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PAPER

Design, Synthesis and Biological Evaluation of Novel Unsymmetrical Azines as Quorum Sensing Inhibitors

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Abstract: Targeting Quorum sensing signals using Quorum Sensing inhibitors has opened new avenues for the application of known antibiotics. In this context, twenty five unsymmetrical azines were synthesised and evaluated as quorum sensing inhibitors. An efficient one pot procedure was adopted that directly link 3-Methyl-2-(methylthio)benzo[d]thiazol-3-ium salt, hydrazine hydrate and substituted aldehyde to give the designed compounds. The synthesized compounds were preliminarily tested for their potential to inhibit CviR receptor based QS signals in *Chromobacterium violaceum*. The bioassay screening results suggested that two compounds exhibited potent QS inhibition activity against CviR receptor showing violacein inhibition (>50%) at 200 µM. Further, the putative positive hits were checked for their potential to inhibit LasR receptor based QS using *PlasB-gfp*(ASV) biomonitor strain of *Pseudomonas aeruginosa*. These compounds were found to inhibit the QS mediated GFP signals in a dose dependant manner. Two active compounds were also exhibited biofilm clearance at 50 µM concentration. Docking studies were performed to examine their potential to bind to LasR protein of *Pseudomonas aeruginosa*.

Introduction

Mortality rate due to bacterial infection is increasing annually making it a major area of focus for the researchers.[1] In this direction, many antibacterial compounds have been studied but in most cases, the bacteria develop resistance either due to modification of the single amino acid at the target site or due to increased activity of enzymes that are capable of degrading antimicrobial compounds[2] or due to biofilm formation.[3]

Gram-negative bacteria communicate with each other by releasing chemical signals termed as autoinducers and this process of cell to cell communication is known as 'quorum sensing'.[4] This communication occurs by releasing various chemical signals such as *N*-acyl-*L*-homoserine lactones (AHLs) to monitor and coordinate their genome expression in a cell density dependent manner.[5,6,7] These AHLs consist of homoserine lactone rings attached via amide bonds to alkyl/acyl chains containing 4 to 18 carbons (Figure 1). The invading pathogen has to reach a critical cell population density sufficient to overcome the host defence for the development of infection. As the cell density increases, concentration of these signal molecules increases and upon reaching a threshold level (when the population is "quorate"), the population activates a cellular response.[8] Quorum sensing controls the expression of virulence factors which includes the secretion of extracellular proteases, pectinase, biosurfactant as well as the formation of biofilm.[9] The frequent occurrence of the resistant pathogenic strains gradually decrease the effectiveness of the traditional antibiotic treatment hence there is an urgent need to develop new class of molecules to target quorum sensing.

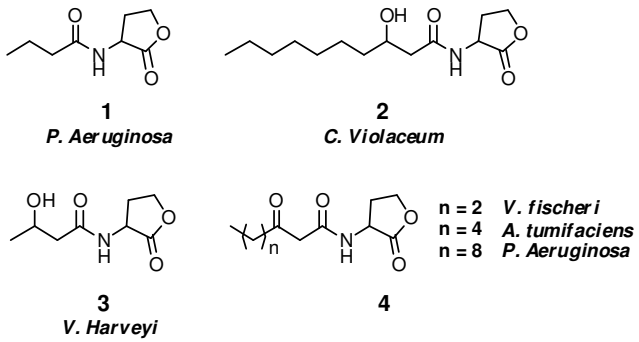

Figure 1: N-acyl-L-homoserine lactones (AHLs) from various Gram negative bacteria.

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Pseudomonas aeruginosa is a ubiquitous gram negative bacterium that is responsible for many opportunistic, nosocomial[10] and chronic infections. They are the major cause of infection in cystic fibrosis patients,[11] AIDS patient,[12] burn victims[13] and neutropenic cancer patients.[14] *Pseudomonas aeruginosa* has three main QS systems: the first two systems are LasR-LasI[15,16] and RhlR-RhlI[17,18] which use Acyl Homoserine Lactones (AHLs) as signal molecules. Both systems together regulate virulence factors. The third QS system, known as PQS (Pseudomonas Quinolone Signal) which consist of PqsR as virulence regulator and uses another kind of signalling molecule, 2-heptyl-3-hydroxy-4-quinolone (HHQ).[19–22]

Many AHL analogues were reported as QS inhibitors, most of them belong to lactone, thiolactone and furanone class (Figure 2).[23–26] These AHL analogues have inherent limitation as QS inhibitors due to (i) rapid degradation in the presence of mammalian lactonases[27,28] (ii) high specificity towards their cognate receptors, which make these compounds as autoinducers[29] (iii) breakdown products of AHL analogues can be toxic, etc.[30] Recently, already known drugs *viz.* salicylic acid (**13**), nifuroxazide (**14**) and chlorzoxazone (**15**) have been identified as *Pseudomonas aeruginosa* quorum-sensing inhibitors (LasR) through structure based virtual screening approach.[31] Hartman *et al*. have reported PqsR antagonist (**16a** and **16b**)[32] which inhibit the third QS system i.e. PqsR in *Pseudomonas aeruginosa* (Figure 3).

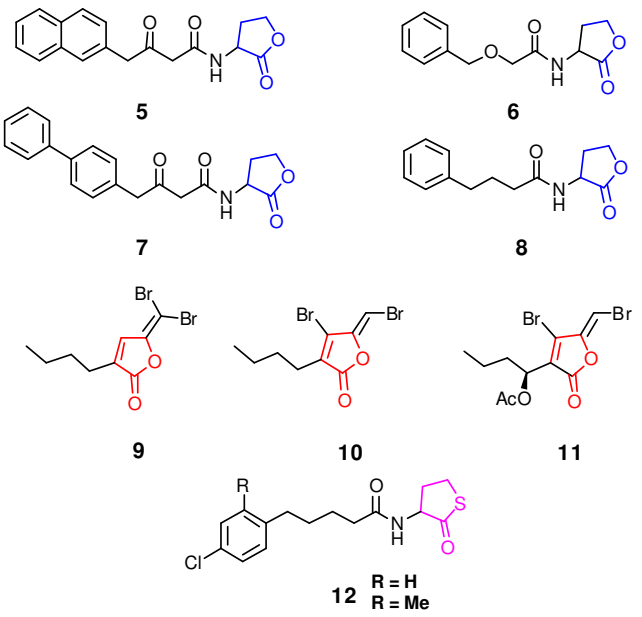


Figure 2: Quorum-sensing inhibitors derived from Acylated Homoserine Lactones (AHL). **5-8** belongs to lactone class, **9-11** belongs to furanone class and **12** belong to thiolactone class.

Inspired from nifuroxazide (**14**) and considering the limitations of analogues of AHL, we have designed unsymmetrical azines (**17a-y**) as structurally unrelated class of QS inhibitors (Figure 3 and Figure 4). Azines are a functional class of organic compounds with the general formula R^1R^2C=N-N=CR^3R^4. The unsymmetrical azines contain different substituents on the two sides of the C=N-N=C frame. Azine derivatives have been studied for antibacterial,[33] antifungal,[34] antifilarial,[35] anticancer,[36] opiate antagonist[37] and molluscacidal[38] activities. Thus, the azine class of compounds can be considered as safe for the development of drug candidate. However, its other derivatives such as hydrazine, azo and hydrazide were reported in literature as a reactive functional group and thus should be avoided during drug discovery.[39] Apart from biological activity, tautomerism in azine is also reported.[40] Majority of the azines reported in literature are symmetric ones, which can be obtained by dimerization. In this report, the focus is on the synthesis of unsymmetrical azines which can not follow a dimerization approach. Azines **17a-y** were prepared by simple and efficient methods (method A and method B) in moderate to good yield. These azines were screened against two gram-negative strains viz. *Chromobacterium violaceum* and *Pseudomonas aeruginosa*. All compounds were initially tested for their potential to inhibit CviR receptor based QS in *Chromobacterium violaceum*. The bioassay screening results suggested that two compounds viz **17j** and **17n** exhibit moderate to good QS inhibition activity against CviR receptor. These hits were found to have potential to inhibit LasR receptor based QS mediated GFP production in dose dependant manner. Compounds **17j** and **17n** significantly resulted in biofilm clearance. Finally, docking analysis was carried out to understand the binding mode and the interactions within the active site of LasR protein.

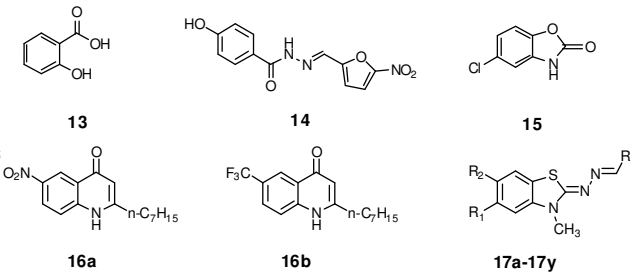


Figure 3: Salicylic acid (**13**), Nifuroxazide (**14**), Chlorzoxazone (**15**) as *Pseudomonas aeruginosa* quorum sensing inhibitors; PqsR antagonists (**16a** and **16b**) and designed azines **17a-y**.

Results and discussion

Designing of unsymmetrical azines as LasR antagonist

From the knowledge of the interactions of natural bound ligand (OdDHL) to AHL binding domain in LasR, pharmacophore model was developed by Taboureau *et al*[41] for OdDHL. Five point pharmacophore model suggested that lactone ring constitute aromatic box, amide linkage constitute polar box and the carbon chain constitute hydrophobic box (Figure 4). This model suggests that in order to interact with the AHL binding domain, ligand

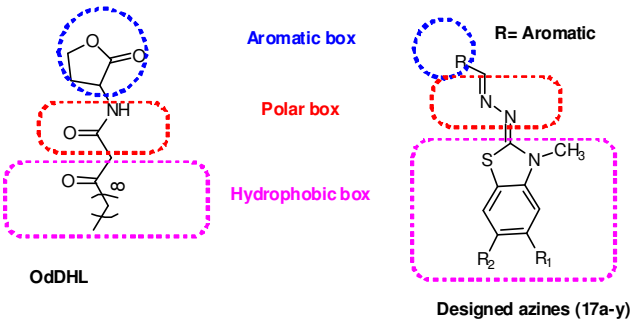
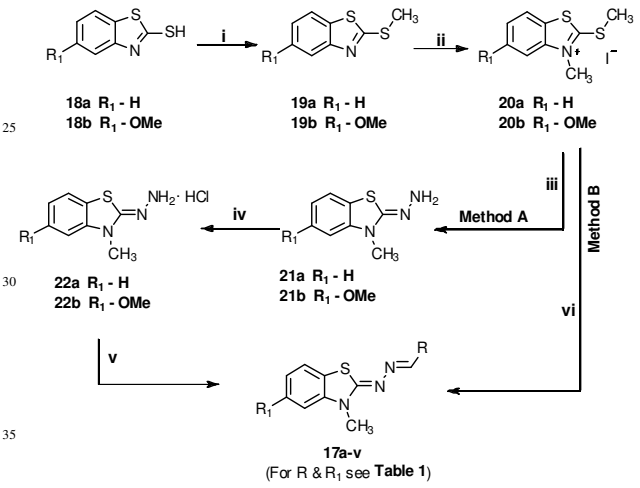


Figure 4: Designing strategy for unsymmetrical azines (**17a-y**).

must possess aromatic box and hydrophobic box linked by polar region. Thus, while designing novel QS inhibitors targeting LasR we proposed unsymmetrical azines which contain benzothiazole nucleus and aryl moiety linked by azine as a spacer (Figure 4). We hypothesized that the benzothiazole nucleus may form hydrophobic region, azine may represent the polar region and aryl moiety may form aromatic box and may show favourable interactions with the amino acids in AHL binding domain. To verify the hypothesis, docking studies of designed azines were carried out and compared with the OdDHL. Docking studies showed that the benzothiazole nucleus occupy hydrophobic region, where as azines spacer is important for hydrogen bond interactions with the amino acids in AHL binding domain. Docking studies revealed that the designed azines showed comparable interactions and docking score with that of OdDHL.

Chemistry

We have carried out the synthesis of **17a-y** by two different methods, method A and method B (Scheme 1 and Scheme 2) respectively. Azines 1**7a-v** were synthesized by adopting scheme 1.

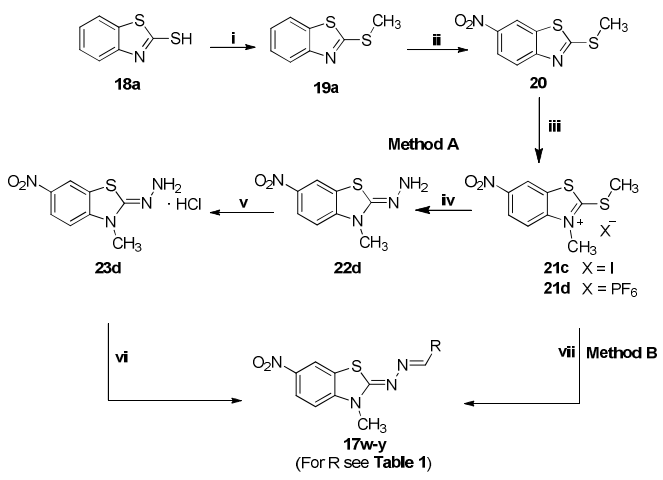


Scheme 1: Reaction and conditions: (i) CH_3I, K_2CO_3, DMF, rt, 30 min, 80% (ii) CH_3I, 50-60 °C, 12 h, 65% (iii) $NH_2NH_2.H_2O$, TEA, MeCN, rt, 2 h iv) MeOH, 1N HCl, 50 °C, 30 min, 90% (v) RCHO, Ethanol, reflux, 10-12 h, 65-95% (vi) **20**, $NH_2NH_2.H_2O$, DMF, rt, 3 h → RCHO, Ethanol, reflux, 12 h (60-80%).

Synthesis starts with readily available 2-mercaptobenzothiazole (**18a**)/5-methoxy-2mercaptobenzothiazole (**18b**), which on treatment with methyl iodide, get convert to **19a**/**19b**.[42] Compound **19a** and **19b** were converted to their iodide salts **20a** and **20b** in moderate yield (~65%) by heating with methyl iodide for 24 h.[43,44] Compound **20** is a common intermediate for the synthesis of azines **17a-v** using method A and method B. Intermediates **20a** and **20b** were reacted with hydrazine hydrate in the presence of triethylamine as a base to afford hydrazone **21a** and **21b**.[45] Since these hydrazones were degrading during work up and hence were converted to their hydrochloride salts (**22a** and **22b**), which upon coupling with substituted aldehydes gave desired azines **17a-v**.[46]

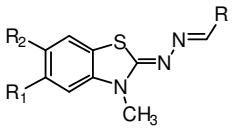
Unsymmetrical azines **17w-y** have the nitro substituent at R^2 and were prepared by scheme 2. The synthetic scheme starts with the nitration of **19a** by using H_2SO_4/HNO_3 as a nitrating mixture to give **20**.[47,48] Methylation of **20** with methyl iodide afforded compound **21c** with poor yield (< 20%), thus methylation of **20** was carried out by changing the methylating agent to dimethyl sulphate. Compound **20** was heated with dimethyl sulphate and the product was precipitated by potassium hexafluorophosphate[45] to get 80% of **21d**. Finally, compounds **17w-y** were prepared by same methods A and B given in Scheme 2.

The hydrazone intermediate (**21a**, **21b** and **22d**) in method A suffers from several drawbacks such as cumbersome work-up procedure, hydrolytic instability and thus difficulty in isolation. To circumvent this, we developed method B, in which salt (**20a/20b/21d**) was treated with hydrazine hydrate in DMF at room temperature for 3 h. This leads to the *in situ* generation of hydrazone. After 3 h, substituted aldehydes and ethanol were added to the reaction mixture and allowed to reflux for 6-12 h to get desired azines **17a-17y** (Table 1). The reaction mixture was allowed to cool and then filtered to get crude product. The desired products were purified by silica gel column chromatography and obtained in moderate to good yield. The synthesized compounds were characterized by means of ^1H NMR, ^{13}C NMR and HRMS (+ESI).



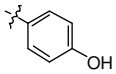
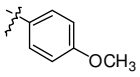
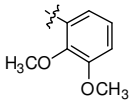
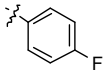
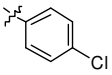
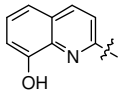
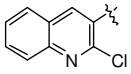
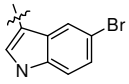
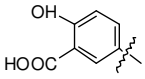
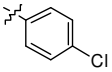
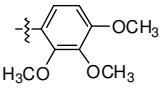
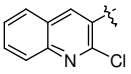
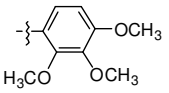
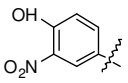
Scheme 2*:* Reaction and conditions: (i) CH_3I, K_2CO_3 DMF, rt, 30 min, 80% (ii) HNO_3, H_2SO_4, (0-10 °C), 75% (iiia) CH_3I, 50 °C, 12 h (iiib) $(CH_3)_2SO_4$, 180 °C, 90 min; H_2O, KPF_6, rt, 5min (iv) $NH_2NH_2.H_2O$, TEA, MeCN, rt, 3 h, 62% (iv) MeOH, 1N HCl, 50 °C, 30 min, 90% (v) RCHO, Ethanol, reflux, 10-12 h, 60-70% vii) **21d**, $NH_2NH_2.H_2O$, DMF, rt, 2 h → RCHO, Ethanol, reflux, 12 h (60-75%).

Table 1: Unsymmetrical Azines **17 a**-**y** and CviR inhibition at 200 µM concentration.



17a-y

Entry	R_1	R_2	R	% Inhibition
17a	H	H		41.6 ± 0.05

17b	H	H		27.6 ± 0.06
17c	H	H		44.7 ± 0.02
17d	H	H		25.3 ± 0.06
17e	H	H		18.4 ± 0.09
17f	H	H		23.6 ±0.03
17g	H	H		NA
17h	H	H		15.1 ± 0.07
17i	H	H		NA
17j	H	H		57.4 ± 0.04
17k	H	H		15.2 ± 0.02
17l	H	H		NA
17m	H	H		4.2 ± 0.05
17n	H	H		60.8 ± 0.02
17o	H	H		NA
17p	H	H		20.1 ± 0.008
17q	OCH_3	H		NA
17r	OCH_3	H		5.9 ± 0.01
17s	OCH_3	H		7.7 ± 0.02
17t	OCH_3	H		NA
17u	OCH_3	H		6.1 ± 0.01
17v	OCH_3	H		NA
17w	H	NO_2		15.7 ± 0.04
17x	H	NO_2		21.9 ± 0.04
17y	H	NO_2		40.0 ± 0.04
4-NPO				43.73 ± 0.03

Biology

QS inhibition activity against CviR receptor

Chromobacterium violaceum is a gram-negative bacterium that produces the purple pigment violacein in response to the presence of the AHL N-hexanoyl homoserine lactone (C6-HSL). The production of violacein is regulated by acyl HSL-mediated QS. Thus, inhibition of HSL-mediated QS system leads to a decrease in the violacein production.[49] In order to see the effect of synthesised azines **17a-y** on HSL-mediated QS system, they were preliminarily checked against CviR receptor based QS in *Chromobacterium violaceum*. The bioassay screening results (Table 1) suggested that the compounds **17j** and **17n** exhibit QS inhibition against CviR receptor. They have shown more than 50% inhibition of purple pigment violacein production by *Chromobacterium violaceum* (CV12472) (Figure 5). A known QS inhibitor, 4-nitropyridine-N-oxide (4-NPO) was also included at 200 µM as the positive control. The inhibitory effect of DMSO control (0.2%) was examined in similar fashion. The two active compounds (**17j** and **17n**) do not possess any substitution on benzene ring of benzothiazole. Substitution at C5/C6 of benzothiazole with an electron donating group (OMe) or electron withdrawing group (NO_2) decreases the percentage inhibition. It means, benzothiazole nucleus cannot tolerate either electron donating (OMe) or withdrawing (NO_2) group at C5/C6 position. On the other hand, hydrophobic groups (OMe, F, Cl) on the benzene ring (**17d-g**) leads to a decrease in activity. When R is heterocyclic nucleus, different patterns of inhibition were observed. The inhibition >50% was observed for **17j** and **17n** which contain chloroquinoline and imidazole nucleus as R group respectively.

Effect of 17j and 17n on QS mediated GFP production in *PlasB-gfp*(ASV)

The two azines, **17j** and **17n** were assayed against *Pseudomonas aeruginosa* MH602 lasB reporter strain *PlasB-gfp*(ASV) following the protocol described by Givskov *et al*. with fewer modifications. AHL signals production by this reporter strain leads to an increase in unstable green fluorescent protein production (GFP) as a function of QS system. Compounds that

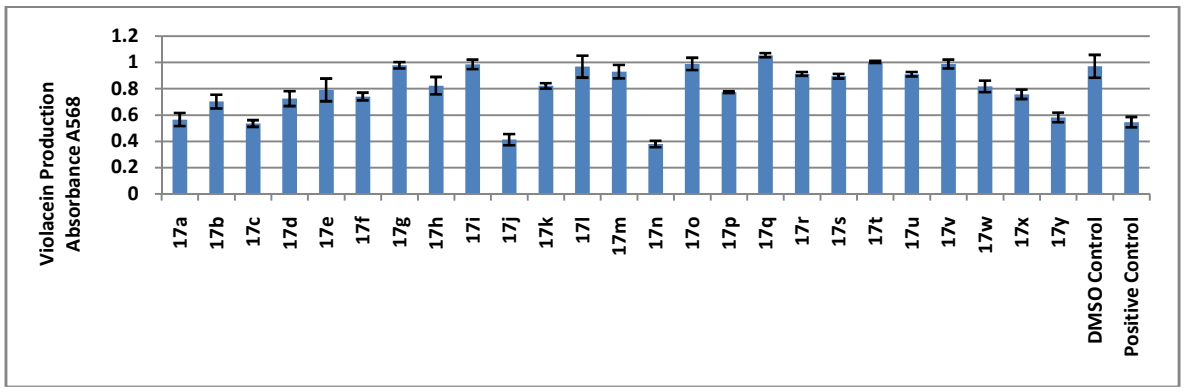


Figure 5: Effect of synthesized Unsymmetrical Azines (**17a**-**17y**) were tested on violacein production by *Chromobacterium violaceum* CV12472 in liquid bioassay. Violacein was extracted as described and quantified by absorbance measurements at 568 nm. Compounds were used at 200 µM concentrations. DMSO-treated cultures were used as negative control (0.2%). A known QSI, 4-NPO (+ve control) also included in this experiment. On comparing the extent of violacein production as compared to the control condition, it was found that compounds **17j** and **17n** have shown good activity.

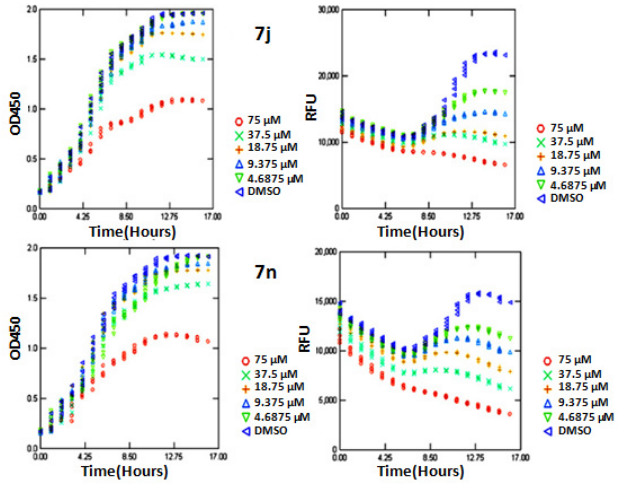


Figure 6: QS inhibition assay of compounds **17j** and **17n** for QS mediated GFP production in *Pseudomonas aeruginosa* MH602 lasB reporter strain (*PlasB-gfp*(ASV)). (A) OD as a function of time. (B) RFU (Relative Fluorescence Units) as a function of time. These compounds reduce the RFU level (GFP fluorescence units divided by OD450) at various concentrations (right), indicating inhibition of LasR, while displaying no effect on growth (left). Growth is displayed as increase in OD450 compared to the initial level. Results are representative of three independent experiments. The final concentration of the DMSO in the experiments is 0.2% and an equivalent concentration was used as negative control.

inhibits bacterial QS systems reduce the expression of GFP. The dose dependant effect of the compounds was checked in the concentration range 5.0-75 µM as shown in Figure 6. Compounds **17j** and **17n** resulted in reduction in GFP production as compared to DMSO control condition without significant effect on the growth of the organism.

Antibiofilm activity

QS system in *Pseudomonas aeruginosa* plays a central role to regulate virulence and biofilm formation. Biofilm make the organism resistant to all known antimicrobial agents making the pseudomonal infections complicated and life threatening. To elucidate whether the active azines inhibit QS mediated biofilm formation or not, we checked the effect of **17j** and **17n** on *Pseudomonas aeruginosa* MH602 lasB reporter strain *PlasB-gfp*(ASV). For this study, biofilms were developed on microscopic cover slips. Three days old biofilms on analysis have shown that the two compounds **17j** and **17n** have significantly reduced the biofilms formation as compared to the DMSO control condition where fully matured biofilms were formed (Figure 7).

Molecular docking analysis

Molecular docking studies have been carried out in order to understand the nature of interactions between the unsymmetrical azines (**17j** and **17n**) and QS receptor. The LasR structure

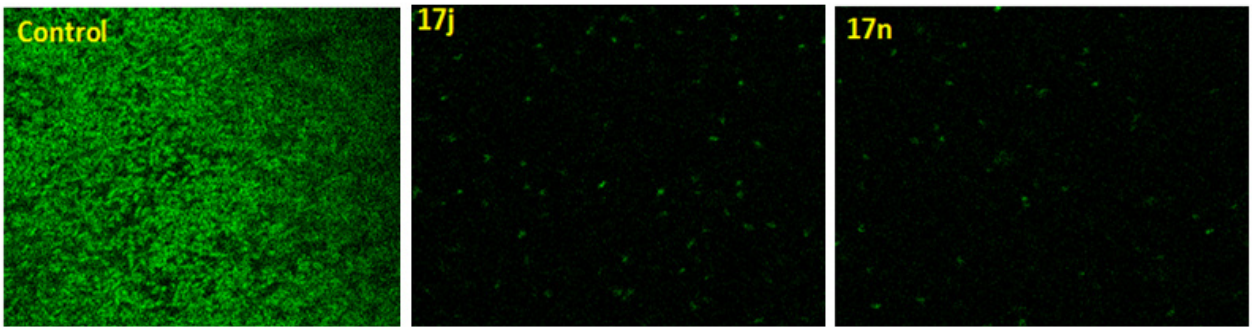


Figure 7: Inhibition of OdDHL-mediated signalling in *Pseudomonas aeruginosa* biofilm carrying *plasB-gfp*(ASV). a) Three days old mature biofilm in the presence of equivalent DMSO condition b) Inhibition of biofilm in the presence of compound **17n** at 50 µM c) biofilm inhibition by compound **17j** at 50 µM concentrations. The final concentration of the DMSO in the experiments is 0.2% and an equivalent concentration was used as negative control. Biofilms were visualized at 60X using Nikon Confocal Microscopes and images were analyzed using NIS-elements software.

contains four monomers of the ligand binding domain, each complex with one OdDHL ligand. The active binding site is conserved between the β-sheet and the external α-helical face made up of α3, α4 and α5 where natural ligand OdDHL binds. The docking results suggested that the hydrogen bonding interaction and hydrophobic interactions to the amino acid residues Ser 129, Tyr 56, Trp 60 and Tyr 47 plays an important role in binding to the OdDHL binding site. The imine nitrogen (C=N) of both the azines (**17j** and **17n**) are showing hydrogen bonding with the Ser 129 (1.4-2.4 Å) and the benzothiazole nucleus is occupying hydrophobic region by forming π-π stacking with Tyr 56 and Trp 60 (Figure 8). The docking pose of **17j** is perfectly overlapping with the natural ligand, OdDHL (Figure 8). Although, these azines are not analogues of OdDHL but they bear pharmacophoric features present in OdDHL. For example, benzothiazole nucleus in active azines overlaps with the hydrophobic acyl chain in OdDHL and thus forming hydrophobic interactions same as OdDHL. The azine spacer -C=N-N=C- overlap with the -CONH constituting polar region and aryl moeity overlap with the lactone ring of OdDHL constituting aromatic box and binds to the same region where lactone ring of OdDHL binds. Thus, these azines very well fit in the active site and hence inhibited QS system.

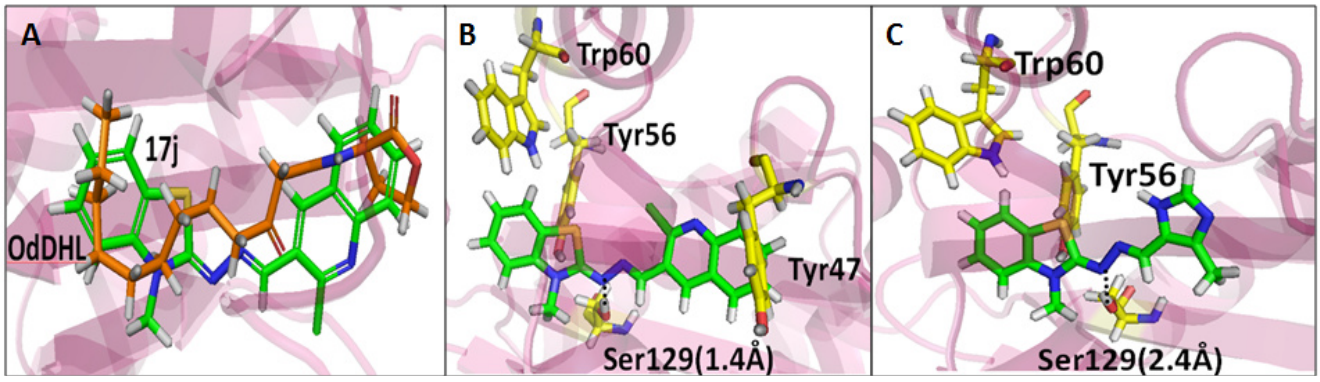


Figure 8: Docking pose of active benzothiazole-hydrazone azines **17j** and **17n** in *Pseudomonas aeruginosa* LasR ligand binding domain. A) Represents the overlapping of natural ligand OdDHL (orange) with the **17j** (green). B, C) Represents binding of **17j** and **17n** (green) with the LasR ligand binding domain respectively. The black dotted line (**.....**) the represents hydrogen bond interaction with the amino acid (yellow) in the vicinity of active site.

Conclusions

Twenty five unsymmetrical azines (**17a-y**) were synthesised, biologically evaluated and docked into LasR receptor to study the binding interactions such as hydrogen bonding, hydrophobic interactions. Here, we have described an efficient one pot synthesis of azines (**17a-y**) by coupling 3-methyl-2-(methylthio)benzo[d]thiazol-3-ium (**20a/20b/21d**), hydrazine hydrate and substituted aldehyde. Initially biological assays (QS inhibition) were performed using *Chromobacterium violaceum* CV12472 strain. Two azines (**17j** and **17n**) showed (>50%) QS inhibitory activity at 200 µM concentration. These active azines were further found to inhibit LasR receptor based QS that has been checked using *plasB-gfp*(ASV) based bioassay. This study is also supported by the results of the molecular docking study, which shows that these azines binds to ligand binding domain of LasR protein. Azines **17j** and **17n** have shown significant biofilm inhibition for *Pseudomonas aeruginosa* at final concentration of 50 µM. Thus, these azines can be consider as hits for the development of the potential quorum sensing inhibitors which could be used in conjugation of known antibiotics to curb the pathogenesis of *Pseudomonas aeruginosa.*

Experimental

Chemistry

The reagents and chemicals required for the study were procured from Sigma-Aldrich (St. Louis, MO, USA) and Alfa Aesar (Johnson Matthey Company, Ward Hill,, MA, USA). All the reagents were used without further purification unless otherwise mentioned. The progress of the reaction was monitored by Thin Layer Chromatography (TLC) performed on silica gel aluminium plates and visualization was done by UV light. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance DX spectrometer at 400 MHz respectively, with TMS as an internal standard. The ^{1}H NMR and ^{13}C NMR was recorded for $CDCl_3$ at 7.26 ppm and 77.00 ppm respectively. The ^{1}H NMR and ^{13}C NMR was recorded for DMSO-d_6 at 2.50 ppm and 39.51 ppm respectively. Chemical shift (δ) are reported in part per million (ppm). Coupling constants (J) were reported in hertz (Hz). The abbreviations used to characterize the signals are as follows: s = singlet, m = multiplet, d = doublet, dd = doublet of doublet, dt = doublet of triplet, t = triplet, q = quartet. Mass spectra were measured with High Resolution Mass Spectra (HRMS). Melting points were determined with an Electro thermal melting point apparatus.

2-(methylthio)benzo[d]thiazole (19a)

2-Mercaptobenzthiazole (5.00 g, 29.94 mmol) (**18a**) was dissolved in DMF (2 mL) and to the reaction mixture K_2CO_3 (0.5 g, 119.76 mmol) was added. The reaction mixture was allowed to stir for few minutes then CH_3I (1.77 mmol) was added dropwise at lower temperature (0-10 °C). The reaction mixture was stirred at room temperature for 1 h. The completion of reaction was monitored by TLC. The reaction solvent was removed by rotary evaporation and the product was diluted with water and extracted with ethyl acetate (3×70 mL).The organic layer was concentrated to give yellow liquid as a desired product (4.30 g, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ2.77 (s, 3H, SC**H**$_3$), 7.27 (t, J = 8 Hz, 1H,

Ar**H**), 7.41 (t, *J* = 8 Hz, 1H, Ar**H**), 7.72 (d, *J* = 8 Hz, 1H, Ar**H**), 7.88 (d, *J* = 8 Hz, 1H, Ar**H**); ^{13}C NMR (100 MHz, $CDCl_3$): δ 15.95, 120.65, 121.38, 124.10, 126.08, 135.16, 153.37, 168.10; MALDI *m/z* calculated for $C_8H_7NS_2$ 181.002, Found: 182.05 $[M+H]^+$.

5-Methoxy-2-(methylthio)benzo[d]thiazole (19b)

5-Methoxybenzo[d]thiazole-2-thiol (**18b**) (5.00 g, 25.30 mmol) was dissolved in DMF (10 mL) and to the mixture K_2CO_3 (14.01g, 101.52 mmol) was added. The reaction mixture was allowed to stir for few minutes then CH_3I (10.70 g, 75.90 mmol) was added dropwise at lower temperature. The reaction mixture was stirred at room temperature for 30 min (TLC). The solvent was removed by rotary evaporation and the product was diluted with water and extracted with ethyl acetate (3×70 mL). The organic layer was filtered through bed of Na_2SO_4 and concentrated to afford brown crystalline solid as a desired product (5.08 g, 95%).^{1}H NMR (400 MHz, $CDCl_3$) δ2.80 (s, 3H, -SC**H**$_3$), 3.87 (s, 3H, -OC**H**$_3$), 6.95 (dd, *J* = 11.2 Hz, 2.4 Hz, 1H, Ar**H**), 7.41 (d, *J* = 2.4 Hz 1H, Ar**H**), 7.60 (d, *J* = 8.7 Hz, 1H, Ar**H**); ^{13}C NMR (100, MHz, $CDCl_3$) δ 15.98, 55.62, 104.50, 11 3.78, 121.14, 126.78, 154.62, 158.96, 169.27; MALDI *m/z* calculated for $C_9H_9NOS_2$ 211.0126, Found 212.022 $[M+H]^+$

3-Methyl-2-(methylthio)benzo[d]thiazol-3-ium iodide (20a)

Thioether (**19a**) (4.30 g, 23.70 mmol) was heated with CH_3I (13.39 g, 95.02 mmol) at 50-60 °C for 12 h. After completion of reaction the yellow salt was washed with diethyl ether to remove unreacted thioether (4.97 g, 65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ3.16 (s, 3H, -SC**H**$_3$), 4.18 (s, 3H, -NC**H**$_3$), 7.75 (t, *J* = 8 Hz, 1H, Ar**H**), 7.87 (t, *J* = 8 Hz, 1H, Ar**H**), 8.11 (d, *J* = 8 Hz, 1H, Ar**H**), 8.26 (d, *J* = 8 Hz, 1H, Ar**H**); ^{13}C NMR (100 MHz, $CDCl_3$) δ17.24, 35.61, 115.11, 123.30, 127.10, 128.51, 129.38, 142.79, 181.85; MALDI *m/z* calculated for $C_9H_{10}NS_2$ 196.0255, Found 196.085 $[M]^+$.

5-Methoxy-3-methyl-2-(methylthio)benzo[d]thiazol-3-ium iodide (20b):

Thioether (**19b**) (5.08 g, 24.07 mmol) was heated with CH_3I (13.57 g, 96.28 mmol) at 50-60 °C for 12 h. After completion of reaction the yellow salt was washed with diethyl ether to remove unreacted thioether. The product obtained is a yellow solid (5.36 g, 63%). ^{1}H NMR (400 MHz, DMSO-d_6) δ3.09 (s, 3H, -SC**H**$_3$), 3.95 (s, 3H, -NC**H**$_3$), 4.09 (s, 3H, -OC**H**$_3$), 7.34 (dd, *J* = 11.36 Hz, 2.3 Hz, 1H, Ar**H**), 7.71 (d, *J* = 2.32 Hz, 1H, Ar**H**), 8.22 (d, *J* = 9 Hz, 1H, Ar**H**), ^{13}C NMR (400 MHz, DMSO-d_6 δ 18.49, 37.00, 56.93, 99.97, 116.68, 120.44, 125.95, 144.52, 16 1.06, 181.65; MALDI *m/z* calculated for $C_{10}H_{12}NOS_2$ 226.036, Found 226.07 $[M]^+$.

2-(methylthio)-6-nitrobenzo[*d*]thiazole (20): Compound **19a** (5.0 g, 22.22 mmol) was dissolved in 5 mL of H_2SO_4 and a mixture of H_2SO_4 (1.09 g, 11.22 mmol) and HNO_3 (0.55 g, 8.88 mmol) was slowly added while cooling the reaction mixture at 0 °C. The solution was stirred in the cold for 1 h The solution is then poured on to ice, the precipitate formed was filtered, washed with water and aqueous ammonia solution to get crude yellow solid. The crude compound was purified by column chromatography over silica gel using 5% ethyl acetate-hexane as eluent to give yellow solid (4.66 g, 75%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.85 (s, 3H, -SC**H**$_3$), 7.91 (d, *J* = 8.9 Hz, 1H, Ar**H**), 8.31 (dd, *J* = 11.2 Hz, 2.3 Hz, 1H, Ar**H**), 8.69 (d, *J* = 2.28 Hz, 1H, Ar**H**); ^{13}C NMR (100 MHz, $CDCl_3$) δ16.0, 117.41, 121.19, 121.95, 135.57, 144.03, 157.10, 175.11; MALDI *m/z* calculated for $C_8H_6N_2O_2S_2$ 225.987, Found 225.950 $[M+H]^+$

(*Z*)-2-Hydrazono-3-methyl-2,3-dihydrobenzo[*d*]thiazole (21a):

Salt (**20a**) (4.97 g, 15.4 mmol) was dissolved in mixture of acetonitrile and to the reaction mixture solution of triethylamine (3.11 g, 30.86 mmol) in acetonitrile was added followed by solution of hydrazine hydrate (7.7 g, 154 mmol) in acetonitrile. The reaction mixture was stirred for 2 h. The completion of reaction was monitored by TLC. The solvent was removed by rotary evaporation and the product was diluted with water and extracted with ethyl acetate (3×70 mL). The organic layer was filtered through bed of Na_2SO_4 and concentrated to afford solid as a crude product. The crude compound was purified by column chromatography over silica gel using 10% ethyl acetate-hexane as eluent to yield white crystalline solid (1.96 g, 72%). ^{1}H NMR (400 MHz, $CDCl_3$) δ3.51 (s, 3H, -NC**H**$_3$), 6.96 (d, *J* = 8 Hz, 1H, Ar**H**), 7.02 (t, *J* = 8 Hz, 1H, ArH), 7.25 (t, *J* = 8 Hz, 1H, ArH), 7.39 (d, *J* = 8 Hz, 1H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ31.95, 108.60, 121.04, 122.13, 124.62, 125.91, 141.31, 160.62, 163.87; MALDI m/*z* calculated for $C_8H_9N_3S$ 179.051, Found 179.079 $[M]^+$.

(*Z*)-2-Hydrazono-5-methoxy-3-methyl-2, 3-dihydrobenzo [*d*]thiazole (21b): Salt (**20b**) (5.08 g, 14.3 mmol) was dissolved in acetonitrile and to the reaction mixture, triethylamine (2.89 g, 28.7 mmol) was added followed by solution of hydrazine hydrate (7.15 g, 143.0 mmol) in acetonitrile. The reaction mixture was stirred for 2 h. The completion of reaction was monitored by TLC. The product was diluted with water and extracted with ethyl acetate (3×70 mL) and then washed with 1N HCl three times. The crude compound was purified by column chromatography over silica gel using 5% ethyl acetate-hexane as eluent to yield white crystalline solid (1.94 g, 65%).

3-Methyl-2-(methylthio)-6-nitrobenzo[d]thiazol-3-ium hexafluorophosphate (21d):

Compound **20** (4.66 g, 20.77 mmol) was treated with Me_2SO_4 (13.04 g, 103.5 mmol). The mixture was heated at 180 °C for 90 min and then cooled to room temperature. Water (20 mL) and KPF_6 (19.04 g, 103.5 mmol) were added leading to the precipitation of a brown solid, which was separated by filtration, washed successively three times with water (10 mL) and three times with EtOAc (5 mL), and then dried to obtained cream colour solid (6.76 g , 85%); ^{1}H NMR (400 MHz, MeOD) δ3.22 (s, 3H, -SC**H**$_3$), 4.24 (s, 3H, -NC**H**$_3$), 8.29 (d, *J* = 9.2 Hz, 1H, Ar**H**), 8.70 (d, *J* = 11.52 Hz, 1H, Ar**H**), 9.21 (d, *J* = 2.2 Hz, 1H, Ar**H**), ^{13}C NMR (100 MHz, MeOD 17.42, 36.05, 116.04, 119.73, 124.42, 129.3, 146.13; MALDI *m/z* calculated for $C_9H_9N_2O_2S_2$ 241.010; Found 241.094 $[M]^+$.

2-Hydrazono-3-methyl-2,3-dihydrobenzo[d]thiazole hydrochloride (22a):

Hydrazone **21a** (1.96 g, 9.11 mmol) was dissolved in methanol and conc. HCl (2 mL) was added to the reaction mixture. The reaction mixture was heated at 50 °C for 1 h. The cooling of reaction mixture leads to the formation of yellow precipitates. The precipitates were filtered to obtained yellow solid **22a** (2.11 g, 90%); ^{1}H NMR (400 MHz, MeOD) δ3.53 (s, 3H, -NC**H**$_3$), 3.85 (brs, 2H, -N**H**$_2$), 7.20-7.24 (m, 1H, Ar**H**), 7.43-7.46 (m, 2H,

Ar**H**), 7.81 (d, *J* = 8 Hz, 1H, Ar**H**);^{13}C NMR (100 MHz, MeOD) δ 31.22, 111.19, 121.32, 122.84,127.32, 140.16; MALDI *m/z* calculated for $C_8H_9N_3S$ 179.051; Found 179.07 $[M]^+$.

***(Z)*-2-Hydrazono-5-methoxy-3-methyl-2,3-dihydrobenzo [*d*]thiazole hydrochloride (22b)**: Hydrazone **21b** (1.94 g, 7.91 mmol) was dissolved in methanol and 2 mL conc. HCl was added to the reaction mixture. The reaction mixture was heated at 50 °C for 1 h. The solvent was removed by rotary evaporation to yield yellow crystalline solid **22b** (2.04 g, 90%). ^{1}H NMR (400 MHz, MeOD) δ3.55 (s, 3H, -NC**H**$_3$), 3.83 (s, 3H, -OC**H**$_3$), 6.83 (d, J = 8 Hz, 1H, Ar**H**), 7.03 (s,1H, Ar**H**), 7.69 (d, *J* = 8 Hz, 1H, Ar**H**), ^{13}C NMR (100 MHz, MeOD) δ 39.34, 56.29, 98.08, 123.99, 141. 94, 159.97; MALDI *m/z* calculated for $C_9H_{11}N_3OS$ 209.062 Found 210.098 $[M+H]^+$.

(*Z*)-2-Hydrazono-3-methyl-6-nitro-2,3-dihydrobenzo[d] thiazole (22d): Salt (**21d**) (6.76 g, 17.52 mmol) was dissolved in acetonitrile. To the reaction mixture, solution of triethylamine (3.54 g, 35.05 mmol) in acetonitrile was added followed by solution of hydrazine hydrate (8.76 g, 175.2 mmol) in acetonitrile. The reaction mixture was stirred for 2 h. The completion of reaction was monitored by TLC. The solvent was removed by rotary evaporation and the product was diluted with water and extracted with ethyl acetate (3×70 mL). The organic layer was filtered through bed of Na_2SO_4 and concentrated to afford solid as a crude product. The crude compound was purified by column chromatography over silica gel using 15% ethyl acetate-hexane as eluent to yield yellow crystalline solid (1.96 g, 50%).

(*Z*)-2-Hydrazono-3-methyl-6-nitro-2,3-dihydrobenzo[d] thiazole hydrochloride (23d)

Hydrazone (**22d**) (1.96 g, 8.77 mmol) was dissolved in methanol and 2 mL conc. HCl was added to the mixture. The mixture was heated at 50 °C for 1 h. The solvent was removed from reaction mixture to yield yellow crystalline solid (1.97 g, 87%) ^{1}H NMR (400 MHz, MeOD) δ3.67 (s, 3H, -NC**H**$_3$), 7.61-7.63 (d, *J* = 8 Hz, 1H, Ar**H**), 8.42-8.44 (d, *J* = 8 Hz, 1H, Ar**H**), 8.81 (s, 1H, Ar**H**); ^{13}C NMR (100 MHz, MeOD δ 32.70, 112.44, 120.48, 124.28, 125.28, 144.60, 146.92; MALDI *m/z* calculated for $C_8H_8N_4O_2S$ 224.036 Found 225.440 $[M+H]^+$.

General procedure for synthesis of compounds (17a-y) by method B:

Salt (**20a/20b/21d**) was treated with hydrazine hydrate in DMF at room temperature for 3 h. This leads to the *in situ* generation of hydrazone (GC-MS). After 3 h, substituted aldehydes and ethanol were added to the reaction mixture and allowed to reflux for 6-12 h to get desired azines **17a-y**. The reaction mixture was allowed to cool and then filtered to get crude product. The crude compound was purified by column chromatography over silica gel using ethyl acetate-hexane as eluent to yield the desired products in moderate to good yield.

2-(Benzylidenehydrazono)-3-methyl-2,3-dihydrobenzo[d] thiazole (17a):White crystalline solid (208 mg, 78%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ3.62 (s, 3H, NC**H**$_3$), 7.13-7.18 (m, 1H, Ar**H**), 7.34-7.48 (m, 5H, Ar**H**), 7.67 (d, *J* = 8 Hz, 1H, Ar**H**), 7.76 (d, *J* = 8 Hz, 2H, Ar**H**), 8.46 (s, 1H, N=C**H**). C^{13} NMR (100 MHz, DMSO-*d6*) δ31.77, 111.04, 122.70, 122.94, 123.89, 127. 17, 127.61, 129.29, 130.48, 135.07, 141.15, 152.68, 167.31; HRMS (+ESI) *m/z* calculated for $C_{15}H_{13}N_3S$, 267.0830 Found 268.0904 $[M+H]^+$.

3-Methyl-2-((pyridine-4-ylmethylene)hydrazono)-2,3-dihydrobenzo[d]thiazole (17b): Pale yellow solid (193 mg, 72%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ3.68 (s, 3H, NC**H**$_3$), 7.22 (t, *J* = 8 Hz, 1H, Ar**H**), 7.42-7.46 (m, 2H, Ar**H**), 7.74 (d, *J* = 8 Hz, 1H, Ar**H**), 8.14 (d, *J* = 4 Hz, 2H, Ar**H**), 8.51 (s, 1H, N=C**H**), 8.81 (d, *J* = 4 Hz, 2H, Ar**H**); ^{13}C NMR (100 MHz, DMSO-*d6*) δ31.92, 111.83, 123.09, 123.22, 123.46, 124.01, 140.95, 143.55, 147.21, 171.59; HRMS (+ESI) *m/z* calculated for $C_{14}H_{12}N_4S$ 268.0783, Found 269.0869 $[M+H]^+$.

4-((3-Methylbenzo[d]thiazol-2(3H)- ylidene)hydrazono) methyl)phenol (17c): Yellow solid (226 mg, 80%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 3.51 (s, 3H, NC**H**$_3$), 6.86 (d, *J* = 8 Hz, 2H, Ar**H**), 7.13-7.17 (m, 1H, Ar**H**), 7.34-7.38 (m, 2H, Ar**H**), 7.60 (d, *J* = 8 Hz, 2H, Ar**H**), 7.68 (d, *J* = 8Hz, 1H, Ar**H**), 8.38 (s, 1H, N=C**H**); ^{13}C NMR (100 MHz, DMSO- *d6*) δ 31.91, 111.10, 122.78, 122.98, 123.87, 125.82, 127.24, 129.51, 141.12, 152.91, 160.07, 166.07; HRMS (+ESI) *m/z* calculated for $C_{15}H_{13}N_3OS$ 283.0779, Found 284.0902 $[M+H]^+$.

`2-((4-Methoxybenzylidene)hydrazono)-3-methyl-2,3-dihydrobenzo[d]thaizole (17d): Yellow solid (274 mg, 79%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ3.60 (s, 3H, NC**H**$_3$), 3.80 (s, 3H, OC**H**$_3$), 7.02 (d, *J* = 8 Hz, 2H, Ar**H**), 7.14.(t, *J* = 8 Hz, 1H, Ar**H**), 7.33-7.39 (m, 2H, Ar**H**), 7.65-7.71 (m, 3H, Ar**H**), 8.41 (s, 1H, N=C**H**); ^{13}C NMR (100 MHz, DMSO- *d6*) δ 31.79, 55.79, 111.00, 114.81, 122.69, 122.94, 123.86, 127.19, 127.52, 129.29, 141.14, 152.51, 161.36, 166.39; HRMS (+ESI) *m/z* calculated for $C_{15}H_{15}N_3OS$ 297.0935 Found: 298.1019 $[M+H]^+$.

2-((2,3-Dimethoxybenzylidene)hydrazono)-3-methyl-2,3-dihydrobenzo[d]thiazole (17e): Yellow solid (241 mg, 74%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ3.63 (s, 3H, NC**H**$_3$), 3.79 (s, 3H, OC**H**$_3$), 3.83 (s, 3H, OC**H**$_3$), 7.10-7.18 (m, 3H, Ar**H**), 7.36-7.40 (m, 2H, Ar**H**), 7.47 (dd, *J* = 2 Hz, 9 Hz, 1H, Ar**H**), 7.68 (d, *J* = 8 Hz, 1H, Ar**H**), 8.63 (s, 1H); ^{13}C NMR (100 MHz, DMSO *d6*) δ31.85, 56.23, 61.69, 111.17, 114.69, 117.68, 122.84, 122.97, 12 3.89, 124.84, 127.23, 128.26, 141.09, 147.94, 148.35, 153.23, 16 7.46; HRMS (+ESI) *m/z* calculated for $C_{17}H_{17}N_3O_2S$ 327.1041, Found 328.1169 $[M+H]^+$.

2-((4-Chlorobenzylidene)hydrazono)-3-methyl-2,3-dihydrobenzo[d]thiazole (17f): Yellow solid (243 mg, 81%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ3.60 (s, 3**H**, NCH$_3$), 7.12-7.17 (m, 1H, Ar**H**), 7.33-7.39.(m, 2H, Ar**H**), 7.52 (d, *J* = 8 Hz, 2H, Ar**H**), 7.65 (d, *J* = 8 Hz, 1H), 7.77 (d, *J* = 8 Hz, 2H, Ar**H**), 8.43 (s , 1H, N=C**H**); ^{13}C NMR (100 MHz, DMSO-*d6*) δ31.65, 111.00, 122.68, 122.91, 123.84, 127.17, 129.13, 129.38, 134.10, 134.78, 141.13, 151.35, 167.65; HRMS (+ESI) *m/z* calculated for $C_{15}H_{12}ClN_3S$ 301.0440 Found: 302.0522 $[M+H]^+$.

2-((4-Fluorobenzylidene)hydrazono)-3-methyl-2,3-dihydro benzo[d]thiazole (17g): White solid (196 mg, 69%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ3.60 (s, 3H, NC**H**$_3$), 7.14 (t, *J* = 8 Hz, 1H, Ar**H**), 7.27-7.38.(m, 4H, Ar**H**), 7.65 (d, *J* = 8 Hz, 1H, Ar**H**), 7.78-7.82 (m, 2H, Ar**H**), 8.44 (s, 1H, N=C**H**); ^{13}C NMR (100 MHz, DMSO-*d6*) δ31.72, 111.01, 116.25, 116.47, 122.69, 122.9 1, 123.83, 127.17, 129.66, 131.68, 141.11, 151.49, 162.32, 164.7 8, 167.30 ; HRMS (+ESI) *m/z* calculated for $C_{15}H_{12}FN_3S$ 285.0736, Found 286.0835 $[M+H]^+$.

3-Methyl-2-((pyridine-2-yl-methylene)hydrazono)-2,3-dihydrobenzo[d]thiazole (17h): Orange colour solid (187 mg, 70%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 3.68 (s, 3H, NC**H**$_3$), 7.21 (t, *J* = 8 Hz, 1H, Ar**H**), 7.39-7.47 (m, 2H, Ar**H**), 7.72-7.79 (m, 2H, Ar**H**), 8.23 (d, *J* = 8 Hz, 1H, Ar**H**), 8.34 (t, *J* = 8 Hz, 1H, Ar**H**), 8.53 (s, 1H, N=C**H**), 8.77(d, *J* = 8Hz, 1H, Ar**H**); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 31.33, 111.71, 122.84, 123.05, 123.29, 123.99, 125.86, 127.35, 140.96, 143.23, 145.56, 170.38; HRMS (+ESI) *m/z* calculated for $C_{14}H_{12}N_4S$ 268.0782, Found 269.0867 [M+H]$^+$.

2-(3-Methylbenzo[d]thiazol-2(3H)ylidene) hydrazono) methyl)quinolin-8-ol (17i): Fine orange solid (263 mg, 79%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 3.71 (s, 3H, NC**H**$_3$), 7.21 (t, *J* = 8 Hz, 1H, Ar**H**),7.37-7.48 (m, 5H, Ar**H**), 7.55-7.61 (m, 1H, Ar**H**), 7.74 (d, *J* = 8 Hz, 1H, Ar**H**), 8.27 (d, *J* = 8 Hz, 1H, Ar**H**), 8.70 (s, 1H, Ar**H**), 8.72 (s, 1H, N=C**H**); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 31.95, 111.66, 118.66, 123.05, 123.26, 124.08, 127.34, 129. 42, 141.07; HRMS (+ESI) *m/z* calculated for $C_{18}H_{14}N_4OS$ 334.0888, Found 335.0982 [M+H]$^+$.

2-((2-Chloroquinolin-3-yl)methylene)hydrazono)-3-methyl-2,3dihydrobenzo[d]thiazole (17j): Yellow solid (246 mg, 70%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 3.62 (s, 3H, NC**H**$_3$), 7.14-7.17 (m, 1H, Ar**H**), 7.35-7.40 (m, 2H, Ar**H**), 7.68 (d, *J* = 8 Hz, 1H, Ar**H**), 7.97-8.01 (m, 1H, Ar**H**), 8.52 (s, 1H, N=C**H**), 8.69 (d, *J* = 8 Hz, 1H, Ar**H**), 8.84 (d, *J* = 8Hz, 1H, Ar**H**), 9.08 (s, 1H, Ar**H**); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 31.63, 111.24, 122.85, 122. 93, 123.84, 127.15,127.19,134.20, 140.32, 141.06, 142.26, 143.1, 147.01, 169.47; HRMS (+ESI) *m/z* calculated for $C_{18}H_{13}ClN_4S$ 352.0549, Found 353.0635 [M+H]$^+$.

3-Methyl-2-(pyridine-3-ylmethylene)hydrazono)-2,3-dihydrobenzo[d]thiazole (17k):Yellow solid (174 mg, 65%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 3.62 (s, 3H, NC**H**$_3$), 7.35-7.38 (m, 2H, Ar**H**), 7.66-7.71 (m, 2H, Ar**H**), 7.85 (t, *J* = 8 Hz, 1H, Ar**H**), 7.97 (d, 1H, Ar**H**), 8.17 (s, 1H, Ar**H**), 8.70 (s, 1H, N=C**H**), 8.89 (d, *J* = 8 Hz, 1H, Ar**H**); ^{13}C NMR (100 MHz, DMSO-*d6*): δ 31.61, 111.05, 122.71, 122.83, 123.99, 127.17, 127.40, 128.18, 128.29, 129.23, 131.95, 135.95, 141.20, 147.33, 147.51, 168.82; HRMS (+ESI) *m/z* calculated for $C_{14}H_{12}N_4S$ 268.0783, Found 269.0869 [M+H]$^+$.

2-(4-Bromoindolin-3-ylidene)hydrazono)-3-methyl-2,3-dihydrobenzo[d]thiazole (17l): White solid (311 mg, 81%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 3.68 (s, 3H, NC**H**$_3$), 7.16-7.20 (m, 1H, Ar**H**), 7.36-7.43 (m, 3H, Ar**H**), 7.47(d, *J* = 8 Hz, 1H, Ar**H**), 7.79 (d, *J* = 8 Hz 1H, Ar**H**), 7.94 (d, *J* = 4 Hz, 1H, Ar**H**), 8.40 (d, *J* = 4 Hz, 1H, Ar**H**), 8.71 (s, 1H, N=C**H**);^{13}C NMR (100 MHz, DMSO-*d6*) δ 32.15, 111.12, 111.79, 113.87, 114.59, 122.76, 123.17, 123.73, 124.54, 125.73, 126.43, 127.30, 133.16, 136.37, 141.29, 149.49, 164.44; HRMS (+ESI) *m/z* calculated for $C_{17}H_{13}BrN_4S$ 386.0044, Found 387.0091 [M+H] $^+$.

3-Methyl-2-((thiophen-2-ylmethylene)hydrazono)-2,3-dihydrobenzo[d]thiazole (17m): Light Green Solid (218 mg, 80%; ^{1}H NMR (400 MHz, DMSO-*d6*) δ 3.59 (s, 3H, NC**H**$_3$), 7.11-7.15 (m, 2H, Ar**H**), 7.32-7.38 (m, 2H, Ar**H**), 7.44 (d, *J* = 4 Hz, 1H, Ar**H**), 7.63-7.67 (m, 2H, Ar**H**), 8.60 (s, 1H, N=C**H**); ^{13}C NMR (100 MHz, DMSO-*d6*): δ 30.98, 110.11, 121.83, 122.27, 123.54, 126.46, 127.75, 128.33, 129.98, 139.75, 140.83, 146.76, 165.80; HRMS (+ESI) *m/z* calculated for $C_{13}H_{11}N_3S_2$ 273.0394, Found 274.0463 [M+H]$^+$.

3-Methyl-2-(((4-methyl-1H-imidazol-5yl)methylene)hydrazono)-2,3-dihydrobenzo[d] thiazole (17n): Yellow solid (211 mg, 78%); ^{1}H NMR (400 MHz, DMSO-*d6*) δ 2.50 (s, 3H, C**H**$_3$), 3.59 (s, 3H, NC**H**$_3$) 7.13 (t, *J* = 8 Hz, 1H, Ar**H**), 7.33-7.38 (m, 2H, Ar**H**), 7.66 (d, *J* = 8 Hz, 1H, Ar**H**), 8.41 (s, 1H, N=C**H**), 9.07 (s, 1H, Ar**H**); ^{13}C NMR (100 MHz, DMSO-*d6*): δ 10.40, 31.50, 110.93, 122.55, 123.82, 124.83, 127.10, 129.79, 134.48, 140.58, 141.14, 167.89; HRMS (+ESI) *m/z* calculated for $C_{13}H_{13}N_5S$ 271.0892, Found 294.0782 [M+Na]$^+$.

3-Methyl-2-(((4-methylthiazol-2- yl)methylene)hydrazono) 2,3-dihydrobenzo[d]thiazole (17o): Pale yellow solid (237 mg, 75%); ^{1}H NMR (400 MHz, DMSO-*d6*) δ2.46 (s, 3H, C**H**$_3$), 3.79 (s, 3H, NC**H**$_3$), 7.13-7.21 (m, 1H, Ar**H**), 7.33-7.44 (m, 2H, Ar**H**), 7.53 (s, 1H, Ar**H**), 7.69 (d, *J* = 8 Hz, 1H, Ar**H**), 8.08 (s, 1H, N=C**H**); ^{13}C NMR (100 MHz, DMSO-*d6*): δ 16.31, 31.78, 110.95, 120.00, 122.59, 123.65, 126.73, 140.40, 141.61, 151.70, 155.33; HRMS (+ESI) *m/z* calculated for $C_{13}H_{12}N_4S_2$ 288.0503, Found 311.0397 [M+Na]$^+$.

2-Hydroxy-5-(((3-methylbenzo[d]thiazol-2(3H)-ylidene) hydrazono)methyl)benzoic acid (17p): Yellow colour (258 mg, 79%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 3.61 (s, 3H, NC**H**$_3$), 7.06 (d, *J* = 8 Hz, 1H, Ar**H**) 7.12-7.14 (m, 1H, Ar**H**),7.33-7.38 (m, 2H, Ar**H**), 7.68 (d, *J* = 8 Hz, 1H, Ar**H**), 7.91 (dd, *J* = 4 Hz, 10 Hz, 1H, Ar**H**), 7.90. (d, *J* = 8 Hz, 1H, Ar**H**), 8.17 (s, 1H, O**H**), 8.44 (s, 1H, N=C**H**); ^{13}C NMR (100 MHz, DMSO-*d6*): δ 31.17, 110.98, 113.87, 118.35, 122.98, 123.89, 126.52, 127.13, 129.92, 134.25, 141.17, 151.64, 162.86, 166.76, 171.97; HRMS (+ESI) m/z calculated for $C_{16}H_{13}N_3O_3S$ 327.0677, Found 328.0765 [M+H]$^+$.

2-((4-Chlorobenzylidene)hydrazono)-5-methoxy-3-methyl-2,3-dihydrobenzo[d]thiazole (17q): Yellow colour solid (235 mg 71%); ^{1}H NMR (400 MHz , DMSO-*d6*): δ3.60 (s, 3H, NC**H**$_3$), 3.82 (s, 3H, OC**H**$_3$), 6.73-6.75 (dd, *J* = 2.4 Hz, 8 Hz, 1H, Ar**H**) 6.96-6.97 (d, *J* = 2.4 Hz, 1H, Ar**H**),7.50-7.53 (m, 3H, Ar**H**), 7.75-7.77 (d, *J* = 8 Hz, 2H, Ar**H**), 8.43 (s, 1H, N=C**H**); ^{13}C NMR (100 MHz, DMSO-*d6*): δ 31.81, 56.14, 97.71, 109.03, 114.88, 123.40, 129.10, 129.36, 134.75, 142.32, 151.24, 159.56, 168.63; HRMS (+ESI) calculated for $C_{16}H_{14}ClN_3OS$ 331.0546, Found 332.0633 [M+H]$^+$

5-Methoxy-3-methyl-2-((3,4,5-trimethoxybenzylidene) hydrazono)-2,3-dihydrobenzo[d]thiazole (17r): Light yellow solid (301 mg, 85%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 3.61 (s, 3H, NC**H**$_3$), 3.71 (s, 3H, OC**H**$_3$), 3.83 (s, 6H, OC**H**$_3$), 6.72-6.75 (dd, *J* = 4 Hz, 8 Hz, 1H, Ar**H**) 6.96-6.97 (d, *J* = 2 Hz, 1H, Ar**H**), 7.08 (s, 2H, Ar**H**), 7.53-7.55 (d, *J* = 8 Hz, 1H, Ar**H**), 8.37 (s, 1H, N =C**H**); ^{13}C NMR (100 MHz, DMSO-*d6*): δ 31.85, 56.14, 60.60, 97.65, 104.79, 108.99, 114.93, 123.42, 130.64, 139.64, 142.38, 152.41, 153.59, 159.56, 167.92; HRMS (+ESI) *m/z* calculated for $C_{19}H_{21}N_3O_4S$ 387.1252, Found 388.1337 [M+H]$^+$

2-((2-Chloroquinolin-3-yl)methylene)hydrazono)-5-methoxy-3-methyl-2,3-dihydrobenzo[d]thiazole (17s): Yellow solid (298 mg, 78%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 3.65 (s, 3H, NC**H**$_3$), 3.84 (s, 3H, OC**H**$_3$), 6.78-6.81 (dd, *J* = 4 Hz, 12 Hz, 1H, Ar**H**), 7.04-7.05 (d, *J* = 4 Hz, 1H, Ar**H**) 7.57-7.68 (m, 2H, Ar**H**), 8.14-8.20 (m, 2H, Ar**H**), 8.41 (s, 1H, N =C**H**), 8.72- 8.73 (d, *J* = 4, 1H, Ar**H**); ^{13}C NMR (100 MHz, DMSO-*d6*) 31.22, 55.76, 97.43, 108.75, 122.79, 126.89, 127.66, 127.75, 128.66, 131.39, 135.42, 146.76, 147.01, 148.42, 159.25, 169.34; HRMS

(+ESI) (*m/z*) calculated for $C_{19}H_{15}ClN_4OS$ 382.0655, Found 382.0618 $[M]^+$.

(*E*)-5-Methoxy-3-methyl-2-((*E*)-(pyridine-3-yl-methylene) hydrazono)-2,3-dihydrobenzo[d]thiazole (17t): Pale yellow solid (190 mg, 64%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 3.61 (s, 3H, NC**H**$_3$), 3.83 (s, 3H, OC**H**$_3$), 6.74-6.77 (dd, *J* = 4 Hz, 8 Hz, 1H, Ar**H**), 6.99 (d, *J* = 2.4 Hz, 1H, Ar**H**), 7.53-7.55 (d, *J* = 8 Hz, 1H, Ar**H**),7.84-7.86 (m, 1H, Ar**H**), 8.49-8.54 (m, 2H, Ar**H**), 8.76-8.77 (d, *J* = 4 Hz, 1H, Ar**H**), 9.03 (s, 1H, N=C**H**); ^{13}C NMR (100 MHz, DMSO-*d6*): δ31.13, 55.78, 97.42, 108.66, 122.80, 125.41, 137.13, 141.90, 144.59, 146.11, 147.76, 159.24; HRMS (+ESI) (*m/z*) calculated for $C_{15}H_{14}N_4OS$: 298.0888, Found 299.0971 $[M+H]^+$

5-Methoxy-3-methyl-2-((thiophene-3-ylmethylene) hydrazono)-2,3-dihydrobenzo[*d*]thiazole (17u): Light green solid (239 mg, 79%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ3.58 (s, 3H, NC**H**$_3$), 3.82 (s, 3H, OC**H**$_3$), 6.72-6.74 (dd, *J* = 2.4 Hz, 8 Hz, 1H, Ar**H**) 6.95-6.96 (d, *J* = 4 Hz, 1H, Ar**H**), 7.13-7.15 (m, 1H, Ar**H**), 7.43-7.44 (d, *J* = 4 Hz, 1H, Ar**H**), 7.52-7.54 (d, *J* = 8 Hz, 1H, Ar**H**), 7.62-7.63 (d, *J* = 4 Hz, 1H, Ar**H**), 8.59 (s, 1H, N=C**H**); ^{13}C NMR (100 MHz, DMSO-*d6*): δ31.77, 56.14, 97.61, 108.90, 114.90, 123.44, 128.41, 129.12, 131.04, 139.99, 142.36, 147.16, 159.54, 167.48; HRMS (+ESI) (*m/z*) calculated for $C_{14}H_{13}N_3OS_2$ 303.0500, Found 304.0596 $[M+H]^+$.

(*E*)-5-Methoxy-3-methyl-2-((*E*)-(pyridine-4-yl-methylene) hydrazono)-2,3-dihydrobenzo[d]thiazole (17v): Brown solid (202 mg, 68%; ^{1}H NMR (400 MHz, DMSO-*d6*): δ 3.67 (s, 3H, NC**H**$_3$), 3.85 (s, 3H, OC**H**$_3$), 6.81-6.83 (d, *J* = 8 Hz, 1H, Ar**H**), 7.07 (s, 1H, Ar**H**),7.59-7.61 (d, *J* = 8 Hz, 1H, Ar**H**), 8.05-8.03 (d, *J* = 8 Hz, 2H, Ar**H**), 8.48 (s, 1H, =C**H**), 8.79-8.80 (d, *J* = 4 Hz, 1H, Ar**H**); ^{13}C NMR (100 MHz, DMSO-*d6*): δ, 31.13, 55.78, 97.42, 108.66, 122.80, 125.41, 137.13, 141.97, 144.59, 146.13, 147.76, 159.24; HRMS (+ESI) (*m/z*) calculated for $C_{15}H_{14}N_4OS$: 298.0888, Found 299.0954 $[M+H]^+$

(*E*)-3-Methyl-5-nitro-2-((*E*)-(3,4,5 trimethoxybenzylidine) hydrazono)-2,3-dihydrobenzo[*d*]thiazole (17w): Orange solid (289 mg, 72%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 3.63 (s, 3H, NC**H**$_3$), 3.72 (s, 3H, OC**H**$_3$), 3.85 (s, 6H, OC**H**$_3$), 7.12 (s, 2H, Ar**H**), 7.43-7.44 (d, *J* = 8.8 Hz, 1H, Ar**H**), 8.20-8.23 (dd, *J* = 2 Hz, 9.2 Hz ,1H, Ar**H**), 8.38 (s, 1H, N=C**H**), 8.61 (d, *J* = 1.6 Hz, 1H, Ar**H**); ^{13}C NMR (100 MHz, DMSO-*d6*): δ 31.43, 56.18, 60.18, 105.41, 109.54, 118.09, 123.06, 125.11, 129.93, 140.22, 141.81, 146.30, 153.25, 154.48, 165.77; HRMS (+ESI) calculated for $C_{18}H_{18}N_4O_5S$ 402.0997, Found 425.0893 $[M+Na]^+$.

(*E*)-3-Methyl-6-nitro-2-((*E*)-(pyridin-4-ylmethylene) hydrazono)-2,3-dihydrobenzo[d]thiazole (17x): Pale yellow solid (192 mg, 61%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 3.66 (s, 3H, NC**H**$_3$), 7.59-7.61 (d, *J* = 12 Hz, 1H, Ar**H**), 8.13-8.14 (d, *J* = 4 Hz, 2H, Ar**H**), 8.27-8.30 (dd, *J* = 2 Hz, 12 Hz, 1H, Ar**H**), 8.60 (s, 1H, Ar**H**), 8.72 (d, *J* = 2.4 Hz, 1H, Ar**H**), 8.86-8.87 (d, *J* = 4 Hz, 1H, Ar**H**); ^{13}C NMR (100 MHz, DMSO-*d6*): δ37.15, 115.98, 116.31, 118.85, 121.72, 123.83, 124.57, 128.79, 130.20, 147.30, 150.86, 154.08, 155.53. HRMS (+ESI) calculated for $C_{14}H_{11}N_5O_2S$ 313.0633, Found 314.0724 $[M+H]^+$.

4-((*E*)-((*E*)-(3-Methyl-6-nitrobenzo[d]thiazol-2(3H)-ylidene)hydrazono)methyl)-2-nitrophenol (17y): Brown solid (268 mg, 72%); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 3.56 (s, 3H, NC**H**$_3$), 7.22-7.24 (d, *J* = 8 Hz, 1H, Ar**H**), 7.44-7.46 (d, *J* = 8 Hz, 1H, Ar**H**), 7.95-7.97 (d, *J* = 8 Hz, 1H, Ar**H**), 818-8.27 (m, 2H, Ar**H**), 8.45 (s, 1H, Ar**H**), 8.61 (s, 1H, Ar**H**); HRMS (+ESI) (*m/z*) calculated for $C_{14}H_{11}N_5O_2S$ 373.0480, Found 373.0478 $[M]^+$.

Biology

Assessment of QSI activity using *Chromobacterium violaceium* based Bioassay:

All the synthesized unsymmetrical azines (**17a-y**) were preliminarily tested against *Chromobacterium violaceium* CV12472 based bioassay for their inhibition of violacein production, with compound 4-NPO (4-nitropyridine-N-oxide) at final concentration of 50 µM being included in this study as positive control. We have performed the liquid bioassay to quantify the violacein contents in presence of the synthesized azines (**17a-y**) which is also indicative of the percentage inhibition being calculated by comparing the inhibition scores of the synthesized compounds with the equivalent DMSO (control) condition for the violacein inhibition. The percentage inhibition mentioned in the table 1, has been calculated based on the absorbance values taken at 568 nm as compared to the negative control DMSO condition which has not resulted in any significant inhibition for violacein production. Both the QS inhibition assay and quantification of the violacein contents were performed as described by Singh *et al*., with fewer modifications.[50] The sixteen hour grown culture is 1:100 diluted with sterile PBS and 20 µL of this diluted culture was added to 1450 µL of LB broth in test tubes, added with 30 µL stock solutions (10 mM) of the each test compounds (**17a-y**) resulting in final concentration of 200 µM and incubated for 36 h at 30°C. Subsequently the violacein is extracted by adding 1000 µL solution of butanol and acetone (3:1) to the test tubes, kept for 10 min in a lukewarm water and afterwards vortex for 5 min. 1 mL of this solution was centrifuged at 14000 rpm for 5 min. and then 200 µL of the supernatant was added to the 96-well plate. The absorbance for the extracted violacein was measured at 568 nm using BioTek Power Wave XS2 plate reader using Gen 5 1.10 software. All the experiments were done in triplicate, and data is recorded for two independent sets of experiments**.**

Assessment of QSI activity using *pLasB-gfp*(ASV):

The leads found active in the preliminary assay using *Chromobacterium violaceum* were further checked for the QS inhibitory potential using *pLasB-gfp*(ASV) as described by the group of Michael Givskov with very fewer modifications.[26,51] In test tube, 10 mL of Luria Bertani broth inoculated with *pLasB-gfp* (ASV) strain and kept for overnight incubation at 37°C. An overnight grown culture is diluted 1:40 (about 125 µL of O/N culture added to 4.875 mL of LB broth). In each row, 170µL of the LB broth was added to 1st well and 100µL to the rest of the wells in 96 well micro-titre plate. 30 µL from 1mM stock of the compounds **17j** and **17n** were added to the first well in each row and 100 µL serially transferred to the next wells upto the fifth well. The last well in each row was added with DMSO as a negative control. Finally, 100 µL of 1:40 diluted O/N culture was added to all the wells resulting in 1:80 dilution of the culture and a concentration dose (75, 37.5, 18.75, 9.375 and 4.6875) of **17j** and **17n**. The effect of the compounds **17j** and **17n** on the growth and gfp expression was measured by taking OD at 450 nm and

fluorescence with excitation of 485 nm and emission at 535 nm.

Effect of compounds on *pLasB-gfp*(ASV) Biofilms:

The biofilms of *plasB-gfp*(ASV) were established on microscopic coverslips. 10 µL of an overnight grown culture was added to chamber having 200 µL of the LB broth and this assembly was kept for incubation at 37°C. After every six hours the media was replaced with fresh LB broth. About after 24 h of incubation the chambers were added with 10 µL of 1mM each compound and then again kept for another 24 h incubation. The three day old biofilms were stained with propidium iodide atleast for 12 h prior to visualization and washed twice with PBS to remove the unattached cells were fixed using fixative. The biofilms on coverslips were visualized on Nikon Confocal microscope using NIS-elements software.

Molecular docking

Molecular docking analysis was carried out using Glide 5.8 module in maestro 9.3[52,53] to study the binding potential of the active compounds in the active site of LasR protein. The docking calculations were carried out using X-ray crystal structure of LasR protein (PDB code: 2UV0, resolution: 1.8 Å) bound to its natural ligand OdDHL.[54] The natural ligand OdDHL binds in the Ligand Binding Domain (LBD). The docking protocol was standardized by performing the redocking of bound ligand OdDHL, The lactone head group of OdDHL forms H-bond with nearby Trp 60 residue, while the amide group shows H-bonding with Tyr 56 and Asp 73 residues.

Protein and Ligand structure Preparation

The structure of LasR protein (PDB code: 2UV0) bound to its natural ligand OdDHL was taken from protein data bank. The protein structures were prepared using the Protein Preparation Wizard incorporated in Schrodinger package maestro version 9.3.5. This tool helps in (i) elimination of water molecules (ii) assigning right bond orders to amino acids and (iii) adds hydrogen atoms to the protein. Impref (Impact Refinement module) minimization was done upto a state where the average root mean square deviation (RMSD) of all the atoms comes down to 0.3 Å. For the preparation of ligands, Lig-prep module of Maestro was utilized where low energy ionized states of the ligands within a pH range of 7.0 ± 2.0 were generated using the OPLS (Optimized Potentials for Liquid Simulations) 2005 force field. [52]

Receptor Grid generation

Receptor Grid generation module of GLIDE (Grid-based Ligand Docking with Energetics) software was used to generate the grid for molecular docking purpose. The co-crystallized synthetic ligand OdDHL was used as a reference for grid generation. The inner grid box of 10 Å was defined around the centroid of bound ligand, whereas the outer box was extended upto 20 Å.

Ligand Docking

For the validation of docking protocol, bound ligand was extracted and then re-docked to generate the same docking pose as found with its co-crystallize form. Finally, the set of optimized ligands were docked using Ligand Docking module of GLIDE, they were analyzed based on their GLIDE docking score and intermolecular interactions.

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Notes and references

Electronic Supplementary Information (ESI) available: ^{1}H NMR, ^{13}C NMR Spectra, HRMS (+ESI) Spectra (**S1**-**S50**).

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Design, Synthesis and Biological Evaluation of Novel Unsymmetrical Azines as Quorum Sensing Inhibitors

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In this report, novel unsymmetrical azines have been designed and synthesised by using one pot approach. Further, they were evaluated as quorum sensing inhibitors.