CHAPTER 3

Short Hybrid Peptides Incorporating β- and γ-Amino Acids as Antimicrobial Agents

3.1 Introduction


Herein, we describe the synthesis, characterization and antimicrobial activities of small hybrid peptides containing Orn/Lys together with β,β-disubstituted-β-amino acids, β3,3-
Ac₆c & β³⁻³-Pip(Ac); β,β-disubstituted-γ-amino acid, gabapentin (Gpn) and monosubstituted γ-amino acids, γ⁴⁻L-Phe and γ⁴⁻L-Leu, respectively. Figure 3.1 shows the chemical structures of hybrid peptides P1-P8.

**Figure 3.1:** Chemical structures of hybrid peptides, LA-Lys-PEA, P1; LA-Lys-β³⁻³Ac₆c-PEA, P2; LA-Orn-β³⁻³Ac₆c-PEA, P3; LA-Lys-Gpn-PEA, P4; LA-Orn-Gpn-PEA, P5; LA-Lys-γ⁴⁻L-Phe-PEA, P6; LA-γ⁴⁻L-Leu-Lys-PEA, P7; LA-β³⁻³Pip(Ac)-Lys-PEA, P8.

### 3.2 Experimental section

All the reagents for chemical synthesis were obtained from Sigma Aldrich, Novabiochem and Alfa aesar. The anhydrous solvents were purchased from Sigma Aldrich and Fisher Scientific. The reactions were monitored using thin-layer chromatography (TLC) on 0.25 mm silica gel 60 F₂₅₄ plates coated on aluminium sheet (E. Merck) and visualized using
UV light (254 nm) and ninhydrin as developing agent. The coupling reactions were mediated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCl.HCl) /1-hydroxybenzotriazole (HOBt) in dry dimethylformamide (DMF)/or dry dichloromethane (DCM) in presence of N-methylmorpholine (NMM). Purification of compounds was carried out by column chromatography using silica gel 60-120 mesh stationary phase. \(^1\)H NMR and \(^{13}\)C NMR spectra (with chemical shifts expressed in \(\delta\) and coupling constants in Hertz) were recorded on Bruker DPX, 400 instruments using CDCl\(_3\) as the solvents with TMS as internal standard. High resolution mass spectra (HRMS) were recorded on Agilent Technologies 6540 instrument.

### 3.2.1 Synthesis of \(\beta,\beta\)-disubstituted \(\beta\)-amino acid

The \(\beta,\beta\)-disubstituted-\(\beta\)-amino acids, \(\beta^{3,3}\)Ac\(_6\)c and \(\beta^{3,3}\)Pip(Ac) were synthesised according to the procedure reported in literature (Wani et al 2013).

### 3.2.2 Synthesis of monosubstituted \(\gamma\)-amino acids, Boc-\(\gamma^4\)-L-Phe-OH/ Boc-\(\gamma^4\)-L-Leu-OH

Scheme 3.1

Reagents and conditions: a) Dry DCM, Meldrum's acid, DMAP, EDC 0\(^\circ\)C to rt, 8 h; b) Dry DCM, CH\(_3\)COOH, NaBH\(_4\), 0\(^\circ\)C to rt, 1 h; c) Toluene, reflux, 1 h; (d) MeOH, 2N-NaOH, 1 h, rt.

Monosubstituted \(\gamma\)-amino acids were synthesised by the procedure reported in the literature (Smrcina et al 1997). Briefly, Boc-L-Phe-OH/Boc-L-Leu-OH was coupled with Meldrum’s acid at 0\(^\circ\)C in DCM using EDC and 4-dimethylamino pyridine (DMAP) to yield the intermediate 2, which was reduced by using sodium Borohydride (NaBH\(_4\)) in glacial acetic acid to yield 3. Further the intermediate 3, was refluxed in toluene for 3 h to
yield 4. The saponification was performed using 1N-NaOH in dry methanol for 30 min at 30°C to yield the desired product 5 (Boc-γ^4-L-Phe-OH/ Boc-γ^4-L-Leu-OH).

3.2.3 Synthesis of hybrid peptides P1-P8

Synthesis of LA-Lys(Cbz)-PEA, P1

Boc-Lys(Cbz)-OH (1.14 g, 3.0 mmol) was dissolved in 5.0 ml of DCM was added NMM (0.5 ml, 5 mmol) followed by the addition of EDC (0.573 g, 3.0 mmol) and phenethyl-amine (PEA) (0.6 ml, 5.0 mmol) at 0°C and stirred the reaction mixture for 12 h. The progress of the reaction was monitored using TLC at regular intervals. The solvent was evaporated and the residue was extracted with ethyl acetate and was washed successively with 2N-HCl (3×10 ml), 2M-Na₂CO₃ (3×10 ml) and brine solution. The combined organic layer was dried over anhydrous sodium sulphate and evaporated under vacuum to yield Boc-Lys(Cbz)-PEA (1), which was deprotected using 30% TFA in DCM to yield the peptide free base H₂N-Lys(Cbz)-PEA.

Scheme 3.2

Reagents and conditions: a) Dry DCM, NMM, EDC, PEA, 0°C to rt, 12 h; b) TFA in DCM, 2 h; c) Dry DMF, NMM, EDC, HOBT, Lauric acid, 0°C to rt, 24 h; d) 10% Pd/C in dry MeOH, 12 h.

Lauric acid (LA) (0.4 g, 2.0 mmol) was dissolved in 3.0 ml of dry DMF was added NMM (0.3 ml, 3.0 mmol) followed by the addition of EDC (0.38 g, 2.0 mmol)/HOBT (0.27 g,
2.0 mmol) the peptide free base H₂N-Lys(Cbz)-PEA was added into the reaction mixture and stirred the reaction for 24 h. The progress of the reaction was monitored using TLC at regular intervals. The reaction was worked up as described for 1 to yield the LA-Lys(Cbz)-PEA, which was purified by column chromatography over silica gel (60-120 mesh) to yield a white solid of LA-Lys(Cbz)-PEA.

LA-Lys(Cbz)-PEA (0.28 g, 0.5 mmol) was dissolved in 5.0 ml of dry methanol and was added 10% Pd/C (100 mg) to the reaction and stirred the reaction for 12 h under H₂ balloon. The progress of the reaction was monitored by using TLC at regular intervals. After completion of the reaction, filter the reaction mixture through Whatman filter paper and washed with 2-3 times with methanol. The filtrate was evaporated under vacuum to yield the desired product LA-Lys-PEA, P1. ¹H NMR (400 MHz, CDCl₃): δ 7.22 (m, 5H), 5.44 (s, 1H), 4.41 (dd, J = 36.0, 6.3 Hz, 1H), 3.47 (m, 3H), 2.78 (d, 3H), 2.32–2.06 (m, 4H), 1.54 (d, 4H), 1.25 (s, 21H), 0.88 (s, 3H). ¹³C NMR (126 MHz, CDCl₃): δ 175.80, 173.27, 171.99, 138.88, 128.74, 128.51, 126.39, 52.91, 51.88, 40.80, 40.66, 36.56, 36.00, 35.66, 31.93, 29.63, 29.59, 29.51, 29.44, 29.37, 29.36, 29.27, 25.71, 25.69, 25.59, 22.71, 14.16. HRMS-ESI: M_cal = 431.35; M_obs = 432.35[M+H]⁺.

**Synthesis of hybrid peptides P2-P6**

Scheme 3.3

Reagents and conditions: a) Dry DCM, NMM, EDC, PEA, 0°C to rt, 12 h; b,c) 30% TFA in DCM, Dry DCM, NMM, EDC, Boc-Lys(Cbz)-OH/BocOrn(Cbz)-OH, 0°C to rt, 24 h; d,e) 30% TFA in DCM, Dry DMF, NMM, EDC, HOBt, Lauric acid, 0°C to rt, 36 h; f) 10% Pd/C in dry MeOH, 12 h.

Boc-β³⁻Ac₆-OH/Boc-Gpn-OH (5.0 mmol) was dissolved in 5.0 ml of dry DCM was added NMM followed by the addition of EDC (5.0 mmol) and PEA (5.0 mmol) at 0°C
and stirred the reaction mixture for 12 h. The progress of the reaction was monitored using TLC at regular intervals. The solvent was evaporated and the residue was extracted with ethyl acetate and was washed successively with 2N-HCl (3×10 ml), 2M-Na₂CO₃ (3×10 ml) and brine solution. The combined organic layer was dried over anhydrous sodium sulphate and evaporated in vacuo to yield Boc-β³³Ac₆c-PEA/Boc-Gpn-PEA. Which was deprotected by 30% TFA in DCM yield the peptide free base H₂N-β³³Ac₆c-PEA/H₂N-Gpn-PEA.

Boc-Lys(Cbz)-OH/Boc-Orn(Cbz)-OH (3.0 mmol) was dissolved in 5.0 ml of dry DCM was added NMM followed by the addition of EDC (3.0 mmol)/HOBt (3.0 mmol). The peptide free base H₂N-β³³Ac₆c-PEA/H₂N-Gpn-PEA was added into the reaction mixture and stirred the reaction mixture for 24 h. The progress of the reaction was monitored by using TLC at regular intervals. The reaction was worked up as described in P1 to yield the Boc-Lys(Cbz)/Orn(Cbz)-β³³Ac₆c-PEA/Boc-Lys(Cbz)/Orn(Cbz)-Gpn-PEA. Which was deprotected by 30% TFA in DCM to yield the peptide free base H₂N-Lys(Cbz)/Orn(Cbz)-β³³Ac₆c-PEA/H₂N-Lys(Cbz)/Orn(Czb)-Gpn-PEA.

Lauric acid (2.0 mmol) was dissolved in 3.0 ml of dry DMF and was added NMM followed by the addition of EDC (2.0 mmol)/HOBt (2.0 mmol), the peptide free base H₂N-Lys(Cbz)/Orn(Cbz)-β³³Ac₆c-PEA/H₂N-Lys(Cbz)/Orn(Czb)-Gpn-PEA was added into the reaction mixture and stirred the reaction for 24 h under nitrogen conditions. The progress of the reaction was monitored using TLC at regular intervals. The reaction was worked up as described in P1 to yield the LA-Lys(Cbz)/Orn(Cbz)-β³³Ac₆c-PEA/LA-Lys(Cbz)/Orn(Czb)-Gpn-PEA. Which was purified by column chromatography over silica gel (60-120 mesh) to yield a white solid of LA-Lys(Cbz)/Orn(Cbz)-β³³Ac₆c-PEA/LA-Lys(Cbz)/Orn(Czb)-Gpn-PEA.

LA-Lys(Cbz)/Orn(Cbz)-β³³Ac₆c-PEA/LA-Lys(Cbz)/Orn(Czb)-Gpn-PEA (1.0 mmol) was dissolved in 5.0 ml of dry methanol and was added 10% Pd/C (100 mg) to the reaction and stirred the reaction for 12 h under H₂ balloon. The progress of the reaction was monitored by using TLC at regular intervals. After completion of the reaction filter the reaction mixture through Whatman filter paper and washed with 2-3 times with methanol. The filtrate was evaporated under vacuum to yield the desired product LA-Lys/Orn-β³³Ac₆c-PEA/LA-Lys/Orn-Gpn-PEA, P2-P5. The peptide P6, was synthesized
by the same procedure described for peptide P2, where $\gamma^L$-Phe was used in place of gabapentin (Gpn).

**N-(6-amino-1-oxo-1-((1-(2-oxo-2-(phenethylamino)ethyl)cyclohexyl)amino)hexan-2-yl)dodecanamide, P2**

$^1$H NMR (400 MHz, DMSO): $\delta$ 7.95 (d, $J = 6.0$ Hz, 1H), 7.68 (s, 1H), 7.28 (m, 3H), 7.20 (m, 3H), 4.17 (m, 1H), 3.27 (m, 2H), 2.72 (dd, $J = 14.0$, 6.8 Hz, 3H), 2.35 (d, $J = 13.1$ Hz, 1H), 2.17–2.10 (m, 3H), 2.02 (d, $J = 11.5$ Hz, 6H), 1.71–1.09 (m, 34H), 0.85 (d, $J = 6.1$ Hz, 3H).

$^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 174.22, 172.29, 172.25, 170.80, 139.08, 128.83, 128.80, 128.55, 126.45, 55.31, 53.72, 40.63, 39.20, 36.39, 36.32, 35.41, 35.05, 34.67, 31.94, 31.52, 29.71, 29.67, 29.61, 29.51, 29.41, 29.39, 26.56, 25.80, 25.31, 22.70, 22.30, 21.68, 14.17, 14.13. HRMS-ESI: $M_{\text{Cal}}$= 570.44; $M_{\text{Obs}}$=571.45[M+H]$^+$. 

**N-(5-amino-1-oxo-1-((1-(2-oxo-2-(phenethylamino)ethyl)cyclohexyl)amino)pentan-2-yl) dodecanamide, P3**

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.26 (m,5H), 7.00 (d, $J = 5.9$ Hz, 1H), 6.35 (s, 1H), 6.23 (s, 1H), 4.16 – 4.08 (m, 1H), 3.58 (m, 2H), 3.41 (m, 1H), 2.81 (dd, $J = 15.2$, 7.8 Hz, 4H), 2.70 (d, $J = 13.4$ Hz, 2H), 2.19 (m, 3H), 1.33 (m, 30H), 0.88 (t, $J = 6.3$ Hz, 3H).

$^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 174.40, 172.18, 171.41, 139.09, 128.80, 128.45, 126.32, 52.59, 45.73, 43.01, 40.58, 40.35, 37.23, 36.59, 35.61, 34.27, 34.15, 31.90, 31.35, 29.64, 29.62, 29.53, 29.38, 29.34, 22.71, 21.71, 21.64, 14.30. HRMS-ESI: $M_{\text{Cal}}$=5576.43; $M_{\text{Obs}}$=557.44[M+H]$^+$. 

**N-(6-amino-1-oxo-1-(1-(2-oxo-2-(phenethylamino)ethyl)cyclohexyl) methyl) amino) hexan-2-yl)dodecanamide, P4**

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.96 (s, 1H), 7.24 (m, 5H), 7.03 (d, $J = 6.9$ Hz, 1H), 4.54 (s, 1H), 3.50 (t, $J = 9.6$ Hz, 2H), 3.21 (dd, $J = 13.5$, 6.2 Hz, 1H), 3.09 (dd, $J = 13.6$, 5.7 Hz, 1H), 3.01 (s, 1H), 2.85 (t, $J = 6.9$ Hz, 2H), 2.25 (t, $J = 7.4$ Hz, 2H), 2.10 (s, 2H), 1.60 (s, 3H), 1.54 – 1.16 (m, 30H), 0.87 (t, $J = 6.3$ Hz, 3H). 

$^{13}$C NMR (101 MHz, CDCl$_3$): $\delta$ 174.40, 172.18, 171.41, 139.09, 128.80, 128.45, 126.32, 52.59, 45.73, 43.01, 40.58, 40.35, 37.23, 36.59, 35.61, 34.27, 34.15, 31.90, 31.35, 29.64, 29.62, 29.53, 29.38, 29.34,

N-(5-amino-1-oxo-1-(1-(2-oxo-2-(phenethylamino)ethyl)cyclohexyl)methyl)amino)pentan-2-yl)dodecanamide, P5

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.71 (s, 1H), 7.25 (m, 5H), 6.50 (d, $J = 7.7$ Hz, 1H), 4.50(m, 1H), 3.59 – 3.49 (m, 3H), 3.22 (dd, $J = 13.6$, 6.5 Hz, 2H), 3.07 (dd, $J = 13.6$, 6.0 Hz, 2H), 2.85 (t, $J = 6.9$ Hz, 3H), 2.38 (d, $J = 6.2$ Hz, 2H), 2.20(m, 3H), 2.05 (m,3H), 1.25 (s, 31H), 0.87 (t, $J = 6.5$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 174.78, 174.28, 172.74, 140.48, 130.24, 129.90, 127.78, 54.87, 54.60, 53.43, 42.03, 38.64, 38.18, 37.11, 35.93, 35.61, 34.34, 33.34, 31.09, 31.07, 31.00, 30.92, 30.86, 30.83, 30.79, 28.45, 27.37, 27.31, 24.78, 24.12, 22.95, 15.57. HRMS-ESI: M_{cal}=570.44; M_{obs}=571.45[M+H]^+.

N-(6-amino-1-oxo-1-((5-oxo-5-(phenethylamino)-1-phenylpentan-2-yl)amino)hexan-2-yl) dodecanamide, P6

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.33–7.07 (m, 10H), 6.61 (s, 1H), 6.54 (d, $J = 8.1$ Hz, 1H), 6.24 (d, $J = 7.0$ Hz, 1H), 4.24 (q, $J = 6.7$ Hz, 6.7 Hz, 1H), 4.01 (s, 18H), 3.55 (m, 2H), 3.42 (m, 2H), 2.82(t, $J = 6.5$ Hz, 3H), 2.69 (dd, $J = 13.7$, 7.1 Hz, 2H), 1.83 – 1.63 (m, 3H), 1.54 (d, $J = 28.3$ Hz, 4H), 1.35 – 1.15 (m, 21H), 0.87 (d, $J = 6.7$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 173.80, 171.77, 156.30, 138.68, 136.33, 128.73, 128.59, 128.53, 128.11, 128.05, 126.53, 66.54, 52.78, 40.67, 40.41, 36.40, 35.48, 31.92, 29.65, 29.63, 29.55, 29.49, 29.39, 29.36, 29.32, 25.66, 22.70, 22.37, 14.15. HRMS-ESI: M_{cal}= 606.44; M_{obs}=607.45[M+H]^+.

Synthesis of peptides P7 and P8

Boc-Lys(Cbz)-OH (3.0 mmol) was dissolved in 5.0 ml of DCM was added NMM followed by the addition of EDC (3.0 mmol) and PEA (5.0 mmol) at 0°C and stirred the reaction mixture for 12 h. The progress of the reaction was monitored using TLC at regular intervals. The solvent was evaporated and the residue was extracted with ethyl acetate and was washed successively with 2N-HCl (3×10 ml), 2M-Na$_2$CO$_3$ (3×10 ml) and brine solution. The combined organic layer was dried over anhydrous sodium sulphate
Scheme 3.4

Reagents and conditions:  
- a) Dry DCM, NMM, EDC, PEA, 0°C to rt, 12 h;  
- b, c) 30% TFA in DCM, Dry DCM, NMM, EDC, Boc-γ^4-Leu-OH, 0°C to rt, 24 h;  
- d, e) 30% TFA in DCM, Dry DMF, NMM, EDC, HOBT, Lauric acid, 0°C to rt, 24 h;  
- f) 10% Pd/C in dry MeOH.

and evaporated in vacuo to yield Boc-Lys(Cbz)-PEA (1). Which was deprotected using 30% TFA in DCM to yield the peptide free base H_2N-Lys(Cbz)-PEA.

Boc-γ^4-Leu-OH (3.0 mmol) was dissolved in 5.0 ml of dry DCM was added NMM followed by the addition of EDC (3.0 mmol)/HOBT (3.0 mmol). The peptide free base H_2N-Lys(Cbz)-PEA was added into the reaction mixture and stirred the reaction mixture for 24 h. The progress of the reaction was monitored using TLC at regular intervals. The reaction was worked up as described in P1 to yield the Boc-γ^4-Leu-Lys(Cbz)-PEA. Which was deprotected using 30% TFA in DCM to yield the peptide free base H_2N-γ^4-Leu-Lys(Cbz)-PEA.

Lauric acid (2.0 mmol) was dissolved in 3.0 ml of dry DMF was added NMM followed by the addition of EDC (3.0 mmol)/HOBT (3.0 mmol), the peptide free base H_2N-γ^4-Leu-Lys(Cbz)-PEA was added into the reaction mixture and stirred the reaction for 24 h under nitrogen conditions. The progress of the reaction was monitored using TLC at regular intervals. The reaction was worked up as described in P1 to yield the LA-γ^4-Leu-Lys(Cbz)-PEA, which was purified by column chromatography over silica gel (60-120 mesh) to yield a white solid of LA-γ^4-Leu-Lys(Cbz)-PEA. The benzylxycarbonyl group was deprotected using 10% Pd/C in dry methanol to yield the final peptide LA-γ^4-Leu-
Lys-PEA. The peptide **P8** was synthesized by the same procedure, where \(\beta^{3,3}\text{Pip(Ac)}\) was used in place of \(\gamma^{4}\text{-Leu}\) to afford LA-\(\beta^{3,3}\text{Pip(Ac)}\)-Lys-PEA.

**N-(1-(6-amino-1-oxo-1-(phenethylamino)hexan-2-yl)amino)-6-methyl-1-oxoheptan-4-yl) dodecanamide, P7**

\(^1\text{H NMR (400 MHz, CDCl}_3\): \(\delta 7.26-7.18\) (m, 5H), 6.92 (s, 1H), 5.96 (d, \(J = 8.0\text{Hz}\), 1H), 5.72 (s, \(J = 8\text{Hz}\), 1H), 4.39 (s, 1H), 3.93 (s, 1H), 3.45 (s, 3H), 2.80 (s, 3H), 2.54 (s, 3H), 2.18 (s, 3H), 1.61 (s, 8H), 1.25 (s, 21H), 0.88 (s, 9H). \(^{13}\text{C NMR (101 MHz, CDCl}_3\): \(\delta 202.45, 196.30, 172.96, 128.78, 128.74, 128.42, 126.29, 126.26, 69.02, 66.84, 63.82, 53.38, 47.08, 46.87, 46.75, 40.83, 40.52, 40.30, 40.10, 40.09, 39.88, 36.91, 35.66, 35.61, 33.05, 33.01, 32.94, 32.88, 31.88. HRMS-ESI: \(M_{\text{cal}}=572.46; M_{\text{obs}}=573.47[M+H]^+\).**

**N-(1-acetyl-4-((6-amino-1-oxo-1-(phenethylamino)hexan-2-yl)amino)-2-oxoethyl) piperidine-4-yl)dodecanamide, P8**

\(^1\text{H NMR (400 MHz, CDCl}_3\): \(\delta 7.33-7.13\) (m, 6H), 6.81 (s, 1H), 6.26 (s, 1H), 4.29 (s, 1H), 4.14 (s, 1H), 3.6–3.29 (m, 5H), 3.27 (d, \(J = 6.5\text{ Hz}\), 1H), 2.97 (dd, \(J = 30.4, 13.6\text{ Hz}\), 1H), 2.76 (m, 2H), 2.74–2.64 (m, 1H), 2.63-2.48 (m, 1H), 2.38 (s, 1H), 2.14 (m, 3H), 2.08 (d, \(J = 2.7\text{ Hz}\), 1H), 2.06 (s, 3H), 1.58 (m, 9H), 1.24 (s, 19H), 0.88 (s, 3H). \(^{13}\text{C NMR (126 MHz, CDCl}_3\): \(\delta 174.28, 171.70, 169.12, 138.95, 128.79, 128.77, 128.73, 128.68, 128.59, 128.55, 128.51, 128.20, 126.36, 53.47, 53.45, 53.38, 53.34, 53.33, 53.29, 40.80, 37.41, 35.63, 31.93, 29.70, 29.65, 29.60, 29.49, 29.38, 25.83, 22.69, 21.41, 14.17. HRMS-ESI: \(M_{\text{cal}}=613.45; M_{\text{obs}}=614.46[M+H]^+\).**
3.2.4 Spectral data

Figure 3.2: $^1$H and $^{13}$C NMR of LA-Lys-PEA, P1 in DMSO-$d_6$. 
Figure 3.3: $^1$H and $^{13}$C NMR of LA-Lys-$\beta^{13}$Ac$_6$-PEA, P2 in DMSO-d$_6$. 
Figure 3.4: $^1$H and $^{13}$C NMR of LA-Orn-$\beta^{3, 3}$Ac$_6$-PEA, P3 in DMSO-d$_6$.
Figure 3.5: $^1$H and $^{13}$C NMR of LA-Lys-Gpn-PEA, P4 in DMSO-d$_6$
Figure 3.6: $^1$H and $^{13}$C NMR of LA-Orn-Gpn-PEA, P5 in DMSO-d$_6$
Figure 3.7: $^1$H and $^{13}$C NMR of LA-Lys-$\gamma$-L-Phe-PEA, P6
3.2.5 Antimicrobial activity of hybrid peptides

To determine the antimicrobial activity of the peptides, MIC and MBC values were evaluated by broth microdilution against Bacillus subtilis (MTCC 121), Pseudomonas aeruginosa (MTCC 424), Salmonella typhimurium (MTCC 98), Escherichia coli (MTCC 118), Klebsiella pneumonia (MTCC 109), Staphylococcus aureus (MTCC 737) by method described by Clinical Laboratory and Standards Institute (CLSI) with slight modification (Steinberg et al. 1997). Briefly, the bacterial colonies were grown in Mueller-Hinton broth medium to mid logarithmic phase then bacteria were adjusted to a 0.5 McFarland turbidity standard with OD at 600 nm to 0.321 which corresponds to $1.5 \times 10^8$ CFU/mL followed by serial dilutions to give $4 \times 10^4$ CFU/mL. The 50 μL aliquots of these bacteria were added to 96 well flat bottom microtiter plates containing 150 μL of peptides at different concentration (50 μM, 25 μM, 12.5 μM, 6.25 μM, 3.12 μM, 1.56 μM and 0.78 μM) and mixture were incubated at 37°C for 12-16 h. Appropriate positive (Streptomycin) and blank controls (virgin media) were used. For determining MIC and MBC, the 50 μL of sample was taken from well having non-visible growth was spread on MH agar plates and incubated overnight at 37°C. The lowest concentration at which there was no visible growth was considered as minimum inhibitory concentration (MIC). For determining MBC, the 50 μL of sample was taken from well having non-visible growth was spread on MH agar plates and incubated overnight at 37°C. The lowest concentration at which 99.9% of the pathogen was killed was considered as the minimum bactericidal concentration (MBC).

3.2.6 Time kill kinetics assay

P. aeruginosa and S. aureus were grown to mid logarithmic phase and then diluted to $10^4$ CFU/ml suspensions. The diluted bacterial suspension was incubated with different concentration of peptides P2, P3, P4, P5 and P6 for 10, 30, 60 and 120 min respectively. After the incubation, the mixture was diluted to $10^2$ times and spread on MH agar plates. After incubation at 37 °C for 24 h, the number of colonies was counted (Choi et al. 2009).

3.2.7 Fluorescence microscopy

The effect of peptides (P2, P3, P4 and P5) on bacterial membrane integrity of P. aeruginosa and S. aureus was assessed by fluorescence microscopy using 4′,6-diamidino-
2-phenylindole dihydrochloride (DAPI) and propidium iodide (PI). PI can only pass through damaged membrane and therefore stain only dead cells whereas DAPI is able to dye all bacteria cells regardless of their viabilities. The bacterial cultures were grown to mid logarithmic phase and incubated with peptide **P3** and **P5** with 8 μM concentrations at 37°C for 2 h. The cells were collected by centrifugation at 5000 g for 15 min, washed with PBS then incubated with PI (5 μg/mL) for 15 min at 0°C in dark. Unbound PI was removed by washing the cells with PBS. Then cells were incubated with DAPI (10 μg/mL) for 15 min at 0°C in dark. The cultures without peptide treatment were designated as controls. The stained bacteria were observed under an inverted microscope equipped with digital camera (Olympus Imaging Corp., Center Valley, PA, USA) (Li et al 2013).

**3.2.8 Hemolytic assay**

The hemolytic activity means amount of hemoglobin released by the lysis of human erythrocytes (Conlon et al 2003). The human red blood cells (RBC) were freshly collected and washed with sterile phosphate buffered saline (PBS) and centrifuged at 1000 g for 10 min at 4°C until the upper solution became clear. RBCs were diluted to 5% suspension and mixed with peptides at different concentrations (500 to 7.81 μM) in a 96 well plate. PBS and 0.1% Triton X-100 were used as negative and positive controls, respectively. The mixture was incubated at 37°C for 1 h and centrifuged at 2000 rpm for 20 min. The supernatant was transferred to a 96 well plate and absorbance was measured at 360 nm on Microplate Spectrophotometer (Thermo Scientific Multiskan GO Microplate Spectrophotometer). The percentage of hemolysis was calculated using the following formula: 100% hemolysis = 100 (Abs_{peptide}-Abs_{PBS}) / (Abs_{triton X}-Abs_{PBS})

**3.3 Results and Discussion**

All the peptides **P1-P8** were evaluated for the antimicrobial activity against Gram-positive *Staphylococcus aureus* (MTCC 737), *Bacillus subtilis* (MTCC 121), *Salmonella typhimurium* (MTCC 98) and Gram-negative *Klebsiella pneumonia* (MTCC 109), *Pseudomonas aeruginosa* (MTCC 424) and *Escherichia coli* (MTCC 118) bacterial strains. The *in vitro* antimicrobial activities of all the peptides are listed in Table 3.1. Among all, peptides **P2, P3, P4 and P5** exhibited potent antimicrobial activities against
Gram-negative bacteria, *P. aeruginosa* (MIC: 6.25 µM). In addition, peptides P2, P3 and P5 showed the antimicrobial activities against Gram-positive bacteria, *S. aureus* (MIC: 6.25 µM).

To understand the efficacy of peptides, time kill assay was used to investigate the ability of peptides P2, P3, P4, P5 and P6 to kill both *S. aureus* (MTCC 737) and *P. aeruginosa*. Time kill assay was used to investigate the ability of hybrid peptides P2, P3, P4 and P5 to kill both Gram-positive *S. aureus* (MTCC 737) and Gram-negative *P. aeruginosa* (MTCC 424) bacteria. These peptides were tested at concentration equal to 0.5xMIC, MIC and 2xMIC. After two hours of treatment, the cell viability was determined by Figure 3.8 shows the treatment of peptides P2 and P4 with *P. aeruginosa* and *S. aureus* in dose response curve. Peptides P2 and P4 completely eradicate both *S. aureus* and *P. aeruginosa* at 12.5 µM but P4 took half time in comparison to P2 against both the pathogens. The treatment of peptides P3, P5 and P6 with *P. aeruginosa* and *S. aureus* are shown in Figure 3.9. Mostly, they were bacteriostatic.

### Table 3.1: Antimicrobial activity of hybrid peptides (P1-P8). Microorganisms used were *Staphylococcus aureus* MTCC 737; *Bacillus subtilis* MTCC 121; *Salmonella typhimurium* MTCC 98; *Klebsiella pneumonia* MTCC 109; *Pseudomonas aeruginosa* MTCC 424; *Escherichia coli* MTCC 118; Streptomycin (µg/mL) and blank (virgin media) were used as positive and negative control.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th><em>Staphylococcus aureus</em></th>
<th><em>Bacillus subtilis</em></th>
<th><em>Salmonella typhimurium</em></th>
<th><em>Klebsiella pneumonia</em></th>
<th><em>Pseudomonas aeruginosa</em></th>
<th><em>Escherichia coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td>MIC&lt;a&gt;</td>
<td>MBC&lt;br&gt;</td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
<td>MBC</td>
</tr>
<tr>
<td>P1</td>
<td>&gt;50</td>
<td>-</td>
<td>25</td>
<td>50</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>P2</td>
<td>6.25</td>
<td>12.5</td>
<td>-</td>
<td>12.5</td>
<td>-</td>
<td>12.5</td>
</tr>
<tr>
<td>P3</td>
<td>6.25</td>
<td>12.5</td>
<td>-</td>
<td>12.5</td>
<td>-</td>
<td>12.5</td>
</tr>
<tr>
<td>P4</td>
<td>25</td>
<td>50</td>
<td>12.5</td>
<td>-</td>
<td>12.5</td>
<td>25</td>
</tr>
<tr>
<td>P5</td>
<td>6.25</td>
<td>-</td>
<td>12.5</td>
<td>-</td>
<td>12.5</td>
<td>6.25</td>
</tr>
<tr>
<td>P6</td>
<td>12.5</td>
<td>25</td>
<td>25</td>
<td>12.5</td>
<td>-</td>
<td>12.5</td>
</tr>
<tr>
<td>P7</td>
<td>25</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>&gt;50</td>
<td>25</td>
</tr>
<tr>
<td>P8</td>
<td>50</td>
<td>-</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>0.623</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>-</td>
<td>0.623</td>
<td>-</td>
<td>0.623</td>
<td>-</td>
<td>0.623</td>
</tr>
</tbody>
</table>

<a>MIC, minimum inhibitory concentration in µM</a>

<b>MBC, minimum bactericidal concentration in µM</b>
In order to investigate the mechanism of bactericidal action of peptides, membrane permeabilization studies were performed. The disruption of membrane of *P. aeruginosa* and *S. aureus* by peptides **P2** and **P4** were carried out by fluorescence microscopic studies using 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) as staining reagents. DAPI is a membrane-permeable dye that can cross membranes, regardless of cell viability, however PI can only stain cells when cell membranes are damaged.

**Figure 3.8:** Time-kill curves of peptides **P2** and **P4** for A) *P. aeruginosa* B) *S. aureus*. The killing activity of peptides against these strains was monitored for the first 2 h. The peptide concentration used in the experiment were untreated control (purple), 0.5 MIC (green), MIC (red) and 2x MIC (blue).

**Figure 3.9:** Time-kill curves of **P3**, **P5** and **P6** for A) *P. aeruginosa* B) *S. aureus* respectively. The killing activity of peptides against these strains was monitored for the first 2 h. The peptide concentration used in the experiment were untreated control (purple), 0.5 MIC (green), MIC (red), and 2x MIC (blue).
Figure 3.10 and 3.11 show the fluorescence micrograph of *P. aeruginosa* and *S. aureus* after treatment for 2 h. As shown in Figure 3.10 the treatment of bacteria with peptides **P2** and **P4** resulted in visibility of red-fluorescent bacteria under the PI channel, suggesting the membranes of both *P. aeruginosa* and *S. aureus* were damaged. In addition, the treatment with **P3** and **P5** led to significant aggregation of *S. aureus* is believed to arise from dissipation of membrane potential due to membrane disruption as shown in Figure 3.11 (Wu et al 2012, Padhee et al 2014).

![Fluorescence micrographs of *P. aeruginosa* and *S. aureus*.](image)

**Figure 3.10:** Fluorescence micrographs of *P. aeruginosa*. 1st row: untreated control; 2nd row: treated with 8 µM of **P2** for 2 h; 3rd row: treated with 8 µM of **P4** for 2 h. and *S. aureus*. 1st row: untreated control; 2nd row: treated with 8 µM of **P2** for 2 h; 3rd row: treated with 30 µM of **P4** for 2 h.

![Fluorescence micrographs of *P. aeruginosa* and *S. aureus*.](image)

**Figure 3.11:** Fluorescence micrographs of *P. aeruginosa* Row 1) untreated control 2) treated with 8 µM of **P3** 3) treated with 4 µM of **P5** and *S. aureus* Row 1) untreated control 2) treated with 8 µM of **P3** 3) treated with 8 µM of **P5**.
Further, all the peptides were checked for their hemolytic properties at different concentration. The hemolytic activities, i.e. the concentration needed to induce 50% (HC_{50}) and 10% (HC_{10}) haemoglobin release of all the peptides are summarized in Table 3.2. Among all the active peptides, peptide P4 exhibited lowest hemolytic activity at HC_{50} and HC_{10}.

<table>
<thead>
<tr>
<th>Compound</th>
<th>HC_{50}</th>
<th>HC_{10}</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>253.42</td>
<td>77.98</td>
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<tr>
<td>P2</td>
<td>29.57</td>
<td>&lt;7.812</td>
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<tr>
<td>P3</td>
<td>81.07</td>
<td>&lt;7.812</td>
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<tr>
<td><strong>P4</strong></td>
<td>&gt;500</td>
<td><strong>175.06</strong></td>
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<tr>
<td>P5</td>
<td>78.05</td>
<td>&lt;7.812</td>
</tr>
<tr>
<td>P6</td>
<td>203.62</td>
<td>&lt;7.812</td>
</tr>
<tr>
<td>P7</td>
<td>&gt;500</td>
<td>308.64</td>
</tr>
<tr>
<td>P8</td>
<td>&gt;500</td>
<td>313.45</td>
</tr>
</tbody>
</table>

### 3.4 Conclusions

In conclusion, we investigated the potent antimicrobial properties of short hybrid peptides containing β- and γ-amino acids. The peptides involving β,β-disubstituted-β-amino acid (β^{3,3}-Ac_{6}c) and β,β-disubstituted-β-amino (Gpn) together with lysine (Lys) display rapid killing and membrane disruption of Gram-positive (S. aureus) and Gram-negative (P. aeruginosa) pathogens. Further, peptide P4 exhibited lowest hemolytic activity among all the active peptides. The present study can be extended to develop potent antimicrobial agents by introducing β,β-disubstituted-β-amino acid and β,β-disubstituted-γ-amino acids in shorter peptide sequences.