CHAPTER 4

Piperic Acid (PA) & 4-Ethylpiperic Acid (EPA) Amides with α-, β- and γ-Amino Acids as Potent NorA Efflux Pump Inhibitors

4.1 Introduction

Multidrug resistant (MDR) pathogenic bacteria represents one of the main causes for the failure of antimicrobial drugs (Hancock et al 1999, Taubes et al 2008, Gorden et al 2005, Giauliani et al 2007, Fjell et al 2012, Yount et al 2012). These pathogens often emerge after the long-term use of antimicrobial drugs. It is commonly characterized by the resistance to a series of unrelated compounds (Simmon et al 1994). The most important form of MDR is mediated by membrane efflux proteins such as P-glycoprotein (P-gp) in mammalian cells (Marquez et al 2005) and the NorA pump in Staphylococcus aureus (Lynch et al 2006). Reduced permeability of the bacterial envelope and/or extrusion of the drug from the cell through membrane-based efflux proteins are one of the major mechanisms of MDR. NorA is a Staphylococcus aureus efflux pump that gives reduced susceptibility to many structurally unrelated compounds i.e. fluoroquinolones, biocides and dyes resulting in a multidrug resistant (MDR) phenotype. Efflux pumps are involved in ~60% of global nosocomial infectious (WHO press release, WHO, Geneva 2000). These are responsible for the extrusion of toxic compounds including antibacterials encountered in the bacterial environment (Marquez et al 2005). Efflux Pump Inhibitors (EPI) have been proved to enhance the activity of several antibiotics identified as the substrate of these efflux pumps by blocking their removal from the core of bacterial cells (Lomovskaya et al 1999, Lynch et al 2006). It has been reported that efflux is an important mechanism of resistance in many clinically relevant pathogens, notably, Streptococcus pneumoniae, Pseudomonas aeruginosa and Neisseria gonorrhoeae (Ross et al 1990, Guay et al 1993). Staphylococcus aureus (S. aureus) has shown ability to acquire resistance to several antibacterial drugs currently available in the market. The pathogen is known to possess several efflux pumps, notably NorA, TetK, MsrA and
MdeA, which transports fluoroquinolones, tetracyclines, macrolides and mupirocin drugs, respectively (Litzinger et al 1997, Stavri et al 2007).

Most of the efflux pump inhibitors have been explored from piperine (Litzinger et al 1997), an alkaloid, present in *Piper nigrum*-L (black pepper). It has been reported that the piperine, a phytochemical potentiator of ciprofloxacin against *Staphylococcus aureus* (Khan et al 2006). Piperine is known for its different biological and pharmacological activities i.e ant apoptotic (Choi et al 2007), antibacterial (Umadevi et al 2013) antidepressant (Lee et al 2005), antifungal (Umit et al 2008), anti-inflammatory (Wei et al 2004), anti-metastatic (Pradeep et al 2002), antioxidative, antitumor (Sunila et al 2004) and antithyroid (Panda et al 2003) as well as for its efflux pump inhibitory (EPI) activity (Mirza et al 2011). Piperine enhances the bioavailability of many drugs through a number of mechanisms (Atal et al 1985, Patil et al 2011). The structural analogs of piperine as well as piperoyl amides have been reported as inhibitor of bacterial NorA efflux pump against *S. aureus* (Kumar et al 2008, Sangwan et al 2008). Figure 4.1 shows some examples NorA efflux pump inhibitors reported in the literature (Marquez et al 2005, Handzlik et al 2013, Kumar et al 2008).

**Figure 4.1:** Chemical structures of known NorA efflux pump inhibitors.
Based on the previous studies with piperine and its analogs, we envisaged to synthesise the amides of piperic acid (PA) and 4-ethylpiperic acid (EPA) with α-, β- and γ-amino acid esters (1-20). L-Ala, L-Val, L-Leu, L-Phe, L-Pro, L-Trp and L-tert-Leu were used as α-amino acid, while β\(^{3,3}\)-Ac\(_{6c}\), 4-ethyl-β\(^{3,3}\)-Ac\(_{6c}\), 4-tert-butyl-β\(^{3,3}\)-Ac\(_{6c}\), β\(^{3,3}\)-Pip(Bzl) and β\(^{3,3}\)-Pip-OH as β-amino acids. β-Amino acids have been reported as an important element of biologically active natural products such as taxol, bleomycin and cytotoxic microcystin (Kudo et al 2014). Gabapentin, an anti-epileptic drug used in neuropathic pain (Rosenberg et al 1997), was used as γ-amino acid. Figure 4.2 shows the chemical structures of piperic acid (PA), 4-ethylpiperic acid (EPA) and their amides 1-20. All the amides were evaluated for EPI activity against wild type strain S. aureus SA-1199 having normal expression level of NorA as well as NorA over expressing strain S. aureus SA-1199B.

![Chemical structures of piperine, piperic acid (PA), 4-ethylpiperic acid (EPA) and their amides (1-20)](image-url)

**Figure 4.2:** Chemical structures of piperine, piperic acid (PA), 4-ethylpiperic acid (EPA) and their amides (1-20)
4.2 Experimental section

All the reagents for chemical synthesis were obtained from Sigma Aldrich, Novabiochem and Alfa aesar. The solvents used in reactions were distilled and dried prior to use. All the chemical reactions were monitored using thin layer chromatography (TLC) on 0.25 mm silica gel 60 F_{254} plates coated on aluminium sheet (E. Merck) using ultra-violet (UV) light as a visualizing agent and ninhydrin as developing agent. The coupling reactions were mediated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI.HCl)/1-hydroxybenzotriazole (HOBt) in dry dimethylformamide (DMF) /or dry dichloromethane (CH_{2}Cl_{2}) 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.

4.2.1 Synthesis of piperic acid [5-(3,4-methylenedioxyphenyl)-2E,4E pentadienoic acid, PA]

Piperine 1 (25.0 mmol, 7.12 g,) was dissolved in 50 ml of ethylene glycol and was added potassium hydroxide (110 mmol, 6.2 g). The reaction mixture was refluxed for 48 h at 180\(^{\circ}\)C. The progress of the reaction was monitored using TLC. After completion of the reaction, the solvent was evaporated and ice cold water (50 ml) was added followed by the addition of 2N-HCl to maintain the pH~4.0. Yellow precipitate was collected, filtered and washed with cold water and recrystallised from ethanol to yield piperic acid 2 as pale yellow crystals (Scheme 4.1). mp 217 \(^{\circ}\)C. \(^1\)H NMR (500 MHz, DMSO-d\(_6\)): \(\delta\) 7.27 (dd, \(J = 15.3, 8.9\) Hz, 2H), 7.00 (d, \(J = 7.8\) Hz, 1H), 6.94 (m, 3H), 6.05 (s, 2H), 5.94 (d, \(J = 15.2\) Hz, 1H). \(^{13}\)C NMR (126 MHz, DMSO): \(\delta\) 167.95, 147.97, 147.93, 143.87, 139.25,
130.56, 124.98, 122.97, 122.15, 108.46, 105.62, 101.31. HRMS-ESI: M_{Cal}=218.21; M_{Obs} =219.32[M+H]^+.

4.2.2 Synthesis of 4-ethylpiperic acid [2E, 4E-4-(benzo[d][1,3]dioxol-5-ylmethyl ene)-hex-2-enoic acid, EPA]

Scheme 4.2

Reagents and conditions: a) C_3H_7I, Mg/Et_2O, 0 °C to room temp.; b) Dry DMF/POCl_3, 0 °C to rt.; c) BrPPh_3CH_2COOEt, NaH/C_6H_6, 0 °C to rt.; d) NaOH/MeOH, reflux, H_3O^+.

Pipernal (5.0 g, 30 mmol) was dissolved in dry diethyl ether followed by the addition of an ethereal solution of Grignard reagent prepared from magnesium metal (0.84 g, 35 mmol) and n-propyliodide (3.0 ml, 40 mmol). The reaction mixture was stirred for 1 h at room temperature. After completion of reaction, saturated aqueous solution of ammonium chloride (10 ml) was added followed by dilution with 100 ml of water and extracted with diethyl ether (2 x 100 ml). The combined organic layers was washed with water (2 x 25 ml), dried over anhydrous sodium sulfate and concentrated under vacuum to yield the semisolid 1-benzo[d][1,3]dioxol-6-yl)propan-1-ol, 1 (5.7g, 94%).

The solution of 1 (5.0 g, 25.5 mmol) in dry DMF (10 ml) was slowly added to phosphorus oxychloride (8.0 ml) in dry DMF (12 ml) at 0°C. The reaction mixture was stirred for 2 h, allowed to attain room temperature followed by heating for 36 h at 40°C. The completion of the reaction was monitored by using TLC. The reaction mixture was poured into 500 ml of ice-cold water, neutralized with alkaline solution and extracted with ethyl acetate (3x100 ml). The combined organic layers was washed with water, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to yield gummy
material, which was purified by column chromatography over silica gel (60-120 mesh) to yield (E)-2-(benzo[d][1,3]dioxol-6-yl)methylene) butanal, 2 (4.1 g, 72%).

A solution of triethylphosphonoacetate (4.5 g, 20 mmol) in diethyl ether (20 ml) was added to the precooled solution of sodium hydride (0.6 g, 25 mmol) in diethyl ether (50 ml) under nitrogen conditions. The reaction mixture was stirred at 0°C for 30 min. After 30 min, a solution of 2 (4.0 g, 19.4 mmol) in diethyl ether (20 ml) was added drop wise to the reaction mixture and stirred for 4 h. The reaction mixture was diluted with 50 ml of 2N-HCl and extracted with diethyl ether (3x50 ml). The combined organic layers was washed with water (3x30 ml), dried over anhydrous Na₂SO₄ and evaporated under reduced pressure. The residue was purified by column chromatography over silica gel using hexane/ethyl acetate (8:2) to afford the compound (2E,4E)-ethyl 4-((benzo[d][1,3]dioxol-6-yl)methylene)hex-2-enoate, 3 (4.6 g, 75%).

A 2N-NaOH solution (4.0 ml) was added to a solution of 3 (2.0 g, 7.2 mmol) in 30 ml of methanol and the reaction was refluxed for 2 h. After completion of the reaction which was monitored by using TLC, the solvent was evaporated and the residue was diluted with 10 ml of water, acidified with 2N-HCl and extracted with ethyl acetate (3x25 ml). The combined organic layer was washed with water, dried over anhydrous Na₂SO₄ and evaporated under reduced pressure. The residue was crystallized in ethyl acetate to yield colourless crystalline solid of 4-ethylpiperic acid, EPA (1.3 g, 72%) (Scheme 4.2).

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\begin{align*}
{^1}H \text{ NMR (500 MHz, CDCl₃): } & \text{ (Yield: 72%) } \delta 7.09 (d, J = 15.7 \text{ Hz, } 1\text{H}), 6.63 (s, 1\text{H}), 6.59 (d, J = 7.9 \text{ Hz, } 2\text{H}), 6.42 (s, 1\text{H}), 5.77 (s, 2\text{H}), 5.71 (d, J = 15.7 \text{ Hz, } 1\text{H}), 2.27 (q, J = 7.5 \text{ Hz, } 2\text{H}), 0.96 (t, J = 7.5 \text{ Hz, } 3\text{H}) \\
{^{13}}C \text{ NMR (126 MHz, CDCl₃): } & \delta 173.71, 153.55, 152.65, 152.15, 143.58, 142.50, 135.21, 128.53, 122.01, 113.55, 113.21, 106.12, 24.98, 18.11. \\
\text{HRMS-ESI: } & M_{\text{Cal}} = 246.26; M_{\text{Obs}} = 247.27 [M+H]^+.
\end{align*}
\]

4.2.3 Synthesis of piperic acid and 4-ethylpiperic acid amides (1-18)

Synthesis of piperic acid (PA) amides (1-6)

Piperic acid (0.218 g, 1.0 mmol) was dissolved in 2.0 ml of dry DCM and cooled in an ice bath while stirring. NMM (3.0 mmol) and EDC (0.191 g, 1.0 mmol) were added into the reaction mixture. Then amino acid ester hydrochloride (1.2 mmol) was added into the
reaction mixture and stirred the reaction for 16 h. The progress of the reaction was monitored using TLC at regular intervals. After completion of the reaction, the solvent was evaporated and the residue dissolved in ethyl acetate. The organic layer was washed successively with 2N-HCl (3×10 ml), 2M-Na₂CO₃ (3×10 ml) and brine. The combined organic layer was dried over anhydrous sodium sulphate and evaporated in vacuo to yield PA-Xxx-OMe (Where Xxx = L-tert-Leu, β⁵₃₆-Ac₆₄c, 4-ethyl-β⁵₃₆-Ac₆₄c, 4-tert-butyl-β⁵₃₆-Ac₆₄c, β⁵₃₆-Pip(Bzl) and Gpn, respectively which were purified by column chromatography over silica gel (60-120 mesh) to yield the PA-Xxx-OMe, 1-6 (Scheme 4.3).

Methyl(S)-2-((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dienamido)-3,3-dimethyl butaneate, 1

¹H NMR (400 MHz, CDCl₃): (Yield: 82%) δ 7.38 (dd, J = 14.1, 11.4 Hz, 1H), 6.99 (s, 1H), 6.90 (d, J = 8.0 Hz, 1H), 6.83–6.74 (m, 2H), 6.68 (dd, J = 15.1, 11.1 Hz, 1H), 6.05 (d, J = 8.6 Hz, 1H), 5.98 (s, 2H), 4.61 (d, J = 9.5 Hz, 1H), 3.74 (s, 3H), 1.25 (s, 1H), 1.00 (s, 9H). ¹³C NMR (126 MHz, CDCl₃): δ 172.25, 165.83, 148.19, 148.17, 142.01, 139.41, 130.74, 124.49, 122.78, 122.51, 108.49, 105.68, 101.30, 59.99, 51.87, 51.85, 35.13, 35.00, 26.52. HRMS-ESI: M₀cal=345.15; M₀obs=346.164[M+H]⁺.
Methyl 2-(1-((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dienamido)cyclohexyl)acetate, 2

$^1$H NMR (400 MHz, CDCl$_3$): (Yield: 58%) $\delta$ 7.26 (s, 1H), 7.23 (d, $J$ = 4.3 Hz, 1H), 6.91 (s, 1H), 6.82 (d, $J$ = 8.0 Hz, 1H), 6.71 (d, $J$ = 7.9 Hz, 1H), 6.65 (d, $J$ = 15.1 Hz, 1H), 6.61 – 6.55 (m, 1H), 5.91 (s, 2H), 5.86 (d, $J$ = 14.8 Hz, 1H), 5.36 (s, 1H), 3.56 (s, 3H), 2.85 (s, 2H), 1.24 – 1.16 (m, 10H). $^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 170.83, 165.15, 147.48, 139.66, 137.34, 130.26, 123.85, 123.28, 121.75, 113.36, 107.69, 104.92, 100.50, 53.80, 50.68, 34.05, 33.09, 31.20, 29.19, 24.62, 21.91, 20.60 HRMS-ESI: $M_{Cal}=371$.17; $M_{Obs}=372$.17 [M+H]$^+$. 

Methyl 2-(1-((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dienamido)-4-ethylcyclohexyl)acetate, 3

$^1$H NMR (400 MHz, CDCl$_3$): (Yield: 48%) $\delta$ 6.91 (s, 1H), 6.82 (d, $J$ = 8.0 Hz, 1H), 6.77 (d, $J$ = 8.9 Hz, 1H), 6.71 (d, $J$ = 8.0 Hz, 1H), 6.66 (s, 1H), 6.61 (d, $J$ = 10.6 Hz, 1H), 6.54 (d, $J$ = 8.9 Hz, 1H), 5.82 (d, $J$ = 14.8 Hz, 1H), 5.65 (s, 1H), 5.23 (s, 2H), 3.64 (s, 3H), 2.90 (s, 2H), 1.24 – 1.17 (m, 10.9H), 1.14 (q, $J$ = 7.0 Hz, 2H), 0.80 (t, $J$ = 6.6 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 172.35, 148.23, 140.51, 139.32, 138.65, 126.99, 126.43, 124.71, 124.35, 123.39, 122.61, 119.28, 114.02, 109.30, 108.32, 101.31, 101.09, 55.01, 51.33, 37.92, 34.15, 33.76, 32.01, 31.53, 29.55, 28.01, 22.59, 14.05, 11.56. HRMS-ESI: $M_{Cal}=399$.20; $M_{Obs}=400$.21 [M+H]$^+$. 

Methyl 2-(1-((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dienamido)-4-(tert-butyl)cyclohexyl)acetate, 4

$^1$H NMR (400 MHz, CDCl$_3$): (Yield: 87%) $\delta$ 7.23 (d, $J$ = 10.6 Hz, 1H), 6.90 (d, $J$ = 1.2 Hz, 1H), 6.81 (dd, $J$ = 8.0, 1.2 Hz, 1H), 6.70 (d, $J$ = 8.0 Hz, 1H), 6.66 (s, 1H), 6.60 (d, $J$ = 10.6 Hz, 1H), 5.90 (s, 2H), 5.83 (d, $J$ = 11.2 Hz, 1H), 5.72 (s, 1H), 3.58 (s, 3H), 3.41 (q, $J$ = 7.0 Hz, 1H), 2.90 (s, 2H), 2.21 (d, $J$ = 13.0 Hz, 2H), 1.13 (dd, $J$ = 14.3, 7.3 Hz, 6H). 0.79 (s, 9H). $^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 172.17, 165.75, 148.13, 140.57, 138.61, 131.02, 124.69, 124.34, 122.55, 108.49, 105.72, 101.30, 54.85, 51.43, 47.32, 36.58, 35.41, 33.86, 32.27, 29.68, 27.52, 23.13, 22.67, 14.15. HRMS-ESI: $M_{Cal}=427$.23; $M_{Obs}=428$.24[M+H]$^+$.
Methyl 2-(4-(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dienamido)-1-benzylpiperic in-4-yl]acetate, 5

$^1$H NMR (400 MHz, CDCl$_3$): δ 7.31 (d, $J = 4.1$ Hz, 4H), 6.98 (s, 1H), 6.89 (d, $J = 7.7$ Hz, 1H), 6.82-6.76 (m, 2H), 6.74 (s, 1H), 6.72–6.63 (m, 1H), 5.98 (s, 2H), 5.93 (d, $J = 14.9$ Hz, 1H), 5.41 (s, 1H), 3.62 (s, 3H), 3.50 (2H), 2.94 (s, 2H), 2.67 (d, 2H), 2.38 (d, $J = 12.9$ Hz, 2H), 2.28 (s, 2H), 1.77 (s, 5H), 1.26 (s, 1H). $^{13}$C NMR (126 MHz, CDCl$_3$): δ 171.52, 166.12, 148.25, 140.89, 138.94, 130.86, 129.24, 128.31, 127.19, 124.56, 123.77, 122.72, 108.52, 105.78, 101.30, 63.03, 52.62, 51.41, 49.06, 34.46. HRMS: M$_{Cal}$=463.22; M$_{Obs}$=463.22.[M+H]$^+$.  

Methyl 2-[1-(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dienamido)methyl]acetate, 6

$^1$H NMR (400 MHz, CDCl$_3$): (Yield: 54%) δ 7.35 (d, $J = 4.5$ Hz, 1H), 6.98 (d, $J = 1.2$ Hz, 1H), 6.92–6.87 (m, 1H), 6.78 (d, $J = 8.0$ Hz, 1H), 6.73 (d, $J = 11.4$ Hz, 1H), 6.70–6.59 (m, 1H), 6.40 (s, 1H), 5.98 (s, 2H), 3.70 (s, 3H), 3.38 (d, $J = 6.5$ Hz, 2H), 2.34 (s, 2H), 1.87–1.11 (m, 10H). $^{13}$C NMR (126 MHz, CDCl$_3$): δ 173.29, 165.99, 148.34, 140.83, 130.87, 124.68, 123.52, 122.57, 108.55, 105.90, 101.36, 51.66, 46.38, 41.96, 37.92, 34.35, 29.55, 25.78, 21.56. HRMS-ESI: M$_{Cal}$=385.18; M$_{Obs}$=386.19[M+H]$^+$.  

Synthesis of 4-ethy1piperic acid (EPA) amides (7-18)

Scheme 4.4

Reagents and conditions: a) Dry DCM, NMM, EDC, amino acid ester hydrochloride (L-Ala-OMe.HCl, L-Val-OMe.HCl, L-Leu-OMe.HCl, L-tert.Leu-OMe.HCl, L-Phe-OMe.HCl, L-Pro-OMe.HCl, L-tryp-OMe.HCl) 0°C to rt, 16 h; b) Dry DCM, NMM, EDC, β amino acid ester hydrochloride ($\beta^{3,3}$Ac$_6$-OMe.HCl, 4-Ethyl-$\beta^{3,3}$Ac$_6$-OMe.HCl, 4-tert.butyl-$\beta^{3,3}$Ac$_6$-OMe.HCl, $\beta^{3,3}$Pip(Bzl)Ac$_6$-OMe.HCl) 0°C to rt, 16 h; c) Dry DCM, NMM, EDC, Gpn-OMe.HCl 0°C to rt, 16 h.
4-Ethylpiperic acid (0.246 g, 1.0 mmol) was dissolved in 2.0 ml of dry DCM and cooled in an ice bath while stirring. NMM (3.0 mmol) and EDC (0.191 g, 1.0 mmol) were added into the reaction mixture. After that amino acid ester hydrochloride (1.2 mmol) was added into the reaction mixture and stirred the reaction for 16 h. The progress of the reaction was monitored using TLC at regular intervals. After completion of the reaction, the solvent was evaporated and the residue dissolved in ethyl acetate. The organic layer was washed successively with 2N-HCl (3×10 ml), 2M-Na₂CO₃ (3×10 ml), and brine. The combined organic layer was dried over anhydrous sodium sulphate and evaporated under vacuum to yield EPA-Xxx-OMe (Where Xxx = L-Ala, L-Val, L-Leu, L-Phe, L-Pro, L-Trp and L-tert-Leu, β₃,₃-Ac₆c, 4-ethyl-β₃,₃-Ac₆c, 4-tert-butyl-β₃,₃-Ac₆c, β₃,₃-Pip(Bzl) and Gpn respectively, which was purified by column chromatography over silica gel (60-120 mesh) to yield the EPA-Xxx-OMe, 7-18 (Scheme 4.4).

Methyl (2E, 4E)-4-(benzo[d][1,3]dioxol-5-ylmethylene)hex-2-enoyl)-L-alaninate, 7

1H NMR (400 MHz, CDCl₃): (Yield: 55%) δ 7.30 (dd, J = 15.4 Hz, 9.4, 1H), 6.86 (s, 1H), 6.82 (s, 2H), 6.66 (s, 1H), 6.21 (s, 1H), 5.99 (s, 2H), 5.95 (s, 1H), 4.73 (q, J = 7.2 Hz, 1H), 3.78 (s, 3H), 2.50 (q, J = 7.5 Hz, 1H), 1.47 (d, J = 7.1 Hz, 3H), 1.19 (t, J = 7.5 Hz, 3H; 13C NMR (126 MHz, CDCl₃): δ 173.84, 165.84, 147.78, 145.99, 147.15, 138.78, 137.42, 123.70, 118.29, 108.87, 108.41, 101.23, 52.57, 48.16, 20.40, 18.71, 13.43; HRMS-ESI: M_cal=331.14; M_obs=332.14[M+H]⁺.

Methyl ((2E,4E)-4-(benzo[d][1,3]dioxol-5-ylmethylene)hex-2-enoyl)-L-valinate, 8

1H NMR (400 MHz, CDCl₃): (Yield: 65%) δ 7.31 (dd, J = 15.4 Hz, 9.4, 1H), 6.86 (s, 1H), 6.83 (s, 2H), 6.66 (s, 1H), 6.08 (d, J = 8.6 Hz, 1H), 6.01 (s, 1H), 5.99 (s, 2H), 4.71 (dd, J = 8.8, 5.0 Hz, 1H), 3.76 (s, 3H), 2.51 (q, J = 7.5 Hz, 2H), 2.21 (td, J = 13.6, 6.7 Hz, 1H), 1.20 (t, J = 7.6 Hz, 3H), 0.96 (dt, J = 13.7, 6.9 Hz, 6H), 13C NMR (126 MHz, CDCl₃): δ 172.87, 166.28, 147.78, 147.14, 145.98, 138.85, 137.32, 130.86, 123.68, 118.39, 108.86, 108.43, 101.30, 57.10, 52.3, 31.5, 20.2, 18.7, 13.45. HRMS-ESI: M_cal=359.17; M_obs=360.18 [M+H]⁺.
Methyl ((2E,4E)-4-(benzo[d][1,3]dioxol-5-ylmethylene)hex-2-enoyl)-L-leucinate, 9

$^1$H NMR (400 MHz, CDCl$_3$): (Yield: 62%) $\delta$ 7.23 (d, $J = 15.4$ Hz, 1H), 6.77 (s, 1H), 6.75 (s, 2H), 6.58 (s, 1H), 5.91 (s, 2H), 4.05 (q, $J = 7.1$ Hz, 1H), 3.68 (s, 3H), 3.41 (q, $J = 7.0$ Hz, 2H), 2.47–2.37 (m, 2H), 1.17–1.08 (m, 3H), 0.90 (dd, $J = 7.6, 6.2$ Hz, 6H).

$^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 174.00, 166.23, 147.75, 147.12, 146.06, 138.80, 137.34, 134.26, 130.84, 123.67, 118.28, 108.85, 108.37, 101.20, 52.34, 50.79, 41.84, 24.88, 22.85, 21.96, 20.38, 13.40.

HRMS-ESI: $M_{Cal}$=373.18; $M_{Obs}$=374.19[M+H]$^+$.

Methyl(S)-2-(2E,4E)-4-(benzo[d][1,3]dioxol-5-ylmethylenehex-2-enamido)-3,3-dimethylbutanoate, 10

$^1$H NMR (400 Hz, CDCl$_3$): (Yield: 75%) $\delta$ 7.31 (d, $J = 15.4$ Hz, 1H), 6.84 (d, $J = 14.2$ Hz, 3H), 6.66 (s, 1H), 6.07 (d, $J = 9.3$ Hz, 1H), 5.99 (d, $J = 1.0$ Hz, 2H), 5.96 (s, 1H), 4.64 (d, $J = 9.4$ Hz, 1H), 3.75 (d, $J = 1.0$ Hz, 3H), 2.56–2.44 (m, 2H), 1.63 (s, 1H), 1.30–1.24 (m, 2H), 1.20 (t, $J = 7.5$ Hz, 3H), 1.02 (s, 9H).

$^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 172.41, 166.05, 147.74, 147.22, 146.18, 138.72, 137.36, 130.87, 123.71, 118.32, 108.87, 108.34, 101.23, 60.06, 51.90, 35.12, 26.68, 20.36, 13.21. HRMS-ESI: $M_{Cal}$=373.18; $M_{Obs}$=374.19 [M+H]$^+$.

(2E,4E)-4-(benzo[d][1,3]dioxol-5-ylmethylenehex-2-enoyl)-L-phenylalaninate, 11

$^1$H NMR (400 MHz, CDCl$_3$): (Yield: 67%) $\delta$ 7.36–7.20 (m, 5H), 7.12 (d, $J = 15.4$ Hz, 1H), 6.85 (s, 1H), 6.81 (s, 2H), 6.65 (s, 1H), 6.09 (s, 1H), 5.97 (s, 2H), 5.93 (d, $J = 15.4$ Hz, 2H), 5.29 (s, 1H), 5.02 (dd, $J = 13.2, 5.7$ Hz, 1H), 3.74 (s, 3H), 2.48 (q, $J = 7.4$ Hz, 2H), 1.16 (t, $J = 7.5$ Hz, 3H); $^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 172.30, 165.80, 147.95, 147.18, 146.22, 138.78, 137.55, 135.94, 130.81, 129.37, 128.60, 127.14, 123.73, 118.14, 108.92, 108.42, 101.32, 53.35, 52.42, 37.95, 20.39, 13.35; HRMS-ESI: $M_{Cal}$=407.17; $M_{Obs}$=408.17[M+H]$^+$.

Methyl (2E,4E)-4-(benzo[d][1,3]dioxol-5-ylmethylenehex-2-enoyl)-D-prolinate, 12

$^1$H NMR (400 MHz, CDCl$_3$): (Yield: 85%) $\delta$ 7.39 (d, $J = 15.3$ Hz, 1H), 6.86 (s, 1H), 6.82 (s, 2H), 6.67 (s, 1H), 6.26 (d, $J = 15.2$ Hz, 1H), 4.60 (dd, $J = 8.1, 4.0$ Hz, 1H), 4.20 (dd, $J = 14.4, 7.2$ Hz, 1H), 3.78 – 3.72 (s,3H), 3.72 – 3.59 (m, 2H), 2.52 (q, $J = 7.5$ Hz, 2H), 2.27–2.09 (m, 2H), 2.08–1.97 (m, 2H), 1.33–1.24 (m, 1H), 1.19 (t, 8.7 Hz, 3H); $^{13}$C NMR
(126 MHz, CDCl$_3$): $\delta$ 173.04, 166.04, 165.50, 147.76, 147.26, 147.13, 139.08, 137.55, 130.89, 123.72, 116.16, 116.05, 108.87, 108.39, 101.22, 67.09, 65.88, 59.02, 52.28, 46.95, 29.21, 24.86, 20.48, 15.29, 14.20, 13.46; HRMS-ESI: $M_{\text{Cal}}$=357.15; $M_{\text{Obs}}$=358.16 [M+H]$^+$

Methyl (S)-2-(2E,4E)-4-(benzo[d][1,3]dioxol-5-ylmethylene)hex-2-enamido)-3-(3H-indol-2-yl) propanoate, 13

$^1$H NMR (400 MHz, CDCl$_3$): (Yield: 68%) $\delta$ 8.13 (s, 1H), 7.55 (d, $J = 7.8$ Hz, 1H), 7.37 (d, $J = 7.9$ Hz, 1H), 7.30 (s, 1H), 7.20 (t, $J = 7.4$ Hz, 1H), 7.11 (t, $J = 7.4$ Hz, 1H), 7.02 (s, 1H), 6.86 (s, 1H), 6.83 (s, 2H), 6.64 (s, 1H), 5.90 (d, $J = 7.2$ Hz, 1H), 5.99 (s, 2H), 5.88 (d, $J = 15.9$ Hz, 1H), 5.30 (s, 1H), 5.11 (s, 1H), 3.72 (s, 3H), 3.40 (s, 2H), 2.46 (q, $J = 7.2$ Hz, 2H), 2.04 (s, 1H), 1.15 (t, $J = 7.3$ Hz, 3H). $^{13}$C NMR (101 MHz, CDCl$_3$): $\delta$ 172.50, 165.97, 147.80-147.60, 147.21, 145.95, 138.86, 137.35, 136.09, 130.80, 123.81 (s), 122.82, 122.14, 119.81, 118.83, 118.47, 111.17, 110.25, 108.81, 108.25, 101.11, 53.08, 29.61, 27.76, 20.13 (s), 13.36. HRMS-ESI: $M_{\text{Cal}}$=446.18; $M_{\text{Obs}}$=447.19[M+H]$^+$.

Methyl 2-(1-(2E,4E)-4-(benzo[d][1,3]dioxol-5-ylmethylene)hex-2-enamido)cyclohexyl)acetate, 14

$^1$H NMR (400 MHz, CDCl$_3$): (Yield: 60%) $\delta$ 7.23 (d, $J = 15.4$ Hz, 1H), 6.85 (s, 1H), 6.82 (s, 2H), 6.63 (s, 1H), 5.98 (s, 2H), 5.93 (d, $J = 15.3$ Hz, 1H), 5.47 (s, 1H), 3.63 (s, 3H), 2.94 (s, 2H), 2.49 (q, $J = 7.5$ Hz, 2H), 1.70–1.44 (m, 10H), 1.19 (t, $J = 7.5$ Hz, 3H); $^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 171.79, 165.97, 147.76, 147.76, 147.02, 144.79, 138.89, 136.73, 130.96, 123.58, 119.81, 108.84, 108.37, 101.20, 65.89, 54.60, 51.41, 41.75, 34.95, 25.48, 21.64, 20.53, 15.34, 13.55. HRMS-ESI: $M_{\text{Cal}}$=399.20; $M_{\text{Obs}}$=400.21[M+H]$^+$.

Methyl 2-(1-(2E,4E)-4-(benzo[d][1,3]dioxol-5-ylmethylene)hex-2-enamido)-4-ethyl cyclohexyl acetate, 15

$^1$H NMR (400 MHz, CDCl$_3$): (Yield: 57%) $\delta$ 7.22 (d, $J = 15.4$ Hz, 1H), 6.83 (d, $J = 13.3$ Hz, 3H), 6.63 (s, 1H), 5.96 (d, $J = 12.5$ Hz, 2H), 5.90 (d, $J = 15.4$ Hz, 1H), 5.77 (s, 1H), 3.65 (s, 3H), 2.99 (s, 2H), 2.48 (q, $J = 7.4$ Hz, 2H), 2.15 (d, $J = 13.1$ Hz, 2H), 1.81 (dd, $J = 17.5$, 7.7 Hz, 2H), 1.71 (d, $J = 7.6$ Hz, 3H), 1.27-1.11 (m, 10H), 0.89 (t, $J = 6.8$ Hz, 3H); $^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 172.25, 166.06, 147.74, 147.01, 146.98, 144.67,
139.00, 136.59, 131.01, 123.59, 119.96, 108.82, 108.36, 101.18, 55.04, 51.36, 37.81, 34.22, 29.82, 28.66, 28.10, 20.49, 13.56, 11.65. HRMS-ESI: $M_{\text{Cal}} = 427.23$; $M_{\text{Obs}} = 428.24$ [M+H]$^+$. 

**Methyl2-(1-((2E,4E)-4-(benzo[d][1,3]dioxol-5-ylmethylene)hex-2-enamido)-4-(tert-butyl)cyclohexyl)acetate, 16**

$^1$H NMR (400 MHz, CDCl$_3$): (Yield: 75%) $\delta$ 7.24 (d, $J = 12.1$ Hz, 1H), 6.83 (m, 3H), 6.63 (s, 1H), 5.98 (s, 2H), 5.87 (d, $J = 12.1$ Hz, 1H), 5.81 (s, 1H), 3.66 (s, 3H), 2.98 (s, 2H), 2.48 (q, $J = 7.5$ Hz, 2H), 2.30 (d, $J = 12.9$ Hz, 2H), 1.80-1.63 (m, 7H), 1.18 (t, $J = 7.5$ Hz, 3H), 0.87 (s, 10H); $^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 172.31, 168.53, 166.09, 147.64, 146.91, 144.75, 138.95, 136.50, 131.00, 123.55, 120.08, 108.85, 108.33, 101.18, 54.88, 51.52, 47.34, 36.50, 35.51, 32.40, 29.66, 27.44, 23.09, 20.45, 13.45. HRMS-ESI: $M_{\text{Cal}} = 455.26$; $M_{\text{Obs}} = 456.27$ [M + H]$^+$. 

**Methyl 2-(4-(2E,4E)-4-(benzo[d][1,3]dioxol-5-ylmethylene)hex-2-enamido)-1-benzylpiperidin-4-yl)acetate, 17**

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.36–7.21 (m, 8), 6.84 (s, 1H), 6.81 (dd, $J = 7.2$, 5.2 Hz, 2H), 6.65 (d, $J = 14.7$ Hz, 1H), 6.02 (s, 2H), 5.95 (d, $J = 5.8$ Hz, 2H), 5.72 (d, $J = 14.7$ Hz, 1H), 3.61 (s, 3H), 3.52 (s, 2H), 2.96 (s, 2H), 2.65 (d, $J = 11.6$ Hz, 2H), 2.55 – 2.45 (q, 2H), 2.43 – 2.23 (m, 4H), 1.77 (m, 2H), 1.22 – 1.12 (t, $J = 7.5$ Hz, 3H); $^{13}$C NMR (101 MHz, CDCl$_3$): $\delta$ 171.55, 168.44, 166.39, 147.77, 147.23, 147.08, 146.68, 144.95, 138.91, 138.75, 137.63, 136.85, 136.80, 130.95, 130.77, 129.16, 128.26, 127.14, 123.69, 123.56, 119.69, 117.86, 108.86, 108.24, 101.08, 63.07, 52.68, 51.47, 48.87, 34.54, 20.56, 13.47. HRMS-ESI: $M_{\text{Cal}} = 490.60$; $M_{\text{Obs}} = 491.60$ [M+H]$^+$. 

**Methyl 2-(1-(2E,4E)-4-(benzo[d][1,3]dioxol-5-ylmethylene)hex-2-enamido) methyl)cyclohexyl)acetate, 18**

$^1$H NMR (400 MHz, CDCl$_3$): (Yield: 80%) $\delta$ 7.31 (s, 1H), 7.27 (d, $J = 1.8$ Hz, 1H), 6.84 (m, 3H), 6.65 (s, 1H), 6.36 (t, $J = 6.5$ Hz, 2H), 5.98 (s, 2H), 5.93 (s, 1H), 3.71 (s, 3H), 3.39 (d, $J = 6.5$ Hz, 2H), 2.50 (q, $J = 7.4$ Hz, 2H), 2.36 (s, 2H), 1.8 – 1.28 (m, 10H), 1.19 (t, $J = 7.5$ Hz, 3H); $^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 173.83, 166.65, 147.74, 147.03, 145.16,

4.2.4 Synthesis of Ac-$\beta^{3,3}$-Pip(EPA)-Aib-OMe, 19

Scheme 4.5

Reagents and conditions: (a) $\text{Py/(Ac)}_2\text{O}$; (b) Dry DCM, NMM, EDC, Aib-OMe.HCl, $^0\text{C}$ to rt, 16 h; (c) 30% TFA in DCM, 3 h; (d) Dry DCM, NMM, EDC, 4-ethylpiperic acid (EPA), HOBt, $^0\text{C}$ to rt, 24 h.

$\beta^{3,3}$Pip(Boc)-OH (5.0 mmol, 1.29 g) was dissolved in 1.0 ml of pyridine followed by the addition of acetic anhydride (7.0 mmol, 0.8 ml) and stirred the reaction for 5 h. The reaction was monitored using TLC, the reaction was quenched with saturated Na$_2$CO$_3$ solution, followed by acidification and was extracted with EtOAc (3×15 ml) and brine solution. The organic layer was dried over anhydrous sodium sulphate and evaporated in vacuo. The residue was filtered through a sintered funnel and was washed with ethyl acetate to give the desired compound Ac-$\beta^{3,3}$Pip(Boc)-OH, 1, which was used further without purification.

Ac-$\beta^{3,3}$Pip(Boc)-OH, 1 (2.0 mmol, 0.6 g) was dissolved in dry DCM and cooled in an ice bath while stirring. NMM (10 mmol, 0.5 ml), EDC (2.0 mmol, 0.382 g) was added into the reaction mixture. Aib-OMe.HCl (2.2 mmol, 0.305 g) was added into the reaction mixture and stirred the reaction for 16 h. The reaction was worked up as described in amide 1. Which was purified by column chromatography using silica gel (60-120 mesh).
to give the desired product Ac-β³³Pip(Boc)-Aib-OMe, 2. Which was deprotected using 30% TFA in DCM and the deprotection was monitored by TLC. After 3 h, solvent was evaporated to yield, 3. The peptide free base, 3 was added to a precooled solution of EPA (1.0 mmol, 0.246 g) in 2.0 ml of dry DCM followed by the addition NMM (3.0 mmol, 0.3 ml), EDC (1.0 mmol, 0.191 g) and HOBt (1.0 mmol, 0.135 g). The reaction mixture was allowed to attain room temperature and was stirred for 24 h. Progress of the reaction was monitored using TLC at regular intervals. The reaction was worked up as described for amide 1. Which was purified by column chromatography using silica gel (60-120 mesh) to give the desired product Ac-β³³-Pip(EPA)-Aib-OMe, 19 (Scheme 4.5)

Methyl 2-(2-(4-acetamido-1-(2E,4E)-4-(benzo[d][1,3]dioxol-5-ylmethylene) hex-2-enoyl) piperidin-4-yl)acetamido)-2-methyl propanoate, 19

¹H NMR (400 MHz, CDCl₃): (Yield: 64%) δ 7.32 (d, J = 15.1 Hz, 1H), 6.85 (d, J = 6.8 Hz, 1H), 6.82 (m, 2H), 6.66 (s, 1H), 6.37 (d, J = 15.1 Hz, 1H), 6.18 (s, 1H), 5.99 (d, J = 1.4 Hz, 2H), 5.69 (s, 1H), 4.19 (s, 1H), 3.74 (s, 3H), 3.40 (s, 1H), 3.21 (s, 1H), 2.69 (d, J = 19.4 Hz, 2H), 2.51 (d, J = 7.3 Hz, 2H), 2.38 (s, 1H), 2.30 (s, 1H), 2.04 (s, 3H), 1.74 (s, 4H), 1.48 (s, 6H), 1.19 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃): δ 174.74, 171.89, 169.44, 166.20, 147.76, 147.14, 147.10, 139.10, 137.02, 130.84, 123.67, 115.16, 108.90, 108.45, 108.39, 101.26, 56.16, 53.88, 52.57, 43.54, 41.82, 35.40, 34.76, 29.70, 25.09, 24.35, 20.45, 13.51, 13.49. HRMS-ESI: MCal=527.26; MObs=528.26[M+H]⁺.

4.2.5 Synthesis of Valeryl-β³³Pip(EPA)-NH-NH-Ph, 20

β³³Pip(Boc)-OH (5.0 mmol, 1.3 g) was dissolved in 5.0 ml of 1,4-dioxane and 2.0 ml of 2N-NaOH. Valeric anhydride (7.0 mmol, 1.4 ml) was added to the reaction mixture stirred for 5 h. The progress of reaction was monitored using TLC at regular intervals. After completion of the reaction, solvent was evaporated, the residue was diluted with 10 ml of water, acidified with 2N-HCl and extracted with ethyl acetate (3x10 ml). The combined organic layer was washed with brine, dried over anhydrous sodium sulphate and evaporated in vacuo to yield 1.

Valeryl-β³³Pip(Boc)-OH, 1 (2.0 mmol, 0.684 g) was dissolved in 2.0 ml of dry DCM and cooled in an ice bath while stirring. NMM (5 mmol, 0.5 ml), EDC (2.0 mmol, 0.382 g)
Scheme 5.6

Reagents and conditions: a) 1,4 dioxane, 2N-NaOH, Valeric anhydride at rt, 6 h; b) Dry DCM, NMM, EDC, Phenylhydrazine 0 °C to rt, 12 h; c) 30% TFA/DCM; d) Dry DMF, NMM, EDC, 4-ethylpiperic acid (EPA), HOBt, 5°C to rt, 24 h.

were added into the reaction mixture. After that phenylhydrazine (2.2 mmol, 0.2 ml) was added dropwise and stirred the reaction for 24 h. The progress of the reaction was monitored using TLC at regular intervals. After completion of the reaction, the solvent was evaporated and the residue was dissolved in ethyl acetate (15 ml). The organic layer was washed successively with 2N-HCl (3×10 ml), 2M-Na$_2$CO$_3$ (3×10 ml) and brine. The combined organic layer was dried over anhydrous sodium sulphate and evaporated in vacuo. Which was purified by column chromatography over silica gel (60-120 mesh) to yield the desired product 2. Valeryl-$\beta^{3,3}$Pip(Boc)-NH-NH-Ph, 2 (1.0 mmol, 0.43 g) was deprotected by 2.0 ml of 30% TFA in DCM and the deprotection was monitored by TLC. After 3 h, solvent was evaporated to yield 3. The peptide free base 3 was added to a precooled solution of EPA (1.0 mmol, 0.246 g) in 2.0 ml of dry DCM followed by the addition NMM (3.0 mmol, 0.3 ml), EDC (1.0 mmol, 0.191 g) and HOBt (1.0 mmol, 0.135 g). The reaction mixture was allowed to attain room temperature and was stirred for 24 h. Progress of the reaction was monitored using TLC at regular intervals. The reaction was worked up as described for 2. Valeryl-$\beta^{3,3}$Pip(EPA)-NH-NH-Ph 20, was obtained as
a gummy material, which was purified by column chromatography over silica gel (60-120 mesh) to yield a white solid Valeryl-β³³Pip(EPA)-NH-NH-Ph, 20 (0.19 g, 66%) (Scheme 4.6).

N-(1-((2E,4E)-4-(benzo[d][1,3]dioxol-5-ylmethylen)hex-2-enoyl)-4-(2-oxo-2-(2-phenylhydrazinyl)ethyl)piperidin-4-yl)pentanamide, 20

\(^1\)H NMR (400 MHz, CDCl\(_3\)): (Yield: 64%) \(\delta\) 8.00 (s, 1H), 7.33 (d, \(J = 15.1\) Hz, 1H), 7.21 (t, \(J = 7.9\) Hz, 2H), 6.90 (t, \(J = 7.3\) Hz, 1H), 6.85 (s, 1H), 6.82 (s, 2H), 6.65 (s, 1H), 6.40 – 6.33 (d, 1H), 6.13 (d, \(J = 4.3\) Hz, 1H), 5.99 (s, 2H), 5.64 (s, 1H), 4.23 (s, 1H), 3.75 (s, 1H), 3.49 (s, 1H), 3.36 (s, 1H), 3.16 (s, 1H), 2.51 (q, \(J = 7.4\) Hz, 2H), 2.25–2.14 (m, 2H), 1.76 (m, 2H), 1.68 (s, 5H), 1.59 (m, 2H), 1.35 (m, 2H), 1.22–1.10 (m, 2H), 0.90 (t, 8.0 Hz, 3H); \(^13\)C NMR (126 MHz, CDCl\(_3\)): \(\delta\) 174.65, 170.25, 166.15, 148.06, 147.77, 147.40, 147.14, 139.10, 137.21, 130.86, 129.19, 123.71, 121.26, 115.02, 113.55, 108.89, 108.46, 101.20, 67.95, 67.11, 65.93, 60.48, 53.55, 41.41, 37.37, 27.87, 25.59, 22.46, 20.53, 15.33, 13.84, 13.53; HRMS-ESI: \(M_{\text{cal}}=560.29\); \(M_{\text{obs}}=561.30\) [M+H]
4.2.6 Spectral data

**Figure 4.3:** $^1$H and $^{13}$C NMR of piperic acid, PA in DMSO-$d_6$. 
Figure 4.4: $^1$H and $^{13}$C NMR of 4-ethylpiperic acid, EPA in DMSO-d$_6$. 
Figure 4.5: $^1$H and $^{13}$C NMR of PA-$\beta^{1,3}$-Pip(Bzl)-OMe, 5 in CDCl$_3$
Figure 4.6: $^1$H and $^{13}$C NMR of PA-Gpn-OMe, 6 in CDCl$_3$
Figure 4.7: $^1$H and $^{13}$C NMR of EPA-L-Ala-OMe, 7 in CDCl$_3$
Figure 4.8: $^1$H and $^{13}$C NMR of EPA-L-Pro-OMe, 12 in CDCl$_3$
Figure 4.9: $^1$H and $^{13}$C NMR of EPA-$\beta^{13}$-Ac$_{6c}$-OMe, 14 in CDCl$_3$
Figure 4.10: $^1$H and $^{13}$C NMR of EPA-Gpn-OMe, 18 in CDCl$_3$
Figure 4.11: $^1$H and $^{13}$C NMR of Valeryl-$\beta^{1,3}$Pip(EPA)-NH-NH-Ph, 20 in CDCl$_3$
4.2.7 Efflux pump inhibitory activity

The efflux pump inhibitory activity (EPI) was evaluated by using checkerboard and accumulation/efflux assay using fluorescent dyes. Using checkerboard assay the potential EPIs was screened and tested at different concentration of ciprofloxacin in Muller Hinton broth. To determine the efflux pump inhibitory activity of compounds in the presence of ciprofloxacin, a NorA efflux pump overexpressing strain of *S. aureus* 1199B and wild type *S. aureus* 1199 were used in this study. In brief, MIC of ciprofloxacin was determined by broth checkerboard method in microtitre plate using serial dilutions in mullerhinton broth. Compounds were tested at seven concentrations (50-0.7µM) in the presence of two fold serial dilution of ciprofloxacin ranging 32-0.06µg/ml. Inoculum was prepared from overnight grown bacterial culture by adjusting its density to 0.5 McFarland (~ 1.5 × 10^8 CFU/ml of *Escherichia coli*) in normal saline solution. This cell suspension was diluted 1:100 and 100 µl of it was used to inoculate microtitre plate achieving a final cell density equal to 5 × 10^5 CFU/ml. Plates were then incubated at 37°C for 24 h. MIC was read visually and defined as the lowest concentration of drug inhibiting the growth of bacteria as evident from the absence of turbidity.

4.2.8 Ethidium bromide efflux and accumulation

Inhibition of efflux and accumulation was determined using *Staphylococcus aureus* 1199B by method reported earlier (Brenwald *et al* 1998). In brief bacteria was grown overnight on tryptic soya agar (TSA) to be used for inoculums. Bacterial suspension having optical density of 0.2 was prepared in uptake buffer (NaCl, 110 mM, KCl, 7 mM; NH₄Cl, 50 mM; Na₂HPO₄, 0.4 mM; Tris base, 52 mM; glucose, 0.2%, adjusted the solution to pH~7.5 with HCl). Bacterial suspension was loaded with 2 µg/ml ethidium bromide for 30 min followed by centrifugation. Cells were resuspended in fresh uptake buffer alone or with test compound (EPI) at MEC & 50 µg/ml. Reserpine and piperine at their MEC i.e. 25 and 50 µg/ml were taken as standard EPI. Fluorescence loss with time was recorded at an interval of 3 min for 30 min at an excitation and emission wavelength of 530 and 600 nm respectively using multimode reader Infinite 200 Pro (Tecan Mannedorf, Switzerland). Accumulation inhibition was also done in a similar fashion.
using ethidium bromide loaded cell suspension and test compounds were added after 30 minutes of ethidium bromide loading followed by immediate plate reading.

4.3 Results and Discussion

All the amides of piperic acid (PA) and 4-ethylpiperic acid (EPA) 1-20 were evaluated for their efflux pump inhibitory activity in combination with ciprofloxacin drug on a NorA overexpressing strain of *S. aureus* as well as wild type strain having normal expression level of NorA, and further tested for reduction in MIC of ethidium bromide using SA1199B (NorA overexpressing) and SA1199 (wild type *S. aureus*). Minimum inhibitory concentration (MIC) potentiation studies of ciprofloxacin in combination with compounds 1-20 against *S. aureus* 1199, which is a wild type strain having normal expression level of NorA, shows the activity as EPIs (Table 4.1).

Among all the compounds, the EPA amide with L-Proline, 12 was found to be the most potent compound with 16 fold decrease in MIC of ciprofloxacin with minimum effective concentration (MEC₄; four fold reduction in MIC) at 3.12 µg/mL. In addition, EPA derived amides 13 and 20 exhibited MEC₄ at 6.25 µg/mL and showed fourfold reduction in MIC of the drug. EPA derived amide 10, and PA derived amides 5 and 6 also showed 4-fold reduction in the MIC of the ciprofloxacin at 12.5 µg/mL.

The compounds having MEC₄ at 12.5 µg/mL or lower than it were further tested for reduction in MIC of ethidium bromide using SA1199B (NorA overexpressing) and SA1199 (wild type *S. aureus*). Tables 4.2 and 4.3 shows the activity of ethidium bromide in combination with 4, 6, 10, 11, 12, 13, and 18 against *S. aureus* 1199B and *S. aureus* 1199, respectively. As expected, NorA overexpressing *S. aureus* 1199B exhibited higher MIC against ethidium bromide compared to the wild type *S. aureus* 1199. Of these compounds, 12 and 13 exhibited the MEC₄ at 3.12 µg/mL in ethidium bromide against SA1199B, while MEC₄ was 25 and 50 µg/ml for the same compounds against SA1199. Further, 6 exhibited eight fold reduction in MIC at 3.12 µg/mL against SA1199B, while 4-fold reduction in MIC was observed at 12.5 µg/ml against SA1199. In case of 11 4-fold reduction in MIC at 6.25 µg/mL was observed against SA1199B.
Table 4.1: Activity of ciprofloxacin in combination with piperic acid (PA) and 4-ethylpiperic acid (EPA) amides, 1-20

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<th>MIC of ciprofloxacin against <em>S. aureus</em> 1199B at 12.5 µg/ml of respective EPI</th>
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<th>With EPI</th>
<th>Fold Reduction</th>
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<tr>
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No antibacterial activity of EPIs was observed at 50 µg/ml that was the highest concentration tested. For determining potentiation of ciprofloxacin, EPI were tested at concentration range of 50–0.8 µg/ml.

* MEC₄, minimum effective concentration at which MIC is four fold reduced.

Further to confirm the inhibitory mechanism of most potent EPIs, ethidium bromide efflux and accumulation assay were used as described in biological evaluation studies (Brenwald *et al* 1998). The most potent compounds, 12 and 20 were studied along with piperine and reserpine as standard EPIs. These EPIs inhibited the efflux of ethidium bromide effectively in a concentration dependent mode with maximum inhibition at 50 µg/mL resulting in significant decrease in fluorescence over the time period of 30min (Figure 4.3).
Similarly, both the compounds were assessed for ethidium bromide accumulation in the presence and absence of EPI. Introduction of EPI after 30 min resulted in the sharp increase in the fluorescence (Figure 4.4). From EPI screening and ethidium bromide assay, it was concluded that EPA amide with L-proline exhibited EPI activity (MEC$_4$ =3.12 µg/mL) which is four fold better than of EPA piperidine (SK-20) having MEC$_4$ at 12.5 µg/ml (Kumar et al 2008).The compound 20, in which β-amino acid, β$_{3,3}$-Pip-OH is conjugated with EPA, also exhibited better EPI activity (MEC$_4$ =6.25 µg/mL as compared to SK-20. In addition, 6 an amide of piperic acid with Gpn, shows similar EPI activity (MEC$_4$ =12.5) µg/mL like SK-20.

Table 4.2: Potentiation of ethidium bromide MIC by active derivatives using SA1199B

<table>
<thead>
<tr>
<th>Compound</th>
<th>MEC$_4$</th>
<th>MIC of EtBr against <em>S. aureus</em> 1199B</th>
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<td>Without EPI</td>
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<tr>
<td>SK-20</td>
<td>12.5</td>
<td>16</td>
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</tbody>
</table>

Table 4.3: Potentiation of ethidium bromide MIC by active derivatives using SA1199

<table>
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<tr>
<th>Compound</th>
<th>MEC$_4$</th>
<th>MIC of EtBr against <em>S. aureus</em> 11999</th>
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<td>Without EPI</td>
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<tr>
<td>6</td>
<td>12.5</td>
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<td>SK-20</td>
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</table>
Figure 4.3: Ethidium bromide efflux of 12 and 20 using NorA over expressing SA-1199B.

Figure 4.4: Ethidium bromide accumulation of 12 and 20 using NorA overexpressing SA-1199B.

4.5 Conclusions
Piperic acid and 4-ethylpiperic acid amides with amino acids (i.e. α-, β- and γ-amino acids) when tested in combination with ciprofloxacin have shown to exhibit efflux pump inhibitory activity property against ciprofloxacin resistant *Staphylococcus aureus*. Compounds 5, 6, 12, 18 & 20 were found to be the active compounds, and 12 displayed 16-fold reductions in the MIC of ciprofloxacin against NorA overexpressing strain of *S. aureus* (S.A1199B). Further, through ethidium bromide efflux inhibition and accumulation assays, these compounds have shown potent NorA inhibitors. The present study can be extended to develop the potent EPIs based on conjugation of piperic and 4-ethylpiperic acid with β,β-disubstituted-β- and γ-amino acids.