Synthesise and Evaluation of Novel Triazoles As Potential Dual Inhibitor of Growth and Efflux Inhibition In Methicillin Resistant Staphylococcus Aureus

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ABSTRACT

Efflux inhibition is proven bacterial machinery responsible for removal of bacterial wastage including antibiotics. Recently, efflux inhibitors (EI) have been tested with encouraging results as an adjuvant therapy for treatment of various bacterial invasions especially methicilline-resistant *Staphylococcus aureus* (*MRSA*). Although, EI have emerged as innovative approach of treatment for several multi drug resistant bacterial infections including tuberculosis, toxicity profile limits their wider use. To address this issue, we have attempted synthesizing hybrid molecules those results by combining known EI and triazole. This synthesis was aimed to arrive at structure that possesses pharmacophore from known EI. Synthesized molecules were evaluated as growth inhibitors (GI) and Efflux inhibitor of *S. aureus*. Pharmacologically active compounds were then tested for their cytotoxicity to further narrow down search. Most active compounds 209, 210, 217 & 228 were then tested for their GEI action against *MRSA*. We arrived at compound 210 as most potent dual inhibitor.

Keywords: *MRSA*, triazole, resistance, PDST210, dual inhibition.

INTRODUCTION

Currently, the global armamentarium of antibacterial drugs is insufficient to address the growing populations of patients with methicilline resistant *S. aureus (MRSA)*.[1] It is also stated that 10% of the world's population is infected with *S. aureus* and 50% of which is expected to develop resistance at some point in their lives if not treated properly.[2]

Above mentioned scenario recommends drug discovery to be synergistic; approach to target newer molecular mechanism and novel NCE which can be combination of pharmacophores of earlier proven entities. Current drug pipeline lacks this innovation[3-5] making "so called" newer NCE vulnerable to mutation based resistance (MBR).

Reports suggests that along with MBR, efflux pump also plays vital role in development of resistance by *MRSA*.[6-8] Intrinsic resistance of *S. aureus* to standard antimicrobial therapies has been attributed primarily to its lipid-rich cell wall composition which limits drug penetration. Efflux pumps, which reduce the passage of antimicrobials across the bacterial cell wall, have been identified as an additional mechanism of resistance. The recent identification of clinical isolates of *MRSA* that have an efflux component as part of their resistance phenotype represents an exciting new field in therapeutics. Along-with detoxification of intracellular metabolites and cellular homeostasis,[9] efflux pumps does contribute for intrinsic and acquired drug resistance in many bacterial pathogens. Usually they confer low-to-intermediate level of resistance; however, the constant pressure of subinhibitory concentrations of the antibiotic promotes the selection of spontaneous mutants.[9] Based on the literature, we state efflux pumps are crucial in a) low level direct drug resistance, b) high level indirect (through effluxing drug from within cell) drug resistance, c) they are effectors of the innate drug-resistance machinery and d) if efflux pumps are inhibited, improvement or restoration of activity of old drug is observed.

Thioridazine,[10-15] chlorpromazine[16,17] and Verapamil[18] have shown efflux pumps as area of opportunity in antibacterial agents but have not evolved toward clinical usage due to potential toxicities. The development of agents with low toxicity profiles that target drug efflux pumps may be clinically useful in reducing the global threat of *MRSA*. Based on recent reports[19-22], we have embarked with present series to identify and evaluate novel compounds for their dual inhibitory action; growth and efflux inhibition (GEI).

MATERIALS AND METHOD

General Instrumentation

The melting points were recorded on electrothermal apparatus and are uncorrected. ^{1}H NMR spectra on a Bruker Avance 500 MHz and ^{13}C NMR spectra on Bruker Avance 300 MHz instrument using DMSO-d_6 as solvent using TMS as internal standard; the chemical shifts (δ) are reported in ppm and coupling constants (*J*) are given in Hertz. Signal multiplicities are represented by s, d, t, ds, dd, m, tt, and br. Mass spectra (m/z) were recorded on an ESI-TOF mass spectrometer (Bruker Micro TOF), and reported mass values are within the error limits of 5 ppm mass units. Elemental analysis was performed on a Heracus CHN-Rapid Analyser. Analysis indicated by the symbols of the elements of functions was within ±0.4% of the theoretical values. The purity of the compounds was checked on silica gel coated Al plates (Merck).

Synthesis of *(3-Bromomethyl-5-mercapto-[1,2,4]triazol-4-yl)-carbamic acid tert-butyl ester* **3.**

To a solution of 4-amino-5-bromomethyl-4*H*-[1,2,4]triazole-3-thiol[12] (15g,   0.071mmol), in dichloromethane (150mL), was added triethylamine (10.87g, 0.107mmol), dropwise, at 0 °C. The reaction mixture was stirred for 10 min. Then, di-tert-butyl dicarbonate (18.77g, 0.086mmol) was added to reaction mixture at 0 °C. After 30 min, reaction mixture was allowed to stir at room temperature overnight. Progress of the reaction was monitored by TLC. After completion of reaction, the reaction mixture was washed with water (50mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford colorless oily product.

(3-Bromomethyl-5-mercapto-[1,2,4]triazol-4-yl)-carbamic acid tert-butyl ester **3.**Yield 78%; mp = 176-181 °C;^{1}H NMR (500 MHz, DMSO-d_6) δ 1.40-1.43 (s, 9H, CH_3), 4.55-4.57 (s, 2H, CH_2Br), 8.30-8.34 (bs, 1H, NH), 12.83 (s, 1H, SH); HRMS [ESI]: calculated for $C_8H_{13}BrN_4O_2S$ [M^+]: 309.183; Found: 309.156.

Synthesis of *(3-Benzyl sulfanyl-5- bromomethyl-[1,2,4]triazol-4-yl)-carbamic acid tert-butyl ester* **4.**

To a solution of (3-bromomethyl-5-mercapto-[1,2,4]triazol-4-yl)-carbamic acid tert-butyl ester 915g, 0.084mmol) in *N,N*-dimethyl formamide (75 mL), was added potassium carbonate (15.4g, 0.111mmol) at room temperature. After stirring for 10 min, Benzyl bromide (9.96g, 0.058mmol) was added to reaction mixture at room temperature. The resulting mixture was allowed to stir at room temperature for next 4 hours. Progress of the reaction was monitored by TLC. After completion of reaction, the contents were poured on to ice-cold water (375 mL) and stirred for 15 min. It was then extracted with ethyl acetate (3X150 mL). Organic layer was washed with water (150ml), dried over anhydrous Na_2SO_4, filtered, concentrated and purified by column chromatography using silica gel, to get yellowish oily product.

(3-Benzyl sulfanyl-5- bromomethyl-[1,2,4]triazol-4-yl)-carbamic acid tert-butyl ester **4.** Yield 69%; mp = 158-163 °C; ^{1}H NMR (500 MHz, DMSO-d$_6$) δ 1.41-1.45 (s, 9H, CH$_3$), 4.21-4.24 (s, 2H, CH$_2$), 4.52-4.54 (s, 2H, CH$_2$Br), 7.08-7.17 (m, 5H, ArH), 8.32-8.35 (bs, 1H, NH); HRMS [ESI]: calculated for C$_{15}$H$_{19}$BrN$_4$O$_2$S [M$^+$]: 399.306; Found: 399.328.

Synthesis of 3-(2,3-dichlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one 7.

2,3-Dichlorobenaldehyde (0.072mmol) was added to a suspension of 1-(2-hydroxyphenyl)ethanone (5.0g, 0.036mmol), in ethanol (50 mL). The mixture was stirred at room temperature overnight. Progress of reaction was monitored by TLC. After completion of reaction, the contents were poured on to ice cold water and acidified with hydrochloric acid (2M) till pH~4. Solid thus obtained was filtered, dried and purified by recrystallization from ethanol to obtain 3-(substituted-phenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one.

3-(2,3-Dichlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one 7. Yield 73%; mp = 122-127 °C; ^{1}H NMR (500 MHz, DMSO-d$_6$) δ 4.92-4.93 (s, 1H, OH), 6.92-6.96 (d, 1H, ArH), 7.02-7.05 (m, 1H, ArH), 7.09-7.12 (d, 1H J=4MHz, ArH), 7.16-7.19 (d, 1H J=4MHz, ArH), 7.24-7.26 (s, 1H, ArH), 7.32-7.37 (d, 1H J=2MHz, CH=CH), 7.41-7.44 (m, 1H, ArH), 7.67-7.69 (d, 1H, ArH), 8.15-8.19 (d, 1H J=2MHz, CH=CH); HRMS [ESI]: calculated for C$_{15}$H$_{10}$Cl$_2$O$_2$[M$^+$]: 293.145; Found: 293.127.

Procedure for synthesis of {3-Benzyl sulfanyl-5-[2-(3-2,3-dichlorophenyl-acryloyl)-phenoxymethyl]-[1,2,4]triazol-4-yl}-carbamic acid tert-butyl ester 8.

To a solution of 3-(2,3-dichlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one **7** (0.013mmol) in *N,N*-dimethyl formamide (20mL) was added potassium carbonate (2.22g, 0.016mmol) at room temperature. After stirring for 10 min, a solution of (3-Benzyl sulfanyl-5- bromomethyl-[1,2,4]triazol-4-yl)-carbamic acid tert-butyl ester **4** in *N,N*-dimethyl formamide (10mL), was added to reaction mixture at room temperature. The resulting mixture was allowed to stir at room temperature overnight. Progress of the reaction was monitored by TLC. After completion of reaction, the contents were poured on to ice cold water, stirred for 15 min. It was then filtered under suction, dried under high vacuum to get off white solid product.

{3-Benzylsulfanyl-5-[2-(3-2,3-dichlorophenyl-acryloyl)-phenoxymethyl]-[1,2,4]triazol-4-yl}-carbamic acid tert-butyl ester 8. Yield 68%; mp = 135-139 °C;^{1}H NMR (500 MHz, DMSO-d$_6$) δ1.42-1.46 (s, 9H, CH$_3$), 4.23-4.25 (s, 2H, CH$_2$), 5.23-5.27 (s, 1H, CH$_2$), 6.91-6.95 (d, 1H, ArH), 7.01-7.06 (m, 6H, ArH), 7.10-7.13 (d, 1H J=4MHz, ArH), 7.17-7.20 (d, 1H J=4MHz, ArH), 7.25-7.27 (s, 1H, ArH), 7.33-7.36 (d, 1H J=2MHz, CH=CH), 7.42-7.44 (m, 1H, ArH), 7.68-7.70 (d, 1H,

ArH), 8.17-8.21 (d, 1H J=2MHz, CH=CH), 8.30-8.35 (br, 1H, NH); HRMS [ESI]: calculated for $C_{30}H_{28}Cl_2N_4O_4S$ [M$^+$]: 611.539; Found: 611.524.

Synthesis of *5-((2-(5-(2,4-dichlorophenyl)-4,5-dihydroisoxazol-3-yl)phenoxy)methyl)-4-amino-1,2,4-triazole-3-thiol* PDST128.

Cyclization:

{3-Benzylsulfanyl-5-[2-(3-2,4-dichlorophenyl-acryloyl)-phenoxymethyl]-[1,2,4]triazol-4-yl}-carbamic acid tert-butyl ester **8** (0.0018moles) and hydroxyamine(0.0036moles) were heated in triethylamine (15mL). As soon as, the solution starts bumping (10-15 min), heating was stopped. The reaction mixture was cooled and poured onto ice-cold water. The product thus obtained was filtered, washed with water and recrystallized from ethanol to give Boc and benzyl protected **PDST128**.

Debenzylation:

One of the Boc and benzyl protected **PDST128** (0.0012moles) was dissolved in Methanol (10 mL) and 10% Pd/C (50%wet) catalyst (2 times) was added into this solution. The resulting mixture was kept in shaker under hydrogen pressure of 80psi for 4 hours. Progress of the reaction was monitored by TLC. After completion of reaction, reaction mixture was filtered through celite under suction, washed with methanol. Filtrate and washings were mixed together and concentrated under reduced pressure, dried under high vacuum to get colourless oily debenzylated but Boc protected **PDST128** product.

Boc deprotection:

One of the Boc protected **PDST128** (0.0009moles) was dissolved in dichloromethane (2.5 mL). This solution was cooled to -10°C. Trifluoro acetic acid (2.5 mL) was added slowly, drop wise to this solution at -10°C over a period of 10 min. The resulting mixture was allowed to stir at -10°C for 1 hour .Progress of the reaction was monitored by TLC. After completion of reaction, the reaction mass was poured onto Petroleum ether (50 mL), stirred for 5 min, decanted. This process was repeated thrice. The thick oily material was then poured onto diethyl ether (50ml), stirred for 15 min, decanted. This process was also repeated thrice. Finally, the solid obtained was dried under high vacuum to give **PDST128**.

Compounds PDST201-PDST232.

Mixture of triazole **PDST128** (0.01 mol) and Na_2CO_3 (1 mol) in ethanol was treated drop-wise with an equimolar amount of the acid/acetyl chloride at 0 °C, which was stirred for 30-45 min. Progress of the reaction was monitored by TLC. After completion of reaction, the reaction mixture was washed with water (50mL), dried over anhydrous Na_2SO_4, filtered and concentrated under

reduced pressure to afford white product. The precipitate was then filtered, washed thoroughly with water and crystallized to yield **PDST201**-**PDST203**.

Compounds PDST204-PDST206.

A solution of triazoles **PDST201**-**PDST203** (0.01 mol), (0.01 mol) of K_2CO_3 and ethyl 2-bromoacetate (0.01 mol) was prepared. The reaction was stirred at 40 °C for 2h. Progress of the reaction was monitored by TLC. After completion of reaction, the reaction mixture was washed with water (50mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford white product. The precipitate was then filtered, washed thoroughly with waterand crystallized to yield **PDST204**-**PDST206**.

Compounds **PDST207-PDST232.**

A solution of triazoles **PDST201**-**PDST203** (0.01 mol), (0.01 mol) of K_2CO_3 and alkyl halide (0.01 mol) was prepared. The reaction was stirred at 40 °C for 4h. Progress of the reaction was monitored by TLC. After completion of reaction, the reaction mixture was washed with water (50mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford white product. The precipitate was then filtered, washed thoroughly with water and crystallized to yield **PDST207**-**PDST232**.

PDST201. Yield 71%; mp = 152-157 °C; White solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.21-2.24 (s, 3H, CH$_3$), 3.92-3.96 (t, 1H, CH), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for $C_{26}H_{22}Cl_2N_6O_2S$ [M$^+$]: 553.46; Found: 553.43.

PDST202. Yield 58%; mp = 141-146 °C; Biege solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 3.92-3.96 (t, 1H, CH), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 7.44-7.47 (m, 2H, ArH), 7.51-7.54 (m, 1H, ArH), 7.86-7.89 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for $C_{31}H_{24}Cl_2N_6O_2S$ [M$^+$]: 615.53; Found: 615.50.

PDST203. Yield 63%; mp = 136-141 °C; White solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 3.92-3.96 (t, 1H, CH), 4.27-4.29 (s, 2H, CH$_2$), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for $C_{26}H_{21}Cl_3N_6O_2S$ [M$^+$]: 587.91; Found: 587.95.

PDST204. Yield 66%; mp = 168-173 °C; White solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.81-185 (d, 1H, CH$_2$), 1.30-1.34 (t, 3H, CH$_3$), 2.01-2.04 (d, 1H, CH$_2$), 2.21-2.24 (s, 3H, CH$_3$), 3.92-3.96 (t, 1H, CH), 3.85-3.88 (s, 2H, CH$_2$), 4.14-4.16 (q, 2H, CH$_2$), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH); HRMS [ESI]: calculated for C$_{30}$H$_{28}$Cl$_2$N$_6$O$_4$S [M$^+$]: 639.55; Found: 639.52.

PDST205. Yield 62%; mp = 159-164 °C; White solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.81-185 (d, 1H, CH$_2$), 1.30-1.34 (t, 3H, CH$_3$), 2.01-2.04 (d, 1H, CH$_2$), 3.92-3.96 (t, 1H, CH), 3.85-3.88 (s, 2H, CH$_2$), 4.14-4.16 (q, 2H, CH$_2$), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 7.44-7.47 (m, 2H, ArH), 7.51-7.54 (m, 1H, ArH), 7.86-7.89 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH); HRMS [ESI]: calculated for C$_{35}$H$_{30}$Cl$_2$N$_6$O$_4$S [M$^+$]: 701.62; Found: 701.66.

PDST206. Yield 67%; mp = 152-157 °C; Buff solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.81-185 (d, 1H, CH$_2$), 1.30-1.34 (t, 3H, CH$_3$), 2.01-2.04 (d, 1H, CH$_2$), 3.92-3.96 (t, 1H, CH), 3.85-3.88 (s, 2H, CH$_2$), 4.14-4.16 (q, 2H, CH$_2$), 4.27-4.29 (s, 2H, CH$_2$), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH); HRMS [ESI]: calculated for C$_{30}$H$_{27}$Cl$_3$N$_6$O$_4$S [M$^+$]: 674.00; Found: 674.04.

PDST207. Yield 55%; mp = 168-173 °C; White solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.21-2.24 (s, 3H, CH$_3$), 2.47-2.49 (s, 3H, CH$_3$), 3.92-3.96 (t, 1H, CH), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for C$_{27}$H$_{24}$Cl$_2$N$_6$O$_2$S [M$^+$]: 567.49; Found: 567.45.

PDST208. Yield 54%; mp = 156-161 °C; White solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.30-1.33 (t, 3H, CH$_3$), 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.21-2.24 (s, 3H, CH$_3$), 3.41-3.45 (s, 2H, COCH$_2$CO), 3.92-3.96 (t, 1H, CH), 4.09-4.12 (s, 2H, CH$_2$), 4.12-4.16 (q, 2H, CH$_2$), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for C$_{32}$H$_{30}$Cl$_2$N$_6$O$_5$S [M$^+$]: 681.59; Found: 681.53.

PDST209. Yield 74%; mp = 148-153 °C; White solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.22-1.25 (t, 3H, CH$_3$), 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.21-2.24 (s, 3H, CH$_3$), 2.93-2.96 (q, 2H, CH$_2$), 3.92-3.96 (t, 1H, CH), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m,

1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for $C_{28}H_{26}Cl_2N_6O_2S$ [M$^+$]: 581.52; Found: 581.54.

PDST210. Yield 69%; mp = 173-178 °C; Biege solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 0.94-0.97 (t, 3H, CH$_3$), 1.73-1.76 (m, 2H, CH$_2$), 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.21-2.24 (s, 3H, CH$_3$), 2.93-2.95 (t, 2H, CH$_2$), 3.92-3.96 (t, 1H, CH), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for $C_{29}H_{28}Cl_2N_6O_2S$ [M$^+$]: 595.54; Found: 595.58.

PDST211. Yield 70%; mp = 177-182 °C; White solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.21-2.24 (s, 3H, CH$_3$), 3.61-3.64 (d, 2H, CH$_2$), 3.92-3.96 (t, 1H, CH), 5.23-5.24 (s, 2H, CH$_2$), 5.03-5.05 (d, 1H, CH=C*H*$_2$), 5.11-5.15 (d, 1H, CH=C*H*$_2$), 5.95-5.98 (m, 1H, C*H*=CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for $C_{29}H_{26}Cl_2N_6O_2S$ [M$^+$]: 593.53; Found: 593.56.

PDST212. Yield 72%; mp = 155-160 °C; White solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.71-1.75 (d, 3H, CH$_3$), 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.21-2.24 (s, 3H, CH$_3$), 3.92-3.96 (t, 1H, CH), 5.23-5.24 (s, 2H, CH$_2$), 5.41-5.44 (m, 1H, CH=C*H*), 6.14-6.16 (d, 1H, C*H*=CH), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for $C_{29}H_{26}Cl_2N_6O_2S$ [M$^+$]: 593.53; Found: 593.51.

PDST213. Yield 76%; mp = 162-167 °C; Buff solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.01-1.05 (d, 6H, CH$_3$), 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.11-2.16 (m, 1H, CH), 2.21-2.24 (s, 3H, CH$_3$), 2.89-2.92 (d, 2H, CH$_2$), 3.92-3.96 (t, 1H, CH), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for $C_{30}H_{30}Cl_2N_6O_2S$ [M$^+$]: 609.57; Found: 609.55.

PDST214. Yield 74%; mp = 146-151 °C; Buff solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 0.94-0.97 (t, 3H, CH$_3$), 1.33-1.36 (m, 2H, CH$_2$), 1.65-1.69 (m, 2H, CH$_2$), 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.21-2.24 (s, 3H, CH$_3$), 2.93-2.96 (t, 2H, CH$_2$), 3.92-3.96 (t, 1H, CH), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for $C_{30}H_{30}Cl_2N_6O_2S$ [M$^+$]: 609.57; Found: 609.54.

PDST215. Yield 75%; mp = 142-147 °C; White solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.21-2.24 (s, 3H, CH$_3$), 3.92-3.96 (t, 1H, CH), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (m, 3H, ArH), 7.16-7.19 (m, 2H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for C$_{32}$H$_{26}$Cl$_2$N$_6$O$_2$S [M$^+$]: 629.56; Found: 629.51.

PDST216. Yield 60%; mp = 152-157 °C; Buff solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.47-2.49 (s, 3H, CH$_3$), 3.92-3.96 (t, 1H, CH), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 7.44-7.47 (m, 4H, ArH), 7.51-7.54 (m, 2H, ArH), 7.86-7.89 (m, 4H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for C$_{32}$H$_{26}$Cl$_2$N$_6$O$_2$S [M$^+$]: 629.56; Found: 629.59.

PDST217. Yield 62%; mp = 154-159 °C; Buff solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.30-1.33 (t, 3H, CH$_3$), 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 3.41-3.45 (s, 2H, COCH$_2$CO), 3.92-3.96 (t, 1H, CH), 4.09-4.12 (s, 2H, CH$_2$), 4.12-4.16 (q, 2H, CH$_2$), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 7.44-7.47 (m, 2H, ArH), 7.51-7.54 (m, 1H, ArH), 7.86-7.89 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for C$_{37}$H$_{32}$Cl$_2$N$_6$O$_5$S [M$^+$]: 743.66; Found: 743.68.

PDST218. Yield 68%; mp = 181-186 °C; White solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.22-1.25 (t, 3H, CH$_3$), 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.93-2.96 (q, 2H, CH$_2$), 3.92-3.96 (t, 1H, CH), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 7.44-7.47 (m, 2H, ArH), 7.51-7.54 (m, 1H, ArH), 7.86-7.89 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for C$_{33}$H$_{28}$Cl$_2$N$_6$O$_2$S [M$^+$]: 643.59; Found: 643.54.

PDST219. Yield 68%; mp = 164-169 °C; White solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 0.94-0.97 (t, 3H, CH$_3$), 1.73-1.76 (m, 2H, CH$_2$), 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.93-2.95 (t, 2H, CH$_2$), 3.92-3.96 (t, 1H, CH), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 7.44-7.47 (m, 2H, ArH), 7.51-7.54 (m, 1H, ArH), 7.86-7.89 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for C$_{34}$H$_{30}$Cl$_2$N$_6$O$_2$S [M$^+$]: 657.61; Found: 657.64.

PDST220. Yield 64%; mp = 188-193 °C; White solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 3.61-3.64 (d, 2H, CH$_2$), 3.92-3.96 (t, 1H, CH), 5.03-5.05 (d, 1H, CH=C*H*$_2$), 5.11-5.15 (d, 1H, CH=C*H*$_2$), 5.23-5.24 (s, 2H, CH$_2$), 5.95-5.98 (m, 1H, C*H*=CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 7.44-7.47 (m, 2H, ArH), 7.51-7.54 (m, 1H, ArH), 7.86-7.89 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for C$_{34}$H$_{28}$Cl$_2$N$_6$O$_2$S [M$^+$]: 655.6; Found: 655.63.

PDST221. Yield 66%; mp = 187-192 °C; Biege solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.71-1.75 (d, 3H, CH$_3$), 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 3.92-3.96 (t, 1H, CH), 5.23-5.24 (s, 2H, CH$_2$), 5.41-5.44 (m, 1H, CH=C*H*), 6.14-6.16 (d, 1H, C*H*=CH), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 7.44-7.47 (m, 2H, ArH), 7.51-7.54 (m, 1H, ArH), 7.86-7.89 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for C$_{34}$H$_{28}$Cl$_2$N$_6$O$_2$S [M$^+$]: 655.6; Found: 655.67.

PDST222. Yield 70%; mp = 169-174 °C; yellowish solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.01-1.05 (d, 6H, CH$_3$), 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.11-2.16 (m, 1H, CH), 2.89-2.92 (d, 2H, CH$_2$), 3.92-3.96 (t, 1H, CH), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 7.44-7.47 (m, 2H, ArH), 7.51-7.54 (m, 1H, ArH), 7.86-7.89 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for C$_{35}$H$_{32}$Cl$_2$N$_6$O$_2$S [M$^+$]: 671.64; Found: 671.66.

PDST223. Yield 63%; mp = 153-158 °C; Biege solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 0.94-0.97 (t, 3H, CH$_3$), 1.33-1.36 (m, 2H, CH$_2$), 1.65-1.69 (m, 2H, CH$_2$), 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.93-2.96 (t, 2H, CH$_2$), 3.92-3.96 (t, 1H, CH), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 7.44-7.47 (m, 2H, ArH), 7.51-7.54 (m, 1H, ArH), 7.86-7.89 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for C$_{35}$H$_{32}$Cl$_2$N$_6$O$_2$S [M$^+$]: 671.64; Found: 671.68.

PDST224. Yield 65%; mp = 161-166 °C; Buff solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 3.92-3.96 (t, 1H, CH), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (m, 3H, ArH), 7.16-7.19 (m, 2H, ArH), 7.22-7.26 (m, 2H, ArH), 7.44-7.47 (m, 2H, ArH), 7.51-

7.54 (m, 1H, ArH), 7.86-7.89 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for $C_{37}H_{28}Cl_2N_6O_2S$ [M$^+$]: 691.63; Found: 691.67.

PDST225. Yield 63%; mp = 163-168 °C; buff solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.47-2.49 (s, 3H, CH$_3$), 3.92-3.96 (t, 1H, CH), 4.27-4.29 (s, 2H, CH$_2$Cl), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for $C_{27}H_{23}Cl_3N_6O_2S$ [M$^+$]: 601.93; Found: 601.98.

PDST226. Yield 64%; mp = 167-172 °C; White solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.30-1.33 (t, 3H, CH$_3$), 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 3.41-3.45 (s, 2H, COCH$_2$CO), 3.92-3.96 (t, 1H, CH), 4.09-4.12 (s, 2H, CH$_2$), 4.12-4.16 (q, 2H, CH$_2$), 4.27-4.29 (s, 2H, CH$_2$Cl), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for $C_{32}H_{29}Cl_3N_6O_5S$ [M$^+$]: 716.03; Found: 716.06.

PDST227. Yield 67%; mp = 150-155 °C; White solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.22-1.25 (t, 3H, CH$_3$), 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.93-2.96 (q, 2H, CH$_2$), 3.92-3.96 (t, 1H, CH), 4.27-4.29 (s, 2H, CH$_2$Cl), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for $C_{28}H_{25}Cl_3N_6O_2S$ [M$^+$]: 615.96; Found: 615.92.

PDST228. Yield 68%; mp = 149-154 °C; White crystals; ^{1}H NMR (500 MHz, CDCl$_3$) δ 0.94-0.97 (t, 3H, CH$_3$), 1.73-1.76 (m, 2H, CH$_2$), 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.93-2.95 (t, 2H, CH$_2$), 3.92-3.96 (t, 1H, CH), 4.27-4.29 (s, 2H, CH$_2$Cl), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for $C_{29}H_{27}Cl_3N_6O_2S$ [M$^+$]: 629.99; Found: 629.94.

PDST229. Yield 58%; mp = 177-182 °C; Biege solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 3.61-3.64 (d, 2H, CH$_2$), 3.92-3.96 (t, 1H, CH), 4.27-4.29 (s, 2H, CH$_2$Cl), 5.03-5.05 (d, 1H, CH=C*H*$_2$), 5.11-5.15 (d, 1H, CH=C*H*$_2$), 5.23-5.24 (s, 2H, CH$_2$), 5.95-5.98 (m, 1H, C*H*=CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for $C_{29}H_{25}Cl_3N_6O_2S$ [M$^+$]: 627.97; Found: 627.93.

PDST230. Yield 75%; mp = 168-173 °C; White solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.71-1.75 (d, 3H, CH$_3$), 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 3.92-3.96 (t, 1H, CH), 4.27-4.29 (s, 2H, CH$_2$Cl), 5.23-5.24 (s, 2H, CH$_2$), 5.41-5.44 (m, 1H, CH=C*H*), 6.14-6.16 (d, 1H, C*H*=CH), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for C$_{29}$H$_{25}$Cl$_3$N$_6$O$_2$S [M$^+$]: 627.97; Found: 627.94.

PDST231. Yield 69%; mp = 194-199 °C; Buff solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.01-1.05 (d, 6H, CH$_3$), 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.11-2.16 (m, 1H, CH), 2.89-2.92 (d, 2H, CH$_2$), 3.92-3.96 (t, 1H, CH), 4.27-4.29 (s, 2H, CH$_2$Cl), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for C$_{30}$H$_{29}$Cl$_3$N$_6$O$_2$S [M$^+$]: 644.01; Found: 644.00.

PDST232. Yield 59%; mp = 212-217 °C; White solid; ^{1}H NMR (500 MHz, CDCl$_3$) δ 0.94-0.97 (t, 3H, CH$_3$), 1.33-1.36 (m, 2H, CH$_2$), 1.65-1.69 (m, 2H, CH$_2$), 1.81-185 (d, 1H, CH$_2$), 2.01-2.04 (d, 1H, CH$_2$), 2.93-2.96 (t, 2H, CH$_2$), 3.92-3.96 (t, 1H, CH), 4.27-4.29 (s, 2H, CH$_2$Cl), 5.23-5.24 (s, 2H, CH$_2$), 6.43-6.47 (m, 2H, ArH), 6.54-6.58 (m, 1H, ArH), 6.80-6.84 (m, 2H, ArH), 7.01-7.03 (m, 3H, ArH), 7.11-7.15 (d, 1H, ArH), 7.22-7.26 (m, 2H, ArH), 8.22-8.29 (br, 1H, NH), 12.5 (s, 1H, SH); HRMS [ESI]: calculated for C$_{30}$H$_{29}$Cl$_3$N$_6$O$_2$S [M$^+$]: 644.01; Found: 644.06.

Pharmacology

Strains, growth conditions and reagents.

Two bacterial strains differed in virulence were used primarily in this study: *S. aureus* ATCC700081 and Methicillin-resistant *S. aureus* ATCC27292T. *S. aureus* strains were maintained in Middlebrook broth medium. Bacteria- specific Middlebrook 7H9 and 7H10 media were obtained from Difco (Detroit, MI, USA) supplemented with 0.2% glycerol, 0.05% Tween 80 and 10% ADS supplement. Thioridazine (TZ), verapamil (VP), isoniazid (INH), rifampicin (RIF), amikacin (AMK), ofloxacin (OFX), EtBr, phosphate-buffered saline (PBS), and glucose were purchased from Sigma-Aldrich (India). All solutions were prepared in deionized water, except rifampicin and the PDST201-232, which were prepared in DMSO. All solutions were prepared on the day of the experiment

Determination of Minimum Inhibitory Concentration.

For *S. aureus* the determination of the MICs of synthesized compounds, the efflux inhibitors TZ and VP, and the efflux substrate ethidium bromide were conducted by the 96-well broth microdilution method as described earlier.

Evaluation of efflux inhibitory activity of compounds by real-time fluorometry.

The EtBr accumulation and efflux by the bacterial strains was assessed on a real-time basis using a fluorometric method, as previously described *S. aureus* and methicillin-resistant *S. aureus* were grown as described above, with the addition of 0.05% Tween 80 to the growth medium.

Cytotoxicity screening

In vitro cytotoxicity assays were evaluated against human monocyte-derived macrophages as described earlier.

RESULTS AND DISCUSSION

Chemistry

Chemical class of triazole is cost effective nucleus which has proven its commercial viability. Thus by undertaking present series, we can state to have a cost effective drug candidate. The synthetic route was followed as reported (Figure 2).[23]

As detailed literature discussing SAR on efflux inhibitors as methicillin-resistant *S. aureus* is lacking, we initiated our synthesis based on Ligand-based Drug Design approach. Two of most studied efflux inhibitors were identified for drug designing, Thioridazine (TZ) and Verapamil (VP) wherein TZ is also explored in depth for its enhancer activity along-with other anti- methicillin-resistant *S. aureus* agents.[24,25] Phenothiazine ring is crucial in conferring neuroleptic activity for TZ while efflux systems are known to affine to basic amino group. Given these premises, we embarked at a structure which reflects parts of these two pharmacophores with couple of variations. The triazole core is well known privileged nucleus in recent drug discovery as it's a nifty heterocycle with a wide range of activities like antibacterial, antifungal, antiviral and also antimycobacterial. Many reports are available confirming fused or linked triazole emerging as novel antitubercular agent.[26-29] Most of the studies have also suggested amino and mercapto position's availability for extensive variation for modulation of activity. Thus combination of hybrid design linked with triazole was finalized to arrive at dual inhibitors. (figure 1)
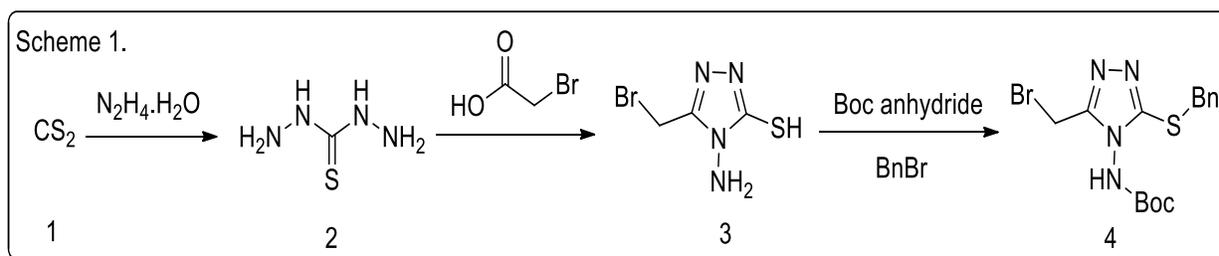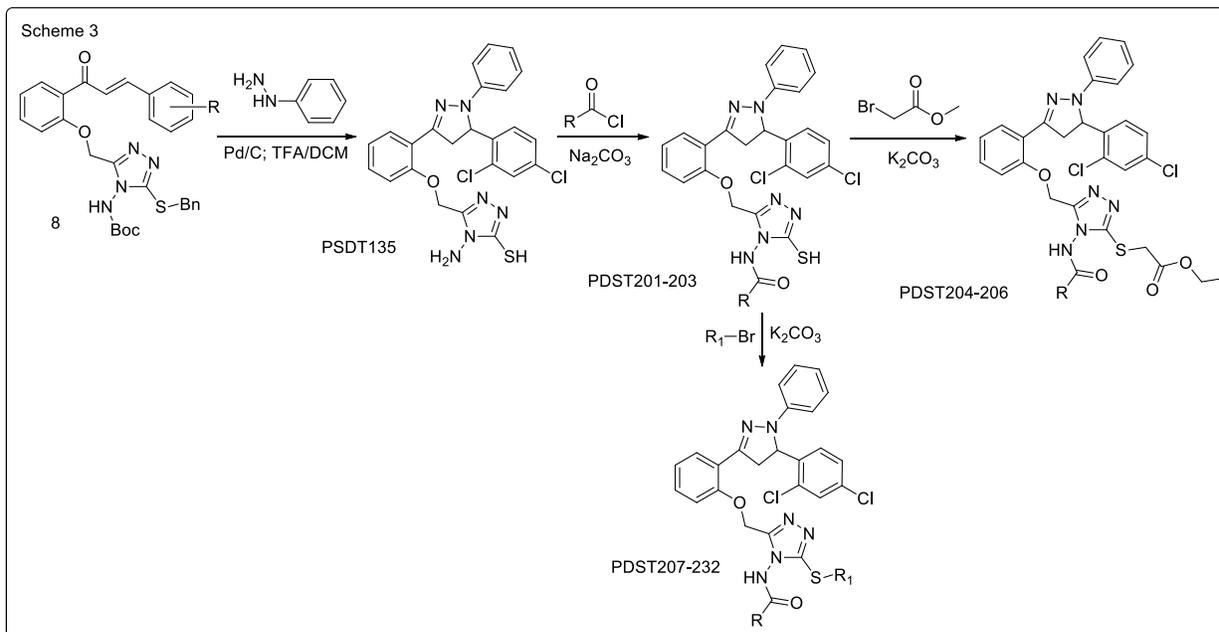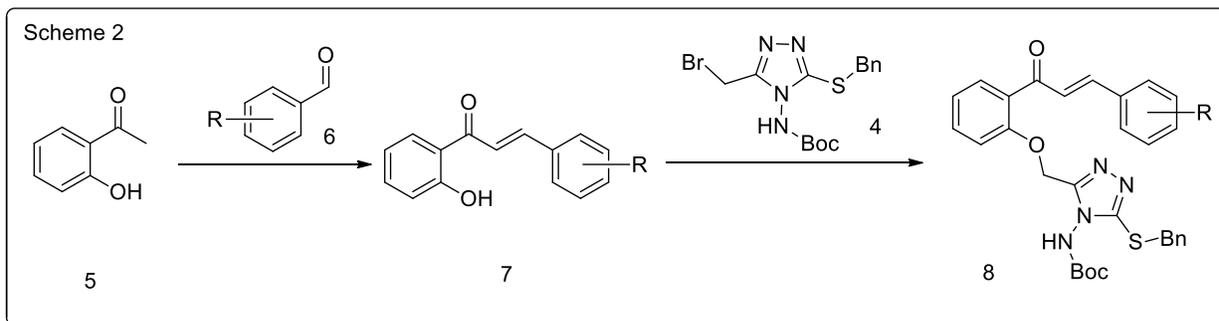
Scheme 1.

Scheme 1: Route of synthesis for the compounds PDST201-232.

Target structure we have designed was combination of 3 precursors viz., protected triazole (4), a chalcone (7) and heterocycle. In brief, initially hydrazide (1) was synthesized by reacting carbon disulfide with hydrazine hydrates. Bromo acetic acid was then treated with hydrazine (2) to produce 4-amino-3-bromomethyl-5-mercapto-(1,2,4)-triazole (3). This conversion did not yield products for several time at the addition was tricky. After several trial and error we identified root cause is the way and speed of addition of reactants. Slow, drop wise addition of bromo acetic acid over 30 mins to hydrazine provided compound 3. Amino and mercapto groups which served as reactive terminals were then protected by Boc anhydride and benzyl bromide respectively to furnish protected triazole (4). Second precursor, chalcone (7) was prepared by reacting 2-hydroxy acetophenone (5) with 2,4-dichlorobenzaldehyde (6). These triazolyl-chalocones (8) were then cyclized using hydroxylamine and further deprotected at amino and thio function by Pd/C and then TFA/DCM to furnish compound PDST128. This compound has shown good inhibition against

primary screening. This compound is under further screening at our lab and currently out of scope of this manuscript. Meanwhile, it was thought worthwhile to develop SAR related to PDST128.

In attempt to do so, we first reacted free amino group and converted to substituted amide (201-203). On observing promising activity of amide compounds, it was further decided to alter reactive thiol group. As initial transformation, esterification was performed to produce PDST204-206. As these compounds shown detrimental effects on inhibitory action, program for ester linkage was scrapped and instead chain elongation strategy was considered. Starting substitutions with smallest linkage, methyl group we extended to ethyl, propyl and n-butyl which have shown betterment of activity. We also synthesized compounds having *a*. appendage with double bond, *b*. branching instead of elongation and *c*. ring insertion but were shown diminishing activity. Thus to develop a concluding SAR for this series, we synthesized PDST207-232 and were evaluated. Most of the conversion were smooth except mentioned ones and gave products with high purity.

Pharmacology

An urgent need for effective alternative methicillin-resistant *S. aureus* treatment, especially for the severe destructive and disseminated forms of *S. aureus* initiated attempts to design and develop compounds with inhibitory activity. Current manuscript reveals part 3 of series wherein a hybrid molecule is coupled with triazole that are aimed as potent and rationally designed GEI (growth and efflux inhibitor). Total of 32 compounds were synthesized in this experiment and all underwent inhibitory assay. All the compounds were initially tested against *S. aureus* reference strain to achieve faster and cheaper results. Molecules, which have shown inhibitory activity better than TZ were further tested for cytotoxicity. Compounds with better inhibition and lesser cytotoxicity were then assessed on methicillin-resistant *S. aureus* and further for synergistic profile with first and second line antibacterial agents.

Preliminary inhibitory assessment

Growth inhibition of *S. aureus*.

As mentioned earlier, we have observed promising dual inhibitory activity of PDST128 from our in-house cluster of compounds. To further explore SAR of this compound, we initiated current synthetic scheme. As our strategy, we initiated exploring the structure-activity relationship of the **PDST** series to determine its potential for progression as a drug candidate. Therefore, we designed, synthesized and tested a number of analogs in this process. In systematic modification, we initially applied amidation at amino group represented by R, while the second variation was esterification and then chain elongation at thiol group which was represented by R_1.

Evaluation of SAR initiated with examining effect of amidation at amino group of traizole. To begin with, three segments of varied length and bulk were tried examples are methyl, phenyl and chloromethyl carbonyl groups. This was undertaken to define the structure activity relationships influencing potency of carbon length and bulkiness of group. Striking with good inhibition, all three compounds suggested amino substitution was allowed but none of them was as active as parent compound PDST128. Thus it was thought to test substitution of thiol group keeping amino group intact. This attempt showed complete loss of activity, suggesting, thiol alone cannot be substituted. The results are not included here and considered to be out of scope of this manuscript.

Introduction of ester at the mercapto is well known to increase anomeric stability, modulate ring pucker and binding affinity as well as enhance pharmacokinetic properties.[30, 31] Thus it was thought worthwhile to synthesize molecule with ester linkage (204-206) but to our disappointment, this substitution led to diminishing activity. Diminished activity of 204-206 suggests steric clash between the ester appendages and a putative protein responsible for uptake or association.

Next set of compounds synthesized was to check whether the chain variation at mercapto can be substituted with improvement in activity. Strategy to design this series was based on overall 2D and 3D-analysis of chemical structures of these derivatives that showed electron-donating groups induced an enhancement in the activity. Especially smaller alkyl groups were observed to be critical for better inhibition. Attempt to test derivative with critical alkyl chain length was encouraging, showing good inhibition. Besides, substitution with ethyl acetoacetate, branching and phenyl group seems to decrease antibacterial activity.

Out of three series of nine compounds, one with methyl have shown better activity while with phenyl group shown lesser than methyl and series with chloromethyl carbonyl have shown least inhibition. Thus we could state entities with modification with methyl substitutions have shown better inhibition than TZ and can be critical.

To further comment on structural consequences, we state compound 207-215 with link alteration have shown two most promising analogs. Compound 210 is exceptionally potent inhibiting growth as well as efflux compared to TZ which is 5 fold better. The structural modification here stated now have prompted requirement of methyl group attachment to phenyl ring as favored substitution. Due to the reduced solubility and the low antibacterial activity, it was not possible to determine the exact MIC values for some compounds such as 201, 221, 223, 226 & 232.

Efflux inhibition

After determining their MICs the compounds were tested for their capability to inhibit efflux of EtBr from *S. aureus* by real-time fluorometry, using TZ and VP as controls (Table 1, relative final

fluorescence (RFF) values). According to a widely used protocol for this kind of assay, not to compromise the cellular viability, all of the compounds were tested at ½ of their MIC. For those compounds for which the exact MIC could not be determined (above reported), the last concentration value that could be technically determined was considered ½ of the MIC. Although SAR was developed with limited compounds, still we can concise our results on SAR of efflux inhibitory effect. From these initial SAR studies based on 5 series, we determined that the key substituents for activity enhancement are: a acetamido group at the amino position and a two or three carbon chain at the thiol position of the triazole ring. We used this knowledge to further identify their effect on methicillin-resistant *S. aureus* and then to observe synergistic effect, if any.

Cytotoxicity

Compounds 205, 207, 208, 209, 210, 211, 216, 218, 219, 225, 227 and 228 were emerged as most active compounds of the series. These were evaluated against human-monocyte derived macrophages to assess their ex vivo cytotoxicity toward eukaryotic cells (Table 1).

We here noticed that, with some of the compounds i.e., 205, 207, 208, 211, 216, 219, 225 and 227 have been proved to be toxic when compared with TZ. Compounds 218 and 228, (IC_{50} 1.21 ± 0.05 µg/mL and 1.77 ± 0.42 µg/mL, respectively) are 2-3-fold less cytotoxic than TZ ($IC_{50} = 5.5$ µg/mL), whereas compounds 209 & 210 ($IC_{50} = 16.22 \pm 0.51$ µg/mL and 119.03 ± 0.34 µg/mL) were found to be safe towards human macrophages. Overall, these preliminary data clearly suggest that the replacement of the phenothiazine core with other ring systems allows maintaining the efflux inhibitory activity with the possibility to decrease the cytotoxicity. Hence, compounds 209, 210, 218 & 228 that have shown better inhibition and less cytotoxicity than TZ were considered to have better inhibitory profile against methicillin-resistant *S. aureus* strain.

Table 1: Anti-bacterial and efflux inhibitory activity of PDST201-232 against *S. aureus* and their toxicity index towards human monocyte derived macrophages.

ID	R	R_1	*S. aureus* MIC (µg/mL)[a]	REF ± SD[b]	IC_{50}[c] µg/mL(µM)
128	--		32	0.98 ± 0.078*	1.7
201	CH_3		>512[d]	0.12 ± 0.016	ND[e]
202	C_6H_5		128	0.30 ± 0.027	1.5
203	$COCH_2Cl$		64	2.18 ± 0.16**	1.4
204	CH_3		256	0.17 ± 0.007	ND
205	C_6H_5		16	1.06 ± 0.074*	1.1
206	$COCH_2Cl$		256	0.33 ± 0.08	ND
207	CH_3	$-CH_3$	16	1.23 ± 0.56	6.3
208	CH_3	$-CH_2COCH_2CO_2Et$	8	3.26 ± 0.06	5.8
209	CH_3	$-C_2H_5$	2	16.22 ± 0.51**	87.6

210	CH$_3$	-C$_3$H$_7$	1	119.03 ± 0.34***	129.4
211	CH$_3$	-CH$_2$CH=CH	8	0.98 ± 0.32*	2.6
212	CH$_3$	-CH=CH-CH$_3$	32	0.20 ± 0.04	ND
213	CH$_3$	-CH$_2$-CH(CH$_3$)-CH$_3$	128	0.11 ± 0.07	ND
214	CH$_3$	-(CH$_2$)$_3$-CH$_3$	128	0.13 ± 0.03*	2.6
215	CH$_3$	-C$_6$H$_5$	128	0.14 ± 0.04	ND
216	C$_6$H$_5$	-CH$_3$	16	0.41 ± 0.05	1.5
217	C$_6$H$_5$	-CH$_2$COCH$_2$CO$_2$Et	256	0.16 ± 0.03*	ND
218	C$_6$H$_5$	-C$_2$H$_5$	8	1.21 ± 0.05	1.9
219	C$_6$H$_5$	-C$_3$H$_7$	4	5.13 ± 0.29*	18.6
220	C$_6$H$_5$	-CH$_2$CH=CH	128	0.32 ± 0.05	ND
221	C$_6$H$_5$	-CH=CH-CH$_3$	128[d]	0.23 ± 0.06	ND
222	C$_6$H$_5$	-CH$_2$-CH(CH$_3$)-CH$_3$	512	0.21 ± 0.07	ND
223	C$_6$H$_5$	-(CH$_2$)$_3$-CH$_3$	>512[d]	0.12 ± 0.09	2.1
224	C$_6$H$_5$	-C$_6$H$_5$	256	0.15 ± 0.04	ND
225	COCH$_2$Cl	-CH$_3$	16	0.37 ± 0.06	2.2
226	COCH$_2$Cl	-CH$_2$COCH$_2$CO$_2$Et	>256[d]	0.10 ± 0.02	ND
227	COCH$_2$Cl	-C$_2$H$_5$	16	0.39 ± 0.03	3.1
228	COCH$_2$Cl	-C$_3$H$_7$	8	1.77 ± 0.42*	29.7
229	COCH$_2$Cl	-CH$_2$CH=CH	64	0.19 ± 0.08	ND
230	COCH$_2$Cl	-CH=CH-CH$_3$	128	0.25 ± 0.01	ND
231	COCH$_2$Cl	-CH$_2$-CH(CH$_3$)-CH$_3$	256	0.26 ± 0.06*	ND
232	COCH$_2$Cl	-(CH$_2$)$_3$-CH$_3$	>128[d]	0.19 ± 0.04	2.1
TZ			31	0.88 ± 0.1*	5.5
VP			776	2.20 ± 0.12**	51.33

[a]Determined by microdilution; [b]Relative final fluorescence based on accumulation of EtBr at 0.25 µg/mL; the results are presented as the average of three independent assays plus standard deviation (± SD). The results were considered significant when *P<0.05 and highly significant when **P<0.01 and ***P<0.001; [c]Index of Cytotoxicity (IC) determined in human monocyte derived macrophages; [d]Due to the reduced solubility of the compounds it was not possible to test at higher concentrations; [e]not determined.

Table 2: Screening of anti-bacterial and efflux inhibitory activity of selected compounds against *methicilline resistant S. aureus*.

ID	*methicilline resistant S. aureus*	
	MIC (µg/mL)[a]	REF ± SD[b]
209	32	0.30 ± 0.02
218	16	2.09 ± 0.08**
228	64	0.21 ± 0.01
210	8	8.26 ± 0.03***
TZ	16	0.25 ± 0.04
VP	128	1.98 ± 0.03**

[a]Determined by microdilution; [b]Relative final fluorescence (RFF) based on accumulation of EtBr at 0.5 µg/mL; the results are presented as the average of three independent assays plus standard

deviation (± SD). The results were considered significant when *P<0.05 and highly significant when **P<0.01 and ***P<0.001; ᶜDue to the reduced solubility of the compounds it was not possible to test at higher concentrations.

CONCLUSION

Although there are reports on efflux inhibitors role in controlling drug resistance, not much of efforts have been made by medicinal chemists. Marketed or recently identified efflux inhibitors (e.g. TZ) poses threat of side effect and toxicity limiting their use as adjuvant or dual inhibitor. This is first report aimed at synthesizing hybrid molecule of TZ, VP and triazole and their evaluation as dual inhibitors. Hit compound 210 have found to show very less toxicity compared to TZ towards human macrophages (16 folds). This compound also displayed higher growth inhibition in both *S. aureus* & methicillin-resistant *S. aureus*. Compound 210 have demonstrated its synergistic effect with first line and second line antitubercular agents. Considering the data on inhibition of EtBr efflux, coupled to the antibacterial activity, it might be claimed that the synergetic activity is due to its ability to hamper the intrinsic bacterial drug efflux. Altogether, these findings provide a solid base to further investigate compound 210 as a booster of the antibacterial chemotherapy when associated with first- and second-line drugs, by virtue of its capability to block the intrinsic efflux activity of bacteria. This might represent a lateral approach toward the cure of another lethal disease, i.e. TB.

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