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Solid-Phase Synthesis and Biological Evaluation of *N*-Dipeptido L-Homoserine Lactones as Quorum Sensing Activators

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Bacteria use small signaling molecules to communicate in a process termed "quorum sensing" (QS), which enables the coordination of survival strategies, such as production of virulence factors and biofilm formation. In Gram-negative bacteria, these signaling molecules are a series of N-acylated L-homoserine lactones. With the goal of identifying non-native compounds capable of modulating bacterial QS, a virtual library of *N*-dipeptido L-homoserine lactones was screened in silico with two different crystal structures of LasR. The 30 most promising hits were synthesized on HMBA-functionalized PEGA resin and released through an efficient acid-mediated cyclative release mechanism. Subsequent screening for modulation of QS in *Pseudomonas aeruginosa* and *E. coli* identified six moderately strong activators. A follow-up library designed from the preliminary derived structure–activity relationships was synthesized and evaluated for their ability to activate the QS system in this bacterium. This resulted in the identification of another six QS activators (two with low micromolar activity) thus illuminating structural features required for QS modulation.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen associated with a major part of hospital-acquired infections and lung infections in cystic fibrosis patients.[1,2] Unfortunately, this bacterium shows growing resistance toward classical antibiotics.[3] Thus, new strategies towards the treatment of infections of *P. aeruginosa* are urgently needed.

P. aeruginosa uses a quorum sensing (QS) system to control the formation of drug-resistant biofilm and to generate the virulence factors that are responsible for extensive tissue damage in mammals.[2,4–8] The biofilm consists primarily of extracellular polymeric matrix material that embeds the bacterial cells and makes it difficult for the immune system (as well as antibiotics) to eradicate the bacteria.[9,10] By modulating the QS system, pathogenic bacteria can be sensitized to immune responses and antibiotics.[11] Furthermore, as QS is not directly involved in biological processes that are essential for bacterial survival, modulation of QS does not impose selective pressure for the evolution of resistant mutants.

QS is an intercellular communication process used in Gram-positive and Gram-negative bacteria. The process is driven by small signaling molecules ("autoinducers") that allow the bacteria to monitor the size of their population and control behaviors that are beneficial for the group.[12,13] The most common class of autoinducers in Gram-negative bacteria is *N*-acylated L-homoserine lactones (AHLs). These signaling molecules are continuously synthesized in low concentrations by a synthase enzyme (LuxI-type synthase) and diffuse into the extracellular fluid. The extracellular concentration of AHL increases as the bacterial population grows, and once an intracellular concentration threshold is reached, AHL binds to an intracellular receptor (LuxR-type transcriptional regulator), which functions as a transcription factor. The AHL/LuxR complex dimerizes and activates transcription of the *lux* gene cluster, including the *luxI*-type gene, thus generating a positive feedback loop.[14]

The QS system of *P. aeruginosa* is complex; it comprises at least two AHL molecules and three LuxR-type receptors (LasR, RhlR, and QscR).[15] The LasR system upregulates the RhlR system, whereas the QscR system represses the LasR and the RhlR systems. The signaling molecule used in the LasR system is *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL); the RhlR system uses *N*-butanoyl-L-homoserine lactone (BHL). Each

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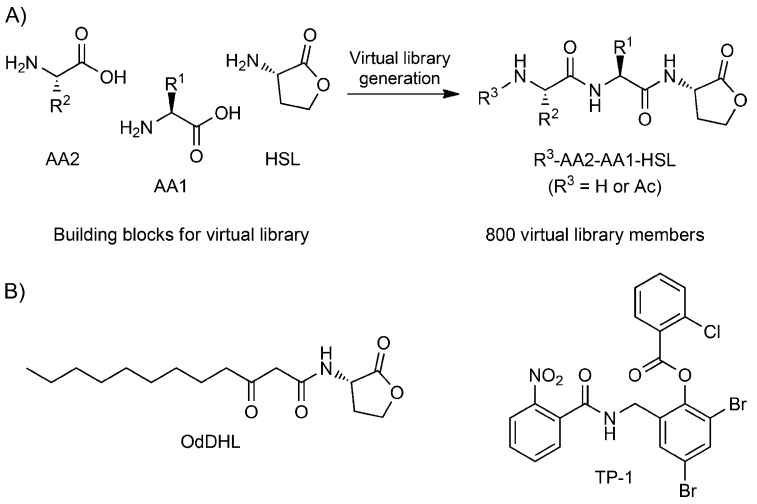
of these ligand/receptor pairs constitutes a potential target for QS modulation.[16,17]

Successful searches for QS modulators have centered around AHL structural analogues; these fall into one of two categories: either the lactone moiety or the acyl tail is varied.[16–18] So far, the synthesis of AHL analogues with non-native head groups has provided only a few active modulators of the LuxR-type proteins in *P. aeruginosa*.[19–25] However, studies on AHL analogues with structural variations in the acyl chain have led to several very potent LasR antagonists and agonists.[16,19,26–34] By simply changing the length or degree of saturation of the alkyl chain, agonists with nanomolar activity have been discovered. Both agonists and antagonists are valuable chemical tools for the study of QS pathways.[17]

Recently, crystal structures of the LasR protein have allowed computational and more rational design of new QS modulators.[26,35,36] In silico screening to identify structures that display strong binding interactions with the ligand-binding domain of LasR would allow the selective synthesis of compounds with a high probability of either activating or inhibiting LasR. Thus, we set out to investigate the QS modulating activity of *N*-dipeptido-L-homoserine lactones as AHL analogues. In this structure, the acyl chain of the natural ligand is replaced with a short peptide chain (Scheme 1). We envisioned that peptides of this type could be easily prepared by high-throughput solid-phase synthesis strategies and display favorable cell-penetrating properties by keeping the peptide chain short. This is critically important for novel QS modulators as the LasR protein so far has resisted stable isolation for biochemical screening assays. We report an in silico screening study of a virtual combinatorial peptide AHL library against the ligand-binding domain of LasR, and solid-phase synthesis and biological evaluation of compounds that modulate QS in vivo.



Scheme 1. A) Generation of a virtual library of HSL-containing tripeptides with the 20 canonical amino acids and L-homoserine lactone. B) Structures of OdDHL and TP-1.

Results and Discussion

In silico screening

A virtual library based on L-homoserine lactone (HSL) and the 20 canonical amino acids (AA) as building blocks was generated (Scheme 1).

By varying the two amino acids, 400 different structures were obtained. These were investigated both as the free amines and as the N-acetylated analogues. The docking was performed to the ligand-binding domain of LasR obtained from two crystal structures (PDB IDs: 2UV0 and 3IX4). As reference, docking of the cocrystallized ligand OdDHL was included in the screen against 2UV0, and the triphenyl mimic TP-1 was used in the screen against 3IX4.

Generally, the docking scores observed for the virtual library members were high compared to OdDHL for the 2UV0 structure, and similar to the score of TP-1 for the 3IX4 structure (Table 1). Structures with a free N terminus tended to give the highest docking scores. Hits for the 2UV0 crystal structure mainly contained amino acids with small, nonaromatic side chains at the AA_1 position, whereas aromatic side chains dominated at this position for 3IX4 structure. This can be explained by displacement of Arg61 in the 3IX4 structure compared to the 2UV0 structure.

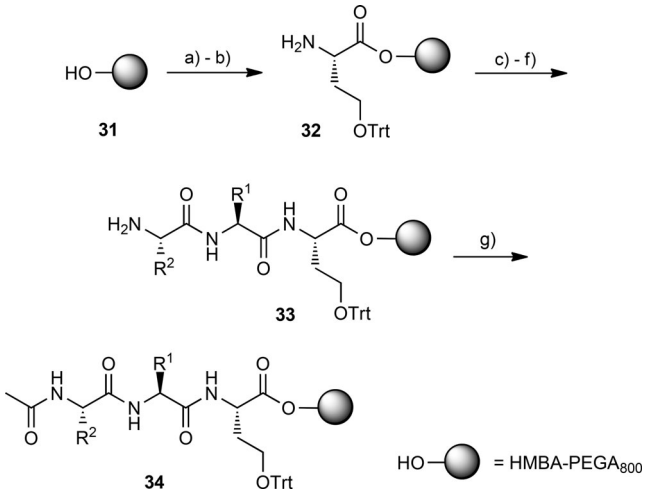
Table 1. Docking hits for the two LasR crystal structures.

Compound	Structure	PDB ID	Docking score
OdDHL	–	2UV0	−7.34
1	Phe-Gly-HSL	2UV0	−11.19
2	*N*-Ac-Phe-Asp-HSL	2UV0	−11.06
3	*N*-Ac-His-Cys-HSL	2UV0	−11.04
4	Tyr-Ser-HSL	2UV0	−10.85
5	Tyr-Gly-HSL	2UV0	−10.84
6	Trp-Ala-HSL	2UV0	−10.35
7	*N*-Ac-Phe-Ser-HSL	2UV0	−9.93
8	Phe-Pro-HSL	2UV0	−9.92
9	Phe-Ala-HSL	2UV0	−9.81
10	*N*-Ac-Phe-Gly-HSL	2UV0	−9.76
11	*N*-Ac-Phe-Ala-HSL	2UV0	−9.65
12	Phe-Asp-HSL	2UV0	−9.54
TP-1	–	3IX4	−12.63
13	Trp-Tyr-HSL	3IX4	−12.43
14	Trp-Phe-HSL	3IX4	−12.16
15	Lys-Trp-HSL	3IX4	−12.12
16	*N*-Ac-Trp-Trp-HSL	3IX4	−12.03
17	Trp-Lys-HSL	3IX4	−12.01
18	Tyr-Tyr-HSL	3IX4	−11.78
19	Phe-Trp-HSL	3IX4	−11.44
20	Met-Trp-HSL	3IX4	−11.42
21	Phe-Tyr-HSL	3IX4	−11.42
22	Trp-Ile-HSL	3IX4	−11.40
23	Trp-His-HSL	3IX4	−11.33
24	Trp-Leu-HSL	3IX4	−11.09
25	*N*-Ac-Phe-Met-HSL	3IX4	−11.08
26	Gln-Trp-HSL	3IX4	−10.76
27	Trp-Val-HSL	3IX4	−10.75
28	Met-Tyr-HSL	3IX4	−10.71
29	*N*-Ac-Phe-Tyr-HSL	3IX4	−10.66
30	*N*-Ac-His-Tyr-HSL	3IX4	−10.64

The docked compounds generally displayed two different binding modes (Figure 1): in one the HSL head group was placed similarly to the natural ligand and interacted with Tyr93, Asp73, and Trp60 (Figure 1 C and D); in the other the HSL group was shifted to a close-by subpocket, thereby facilitating interactions between the terminal amine and Asp73 (Figure 1 A and B). In both cases, an aromatic group was located in a pocket formed by generally hydrophobic residues (e.g., Gly38, Leu40, Tyr47, Val76, Cys79, Leu125, and Gly126) thereby forming π-stacking interactions with Tyr47 (for a detailed overview of the LasR ligand binding site please see Skovstrup et al.[37]). Interestingly, there was no overlap of the hits between the different crystal structures. Of the 40 best hits, 30 were selected for synthesis and biological evaluation.

Chemistry

With the aim of synthesizing a peptide library, it was decided to develop a solid-phase synthesis strategy based on amino-functionalized PEG-based resins ($PEGA_{800}$ and ChemMatrix). The resin was prepared for synthesis by attachment of the HMBA linker by using an *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(benzotriazol-1-yl)-uronium tetrafluoroborate (TBTU)-mediated amide coupling procedure (Scheme 2).[37] The first step in the synthesis was



Scheme 2. Synthesis of solid-supported tripeptides. Reagents and conditions: a) *N*-Fmoc-*O*-Trt-L-Hse-OH, MSNT, MeIm, CH_2Cl_2, 1 h (×2); b), d), f) 20% piperidine (DMF), 20 min; c), e) Fmoc-AA-OH, TBTU, NEM, DMF, 2 h; g) 20% Ac_2O (DMF), 30 min.

coupling of *N*-Fmoc-*O*-Trt-HSe-OH by using an optimized MSNT protocol for ester formation.[38] Fmoc deprotection with 20% piperidine in DMF was followed by two rounds of TBTU-mediated couplings with selected AAs to give **33**. For the N-acetylated analogues the resin was treated with 20% Ac_2O in DMF to give **34**.

Solid-supported peptides with an ester-linked *O*-Trt-L-homoserine residue in the first position would, after Trt deprotection, allow acid-mediated cyclative release of the desired product from the solid phase. A similar strategy by Blackwell and

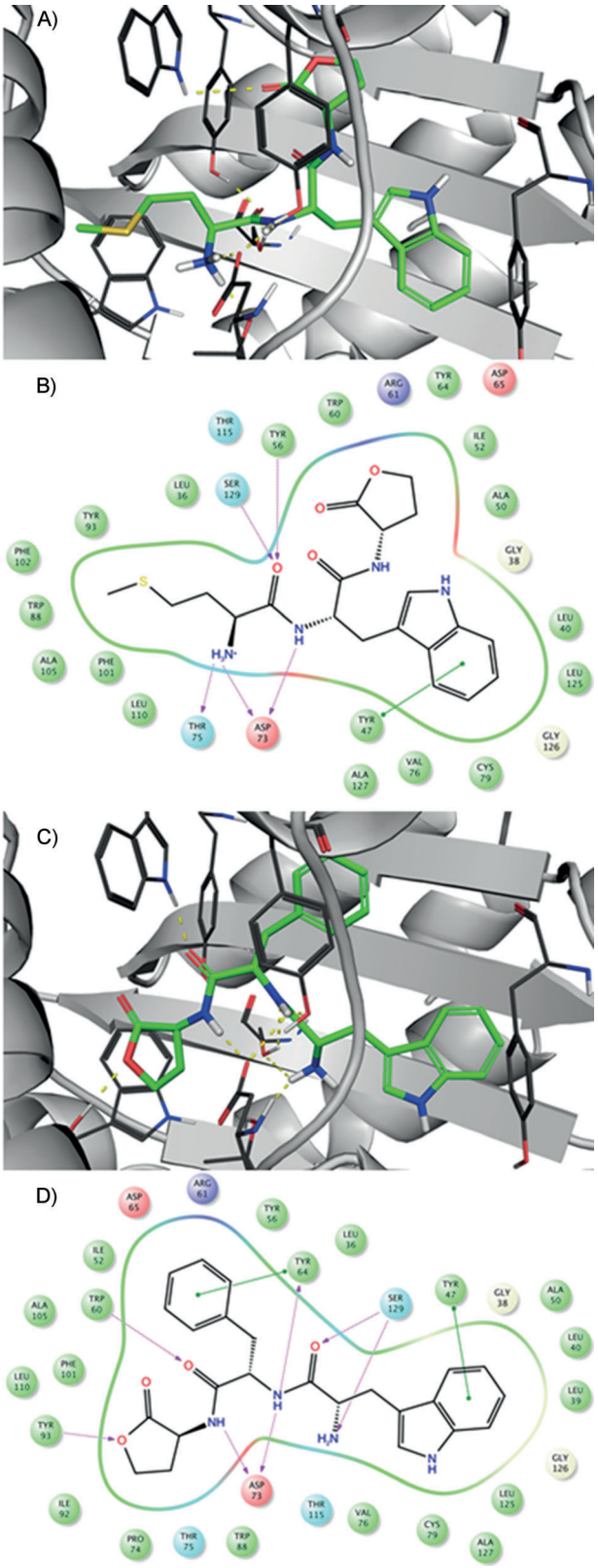
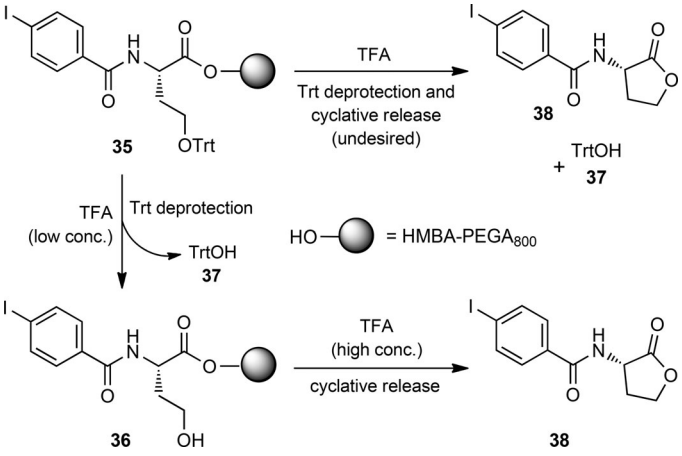


Figure 1. A) and C) 3D binding conformations of **31** and **24** in the LasR binding pocket from the 3IX4 crystal structure. B) and D) 2D ligand–protein interactions. Pink arrows represent hydrogen bonds to a protein side chain; green lines represents π–π interactions.

co-workers used TFA vapor to release HSLs from an 3,5-dichloro-4-hydroxybenzoic acid linker attached to a planar solid support.[31] We needed a more robust linker construct to meet this particularly challenging part of the synthetic process to obtain preparative amounts sufficient for HPLC purification and scale-up. As discussed below, the HMBA linker proved very well suited for this synthetic approach, thus allowing multistep peptide coupling and deprotection reactions.

For standard synthesis, beads, various solvent systems, TFA concentrations, and reaction times were investigated in a model system (Scheme 3, **35**) in order to find conditions meeting these requirements. We expected that acidic treatment with TFA in low concentrations would be sufficient to remove the Trt protecting group from the homoserine (Scheme 3, lower pathway). The released Trt alcohol **37** could then be removed by filtration; subsequent treatment of the resin with a high concentration of TFA would then provide **38**. For this strategy to be successful, it was important than the Trt removal could be performed at TFA concentrations that did not affect cyclative release of the product as this would result in loss of product during filtration (Scheme 3, upper pathway).

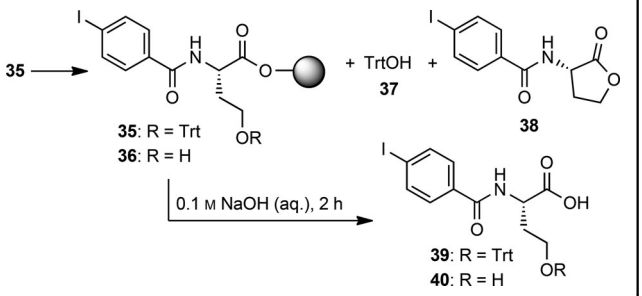


Scheme 3. Model system for the identification of optimal conditions for selective Trt-deprotection.

Following TFA treatment, the filtrate was analyzed by HPLC to determine the ratio between the Trt alcohol **37** and the released HSL **38**. To investigate whether Trt deprotection was complete, the resin was subsequently subjected to aqueous sodium hydroxide to release the substrate from the resin and to determine the ratio between the carboxylic acids **39** and **40** that result from cleavage of **35** and **36**, respectively.

Selected results from the screening of reaction conditions are reported in Table 2. The best results were observed when using water as solvent, and the optimal conditions were concluded to be treatment with 20% aqueous TFA for 10 min (entry 5). Lower TFA concentrations resulted in incomplete removal of the Trt group, whereas higher concentrations resulted in both Trt deprotection and cyclative release of product. The developed protocol was applied to the library of solid-supported tripeptides (Scheme 4). After the deprotection step, the resin was washed once with acetonitrile, followed by treatment with 95% aqueous TFA for 2 h to provide the desired product by cyclative release. The selective Trt deprotection proved to be somewhat substrate dependent, albeit bringing about cyclative release in synthetically useful yields (26–95%; see the Supporting Information for details). The synthesized compounds were easily purified by preparative RP-HPLC prior to biological screening.

Table 2. Optimization study for selective Trt deprotection (selected results).

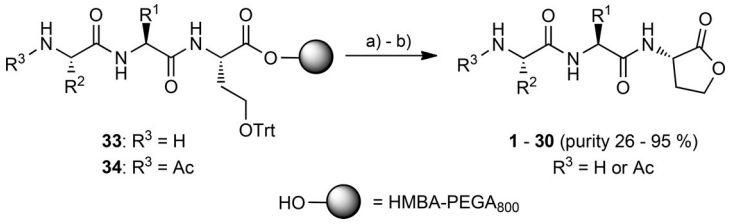


	Aqueous TFA [%]	t_{react} [min]	Ratio **37**:**38**[a,b]	Ratio **39**:**40**[a]
1	5	10	>15:1	5:1
2	5	30	>15:1	16:1
3	10	10	>15:1	1:1
4	10	30	10:1	1:3
5	**20**	**10**	**10:1**	**<1:15**
6	20	30	7:1	<1:15
7	50	10	5:1	<1:15
8	50	30	3:1	<1:15

[a] Determined by RP-HPLC. [b] Corrected for UV absorption at 215 nm.

Biological screening

To determine their QS modulating activity, the synthesized compounds were screened by means of cell-based LasR inhibition and activation assays. Six compounds were identified as LasR activators with EC_{50} values in the low micromolar range (Table 3); none of the compounds displayed LasR-inhibiting activity. The most active compounds were **19** and **20** (EC_{50} 4 and 6 μM, respectively). A clear trend was observed in the structures of the active compounds: an amino acid with an aromatic side chain in the first position and a free N terminus were important for the activation of LasR.

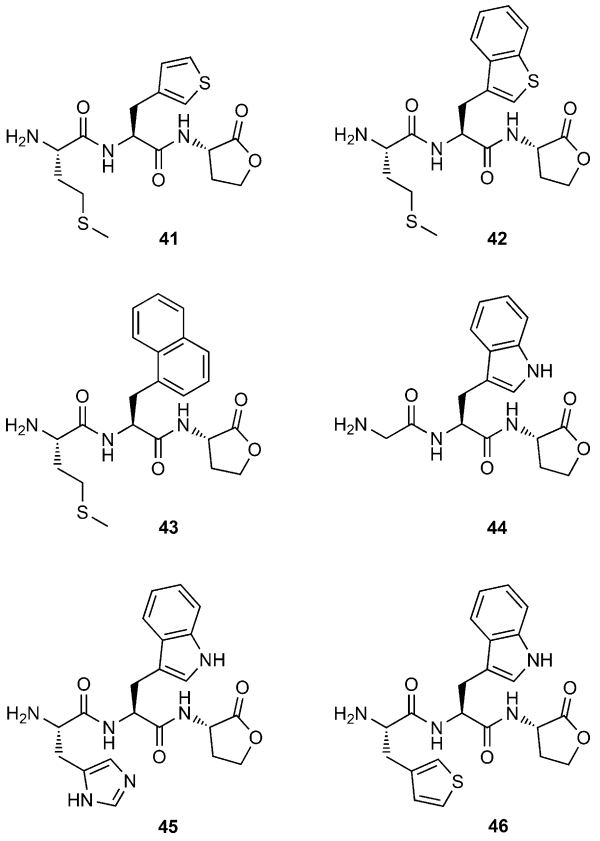


Scheme 4. Trt deprotection and cyclative release of library compounds. Reagents and conditions: a) 20% aqueous TFA, 10 min; b) 95% aqueous TFA, 2 h.

Table 3. EC_{50} values of the most active *N*-dipeptido L-homoserine lactones showing in vitro QS activation (LasR agonism) in an *E. coli* reporter strain.

	Compound	EC_{50} [μM]		Compound	EC_{50} [μM]
1	**OdDHL**	0.009	5	**20**	6
2	**14**	20	6	**21**	12
3	**18**	16	7	**28**	25
4	**19**	4			

Based on the initial screening results, follow-up compounds were designed and synthesized. Analogues of the QS agonists (Table 3) were explored by replacing tryptophan with unnatural aromatic and heteroaromatic amino acids, for example 3-(3-thienyl)alanine, 3-(3-benzothienyl)alanine and 3-(1-naphthyl)alanine. N-acetylated analogues of **19** and **20** and analogues in which the amino acid in the AA_2 position was varied with both canonical and unnatural amino acids were also investigated. Finally, the library also included diastereoisomeric variants of **19** and **20**, synthesized from the corresponding D-amino acids (synthesis protocols in Schemes 2 and 4). By subjecting the ChemMatrix resin to extensive washings with water, DMF, and dichloromethane after the Trt deprotection step, products (including **41**–**46**) were obtained with high purity (>95%) after the cyclative release, thereby illustrating the usefulness of the overall synthesis route, and the advantage of the cyclization–cleavage strategy. The compounds were evaluated in a LasR activation assay (Table 4), and the screening identified another



Scheme 5. Structures of active compounds from follow up studies.

Table 4. EC_{50} values of structurally related *N*-dipeptido L-homoserine lactones showing in vitro QS activation (LasR agonism) in an *E. coli* reporter strain.

	Compound	EC_{50} [μM]		Compound	EC_{50} [μM]
1	**41**	30	4	**44**	20
2	**42**	6		**45**	1.5
3	**43**	5	6	**46**	1.3

six LasR activators (including **45** and **46**) with improved EC_{50} values compared to the initial library (Scheme 5). Neither N-acetylated analogues nor compounds containing D-amino acid residues showed any activity.

Based on the screening results, it is concluded that a large aromatic amino acid in the AA_1 position is favored for LasR activation. Such amino acids include tryptophan and the unnatural benzothienyl- and naphthyl-substituted alanines. When the AA_1 position was occupied by one of these amino acids, compounds with various functional groups in the side chain of AA_2 displayed activating properties; the most potent were those with a small heterocyclic motif at this position.

Conclusions

We screened in silico a virtual library of *N*-dipeptido L-homoserine lactones against two different crystal structures of LasR, and thereby identified several compounds with potential for modulation of QS in *P. aeruginosa*. To assist these efforts, we developed an efficient protocol for solid-phase synthesis of *N*-peptido-homoserine lactones on ChemMatrix resin. All compounds were screened for their effects on activation and inhibition of LasR in cell-based assays. The structures of hits identified in the LasR activation assay were used to design further sets of analogues, some of which were highly potent (low micromolar EC_{50} for QS activation). Intense research efforts in academic laboratories have recently been directed towards QS in Gram-negative bacteria, and several non-native autoinducers capable of antagonizing or agonizing QS in these organisms have been reported. The dipeptido homoserine lactones in this study are among the most potent agonists reported to date.

Experimental Section

In silico screening: The LasR crystal structures (PDB IDs: 2UV0 and 3IX4) were prepared for molecular docking by the Protein Preparation Wizard of Maestro software.[39] (This involves the addition of hydrogen atoms and optimization of the hydrogen bonding networks throughout the protein structure.) Only one protein monomer was retained (chain E; the four monomers are, however, very similar around the ligand), and all water molecules were removed. The peptide library was manually created and docked into the two LasR ligand binding sites by Glide (Maestro) by using SP precision and default settings, but with rotatable hydroxyl groups.[40]

Chemistry: Experimental details for the synthesis of *N*-dipeptido L-homoserine lactones are provided in the Supporting Information.

Reporter strains: Two bacterial reporter strains were used for measuring modulation of QS. For measuring antagonist activity we used a *P. aeruginosa* PAO1 strain harboring a plasmid pMHLAS,[41] which contain P*lasB-gfp*(ASV) and P*lac-lasR* fusions divergently and separately transcribed. For measuring agonist activity an *E. coli* MT102 strain harboring the same plasmid was used.[41] The strains were grown in ABT minimal medium (B medium with thiamine (2.5 mg L^{-1}))[42] supplemented with A10 (10%), glucose (0.5%, *w/v*), and Casamino acids (0.5%, *w/v*) for 16 h at 30 °C with shaking (180 rpm).

Biological assays: The biological assays were conducted in 96-wells microtiter dish (Black Isoplate, PerkinElmer) as previously described.[43] Growth and green fluorescent protein (GFP) expression were monitored by using a Victor X4 multilabel plate reader (PerkinElmer; 34 °C, measurement every 15 min over 14 h). Growth was measured as absorbance at 450 nm. GFP expression was measured as fluorescence at $\lambda_{ex}=485$ nm and $\lambda_{em}=535$ nm. The EC_{50} values were calculated from curves showing the GFP expression.

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Keywords: cyclative release · homoserine lactones · peptides · *Pseudomonas aeruginosa* · quorum sensing · solid-phase synthesis

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