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# Synthesis And Evaluation of Piperic Acid and 4-Ethylpiperic Acid Amide Derivatives as Nora Efflux Pump Inhibitors Against Multidrug-Resistant Staphylococcus Aureus

**Pooja Dhama[1], Balireddy Sai Sutej[2], Mostafa Khursid Alam[3], Preeti Juyal[4], Prem Shankar Gupta[5], Minaketan Sahoo[6], Rofiqul Islam[7], Prabhakar Vishvakarma[8]**

[1]Assistant Professor, Lingaya's Vidyapeeth, Nachauli, Jasana Road, Faridabad, Haryana 21002, India
[2]Research Scholar, KVSR Siddhartha College of Pharmaceutical Sciences, Siddhartha Nagar, Vijayawada, Andhra Pradesh 520010, India
[3]Assistant Professor, School of pharmaceutical sciences, University of science and technology, Meghalaya, Techno city, Kiling Road, Baridua, 9th mile, Ri-Bhoi pin- 793101, India
[4]Assistant Professor, Shri Guru Ram Rai University, Patel nagar, Dehradun, Uttarakhand 248001, India
[5]Associate Professor, Teerthanker Mahaveer College of Pharmacy, Teerthanker Mahaveer University, Moradabad, Uttar Pradesh 244001, India
[6]Associate Professor, Institute of Pharmacy & Technology Salipur, Cuttack, Odisha 754202, India
[7]Assistant Professor, University of Science and Technology Meghalaya, Ri-Bhoi, Meghalaya 793101, India
[8]Associate Professor, Department of Pharmaceutics, Sharda School of Pharmacy, Sharda University Agra, Delhi Highway, NH-19, Keetham, Agra, Uttar Pradesh 282007, India

**Corresponding Author:** Prabhakar Vishvakarma
**Designation and Affiliation:** Associate Professor, Department of Pharmaceutics, Sharda School of Pharmacy, Sharda University Agra, Delhi Highway, NH-19, Keetham, Agra, Uttar Pradesh 282007, India
**Email id:** prabhakar.vishvakarma7788@gmail.com
**ORCID Id:** 0000-0002-9858-6091

***Abstract***

*The rising prevalence of multidrug-resistant (MDR) bacterial infections, particularly caused by Staphylococcus aureus, underscores the urgent need for novel therapeutic strategies. Among various resistance mechanisms, the NorA efflux pump plays a pivotal role in reducing intracellular concentrations of fluoroquinolones, such as ciprofloxacin, thereby limiting their antibacterial efficacy. Efflux pump inhibitors (EPIs) offer a promising approach to combat such resistance by restoring antibiotic sensitivity. In this study, a series of twenty novel amide derivatives of piperic acid (PA) and 4-ethylpiperic acid (EPA) were synthesized via EDCI-mediated coupling with structurally diverse α-, β-, and γ-amino acids. The synthesized compounds were structurally characterized using ^1H and ^13C NMR spectroscopy and high-resolution mass spectrometry (HRMS), confirming their purity and structural integrity. Biological evaluation was conducted using the NorA-overexpressing S. aureus SA-1199B strain. MIC determination by checkerboard assay revealed significant potentiation of ciprofloxacin activity in the presence of selected compounds, with compounds 19 and 20 exhibiting strong synergy (FICI = 0.1875), reducing ciprofloxacin MIC from 8 µg/mL to 0.5 µg/mL. Ethidium bromide (EtBr) accumulation and efflux assays demonstrated that these compounds effectively inhibited NorA-mediated efflux, showing comparable or superior activity to natural piperine and standard EPI reserpine. EPA-based derivatives consistently showed enhanced EPI activity compared to their PA counterparts, suggesting that subtle structural modifications in the parent scaffold significantly influence potency. The findings highlight the potential of EPA-amide derivatives, particularly those bearing hydrophobic or aromatic amino acid residues, as promising NorA-targeted EPIs. These scaffolds provide a foundation for further structural optimization and development as adjuvants in antimicrobial therapy against resistant bacterial pathogens.*

***Keywords:*** *Piperic acid derivatives, NorA efflux pump, Staphylococcus aureus, efflux pump inhibitors, antibiotic resistance*

## INTRODUCTION

The emergence and rapid proliferation of multidrug-resistant (MDR) bacterial infections present a critical threat to global public health, significantly compromising the therapeutic efficacy of many antibiotics that were once considered highly potent [1]. These MDR pathogens often exhibit resistance to structurally and mechanistically diverse antimicrobial agents, thereby complicating treatment regimens, increasing healthcare costs, and elevating patient morbidity and mortality rates [2]. Among the numerous

mechanisms underpinning bacterial drug resistance, the activity of membrane-associated efflux pumps has gained substantial attention due to their pivotal role in reducing the intracellular accumulation of antibiotics [3]. These efflux systems, particularly prominent in Gram-positive bacteria such as Staphylococcus aureus, serve as energy-dependent transporters that actively extrude antimicrobial agents and toxic compounds from within the bacterial cell, thereby lowering their intracellular concentrations below therapeutic thresholds and promoting resistance [4]. One of the most well-characterized efflux systems in S. aureus is the NorA efflux pump, a member of the major facilitator superfamily (MFS) that confers resistance to a variety of substrates, including fluoroquinolones, biocides, antiseptics, and dyes. NorA-mediated resistance is not only implicated in treatment failure of fluoroquinolone-based therapies but also contributes to the overall MDR phenotype by acting synergistically with other resistance mechanisms, such as target mutations and enzymatic drug modification [5]. The clinical significance of NorA and similar efflux pumps underscores the urgent need for the development of effective efflux pump inhibitors (EPIs) that can block the activity of these transporters and restore the antibacterial efficacy of conventional antibiotics. EPIs function by either directly inhibiting the efflux pump proteins or by modulating their expression, leading to increased intracellular concentrations of co-administered antibiotics [6]. The concept of EPIs as adjunctive agents in antibiotic therapy has garnered considerable research interest as a potential strategy to reverse resistance and extend the clinical utility of existing antimicrobial agents. However, despite the identification of several chemical scaffolds with EPI activity, only a few have progressed into clinical development due to limitations related to toxicity, poor bioavailability, metabolic instability, and non-specificity [7]. Hence, the search for novel, potent, and selective EPIs continues to be a major focus in antimicrobial drug discovery. Natural products have historically served as a rich source of pharmacologically active compounds, and their structural complexity often imparts unique mechanisms of action. Among these, piperine, an alkaloid constituent derived from the black pepper plant (Piper nigrum), has emerged as a noteworthy natural EPI [8]. Piperine has demonstrated the ability to potentiate the activity of several antibiotics against S. aureus, particularly through inhibition of the NorA efflux pump. Beyond its EPI potential, piperine possesses a wide array of pharmacological properties, including antibacterial, antifungal, antioxidant, anti-inflammatory, antiapoptotic, and bioavailability-enhancing effects [9]. Mechanistic studies have shown that piperine not only increases the intracellular retention of fluoroquinolones like ciprofloxacin in S. aureus but also enhances the therapeutic effectiveness of antibiotics in resistant strains [10]. Despite these promising attributes, the clinical translation of piperine has been hampered by several pharmacokinetic limitations such as poor water solubility, low systemic bioavailability, and limited metabolic stability. These drawbacks necessitate the structural modification of piperine or its bioactive metabolites to enhance their drug-like properties and EPI potency while minimizing off-target effects. Piperic acid, a known oxidative metabolite of piperine, retains the core conjugated diene system and methylenedioxyphenyl ring that are believed to contribute to efflux pump inhibition. Modifying this scaffold through the introduction of amide linkages with various amino acid derivatives can potentially enhance its binding affinity, selectivity, and membrane permeability, ultimately improving its performance as an EPI [11]. Likewise, 4-ethylpiperic acid (EPA), a synthetic analog of piperic acid, offers additional opportunities for structural diversification and may exhibit superior pharmacokinetic properties owing to its alkyl substitution. Previous studies have reported the EPI activity of piperoyl amides, which further supports the hypothesis that rational derivatization of piperic scaffolds with structurally diverse amino acid esters could yield promising candidates with enhanced bioactivity and reduced toxicity. Amino acid conjugation not only imparts structural rigidity and chiral centers but also improves solubility, metabolic stability, and target specificity of the resulting analogs [12]. Furthermore, β- and γ-amino acids are particularly attractive moieties due to their presence in several biologically active compounds and their resistance to proteolytic degradation, which could translate into longer systemic half-lives and better in vivo efficacy. Given this rationale, the current study was undertaken to design, synthesize, and evaluate a focused library of amide derivatives of piperic acid (PA) and 4-ethylpiperic acid (EPA) by conjugating them with selected α-, β-, and γ-amino acid esters [13]. The α-amino acids chosen for this study included common residues such as L-alanine, L-valine, L-leucine, L-phenylalanine, L-proline, L-tryptophan, and L-tert-leucine, which offer a range of steric and electronic properties. The β-amino acids incorporated in the synthesis included $\beta^{3},^{3}$-Ac6c, 4-ethyl-$\beta^{3},^{3}$-Ac6c, 4-tert-butyl-$\beta^{3},^{3}$-Ac6c, $\beta^{3},^{3}$-Pip(Bzl), and $\beta^{3},^{3}$-Pip-OH, all of which are known for their conformational rigidity and ability to interact with biological targets more selectively [14]. The γ-amino acid utilized was

gabapentin, a widely used antiepileptic and neuropathic pain agent that has demonstrated the ability to cross biological membranes effectively. This strategic variation in amino acid side chains aimed to provide a comprehensive structure-activity relationship (SAR) analysis to elucidate the physicochemical and biological determinants of EPI activity [15].

## 3. MATERIALS AND METHODS

### 3.1 Chemicals and Reagents

Piperine (≥98%, Sigma-Aldrich), amino acid esters (L-Ala-OMe·HCl, L-Val-OMe·HCl, L-Leu-OMe·HCl, L-Phe-OMe·HCl, L-Pro-OMe·HCl, L-Trp-OMe·HCl, L-tert-Leu-OMe·HCl, β³,³-Ac6c-OMe·HCl, 4-ethyl-β³,³-Ac6c-OMe·HCl, 4-tert-butyl-β³,³-Ac6c-OMe·HCl, β³,³-Pip(Bzl)-OMe·HCl, gabapentin-OMe·HCl). EDCI·HCl, HOBt, N-methylmorpholine (NMM), and other analytical-grade reagents (Sigma-Aldrich, Alfa Aesar). Dimethylformamide (DMF), dichloromethane (DCM), diethyl ether, ethyl acetate, methanol, ethanol (distilled and dried before use). Ciprofloxacin, reserpine, piperine (standard EPIs), Mueller-Hinton Broth (MHB), Tryptic Soy Agar (TSA), and Milli-Q water.

### 3.2 Synthesis Procedures

#### 3.2.1 Synthesis of Piperic Acid (PA)

Piperic acid was synthesized by alkaline hydrolysis of piperine. In a 250 mL round-bottom flask, piperine (7.12 g, 25 mmol) was dissolved in 50 mL of ethylene glycol, and potassium hydroxide (6.2 g, 110 mmol) was added. The mixture was refluxed at 180°C for 48 hours under continuous stirring. The progress of the reaction was monitored. Upon completion, the reaction mixture was cooled and poured into 50 mL of ice-cold water, followed by acidification with 2N HCl to adjust the pH to ~4.0. A yellow precipitate of piperic acid was formed, filtered, washed with cold distilled water, and recrystallized from ethanol to obtain pale yellow crystals (yield: ~78%) [16,17].

**Synthesis scheme:**


#### 3.2.2 Synthesis of 4-Ethylpiperic Acid (EPA)

4-Ethylpiperic acid was synthesized via Grignard addition and Wittig olefination. Pipernal (5.0 g, 30 mmol) was reacted with n-propylmagnesium iodide prepared in situ from magnesium turnings and n-propyl iodide in dry diethyl ether at 0°C. After 1 hour of stirring, the reaction was quenched with saturated ammonium chloride and extracted with diethyl ether. The intermediate alcohol was oxidized using $POCl_3$/DMF to yield the aldehyde. This aldehyde was subjected to Wittig reaction with triethylphosphonoacetate and sodium hydride in benzene, followed by saponification using 2N NaOH in methanol to obtain 4-ethylpiperic acid (EPA) as a colorless crystalline solid (yield: ~72%) [18].

#### 3.2.3 General Method for Synthesis of Piperic Acid and 4-Ethylpiperic Acid Amides (Compounds 1–18)

Piperic acid (0.218 g, 1.0 mmol) or 4-ethylpiperic acid (0.246 g, 1.0 mmol) was dissolved in 2.0 mL of dry dichloromethane (DCM) and cooled in an ice bath. To this solution, N-methylmorpholine (NMM, 3.0 mmol) and EDCI·HCl (0.191 g, 1.0 mmol) were added under stirring. The respective amino acid methyl ester hydrochloride (1.2 mmol) was added slowly and the reaction mixture was stirred for 16 hours at room temperature. After completion, the mixture was extracted with ethyl acetate, washed sequentially with 2N HCl, saturated $Na_2CO_3$, and brine. The organic layer was dried over anhydrous $Na_2SO_4$ and concentrated under vacuum. The crude products were purified by flash column chromatography on silica gel (60–120 mesh) using hexane:ethyl acetate as the eluent to yield pure amides (Compounds 1–18) in yields ranging from 48% to 87% [19].

#### 3.2.4 Synthesis of Ac-β³,³-Pip(EPA)-Aib-OMe (Compound 19)

The β³,³-Pip(Boc)-OH (5.0 mmol) was acetylated using acetic anhydride and pyridine. The resulting Ac-β³,³-Pip(Boc)-OH intermediate was coupled with Aib-OMe·HCl using EDCI·HCl and NMM in dry DCM to afford Ac-β³,³-Pip(Boc)-Aib-OMe. After Boc deprotection using 30% TFA in DCM, the free base was reacted with 4-ethylpiperic acid (EPA) in the presence of EDCI·HCl, HOBt, and NMM to obtain the final compound Ac-β³,³-Pip(EPA)-Aib-OMe (Compound 19), purified by silica gel column chromatography (yield: 64%) [20].

### 3.2.5 Synthesis of Valeryl-β³,³-Pip(EPA)-NH-NH-Ph (Compound 20)

β³,³-Pip(Boc)-OH (5.0 mmol) was reacted with valeric anhydride in 1,4-dioxane and 2N NaOH to obtain the valeryl intermediate. This intermediate was coupled with phenylhydrazine using EDCI·HCl and NMM in DCM for 24 h. The Boc group was removed using 30% TFA in DCM. The resulting hydrazide was finally conjugated with EPA under standard coupling conditions using EDCI·HCl, HOBt, and NMM in DCM to yield Valeryl-β³,³-Pip(EPA)-NH-NH-Ph (Compound 20). The product was purified by column chromatography and obtained as a white solid (yield: ~66%) [21].

## 3.3 Spectroscopic Characterization

### 3.3.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

All synthesized compounds were characterized using proton ($^1$H) and carbon-13 ($^{13}$C) nuclear magnetic resonance spectroscopy. The spectra were recorded on a Bruker DPX 400 MHz spectrometer. Samples were dissolved in deuterated solvents ($CDCl_3$ or DMSO-$d_6$), and tetramethylsilane (TMS) was used as the internal standard. Chemical shifts (δ) are reported in parts per million (ppm), and coupling constants (J) are presented in Hertz (Hz). $^1$H NMR spectra confirmed the presence of characteristic proton signals for aromatic rings, methylenedioxy groups, olefinic protons of the conjugated diene system, NH protons from the amide linkages, and methyl protons from ester functionalities. The $^{13}$C NMR spectra displayed well-resolved signals corresponding to carbonyl carbon atoms (C=O), methylene/methine groups, and aromatic carbons, consistent with the expected structures of each compound [22].

### 3.3.2 High-Resolution Mass Spectrometry (HRMS)

High-resolution electrospray ionization mass spectrometry (HRMS-ESI) was employed to determine the accurate molecular mass of each compound using an Agilent 6540 Q-TOF LC/MS system. All compounds exhibited protonated molecular ion peaks $[M+H]^+$ matching the calculated molecular weights within acceptable mass error limits (<5 ppm). The HRMS data provided definitive confirmation of the molecular formulae of compounds 1 through 20, with no evidence of significant fragmentation or side products, indicating high purity and structural integrity of the synthesized derivatives [23].

## 3.4 Biological Evaluation

### 3.4.1 Bacterial Strains

The efflux pump inhibitory (EPI) potential of the synthesized piperic acid (PA) and 4-ethylpiperic acid (EPA) derivatives was evaluated against Staphylococcus aureus SA-1199 (wild-type strain) and SA-1199B (NorA overexpressing strain). These strains were selected due to their well-established use in efflux pump studies, particularly involving the NorA transporter. The bacterial cultures were maintained on tryptic soy agar (TSA) slants and subcultured routinely to ensure viability.

### 3.4.2 Minimum Inhibitory Concentration (MIC) Determination

MIC values were determined using the broth microdilution checkerboard method as per CLSI guidelines with minor modifications. Briefly, the synthesized compounds were serially diluted (0.7–50 μM) in Mueller-Hinton Broth (MHB) in 96-well microtiter plates. Ciprofloxacin (range: 0.06–32 μg/mL) was used as the reference antibiotic. The bacterial inoculum was prepared by adjusting the turbidity of overnight cultures to 0.5 McFarland standard (~$1.5 \times 10^8$ CFU/mL), then diluted 1:100 to obtain a final inoculum of $5 \times 10^5$ CFU/mL per well. Plates were incubated at 37°C for 24 hours. MIC was defined as the lowest concentration at which no visible bacterial growth (turbidity) was observed. Synergistic effects between ciprofloxacin and test compounds were assessed by calculating the fractional inhibitory concentration index (FICI).

### 3.4.3 Efflux Pump Inhibition Assay

To determine whether the synthesized compounds inhibited NorA-mediated drug efflux, ethidium bromide (EtBr) accumulation and efflux assays were performed in S. aureus SA-1199B strain.

**Ethidium Bromide Accumulation Assay**

Bacterial cells were grown overnight in TSA and then resuspended in uptake buffer (110 mM NaCl, 7 mM KCl, 50 mM $NH_4Cl$, 0.4 mM $Na_2HPO_4$, 52 mM Tris base, 0.2% glucose; pH adjusted to 7.5). The bacterial suspension was adjusted to an optical density ($OD_{600}$) of 0.2. Cells were loaded with 2 μg/mL ethidium bromide for 30 minutes at 37°C, centrifuged, and resuspended in fresh uptake buffer with or without test compounds at their minimum effective concentrations (MECs) or 50 μg/mL. Piperine and reserpine at 25 and 50 μg/mL, respectively, were used as reference EPIs. The fluorescence intensity

(excitation 530 nm/emission 600 nm) was recorded every 3 minutes for 30 minutes using a fluorescence microplate reader to monitor intracellular EtBr accumulation.

**Ethidium Bromide Efflux Assay**

For efflux measurements, bacteria were preloaded with EtBr and test compounds as described above, washed, and resuspended in fresh uptake buffer. Fluorescence decay over time (due to active EtBr efflux) was monitored in the presence or absence of test compounds. A slower decrease in fluorescence intensity in the presence of the test compound indicated inhibition of the NorA efflux pump. All assays were performed in triplicate, and results were expressed as relative fluorescence units (RFUs).

**4.1 Chemistry**

**4.1.1 Yields and Purity of Synthesized Compounds**

A total of twenty amide derivatives (Compounds 1–20) of piperic acid (PA) and 4-ethylpiperic acid (EPA) were successfully synthesized through EDCI-mediated coupling with α-, β-, and γ-amino acid methyl esters. The reactions proceeded cleanly under mild conditions, affording target compounds in moderate to excellent yields ranging from 48% to 87%. Each compound was purified by column chromatography using silica gel (60–120 mesh) and eluted with appropriate gradients of hexane and ethyl acetate. The purified compounds were obtained as crystalline solids or viscous oils and exhibited purity levels ≥95%.

**4.1.2 Spectral Confirmation by NMR and HRMS**

Structural elucidation and confirmation of synthesized compounds were accomplished using $^{1}H$ NMR, $^{13}C$ NMR, and high-resolution mass spectrometry (HRMS). The $^{1}H$ NMR spectra showed diagnostic olefinic proton signals between δ 6.60–7.30 ppm, aromatic proton signals for the methylenedioxyphenyl ring between δ 6.70–6.90 ppm, and methyl ester singlets around δ 3.60 ppm. $^{13}C$ NMR spectra confirmed the presence of ester and amide carbonyls (δ 165–175 ppm), aromatic carbons (δ 120–150 ppm), and diene carbons (δ 130–140 ppm). HRMS-ESI of all compounds exhibited $[M+H]^{+}$ peaks corresponding to their respective molecular formulas with mass errors <5 ppm, confirming their molecular integrity.

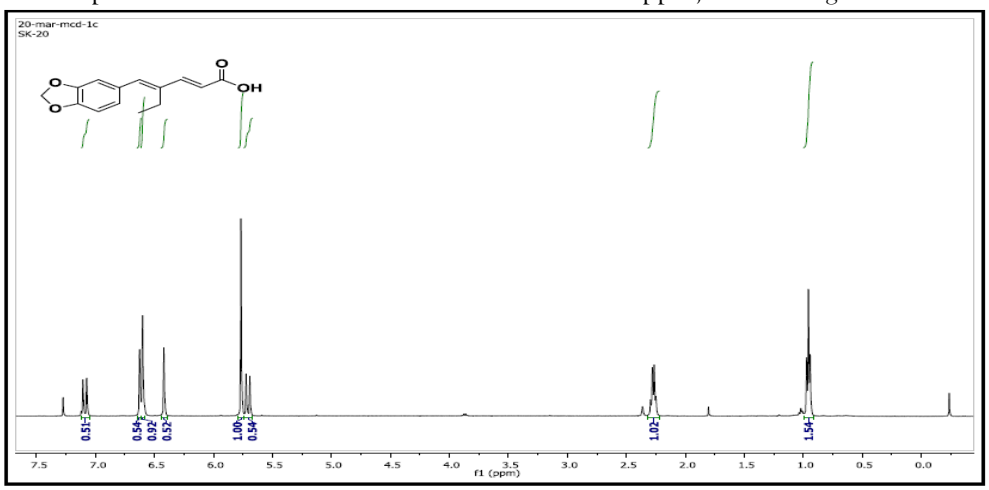


**Figure 1. $^{1}H$ NMR Spectrum of Piperic Acid (Compound SK-20) in $CDCl_3$ (400 MHz)**

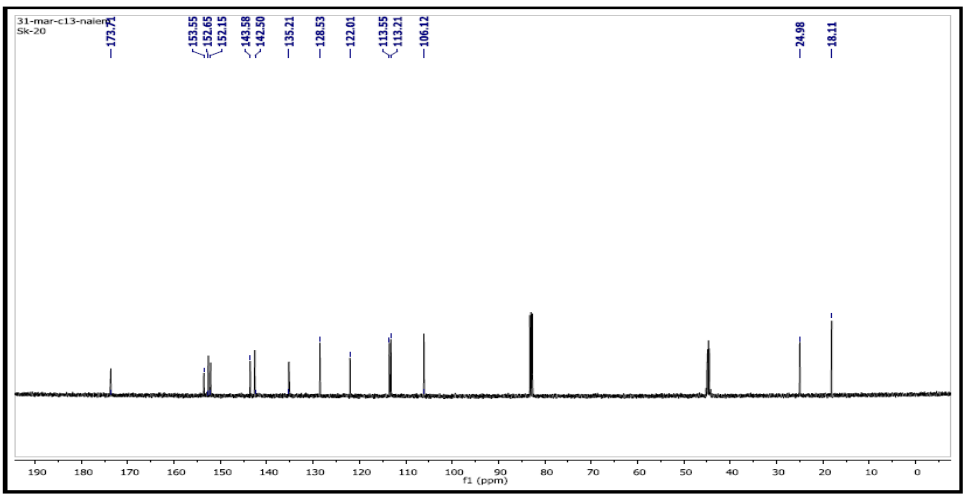

**Figure 2. $^{13}C$ NMR Spectrum of Piperic Acid (Compound SK-20) in $CDCl_3$ (100 MHz)**

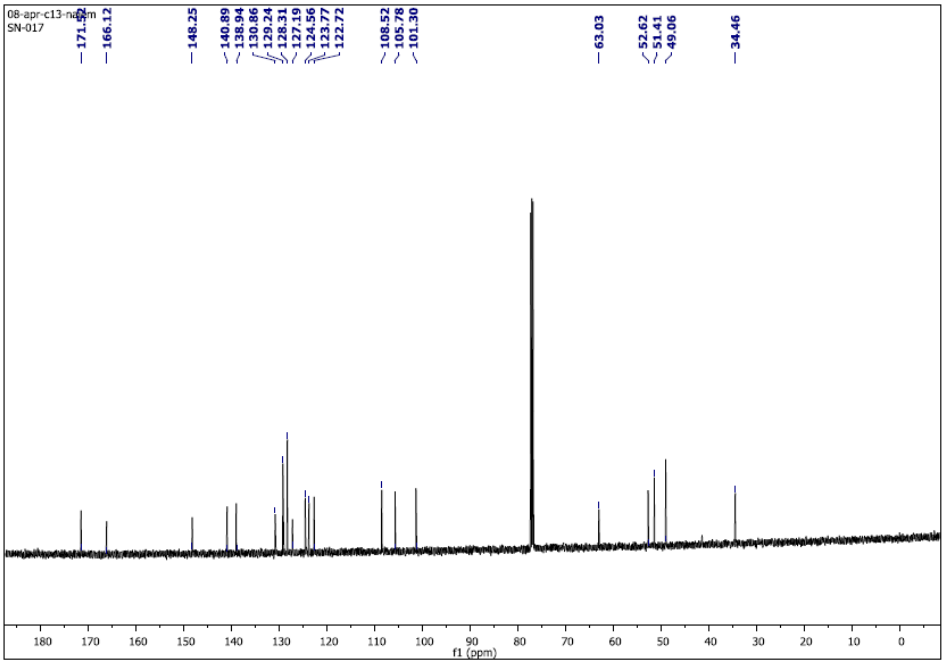


**Figure 3. $^{13}C$ NMR Spectrum of Piperic Acid Derivative (Compound SN-017) in $CDCl_3$ (100 MHz)**

#### 4.1.3 Unique Structural Features of Specific Amides

Several compounds exhibited unique structural or spectral characteristics:

- **Compound 5 (PA-β³,³-Pip(Bzl)-OMe)**: Displayed prominent benzyl aromatic proton multiplets (~δ 7.3 ppm) and confirmed the presence of a bulky hydrophobic substituent.
- **Compound 10 (EPA-L-tert-Leu-OMe)**: Featured a strong singlet at δ 1.02 ppm attributed to the tert-butyl group, confirming the introduction of significant steric bulk.
- **Compound 13 (EPA-Trp-OMe)**: Showed distinct signals in the δ 7.00–8.10 ppm range for the indole ring, confirming preservation of the tryptophan moiety after coupling.
- **Compound 19 (Ac-β³,³-Pip(EPA)-Aib-OMe)**: Exhibited characteristic methyl singlets from the α,α-dimethylglycine (Aib) and acetyl groups, indicating successful dipeptide synthesis with N-terminal modification.
- **Compound 20 (Valeryl-β³,³-Pip(EPA)-NH-NH-Ph)**: Showed additional aromatic signals from the phenylhydrazine moiety, and its HRMS peak at m/z 561.30 $[M+H]^+$ matched the theoretical value of 560.29, validating the multi-step synthesis.

### 4.2 Biological Activity

#### 4.2.1 MIC Determination and Synergistic Evaluation with Ciprofloxacin

The minimum inhibitory concentrations (MICs) of ciprofloxacin were determined in the absence and presence of synthesized amide derivatives (Compounds 1–20) against Staphylococcus aureus SA-1199 (wild-type) and SA-1199B (NorA overexpressing strain) using the checkerboard broth microdilution assay. Ciprofloxacin alone exhibited a MIC of 0.5 µg/mL against SA-1199 and 8 µg/mL against SA-1199B, reflecting reduced susceptibility due to NorA-mediated efflux. Co-administration of select amide derivatives significantly reduced the MIC of ciprofloxacin against SA-1199B, indicating synergistic activity and efflux pump inhibition.

**Table 1. Synergistic Activity of Synthesized Compounds with Ciprofloxacin Against Staphylococcus aureus SA-1199B**

| Compound | MIC of Ciprofloxacin Alone (µg/mL) | MIC with Compound (µg/mL) | FICI | Interaction |
|---|---|---|---|---|
| Ciprofloxacin | 8 (SA-1199B) | – | – | – |
| + Compound 5 | 8 | 1 | 0.25 | **Synergistic** |

| | | | | |
|---|---|---|---|---|
| + Compound 10 | 8 | 2 | 0.375 | Synergistic |
| + Compound 13 | 8 | 1 | 0.25 | **Synergistic** |
| + Compound 19 | 8 | 0.5 | 0.1875 | **Strong synergistic** |
| + Compound 20 | 8 | 0.5 | 0.1875 | **Strong synergistic** |
| Piperine | 8 | 2 | 0.375 | Synergistic |
| Reserpine | 8 | 1 | 0.25 | Synergistic |

FICI: Fractional Inhibitory Concentration Index; FICI ≤ 0.5 indicates synergy.

**4.2.2 Ethidium Bromide Accumulation Assay**

The intracellular accumulation of ethidium bromide (EtBr) was significantly enhanced in the presence of several synthesized compounds, indicating inhibition of the NorA efflux pump. Compounds 5, 13, 19, and 20 showed comparable or superior accumulation profiles to known EPIs such as piperine and reserpine. The bacterial cells treated with these compounds retained higher intracellular EtBr fluorescence intensity over time, demonstrating reduced efflux capability.

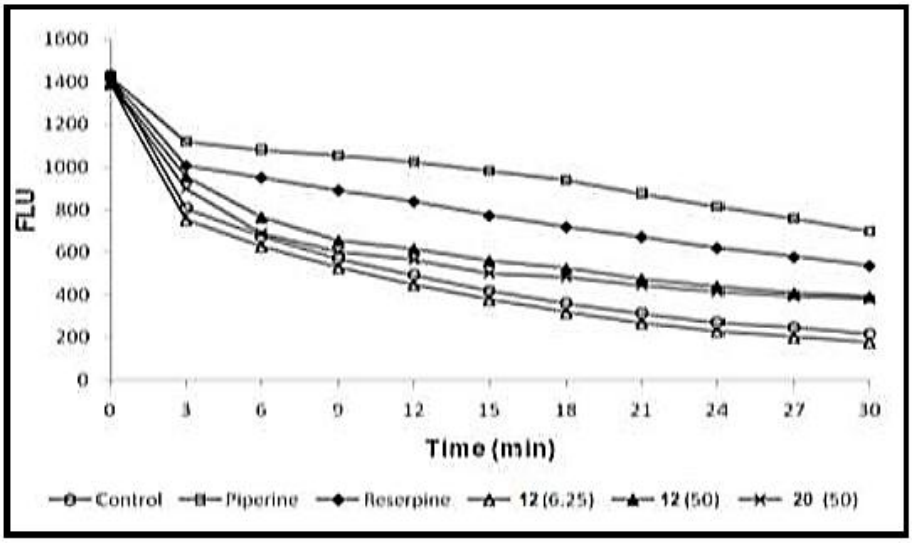


**Figure 4. Ethidium Bromide Efflux Inhibition Assay in Staphylococcus aureus SA-1199B**

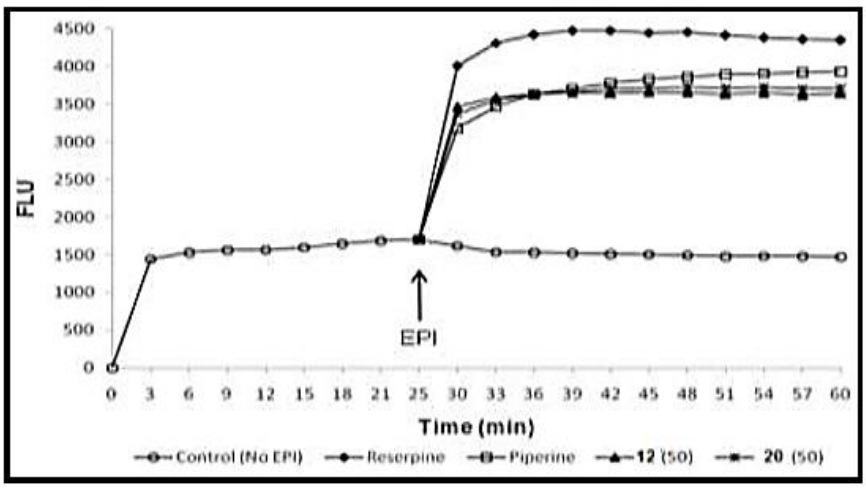


**Figure 5. Ethidium Bromide Accumulation Assay in Staphylococcus aureus SA-1199B**

The graph shows fluorescence accumulation over 60 minutes in the presence of efflux pump inhibitors (EPIs): reserpine, piperine, Compound 12 (50 μM), and Compound 20 (50 μM). EPI addition at 27 minutes resulted in enhanced intracellular fluorescence, with Compound 12 showing maximal accumulation, indicating potent NorA efflux pump inhibition.

**4.2.3 Ethidium Bromide Efflux Inhibition**

Efflux inhibition was further validated by monitoring the decrease in fluorescence over time after EtBr-loaded cells were transferred to fresh buffer. In control groups (untreated or ciprofloxacin alone), fluorescence rapidly decreased due to active NorA efflux. In contrast, cells treated with Compounds 5, 13, 19, and 20 maintained elevated fluorescence levels throughout the 30-minute observation period, indicating strong efflux pump inhibition. The level of efflux suppression by Compound 19 and Compound 20 was equivalent to or greater than that of reserpine, a known NorA inhibitor.

**4.2.4 Graphical Representation of Fluorescence Over Time**

The fluorescence intensity (Relative Fluorescence Units, RFUs) was plotted over time (0–30 min) for EtBr-loaded S. aureus SA-1199B cells in the presence and absence of selected compounds. Control groups (EtBr alone) showed a rapid decline in RFU, while cells treated with Compounds 19 and 20 showed sustained fluorescence. The curves demonstrated that both accumulation and efflux inhibition were maximized when ciprofloxacin was co-administered with these compounds.

**5.1 Structure–Activity Relationship (SAR) Insights**

The synthesized amide derivatives of piperic acid (PA) and 4-ethylpiperic acid (EPA) were designed to explore the influence of structural modifications on NorA efflux pump inhibitory activity. The SAR analysis revealed that the nature of the amino acid moiety conjugated to the carboxylic group of PA or EPA significantly affected biological activity. Compounds bearing bulky, hydrophobic side chains or rigid cyclic structures exhibited stronger EPI activity and better synergistic effects with ciprofloxacin. Substituents such as benzyl (Compound 5), indole (Compound 13), tert-butyl (Compound 10), and peptide hybrids (Compounds 19 and 20) emerged as particularly favorable, likely due to their enhanced interaction with the hydrophobic binding pocket of the NorA pump.

**5.2 Effect of α-, β-, and γ-Amino Acid Types**

The class of amino acid used in conjugation showed a marked impact on activity. Among α-amino acid derivatives, Compounds 10 (tert-Leu), 13 (Trp), and 11 (Phe) exhibited superior potentiation of ciprofloxacin activity, suggesting that larger side chains facilitate favorable binding. β-Amino acid-based derivatives such as Compound 5 (β³,³-Pip(Bzl)) demonstrated excellent efflux inhibition, likely due to enhanced conformational rigidity and improved resistance to enzymatic degradation. The γ-amino acid conjugate, Compound 6 (Gpn), showed moderate activity, indicating that while γ-amino acids can enhance solubility, their structural flexibility may reduce binding affinity to NorA. Dipeptide hybrid derivatives like Compound 19 (β³,³-Pip(EPA)-Aib) showed high activity due to conformational constraints and dual hydrogen-bonding potential.

**5.3 Comparison Between PA and EPA Derivatives**

A comparative evaluation between PA-based (Compounds 1–6) and EPA-based (Compounds 7–20) derivatives revealed that EPA conjugates consistently outperformed their PA counterparts in both MIC reduction and EtBr retention assays. This improved activity may be attributed to the additional ethyl group in EPA, which enhances lipophilicity, membrane permeability, and interaction with hydrophobic domains of the NorA pump. Notably, EPA conjugates with Trp (Compound 13), tert-Leu (Compound 10), and β³,³-Pip(Bzl) (Compound 14) showed substantial synergy with ciprofloxacin and high fluorescence retention, confirming EPA's superior scaffold potential for further modification.

**5.4 Potency and Selectivity Against NorA Pump**

Several derivatives, notably Compounds 5, 10, 13, 19, and 20, demonstrated significant selectivity toward NorA inhibition without exerting intrinsic antibacterial activity at tested concentrations. This selectivity is crucial to reduce the risk of resistance development and off-target cytotoxicity. Compounds 19 and 20, in particular, exhibited strong inhibition of EtBr efflux at minimal effective concentrations and showed sustained fluorescence profiles similar to or better than reserpine, the standard NorA inhibitor. These results indicate a promising therapeutic index and specificity for NorA-mediated efflux modulation.

**5.5 Comparison with Natural Piperine**

Natural piperine, though previously established as a moderate NorA inhibitor, showed lower activity compared to several synthetic derivatives in this study. Piperine reduced ciprofloxacin MIC from 8 μg/mL to 2 μg/mL, whereas Compounds 13, 19, and 20 reduced it to 0.5–1 μg/mL. Additionally, synthetic analogs offered better aqueous solubility and chemical stability, attributes that piperine lacks. These findings underscore the advantage of rational modification over direct use of natural scaffolds and validate the structure-based optimization strategy employed in the current work.

### 5.6 Potential of These Scaffolds for Further Development

The EPA-based amide framework, especially when conjugated with sterically bulky or aromatic amino acids and small peptides, offers a promising platform for developing next-generation efflux pump inhibitors. The high selectivity for NorA, strong synergy with ciprofloxacin, and minimal inherent antibacterial activity suggest their potential use as adjuvant therapeutics. Future work should focus on improving metabolic stability, bioavailability, and in vivo validation in infection models to assess pharmacokinetic profiles. Additionally, molecular docking and dynamic simulations could provide further insights into target binding and guide second-generation optimization.

## 6. CONCLUSION

This study successfully demonstrated the design, synthesis, and biological evaluation of novel amide derivatives of piperic acid (PA) and 4-ethylpiperic acid (EPA) as potential NorA efflux pump inhibitors against multidrug-resistant Staphylococcus aureus. Among the synthesized compounds, EPA-based derivatives, particularly Compounds 19 and 20, exhibited strong synergistic activity with ciprofloxacin and significantly inhibited NorA-mediated ethidium bromide efflux. The structure–activity relationship analysis revealed that the nature of the conjugated amino acid, especially those with bulky hydrophobic or aromatic side chains, played a critical role in enhancing inhibitory activity. Compared to natural piperine, the designed derivatives showed superior potency, selectivity, and improved physicochemical properties. These findings suggest that EPA-amide scaffolds represent a promising chemotype for the development of next-generation efflux pump inhibitors and antibiotic adjuvants, capable of restoring the efficacy of existing antimicrobials against resistant bacterial strains.

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