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Molecules and Cells

Development of Inhibitors against TraR Quorum-Sensing System in *Agrobacterium tumefaciens* by Molecular Modeling of the Ligand-Receptor Interaction

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The quorum sensing (QS) inhibitors that antagonize TraR, a receptor protein for *N*-3-oxo-octanoyl-L-homoserine lactones (3-oxo-C8-HSL), a QS signal of *Agrobacterium tumefaciens* were developed. The structural analogues of 3-oxo-C8-HSL were designed by *in silico* molecular modeling using SYBYL packages, and synthesized by the solid phase organic synthesis (SPOS) method, where the carboxamide bond of 3-oxo-C8-HSL was replaced with a nicotinamide or a sulfonamide bond to make derivatives of *N*-nicotinyl-L-homoserine lactones or *N*-sulfonyl-L-homoserine lactones. The *in vivo* inhibitory activities of these compounds against QS signaling were assayed using reporter systems and compared with the estimated binding energies from the modeling study. This comparison showed fairly good correlation, suggesting that the *in silico* interpretation of ligand-receptor structures can be a valuable tool for the pre-design of better competitive inhibitors. In addition, these inhibitors also showed anti-biofilm activities against *Pseudomonas aeruginosa*.

INTRODUCTION

Controlling the virulence of pathogenic bacteria is one of the biggest issues in medicine and microbiology (Kim et al., 2008a; Rasmussen and Givskov, 2006). Among many pathogenic bacteria, *Agrobacterium tumefaciens* and *Pseudomonas aeruginosa* are common pathogens in plants and animals and have been targets of interest in virulence-control studies. *A. tumefaciens* is the causative agent of crown gall tumors in plants, a disease of roots and stems that directly transforms plant cells at wound sites (White and Winans, 2007). *P. aeruginosa*, a multi-host pathogen, is also an opportunistic human pathogen, causing a wide variety of infections including cystic fibrosis, microbial keratitis, and burn wound infections (Willcox et al., 2008). *P. aeruginosa* is also notorious for the vigorous development of biofilms, which are surface-attached slime layers composed of microorganisms and extracellular polymeric substances (EPS) secreted by the microorganisms (Passador et al., 1993). The *P. aeruginosa* cells in biofilms are able to protect themselves from attacks by most antimicrobial agents, physical shearing forces, and host immune systems during infection (Kolter and Greenberg, 2006).

Understanding the quorum sensing (QS) mechanism is important in order to solve the problems caused by bacterial virulence and biofilm formation, since both are governed by the QS systems in many pathogenic bacteria (Daniels et al., 2004). QS is a mechanism in which bacteria communicate with each other and sense cell density through small diffusible signal molecules, such as *N*-acyl-L-homoserine lactones (acyl-HSL) or oligopeptides (Henke and Bassler, 2004). For the QS, bacteria release signaling molecules or autoinducers (AIs) to the outside of the cells, and as the bacteria grow to high cell density, the signal molecules accumulate above a certain threshold concentration. The signaling molecules then dock with their cognate receptor proteins, and the signal-receptor complexes regulate expression of various genes involved in virulence factor production, biofilm formation, and the infectious process. Therefore, to better inhibit bacterial propagation and infection, an understanding of the bacterial QS signal molecules and their interaction with receptor proteins is crucial (Rasmussen and Givskov, 2006).

Gram-negative bacteria produce multiple acyl-HSLs as the main signaling molecules for the QS. *A. tumefaciens* and *P. aeruginosa* produce *N*-3-oxo-octanoyl-L-HSL (3-oxo-C8-HSL) and *N*-3-oxo-dodecanoyl-L-HSL (3-oxo-C12-HSL), respectively, as the trigger to initiate the subsequent QS cascade (Fuqua

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and Greenberg, 2002; Henke and Bassler, 2004). Therefore, to efficiently inhibit the harmful consequences of the QS, the function of these signals should be antagonized. Recently, many laboratories have studied synthetic QS antagonists by modifying the molecular structure of acyl-HSLs (Baveja et al., 2004; Kim et al., 2007; 2008b; 2009; Kline et al., 1999; Manefield et al., 2002; Persson et al., 2005; Smith et al., 2003). For this, molecular modeling studies are suggested to be very beneficial for understanding the nature of the interactions between synthetic antagonists and receptor proteins (Kim et al., 2007; 2008b; 2009; Sakowski et al., 2001).

In order to develop better inhibitors against the QS of *A. tumefaciens*, structural analogues of 3-oxo-C8-HSL were designed by *in silico* molecular modeling using the FlexX program (Kim et al., 2007; 2008b; 2009; Sakowski et al., 2001) and synthesized by the solid phase organic synthesis (SPOS) method. To evaluate our modeling study, the molecular binding poses and energies between newly synthesized inhibitor candidates and TraR, a 3-oxo-C8-HSL receptor protein in *A. tumefaciens*, were predicted and compared with the *in vivo* inhibitory activities measured in bioassay systems. From our results, we suggest that the *in silico* interpretation of ligand-receptor structures can be a valuable tool for the pre-design of better competitive inhibitors.

MATERIALS AND METHODS

Chemical synthesis

Figure 1 shows the entire procedure for synthesizing new QS inhibitors that are derivatives of *N*-nicotinyl-L-HSL and *N*-sulfonyl-L-HSL. These were synthesized using the solid-phase organic synthesis (SPOS) method (Ko et al., 1998; Scott et al., 1996). Aminomethyl polystyrene resin and other chemicals were purchased from Beadtech Inc. (Korea) and Sigma-Aldrich Chemical Company.

Preparation of HSL derivatives

Amines of 2, 3, and 8 were protected through the brief reaction with formic acid (Fig. 1A), and 3-ethylenedioxooctanoic acid (9) was synthesized as described elsewhere (Geske et al., 2005; Smith et al., 2003), as shown in Fig. 1B. Figure 1C shows all R-donors used in the solid-phase synthesis (Fig. 1D). The 10 *g* (22 mmol) of aminomethyl polystyrene resin (10, AM PS, 200-400 mesh, 2.2 mmol/g, Beadtech Inc., Korea) were swollen in 100 mL of N-methyl-2-pyrrolidone (NMP) in a 3-neck flask (250 ml). To introduce methionine residues onto the resin, *N*-Fmoc-methionine (14.86 *g*, 40 mmol), 1-hydroxybenzotriazole (HOBt, 5.33 *g*, 40 mmol), diisopropyl ethylamine (DIEA, 5.17 *g*, 40 mmol), and benzotriazol-1-yl-oxy-tris (dimethylamino) phosphonium hexafluorophosphate (BOP, 17.69 *g*, 40 mmol), which acted as a coupling agent, were added to the flask. The solution was stirred at room temperature for 24 h, and the reaction was monitored by ninhydrin color test. After filtering the *N*-Fmoc methionine-loaded resin (11), the resin was washed 2-3 times with NMP, methylene chloride (MC), and MeOH, and dried under vacuum (mass increase: 8.17 *g*, yield: 99.8%). The amide bond formation by the coupling reaction was confirmed in the FT-IR spectrum, which showed amide peaks at 1,712 and 1,660 cm^{-1}. To remove the Fmoc group of the N-Fmoc methionine-loaded resin, the resin (11 *g*) was treated with piperidine/dimethyl formamide (DMF) (20%, v/v) twice at room temperature for 1 h.

The resin was filtered and washed 2-3 times with DMF, MC, and MeOH, and then dried under vacuum before its final mass was measured (7.13 *g*). The ninhydrin test indicated the presence of the amine group, and the amide band disappeared in the FT-IR spectrum (1712 cm^{-1}). The Fmoc-removed resin (12, 500 mg) was swollen in NMP (15 ml) in each of nine filtered reactors (Libra tube RT-20 M, Beadtech Inc., Korea). The resin was reacted with nicotinoic acid (1, 2, 3, 4, 5) or sulfonic acid (6, 7, 8) derivatives or 3,3-ethylenedioxooctanoic acid (9) (2.8 mmol) with HOBt (378.28 mg, 2.8 mmol), BOP (1238.44 mg, 2.8 mmol), and DIEA (361.9 mg, 2.8 mmol). The reactions were carried out at room temperature for 24 h, and then filtered and washed with NMP, MC, and MeOH, and dried under vacuum. The yields of the reactions were monitored through the mass increase (80-87%). The acid-protected amine-containing resins (13-1) were then hydrolyzed with THF/1 N NaOH (1:1). After hydrolysis, each reaction product was filtered and washed with NMP, MC and MeOH, and dried under vacuum. To prepare HSLs, the resin (13) was treated with BrCN (860 mg, 8 mmol) and trifluoroacetic acid (TFA, 5%) in $CHCl_3$/water (10 ml/5 ml) in each of the 9 filtered reactors. The products of HSL derivatives were chemically cleaved from the beads twice for 12 h and the resin was filtered and washed 2-3 times with $CHCl_3$. The solutions were collected in a round-bottom flask (100 mL) and extracted several times with $CHCl_3$ and brine, and $CHCl_3$ was then evaporated. Finally, the HSL products (14) were obtained from the AM PS resin in a yield ranging from 64% to 74% [14a (70%), 14b (72%), 14c (73%), 14d (74%), 14e (71%), 14f (69%), 14g (68%), 14h (64%)].

Molecular modeling study

In order to examine the binding energies of the newly synthesized QS inhibitor candidates, molecular modeling studies were performed on the inhibitor-TraR interactions using SYBYL packages (SYBYL molecular modeling software; Tripos, USA). FlexX dockings of the inhibitors were carried out using the Run-Multiple ligand option of FlexX as previously described (Kim et al., 2007; 2008b; 2009; Sakowski et al., 2001). Optimal conformational poses were selected from several possibilities based on the values obtained for the root-mean-square (RMS) deviation from the reference structures that were revealed by X-ray crystallography (Zhang et al., 2002).

Bioassay

The inhibitory activities of the newly synthesized inhibitor candidates were assayed in both solid and liquid media systems. For solid plate assays, *A. tumefaciens* KYC6, a 3-oxo-C8-HSL producer, and *A. tumefaciens* A136, an acyl-HSL-biosensor strain, were used as the signal generator and detector, respectively, as previously described (Fuqua and Winans, 1996; Fuqua et al., 1995; McLean et al., 1997; 2004). These two strains were streaked 1-2 cm apart and cultivated on a plate containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for colorimetric detection. X-gal turns blue when degraded by the β-galactosidase expressed by *A. tumefaciens* A136 upon exposure to the 3-oxo-C8-HSL diffused from *A. tumefaciens* KYC6. Thus, we can measure the strength of the response to 3-oxo-C8-HSL by the color intensity and measure the inhibition of this response upon addition of synthetic inhibitors.

The liquid assay was used for more quantitative measurement of the inhibition (Joshi and Khosla, 2003). Briefly, *A. tumefaciens* A136 was cultured in Luria-Bertani (LB) media with 50 μg/mL spectinomycin and 4.5 μg/ml tetracycline at 30°C. The overnight culture was diluted 1:100 and grown to OD_{600} = 0.3. The 3-oxo-C8-HSL and synthesized inhibitors were added at the indicated concentrations. After 2-h incubation at 30°C, β-galactosidase activity was assayed with the Tropix-plus kit (T1011, Applied Biosystems, USA) according to the manufac-

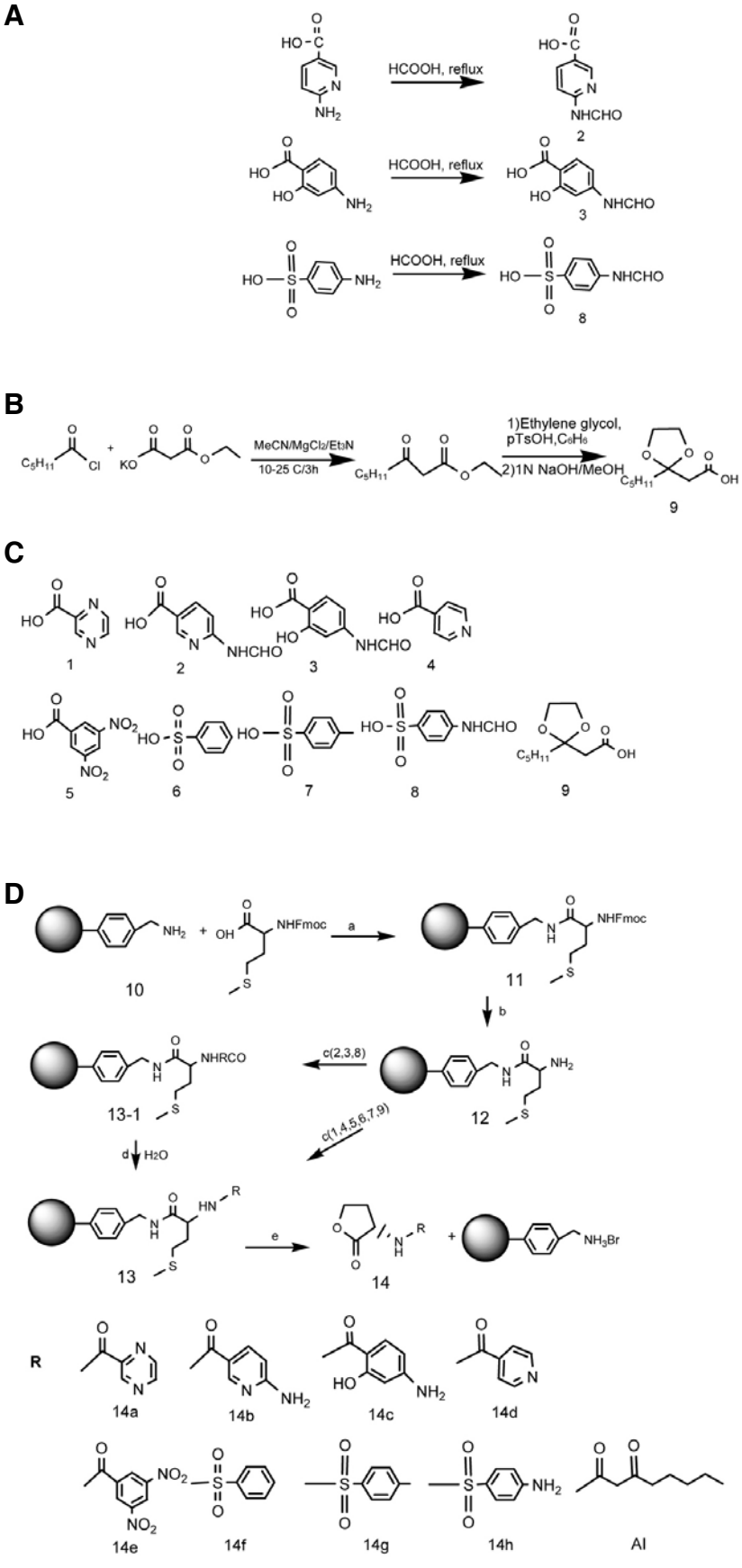

Fig. 1. Synthesis of the derivatives of *N*-nicotinyl-L-homoserine lactones and *N*-sulfonyl-L-homoserine lactones as new QS inhibitors. (A) Protection of amines with formic acid. (B) Synthesis of 3,3-ethylene-dioxooctanoic acid. (C) Acids used for synthesis of antagonist candidates and AI. (D) Synthesis of new analogues. Reagents and conditions: a) DIEA, HOBt, BOP, NMP, 25°C; b) 20% piperidine/DMF, 25°C; c) DIEA, HOBt, BOP, nicotinyl chloride or sulfonyl chloride, NMP, 25°C; d) THF/1N NaOH (1:1); e) BrCN, CF_3COOH, $CHCl_3$/H_2O, 25°C.

turer's instructions. Luminescence was normalized to cell density at OD_{600}, and the final activities were presented as relative luminescence/OD_{600} (McLean et al., 2004).

Antifouling and biofilm formation assay

In order to investigate the inhibition of biofilm formation by the synthesized inhibitor candidates, green fluorescent protein (GFP)-expressing *P. aeruginosa* PAO1 strain (PAO1-GFP) was kindly obtained from the Center for Biofilm Engineering, Montana State University. Biofilm of *P. aeruginosa* was formed in a CDC (Center for Disease Control) reactor system (Biosurface Technologies Inc., USA). PAO1-GFP cells were cultured in 1/10 tryptic soy broth (TSB) for 20 h at 37°C. This culture was diluted 1/100 in 1/100-strength TSB, with or without 20 μg/ml inhibitor, in the sterilized CDC reactor. Glass coupons were used as surface material for biofilm formation, and the initial amount of PAO1 cells was about 10^5 CFU/ml. The reactor was operated in batch mode for 24 h at 100 rpm at room temperature. After 24 h, the reactor was connected by a nutrient feed line to the carboy containing 1/300-strength TSB with or without 1 μM inhibitor, and further operated in continuous flow mode at a flow rate of 10 ml/min for 24 h. The glass coupons were collected in 10 ml of phosphate-buffered saline (PBS, pH 7.0) and prepared for epifluorescence microscope (Nikon H550L, Japan) analysis. A portion of the each biofilm on the coupons was detached by brief sonication and used for CFU counting (Speranza et al., 2004).

RESULTS

Synthesis of QS inhibitor candidates

In many studies of synthetic QS inhibitor development, researchers have employed the solution-phase synthesis method (Castang et al., 2004). In our previous effort to develop QS inhibitors of various bacteria, we used the solid phase organic synthesis (SPOS), which has some advantages over the solution phase synthesis, including higher yield and easier combinatorial synthesis (Kim et al., 2007; 2009). In particular, since molecules with an L-type enantiomer at the asymmetric carbon atom show a higher degree of antagonizing activity, this method is particularly beneficial in synthesizing the desired 3-D structure (Ko et al., 1998). Using SPOS methods, 5 analogues of N-nicotinyl-L-HSL and 3 analogues of N-sulfonyl-L-HSL were synthesized by substituting the alkyl tail portion of acyl-HSL with various functional groups of hetero ring moieties (Fig. 1). In the case of N-sulfonyl-L-HSL, replacing the carbonyl group with a sulfonyl group was expected to enhance the ligand flexibility, because the distance between the sulfur and the aromatic carbon is longer than that between the carbonyl carbon and the aromatic carbon (1.78 Å and 1.57 Å, respectively).

Design of QS inhibitor candidates and modeling of the ligand-TraR interaction

We previously investigated the interaction between TraR and its ligand, 3-oxo-C8-HSL, in 3-D space using SYBYL and FlexX programs (Kim et al., 2007), which yielded reliable RMS deviation (0.62 Å) in comparison with the structure of the TraR-3-oxo-C8-HSL complex determined by X-ray crystallography (Zhang et al., 2002). In that study, the structure of 3-oxo-C8-HSL was divided into three sub-parts: a lactone head, a body (amide bond and two nearby carbonyl groups), and a hydrocarbon tail. In this way, we demonstrated various interactions including hydrogen bonding (H bonds) and hydrophobic interactions between each sub-part of 3-oxo-C8-HSL and the active site residues of TraR (Kim et al., 2007). Briefly, H-bonds were formed between the carbonyl oxygen of the lactone head and the ε nitrogen of Trp57, and between N-H of the body and the carbonyl oxygen of Asp70. Two carbonyls of the body portion interact with Tyr53 and Thr129. The acyl tail is stabilized by hydrophobic interaction in a large pocket that has few polar groups and is surrounded by residues Tyr53, Leu40, Tyr61, and Phe62. The small pocket is surrounded by residues including Thr129, Met127, and Leu40, which stabilize the head and body parts by H-bonds and hydrophobic interactions.

Table 1. Binding energies (docking score) of QS inhibitors designed in this study

Ligand	Binding energy (kcal/mol)
AI	-12.78
14a	-18.60
14b	-18.87
14c	-19.84
14d	-18.40
14e	-18.43
14f	-19.79
14g	-20.83
14h	-19.19

Based on this interaction model, we designed new inhibitor candidates by replacing the body and tail parts with nicotinyl or sulfonyl groups. Using the SYBYL and FlexX computer programs, the interactions between the new compounds and TraR were examined; all compounds were sketched and minimized using the Tripos force field until the RMS gradient was less than 0.05. As listed in Table 1, all compounds had better docking scores than 3-oxo-C8-HSL, suggesting that all could have better binding to TraR. Our compounds were classified into three groups based on their tail structures and the predicted binding poses with TraR. Group A compounds (14a, 14b, 14d) had an aromatic group instead of the tail part (Fig. 1D), which fit in the small pocket surrounded by Thr129, Met127, and Leu40 (Fig. 2A). Group B compounds (14c, 14e) had polar groups on the aromatic ring (Fig. 1D), forming an H-bond with the pocket residues (Fig. 2B). In Group C compounds (14f, 14g, and 14h), C = O was substituted by SO_2 (Fig. 1D), which altered flexibility.

The docking poses of Group A compounds are depicted in Fig. 2A. While the lactone head of 14a was positioned similarly to that of 3-oxo-C8-HSL, those of 14b and 14d were positioned a bit differently. This difference was most likely caused by the different orientation of the pyrimidine, pyridine, and pyridinamine rings of 14a, 14b, and 14d in the small pocket to maximize hydrophobic interaction with residues surrounded by Thr129, Leu40, and Met127. However, there was no significant difference in the binding scores (Table 1).

The modeling study suggested that Group B compounds might form an H-bond with the polar residue in the small binding pocket. As depicted in Fig. 2B, 14c formed strong H-bonds with Tyr53 and Leu40, whereas Tyr53 did not form an H-bond with 3-oxo-C8-HSL. In the case of 14e, the H-bond between NO_2 and Leu40 was slightly influenced by steric hindrance caused by Met127. Therefore, 14e had a lower binding score than 14c (Table 1). In addition to H-bonds, the hydrophobic interaction in the small pocket was an important stabilizing factor for Group B compounds.

Group C compounds, 14f, 14g, and 14h, also formed H-bonds with Tyr53, and were surrounded by residues such as Tyr53, Leu40, Tyr61, and Phe62, as shown in Fig. 2C. Pecu-

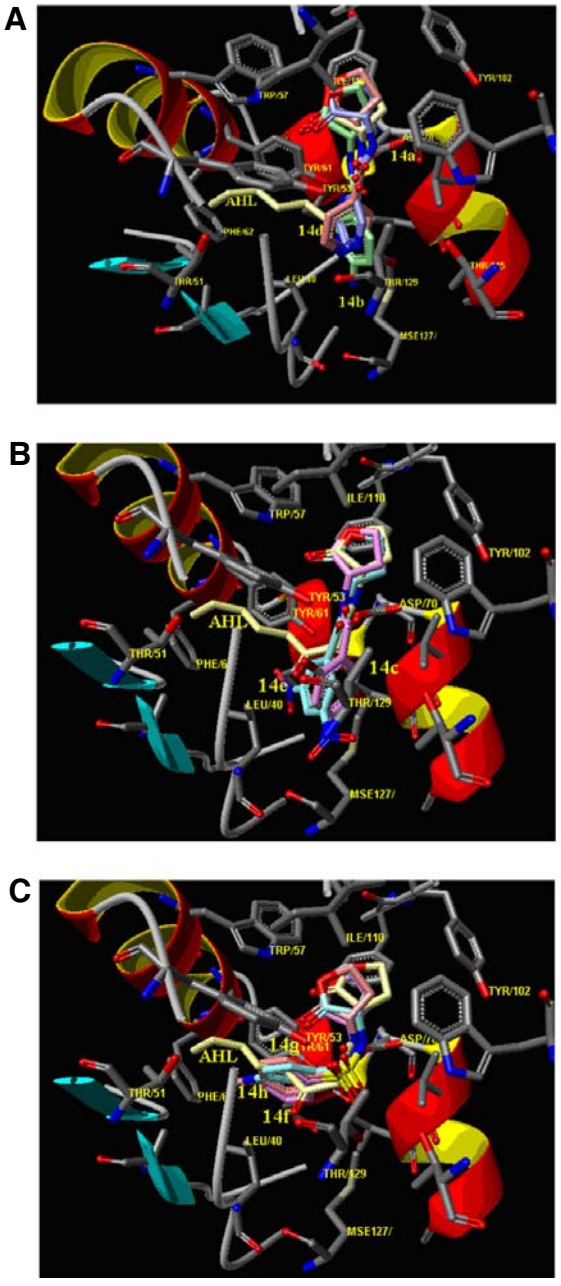

Fig. 2. The best-docked pose of QS inhibitors in the binding pocket of TraR from the FlexX modeling study. Residues are represented as sticks, and 3-oxo-C8-HSL as ball-and-sticks. Oxygens, nitrogens, and carbons are red, blue, and grey, respectively. (A) 14a, 14b, and 14d (Group A). (B) 14c and 14e (Group B). (C) 14f, 14g, and 14h (Group C). Hydrogen atoms were omitted for clarity.

liarly, their aromatic groups were not positioned in the small pocket. Instead, they maximized the hydrophobic interaction with residues in the large pocket, allowing better binding energies (Table 1).

In vivo inhibitory activities and antifouling effects of the inhibitor candidates

Bioassays in solid plates and liquid culture systems were performed to investigate the *in vivo* inhibitory effects of the eight

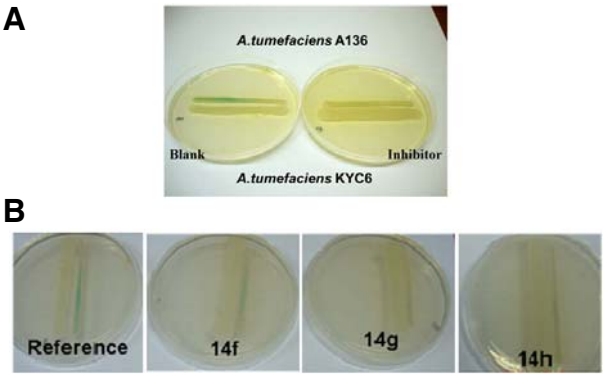

Fig. 3. Plate detection of the QS inhibitors. (A) Solid plate bioassay without (left) or with a synthetic inhibitor (14h, 1 μM, right). (B) Bioassay with the inhibitor candidates (*N*-nicotinyl or sulfonyl-L-HSL derivatives). The addition of candidates lessened the blue color compared to no addition control.

inhibitor candidates. Figure 3A shows the control of the solid plate assay, in which the sensor strain, *A. tumefaciens* A136 (top streak), sensed the 3-oxo-C8-HSL produced by the signal producing strain, *A. tumefaciens* KYC6 (bottom streak). In the presence of our inhibitor candidates in this system, the blue color of the streaks of the sensor strain was significantly reduced compared with no addition control (Fig. 3B). Thus, the inhibitors effectively inhibited the QS response of *A. tumefaciens*.

To better quantify the inhibition, we carried out liquid bioassays in which *A. tumefaciens* A136, the sensor strain, was cultivated in liquid media and responded to a fixed concentration of 3-oxo-C8-HSL with co-addition of inhibitors. The concentration of 3-oxo-C8-HSL was adjusted to 1 nM for half-maximal induction, which induces β-galactosidase expression almost 100-fold without inhibitors. We set this level to 100 and measured the relatively reduced level resulting from the co-addition of inhibitors. As shown in Fig. 4A, our inhibitor candidates significantly reduced the QS response by 45% to 65%. The best results were observed with 14c and 14g, which also had the best FlexX docking scores. There was fairly good correlation between the *in vivo* inhibition and the docking score, as shown in Fig. 4B.

We also examined the antifouling activities of our compounds. Since the biofilm formation of *P. aeruginosa* has been well documented as a QS-dependent process, we investigated whether our compounds could interfere with the QS and biofilm formation of *P. aeruginosa* cells. When added to the biofilm reactor, our compounds significantly reduced the adhesion of *P. aeruginosa* cells to the glass surface by 50-81% (Fig. 5A). Figure 5B shows a microscopic view of the antifouling test, where 14g was used as the anti-biofilm agent. These results show that our compounds efficiently prevented *P. aeruginosa* biofilm formation, presumably through inhibition of the QS signaling.

DISCUSSION

In this study, the acyl tail part of 3-oxo-C8-HSL was substituted with various aromatic moieties, or N-nicotinyl and N-sulfonyl groups. These QS inhibitor candidates were synthesized by the solid phase organic synthesis (SPOS) method. Our results suggested that these eight compounds have the potential to disturb the bacterial QS system and inhibit biofilm formation.

We expected that the presence of an aromatic ring would fa-

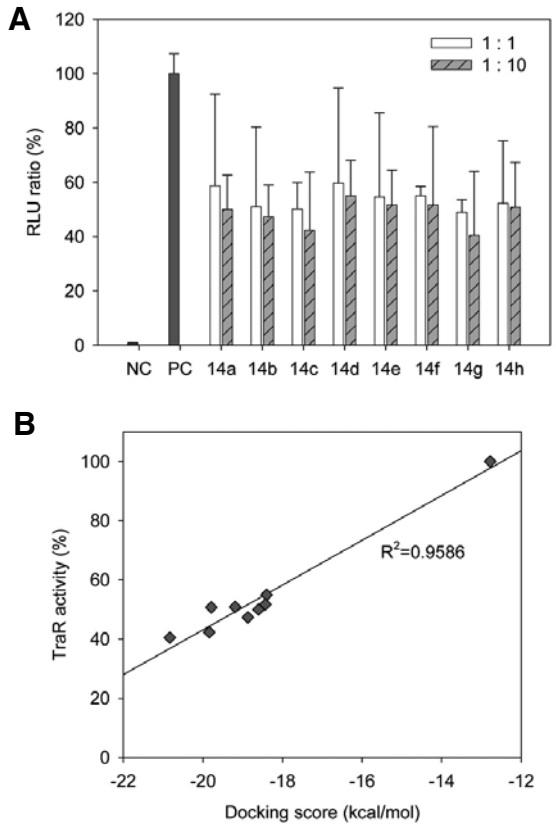

Fig. 4. Measurement of QS inhibition in liquid bioassay. (A) To quantify the QS inhibition by the inhibitor candidates in liquid bioassay, *A. tumefaciens* A136 cells were grown for 2 h in the presence of 1 nM 3-oxo-C8-HSL and 1 nM (equimolar) or 10 nM (10-fold molar excess) of each inhibitor, and assayed for β-galactosidase activity. NC, negative control without 3-oxo-C8-HSL; and PC, positive control with only 1 nM 3-oxo-C8-HSL. (B) Correlation between the docking scores from Table 1 and the *in vivo* inhibition obtained from (A). For this plot, the inhibition data obtained from the 10-fold molar excess of inhibitors were used.

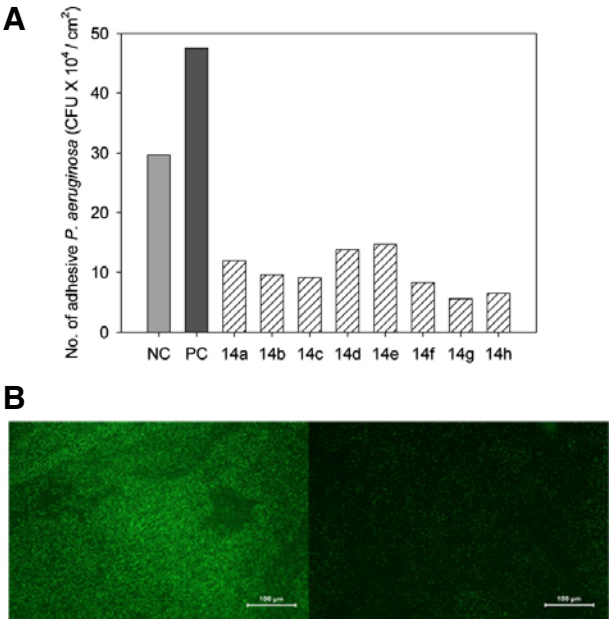

Fig. 5. (A) Anti-adhesion test of *Pseudomonas aeruginosa* with the inhibitor candidates (1 μM). NC (negative control): no addition; PC (positive control): 0.1 μM 3-oxo-C8-HSL only. (B) Micrographs of *P. aeruginosa* biofilms grown on glass slides after 48 h culture without inhibitor (left) or with inhibitor 14 g (1 μM) (right).

cilitate a specific interaction with TraR, because many nicotinyl- and sulfonyl-L-HSL analogues bearing an aromatic ring inhibited TraR in a previous study (Reverchon et al., 2002). In the case of N-sulfonyl-L-HSL, we expected that the sulfonyl group could enhance the flexibility of the ligand because the distance between the sulfur and the aromatic carbon is longer than that between the carbonyl carbon and the aromatic carbon. To confirm these postulations, we carried out molecular modeling studies using SYBYL packages. As expected, 14f, 14g, and 14h, which have SO_2-substitutions showed better binding energy (lower docking score) than others. The 14g had the best binding energy. Finally, we performed *in vivo* bioassays to assess the QS inhibition and biofilm prevention by our inhibitor candidates, and the bioassays showed good consistency and correlation with the molecular modeling. Therefore, we suggest that molecular modeling is an effective way to design new QS antagonists.

Our inhibitors also showed good inhibitory activity toward *Pseudomonas* biofilm formation, which is a well-known QS-dependent process. In this process, LasR, the QS receptor most responsible for biofilm formation in *P. aeruginosa*, senses 3-oxo-C12-HSL, which differs from the signal for TraR, 3-oxo-C8-HSL; this result was quite intriguing. However, this result does not conflict with our modeling study. Since the structural difference between 3-oxo-C8-HSL and 3-oxo-C12-HSL is the length of the acyl tail, the structures of TraR and LasR must differ significantly in the large pocket where the acyl tails are positioned. However, as shown in Figs. 2A, 2B, and 2C, our inhibitors mostly interact with residues in the small pocket and they have no long protrusion toward the large pocket. Therefore, we believe that our inhibitors could ignore the structural difference in the large pockets of TraR and LasR and have a wider range of QS inhibition. This may be a good clue for the development of QS inhibitors toward a broad range of gram negative bacteria, suggesting that the small pocket of QS receptor proteins may be a better target for wider QS inhibition.

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