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Synthetic homoserine lactone analogues as antagonists of bacterial quorum sensing

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ABSTRACT

Quorum sensing (QS) is a density-dependent form of cell-cell communication that triggers the functional coordination of cooperative behaviors such as the production of virulence factors and biofilm formation. Quorum quenching (QQ) refers to all processes involved in the disruption of QS and is regarded as a promising strategy for treating bacterial infections. Herein, four compounds with closely related chemical structures to homoserine γ-lactone were synthesized and fully characterized. The compounds are termed TGK-series compounds. These compounds were subsequently tested in their QS inhibition activity using an *E. coli* Top 10 QS biosensor strain, a GFP QS reporter, that probes the capacity of bacteria to detect their cognate autoinducer *N*-(3-oxohexanoyl)-homoserine lactone ($3OC_6HSL$) substrate by means of a single intracellular protein LuxR. All TGK-series compounds were found to significantly inhibit the ability of bacteria to produce GFP but without exerting toxicity when applied at a concentration of 50 μM. In parallel, the interaction of TGK-series compounds with LuxR were studied by molecular docking simulations. These studies revealed that TGK-series compounds bound to the natural substrate *N*-(3-oxo-octanoyl)-L-homoserine lactone (OOHL) binding site and that the binding ability of the compounds with the TraR protein (a surrogate of LuxR) was even more favorable in comparison with the natural substrate. It was also uncovered that TGK-series compounds form stronger hydrophobic interactions with the TraR protein than $3OC_6HSL$ does, thus providing a rationale for the enhancement of the QQ activity of the synthetic TGK-series compounds. This study will serve to guide future works aimed to design promising novel QS inhibitor candidates on a rational basis.

1. Introduction

Traditional antibiotics have the ability to kill bacteria or inhibit their growth and have been highly effective over several decades. However, the deployment of novel antibiotics to supplement the market is still an urgent issue for scientists and industries due to the significance of antibiotic resistance. Even worse, only a few new antibiotics have been discovered in the last 40 years and bacteria have already developed antibiotic resistance against them [1]. Pathogenic bacteria are harmful to human health primarily because of the production of virulence factors that can cause damage to host tissues. Therefore, targeting the regulation of virulence expression is a promising approach for the development of new therapies [2,3]. Bacteria have evolved a mechanism of orchestrating their phenotypic behavior by the synthesis of small signaling molecules, called autoinducers (AIs), which can be detected by intracellular and membrane proteins. This form of "bacterial language" for cell-cell communication is known as quorum sensing (QS). The concentration of AIs in the environment is proportional to the bacterial cell density, hence, QS enables bacteria to regulate their collective behaviour dependent on the cell density of

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their environment. *N*-acyl homoserine lactones (AHLs) are the most common class of AIs used by a large number of gram-negative bacteria. AHLs diffuse into the cytosol of bacteria and bind to LuxR, a family of proteins that upon binding to AIs act as transcriptional activators of genes that result in biofilm formation, sporulation, genetic competence, swarming motility, bioluminescence, and toxins production [4]. There are different classes of known AIs that mediate bacterial QS. Some are general and are used by different species, while others are rather specific and exist only in a given genus (*e.g., Pseudomonas*). Also, there are AIs that mediate crosstalk communication among different species of gram-positive and gram-negative bacteria [5].

The gram-negative bacterium *Vibrio fischeri* uses the LuxR/LuxI system to regulate bioluminescence as a direct consequence of QS activity. In this system, *N*-(3-oxohexanoyl)-homoserine lactone ($3OC_6HSL$), a specific type of AHL, is synthesized by LuxI synthase which catalyzes a reaction between *S*-adenosylmethionine (SAM) and an acyl carrier protein (ACP). When the cell density exceeds a critical threshold level $3OC_6HSL$ will bind the LuxR protein and make the protein fold during dimerization processes. As the AIs accumulate, the ligand-receptor complex binds with DNA to activate the transcription of target genes, including *luxI* and the *luxCDABE* operon, leading to bioluminescence production [6,7].

Inhibition of QS is considered a novel approach for dealing with bacterial infections. As inhibition of QS does not affect cell survival, the target bacteria are thought to be less prone to selection pressure to develop antibacterial resistance. On the other hand, strategies that inhibit QS are generically known as “quorum quenching” (QQ) and offer the potetial to block virulence factor production, including biofilm formation [8]. Therefore, QQ holds promise as a powerful strategy to contribute to mitigating antibacterial resistance and is presented as a promising tool in different fields such as medicine and aquaculture. Besides, an anti-virulence compound could indirectly influence antimicrobial resistance development by lowering the use of antimicrobials. Different mechanisms of QQ have been explored, namely: (i) discovering new natural competitors that bind to AI receptors; (ii) using enzymes to degrade AIs; (iii) reducing the activity of AI synthases or AI cognate receptor proteins; (iv) blocking the production of AIs; and (v) synthesizing new compounds as analogues of the cognate AIs that compete with the cognate AIs for the receptor binding site in the detection system (e.g., LuxR proteins) [9,10,11].

Many quorum sensing inhibiting (QSI) compounds have been discovered from natural sources, such as food, prokaryotic organisms, animals, plants, marine organisms, and fungus [12–16]. However, the rather low abundance of natural compounds with potent QS inhibition activity has resulted in turning the attention to chemically synthetic compounds. Several QS inhibition strategies are being pursued in this regard to synthesize compounds to target the biosynthetic pathways of QS, such as modifications in the side chain or ring moiety of AHL, or in the signal production [17–20]. Kim Cheoljin *et al* synthesized eleven QS antagonists and found that ten new analogs of AHLs or *N*-Sulfonyl homoserine lactone derivatives were shown to act as antagonists against *N*-(3-oxooctanoyl)-L-homoserine lactone. Moreover, using molecular modelling they found hydrophobic interactions are an important stabilizing factor [21].

Computer-based (*in silico*) molecular modelling can be used as an important tool for screening and identifying compounds with potential QS inhibitory activity [22–25]. The detailed in-depth knowledge of how a substrate interacts with the protein can be exploited to design or screen compounds with inhibitory activity. Furthermore, the method GRID-molecular interaction fields (GRID-MIFs) combined with docking studies has been successfully used for identifying the property of binding sites, binding modes, and the interaction of compounds.

In our study, four compounds (TGK-series) were synthesized and their QS inhibition activity tested with an *Escherichia coli* (*E. coli*) QS biosensor strain. The scientific basis of choosing these compounds lies in the unique structural feature that these compounds possess in common with that of some of the known QS modulators. For example, these compounds have an exocyclic double bond next to the lactam nitrogen just like the one present in bromated furanones produced by the *Delisea pulchra* alga. These furanones are known inhibitors of bacterial colonization on the algae [26]. Also, nitrogen analogues of AHLs, such as γ-lactam which is present in TGK-series compounds are also reported to have biofilm inhibitory activities [27,28]. Moreover, there are reports that compounds based on thiolactone containing an aryl ring on the side chain inhibit quorum sensing in *Pseudonomas aeruginosa* and biofilm formation [29]. The results show that all the four tested TGK-series compounds are promising candidates as QS inhibitors. Additionally, none of these compounds affects the growth nor viability of bacteria. The tested candidates were further analyzed by molecular docking and GRID-MIFs methods. To this end, the TraR protein was used due to its close homology and similar properties as LuxR [30]. Frezza *et al* have indicated that the use of TraR as a surrogate of LuxR in molecular simulations is appropriate due to the similar binding modes of LuxR/$3OC_6HSL$ and TraR/OOHL complexes within the ligand-binding site [31]. The docking and GRID-MIFs suggested that QS inhibition can be achieved by the formation of an additional hydrophobic interaction in the TraR protein-ligand *N*-(3-oxo-octanoyl)-L-homoserine lactone(OOHL) binding cavity.

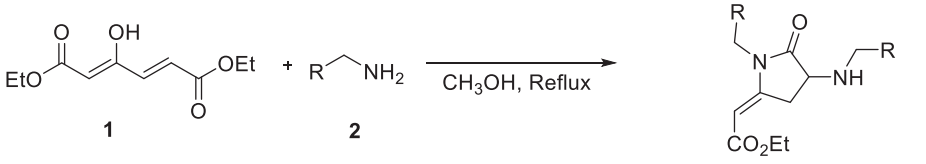
2. Materials and methods

2.1. Materials

$3OC_6HSL$ and all other chemicals were of analytical grade and, unless otherwise stated, were purchased from Sigma-Aldrich, Steinheim, Germany. Milli-Q water was used throughout.

2.2. Chemical synthesisof substituted γ-lactams (TGK-series) compounds

2.2.1. General procedure for the synthesis of substituted γ-lactams (TGK1-TGK4 [32])



TGK1: R = 4-Me-C_6H_4
TGK2: R = 2-thiophenyl
TGK3: R = 2-furanyl
TGK4: R = Ph

To a solution of **1** [33]. (1.0 equiv.) in methanol, amine **2** (2.5 equiv.) was added. The reaction mixture was, unless otherwise stated, allowed to stir at reflux condition. After 9 h, the solvent was removed in a rotavapor. The residue was loaded on a silica gel column. It was

eluted with ethyl acetate/hexanes mixture to obtain the pure product.

2.2.2. Characterization of (Z)-Ethyl 2-(1-(4-methylbenzyl)-4-((4-methylbenzyl)amino)-5-oxopyrrolidin-2-ylidene) acetate (TGK1)

Prepared by the general procedure above; yield = 55%; yellow liquid; R_f = 0.15 in 1:1 EtOAc/hexanes; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.25–7.21 (m, 2H), 7.15–7.08 (m, 6H), 5.22 (s, 1H), 4.70 (d, J = 15.6 Hz, 1H), 4.64 (d, J = 15.6 Hz, 1H), 4.11 (q, J = 7.2 Hz, 2H), 3.92 (d, J = 13.2 Hz, 1H), 3.79 (d, J = 12.8 Hz, 1H), 3.67–3.59 (m, 2H), 3.01–2.92 (m, 1H), 2.33 (s, 3H), 2.31 (s, 3H), 1.24 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.9, 167.0, 156.6, 137.5, 136.9, 135.9, 131.5, 129.4, 129.2, 128.2, 127.1, 93.4, 59.6, 54.7, 51.1, 44.0, 33.1, 21.0, 14.3; IR (KBr): υ 3297, 2984, 2924, 1626, 1312, 1034, 814 cm^{-1}; HRMS: calcd for $C_{24}H_{28}N_2O_3$ $[M + H]^+$ 393.2179, found 393.2178.

2.2.3. (Z)-Ethyl 2-(5-oxo-1-(thiophen-2-ylmethyl)-4-((thiophen-2-ylmethyl)amino)pyrrolidin-2-ylidene) acetate (TGK2)

Prepared by the general procedure above; yield = 62%; yellow liquid; R_f = 0.09 in 1:3 EtOAc/hexanes; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.24–7.21 (m, 2H), 7.02–7.01 (m, 1H), 6.96–6.93 (m, 3H), 5.39 (s, 1H), 4.90 (d, J = 15.6 Hz, 1H), 4.81 (d, J = 16.0 Hz, 1H), 4.16 (q, J = 7.2 Hz, 2H), 4.14 (d, J = 14.0 Hz, 1H), 4.07 (d, J = 14.0 Hz, 1H), 3.69–3.60 (m, 2H), 2.98–2.90 (m, 1H), 2.12 (br s, 1H), 1.27 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.1, 166.9, 155.8, 142.3, 136.6, 127.0, 126.9, 126.8, 125.7, 125.6, 125.0, 93.4, 59.8, 54.4, 45.9, 39.1, 33.1, 14.4 ; IR (neat): υ 3412, 3055, 2986, 1630, 1265, 1140, 746 cm^{-1}; HRMS: calcd for $C_{18}H_{20}N_2O_3S_2$ $[M+H]^+$ 377.0994, found 377.0996.

2.2.4. (Z)-Ethyl 2-(1-(furan-2-ylmethyl)-4-((furan-2-ylmethyl)amino)-5-oxopyrrolidin-2-ylidene) acetate (TGK3)

Prepared by the general procedure above; yield = 48%; yellow liquid; R_f = 0.13 in 1:3 EtOAc/hexanes; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35 (dd, J = 14.4, 0.8 Hz, 2H), 6.31–6.28 (m, 3H), 6.22–6.21 (m, 1H), 5.45 (s, 1H), 4.76 (d, J = 15.6 Hz, 1H), 4.62 (d, J = 15.6 Hz, 1H), 4.16 (q, J = 7.2 Hz, 2H), 3.96 (d, J = 14.4 Hz, 1H), 3.85 (d, J = 14.8 Hz, 1H), 3.66–3.57 (m, 2H), 2.92–2.88 (m, 1H), 2.13 (br s, 1H), 1.28 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.1, 167.0, 156.1, 152.4, 147.9, 142.6, 142.3, 110.4, 110.1, 108.9, 107.8, 93.4, 59.7, 54.3, 43.8, 37.3, 32.9, 14.4; IR (neat): υ 3327, 3055, 2986, 1630, 1422, 1265, 1148, 748 cm^{-1}; HRMS: calcd for $C_{18}H_{20}N_2O_5$ $[M + H]^+$ 345.1451, found 345.1451.

2.2.5. (Z)-Ethyl 2-(1-benzyl-4-(benzylamino)-5-oxopyrrolidin-2-ylidene) acetate (TGK4)

Prepared according with the general procedure above, but at room temperature for 24 h; yield = 72%; yellow liquid; R_f = 0.42 in 1:1 EtOAc/hexanes; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35–7.25 (m, 8H), 7.21–7.19 (m, 2H), 5.22 (t, J = 1.6 Hz, 1H), 4.75 (d, J = 15.2 Hz, 1H), 4.69 (d, J = 15.2 Hz, 1H), 4.11 (q, J = 7.2 Hz, 2H), 3.97 (d, J = 12.8 Hz, 1H), 3.85 (d, J = 12.8 Hz, 1H), 3.70–3.62 (m, 2H), 3.03–2.94 (m, 1H), 2.06 (br s, 1H), 1.24 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.9, 166.9, 156.5, 139.0, 134.5, 128.8, 128.6, 128.3, 127.8, 127.4, 127.1, 93.6, 59.7, 54.9, 51.5, 44.2, 33.1, 14.3; IR (neat): υ 3337, 3055, 2986, 1630, 1265, 1148, 743 cm^{-1}; HRMS: calcd for $C_{22}H_{24}N_2O_3$ $[M+H]^+$ 365.1866, found 365.1869.

3. Methods

3.1. Bacterial strains

The BioBrick standard biological sequence BBa_T9002 ligated into vector psb1a3 (http://partsregistry.org/Part:BBa_T9002), was a gift from Prof. Anderson (UC Berkeley, USA). The sequence BBa_T9002 was introduced by chemical transformation into *E. coli* Top 10 (Invitrogen, Life Technologies Co., UK) and stored as a 30% glycerol stock at −80 °C. The sequence BBa_T9002 comprised the transcription factor (LuxR), which is constitutively expressed but only active in the presence of the exogenous cell-cell signaling molecule $3OC_6HSL$. At an adequate concentration, two molecules of $3OC_6HSL$ bind to two molecules of LuxR and activate the expression of green fluorescent protein (GFP) (output) under the control of the *lux* pR promoter from *Vibrio fischeri*. The fluorescence biosensor was calibrated for different $3OC_6HSL$ concentrations as described in our previous paper [34].

3.2. Growth media and conditions

Bacterial were grown in Luria-Bertani (LB) medium and M9 minimal medium purchased from Becton, Dickinson and Company, Germany. We inoculated 10 mL of LB broth supplemented with 200 μg/mL ampicillin with a single colony from a freshly streaked plate of Top 10 containing BBa_T9002 and incubated the culture for 18 h at 37 °C, shaking at 100 rpm. Each culture was then diluted 1:1000 into 20 mL M9 minimal medium supplemented with 0.2% casamino acids and 1 mM thiamine hydrochloride plus 200 μg/mL ampicillin (AppliChem GmbH, Germany). The culture was maintained under the same conditions until the OD_{600} reached 0.15 (~5h). We then mixed 500 μL culture and 500 μL 30% sterile glycerol together in white plastic vials and stored them at −80 °C. For the biosensor assay, we prepared 40 μL from the white plastic vials with 20 mL of M9 medium plus 200 μg/mL ampicillin. The culture was used for the biosensor assay until the OD_{600} reached 0.04–0.07 (~4h).

3.3. Biosensor assay measurement

TGK-like compounds were evaluated for their ability to inhibit the production of GFP through competing with $3OC_6HSL$ in the *E. coli* Top 10 strain QS system described above. For the preparation of the biosensor assay, 180 μL of bacteria were pipetted into 96-well plates in the presence of 10 μL of $3OC_6HSL$ (10 nM) and 10 μL of TGK-like compounds, with concentrations ranging from 10 nM to 1 mM. The final inhibitor concentrations therefore ranged from 0.5 nM to 50 μM. Biosensor assays were run using flat-bottomed 96-well plates (Greiner Bio-One, cat. # M3061) and GFP expression was quantified using a Safire Tecan-F129013 Microplate Reader (Tecan, Crailsheim, Germany) at 37 °C. Fluorescence measurements were taken automatically using the following procedure: λ_{ex} = 480 nm and λ_{em} = 510 nm, 40 μs, 10 flashes, gain 100, top fluorescence. We also checked if the compounds inhibit bacterial growth by absorbance measurements (OD_{600}) (λ = 600 nm absorbance filter, 10 flashes) and shaking (5 s, orbital shaking, high speed) at different concentrations. A negative control containing only 180 μL of bacteria and 20 μL of milliQ water was performed in the absence of $3OC_6HSL$ for testing the auto-fluorescence. A positive control was performed by adding 10 μL of $3OC_6HSL$ (10 nM) and 10 μL of milliQ to 180 μL of bacteria. All measurements were done with the previously described microplate reader for 4.5 h and the experiments were carried out in triplicate.

3.4. Molecular modeling

In-silico study The 3D structures of TGK-series compounds were generated using Marvin sketch v6.1.3 (ChemAxon Ltd, Hungary). The 3D crystal structure of *Agrobacterium tumefaciens* TraR was retrieved from the Protein Data Bank (PDB ID 1L3L) [30]. The protein structure was prepared by adding all missing polar hydrogen atoms and by removing water molecules except for one conserved water molecule in the binding pocket which plays an important role in the interaction. The structure was energy minimized with Tripos SYBYL-X 1.1 by using the Powell method with a termination gradient of 0.05 and max iterations of 1000. Before docking, structures of compounds were prepared by adding polar hydrogens, assigning charges and providing full

flexibility to the TGK-series compounds by setting the number of torsions. Docking sites in the receptor included the entire active site thus providing enough space for ligand translational and rotational walks. GRID-MIFs were generated at the TraR binding site using the Autogrid program inbuilt in AutoDockTools 1.5.6 [35]. The Lamarckian Genetic Algorithm was applied with default settings to search for the best conformations and a maximum of 50 conformations was generated for each TGK compound [36]. Binding scores between the ligand and protein were evaluated using the AUTODOCK utility *autoscorer* considering hydrogen bond forces, electrostatic forces, van der Waals forces, solvation energy, and entropy. The molecular surface area of TGK-series compounds was calculated with Marvin sketch v6.1.3. Receptor surface pocket size was calculated with CASTp [35]. USCF Chimera version 1.8 was used for structural analysis, visualization, and image generation [37].

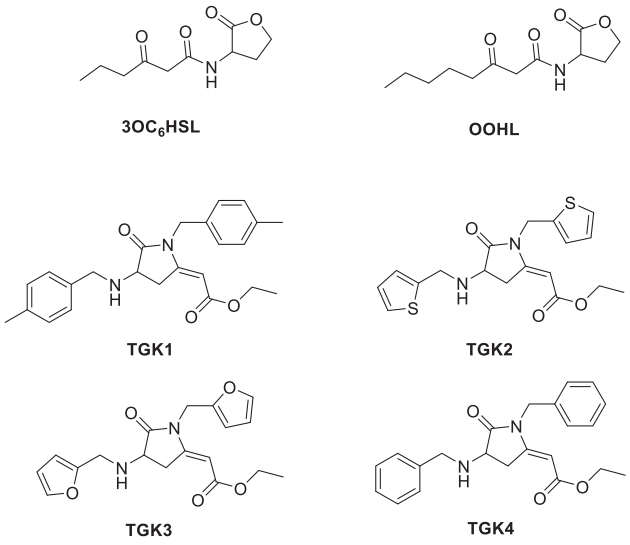
3.5. Statistical analysis

All experiments were performed in triplicates to validate reproducibility and p values were calculated statistically using the Student's t test. Values were expressed as mean ± SD. Comparison analysis was performed between tests and control.

4. Results

4.1. QS inhibition activity of TGK-series compounds

The TGK-series compounds show moderate similarity with the natural HSL based QS modulators. The marked differences of the TGK-series compounds from the natural modulators is that they are lactams and have additional *N*-substituent and α,β -unsaturated ester functions on the lactam. TGK-series compounds also have an *N*-alkyl substituent on the amine nitrogen unlike the *N*-acyl in the natural substrates. Moreover, they can be synthesized in a straightforward way starting from commercially available ethyl propiolate in three steps. Treatment of ethyl propiolate with catalytic DABCO resulted in diethyl (*E*)-hex-2-en-4-ynedioate in excellent yield which on gold-catalyzed hydration resulted in diethyl (*E*)-4-oxohex-2-enedioate **1** in 85% yield [33]. Treatment of 1 with different benzylamines under reflux resulted in the formation of TGK-series compounds in moderate yields [32]. The TGK-series compounds (Scheme 1) were evaluated for their potential ability to interfere with the GFP fluorescence induced by $3OC_6HSL$ in the *E. coli* Top 10 strain QS system. This strain was chemically transformed with the plasmid pSB1A3-BBa T9002, which comprised the *luxR* gene and coded for the transcriptional factor LuxR under the control of the pTetR promoter being expressed in a constitutive manner. The strain could express GFP upon exogenous addition of $3OC_6HSL$ and respond at concentrations of < 1 nM [34].



Scheme 1. Structure of $3OC_6HSL$, OOHL and TGK-series compounds.

The four synthetic TGK-series compounds (Scheme 1) bearing two rings attached to the homoserine lactam ring with short carbon chains displayed QS antagonist activity, as evidenced by the significant inhibition of GFP production. No pronounced toxic effects on bacterial growth were indicated as traces of time-dependency of the optical density (OD_{600}) showed almost no difference from the trace corresponding to the untreated control (Left panels in Fig. 1a–d). By contrast, the fluorescence intensity normalized by the OD_{600} (Right panels in Fig. 1a–d), showed a consistent concentration-dependent decreasing trend for the four compounds. Given the relatively short timeframe of the assay (4.5 h) and the limited range of concentrations explored, it is difficult to affirm conclusively that the compounds do not have antibacterial activity. Determinations of the minimum inhibitory concentrations (MIC) by expanding the range would have been informative in this regard. However, when compared with the results of our previous studies obtained using the same *E. coli* QS GFP reporter strain and experimental protocol, it is found that the TGK-series compounds show a similar low toxic profile as that of other well-known established anti-QS compounds (e.g., *trans*-cinnamaldehyde, Qin *et al* 2018) [38]. This pattern is in sharp contrast with that of compounds with notorious antibacterial activity even when applied at much lower concentrations (e.g., quercetin and baicalein, Ownega *et al* 2018) [39]. Fig. 2 shows the end-point data of the relative QS activity for all the TGK-series compounds. Compounds TGK2, TGK3 and TGK4 that have no further substituents in the rings attached to the homoserine lactone ring reduced the QS activity to about the same magnitude, namely to 53.5% ($p \leq 0.01$), 56.9% ($p \leq 0.01$) and 57.9% ($p \leq 0.01$), respectively. A somewhat stronger reduction to 47.9% ($p \leq 0.001$) in QS activity was achieved by the TGK1 compound that has two benzene rings each with a methyl substituent at the para- position with respect to the methylene connected to the nitrogen of the molecule.

4.2. Molecular modeling with four TGK-like compounds

The crystal structure of the TraR protein bound with the pheromone OOHL has been solved; the natural substrate (OOHL) in the TraR binding cavity forms three hydrogen bonds with the amino acid residues Y53+ W57+ D70+ another hydrogen bond with conserved water and hydrophobic interactions with several nearby residues. Besides, from the substrate-bound enzyme structure (1L3L.pdb), it is known that the binding site of the TraR receptor is compact and it binds the substrate completely enclosed in the cavity without any solvent contact [30]. Hence, to confirm that TGK-series compounds fit in the compact cavity, we measured the binding site pocket volume and its surface area as well as the surface area of the TGK-series compounds. Calculations show that the size of all the TGK-series compounds was within the range of the binding pocket site size (Table 1) and strongly suggested that TGK-series compounds can fit in the binding pocket site of the substrate (OOHL).

To further investigate the property of the binding site and to identify the most favorable interaction sites, GRID-MIFs were generated at the binding site using hydrophobic donor and acceptor molecular probes. We revealed several donor/acceptor sites and a large hydrophobic patch at the binding pocket of the TraR receptor (Fig. 3). This result suggested that hydrophobic interactions are the predominant type of interaction at the binding site, and might be important for biological activity. Thus, the results indicate that the occurrence of ligand-containing aromatic rings or alkyl chains would be favorable complementary groups. This is consistent with the physicochemical properties of our TGK-series compounds.

Moreover, to determine the binding affinity of TGK-series compounds with the OOHL, docking studies were carried out at the active

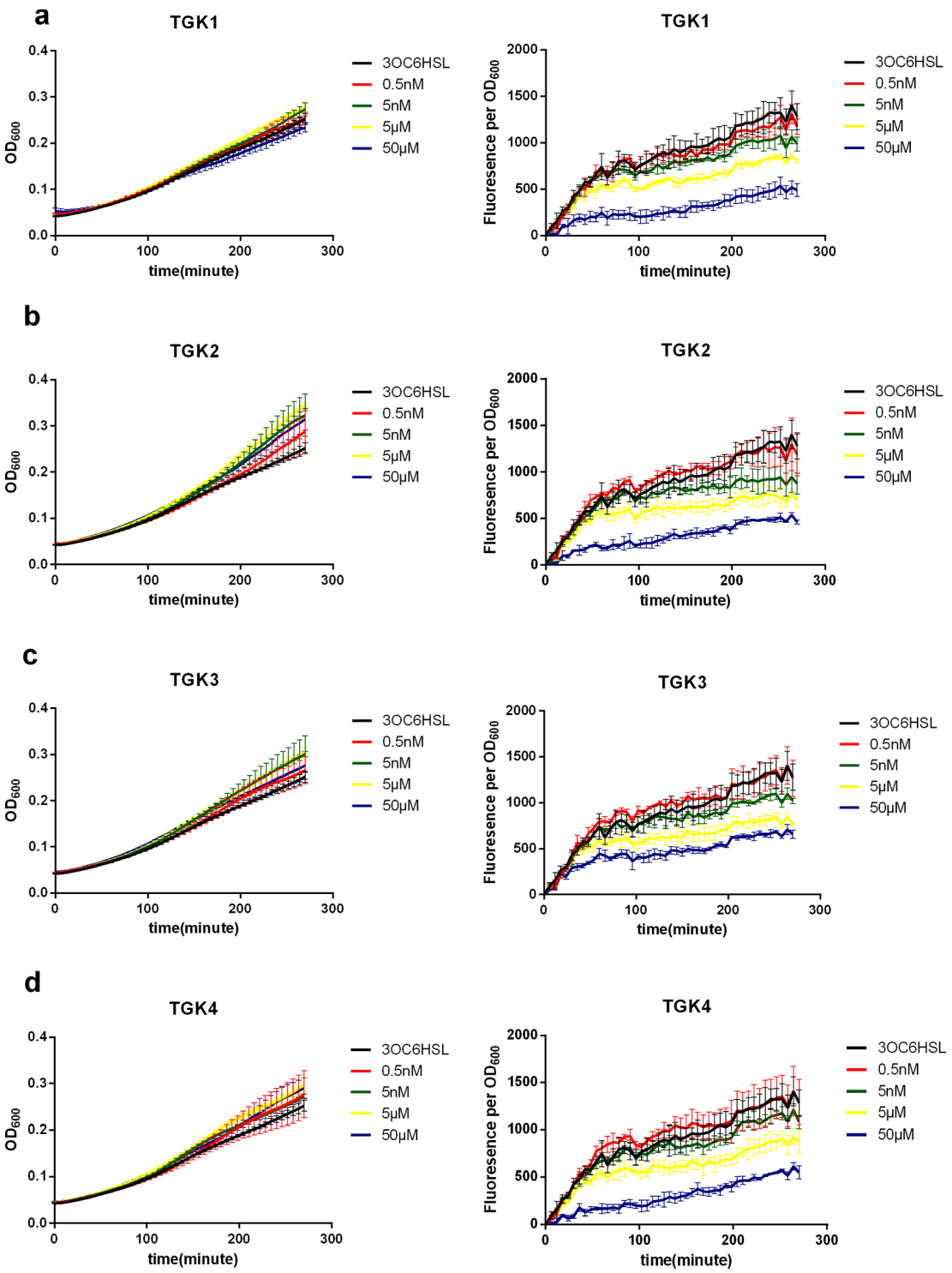


Fig. 1. Dependence of the growth (OD_{600}) and normalized fluorescence intensity/OD_{600} responses during the growth of the *E. coli* biosensor upon treatment with varying concentrations of: (a) TGK1; (b) TGK2; (c) TGK3; and (d) TGK4 at 0.5 nM, 5 nM, 5 μM and 50 μM. The individual traces represent the average of three independent biological experiments with at least three technical replicates.

site of the receptor. From simulations, the best conformation of compounds were selected based on the lowest binding score as well as on their similarity with the conformation of the natural substrate in the crystallographic structure. The outcome of our docking process shows that OOHL adopts the same conformation of the crystallographic natural substrate and the docked one, with rmsd 0.9 Å (Fig. S1a). This conformation is involved in almost similar interactions as reported in the crystallographic structure (Fig. S1a–c) [30], thus indicating the accuracy of our docking procedures. Results from docking studies with the TGK-series compounds have shown that the conformation of all four TGK-series compounds are the same at the binding site and in all cases, they occupy the whole space in the binding site (Fig. 4 a,b). From the molecular docking scores are summarized in Table 1. From the obtained results, its evident that the binding affinities of TGK-series compounds are greater than those of cognate natural ligand (OOHL) of TraR. However, the binding affinities of TGK1 and TGK4 (group1) were

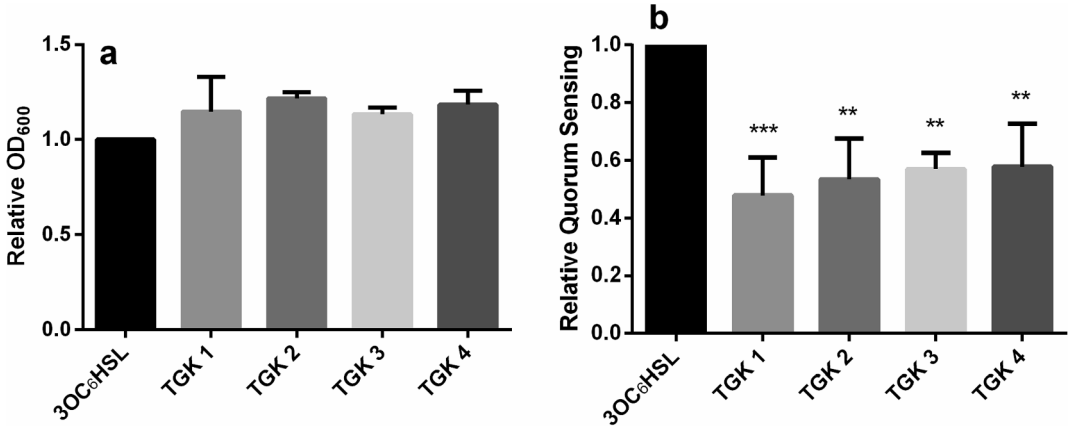


Fig. 2. Effect of addition of TGK-series compounds (50 μM) to the *E. coli* Top 10 QS AHL-sensitive strain on: (a) Relative OD_{600}; and (b) Relative QS response (*i.e.*, the normalized intensity fluorescence per OD_{600}) relative to the control ($3COC_6HSL$ 0.5 nM). Values represent the average data (± S.D., $n = 3$) of the last 100 min of the incubation period ($^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ compared to control group).

Table 1
Results of *in silico* molecular docking calculations between OOHL and TGK-series compounds, and *Agrobacterium tumefaciens* TraR protein (PDB ID 1L3L).

Receptor/compounds	Surface area Angstrom (Å)	Binding Energy (kcal/mol)
Receptor (Pocket)	659.2	
OOHL	395	−8.04
TGK1	614	−12.59
TGK2	484	−10.26
TGK3	499	−9.83
TGK4	560	−11.97

within a similar range, whereas docking scores for TGK2 and TGK3 (group2) were quite close. Nevertheless, clear differences were observed in the binding affinity of group 1 and group 2 TGK-series compounds, thus indicating that group 1 compounds have a greater binding affinity than group 2 compounds. Moreover, TGK1 and TGK4 molecular structures share similar features, thus both are involved in similar types of interactions. As shown in Fig. 4c, TGK1 formed three hydrogen bonds with amino acid residues Y61, D70 and T129, and possibly one with Y53, and is involved in strong hydrophobic/van der Waals interactions with several nearby residues (Fig. 4e). Based on structural analysis, the aromatic residues Y61, W85, and F101 (Fig. 4d) are involved in stacking interactions with the benzene rings of TGK1 and TGK4, thus playing an important role in the positioning and in defining the conformation of TGK-series compounds. Despite TGK1 showing a very similar docked conformation, the measured binding affinity was slightly higher than that of TGK4, indicating that the substitution of the methyl group on the benzene ring in TGK1 is a favorable functional group and thus increases interactions. Furthermore, TGK2 and TGK3 are more or less similar compounds, both containing a cyclopentane ring. Enzyme-substrate interaction maps have shown that sulphur and oxygen atoms present in the cyclopentane (S, O) ring form an extra hydrogen bond with W57 (Fig. S2a and S3a). However, the binding affinities measured for TGK2 and TGK3 were lower than that of TGK1 and TGK2. Our *in silico* results concurred well with the experimental data, where the measured QS inhibition was highest for TGK1. Our analysis suggested that due to a comparatively larger surface area and strong hydrophobic interactions, TGK1 proved a stronger QS inhibitor than TGK2, TGK3, and TGK4.

5. Discussion

In this work, we have synthesized QS inhibitors that are able to bind to the LuxR protein responsible for the "hearing" response of QS [34]. The studied compounds were a TGK-series that exhibit structural similarity to the cognate native ligand of LuxR, $3OC_6HSL$. Previous studies showed that the length of the acyl chain of AHL plays a critical role in the QS inhibition activity regulated by LuxR [11]. In this study, we showed that the size of the TGK-series compounds were within the range of that of the binding site pocket, and the physicochemical properties of the TGK-series compounds match the receptor GRID-MIFs; consequently, the geometry of the TGK-series compounds fit well in the OOHL binding cavity of TraR. Docking studies were used to characterize the underlying interaction between the synthesized TGK-series compounds and the receptor. Results showed that (Table 1) TGK-series compounds adopt the same conformation at the active site as the natural substrate (OOHL) and are involved in similar interactions. Although the binding affinity of TGK-series compounds was predicted to be higher than that of the natural substrate (OOHL), we showed that the aromatic functional group is responsible for increasing affinity due to increased hydrophobic interactions with active site residues. However,

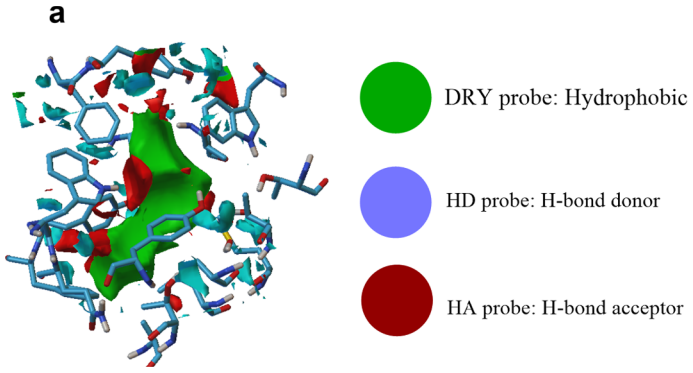


Fig. 3. (a) GRID-MIFs for TraR protein with DRY probe (green) showing favorable hydrophobic interaction sites. GRID-MIFs for TraR protein with HD (blue) and HA (red) probes showing favorable hydrogen bonds (blue for hydrogen bond donor and red for hydrogen bond acceptor) binding sites. (b) Natural substrate OOHL. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

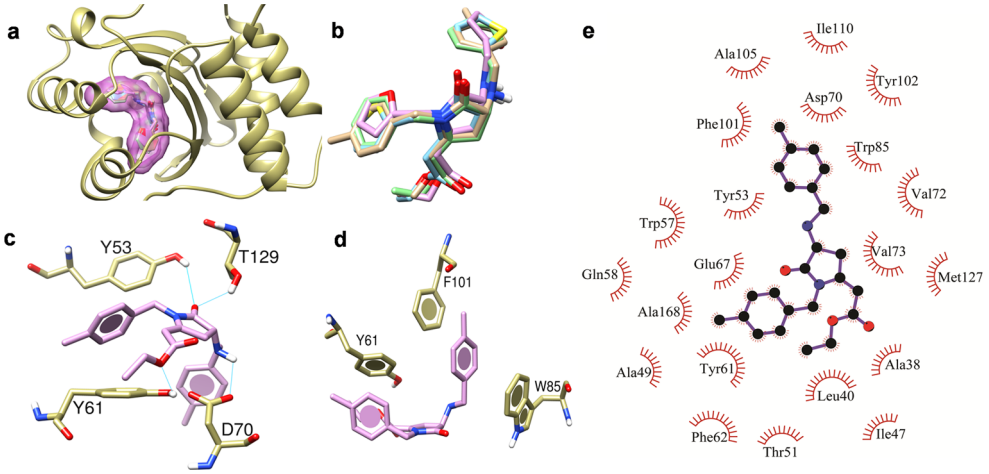


Fig. 4. (a, b) Superimposed conformation of TGK1, TGK2, TGK3 and TGK4 at the binding site. (c) Hydrogen bond interaction between TGK1 and receptor. (d) Stacking interactions between benzene ring and amino acid residues Y61, W85, F101. (e) 2D Hydrophobic interaction map between TGK1 and receptor.

TGK1 was identified as the most promising candidate suggesting that benzene rings with para- substituted methyl groups further enhances the capacity for hydrophobic interactions with the receptor.

Based on our results, we suggest that a stronger hydrophobic interaction between the TraR and TGK-series compounds, in comparison to the natural ligand OOHL, probably correlated with the enhanced affinity for the hydrophobic protein pocket, thus exhibiting significant QS inhibition activity. Our experimental results using the QS biosensor activity assay are consistent with the overall predictions of the *in silico* docking simulations. The TGK-series compounds somehow mimic $3OC_6HSL$ in the length of the alkyl chain (See Scheme 1) thus suggesting that the incorporation of two aromatic functional groups into the lactone ring in the "tail" of the molecules are responsible for the enhancement of their QS inhibitory activity [21].

6. Conclusion

In this work, we have synthesized four homoserine lactone-like TGK-series compounds and evaluated them in their ability to inhibit the AHL-regulated QS systems using a constructed *E. coli* QS biosensor strain. We showed that the TGK-series compounds exhibited strong QS inhibition ability which can be explained by our molecular modeling results. Thus, we propose that TGK-series compounds are promising QS inhibitors. This study will serve to guide future works aimed to design novel QS inhibitors. Future studies might deepen the biological significance of the present findings by measuring alternative outputs (e.g., virulence factors) from wildtype strains.

Declaration of Competing Interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2020.103698.

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