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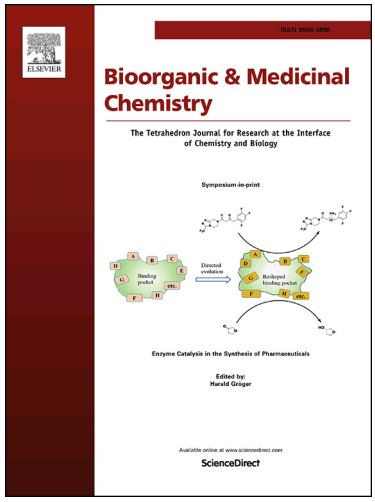

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Synthesis of amides from (E)-3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid and substituted amino acid esters as NorA efflux pump inhibitors of *Staphylococcus aureus*

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Keywords: NorA efflux pump inhibitors; Tetralone; Amino acids; Ciprofloxacin; *Staphylococcus aureus*; Antibacterial; Ethidium bromide.

List of Abbreviations:

CPX: Ciprofloxacin

DCM: Dichloromethane

DMF: Dimethylformamide

EtBr: Ethidium bromide

EDCI.HCl: 1-ethyl-3(3,3-dimethylaminopropyl) carbodiimide hydrochloride

EPI: Efflux pump inhibitor

Et_3N: Triethylamine

GABA: Gamma-aminobutyric acid

HRMS: High resolution mass spectra

MEC: Minimum effective concentration

MFS: Major facilitator family

MIC: Minimum inhibitory concentration

$POCl_3$: Phosphorus oxychloride

SA: *Staphylococcus aureus*

SAR: Structure activity relationship

$SOCl_2$: Thionyl chloride

TLC: Thin layer chromatography

ABSTRACT

Inhibitors for NorA efflux pump of *Staphylococcus aureus* have attracted the attention of many researchers towards the discovery and development of novel efflux pump inhibitors (EPIs). In an attempt to find specific potent inhibitors of NorA efflux pump of *S. aureus*, a total of 15 amino acid conjugates of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**4-18**) were synthesized using a simple convenient synthetic approach and bioevaluated against NorA efflux pump. Two compounds **7** and **8** (each having MEC of 1.56 µg/mL) were found to restore the activity of ciprofloxacin through reduction of the MIC elucidated by comparing the ethidium bromide efflux in dose dependent manner in addition to ethidium bromide efflux inhibition and accumulation study using NorA overexpressing strain SA-1199B. Most potent compounds among these were able to restore the antibacterial activity of ciprofloxacin completely against SA-1199B. Structure activity relationship (SAR) studies and docking study of potent compounds **7** and **8** could elucidate the structural requirements necessary for interaction with the NorA efflux pumps. On the whole, compounds **7** and **8** have ability to reverse the NorA efflux mediated resistance and could be further optimized for development of potent efflux pump inhibitors.

1. Introduction

Bacterial infections are considered as major cause of various widespread human diseases, with many of them life-threatening,[1,2] and the reason for it being that bacteria have evolved various mechanisms for survival under unfavourable and toxic environmental conditions,[3,4] resulting thereby to drug resistance. Mechanisms that are found responsible for microbial drug resistance include alteration of drug targets, enzymatic inactivation of antimicrobials, activation of alternate pathways to avoid antimicrobial action and change in permeability (efflux/influx) of drugs such as β-lactams, macrolides, tetracyclines and

fluoroquinolone.[5,6] The alarming rise of active drug efflux mediated multidrug resistance (MDR) pathogenic bacteria is becoming a major worldwide attention in recent scenario. It has also been found that the occurrence of efflux mediated resistance is overshadowed when compared with other mechanisms such as target alternation,[7,8] irrespective of the fact that the efflux pumps are prominent in terms of both their high efficiency of drug extrusion and broad substrate specificities, underlying their roles in multidrug resistance.[9]

Many of the antibiotics/chemotherapeutic agent are found to be substrates of efflux pumps, which results in extrusion of drugs out of the cells.[9-11] The over expression of membrane surface proteins of major facilitator family (MFS) has shown resistance against various fluoroquinolones in *Staphylococcus aureus*.[11-13] Inhibiting the efflux pumps is regarded as viable approach to combat multidrug resistance as it would help to restore the potency of ineffective antibiotics due to efflux. Additionally it would reduce the emergence of antibiotic resistance and improvement of antibacterial potency at low concentration.[13,14] Therefore, intensive research for the discovery and development of new active EPIs is a promising avenue to tackle this complex panorama which could competently restrain the multidrug resistance.[14,15]

The fascinating potential effect of NorA inhibition involves the delay, prevention or reduction of fluoroquinolone resistance in multidrug resistant strains of *S. aureus,* and the inhibition has been achieved through the application of natural products such as piperine,[16,17] reserpine,[16,18] carnosic acid,[10,19] berberine,[16,20] chalcones,[16,21] capsaicin[22], boeravinone B[23] (Fig. 1) and other natural products[15] and as well as through the application of synthetic compounds.[24-31] Use of semi-synthetic derivatives of natural products have also afforded EPIs that restore the antibacterial activity of ciprofloxacin against NorA overexpressing *S. aureus*.[32-34]

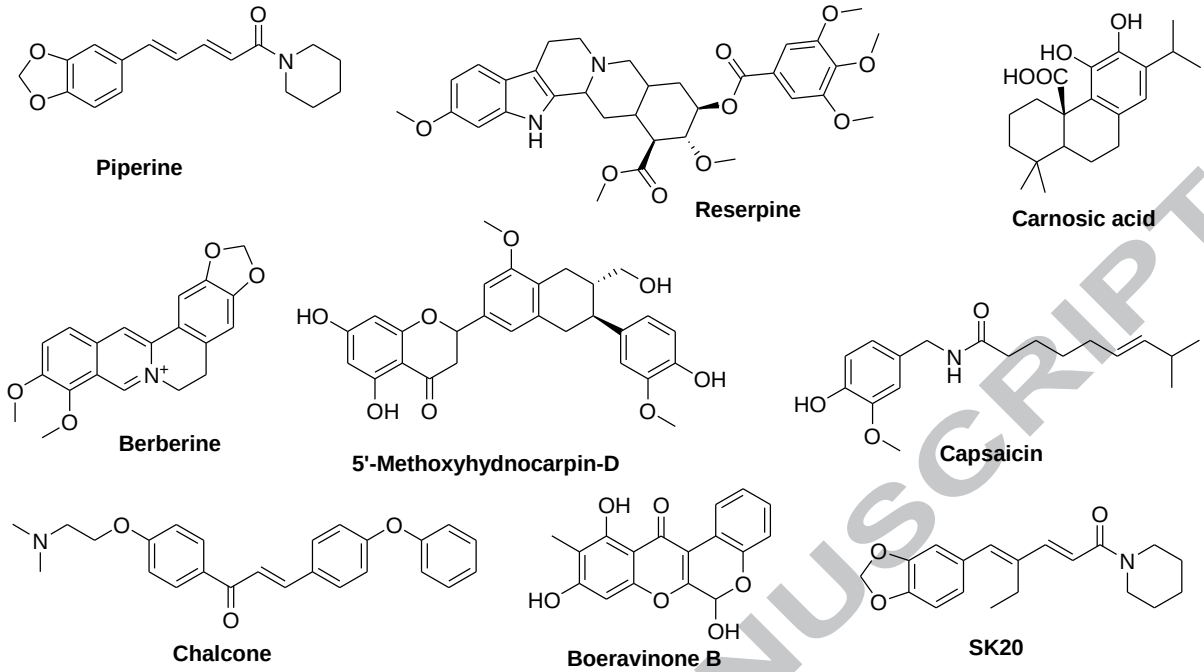


Fig. 1. Chemical structures of known NorA efflux pump inhibitors

In our earlier work, we used amino acid esters and different amines for the preparation of amides of piperic acid and 4-ethylpiperic acid which resulted in the identification of EPIs active against overexpressing NorA efflux pump of *S.aureus.*[28,30,33,34] In continuation of this ongoing exploratory work towards the identification/ development of potent EPIs, we set out to prepare synthetic amides of amino acid ester derivatives of chloro dihydronapthyl scaffold (Fig. 2) using α tetralone as the starting material and evaluate the inhibitory potential of the amides against overexpressing NorA efflux pump. Herein, we report the synthesis of chloro dihydronapthyl substituted amides of amino acid ester derivatives (Fig. 2) and their property as EPIs. Additionally, the amides of amino acid ester linkage are observed to be more stable over simple amide linkage, which signify our present work is more perceptive and preferable.[35] The structure activity relationship of these analogues and the docking studies of three potent analogues **7**, **8** and **14** to understand the interaction with the efflux pump are also described.

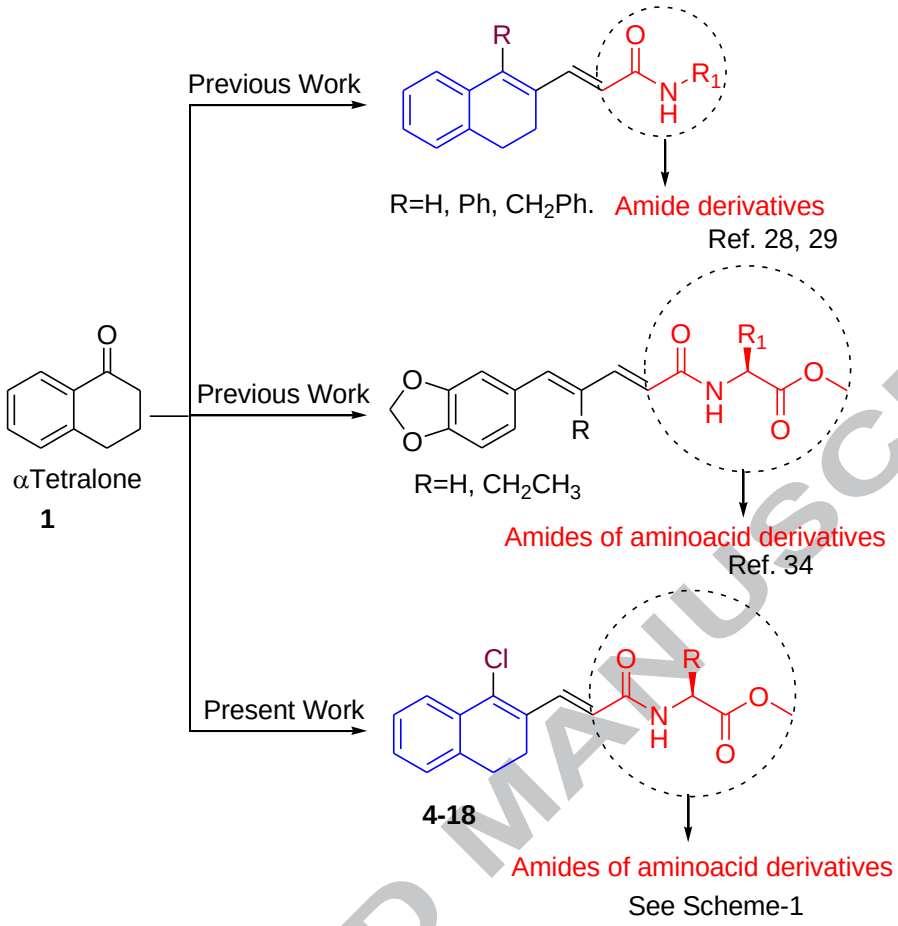


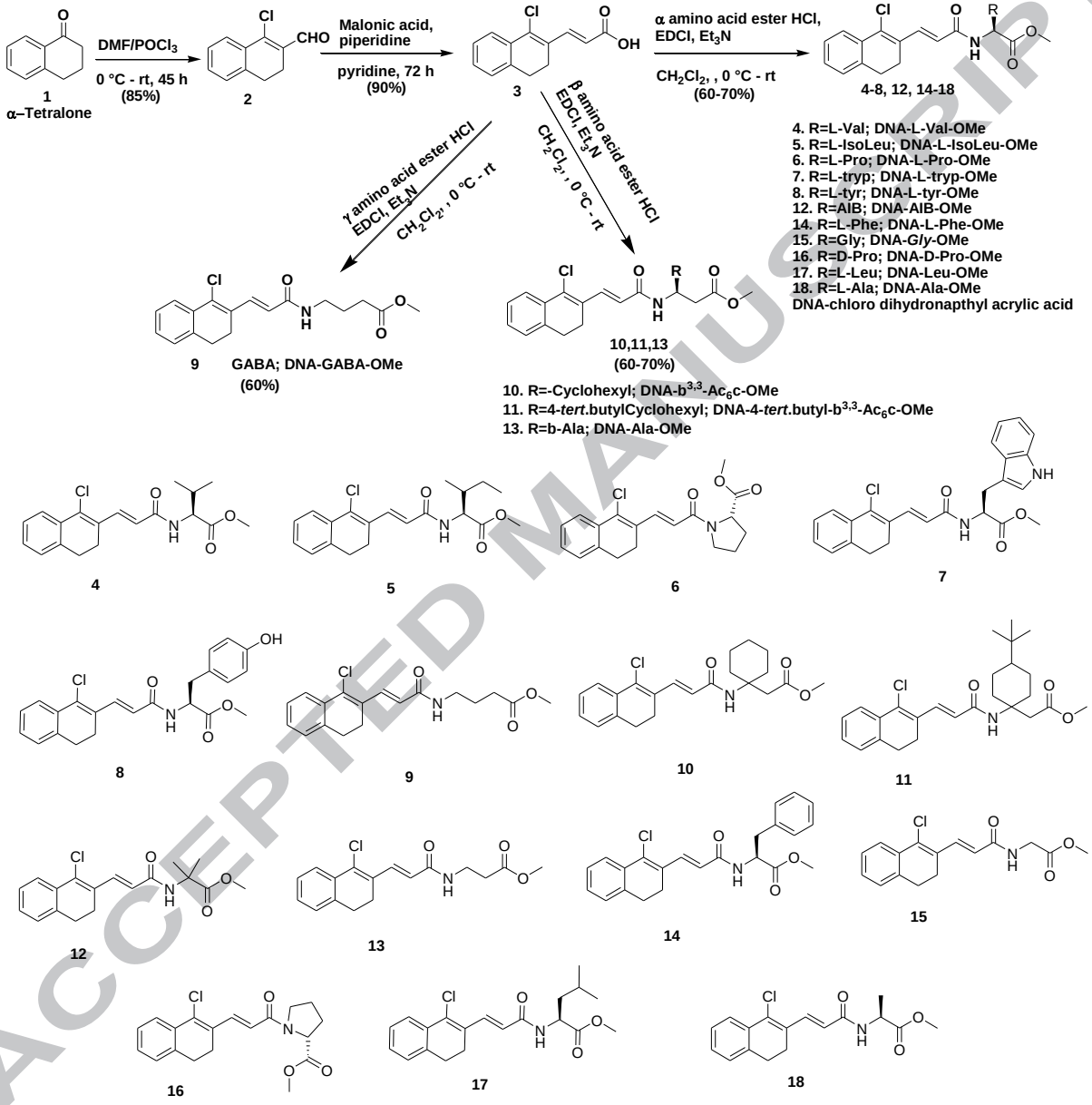
Fig. 2. Design of amides of amino acid ester derivatives of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid from α-tetralone.

2. Results and Discussion

2.1. Chemistry

The synthesis of the 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**3**) and it's corresponding amino acid derivatives (**4-18**) were performed by following the synthetic strategy[29] depicted in Scheme 1 . Starting from α-tetralone in dry dimethylformamide (DMF) as solvent and $POCl_3$ was added drop wise to it and stirred at room temperature to obtain 1-chloro-3,4-dihydronaphthalene-2-carbaldehyde (**2**) in good yield (85%). Condensation of intermediate product **2** with malonic acid in pyridine and piperidine at room temperature for 72 h and after usual work up afforded the key intermediate **3** in 90% yield. Finally the

compound **3** in dry dichloromethane (DCM) in addition with triethylamine (Et_3N) and EDCI was condensed with different α, β and γ amino acid methyl esters to obtained diverse dihydronapthyl substituted amino acid derivatives (**4-18**).



Scheme 1. Synthesis of α, β, and λ amino acid esters amides (**4-18**).

The α-amino acid methyl esters were used such as L-Val, L-Isoleu, L-Pro, L-Trp, L-Tyr, AIB, L-Phe, L-Gly, D-Pro, L-Leu, L-Ala (Scheme 1) for synthesis of compounds **4-8**, **12**, **14-18** respectively, where in β,β-disubstituted β-amino acids were used as β-amino acids such as

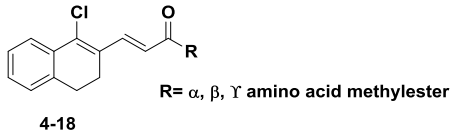
$\beta^{3,3}$-Ac_6c-OMe.HCl, *tert*-butyl-$\beta^{3,3}Ac_6$c-OMe.HCl and β-Ala-OMe.HCl (Scheme 1) for synthesis of compounds **10, 11, 13** respectively and gamma aminobutyric acid (GABA) as γ-amino acid (Scheme 1**)** for compound **9** in the present work. The amides of amino acid esters play an important role in biological activity and β-amino acid ester coupled 4-ethyl piperic acid derivatives were found quite promising activity against ciprofloxacin resistant *S. aureus.*[34] Beside this, β-amino acids and its derivatives have been also reported as an important constituent of biologically active natural products such as cispentacin, amipurimycin, oryzoxymycin, capreomycidine, bleomycin and cytotoxic microcystin[35,36] and γ-amino acid as a neurotransmitter.[37]

2.2. Bioevaluation study

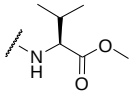
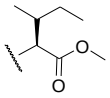
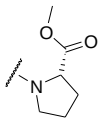
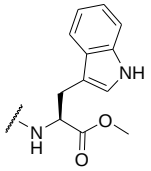
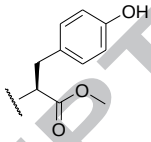
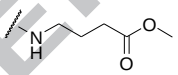
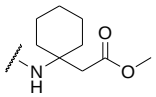
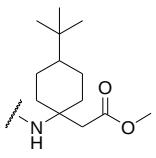
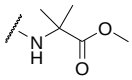
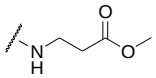
The synthesized series of amino acid amides of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**4-18**) (Scheme 1) were bioevaluated for ciprofloxacin synergistic activity using checkerboard method (Table 1).

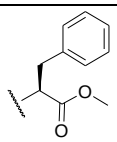
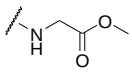
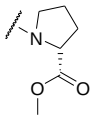
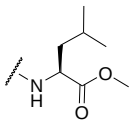
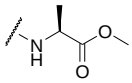
Microbiological evaluation involved the determination of antimicrobial as well as synergistic activity against SA-1199B (NorA overproducing) (Table 1). There was no significant enhancement of MIC using SA-1199 (wild type parent of SA-1199B) and SA-K1758 (*norA* gene knockout) using ciprofloxacin as NorA substrate (Table S1, supporting information). From these results it is clear that none of the compound potentiated the ciprofloxacin against SA-K1758 proves their specific role as NorA inhibitors. Only a single compound (**16**) among all in this study, have intrinsic antimicrobial activity at 50 µg/mL, so it was not further taken for other studies as the synergistic activity might be due to antistaphylococcal activity. Four potent hit candidates **4**, **6**, **7** and **8** were identified as good EPIs (Table 1) which were comparable with standard efflux pump inhibitors like piperine, SK-20 and reserpine with MEC 3.12, 6.25, 1.56, and 1.56 µg/mL respectively.

Table 1. Synthesized chloro-dihydronapthyl substituted amides of amino acid ester derivatives with minimum effective concentration against *S. aureus* SA-1199B



4-18

Compound code	R=	Amino acid	MEC in μg/mL	% EtBr efflux inhibition[#]
4		L-Val-OMe	**3.12**	67.21
5		L-Isoleu-OMe	12.5	58.24
6		L-Pro-OMe	**6.25**	**80.33**
7		L-Trp-OMe	**1.5**	**83.87**
8		L-Tyr-OMe	**1.5**	**85.24**
9		GABA-OMe	>50	ND
10		$\beta^{3,3}$-Ac_6c-OMe	>50	ND
11		4-ethyl- $\beta^{3,3}$-Ac_6c-OMe	>50	ND
12		AIB-OMe	12.5	41.35
13		β-alanine-OMe	25	ND

14		L-Phe-OMe	12.5	48.04
15		L-Gly-OMe	>50	ND
16		D-Pro-OMe	12.5	52.59
17		L-Leu-OMe	12.5	56.23
18		L-Ala-OMe	25	ND
Reserpine	--	--	25	83.06
Piperine	--	--	50	58.46
SK-20	--	--	6.25	68.85

[#] Percentage EtBr efflux inhibition, percentage only calculated for compounds showing MEC ≤ 12.5 μg/mL, MEC is minimum effective concentration.

From the activity profile among the α-amino acid coupled derivatives, except for compound **15** (Glycine), promising NorA efflux pump inhibitory activity was observed for all other analogues. Among α-amino acid coupled derivatives, the amide of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid with L-Trp (**7**) and L-Tyr (**8**) were found to be the most potent showing sixteen fold reduction in the MIC of ciprofloxacin at 25 and 50 μg/mL respectively with minimum effective concentration (MEC hereafter) of 1.56 μg/mL against SA-1199B (Fig. 3a) and comparatively high MEC required for SA-1199 (Fig. 3b). As expected there was no effect on the MIC of SA-K1758 (Fig. 3c). Further the amides of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid with L-proline and D-proline (**6** and **16**) were active, but the amide of L-proline showing promising activity with MEC values of 6.25 and 50 μg/mL against SA-1199B and SA-1199. Moreover, the L-Valine coupled derivative (**4**) is showing promising activity with MEC of 3.12 μg/mL which was found to be two folds more potent than the piperine analogue.[33] The other α–amino acid ester coupled derivatives L-

Isoleu (**5**), L-AIB (**12**), L-Phe (**14**) and L-Leu (**17**) showed MEC of 12.5 µg/mL and up to two fold reduction in MIC of ciprofloxacin against SA-1199.

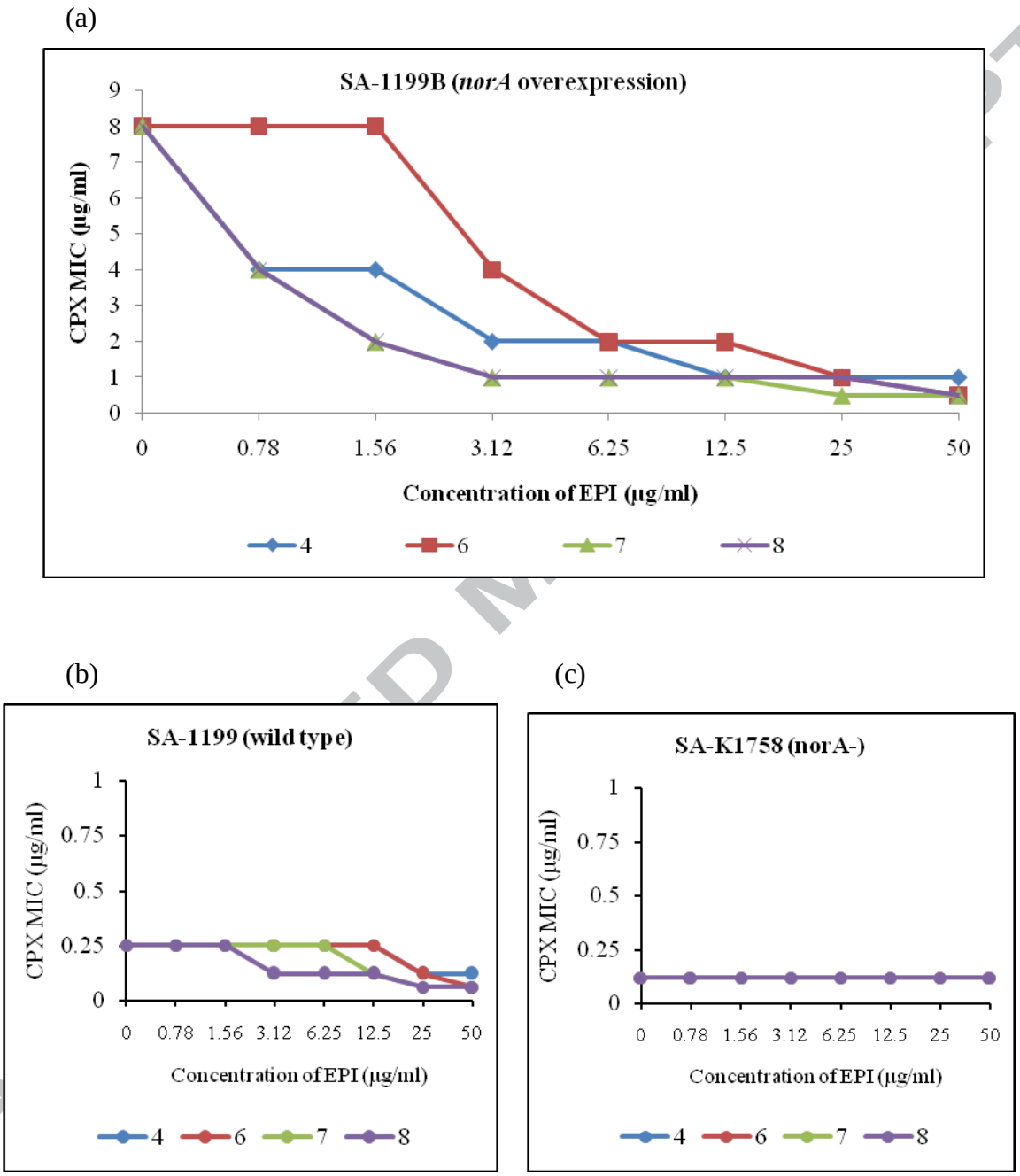


Fig. 3. Isobolograms of ciprofloxacin potentiation in the presence of different concentrations of selected compounds using NorA overexpressing *S. aureus* SA-1199B (a), wild type SA-1199 (b) and NorA knockout strain SA-K1758 (c).

Among β-amino acid coupled derivatives (**10**, **11**, and **13**), amino acid amides of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid with β,β-disubstituted β-amino acids (i.e $\beta^{3,3}Ac_6c$; **10**, *tert*-butyl-$\beta^{3,3}Ac_6c$; **11**) were not active, while un-substituted β-amino acid i.e. β-Ala (**13**) displayed MEC of 25 μg/mL equipotent to that of L-Ala coupled derivative **18**, the only compound that showed moderate activity. The amide of un-substituted γ-amino acid (i.e γ-aminoisobutyric acid; compound **9**) was found inactive (Table 1).

From the bio evaluation studies, it was observed that all α-amino acid ester conjugated derivatives of chloro dihydronapthyl moiety displayed significant potentiating activity of the drug, while β and γ-amino acid ester conjugated derivatives showed moderate to low activity against NorA efflux pump. Furthermore, the amino acid conjugates with hydrophilic group containing N-heterocycle aromatic compound **7** and hydrophilic aromatic substitution compound **8** were found to be most effective among all α-amino acid ester conjugated derivatives (Table 1). Further, 5-membered saturated heterocycle L-proline and D-proline conjugated compounds **6** and **16** analogues were also found to be equipotent against NorA efflux pump than those of other straight chain amino acid conjugated analogues like compounds **5**, **12**, **15**, **17**, and **18** (Table 1).

Most potent compounds having MEC ≤ 12.5 μg/mL (compounds **4**, **5**, **6**, **7**, **8**, **12**, **14**, **16**, and **17**) were further assessed at a single concentration of 50 μM for their percentage inhibition of ethidium bromide (EtBr hereafter) efflux (Table 1). Among these compounds **4**, **6**, **7** and **8** were found to have EtBr efflux inhibition of more than 65%. These compounds were further assessed at concentrations ranging from 6.25-50 μM to enumerate dose response curve in comparison with reference compounds SK-20, reserpine and piperine (Fig. 4). From the results it was concluded that compounds **6**, **7** and **8** were most potent showing EtBr efflux inhibition of more than 80% which is comparable to reserpine (a known efflux pump

inhibitor) at equimolar concentrations. These results further strengthen the synergistic activity results where these compounds were found most potent with the exception of compound **4** where the EtBr efflux percentage was 67%.

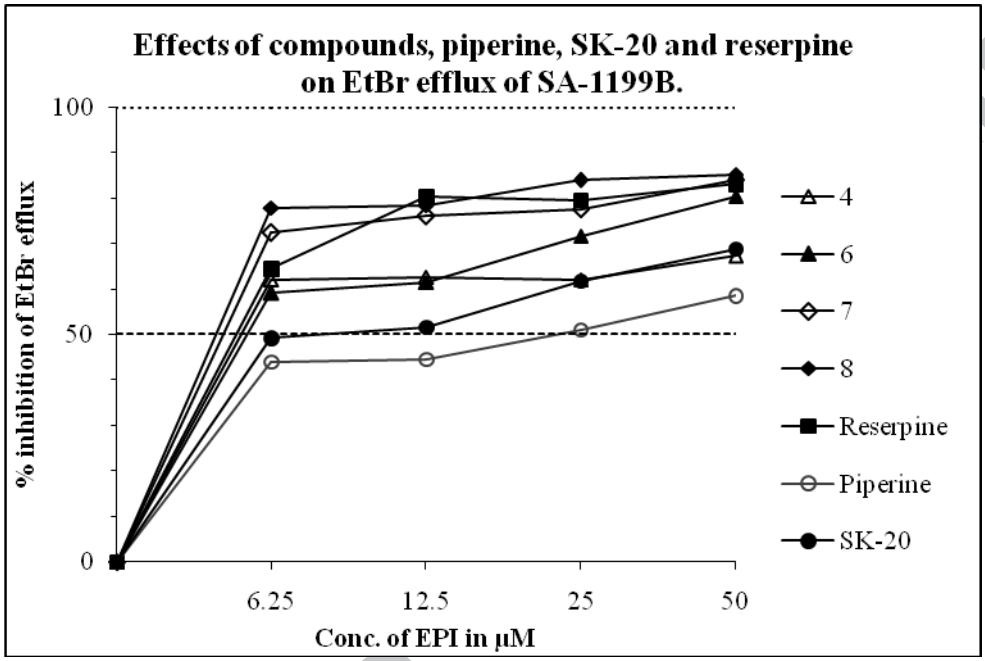


Fig. 4. Effect of selected compounds **4**, **6**, **7** and **8** along with standard EPIs on percent ethidium bromide efflux against *S. aureus* SA-1199B.

The most potent inhibitors were selected for further studies pertaining to their potential for EtBr efflux inhibition and accumulation studies for longer duration. These studies included the previously reported EPI piperine, and standard EPI i.e. reserpine for comparative study. All the molecules were assessed at an equimolar concentration of 25 µM in both assays for better comparison. Both of these compounds were found equipotent to reserpine and bit superior to piperine for a time period of 30 min (Fig. 5). In case of accumulation studies compound **8** was found superior to prior known EPIs while compound **7** showed comparatively lower activity (Fig. 5).

The most potent compounds (**7** and **8**) were found noncytotoxic to hepatic cells when assessed at 100 µM (>10X MEC) to predict their safety to mammalian cells.

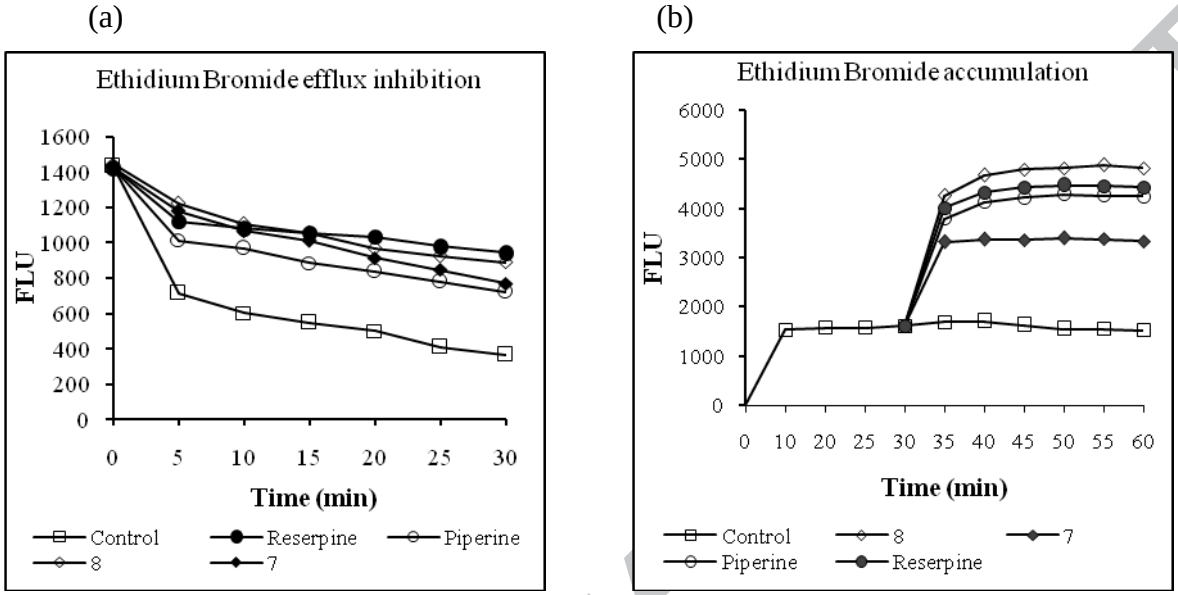


Fig. 5. Impact of compound **7** and **8** on ethidium bromide efflux inhibition (a) and accumulation (b) against SA-1199B

2.3. Molecular modelling studies for NorA

Further, in order to rationalize the *S. aureus* NorA efflux pump inhibition by active compounds **7**, **8**, **14** and piperine (known inhibitor), molecular docking studies were carried out with the built homology model. The binding site of NorA efflux pump was prepared using the iterative minimization cycles in prime (module) as reported previously.[22,38] The model was further validated by the ramachandran plot which states that more than 92.0 % residues are in conformational favoured region. The amino acid (AA) residues Ile19,Val 22, Ile 23, and Val 44 of the protein were found involved in the interaction with all the four ligands namely A (piperine), B (compound **7**), C (compound **8**) and D (compound **14**). Further, Asn 137, Phe 140, Leu 218, Phe 306, Asn 340 and Phe 341residues of the protein were found involved in the interaction with B, C, and D but not with ligand A. Importantly,

it was observed that Phe 140 of the protein is involved in π- π interaction with B and Phe 306 with ligand C, an effect found absent with A and D. Furthermore, Asn 340 is seen involved in the H-bonding with the ligand B (Bond length 1.81 angstrom) and Gln 51 residue with ligand C (Bond length 2.30 angstrom), and apparently these biological interactions play an important role in the display of NorA efflux pump inhibition (Fig. 6).[22]

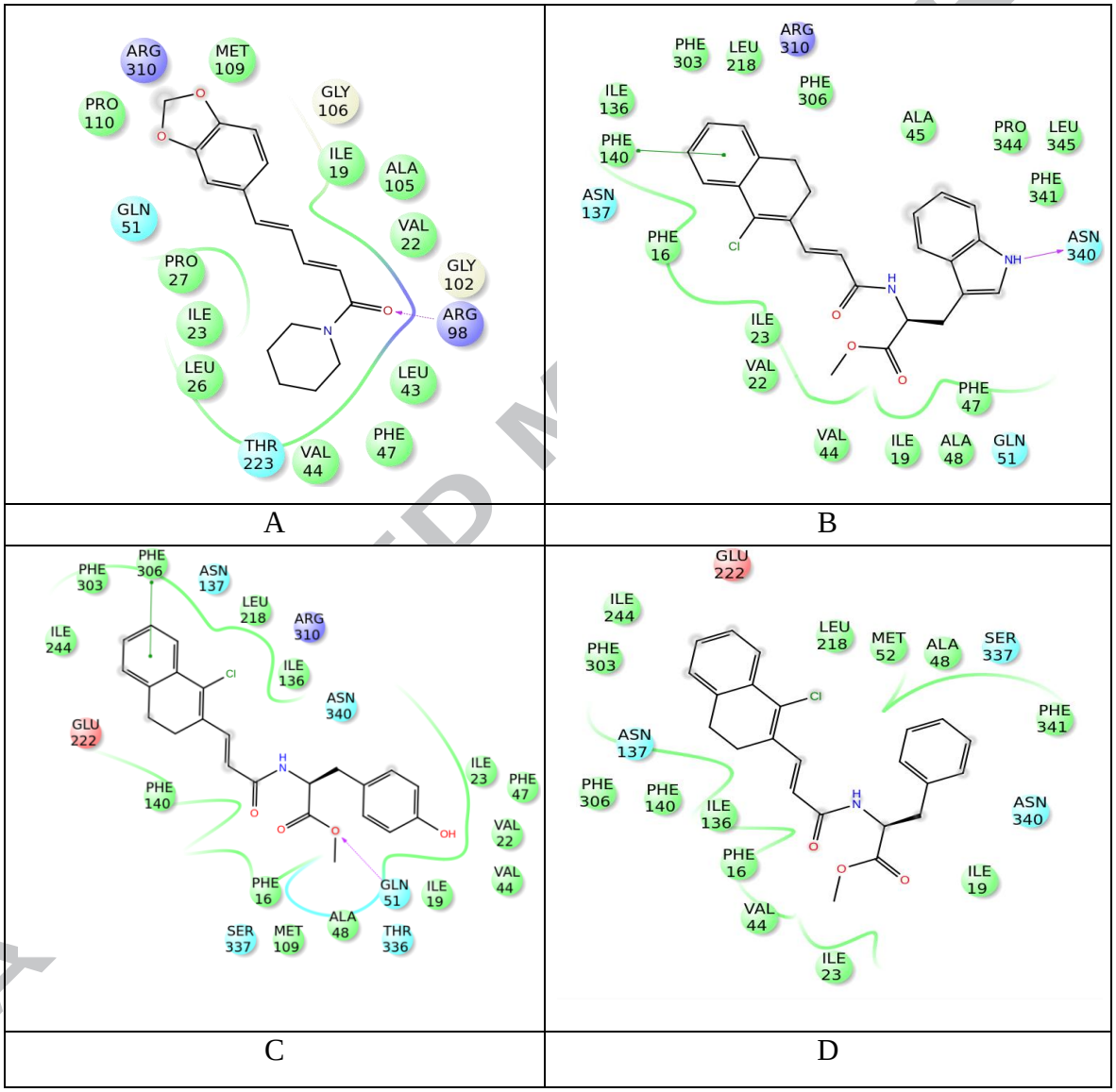


Fig. 6. Interaction of A (piperine), B (compound **7**), C (compound **8**) and D (compound **14**) with binding site of NorA at site-1. H-bond formation between Asn 340 with B, and Gln 51 with C, and π- π interaction of Phe 140 with B and Phe 306 with C strengthens the interactions of the protein/ligand complex and contribute towards the display of high EPI activity of these compounds.

3. CONCLUSION

In conclusion, herein, a simple convenient synthetic approach for the synthesis of fifteen amino acid amides of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid is established, with eleven of them identified as potent NorA efflux pump inhibitory activity. Eight compounds **4**, **6**, **7**, **8**, **12**, **14**, **16**, **17** of eleven compounds have shown MEC ≤ 12.5μg/mL and among these compounds **4** and **6** were found MEC values of 3.12 μg/mL and 6.25 μg/mL respectively against SA-1199B. The compound **4** has only two fold reduction in MIC of ciprofloxacin while MEC of 50 μg/mL for compound **6** against wild type strain SA-1199. However, compounds **7** and **8** were found most potent candidates, able to reduce the MIC of ciprofloxacin by sixteen fold against a NorA overexpressing strain of *S. aureus* SA-1199B in a concentration dependent manner and were devoid of intrinsic activity. All these compounds discussed above are 4-32 folds more active than previously known EPIs reserpine, piperine, verapamil and SK-20. The fluorescence-based efflux studies of ethidium efflux inhibition and accumulation potentiation using NorA-overproducing *S. aureus* cells in the presence of compounds **7** and **8** showed reduced efflux and increased accumulation in the presence of most potent compounds and standard EPIs reserpine and piperine, established inhibition of the efflux mechanism and confirmation of its role as inhibitors of ciprofloxacin efflux from bacterial cells. Ethidium bromide efflux inhibition percentage also has shown these molecules to be superior to reserpine, SK-20 and piperine. SAR studies also revealed that α-amino acid coupled derivatives **7** and **8** containing hydrophilic aromatic groups were found to be most potent among all. Finally, significant chlorodihydronapthyl scaffold analogues with potent NorA EPI activity were identified and their safety profiling may give a new life to old antibiotics.

4. Experimental

4.1. General Methods

All the reagents for chemical synthesis were obtained from Sigma Aldrich Bengaluru, India and the solvents used in reactions were distilled and dried prior to use to perform the present work. The coupling reactions were mediated by 1-ethyl-3(3,3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI.HCl) in presence of triethyl amine. The acid ester hydrochlorides of amino acids were prepared by the reaction of thionyl chloride ($SOCl_2$) in dry methanol. All the chemical reactions were monitored by TLC on 0.25 mm silica gel 60 F254 plates (E. Merck) visualized using UV illumination and 2% ceric ammonium sulfate solution and dragendorff reagent as spraying reagents for detection of the spots on the TLC. Purification of all synthetic derivatives was carried out by column chromatography using Silica gel 60-120 mesh (HiMedia laboratories Pvt Ltd, Nashik, India). ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker DPX 400 and DPX 500 instruments (Bruker Switzerland) using $CDCl_3$ or CD_3OD as the solvents with TMS as internal standard. The chemical shifts were expressed in δ ppm and coupling constants in Hertz. The abbreviations used are as follows: s, singlet; bs, broad singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet. The spectral data are consistent with the assigned structures. High resolution mass spectra (HRMS) and MS were recorded on Agilent Technologies 6540 instrument (Agilent Technologies, Santa Clara, California, United States).

4.2. Synthesis

*4.2.1. Synthesis of 1-chloro-3,4-dihydronaphthalene-2-carbaldehyde [$C_{11}H_9ClO$] (**2**)*

To a chilled solution of α-tetralone (**1**) (5 g) in dry dimethylformamide (15 mL), $POCl_3$ (7.5 mL), was added drop wise at 0-5°C for 0.5-1 h and the resulting reaction mixture was stirred at room temperature for 45 hrs.[29] The reaction mixture was poured into ice-cold

water (150 mL) containing sodium acetate (10 g). The contents were extracted with ethyl acetate (3x 100 mL). The combined extract were pooled and washed with water (3x 50 mL), dried over anhydrous sodium sulfate (Na_2SO_4) and concentrated on rotary vapour under reduced pressure to give crude product which was purified on silica gel column using petroleum ether and ethyl acetate (99:1) as an eluent to give yellow oily liquid compound 1-chloro-3,4-dihydronaphthalene-2-carbaldehyde (**2**) with 85% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.38 (s, 1H), 7.85 (d, J = 8.0 Hz, 1H), 7.39 - 7.31 (m, 2H), 7.22 (d, J = 8.0 Hz, 1H), 2.84 (t, J = 7.6 Hz, 2H), 2.63 (t, J = 7.6 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 190.64, 145.90, 138.96, 132.03, 131.93, 131.43, 127.74, 127.11, 126.31, 27.00, 21.58; HRMS *m/z* $[M+H]^+$ calcd for $C_{11}H_9ClO$: 193.0415, found: 193.0405.

*4.2.2. Synthesis of (E)-3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**3**)*

To a mixture of 1-chloro-3,4-dihydronaphthalene-2-carbaldehyde (**2**) (5 g) in pyridine (15 mL) and piperidine (2.5 mL), malonic acid was added and kept the reaction mixture at room temperature for 72 h. Later the reaction mixture was heated on water bath for 6 h. Then the reaction mixture poured into a conical flask and 50% of aqueous HCl solution was added to precipitate the compound.[29] The precipitate was filtered and crystallized in petroleum ether and ethyl acetate to yield pale yellowish solid pure compound 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**3**) with 90% yield. ^{1}H NMR (400 MHz, CD_3OD) δ 8.15 (d, J = 15.8 Hz, 1H), 7.74 (d, J = 8.0 Hz, 1H), 7.31 – 7.25 (m, 2H), 7.19 – 7.17 (d, J = 8.0 Hz, 1H), 6.09 (d, J = 15.8 Hz, 1H), 2.92 – 2.88 (t, J = 7.6 Hz, 2H), 2.64 – 2.60 (t, J = 7.6 Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 169.42, 141.57, 137.13, 135.26, 132.71, 130.16, 129.23, 127.26, 126.79, 125.79, 119.90, 27.21, 24.81; HRMS *m/z* $[M+H]^+$ calcd for $C_{13}H_{11}ClO_2$: 235.0520, found: 235.0520.

*4.2.3. General procedure for synthesis of chloro-dihydronapthyl substituted amino acid derivatives (**4-18**)*

To a mixture of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**3**) (0.427 mmol) was dissolved in 2.0 mL of dry dichloromethane (DCM) and cooled in an ice bath while stirring. Triethylamine (0.854 mmol) and EDCI.HCl (0.513 mmol) were added into the reaction mixture followed by the addition of amino acid ester hydrochloride (0.513 mmol) and the reaction was stirred for 16 h.[34] 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- Na_2CO_3 (3×10 mL), and brine. The combined organic layer was dried over anhydrous sodium sulfate and evaporated in rotavapour to yield dihydronapthyl substituted amino acid derivatives **4-18**, which was purified by column chromatography over silica gel (60-120 mesh) with yield of 60-70%.

*4.2.3.1. Synthesis of methyl (E)-(3-(1-chloro-3,4-dihydronaphthalen-2-yl)acryloyl)-L-valinate (**4**).* Following the general procedure of section 4.2.3, the desired compound **4** was synthesized by condensation of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**3**) (100 mg, 0.427 mmol) and L-Valine methyl ester hydrochloride (85 mg, 0.513 mmol) to obtain after purification by column chromatography (silica gel/CH_2Cl_2/MeOH 98.5/1.5) a pale yellowish solid in 65% yield. ^{1}H NMR (500 M Hz, $CDCl_3$) δ 8.06 (d, J = 15.5 Hz, 1H), 7.70 (d, J = 7.5 Hz, 1H), 7.24 (m, 2H), 7.14 (d, J = 6.9 Hz, 1H), 6.28 (s, 1NH), 6.14 (d, J = 15.5 Hz, 1H), 4.71 (dd, J = 8.8, 5.0 Hz, 1H), 3.75 (s, 3H), 2.86 (t, J = 7.9 Hz, 2H), 2.57 (t, J = 7.9 Hz, 2H), 2.21 (dq, J = 13.6, 6.8 Hz, 1H), 0.97 (d, J = 6.8 Hz, 3H), 0.94 (d, J = 6.9 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.94, 165.80, 138.34, 137.02, 134.75, 133.08, 130.03,

129.14, 127.11, 126.88, 125.87, 121.82, 57.34, 52.27, 31.52, 27.40, 25.11, 19.03, 17.92; HRMS *m/z* $[M+H]^+$ calcd for $C_{19}H_{22}ClNO_3$: 348.1361, found: 348.1380.

*4.2.3.2. Synthesis of methyl ((E)-3-(1-chloro-3,4-dihydronaphthalen-2-yl)acryloyl)-L-alloisoleucinate (**5**).* Following the general procedure of section 4.2.3, the desired compound **5** was synthesized by condensation of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**3**) (100 mg, 0.427 mmol) and L-isoleucine methyl ester hydrochloride (95 mg, 0.513 mmol) to obtain after purification by column chromatography (silica gel/CH_2Cl_2/MeOH 98.5/1.5) a pale yellowish solid in 65% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 (d, J = 15.5 Hz, 1H), 7.72 (d, J = 8.7 Hz, 1H), 7.25 (m, 2H), 7.15 (d, J = 6.7 Hz, 1H), 6.23 (s, 1NH), 6.14 (d, J = 15.5, 5.7 Hz, 1H), 4.75 (dd, J = 8.6, 5.0 Hz, 1H), 3.76 (s, 3H), 2.88 (t, J = 7.7 Hz, 2H), 2.59 (t, J = 7.8 Hz, 2H), 1.95 (m, 1H), 1.49 (m, 1H), 1.22 (m, 1H), 0.94 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.79, 165.69, 138.31, 137.00, 134.65, 132.98, 130.06, 129.06, 127.07, 126.90, 125.87, 121.90, 56.64, 52.25, 38.20, 27.44, 25.34, 25.12, 15.49, 11.60; HRMS *m/z* $[M+H]^+$ calcd for $C_{20}H_{24}ClNO_3$: 362.1517, found: 362.1508.

*4.2.3.3. Synthesis of methyl (E)-(3-(1-chloro-3,4-dihydronaphthalen-2-yl)acryloyl)-L-prolinate (**6**).* Following the general procedure of section 4.2.3, the desired compound **6** was synthesized by condensation of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**3**) (100 mg, 0.427 mmol) and L-proline methyl ester hydrochloride (87 mg, 0.513 mmol) to obtain after purification by column chromatography (silica gel/CH_2Cl_2/MeOH 98.5/1.5) a pale yellowish solid in 60% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.17 (d, J = 15.4 Hz, 1H), 7.72 (d, J = 7.1 Hz, 1H), 7.25 (m, 2H), 7.15 (d, J = 6.6 Hz, 1H), 6.40 (d, J = 15.3 Hz, 1H), 4.60 (dd, J = 8.5, 4.2 Hz, 1H), 3.75 (s, 3H), 3.67 (m, 2H), 2.88 (t, J = 7.7 Hz, 2H), 2.62 (t, J = 7.7 Hz, 2H), 2.22 (m, 1H), 2.12 (m, 1H), 2.03 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.82,

165.14, 139.66, 137.07, 134.69, 133.06, 130.40, 129.13, 127.08, 126.91, 125.96, 119.46, 59.17, 52.39, 47.01, 29.18, 27.39, 25.24, 24.90; HRMS *m/z* $[M+H]^+$ calcd for $C_{19}H_{20}ClNO_3$: 346.1204, found: 346.1204.

*4.2.3.4. Synthesis of methyl (E)-(3-(1-chloro-3,4-dihydronaphthalen-2-yl)acryloyl)-L-tryptophanate (**7**).* Following the general procedure of section 4.2.3, the desired compound **7** was synthesized by condensation of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**3**) (100 mg, 0.427 mmol) and L-tryptophan methyl ester hydrochloride (130 mg, 0.513 mmol) to obtain after purification by column chromatography (silica gel/CH_2Cl_2/MeOH 98.5/1.5) a yellowish solid in 60% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (s, 1H), 8.06 (d, *J* = 15.6 Hz, 1H), 7.73 (d, *J* = 6.0 Hz, 1H), 7.55 (d, *J* = 4.1 Hz, 1H), 7.36 (d, *J* = 8.1 Hz, 1H), 7.25 (m, 2H), 7.18 (m, 2H), 7.12 (m, 4.0 Hz, 1H), 7.01 (d, *J* = 2.3 Hz, 1H), 6.14 (d, *J* = 7.6 Hz, 1H), 6.02 (d, *J* = 15.5 Hz, 1H), 5.10 (dt, *J* = 7.9, 5.3 Hz, 1H), 3.72 (s, 3H), 3.40 (t, *J* = 5.0 Hz, 2H), 2.86 (t, *J* = 7.4 Hz, 2H), 2.53 (t, *J* = 7.5 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.29, 169.42, 142.05, 140.79, 140.00, 138.50, 136.79, 133.90, 132.91, 131.55, 130.90, 130.74, 129.69, 126.70, 126.09, 125.75, 123.58, 122.49, 115.14, 113.89, 57.10, 56.25, 31.56, 31.22, 28.90; HRMS *m/z* $[M+H]^+$ calcd for $C_{25}H_{23}ClN_2O_3$: 435.1470, found: 435.1458.

*4.2.3.5. Synthesis of methyl (E)-(3-(1-chloro-3,4-dihydronaphthalen-2-yl)acryloyl)-L-tyrosinate (**8**).* Following the general procedure of section 4.2.3, the desired compound **8** was synthesized by condensation of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**3**) (100 mg, 0.427 mmol) and L-tyrosine methyl ester hydrochloride (111 mg, 0.513 mmol) to obtain after purification by column chromatography (silica gel/CH_2Cl_2/MeOH 98.5/1.5) a pale yellowish solid in 60% yield. ^{1}H NMR (400 MHz, $CDCl_3$ with 2 drops of CD_3OD) δ 8.05 (d, *J* = 15.5 Hz, 1H), 7.73 (d, *J* = 6.7 Hz, 1H), 7.26 (m, 3H), 7.17 (d, *J* = 6.8 Hz, 2H), 6.97 (d, *J*

= 8.4 Hz, 3H), 6.76 (d, *J* = 8.5 Hz, 3H), 6.14 (d, *J* = 15.5 Hz, 1H), 4.92 (s, 1H), 3.74 (s, 4H), 3.08 (m, 3H), 2.88 (t, 7.6 Hz, 3H), 2.59 (t, *J* = 7.9 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.48, 166.26, 155.83, 138.24, 136.94, 134.67, 132.79, 130.16 (2C), 130.05, 129.06, 127.01, 126.79, 126.69, 125.72, 121.50, 115.39 (2C), 53.63, 52.54, 36.83, 27.33, 24.91; HRMS *m/z* $[M+H]^+$ calcd for $C_{23}H_{22}ClNO_4$: 412.1310, found: 412.1332.

*4.2.3.6. Synthesis of methyl (E)-4-(3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylamido) butanoate (**9**).* Following the general procedure of section 4.2.3, the desired compound **9** was synthesized by condensation of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**3**) (100 mg, 0.427 mmol) and GABA methyl ester hydrochloride (72 mg, 0.513 mmol) to obtain after purification by column chromatography (silica gel/CH_2Cl_2/MeOH 98.5/1.5) a pale yellowish solid in 60% yield. ^{1}H NMR (500 MHz, $CDCl_3$) *δ* 8.0 (d, *J* = 15.5 Hz, 1H), 7.71 (d, *J* = 8.7 Hz, 1H), 7.24 (m, 2H), 7.13 (d, *J* = 6.5 Hz, 1H), 6.34 (s, 1NH), 6.09 (d, *J* = 15.5 Hz, 1H), 3.66 (s, 3H), 3.40 (dd, *J* = 12.7, 6.6 Hz, 2H), 2.84 (t, *J* = 7.6 Hz, 2H), 2.55 (t, *J* = 7.7 Hz, 2H), 2.40 (t, *J* = 7.1 Hz, 2H), 1.89 (dt, *J* = 13.8, 6.9 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) *δ* 174.18, 166.43, 137.53, 136.89, 134.25, 132.98, 130.13, 129.02, 127.06, 126.90, 125.78, 122.50, 51.78, 39.30, 31.58, 27.36, 25.11, 24.59; HRMS *m/z*$[M+H]^+$ calcd for $C_{18}H_{20}ClNO_3$: 334.1204, found: 334.1209.

*4.2.3.7. Synthesis of methyl (E)-2-(1-(3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylamido)cyclohexyl)acetate (**10**).* Following the general procedure of section 4.2.3, the desired compound **10** was synthesized by condensation of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**3**) (100 mg, 0.427 mmol) and cyclohexyl methyl ester hydrochloride (88 mg, 0.513 mmol) to obtain after purification by column chromatography (silica gel/CH_2Cl_2/MeOH 98.5/1.5) a yellowish solid in 60% yield. ^{1}H NMR (500 MHz,

$CDCl_3$) δ 8.02 (d, J = 15.4 Hz, 1H), 7.71 (d, J = 7.5 Hz, 1H), 7.25 (m, 2H), 7.15 (d, J = 7.0 Hz, 1H), 6.11 (d, J = 15.4 Hz, 1H), 5.66 (s, 1NH), 3.63 (s, 3H), 2.94 (s, 2H), 2.86 (t, J = 7.4 Hz, 2H), 2.57 (t, J = 7.5 Hz, 2H), 2.32 (s, 2H), 1.53 (m, 8H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.90, 165.79, 137.24, 136.89, 134.11, 133.09, 130.14, 129.03, 127.04, 126.88, 125.78, 123.41, 54.71, 51.37, 41.64, 34.84 (2C), 27.42, 25.51, 25.15, 2 x 21.65; HRMS *m/z* $[M+H]^+$ calcd for $C_{22}H_{26}ClNO_3$: 388.1674, found: 388.1677.

*4.2.3.8. Synthesis of methyl (E)-2-(4-(tert-butyl)-1-(3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylamido)cyclohexyl acetate (**11**).* Following the general procedure of section 4.2.3, the desired compound **11** was synthesized by condensation of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**3**) (100 mg, 0.427 mmol) and 4-*tert* butyl cyclohexyl methyl ester hydrochloride (117 mg, 0.513 mmol) to obtain after purification by column chromatography (silica gel/CH_2Cl_2/MeOH 98.5/1.5) a white solid in 60% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, J = 15.4 Hz, 1H), 7.74 (d, J = 7.5, 1.4 Hz, 1H), 7.23 (m, 2H), 7.14 (d, J = 6.8 Hz, 1H), 6.05 (d, J = 15.4 Hz, 1H), 5.96 (s, 1NH), 3.66 (s, 3H), 2.98 (s, 2H), 2.86 (t, J = 7.7 Hz, 2H), 2.57 (t, J = 7.6 Hz, 2H), 2.28 (d, J = 12.7 Hz, 2H), 1.75 (dd, J = 28.2, 14.4 Hz, 4H), 1.25 (dq, J = 9.8, 5.2 Hz, 1H), 1.14 (dd, J = 22.1, 11.5 Hz, 3H), 0.87 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.40, 165.50, 136.99, 136.89, 133.94, 133.12, 130.09, 128.95, 127.01, 126.86, 125.75, 123.86, 55.10, 51.62, 47.46, 36.67, 35.49 (2C), 32.35, 27.50 (3C), 27.43, 25.09, 23.24 (2C); HRMS *m/z* $[M+H]^+$ calcd for $C_{26}H_{34}ClNO_3$: 344.2300, found: 344.2306.

*4.2.3.9. Synthesis of methyl (E)-2-(3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylamido)-2-methylpropanoate (**12**).* Following the general procedure of section 4.2.3, the desired compound **12** was synthesized by condensation of 3-(1-chloro-3,4-dihydronaphthalen-2-

yl)acrylic acid (**3**) (100 mg, 0.427 mmol) and Aib methyl ester hydrochloride (98 mg, 0.513 mmol) to obtain after purification by column chromatography (silica gel/CH_2Cl_2/MeOH 98.5/1.5) a pale yellowish solid in 60% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (d, *J* = 15.5 Hz, 1H), 7.71 (d, *J* = 8.6 Hz, 1H), 7.24 (m, 2H), 7.14 (d, *J* = 6.7 Hz, 1H), 6.40 (s, 1H), 6.11 (d, *J* = 15.5 Hz, 1H), 3.77 (s, 3H), 2.86 (t, *J* = 7.7 Hz, 2H), 2.57 (t, *J* = 7.8 Hz, 2H), 1.62 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.14, 165.20, 137.80, 136.94, 134.53, 133.00, 130.08, 129.00, 127.04, 126.89, 125.82, 122.49, 56.76, 52.68, 27.46, 25.15, 24.83 (2C); HRMS *m/z* $[M+H]^+$ calcd for $C_{18}H_{20}ClNO_3$: 334.1204, found: 334.1201.

*4.2.3.10. Synthesis of methyl (E)-3-(3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylamido) propanoate (**13**).* Following the general procedure of section 4.2.3, the desired compound **13** was synthesized by condensation of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**3**) (100 mg, 0.427 mmol) and β alanine methyl ester hydrochloride (71 mg, 0.513 mmol) to obtain after purification by column chromatography (silica gel/CH_2Cl_2/MeOH 98.5/1.5) a brownish semisolid solid in 70% yield. ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 8.02 (d, *J* = 15.5 Hz, 1H), 7.68 (d, *J* = 7.1 Hz, 1H), 7.22 (m, 2H), 7.11 (d, *J* = 6.6 Hz, 1H), 6.83 (s, 1H), 6.14 (d, *J* = 15.5 Hz, 1H), 3.68 (s, 3H), 3.62 (m, 2H), 2.82 (t, *J* = 7.6 Hz, 2H), 2.62 (t, *J* = 6.2 Hz, 2H), 2.54 (t, *J* = 7.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 172.98, 166.29, 137.50, 136.88, 134.20, 132.89, 130.19, 129.00, 127.05, 126.85, 125.71, 122.57, 51.76, 35.25, 33.92, 27.32, 25.11; HRMS *m/z* $[M+H]^+$ calcd for $C_{17}H_{18}ClNO_3$: 320.1048, found: 320.1051.

*4.2.3.11. Synthesis of methyl (E)-(3-(1-chloro-3,4-dihydronaphthalen-2-yl)acryloyl)-L-phenylalaninate (**14**).* Following the general procedure of section 4.2.3, the desired compound **14** was synthesized by condensation of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**3**) (100 mg, 0.427 mmol) and L-phenylalanine methyl ester hydrochloride

(111 mg, 0.513 mmol) to obtain after purification by column chromatography (silica gel/CH_2Cl_2/MeOH 98.5/1.5) a white solid in 60% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05 (d, *J* = 15.5 Hz, 1H), 7.72 (d, *J* = 5.9 Hz, 1H), 7.26 (m, 6H), 7.12 (dd, *J* = 16.8, 7.4 Hz, 4H), 6.05 (m, 2H), 5.01 (dd, *J* = 13.2, 5.6 Hz, 1H), 3.76 (s, 3H), 3.19 (dd, *J* = 8.5, 5.6 Hz, 2H), 2.86 (t, 2H), 2.55 (t, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.11, 165.46, 138.37, 136.98, 135.85, 134.78, 132.95, 130.03, 129.35, 129.12, 128.64, 127.17, 127.09, 126.93, 125.89, 121.69, 53.33, 52.48, 37.92, 27.37, 25.13; HRMS *m/z* $[M+H]^+$ calcd for $C_{23}H_{22}ClNO_3$: 396.1361, found: 396.1349.

*4.2.3.12. Synthesis of methyl (E)-(3-(1-chloro-3,4-dihydronaphthalen-2-yl)acryloyl) glycinate (**15**).* Following the general procedure of section 4.2.3, the desired compound **15** was synthesized by condensation of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**3**) (100 mg, 0.427 mmol) and glycine methyl ester hydrochloride (64 mg, 0.513 mmol) to obtain after purification by column chromatography (silica gel/CH_2Cl_2/MeOH 98.5/1.5) a pale yellowish solid in 70% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.08 (d, *J* = 15.6 Hz, 1H), 7.74 (d, *J* = 7.5 Hz, 1H), 7.26 (m, 2H), 7.16 (d, *J* = 6.8 Hz, 1H), 6.18 (s, 1NH), 6.14 (d, *J* = 15.5 Hz, 1H), 4.19 (d, *J* = 5.1 Hz, 2H), 3.79 (s, 3H), 2.89 (t, *J* = 7.7 Hz, 2H), 2.59 (t, *J* = 7.8 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.62, 166.11, 138.42, 136.94, 134.89, 132.93, 129.99, 129.14, 127.10, 126.93, 125.90, 121.47, 52.53, 41.56, 27.48, 25.16; HRMS *m/z* $[M+H]^+$ calcd for $C_{16}H_{16}ClNO_3$: 306.0891, found: 306.0887.

*4.2.3.13. Synthesis of methyl (E)-(3-(1-chloro-3,4-dihydronaphthalen-2-yl)acryloyl)-D-prolinate (**16**).* Following the general procedure of section 4.2.3, the desired compound **16** was synthesized by condensation of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**3**) (100 mg, 0.427 mmol) and D-proline methyl ester hydrochloride (87 mg, 0.513 mmol) to

obtain after purification by column chromatography (silica gel/CH_2Cl_2/MeOH 98.5/1.5) a pale yellowish solid in 70% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.11 (d, *J* = 15.3 Hz, 1H), 7.68 (d, *J* = 7.8 Hz, 1H), 7.20 (m, 2H), 7.11 (d, *J* = 7.0 Hz, 1H), 6.37 (d, *J* = 15.3 Hz, 1H), 4.55 (dd, *J* = 8.5, 4.1 Hz, 1H), 3.77 (m, 1H), 3.68 (s, 3H), 3.63 (dt, *J* = 9.3, 7.1 Hz, 1H), 2.83 (t, *J* = 7.8 Hz, 2H), 2.55 (t, *J* = 7.8 Hz, 2H), 2.18 (m, 1H), 2.08 (m, 1H), 1.99 (dd, *J* = 15.2, 8.9 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.85, 164.93, 139.44, 136.90, 134.68, 132.95, 130.49, 129.07, 127.09, 126.88, 125.80, 119.53, 59.11, 52.24, 47.00, 29.16, 27.35, 25.22, 24.87; HRMS *m/z* calcd for $C_{19}H_{20}ClNO_3$ $[M+H]^+$ 346.1204, found: 346.1204.

*4.2.3.14. Synthesis of methyl (E)-(3-(1-chloro-3,4-dihydronaphthalen-2-yl)acryloyl)-L-leucinate (**17**).* Following the general procedure of section 4.2.3, the desired compound **17** was synthesized by condensation of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**3**) (100 mg, 0.427 mmol) and L-leucine methyl ester hydrochloride (87 mg, 0.513 mmol) to obtain after purification by column chromatography (silica gel/CH_2Cl_2/MeOH 98.5/1.5) a brownish semisolid in 70% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.03 (d, *J* = 15.5 Hz, 1H), 7.63 (d, *J* = 7.5 Hz, 1H), 7.20 (m, 2H), 7.11 (d, *J* = 6.4 Hz, 1NH), 6.80 (d, *J* = 8.2 Hz, 1H), 6.14 (d, *J* = 15.5 Hz, 1H), 4.79 (m, 1H), 3.74 (s, 3H), 2.83 (t, *J* = 7.6 Hz, 2H), 2.53 (t, *J* = 7.6 Hz, 2H), 1.70 (m, 2H), 1.69 (m, 1H), 0.94 (t, *J* = 6.9 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.38, 165.99, 138.21, 137.11, 134.50, 132.95, 130.17, 129.07, 127.05, 126.86, 125.77, 121.98, 52.42, 50.93, 41.44, 27.36, 25.01, 24.92, 22.92, 21.81; HRMS *m/z* $[M+H]^+$ calcd for $C_{20}H_{24}ClNO_3$: 362.1517, found: 362.1521.

*4.2.3.15. Synthesis of methyl (E)-(3-(1-chloro-3,4-dihydronaphthalen-2-yl)acryloyl)-L-alaninate (**18**).* Following the general procedure of section 4.2.3, the desired compound **18** was synthesized by condensation of 3-(1-chloro-3,4-dihydronaphthalen-2-yl)acrylic acid (**3**)

(100 mg, 0.427 mmol) and L-alanine methyl ester hydrochloride (71 mg, 0.513 mmol) to obtain after purification by column chromatography (silica gel/CH_2Cl_2/MeOH 98.5/1.5) a white solid in 70% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (d, *J* = 15.5 Hz, 1H), 7.67 (d, *J* = 6.9 Hz, 1H), 7.22 (m, 1H), 7.12 (d, *J* = 6.5 Hz, 1H), 6.51 (d, *J* = 7.4 Hz, 1H), 6.11 (d, *J* = 15.5 Hz, 1H), 4.72 (p, *J* = 7.2 Hz, 1H), 3.75 (s, 1H), 2.84 (t, *J* = 7.5 Hz, 2H), 2.54 (t, *J* = 7.8 Hz, 2H), 1.44 (d, *J* = 7.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.69, 169.32, 141.97, 140.79, 138.43, 136.77, 133.91, 132.89, 130.90, 130.71, 129.67, 125.73, 56.39, 52.05, 31.20, 28.91, 22.31; HRMS *m/z* $[M+H]^+$ calcd for $C_{17}H_{18}ClNO_3$: 320.1048, found: 320.1063.

4.3. Biology

4.3.1. Bacterial strains and growth conditions media

To determine the ciprofloxacin potentiation various strains of *S. aureus* employed were SA-1199B (overexpressing *norA* and also possesses an A116E GrlA substitution) and its isogenic parent SA-1199 (wild-type).[39] In addition NorA knockout strain SA-K1758 (*norA*-deleted) was also used. Cation-adjusted Mueller–Hinton broth (MHB; Becton–Dickinson, Cockeysville, MD, USA) was used for combination studies and tryptone soya agar was used to grow strains for ethidium bromide studies.

4.3.2. Minimum inhibitory concentration and efflux pump inhibitory activity

Minimum inhibitory concentration (MIC) of compounds was determined by microdilution technique according to method described previously according to CLSI guidelines.[40] MIC is defined as concentration at which there is no visible growth. While synergistic activity is described in terms of minimum effective concentration (MEC) which is minimum concentration required to effectively reduce the MIC by fourfold. NorA efflux pump inhibitory activity of compounds was determined using checkerboard microdilution technique by previously known method.[41] Ciprofloxacin (NorA substrate) potentiation

activity of compounds was first determined using SA-1199B, SA-1199 and SA-K1758. Compounds were tested at seven concentrations (50-0.7 µg/mL) in the presence of twofold serial dilution of ciprofloxacin ranging 32-0.06 µg/mL using broth checkerboard microdilution. Piperine and SK-20 served as standard and parent EPIs for reference purpose.

4.3.3 Ethidium bromide efflux and accumulation

Efflux of ethidium bromide from SA-1199B in presence of selected compounds was assessed by fluorescence bases assay describes previously.[42] In brief efflux of ethidium bromide was compared in presence of various concentrations of compounds ranging from 0 – 50 µM over a period of 5 min. Effect of piperine, reserpine and SK-20 was also studied in parallel. Experiments were conducted in triplicate and mean values plotted to calculate percent inhibition in comparison to absence of any inhibitor. In a similar way two compounds 7 and 8 and standard EPIs (Piperine, reserpine and SK-20) were assessed for a total time 30 min (5 min intervals) for the ethidium bromide efflux inhibition.[42] Additionally ethidium bromide accumulation was also studied using ethidium bromide preloaded cells to see the impact of EPI on accumulation efficiency. Fluorescence loss or gain with time was recorded at an interval of 5 min over the total time at an excitation and emission wavelength of 530 and 600 nm respectively using multimode reader Infinite 200 Pro (Tecan Mannedorf, Switzerland).

4.3.4 Evaluation of the cytotoxicity of the compounds

Human HepG2 cell line was used to determine the cytotoxic profile of the compounds using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay in DMEM (GlutaMax, Life technologies, USA) media with high glucose (4.5 g/mL) according to method described previously.[43] Monolayer (1×10^6 cells/well) was exposed to 50 and 100

μM concentrations of the compounds in triplicates and incubated for 24 h (37°C; 5% CO_2). MTT was added at a concentration of 2.5 mg/mL dissolved in DMSO, and cell viability was determined by measuring the absorbance of the reduced formazan at 570 nm. Cytotoxicity is reported as CC_{50}, the concentration that causes a 50% reduction in cell viability. Tamoxifen was used as a positive control at a concentration of 10 and 20 μg/mL and healthy cells as negative control. The percent cytotoxicity of the compounds was calculated according to standard methods using tamoxifen as positive control and healthy cells as a negative control.

4.4 Procedure of molecular modelling

4.4.1 Selection of template and model preparation

The protein sequence of *S. aureus* NorA pump was retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov) and a BLAST search (http://blast.ncbi.nlm.nih.gov) was performed against the Protein Data Bank (PDB) with the default parameters to select best template for building homology model.[44] Model was prepared by Modeller 9.14 software using the glycerol-3-phosphate transporter pump from *Escherichia coli* (PDBID: 1PW4) as a template as it was showing highest similarity and identity with the target protein.[21] Best model was selected on the basis of least dope score energy which was obtained from the modelling experimental results.

4.4.2 Validation of homology model

The homology models of *S. aureus* NorA efflux pump were validated with the results obtained from ERRAT and Ramachandran plots.[42] The root-mean-square deviation (RMSD) calculated between the template and PDB with homology model was checked for the deviation from the original PDB structure for the 3D structure validation.

4.4.3 Docking

Molecular modelling of potent EPIs **7**, **8**, **14** with *S. aureus* NorA efflux pump homology model was carried out using Schrodinger 2015-1 molecular modelling suite. Homology model NorA protein was prepared and minimized by the protein preparation wizard tool. The grid file was constructed using reserpine binding residues to define active site around the residues important for NorA efflux pump inhibition.[22,45] The docking studies were carried out using XP scoring function of Glide as per previously reported.[22] Due to the unavailability of accurate binding site of substrates and inhibitors to the NorA efflux pump, the probable binding sites for the compounds were identified by using site-map module in the Schrodinger software. With the analysis of binding energy, the active site residues and the residues of the side chain which involved in the interaction with the ligand was demonstrated initially to validate the docking protocol.[22,44] The alignments of docked poses were found to be similar to the pose of the ligand reserpine, thus demonstrating the accuracy of the docking.[22] The residues obtained through docking studies were within the distance of 4 Å from the ligand and could be involved in the direct and indirect interaction with the drug target.

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Supporting Information

The ^{1}H NMR, ^{13}C NMR, DEPT135, HRMS spectral data and validation of homology model were provided in the supporting information section.

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Highlights

- Synthesis of amino acid conjugates of 3-(1-chloro-3,4-dihydronaphthalen-2-yl) acrylic acid.
- Screening for NorA inhibitors against *Staphylococcus aureus*
- The potent inhibitors displayed MEC of 1.56 μg/mL against NorA overexpressing strain SA-1199B
- Mechanism for NorA specificity elucidated by ethidium bromide efflux.

Graphical Abstract