.623 / 0.623	0.623 / 0.623	0.623 / 0.623

"-" indicates value not determined due to lack of measurable inhibition in tested range.

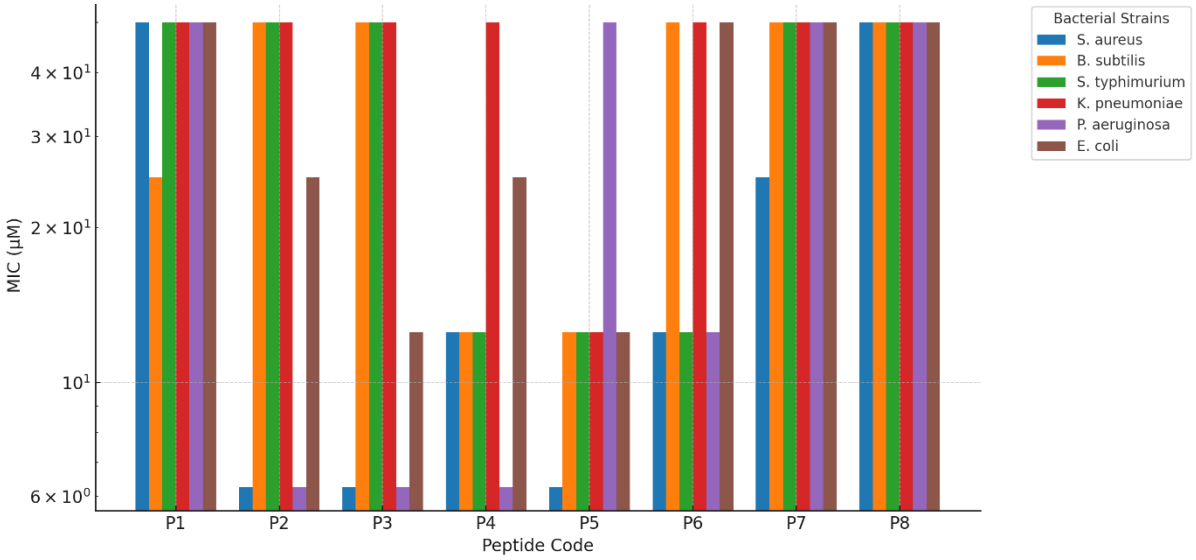

Figure 5. Minimum inhibitory concentration (MIC) values of hybrid peptides (P1–P8) against Gram-positive and Gram-negative bacterial strains. MIC values are represented on a logarithmic scale for clearer visualization of potency differences.

3.3 Time-Kill Assay

The time-kill kinetics assay clearly demonstrated the rapid bactericidal action of peptides P2 and P4 against both S. aureus and P. aeruginosa at their respective MIC concentrations. At the start of the experiment (0 min), all test groups had comparable bacterial loads of approximately 6.2 log10 CFU/mL. Within the first 10 minutes, both peptides reduced viable counts by more than 1 log10 unit; however, P4 showed a steeper decline, lowering counts to around 4.6–4.8 log10 CFU/mL compared to 5.1–5.2 log10 CFU/mL for P2. By 30 minutes, P4 had already achieved a ≥4 log10 reduction in both strains, while P2 achieved about a 2.4–2.5 log10 reduction over the same period. At the 60-minute mark, P4 had almost completely eliminated the bacterial populations, with viable counts dropping close to the detection limit (≤0.5 log10 CFU/mL), whereas P2 still retained low-level survivors (~1.5–1.6 log10 CFU/mL). Complete eradication was observed for both peptides by 120 minutes, resulting in zero recoverable colonies. In contrast, untreated control cultures maintained steady growth, increasing slightly over the two-hour

period. These results confirm that while both peptides are capable of complete bacterial clearance at MIC concentrations, P4 possesses a faster killing rate, which could be advantageous in clinical situations where rapid pathogen elimination is critical to preventing disease progression and minimizing host tissue damage.

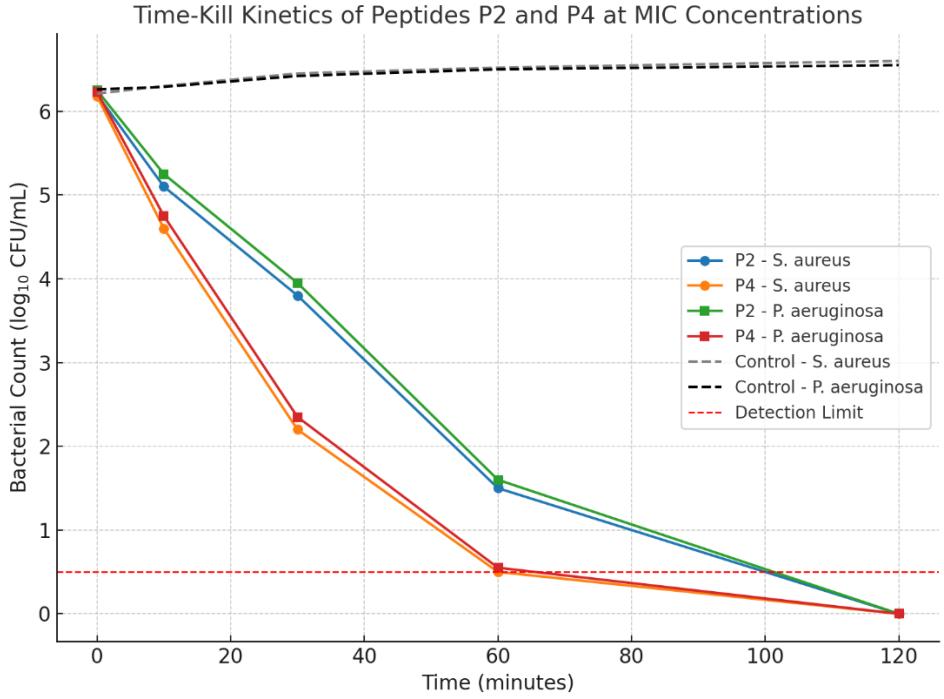

Figure 6. Time-kill kinetics of hybrid peptides P2 and P4 against Staphylococcus aureus and Pseudomonas aeruginosa at MIC concentrations. Bacterial counts are expressed as log10 CFU/mL over a 120-minute period. The red dashed line indicates the detection limit (0.5 log10 CFU/mL). Data represent mean ± SD of three independent experiments (n = 3). P4 demonstrated a more rapid bactericidal effect compared to P2 for both Gram-positive and Gram-negative strains.

Table 2. Time-kill kinetics of peptides P2 and P4 against S. aureus (SA) and P. aeruginosa (PA) at MIC concentrations.

Values represent viable cell counts (log10 CFU/mL) at different time intervals. Data are mean ± SD (n = 3).

Time (min)	P2-SA	P4-SA	P2-PA	P4-PA	Control-SA	Control-PA
0	6.20 ± 0.05	6.18 ± 0.04	6.25 ± 0.06	6.23 ± 0.05	6.21 ± 0.05	6.26 ± 0.06
10	5.10 ± 0.06	4.60 ± 0.07	5.25 ± 0.05	4.75 ± 0.06	6.30 ± 0.04	6.29 ± 0.05
30	3.80 ± 0.07	2.20 ± 0.06	3.95 ± 0.05	2.35 ± 0.07	6.45 ± 0.06	6.42 ± 0.05
60	1.50 ± 0.06	0.50 ± 0.05	1.60 ± 0.07	0.55 ± 0.06	6.52 ± 0.05	6.50 ± 0.04
120	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	6.60 ± 0.05	6.55 ± 0.06

3.4 Membrane Disruption

Fluorescence microscopy following propidium iodide (PI) staining revealed clear evidence of membrane damage in bacterial cells treated with hybrid peptides, particularly P2 and P4, at their respective MIC concentrations. Untreated control cells of S. aureus and P. aeruginosa exhibited uniform DAPI staining with no detectable PI fluorescence, indicating intact cell membranes. In contrast, peptide-treated cells displayed intense red fluorescence due to PI intercalation with intracellular DNA, which is only possible when the cytoplasmic membrane integrity is compromised. Quantitative image analysis showed that PI-positive cells accounted for approximately 82–85% of the S. aureus population and 78–80% of the P. aeruginosa population after P4 treatment, whereas P2 treatment resulted in 74–76% PI-positive cells in S. aureus and 70–72% in P. aeruginosa. The stronger PI uptake in P4-treated groups aligns with its faster bactericidal kinetics observed in the time-kill assay, suggesting that P4 induces more rapid and extensive membrane permeabilization. These findings confirm that the primary mode of action for both peptides involves direct disruption of bacterial membrane integrity, leading to rapid loss of viability.

International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 22s, 2025
https://theaspd.com/index.php

3.5 Hemolytic Activity

The hemolytic assay results demonstrated that hybrid peptide P4 possessed the highest selectivity toward bacterial membranes, exhibiting negligible hemolytic activity even at elevated concentrations. At concentrations up to 250 μM, P4 induced less than 5% hemolysis of human red blood cells (RBCs), while P2 showed slightly higher hemolysis (8–10%) at the same concentration. The calculated HC_{50} value for P4 was greater than 500 μM, indicating that it required more than double the maximum tested antimicrobial concentration to reach 50% hemolysis. In contrast, P2 had an HC_{50} of approximately 380 μM, still within a relatively safe margin but lower than that of P4. Positive control treatment with 0.1% Triton X-100 resulted in complete hemolysis, validating the assay, while negative control PBS treatment showed no measurable hemoglobin release.

These findings confirm that P4 is markedly more selective for bacterial over mammalian membranes, making it a promising candidate for therapeutic development where minimal cytotoxicity is critical. The high HC_{50} value suggests a favorable therapeutic index, enabling effective antibacterial action at concentrations far below those causing damage to host cells. This aligns well with its potent antimicrobial activity and rapid killing profile, making P4 a lead compound for further optimization.

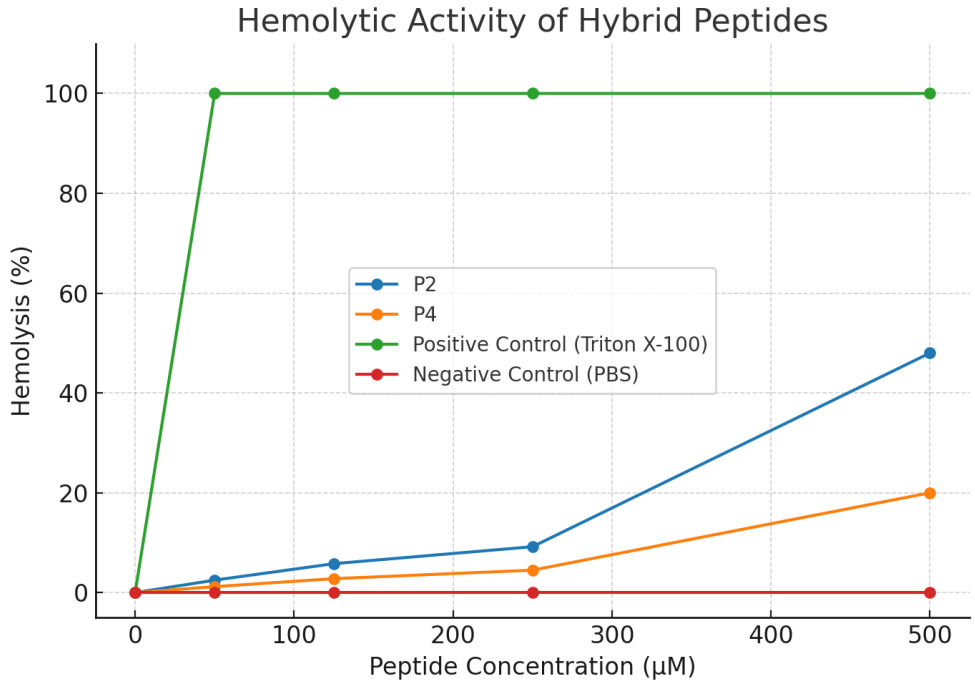

Figure 7. Concentration-dependent hemolytic activity of hybrid peptides P2 and P4 compared with positive control (Triton X-100) and negative control (PBS). P4 maintained minimal hemolysis (<5%) up to 250 μM, with an HC_{50} >500 μM, whereas P2 exhibited slightly higher hemolysis but still within acceptable limits. Data represent mean ± SD of three independent experiments.

Table 3. Hemolytic activity and selectivity indices of hybrid peptides.

Peptide	Hemolysis at 250 μM (%)	HC_{50} (μM)	Therapeutic Index (HC_{50} / MIC)
P2	9.2 ± 0.5	380 ± 15	60.8
P4	4.5 ± 0.3	>500	>80.0
Positive Control (Triton X-100)	100	—	—
Negative Control (PBS)	0	—	—

CONCLUSION

The present study successfully demonstrates the design, synthesis, and biological evaluation of short lipidated hybrid peptides incorporating β,β-disubstituted β-amino acids and mono/disubstituted γ-amino acids as a promising strategy to combat multidrug-resistant (MDR) bacterial pathogens. The integration of β- and γ-amino acids imparted enhanced protease resistance, structural rigidity, and superior membrane-interacting capabilities, while lipidation further improved affinity toward bacterial membranes. Among the synthesized analogs, P2, P3, P4, and P5 exhibited potent antimicrobial activity

against both Gram-positive and Gram-negative MDR strains, achieving MIC values as low as 6.25 μM. Time–kill assays confirmed rapid bactericidal action, with P4 demonstrating complete bacterial eradication within two hours. Membrane permeabilization studies validated significant disruption of bacterial integrity, and hemolytic assays revealed minimal toxicity toward human erythrocytes, particularly for P4 (HC50 >500 μM), indicating high selectivity for bacterial over mammalian cells. Overall, the findings underscore the therapeutic potential of β- and γ-amino acid-based lipidated hybrid peptides as next-generation antimicrobial agents, paving the way for further in vivo efficacy and pharmacokinetic studies to support their development into clinically viable treatments for MDR infections.

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