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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

A 4-(*o*-methoxyphenyl)-2-aminothiazole: an anti-quorum sensing compound

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Abstract The 4-(*o*-methoxyphenyl)-2 aminothiazole was synthesized by reacting *o*-methoxyacetophenone, iodine, and thiourea; and characterized by spectral UV–Visible, Infra-Red (IR), H^1 nuclear magnetic resonance, C^{13}NMR, gas chromatography-mass spectroscopy, and thermal analyses. From the thermo gravimetric-differential thermal analysis curve, various kinetic parameters like order of reaction (*n*), energy of activation (*E*), pre-exponential factor (*Z*), entropy of activation (ΔS), and free energy change (*G*) have been calculated using Coats–Redfern, MacCallum–Tanner, and Horowitz–Metzger methods. The compound was evaluated for antibacterial activity against *Bacillus subtilis* 2063 and *Escherichia coli* 2931, and its mechanism of action was studied by fluorescence microscopy and scanning electron microscopy. A novel functional application of MPAT, a quorum sensing mediated inhibition of biofilm was studied in *Pseudomonas aeruginosa*, which is hitherto un-attempted.

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Introduction

Thiazole derivatives are an important class of heterocyclic compounds that occupies important position in medicinal chemistry by exhibiting a wide range of bioactivities. As medicines, many of them have displayed excellent antibacterial and antifungal (Ulusoy *et al.*, 2002; Kaplancikli *et al.*, 2004), anti-HIV (Al-Saddi *et al.*, 2008), hypertension (Tripathi, 2003), anti-inflammatory (Karpov *et al.*, 2001), anticancer (Baselt *et al.*, 2008), and anti-convulsant (Karade *et al.*, 2008) activities. Thus, thiazoles and their derivatives, particularly the MPAT nucleus have attracted continuing interest over the years because of their varied biological activities. In fact, literature review reveals that MPAT is used as a template for the development and designing of more potent therapeutic agents, and recent sprung in the chemistry of MPAT has arose owing to its role in treatment of neurological diseases and modulators of transcriptional repression for treatment of Huntington's disease (Samantha *et al.*, 2008). Thus, the observed biological application of MPAT and its derivatives has prompted us to synthesize and characterize (spectral and thermal analysis) MPAT and evaluate its biological importance as well. We explored the most wide application of MPAT, "an antibacterial property." We have not only studied the biological application of (antibacterial) MPAT, but also have introduced a new role of MPAT, "an anti-QS agent." The QS is a mechanism by which bacteria assess their population density through the secretion and sensing of small signal molecules, the concentration of which in the local environment indicates the number of neighboring

cells of the same species. When this concentration exceeds a certain threshold, bacteria are triggered to express phenotypic traits associated with infection, including biofilm formation and the production of virulence factors. *Pseudomonas aeruginosa* is an example of opportunistic human pathogen and is implicated in the infection of many tissues through biofilm formation, including the lungs of patients suffering from cystic fibrosis, where it is regarded as one of the major causes of mortality associated with the condition. The *P. aeruginosa* infections are generally associated with a high degree of mortality, regardless of appropriate antimicrobial treatment regimes (Moreau *et al.*, 2008). In fact, the Center for Disease Control and Prevention (CDCP) has estimated that 65 % of human bacterial infections involve biofilms (Rossolini, 2005). Therefore, in the present communication, we report synthesis, characterization, and evaluation of antibacterial activity of MPAT against *Bacillus subtilis* and *Escherichia coli.* We have not only studied its biological application, but also studied the mechanism of action of MPAT and introduced a new function of MAPT, "as an anti-QS agent" for inhibiting the formation of biofilm in *P. aeruginosa.*

Results and discussion

Chemistry

The compound MPAT, molecular formula ($C_{10}H_{10}N_2OS$), is a colorless crystalline solid having melting point (m.p.) 0.106 °C. It is soluble in common organic solvent like acetone, alcohol, chloroform, carbon tetrachloride, etc.

The GC-MS of MPAT showed molecular ion peak at 206 (relative intensity 100 %) and confirmed the molecular formula of MPAT as $C_{10}H_{10}N_2OS$. The molecular ion undergoes fragmentation (Figs. 1, 2) to give a fragment having m/z 164 (relative intensity 54.45 %). This fragment further undergoes decomposition to give smaller fragments, represented as (relative intensity %): 146 (38.61), 131 (72.27), 121 (50.49), 104 (18.81), 91 (24.75), 77 (55.44), 63 (21.78), 51 (24.32), and 45 (34.65).

The TG-DTA curve of MPAT was depicted in Fig. 3. The compound undergoes decomposition in two stages (stage—I 160.59–327 °C and stage—II 350–487 °C). The stage—I showed weight loss 91.56 % and stage—II showed weight loss 5.791 %. The major weight loss occurred in stage I only. Two DTA (endothermic) peaks were observed at 100 and 230 °C, and one DTA peak (exothermic) was observed at 550 °C. The various kinetic parameter viz., E, n, Z, ΔS, and G have been calculated for stage I (major weight loss 91.56 % occurs in this stage) by C.R., (Coats and Reddfern, 1964), M.T. (MacCallum and Tanner, 1970), and H.M. method (Horowitz and Metzger, 1963), and represented as Eqs. 1, 2, and 3, respectively.

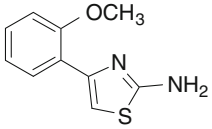

Fig. 1 Chemical structure of 4-(*o*-methoxyphenyl)-2-aminothiazole

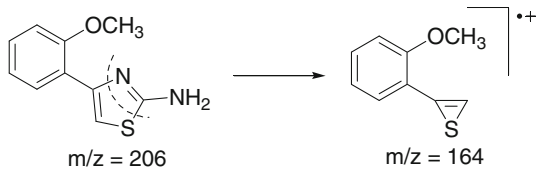

Fig. 2 Mass spectral fragmentation scheme of 4-(*o*-methoxyphenyl)-2-aminothiazole

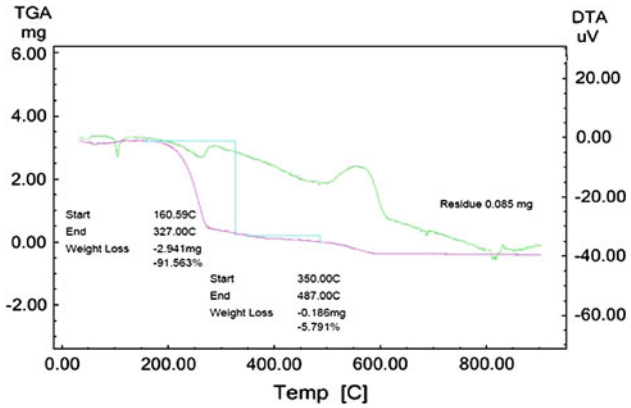

Fig. 3 TG-DTA curves of 4-(*o*-methoxyphenyl)-2 aminothiazole

$$log\left[\frac{1-(1-\propto)^{1-n}}{(1-n)T^2}\right] = \log\frac{ZR}{Eq}\left[1-\frac{2RT}{E}\right]-\frac{E}{2.303R}\cdot\left[\frac{1}{T}\right] \quad (1)$$

$$log\left[\frac{1-(1-\propto)^{1-n}}{(1-n)}\right] = \log\frac{ZE}{Rq}-0.485E^{0.435} - \frac{0.449+0.217E}{T}.10^3 \quad (2)$$

$$log\left[\frac{1-(1-\propto)^{1-n}}{(1-n)}\right] = \log\frac{ZRTs^2}{Eq}-\frac{E}{2.303RTs} + \frac{E\theta}{2.303RTs^2} \quad (3)$$

In all equations, α fraction decomposed, q heating rate, T absolute temperature, T_s temperature at half weight loss, and $\theta = (T - T_s)$. From the calculated values of E and Z; the values of ΔS and G were determined.

The values of n, E, Z, ΔS, and G calculated by the three methods were in accordance with each other (Table 1). The

Table 1 Calculation of kinetic parameters by (C.R.), (M.T.), and (H.M.) methods

Kinetic parameter	C.R.	M.T.	H.M.
n	1.42	1.4	1.77
E	28.95	33.09	36.19
Z	4.82×10^8	3.19×10^6	7.49×10^9
ΔS	−10.02	−15.04	−7.28
G	23.7144	25.2267	32.3801

E kcal/mol; z s^{-1}; ΔS Jk^{-1} mol^{-1}; G kcal mol^{-1}

value of E was lying in range of 28–36 kcal/mol which is sufficiently high and suggests MPAT a thermally stable compound.

Antibacterial studies

Antibiotics have been extensively used in the treatment of infectious diseases. The lethality of antibiotics have been exploited in clinical and laboratory approaches, and their specific targets in bacterial physiology have been elucidated. However, along with wide usages of antibiotics, the problem of increasing resistance in microbes arose, resulting in dramatically reduced therapeutic effectiveness (Davies, 1994). Pathogenic microbes have rapidly evolved efficient mechanisms of resistance, including increased efflux, enzymatic inactivation, target modification, or biofilm formation (Davies, 2006). So, there has been a spring in the search for novel compound against pathogenic microbes, and chemistry of MPAT is one of the answer to the aforementioned problem. In fact, 2-MPAT, a template for the synthesis of variety of such drugs, has been synthesized and its derivatives used against pathogenic organisms (as mentioned somewhere). The MPAT was synthesized in the laboratory by reacting *o*-methoxyacetophenone, iodine, and thiourea, and its biological activities were studied by assaying the antibacterial property using two different groups of model organisms, Gram-positive *B. subtilis* and Gram-negative *E. coli*. We have not restricted our studies only to its biological property; we have also explored the possible mechanism of its action. Finally, for the first time, an unexplored role of MPAT as an anti-QS agent in impeding the formation of biofilm in *P. aeruginosa* PA 01 is communicated wide this report.

We have studied the antibacterial action of MPAT by determining the minimum inhibitory concentration (MIC) by dye reduction method and minimum bactericidal concentration (MBC) by counting the number of viable organisms as colony forming units per milliliter (cfu/ml), which are the lowest concentrations of a compound that can inhibit bacterium growth or kill 99.9 % of organisms (3log), respectively, in comparison to control. MIC determination by dye reduction method uses a redox dye, resazurine, which shows a deep blue color in its oxidized state and a purple color in its reduced state. When the bacterial cells are viable and metabolically active, i.e., in growing stage, oxidative enzymes present inside the cells reduced the dye to purple color; however, when the cells are metabolically inactive, the deep blue color of dye remains unchanged. Thus, the as-characterized MPAT showed a reported biological property, "an antibacterial agent." During the determination of MIC when the cells of the test organism were subjected to decreasing concentration of MPAT in Muller Hinton (MH) broth and incubated for 12 h, the blue color of the dye decreased and showed an increase in the pink coloration, the highest in control, indicating that MPAT was acting as an antibacterial agent. The break point (the first concentration) at which there was a change in color of dye from pink to blue, a MIC value, was 250 μg/ml for *B. subtilis* and 500 μg/ml for *E. coli*. The MIC determination gives information of the compound as a bacteriostatic agent; however, it does not give any idea of the compound as bactericidal agent, as well. The bactericidal property of compounds is far better over bacteriostatic as former property irreversibly inhibits the growth of bacteria.

In order to know the bactericidal property of the MPAT, determination of the MBC was carried out. The MBC value gives information on the viable number of cells and is determined as colony forming units per milliliter (cfu/ml). When an aliquot from all MIC-wells were spread on the MH agar plate, and incubated for 24 h to develop colony, if any, it was found that there was a reduction in the number of viable cells as concentration of the compound increased and the first concentration of the MPAT that reduces the number of viable cells to or more than 99.9 % (3log or more than 3log) was also 250 μg/ml for *B. subtilis* and 500 μg/ml for *E. coli* (Fig. 4). In comparison to commercial available erythromycin and penicillin, the antimicrobial activity of the MPAT was less effective. However, MPAT can be further derivatized to improve the antibacterial activity in comparison to other commercial available drugs.

It is to mention that there was a scarcity of knowledge about the mechanism of antibacterial action of MPAT. Except a report of Pappenberger *et al.*, 2007, wherein the action of MPAT has been predicted based on structure-based biophysical interaction with ketosynthase (KS) domain of bacterial fatty acid synthases (FAS) complex, there are no reports of the mechanism of action of MPAT. Since the action of MPAT as an inhibitor of fatty acid synthases is known, and the fact that there are reports of antibacterial action of antibiotics by generation of reactive oxygen species (ROS) (Ines Albesa *et al.* 2004; Daniel *et al.*, 2009), we have explored the possibility of generation

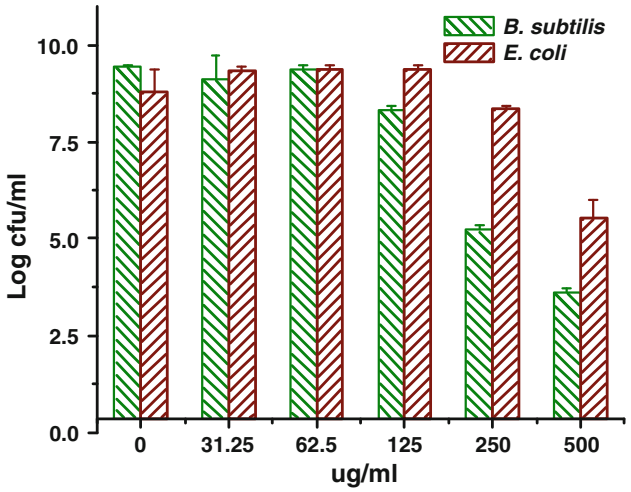

Fig. 4 An antibacterial activity of MPAT against *B. subtilis* and *E. coli*. The cultures were set up at initial inoculums of 1×10^5 in Muller Hilton broth containing various concentrations (0.031-25–0.5 mg/ml) of compound, and incubated for 12 h at 180 rotation per minute (r.p.m) and 37 °C. A 3log reduction occurred at 250 μg/ml for *B. subtilis* while it was 500 μg/ml for *E. coli*. *Error bars* represent the standard deviation ($n = 3$)

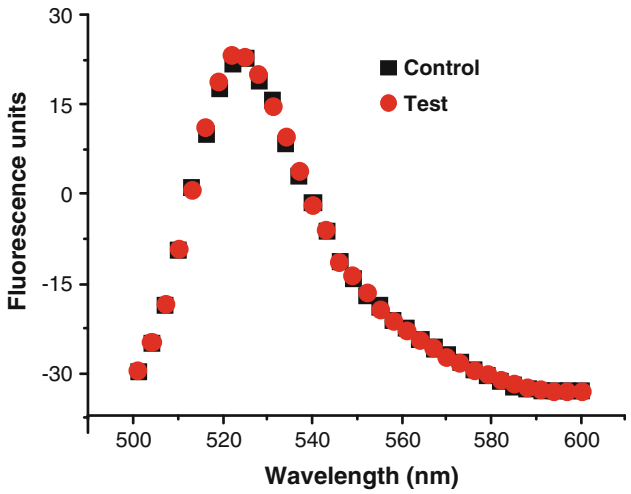

Fig. 5 Measurement of ROS generation. The cells of *B. subtilis* (1×10^7 cells/ml) after treating with H_2DFFDA, showed a similar generation of fluorescence units in control and at MIC_{50} value, 200 μg/ml of MPAT (Data for *E.coli* is similar and not shown)

of ROS as a mechanism of action of MPAT. The ROS are known to cause oxidative damage to important cellular processes (Imlay, 2003). We have studied the generation of ROS in *B. subtilis* only. The generation of ROS in cells of *B. subtilis* in the presence of MPAT was studied by fluorescent dye based spectroscopy method, using 5-(and-6)-carboxy-2,7-dihydrodifluorofluorescein diacetate (H_2DFFDA). In this assay, hydrophobic DCFH-DA molecules readily penetrate cellular membranes and were hydrolyzed by intracellular esterases to yield dichlorofluoroscein (DCFH), a nonfluorescent compound; DCFH can then be oxidized by ROS to the fluorescent compound dichlorofluorescein, DCF.

When the cells of *B. subtilis* were subjected to the sub-inhibitory concentration of compound, generation of fluorescence inside cells remained same as in control cells (Fig. 5), thus, indicating that there was no generation of ROS inside the cells in the presence of MPAT. Since the action of MPAT on target organisms was tested in nutrient rich medium, it is likely that the generated ROS is quenched by the nutrient rich medium, thus, was not detected under spectrophotometer. The primary target for the generated ROS is cell membrane, leading to the distortion of cell shape, so we further confirmed negative results of ROS generation on the cell membrane by observing the morphology of cells under high-resolution scanning electron microscopy (SEM).

As excepted we did not observe any change in the cell shape in the presence of MPAT (Fig. 6, right panel); its morphology remained intact, as observed for control cells (Fig. 6, left panel). Thus, the action of MPAT is not mediated by ROS, i.e., its antibacterial action is independent of ROS generation and is likely to be mediated by the inhibition of elongation step catalyzed by KS domain of FAS complex.

Having succeeded in synthesizing MPAT and showing an antibacterial property to MPAT, we were also aware of the growing resistance of pathogenic organism to presently used array of drugs. Since the therapeutics actions of these drug molecules were inhibiting the growth of microorganisms, the later have evolved systems to overcome the inhibitory action of antimicrobials, and generation of MRS is a recent example of such misuse of antimicrobials. So, there is a strategic need to develop an antimicrobial agent that will not give any selective evolutionary advantage to pathogenic microorganisms to develop resistance, and anti-QS is a best answer to the aforementioned problem. Bacterial QS refers to the ability of bacteria to control gene expression through the detection of a minimal threshold stimulatory concentration of certain chemicals called auto-inducer (s), which are secreted by self and/or other bacteria (Waters and Bassler, 2005; Miller and Bassler, 2001). The QS has been implicated in the control of bacterial behaviors such as the formation of biofilm, latter play an important role in infectious disease (Hammer and Bassler, 2003; Cvitkovitch *et al.*, 2003). Biofilms are formed when planktonic bacteria adhere to a surface and initiate the formation of a microcolony that exists as a community encased in a protective extracellular matrix (Musk and Hergenrother, 2006; Donlan and Costerton, 2002). It is estimated that biofilms account for up to 80 % of microbial infections in the body (Davies, 2003; Costerton *et al.*, 1999). Biofilms also underlie importunate infections of implanted medical devices. Within a biofilm, bacteria display differential gene expression and are upward of 1000-times more resistant to conventional

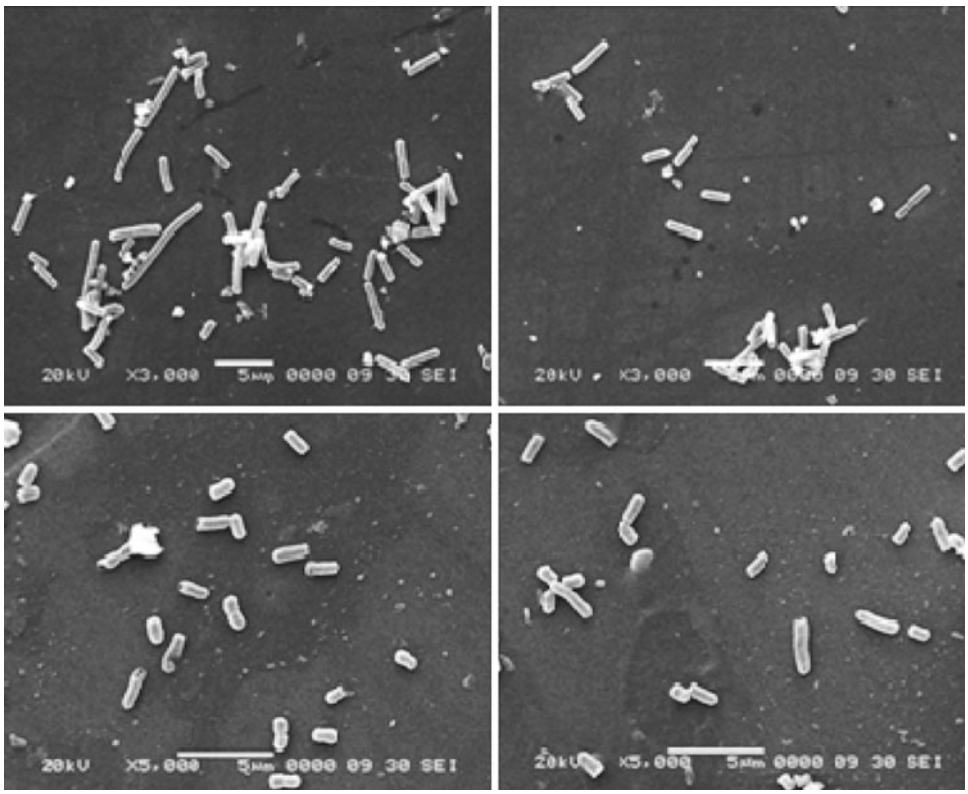

Fig. 6 The SEM images of cells of *B. subtilis* and *E. coli*. The cell surface morphology of *B. subtilis* and *E. coli* in the presence of MPAT (*right panel*), respectively, remained unchanged. Corresponding controls were shown in *left panel*

antibiotic treatment (Rasmussen and Givskov, 2006). Current efforts toward small molecule-based strategies to control biofilm formation have focused almost exclusively on inhibiting QS, a signaling cascade that is critical for bacterial communication (Smith *et al.*, 2003; Geske *et al.*, 2005). Since QS systems regulate fundamental virulence processes in many pathogenic bacteria, interfering with this cell-to-cell communication mechanism is a rational strategy to attenuate their virulence with the hope of developing a new drug arsenal to counterbalance the emergence of antibiotic-resistant pathogens (Bjarnsholt and Givskov, 2008; Gonzalez and Keshavan, 2006; McDougald *et al.*, 2007). Anti-QS compounds have been identified from natural sources (Givskov *et al.*, 1996; Gram *et al.*, 1996; Manefield *et al.*, 1999; Skindersoe *et al.*, 2008a, b) and chemical synthesis programs (Ishida *et al.*, 2007; Muh *et al.*, 2006); however, there is no mention of MPAT as the template for the synthesis of anti-QS agents, so we have taken up a study to explore a possible role of MPAT as an anti-QS agent in inhibiting/impeding the formation of biofilm in *P. aeruginosa*.

When the cells of *P. aeruginosa* were subjected sub-inhibitory concentration of MPAT, there was a decrease in the biofilm formation. When the cells of *P. aeruginosa* were seen under SEM, matrix covered uniform cells were observed i.e., cells embedded in a polysaccharides, a typical of biofilm structure (Fig. 7a). However, in the presence of MPAT, cells of *P. aeruginosa* were seen as uncovered structures, with scattered and exposed cells (Fig. 7b). An enmesh observed under SEM is a complex of polysaccharides, synthesized by growing cells in response to QS; however, since in the presence of MPAT this QS is disturbed, so an enmesh of the structures was not observed under SEM.

Quantitative determination of the inhibition of QS was carried out by crystal violet retention assay, since crystal violet has a high affinity toward the polysaccharides, in 24-well microtiter polypropylene plates under static condition. When *P. aeruginosa* were grown in different concentration of MPAT, it was found that at 25–100 μg/ml, there was a decrease in formation of biofilm as compared to control (Figs. 8, 9).

However, at increasing concentration, there was a decrease in the number of viable cells (data not shown). Therefore, 25–100 μg/ml of MPAT significantly arrested biofilm formation without affecting viability, whereas concentration above it inhibited the growth of the organism itself. Since expression of many virulence factors and establishment of chronic infections are regulated by QS (Van Delden and Iglewski, 1998) and disruption of QS resulted in reduced virulence in *P. aeruginosa*, QS inhibitors are expected to reduce the pathogenicity of *P. aeruginosa* (Hentzer *et al.*, 2005).

The advantage of the anti-QS approach to control infection is that there are few evolutionary forces that select for resistance. In other words, natural selection does not come into play and resistant strains will be unlikely to occur.

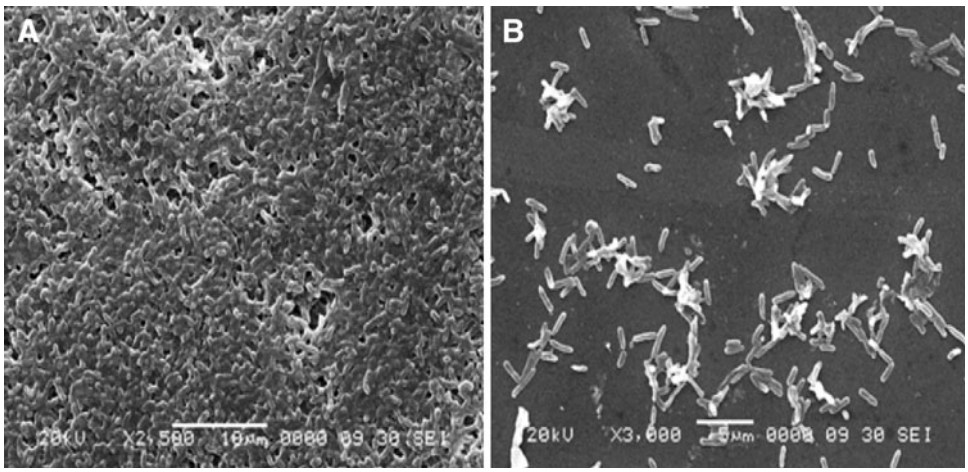

Fig. 7 Effect of MPAT on the formation of Biofilm in *P. aeruginosa.* Cells of *P. aeruginosa* (mid log phase, 1×10^5 cells/ml) in the presence of 50 µg/ml of compound were seen as sparsely distributed as opposed to control wherein they were observed as compact mass of structures surrounded by matrix

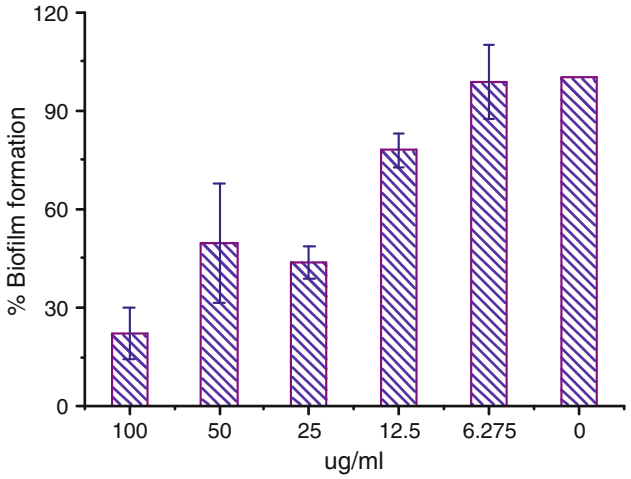

Fig. 8 Quantification of the biofilm. The cultures of *Pseudomonas aeruginosa* PA01 were set up at initial inoculums of 1×10^6 in Muller Hilton at 37 °C in the presence of various concentration of MPAT. After removal of planktonic cells, formed biofilm was treated with 0.1 % crystal violet and extracted in acetic acid to read at 595 nm. At 50 µg/ml of compound, there was a reduction in biofilm by about 59 %. *Error bars* represent the standard deviation ($n = 3$)

Therefore, QS inhibition offers new hope in combating the multiple antibiotic-resistance in clinical bacteria. This approach is highly attractive because it does not impose harsh selective pressure for the development of resistance with antibiotics because QS is not directly involved in processes essential for growth of bacteria. The negative effect of MPAT on formation of biofilm is not caused by the inhibition of growth but rather by disruption of QS signaling systems. This is important because when the growth is not affected, there is no selective pressure for the development of resistant bacteria. Inhibition of bacterial QS, rather than bactericidal or bacteriostatic strategies, may find application in many different fields, such as medicine, agriculture, and food technology (Dickschat, 2010).

Experimental

All the chemicals used were of A. R. Grade. The solvents were dried according to the standard procedures and distilled before use. UV–Visible spectrum was recorded in ethanol on Perkin Elmer UV–visible 25 lamba spectrometer. IR spectrum was recorded in KBr pellets on Shimadzu FT-IR 8400 spectrometer. H^1NMR spectrum was recorded in $CDCl_3$ using TMS as the standard on Varian 300 MHz spectrometer. GC-MS was recorded on Shimadzu GC-MS QP 5050 mass spectrometer. TGA-DTA curve was recorded on V2 4F TA thermal analyzer at the heating rate 10 °C per minute in nitrogen atmosphere.

Synthesis of 4-(o-methoxyphenyl)-2-aminothiazole

A mixture of *o*-methoxyacetophenone (0.05 mol), iodine (0.1 mol), and thiourea (0.1 mol) was refluxed on water bath for 8 h and again 12–16 h after removal of condenser. The crude product was kept in contact with ether with occasional shaking for 48 h. The ether layer was removed and reaction product was treated with sodium thiosulphate solution to remove traces of iodine. The product was boiled

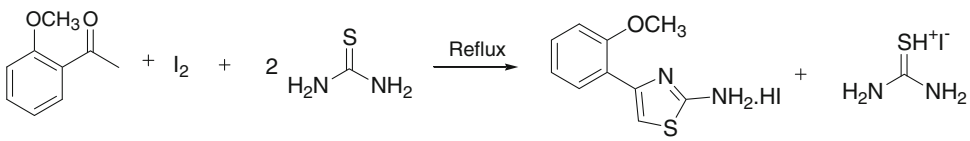

Fig. 9 Reaction of *o*-methoxyacetophenone, iodine, and thiourea

with water and then filtered. The filtrate was treated with concentrated ammonia to obtain MPAT, which was recrystallized from 50 % ethanol and dried under reduced pressure (Dash and Raut, 1955).

Spectral analysis

The UV–Visible spectrum of MPAT exhibits λ_{max} at 296.15 nm. The literature survey reveals that MPAT and other aromatic compounds with comparable structures exhibit λ_{max} at ~275 and ~300 nm, respectively (Mathews and Gregory, 1952; Convover and Tarbell, 1950).

The IR spectrum of MPAT in KBr shows ν(NH_2), ν (C=N), ν (C–O), and ν (C–S–C) modes at ~3358, ~1649, ~1547, and ~754 cm^{-1}, respectively. Phenyl and thiazole ring vibration occur at ~1500, ~1267, and ~1024 cm^{-1}. The spectral data is in accordance with earlier reports (Saydam and Yilmaz, 2005; Kovacic, 1967).

The H^1NMR spectrum of MPAT shows signals at ($CDCl_3$, TMS, δppm) 3.88 δ (3 H, S, Ar–OCH_3), 6.93 δ (1 H, d, Ar–H), 6.96 δ (1 H, d, Ar–H), 6.99 δ (2 H, S, Ar–NH_2), 7.15 δ (1 H, t, Ar–H), 7.26 δ (1 H, t, Ar–H), and 8.03 δ (1 H, s, thiazole ring). The spectral data is in agreement with earlier results (Silvestein and BasslerGCandMorill, 1991). The C^{13}NMR spectrum of MPAT shows signals at ($CDCl_3$, TMS, δppm) 165.88, 156.67, 146.61, 129.66, 128.27, 123.20, 120.63, 110.91, 107.32, and 55.27.

Antibacterial study

Determination of MIC and MBC

An antibacterial study was carried out using two different organisms, Gram-positive, *Bacillus subtilis* NCIM 2063 and Gram-negative, *Escherichia coli* NCIM 2931. The antibacterial study was carried out by dye reduction method. In short, a 24-well microtiter plates containing 1 ml Muller Hinton (MH) broth (Hi-Media Mumbai, India), with MPAT (in the concentration range of 31.25–500 μg/ml) dissolved in dimethyl sulfoxide were inoculated with test strains (final cell density of 5×10^4 cfu/ml) and incubated at 37 °C for 15 h. The lowest concentration of compound showing no change in color (no growth) was considered as the minimum inhibitory concentration. The MBC was measured by preparing serial dilutions from the MIC assay and plating the dilutions on MH agar plates. The MBC is defined as the minimum concentration at which there was a 3log reduction in the cfu. The data were recorded as survival rates (cfu/ml), based on 100 % survival for the untreated control. All MIC and MBC values reported were based on three experimental repeats.

Determination of ROSs

The ROS generated by MPAT inside the cells were determined by a spectrofluorometric assay, using the intracellular oxidation of DCFH-DA. The cells of *B. subtilils* and *E.coli* were grown to mid log phase (1×10^7 cells/ml) and treated with MPAT (MIC_{50} value) for 3 h at 37 °C and 150 r.p.m. The cells were collected by centrifugation at 10,000 gravitational force (*g*) for 15 min at 4 °C. The pellets were then washed three times with 0.1 M phosphate buffer (pH 7.4) and incubated with 25 μM DCFH-DA for 30 min. At the end of the incubation, cells were washed with PBS again. The fluorescence of dichlorofluoroscein (DCF), which is the oxidized product of dichlorofluoroscein (DCFH, hydrolyzed from DCFH-DA by intracellular esterases), was detected using a Varian fluorescence spectrophotometer, using an excitation of 485 nm and an emission of 530 nm. The DCF concentration in cells not exposed to compound was used as a control.

Determination of structural change

SEM was used to analyze the surface morphology of cells after exposed to MPAT. The cells of *B. subtilils* and *E. coli* were grown to mid log phase (1×10^7 cells/ml) and treated with MPAT (MIC_{50} value, 150 μg/ml) for 3 h at 37 °C and 150 r.p.m. The cells were collected by centrifugation at 10,000 gravitational forces (*g*) for 15 min at 4 °C. The pellets were then washed three times with 0.1 M phosphate buffer (pH 7.4), and fixed in 0.1 M phosphate buffer (pH 7.4) containing 2.5 % glutaraldehyde at 4 °C for 4 h. After rinsing twice with 0.1 M phosphate buffer (pH 7.4), the pellets were dehydrated in ethanol serials (10, 30, 50, 70, 80, 90, and 100 %, 15 min per step), and then dried in air. Finally, the images were obtained using the Joel, JSM 6360A (Japan) SEM at 1.5 kV.

Antiquorum sensing

Morphology of biofilm

P. aeruginosa was grown overnight in Lurai Bertani (LB) broth at 37 °C with agitation. After growth, the culture was diluted with LB medium (OD_{600} 0.02), and 50 μl of the diluted culture was added to 950 μl of LB medium supplemented with 25 μg/ml of MPAT and were incubated statically for 18 h at 37 °C in 8-well glass chamber slide. After incubation, planktonic bacteria were discarded, and the biofilms were washed three times with cocadylate buffer (0.1 M, pH 7.4). Biofilms formed on glass plates were fixed in 2 % glutaraldehyde in 0.1 M cocdylate buffer (pH 7.4) for 4 h at 4 °C. After thorough washing with cocadylate buffer, samples were dehydrated in a series of

ethanol solutions (10–100 %). The samples were dried, mounted on aluminum stubs with conductive carbon cement, and then coated with a gold film. Samples were observed with a Hitachi 14800 SEM at 1.5 kV.

Quantification of biofilm

P. aeruginosa was grown overnight in LB medium at 37 °C with agitation. After growth, the culture was diluted with LB medium (OD_{600} 0.02), and 50 µl of the diluted culture was added to 940 µl of LB medium supplemented with 6.25–100 µg/ml of MPAT and were incubated statically for 18 h at 37 °C in 24-well polystyrene plates. After incubation, planktonic bacteria were discarded, and the biofilms were washed three times with phosphate buffered saline buffer. Washed biofilms were fixed with 1 ml of methanol (99 %). After 15 min, the methanol was discarded, and the plates were dried at room temperature. Crystal violet (0.1 % in water) was then added to each well (1 ml/well), and the plates were incubated for 15 min at room temperature. Crystal violet was then discarded, and stained biofilms were washed three times with 1 ml of water. Acetic acid (33 % in water) was added to the stained biofilms (2 ml) to solubilize the crystal violet, and the absorbance of the solution was read at 590 nm with a Shimadzu spectrophotometer (Japan).

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