Hybrid N-Acylcysteines as Dual-Acting Matrix Disruptive and Anti-Quorum Sensing Agents Fighting *Pseudomonas aeruginosa* Biofilms: Design, Synthesis, Molecular Docking Studies, and *In Vitro* Assays

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**ABSTRACT:** Biofilms facilitate the pathogenesis of life-threatening *Pseudomonas aeruginosa* infections by coating mucosal surfaces or invasive devices and offer protection from antimicrobial therapy and the host immune response, thus increasing mortality rates and financial burden. Herein, new hybrid N-acylcysteines (NAC) incorporating selected acyl groups from organic acids and their derivatives, which are capable of quenching pathogen quorum sensing (QS) systems, were designed and their antibiofilm activity and anti-QS were evaluated. N-acylcysteines (4a–h) were synthesized and characterized by 1H NMR and 13C NMR, and their purity was confirmed by elemental analyses. N-(4-Hydroxy-3,5-dimethoxybenzoyl)-L-cysteine (4d) and N-(4-methoxybenzoyl)-L-cysteine (4h) showed a higher antibiofilm activity against PAO1 biofilms than the rest of the targets and the standard NAC. They showed 83 and 82% inhibition of biofilms at 5 mM and eradicated mature biofilms at 20 mM concentrations (NAC biofilm inhibition = 66% at 10 mM and minimum biofilm eradication concentration = 40 mM). This was confirmed via visualizing adherent biofilm cells on catheter pieces using scanning electron microscopy. In the same vein, both 4d and 4h showed the highest docking score with the QS signal receptor protein LasR (−7.8), which was much higher than that of NAC (−5) but less than the score of the natural agonist N-(3-oxododecanoyl)-L-homoserine (OdDHL) (−8.5). Target 4h (5 mM) decreased the expression of quorum sensing encoding genes in *P. aeruginosa* PAO1 strain by 53% for *pslA*, 47% for *lasI* and *lasR*, and 29% for *filC*, lowered PAO1 pyocyanin production by 76.43%, completely blocked the proteolytic activity of PAO1, and did not affect PAO1 cell viability. Targets 4d and 4h may find applications for the prevention and treatment of biofilm-mediated *P. aeruginosa* local infections of the skin, eye, and wounds. N-(4-Methoxybenzoyl)-L-cysteine 4h is a promising dual-acting matrix disruptive and anti-QS antibiofilm agent for further investigation and optimization.

1. **INTRODUCTION**

Microbial biofilms are formed when microbial cells are encased in a multicomponent extracellular matrix containing polysaccharides, proteins, and nucleic acids ([Figure 1](#)). Microorganisms often live in biofilms, which provide many advantages to their inhabitants: (i) they serve as a matrix for the exchange of metabolites, genetic units, and signaling molecules between cells and (ii) they protect their inhabitants from their natural predators and antimicrobial agents. Microbial biofilms are involved in the majority of infections that are related to mechanical ventilators, urinary catheters, and intravascular devices as well as chronic wound infections. Such biofilms complicate the treatment of microbial infections by improving microbial ability to evade the host immunity and resist the action...
of antimicrobial chemotherapeutics. The biofilm-mediated microbial resistance may be attributed to the hindered penetration of antimicrobial agents across the matrix of the biofilm and reduced oxygen and nutrients content, in addition to a slower metabolism. In addition, persister cells, high bacterial cell density, and activation of the general stress response are other factors that might contribute to the biofilm-mediated microbial resistance.

*Pseudomonas aeruginosa* is a highly life-threatening Gram-negative bacterium. It causes fatal nosocomial infections especially in hospitalized and immunocompromised patients. In 2017, WHO listed *P. aeruginosa* as one of the top priority pathogens representing an urgent threat that necessitates extensive research for the discovery of novel antibiotics. Biofilms facilitate the pathogenesis of *P. aeruginosa* infections by coating mucosal surfaces or invasive devices and offer protection from antimicrobial therapy and the host immune response, thus increasing mortality rates and financial burden.

Cystic fibrosis patients are liable to a common biofilm-mediated chronic lung infection caused mainly by *P. aeruginosa*. In patients with burn wound infections, *P. aeruginosa* is a common etiological agent that causes morbidity. *P. aeruginosa* biofilms were found on contact lenses as well as lens storage cases in wearers suffering from microbial keratitis. At comparable concentrations, NAC (N-acetylcysteine) was able to treat chronic wounds before and after the formation of *P. aeruginosa* biofilms. It has been estimated that biofilms are associated with poor wound healing in millions of patients in the USA, resulting in economic consequences estimated at over $25 billion annually.

NAC is a drug with mucolytic activity and has been reported as a matrix disruptive antibiofilm agent. NAC was reported as an antibiofilm agent capable of hindering the production of biofilms by different Gram-negative bacteria such as *Escherichia coli*, *Klebsiella pneumonia*, *Enterobacter cloacae*, *Proteus spp.*, *Pseudomonas mendocina*, *Acinetobacter baumannii*, *Prevotella intermedia*, *P. aeruginosa*, and quinolone-resistant *P. aeruginosa*. NAC also showed antibiofilm activity toward Gram-positive bacteria including *Enterococcus faecalis*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* as well as yeast, e.g., *Candida albicans*. The antibiofilm activity of NAC is mediated by the prevention of the production of extracellular polysaccharide (EPS) matrixes and characterized by reduction of biofilm mass. NAC was also able to promote the disruption of mature biofilms.

NAC has three functional groups: (i) sulphurhydryl group (−SH), (ii) acetamido group (−NHCOCOCH₃), and (iii) carboxylic acid (−COOH). The sulphurhydryl group of NAC is very important for its mucolytic where its interaction with the disulphide bonds of mucoproteins breaks bronchial mucus into a more fluid form. The sulphurhydryl group is important for the antimicrobial activity of NAC, which might be mediated by the competitive inhibition of bacterial utilization of cysteine or by the reaction of its sulphurhydryl group with bacterial membranes. The conversion of the NAC sulphurhydryl group to a thioether bond with poly(vinylsilsesquioxanes) and poly(methylvinylsiloxanes) exhibited a lower antibacterial activity. Nonetheless, both polymers retained NAC ability to eradicate mature biofilms.

Despite the scarcity of medicinal chemistry research on NAC, the following SAR map of NAC was concluded based on previous literature reports (Figure 2). Martins-Green et al. compared the antibiofilm activity of NAC to analogous compounds, namely, N-acetylserine, glutathione, cysteine (Cys), and N-acetylcysteine amide (NACA). They concluded that the acetyl and carboxylic acid functional groups are very important for the antibiofilm activity of NAC, which might be mediated by the competitive inhibition of bacterial utilization of cysteine or by the reaction of its sulphurhydryl group with bacterial membranes. The replacement of the NAC sulphurhydryl group by a thioether bond with poly(vinylsilsesquioxanes) and poly(methylvinylsiloxanes) exhibited a lower antibacterial activity. Nonetheless, both polymers retained NAC ability to eradicate mature biofilms.

Organic acids and their derivatives were reported as agents capable of quenching pathogen quorum sensing (QS) machinery that is vital for biofilm formation. Vivanco and

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**Figure 1.** Composition of the EPS matrix: polysaccharides, enzymes, structural proteins, eDNA, and lipids. Reproduced with permission from ref 11. Copyright [2020/Frontiers] [Frontiers In Microbiology/Frontiers].

**Figure 2.** Importance of functional groups for the antibiofilm activity of NAC.
co-workers reported that salicylic acid and benzoic acid (Figure 3A,B) downregulated the virulence factors (pyocyanin production as well as elastase and proteases activities) of P. aeruginosa.

Castillo-Juarez et al. demonstrated that 2-amino-6-chlorobenzoic acid (Figure 3C) and other anthranilic acid analogs interfered with pyocyanin and elastase production and enhanced the survival of infected mice via competitive inhibition of PqsA, a coenzyme A ligase, which represents the first step among other steps in the QS system of P. aeruginosa.

Syringic acid (Figure 3D) and other phenolic carboxylic acids were reported as QS inhibitors that can modulate N-acylhomoserine lactone (AHLs) activity and reduce EPS.

4-Methoxybenzoic acid (Figure 3E) was able to (i) inhibit QS-mediated virulence factors including protease, elastase, and pyocyanin, (ii) reduce biofilm matrixes, and (iii) inhibit DNA transcripts of P. aeruginosa.

4-Hydroxybenzoic acid is a phenolic acid found in many medicinal plants (Figure 3F) and was able to regulate Staphylococcus biofilm formation and viability and is a possible virulence attenuating agent that could suppress T3SS of Pst DC3000. 4-Chlorobenzoic acid (Figure 3G) decreased the viability of Gram-positive and Gram-negative bacteria. In addition, ethyl-2-((4-chlorobenzoyl) acetate (Figure 3H) quenched quorum sensing machinery via competition with N-acyl homoserine lactones.

Since NAC did not previously show quorum sensing inhibitory activity, we aimed at the replacement of its acetyl group with acyl groups from known inhibitors of bacterial quorum sensing such as organic acids and a β-ketoester (Figure 4) to produce dual-acting hybrid targets 4a–h. These hybrid N-acylcysteines 4a–h carry the structural features of NAC (—COOH, —SH, and —NHCO—) and aroyl groups from QS quenchers. Since N-acylbenzotriazoles can selectively acylate the amino and the thiol groups of cysteine, they were used in the preparation of N-acylcysteines 4a–h.

The antibacterial activity (MICs) of targets 4a–h and the reference NAC were determined against P. aeruginosa ATCC 9027 (a biofilm-producing standard strain) using the broth microdilution method according to CLSI. Then, the ability of sub-MICs of the targets to inhibit P. aeruginosa biofilm formation and eradication was assessed. To investigate the effect of the new aroyl groups on anti-QS activity, targets 4a–h were docked in QS signal receptor protein LasR and their scores were compared with the natural ligand N-(3-oxododecanoyl)-L-homoserine. The target that showed the highest antibiofilm activity and the best binding mode (4h) was examined to explore its ability to inhibit P. aeruginosa virulence factors, namely, pyocyanin production as well as protease activity. Also, the effect of the most active target on the expression of genes encoding quorum sensing was assessed by quantitative RT-PCR.

2. RESULTS AND DISCUSSION

2.1. Chemistry.

2.1.1. Preparation of N-Acylcysteines 4a–h. N-Acylbenzotriazoles 3a–h intermediates were prepared in 76–90% yield via the reaction of carboxylic acids 2a–h with 1H-benzotriazole according to reported procedures. 4-Methoxybenzoic acid (Figure 3E) was able to (i) inhibit QS-mediated virulence factors including protease, elastase, and pyocyanin, (ii) reduce biofilm matrixes, and (iii) inhibit DNA transcripts of P. aeruginosa. 4-Hydroxybenzoic acid is a phenolic acid found in many medicinal plants (Figure 3F) and was able to regulate

Figure 4. Design of novel N-acylcysteines 4a–h.
initial S-acylation of cysteine followed by N- to S-acyl transfer by a five-membered transition state, thus decreasing the possibility of diacylation. Interestingly, targets (4c and 4d) were prepared in excellent yields (93 and 91%, respectively) without the need for protection of the free amino group of 3c and 3d. Likewise, targets 4f and 4h were prepared in 91 and 87% yields, respectively, without the need for protection of the phenolic hydroxyl groups of 3f and 3h.

2.1.2. Molecular Docking Studies. AutoDock VINA implicated in the PyRx 0.8 tool was used to examine the molecular docking interaction of the synthesized N-acylcysteine 4a–h and QS receptor. The crystal structure (PDB code: 2UV0) of the QS signal receptor protein LasR with the agonist N-(3-oxododecanoyl)-L-homoserine (OdDHL) was used for this study. The docking reliability was supported by docking back the normal agonist OdDHL into the protein as a control. The docking scores are given in Table 1, where the produced docked complexes were investigated based on binding affinities values (kcal/mol) and the pattern of bonding interaction (electrostatic, hydrogen, and hydrophobic).

Docking of OdDHL into the LasR receptor gave a pose that form the reported conventional hydrogen bonds along with other types of interactions with Thr75, Ser129, Tyr56, Tyr 93, Asp 73, and Trp60 in the X-ray structure; also, the long acyl chain extends into the cavity lined with hydrophobic residues, e.g., Leu-40, Tyr-47, Cys-79, Gly-126, Ala-127, Ala-70, Ala-127, Gly-38, and Thr115 (Figure 5).

Results presented in Table 1 show desirable binding energies with LasR and reveal how the substitution pattern on the benzoyl part is likely to control binding to LasR, which could explain their effects as quorum sensing inhibitors. N-Benzoylcysteine formed only one H-bond with Arg 61; it occupied the same core as the native ligand lactone ring and showed electrostatic interaction with Asp 73, Ser129, Thr75, Tyr 56, Leu 36, Tyr64, and Ala127 (Figure 6A). In contrast to

Table 1. Molecular Docking Scores of N-Acylcysteines 4a–h

| compound | docking score (kcal/mol) |
|----------|-------------------------|
| 4a       | -6.9                    |
| 4b       | -7.6                    |
| 4c       | -7.1                    |
| 4d       | -7.8                    |
| 4e       | -6.7                    |
| 4f       | 7.2                     |
| 4g       | -7.4                    |
| 4h       | -7.8                    |
| NAC      | -5                      |
| ref      | -8.5                    |

Figure 5. 3D representation of OdDHL in the LasR receptor.
the native ligand, the cysteine portion of the designed compounds extended in the hydrophobic cavity of LasR, forming two hydrogen bonds with Tyr 47 and Gly38, in addition to van der Waals interactions with Leu-40, Gly-126, Ile-52, Leu-125, and Leu-39. This new mode of interaction may prevent the correct formation of the hydrophobic core of LasR.

Figure 6. (A) 3D visualization of compound 4a in the LasR receptor, (B) 3D visualization of compound 4b in the LasR receptor, and (C) 3D visualization of compound 4f in the LasR receptor.

Figure 7. (A) 3D visualization of compound 4c in the LasR receptor, (B) 3D visualization of compound 4g in the LasR receptor, and (C) 3D visualization of compound 4h in the LasR receptor.
which is mediated by the long acyl chain of the native inducer that controls the folding of the central $\beta$-sheet. This could result in an unstable protein and the observed QSI activity. Introducing amino or hydroxyl groups at the 4-position of the benzoyl ring led to a higher docking score ($-7.6$ and $-7.2$ kcal/mol, respectively) compared to the unsubstituted analog ($-6.9$ kcal/mol). Like $N$-benzoylcysteine, $4C$, $4h$ (Figure 6B,C) formed one H-bond with Arg 61; also, $\pi$-anion and $\pi$-$\pi$ stacked interactions with Asp 73 and Tyr 64 were observed in both compounds side by side with various van der Waals interactions. Amino group protons participate in an unfavorable donor–donor interaction with Thr 75 and Tyr 56, while the hydroxyl group forms a H-bond with Thr 75, Thr 115, and Ser 129.

The methoxy group at the para position of the benzoyl part exhibited the best docking score of $-7.8$ kcal/mol. A comparative analysis of the pocket interactions revealed $\pi$–anion interactions between $4h$ and Asp 73 while $\pi$–$\pi$ stacked interaction with Tyr 64. Additionally, $\pi$–alkyl interaction was elicited with Leu 36 and Ala 127 while a H-bond was observed with Arg 61 and Thr 115 (Figure 7A). In addition to these interactions, several van der Waals interactions collectively anchor compound $4h$ within a LasR binding pocket. Introducing chloride at the para position (Figure 7B) of compound $4g$ gives a score of $-7.4$ kcal/mol, which is near to that of the amino, hydroxyl, or even methoxy groups, thus reflecting the ability of the LasR pocket to accommodate these types of substitutions.

Additional substitution with an amino group at the ortho position of compound $4g$ as in the case of compound $4c$ lowered the docking score to $7.1$ kcal/mol and hindered the formation of a hydrogen bond with Tyr 47 observed with $4g$ while keeping other patterns of interactions like $4g$ (Figure 7c). This may reflect that substitution at the ortho position is not optimum for activity; this observation is strengthened by examining the interaction of compound $4e$, which has only a hydroxyl group at the ortho position of the benzoyl part with LasR. In compound $4e$, the docking score decreased to $6.7$ kcal/mol and formed one H-bond with Arg 61 via its hydroxyl group, not with the carbonyl group of the benzoyl part like the rest of the designed compound, thus preventing anchoring the compound deeply in the LasR pocket occupied with the lactone ring part of the autoinducer compared to analog $4f$ with the hydroxyl group in the para position (Figure 8); thus, it cannot form H-bonds with Thr75, Thr115, and Ser129.

Target $4d$ has a higher docking score of $-7.8$ kcal/mol compared to $4f$, indicating that the additional 3,5-dimethoxy groups maintained a better binding pattern with LasR (Figure 9).

![Figure 8](https://doi.org/10.1021/acsomega.2c01667) Overlapping poses of $4e$ (yellow) and $4f$ (cyan) in the LasR receptor.

![Figure 9](https://doi.org/10.1021/acsomega.2c01667) 3D representation of intermolecular interactions exhibited by $3e$.

### 2.2. Microbiology

#### 2.2.1. Antibacterial Activity of $N$-Acetyl-l-cysteine and Synthetic Derivatives against *P. aeruginosa* (PAO1)

$N$-Acetyl-l-cysteine showed bacteriostatic activity against PAO1 at 40 mM. The synthetic derivatives showed variable bacteriostatic activities. Compounds $4d$ and $4h$ exhibited higher bacteriostatic activities than NAC. Their MIC values were lower (20 mM) than that of NAC (40 mM). The rest of the targets showed similar MIC values to NAC (40 mM) (Table 2).

**Table 2. Antibacterial and Biofilm-Inhibiting Activities of NAC and Targets 4a–h**

| compounds | MIC (mM) | 1/4 MIC (mM) | OD$_{590}$ (mean ± SD) | % inhibition of biofilm formation |
|-----------|----------|--------------|------------------------|---------------------------------|
| control (no drug) | NA | 0.452 ± 0.005 | NA |
| NAC | 40 | 0.152 ± 0.008 | 66.33% |
| 4a | 40 | 0.138 ± 0.008 | 69.51% |
| 4b | 40 | 0.266 ± 0.003 | 42.55% |
| 4c | 40 | 0.267 ± 0.010 | 41.14% |
| 4d | 20 | 0.079 ± 0.004 | 82.62% |
| 4e | 40 | 0.169 ± 0.002 | 62.73% |
| 4f | 40 | 0.134 ± 0.005 | 70.33% |
| 4g | 40 | 0.095 ± 0.0005 | 79.08% |
| 4h | 20 | 0.0863 ± 0.0012 | 81.93% |

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the acetyl group of NAC with the benzoyl group of 4a led to 70% inhibition of biofilm formation, which is comparable to the antibiofilm activity of NAC (66% inhibition) at 10 mM. Substitution of the benzoyl group with 3,5-dimethoxy-4-hydroxy (4d) and 4-methoxy (4h) substituents resulted in the most potent compounds in the series wherein they inhibited biofilm formation by 82 and 83%, respectively, at 1/2 the concentration of 4b and NAC (5 mM). Interestingly, both 4d and 4h showed the highest docking score (~7.8) in the QS signal receptor protein LasR. While the substitution of the benzoyl group with the 4-hydroxy group gave equipotent compounds MBEC (mM) for the tested sample and the untreated one. Target 4h reduced the production of pyocyanin by 76.43% (Figure 11B).

2.2.6. Target 4h Reduced Pyocyanin Production. Pyocyanin (PCN) is a characteristic pigment that *P. aeruginosa* produces as a secondary metabolite. PCN facilitates biofilm formation via an eDNA-related mechanism. The ability of *P. aeruginosa* to produce its characteristic pyocyanin pigment was assessed both in the treated sample and the untreated one. Target 4h reduced the production of pyocyanin by 76.43% (Figure 11B).

2.2.7. Effect of Target 4h on PAO1 Protease Activity. To assess the inhibiting activity of 4h against protease activity in *P. aeruginosa* PAO1, the supernatants of treated and untreated cultures were added to the wells in skim milk agar plates and the clear zones due to protease activity were measured. Interestingly, 4h blocked entirely the protease activity of PAO1 at 5 mM (Figure 11C).

2.2.8. Target 4h Downregulated the Expression of Quorum Sensing Genes in *P. aeruginosa*. The impact of 4h on the expression of *P. aeruginosa* PAO1 virulence genes, namely, *pslA*, *lasI lasR*, and *filC*, was evaluated by employing qRT-PCR (Figure 11D). The 2-ΔΔCt method was applied to compare the expression of QS genes in both 4h-treated and untreated PAO1. Interestingly, the expression of all tested genes was significantly reduced in the presence of 5 mM 4h compared to the control PAO1 culture. The downregulation of the genes was 53% for *pslA*, 47% for each of *lasI* and *lasR*, and 29% for *filC*.

### 3. CONCLUSIONS

In conclusion, the hybridization of substituted benzoic acid and its derivatives with NAC improved its antibiofilm activity and enhanced its anti-QS activity as shown by molecular docking and *in vitro* studies. In the current work, *N-(4-methoxybenzoyl)-cysteine* (4h) was the most promising target, showing the best docking score, binding mode, and % inhibition of PAO1 biofilm production and the capability of disrupting mature PAO1 biofilms at 20 mM. Target 4h is also able to downregulate quorum sensing encoding genes in *P. aeruginosa* by 53% *pslA*, 47% for *lasI* and *lasR*, and 29% for *filC*, reduce pyocyanin production, and completely block protease activity of PAO1. The results reported herein pave the way for future follow-up studies for the application of 4d and 4h in local biofilm-mediated *P. aeruginosa* infections. In addition, more N-acylcysteines can be synthesized in search of optimized potency.

### 4. EXPERIMENTAL SECTION

4.1. Chemistry. 4.1.1. General. Solvents and fine chemicals were obtained from commercial sources and utilized with no additional purification. A Fisher melting apparatus was used to determine melting points that are uncorrected. A JEOL a 500 MHz NMR spectrometer at the Faculty of Science, Mansoura University, was used to record the 1H NMR (500 MHz) and 13C NMR (125 MHz) spectra using DMSO-d_6, D_2O, and CDCl_3 as solvents. On the other hand, a Bruker 400 MHz NMR spectrometer at the Faculty of Science, Zagazig University, was used to record the 1H NMR (400 MHz) and 13C NMR (100 MHz) spectra using DMSO-d_6 as a solvent. Chemical shifts (δ) are given in ppm, and coupling constants (J) are reported in Hz. Elemental analyses were performed at the Regional Center for Mycology & Biotechnology, Al-Azhar University, on a Thermo

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**Table 3. Biofilm-Eradicating Activities of NAC and Selected Derivatives**

| compounds | MBEC (mM) |
|-----------|-----------|
| NAC       | 40        |
| 4a        | 40        |
| 4d        | 20        |
| 4f        | 40        |
| 4g        | 40        |
| 4h        | 20        |
Figure 10. SEM visualization of biofilms in the presence and absence of MBECs of tested agents. The tested agents could inhibit biofilm formation as seen from the very few or nearly absent biofilm cells (×5000) as compared to the compact cells embedded in the biofilm matrix in the case of the control PAO1 biofilm.

Figure 11. (A) Effect of 4h (5 mM) on PAO1 growth. (B) Effect of 4h (5 mM) against pyocyanin production in P. aeruginosa. (C) Effect of 4h against protease activity in P. aeruginosa. (D) Downregulation of QS encoding genes by RT-qPCR in the presence of 5 mM 4h compared to untreated PAO1.
Synthesis of N-Acylcysteines 4a–h. N-Acylbenzotriazoles were synthesized according to reported procedures. The NMR spectra of the reported N-acetylbenzotriazoles and their melting points were consistent with their reported values.

4.1.2. Procedure for the Synthesis of N-Acylcysteines 4a–h. A solution of N-acetyl/arylacetylbenzotriazole (1 mmol) in acetonitrile (7.5 mL) was added to a solution of l-cysteine (0.121 g, 1 mmol) in water (2.5 mL) and triethylamine (0.14 mL, 1 mmol). The reaction mixture was stirred for 1.5–2 h until the disappearance of N-acetyl/arylacetylbenzotriazole spot from TLC. The solvent was evaporated under reduced pressure. Water (5 mL) was added to the residue and extracted with ethyl acetate (10 mL, 3x). The ethyl acetate layer was washed with 2 N HCl and extracted with ethyl acetate (10 mL, 3x). The ethyl acetate layer was then added to CH2Cl2, and the precipitate formed was dried under vacuum to afford targets 3a–h.

1H-Benzo[d][1,2,3]triazol-1-yl-(4-hydroxy-3,5-dimethoxyphenyl) Methanone (3d). White microcrystals, yield 0.227 g (76%); mp 109–111 °C. 1H NMR (500 MHz, DMSO-d6) δ 9.80 (s, 1H, OH), 8.28 (d, J = 8.0 Hz, 1H, Ar-H), 8.26 (d, J = 8.0 Hz, 1H, Ar-H), 7.81 (t, J = 7.8 Hz, 1H, Ar-H), 7.64 (t, J = 7.8 Hz, 1H, Ar-H), 7.52 (s, 2H, Ar-H), 3.85 (s, 3H, OCH3). 13C NMR (125 MHz, DMSO-d6) δ 165.4 (CO), 147.4 ( =C-CH3), 145.1 (C=O), 142.0 (C=O), 132.1 (C=O), 130.5 (Ar-C), 126.4 (Ar-C), 120.4 (Ar-C), 114.4 (Ar-C), 110.1 (Ar-C), 56.3 (OCH3). Anal. calc'd for C10H11NO4S: C, 47.94; H, 5.08; N, 4.69; S, 10.7%. Found: C, 47.85; H, 4.65; N, 5.35; S, 11.0%.

N-(2-Amino-4-chlorobenzoyl)-l-cysteine (4c). Buff microcrystals, yield 0.25 g (91%); mp 85–87 °C. 1H NMR (500 MHz, DMSO-d6) δ 7.91 (d, J = 7.0 Hz, 1H, NH), 7.50 (d, J = 10.0 Hz, 1H, OC-(ortho-Ar-H)), 6.72 (s, 1H, H-N-(ortho-Ar-H)), 6.64 (s, 2H, NH2), 6.50 (d, J = 10.0 Hz, 1H, Ar-H), 4.29 (s, br, 1H, CH-COOH), 3.43 (d, br, 1H, HS-CH2), 3.08 (dd, J = 13.0, 6.5 Hz, 1H, HS-CH2). 13C NMR (125 MHz, DMSO-d6) δ 172.7 (COOH), 167.7 (CO-NH), 151.0 (=C-NH2), 136.3 (C=Cl), 130.0 (Ar-C), 111.5 (Ar-C), 114.3 (Ar-C), 113.0 (Ar-C), 52.4 (C-CH-COOH), 8.5 (HS-CH2). Anal. calc'd for C8H9ClN2O3S: C, 43.72; H, 4.04; N, 10.20; S, 11.84%.

N-(4-Hydroxy-3,5-dimethoxybenzoyl)-l-cysteine (4d). Buff microcrystals, yield 0.26 g (86%); mp 76–78 °C. 1H NMR (500 MHz, DMSO-d6) δ 8.98 (s, 1H, OH), 8.62 (d, J = 7.5 Hz, 1H, NH), 7.19 (s, 2H, Ar-H), 4.73–4.68 (m, 1H, -CH(COOH)), 3.79 (s, 6H, m-OCH3), 3.29 (dd, J = 13.5, 4.5 Hz, 1H, HS-CH2), 3.11 (dd, J = 13.5, 10.0 Hz, 1H, HS-CH2), 1.17 (s, 1H, SH). 13C NMR (125 MHz, DMSO-d6) δ 172.4 (COOH), 166.1 (CONH), 147.4 (=C(OCH3)), 138.9 (=C(OH)), 123.4 (=COCO), 105.3 (Ar-C), 56.1 (OCH3), 52.0 (C-CH-COOH), 14.2 (HS-CH2). Anal. calc'd for C10H11NO5S: C, 47.83; H, 4.50; N, 4.65, S, 10.64. Found: C, 47.83; H, 4.65; N, 4.58; S, 11.0%.

N-(4-Hydroxybenzyl)-l-cysteine (4e). Oily, yield 0.22 g (91%). 1H NMR (500 MHz, DMSO-d6) δ 9.10 (s, 1H, NH), 7.89 (d, J = 7.5 Hz, 1H, Ar-H), 7.39 (d, J = 7.5 Hz, 1H, Ar-H), 6.94–6.87 (m, 2H, Ar-H), 4.74 (d br, 1H, CH-COOH), 3.52 (dd, J = 13.5, 3.5 Hz, 1H, HS-CH2), 3.10 (dd, J = 13.5, 8.5 Hz, 1H, HS-CH2), 1.24 (s, 1H, SH). 13C NMR (125 MHz, DMSO-d6) δ 172.3 (COOH), 168.7 (HNCO), 159.2 (HO-C), 133.8 (Ar-C), 128.9 (Ar-C), 118.9 (Ar-C), 117.4 (Ar-C), 116.0 (Ar-C), 52.4 (HN-CH(COOH)), 12.5 (HS-CH2). Anal. calc'd for C8H11NO3S: C, 49.78; H, 4.60; N, 5.81, S, 13.29. Found: C, 49.89; H, 4.65; N, 5.86; S, 13.37%.

N-(4-Hydroxybenzyl)-l-cysteine (4f). Sticky, yield 0.21 g (87%). 1H NMR (500 MHz, D2O) δ 7.54 (d, J = 9.0 Hz, 2H, OC-(ortho-Ar-H)), 6.76 (d, J = 9.0 Hz, 2H, OC-(meta-Ar-H)), 4.62 (dd, J = 8.5, 5.0 Hz, 1H, CH-COOH), 3.30 (dd, J = 14.0, 5.0 Hz, 1H, HS-CH2), 3.01 (dd, J = 14.0, 8.5 Hz, 1H, HS-CH2). 13C NMR (125 MHz, D2O) δ 178.5 (COOH), 170.8 (=C-CO2-), 160.8 (=C-OH), 134.3 (Ar-C), 131.0 (Ar-C), 116.9 (Ar-C), 56.4 (=C-COOH), 9.9 (HS-CH). Anal. calc'd for C10H11NO3S: C, 49.78; H, 4.60; N, 5.81, S, 13.29. Found: C, 49.84; H, 4.67; N, 5.88; S, 13.35%.

N-(4-Chlorobenzyl)-l-cysteine (4g). Sticky, yield 0.21 g (80%). 1H NMR (400 MHz, DMSO-d6) δ 8.95 (s, 1H, NH), 7.96 (d, J = 8.0 Hz, 2H, CO-(ortho-Ar-H)), 7.55 (d, J = 8.0 Hz, 2H, CO-(meta-Ar-H)), 4.63 (br d, 1H, CH-COOH), 3.32–3.14 (m, 1H, CH-CH3), 3.05–2.68 (m, 1H, CH-CH2), 1.20 (s, 1H, SH). 13C NMR (101 MHz, DMSO) δ 172.4 (COOH), 165.9 (=C-CO2-), 136.8 (=C-CO2-), 132.9 (=C-CO2-), 129.8 (Ar-C), 128.8 (Ar-C), 56.1 (CH-COOH), 8.9 (CH-SH). Anal. calc'd for C10H11ClNO3S: C, 46.25; H, 3.38; N, 5.39; S, 12.34 Found: C, 46.35; H, 3.92; N, 5.44; S, 12.40%.

N-(4-Methoxybenzyl)-l-cysteine (4h). White microcrystals, yield 0.24 g (94%); mp 65–66 °C. 1H NMR (500 MHz, DMSO-d6) δ 8.47 (d, J = 7.5 Hz, 1H, NH), 7.82 (d, J = 8.9 Hz, 2H, OC-
(ortho- Ar- H)), 6.98 (d, J = 8.9 Hz, 2H, OC-(meta- Ar- H)), 4.68–4.64 (m, 1H, -(CH2(CO0H)))), 3.79 (s, 3H, OCH3), 3.37 (dd, J = 13.6, 3.9 Hz, 1H, HS-CH2), 3.07 (dd, J = 13.6, 10.1 Hz, 1H, HS-CH2), 1.17 (s, 1H, SH). 1H NMR (125 MHz, DMSO-d6) δ 172.4 (COOH), 165.8 (CONH), 161.7 (=C(OCH3)), 129.2 (Ar-C), 126.1 (Ar-C), 113.5 (Ar-C), 55.4 (-CH2SH). Anal. calcd for C11H13NO4S: C, 51.75; H, 5.13; N, 5.49; S, 12.56. Found: C, 51.87; H, 5.19; N, 5.54; S, 12.63%.

4.1.3. Computational Methodology. The crystal structure (PDB code: 2UVO) of the QS signal receptor protein LaS receptor with the natural ligand 3- (3-oxododecanoyl)-1-homoserine (OdDHL) was obtained from the protein data bank (PDB). In preparation for molecular docking, all nonstandard residues that include water were deleted followed by the addition of hydrogens. Marvin Sketch software was used to create the two-dimensional structures of the synthesized compounds. Three-dimensional structures of the targets 4a–h were built by optimization of the energy of their two-dimensional structures using Avogadro 1.2.0 software. The steepest descent algorithm was used for the optimization of molecular geometries of compounds in preparation for their molecular docking. AutoDock VINA (implicated in the PyRx 0.8 tool) was used to perform docking of compounds into LaS receptor (the QS signal receptor protein). The included grid box coordinates of molecular docking were as follows: center: X = 23.032, Y = 16.29, and Z = 79.71 and dimensions: X = 16.45, Y = 17.62, and Z = 19.97. The molecular interactions of the docked complexes were viewed by Discovery Studio Visualizer.

4.2. Antimicrobial and Antibiofilm Assays. 4.2.1. Media and Chemicals. The media utilized in this study were tryptone soya broth and Mueller Hinton broth (Oxoid, Hampshire, UK). Dimethyl sulfoxide (DMSO) and N-acetyl-L-cysteine were purchased from Sigma (St. Louis, USA). Other chemicals were of pharmaceutical grade.

4.2.2. Bacterial Strains. P. aeruginosa PAO1 strain was kindly supplied by the Department of Microbiology, Faculty of Pharmacy, Mansoura University.

4.2.3. Determination of Minimum Inhibitory Concentration (MIC). The minimum inhibitory concentrations of NAC and the synthetic N-acetylceysteines 4a–h were determined by applying the broth microdilution method. Serial (two-fold) dilutions of the tested compounds were prepared in Mueller-Hinton broth to produce dilutions of 80, 40, 20, 10, 5, 2.5, and 1.25 mM. Aliquots of 100 μL of the dilutions were added to the wells of a 96-well microtiter plate. Then, aliquots were added in volumes of 100 μL of PAO1 suspension prepared in Mueller-Hinton broth with an approximate cell density of 1 × 10^6 CFU/mL. The microtiter plate was incubated at 37 °C for 24 h, and the least concentration of each of the compounds that could inhibit the growth was considered the minimum inhibitory concentration of this compound.

4.2.4. Biofilm Inhibition Assay. The ability of the tested agents to inhibit biofilm formation was assessed by using the modified method of Stepanovic et al. using 1/4 MIC of the tested agents. PAO1 was grown overnight in TSB and then diluted to produce a suspension with cell density approximating 10^8 CFU/mL. Then, aliquots of 5 mL of the prepared suspension were delivered into 15 mL Falcon tubes containing pieces of a urinary catheter (1 cm in length) in the presence and absence of 1/4 MIC of the tested agents. The tubes were incubated for 24 h at 37 °C. Then, the catheter pieces were removed and washed three times using sterile phosphate-buffered saline (PBS, pH 7.2) to remove any planktonic cells. Methanol (99%) was added for 20 min to fix the adherent cells. The catheter pieces were stained with crystal violet (0.5%) for 20 min. The non-adherent stain was removed by washing with distilled water. Then, glacial acetic acid (33%) was used to solubilize the dye. The absorbance of solubilized stain was measured at 590 nm with a Biotek Spectrofluorimeter (Biotek, USA), and the percentage of inhibition was calculated.

4.2.5. Determination of Minimum Biofilm Eradication Concentration (MBEC). To determine the biofilm-eradicating activities of NAC and targets that showed similar or higher biofilm inhibition rates than NAC, the MBEC was determined by employing a modified method of Ceri et al. P AO1 suspension was prepared in TSB (1 × 10^6 CFU/mL) and then diluted with TSB to a cell density of (1 × 10^6 CFU/mL). Biofilms were formed at first by adding aliquots of 100 μL to the wells of microtiter plates that were incubated at 37 °C for 24 h. After removal of non-attached cells and washing the wells with sterile PBS, aliquots of 100 μL of the tested agents’ dilutions were added to the wells of the plates that were incubated at 37 °C for 24 h. The contents were removed, and the wells were washed before re-suspending the attached cells by adding aliquots of 100 μL of PBS and scraping the sides of the wells with a pipette tip. To calculate MBEC, 10 μL from each of the wells was plated on tryptone soy agar (TSA) plates that were incubated at 37 °C for 24 h. MBECs were the lowest concentrations that inhibited growth on TSA.

4.2.6. Scanning Electron Microscopy of Catheter Pieces. To visualize the eradicating effects of the tested agents 4a, 4d, 4f, 4g, and 4h and NAC on biofilm formation by P. aeruginosa PAO1 strain, the adherent biofilm cells formed on catheter pieces in the same way as in the biofilm inhibition assay but in the presence and absence of MBECs of the tested agents. The catheter pieces were left overnight to dry, and then the biofilms were fixed using 2.5% glutaraldehyde in PBS (0.2 M, pH 7.4). Then, they were prepared for examination using a scanning electron microscope as previously described.

4.2.7. Determination of the Effect of Sub-MIC of 4h on the Growth of Bacteria. The potential effect of 1/4 MIC (5 mM) of 4h on PAO1 growth was detected using Nalca et al. method. First, PAO1 was grown overnight and this culture was used to inoculate LB broth containing 4h and control LB broth. The optical densities of both cultures were measured after overnight incubation at 37 °C using a Biotek Spectrofluorimeter (Biotek, USA) at 600 nm. The sub-inhibitory concentration of 4h (5 mM) was used to assess its potential anti-virulence as well as its quorum sensing inhibiting activities against P. aeruginosa.

4.2.8. Pyocyanin Assay. The production of pyocyanin (blue-green pigment) by 4h (5 mM)-treated PAO1 and the control PAO1 strain cultures was assessed using the reported procedures by Das and Mane. A PAO1 culture in LB broth was prepared with an optical density of 0.4 at 600 nm. LB broth tubes (1 mL) with and without 4h were inoculated with 10 μL aliquots of PAO1 suspension, and the tubes were incubated for 48 h at 37 °C. The supernatants were separated by centrifugation at 10,000 rpm for 10 min. Pyocyanin was measured in the supernatants spectrophotometrically at 691 nm. The test was made in triplicate.

4.2.9. Protease Assay. To determine the effect of 4h (5 mM) on the protease activity of PAO1 strain, the skim milk agar method was employed. The supernatants of 4h-treated PAO1 culture and control culture were obtained by centrifugation. Aliquots of 100 μL were added to wells in skim milk agar plates.
that were incubated overnight at 37 °C. The clear zones due to protease activity were measured, and the test was made in triplicate.

4.2.10. Total RNA Extraction for qRT-PCR and Quantitative RT-PCR of QS Genes. The ability of 4h (5 mM) to downregulate quorum sensing genes was assessed by RNA extraction of 4h-treated and untreated cultures of PAO1 using a Gene Jet RNA Purification Kit (Thermo Scientific, USA) following the instructions of the manufacturer. Control and treated PAO1 cultures were prepared and centrifuged at 12000g for 2 min to collect the pellets. The pellets were then resuspended in Tris-EDTA buffer containing lysozyme (100 μL) and incubated at 25 °C for 5 min. Lysis buffer containing B-mercaptoethanol was added and mixed well. RNA was eluted after purification with 100 μL of nuclease-free water and was stored at −70 °C until use.

qRT-PCR was used to assess the relative expression levels of QS genes in PAO1 strain treated and untreated with 4h using the primers listed in Table 4.47 The housekeeping gene rpoD was used to normalize the relative expression level of tested genes. A StepOne Real-Time PCR system (Applied Biosystems, USA) was used, employing a SensiFAST SYBR Hi-ROX One-Step Kit protocol (Bioline, UK). Agarose gel electrophoresis and melting curve analysis of products were used to confirm the specificity and efficiency of the primers. The ability of 4h to inhibit and change the expression of QS genes in PAO1 strain was determined at a value of 4h ≤ 0.05.

Table 4. List of Primers Used in RT-PCR

| gene name | primer sequence |
|-----------|-----------------|
| rapD (F)  | 5′-CGAACCTGGTTGCGGACTT-3′ |
| rapD (R)  | 5′-CGGAGAGGCCTCAAGGATAC-3′ |
| lasI (F)  | 5′-CGGACACATCTGGGAACTCA-3′ |
| lasI (R)  | 5′-CGGACGGATCATCCT-3′ |
| lasR (F)  | 5′-CTGTTGAGTGGCAAGGACTAC-3′ |
| lasR (R)  | 5′-AAGTCTGGTTGCCGATGG-3′ |
| fliC (F)  | 5′-GGCTAACAAAGAGATTGCCCT-3′ |
| fliC (R)  | 5′-TTCCGTCGGAGCGATGGTAAGAAC-3′ |
| PslA (F)  | 5′-AGATCAAGAAAACGCCGTAAG-3′ |
| PslA (R)  | 5′-TGTAAGATGCACCCACACCG-3′ |

"F = Forward, R = Reverse.

was used to normalize the relative expression level of tested genes. A StepOne Real-Time PCR system (Applied Biosystems, USA) was used, employing a SensiFAST SYBR Hi-ROX One-Step Kit protocol (Bioline, UK). Agarose gel electrophoresis and melting curve analysis of products were used to confirm the specific PCR amplification, and the comparative threshold cycle (ΔΔCt) method was used to calculate the relative gene expression. 44

4.3. Statistical Analysis. One-way ANOVA tests, Student t-tests, and Graph Pad Prism 5 were used to study the significance of the inhibitory activities of 4h against the virulence factors of P. aeruginosa PAO1 strain. P values < 0.05 were considered statistically significant.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c01667.

Scans of 1H NMR and 13C NMR spectra and 2D binding modes of 4a–h with QS signal receptor protein LasR (PDF)

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