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Synthesis, Biological and Molecular Docking Studies of New Polysubstituted 2-Amino-3-Cyano-4H-Chromene Derivatives

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A series of new substituted 2-amino-3-cyano-4H-chromene derivatives have been synthesised by one pot multicomponent reaction of substituted benzaldehyde, malononitrile and 4-chloro resorcinol. All the synthesised compounds have been characterised using FT-IR, ^{1}H NMR, ^{13}C NMR and HR-MS spectra and screened for bioactivities such as swarming behaviour, biofilm formation, biofilm disruption, urease production in *Proteus mirabilis*. Whereas swarming inhibition was shown by all synthesized chromene derivatives, biofilm inhibition was shown only by nitrophenyl and fluorophenyl substituted chromene derivatives. Interestingly, urease activity was shown by meta substituted nitrophenyl and fluorophenyl derivatives. Quantitative polymerase chain (qPCR) reaction used to investigate the molecular mechanism of most active chromene derivatives showed prominent down regulations of urease enzyme genes. Molecular docking study against urease supported the observed bioactive effect and provide valuable insights into the binding modes and affinities of these new chromene derivatives. The per-residue interaction analysis proposed the involvement of non-bonded (steric and electrostatic) and bonded (hydrogen bonding and pi-pi stacking) interactions with the active site.

1. Introduction

Urinary tract infections (UTIs) are some of the most common bacterial infections, affecting several people each year worldwide. More than 80% of urinary tract infections are due to the use of indwelling urethral catheters. Catheter Associated Urinary Tract Infections (CAUTI) are most frequent found in hospitalized patients.[1] Most frequent etiological agents of urinary tract infection are gram negative bacteria belonging to the family Enterobacteriaceae. Among the hundreds of known species within the Enterobacteriaceae, *Proteus mirabilis* is most frequent etiological agent to cause catheter associated UTI (CAUTI).[2] Patients with long term catheterization are more susceptible to infection of *P. mirabilis*.[3] The bacterium *P. mirabilis* is also responsible for the formation of 15% of urinary stone diseases, as it is the second to *E. coli* as a common cause of urinary tract infection. The infections of *P. mirabilis* are often persistent and difficult to treat, since dense crystalline biofilm protects the bacteria from the action of antibiotics. Furthermore, in last two decades, *P. mirabilis* are able to resist many antimicrobial drugs including beta-lactamses which have made treatment process very challenging.[4]

P. mirabilis produces several virulence factors which may lead to serious complications such as cystitis, acute pyelonephritis, and even death of patients.[5,6] *P. mirabilis* can form biofilms on biotic as well as abiotic surfaces including catheter surface. Biofilm formation facilitates the survival and acclimatization to hostile conditions of the external environment and protect from host immune system.[7,8] The crystalline biofilms lead encrustation and block the urinary flow of indwelling catheters in patients with UTIs.[7] Swarming differentiation and migration is another virulence attribute of *P. mirabilis* which facilitate the movement of bacterium over solid catheter surface.[9] Swarming ability causes initiation of infection by the migration of *P. mirabilis* from intestine to urinary tract.[6] Urease, an nickel containing enzyme, is associated with formation of urinary crystalline stones as it catalyses hydrolysis of urea into ammonium ions and carbon dioxide.[10,11] Increase in ammonium ions causes increase in alkalinity of urine to form the crystals of carbonate apatite and magnesium struvite. This crystals precipitate to form urinary stones after nucleation process.[12] The crystalline biofilm and exopolysaccharides (EPS) protect bacteria from antibiotics and immune cells of the host.[13] The fully developed crystalline biofilm eventually causes catheter obstruction.[12] Considering all these consequences, a new mode

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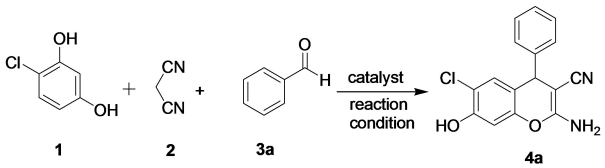
of action for treatment is urgently needed. By suppressing or counteracting the expression of virulence factors, the pathogenicity of bacteria can be reduced, making it easier for the immune system to fight them off.[14]

Chromene derivatives are important heterocyclic compounds that are widely present in natural products.[15] Due to their excellent pharmacological and biological activities, chromene and its derivatives have been recognized as 'medicinal scaffolds'.[16] Amongst all the chromene derivatives, 2-amino-3-cyano-4H-chromene is especially attractive for synthesis because of its high reactivity and excellent medicinal applications such as antioxidant, antimicrobial, anti-inflammatory, antimalarial, antitumor, HIV-fighting and anticancer.[17–21] Also, chromene derivatives shown excellent biological activities like antiproliferative,[22,23] antifungal activities,[24,25] Antiviral activity,[26] antiangiogenic activity.[27] Based on aforementioned information, the present study was attempted to synthesize bioactive chromene derivatives and explore the bioactive potentials of 2-amino-3-cyano-4H-chromene derivatives against the virulence factors of *P. mirabilis*. The effect of chromenes derivatives on the swarming, biofilm formation, urease activity and the mechanism thereof by qPCR was studied in present study.

Table 1. Optimization of the reaction condition for the synthesis of 2-amino-6-chloro-7-hydroxy-4-phenyl-4H-chromene-3-carbonitrile.[a]



1 2 3a 4a

Entry	Catalyst	Amount of catalyst (mol %)	Time (h)	Yield (%) [b]
1	NaOH	10	2	92
2	**NaOH**	**15**	**2**	**96**
3	KOH	10	2	72
4	KOH	15	2	78
5	K_2CO_3	10	3	68
6	K_2CO_3	15	3	70
7	$NaHCO_3$	10	3	62
8	$NaHCO_3$	15	3	66
9	$NaOCOCH_3$	10	3	60
10	$NaOCOCH_3$	15	3	62

[a] Reaction conditions: benzaldehyde (2 mmol), malononitrile (2 mmol), 4-chloro resorcinol (2 mmol) solvent water, 60 °C; [b] isolated yields.

2. Results and Discussion

2.1. Chemistry

The successful synthesis of 2-amino-3-cyano-4H-chromene derivatives with excellent yield was achieved by one pot three component reaction of 4-chloro resorcinol and malononitrile with versatile aldehyde using suitable catalyst in water as greener solvent at 60 °C for the appropriate time. To carry out this reaction, suitable catalyst and reaction medium was studied, and results are presented. Initially optimisation of the reaction condition was carried out in the presence of numerous catalysts via one pot three component reaction of 4-chloro resorcinol (**1**) (2 mmol), malononitrile (**2**) (2 mmol) and benzaldehyde (**3 a**) (2 mmol) in aqueous medium to obtain the product 2-amino-4H-chromene-3-carbonitrile (**4 a**) considered as model reaction (Table 1). To find out the efficiency of catalyst to synthesize chromene, we have explored model reaction with various catalyst such as NaOH, KOH, K_2CO_3, $NaHCO_3$, $NaOCOCH_3$ as catalyst.

Initially, we have used strong inorganic bases as a catalyst to prepare 2-amino-4H-chromene. With the use of NaOH (10 mol %), the product 4a was obtained in 92 % yield (Table 1, entry 1). Whereas the concentration of NaOH was increased up to 15 mol %, the product 4a was obtained in 96 % yield (Table 1, entries 2). Furthermore, we have used KOH (10 and 15 mol %) under same reaction condition, product 4a was obtained in 72 and 78 % yield, respectively (Table 1, entries 3–4). However, the weak inorganic bases K_2CO_3, $NaHCO_3$ and $NaOCOCH_3$ (10 and 15 mol %) showed less catalytic activity and afford lesser yield i. e., 60–70 % (Table 1, entries 5–10) and showed some undesirable spot-on TLC. Out of all the catalyst used for the synthesis of chromene, NaOH (15 mol %) exhibits highest catalytic activity and could afford 96 % yield of the corresponding 2-amino-4H-chromene-3-carbonitrile (**4 a**) (Table 1, entry 2).

To determine optical amount of catalyst, model reaction was performed by using 5, 8,10, 15 and 20 mol % of NaOH and results are summarised in Table 2. It was observed that, good yield of the product 4a was obtained with amount of catalyst less than 20 mol % (Table 2, entries 1–4). More yield (96 %) of the desired product 4a was obtained with 15 mol % of the NaOH (Table 2, entries 4). Further, increase in the amount of NaOH up to 20 mol %, there is no significant effect on product yield (Table 2, entries 5). The model reaction was also carried out using different solvent like methanol, dioxane, dichloromethane, tetrahydrofuran in presence of NaOH at 60 °C, but low yield of product obtained. The study showed that 15 mol % of the NaOH is efficient to prepare the chromene with excellent yield. In conclusion, for the synthesis of 2-amino-4H-chromene-3-carbonitrile **4 a**, NaOH (15 mol %) was used as a catalyst in

Table 2. Effect of amount of catalyst on the model reaction.[a]

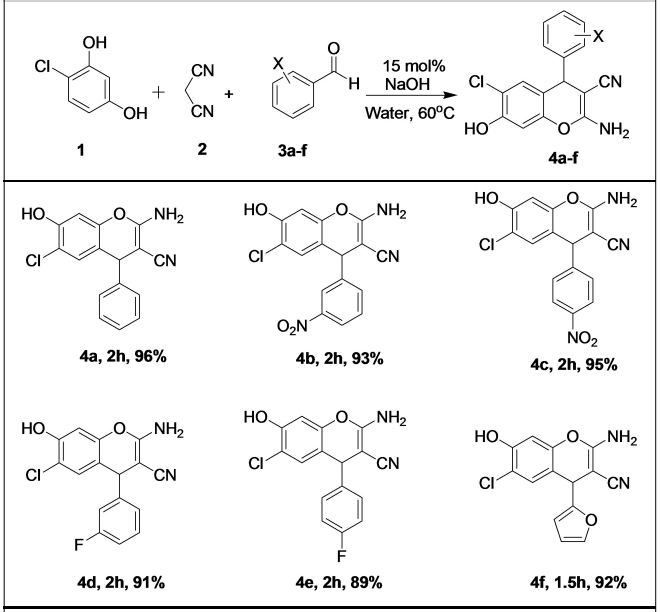
Entry	Catalyst (mol)%	Time (h)	Yield (%) [b]
1	5	2	70
2	8	2	85
3	10	2	90
4	15	2	96
5	20	2	96

[a] Reaction conditions: benzaldehyde (2 mmol), malononitrile (2 mmol), 4-chloro resorcinol (2 mmol) solvent water, 60 °C; [b] isolated yields.

aqueous medium at 60 °C are the appropriate reaction conditions have been optimized.

With the optimised reaction condition in hand, we investigated scope of various aromatic aldehyde for the synthesis of chromene by the one pot three component reaction of activated benzaldehyde with malononitrile and 4-chloro resorcinol using 15 mol% NaOH in aqueous medium for the appropriate time. The results are summarised in Table 3. Initially, reaction was carried out using benzaldehyde to obtain the desired product with 96% yield (Table 3, entry 4a). When strong electron withdrawing group such as nitro group on para and meta position on benzaldehyde are well tolerated and obtained the desired product in 93 and 95% yield, respectively. (Table 3, entries 4b–4c). Further, fluoro group on meta and para position in benzaldehyde also gives the desired product in 91 and 89% yield (Table 3, entries 4d–4e). Also, hetero-aryl benzaldehyde like furfural reacted smoothly with 4-chloro resorcinol and malononitrile to afford the desired product in 92% yields (Table 3, entry 4f). It is observed that different functional groups on the aromatic aldehydes such as halogen, nitro and hetero-aromatic aldehyde smoothly reacted with the malononitrile and 4-chloro resorcinol to afford the desired product 2-amino-3-cyano-4H-chromenes derivatives (**4a**–**4f**) with excellent yields (89 to 96%). The newly synthesized chromene (**4a**–**4f**) were fully characterised by standard spectroscopic techniques such as IR, ^{1}H NMR, ^{13}C NMR and mass spectral analysis.

Table 3. Substrate scope for the synthesis of 2-amino-3-cyano-4H-chromene derivatives.[a]



[a] Reaction conditions: aromatic aldehydes (2 mmol), malononitrile (2 mmol), 4-chloro resorcinol (2 mmol), NaOH (15 mol%), water, 60 °C; [b] isolated yields.

2.2. Biological Assay

2.2.1. Inhibition of Swarming by 2-Amino-3-Cyano-4H-Chromene Derivatives

Swarming activity of *P. mirabilis* is associated with migration of bacteria over the solid surface. *P. mirabilis* form concentric ring like bull's eye patterns on solid agar surfaces due to their swarming ability.[28] In our results, the chromene derivatives inhibited the swarming activity (figure 1). In presence of chromene derivatives (4a–f), the formation of concentric ring like patterns on solid agar surfaces were inhibited, indicating inhibition of swarming activities in *P. mirabilis.* However, in control sample, such concentric ring like patterns were observed on the solid agar surfaces. Till date only a few reports are available on the inhibition of swarming activities in *P. mirabilis.* Essential oils extracted from medicinal plants native to Iran have inhibited swarming behaviours in *P. mirabilis* at 1.25–11.50% w/w or 01.–0.4 mg/mL concentrations.[29] In other reports, resveratrol[30] and *p*-nitrophenyl glycerol[31] and mono-

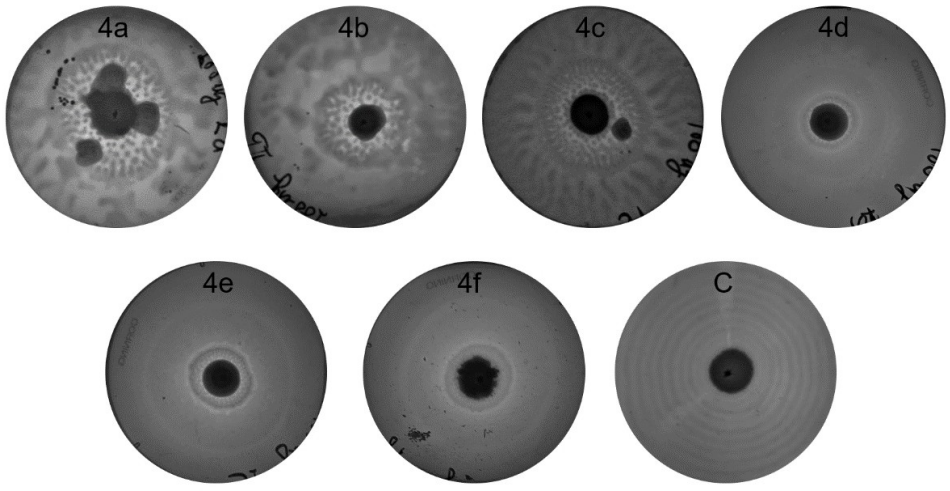


Figure 1. Effect of chromene derivatives on swarming behaviour of *P. mirabilis.* Formation of concentric rings were inhibited in *P. mirabilis* at (100 μg/mL) after 16 h for all derivatives. Control shows concentric rings.

terpenes completely inhibited swarming activity at 60, 200, and 500–750 μg/mL, respectively.

Ours is the first study which explores synthetic small molecules derived from 2-amino-3-cyano-4H-chromene derivatives for inhibiting swarming behaviours in *P. mirabilis*.

2.2.2. Inhibition of Biofilm

Major attribute of pathogenesis of *P. mirabilis* is crystalline biofilm formation.[14] Formation of crystalline biofilm on the catheter surface causes catheter blockage and kidney infection. The bacterial cells present in biofilm can change their proteome to adopt in the sessile state with low metabolic level and down-regulated cellular activities.[32] In mature biofilm, the bacterial cells were embedded in exopolysaccharide which blocks the various drugs before reaching the bacterial cell wall.[33] Due to this, the eradication of mature *P. mirabilis* biofilms are prerequisite. In current study, chromene derivatives 4d and 4e inhibited biofilm with minimum inhibitory concentration value of 100 μg/mL (figure 2). About 40–45% biofilm was inhibited at minimum inhibitory concentration around 100–200 μg/mL. In other word, fluorophenyl substitutions on 2-amino-3-cyano-4H-chromene (4d and 4e) gives potent antibiofilm. Nitrophenyl substitutions on 2-amino-3-cyano-4H-chromene (4b and 4c) are also bioactive, but at higher concentrations. Also, antibiofilm results of chromene derivatives compared with standard drug vancomycin. Vancomycin at 25 ug/mL has showed good antibiofilm activity. Biofilm inhibition assay of 2-amino-3-cyano-4H-chromene derivatives, their MIC values and results of docking studies are shown in Table 4.

2.2.3. Disruption of Preformed Biofilm

The mature biofilm promotes the urinary tract infection and responsible for the urinary stone formation. Therefore, we also studied the disruption of preformed biofilm. Unlike biofilm

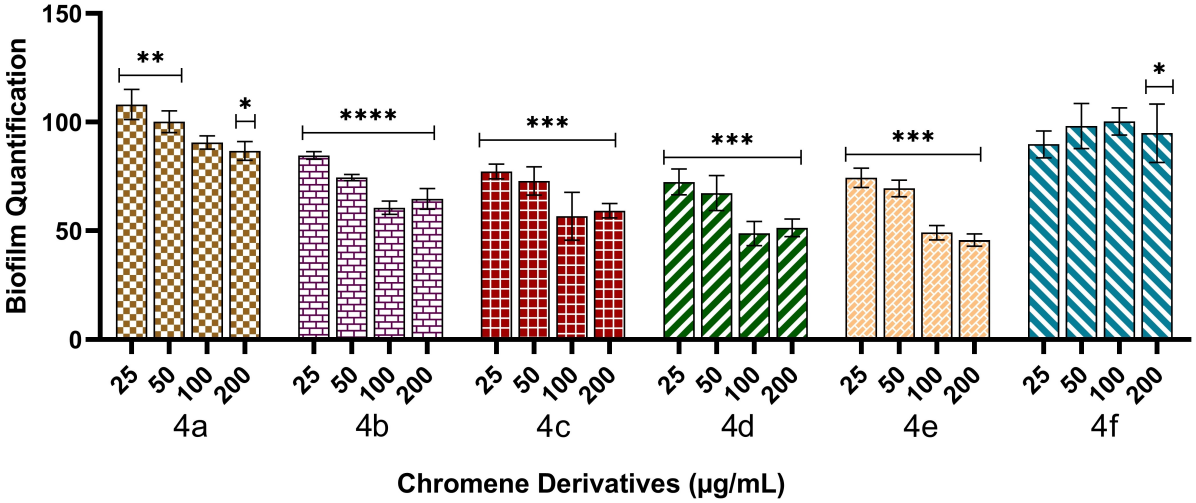


Figure 2. Inhibition of biofilm formation by chromene derivatives at different concentration after 16 h. Biofilm inhibition was assessed by quantifying the metabolic activities of cells by in the biofilm MTT assay. Percent viability is calculated in comparison to control biofilm (without chromene derivative). Data are presented as means ± SD. * indicates statistical significance ($p < 0.05$). Compounds 4d and 4e formed lowest biofilm.

Table 4. Biofilm Inhibition assay and results of docking studies of 2-amino-3-cyano-4H-chromene derivatives.

Comp	Concentration (μg/mL)				MIC Value	Glide Score	Glide binding energy (Kcal/mol)	Hydrogen bonding (Å)	Pi-stacking (Å)
	25	50	100	200					
4a	Inactive	Inactive	Inactive	13.225	>200	−7.321	−41.805	Asp224(2.154), Arg339(2.411)	–
4b	15.260	25.493	39.377	35.308	>200	−7.494	−42.267	Asp224(1.865)	Arg339(2.223), His323(2.483)
4c	22.681	27.049	43.267	40.754	>200	−7.490	−42.155	Asp224(2.011)	Arg339(2.952), His323(2.471)
4d	27.528	32.675	51.226	48.653	100	−8.886	−47.036	Asp224(2.089), Arg339(2.178)	Arg339(2.178), His249(2.804)
4e	25.613	30.460	50.867	54.278	100	−8.925	−48.127	Asp224(1.757), Arg339(2.423)	Arg339(2.424), His249(2.862)
4f	Inactive	Inactive	Inactive	Inactive	>200	−7.553	−43.775	Asp224(2.127), Arg339(2.377), Arg339(2.589)	–

inhibition, the preformed biofilm was inhibited (about 40%) at higher concentration, about equal to 200 μg/mL or more concentrations (figure 3). A higher concentration of chromene derivatives were required because the EPS of the preformed biofilm may hindered the diffusion of derivatives, and eventually the effective concentrations of the bioactive derivatives to act on the cells enclosed inside the EPS.

We further assessed the effect of the most bioactive chromene derivatives (4e) on preformed biofilm by observing the structure of biofilm under scanning electron microscopy (SEM) (figure 4). In SEM, the control biofilm was observed as a thick uniform layer of cells. The observed surface of the biofilm appears smooth due to EPS cover over the cells (cells were embedded in EPS). However, when the preformed biofilm was treated with derivative 4e and observed under SEM we could observed that, towers of cells and surfaces of the towers were rough, i.e., cells were not covered with EPS (EPS production could have been inhibited or EPS were removed due to the treatment with derivative 4e).

Across literature, we could find only few reports on the biofilm inhibition in *P. mirabilis*. Allicin at 32 μg/mL showed the maximum reduction in biofilm development by 33.8% in *P. mirabilis* strain,[34] 0.4 mg/mL ascorbic acid and 0.02 μg/mL rutoside assisted the therapeutic potential of chemotherapeutic agents (conc 1–4 μg/mL) in inhibiting biofilm formation in *P. mirabilis*,[35] and 25–100 mg/mL of ethanol extract of propolis on biofilm formation and concentrations of 25–50 mg/mL of ethanol extract of propolis on formed biofilm has been shown

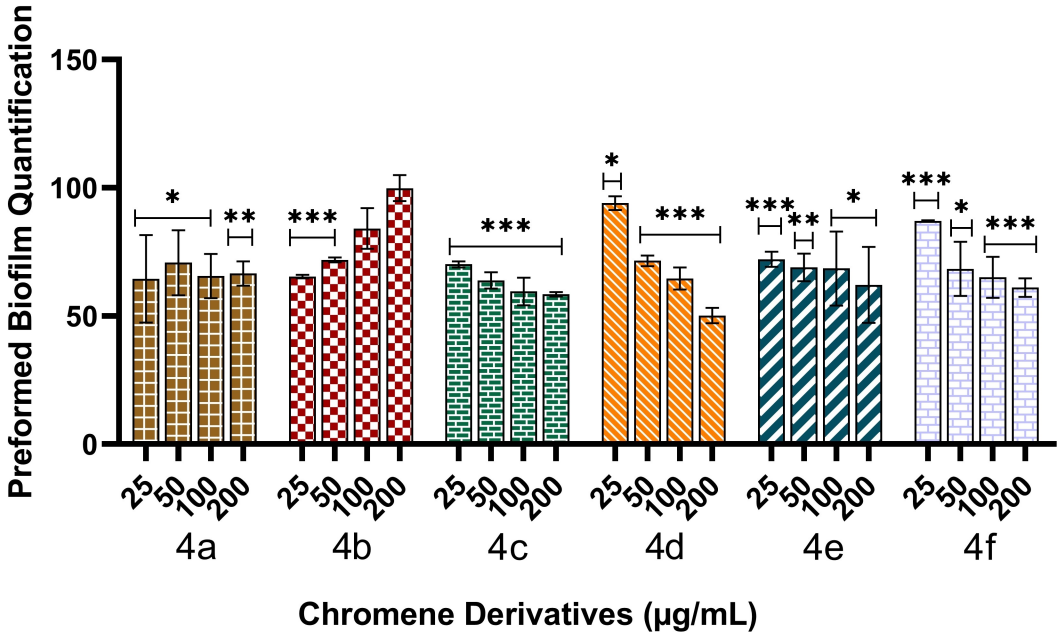


Figure 3. Quantitative assessment of effect of chromene derivative on preformed biofilm. Performed biofilm quantification was performed by calculating percent viability of cells inside the biofilm by MTT assay and is calculated in comparison to control biofilm (without chromene derivatives). Data are presented as means ± SD. * indicates statistical significance ($p < 0.05$). The preformed biofilm in presence of compounds 4d&4e is less. Increase in the % of preformed biofilm at 200 μg/mL for compound 4b may be due to precipitation/colour of the drug and subsequent increase in the optical density due to aggregation/colour of the drug.

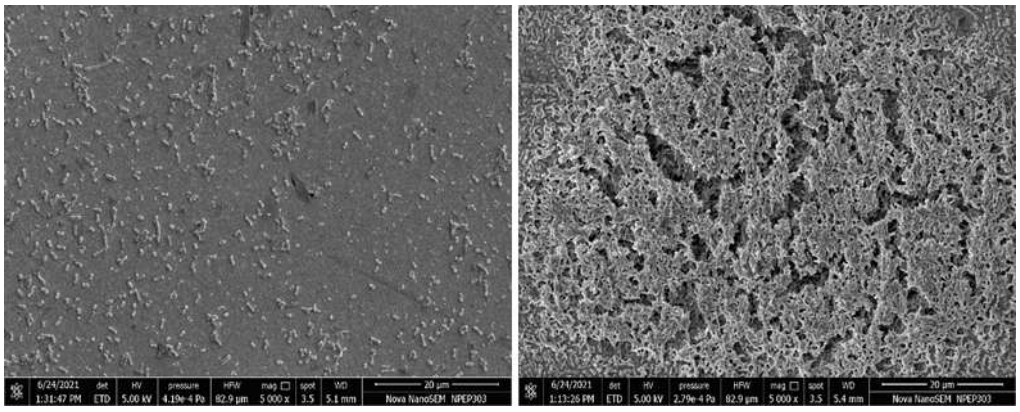


Figure 4. SEM micrographs of the biofilm. Control preformed biofilm (right) and performed biofilm was treated with 4e (left). (5000X magnification, scale 20 μm). Whereas uniform and confluent mass of biofilm in control is seen, rough and irregular patch of aggregated cells is visualized in presence of 4e chromene derivatives.

to be effective in inhibiting biofilm in *P. mirabilis*.[36] In our study, we used synthetic molecules, 2-amino-3-cyano-4H-chromene derivatives, and which has inhibited around 40–45% biofilm (formation or preformed), which is better than the reported studies. However, in comparison to the standard drug vancomycin, the biofilm formation and disruption was better over chromene bioactive derivatives of the study.

2.2.4. Inhibition of Urease Activity by 2-Amino-3-Cyano-4H-Chromene Derivatives

The main function of urease in *P. mirabilis* is to provide nitrogen in the form of ammonia, which increases the pH of the urine, promoting the formation of struvite and apatite crystals. The crystals are embedded in the biofilm of *P. mirabilis* and attach to the catheter's lumen and balloon, preventing urine from flowing into the drainage bag. Often, crystalline biofilm networks are resistant to antibiotics and are difficult to treat. Therefore, we decided to study the urease inhibition activity of the chromene derivatives. As can be seen from figure 5, 4b and 4d inhibited the urease production. In other words, the nitrophenyl and fluorophenyl derivatives in which the nitro or fluoro were present on *meta* position, were most effective in inhibiting the urease activity. In comparison to control (which is considered as 100%), 4b and 4d inhibited urease activity by 40–45%.

Recent studies have shown that most of the synthetic compounds which are structurally similar to urea may act as potent urease inhibitors. Many urease inhibitor synthetic compounds were screened by performing *in-vitro* assay using urease enzyme, however, only synthetic compounds such as aminomethyl phosphonic and aminomethyl (*P*-methyl) phosphinic acids,[37] N-substituted aminomethanephosphonic and aminomethane-*P*-methylphosphinic acids[38] thiobarbiturates[39] were shown to inhibit ureases activity. The inhibition of urease activity has direct link with the reduction in the formation of crystalline biofilm in *P. mirabilis*.

2.2.5. Gene Expression Studies

In order to gain a deeper understanding of the inhibition of virulence factors in *P. mirabilis*, we performed qPCR to analysis the expression of prominent genes which play a significant role in virulence of *P. mirabilis* by using sets of primer as mentioned in Table 5.

A qPCR is a technique, which quantifies the expression of genes. In qPCR the fold change in gene expression was determined by normalizing the expression with respect to RpoA (housekeeping gene). Fold change in the gene expression after treating *P. mirabilis* with chromene derivative 4e is shown as figure 6. In qPCR studies, the expression of fimbrial genes *MrpC* was downregulated, whereas *MrpH* and *PmfA* were upregulated in presence of chromene derivative 4e. The genes responsible for urease activity *UreA*, *UreB*, *UreC* were downregulated. The genes of transporter system, *PstA*, *PstB*, *PstC*, *PhoU* and *LptB* were downregulated.

Fimbrial genes *MrpC, MrpH* and *PmfA*, has been shown to help *P. mirabilis* for adhering to surfaces, and this function is often essential for pathogens to gain a foothold in the host, and inhibiting fimbria-associated genes were shown to interfere with swarming ability of the organism.[40] Whereas the down regulation of *MrpC* is in accordance to our expectation, the up regulations of *MrpH* and *PmfA* is at present needs further investigation. Two component signaling system regulates gene expression in response to external stimuli. Chromene derivatives upregulate the signaling protein *RcsB* and *RcsD*, causing

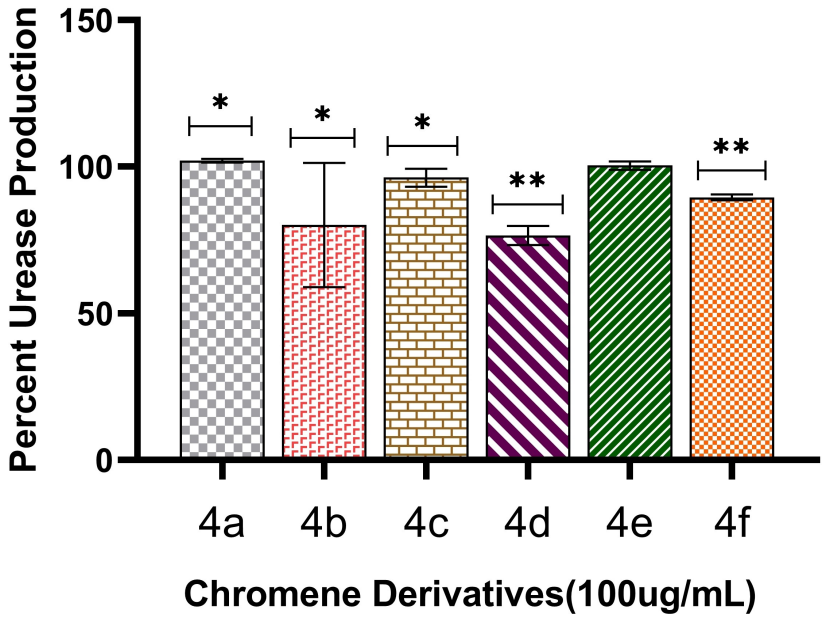


Figure 5. Effect of chromene derivatives on urease activity of *P. mirabilis* after 24 h. Percent urease production of each derivative was compared with urease activity at 24 h and express as ± mean. Statistical significance * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$. Compounds 4b and 4d showed less urease activity.

Table 5. List of virulence factors with specific primers used for real-time PCR.

Virulence factor	Role in Biofilm	Gene	Primer Pair
Fimbriae (MR/P, PMF)	Adherence to bowman's capsule and tubular basement of kidney	*MrpA*	FP TGTTGCGGGTTCTGCTTTAG RP CGAGAATTACTACGGCCACC
		MrpH	FP CTCTGATAGTGGGCGGGTAC RP GAGCTACCCGTCCATTCGT
	Recognition of bladder specific receptor	*PmfA*	FP GGCGCAGGTATTGGCTTAAT RP CTTGGAATTCACCTGGCGTT
Urease	Increase surrounding pH to impel crystalline stone formation	*UreA*	FP GCCTTGATTAGTTGCGCCAT RP CGGGGAAAGTGCACTCTACT
		UreB	FP ACAAGTCGGCTCTCATTACC RP TGCAAAAGCCACTAACTCAACA
		UreC	FP TACACGATTAACCCGGCACT RP ACGATAATGAACCGGTTGCG
RNA-binding proteins	Inhibit swarming and virulence factor expression	*RsmA*	FP GCACGCTTAGAAGTTCACCC RP CCACTAGGCGATTAACCACC
Rcs phosphorelay	Repress the master regulator of flagella FlhDC, Regulate capsule synthesis	*RcsB*	FP CCGACCTATCTATGCCTGGT RP GCTTTAGGTAGATCAGCAGGC
		RcsD	FP CCGCTATCACGCTAACCAAC RP CGTTTGCAAAATAACCGGCT
Pst Transporter	Enhance the biofilm formation	*PstA*	FP TTTGATGGCATGTCTTGGGC RP AACAACAATCGATGGGGCTG
		PstB	FP GCGTGCTGAAGGTGAAATCT RP CATTTCAGCGCGAGACAGTT
		PstC	FP TTGTGGACGAAAGAGTGGGA RP CAAAGAGCGGCGCAAAGATA
		PhoU	FP TCCTTAGGCCAACATGCGAT RP TCAGTACGCTCGGGATAGTG
Lipopoly-saccharide	Maintain structural integrity, helps in biofilm formation	*LptB*	FP GCCGTCGTGTAGAGATAGCA RP TCGCGTACATTATGGTCGGT

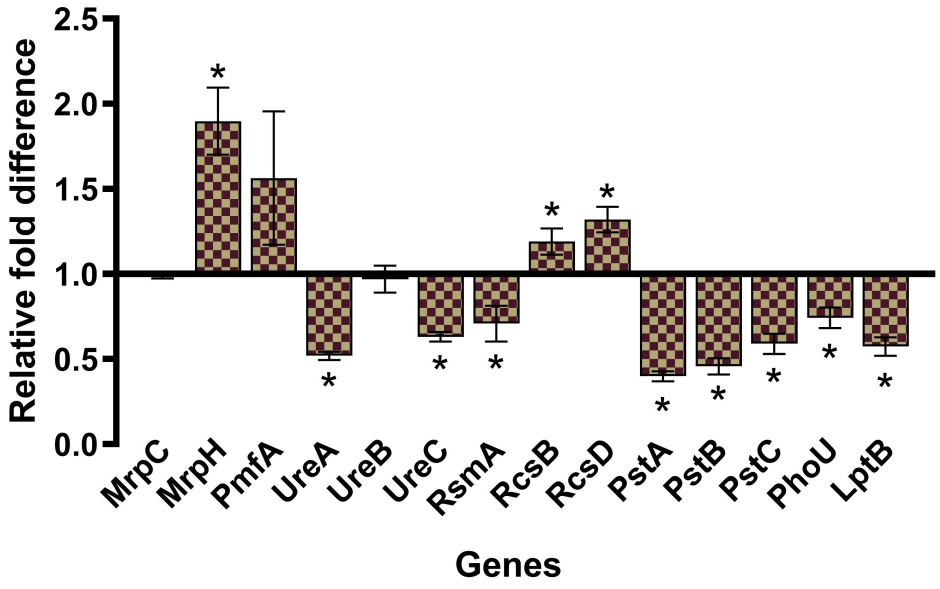


Figure 6. Gene expression analysis of preformed biofilm disruption of *P. mirabilis* in presence of chromene derivative 4e. Expression of genes associated with virulence factors of *P. mirabilis* was assessed. Data are expressed as means ± SD. * indicates statistical significance ($p < 0.05$). X axis indicates the name of gene and Y axis denote relative fold difference in gene expression between 4e treated sample and control. (> 1: upregulated; < 1: downregulated.

them to reduce the swarming activity by repressing the expression of *FlhDC*, the master regulator of swarming motility.[41]

Phosphorous is important component of various biomolecules and component of small cofactor molecules. The Pst transport system is a phosphate uptake mechanism which is

induced at low extracellular Pi concentration. Pst transport system is member of ABC transporters. The Peptide PhoU act as negative regulator of Pho-regulon which lies upstream to Pst operon[42], Pst transporter involved in various pathogenic factors include regulation of invasion,[43] colonization[44,45] biofilm formation,[46] and antibiotic resistance.[47] Mutation in genes involved in Pst transport system shows abnormal biofilm development.[42] The downregulation of Pst genes in presence of chromene derivative indicates that, the chromene were inhibiting the biofilm development in *P. mirabilis.* The gene UreABC encodes for α, β and γ which are three major components of Urease. In this study, urease activity of *P. mirabilis* is moderately affected in presence of urease. In gene expression analysis, these genes are downregulated in presence of chromene derivative 4e which shows chromene reducing urease activity and the subsequent pathogenicity of *P. mirabilis.*

2.3. Computational Study

2.3.1. Molecular Docking Study

In order to rationalize the observed antimicrobial activity and gain an insight into the mechanism of inhibition, molecular docking of novel substituted 2-amino-3-cyno-4H-chromene derivatives (4a–4f) onto the crystal structure of urease was carried out. Since the crystal structures of *P. mirabilis* urease are not available, however, the active site is well conserved, the crystal structure of urease (PDB ID: 4UBP) from *Sporosarcina pasteurii* (S. pasteurii) was chosen for docking. A perusal of docking solutions indicates that all the chromene derivatives (4a–4f) were embedded well into the active site of urease at co-ordinates close to the co-crystallized ligand producing an average docking score of −7.944 and binding energy of −44.194 kcal/mol. Also, their binding affinities corroborated with the observed antimicrobial activities (Table 4). A detailed investigation of per-residue interaction could shed light on the crucial thermodynamic interactions influencing the binding affinities which is elaborated for one of the most active analog 4e in next section.

Visual inspection of the lowest energy docked conformation of 4e showed that it could snugly fit at the active site of Urease with a significant higher binding affinity (Glide score of −8.925 and Glide binding energy: −48.127 kcal/mol (Figure 7). The major driving force for the mechanical interlocking of 4e was seen to be a network of favourable van der Waals interactions with 2-amino-3-cyano-4H-chromene scaffold through Ala366(−2.878 kcal/mol), Arg339(−1.519 kcal/mol), His323(−3.121 kcal/mol), Cys322(−3.687 kcal/mol), Asp224(−1.522 kcal/mol), Ala170(−2.338 kcal/mol), Lys169(−3.589 kcal/mol) and Glu166(−1.062 kcal/mol) residues while the 4-fluro substituted phenyl side chain engaged in similar type of interactions with Asp363(−1.419 kcal/mol), Gly280(−1.109 kcal/mol), His275(−1.138 kcal/mol), His249(−1.964 kcal/mol) and His222(−1.917 kcal/mol) residues lining the active site of enzyme. The higher binding affinity observed for 4e is also attributed to favourable electrostatic interactions with Met367(−2.611 kcal/mol), Arg339(−3.387 kcal/mol), Cys232(−1.718 kcal/mol), His249(−1.031 kcal/mol), Asp224(−5.962 kcal/mol), Lys169(−1.538 kcal/mol) and Glu166(−1.522 kcal/mol) residues. Along with these non-bonded interactions, 4e was also seen to be engaged in a close hydrogen bonding interactions with Asp224(1.757 Å) through the amino group (−NH−) on the chromene nucleus while the cyano (−CN−) group was engaged in a hydrogen bond with Arg339(2.423 Å) residue. Furthermore, a very prominent pi-pi (π-π) stacking interaction was also observed through fluoro substituted phenyl ring with Arg339(2.424 Å) and His249(2.862 Å) residues which along with hydrogen bonding interactions serve as ''anchor'' to guide the orientation of the ligand into the 3D space of active site. Analysis of the docked conformations of other molecules in the series (Figures 1S–5S) revealed similar bonded and non-bonded interactions which suggest that the 2-amino-3-cyno-4H-chromene scaffold could serve as a pertinent starting point for further optimization to arrive at leads with potent antimicrobial activity.

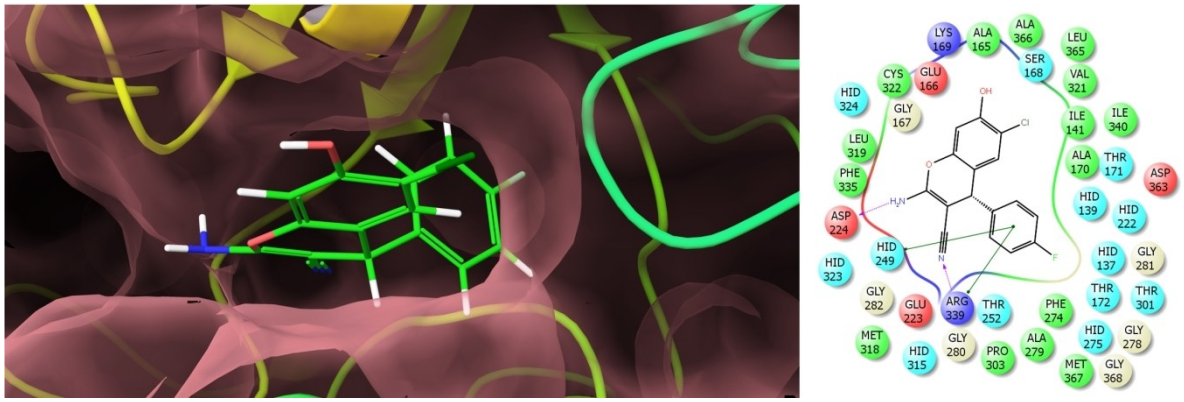


Figure 7. Compound **4e** docked at the active site of urease; 2D interaction diagram on right side showing the hydrogen bonding and pi-pi stacking interactions.

3. Conclusions

In summary, we presented an efficient, mild and rapid method for the synthesis of novel substituted 2-amino-3-cyano-4H-chromene derivatives via one pot three component reaction of substituted aldehyde, malononitrile and 4-chloro resorcinol. Mainly 2H and 4H chromene ring has been used as a template for designing and developing new scaffolds which have potential application in biological activity. Knoevenagel condensation, aldol condensation and Michael addition synthetic method are mostly commonly used for the synthesis of 4H-chromene because of ready availability of starting materials. All the synthesized chromene derivatives evaluated for the bioactivity on the virulence factors of *P. mirabilis.* Furthermore, present work demonstrated that compound 4e could effectively inhibit biofilm formation and biofilm disruption of *P. mirabilis.* Also, all the synthesised compound showing anti-swarming activity and urease activity is moderately inhibited by 2-amino-3-cyano-4H-chromene derivatives. Molecular docking against urease enzyme was found to be agreement with the observed antimicrobial activity and could provide insights into the binding modes of these molecules. The per-residue interaction analysis could identify the most significant thermodynamic interactions which could use to carry out site specific modifications of the 4H-chromene scaffold to arrive at compounds with higher affinity and potency.

Supporting Information

Supporting pdf file contains experimental procedure for the synthesis of compounds 4a–f, experimental protocol for biological activities and computational studies and spectral data (IR, ^{1}H NMR, ^{13}C NMR and HRMS spectra) of the compounds and Molecular docking figures (1S to 5S). The authors have cited additional references within the Supporting Information.[50–59]

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Conflict of Interests

The Authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: 4H-chromene · *Proteus mirabilis* · biofilm formation · urease activity · molecular docking

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